Vážení čtenáři,

toto číslo Chemických listů je věnováno 6. Mezinárodnímu symposiu o biokatalýze a biotransformacích (BIOTRANS 2003) a satelitní konferenci jedné z aktivit „Evropské spolupráce ve vědeckém a technickém výzkumu“ (COST) – Akce D25 (Aplikovaná biokatalýza: stereoselektivní a ekologicky šetrné reakce katalyzované enzymy), které se uskutečnily na Univerzitě Palackého v Olomouci. Obsahem červnového čísla je 324 příspěvků abstrakt přednášek a plakátových sdělení.

Dosud se všechny předcházející konference BIOTRANS konaly v západní Evropě. První byla v roce 1993 v Grazu (Rakousko), a pak vždy po dvou letech v některé ze zemí Evropské unie (Warwick, Velká Británie, 1995; La Grande Motte, Francie, 1997; Giardini-Naxos, Itálie, 1999 a Darmstadt, Německo, 2001). Přidělení konference do České republiky bylo nejen oceněním výsledků českých vědeckých pracovišť zaměřených na tento obor, ale také výzvou organizátorům. Konání symposia v srdci střední Evropy usnadnilo účast vědců ze zemí bývalého východního bloku.

Pro Vás, kteří jste odborníky v jiných oborech chemie, chceme přiblížit tématické zaměření konferencí BIOTRANS. Jako biotransformace se označuje konverze jedné látky (substrátu) na jinou (produkt) pomocí biologického systému. Jde o multidisciplinární obor využívající poznatky a metodiky organické chemie, biochemie, molekulární biologie, ale také imunologie a medicíny. Výsledky lze přímo aplikovat ve všech oblastech biotechnologie. Hlavními oblastmi využití jsou (i) chemické modifikace cizorodých látek (xenobiotik) v živém organismu, (ii) užití enzymů, mikroorganismů, frakcí nebo kultur živočišných a rostlinných buněk v chemickém výzkumu a průmyslových technologiích a (iii) odstraňování škodlivých a toxických látek z životního prostředí biodegradací.

Rozmach biotransformací začal v poslední třetině minulého století díky tomu, že byly popsány nové enzymy a jimi katalyzované reakce. Tyto objevy umožnily vývoj nových metod a technologií až k současnému širokému využití biokatalýzy v průmyslu. Biotransformace významně přispívají k zavedení technologií, které šetří životní prostředí a tak splňují požadavky na trvale udržitelný vývoj lidské společnosti.

Biokatalýza je základem „ekologické chemie“, která nahrazuje toxické katalyzátory, organická rozpouštědla a extrémní reakční podmínky, vedoucí k velkému množství toxických odpadů, biokatalyzátory – enzymy, které většinou působí ve vodném prostředí a mírných teplotách. Enzymy aplikované v biokatalýze jsou zpravidla schopny transformovat široké spektrum látek, které nejsou jejich přirozenými substráty. Základní výhodou enzymů je jejich vysoká stereospecifita, která umožňuje přípravu opticky čistých látek. Všechny biokatalyzátory jsou plně regenerovatelné, např. kultivací mikroorganismů. Vývoj nových metod umožňuje užití enzymů také v nevodném prostředí. Imobilizované enzymy, membránové reaktory a multienzymové systémy mají průmyslové využití. Současný rozvoj biotransformací by nebyl možný bez interakcí s novými molekulárními

Dear readers

This issue of Chemické Listy is devoted to the 6th International Symposium on Biocatalysis and Biotransformations (BIOTRANS 2003) and to the satelite Workshop of European network COST D25 (Applied biocatalysis: stereoselective and environmentally-friendly reactions catalysed by enzymes) which were held at Palacký University in Olomouc. June issue contains 324 abstracts of lectures and posters.

All the previous BioTrans conferences were held in Western Europe: in Graz (A) 1993, Warwick (UK) 1995, La Grande Motte (F) 1997, Giardini-Naxos (I) 1999 and Darmstadt (D) 2001. The allocation of the conference to the Czech Republic implied not only an acknowledgement of the Czech science but also posed a significant challenge to the organizers. Location in the heart of central Europe facilitated the participation of scientists from central and Eastern Europe.

For those who are not experts, the following information should provide an idea of the scientific orientation of BioTrans conferences, Biotransformation consists in the conversion of one substance (substrate) to another (product) by a biological system. This definition suggests the multidisciplinary nature of this research field. Indeed, the studies of biotransformations use a variety of approaches ranging from chemistry, biochemistry and molecular biology to immunology and medicine. The results are directly applicable in all branches of biotechnology. The main fields of interest are (i) conversion of pharmaceuticals and other foreign compounds (xenobiotics) in living organism, (ii) application of enzymes, microorganisms, animal and plant cells and cellular fractions in chemical and biochemichal research and industrial technologies and (iii) removal of harmful or toxic compounds from the environment by biodegradation. Currently all these research fields undergo a rapid development.

The boom of biotransformations began in 1970s and 1980s owing to the reports of novel biocatalysts and novel enzymatic reactions. These discoveries led to the development of new methods and technologies up to the current broad industrial use of biocatalysis. Biotransformations significantly contribute to the establishment of environmentally friendly technologies that meet the demands of sustainable development of the human society.

Biocatalysis is the basis of the “green chemistry” that replaces toxic catalysts, organic solvents and extreme reaction conditions, leading to large amounts of toxic wastes, by biocatalysts – enzymes that are mostly utilizable in aqueous environment and at mild temperatures. In general, enzymes applicable in biocatalysis are able to transform a broad spectrum of unnatural substrates. The principal benefit of enzymes is their high stereospecificity that makes the preparation of optically pure compounds possible. All biocatalysts are fully renewable by, e.g., cultivation of microorganisms. Newly developed approaches enable to apply biocatalysts in a non-aqueous environment that is frequently required by the protocols of organic synthesis. The current development of biotransformations would not be possible without novel molecular techniques such as molecular genetics, protein crystallography,

technikami používanými v molekulární genetice, proteomice a farmakologii, metodami analytické a organické chemie. Tento mnohostranný přístup vedl k objevům nových typů reakcí, stovek nových enzymů a konečně k vývoji nových typů biokatalyzátorů jako katalytických protilátek, nukleoproteinových komplexů (ribozymů) a kovalentně modifikovaných enzymů. Při optimalizaci a modifikaci enzymů se významně uplatnila technika přesunu genů (gene shuffling). V současné době asi 70 % „katalytických" průmyslových procesů užívá jako katalyzátory enzymy. To svědčí o ekonomickém významu těchto výrobních postupů.

Věříme, že obsah tohoto čísla, i přes své odlišné zaměření, bude pro čtenáře zajímavý a v mnohém poučný. Čtenář má možnost konfrontovat výsledky jednotlivých pracovišť z oboru biotransformací, orientovat se v perspektivách tohoto vědního odvětví či se inspirovat při přípravě svých vývojových a průmyslových projektů.

Vladimír Křen
Vilím Šimánek

spectral methods, proteomics, and modem advances of organic chemistry and pharmacology. This multidisciplinary approach leads to the discovery of new reaction types, hundreds of new enzymes and finally to the development of new types of biocatalysts, such as catalytic antibodies, nucleoprotein complexes (ribozymes) and covalently modified enzymes. The techniques of "gene shuffling" (rearrangement of genes) are of utmost importance for the optimization and modification of enzymes,

It is estimated that at present 70 % of all "catalytic procedures" in industry are mediated by enzymes or other biocatalysts. This reflects an immense economic impact of these applications.

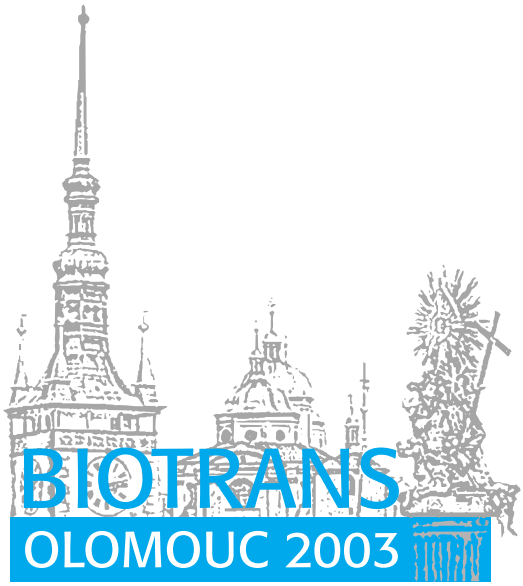
We hope very much that the content of this issue, despite its specificity, will be not only interesting for the reader but will provide new information on recent results of leading laboratories working in the field of biotransformation. It should also help to get a deeper insight into this scientific area and to obtain inspiration to the design of novel industrial procedures.

Vladimír Křen
Vilím Šimánek

6th International Symposium on Biocatalysis and Biotransformations

together with

COST D25 Workshop



June 28 – July 3, 2003

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L001 CREATING ENANTIOSELECTIVE HYDROLASES FOR ORGANIC SYNTHESIS: COMBINING RATIONAL AND RANDOM METHODS

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Keywords: enantioselectivity, STAR mutagenesis, *Pseudomonas fluorescens* esterase

Although it is difficult to design more enantioselective enzymes, it is also difficult to evolve them by directed evolution because one must screen thousands of mutants. The flaw in directed evolution is random mutagenesis, whether error-prone PCR, DNA shuffling or related methods. In a typical enzyme, <10 % of the amino acid residues are within the active site and most lie 18 ± 3 Å away from the active site. For this reason, random mutagenesis is biased toward mutations far from the active site. On the other hand, to change catalytic properties such as enantioselectivity, mutations in or near the active site are more effective than distant ones. We propose that focusing mutations into the active site is a more effective approach to changing the catalytic properties of an enzyme. We call this structure-targeted random mutagenesis, or STAR mutagenesis. We used this strategy to dramatically improve the moderate enantioselectivity (E = 12(S)) of a *Pseudomonas fluorescens* esterase (PFE) toward methyl 3-bromo-2-methylpropionate (MBMP). A homology model identified four amino acid residues close to the chiral acyl group of the substrate: Trp29, Val122, Phe199, and Val226. Saturation mutagenesis at each site followed by screening for altered enantioselectivity using Quick E identified seven different point mutants with 3–5 times higher enantioselectivity than wild type. This success rate of this method (18 % of the active mutants showed increased enantioselectivity) is dramatically higher than the success rate using random mutagenesis ($<$ 1 %). Further double mutants show even higher enantioselectivity.

L002 BIOCATALYSED ASYMETRIC BAEYER VILLIGER OXIDATION USING WHOLE CELLS

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Keywords: asymmetric Baeyer-Villiger oxidation, enantioselectivity, biocatalysis

In 1899, Adolf von Baeyer, working together with Victor Villiger, mixed in an Erlenmeyer flask 15 g of natural menthone with 38 g of anhydrous potassium persulfate, under cooling… Further work up led to a new crystalline compound which was shown to be the corresponding lactone. This was the first observed chemical oxidation of a ketone to a lactone, and the beginning of the story of a new reaction known as being „the Baeyer-Villiger oxidation“. Since then, numerous applications of this reaction have been described for fine organic synthesis.

Interestingly, this type of oxidation has also been postulated, in 1953, as a biological step in steroid degradation. In 1981, Schwab described – at an analytical scale – the very interesting feature that a newly purified microbial enzyme, i.e. cyclohexanone monooxygenase, could lead to asymmetric oxidation of α-deuterocyclohexanone. To the best of our knowledge, this was the first asymmetric Baeyer-Villiger oxidation process known to date.

Further efforts – carried out to explore this new possible biocatalytic route to enantiopure lactones – have been developed by several research teams at a laboratory scale within the last fifteen years, using either whole cells or isolated enzymes. This led to the very important demonstration that these biocatalysts can indeed be efficiently used to achieve the enantioselective Baeyer-Villiger oxidation of various ketones.

Only recently (1994) the first transition metal catalysed asymmetric Baeyer-Villiger oxidation was described. In spite of some recent improvements by Katsuki et al., the results obtained using this conventional chemistry approach are still unsatisfactory in terms of substrate and enantioselectivity. Thus it appears that, at the present time, biocatalysis is by far the best tool for achieving the asymmetric Baeyer-Villiger oxidation[1–4].

The aim of this presentation will be to present a survey of our efforts aimed at developing this type of approach and to illustrate its potential to perform the synthesis of some targets of interest in enantiopure form. Recent results of our efforts aimed at exploring the possibility to scale up such a process to a (mini) pilot scale will also be described.

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L003 THE IMPORTANCE OF SUBSTRATE ENGINEERING IN BIOCATALYSIS

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Keywords: substrate engineering, enzymatic cyanohydrin reaction, biohydroxylation, docking/protecting group principle

Very often biocatalytic transformations require an optimisation, engineering (e), of the reactions. The possibilities are: Choice of reaction conditions (reaction e.), selection and modification of the enzyme applied (enzyme e.), influencing the cell metabolism for whole cell transformations (metabolic e.), transformation of the substrate to molecules accepted best by the biocatalyst (substrate e.)

After a general overview on the possibilities of substrate engineering the lecture will focus on the application of this principle to two biocatalytic transformations: Modification of the substrate for enzymatic cyanohydrin reactions and the optimisation of biohydroxylation reactions by application of the principle of docking/protecting groups[1–4].

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L004 ENZYME ASSISTED SYNTHESES: FROM BIOACTIVE MOLECULES TO THE BIOCONVERSION OF RENEWABLE RESOURCES

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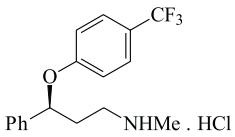
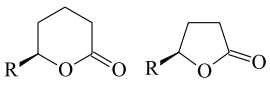
Keywords: bioactive compounds, enantiomers, lipases, pharmaceuticals, renewable resources

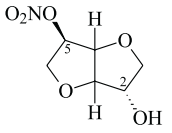
Enzymes have emerged in recent years as highly efficient catalysts in organic syntheses. This is particularly true for esterhydrolases (esterases, lipases) many of which ideally combine the required high reaction selectivities with the synthetically so important broad substrate tolerance. As demonstrated in many laboratories including ours they are ideally suited for the preparation of enantiomerically pure hydroxy compounds due to their ability to differentiate between i) enantiomers, ii) enantiotopic groups attached to prochiral centers and iii) enantio-topic groups in *meso*-compounds. Using these three modes of substrate recognition in the first part of the lecture enzyme assited syntheses of several classes of compounds with known or potential biological activities will be described. Thus resolutions of racemates are leading to building blocks for pharmaceuticals[1–3] (e. g. fluoxetine, isosorbide-5-mononitrate, aromatase inhibitors), flavour compounds[4,5] (γ- and δ- lactones) and D-aminoacids[6].



(*S*)-Fluoxetine



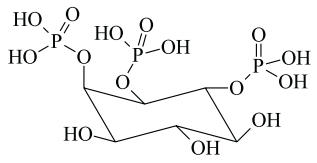
δ - and γ - lactones



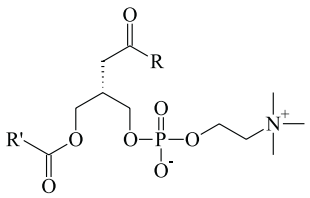
Isosorbide-5-mononitrate

Differentiation of the enantiotopic hydroxy groups in 2-substituted 1,3-diols leads to chiral C-3 building blocks, mimics of diacylglycerols (second messengers) and carba-analogues of phospholipids[7,8]. Differentiation of enantiotopic hydroxygroups in suitably protected derivatives of achiral *myo*-inositol leads to enantiomerically pure building blocks

for *myo*-inositol phosphates – important second messengers[9, 10].

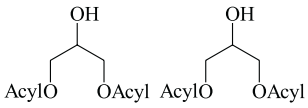


1,2,6-*myo*-Inositoltriphosphate

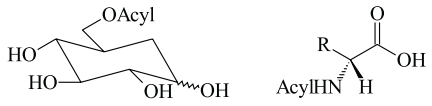


carba - analogues of phospholipids

In connection with recent efforts related to the development of environmentally friendly processes (Green Chemistry) the bioconversion also of commodities such as plant derived bulk materials – so called renewable resources – is increasingly addressed in numerous laboratories. Agricultural crops represent a considerable reservoir of useful and low cost raw materials such as fats and oils, proteins and carbohydrates. By selective combination of their molecular constituents (fatty acids, glycerol, amino acids, saccharides) a wide variety of surface active materials can be prepared, all of them – due to their molecular structures – being highly biodegradable. Lipases are well established biocatalysts for the selective formation of ester and amide bonds and thus ideally suited for the preparation of combination products with surface active properties such as partial glycerides[11–13], sugar esters and *N*-acylated amino acids. In the lecture the employed methodologies for the enzymatic preparation of these materials will be discussed in detail.



partial glycerides



sugar esters N-acylated aminoacids

The resulting products are useful both as emulsifiers in food applications, excipients in pharmaceutical formulations and as mild and skin friendly detergents for cosmetic uses.

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L005 ENZYME 'BUILDING BLOCKS' FOR NANO-STRUCTURED COMPOSITE MATERIALS

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Keywords: enzyme, building block, nano-particle, protein-based composite material

The design, fabrication, characterization and applications of nano-structures, has recently gained much attention. The use of biological macromolecules such as DNA and proteins, as 'building blocks' for the construction of functional nano-structures via self-assembly offers several advantages, including homogeneous large sized 'building blocks' molecular population, self-assembly mediated by biological recognition, and a sound basis of structural and biochemical data as well as available tools for production, isolation and characterization. The use of enzymes as 'building blocks' – either as single molecule or as protein crystal or scaffold – may be par-

ticularly attractive due to their well characterized molecular structures, chemical surface compositions and biological activity.

Three different modes of application of enzymes as 'building blocks' will be described:

A. Enzyme crystals as protein made templates for the preparation of novel composite materials: a unique new family of composite materials, comprised of three dimensional highly ordered alternating arrays of nano-scale biological moiety (the protein) and synthetic-organic, inorganic-ceramic or metallic nano-scale moiety, embedded within the crystal cavities;
B. Enzyme molecules serving as core for the fabrication of a new type of nano-particles comprised of a protein core and a grafted fur-like synthetic polymeric shell;
C. Enzymes and other protein molecules and arrays serving as template for the fabrication of metallic nano-particles comprised of a protein core and a thin metallic surface made by a new controlled electroless deposition.

The design, fabrication and characterization of the above mentioned new composite materials will be described. Our results demonstrated feasibility of the three methodologies, paving the way to the fabrication and use of a new family of protein-based composite materials.

L006 PATHWAY ENGINEERING FOR PRODUCTION OF 1,3-PROPANEDIOL FROM GLUCOSE

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Keywords: 1,3-propanediol, *E. coli,* biocatalyst development, metabolic engineering

At present, there is limited production of 1,3-propanediol, a monomer highly desired for copolymerization with terephthalic acid to make 3GT polyester (Sorona™). A joint team of scientists at Genencor and DuPont have developed a breakthrough bioprocess which promises to provide 1,3-propanediol at a scale and price appropriate for a commodity chemical. The process employs an engineered *E. coli* microorganism and utilizes the renewable starting material glucose as its feedstock, converting glucose into 1,3-propanediol in a single organism. We will describe (i) the general scheme for the construction of the biocatalyst and (ii) the subsequent approach to maximize carbon and reducing equivalent throughput *via* metabolic engineering. We will highlight the integration of metabolic flux analysis, DNA macroarrays, traditional protein biochemistry, molecular biology and fermentation in the biocatalyst development.

L007 BIOCATALYTIC ASYMMETRIC HYDROGEN TRANSFER

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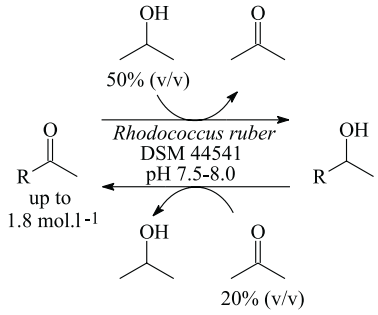
Keywords: alcohol dehydrogenase, *Rhodococcus ruber*, ketone, assymetric reduction

An alcohol dehydrogenase from *Rhodococcus* sp. was employed in a biocatalytic hydrogen-transfer process[1] for the asymmetric reduction of ketones (at the expense of 2-propanol as cosubstrate/hydrogen donor) or *via* oxidative kinetic resolution of *rac*-*sec*-alcohols using acetone as co-substrate as depicted in the Scheme below[2,3]. The feasibility of these processes depends on the exceptional enzyme-stability toward high co-substrate concentrations, i.e. 2-propanol (50 % v/v) for reduction and acetone (20 % v/v) for oxidation, respectively[4]. Using these co-substrates, cofactor recycling becomes trivial, especially when using whole (resting) cells as the catalyst. The scope and limitations of this biocatalytic approach for the oxidative kinetic resolution of *sec*-alcohols as well as the asymmetric reduction of ketones to furnish both stereoisomers of the corresponding non-racemic alcohols will be discussed[5]. The applicability of the oxidation as well as of the reduction will be demonstrated for various products of the flavour and pharmaceutical industry.



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L008 NOVEL BIOCATALYST FOR THE PRODUCTION OF ENANTIOMERICALLY PURE AMINES AND AMINO ACIDS

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Keywords: chiral amines, amino acids, penicillin acylase, peptide deformylase, amidase, aminopeptidase, kinetic resolution, chiral synthesis

Biocatalysis has developed into a well-recognized, valuable tool for fine chemicals manufacturing[1,2]. Crucial to development of novel processes for fine chemicals production, which integrate single or even multiple biocatalytic steps, is the availability of biocatalysts with the desired activity, selectivity and process stability enabling the industrial synthesis of the desired enantiomer of chiral compounds[3].

DSM has developed proprietary biocatalyst platforms for production of a variety of enantiomerically pure fine chemicals. These platforms include a collection of amidases and acylases for the synthesis of α- and β-amino acids, dipeptides, chiral amines and derivatives thereof. Several examples of the use of these biocatalyst platforms will be presented.

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L009 DERACEMISATION OF CHIRAL AMINES USING ENZYMES OBTAINED BY DIRECTED EVOLUTION

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Keywords: deracemisation, enantioselective enzyme, non-selective chemical reagent, directed evolution techniques

Emphasis is increasingly being placed upon the development of novel enzyme-catalysed processes that are able to deliver the product in yields approaching quantitative and high optical purities. In this context, dynamic kinetic resolutions, enantioconvergent reactions and asymmetric transformations have recent considerable attention in recent years. In order to extend the range of chiral molecules that are amenable to dynamic kinetic resolutions, it has been necessary to discover new reagents for the racemisation of chiral molecules under conditions that are compatible with enzyme catalysis. Particularly impressive advances have been made in the DKR of secondary alcohols using transition metal based catalysts for racemisation.

This lecture will describe a new approach that we are developing for the deracemisation of racemic mixtures of chiral compounds. Deracemisation of racemic mixtures represents a new and highly efficient approach to the synthesis of a wide range of chiral, optically pure, intermediates for pharmaceutical and fine chemical end products. The principle of the deracemisation strategy is to combine an enantioselective enzyme with a non-selective chemical reagent for the stereoinversion of enantiomers. Figure 1 illustrates the conversion of D- to L-α-amino acids by carrying out sequential cycles of oxidation/reduction using a D-selective amino acid oxidase together with *in situ* reduction of the intermediate imine **2**.

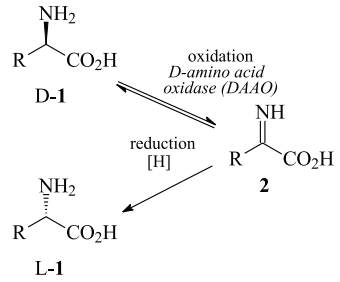

Fig. 1. Stereoinversion of D- to L-amino acids using a D-amino acid oxidase in combination with a chemical reducing agent.

The deracemisation approach has been successfully applied to both L- (ref.[1]) and D-α-amino acids[2] and more recently we have further extended the method to include chiral amines[3]. A key aspect of the deracemisation of amines was the application of 'directed evolution' techniques to select for enzymes with optimised characteristics (*e.g.* enantioselectivity, solvent stability, substrate range, tolerance of high product concentration) for applications in large scale synthesis.

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L010 METAGENOMICS, GENE DISCOVERY AND THE 'IDEAL BIOCATALYST'

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Keywords: metagenome, gene discovery, biocatalysts

Within the new paradigm where process conditions are no longer the defining or limiting constraints on successful biocatalytic processes and the biocatalyst can be found or made to fit the purpose, the technologies of gene discovery and enzyme engineering are vitally important[1]. Here we focus on the molecular technologies both in use and under development for the discovery of genes from 'metagenomic' resources.

The metagenome constitutes the total complement of genomes (and genes) available in a particular environment. It is now widely accepted that, in any microbial environment, less than 1 % of the extant species diversity has ever been cultured. It therefore follows that effective access to the metagenome, and the genes therein, must depend on technologies that lie outside classical microbial isolation.

The first of these technologies (metagenomic or 'multiplex' cloning[2] has been implemented with considerable success over the past 5 years. The current applications of this method, together with its limitations and capacity for further evolution, will be described. Newly evolving methods for direct access to the metagenome, including PCR-dependent methods and sequencing approaches, will be presented.

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L011 COMPLEMENTARY AND SYNERGISTIC APPLICATION OF ENZYME AND MOLECULAR CATALYSIS

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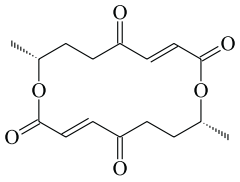
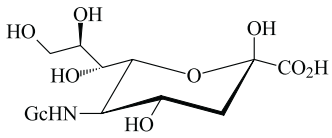
Keywords: lipase, nitrile hydratase, amidase, protease, yeast-mediated reduction

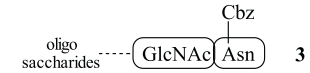
An important aspect in the enzyme-catalyzed production of chemicals is the complementary and synergistic use of chemo-enzymatic procedure so that we can draw a reasonable and straightforward blueprint towards the target molecules. Our early achievements were the syntheses of (*R*,*R*)-pyrenophorin (**1**, 1995) and *N*-glycolylneuraminic acid (NeuGc, **2**, 1997). In the former, the introduction of chirality and functional group transformation were effectively performed by whole-cell enzymes.



(*R*,*R*)-pyrenophorin **1**



NeuGC **2**



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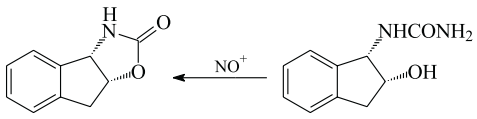
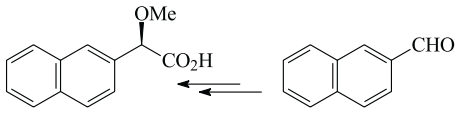


4 **5**



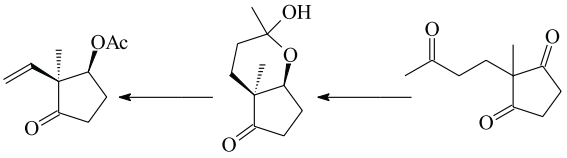
(*R*)-2-NMA **6** **7**

The latter case prompted us the chemo-enzymatic transformation with minimally protected polyfunctional molecules, exemplified as the synthesis of glycopeptide intermediate (**3**). Papain was efficiently used for the deprotection of carboxylic ester under mild conditions.

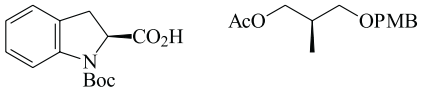
Of course, the development of new chemical reaction, to be coupled with enzymes as key steps, was necessary, as shown in the synthesis of an oxazolidinone (**4**: nitrosation-deaminocyclization) and (*R*)-2-NMA (**6**: one-pot cyanohydrin acetate formation) from **5** and **7**, respectively.

The design of the substrates and the device of subsequent chemical transformation are the clue to overcome the frequently encountered two obstacles, the low accessibility to the proper substrates and the narrow substrate specificity of the enzymes. This topic is presented in the synthesis of **8** from **10** *via* a cyclic hemiacetal (**9**).

Finally, to minimize the product inhibition problem of enzyme-catalyzed hydrolysis, the release of inhibitory by-product (for **11**), and the enantiomeric enrichment of substrate (for **12**) prior to the reaction by either another chemical or enzymatic way, were studied.



8 **9** **10**



11 **12**

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L012 APLICATION OF BACTERIA AND FUNGI AS BIOCATALYSTS FOR THE PREPARATION OF OPTICALLY ACTIVE HYDROXYPHOSPHONATES

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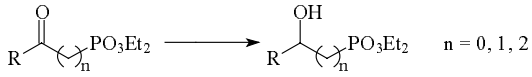
Keywords: hydroxyalkanephosphonate, oxoalkanephosphonate, stereoselective reduction, microbial whole cells

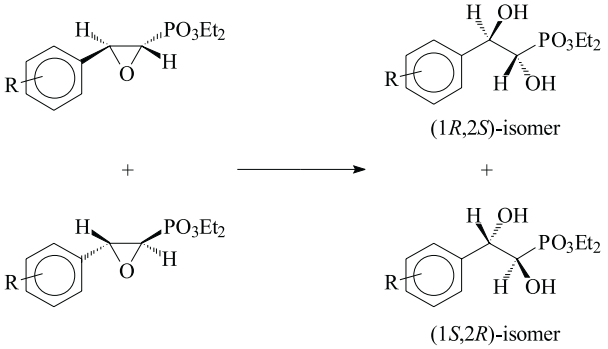
Biotransformations offer an effective and sometimes preferable alternative to the standard synthesis of fine chemicals, especially in the case when optically active products are desirable. Hydroxyalkanephosphonates could be considered as analogues of hydroxy acids and are obtained by replacement of carboxylic acid moiety by phosphonic acid one. This approach quite frequently results in promising biologically active compounds, application of which range from medicine to agriculture. Moreover, they may serve as substrates for the preparation of aminoalkanephosphonic acids, mimetic of amino acids, which display even more promising physiological activity. For proper evaluation of these activities availability of enantiomerically pure compounds is indispensable.

The use of biotransformations for the preparation of optically active phosphonic acid derivatives has been only scarcely reported in the literature and therefore we have undertaken studies on the use of microbial whole cells as biocatalysts for their preparation. The first approach considered stereoselective reduction of oxoalkanephosphonates using baker's yeast and some other strains of fungi.



This reaction afforded the desired product in satisfactory yields and with high optical purities when β– and γ–oxoalkanephosphonic (n = 1 or 2) acids were used as substrates. Diethyl 1-oxolakyphosphonates (n = 0) are, however, extremely unstable in aqueous solutions and bioreductions of this class of compounds were carried out in anhydrous hexane using lyophilized or immobilized microbial cells.

1,2-Dihydroxyalkanephosphonates constitute a small class of compounds preparation of which is still challenging and desirable, with their biological activity awaiting determination. We have, therefore, also tested ability of several strains of fungi and bacteria to carry out stereoselective hydrolysis of *trans*-epoxyethanephosphonates in order to obtain corresponding 1,2-dihydroxyalkanephosphonic acids.



The stereochemical course of the reaction was determined by means of ^{31}P NMR using quinine as a chiral selector. Contrary to the chemical hydrolysis biocatalysis resulted preferably in *erythro*-1,2-dihydroxylakanephosphonates, which were obtained in good yields and with enantioselectivity strongly dependent on the structure of the used substrate and the kind of the applied microorganism. We are, however, unable to determine, which of the two formed isomers (1*S*,2*R*) or (1*R*,2*S*) is the major product of this hydrolysis.

L013 BIOTRANSFORMATION OF IMMUNOSUPPRESSIVE COMPOUNDS

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Keywords: cyclosporin A, bioconversion, metabolite, biological activity

Cyclosporin A, a lipophilic cyclic undecapeptide produced by a fungus *Tolypocladium inflatum*, has been selected

as a model molecule to discuss the biotransformation of cyclophilin binding-immunosuppressive compounds. A metabolism of cyclosporin A (immunosuppressive compounds) occurs predominantly in the liver, the bowel, and to lesser degree in kidney by cytochrome P450 CYP3A4 oxidase. Biotransformation is initiated by oxidative attack at five main points on the molecular face opposite to cyclophilin binding site. Mathematical analysis predicts 41 mono-, di- or tri-oxidized derivatives. All metabolites retain the intact cyclic oligopeptide structure of the parent drug. Structural modifications consist of mono- and dihydroxylation, *N*-demethylation and intramolecular cyclization. *In vitro* studies utilizing liver microsomes show that the first oxidation products of CsA are the primary metabolites M1, M9, and M4N, produced by stepwise biotransformation beginning with a single conversion: either a region-specific hydroxylation at the n or gamma positions of amino acid 1, or a demethylation at amino acid 4. Further oxidation of these compounds then produces a second group of metabolites which exhibit combined biotransformation, such as M19 characterized by dihydroxylation at both n and gamma position of amino acid 1 and 9, M49 which is oxidized at position 4 and 9, or M4N9 which is oxidized at position 4 (N) and 9. Other secondary metabolites include M4N69 and M69 found in urine, both of which are oxidized at position 6 and may represent further oxidation products of M4N9 and M9, respectively. M1AL, an aldehyde obtained by oxidation of primary alcohol M1, and M1A, the corresponding acid first isolated as the bile acid metabolite Ma. Both of the later can be further oxidized in positions 4,4 (N), 6, and 9 or cyclized at position 1. A third conversion mechanism is intramolecular tetrahydrofuran ether formation at amino acid 1, producing M1C, while the ultimate formation of linear CsA metabolites formed as gamma lactones has also been predicted and at least one of these has been produced synthetically.

In addition to oxidized metabolites, Cs A is metabolized by conjugation of the hydroxyl group at MeBmt amino acid, represented by e.g. water soluble sulfate conjugate.

The purpose of the lecture is not only to summarize existing pathways of bioconversion and known metabolites of immunosuppressive drugs but also to discuss the biological activities and potential involvement of metabolites in primary and side effects, and in toxicity of immunosuppressive compounds.

L014 ENZYMATIC CATALYSIS IN ORGANIC SYNTHESIS

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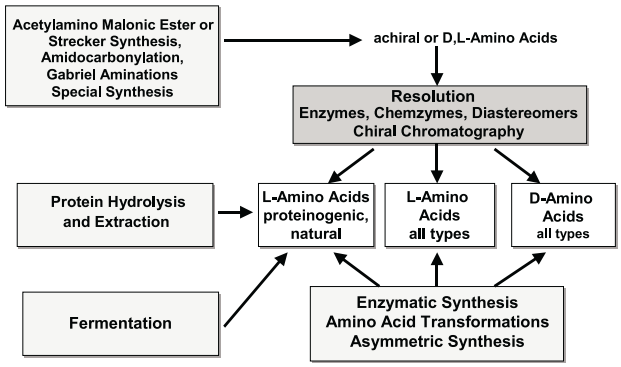
Keywords: enzymes, biocatalysis, organic synthesis, amino acid

The world of biocatalysis has undergone significant changes during the last decades. Enzymes are showing up in many more organic syntheses and a number of important new industrial processes coming on line. The search for better, enantiomerically pure biologically active compounds and their intermediates is forcing many chemists to use enzymes. Ever increasing demands for environmentally friendly processes push in the same direction.

Rapidly developing technologies for discovering new enzymes and optimizing them by genetic engineering are offering new opportunities. So we can respond more rapidly and effectively to new synthetic needs with biocatalytic solutions.

This paper will focus on optimized and new biocatalytic methods especially for synthesizing amino acids and derivatives.



L015 DIRECTED MOLECULAR EVOLUTION TECHNOLOGIES: IMPROVING GENES, PATHWAYS, AND WHOLE ORGANISMS FOR BIOCATALYSIS, FERMENTATION, AND PROCESS APPLICATIONS

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Keywords: biocatalyst, development, stability, stereospecificity, technology

Few naturally occurring enzymes and fermentation strains are optimal for industrial use. Codexis, Inc., a subsidiary of Maxygen, Inc., is using its proprietary technologies to enable and develop improved biocatalysts, strains and processes. By integrating recursive DNA sequence recombination (DNA shuffling) with process-appropriate screening methodology, improvements in the catalytic performance of enzymes, pathways, and whole organisms have been realized and successfully commercialized. Using this technology, multiple enzyme traits can be co-evolved, to create superior biocatalysts with increased activity, stability and preferred chemo-, regio- and stereospecificity under desirable process conditions. In addi-

tion, many industrial opportunities require the coordination of multiple enzymatic steps and the evolution of complex biological pathways. This presentation will give an overview of the technology and will describe examples of our recent efforts in the area of biocatalysis and fermentation.

L016 DISCOVERY AND DEVELOPMENT OF ENZYMES FOR STEREOSELECTIVE REDOX REACTIONS

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Keywords: stereoselective redox biotransformation, ketone, large scale

This talk will focus on our recent advances in developing stereoselective redox biotransformations by marrying genome sequence data with novel chemical reactions and bioprocesses. Specific examples will include asymmetric ketone reductions by baker's yeast enzymes and ketone oxidations by a bacterial flavin monooxygenase. Methods to discover enzymes with the optimal combination of rapid reaction rates and stereospecificities will also be discussed, along with strategies to carry out the reactions economically on large scales. Our goal is to devise a set of generally applicable, integrated strategies that can be used to solve problems in the pharmaceutical, agrochemical and polymer chemicals sectors.

L017 BIOCATALYSIS IN IONIC LIQUIDS

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Keywords: ionic liquids, enzyme catalysis, membrane, nanofiltration

During the past decade, ionic liquids (IL's) have gained increasing attention for performing all types of reaction with sometimes remarkable results[1, 2]. IL's are salts with melting points below 100 °C; they have no measurable vapor pressure making them ideal tools for clean and sustainable processes. Applications for ionic liquids in electrochemsitry, solar cells, sensors, supported liquid membranes[3], homogeneous catalysis and even protein renaturation[4] have been reported. It also has been demonstrated by numerous groups that ionic liquids can be used as novel biocompatible solvents. An overview is given in two recent reviews[5, 6].

There are basically three modes of operation: Use of the IL as co-solvent, as pure solvent or in a biphasic system. In many cases improved stability of the biocatalyst has been reported compared to traditional solvents. Improved (enatio)selectivity for especially lipases has been observed.

The contribution will focus on areas where ionic liquids may be used advantageously by making use of their special mixing and solvation properties[7]. It is very likely that these special properties will be essential for further fostering the use of ionic liquids in novel applications. Another topic to be addressed is the problem of impurities and analytical tools in order to guarantee a special purity necessary for biocatalysis – which is different from what is required for transition metal catalysis.

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L018 GLUCOSYL TRANSFER BY SACCHARIDE PHOSPHORYLASES: COMPONENTS OF MECHANISM AND APPLICATIONS IN (GLYCO)BIOTECHNOLOGY

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Keywords: saccharide phosphorylase, trehalose phosphorylase, cellobiose phosphorylase, synthesis of glucosides

When classified according to their enzymic reactions, saccharide phosphorylases constitute a heterogeneous class of glucosyl transferases (EC 2.4.1) that catalyse reversible glucosyl transfer to and from phosphate. They are intracellularly located and in the environment of the cell where relatively high phosphate concentrations prevail, the direction of phosphorolysis of glucosidic bonds is favoured thermodynamically. The physiological role of saccharide phosphorylases is effectively catabolic and pertains to conversion of glucosides just taken up into the cytoplasm or stockpiled therein as a reserve carbohydrate (glycogen) and for other functions (α, α-trehalose). Phosphorolysis releases one activated glucosyl molecule as glucose 1-phosphate per cleaved glucosidic bond and is, clearly, more energy-conserving than hydrolysis of the same bond. The most widely distributed saccharide phosphorylase in nature is α-glucan (glycogen, starch, maltodextrin) phosphorylase, which appears to be present in almost all organisms and cell types. By contrast, disaccharide phosphorylases are found in relatively few microbial taxae and obviously have been selected for special physiological needs and non-conventional metabolic pathways.

The first part of this paper will provide a classification of the group of saccharide phosphorylases based on sequence, substrate specificity, and reaction mechanism. In the second part, kinetic and mechanistic components of enzymic glucosyl transfer catalysed by trehalose phosphorylase from the fungus *Schizophyllum commune* will be described based on evidence obtained with substrate analogues and inhibitors[1–3]. The stereochemistry of the reaction of this trehalose phosphorylase, which converts α, α-trehalose and phosphate, likely the mono-anion, into α-D-glucose 1-phosphate and α-D-glucose, is one of retention of configuration at the anomeric centre. The implications of the observed stereochemical control on catalytic mechanism are discussed. Finally, we will present principles of phosphorylase-catalysed synthesis of glucosides and point out potential and current limitations, using *Cellulomonas uda* cellobiose phosphorylase[4] as an example.

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L019 ASYMMETRIC SYNTHESIS OF LONG-CHAIN POLYKETIDES BASED ON ENZYMATIC DESYMMETRIZATION OR RESOLUTION

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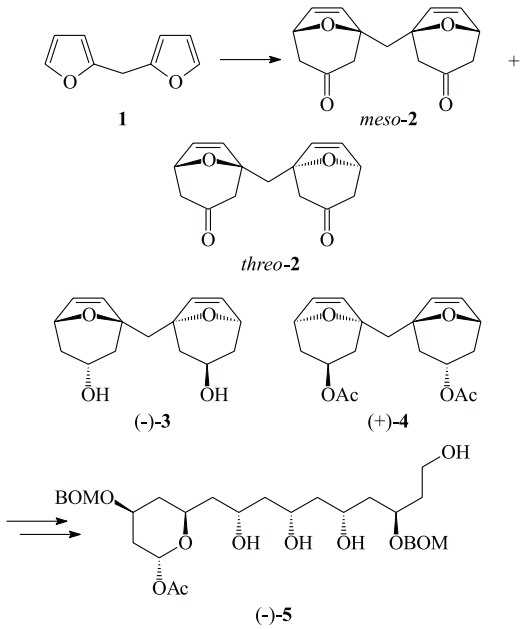
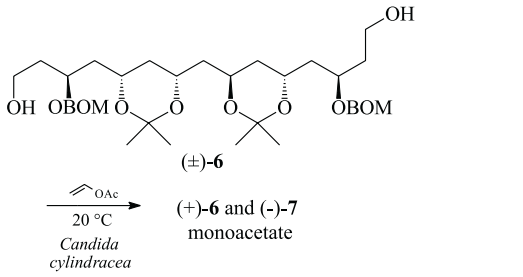
Keywords: double chain elongation, lipase-catalyzed acetylation, 1,3-polyols

A great variety of natural products of biological interest include polyketides (1,3-polyoxo, 1,3-polyols, aldols). We have proposed a new, non-iterative asymmetric synthesis of long-chain 1,3-polyols starting from the readily available 2,2'-methylenedifuran (**1**) (ref.[1]). The method relies upon the double [4+3]-cycloaddition of 2-oxyallyl cation intermediates[2] giving, after reductive work-up, 45:55 mixture of *meso*-**2** and (±)-*threo*-**2** that are readily separated. Diol (±)-**3** has been resolved kinetically with *Candida cylindracea* lipase-catalyzed transeserification giving (-)-3 (30 % yield, 98 % ee) and (+)-**4** (30 % yield, 98 % ee)[3]. These compounds can be converted in a few steps into several stereomeric polyketides, e.g. into (-)-**5**.



In a few steps *meso*-**2** is converted into racemic (±)-**6**, which has been resolved kinetically by lipase-catalyzed acetylation.



This work was supported by the Swiss National Science Foundation, OFES (Bern), Flores Valles-UCM (Madrid), COSTD25/001/02.

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L020 CHEMO-ENZYMATIC APPROACH TO STATIN-SIDE CHAIN BUILDING BLOCKS

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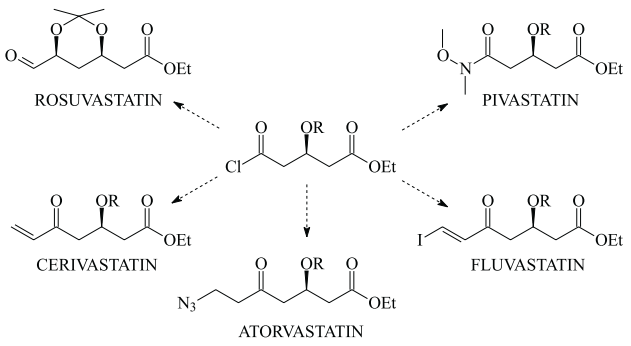
Keywords: statin-side chain, enzymatic desymmetrization

The statin family is a class of pharmaceuticals, derived from the natural mevilonolacton, known as strong HMG-CoA-R-inhibitors. Although the different congeners are marketed by a number of companies there is still a need for an efficient synthesis of highly optically pure precursors[1, 4].



We designed our approach to get ready access to a common precursor, which can be used for the preparation of a series of side chain building blocks (see scheme). For us, a resolution for the synthesis of bulk material did not seem an economic starting point. So we resided to an enzymatic desymmetrization of the symmetrical glutaric acid esters[2]. This way an almost quantitative yield of optically pure "acid/ester" is accessible in contrast to a 50%-yield obtainable *via* a resolution of various starting compounds. The resulting acid is transformed into the corresponding acid chloride, which serves as key intermediate to a variety of statin-side chain building blocks[3, 4].

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L021 PRODUCTION OF A FUNGAL α-L-RHAMNOSIDASE LIBRARY AND ITS USE FOR NATURAL GLYCOSIDE MODIFICATION

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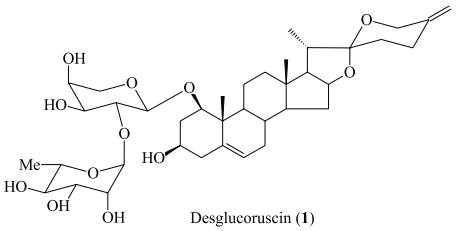
Keywords: α-L-rhamnosidase, enzyme library, natural glycosides

Screening of 16 different fungal strains was performed for the production of α-L-rhamnosidases under different cultivation conditions, using L-rhamnose or some flavonoid glycosides (rutin, hesperidin and naringin) as specific inductors. No significant constitutive production of α-L-rhamnosidases were detected in non-induced cultures, whereas high levels of glycosidases activities were obtained using different inductors with the various species and also within different strains of the same species. Series of new species, so far unknown for the α-L-rhamnosidase production, were identified.

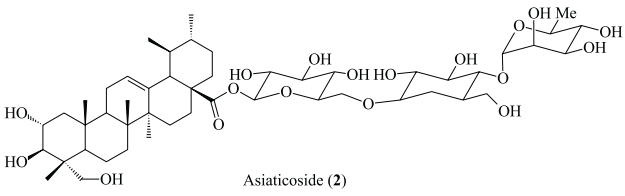
Over 30 different α-L-rhamnosidase preparations were recovered by ammonium sulfate precipitation. Substrate specificity of this α-L-rhamnosidase library was tested with vari-

ous L-rhamnose-containing natural compounds (flavonoids, terpenoids and saponins). Most of the enzymatic preparations showed broad substrate specificity, and some of them were also acting on sterically hindered substrates (e.g., quercitrin). Screening the library under different reaction conditions showed the coexistence in the same preparation of more than one α-L-rhamnosidase activity, possessing different substrate specificity and different stability towards organic solvents.

In order to exploit this enzymatic library for the modification of substrates carrying α-L-rhamnose moieties linked either to α-L-arabinopyranosidic (e.g., desglucoruscin, **1**) or to β-D-glucopyranosidic (e.g., asiaticoside, **2**) residues, the presence of contaminating glycosidase activities, particularly of α-L-arabinosidases and β-D-glucosidases was investigated. The latter enzymes were observed in several preparations, whereas the content of α-L-arabinosidases was generally quite low.



Desglucoruscin (**1**)



Asiaticoside (**2**)

The selective derhamnosylation of desglucoruscin was investigated. The enzyme obtained by rhamnose induction of the *Aspergillus niger* K2 CCIM strain showed high activity towards this substrate and negligible α-L-arabinosidase activity, so it was chosen for the selective derhamnosylation reaction, which provided the desired product in 70 % yield.

Support by the joint projects between CNR and AV CR, COST D25/0001/01, grants 203/01/1018 from GACR, and OC D25.002 from MSMT is highly acknowledged.

L022 OLIGOSACCHARIDE AND POLYSACCHARIDE SYNTHESIS AND MODIFICATION USING ENZYME TECHNOLOGY

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Keywords: oligosaccharides, polysaccharide hydrolysis, reversed hydrolysis

A large body of information indicates that polysaccharides from a wide variety of sources have beneficial effects on the human immune system. Although some polysaccharides such as schizophyllan and lentinan have been studied in some detail as antitumour agents, there is a general lack of knowledge of the types of structure that are most effective in interacting with the immune system.

Certain oligosaccharides enhance or elicit the synthesis of defence in plants and fungi[1]. Recently it has been shown that some oligosaccharides can influence the intrinsic human immune system[2], providing hints at structure-function relationships. The size (degree of polymerisation) and shape of oligosaccharides seem important for immunostimulatory activity. The most effective materials tested so far have approximately helical structures and D. P. of 7. Further investigation of structure-function relationships is stultified by the unavailability of sufficient quantities of other oligosaccharides.

Enzyme technology provides possible routes to the preparation of novel oligosaccharides and to the modification of polysaccharide structures. Partial hydrolysis of polysaccharides by hydrolases or lyases is feasible, either to produce oligosaccharides or to modify side chains. Synthesis of oligosaccharides by the “reversed hydrolysis” of mixtures of sugars by endo-enzymes such as bacterial α-amylases is possible. Isolation of individual oligosaccharides from complex mixtures may be an even greater challenge than synthesising them in the first place.

Availability of suitable enzymes and enzyme technologies for oligosaccharide production and polysaccharide modification will be discussed.

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L023 GLYCOMIMETICS AS SELECTIVE TOOLS FOR GLYCOSIDASES: PYRROLIDINE-POLYAMINES AS GLYCOSIDASE INHIBITORS AND CYANODEOXY GLYCOSIDES AS SUBSTRATES FOR GLYCOSIDASES AND NITRILE HYDRATING ENZYMES

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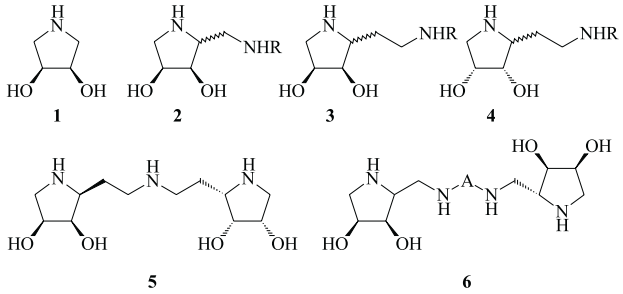
Keywords: pyrrolidines, pyrrolidine-polyamines, enzymatic inhibition, sugar mimetics, nitrilases, cyanodeoxy glycosides

Derivatives of 1,4-dideoxy-1,4-iminoalditols (hydroxylated pyrrolidines) constitute an important class as glycosidase inhibitors[1], however, in many instances they lack selectivity presenting a wide range of enzymatic inhibition. The selectivity in enzyme inhibition can be improved by providing the iminosugar with some information of the aglycon that is liberated during the enzymatic hydrolysis, in addition to the information about the structure of the glycosyl moiety that is cleaved and that mimick the oxocarbenium ion.

Looking for an easy and efficient approach of increasing activity and selectivity in enzyme inhibition, we envisionned the attachment of aryl(alkyl)amino groups to the pyrrolidine moiety: additional amino groups could be able of increasing electrostatic interactions with the carboxylate groups of the enzyme, and lipophilic moieties could be capable of establishing stabilizing hydrophobic interactions with the active site of the enzyme.



In this communication we present the synthesis and enzymatic inhibition of several pyrrolidine-diamines of type **2**, **3** and **4,** two pyrrolidine-triamines **5** and *ent*-**5** and several pyrrolidine-tetramines **6,** and demonstrate their better enzymatic inhibitory properties compared to the simple *meso*-3,4-dihydroxypyrrolidine (**1**).

On the other hand, as a part of a program related with the use of nitrile-hydrolyzing enzymes as tools in organic synthesis, we are presenting a convenient route for the synthesis of *p*-nitrophenyl 2-acetamido-2-deoxy-cyanoglycosides **12** and **13**. The strategy is based on the use of butanediacetal (BDA) protecting groups[2].

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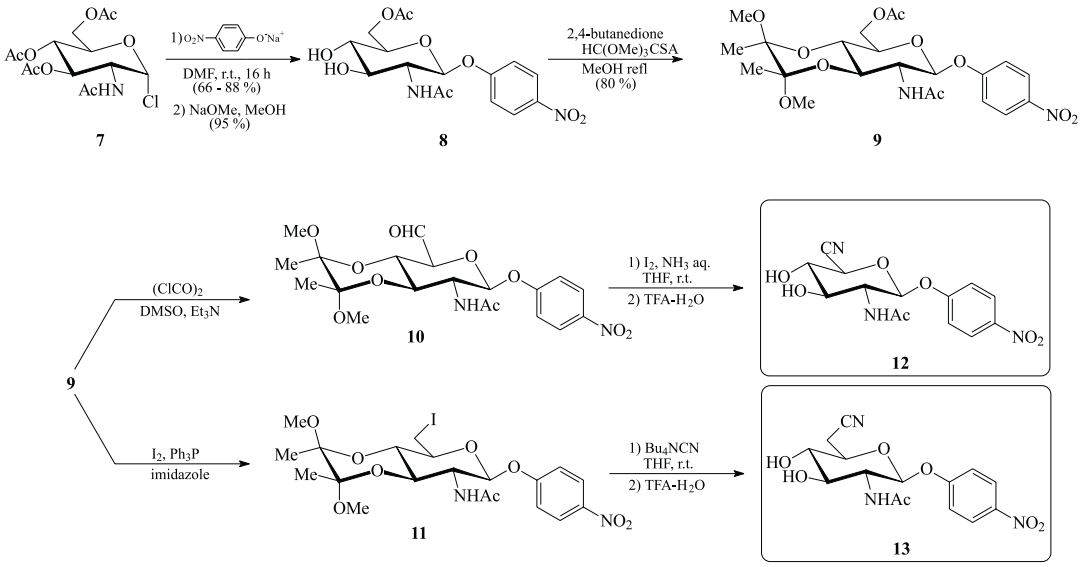

L024 MONITORING BIOTRANSFORMATIONS IN ACRYLIC FIBRES

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KEYWORDS: nitrilase, dye uptake, enzyme stability, sorbitol

Most methods to modify the polymer surface require strong chemical agents. Nitrilase from *Rhodococcus rhodochous* (EC 3.5.5.3) was used to change surface properties of acrylic fibres (PAN) and improve dyeability. The nitrile groups in PAN can be transformed into the corresponding carboxylic acid yielding ammonia by enzyme action. The stability of industrial enzyme under various conditions is an important characteristic. Therefore, it is necessary to develop techniques for enhancing the stability of enzymes. Several polyols were studied, being sorbitol selected because its addition improved the enzyme stability and activity. Organic solvents were also used, to help the enzyme penetration into the polymer structure. Due to enzymatic modification, the acrylic fibers became more hydrophilic and dye uptake was enhanced at temperatures below glass transition (60–80 °C). At 80 °C, an increase on dye uptake of 200 % was achieved when the enzymatic treatment, at the best conditions, was carried out.

L025 BIOTRANSFORMATION OF AZO-COMPOUNDS WITH NEW ALKALITHERMOSTABLE OXIDOREDUCTASES

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Keywords: azo-reductase, peroxidase, laccase, alkalithermostable

Many industrial processes involving transformation of azo-compounds are carried out at high pH-values and high temperatures thus limiting the applications of existing oxidoreductases. In this paper, we describe new alkalithermostable oxidoreductases and new applications based on investigation of their reaction mechanisms. We isolated various *Bacillus* strains growing at high pH values (10.5) and high temperatures (65 °C). While intracellular oxidoreductases of these organisms show high stabilities under these conditions, enzymes such as laccases contained in the spores are even more stable. Here, we describe the 62 kDa intracellular azo-reducatase and the dimeric 165 kDa peroxidase purified from a new *Bacillus* strain and the 33 kDa spore laccase from this organisms. At pH 8.5 all three enzymes show half life times longer than a day. MALDI-TOF peptide mass mapping revealed some similarity to proteins from other alkalithermostable organisms. The NADH dependent azo-reductase reduced the azo-bonds of many structurally different azo-compounds yielding the corresponding amines as shown by LC-MS analysis. To study the substrate specificity of this enzyme we have synthesized 22 model azo-compounds only differing in the type and position of substituent on the aromatic ring. Electron withdrawing substituents in *ortho* and *para* position of the aromatic ring enhanced the reaction with highest values obtained with $-NO_3$ substituents. Azo-reductase and oxidase activities are retained when whole cells are used for biotransformation which might have advantages in case of azo-reductase in terms of co-factor regeneration. Comparing the intracellular peroxidase with the spore laccase on various phenolic azo-compounds the latter enzyme was able to oxidize a wider range of substrates. We further demonstrate, that even with one and the same substrate, by varying the reaction conditions and redox-mediator used, either degradation or dimerisation and polymersation can be achieved. In contrast to the azo-reductase, electron donating methyl and methoxy substituents seemed to enhance laccase activity while electron withdrawing chloro, fluoro and nitro substituents decreased oxidation rate. Based on the presented knowledge on reaction mechanisms potential applications of the enzymes such as oxidative coupling of phenolic compounds or covalent attachment of azo-compounds on fibres are discussed.

L026 AMIDASE/NITRILE HYDRATASE OPERON FROM *Agrobacterium tumefaciens*

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Keywords: nitrile hydratase, amidase, *Agrobacterium tumefaciens*, chromosome walking

Nitriles (R-CN) can be degraded by the sequential action of nitrile hydratase (EC 4.2.1.84) ($R\text{-}CN + H_2O \rightarrow R\text{-}CONH_2$) and amidase (EC 3.5.1.4) ($R\text{-}CO\,NH_2 + 2H_2O \rightarrow R\text{-}COOH + NH_3$).

Agrobacterium tumefaciens is a Gram negative bacterium known to produce the Crown Gall disease in dycotiledonous plants. Amidase (Amd) and nitrile hydratase (NHase) activity has been previously described in this genus.

Conserved regions were identified from multiple aligned α subunit protein sequences and PCR oligonucleotide primers were designed using a consensus-degenerate hybrid strategy. A DNA fragment of about 230 bp was successfully amplified and sequencing confirmed its relation with the other known nitrile hydratase sequences. Several chromosome walking steps were performed, starting from the known sequence. The obtained fragments were cloned and sequenced, allowing a 3300 bp assembly that includes the complete sequences of amidase, nitrile hydratase α and β subunits, and a downstream sequence with high similarity to P14K (nitrile hydratase associated protein) from *Pseudomonas putida*. Unlike what is found in most organisms known to possess the Amd/NHase operon, amidase in *A. tumefaciens* is not located contiguously upstream the nitrile hydratase α subunit. A circa 1200 bp sequence which includes a segment with high similarity with unknown hypothetical proteins in databases, can be found between amidase and nitrile hydratase.

L027 ENANTIOSELECTIVE HYDROLYSIS OF MANDELONITRILE

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Keywords: nitrilase, nitrile hydratase, enantioselectivity, nitrile hydrolysis

Nitriles are useful starting compounds for the enzymatic synthesis of amides and carboxylic acids[1]. The enzymatic hydrolysis of nitriles follows two possible pathways. Nitrilases (EC 3.5.5.1) catalyze the conversion of nitriles to the corresponding acids directly, whereas nitrile hydratases (EC 4.2.1.84) catalyze the hydration of nitriles to their amides, which subsequently might be converted to the carboxylic acids by corresponding amidases (EC 3.5.1.4). Enantiopure mandelamide and mandelic acid are industrially important chiral synthons, which can be produced by racemic resolution of using the above mentioned enzymes.

Due to the reverse chemical reaction of benzaldehyde with cyanide in phosphate buffer to form *rac*-mandelonitrile[2] a theoretically 100 % yield of enantiopure mandelamide or mandelic acid is possible by racemic resolution. Results using more than 30 new isolated microorganisms are presented which were able to convert *rac*-mandelonitrile enantioselectively to either (*S*)-mandelamide (*ee* > 90 %) or (*R*)-mandelic acid (*ee* > 99 %), respectively.

The most suitable bacterial strain with (*S*)-selective nitrile hydtratase activity was further investigated for nitrile hydratase production in a bioreator cultivation (5-l-scale; yield 53,400 nkat.l^{-1}). Various biotransformation experiments with *rac*-mandelonitrile as the substrate using either partially purified nitrile hydratase or whole cells in 40-ml-scale are presented and discussed.

This project is sponsored by the Federal Ministry of Education and Research (AZ: -31P2762) and Solvent Innovation GmbH, Cologne.

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L028 CRYSTAL STRUCTURE AND MUTATIONAL STUDY OF ENANTIOSELECTIVE PROTONATION CATALYTIC ANTIBODY 14D9

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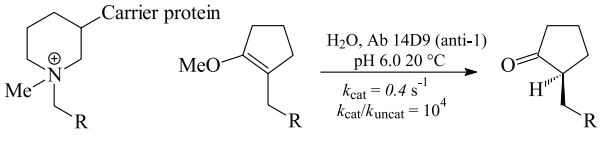
Keywords: antibody 14D9, expression, X-ray crystallography, active site, enantioselectivity

Catalytic antibody 14D9 is a monoclonal antibody obtained from immunization against hapten **1**, and it catalyzes the enantioselective protonation of enol ethers (*e.g.* **2**) to produce optically pure carbonyl compounds (*e.g.* **3**)[1–5]. Mechanistic studies of the reaction showed that an acidic residue with pK_a = 4.5, presumably a carboxylate, is largely responsible for catalysis. A number of related acid catalyzed reactions, such as the hydrolysis of acetals and epoxides, are also catalyzed by this antibody.



1 **2** (*S*)-**3**

The gene fragments of antibody 14D9 and its close relative 19C9 were cloned using phage display methods and expressed as chimeric Fab in *E. coli*. The structure of the 14D9 apo form and the 19C9 complex with hapten **1** were solved by X-ray crystallography. These two antibodies show very similar active sites, with the critical catalytic residue being

AspH101, as confirmed by site-directed mutagenesis. Transition state docking experiments indicate that the catalytic machinery consists in a unique triangle hydrogen-bond network involving a water molecule. Chain shuffling experiments show that catalytic activity is controlled remotely by a hydrogen-bonding residue on the light chain of the antibody interacting directly with the catalytic residue AspH101. The active site geometry explains the origin of the high enantioselectivity observed with antibody 14D9.

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L029 ENCAPSULATION OF *Hevea brasiliensis* HYDROXYNITRILE LYSASE IN SOL-GELS

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Keywords: hydroxynitrile lyase, oxynitrilase, sol-gel, cyanohydrin, enantioselective catalysis

Cyanohydrins are versatile building blocks for pharmaceutical and agricultural chemistry. Many different approaches for their enantioselective synthesis, such as transition metal catalysis, diketopiperazine catalysis and lipase catalysed kinetic resolutions, have been described[1]. The utilization of hydroxynitrile lyases (HNL), the enzymes that in nature catalyse the degradation of cyanohydrins, has been particularly successful (Scheme 1)[1]. Indeed, the HNL from *Hevea brasiliensis* (*Hb*HNL), which catalyses the formation of *S*-cyanohydrins, is even applied on an industrial scale[2].

Scheme 1. **In a one-pot procedure *Hb*HNL catalyses the destruction of acetone cyanhydrin and the *S*-selective formation of a chiral cyanohydrin**

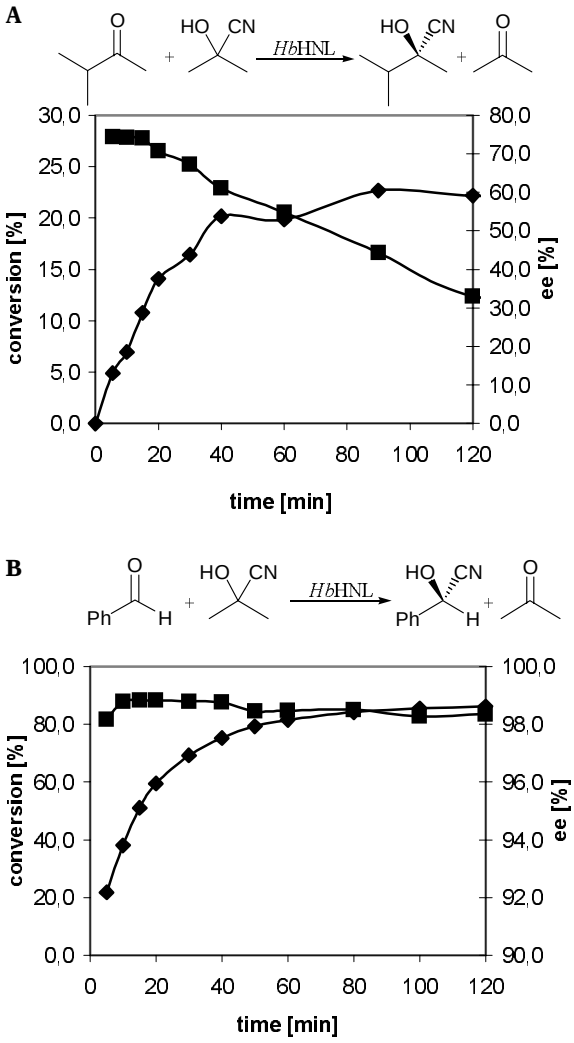

Fig. 1. **Reactions catalysed by sol-gel encapsulated *Hb*HNL at 25 °C in diisopropyl ether; A:** isopropylmethyl ketone 1:3 acetone cyanohydrin; **B:** benzaldehyde 1:3 acetone cyanohydrin

In order to improve the recycling of enzymes and to facilitate their use in organic solvents, it can often be favourable to immobilise them. When *Hb*HNL is immobilized via adsorption on celite, it retains high activities, however, it cannot be used in pure organic solvents but only in mixtures with water[3]. Immobilization as a CLEA lead to significant decrease in activity[4]. We therefore utilized the sol-gel approach. Unlike the methods described earlier the sol-gels encapsulate the enzyme without modifying its structure. When *Hb*HNL was encapsulated in a sol-gel it retained 65 % of its activity with unchanged enantioselectivity (Fig. 1 – A, B).

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L030 SILICA AEROGELS AS ENCAPSULATION MEDIA OF LIPASES

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Keywords: immobilization, lipase, aerogel, silica

Silica aerogels are derived from wet silica gels by supercritical drying. This is a technique which consists in bringing the material beyond the critical point of the liquid contained in the pores. This liquid is next transformed to a supercritical fluid which can be evacuated as a gas. Because no meniscus is present at the exit of the gel pores, no capillary contraction occurs in the best case, so that the dry material which is obtained is very porous. Aerogels with pores occupying up to 98% of a sample volume have been reported. This is much higher than in xerogels which are obtained from the same wet gels, except that drying is done by evaporation. This is also better than the cryogels, which are again obtained from the same wet silica gels by freezing and sublimation of the solidified solvent under vacuum (i.e. lyophilisation). Supercritical drying alleviates the occurrence of compressive mechanical stresses on the gel network as well as on embedded enzymes, cells or bacteria. It also makes it possible to avoid the growth of ice crystals during freeze drying, which induces mechanical stresses on the silica network and the embedded material. Besides, supercritical drying can be done with a gel previously filled with liquid CO_2, an inert compound which has a low temperature critical point ($T_c \approx 31.1$ °C, $P_c \approx 7.38$ MPa), in conditions friendly to enzymes.

For aerogel as well as for xerogel immobilization, the enzyme to be encapsulated needs not be adsorbed or attached or made to diffuse inside a preexisting solid support. Instead, the entrapping network is built about the enzyme, itself dispersed in at least a partly aqueous media. The embedding network is formed as a result of hydrolysis and condensation reactions from a silicon precursor which can be an alkoxide such as tetramethylorthosilicate $Si(OCH_3)_4$ or sodium silicate Na_2SiO_3.

Sol-gel encapsulation requires to select chemical conditions which are not harmful to the enzymes, in terms of solvent nature, pH or sol-gel hydrolysis products such as a an alcohol. In the latter case, the alcohol can be largely eliminated by evaporation prior to enzyme addition and gelation, by performing a separate pre-hydrolysis step. The solvent exchange procedure for liquid CO_2 is another critical step, which must be adapted to the enzyme.

Nevertheless, when successful, the aerogel medium can magnify the activity of the enzyme, in particular for performing catalysis in organic solvents. First, the aerogel pore structure is less prone to limitation by diffusion of the substrates and products than xerogels. Secondly, aggregation of the enzyme is made impossible by the gel network. At last, the range of available silicon precursors of the type $Si(OR)_nX_{n-4}$ with a different functionality X, is large. This makes it possible to tailor the affinity of the medium which surrounds the enzyme, for instance the hydrophilic to hydrophobic balance, so that the aerogel modify the kinetics by displacing the substrate concentrations about the enzyme

The communication will present the research in this direction with the lipase from *Burkholderia cepacia* and its application to esterification and transesterification reactions.

L031 MULTISTEP CATALYSIS BY WHOLE DEHYDRATED CELLS IN A SOLID-GAS BIOREACTOR: INFLUENCE OF OPERATING PARAMETERS ON DEPOLLUTION EFFICIENCY

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Keywords: gas phase, multi-step reaction, whole cells, depollution

Volatile organic compounds (VOCs) are widely produced and used both in industrial and domestic activities. Among the VOCs, halogenated organic compounds constitute one of the largest groups of environmental pollutants and many of them have been designated as priority pollutants by the United State Environment Protection Agency (EPA) because of their recalcitrance, toxicity, carcinogenicity and potential teratogenicity[1]. Consequently there has been a great number of papers dealing with depollution processes, biological ones becoming more and more popular since they offer a cost effective and environmentally friendly solution[2]. Nevertheless most of papers dealing with biological waste air treatment have concern biofilters (solid/liquid/gas bioreactor) and there have been less reports on waste air treatment by direct treatment in a solid/gas bioreactor. Yet this kind of process could be of interest for transformation of volatile organic compound. Indeed solid/gas catalysis does not need solubilisation of compounds since substrates are directly treated in the gas phase. This could be of great interest in treatment of volatile organic compounds (VOCs) that often have low solubility in water and relatively high volatility. Solid/gas technology has been mainly applied to isolated enzymes for synthesis[3,4] but it has been recently shown that whole cells can be used for bioconversion[5,6]. It also appeared us interesting to investigate if

whole-dehydrated cells could be used as catalyst for the depollution of gaseous effluents. We particularly focused on the opportunity to catalyze multistep reactions.

We choose the biodegradation of 1-chlorobutane by lyophilized cells of *Rhodococcus erythropolis* NCIMB 13064 as a model reaction. We first studied the ability of cells to metabolize this compound in the aqueous phase and determine the degradation products and the enzymes involved. We then compared this behavior with those of dehydrated cells in the gas phase. We first studied the dehalogenation step often limiting in depollution processes.

We observed that dehydrated cells of *Rhodococcus erythropolis* can catalyze the conversion of 1-chlorobutane in 1-butanol directly in the gas phase at interesting rates compared to pure enzyme in the gas phase[7] or to resting cells in the aqueous phase. Effect of operating parameters such as water activity, temperature and residence time in the bioreactor, on activity and stability of the catalyst has been studied. Dehalogenase activity and stability of cells were found to depend on the amount of HCl produced. We then studied the transformation of 1-butanol in various products (aldehyde, acid) by the dehydrated cells in gas and aqueous phase.

To finish, we extended our study to other products chlorinated or brominated compounds and observed that dehydrated cells of *Rhodococcus erythropolis* are able to convert a range of halogenated compounds in the gas phase. This ability is discussed according to the carbon chain length and position of the halogenated atom.

This work shows that dehydrated cells can be used directly in the gas phase to catalyze multistep reactions and that they can be used for depollution.

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L032 ENZYMATIC STEREOSELECTIVE ALDOL CONDENSATIONS IN HIGHLY CONCENTRATED EMULSIONS (GEL EMULSIONS)

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Keywords: w/o gel emulsions, DHAP-aldolases, *N*-protected amino aldehydes, iminocyclitols

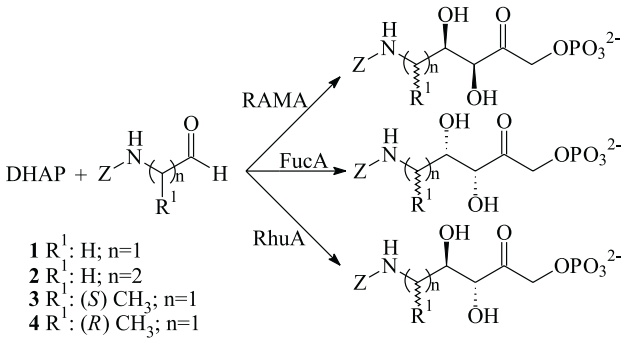
Highly concentrated emulsions (gel emulsions) are liquid-liquid dispersions with an internal phase volume fraction larger than 0.74, the critical value of the most compact arrangement of spheres of equal radius. Hence, their structure consists of closed-packed droplets, deformed and/or polydisperse, with radii typically of a few microns, separated by a thin film of continuous phase, a type of structure that resembles gas-liquid foams[1]. The advantages of W/O gel emulsions as reaction media are: a) they have a large interfacial area; b) they can solubilize large quantities of both hydrophilic and hydrophobic compounds; c) they can be formulated with a large amount of water (*i. e.* as much as 99 % w/w) and very low surfactant concentration (< 0.5 % w/w). These features make them especially attractive as reaction media when substrates of opposite solubility properties have to be put in contact for reaction. This is, in many instances, the situation in aldolase-catalyzed carbon-carbon bond formation and, particularly, with dihydroxyacetone phosphate (DHAP)-dependent aldolases. Whilst the donor DHAP is fully soluble in aqueous media and insoluble in organic solvents including the polar ethanol or methanol, the solubility of the acceptor aldehyde is generally reverse. In this communication, W/O gel emulsions were investigated as reaction media for aldolase-catalyzed reactions of DHAP with model hydrophobic aldehyde acceptors and thereafter applied to the stereodivergent synthesis of iminocyclitols from *N*-protected amino aldehydes.

W/O gel emulsions of the ternary water/poly(oxyethylene) tetradecyl ether surfactant ($C_{14}E_4$)/aliphatic hydrocarbon systems with 90 wt% water, stable at the reaction temperature, 25 °C, were chosen as reaction media[2,3]. In these systems, the enzymatic activity and product yield were studied as a function of the reaction temperature and the aliphatic hydrocarbon (i. e., the oil) chain length. The aldolic condensations of DHAP and either phenylacetaldehyde or benzyloxyacetaldehyde catalyzed by fructose-1,6-diphosphate aldolase from rabbit muscle (RAMA) were used as model reaction. It was observed that the stability of RAMA in W/O gel emulsions was improved by 7 and 25-fold compared to

that in aqueous medium or conventional dimethylformamide/water 1/4 v/v mixture, respectively. It was found that the equilibrium yields and enzymatic activity depended on both the aldehyde partitioning between the continuous and dispersed phases and the water-oil interfacial tension ($\gamma_{w\text{-}o}$). The highest enzymatic activities were achieved in W/O gel emulsion systems with the lowest water-oil interfacial tension. The equilibrium yield depended on the $\gamma_{w\text{-}o}$ for the hydrophobic phenylacetaldehyde, and on the partition coefficient for the more hydrophilic benzyloxyacetaldehyde. Optimum equilibrium product yields (65–70 %) were achieved at either the lowest water-oil interfacial tension or partition coefficient values[4].

Under the best reaction conditions, the stereodivergent synthesis of selected iminosugars was accomplished by enzymatic aldol condensation of DHAP with hydrophobic and poor water-soluble *N*-benzyloxycarbonyl amino aldehydes **1**, **2**, **3** and **4** (Figure).

RAMA, type II recombinant L-rhamnulose-1-phosphate aldolase (RhuA) and fuculose-1-phosphate aldolase (FucA) both from *E. coli* were used as catalysts (Figure). The reaction yields in gel emulsions and in a conventional DMF/water 1:4 mixture as well as the diastereomeric excesses obtained for each aldolase and acceptor aldehyde will be discussed.



1 R^1: H; n=1
2 R^1: H; n=2
3 R^1: (*S*) CH_3; n=1
4 R^1: (*R*) CH_3; n=1

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L033 NEW COMPUTATIONAL ANALYSIS OF *Burkholderia cepacia* LIPASE ENANTIOSELECTIVITY: MODELLING OF THE ENANTIOMER TRAJECTORY INTO THE ACTIVE SITE

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Keywords: enantioselectivity, lipase, molecular modelling, enantiomer trajectory

To reduce the cost of development of enantiopure molecules such as pharmaceuticals, many efforts are focused on the understanding of the molecular features that control enzyme enantioselectivity[1–7]. The aim is to develop reliable predictive methods and to guide substrate and/or enzyme modifications to enhance kinetic resolution. Lipases are serine hydrolases (EC 3.1.1.3) frequently used in organic synthesis to catalyse asymmetric hydrolysis, esterification or transesterification of a wide range of substrates yielding optically pure compounds.

Our study focused on lipase-catalysed resolution of (*R,S*)-2-bromophenyl acetic acid ethyl ester, used for the synthesis of enantiomerically pure pharmaceutical molecules. Thus, by molecular modelling, we attempted to identify the molecular processes responsible for *Burkholderia cepacia* lipase selectivity for (*R*)-2-bromophenyl acetic acid ethyl ester ($E = 57$). The approach used, had never been described in the literature. For the first time, the trajectory of each enantiomer to the active site was mapped and the energy of enzyme/substrate interactions was calculated along the path. On the basis of interaction energy, we showed that the enzyme active site is less accessible to the *S*-enantiomer than to the *R*-one. A hydrophobic network of amino acids with pivoting side chains (Val, Leu), covering the sides of the active site, seems to play a role in driving the substrate to the active site. In particular, two amino acids Val266 and Leu17 form a bottleneck. We suggest that this structural fracture influences the discrimination of *R,S*-enantiomers. The determination of the enantioselectivity of the mutant V266L with a side chain more bulky at this position, supported this assumption. In fact, for this mutant, the size of the bottleneck is reduced, and the enantioselectivity was found to be higher than 200.

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L034 DEVELOPMENTS OF RESOLUTION REACTIONS CATALYSED BY LIPASES IN NON-CONVENTIONAL MEDIA: PRODUCTION OF CHIRAL CARBOXYLIC ACIDS POSSESSING BIOLOGICAL ACTIVITY

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Keywords: 2-substituted-propanoic acids, lipases, resolution, solvent engineering, ionic liquids

Lipases are widely studied enzymes because they are commercially available, easy to handle, don't require cofactors, and have good stability and catalytic activity in non-conventional media. They accept a broad range of substrates, while retaining high enantioselecitivity.

This work provides a review of the evolutionary developments of lipase catalysed resolution reactions of 2-substituted-alkanoic acids as examples of chiral carboxylic acids in non-conventional media including results from our labratory obtained in different organic solvents in the enantioselective esterification of 2-substituted propanoic acid derivatives. The most studied substrates among these compounds are the 2-aryl-substituted-propanoic acid derivatives (called as profens), as Naproxen [2-(6-methoxy)propanoic acid] and Ibuprofen [2-(4-isobuthylphenyl)propanoic acid] – nonsteroidal anti-inflammatory drugs[1]. However among the other 2-substituted-propanoic acids, intermediers of pesticide production (2-halo-substituted-propanoic acid derivatives), and bioflavors (2-alkyl-substituted-propanoic acid derivatives) can be found[2].

The esterification, transesterification and thiotransesterification reactions of these compounds in organic solvents are all well established. Lipase screening showed, that *Candida rugosa* lipase is one of the most selective enzyme toward 2-substituted propanoic acids, but some other lipase are also proved to be good catalyst in these reactions. Enzyme pretreatments and purification were shown to enhance the selectivity of enzymes.

There have been much effort in order to study the role of different substituents of the substrate molecules (electron donor, electron acceptor), the solvent properties (hydrophobicity, polarity, chemical structure), the alcohol chain lenght, water activity and the reaction conditions, as temperature, and the pressure in the case of supercritical medium.

In most of the cases, higher enantioselectivity (but lower yield) can be attained in hydrophilic solvents, and at lower water activities with the same enzyme catalyst[3]. The reason of the water activity dependence of enantioselectivity is that the reaction rate of the two substrate enantiomers varies differently for the alteration of water activity[4]. Investigation with chiral solvents showed, that a certain interaction can be formed between solvent and enzyme molecules resulting in an altered lipase activity, however the enantioselectivity obtained in the *(R)*-, and *(S)*-solvent isomer was similar[5]. Promising results has been obtained in ionic liquids in the resolution reactions of 2-chloro-propanoic-acids considering the enzyme enantioselectivity, although these reactions are still under investigations[6]. Further improvements in substrate recognition (enantioselectivity) of enzymes can be achieved by selecting substrates, (substituents) since the greater the substituents size, the higher the enantioselectivity is observed.

From the experiments, it can be concluded, that solvent engineering is a powerful tool for enhancing enzyme activity and selectivity in these reactions systems, and using together with lipase engineering, the above biologically active molecules can be enantioselectively produced.

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L035 LEARNING FROM DIRECTED EVOLUTION: INSIGHTS IN LIPASE ENANTIOSELECTIVITY

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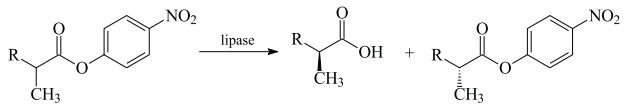
Keywords**:** directed evolution, lipase, mutants, enantioselectivity, structure, simulation

Bacterial lipases are widely used biocatalysts for the kinetic resolution of racemic chiral esters via enantioselective deacylation[1]. Recently we have demonstrated that directed evolution can be used to create mutant enzymes with improved enantioselectivity[2]. Specifically, the kinetic resolution of the ester **1** catalyzed by the lipase from *Pseudomonas aeruginosa* was studied.



1 **2** **3**

These results indicate that with directed evolution the enantioselectivity of an enzyme can be improved and even reversed. The overall catalytic mechanism of lipases is analogous to that of serine proteases, but the factors governing enantioselectivity have not yet been established. Based on the structure of *Pseudomonas aeruginosa* lipase[3], a model of the bound substrate allowed for locating the position of the substitutions and for the rationalization of their effects. The key mutations for reversal of enantioselectivity are located in the active site of the enzyme, whereas the additional mutations in the optimized *R*-selective mutants are found in the surrounding helices. To understand the influence of these mutations on the enantioselectivity we used different theoretical approaches ranging from QM calculations on small model systems to classical MD-simulations of the full enzyme. We identified several cooperative sets of amino acids linking remote mutations to enantioselective substrate recognition.

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L036 ENANTIODIVERGENT BAEYER-VILLIGER OXIDATIONS BY RECOMBINANT WHOLE CELLS

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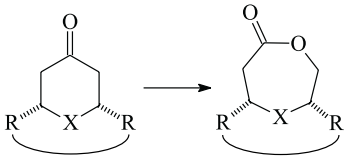
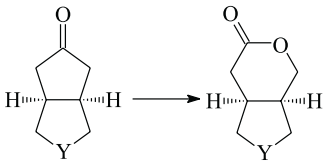
Keywords: Bayer-Villigerase, cyclohexanone monooxygenase, recombinant whole-cells

Flavin dependent monooxygenases represent highly versatile biocatalysts for the Baeyer-Villiger oxidation of cyclic ketones to lactones[1]. However, this class of enzymes is dependent on cofactors, which complicates their utilization for chemical transformations. One way to overcome the obstacle of cofactor recycling is to use whole-cells instead of isolated enzymes. Advances in molecular biology enabled the development of recombinant systems with significantly improved selectivity for the desired biotransformation and with higher efficiency compared to the native strains. Recently, we designed an *Escherichia coli* based overexpression system for the most extensively studied Baeyer-Villigerase to date, cyclohexanone monooxygenase (CHMO) from *Acinetobacter* sp. NCIMB 9871 (E.C. 1.14.13.22), confirming this concept of an easy-to-use biocatalyst[2].



1 X = O, NPG, CH_2, C-alkyl$_2$



2 Y = CH_2, CHOMe, CHCl

With an increasing number of genome sequences available, a substantial number of Baeyer-Villiger monooxygenases have been identified and cloned for applications in biotransformations. Hence, one of the key aspect in biocatalysis can be addressed to access both antipodal forms of a desired product lactone.

With this contribution, recent result by our group in identifying and characterizing enantiodivergent Baeyer-Villigerases – expressed in recombinant whole-cells – with overlapping substrate profiles will be presented. Biooxidations of precursors **1** and **2** will be discussed, with the obtained product lac-

tones representing valuable precursors for the synthesis of natural compounds[3].

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L037 BENEFITS OF WHOLE CELL OXIDATION OF AROMATICS IN MANUFACTURING OF FINE CHEMICALS AND PHARMACEUTICALS

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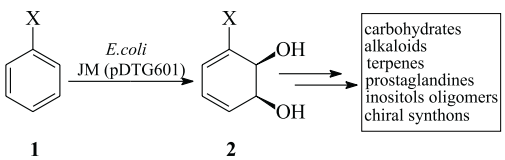
Keywords: cyclohexadiene diols, enantioselective synthesis, pancratistatin, morphine

In the late 1960's Gibson and coworker discovered that whole cell oxidation of aromatic compounds with mutant strains of *Pseudomonas putida* yields in the formation of *cis*-cyclohexadiene diols[1]. Genes responsible for this process were successfully cloned in recombinant *Escherichia coli* JM109 (pDTG601) and a large number of cyclohexadiene diols are available in good yield and excellent enantiopurity. Those cyclohexadiene diols proved to be useful intermediates in chemoenzymatic approaches to alkaloids, carbohydrates and a variety of chiral synthons[2].

The identity of new metabolites of toluene dioxygenase and the progress in the synthesis of morphine[3] and pancratistatin[4], an important member of the *Amaryllidaceae* alkaloids, will be disclosed (see Scheme). In all of the projects discussed the emphasis will be placed on the efficiency of combining traditional organic chemistry and biotechnology.



1 **2**

X = alkyl, aryl, halogen

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L038 REGIO- AND STEREOSELECTIVE BIOTRANSFORMATIONS WITH *Sphingomonas* SP. HXN-200

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Keywords: biohydroxylation, epoxide hydrolase, *trans*-dihydroxylation, P450 monooxygenase, *Sphingomonas* sp. HXN-200

We are interested in discovery and development of new biocatalyst for practical synthesis. We selected an area as particularly challenging to organic synthesis, namely regio- and stereoselective hydroxylation of non-activated carbon atom. This was tackled by isolating and identifying strains capable of such oxidation. Many alkane-degrading bacteria were found to catalyze the regio- and stereoselective hydroxylation of *N*-benzyl-pyrrolidine[1]. Among them, *Sphingomonas* sp. HXN-200 showed high activity and enantioselectivity and was chosen for further investigation. Cells of *Sphingomonas* sp. HXN-200 were easily prepared in large amounts and can be stored at – 80 °C for two years without significant loss of activity. The easy to handle frozen/thawed cells were successfully used for routine biohydroxylation[2]. Moreover, rehydrated lyophilized cell powder were demonstrated, for the first time, to catalyze the hydroxylation without addition of cofactor[3], i.e. that they are metabolically functional. High yield and high product concentration were also achieved with growing cells as biocatalyst[2].

Sphingomonas sp. HXN-200 catalyzed the hydroxylation of a broad range of substrates, such as *N*-substituted pyrrolidines, piperidines, azetidines, 2-pyrrolidinones, and 2-piperidinones, with high activity, high yield, excellent regioselectivity, and good to excellent enantioselectivity[2–5], representing by far the best enzyme for these reactions reported thus far. The hydroxylation products are useful phar-

maceutical intermediates and difficult to make by chemical syntheses.

Sphingomonas sp. HXN-200 was found to contain an NADH-dependent soluble P450 monooxygenase. The P450pyr and ferredoxin components were purified, and their *N*-terminal sequences and molecular weights were determined. The purified components are not active in isolation, but mixing of the components restores the hydroxylation activity. The gene of P450pyr was identified and sequenced, and a homology structure model was established. Docking substrates on to the structure model provided the first insight into the regio- and enantioselectivity. A recombinant strain encoding the genes of the monooxygenase components was engineered giving the desired hydroxylation activity.

Sphingomonas sp. HXN-200 was found to contain an enantioselective epoxide hydrolase. Hydrolysis of *N*-benzyloxycarbonyl-3,4-epoxy-pyrrolidine and cyclohexene oxide with *Sphingomonas* sp. HXN-200, respectively, gave the corresponding vicinal *trans*-diols in high ee and yield[6]. This represents the first example of enantioselective hydrolysis of a *meso*-epoxide with a bacterial epoxide hydrolase. High enantioselectivity was also observed in hydrolysis of several chiral epoxides.

Recently we found that *Sphingomonas* sp. HXN-200, as the first bacterial catalyst, catalyzed the enantioselective *trans*-dihydroxylation of non-activated C-C double bond of alicyclic compounds giving the corresponding *trans*-diol in high ee and high yield.

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L039 SAFE MODIFICATION OF ENZYME SUBSTRATE SPECIFICITY BY ENGINEERING OF SURFACE RESIDUES IN THE ENTRANCE TUNNEL

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Keywords: haloalkane dehalogenase, *Sphingomonas paucimobilis*, site-directed mutagenesis, substrate specificity

Hydrolysis of haloalkanes to their corresponding alcohols and inorganic halides is catalysed by α/β-hydrolases called haloalkane dehalogenases. Their activity and specificity is not optimal for industrial applications (e.g., bioremediation, biosensors and organic synthesis) and numerous projects have been initiated to engineer their catalytic properties. Haloalkane dehalogenase LinB from *Sphingomonas paucimobilis* UT26 is the enzyme involved in the degradation of the important enviromental polutant γ-hexachlorocyclohexane. This enzyme hydrolyses a broad range of halogenated aliphatic compounds[1].

The amino acid residue in position 177 of LinB is positioned in the mouth of the entrance tunnel leading the enzyme active site and is pointing directly to this tunnel[2]. It was predicted to be a key residue determining the substrate specificity. L177 of the wild type enzyme was replaced by every other amino acid and the effect of mutations on enzyme specificity was studied. Two protein variants (L177E and L177N) from nineteen prepared proteins could not be overexpressed in *E. coli*, while other two variants (L177P and L177I) did not show activity with any tested substrate. Successfully purified enzymes were kinetically characterised by determination of their specific activities with twelve different substrates and steady-state kinetic parameters with two substrates: 1-chlorobutane and 1,2-dibromoethane. Dehalogenation of 1-chlorobutane showed typical Michaelis-Menten dependence, while dehalogenation of 1,2-dibromoethane showed substrate inhibition.

Systematic exploration of the data was achieved by the Principal Component Analysis. The analysis resulted in two biologically interpretable principal components all together explaining 63 % of the data variance. The first statistically significant component explained 44 % of the data variance and ordered the protein variants approximately according to the size of amino acid residue introduced to the position 177, while the second principal component explained 19 % of data variance and ordered the protein variants according to the polarity of amino acid residue introduced to the position 177

(Fig. 1). Along diagonal were the proteins ordered according to their overall activity with 8 substrates. Position 177 of LinB was found to be highly tolerable for introduction of different amino acid residues. The study demonstrates that modification of catalytic properties of enzymes using site-directed mutagenesis of entrance tunnel residues represents a safe strategy for rational engineering of substrate specificity.

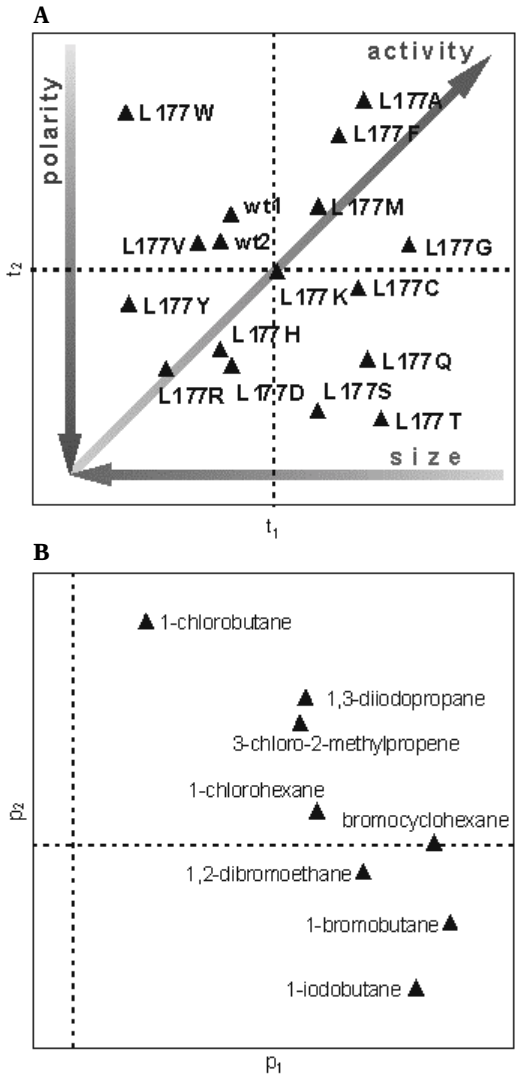

Fig. 1. **The score plot (A) and the loading plot (B) of first two components from the principal component analysis of specific activities measured for eight halogenated substrates;** the arrows in the score plot indicate that the protein variants are ordered approximately by the size of amino acid introduced to the position 177 along the first component, by the polarity along the second principal component and by the overall activity along diagonal.

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L040 FORMATE DEHYDROGENASE: FROM WILD-TYPE ENZYME TO ROBUST BIOCATALYST FOR NAD(P)H REGENERATION

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Keywords: formate dehydrogenase, coenzyme regeneration, protein engineering, expression

The contribution of drugs based on optically pure enantiomers shows a tendency to rise compared to those based on racemic and non-chiral chemicals. Among 500 best selling medications in 2000, the contribution of single enantiomers reached 58 % with the sale volume of 107.1 billion dollars[1]. The predicted growth of the chiral drugs market in the next 3 years is estimated as 130 to 172 billion dollars.

All dehydrogenases are characterized by the high specificity of hydride transfer from the coenzyme to a substrate and thus, can be successfully used for synthesis of chiral compounds. However, dehydrogenase applications based on the use of reduced cofactors, NADH or NADPH, is commercially unfair because of the high price of these reagents. The problem solution is thought to be in the introduction of an additional enzyme responsible for $NAD(P)^+$ regeneration *in situ*[2]. Various cofactor regeneration enzyme-substrate systems like alcohol dehydrogenase-propanol, glucose dehydrogenase-glucose, etc., were probed for the purposes of chiral synthesis over the past three decades. The summary of this tremendous work can be found in reviews[3, 4]. The comparison of various regeneration systems unequivocally demonstrated the superiority of NAD^+-dependent FDH from methylotrophic microorganisms. Only this enzyme meets all the criteria for the universal catalyst of NAD(P)H regeneration:

1. Wide pH-optimum for catalytic activity. FDH activity is unchanged within the range of pH 5.5–11.0, and Michaelis constants for NAD^+ and formate are constant in the range of pH 6.0–9.5 (ref.[5]).
2. Providing the maximum yield of a target product. The reaction catalyzed by FDH is irreversible, and provides the conversion degree of 98–100 % in all cases studied.
3. Low cost of a substrate for NADP(H) regeneration, the absence of substrate and product inhibition, simplicity of substrate and product removal while purifying the target product. Sodium and ammonium formate are cheap and do not inhibit dehydrogenases catalyzing the basic synthetic reaction. Carbon dioxide, the product of FDH-catalyzed reaction, has no inhibition effect on majority of

dehydrogenases and does not interfere with the target product purification.

The listed above factors position FDH as almost an ideal candidate for the regeneration of the reduced cofactor. The enzyme disadvantage is a comparatively low specific activity, i.e. 6–7 and 10 U per mg of protein for the yeast and bacterial FDHs, respectively. Another drawback is the limited coenzyme specificity of FDH. Unfortunately, there are no $NADP^+$-specific FDH found in nature so far. Operating stability of FDHs is also rather low. FDHs have essential cysteine residues[6,7] and chemical modification of this residues by impurities results in enzyme inactivation.

The superiority of FDH over the other dehydrogenases ensured its introduction into practice. Currently it is used in a number of large-scale production processes (dozens and hundreds of tons) of synthetic chiral compounds, like the Degussa process of *tert*-L-leucine production[8]. The mostly used is the FDH from yeast *Candida boidinii* (CboFDH). The cultivation of the original yeast strain has been optimized to give the maximum yield of activity 3–5 kUnits per liter per day; the scale-up FDH purification and production method has been developed up to the range of million Units[9]. However, the production cost of CboFDH in accordance with the above method is still rather high and limits the enzyme application for chiral synthesis. In this context, we have developed the process of production of NAD(P)H regeneration biocatalysts based on mutant forms of recombinant FDH from *Pseudomonas sp. 101* (PseFDH) expressed in *E. coli*. The following list of tasks has been solved:

1. Enzyme time/space yield has been increased under the optimized cultivation conditions up to values 30 000 Units per liter per day and higher. Cultivation process has been scaled up to values 5–10 million Units per day.
2. Simplified scale-up protocol for the enzyme purification has been developed.
3. Kinetic properties of PseFDH and its stability toward elevated temperatures and chemical denaturants has been improved. Affinity of PseFDH to coenzyme was improved twice. Thermal stability of the enzyme was increased 70 fold. Replacement of essential Cys residues by Ala and Ser resulted in increase of chemical stability of PseFDH at least two orders of magnitude[7]. Similar improvement of chemical stability was achieved with CboFDH (ref.[6]).
4. Different types of PseFDH specific to $NADP^+$ have been constructed using protein engineering methods. The mutant enzyme has practically the same kinetic properties as wild type PseFDH. Experiments for changing coenzyme specificity of FDH from *C. methylica* and *S. cerevisiae* did not result in enzymes with appropriate kinetic properties and stability[10,11].

All tasks could be solved only in tight connection to each other. For instance, to increase the enzyme yield in the course of cultivation (Task 1) one has to use recombinant *E. coli* strains providing the production of the target protein at the level of 40–50 % of the total soluble protein, i.e. the level that can never be reached with the use of natural strains. The increase in the target enzyme content in the biomass plays an important role for the lowering the purification costs (Task 2). On the other hand, to get the high enzyme content in the biomass as an active protein one needs to enhance its stability (Task 3). The production of recombinant mutant FDH with high thermal stability (Task 3) allowed us to introduce a step of heat treatment of cell-free extract at temperatures >55 ^{O}C into the purification process to remove impurities of *E. coli* proteins.

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L041 ENANTIOSELECTIVE OXIDATION EMPLOYING ADH AND NADH OXIDASE: COFACTOR REGENERATION OF BOTH NAD^+ FROM NADH AND $NADP^+$ FROM NADPH

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Keywords: enzymes, cofactor regeneration, NADH oxidase, (*R*)-alcohol dehydrogenase

Dehydrogenases with their superb enantioselectivity can be employed advantageously to prepare enantiomerically pure alcohols, hydroxy acids, and amino acids. For economic syntheses, however, the co-substrate of dehydrogenases, the NAD(P)(H) cofactor, has to be regenerated[1]. A possible so-

lution of regenerating $NAD(P)^+$ from NAD(P)H is oxidation of NAD(P)H with concomitant reduction of oxygen catalyzed by NADH oxidase (E.C. 1.6.–.–) which can reduce O_2 either to undesirable H_2O_2 or to innocuous H_2O. We have found and characterized such an NADH oxidase from *Lactobacillus sanfranciscensis* with hitherto only machine-annotated NADH oxidase function[2]. We have overexpressed the corresponding proteins and could prove the annotated function to be correct. As demonstrated with a more sensitive assay than employed previously, the two novel NADH oxidases reduce O_2 to H_2O.

We employ NADH oxidase from *L. sanfranciscensis* and (*R*)-ADH from *Lactobacillus brevis* to perform enantioselective oxidation of a variety of substrates with regeneration of both NAD^+ and $NADP^+$ cofactors from their reduced precursors. Whereas the wildtype (*R*)-ADH from *L. brevis* accepts $NADP(^+)(H)$ only, its G37D mutant strongly prefers $NAD(^+)(H)$. After optimized purification of both NADH oxidase and (*R*)-ADH with high yields to remove impurities likely to interfere in the oxidation-regeneration cycle, NADH oxidase was coupled with wildtype-ADH from *L. brevis* on NADP(H) and mutant ADH from *L. brevis* on NAD(H) to convert racemic phenylethanol to (*S*)-phenylethanol and acetophenone. Depending on the relative concentration of alcohol to cofactor, up to more than 100 turnovers were observed. We believe that this is the first demonstration of a regeneration scheme for both NAD^+ from NADH and $NADP^+$ from NADPH with the same enzyme.

Enzymes play an increasingly important role as catalysts in the synthesis of fine chemicals and pharmaceutical intermediates. One of the main drawbacks of enzymes often is their lack of adequate stability under reacting conditions (operating stability). We have investigated the stability of several dehydrogenases. We find that dehydrogenases are deactivated by chaotropes but not kosmotropes: the deactivation constant k_d correlates well with the Jones-Dole coefficient B, a virial coefficient derived from the relative viscosity η/η_0 of a salt solution compared to water, rather than the surface tension increment $\Delta\sigma$ (ref.[3, 4]). As most current theories for quantitative explanantion of the lyotropic series (Hofmeister series) are based on the surface tension increment $\Delta\sigma$, they do not seem to be satisfactory and novel theories seem to be required.

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L042 MOLECULAR HYDROGEN AS EFFICIENT REDUCTION EQUIVALENT FOR BIOCATALYSIS?

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Keywords: *Pyrococcus furiosus* hydrogenase, NADPH, cofactor regeneration, molecular hydrogen, enantioselective reduction

Oxidoreductases that catalyze the enantioselective reduction of prochiral ketones require NADH or NADPH as cofactors, supplying the needed reduction equivalents. Since these nicotinamide cofactors are too expensive to use them stoichiometricly, efficient cofactor regeneration is needed. Additionally, also an economic method for the production of the reduced cofactors is required. One way is the application of formate dehydrogenase, utilizing formate to reduce $NAD(P)^+$. But since the formate dehydrogenase is inhibited by NADPH, no high product concentrations of the reduced nicotinamide cofactors can be reached in view of its production[1–4].

For the production and regeneration of NADPH a hydrogenase from the hyperthermophilic strain of the archaeon *Pyrococcus furiosus* is applied (Fig. 1), utilizing cheap molecular hydrogen. By this method no side product is produced. Additionally, any excess of the gaseous reduction reagent H_2 can be easily separated.

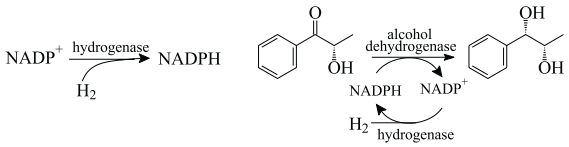

Fig. 1. **Generation and regeneration of NADPH with hydrogenase from *Pyrococcus furiosus***

NADPH was produced in an especially developed continuously operated enzyme membrane reactor for over 120 h (84 residence times) at a conversion of > 80%.

The hydrogenase was additionally applied for the cofactor regeneration in a coupled system with an alcohol dehydrogenase (Fig. 1) catalyzing the enantioselective reduction of (*S*)-2-hydroxy-1-phenylpropanone to the corresponding (1*S*, 2*S*)-diol with de = 98%. This reaction was carried out as a repetitive batch regarding the enzyme.

These results show that there is a high potential in utilizing the hydrogenase from *Pyrococcus furiosus.* With molecular hydrogen one of the cheapest redox equivalents is now available for cofactor regeneration. Additionally it enables a side-product free NADPH generation. The next step will be the scale-up of the NADPH production and the increase of the total turnover number in the case of cofactor regeneration.

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L043 A NOVEL PEROXIDASE-CATALYZED REACTION. SUBSTRATE SPECIFICITY AND KINETIC STUDIES

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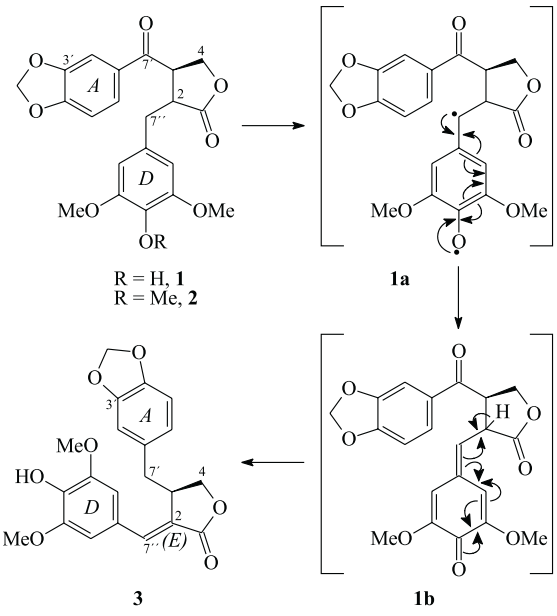
Keywords: peroxidases, dibenzylbutanolides, podophyllotoxin, kinetic studies

A purified peroxidase from plant cell cultures of *Cassia didymobotrya* was shown to catalyze a peculiar dehydrogenation reaction of dibenzylbutanolide **1**, postulated intermediate in the biosynthesis of podophyllotoxin, converting it to corresponding benzylidene-benzoyl-γ-butyrolactone (**3**, 60 % yield) following the classical kinetics of peroxidases. By contrast, **2**, was recovered as such, when exposed to the enzymatic reaction. The enzymatic mechanism can explain the obtainment of **3**. Presumably the double bond formation is initiated by hydrogen atom abstraction to form a benzylic radical and the latter proceeds to give the diradical intermediate **1a**. This, in turn, by a conventional peroxidase-catalyzed process, can clearly lead to *p*-quinoid intermediate **1b**, which is expected to rapidly rearrange to the more stable aromatic compound **3**.



L044 SYNTHESIS OF OPTICAL ANTIPODES OF α-AMINO-β-HYDROXYACIDS USING D- AND L-THREONINE ALDOLASES

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Keywords: D-threonine aldolase, L-threonine aldoldase, α-amino-β-hydroxy acids, kinetic resolution, chiral synthesis

DSM is a leading company in the production of chiral intermediates for the pharmaceutical industry and many of our production processes for these intermediates involve biocatalysis. To expand our position in biocatalysis, DSM is developing novel biocatalyst collections (platforms) and is scouting for novel applications of unknown and known enzymes. Threonine aldolases are pyridoxal phosphate dependent enzymes that are capable of coupling glycine to a broad range of aldehydes, forming α-amino-β-hydroxy acids. Both D- and L-specific threonine aldolases are known, which allows the preparation of both the D- and L-α-amino-β-hydroxy acids.

We have cloned an L-threonine aldolase gene from *Pseudomonas putida* NCIMB 12565 (ref.[1]) and a D-threonine aldolase gene from *Achromobacter xylosoxidans* IFO 12669 (ref.[2]) and expressed them in *E. coli*. Sequencing and analysis of the genes revealed that the cloned D-threonine aldolase gene from *A. xylosoxidans* IFO 12669 showed 91 % and 90 % identity to D-threonine aldolases of *Arthrobacter* sp. strain DK-38 (ref.[3]) and *Xanthomonas oryzae*[4], respectively. In contrast, it showed only 58 % identity to the published sequence of D-threonine aldolase of *A. xylosoxidans* IFO 12669 (ref.[2]), due to errors in the published nucleotide sequence.

The cloned D- and L-threonine aldolases were used in the resolution of phenylserine derivatives and the direct synthesis of a limited number of α-amino-β-hydroxy acids. The enantiomeric ratio E was greater than 200 for most of the tested substrates.

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L045 STUCTURAL INVESTIGATIONS OF FUNGAL α-*N*-ACETYLHEXOSAMINIDASES USEFUL IN SYNTHESES OF NEW UNIQUE OLIGOSACCHARIDES

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Keywords: β-*N*-acetylhexosaminidase, *Aspergillus oryzae*, molecular cloning, molecular model

β-*N*-Acetylhexosaminidase from *Aspergillus oryzae* CCF1066 is a robust extracellular (secreted) enzyme used in enzymatic syntheses of oligosaccharides and biotechnology[1,2]. We studied its structure and biology. Sequencing and molecular cloning of the enzyme revealed that it had 600 amino acids (including 6 cysteins and 6 sites of *N*-glycosylation). Enzyme is composed of cleaved signal peptide, the propeptide sequence involved in the regulated secretion, the inactive zincin domain, and the catalytical domain belonging to family 20 of glycohydrolases. Molecular model of the enzyme has been created based on homology modeling with the two related crystallized enzymes. The enzyme was found to be able to accept and even transglycosylate modified glucosamine residues at position C-6 and C-2. Predictions from the molecular model were in good correlation with the experimental values for the cleavage of chitobiose and other substrates. In particular, the inability of this enzyme to cleave oligosaccharides bearing the reducing ManNAc residues due to steric clash and distortion in the substrate binding site has been used for the synthesis of oligosaccharides with unique immunomodulatory properties, such as GlcNAcβ1->4ManNAc or even GalNAcβ1->4GlcNAcβ1->5ManNAc (ref.[3]).

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L046 INFLUENCE OF WATER ON HYDROLASES IN ORGANIC MEDIA

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Keywords: hydrolase, lipase, organic media, water activity, immobilization

In organic media hydrolases can be successfully used to catalyse reversed hydrolytic reactions and various types of transferase type reactions (tranesterification, transpeptidation and transglycosylation). The catalytic activities of the enzymes and the yields obtained are influenced to a large extent by the water activity of the reaction medium. The water activity dependence is quite different for different types of hydrolases and this influnces their usefulness for synthesis. A low water activity is often desired because of high equilibrium yields and high ratios between transferase activity and hydrolytic activity. Most lipases can be used at water activities below 0.1 and are thus useful for synthesis[1]. On the other hand, glycosidases need a high water activity to be active and the intrinsic selectivity of the enzymes for transglycosylation decreases with decreasing water activity[2]. Proteases have intermediate properties in relation to the other two groups of hydrolases[3].

Within the lipase group there are relatively large differences in water depedence. They normally express maximal activity in emulsions. Under optimal conditions in purely organic medium (pure tributyrin) lipase B from *Candida antarctica* (CALB) expressed 49 % of its activity in emulsion, while the lipase from *Thermomyces lanuginosa* (TLL) in the same medium expressed only 9.2 % of its activity in emulsion[4]. Lipases were tried early when the research area of biocatalysis in organic media developed and it was sometimes believed that typical lipases, which have large lid regions and are interfacially activated by undissolved hydrophobic substrates, are especially well suited for organic media. However, the present study shows that TLL, which is a “typical” lipase, according to this definition, in fact seems to be less well

adapted for catalysis in organic media than CALB, which is a more atypical lipase.

Since most lipases accept a wide range of substrates and because of the advantageous catalytic properties discussed above, it is of special importance to optimise lipase performance in organic media. The catalytic activity of different preparations of the same lipase can vary within several orders of magnitude. In a comparative study, it was shown that maximal activity was obtained with lipases adsorbed on porous polypropylene[5]. Other good preparations methods were immobilisation in sol gel and formation of surfactant complexes.

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L047 ACETYLATION OF CARBOHYDRATES CATALYZED BY AN ACETYLXYLAN ESTERASE IN MICROEMULSIONS

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Keywords: acetylxylan esterase, transacetylation, carbohydrates, microemulsion

Acetylxylan esterases (EC 3.1.1.72) are common accessory enzyme components of microbial xylanolytic systems[1, 2]. Their natural role is to liberate acetic acid from acetyl glucuronoxylan, to make the xylan main chain accessible for productive binding with endo-β-1,4-xylanases. There is general interest to find out whether these enzymes could work in a reverse way, that is, to acetylate carbohydrates. The driving force of research in this direction is the ability of acetylxylan esterases to operate on polymeric substrates and their regioselectivity of deacetylation of xylopyranosyl residues in the xylan main chain at positions 2 and 3 (ref.[3, 4]). Thus these enzymes may have a potential to catalyze regioselective acetylation of polysaccharides and polysaccharide-containing materials. Various conditions were applied in this work to test the ability of acetylxylan esterase from a wood-rotting fungus *Schizophyllum commune* to catalyze acetyl group transfer to methyl β-D-xylopyranoside. The best performance of the enzyme was observed in a detergent-containing microemulsion system, *n*-hexane-vinyl acetate-sodium dioctyl sulfosuccinate-water, at the water-detergent ratio of about 4-5. More than 60 % conversion of methyl β-D-xylopyranoside to mono-, di- and triacetylated derivatives was achieved, however, with a low acetylation regioselectivity . The degree of acetylation of the acceptor corresponded to thermodynamic equilibrium between the acceptor and reaction products. Under identical experimental conditions the enzyme acetylated to a similar degree other carbohydrates such as methyl β-D-cellobioside, cellobiose, cellotetraose, mannobiose, mannopentaose, mannohexaose and xylooligosaccharides up to xylopentaose. With partially soluble acetyl group acceptors, acetylations proceeded also in heterogeneous phase. The formation of partially acetylated derivatives was monitored by TLC, ^{1}H-NMR spectroscopy and electrospray mass spectrometry. Evidence for a single to fourfold acetylation of oligosaccharides was obtained. This is the first example of transacetylation to carbohydrates catalyzed by an acetylxylan esterase and a carbohydrate esterase of family 1. The results stimulate our further interest in the potential of carbohydrate esterases to modify hydrophilic properties of natural polymers.

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L048 ENANTIOSPECIFIC LIPASE-CATALYZED REACTIONS AT HIGH TEMPERATURES UP TO 120 °C IN AUTOCLAVE

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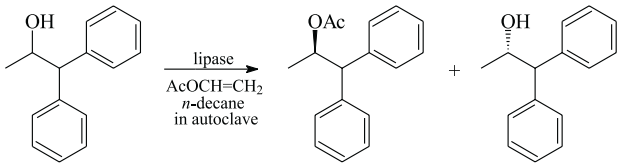
Keywords: high temperature, lipase, mechanism, enantioselectivity

Potential capabilities of enzymes may be developed by increasing or decreasing the reaction temperature. The fact that the lipase-catalyzed transesterifications proceed enantioselectively even at –60 °C is one of the most remarkable examples of the temperature effect[1]. Here we report that lipase-catalyzed transesterifications proceeded at high temperatures up to 120 °C in an enantiospecific manner.

We have previously reported that 1,1-diphenyl-2-propanol (**1**) showed no reactivity for a lipase[2]. High temperature may force **1** to get over the high-energy transition state. We used a Toyonite-immobilized lipase that is commercially available, lipase PS-C "Amano" II (Amano Enzyme Inc.), for the high-temperature biocatalysis. We used *n*-decane as solvent, having a high boiling point (174 °C), and a stainless autoclave as a reaction vessel to suppress the evaporation of vinyl acetate. The lipase-catalyzed kinetic resolutions of **1** were conducted with vinyl acetate (2 equiv.) at 30–120 °C (Scheme). The results are listed in Table I.



1 (*R*)-**2** (*S*)-**1**

Table I
Enantiospecific lipase-catalyzed kinetic resolutions of **1** at high temperatures

		% yield (% ee)		
temp (°C)	c *(%)*	*(*R*)-2*	*(S)-1*	E *value*
30	3	– (–)	– (–)	–
40	16	13 (>98)	74 (16)	>116
50	23	17 (>98)	70 (27)	>129
60	27	20 (>98)	60 (39)	>145
70	32	27 (>98)	58 (46)	>156
80	39	31 (>98)	52 (58)	>179
90	39	33 (>98)	52 (59)	>181
100	30	29 (>98)	61 (42)	>150
110	21	25 (>98)	62 (28)	>130
120	12	13 (>98)	71 (14)	>114

Although the conversion was only 3 % at 30 °C after 3 h, it was increased with increasing the reaction temperature to reach 39 % at 80 and 90 °C. Further increase in the temperature resulted in lower conversions. The obtained ester **2** was optically pure (> 98 % ee) in all cases measured (except 30 °C). It should be noted that the enzymatic catalysis proceeded enantiospecifically even at 120 °C, a sterilization temperature for microorganisms. We also examined vinyl hexanoate because its boiling point is higher than 120 °C. In this case, the reaction was conducted at 120 °C in a test tube with a rubber septum. As a result, the corresponding ester was obtained in > 98 % ee and 8 % yield.

The enantioselectivity for **1** was excellent even at high temperatures. The transition-state model that we have previously proposed[3] suggests that the reactivity of the unfavorable enantiomer, (*S*)-**1**, is completely suppressed by a severe steric repulsion between the diphenyl moiety of (*S*)-**1** and the protein wall and/or by a conformational strain which are caused in the transition state, leading to the perfect enantiomeric purity of (*R*)-**2** even at 120 °C.

In summary, lipase PS-C "Amano" II is a robust biocatalyst useful for organic synthesis. The lipase-catalyzed reactions for **1** proceeded at 40–120 °C to give enantiopure (*R*)-**2**, with the highest conversion at 80–90 °C. Since the lipase-catalyzed enantioselective transesterifications proceed at –60 °C using the same enzyme as reported previously[1], it follows that the single enzyme can show the catalytic function at a very wide range of temperatures from –60 to 120 °C.

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L049 NEW APPROACHES IN ELECTROENZYMOLOGY: COUPLING HOMOGENEOUS AND ENZYME CATALYSIS FOR HIGHLY SPECIFIC HYDROXYLATIONS, EPOXIDATIONS AND HYDROGENATIONS

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Keywords: electroenzymology, asymmetric synthesis, cofactor regeneration, monooxygenase, homogeneous catalysis

The use of isolated oxidoreductases as catalysts in organic synthesis allows the uncoupling of catalyst preparation from its application and independent optimization of both processes – and it requires an economical supply of redox equivalents. Case specific regeneration solutions have been described for individual enzyme classes like dehydrogenases, monooxygenases and peroxidases. However, a more universally applicable redox catalyst is still missing.

We use the organometallic complex pentamethylcyclopentadienyl bipyridin rhodium ($[Cp^*Rh(bpy)(H_2O)]^{2+}$). It exhibits a high tolerance towards extreme reaction conditions with respect to temperature, buffer composition, and pH (ref.[1]).

The complex was applied for the enzyme-free regeneration of reduced nicotinamide coenzymes (NADH and NADPH). It catalyzes the transfer of reduction equivalents either from a cathode or formate to the oxidized nicotinamide coenzyme. We have successfully applied this concept of enzyme-free regeneration to monooxygenase-catalyzed regiospecific phenol hydroxylation, asymmetric epoxidation of

styrene to *S*-styrene oxide (ee > 99%) and enantiospecific hydrogenations catalyzed by a new thermostable dehydrogenase (recombinant from *Thermus* sp, Schmid et al., unpublished). Products were synthesized up to a gram scale in biotransformations with productivities up to 3 $g.l^{-1}.h^{-1}$, stable over 10 h in batch or continuous mode.

Direct regeneration of oxidoreductases has also been accomplished. The FAD-dependent oxygenase component StyA of styrene monooxygenase[2] has been regenerated directly. Thus, the native reductase component StyB, the nicotinamide coenzyme and a putative NADH regeneration system could be omitted from the reaction while preserving the enantio- and regiospecificity of the oxygenase.

Direct, non-enzymatic reduction of heme-iron has been shown for cytochrome C. The catalytically active oxyferryl species can be generated by a hydrogen peroxide shunt pathway. *In situ* supply of H_2O_2 in appropriate amounts (minimizing the hazardous effect of H_2O_2 on enzymatic activity) was achieved with $[Cp^*Rh(bpy)(H_2O)]^{2+}$.

The presentation will address basic concepts, perspectives, and process engineering solutions for coupling mediator based homogeneous catalysis to enzyme catalysis.

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L050 EQUILIBRIUM OF REACTIONS ON SOLID-PHASE SUBSTRATES CAN BE SHIFTED FOR USEFUL ENZYMATIC SYNTHESES

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Keywords: solid-phase, equilibrium shift, amide synthesis, solid support

Enzymatic reactions are of increasing interest in solid-phase chemistry, especially with selectively cleavable protecting groups and linkers. However, most studies have found disappointing yields, and/or used relatively large amounts of enzyme and reaction times. There is a need for more fundamental study of the effects on thermodynamics and kinetics when substrates are attached to solid supports. By understanding the reasons for slower rates and/or lower equilibrium conversions we can identify ways to improve them. Understanding such systems will also be useful for other purposes, such as on-bead screening and some situations in living organisms.

By recent experimental and theoretical studies, we have found that equilibrium positions can be significantly altered when one reactant is attached to a solid phase, compared to a condensation in free solution. So far we have studied the synthesis of amides between solid-phase amino groups and an acid in solution. The main example has used the enzyme thermolysin and amino acid residues attached to the support beads of $PEGA_{1900}$, with observed conversions to amide of over 99 % in fully aqueous media[1].

Three factors may contribute to the shift in equilibrium[2]:

1) The mass action effect of using a high concentration of the acid – the solid phase product makes removal of the excess easier. This is theoretically trivial, practically useful, but is not the only important factor.
2) Unfavourable hydrophobic hydration of groups in the acid will be reduced when this becomes linked to the surface. Contact with water will be reduced, and what remains will be already more ordered by the nearby surface. This effect can be very large, with examples already showing over 10^4-fold increase in the equilibrium ratio of amide to amine[2]. The effect is of course largest when the acid is most hydrophobic, for example an amino acid with hydrophobic side chain and/or protecting group.
3) Ionisation of free amino groups will be suppressed when they are close together inside the solid particles. Protonation of the free amino group contributes significantly to making amide hydrolysis favourable in aqueous solution, so its suppression will shift the equilibrium towards synthesis. The magnitude of this effect is theoretically expected to be greater in media of lower ionic strength and pH, and a quantitative model is under development (unpublished). Experiments have shown that this effect is relatively small with PEGA beads at ionic strength around 0.2 M (ref.[2]).

This shift in equilibrium position can be exploited preparatively. Hence coupling to the free amino group of a solid-attached phenylalanine residue proceeds to high yields, 99 % in the case of Fmoc-Gly, Leu or Phe, for example, and 70 % even with Fmoc-Asp (ref.1). With dissolved Phe-OMe, and the same (saturated) concentrations of acids, conversions range from 0.1 to 2.3 % (ref.2). This shift in equilibrium probably also explains some of the poor yields observed when attempts have been made to hydrolyse solid-phase amides. These may reflect an unfavourable equilibrium for complete hydrolysis, rather than slow kinetics or inaccessibility to the enzyme, as has been believed.

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L051 DEVELOPING NOVEL BIOCATALYSTS FOR MANUFACTURING OPTICALLY ACTIVE COMPOUNDS

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Keywords: functional genomic screening, gene directed evolution, alcohol dehydrogenases

Developing novel biochemical processes for the production of active chiral molecules enables pharmaceutical and agrochemical industries to generate novel intellectual property rights and enhance their competitiveness. This approach has now become possible within the required timelines and budgets because of the recent developments in biotechnology. Protéus has developed proprietary technologies for functional genomic screening (Phenomics™, CLIPS-O™) which enables the rapid discovery of novel biocatalysts that are present in natural biodiversity. Biodiversity from extreme environments has proven to be particularly rewarding for industrial biocatalysis. The catalytic properties of these enzymes can then further be improved using L-Shuffling™, a high throughput proprietary directed evolution technology. By combining these two approaches, high performance catalysts can be delivered to the industry within unprecedented accelerated timeframe.

As an example, we will describe the discovery of alcohol dehydrogenases (ADHs). ADHs are of particular interest for catalyzing the reduction of ketones into enantiomerically pure alcohols. The use of proper technologies and strategies enables the rapid screening of the natural biodiversity that results in the isolation and cloning of ADHs covering a broad substrate range. This set of ADHs can then be used as a „toolbox“ to rapidly screen for activity on substrates of industrial interest. However, the performances of natural enzymes might not always reach the industrial standard required for setting up a competitive large-scale industrial manufacturing process. We will discuss L-Shuffling™, a proprietary gene directed evolution developed by Protéus which provides reduced-to-practice means to „breed“ and optimize new, non natural enzymes, allowing to rapidly develop novel biocatalysts and fulfil virtually any industrial requirements.

L052 RATIONAL REDESIGN OF A LIPASE INTO A LYASE

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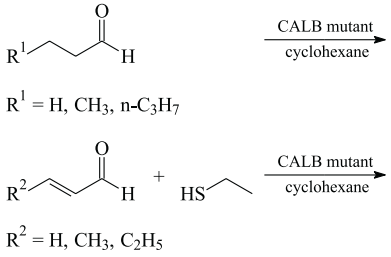
Keywords: rational engineering, aldol additions, Michael additions, *Candida antarctica* lipase B

Enzymes are efficient catalysts in synthetic chemistry and their catalytic activity with unnatural substrates in organic reaction media is an area attracting much attention. Protein engineering has opened the possibility to rationally alter the reaction specificity of enzymes and allow for new reactions to take place in their active sites. We have used this strategy on the well-studied active-site scaffold offered by the serine hydrolase *Candida antarctica* lipase B (CALB, EC 3.1.1.3) to achieve catalytic activity for reactions unnatural to this enzyme, such as aldol additions[1] and Michael additions. The catalytic reactions were first studied in detail by means of quantum chemical calculations in model systems. These calculations showed that the reactions were possible in the active site of a CALB mutant where the nucleophilic active site serine was removed. The predictions from the quantum chemical calculations were then challenged by experiments. Consequently, Ser105 in CALB was targeted by site-directed mutagenesis to create enzyme variants lacking the nucleophilic feature of the active site. The experiments clearly showed an increased reaction rate when the aldol and Michael addition reactions were catalyzed by the mutant enzymes. The reactions were run in cyclohexane with the substrates indicated in Figure. We have shown that the new catalytic activity, harbored in the stable protein scaffold of *Candida antarctica* lipase B, allows aldol and Michael additions of substrates that can not be catalyzed by traditional aldolases.



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L053 CHEMOENZYMATIC SYNTHESIS OF THE CHIRAL SIDE-CHAIN OF ATORVASTATIN

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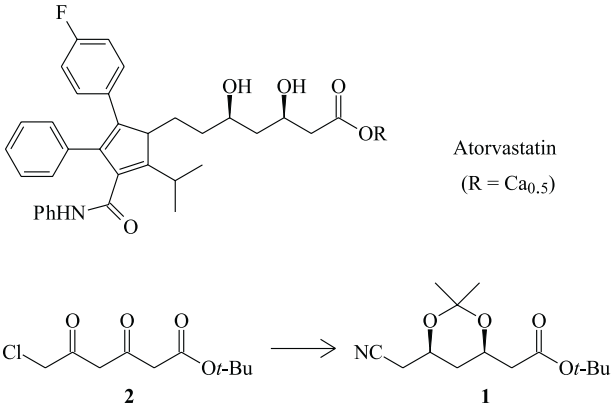
Keywords: atorvastatin, HMG-CoA reductase inhibitors, alcohol dehydrogenase, enantioselective reduction, cyanation

Atorvastatin is an important lipid lowering drug for the treatment of atherosclerosis and other diseases connected to hyperlipidaemia, e.g. coronary heart disease[1]. The isopropylidene-protected dihydroxy ester **1** is the key chiral intermediate of an economical convergent route to this fully synthetic mevinic acid derivative[2].



In this presentation, we report a short and highly enantioselective synthesis of the isopropylidene-protected dihydroxy ester **1** (>99 % *ee*, *dr* > 200 : 1). Crucial step of our strategy is an enzymatic reduction of the achiral diketo ester **2** by means of readily available *Lactobacillus brevis* alcohol dehydrogenase[3]. A one-step synthesis of diketo ester **2**, a laboratory scale-up of the NADP-dependent enzymatic reduction (75g scale), and a high-yielding method for performing the unexpected difficult cyanation of the enzymatic reduction product will be presented.

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L054 ENGINEERING AN ENTIRELY ENZYMATIC PROCESS FOR L-ASCORBIC ACID PRODUCTION FROM D-GLUCURONIC ACID

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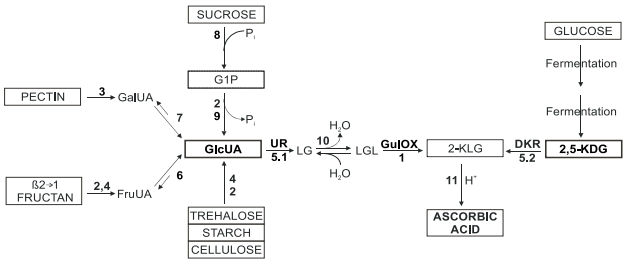
Keywords: L-ascorbic acid, vitamin C, D-uronate reductase, L-gulono-1,4-lactone oxidase

Vitamin C has a very large market share both in the food and drinks industries (more than 50 %) and in the pharmaceutical industries. In addition to the well established chemical process for L-ascorbic acid production[1], a multitude of other chemical or fermentative routes have been developed[2].

Since the Reichstein process is largely a chemical one, there is increasing pressure (e.g. environmental concerns and legislation) to develop efficient and cheap biological alternatives. However, no entirely enzymatic process is documented in the literature to date. Possible routes of this kind are sketched out in Figure 1.



1 L-Gulonolactone oxidase
2 Chemical oxidation
3 Polygalacturonase/Pectin methylesterase
4 Chemical hydrolysis
5.1 Uronate reductase
5.2 2,5-Diketogluconic acid reductase
6 Uronic acid isomerase
7 C_4 epimerase (UDP activated ?)
8 Sucrose phosphorylase
9 Alkaline phosphatase
10 Lactonase
11 Enolisation

Fig. 1**. Reaction scheme for the enzymatic synthesis of L-ascorbic acid *via* D-glucuronic acid (GlcUA) and 2,5-diketo-D-gluconic acid (2,5-KDG), resp. Routes leading to these key intermediates are also integrated;** Fru UA = fructuronic acid, GalUA = galacturonic acid, 2-KLG = 2-keto-L-gulonic acid, LG = L-gulonic acid, LGL = L-gulono-1,4-lactone

By imitating in part the biological pathway, Vitamin C synthesis was started from D-glucuronic acid, which can be prepared chemo-enzymatically from renewable materials, for example from sucrose via glucose 1-phosphate. A three-step process was established:

1. D-Glucuronic acid (0.75 M) was reduced and inverted to L-gulonic acid by NADPH-dependent uronate reductase from *S. cerevisiae* YNN295 cloned into *E. coli* JM109 and expressed under the control of the tac-promotor[3]. The native

coenzyme is regenerated by NAD(P)-dependent glucose dehydrogenase from *Bacillus cereus* in a charged ultrafiltration membrane reactor[4]. Product yield is more than 90 %, the total turnover number of NADP/H was about 10.000.

2. L-Gulono-1,4-lactone was obtained by chemical lactonisation in quantitative yield; a lactonase-catalyzed step is under study.

3. Finally, the lactone is oxidized by a cytosolic fungal oxidase (GulOx) to 2-keto-L-gulonic acid and rearranged to L-ascorbic acid. A tubular enzyme membrane reactor is used to supply oxygen and to regenerate the cofactor effectively. GulOx is now being cloned. In a series of discontinuous experiments (Table I) optimal conditions for maximal substrate transformation (98,6 %) and Vitamin yield (43.1 $g.l^{-1}$) were determined. GulOx stability allows for repeated batch operation. Possible improvements towards an entirely enzymatic production of L-ascorbic acid will be discussed.

Table I
Comparative results of discontinuous transformation of L-gulono-1,4-lactone to L-ascorbic acid by fungal GulOx under different experimental conditions

Expt. No	L-Gulono--1,4-lactone	GulOx	Catalase	L-Ascorbic acid		GulOx act remaining
	pH	[mM]	[U.ml^{-1}]	[kU.ml^{-1}]	[%] (h)	[g.l^{-1}]	[%]
1	4.0	20	1	0	0	0	0
2	4.0	200	1	0.5	0	0	0
3	4.0	200	1	1.0	18.4 (6)	4.8	18
4	5.0	200	1	1.0	56.6 (22)	14.2	96
5	5.0	200	2.5	1.0	79.6 (22)	22.0	95
6	6.0	200	2.5	1.0	98.6 (23)	24.1	98
7	6.0	300	2.5	1.0	96.4 (21)	33.6	92
8	6.0	500	2.5	1.0	83.9 (24)	43.1	96
9	4.0	200	1	1.0	46.1 (23)	12.4	0
10	5.0	200	1	1.0	62.6 (22)	16.3	78
11	6.0	200	2.5	1.0	97.4 (22)	22.2	97

Optimal conditions for maximal substrate conversion (98,6 %) were 200 mM of L-gulono-1,4-lactone; pH 6.0 (experiment 6). At 500 mM substrate a final L-ascorbic acid concentration of 43.1 $g.l^{-1}$ (≡ 84 % conversion) was possible (expt. 8). Loss of GulOx activity was 2 and 4 %, resp.

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L055 NEW BIOCATALYSTS FROM THE METAGENOME: ALPHA/BETA HYDROLASE FOLD ENZYMES

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Keywords: metagenome, enzyme libraries, screening

The alpha/beta-hydrolase family comprises many different enzyme classes which share the same tertiary protein fold. Some of these enzymatic activities are of major interest to biotechnological industries: Lipases and esterases are today the most often used enzymes in organic synthesis of fine chemicals[1]. Epoxide hydrolases are of high value because of the possible production of enantiopure epoxides or their corresponding vicinal diols[2]. Haloperoxidases have received increasing attention due to their ability to halogenate a variety of commercially important compounds. Additionally, these enzyme might be interesting for their sulfoxidation and epoxidation capabilities[3]. However, the availability of appropriate biocatalysts of many of these enzyme classes for changing industrial applications is still limited.

Our aim is therefore to provide novel enzyme libraries by accessing the functional sequence space of biodiversity in order to facilitate the identification of suitable biocatalysts for specialized applications. Limited to screening strain collections biotechnology has missed to evaluate up to 99 % of existing microbial resources. This was due to the inability to cultivate most microorganisms. Novel strategies of directly cloning „metagenome DNA“ as the genetic blueprints of entire microbial consortia are becoming increasingly applicable to circumvent this restriction[4].

Screening of metagenomic libraries by both a sequence homology based approach and an activity based approach has led to the identification of several new and highly diverse enzymes of the alpha/ beta hydrolase family.

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P001 FLEXIBILITY OF LIPASE BROUGHT ABOUT BY REACTION TEMPERATURE AND ADDITIVES CONTROLS ITS ENANTIOSELECTIVITY IN ORGANIC SOLVENTS: A RATIONAL APPROACH FOR OPTIMIZATION OF ENANTIOSELECTIVITY FOR GIVEN ENZYMATIC REACTION

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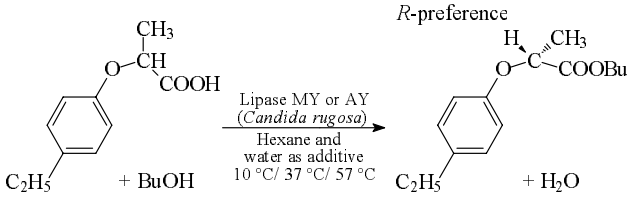
Keywords: chiral resolution, enantioselectivity, lipase, conformational flexibility, EPR spectroscopy

Since the basic discovery showing enzymatic activity even in organic solvents by Klibanov et. al.[1], a number of strategies for enhancing the enantioselectivity and activity of enzymes have appeared. An ultimate goal, especially for organic chemists is to control rationally the enantioselectivity of enzyme as a function of experimental conditions. It is generally accepted that the flexibility of enzymes plays an important role in the discrimination between the enantiomers of the substrate used[2]. Further developments, however, are waited a model showing how to control the enzyme's enantioselectivity by its flexibility.

In our recent studies, we have reported a relationship between the flexibility of enzyme brought about by the additive such as DMSO (ref.[3]) or the solvent nature[4] and its enantioselectivity for enzyme-catalyzed reactions, the flexibility of which was estimated by EPR spectroscopy. Here, our discussion is focused mainly on the effects of the reaction temperature on the flexibility of lipase, because the reaction temperature is also anticipated to strongly affect flexibility of enzyme, resulting in altering its enantioselectivity.



The enantioselectivity for two kinds of *Candida rugosa* lipases-catalyzed reactions was investigated under the various temperature conditions (see scheme). The behavior of the obtained enantioselectivity as a function of the reaction temperature showed an opposite trend for two kinds of lipases in the different preparations form *Candida rugosa*, high temperature-induced high enantioselectivity and low tempertureinduced high enantioselectivity[5]. The opposite trend can be ascribed to the experimental results that the lipases have the individual optimum flexibilities to produce their maximal enantioselectivities.

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P002 DIFFERENT STRATEGIES FOR THE BIOCATALYTICAL CHARACTERIZATION OF *Candida rugosa* LIPASES IN ORGANIC SOLVENTS

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Keywords: isoenzymes, characterization, *C. rugosa* lipase, acyl transfer, organic solvents

It is well known that the different composition of *Candida rugosa* lipases (CRL), depending on the origin of the sample (concentration and isoenzymes percentage) leads to some reproducibility problems described when performing asymmetric synthesis in organic solvents[1,2]. Thus, for rationalizing such processes, a previous synthetical characterization of the catalyst behaviour in organic media should be performed.

In the present work we report several new and easy-to-perform methodologies with the aim of carrying out a complete characterization of *C. rugosa* lipases for biocatalytical purposes. Thus, as a quick first test, the heptyl oleate synthesis turns out to be a useful tool for discriminating between lipases and esterases in non-aqueous media[3]. For that process, while initial rate is directly related to the concentration of lipases in the crude sample, the final yield is mainly dependent on both the water produced and the capability of the

crude biocatalyst for distributing such water molecules. This influence can be qualitatively predicted through water sorption isotherms and thermogravimetric and differential thermal analysis (TGA/DTA).

However, in the measurement of the concentration of lipases by kinetical methods, under heterogeneus biocatalysis, some other parameters (such as solvent, temperature, stirring speed or water activity) play an important role in the initial rate. In that sense, it is necessary to find a test reaction where the final yield could be dependent (mainly) on the concentration of lipases. This is possible by using the transesterification of 1-heptanol with vinyl acetate in *n*-hexane; in fact, the acetaldehyde produced as secondary product is toxic for *C. rugosa* lipases[4], and thus the different final yield obtained by using variable amounts of CRL shows a linear dependence on the concentration of lipases. Furthermore, by simply changing the alcohol employed (using cyclohexanol) the proportion of the isoenzyme Lip3 (also described as a cholesterol esterase[5]) can be semi-quantitatively estimated by comparison of the final yields obtained in the transesterification of both alcohols. As an additional confirmation, those reactions catalyzed by the pure recombinant Lip1 isoenzyme show a much lower rate for the transesterification of cyclohexanol *versus* that obtained for 1-heptanol. Therefore, we must conclude that cyclic secondary alcohols are mainly acylated by Lip3, and thus, being Lip3 a cholesterol esterase[5], the different catalytical rates obtained for the acylation of several cyclic alcohols should be rationalize by comparison of the cholesterol molecule with different cycloalkanols tested. In addition, this fact could explain why the Kazlauskas' rule[6] is valid, in *C. rugosa* lipases, only for cyclic alcohols, and not for acyclic secondary ones.

Finally, the strategy of characterization is succesfully tested in the transesterification of 3-methyl-2-butanol with vinylacetate, where it is shown that final yield obtained is once again mainly dependent on the relative proportion of Lip3 in the crude *C. rugosa* biocatalyst.

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P003 INFLUENCE OF ALKYL-SUBSTITUTED SILANE PRECURSORS OF SILICA GELS ON THE ENZYMATIC ACTIVITY OF IMMOBILIZED MICROBIAL LIPASE FROM *Candida rugosa*

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Keywords: lipase, sol-gel, immobilized lipase, *Candida rugosa*

Investigations are being carried out to optimize the procedure for lipase encapsulation for application in the modification of vegetable oils. In this study, we present a method to immobilize lipase by entrapment in chemically inert hydrophobic silica gels, which are prepared by the hydrolysis of alkyl-substituted silanes like tetraethoxysilane (TEOS), methyltrimethoxysilane (MTMS), and polydimethysiloxane (PDMS) in the presence of enzyme and additives such as polyethyleneglycol (PEG, PM-1450) or polyvinylalcohol (PVA). For the precursor TEOS a novel methodology was established under nitrogen inert atmosphere and used an aqueous solution of lipase, hydrochloric acid, ultra-pure water, ammonium hydroxide, ethanol, and stabilizing additive (PEG 1450). For the gel precursors MTMS and PDMS, a typical immobilization procedure uses: an aqueous solution of lipase, sodium fluoride as a catalyst, alkoxysilane derivative precursors, and additives (PEG-1450 and PVA).

To evaluate these immobilization procedures, lipase from *Candida rugosa* immobilized in different gel matrixes was fully characterized with respect to their morphological properties (i. e., particle size, surface area and pore size distribution) and used in ester hydrolysis and synthesis. The coupling yields for the lipase immobilized by encapsulation using MTMS and MTMS/PDMS as precursors, was very poor (9.0 %). These data suggest that these precursors may cause conformational changes of the enzyme chain upon its adhesion to the support surface. The latter probably caused steric hindrance which may render certain regions of the enzyme molecule inaccessible to the substrate (olive oil). In this case, the addition of an additive did not produce beneficial effect. However, the positive effect of the PEG-1450 was noticed for the derivatives prepared by the encapsulation technique using TEOS as precursor. The highest coupling yield (31.98 %) was observed for the immobilized derivative obtained by lipase encapsulation in the presence of the PEG-1450; confirming the efficiency of this kind of additive[1, 2].

Further information on the catalytic activity was obtained by testing the derivatives prepared in synthetic applications, that is, in esterification reactions with *n*-butanol and butyric acid, and a different activity dependence was found. Better performance was achieved by derivatives resulting from the encapsulation of *Candida rugosa* lipase in the gels prepared with MTMS as precursor, in the presence of PEG (PM-1450). This lipase preparation exhibits an increased esterification activity (154.85 $\mu mol.g^{-1}.min^{-1}$) that is 3 times greater than that prepared with TEOS (51.98 $\mu mol.g^{-1}.min^{-1}$), and 1.74 times greater than that prepared with MTMS/PDMS (88.79 $\mu mol.g^{-1}.min^{-1}$) as precursor.

The results presented here allowed a deeper understanding of the process of encapsulation of commercial *Candida rugosa* lipase in the presence of an additive, using TEOS and MTMS as precursors.

The authors acknowledge financial assistance from CAPES, CNPq and Fundação Araucária.

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P004 SUBSTRATE-ASSISTED CATALYSIS IN A REDESIGNED HYDROLASE TO INCREASE ENANTIOSELECTIVITY

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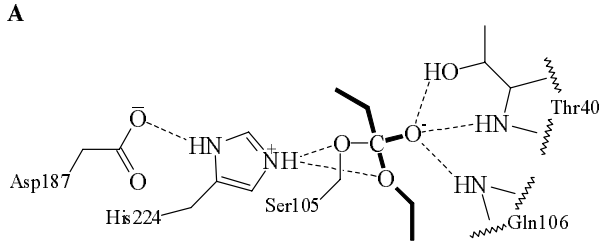
Keywords: substrate-assisted catalysis, rational design, enantioselectivity, *Candida antarctica* lipase B, isothermal titration calorimetry

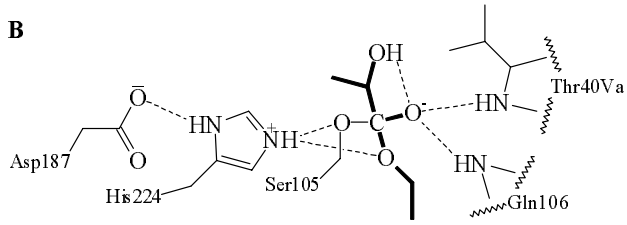
We have changed the enantioselectivity of a hydrolase towards substrates carrying a hydrogen-bond donor. A mutation in the oxyanion hole allows the hydrogen-bond donor of the substrate to stabilize the transition state. By this means the enantioselectivity was improved compared to that of the wild type enzyme.

In our experiments we used lipase B from *Candida antarctica*. It has a catalytic machinery that consists of the triad Ser-His-Asp (ref.[1]). In the active site is also the oxyanion hole, a spatial arrangement of hydrogen-bond donors, which plays a major role in the stabilisation of the transition state conformation[2]. The oxyanion is stabilized by two backbone amide hydrogen atoms and the side chain hydroxyl group of Thr40 (ref.[3]) **(A)**. By changing threonine 40, we have removed one of the hydrogen-bond donors capable of stabilizing the transition state. With substrates carrying a hydroxyl group we have performed substrate-assisted catalysis in the modified enzyme and increased the enantioselectivity greatly[4] **(B)**.

A



B



To further understand what determines the enzyme specificity we now measure how the point mutation affects transition state stabilization (k_{cat}) and substrate binding (K_M). We do this with substrates carrying either a hydroxyl or an amino group as potential hydrogen-bond donor. The catalytic constants are determined with isothermal titration calorimetry measurements.

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P005 STABILIZATION OF AN INTRACELLULAR *Mucor circinelloides* LIPASE FOR APPLICATION IN NON-AQUEOUS MEDIA

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Keywords: immobilization, membrane-bound lipase, *Mucor circinelloides*

The membrane-bound *M. circinelloides* lipase displays very high synthetic activity and can be used for synthesis of various esters, as was described elsewhere[1–3]. Different methods of this enzyme stabilization, facilitating its application in a milieu of organic solvents for synthesis of esters of higher fatty acids and aliphatic alcohols, glycerol and saccharides, were tested. Within the scope of these investigations, the lipase was isolated from the mycelium, purified and immobilized on octyl- and palmityl-cellulose, diatomaceous earth, porous glass and silica aerogel[4], and immobilized *in situ* in the mycelium (by means of de-fatting and de-hydration of the mycelium with acetone). The membrane-bound lipase was also entrapped in carragenan[4], polyvinyl alcohol cryogel and chitosan. Preparations of the immobilized lipase, in a form of mycelium pellets stabilized by treatment with glutaraldehyde, polyvinyl alcohol and alginate, were also obtained. The problems related to an application of the lipase entrapped in the PVA cryogel in organic solvents have been already presented[5]. In this work, the results of esters (sucrose, glucose, butyl and propyl oleates and caprylates) synthesis, carried out in petroleum and di-*n*-pentyl ethers, and catalyzed by various preparations of the immobilized lipase, have been compared. The lipase isolated from the cells (solubilization with sodium cholate) and immobilized on solid carriers showed a weak catalytic activity. An effective method of *M. circinelloides* lipase stabilization was found to be an entrapment of the membrane-bound enzyme in poly(vinyl pyrrolidone)-containing chitosan granules, prepared by using the phase inversion method. This method ensured the high mechanical and chemical resistance of the biocatalyst beads. Chitosan beads do not shrink in organic solvents, in contrast to PVA-gel beads. Moreover, an activation of the chitosan-biocatalyst beads during the initial batches of their iterative use for esters synthesis was observed. The maximum yields of butyl oleate and sucrose caprylate synthesis were 90 % and 75 %, respectively. The lipase preparations with high synthetic activity can be also produced by cross-linking of the *M. circinelloides* mycelium pellets (harvested from optimal medium inoculated with the suspension of sporangiospors in 0.1 % Triton X-100) with glutaraldehyde. Cross-linking of the lipase-containing pellets with PVA and glutaraldehyde, provides both high activity (approximately 3.3 $\mu kat.g^{-1}$) and operational stability of the catalyst for 120 hrs of sucrose caprylate synthesis. Water released during esters synthesis should be removed from the beads in order to provide the satisfactory yield of their repeated usage. The synthesis yield can be significantly enhanced by optimization of the reaction conditions.

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P006 SUGAR ESTERS SYNTHESIS BY A MEMBRANE-BOUND *M. circinelloides* LIPASE IN MICROREACTOR EQUIPPED WITH THE WATER ACTIVITY – MONITORING SENSOR

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Keywords: saccharide esters, *Mucor circinelloides* lipase, mathematical modelling

Esters of saccharides and fatty acids belong to a class of natural, “green”, nonionic surfactants. They are synthesized from natural components of foods and show prebiotic properties, enabling their application in food production as dietetic fats, emulsifiers, plasticizers and as antimicrobial and protective coatings for fruits[1]. The mycelium bound lipase was prepared by dehydration of *Mucor circinelloides* mycelium with acetone[2,3]. The lipase displays high stability in the medium of apolar organic solvents at 100 °C and shows high activity in hydrolysis and synthesis of esters[4]. The reactions of the sugar fatty acid esters synthesis were carried out at 50 °C in 37-ml microreactor equipped with a stirrer (120 rpm), containing 2 mmol of sugar, 2 mmol of fatty acid and 0.2 g of the lipase suspended in 10 ml of the mixture of di-*n*-pentyl ether and petroleum ether, and modified with astaxanthin or carotene, acting as activator or inhibitor of the enzyme depen-

dently on the concentration in the reaction mixtures. Free fatty acids were titrated with 0.05 M NaOH up to pH 10.0 using TitroLine (Schott) titrator. The esters concentration was determined using HPLC (ODS: 250 × 5.6 mm column, the eluent: acetonitrile-water (8 : 2), refractometric detector). Changes in thermodynamic water activity and temperature of reaction medium throughout ester synthesis were monitored using the water activity digital sensor AwVC-DIO (Rotronik) coupled with a computer (Fig. 1).

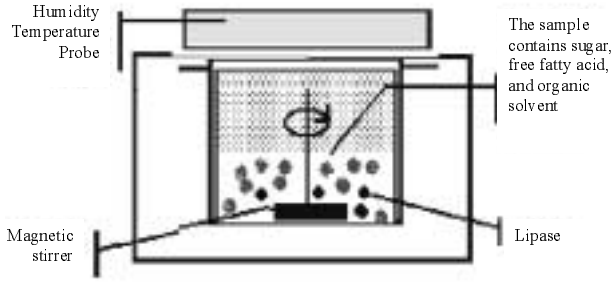

Fig. 1. **The scheme of enzymatic reactor equipped with an on line water activity (a_w) measuring probe**

The effect of molar ratio of the substrates in the reaction mixtures on the lipase activity was studied. The optimal molar ratio of caprylic acid and sucrose was found to be 9 : 5 and the corresponding degree of the sucrose conversion in di-*n*-pentyl ether medium exceeded 90 %. The dependence of water activity of bi-phasic systems di-*n*-pentyl ether – water on the phase volume coefficient $A = V_{org}/V_{water}$ (were V_{org} – organic phase volume; V_{water} – water phase volume) and an effect of water activity on the saccharide esters synthesis yield were also examined. The invented mathematical model of esters synthesis in a biphasic system was experimentally verified by sucrose and glucose esters synthesis[5]. Modelling was done by using the Mathematica 4.2 program.

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P007 NEW EFFICIENT ENZYMATIC PROCEDURES CATALYSED BY LIPASES FOR THE SYNTHESIS OF VALUABLE REGIOPROTECTED PRECURSORS OF D-FRUCTOSE IN PRODUCTION OF SUGAR DERIVATIVES

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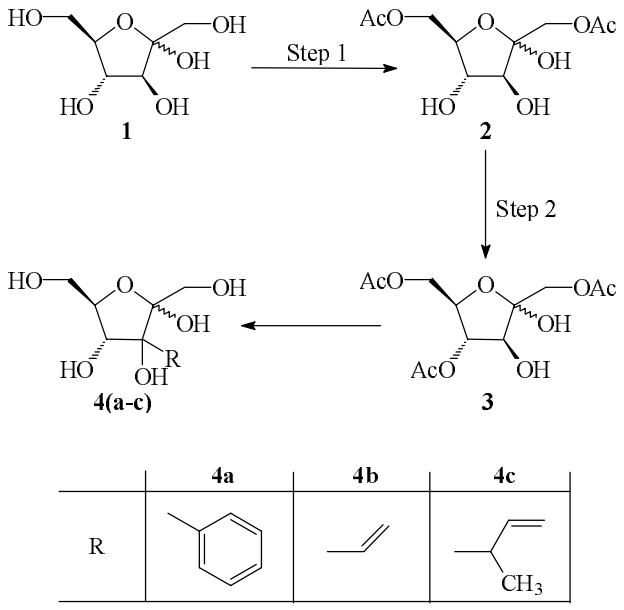
Keywords: fructose, lipase, regioselectivity, enzyme inhibitors, sugar derivatives

Carbohydrates have long been and still are important sources of chirality in the synthesis of many biological active compounds. Furthermore, many of them are components of different natural products which display powerful biological properties[1]. For these reasons many studies have been reported about chemical transformations, especially at anomeric carbons. Regioprotected derivatives of carbohydrates are important intermediates towards the synthesis of very valuable compounds. Conventional chemical methods often are not able to achieve this task and can require the use of toxic solvents and inorganic catalysts that leave residual traces in the final product; furthermore they often require difficult and expensive purification procedures. Conversely enzymatic processes offer important alternatives for the synthesis of these molecules. In particular lipases and proteases have been exploited with success in the preparation of regioprotected sugar derivatives[2].

D-Fructose **(1)** is a very sweet sugar and it is greatly abundant in the natural saccharides that exhibit interesting biological activities. We propose in this study new biocatalysed routes towards the synthesis of different regioprotected fructose derivatives. A two step enzymatic process for the synthesis of 1,4,6-triacetyl fructofuranose **(3)** has been developed: in a first step D-fructose was acetylated in tetrahydrofurane using lipase from *Candida antarctica* (Novozyme) as the catalyst to give the regioselectively protected 1,6-diacetyl fructofuranose **(2)** as sole product. In a second step the recovered diester **2** was selectively acetylated at the C-4 hydroxy group using lipase from *Candida rugosa* in *tert*-butylmethylether to give compound **3** that**,** bearing a free OH function at C-3, is a valuable starting material for further selective chemical transformations. For example oxidation of C-3 hydroxy group, followed by coupling via the Grignard reaction allows to synthesise interesting sugar derivatives **(4a-c)** of potential application as enzyme inhibitors.

Subsequently the C-4-OH preference observed in the recognition of *C. rugosa* lipase was exploited, *via* alcoholysis reaction, for the synthesis of rare fructose isomers.



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P008 EFFECT OF DIVERSE LIPASES ON THE TRANSESTERIFICATION OF GRAPE SEED OIL WITH SOLKETAL AND GLYCIDOL

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Keywords: *Aspergillus flavus*, *Rhizopus oryzae*, biocatalysts, esterification, solketal, glycidol

The enzymatic transesterification is a potentially attractive route for the modification of the physical and chemical properties of vegetable oils. This reaction can also be used to obtain some compounds that can render monoacylglycerides. These reactions usually take place with high regio and/or enantioselectivity. Moreover, the mild conditions needed to carry out these reactions permits the preparation of products with high yields without the formation of secondary products that sometimes are very difficult to remove.

In this study is described the influence of the biocatalyst on the transesterification of grape seed oil with solketal and glycidol. Two own catalysts, prepared from resting-cells of *Aspergillus flavus* and *Rhizopus oryzae*, and five commercial enzymes, Amano Lipase AYS *(Candida rugosa)* (Aldrich), Novozym 435 (Novo Nordisk), Amano Lipase A *(Aspergillus niger)* (Aldrich), Amano Lipase PS *(Pseudomonas cepacia)* (Aldrich), Lipozyme, immobilized *(Mucor miehei)* (Fluka), were used to carry out this reaction. In a 12 ml vial were added 300 mg of grape seed oil, 135 mg of solketal, 30 mg of biocatalyst and 4 ml of isooctane. The mixture was then magnetically stirred at 50 °C for 196 h. Samples were taken at 24, 48, 72, 96, 144 and 196 h and analyzed by HPLC-RP. Similar experiments were carried out using glycidol instead of solketal as reagent for the transesterifiaction of the vegetable oil.

Among the enzymes assayed, *Rhizopus oryzae* gave the best results. Thus, the solketal derivative yield was 11 $mg.ml^{-1}$ at 72 h reaction. Although the same biocatalyst gave the highest transformation when glycidol was used, the yield was much lower.

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P009 ASSESSMENT OF ACYLGLYCEROL COMPOSITION, OXIDATION PRODUCTS AND FREE FATTY ACIDS IN LIPASE-CATALYSED TRANSESTERIFIED FATS RICH IN OMEGA-3 POLYUNSATURATED FATTY ACIDS

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Keywords: acylglycerols, free fatty acids, immobilized lipase, oxidation products, transesterification

In a previous work[1], a commercial immobilized lipase from *Thermomyces lanuginosa*, Lipozyme™ TL IM, was used

in batch reactors, for the transesterification of fat blends. Mixtures of palm oil stearin (POS), palm kernel oil (PK) and two different commercial concentrates of triglycerides enriched in ω-3 polyunsaturated fatty acids, EPAX 4510TG and EPAX 2050TG, were used for the production of margarines basestocks.

Runs were carried out with values of reaction medium composition, temperature *(T)* and reaction time *(t)* given by a central composite rotatable design (CCRD).

The free fatty acids (FFA) were assayed. These are released in fat hydrolysis and in the first step of lipase-catalysed transesterifications. An increase in FFA was observed and the final values varied between 5.0 % and 9.5 %, as compared to values lower than 0.5 % in the initial blends. FFA formation was independent of the medium composition, and also of reaction temperature and time. This suggests that the production of FFA is mainly a result of the mechanism of lipase-catalysed transesterification. However, the formation of oxidation products was negligible during the time course of the reaction.

The interchange of acyl groups amongst glycerides was followed by non-aqueous reverse-phase high performance liquid chromatography (HPLC) with refractive index detection. "Consumption" of TG was accompanied by the formation of other TG and also mono and diglycerides, depending on the initial medium composition and reaction conditions. When the transesterified fat was treated with alumina, the peaks corresponding to the formation of mono and diglycerides disappeared.

Supported by the FEDER and Fundação para a Ciência e Tecnologia, Portugal, (Research Project POCTI/39168/AGR/2001).

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P010 LIPASE-CATALYZED ESTERIFICATION OF GLYCEROL WITH ALIPHATIC AND SUBSTITED AROMATIC ACID ANHYDRIDES

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Keywords: *Candida antarctica*, esterification, lipase, monoacylglycerol, organic solvent

Due to their emulsifying activity and wide antimicrobial spectrum, monoacylglycerols (MAGs) are by far the most commonly used surfactants[1]. They are also useful chiral synthons and a perspective material for the nanotechnology[2]. Currently, there have been a great interest in the synthesis of optically active monoacylglycerols by enzymatic reactions[3,4]. The use of lipases for this purpose has become an attractive alternative to the bulky chemical asymmetric synthesis.

Recently, we have reported an efficient synthesis of *(R)*-α-monobenzoyl glycerol (MBG) by using carrier-fixed Chirazyme® L-2 *(Candida antarctica)* in 1,4-dioxane as the most suitable solvent and benzoic anhydride as the acyl donor[4]. After optimization of reaction conditions we have synthesized *(R)*-α-MBG with high optical purity in a large scale[5]. Since this reaction does not cause the formation of water or alcohol, it has been completely shifted towards the esterification. By this reason, we have found benzoic anhydride is more effective as an acyl donor for synthesis of MAGs than some alkanoic and aromatic acid esters[6]. We therefore expected that the replacement of the acyl donors with their anhydrides would increase the yield and optical purities of MAGs produced by the enzyme reaction. In this study we examined various aliphatic and aromatic anhydrides $(RCO)_2O$ as substrates for the lipase-catalyzed esterification of glycerol (Fig. 1). We synthesized several anhydrides (R = cyclohexane, $Ph(CH)_2$, $PhCH_2$, *p*-Me-Ph, *p*-MeO-Ph, *p*-Cl-Ph, *p*-NO_2-Ph) and used another commercially available ones (R = Ph, Me, Et, *n*-Pr, *i*-Pr, *n*-Bu, *i*-Bu, *t*-Bu). Under optimal conditions (100 mM glycerol, 100 mM acid anhydride, dioxane, 15 °C) Chirazyme® L-2 was active towards all the anhydrides to give the corresponding MAGs. The enzyme preferred acting on the aliphatic substrates, especially acetic and propionic anhydrides which afforded the short-chain monoacylglycerols. The yields and enantiomeric excess were determined for aliphatic MAGs by GLC or HPLC without derivatization and for aromatic acylglycerols by HPLC of their acetonides. The authentic samples were synthesized by reaction of *(R,S)*-solketal and acid anhydrides followed by suitable deprotection of the resulted compounds.

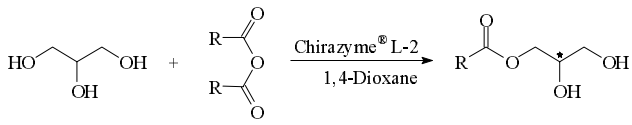

Fig 1. **Enzymatic esterification of glycerol by Chirazyme® L-2**

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P011 LIPASE-CATALYZED DIRECT CONDENSATION OF L-ASCORBIC ACID AND FATTY ACIDS IN IONIC LIQUIDS WITH ASSISTANCE OF HYDROPHOBIC ADDITIVES

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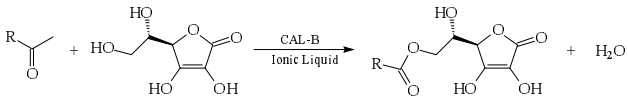
Keywords: ionic liquid, lipase, L-ascorbyl fatty acid ester

L-Ascorbic acid (Vitamin C) is a potentially useful compound for food and cosmetic chemistry because of its high reducing activity. However, it dissolves only in water and thus cannot be used in applications that require solubility in fats. Modification to its fatty acid esters would enable its use as an antioxidant. Researchers synthesized L-6-*O*-ascorbyl fatty acid esters under a mild reaction conditions such as lipase-catalyzed esterification, but the poor solubility of L-ascorbic acid in nonpolar organic solvents prevented an efficient synthesis. Polar ionic liquids permit a lipase-catalyzed esterification with high conversion because they readily dissolve L-ascorbic acid (e. g. ~ 130 mg.ml^{-1} in sBMIM × BF_4 at 60 °C) and to some extent fatty acids, but do not inactivate the lipase. The product, L-ascorbyl fatty acid esters, inhibited the reaction by precipitating on the lipase particles. To avoid a product inhibition, we added a hydrophobic additive such as hexane or polypropylene. With assistance of these additives, lipase B from *Candida antarctica* (immobilized on macroporous acrylic resin) catalyzed a direct esterification of ascorbic acid with 83 % conversion and 65 % yield to produce L-ascorbyl 6-oleate.



R = $(CH_2)_7CH=CH(CH_2)_7CH_3$

P012 CORK POLYESTERS AND THEIR BUILDING BLOCKS FROM PLANT OILS BY ENZYME CATALYSIS

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Keywords: cork, long chain dicarboxylic acid derivatives, synthesis, Novozym 435®

Long chain unsaturated, epoxidized and hydroxylated dicarboxylic acids as well as ω-hydroxy carboxylic acids are polymer building blocks of polyesters occurring in suberin, the main constituent of cork from cork oac[1]. We studied the synthesis of these and analogous structures and further unusual polyesters on the base of vegetable oils.

Natural unsaturated fatty acid methyl esters obtained from plant oils like rapeseed oil, crambe oil or castor oil were converted by metathesis with ethylene or pyrolysis to terminal unsaturated fatty acid esters (C_{10}, C_{14} and C_{11}). Cometatheses of these esters with equimolar amounts of their analogous alcohols yielded symmetrically unsaturated diols, hydroxy fatty acids and dicarboxylic acid derivatives (ratio: 1/2/1). The internal unsaturated derivatives were epoxidized using our new method of chemo-enzymatic epoxidation with methyl acetate/H_2O_2 and Novozym 435®, the immobilized lipase B from *Candida antarctica*, as biocatalyst[2].

Enzymatic hydrolysis of the epoxides under catalysis of epoxide-hydrolase which was prepared from soybeen as aceton-powder, demonstrate that this method can be used for selective epoxide-ring-opening with water. Alternatively the preparative synthesis of the diols could be achieved by dihydroxylation of the olefins using formic acid/H_2O_2 and following alkaline saponification. Similar to our enzymatic polycondensation of long chain dicarboxylic acids with diols[3], the mixtures mentioned above were polycondensated using Novozym 435® as catalyst. Due to the selectivity of the enzyme and equimolar amounts of acid- and primary hydroxyl-groups in the mixtures of diol-, hydroxy fatty acid- and dicarboxylic acid derivatives, polycondensation reactions led to linear polyesters (Mw up to 55,000 g.mol^{-1}).

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P013 LIPASE-MEDIATED PREPARATION OF CHIRAL BUILDING BLOCKS FOR TERPENES

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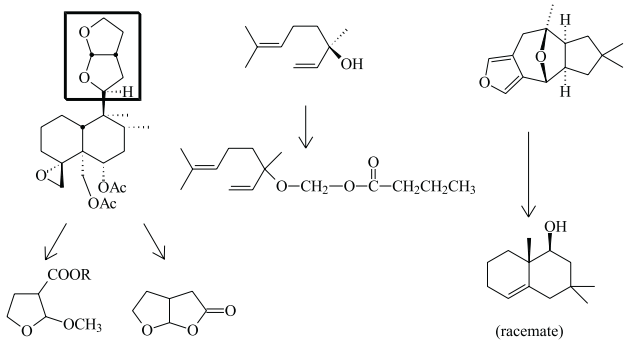
Keywords: terpenes, chiral building blocks, lipases

Terpenes are one of the major classes of secondary metabolites, widespread in the animal and plant kingdom as well as in microorganisms. Their natural functions range from chemical communication (*e. g.*, fragrances of flowering plants) to chemical defence (*e. g.*, insect antifeedants). Isolation from Nature is often not feasible for practical applications, so their total synthesis has been studied extensively. Almost all terpenes have one or more chiral carbon atoms and their absolute configuration is usually essential for biological activity, so the total syntheses have to be stereoselective.

This poster summarises our work on the lipase-mediated kinetic resolution of chiral building blocks for the synthesis of several terpenes.



P014 BIOGENERATION OF LIPOPHENOL COMPOUNDS IN HEXANE MEDIUM USING SELECTED LIPASES AND SUBSTRATE MODELS

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Keywords: phenolic compounds, triacylglycerols, lipophenols, lipases, esterification

Lipophenols are products of an esterification reaction between phenolic compounds and fatty alcohols, fatty acids or triacylglycerols. Since phenolic acids are hydrophilic, they exhibit little stability and solubility in different organic solvent systems and therefore have limited antioxidant efficiency in stabilizing oils and fats[1]. However, enzymatic esterification of phenolic compounds with free fatty acids and their acylglycerol esters could therefore be used to modify these physical properties by producing lipophenols with different solubility characteristics and making them more useful as food lipophilic antioxidants and emulsifiers[2]. Enzymes can act as biocatalysts in nearly anhydrous organic solvents, which is due to the fact that the water needed for enzymatic activity is bound tightly to the enzyme molecule even the bulk water is replaced with organic solvent[3].

These lipophenols have the potential to become excellent nutraceutical and functional products since they would posses the combined health benefits of polyunsaturated fatty acids and the phenolic antioxidant activity.

The biogeneration of lipophenols by enzymatic esterification of phenolic compounds, catechin and catechol, with the fatty acid caprylic and its triacylglycerol ester, tricaprylin, in organic solvent medium model, hexane, was investigated. Commercial lipase obtained from *Rhizopus niveus* (Lipase N) as well as immobilized ones obtained from *Mucor miehei* (Lipozyme IM) and *Candida antarctica* (Novozym 435) were used in throughout this study.

In order to determine the optimal conditions for enzymatic biocatalysis, the effect of reaction time, incubation temperatures and agitation speeds on enzyme activity were investigated. The optimal temperature for lipase activity was determined to be 37.5 °C for Lipase N and 55 °C for Lipozyme IM and Novozym 435. Maximum hydrolysis of tricaprylin, 1.7 μmol free fatty acid per ml, was obtained after 1.5 days of reaction time with Lipase N. However, Lipozyme showed higher hydrolytic activity after 1 and 4 days of reaction time with 8.1 and 8.8 μmol free fatty acid per ml, respectively, whereas Novozym 435 showed such activity after 2 and 9 days of reaction time, with 4.0 and 6.1 μmol free fatty acid per ml, respectively.

The high-performance liquid chromatography (HPLC) analysis indicated that there was no formation of lipophenol molecules from tricaprylin and catechin as substrates when Lipase N was used as biocatalyst. However, there were 47.8 and 37.3 % relative esterification yields using Lipozyme IM and Novozym 435 after 4 and 8 days of reaction time, respectively.

The use of caprylic acid and catechin as substrates resulted in relative esterification yields of 44.0 and 54.0 % after 3 and 1 days of reaction time, when Lipozyme IM and Novozym 435 were used, respectively, as biocatalysts. The relative esterification yields of tricaprylin and catechol, used as substrates, were 12.5 and 33.7 % after 8 days of reaction time using Lipozyme IM and Novozym 435, respectively, as biocatalysts. In addition, the use of caprylic acid and catechol, used as substrates, resulted in relative esterification yields of 26.3 and 70.4 % after 1 and 6 days of reaction time, using Lipozyme IM and Novozym 435, respectively, as biocatalysts.

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P015 A DIRECT ENZYMATIC ROUTE TO ENANTIOPURE ALICYCLIC β-AMINO ACIDS

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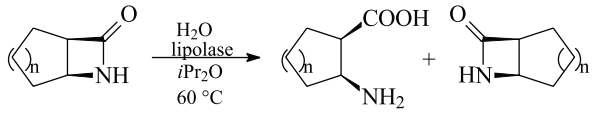
Keywords: cispentacin, β-amino acid, β-lactam, enantioselective ring-opening, Lipolase

The β-amino acids and β-lactams are of biological and chemical importance[1]. They can be used as building blocks for the synthesis of modified peptides with increased activity and stability, and in drug research. Consequently, in the past few years a large number of syntheses have been developed for enantiopure alicyclic β-amino acid derivatives. One good possibility for the preparation of enantiopure unactivated β-lactams is an enantioselective enzyme-catalyzed hydrolysis of β-lactams[2].

We now report a highly efficient and very simple method for the enantioselective ring opening of unactivated alicyclic β-lactams (±)–**1** – (±)–**4** (n = 1, 2, 3, 4), yielding the ring-opened valuable β-amino acids **1a** – **4a** (e. g. cispentacin) and unreacted β-lactam enantiomers **1b** – **4b** in an organic medium.



(±)-1 - (±)-4 **1a - 4a** **1b - 4b**

High enantioselectivity (*E* > 200) was observed when the Lipolase (lipase B from *Candida antarctica*)-catalyzed reactions were performed with H_2O (1 equiv.) in diisopropyl ether at 60 °C. The products, obtained in good chemical yield (36–47 %), could be easily separated. Transformations by ring opening of β-lactams **1b** – **4b** with 18 % aqueous HCl resulted in the corresponding β-amino acid hydrochloride enantiomers (ee ≥ 99 %).

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P016 APPROACH FOR THE DYNAMIC KINETIC RESOLUTION OF CYCLIC α-AMINO ESTERS

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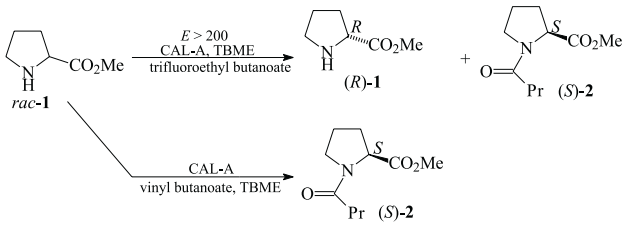
Keywords: DKR, CAL-A, amino acid, resolution

The present research introduces a dynamic kinetic resolution method based on the acylation of the amino group of amino acids with vinyl butanoate catalysed by *Candida antarctica* lipase A (CAL-A) in *tert*-butyl methylether. Racemization is induced by acetaldehyde through Schiff's base formation. Inexpensive and readily available methyl ester of proline (*rac*-**1**) was chosen as a model compound based on our previous work with methyl pipecolinate[1].



In order to develop a satisfactory method of dynamic kinetic resolution where product is formed with high yield and enantiomeric excess, the resolution reaction should be highly enantioselective and racemization of the starting material should take place fast enough. The normal kinetic resolution of *rac*-**1** with trifluoroethyl butanoate by CAL-A showed excellent enantioselectivity (*E* > 200). This prompted that also the resolution with vinyl butanoate could proceed with high enantioselectivity. In this case the racemizing agent, acetaldehyde, is elegantly released during the course of the reaction. Under optimised conditions the resolution led to highly enantiopure (ee = 97,5 %) product (*S*)-**2** with 90 % yield.

The developed method was also tested with other amino acids. The results and mechanisms are presented and discussed in the poster.

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P017 ACTIVATION AND PEPTIDE BOND FORMATION BY LIPASE-CATALYZED ACYL TRANSFERS

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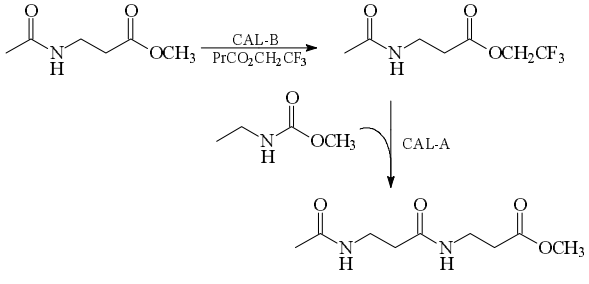
Keywords: lipase, enantioselective, dipeptide, kinetic resolution

Enzymes have gained acceptance as asymmetric catalysts in organic chemistry, especially with the aim of the preparation of enantiopure compounds. Our focus has long been on the lipase-catalyzed kinetic resolution of various types of β-amino esters employing lipases as catalysts[1–3]. In these reactions, CAL-A (lipase A from *Candida antarctica*) and CAL-B (lipase B from *Candida antarctica*), two lipases, which behave in highly different manners, have been especially useful. Thus, CAL-A is exceptionally effective and enantioselective for asymmetric acylation of β-amino esters in organic solvents whereas CAL-B shows various degrees of chemo- and enantioselectivity under the same conditions, substrate structure being a tool to control between the acylation of the amino group and interesterification of the ester group.

Enzymatic peptidomimetics synthesis is ever gaining greater significance because of the potential applications in pharmaceutical industry. In this work, we now report the application of CAL-A and CAL-B catalyses on the preparation of dipeptides through reactions where an amino ester is first activated by lipase-catalyzed interesterification with 2,2,2-trifluoroethyl ester and then a peptide bond is created by the lipase-catalyzed acylation of the amino group of another amino ester. The strategy of peptide synthesis is from *N*-terminus to *C*-terminus as is shown below by using achiral β-alanine methyl ester as a model substrate. Racemic and enantiopure β-amino esters as substrates will be described.



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P018 "EASY ON-EASY OFF TECHNOLOGY": A FULLY ENZYMATIC METHOD FOR KINETIC RESOLUTION OF CHIRAL AMINES

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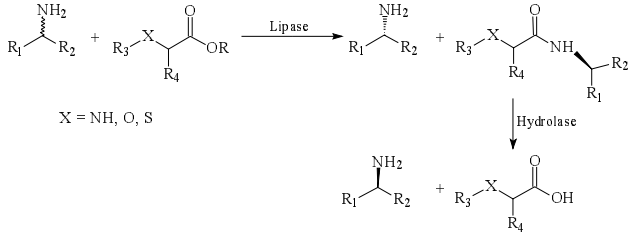
Keywords: enzymatic amine resolution, lipase, hydrolase

Enantiopure amines are used in the fine chemical industry as resolving agents, chiral auxiliaries and chiral synthetic building blocks. Some common methods to produce enantiopure amines are the crystallisation of diastereomeric salts and enantioselective reductive amination. Enzymatic resolution through enantioselective acylation, is another well-developed method. However, the chemical deacylation step to liberate the free chiral amine requires harsh reaction conditions that sometimes are incompatible with certain amines.

"Easy On-Easy Off Technology" is a newly elaborated and fully enzymatic strategy for chiral amine resolution, which involves a lipase-catalyzed acylation followed by a hydrolase catalyzed deacylation[1,2]. The enzymatic cleavage of the amide is quite facile due to the activating effect of the heteroatom in the acyl moiety. Thus, some results of amines resolution using this method will be presented.



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P019 CAL-B CATALYZED RESOLUTION OF 2-PHENYLCYCLOALKANAMINES

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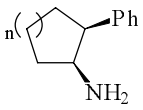
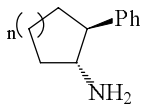
Keywords: resolution, cycloalkanamines, aminolysis, lipase

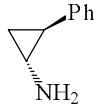
2-Phenylcycloalkanamines are interesting compounds because of their pharmacological properties and synthetic applications. For example, *cis* and *trans*-isomers of 2-phenylcyclopentanamine and 2-phenylcyclohexanamine are building blocks of potent hypoglycemic agents[1] and potentiators of AMPA receptors[2]. In addition, *trans*-2-phenylcyclopentanamine, "cypenamine", and 2-phenylcyclopropylamine, "tranylcypromine" are well-known antidepressives. In most cases, the pharmacological activities of these amines are related to the configuration of the stereogenic centers.



(±)-**1** : n=1
(±)-**3** : n=2



(±)-**2** : n=1
(±)-**4** : n=2



(±)-**5**

In the last years, we have investigated the utility of some lipases, specially, the lipase B from *Candida antarctica* (CAL-B) to catalyze the aminolysis of esters[3,4]. In these processes, optically active esters, amines and amides have been obtained. In the present work, we describe the resolution of some racemic 2-phenylcycloalkanamines following the methodology developed in our research group. Thus, the resolution of (±)–**1-5** was carried out by CAL-B catalyzed enantioselective acylation, using the most simple reaction conditions, that is, employing ethyl acetate as the acyl donor and solvent. Under these conditions the enzyme catalyzed acylation of the *(R)*-enantiomer of the amine preferentially, the resulting *(R)*-acetamides and the remaining *(S)*-amines being easily separated by selective extraction and isolated in very high yields (>85 %).

From the conversion values and enantiomeric excesses showed in Table I, it can be deduced that *trans*-isomers **2** and **4** are more suitable substrates for the enzyme in comparison with the *cis*-isomers **1** and **3**. In addition, the size of the cycle plays a key role in the enantioselectivity of these reactions, cyclopentanamines being transformed with higher *E* values than the corresponding cyclohexyl analogous. It is of note the high rate of conversion and the low *E* value achieved in the reaction with 2-phenylcyclopropanamine.

Table I
Conversion values and enantiomeric excesses of **1–5**

Amine	*t* (h)	*c* (%)	remaining susbstrate	E. e.[a]	acetamide	E. e[a].	*E*[b]
(±)–**1**	24	28	*(S,S)*-1	33	*(R,R)*-**6**	85	16
(±)–**2**	6.5	50	*(S,R)*-2	98	*(R,S)*-**7**	97	>200
(±)–**3**	47	5	*(S,S)*-3	1	*(R,R)*-**8**	13	1
(±)–**4**	20	41	*(S,R)*-4	67	*(R,S)*-**9**	98	200
(±)–**5**	2	90	*(S,R)*-5	96	*(R,S)*-**10**	11	3

[a] determined by chiral HPLC,
[b] enantiomeric ratio calculated according to Sih et al.[5]

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P020 TWO CHEMOENZYMATIC SYNTHESES OF BOTH ENANTIOMERS OF *trans*-CYCLOPENTANE-1,2-DIAMINE

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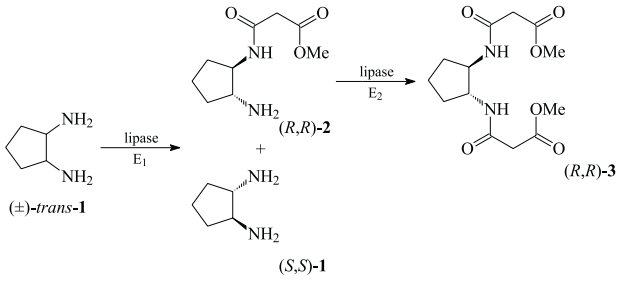
Keywords: diamines, enzymatic resolution, lipases

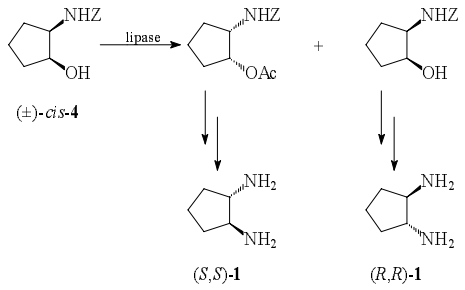
In recent years, chiral synthetic diamine derivatives have been employed as medicinal agents, in particular in chemotherapy[1]. Their use in organic synthesis has also increased considerably, specially in the field of catalytic asymmetric induction[2]. The easy availability of both enantiomers of the target compound in very high ee is one of the limitations for the use of some optically active diamines. Lipase-catalyzed kinetic resolution of racemates is one the most frequently used strategies for the preparation of optically active compounds[3]. Here we describe two different approaches to enantiomerically pure *trans*-cyclopentane-1,2-diamine **(1)**. The first one (Scheme 1) is through a one-pot sequential biocatalyitc resolution of racemic diamine. This strategy had been successfully used for the preparation of optically active *trans*-cyclohexane-1,2-diamine[4]. The enantioselectivities[5] of both steps have been studied and we conclude enantiopure products cannot be obtained with the first aminolysis reaction

(E_1 = 21), but the second biocatalytic process (E_2 > 200) is enantioselective enough for obtaining substrate and product both in enantiomerically pure forms[6].



Scheme 1

On the other hand, we had previously described the efficient resolution of (±)-*cis*-*N*-Cbz-2-aminocyclopentanol[7]. In this case product and substrate were recovered in 99 % ee. We carried out an alternative chemoenzymatic synthesis of **1** using these enantiomers as starting material (Scheme 2).



Scheme 2

Further synthetic applications of the enantiomerically pure obtained compounds are under investigation in our laboratory.

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P021 CALB-CATALYZED ASYMMETRIC AMINOLYSIS AND AMMONOLYSIS OF PROCHIRAL GLUTARATES

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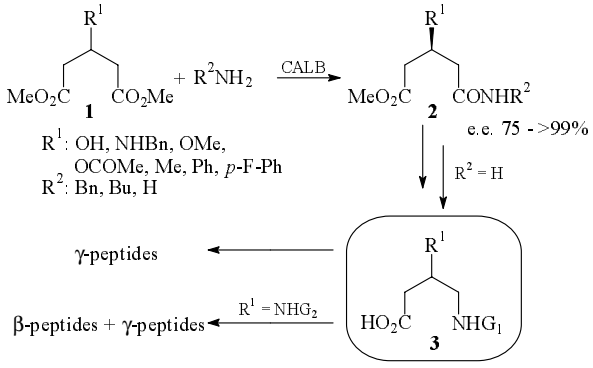
Keywords: desymmetrization, glutarates, ammonolysis, aminolysis, lipase

The enzymatic desymmetrization of *meso* and prochiral compounds constitutes an elegant approach to the synthesis of enantiomerically pure compounds[1]. This methodology has attracted considerable interest as it avoids the inherent 50 % yield limit and the difficult separations often encountered in the resolution of racemates. Enzymatic hydrolysis, transesterification and lactonization of prochiral diesters and diols have been largely applied to prepare chiral synthons in high enantiomeric excesses. However, the potential of enzymes to catalyze the aminolysis and ammonolysis of prochiral substrates has been recently reported by us. Lipase B from *Candida antarctica* (CALB) has shown to be an exceedingly effective catalyst either for the asymmetric aminolysis and ammonolysis of dimethyl 3-hydroxyglutarate (R^1 = OH, Scheme), thus affording enantiopure monoamidation products in very high yield[2].

Following on from these studies, we envisioned to generalize this methodology to different dimethyl 3-substituted glutarates, **1**, and to study the scope of this biocatalytic process as well as the effect of such substitution in the efficiency and enantioselectivity of the reaction.



CALB always showed a clear preference toward the *pro-R* ester group, leading to monoamides of *S* configuration, **2**. Diamides were never detected. However, both chemical yield and enantiomeric excesses strongly depends on the substrate structure (R^1) and, in a lesser extent, on the nucleophile (R^2). Glutarates with a heteroatom at C-3 position with possible hydrogen bonding acceptor behaviour, showed higher chemical yields and e.e.'s. In all the tested conditions, dimethyl 3-methylglutarate showed the poorer results. Aromatic derivatives were less reactive although high e.e.'s can be obtained when the reaction conditions are optimized.

Optically active monoamides **2** (R^2 = H) are interesting chiral compounds for the preparation of different *β*-substituted *γ*-amino acids **3**, which are useful starting materials in the synthesis of non-natural *β*- and *γ*-peptides.

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P022 KINETIC RESOLUTION OF NITRO-ALDOL ADDUCTS

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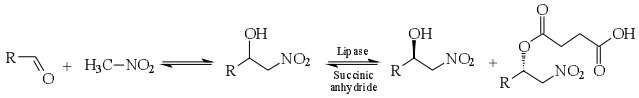
Keywords: β-nitro alcohols, chiral synthesis, succinic anhydride

The aldol reaction is one of the most important methods for C-C bond formation. An aldol-type condensation between an aldehyde and a nitro alkane (Henry reaction) will result in a β-nitro alcohol. The control of the formed stereochemistry is of importance for many synthetical purposes. Two routes that can be followed are direct chiral synthesis from prochiral substrates or resolution of the formed secondary alcohol.



Chiral nitro alcohols can be reduced to the hydroxy amines or undergo further C-C bond formation first on the α-carbon, giving acces to a wide variety of important chiral intermediates.

In this work the applicability of lipase catalysed resolution is studied for obtaining enantiopure β-nitro alcohols. The use of succinic anhydride as the acyl donor has some benefits for downstream product separation and showed much higher enantiomeric ratios for this type of components compared to other commonly used donors like vinyl acetate.

P023 ENANTIOSELECTIVE SYNTHESIS OF 1,4-DIHYDROPYRIDINE DERIVATIVES USING LIPASES

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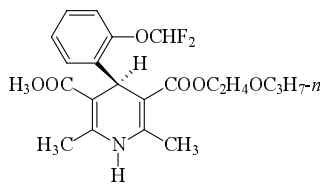
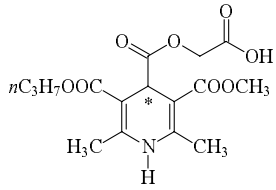
Keywords: 1,4-dihydropyridines, lipases, asymmetrisation, kinetic resolution

1,4-Dihydropyridines (1,4-DHPs) are an important class of drugs since they possess a wide range of biological activities. Since it is well known that enantiomers differ in their biological effects, we have been studying chemoenzymatic routes towards enantiopure building blocks for chiral 1,4-DHPs.

Since ester groups that are connected directly to the 1,4-DHP ring are not reactive towards enzymes, we have used spacers attached to a hydrolysable group in order to turn 1,4-DHPs into substrates[1]. We have found before that the $-CH_2O-$ spacer is advantageous in these reactions, since it splits off formaldehyde spontaneously after hydrolysis of the adjacent ester. In the same studies[1], an isobutyric acid ester was found to give the highest stereorecognition, because it has the optimum amount of steric hindrance. This spacer and ester are now used in a new versatile route for making 4-[2-(difluoromethoxy)phenyl]-1,4-DHP-3,5-diesters, as well as in some studies on 1,4-DHP-3,4,5-triesters (isonicotinic acid derivatives).



4-[2-(difluoromethoxy)phenyl] 1,4-DHP-3,5-diester



1,4-DHP-3,4,5-triester

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P024 *Candida rugosa* LIPASE-CATALYSED KINETIC RESOLUTION OF POLYCYCLIC ANALOGUES OF 1,4-DIHYDROPYRIDINES

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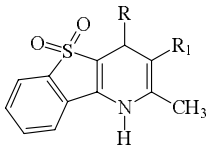
Keywords: 1,4-dihydrobenzthieno[3,2-b]pyridine-5,5-dioxide, 1,4-dihydropyridine, kinetic resolution, lipase

1,4-Dihydropyridines (1,4-DHPs) are an important class of calcium channel antagonists. Novel activities of 1,4-DHPs, such as neuroprotective, antineurodegenerative, cognition and memory enhancing, antidiabetic, anti-inflammatory and antiviral have been also described. Chirality plays an important role in the biological activity of 1,4-DHPs. The use of enantiopure and racemic drugs is regulated by specific rules in EC (ref.[1]). Classical Hantzsch synthesis of 1,4-DHPs is not enantioselective, so enantioselective synthetic methodologies have to be developed.

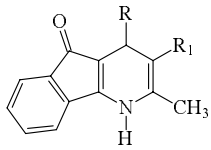
Polycyclic analogues of 1,4-DHPs in enantiopure form are desired for extended pharmacological studies, since racemic 1,4-dihydrobenzothieno[3,2-*b*]pyridine-5,5-dioxides **1** and 5-oxo-4,5-dihydro-1,4-indeno[1,2-*b*]pyridines **2** have exhibited coronary dilating and anticancer activities; they have been also found as active glutathione *S*-transferase inhibitors.

The standard resolution technique of monocyclic 1,4-DHPs, such as incorporation of an enzymatically labile acyloxymethyl group[2–4] has been successfully applied for the kinetic resolution of 3-(isobutyryloxy)methyl 4-[2-(difluoromethoxy)phenyl]-2-methyl-5,5-dioxo-1,4-dihydrobenzothieno[3,2-*b*]pyridine-3-carboxylate **3** (see Scheme).

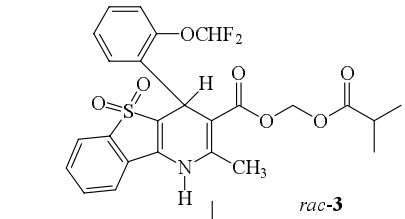
Careful screening yielded *Candida rugosa* lipase (CRL) as the preferred biocatalyst. A remarkably good *E*-value was obtained for the CRL-catalysed resolution of this bulky 1,4-DHP derivative. The enantioselectivity of *C. rugosa* lipase can be improved by changing the reaction medium and the temperature. The transition from water-saturated IPE to a solution of *n*-butanol in toluene that was water-saturated at 45 °C resulted in an increase of enantiomeric ratio from $E = 12$ to $E = 28$. More derivatives of the polycyclic 1,4-DHP will be studied.



1

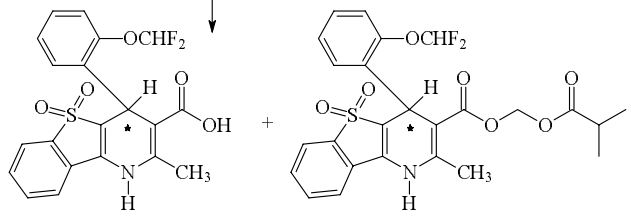


2



rac-**3**

Candida rugosa lipase E = 28



(-)-**4** (+)-**3**

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P025 ENZYME-CATALYZED KINETIC RESOLUTION OF PIPERIDINE HYDROXY-ESTER REGIOISOMERS

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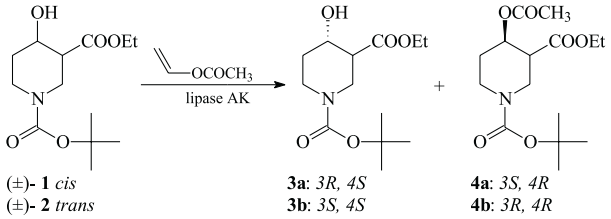
Keywords: piperidines, regioisomers, kinetic resolution, organic solvents, lipase AK

Esters and hydroxyesters possessing piperidine ring among alkaloids and their analogues can modify the neurotransmission resulting in wide-ranging effects on the central nervous system[1, 2].

Our aim was to obtain all four enantiomers of 1-(*tert*-butyl) 3-ethyl 4-hydroxypiperidine-1,3-dicarboxylate **(1, 2)** and of its regioisomer, 1-(*tert*-butyl) 4-ethyl 3-hydroxypiperidine-1,4-dicarboxylate through lipase-catalyzed kinetic resolution in organic media.



High enantioselectivity (E > 200) was observed in the acylation reaction of (±)-*cis* and *trans* 1-(*tert*-butyl) 3-ethyl 4-hydroxypiperidine-1,3-dicarboxylate **(1, 2)** using vinyl-acetate as acyl donor and *Pseudomonas fluorescens* lipase (lipase AK) as a catalyst in diisopropyl ether at 45 °C. In the lipase AK-catalyzed acylation of the sterically more hindered (±)-*cis* 1-(*tert*-butyl) 4-ethyl 3-hydroxypiperidine-1,4-dicarboxylate no product formation was observed even at higher temperature (60 °C). From the lipases screened only *Candida antarctica* A catalysed the reaction with low enantioselectivity.

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P026 SYNTHESIS OF ENANTIOMERS OF PROLINE-RELATED COMPOUNDS *via* HYDROLYTIC ENZYME-CATALYZED KINETIC RESOLUTION

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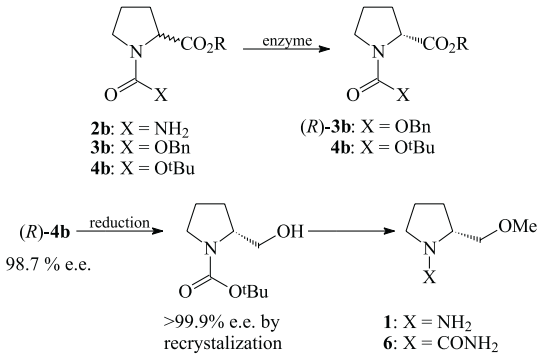
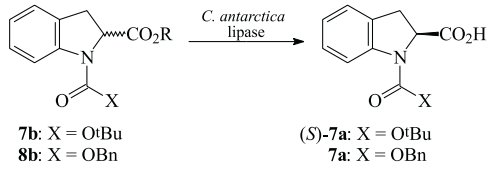
Keywords: *C. antarctica* lipase, protease, cyclic amino acid, hydrolysis, kinetic resolution

We examined approaches towards the preparations of SAMP and RAMP (1-amino-2-methoxymethylpyrrolidine **1**, important chiral auxiliaries) *via* enzyme-catalyzed enantiomeric resolution of racemic intermediates. *B. licheniformis* protease (subtilisin) preferentially hydrolyzed the (*R*)-carbamoylproline ester **(2b)** with an enantiomeric ratio *(E)* of 10. To a hydrophobic *N*-Cbz proline ester **(3b)**, subtilisin showed lower selectivity (E 2.8), and contrary to that, a purified earthworm protease isozyme *(Lumbricus rubellus)* showed the preference of (*S*)-enantiomer (E 13.6). Diverse enantiomeric preference was observed between purified isozymes. In turn, *C. antarctica* lipase B (Chirazyme L-2) was effective for the enantioselective hydrolysis of *N*-Cbz and *N*-Boc proline esters **(4b)** with E > 100 (*S* preferred). The methyl ester **(4b)** was obtained in 49 % yield (98.7 % ee) through a preparative-scale enzyme-catalyzed resolution.



C. antarctica lipase-catalyzed hydrolysis could also be applied on the bicyclic substrates. Enantioselectivities (E >100) were shown in the case of the hydrolysis of *N*-Boc **(7b)** and *N*-Cbz indoline-2-carboxylic esters **(8b)**. An elevated reaction temperature over melting point of the substrate was required, so that the reaction proceeds promptly up to nearly 50 % conversion, to give enantiomerically pure acid **7a** (49 % yield, >99.9 % ee) and ester **7b** (50 % yield, 97.6 % ee).



P027 LIPASE-CATALYSED RESOLUTION OF CYCLIC β-HYDROXYESTERS

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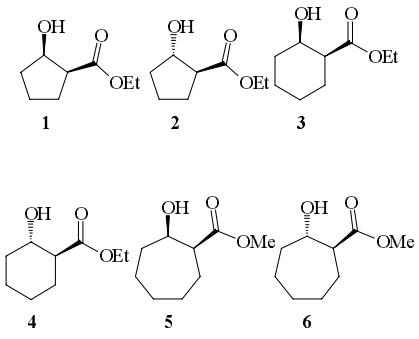
Keywords: lipase, resolution, *Candida antarctica*, cyclic β-hydroxy esters

The importance of optically active β-hydroxy acid derivatives as versatile building blocks for the synthesis of biologically active compounds is well established[1]. Amongst them,

those bearing a substituent in α-position are especially interesting, since they are bifunctionalised molecules with two vicinal stereogenic centres. Biocatalytic approaches to these compounds have been mainly *via* enantio- and diastereoselective reduction of the corresponding β-keto esters[2], amides or nitriles using whole cells, or by kinetic resolution of the racemic mixtures with hydrolases (*i. e.* lipases or esterases)[3]. Although in recent years, a number of elegant strategies have been developed to address the problems associated with the above mentioned methods, β-hydroxy acids are still not readily available. Hence, an efficient methodology for their preparation is still required.



Encouraged by the excellent results obtained in our research group in the enzymatic kinetic resolution of cyclic amino alcohols[4] and diamines[5], over the last few years, we decided to examine the lipase-catalysed enantioselective acylation of the cyclic β-hydroxy esters **1-6** . The resolution of the racemic alcohols (±)–**1-6** was carried out by CALB-catalysed enantioselective acylation using vinyl acetate (VA) as the acyl donor and *tert*-butyl methyl ether as the solvent. In all cases the acyl donor was used in moderate excess (molar ratio VA: alcohol 3 : 1), and the temperature was kept at 30 °C. This acylation procedure afforded excellent enantioselectivities (E = 200) reaching in all cases (apart from substrate (±)–**3**) the maximal conversion of 50 %, at which both substrate and product were isolated enantiomerically pure in high yields. Quite surprisingly, the activity of CALB towards substrate (±)–**3** was very low (and thus unsatisfactory for preparative purposes). After two days, a conversion of only 5 % was reached, although it should be pointed out that this enzyme showed again complete enantiodiscrimination, (product (+)–**3** was obtained in >99 % ee. After testing a number of different solvents and higher temperatures without success, we considered the possibility of using a biocatalyst whose binding pocket can accept more sterically hindered substrates. Another isozyme from *Candida antarctica* (CALA) belongs to the group of lipases which are highly active towards tertiary alcohols. Very satisfyingly, under the reaction conditions described above for CALB-catalysed resolution, a 34 % conversion was reached after just 30 minutes affording product (+)–**3** in >99 % ee and the remaining substrate in 51 % ee.

Thus, we have shown that lipases from *Candida antartica* are excellent biocatalysts for the enantioselective transesterification of five to seven-membered ring β-hydroxy esters. This constitutes for the first time, a direct access to all four stereoisomers of these interesting building blocks in enantiopure form and high yield.

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P028 CHEMOENZYMATIC SYNTHESIS OF OPTICALLY PURE α-HYDROXYALDEHYDES AND KETONES

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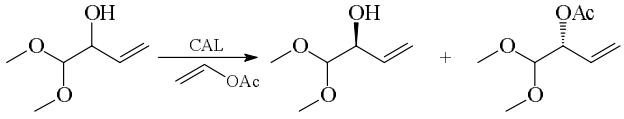
Keywords: enzymatic kinetic resolution, lipase, enol ester epoxide

We have been interested, for years, in the possibility to use transketolase and fructose-1,6-diphosphate aldolase for the production of ketoses with the (3*S*,4*R*) D-*threo* configuration[1, 2]. These enzymes accept a wide range of aldehydes as acceptor substrates. Transketolase appears very enantioselective towards α-hydroxyaldehydes. However when a racemic aldehyde is used as substrate, fructose-1,6-diphosphate aldolase gives both diastereoisomers. We have therefore decided to study the enantioselective synthesis of α-hydroxyaldehydes. Two methodologies have been developped, both based on lipase-catalysed kinetic resolution.

Alcohol **1**, easily prepared from glyoxal dimethyl acetal is a versatile synthon for applications with aldolase and trans-

ketolase catalysed reactions. This alcohol can be converted into α-hydroxyaldehydes by ozonolysis of the double bond or hydrolysis of the acetal moiety. The kinetic resolution of this product was accomplished by acylation with vinyl acetate in the presence of *Candida antarctica* lipase.



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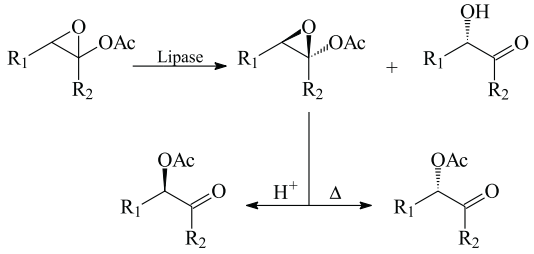
Enol ester epoxides are usefull intermediates in organic synthesis. They can rearrange to α-acyloxyaldehydes or ketones under thermal or acidic conditions[3]. The chemical enantioselective synthesis of enol ester epoxides has been reported. We studied the enzymatic kinetic resolution of these compounds. The epoxides are prepared by epoxidation of enol esters or oxidation of α,β-unsaturated ketones. The lipase-catalysed hydrolysis of enol esters epoxides gives the desired α-hydroxy carbonyl compounds. The remaining chiral epoxides can undergo stereospecific rearrangements to the enantiomerically enriched α-acyloxy carbonyl compounds.



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P029 THE ENANTIOSELECTIVITY CHANGES WITH SUBSTRATE CONVERSION DURING KINETIC RESOLUTION OF SECONDARY ALCOHOLS AND THEIR BUTANOATES CATALYSED BY CALB

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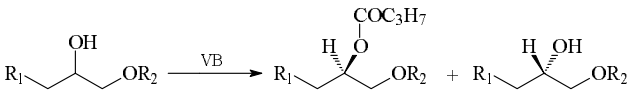
Keywords: kinetic resolution, *Candida antarctica* lipase, hydrolysis, transesterification

Enzyme catalyzed kinetic resolution is characterized by the enantiomeric ratio (*E*-value), which, according to the theory, should be constant during the reaction and independent of the degree of conversion[1]. However, *E*-values increasing with conversion, both in transesterifications[2] and hydrolysis[3, 4] have been reported.



R_1 = Me, Et, Br, Cl
R_2 = Ph, CH_2Ph

The substrate structure has been varied as follows: R_1 = = Me, R_2 = Ph **(1a, 1b)**, R_1 = Et, R_2 = Ph **(2a, 2b)** R_1 = Br, R_2 = Ph **(3a, 3b)**, R_1 = Me, R_1 = Cl, R_2 = Ph **(4a, 4b)** R_1 = = Cl, R_2 = CH_2Ph **(5a, 5b)** where **1a**-**5a** are the alcohols and **1b**-**5b** are the respective butanoates. During kinetic resolution of **1**-**5** catalyzed by *Candida antarctica* lipase B the *E*-value was found to decrease with increasing degree of conversion when the reaction was transesterification. However, when the resolution was performed as hydrolysis of the corresponding butanoates, the *E*-values increased by increasing conversion. Since the composition of the reaction mixture changes during the resolution process, the observed effect may be due to enantioselective inhibition or activation either by substrate or product.

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P030 THIOL FUNCTIONALIZATION OF ALCOHOLS BY CHEMOSPECIFIC LIPASE CATALYSIS

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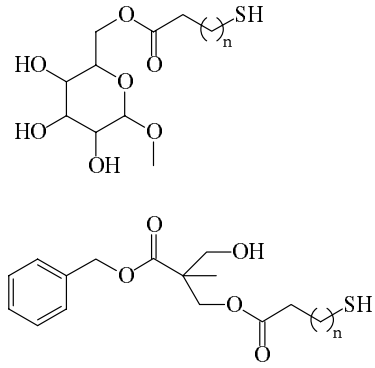
Keywords: thiol functionalization, lipase, chemospecificity

Thiols are highly interesting compounds due to their ability to form self-assembled monolayers (SAMs) on gold surfaces, with applications in areas such as biosensors and nano-electronics. They also have widespread interest in material science in form of polymers with thiol functionalities. Thiol functionalization of alcohols can be performed via ester bond formation using *Candida antarctica* lipase B. This study is focused on the acylation of methyl-β-D-glucopyranoside and the benzyl ester of bis(hydroxy)-propionic acid using 3-mercaptopropionic acid and γ-thiobutyrolactone as acyl donors. This resulted in the thiol functionalized compounds shown below. In contrast to acyl donors such as lactones and hydroxy acids no polymerisation were observed with the corresponding thiolactone and mercapto acid.



n = 1 for 3-mercaptopropionic acid, n = 2 for γ-thiobutyrolactone

P031 *Candida antarctica* B LIPASE CATALYSED ALCOHOLYSIS OF 3',5'-DIACETYL-2'--DEOXYNUCLEOSIDES

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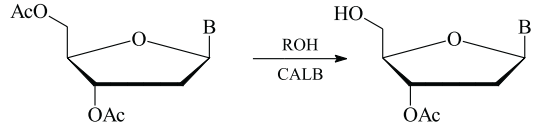
Keywords: deacylation, enzymatic alcoholysis, lipases, nucleosides

Biotransformations catalysed by hydrolytic enzymes provide convenient methods to achieve regioselective transformations in the sugar moiety of nucleosides. Most of the examples described and reviewed deal with the enzymatic regioselective acylation and alkoxycarbonylation of nucleosides[1,2]; in a lesser extent, the hydrolase-catalysed deacylation of nucleosides has also been studied, mainly through enzymatic hydrolysis[1–3].

Over the last years we have been studying the enzymatic deacylation of peracylated ribonucleosides through enzymatic alcoholysis and we have found that *Candida antarctica* B lipase catalyses efficiently the formation of the corresponding 2',3'-di-*O*-acylribonucleosides[4–6]. Taking into account these results, we considered of interest to study the *Candida antarctica* B lipase (CAL B)-catalysed alcoholysis of 2',3'-di--*O*-acetyl-2'deoxy-nucleosides **(1a-e)** :



1a-e **2a-e**

a B: Uracil
b B: Thymine
c B: Cytosine
d B: Guanine
e B: Adenine

R: C_2H_5-, *n*-C_3H_7-, *n*-C_4H_9-

According to the regioselectivity displayed by CAL B in the enzymatic acylation and deacylation of nucleosides[1–6], the selective cleavage of the 5'-*O*-acetate of the substrates was observed, giving the corresponding 3'-*O*-acetyl-2'-deoxynucleosides **2a-e** . The enzymatic butanolysis of 3',5'-di-*O*--acetyl-2'deoxyguanosine **(1d)** afforded the best result: after 48 hours at 30 °C, **2d** was formed in 96 %.

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P032 INFLUENCE OF RING D SUBSTITUTION ON LIPASE-CATALYSED DEACETYLATION OF STEROID DERIVATIVES

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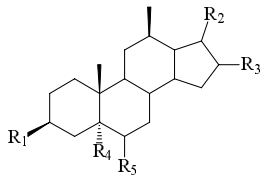
Keywords: androstane, pregnane, deacetylation, lipase-catalysed

The pharmaceutical properties and high cost of steroids made the research on these natural compounds a noteworthy achievement. In the last years, highly selective synthetic procedures for the transformation of complex polyfunctional steroids were developed. Among them, the enzyme-catalysed approach was applied succesfully in regioselective acylation and deacylation reactions producing new compounds with added value to their physiological properties[1–4]. In previous works we have reported the enzyme-catalysed alcoholysis of steroids 3β-acetates containing labile funcional groups in the molecule[5] and the regioselective deacetylation of some androstane derivatives[6]. We have observed that lipases from two yeasts catalysed the alcoholysis of acetyl groups located at different positions of the steroid skeleton. *Candida rugosa* lipase (CRL) and *Candida antarctica* lipase (CAL) have affinity for different regions of the rigid steroid molecule. While CRL removed acetyl groups situated in ring A, CAL was preferentially active on substituents located in ring D. Considering the good performance of CAL in alcoholysis of 16β-*O*-Ac with a carbonyl group at C(17) **(1a)** and the poor activity when the keto at C(17) is replaced by an acetoxy group, the activity of CAL seems to be conditioned by the occurrence of an sp^2-hybridized C-atom in ring D (ref.[6]).

To learn more about the regio and stereoleselective behavior of lipases in the deacetylation of steroids we have performed this reaction using different androstanes and pregnanes **2a**-**10a** as substrates. These substrates were prepared in our laboratory, some of them being novel steroidal compounds.



1a R_1 = AcO; R_2 = =O; R_3 = β-AcO; R_4 = R_5 = H
2a R_1 = AcO; R_2 = =O; R_3 = α-AcO; R_4 = R_5 = H
3a R_1 = AcO; R_2 = $=CH_2$; R_3 = β-AcO; R_4, R_5 = Δ^5
4a R_1 = AcO; R_2 = $=CH_2$; R_3 = α-AcO; R_4, R_5 = Δ^5
5a R_1 = TBDMSO; R_2 = $=CH_2$; R_3 = α-AcO; R_4, R_5 = Δ^5
6a R_1 = AcO; R_2 = (*E*) $=CHCH_3$; R_3 = β-AcO; R_4, R_5 = Δ^5
7a R_1 = AcO; R_2 = (*E*) $=CHCH_3$; R_3 = α-AcO; R_4, R_5 = Δ^5
8a R_1 = AcO; R_2 = (*E*) $=CHCH_2OAc$; R_3 = H; R_4, R_5 = Δ^5
9a R_1 = TBDMSO; R_2 = (*Z*) $=CHCH_2OAc$; R_3 = α-AcO; R_4, R_5 = Δ^5
10a R_1 = AcO; R_2 = (*Z*) $=CHCH_2OAc$; R_3 = α-AcO; R_4, R_5 = Δ^5

CAL was not very stereoselective in the deacetylation of position 16 of androstanes. On treatment of 16α-*O*-Ac **2a** with this lipase, the 3β-acetate of **2a**, unknown till now, was obtained in such a good yield as we had previously obtained with 3β-acetate from **1a** (ref.[6]).

The replacement of the carbonyl group by a carbon-carbon double bond at position 17 showed a remarkable decrease or no activity at all of CAL on the α and β 16-*O*-Ac of substrates **3a**-**7a** . The corresponding 3β-acetate-16-hydroxy compounds were obtained in poor yield or not obtained at all. The primary acetyl group in **8a** was easily removed by both enzymes (CAL and CRL). CAL kept its regioselectivity in the formation of the 3β-monoacetyl derivative.

In **9a** and **10a** the primary acetyl group in carbon 21 was more reactive than the 16α-*O*-Ac. This result shows that the *tert*-butyldimethylsilyloxy in 3β position **(9a)** does not produce inactivation of the enzyme.

The behavior of CRL was the expected one in all substrates being removed the acetyl group in position 3β in the acetylated derivatives. By enzymatic catalysis with CRL the *tert*-butyldimethylsilyl derivatives **5a** and **9a** remained unaltered.

In conclusion, we show that lipases from *C. rugosa* and *C. antarctica* removed acetyl groups in a regioselective fashion from di- and triacetylated androstane and pregnane derivatives. These results could lead us to say that the substitution in ring D with a polar carbon double bond such as the carbonyl group could be necessary for CAL activity. Some of the reported compounds were proved to be useful intermediates in the synthesis of biologically active steroids.

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P033 DYNAMIC RESOLUTION OF *(R,S)*-NAPROXEN 2,2,2-TRIFLUORO ESTER *via* LIPASE-CATALYZED HYDROLYSIS IN MICRO-AQUEOUS ISOOCTANE

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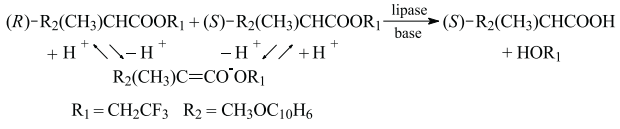
Keywords: dynamic resolution, lipases, naproxen, hydrolysis

Candida rugosa lipases immobilized on polypropylene powders were employed as the biocatalyst for the enantioselective hydrolysis of *(R,S)*-naproxen 2,2,2-trifluoroethyl ester in micro-aqueous isooctane at 45 °C, in which TBD immobilized on polystyrene crosslinked with 2 % DVB was added as an in situ racemization catalyst for the remaining *(R)*-ester (Scheme). The kinetic behaviors of the lipase and the base were first investigated, respectively, by considering of the enzyme stability and product inhibition as well as the base deactivation. The results for the dynamic resolution process were then compared with the kinetic resolution process without adding the base. The results were also compared with the dynamic resolution process by employing *(R,S)*-naproxen 2,2,2-trifluorothioethyl ester and trioctylamine as the substrate and the base, respectively, for producing the desired *(S)*-naproxen.



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P034 ENZYMATIC RESOLUTION OF TERPENOID LACTAMS TOWARDS NEW CHIRAL COMPOUNDS WITH POTENTIAL NEUROACTIVITY

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Keywords: (+)-3-carene, chiral compounds, terpenoid lactams, enzymatic resolution, pharmacological activity

Optical active lactams are valuable building blocks in organic synthesis and find wide application as synthons for biologically active compounds. It is known that some of them are resoluted into stereoisomers by lipase-catalysed transesterification[1, 2].

We have obtained the isomeric mixtures of bicyclic terpenoid lactams, starting from (+)-3-carene, naturally occurring monoterpene, component of many turpentines[3], followed by conversion into new amino acids[4]. Pharmacological studies showed that these amino acids possess neuromodulatory activity[5].

As a continuation of our investigation we have developed lipase-catalyzed resolution process for diastereoisomeric mixture of terpenoid lactams which are intermediates in the synthesis of novel chiral piracetam analogues with nootropic activity.

Screening of lipases and the effects of solvent, influence of donor group and temperature will be widely discussed with special emphasis on their biosynthetic aspects.

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P035 A NEW CHEMOENZYMATIC METHOD FOR THE SYNTHESIS OF *(S)*-(+)-ZOPICLONE

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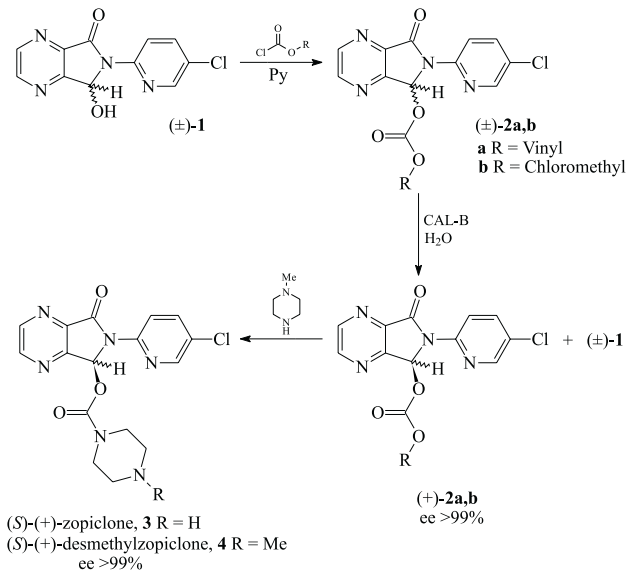
Keywords: hydrolysis, lipase, zopiclone, hypnotic, pharmaceutical industry

Racemic zopiclone is a hypnotic agent of the cyclopyrrolone class, which has been commercialized for the treatment of insomnia[1]. However, the *(S)*-configured enantiomer is more active and less toxic than the *(R)*-isomer. In a previous report we described a chemoenzymatic method for the preparation of enantiomerically pure *(S)*-(+)-zopiclone, **3** (Scheme)[2]. The key step in this procedure is the resolution of the vinyl carbonate intermediate (±)–**2a** by lipase-catalyzed hydrolysis. Although the enzymatic step is efficient, two facts make the method expensive for the kilogram preparation of enantiopure *(S)*-(+)-zopiclone. Firstly, the high cost of vinyl chloroformate as a reagent and, secondly, the low 30 % yield of the last step in the synthesis, i. e. the treatment of the vinyl carbonate (+)–**2a** with *N*-methylpiperazine.



Herein, we describe an improvement of this method based on the preparation and enzymatic resolution of a new carbonate intermediate (±)–**2b**, via *Candida antarctica* lipase B-catalyzed hydrolysis. The *(R)*-configured product **1** of the enzymatic reaction undergoes spontaneous racemization in the reaction medium. Therefore, it can be directly recycled after work-up of the enzymatic reaction. Thus, the overall formal yield of the enzymatic process is 100 %, even though this enzymatic step is a kinetic resolution process.

It is noteworthy the low cost of the reagents employed in the synthesis of (±)–**2b** as well as the high yield of the last step in the synthesis of *(S)*-(+)-zopiclone by the reaction of the enantiomerically pure carbonate (+)–**2b** with *N*-methylpiperazine.

This methodology has been also successfully used in the synthesis of *(S)*-(+)-desmethylzopiclone, **4**, a metabolite of zopiclone, which has been recently reported as an interesting compound for its biological activities in preclinical anxiolytic evaluation[3].

Taking into account the simplicity and easy scale-up of lipase-catalyzed reactions, it is noteworthy the applicability of this method to the industrial preparation of the hypnotic *(S)*-(+)-zopiclone.

Furthermore, in order to prove the economic efficiency of this biocatalytic method, we are investigating the feasibility of reusing the immobilized *Candida antarctica* lipase B.

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P036 ALCOHOL AND ACYL DONOR STRUCTURE EFFECT ON THE ENZYMATIC ACYLATION OF FLAVONOIDS

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Keywords: flavonoids, fatty acids, enzymatic, acylation, *Candida antarctica*

Flavonoids are natural compounds with interesting properties due to their antioxidant, antimicrobial, anticarcinogenic activities[1]. However, they are characterised by a low solubility and stability, which could limit their use. In view to improve these properties chemical and enzymatic acylations with lipophilic groups have been suggested[2,3]. The aim of this work is to investigate the behaviour of enzymatic acylation of different flavonoids and to study the carbon chain length effect of the acyl donor (fatty acids).

The enzymatic acylations were led in *tert*-amyl alcohol as solvent at 60 °C and with immobilized lipase of *Candida antarctica* as catalyst. The substrate used in this study are rutin, hesperidin and esculin as flavonoids and caproic, pelargonic, oenanthic, lauric, myristic, palmitic, stearic and oleic acid as acyl donor. The reactions were conducted in Chemspeed automated parallel synthesis workstation at low level of water content in the reaction medium (< 300 ppm). The substrates and products were quantified by HPLC system equipped by UV, RI and LS detectors. The structures of some obtained products were characterised by ^{1}H NMR analysis.

The enzymatic acylation of the three flavonoids tested indicated that the performance of this reaction depends of the presence or not of a primary hydroxyl group on the glycosyl moiety. The highest conversion yields was obtained with esculin and it is about of 82 %. When rutin was acylated with different fatty acids, the results showed that the conversion yield increases with carbon chain length up to C12. For higher carbon number, no effect was detected except for C14. The ^{1}H NMR analysis showed that for glycosylated flavonoids the acylation took place on the glycoside moiety.

Flavonoids acylation performance was affected by alcohol and acyl donors structure. The highest conversions (> 80 %) were obtained for glycosylated flavonoids with a primary hydroxyl group. Only fatty acids with short carbon chain affect the kinetic of this reaction. These enzymatically acylated flavonoids will be evaluated for their properties and biological activities.

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P037 ENZYMATIC PREPARATION OF FLAVONOID DERIVATIVES

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Keywords: flavonoid, antioxidant, esterification

The flavonoids constitute a large class of compounds, ubiquitous in plants, containing a number of phenolic hydroxyl groups attached to ring structures, conferring the antioxidant activity. The practical exploitation of their antioxidant properties, for instance as protective agents for fats and oils against oxidation, is limited by the hydrophilic nature of these compounds that may hinder solubility in lipidic matrices. This problem can be solved by esterification of the polyhydroxylated molecules catalysed by enzymes, in organic solvent[1, 2, 3].

In this work acylated derivatives of plant derived flavonoids as catechin, hesperetin, were synthesised in a reaction catalysed by lipase B from *Candida antarctica*, using *tert*-butanol as solvent whereas vinyl acetate and vinyl laurate were used as the acyl donors. The various parameters affecting the reactions were extensively studied.

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P038 DIRECTED EVOLUTION OF A *Pseudomonas fluorescens* LIPASE FOR RESOLUTION IN ORGANIC SOLVENTS

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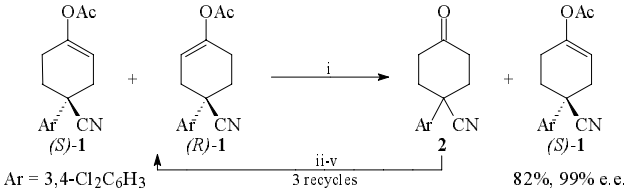
Keywords: lipase, directed evolution, organic solvents

A novel biotransformation has been developed in our laboratory for the preparation of homochiral enol acetates derived from prochiral ketones. 4-Cyano-4-aryl substituted cyclohex-1-enyl acetates such as 1 have been resolved with *Pseudomonas fluorescens* lipase (PFL) by transesterification with *n*-butanol in THF giving good to excellent enantiomeric purities (e.e.'s) for the chiral enol acetates. With an efficient recycling method (Scheme), even with a relatively low *E* value of 13, it is possible to isolate enantiomerically pure enol acetate *(S)*-1 in 82 % yield after 4 cycles[1, 2]. This is a successful approach and has been scaled up to provide synthetically useful quantities (20 g) of the *(S)*-enol ester 1 which was used for the synthesis of a tachykinin NK-2 antagonist used in the treatment of neuro-inflammatory conditions[3, 4].

The aim of this project is to use this lipase resolution as a model system for developing a high throughput directed-evolution strategy for obtaining lipases with improved enantioselectivity for use in organic solvents. The *Pseudomonas fluorescens* lipase gene, *lipA*, has been cloned with a histidine tag, expressed in E. coli, purified under denaturing conditions and refolded directly on the Ni-NTA resin. This gene will be subjected to a radical mutagenesis protocol (ep-PCR) to

generate a library of mutant enzymes. Due to the tag, the enzymes can be absorbed from the crude cell lysate using Ni-NTA coated microtitre plates, facilitating a high-throughput enzyme screening strategy in organic solvents. The e. e. screen is based on the use of pseudo-racemic d^3-labelled enol ester 1 with electrospray ionisation mass spectrometry (ESI-MS) to detect M and M + 3 (ref.[5]). A protocol is being developed to maximise the spectrum of mutations in the *lipA* gene, thereby increasing the probability of creating and isolating mutant enzymes with improved enantioselectivity. Interesting mutants will then be sequenced to identify key mutations and may be subjected to further rounds of random mutagenesis or gene shuffling.



i: Pseudomonas fluorescens lipase (PFL), nBuOH, THF; ii: Remove PFL; iii: add isopropenyl acetate and ^{t}BuOK; iv: Add Dowex H^+ resin; v: Remove resin and add PFL and nBuOH

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P039 PRESERVING THE ACTIVITY OF LIPASE FROM *Pseudomonas fluorescence* BY HYDROPHYLISATION IN THE PRESENCE OF SODIUM DODECYL SULFATE

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Keywords: lipase, thermoinactivation, chemical modification

The industrial interest in lipase activity is very high, especially as component of household detergents. Especially the temperature stability of enzymes used in laundry detergents is an important factor. Enzyme engineering is a variable tool to enhance the thermostability of enzymes.

The hydrophile-lipophile balance (HLB) of an enzyme seems to be essential for most of its properties (recognition of substrate, binding, stability, etc). In our work, the HLB of lipase (Lip) from bacteria *Pseudomonas fluorescence* has been changed by chemical modification. Lipase was modified by glucose with primary amino group of lysine and the Schiff base formed in the reaction was reduced by the addition of sodium borohydride.

The comparative study of the behavior of the native Lip and Lip-Glu modified by hydrophylisation showed that glucosylation of lipase does not affect on the stability of these enzymes in buffer solution. The rate of thermoinactivation of lipases at 58 °C is the same.

In the presence, however, of sodium dodecyl sulfate (SDS) both lipases are less stable, but hydrophylisation of lipase provides a better protection for thermoinactivation. This stabilizing effect (ratio of the rate constant of thermoinactivation of the modified and native enzymes) is about 2-fold.

P040 REAL-TIME MONITORING OF LIPASE-CATALYSED CARBOHYDRATE MODIFICATION BY ^{1}H-NMR SPECTROSCOPY

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Keywords: enzymatic, NMR spectroscopy, lipase, carbohydrate

Nuclear magnetic resonance (NMR) spectroscopy is a non-invasive analytical method that can be used to study biocatalytic reactions *in situ*. In this study, ^{1}H-NMR was used to monitor on-line lipase-catalysed transesterification reactions in organic solvents without the need to sample the reaction medium. The synthesis of acryl esters of a range of monosaccharides and their α-methoxy derivatives catalysed by lipase (Novozym 435) in *tert*-butan(ol-d) has been monitored. The time-course of sugar acrylate and acetaldehyde formation as well as the disappearance of vinyl acrylate was monitored, allowing for simultaneous quantification of each reactant and product species in the reaction mixture. The kinetic parameters obtained from real-time ^{1}H-NMR data were comparable with those obtained by classical methods consisting of sampling the reaction mixture and quantitative determination by GC.

This study shows that ^{1}H-NMR can be used for real-time monitoring of heterogeneous biocatalytic reactions.

P041 CLONING AND CHARACTERIZATION OF A NOVEL LIPASE FROM *Archaeoglobus fulgidus* DSM 4304

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Keywords: *A. fulgidus*, hyperthermophilic, lipase, alcalophilic

A novel lipase was identified in the genome of the hyperthermophilic archaeon *Archeaoglobus fulgidus*[1]. *Archaeoglobus fulgidus* is a sulphur-metabolizing organism with an optimal growth temperature of 83 °C. We have cloned and overexpressed a novel lipase from this organism in *E. coli* and purified it using immobilized metal affinity chromatography combined with anion exchange chromatography.

The activity measurements performed in a pH-Stat and spectrophotometrically demonstrated that this enzyme preferentially uses *p*-nitrophenylesters and esters of short chain fatty acids as substrates. Tributyrin was only weakly hydrolysed while triolein and other typical substrates for lipases were not used. The location of the catalytic triad Ser-Asp-His was identified by site-directed mutagenesis and the properties of the enzyme concerning the temperature and pH stability have been evaluated. The enzyme showed optimal activity and stability at pH values of 10–11. The temperature dependent activity measurements revealed a temperature optimum of 70 °C and a rapid loss of activity at higher temperatures. This leads to the assumption that compatible solutes of *Archaeoglobus fulgidus* like di-glycerolphosphate are a crucial factor for the temperature stabilization of the enzyme *in vivo* as it is in other thermophilic organisms[2].

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P042 PRODUCTION OF ETHYL BUTYRATE BY LIPASE-CATALYSED ESTERIFICATION IN BATCH AND IN CONTINUOUS REACTORS

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Keywords: batch reactor, continuous reactor, esterification, lipase, polyurethane

Ethyl butyrate is an important flavouring ester widely used in food and pharmaceutical products. The aim of this study was the production of ethyl butyrate in *n*-hexane catalysed by *Candida rugosa* lipase immobilized in biocompatible hydrophilic polyurethane foams prepared with the pre-polymers "Hypol FHP 2002™" (toluene diisocyanate) and "Hypol FHP 5000™" (diphenylmethane-4,4'-diisocyanate). The immobilised preparations were used in batch and in continuous reactors.

Batch experiments were conducted in 12 cm^3 of *n*-hexane solutions, at 30 °C under magnetic stirring, following a central composite rotatable design, as a function of the initial butyric acid concentration, *A* (0.078–0.57 M) and of the molar ratio ethanol/acid, *R* (0.43–2.27).

For both immobilised preparations, at 18 hours reaction time, production of ethyl butyrate and conversion could be fitted to flat or convex surfaces, described by first or second order polynomial models.

Theoretically, a maximum ester production of 0.23M is expected after 18 hours reaction time, at 0.35 M of *A* and *R* equal to 1.5, for the lipase in "FHP 2002™". When the immobilized lipase in "FHP 5000™" foam is used, a maximum of 0.27M ethyl butyrate is expected at 0.53 M of *A* and 0.78 of *R*. In reality, with these initial substrates concentrations, 0.28 M and 0.32 M ethyl butyrate concentrations were achieved, after 48 hours reaction time, when the lipase was immobilised in "FHP 2002™" and "FHP 5000™" foams, respectively. These concentrations correspond to conversions of 80.7 % and 78.2 %, respectively.

For both immobilized preparations, the highest conversion values were observed in acid-limited reactions, namely for *A* equal to 0.078 M and *R* equal to 1.5. Under these conditions, after 15 hours reaction time, 74.4 % and 97.1 % conversions were attained with the lipase in "FHP 2002™" and "FHP 5000™, respectively. However, these high conversions correspond to very low concentrations of ester (0.058 M and 0.076 M, respectively) and therefore to low productivities.

For the continuous process, an up-flow (0.1 $ml.min^{-1}$) fixed-bed reactor consisting of a thermostated (30 °C) glass

column (16 mm internal diameter; 20 cm height) filled with the immobilized biocatalyst (about 60 mg of lipase per ml of reaction medium) was used. The reaction mixture residence time was, in average, 260 min. As in batch reactions, when the reaction medium composed by 0.078 M *A* and 1.35 *R* was used, high conversions and low ester concentrations were also obtained with both immobilised preparations.

The bioreactor was continuously operated for 1 month. During this period, no activity decay was observed with either immobilized preparation.

Using the reaction medium formulation predicted to maximize ester production, the ethyl butyrate concentration obtained with the biocatalyst in "FHP 2002™" was only 0.097 M, corresponding to a conversion of 27.6 %. These unexpected results might be ascribed to an insufficient residence time, rather than to an inactivation of the biocatalyst.

The authors are grateful to Amano Enzyme Europe, for the gift of lipase AY and to Dow Chemical Company, U. K., for the gift of the polyurethane pre-polymers.

P043 PRODUCTION OF MARGARINE BASESTOCKS BY TRANSESTERIFICATION OF BLENDS OF THREE VEGETABLE FATS CATALYZED BY A COMMERCIAL IMMOBILIZED LIPASE

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Keywords: lipase, margarine, response surface modelling, transesterification

The functional properties of fats are determined by the distribution pattern of fatty acid radicals in their molecules. The modification of that pattern, by transesterification, is a route to improve certain properties of natural fats and to implement their nutritional value. This is of much interest for applications in margarine, confectionary and bakery industries, as well as for pharmaceutical purposes.

In this study, a commercial immobilized thermostable 1,3-specific lipase from *Rizomucor miehei*, "Lipozyme™ IM", kindly donated by Novozymes, Denmark, was used as a catalyst for the transesterification of fats in a solvent free medium. The reaction medium was composed of palm oil stearin (POS), palm kernel oil (PK) and rapeseed oil (RP). Transesterification reactions were carried out in capped 100 ml cylindrical thermostated reactors, under magnetic stirring with a biocatalyst load of 5 % *(w/w)*. The biocatalyst was used at its original water activity (a_w) of 0.248, at 25 °C.

A set of 26 experiments was carried out following a Central Composite Rotatable Design, as a function of 4 variables: POS and RP content (reaction medium formulation), temperature *(T)* and reaction time *(t)*. Temperature varied from 55 °C to 75 °C; *t* from 15 to 105 minutes; POS content from 45 % to 85 % *(w/w)* and RP from 5 % to 25 % *(w/w)*. The proportion of PK used was calculated from the amounts of the other two fats. Five levels were considered for each variable inside the tested range.

The reaction time course was indirectly followed by the "Solid Fat Content" of the blend assayed by NMR at different temperatures ($SFC_{10\,°C}$; $SFC_{20\,°C}$ and $SFC_{35\,°C}$), which are related with the rheological behaviour of fats at storage ($SFC_{10\,°C}$), packing ($SFC_{20\,°C}$) and consumption temperatures ($SFC_{35\,°C}$), respectively. The $SFC_{35\,°C}$ of transesterified fats should be lower than that of their original counterparts, to prevent a sandy and coarse texture. Also, the extent of the competing reactions of hydrolysis and lipid oxidation was evaluated along the transesterification reaction.

Reaction time showed to have significant linear and/or quadratic effects on all SFC values and on the formation of oxidation products (initial and final oxidation products). A linear effect of reaction temperature on $SFC_{35\,°C}$, on FFA and oxidation products was also observed. SFC values showed to depend on the initial POS and on RP content. Also, a linear effect of the initial POS concentration on the formation of oxidation products was observed. Only the linear interactions ($t \times T$) and ($t \times POS$) were significant on $SFC_{20\,°C}$ and $SFC_{35\,°C}$.

Response surfaces, described by first or second order polynomials, can be well fitted to the final SFC values, $SFC_{35\,°C}$ reduction and oxidation products. The lowest $SFC_{35\,°C}$ values (lower than 8) and highest reduction levels of $SFC_{35\,°C}$ (higher than 50 %) were achieved under temperatures and reaction times higher than 65 °C and 70 minutes, respectively, and for POS between 45 and 60 %.

Starting from blends with 0.2 % FFA, transesterified fats with 2 % to 4 % FFA were obtained. A lack of fit was observed between FFA experimental data and the model. This suggests that the production of FFA is mainly a result of the first-step of lipase-catalysed transesterification and not very much dependent on reaction conditions[1].

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P044 RESPONSE SURFACE MODELLING OF THE TRANSESTERIFICATION OF FAT BLENDS, RICH IN ω-3 POLYUNSATURATED FATTY ACIDS, BY A COMMERCIAL IMMOBILIZED LIPASE

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Keywords: hardness, immobilized lipase, modelling, solid fat content, transesterification

In this study, a commercial immobilized thermostable lipase from *Thermomyces lanuginosa*, Lipozyme™ TL IM, kindly donated by Novozymes, Denmark, was used for the transesterification of fats in a solvent free medium, starting from different blends. The reaction medium was composed of palm oil stearin (POS), palm kernel oil (PK) and a commercial concentrate of triglycerides enriched in omega-3 fatty acids (ω-3 PUFA). The reaction medium formulation, temperature *(T)* and reaction time *(t)* varied according to a central composite rotatable design (CCRD). In CCRD 1, the commercial concentrate ("EPAX 2050TG") contained 20 % eicosapentaenoic (EPA) and 50 % docosahexaenoic acids (DHA). In CCRD 2, the concentrate "EPAX 4510TG", with c. a. 45 % EPA and 10 % DHA was used. Transesterification reactions were carried out in small cylindrical thermostated reactors, under magnetic stirring with a biocatalyst load of 5 % (w/w). The reaction time course was indirectly followed by the "Solid Fat Content" of the blend assayed by NMR at different temperatures ($SFC_{10\,°C}$; $SFC_{20\,°C}$; $SFC_{30\,°C}$ and $SFC_{35\,°C}$). A decrease in $SFC_{35\,°C}$ is desirable since this parameter is related to the decrease in the extent of fat crystallisation at the same temperature. Hardness of the fats was assayed at 20 °C by a TA-XT2 Texture Analyzer, using a 8 mm stainless steel cylinder probe. The SFC values of the transesterified fats depended on the POS and EPAX content, on the reaction temperature and time, and also on the linear interactions between these variables, for both experimental designs. The transesterification resulted in higher hardness values for high POS and low EPAX content, high *t* and *T* values. Response surfaces, described by first or second order polynomials, can be well fitted to the final SFC values, SFC reduction and final hardness values, either when "EPAX 2050TG" or "EPAX 4510", were used. Hardness at 20 °C can be described by second order polynomials as a function of $SFC_{10\,°C}$ or $SFC_{20\,°C}$.

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P045 THE INTERACTION BETWEEN *Candida antarctica* LIPASE AND BRANCHED CHAIN FATTY ACIDS: A KINETIC AND MOLECULAR MODELLING STUDY

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Keywords: lipase, branched chain fatty acids, molecular modeling, kinetic resolution, esterification

Branched chain fatty acids are widespread in Nature, occurring in several plant and animal tissues. They play an important role in the flavour and fragrance of mutton and sheep's cheese[1]. Most of them are chiral, depending on the substituent and its position at the chain. Earlier research in our laboratory has revealed that immobilised *Candida antarctica* lipase B (Novozym 435®) is the best catalyst for the kinetic resolution of 4-methyloctanoic acid. An enantiomeric ratio *(E)* of 57 was obtained in a direct esterification reaction with ethanol in a solventless system[2] which is quite high regarding the distance between the chiral centre and the position where the enzymatic attack takes place (the carbonyl group).

In order to gain more information about enzymatic stereorecognition at more or less remote chiral centres, we have synthesised all positional isomers of methyloctanoic acid and subjected them to Novozym 435®-mediated esterification with ethanol under the same conditions as previously used for 4-methyloctanoic acid. Remarkably, there appeared to be no clear relation between the distance to the chiral centre and the enantiomeric ratio. Using molecular dynamics we were able to explain our results and give a good estimate of *E*-values.

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P046 *Georichum candidum* LIPASE: ACTIVATION AND ITS ENANTIOSELECTIVITY TOWARDS XENOBIOTIC SUBSTRATES

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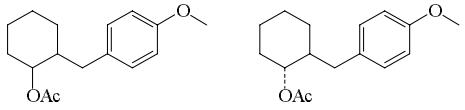
Keywords: *Geotrichum candidum*, lipase, enantioselectivity

Production of lipase by four strains of *Geotrichum candidum* (48, 0302, 4012, 4013) in liquid medium was investigated under addition of olive oil, which has been used as activator of the activity.

Calculated differences in lipase activity values were used as a basis for optimizing of fermentation conditions. Olive oil and peptone were the best sources of carbon and nitrogen. Majority of activated lipases represented extracellular lipases, which penetrated through cellular membrane into the medium. The activity of intracellular lipases was determined as well. The most remarkable growth of the cell biomass was also observed using a medium with peptone as a source of nitrogen. The highest specific activity of all activated lipases was found in the second half of the period of exponential (log) phase cultures. Strain 48 displayed the highest extracellular and intracellular lipase activity after 80 h of growth on a shaker at 30 °C. All activated lipases were used as biocatalysts of the hydrolytic resolution of racemic *cis*- and *trans*-isomers of 2-(4-methoxybenzyl)cyclohex-1-yl acetates (**1** and **2**)[1]. Enantiomeric purity and absolute configuration of the products, chiral isomers of 2-(4-methoxybenzyl)cyclohexanol, were studied.



1 **2**

The substrate **2** was more favoured by most of the activated lipases employed due to the higher conversion rates found. The optimal enantioselectivity of the enzymes were found when the compound **2** was used as the substrate of hydrolytic reaction.

The authors thank Ministry of Education of the Czech Republic for financial support of this research through the COST D13.10 project, a part of the COST D13/0014/01 network.

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P047 NEW EFFICIENT LIPASE FROM *Yarrowia lipolytica* FOR THE RESOLUTION OF 2-SUBSTITUTED CARBOXYLIC ACID ESTERS

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Keywords: lipase, *Yarrowia lipolytica* culture, enantioselectivity

A new extracellular lipase from the yeast *Yarrowia lipolytica* was recently characterized[1]. This acid resistant and thermostable lipase was demonstrated to be of great interest in lipid waste process and in human pancreatic deficiency treatments[2]. Moreover, *Y. lipolytica* appears to be one of the most attractive organisms for heterologous enzyme production and new expression plasmids have been developed[3]. It combines the facility of single cell use, of high secretion abilities and efficient tools for post-translational modifications[4]. New *Y. lipolytica* strains were constructed by introducing multiple copies of the lipase gene in the genome (~15 copies/genome)[5]. The lipase gene is under the control of a promoter POX2 inducible by oleic acid.

A mineral medium was developed for lipase production which fulfilled *Y. lipolytica* nutritional requirements and enabled in fed-batch mode 100 $g.l^{-1}$ of biomass to be obtained with the tremendous production of 60 000 lipase $U.ml^{-1}$ (1U = 1 micromol triolein hydrolyzed per minute). The lipase represented the main enzyme in the broth.

New potentialities of this lipase were discovered in the field of resolution of racemic compounds. *Y. lipolytica* lipase is active and selective for resolution of 2-bromo phenyl and tolyl acetic acid esters. These types of compounds are important intermediates in the synthetic pathways of drugs such as prostaglandin, prostacyclin, semi-synthetic penicillin, thiazolium salts, etc. Methyl and ethyl ester derivatives of 2-bromo-*o*-tolylacetic acid are used as precursors for the synthesis of analgesics, and non-peptide angiotensin II-receptor antagonists[6]. Activity and enantioselectivity performances of *Y. lipolytica* lipase were compared with those of *Burkholderia cepacia* lipase, which is one of the preferred enzymes for

these reactions[6]. *Y. lipolytica* lipase has shown higher activities, an inverse enantioselectivity preference (*S* preference) and similar enantioselectivity.

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P048 ENANTIOSELECTIVITY IMPROVEMENT OF *Yarrowia lypolitica* LIPASE FOR RESOLUTION OF 2-SUBSTITUTED CARBOXYLIC ACID ESTERS

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Keywords: site-directed mutagenesis, lipase, *Yarrowia lipolytica*, enantioselectivity, 2-substituted carboxylic acid esters

A new extracellular lipase from the yeast *Yarrowia lipolytica* was recently demonstrated as efficient for resolution of 2-bromo phenyl and tolyl acetic acid esters (intermediates in the synthetic pathways of drugs such as prostaglandin, prostacyclin, semi-synthetic penicillin, thiazolium salts, etc.) and resolution of nonsteroidal anti-inflammatory drugs (ibuprofen, ketoprofen, naproxen) (see **P047**).

The objective of this work was to improve the lipase enantioselectivity by site-directed-mutagenesis. An original approach was used because of the non availability of the 3D-structure. By homology modelling (primary and secondary structure) with *Rhizomucor miehei* lipase (identity 32 %) the 3D-structure of the lipase from *Y. lipolytica* was determined *in-silico*. Four potential targets for mutagenesis were identified using this structural model. Twenty five variants of the protein were constructed containing one, two or three amino acid changes. An original reaction medium was developed in order to easily and rapidly quantify enantioselectivity from an Erlenmeyer culture of a *Y. lipolytica* strain containing only one copy of the variant lipase gene in its genome.

Enantioselectivity was increased 12 times (E = 48) with only one amino acid change for resolution of the 2-bromophenylacetic acid ethyl ester, and 5 times (E = 40) with two amino acid changes for resolution of 2-bromo-*o*-tolylacetic acid ethyl ester. Surprisingly, this improvement in enantioselectivity was accompanied by an tremendous increase in activity (fourteen and one hundred times respectively for the two substrates). One mono mutant enabled enantioselectivity to be reversed for both substrates.

A molecular model, based on accurate kinetic studies, is proposed to explain the observed enantioselectivity and activity gains.

P049 MOLECULAR MODELING STUDIES ON THE ENANTIOSELECTIVE ESTERIFICATION OF (+/-)-PERILLYL ALCOHOL CATALYSED BY LIPASES

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Keywords: lipase, enantioselectivity, structure-function relationship, molecular dynamics simulation

The use of biocatalysis by employing hydrolytic enzymes such as lipases, esterases and proteases in non-aqueous media for the synthesis of compounds of biological interest has gained particular interest during the last years mainly because of the advantages they present over the use in aqueous solutions. One of the most important properties of enzymes is the enantioselectivity they present towards a variety of substrates. In this work several lipases have been tested as far as their catalytic efficiency is concerned in the esterification of the two enantiomeric forms of perillyl alcohol, *(S)*-(–) and *(R)*-(+) and the findings showed a different preference of the enzymes towards the two enantiomers.

In an attempt to explain the molecular basis of the enantioselectivity, we have initiated molecular dynamics simulation by using the program CNS based on the known X-ray crystal structures of the lipases used.

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P050 ENZYME STORAGE STABILITY IN ORGANIC SOLVENTS

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Keywords: stability, lipase, stability, organic solvents, transesterification

This laboratory has recently reported the low storage stability of different preparations of subtilisin Carlsberg suspended in several organic solvents[1]. It was also reported that the highly efficient co-lyophilized preparation with methyl-beta cyclodextrin[2,3] losses its activity most rapidly (as compared to the CLEC and lyophilized powder). Curiously, the enantioselectivity of all preparations is conserved during the long incubation period (7 days).

To explain the reasons for these observations, we decided to study the stability of a very different enzyme: a lipase. The data to be presented compares the above mentioned results with the storage stability of different preparations (colyophilized with MßCD, CLEC and lyophilized) of a lipase, measured in different organic solvents. The substrates used for these transesterification reactions were *sec*-phenetyl and benzyl alcohols, and vinyl butyrate. Our results show that under certain conditions, and for some preparations, the lipase is very stable, and actually in some cases its activity increases after the first day of incubation in one organic solvent. An explanation for these observations will be presented.

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P051 CHARACTERIZATION OF ENRICHED LIPOXYGENASE EXTRACT FROM *Aspergillus niger* IN TERMS OF ACTIVITY AND SPECIFICITY

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Keywords: lipoxygenase, *Aspergillus niger*, activity, specificity, flavor precursors

Lipoxygenases (EC 1.13.11.12) are distributed widely in nature and are responsible for the enzymatic conversion of 1*(Z)*,4*(Z)*-pentadiene containing PUFAs into stereospecific hydroperoxide isomer(s), by antarafacial insertion of molecular oxygen at the methylene carbon[1]. However, there has been a paucity of studies investigating the role of this dioxygenase in microorganisms[2]. LOX has been implicated in the biogeneration of volatile desirable and undesirable flavor compounds in foods, stemming from an initial oxidation of polyunsaturated fatty acid[3]. There is an ongoing research work in our laboratory aimed at the biotechnological applications of microbial enzymes for the production of natural flavor precursors and flavor compounds from lipids[4].

The mold *Aspergillus niger* was grown and cultivated on day 6 which corresponded to its maximal dry biomass and lipoxygenase activity. Mycelia were washed of the growing media, and pH adjusted with sodium phosphate buffer. Lipoxygenase (LOX) enzymatic extract was recovered and partially purified by ammonium sulfate precipitation. Enzymatic fraction precipitated by ammonium sulfate at 30–70 % of saturation showed the highest LOX activity, with a purification fold of 2.2. The enriched enzymatic fraction was assayed for its LOX activity using selected polyunsaturated fatty acids (PUFAs) as substrates, including linoleic, linolenic and, arachidonic acids. Two pH optima were determined, in the acidic range (pH 5.0) and in the basic range (pH 10.5). The K_m and V_{max} values indicated that LOX from *A. niger* displayed preferential activities toward linolenic and linoleic acids at low and high pH, respectively. Using high-performance liquid chromatography (HPLC) and gas chromatography/mass spectrometry (GC/MS) analyses, the amounts of hydroperoxides produced were separated and quantified. Substrate specificity of the microbial LOX demonstrated a preferential affinity for the free linoleic acid over its mono-, di- and triacylglycerol esters. It was also shown that the LOX activity of *A. niger* produced all positional isomers (monohydroperoxy regioisomers) of the assayed PUFAs. In addition, there was a predominance of conjugated diene

hydroperoxides, with approximately 60 % of end products. Significant production of the unconjugated 10-hydroperoxide of linoleic and linolenic acids was demonstrated for this enriched LOX extract, ranging from 15 to 21 % of total hydroperoxide produced, with a greater proportion attributed to the more saturated fatty acid among the assayed PUFAs.

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P052 SELECTIVE ENZYMATIC ACYLATION OF *N*-ACETYLHEXOSAMINES AND THEIR DERIVATIVES

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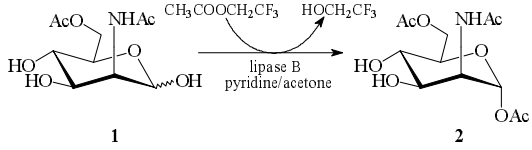
Keywords: enzymatic acylation, lipase, 1-*O*-acetates

Selective chemical modifications of carbohydrates always involve multistep reaction sequences, and despite high yield in each reaction step (e. g. 90 %), the overall yield is usually quite low (e. g. after 5 steps the total yield is only 59 %), regardless labour and material costs. Therefore, one-step selective modification, such as enzymatic acylation, is always advantageous.

The goal of our project was to acylate selectively *N*-acetylhexosamines using enzymes. For this aim we tested lipases (lipase PS from *Burkholderia cepacia*, lipase B from *Candida antarctica*, porcine pancreatic lipase) and protease subtilisin from *Bacillus licheniformis*. Acetylation and butyrylation of GalNAc, ManNAc and *p*-NP-*β*-GalNAc were carried out. Acylation of GalNAc with subtilisin yielded 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose and interestingly also its furanose form, i. e. 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactofuranose, which were obtained in a pure and stable form. Acylation of *p*-NP-*β*-GalNAc with lipase B yielded selectively *p*-nitrophenyl 2-acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranoside. Acylation of ManNAc was considerably less feasible than that of GalNAc. ManNAc could be acylated exclusively by subtilisin to afford 2-acetamido-6-*O*-acyl-2-deoxy-D-mannopyranose. However, butyrylation with the same enzyme (trichloroethyl butyrate as donor) proceeded further to 2-acetamido-3,6-di-*O*-butyryl-2-deoxy-D-mannopyranose. 2-Acetamido-6-*O*-acetyl-2-deoxy-D-mannopyranose **(1)** could be further acylated also by other enzymes, e. g. lipase B, yielding a quite unusual anomeric diacylderivative 2-acetamido-1,6-di-*O*-acetyl-2-deoxy-a-D-mannopyranoside **(2)**. 2-Acetamido-6-*O*-butyryl-2-deoxy-D-mannopyranose treated with lipase B gave 2-acetamido-4,6-di-*O*-butyryl-2-deoxy-D-mannopyranose.



1 **2**

Support by the COST D25/0001/02, Ministry of Education of the Czech Republic OC D 25.002, by the grant 203/01/1018 from the Grant Agency of the Czech Republic and the bilateral project between CNR and Academy of Sciences of the Czech Republic is acknowledged.

P053 NEW TRANSGLYCOSYLATION REACTIONS WITH MODIFIED SUBSTRATES CATALYSED BY β-*N*-ACETYLHEXOSAMINIDASES

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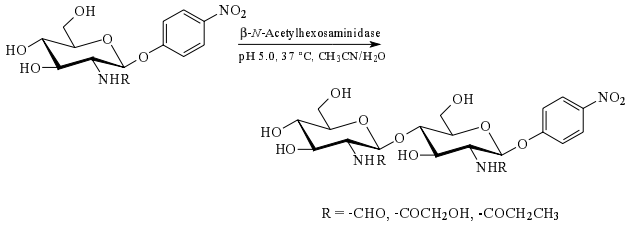
Keywords: β-*N*-acetylhexosaminidase, modified substrates, transglycosylation

There have been several hints in the literature suggesting that glycosidases exhibit a broad substrate specificity, which enables them to cleave and even to transfer modified glycosidic moieties yielding oligosaccharides[1–3]. In our project we have studied five *N*-acyl modified glycosides considering their ability to serve as glycosyl donors for fungal β-*N*-acetylhexosaminidases from our library.

The *p*-nitrophenyl glycosides bearing different substituents at the 2-C amino group instead of the common acetyl (formyl, glycoloyl, propionyl, trifluoroacetyl and hydrogen)

were tested as substrates for 32 β-*N*-acetylhexosaminidases belonging to the genera of *Acremonium*, *Aspergillus*, *Penicillium* and *Talaromyces*. The enzymes were considered regarding the following criteria: hydrolysis and transglycosylation potential, yield of transglycosylation products and selectivity and stability in acetonitrile, used as a cosolvent due to the limited solubility of substrates. On the basis of the screening, β-*N*-acetylhexosaminidases from *A. oryzae*, *P. oxalicum* and *Talaromyces flavus* were selected for the catalysis of semi-preparative transglycosylations, which gave yield to *p*-nitrophenyl oligosaccharides. This rather novel concept widely extends the synthetic capability of glycosidases.



Support by the grant 203/01/1018 and 204/02/P096 from the Grant Agency of the Czech Republic and Institutional Research Concept No. AV0Z5020903 is acknowledged.

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P054 *N*-ACETYLMANNOSAMINE CONTAINING SACCHARIDES: PRODUCTION USING β-*N*-ACETYLHEXOSAMINIDASES AND THEIR SEPARATION BY ION-EXCHANGE/EXCLUSION CHROMATOGRAPHY

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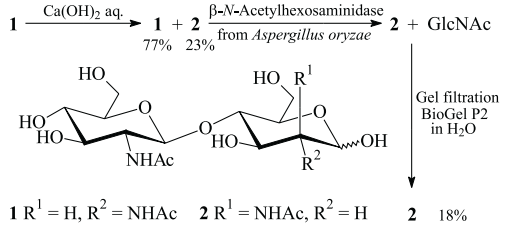
Keywords: acetamidosugars, alkali catalysed epimerization, *Aspergillus oryzae*, separation

Derivatives of 2-acetamido-2-deoxy-D-mannopyranose (ManNAc) are quite rare in nature, however they are rather important. β-ManNAc occurs in some surface structures of bacteria, e. g., those attaching teichoic acid. Occurrence of ManNAc is often linked to virulence and evading from the immunity surveillance. ManNAc and its derivatives were identified to be strong activators of the natural killer cells (NK cells)[1]. Preparation of the ManNAc derivatives, especially of those with β-configuration is one of the greatest challenge of the carbohydrate synthetic chemistry.

GlcNAcβ(1→4)ManNAc **(2)** was prepared from chitobiose **(1)** by 2-epimerization under catalysis of $Ca(OH)_2$. Resulting mixture is hardly separable only by analytical HPLC. Chitobiose in reaction mixture was selectively removed by hydrolysis β-*N*-acetylhexosaminidase from *Aspergillus oryzae*; GlcNAcb(1→4)ManNAc **(2)** is resistant to the enzyme hydrolysis. Resulting disaccharide can be then easily isolated by gel filtration. The enzyme was cloned and the molecular modelling explains the mode of discrimination of these two saccharides.



New, original and more effective methodology based on separation of the GlcNAc and ManNAc derivatives in borate buffer on the various gel materials (Sephadex, Toypearl, Cellufine) was developed. This method enables effective large-scale production of rather rare oligosaccharides containing ManNAc with important immunoactivity. This method has been extended to all common aminosugars (GlcNAc, GalNAc, ManNAc, TalNAc and their di- and trisaccharides). Analogous disaccharide as GalNAcβ(1→4)ManNAc and trisaccharides GlcNAcβ(1→4)GlcNAcβ(1→4)ManNAc and GalNAcβ(1→4)GlcNAcβ(1→4)ManNAc were prepared by the above methodologies as well.

This work was supported by COST Action D25, D13 and Grant Agency of the Czech Republic (grant No. 203/01/1018).

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P055 ENZYMATIC MODIFICATION OF A MACROCYCLIC COMPOUND WITH *N*-ACETYLGLUCOSAMINE

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Keywords: *N*-acetylhexosaminidase, *N*-acetylglucosamine, transglycosylation, rifampicin

Macrocyclic lactones and lactames are known as potent antibiotic and anti-cancer drugs. Since glycosidic side chains play an important role for the specifity of these structures, we want to generate a compound library of enzymatically modified macrocycles as part of a screening system for novel anti-infective and anti-cancer agents.

As a first attempt, we investigated the enzymatic transfer of the β-*N*-acetylglucosamine residue (GlcNAc) onto the secondary or the phenolic OH-residues of rifampicin as a model substrate (Fig. 1).

N-Acetylhexosaminidases, which usually catalyze the hydrolysis of terminal, non-reducing GlcNAc residues, are also capable of catalyzing the formation of GlcNAc derivatives both via reverse hydrolysis and transglycosylation[1–2].

Several examples for enzymatic GlcNAc transfer using the activated donor *p*-nitrophenyl-GlcNAc have been reported. However, only a few non-sugar compounds have been described as glycosyl acceptors, e. g. ergot alkaloids and vitamins with low yields below 15 % (ref.[3–4]).

We tested several conditions for the enzymatic modification of rifampicin, employing the commercially available *N*-acetylhexosaminidases from *Canavalia ensiformis* (jackbeans) and the fungus *Aspergillus oryzae*.

To increase the solubility of rifampicin, organic co-solvents, namely acetonitrile and *tert*-butanol, were used in varying amounts. Ammonium sulfate was added in order to reduce the water activity in the medium. The reaction mixtures were analyzed with HPLC.

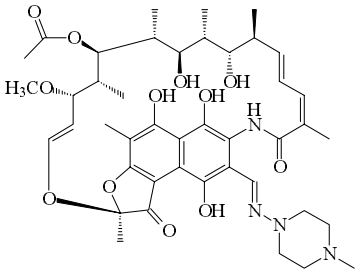

Fig. 1. **Structure of rifampicin**

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P056 α-L-RHAMNOSIDASES AND THEIR USE IN SELECTIVE TRIMMING OF NATURAL COMPOUNDS

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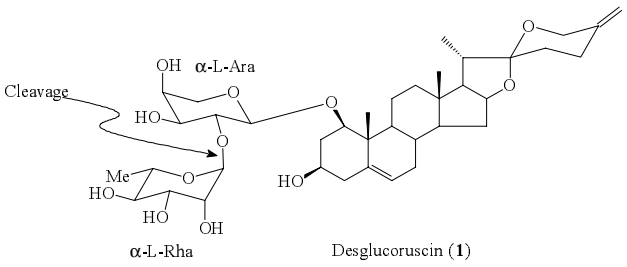
Keywords: α-L-rhamnosidases, desglucoruscin, selective deglycosylation

The pharmacological properties of ruscosides, in particular their anti-inflammatory activity, encouraged us to study the enzymatic modification of this family of natural glycosides in order to obtain new derivatives for testing. Specifically, we concentrated on desglucoruscin **(1)** and, in addition to other biotransformations (e. g. selective acylations in organic solvents), we were interested in changing the carbohydrate moiety of this molecule. The repertoire of commercially available α-L-rhamnosidases is quite limited and it did not give satisfactory results.

Therefore, a series of fungal strains was screened for the production of α-L-rhamnosidase (EC 3.2.1.40) in the presence of various inducers. Besides α-L-rhamnose, flavonoid glycosides (e. g. rutin, hesperidin or naringin) were used as inducers. None of the strains produced the enzyme constitutively, the induction was always necessary. All preparations were tested for a selective derhamnosylation of desglucoruscin **(1)** with respect to the undesired α-L-arabinosidase activity.

The substrate specificity of selected enzymes was tested. Experiments with different substrates with water-miscible solvents indicated the presence of isoenzymes having a different specificity and stability. The enzyme from *Aspergillus niger* K2 CCIM induced by L-rhamnose proved to be highly active towards **1** and stable in organic solvents. The contamination with α-L-arabinosidase was negligible. Consequent-

ly, it was chosen for a preparatory derhamnosylation of **1**, which yielded the desired product in 70 % conversion.



COST D25/0001/02, Grant Agency of the Czech Republic, grant no. 203/01/1018 and Ministry of Education of the Czech Republic OC D 25.002 are gratefully acknowledged.

P057 NARINGINASE – ACTIVITY AND STABILITY OF A BITTER SWEET α-RHAMNOPYRANOSIDASE, FREE AND IMMOBILIZED

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Keywords: naringin, naringinase, kinetic parameters, immobilisation, calcium alginate

Naringinase, an α-rhamnopyranosidade from *Penicillium decumbens*, is commonly used to debitter grapefruit juice. This enzyme does not occur in mammalian biology which turn it particularly important for application to prodrug activation. A full understanding of the enzymology of naringinase is essential for its major applications.

Flavonoids, namely naringin from citrus, are functional chemicals with important properties in the fields of health care, food, and agriculture. They possess anticarcinogenic activity, act as natural pest control agents and as chemotaxonomic markers.

Naringin, the principal bitter flavonone glycoside and the primary bitter component in grapefruit juice, can be hydrolyzed by α-L-rhamnosidase into rhamnose and prunin. Prunin, with a one third bitterness ratio to naringin can be further hydrolyzed by the β-D-glucosidase into glucose and tasteless naringenin.

Naringinase provides the activities of both enzymes, α-L-rhamnosidase and β-L-glucosidase[1]. In the past, the high cost and limited availability of naringinase has restrained its industrial application. Recently, the gene of α-L-rhamnosidase has been cloned and expressed with marked activity in *Escherichia coli*[2]. The recombinant α-L-rhamnosidase, in naringinase provides an economical and easily available source of debittering enzymes and reveals a practical revolution in industrial debittering of grapefruit juices, having also an important impact in the pharmaceutical industry.

The aim of this work was the study of the kinetic parameters of naringinase under different conditions and its potential applications to the degradation of naringin in biological systems. The bioconversion of naringin in standard solutions (acetate buffer, pH 4), on an orbital shaker at 200 rpm, was carried out at different: initial naringin concentrations, naringinase concentration, incubation time, pH and temperature (25, 30, 40 and 50 °C).

Initial rates of naringin conversion were calculated by linear regression of the 5 data-points during the first 15 minutes reaction time. The fit of Michaelis-Menten model to experimental data was carried out using a nonlinear curve-fit program. Kinetic studies showed that the free enzyme had an optimum temperature at 40 °C, a Michaelis-Menten constant (K_m) of 130 μg.ml^{-1} and a maximum velocity (V_{max}) of 12.8 μg.ml^{-1}.min^{-1}, at 40 °C.

The activity and the operational stability of immobilised naringinase depend on several parameters such as the type of support and the immobilisation method. Naringinase was immobilised by entrapment in calcium alginate beads. Several parameters were studied, namely: naringinase loading, incubation time, pH, initial naringin concentrations, and temperature. Operational stability was evaluated.

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P058 ENZYMATIC GLYCOSYLATION OF CARMINIC ACID

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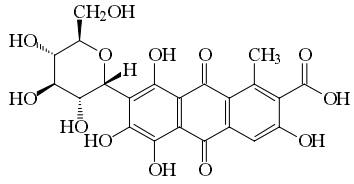
Keywords: enzymatic, glycosylation, carminic acid, transglycosylation

For the pharmacological effect of many natural products glycosylation plays an important role. The aim of the EU-Project COMBIOCAT (EC Framework programme 5) is the synthesis of novel macrolids, glycosylation and screening for biological activity. Carminic acid is a natural product and used as a red colorant. It was used as a testing material for enzymatic glycosylation.

Within our work we tested different glycosidases for their ability to glycosylate carminic acid. From literature no glycosylated carminic acid derivates are known. Our results with six glycosidases are listed in Table I.

The reaction mixtures consisted of: Carminic acid, 100 mM phosphate buffer pH 6 and depending on the reaction type different donors (disaccharides, nitrophenylglycosides or the monosaccharides). Reactions were carried out in microtubes at 37 °C (75 °C for ß-Glucosidase Cel B from *Pyrococcus furiosus*). For analysis we used HPLC with a RP C-18 column. Mass spectroscopy was used for identification of the peaks. Four different single glycosylated derivatives of carminic acid could be proved. Yields obtained range from 1 % up to 13 %, reaction conditions were not fully optimized.



Carminic Acid

Table I
Enzymes used for the experiments and results

Enzyme	Nitrophenylglycoside as donor	Disaccharide as donor	Reverse hydrolysis
β-Glucosidase from Almonds	+	+	O
β-Glucosidase Cel B from *Pyrococcus furiosus*	+	++	O
β-Glucosidase Rec from *Saccharomyces cerevisiae*	+	O	O
β-Galactosidase from *Bacillus circulans*	+	++	O
α-Galactosidase from *Aspergillus oryzae*	+	+	O
α-Mannosidase from Jack Beans	+	–	O

+ glycosylated carminic acid found, – no reaction occurred, O – only traces of the product were found

P059 MEDIUM ENGINEERING FOR THE THERMOSTABLE β-GLUCOSIDASE FROM *Pyrococcus furiosus*

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Keywords: thermostable glycosidase, β-glucosidase, glucosylglycerol

Beyond the natural hydrolysis activity the recombinant β-glucosidase from *Pyrococcus furiosus* shows a remarkable transglucosylation activity. Furthermore, both the thermostable properties and the less specificity towards aglycones makes the enzyme interesting for synthetic purposes[1].

This work focuses on the enzymatic condensation of β-D-glucose with glycerol. The resulting products 2-*O*-β-D--glucosylglycerol, (2*R*) and (2*S*)-3-*O*-ß-D-glucosylglycerol (GG) have shown attractive properties, especially in anti-tumor promoting studies[2]. They might also find application in food technology and as pharmaceuticals[3]. Within this work the aim was to optimise the yield by using the following strategies: (i) Find a way to suppress the hydrolysis activity (Fig. 1), (ii) stabilise the product yield in preventing the secondary hydrolysis (Fig. 1), (iii) explore optimum conditions for enzyme activity and stability.

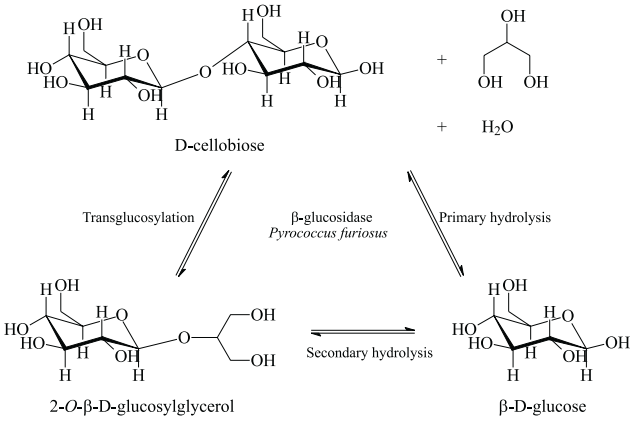

Fig. 1. **Scheme of the investigated reaction system**

As glycone we used D-cellobiose, which is an easily available raw material for biocatalytical transglycosylations with glucosidases. The reaction mixture consisted of: D-cellobiose, β-D-glucose, GG, glycerol and acetate buffer (50 mM). These substrates and products could be analysed by HPLC using an organic acid analysis column (Aminex HPX – 87 H).

First studies showed promising results in suppressing the competing hydrolysis and optimising the enzyme activity. By increasing the amount of glycerol in the reaction mixture, the hydrolysis could be suppressed to a great extent. The enzyme activity could be improved by optimising the cellobiose concentration and the reaction temperature. At 75 °C the maximum of enzyme activity is limited by solubility of cellobiose which was 0.58 mol.l^{-1}. Rising the temperature up to 95 °C, the solubility of the disaccharide could almost be doubled to 1.12 mol.l^{-1}. Glucosidase activity and product maxima could be increased significantly.

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P060 ENZYMATIC SYNTHESIS OF FUNCTIONAL FOOD INCREDIENTS FROM LACTOSE USING β-GLYCOSIDASE (EC 3.2.1.21) FROM *Pyrococcus furiosus*

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Keywords: lactose, glycoconjugate, β-glycosidase, functional food

During cheese production the disaccharide lactose accumulates to an amount of approximately 4 million tons annually[1]. Only about 25 % of this quantity is used for further applications and the large residual amount causes increasing environmental problems by its disposal[2]. Besides, around 70 % of the world population is lactose intolerant, leading to heavy gastrointestinal problems after consumption of food containing lactose[3].

The aim of our work was to obtain valuable conversion products of enzymatic treated lactose that possesses novel functional food properties.

As already described by Petzelbauer *et al*., a thermostable β-glycosidase from *Pyrococcus furiosus* was successfully used for lactose hydrolysis and galacto-oligosaccharide (GalOS) formation respectively[4]. The major GalOS formed during lactose hydrolysis were identified as (1-3)- and (1-6)-linked di-, tri- and tetrasaccharides in a maximum yield of 30 % depending on initial lactose concentration[5].

We used free and immobilised β-glycosidase from *Pyrococcus furiosus* with lactose as glycosyl donor and different sugar and non-sugar compounds as acceptors. The free enzyme has about 60 % β-galactosidase activity referring to its β-glucosidase activity and a half life time of 85 h at 100 °C (ref.[6, 7]).

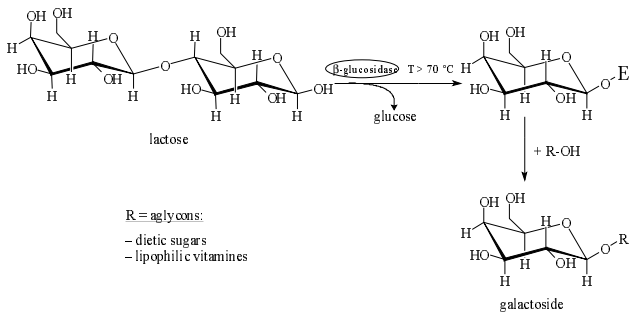

Fig. 1. **Reaction scheme of transglycosylation using β-glucosidase from *Pyrococcus furiosus*.** R-OH could be a primary, secondary or tertiary hydroxylgroup, E = enzyme.

The biotransformations (10 ml scale) were performed at 75 °C and pH 5 in aqueous media, in case of lipophilic aglycons organic solvents were added. Products obtained were analyzed by TLC, HPLC and NMR after preparative HPLC.

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P061 SYNTHESIS OF NOVEL FOOD ADDITIVES, UTILISING α- AND β-GALACTOSIDASES WITH NATURAL AND ARTIFICIAL DONOR SUBSTRATES

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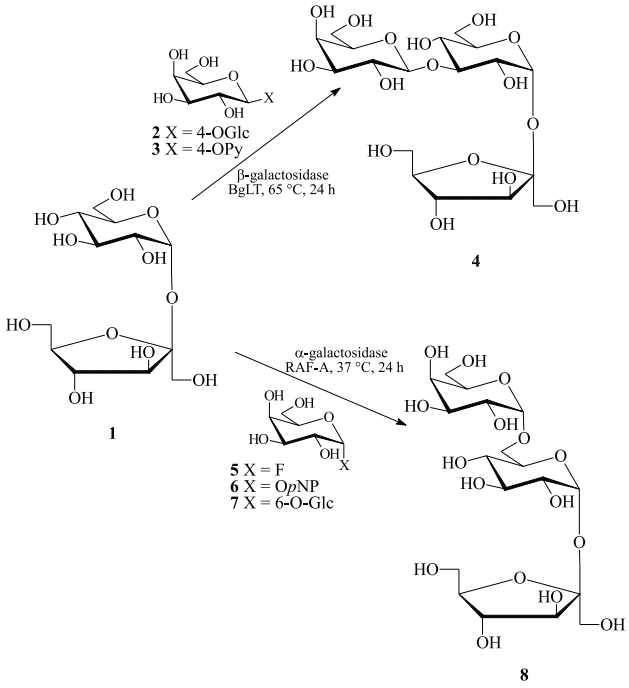
Keywords: galactosidase, transgalactosylation, biologically active carbohydrates, non-cariogenic sweeteners, bifidogenic sugars

The increasing interest in biologically active carbohydrates and novel food components, in particular the synthesis of non-cariogenic sweeteners and bifidogenic sugars, constitutes a field of significant interest. Herein we want to report the synthesis of new oligosaccharides based on known ingredients in food utilising α- as well as β-galactosidases and natural and artificial donor substrates.

For example sucrose **(1)** as acceptor was reacted with lactose **(2)** or 4-hydroxypyridinol-β-D-galactopyranoside **(3)** catalysed by β-galactosidase BgLT. By using donor **2** a maximum yield of 31 % of product **4** was obtained and in case of donor **3** the yield was 12 %. Further on sucrose **(1)** as acceptor was reacted with α-galactopyranosyl fluoride **(5)**, α-para-nitrophenylgalactopyranoside **(6)** and melibiose **(7)** catalysed by α-galactosidase RAF-A gave product **8** in 23 %, 24 % and 19 % yield, respectively.



P062 ENZYMATIC REMOVAL OF LACTOSE FROM GALACTO-OLIGOSACCHARIDE MIXTURES

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Keywords: galacto-oligosaccharides, β-galactosidase, cellobiose dehydrogenase

We developed an efficient method for removing remaining lactose and monosaccharides from lactose-derived galacto-oligosaccharide mixtures (GOS). The initial sugar mixture was obtained by enzymatic transgalactosylation of a lactose solution (270 g.l^{-1}) at 70 °C using recombinant β-glycosidase from the Archaeon *Sulfolobus solfataricus*. At the optimum reaction time for oligosaccharide yield, it contained 46 % monosaccharides, 13 % lactose and 41 % GOS. Lactose was selectively oxidized into lactobionic acid by using cellobiose dehydrogenase from *Sclerotium rolfsii* which displays a ≈ 100-fold preference for reaction with lactose compared to reaction with GOS. Oxidation of lactose was coupled to reduction of 2,6-dichloro-indophenol (DCIP) which was added in catalytic concentrations. The reduced redox mediator was regenerated continuously by fungal laccase-catalysed reduction of molecular oxygen into water. Anion exchange chromatography was employed to remove lactobionic acid, DCIP, other ions and monosaccharides. The final product contained 96.7 % GOS, 1.2 % lactose and 2.1 % monosaccharides. The yield accounted for 25 % of original lactose.

P063 β-GALACTOSIDASES FROM *Lactobacilli* STRAINS

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Keywords: *Lactobacilli*, β-galactosidase, galacto-oligosaccharides, transgalactosylation

A number of *Lactobacilli* strains were tested for their ability to utilize and grow on galacto-oligosaccharides and for the presence of β-galactosidases. The tested strains showed the ability to grow well on two commercial galacto-oligosaccharides compared to that on glucose. The *in vitro* results suggest that these galacto-oligosaccharides will promote the growth of these strains *in vivo* if they are consumed in combination with the products containing these galacto-oligosaccharides. β-Galactosidases extracted from these strains can be used for the production of galacto-oligosaccharides by transgalactosylation of lactose[1,2]. The formation of galacto-oligosaccharides by transgalactosylation of lactose is shown in Fig. 1. These 'prebiotic' galacto-oligosaccharides might be used for human and animal food applications.

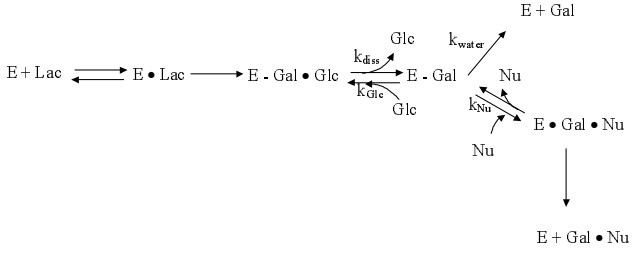

Fig. 1. **Formation of galacto-oligosaccharides by transgalactosylation of lactose catalyzed by β-galactosidases;** E – enzyme (β-galactosidase), Lac – lactose, Gal – galactose, Glc – glucose, Nu – galactosyl acceptor

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P064 OPTIMIZATION OF ENZYMIC HYDROLYSIS AND TRANSGALACTOSYLATION IN ACID WHEY

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Keywords: β-galactosidase, lactose, galacto-oligosaccharides, whey

β-Galactosidase (EC 3.2.1.23) is a hydrolase which attacks the *O*-glucosyl group of milk sugar. Galactosyl transfer to other sugars accompanies lactose hydrolysis into monosaccharides-glucose and galactose[1]. Thereby galacto-oligosaccharides containing from two to seven units are formed. Ingestion of transgalactosylated oligosaccharides induces a significant increase of probiotic intestinal bacteria and this change is responsible for several beneficial physiological effects[2]. In addition, many people are lactose intolerant and have gastrointestinal problems because of the lactose consumption. Therefore, low-lactose but high-oligosaccharides dairy product might provide more than nutrition and could be recognized as "functional food" (ref.[3]).

In this study the effect of enzyme type and concentration, temperature and hydrolysis time on oligosaccharides production was investigated. The objective was to find the optimal conditions for the enzymic reactions in concentrated (RO) acid whey. β-Galactosidases from *Aspergillus oryzae* (Amano F, Tolerase, Validase) were used, the conditions investigated were the temperatures of 12, 20 and 43 °C and the enzyme concentrations 0.003, 0.005, 0.01, 0.02 and 0.03 %. During the enzymic reaction the samples were removed periodically, heated in boiling water to inactivate the enzyme and after protein precipitation analyzed for saccharides content with High Performance Liquid Chromatography (Ostion LG KS 0800 Ca^{2+} column, elution by deionized water, ELS detection). The maximum of oligosaccharides is formed early on (at low degree of hydrolysis – DH) under the all conditions (Amano F: 15–30 % DH, Tolerase and Validase: 20–55 % DH). The choice of conditions depends on demands. The temperature of 20 °C is critical because of microbial growth; the temperature of 12 °C is energetic particular because of cooling and of long time necessity. The temperature of 43 °C is not so much microbial critical as 20 °C and allows a shorter time and lower enzyme concentrations as well. Indispensable content of oligosaccharides in time of 70 % DH at least was obtained with enzyme Tolerase (43 °C, 7 hr, 0.01 % E) and Validase (43 °C, 12 hr, 0.003 % E or 43 °C, 1 hr, 0.03 % E). These are the optimal conditions for obtaining of combined effect. By described procedures we obtained the low-lactose high-oligosaccharides product.

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P065 INFLUENCE OF SODIUM AND CALCIUM IONS ON THE KINETICS OF THERMAL AND ACID INACTIVATION OF α-AMYLASE

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Keywords: enzyme inactivation, thermal, pH, kinetics, mechanism

The inactivation behaviour of a technical preparation α-amylase, Maxamyl from Gist-Brocades, was investigated in a broad range of conditions. The thermal inactivation study was conducted in the temperature interval of 70–95 °C at pH 5.3 in four different solutions: distilled water in the presence or absence of calcium ions, respectively and acetate buffer both with and without calcium ions. The experiments were carried out in a batch mode with frequent assaying until almost complete loss of activity. The inactivation curves exhibited a biphasic inactivation pattern at lower temperatures whereas first-order behaviour was demonstrated at higher temperatures. Compared to the previous study of glucoamylase[1], the deviation from the first order was less pronounced even in those experiments where the incubation had to be extended for quite a long period (up to 3600 min).

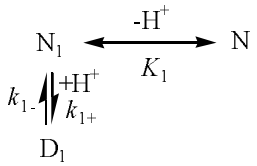
From several mechanisms considered, a consistent description of experimental data was achieved using the kinetic form of the Lumry-Eyring mechanism,

$$N \underset{k_{1-}}{\overset{k_{1+}}{\rightleftarrows}} D \xrightarrow{k_2} I$$

where N is the native enzyme form, D is the denatured form and I is the irreversibly inactivated form. The temperature dependence of the rate constants of this mechanism was expressed through the Arrhenius equation and incorporated

in the mathematical model characterizing the temporal changes of individual enzyme forms at different temperatures. Using the so-called multi-temperature evaluation, based on a simultaneous fit of all available inactivation data, the kinetic parameters, the rate constants at the reference temperature and activation energies, were estimated. The comparison of estimated parameters for different solutions clearly demonstrated the stabilizing effect of calcium and sodium ions.

The acid inactivation of α-amylase was carried in the same four types of solutions in the pH-range of 3.3–4.5 at 30 °C. The individual inactivation curves could be well fitted with first-order kinetics but the rate constants strongly increased with more acidic pH. Several mechanisms were suggested from which models were derived that were used to fit all inactivation curves simultaneously. The validated mechanism had the following form,



where the subscript 1 denotes the acidic forms of the enzyme. The protonation/deprotonation reaction between the neutral and acidic native forms had a character of a fast equilibrium reaction characterized by the equilibrium constant K_1.

The denaturation of the acidic native form had a kinetic character and was considered to be an acid-catalyzed reaction. The calcium and sodium ions exhibited also a significant stabilization effect toward acid inactivation.

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P066 GLUCOSYLATION OF ALKYLGLUCOSIDES WITH ENZYMATIC CATALYSTS FROM *Leuconostoc mesenteroides*

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Keywords: alkylpolyglucosides, glucansucrases, *Leuconostoc mesenteroides*, acceptor reaction

Alkylpolyglucosides (APG) are a class of non ionic surfactants of great interest[1]. Indeed, they are synthesized from renewable materials (starch and oil), are non toxic and ecologically safe. In addition, APG present numerous physicochemical properties, which render them very useful for various applications as detergents and cosmetics. These properties are closely related to the hydrophilic/lipophilic balance (HLB), whose value depends on the size of both the alkyl and glucosidic moieties. Whereas the size of the hydrophobic part of the molecule can be easily modified by chemical way, glucosidic chain length can't be increased easily *via* chemical synthesis. Consequently, APG with high HLB cannot be easily available.

Glucansucrases (E.C. 2.4.1, GS) from *Leuconostoc mesenteroides* are natural catalysts for glucosylation tool: in the presence of sucrose as substrate, they are able to transfer the glucosyl residue from this donor to an acceptor molecule, yielding series of oligosaccharides[2].

In our study, three GS have been tested to glucosylate α-butylglucopyranoside (α-BG). These enzymes have been selected for their specificities to catalyse the synthesis of different types of glucosidic linkage. All the GS tested glucosylated α-BG, yielding APG with polymerisation degree up to 8. The glucosylation efficiency of the reaction was improved by increasing the sucrose/acceptor molar ratio. Structural characterization of some of these APG using LC-MS and NMR techniques showed that various kind of regio-isomers can be formed, owing to the GS used.

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P067 ENZYMATIC GLUCOSYLATION OF 1,5-ANHYDRO-D-FRUCTOSE BY GLUCANSUCRASES FROM *Leuconostoc mesenteroides*

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Keywords: anhydrofructose, glucansucrases, *Leuconostoc mesenteroides*, acceptor reaction

Deffieux *et al.* were the first to demonstrate the presence of 1,5-anhydro-D-fructose (AF) in fungi as a precursor of the

prebiotic microthecin[1]. Since, this molecule has proved to occur in numerous other organisms (morels, red algae, *E. coli*, rat liver tissue). Its presence results from the degradation of starch or glycogen by the action of α-1,4-glucan lyase (EC 4.2.2.13)[2]. The great interest of the molecule is related to its particular structure: no anomeric carbon, but two hydroxyl groups (primary and secondary) and a pro-chiral center . This renders AF very attractive as a chemical synthon, and is responsible for its anti-oxidant and anti-diabetic properties.

To further extend AF properties, enzymatic glucosylation of AF has been envisaged. Glucansucrases (EC 2.4.1, GS) from *Leuconostoc mesenteroides* catalyse glucosyl residue transfer from sucrose to an acceptor, yielding series of oligosaccharides with the acceptor at the reducing end[3].

In our study, AF has been tested as an acceptor for three of these GS, which have been selected for their distinctive regio-specificities, in the prospect of synthesizing various glucosylation products. Thus, all the GS tested have recognized AF as an acceptor, yielding glucosylation products with polymerisation degree up to 5. Glucosylation reaction has also been optimised leading to yields up to 80 %. Besides, owing to the catalyst used, different regio-isomers are synthesized.

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P068 INCREASED THERMOSTABILITY OF *Thermomyces lanuginosus* β-XYLANASE BY DIRECTED EVOLUTION

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Keywords: directed evolution, xylanase, *Thermomyces lanuginosus*

Most natural enzymes are unsuitable for biotechnological processes since they have evolved over millions of years to acquire their specific biological functions. Such enzymes are often genetically altered to suit the rigours of industrial processes. Directed evolution is one such strategy and makes use of iterative rounds of random mutagenesis, screening and recombination to enhance the existing properties of enzymes. *Thermomyces lanuginosus* is a thermophilic fungus that produces high levels of a thermostable xylanase and although many of its hemicellulases have been extensively characterized on a physiological level, they have limited application in industry. The xylanase gene from *T. lanuginosus* DSM 5826 was functionally expressed in *E. coli* as a LacZ fusion protein[1]. Here we show that both the thermostability and activity of this cloned *xyn A* was improved by error-prone PCR using different concentrations of $MnCl_2$. Transformed colonies were first selected for xylanase production on 0.4 % Remazol Brilliant Blue Xylan and then screened at different temperatures for improved stability and activity. After the first round of screening, four mutants showed slight improvement in both stability and activity and were subjected to further mutagenesis, using low concentrations of $MnCl_2$. Three mutants with markedly enhanced stability were obtained. One mutant, 2B7-10, exhibited a two-fold higher activity than the wild type *xynA* and retained 71 % of its activity after treatment at 80 °C for 60 min. The other two mutants retained almost 65 % of their activity when treated under the same conditions, but showed a reduction in activity in comparison to their first generation parent mutants. These genes are currently being sequenced to determine their resulting mutations and we are attempting to further enhance the properties of the xylanases using DNA shuffling, between the variants.

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P069 CHARACTERIZATION OF PYRANOSE DEHYDROGENASE FROM *Agaricus xanthoderma*

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Keywords: pyranose dehydrogenase, oxidation, keto sugars, lignin

Pyranose dehydrogenase (PDH) from *Agaricus xanthoderma* is a new type of oxidoreductase, that was only recently discovered and is of interest for both biochemistry and biocatalysis. PDH so far has not received an EC number.

Pyranose dehydrogenase from the mushroom *A. xanthoderma* was purified to apparent homogenity and subsequently

characterized. PDH is a weakly glycosylated monomeric protein, that is actively secreted into the extracellular fluid. PDH has a native molecular mass of ~58 kDa as determined by gel filtration. The organism forms at least 5 isoforms with isoelectric points ranging from 4.2 to 4.5.

Pyranose dehydrogenase is believed to be involved in lignocellulose breakdown by interconnecting sugar and lignin metabolism through its donor/acceptor specificities.

PDH does not accept oxygen as electron acceptor but instead reduces predominantly various quinones, natural and toxic products of lignin degradation. Pyranose dehydrogenase exhibits an extremly broad sugar substrate specificity, whereas it is rather limited concerning the electron acceptors it reduces. In addition to monosaccharides, both disaccharides and oligosaccharides can serve as electron donor substrates and are oxidised, depending on the saccharide and the incubation time, to the corresponding 2-ketoaldoses, 3-ketoaldoses and 2,3-diketoaldoses. Oxidation can thus occur at C2 and/or C3. The so formed ketosaccharides are useful synthons for carbohydrate chemistry and will play an important role in food industry.

P070 CONTINUOUS OXIDATION OF GLUCOSE BY PYRANOSE OXIDASE: REACTION ENGINEERING AND PROCESS DEVELOPMENT

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Keywords: pyranose oxidase, 2-ketoglucose, enzym reactor, *Trametes multicolor*

Pyranose oxidase (P2O), a tetrameric flavoenzyme found in wood degrading fungi, catalyzes the C-2 oxidation of several aldopyranoses, including glucose and some monosaccharides commonly found in lignocellulose, to the corresponding 2-keto derivatives. During this oxidation electrons are transferred to oxygen to yield hydrogen peroxide. Pyranose oxidase was regarded as the key biocatalyst in the Cetus process, in which pure crystalline fructose was produced from glucose via the intermediate 2-ketoglucose. Traditionally, pyranose oxidase has been utilized in biotransformations as an immobilized preparation. We decided to use the free enzyme in order to exploit its full catalytic potential and to avoid possible mass transfer limitations that frequently occur in heterogeneous systems due to diffusion in particles. The soluble enzyme from the fungus *Trametes multicolor* proved to be exceptionally stable even under operational conditions, provided that hydrogen peroxide was continuously destroyed. Hence, the biocatalyst could be successfully reutilized for several cycles of discontinuous substrate conversion followed by separation of the enzyme by ultrafiltration. For the continuous P2O-catalyzed conversion of sugars a novel plug-flow enzyme reactor with bubble-free aeration was developed in our laboratory. The performance of microporous Teflon tubings was compared to that of silicon tubings. This continuous enzyme reactor was successfully operated for more than 2 weeks with an average productivity of 3.5 g of 2-ketoglucose per liter per hour.

P071 SYNTHESIS AND EVALUATION OF UNNATURAL SUGAR NUCLEOTIDES AS DONOR SUBSTRATES IN GLYCOSYLTRANSFERASE--CATALYZED REACTIONS

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Keywords: glycosyltransferase, donor substrate, analogue, sugar nucleotide

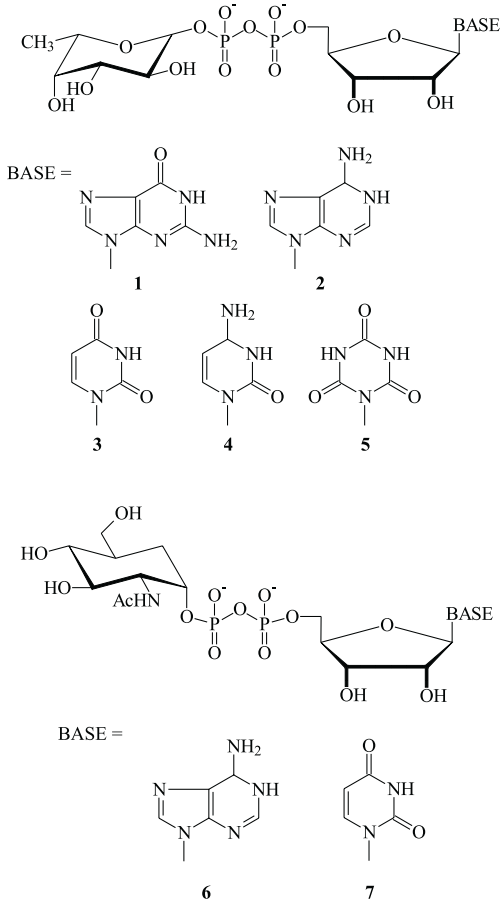
The availability of cloned glycosyltransferases has made enzymatic glycosylation a realistic alternative to chemical synthesis. However, a severe drawback to this approach is the requirement for sugar nucleotides. *In situ* regeneration of the sugar nucleotides is the most elegant way of using these cofactors, but it calls for additional enzymes and complicates matters. From a preparative point of view, donor substrates with a simpler structure than sugar nucleotides, which would be prepared at a lower cost, would be highly desirable. This question can be addressed according to two independent approaches, searching either for direct nucleoside analogues or diphosphate bond mimics. We chose to tackle the problem by first looking at the possible changes of the base in the nucleotide part of the molecule.

Therefore, we describe here the chemical synthesis of four unnatural sugar nucleotides **2**, **3**, **4**, **5**, analogues to GDP-Fuc **1**, the natural fucose donor, and the sugar nucleotide **6**, analogue to UDP-GlcNAc **7**, the natural *N*-acetylglucosamine donor. These syntheses rely on the coupling of fucose-β-1-phosphate or *N*-acetylglucosamine-α-1-phosphate with the nucleotide activated as morpholidate[1].

The new compounds **3**, **4**, **5**, as well as compound **2** already known[2] were tested as donor substrates of a recombinant human α(1-3/4)fucosyltransferase (FucTIII)[3] and compound **6** as a donor substrate of a recombinant bacterial β(1-3)*N*-acetylglucosaminyltransferase[4]. Their ability to serve as donors was evaluated in simple enzymatic assays using fluorescent oligosaccharide acceptors.

According to the kinetic constants, UDP-Fuc **3** turned out to be as efficient a substrate of FucTIII as ADP-Fuc **2** (ref.[5]). Analogue **5,** easily prepared from cyanuric acid, a very cheap starting material, exhibited a low affinity towards FucTIII, but a higher V_{max} than CDP-Fuc **4**, a poor substrate. Furthermore the purine sugar nucleotide **6** turned out to be a very poor donor substrate of the bacterial *N*-acetylglucosaminyltransferase, whereas conversely the pyrimidine sugar nucleotide **3** is well recognized as a substrate of FucTIII.

In conclusion fucose can be effectively transferred from the GDP-Fuc analogues **2**, **3** and even **5** to oligosaccharide acceptors in the α(1-3/4)fucosyltransferase-catalyzed reaction.



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P072 FRUCTOSE-1,6-DIPHOSPHATE ALDOLASE MEDIATED SYNTHESES OF AMINOCYCLITOLS, ANALOGUES OF VALIOLAMINE

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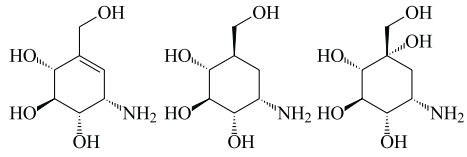
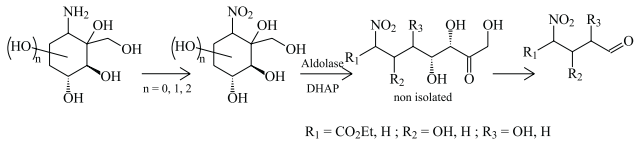
Keywords: aldolase, aminocyclitols, valiolamine analogues, inhibitors, glycosidases

Besides azasugars like nojirimycine and mannojirimycine, or aminocyclopentitols like mannostatine A, six members ring aminocyclitols appear as potential inhibitors of glycosidases. Valienamine, validamine and valiolamine found in the structure of antibiotics like validamycin or antidiabetic agents like acarbose or voglibose, are natural products[1].



Numerous syntheses of sugar analogues based on the utilisation of aldolases and transketolase have been published, but only few of them have concerned cyclitols[2–4]. In this communication, we will present our work concerning the condensation of dihydroxyacetone phosphate (DHAP) with nitroaldehydes catalysed by the fructose-1,6-diphosphate aldolase.



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P073 NEW FRUCTOSYLTRANSFERASES FOR POTENTIAL INDUSTRIAL APPLICATIONS

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Keywords: fructosyltransferase, levansucrase, sucrose

Glycosyltransferases catalyse the transfer of sugar moieties. Most of the so far known enzymes belong to the Leloir-type and therefore require activated sugar derivatives such as UDP-glucose. These substances are too expensive for usage in food industry.

Only few glycosyltransferases are known which are able to transfer sugar moieties just by utilisation of high energy glycosidic bonds. For instance the dextransucrase from *Leuconostoc mesenteroides*[1] and the levansucrase from *Rahnella aquatilis*[2] can utilise sucrose as sole substrate for polysaccharide formation.

The especially promising market in the food sector, the pre- and synbiotics for use as functional food, constitutes the need for new enzymes implementable in industrial processes.

After investigating sucrose containing habitats in a semi automated screening (microplate scale), several micro-organisms with either levan- or inulinsucrase activity were obtained. Those strains, showing highest fructosyltransferase activity in the screening assay were compared to the reference organism *Rahnella aquatilis*, concerning activity and formed products.

This work is financially supported by the DBU (Az: 13066).

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P074 EXPRESSION OF *Fusarium oxysporum* LACTONASE GENE IN *Aspergillus oryzae*

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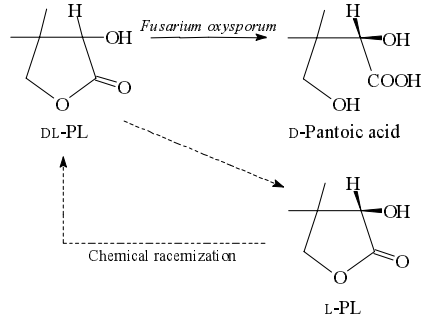
Keywords: lactonase, optical resolution, pantoyl lactone, *Fusarium oxysporum*, *Aspergillus oryzae*

There is a group of esterase-family enzymes, *i. e.* lactonases, which catalyze the hydrolysis of the intramolecular ester bonds of lactone compounds. The reactions catalyzed by lactonases, as well as those by lipases and other esterase-family enzymes, sometimes show stereoselectivities, and thus should be applicable to the synthesis of useful compounds. For example, a novel lactonase from *Fusarium oxysporum*, which was previously found in our laboratory[1], catalyzed the stereoselective hydrolysis of D-pantoyl lactone (D-PL), and enabled the large-scale optical resolution of DL-PL. The enzymatic resolution of DL-PL using the Fusarium cells has already come into practical use, and it has been shown that the novel enzymatic process is highly satisfactory not only from an economic aspect but also an environmental one.



Here, we report the overexpression of the *Fusarium* lactonase gene in a heterologous fungus, *Aspergillus oryzae*, and its application to the enzymatic resolution of DL-PL as the enzyme source with higher potential.

The lactonase genomic gene of *F. oxysporum* contains five introns and presumed NH_2-terminal signal peptide. In order to determine whether *A. oryzae* can recognize these splice junctions and the signal peptide, we constructed three plasmids. The plasmid pNAN-PC bears the cDNA coding the mature lactonase. pNAN-XC and pNAN-XG bear, respectively, the cDNA and genomic DNA coding full-length form of the lactonase including the signal peptide. The transformants harboring each plasmid could produce the lactonase, but the structures of the recombinant enzymes were different from each other. While the molecular mass of the lactonases of pNAN-XC and pNAN-XG transformants were estimated to be 60 kDa, which was identical to that of the wild type enzyme, the pNAN-PC transformant produced an extra protein of 51 kDa. Deglycosylation analysis with glycopeptidase revealed that the difference of the molecular mass arise from the sugar content of each recombinant enzyme.

The mycelia of the transformants were immobilized by calcium alginate gel, and used as catalyst for asymmetric hydrolysis of DL-PL. When the immobilized mycelia of the pNAN-XG transformant were incubated with 35 % DL-PL solution for 24 h, 49.1 % of the initial amount of DL-PL was converted to D-pantoic acid, stereoselectively.

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P075 CLONING OF THE GENE ENCODING FOR RHAMNOGALACTURONASE BY A CONSTITUTIVE MUTANT OF *Penicillium* STRAIN

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Keywords: pectinase, mutagenesis, rhamnogalacturonase, PCR

The production of pectinases was studied in fungi and one of them, *Penicillium occitanis*, was chosen for nitrous acid mutagenesis. Based on hexadecyl-trimethyl-ammonium (CTAB) staining and after only one round of mutagenesis, an interesting mutant, CT1, was selected. It secretes about 50 times more pectinase than the wild-type strain (CL100) but not cellulases or other hydrolases. In comparison with another already known mutant of the same strain (the Pol 6 mutant) CT1 is not only genetically stable and sporulating, but also able to secrete high amounts of pectinases on local substrates such as "orange peel" and "gruel". The most interesting feature of this mutant is its constitutivity: it produces the same specific activity of pectinases on citrus pectin as well as on glycerol or glucose, which are potent repressors of pectinolytic activities in *Penicillium* and many other fungi.

In order to get molecular information on the other pectinolytic genes in this mutant, namely the disbranching enzymes, we started by the isolation of a rhamnogalacturonase gene by PCR strategy using genomic DNA and a couple of rhamnogalacturonase consensus primers. This fragment will be used as a probe for mRNA analysis.

P076 PREPARATION OF A CYCLODEXTRIN GLUCANOTRANSFERASE FROM THE BACTERIAL ISOLATE BT3-2: TOWARDS THE PRODUCTION OF LARGE RING CYCLODEXTRINS

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Keywords: cyclodextrin glucanotransferase, coupling, cycling, large ring cyclodextrins

Cyclodextrin glucanotransferase (CGTase) was recently confirmed produce not only small cyclodextrins such as CD6, CD7 and CD8, but also large ring cyclodextrins (LR-CD) with a degree of polymerisation (DP) up to at least 60 (ref.[1, 2]). CGTases differ not only with regard to the equilibrium amounts of cyclodextrins produced but also with regard to the courses by which they are formed[3]. CGTase from bacterial isolate BT3-2 was purified to homogeneity using the DEAE sepharose 6FF and resource Q chromatography. About 39 % of the enzyme activity could be recovered. Characterization of the enzyme showed a temperature optimum of 60 °C and a pH optimum of 6.0. The synthesis of LR-CD by the purified enzyme from bacterial isolate BT3-2 was investigated using synthetic amylose as the substrate. After 20 hours incubation at 60 °C in 50 mM acetate buffer (pH 5.5), LR-CD with DP up to 50 could be detect under the experiment conditions. LR-CD from CD9 to CD21 were quantified using HPAEC chromatography. This confirmed that the production of LR-CD at the initial stage is the common feature of CGTase. Adjust the reaction condition of the CGTase could produce LR-CD.

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P077 PROTEASE CATALYSED TRANSESTERIFICATION OF SUCROSE AND CYCLODEXTRINS

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Keywords: protease, transesterification, sucrose, cyclodextrin, solvents

Three proteases were investigated for their catalytic properties regarding synthesis of fatty acid esters of sucrose and cyclodextrins in DMSO and other organic solvents.

Sucrose laurate esters were synthesised from sucrose and vinyl laurate in organic solvents using an alkaline protease AL-89 from a new alkalophilic strain, *Bacillus pseudofirmus* AL-89. Maximum synthetic activity was observed in DMF mixed 1 : 1 (v/v) with either DMSO or pyridine in the presence of 7.5 % (v/v) water at pH 7–10. With protease AL-89 esterification occurred predominantly at the 2-*O*-position while subtilisin A catalysed monoester predominantly at the 1'-*O*-position. In the absence of enzyme, buffer salts cata-

lysed nonspecific synthesis of a number of esters. Nonspecific catalysis was also observed upon inhibition of protease AL-89 using a serine protease inhibitor as well as inactivation at pH above 10 (ref.[1]).

Thermolysin catalysed the formation of sucrose esters from sucrose and vinyl laurate in DMF and DMSO respectively, with 2-*O*-lauroyl-sucrose as the main product and a specific activity in DMSO of 53 $nmol.min^{-1}.mg^{-1}$. Transesterification reactions are normally observed only when the mechanism involves an acyl enzyme intermediate, as with lipases or serine proteases, and not with metalloproteases like thermolysin. A possible reason could be the affinity of the active site of thermolysin for sugar moieties, as for the potent inhibitor phosporamidon. The reaction was not catalysed by other proteins under the same conditions, and was inhibited by removal of the active site zinc[2].

Vinyl fatty acid esters were used as acyl donors for esterification of cyclodextrins in DMSO and other solvents. The cyclodextrin esters obtained were isolated and characterized by FTIR, NMR and mass spectroscopy. The position and degree of substitution depended on the choice of protease and substrate, with Thermolysin regioselective substitution of β-cyclodextrin at the glucose C2 position was obtained.

Protease activity and regioselectivity is discussed regarding the effect of polar, aprotic solvents on the solubility and conformation of carbohydrates and proteins.

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P078 ALKYL-β-GLYCOSIDE SYNTHESIS USING β-GLYCOSIDASES FROM FILAMENTOUS FUNGUS

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Keywords: alkyl-glucoside, alkyl-xyloside, β-glucosidase, β-xylosidase, filamentous fungus

The importance of alkyl-glycosides[1], in general, has been demonstrated for many industrial applications as non-ionic surfactants. β-Glycosidases, β-glucosidase and β-xylosidase, were used in the synthesis of alkyl-glycosides by transglycosylation reaction with long-chain alcohols. The medium is biphasic[2] (alcohol/aqueous) in which the aqueous phase (pH 4 to 5) contains 5 U enzyme and 50 mg sugar substrate per ml. The synthesis was studied under different conditions with primary and secondary alcohols as substrates, in presence of free or immobilized enzyme. Different supports were tested for immobilization. β-Glucosidase and β-xylosidase were immobilized by adsorption on Duolite, Amberlite, Celite and DEAE-sepharose, and also by entrapment in polyacrylamide gel or reticulation using glutaraldehyde. We used cellobiose and xylan respectively for the synthesis of alkyl--glucosides and alkyl-xylosides.

Table I summarizes some results of transglycosylation reaction catalyzed by β-glycosidases from *Sclerotinia sclerotiorum* compared to activity from other source. Highest yields were obtained when using xylan and C_4 to C_6-alcohols in presence of enzyme extract from *S. sclerotiorum*. The reaction produced alkyl-β-xyloside, alkyl-β-xylobioside as well as small amounts of alkyl-β-xylotrioside and alkyl-β-xylotetraoside identified by MS. HPLC analysis quantified the production of alkyl-β-xyloside and alkyl-β-xylobioside (Prontosil C18-AQ, refractometer). Up to 22 mM isoamyl-xyloside and 14 mM isoamyl-xylobioside were produced from isoamyl alcohol and xylan. When using xylan and hexan-1-ol as substrates the synthesis reaction takes around 3 days. Two enzymes are involved in this reaction. After hydrolysis of xylan with endo-xylanase, the β-xylosidase is able to catalyze transxylosylation between partially hydrolyzed xylan and alcohol with more than 20 % conversion yield. Alkyl-glycosides were applied in detergents and pharmaceuticals as biosurfactants.

The used enzymes are produced by a plant pathogenic fungus, and participate to the degradation of polysaccharides (as cellulose and xylan) among other hydrolyzing enzymes that damage the plant cell-walls (xylanases, pectinases, cutinases, esterases, endoglucanases…). The production of β-glucosidase and β-xylosidase was optimized in presence of different carbon sources[3]. This had influence not only on the enzyme concentration but also on the iso-enzyme production. Enzyme activities and stabilities were investigated under different conditions after purification achieved on ion exchange and gel filtration chromatography.

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Table I
Alkyl-xyloside and alkyl-glucoside synthesis in biphasic medium

	Butan-1-ol[a]	Isoamyl alcohol[a]	Me-2-butan-2-ol[a]	Pentan-2-ol[a]	Pentan-1-ol[a]
Alkyl-β-glycoside[e]	2.1	21	0.9	14.2	17
Alkyl-β-glycobioside[e]	1	13.6	0.5	2.2	11

	Hexan-1-ol[a]	Hexan-1-ol[b]	Hexan-1-ol[c]	Octan-1-ol[a]	Octan-1-ol[d]
Alkyl-β-glycoside[e]	9.7	1.1	12	2.8	2.5
Alkyl-β-glycobioside[e]	3.8	nd	nd	2.1	nd

[a]Synthesis of alkyl-xyloside in presence of β-xylosidase from *S. sclerotiorum* in biphasic medium alcohol/aqueous (4/1) pH 4, 50 °C, 250 rpm, 10 mg.ml^{-1} xylan oat spelt; [b]synthesis of alkyl-xyloside in presence of immobilized β-xylosidase from *S. sclerotiorum* on Celite 545 in biphasic medium alcohol/aqueous (4/1) pH 4, 50 °C, 250 rpm, 10 mg.ml^{-1} xylan oat spelt; [c]synthesis of alkyl-glucoside in presence of β-glucosidase from *S. sclerotiorum* in biphasic medium alcohol/aqueous (4/1) pH 5, 50 °C, 250 rpm, 150 mg.ml^{-1} cellobiose; [d]synthesis of alkyl-xyloside in presence of β-xylosidase from *Trichoderma reesei* in biphasic medium alcohol/aqueous (4/10) pH 4.5, 40 °C, 300 rpm, 14.3 mg.ml^{-1} xylan oat spelt; [e]alkyl-β-xyloside or alkyl-β-glucoside in mmol.l^{-1}

P079 PRODUCTION OF FUNGAL β-*N*-ACETYLHEXOSAMINIDASE – EFFECTS OF VARIOUS INDUCTORS

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Keywords: *Aspergillus oryzae*, β-*N*-acetylhexosaminidase, *N*-acetyl-D-glucosamine, induction, secretion

Fungal β-*N*-acetylhexosaminidases are widely known for their participation in the process of extracellular digestion of chitin. They are also important tool in biotechnology due to its ability to carry out enzymatic synthesis of oligosaccharides[1]. β-*N*-Acetylhexosaminidases from *Aspergillus oryzae* CCF1066 – enzyme showing interesting properties in chemoenzymatic reactions[2] – was isolated from the medium and sequenced using both protein chemistry and molecular cloning of the corresponding gene. We also studied the details of induction and intracellular transport of the enzyme. We have found that a broad range of chitin-related compounds can be used for induction of the enzyme's biosynthesis. However, only the end cleavage product of the enzyme – *N*-acetylglucosamine, induced accelerated secretion of the enzyme, which was characteristic by high production of the enzyme accompanied by higher total protein secretion. Moreover, distinct changes in mycelliar morphology were observer upon induction with *N*-acetylglucosamine.

A characteristic peptide motif was found in the N-terminal part of the enzyme. We proposed that this motif may be involved in the accelerated secretion of the enzyme, by which the enzyme is released from the intracellular stores depending on the extracellular concentration of its end cleavage product.

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P080 LIPASES-CATALYSED PREPARATION OF REGIOSELECTIVELY ACETYLATED 4-NITROPHENYL GLYCOSIDES

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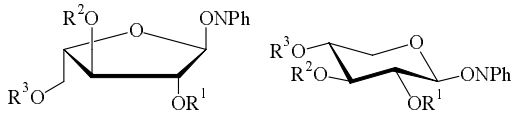
Keywords: lipase, regioselective deacetylation or acetylation, 4-nitrophenyl glycoside

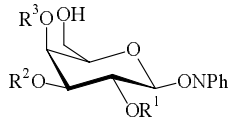
Development of economically feasible and ecologically friendly processes of bioconversion of renewable plant biomass requires detail knowledge of catalytic properties of microbial glycosyl hydrolases and esterases involved in biodegradation of plant cell walls. In this connection, there is an increasing demand for variety of regioselectively acetylated 4-nitrophenyl glycosides that can serve as precursors for chromogenic substrates important for investigation of catalytic properties of the enzymes and for elaboration of simple methods for enzyme monitoring. In this work we present summary of results of lipase-catalysed preparations of di-*O*-

and mono-*O*-acetates of 4-nitrophenyl α-L-arabinofuranoside **1-7** , 4-nitrophenyl β-D-xylopyranoside **8-12** and 2,3,4-tri-*O*--acetate of 4-nitrophenyl β-D-galactopyranoside **13** (Figure). The acetates of NPh glycosides **1** and **13** were produced by selective hydrolysis of per-*O*-acetylated NPh glycosides by lipase AY *(Candida rugosa)* or Lipolyve CC *(Candida cylindracea)* in phosphate buffer. Diacetates **2**, **3**, **8** and **9** were obtained by regioselective acetylation of the corresponding glycoside in the presence of Lipase PS *(Burkholderia cepacia)* and vinylacetate in organic solvent. Variation of polarity of organic solvents was found to have an effect on regioselectivity of di-*O*-acetylation and on the yields of products **8** and **9**. The influence of reaction period and organic solvents on the main production of monoacetates **10-12** during lipase PS acetylation will be also presented. We were able to isolate monoacetate **6** as the main product by controlling the lipase PS acetylation of NPh α-L-arabinofuranoside. Finally, we prepared monoacetates **4** and **5** by enzymatic hydrolysis of primary position of diacetates **2** and **3**. The diacetates **1-3** , **7-9** and triacetate **13** were used for the syntheses of saccharidic ferulates[1, 2] through enzymatic protection, feruloylation and chemical deacetylation. We intend to use monoacetates **4-6** and **10-12** to study substrate specifity and substrate structure requirements of α-L-arabinofuranosidases and β-D-xylosidases.



NPh = 4-nitrophenyl

1 R^1=Ac, R^2=Ac, R^3=H
2 R^1=Ac, R^2=H, R^3=Ac
3 R^1=H, R^2=Ac, R^3=Ac
4 R^1=Ac, R^2=H, R^3=H
5 R^1=H, R^2=Ac, R^3=H
6 R^1=H, R^2=H, R^3=Ac
7 R^1=Ac, R^2=Ac, R^3=H
8 R^1=Ac, R^2=H, R^3=Ac
9 R^1=H, R^2=Ac, R^3=Ac
10 R^1=Ac, R^2=H, R^3=H
11 R^1=H, R^2=Ac, R^3=H
12 R^1=H, R^2=H, R^3=Ac



13 R^1=R^2=R^3=Ac

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P081 KINETICS AND THERMODYNAMICS OF ENANTIOSELECTIVE ALCOHOL RELEASE STEP IN LIPASE-CATALYZED HYDROLYSIS OF SYNTHETIC ESTERS

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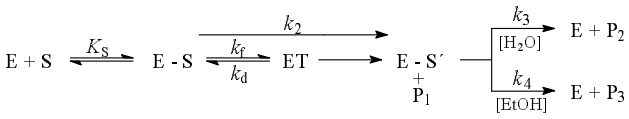
Keywords: acylation, nucleophile, tetrahedral intermediate, transition state

We present the results of kinetic and thermodynamic investigations of alcohol release step in the *Candida antarctica* lipase B (CALB)-catalyzed hydrolysis of the acetates of the both single enantiomers of a series of chiral primary and secondary alcohols in sufficiently emulsified reaction mixtures of water-insoluble substrates and discuss the mechanism of action of the enzyme including rate determining enantioselective catalysis in the step.

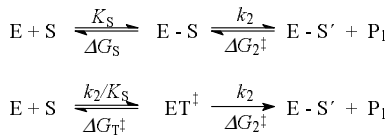
The partition experiments shown in Scheme 1 has successfully determined deacylation rate constant k_3 being 4×10^3 s^{-1} using ethanol as a nucleophile. The value, extremely larger than k_{cat}s obtained, has demonstrated quantitatively the acylation being the rate determining step and $k_{cat} = k_2$ and $K_m = K_S$ for almost all the substrates examined.

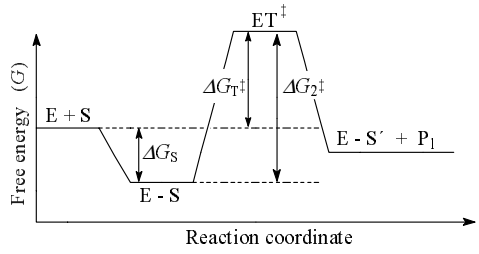
Measuring k_2, K_S and k_2/K_S values for each substrates above at 40 °C, we have determined thermodynamic parameters associated with these kinetic constants (Scheme 2) of the both enantiomers of the two substrates **1** and **2** exhibited in Table I.

The results in Table I clearly indicate that great enantioselectivity of the secondary alcohol is predominantly attributed to the large enthalpy difference of activation between the two enantiomers, whereas small enantioselectivity of the primary alcohol is mainly due to the difference in entropic factor of activation. Table II A shows the binding feature of *(R)*-**2** in the ES complex is different from that of *(S)*-**1**, although the strength of binding appears to be similar to each other. The tiny enthalpy of activation for *(R)*-**2** in Table II B indicates the substrate was bound favorably to release the enthalpy greatly in the transition state. This may imply the rate determining step is the formation of tetrahedral intermediate (ET) in the alcohol release step. On the other hand, rather high entropy of activation of *(S)*-**1** may suggest the release of solvent shell water in the transition state and rate determining breakdown of ET.



Scheme 1





Scheme 2

Table I
Thermodynamic parameters of CALB for enantiometric ratio $(k_2/K_S)^R/(k_2/K_S)^S = E_{R-S}$ at 313 K

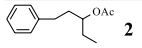
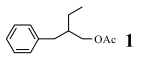
Substrate		$\Delta\Delta G_T^I$ (kcal.mol^{-1})	$\Delta\Delta H_T^I$ (kcal.mol^{-1})	$T\Delta\Delta S_T^I$ (kcal.mol^{-1})	$\Delta\Delta S_T^I$ (cal.mol.T^{-1})
	**2**	–5.2	–9.3	–4.1	–13.0
	**1**	1.1	–1.1	–2.2	–7.0

Table II
Thermodynamic parameters of CALB for hydrolysis of single enantiomers of **2** and **1** at 313K. Date shown are only for fast-reacting enantiomers

(A)

			ΔG_S (kcal.mol^{-1})	ΔH_S (kcal.mol^{-1})	$T\Delta S_S$ (kcal.mol^{-1})	ΔS_S (kcal.mol^{-1}.T^{-1})
$1/K_S$	**2**	*R*	–5.0	–4.1	0.93	3.0
	1	*S*	–4.2	–9.0	–4.8	–15.2

(B)

			ΔG^I (kcal.mol^{-1})	ΔH^I (kcal.mol^{-1})	$T\Delta S^I$ (kcal.mol^{-1})	ΔS^I (kcal.mol^{-1}.T^{-1})
k_2	**2**	*R*	17.0	5.0	–12.0	–38.0
	1	*S*	17.6	14.3	–3.3	–10.5
k_2/K_S	**2**	*R*	12.1	0.96	–11.1	–35.0
	1	*S*	13.3	5.3	–8.0	–26.0

P082 SUBSTRATE PROFILES OF NITRILE HYDROLYSING BIOCATALYSTS

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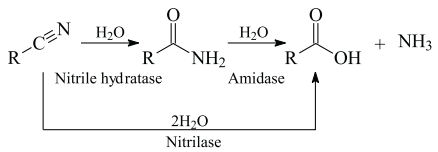
Keywords: biocatalysis, nitrilase, nitrile hydratase, amidase, substrate profile

We investigated biocatalytic reactions to convert aromatic and arylaliphatic nitriles to the structurally related amide or acid. Each biocatalyst exhibited a distinctive substrate selectivity profile, related to the length of the aliphatic chain of the arylaliphatic nitrile, and the position of substituents on the aromatic ring or aliphatic chain[1, 2]. The cell free nitrilases exhibited a narrower substrate range than the resting whole cells of *Rhodococci* with nitrile hydratase activity.



Based on this information, we were able to provide an initial model of the enzyme active sites using quantitative structure-activity relationships and predict additional substrates.

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P083 SYNTHESIS OF ENANTIOPURE CARBOXYLIC ACIDS USING NITRILASE CATALYSIS

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Keywords: nitrile, nitrilase, enantiopure carboxylic acid

Nitriles are versatile synthetic intermediates due to the ease with which they can be obtained and subsequently hydrolysed, yielding the corresponding amides and acids. The products from nitrile hydrolysis have a great potential as building blocks for the life science industries (pharmaceuticals and agrochemicals), provided they are enantiopure.

Biocatalytic procedures for the hydrolysis of nitriles (Fig. 1) have numerous advantages when compared to the chemical pathways, as regards reaction conditions, chemo-, regio- and stereoselectivity.

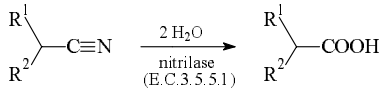

Fig. 1: **Enzymatic nitrile hydrolysis**

The principles have been demonstrated by Yamamoto who produced *(R)*-mandelic acid from mandelonitrile using *A. faecalis* cells[1]. Our goal is to create novel methods for synthesizing commercially relevant, enantiopure carboxylic acids with the use of nitrilases produced by either naturally selected or genetically engineered strains.

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P084 BIOTRANSFORMATION OF *N*-PROTECTED β-AMINO NITRILES TO β-AMINO ACIDS

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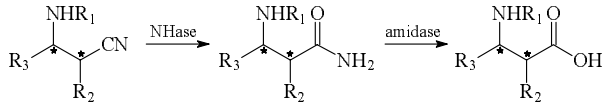
Keywords: β-amino nitrile, β-amino acid, biotransformation, nitrile hydratase

In the last decade, β-amino acids have been recognized to have antibiotic, antifungal, cytotoxic and other pharmacological effects[1]. Recent work by several authors[2] has demonstrated that carboxylic acids can be prepared from nitriles using isolated enzymes or whole cell systems of *Rhodococci* under very mild conditions.

We wish to report a new protocol for the preparation of *N*-protected β-amino acids and carboxamides using whole cells of *Rhodococcus* sp. R312 and *Rhodococcus erythropolis* NCIMB 11540, both containing the nitrile hydratase/amidase system.



Alicyclic as well as aliphatic β-amino nitriles were prepared bearing different *N*-protecting groups. Screening experiments revealed that the *N*-protecting group has a significant influence on the biotransformation. Moreover, alicyclic substrates are better accepted than their aliphatic counterparts. Isolated yields were determined of those substrates which showed promising screening results.

In summary, we have developed a novel approach to β-amino acids and amides, some of which have not been reported in the literature to date. Extensions of our work are due.

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P085 NITRILE HYDRATASE-CATALYSED TRANSFORMATIONS OF GLYCOSYL CYANIDES

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Keywords: nitrile hydratase, *Rhodococcus*, glycosyl cyanide

Rhodococcus equi strain A4 is a versatile biocatalyst for hydrolysis of nitriles and amides. The purified nitrile hydratase of this microorganism has a very broad substrate specificity towards a wide range of nitriles[1, 2]. Fungal strain *Aspergillus niger* K10 also seems to be a promising source of nitrile hydratase. Saccharides bearing a nitrile moiety have been rarely demonstrated as substrates of nitrile hydratases[3, 4]. The enzymes examined by us showed activity towards such nitriles.

2,3,4,6-Tetra-*O*-acetyl-β-D-glucopyranosyl cyanide **(1)**; 2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl cyanide **(2)**; 4,5,7--tri-*O*-acetyl-2,6-anhydro-3-deoxy-D-*lyxo*-hept-2-enononitrile **(3)** were tested as substrates for both purified nitrile hydra-

tase and whole cells of *Rhodococcus or Aspergillus*. 2,6-Anhydro-3-deoxy-D-*lyxo*-hept-2-enononitrile **(4)** was prepared by deacetylation of **3** and used as a substrate for the purified enzyme only. In case of *Rhodococcus*, glycosyl cyanides **1** and **3** were transformed into the corresponding amides and at the same time partially deacetylated due to presence of an esterase activity in this strain. However, biotransformation of **1** was slower and the major product was the deacetylated glycosyl cyanide. *Aspergillus* transformed only glycosyl cyanide **3** and produced deacetylated amide. By using purified nitrile hydratase, both **3** and **4** were converted into 2,6-anhydro-3--deoxy-D-*lyxo*-hept-2-enonoamide. Deacetylation of **3** occurred because of remaining esterase activity in nitrile hydratase. Hydration of the cyano group into amide in **1** was very slow as that catalyzed by whole cells. This suggests that substitution on C-2 decreased nitrile hydratase activity. Galactosyl cyanide **2** was substrate neither for nitrile hydratase nor for microorganisms tested.

Financial support of this work from the Grant Agency of the Academy of Sciences of the Czech Republic (project A4020213/2002), Ministry of Education of the Czech Republic (project OC DC25.001), COST D25/0002/02 and Institutional Research Concept no. AV0Z5020903 is gratefully acknowledged.

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P086 ENZYMATIC HYDROLYSIS OF NITRILES USING *Aspergillus niger* K10

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Keywords: nitrile hydratase, amidase, substituted benzonitriles, 2-substituted acrylonitriles

Data published on fungal nitrile-converting enzymes have been scarce to date. Hydrolysis of 3-indoleacetonitrile, 2-aminonitriles, di- and trinitriles was demonstrated with filamentous fungi. Detection of carboxylic acids as products supported the opinion that nitrilases were involved in these biotransformations[1–3]. Indeed, a nitrilase accepting aromatic nitriles as substrates was purified from *Fusarium solani*[4]. However, the only purified nitrile hydratase from a filamentous fungus was that from *Myrrothecium verrucaria*[5]. This enzyme showed a narrow substrate specificity for cyanamide.

Aspergillus niger K10 studied in the present work produced carboxylic acids from most aromatic and arylaliphatic nitriles tested. However, formation of picolinamide from 2-cyanopyridine and 4-cyanobenzamide from 1,4-dicyanobenzene suggested that the fungus metabolized nitriles via the nitrile hydratase/amidase pathway. Steric hindrances in these nitriles probably prevented the amidase from hydrolysis of the amide intermediates.

Benzonitrile (at 10 mM) was the superior substrate of the fungus, being totally hydrolyzed within 3 h. *Meta*- and *para*-substituted benzonitriles and 2-, 3- and 4-cyanopyridine were converted at a significantly lower rate than benzonitrile while *ortho*-substituted benzonitriles (2-tolunitrile, 1,2-dicyanobenzene) were not substrates of the nitrile hydratase. 3-Chlorobenzonitrile as a substrate with an electron-withdrawing substituent was hydrolyzed more rapidly than 3-tolunitrile and 3-hydroxybenzonitrile, i. e. compounds bearing electron-donating substituents. Small arylaliphatic nitriles such as 2-phenylacetonitrile and 2-thiopheneacetonitrile were also hydrolyzed rapidly, but more bulky molecules such as 3-indoleacetonitrile or nitriles substituted at C-2 such as 2-phenylpropionitrile were poor substrates. 2-Substituted acrylonitriles, e. g. 2-(1-hydroxy-1-phenylmethyl)-acrylonitrile and 2-(2-hydroxy-3,3-dimethoxy-propyl)-acrylonitrile, were converted at a slower rate than aromatic substrates. The biocatalyst applied either as whole cells or cell extract was functional at both alkaline and acidic pH values.

Financial support from the Grant Agency of the Academy of Sciences of the Czech Republic (project A4020213/2002), MSMT (Barrande 2003-020-2, ME 579, OC D25.001), COST D25/0002/02 and the Institutional Research Concept No. AVOZ5020903 is gratefully acknowledged.

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P087 FAST SCREENING OF NITRILE HYDRATASES ON COLONY LEVEL

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Keywords: nitrile hydratase, screening, high throughput

Nitrile hydratases catalyze the addition of water to nitriles forming amides, which are not further hydrolyzed, as is the case for nitrilases[1–5]. Both, substrate and reaction product are not easily detectable, especially when taking the case of a cell background. We therefore applied an amidase for the detection of the amide, released by the enzyme reaction. This sensing enzyme shows sufficient selectivity towards the primary amide compound, leaving behind the biological matrix. Hydroxamic acid for detection as colored iron compound is formed, as the sensing step is done in the presence of hydroxylammonium chloride. In a second step the gene for the expression of the accessory amidase has been integrated into an *E. coli* chromosome, thus forming a screening strain for the easy detection of nitrile hydratase expression from plasmid libraries transformed to this strain. We demonstrated the usefulness of this novel method of screening for the fast identification of nitrile hydratase activity among bacteria. Furthermore we managed to identify nitrile hydratase active clones in chromosomal gene libraries.

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P088 IN SITU SYNTHESIS OF PROTECTED CYANOHYDRINS USING OXYNITRILASE

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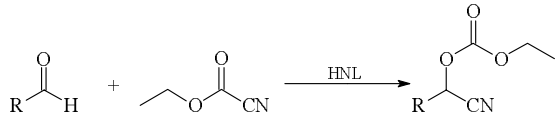
Keywords: hydroxynitrile lyase, oxynitrilase, cyanohydrins, ethyl cyanoformate

The enantioselective formation of cyanohydrins from aldehydes and ketones catalyzed by hydroxynitrile lyases represents a method to produce versatile chiral building blocks with high optical purities as precursors for the synthesis of biologically active molecules such as α-hydroxy carboxylic acids, α-hydroxy ketones or β-amino alcohols[1, 2]. Up to now only HCN and acetone cyanohydrin were used as cyanide sources. This prompted us to investigate the synthetic potential of ethyl cyanoformate as a reagent consisting of both the cyanide group and a protecting functionality for the *in situ* derivatization of the cyanohydrin formed in this reaction.



Due to the fact that some cyanohydrins are labile this concept opens the possibility to obtain protected cyanohydrins and hence stable products with high enantiomeric excess from an enzyme catalyzed one pot procedure. Mechanistical considerations based on NMR-studies and experimental results will be disclosed in more detail in this presentation.

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P089 ENZYME CATALYSED CYANOHYDRIN FORMATION FROM HETEROCYCLIC KETONES

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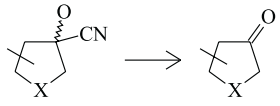
Keywords: hydroxynitrile lyase, oxynitrilase, cyanohydrins

Cyclopentenone cyanohydrin glycosides are known from nature[1], cyclobutanone derivatives were synthesised enzyme catalysed recently[2]. These results prompted us to investigate diverse derivatives of methyltetrahydrofuranon and methyltetrahydrothiophenon. This new class of substrates reacted with HCN under the catalytic action of hydroxynitrile lyase (HNL) from *Hevea brasiliensis* as well as *Prunus amygdalus* according to known procedures[3]. Depending on the methyl position, the heteroatom, and various reaction conditions different distributions of diastereomers were obtained.



X = S or O

For structure elucidation after enzymatic conversion further transformations were performed.

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P090 FOLLOW-UP CHEMISTRY OF ENZYMATICALLY PRODUCED OPTICALLY PURE FERROCENYL CYANOHYDRINS

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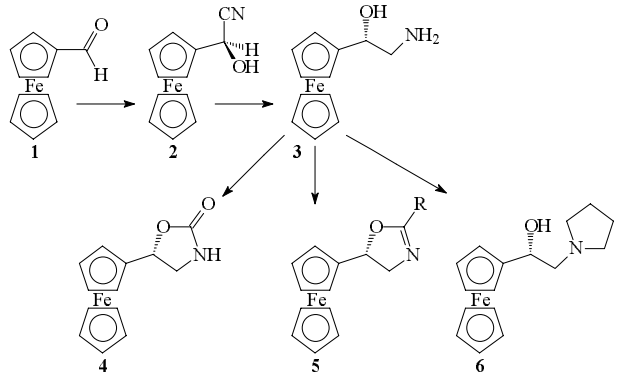
Keywords: hydroxynitrile lyase, ferrocene, cyanohydrins

Based on the *Hevea brasiliensis* hydroxynitrile lyase catalysed transformation of ferrocene-carbaldehyde **1** to the corresponding *(R)*-cyanohydrin **2** (99 % ee, 98 % yield)[1] several follow-up reactions were performed. Reduction of the unprotected cyanohydrin constitutes a convenient and high-yielding access to enantiomerically pure ferrocenyl amino alcohol **3**. Amino alcohols of similar structure have attracted attention as catalysts for the enantioselective addition of dialkylzinc--compounds to aldehydes[2] and as chiral ligands for ruthenium catalysed asymmetric hydrogenation[3]. Furthermore, amino alcohol **3** can easily be transformed into several interesting heterocyclic compounds such as oxazolidinone **4**, oxazoline **5** and pyrrolidine **6**, which may find applications as chiral ligands and auxiliaries for asymmetric synthesis.



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P091 EFFICIENT SYNTHESIS OF OPTICALLY ACTIVE CYANOHYDRINS USING *R*-OXYNITRILASE CLEA

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Keywords: oxynitrilase, cross-linked enzyme aggregate, enantiopurity, cyanohydrin

The enantioselective hydrocyanation of aldehydes in the presence of an oxynitrilase (hydroxynitrile lyase, EC 4.1.2.10) is an established concept in the biocatalytic production of fine chemicals. The competing, uncatalyzed background reaction, which erodes the *ee* of the product, is an inherent problem in such procedures and necessitates a careful tuning of the reaction conditions. We reasoned that the use of a highly active, immobilized biocatalyst would obviate the background reaction in a straightforward manner. Thus, we have prepared a cross-linked enzyme aggregate (CLEA), of *(R)*-oxynitrilase from almonds *via* its precipitation and subsequent cross-linking using glutaraldehyde. The resulting enzyme preparation very efficiently catalyzed the synthesis of of cyanohydrins in a micro-aqueous organic solvent. Under these conditions, the uncatalyzed background reaction was considerably reduced, which greatly benefitted the enantiopurity of the obtained cyanohydrin, especially in the case of slow-reacting substrates, such as *trans*-cinnamaldehyde and bulky *ortho*-substituted benzaldehyde derivatives. Furthermore, the immobilized catalyst could be easily separated from the reaction mixture and recycled for at least ten times, offering an additional advantage over the traditional two-phase system using free enzyme. In conclusion, the use of an oxynitrilase CLEA results in a considerably improved procedure.

P092 ONE POT CONVERSION OF BENZALDEHYDE INTO MANDELIC ACID USING CLEA TECHNOLOGY

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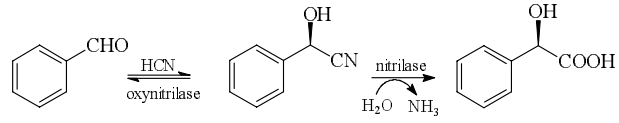
Keywords: CLEA, mandelic acid, dextran, nitrilase, oxynitrilase

Enantio-pure mandelic acid is a very important compound from industrial point of view because it is the precursor of interesting semisynthetic antibiotics. Thus, an asymmetric synthesis starting from a very simple compound such as benzaldehyde would be very interesting. We have investigated a two step bienzymatic procedure for mandelic acid. In the first step, benzaldehyde and HCN are transform in the presence of oxynitrilase. In the second step, the hydrolysis of previously formed mandelonitrile is catalysed by a nitrilase to produce mandelic acid.



The limited stability of the intermediate product, mandelonitrile at neutral or basic pH, is a major obstacle. Hence, we have investigated the simultaneous use of immobilised oxynitrilase and nitrilase in a one-pot procedure.

The enzymes were immobilised as cross-linked enzyme aggregates because these are composed of pure protein and, hence, are highly active. Moreover, the costs of the supports are avoid and CLEAs can be used in organic media[1,2].

By optimisation of the reaction parameters, a quantitative conversion of benzaldehyde into mandelic acid was obtained.

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P093 BIOCATALYTIC CONVERSIONS OF UNNATURAL SUBSTRATES BY RECOMBINANT ALMOND *R*-HNL

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Keywords: HNL, prunus, isoenzyme, cyanohydrine

We have cloned genes coding for isoenzymes of *R*-oxynitrile lyase (*R*-HNL) from almonds and showed expression of active enzyme by the host *Pichia pastoris* (EP1223220).

The enzymes have been optimised for highly efficient enzymatic conversion of unnatural substrates.

Successful examples, which were scaled up to g-scale product formation of industrially relevant cyanohydrins will be shown.

P094 CYANOHYDRIN FORMATION USING WILDTYPE AND MUTANT HNL AS A STARTING POINT FOR FURTHER TRANSFORMATIONS

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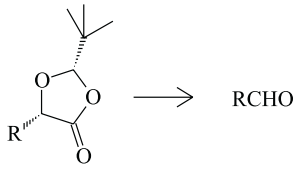
Keywords: hydroxynitrile lyase, *Hevea brasiliensis*, cyanohydrin

Different rigid aldehydes were converted using the hydroxynitrile lyase (HNL) from *Hevea brasiliensis* according to our published procedure[1]. Both the wild type enzyme and mutants were used to prepare the corresponding cyanohydrins achieving high optical purity and yield.



Consequently, after the enzymatic conversion, the cyanohydrins were hydrolysed to the corresponding hydroxy acids and further follow-up chemistry using the method developed by Seebach[2] gave a new approach to versatile optically pure precursors for agrochemicals and pharmaceuticals.

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P095 SUBSTRATE SPECIFICITY OF MUTANTS OF THE HYDROXYNITRILE LYASE FROM *Manihot esculenta*

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Keywords: cyanohydrins, hydroxynitrile lyase, mutants, stereoselectivity, substrate specificity

For the preparation of *(R)*-cyanohydrins, *(R)*-PaHNL from bitter almonds and for the synthesis of *(S)*-cyanohydrins, the recombinant HNLs from cassava (MeHNL) and rubber tree (HbHNL) turned out to be the best biocatalysts[1].

The three-dimensional structures of HbHNL (ref.[2]) and MeHNL (ref.[3]) show a topology that is related to but still distinct from α/β hydrolases. In the active site of MeHNL, Ser80 is involved in binding the substrate carbonyl group, with participation of Thr11, whereas His236 is proposed to act as a general base. From kinetic measurements of the mutants Ser80Ala, Thr11Ala and Cys81Ala combined with structural data a plausible mechanism of cyanogenesis can be deduced.

Several tryptophan128-substituted mutants of the hydroxynitrile lyase from *Manihot esculenta* (MeHNL) are constructed and applied in the MeHNL-catalyzed addition of HCN to various aromatic and aliphatic aldehydes as well as to methyl and ethyl ketones to yield the corresponding cyanohydrins. The mutants, especially MeHNL-W128A, are in most cases superior to the wild-type (wt) enzyme when diisopropyl ether is used as the solvent. Substitution of tryptophan128 by an alanine residue enlarges the entrance channel to the active site of MeHNL and thus facilitates access of sterically demanding substrates to the active site, as clearly demonstrated for aromatic aldehydes, especially 3-phenoxybenzaldehyde. These experimental results are in accordance with the X-ray crystal structure of MeHNL-W128A. Aliphatic aldehydes, surprisingly, do not demonstrate this reactivity dependence of mutants on substrate bulkiness. Comparative reactions of 3-phenoxybenzaldehyde with wtMeHNL and MeHNL-W128A in both aqueous citrate buffer and a two-phase system of water/methyl *tert*-butyl ether again reveal the superiority of the mutant enzyme: 3-phenoxybenzaldehyde was converted quantitatively into a cyanohydrin nearly independently of the amount of enzyme present, with a space-time yield of 57 $g.l^{-1}.h^{-1}$.

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P096 SUBSTRATE SPECIFICITY OF MUTANTS OF HYDROXYNITRILE LYASE FROM *Hevea brasiliensis* AND HETEROLOGOUS EXPRESSION OF THE ENZYME VARIANTS IN *Pichia pastoris*

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Keywords: hydroxynitrile lyase, *Hevea brasiliensis*, site directed mutagenesis, mutant characterization

The hydroxynitrile lyase from the tropical rubber tree *Hevea brasiliensis* (HbHNL) catalyzes the decomposition of α-hydroxynitrile acetone cyanohydrin into HCN and acetone during cyanogenesis of damaged plants. HbHNL is of practical importance as biocatalyst for the reverse reaction of cyanogenesis, as this enzyme can also be used for stereoselective synthesis of a wide range of *(S)*-cyanohydrins by addition of HCN to aldehydes or ketones. Chiral cyanohydrins have attracted much attention as components or intermediates of numerous pharmaceuticals and agrochemicals. HbHL shows high stereoselectivity, but rather low substrate specificity, which offers a wide range of industrial applications of HbHNL and the catalysed reaction represents one of the few industrially relevant examples of enzyme mediated C-C coupling reactions.

The catalytic triad (Ser80, His235, Asp207) has already been determined by mutational analysis[1], allowing subsequently the formulation of the mechanism of enzyme-catalysed cyanohydrin formation or cleavage[2]. Recently a number of amino acids, proposed to be involved in reaction mechanism of HbHNL were also changed by site directed mutagenesis. Additional we constructed a set of HbHNL mutants with improved features like substrate acceptance. All the mutated HbHNLs were expressed in an appropriate *Pichia pastoris* expression strain to gain high yield of the recombinant proteins. This high level expression allowed efficient biochemical and functional characterisation of the HbHNL mutants, providing a more detailed picture of the enzyme properties and relationship between structure and function of the HbHNL enzyme.

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P097 CLONING OF A NITRILASE GENE FROM THE CYANOBACTERIUM *Synechocystis* SPP. PCC6803 AND HETEROLOGOUS EXPRESSION AND CHARACTERIZATION OF THE ENCODED PROTEIN

UTE HEINEMAN, DIRK ENGELS, and **ANDREAS STOLZ**

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Keywords: nitrilase, gene cloning, heterologous expression, purification, enzyme activity

The gene encoding a putative nitrilase was identified in the genome sequence of the photosynthetic blue-green bacterium *Synechocystis* spp. PCC6803. The gene was amplified by PCR and cloned into an expression vector. The encoded protein was heterologously expressed in the native form and as a His-tagged protein in *Escherichia coli* and the recombinant strains shown to convert benzonitrile to benzoate. The active enzyme was purified to homogeneity and shown by gel filtration to consist probably of 10 subunits. The purified nitrilase converted various aromatic and aliphatic nitriles. The highest enzyme activity was observed with fumarodinitrile, but also some rather hydrophobic aromatic (*e. g.* naphthalenecarbonitrile), heterocyclic (*e. g.* indole-3-acetonitrile) or long-chain aliphatic (di)nitriles (*e. g.* octanoic acid dinitrile) were converted with higher specific activities than benzonitrile. From aliphatic dinitriles with less than six carbon atoms only one mole of ammonia was released per mole of dinitrile and thus presumably the corresponding cyanocarboxylic acids formed. The purified enzyme was active in the presence of a wide range of organic solvents and the turn-over rates of dodecanonitrile and naphthalenecarbonitrile were increased in the presence of water-soluble and water-immiscible organic solvents.

P098 DIVERSITY OF NITRILE HYDRATASE ENZYMES FROM GEOGRAPHICALLY DISTINCT *Rhodococcus erythropolis* STRAINS

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Keywords: nitrile hydratase, enzyme diversity, *Rhodococcus erythropolis*, aminonitrile, biotransformation

The investigation of microorganisms at the infraspecific level is crucial in the context of biotechnology discovery because many sought-after properties are known to be strain as opposed to species determined[1]. In our study, considerable molecular diversity was found among nitrile hydratase (NHase) enzymes of geographically distinct *Rhodococcus erythropolis* strains demonstrating the extent of such infra-species variability[2]. The genetic diversity of the NHases was screened by PRS analysis, a molecular fingerprinting technique used as a pre-sequencing step that combines polymerase chain reaction (PCR), restriction fragment length polymorphism (RFLP) and single-strand conformational polymorphism (SSCP)[3–5]. The enzyme genes revealed unique PRS patterns that mostly correlated with the distinct geographical sites studied indicating that *R. erythropolis* from widespread locations possess NHase genes that are not globally mixed. A selection of NHase PRS patterns were sequenced and nucleotide sequences showed high similarities. Phylogenetic analysis revealed these enzymes were related to Fe-type NHases. The alignment of the deduced amino acid sequences of the studied NHases α and β subunits revealed diverse positions with variable residues. The substituted residues were located at neighbouring or very close positions to regions not directly related to the enzymes active site but that were completely conserved among published Fe- and Co-type NHases, highlighting the potential significance of these substitutions on the structure, activity and substrate specificity of the final active enzymes. The deduced amino acid NHase sequence of a particular *R. erythropolis* strain showed one amino acid substitution at a region that is highly conserved among all NHases reported in literature, suggesting this change could be responsible for its different catalytic activities. Currently this strain is being studied in more detail for its ability to convert nitriles, particularly α-aminonitriles.

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P099 GLUTARYL-7-ACA ACYLASE: A NEW TOOL FOR THE BIOCATALYZED KINETIC RESOLUTION OF RACEMIC AMINES AND ALCOHOLS

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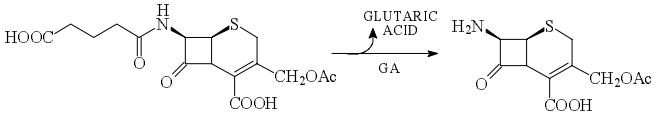
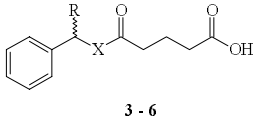
Keywords: glutaryl-7-ACA acylase, kinetic resolution, enantioselectivity

The enzymes D-amino acid oxidase and glutaryl-7-ACA acylase (GA) are currently utilised for the industrial production of 7-aminocephalosporanic acid (7-ACA, **2**), an important precursor of semisyntetic cephalosporins. Specifically, GA is devoted to the cleavage of the amide bond between glutaric acid and 7-ACA in the intermediate glutaryl-7-ACA **(1)**.

The synthetic performances of this enzyme towards **1** have been widely investigated, while very little is known on GA substrate specificity. We have found that an industrial GA is very specific for the acyl moiety that has to be released, the glutaryl derivatives being by far the best substrates[1,2]. On the other hand, this enzyme accepts a wide variety of "leaving groups". Not only *N*-glutarate of β-lactam derivatives, but also *N*-glutaryl aminoacids as well as *N*-glutarylamides (aromatic and aliphatic) could be hydrolysed by GA, which, additionally, showed a significant esterase activity. More notably, GA-catalysed hydrolyses were highly enantioselective, as exemplified with the racemic compounds **3**-**6** .



1 **2**



3 - 6

3 R = $COOCH_3$, X = NH
4 R = CH_3, X = NH
5 R = $COOCH_3$, X = O
6 R = CH_3, X = O

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P100 SUBSTRATE TOLERANCE OF GLUTARYL ACYLASE

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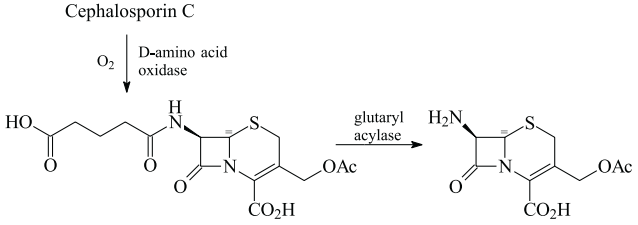
Keywords: glutaryl acylase, substrate tolerance

Glutaryl acylase (GA) is an enzyme that belongs to the *Ntn* hydrolase superfamily and exhibits a close structural relationship to penicillin G acylase (PGA)[1]. GA is of technical importance for the generation of 7-aminocephalosporanic acid (7-ACA) **(1)** from natural Cephalosporin C produced by fermentation[2]. Despite its commercial availability, applications of GA in synthetic organic chemistry are lacking so far.



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In this work we have investigated the substrate tolerance of the GA from *E. coli* to evaluate its suitability for preparative applications, particularly for racemate resolutions of chiral amines or alcohols, or for enzymatic protective group chemistry. For this purpose, several series of substrate analogs were screened by small-scale preparative reactions. Structural variations included amines and alcohols with different aromatic, aliphatic, or multiply substituted side chains. Additionally, the acyl moiety was investigated for acceptance of structural variations. The investigation furnished a fluorogenic substrate that proved suitable for sensitive assays in high-throughput mode for screening of GA activity.

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P101 NEW APPLICATION OF AMINOACYLASE I – ENANTIOSELECTIVE CONVERSIONS OF AMINO ACIDS CARBOXYLIC DERIVATIVES

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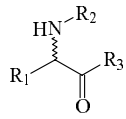
Keywords: aminoacylase, amino acids resolution, enantioselective conversion

Aminoacylase I (*N*-acyl-L-amino-acid amidohydrolase, E.C. 3.5.1.14) is a readily available and inexpensive enzyme with a relaxed substrate specificity[1] that is used in the industrial production of enantiopure L-amino acids from their *N*-acyl derivatives[2,3]. In organic and water-organic medium aminoacylase was shown to be able to mediate the reverse reaction, acylation of L-amino acids, and also to perform the enantioselective irreversible acyl transfer from activated acyl donor to alcohols and amines. In general, typical substrate for the aminoacylase-catalyzed reactions can be presented by structure **(1)**. Until now, near all scientific and industrial applications of aminoacylase-family exploited the ability of these enzymes to cleave or synthesise the *N*-acyl bond of amino group (R_2 moiety).



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In presented investigation we have shown several principally new abilities of Aminoacylase I – family enzymes to perform the enantioselective conversions of the carboxyl derivatives of different amino compounds (R_3 moiety of structure **1**). Particularly, aminoacylase was shown to be able to hydrolyse the amides and esters of natural and non-natural amino acids (including beta-amino acids) with surprisingly high enantioselectivity. The reaction rates of these conversions are comparable, and in some cases much faster, than hydrolysis of "traditional" for aminoacylase *N*-acetyl derivatives of corresponding compounds.

We believe that presented invention can be of high interest for the development of new effective and inexpensive methods for the obtaining of optically active amino compounds, including non-natural amino acids and their derivatives.

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P102 PENICILLIN ACYLASE-CATALYZED RESOLUTION OF AMINES IN AQUEOUS MEDIUM

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Keywords: chiral amine, enantioselective acylation, penicillin acylase, *Alcaligenes faecalis*

The new principal strategy of effective and enantioselective acylation of amines in aqueous medium based on the unique catalytic properties, stability and enantioselectivity of relatively unknown penicillin acylase from *Alcaligenes faecalis* has been suggested recently[1]. In contrast to lipase-catalyzed acylation in organic solvents, acylation of reactive amines in aqueous medium by *Alcaligenes* penicillin acylase is a very fast and chemoselective process not accompanied by their spontaneous acylation and accumulation of by-products. Acylated amine (active form) can be easily isolated and subjected to the subsequent stereoselective deacylation by the same enzyme. Two enzymatic steps - acylation and deacylation can be integrated into an original biocatalytic process for effective production of both amine enantiomers. As a result double chiral control is imposed at this type of biocatalytic resolution.

Detailed kinetic analysis of acyl transfer to racemic amines catalyzed by *Alcaligenes* penicillin acylase in aqueous medium helped to find out critical factors delimiting effectivity of enzymatic acylation. At an adequate management enzymatic acylation in aqueous medium appears to be very effective for synthetic as well as for resolution purposes. Using different acyl donors (phenylacetamide and its structural analogues *R*-phenylglycine amide, *R*- and *S*-mandelamide, which are totally stable to non-enzymatic hydrolysis and do not spontaneously acylate amines) allowed improving enantioselectivity and effectivity of acylation (synthesis/hydrolysis ratio and rate of enzymatic reaction). Examples of nearly quantitative conversion (more than 95 %) of both amine and acyl donor and extremelly enantioselective (E > 1000) penicillin acylase-catalyzed acylation have been documented. Resolution of chiral amines catalyzed by *Alcaligenes* penicillin acylase in aqueous medium can become a practical alternative to the existing methods. The possibility to exploit stabilized enzyme preparations for these purposes has been examined.

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P103 RESOLUTION OF *(R,S)*-PHENYLGLYCINONITRILE BY PENICILLIN ACYLASE-CATALYZED ACYLATION IN AN AQUEOUS MEDIUM

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Keywords: penicillin acylase, phenylglycinonitrile, enzymatic resolution, enantiomers

α-Aminonitriles are crucial intermediates in production of corresponding amides and acids. Within the frames of numerous applications of these compounds in fine chemistry phenylglycinonitrile is of special interest since its derivative, *(R)*-phenylglycine amide, serves as an acyl chain donor in a biocatalytic ampicillin and cephalexin synthesis[1]. However, each particular application demands only one enantiomer and hence a problem of chiral resolution arises since α-aminonitriles are available via Strecker reaction only in a racemic form. The current strategies tackle this problem by using nitrilases or combining nitrile hydratases and amidases. The former, nitrilases, perform enantioselective conversion of a nitrile directly into carboxylic acid while the latter imply (as a rule) nonspecific hydration of a nitrile to an amide with consequent amidase-catalyzed hydrolysis of a single enantiomer[2,3]. Despite a variety of strategies to cultivate strains, harboring activity towards given nitrile, are available[4,5], still industrial applications of biocatalytic nitrile conversions are handicapped due to the lack of properly formulated biocatalyst: immobilized cells are prone to loose activity when recycling,[6] and purified enzymes are very capricious[7]. In addition only low concentrations of substrates could be converted by currently available techniques: the best examples show productivity (the so-called space-time yield) of 0.1–0.3 kg of product per m^3 of reactor space per hour, which is quite low for industrial purposes[4,6]. With respect to phenylglycinonitrile there have been a number of studies on nitrilase and hydratase/amidase catalyzed resolution[6–9]. It was shown, that the latter approach works better with respect to yields and

enantioselectivity. However, productivity of the process was still moderate even at high catalyst loading[6].

We suggest an alternative route of chiral phenylglycinonitrile resolution based on the enzymatic recognition of α-amino- rather than nitrile-functionality. Namely, we have found that the aminogroup of the nitrile could be acylated enantioselectively and with nearly quantitative yield. For this purpose penicillin acylase from *E. coli*, an enzyme, well known for its affinity towards *(S)*-phenylglycine derivatives as external nucleophiles[10–13], was used with phenylacetic acid as an acylating agent in an aqueous medium. Proposed approach allows converting high substrate concentrations and enables easy separation of non-reactive *(R)*-phenylglycinonitrile from acylated *(S)*-form by simple filtration due to very low solubility of the latter. As a result removal of *(S)*-phenylglycinonitrile from the reaction sphere is almost complete and irreversible, favoring kinetics of the process and making possible high degrees of requested conversion. Proposed approach is characterized by high space-time yield and extends the scopes of enzymatic synthesis in an aqueous medium.

We would like to acknowledge financial support by the Russian Foundation for Basic Research (03-04-48472, 03-04-06307), INTAS (2001-0673) and DSM Research.

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P104 CHARACTERISTICS OF PENICILLIN G AMIDASE AND D-AMINO ACID OXIDASE IN IONIC LIQUIDS

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Keywords: ionic liquids, PGA, DAAO, activity, biocatalysis

Ionic liquids are recently explored as reaction media for biocatalytic reactions[1]. The well-investigated hydrolytic enzymes, such as lipases, proteases or glycosidases were chiefly examined in this unconventional media[2].

Our aim was to study the feasibility of using ionic liquids as reaction media for two enzymes, which were up to now not tested in this environment: namely penicillin G amidase (penicillin G acylase, PGA, EC 3.5.1.11) and D-amino acid oxidase (DAAO, EC 1.4.3.3).

Penicillin G amidases are involved in the industrial production of semisynthetic penicillins and cephalosporins, which remain the most widely used group of antibiotics[3]. We examine the application of PGA in ionic liquids to overcome some limitations of the conventional process. In the synthesis of semi-synthetic penicillins the major drawback such as substrate/product solubility and selectivity (ratio of hydrolysis > synthesis) limits the process yield.

In the other part, the application of an immobilized DAAO from *Trigonopsis variabilis* for racemate resolution of D/L-amino acids in ionic liquids was investigated. Here, the higher oxygen solubility in ionic liquids is advantageous, since oxygen is a limiting factor in the aqueous system[4].

The activity and operational stability of PGA and DAAO in different ionic liquids as well as in organic solvents were studied for comparison. The applied ionic liquids have a high degree of purity, since ionic liquids with impurities caused a pH-shift in the reaction medium (up to 5 pH units). For both enzymes the following water miscible solvents were used: [BMIM][$OcSO_4$], [BMIM][BF_4], dimethyl formamide (additionally methanol for PGA and *n*-propanol for DAAO). Further, DAAO was also investigated with water immiscible solvents e. g. [BMIM][BTA], [BMIM][PF_6] and *n*-hexane.

We express our thanks to BC Biochemie, Kundl, Austria for providing the immobilized DAAO, to our co-operation partner Solvent Innovation GmbH, Köln, Germany and the BMBF for financial support.

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P105 IMMOBILISED PENICILLIN AMIDASE (*E. coli*) ONTO MAGNETIC, MICRO, NON-POROUS CARRIERS: CHARACTERISATION IN MODEL REACTIONS

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Keywords: magnetic carriers, imobilisation, penicillin amidase, (enantio)selectivity

Different types of polymer magnetic beads – commercial poly(vinyl alcohol) donated by "Chemagen"(Baesweiler, Germany) and in house produced poly(methyl methacrylate-divinylbenzene) and poly(vinyl acetate-divinylbenzene), were activated with epoxy- or amino-spacer and tested as matrices for covalent attachment of penicillin amidase *(E. coli)* as a model system. The small size (1–6 μm) of the particles, their large surface area and practically non-porous nature result in reduced mass transfer limitations during the processes catalysed by enzymes immobilized onto them. Another advantage is the easy separation of the immobilised biocatalysts after completion of the reaction by mean of a magnetic field[1]. The immobilisation conditions were varied to optimise the immobilisation yield, stability and catalytic efficiency of the attached penicillin amidase. Its catalytic properties were characterised in hydrolytic and kinetically controlled synthetic reactions[2]. The enzyme immobilised onto poly(vinyl alcohol) magnetic beads has k_{cat} for the hydrolysis of penicillin G the same order of magnitude as the free enzyme. The K_m value is higher compared to the free enzyme, but an order of magnitude lower compared to enzymes immobilised on the widely used larger (~ 100 μm) porous carriers. The immobilised penicillin amidase was tested as a catalyst for the kinetically controlled synthesis of cephalexin from *R*-phenylglycine amide and 7-aminodeacetoxycephalosporanic acid. Its selectivity in the condensation reaction was higher compared to the conventional carriers. Another important property of the immobilised biocatalysts is their enantioselectivity[3]. For the studies immobilised penicillin amidase was tested for the condensation of *R*-phenylglycine amide with *S*- and *R*-phenylalanine and for the hydrolysis of racemic penylacetyl-phenylalanine. The enantiomeric ratio *E* was reduced compared to the free enzyme, but improved compared to enzyme immobilised onto carriers with limited mass transfer.

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P106 EXPRESSION CLONING OF ENVIRONMENTAL DNA FOR THE DISCOVERY OF NEW PENICILLIN AMIDASES

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Keywords: environmental DNA, expression cloning, penicillin amidase, screening

The metagenome constitutes a vast and almost unexplored pool of new genes and, consequently, potential new biocatalysts. We used microorganisms indigenous to different types of soil and sediment as a source of DNA to construct environmental gene banks in *E. coli*, which were subsequently screened for clones encoding penicillin amidase activity. In one case, DNA was isolated from soil without any intermediate cultivation step to preserve a high degree of genetic diversity, which was demonstrated by DGGE analysis of the starting material and the presence of various enzymatic activities in the resulting gene bank[1]. When screening about 200 Mb of environmental DNA, clones exhibiting glucosidase (2), β-lactamase (4), amylase (1), and amidase activity (2) were found. To possibly reduce the number of clones required to find one of our actual target enzymes, DNA was also isolated from enrichment cultures, in which one single or a mixture of different aromatic and non-aromatic acetamide and glycine amide derivatives were supplied as sole nitrogen source. Amplified gene libraries were searched, using a three-step screening procedure that started with growth selection of amidase-expressing clones (> 200,000 individual clones input), followed by colorimetric screening of positives in microtiter plates (300 clones), and a final round of HPLC analysis, in which a limited number of different clones (7) were tested for their capacities in β-lactam antibiotic synthesis.

This procedure, although carried out on a relatively small scale, resulted in the isolation of 7 new amidases including one with penicillin amidase activity. Results from sequence analysis and detailed biochemical and kinetic characterization of the recovered enzymes will be presented.

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P107 DIRECTED EVOLUTION OF PENICILLIN ACYLASES TO IMPROVE THE SYNTHESIS OF β-LACTAM ANTIBIOTICS

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Keywords: directed evolution, penicillin acylase

Penicillin acylase of *Escherichia coli* is capable of hydrolyzing the antibiotic Penicillin G into phenylacetic acid and 6-aminopenicillanic acid (6-APA), but the enzyme can also be used in the reverse reaction, thus the coupling of a synthetic acylgroup to 6-APA. The aim of this project is to improve these synthetic properties, so penicillin acylase can be used more efficient in the production of semi-synthetic antibiotics.

To achieve this goal we used family-gene shuffling[1] combined with error prone PCR. In this directed evolution experiment the 3 genes of *Escherichia coli, Kluyvera cryocrescens* and *Providencia rettgeri* were used. These genes encode proteins that have more then 60 % sequence identity.

To check if our protocol resulted in hybrids between the different genes, we have performed a DNA-restriction with *Rsa*I. The three genes show a different pattern on an agarose gel after restriction. Four out of 15 randomly picked transformants had a restriction pattern that suggests the shuffled genes are hybrids. DNA sequencing confirmed that these four genes are indeed hybrid genes.

Gene shuffling generates a large number of mutants and to find improved mutants efficient selection and rapid screening methods are of utmost importance. To eliminate screening of inactive mutants a growth selection is used. The screening is done with the use of HPLC coupled to an pipetting robot. Screening resulted in three enzymes with improved synthetic properties. These enzymes have better synthesis over hydrolysis ratio, while retaining the high activity of the wild type enzyme. With the means of site-directed mutagenesis also mutants with better a better synthesis over hydrolysis ratio have been made, but all of these mutants have a 10-fold reduced activity.

Family-gene shuffling proves to be a good way to make penicillin mutants with improved synthetic properties, while retaining the high activity of the wild type enzyme.

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P108 HOMOLOGY MODEL OF PENICILLIN ACYLASE FROM *Alcaligenes faecalis* AND *in silico* EVALUATION OF ITS SELECTIVITY

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Keywords: penicillin acylase, homology modelling, enzyme models, enzyme selectivity

Among industrially employed enzymes, penicillin acylase is one of the most widely studied and used, since the β-lactam antibiotics industry has replaced the traditional chemical multistep process for the production of 6-aminopenicillanic acid by an enzymatic one, just using penicillin acylase (PA)[1]. The most common source of commercially available penicillin acylase is *Escherichia coli* (PA-EC), though the same enzyme from *Alcaligenes faecalis* (PA-AF) has recently received attention in the literature[2–4], especially for its high synthetic efficiency in enantioselective synthesis and its high thermostability.

Since up to date no crystallographic data are available for PA-AF, a tridimensional model of PA-AF was built up by means of homology modelling using three different crystal structures of penicillin acylase from various sources. An *in silico* selectivity study was performed to compare this homology model to the PA-EC in order to point out selectivity differences between the two enzymes. The GRID/PCA technique, that conjugates molecular mechanics with multivariate analysis and was originally thought for drug design, was applied to identify the regions of the active sites where the PAs potentially engage different interactions with ligands. GRID/PCA method is able to point out differences and also to evaluate their entity (nature and strength) after having cleared away the “noise” of non-significant differences. It proved to be a very effective tool for the comparison of the two structures on a rational basis, while speeding up the computational analysis. The structural differences pointed out by GRID/PCA were further analysed and confirmed by molecular docking simulations. The PA-AF homology model provided the structural basis for the explanation of the different enantioselectivity of the enzyme previously demonstrated experimentally and reported in the literature. Furthermore,

a different selectivity towards the 4-hydroxyphenylacetic residue was predicted on the basis of a change in the aminoacid sequence in position B:67.

The PA-AF tridimensional homology model represents a valuable tool for fully exploiting this attractive and efficient biocatalyst, especially in enantioselective acylations of amines.

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P109 NOVEL EPOXIDE HYDROLASES IDENTIFIED BY GENOME ANALYSIS

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Keywords: epoxide hydrolase, enantioselectivity, sequence database screening

With recent progress in sequencing of whole genomes, a large amount of data has become available that can be screened for promising genes encoding biotransformation enzymes. We have explored enzymes that share the same canonical α/β-hydrolase fold and contain a Ser/Asp-His-Acid catalytic triad. The different families were distinguished using multiple sequence alignments and phylogenetic analysis. This analysis was done using both sequences of well-characterised proteins and sequences obtained with a BLAST search using as a query the amino acid sequence of an enzyme of known function. The focus was on epoxide hydrolases, since they can be used in kinetic resolutions of epoxides and are therefore of interest to biocatalytic applications.

The multiple sequence alignments and phylogenetic analysis revealed that at least three families of epoxide hydrolases can be distinguished in addition to three families of proteins that consist only of putative sequences that share the conserved residues and motifs of epoxide hydrolases. The motifs present were the Asp-His-Asp catalytic triad, at least one putative ring-opening tyrosine, and the G-x-G-x-S and H-G-x-P motifs.

To test their suitability in biocatalytic applications, some genes of a subclass of family 2, to which also the plant and mammalian cytosolic epoxide hydrolases belong, were cloned as a Maltose Binding Protein (MBP) N-terminal fusion and expressed *in Escherichia coli*, in order to prevent possible inclusion body formation and facilitate purification. The putative epoxide hydrolases from *Bacillus subtilis* (Bsueh), *Deinnococcus radiodurans* (Draeh) and *Nostoc punctiforme* (Npueh1) all showed activity towards pNSO and/or pNPGE with opposite enantiopreference as compared to the well-studied epoxide hydrolases from *A. radiobacter* AD1 (ref.[1]), *Aspergillus niger*[2] and *Rhodotorula glutinis*[3]. One example of an enantioselective conversion of phenyl glycidyl ether with the same enantiopreference as the three newly cloned enzymes is the whole cell biocatalyst *Bacillus megaterium* ECU1001 (ref.[4]). These results show that in addition to screening of large culture collections of microorganisms for epoxide hydrolase activity, the *in silico* available sequence space can be successfully screened for new enantioselective epoxide hydrolases with complementing stereospecific properties as compared to many of the well known cloned microbial enzymes.

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P110 IMMOBILISATION AND STABILIZATION OF THE *A. niger* EPOXIDE HYDROLASE. A NOVEL BIOCATALYTIC TOOL FOR REPEATED-BATCH HYDROLYTIC KINETIC RESOLUTION OF EPOXIDES

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Keywords: epoxide hydrolase, immobilisation, *Aspergillus niger*, eupergit, resolution

Studies aimed to immobilise the *Aspergillus niger* epoxide hydrolase were performed[1]. The use of conventional approaches – i. e. of commercially available supports and classical methodologies – only led to low stabilisation and unsatisfactory enzymatic activity recovery. Therefore, a new strategy based on the use of a "second generation" type of support allowing multi-point covalent immobilization – i. e. Eupergit C activated with ethylene diamine (Eupergit C/EDA)[2] and of an adequate experimental procedure, allowed to prepare an immobilized biocatalyst with 70 % retention of the initial enzymatic activity and a stabilisation factor of about 30. Interestingly, this biocatalyst also led to a noticeable increase of the E value for the resolution of two test substrates – i. e. styrene oxide and *p*-Cl-styrene oxide. This was improved from about 25 to 56 and from 40 to 100, respectively. A typical repeated batch experiment indicated that the thus immobilized enzyme could be re-used for over 12 cycles without any noticeable loss of enzymatic activity and enantioselectivity. This therefore opens the way to the use of an "heterogeneous" methodology for achieving the preparation of various enantiopure epoxides *via* biocatalysed hydrolytic kinetic resolution.

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P111 A HIGH-PERFORMANCE EPOXIDE HYDROLASE REACTOR. APPLICATION TO THE PREPARATIVE SCALE SYNTHESIS OF AZOLE ANTIFUNGAL AGENTS KEY SYNTHONS

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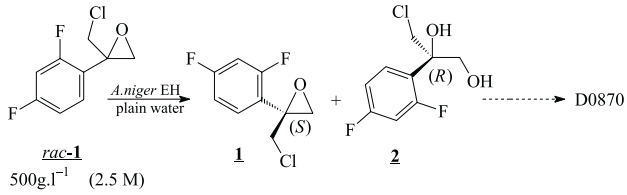
Keywords: epoxide hydrolase, D0870, resolution, *Aspergillus niger*

In this presentation we describe a high performance preparative scale resolution of *rac*-**1** – at a very high substrate concentration (i. e. 500 g.l^{-1}, 2.5 M) – using the fungal *Aspergillus niger* epoxide hydrolase[2]. This affords both *(S)*-chloroepoxide **1** and *(R)*-chloro-diol **2** in enantiopure form[3]. The thus obtained products both allow the formal synthesis of the enantiopure eutomer of D0870, an efficient bis-triazole antifungal agent[4].



During the scale up of this bioreactor, a surprising reaction rate enhancement was observed at high substrate concentration. A comparative study with the enzymatic resolution of styrene oxide using the same enzyme showed that the formed diol **2** was a strong activator.

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P112 PREPARATIVE SCALE ENZYMATIC KINETIC RESOLUTION OF GLYCIDYL ACETAL DERIVATIVES USING THE *A. niger* EPOXIDE HYDROLASE

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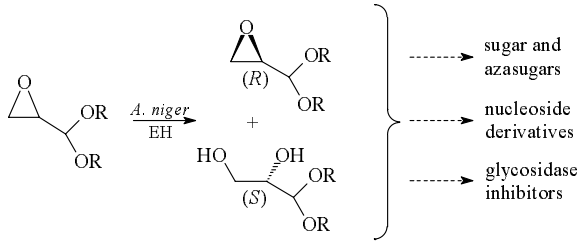
Keywords: epoxide hydrolase, glycidyl acetal, resolution, *Aspergillus niger*

The general objective of our work is to develop efficient biocatalytic approaches for the synthesis of enantiopure epoxides (or vicinal diols) by performing the hydrolytic kinetic resolution of racemic epoxides using an epoxide hydrolase (EH)[1].

Glycidyl dialkyl acetals are very interesting chiral building blocks because these molecules are bearing two chemically different reactive sites, i. e. an oxirane ring and a protected

aldehyde moiety. They have been used as C3 chirons to carry out the synthesis of various bioactive compounds like for example azasugars, nucleoside derivatives or glycosidase inhibitors. In this presentation we propose a preparative scale enzymatic process, implying an epoxide hydrolase as biocatalyst, to synthetise these compounds in enantiopure form.



The biocatalyzed hydrolytic kinetic resolution of some glycidyl dialkyl acetals i. e. ethyl-, isopropyl- and 2,2-dimethylen acetals derivatives by the *Aspergillus niger* epoxide hydrolase has been explored[1,2]. A comparative study using whole cells, crude extracts of the wild type strain, as well as partially purified recombinant (immobilized) enzyme to hydrolyse these substrates has been carried out. From this study it appeared that the preparation of the two enantiomers of these products could be easily achieved at a very high substrate concentration (>200 $g.l^{-1}$). A preparative multi-gram scale resolution showing the feasibility and the interest of this enzymatic process will be described.

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2. Archelas A., Furstoss R.: Curr. Opin. Chem. Biol. *5*, 112 (2001).

P113 PREPARATION AND PROPERTIES OF IMMOBILIZED EPOXIDE HYDROLASE FROM *Aspergillus niger*

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Keywords: *Aspergillus niger*, epoxide hydrolase, enantioselective hydrolysis, immobilization

Chiral epoxides and vicinal diols are high-value intermediates for the synthesis of biologically active pharmaceutical compounds. In the last years, a great interest has been devoted to biocatalytic approach involving the hydrolytic activity of epoxide hydrolases (EC 3.3.2.3) to prepare these important building blocks in enantiopure form[1–2]. The enzymatic way is an interesting alternative approach to the chemical methods to obtain enantiopure epoxides. A research work done in our laboratory has demonstrated that epoxide hydrolase (EH) from the *Aspergillus niger* is a very interesting biocatalyst with high activity and enantioselectivity on derivatives of styrene oxide[3]. Bioconversions at a multi-gram scale have been demonstrated using the free enzyme[4]. However, the enzyme was not stable in these conditions and no recycle was possible. The lack of biocatalyst recovery hampered the practical use of the process at higher scale level. Thus, the EH from *Aspergillus niger* was immobilized as a mean to get an easy enzyme recovery, as a goal to obtain a more stable biocatalyst and as a way to run continuous reactors[5]. Simple immobilization by adsorption onto DEAE--cellulose was used in this study and EH activity was tested by hydrolysis of racemic *para*-chlorostyrene oxide *p*CSO. The protein loading varied from 2 to 50 $mg.g^{-1}$ of support. The retention of activity was 70 % in the range of 2–10 $mg.g^{-1}$ of immobilized proteins and further decreased due to diffusional limitations. The optimal amount of 10 $mg.g^{-1}$ was selected, in these conditions the immobilization yield on activity was 99 %. Analysis of the adsorption isotherm of EH onto DEAE-cellulose using the Langmuir model resulted in the following constants: K_L = 12.7 $mg.ml^{-1}$, *[A]* max = 124 $mg.g^{-1}$ which suggested that the enzyme had a high affinity for the support. The temperature where the activity was maximal (40 °C) and the activation energy (38.8 $kJ.mol^{-1}$) for the immobilized EH were similar to those for the free EH. The effect of temperature on enzyme stability showed no clear stabilization upon immobilization. The optimal pH was about one unit less (6.5 and 7.5) for the immobilized EH than for the free enzyme. This difference was explained by a microenvironmental effect due to the positive charges of the DEAE cellulose.

The diminution of the specificity constant, k_{cat}/K_{Mapp}, for *p*CSO after immobilization came from variations of both K_{Mapp} compared to k_{cat}. The immobilization slightly affected the enantioselectivity of EH as well as temperature of reaction with E = 29 at 27 °C and E = 60 at 4 °C. The operational half-life of the immobilized EH in continuous packed-bed reactor at 4 °C was 27 days (substrate concentration of 4 mM). This catalyst was tested in repeated batches to achieve the resolution of racemic *p*CSO in monophasic and biphasic conditions. At 4 mM (monophasic system) significant recovery of activity (80 %) was observed after 7 runs. More interestingly, from a preparative point of view, at a concentration of 2M (306 $g.l^{-1}$, biphasic condition) a high enantiomeric excess of the residual epoxide could be always reached after seven cycles, the reaction time being only increased from 4 to 30 h.

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P114 BIOCATALYTIC PREPARATION OF OPTICALLY PURE EPOXIDES AND ALCOHOLS

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Keywords: epichlorohydrin, styrene oxide, epoxide hydrolase, halohydrin dehalogenase

Optically pure epoxides are important building blocks for the production of a wide range of pharmaceutical compounds. Besides synthetic methods using asymmetric catalysis, some biocatalytic methods have been described. We investigated biocatalytic applications of two enzymes obtained from the epichlorohydrin degrading bacterium *Agrobacterium radiobacter* AD1. Two enzymes are involved in the degradation of epichlorohydrin, an epoxide hydrolase (Fig. 1, steps a and c), and a halohydrin dehalogenase (step b). Halohydrins can be considered as direct precursors of epoxides since ring-closure of an optically pure halohydrin generally yields an optically pure epoxide.

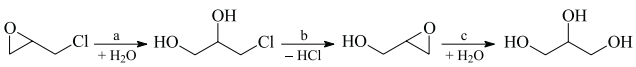

Fig. 1. **Degradation route of epichlorohydrin by *Agrobacterium radiobacter* AD1 by epoxide hydrolase** (a, c), **and halohydrin dehalogenase** (b).

Both enzymes have been brought to overexpression making them available in large quantities for biocatalytic applications. With the epoxide hydrolase several optically pure aromatic epoxides can be obtained by kinetic resolution with moderate to high enantioselectivity. Aliphatic epoxides such as epichlorohydrin were converted without any enantioselectivity.

The halohydrin dehalogenase enantioselectively dehalogenated 2,3-dihalo-1-propanols and aromatic halohydrins such as 2-chloro-1-phenylethanol. Because of the reversibility of the enzyme action, epoxide hydrolase was added to draw the kinetic resolutions to completion. In this way an e. e. > 99 % was reached for all tested halohydrins. This high activity and enantioselectivity of a halohydrin dehalogenase towards aromatic halohydrins has not been described before.

Using the chromogenic substrate *p*-nitrostyrene oxide, we determined the activity and equilibrium constant of the reversible ring opening with a variety of halides and nucleophiles. The halohydrin dehalogenase catalysed the highly enantioselective (E > 200) and regioselective azidolysis of substituted styrene oxides. The high β-regioselectivity (>95 % β-selective) of the enzyme-catalysed reaction is opposite to the observed selectivity in the non-catalysed azide ion ring opening. We have also examined two distinct halohydrin dehalogenases from other organisms in this investigation. The recombinant enzymes from *Mycobacterium* sp. GP1 and *Arthrobacter* sp. AD2 also catalyzed the azidolysis of *p*-nitrostyrene oxide, but the E-value was lower than 5.

P115 IMPROVING THE BIOCATALYTIC PROPERTIES OF A HALOHYDRIN DEHALOGENASE BY MODIFYING THE HALIDE BINDING SITE

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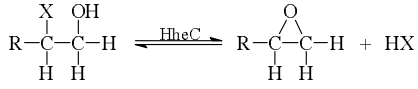
Keywords: halohydrin dehalogenase, halide binding, steady-state kinetics, stopped-flow fluorescence, enantioselectivity

Halohydrin dehalogenases, which catalyze the conversion of halohydrins to the corresponding epoxides, have been purified from several bacterial strains. Due to their high enantioselectivity, these hydrolytic enzymes represent promising biocatalytic tools.



Recently we have cloned the gene encoding the halohydrin dehalogenase from *A. radiobacter* AD1 (HheC)[1]. The kinetic mechanism of HheC has been studied by steady state and pre-steady state kinetic analysis[2]. The halide release step was identified as the slowest step in the catalytic cycle. X-ray crystallographic data of the enzyme reveals that Trp249, Tyr187, and Asn176 form hydrogen bonds, which can modulate halide release. In order to investigate the effect of these

residues on the catalytic activity of the enzyme, several mutants have been constructed (W249F, Y187F, N176A, and N176D). Steady-state kinetic studies revealed that, compared with wild-type enzyme, several mutants displayed a higher k_{cat} value with some tested substrates. Moreover, stopped-flow fluorescence experiments showed that both binding and release of halide with these mutant enzymes is much faster than with wild-type HheC. Interestingly, it was found that also the enantioselectivity towards *p*-nitro-2-bromo-1-phenylethanol could be significantly improved by one of these mutations.

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P116 COMPUTER-ASSISTED ENGINEERING OF HALOALKANE DEHALOGENASES FOR ENVIRONMENTAL APPLICATIONS

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Keywords: biosensor, bioreactor, computer design, dehalogenation, protein engineering

Haloalkane dehalogenases (E.C. 3.8.1.5) utilize water as the only co-substrate to transform haloalkanes into inorganic halides and alcohols. The industrial production of halocarbons and the persistence of these compounds in the environment has lead to interest in dehalogenating enzymes for bioremediation purposes. Dehalogenase-containing bacteria are being used as an inoculum in the bioreactor for decontamination of ground water polluted by 1,2-dichloroethane[1]. Technologies utilising the haloalkane dehalogenases for removal of side-products from chemical synthesis of propylene oxide, epichlorohydrin and butylene oxide[2] and for biosensors allowing on-line monitoring of the presence of halogenated contaminants in the environment are under development. Catalytic properties and thermostability of natural enzymes is not optimal for these practical applications.

This project aims to study structure-function relationships and rationally re-design haloalkane dehalogenases. The major objectives of the project are: (i) to understand the structural determinants of catalytic activity and substrate specificity of these enzymes, (ii) to design mutant proteins with modified catalytic properties, (iii) to construct such mutants using DNA-recombinant technology and (iv) to characterise them structurally and functionally. To meet these objectives, theoretical (i. e., bioinformatics, computer modelling) and experimental (i. e., molecular biology, enzymology and X-ray crystallography) methods are being employed in parallel. The presentation will demonstrate the benefits of such a combined approach not only for the construction of more efficient biocatalysts, but also for better understanding of fundamental principles of enzymatic catalysis that are applicable also to other enzymes[3–7].

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P117 APPLICATION OF DEGENERATE OLIGONUCLEOTIDE GENE SHUFFLING FOR CONSTRUCTION OF HYBRID HALOALKANE DEHALOGENASE

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Keywords: haloalkane dehalogenase, *in vitro* recombination, construct, substrate specificity

Halogenated organic compounds constitute one of the largest groups of environmental pollutants as a result of their widespread use in industry and agriculture. Haloalkane dehalogenases hydrolytically convert halogenated aliphatic compounds to corresponding alcohols. Enhancement of catalytic properties of environmentally important enzymes using *in vitro* evolution techniques is one of the obvious goal of biotechnology.

Our study was undertaken to construct and preliminary characterise hybrid haloalkane dehalogenases. Four genes cloned from different bacteria were used in this study: *dhlA* cloned from *Xanthobacter autotrophicus* GJ10 (ref.[1]), *linB* from *Sphingomonas paucimobilis* UT26 (ref.[2]), *dhaA* cloned from *Rhodoccous erythropolis* NCIMB13064 (ref.[3]) and *dhmA* from *Mycobacterium avium* N85 (ref.[4]). Considering relatively low level of homology among parental genes, Degenerate Oligonucleotide Gene Shuffling techniques was selected as an effective tool for of haloalkane dehalogenase genes[5].

Altogether twelve different combinations were constructed using one pair of degenerate oligonucleotides, cloned into pAQN vector and hybrid proteins were overexpressed in *Escherichia coli* BL21(DE3). Preliminary screening of dehalogenating activity was conducted with resting cells and six substrates representing different classes of halogenated aliphatic compounds. Four out of twelve hybrid proteins keep good expression and ten proteins showed obvious catalytic activity. Some hybrid proteins showed dehalogenating activity despite of changes in position of catalytic residues within the active site. Comparison of relative activities determined for the hybrid enzymes with the activities of wild type enzymes suggests that constructs do not possess novel substrate specificities.

Work on more efficient expression systems to obtain higher expression level of hybrid haloalkane dehalogenase is under progress recently.

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P118 KINETICS AND SPECIFICITY OF HALOALKANE DEHALOGENASE LinB FROM *Sphingomonas paucimobilis* UT26

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Keywords: haloalkane dehalogenase, *Sphingomonas paucimobilis*, *Xanthobacter autotrophicus*, reaction pathway, covalent alkyl-enzyme intermediate

Steady-state and transient-state kinetic methods were applied to solve reaction pathway, to identify reaction intermediate and to specify the rate limiting step of catalytic action of haloalkane dehalogenase LinB from bacterial strain *Sphingomonas paucimobilis* UT26 (ref.[1]). The steady-state experiments involved direct monitoring of LinB activity by isothermal titration calorimetry and initial rate of product formation measurements using gas chromatography. Stopped-flow fluorescence and rapid-quench-flow techniques were applied for the transient-state kinetics measurements. Additionaly, steady-state inhibition experiments and transient-state binding experiments were employed to find out leaving ability of both products (halide and alcohol) during dehalogenation reaction.

The results showed that export of products as well as import of substrates into the active site of LinB are fast processes reaching rapid equilibrium. This fast exchange of the ligands between the active site and bulk solvent can be explained by wide opening of the entrance tunnel and large active site of LinB. In contrary, the release of the halide ion from narrow active site after the reaction was found to be

slow rate limiting step for another haloalkane dehalogenase, enzyme DhlA from *Xanthobacter autotrophicus* GJ10 (ref.[2]). The actual cleavage of the carbon-halogen bond was found to be fast step in both enzymes. Further the results confirmed, that the reaction proceeds via a covalent alkyl-enzyme intermediate. Using bromocyclohexane, chlorocyclohexane and 1-chlorohexane as model substrates, hydrolysis of this intermediate was found to be the slowest step in the catalytic cycle of LinB. The alkyl-enzyme complex was highly accumulated due to the fast dehalogenation step following the slow hydrolyses of this intermediate. The study provides a basis for the analysis of kinetic steps in hydrolysis of environmentally important substrates by the action of LinB.

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P119 COMPARISON OF FOUR YEAST PYRUVATE DECARBOXYLASES FOR *R*-PHENYLACETYLCARBINOL PRODUCTION

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Keywords: biotransformation, enzyme stability, pyruvate decarboxylase, *R*-phenylacetylcarbinol

The chiral intermediate *R*-phenylacetylcarbinol (PAC) is the precursor in the production of ephedrine and pseudoephedrine. Commercially, it is synthesized through biotransformation of benzaldehyde and pyruvate by fermenting yeast *Saccharomyces cerevisiae*. Alternative use of a cell free system, e. g. *Candida utilis* pyruvate decarboxylase (PDC), has the advantage of higher PAC yields and concentrations. For both options, issues regarding PDC stability, activity and by-product formation are still of key concern.

This study investigates PDC from 4 yeast strains i. e. *C. utilis*, *C. tropicalis*, commercial PAC producer *S. cerevisiae* and thermotolerant *Kluyveromyces marxianus*. PDC was produced and investigated with respect to PAC production as well as stability at 23 °C using whole cell and/or crude extract preparations as catalysts.

All strains were grown in shake flasks with 90 $g.l^{-1}$ glucose medium and harvested when glucose concentration fell below 10 $g.l^{-1}$, which was within 10–15 hours. Between 120 and 250 U PDC/g dry cell weight were produced.

PAC production in an aqueous/organic two-phase system with whole cells at 23 °C resulted in the highest PAC concentration with *C. utilis* and in the lowest by-product acetoin formation with *C. tropicalis*. In a single phase system, cell free *C. tropicalis* PDC also formed the lowest amount of by-product acetoin. Lower acetoin formation in both systems indicates that the formation of this by-product is a characteristic of the PDC itself.

C. utilis and *S. cerevisiae* PDC were very stable with half-lives of nearly two weeks at 23 °C. The half-lives were even slightly longer in whole cells. In contrast *C. tropicalis* PDC was very unstable (half-life 3 days in crude extract and less than one day in whole cells). The deactivating effect of 50 mM benzaldehyde was confirmed, generally reducing the half-lives by 50 % or more. *K. marxianus* PDC was stabilized by the addition of protease inhibitors while these had no influence on PDC stability for the other strains.

In summary, *C. utilis* PDC was confirmed to be the best catalyst for PAC production with high stability (half-life of nearly two weeks at 23 °C) and highest final PAC concentrations. *C. tropicalis* PDC had the advantage of lower by-product acetoin formation but was very unstable and yielded less PAC.

P120 IMPROVED PRODUCTION OF *Candida utilis* PYRUVATE DECARBOXYLASE FOR BIOTRANSFORMATION

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Keywords: pyruvate decarboxylase, *Candida utilis*, pH, *R*-phenylacetylcarbinol

Pyruvate decarboxylase (PDC) catalyses the decarboxylation of the glycolysis end product pyruvate to acetaldehyde and carbon dioxide as the penultimate reaction of ethanol fermentation. This same enzyme is used industrially in the synthesis of enantiomerically pure *R*-phenylacetylcarbinol (*R*-PAC), which is the precursor for the chemical synthesis of ephedrine and pseudoephedrine.

After a comprehensive screen of PDC from various sources (Rosche et al 2003, submitted for publication), *Candida utilis* PDC is currently regarded as the best enzyme for biotransformation in terms of enzyme stability and *R*-PAC

concentration. Since *R*-PAC production depends on the activity of the PDC enzyme, it is of interest to optimise PDC production for the biotransformation. Higher PDC activity level will be beneficial as it can enhance *R*-PAC production and contribute significantly in the development of an economically competitive process.

Candida utilis PDC enzyme production was carried out in 5l batch and fed-batch bioreactors under controlled conditions using minimal medium. A preliminary study on PDC production was carried out in shake flasks with low agitation and limited buffering. The final PDC carboligase activity of 333 $U.g^{-1}$ dry cell was higher than previously achieved activity of 86 $U.g^{-1}$ dry cell (Sandford et al. 2003, submitted for publication). The aim of this study is to determine and optimise conditions for PDC production in a bioreactor.

This project was focusing on the effect of aeration and pH to PDC production. Decreasing aeration in comparison to the previously established protocol (aerobic growth phase followed by a fermentative phase) resulted in an increase of PDC activity to 160 $U.g^{-1}$ dry cell when one third of glucose was consumed. Afterwards the PDC activity remained constant throughout the process. However, when the culture was allowed to shift pH from 6.0 to 2.9 at this time point, the PDC carboligase activity was drastically increased to 335 $U.g^{-1}$ dry cell at the end of the process with a biomass of 9.1 $g\text{-}DCW.l^{-1}$. The drop in pH was caused by the yeast, and most likely by the secretion of pyruvic acid.

In order to investigate if there is an optimum pH for PDC production, three identical bioreactors were set up in parallel at constant pH of 3, 4, and 5. The resultant PDC carboligase activities had shown no significant differences to each other and to a process at pH 6. This is strong evidence that not the pH itself, but the pH shift was responsible for the increased PDC activity.

P121 LACTATE RACEMASE AS A VERSATILE TOOL FOR THE RACEMIZATION OF α-HYDROXYCARBOXYLIC ACIDS

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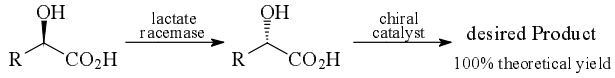
Keywords: lactate racemase, racemization, α-hydroxycarboxylic acids

The transformation of a racemate into a single stereoisomeric product in 100 % theoretical yield has become a prime target for the industrial production of chiral nonracemic materials[1]. One powerful method to reach this goal makes use of a stepwise kinetic resolution which is based on the enzyme-catalyzed racemization of the non-reacted enantiomer. Thus both enantiomers of a racemate can be converted into the desired product in 100 % theoretical yield[2].



Previous studies have shown that mandelate racemase is a versatile tool for the biocatalytic racemization of β,γ-unsaturated α-hydroxy acids. However, aliphatic α-hydroxycarboxylic acids are not accepted[3]. In order to circumvent this limitation, we were aiming at the enzymatic racemization of aliphatic substrate analogues. A screening for lactate racemase activity was initiated starting from scarce literature data[4–6]. In our ongoing screening for lactate racemase activity we identified several active strains possessing a desired broad substrate spectrum for α-hydroxycarboxylic acids, which could not be transformed by mandelate racemase.

This work was performed within the Spezialforschungsbereich Biokatalyse (project # F-115) and financial support by the FWF (Vienna) is gratefully acknowledged.

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P122 DEVELOPMENT OF A MULTI-ENZYMATIC SYSTEM FOR C-C BOND FORMATION

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Keywords: aldolase, aldol reaction, dihydroxyacetone kinase, dihydroxyacetone phosphate, enzyme catalysis

Aldolases have attracted the interest of organic chemists because their ability to catalyze the formation of C-C bonds by an aldol addition reaction between an aldehyde and a ketone, with a high degree of stereochemical control[1]. Dihydro-

xyacetone phosphate (DHAP)-dependent aldolase produce 2-keto-3,4-dihydroxy adducts and, with some exceptions[2], they control the configuration of the newly formed stereogenic centers. An additional advantage of these enzymes is that they are stereocomplementary, that is, their use allows the synthesis of the four possible diaestereoisomers for a given pair of substrates. DHAP-dependent aldolases have shown their utility in the synthesis of carbohydrate, carbohydrate--like structures or non-carbohydrate compounds.

One limitation of these enzymes is that they practically only accept DHAP as donor substrate. DHAP to be used as aldolase substrate can be efficiently obtained by chemical[3] and enzymatic[4] synthesis. Recently, it has been shown that DHAP can be also produced by enzymatic phosphorylation of dihydroxyacetone (DHA) catalyzed by the enzyme dihydroxyacetone kinase (DHAK) from *Schizosaccharomyces pombe*[5].

Here, we describe a new multi-enzymatic system for one--pot C-C bond formation, based in the use of a recombinant DHAK from *Citrobacter freundii*. Thus, DHA phosphorylation is coupled with the aldolic condensation catalysed by the DHAP-dependent aldolase. The multi-enzymatic system is completed with the *in situ* regeneration of ATP catalysed by acetate kinase.

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P123 AMINOTRANSFERASES FOR THE PRODUCTION OF UNNATURAL AMINO ACIDS: APPLICATION TO GLUTAMIC ACID ANALOGUES

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Keywords: aminotransferase, transamination, glutamic acid

Aminotransferases are becoming useful catalysts for the stereoselective synthesis of rare or non-proteinogenic amino acids[1]. This emergence is due to the variety of aminotransferases available and to their broad substrate specificity. Furthermore, these enzymes are characterised by high turnover numbers and do not require external cofactor recycling. However, aminotransferases catalyse equilibrated reactions and this drawback has to be overcome for biotechnological applications.

Our studies in this field are focused on the production of L-glutamic acid (Glu) analogues. Indeed, Glu is widely accepted as the major excitatory transmitter within the central nervous system where it acts at multiple subtypes of ionotropic and metabotropic receptors[2]. Glu analogues behaving as selective agonists or antagonists are helpful to elucidate the properties of these receptors[3] and could even bring some therapeutic effects. New methodologies to access this class of compounds have attracted considerable attention from chemists researchers in recent years.

We have developed a chemo-enzymatic approach based on transamination of α-ketoglutaric acid (KG) analogues catalysed by aspartate aminotransferase (AAT, EC 2.6.1.1)[4]. A close analogue of aspartic acid (cysteine sulfinic acid) is used as the amino donor substrate thus providing a shift of the transamination equilibrium and simplifying glutamic acids isolation.

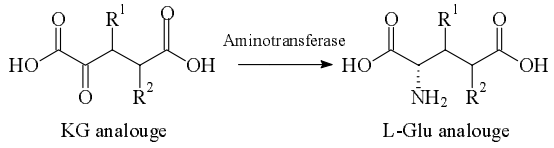

Fig. 1. **Production of L-Glu analogues by enzymatic transamination**

We have designed efficient methods for the chemical synthesis of diversely substituted KGs. Most of these analogues are readily converted into L-glutamic acids with AAT from porcine heart or *E. coli*. Surprisingly, this enzyme presents a broad substrate specificity, specially towards 4-substituted KGs bearing alkyl or polar functionalised groups. Furthermore, AAT shows a marked enantioselectivity for most substrates which allows the kinetic resolution of racemic substituted KGs.

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P124 OXIDATION OF POLY(ETHYLENE GLYCOLS) BY ALCOHOL OXIDASE FROM *Pichia pastoris*

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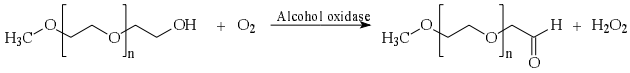
Keywords: alcohol oxidase, *Pichia pastoris*, poly(ethylene glycol), oxidation

Alcohol oxidase (AO) has been isolated from several yeast sources. It was found that AO from methylotropic yeast *Pichia pastoris* has the most wide substrate specificity. Numerous primary alcohols may be oxidized to the corresponding aldehydes by using this enzyme as a catalyst[1].

We present here oxidation of monomethylethers of poly(ethylene glycols) catalyzed by AO from *P. pastoris*:



1 **2**

Relative initial rates of the oxidation reactions are given in the Table I. Two aliphatic alcohols (methanol and butanol) are included for comparison. Oxidation rate is high enough even for long chain alcohols **(1)**. Similar results were obtained by using AO immobilized on macroporous cellulose carrier.

Oxidation rate is falling with the time course of the reaction. It may be caused by product inhibition[2]. TRIS buffer was proposed to alleviate this inhibition[2]. But we found that TRIS itself is oxidized by AO from *P. pastoris*.

We tested three chemical oxidation methods for conversion of poly(ethylene glycol) monomethylethers **(1)** to aldehydes **(2)**: oxidation by oxalyl chloride[3], action by TEMPO radical (2,2,6,6-tetramethylpiperidin-1-yloxy radical) in the presence of sodium hypochlorite[3] or by BAIB ([bis(acetoxy)-iodo]benzene) and potassium bromide as cooxidants[4]. All these chemical oxidation methods were not perfect because of undesirable side products, difficult separation or cross-linking. Oxidation of poly(ethylene glycol) monomethyl ethers **(1)** by using enzymatic method would be very attractive because of absence of oligomeric side-products which are hardly separable.

Poly(ethylene glycol) monomethyl ether aldehydes **(2)** were used for reductive alkylation of chitosan leading to water-soluble comb-shaped graft copolymers. Positive results were obtained.

Table I
Relative rates of oxidation of alcohols by AO

Substrate	Relative rate of oxidation[a], %
Methanol	100
n-Butanol	58
1, n = 1	42
1, n = 2	30
1, n ≈ 8 (M = 350)	17
1, n ≈ 17 (M = 750)	12
1, n ≈ 25 (M = 1100)	14

[a]0.1M phosphate buffer, pH = 7.3, 30 °C, substrate concentration 0,1M

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P125 SCALE UP OF BIOCATALYTIC SYNTHESIS OF CHIRAL FINE CHEMICALS ON THE EXAMPLE OF ENANTIOPURE 2,3-BUTANEDIOL

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Keywords: 2,3-butanediol, kilogram scale, bioconversion, alcohol dehydrogenase, chiral synthesis

Enantiomeric pure fine chemicals like (2*S*,5*S*)-2,5-hexanediol are very precious educts for the production of pharmaceuticals and agrochemicals such as various chiral phosphine ligands like the DuPhosLigand. The stereospecific *de novo* synthesis of these compounds by organic chemistry however is a very complicated and expensive process, often requiring lots of chemical steps. The yields involved are very poor in most cases because of the reactions or product losses by changes of reaction conditions between successive reaction steps.

Another chemical approach to get enantiomeric pure compounds is the resolution of racemic mixtures, in which on principle the half of educt is lost.

On the other hand only one enantiomer of an interesting substance can be produced in just one reaction by bioconver-

sion of prochiral compounds using different enantioselective enzymes. Because of the very high enantioselectivity (>99 %) of an appropriate enzyme and a comparatively simple down stream processing this kind of synthesis is the method of choice for providing a broad variety of chiral compounds. Catalytic active proteins are taken from the nearly inexhaustible variety of nature: yeasts, plants, fungi and bacteria from different habitats were screened for interesting activities in reduction of prochiral ketones, e. g. the cheap 2,5-hexanedione.

The use of alcohol dehydrogenases for these productions is one of the specialities of JFC. Bacteria strains constructed by methods of biotechnology allow to obtain these proteins in large amounts for efficient production of chiral fine chemicals.

A rare and often requested compound is the chiral 2,3-butanediol in all of its different stereoisomers (2*S*,3*S*)-, (2*R*,3*R*)- and *meso*-butanediol. These substances play an important role as e. g. part of chiral catalysts in pure chemical asymmetric reactions. They are expensive and accessible in small amounts only. Using above mentioned enzyme systems JFC is able to provide all these stereoisomers of 2,3-butanediol with an enantiomeric and diastereomeric excess higher then 99 % in a kilogram scale. This increase of production scale permits a new order of following applications.

P126 PRODUCTION OF ENANTIOPURE (2*R*)-PIPERIDINE DERIVATIVES IN HIGH YIELDS BY ENZYME-CATALYZED DYNAMIC KINETIC RESOLUTION

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Keywords: pipecolic acid, biotransformation, dynamic kinetic resolution, chiral building block, cofactor regeneration

The piperidine ring is an ubiquitous structural feature occuring in numerous secondary metabolites (e. g., alkaloids, nonproteinogenic amino acids and respective peptides) and biologically active compounds (e. g., anesthetics, analgetics immunosupressor agents)[1]. Enantiomerically pure pipecolic acid **1** (piperidine-2-carboxylic acid, **Pip**) and its derivatives are important building blocks for the introduction of the chiral 2-alkyl-piperidine motif into these types of compounds. Surprisingly, there exists up to now no powerful chemical or biocatalytical process for the production of **Pip** derivatives which provides high yields and at the same time a high enantiomeric purity of the desired product[2]. Therefore, the main focus in the present work was the development of a DKR process ("dynamic kinetic resolution"), which enabled 100 % yield of enantiopure product[3]. For this purpose, the spontaneous racemization of *N*-*p*-toluenesulfonyl pipecolic aldehyde *(rac)*-**2** should be coupled with a preferably enantiospecific biotransformation process. After a screening for oxidoreductase activity employing microorganisms as well as isolated enzymes, the bioreduction of **2** with an alcohol dehydrogenase proved highly active and enantiospecific. The bioreduction was coupled with an enzymatic NADH regeneration method and the reaction kinetics of the resulting batch-system (Fig. 1) was analyzed. Specific limitations of the investigated process could be identified. After optimization of the reaction conditions, optically pure *N*-*p*-toluenesulfonyl-(2*R*)-(hydroxymethyl)-piperidine (2*R*)-**3** was synthesized by means of a preparative scale conversion in 73 % yield. The compound by itself is a valuable and configurationally stable synthetic building block, or it can easily be converted to D-pipecolic acid by chemical means.

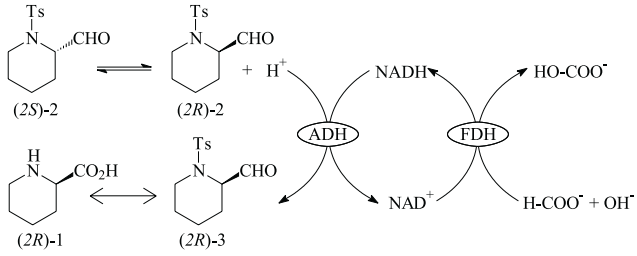

Fig. 1. **Enantioselective biotransformation process for the production of pipecolic acid derivative (2*R*)-3.** ADH: Alcohol dehydrogenase, FDH: Formate dehydrogenase, Ts: *p*-Toluenesulfonyl

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P127 ASYMMETRIC TRANSFER HYDROGENATION PROCESS BY PHENYLACETALDEHYDE REDUCTASE TO PRODUCE CHIRAL ALCOHOLS

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Keywords: phenylacetaldehyde reductase, NADH regeneration, chiral alcohol, asymmetric reduction, *Corynebacterium* sp.

Phenylacetaldehyde reductase (PAR) produced by styrene-assimilating *Corynebacterium (Rhodococcus)* strain ST-10 was used to synthesize chiral alcohols. This enzyme with a broad substrate range reduced various prochiral aromatic ketones and β-ketoesters to yield optically active secondary alcohols with an enantiomeric purity of more than 98 % enantiomeric excess (e. e.)[1]. The *E. coli* recombinant cells which expressed the *par* gene could efficiently produce important pharmaceutical intermediates; *(R)*-2-chloro-1-(3--chlorophenyl)ethanol (28 mg.ml^{-1}) from *m*-chlorophenacyl chloride, ethyl *(R)*-4-chloro-3-hydroxy butanoate (28 mg.ml^{-1}) from ethyl 4-chloro-3-oxobutanoate and (*S*)-*N*-*tert*-butoxycarbonyl(Boc)-3-pyrrolidinol from *N*-Boc-3-pyrrolidinone (51 mg.ml^{-1}), with more than 86 % yields. The high yields were due to the fact that PAR could concomitantly reproduce NADH in the presence of 3–7 % (v/v) 2-propanol in the reaction mixture. We have established a practical asymmetric hydrogen transfer process using 2-propanol as the hydrogen donor by phenylacetaldehyde reductase (PAR) expressed in *E. coli* cells (Fig. 1)[2].

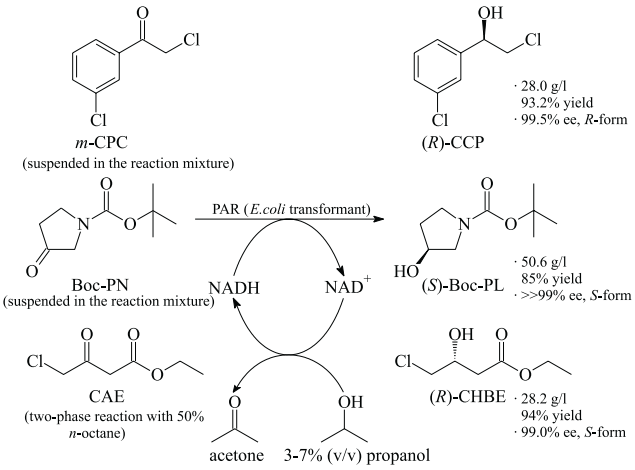

Fig. 1. **Asymetric transfer hydrogenation process by recombinant PAR**

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P128 TWO NOVEL REDUCTASES CATALYZING THE STEREOSPECIFIC REDUCTION OF C=C AND C=O BONDS

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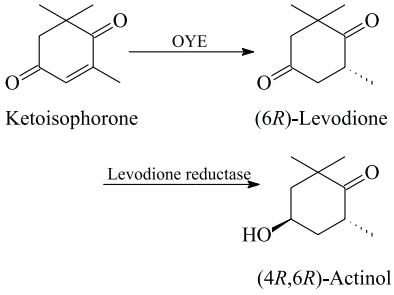
Keywords: carbonyl reductase, Old-Yellow-Enzyme, asymmetric reduction

The demands for optically active drugs has increased owing to the efficacy of these drugs and the market pressure for safe chemical compounds. Therefore, chiral starting materials have also been increasingly required in pharmaceutical and agrochemical fields. A biological method is often adopted for the synthesis of optically active compounds, because biocatalysts express high stereospecifities. Stereospecific dehydrogenase/reductase might be one of the useful biocatalysts for the synthesis of chiral compounds. Recently, two novel microbial reductases catalyzing C=C or C=O reduction were found.

Candida macedoniensis was found to catalyze the hydrogenation of C=C bond of ketoisophorone (2,6,6-trimethyl-2--cyclohexene-1,4-dione), and to specifically produce (6*R*)-levodione (2,2,6-trimethylcyclohexane-1,4-dione). The enzyme catalyzing this stereospecific conversion was identified as one of the Old-Yellow-Enzyme (OYE) family proteins[1]. This is the first application of OYE to the production of chiral compounds.

Carbonyl reductase of *Corynebacterium aquaticum*, which catalyzed the asymmetric reduction of (6*R*)-levodione to (4*R*,6*R*)-actinol (4-hydroxy-2,2,6-trimethylcyclohexanone), was also found. The enzyme, levodione reductase, was highly activated by monovalent cations, and shown to belong to the short-chain alcohol dehydrogenase/reductase family[2, 3].

By combination of these two reductases, enzymatic production of doubly chiral compound, (4*R*,6*R*)-actinol, which is a useful chiral intermediate for the synthesis of naturally--occurring optically active compounds such as zeaxanthin and xanthoxin, from prochiral compound, ketoisophorone, was established[4].



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P129 ENANTIOSELECTIVE ENZYMATIC AND CHEMICAL HYDROLYSIS OF *sec*-ALKYL SULFATE ESTERS

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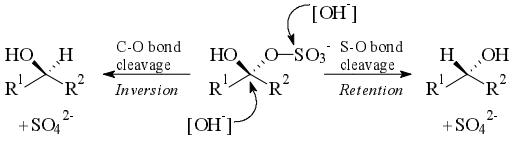
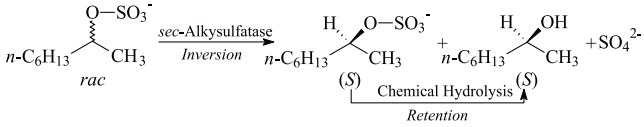
Keywords: *sec*-alkylsulfatase, enantioselective hydrolysis, enantioconvergent process

Sulfatases catalyze the hydrolytic cleavage of the sulfate ester bond[1] *via* retention or inversion of configuration[2, 3] depending on the type of enzyme.

Using a bacterial alkylsulfatase from *Rhodococcus ruber* DSM 44541, biohydrolysis of *sec*-alkyl sulfate esters proceeds in an enantioselective fashion *via* inversion of configuration[4]. Thus, a homochiral product mixture can be obtained from a racemate. In order to gain a single enantiomeric pure *sec*-alcohol product, the remaining *(S)*-sulfate ester has to be hydrolyzed under retention of configuration at the chiral carbon atom.





By combining the two independent selective reactions proceeding through opposite stereochemical pathways, an enantioconvergent process can be achieved[5, 6].

This work was performed within the Spezialforschungsbereich Biokatalyse (project # F-115) and financial support by Degussa AG (Frankfurt) and the FWF (Vienna) is gratefully acknowledged.

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P130 LACCASE-NITROXYL RADICAL CATALYZED OXIDATION OF ALCOHOLS: MECHANISTIC INVESTIGATIONS

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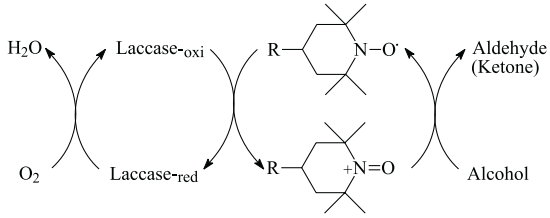
Keywords: laccase, TEMPO, alcohol oxidation

Laccase-mediator systems that catalyze oxidation of alcohols have drawn increasing attention in organic synthesis. Nitroxyl radical 2,2,6,6-tetramethylpiperidinyloxy (TEMPO) was shown to be the most effective mediator of laccase catalyzed oxidation of alcohols[1, 2]. It seems likely that oxoammonium ions, which can be formed in-situ, are the actual oxidants. Disadvantage of the laccase-TEMPO system are the long reaction time and the large amounts of TEMPO (up to 30 mol %) required.

In order to understand and optimize the system, we have performed mechanistic investigations on the fungal laccase (from *Coriolus versicolor*) catalyzed oxidation of alcohols in the presence of TEMPO and its derivatives. No oxidation took place in the absence of either TEMPO, laccase, or oxy-

gen. Results obtained from kinetic isotope studies and reaction kinetics underline the intermediacy of oxoammonium ions. One of the problems we identified is that the oxoammonium, formed via oxidation of TEMPO, is unstable in acidic acetate buffer. A way to address this problem is the use of two-phase systems. We will present our results on the use of laccase-TEMPO in two phase systems.



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P131 OXIDATIVE COUPLING OF NATURAL PHENOL DERIVATIVES CATALYZED BY LACCASES

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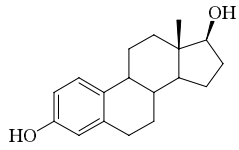
Keywords: laccase, oxidoreductases, estradiol, resveratrol, catechin

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) are multicopper enzymes that catalyze the one-electron oxidation of a wide variety of substrates, i. e. phenols and aromatic amines, with the concomitant reduction of molecular oxygen to water. Recent reports describe the use of laccases for the oxidation of different classes of organic compounds using suitable "mediators" (ref.[1]). Conversely, literature data on the laccase-mediated oxidation of complex natural compounds are quite scant. For instance, 30 years ago a laccase from *Polyporus versicolor* was used for the oxidation of steroid hormones (i. e. β-estradiol **1**) in emulsion of water and organic solvents to give a mixture of isomeric dimers[2].

In the frame of our general interest in the biocatalyzed formation of carbon-carbon bonds, we are studying the performances of laccases in the oxidative coupling of natural compounds in water solution or in the presence of organic cosolvents. In this report we will present the results obtained using the laccases from *Myceliophtora thermophyla* and from *Trametes versicolor* for the oxidation of the phenolic derivatives β-estradiol **(1)**, resveratrol **(2)** and catechin **(3)**.



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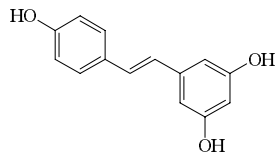

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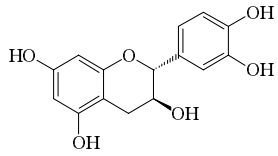

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P132 BIOCATALYSIS OF ENDOGENOUS APPLE POLYPHENOL OXIDASE IN ORGANIC SOLVENT MEDIA USING SELECTED SUBSTRATES

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Keywords: endogenous, polyphenol oxidase, phenolics, apple, biocatalysis

Enzymatic browning is catalyzed by a group of enzyme known as polyphenol oxidases (PPO; EC 1.14.18.1), which are copper-containing oxido-reductase enzymes. Although the biocatalysis of PPO has been investigated in aqueous media[1], its use as a catalyst is limited because of the instability of *o*-quinones[2]. However, the use of organic solvents has been regarded as potential reaction media for PPO, particularly, for the production of natural stable pigments of selected color intensity[3–5].

Endogenous phenolic compounds as well as PPO enzymatic extract were recovered from apple fruit. The endogenous apple PPO was enriched by acetone precipitation. The biocatalysis of enriched apple PPO in selected organic

solvent media, including hexane, heptane, dichloromethane and toluene, using model substrates, chlorogenic acid and catechin, as well as endogenous apple phenolic compounds. The optimum enzyme concentration, optimum reaction temperature, optimum pH as well as kinetics parameters such as K_m and V_{max} were investigated. The result indicated that there was a 4.7 fold increase in PPO activity, for the enriched enzymatic extract, in organic solvent media compared to that in the aqueous one. In addition, the experimental findings showed that there were significant increases in PPO activity, 4.2 to 4.5-fold and 1.5 to 2.4-fold, in the reaction media of hexane and heptane, respectively.

Using endogenous apple phenolic compounds, catechin and chlorogenic acid as substrates, the results demonstrated that the K_m values for apple PPO activity in hexane medium were 0.58, 0.76 and 0.74 mM, respectively, and those of V_{max} were 5.34×10^{-3}, 4.17×10^{-3} and 7.14×10^{-3} ΔA/μg protein/sec. Using the same substrates, the results demonstrated that the K_m values for apple PPO activity in hexane medium were 0.55, 0.44 and 1.19 mM, respectively, and those of V_{max} were 1.13×10^{-3}, 1.15×10^{-3} and 0.77×10^{-3} ΔA/μg protein/sec. The results indicated that hexane is more appropriate for the biocatalysis of PPO than heptane, with catalytic efficiency ranging from 5.48 to 9.69 compared to 0.64 to 2.61, respectively.

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P133 BIOCATALYSIS OF CHLOROPHYLLASE IN ORGANIC SOLVENT MEDIUM-CONTAINING CANOLA OIL MODEL SYSTEM

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Keywords: chlorophyllase, biocatalysis, chlorophyll, pheophytin, canola oil

The green color in immature oilseeds is due to the presence of the photosynthetic pigment chlorophyll. Oil seeds contain variable amounts of chlorophyll depending on their maturity. During extraction, there remains in the oil a proportion of the chlorophyll that is very difficult to remove by conventional bleaching methods. Oils with high chlorophyll levels require special treatment during refining to become acceptable in terms of color, free fatty acid content, peroxide value and flavor stability[1]. Theoretically, vegetable oils containing chlorophylls could be decolorized much more efficiently using chlorophyllase (chlorophyll-chlorophyllidohydrolase, EC 3.1.1.14); the enzyme catalyzes the replacement of the phytol group in chlorophyll by hydrogen atom in the chlorophyllides[2]. There is increasing interest in the biotechnological application of chlorophyllase for the removal of green pigments from edible oil, which should be a potential alternative to the conventional bleaching technique[3].

Partially purified chlorophyllase, obtained from the alga *Phaeodactylum tricornutum*, was assayed for its hydrolytic activity in an aqueous/miscible organic solvent system containing refined-bleached-deodorized (RBD) canola oil, using chlorophyll and pheophytin as substrate models. The effects of a wide range of oil contents, acetone concentrations, enzyme concentrations, chlorophyll and pheophytin concentrations, incubation temperatures and agitation speeds on the enzyme activity were investigated. The optimum reaction conditions for chlorophyllase biocatalysis were determined to consist of 20 % oil, 10 % acetone and a 200 rpm agitation speed with optimum temperatures and substrate concentrations of 35 °C and 12.6 μM for chlorophyll, and 30 °C and 9.3 μM for pheophytin. The results indicated that the presence of 10 % acetone in the reaction medium increased the hydrolytic activity of chlorophyllase by 1.5 and 1.8 times, respectively, using chlorophyll and pheophytin as substrates. However, the presence of 30 % RBD canola oil decreased the hydrolytic activity of chlorophyllase by 3.8 and 4.1 times, using chlorophyll and pheophytin as substrates, respectively. The experimental findings showed that RBD canola oil has an inhibitory effect on chlorophyllase activity, whereas acetone at low concentrations acted as an activator for the enzyme and an inhibitor at higher ones. Moreover, chlorophyllase showed a limited affinity towards pheophytin compared to that obtained with chlorophyll.

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P134 COMBINED ALKENE MONOOXYGENASE AND EPOXIDE HYDROLASE BIOCATALYSTS FOR TWO STEP TRANSFORMATIONS

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Keywords: alkene monooxygenases, epoxide hydrolase, stereospecificity

Alkene specific monooxygenases are useful biocatalysts for the production of chiral epoxides. However, the toxicity and aqueous phase lability of the accumulated epoxides can be a problem in larger scale processes. Although this may be addressed using 2 phase systems, an alternative strategy is to trap the stereocentre(s) introduced by the monooxygenase by reaction with a second enzyme, such as an epoxide hydrolase.

The stereospecificity of alkene monooxygenases usually varies with enzyme and substrate. As a long-term goal it may be feasible to tailor variants to specific substrates, based on a common expression platform. However, the two step strategy outlined above, also lends itself to improving product enantiopurity by either selective hydrolysis of one of the enantiomers or enantioconvergent hydrolysis, possibly using combinations of epoxide hydrolases.

In this poster we will present preliminary results from a project based on this two step strategy using a number of epoxide producing monooxygenases and recombinant limonene epoxide hydrolase.

P135 STYRENE MONOOXYGENASE IS A VERSATILE BIOCATALYST FOR ENANTIOSPECIFIC EPOXIDATION REACTIONS IN CELL-FREE SYSTEMS

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Keywords: styrene monooxygenase, epoxidation reactions, cell-free application, expanded bed chromatography, two-liquid phase

Styrene monooxygenase is an excellent biocatalyst for enantiospecific epoxidation reactions[1,2] and has recently been preliminary characterized[3]. Using recombinant *E. coli* expressing the two components StyA (oxygenase) and StyB (reductase) of styrene monooxygenase gram amounts of various enantiopure styrene oxide derivatives have been prepared[4].

The oxygenase component StyA was produced and purified on technical scale for preparative cell-free applications. 15 g of enriched StyA were produced from recombinant *E. coli* via expanded bed anion exchange chromatography using a DEAE® streamline™ matrix. The enzyme was obtained by one purification step with a purity up to 70 % and an overall yield of over 90 %.

Cell-free biotransformations were performed in a two-liquid phase system with dodecane as organic phase serving as reservoir for the substrate and product-sink. Formate dehydrogenase was used for the regeneration of the cofactor NADH (Fig. 1). Styrene oxide production remained stable over more than 7 hours, yielding 80 mM styrene oxide (>80 % conversion).

Overall, we have herewith established an integrated process for the preparative synthesis of various enantiopure epoxides including efficient and simple large-scale preparation of the biocatalyst.

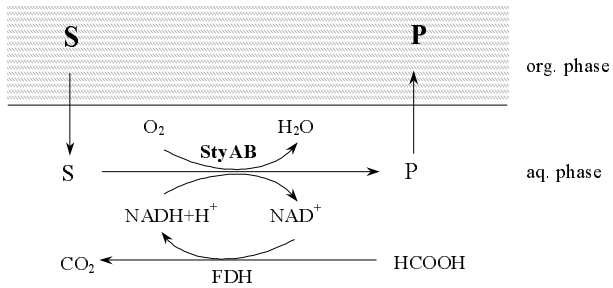

Fig. 1. **Biotransformation principle.** Application of styrene monooxygenase (StyAB) in organic/aqueous emulsions with regeneration of the cofactor NADH from NAD^+ by formate dehydrogenase (FDH). Substrate (S) is continuously supplied via the organic phase and converted in the aqueous phase to the product (P).

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P136 2-HYDROXYBIPHENYL 3-MONOOXYGENASE: LARGE SCALE PREPARATION AND CELL FREE APPLICATION IN EMULSIONS THE APPLICATION OF CROSS-LINKED ENZYME PRECIPITATES (CLEPS)

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Keywords: biotransformation, biphasic catalysis, cofactor, enzyme catalysis, oxidoreductase

Catalysis of specific hydroxylation reactions of organic compounds is difficult by chemical means. Therefore the interest in biocatalysts catalyzing specific hydroxylations has grown in recent years simultaneous to the interest in the application of oxygenases. As oxygenases require expensive cofactors, usually NAD(P)H, in stoichiometric amounts, these enzymes are mostly used in whole cell biotransformations[1,2]. However, independent optimization of enzyme production and biotransformation reactions can better be achieved by *in vitro* approaches.

The soluble and NADH dependent flavoprotein 2-hydroxybiphenyl 3-monooxygenase (HbpA) from *Pseudomonas azelaica* HBP1 is catalyzing the hydroxylation of *ortho*-substituted phenols to corresponding 3-substituted catechols with absolute regiospecificity[3]. HbpA was partially purified on large scale by expanded bed adsorption (EBA) chromatography[4].

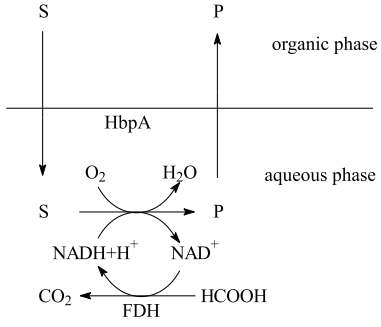

Fig. 1. **Reaction cycle catalysed by HbpA and FDH in aqueous-organic two phase systems**

Together with the commercially available cofactor regeneration enzyme formate dehydrogenase (FDH) both enzymes were precipitated and cross-linked. These cross-linked enzyme precipitates (CLEPs) were applied for the biotransformation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl in aqueous-organic two phase systems (Fig. 1). A productivity of 0.18 $g.l^{-1}.h^{-1}$) could be maintained over 9 hours.

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P137 BIOCATALYST DEVELOPMENT FOR THE PRODUCTION OF *cis*-DIOLS USING CHLOROBENZENE DIOXYGENASE

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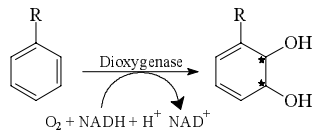
Keywords: chlorobenzene dioxygenase, *cis*-diols, biotransformation, scale-up

Aromatic ring dioxygenases are multicomponent enzyme systems that add molecular oxygen to the aromatic nucleus to form arene *cis*-diols (Scheme 1). The broad substrate range and the high regio- and enantioselectivities of dioxygenases make these enzymes useful not only for bioremediation, but also for biocatalytic synthesis of chiral synthons for the production of biologically active chemicals and pharmaceuticals[1].



Scheme 1. **Oxidation of aromatic compounds *via* dioxygenases**

Chlorobenzene dioxygenase (CDO) of *Pseudomonas* sp. P51 consists of 3 components, NADH-ferredoxin reductase, ferredoxin, and terminal oxygenase component, which is composed of a small and a large subunit[2]. It was shown that CDO can oxidize different classes of aromatic compounds to the corresponding *cis*-dihydrodiols[3]. Powerful and stable expression system is required to develop an efficient process for the production of *cis*-dihydrodiols. Therefore we constructed a new expression system pTEZ30 harboring the CDO genes under strict control of the P*alk* promoter which is derived

from *Pseudomonas oleovorans* GPo1 and confers kanamycin resistance. We also showed the potential of this new expression system for efficient synthesis of chlorobenzene dioxygenase for the scale-up of *cis*-dihydrodiol production.

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P138 SCREENING AND OPTIMISATION OF COMMERCIAL ENZYMES FOR THE ENANTIOSELECTIVE HYDROLYSIS OF *(R,S)*-NAPROXEN ESTER

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Keywords: lipase, esterase, naproxen, biocatalysis, hydrolysis, optimisation

The purpose of this study was the identification of suitable lipase or esterase for enantiomeric resolution of *(R,S)*-naproxen.

The aim was to find an enzyme that yields *(S)*-naproxen with an enantiomeric excess of more than 98 %, an enantiomeric ratio *(E)* of greater than 100, and substrate conversion in excess of 40 %. Commercially available enzymes were screened, and selected for optimisation of enantioselectivity through statistically designed experiments on the reaction conditions.

Optimisation efforts resulted in a more than 20-fold improvement of activity, while the excellent enantioselectivity of the enzymes was maintained. In particular, the addition of PEG 1000 as a co-solvent improved conversion rates 10-fold.

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P139 ASYMMETRIC TRANSFORMATION OF ENOL ACETATES WITH ESTERASES FROM *Marchantia polymorpha*

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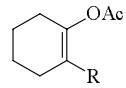
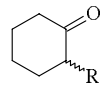
Keywords: esterase, enol acetate, asymmetric hydrolysis, chiral ketone

Optically active α-substituted ketone derivatives are widely employed as chiral synthons in asymmetric syntheses. Recently, it has been found that yeast[1,2] and cultured cells of *M. polymorpha*[3] were capable of performing the hydrolysis of α-alkylated cyclohexanone enol esters to give α-substituted chiral ketones. We have now investigated the enzymes which are able to catalyze the asymmetric hydrolysis of enol acetates.

A Butyl-Toyopearl column chromatography of the crude enzyme preparation gave a good separation of the two different esterases. Further purification by chromatography on a Diethylaminoethyl-Toyopearl column and then a Sephadex G-75 column gave homogeneous esterases: Esterase I, molecular mass ca 54000, dimeric form composed of two identical subunits; Esterase II, molecular mass ca 45000, dimeric form composed of two identical subunits. The amino termini of these esterases were blocked. The esterases, therefore, were digested with protease and the internal amino acid sequences of the peptide fragments obtained from Esterase I were not similar to those of any hydrolytic enzymes.



1: R = Me
2: R = Et
3: R = *n*-Pr
4: R = *n*-Pnt
5: R = Bnz



6: R = Me
7: R = Et
8: R = *n*-Pr
9: R = *n*-Pnt
10: R = Bnz

Several cyclohexanone enol acetates **(1-5)** were subjected to enzymatic hydrolysis with these esterases to clarify the effect of various substituents at β-position to the acetoxyl group on the enantiomeric ratio and the catalytic activity of enzymes. As shown in Table I, hydrolysis of enol acetates, **1** and **2**, by Esterase I gave the corresponding optically active ketones (**6** and **7**). The chiral preference of Esterase I was re-

tained among these substrates: the protonation of the enol intermediates from **1** and **2** occurred preferentially from the same enantiotopic face of the C-C double bond. However, when *n*-propyl, *n*-pentyl and benzyl groups were introduced into the β-position to the acetoxyl group of the substrates **(3-5)** , the corresponding products having opposite configuration were obtained. This result indicates that the stereoselectivity of Esterase I in the protonation of these enol intermediates is reversed by long chain (C > 3) and bulky substituents at the β-position to the acetoxyl group. On the other hand, the conversion yield and enantiomeric purity in the hydrolysis of enol acetates **(1-5)** with Esterase II are very low in comparison to the case of Esterase I. However, the stereoselectivity in the hydrolysis with Esterase II was opposite to that with Esterase I.

Table I
Enantioselectivity in the hydrolysis of enol acetates by Esterases I and II

Substrate	Product	Esterase I			Esterase II		
		Conv. (%)	e. e.	Config.[a]	Conv. (%)	e. e.	Config.[a]
1	**6**	>99	>99	*S*	4	4	*R*
2	**7**	>99	14	*S*	3	2	*R*
3	**8**	>99	>99	*R*	5	4	*S*
4	**9**	20	26	*R*	16	7	*S*
5	**10**	15	>99	*S*	11	14	*R*

[a]Preferred configuration at the α-position to the carbonyl group of the products

Thus, two hydrolytic enzymes were isolated from cultured cells of *M. polymorpha* and were confirmed to be capable of discriminating the enantiotopic face of the C-C double bond of the enol intermediate in the hydrolysis. The enantioselectivities in the protonation of the enol intermediate were opposite between these enzymes. The enantioselectivity of both enzymes reversed in the hydrolysis of the substrates with long side chain and bulky benzyl group at the α-position of enol acetates, compared with the substrates having short side chains.

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P140 ENZYME-MEDIATED ENANTIOSELECTIVE HYDROLYSIS OF PEG-TAGGED CARBONATES

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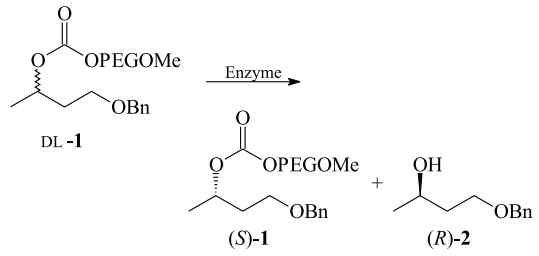
Keywords: enzymatic hydrolysis, PEG, PPL, esterase

In the enzymatic hydrolysis of racemic acyl compounds, usual work-up procedures including the separation of the mixture into the remaining substrate and the resulting alcohol spend a lot of time and waste much amount of solvents. In order to avoid the tedious steps, we have developed a new type of the enzymatic hydrolysis of PEG-tagged carbonates as the unique substrates. Because of the amphiphilic character, the reaction could be easily monitored by TLC and NMR analysis and the purification steps could be simple.

A MeOPEG (Mw = 750)-tagged carbonate DL-**1** was used as the substrate of the screening test. Amongst the 12 commercially available enzymes, some esterase and PPL hydrolyzed the carbonate **1** to give the corresponding alcohol **2**. Finally, PPL was chosen as the best enzyme. As expected, the recovered substrate *(S)*-**1** was easily separated from the resulting alcohol *(R)*-**2** by using the minimum amount of silica gel and organic solvents. In the reaction of the substrate supported with MeOPEG (Mw = 550) for 24 h at 10 °C, the conversion and *E* value were 0.10 and 32, respectively.



P141 *T. reesei* ACETYL ESTERASE CATALYZED TRANSESTERIFICATION IN WATER

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Keywords: esterases, transesterification, acylation, *Trichoderma reesei*

Hydrolases are group of enzymes catalyzing hydrolytic reactions. Introducing of organic reaction media has made it possible to use these enzymes efficiently for reversed hydrolytic reactions and transferase-type reactions as well[1]. Water in these organic systems is often believed to shift equilibrium in favor to hydrolysis[2].

Contrary to numerous studies proving essential importance of low or minimal water content in such reactions[3,4], partially purified *Trichoderma reesei* RUT-C30 acetyl esterase preparation was found to catalyze acyl transfer reactions not only in organic solvents, but also in mixtures of organic solvents/water and even in water. Using different acyl donors, the best results were obtained using vinyl acetate. As acetyl acceptors, variety of hydroxyl bearing compounds in their water solutions were used. Conversion and number of newly formed acetates varied according acceptor used. Conversions over 50 % were observed by majority of most common monosaccharides, their methyl and deoxy derivatives and oligosaccharides. In several cases, *T. reesei* acetyl esterase catalyzed transesterification exhibited strict regioselectivity, leading to only one acetyl derivative. Preparative potential of described enzymic transesterification in water was demonstrated by transacetylation of methyl β-D-glucopyranoside and *p*-nitrophenyl β-D-glucopyranoside, yielding 56.4 % of methyl 3-*O*-acetyl β-D-glucopyranoside and 70.2 % *p*-nitrophenyl 3-*O*-acetyl β-D-glucopyranoside as the only products of the reactions.

This new enzymaticaly catalyzed transacetylation in water opens new area in chemoenzymatic synthesis. Its major advantages are easy and regioselective esterifying of polar compounds, high yields, low enzyme consumption and elimination of use of toxic organic solvents.

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P142 PRELIMINARY X-RAY CRYSTAL STRUCTURE INFORMATION OF AN ESTERASE FROM *Pseudomonas fluorescens*

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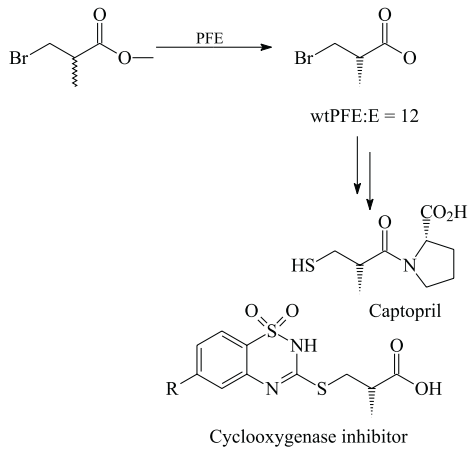
Keywords: X-ray structure, *Pseudomonas fluorescens* esterase, enantioselectivity

An aryl esterase from *Pseudomonas fluorescens*[1] has been employed in the resolution of 3-bromo-2-methyl-propionic acid methyl ester, an important building block in the synthesis of Captopril and a potent cyclooxygenase inhibitor[2] (Figure). Significant improvements to the enantioselectivity seen by the wild type enzyme have been made by site directed mutagenesis. The preliminary X-ray crystal structure of both the wild type and several mutants of interest have been elucidated. The wild type enzyme as well as mutants crystallize in high $(NH_4)_2SO_4$ concentrations as a dimer and belong to the R3 space group. The unit cell has dimensions of 147.5 Å × × 147.5 Å × 131.9 Å with angles of $\alpha = \beta = 90°$, $\gamma = 120°$. Active site analysis of the crystal structure of the wild type and of mutants will hopefully lead to understanding of the origins of improvements in enantioselectivity.



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P143 ENANTIOPURE SULFINAMIDES VIA SUBTILISIN-CATALYZED KINETIC RESOLUTION OF *N*-ACYLSULFINAMIDES

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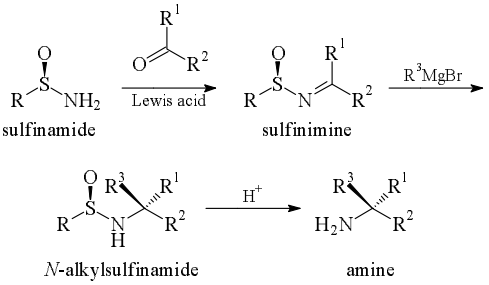
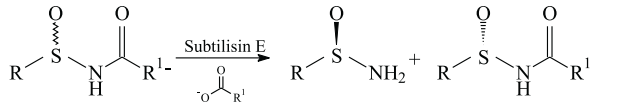
Keywords: sulfinamides, subtilisin, enantioselective, chiral auxiliary

Sulfoxides have been widely used as chiral auxiliaries in organic synthesis[1]. Some of the most interesting new sulfoxide auxiliaries are the sulfinimines (*N*-sulfinyl imines), which are chiral auxiliaries for the imine group and used for the synthesis of amines and related compounds[2]. The simplest route to prepare a sulfinimine is by the direct condensation of an aldehyde or ketone with an enantiopure sulfinamide (Scheme 1). Therefore the utility of these chiral auxiliaries is dependent upon concise methods of sulfinamide preparation[3].



Scheme 1. **Stereoselective synthesis of an amine from enantiopure sulfinamide**

Through our continuing efforts to design new methods to enantiopure materials, we have developed a novel enzyme-catalyzed route to enantiomerically pure sulfinamides. Subtilisin E (bacterial serine protease) catalyzes the hydrolysis of various *N*-chloroacetyl- and *N*-hydrocinnamoyl-sulfinamides with very high enantioselectivity ($E > 150$) toward the *(R)*-enantiomer (Scheme 2). Our results show subtilisin E does not favour the enantiomer predicted with empirical rules for proteases[4]. Through substrate engineering and molecular modelling experiments we have revealed the molecular basis for the acyl group selectivity and reverse enantioselectivity demonstrated by subtilisin E. Experiments suggest the acyl group is important for substrate binding and the enantioselectivity arises because the slow-reacting *(S)*-enantiomer binds in a nonproductive orientation. Large-scale resolution experiments of *N*-chloroacetyl-2-mesitylenesulfinamide and *N*-hydrocinnamoyl-*p*-chlorobenzenesulfinamide with subtilisin E gave their corresponding *(R)*-sulfinamides in high yield and enantiomeric excess. As well, the enzyme demonstrates a wide acceptance of aryl sulfinamide substrates and has been used to resolve several sulfinamides that were previously unavailable.



Scheme 2. **Subtilisin E-catalyzed resolution of *N*-acylsulfinamides**

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P144 UTILIZATION OF MICROBIAL PROTEASES FOR PEPTIDE SYNTHESIS IN ORGANIC MEDIA

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Keywords: *Aspergillus* proteases, *Bacillus* proteases, carbamoylmethyl ester, pH effect, protease-catalyzed peptide synthesis

Microbial proteases from a variety of sources are commercially available. Although they possess potential for organic syntheses, they have hitherto been employed mainly for food processing and as detergent ingredients. Protease-catalyzed peptide synthesis has many advantages over chemical methods, while narrow substrate specificity and the secon-

dary hydrolysis of a growing peptide are counted as major drawbacks. We have recently reported on the broadening of substrate tolerance of a mammalian protease α-chymotrypsin by using such activated esters as the carbamoylmethyl (Cam) ester as an acyl donor in the so-called kinetically controlled peptide bond formation[1]. As a part of our continuing research on the effective use of microbial proteases for organic syntheses, we have investigated peptide bond formation using some *Aspergillus* and *Bacillus* proteases. We first examined proteases from *A. melleus* (Amano protease P) and *A. oryzae* (Amano protease A). A series of *Z*-amino acid Cam esters were allowed to react with amino acid amides. With these *Aspergillus* proteases, aimed-at peptides were obtained generally in rather high yields in the mixed solvent of 1,1,1,3,3,3--hexafluoro-2-propanol and DMF (1 : 1, v/v)[2]. We next examined *B. licheniformis* protease (subtilisin Carlsberg). Couplings were carried out in anhydrous acetonitrile in the presence of the immobilized protease on Celite. We observed that the peptide yield changed significantly upon the pH of the buffer solution from which the immobilized protease was prepared; the maximal peptide yield was obtained with the preparation from pH 10.7 (ref.[3]). With the Cam ester as the acyl donor, D-amino acid amides were as good amine nucleophiles as the L-counterparts (Table I).

Table I
B. licheniformis protease-catalyzed couplings of *Z*-L-Ala--OCam with Xbb-NH_2 (after 4 h)

Xbb	Peptide (%)	Xbb	Peptide (%)
L-Ala	95.4	D-Ala	97.1
L-Leu	93.8	D-Leu	87.7
L-Phe	79.9	D-Phe	91.1

A series of dipeptide syntheses and several segment condensations including the synthesis of the Leu-enkephalin sequence were achieved generally in high yields. We examined also *Bacillus subtilis* protease (Amano protease N) as a catalyst for both kinetically and thermodynamically controlled peptide syntheses in acetonitrile with low water content. In several cases, the latter approach using carboxyl components bearing a free carboxyl group proved to be superior to the former.

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P145 TRYPSIN ASSISTED SEMISYNTHESIS OF HUMAN INSULIN ANALOGUES

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Keywords: desoctapeptideinsulin, trypsin, semisynthesis, opioid peptides, aminopeptidase substrates

Hundreds of analogues of Human insulin have been prepared by trypsin assisted semisynthesis, using the large insulin fragment – desoctapeptide insulin- and a series of synthetic analogues of the B^{23}–B^{30} sequence of the insulin peptide chain. Some of them were highly potent in tests *in vitro* (glucose transport, thymidine incorporation into DNA), nevertheless their potency *in vivo* was not higher than that of natural insulins. The same approach was recently used for the semisynthesis of another class of insulin derivatives.

We used desoctapeptide insulin for the trypsin catalyzed semisynthesis of peptide hormonogens of opioid peptides and masked substrates of aminopeptidases[1, 2]. Both groups of compounds were characterized by analytical RP-HPLC, mass spectrometry, capillary electrophoresis and by metabolic stability assays.

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P146 DISCOVERY OF A NOVEL HEME-CONTAINING LYASE, PHENYLACETALDOXIME DEHYDRATASE, FROM MICROORGANISMS AND ITS APPLICATION TO ORGANIC SYNTHESIS

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Keywords: aldoxime, nitrile, dehydratase, metabolism, microorganisms

Dehydration of aldoximes, which are easily prepared from aldehydes and hydroxylamine, is a useful method for synthesizing nitriles, although many of the chemical procedures require harsh reaction conditions. In plants, aldoximes are considered to be intermediates in the biosynthesis of certain biologically active compounds such as indoleacetic acid, cyanogenic glucosides, and glucosinolates. However, very little is known about the aldoxime degrading enzyme: it has never been purified to homogeneity nor characterized in detail. We focused on microbial aldoxime metabolism not only to apply the enzyme to organic synthesis, but also to study possible relationships between aldoxime-dehydration and nitrile-degrading enzymes and their functions in microorganisms (Fig. 1).

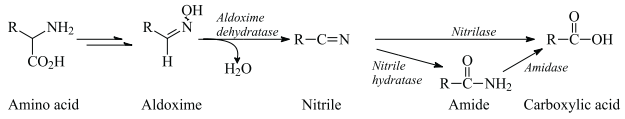

Fig. 1. **Aldoxime-nitrile pathway in microorganisms**

We screened for aldoxime-degrading microorganisms from soil and isolated *Bacillus* sp. OxB-1 (ref.[1]) and *Rhodococcus* sp. YH3-3 (ref.[2]), which degrades *Z*-phenylacetaldoxime (*Z*-PAOx) and *E*-pyridine-3-aldoxime (*E*-PyOx), respectively, by an acclimation culture technique. They metabolized aldoximes through nitriles into the corresponding carboxylic acid by a combination of a novel aldoxime dehydratase and nitrile-hydrolyzing enzymes[1, 2]. By using the dehydratase, the enzymatic synthesis of nitriles from aldoximes under mild conditions (pH 7–8, 30 °C) were achieved for the first time[3, 4].

We purified the enzyme from *Bacillus* sp. OxB-1 and studied its enzymological properties[5]. The enzyme, named "PAOx dehydratase (Oxd, EC 4.2.1.-)", is quite unique because it catalyzes a simple dehydration reaction, yet contains heme *b* as a prosthetic group and requires FMN or SO_3^{2-} as an electron acceptor. The gene *(oxd)* coding for the enzyme was shown to be linked with the gene *(nit)* of a nitrilase, which participates in aldoxime metabolism in the organism[5]. The enzyme was expressed in a soluble and active form by the recombinant *E. coli* under the control of *lac* promoter in the pUC18 vector[6]. The production of active enzyme was markedly enhanced by increasing the volume of culture medium (1 liter in 2-liter flask). Under optimized conditions, the enzyme was produced in an active and soluble form at 15,000 U per liter of culture, which is about 1,500-fold higher than the amount produced by the wild-type strain. Moreover, the enzyme comprised over 40 % of total extractable cellular protein. The overproduced Oxd was useful for the high-yield synthesis of nitriles from aldoximes[4].

We also investigated the distribution of aldoxime dehydratase and the relationship between aldoxime-degrading enzymes and nitrile-degrading enzymes among microorganisms[7, 8]. All the aldoxime degraders possessed nitrile-hydrolyzing activities. On the other hand, all of the nitrile-degraders described in the literature possessed aldoxime-degrading activities. Thus, we showed the co-existence of aldoxime dehydratase and nitrile degrading enzymes and elucidated the role of nitrile degrading enzymes in aldoxime metabolism in microorganisms, permitting us to postulate a novel "aldoxime-nitrile pathway" (Fig. 1)[9].

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P147 NEW CHROMOGENIC SUBSTRATES OF PARAOXONASE (PON1)

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Keywords: paraoxonase, arylesterase, chromogenic substrate

The study of lipid peroxidation is a rapidly growing field in medicine and biology, spurred by increasing evidence that lipid oxidation is involved in the pathogenesis of many chronic diseases, e. g. atherosclerosis and aging[1,2]. Paraoxonase (PON1) is a high density lipoprotein (HDL)-associated serum enzyme with peroxidase activity[3]. It was found to protect low density lipoproteins (LDL) from oxidative modifications by hydrolyzing phospholipid hydroperoxides.

PON1 was first discovered for its role in detoxifying organophosphates and its name reflects its ability to hydrolyze paraoxon, a metabolite of the insecticide parathion. Although it was then classified as aryldialkylphosphatase PON1 can also hydrolyze aromatic carboxylic esters[4].

Thus the understanding of the properties of this enzyme which have both physiological and toxicological importance is the subject of extensive research. Herein we present the screening of synthetic chromogenic substrates for monitoring the arylesterase-like activity of PON1.

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P148 A FLUOROGENIC ASSAY FOR TRANSKETOLASE FROM *Saccharomyces cerevisiae*

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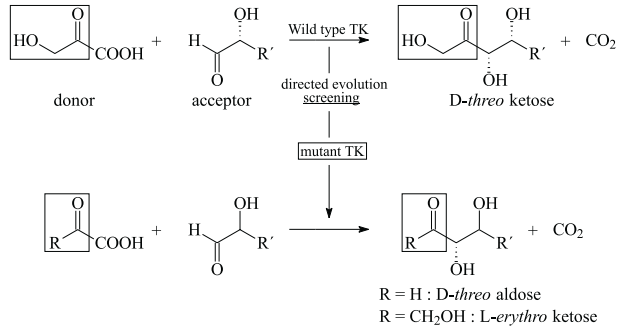
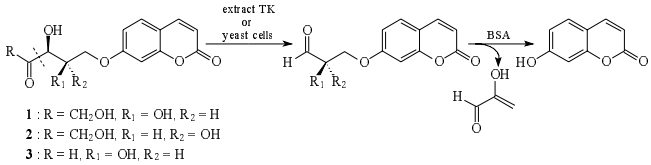
Keywords: transketolase, enzyme catalysis, fluorescence spectroscopy

Saccharomyces cerevisiae recombinant transketolase (TK)[1] is a useful catalyst for ketose synthesis due to the stereo-controlled formation of the C3–C4 bond. It catalyzes the transfer of a ketol unit from β-hydroxypyruvic acid to an aldehyde to generate D-*threo* (3*S*,4*R*) ketose. The decarboxylation of this donor substrate and subsequent loss of carbon dioxide make the overall condensation reaction irreversible. A wide range of aldehydes as ketol acceptors has been used for the obtention of various ketoses[2–4]. The enzyme appears highly specific for ketol donor substrates and for hydroxyaldehyde substrates with the *(R)* configuration.



In order to modify these TK properties, we examined the possibility of altering the substrate specificity of this enzyme by directed evolution. We are interested in obtaining variants of TK able to accept glyoxylate as donor substrate to obtain D-*threo* aldoses or able to accept *(S)*-hydroxyaldehyde substrates to obtain L-*erythro* ketoses.

An efficient screening or selection system is an absolute prerequisite for identifying the evolved enzyme variants that display improved properties. Here we report an assay allowing us to detect TK activities *in vitro* and *in vivo* by fluorescence in solution according to the Reymond's test[5]. We examined the use of a fluorogenic substrate 1 of wild type TK, which is itself non fluorescent but release a fluorescent product umbelliferon[6]. This assay could be used to screen mutants with the appropriate fluorogenic substrates 2 or 3.



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P149 OPTIMIZATION OF SPECTROPHOTOMETRIC METHOD SUITABLE FOR ASSESSING PRIMARY AMINO GROUPS IN DAIRY PROTEINS AND MONITORING COURSE OF ENZYMATIC HYDROLYSIS

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Keywords: *o*-phtaldialdehyde, hydrolysis, whey proteins

Another use of whey proteins in food chemistry is highly requested. One of the best known methods of modifying whey proteins is enzymatic hydrolysis by which is possible to produce a wide range of hydrolysates with desired properties available furthermore for producing functional foods. Spectrophotometric determination of primary amino groups based on reaction *o*-phtaldialdehyde (OPA) reagent with primary amino groups in presence of thiol group is widely used in analysis of amino acids, peptides and proteins and by the use of this method is possible to monitore the course of enzymatic hydrolysis and its degree, respectively. Methods published in the past usually had some disadvantages (e. g. instability of 1-alkyl-2-alkylisoindols emerging during reaction), therefore new modified recipe of reactant mixture of OPA and *N*-acetyl-L-cysteine was suggested and proved[1]. This mixture gives stable light absorbing product emerging during the reaction and the reaction provides more precise results and saves chemicals. Another advantage of this method is substitution of bad smelling 2-mercaptoethanol by *N*-acetyl--L-cysteine like a donor of thiol group. Several amino acids (Arg, Glu, Gly, His, Lys, Pro and standard equimolar solution; in different concentrations), whey protein hydrolysates and urea were chosen and response to the reaction with OPA were investigated. The reaction worked under laboratory conditions, absorbance was measured at λ = 335 nm (absorbance maximum of 1-alkylthio-2-akylisoindols) after 2 min. The product of reaction was stable at least 90 min. The value of ε for all measured amino acids in water solutions varied between 6700–7300 $cm^{-1}.l.mol^{-1}$ except Lys (double response becouse of 2 amino groups in structure) and Pro (no reaction). Calibration curves were linear in range 0.0–0.35 $mmol.l^{-1}$ of amino acids in water solution. Also the influnce of storage conditions to stability of OPA reagent were investigated. These experiments showed that storage of OPA reagent in refrigerator provides more stability (at least 11 days) and more economic application.

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P150 PRODUCTION OF BENZALDEHYDE FROM PHENYLALANINE CATALYSED BY A BIENZYMATIC OXIDASE-PEROXIDASE SYSTEM

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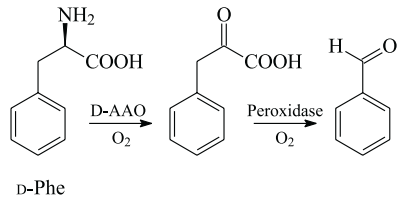
Keywords: aroma, peroxidase, amino acid oxidase, benzaldehyde, phenylalanine

Benzaldehyde is the major component of bitter almond aroma. It is the second most important flavour molecule (after vanillin), and is used as a key ingredient in cherry and other natural fruit flavours. Benzaldehyde is produced on an industrial scale by oxidation of aromatic hydrocarbons. There is however an increasing preference of consumers for "natural" food additives[1]. Thus, natural benzaldehyde is mainly extracted or processed from essential oils of higher plants. Biotechnology can represent an alternative: several publications report the biotransformation of phenylalanine to benzaldehyde by cultures of either moulds or microorganisms[2]. Phenylalanine can also be converted to benzaldehyde by cell free extracts[3]. In both cases, yields are low, and benzaldehyde has to be purified from complex mixtures of by-products.

We report a new bioconversion of D-phenylalanine to benzaldehyde catalysed by two coupled commercially available pure enzymes.



D-Phe

D-Phe is first oxidised by a D-aminoacid oxidase (immobilised, from *Trigonopsis variabilis*, Fluka*)* to phenypyruvic acid, which is converted *in situ* to benzaldehyde by a peroxidase (from *Coprinus cinereus*, Novozymes). After extraction from the aqueous reaction mixture, the produced benzaldehyde appears to be pure by GC and ^{1}H NMR. Yield borders 35 %. Racemic phenylalanine can be used as well with the same yield (relatively to D-Phe). Starting from natural L-phenylalanine implies that the amino acid is first racemised by a classical treatment with acetic anhydride and aqueous sulphuric acid. The crude DL-Phe prepared by this method is also a satisfactory substrate of DAO.

Experimental conditions and studies on mechanism of the transformation will be discussed.

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P151 ENZYMATIC SYNTHESIS OF NATURAL VANILLIN

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Keywords: vanillin, vanillyl-alcohol oxidase, mutants, characterization

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a widely used flavour compound in food and personal products. Vanillin is mainly produced by chemical synthesis and less than 1 % of its total demand is obtained from the curing of the beans of the orchid *Vanilla planifolia*.

With the increasing interest in natural products alternative processes are being developed to produce natural vanillin. Among others, one of the approaches includes the use of isolated enzymes. In an earlier study[1], we showed that the flavoprotein vanillyl-alcohol oxidase (VAO) can be used for the two-step enzymatic synthesis of vanillin from the natural precursor creosol (2-methoxy-*p*-cresol). Although creosol is a very poor substrate for VAO, protein structural analysis did not reveal any obvious reason for this poor reactivity[2]. Therefore, we started an error-prone PCR based random mutagenesis approach screening for VAO variants with improved creosol reactivity. After a single round of mutagenesis, four single-point mutants were selected for a detailed structural and functional characterization. The evolved VAO variants displayed remarkable substrate specificities, being active with creosol but not with cresol. The enhanced reactivity with creosol is ascribed to the destabilization of an abortive covalent adduct between the substrate and the FAD cofactor[1]. All mutated residues are located in loop regions outside the active site and their replacements do not cause any significant structural perturbation. This suggests that the changes in substrate specificity are caused by dynamic coupled motions which cannot easily be directed by rational redesign.

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P152 CHEMOENZYMATIC ASYMMETRIC TOTAL SYNTHESIS OF AN AROMA CONSTITUENT OF JAMAICAN RUM AND OF (+)-PESTALOTIN

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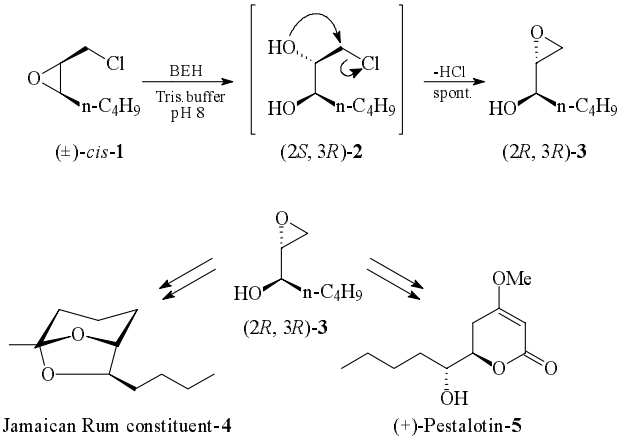
Keywords: bacterial epoxide hydrolases, enantioconvergent, cascade-reaction

A powerful method to obtain enantiopure epoxides or vicinal diols makes use of bacterial epoxide hydrolases (BEH)[1]. This approach was used for a short chemoenzymatic synthesis of two natural products – an aroma component of Jamaican Rum **4** and the gibberelin synergist (+)-pestalotin **5**.

Key step of the synthesis consists of an asymmetric enzymatic hydrolysis of halomethyl oxirane (±)-*cis*-**1** with BEH to furnish the corresponding diol (2*S*, 3*R*)-**2**. The latter spontaneously undergoes an intramolecular *exo-tet*-cyclization[2] ("Payne-type-rearrangement") to give epoxyalcohol (2*R*, 3*R*)-**3** in good optical purity and yield. Overall, this sequence represents an enzyme-catalyzed cascade-reaction. Since the biohydrolysis proceeds in an enantioconvergent fashion, the occurrence of an unwanted stereoisomer was avoided[3, 4]. Epoxy alcohol **3** served as the central building block for the synthesis of **4** and **5**.



This work was performed within the Spezialforschungsbereich Biokatalyse (project # F-104) and financial support by the FWF (Vienna) is gratefully acknowledged.

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P153 ENZYME-GENERATED RADICALS AS A TOOL TO PRODUCE NATURAL COMPOUNDS. THE CASE OF THE PRODUCTION OF CAROTENOID-DERIVED AROMA COMPOUNDS

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Keywords: radicals, β-carotene, β-ionone, xanthine oxidase

The production of natural compounds requires “natural” processes, i. e. processes in which the substrate, catalysis and treatments are “natural”. The use of radical mediated reactions seems thus difficult to carry out in this frame. However, some enzymes are able to generate radical species. It is the case with xanthine oxidase, an enzyme taken from membrane of milk fatty globules.

We have investigated the possibility of modulating the synthesis of radical species in modifying the substrate of the enzyme and monitored this effect on the degradation of β-carotene and the synthesis of β-ionone. By using various aldehydes and xanthine, it was possible to generate the superoxide anion (from xanthine) but also alkyl radicals (from butanal) and oxygenated radicals (from acetaldehyde) that exhibited various effects on the degradation. With the superoxide anion, the reaction was occurring at the same rate as autooxidation whereas the two aldehydes had a strong effect on the rate of degradation of carotene and also of ionone.

P154 EFFECT OF CULTURE MEDIUM COMPOSITION ON THE BIOGENESIS OF THE NATURAL FLAVOR 1-OCTEN-3-OL BY *P. camemberti*

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Keywords: bioconversion, *P. camemberti*, lipoxygenase, hydroperoxide lyase, 1-octen-3-ol

The production of certain natural flavor compounds, including C5 to C9 aliphatic alcohols and carbonyl compounds, results from a biosynthetic pathway involving several enzymatic activities, in particular lipoxygenase and hydroperoxide lyase, and lipid components as substrates. This biosynthetic pathway is initiated by the oxygenation of PUFAs possessing a 1*(Z)*, 4*(Z)*-pentadiene moiety to produce stereo- and regio-specific hydroperoxides (HPODs), which in their turn are converted into volatile alcohols, aldehydes, alkanes or alkenes, ranging from five to nine carbons, and non-volatile corresponding oxoacids[1].

The effects of linoleic acid induction on the biomass production as well as on the changes in glucose consumption, pH values and 1-octen-3-ol profiles during the growth of *Penicillium camemberti* were previously investigated by our group[2, 3]. In addition, these changes as well as the changes in 1-octen-3-ol profiles were also investigated by our group during the growth of *P. camemberti* on synthetic medium containing free linoleic acid[4]. In the present work, the effects of the addition of soybean oil in the same defined synthetic medium and two dairy media on the biomass production of *P. camemberti*, lactose consumption, pH values and the production of 1-octen-3-ol were investigated.

The addition of refined soybean oil to the defined synthetic medium enhanced the production of 1-octen-3-ol, *in vitro*, by a factor of 8 compared to that obtained with the same medium but containing linoleic acid. The milk and acid-gelified milk, used as culture medium, enhanced the production, *in vitro*, of 1-octen-3-ol respectively by 1.2- and 3-fold compared to the defined synthetic medium containing linoleic acid. However, these productions of 1-octen-3-ol on dairy media were smaller than those obtained with the enzymatic extract obtained from the biomass of *P. camemberti*, grown on the defined synthetic medium but supplemented with soybean oil.

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P155 GENETIC ENGINEERING OF THE γ-OXIDATION PATHWAY IN THE YEAST *Yarrowia lipolytica* TO INCREASE THE PRODUCTION OF AROMA COMPOUNDS

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Keywords: γ-oxidation, acyl-CoA oxidase, γ-decalactone

As many yeast species, *Yarrowia lipolytica* is able to transform ricinoleic acid (a hydroxylated C18 fatty acid) into γ-decalactone, a fruity and creamy aroma compound[1]. Unfortunately, this species is also able to degrade the produced lactone. The pathway of biotransformation involves γ-oxidation and requires the lactonisation at the C10 level (when the hydroxy group is in γ -). *Y. lipolytica* possesses a five-member family of acyl-CoA oxidases (Aox1 to 5), the enzyme catalysing the first step of γ-oxidation, some of which are long--chain specific (Aox2)[2] or short-chain specific (Aox3)[3, 4]. In a previous paper, we have tried to decrease the lactone degradation by constructing a strain with no more activity on short-chain substrates[5]. However, this strain was growing and biotransforming very slowly. In this study, we have constructed strains without acyl-CoA oxidase activity for short-chain substrates but with increased activity on long chains. These strains are able to grow at the same rate as the wild type but produce about 10 times more in only 48 hr, and this amount does not significantly decrease in 250 hr.

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P156 SYNTHETIC STUDIES ON HELIANNANE SESQUITERPENES VIA CHEMOENZYMATIC TRANSFORMATION

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Keywords: heliannuol E, heliannuol C, sesquiterpene, lipase, enantioselective synthesis

(-)-Heliannuols E and C are naturally occurring sesquiterpenes exhibiting allelopathic activity[1]. We report herein the enantioselective total syntheses of heliannuols E (ref.[2]) and C employing chemoenzymatic transformations of the prochiral diols **1** and **3** into the optically enriched acetate **2** and **4** as the key reaction steps (Fig. 1). It should be noted that the absolute stereochemistry of heliannuol C was firmly established by the completion of the first total synthesis.

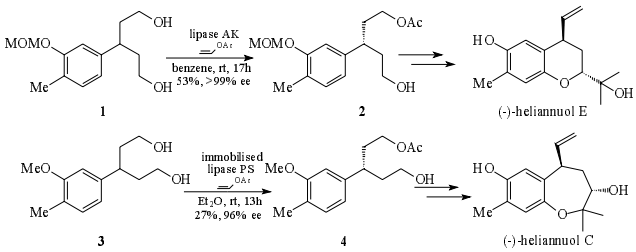

Fig. 1. **Syntheses of heliannuols E and C**

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P157 REGIOSELECTIVE ENZYMATIC ACYLATION OF POLYHYDROXYLATED SESQUITERPENOIDS

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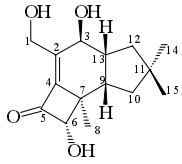
Keywords: sesquiterpenoids, tsugicoline, sterpurane, illudin, lipase

A number of *Basidiomycetes* are known to produce sesquiterpenoids with a protoilludane **(1-3)** or an illudane **(4)** skeleton. As a part of our continuing search for biologically active metabolites, we have recently described the isolation of tsugicoline A **(1)** and its transformation into the sterpurane derivative **(2)** and the 2-amino-tsugicoline E **(3)** (ref.[1]). The anomalous presence of a carbonyl function in the four member ring of **1** is the key to understand the reactivity of this interesting metabolite and led to an easy opening of the C(6)–C(7) bond to give, under suitable conditions, compounds **2** and **3**. The illudane sesquiterpenes illudine S **4**, a potent cytotoxic compound, was also isolated by us from a strain of *Omphalotus olearius*[2].

In order to extend the number of available derivatives of these compounds, we have considered the possibility to carry out their enzymatic acylations in a comparative study. In this report we will describe the results obtained using a set of different lipases for the regioselective acylation of **1-4** .



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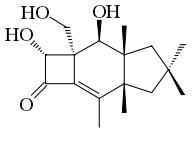

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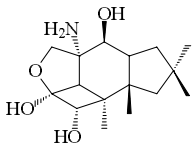

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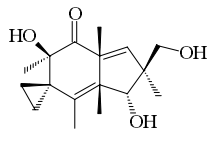

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P158 SYNTHESES AND ENZYMATIC EVALUATION OF SUBSTRATE ANALOGS OF MEDIUM-CHAIN PRENYL DIPHOSPHATE SYNTHASE

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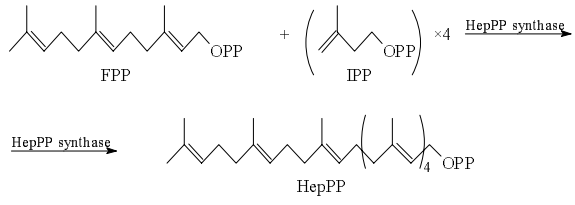
Keywords: farnesyl diphosphate, prenyl diphosphate synthase, inhibitor, heptaprenyl diphosphate, substrate analog

Heptaprenyl diphosphate (HepPP) synthase (EC 2.5.1.30) catalyzes the condensation of isopentenyl diphosphate (IPP) with *(E,E)*-farnesyl diphosphate (FPP) to afford HepPP with chain lengths of C_{35}, which is the precursor of menaquinone-7 (Scheme 1)[1].

HepPP synthase from *Bacillus subtilis* has been shown to be composed of two dissociable components, I and II. Each component has no catalytic activity as the prenyl chain elongating enzyme unless they are combined[2]. There is little knowledge about the substrate recognition site of HepPP synthase. Especially, we are interested in the steric course of the enzyme reaction. So we prepared some substrate analogs (Fig. 1, **1a-1k, 6, 7)** and carried out the enzyme reaction using these analogs. It has been reported that 3-methyl group at allylic substrate is very important for prenyl diphosphate synthase reaction[3]. So we also prepared some 3-desmethyl analogs (Fig. 1, **2-5**) to study the inhibitory effect to the enzyme.



Scheme 1. **HepPP synthase reaction**

We carried out the enzyme reaction using the artificial substrates to investigate the substrate specificity of HepPP synthase from *B. subtilis*. The reaction of IPP with the analogs **1a-j** , which have the various chain lengths of alkyl group at 4-position of DMAPP, showed that the analog **1i** was the most reactive, which has just same chain length as one of FPP, while **1h** or **1j**, the chain length of which is shorter or longer by one methylene than **1i**, respectively, is less reactive than **1i**. These results suggested that the enzyme exactly recognizes the chain length of allylic substrate.

The analogs **2-5** have no methyl group at 3-position of the allylic substrate. These analogs showed no reactivity for the enzyme[3]. On the other hand, these analogs **2** or **4** had inhibitory effect to the enzyme. Especially, the analog **2** showed the stronger inhibitory effect than **4** (Fig. 2). These results suggested that HepPP synthase preferred the *E*-isomer and exactly recognized the stereochemistry of the allylic primer.

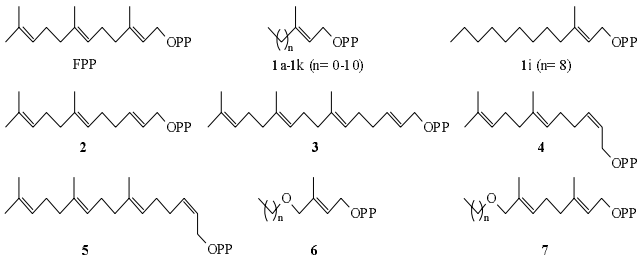
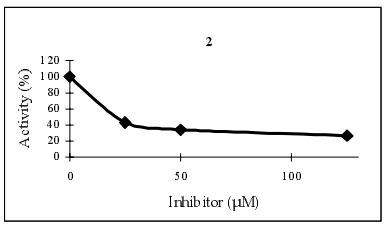


Fig. 1. **Artificial substrate analogs**



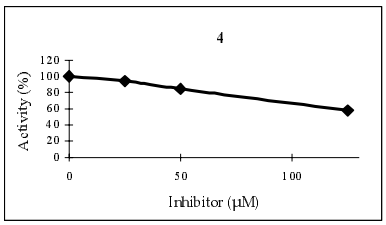


Fig. 2. **Inhibitory effects of 2 and 4 to HepPP synthase**

The analogs **6** (n = 0, 1, 2, 3) or **7** (n = 0, 1, 2, 3) are the DMAPP or GPP analogs having oxygen atom in their prenyl chain, respectively. The analog **6** (n = 0, 1, 2 or 3) can be hardly accepted, but **7** (n = 0, 1, 2 or 3) can be easily accepted by HepPP synthase. These findings are very interesting because FPP synthase from *Bacillus stearothermophillus* hardly accepted all these analogs containing oxygen atom in their prenyl chain, though both synthases belong to the same *E*-type prenyltransferase family.

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P159 SUBSTRATE SPECIFICITIES OF SEVERAL PRENYLCHAIN ELONGATING ENZYMES WITH RESPECT TO 4-METHYL-4-PENTENYL DIPHOSPHATE

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Keywords: substrate specificity, prenylchain elongating enzyme, farnesyl diphosphate synthase, homogeraniol, homolog

Prenyltransferase reaction proceeds with the condensation of an allylic prenyl diphosphate with isopentenyl diphosphate (IPP) stereospecifically and the condensation terminates precisely until the elongation of prenyl chain reaches certain length according to the specificities of individual enzymes.

Ogura *et al.*[1,2] reported that 4-methyl-4-pentenyl diphosphate (homoIPP) was accepted as a homoallylic substrate for a porcine liver farnesyl diphosphate (FPP) synthase, and that the products derived from the reaction of homoIPP with geranyl diphosphate (GPP) or with dimethylallyl diphosphate (DMAPP) were only *Z*-homofarnesyl- or *Z*-homogeranyl diphosphate, respectively.

Recently, antiproliferative terpene derivatives such as *(Z)*-4,8-dimethylnon-3-en-sodium sulfate **(1)** or 3,7,11,15-tetramethylhexadecan-1,19-sodium disulfate **(2)** have been isolated from marine organisms such as sea squirts, Japanese name “hoya” (ref.[3,4]). In order to synthesize several carbon skeleton homologs of compound **1**, we examined the applicability and substrate specificities of some prenylchain elongating enzymes with respect to homoIPP.

As the result, the alcohol derived from the reaction of DMAPP with homoIPP by use of a recombinant *Bacillus stearothermophilus* FPP synthase afforded in higher yields; *Z*-homoGOH (yield: 45.9 %) and *E*-homoGOH (25.5 %) as shown in Fig. 1, comparing with that of *Z*-homoGOH (yield; 1 % or less, no *E*-product) by the porcine liver FPP synthase. Substrate specificities of other types of prenylchain elongating enzymes will also be presented.

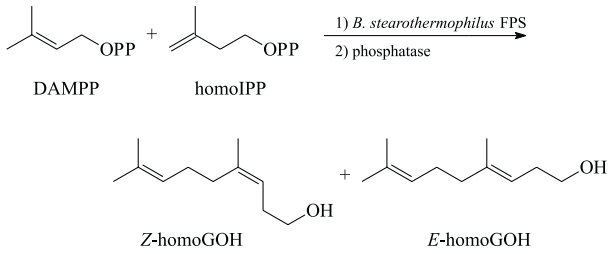

Fig. 1. **Reaction of homoIPP with DMAPP by use of *B. stearothermophilus* FPS.**

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P160 EXTRACELLULAR STEROL OXIDASE OF *Mycobacterium vaccae*

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Keywords: sterol oxidase, *Mycobacterium vaccae*, isolation, purification

Sterol oxidase (SO) catalyzes dehydrogenation of 3-hydroxy steroids at C-3 followed by $\Delta^{5(6)}$ to $\Delta^{4(5)}$ isomerisation, thus modifying 3-hydroxy-5-en to 3-keto-4-en moiety. This conversion is one of key reactions at sterol to C-17-keto-steroid transformation by *Mycobacterium* strains. In spite the reaction is known since 50th, no systematic investigation on the properties of the enzymes from sterol utilizing mycobacteria was published. It was unclear if the same enzyme responsible for both dehydrogenation at C-3 and $\Delta^{5(6)}$ to $\Delta^{4(5)}$ isomerization, as well as if the enzyme represent short-chain dehydrogenase, or mixed function oxidase. No extracellular SO from mycobacteria was reported so far.

In this work, the extracellular sterol oxidase was isolated from cell-free cultivation broth of sterol transforming *Mycobacterium vaccae VKM Ac-1815D*. Along with 3-hydroxysteroid dehydrogenase and $\Delta^{5(6)}$ to $\Delta^{4(5)}$ isomerase activity, 1(2)-dehydrogenase and 17β-dehydrogenase activities were revealed in cell-free cultivation broth. The SO was purified 100-fold using hollow fiber concentration, chromatography on DEAE-Toyopearl, hydroxyapatite Bio-Gel HTP and Bio-Gel A-0.5 M double filtration. In the presence of NADH or NADPH the enzyme expressed 3-hydroxy steroid dehydrogenase, $\Delta^{5(6)}$ to $\Delta^{4(5)}$ isomerase and 6-hydroxylase activities. The enzyme activity of 86.7 $\mu mol.min^{-1}.g^{-1}$ was observed towards dehydroepiandrosterone. Two SO isoforms were revealed by Bio-Gel A-0.5M filtration. The molecular weight of SO by SDS-electrophoresis was determined as 60 ± 4 kDa, K_m for dehydroepiandrosterone averaged 4.1×10^{-4} M.

P161 21-ACETOXY-PREGNA-4(5),9(11),16(17)-TRIENE--21-OL-3,20-DIONE BIOCONVERSION BY *Nocardioides simplex* VKM AC-2033D

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Keywords: steroid, 1(2)-dehydrogenation, deacetylation, 21-acetoxy-pregna-1(2),4(5),9(11),16(17)-tetraene-21-ol--3,20-dione, bioconversion

Microbial 1(2)-dehydrogenation of acetylated 9(11)-dehydrosteroids is of great significance for the synthesis of modern fluoroglucocorticoids[1]. Recently, we studied the metabolic pathways of pregna-4,9(11)-diene-17α,21-diol--3,20-dione 21-acetate and 17,21-diacetate by *Nocardioides simplex* VKM Ac-2033D. As shown, 1(2)-dehydrogenation was accompanied by deacetylation, 20β-reduction and non--enzymatic migration of acyl group from position 17 to 21 (in case of diacetate). The conditions providing predominant accumulation of 1(2),4,9(11)-triene acetates were determined[2].

It was of special interest to find out whether the introduction of 16(17)-double bond in 21-acetylated 4(5),9(11)-diene steroid would influence 1(2)-dehydrogenation and deacetylation by *N. simplex*. In order to clear structure/activity relationship and full biocatalytic potential of this organism in respect to acetylated 9(11)-dehydrosteroids, the conversion of 21--acetoxy-pregna-4(5),9(11),16(17)-triene-21-ol-3,20-dione **(1)** was investigated. The biotransformation of similar steroids has never been studied so far.

21-Acetoxy pregna-1(2),4(5),9(11),16(17)-tetraene-3,20--dione **(2)**, pregna-4(5),9(11),16(17)-triene-21-ol-3,20-dione **(3)** and pregna-1(2),4(5),9(11),16(17)-tetraene-21-ol-3,20--dione **(4)** were revealed as major metabolites at the conversion of **1** by *N. simplex*. The structure of metabolites was confirmed by MS and H^1-NMR. Unlike inducible 1(2)-dehydrogenase, the constitutive esterase activity was shown. The presence of both soluble and membrane associated steroid esterases was shown by cell fractionation experiments. The conditions were found providing full substrate **(1)** conversion with 92 % molar yield of acetylated 1(2)-dehydroanalogue **(2)**.

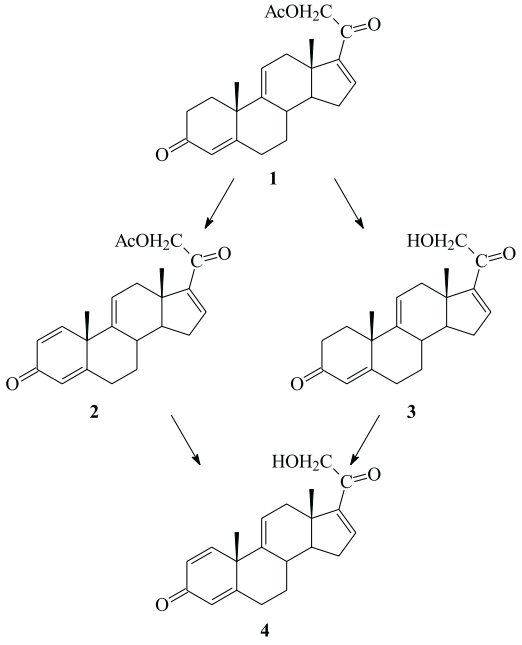

Fig. 1. **Proposed scheme of 21-acetoxy-pregna-4(5),9(11),16(17)--triene-21-ol-3,20-dione (1) bioconversion by *Nocardioides simplex* cells**

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P162 MICROBIAL CONVERSION OF STEROL-ENRICHED FRACTIONS OF SOYBEAN OIL PRODUCTION WASTE BY *Mycobacterium* SP. VKM AC-1817D

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Keywords: biotransformation, soybean extract residue, 9α--hydroxyandrostenedione, sitosterol

Different approaches were undertaken to obtain C_{17}-ketosteroids from industrial wastes of the pulp and paper industry – fractions of tall oil products without isolation and purification of phytosterols. Best results ensued at the conversion of sterol-rich tall-oil unsaponifiable isolates to androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione, with total molar yield of 55 % at substrate loading of 2.5 mM (ref.[1, 2]).

In the present study soybean extract residue (scum), a waste of soybean oil production, was estimated as a raw material for C_{17}-ketosteroid production. As a model process, bioconversion of scum to 9α-hydroxyandrost-4-ene-3,17-dione (9-OH-AD) by *Mycobacterium* sp. VKM Ac-1817D was studied. Scum contained ~14 % sterols comprised mostly by sitosterol, stigmasterol and campesterol.

The bioconversion of scum to 9-OH-AD without intermediate isolation of sterols was characterized by a long-term lag-period followed by drastic increase of 9-OH-AD accumulation. The pre-treatment of scum by either microbial, or chemical single step procedures allowed to reach the productivity comparable with that at the use of high quality tall sitosterol. 9-OH-AD molar yield of about 70 % was obtained at the conversion of scum preparation contained 10 $g.l^{-1}$ sterols. Time courses of bioconversions of scum preparations obtained by mostly effective single or double step pre-treatment techniques are shown on the following Fig. 1.

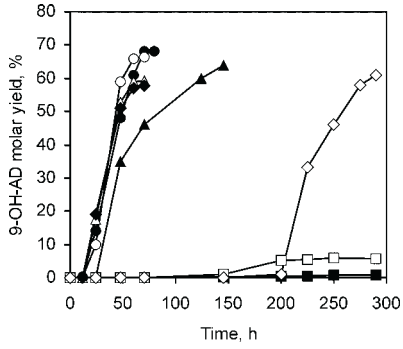

Fig. 1: **Time course of 9-OH-AD at microbial conversions of tall sitosterol (●) and scum preparations obtained by different techniques**; C1 (○), C2 (▲) – crystallisation from ethyl alcohol; (■) F1, (□) F2 – freeze-sedimentation; (◇) H1 - hydrolysis-extraction; F1C1 (◆) – crystallisation of the preparation F1 from ethyl alcohol; C2.1 (△) – re-crystallisation of the preparation C2 from ethyl alcohol

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P163 SITOSTEROL SIDE-CHAIN CLEAVAGE IN AN ORGANIC-AQUEOUS TWO-LIQUID PHASE SYSTEM WITH CHRYSOTILE IMMOBILIZED MYCOBATERIAL CELLS

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Keywords: sitosterol, androstenedione, bioconversion, chrysotile, immobilized cells

The use of organic-aqueous two-liquid phase systems is a well-established approach for the biotransformation of poorly water-soluble compounds. Cell membranes integrity may however be tampered due to the toxic action of the organic phase. Cell immobilization has been shown to reduce the deleterious action of the organic solvent, by providing a protective microenvironment[1]. Chrysotile, an inexpensive mineral material has been successfully used for the immobilization of yeast cells in aqueous-based media[2]. This work aims to evaluate the bioconversion of sitosterol to androstenedione (AD) in a dioctyl phtalate (DOP)/phosphate buffer, using mycobacterial cells immobilized on chrysotile. An increase in final product yield, from 60 % to 90 %, was observed when the immobilized form of the biocatalyst was used, as compared to free cells, in batch bioconversion. The development of a continuous system, based in a CSTR operation mode, was assessed. A flow rate of 0.03 $ml.min^{-1}$ (residence time of 40 h) led to a consistent conversion yield around 50 %, for a substrate concentration of 12 mM, during a one-week operation period. Increasing the flow rate to 0.05 $ml.min^{-1}$ (residence time of 20 h) led to a decrease in the conversion yield to around 25 %, which was nevertheless kept constant for a 30-days working period, suggesting high operational stability. The results obtained with the continuous experimental set-up suggests this approach could be considered for the development of an effective continuous bioconversion system for the production of AD from sitosterol.

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P164 SITOSTEROL SIDE-CHAIN CLEAVAGE BY FREE MYCOBACTERIAL RESTING CELLS IN ORGANIC MEDIA: ASSESSMENT KEY OPERATIONAL PARAMETERS

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Keywords: biotransformation, sterol side-chain cleavage, organic solvent, *Mycobacterium*, resting cells

The selective side-chain cleavage of β-sitosterol by *Mycobacterium* sp. NRRL B-3805 free cells is a well established multi-enzymatic process for the production of pharmaceutical steroid precursors, namely 4-androstene-3,7-dione (AD) and 1,4-androstadiene-3,7-dione (ADD). Since substrate and products are hydrophobic, the use of organic solvents as bioconversion media is one way to overcome low volumetric productivity, intrinsic to conventional aqueous-based biotransformation of such compounds.

In this work, bis(2-ethylhexyl)phthalate (BEHP) was used as reaction medium in a suspended-cell system with low water content as this solvent was previously described as bio-

compatible for this system, allowing high AD and ADD yields[1]. The effect of relevant operational parameters on product yield and reaction rate was evaluated. Namely, variations in the biocatalyst/substrate mass ratio, the amount of water added to the medium and the aeration rate were studied. Off-line monitoring of sitosterol consumption and product formation was performed by HPLC analysis.

Forced aeration of the bioconversion medium favoured product yield. The need to maintain a minimum amount of water in the medium as to retain biocatalytic activity was also evidenced. Increasing the biomass to substrate ratio led to an increase of reaction rate up to saturation level. Product degradation was observed in extented bioconversion runs, suggesting residual activity of sterol ring structure degrading enzymes, and highlighting the need for close monitoring of the bioconversion process so as to avoid such unwanted reactions.

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P165 OPTIMIZATION OF ANDROSTENEDIONE PRODUCTION IN AN ORGANIC-AQUEOUS TWO-LIQUID PHASE SYSTEM

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Keywords: androstenedione, biotransformation, sitosterol side-chain cleavage, *Mycobacterium* sp. NRRL B-3805, organic-aqueous phase system

The use of a biocompatible water-immiscible organic phase as a substrate and product pool has been acknowledged as an effective tool to overcome the low volumetric productivity of aqueous bioconversion systems involving hydrophobic compounds. This approach has been successfully used for the specific side-chain cleavage of sitosterol to androstenedione using *Mycobacterium* sp. NRRL B-3805 cells[1]. Although extensive work has been performed regarding the selection of a biocompatible organic[1–3], few information is available on the effect of aqueous phase composition in the sterol side-chain cleavage activity of mycobaterial cell[2]. This work aims to fill in such gap, through a systematic evaluation of the effect of pH, buffer composition and concentration in catalytic activity. Biocatalytic activity was not significantly affected when buffered solutions with concentration ranging from 20 mM to 100 mM were used. Best results were obtained with phosphate and Tris-HCl buffer solutions, with the highest bioconversion rates being observed in slightly basic bioconversion media (pH within 7.5 to 8). Temperatures in the range 30 °C to 35 °C favored bioconversion rate, whereas low catalytic activity was observed at 20 and 40 °C. Increasing stirring speed up to 500 rpm also favored bioconversion rate. The effect of biomass concentration in product yield is being currently evaluated.

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P166 ENANTIOSELECTIVE SYNTHESIS OF (+) AND (-)-*(Z)*-7,15-HEXADECADIEN-4-OLIDE, THE SEX PHEROMONE OF THE YELLOWISH ELONGATE CHAFER, *Heptophilla picea*

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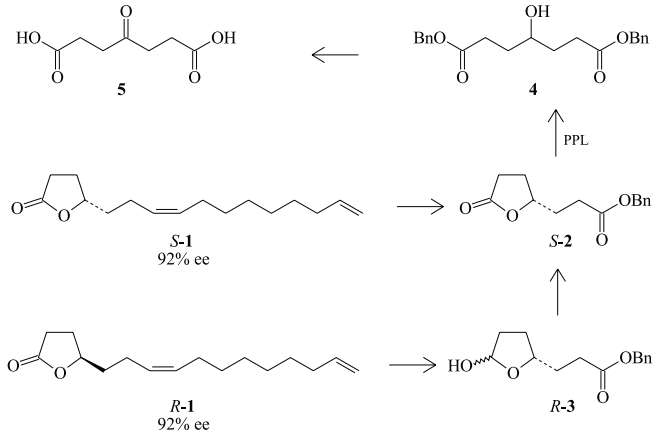
Keywords: biocatalysis, lipase, butyrolactone, pheromone, *Heptophilla picea*

Optically active γ-butyrolactones are valuable building blocks in organic synthesis and find application as synthons in natural products synthesis[1]. Isolated enzymes, such as lipases, have been exploited for preparing optically active lactones[2]. In 1996 Leal et *al.*[3] isolated and identified (4*R*,7*Z*)-7,15-hexadecadien-4-olide **(1)** as a the female-produced sex pheromone of yellowish elongate chafer *(Heptophilla picea)*, which is an agriculture pest that causes losses in tea and flower production in Japan. Compound *R*-**1** was prepared from L-malic acid in 14 steps and the synthetic compound showed spectroscopic data and biological activity identical to those of the natural material[4]. In this communication we report a short and versatile enantioselective synthesis of both enantiomers of **1** using a lipase-catalyzed lactonization in the key step. Scheme 1 shows the retrosynthetic analysis for *R* and *S (Z)*-7,15-hexadecadien-4-olide:



Scheme 1: **Retrosynthetic analysis for *R* and *S* (Z)-7,15-hexadecadien-4-olide**

Both enantiomers were prepared starting from the commercially avaiable 4-ketopimelic acid **(5)**. The enantioselective reactions were performed with porcine pancreatic lipase (PPL) in diethyl ether, and after few synthetic steps, both were prepared in good enantiomeric excess. In conclusion, we have described a convenient and useful enantioselective synthesis of *R* and *S-(Z)*-7,15-hexadecadien-4-olide through lipase-catalyzed enantioselective lactonization. In addition, this fact shows that compound *S*-**3** is a versatile building block for the synthesis of naturally occurring γ-butyrolactones.

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P167 ENZYMATIC BAEYER-VILLIGER OXIDATION OF NEW 2- AND 3-SUBSTITUTED CYCLOHEXANONES

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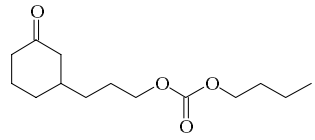
Keywords: Baeyer-Villiger oxidation, cyclohexanone monooxygenase, formate dehydrogenase, hydroxypropylcyclohexanones

Baeyer-Villiger oxidations of alkyl-substituted cyclohexanones have been investigated extensively using both whole cells and isolated cyclohexanone monooxygenase from *Acinetobacter* NCIMB 9871 (ref.[1, 2]) or a recombinant baker's yeast[3] and a recombinant *Escherichia coli* strain[4], respectively. Here we present new examples of the conversion of 2- and 3-substituted cyclohexanones which possess functional groups in the side chain by the system cyclohexanone monooxygenase (CHMO)/formate dehydrogenase (FDH)[5, 6].

2- and 3-hydroxypropylcyclohexanone were oxidised with this enzyme system successfully. These ketones were esterified with several dicarboxylic acids with the purpose to find out a suitable linker system for binding of substituted cyclohexanones to a resin. In kinetic assays could be shown that most of such esters, like e. g. compound **1**, were accepted as substrates of the cyclohexanone monooxygenase.



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P168 ENANTIODIVERGENT BAEYER-VILLIGER OXIDATION OF FUNCTIONALIZED PROCHIRAL CYCLOHEXANONE DERIVATIVES UTILIZING RECOMBINANT CELLS

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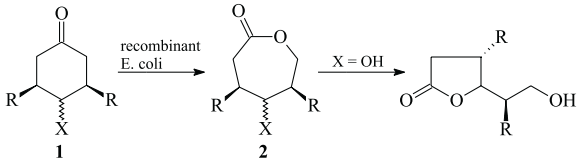
Keywords: Baeyer-Villiger oxidation, lactone, enantioselectivity, protein sequence analysis

Beside the enantioselective organo-metal catalyzed Baeyer-Villiger oxidation[1] the microbial Baeyer-Villiger oxidation[2] has become a powerful tool to synthesize asymmetric lactones as interesting intermediates in organic chemistry and frequently encountered precursors in enantioselective synthesis. The importance of enantioselective microbial reactions has grown in recent years due to an increased need for "green chemistry" approaches in industrial synthesis. Especially the field of chiral Baeyer-Villiger oxidations is one of the representative domains for biocatalysis.

In this study we present whole-cell mediated Baeyer-Villiger reactions on preparative scale using recombinant organisms as facile tools for organic chemists. Four expression systems for flavin dependent monooxygenases from *Acinetobacter* sp., *Comamonas* sp., and *Brevibacterium* sp. (I + II) were investigated for their substrate acceptance on prochiral 3,4,5 functionalized carbocyclic ketones **1**.



X = H, CH_3, $=CH_2$, *cis*-OH
trans-OH, *cis*-Cl, *trans*-Cl
R = H, CH_3

In this poster we present the results of the microbial Baeyer-Villiger oxidation of compounds **1**, which lead to lactones **2** in high optical purities and potential enantiodivergence. The influence of substrate polarity and sterical aspects of substituents R and X will be discussed in detail. The enantioselectivity of the enzymatic transformation, will be compared with protein sequence analysis of all four different expression systems.

A diastereoselective synthetic route to compounds **1** will be outlined together with potential applications of product lactones **2** in natural product and bioactive compound synthesis.

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P169 MICROBIAL BAEYER-VILLIGER OXIDATION OF PROCHIRAL TETRAHYDROPYRANONS USING RECOMBINANT WHOLE-CELLS

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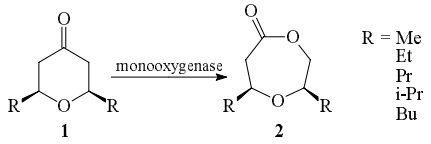
Keywords: Baeyer-Villigerase, heterocyclic ketone, stereoselectivity

Baeyer-Villigerases have been proven to be a versatile tool for the conversion of cyclic ketones to lactones. A key feature beside the regio- and chemoselectivity is the possibility of introducing chirality on a large number of non-natural substrates[1].

Based on our previous reports on recombinant whole cell mediated oxidations[2] of carbocyclic prochiral ketones[3], we are currently expanding our substrate profiling on heterocyclic substrates[4].



In this contribution we discuss our latest results on the conversion of heterocyclic ketones of type **1** to the corresponding lactones **2**. A detailed discussion of the synthetic approach towards the required tetrahydropyranone substrates will be presented. The stereoselectivity of the microbial transformation will be studied together with a survey of spatial requirements of the active site of the enzymes investigated based on substrate acceptance.

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P170 MICROBIAL BAEYER-VILLIGER OXIDATION: SYNTHESIS OF OPTICALLY ACTIVE GEISSMAN-WAISS LACTONE

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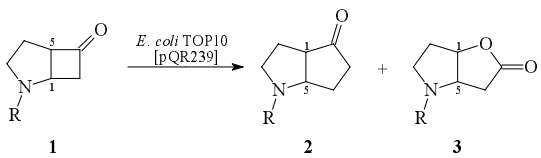
Keywords: whole cells biotransformation, asymmetric Baeyer-Villiger oxidation, Geissman-Waiss lactone

Pyrrolizidine alkaloids (PAs) are natural products containing an azabicyclic[3.3.0]heptane structural core. These compounds have a wide range of biological and pharmacological activity with therapeutic potential[1].

In the course of our studies on microbial Baeyer Villiger oxidation, we decided to investigate the biotransformation of the protected 2-azabicyclo[3.2.0]heptan-6-one **1**. It could be a direct route to obtain the Geissman-Waiss lactone (2-oxa-6-azabicyclo[3.3.0]octan-3-one) **3**, an important intermediate for preparing PAs necine base family[2] Baeyer-Villigerases (enzymes catalysing BV oxidation), in particular the cyclohexanone monooxygenase (CHMO), exhibit a broad substrate versatility[3], but only few examples of containing nitrogen compounds were described until now[4]. Following a methodology developed in our laboratory, we used a whole cell process involving recombinant *Escherichia coli*[5] as well as wild type strains.



1 **2** **3**

R = COOBn

Similarly to the well known bicyclo[3.2.0]hept-2-en-6-one[6] biotransformation, ketone **1** was oxidized to a mixture of the regioisomeric and optically active lactones **2** and **3**, the relative proportion of these lactones depends on the substrate and the microorganism employed.

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P171 MICROBIAL BAEYER-VILLIGER OXIDATION: A DYNAMIC KINETIC RESOLUTION USING A HETEROGENEOUS RACEMISATION CATALYST

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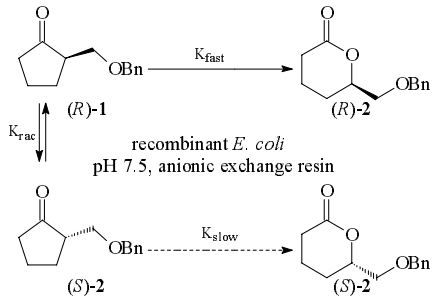
Keywords: dynamic resolution, whole cell biotransformation, asymmetric Baeyer-Villiger oxidation, heterogeneous catalysis, exchange resins

The conventional kinetic resolution is a powerful tool in asymmetric organic synthesis allowing to prepare enantiopure compounds starting from racemic substrates. However, the maximum theoretical yield of such process is intrinsically limited to 50 %. One way to circumvent this problem is the set up of a dynamic kinetic resolution process, which theoretically permits to reach a 100 % chemical yield as well as a 100 % ee starting from a racemate[1].

The Baeyer-Villiger (BV) oxidation of ketones is an important and interesting reaction because of its large number of applications. Asymmetric BV oxidation the ketones using organometallic reagents have only recently been described with moderate success[2]. On the contrary, the utilisation of enzymes (BV monooxygenases) offers efficient access to enantiomerically pure lactones and is considered an interesting "green chemistry" alternative to conventional catalysis[3].

We have recently described the first example of a dynamic kinetic resolution process applied to the microbiological Baeyer-Villiger (BV) oxidation of 2-benzyloxymethylcyclopentanone **(1)**. In this case, the racemisation process was achieved by a basic-catalysed method, at pH 9 (ref.[4]). However, only 0.3 $g.l^{-1}$ of ketone **1** could be transformed because of the extreme biotransformation conditions. In order to improve this result, we explored the possible racemisation of

1 using a procedure based on the application of heterogeneous catalysts. Using this approach, the (bio)oxidation of racemic ketone **1** by *E. coli* TOP10 [pQR239] (at higher concentration) afforded nearly enantiopure (*R*)-6-benzyloxymethyl-tetrahydropyran-2-one **(2)** in a yield of about 84 %.



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P172 MICROBIAL BAEYER-VILLIGER OXIDATION: A PROCESS CONCEPT COMBINING BIOTRANSFORMATION AND SOLID PHASE EXTRACTION IN A NOVEL WAY

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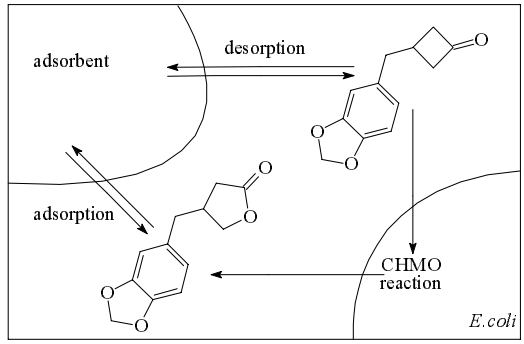
Keywords: asymmetric Baeyer-Villiger oxidation, adsorbent, whole cell biotransformation, preparative scale

During the last decades, enzymatic Baeyer-Villiger oxidation proved to be a successful tool in asymmetric synthesis which is well-known for its excellent enantioselectivities. A number of interesting synthetic applications has been published including the oxidation of bicyclic ketones and prochiral 3-substituted cyclobutanones[1].

The use of whole cells, avoiding the cofactor recycling necessity (the so-called “Baeyer-Villigerases” mostly belong to an NADPH-dependent flavoenzyme family), is the method of choice for preparative scale synthesis[2,3]. However, up to now, the use of this approach at a larger scale is hampered by the necessity to work at low substrate concentration, primarily due to inhibition phenomena or low solubilities of the organic substrate and product compounds.



Scheme 1. **Two-in-one concept of substrate feeding and product recovery**

The present work demonstrates on two substrates of the above mentioned families – namely bicyclo[3.2.0]hept-2-en-6--one and 3-piperonylcyclobutanone – how we have overcome this limitation by integrating solid phase extraction techniques into the process[4]. Ketone is loaded onto an adsorbent carrier material which is able to release it into the liquid phase in a way matching the needs of the cells during biotransformation. Likewise, the formed lactone is readsorbed onto the carrier to keep product concentration low. This “two-in-one” concept permits not only simple downstreaming processing but also intensified exploitation of biocatalyst productivity (Scheme 1).

We used whole cells of *E. coli* TOP10 [pQR239] into which had been cloned and overexpressed the cyclohexanone monooxygenase from *Acinetobacter calcoaceticus* NCIMB 9871 (ref.[5,6]). A wide range of adsorbent materials has been tested on the two substrates which differ in their adsorption/desorption behaviour. Biotransformations have been carried out in a laboratory scale reactor set up for this purpose and demonstrate the increase in productivity for biocatalytic Baeyer-Villiger oxidation on larger scale and for nearly enantiopure products.

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P173 MICROBIAL BAEYER-VILLIGER OXIDATION OF BICYCLO[4.3.0]- AND BICYCLO[3.3.0] KETONES USING RECOMBINANT WHOLE-CELLS

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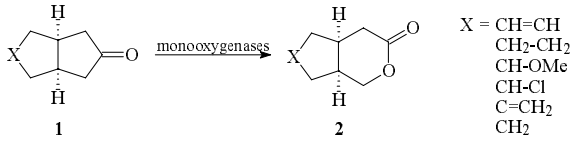
Keywords: biocatalysis, Baeyer-Villiger oxidation, monooxygenase, enatioselective synthesis, enantiodivergence

Over the last few years Baeyer-Villigerases have gathered increased attention for their ability to convert a large number of cyclic ketones in a regio- and chemoselective manner. But the most interesting feature for a synthetic chemist is the possibility to introduce chirality. The chiral lactones obtained, represent useful intermediates for the synthesis of natural products[1].

Based on our previous reports on recombinant whole cell mediated oxidations[2], we were interested in the prepartion of chiral lactones of type **2**.



1 **2**

In this contribution we discuss our results for the conversion of bicyclic ketones of type **1**. Both 5- and 6-membered fused carbocycles represent substrates for *Cyclohexanone monooxygenase* (EC 1.14.13.22)[3] and *Cyclopentanone monooxygenase* (EC 1.14.13.16)[4]. The stereoselectivity of the microbial transformation will be studied together with a survey of spatial requirements of the active site of the enzymes investigated based on substrate acceptance. This enables a novel approach to yohimbine-type alkaloids as potential α_2-adrenoceptor antagonists. An improved synthetic procedure to the corresponding ketones **1** will be presented together with conditions for the whole-cell biotransformation.

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P174 ENGINEERING A NADH-SPECIFIC BAEYER-VILLIGER MONOOXYGENASE

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Keywords: Baeyer-Villiger monooxygenase, coenzyme specificity, NAD(P)H, flavin, sulfoxidation

Baeyer-Villiger monooxygenases catalyse both nucleophilic and electrophilic oxygenation reactions. Both types of reactions can proceed with exquisite selectivity indicating that these enzymes can be of great value for the synthesis of interesting fine chemicals. So far, only a limited number of Baeyer-Villiger monooxygenases have been identified from bacteria and fungi. Recently, we have cloned the gene encoding 4-hydroxyacetophenone monooxygenase (HAPMO) from *Pseudomonas fluorescens* ACB into *E. coli*[1]. The enzyme efficiently catalyses Baeyer-Villiger oxygenation reactions of a variety of ketones and aldehydes (Fig. 1)[2]. Except for converting carbonyl compounds, HAPMO also efficiently catalyses enantioselective sulfoxidation reactions

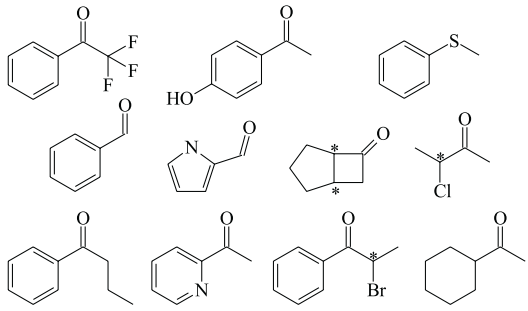

Fig. 1. **Several HAPMO substrates**

HAPMO is member of a recently recognised sequence--related flavin-dependent monooxygenase enzyme family[3]. As has been observed for all other family members, HAPMO is highly specific for NADPH. Since a switch in coenzyme specificity towards NADH would be beneficial for biocatalytic applications, we have started a study that aims at identifying the residues that modulate the coenzyme specificity. Alignment of the HAPMO sequence with sequences from characterised Baeyer-Villiger monooxygenases revealed several conserved arginine and lysine residues. It is known that arginines and lysines are often involved in binding the 2'-phosphate moiety of NADPH. Therefore, we probed the function of these conserved basic residues by site-directed and random-mutagenesis. By this, we have been able to engineer a HAPMO variant which displays a significantly increased activity with NADH. By a similar approach, we could also alter the coenzyme specificity of another sequence related Baeyer-Villiger monooxygenase; cyclohexanone monooxygenase. In conclusion, we have identified several residues that determine the coenzyme specificity of Baeyer-Villiger monooxygenases. However, to switch the coenzyme specificity of BVMOs from NADPH to NADH in terms of catalytic efficiency, more residues need to be mutated.

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P175 DIRECTED EVOLUTION OF ENANTIOSELECTIVITY OF CYCLOHEXANONE – MONOOXYGENASE

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Keywords: cyclohexanone-monooxygenase, Baeyer-Villiger, directed evolution

Cyclohexanone – monooxygenase (CHMO) from *Acinetobacter* sp. *9871*, which is able to catalyse reactions of the Baeyer-Villiger-type and the oxidation of heteroatoms, has been overexpressed in *E. coli* as host organism. CHMO has been successfully applied in asymmetric synthesis and in kinetic resolutions[1]. Many substituted cyclic ketones have been converted into the corresponding lactones in high enantiomeric excess.

Directed evolution is a powerful tool to enhance enantioselectivity for those substrates in which the wild type enzyme gives only low *ee*'s (ref.[2]). 4-Hydroxycyclohexanone has been chosen as substrate for the directed evolution of enantioselectivity since it shows an *ee* of only 9.6 % with the CHMO – wild type.

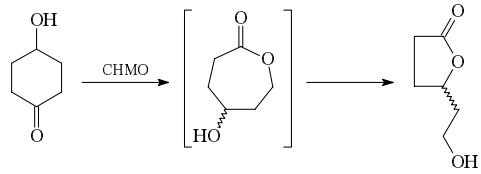

Fig. 1. **Baeyer-Villiger oxidation of 4-hydroxycyclohexanone**

GC screening[3] enables us to screen up to 500 mutants per day. In the first round of mutagenesis, using error prone PCR, mutants with enhanced and reversed enantioselectivity have been detected.

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P176 BIOHYDROXYLATIONS OF KETONES AND ALDEHYDES WITH *Sphingomonas* SP. HXN-200 USING THE DOCKING/PROTECTING GROUP CONCEPT

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Keywords: biohydroxylation, *Sphingomonas* sp. HXN-200, docking/protecting group concept

Sphingomonas sp. HXN-200 has been found to be a very potent and easy to use hydroxylating microorganism[1–5]. In combination with the docking and protecting group concept, which was developed to ease the biohydroxylation of compounds containing nonactivated carbon atoms[6,7], the hydroxylation of a number of substance classes such as amines[1,3,4], amides[2,5], and carboxylic acids[8] could be achieved with this microorganism. In a few cases it was also possible to biohydroxylate ketones and aldehydes which were protected as *N*-benzoylated spirooxazolidines and oxazolidines. However, with most of the tested substrates, no or very little product formation was observed. For this reason, more appropriate docking/protecting groups are required for these substance classes. Indeed, the nature of the docking/protecting group has been found to have a significant influence[5] on hydroxylation regio- and stereoselectivity in other substrates, this adding impetus to our investigations.

Results will be disclosed in detail in this presentation.

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P177 INVESTIGATIONS INTO THE BIOHYDROXYLATION OF CHIRAL ALCOHOLS EMPLOYING THE DOCKING/PROTECTING GROUP CONCEPT

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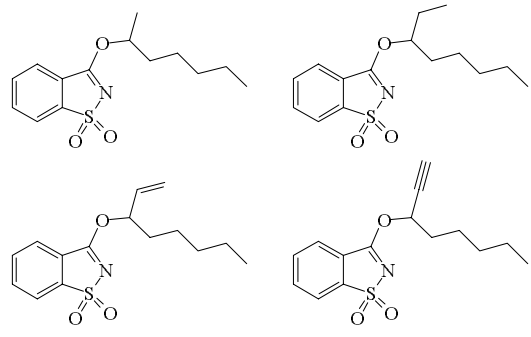
Keywords: biohydroxylation, docking/protecting group concept, alcohols

The development of the docking/protecting group concept has proved to be very useful for the biohydroxylation of nonactivated carbon atoms belonging to a variety of substance classes such as alcohols, aldehydes, ketones and carboxylic acids[1–3].

This work focuses on the biohydroxylation of open-chain alcohols protected as isosaccharine derivatives (Figure). On the basis of preliminary screening results with a range of bacteria and fungi, the most promising biohydroxylating microorganisms for each examined compound were selected, and the best biotransformations repeated on a preparative scale.



The results of these biotransformation experiments will be disclosed in more detail in this presentation.

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P178 EFFICIENT ENANTIOSELECTIVE BIO-OXIDATION OF *sec*-ALCOHOLS

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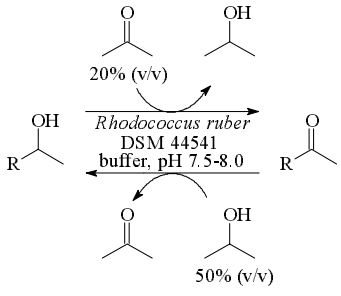
Keywords: alcohol dehydrogenases, *sec*-alcohol, Oppenauer--oxidation

In general, asymmetric biocatalytic hydrogen-transfer is based on alcohol dehydrogenases. By comparison of the biocatalytic approach with the conventional Oppenauer-oxidation and transition-metal catalysed hydrogen-transfer, the advantages of biocatalysis are the intrinsic asymmetry of enzymes[1], absence of side reactions[2] and essentially mild reactions conditions[3].

Whole resting cells of *Rhodococcus ruber* DSM 44541 are able to oxidise *sec*-alcohols at the expense of a sacrificial ketone (e. g. acetone) as hydrogen-acceptor. The process could be easily switched into the reverse mode to reduce ketones to *sec*-alcohols by replacing the auxiliary ketone with 2-propanol as hydrogen-donor.

The microorganism prefers to oxidise medium-chain alcohols and reduce ketones with the functional group in (ω-1)-position.



The substance tolerance is rather broad and encompasses not only simple *sec*-*n*-alkanols, but also a variety of substrates bearing alicyclic or aromatic functional groups. This poster focuses especially on diols in various positions of the substrate. Best results were obtained when one alcohol moiety is located in the (ω-1)-position.

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P179 BIOHYDROXYLATIONS OF BICYCLIC SATURATED γ-LACTONES WITH THE SUBSTITUTED CYCLOHEXANE SYSTEM

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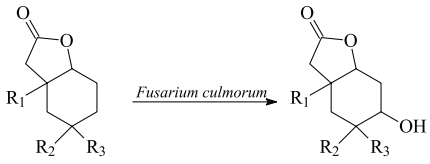
Keywords: lactones, biotransformation, hydroxylation, *Fusarium culmorum*

Many antifeedants with the lactone moiety isolated from the natural sources possess additional functional group, mainly hydroxy or acetoxy. Because of our interest in synthesis of structural analogues of natural antifeedants we have been studing methods for an efficient and regiospecific hydroxylation of lactones.One of the most used and attractive methods of introduction of such group in the unactivated position of molecule is a microbial oxidation of C-H bond to the alcohol catalysed by cytochrome P450 monooxygenases[1, 2].

On the basis of earlier studies with biohydroxylations of γ-lactones[2], we applied some fungi to biotransformate saturated γ-lactones with cyclohexane ring substituted with various number of methyl groups **(1 a, b)**.

The most effective transformation took place when different species *Fusarium culmorum* were used. The fungi were cultivated at 25 °C in Erlenmayer flasks, after 5 days the substrates dissolved in acetone were added to the grown cultures. The biotransformations were being continued for 14 days. The products were extracted with ethyl ether and analysed by GC and TLC. Then they were isolated and purified by column chromatography. Their structures were established on the basis of ^{1}H NMR and IR data.



1a,b **2a,b**

a: R_2,R_3 = CH_3; R_1 = H
b: R_1,R_2,R_3 = CH_3

The products of biotransformation of γ-lactone **1 a, b** were identified as the hydroxylactones **2 a, b** respectively, with the hydroxy group located at the C-3 in equatorial position.

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P180 SOLVENTLESS YEAST MEDIATED REACTIONS

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Keywords: yeast, solventless, reduction, stereoselective

Yeast is capable of catalysing a wide range of reactions in a highly stereoselective manner[1]. The almost mandatory use of aqueous reaction media for this reaction causes problems associated with substrate solubility and product isolation. The use of an organic solvent for yeast mediated reactions greatly simplifies the isolation process and generally leads to higher isolated yields and enantioselectivity. For example, the yeast mediated reduction of β-keto esters in petroleum ether proceeds with isolated yields of up to 96 % and ee values of >99 % (ref.[2]).

We have now discovered that yeast reactions take place very efficiently if the substrate is simply added to moistened yeast. This is effectively a solventless reaction since only 0.8 ml water/g yeast is required, which is just sufficient to "wet" the yeast but not sufficient to form a distinct water layer. This reaction system is more reactive towards substrates than either a water or an organic solvent based system.

Using this system we have been able to reduce β-keto esters, β-keto amides, nitrostyrenes and enol ethers with high enantioselectivity and good isolated yield. Generally the isolated yields are better than those obtained using other yeast reaction systems.

The scope of this new reaction system along with the various factors (water content, temperature, substrate concentration, etc.) which influence the reaction outcome will be discussed.

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P181 ENANTIOSELECTIVE ENZYMIC REDUCTION OF A PROCHIRAL CYCLIC KETONE USING YEASTS

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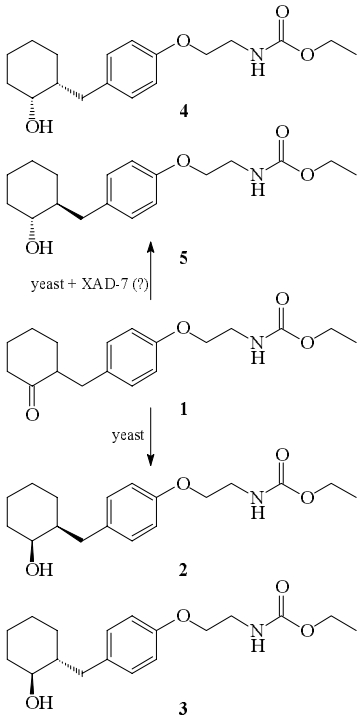
Keywords: *Geotrichum candidum*, *Saccharomyces cerevisiae*, ketone, bioreduction, polymer

Various synthetic tricks have been applied to stimulate microorganisms in producing opposite enantiomers of target structures under modified conditions[1]. One of the most often used ways is to introduce an auxiliary substituent, assisting in changing bulkiness of substituents at the stereogenic center, and, consequently, stereopreference during enzymic reduction. The auxiliary substituent can easily be removed or transformed once the new chiral center is contructed and the new product is obtained. This approach is usually applicable in the synthesis of chiral synthons or intermediates, however, can be hardly applied, when chirality is introduced into prochiral molecule in the final synthetic step. In such cases, change of environment (conditions or medium used) of the enzymic procedure has been studied. One of such approaches is to apply different immobilization factor to the microorganism, which may result in changing stereospecific ability of enzyme mediating the target process.

A hydrophobic polymer (Amberlite XAD-7)[2] has been used as auxiliary additive in the final step of the synthesis of enantiomerically pure stereoisomers **2** and **3** of ethyl *N*-{2-{4-[(2-hydroxycyclohexyl)methyl]phenoxy}ethyl}carbamate, an insect pest management agent, accessible from the parent ethyl *N*-{2-{4-[(2-oxocyclohexyl)methyl]phenoxy}ethyl}-carbamate **(1)**. In the former modification, absence of the polymer results in expected product bearing *(S)* absolute configuration at the C(2)-OH rising stereocenter. In the latter modification, addition of the polymer to the mixture is expected to immobilize yeast cell culture *in situ*, and to enable enzymic reaction to produce opposite stereoisomers **4** and **5** of the products in comparison with the former process. Se-

veral strains of the yeasts *Geotrichum candidum* and *Saccharomyces cerevisiae* have been subjected to this study.



This research was supported by the grants GA AVČR S4055104 and GAČR 203/02/0166.

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P182 BAKER'S YEAST MEDIATED ASYMMETRIC REDUCTION OF CINNAMALDEHYDE DERIVATIVES

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Keywords: asymmetric reduction, cinnamaldehydes, baker's yeast

The enantioselective reduction of cinnamaldehyde derivatives is an attractive strategy to prepare various optically active multifunctional molecules that can be used as chiral building blocks for the synthesis of some HIV-protease inhibitors[1]. The asymmetric reduction of cinnamaldehydes **1a-c** mediated by baker's yeast *(Saccharomyces cerevisiae)* furnished alcohols **2a** and **2b** in excellent enantiomeric excesses and yields (Table I).

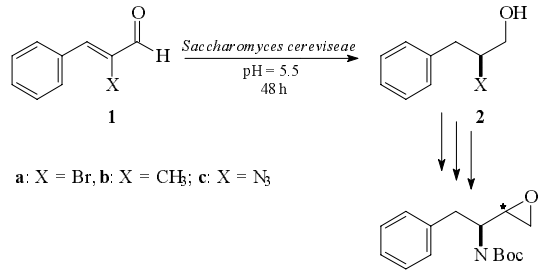

Table I
Reduction of cinnamaldeydes **1a–c** by baker's yeast[a]

Aldehyde	Alcohol	Yield (%)	$[\alpha]_D^{20}$	e. e.[c] (%)	Configuration
1a	**2a**	98	–22.5 (*c* 5, $CHCl_3$)[b]	>99	(*S*)
1b	**2b**	99	–11.0 (*c* 4.6, benzene)[b]	>99	(*S*)
1c	**2c**	98	–7.6 (*c* 0.75, $CHCl_3$)[b]	–	–

[a]T = 30 °C, 48 h, pH = 5.5, 4.7 mmol/25g (aldehyde/baker's yeast), 10.4 g of glucose, 250 ml H_2O (2.0 ml of EtOH); [b]Values of *c* (g /100 ml); [c]Determined by GC-MS analysis (capillary chiral column CHIRASIL-DEX)

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P183 ENANTIOSELECTIVE OXIDATION AND REDUCTION OF ACYCLIC COMPOUNDS BY A YEAST

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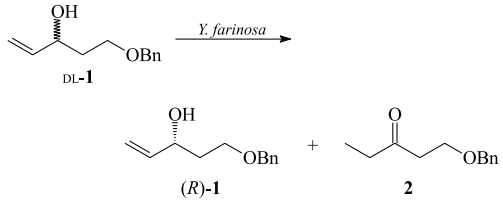
Keywords: yeast, oxidation, reduction, acyclic alcohols, acyclic ketones

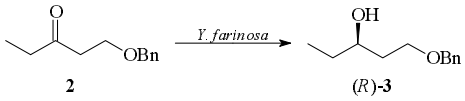
Optically active acyclic alcohols are very useful in synthesis of natural products. We have reported that *Yamadazyma*

farinosa IFO10896, a yeast, catalyzes the enantioselective oxidation of cyclic allyl alcohols to afford the optically active form[1]. Herein, we will report the enantioselective oxidation and reduction of acyclic compounds by the yeast.

First, the oxidation of an acyclic allyl alcohol DL-**1** was examined. As expected, the reaction proceeded with high enantioselectivity as well as that of cyclic compounds to give optically pure *(R)*-**1**. Interestingly, the microbial reduction of C = C double bond of the corresponding ketone also occurred in the reaction system to afford the saturated ketone **2**.



Second, we tried the reduction of the resulting **2** using *Y. farinosa*. While the reduction under aerobic conditions proceeded without enantioselectivity, asymmetric reduction of **2** occurred under anaerobic conditions to afford *(R)*-**3** with high ee.



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P184 ASYMMETRIC REDUCTION OF KETONES BY PHOTOSYNTHETIC ORGANISMS

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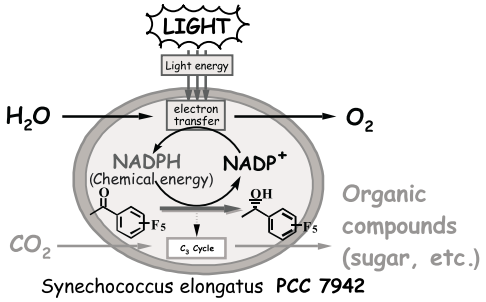
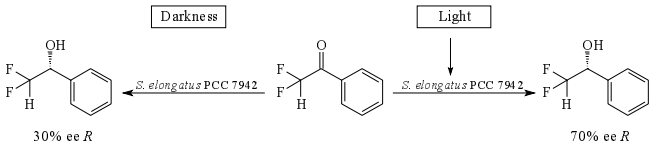
Keywords: photosynthetic organisms, asymmetric reduction, ketone, light-mediated, light-controlled

Biocatalytic reduction is a useful tool for obtaining optically active alcohols, and many scientists have studied reactions using isolated enzymes, microbes and plant cell cultures as biocatalysts. Reduction of substrates usually requires a large input of energy, and in microbial reductions, carbohydrates such as sugars have been used to recycle the coenzyme. These carbohydrates are generated through photosynthesis with sunlight energy. In other words, we have been indirectly using light energy for asymmetric reduction[1–3].

Now we propose the direct use of light energy for such reactions by using biocatalyst that fall into a new category, the photosynthetic organisms, because they can directly use light energy. Reduction of artificial ketones such as acetophenone derivatives by *Synechococcus elongatus* PCC 7942 proceeds smoothly by the aid of light. The efficiency of the reaction is very high since the coenzyme, NADPH is regenerated by using light energy.



In the reduction by *S. elongatus* PCC 7942, the stereochemical course of asymmetric reduction of ketones is largely regulated by light. Thus, we find that enantioselectivity in the reduction of α,α-difluoroacetophenone by *S. elongatus* PCC 7942 increases as a result of illumination by fluorescent light. Furthermore, DCMU, an inhibitor of photosynthesis affects to the stereoselectivity under illumination, and decreases the enantioselectivity.



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P185 ASYMMETRIC REDUCTION BY *Geotrichum candidum* IN SUPERCRITICAL CARBON DIOXIDE USING SEMI-CONTINUOUS FLOW REACTOR

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Keywords: supercritical carbon dioxide, alcohol dehydrogenase, *Geotrichum candidum*, asymmetric reduction, chiral alcohols

Supercritical carbon dioxide (scCO2) has been used as a solvent for organic synthesis, extraction and chromatography due to it's environmentally benign nature, unique characteristics and high functionalities. The use of biocatalysts in scCO2 for organic synthesis has the additional attraction of combining a natural catalyst with a natural solvent. Moreover, if flow scCO2 reactors that discharge products and CO2 from a column packed with biocatalysts are used, the problem of product extraction, which obstructs the practical use of enzymes in an aqueous solvent for organic synthesis, can be solved because CO2 can be removed easily, whereas, with batch reactors, the separation of biocatalysts from the products is necessary after depressurization.

Although flow scCO2 systems using hydrolytic enzymes have been developed, no study has been conducted using dehydrogenases[1]. We used the resting cell of *Geotrichum candidum* as a catalyst for the reduction of ketones in a semi-continuous flow process using scCO2 for the first time. The reduction of cyclohexanone was successful, and the biocatalyst could be used repeatedly. It was also suitable for the asymmetric reduction of *o*-fluoroacetophenone, and resulted in excellent enantioselectivity (ee >99 %) and higher productivity than that of the corresponding batch process.

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P186 STUDY OF SUBSTRATE SPECIFICITY ON THE REDUCTION OF BICYCLIC DIKETONES WITH A YEAST STRAIN, *Torulaspora delbrueckii* IFO10921

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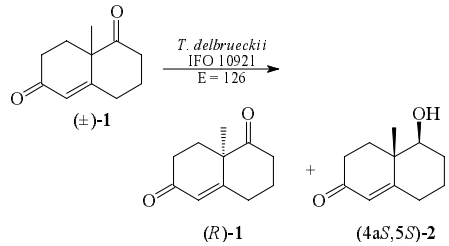
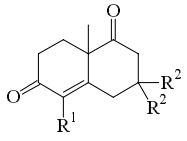
Keywords: kinetic resolution, yeast-mediated reduction, substrate specificity, whole cell biocatalyst

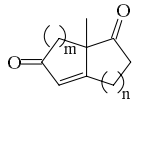
Both enantiomers of Wieland-Miescher ketone **1** in a highly enantiomerically enriched form became readily available by a newly-developed kinetic resolution with yeast--mediated reduction[1]. From a screening of yeast strains, *Torulaspora delbrueckii* IFO 10921 was selected. The collected stationary-phase cells of this strain, obtained by an incubation in a glucose medium, smoothly reduced only the isolated carbonyl group of the *(S)*-enantiomer, while the *(R)*-enantiomer remained intact (*E*: 126). Starting from both enantiomers (*ca.* 70 % ee) prepared by an established proline-mediated asymmetric Robinson annulation, the reduction with *T. delbrueckii* gave the *(R)*-enantiomer (98 % ee) and the corresponding alcohol **2** (94 % ee, 94 % de) in preparative scale in nearly quantitative yields.



Substrate specificity on the related compounds was further studied. Compared with a high level of recognition for Wieland-Miescher ketone **1**, the introduction of substituents on the octahydronaphthalene skeleton as well as the structural change into an octahydroindene skeleton retarded the enzymatic reduction and the enantiomeric ratio fell in 5–16. Further structural variation into a bicyclo[3.3.0] skeleton led to an exclusive 1,4-conjugate reduction of the α,β-unsaturated carbonyl group, and the above results suggested the participation of plural oxidoreductive enzymes in the whole cell.



3 (R^1 = Me, R^2 = H)
4 (R^1 = H, R^2 = Me)



5 (m = 2, n = 1)
6 (m = 1, n = 2)
7 (m = 1, n = 1)

An air-dried preparation of these cells was very effective both in terms of activitiy and enantioselectivity, with a high retention of a co-factor regeneration system. The *E* value (123) was nearly equal with that of the freshly harvested cells (126). Moreover, these air-dried cells showed a long-term stability. The activity was almost same, even after storage in a refrigerator (4 °C) for sixty days. In this way, by virtue of preservable whole cells of this yeast strain, the applicability of this yeast-mediated reduction in synthetic organic chemistry was greatly increased.

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P187 APPLICATION OF *Torulaspora delbrueckii*--MEDIATED REDUCTION IN NATURAL PRODUCT SYNTHESIS

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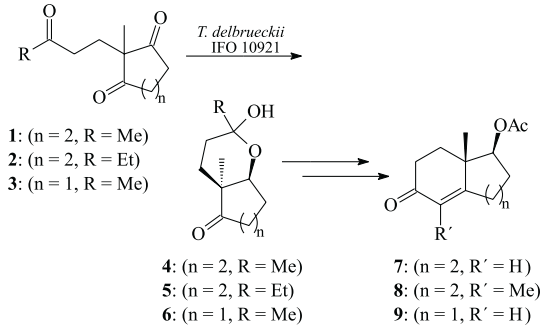
Keywords: asymmetric reduction, yeast-mediated reduction, substrate specificity, quaternary asymmetric center, radical--mediated β-fragmentation

Torulaspora delbrueckii IFO10921 catalyzes the enantiotopic group-selective reduction of triketone **1**, the prochiral precursor of Wieland-Miescher ketone, to give a stereomerically pure form of bicyclic hemiacetal **4**, which is the equivalent of the resulting hydroxyketone[1].

Through a study of substrate specificity among 2,2-disubstituted cycloalkane-1,3-diones **2** and **3**, there were found some good substrates to give the equivalents of the resulting hydroxyketone by yeast-mediated reduction. These products were isolated as cyclic hemiacetals **5** and **6**. The subsequent chemical transformation to **7**, **8** and **9** warranted the stereochemistry and the stereochemical purity of the yeast-mediated reduction products, with considerable values as the starting materials for natural product synthesis.

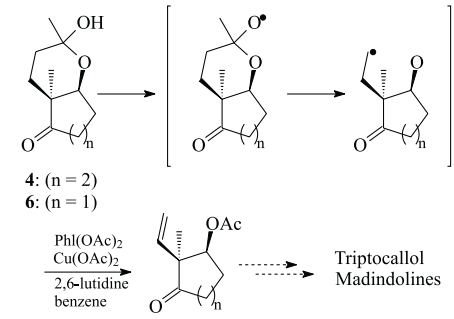


1: (n = 2, R = Me)
2: (n = 2, R = Et)
3: (n = 1, R = Me)

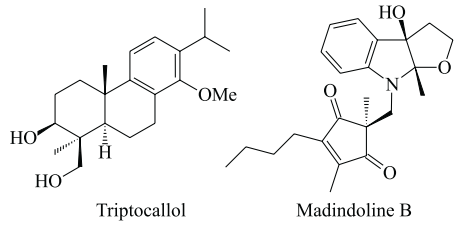
4: (n = 2, R = Me)
5: (n = 2, R = Et)
6: (n = 1, R = Me)

7: (n = 2, R′ = H)
8: (n = 2, R′ = Me)
9: (n = 1, R′ = H)

Also the above hemiacetals **4** and **6** served as the unique precursor of a sterically congested β-acetoxyketones containing a quaternary asymmetric center with three independent functional groups, by means of C-C bond cleavage through a β-fragmentation of alkoxy radicals. The resulted β-acetoxyketones **10** and **11** are expected for the multi-functional building blocks, and attempts to apply on the natural product syntheses, such as triptocallol and madindolines are now under way.



4: (n = 2)
6: (n = 1)



Triptocallol Madindoline B

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P188 BIOREDUCTION OF ACETOPHENONES AND DERACEMIZATION OF ARYL ETHANOLS BY FUNGI

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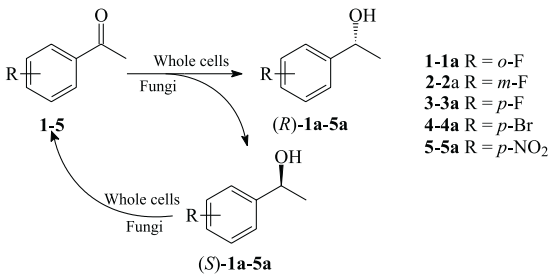
Keywords: bioreduction, deracemization, whole cells, chiral alcohol, biocatalysts

Biocatalysis is one of the most important methods for the preparation of optically active compounds with high stereoselectivity[1]. Among the biocatalysed reactions, we can mention the asymmetric reduction of ketones or kinetic resolution of racemic alcohols by whole cells or isolated enzymes, leading to chiral alcohols which are of great synthetic utility in view of the possible transformation of alcohols into other functionalities[1]. Recently, we have explored new microorganism strains, native of the Brazilian rain forests, for synthetic purposes, for instance in the oxidation of sulfides[2] and hydrolysis of epoxides[3]. As a part of this program, we initiated the search for microorganisms from tropical rain forests with oxidoreductase activity. The prochiral ketones **1-5** were reduced by whole cells cultures of *Rhizopus oryzae* CCT 4964, *Aspergillus terreus* CCT 3320 and *Aspergillus terreus* CCT 4083 (Scheme 1 and Table I)[4].



Scheme 1. **Mechanistic pathway proposed for the biotransformation of acetophenones and aryl ethanols by whole cells of fungi**

Different selectivities were observed for each of the mentioned microorganism. The deracemization of alcohols **1a-5a** was achieved with high enantiomeric excess when whole cells of *Aspergillus terreus* CCT 3320 and *Aspergillus terreus* CCT 4083 were used (Scheme 1 and Table II). The enantioselectivity was anti-Prelog in some cases. In conclusion, we developed an efficient method to reduce acetophenones to the corresponding alcohols using whole cells of new strains of fungi native of the rain forests, which proved to be excellent biocatalysts for this purpose. In addition, *A. terreus* CCT 3320 and *A. terreus* CCT 4083 promoted the efficient deracemization of aryl ethanols. These results demonstrate that the investigated microorganisms have a great bioenzymatic potential to perform reduction and deracemization reactions. Further studies with these microbial cells of native Brazilian fungi are in progress in our group.

Table I
Reduction of acetophenones by whole cells of fungi

#	*Aspergillus terreus* CCT 3320			*Aspergillus terreus* CCT 4083			*Rhizopus oryzae* CCT 4964		
ketone	t	c (%)	*e. e.* (%)*	t	c (%)	*e. e.* (%)*	t	c (%)	*e. e.* (%)*
1	3	69	65 *(S)*[a]	3	91	57 *(S)*[a]	1	91	98 *(S)*
	17	98	72 *(R)*[a]	17	94	66 *(R)*[a]	3	92	>99 *(S)*[b]
2	3	45	76 *(R)*	3	63	26 *(S)*	1	91	57 *(S)*
	6	91	>99 *(R)*[c]	8	75	83 *(S)*	3	92	62 *(S)*
3	3	35	94 *(S)*	3	52	70 *(S)*	1	23	76 *(R)*
	8	29	97 *(S)*[d]	8	31	22 *(S)*	3	84	66 *(R)*
4	2	55	81 *(R)*	1	96	91 *(R)*[a]	–	–	–
	5	57	70 *(R)*	11	98	65 *(S)*[a]	–	–	–
5	1	98	82 *(S)*	1	100	53 *(S)*	1	86	79 *(R)*
	6	100	96 *(S)*	9	99	51 *(S)*	3	63	87 *(R)*

t – time (days), c – conversion in alcohol, *e. e* – enantiomeric excess, [a]deracemization occured, [b]isolated yield *(S)*-(-)-**1a**: 90 %, [c]isolated yield *(R)*-(+)-**2a** 59 %, [d]isolated yield *(S)*-**3a** 18 %,. **e. e.* and conversion calculated by GC analysis (capillary column: chiral Chirasil-Dex CB β-cyclodextrin).

Table II
Deracemization of phenyl ethanols by whole cells of fungi

#	*Aspergillus terreus* CCT 3320				*Aspergillus terreus* CCT 4083			
alcohol	t	c (%)	c* (%)	*e. e.* (%)**	t	c (%)	c* (%)	*e. e.* (%)**
1a	3	4	8	21 *(S)*	3	–	4	14 *(S)*
	5	7	10	51 *(S)*	5	–	10	25 *(S)*
	8	2	6	61 *(S)*	8	–	21	52 *(S)*
2a	3	18	82	68 *(R)*	3	14	86	75 *(S)*
	5	12	88	>99 *(R)*	5	18	82	95 *(S)*
	7	12	88	>99 *(R)*[a]	7	16	84	97 *(S)*[b]
4a	4	32	68	70 *(R)*	3	12	88	6
	6	37	63	74 *(R)*	5	12	88	8
	9	43	57	74 *(R)*	17	21	79	>99 *(S)*
5a	4	98	02	–	5	18	82	84 *(R)*
	6	100	00	–	6	15	85	95 *(R)*
	9	100	00	–	7	14	86	>99 *(R)*

T – time (days), c – conversion in acetophenone, c* – conversion in alcohol, *e. e.*: enantiomeric excess, [a]isolated yield *(R)*-(+)-**2a**: 35 %, [b]isolated yield *(S)*-(-)-**2a** 59 %, ***e. e.* and conversion calculated by GC analysis (capillary column: chiral Chirasil-Dex CB β-cyclodextrin)

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P189 ENANTIOSELECTIVE REDUCTION OF 1-(1,3-BENZODIOXOL-5-YL)-2-HALO-1--ETHANONES BY *Rhodotorula glutinis* CCT 2182

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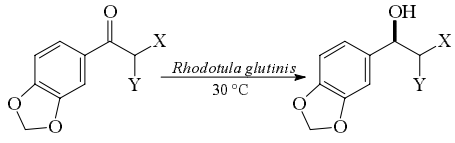
Keywords: asymmetric reduction, 1,2-aminoalcohols, *Rhodotorula glutinis*

The stereoselective synthesis of optically pure 1,2-aminoalcohols is an important issue since these compounds are versatile chiral building blocks for the synthesis of chemotherapeutic drugs, chiral auxiliaries and other chiral intermediates in organic synthesis[1,2].

Table I shows that *Rhodotorula glutinis* CCT 2182 was able to convert 1-(1,3-benzodioxol-5-yl)-2-halo-1-ethanones **1a-d** into the corresponding *(R)*-halohydrines **2a-d** in good yields and excellent enantiomeric excesses. However, the yeast was unable to reduce ketone **1e**. The *(R)*-halohydrines **2a-b** may be used as raw materials for the preparation of the pharmaceuticals *(R)*-(-)-epinefrine, *(R)*-(-)-norepinefrine and *(R)*-(-)-isoproterenol.



1 **2**

a: X = Cl, Y = H
b: X = Br, Y = H
c: X = Cl, Y = Cl
d: X = Br, Y = Br
e: X = H, Y = H

Table I
Asymmetric reduction of ketones **1a–e** by *Rhodotorula glutinis* CCT 2182[a]

Ketone	Alcohol	Yield (%)	$[\alpha]_D^{20b}$	e. e.[c] (%)	Configuration
1a	**2a**	98	–36.0	>99	*(R)*
1b	**2b**	57	–33.2	>99	*(R)*
1c	**2c**	88	–19.0	76	*(R)*
1d	**2b**	92	–11.7	72	*(R)*
1e	**2e**	–	–	–	–

[a]T = 30 °C, 24 h, 1 mmol/5 g (ketone/*Rhodotorula glutinis*), 100 ml H_2O (1.5 ml of EtOH); [b]*c* 1, $CHCl_3$; [c]determined by GC-MS analysis (capillary chiral column CHIRASIL-DEX)

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P190 HIGHLY EFFICIENT EXTRACTIVE BIOCATALYSIS WITH AMBERLITE XAD-7 IN THE ASYMMETRIC REDUCTION OF ENONES BY *Pichia kluyveri* AND *Rhodotorula glutinis*

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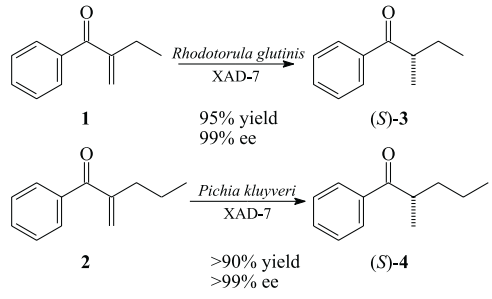
Keywords: extractive biocatalysis, asymmetric reduction, α-methyleneketones, non-conventional yeasts, Amberlite XAD-7

Chiral α-substituted ketones are versatile building blocks for the synthesis of natural products, especially in pheromone synthesis. Although it has been generally accepted that enzymatic transformations are suitable methods to obtain chiral compounds, little is known about stereoselective α-substituted ketone synthesis through α-methyleneketone reduction[1].

Herein, we describe the high regio- and enantioselective reduction of enones **1** and **2** to the corresponding saturated ketones **3** and **4**, by the yeasts *Pichia kluyveri* and *Rhodotorula glutinis* using Amberlite XAD-7 as adsorbing resin (extractive biocatalysis).



We found that the use of Amberlite XAD-7 as adsorbent of the enones played a crucial role in the reaction profile with two biocatalysts. In the absence of XAD-7 poor yields and enantiomeric excesses were achieved. On the other hand, growing amounts of XAD-7 sensibly improved the yields and ee's of the products. Best results with adsorbed enones **1** and **2** onto XAD-7 were achieved with *Rhodotorula glutinis* and *Pichia kluyveri* as biocatalysts, respectively.

In conclusion, the maintenance of low concentrations of the substrates in the aqueous phase by the use XAD-7 as adsorbing resin in the course of the reduction of enones **1** and **2** by suitable yeasts allowed us to obtain ketones **3** and **4** in high yields and enantiomeric excesses. Therefore, the extractive biocatalysis method proves again to be a powerful tool when inicial concentration and toxicity of the substrate are the limiting issues in the reaction[2].

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P191 ENZYMATIC DYNAMIC KINETIC RESOLUTION OF (±)-2-HYDROXY-1-INDANONE BY *Trichosporon cutaneum*: A SHORTCUT TO HOMOCHIRAL (1*S*,2*R*)-1,2-INDANDIOL

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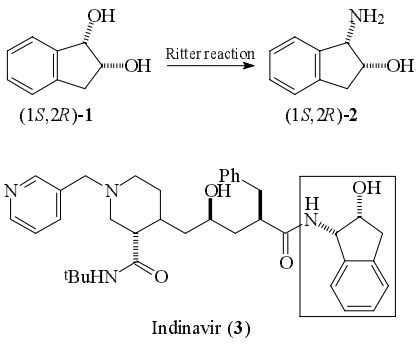
Keywords: enzymatic dynamic kinetic resolution, asymmetric reduction, (1*S*,2*R*)-1,2-indandiol, *Trichosporon cutaneum*

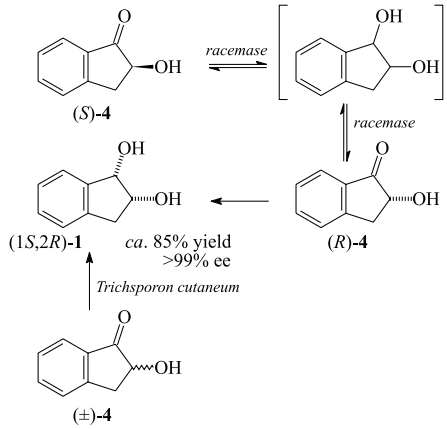
The (1*S*,2*R*)-1,2-indandiol **(1)** is a well stablished precursor to the (1*S*,2*R*)-1-amino-2-indanol **(2)**, a key raw material in the synthesis of the leading HIV-I protease inhibitor oligopeptide mimic Indinavir **(3)** (Scheme 1). Hitherto, only few biocatalytic roots to **1** have been delivered, *i. e.*: (i) microbial asymmetric dioxygenation of indene[1] and (ii) *Trichosporon cutaneum* MY 1506-mediated asymmetric reduction of 1,2-indanedione[2]. In turn, diol **1** can be promptly converted to **2** through a Ritter reaction[3].



Scheme 1

We wish to report a highly efficient enantio-convergent preparation of homochiral (1*S*,2*R*)-1,2-indandiol **(1)** through a enzymatic dynamic kinetic resolution of (±)-2-hydroxy-1--indanone **(4)** with stereoselective reduction mediated by resting cells of the non-conventional yeast *Trichosporon cutaneum* CCT 1903 (TC). Accordingly, when 1 g of racemic benzoin **4** was added to a slurry of resting cells of TC and glucose in distilled water, we were able to recover the (1*S*,2*R*)-1,2-indandiol **(1)** in *ca.* 85 % isolated yield plus >99 % e. e. after 3–4 days of incubation on an orbital shaker (150 rpm) at 28 °C (Scheme 2).

The intervention of a highly active racemase acting in the enzymatic dynamic kinetic resolution of the (±)-2-hydroxy--1-indanone **(4)** is evoked (Scheme 2). In conclusion, we have disclosed a valuable shortcut to homochiral (1*S*,2*R*)-1,2-indandiol in comparison with the previously reported method[2].



Scheme 2

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P192 ASYMMETRIC HYDROGENATION OF NITROALKENES USING *Clostridium sporogenes*

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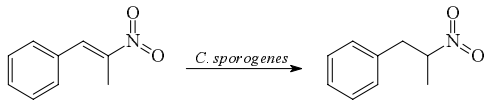
Keywords: asymmetric hydrogenation, nitroalkenes, *Clostridium sporogenes*, anaerobic bacteria

Asymmetric hydrogenation of nitroalkenes would be extremely valuable for the synthesis of chiral building blocks. In general, these substrates cannot be reduced enantioselectively by metal catalysts, whilst Baker's yeast and aerobic microorganisms have a limited substrate range and reduce the carbon-carbon double bond with very low efficiency. We have developed several anaerobic bacteria as novel biocatalysts which reduce nitroalkenes with good enantioselectivity and efficiency, and we are now studying the optimisation of the reactions.

Initially, we optimized the growth conditions for production of *C. sporogenes* to obtain maximal biocatalytic activity in harvested cells. Although glucose supported better growth than phenylalanine, the rate of cinnamate reduction was approximately 2-fold higher after growth on phenylalanine than on glucose. Addition of phenylalanine or cinnamate to cultures growing on glucose did not improve growth or increase the rate of cinnamate reduction using harvested cells. When *C. sporogenes* was grown in chemostat culture, the rate of cinnamate reduction increased with pH and with decreasing dilution rate. The reaction conditions for C = C reduction using harvested cells were also optimised. The efficiency of electron donors for reducing cinnamate was in the order hydrogen > phenylalanine > glucose.



The optimised biocatalyst production and reaction systems were used for the reduction of 2-nitro-1-phenyl-1-propene, using an anaerobic two liquid phase reaction system to protect the microorganism from substrate toxicity. The enantiopurity of the nitroalkane product depended on the growth substrate used to produce the biocatalyst and on the substrate concentration in the reaction. We are currently investigating the reasons for the varying enantioselectivity, with the aim of developing methods to control enantiopurity of the nitroalkane product.

P193 MULTI BIOREACTION SCREENING, A TOOL TO DISCOVER NEW ENZYMATIC ACTIVITIES

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Keywords: multi bioreaction screening, *Serratia rubidea*, *Saccharomyces cereviseae*

Biocatalysis has been pushed towards high throughput screening (HTS)[1, 2] in order to monitor enzymatic activity of libraries of natural or modified microorganisms. Among the most popular HTS methodologies are the high performance liquid chromatography and gas chromatography associated to mass spectrometry. Reagent based assays are also good alternatives which require the modification of the substrate by inserting a fluorogenic or chromogenic moieties. In principle all are efficient and are based on one reaction and several microorganisms or several enzymes (namely in 96 or 398 microtiter plates). Hoping to contribute with this worldwide trend we visualized a high throughput screening of reactions namely several substrates possessing different functional groups to be simultaneously by several microorganisms. This would increase the speed of the HTS *n* times, where *n* is the number of added substrates.

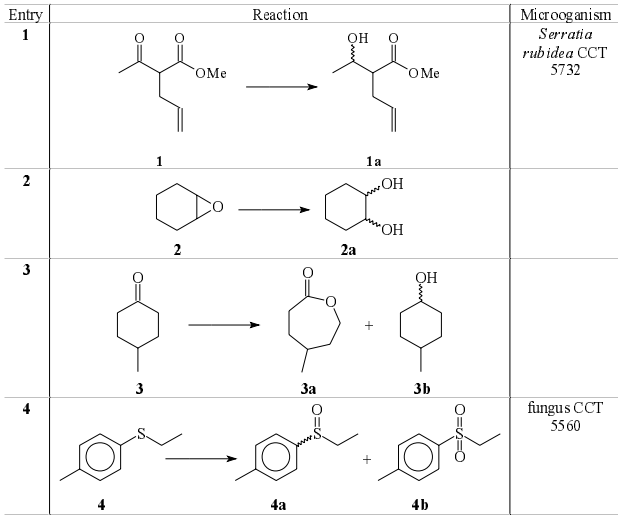
To test the effectiveness of the methodology we have selected 2 Brazilian microorganisms (*Serratia rubidea* CCT 5732 and the fungus CCT 5560) under study in our laboratory and one strain of *Saccharomyces cereviseae* DSM 0195. We selected substrates **1-4** with the objective of simultaneously monitoring ketone oxidation and reduction, sulfoxidation and epoxide hydrolysis. These reactions had been previously studied detecting microorganisms that could better perform them in terms of reaction time, yield and enantiomeric excesses. In biotransformations the substrate concentration has to be below the minimum inhibitory concentration (MIC) but taking into consideration that each reagent will act individually we could add 4 times the usual amount of xenobiotics to the cell suspensions and none of the individual substrates would be above the MIC. Notwithstanding this rationale we added only 50 mg of xenobiotic (mixture of compounds **1-4**) per 2 g (wet weight) of MO cells.

The reactions were monitored by GC/MS and indeed the *Serratia rubidea* CCT 5732 known to have oxidoredutase activity preferentially transformed substrate **1** into *syn* and *anti* **1a** in 2 hours leaving the remaining substrates with little or no modification. The same behavious was observed when treating the reaction individually (Table I). The reaction monitoring was continued for 20 hr when 1a was degraded and phenyltioderivatives was observed.

Fungus CCT 5560 had been previously selected as a good microorganism for sulfoxidation yielding chiral sulfoxides in

good yield and high enantiomeric excess. During this multibioreaction screening this same fungus was also selected for sulfoxidation just like in the individual screening.

Table I
Multibioreaction screening



Finally we are conscious that this methodology requires optimization but the central idea is sound and proved to be correct allowing the screen of a chemical mixture and reaching the same conclusion of individual substrate screening but n times faster were n is the number of added substrates.

The authors are indebted to FAPESP, CNPq and CAPES for grants and scholarship.

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P194 ENANTIOSELECTIVE OXIDATION OF SULFIDES AND SULFINIC ACID ESTER WITH THE AID OF BRAZILIAN MICROORGANISMS

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Keywords: organosulfur compounds oxidation, chiral sulfinates, chiral sulfoxides

The asymmetric oxidation of sulfide is one of the most convenient route to chiral sulfoxides which depends on chiral environments (electrodes[1], clays[2], helices[3], cyclodextrins[4]) and this is either limited to specific substrates or gives products in only moderate to low enantiomeric excesses. A new horizon was envisaged with Kagan-Modena's method using the modified Sharpless reagent[5,6] and giving access to useful chiral sulfoxides. The classic Andersen's chiral sulfoxide synthesis relies on the nucleophilic substitution on diastereomerically pure *(S,S)* menthyl *p*-toluenesulfinate[7] but high enantiomeric excesses are mainly obtained with *p*-tolyl derivatives. Focusing on the enzymatic oxidation of organo sulfur derivatives by Brazilian microorganisms as an alternative way to obtain chiral sulfoxides we have selected two major approaches: one by direct oxidation of sulfide derivatives and the second by an enzymatic Andersens's synthesis, that is, kinetic resolution of sulfinates viewing the production of chiral sulfoxides. In a previous program we obtained chiral sulfoxides in high enantiomeric excesses from organosulfur compounds using fungi whole cells either resting or immobilized. The conversions were not satisfactory due to the subsequent transformation of one of the sulfoxides into sulfone. At the time the selected microorganisms produced sulfoxides with *(S)* configuration from a wide range of benzyl alkyl and phenyl alkyl sulfide[8,9]. To access the oxidation of sulfides with little or no production of sulfone derivatives we have screened novel microorganisms using substrate **1**. The results are summarized in Table I.

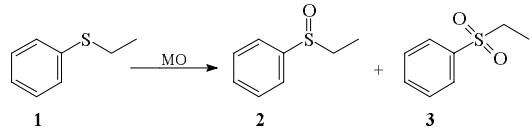

Table I
Biotransformation of phenyl ethyl sulfide **1** with Brazilian microorganisms

Entry	Microorganism	C (%)	2 (%)	3 (%)	ee (%)	Conf.
1	*Geotrichum candidum* CCT 5551	100	32	68	65	*S*
2	*Aspergillus niger* CCT 1435	100	34	66	76	*S*
3	Rhizopus oryzae CCT 4964	100	100	–	62	*S*
4	Fungi CCT 5551 (*Trigona sp*. bee)	97	97	–	68	*R*
5	*Nodulisporium sp.* CCT 5552	100	32	68	45	*R*
6	Fungus CCT 5553	75	71	4	>99	*R*
7	Fungus CCT 5560	83	83	–	85	*R*
8	Fungus CCT 5630	100	67	33	3	*S*
9	Fungus CCT 5632	80	80	–	>0	*R*
10	Fungus CCT 5635	100	96	4	53	*R*
11	Fungus CCT 5661	98	74	24	96	*S*
12	*Aspergillus ochraceus*	96	69	27	38	*S*

Thus fungus CCT 5553 isolated from a *Trigona* sp bee[10] was selected as the best candidate to transform sulfide **1** into sulfoxide **2** in >99 ee and 75 % conversion. Reaction scale up allowed a gram scale production of **2** which will be used in asymmetric synthesis. For the second approach we focused on the resolution of racemic sulfinates and sulfinates we first tested the stability of **4** and **5** in aqueous medium but only **5** did not hydrolyzed spontaneously. Therefore substrate **5** was

used to screen the enzymatic activity present in several microorganisms (Table II). As it can be appreciate by scrutinizing Table II, hydrolysis and oxidation are two competitive reactions. In all cases the hydrolysis was not enantioselective while sulfinate **5** of high enantiomeric purity was obtained by oxidative kinetic resolution. Notwithstanding this apparent sucesss the conversions obtained so far are not acceptable. Finally our results further improved the access to **2** in gram-scale with the alternative of choosing **2-***(R)* or the **2-***(S)* configuration. The biocatalytic method of obtaining chiral sulfoxides using the chiral sulfinate was also investigated by selecting microorganisms that resolved sulfinate **5**.

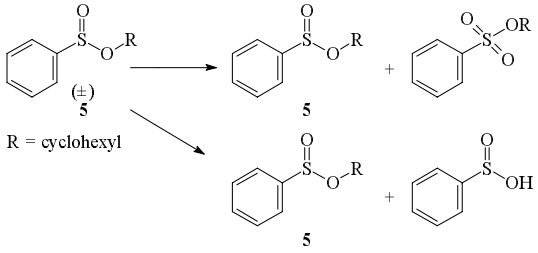

Table II
Kinetic resolution of sulfinates 5 with Brazilian microorganisms

Entry	Microorganism	5 ee (%)	Reaction
1	*Geotrichum candidum* CCT 5551	95	oxidation
2	*Aspergillus niger* CCT 1435	–	hydrolysis
3	*Rhizopus oryzae* CCT 4964	25	oxidation
4	*Aspergillus terreus* CCT 3320	95	oxidation
5	*Nodulisporium sp.* CCT 5552	–	hydrolysis
6	Fungus CCT 5553	44	oxidation
7	Fungus CCT 5560	–	hydrolysis
8	*Aspergillus ochraceus*	99	oxidation

The authors are indebted to FAPESP and CNPq for grants and scholarships, respectively.

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P195 ASYMMETRIC SYNTHESIS OF ARYLSELENOALCOHOLS BY MEANS OF THE REDUCTION OF ORGANOSELENO ACETOPHENONES BY FUNGI

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Keywords: bioreduction, organoselenium, ketone, chiral, alcohol

In recent years chiral organoselenium compounds have been prepared and some of them were applied in asymmetric synthesis[1]. This concern constitutes a new trend in this field of organoelemental chemistry. Thus, the search for efficient methods for preparing organoselenium compounds with high enantiomeric purities is an important goal in this area of chemistry. In this context, biocatalysis has attracted much attention for being an important method in the preparation of optically active compounds with high stereoselectivity under environmentally friendly conditions[2]. In this communication we report the bioreduction of organoseleno acetophenones **3a-f** with whole cells of fungi (Fig. 1).

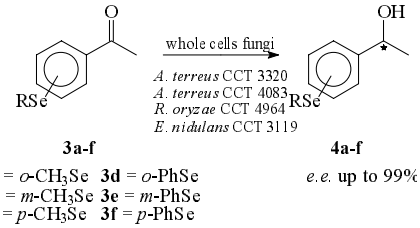

Fig. 1. **Asymmetric bioreduction of seleno ketones 3a–f with whole cells fungi**

We screened for microorganisms which are able to perform the reduction of the carbonyl group. The biotransformation was performed by re-suspending the wet cells of the appropriate fungi (4 g) in a phosphate buffer solution (50 ml, pH 7.0, 0.1 M) in a 125-ml Erlenmeyer flask. To these cells suspensions, solutions of ketones **3a-f** (20 mg) in ethanol (0.5 ml) were added and the mixtures were incubated in a rotary shaker (170 rpm) at 32 °C for times indicated in Table I. The progress of the biotransformation was monitored by GLC. The results are summarized in the Table I. As can be observed, two of the six seleno ketones **3**, namely *ortho*-methylseleno acetophenone **3a** and *ortho*-phenylseleno acetophenone **3d**, were unreactive toward the four investigated fungi under the bioreduction conditions employed. The other selenoacetophenones were reduced with good conver-

sion rates and ee. The results obtained in the bioreduction of seleno ketones **3c**, **3e** and **3f** showed that the fungus *Rhizopus oryzae* CCT 4964 gave the best values of conversion and enantiomeric excess for preparation of organoseleno-α--methylbenzyl alcohol **4**. To our knowledge this is the first reported biotransformatiom of a selenium compound for synthetic purposes.

Table I
Bioreduction of seleno ketones

Entry	Substrate	Whole cells fungi	Time (day)	Conversion (%)[a]	Product **4** ee (%)[b]
1	**3a**	*Rhizopus oryzae* CCT 4964	7	n. c.	–
2	**3b**	*Rhizopus oryzae* CCT 4964	2	99 (55)[c]	94
3	**3c**	*Rhizopus oryzae* CCT 4964	2	91 (50)[c]	96
4	**3d**	*Rhizopus oryzae* CCT 4964	7	n. c.	–
5	**3e**	*Rhizopus oryzae* CCT 4964	7	90 (27)[c]	87
6	**3f**	*Rhizopus oryzae* CCT 4964	7	85 (25)[c]	71
7	**3a**	*Aspergillus terreus* CCT 4083	7	n. c.	–
8	**3b**	*Aspergillus terreus* CCT 4083	2	76	90
9	**3c**	*Aspergillus terreus* CCT 4083	2	86	55
10	**3d**	*Aspergillus terreus* CCT 4083	7	n. c.	–
11	**3e**	*Aspergillus terreus* CCT 4083	9	21	47
12	**3f**	*Aspergillus terreus* CCT 4083	9	12	45
13	**3a**	*Aspergillus terreus* CCT 3320	7	n. c.	–
14	**3b**	*Aspergillus terreus* CCT 3320	2	75	86
15	**3c**	*Aspergillus terreus* CCT 3320	10	75	95
16	**3d**	*Aspergillus terreus* CCT 3320	7	n. c.	–
17	**3e**	*Aspergillus terreus* CCT 3320	7	41	99
18	**3f**	*Aspergillus terreus* CCT 3320	7	n. c.	–
19	**3a**	*Emericella nidulans* CCT 3119	7	n.c.	–
20	**3b**	*Emericella nidulans* CCT 3119	3	9	67
21	**3c**	*Emericella nidulans* CCT 3119	5	99	99
22	**3d**	*Emericella nidulans* CCT 3119	7	n. c.	–
23	**3e**	*Emericella nidulans* CCT 3119	10	n. c.	–
24	**3f**	*Emericella nidulans* CCT 3119	7	n. c.	–

[a]Conversion determined by GC, isolated yield in parentheses, n. c. = = no conversion, [b]determined by GC analysis using a chiral column (Chirasil-Dex CB β-cyclodextrin 25 m × 0.25 mm) **(4a-d)** , by chiral HPLC analysis after reaction with 3,5-dinitrobenzoyl chloride (column Astec-Cyclobond I 2000 SN: hexane/isopropanol = 99/1) **(4e, 4f)**, [c]100 mg of substrate was used

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P196 ENANTIOSELECTIVE SULFIDE OXIDATION CATALYSED BY RECOMBINANT *Escherichia coli* WHOLE CELLS

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Keywords: whole-cells, cyclohexanone monooxygenase, hydrophobic resin

A recombiant strain of *Escherichia coli* (TOP 10 pQR239)[1] expressing cyclohexanone monooxygenase (CHMO) from *Acinetobacter calcoaceticus* sp. NCIMB 9871 has been used as whole cell biocatalyst for the oxidation of 1,3-dithiane[2,3] to the corresponding enantiopure *(R)*-sulfoxide (ee > 98 %) (Fig. 1).

CHMO suffers from substrate and product inhibition and, furthermore, 1,3-dithiane shows low solubility in the biotransformation medium. These limitations have been overcome by using a polymeric hydrophobic resin[4]. After a series of substrate/product binding tests on a variety of resins with different hydrophobic properties, we choose SP207 (Sepabeads – Resindion). This acrylic resin was able to adsorb 1,3-dithiane, thus acting as a reservoir for high substrate concentrations. Furthermore, the product was also adsorbed onto the resin, thus allowing to remove it from the reaction mixture as it formed.

This approach has made it possible to increase 1,3-dithiane concentration in the reaction medium from 1 g.l^{-1} to 5 g.l^{-1} with complete substrate conversion.

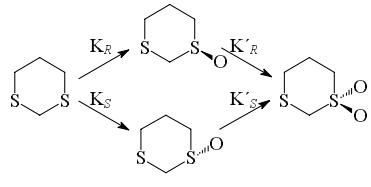

Fig 1. **CHMO catalysed oxidation of 1,3-dithiane to *(R)*-sulfoxide**; sulfone started forming only after complete substrate consumption.

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P197 APPLICATION OF BACTERIAL ALDEHYDE OXIDATION SYSTEM FOR SIMPLE PREPARATION OF USEFUL ACIDS

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Keywords: application, bacterial aldehyde oxidation, useful acids

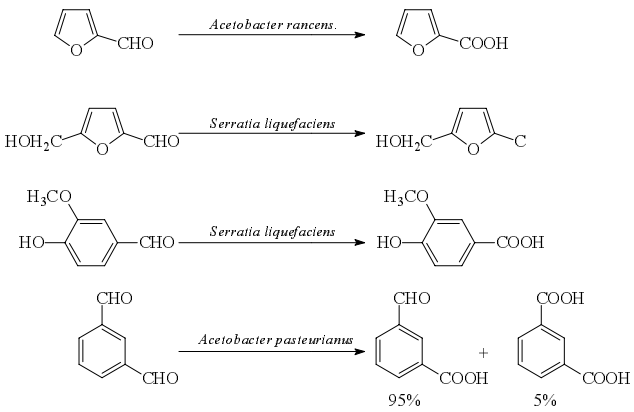
For the accomplishment of "green chemistry", the environmental friendly bioprocess should be introduced into chemical industry. The bacterial aldehyde oxidation system has been applied for the synthesis of various useful acids.

We surveyed the powerful aldehyde-oxidizing bacteria through the conventional enrichment culture technique. In addition to *Acetobacter* and *Gluconobacter* strains, we found that *Pseudomonas fluorescens* and *Serratia liquefaciens* exhibit the powerful aldehyde oxidation activity. We apply these bacterial aldehyde oxidation system for the rapid and convenient preparation of various useful acids from the corresponding aldehyde.

1) Syntheses of 2-furancarboxylic acid and 5-hydroxymethylfuran-2-carboxylic acid: Furfural and 5-hydroxymethyl-2-furaldehyde are abundant natural renewable resources obtained easily through the heat-distillation of pentosan and hexosan treated with the diluted sulfuric acid, respectively. 2-Furancarboxylic acid is used for the synthesis of various pharmaceuticals, perfumes, agricultural chemicals and resins. Although several synthetic methods of 2-furancarboxylic acid from furfural have been reported, there have been some problems to be resolved, particularly, for its production on a large scale. We focused on the bacterial aldehyde oxidizing capability. *Acetobacter rancens* and *Gluconobacter cerinus* oxidized furfural to produce 2-furancarboxylic acid. The accumulation of 2-furancarboxylic acid was 110 $g.l^{-1}$ with 83 % molar conversion yield for 30 h with the resting cells of *Acetobacter rancens*. *Serratia liquefaciens* converted 5-hydroxymethyl-2-furaldehyde into 5-hydroxymethylfuran-2-carboxylic acid. The accumulation of 5-hydroxymethylfuran-2-carboxylic acid reached 32.6 $g.l^{-1}$ with 88 % molar conversion yield for 26 h. The hydroxymethyl group of 5-hydroxymethyl-2-furaldehyde was inert and the oxidation was very specific to aldehyde. Compared to the oxidation of 5-hydroxymethyl-2-furaldehyde by chloroperoxidase[1], *Serratia liquefaciens* oxidation activity was very specific to aldehyde.

2) Synthesis of vanilinic acid: We also established the efficient conversion of vanillin into vanillic acid. Among tested aldehyde-oxidizing bacteria, *Serratia liquefaciens* showed highest productivity of vanillic acid. The accumulation of vanillic acid was 70.6 $g.l^{-1}$ with 89 % molar conversion yield for 12 h.

3) Selective oxidation of dialdehyde (*o*, *m*, and *p*-phthaladehyde): The selective oxidation of dialdehydes such as *o*, *m*, and *p*-phthaladehyde was also examined using aldehyde-oxidizing bacteria. When the oxidation of *m*-phthaladehyde as a substrate was examined using *Acetobacter pasteurianus* resting cells, *m*-phthalaldehydic acid was preferentially formed with 95 % conversion, and then formation of *m*-phthalic acid was only 5 %. The accumulation of *m*-phthalaldehydic acid reached 36.8 $g.l^{-1}$ with 88 % molar conversion yield for 40 h. On the other hand, the selectivity of oxidation of *p*-phthaladehyde was poor and the oxidation of *o*-phthaladehyde almost did not proceed.



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P198 MICROBIAL DERACEMIZATION OF α-SUBSTITUTED CARBOXYLIC ACIDS

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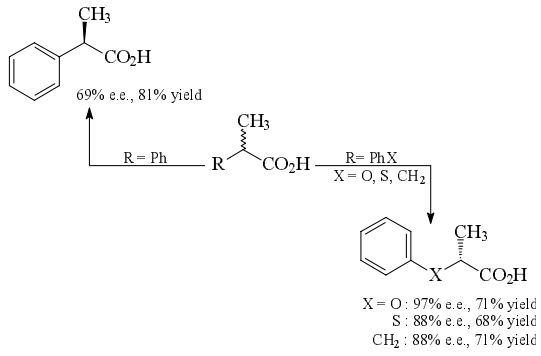
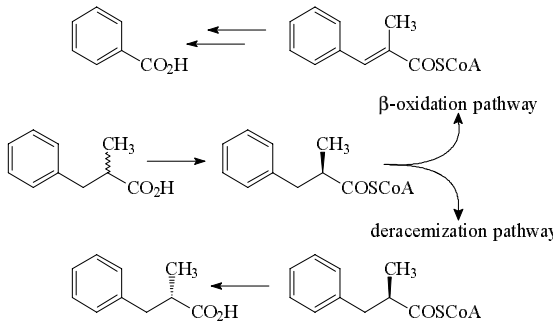
Keywords: deracemization, chiral inversion, suppression of metabolism, reaction mechanism, α-substituted carboxylic acids

Biocatalytic methods such as kinetic resolution for racemates provide you with an easy access to the optically active compounds. Unfortunately, however, these techniques are not ultimate methods obtaining the optically active products. These have disadvantages. In case of kinetic resolution, the maximum yield of the desired enantiomer is limited to 50 %, although this technique is already well-established to prepare the optically active products from their racemates. Recently, we have reported a new approach obtaining the optically active *(R)*-2-aryl- and *(R)*-2-aryloxypropanoic acid starting from their racemates by the aid of the growing cells of *Nocardia diaphanozonaria* JCM3208 (Scheme 1)[1]. This method is known as deracemization reaction and is capable of overcoming the drawback of the kinetic resolution process. Theoretically, deracemization reaction could give the desired enantiomer in 100% yield. It means that the synthesis of racemates is almost equal to the synthesis of optically active compounds and this concept is entirely different from the commonly accepted one in the asymmetric synthesis.



Scheme 1

In this poster, we would like to present the new design of reaction conditions to suppress the metabolic reactions for two types of compounds, 2-phenylthiopropanoic acid and 2-methyl-3-phenylpropanoic acid, and to proceed preferentially the deracemization reaction (Scheme 1). In addition, the investigation of the reaction mechanism using the cell free extracts as well as the whole cells indicate that this deracemization process is competitive reaction against the β-oxidation pathway of fatty acid metabolism (Scheme 2). Also, it was found that new types of enzymes take part in this system, although the reaction proceeds by way of the same mechanism as that in rat liver[2].



Scheme 2

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P199 MICROBIAL DERACEMISATION OF β-HYDROXY ESTERS – AN IMPORTANT STRATEGY TOWARDS VARIOUS CHIRAL INTERMEDIATES

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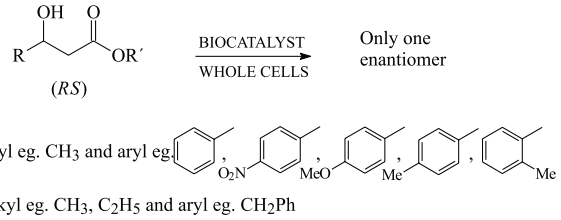
Keywords: deracemisation, β-hydroxy esters, microbial whole cells

Chiral β-hydroxy esters are important building blocks in organic synthesis especially in the preparation of natural and pharmaceutical products[1]. Several chemical[2] and biocatalytic[3] methods are known for preparing optically pure β-hydroxy esters. Among the biocatalysts, lipases, generally known for esterification and transesterifications[4] are extensively employed for the resolution[5] of secondary alcohols. The disadvanages of the reported methods are mainly low yields (maximum 50 %) and separation of the products after resolution or the use of expensive cofactors for isolated enzyme mediated asymmetric reduction. Deracemisation[6] is an attractive alternative as it permits the conversion of racemic

β-hydroxy esters to optically pure hydroxy esters in high enantiomeric excess as well as high yield. It is a novel approach for the synthesis of optically pure β-hydroxy esters . The starting materials for this reaction are racemic β-hydroxy esters[7]. This study will highlight the biocatalytic synthesis of optically pure β-hydroxy esters by deracemisation using microbial whole cells. The substrate specificity of the biocatalyst will be presented using a variety of aryl and alkyl β-hydroxy esters. A possible mechanism of the microbial deracemisation reaction will also be presented.



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P200 MICROBIAL MODELS OF ANIMAL DRUG METABOLISM: MICROBIAL PREPARATION AND IDENTIFICATION OF HYDROXYLATED METABOLITES OF IRBESARTAN

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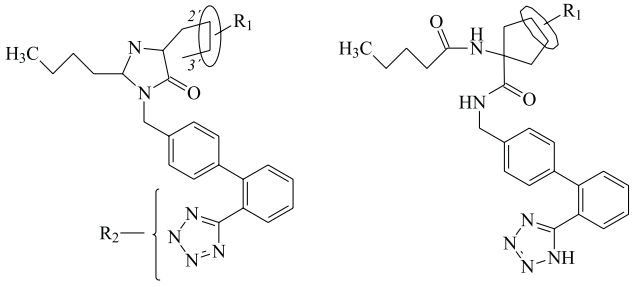
Keywords: drug metabolism, irbesartan, bacteria, *N*-glycosylation, hydroxylation

Irbesartan **(1)**, a recently developed and highly selective orally active non-peptidic angiotensin II receptor antagonist, is metabolized in animals and humans to give at least eight urinary metabolites[1–3]. Two of the minor metabolites correspond to monohydroxylated derivatives resulting from the oxidation of the spirocyclopentane ring. The stereochemical features of these metabolites have not been fully elucidated: indeed, symmetrical positions (2' and 3') on the cyclopentane ring are enantiotopic, due to a symmetry plane involving the substituted spiroheterocyclic ring. Hydroxylation on each of these positions, on either face, should generate enantiomeric pairs of *cis*- or *trans*-hydroxylated derivatives.



1: R_1 = H; R_2 = H
2: R_1 = OH; R_2 = H
3: R_1 = H; R_2 = 1-glycosyl

4: R_1 = OH

We describe some results about the biotransformation of irbesartan by various microbial species in order to prepare some of its metabolites in sufficient amounts to complete the determination of their structural and stereochemical characteristics. Depending on the strain used, each isomer **(2)** could be obtained in good yield together with the corresponding hydrolysed (open form) metabolite **(4)**. In addition, a *N*(tetrazole)-glycosylated metabolite **(3)**, analogous to the *N*-glucuronyl animal metabolite, was produced by some strains and fully characterized.

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P201 A HIGH THROUGHPUT SCREENING METHOD FOR THE STUDY OF MICROBIAL METABOLISM OF XENOBIOTICS AND THE GENERATION OF MOLECULAR DIVERSITY

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Keywords: biocombinatorial, microorganisms, microplate, metabolism, molecular diversity

A fast 96-well microplate system has been developed to explore the biotransformation activities of various microorganisms (fungi, yeasts, bacteria) upon synthetic or natural compounds of interest in the pharmaceutical, agrochemical or cosmetic fields. This miniaturized method, associated with sensitive and efficient microanalyses of the products formed (LC, GC-MS, LC-MS…), allows to select the best strains 1) to study the formation of microbial metabolites[1]; 2) to generate molecular diversity, in a biocombinatorial approach[2].

Several examples of this technique will be described, and compared with the corresponding results obtained in classical incubation conditions.

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P202 PREDICTIVE BIOTRANSFORMATION OF POTENTIAL TOXIC CHEMICALS

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Keywords: predictive biotransformation, toxic chemicals, functional groups, enzymes

Over 18 million chemical substances are known, with more than 65,000 currently used in commerce. For past several decades, considerable progress has been made towards understanding environmental fates of these naturally occurring and synthetic compounds. The important role of microorganisms in these transformations and mineralization has been clearly established. Biodegradability is one of the most important characteristics of an organic compound to assess its life and fate in the environment[1]. For mineralization of a chemical to occur, several enzymes must act sequentially to transform or breakdown chemicals to simpler molecules that enter intermediary metabolism. With expert knowledge of enzyme substrate specificity, sequential steps in the metabolism of naturally occurring compounds, and catabolic pathways, one can identify structural features of synthetic chemicals that are likely to be substrates for enzyme(s)[2].

The ability to predict biodegradability of chemicals in the environment is of increasing importance due to concerns about the persistence and toxicity of the parent compounds and/or their metabolites in addition to high cost of empirically assessing their fate. The predictive information of how a particular chemical compound may be degraded has enormous implications for industry as well as for regulatory agencies[3]. Though, the biodegradability of chemicals in the environment is largely predicted on their ability to serve as substrates for microbial enzymes, enzymatic mechanisms have been determined for only a small percentage of these chemicals.

Focusing on the above aspect, the present study was carried out to predict plausible biotransformation routes for potential toxic chemicals using chemical heuristics and information resources (published and unpublished scientific literature). The chemical substances were chosen based on the extent of utilization by chemical industries and the listing in hazardous substances registers. The predictions were based within a framework of certain rules: (i) prediction of one possible chemical route, (ii) prediction of one possible biochemical route – a sequence of plausible enzymatic reactions, (iii) prediction of all possible biochemical routes to intermediary metabolism, and (iv) evaluation of biochemical reactions for chemical feasibility.

The first step is to predict the possible transformation of the most reactive site (in the test chemical substance) based

on chemical heuristics. The second step is to examine the scientific/experimental literature to verify the occurrence of the above transformation biochemically. Deriving on the similarity of this transformation to the known enzymatic reactions, an enzyme and its characteristics are assigned to the step catalyzing the biotransformation. Similar process is followed further on the test molecule and a predictive pathway is proposed until the final product formed enters the intermediary metabolism[4].

Conversion of an aromatic isocyanate to an aromatic amine is an example of such biotransformation route, which has been contributed under the present study and accepted as a rule (BTrule) in Pathway Prediction System, developed at University of Minnesota Biocatalysis and Biodegradation Database (UMBBD)[5]. As of now, predictive biodegradation pathways have been studied for allyl amine (107-11-9), allyl alcohol (107-18-6), benzyl sulfide (538-74-9), glycolonitrile (107-16-4), methylene diphenyl diisocyanate (101-68-8) and toluene diisocyanate (584-84-9) which would be presented.

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P203 INDUCIBLE AND CONSTITUTIVE CYTOCHROMES P-450 INVOLVED IN OXIDATION OF TERFENADINE BY *Streptomyces platensis*

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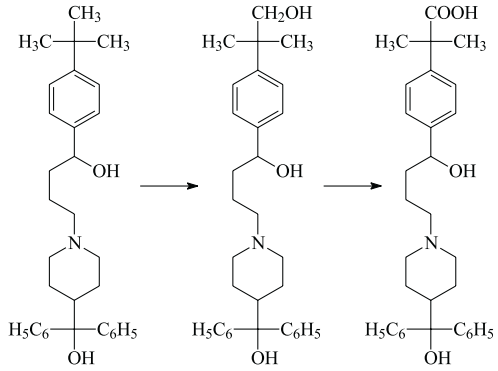
Keywords: *Streptomyces*, hydroxylation, oxidation, cytochromes, inducible

In the course of the preparation of the fexofenadine **3**, which is the active metabolite of terfenadine **1**, an antihistaminic drug, it has been found that *S. platensis* NRRL 2364 was able to oxidise the *t*-butyl group of **1** to give two products[1]. The hydroxylation of one methyl group gave the alcohol derivative **2** (so-called triol) and a subsequent oxidation could be observed and furnished the fexofenadine **3** in variable amounts.



Terfenadine **1** Triol **2** Fexofenadine **3**

We report here our investigations concerning the enzymes involved in these transformations. We have showed that two cytochromes P-450 catalysed the oxidation of terfenadine **1**. One was present only when the microorganism grew in soybean-peptone-containing culture medium, which was able to transform terfenadine **1** in fexofenadine **3** in short time incubation. The activity of this enzyme was higher after 48 hours of cultivation and decreased rapidly.

The second enzyme, more stable, was present in *S. platensis* grown in culture medium without soybean peptone and was able to transform terfenadine **1** and triol **2**. However, the oxidation of triol took place only without terfenadine in incubation medium. When the incubation of triol **2** was performed under oxygen-18, one atom of oxygen-18 was incorporated into fexofenadine. These observations suggest that the monooxygenase, which catalysed the hydroxylation of terfenadine was implicated in the oxidation of triol in fexofenadine. In order to optimise the formation of triol **2** we also studied the conditions of incubation (pH, temperature, concentration).

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P204 BIOTRANSFORMATION OF TERFENADINE, EBASTINE AND ANALOGUES BY SOME MICROORGANISMS

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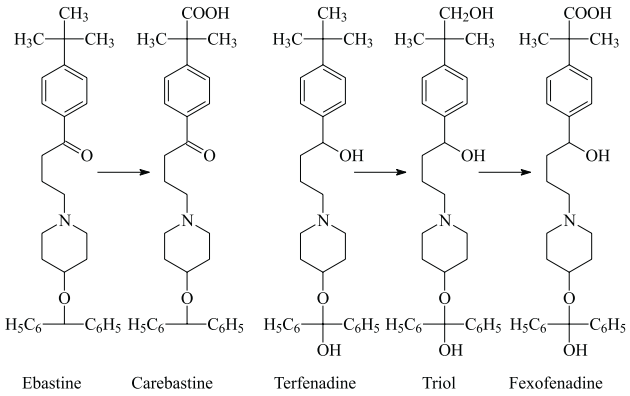
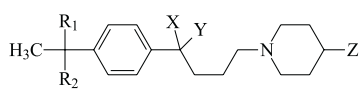
Keywords: microorganism, hydroxylation, oxidation, cytochrome P450

Terfenadine and ebastine, two antihistaminic drugs, are oxidised by hepatic cytochromes P-450 to fexofenadine and carebastine respectively, which are responsible for the pharmacological effects. Described chemical synthesis of fexofenadine and carebastine are laborious and products are obtained in low yields compared to synthesis of terfenadine and ebastine. Thus, the microbial oxidation of the *tert*-butyl groups of terfenadine and ebastine has been developed as an alternative method.



Cunninghamella echinulata is efficient to oxidise ebastine to carebastine[1] but transforms terfenadine into triol[2], the hydroxylated intermediate. The formation of fexofenadine has been reported[3] by oxidation with the bacteria *Streptomyces platensis* and the fungi *Absidia corymbifera*.

We present here our study concerning the biotransformation of several analogues of formula **1**. The aim of this work was to investigate the structural requirements necessary for the oxidative enzymatic activity of these microorganisms.



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P205 IS THE OXIDATIVE CLEAVAGE OF HETEROCYCLIC NAPHTHOQUINONES IN *Streptomyces* CATALYZED BY A MEMBER OF THE HYDROQUINONE-EPOXIDASE FAMILY?

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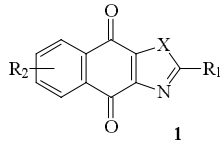
Keywords: dioxygenase, hydroquinone-epoxidase, *Streptomyces*, naphthoquinone, heteroaromatic

We have recently demonstrated that several *Streptomyces* strains are able to oxidatively cleave a large number of heterocyclic naphthoquinones (**1**, X = S, O, N) into isomeric carboxylic bis-(hetero)aromatic ketones (**2** or **3**) by a formal H_2O_2 addition, which is the result of a dioxygenase-type mechanism, as shown by the simultaneous incorporation of both oxygen atoms from an O_2 molecule[1]. The mechanism of this reaction will be compared to the hydroquinone-epoxydase reaction catalyzed by several *Streptomyces* strains with polyketide antibiotic precursors as substrates (antibiotic LL-C10037α (ref.[2]), tetracenomycin C, elloramycin[3], etc…) and the formation of a non-epoxide cleaved product will be discussed.



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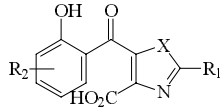

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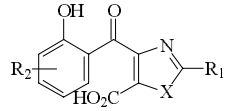

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P206 BIPHASIC BIOCONVERSION OF NAPHTHALENES INTO DIHYDRODIOLS: SOLVENT EFFECTS AND SUBSTRATE DIFFERENCES

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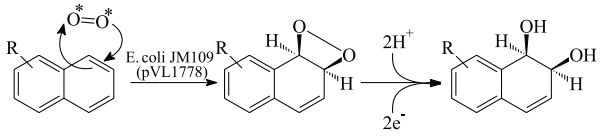
Keywords: dioxygenase, bioconversion, whole cell, naphthalene dihydrodiols, solvent effects

Since many years, we have been interested in whole cell bioconversions of aromatic hydrocarbons into derivatives that can be of interest as synthetic intermediates. We have developed recombinant bacteria containing oxygenases from *Pseudomonas* strains. In particular, we isolated the naphthalene dioxygenase of *P. fluorescens* N3 responsible of the conversion of substituted naphthalenes into the corresponding dihydrodiols with unique absolute configuration.



In order to increase the bioconversion yield and the system performance we studied the possibility of using a culture where the substrate is dissolved in an appropriate hydrophobic solvent. Preliminary results concerned the effects on the naphthalene transformation. Now, to further our understanding, we extend our experiments to some substituted naphthalenes. In this perspective, several flask transformations and some reactor experiments have been performed. The results show that the solvent role is highly affected by the substrate used and that some other important bioconversion variables influence the transformation. It appears clear that substrate bioavailability is definitely an essential condition, which is influenced by the solvent selection, the solvent-water phase ratio, the presence of surfactants, and, not last, the transfer mechanism. On the other hand, the absolute yield also depends on the possibility of maintaining the enzyme activity for long enough time. Our results will be presented and discussed together with future directions and developments.

In this respect, we began a series of reactor experiments using a multivariate approach where the role of some variables has been explicitly investigated.

P207 BIOTRANSFORMATION OF PYRIDINES WITH *Pseudomonas* SP STRAIN NCIB 9816-4

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Keywords: dioxygenases, *cis*-dihydroxylation, pyridines, naphthalene *cis*-dihydrodiol dehydrogenase

Bacterial degradation of aromatic compounds starts with stereospecific dioxygenation catalyzed by dioxygenases with formation of *cis*-dihydro-dihydroxy compounds. In wild strains of microorganisms these diols undergo dehydrogenation by *cis*-diol-dehydrogenases (DDH) to corresponding catechols. In mutant strains deprived of DDH, intermediate *cis*-dihydro-diols can be obtained as sole products. These *cis*--diols are valuable synthons in preparation of useful synthetic intermediates[1].

More than ten years ago Nikolai S. Zefirov (Moscow State University) suggested that application of such dioxygenase-catalyzed reaction to pyridines could lead to creation of enzymatic approach to synthesis of the naturally-occurring polyhydroxylated piperidine alkaloids[2]. By this time it was already known that bacterial catabolism of pyridines does not involve dioxygenation as a first step[3]. That is why microorganisms containing aromatic dioxygenases were chosen to explore such reaction on pyridines. However already first steps in this direction showed that the more electron poor pyridine ring is an unsuitable substrate for dioxygenation. For example, it was shown that *Pseudomonas* sp. NCIB 9816-4 containing naphthalene dioxygenase (NDO) transformed 4-pyridinecarboxaldehyde to isonicotinic acid and 4-hydroxymethylpyridine without any oxidation of heteroaromatic ring[4].

However when 1-methyl-2-pyridone (where electron-releasing substituents are present) was chosen as a substrate for NDO, dioxygenation led to corresponding dihydro-dihydroxypyridones (L. M., unpublished results), the structure of which and absolute stereochemistry were determined as *cis*-(5*S*,6*S*)-1-methyl-5,6-dihydroxy-5,6-dihydro-2-pyridone

(1) and *cis*-(3*S*,4*S*)-1-methyl-3,4-dihydroxy-3,4-dihydro-2-pyridone **(2)**[5, 6].

It was shown that besides NDO this reaction can be also catalyzed by toluene and biphenyl dioxygenases[6]. However, all these reactions were carried out by mutant strains of microorganisms – of which there are not many. Moreover those dioxygenases cannot accept the more polar, than 1-methyl-2--pyridone, pyridines as substrates.

Now we have found that *cis*-dihydro-dihydroxypyridones **1** and **2** can also be obtained successfully with *Pseudomonas* sp. NCIB 9816-4 (a wild-type strain containing both NDO and naphthalene *cis*-dihydrodiol dehydrogenase (NDD).

What is even more important, we have not found any traces of products of aromatization: dihydroxypyridones or phenols. This clearly indicates that dihydro-diols **1** and **2** are not substrates for NDD.

This finding allows us to broaden the circle of microorganisms, which can be applied for dioxygenation of various pyridine derivatives.

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P208 CARBAZOLE HYDROXYLATION BY *Aspergillus flavus* VKM Ac-1024

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Keywords: biotransformation, carbazole, hydroxycarbazoles

Carbazole (Cz) is the tricyclic aromatic *N*-heterocyclic compound well known as a highly persistent environmental pollutant. On the other hand, its hydroxylated derivatives represent valuable pharmaceutical precursors[1].

The strain of *Aspergillus flavus* VKM Ac-1024 was selected among 300 species as showed high hydroxylase activity towards *N*-heterocyclic compounds. No 4- or 5-hydroxy indoles were formed from indole, while 1-benzoyl indole was converted by introducing hydroxyl function at the position 4 followed by cleavage of benzoyl substituent to form 4-hydroxy indole as a major product[2].

In the present study biocatalytic potential of this organism in respect of Cz and its *N*-substituted derivatives was estimated. The structure of metabolites was determined by H1-NMR, MS and GLC analyses.

3-Hydroxy carbazole was revealed as a major bioconversion product, while 1-hydroxy and 2-hydroxy carbazoles were formed in minorities at Cz conversion. The hydroxylation position shifted to preferable accumulation of 2-hydroxy carbazole and the formation of double hydroxylated 2,6- and 2,7-dihydroxy carbazoles in the presence of 1-benzoyl indole. The effect was never described so far. The conversion of *N*-substituted carbazole derivatives was found to depend on the type of substituent. No conversion of *N*-benzoyl carbazole was observed, while *N*-acetyl carbazole was transformed to carbazole and its 2- and 3-hydroxy derivatives.

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P209 ONE-POT EXTRACTION-SOLVOLYSIS OF TRIACYLGLYCERIDES CATALYZED BY *Rhizopus oryzae* RESTING-CELLS IN SOLVENT AND SOLVENT-FREE MEDIA

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RAMON CANELA[a, b], and MERCÈ TORRES[c]

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Keywords: resting-cells, *Rhizopus oryzae*, hydrolysis, esterification, one-pot extraction

The synthesis of heterocyclic hydrocarbons with one or several hydroxy groups in a ring is one of the most challenging tasks in modern organic chemistry. Biocatalysis allows obtaining of ring hydroxylated *N*-heterocycles not accessible by conventional chemical synthesis.

Acylglycerides are usually extracted from plant or animal material using physico-chemical or physical methods. Crude extracts are then purified and finally modified[1]. Enzymatic transformations are a potentially attractive method for the modification of the physical and chemical properties of edible

fats and oils[2]. The mild conditions associated with these transformations should permit one-pot extraction and transformation of acylglycerides to the desired compounds. Thus, Klass and Warwel have recently proposed using a commercial immobilized enzyme, deposited at the bottom of a soxhlet--apparatus, to transesterify triacylglycerides extracted from the oilseed-reservoir[3]. For the present study, an endophytic fungus isolated from a plant was selected. The ability of its resting-cells to carry out the one-pot extraction and transformation of acylglycerides in solvent and solvent-free systems was then studied.

Milled oilseeds or coffee grounds and fungal resting-cells were shaken at 50 °C. An appropriate amount of water and/or alcohol was added at the due time. An organic solvent was added either at starting or near the end of the process. The resulting organic solution was recovered by filtration and evaporated. Aliquots of the residue were dissolved and analyzed.

The one-pot extraction-solvolysis produced a 56–99 % of crude extract containing 33–95 % fatty acid esters and 3–75 % free fatty acids. The amount of water present in each material, the solvent, the alcohol, and the hardness of the solid material used have influence on the final results of the described process.

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P210 NOVEL HYDROLASES FROM THERMOFILIC FUNGI FOR STEREOSELECTIVE BIOTRANSFORMATIONS

VIKTÓRIA BÓDAI[a, b], CSABA PAIZS[c], MONICA TOŞA[c], CORNELIA MAJDIK[c], SAROLTA PILBÁK[c], LAJOS NOVÁK[a], FLORIN-DAN IRIMIE[c], GYÖRGY SZAKÁCS[b], and LÁSZLÓ POPPE[a]

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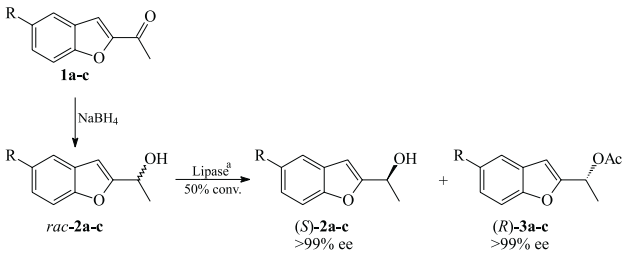
Keywords: hydrolases, thermophilic filamentous fungi, enzyme catalysis, kinetic resolution

By a novel method involving an enzymatic alcoholysis as key step benzofurane derivatives were prepared[1]. From the benzofurane-2-yl ketones **(1a-c)** obtained by this method, asymmetric baker's yeast reductions were performed, resulting the corresponding alcohols (*(S)*-**2a-d)** of moderate enantiomeric purities[2].

As an alternative to this bioreduction, the racemic alcohols **(2a-c)** were acylated in a highly enantiomer selective way (Scheme). For this transformations, a series of novel hydrolases from thermophilic fungi were produced and tested as biocatalysts[3].



R = H, Br, NO_2

[a] cca 80 different lipases were tested as biocatalysts in acylation

The lipase-catalysed acylation results were extended to further heterocyclic compounds. Using the appropriate lipases, some of these highly enantiopure compounds were also produced in a continuously operating bioreactor – extractor cascade.

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P211 NOVEL HYDROLASES FROM THERMOPHILIC FILAMENTOUS FUNGI FOR ENANTIOMER AND ENANTIOTOPIC SELECTIVE BIOTRANSFORMATIONS

VIKTÓRIA BÓDAI[a, b], GYÖRGY SZAKÁCS[b], CSABA PAIZS[c], SAROLTA PILBÁK[c], RÉKA PEREDI[d], JÓZSEF BÁLINT[d], GABRIELLA EGRI[d], LAJOS NOVÁK[a], JÓZSEF DUKAI[e], and **LÁSZLÓ POPPE**[a]

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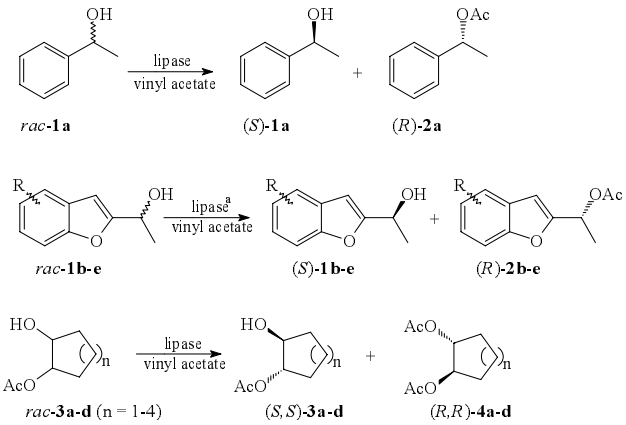
Keywords: hydrolases, thermophilic filamentous fungi, enzyme catalysis, kinetic resolution, asymmetric catalysis

A series of thermophilic filamentous fungi were cultivated under different conditions and were assayed for lipase/carboxylesterase activities[1]. The enzyme preparations were tested as biocatalysts in organic solvents. Kinetic resolution of racemic aryl/hetaryl methyl carbinols (*rac*-**1**)[1] or racemic *trans*-cycloalkane-1,2-diol monoacetates (*rac*-**3**)[2] (Fig. 1) and desymmetrisation of 2-acyloxypropan-1,3-diols **(5)**[1] (Fig. 2) by acetylation with vinyl acetate were chosen for testing the biocatalytic abilities of these preparations.



a – cca 70 different lipases were tested as biocatalyst in acylation

Fig. 1. **Kinetic resolution of racemic alcohols**

First, acetylation of racemic 1-phenylethanol (*rac*-**1a**) was performed[1]. Then the lipase-catalysed acylation was extended to further heterocyclic compounds, such as 1-(benzofuran-2--yl)ethanols (*rac*-**1b-e**). Using the appropriate lipases, some of these highly enantiopure compounds were also produced in a continuously operating bioreactor – extractor cascade. Next, kinetic resolution of a series of racemic *trans*-cycloalkane-1,2-diol monoacetates (*rac*-**2a-d**) was performed.

Finally, asymmetric acylation of 2-acyloxypropan-1,3--diols **(5a, b)** have been chosen for testing the enantiotopic selectivities of the novel biocatalysts.

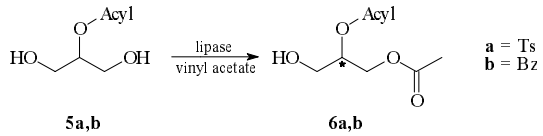


Fig. 2. **Asymmetric acetylation of prochiral 1,3-diols**

In summary, the tested biocatalysts proved to be superior over the commercially available enzymes with respect to the degree of enantiomer selectivity, and they exhibited a wider range of enantiotopic selectivity than the most common commercial enzymes.

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P212 CLONING AND EXPRESSION IN *E. coli* OF THE GENE ENCODING *Streptomyces* PMF PLD, A PHOSPHOLIPASE D WITH HIGH TRANSPHOSPHATIDYLATION ACTIVITY

DANIELA MONTI[a], CARLO ZAMBONELLI[a], PAOLA MORANDI[b], MARIA ANTONIETTA VANONI[b], GABRIELLA TEDESCHI[c], ROSSANA DI LORENZO[a], STEFANO SERVI[a, d], BRUNO CURT[b], and GIACOMO CARREA[a]

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Keywords: phospholipase D, *Streptomyces* PMF, cloning, heterologous expression

Phospholipases D (PLDs) hydrolyze phospholipids to yield phosphatidic acid and the corresponding alcohol and catalyze a transesterification (transphosphatidylation) reaction when alcohol is present as a nucleophilic donor. Bacterial forms of PLDs have shown to be suitable as biocatalysts for the synthesis of phospholipid derivatives of industrial interest.

Recently, PLD from *Streptomyces* PMF, an enzyme with a high transphosphatidylation activity, was purified and its crystallographic structure was solved at 1.4 Å.

A 315 bp fragment of the *pld* gene of *S.* PMF was amplified by PCR using chromosomal DNA as template and a pair of heterologous primers based on *S. antibioticus pld* gene sequence. The complete *pld* gene was isolated by colony hybridization and sequenced. DNA sequence analysis revealed a significant similarity with known *pld* gene sequences and showed the presence of highly conserved sequence motifs, namely the HKD motifs, shared by other members of the PLD superfamily. In order to promote the secretion of the protein into the medium, the mature PLD gene was fused in a pET derivative with the PelB signal sequence, and expression was performed in *E. coli* BL21(DE3)pLysE cells after induction with IPTG. Recombinant PLD activity detected in the culture supernatant was purified to homogeneity and structural and functional analyses confirmed the identity of the recombinant PLD with the wild-type protein.

P213 INVERSION OF THE ENANTIOSELECTIVITY OF ARYLMALONATE DECARBOXYLASE BY POINT MUTATION

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Keywords: arylmalonate decarboxylase, asymmetric decarboxylation, inversion of the enantioselectivity, point mutation

We have demonstrated that arylmalonate decarboxylase (AMDase; EC. 4.1.1.76) catalyzes asymmetric decarboxylation of arylmalonates to give optically pure *(R)*-α-arylpropionates. This enzyme has four cysteine residues and one of which, Cys188, is estimated to be located in the active site. It is estimated that it delivers a proton from the *si*-face of the intermediate enolate to give *(R)*-product (Fig. 1)[1].

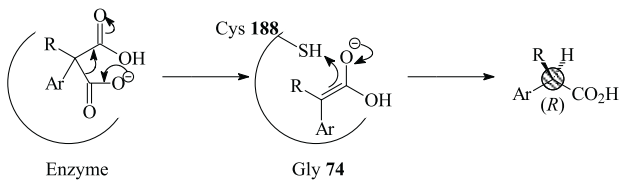

Fig. 1. **Active site of AMDase**

From the result of the data base searches, it is revealed that AMDase has some homology with Glu racemase. Glu racemase has a pair of Cys, 73 and 188, in the active site, which are estimated to provide a proton from both side. On the other hand AMDase has only one Cys at 188, and this is the reason why this enzyme gives optically pure products (Fig. 2). The estimated 3D-structure of AMDase suggested that Ser71 is in the opposite side of Cys188 (Fig. 3).

Glu racemase	-NDPI ~	ALVIA**C**NTAT ~	LVG**C**THFPL ~
Asp racemase	MFFSI ~	FIIMP**C**NTAH ~	VILG**C**TELSL ~
Hyd* racemase	MKVIN ~	AFVIA**C**WGDP ~	ILLG**C**AGMAE ~
Mal* isoracemase	---MK ~	VMAYA**C**LVAI ~	LSA-**C**VQMPS ~
AMDase	-MQAS ~	VVSLM**G**TSLS ~	ILLS**C**GGLLT ~
		74	**188**

Hyd* – hydantoin, Mal* – maleate

Fig. 2. **Homology of AMDase with some enzymes**

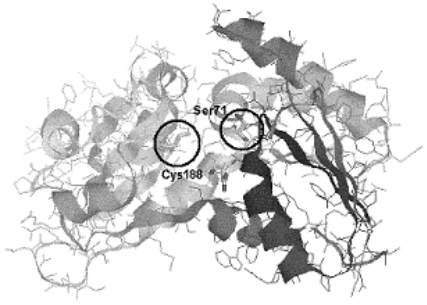

Fig. 3. **Estimated 3D-structure of AMDase**

Then we expected that introduction of one Cys around 71 to 76 and replacement of Cys188 with less acidic Ser might invert the enantioselectivity of the enzyme. Thus we prepared 6 mutants in which Cys188 was changed with Ser, and either one of the amino acid from 71 to 76 was changed with Cys. As the result, two double mutants, S71C/C188S and G74C/ /C188S exhibited decarboxylation activity and gave the opposite enantiomer of the products from wild type enzyme.

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P214 CHIRAL ALCOHOL PRODUCTION BY β-KETOESTER REDUCTASE FROM *Penicillium citrinum* COUPLED WITH REGENERATION SYSTEM OF NADPH

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Keywords: reductase, aldo-keto reductase super family, mutation

We isolated NADPH-dependent alkyl 4-halo-3-oxobutanoate reductase (RPC) from *Penicillium citrinum*. RPC catalyzed the reduction of methyl 4-bromo-3-oxobutanoate (BAM) to methyl *(S)*-4-bromo-3-hydroxybutanoate (BHBM). The product, *(S)*-BHBM, is a potential key intermediate for the synthesis of a variety of HMG CoA reductase inhibitors.

The *rpc* gene comprises 978 bp and encodes a polypeptide of 36.7 kDa. The deduced amino acid sequence was found to be similar to well-known sequences of the aldo-keto reductase superfamily. The *rpc* gene and glucose dehydrogenase gene *(gdh)* to regenerate cofactor were overexpressed in *Escherichia coli*. The recombinant *E. coli* cells produced *(S)*-BHBM from BAM in the presence of an NADPH-regeneration system.

To increase thermostability of RPC, the recombinant RPC expressed in *E. coli* was evolved by the error-prone polymerase chain reaction (epPCR) method. We isolated three mutants (A3-49, A8-39 and T1-99) with increased thermostability. Mutant T1-99 was improved not only thermostability but also enantioselectivity. Changes in the amino acid sequence of the mutant enzymes were identified by analyzing the nucleotide sequence of the genes. Mutant T1-99 had two amino acid changes (L54Q and R104C).

The effect of each amino acid residue on the thermostability and enantioselectivity of RPC was investigated. The only mutant L54Q revealed higher thermostability and enantioselectivity than those of wild-type RPC. An alignment of the deduced RPC amino acid sequence with other aldo-keto reductases suggested that L54 is close to the active site of the enzyme[1]. K_m value of the mutant L54Q was estimated to be similar to that of the wild-type enzyme, but K_{cat} value was slightly decreased.

Then L54 was subjected to saturation mutagenesis to give ten different mutants with increased thermostability. Fourteen different mutants catalyzed the formation of *(S)*-BHBM with higher enantioselectivity than the wild-type enzyme.

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P215 HETEROLOGOUS EXPRESSION AND SITE DIRECTED MUTAGENESIS OF ALKENE MONOOXYGENASES FOR IMPROVED ACTIVITY AND STEREOSELECTIVITY

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Keywords: alkene monooxygenase, site directed mutagenesis, stereoselectivity

Alkene monooxygenases are found in bacteria isolated on low molecular weight alkenes (eg ethene, propene), and convert the alkene to an epoxide as the first step in metabolism. They are part of the bi-nuclear non-haem iron family of oxygenases which includes methane monooxygenase and attack the alkene double bond to form epoxides, selectively, with no reactivity against alkanes[1]. Depending on the enzyme and substrate, these enzymes can exhibit moderate to good stereoselectivity, forming *R*-epoxides from pro-chiral 1-alkenes. To date, alkene monooxygenases have only been found in a narrow range of bacteria including the high GC Gram positive *Mycobacteria* sp. and *Rhodococcus* sp. and the Gram negative *Xanthobacter autotrophicus*. These are relatively slow growing organisms.

Despite the fact that homologues such as toluene monooxygenase are actively expressed in *E. coli*, it has proved difficult to express alkene monooxygenases in this host although expression can be obtained in more closely related hosts (eg other high GC Gram positive organisms). This paper will describe progress towards expression of the enzyme from *X. autotrophicus* in *E. coli* and site directed mutagenesis of a mycobacterial enzyme expressed in *M. smegmatis*. By sequence alignment and comparison with the known 3D structure of methane monooxygenase[2], residues have been identified which exhibit a systematic variation between different types of enzyme (aromatic, alkene, methane), and other residues identified which vary systematically with stereoselectivity of alkene epoxidation. Mutagenesis of these residues is starting to provide some insight into the differences between alkene and alkane oxidation and control of stereoselectivity.

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P216 DIRECTED EVOLUTION OF *dszABC* OPERON FROM *Rhodococcus* SP. DS7: EVALUATION OF THE SELECTED MUTANTS FOR BIODESULFURIZATION ACTIVITY ON DIFFERENT SUBSTRATES

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Keywords: biodesulfurization, directed evolution, substrate specificity

The combustion of sulfur-containing fossil fuels produces environmentally hazardous SO_x gases that released in the atmosphere generate air pollution and acid rain. Conventional hydrodesulfurization (HDS) of petroleum fractions involves an inorganic catalyst and hydrogen under conditions of high temperature and pressure to produce hydrogen sulfide and a desulfurized compound. Even though HDS is an effective and well-understood technology, heterocyclic sulfur compounds cannot be completely removed. The slow-reacting, recalcitrant components all belong to the class of dibenzothiophenes, like dibenzothiophene (DBT) and its alkylated derivatives. Since the 1980s several microorganisms able to selectively desulfurize DBTs have been isolated and most of them are Gram-positive like *Rhodococcus* sp. strain IGTS8 described by Kilbane[1] capable to remove sulfur from DBT in a C-S bond-targeted fashion to produce 2-hydroxybiphenyl (HBP) and sulfite. Therefore the application of a biodesulfurization process using a DBT-desulfurizing microorganism following HDS is an attractive possibility for achievement of deeper desulfurization but the substrate specificity is a strong limitation.

The conversion of DBT to HBP is catalyzed by a multienzyme pathway proceeding *via* two cytoplasmic monooxygenases (DszC and DszA) supported by a flavin reductase (DszD) and a desulfinase (DszB). This pathway resembles other sulfur removing pathways with the feature that the carbon skeleton is not mineralized.

With the aim to generate new enzymes able to better degrade DBTs alkylated derivatives, an *in vitro* evolution approach has been applied to the *dsz* operon from *Rhodococcus* sp. DS7, a DBT-degrading strain isolated in our laboratories[2].

Using epPCR a library of 5×10^5 mutants has been created and over 10^4 mutants have been screened for their ability to metabolize different substrates.

Data concerning the biodesulfurization activity of a number of selected mutants will be presented.

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P217 MASTERING POX GENOTYPE FOR FATTY ACID TRANSPORT AND ACCUMULATION IN THE YEAST *Yarrowia lipolytica*

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Keywords: *Yarrowia lipolytica*, lipids, acyl-CoA oxidase, protrusion, single cell oil

Due to its ability to produce different metabolites such as citrate, and to secrete numerous proteins, the yeast *Yarrowia lipolytica* has been used in industrial processes. It is also able to grow on and degrade alkanes and to use fatty acids as carbon sources, which makes it a promising tool for biotechnological uses[1]. Its capacity to degrade oils of different origins makes it a potential de-polluting agent and for single cell oil production (SCO)[2].

Y. lipolytica could be used for γ-decalactone (peach flavor) production. This yeast can be used for lipase production[3,4]. The *Y. lipolytica* lipase Lip2p was shown to be efficient for carboxylic esters resolution.

Degradation of alkanes involves three enzymatic steps in endoplasmic reticulum to produce a fatty acid. Similarly, degradation of triglycerides is performed by secreted lipases which release the corresponding fatty acids, which are then metabolized via β-oxidation.

The initial step of peroxisomal β-oxidation is catalyzed by the acyl-CoA oxidases (Aoxs) encoded by the *POX* genes. *Y. lipolytica POX* set is the most complex among the yeasts studied to date, as it comprises five different genes encoding Aoxs.

Functions of different acyl-CoA oxidases have been investigated by successive gene disruptions (construction of mono-, bi-, tri- and tetradisruptants)[5]. These genes encode for proteins exhibiting different specificities with respect to the chain lengths of the acyl-CoA substrates. Aox3p and Aox2p from *Yarrowia lipolytica* were bacterially expressed, purified and their activity as a function of substrate chain length was established[6, 7]. Using DNA shuffling techniques, we have constructed 11 chimeras between Aox2p and Aox3p, two of them exhibit broad chain length specificity. Tri and tetra- disruptants were transformed with Aox2p, Aox3p and chimera encoding genes. Growth in oleic acid (0.1–5 %), peroxisomes and lipid bodies proliferation was followed during a 60 h time course. We determined cellular, medium and surface lipid composition by TLC and GC/MS. In addition, cell surface and organelles formation were analyzed by scanning and transmission electron microscopy.

Our results demonstrate the induction of protrusions on yeast cell surface involved in oil droplet fixation. In those strains, peroxisome proliferation and size were not significantly modified. In contrast, *POX* genotype affected dramatically lipid bodies formation (few lipid bodies up to giant lipid bodies), alters lipid composition (no triglycerid accumulation up to high TG accumulation).

These demonstrates that mastering *POX* genotype allowed us to construct strains with high lipid content which could be used for.

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P218 PRODUCTION OF RUBBER MOLECULES WITH RECOMBINANT *Hevea brasiliensis* RUBBER TRANSFERASE

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Keywords: rubber transferase, *cis*-prenyl chain elongation, c-DNA cloning, *Hevea brasiliensis*, polyprenols

Natural rubber from *Hevea brasiliensis* is the high molecular weight polymer of isoprene units with *cis*-configuration. The enzyme responsible for the *cis*-1,4-polymerization of isoprene units has been shown as a particle-bound rubber transferase, but no gene encoding this enzyme has been cloned in rubber-producing plants. Using sequence information of the conserved regions of *cis*-prenyltransferases cloned recently from *Micrococcus luteus* B-P 26 (ref.[1]), *Escherichia coli* (ref.[2, 3]), yeast[4] and *Arabidopsis thaliana* (ref.[5, 6]), we have isolated and characterized the cDNA for the rubber transferase from *Hevea brasiliensis*. Sequence analysis revealed that all five highly conserved regions among *cis*-prenyltransferases[7] are found in this protein sequences, which are totally different to those of *trans*-prenyl chain elongating enzymes[8].

In vitro rubber production using the recombinant enzyme overexpressed in *E. coli* cells revealed that the rubber transferase catalyzed the formation of medium chain and long chain polyisoprenes with approximate molecular size of $2 \times 10^3 \sim 1 \times 10^6$ Da in the presence of washed bottom fraction particles. Neither the recombinant enzyme nor washed bottom fraction particles alone showed significant activity for rubber production. This result suggested that *Hevea* rubber transferase might require some activation factors in the washed bottom fraction particle for the production of high molecular weight rubber molecules.

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P219 GEL-STABILIZED TWO-PHASE SYSTEMS: NEW APPROACHES TO THE ENZYMATIC SYNTHESIS OF HYDROPHOBIC FINE CHEMICALS

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Keywords: enzyme, entrapment, gel, hydrophobic compound

Within the last decades, enzyme catalysis proved to be a versatile tool in the synthesis of chemical compounds, mainly due to the broad substrate range and selectivity of enzymes. The technical application, however, is often restricted to the production of fairly water-soluble molecules as hydrophobic compounds are only inefficiently converted in aqueous phases, while many enzymes are instable in the presence of non-aqueous media such as organic solvents. Thus, the use of enzymes for the synthesis of hydrophobic molecules, which are known to be important building blocks of fine chemicals, pharmaceuticals or agrochemicals, requires the development of alternative reaction techniques.

In this work, gel-stabilized two-phase systems, meaning two-phase systems, consisting of a gellified aqueous phase and a non water-miscible organic solvent, were investigated. Enzymes from different classes, an alcohol dehydrogenase, a lipase, and a decarboxylase, were entrapped in the natural or synthetic polymer matrices and characterized with regard to their stability, activity, and/or selectivity in the synthesis of hydrophobic chiral alcohols, aroma esters and chiral hydroxy ketones, respectively. It was demonstrated that the stability of an alcohol dehydrogenase with a usually high sensitivity to the presence of organic solvents was strongly enhanced. Loss of its cofactor during gel preparation was avoided by the use of an emulsion technique, and cofactor regeneration was performed within the matrix by coupling the reduction of ketones to chiral alcohols with the oxidation of isopropanol. The stereoselectivity of both, the alcohol dehydrogenase and the decarboxylase was unaffected by the system features, and the product yield was high with all three enzymes. The overall productivity of the reaction systems not only depended on the entrapped biocatalysts, but also on the polymer type used as the aqueous phase.

P220 SOL-GEL BIOCOMPOSITE MATERIALS AS SOLID-PHASE BIOCATALYSTS

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Keywords: sol-gel, silica, entrapment, hydrolases

One of the most important issues when using enzymes for developing active biocatalysts is whether they remain functional, during immobilization and over time, and to what degree. The entrapment of proteins into sol-gel derived silica glass matrices (monoliths, thin films, powders) has been proposed as a potential generic method for obtaining stable encapsulated biomolecules[1–3]. The accessibility of substrates to the entrapped enzyme is determined largely by the pore size and the electrostatic interactions with the silica matrix, which can be altered by various methods[4,5]. We have employed variation of sol-gel synthesis conditions, entrapped enzyme concentration and silane:solvent:water ratio, manipulation of aging conditions and use of molecular templating agents to promote larger pores and to maximise accessibility.

The present study is focused on the use of polymer or small molecule additives to enhance the stability of some hydrolytic enzymes. A combined method of sol-gel entrapment/deposition on inorganic support has been used to reduce structural compresion and internal mass-transfer limitations. Alcalase was immobilised by entrapment in sol-gel derived silica deposited on celite, alumina, zeolite and silica. An increase in activity was achieved, especially with celite. These results suggest that the microenvironment experienced by the entrapped biomolecule can be manipulated by dispersing organic dopants that stabilise the enzyme protein, preserving its biochemical function.

The enzyme accessibility and structure (native or unfolded), and the charge and polarity of the local environment affect the catalytic properties of the molecule (catalytic constant k_{cat} and Michaelis constant K_m).

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P221 IMMOBILISATION OF P450 BM-3 AND AN NADP$^+$ COFACTOR RECYCLING SYSTEM: TOWARDS A TECHNICAL APPLICATION OF HEME-CONTAINING MONOOXYGENASES IN FINE CHEMICAL SYNTHESIS

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Keywords: biotransformation, cofactor-recycling, cytochrome P450 BM-3, immobilisation, sol-gel

Cytochrome P450 monooxygenases are potentially a very useful class of hydroxylation catalysts; they are able to introduce oxygen at activated and non-activated carbon-hydrogen bonds[1]. These hydroxylation reactions can lead to regio- and/or stereochemically pure compounds. Stereo- and regiospecific hydroxylations are a task very difficult to perform by traditional organic chemistry. However, this potential is lowered by the intrinsic low activity and inherent instability of cytochromes P450. Additionally P450-catalysed biotransformations require a constant supply of NAD(P)H, making the process an expensive one.

P450 BM-3 (EC 1.14.14.1) and various mutants thereof were shown to accept a broad range of substrates[2]. To render these catalysts more suitable for industrial biocatalysis, the immobilisation of P450 BM-3 (CYP 102) from *Bacillus megaterium* in a sol-gel matrix[3] derived from TEOS was combined with a cofactor recycling system based on NADP+-dependent formate dehydrogenase (EC 1.2.1.2) from *Pseudomonas* sp. 101 (ref.[4]) and tested for practical applicability. Sol-gel immobilised P450 BM-3 showed enhanced properties with respect to long-term stability and activity. Beside oxidation of the model substrate *p*-nitrophenoxydecanoic acid (10-pNCA)[5], this approach was used for the conversion of *β*-ionone into 4-hydroxy-*β*-ionone using a sol-gel immobilised P450 BM-3 mutant and sol-gel immobilised FDH for *in situ* regeneration of NADPH.

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P222 ENZYMATIC SYNTHESIS OF OLIGOSACCHARIDES ON A DENDRIMERIC SOLUBLE SUPPORT

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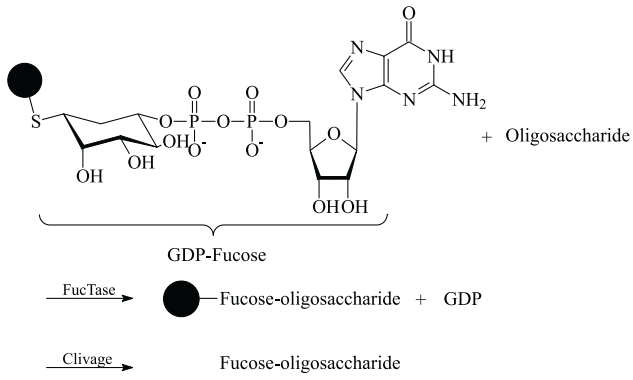
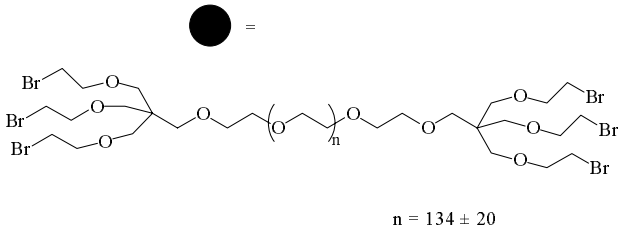
Keywords: supported synthesis, dendrimeric support, nucleotide-sucre, fucosyltransferase

The advantages of enzymatic carbohydrate synthesis are nowdays well demonstrated. The major problem in this strategy remains the purification of the products. In order to simplify this crucial step, our solution is using supported enzymatic chemistry either with immobilized acceptor as already reported by us[1] or donor as shown here.

We are aiming towards the successful total synthesis of a supported GDP-fucose – substrate of the fucosyltransferase – in order to synthesize fucosylated oligosaccharides without the need to attach at each time the acceptor on the support as shown in the scheme below.

The dendrimeric support developed in our laboratory is a brominated derivative of polyethylene glycol. This allows the facile coupling of the sugar to the polymer by nucleophilic substitution. Here, we report the total synthesis of the supported GDP-fucose on the soluble dendrimeric polymer and its enzymatic activity towards the α(1-4)-fucosyltransferase.





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P223 IMMOBILIZATION OF INULINASE FOR SUCROSE HYDROLYSIS

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Keywords: biotransformation, Ca-alginate entrapment, enzyme immobilization, inulinase

Sucrose hydrolysis is currently performed using invertases. Research work, aiming at improving the efficiency of this bioconversion process has revolved around the use of immobilized invertases. Recently a different approach, the use of a new biocatalyst, has been suggested. This is based on the activity for sucrose hydrolysis evidenced by crude enzyme preparations of inulinases from *Aspergillus ficuum*[1]. Still, the use of an immobilized form of the biocatalyst is advised, due to the many advantages this approach provides. In this work, a screening of immobilized forms of a commercial inulinase preparation for sucrose hydrolysis was performed. The highest immobilization yields were obtained with inulinase entrapped in Ca-alginate beads. Increasing the concentration of Ca from 1.5 % to 4 % led to a 15-fold increase in the immobilization yield. This was ascribed to a reduction in enzyme leakage of the enzyme preparation, despite a foreseeable increase in diffusional resistance for substrate migration within the beads. No significant shifts in optimal pH or temperature were observed when the free and immobilized forms of the biocatalyst were compared.

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P224 IMPROVING GERANYL ACETATE SYNTHESIS IN SUPERCRITICAL FLUIDS WITH ZEOLITES

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Keywords: Novozym 435, *Candida antartica* lipase B, zeolite, salt hydrate, supercritical fluid, water activity

Zeolite molecular sieves are very commonly used as *in situ* drying agents in reaction mixtures of enzymes in non-aqueous media. Recently, we have shown that zeolites can have acid-base effects on enzymes in low-water media, resulting from their ionexchange ability[1]. We have also demonstrated that pairs of salt hydrates, commonly used to fix water activity (a_w) *in situ*, can have similar effects[2]. Here, we studied the impact of zeolites NaA and NaY on an immobilized enzyme (Novozym 435) in an esterification reaction, in supercritical ethane and supercritical CO_2. To avoid drying effects of the zeolites, these were pre-equilibrated to the a_w of the experiments. The experiments that were done in the presence of zeolite NaA yielded higher initial rates and higher conversion than assays performed in the presence of pairs of salt hydrates, especially at the a_w values of 0.1 and 0.75. These results are explained on the basis of acid-base effects of both the zeolites and the salt hydrate pairs.

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P225 CROSSLINKING OF PROTEINS BY PEROXIDASE-MEDIATED OXIDATIVE DEHYDROGENATION IN THE PRESENCE OF EXOGENOUS PHENOLS

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Keywords: enzymatic, crosslinking, proteins, peroxidase

Cross-linking of proteins is of great interest to the food industry as a way to enhance protein functionality, thereby increasing their value. Use of enzymes for protein cross-linking is a viable alternative to the chemical routes, since enzymatic reactions are highly specific and require mild reaction conditions, thus minimising the risks of both protein denaturation and formation of possible toxic side products.

The present paper reports a systematic study on the cross-linking of proteins by peroxidase-mediated oxidative dehydrogenation in the presence of phenols as low molecular weight hydrogen donors. Two globular proteins, α-lactalbumin and bovine serum albumin (BSA), and β-casein, a protein with a flexible open structure, have been used as model proteins. A range of mono-, di-, and polyphenols with different ring substitution pattern has been used as the second hydrogen donor. Both the phenolic side-chain of tyrosine (Tyr) residues in proteins and the free (poly)phenols react with the active compounds I and II of peroxidase to generate reactive radicals that react further with each other to form homo- and hetero-products. Under controlled reaction conditions, the reaction can be stirred toward synthesis of high molecular weight (HMW) protein-phenol hetero-conjugates.

Full conversion of β-casein into high molecular weight polymers has been achieved. However, peroxidase-phenol mediated crosslinking of BSA and α-lactalbumin resulted only in partial protein conversion and formation of oligomers. This difference in reactivity has been associated with the specific conformation of each protein that restricts the accessibility of tyrosyl moieties for reaction. Conditions have been found to induce controlled conformational changes of BSA and α-lactalbumin to increase their conversion into polymers.

Different product patterns and conversions have been obtained by using a range of substituted mono- and polyphenols. *Ortho*- and *para*-diphenols are the most efficient crosslinking agents in the reactions studied. Bulky substituents in *ortho*- and *para*-positions of the aromatic ring reduce the reactivity of phenols due to steric hindrance. The reactivity of phenols in the peroxidase-mediated crosslinking of proteins has been correlated with their redox and ionisation potential.

Based on the results of this study and the products identified in model studies with a tyrosine-containing peptide[1,2], a tentative model for peroxidase-mediated protein cross-linking in the presence of phenols is proposed. The effect of crosslinking on the functional properties of the proteins is also discussed.

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P226 INVESTIGATION OF KINETICS OF IMMOBILIZED LIVER ESTERASE BY FLOW CALORIMETRY

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Keywords: flow calorimetry, kinetics, pig liver esterase, reaction-diffusion system

Flow calorimetry (FC) was used to estimate kinetic parameters of phenyl acetate hydrolysis by pig liver esterase immobilised in alginate gel. Pig liver esterase belongs to the group of hydrolytic enzymes as carboxyl esterases, aryl esterases, lipases, acetyl esterases, cholin esterases and cholesterol esterases, which are widely distributed in animals, plants and microorganism and show wide substrate tolerance with highest activity towards soluble state of its substrate. They are usually stable and even active in organic solvents. Because of high regio- and stereo-specificity, they are attractive biocatalysts for the production of optically pure components. Esterases preferentially break ester bond of shorter chain fatty acid. Methanol and other weakly acid alcohol are highly effective nucleophiles toward the acyl group in reactions catalyzed by esterases in order to raise the reaction rate[1]. Probably the most well-known up to date application is the production of vanillin from ferulic acid released by carboxyl esterase from plant cell wall polysaccharides such as pectin or xylan[2]. The hydrolysis of the matching substrate can obey substrate activation[3] or inhibition[4] or can follow the Michaelis-Menten kinetics[5]. The hydrolysis of phenyl acetate by pig liver esterase was shown to exhibit substrate inhibition. In the present work the course of enzymatic reaction was measured in steady-state (single flow mode) and non-steady-state (total recycling of reaction solution). From the single flow mode the dependence of steady-state thermometric signal on substrate was obtained and from the recycling mode the time dsependence of thermometric signal representing the substrate consumption in recycling section within the flow calorimeter system (Fig. 1) was obtained. The experimental data were treated by mathematical modelling, based on material and heat balances. In the mathematical model the calorimeter column was divided into five differential sections. The mathematical model represented a set of partial and ordinary differential and algebraic equations. Spatial derivatives in balance equations were discretized by finite difference Crank-Nicholson scheme and the FC column was solved by orthogonal collocation method using three interior collocation points within the particle with immobilized esterase. After introducing dimensionless parameters, the resulting set of ordinary differential and algebraic equations was solved

using Athena Visual Workbench software package (Stewart and Associates Engineering Software).

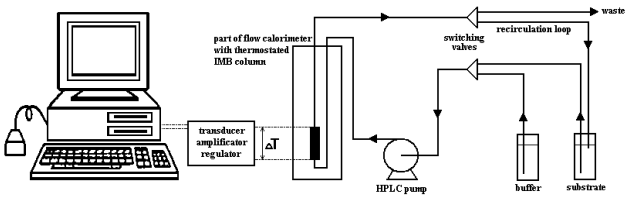

Fig. 1. **Experimental set-up for flow calorimetry**

The evaluation of data from total recycling led to the quantification of transformation parameter α, which enabled us to rewrite thermometric signals from steady-state experiment into steady-state reaction rates. Thus, the steady-state thermometric signal dependence on substrate concentration could be transformed to steady-state reaction rate dependence on substrate concentration. Afterwards, the latter dependence was optimized in order to obtain the true kinetic parameters of immobilized pig liver esterase.

The proposed technique enabled to determine intrinsic kinetic parameters of substrate inhibited enzyme reactions influenced by internal particle diffusion directly from calorimetric data, without using any additional analytical technique.

Conference presentation assisted by RHODIA company.

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P227 TAURINE CHLORINATION BY MYELOPEROXIDASE/H_2O_2/CL^- SYSTEM: A KINETIC STEADY-STATE STUDY

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Keywords: myeloperoxidase, steady-state kinetics, hypochlorous acid

Myeloperoxidase, MPO, joins the homologous mammalian peroxidase family, but differs from others in its physical and chemical properties. The most characteristic reactivity is its ability to catalyze the oxidation of chloride, Cl^-, by hydrogen peroxide, H_2O_2, to form the potent oxidant and bactericidal agent hypochlorous acid[1].

Available information shows that efforts on studying the kinetics of halogenation activity of peroxidases have been focused on the analysis of initial rates[2,3]. The use of this approach avoids the complexity of taking into account other side reactions also undertaken by this enzymatic system. Nevertheless, data obtained under pre-steady-state conditions yield knowledge only about the elementary steps leading to the formation of a halogenating agent or to a substrate halogenation.

Kinetic measurements after steady-state achievement incorporate all feasible concurrent processes, *i. e.* data so obtained permit a more accurate description of *in vivo* halogenation activity, and results can be better extrapolated to biological media.

The main findings attained for the rate of taurine chlorination catalyzed by the human MPO/H_2O_2/Cl^- enzymatic system are presented. Figure 1 shows a typical kinetic trace. The equilibrium between MPO compounds I and II

$$\text{MPO-I} + H_2O_2 \rightleftarrows \text{MPO-II} + HO_2^{\cdot}$$

has been here proved to strongly affect to the chlorination rate, provoking the wave shaped profile of kinetic traces. It should be considered the most important by-process influencing steady-state rate.

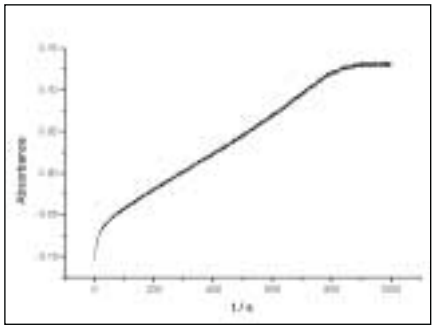

Fig. 1. **Kinetic trace obtained at λ = 252 nm**; [MPO] = 100 nM, [H_2O_2] = 0.8 mM, [Cl^-] = 100 mM, [taurine] = 10 mM, pH = 5.5, T = 298K

The dependence of reaction rate on the H_2O_2 concentration and the acidity of the medium is sketched in Figure 2. This values would correspond to the effective rate at which taurine is chlorinated *in vivo*, allowing the optimal conditions for the chlorination activity of MPO to be revealed.

In contrast with published data based on initial rate measurements, where rate of chlorination is enhanced with increasing pH value and/or H_2O_2 concentration[3,4], these data suggest that MPO chlorination activity reaches a maximum when [H_2O_2] ≈ 0.25 mM and pH ≈ 5.0. The present study

sheds light on the optimal conditions for *in vivo* studies of MPO-catalyzed chlorination reactions.

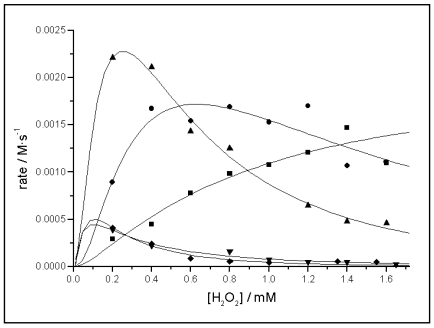

Fig. 2. **pH and [H_2O_2] influence on steady state rate value for taurine chlorination;** pH = 4.0 (■), 4.5 (●), 5.0 (▲), 5.5 (▼), and 6.0 (♦)

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P228 THE ELECTROCHEMISTRY OF HAEM PROTEINS IN NONAQUEOUS SOLVENTS

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Keywords: microperoxidase, cytochrome c, redox potential, thermodynamics, nonaqueous solvent

Enzymes such as trypsin and subtilisin are now widely used as efficient catalysts in nonaqueous solvents[1]. The use of redox enzymes is much less widespread, mainly due to their instability which arises from the need for an exogeneous oxidant, typically hydrogen or alkyl peroxides. We have investigated the electrochemistry of the haem proteins, microperoxidase and cytochrome c in a range of nonaqueous solvents. Dramatic shift in the oxidation potential of cytochrome c can occur, e. g. 900 mV in acetonitrile and ethyl acetate vs. 70 mV in aqueous buffer[2]. In acetonitrile, the heam is irreversibly oxidised, in ethyl acetate, an amino acid. The changes in redox potential of microperoxidase are much less pronounced, e. g. increasing by 100 mV in ethanol (vs. buffer). The thermodynamics of reduction of cytochrome c in glycerol were significantly changed, with $\Delta H^{\circ'}_{rc}$ decreasing from –35.6 to –47.7 kJ.mol^{-1} and $\Delta S^{\circ'}_{rc}$ from –35.2 to –76.8 J.K^{-1}.mol^{-1} (ref.[3]). By contrast, for microperoxidase, $\Delta S^{\circ'}_{rc}$ increased from 5 to 77 J.K^{-1}.mol^{-1}, while $\Delta H^{\circ'}_{rc}$ increased from 36 to 54 kJ.mol^{-1} (ref.[4]). The activation energy for reduction of cytochrome c was significantly increased from 5.7 kJ.mol^{-1} in aqueous buffer to 44.2 kJ.mol^{-1} in glycerol. These results demonstrate that enveloping the haem in an amino acid pocket can significantly affect the redox properties of the haem in nonaqueous solvents.

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P229 THE STUDY OF ELECTROCHEMICAL PROPERTIES OF THE REDOX ENZYMES IN ORGANIC SOLVENTS

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Keywords: mediated bioelectrocatalysis, galactose oxidase, immobilization, solvents

Galactose oxidase was used as a model enzyme for the investigation of effect of organic solvents on the electrochemical properties of the redox enzymes. The aim of this work was to obtain a stable response from the enzyme in organic solvent and todetermine any changes that occurred in electron transfer kinetics.

Cyclic voltammetry was used to measure rates of electron transfer. Ferrocene derivatives and some quinones were used as mediators for galactose oxidase. For experiments in organic media the enzyme was immobilized on the glassy carbon electrode by cross-linking with glutaraldehyde in the presence of bovine serum albumin and Nafion[1]. 3-Methoxybenzyl alcohol was used as the substrate for galactose oxidase. The affect of immobilization procedure on the response of enzyme electrode was studied. The experiments were carried out in carbonate buffer (pH = 10.8), 1,1'-dimethylsulfoxide, 1,1'-dimethylacetamide, 1,1'-dimethylformamide[2].

The results showed that the presence of Nafion in the electrode membrane increased the mechanical stability of the enzyme electrode. The enzyme film adhered better to the electrode surface. The response of the mediator was irreversible in the organic solvents – no reduction peak was observed. Bovine serum albumin improved the reproducibility of the catalytic current obtained. Experiments with dry solvents and with 10 % water added were carried out. No chan-

ges in enzyme electroderesponse were observed. In 30 % dimethylacetamide enzyme denatured rapidly. Naphthoquinone and dimethylquinone did not function as mediators of electron transfer between the active site of galactose oxidase and electrode. Ferrocene dimethanol, ferrocene methanol, ferrocene monocarboxylic acid were found to be good electron mediators for galactose oxidase. The electron transfer constant for galactose oxidase and ferrocene monocarboxylic acid was found to be $7.5 \times 10^5\ M^{-1}.s^{-1}$ in buffer[3].

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P230 THE IDEAL BIOCATALYST: THE NEW APPROACH AND ITS APPLICATION IN BIOCATALYSIS PROCESS DEVELOPMENT

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Keywords: biocatalyst, biochemical engineering

Recently, we reviewed a new approach in developing novel biocatalysts for industrial purposes[1]. The use of enzymes as biocatalysts in the industrial manufacture of fine chemicals and pharmaceuticals has enormous potential, but application has hitherto been limited largely by evolution-led catalyst traits. The advent of designer biocatalysts, produced by informed selection and mutation, through recombinant DNA technology, has enabled production of improved, process--compatible enzymes. The design and development of practical biocatalysts is being advanced and shaped by progress in protein structure-function studies, understanding of protein stability, conformational stability and activity. Modern molecular techniques have allowed us to visualize catalytic systems that approach the functional ideal. Biocatalysts can be now designed in a paradigm where the process conditions are no longer the defining or limiting constraints on successful biocatalytic processes, and the biocatalyst can be made to fit the purpose. To fully realize the potential of designer enzymes in industrial applications, it is necessary to tailor the catalyst properties so that they are optimal not only for a given reaction, but also in the context of the industrial process in which the enzyme is applied.

This paper will discuss these approaches, illustrating by using examples of biocatalyst development in the authors' current projects, and focusing most specifically on biocatalytic systems for oxidation of aromatic compounds and hydrolysis of cyclic amides (hydantoins). In the case of oxidase systems the products are potential antioxidants, and in the case of hydantoinases, the products are optically pure amino acids destined for use as pharmaceuticals. In both cases, reaction engineering approaches have resulted in effective biotransformation systems.

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P231 OPPORTUNITIES AND CHALLENGES FOR BIOCATALYSIS IN THE PHARMACEUTICAL INDUSTRY

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Keywords: biocatalysis, high throughout screening, tertitiary alcohols, secondary amines, kinetic resolution

Some of the major opportunities and challenges facing the field of biocatalysis within the pharmaceutical industry will be presented. The use of genome sequencing, bioinformatics and cloning to rapidly construct new and functionally diverse enzyme libraries, as well as the application of advanced HTS technologies towards the mining of these libraries and subsequent reaction optimization, will be briefly discussed. Opportunities for collaboration with second-generation enzyme suppliers will also be outlined. In addition, the lack of efficient methods for the synthesis of optically active secondary amines and tertiary alcohols, a major challenge facing the pharmaceutical industry today, will be addressed through the presentation of a general chemo-enzymatic resolution approach that has been developed and validated for a number of major blockbuster drugs within Pfizer. Furthermore, challenges facing the use of new enzymes, including nitrilases, epoxidases, transaminases, reductases, hydroxylases, glycosylases, and cyclases, will also be discussed. Finally, a general high throughput screening approach that has been validated for the enzymatic resolution of a number of key intermediates within Pfizer will be summarized, stressing the need for such a protocol in routinely evaluating the overlooked yet powerful technique of solvent engineering in enzymatic hydrolytic reactions[1].

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P232 FROM FERMENTATION ENGINEERING TO GENETICALLY ENGINEERED HOST CELLS: DIFFERENT APPROACHES FOR OPTIMIZATION OF BACTERIAL GLYCOSYLTRANSFERASES PRODUCTION

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Keywords: gene expression, glycosyltransferases, immobilization, protein engineering, protein folding

Glycosyltransferases have become powerful tools for the synthesis of oligosaccharides through their strict control over the stereo- and regioselectivity of glycosidic bond formation[1]. One major drawback of glycosyltransferases in synthesis is their limited availability. The cloning and over-expression of bacterial glycosyltransferases is one alternative to overcome this problem.

To make available an enzyme for synthetic purposes is necessary to develop efficient and cost-effective large-scale production strategies. Three factors are of main importance to successfully attempt to this goal: i) the expression system, ii) the purification process, and iii) the stability of the recombinant enzyme under the reaction conditions.

The final aim of the expression system must be to obtain the maximum yield of active enzyme. For large-scale production of proteins that are limited in size and do not require substantial post-translational modifications (e. g. glycosylation), prokaryotic expression systems, and in particular *Escherichia coli*, are the most attractive ones because of their ability to grow rapidly and at high cell density in fermentation processes. However, when a heterologous protein is over-expressed in *E. coli* misfolding and aggregation happen frequently, driving the recombinant protein into inactive aggregates known as inclusion bodies (IB)[2]. Since the propensity to aggregate is strongly dependent on the particular protein being expressed, it is obvious that there is not a general protocol to prevent the formation of IB's. Formation of IB's can be avoided modifying the culture conditions (fermentation engineering), modifying the protein to be expressed (protein engineering) and/or manipulating the host cells (genetic engineering).

Once the recombinant enzyme is successfully produced, it is necessary to face the downstream process for its purification. When the recombinant enzyme preparation is to be used as biocatalyst, one affinity chromatographic step can give the required purity degree. Immobilized Metal Ion Affinity Chromatography (IMAC) can also be used for enzyme immobilization[3], since the adsorption of the protein to the chromatographic resin is not by the enzyme active center.

In this presentation we described our work[4–6] to face the three factors described above for the case of the α-1,6-FucT from *Rhizobium* sp.: i) optimization of the expression yield of properly folded and active recombinant enzyme, ii) the purification scheme, and iii) the stability of the recombinant enzyme.

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P233 SYNERGISTIC ASSOCIATION OF BACTERIA AND A GREEN MICROALGA FOR THE BIODEGRADATION OF AROMATIC POLLUTANTS

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Keywords: aromatic pollutants, biodegradation, microalgae, photobioreactor, synergistic association

In nature, most microalgae are found in association with other microorganisms. Indeed, they produce the molecular oxygen that is used as electron acceptor by the heterotrophic aerobic microflora. In return, the carbon dioxide released during the mineralization process completes the photosynthetic cycle. The mutualistic relationships between algae and other microorganisms form the basis of the carbon turnover in natural ecosystems and in artificial freshwater envi-

ronments (aerobic stabilization ponds for the treatment of domestic wastewater).

In this context, our study was conducted to determine the potential of algae-bacteria combinations for the degradation of aromatic pollutants, with the aim of using these microcosms for the treatment of toxic and/or recalcitrant organic compounds contaminating aqueous media. The green microalga *Chlorella sorokiniana* was thus mixed with different aromatic degrading bacteria. Salicylic acid (sodium salt), phenol and phenanthrene were tested as model pollutants for representing a wide range of aqueous solubility and toxicity. In the various tested microcosms, the synergistic association between algae and bacteria was clearly established in a photosynthesis-helped biodegradation process as shown by the removal of pollutants. Simultaneously to this study, we designed and built our own photobioreactor. This reactor was investigated with biofilms in a continuous mode of operation both for the production of photosynthetic oxygen by *C. sorokiniana* and for the degradation of the aromatic pollutants. This reactor appeared to be an efficient tool for these two applications[1,2]. The experimental set-up as well as some major results will be presented in this communication.

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P234 STUDIES ON BIOTRANSFORMATIONS OF HARD CARBON COATINGS (DLC & NCD)

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Keywords: carbon coatings, microorganisms, biotransformation

The carbon thin film coatings (DLC – diamond-like carbon, NCD – nanocrystalline diamond) are used for improvement of surface properties of metals and ceramics devices that are applied in biotechnology, electrochemistry, nanochemistry and medicine. They have very attractive properties such as high wear resistance and hardness, chemical inertness and very low friction coefficients[1,2]. Hard carbon coatings consist mainly of the tetragonally sp^3 – coordinated carbon atoms (typical of diamond), the trigonally sp^2 – coordinated carbons (typical of graphite) as well as some sp^1 – coordinated ones[3]. The response of different animal cells (mouse fibroblasts, macrophages, endothelial cells, neutrophils and other) to diamond surfaces was studied[4]. However, there is no information about the behavior of these coatings in the presence of microbes and their metabolites. Therefore, an effect of various physiologically active microorganisms on surfaces covered with diamond-like coatings has been studied. Thin carbon films on medical stainless steel AISI 316 L have been prepared using the radio frequency plasma chemical vapor deposition (RF PCVD) method. The samples covered with carbon coatings were inoculated with various fungi *(A. niger, Chaetomium globosum, M. circinelloides, Paecilomyces variotii, Penicillium ochrochloron, Trichoderma viride, Fusarium oxysporum, Phanerochaete chrysosporium)* and bacteria (e. g. *L. delbrueckii*, *Ps. fluorescens*). Some of the selected strains can solubilize the lignite (brown coal) and are able to grow in petrol-oil[5,6]. The carbon layers before and after the growth of the microorganisms were examined by means of fluorescence microscope, scanning electron microscopy (SEM) and Raman spectroscopy. It was found that most of the tested filamentous fungi and some bacterial cells strongly adhered to the surfaces covered with DLC coatings during cultivation. Furthermore, changes in the color of DLC – films from blue to yellow or metallic, after the growth of the majority of the microscopic fungi, were observed using optical and metallographic microscopes. The strong influence of some of the tested microorganisms on carbon coatings deposited on medical stainless steel was confirmed using SEM microscopy (connected with a X-ray microanalyzer) analysis. It shows differences in the concentration of certain elements present in surface layers of the samples before and after subjecting them to treatment with microorganisms. Some of the microorganisms very strongly attacked the surface of carbon coatings and changed their structure. Raman spectroscopy – the most appropriate to characterize the graphite phase – proved that the microorganisms can remove (or modify?) some graphite phase from DLC coatings.

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P235 CELL ADAPTATION TO SUBSTRATE, SOLVENT AND PRODUCT: A SUCCESSFUL STRATEGY TO OVERCOME PRODUCT INHIBITION IN A BIOCONVERSION SYSTEM

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Keywords: biotransformation, (-)-carveol, (-)-carvone, column reactor, viability

Rhodococcus erythropolis DCL14 cells can produce three different carveol dehydrogenases, each of them dependent on a different co-factor[1]. When the cells grow on limonene or cyclohexanol the major activity is NAD-dependent. Cell viability is therefore an issue, which makes both reactor configuration and operation conditions important. These cells are able to "resolve" a diastereomeric mixture of (-)-carveol, two products being obtained: (i) (-)-carvone and (ii) isomerically resolved (-)-*cis*-carveol.

Both in a mechanically stirred direct contact reactor and in a membrane reactor, the reaction practically stopped when the carvone concentration reached 50 mM (ref.[2]). An attempt to adapt the cells was thus carried out. A 130 ml glass column was tested as an air-driven direct contact bioreactor. *n*-Dodecane 50 mM in carveol was recirculated at 50 $ml.h^{-1}$ through the column containing the aqueous phase. At this circulation rate the contact time between the cells and the substrate is low enough so that the biotransformation takes place to a small extent only.

In the first run, recirculation was stopped after 20 h, the whole organic volume was allowed to enter the column and air was injected at the bottom of the column at 29 $ml.min^{-1}$ (0.3 vvm). A good emulsion was formed and the whole of the cell population had migrated towards the organic phase after 10 h. The aqueous phase became completely cell free. The cells were able to carry out the biotransformation for at least 310 h. At this time carvone production reached 94 mM.

As a further attempt to adapt the cells to the presence of solvent, substrate and product, an assay was carried out in which *n*-dodecane containing the substrate was recirculated, as described above, for 136 h. This long adaptation period was actually a successful strategy, allowing accumulation of carvone up to 259 mM, thus overcoming the product inhibition problems previously encountered.

The results suggest that if cells are allowed to stay in contact with solvent, substrate and slow increasing concentrations of carvone, they are able to adapt. In fact, in the second run 69 % of the cells in the organic phase were viable after 310 h, while in the first run, only 49 % of the cells were viable after the same period. Somehow, cells in *n*-dodecane were protected from the toxic effect of carvone.

To confirm wheter the adaptation period was indeed responsible for an increase in productivity, a run was carried out in which the cells were allowed to adapt during 50 h. The column worked for 818 h and a concentration of carvone of 178 mM was attained. A plot of the carvone concentration obtained in each assay *vs.* the adaptation period gave a logarithmic shaped curve. The trendline that best fits the results has a *R*-square value of 0.999.

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P236 TOWARDS THE BIO-PRODUCTION OF *trans*-CARVEOL AND CARVONE FROM LIMONENE

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Keywords: limonene, carveol, carvone, *Rhodococcus opacus*

The strain *Rhodococcus opacus* PWD4 was found to hydroxylate D-limonene, yielding (+)-*trans*-carveol and traces of (+)-carvone, when grown on toluene[1]. In the present work we studied the activity shown by these cells when grown on different carbon sources, in both single aqueous and biphasic systems, the latter allowing to overcome the low solubilities of both substrate and products[2].

Cells were grown on 0.125 and 0.25 % (v/v) of ethanol, limonene and toluene as single carbon sources. In both aqueous and aqueous:organic systems, at 28 °C and 200 rpm, the highest initial *trans*-carveol production rate was observed for cells grown on limonene. In biphasic systems, the activity shown by the cells grown on 0.125 % of limonene is 2.7 and 3.11 times higher than that shown by cells grown on toluene and ethanol 0.125 %, respectively. Furthermore, the carveol production rate in biphasic systems is 10 times higher than in aqueous systems. In aqueous:organic systems, the production of *trans*-carveol is higher than the production of carvone, but the ratio between the production of carveol and carvone depends on the carbon source used: 14.9, 3.4, 7.5 and 28.7 for cells grown on 0.125 % ethanol, 0.125 % limonene, 0.125 % toluene and 0.25 % toluene, respectively.

The effect of the initial limonene concentration on the growth medium was evaluated by adding 5, 10, 20, 40, 80 and 160 μl of limonene to 25 ml of medium. The carvone production rate decreased with increasing amounts of limonene

added until 40 μl, and increased with higher amounts. The maximums were achieved for 5, 80 and 160 μl of limonene added.

The production rate of both *trans*-carveol and carvone increased with the initial percentage of limonene. Changing the limonene concentration, referred to the aqueous phase, from 20 to 150 mM led to a 5.9 fold increase in *trans*-carveol and a 2.8 fold increase in carvone production.

Production was also tested in an aqueous(20 ml):dodecane(4 ml) biphasic bioreactor with limonene supplied through the air stream. The reactor worked at 28 °C and was agitated at 200 rpm. Using cells grown on toluene, the carvone production rate became 18 times higher than the *trans*--carveol production rate. The increase was not that high with cells grown on limonene. However, using the latter in a similar aerated bioreactor but with an initial limonene concentration of 50 mM, the carvone production rate was 141 times higher than the *trans*-carveol production rate.

The results indicate that the conditions of growth, as well as those of biotransformation, may greatly influence the limonene degradation carried out by *R. opacus* PWD4 cells.

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P237 BIODEGRADATION OF HYDROCARBONS UNDER SALINE AND NON-SALINE CONDITIONS AT 15 AND 28 °C

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Keywords: hydrocarbons degradation, bioremediation, *Rhodococcus erythropolis*, saline conditions

The bioremediation of sites contaminated with hydrocarbons, resulting from *e. g.* spillage of diesel or jet fuels, maybe difficult due to low ambient temperatures or saline conditions. The ability of *Rhodococcus erythropolis* DCL14 cells to degrade hydrocarbons at 15 and 28 °C was assessed, as well as their capacity to carry out the degradation under saline conditions. The temperatures were chosen with reference to the minimum and maximum average temperatures observed in the Atlantic, Pacific and Indian oceans between 0 and 55° of latitude.

The hydrocarbons tested were *n*-hexane, cyclohexane, toluene, *n*-octane, *iso*-octane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane at initial concentrations of 0.125 and 0.25 % (v/v).

In the absence of NaCl, at 15 °C, all growth curves reached the end of the exponential phase after 90 h, except when *n*-tetradecane (100 h) and toluene 0.125 % (125 h) were used as carbon source. The final optical density (O. D.) at 600 nm observed ranged from 3.5 to 7. When toluene was used at an initial concentration of 0.25 %, almost no growth was observed, indicating that the cells were unable to degrade toluene at this concentration at 15 °C. At 28 °C, the end of the exponential phase was attained after 30 h for most of the hydrocarbons tested. The exceptions occurred when the cells were in the presence of 0.25 % *iso*-octane (around 50 h) and 0.125 % toluene (around 60 h). The final optical density obtained ranged between 4.5 and 7.5. Once again, almost no toluene degradation was observed at an initial concentration of 0.25 %.

The composition of the cell membrane supposedly varies with the carbon source used and hence cell hydrophobicity should also be affected. Cell hydrophobicity was determined by the MATH (Microbial Adhesion To Hydrocarbons) test[1]. At 15 °C, high hydrophobicities were observed for cells grown on toluene, *iso*-octane and *n*-tetradecane while those grown on *n*-hexadecane presented lower hydrophobicity. Cells grown at 28 °C showed high hydrophobicity when grown on *n*-tetradecane and *n*-hexadecane, whilst lower values were obtained with cells grown on *n*-hexane, toluene and *n*-octane. The results suggest that there is no direct relation between cell hydrophobicity and the carbon number.

Three NaCl concentrations were used to assess the possibility of using *R. erythropolis* cells in saline media: 1.0, 1.95 and 2.5 %. The carbon sources tested were the following: *n*-hexane, *iso*-octane and *n*-octane 0.125 %. At 15 °C, the *lag* phase was around 50 h, except for *n*-octane in which the cells needed nearly 100 h to start growing. The final O. D., at 600 nm, ranged between 2.0 and 3.0. The *lag* phase increased to around 160 h when the initial concentration of salt increased to 1.95 %.

Using *n*-octane, an increase in salt concentration led to an increase in cell hydrophobicity. In general, it was observed that the higher the carbon number of the carbon source, the lower the influence of NaCl concentration on cell hydrophobicity.

Thus, *R. erythropolis* DCL14 cells are fairly good biocatalysts for degrading hydrocarbons in both relatively cold and warm environments even under saline conditions. Several other strains of the genus *Rhodococcus* have been found to metabolise environmental contaminants, using various alkane-catabolic pathways[2].

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P238 BIODEGRADATION OF MOTOR OILS AT 16 AND 28 °C BY *Rhodococcus erythropolis* DCL14

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Keywords: oil degradation, bioremediation, *Rhodococcus erythropolis*

Motors are widely present in our environment and accidental oil spills are frequent. Microorganisms able to degrade motor oils under different environmental conditions should be a good tool for *in situ* bioremediation.

Rhodococcus erythropolis DCL14 cells were tested as degraders of four and two-stroke engine oils at 15 and 28 °C. Since these oils are mainly used by cars, motocycles and boats, this temperature range covers the majority of conditions under which the spills occur. The percentages of oil tested were the following: 0.125, 0.25, 0.5, 1 and 2 % (v/v).

At 28 °C, when cells were incubated with Shell Helix standard 20W-50 oil for four-stroke engines, the optical density (O. D.) measured at 600 nm doubled in the first 20 h with each of the tested percentages. The growth rate decreased with the increase of the initial oil fraction. After the first 30 h, the cells formed clusters which were visible under the naked eye. Thus, thereafter, the quantification of growth, by the measurement of the optical density, became impossible. Using Mobil Super 2T for two-stroke engines as sole carbon source, the velocity of growth decreased with the increase in oil fraction: after 43 h the O. D. at 600 nm had tripled for concentrations lower than 0.5 % and only slightly increased for 1 and 2 %. Cell clustering was observed after 50 h under naked eye.

At 15 °C, very small clusters were visible after 60 h in all growth runs with both oils.

Images of the different culture media, obtained with an optical microscope, showed cells surrounding the oil droplets. The higher the oil fraction, the larger the cell clusters were.

The MATH hydrophobicity test[1] showed that, for both oils, cells were less hydrophobic when grown on 0.5 % oil fraction. The most hydrophobic cells were those grown on either the lowest (0.125 %) or the highest (2 %) initial oil fraction. Futhermore, cells grown on Mobil Super 2T were more hydrophobic than those uptaking Shell Helix standard, at identical initial fractions.

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P239 PRELIMINARY STUDIES ON THE OPTIMISATION OF FERMENTATION PROCESSES IN BATCH CULTURE FOR THE PRODUCTION OF ERYTHROMYCIN

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Keywords: erythromycin, fermentation, *Saccharopolyspora erythraea*, fermentor, adsorption

Erythromycin is a broad spectrum polyketide antibiotic, specially indicated in the treatment of respiratory and skin complains. It has a similar activity spectrum to the penicillin and are used by penicillin-sensitive people to combat Gram-positive bacteria, and in addition used against *Mycoplasma*, *Campylobacter* and *Legionella*. In turn erythromycin is the starting material for second and third generation semi-synthetic derivatives. Erythromycin is a 14-carbon macrolide, produced by fermentation of *Streptomyces erythreus* or *Saccharopolyspora erythraea*. The new findings, effective completion of the genome sequence for the bacterium *S. erythrae*, offer a host of possibilities for production of novel antibiotics, immunosupressants and anti-cancer compounds, all based on polyketide starting materials.

Optimising the fermentation conditions, by selecting adequate parameters like the effect of medium composition, pH, temperature, oxygen levels, ratio C/N, is therefore an important aspect for the successful operation of any fermentor.

The aim of this work was the study of production and recovery of erythromycin from fermentation broth by selective adsorption on neutral resins (Amberlite™ XAD-4, XAD-7 and XAD-16) and anion exchange resin (Amberlite™ XAD-410). In previous work these resins were used in adsorption studies of erythromycin from standard solutions[1].

Erythromycin was produced by fermentation, from *S. erythraea* in a 3 l fermentor. Preliminary studies have been carried out in a batch mode for the optimisation of fermentation conditions. Cultures of *S. erythraea* were grown in various media formulations, in shake flasks. Culture growth was compared in terms of specific rate cell growth, duplication time and fermentation time. Erythromycin was analysed by sphectrophotometry, at 280 nm and by HPLC according to the method of USP (ref.[2]).

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P240 USE OF NITRILE HYDRATASE FROM *Brevibacterium imperiale CBS 498-74* RESTING CELLS FOR PROPRIOAMIDE PRODUCTION: A STUDY IN UF-MEMBRANE REACTORS

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Keywords: *Brevibacterium imperiale* growth, nitrile hydratase, proprionitrile biotransformation, enzyme kinetics, UF-membrane reactor

The bioconversion of proprionitrile into proprioamide was catalyzed by the nitrile hydratase present in resting cells of *Brevibacterium imperiale CBS 498-74*. This strain follows a two-step degradation pathway of nitriles, which involves nitrile hydratase and amidase, and presents an high nitrile hydratase activity while amidase activity is negligible and therefore theoretical 100 % conversion yields are possible. The influence of various parameters on microorganism growth for the production of nitrile hydratase was already tested[1, 2].

B. imperiale CBS 498-74 (new classification, *Microbacterium imperiale*) was obtained at the optimum initial glucose concentration for nitrile hydratase (NHase) production, 5 $g.l^{-1}$, in a shake flask (220 $rev.min^{-1}$) at 28 °C for culture periods of up to 150 h. The highest NHase productivity (U per ml of broth per h) was reached after 24 h of incubation. Specific activities in the cell were found to be: 34.41 $U.mg^{-1}_{DW}$. NHase activity in the whole cell suspension was tested following the biotransformation of proprionitrile (50 mM) into proprioamide at 20 °C in 50 mM sodium phosphate buffer, pH 7.0. The kinetic parameters, K_m and V_{max}, were evaluated at different temperatures and resulted respectively at 10 °C: 61.0 mM and 10.36 $\mu mol.min^{-1}$ and at 20 °C 88.9 mM and 13.98 $\mu mol.min^{-1}$. The activation energy, E_{act}, was also evaluated.

In this study use was made of both differential and integral UF-membrane reactors for kinetic characterisation of the reaction. The laboratory scale membrane bioreactor was a commercially available flat membrane cell Amicon Mod. 52 and the UF-membranes was FS81PP. The membrane resistance to chemicals was fair at proprionitrile and proprioamide concentrations up to 100 mM and 1 M respectively. No rejection of solute was determined. Membranes totally retained the resting cells and no fouling was observed working with 2.3 mg and 5.7 mg of biocatalyst under stirring conditions[3]. Membrane compaction was responsible for flux loss during the first 3–4 hours of operation.

The enzymatic reaction was operated at 5 °C and 10 °C. Substrate concentration ranged from 100 mM to 500 mM. Proprionitrile conversion as high as 76.6 % was attained using 32.7 µg of cells per ml in a continuously operating reactor. The laboratory scale membrane bioreactor, continuously operating, allowed to study the dependence of enzyme deactivation on the substrate concentration and process time[4]. In this study we report on the irreversible damage of NHase activity caused by high proprionitrile substrate concentration.

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P241 BIOTRANSFORMATIONS FOR THE PRODUCTION OF OPTICALLY PURE 1,2-*O*-ISOPROPYLIDENE GLYCEROL

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Keywords: solketal, microbial hydrolysis, carboxylesterase, enantioselective biotransformation

Biotransformation techniques have evolved such that the synthetic chemist can utilize biocatalysts just as many other synthetic reagents are used. Natural microbial diversity and new techniques for further diversification offer an almost inexhaustible source of biocatalysts.

The major goal of this work has been the obtainment of 1,2-*O*-isopropylidene glycerol (also called solketal or IPG) as optically pure molecule by microbial hydrolysis of the corresponding racemic esters. This biotransformation is difficult to achieve with high enantioselectivity using commercially available enzymes. Different techniques have been exploited for improving the selectivity and/or productivity of the biotransformation, such as medium manipulation (e. g. binding agents or organic solvents), biocatalyst manipulation (e. g. lyophilised cells, thermal pre-treatment, immobilization etc.) or reactor engineering (e. g. membrane reactors). Carboxylesterases responsible for the enantioselective biotransformations have been (partially) purified.

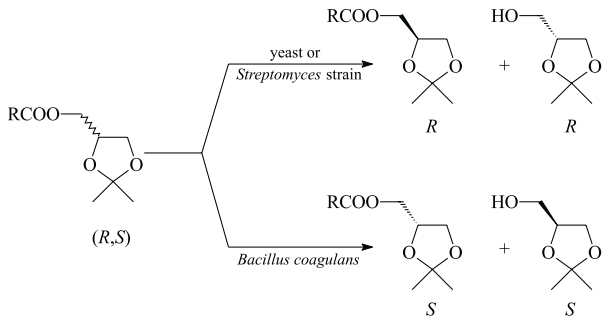

Fig. 1. **Simple membrane reactors allowed for multi-gram production of optically pure solketal**

P242 OPTIMIZATION OF METHYLENEDIOXYPHENYL-ACETONE CHIRAL BIOREDUCTION

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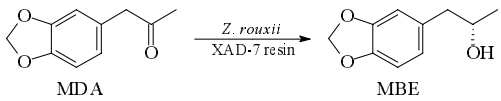
Keywords: *Zygosaccharomyces rouxii*, bioreduction, resin

Talampanel is a non-competitive antagonist of AMPA receptor, and it is a possible drug for treatment of epilepsy and cerebrovascular ischemia. The first step of an efficient synthesis of Talampanel is the reduction of 3,4-methylenedioxyphenyl-acetone (MDA) to *(S)*-α-methyl-1,3-benzodioxole-5-ethanol (MBE) accomplished with *Zygosaccharomyces rouxii* in the presence of XAD-7 resin[1]. The application of the hydrophobic polymeric adsorbent resulted in low and non-toxic concentration of both the substrate and the product in the water phase (< 6 $g.l^{-1}$). *Z. rouxii* was chosen because it tolerated higher substrate and product concentration and had a higher productivity number in comparison to the other tried yeasts[2].



A low cost fermentation medium without any component of animal origin was developed to produce *Z. rouxii* biomass. The control of pH and dissolved oxygen concentration, the temperature, the antifoam system, the time of harvest, the rate of inoculations and the number of seed steps were also investigated. The fermentation process in 1000-l fermentor provided cell pastes possessing a satisfactory ketoreductase activity (95–98 %) and an excellent enantioselectivity.

A method was developed to examine the enzyme activity and to determine the key parameters of the bioreduction. It was found that enantioselectivity was independent of the examined conditions of reduction. The yield was very sensitive to both the quality of cell paste and conditions of bioreduction except the temperature and the aeration. This *in vivo* enzyme system is extremely sensitive to the change in the concentration of the ingredients.

Z. rouxii cell pastes were also used in pilot plant scale bioreduction in 5 consecutive batches with 200 liter total volume each. The reductions were performed in a modified Rosenmund agitated filter-dryer filled with Amberlite XAD-7 HP resin. The obtained yields were similar to our results carried out in our laboratories.

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P243 COMPARISION OF 1,3-DIHYDROXYACETON AND L-ERYTHRULOSE PRODUCTION OF *Gluconobacter oxydans* ATCC 621H

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Keywords: *Gluconobacter oxydans*, fermentative production, 1,3-dihydroxyaceton, L-erythrulose

Gluconobacters are well known for their capacity of oxidising a series of different sugar alcohols. The valuable molecules: 1,3-dihydroxyaceton and L-erythrulose are the products of the fermentation on glycerol and *meso*-erythritol, respectively. Here we report the systematic investigation and comparision of the dihydroxyaceton and erythrulose production capacities of *Gluconobacter oxydans* ATCC 621H.

The influence of carbon source concentration between 30 to 280 $g.l^{-1}$ on the cell mass production, product concentration, yield and productivity was investigated by orthogonal shake-flask experimental designs. 160 $g.l^{-1}$ erythritol was considered to be the optimal starting concentration whereas the ideal glycerol concentration was significantly lower. To increase cell mass production, a series of supplementary compounds were evaluated with the aid of a Plackett-Burman design. To determine the inhibitory level of product concentration, fed-batch fermentations were carried out, where the substrate supply was controlled by the dissolved oxygen level in the fermentation broth. Inhibitory product concentrations were determined for circumstances among limiting as well as for surplus substrate concentrations. A series of NAD^+ and

PQQ-dependent dehydrogenase activities were also determined from the disrupted cells in order to gain deeper knowledge of the activation mechanism of *Gluconobacter* enzymes. These results establish the possibility of the effective fermentative production of dihydroxyaceton and erythrulose as well as the enzymes responsible for their production.

P244 BIOPROCESS DEVELOPMENT FOR EPHEDRINE PRODUCTION

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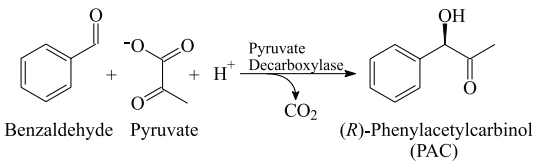
Keywords: biotransformation, pyruvate decarboxylase, *(R)*-phenylacetylcarbinol, ephedrine, pseudoephedrine, two-phase process

The chiral pharmaceuticals ephedrine and pseudoephedrine are currently produced commercially via a biotransformation of benzaldehyde by fermenting baker's yeast followed by chemical catalysis (Fig. 1). The biotransformation is catalyzed by a side-reaction of the enzyme pyruvate decarboxylase (PDC), which transfers enzyme-bound "active acetaldehyde" onto benzaldehyde to form *(R)*-phenylacetylcarbinol (PAC). Acetaldehyde and acetoin can be formed as by-products.

BIOTRANSFORMATION:



CHEMICAL SYNTHESIS:

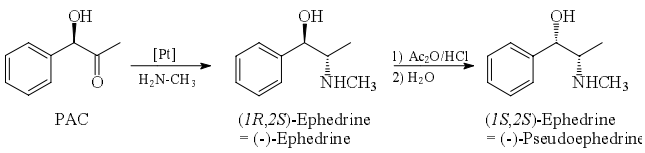


Fig. 1. **Ephedrine production by sequential biotransformation and chemical synthesis**

Screening of filamentous fungi[1] and yeast for cell-free PAC production resulted in a number of efficient PDCs e. g. from *Rhizopus javanicus*, *Candida utilis* and *Candida tropicalis*. *Candida utilis* PDC was more stable and formed less by-product acetoin than PDC from the filamentous fungus *Rhizopus javanicus*.

Enzyme stabilization and pH control were identified as crucial factors for increasing final PAC levels[2], which have previously been limited to 28.6 $g.l^{-1}$ in an enzymatic process[3]. With improved buffering partially purified PDC produced 50 $g.l^{-1}$ PAC in a simple batch biotransformation[3].

For further process enhancement, loss of substrate pyruvate was minimized. The loss resulted from two phenomena: temperature dependant non-enzymatic concentration decrease due to the cofactor Mg^{2+} and formation of by-products acetaldehyde and acetoin by PDC. The molar yield of PAC on consumed pyruvate increased from 59 % (*R. javanicus* PDC, 20 mM Mg^{2+}) to 74 % (*R. javanicus* PDC, 0.5 mM Mg^{2+}) by lowering the Mg^{2+} concentration, and increased further to 89 % (*C. utilis* PDC, 0.5 mM Mg^{2+}) by changing the source of PDC.

An aqueous/organic two-phase system was designed to overcome enzyme deactivation by benzaldehyde, acetoin and PAC and to decrease inhibition by acetaldehyde. Under optimized conditions 141 $g.l^{-1}$ PAC was produced in the organic phase with additional 19 $g.l^{-1}$ formed in the aqueous phase using *Candida utilis* PDC. Significant increases in PAC per unit of enzyme were achieved[4]. Utilizing whole cells instead of partially purified PDC further reduced the catalyst cost. Additionally PDC production in *Candida utilis* was drastically enhanced through a novel pH shift process.

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P245 PROCESS DEVELOPMENT FOR *R*-PHENYLACETYLCARBINOL (PAC) PRODUCTION IN AQUEOUS/ORGANIC TWO-PHASE BIOTRANSFORMATION

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Keywords: biotransformation, enzyme stability, pyruvate decarboxylase, *R*-phenylacetylcarbinol

R-Phenylacetylcarbinol (PAC) is a precursor for the pharmaceuticals *(1R, 2S)* ephedrine and *(1S, 2S)* pseudoephedrine. PAC is commonly synthesized by a biotransformation process utilizing benzaldehyde and pyruvate as substrates and an enzyme, pyruvate decarboxylase (PDC), as catalyst. Main by-products associated with the biotransformation are acetaldehyde, acetoin and benzyl alcohol.

Based on the amount of benzaldehyde that is used in the biotransformation, there are three basic types of system that should be applied (in the order of increasing benzaldehyde concentration): (1) Aqueous phase, (2) Benzaldehyde emulsion[1] and (3) Aqueous/organic phase system[2].

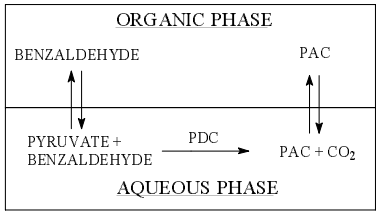

Fig. 1. **Organic/aqueous two-phase system for *R*-PAC production**[2]

It was previously found that benzaldehyde, PAC and acetoin inactivated PDC while acetaldehyde had an inhibiting effect (Sandford et al., submitted). It is also known that PDC is most stable at low temperature, neutral pH and in the presence of high concentrations of MOPS buffer or other additives like glycerol or salt[1].

In this study, the effects of further process parameters on PAC production were investigated in the aqueous and aqueous/organic phase system. It was found in the aqueous phase system that agitation rate and enzyme level had no effect on PDC deactivation as long as foam formation was prevented. The deactivating effect of benzaldehyde was confirmed while octanol had no considerable effect. However, it was observed that deactivation by benzaldehyde was enhanced in the presence of octanol.

It was further found in the aqueous/organic phase system that there was no strong additional deactivating effect by high concentration of benzaldehyde (1.46 M) contained in the octanol phase. This implies efficient protective effect of the octanol phase, which prevents the strong inactivation that has been reported previously for a benzaldehyde emulsion system.

Lowering the ratio of organic to aqueous phase volume from 1 : 1 to 0.43 : 1 in the two-phase biotransformation while maintaining the total concentrations of enzyme and substrates resulted in 11 % higher overall PAC and lower overall concentration of the by-product acetoin. In addition, the PAC was highly concentrated in the organic phase with 1400 mM PAC in octanol in comparison to approximately 800 mM when using the 1 : 1 ratio. Hence, application of less organic phase has the potential for reduction in production cost (since less solvent is used) and more efficient downstream processing (since the product is more concentrated).

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P246 PREPARATION OF AN EPHEDRINE CHIRAL SYNTHON BY BAKER'S YEAST REDUCTION OF 1-PHENYL-1,2-PROPANEDIONE

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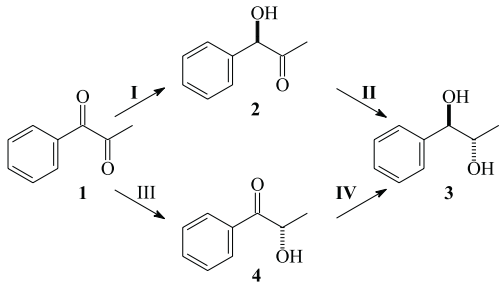
Keywords: *(R)*-1-hydroxy-1-phenyl-2-propanone, baker's yeast reduction, enantioselective reduction

(R)-1-Hydroxy-1-phenyl-2-propanone **2** is an important chiral synthon that has been used in the manufacture of the pharmaceuticals (-)-ephedrine and (-)-pseudoephedrine[1]. The most commonly used method for production of **2** is biological, in which strains of *Saccharomyces cerevisiae* and *Candida utilis* are employed to mediate the acyloin condensation of benzaldehyde and pyruvic acid[2].

Another promising biological alternative for production of **2** is the baker's yeast reduction of 1-phenyl-1,2-propanedione **1**, which gives a mixture of optically active **2**, plus (1*R*,2*S*)-1-phenyl-1,2-propanediol **3**, and (*S*)-2-hydroxy-1--phenyl-1-propanone **4** (Scheme)[1]. Considering that compounds **2** and **4** are barely separable by silica gel column chromatography, the aim of this work is to improve the regio-



selectivity of the reduction of **1** to obtain compound **2**. The previously published[3] scheme shows compounds **2** and **4** as intermediates in the baker's yeast reduction of **1** to **3**. As our interest is the isolation of the intermediate **2**, we performed a study of this reaction to maximize the yields of **2** free of **4**. Thus, samples were withdrawn from the reaction mixture at appropriate intervals and analyzed by gas chromatography. During this study, we observed that the value of the **2/4** ratio varied with the velocity of reaction flask agitation and thus an anaerobic procedure was performed. Figure 1 shows that compound **2** may be obtained free from **4** at 90 min of reaction using a pre-treated baker's yeast (nitrogen was bubbled during 60 min followed by 20 min of oxygen bubbling into a suspension formed by baker's yeast and water). Therefore, we use this process to prepare an easily separable mixture of **2** (28–31 % isolated yield, 96 % e. e.) and **3** (42–62 % isolated yield, 99 % e. e.).

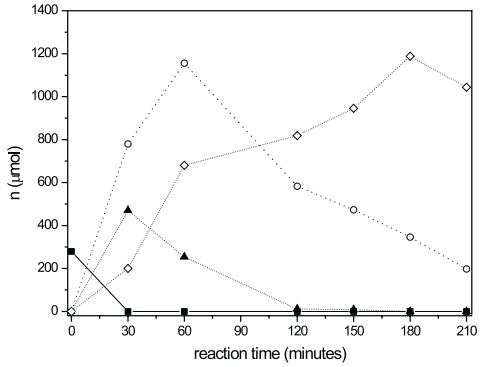


Fig. 1. **Conversion of 1 (■) to 2 (○), 3 (◊) and 4 (▲) mediated by pre-treated baker's yeast.**

In conclusion, the reduction of **1** by anaerobically pre-treated baker's yeast afforded an easily separable mixture of **2** and **3**, almost free from **4**, in reasonable yields and excellent e. e. at 90 min of reaction.

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P247 WHOLE-CELL BIOCONVERSION OF L-PHENYLALANINE TO 2-PHENYLETHANOL WITH YEASTS: MEDIUM OPTIMIZATION USING A GENETIC ALGORITHM

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Keywords: genetic algorithm, 2-phenylethanol, aroma, yeast

Various food-grade yeasts produce 2-phenylethanol among other volatile products useful as flavor and fragrance compounds. By adding the amino acid L-phenylalanine to the medium the yield of the desired product can be significantly increased as the yeasts convert the precursor via a cascade of three enzymatic steps (Ehrlich pathway) to 2-phenylethanol[1].

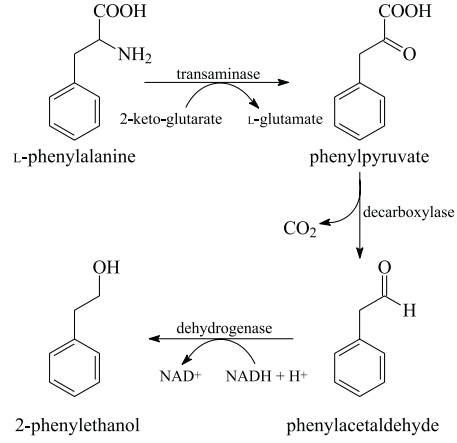

Fig. 1. **Ehrlich pathway**

To avoid inhibition by 2-phenylethanol the product has to be removed continuously from the bioprocess. On shake-flask scale this is done by an aqueous-organic two-phase system with oleyl alcohol as the extractive phase.

In a screening of fourteen yeasts *Kluyveromyces marxianus* CBS 600 was found to be the most productive strain. In

a medium with molasses as carbon source it produced approx. 3 $g.l^{-1}$ 2-phenylethanol. This medium also contained a complex source of vitamins and other supplements which is expensive and cannot be used in a technical process.

A genetic algorithm was used to improve medium composition and process temperature towards higher product yields. Special emphasis was on varying single nutrients of the complex source to distinguish between those components which play a key role for the desired bioconversion and those which could be left out without impairing the results.

The optimization was carried out by varying ten parameters affording twenty individuals (in parallel experiments) per generation. In the course of five generations two local maxima corresponding to two significantly different medium compositions appeared, where the product concentration was increased by 58 % and 30 %, respectively. As the former medium is significantly more expensive than the latter, only an overall economic analysis of both variants will identify the "optimal" medium.

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P248 PRODUCTION OF ALCOHOLS AND ALDEHYDES BY BAKER'S YEAST IN A SOLID/GAS REACTOR

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Keywords: yeast, alcohol dehydrogenase, solid/gas catalysis, aldehydes

Industrial applications of baker's yeast include the production of ethanol[1,2], chiral alcohols and ketones[3,4] in aqueous or organic media. Nevertheless these processes often present limitations such as a low productivity, a low yield or a high sensitivity to high products and substrate concentrations. The use of non-conventional media and especially the gas phase could overcome some of these limitations.

Recently, the use of *Saccharomyces cerevisae* in the gaseous phase has been successfully reported for the production of alcohols and aldehydes[5,6]. This system presents the following advantages: (i) Cofactor regeneration is made *in situ*, (ii) no addition of cofactor is required, (iii) this system is cheaper than a system using crystallised ADH/cofactor.

The aim of this work is to better understand the influence of physico-chemical parameters on activity of this catalyst and to better understand phenomenon involved in a such system.

We then studied factors affecting catalysis and more particularly existence of possible diffusional limitations. Indeed the use of whole cells implies the presence of wall and membranes which may limit the gas diffusion. In order to check if there are diffusional limitations when using whole cells, walls were disrupted by sonication and the effect of this treatment on activity of cells has been studied. The preparation was tested at different water activities and the effect of cofactor addition is also presented.

As the preliminary tests was carried out with ethanol and butanal, this system may be an alternative method for the production of enantiopure compounds and aromas for the chemical and food industry and a best comprehension of the mechanism of YADH in solid/gas reactor is necessary to optimise the reaction.

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P249 ACTION OF HYDROLYTIC ENZYMES ON SYNTHETIC FIBRES

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Keywords: synthetic fibre, nitrilase, protease, esterase, cutinase, amidase

Enzymes are natural catalysts, promoting a great variety of chemical reactions. For this reason, enzymes have an important role on textile industry as substitutes of some chemical agents with negative effects on the environment. This work intend to study the enzymes capacity to alter surface properties of synthetic fibres. The hydrolytic enzymes nitrilases, proteases and esterases promote the cleavage of nitrile,

amide and ester groups, respectively, into carboxylic acids. These reactions can be very useful for the treatment of synthetic fibres containing these groups. The enzymatic action causes an increase of charged groups at the surface, improving water absorption (increase hydrofility) and dyeability.

Due to enzymatic modification, the acrylic fibers became more hydrophilic and dye uptake was enhanced at temperatures below glass transition. Nitrilase action on PAN fibres was monitored by measuring the release of ammonia and by FTIR detection of the formed of carboxylic groups by diffuse reflectance.

Cutinase were used on polyester fibres. The esterase action on polyester fibres promotes an increase of OH and COOH end groups. The OH end groups can be detected by a titration method and measuring the K/S spectrophotometrically after dyeing with a cotton reactive dye. Similarly enzyme preparations showing amidase activity towards polyamide fibres, promote an increase of NH_2 and COOH end groups. The amine groups were detected by a titration method with an increase of the molecular mass on treated samples. When samples were dyed with a wool reactive dye uptake was enhanced at temperatures below glass transition of polyamide.

P250 NOVEL ENZYME APPLICATIONS ON COTTON CELLULOSE

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Keywords: lipase, protease, hexokinases, cotton fabrics

Lipases and proteases were used to restore partially the strength loss of cotton fabrics, cross-linked with respectively 1,2,3,4-butanetetracarboxylic acids and *N*-hydroxymethyl acryl amide. Nearly one half of the strength loss of the fabrics could be restored by means of enzymatic hydrolysis at low temperature and neutral pH, while the crease-resistance effect decreased only slightly. In another application an enzymatically catalysed phosphorylation of cotton cellulose was achieved using hexokinases in the presence of a phosphate donor adenosine-5'-triphosphate. The enzymatic modification provided a new, reactive type cellulose substrate with improved dyeability and flame-resistance.

P251 ENZYMATIC DYEING OF KERATINOUS MATERIALS

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Keywords: keratinous fibre, laccase, protein disulfide isomerase

This study reports on the dyeing of keratinous materials using appropriate enzymatic systems – laccases and protein disulfide isomerase. The enzymatic dyeing was performed as a batchwise process at the temperature and pH of maximum enzyme activity. Laccases generate the colour "in situ" starting from low molecular colourless compounds – dye precursor and dye modifiers. Different hues and depth of shades could be achieved varying the concentration of the modifiers and the time of laccase treatment. Protein disulfide isomerases, based on their ability to catalyze thiol-disufide exchange, including oxidation, reduction and rearrangement, were used for covalent fixation of novel cysteine-modified dyes on keratinous fibres.

P252 CHEMO ENZYMATIC PREPARATION OF D-ALLOISOLEUCINE

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Keywords: D-alloisoleucine, subtilisin, diastereoselective hydrolysis

D-Alloisoleucine is a non-proteinogenic amino acid found as a component unit in a number of biologically active depsipeptides, useful intermediates in the synthesis of oxytocin analogues of isostatins and of natural cytotoxic compounds like tamandarins.

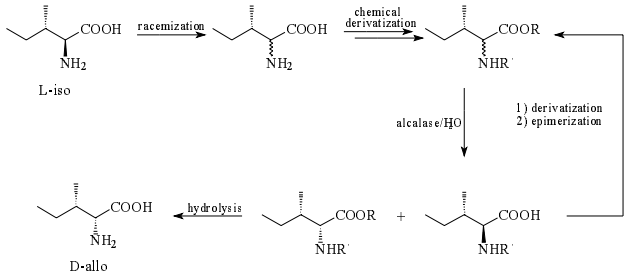
We report here a practical approach to D-alloisoleucine starting from a mixture of L-isoleucine and the D-allo stereoisomer, with an approach both chemical and enzymatic. The latter is based on the use of an industrial hydrolytic enzyme developed for detergency.

Thus L-isoleucine was epimerised and the diastereoisomeric mixture was transformed in a number of *N*-acyl-*O*-es-

ter derivatives including protecting groups usually employed in peptide synthesis.

Hydrolysis in water gave excellent separation of the two diastereoisomers allowing the obtainment of D-alloisoleucine derivatives in the maximum allowable yield in high enantiomeric excess.

During the elaboration of the substrates opportunities to effect the separation of the diastereoisomers *via* crystallization were successfully explored.



P253 PHOSPHOLIPASE D CATALYSED SYNTHESIS OF PHOSPHATIDYLSERINE IN A HOLLOW-FIBER MEMBRANE REACTOR

PAOLA D'ARRIGO, **EZIO FASOLI**, GIUSEPPE FANTONI PEDROCCHI, STEFANO SERVI, and DAVIDE TESSARO

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Keywords: phospholipase D, hollow fiber membrane, transphosphatidylation

Phospholipase D obtained from a culture of *Streptomyces* sp. PMF, was immobilised in a hollow fiber membrane by ultrafiltration. The reactor was used to contact an organic solution containing phosphatidylcholine (PC, 50 $g.l^{-1}$) and a water solution at different pH and the rate of formation of the hydrolysis product (phosphatidic acid, PA) was measured. Subsequently the water phase was replaced with a 3M water solution of L-serine and the rate of transphosphatidylation at different pH was evaluated. The reaction was complete in a 48 h period. The formation of the hydrolysis product was minimized working at pH 4.5 were hydrolysis rate is minimal. The operational stability of the system was excellent during a period of several month.

Although the space-time yield of phosphatidylserine formation is lower than in a byphasic CSTR system, the purity of the product and the enzyme consumption is advantageous.

P254 BIOSYNTHESIS OF SESQUITERPENE LACTONES IN CHICORY AND APPLICATION OF THE ENZYMES INVOLVED

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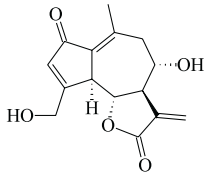
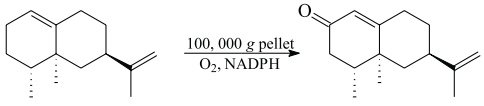
Keywords: sesquiterpenes, biosynthesis, hydroxylation, nootkatone

The edible sprouts of chicory (*Cichorium intybus* L.) are used as a vegetable and are well-known for their slightly bitter taste, which originates from sesquiterpene lactones (*e. g.*, lactucin). Especially the roots contain high concentrations of these bitter principles, which makes them unsuitable for use as cattle feed. However, we have demonstrated that the enzymes responsible for the biosynthesis of the sesquiterpene lactones are still present and active in the roots after the harvest of the sprouts. The first steps in the biosynthetic route have been elucidated by us[1–3].

One of the enzymes involved, the (+)-germacrene A hydroxylase, appears to possess a broad substrate specificity coupled to a high regioselectivity, making this enzyme an attractive catalyst for the hydroxylation of terpenes in flavour and fragrance industry. An especially interesting reaction is the one-step conversion of (+)-valencene into nootkatone[4], a much sought flavour component of grapefruit.



Lactucine



(+)-Valencene Nootkaone

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P255 ENANTIOSELECTIVE HYDROLYSIS OF 1-OXASPIRO[2.5]OCTANES BY YEAST EPOXIDE HYDROLASE

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Keywords: spiro epoxides, epoxide hydrolase, kinetic resolution

Naturally occurring compounds with antimicrobial or antitumor activity in many cases contain an epoxide group in a spiro attachment to a tetrahydropyran or cyclohexane ring. The epoxide moiety is generally assumed to be essential for the biological activity of these compounds. Oxaspiro compounds may thus be assumed to have significant therapeutic potential and their preparation in enantiopure form will be of interest[1,2]. Various enantiopure epoxides can be obtained effectively by kinetic resolution using epoxide hydrolases. A powerful tool for this method is the yeast epoxide hydrolase (YEH) of *Rhodotorula glutinis*, which can accept structurally diverse molecules as substrates[3]. We have therefore used the YEH to investigate the kinetic resolution of oxaspiro compounds. The structural requirements of substrates for YEH are studied in more detail by using a range of methyl substituted 1-oxaspiro[2.5]octanes (Fig. 1).

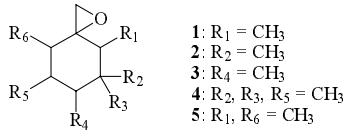

Fig. 1. **Methyl substituted 1-oxaspiro[2.5]octanes used as substrates for the YEH of *Rhodotorula glutinis***

Hydrolysis of the tested substrates by the YEH of *Rhodotorula glutinis* was strongly influenced by the position and number of methyl substituents. The initial reaction rate was found maximal for compound **3** and was comparable to the rate for the unsubstituted 1-oxaspiro[2.5]octane. The rate of hydrolysis decreased when methyl substituents were positioned closer to the epoxide group. A similar affect was observed by increasing the number of methyl substituents. No hydrolysis was observed when two methyl groups were placed on both positions next to the epoxide group (compound **5**). Enantioselectivities increased from moderate to high in the hydrolysis of substrates **4**, **2** and **1**. Based on the present results, we will further explore YEH-catalyzed kinetic resolutions of more complex oxaspiro compounds.

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P256 DYNAMIC KINETIC RESOLUTION OF ALCOHOLS: COMPLEMENTARY REACTIONS IN STEREOSELECTIVITY

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Keywords: alcohols, dynamic kinetic resolution, enzyme--metal combo, catalysis

The complete transformation of a racemic mixture into a single enantiomer is one of the current challenging problems in asymmetric synthesis. The dynamic kinetic resolution (DKR) of racemic substrates by enzyme-metal combo-catalysis provides a useful solution to this problem. In this approach, the enzymatic resolution of racemic substrate is coupled in situ with metal-catalyzed racemization of substrate, leading to the almost complete conversion of the racemic substrates to enantiomerically-enriched single products. The *R*-selective DKR of alcohols can be achieved by employing lipase-ruthenium combo catalysis. A wide range of simple and functionalized alcohols have been successfully resolved by using this method[1–10]. The *S*-selective DKR as a complementary process is more challenging because few enzymes are available to show satisfactory *S*-selectivity together with good activity and stability in organic solvents.

Some proteases have been examined as the resolution catalysts for the *S*-selective DKR. In the meeting, we wish to present some of the preliminary results from these studies.

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P257 APPLICATIONS OF IONIC LIQUIDS IN BIOCATALYSIS: ENHANCEMENT OF ENZYME ACTIVITY AND SELECTIVITY

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Keywords: ionic liquid, biocatalysis, activity enhancement, selectivity enhancement

Room-temperature ionic liquids are attracting growing interest as alternative reaction media for chemical transformations[1]. They are particularly promising as solvents for catalysis. Their use can enhance activity, selectivity, and stability of catalysts. As a part of our "green chemistry" research program, we became interested in ionic liquids as alternative solvents for biotransformations using cell-free enzymes[2–7]. In our first study, we examined lipase-catalyzed reactions in ionic liquids to see if enzyme exhibits any enhanced activity and enantioselectivity. It was observed that the enzyme enantioselectivity increased by 1.5 to 2-fold, but the enzyme activity remained unchanged[8]. In our second study, we examined the activity and selectivity of ionic liquid-coated enzyme (ILCE) in organic solvents. It was found that ILCE had better enantioselectivity and similar activity compared to its native counterpart[9]. Very recently, we have examined the potential of ionic liquids as lyoprotectants and supporters for enzyme activation and stabilization. In the meeting, we will present the results from these studies with some experimental detail.

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P258 *Acinetobacter* SP. LIPASE AND ITS APPLICATION TO RESOLVE 1,3-DIOXOLANE

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Keywords: lipase, *Acinetobacter* sp., reactivity, immobilization

Acinetobacter sp. lipase (called as SY-01) was selected after screening from soils to resolve the target molecule 1,3-dioxolane which is containing quaternary chiral center. This new enzyme showed specific reactivity that the existing enzymes did not show. Research to immobilize wild type SY-01 enzyme to various supporters was proceeded and optimized. Study to optimize the resolution of racemic mixtures was progressed according to changing pH, reaction temperature, reaction time, substrate concentration, and the use of various reaction solvents.

P259 SCREENING AND SYNTHETIC APPLICATION OF NEW BACTERIAL ALCOHOL DEHYDROGENASE FOR ENANTIOSELECTIVE REDUCTION OF β-KETO ESTERS

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Keywords: alcohol dehydrogenase, reduction, β-keto ester

We are working on the screening of new biocatalysts for enantioselective reduction of β-keto esters by use of microtiter plate for parallel growth and bioconversion[1,2]. We established an analytic system which enables the fast determination of conversion and enantioselectivity of the reactions. Several bacteria strains were found to catalyze the reduction of a series of β-keto esters to the corresponding β-hydroxy esters with high activity and high enantioselctivity. Most important asset was finding of strains with opposite enantioselectivity, which allows the production of both *S*- and *R*-enantiomers of the target molecules. The detail results will be presented.

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P260 ENANTIOSELECTIVE *trans*-DIHYDROXYLATION OF NON-ACTIVATED C-C DOUBLE BOND AND ENANTIOSELECTIVE HYDROLYSIS OF RACEMIC AND *meso*-EPOXIDES WITH *Sphingomonas* SP. HXN-200

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Keywords: *trans*-dihydroxylation, epoxide hydrolase, *Sphingomonas* sp. HXN 200

Enantioselective *trans*-dihydroxylation of non-activated C-C double bond is a useful method for preparing *trans*-diols. Several fungi containing monooxygenase and hydrolase are known to catalyze such transformations of several acyclic terpenes, limonene and α-terpinene[1]. However, enantioselective *trans*-dihydroxylation of non-terpene substrates has thus far been unsuccessful[2]. Here, we report that *Sphingomonas* sp. HXN-200, an alkane-degrading bacterium, catalyses the enantioselective *trans*-dihydroxylation of non-activated C-C double bond of heterocyclic compounds giving the corresponding *trans*-diols in high yield and high ee.

Sphingomonas sp. HXN-200 was found to contain a soluble epoxide hydrolase. Hydrolysis of meso-epoxide *N*-benzyloxycarbonyl-3,4-epoxy-pyrrolidine and cyclohexene oxide gave the corresponding trans-diol in high ee and high yield[3], which is the first example of a bacterial EH catalyzing a meso-epoxide. Hydrolysis of several racemic epoxides with HXN-200 showed good enantioselectivity. The optical active epoxides and *trans*-diols prepared from these methods are useful synthetic intermediates that are difficult to make by classic chemistry.

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P261 PURIFICATION AND MODELLING OF A SOLUBLE P450 MONOOXYGENASE IN *Sphingomonas* SP. HXN-200

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Keywords: biohydroxylation, emzyme purification monooxygenase, P450, modeling

We have recently developed *Sphingomonas* sp. HXN-200 as a highly active and easy to handle biocatalyst for regio- and stereoselective hydroxylation of pyrrolidines[1,3], pyrrolidine--2-ones[2], piperidines[4], azetidines[4], and piperidin-2-ones[5], to prepare the corresponding hydroxylated compounds that are useful pharmaceutical intermediates. We found that *Sphingomonas* sp. HXN-200 contains a soluble NADH-dependent P450 monooxygenase. Enzyme components were purified by standard chromatography procedures (ion-exchange chromatography, hydrophobic interaction chromatography, gel filtration, etc.). The purified monooxygenase component is not active by itself. However, combination with other component restores the hydroxylation activity. MS and *N*-terminal se-

quences were determined. Genes of $P450_{pyr}$ was identified and sequenced. A homology model with $P450_{terp}$ as template was established. Combined with the experiment data, the interaction of the enzyme with substrates was investigated with docking program.

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P262 β-*N*-ACETYLHEXOSAMINIDASE-CATALYSED SYNTHESIS OF NON-REDUCING OLIGOSACCHARIDES

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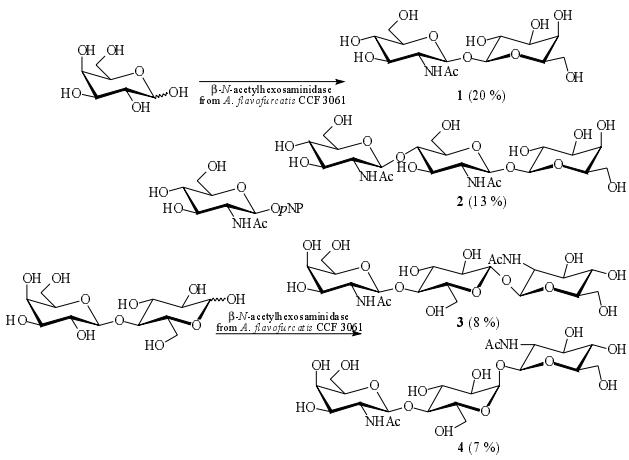
Keywords: β-*N*-acetylhexosaminidase, non-reducing saccharides, enzymatic transglycosylation, *Aspergillus flavofurcatis*, glycosylation regioselectivity

Large panel of fungal β-*N*-acetylhexosaminidases was tested for the regioselectivity of the β-GlcNAc transfer onto Gal-type acceptors (D-galactose, lactose, *N*-acetylgalactosamine). Unique, non-reducing disaccharide and trisaccharides β-D-Glc*p*NAc-(1↔1)-β-D-Gal*p* **(1)**, β-D-Glc*p*NAc-(1→4)-β-D--Glc*p*NAc-(1↔1)-β-D-Gal*p* **(2)**, β-D-Gal*p*-(1→4)-β-D-Glc*p*-β--(1↔1)-β-D-Glc*p*NAc **(3)** and β-D-Gal*p*-(1→4)-β-D-Glc*p*-α--(1↔1)-β-D-Glc*p*NAc (**4**) were synthesized under catalysis of β-*N*-acetylhexosaminidase from *Aspergillus flavofurcatis* CCF 3061 and D-galactose and lactose as acceptors. Disaccharide β-D-Glc*p*NAc-(1→6)-β-D-Gal*p* was produced exclusively with β-*N*-acetylhexosaminidase from *Aspergillus tamarii* CCF 1665. The use of *N*-acetylgalactosamine as an acceptor afforded only β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*NAc.



This project was supported by grants from Czech National Granting Agency No. 203/01/1018, and No. 204/02/P096/A, MSMT grant ME 371 and Research Concept No. AVOZ5020903.

P263 CHEMICAL BIOMIMETIC SYNTHESIS AND ENZYMATIC CONVERSIONS OF THE BIOGENIC PRECURSORS ARTEMISINIC ACID AND ARTEANNUIN B

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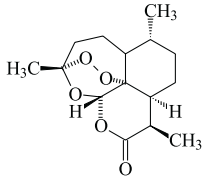
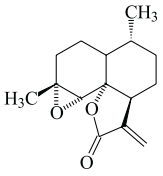
Keywords: artemisinin, antimalarial drugs, enzymatic conversions

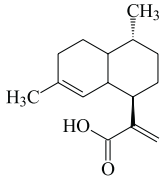
Malaria is one of the most important killer diseases of the tropical world. It has been responsible for over two million deaths per annum and has become a major obstacle in the development and prosperity of third-world nations. Considerable efforts to eradicate malaria are impeded by multi-drug resistant strains. Artemisinin **(1)** is an important antimalarial drug that meets the dual challenge posed by drug-resistant parasites and rapid progression of malaria illness. Artemisinin is extracted from *Artemisia annua*. This herb has been used for malaria therapy in China for over 1000 years. *Artemisia annua* is a temperate plant and is not available in abundance to the tropical regions where malaria is highly endemic. Unfortunately, the low natural abundance (0.05–1.1 %) of artemisinin and its complex structure make total synthesis very difficult and certainly not adaptable to industrial production at low cost[1, 2].



1 Artemisinin

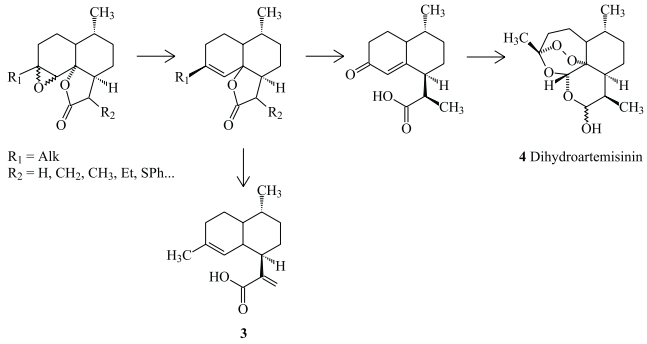


2 Arteannuin B



3 Artemisininc acid

Hence, we have been led to consider a biotechnological approach by employing cell-free extracts of *Artemisia annua* leaves as an alternative to produce artemisinin from biogenic precursors. Indeed, these biosynthetic precursors, arteannuin B **(2)** and artemisinic acid **(3)**, occur of levels 8–10 times higher than artemisinin in plants[3]. Artemisinic acid has the added advantage of facile isolation from the plant *via* base extraction.



R_1 = Alk
R_2 = H, CH_2, CH_3, Et, SPh...

4 Dihydroartemisinin

3

We are currently developing novel routes to arteannuin B and analogues from dihydroartemisinin **(4)** and **(3)** to generate novel bioactive artemisinin derivatives. The biotransformation of these substrates by using plant cell-free extracts will be reported.

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P264 POLYMERIC MEMBRANES SYNTHESIS FOR POTABLE WATER PRODUCTION

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Keywords: membrane synthesis, *E. coli*, potable water

The objective of this work was a study of polymeric asymmetric membranes synthesis for the *Escherichia coli* bacteria removal getting a production of potable water. In spite of the known concept of the water being an infinite resource, it is a finite one and it is becoming rare, due a lot of factors, such as, source contaminations, demographic explosion and the natural wood degradation. Consequently, urge a rational management of waters and the domain of techniques for their treatments. In this work we tried to present an alternative process for treating water contaminated.

The process used in this work for preparing the membranes was the inversion phase, and the technique was the precipitation by immersion. The membranes were synthesized from a polymer solution of dimetylformamide (DMF), using either the polymers polysulfone or polyvinylidene fluoride (PVDF). In addition, the salt KCl was used as an additive in the polymeric solution. The membranes support used in the synthesis was polyester-polypropylene sheet, and the preparation of the asymmetric membranes was made by spreading the polymeric solution (polymer + solvent + salt) as a thin film, on the support, which was previously adhered in a glass plate by a nylon string. This film was introduced in some water baths at 20 °C. The last stage was the dry of the membranes. For verifying the performance of the synthesized membranes, some samples of deionised water were contaminates with the *E. coli* bacteria with a bacterial mass of 10^7–10^8 CFU.ml^{-1}. These contaminated samples were filtered in a dead end filtration module. In this module the synthesized membranes were placed on a perforated metal plate and magnetic agitation was kept to avoid the deposition of material on the membrane surface. The operation pressure was of 3 bar.

The permeate was analyzed in terms of the retention of the *E. coli* (microbial measure) and also the flux of permeate.

Analyzing the results, it is possible to state that the aim of this work was reached because, not only was got a great *E. coli* removal, resulting in potable water, but also kept a satisfactory permeate flux. All synthesized membranes gave good results, reaching an *E. coli* removal between 95 and 100 %, and permeate flux above 200 l.hm^2. The results obtained can be considered good because the synthesized membranes were tested with a mass bacterial of *E. coli* (10^7–10^8 CFU) well

above of the commonly found in waters, and this mass bacterial is difficult to be removed.

P265 SPECIFICITY OF FUNGAL KERATINOLYTIC PROTEASES

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Keywords: fungal keratinases, cleavage sites, catalytic properties, enzyme stability

Enzymes that hydrolyse scleroproteins, keratins, are among most powerful proteolytic enzymes. They are synthesized by some insects and microorganisms and are mostly serine type proteases active in alkaline environment. Apart from bacteria and dermatophytes, producers of keratinases can also be found among common non-pathogenic fungi as shown in our screening experiments[1]. The most promising fungi were cultivated under conditions promoting keratinase synthesis. The enzymes of *Doratomyces microsporus*[2] and *Paecilomyces marquandii* were purified and characterised. Both of them are most active at pH 8, but differ in optimal temperature that is 45 °C for the former and 60–65 °C for the latter. Purified enzymes are susceptible to autolysis, however, the keratinase of *P. marquandii* is notably more stable than that of *D. microsporus*. In order to determine the preferred peptide cleavage sites, the activity of the enzymes was tested on synthetic peptides as well as on the oxidised insulin B-chain. Results on the synthetic substrates showed that both keratinases exhibited similar substrate specificity. They were most active on Suc-Ala-Ala-Pro-Phe-pNa (AAPF) that has a non-polar aromatic amino acid at the cleavage site, while Suc-Ala-Ala-Ala-pNa with the non-polar aliphatic amino acid at the cleavage site was far less affected. Hydrolysis of Ac-Tyr-OEt indicated esterase activity of both keratinases. Neither of the enzymes hydrolysed Bz-Arg-pNa, Bz-Tyr-pNa or FA-Leu-Gly-Pro-Ala-OH which are specific synthetic substrates for trypsin, collagenase and chymotrypsin, respectively. Catalytic properties on AAPF obtained by a non-linear regression based on the Michaelis-Menten equation are presented in Table I.

Table I
Catalytic properties of two fungal keratinases on Suc-Ala-Ala-Pro-Phe-pNa

Enzyme	K_m (μM)	V_{max} (μmol.min^{-1})	E_{tot} (μmol)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}.min^{-1})
Keratinase of *D. microsporus*	1027 ± 169	0.070 ± 0.004	1.33 E-4	526	0.51
Keratinase of *P. marquandii*	165 ± 18	0.044 ± 0.001	1.79 E-5	2458	14.90

Specificity of hydrolysis was determined on the oxidized insulin B-chain by incubating enzyme/substrate mixture at 45 °C for 20 min and for 12 h. Cleavage sites were compared to those published for Proteinase K, the only commercially available keratinolytic protease of fungal origin, as well as for some other known serine proteases. The results are presented in Table II.

Table II
Cleavage sites on the oxidised insulin B-chain by keratinases of *Doratomyces microsporus* and *Paecilomyces marquandii* in comparison with those described for some known proteinases[3,4]

	1	5	10	15	20	25	30
Oxidised insulin B-chain	**F V N Q H L C G S H L V E A L Y L V C G E R G F F Y T P K A**						
Keratinase of *D. microsporus*	↑	↑↑↑↑	↑	↑↑	↑↑↑↑↑↑	↑↑ ↑	↑↑
Keratinase of *P. marquandii*	↑ ↑	↑	↑ ↑	↑ ↑ ↑	↑	↑↑ ↑	
Proteinase K (ref.[3])	↑↑↑	↑ ↑ ↑	↑	↑↑↑	↑↑↑	↑↑↑	
Subtilisin[3]	↑	↑ ↑	↑↑↑			↑	
Trypsin[4]						↑	↑
Elastase[4]			↑	↑		↑	

Compared to other proteases, fungal keratinases including Proteinase K show very broad specificity that may be responsible for their high activity against keratin filaments.

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P266 IS THE AROMA COMPOUND γ-DECALACTONE PRODUCED BY YEAST IN RESPONSE TO A MEMBRANE-RIGIDIFYING STRESS INDUCED BY THE BIOTRANSFORMATION MEDIUM?

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Keywords: γ-decalactone, biotransformation, yeast cells, biological membranes

The peach-like aroma compound γ-decalactone is produced biotechnologically through the biotransformation of ricinoleic acid (RA) by some yeast species. In order to better understand the interactions occurring during the process between the lactone and the yeast cells, the influence of the lactone on the physical properties of cell membranes and model phospholipids has been studied. The producing yeast *Yarrowia lipolytica* was used as a model cell in this study. γ-Decalactone strongly increased membrane fluidity *in vivo* and decreased in a concentration-dependent manner, the phase transition temperature (*T*m) of the deuterated phospholipid DMPC-d27 (ref.[1–3]). This indicates that the lactone exhibits an important membrane-fluidizing action. On the other hand, the hydroxylated C18 fatty acid (RA) used as the precursor of the biotransformation, increased the *T*m of DMPC-d27, i. e. it rigidified the phospholipid bilayers. These observations bring about the hypothesis that the production of the lactone, a compound that is presently considered as a secondary metabolite, in fact could be stimulated in order to enable a homeoviscous adaptation of cell membranes and so to permit an optimal cell development in the medium. From this point of view, new strategies may be elaborated to improve γ-decalactone production yields.

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P267 GLYCOMIMETICS AS SELECTIVE TOOLS FOR ENZYME INHIBITION

ANA T. CARMONA[a], FLORENCE POPOWYCZ[b], SANDRINE GERBER-LEMAIRE[b], ELIAZAR RODRIGUEZ-GARCIA[b], PIERRE VOGEL[b], and **INMACULADA ROBINA**[a]

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Keywords: pyrrolidines, enzymatic inhibition, sugar mimetics

Sugar mimetics have shown to be inhibitors of enzyme-promoted hydrolysis of C-O glycosidic bonds exhibiting interesting applications as antibacterial, antiviral and anticancer agents. Among them, 1,4-dideoxy-1,4-iminoalditols (hydroxylated pyrrolidines) constitute an important type of compounds with a well-known importance as glycosidase inhibitors[1]. However, in many instances they are not selective presenting a wide range of enzymatic inhibition.

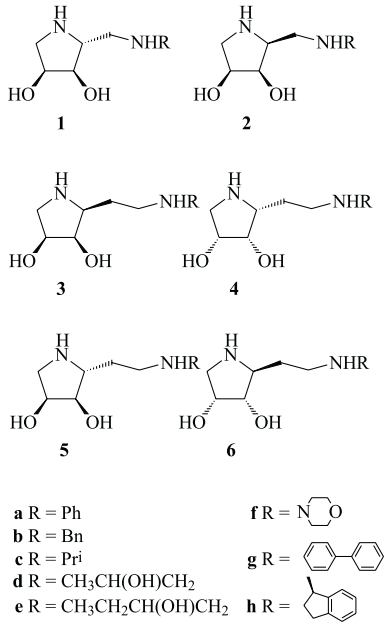
The selectivity in enzyme inhibition can be improved by providing the iminosugar with some information of the structure of the glycosyl moiety that is cleaved in the enzymatic hydrolysis and of the aglycon itself. Therefore, the introduction of additional groups in the iminosugar joined by hydrolytically stable C-C links could led to new more potent, more selective and hydrolytically stable enzyme inhibitors. Lipophilic moieties are important structural features because they, additionally, have the advantage of their permeability through membranes, which is an important requirement for a compound to become a useful drug.

We have found that diamines of type **1** with aryl(alkyl)-aminomethyl chains can be higly selective and competitive inhibitors of α-mannosidases and we have reported[2] a quick combinatorial approach for their preparation.

In this commuincation we report on the influence on the enzymatic activity of three stereogenic centers in the pyrrolidine ring and the influence of the methylene spacer between the nitrogen of the pyrrolidine ring and of the aminoalkyl chain. To achieve this goal, we describe the synthesis and enzymatic evaluation of derivatives of (2*S*,3*R*,4*S*), (2*R*,3*R*,4*S*), (2*R*,3*S*,4*R*) and (2*S*,3*S*,4*R*)-2-alkyl(aryl)aminoethyl-3,4-dihydroxypyrrolidines **(3-6)** and of (2*S*,3*R*,4*S*)-2-(*N*-alkyl(aryl)-aminomethyl-3,4-dihydroxypyrrolidines **(2)**.

Enzymatic inhibitory studies of diamines **2-6** indicate that the activity and selectivity in enzyme inhibition are influenced by the absolute configuration of the stereogenic centers in the pyrrolidine. Thus, (2*S* and 2*R*, 3*R*, 4*S*)-2-alkyl(aryl)aminoethyl **3** and **5** and (2*S* and 2*R*, 3*R*, 4*S*)-2-alkyl(aryl)aminomethyl **1** and **2** have shown to be moderate-to-good inhibitors of β-mannosidases, while derivatives **4** and **6** of configuration (2*R* and 2*S*, 3*S*, 4*R*) were inactive towards those enzymes presenting good inhibitory values towards β-galactosidases

and β-glucosidases or α-L-fucosidases. The spacer between the two nitrogens (aminomethyl *vs.* aminoethyl side chains), and the substituent (alkyl *vs.* aryl) have also a remarkable influence.



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P268 PREPARATION OF DEAZAPURINE NUCLEOSIDES BY MICROBIAL TRANSGLYCOSYLATION

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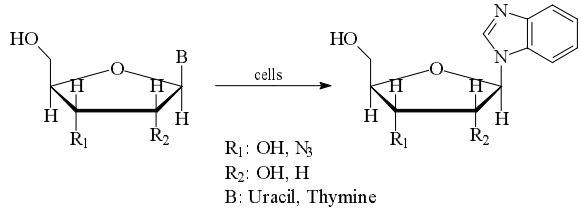
Keywords: deazapurine nucleosides, microbial transglycosylation

Benzimidazole nucleosides have shown inhibitory effect on human cytomegalovirus infections[1], herpes viruses[2] and other RNA viruses and in some cases possess mutagenic properties. The chemical synthesis of these nucleosides is complex and stereounspecific and affords low yields[3].

To our best knowledge, only ribo- and deoxyribofuranosyl nucleosides of benzimidazole have been previously synthesised[4] using microbial transglycosylation and guanosine as starting material.

Based on our experience[5, 6], benzimidazole nucleosides carrying natural and modified sugar moieties were prepared starting from available pyrimidine ones. The selected microorganisms were screened from our bacterial collection, affording yields between 60–90 % after optimisation of the reaction conditions.



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P269 A SURPRISING DOUBLE OXIDATION OF 7-PHENYLBICYCLO[3.2.0]HEPT-2-EN-6-ONE DERIVATIVES BY *Cunninghamella echinulata* NRRL 3655

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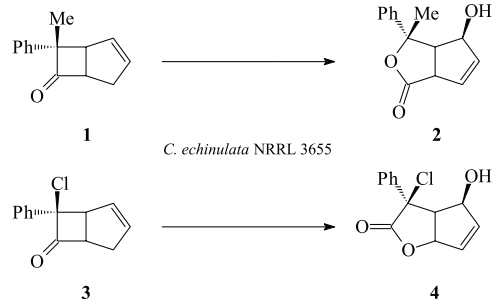
Keywords: Baeyer-Villiger oxidation, *Cunninghamella echinulata*

The fungus *Cunninghamella echinulata* NRRL 3655 has proved useful for the Baeyer-Villiger oxidation of bicyclo [3.2.0]hept-2-en-6-one owing to its ability to catalyse a resolution of the starting material[1], in contrast to the enantiodivergent biotransformation to equimolar quantitities of 2-oxa and 3-oxa lactones observed with prokaryotic Baeyer-Villger monooxygenase enzymes[2,3]. On application of this catalyst to the transformation of 7-phenylbicyclo-[3.2.0]hept-2-en-6-one derivatives, a surprising double oxidation has been observed. 7-*endo*-phenyl, 7-*exo*-methylbicyclo-[3.2.0]hept-2-en-6-one **1** was transformed to the 3-oxa lactone derivative with concomitant allylic hydroxylation to yield hydroxylactone **2**. Contrastingly, 7-*endo*-phenyl, 7-*exo*-chlorobicyclo[3.2.0]hept-2-en-6-one **3** was again transformed to a hydroxylactone, but in this case oxygen insertion occurred to give the 2-oxa derivative **4**.



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