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.

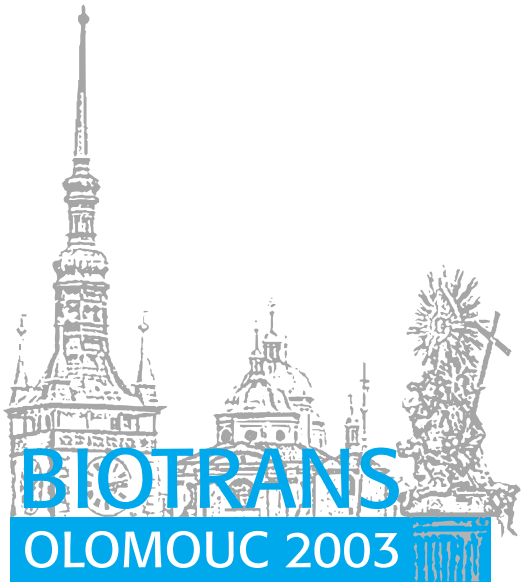
We hope very much that the content of this issue, despite its specificity, will be not only interesting for the reader but will provide new information on recent results of leading laboratories working in the field of biotransformation. It should also help to get a deeper insight into this scientific 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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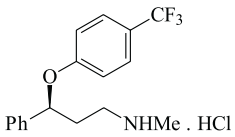
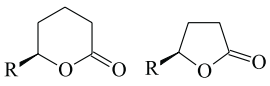
Keywords: bioactive compounds, enantiomers, lipases, pharmaceuticals, renewable resources

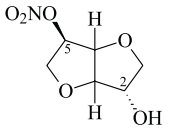
Enzymes have emerged in recent years as highly efficient catalysts in organic syntheses. This is particularly true for esterhydrolases (esterases, lipases) many of which ideally combine the required high reaction selectivities with the synthetically so important broad substrate tolerance. As demonstrated 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



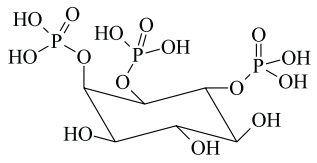
δ - and γ - lactones



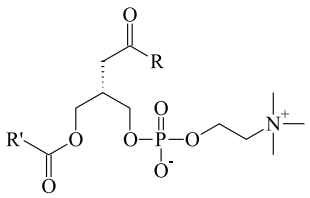
Isosorbide-5-mononitrate

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].

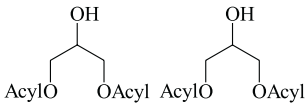


1,2,6-*myo*-Inositoltriphosphate

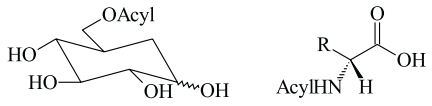


carba - analogues of phospholipids

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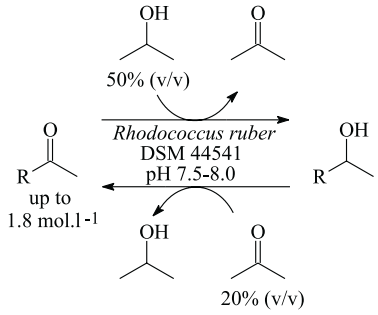
Keywords: alcohol dehydrogenase, *Rhodococcus ruber*, ketone, assymetric reduction

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**.

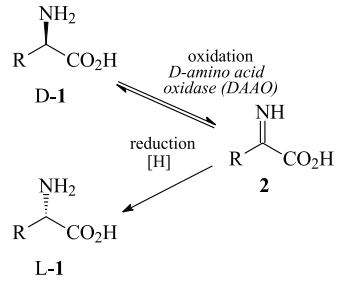

Fig. 1. Stereoinversion of D- to L-amino acids using a D-amino acid oxidase in combination with a chemical reducing agent.

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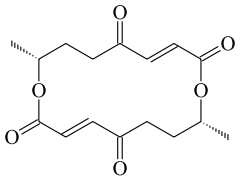
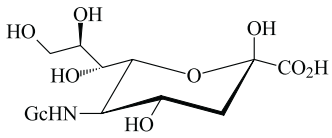
Keywords: lipase, nitrile hydratase, amidase, protease, yeast-mediated reduction

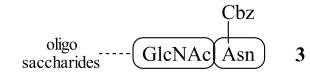
An important aspect in the enzyme-catalyzed production of chemicals is the complementary and synergistic use of chemo-enzymatic procedure so that we can draw a reasonable and straightforward blueprint towards the target molecules. Our early achievements were the syntheses of (*R*,*R*)-pyrenophorin (**1**, 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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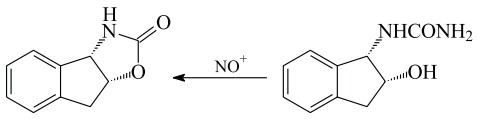
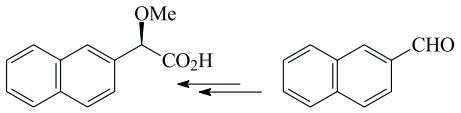


4 **5**



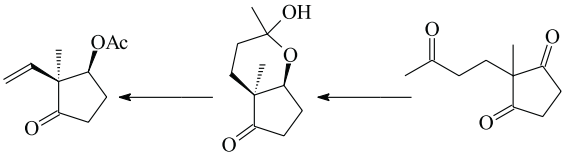
(*R*)-2-NMA **6** **7**

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.

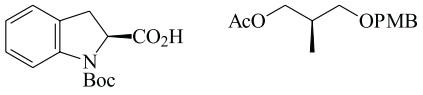
Of course, the development of new chemical reaction, to be coupled with enzymes as key steps, was necessary, as shown in the synthesis of an oxazolidinone (**4**: nitrosation-deaminocyclization) and (*R*)-2-NMA (**6**: one-pot cyanohydrin acetate formation) from **5** and **7**, respectively.

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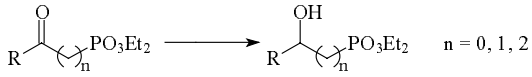
Keywords: hydroxyalkanephosphonate, oxoalkanephosphonate, stereoselective reduction, microbial whole cells

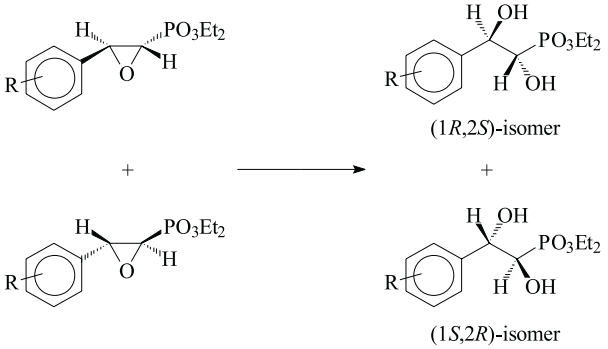
Biotransformations offer an effective and sometimes preferable alternative to the standard synthesis of fine chemicals, especially in the case when optically active products are desirable. Hydroxyalkanephosphonates could be considered as analogues of hydroxy acids and are obtained by replacement of carboxylic 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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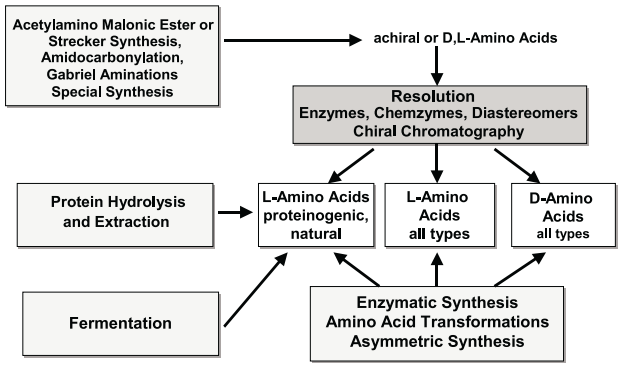
Keywords: enzymes, biocatalysis, organic synthesis, amino acid

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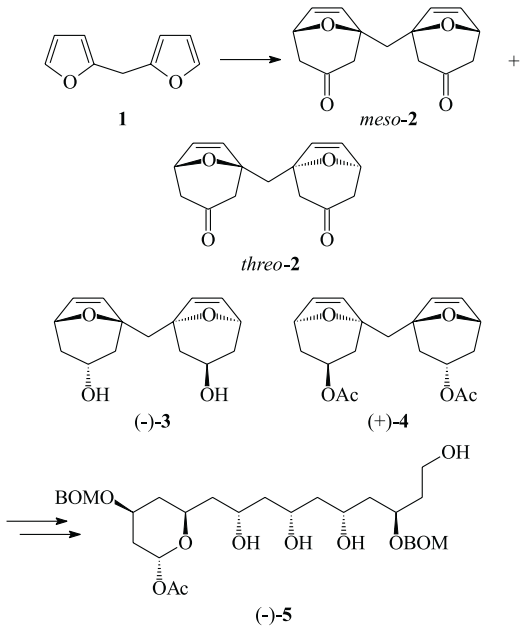
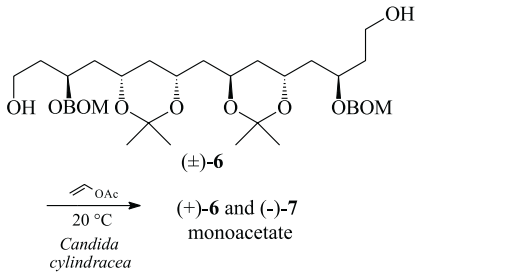
Keywords: double chain elongation, lipase-catalyzed acetylation, 1,3-polyols

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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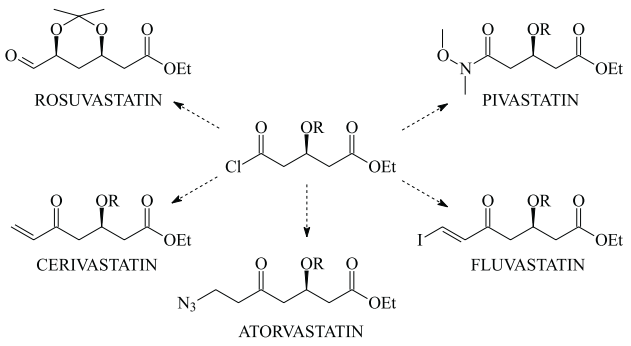
Keywords: statin-side chain, enzymatic desymmetrization

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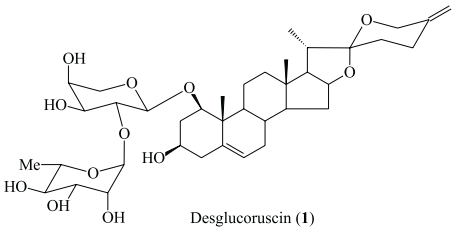
Keywords: α-L-rhamnosidase, enzyme library, natural glycosides

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.

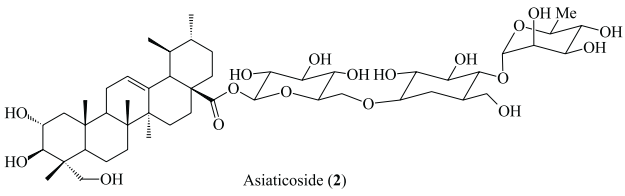
Over 30 different α-L-rhamnosidase preparations were recovered by ammonium sulfate precipitation. Substrate specificity of this α-L-rhamnosidase library was tested with vari-

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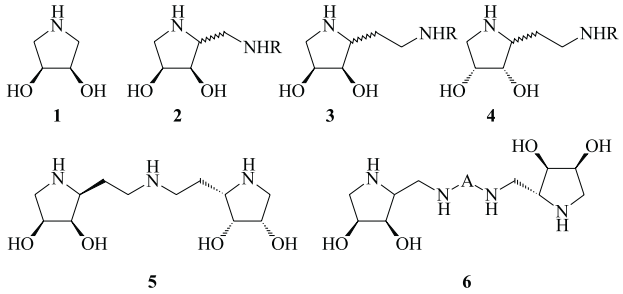
Keywords: pyrrolidines, pyrrolidine-polyamines, enzymatic inhibition, sugar mimetics, nitrilases, cyanodeoxy glycosides

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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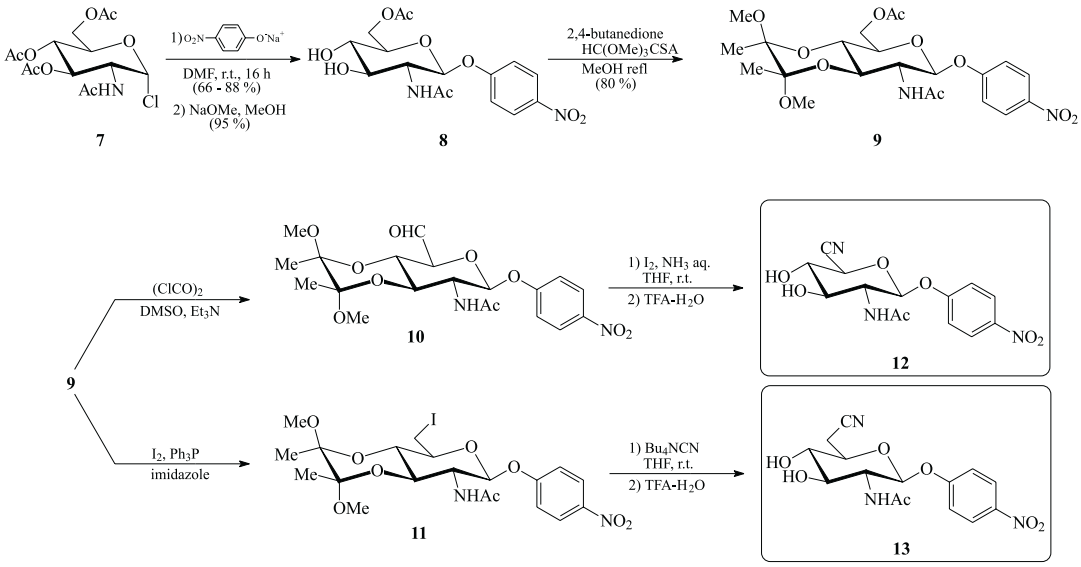

L024 MONITORING BIOTRANSFORMATIONS IN ACRYLIC FIBRES

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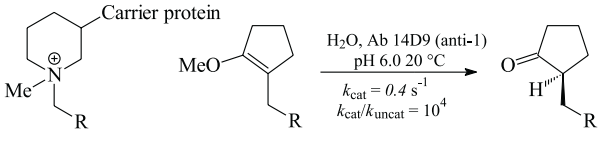
Keywords: antibody 14D9, expression, X-ray crystallography, active site, enantioselectivity

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**

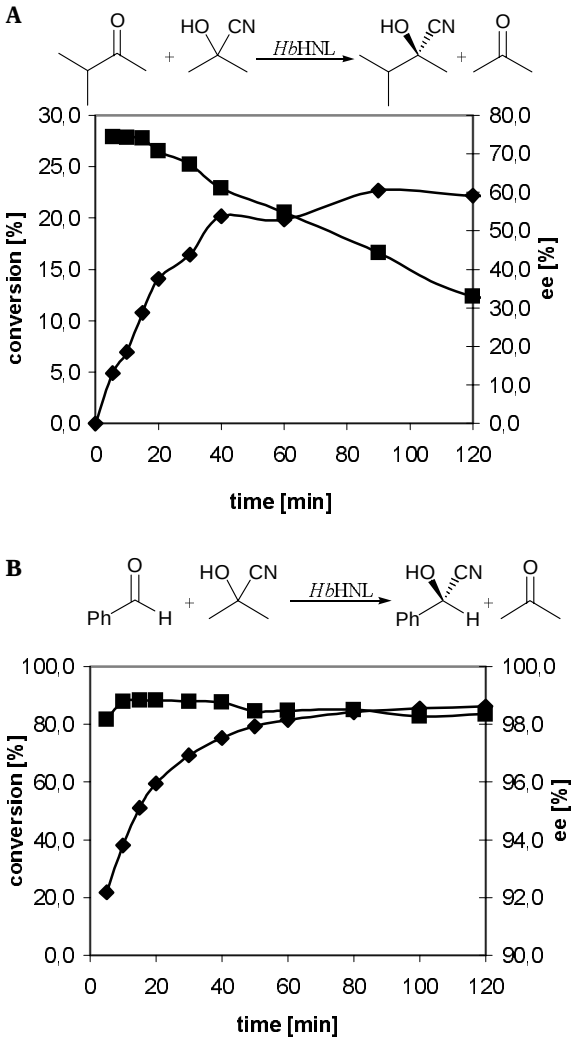

Fig. 1. **Reactions catalysed by sol-gel encapsulated *Hb*HNL at 25 °C in diisopropyl ether; A:** isopropylmethyl ketone 1:3 acetone cyanohydrin; **B:** benzaldehyde 1:3 acetone cyanohydrin

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

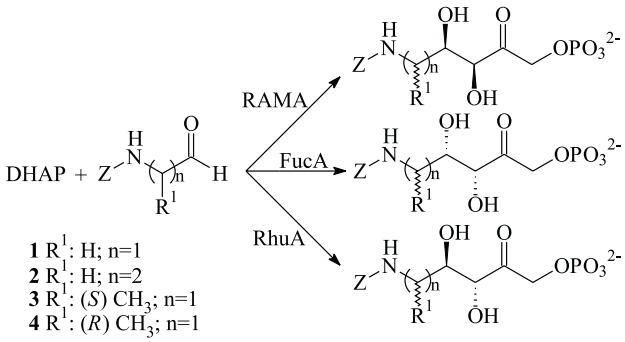
Highly concentrated emulsions (gel emulsions) are liquid-liquid dispersions with an internal phase volume fraction larger than 0.74, the critical value of the most compact arrangement of spheres of equal radius. Hence, their structure consists of closed-packed droplets, deformed and/or polydisperse, 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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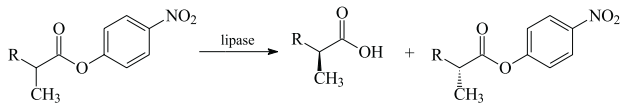
Keywords**:** directed evolution, lipase, mutants, enantioselectivity, structure, simulation

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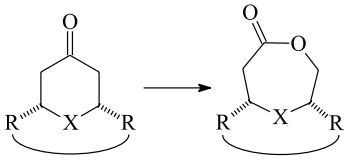
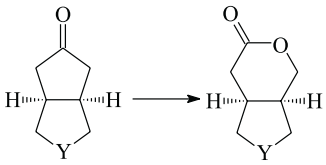
Keywords: Bayer-Villigerase, cyclohexanone monooxygenase, recombinant whole-cells

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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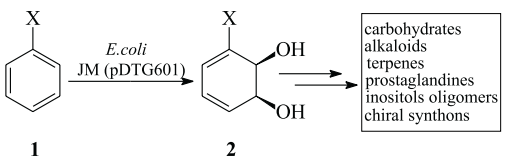
Keywords: cyclohexadiene diols, enantioselective synthesis, pancratistatin, morphine

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.

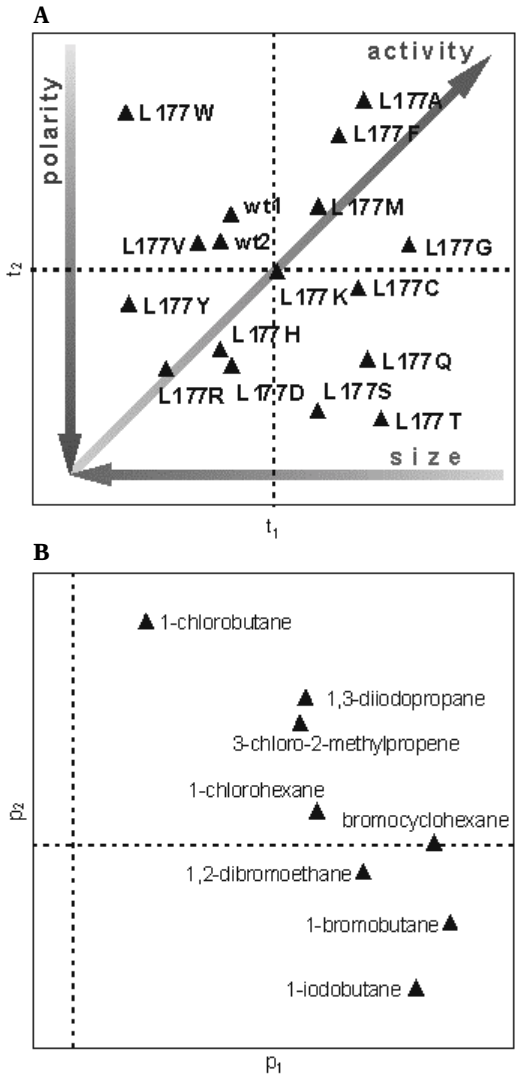

Fig. 1. **The score plot (A) and the loading plot (B) of first two components from the principal component analysis of specific activities measured for eight halogenated substrates;** the arrows in the score plot indicate that the protein variants are ordered approximately by the size of amino acid introduced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

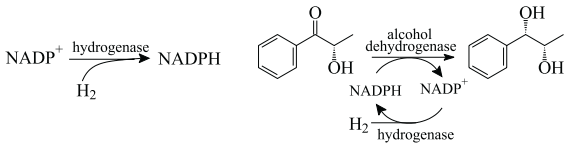

Fig. 1. **Generation and regeneration of NADPH with hydrogenase from *Pyrococcus furiosus***

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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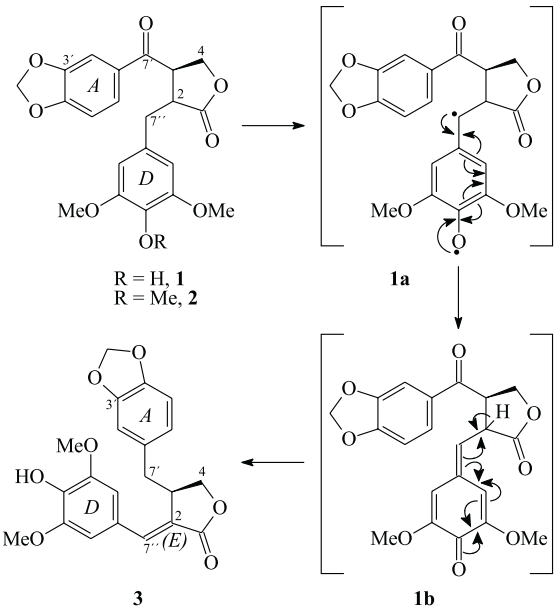
Keywords: peroxidases, dibenzylbutanolides, podophyllotoxin, kinetic studies

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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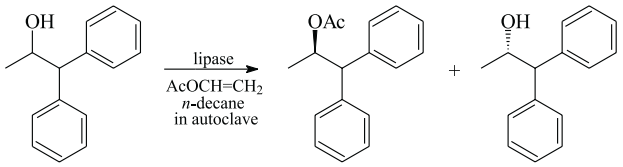
Keywords: high temperature, lipase, mechanism, enantioselectivity

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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