P214 CHIRAL ALCOHOL PRODUCTION BY β-KETOESTER REDUCTASE FROM *Penicillium citrinum* COUPLED WITH REGENERATION SYSTEM OF NADPH

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Keywords: reductase, aldo-keto reductase super family, mutation

We isolated NADPH-dependent alkyl 4-halo-3-oxobutanoate reductase (RPC) from *Penicillium citrinum*. RPC catalyzed the reduction of methyl 4-bromo-3-oxobutanoate (BAM) to methyl *(S)*-4-bromo-3-hydroxybutanoate (BHBM). The product, *(S)*-BHBM, is a potential key intermediate for the synthesis of a variety of HMG CoA reductase inhibitors.

The *rpc* gene comprises 978 bp and encodes a polypeptide of 36.7 kDa. The deduced amino acid sequence was found to be similar to well-known sequences of the aldo-keto reductase superfamily. The *rpc* gene and glucose dehydrogenase gene *(gdh)* to regenerate cofactor were overexpressed in *Escherichia coli*. The recombinant *E. coli* cells produced *(S)*-BHBM from BAM in the presence of an NADPH-regeneration system.

To increase thermostability of RPC, the recombinant RPC expressed in *E. coli* was evolved by the error-prone polymerase chain reaction (epPCR) method. We isolated three mutants (A3-49, A8-39 and T1-99) with increased thermostability. Mutant T1-99 was improved not only thermostability but also enantioselectivity. Changes in the amino acid sequence of the mutant enzymes were identified by analyzing the nucleotide sequence of the genes. Mutant T1-99 had two amino acid changes (L54Q and R104C).

The effect of each amino acid residue on the thermostability and enantioselectivity of RPC was investigated. The only mutant L54Q revealed higher thermostability and enantioselectivity than those of wild-type RPC. An alignment of the deduced RPC amino acid sequence with other aldo-keto reductases suggested that L54 is close to the active site of the enzyme[1]. K_m value of the mutant L54Q was estimated to be similar to that of the wild-type enzyme, but K_{cat} value was slightly decreased.

Then L54 was subjected to saturation mutagenesis to give ten different mutants with increased thermostability. Fourteen different mutants catalyzed the formation of *(S)*-BHBM with higher enantioselectivity than the wild-type enzyme.

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P215 HETEROLOGOUS EXPRESSION AND SITE DIRECTED MUTAGENESIS OF ALKENE MONOOXYGENASES FOR IMPROVED ACTIVITY AND STEREOSELECTIVITY

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Keywords: alkene monooxygenase, site directed mutagenesis, stereoselectivity

Alkene monooxygenases are found in bacteria isolated on low molecular weight alkenes (eg ethene, propene), and convert the alkene to an epoxide as the first step in metabolism. They are part of the bi-nuclear non-haem iron family of oxygenases which includes methane monooxygenase and attack the alkene double bond to form epoxides, selectively, with no reactivity against alkanes[1]. Depending on the enzyme and substrate, these enzymes can exhibit moderate to good stereoselectivity, forming *R*-epoxides from pro-chiral 1-alkenes. To date, alkene monooxygenases have only been found in a narrow range of bacteria including the high GC Gram positive *Mycobacteria* sp. and *Rhodococcus* sp. and the Gram negative *Xanthobacter autotrophicus*. These are relatively slow growing organisms.

Despite the fact that homologues such as toluene monooxygenase are actively expressed in *E. coli*, it has proved difficult to express alkene monooxygenases in this host although expression can be obtained in more closely related hosts (eg other high GC Gram positive organisms). This paper will describe progress towards expression of the enzyme from *X. autotrophicus* in *E. coli* and site directed mutagenesis of a mycobacterial enzyme expressed in *M. smegmatis*. By sequence alignment and comparison with the known 3D structure of methane monooxygenase[2], residues have been identified which exhibit a systematic variation between different types of enzyme (aromatic, alkene, methane), and other residues identified which vary systematically with stereoselectivity of alkene epoxidation. Mutagenesis of these residues is starting to provide some insight into the differences between alkene and alkane oxidation and control of stereoselectivity.

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P216 DIRECTED EVOLUTION OF *dszABC* OPERON FROM *Rhodococcus* SP. DS7: EVALUATION OF THE SELECTED MUTANTS FOR BIODESULFURIZATION ACTIVITY ON DIFFERENT SUBSTRATES

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Keywords: biodesulfurization, directed evolution, substrate specificity

The combustion of sulfur-containing fossil fuels produces environmentally hazardous SO_x gases that released in the atmosphere generate air pollution and acid rain. Conventional hydrodesulfurization (HDS) of petroleum fractions involves an inorganic catalyst and hydrogen under conditions of high temperature and pressure to produce hydrogen sulfide and a desulfurized compound. Even though HDS is an effective and well-understood technology, heterocyclic sulfur compounds cannot be completely removed. The slow-reacting, recalcitrant components all belong to the class of dibenzothiophenes, like dibenzothiophene (DBT) and its alkylated derivatives. Since the 1980s several microorganisms able to selectively desulfurize DBTs have been isolated and most of them are Gram-positive like *Rhodococcus* sp. strain IGTS8 described by Kilbane[1] capable to remove sulfur from DBT in a C-S bond-targeted fashion to produce 2-hydroxybiphenyl (HBP) and sulfite. Therefore the application of a biodesulfurization process using a DBT-desulfurizing microorganism following HDS is an attractive possibility for achievement of deeper desulfurization but the substrate specificity is a strong limitation.

The conversion of DBT to HBP is catalyzed by a multienzyme pathway proceeding *via* two cytoplasmic monooxygenases (DszC and DszA) supported by a flavin reductase (DszD) and a desulfinase (DszB). This pathway resembles other sulfur removing pathways with the feature that the carbon skeleton is not mineralized.

With the aim to generate new enzymes able to better degrade DBTs alkylated derivatives, an *in vitro* evolution approach has been applied to the *dsz* operon from *Rhodococcus* sp. DS7, a DBT-degrading strain isolated in our laboratories[2].

Using epPCR a library of 5×10^5 mutants has been created and over 10^4 mutants have been screened for their ability to metabolize different substrates.

Data concerning the biodesulfurization activity of a number of selected mutants will be presented.

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P217 MASTERING POX GENOTYPE FOR FATTY ACID TRANSPORT AND ACCUMULATION IN THE YEAST *Yarrowia lipolytica*

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Keywords: *Yarrowia lipolytica*, lipids, acyl-CoA oxidase, protrusion, single cell oil

Due to its ability to produce different metabolites such as citrate, and to secrete numerous proteins, the yeast *Yarrowia lipolytica* has been used in industrial processes. It is also able to grow on and degrade alkanes and to use fatty acids as carbon sources, which makes it a promising tool for biotechnological uses[1]. Its capacity to degrade oils of different origins makes it a potential de-polluting agent and for single cell oil production (SCO)[2].

Y. lipolytica could be used for γ-decalactone (peach flavor) production. This yeast can be used for lipase production[3,4]. The *Y. lipolytica* lipase Lip2p was shown to be efficient for carboxylic esters resolution.

Degradation of alkanes involves three enzymatic steps in endoplasmic reticulum to produce a fatty acid. Similarly, degradation of triglycerides is performed by secreted lipases which release the corresponding fatty acids, which are then metabolized via β-oxidation.

The initial step of peroxisomal β-oxidation is catalyzed by the acyl-CoA oxidases (Aoxs) encoded by the *POX* genes. *Y. lipolytica POX* set is the most complex among the yeasts studied to date, as it comprises five different genes encoding Aoxs.

Functions of different acyl-CoA oxidases have been investigated by successive gene disruptions (construction of mono-, bi-, tri- and tetradisruptants)[5]. These genes encode for proteins exhibiting different specificities with respect to the chain lengths of the acyl-CoA substrates. Aox3p and Aox2p from *Yarrowia lipolytica* were bacterially expressed, purified and their activity as a function of substrate chain length was established[6, 7]. Using DNA shuffling techniques, we have constructed 11 chimeras between Aox2p and Aox3p, two of them exhibit broad chain length specificity. Tri and tetra- disruptants were transformed with Aox2p, Aox3p and chimera encoding genes. Growth in oleic acid (0.1–5 %), peroxisomes and lipid bodies proliferation was followed during a 60 h time course. We determined cellular, medium and surface lipid composition by TLC and GC/MS. In addition, cell surface and organelles formation were analyzed by scanning and transmission electron microscopy.

Our results demonstrate the induction of protrusions on yeast cell surface involved in oil droplet fixation. In those strains, peroxisome proliferation and size were not significantly modified. In contrast, *POX* genotype affected dramatically lipid bodies formation (few lipid bodies up to giant lipid bodies), alters lipid composition (no triglycerid accumulation up to high TG accumulation).

These demonstrates that mastering *POX* genotype allowed us to construct strains with high lipid content which could be used for.

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P218 PRODUCTION OF RUBBER MOLECULES WITH RECOMBINANT *Hevea brasiliensis* RUBBER TRANSFERASE

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Keywords: rubber transferase, *cis*-prenyl chain elongation, c-DNA cloning, *Hevea brasiliensis*, polyprenols

Natural rubber from *Hevea brasiliensis* is the high molecular weight polymer of isoprene units with *cis*-configuration. The enzyme responsible for the *cis*-1,4-polymerization of isoprene units has been shown as a particle-bound rubber transferase, but no gene encoding this enzyme has been cloned in rubber-producing plants. Using sequence information of the conserved regions of *cis*-prenyltransferases cloned recently from *Micrococcus luteus* B-P 26 (ref.[1]), *Escherichia coli* (ref.[2, 3]), yeast[4] and *Arabidopsis thaliana* (ref.[5, 6]), we have isolated and characterized the cDNA for the rubber transferase from *Hevea brasiliensis*. Sequence analysis revealed that all five highly conserved regions among *cis*-prenyltransferases[7] are found in this protein sequences, which are totally different to those of *trans*-prenyl chain elongating enzymes[8].

In vitro rubber production using the recombinant enzyme overexpressed in *E. coli* cells revealed that the rubber transferase catalyzed the formation of medium chain and long chain polyisoprenes with approximate molecular size of $2 \times 10^3 \sim 1 \times 10^6$ Da in the presence of washed bottom fraction particles. Neither the recombinant enzyme nor washed bottom fraction particles alone showed significant activity for rubber production. This result suggested that *Hevea* rubber transferase might require some activation factors in the washed bottom fraction particle for the production of high molecular weight rubber molecules.

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P219 GEL-STABILIZED TWO-PHASE SYSTEMS: NEW APPROACHES TO THE ENZYMATIC SYNTHESIS OF HYDROPHOBIC FINE CHEMICALS

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Keywords: enzyme, entrapment, gel, hydrophobic compound

Within the last decades, enzyme catalysis proved to be a versatile tool in the synthesis of chemical compounds, mainly due to the broad substrate range and selectivity of enzymes. The technical application, however, is often restricted to the production of fairly water-soluble molecules as hydrophobic compounds are only inefficiently converted in aqueous phases, while many enzymes are instable in the presence of non-aqueous media such as organic solvents. Thus, the use of enzymes for the synthesis of hydrophobic molecules, which are known to be important building blocks of fine chemicals, pharmaceuticals or agrochemicals, requires the development of alternative reaction techniques.

In this work, gel-stabilized two-phase systems, meaning two-phase systems, consisting of a gellified aqueous phase and a non water-miscible organic solvent, were investigated. Enzymes from different classes, an alcohol dehydrogenase, a lipase, and a decarboxylase, were entrapped in the natural or synthetic polymer matrices and characterized with regard to their stability, activity, and/or selectivity in the synthesis of hydrophobic chiral alcohols, aroma esters and chiral hydroxy ketones, respectively. It was demonstrated that the stability of an alcohol dehydrogenase with a usually high sensitivity to the presence of organic solvents was strongly enhanced. Loss of its cofactor during gel preparation was avoided by the use of an emulsion technique, and cofactor regeneration was performed within the matrix by coupling the reduction of ketones to chiral alcohols with the oxidation of isopropanol. The stereoselectivity of both, the alcohol dehydrogenase and the decarboxylase was unaffected by the system features, and the product yield was high with all three enzymes. The overall productivity of the reaction systems not only depended on the entrapped biocatalysts, but also on the polymer type used as the aqueous phase.

P220 SOL-GEL BIOCOMPOSITE MATERIALS AS SOLID-PHASE BIOCATALYSTS

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Keywords: sol-gel, silica, entrapment, hydrolases

One of the most important issues when using enzymes for developing active biocatalysts is whether they remain functional, during immobilization and over time, and to what degree. The entrapment of proteins into sol-gel derived silica glass matrices (monoliths, thin films, powders) has been proposed as a potential generic method for obtaining stable encapsulated biomolecules[1–3]. The accessibility of substrates to the entrapped enzyme is determined largely by the pore size and the electrostatic interactions with the silica matrix, which can be altered by various methods[4, 5]. We have employed variation of sol-gel synthesis conditions, entrapped enzyme concentration and silane:solvent:water ratio, manipulation of aging conditions and use of molecular templating agents to promote larger pores and to maximise accessibility.

The present study is focused on the use of polymer or small molecule additives to enhance the stability of some hydrolytic enzymes. A combined method of sol-gel entrapment/deposition on inorganic support has been used to reduce structural compresion and internal mass-transfer limitations. Alcalase was immobilised by entrapment in sol-gel derived silica deposited on celite, alumina, zeolite and silica. An increase in activity was achieved, especially with celite. These results suggest that the microenvironment experienced by the entrapped biomolecule can be manipulated by dispersing organic dopants that stabilise the enzyme protein, preserving its biochemical function.

The enzyme accessibility and structure (native or unfolded), and the charge and polarity of the local environment affect the catalytic properties of the molecule (catalytic constant k_{cat} and Michaelis constant K_m).

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P221 IMMOBILISATION OF P450 BM-3 AND AN NADP$^+$ COFACTOR RECYCLING SYSTEM: TOWARDS A TECHNICAL APPLICATION OF HEME-CONTAINING MONOOXYGENASES IN FINE CHEMICAL SYNTHESIS

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Keywords: biotransformation, cofactor-recycling, cytochrome P450 BM-3, immobilisation, sol-gel

Cytochrome P450 monooxygenases are potentially a very useful class of hydroxylation catalysts; they are able to introduce oxygen at activated and non-activated carbon-hydrogen bonds[1]. These hydroxylation reactions can lead to regio- and/or stereochemically pure compounds. Stereo- and regiospecific hydroxylations are a task very difficult to perform by traditional organic chemistry. However, this potential is lowered by the intrinsic low activity and inherent instability of cytochromes P450. Additionally P450-catalysed biotransformations require a constant supply of NAD(P)H, making the process an expensive one.

P450 BM-3 (EC 1.14.14.1) and various mutants thereof were shown to accept a broad range of substrates[2]. To render these catalysts more suitable for industrial biocatalysis, the immobilisation of P450 BM-3 (CYP 102) from *Bacillus megaterium* in a sol-gel matrix[3] derived from TEOS was combined with a cofactor recycling system based on NADP+ -dependent formate dehydrogenase (EC 1.2.1.2) from *Pseudomonas* sp. 101 (ref.[4]) and tested for practical applicability. Sol-gel immobilised P450 BM-3 showed enhanced properties with respect to long-term stability and activity. Beside oxidation of the model substrate *p*-nitrophenoxydecanoic acid (10-pNCA)[5], this approach was used for the conversion of *β*-ionone into 4-hydroxy-*β*-ionone using a sol-gel immobilised P450 BM-3 mutant and sol-gel immobilised FDH for *in situ* regeneration of NADPH.

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P222 ENZYMATIC SYNTHESIS OF OLIGOSACCHARIDES ON A DENDRIMERIC SOLUBLE SUPPORT

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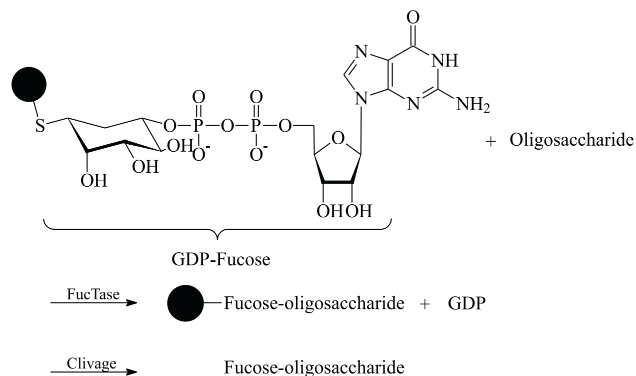
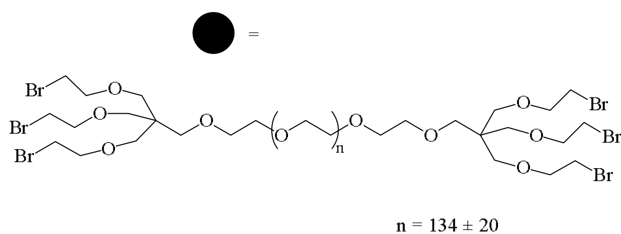
Keywords: supported synthesis, dendrimeric support, nucleotide-sucre, fucosyltransferase

The advantages of enzymatic carbohydrate synthesis are nowdays well demonstrated. The major problem in this strategy remains the purification of the products. In order to simplify this crucial step, our solution is using supported enzymatic chemistry either with immobilized acceptor as already reported by us[1] or donor as shown here.

We are aiming towards the successful total synthesis of a supported GDP-fucose – substrate of the fucosyltransferase – in order to synthesize fucosylated oligosaccharides without the need to attach at each time the acceptor on the support as shown in the scheme below.

The dendrimeric support developed in our laboratory is a brominated derivative of polyethylene glycol. This allows the facile coupling of the sugar to the polymer by nucleophilic substitution. Here, we report the total synthesis of the supported GDP-fucose on the soluble dendrimeric polymer and its enzymatic activity towards the α(1-4)-fucosyltransferase.





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P223 IMMOBILIZATION OF INULINASE FOR SUCROSE HYDROLYSIS

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Keywords: biotransformation, Ca-alginate entrapment, enzyme immobilization, inulinase

Sucrose hydrolysis is currently performed using invertases. Research work, aiming at improving the efficiency of this bioconversion process has revolved around the use of immobilized invertases. Recently a different approach, the use of a new biocatalyst, has been suggested. This is based on the activity for sucrose hydrolysis evidenced by crude enzyme preparations of inulinases from *Aspergillus ficuum*[1]. Still, the use of an immobilized form of the biocatalyst is advised, due to the many advantages this approach provides. In this work, a screening of immobilized forms of a commercial inulinase preparation for sucrose hydrolysis was performed. The highest immobilization yields were obtained with inulinase entrapped in Ca-alginate beads. Increasing the concentration of Ca from 1.5 % to 4 % led to a 15-fold increase in the immobilization yield. This was ascribed to a reduction in enzyme leakage of the enzyme preparation, despite a foreseeable increase in diffusional resistance for substrate migration within the beads. No significant shifts in optimal pH or temperature were observed when the free and immobilized forms of the biocatalyst were compared.

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P224 IMPROVING GERANYL ACETATE SYNTHESIS IN SUPERCRITICAL FLUIDS WITH ZEOLITES

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Keywords: Novozym 435, *Candida antartica* lipase B, zeolite, salt hydrate, supercritical fluid, water activity

Zeolite molecular sieves are very commonly used as *in situ* drying agents in reaction mixtures of enzymes in non-aqueous media. Recently, we have shown that zeolites can have acid-base effects on enzymes in low-water media, resulting from their ionexchange ability[1]. We have also demonstrated that pairs of salt hydrates, commonly used to fix water activity (a_W) *in situ*, can have similar effects[2]. Here, we studied the impact of zeolites NaA and NaY on an immobilized enzyme (Novozym 435) in an esterification reaction, in supercritical ethane and supercritical CO_2. To avoid drying effects of the zeolites, these were pre-equilibrated to the a_W of the experiments. The experiments that were done in the presence of zeolite NaA yielded higher initial rates and higher conversion than assays performed in the presence of pairs of salt hydrates, especially at the a_W values of 0.1 and 0.75. These results are explained on the basis of acid-base effects of both the zeolites and the salt hydrate pairs.

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P225 CROSSLINKING OF PROTEINS BY PEROXIDASE-MEDIATED OXIDATIVE DEHYDROGENATION IN THE PRESENCE OF EXOGENOUS PHENOLS

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Keywords: enzymatic, crosslinking, proteins, peroxidase

Cross-linking of proteins is of great interest to the food industry as a way to enhance protein functionality, thereby increasing their value. Use of enzymes for protein cross-linking is a viable alternative to the chemical routes, since enzymatic reactions are highly specific and require mild reaction conditions, thus minimising the risks of both protein denaturation and formation of possible toxic side products.

The present paper reports a systematic study on the cross-linking of proteins by peroxidase-mediated oxidative dehydrogenation in the presence of phenols as low molecular weight hydrogen donors. Two globular proteins, α-lactalbumin and bovine serum albumin (BSA), and β-casein, a protein with a flexible open structure, have been used as model proteins. A range of mono-, di-, and polyphenols with different ring substitution pattern has been used as the second hydrogen donor. Both the phenolic side-chain of tyrosine (Tyr) residues in proteins and the free (poly)phenols react with the active compounds I and II of peroxidase to generate reactive radicals that react further with each other to form homo- and hetero-products. Under controlled reaction conditions, the reaction can be stirred toward synthesis of high molecular weight (HMW) protein-phenol hetero-conjugates.

Full conversion of β-casein into high molecular weight polymers has been achieved. However, peroxidase-phenol mediated crosslinking of BSA and α-lactalbumin resulted only in partial protein conversion and formation of oligomers. This difference in reactivity has been associated with the specific conformation of each protein that restricts the accessibility of tyrosyl moieties for reaction. Conditions have been found to induce controlled conformational changes of BSA and α-lactalbumin to increase their conversion into polymers.

Different product patterns and conversions have been obtained by using a range of substituted mono- and polyphenols. *Ortho*- and *para*-diphenols are the most efficient crosslinking agents in the reactions studied. Bulky substituents in *ortho*- and *para*-positions of the aromatic ring reduce the reactivity of phenols due to steric hindrance. The reactivity of phenols in the peroxidase-mediated crosslinking of proteins has been correlated with their redox and ionisation potential.

Based on the results of this study and the products identified in model studies with a tyrosine-containing peptide[1, 2], a tentative model for peroxidase-mediated protein cross-linking in the presence of phenols is proposed. The effect of crosslinking on the functional properties of the proteins is also discussed.

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P226 INVESTIGATION OF KINETICS OF IMMOBILIZED LIVER ESTERASE BY FLOW CALORIMETRY

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Keywords: flow calorimetry, kinetics, pig liver esterase, reaction-diffusion system

Flow calorimetry (FC) was used to estimate kinetic parameters of phenyl acetate hydrolysis by pig liver esterase immobilised in alginate gel. Pig liver esterase belongs to the group of hydrolytic enzymes as carboxyl esterases, aryl esterases, lipases, acetyl esterases, cholin esterases and cholesterol esterases, which are widely distributed in animals, plants and microorganism and show wide substrate tolerance with highest activity towards soluble state of its substrate. They are usually stable and even active in organic solvents. Because of high regio- and stereo-specificity, they are attractive biocatalysts for the production of optically pure components. Esterases preferentially break ester bond of shorter chain fatty acid. Methanol and other weakly acid alcohol are highly effective nucleophiles toward the acyl group in reactions catalyzed by esterases in order to raise the reaction rate[1]. Probably the most well-known up to date application is the production of vanillin from ferulic acid released by carboxyl esterase from plant cell wall polysaccharides such as pectin or xylan[2]. The hydrolysis of the matching substrate can obey substrate activation[3] or inhibition[4] or can follow the Michaelis-Menten kinetics[5]. The hydrolysis of phenyl acetate by pig liver esterase was shown to exhibit substrate inhibition. In the present work the course of enzymatic reaction was measured in steady-state (single flow mode) and non-steady-state (total recycling of reaction solution). From the single flow mode the dependence of steady-state thermometric signal on substrate was obtained and from the recycling mode the time dsependence of thermometric signal representing the substrate consumption in recycling section within the flow calorimeter system (Fig. 1) was obtained. The experimental data were treated by mathematical modelling, based on material and heat balances. In the mathematical model the calorimeter column was divided into five differential sections. The mathematical model represented a set of partial and ordinary differential and algebraic equations. Spatial derivatives in balance equations were discretized by finite difference Crank-Nicholson scheme and the FC column was solved by orthogonal collocation method using three interior collocation points within the particle with immobilized esterase. After introducing dimensionless parameters, the resulting set of ordinary differential and algebraic equations was solved

using Athena Visual Workbench software package (Stewart and Associates Engineering Software).

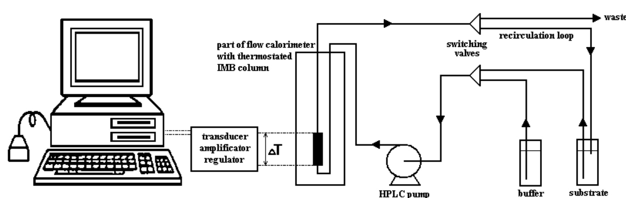

Fig. 1. **Experimental set-up for flow calorimetry**

The evaluation of data from total recycling led to the quantification of transformation parameter α, which enabled us to rewrite thermometric signals from steady-state experiment into steady-state reaction rates. Thus, the steady-state thermometric signal dependence on substrate concentration could be transformed to steady-state reaction rate dependence on substrate concentration. Afterwards, the latter dependence was optimized in order to obtain the true kinetic parameters of immobilized pig liver esterase.

The proposed technique enabled to determine intrinsic kinetic parameters of substrate inhibited enzyme reactions influenced by internal particle diffusion directly from calorimetric data, without using any additional analytical technique.

Conference presentation assisted by RHODIA company.

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P227 TAURINE CHLORINATION BY MYELOPEROXIDASE/H_2O_2/CL^- SYSTEM: A KINETIC STEADY-STATE STUDY

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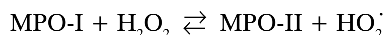
Keywords: myeloperoxidase, steady-state kinetics, hypochlorous acid

Myeloperoxidase, MPO, joins the homologous mammalian peroxidase family, but differs from others in its physical and chemical properties. The most characteristic reactivity is its ability to catalyze the oxidation of chloride, Cl^-, by hydrogen peroxide, H_2O_2, to form the potent oxidant and bactericidal agent hypochlorous acid[1].

Available information shows that efforts on studying the kinetics of halogenation activity of peroxidases have been focused on the analysis of initial rates[2, 3]. The use of this approach avoids the complexity of taking into account other side reactions also undertaken by this enzymatic system. Nevertheless, data obtained under pre-steady-state conditions yield knowledge only about the elementary steps leading to the formation of a halogenating agent or to a substrate halogenation.

Kinetic measurements after steady-state achievement incorporate all feasible concurrent processes, *i. e.* data so obtained permit a more accurate description of *in vivo* halogenation activity, and results can be better extrapolated to biological media.

The main findings attained for the rate of taurine chlorination catalyzed by the human MPO/H_2O_2/Cl^- enzymatic system are presented. Figure 1 shows a typical kinetic trace. The equilibrium between MPO compounds I and II



$$\text{MPO-I} + H_2O_2 \rightleftarrows \text{MPO-II} + HO_2^{\cdot}$$

has been here proved to strongly affect to the chlorination rate, provoking the wave shaped profile of kinetic traces. It should be considered the most important by-process influencing steady-state rate.

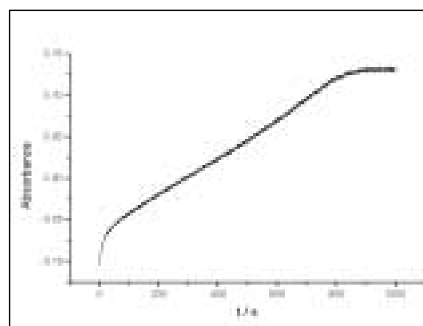


Fig. 1. **Kinetic trace obtained at λ = 252 nm**; [MPO] = 100 nM, [H_2O_2] = 0.8 mM, [Cl^-] = 100 mM, [taurine] = 10 mM, pH = 5.5, T = 298K

The dependence of reaction rate on the H_2O_2 concentration and the acidity of the medium is sketched in Figure 2. This values would correspond to the effective rate at which taurine is chlorinated *in vivo*, allowing the optimal conditions for the chlorination activity of MPO to be revealed.

In contrast with published data based on initial rate measurements, where rate of chlorination is enhanced with increasing pH value and/or H_2O_2 concentration[3, 4], these data suggest that MPO chlorination activity reaches a maximum when [H_2O_2] ≈ 0.25 mM and pH ≈ 5.0. The present study

sheds light on the optimal conditions for *in vivo* studies of MPO-catalyzed chlorination reactions.

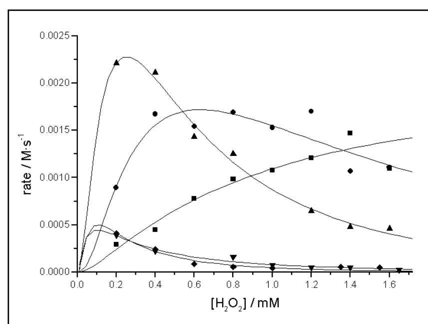


Fig. 2. **pH and [H_2O_2] influence on steady state rate value for taurine chlorination;** pH = 4.0 (■), 4.5 (●), 5.0 (▲), 5.5 (▼), and 6.0 (♦)

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P228 THE ELECTROCHEMISTRY OF HAEM PROTEINS IN NONAQUEOUS SOLVENTS

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Keywords: microperoxidase, cytochrome c, redox potential, thermodynamics, nonaqueous solvent

Enzymes such as trypsin and subtilisin are now widely used as efficient catalysts in nonaqueous solvents[1]. The use of redox enzymes is much less widespread, mainly due to their instability which arises from the need for an exogeneous oxidant, typically hydrogen or alkyl peroxides. We have investigated the electrochemistry of the haem proteins, microperoxidase and cytochrome c in a range of nonaqueous solvents. Dramatic shift in the oxidation potential of cytochrome c can occur, e. g. 900 mV in acetonitrile and ethyl acetate vs. 70 mV in aqueous buffer[2]. In acetonitrile, the heam is irreversibly oxidised, in ethyl acetate, an amino acid. The changes in redox potential of microperoxidase are much less pronounced, e. g. increasing by 100 mV in ethanol (vs. buffer). The thermodynamics of reduction of cytochrome c in glycerol were significantly changed, with $\Delta H^{\circ\prime}_{rc}$ decreasing from −35.6 to −47.7 kJ.mol^{-1} and $\Delta S^{\circ\prime}_{rc}$ from −35.2 to −76.8 J.K^{-1}.mol^{-1} (ref.[3]). By contrast, for microperoxidase, $\Delta S^{\circ\prime}_{rc}$ increased from 5 to 77 J.K^{-1}.mol^{-1}, while $\Delta H^{\circ\prime}_{rc}$ increased from 36 to 54 kJ.mol^{-1} (ref.[4]). The activation energy for reduction of cytochrome c was significantly increased from 5.7 kJ.mol^{-1} in aqueous buffer to 44.2 kJ.mol^{-1} in glycerol. These results demonstrate that enveloping the haem in an amino acid pocket can significantly affect the redox properties of the haem in nonaqueous solvents.

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P229 THE STUDY OF ELECTROCHEMICAL PROPERTIES OF THE REDOX ENZYMES IN ORGANIC SOLVENTS

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Keywords: mediated bioelectrocatalysis, galactose oxidase, immobilization, solvents

Galactose oxidase was used as a model enzyme for the investigation of effect of organic solvents on the electrochemical properties of the redox enzymes. The aim of this work was to obtain a stable response from the enzyme in organic solvent and todetermine any changes that occurred in electron transfer kinetics.

Cyclic voltammetry was used to measure rates of electron transfer. Ferrocene derivatives and some quinones were used as mediators for galactose oxidase. For experiments in organic media the enzyme was immobilized on the glassy carbon electrode by cross-linking with glutaraldehyde in the presence of bovine serum albumin and Nafion[1]. 3-Methoxybenzyl alcohol was used as the substrate for galactose oxidase. The affect of immobilization procedure on the response of enzyme electrode was studied. The experiments were carried out in carbonate buffer (pH = 10.8), 1,1'-dimethylsulfoxide, 1,1'-dimethylacetamide, 1,1'-dimethylformamide[2].

The results showed that the presence of Nafion in the electrode membrane increased the mechanical stability of the enzyme electrode. The enzyme film adhered better to the electrode surface. The response of the mediator was irreversible in the organic solvents – no reduction peak was observed. Bovine serum albumin improved the reproducibility of the catalytic current obtained. Experiments with dry solvents and with 10 % water added were carried out. No chan-

ges in enzyme electroderesponse were observed. In 30 % dimethylacetamide enzyme denatured rapidly. Naphthoquinone and dimethylquinone did not function as mediators of electron transfer between the active site of galactose oxidase and electrode. Ferrocene dimethanol, ferrocene methanol, ferrocene monocarboxylic acid were found to be good electron mediators for galactose oxidase. The electron transfer constant for galactose oxidase and ferrocene monocarboxylic acid was found to be $7.5 \times 10^5 M^{-1}.s^{-1}$ in buffer[3].

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P230 THE IDEAL BIOCATALYST: THE NEW APPROACH AND ITS APPLICATION IN BIOCATALYSIS PROCESS DEVELOPMENT

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Keywords: biocatalyst, biochemical engineering

Recently, we reviewed a new approach in developing novel biocatalysts for industrial purposes[1]. The use of enzymes as biocatalysts in the industrial manufacture of fine chemicals and pharmaceuticals has enormous potential, but application has hitherto been limited largely by evolution-led catalyst traits. The advent of designer biocatalysts, produced by informed selection and mutation, through recombinant DNA technology, has enabled production of improved, process-compatible enzymes. The design and development of practical biocatalysts is being advanced and shaped by progress in protein structure-function studies, understanding of protein stability, conformational stability and activity. Modern molecular techniques have allowed us to visualize catalytic systems that approach the functional ideal. Biocatalysts can be now designed in a paradigm where the process conditions are no longer the defining or limiting constraints on successful biocatalytic processes, and the biocatalyst can be made to fit the purpose. To fully realize the potential of designer enzymes in industrial applications, it is necessary to tailor the catalyst properties so that they are optimal not only for a given reaction, but also in the context of the industrial process in which the enzyme is applied.

This paper will discuss these approaches, illustrating by using examples of biocatalyst development in the authors' current projects, and focusing most specifically on biocatalytic systems for oxidation of aromatic compounds and hydrolysis of cyclic amides (hydantoins). In the case of oxidase systems the products are potential antioxidants, and in the case of hydantoinases, the products are optically pure amino acids destined for use as pharmaceuticals. In both cases, reaction engineering approaches have resulted in effective biotransformation systems.

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P231 OPPORTUNITIES AND CHALLENGES FOR BIOCATALYSIS IN THE PHARMACEUTICAL INDUSTRY

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Keywords: biocatalysis, high throughout screening, tertitiary alcohols, secondary amines, kinetic resolution

Some of the major opportunities and challenges facing the field of biocatalysis within the pharmaceutical industry will be presented. The use of genome sequencing, bioinformatics and cloning to rapidly construct new and functionally diverse enzyme libraries, as well as the application of advanced HTS technologies towards the mining of these libraries and subsequent reaction optimization, will be briefly discussed. Opportunities for collaboration with second-generation enzyme suppliers will also be outlined. In addition, the lack of efficient methods for the synthesis of optically active secondary amines and tertiary alcohols, a major challenge facing the pharmaceutical industry today, will be addressed through the presentation of a general chemo-enzymatic resolution approach that has been developed and validated for a number of major blockbuster drugs within Pfizer. Furthermore, challenges facing the use of new enzymes, including nitrilases, epoxidases, transaminases, reductases, hydroxylases, glycosylases, and cyclases, will also be discussed. Finally, a general high throughput screening approach that has been validated for the enzymatic resolution of a number of key intermediates within Pfizer will be summarized, stressing the need for such a protocol in routinely evaluating the overlooked yet powerful technique of solvent engineering in enzymatic hydrolytic reactions[1].

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P232 FROM FERMENTATION ENGINEERING TO GENETICALLY ENGINEERED HOST CELLS: DIFFERENT APPROACHES FOR OPTIMIZATION OF BACTERIAL GLYCOSYLTRANSFERASES PRODUCTION

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Keywords: gene expression, glycosyltransferases, immobilization, protein engineering, protein folding

Glycosyltransferases have become powerful tools for the synthesis of oligosaccharides through their strict control over the stereo- and regioselectivity of glycosidic bond formation[1]. One major drawback of glycosyltransferases in synthesis is their limited availability. The cloning and over-expression of bacterial glycosyltransferases is one alternative to overcome this problem.

To make available an enzyme for synthetic purposes is necessary to develop efficient and cost-effective large-scale production strategies. Three factors are of main importance to successfully attempt to this goal: i) the expression system, ii) the purification process, and iii) the stability of the recombinant enzyme under the reaction conditions.

The final aim of the expression system must be to obtain the maximum yield of active enzyme. For large-scale production of proteins that are limited in size and do not require substantial post-translational modifications (e. g. glycosylation), prokaryotic expression systems, and in particular *Escherichia coli*, are the most attractive ones because of their ability to grow rapidly and at high cell density in fermentation processes. However, when a heterologous protein is over-expressed in *E. coli* misfolding and aggregation happen frequently, driving the recombinant protein into inactive aggregates known as inclusion bodies (IB)[2]. Since the propensity to aggregate is strongly dependent on the particular protein being expressed, it is obvious that there is not a general protocol to prevent the formation of IB's. Formation of IB's can be avoided modifying the culture conditions (fermentation engineering), modifying the protein to be expressed (protein engineering) and/or manipulating the host cells (genetic engineering).

Once the recombinant enzyme is successfully produced, it is necessary to face the downstream process for its purification. When the recombinant enzyme preparation is to be used as biocatalyst, one affinity chromatographic step can give the required purity degree. Immobilized Metal Ion Affinity Chromatography (IMAC) can also be used for enzyme immobilization[3], since the adsorption of the protein to the chromatographic resin is not by the enzyme active center.

In this presentation we described our work[4–6] to face the three factors described above for the case of the α-1,6-FucT from *Rhizobium* sp.: i) optimization of the expression yield of properly folded and active recombinant enzyme, ii) the purification scheme, and iii) the stability of the recombinant enzyme.

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P233 SYNERGISTIC ASSOCIATION OF BACTERIA AND A GREEN MICROALGA FOR THE BIODEGRADATION OF AROMATIC POLLUTANTS

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Keywords: aromatic pollutants, biodegradation, microalgae, photobioreactor, synergistic association

In nature, most microalgae are found in association with other microorganisms. Indeed, they produce the molecular oxygen that is used as electron acceptor by the heterotrophic aerobic microflora. In return, the carbon dioxide released during the mineralization process completes the photosynthetic cycle. The mutualistic relationships between algae and other microorganisms form the basis of the carbon turnover in natural ecosystems and in artificial freshwater envi-

ronments (aerobic stabilization ponds for the treatment of domestic wastewater).

In this context, our study was conducted to determine the potential of algae-bacteria combinations for the degradation of aromatic pollutants, with the aim of using these microcosms for the treatment of toxic and/or recalcitrant organic compounds contaminating aqueous media. The green microalga *Chlorella sorokiniana* was thus mixed with different aromatic degrading bacteria. Salicylic acid (sodium salt), phenol and phenanthrene were tested as model pollutants for representing a wide range of aqueous solubility and toxicity. In the various tested microcosms, the synergistic association between algae and bacteria was clearly established in a photosynthesis-helped biodegradation process as shown by the removal of pollutants. Simultaneously to this study, we designed and built our own photobioreactor. This reactor was investigated with biofilms in a continuous mode of operation both for the production of photosynthetic oxygen by *C. sorokiniana* and for the degradation of the aromatic pollutants. This reactor appeared to be an efficient tool for these two applications[1,2]. The experimental set-up as well as some major results will be presented in this communication.

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P234 STUDIES ON BIOTRANSFORMATIONS OF HARD CARBON COATINGS (DLC & NCD)

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Keywords: carbon coatings, microorganisms, biotransformation

The carbon thin film coatings (DLC – diamond-like carbon, NCD – nanocrystalline diamond) are used for improvement of surface properties of metals and ceramics devices that are applied in biotechnology, electrochemistry, nanochemistry and medicine. They have very attractive properties such as high wear resistance and hardness, chemical inertness and very low friction coefficients[1,2]. Hard carbon coatings consist mainly of the tetragonally sp^3 – coordinated carbon atoms (typical of diamond), the trigonally sp^2 – coordinated carbons (typical of graphite) as well as some sp^1 – coordinated ones[3]. The response of different animal cells (mouse fibroblasts, macrophages, endothelial cells, neutrophils and other) to diamond surfaces was studied[4]. However, there is no information about the behavior of these coatings in the presence of microbes and their metabolites. Therefore, an effect of various physiologically active microorganisms on surfaces covered with diamond-like coatings has been studied. Thin carbon films on medical stainless steel AISI 316 L have been prepared using the radio frequency plasma chemical vapor deposition (RF PCVD) method. The samples covered with carbon coatings were inoculated with various fungi *(A. niger, Chaetomium globosum, M. circinelloides, Paecilomyces variotii, Penicillium ochrochloron, Trichoderma viride, Fusarium oxysporum, Phanerochaete chrysosporium)* and bacteria (e. g. *L. delbrueckii*, *Ps. fluorescens*). Some of the selected strains can solubilize the lignite (brown coal) and are able to grow in petrol-oil[5,6]. The carbon layers before and after the growth of the microorganisms were examined by means of fluorescence microscope, scanning electron microscopy (SEM) and Raman spectroscopy. It was found that most of the tested filamentous fungi and some bacterial cells strongly adhered to the surfaces covered with DLC coatings during cultivation. Furthermore, changes in the color of DLC – films from blue to yellow or metallic, after the growth of the majority of the microscopic fungi, were observed using optical and metallographic microscopes. The strong influence of some of the tested microorganisms on carbon coatings deposited on medical stainless steel was confirmed using SEM microscopy (connected with a X-ray microanalyzer) analysis. It shows differences in the concentration of certain elements present in surface layers of the samples before and after subjecting them to treatment with microorganisms. Some of the microorganisms very strongly attacked the surface of carbon coatings and changed their structure. Raman spectroscopy – the most appropriate to characterize the graphite phase – proved that the microorganisms can remove (or modify?) some graphite phase from DLC coatings.

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P235 CELL ADAPTATION TO SUBSTRATE, SOLVENT AND PRODUCT: A SUCCESSFUL STRATEGY TO OVERCOME PRODUCT INHIBITION IN A BIOCONVERSION SYSTEM

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Keywords: biotransformation, (-)-carveol, (-)-carvone, column reactor, viability

Rhodococcus erythropolis DCL14 cells can produce three different carveol dehydrogenases, each of them dependent on a different co-factor[1]. When the cells grow on limonene or cyclohexanol the major activity is NAD-dependent. Cell viability is therefore an issue, which makes both reactor configuration and operation conditions important. These cells are able to "resolve" a diastereomeric mixture of (-)-carveol, two products being obtained: (i) (-)-carvone and (ii) isomerically resolved (-)-*cis*-carveol.

Both in a mechanically stirred direct contact reactor and in a membrane reactor, the reaction practically stopped when the carvone concentration reached 50 mM (ref.[2]). An attempt to adapt the cells was thus carried out. A 130 ml glass column was tested as an air-driven direct contact bioreactor. *n*-Dodecane 50 mM in carveol was recirculated at 50 $ml.h^{-1}$ through the column containing the aqueous phase. At this circulation rate the contact time between the cells and the substrate is low enough so that the biotransformation takes place to a small extent only.

In the first run, recirculation was stopped after 20 h, the whole organic volume was allowed to enter the column and air was injected at the bottom of the column at 29 $ml.min^{-1}$ (0.3 vvm). A good emulsion was formed and the whole of the cell population had migrated towards the organic phase after 10 h. The aqueous phase became completely cell free. The cells were able to carry out the biotransformation for at least 310 h. At this time carvone production reached 94 mM.

As a further attempt to adapt the cells to the presence of solvent, substrate and product, an assay was carried out in which *n*-dodecane containing the substrate was recirculated, as described above, for 136 h. This long adaptation period was actually a successful strategy, allowing accumulation of carvone up to 259 mM, thus overcoming the product inhibition problems previously encountered.

The results suggest that if cells are allowed to stay in contact with solvent, substrate and slow increasing concentrations of carvone, they are able to adapt. In fact, in the second run 69 % of the cells in the organic phase were viable after 310 h, while in the first run, only 49 % of the cells were viable after the same period. Somehow, cells in *n*-dodecane were protected from the toxic effect of carvone.

To confirm wheter the adaptation period was indeed responsible for an increase in productivity, a run was carried out in which the cells were allowed to adapt during 50 h. The column worked for 818 h and a concentration of carvone of 178 mM was attained. A plot of the carvone concentration obtained in each assay *vs.* the adaptation period gave a logarithmic shaped curve. The trendline that best fits the results has a R-square value of 0.999.

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P236 TOWARDS THE BIO-PRODUCTION OF *trans*-CARVEOL AND CARVONE FROM LIMONENE

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Keywords: limonene, carveol, carvone, *Rhodococcus opacus*

The strain *Rhodococcus opacus* PWD4 was found to hydroxylate D-limonene, yielding (+)-*trans*-carveol and traces of (+)-carvone, when grown on toluene[1]. In the present work we studied the activity shown by these cells when grown on different carbon sources, in both single aqueous and biphasic systems, the latter allowing to overcome the low solubilities of both substrate and products[2].

Cells were grown on 0.125 and 0.25 % (v/v) of ethanol, limonene and toluene as single carbon sources. In both aqueous and aqueous:organic systems, at 28 °C and 200 rpm, the highest initial *trans*-carveol production rate was observed for cells grown on limonene. In biphasic systems, the activity shown by the cells grown on 0.125 % of limonene is 2.7 and 3.11 times higher than that shown by cells grown on toluene and ethanol 0.125 %, respectively. Furthermore, the carveol production rate in biphasic systems is 10 times higher than in aqueous systems. In aqueous:organic systems, the production of *trans*-carveol is higher than the production of carvone, but the ratio between the production of carveol and carvone depends on the carbon source used: 14.9, 3.4, 7.5 and 28.7 for cells grown on 0.125 % ethanol, 0.125 % limonene, 0.125 % toluene and 0.25 % toluene, respectively.

The effect of the initial limonene concentration on the growth medium was evaluated by adding 5, 10, 20, 40, 80 and 160 μl of limonene to 25 ml of medium. The carvone production rate decreased with increasing amounts of limonene

added until 40 μl, and increased with higher amounts. The maximums were achieved for 5, 80 and 160 μl of limonene added.

The production rate of both *trans*-carveol and carvone increased with the initial percentage of limonene. Changing the limonene concentration, referred to the aqueous phase, from 20 to 150 mM led to a 5.9 fold increase in *trans*-carveol and a 2.8 fold increase in carvone production.

Production was also tested in an aqueous(20 ml):dodecane(4 ml) biphasic bioreactor with limonene supplied through the air stream. The reactor worked at 28 °C and was agitated at 200 rpm. Using cells grown on toluene, the carvone production rate became 18 times higher than the *trans*-carveol production rate. The increase was not that high with cells grown on limonene. However, using the latter in a similar aerated bioreactor but with an initial limonene concentration of 50 mM, the carvone production rate was 141 times higher than the *trans*-carveol production rate.

The results indicate that the conditions of growth, as well as those of biotransformation, may greatly influence the limonene degradation carried out by *R. opacus* PWD4 cells.

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P237 BIODEGRADATION OF HYDROCARBONS UNDER SALINE AND NON-SALINE CONDITIONS AT 15 AND 28 °C

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Keywords: hydrocarbons degradation, bioremediation, *Rhodococcus erythropolis*, saline conditions

The bioremediation of sites contaminated with hydrocarbons, resulting from *e. g.* spillage of diesel or jet fuels, maybe difficult due to low ambient temperatures or saline conditions. The ability of *Rhodococcus erythropolis* DCL14 cells to degrade hydrocarbons at 15 and 28 °C was assessed, as well as their capacity to carry out the degradation under saline conditions. The temperatures were chosen with reference to the minimum and maximum average temperatures observed in the Atlantic, Pacific and Indian oceans between 0 and 55° of latitude.

The hydrocarbons tested were *n*-hexane, cyclohexane, toluene, *n*-octane, *iso*-octane, *n*-dodecane, *n*-tetradecane and *n*-hexadecane at initial concentrations of 0.125 and 0.25 % (v/v).

In the absence of NaCl, at 15 °C, all growth curves reached the end of the exponential phase after 90 h, except when *n*-tetradecane (100 h) and toluene 0.125 % (125 h) were used as carbon source. The final optical density (O. D.) at 600 nm observed ranged from 3.5 to 7. When toluene was used at an initial concentration of 0.25 %, almost no growth was observed, indicating that the cells were unable to degrade toluene at this concentration at 15 °C. At 28 °C, the end of the exponential phase was attained after 30 h for most of the hydrocarbons tested. The exceptions occurred when the cells were in the presence of 0.25 % *iso*-octane (around 50 h) and 0.125 % toluene (around 60 h). The final optical density obtained ranged between 4.5 and 7.5. Once again, almost no toluene degradation was observed at an initial concentration of 0.25 %.

The composition of the cell membrane supposedly varies with the carbon source used and hence cell hydrophobicity should also be affected. Cell hydrophobicity was determined by the MATH (Microbial Adhesion To Hydrocarbons) test[1]. At 15 °C, high hydrophobicities were observed for cells grown on toluene, *iso*-octane and *n*-tetradecane while those grown on *n*-hexadecane presented lower hydrophobicity. Cells grown at 28 °C showed high hydrophobicity when grown on *n*-tetradecane and *n*-hexadecane, whilst lower values were obtained with cells grown on *n*-hexane, toluene and *n*-octane. The results suggest that there is no direct relation between cell hydrophobicity and the carbon number.

Three NaCl concentrations were used to assess the possibility of using *R. erythropolis* cells in saline media: 1.0, 1.95 and 2.5 %. The carbon sources tested were the following: *n*-hexane, *iso*-octane and *n*-octane 0.125 %. At 15 °C, the *lag* phase was around 50 h, except for *n*-octane in which the cells needed nearly 100 h to start growing. The final O. D., at 600 nm, ranged between 2.0 and 3.0. The *lag* phase increased to around 160 h when the initial concentration of salt increased to 1.95 %.

Using *n*-octane, an increase in salt concentration led to an increase in cell hydrophobicity. In general, it was observed that the higher the carbon number of the carbon source, the lower the influence of NaCl concentration on cell hydrophobicity.

Thus, *R. erythropolis* DCL14 cells are fairly good biocatalysts for degrading hydrocarbons in both relatively cold and warm environments even under saline conditions. Several other strains of the genus *Rhodococcus* have been found to metabolise environmental contaminants, using various alkane-catabolic pathways[2].

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P238 BIODEGRADATION OF MOTOR OILS AT 16 AND 28 °C BY *Rhodococcus erythropolis* DCL14

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Keywords: oil degradation, bioremediation, *Rhodococcus erythropolis*

Motors are widely present in our environment and accidental oil spills are frequent. Microorganisms able to degrade motor oils under different environmental conditions should be a good tool for *in situ* bioremediation.

Rhodococcus erythropolis DCL14 cells were tested as degraders of four and two-stroke engine oils at 15 and 28 °C. Since these oils are mainly used by cars, motocycles and boats, this temperature range covers the majority of conditions under which the spills occur. The percentages of oil tested were the following: 0.125, 0.25, 0.5, 1 and 2 % (v/v).

At 28 °C, when cells were incubated with Shell Helix standard 20W-50 oil for four-stroke engines, the optical density (O. D.) measured at 600 nm doubled in the first 20 h with each of the tested percentages. The growth rate decreased with the increase of the initial oil fraction. After the first 30 h, the cells formed clusters which were visible under the naked eye. Thus, thereafter, the quantification of growth, by the measurement of the optical density, became impossible. Using Mobil Super 2T for two-stroke engines as sole carbon source, the velocity of growth decreased with the increase in oil fraction: after 43 h the O. D. at 600 nm had tripled for concentrations lower than 0.5 % and only slightly increased for 1 and 2 %. Cell clustering was observed after 50 h under naked eye.

At 15 °C, very small clusters were visible after 60 h in all growth runs with both oils.

Images of the different culture media, obtained with an optical microscope, showed cells surrounding the oil droplets. The higher the oil fraction, the larger the cell clusters were.

The MATH hydrophobicity test[1] showed that, for both oils, cells were less hydrophobic when grown on 0.5 % oil fraction. The most hydrophobic cells were those grown on either the lowest (0.125 %) or the highest (2 %) initial oil fraction. Futhermore, cells grown on Mobil Super 2T were more hydrophobic than those uptaking Shell Helix standard, at identical initial fractions.

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P239 PRELIMINARY STUDIES ON THE OPTIMISATION OF FERMENTATION PROCESSES IN BATCH CULTURE FOR THE PRODUCTION OF ERYTHROMYCIN

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Keywords: erythromycin, fermentation, *Saccharopolyspora erythraea*, fermentor, adsorption

Erythromycin is a broad spectrum polyketide antibiotic, specially indicated in the treatment of respiratory and skin complains. It has a similar activity spectrum to the penicillin and are used by penicillin-sensitive people to combat Gram--positive bacteria, and in addition used against *Mycoplasma*, *Campylobacter* and *Legionella*. In turn erythromycin is the starting material for second and third generation semi-synthetic derivatives. Erythromycin is a 14-carbon macrolide, produced by fermentation of *Streptomyces erythreus* or *Saccharopolyspora erythraea*. The new findings, effective completion of the genome sequence for the bacterium *S. erythrae*, offer a host of possibilities for production of novel antibiotics, immunosupressants and anti-cancer compounds, all based on polyketide starting materials.

Optimising the fermentation conditions, by selecting adequate parameters like the effect of medium composition, pH, temperature, oxygen levels, ratio C/N, is therefore an important aspect for the successful operation of any fermentor.

The aim of this work was the study of production and recovery of erythromycin from fermentation broth by selective adsorption on neutral resins (Amberlite™ XAD-4, XAD-7 and XAD-16) and anion exchange resin (Amberlite™ XAD-410). In previous work these resins were used in adsorption studies of erythromycin from standard solutions[1].

Erythromycin was produced by fermentation, from *S. erythraea* in a 3 l fermentor. Preliminary studies have been carried out in a batch mode for the optimisation of fermentation conditions. Cultures of *S. erythraea* were grown in various media formulations, in shake flasks. Culture growth was compared in terms of specific rate cell growth, duplication time and fermentation time. Erythromycin was analysed by sphectrophotometry, at 280 nm and by HPLC according to the method of USP (ref.[2]).

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P240 USE OF NITRILE HYDRATASE FROM *Brevibacterium imperiale CBS 498-74* RESTING CELLS FOR PROPRIOAMIDE PRODUCTION: A STUDY IN UF-MEMBRANE REACTORS

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Keywords: *Brevibacterium imperiale* growth, nitrile hydratase, proprionitrile biotransformation, enzyme kinetics, UF-membrane reactor

The bioconversion of proprionitrile into proprioamide was catalyzed by the nitrile hydratase present in resting cells of *Brevibacterium imperiale CBS 498-74*. This strain follows a two-step degradation pathway of nitriles, which involves nitrile hydratase and amidase, and presents an high nitrile hydratase activity while amidase activity is negligible and therefore theoretical 100 % conversion yields are possible. The influence of various parameters on microorganism growth for the production of nitrile hydratase was already tested[1,2].

B. imperiale CBS 498-74 (new classification, *Microbacterium imperiale*) was obtained at the optimum initial glucose concentration for nitrile hydratase (NHase) production, 5 $g.l^{-1}$, in a shake flask (220 $rev.min^{-1}$) at 28 °C for culture periods of up to 150 h. The highest NHase productivity (U per ml of broth per h) was reached after 24 h of incubation. Specific activities in the cell were found to be: 34.41 $U.mg_{DW}^{-1}$. NHase activity in the whole cell suspension was tested following the biotransformation of proprionitrile (50 mM) into proprioamide at 20 °C in 50 mM sodium phosphate buffer, pH 7.0. The kinetic parameters, K_m and V_{max}, were evaluated at different temperatures and resulted respectively at 10 °C: 61.0 mM and 10.36 $\mu mol.min^{-1}$ and at 20 °C 88.9 mM and 13.98 $\mu mol.min^{-1}$. The activation energy, E_{act}, was also evaluated.

In this study use was made of both differential and integral UF-membrane reactors for kinetic characterisation of the reaction. The laboratory scale membrane bioreactor was a commercially available flat membrane cell Amicon Mod. 52 and the UF-membranes was FS81PP. The membrane resistance to chemicals was fair at proprionitrile and proprioamide concentrations up to 100 mM and 1 M respectively. No rejection of solute was determined. Membranes totally retained the resting cells and no fouling was observed working with 2.3 mg and 5.7 mg of biocatalyst under stirring conditions[3]. Membrane compaction was responsible for flux loss during the first 3–4 hours of operation.

The enzymatic reaction was operated at 5 °C and 10 °C. Substrate concentration ranged from 100 mM to 500 mM. Proprionitrile conversion as high as 76.6 % was attained using 32.7 μg of cells per ml in a continuously operating reactor. The laboratory scale membrane bioreactor, continuously operating, allowed to study the dependence of enzyme deactivation on the substrate concentration and process time[4]. In this study we report on the irreversible damage of NHase activity caused by high proprionitrile substrate concentration.

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P241 BIOTRANSFORMATIONS FOR THE PRODUCTION OF OPTICALLY PURE 1,2-*O*-ISOPROPYLIDENE GLYCEROL

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Keywords: solketal, microbial hydrolysis, carboxylesterase, enantioselective biotransformation

Biotransformation techniques have evolved such that the synthetic chemist can utilize biocatalysts just as many other synthetic reagents are used. Natural microbial diversity and new techniques for further diversification offer an almost inexhaustible source of biocatalysts.

The major goal of this work has been the obtainment of 1,2-*O*-isopropylidene glycerol (also called solketal or IPG) as optically pure molecule by microbial hydrolysis of the corresponding racemic esters. This biotransformation is difficult to achieve with high enantioselectivity using commercially available enzymes. Different techniques have been exploited for improving the selectivity and/or productivity of the biotransformation, such as medium manipulation (e. g. binding agents or organic solvents), biocatalyst manipulation (e. g. lyophilised cells, thermal pre-treatment, immobilization etc.) or reactor engineering (e. g. membrane reactors). Carboxylesterases responsible for the enantioselective biotransformations have been (partially) purified.

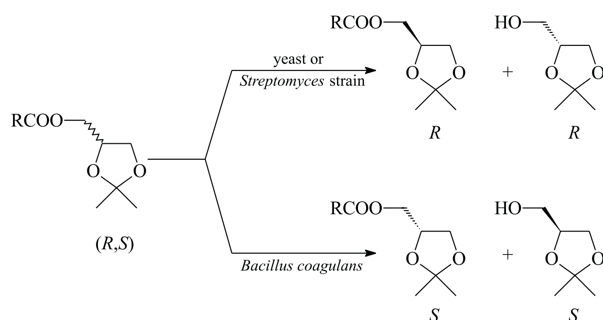

Fig. 1. **Simple membrane reactors allowed for multi-gram production of optically pure solketal**

It was found that enantioselectivity was independent of the examined conditions of reduction. The yield was very sensitive to both the quality of cell paste and conditions of bioreduction except the temperature and the aeration. This *in vivo* enzyme system is extremely sensitive to the change in the concentration of the ingredients.

Z. rouxii cell pastes were also used in pilot plant scale bioreduction in 5 consecutive batches with 200 liter total volume each. The reductions were performed in a modified Rosenmund agitated filter-dryer filled with Amberlite XAD-7 HP resin. The obtained yields were similar to our results carried out in our laboratories.

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P242 OPTIMIZATION OF METHYLENEDIOXYPHENYL-ACETONE CHIRAL BIOREDUCTION

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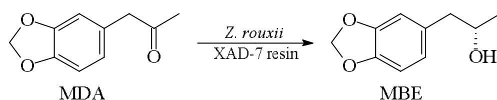
Keywords: *Zygosaccharomyces rouxii*, bioreduction, resin

Talampanel is a non-competitive antagonist of AMPA receptor, and it is a possible drug for treatment of epilepsy and cerebrovascular ischemia. The first step of an efficient synthesis of Talampanel is the reduction of 3,4-methylenedioxyphenyl-acetone (MDA) to *(S)*-α-methyl-1,3-benzodioxole-5-ethanol (MBE) accomplished with *Zygosaccharomyces rouxii* in the presence of XAD-7 resin[1]. The application of the hydrophobic polymeric adsorbent resulted in low and non-toxic concentration of both the substrate and the product in the water phase (< 6 $g.l^{-1}$). *Z. rouxii* was chosen because it tolerated higher substrate and product concentration and had a higher productivity number in comparison to the other tried yeasts[2].



A low cost fermentation medium without any component of animal origin was developed to produce *Z. rouxii* biomass. The control of pH and dissolved oxygen concentration, the temperature, the antifoam system, the time of harvest, the rate of inoculations and the number of seed steps were also investigated. The fermentation process in 1000-l fermentor provided cell pastes possessing a satisfactory ketoreductase activity (95–98 %) and an excellent enantioselectivity.

A method was developed to examine the enzyme activity and to determine the key parameters of the bioreduction.

P243 COMPARISION OF 1,3-DIHYDROXYACETON AND L-ERYTHRULOSE PRODUCTION OF *Gluconobacter oxydans* ATCC 621H

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Keywords: *Gluconobacter oxydans*, fermentative production, 1,3-dihydroxyaceton, L-erythrulose

Gluconobacters are well known for their capacity of oxidising a series of different sugar alcohols. The valuable molecules: 1,3-dihydroxyaceton and L-erythrulose are the products of the fermentation on glycerol and *meso*-erythritol, respectively. Here we report the systematic investigation and comparision of the dihydroxyaceton and erythrulose production capacities of *Gluconobacter oxydans* ATCC 621H.

The influence of carbon source concentration between 30 to 280 $g.l^{-1}$ on the cell mass production, product concentration, yield and productivity was investigated by orthogonal shake-flask experimental designs. 160 $g.l^{-1}$ erythritol was considered to be the optimal starting concentration whereas the ideal glycerol concentration was significantly lower. To increase cell mass production, a series of supplementary compounds were evaluated with the aid of a Plackett-Burman design. To determine the inhibitory level of product concentration, fed-batch fermentations were carried out, where the substrate supply was controlled by the dissolved oxygen level in the fermentation broth. Inhibitory product concentrations were determined for circumstances among limiting as well as for surplus substrate concentrations. A series of NAD^+ and

PQQ-dependent dehydrogenase activities were also determined from the disrupted cells in order to gain deeper knowledge of the activation mechanism of *Gluconobacter* enzymes. These results establish the possibility of the effective fermentative production of dihydroxyaceton and erythrulose as well as the enzymes responsible for their production.

P244 BIOPROCESS DEVELOPMENT FOR EPHEDRINE PRODUCTION

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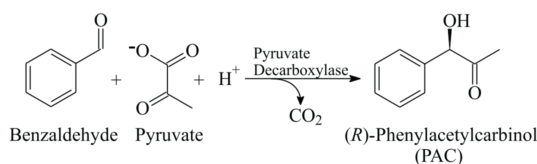
Keywords: biotransformation, pyruvate decarboxylase, *(R)*-phenylacetylcarbinol, ephedrine, pseudoephedrine, two-phase process

The chiral pharmaceuticals ephedrine and pseudoephedrine are currently produced commercially via a biotransformation of benzaldehyde by fermenting baker's yeast followed by chemical catalysis (Fig. 1). The biotransformation is catalyzed by a side-reaction of the enzyme pyruvate decarboxylase (PDC), which transfers enzyme-bound "active acetaldehyde" onto benzaldehyde to form *(R)*-phenylacetylcarbinol (PAC). Acetaldehyde and acetoin can be formed as by-products.

BIOTRANSFORMATION:



CHEMICAL SYNTHESIS:

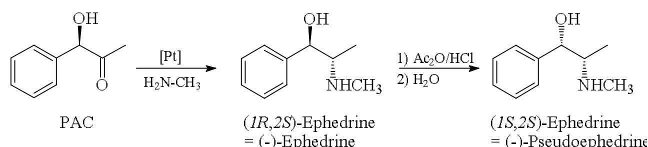


Fig. 1. **Ephedrine production by sequential biotransformation and chemical synthesis**

Screening of filamentous fungi[1] and yeast for cell-free PAC production resulted in a number of efficient PDCs e. g. from *Rhizopus javanicus*, *Candida utilis* and *Candida tropicalis*. *Candida utilis* PDC was more stable and formed less by-product acetoin than PDC from the filamentous fungus *Rhizopus javanicus*.

Enzyme stabilization and pH control were identified as crucial factors for increasing final PAC levels[2], which have previously been limited to 28.6 $g.l^{-1}$ in an enzymatic process[3]. With improved buffering partially purified PDC produced 50 $g.l^{-1}$ PAC in a simple batch biotransformation[3].

For further process enhancement, loss of substrate pyruvate was minimized. The loss resulted from two phenomena: temperature dependant non-enzymatic concentration decrease due to the cofactor Mg^{2+} and formation of by-products acetaldehyde and acetoin by PDC. The molar yield of PAC on consumed pyruvate increased from 59 % (*R. javanicus* PDC, 20 mM Mg^{2+}) to 74 % (*R. javanicus* PDC, 0.5 mM Mg^{2+}) by lowering the Mg^{2+} concentration, and increased further to 89 % (*C. utilis* PDC, 0.5 mM Mg^{2+}) by changing the source of PDC.

An aqueous/organic two-phase system was designed to overcome enzyme deactivation by benzaldehyde, acetoin and PAC and to decrease inhibition by acetaldehyde. Under optimized conditions 141 $g.l^{-1}$ PAC was produced in the organic phase with additional 19 $g.l^{-1}$ formed in the aqueous phase using *Candida utilis* PDC. Significant increases in PAC per unit of enzyme were achieved[4]. Utilizing whole cells instead of partially purified PDC further reduced the catalyst cost. Additionally PDC production in *Candida utilis* was drastically enhanced through a novel pH shift process.

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P245 PROCESS DEVELOPMENT FOR *R*-PHENYLACETYLCARBINOL (PAC) PRODUCTION IN AQUEOUS/ORGANIC TWO-PHASE BIOTRANSFORMATION

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Keywords: biotransformation, enzyme stability, pyruvate decarboxylase, *R*-phenylacetylcarbinol

R-Phenylacetylcarbinol (PAC) is a precursor for the pharmaceuticals *(1R, 2S)* ephedrine and *(1S, 2S)* pseudoephedrine. PAC is commonly synthesized by a biotransformation process utilizing benzaldehyde and pyruvate as substrates and an enzyme, pyruvate decarboxylase (PDC), as catalyst. Main by-products associated with the biotransformation are acetaldehyde, acetoin and benzyl alcohol.

Based on the amount of benzaldehyde that is used in the biotransformation, there are three basic types of system that should be applied (in the order of increasing benzaldehyde concentration): (1) Aqueous phase, (2) Benzaldehyde emulsion[1] and (3) Aqueous/organic phase system[2].

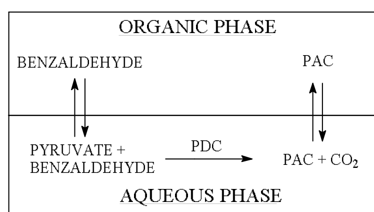

Fig. 1. **Organic/aqueous two-phase system for *R*-PAC production**[2]

It was previously found that benzaldehyde, PAC and acetoin inactivated PDC while acetaldehyde had an inhibiting effect (Sandford et al., submitted). It is also known that PDC is most stable at low temperature, neutral pH and in the presence of high concentrations of MOPS buffer or other additives like glycerol or salt[1].

In this study, the effects of further process parameters on PAC production were investigated in the aqueous and aqueous/organic phase system. It was found in the aqueous phase system that agitation rate and enzyme level had no effect on PDC deactivation as long as foam formation was prevented. The deactivating effect of benzaldehyde was confirmed while octanol had no considerable effect. However, it was observed that deactivation by benzaldehyde was enhanced in the presence of octanol.

It was further found in the aqueous/organic phase system that there was no strong additional deactivating effect by high concentration of benzaldehyde (1.46 M) contained in the octanol phase. This implies efficient protective effect of the octanol phase, which prevents the strong inactivation that has been reported previously for a benzaldehyde emulsion system.

Lowering the ratio of organic to aqueous phase volume from 1 : 1 to 0.43 : 1 in the two-phase biotransformation while maintaining the total concentrations of enzyme and substrates resulted in 11 % higher overall PAC and lower overall concentration of the by-product acetoin. In addition, the PAC was highly concentrated in the organic phase with 1400 mM PAC in octanol in comparison to approximately 800 mM when using the 1 : 1 ratio. Hence, application of less organic phase has the potential for reduction in production cost (since less solvent is used) and more efficient downstream processing (since the product is more concentrated).

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P246 PREPARATION OF AN EPHEDRINE CHIRAL SYNTHON BY BAKER'S YEAST REDUCTION OF 1-PHENYL-1,2-PROPANEDIONE

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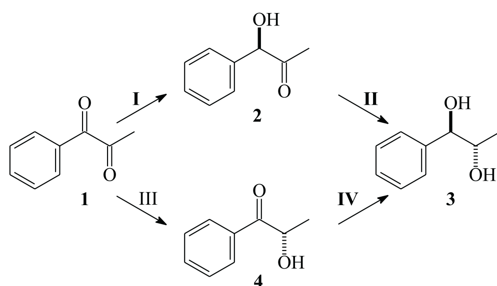
Keywords: *(R)*-1-hydroxy-1-phenyl-2-propanone, baker's yeast reduction, enantioselective reduction

(R)-1-Hydroxy-1-phenyl-2-propanone **2** is an important chiral synthon that has been used in the manufacture of the pharmaceuticals (-)-ephedrine and (-)-pseudoephedrine[1]. The most commonly used method for production of **2** is biological, in which strains of *Saccharomyces cerevisiae* and *Candida utilis* are employed to mediate the acyloin condensation of benzaldehyde and pyruvic acid[2].

Another promising biological alternative for production of **2** is the baker's yeast reduction of 1-phenyl-1,2-propanedione **1**, which gives a mixture of optically active **2**, plus (1*R*,2*S*)-1-phenyl-1,2-propanediol **3**, and (*S*)-2-hydroxy-1-phenyl-1-propanone **4** (Scheme)[1]. Considering that compounds **2** and **4** are barely separable by silica gel column chromatography, the aim of this work is to improve the regio-



selectivity of the reduction of **1** to obtain compound **2**. The previously published[3] scheme shows compounds **2** and **4** as intermediates in the baker's yeast reduction of **1** to **3**. As our interest is the isolation of the intermediate **2**, we performed a study of this reaction to maximize the yields of **2** free of **4**. Thus, samples were withdrawn from the reaction mixture at appropriate intervals and analyzed by gas chromatography. During this study, we observed that the value of the **2**/**4** ratio varied with the velocity of reaction flask agitation and thus an anaerobic procedure was performed. Figure 1 shows that compound **2** may be obtained free from **4** at 90 min of reaction using a pre-treated baker's yeast (nitrogen was bubbled during 60 min followed by 20 min of oxygen bubbling into a suspension formed by baker's yeast and water). Therefore, we use this process to prepare an easily separable mixture of **2** (28–31 % isolated yield, 96 % e. e.) and **3** (42–62 % isolated yield, 99 % e. e.).

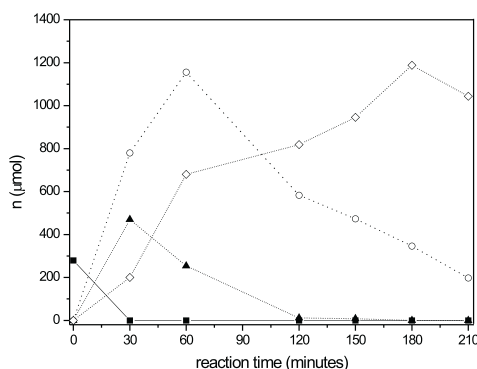


Fig. 1. **Conversion of 1 (■) to 2 (○), 3 (◇) and 4 (▲) mediated by pre-treated baker's yeast.**

In conclusion, the reduction of **1** by anaerobically pre-treated baker's yeast afforded an easily separable mixture of **2** and **3**, almost free from **4**, in reasonable yields and excellent e. e. at 90 min of reaction.

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P247 WHOLE-CELL BIOCONVERSION OF L-PHENYLALANINE TO 2-PHENYLETHANOL WITH YEASTS: MEDIUM OPTIMIZATION USING A GENETIC ALGORITHM

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Keywords: genetic algorithm, 2-phenylethanol, aroma, yeast

Various food-grade yeasts produce 2-phenylethanol among other volatile products useful as flavor and fragrance compounds. By adding the amino acid L-phenylalanine to the medium the yield of the desired product can be significantly increased as the yeasts convert the precursor via a cascade of three enzymatic steps (Ehrlich pathway) to 2-phenylethanol[1].

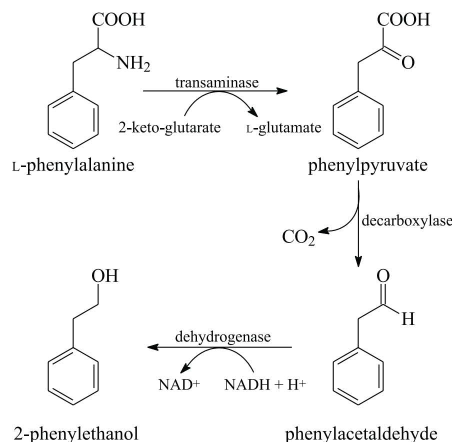

Fig. 1. **Ehrlich pathway**

To avoid inhibition by 2-phenylethanol the product has to be removed continuously from the bioprocess. On shake-flask scale this is done by an aqueous-organic two-phase system with oleyl alcohol as the extractive phase.

In a screening of fourteen yeasts *Kluyveromyces marxianus* CBS 600 was found to be the most productive strain. In

a medium with molasses as carbon source it produced approx. 3 g.l^{-1} 2-phenylethanol. This medium also contained a complex source of vitamins and other supplements which is expensive and cannot be used in a technical process.

A genetic algorithm was used to improve medium composition and process temperature towards higher product yields. Special emphasis was on varying single nutrients of the complex source to distinguish between those components which play a key role for the desired bioconversion and those which could be left out without impairing the results.

The optimization was carried out by varying ten parameters affording twenty individuals (in parallel experiments) per generation. In the course of five generations two local maxima corresponding to two significantly different medium compositions appeared, where the product concentration was increased by 58 % and 30 %, respectively. As the former medium is significantly more expensive than the latter, only an overall economic analysis of both variants will identify the “optimal” medium.

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P248 PRODUCTION OF ALCOHOLS AND ALDEHYDES BY BAKER’S YEAST IN A SOLID/GAS REACTOR

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Keywords: yeast, alcohol dehydrogenase, solid/gas catalysis, aldehydes

Industrial applications of baker’s yeast include the production of ethanol[1, 2], chiral alcohols and ketones[3, 4] in aqueous or organic media. Nevertheless these processes often present limitations such as a low productivity, a low yield or a high sensitivity to high products and substrate concentrations. The use of non-conventional media and especially the gas phase could overcome some of these limitations.

Recently, the use of *Saccharomyces cerevisae* in the gaseous phase has been successfully reported for the production of alcohols and aldehydes[5, 6]. This system presents the following advantages: (i) Cofactor regeneration is made *in situ*, (ii) no addition of cofactor is required, (iii) this system is cheaper than a system using crystallised ADH/cofactor.

The aim of this work is to better understand the influence of physico-chemical parameters on activity of this catalyst and to better understand phenomenon involved in a such system.

We then studied factors affecting catalysis and more particularly existence of possible diffusional limitations. Indeed the use of whole cells implies the presence of wall and membranes which may limit the gas diffusion. In order to check if there are diffusional limitations when using whole cells, walls were disrupted by sonication and the effect of this treatment on activity of cells has been studied. The preparation was tested at different water activities and the effect of cofactor addition is also presented.

As the preliminary tests was carried out with ethanol and butanal, this system may be an alternative method for the production of enantiopure compounds and aromas for the chemical and food industry and a best comprehension of the mechanism of YADH in solid/gas reactor is necessary to optimise the reaction.

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P249 ACTION OF HYDROLYTIC ENZYMES ON SYNTHETIC FIBRES

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Keywords: synthetic fibre, nitrilase, protease, esterase, cutinase, amidase

Enzymes are natural catalysts, promoting a great variety of chemical reactions. For this reason, enzymes have an important role on textile industry as substitutes of some chemical agents with negative effects on the environment. This work intend to study the enzymes capacity to alter surface properties of synthetic fibres. The hydrolytic enzymes nitrilases, proteases and esterases promote the cleavage of nitrile,

amide and ester groups, respectively, into carboxylic acids. These reactions can be very useful for the treatment of synthetic fibres containing these groups. The enzymatic action causes an increase of charged groups at the surface, improving water absorption (increase hydrofility) and dyeability.

Due to enzymatic modification, the acrylic fibers became more hydrophilic and dye uptake was enhanced at temperatures below glass transition. Nitrilase action on PAN fibres was monitored by measuring the release of ammonia and by FTIR detection of the formed of carboxylic groups by diffuse reflectance.

Cutinase were used on polyester fibres. The esterase action on polyester fibres promotes an increase of OH and COOH end groups. The OH end groups can be detected by a titration method and measuring the K/S spectrophotometrically after dyeing with a cotton reactive dye. Similarly enzyme preparations showing amidase activity towards polyamide fibres, promote an increase of NH_2 and COOH end groups. The amine groups were detected by a titration method with an increase of the molecular mass on treated samples. When samples were dyed with a wool reactive dye uptake was enhanced at temperatures below glass transition of polyamide.

P250 NOVEL ENZYME APPLICATIONS ON COTTON CELLULOSE

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Keywords: lipase, protease, hexokinases, cotton fabrics

Lipases and proteases were used to restore partially the strength loss of cotton fabrics, cross-linked with respectively 1,2,3,4-butanetetracarboxylic acids and *N*-hydroxymethyl acryl amide. Nearly one half of the strength loss of the fabrics could be restored by means of enzymatic hydrolysis at low temperature and neutral pH, while the crease-resistance effect decreased only slightly. In another application an enzymatically catalysed phosphorylation of cotton cellulose was achieved using hexokinases in the presence of a phosphate donor adenosine-5'-triphosphate. The enzymatic modification provided a new, reactive type cellulose substrate with improved dyeability and flame-resistance.

P251 ENZYMATIC DYEING OF KERATINOUS MATERIALS

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Keywords: keratinous fibre, laccase, protein disulfide isomerase

This study reports on the dyeing of keratinous materials using appropriate enzymatic systems – laccases and protein disulfide isomerase. The enzymatic dyeing was performed as a batchwise process at the temperature and pH of maximum enzyme activity. Laccases generate the colour "in situ" starting from low molecular colourless compounds – dye precursor and dye modifiers. Different hues and depth of shades could be achieved varying the concentration of the modifiers and the time of laccase treatment. Protein disulfide isomerases, based on their ability to catalyze thiol-disufide exchange, including oxidation, reduction and rearrangement, were used for covalent fixation of novel cysteine-modified dyes on keratinous fibres.

P252 CHEMO ENZYMATIC PREPARATION OF D-ALLOISOLEUCINE

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Keywords: D-alloisoleucine, subtilisin, diastereoselective hydrolysis

D-Alloisoleucine is a non-proteinogenic amino acid found as a component unit in a number of biologically active depsipeptides, useful intermediates in the synthesis of oxytocin analogues of isostatins and of natural cytotoxic compounds like tamandarins.

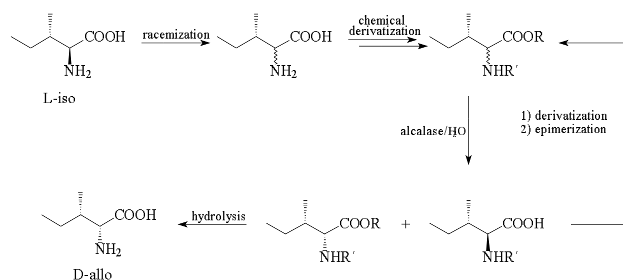
We report here a practical approach to D-alloisoleucine starting from a mixture of L-isoleucine and the D-allo stereoisomer, with an approach both chemical and enzymatic. The latter is based on the use of an industrial hydrolytic enzyme developed for detergency.

Thus L-isoleucine was epimerised and the diastereoisomeric mixture was transformed in a number of *N*-acyl-*O*-es-

ter derivatives including protecting groups usually employed in peptide synthesis.

Hydrolysis in water gave excellent separation of the two diastereoisomers allowing the obtainment of D-alloisoleucine derivatives in the maximum allowable yield in high enantiomeric excess.

During the elaboration of the substrates opportunities to effect the separation of the diastereoisomers *via* crystallization were successfully explored.



P253 PHOSPHOLIPASE D CATALYSED SYNTHESIS OF PHOSPHATIDYLSERINE IN A HOLLOW-FIBER MEMBRANE REACTOR

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Keywords: phospholipase D, hollow fiber membrane, transphosphatidylation

Phospholipase D obtained from a culture of *Streptomyces* sp. PMF, was immobilised in a hollow fiber membrane by ultrafiltration. The reactor was used to contact an organic solution containing phosphatidylcholine (PC, 50 $g.l^{-1}$) and a water solution at different pH and the rate of formation of the hydrolysis product (phosphatidic acid, PA) was measured. Subsequently the water phase was replaced with a 3M water solution of L-serine and the rate of transphosphatidylation at different pH was evaluated. The reaction was complete in a 48 h period. The formation of the hydrolysis product was minimized working at pH 4.5 were hydrolysis rate is minimal. The operational stability of the system was excellent during a period of several month.

Although the space-time yield of phosphatidylserine formation is lower than in a byphasic CSTR system, the purity of the product and the enzyme consumption is advantageous.

P254 BIOSYNTHESIS OF SESQUITERPENE LACTONES IN CHICORY AND APPLICATION OF THE ENZYMES INVOLVED

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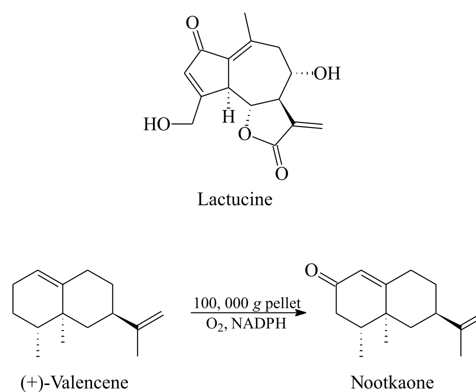
Keywords: sesquiterpenes, biosynthesis, hydroxylation, nootkatone

The edible sprouts of chicory (*Cichorium intybus* L.) are used as a vegetable and are well-known for their slightly bitter taste, which originates from sesquiterpene lactones (*e. g.*, lactucin). Especially the roots contain high concentrations of these bitter principles, which makes them unsuitable for use as cattle feed. However, we have demonstrated that the enzymes responsible for the biosynthesis of the sesquiterpene lactones are still present and active in the roots after the harvest of the sprouts. The first steps in the biosynthetic route have been elucidated by us[1–3].

One of the enzymes involved, the (+)-germacrene A hydroxylase, appears to possess a broad substrate specificity coupled to a high regioselectivity, making this enzyme an attractive catalyst for the hydroxylation of terpenes in flavour and fragrance industry. An especially interesting reaction is the one-step conversion of (+)-valencene into nootkatone[4], a much sought flavour component of grapefruit.



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P255 ENANTIOSELECTIVE HYDROLYSIS OF 1-OXASPIRO[2.5]OCTANES BY YEAST EPOXIDE HYDROLASE

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Keywords: spiro epoxides, epoxide hydrolase, kinetic resolution

Naturally occurring compounds with antimicrobial or antitumor activity in many cases contain an epoxide group in a spiro attachment to a tetrahydropyran or cyclohexane ring. The epoxide moiety is generally assumed to be essential for the biological activity of these compounds. Oxaspiro compounds may thus be assumed to have significant therapeutic potential and their preparation in enantiopure form will be of interest[1,2]. Various enantiopure epoxides can be obtained effectively by kinetic resolution using epoxide hydrolases. A powerful tool for this method is the yeast epoxide hydrolase (YEH) of *Rhodotorula glutinis*, which can accept structurally diverse molecules as substrates[3]. We have therefore used the YEH to investigate the kinetic resolution of oxaspiro compounds. The structural requirements of substrates for YEH are studied in more detail by using a range of methyl substituted 1-oxaspiro[2.5]octanes (Fig. 1).

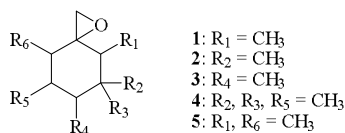

Fig. 1. **Methyl substituted 1-oxaspiro[2.5]octanes used as substrates for the YEH of *Rhodotorula glutinis***

Hydrolysis of the tested substrates by the YEH of *Rhodotorula glutinis* was strongly influenced by the position and number of methyl substituents. The initial reaction rate was found maximal for compound **3** and was comparable to the rate for the unsubstituted 1-oxaspiro[2.5]octane. The rate of hydrolysis decreased when methyl substituents were positioned closer to the epoxide group. A similar affect was observed by increasing the number of methyl substituents. No hydrolysis was observed when two methyl groups were placed on both positions next to the epoxide group (compound **5**). Enantioselectivities increased from moderate to high in the hydrolysis of substrates **4**, **2** and **1**. Based on the present results, we will further explore YEH-catalyzed kinetic resolutions of more complex oxaspiro compounds.

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P256 DYNAMIC KINETIC RESOLUTION OF ALCOHOLS: COMPLEMENTARY REACTIONS IN STEREOSELECTIVITY

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Keywords: alcohols, dynamic kinetic resolution, enzyme--metal combo, catalysis

The complete transformation of a racemic mixture into a single enantiomer is one of the current challenging problems in asymmetric synthesis. The dynamic kinetic resolution (DKR) of racemic substrates by enzyme-metal combo-catalysis provides a useful solution to this problem. In this approach, the enzymatic resolution of racemic substrate is coupled in situ with metal-catalyzed racemization of substrate, leading to the almost complete conversion of the racemic substrates to enantiomerically-enriched single products. The *R*-selective DKR of alcohols can be achieved by employing lipase-ruthenium combo catalysis. A wide range of simple and functionalized alcohols have been successfully resolved by using this method[1–10]. The *S*-selective DKR as a complementary process is more challenging because few enzymes are available to show satisfactory *S*-selectivity together with good activity and stability in organic solvents.

Some proteases have been examined as the resolution catalysts for the *S*-selective DKR. In the meeting, we wish to present some of the preliminary results from these studies.

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P257 APPLICATIONS OF IONIC LIQUIDS IN BIOCATALYSIS: ENHANCEMENT OF ENZYME ACTIVITY AND SELECTIVITY

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Keywords: ionic liquid, biocatalysis, activity enhancement, selectivity enhancement

Room-temperature ionic liquids are attracting growing interest as alternative reaction media for chemical transformations[1]. They are particularly promising as solvents for catalysis. Their use can enhance activity, selectivity, and stability of catalysts. As a part of our "green chemistry" research program, we became interested in ionic liquids as alternative solvents for biotransformations using cell-free enzymes[2–7]. In our first study, we examined lipase-catalyzed reactions in ionic liquids to see if enzyme exhibits any enhanced activity and enantioselectivity. It was observed that the enzyme enantioselectivity increased by 1.5 to 2-fold, but the enzyme activity remained unchanged[8]. In our second study, we examined the activity and selectivity of ionic liquid-coated enzyme (ILCE) in organic solvents. It was found that ILCE had better enantioselectivity and similar activity compared to its native counterpart[9]. Very recently, we have examined the potential of ionic liquids as lyoprotectants and supporters for enzyme activation and stabilization. In the meeting, we will present the results from these studies with some experimental detail.

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P258 *Acinetobacter* SP. LIPASE AND ITS APPLICATION TO RESOLVE 1,3-DIOXOLANE

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Keywords: lipase, *Acinetobacter* sp., reactivity, immobilization

Acinetobacter sp. lipase (called as SY-01) was selected after screening from soils to resolve the target molecule 1,3-dioxolane which is containing quaternary chiral center. This new enzyme showed specific reactivity that the existing enzymes did not show. Research to immobilize wild type SY-01 enzyme to various supporters was proceeded and optimized. Study to optimize the resolution of racemic mixtures was progressed according to changing pH, reaction temperature, reaction time, substrate concentration, and the use of various reaction solvents.

P259 SCREENING AND SYNTHETIC APPLICATION OF NEW BACTERIAL ALCOHOL DEHYDROGENASE FOR ENANTIOSELECTIVE REDUCTION OF β-KETO ESTERS

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Keywords: alcohol dehydrogenase, reduction, β-keto ester

We are working on the screening of new biocatalysts for enantioselective reduction of β-keto esters by use of microtiter plate for parallel growth and bioconversion[1,2]. We established an analytic system which enables the fast determination of conversion and enantioselectivity of the reactions. Several bacteria strains were found to catalyze the reduction of a series of β-keto esters to the corresponding β-hydroxy esters with high activity and high enantioselctivity. Most important asset was finding of strains with opposite enantioselectivity, which allows the production of both *S*- and *R*-enantiomers of the target molecules. The detail results will be presented.

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P260 ENANTIOSELECTIVE *trans*-DIHYDROXYLATION OF NON-ACTIVATED C-C DOUBLE BOND AND ENANTIOSELECTIVE HYDROLYSIS OF RACEMIC AND *meso*-EPOXIDES WITH *Sphingomonas* SP. HXN-200

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

Enantioselective *trans*-dihydroxylation of non-activated C-C double bond is a useful method for preparing *trans*-diols. Several fungi containing monooxygenase and hydrolase are known to catalyze such transformations of several acyclic terpenes, limonene and α-terpinene[1]. However, enantioselective *trans*-dihydroxylation of non-terpene substrates has thus far been unsuccessful[2]. Here, we report that *Sphingomonas* sp. HXN-200, an alkane-degrading bacterium, catalyses the enantioselective *trans*-dihydroxylation of non-activated C-C double bond of heterocyclic compounds giving the corresponding *trans*-diols in high yield and high ee.

Sphingomonas sp. HXN-200 was found to contain a soluble epoxide hydrolase. Hydrolysis of meso-epoxide *N*-benzyloxycarbonyl-3,4-epoxy-pyrrolidine and cyclohexene oxide gave the corresponding trans-diol in high ee and high yield[3], which is the first example of a bacterial EH catalyzing a meso-epoxide. Hydrolysis of several racemic epoxides with HXN-200 showed good enantioselectivity. The optical active epoxides and *trans*-diols prepared from these methods are useful synthetic intermediates that are difficult to make by classic chemistry.

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P261 PURIFICATION AND MODELLING OF A SOLUBLE P450 MONOOXYGENASE IN *Sphingomonas* SP. HXN-200

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Keywords: biohydroxylation, emzyme purification monooxygenase, P450, modeling

We have recently developed *Sphingomonas* sp. HXN-200 as a highly active and easy to handle biocatalyst for regio- and stereoselective hydroxylation of pyrrolidines[1,3], pyrrolidine-2-ones[2], piperidines[4], azetidines[4], and piperidin-2-ones[5], to prepare the corresponding hydroxylated compounds that are useful pharmaceutical intermediates. We found that *Sphingomonas* sp. HXN-200 contains a soluble NADH-dependent P450 monooxygenase. Enzyme components were purified by standard chromatography procedures (ion-exchange chromatography, hydrophobic interaction chromatography, gel filtration, etc.). The purified monooxygenase component is not active by itself. However, combination with other component restores the hydroxylation activity. MS and *N*-terminal se-

quences were determined. Genes of $P450_{pyr}$ was identified and sequenced. A homology model with $P450_{terp}$ as template was established. Combined with the experiment data, the interaction of the enzyme with substrates was investigated with docking program.

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P262 β-*N*-ACETYLHEXOSAMINIDASE-CATALYSED SYNTHESIS OF NON-REDUCING OLIGOSACCHARIDES

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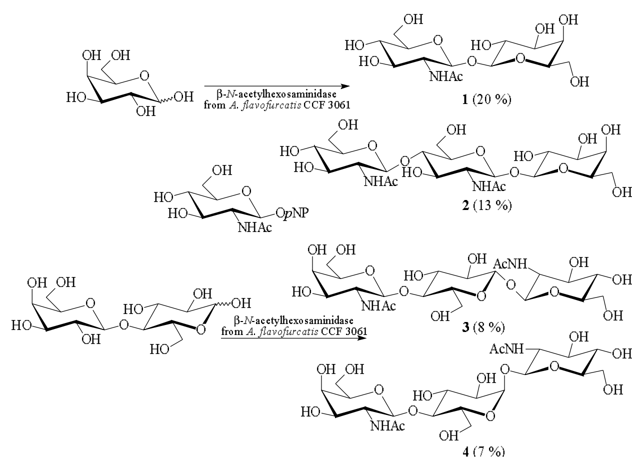
Keywords: β-*N*-acetylhexosaminidase, non-reducing saccharides, enzymatic transglycosylation, *Aspergillus flavofurcatis*, glycosylation regioselectivity

Large panel of fungal β-*N*-acetylhexosaminidases was tested for the regioselectivity of the β-GlcNAc transfer onto Gal-type acceptors (D-galactose, lactose, *N*-acetylgalactosamine). Unique, non-reducing disaccharide and trisaccharides β-D-Glc*p*NAc-(1↔1)-β-D-Gal*p* **(1)**, β-D-Glc*p*NAc-(1→4)-β-D-Glc*p*NAc-(1↔1)-β-D-Gal*p* **(2)**, β-D-Gal*p*-(1→4)-β-D-Glc*p*-β-(1↔1)-β-D-Glc*p*NAc **(3)** and β-D-Gal*p*-(1→4)-β-D-Glc*p*-α-(1↔1)-β-D-Glc*p*NAc **(4)** were synthesized under catalysis of β-*N*-acetylhexosaminidase from *Aspergillus flavofurcatis* CCF 3061 and D-galactose and lactose as acceptors. Disaccharide β-D-Glc*p*NAc-(1→6)-β-D-Gal*p* was produced exclusively with β-*N*-acetylhexosaminidase from *Aspergillus tamarii* CCF 1665. The use of *N*-acetylgalactosamine as an acceptor afforded only β-D-Glc*p*NAc-(1→6)-β-D-Gal*p*NAc.



This project was supported by grants from Czech National Granting Agency No. 203/01/1018, and No. 204/02/P096/A, MSMT grant ME 371 and Research Concept No. AVOZ5020903.

P263 CHEMICAL BIOMIMETIC SYNTHESIS AND ENZYMATIC CONVERSIONS OF THE BIOGENIC PRECURSORS ARTEMISINIC ACID AND ARTEANNUIN B

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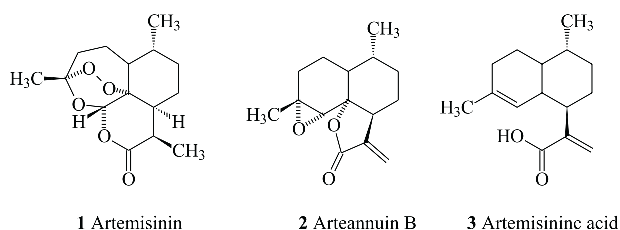
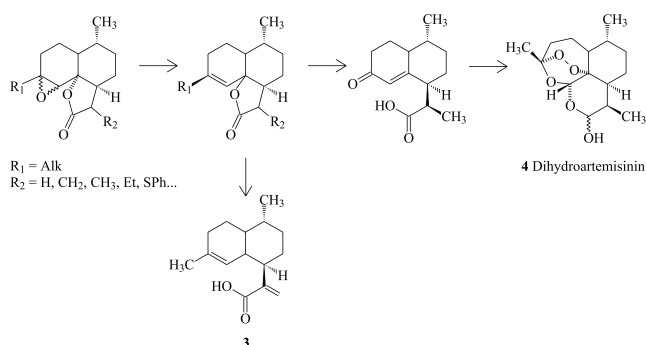
Keywords: artemisinin, antimalarial drugs, enzymatic conversions

Malaria is one of the most important killer diseases of the tropical world. It has been responsible for over two million deaths per annum and has become a major obstacle in the development and prosperity of third-world nations. Considerable efforts to eradicate malaria are impeded by multidrug resistant strains. Artemisinin **(1)** is an important antimalarial drug that meets the dual challenge posed by drug-resistant parasites and rapid progression of malaria illness. Artemisinin is extracted from *Artemisia annua*. This herb has been used for malaria therapy in China for over 1000 years. *Artemisia annua* is a temperate plant and is not available in abundance to the tropical regions where malaria is highly endemic. Unfortunately, the low natural abundance (0.05–1.1 %) of artemisinin and its complex structure make total synthesis very difficult and certainly not adaptable to industrial production at low cost[1, 2].



1 Artemisinin **2** Arteannuin B **3** Artemisininc acid

Hence, we have been led to consider a biotechnological approach by employing cell-free extracts of *Artemisia annua* leaves as an alternative to produce artemisinin from biogenic precursors. Indeed, these biosynthetic precursors, arteannuin B **(2)** and artemisinic acid **(3)**, occur of levels 8–10 times higher than artemisinin in plants[3]. Artemisinic acid has the added advantage of facile isolation from the plant *via* base extraction.



We are currently developing novel routes to arteannuin B and analogues from dihydroartemisinin **(4)** and **(3)** to generate novel bioactive artemisinin derivatives. The biotransformation of these substrates by using plant cell-free extracts will be reported.

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P264 POLYMERIC MEMBRANES SYNTHESIS FOR POTABLE WATER PRODUCTION

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Keywords: membrane synthesis, *E. coli*, potable water

The objective of this work was a study of polymeric asymmetric membranes synthesis for the *Escherichia coli* bacteria removal getting a production of potable water. In spite of the known concept of the water being an infinite resource, it is a finite one and it is becoming rare, due a lot of factors, such as, source contaminations, demographic explosion and the natural wood degradation. Consequently, urge a rational management of waters and the domain of techniques for their treatments. In this work we tried to present an alternative process for treating water contaminated.

The process used in this work for preparing the membranes was the inversion phase, and the technique was the precipitation by immersion. The membranes were synthesized from a polymer solution of dimetylformamide (DMF), using either the polymers polysulfone or polyvinylidene fluoride (PVDF). In addition, the salt KCl was used as an additive in the polymeric solution. The membranes support used in the synthesis was polyester-polypropylene sheet, and the preparation of the asymmetric membranes was made by spreading the polymeric solution (polymer + solvent + salt) as a thin film, on the support, which was previously adhered in a glass plate by a nylon string. This film was introduced in some water baths at 20 °C. The last stage was the dry of the membranes. For verifying the performance of the synthesized membranes, some samples of deionised water were contaminates with the *E. coli* bacteria with a bacterial mass of 10^7–10^8 CFU.ml^{-1}. These contaminated samples were filtered in a dead end filtration module. In this module the synthesized membranes were placed on a perforated metal plate and magnetic agitation was kept to avoid the deposition of material on the membrane surface. The operation pressure was of 3 bar.

The permeate was analyzed in terms of the retention of the *E. coli* (microbial measure) and also the flux of permeate.

Analyzing the results, it is possible to state that the aim of this work was reached because, not only was got a great *E. coli* removal, resulting in potable water, but also kept a satisfactory permeate flux. All synthesized membranes gave good results, reaching an *E. coli* removal between 95 and 100 %, and permeate flux above 200 l.hm^2. The results obtained can be considered good because the synthesized membranes were tested with a mass bacterial of *E. coli* (10^7–10^8 CFU) well

above of the commonly found in waters, and this mass bacterial is difficult to be removed.

P265 SPECIFICITY OF FUNGAL KERATINOLYTIC PROTEASES

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Keywords: fungal keratinases, cleavage sites, catalytic properties, enzyme stability

Enzymes that hydrolyse scleroproteins, keratins, are among most powerful proteolytic enzymes. They are synthesized by some insects and microorganisms and are mostly serine type proteases active in alkaline environment. Apart from bacteria and dermatophytes, producers of keratinases can also be found among common non-pathogenic fungi as shown in our screening experiments[1]. The most promising fungi were cultivated under conditions promoting keratinase synthesis. The enzymes of *Doratomyces microsporus*[2] and *Paecilomyces marquandii* were purified and characterised. Both of them are most active at pH 8, but differ in optimal temperature that is 45 °C for the former and 60–65 °C for the latter. Purified enzymes are susceptible to autolysis, however, the keratinase of *P. marquandii* is notably more stable than that of *D. microsporus*. In order to determine the preferred peptide cleavage sites, the activity of the enzymes was tested on synthetic peptides as well as on the oxidised insulin B-chain. Results on the synthetic substrates showed that both keratinases exhibited similar substrate specificity. They were most active on Suc-Ala-Ala-Pro-Phe-pNa (AAPF) that has a non-polar aromatic amino acid at the cleavage site, while Suc-Ala-Ala-Ala-pNa with the non-polar aliphatic amino acid at the cleavage site was far less affected. Hydrolysis of Ac-Tyr-OEt indicated esterase activity of both keratinases. Neither of the enzymes hydrolysed Bz-Arg-pNa, Bz-Tyr-pNa or FA-Leu-Gly-Pro-Ala-OH which are specific synthetic substrates for trypsin, collagenase and chymotrypsin, respectively. Catalytic properties on AAPF obtained by a non-linear regression based on the Michaelis-Menten equation are presented in Table I.

Table I
Catalytic properties of two fungal keratinases on Suc-Ala-Ala-Pro-Phe-pNa

Enzyme	K_m (μM)	V_{max} (μmol.min^{-1})	E_{tot} (μmol)	k_{cat} (min^{-1})	k_{cat}/K_m (μM^{-1}.min^{-1})
Keratinase of *D. microsporus*	1027 ± 169	0.070 ± 0.004	1.33 E-4	526	0.51
Keratinase of *P. marquandii*	165 ± 18	0.044 ± 0.001	1.79 E-5	2458	14.90

Specificity of hydrolysis was determined on the oxidized insulin B-chain by incubating enzyme/substrate mixture at 45 °C for 20 min and for 12 h. Cleavage sites were compared to those published for Proteinase K, the only commercially available keratinolytic protease of fungal origin, as well as for some other known serine proteases. The results are presented in Table II.

Table II
Cleavage sites on the oxidised insulin B-chain by keratinases of *Doratomyces microsporus* and *Paecilomyces marquandii* in comparison with those described for some known proteinases[3, 4]

	1				5					10					15					20					25					30
Oxidised insulin B-chain	**F**	**V**	**N**	**Q**	**H**	**L**	**C**	**G**	**S**	**H**	**L**	**V**	**E**	**A**	**L**	**Y**	**L**	**V**	**C**	**G**	**E**	**R**	**G**	**F**	**F**	**Y**	**T**	**P**	**K**	**A**
Keratinase of *D. microsporus*	↑		↑	↑	↑	↑			↑		↑	↑		↑	↑	↑	↑	↑	↑		↑	↑		↑		↑	↑			
Keratinase of *P. marquandii*	↑		↑				↑			↑		↑			↑		↑		↑		↑	↑		↑						
Proteinase K (ref.[3])			↑	↑	↑		↑		↑		↑		↑		↑	↑	↑		↑	↑	↑			↑	↑	↑				
Subtilisin[3]				↑					↑		↑				↑	↑	↑								↑					
Trypsin[4]																						↑							↑	
Elastase[4]														↑				↑					↑							

Compared to other proteases, fungal keratinases including Proteinase K show very broad specificity that may be responsible for their high activity against keratin filaments.

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P266 IS THE AROMA COMPOUND γ-DECALACTONE PRODUCED BY YEAST IN RESPONSE TO A MEMBRANE-RIGIDIFYING STRESS INDUCED BY THE BIOTRANSFORMATION MEDIUM?

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Keywords: γ-decalactone, biotransformation, yeast cells, biological membranes

The peach-like aroma compound γ-decalactone is produced biotechnologically through the biotransformation of ricinoleic acid (RA) by some yeast species. In order to better understand the interactions occurring during the process between the lactone and the yeast cells, the influence of the lactone on the physical properties of cell membranes and model phospholipids has been studied. The producing yeast *Yarrowia lipolytica* was used as a model cell in this study. γ-Decalactone strongly increased membrane fluidity *in vivo* and decreased in a concentration-dependent manner, the phase transition temperature (*T*m) of the deuterated phospholipid DMPC-d27 (ref.[1–3]). This indicates that the lactone exhibits an important membrane-fluidizing action. On the other hand, the hydroxylated C18 fatty acid (RA) used as the precursor of the biotransformation, increased the *T*m of DMPC-d27, i. e. it rigidified the phospholipid bilayers. These observations bring about the hypothesis that the production of the lactone, a compound that is presently considered as a secondary metabolite, in fact could be stimulated in order to enable a homeoviscous adaptation of cell membranes and so to permit an optimal cell development in the medium. From this point of view, new strategies may be elaborated to improve γ-decalactone production yields.

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P267 GLYCOMIMETICS AS SELECTIVE TOOLS FOR ENZYME INHIBITION

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Keywords: pyrrolidines, enzymatic inhibition, sugar mimetics

Sugar mimetics have shown to be inhibitors of enzyme-promoted hydrolysis of C-O glycosidic bonds exhibiting interesting applications as antibacterial, antiviral and anticancer agents. Among them, 1,4-dideoxy-1,4-iminoalditols (hydroxylated pyrrolidines) constitute an important type of compounds with a well-known importance as glycosidase inhibitors[1]. However, in many instances they are not selective presenting a wide range of enzymatic inhibition.

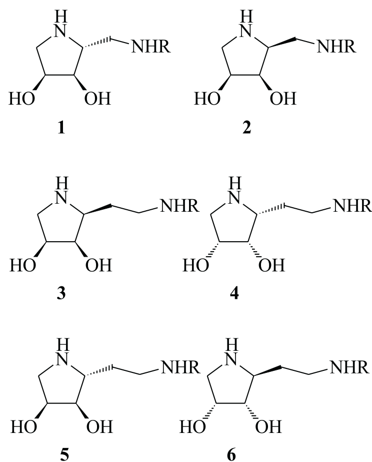
The selectivity in enzyme inhibition can be improved by providing the iminosugar with some information of the structure of the glycosyl moiety that is cleaved in the enzymatic hydrolysis and of the aglycon itself. Therefore, the introduction of additional groups in the iminosugar joined by hydrolytically stable C-C links could led to new more potent, more selective and hydrolytically stable enzyme inhibitors. Lipophilic moieties are important structural features because they, additionally, have the advantage of their permeability through membranes, which is an important requirement for a compound to become a useful drug.

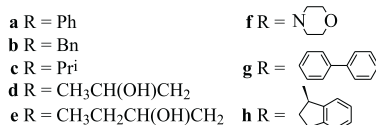
We have found that diamines of type **1** with aryl(alkyl)-aminomethyl chains can be higly selective and competitive inhibitors of α-mannosidases and we have reported[2] a quick combinatorial approach for their preparation.

In this commuincation we report on the influence on the enzymatic activity of three stereogenic centers in the pyrrolidine ring and the influence of the methylene spacer between the nitrogen of the pyrrolidine ring and of the aminoalkyl chain. To achieve this goal, we describe the synthesis and enzymatic evaluation of derivatives of (2*S*,3*R*,4*S*), (2*R*,3*R*,4*S*), (2*R*,3*S*,4*R*) and (2*S*,3*S*,4*R*)-2-alkyl(aryl)aminoethyl-3,4-dihydroxypyrrolidines **(3-6)** and of (2*S*,3*R*,4*S*)-2-(*N*-alkyl(aryl)-aminomethyl-3,4-dihydroxypyrrolidines **(2)**.

Enzymatic inhibitory studies of diamines **2-6** indicate that the activity and selectivity in enzyme inhibition are influenced by the absolute configuration of the stereogenic centers in the pyrrolidine. Thus, (2*S* and 2*R*, 3*R*, 4*S*)-2-alkyl(aryl)aminoethyl **3** and **5** and (2*S* and 2*R*, 3*R*, 4*S*)-2-alkyl(aryl)aminomethyl **1** and **2** have shown to be moderate-to-good inhibitors of β-mannosidases, while derivatives **4** and **6** of configuration (2*R* and 2*S*, 3*S*, 4*R*) were inactive towards those enzymes presenting good inhibitory values towards β-galactosidases

and β-glucosidases or α-L-fucosidases. The spacer between the two nitrogens (aminomethyl *vs.* aminoethyl side chains), and the substituent (alkyl *vs.* aryl) have also a remarkable influence.





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P268 PREPARATION OF DEAZAPURINE NUCLEOSIDES BY MICROBIAL TRANSGLYCOSYLATION

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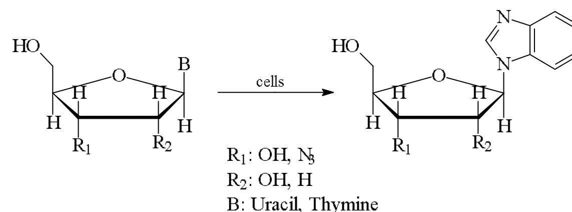
Keywords: deazapurine nucleosides, microbial transglycosylation

Benzimidazole nucleosides have shown inhibitory effect on human cytomegalovirus infections[1], herpes viruses[2] and other RNA viruses and in some cases possess mutagenic properties. The chemical synthesis of these nucleosides is complex and stereounspecific and affords low yields[3].

To our best knowledge, only ribo- and deoxyribofuranosyl nucleosides of benzimidazole have been previously synthesised[4] using microbial transglycosylation and guanosine as starting material.

Based on our experience[5, 6], benzimidazole nucleosides carrying natural and modified sugar moieties were prepared starting from available pyrimidine ones. The selected microorganisms were screened from our bacterial collection, affording yields between 60–90 % after optimisation of the reaction conditions.



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P269 A SURPRISING DOUBLE OXIDATION OF 7-PHENYLBICYCLO[3.2.0]HEPT-2-EN-6-ONE DERIVATIVES BY *Cunninghamella echinulata* NRRL 3655

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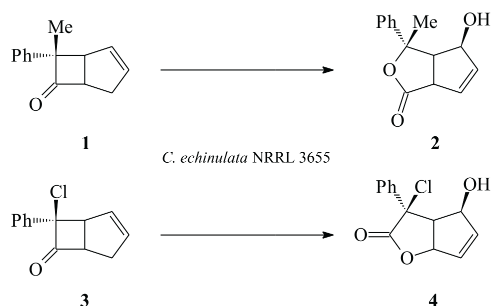
Keywords: Baeyer-Villiger oxidation, *Cunninghamella echinulata*

The fungus *Cunninghamella echinulata* NRRL 3655 has proved useful for the Baeyer-Villiger oxidation of bicyclo [3.2.0]hept-2-en-6-one owing to its ability to catalyse a resolution of the starting material[1], in contrast to the enantiodivergent biotransformation to equimolar quantitities of 2-oxa and 3-oxa lactones observed with prokaryotic Baeyer-Villger monooxygenase enzymes[2, 3]. On application of this catalyst to the transformation of 7-phenylbicyclo-[3.2.0]hept-2-en-6-one derivatives, a surprising double oxidation has been observed. 7-*endo*-phenyl, 7-*exo*-methylbicyclo-[3.2.0]hept-2-en-6-one **1** was transformed to the 3-oxa lactone derivative with concomitant allylic hydroxylation to yield hydroxylactone **2**. Contrastingly, 7-*endo*-phenyl, 7-*exo*-chlorobicyclo[3.2.0]hept-2-en-6-one **3** was again transformed to a hydroxylactone, but in this case oxygen insertion occurred to give the 2-oxa derivative **4**.



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