P117 APPLICATION OF DEGENERATE OLIGONUCLEOTIDE GENE SHUFFLING FOR CONSTRUCTION OF HYBRID HALOALKANE DEHALOGENASE

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Keywords: haloalkane dehalogenase, *in vitro* recombination, construct, substrate specificity

Halogenated organic compounds constitute one of the largest groups of environmental pollutants as a result of their widespread use in industry and agriculture. Haloalkane dehalogenases hydrolytically convert halogenated aliphatic compounds to corresponding alcohols. Enhancement of catalytic properties of environmentally important enzymes using *in vitro* evolution techniques is one of the obvious goal of biotechnology.

Our study was undertaken to construct and preliminary characterise hybrid haloalkane dehalogenases. Four genes cloned from different bacteria were used in this study: *dhlA* cloned from *Xanthobacter autotrophicus* GJ10 (ref.[1]), *linB* from *Sphingomonas paucimobilis* UT26 (ref.[2]), *dhaA* cloned from *Rhodoccous erythropolis* NCIMB13064 (ref.[3]) and *dhmA* from *Mycobacterium avium* N85 (ref.[4]). Considering relatively low level of homology among parental genes, Degenerate Oligonucleotide Gene Shuffling techniques was selected as an effective tool for of haloalkane dehalogenase genes[5].

Altogether twelve different combinations were constructed using one pair of degenerate oligonucleotides, cloned into pAQN vector and hybrid proteins were overexpressed in *Escherichia coli* BL21(DE3). Preliminary screening of dehalogenating activity was conducted with resting cells and six substrates representing different classes of halogenated aliphatic compounds. Four out of twelve hybrid proteins keep good expression and ten proteins showed obvious catalytic activity. Some hybrid proteins showed dehalogenating activity despite of changes in position of catalytic residues within the active site. Comparison of relative activities determined for the hybrid enzymes with the activities of wild type enzymes suggests that constructs do not possess novel substrate specificities.

Work on more efficient expression systems to obtain higher expression level of hybrid haloalkane dehalogenase is under progress recently.

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P118 KINETICS AND SPECIFICITY OF HALOALKANE DEHALOGENASE LinB FROM *Sphingomonas paucimobilis* UT26

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Keywords: haloalkane dehalogenase, *Sphingomonas paucimobilis*, *Xanthobacter autotrophicus*, reaction pathway, covalent alkyl-enzyme intermediate

Steady-state and transient-state kinetic methods were applied to solve reaction pathway, to identify reaction intermediate and to specify the rate limiting step of catalytic action of haloalkane dehalogenase LinB from bacterial strain *Sphingomonas paucimobilis* UT26 (ref.[1]). The steady-state experiments involved direct monitoring of LinB activity by isothermal titration calorimetry and initial rate of product formation measurements using gas chromatography. Stopped-flow fluorescence and rapid-quench-flow techniques were applied for the transient-state kinetics measurements. Additionaly, steady-state inhibition experiments and transient-state binding experiments were employed to find out leaving ability of both products (halide and alcohol) during dehalogenation reaction.

The results showed that export of products as well as import of substrates into the active site of LinB are fast processes reaching rapid equilibrium. This fast exchange of the ligands between the active site and bulk solvent can be explained by wide opening of the entrance tunnel and large active site of LinB. In contrary, the release of the halide ion from narrow active site after the reaction was found to be

slow rate limiting step for another haloalkane dehalogenase, enzyme DhlA from *Xanthobacter autotrophicus* GJ10 (ref.[2]). The actual cleavage of the carbon-halogen bond was found to be fast step in both enzymes. Further the results confirmed, that the reaction proceeds via a covalent alkyl-enzyme intermediate. Using bromocyclohexane, chlorocyclohexane and 1-chlorohexane as model substrates, hydrolysis of this intermediate was found to be the slowest step in the catalytic cycle of LinB. The alkyl-enzyme complex was highly accumulated due to the fast dehalogenation step following the slow hydrolyses of this intermediate. The study provides a basis for the analysis of kinetic steps in hydrolysis of environmentally important substrates by the action of LinB.

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P119 COMPARISON OF FOUR YEAST PYRUVATE DECARBOXYLASES FOR *R*-PHENYLACETYLCARBINOL PRODUCTION

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

The chiral intermediate *R*-phenylacetylcarbinol (PAC) is the precursor in the production of ephedrine and pseudoephedrine. Commercially, it is synthesized through biotransformation of benzaldehyde and pyruvate by fermenting yeast *Saccharomyces cerevisiae*. Alternative use of a cell free system, e. g. *Candida utilis* pyruvate decarboxylase (PDC), has the advantage of higher PAC yields and concentrations. For both options, issues regarding PDC stability, activity and by-product formation are still of key concern.

This study investigates PDC from 4 yeast strains i. e. *C. utilis*, *C. tropicalis*, commercial PAC producer *S. cerevisiae* and thermotolerant *Kluyveromyces marxianus*. PDC was produced and investigated with respect to PAC production as well as stability at 23 °C using whole cell and/or crude extract preparations as catalysts.

All strains were grown in shake flasks with 90 $g.l^{-1}$ glucose medium and harvested when glucose concentration fell below 10 $g.l^{-1}$, which was within 10–15 hours. Between 120 and 250 U PDC/g dry cell weight were produced.

PAC production in an aqueous/organic two-phase system with whole cells at 23 °C resulted in the highest PAC concentration with *C. utilis* and in the lowest by-product acetoin formation with *C. tropicalis*. In a single phase system, cell free *C. tropicalis* PDC also formed the lowest amount of by-product acetoin. Lower acetoin formation in both systems indicates that the formation of this by-product is a characteristic of the PDC itself.

C. utilis and *S. cerevisiae* PDC were very stable with half-lives of nearly two weeks at 23 °C. The half-lives were even slightly longer in whole cells. In contrast *C. tropicalis* PDC was very unstable (half-life 3 days in crude extract and less than one day in whole cells). The deactivating effect of 50 mM benzaldehyde was confirmed, generally reducing the half-lives by 50 % or more. *K. marxianus* PDC was stabilized by the addition of protease inhibitors while these had no influence on PDC stability for the other strains.

In summary, *C. utilis* PDC was confirmed to be the best catalyst for PAC production with high stability (half-life of nearly two weeks at 23 °C) and highest final PAC concentrations. *C. tropicalis* PDC had the advantage of lower by-product acetoin formation but was very unstable and yielded less PAC.

P120 IMPROVED PRODUCTION OF *Candida utilis* PYRUVATE DECARBOXYLASE FOR BIOTRANSFORMATION

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Keywords: pyruvate decarboxylase, *Candida utilis*, pH, *R*-phenylacetylcarbinol

Pyruvate decarboxylase (PDC) catalyses the decarboxylation of the glycolysis end product pyruvate to acetaldehyde and carbon dioxide as the penultimate reaction of ethanol fermentation. This same enzyme is used industrially in the synthesis of enantiomerically pure *R*-phenylacetylcarbinol (*R*-PAC), which is the precursor for the chemical synthesis of ephedrine and pseudoephedrine.

After a comprehensive screen of PDC from various sources (Rosche et al 2003, submitted for publication), *Candida utilis* PDC is currently regarded as the best enzyme for biotransformation in terms of enzyme stability and *R*-PAC

concentration. Since *R*-PAC production depends on the activity of the PDC enzyme, it is of interest to optimise PDC production for the biotransformation. Higher PDC activity level will be beneficial as it can enhance *R*-PAC production and contribute significantly in the development of an economically competitive process.

Candida utilis PDC enzyme production was carried out in 5l batch and fed-batch bioreactors under controlled conditions using minimal medium. A preliminary study on PDC production was carried out in shake flasks with low agitation and limited buffering. The final PDC carboligase activity of 333 $U.g^{-1}$ dry cell was higher than previously achieved activity of 86 $U.g^{-1}$ dry cell (Sandford et al. 2003, submitted for publication). The aim of this study is to determine and optimise conditions for PDC production in a bioreactor.

This project was focusing on the effect of aeration and pH to PDC production. Decreasing aeration in comparison to the previously established protocol (aerobic growth phase followed by a fermentative phase) resulted in an increase of PDC activity to 160 $U.g^{-1}$ dry cell when one third of glucose was consumed. Afterwards the PDC activity remained constant throughout the process. However, when the culture was allowed to shift pH from 6.0 to 2.9 at this time point, the PDC carboligase activity was drastically increased to 335 $U.g^{-1}$ dry cell at the end of the process with a biomass of 9.1 $g\text{-}DCW.l^{-1}$. The drop in pH was caused by the yeast, and most likely by the secretion of pyruvic acid.

In order to investigate if there is an optimum pH for PDC production, three identical bioreactors were set up in parallel at constant pH of 3, 4, and 5. The resultant PDC carboligase activities had shown no significant differences to each other and to a process at pH 6. This is strong evidence that not the pH itself, but the pH shift was responsible for the increased PDC activity.

P121 LACTATE RACEMASE AS A VERSATILE TOOL FOR THE RACEMIZATION OF α-HYDROXYCARBOXYLIC ACIDS

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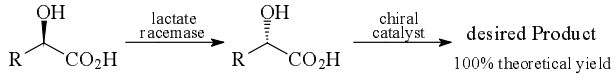
Keywords: lactate racemase, racemization, α-hydroxycarboxylic acids

The transformation of a racemate into a single stereoisomeric product in 100 % theoretical yield has become a prime target for the industrial production of chiral nonracemic materials[1]. One powerful method to reach this goal makes use of a stepwise kinetic resolution which is based on the enzyme-catalyzed racemization of the non-reacted enantiomer. Thus both enantiomers of a racemate can be converted into the desired product in 100 % theoretical yield[2].



Previous studies have shown that mandelate racemase is a versatile tool for the biocatalytic racemization of β,γ-unsaturated α-hydroxy acids. However, aliphatic α-hydroxycarboxylic acids are not accepted[3]. In order to circumvent this limitation, we were aiming at the enzymatic racemization of aliphatic substrate analogues. A screening for lactate racemase activity was initiated starting from scarce literature data[4–6]. In our ongoing screening for lactate racemase activity we identified several active strains possessing a desired broad substrate spectrum for α-hydroxycarboxylic acids, which could not be transformed by mandelate racemase.

This work was performed within the Spezialforschungsbereich Biokatalyse (project # F-115) and financial support by the FWF (Vienna) is gratefully acknowledged.

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P122 DEVELOPMENT OF A MULTI-ENZYMATIC SYSTEM FOR C-C BOND FORMATION

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Keywords: aldolase, aldol reaction, dihydroxyacetone kinase, dihydroxyacetone phosphate, enzyme catalysis

Aldolases have attracted the interest of organic chemists because their ability to catalyze the formation of C-C bonds by an aldol addition reaction between an aldehyde and a ketone, with a high degree of stereochemical control[1]. Dihydro-

xyacetone phosphate (DHAP)-dependent aldolase produce 2-keto-3,4-dihydroxy adducts and, with some exceptions[2], they control the configuration of the newly formed stereogenic centers. An additional advantage of these enzymes is that they are stereocomplementary, that is, their use allows the synthesis of the four possible diaestereoisomers for a given pair of substrates. DHAP-dependent aldolases have shown their utility in the synthesis of carbohydrate, carbohydrate--like structures or non-carbohydrate compounds.

One limitation of these enzymes is that they practically only accept DHAP as donor substrate. DHAP to be used as aldolase substrate can be efficiently obtained by chemical[3] and enzymatic[4] synthesis. Recently, it has been shown that DHAP can be also produced by enzymatic phosphorylation of dihydroxyacetone (DHA) catalyzed by the enzyme dihydroxyacetone kinase (DHAK) from *Schizosaccharomyces pombe*[5].

Here, we describe a new multi-enzymatic system for one--pot C-C bond formation, based in the use of a recombinant DHAK from *Citrobacter freundii*. Thus, DHA phosphorylation is coupled with the aldolic condensation catalysed by the DHAP-dependent aldolase. The multi-enzymatic system is completed with the *in situ* regeneration of ATP catalysed by acetate kinase.

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P123 AMINOTRANSFERASES FOR THE PRODUCTION OF UNNATURAL AMINO ACIDS: APPLICATION TO GLUTAMIC ACID ANALOGUES

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Keywords: aminotransferase, transamination, glutamic acid

Aminotransferases are becoming useful catalysts for the stereoselective synthesis of rare or non-proteinogenic amino acids[1]. This emergence is due to the variety of aminotransferases available and to their broad substrate specificity. Furthermore, these enzymes are characterised by high turnover numbers and do not require external cofactor recycling. However, aminotransferases catalyse equilibrated reactions and this drawback has to be overcome for biotechnological applications.

Our studies in this field are focused on the production of L-glutamic acid (Glu) analogues. Indeed, Glu is widely accepted as the major excitatory transmitter within the central nervous system where it acts at multiple subtypes of ionotropic and metabotropic receptors[2]. Glu analogues behaving as selective agonists or antagonists are helpful to elucidate the properties of these receptors[3] and could even bring some therapeutic effects. New methodologies to access this class of compounds have attracted considerable attention from chemists researchers in recent years.

We have developed a chemo-enzymatic approach based on transamination of α-ketoglutaric acid (KG) analogues catalysed by aspartate aminotransferase (AAT, EC 2.6.1.1)[4]. A close analogue of aspartic acid (cysteine sulfinic acid) is used as the amino donor substrate thus providing a shift of the transamination equilibrium and simplifying glutamic acids isolation.

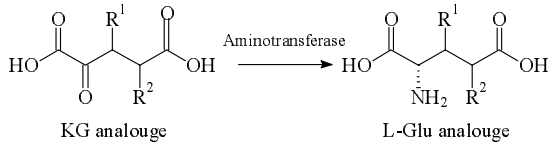

Fig. 1. **Production of L-Glu analogues by enzymatic transamination**

We have designed efficient methods for the chemical synthesis of diversely substituted KGs. Most of these analogues are readily converted into L-glutamic acids with AAT from porcine heart or *E. coli*. Surprisingly, this enzyme presents a broad substrate specificity, specially towards 4-substituted KGs bearing alkyl or polar functionalised groups. Furthermore, AAT shows a marked enantioselectivity for most substrates which allows the kinetic resolution of racemic substituted KGs.

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P124 OXIDATION OF POLY(ETHYLENE GLYCOLS) BY ALCOHOL OXIDASE FROM *Pichia pastoris*

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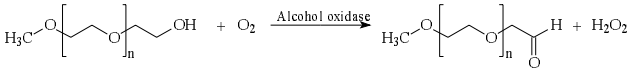
Keywords: alcohol oxidase, *Pichia pastoris*, poly(ethylene glycol), oxidation

Alcohol oxidase (AO) has been isolated from several yeast sources. It was found that AO from methylotropic yeast *Pichia pastoris* has the most wide substrate specificity. Numerous primary alcohols may be oxidized to the corresponding aldehydes by using this enzyme as a catalyst[1].

We present here oxidation of monomethylethers of poly(ethylene glycols) catalyzed by AO from *P. pastoris*:



1 **2**

Relative initial rates of the oxidation reactions are given in the Table I. Two aliphatic alcohols (methanol and butanol) are included for comparison. Oxidation rate is high enough even for long chain alcohols **(1)**. Similar results were obtained by using AO immobilized on macroporous cellulose carrier.

Oxidation rate is falling with the time course of the reaction. It may be caused by product inhibition[2]. TRIS buffer was proposed to alleviate this inhibition[2]. But we found that TRIS itself is oxidized by AO from *P. pastoris*.

We tested three chemical oxidation methods for conversion of poly(ethylene glycol) monomethylethers **(1)** to aldehydes **(2)**: oxidation by oxalyl chloride[3], action by TEMPO radical (2,2,6,6-tetramethylpiperidin-1-yloxy radical) in the presence of sodium hypochlorite[3] or by BAIB ([bis(acetoxy)-iodo]benzene) and potassium bromide as cooxidants[4]. All these chemical oxidation methods were not perfect because of undesirable side products, difficult separation or cross-linking. Oxidation of poly(ethylene glycol) monomethyl ethers **(1)** by using enzymatic method would be very attractive because of absence of oligomeric side-products which are hardly separable.

Poly(ethylene glycol) monomethyl ether aldehydes **(2)** were used for reductive alkylation of chitosan leading to water-soluble comb-shaped graft copolymers. Positive results were obtained.

Table I
Relative rates of oxidation of alcohols by AO

Substrate	Relative rate of oxidation[a], %
Methanol	100
n-Butanol	58
1, n = 1	42
1, n = 2	30
1, n ≈ 8 (M = 350)	17
1, n ≈ 17 (M = 750)	12
1, n ≈ 25 (M = 1100)	14

[a]0.1M phosphate buffer, pH = 7.3, 30 °C, substrate concentration 0,1M

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P125 SCALE UP OF BIOCATALYTIC SYNTHESIS OF CHIRAL FINE CHEMICALS ON THE EXAMPLE OF ENANTIOPURE 2,3-BUTANEDIOL

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Keywords: 2,3-butanediol, kilogram scale, bioconversion, alcohol dehydrogenase, chiral synthesis

Enantiomeric pure fine chemicals like (2*S*,5*S*)-2,5-hexanediol are very precious educts for the production of pharmaceuticals and agrochemicals such as various chiral phosphine ligands like the DuPhosLigand. The stereospecific *de novo* synthesis of these compounds by organic chemistry however is a very complicated and expensive process, often requiring lots of chemical steps. The yields involved are very poor in most cases because of the reactions or product losses by changes of reaction conditions between successive reaction steps.

Another chemical approach to get enantiomeric pure compounds is the resolution of racemic mixtures, in which on principle the half of educt is lost.

On the other hand only one enantiomer of an interesting substance can be produced in just one reaction by bioconver-

sion of prochiral compounds using different enantioselective enzymes. Because of the very high enantioselectivity (>99 %) of an appropriate enzyme and a comparatively simple down stream processing this kind of synthesis is the method of choice for providing a broad variety of chiral compounds. Catalytic active proteins are taken from the nearly inexhaustible variety of nature: yeasts, plants, fungi and bacteria from different habitats were screened for interesting activities in reduction of prochiral ketones, e. g. the cheap 2,5-hexanedione.

The use of alcohol dehydrogenases for these productions is one of the specialities of JFC. Bacteria strains constructed by methods of biotechnology allow to obtain these proteins in large amounts for efficient production of chiral fine chemicals.

A rare and often requested compound is the chiral 2,3-butanediol in all of its different stereoisomers (2*S*,3*S*)-, (2*R*,3*R*)- and *meso*-butanediol. These substances play an important role as e. g. part of chiral catalysts in pure chemical asymmetric reactions. They are expensive and accessible in small amounts only. Using above mentioned enzyme systems JFC is able to provide all these stereoisomers of 2,3-butanediol with an enantiomeric and diastereomeric excess higher then 99 % in a kilogram scale. This increase of production scale permits a new order of following applications.

P126 PRODUCTION OF ENANTIOPURE (2*R*)-PIPERIDINE DERIVATIVES IN HIGH YIELDS BY ENZYME-CATALYZED DYNAMIC KINETIC RESOLUTION

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Keywords: pipecolic acid, biotransformation, dynamic kinetic resolution, chiral building block, cofactor regeneration

The piperidine ring is an ubiquitous structural feature occuring in numerous secondary metabolites (e. g., alkaloids, nonproteinogenic amino acids and respective peptides) and biologically active compounds (e. g., anesthetics, analgetics immunosupressor agents)[1]. Enantiomerically pure pipecolic acid **1** (piperidine-2-carboxylic acid, **Pip**) and its derivatives are important building blocks for the introduction of the chiral 2-alkyl-piperidine motif into these types of compounds. Surprisingly, there exists up to now no powerful chemical or biocatalytical process for the production of **Pip** derivatives which provides high yields and at the same time a high enantiomeric purity of the desired product[2]. Therefore, the main focus in the present work was the development of a DKR process ("dynamic kinetic resolution"), which enabled 100 % yield of enantiopure product[3]. For this purpose, the spontaneous racemization of *N*-*p*-toluenesulfonyl pipecolic aldehyde *(rac)*-**2** should be coupled with a preferably enantiospecific biotransformation process. After a screening for oxidoreductase activity employing microorganisms as well as isolated enzymes, the bioreduction of **2** with an alcohol dehydrogenase proved highly active and enantiospecific. The bioreduction was coupled with an enzymatic NADH regeneration method and the reaction kinetics of the resulting batch-system (Fig. 1) was analyzed. Specific limitations of the investigated process could be identified. After optimization of the reaction conditions, optically pure *N*-*p*-toluenesulfonyl-(2*R*)-(hydroxymethyl)-piperidine (2*R*)-**3** was synthesized by means of a preparative scale conversion in 73 % yield. The compound by itself is a valuable and configurationally stable synthetic building block, or it can easily be converted to D-pipecolic acid by chemical means.

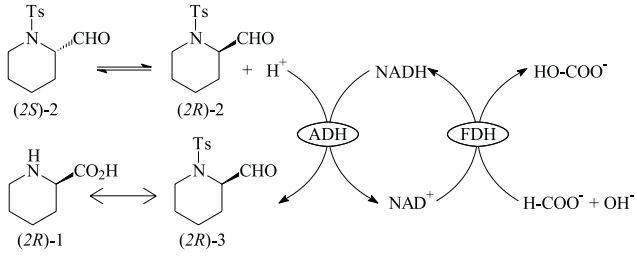

Fig. 1. **Enantioselective biotransformation process for the production of pipecolic acid derivative (2*R*)-3.** ADH: Alcohol dehydrogenase, FDH: Formate dehydrogenase, Ts: *p*-Toluenesulfonyl

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P127 ASYMMETRIC TRANSFER HYDROGENATION PROCESS BY PHENYLACETALDEHYDE REDUCTASE TO PRODUCE CHIRAL ALCOHOLS

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Keywords: phenylacetaldehyde reductase, NADH regeneration, chiral alcohol, asymmetric reduction, *Corynebacterium* sp.

Phenylacetaldehyde reductase (PAR) produced by styrene-assimilating *Corynebacterium (Rhodococcus)* strain ST-10 was used to synthesize chiral alcohols. This enzyme with a broad substrate range reduced various prochiral aromatic ketones and β-ketoesters to yield optically active secondary alcohols with an enantiomeric purity of more than 98 % enantiomeric excess (e. e.)[1]. The *E. coli* recombinant cells which expressed the *par* gene could efficiently produce important pharmaceutical intermediates; *(R)*-2-chloro-1-(3--chlorophenyl)ethanol (28 mg.ml^{-1}) from *m*-chlorophenacyl chloride, ethyl *(R)*-4-chloro-3-hydroxy butanoate (28 mg.ml^{-1}) from ethyl 4-chloro-3-oxobutanoate and (*S*)-*N*-*tert*-butoxycarbonyl(Boc)-3-pyrrolidinol from *N*-Boc-3-pyrrolidinone (51 mg.ml^{-1}), with more than 86 % yields. The high yields were due to the fact that PAR could concomitantly reproduce NADH in the presence of 3–7 % (v/v) 2-propanol in the reaction mixture. We have established a practical asymmetric hydrogen transfer process using 2-propanol as the hydrogen donor by phenylacetaldehyde reductase (PAR) expressed in *E. coli* cells (Fig. 1)[2].

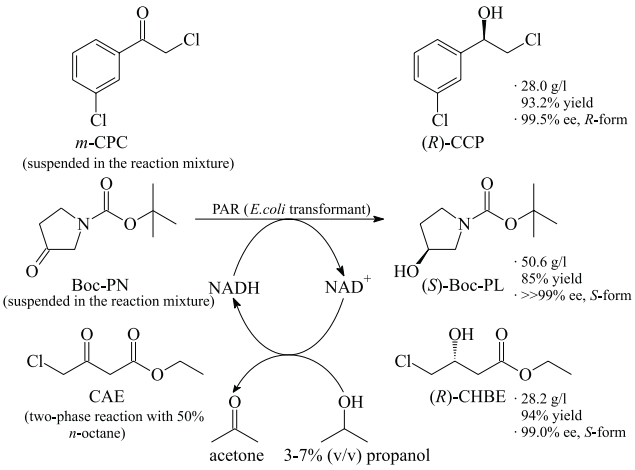

Fig. 1. **Asymetric transfer hydrogenation process by recombinant PAR**

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P128 TWO NOVEL REDUCTASES CATALYZING THE STEREOSPECIFIC REDUCTION OF C=C AND C=O BONDS

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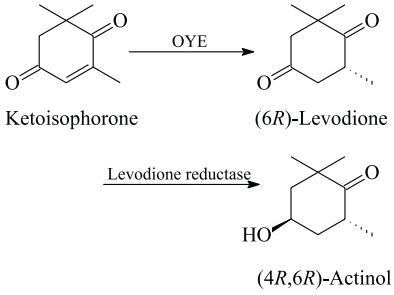
Keywords: carbonyl reductase, Old-Yellow-Enzyme, asymmetric reduction

The demands for optically active drugs has increased owing to the efficacy of these drugs and the market pressure for safe chemical compounds. Therefore, chiral starting materials have also been increasingly required in pharmaceutical and agrochemical fields. A biological method is often adopted for the synthesis of optically active compounds, because biocatalysts express high stereospecifities. Stereospecific dehydrogenase/reductase might be one of the useful biocatalysts for the synthesis of chiral compounds. Recently, two novel microbial reductases catalyzing C=C or C=O reduction were found.

Candida macedoniensis was found to catalyze the hydrogenation of C=C bond of ketoisophorone (2,6,6-trimethyl-2--cyclohexene-1,4-dione), and to specifically produce (6*R*)-levodione (2,2,6-trimethylcyclohexane-1,4-dione). The enzyme catalyzing this stereospecific conversion was identified as one of the Old-Yellow-Enzyme (OYE) family proteins[1]. This is the first application of OYE to the production of chiral compounds.

Carbonyl reductase of *Corynebacterium aquaticum*, which catalyzed the asymmetric reduction of (6*R*)-levodione to (4*R*,6*R*)-actinol (4-hydroxy-2,2,6-trimethylcyclohexanone), was also found. The enzyme, levodione reductase, was highly activated by monovalent cations, and shown to belong to the short-chain alcohol dehydrogenase/reductase family[2, 3].

By combination of these two reductases, enzymatic production of doubly chiral compound, (4*R*,6*R*)-actinol, which is a useful chiral intermediate for the synthesis of naturally--occurring optically active compounds such as zeaxanthin and xanthoxin, from prochiral compound, ketoisophorone, was established[4].



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P129 ENANTIOSELECTIVE ENZYMATIC AND CHEMICAL HYDROLYSIS OF *sec*-ALKYL SULFATE ESTERS

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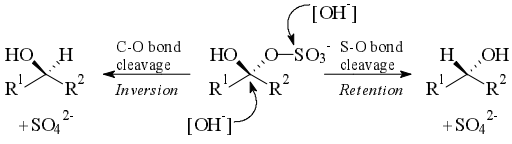
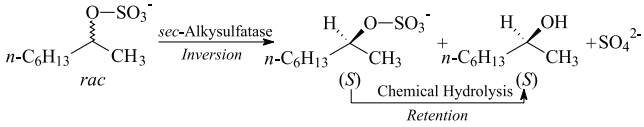
Keywords: *sec*-alkylsulfatase, enantioselective hydrolysis, enantioconvergent process

Sulfatases catalyze the hydrolytic cleavage of the sulfate ester bond[1] *via* retention or inversion of configuration[2, 3] depending on the type of enzyme.

Using a bacterial alkylsulfatase from *Rhodococcus ruber* DSM 44541, biohydrolysis of *sec*-alkyl sulfate esters proceeds in an enantioselective fashion *via* inversion of configuration[4]. Thus, a homochiral product mixture can be obtained from a racemate. In order to gain a single enantiomeric pure *sec*-alcohol product, the remaining *(S)*-sulfate ester has to be hydrolyzed under retention of configuration at the chiral carbon atom.





By combining the two independent selective reactions proceeding through opposite stereochemical pathways, an enantioconvergent process can be achieved[5, 6].

This work was performed within the Spezialforschungsbereich Biokatalyse (project # F-115) and financial support by Degussa AG (Frankfurt) and the FWF (Vienna) is gratefully acknowledged.

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P130 LACCASE-NITROXYL RADICAL CATALYZED OXIDATION OF ALCOHOLS: MECHANISTIC INVESTIGATIONS

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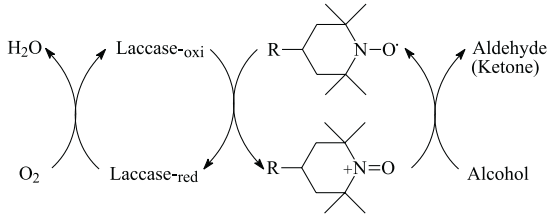
Keywords: laccase, TEMPO, alcohol oxidation

Laccase-mediator systems that catalyze oxidation of alcohols have drawn increasing attention in organic synthesis. Nitroxyl radical 2,2,6,6-tetramethylpiperidinyloxy (TEMPO) was shown to be the most effective mediator of laccase catalyzed oxidation of alcohols[1, 2]. It seems likely that oxoammonium ions, which can be formed in-situ, are the actual oxidants. Disadvantage of the laccase-TEMPO system are the long reaction time and the large amounts of TEMPO (up to 30 mol %) required.

In order to understand and optimize the system, we have performed mechanistic investigations on the fungal laccase (from *Coriolus versicolor*) catalyzed oxidation of alcohols in the presence of TEMPO and its derivatives. No oxidation took place in the absence of either TEMPO, laccase, or oxy-

gen. Results obtained from kinetic isotope studies and reaction kinetics underline the intermediacy of oxoammonium ions. One of the problems we identified is that the oxoammonium, formed via oxidation of TEMPO, is unstable in acidic acetate buffer. A way to address this problem is the use of two-phase systems. We will present our results on the use of laccase-TEMPO in two phase systems.



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P131 OXIDATIVE COUPLING OF NATURAL PHENOL DERIVATIVES CATALYZED BY LACCASES

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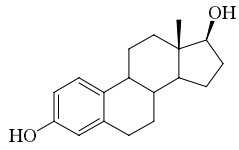
Keywords: laccase, oxidoreductases, estradiol, resveratrol, catechin

Laccases (benzenediol:oxygen oxidoreductases; EC 1.10.3.2) are multicopper enzymes that catalyze the one-electron oxidation of a wide variety of substrates, i. e. phenols and aromatic amines, with the concomitant reduction of molecular oxygen to water. Recent reports describe the use of laccases for the oxidation of different classes of organic compounds using suitable "mediators" (ref.[1]). Conversely, literature data on the laccase-mediated oxidation of complex natural compounds are quite scant. For instance, 30 years ago a laccase from *Polyporus versicolor* was used for the oxidation of steroid hormones (i. e. β-estradiol **1**) in emulsion of water and organic solvents to give a mixture of isomeric dimers[2].

In the frame of our general interest in the biocatalyzed formation of carbon-carbon bonds, we are studying the performances of laccases in the oxidative coupling of natural compounds in water solution or in the presence of organic cosolvents. In this report we will present the results obtained using the laccases from *Myceliophtora thermophyla* and from *Trametes versicolor* for the oxidation of the phenolic derivatives β-estradiol **(1)**, resveratrol **(2)** and catechin **(3)**.



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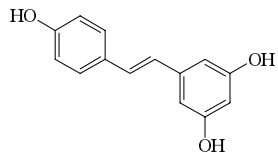

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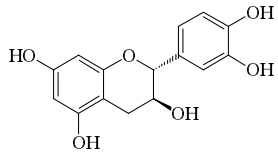

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P132 BIOCATALYSIS OF ENDOGENOUS APPLE POLYPHENOL OXIDASE IN ORGANIC SOLVENT MEDIA USING SELECTED SUBSTRATES

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Keywords: endogenous, polyphenol oxidase, phenolics, apple, biocatalysis

Enzymatic browning is catalyzed by a group of enzyme known as polyphenol oxidases (PPO; EC 1.14.18.1), which are copper-containing oxido-reductase enzymes. Although the biocatalysis of PPO has been investigated in aqueous media[1], its use as a catalyst is limited because of the instability of *o*-quinones[2]. However, the use of organic solvents has been regarded as potential reaction media for PPO, particularly, for the production of natural stable pigments of selected color intensity[3–5].

Endogenous phenolic compounds as well as PPO enzymatic extract were recovered from apple fruit. The endogenous apple PPO was enriched by acetone precipitation. The biocatalysis of enriched apple PPO in selected organic

solvent media, including hexane, heptane, dichloromethane and toluene, using model substrates, chlorogenic acid and catechin, as well as endogenous apple phenolic compounds. The optimum enzyme concentration, optimum reaction temperature, optimum pH as well as kinetics parameters such as K_m and V_{max} were investigated. The result indicated that there was a 4.7 fold increase in PPO activity, for the enriched enzymatic extract, in organic solvent media compared to that in the aqueous one. In addition, the experimental findings showed that there were significant increases in PPO activity, 4.2 to 4.5-fold and 1.5 to 2.4-fold, in the reaction media of hexane and heptane, respectively.

Using endogenous apple phenolic compounds, catechin and chlorogenic acid as substrates, the results demonstrated that the K_m values for apple PPO activity in hexane medium were 0.58, 0.76 and 0.74 mM, respectively, and those of V_{max} were 5.34×10^{-3}, 4.17×10^{-3} and 7.14×10^{-3} ΔA/μg protein/sec. Using the same substrates, the results demonstrated that the K_m values for apple PPO activity in hexane medium were 0.55, 0.44 and 1.19 mM, respectively, and those of V_{max} were 1.13×10^{-3}, 1.15×10^{-3} and 0.77×10^{-3} ΔA/μg protein/sec. The results indicated that hexane is more appropriate for the biocatalysis of PPO than heptane, with catalytic efficiency ranging from 5.48 to 9.69 compared to 0.64 to 2.61, respectively.

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P133 BIOCATALYSIS OF CHLOROPHYLLASE IN ORGANIC SOLVENT MEDIUM-CONTAINING CANOLA OIL MODEL SYSTEM

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Keywords: chlorophyllase, biocatalysis, chlorophyll, pheophytin, canola oil

The green color in immature oilseeds is due to the presence of the photosynthetic pigment chlorophyll. Oil seeds contain variable amounts of chlorophyll depending on their maturity. During extraction, there remains in the oil a proportion of the chlorophyll that is very difficult to remove by conventional bleaching methods. Oils with high chlorophyll levels require special treatment during refining to become acceptable in terms of color, free fatty acid content, peroxide value and flavor stability[1]. Theoretically, vegetable oils containing chlorophylls could be decolorized much more efficiently using chlorophyllase (chlorophyll-chlorophyllidohydrolase, EC 3.1.1.14); the enzyme catalyzes the replacement of the phytol group in chlorophyll by hydrogen atom in the chlorophyllides[2]. There is increasing interest in the biotechnological application of chlorophyllase for the removal of green pigments from edible oil, which should be a potential alternative to the conventional bleaching technique[3].

Partially purified chlorophyllase, obtained from the alga *Phaeodactylum tricornutum*, was assayed for its hydrolytic activity in an aqueous/miscible organic solvent system containing refined-bleached-deodorized (RBD) canola oil, using chlorophyll and pheophytin as substrate models. The effects of a wide range of oil contents, acetone concentrations, enzyme concentrations, chlorophyll and pheophytin concentrations, incubation temperatures and agitation speeds on the enzyme activity were investigated. The optimum reaction conditions for chlorophyllase biocatalysis were determined to consist of 20 % oil, 10 % acetone and a 200 rpm agitation speed with optimum temperatures and substrate concentrations of 35 °C and 12.6 μM for chlorophyll, and 30 °C and 9.3 μM for pheophytin. The results indicated that the presence of 10 % acetone in the reaction medium increased the hydrolytic activity of chlorophyllase by 1.5 and 1.8 times, respectively, using chlorophyll and pheophytin as substrates. However, the presence of 30 % RBD canola oil decreased the hydrolytic activity of chlorophyllase by 3.8 and 4.1 times, using chlorophyll and pheophytin as substrates, respectively. The experimental findings showed that RBD canola oil has an inhibitory effect on chlorophyllase activity, whereas acetone at low concentrations acted as an activator for the enzyme and an inhibitor at higher ones. Moreover, chlorophyllase showed a limited affinity towards pheophytin compared to that obtained with chlorophyll.

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P134 COMBINED ALKENE MONOOXYGENASE AND EPOXIDE HYDROLASE BIOCATALYSTS FOR TWO STEP TRANSFORMATIONS

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Keywords: alkene monooxygenases, epoxide hydrolase, stereospecificity

Alkene specific monooxygenases are useful biocatalysts for the production of chiral epoxides. However, the toxicity and aqueous phase lability of the accumulated epoxides can be a problem in larger scale processes. Although this may be addressed using 2 phase systems, an alternative strategy is to trap the stereocentre(s) introduced by the monooxygenase by reaction with a second enzyme, such as an epoxide hydrolase.

The stereospecificity of alkene monooxygenases usually varies with enzyme and substrate. As a long-term goal it may be feasible to tailor variants to specific substrates, based on a common expression platform. However, the two step strategy outlined above, also lends itself to improving product enantiopurity by either selective hydrolysis of one of the enantiomers or enantioconvergent hydrolysis, possibly using combinations of epoxide hydrolases.

In this poster we will present preliminary results from a project based on this two step strategy using a number of epoxide producing monooxygenases and recombinant limonene epoxide hydrolase.

P135 STYRENE MONOOXYGENASE IS A VERSATILE BIOCATALYST FOR ENANTIOSPECIFIC EPOXIDATION REACTIONS IN CELL-FREE SYSTEMS

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Keywords: styrene monooxygenase, epoxidation reactions, cell-free application, expanded bed chromatography, two-liquid phase

Styrene monooxygenase is an excellent biocatalyst for enantiospecific epoxidation reactions[1,2] and has recently been preliminary characterized[3]. Using recombinant *E. coli* expressing the two components StyA (oxygenase) and StyB (reductase) of styrene monooxygenase gram amounts of various enantiopure styrene oxide derivatives have been prepared[4].

The oxygenase component StyA was produced and purified on technical scale for preparative cell-free applications. 15 g of enriched StyA were produced from recombinant *E. coli* via expanded bed anion exchange chromatography using a DEAE® streamline™ matrix. The enzyme was obtained by one purification step with a purity up to 70 % and an overall yield of over 90 %.

Cell-free biotransformations were performed in a two-liquid phase system with dodecane as organic phase serving as reservoir for the substrate and product-sink. Formate dehydrogenase was used for the regeneration of the cofactor NADH (Fig. 1). Styrene oxide production remained stable over more than 7 hours, yielding 80 mM styrene oxide (>80 % conversion).

Overall, we have herewith established an integrated process for the preparative synthesis of various enantiopure epoxides including efficient and simple large-scale preparation of the biocatalyst.

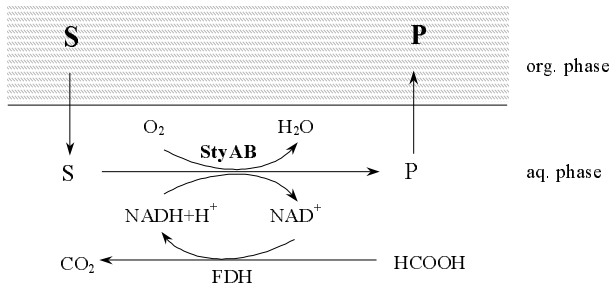

Fig. 1. **Biotransformation principle.** Application of styrene monooxygenase (StyAB) in organic/aqueous emulsions with regeneration of the cofactor NADH from NAD^+ by formate dehydrogenase (FDH). Substrate (S) is continuously supplied via the organic phase and converted in the aqueous phase to the product (P).

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P136 2-HYDROXYBIPHENYL 3-MONOOXYGENASE: LARGE SCALE PREPARATION AND CELL FREE APPLICATION IN EMULSIONS THE APPLICATION OF CROSS-LINKED ENZYME PRECIPITATES (CLEPS)

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Keywords: biotransformation, biphasic catalysis, cofactor, enzyme catalysis, oxidoreductase

Catalysis of specific hydroxylation reactions of organic compounds is difficult by chemical means. Therefore the interest in biocatalysts catalyzing specific hydroxylations has grown in recent years simultaneous to the interest in the application of oxygenases. As oxygenases require expensive cofactors, usually NAD(P)H, in stoichiometric amounts, these enzymes are mostly used in whole cell biotransformations[1,2]. However, independent optimization of enzyme production and biotransformation reactions can better be achieved by *in vitro* approaches.

The soluble and NADH dependent flavoprotein 2-hydroxybiphenyl 3-monooxygenase (HbpA) from *Pseudomonas azelaica* HBP1 is catalyzing the hydroxylation of *ortho*-substituted phenols to corresponding 3-substituted catechols with absolute regiospecificity[3]. HbpA was partially purified on large scale by expanded bed adsorption (EBA) chromatography[4].

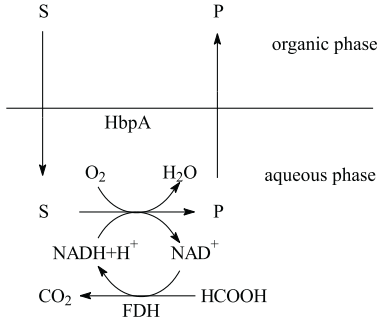

Fig. 1. **Reaction cycle catalysed by HbpA and FDH in aqueous-organic two phase systems**

Together with the commercially available cofactor regeneration enzyme formate dehydrogenase (FDH) both enzymes were precipitated and cross-linked. These cross-linked enzyme precipitates (CLEPs) were applied for the biotransformation of 2-hydroxybiphenyl to 2,3-dihydroxybiphenyl in aqueous-organic two phase systems (Fig. 1). A productivity of 0.18 $g.l^{-1}.h^{-1}$) could be maintained over 9 hours.

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P137 BIOCATALYST DEVELOPMENT FOR THE PRODUCTION OF *cis*-DIOLS USING CHLOROBENZENE DIOXYGENASE

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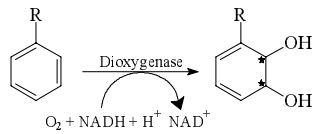
Keywords: chlorobenzene dioxygenase, *cis*-diols, biotransformation, scale-up

Aromatic ring dioxygenases are multicomponent enzyme systems that add molecular oxygen to the aromatic nucleus to form arene *cis*-diols (Scheme 1). The broad substrate range and the high regio- and enantioselectivities of dioxygenases make these enzymes useful not only for bioremediation, but also for biocatalytic synthesis of chiral synthons for the production of biologically active chemicals and pharmaceuticals[1].



Scheme 1. **Oxidation of aromatic compounds *via* dioxygenases**

Chlorobenzene dioxygenase (CDO) of *Pseudomonas* sp. P51 consists of 3 components, NADH-ferredoxin reductase, ferredoxin, and terminal oxygenase component, which is composed of a small and a large subunit[2]. It was shown that CDO can oxidize different classes of aromatic compounds to the corresponding *cis*-dihydrodiols[3]. Powerful and stable expression system is required to develop an efficient process for the production of *cis*-dihydrodiols. Therefore we constructed a new expression system pTEZ30 harboring the CDO genes under strict control of the P*alk* promoter which is derived

from *Pseudomonas oleovorans* GPo1 and confers kanamycin resistance. We also showed the potential of this new expression system for efficient synthesis of chlorobenzene dioxygenase for the scale-up of *cis*-dihydrodiol production.

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P138 SCREENING AND OPTIMISATION OF COMMERCIAL ENZYMES FOR THE ENANTIOSELECTIVE HYDROLYSIS OF *(R,S)*-NAPROXEN ESTER

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Keywords: lipase, esterase, naproxen, biocatalysis, hydrolysis, optimisation

The purpose of this study was the identification of suitable lipase or esterase for enantiomeric resolution of *(R,S)*-naproxen.

The aim was to find an enzyme that yields *(S)*-naproxen with an enantiomeric excess of more than 98 %, an enantiomeric ratio *(E)* of greater than 100, and substrate conversion in excess of 40 %. Commercially available enzymes were screened, and selected for optimisation of enantioselectivity through statistically designed experiments on the reaction conditions.

Optimisation efforts resulted in a more than 20-fold improvement of activity, while the excellent enantioselectivity of the enzymes was maintained. In particular, the addition of PEG 1000 as a co-solvent improved conversion rates 10-fold.

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P139 ASYMMETRIC TRANSFORMATION OF ENOL ACETATES WITH ESTERASES FROM *Marchantia polymorpha*

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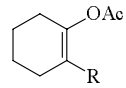
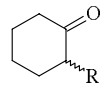
Keywords: esterase, enol acetate, asymmetric hydrolysis, chiral ketone

Optically active α-substituted ketone derivatives are widely employed as chiral synthons in asymmetric syntheses. Recently, it has been found that yeast[1,2] and cultured cells of *M. polymorpha*[3] were capable of performing the hydrolysis of α-alkylated cyclohexanone enol esters to give α-substituted chiral ketones. We have now investigated the enzymes which are able to catalyze the asymmetric hydrolysis of enol acetates.

A Butyl-Toyopearl column chromatography of the crude enzyme preparation gave a good separation of the two different esterases. Further purification by chromatography on a Diethylaminoethyl-Toyopearl column and then a Sephadex G-75 column gave homogeneous esterases: Esterase I, molecular mass ca 54000, dimeric form composed of two identical subunits; Esterase II, molecular mass ca 45000, dimeric form composed of two identical subunits. The amino termini of these esterases were blocked. The esterases, therefore, were digested with protease and the internal amino acid sequences of the peptide fragments obtained from Esterase I were not similar to those of any hydrolytic enzymes.



1: R = Me
2: R = Et
3: R = *n*-Pr
4: R = *n*-Pnt
5: R = Bnz



6: R = Me
7: R = Et
8: R = *n*-Pr
9: R = *n*-Pnt
10: R = Bnz

Several cyclohexanone enol acetates **(1-5)** were subjected to enzymatic hydrolysis with these esterases to clarify the effect of various substituents at β-position to the acetoxyl group on the enantiomeric ratio and the catalytic activity of enzymes. As shown in Table I, hydrolysis of enol acetates, **1** and **2**, by Esterase I gave the corresponding optically active ketones (**6** and **7**). The chiral preference of Esterase I was re-

tained among these substrates: the protonation of the enol intermediates from **1** and **2** occurred preferentially from the same enantiotopic face of the C-C double bond. However, when *n*-propyl, *n*-pentyl and benzyl groups were introduced into the β-position to the acetoxyl group of the substrates **(3-5)** , the corresponding products having opposite configuration were obtained. This result indicates that the stereoselectivity of Esterase I in the protonation of these enol intermediates is reversed by long chain (C > 3) and bulky substituents at the β-position to the acetoxyl group. On the other hand, the conversion yield and enantiomeric purity in the hydrolysis of enol acetates **(1-5)** with Esterase II are very low in comparison to the case of Esterase I. However, the stereoselectivity in the hydrolysis with Esterase II was opposite to that with Esterase I.

Table I
Enantioselectivity in the hydrolysis of enol acetates by Esterases I and II

Substrate	Product	Esterase I			Esterase II		
		Conv. (%)	e. e.	Config.[a]	Conv. (%)	e. e.	Config.[a]
1	**6**	>99	>99	*S*	4	4	*R*
2	**7**	>99	14	*S*	3	2	*R*
3	**8**	>99	>99	*R*	5	4	*S*
4	**9**	20	26	*R*	16	7	*S*
5	**10**	15	>99	*S*	11	14	*R*

[a]Preferred configuration at the α-position to the carbonyl group of the products

Thus, two hydrolytic enzymes were isolated from cultured cells of *M. polymorpha* and were confirmed to be capable of discriminating the enantiotopic face of the C-C double bond of the enol intermediate in the hydrolysis. The enantioselectivities in the protonation of the enol intermediate were opposite between these enzymes. The enantioselectivity of both enzymes reversed in the hydrolysis of the substrates with long side chain and bulky benzyl group at the α-position of enol acetates, compared with the substrates having short side chains.

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P140 ENZYME-MEDIATED ENANTIOSELECTIVE HYDROLYSIS OF PEG-TAGGED CARBONATES

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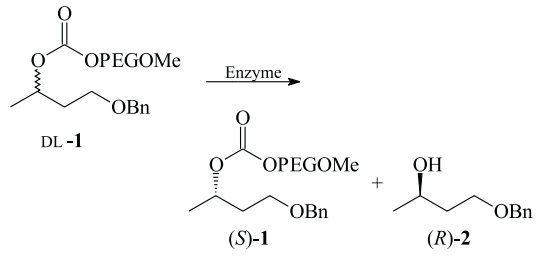
Keywords: enzymatic hydrolysis, PEG, PPL, esterase

In the enzymatic hydrolysis of racemic acyl compounds, usual work-up procedures including the separation of the mixture into the remaining substrate and the resulting alcohol spend a lot of time and waste much amount of solvents. In order to avoid the tedious steps, we have developed a new type of the enzymatic hydrolysis of PEG-tagged carbonates as the unique substrates. Because of the amphiphilic character, the reaction could be easily monitored by TLC and NMR analysis and the purification steps could be simple.

A MeOPEG (Mw = 750)-tagged carbonate DL-**1** was used as the substrate of the screening test. Amongst the 12 commercially available enzymes, some esterase and PPL hydrolyzed the carbonate **1** to give the corresponding alcohol **2**. Finally, PPL was chosen as the best enzyme. As expected, the recovered substrate *(S)*-**1** was easily separated from the resulting alcohol *(R)*-**2** by using the minimum amount of silica gel and organic solvents. In the reaction of the substrate supported with MeOPEG (Mw = 550) for 24 h at 10 °C, the conversion and *E* value were 0.10 and 32, respectively.



P141 *T. reesei* ACETYL ESTERASE CATALYZED TRANSESTERIFICATION IN WATER

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Keywords: esterases, transesterification, acylation, *Trichoderma reesei*

Hydrolases are group of enzymes catalyzing hydrolytic reactions. Introducing of organic reaction media has made it possible to use these enzymes efficiently for reversed hydrolytic reactions and transferase-type reactions as well[1]. Water in these organic systems is often believed to shift equilibrium in favor to hydrolysis[2].

Contrary to numerous studies proving essential importance of low or minimal water content in such reactions[3,4], partially purified *Trichoderma reesei* RUT-C30 acetyl esterase preparation was found to catalyze acyl transfer reactions not only in organic solvents, but also in mixtures of organic solvents/water and even in water. Using different acyl donors, the best results were obtained using vinyl acetate. As acetyl acceptors, variety of hydroxyl bearing compounds in their water solutions were used. Conversion and number of newly formed acetates varied according acceptor used. Conversions over 50 % were observed by majority of most common monosaccharides, their methyl and deoxy derivatives and oligosaccharides. In several cases, *T. reesei* acetyl esterase catalyzed transesterification exhibited strict regioselectivity, leading to only one acetyl derivative. Preparative potential of described enzymic transesterification in water was demonstrated by transacetylation of methyl β-D-glucopyranoside and *p*-nitrophenyl β-D-glucopyranoside, yielding 56.4 % of methyl 3-*O*-acetyl β-D-glucopyranoside and 70.2 % *p*-nitrophenyl 3-*O*-acetyl β-D-glucopyranoside as the only products of the reactions.

This new enzymaticaly catalyzed transacetylation in water opens new area in chemoenzymatic synthesis. Its major advantages are easy and regioselective esterifying of polar compounds, high yields, low enzyme consumption and elimination of use of toxic organic solvents.

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P142 PRELIMINARY X-RAY CRYSTAL STRUCTURE INFORMATION OF AN ESTERASE FROM *Pseudomonas fluorescens*

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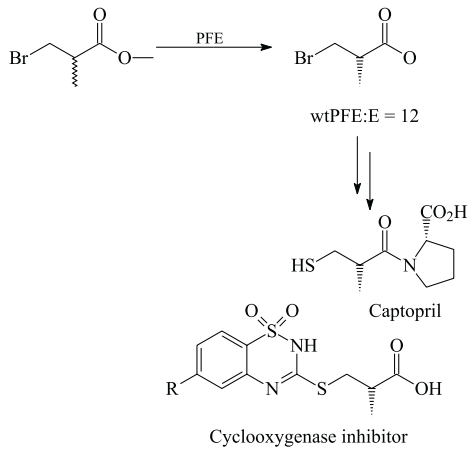
Keywords: X-ray structure, *Pseudomonas fluorescens* esterase, enantioselectivity

An aryl esterase from *Pseudomonas fluorescens*[1] has been employed in the resolution of 3-bromo-2-methyl-propionic acid methyl ester, an important building block in the synthesis of Captopril and a potent cyclooxygenase inhibitor[2] (Figure). Significant improvements to the enantioselectivity seen by the wild type enzyme have been made by site directed mutagenesis. The preliminary X-ray crystal structure of both the wild type and several mutants of interest have been elucidated. The wild type enzyme as well as mutants crystallize in high $(NH_4)_2SO_4$ concentrations as a dimer and belong to the R3 space group. The unit cell has dimensions of 147.5 Å × × 147.5 Å × 131.9 Å with angles of $\alpha = \beta = 90°$, $\gamma = 120°$. Active site analysis of the crystal structure of the wild type and of mutants will hopefully lead to understanding of the origins of improvements in enantioselectivity.



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P143 ENANTIOPURE SULFINAMIDES VIA SUBTILISIN-CATALYZED KINETIC RESOLUTION OF *N*-ACYLSULFINAMIDES

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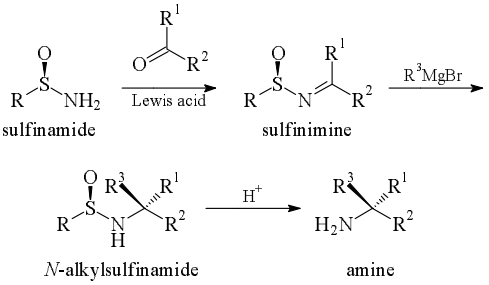
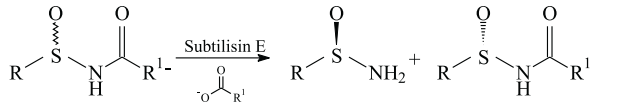
Keywords: sulfinamides, subtilisin, enantioselective, chiral auxiliary

Sulfoxides have been widely used as chiral auxiliaries in organic synthesis[1]. Some of the most interesting new sulfoxide auxiliaries are the sulfinimines (*N*-sulfinyl imines), which are chiral auxiliaries for the imine group and used for the synthesis of amines and related compounds[2]. The simplest route to prepare a sulfinimine is by the direct condensation of an aldehyde or ketone with an enantiopure sulfinamide (Scheme 1). Therefore the utility of these chiral auxiliaries is dependent upon concise methods of sulfinamide preparation[3].



Scheme 1. **Stereoselective synthesis of an amine from enantiopure sulfinamide**

Through our continuing efforts to design new methods to enantiopure materials, we have developed a novel enzyme-catalyzed route to enantiomerically pure sulfinamides. Subtilisin E (bacterial serine protease) catalyzes the hydrolysis of various *N*-chloroacetyl- and *N*-hydrocinnamoyl-sulfinamides with very high enantioselectivity ($E > 150$) toward the *(R)*-enantiomer (Scheme 2). Our results show subtilisin E does not favour the enantiomer predicted with empirical rules for proteases[4]. Through substrate engineering and molecular modelling experiments we have revealed the molecular basis for the acyl group selectivity and reverse enantioselectivity demonstrated by subtilisin E. Experiments suggest the acyl group is important for substrate binding and the enantioselectivity arises because the slow-reacting *(S)*-enantiomer binds in a nonproductive orientation. Large-scale resolution experiments of *N*-chloroacetyl-2-mesitylenesulfinamide and *N*-hydrocinnamoyl-*p*-chlorobenzenesulfinamide with subtilisin E gave their corresponding *(R)*-sulfinamides in high yield and enantiomeric excess. As well, the enzyme demonstrates a wide acceptance of aryl sulfinamide substrates and has been used to resolve several sulfinamides that were previously unavailable.



Scheme 2. **Subtilisin E-catalyzed resolution of *N*-acylsulfinamides**

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P144 UTILIZATION OF MICROBIAL PROTEASES FOR PEPTIDE SYNTHESIS IN ORGANIC MEDIA

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Keywords: *Aspergillus* proteases, *Bacillus* proteases, carbamoylmethyl ester, pH effect, protease-catalyzed peptide synthesis

Microbial proteases from a variety of sources are commercially available. Although they possess potential for organic syntheses, they have hitherto been employed mainly for food processing and as detergent ingredients. Protease-catalyzed peptide synthesis has many advantages over chemical methods, while narrow substrate specificity and the secon-

dary hydrolysis of a growing peptide are counted as major drawbacks. We have recently reported on the broadening of substrate tolerance of a mammalian protease α-chymotrypsin by using such activated esters as the carbamoylmethyl (Cam) ester as an acyl donor in the so-called kinetically controlled peptide bond formation[1]. As a part of our continuing research on the effective use of microbial proteases for organic syntheses, we have investigated peptide bond formation using some *Aspergillus* and *Bacillus* proteases. We first examined proteases from *A. melleus* (Amano protease P) and *A. oryzae* (Amano protease A). A series of *Z*-amino acid Cam esters were allowed to react with amino acid amides. With these *Aspergillus* proteases, aimed-at peptides were obtained generally in rather high yields in the mixed solvent of 1,1,1,3,3,3--hexafluoro-2-propanol and DMF (1 : 1, v/v)[2]. We next examined *B. licheniformis* protease (subtilisin Carlsberg). Couplings were carried out in anhydrous acetonitrile in the presence of the immobilized protease on Celite. We observed that the peptide yield changed significantly upon the pH of the buffer solution from which the immobilized protease was prepared; the maximal peptide yield was obtained with the preparation from pH 10.7 (ref.[3]). With the Cam ester as the acyl donor, D-amino acid amides were as good amine nucleophiles as the L-counterparts (Table I).

Table I
B. licheniformis protease-catalyzed couplings of *Z*-L-Ala--OCam with Xbb-NH_2 (after 4 h)

Xbb	Peptide (%)	Xbb	Peptide (%)
L-Ala	95.4	D-Ala	97.1
L-Leu	93.8	D-Leu	87.7
L-Phe	79.9	D-Phe	91.1

A series of dipeptide syntheses and several segment condensations including the synthesis of the Leu-enkephalin sequence were achieved generally in high yields. We examined also *Bacillus subtilis* protease (Amano protease N) as a catalyst for both kinetically and thermodynamically controlled peptide syntheses in acetonitrile with low water content. In several cases, the latter approach using carboxyl components bearing a free carboxyl group proved to be superior to the former.

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P145 TRYPSIN ASSISTED SEMISYNTHESIS OF HUMAN INSULIN ANALOGUES

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Keywords: desoctapeptideinsulin, trypsin, semisynthesis, opioid peptides, aminopeptidase substrates

Hundreds of analogues of Human insulin have been prepared by trypsin assisted semisynthesis, using the large insulin fragment – desoctapeptide insulin- and a series of synthetic analogues of the B^{23}–B^{30} sequence of the insulin peptide chain. Some of them were highly potent in tests *in vitro* (glucose transport, thymidine incorporation into DNA), nevertheless their potency *in vivo* was not higher than that of natural insulins. The same approach was recently used for the semisynthesis of another class of insulin derivatives.

We used desoctapeptide insulin for the trypsin catalyzed semisynthesis of peptide hormonogens of opioid peptides and masked substrates of aminopeptidases[1, 2]. Both groups of compounds were characterized by analytical RP-HPLC, mass spectrometry, capillary electrophoresis and by metabolic stability assays.

Supported by grants of Ministry of Industry and Trade of the Czech Republic (PZ-22-32, 1997-2000), Ministry of Education of the Czech and Slovak Republics (73/191, 2002-3) and by the Grant Agency of the Academy of Sciences of the Czech Republic (S 40553303, 2003).

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P146 DISCOVERY OF A NOVEL HEME-CONTAINING LYASE, PHENYLACETALDOXIME DEHYDRATASE, FROM MICROORGANISMS AND ITS APPLICATION TO ORGANIC SYNTHESIS

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Keywords: aldoxime, nitrile, dehydratase, metabolism, microorganisms

Dehydration of aldoximes, which are easily prepared from aldehydes and hydroxylamine, is a useful method for synthesizing nitriles, although many of the chemical procedures require harsh reaction conditions. In plants, aldoximes are considered to be intermediates in the biosynthesis of certain biologically active compounds such as indoleacetic acid, cyanogenic glucosides, and glucosinolates. However, very little is known about the aldoxime degrading enzyme: it has never been purified to homogeneity nor characterized in detail. We focused on microbial aldoxime metabolism not only to apply the enzyme to organic synthesis, but also to study possible relationships between aldoxime-dehydration and nitrile-degrading enzymes and their functions in microorganisms (Fig. 1).

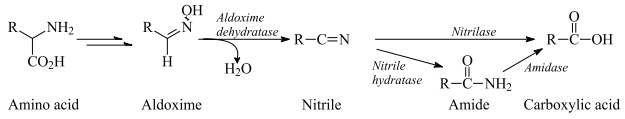

Fig. 1. **Aldoxime-nitrile pathway in microorganisms**

We screened for aldoxime-degrading microorganisms from soil and isolated *Bacillus* sp. OxB-1 (ref.[1]) and *Rhodococcus* sp. YH3-3 (ref.[2]), which degrades *Z*-phenylacetaldoxime (*Z*-PAOx) and *E*-pyridine-3-aldoxime (*E*-PyOx), respectively, by an acclimation culture technique. They metabolized aldoximes through nitriles into the corresponding carboxylic acid by a combination of a novel aldoxime dehydratase and nitrile-hydrolyzing enzymes[1, 2]. By using the dehydratase, the enzymatic synthesis of nitriles from aldoximes under mild conditions (pH 7–8, 30 °C) were achieved for the first time[3, 4].

We purified the enzyme from *Bacillus* sp. OxB-1 and studied its enzymological properties[5]. The enzyme, named "PAOx dehydratase (Oxd, EC 4.2.1.-)", is quite unique because it catalyzes a simple dehydration reaction, yet contains heme *b* as a prosthetic group and requires FMN or SO_3^{2-} as an electron acceptor. The gene *(oxd)* coding for the enzyme was shown to be linked with the gene *(nit)* of a nitrilase, which participates in aldoxime metabolism in the organism[5]. The enzyme was expressed in a soluble and active form by the recombinant *E. coli* under the control of *lac* promoter in the pUC18 vector[6]. The production of active enzyme was markedly enhanced by increasing the volume of culture medium (1 liter in 2-liter flask). Under optimized conditions, the enzyme was produced in an active and soluble form at 15,000 U per liter of culture, which is about 1,500-fold higher than the amount produced by the wild-type strain. Moreover, the enzyme comprised over 40 % of total extractable cellular protein. The overproduced Oxd was useful for the high-yield synthesis of nitriles from aldoximes[4].

We also investigated the distribution of aldoxime dehydratase and the relationship between aldoxime-degrading enzymes and nitrile-degrading enzymes among microorganisms[7, 8]. All the aldoxime degraders possessed nitrile-hydrolyzing activities. On the other hand, all of the nitrile-degraders described in the literature possessed aldoxime-degrading activities. Thus, we showed the co-existence of aldoxime dehydratase and nitrile degrading enzymes and elucidated the role of nitrile degrading enzymes in aldoxime metabolism in microorganisms, permitting us to postulate a novel "aldoxime-nitrile pathway" (Fig. 1)[9].

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P147 NEW CHROMOGENIC SUBSTRATES OF PARAOXONASE (PON1)

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Keywords: paraoxonase, arylesterase, chromogenic substrate

The study of lipid peroxidation is a rapidly growing field in medicine and biology, spurred by increasing evidence that lipid oxidation is involved in the pathogenesis of many chronic diseases, e. g. atherosclerosis and aging[1,2]. Paraoxonase (PON1) is a high density lipoprotein (HDL)-associated serum enzyme with peroxidase activity[3]. It was found to protect low density lipoproteins (LDL) from oxidative modifications by hydrolyzing phospholipid hydroperoxides.

PON1 was first discovered for its role in detoxifying organophosphates and its name reflects its ability to hydrolyze paraoxon, a metabolite of the insecticide parathion. Although it was then classified as aryldialkylphosphatase PON1 can also hydrolyze aromatic carboxylic esters[4].

Thus the understanding of the properties of this enzyme which have both physiological and toxicological importance is the subject of extensive research. Herein we present the screening of synthetic chromogenic substrates for monitoring the arylesterase-like activity of PON1.

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P148 A FLUOROGENIC ASSAY FOR TRANSKETOLASE FROM *Saccharomyces cerevisiae*

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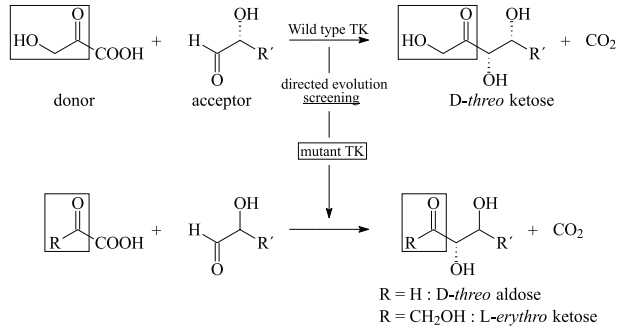
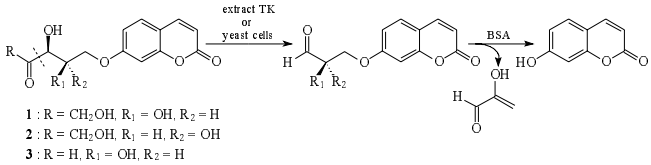
Keywords: transketolase, enzyme catalysis, fluorescence spectroscopy

Saccharomyces cerevisiae recombinant transketolase (TK)[1] is a useful catalyst for ketose synthesis due to the stereo-controlled formation of the C3–C4 bond. It catalyzes the transfer of a ketol unit from β-hydroxypyruvic acid to an aldehyde to generate D-*threo* (3*S*,4*R*) ketose. The decarboxylation of this donor substrate and subsequent loss of carbon dioxide make the overall condensation reaction irreversible. A wide range of aldehydes as ketol acceptors has been used for the obtention of various ketoses[2–4]. The enzyme appears highly specific for ketol donor substrates and for hydroxyaldehyde substrates with the *(R)* configuration.



In order to modify these TK properties, we examined the possibility of altering the substrate specificity of this enzyme by directed evolution. We are interested in obtaining variants of TK able to accept glyoxylate as donor substrate to obtain D-*threo* aldoses or able to accept *(S)*-hydroxyaldehyde substrates to obtain L-*erythro* ketoses.

An efficient screening or selection system is an absolute prerequisite for identifying the evolved enzyme variants that display improved properties. Here we report an assay allowing us to detect TK activities *in vitro* and *in vivo* by fluorescence in solution according to the Reymond's test[5]. We examined the use of a fluorogenic substrate 1 of wild type TK, which is itself non fluorescent but release a fluorescent product umbelliferon[6]. This assay could be used to screen mutants with the appropriate fluorogenic substrates 2 or 3.



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P149 OPTIMIZATION OF SPECTROPHOTOMETRIC METHOD SUITABLE FOR ASSESSING PRIMARY AMINO GROUPS IN DAIRY PROTEINS AND MONITORING COURSE OF ENZYMATIC HYDROLYSIS

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Keywords: *o*-phtaldialdehyde, hydrolysis, whey proteins

Another use of whey proteins in food chemistry is highly requested. One of the best known methods of modifying whey proteins is enzymatic hydrolysis by which is possible to produce a wide range of hydrolysates with desired properties available furthermore for producing functional foods. Spectrophotometric determination of primary amino groups based on reaction *o*-phtaldialdehyde (OPA) reagent with primary amino groups in presence of thiol group is widely used in analysis of amino acids, peptides and proteins and by the use of this method is possible to monitore the course of enzymatic hydrolysis and its degree, respectively. Methods published in the past usually had some disadvantages (e. g. instability of 1-alkyl-2-alkylisoindols emerging during reaction), therefore new modified recipe of reactant mixture of OPA and *N*-acetyl-L-cysteine was suggested and proved[1]. This mixture gives stable light absorbing product emerging during the reaction and the reaction provides more precise results and saves chemicals. Another advantage of this method is substitution of bad smelling 2-mercaptoethanol by *N*-acetyl--L-cysteine like a donor of thiol group. Several amino acids (Arg, Glu, Gly, His, Lys, Pro and standard equimolar solution; in different concentrations), whey protein hydrolysates and urea were chosen and response to the reaction with OPA were investigated. The reaction worked under laboratory conditions, absorbance was measured at λ = 335 nm (absorbance maximum of 1-alkylthio-2-akylisoindols) after 2 min. The product of reaction was stable at least 90 min. The value of ε for all measured amino acids in water solutions varied between 6700–7300 $cm^{-1}.l.mol^{-1}$ except Lys (double response becouse of 2 amino groups in structure) and Pro (no reaction). Calibration curves were linear in range 0.0–0.35 $mmol.l^{-1}$ of amino acids in water solution. Also the influnce of storage conditions to stability of OPA reagent were investigated. These experiments showed that storage of OPA reagent in refrigerator provides more stability (at least 11 days) and more economic application.

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P150 PRODUCTION OF BENZALDEHYDE FROM PHENYLALANINE CATALYSED BY A BIENZYMATIC OXIDASE-PEROXIDASE SYSTEM

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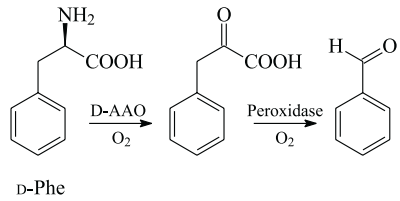
Keywords: aroma, peroxidase, amino acid oxidase, benzaldehyde, phenylalanine

Benzaldehyde is the major component of bitter almond aroma. It is the second most important flavour molecule (after vanillin), and is used as a key ingredient in cherry and other natural fruit flavours. Benzaldehyde is produced on an industrial scale by oxidation of aromatic hydrocarbons. There is however an increasing preference of consumers for "natural" food additives[1]. Thus, natural benzaldehyde is mainly extracted or processed from essential oils of higher plants. Biotechnology can represent an alternative: several publications report the biotransformation of phenylalanine to benzaldehyde by cultures of either moulds or microorganisms[2]. Phenylalanine can also be converted to benzaldehyde by cell free extracts[3]. In both cases, yields are low, and benzaldehyde has to be purified from complex mixtures of by-products.

We report a new bioconversion of D-phenylalanine to benzaldehyde catalysed by two coupled commercially available pure enzymes.



D-Phe

D-Phe is first oxidised by a D-aminoacid oxidase (immobilised, from *Trigonopsis variabilis*, Fluka*)* to phenypyruvic acid, which is converted *in situ* to benzaldehyde by a peroxidase (from *Coprinus cinereus*, Novozymes). After extraction from the aqueous reaction mixture, the produced benzaldehyde appears to be pure by GC and ^{1}H NMR. Yield borders 35 %. Racemic phenylalanine can be used as well with the same yield (relatively to D-Phe). Starting from natural L-phenylalanine implies that the amino acid is first racemised by a classical treatment with acetic anhydride and aqueous sulphuric acid. The crude DL-Phe prepared by this method is also a satisfactory substrate of DAO.

Experimental conditions and studies on mechanism of the transformation will be discussed.

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P151 ENZYMATIC SYNTHESIS OF NATURAL VANILLIN

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Keywords: vanillin, vanillyl-alcohol oxidase, mutants, characterization

Vanillin (4-hydroxy-3-methoxybenzaldehyde) is a widely used flavour compound in food and personal products. Vanillin is mainly produced by chemical synthesis and less than 1 % of its total demand is obtained from the curing of the beans of the orchid *Vanilla planifolia*.

With the increasing interest in natural products alternative processes are being developed to produce natural vanillin. Among others, one of the approaches includes the use of isolated enzymes. In an earlier study[1], we showed that the flavoprotein vanillyl-alcohol oxidase (VAO) can be used for the two-step enzymatic synthesis of vanillin from the natural precursor creosol (2-methoxy-*p*-cresol). Although creosol is a very poor substrate for VAO, protein structural analysis did not reveal any obvious reason for this poor reactivity[2]. Therefore, we started an error-prone PCR based random mutagenesis approach screening for VAO variants with improved creosol reactivity. After a single round of mutagenesis, four single-point mutants were selected for a detailed structural and functional characterization. The evolved VAO variants displayed remarkable substrate specificities, being active with creosol but not with cresol. The enhanced reactivity with creosol is ascribed to the destabilization of an abortive covalent adduct between the substrate and the FAD cofactor[1]. All mutated residues are located in loop regions outside the active site and their replacements do not cause any significant structural perturbation. This suggests that the changes in substrate specificity are caused by dynamic coupled motions which cannot easily be directed by rational redesign.

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P152 CHEMOENZYMATIC ASYMMETRIC TOTAL SYNTHESIS OF AN AROMA CONSTITUENT OF JAMAICAN RUM AND OF (+)-PESTALOTIN

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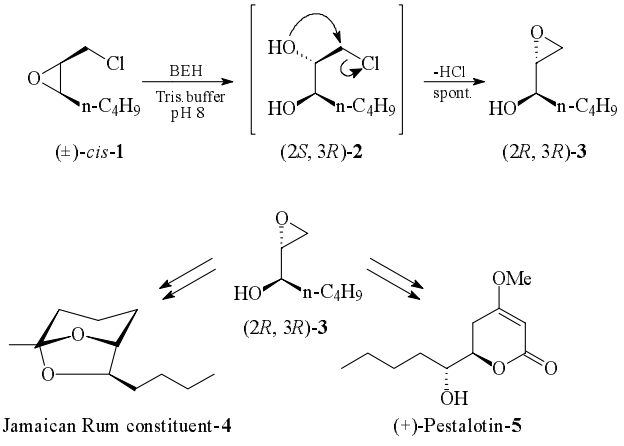
Keywords: bacterial epoxide hydrolases, enantioconvergent, cascade-reaction

A powerful method to obtain enantiopure epoxides or vicinal diols makes use of bacterial epoxide hydrolases (BEH)[1]. This approach was used for a short chemoenzymatic synthesis of two natural products – an aroma component of Jamaican Rum **4** and the gibberelin synergist (+)-pestalotin **5**.

Key step of the synthesis consists of an asymmetric enzymatic hydrolysis of halomethyl oxirane (±)-*cis*-**1** with BEH to furnish the corresponding diol (2*S*, 3*R*)-**2**. The latter spontaneously undergoes an intramolecular *exo-tet*-cyclization[2] ("Payne-type-rearrangement") to give epoxyalcohol (2*R*, 3*R*)-**3** in good optical purity and yield. Overall, this sequence represents an enzyme-catalyzed cascade-reaction. Since the biohydrolysis proceeds in an enantioconvergent fashion, the occurrence of an unwanted stereoisomer was avoided[3, 4]. Epoxy alcohol **3** served as the central building block for the synthesis of **4** and **5**.



This work was performed within the Spezialforschungsbereich Biokatalyse (project # F-104) and financial support by the FWF (Vienna) is gratefully acknowledged.

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P153 ENZYME-GENERATED RADICALS AS A TOOL TO PRODUCE NATURAL COMPOUNDS. THE CASE OF THE PRODUCTION OF CAROTENOID-DERIVED AROMA COMPOUNDS

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Keywords: radicals, β-carotene, β-ionone, xanthine oxidase

The production of natural compounds requires “natural” processes, i. e. processes in which the substrate, catalysis and treatments are “natural”. The use of radical mediated reactions seems thus difficult to carry out in this frame. However, some enzymes are able to generate radical species. It is the case with xanthine oxidase, an enzyme taken from membrane of milk fatty globules.

We have investigated the possibility of modulating the synthesis of radical species in modifying the substrate of the enzyme and monitored this effect on the degradation of β-carotene and the synthesis of β-ionone. By using various aldehydes and xanthine, it was possible to generate the superoxide anion (from xanthine) but also alkyl radicals (from butanal) and oxygenated radicals (from acetaldehyde) that exhibited various effects on the degradation. With the superoxide anion, the reaction was occurring at the same rate as autooxidation whereas the two aldehydes had a strong effect on the rate of degradation of carotene and also of ionone.

P154 EFFECT OF CULTURE MEDIUM COMPOSITION ON THE BIOGENESIS OF THE NATURAL FLAVOR 1-OCTEN-3-OL BY *P. camemberti*

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Keywords: bioconversion, *P. camemberti*, lipoxygenase, hydroperoxide lyase, 1-octen-3-ol

The production of certain natural flavor compounds, including C5 to C9 aliphatic alcohols and carbonyl compounds, results from a biosynthetic pathway involving several enzymatic activities, in particular lipoxygenase and hydroperoxide lyase, and lipid components as substrates. This biosynthetic pathway is initiated by the oxygenation of PUFAs possessing a 1*(Z)*, 4*(Z)*-pentadiene moiety to produce stereo- and regio-specific hydroperoxides (HPODs), which in their turn are converted into volatile alcohols, aldehydes, alkanes or alkenes, ranging from five to nine carbons, and non-volatile corresponding oxoacids[1].

The effects of linoleic acid induction on the biomass production as well as on the changes in glucose consumption, pH values and 1-octen-3-ol profiles during the growth of *Penicillium camemberti* were previously investigated by our group[2, 3]. In addition, these changes as well as the changes in 1-octen-3-ol profiles were also investigated by our group during the growth of *P. camemberti* on synthetic medium containing free linoleic acid[4]. In the present work, the effects of the addition of soybean oil in the same defined synthetic medium and two dairy media on the biomass production of *P. camemberti*, lactose consumption, pH values and the production of 1-octen-3-ol were investigated.

The addition of refined soybean oil to the defined synthetic medium enhanced the production of 1-octen-3-ol, *in vitro*, by a factor of 8 compared to that obtained with the same medium but containing linoleic acid. The milk and acid-gelified milk, used as culture medium, enhanced the production, *in vitro*, of 1-octen-3-ol respectively by 1.2- and 3-fold compared to the defined synthetic medium containing linoleic acid. However, these productions of 1-octen-3-ol on dairy media were smaller than those obtained with the enzymatic extract obtained from the biomass of *P. camemberti*, grown on the defined synthetic medium but supplemented with soybean oil.

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P155 GENETIC ENGINEERING OF THE γ-OXIDATION PATHWAY IN THE YEAST *Yarrowia lipolytica* TO INCREASE THE PRODUCTION OF AROMA COMPOUNDS

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Keywords: γ-oxidation, acyl-CoA oxidase, γ-decalactone

As many yeast species, *Yarrowia lipolytica* is able to transform ricinoleic acid (a hydroxylated C18 fatty acid) into γ-decalactone, a fruity and creamy aroma compound[1]. Unfortunately, this species is also able to degrade the produced lactone. The pathway of biotransformation involves γ-oxidation and requires the lactonisation at the C10 level (when the hydroxy group is in γ -). *Y. lipolytica* possesses a five-member family of acyl-CoA oxidases (Aox1 to 5), the enzyme catalysing the first step of γ-oxidation, some of which are long--chain specific (Aox2)[2] or short-chain specific (Aox3)[3, 4]. In a previous paper, we have tried to decrease the lactone degradation by constructing a strain with no more activity on short-chain substrates[5]. However, this strain was growing and biotransforming very slowly. In this study, we have constructed strains without acyl-CoA oxidase activity for short-chain substrates but with increased activity on long chains. These strains are able to grow at the same rate as the wild type but produce about 10 times more in only 48 hr, and this amount does not significantly decrease in 250 hr.

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P156 SYNTHETIC STUDIES ON HELIANNANE SESQUITERPENES VIA CHEMOENZYMATIC TRANSFORMATION

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Keywords: heliannuol E, heliannuol C, sesquiterpene, lipase, enantioselective synthesis

(-)-Heliannuols E and C are naturally occurring sesquiterpenes exhibiting allelopathic activity[1]. We report herein the enantioselective total syntheses of heliannuols E (ref.[2]) and C employing chemoenzymatic transformations of the prochiral diols **1** and **3** into the optically enriched acetate **2** and **4** as the key reaction steps (Fig. 1). It should be noted that the absolute stereochemistry of heliannuol C was firmly established by the completion of the first total synthesis.

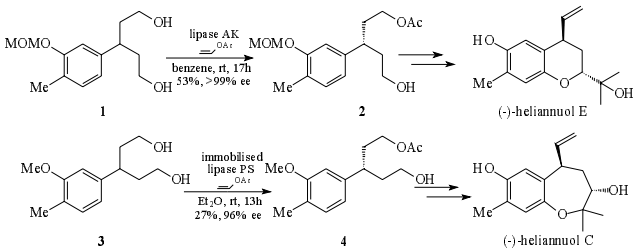

Fig. 1. **Syntheses of heliannuols E and C**

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P157 REGIOSELECTIVE ENZYMATIC ACYLATION OF POLYHYDROXYLATED SESQUITERPENOIDS

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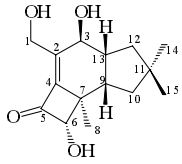
Keywords: sesquiterpenoids, tsugicoline, sterpurane, illudin, lipase

A number of *Basidiomycetes* are known to produce sesquiterpenoids with a protoilludane **(1-3)** or an illudane **(4)** skeleton. As a part of our continuing search for biologically active metabolites, we have recently described the isolation of tsugicoline A **(1)** and its transformation into the sterpurane derivative **(2)** and the 2-amino-tsugicoline E **(3)** (ref.[1]). The anomalous presence of a carbonyl function in the four member ring of **1** is the key to understand the reactivity of this interesting metabolite and led to an easy opening of the C(6)–C(7) bond to give, under suitable conditions, compounds **2** and **3**. The illudane sesquiterpenes illudine S **4**, a potent cytotoxic compound, was also isolated by us from a strain of *Omphalotus olearius*[2].

In order to extend the number of available derivatives of these compounds, we have considered the possibility to carry out their enzymatic acylations in a comparative study. In this report we will describe the results obtained using a set of different lipases for the regioselective acylation of **1-4** .



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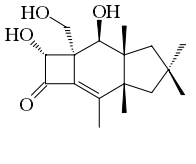

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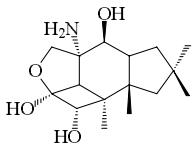

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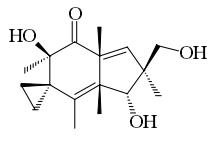

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P158 SYNTHESES AND ENZYMATIC EVALUATION OF SUBSTRATE ANALOGS OF MEDIUM-CHAIN PRENYL DIPHOSPHATE SYNTHASE

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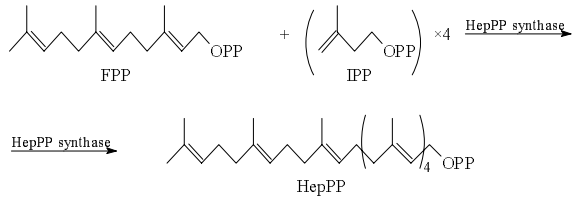
Keywords: farnesyl diphosphate, prenyl diphosphate synthase, inhibitor, heptaprenyl diphosphate, substrate analog

Heptaprenyl diphosphate (HepPP) synthase (EC 2.5.1.30) catalyzes the condensation of isopentenyl diphosphate (IPP) with *(E,E)*-farnesyl diphosphate (FPP) to afford HepPP with chain lengths of C_{35}, which is the precursor of menaquinone-7 (Scheme 1)[1].

HepPP synthase from *Bacillus subtilis* has been shown to be composed of two dissociable components, I and II. Each component has no catalytic activity as the prenyl chain elongating enzyme unless they are combined[2]. There is little knowledge about the substrate recognition site of HepPP synthase. Especially, we are interested in the steric course of the enzyme reaction. So we prepared some substrate analogs (Fig. 1, **1a-1k, 6, 7)** and carried out the enzyme reaction using these analogs. It has been reported that 3-methyl group at allylic substrate is very important for prenyl diphosphate synthase reaction[3]. So we also prepared some 3-desmethyl analogs (Fig. 1, **2-5**) to study the inhibitory effect to the enzyme.



Scheme 1. **HepPP synthase reaction**

We carried out the enzyme reaction using the artificial substrates to investigate the substrate specificity of HepPP synthase from *B. subtilis*. The reaction of IPP with the analogs **1a-j** , which have the various chain lengths of alkyl group at 4-position of DMAPP, showed that the analog **1i** was the most reactive, which has just same chain length as one of FPP, while **1h** or **1j**, the chain length of which is shorter or longer by one methylene than **1i**, respectively, is less reactive than **1i**. These results suggested that the enzyme exactly recognizes the chain length of allylic substrate.

The analogs **2-5** have no methyl group at 3-position of the allylic substrate. These analogs showed no reactivity for the enzyme[3]. On the other hand, these analogs **2** or **4** had inhibitory effect to the enzyme. Especially, the analog **2** showed the stronger inhibitory effect than **4** (Fig. 2). These results suggested that HepPP synthase preferred the *E*-isomer and exactly recognized the stereochemistry of the allylic primer.

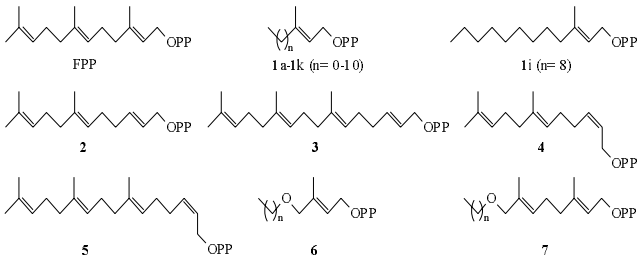
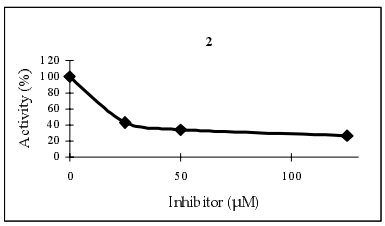


Fig. 1. **Artificial substrate analogs**



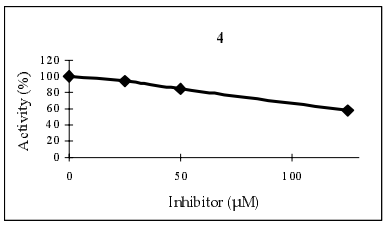


Fig. 2. **Inhibitory effects of 2 and 4 to HepPP synthase**

The analogs **6** (n = 0, 1, 2, 3) or **7** (n = 0, 1, 2, 3) are the DMAPP or GPP analogs having oxygen atom in their prenyl chain, respectively. The analog **6** (n = 0, 1, 2 or 3) can be hardly accepted, but **7** (n = 0, 1, 2 or 3) can be easily accepted by HepPP synthase. These findings are very interesting because FPP synthase from *Bacillus stearothermophillus* hardly accepted all these analogs containing oxygen atom in their prenyl chain, though both synthases belong to the same *E*-type prenyltransferase family.

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P159 SUBSTRATE SPECIFICITIES OF SEVERAL PRENYLCHAIN ELONGATING ENZYMES WITH RESPECT TO 4-METHYL-4-PENTENYL DIPHOSPHATE

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Keywords: substrate specificity, prenylchain elongating enzyme, farnesyl diphosphate synthase, homogeraniol, homolog

Prenyltransferase reaction proceeds with the condensation of an allylic prenyl diphosphate with isopentenyl diphosphate (IPP) stereospecifically and the condensation terminates precisely until the elongation of prenyl chain reaches certain length according to the specificities of individual enzymes.

Ogura *et al.*[1,2] reported that 4-methyl-4-pentenyl diphosphate (homoIPP) was accepted as a homoallylic substrate for a porcine liver farnesyl diphosphate (FPP) synthase, and that the products derived from the reaction of homoIPP with geranyl diphosphate (GPP) or with dimethylallyl diphosphate (DMAPP) were only *Z*-homofarnesyl- or *Z*-homogeranyl diphosphate, respectively.

Recently, antiproliferative terpene derivatives such as *(Z)*-4,8-dimethylnon-3-en-sodium sulfate **(1)** or 3,7,11,15-tetramethylhexadecan-1,19-sodium disulfate **(2)** have been isolated from marine organisms such as sea squirts, Japanese name “hoya” (ref.[3,4]). In order to synthesize several carbon skeleton homologs of compound **1**, we examined the applicability and substrate specificities of some prenylchain elongating enzymes with respect to homoIPP.

As the result, the alcohol derived from the reaction of DMAPP with homoIPP by use of a recombinant *Bacillus stearothermophilus* FPP synthase afforded in higher yields; *Z*-homoGOH (yield: 45.9 %) and *E*-homoGOH (25.5 %) as shown in Fig. 1, comparing with that of *Z*-homoGOH (yield; 1 % or less, no *E*-product) by the porcine liver FPP synthase. Substrate specificities of other types of prenylchain elongating enzymes will also be presented.

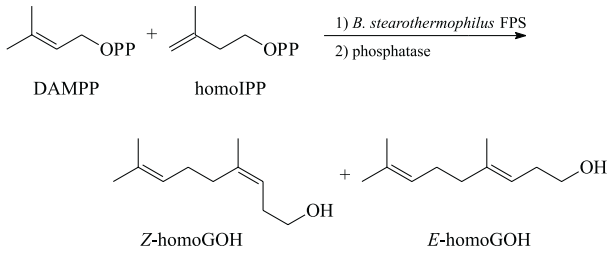

Fig. 1. **Reaction of homoIPP with DMAPP by use of *B. stearothermophilus* FPS.**

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P160 EXTRACELLULAR STEROL OXIDASE OF *Mycobacterium vaccae*

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Keywords: sterol oxidase, *Mycobacterium vaccae*, isolation, purification

Sterol oxidase (SO) catalyzes dehydrogenation of 3-hydroxy steroids at C-3 followed by $\Delta^{5(6)}$ to $\Delta^{4(5)}$ isomerisation, thus modifying 3-hydroxy-5-en to 3-keto-4-en moiety. This conversion is one of key reactions at sterol to C-17-keto-steroid transformation by *Mycobacterium* strains. In spite the reaction is known since 50th, no systematic investigation on the properties of the enzymes from sterol utilizing mycobacteria was published. It was unclear if the same enzyme responsible for both dehydrogenation at C-3 and $\Delta^{5(6)}$ to $\Delta^{4(5)}$ isomerization, as well as if the enzyme represent short-chain dehydrogenase, or mixed function oxidase. No extracellular SO from mycobacteria was reported so far.

In this work, the extracellular sterol oxidase was isolated from cell-free cultivation broth of sterol transforming *Mycobacterium vaccae VKM Ac-1815D*. Along with 3-hydroxysteroid dehydrogenase and $\Delta^{5(6)}$ to $\Delta^{4(5)}$ isomerase activity, 1(2)-dehydrogenase and 17β-dehydrogenase activities were revealed in cell-free cultivation broth. The SO was purified 100-fold using hollow fiber concentration, chromatography on DEAE-Toyopearl, hydroxyapatite Bio-Gel HTP and Bio-Gel A-0.5 M double filtration. In the presence of NADH or NADPH the enzyme expressed 3-hydroxy steroid dehydrogenase, $\Delta^{5(6)}$ to $\Delta^{4(5)}$ isomerase and 6-hydroxylase activities. The enzyme activity of 86.7 $\mu mol.min^{-1}.g^{-1}$ was observed towards dehydroepiandrosterone. Two SO isoforms were revealed by Bio-Gel A-0.5M filtration. The molecular weight of SO by SDS-electrophoresis was determined as 60 ± 4 kDa, K_m for dehydroepiandrosterone averaged 4.1×10^{-4} M.

P161 21-ACETOXY-PREGNA-4(5),9(11),16(17)-TRIENE--21-OL-3,20-DIONE BIOCONVERSION BY *Nocardioides simplex* VKM AC-2033D

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Keywords: steroid, 1(2)-dehydrogenation, deacetylation, 21-acetoxy-pregna-1(2),4(5),9(11),16(17)-tetraene-21-ol--3,20-dione, bioconversion

Microbial 1(2)-dehydrogenation of acetylated 9(11)-dehydrosteroids is of great significance for the synthesis of modern fluoroglucocorticoids[1]. Recently, we studied the metabolic pathways of pregna-4,9(11)-diene-17α,21-diol--3,20-dione 21-acetate and 17,21-diacetate by *Nocardioides simplex* VKM Ac-2033D. As shown, 1(2)-dehydrogenation was accompanied by deacetylation, 20β-reduction and non--enzymatic migration of acyl group from position 17 to 21 (in case of diacetate). The conditions providing predominant accumulation of 1(2),4,9(11)-triene acetates were determined[2].

It was of special interest to find out whether the introduction of 16(17)-double bond in 21-acetylated 4(5),9(11)-diene steroid would influence 1(2)-dehydrogenation and deacetylation by *N. simplex*. In order to clear structure/activity relationship and full biocatalytic potential of this organism in respect to acetylated 9(11)-dehydrosteroids, the conversion of 21--acetoxy-pregna-4(5),9(11),16(17)-triene-21-ol-3,20-dione **(1)** was investigated. The biotransformation of similar steroids has never been studied so far.

21-Acetoxy pregna-1(2),4(5),9(11),16(17)-tetraene-3,20--dione **(2)**, pregna-4(5),9(11),16(17)-triene-21-ol-3,20-dione **(3)** and pregna-1(2),4(5),9(11),16(17)-tetraene-21-ol-3,20--dione **(4)** were revealed as major metabolites at the conversion of **1** by *N. simplex*. The structure of metabolites was confirmed by MS and H^1-NMR. Unlike inducible 1(2)-dehydrogenase, the constitutive esterase activity was shown. The presence of both soluble and membrane associated steroid esterases was shown by cell fractionation experiments. The conditions were found providing full substrate **(1)** conversion with 92 % molar yield of acetylated 1(2)-dehydroanalogue **(2)**.

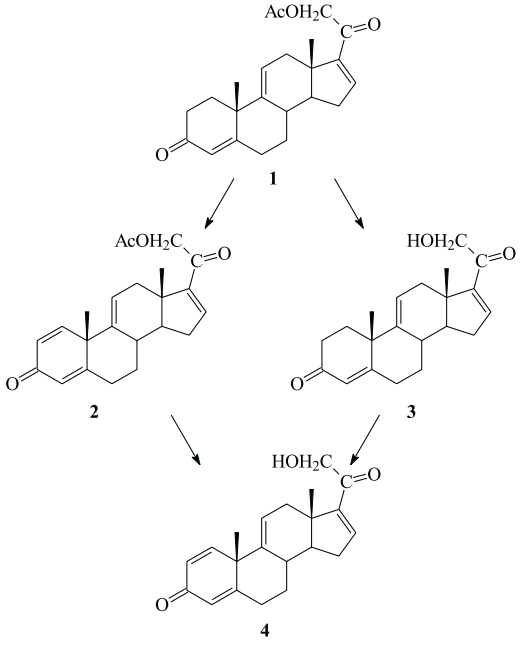

Fig. 1. **Proposed scheme of 21-acetoxy-pregna-4(5),9(11),16(17)--triene-21-ol-3,20-dione (1) bioconversion by *Nocardioides simplex* cells**

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P162 MICROBIAL CONVERSION OF STEROL-ENRICHED FRACTIONS OF SOYBEAN OIL PRODUCTION WASTE BY *Mycobacterium* SP. VKM AC-1817D

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Keywords: biotransformation, soybean extract residue, 9α--hydroxyandrostenedione, sitosterol

Different approaches were undertaken to obtain C_{17}-ketosteroids from industrial wastes of the pulp and paper industry – fractions of tall oil products without isolation and purification of phytosterols. Best results ensued at the conversion of sterol-rich tall-oil unsaponifiable isolates to androst-4-ene-3,17-dione and androsta-1,4-diene-3,17-dione, with total molar yield of 55 % at substrate loading of 2.5 mM (ref.[1, 2]).

In the present study soybean extract residue (scum), a waste of soybean oil production, was estimated as a raw material for C_{17}-ketosteroid production. As a model process, bioconversion of scum to 9α-hydroxyandrost-4-ene-3,17-dione (9-OH-AD) by *Mycobacterium* sp. VKM Ac-1817D was studied. Scum contained ~14 % sterols comprised mostly by sitosterol, stigmasterol and campesterol.

The bioconversion of scum to 9-OH-AD without intermediate isolation of sterols was characterized by a long-term lag-period followed by drastic increase of 9-OH-AD accumulation. The pre-treatment of scum by either microbial, or chemical single step procedures allowed to reach the productivity comparable with that at the use of high quality tall sitosterol. 9-OH-AD molar yield of about 70 % was obtained at the conversion of scum preparation contained 10 $g.l^{-1}$ sterols. Time courses of bioconversions of scum preparations obtained by mostly effective single or double step pre-treatment techniques are shown on the following Fig. 1.

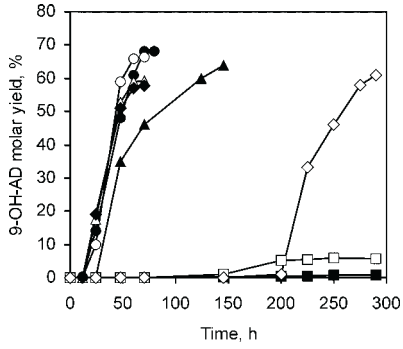

Fig. 1: **Time course of 9-OH-AD at microbial conversions of tall sitosterol (●) and scum preparations obtained by different techniques**; C1 (○), C2 (▲) – crystallisation from ethyl alcohol; (■) F1, (□) F2 – freeze-sedimentation; (◇) H1 - hydrolysis-extraction; F1C1 (◆) – crystallisation of the preparation F1 from ethyl alcohol; C2.1 (△) – re-crystallisation of the preparation C2 from ethyl alcohol

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P163 SITOSTEROL SIDE-CHAIN CLEAVAGE IN AN ORGANIC-AQUEOUS TWO-LIQUID PHASE SYSTEM WITH CHRYSOTILE IMMOBILIZED MYCOBATERIAL CELLS

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Keywords: sitosterol, androstenedione, bioconversion, chrysotile, immobilized cells

The use of organic-aqueous two-liquid phase systems is a well-established approach for the biotransformation of poorly water-soluble compounds. Cell membranes integrity may however be tampered due to the toxic action of the organic phase. Cell immobilization has been shown to reduce the deleterious action of the organic solvent, by providing a protective microenvironment[1]. Chrysotile, an inexpensive mineral material has been successfully used for the immobilization of yeast cells in aqueous-based media[2]. This work aims to evaluate the bioconversion of sitosterol to androstenedione (AD) in a dioctyl phtalate (DOP)/phosphate buffer, using mycobacterial cells immobilized on chrysotile. An increase in final product yield, from 60 % to 90 %, was observed when the immobilized form of the biocatalyst was used, as compared to free cells, in batch bioconversion. The development of a continuous system, based in a CSTR operation mode, was assessed. A flow rate of 0.03 $ml.min^{-1}$ (residence time of 40 h) led to a consistent conversion yield around 50 %, for a substrate concentration of 12 mM, during a one-week operation period. Increasing the flow rate to 0.05 $ml.min^{-1}$ (residence time of 20 h) led to a decrease in the conversion yield to around 25 %, which was nevertheless kept constant for a 30-days working period, suggesting high operational stability. The results obtained with the continuous experimental set-up suggests this approach could be considered for the development of an effective continuous bioconversion system for the production of AD from sitosterol.

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P164 SITOSTEROL SIDE-CHAIN CLEAVAGE BY FREE MYCOBACTERIAL RESTING CELLS IN ORGANIC MEDIA: ASSESSMENT KEY OPERATIONAL PARAMETERS

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Keywords: biotransformation, sterol side-chain cleavage, organic solvent, *Mycobacterium*, resting cells

The selective side-chain cleavage of β-sitosterol by *Mycobacterium* sp. NRRL B-3805 free cells is a well established multi-enzymatic process for the production of pharmaceutical steroid precursors, namely 4-androstene-3,7-dione (AD) and 1,4-androstadiene-3,7-dione (ADD). Since substrate and products are hydrophobic, the use of organic solvents as bioconversion media is one way to overcome low volumetric productivity, intrinsic to conventional aqueous-based biotransformation of such compounds.

In this work, bis(2-ethylhexyl)phthalate (BEHP) was used as reaction medium in a suspended-cell system with low water content as this solvent was previously described as bio-

compatible for this system, allowing high AD and ADD yields[1]. The effect of relevant operational parameters on product yield and reaction rate was evaluated. Namely, variations in the biocatalyst/substrate mass ratio, the amount of water added to the medium and the aeration rate were studied. Off-line monitoring of sitosterol consumption and product formation was performed by HPLC analysis.

Forced aeration of the bioconversion medium favoured product yield. The need to maintain a minimum amount of water in the medium as to retain biocatalytic activity was also evidenced. Increasing the biomass to substrate ratio led to an increase of reaction rate up to saturation level. Product degradation was observed in extented bioconversion runs, suggesting residual activity of sterol ring structure degrading enzymes, and highlighting the need for close monitoring of the bioconversion process so as to avoid such unwanted reactions.

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P165 OPTIMIZATION OF ANDROSTENEDIONE PRODUCTION IN AN ORGANIC-AQUEOUS TWO-LIQUID PHASE SYSTEM

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Keywords: androstenedione, biotransformation, sitosterol side-chain cleavage, *Mycobacterium* sp. NRRL B-3805, organic-aqueous phase system

The use of a biocompatible water-immiscible organic phase as a substrate and product pool has been acknowledged as an effective tool to overcome the low volumetric productivity of aqueous bioconversion systems involving hydrophobic compounds. This approach has been successfully used for the specific side-chain cleavage of sitosterol to androstenedione using *Mycobacterium* sp. NRRL B-3805 cells[1]. Although extensive work has been performed regarding the selection of a biocompatible organic[1–3], few information is available on the effect of aqueous phase composition in the sterol side-chain cleavage activity of mycobaterial cell[2]. This work aims to fill in such gap, through a systematic evaluation of the effect of pH, buffer composition and concentration in catalytic activity. Biocatalytic activity was not significantly affected when buffered solutions with concentration ranging from 20 mM to 100 mM were used. Best results were obtained with phosphate and Tris-HCl buffer solutions, with the highest bioconversion rates being observed in slightly basic bioconversion media (pH within 7.5 to 8). Temperatures in the range 30 °C to 35 °C favored bioconversion rate, whereas low catalytic activity was observed at 20 and 40 °C. Increasing stirring speed up to 500 rpm also favored bioconversion rate. The effect of biomass concentration in product yield is being currently evaluated.

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P166 ENANTIOSELECTIVE SYNTHESIS OF (+) AND (-)-*(Z)*-7,15-HEXADECADIEN-4-OLIDE, THE SEX PHEROMONE OF THE YELLOWISH ELONGATE CHAFER, *Heptophilla picea*

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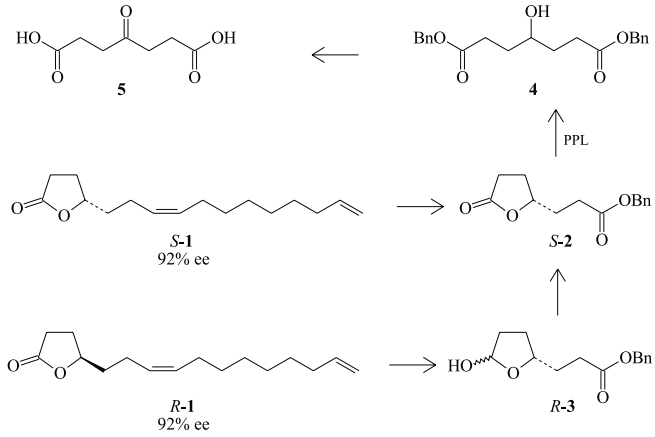
Keywords: biocatalysis, lipase, butyrolactone, pheromone, *Heptophilla picea*

Optically active γ-butyrolactones are valuable building blocks in organic synthesis and find application as synthons in natural products synthesis[1]. Isolated enzymes, such as lipases, have been exploited for preparing optically active lactones[2]. In 1996 Leal et *al.*[3] isolated and identified (4*R*,7*Z*)-7,15-hexadecadien-4-olide **(1)** as a the female-produced sex pheromone of yellowish elongate chafer *(Heptophilla picea)*, which is an agriculture pest that causes losses in tea and flower production in Japan. Compound *R*-**1** was prepared from L-malic acid in 14 steps and the synthetic compound showed spectroscopic data and biological activity identical to those of the natural material[4]. In this communication we report a short and versatile enantioselective synthesis of both enantiomers of **1** using a lipase-catalyzed lactonization in the key step. Scheme 1 shows the retrosynthetic analysis for *R* and *S (Z)*-7,15-hexadecadien-4-olide:



Scheme 1: **Retrosynthetic analysis for *R* and *S* (Z)-7,15-hexadecadien-4-olide**

Both enantiomers were prepared starting from the commercially avaiable 4-ketopimelic acid **(5)**. The enantioselective reactions were performed with porcine pancreatic lipase (PPL) in diethyl ether, and after few synthetic steps, both were prepared in good enantiomeric excess. In conclusion, we have described a convenient and useful enantioselective synthesis of *R* and *S-(Z)*-7,15-hexadecadien-4-olide through lipase-catalyzed enantioselective lactonization. In addition, this fact shows that compound *S*-**3** is a versatile building block for the synthesis of naturally occurring γ-butyrolactones.

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P167 ENZYMATIC BAEYER-VILLIGER OXIDATION OF NEW 2- AND 3-SUBSTITUTED CYCLOHEXANONES

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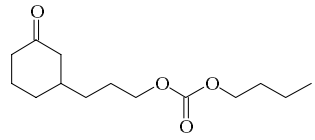
Keywords: Baeyer-Villiger oxidation, cyclohexanone monooxygenase, formate dehydrogenase, hydroxypropylcyclohexanones

Baeyer-Villiger oxidations of alkyl-substituted cyclohexanones have been investigated extensively using both whole cells and isolated cyclohexanone monooxygenase from *Acinetobacter* NCIMB 9871 (ref.[1, 2]) or a recombinant baker's yeast[3] and a recombinant *Escherichia coli* strain[4], respectively. Here we present new examples of the conversion of 2- and 3-substituted cyclohexanones which possess functional groups in the side chain by the system cyclohexanone monooxygenase (CHMO)/formate dehydrogenase (FDH)[5, 6].

2- and 3-hydroxypropylcyclohexanone were oxidised with this enzyme system successfully. These ketones were esterified with several dicarboxylic acids with the purpose to find out a suitable linker system for binding of substituted cyclohexanones to a resin. In kinetic assays could be shown that most of such esters, like e. g. compound **1**, were accepted as substrates of the cyclohexanone monooxygenase.



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