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

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

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

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

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

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

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

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

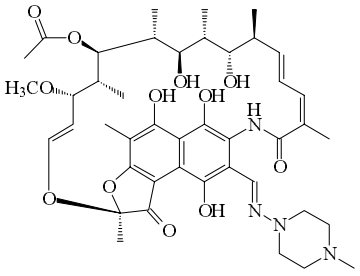

Fig. 1. **Structure of rifampicin**

REFERENCES

1. Scigelova M., Crout D. H. G.: Enzyme Microb. Tech. *25*, 1 (1999).
2. Horsch M., Mayer C., Sennhauser U., Rast D. M.: Pharmacol. Ther. *76*, 1 (1997).
3. Kren V., Scigelova M., Prikrylova V., Havlicek V., Sedmera P.: Biocatalysis *10*, 1 (1994).
4. Kren V., Hunova Z., Halada P., Suzuki Y.: Biosci. Biotechnol. Biochem. *62*, 12 (1998).

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

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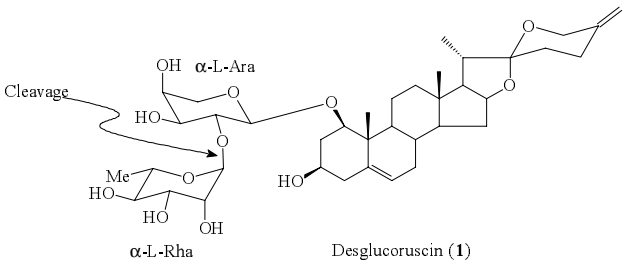
Keywords: α-L-rhamnosidases, desglucoruscin, selective deglycosylation

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

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

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

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




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

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

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

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

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

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

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

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

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

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

REFERENCES

1. Puri M., Marwaha S. S., Kothari R. M. and Kennedy J. F.: Crit. Rev. Biotech. *16*, 145 (1996).
2. Zverlov V. V., Hertel C., Bronnenmeier, K., Hroch A., Kellermann J., Schwarz W. H.: Mol. Microbiol. *35*, 173 (2000).

## P058 ENZYMATIC GLYCOSYLATION OF CARMINIC ACID

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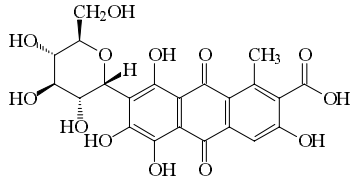
Keywords: enzymatic, glycosylation, carminic acid, transglycosylation

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

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

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



Carminic Acid

Table I
Enzymes used for the experiments and results

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

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

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

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Keywords: thermostable glycosidase, β-glucosidase, glucosylglycerol

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

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

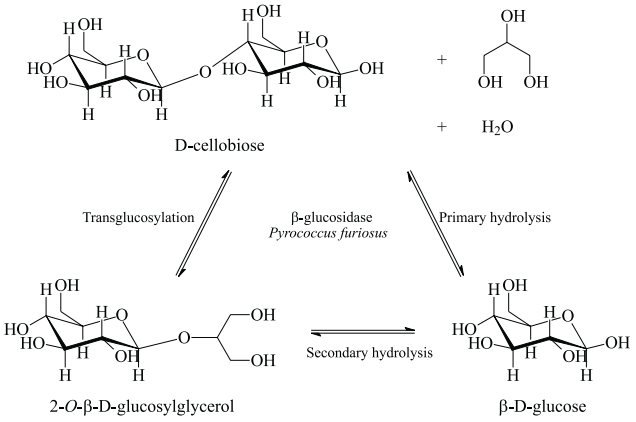


Fig. 1. **Scheme of the investigated reaction system**

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

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

REFERENCES

1. Fischer L., Bromann R., Kengen S. W. M., de Vos W. M., Wagner F.: Bio/Technology *14*, 88 (1996).
2. Colombo D., Scala A., Taino I. M., Toma L., Ronchetti F., Tokuda H., Nishino H., Nagatsu A., Sakakibara J.: Bioorg. Med. Chem. Lett. *6*, 1187 (1996).
3. Takenaka F., Uchiyama H.: Biotechnol. Biochem. *64*, 1821 (2000).

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

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

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

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

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

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

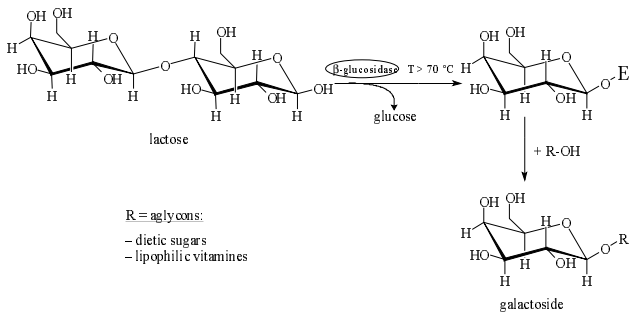


Fig. 1. **Reaction scheme of transglycosylation using β-glucosidase from *Pyrococcus furiosus*.** R-OH could be a primary, secondary or tertiary hydroxylgroup, E = enzyme.

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

REFERENCES

1. Siso M. I. G.: Bioresorce Tech. *57*, 1 (1996).
2. Kulbe K. D.: DMZ *9*, 28 (2002).
3. Harju M.: Bull. Int. Dairy Fed. *212*, 50 (1987).
4. Petzelbauer I., Nidetzky B., Haltrich D., Kulbe K. D.: Biotechnol. Bioeng. *64*, 322 (1998).
5. Petzelbauer I., Zeleny R., Reiter A., Kulbe K. D., Nidetzky B.: Biotechnol. Bioeng. *69*, 140 (2000).
6. Hansson T., Kaper T., van der Oost J., de Vos W. M., Adlercreutz P.: Biotechnol. Bioeng. *73*, 203 (2001).
7. Fischer L., Bromann R., Kengen S. W. M., de Vos W. M., Wagner F.: Biotechnol. *14*, 88 (1996).

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

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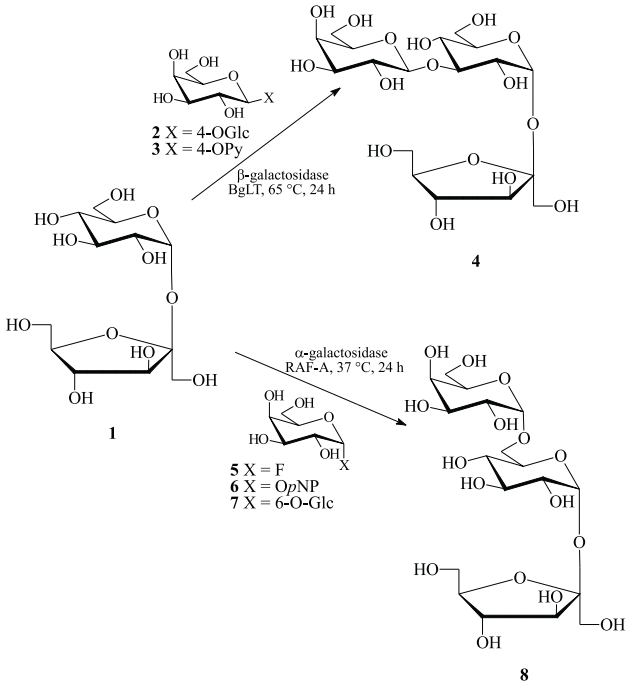
Keywords: galactosidase, transgalactosylation, biologically active carbohydrates, non-cariogenic sweeteners, bifidogenic sugars

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

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




## P062 ENZYMATIC REMOVAL OF LACTOSE FROM GALACTO-OLIGOSACCHARIDE MIXTURES

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

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

## P063 β-GALACTOSIDASES FROM *Lactobacilli* STRAINS

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

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

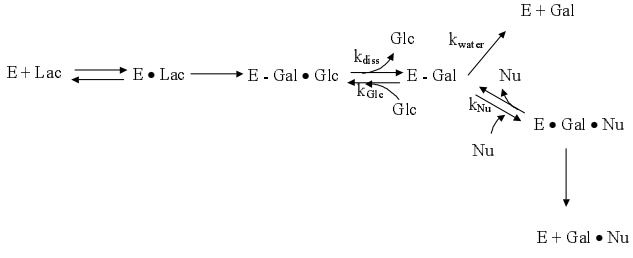


Fig. 1. **Formation of galacto-oligosaccharides by transgalactosylation of lactose catalyzed by β-galactosidases;** E – enzyme (β-galactosidase), Lac – lactose, Gal – galactose, Glc – glucose, Nu – galactosyl acceptor

REFERENCES

1. Pivarnik L. F., Senegal A. G., Rand A. G., in: *Advances in Food and Nutrition research* (Kinsella J. E., Taylor S. L., ed.), vol. 38. Academic Press, New York 1995.
2. Nakayama T., Amachi T.: *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis, and Bioseparation*, pp. 1291–1305. John Wiley, New York 1999.

## P064 OPTIMIZATION OF ENZYMIC HYDROLYSIS AND TRANSGALACTOSYLATION IN ACID WHEY

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

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

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

REFERENCES

1. Smart J. B.: Int. Dairy J. *11*, 19 (2001).
2. Playne M., Crittenden R., Mahoney R. R.: Food Chem. *63*, 147 (1998).
3. Gopal P. K., Sullivan P. A.: Bull. Idf. *313*, 10 (1996).

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

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

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

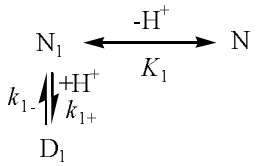
From several mechanisms considered, a consistent description of experimental data was achieved using the kinetic form of the Lumry-Eyring mechanism,

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

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

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

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




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

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

*This work was supported by the Polish KBN grant No. 3T09C 03119. Milan Polakovič was also supported by the Slovak Grant Agency for Science VEGA grant No. 1/0065/03.*

REFERENCES

1. Polakovič M., Bryjak J.: J. Mol. Catal. B: Enzym. *19–20*, 443 (2002).

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

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

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

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

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

REFERENCES

1. Hill K., Rybinsky W., Stoll G.: *Alkyl Polyglucosides*. VCH Publishers Inc, New York 1997.
2. Remaud-Simeon M., Willemot R. M., Sarçabal P., Potocki de Montalk G., Monsan P.: J. Mol. Catal. B: Enzym. *10*, 117 (2000).

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

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

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

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

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

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

REFERENCES

1. Deffieux G., Baute R., Baute M. A., Atfani M., Carpy A.: Phytochemistry *26*, 1391 (1987).
2. Yu S.: Trends Glycosc. Glycotech. *12*, 381 (2000).
3. Remaud-Simeon M., Willemot R. M., Sarçabal P., Potocki de Montalk G., Monsan P.: J. Mol. Catal. B: Enzym. *10*, 117 (2000).

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

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Keywords: directed evolution, xylanase, *Thermomyces lanuginosus*

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

REFERENCES

1. Schlacher A., Holzmann K., Hayn M., Steiner W., Schwab H.: J. Biotechnol. *49*, 211 (1996).

## P069 CHARACTERIZATION OF PYRANOSE DEHYDROGENASE FROM *Agaricus xanthoderma*

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Keywords: pyranose dehydrogenase, oxidation, keto sugars, lignin

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

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

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

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

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

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

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

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

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

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Keywords: glycosyltransferase, donor substrate, analogue, sugar nucleotide

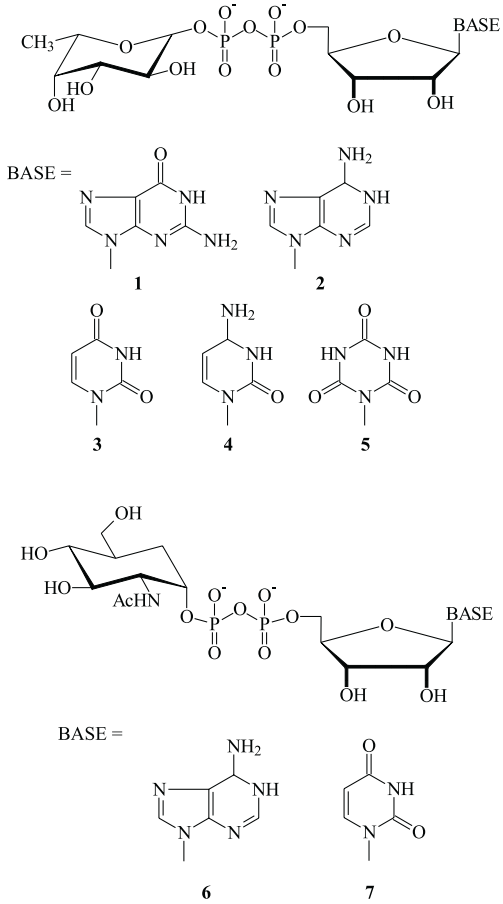
The availability of cloned glycosyltransferases has made enzymatic glycosylation a realistic alternative to chemical synthesis. However, a severe drawback to this approach is the requirement for sugar nucleotides. *In situ* regeneration of the sugar nucleotides is the most elegant way of using these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

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

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

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

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




REFERENCES

1. Moffat J. G.: Methods Enzymol. *8*, 136 (1966).
2. Baisch G., Öhrlein R.: Bioorg. Med. Chem. *5*, 383 (1997).
3. Augé C., Malleron A., Tahrat H., Marc A., Goergen J.-L., Cerutti M., Steelant W. F. A., Delannoy P., Lubineau A.: J. Chem. Soc., Chem. Commun. *2000*, 2017.
4. Wakarchuk W. W., Martin A., Jennings M. P., Moxon E. R., Richards J. C.: J. Biol. Chem. *271*, 19166 (1996).
5. Baisch G., Öhrlein R., Katopodis A.: Bioorg. Med. Chem. Lett. *6*, 2953 (1996).

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

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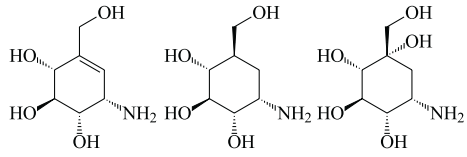
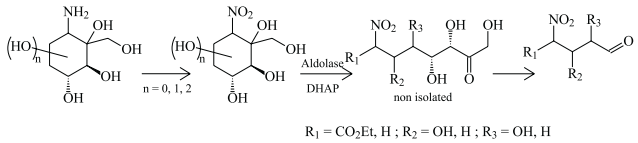
Keywords: aldolase, aminocyclitols, valiolamine analogues, inhibitors, glycosidases

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




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




REFERENCES

1. Ogawa S., in: *Carbohydrate Mimics* (Chapleur Y., ed.), pp. 87–106. Wiley-VCH, Weinheim1998.
2. Chou W. C., Fostch C., Wong C.-H.: J. Org . Chem. *60*, 2916 (1995).
3. Schmid W., Whitesides G. M.: J. Am. Chem. Soc. *112*, 9670 (1990).
4. Gijsen H. J., Wong C.-H.: Tetrahedron Lett. *36*, 7057 (1995).

## P073 NEW FRUCTOSYLTRANSFERASES FOR POTENTIAL INDUSTRIAL APPLICATIONS

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Keywords: fructosyltransferase, levansucrase, sucrose

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

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

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

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

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

REFERENCES

1. Jeanes A., *et al.*: J. Am. Chem. Soc. *76*, 5041 (1954).
2. Ohtsuka K., Hino S., Fukushima T., Ozawa O., Kanematsu T., Uchida T.: Biosci. Biotech. Biochem. *56*, 1373 (1992).

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

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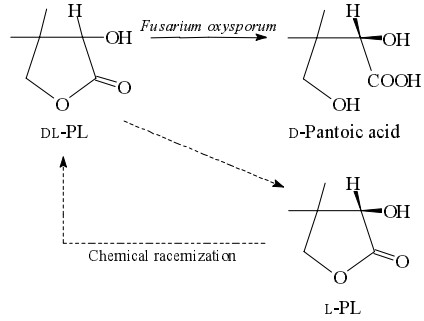
Keywords: lactonase, optical resolution, pantoyl lactone, *Fusarium oxysporum*, *Aspergillus oryzae*

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




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

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

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

REFERENCES

1. Shimizu S., Ogawa J., Cheng M. C., Yamada H.: Eur. J. Biochem. *209*, 383 (1992).

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

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Keywords: pectinase, mutagenesis, rhamnogalacturonase, PCR

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

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

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

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

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

REFERENCES

1. Terada Y., Yanase M., Takata H., Takaha T., Okada S.: J. Biol. Chem. *272*, 15729 (1997).
2. Endo T., Zheng M., Zimmermann W.: Aust. J. Chem. *55*, 39 (2002).
3. Terada Y., Sanbe H., Takaha T., Kitahata S., Koizumi K., Okada S.: Appl. Environ. Microbiol. *6*, 1453 (2001).

## P077 PROTEASE CATALYSED TRANSESTERIFICATION OF SUCROSE AND CYCLODEXTRINS

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Keywords: protease, transesterification, sucrose, cyclodextrin, solvents

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

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

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

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

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

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

REFERENCES

1. Pedersen N. R., Wimmer R., Matthiesen R., Pedersen L. H., Gessesse A.: Tetrahedron: Asymmetry *14*, 667 (2003).
2. Pedersen N. R., Halling P. J., Pedersen L. H., Wimmer R., Matthiesen R., Veltman O. R.: FEBS Lett. *519*, 181 (2002).

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

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

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

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

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

REFERENCES

1. Drouet P., Zhang M., Legoy M. D.: Biotechnol Bioeng. *43*, 1075 (1994).
2. Gargouri M., in: *Liquid Interfaces in Chemical, Biological and Pharmaceutical Applications* (Volkov A., ed.), Vol. 95, pp. 553–584. Marcel Dekker, New York 2001.
3. Smaali I., Gargouri M., Limam F., Fattouch S., Maugard T., Legoy M. D., Marzouki M. N.: Appl. Biochem. Biotechnol., in press.

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

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

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

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

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

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

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

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

*Supported by Ministry of Education of the Czech Republic grant MSM 113100001, by Institutional Research Concept No. A0Z5020903 for the Institute of Microbiology, and by Grant Agency of the Czech Republic grant 203/01/1018. Conference presentation assisted by RHODIA company.*

REFERENCES

1. Křen V., Rajnochová E., Huňková Z., Dvořáková J., Sedmera P.: Tetrahedron Lett. *39*, 9777 (1998).
2. Hušáková L., Herkommerová-Rajnochová E., Semeňuk T., Kuzma M., Rauvolfová J., Přikrylová V., Ettrich R., Plíhal O., Bezouška K., Křen V.: Adv. Synth. Catal., in press.

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

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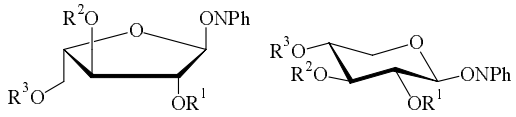
Keywords: lipase, regioselective deacetylation or acetylation, 4-nitrophenyl glycoside

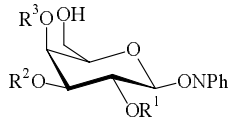
Development of economically feasible and ecologically friendly processes of bioconversion of renewable plant biomass requires detail knowledge of catalytic properties of microbial glycosyl hydrolases and esterases involved in biodegradation of plant cell walls. In this connection, there is an increasing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

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




NPh = 4-nitrophenyl

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




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

REFERENCES

1. Biely P., Mastihubová M., van Zyl W. H., Prior B. A.: Anal. Biochem. *311*, 68 (2002).
2. Mastihubová M., Szemesová J., Biely P.: Tetrahedron Lett. *44*, 1671 (2003).

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

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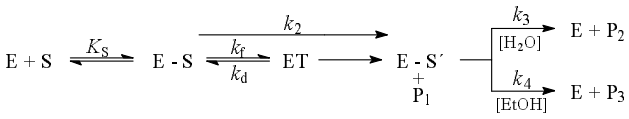
Keywords: acylation, nucleophile, tetrahedral intermediate, transition state

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

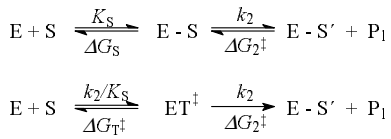
The partition experiments shown in Scheme 1 has successfully determined deacylation rate constant $k_3$ being $4 \times 10^3$ $s^{-1}$ using ethanol as a nucleophile. The value, extremely larger than $k_{cat}$s obtained, has demonstrated quantitatively the acylation being the rate determining step and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

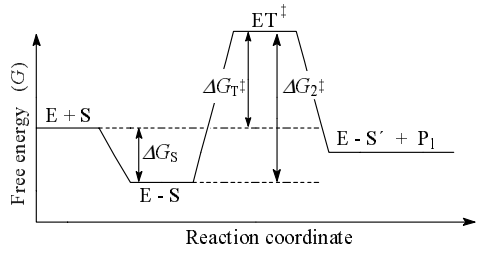
Measuring $k_2$, $K_S$ and $k_2/K_S$ values for each substrates above at 40 °C, we have determined thermodynamic parameters associated with these kinetic constants (Scheme 2) of the both enantiomers of the two substrates **1** and **2** exhibited in Table I.

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




Scheme 1




Scheme 2

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

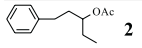
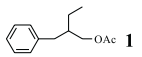
| Substrate | | $\Delta\Delta G_T^I$ (kcal.mol$^{-1}$) | $\Delta\Delta H_T^I$ (kcal.mol$^{-1}$) | $T\Delta\Delta S_T^I$ (kcal.mol$^{-1}$) | $\Delta\Delta S_T^I$ (cal.mol.T$^{-1}$) |
|---|---|---|---|---|---|
|  | **2** | –5.2 | –9.3 | –4.1 | –13.0 |
|  | **1** | 1.1 | –1.1 | –2.2 | –7.0 |

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

(A)

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

(B)

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

## P082 SUBSTRATE PROFILES OF NITRILE HYDROLYSING BIOCATALYSTS

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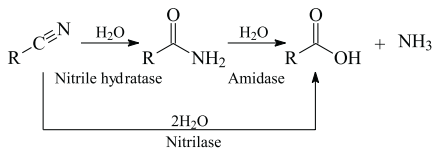
Keywords: biocatalysis, nitrilase, nitrile hydratase, amidase, substrate profile

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




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

REFERENCES

1. Wegman M. A., Heinemann U., Stolz A., van Rantwijk F., Sheldon R. A.: Org. Process Res Dev. *4*, 318 (2000).
2. Wegman M. A., Heinemann U., van Rantwijk F., Stolz A., Sheldon R. A.: J. Mol. Catal. B: Enzym. *11*, 249 (2001).

## P083 SYNTHESIS OF ENANTIOPURE CARBOXYLIC ACIDS USING NITRILASE CATALYSIS

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Keywords: nitrile, nitrilase, enantiopure carboxylic acid

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

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

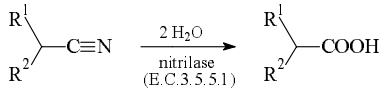


Fig. 1: **Enzymatic nitrile hydrolysis**

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

REFERENCES

1. Yamamoto K., Oishi K., Fujimatsu I., Komatsu K.: Appl. Environ. Microbiol. *51*, 3028 (1991).

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

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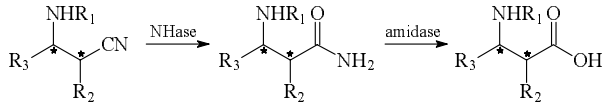
Keywords: β-amino nitrile, β-amino acid, biotransformation, nitrile hydratase

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

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




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

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

REFERENCES

1. Juaristi E.: *Enantioselective Synthesis of β-Amino Acids*. Wiley-VCH, Weinheim 1997.
2. Martinkova L., Kren V.: Biocatal. Biotrans. *20*, 73 (2002); Martinkova L., Klempier N., Preiml M., Ovesna M., Kuzma M., Mylerova V., Kren V.: Can. J. Chem. *80*, 724 (2002).

## P085 NITRILE HYDRATASE-CATALYSED TRANSFORMATIONS OF GLYCOSYL CYANIDES

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Keywords: nitrile hydratase, *Rhodococcus*, glycosyl cyanide

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

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

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

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

REFERENCES

1. Martínková L., Klempier N., Preiml M., Ovesná M., Kuzma M., Mylerová V., Křen V.: Can. J. Chem. *80*, 724 (2002).
2. Přepechalová I., Martínková L., Stolz A., Ovesná M., Bezouška K., Kopecký J., Křen V.: Appl. Microbiol. Biotechnol. *55*, 150 (2001).
3. Yokoyama M., Nakatsuka Y., Sugai T., Ohta H.: Biosci. Biotech. Biochem. *60*, 1540 (1996).
4. DeRaadt A., Griengl H., Klempier N., Stütz A.: J. Org. Chem. *58*, 3179 (1993).

## P086 ENZYMATIC HYDROLYSIS OF NITRILES USING *Aspergillus niger* K10

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

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

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

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

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

REFERENCES

1. Thimann K. V., Mahadevan S.: Arch. Biochem. Biophys. *105*, 133 (1964).
2. Strobel G. A.: J. Biol. Chem. *241*, 2618 (1966); Strobel G. A.: J. Biol. Chem. *242*, 3265 (1967).
3. Asano Y., Ando S., Tani Y., Yamada H., Ueno T.: Agric. Biol. Chem. *45*, 57 (1981).
4. Harper D. B.: Biochem. J. *167*, 685 (1977).
5. Maier-Greiner U. H., Obermaier-Skrobranek B. M. M., *et al.*: Proc. Natl. Acad. Sci. U.S.A. *88*, 4260 (1991).

## P087 FAST SCREENING OF NITRILE HYDRATASES ON COLONY LEVEL

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Keywords: nitrile hydratase, screening, high throughput

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

REFERENCES

1. Fournand D., Bigey F., Arnaud A.: App. Environ. Micro. *64*, 2844 (1998).
2. Hashimoto Y., Nishiyama M., Horinouchi S., Beppu T.: Biosci. Biotechnol. Biochem. *58*, 1859 (1994).
3. Lilly M. D., Clarke P. H., Houldsworth M., Currier J. A., Dunnill P.: Biotechnol. Bioeng. *11*, 283 (1969).
4. Nakasako M., Odaka M., Yohda M., Dohmae N., Takio K., Kamiya N., Endo I.: Biochemistry *38*, 9887 (1999).
5. Nojiri M., Yohda M., Odaka M., Matsushita Y., Tsujimura M., Yoshida T., Dohmae N., Takio K., Endo I.: J. Biochem. *125*, 696 (1999).

## P088 IN SITU SYNTHESIS OF PROTECTED CYANOHYDRINS USING OXYNITRILASE

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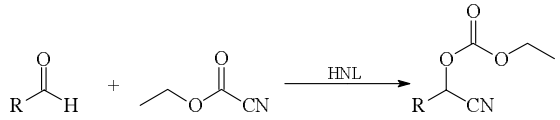
Keywords: hydroxynitrile lyase, oxynitrilase, cyanohydrins, ethyl cyanoformate

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




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

REFERENCES

1. North M.: Tetrahedron: Asymmetry *14*, 147 (2003).
2. Gregory R. J. H.: Chem. Rev. *99*, 3649 (1999).

## P089 ENZYME CATALYSED CYANOHYDRIN FORMATION FROM HETEROCYCLIC KETONES

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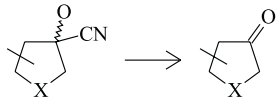
Keywords: hydroxynitrile lyase, oxynitrilase, cyanohydrins

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




X = S or O

For structure elucidation after enzymatic conversion further transformations were performed.

REFERENCES

1. Olafsdottir E. S., Anderson J. V., Jaroszewski J. W.: Phytochemistry *28*, 127 (1989).
2. Gregory R. J. H., Roberts S. M., Barkley, J. V., Coles M. B. H., Hibbs D. E.: Tetrahedron *40*, 7407 (1999).
3. Fechter M. H., Griengl H., in: *Enzyme Catalysis in Organic Synthesis* (Drauz K., Waldmann H. ed.), p. 974, 2nd Eddition. Wiley-VCH, Weinheim 2002.

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

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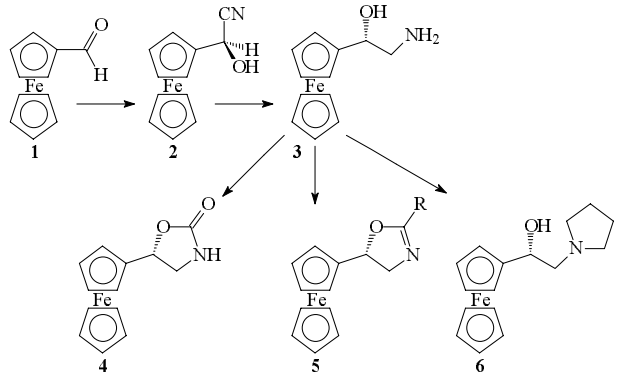
Keywords: hydroxynitrile lyase, ferrocene, cyanohydrins

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




REFERENCES

1. Fröhlich R. F. G., Zabelinskaja-Mackova A. A., Fechter M. H., Griengl H.: Tetrahedron: Asymmetry *14*, 355 (2003).
2. Bastin S., Brocard J., Pelinski L.: Tetrahedron Lett. *41*, 7303 (2000).
3. Patti A., Pedotti S.: Tetrahedron: Asymmetry *14*, 597 (2003).

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

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

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

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

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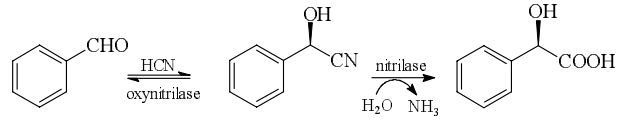
Keywords: CLEA, mandelic acid, dextran, nitrilase, oxynitrilase

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




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

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

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

REFERENCES

1. Cao L., van Rantwijk F., Sheldon R. A.: Org. Lett. *2*, 10 (2000).
2. Cao L., van Langen L. M., van Rantwijk F., Sheldon R. A.: J. Mol. Catal. B: Enzym. *11*, 665 (2001).

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

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Keywords: HNL, prunus, isoenzyme, cyanohydrine

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

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

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

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

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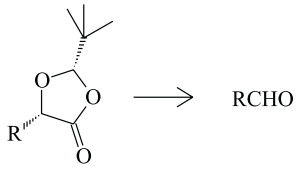
Keywords: hydroxynitrile lyase, *Hevea brasiliensis*, cyanohydrin

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




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

REFERENCES

1. Griengl H., Klempier N., Pöchlauer P., Schmidt M., Shi N., Zabelinskaja-Mackova A. A.: Tetrahedron *54*, 14477 (1998); Griengl H., Schwab H., Fechter M. H.: Trends Biotechnol. *18*, 252 (2000).
2. Seebach D., Naef R., Calderari G.: Tetrahedron *40*, 1313 (1984).

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

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

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

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

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

REFERENCES

1. Effenberger F.: Angew. Chem. Int. Ed. Engl. *33*, 1555 (1994); Effenberger F., Förster S., Wajant H.: Curr. Opin. Biotechnol. *11*, 532 (2000); Schmidt M., Griengl H.: Top. Curr. Chem. *200*, 193 (1999); Gregory R. H.: Chem. Rev. *99*, 3649 (1999).
2. Wagner U. G., Haslacher M., Griengl H., Schwab H., Kratky C.: Structure *4*, 811 (1996).
3. Lauble H., Förster S., Miehlich B., Wajant H., Effenberger F.: Acta Crystallogr. Sect. D *57*, 194 (2001).

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

**BARBARA KRAMMER**, PETER REMLER, KARL GRUBER, MARGARETHA SCHILLER, and HELMUT SCHWAB

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

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

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

REFERENCES

1. Hasslacher M., Kratky C., Griengl H., Schwab H., Kohlwein, S. D.: Prot. Struct. Funct. Genet. *27*, 438 (1997).
2. Zuegg J., Gruber K., Gugganig M., Wagner U. G., Kratky C.: Protein Sci. *8*, 1990 (1999).

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

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

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

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

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

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

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

REFERENCES

1. Bull A. T., Ward A. C., Goodfellow M.: Microbiol. Mol. Biol. Rev. *64*, 573 (2000).
2. Brandão P. F. B., Clapp J. P., Bull A. T.: submitted.
3. Brandão P. F. B., Clapp J. P., Bull A. T.: Environ. Microbiol. *4*, 262 (2002).
4. Brandão P. F. B., Torimura M., Kurane R., Bull A. T.: Appl. Microbiol. Biotechnol. *58*, 77 (2002).
5. Clapp J. P., Mansur I., Dodd J. C., Jeffries P.: Environ. Microbiol. *3*, 273 (2001).

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

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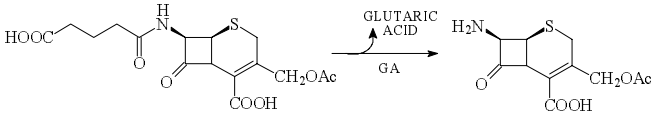
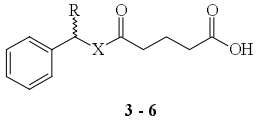
Keywords: glutaryl-7-ACA acylase, kinetic resolution, enantioselectivity

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

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




**1** **2**




**3 - 6**

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

REFERENCES

1. Raimondi S., Forti L., Monti D., Riva S.: Tetrahedron: Asymmetry *14*, 1091 (2003).
2. Raimondi S., Monti D., Pagnoni U. M., Riva S.: Advanced Synthesis & Catalysis, in press.

## P100 SUBSTRATE TOLERANCE OF GLUTARYL ACYLASE

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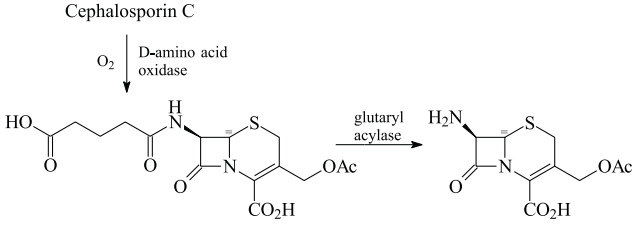
Keywords: glutaryl acylase, substrate tolerance

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




**1**

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

REFERENCES

1. Fritz-Wolf K., Koller K., Lange G., Liesum A., Sauber K., Schreuder H., Aretz W., Kabsch W.: Protein Sci. *11*, 92 (2002).
2. Liese A., Seelbach K., Wandrey C.: *Industrial Biotransformations*, pp. 225 (2000).

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

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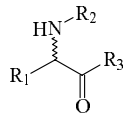
Keywords: aminoacylase, amino acids resolution, enantioselective conversion

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




**1**

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

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

REFERENCES

1. Chenault H. K., Dahmer J., Whitesides G.: J. Am. Chem. Soc. *111*, 6354 (1989).
2. Bommarius A. S., Drauz K., Klenk H., Wandrey C.: Ann. N. Y. Acad. Sci. *672*, 126 (1992).
3. Sato T., Tosa T.: Bioprocess Technol. *16*, 3 (1993).

## P102 PENICILLIN ACYLASE-CATALYZED RESOLUTION OF AMINES IN AQUEOUS MEDIUM

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

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

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

REFERENCES

1. Guranda D. T., van Langen L. M., van Rantwijk F., Sheldon R. A., Švedas V. K.: Tetrahedron: Asymmetry *12*, 1645 (2001).

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

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Keywords: penicillin acylase, phenylglycinonitrile, enzymatic resolution, enantiomers

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

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

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

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

REFERENCES

1. Bruggink A., Roos E. C., de Vroom E.: Org. Process Res. Dev. *2*, 128 (1998).
2. Crosby J., Moilliet J., Parrat J. S., Turner N.: J. Chem. Soc. Perkin Trans. *1*, *1994*, 1679.
3. Sugai T., Yamazaki T., Yokoyama M., Ohta H.: Biosci. Biotechnol. Biochem. *61*, 1419 (1997).
4. Ospiran I., Jarret C., Strauss U., Kroutil W., Orru R. V. A., Felfer U., Willetts A. J., Faber K.: J. Mol. Catal. B: Enzym. *6*, 555 (1999).
5. Stolz A., Trott S., Binder M., Bauer R., Hirrlinger B., Layh N., Knackmuss H.-J.: J. Mol. Catal. B: Enzym. *5*, 137 (1998).
6. Wegman M. A., Heinemann U., van Rantwijk F., Stolz A., Sheldon R. A.: Org. Process Res. Dev. *4*, 318 (2000).
7. Hjort C. M., Godtfredsen S. E., Emborg C. J.: Chem. Tech. Biotechnol. *48*, 217 (1990).
8. Layh N., Hirrlinger B., Stolz A., Knackmuss H.-J.: Appl. Microbiol. Technol. *47*, 668 (1997).
9. Wegman M. A., Heinemann U., van Rantwijk F., Stolz A., Sheldon R. A.: J. Mol. Catal. B: Enzym. *11*, 249 (2001).
10. Didžiapetris R., Drabnig B., Schellenberger V., Jakubke H.-D., Švedas V.: FEBS Lett. *287*, 31 (1991).
11. Didžiapetris R. J., Švedas V. K.: Biomed. Biochim. Acta. *50*, 237 (1991).
12. Švedas V. K., Savchenko M. V., Beltser A. I., Guranda D. F.: Annals N. Y. Acad. Sci. *799*, 659 (1996).
13. Van Langen L. M., van Rantwijk F., Švedas V. K., Sheldon R. A.: Tetrahedron: Asymmetry *11*, 1077 (2000).

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

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

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

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

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

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

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

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

REFERENCES

1. Cull S. G., Holbrey J. D., Vargas-Mora V., Seddon K. R., Lye G. J.: Biotechnol. Bioeng. *69*, 227 (2000).
2. Sheldon R.: Chem. Commun. *2001*, 2399.

3. de Vroom E.: Chimica Oggi, *17*, 65 (1999).
4. Anthony J. L., Maginn E. J., Brennecke J. F.: J. Phys. Chem. *109*, 7315 (2002).

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

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

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

REFERENCES

1. Hoffmann C.:, Franzreb M.: Höll W. H.: IEEE Transact. Apl. Supercond. *12*, 963 (2002).
2. Kasche V.: Enzyme Microb. Technol. *8*, 4 (1986).
3. Galunsky B., Kasche V.: Adv. Synth. Catal. *344*, 1115 (2002).

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

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Keywords: environmental DNA, expression cloning, penicillin amidase, screening

The metagenome constitutes a vast and almost unexplored pool of new genes and, consequently, potential new biocatalysts. We used microorganisms indigenous to different types of soil and sediment as a source of DNA to construct environmental gene banks in *E. coli*, which were subsequently screened for clones encoding penicillin amidase activity. In one case, DNA was isolated from soil without any intermediate cultivation step to preserve a high degree of genetic diversity, which was demonstrated by DGGE analysis of the starting material and the presence of various enzymatic activities in the resulting gene bank[1]. When screening about 200 Mb of environmental DNA, clones exhibiting glucosidase (2), β-lactamase (4), amylase (1), and amidase activity (2) were found. To possibly reduce the number of clones required to find one of our actual target enzymes, DNA was also isolated from enrichment cultures, in which one single or a mixture of different aromatic and non-aromatic acetamide and glycine amide derivatives were supplied as sole nitrogen source. Amplified gene libraries were searched, using a three-step screening procedure that started with growth selection of amidase-expressing clones ( > 200,000 individual clones input), followed by colorimetric screening of positives in microtiter plates (300 clones), and a final round of HPLC analysis, in which a limited number of different clones (7) were tested for their capacities in β-lactam antibiotic synthesis.

This procedure, although carried out on a relatively small scale, resulted in the isolation of 7 new amidases including one with penicillin amidase activity. Results from sequence analysis and detailed biochemical and kinetic characterization of the recovered enzymes will be presented.

REFERENCES

1. Gabor E. M., de Vries E. J., Janssen D. B.: FEMS Microbiol. Ecol., in press.

## P107 DIRECTED EVOLUTION OF PENICILLIN ACYLASES TO IMPROVE THE SYNTHESIS OF β-LACTAM ANTIBIOTICS

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Keywords: directed evolution, penicillin acylase

Penicillin acylase of *Escherichia coli* is capable of hydrolyzing the antibiotic Penicillin G into phenylacetic acid and 6-aminopenicillanic acid (6-APA), but the enzyme can also be used in the reverse reaction, thus the coupling of a synthetic acylgroup to 6-APA. The aim of this project is to improve these synthetic properties, so penicillin acylase can be used more efficient in the production of semi-synthetic antibiotics.

To achieve this goal we used family-gene shuffling[1] combined with error prone PCR. In this directed evolution experiment the 3 genes of *Escherichia coli, Kluyvera cryocrescens* and *Providencia rettgeri* were used. These genes encode proteins that have more then 60 % sequence identity.

To check if our protocol resulted in hybrids between the different genes, we have performed a DNA-restriction with *Rsa*I. The three genes show a different pattern on an agarose gel after restriction. Four out of 15 randomly picked transformants had a restriction pattern that suggests the shuffled genes are hybrids. DNA sequencing confirmed that these four genes are indeed hybrid genes.

Gene shuffling generates a large number of mutants and to find improved mutants efficient selection and rapid screening methods are of utmost importance. To eliminate screening of inactive mutants a growth selection is used. The screening is done with the use of HPLC coupled to an pipetting robot. Screening resulted in three enzymes with improved synthetic properties. These enzymes have better synthesis over hydrolysis ratio, while retaining the high activity of the wild type enzyme. With the means of site-directed mutagenesis also mutants with better a better synthesis over hydrolysis ratio have been made, but all of these mutants have a 10-fold reduced activity.

Family-gene shuffling proves to be a good way to make penicillin mutants with improved synthetic properties, while retaining the high activity of the wild type enzyme.

REFERENCES

1. Crameri A., Raillard S. A., Bermudez E., Stemmer W. P.: Nature *391*, 288 (1998).

## P108 HOMOLOGY MODEL OF PENICILLIN ACYLASE FROM *Alcaligenes faecalis* AND *in silico* EVALUATION OF ITS SELECTIVITY

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Keywords: penicillin acylase, homology modelling, enzyme models, enzyme selectivity

Among industrially employed enzymes, penicillin acylase is one of the most widely studied and used, since the β-lactam antibiotics industry has replaced the traditional chemical multistep process for the production of 6-aminopenicillanic acid by an enzymatic one, just using penicillin acylase (PA)[1]. The most common source of commercially available penicillin acylase is *Escherichia coli* (PA-EC), though the same enzyme from *Alcaligenes faecalis* (PA-AF) has recently received attention in the literature[2–4], especially for its high synthetic efficiency in enantioselective synthesis and its high thermostability.

Since up to date no crystallographic data are available for PA-AF, a tridimensional model of PA-AF was built up by means of homology modelling using three different crystal structures of penicillin acylase from various sources. An *in silico* selectivity study was performed to compare this homology model to the PA-EC in order to point out selectivity differences between the two enzymes. The GRID/PCA technique, that conjugates molecular mechanics with multivariate analysis and was originally thought for drug design, was applied to identify the regions of the active sites where the PAs potentially engage different interactions with ligands. GRID/PCA method is able to point out differences and also to evaluate their entity (nature and strength) after having cleared away the “noise” of non-significant differences. It proved to be a very effective tool for the comparison of the two structures on a rational basis, while speeding up the computational analysis. The structural differences pointed out by GRID/PCA were further analysed and confirmed by molecular docking simulations. The PA-AF homology model provided the structural basis for the explanation of the different enantioselectivity of the enzyme previously demonstrated experimentally and reported in the literature. Furthermore,

a different selectivity towards the 4-hydroxyphenylacetic residue was predicted on the basis of a change in the aminoacid sequence in position B:67.

The PA-AF tridimensional homology model represents a valuable tool for fully exploiting this attractive and efficient biocatalyst, especially in enantioselective acylations of amines.

REFERENCES

1. Wegman M. A., Janssen M. H. A., van Rantwijk F., Sheldon R. A.: Adv. Synth. Catal. *343*, 559 (1995).
2. van Langen L. M., Oosthoek N. H. P., Guranda D. T., van Rantwijk F., Švedas V. K., Sheldon R. A.: Tetrahedron: Asymmetry *11*, 4593 (2000).
3. Guranda D. T., van Langen L. M., van Rantwijk F., Sheldon R. A., Švedas V. K.: Tetrahedron: Asymmetry *12*, 1645 (2001).
4. Švedas V. K., Guranda D. T., van Langen L. M., van Rantwijk F., Sheldon R. A.: FEBS Lett. *417*, 414 (1997).

## P109 NOVEL EPOXIDE HYDROLASES IDENTIFIED BY GENOME ANALYSIS

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Keywords: epoxide hydrolase, enantioselectivity, sequence database screening

With recent progress in sequencing of whole genomes, a large amount of data has become available that can be screened for promising genes encoding biotransformation enzymes. We have explored enzymes that share the same canonical α/β-hydrolase fold and contain a Ser/Asp-His-Acid catalytic triad. The different families were distinguished using multiple sequence alignments and phylogenetic analysis. This analysis was done using both sequences of well-characterised proteins and sequences obtained with a BLAST search using as a query the amino acid sequence of an enzyme of known function. The focus was on epoxide hydrolases, since they can be used in kinetic resolutions of epoxides and are therefore of interest to biocatalytic applications.

The multiple sequence alignments and phylogenetic analysis revealed that at least three families of epoxide hydrolases can be distinguished in addition to three families of proteins that consist only of putative sequences that share the conserved residues and motifs of epoxide hydrolases. The motifs present were the Asp-His-Asp catalytic triad, at least one putative ring-opening tyrosine, and the G-x-G-x-S and H-G-x-P motifs.

To test their suitability in biocatalytic applications, some genes of a subclass of family 2, to which also the plant and mammalian cytosolic epoxide hydrolases belong, were cloned as a Maltose Binding Protein (MBP) N-terminal fusion and expressed *in Escherichia coli*, in order to prevent possible inclusion body formation and facilitate purification. The putative epoxide hydrolases from *Bacillus subtilis* (Bsueh), *Deinnococcus radiodurans* (Draeh) and *Nostoc punctiforme* (Npueh1) all showed activity towards pNSO and/or pNPGE with opposite enantiopreference as compared to the well-studied epoxide hydrolases from *A. radiobacter* AD1 (ref.[1]), *Aspergillus niger*[2] and *Rhodotorula glutinis*[3]. One example of an enantioselective conversion of phenyl glycidyl ether with the same enantiopreference as the three newly cloned enzymes is the whole cell biocatalyst *Bacillus megaterium* ECU1001 (ref.[4]). These results show that in addition to screening of large culture collections of microorganisms for epoxide hydrolase activity, the *in silico* available sequence space can be successfully screened for new enantioselective epoxide hydrolases with complementing stereospecific properties as compared to many of the well known cloned microbial enzymes.

REFERENCES

1. Lutje Spelberg J. H., Rink R., Kellogg R. M., Janssen D. B.: Tetrahedron: Asymmetry *9*, 459 (1998).
2. Morriseau C., Nellaiah H., Archelas A., Furstoss R., Barratti J.: Enzyme Microb. Technol *20*, 446 (1997).
3. Kronenburg N. A. E., de Bont J. A. M., Fischer L.: J. Mol. Catal. B: Enzym. *16*, 121 (2001).
4. Tang Y., Xu J., Ye Q., Schulze B.: J. Mol. Catal. B: Enzym. *13*, 61 (2001).

## P110 IMMOBILISATION AND STABILIZATION OF THE *A. niger* EPOXIDE HYDROLASE. A NOVEL BIOCATALYTIC TOOL FOR REPEATED-BATCH HYDROLYTIC KINETIC RESOLUTION OF EPOXIDES

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Keywords: epoxide hydrolase, immobilisation, *Aspergillus niger*, eupergit, resolution

Studies aimed to immobilise the *Aspergillus niger* epoxide hydrolase were performed[1]. The use of conventional approaches – i. e. of commercially available supports and classical methodologies – only led to low stabilisation and unsatisfactory enzymatic activity recovery. Therefore, a new strategy based on the use of a "second generation" type of support allowing multi-point covalent immobilization – i. e. Eupergit C activated with ethylene diamine (Eupergit C/EDA)[2] and of an adequate experimental procedure, allowed to prepare an immobilized biocatalyst with 70 % retention of the initial enzymatic activity and a stabilisation factor of about 30. Interestingly, this biocatalyst also led to a noticeable increase of the E value for the resolution of two test substrates – i. e. styrene oxide and *p*-Cl-styrene oxide. This was improved from about 25 to 56 and from 40 to 100, respectively. A typical repeated batch experiment indicated that the thus immobilized enzyme could be re-used for over 12 cycles without any noticeable loss of enzymatic activity and enantioselectivity. This therefore opens the way to the use of an "heterogeneous" methodology for achieving the preparation of various enantiopure epoxides *via* biocatalysed hydrolytic kinetic resolution.

REFERENCES

1. Archelas A., Arand M., Baratti J., Furstoss R.: French Patent Application 9905711 (1999), and International Patent Application WO 00/68394 (CAN 133: 360460) (2000). This enzyme is presently commercially available from FLUKA (ref: 71832).
2. Mateo C., Fernandez-Lorente G., Abian O., Fernández--Lafuente R., Guisan J. M.: Biomacromolecules *1*, 739 (2000).

## P111 A HIGH-PERFORMANCE EPOXIDE HYDROLASE REACTOR. APPLICATION TO THE PREPARATIVE SCALE SYNTHESIS OF AZOLE ANTIFUNGAL AGENTS KEY SYNTHONS

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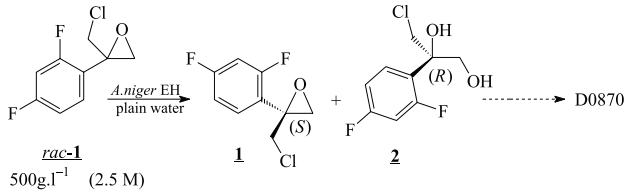
Keywords: epoxide hydrolase, D0870, resolution, *Aspergillus niger*

In this presentation we describe a high performance preparative scale resolution of *rac*-**1** – at a very high substrate concentration (i. e. 500 g.l$^{-1}$, 2.5 M) – using the fungal *Aspergillus niger* epoxide hydrolase[2]. This affords both *(S)*-chloroepoxide **1** and *(R)*-chloro-diol **2** in enantiopure form[3]. The thus obtained products both allow the formal synthesis of the enantiopure eutomer of D0870, an efficient bis-triazole antifungal agent[4].




During the scale up of this bioreactor, a surprising reaction rate enhancement was observed at high substrate concentration. A comparative study with the enzymatic resolution of styrene oxide using the same enzyme showed that the formed diol **2** was a strong activator.

REFERENCES

1. Archelas A., Furstoss R.: Curr. Opin. Chem. Biol. *5*, 112 (2001).
2. Archelas A., Arand M., Baratti J., Furstoss R.: French Patent Application 9905711 (1999) and International Patent Application WO 00/68394 (CAN 133: 360460) (2000). This enzyme is presently commercially available from FLUKA (ref: 71832).
3. Monfort N., Archelas A., Furstoss R.: Tetrahedron: Asymmetry *13*, 2399 (2002).
4. Wardle H. M., Law D., Moore C. B., Mason C., Denning D. W.: Antimicrob. Agents Chemother. *39*, 868 (1995).

## P112 PREPARATIVE SCALE ENZYMATIC KINETIC RESOLUTION OF GLYCIDYL ACETAL DERIVATIVES USING THE *A. niger* EPOXIDE HYDROLASE

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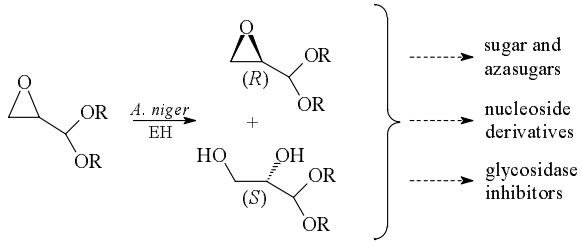
Keywords: epoxide hydrolase, glycidyl acetal, resolution, *Aspergillus niger*

The general objective of our work is to develop efficient biocatalytic approaches for the synthesis of enantiopure epoxides (or vicinal diols) by performing the hydrolytic kinetic resolution of racemic epoxides using an epoxide hydrolase (EH)[1].

Glycidyl dialkyl acetals are very interesting chiral building blocks because these molecules are bearing two chemically different reactive sites, i. e. an oxirane ring and a protected

aldehyde moiety. They have been used as C3 chirons to carry out the synthesis of various bioactive compounds like for example azasugars, nucleoside derivatives or glycosidase inhibitors. In this presentation we propose a preparative scale enzymatic process, implying an epoxide hydrolase as biocatalyst, to synthetise these compounds in enantiopure form.




The biocatalyzed hydrolytic kinetic resolution of some glycidyl dialkyl acetals i. e. ethyl-, isopropyl- and 2,2-dimethylen acetals derivatives by the *Aspergillus niger* epoxide hydrolase has been explored[1,2]. A comparative study using whole cells, crude extracts of the wild type strain, as well as partially purified recombinant (immobilized) enzyme to hydrolyse these substrates has been carried out. From this study it appeared that the preparation of the two enantiomers of these products could be easily achieved at a very high substrate concentration (>200 $g.l^{-1}$). A preparative multi-gram scale resolution showing the feasibility and the interest of this enzymatic process will be described.

REFERENCES

1. Archelas A., Arand M., Baratti J., Furstoss R.: French Patent Application 9905711 (1999) and International Patent Application WO 00/68394 (CAN 133: 360460) (2000). This enzyme is presently commercially available from FLUKA (ref: 71832).
2. Archelas A., Furstoss R.: Curr. Opin. Chem. Biol. *5*, 112 (2001).

## P113 PREPARATION AND PROPERTIES OF IMMOBILIZED EPOXIDE HYDROLASE FROM *Aspergillus niger*

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Keywords: *Aspergillus niger*, epoxide hydrolase, enantioselective hydrolysis, immobilization

Chiral epoxides and vicinal diols are high-value intermediates for the synthesis of biologically active pharmaceutical compounds. In the last years, a great interest has been devoted to biocatalytic approach involving the hydrolytic activity of epoxide hydrolases (EC 3.3.2.3) to prepare these important building blocks in enantiopure form[1–2]. The enzymatic way is an interesting alternative approach to the chemical methods to obtain enantiopure epoxides. A research work done in our laboratory has demonstrated that epoxide hydrolase (EH) from the *Aspergillus niger* is a very interesting biocatalyst with high activity and enantioselectivity on derivatives of styrene oxide[3]. Bioconversions at a multi-gram scale have been demonstrated using the free enzyme[4]. However, the enzyme was not stable in these conditions and no recycle was possible. The lack of biocatalyst recovery hampered the practical use of the process at higher scale level. Thus, the EH from *Aspergillus niger* was immobilized as a mean to get an easy enzyme recovery, as a goal to obtain a more stable biocatalyst and as a way to run continuous reactors[5]. Simple immobilization by adsorption onto DEAE--cellulose was used in this study and EH activity was tested by hydrolysis of racemic *para*-chlorostyrene oxide *p*CSO. The protein loading varied from 2 to 50 $mg.g^{-1}$ of support. The retention of activity was 70 % in the range of 2–10 $mg.g^{-1}$ of immobilized proteins and further decreased due to diffusional limitations. The optimal amount of 10 $mg.g^{-1}$ was selected, in these conditions the immobilization yield on activity was 99 %. Analysis of the adsorption isotherm of EH onto DEAE-cellulose using the Langmuir model resulted in the following constants: $K_L$ = 12.7 $mg.ml^{-1}$, *[A]* max = 124 $mg.g^{-1}$ which suggested that the enzyme had a high affinity for the support. The temperature where the activity was maximal (40 °C) and the activation energy (38.8 $kJ.mol^{-1}$) for the immobilized EH were similar to those for the free EH. The effect of temperature on enzyme stability showed no clear stabilization upon immobilization. The optimal pH was about one unit less (6.5 and 7.5) for the immobilized EH than for the free enzyme. This difference was explained by a microenvironmental effect due to the positive charges of the DEAE cellulose.

The diminution of the specificity constant, $k_{cat}/K_{Mapp}$, for *p*CSO after immobilization came from variations of both $K_{Mapp}$ compared to $k_{cat}$. The immobilization slightly affected the enantioselectivity of EH as well as temperature of reaction with $E$ = 29 at 27 °C and $E$ = 60 at 4 °C. The operational half-life of the immobilized EH in continuous packed-bed reactor at 4 °C was 27 days (substrate concentration of 4 mM). This catalyst was tested in repeated batches to achieve the resolution of racemic *p*CSO in monophasic and biphasic conditions. At 4 mM (monophasic system) significant recovery of activity (80 %) was observed after 7 runs. More interestingly, from a preparative point of view, at a concentration of 2M (306 $g.l^{-1}$, biphasic condition) a high enantiomeric excess of the residual epoxide could be always reached after seven cycles, the reaction time being only increased from 4 to 30 h.

REFERENCES

1. Archelas A., Furstoss R.: Trends biotechnol. *16*, 108 (1998).
2. Archelas A., Furstoss R.: Curr. Opin. Chem. Biol. *5*, 112 (2001).
3. Morisseau C., Nellaiah H., Archelas A., Furstoss R., Baratti J. C.: Enzyme Microb. Technol. *20*, 446 (1997).
4. Manoj K. M., Archelas A., Baratti J., Furstoss R.: Tetrahedron *57*, 695 (2001).
5. Karboune S., Amourache H., Nellaiah C., Morisseau C., Baratti J.: Biotechnol. Lett. *23*, 1633 (2001).

## P114 BIOCATALYTIC PREPARATION OF OPTICALLY PURE EPOXIDES AND ALCOHOLS

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Keywords: epichlorohydrin, styrene oxide, epoxide hydrolase, halohydrin dehalogenase

Optically pure epoxides are important building blocks for the production of a wide range of pharmaceutical compounds. Besides synthetic methods using asymmetric catalysis, some biocatalytic methods have been described. We investigated biocatalytic applications of two enzymes obtained from the epichlorohydrin degrading bacterium *Agrobacterium radiobacter* AD1. Two enzymes are involved in the degradation of epichlorohydrin, an epoxide hydrolase (Fig. 1, steps a and c), and a halohydrin dehalogenase (step b). Halohydrins can be considered as direct precursors of epoxides since ring-closure of an optically pure halohydrin generally yields an optically pure epoxide.

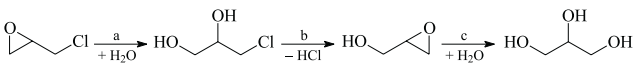


Fig. 1. **Degradation route of epichlorohydrin by *Agrobacterium radiobacter* AD1 by epoxide hydrolase** (a, c), **and halohydrin dehalogenase** (b).

Both enzymes have been brought to overexpression making them available in large quantities for biocatalytic applications. With the epoxide hydrolase several optically pure aromatic epoxides can be obtained by kinetic resolution with moderate to high enantioselectivity. Aliphatic epoxides such as epichlorohydrin were converted without any enantioselectivity.

The halohydrin dehalogenase enantioselectively dehalogenated 2,3-dihalo-1-propanols and aromatic halohydrins such as 2-chloro-1-phenylethanol. Because of the reversibility of the enzyme action, epoxide hydrolase was added to draw the kinetic resolutions to completion. In this way an e. e. > 99 % was reached for all tested halohydrins. This high activity and enantioselectivity of a halohydrin dehalogenase towards aromatic halohydrins has not been described before.

Using the chromogenic substrate *p*-nitrostyrene oxide, we determined the activity and equilibrium constant of the reversible ring opening with a variety of halides and nucleophiles. The halohydrin dehalogenase catalysed the highly enantioselective ($E$ > 200) and regioselective azidolysis of substituted styrene oxides. The high β-regioselectivity (>95 % β-selective) of the enzyme-catalysed reaction is opposite to the observed selectivity in the non-catalysed azide ion ring opening. We have also examined two distinct halohydrin dehalogenases from other organisms in this investigation. The recombinant enzymes from *Mycobacterium* sp. GP1 and *Arthrobacter* sp. AD2 also catalyzed the azidolysis of *p*-nitrostyrene oxide, but the $E$-value was lower than 5.

## P115 IMPROVING THE BIOCATALYTIC PROPERTIES OF A HALOHYDRIN DEHALOGENASE BY MODIFYING THE HALIDE BINDING SITE

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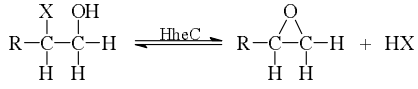
Keywords: halohydrin dehalogenase, halide binding, steady-state kinetics, stopped-flow fluorescence, enantioselectivity

Halohydrin dehalogenases, which catalyze the conversion of halohydrins to the corresponding epoxides, have been purified from several bacterial strains. Due to their high enantioselectivity, these hydrolytic enzymes represent promising biocatalytic tools.




Recently we have cloned the gene encoding the halohydrin dehalogenase from *A. radiobacter* AD1 (HheC)[1]. The kinetic mechanism of HheC has been studied by steady state and pre-steady state kinetic analysis[2]. The halide release step was identified as the slowest step in the catalytic cycle. X-ray crystallographic data of the enzyme reveals that Trp249, Tyr187, and Asn176 form hydrogen bonds, which can modulate halide release. In order to investigate the effect of these

residues on the catalytic activity of the enzyme, several mutants have been constructed (W249F, Y187F, N176A, and N176D). Steady-state kinetic studies revealed that, compared with wild-type enzyme, several mutants displayed a higher $k_{cat}$ value with some tested substrates. Moreover, stopped-flow fluorescence experiments showed that both binding and release of halide with these mutant enzymes is much faster than with wild-type HheC. Interestingly, it was found that also the enantioselectivity towards *p*-nitro-2-bromo-1-phenylethanol could be significantly improved by one of these mutations.

REFERENCES

1. van Hylckama Vlieg J. E. T., Tang L., Lutje Spelberg J. H., Smilda T., Poelarends G. J., Bosma T., van Merode A. E. J., Fraaije M. W., Janssen D. B.: J. Bacteriol. *183*, 5058 (2001).
2. Tang L., Lutje Spelberg J. H., Fraaije M. W., Janssen D. B.: Biochemistry, submitted.

## P116 COMPUTER-ASSISTED ENGINEERING OF HALOALKANE DEHALOGENASES FOR ENVIRONMENTAL APPLICATIONS

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Keywords: biosensor, bioreactor, computer design, dehalogenation, protein engineering

Haloalkane dehalogenases (E.C. 3.8.1.5) utilize water as the only co-substrate to transform haloalkanes into inorganic halides and alcohols. The industrial production of halocarbons and the persistence of these compounds in the environment has lead to interest in dehalogenating enzymes for bioremediation purposes. Dehalogenase-containing bacteria are being used as an inoculum in the bioreactor for decontamination of ground water polluted by 1,2-dichloroethane[1]. Technologies utilising the haloalkane dehalogenases for removal of side-products from chemical synthesis of propylene oxide, epichlorohydrin and butylene oxide[2] and for biosensors allowing on-line monitoring of the presence of halogenated contaminants in the environment are under development. Catalytic properties and thermostability of natural enzymes is not optimal for these practical applications.

This project aims to study structure-function relationships and rationally re-design haloalkane dehalogenases. The major objectives of the project are: (i) to understand the structural determinants of catalytic activity and substrate specificity of these enzymes, (ii) to design mutant proteins with modified catalytic properties, (iii) to construct such mutants using DNA-recombinant technology and (iv) to characterise them structurally and functionally. To meet these objectives, theoretical (i. e., bioinformatics, computer modelling) and experimental (i. e., molecular biology, enzymology and X-ray crystallography) methods are being employed in parallel. The presentation will demonstrate the benefits of such a combined approach not only for the construction of more efficient biocatalysts, but also for better understanding of fundamental principles of enzymatic catalysis that are applicable also to other enzymes[3–7].

REFERENCES

1. Stucki G., Thuer M.: Environ. Sci. Technol. *29*, 2339 (1995).
2. Swanson P. E.: Curr. Opin. Biotech. *10*, 365 (1999).
3. Bohac M., Nagata Y., Prokop Z., Prokop M., Monincova M., Tsuda M., Koca J., Damborsky J.: Biochemistry *41*, 14272 (2002).
4. Bosma T., Damborsky J., Stucki G., Janssen D. B.: Appl. Env. Microbiol. *68*, 3582 (2002).
5. Otyepka M., Damborsky J.: Prot. Sci. *11*, 1206 (2002).
6. Oakley A. J., Prokop Z., Bohac M., Kmunicek J., Jedlicka T., Monincova M., Kuta-Smatanova I., Nagata Y., Damborsky J., Wilce M. C. J.: Biochemistry *41*, 4847 (2002).
7. Nagata Y., Prokop Z., Marvanova S., Sykorova J., Monincova M., Tsuda M., Damborsky, J.: Appl. Env. Microbiol., in press.