Ph.D. thesis booklet

INVESTIGATION OF BIOCATALYSTS AND BIOCATALYTIC PROCESSES AND THEIR SYNTHETIC APPLICATIONS

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1. INTRODUCTION AND AIMS

Development of novel biocatalytic methods is a continuously growing area of chemistry, microbiology and genetic engineering - due to the fact that biocatalysts are selective, easy-to-handle and environmentally friendly. Industrial applications are widespread as biocatalytic steps are already being used to manufacture a wide range of products, including drugs, agricultural chemicals, organics, fine chemicals and plastics. Because the demand for the enantiopure form of the chiral compounds has been increasing rapidly, novel microorganisms and/or their enzymes are subject of screening to produce such chemicals.

The primary aim of my Ph.D. research was to investigate the enzyme production of thermophilic filamentous fungi strains and to study the lipase/carboxylesterase activities applied effectively as a general biocatalyst in organic synthesis practice. Investigation of these enzyme products in common and novel biocatalyc processes was also a part of my research work.

2. SUMMARY OF NOVEL SCIENTIFIC RESULTS

2.1. Shake flask fermentation of thermophilic filamentous fungi[1]

45 thermophilic filamentous fungi strains (including 18 strains which has not been identified yet) were cultivated on two different media and the supernatants were assayed for lipase/carboxylesterase activities using olive-oil, *p*-nitrophenyl palmitate and *p*-nitrophenyl butyrate as substrates.

Shake flask fermentation is the most commonly used, laboratory-scale method for the cultivation of microorganisms, providing results that correlate well with results obtained from submerged fermentation, the technology with wide-spread industrial application.

Among the thermophilic fungal strains selected for our lipase/carboxylesterase study, *Chaetomium thermophilum*, *Humicola grisea*,

Humicola insolens, *Paecilomyces* sp., *Sporotrichum thermophile*, *Talaromyces emersonii*, *Talaromyces thermophilus*, *Thermoascus aurantiacus*, *Thermoascus thermophilus* and *Thermomyces lanuginosus* have been reported for such enzyme production. According to our best knowledge, lipase/carboxyester hydrolase activity of *Myceliophthora thermophila*, *Thielavia terrestris* and *Thermomucor indicae-seudaticae* has not been published yet.

The 90 crude enzyme powders (acetone precipitated supernatants) were tested as biocatalysts in organic solvents.

2.2. Solid state fermentation of thermophilic filamentous fungi

25 strains of thermophilic filamentous fungi exhibiting high enzymatic activity were chosen for cultivation on two different solid state media and the lipase/hydrolase activities in the supernatant were evaluated. It was found that in several cases higher lipase / hydrolase activity could be achieved by solid state fermentation than by the shake flask fermentation used earlier for thermophilic filamentous fungi cultivation. About 45 enzyme products were prepared by drying the fermentation matrix containing the fungal mycelium. These products proved to be inexpensive, easy-to-handle and reliable, therefore, their patenting has been taken into consideration.

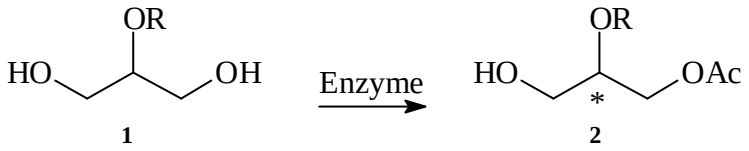
2.3.Testing of biocatalysts in organic syntheses

Although hydrolases (lipases/esterases) were detected in numerous thermophilic fungi strains, only a few have been evaluated as synthetic biocatalyst yet. Therefore, we thought worthwhile to characterize the biocatalytic abilities of enzymes from thermophilic fungi by two typical enantioselective processes. Thus, enantiomer selectivity and the enantiotopic selectivity by desymmetrization were studied both by enzymatic acetylation with vinyl acetate.

The tested biocatalysts proved to be comparable to the commercially available enzymes with respect to the degree of enantiomer selectivity, whereas they exhibited a wider range of enantiotopic selectivity than the most common commercial enzymes.

2.3.1. Investigation of enantiotopic selective biotransformations

*2.3.1.1. Synthesis and enzymatic reactions of 2-acyloxi-1,3-propanediols(**1**)* [1, 2]



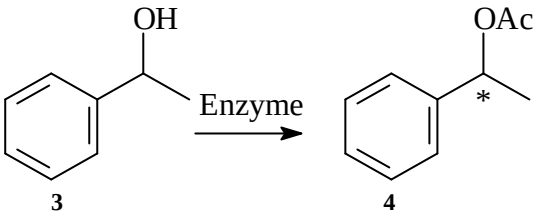
1 **2**

R: **a** benzoyl
b *p*–toluenesulfonyl

The enzyme catalyzed transformation of 2-acyloxi-1,3-propanediols (ie. 2-*O*-acylated glycerol derivatives) has not been studied prior to our work – according to our best knowledge –, even though the general application of the resulting chiral derivatives are promising as compounds for synthetic purposes. My Ph.D. work included the production of 2-benzoyloxypropane-1,3-diol (**1a**) as the first member of this group - by use of commercially available enzymes[1]. Later, the acylation reactions of 2-benzoyloxypropane-1,3-diol (**1a**) and 2-(*p*-toluenesulfonyl)-oxypropane-1,3-diol (**1b**) with vinyl acetate have been chosen for the determination of enantiotopic selectivities of the novel biocatalysts isolated from the thermophilic fungi as well[2]. The investigated biocatalysts proved to be comparable to the commercially available enzymes with respect to the degree of enantiomer selectivity, whereas they achieved wider range of enantiotopic selectivity than the most common commercial enzymes and in some cases the other enantiomer product were formed, both for benzoyl (**1a,** BUTE-7b, *R*, 94 % ee — BUTE-4a, *S*, 71 % ee) and *p*-toluenesulfonyl (**1b,** BUTE-7b, *R*, 4 % ee — BUTE-4a, *S*, 52 % ee) derivatives[2].

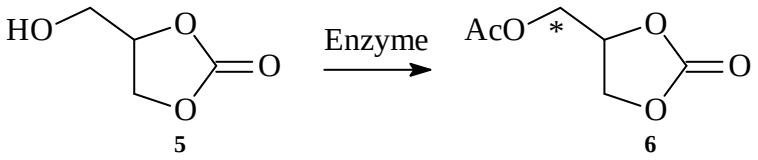
2.3.2. Investigation of enantiomer selective biotransformations

*2.3.2.1. Enzyme catalyzed acylation of 1-phenylethanol(**3**)* [1]



The enantiomers of 1-phenylethanol (**3**) are of interest since they are used as chiral reagents for determination of enantiomeric purity and for resolution of acids or for asymmetric opening of cyclic anhydrides and of epoxides. The enzymatic kinetic resolution of *racemic* 1-phenylethanol (*rac*-**3**) or its esters is well published. Therefore, acetylation of 1-phenylethanol (**3**) with vinyl acetate in hexane was chosen for investigation of enantiomer selectivity of our enzyme preparations. The degree of enantiomer selectivity (*E*) was precisely calculated from the conversion - enantiomeric composition data obtained by GC on chiral stationary phase. A number of our enzyme products performed within the selectivity range of commercially available enzymes, while several others provided products with higher enantiomer purity than the best commercially available enzyme. (**3,** BUTE-3a, *R*, >99 % ee; E>1000).

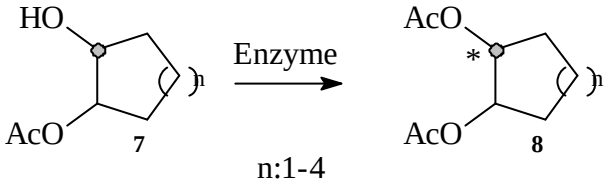
*2.3.2.2. Enzyme catalyzed reactions of glycerol-carbonate(**5**)*



The enzyme catalysed acylation and hydrolysis of *racemic* glycerol-carbonate – where both optical active (*R*)-glycerol-carbonate ((*R*)-**5**) and (*S*)-glycerol-carbonate ((*S*)-**5**) have been obtained in medium selectivity – are known processes using commercial enzymes. Our aim was to assay this transformation by acylation with about 30 commercially available enzymes and

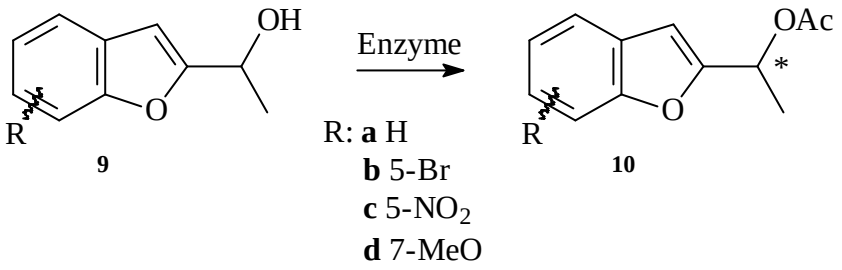
enzyme preparations obtained by shake flask fermentation (about 90) and by solid state fermentation (about 13). Among the tested preparations, BUTE-6a enzyme isolated in our laboratories proved to be more selective compared to the commercially available, most selective enzyme, Lipase PS (*S*, 52 % ee, *E*=5,1) BUTE-6a (*S*, 74 % ee, *E*=10). With the purpose of tracking the reactions, separation of the alcohol and acetate enantiomers was developed using GC on chiral stationary phase.

*2.3.2.3.Enzyme catalyzed reactions of trans-2-acetoxycycloalkan-1-ols(**7**)*[3]



Kinetic resolution of a series of *racemic trans*-cycloalkane-1,2-diol monoacetates (**7**) (cyclopentane, -hexane, -heptane and -octane derivatives) was performed by enantiomer selective transesterification with vinyl acetate catalysed by commercial and own-prepared lipases to yield (*R*,*R*) diacetates ((*R*,*R*)-**8**) and (*S*,*S*) monoacetates ((*S*,*S*)-**7**) in high enantiomeric purity. The enzymes which performed best on the six-membered monoacetate were also quite selective towards the five- and seven-membered analogues, thus enabling the preparation of highly enantiopure (*R*,*R*)-diacetates ((*R*,*R*)-**8**) and (*S*,*S*) monoacetates ((*S*,*S*)-**7**). Unfortunately, acylation of the eight-membered monoacetate gave the (*S*,*S*)-monoacetate ((*S*,*S*)-**7**) only in lower enantiomeric excess. With the purpose of tracking reactions, separation of diacetate and monoacetate enantiomers was developed using GC on chiral stationary phase. Preparative scale enzymatic acylation/hydrolysis were also performed with the best three enzymes. In order to simplify the processing of the preparative reactions and obtain the pure enantiomers the final chromatographic procedure was replaced by an extraction step.

*2.3.2.4. Production and enzymatic reactions of 1-(benzofuran-2-yl)ethanols(***9***)*[4,5]

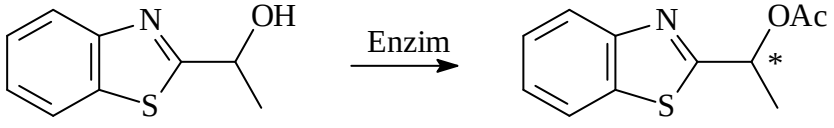


9 **10**

In order to study reduction performed by baker's yeast for the production of hydroxymethyl and acetoxymethyl [4], a novel procedure, including enzymatic ethanolysis, was developed. With the chemical reduction of the intermediate benzofuranyl methyl ketones racemic alcohols were produced and their lipase-catalysed acylation reactions were investigated. The lipase-catalysed acylation of *racemic* 1-(benzofuran-2-yl)ethanols (*rac*-**9**) was performed successfully with significantly high enantiomer selectivity using a number of enzymes, including the commercially available, immobilized Lipozyme TL IM enzyme and the self-isolated

BUTE-3b biocatalyst. The degree of enantiomer selectivity for enzymatic alcoholysis/hydrolysis processes starting from *racemic* 1-acetoxy-1-(benzofuran-2-yl)ethane (*rac*-**10**) was also investigated among various conditions including supercritical CO_2 medium, but lower enantiomer selectivity was measured than that of the acylation process.

*2.3.2.5. Production and enzymatic reactions of 1-(benzthiazol-2-yl)ethanol (***11***)*



11 **12**

Similarly to the benzofurane derivatives, high selectivities were achieved with the enzyme catalyzed acetlyation of 1-(benzthiazol-2-yl)ethanol (**11**).

2.4. Conclusions

According to our initial goals several biocatalysts for general synthetic applications have been produced. Among the enzyme preparations tested in organic synthetic reactions a number of biocatalysts have been found, which catalyze processes at a rate and with a selectivity similar to that of commercially available enzymes. In a few cases, our enzyme preparations exhibited stereoselectivities opposite to those found with other enzymes. Our most significant result was the production of an enzyme preparation (BUTE-3b), which catalyzed many processes with similar or even higher selectivity than the commercially available enzymes. Patenting of this enzyme preparation is being considered.

3. PUBLISHED ARTICLES ON THE SUBJECT OF THE DISSERTATION

3.1. Articles

1. Bódai, V., Peredi, R., Bálint, J., Egri, G., Novák, L., Szakács, Gy., Poppe, L.: Novel Hydrolases from Thermophilic Filamentous Fungi for Enantiomer and Enantiotopic Selective Biotransformations, *Adv. Synth. Catal*., *345*, **2003**, (6-7), 811-818. [**2.18**]
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4. Paizs, Cs.; Tosa, M.; Majdik, C.; Bódai, V.; Novák, L.; Irimie, F.-D.; Poppe, L.: Chemo-enzymic preparation of hydroxymethyl ketones, *J. Chem. Soc., Perkin 1*, *21*, **2002**, 2400-2402. [**2.18**]
5. Paizs, Cs., Tosa, M.; Bódai, V.; Szakács, Gy.; Kmecz, I.; Simándi, B.; Majdik, C.; Novák, L.; Irimie, F.D.; Poppe, L.: Kinetic resolution of 1-(benzofuran-2-yl)ethanols by lipase-catalysed enantiomer selective reactions, *Tetrahedron: Asymmetry*, *14*, **2003**, 1943-1949. [**2.80**]
6. Bódai, V.: Mikroorganizmusok alkalmazása a kémiában, *Környezetvédelem* **2002**, (3-4), 41.

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7. Poppe, L.; Paizs, Cs.; Tosa M.; Majdik C.; Pilbák, S.; Bódai,V.; Szakács, Gy Novák L.; Irimie, F.-D.: Magas enantiomertisztaságú heterociklusos vegyületek eloállítása biokatalitikus módszerekkel; **VIII. Nemzetközi Vegyészkonferencia**, **2002** november 15-17, Kolozsvár, Románia, *Erdélyi Magyar Muszaki Tudományos Társaság* 250-253; (ISBN 973-85809-8-6)
8. Bódai,V.; Paizs, Cs., Tosa M., Majdik C., Peredi, R.; Egri G.; Bálint, J.; Novák L.; Szakács, Gy., Poppe, L.: Termofil fonalasgomba lipázok szintetikus alkalmazásának lehetoségei; **VIII. Nemzetközi Vegyészkonferencia**, **2002** november 15-17, Kolozsvár, Románia, *Erdélyi Magyar Muszaki Tudományos Társaság* 62-65; (ISBN 973-85809-8-6)

9. Bódai, V.; Paizs, C.; Tosa, M.; Majdik, C.; Pilbák, S.; Novák, L.; Florin-Dan Irimie; Szakács, G.; Poppe, L.: Novel hydrolases from thermofilic fungi for stereoselective biotransformations; **BioTrans Olomouc 2003**, jun. 28- jul. 3, 2003, 6th International Symposium on Biocatalysis and Biotransformations, Olomouc, Czech Republic, CHLSAC 97; *Chemické listy 6*, 486; 487; (ISSN 0009-2770)
10. Bódai, V.; Szakács, G.; Paizs, C.; Pilbák, S.; Peredi, R.; Bálint, J.; Egri, G.; Novák, L.; Dukai, J.; Poppe, L.: Novel hydrolases from thermophilic filamentous fungi for enantiomer and enantiotopic selective biotransformations; **BioTrans Olomouc 2003**, jun. 28- jul. 3, 2003, 6th International Symposium on Biocatalysis and Biotransformations, Olomouc, Czech Republic, CHLSAC 97; *Chemické listy 6*, 487; (ISSN 0009-2770)

3.2. Oral presentation

11. Bódai, V.; Paizs, Cs., Tosa M., Majdik C., Peredi, R.; Egri G.; Bálint, J.; Novák L.; Szakács, Gy. Poppe, L.: Termofil fonalasgomba lipázok szintetikus alkalmazásának lehetoségei; **VIII. Nemzetközi Vegyészkonferencia,** 2002. November 15-17, Kolozsvár, Románia,
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13. Poppe, L.; Paizs, Cs.; Tosa M.; Majdik C.; Pilbák, S.; Bódai,V.; Szakács, Gy Novák L.; Irimie, F.-D.: Magas enantiomertisztaságú heterociklusos vegyületek eloállítása biokatalitikus módszerekkel; **VIII. Nemzetközi Vegyészkonferencia**, **2002** November 15-17, Kolozsvár, Románia,
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16. Bódai, V.; Szakács, G.; Paizs, C.; Pilbák, S.; Peredi, R.; Bálint, J.; Egri, G.; Novák, L.; Dukai, J.; Poppe, L.: Novel hydrolases from thermophilic filamentous fungi for enantiomer and enantiotopic selective biotransformations; **BioTrans Olomouc 2003**, 6th International Symposium on Biocatalysis and Biotransformations, Junius 28 - Julius 3, **2003**, Olomouc, Czech Republic.
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18. Kósa R.; Juhász R.; Bódai, V.; Szakács Gy.: Lipáztermelo termofil fonalas gombák szurovizsgálata szilárd fázisú fermentációban **Magyar Mikrobiológiai Társaság 2002 évi nagygyulése**, **2002** October 8-10, Balatonfüred.
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