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Bacterial lipases for biotechnological applications

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Abstract

Lipase genes originating from the Gram-negative bacteria *Serratia marcescens* and *Pseudomonas aeruginosa* were cloned. *S. marcescens* lipase was overexpressed in *Escherichia coli* yielding inclusion bodies which were purified and finally refolded to give enzymatically active lipase. The lipase operon of *P. aeruginosa* consisting of genes *lipA* and *lipH* was cloned behind the T7 ϕ10 promoter and overexpressed in a lipase-negative *P. aeruginosa* strain carrying a chromosomal insertion of the gene encoding T7 RNA polymerase. A 3D structural model was built for *P. aeruginosa* lipase using the coordinates of the *Burkholderia cepacia* lipase structure which has recently been solved in its open conformation by X-ray crystallography. Both lipases have been purified to homogeneity and were tested for their potential to catalyze biotechnologically important reactions. *S. marcescens* lipase stereoselectively hydrolyzed racemic isopropylideneglycerol acetate which is a basic building block in a variety of organic synthesis reactions. *P. aeruginosa* lipase was successfully used for kinetic resolution of chiral alcohols and amines giving enantiomeric excess values of $\geq 95\%$ at reaction rates of 40–50%. Our results demonstrate that both lipases can be produced at levels of 100 mg/l for *S. marcescens* and 150 mg/l for *P. aeruginosa*. The recombinant lipase proteins are promising candidates for biotechnological applications.

Keywords: Lipase; *Serratia marcescens*; *Pseudomonas aeruginosa*; Overexpression; 3D structural model; Kinetic resolution

1. Introduction

The estimated world-wide sales volume for industrial enzymes in 1995 is US$ 1 billion and this volume is foreseen to double until 2005 [1]. At least 75% of all these enzymes are hydrolases, and 90% of them are produced from microorganisms by fermentation. Following proteases and carbohydrases, lipases are considered to be the third largest group based on total sales volume. Their biotechnological potential is relying on their ability to catalyze not only the hydrolysis of a given triglyceride, but also its synthesis from glycerol and fatty acids. The main application fields for lipases include detergents, dairy, diagnostics, oil processing, and biotransformations. Recently, special emphasis is lying on the production of chiral chemicals which serve as basic building blocks in the

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production of pharmaceuticals, agrochemicals, and pesticides or insecticides [2].

In a survey of enzyme supplier companies, 136 companies were listed which produced at least 23 different lipases, however, only 6 of them were of bacterial origin [1]. This underrepresentation stands in a sharp contrast to the actual knowledge in molecular biology and biochemistry of these lipases [3,4]. In this report, we present a summary of recent results obtained with extracellular lipases from the Gram-negative species *Serratia marcescens* and *Pseudomonas aeruginosa*.

S. marcescens lipase is a protein of M_r 64800 which was purified to electrophoretic homogeneity. The structural gene was cloned and sequenced and the mechanism of secretion of this enzyme has been studied [5–7]. However, overexpression of this lipase was not reported. *P. aeruginosa* lipase (M_r: 29000) is encoded by a structural gene *lipA* which forms an operon with a second gene *lipH* encoding a lipase-specific foldase which is involved in lipase folding and/or secretion. Both genes have been overexpressed and the corresponding proteins were purified and biochemically characterized [8]. Recently, experimental evidence was obtained for a cell-density dependent type of regulation called 'quorum sensing' [9] of lipase gene expression involving a regulatory protein LipR [10].

Only few biotechnological applications of these enzymes have been reported. The asymmetric hydrolysis of 3-phenylglycidic acid ester which is a key intermediate in the synthesis of Diltiazem hydrochloride, a widely used coronary vasodilator, was carried out with *S. marcescens* lipase [11]. *P. aeruginosa* lipase was able to convert the diester dimethyl 5(3-(2-(7-chlorochinolin-2-yl)ethyl)phenyl)4,6-dithianoanedioate to its (*S*)-ester acid which is a chiral intermediate in the synthesis of the leukotriene receptor agonist Verlukast [12]. The production of this lipase has been scaled up to pilot plant bioreactors (volume: 1900 l) by Merck US [13]. In order to enable more comprehensive studies on biotechnological applications of these two enzymes, we have constructed overexpression systems for both genes. The overexpressed proteins have been isolated and tested for their ability to catalyze the kinetic resolution of racemic substrates.

2. Results and discussion

2.1. Lipase from Serratia marcescens

2.1.1. Overexpression, purification and refolding [14]

A fragment of chromosomal *S. marcescens* DNA carrying the lipase gene was cloned into the T7-expression vector pET11d and overexpressed in *E. coli* JM109/DE3. The vector contains a T7 promoter and the host strain a chromosomally encoded T7 RNA polymerase which are both inducible by addition of isopropyl-β-D-thiogalactopyranoside (IPTG) [15]. Bacteria were grown until they reached the logarithmic growth phase ($OD_{580} = 0.6$) and lipase gene expression was induced by addition of 0.4 mM IPTG. After further 3 h of growth lipase was produced at about 40% of total cellular protein. However, the overexpressed protein was not secreted but formed insoluble inclusion bodies inside the bacterial cytoplasm. These inclusion bodies were purified by centrifugation, denaturation in 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM Na_2-EDTA, and 8 M urea, and subsequent gel filtration, and were finally refolded to enzymatic activity by rapid dilution in 50 mM Tris–HCl buffer (pH 8.0) containing 1 mM Na_2-EDTA, and 10 mM $CaCl_2$ [14]. Fig. 1 shows an SDS polyacrylamide gel electrophoresis analysis at various stages of the purification procedure. After gel filtration, the silver-stained gel (lanes 4–6) revealed only one major protein band at M_r 64000 (theoretical M_r: 64800). Its identity was proven after blotting of this protein band by determination of the N-terminal amino acid sequence (amino acids no. 1–20) showing 100% identity with the se-

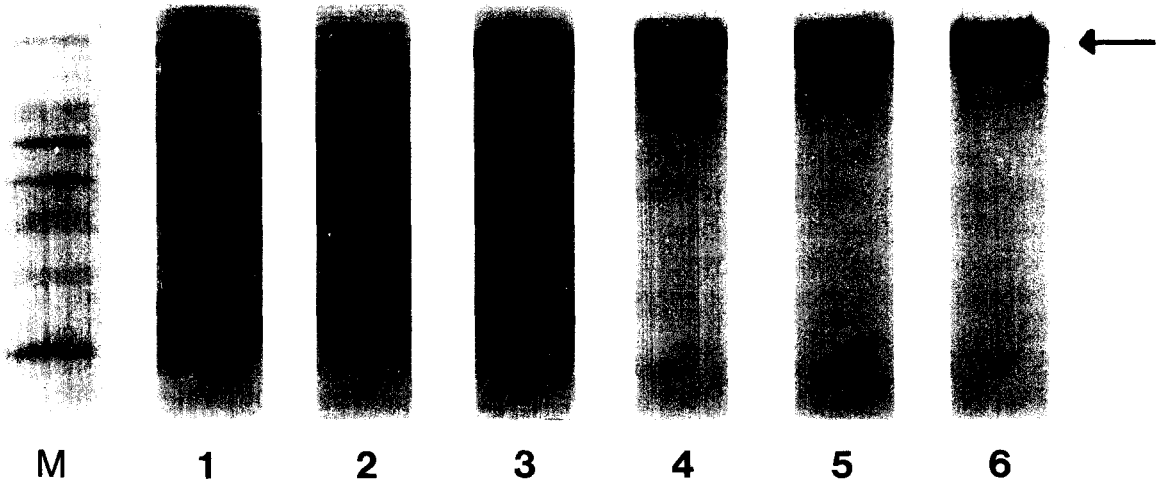


Fig. 1. Purification of *S. marcescens* lipase. Samples obtained at various stages of the purification procedure were analyzed by SDS–PAGE and staining with Coomassie Brilliant Blue R250 (lanes 1, 2, and 3 containing 10 μg of protein) and silver (lanes 4, 5, and 6 containing 5 μg of protein). The band showing lipase (M_r: 64800) is marked with an arrow. Samples were obtained from: cell lysate obtained by ultrasonication (lane 1); soluble fraction (lane 2) and sediment (lane 3) after centrifugation (Sorvall RC5B, Rotor SS34, 6000 *g* for 15 min at 4°C); gel filtration chromatography (Superdex TM200 column) (lane 4); ultracentrifugation (Kontron TGA 65, rotor SW 40, 100000 *g* for 60 min at 4°C) and refolding (lane 5); concentration of refolded lipase by ultrafiltration (lane 6).

quence deduced from the DNA sequence of the cloned gene [5]. The ability to refold extracellular lipase to enzymatic activity enabled us to isolate significant amounts of protein (100 mg per liter of culture) which could be purified in a one-step purification giving electrophoretically homogeneous lipase protein with a yield of 30%. Recently, we have obtained a polyclonal antiserum against this lipase which will be used to study localization inside the bacterial cell. Furthermore, lipase has been subjected to crystallization experiments in order to determine its 3D structure by X-ray crystallography.

2.1.2. Secretion

S. marcescens lipase does not possess an N-terminal signal sequence but instead contains multiple repeats of a glycine-rich consensus sequence (L–X–G–G–X–G–X–D) at the C-terminus. This sequence is indicative for a secretion system which consists of three different proteins, an ABC transporter (ABC), a membrane fusion protein (MFP), and an outer membrane component forming a pore-like structure (OMP) extending from the cytoplasm into the extracellular space. The corresponding genes are organized in an operon with the structural gene followed by the genes coding for ABC and MFP whereas the gene coding for OMP may be separated [16]. We could demonstrate that *S. marcescens* lipase was secreted from *E. coli* if the strain contained a plasmid carrying either the *prt D*, *E*, and *F* genes for metalloprotease secretion in *Erwinia chrysanthemi* or *hlyB*, *C*, and *tolC* genes for α-hemolysin secretion in *E. coli* coding for the ABC, MFP, and OMP proteins [5]. More recently, an operon consisting of three genes named *lipB*, *C*, and *D* was cloned from *S. marcescens*. Expression of these three genes in *E. coli* allowed secretion of both metalloprotease and lipase of *S. marcescens* [7]. Although these authors have studied secretion only in *E. coli*, genes *lipB*, *C*, and *D* presumably encode an ABC-secretion system used in *S. marcescens*. We have recently constructed a *S. marcescens* mutant strain carrying an insertion in the *lipB* gene coding for the ABC-transporter which exhibited a lipase- and protease-negative phenotype [14].

2.1.3. Biotechnological applications

Attempts to use *S. marcescens* lipase for synthesis reactions in organic solvent failed so far. At present, we assume that this lipase may need a water shell surrounding the molecule, and its replacement by organic solvent may cause irreversible denaturation. This view is supported by the finding that freeze-drying of *S. marcescens* lipase led to a decrease in enzyme activity by 75%.

However, this lipase may be useful for kinetic resolution of chiral compounds. Racemic isopropylideneglycerol acetate (IPG) which is a basic building block in a variety of organic synthesis reactions was hydrolyzed at a reaction rate of about 25% giving a 35% enantiomeric excess of the D-enantiomer. Furthermore, we have observed the stereoselective esterification of the racemic alcohol (*R,S*)-2-phenyl-1-propanol at a conversion rate of 31% and an enantiomeric excess of 20% of the ester isomer. At present, we are trying to immobilize *S. marcescens* lipase by using a newly developed method [17] in order to stabilize the enzyme thereby improving its biotechnological applications in organic chemistry.

2.2. Lipase from Pseudomonas aeruginosa

2.2.1. Cloning and overexpression

Initially, we used a two-component T7 system developed by Darzins [18] to overexpress *P. aeruginosa* lipase. The gene coding for T7 RNA polymerase is stably integrated into the chromosome of *P. aeruginosa* strain ADD1976 and a 2.8 Kb *Xmn*I/*Sma*I fragment of *P. aeruginosa* DNA containing genes *lipA* and *H* necessary to obtain enzymatically active lipase were cloned into the *Sma*I-site of plasmid pEB12 giving pEL11 with the lipase genes being expressed from the T7 gene10 promotor. Fig. 2B shows that we obtained an increase in extracellular lipase (mass of enzymatically active protein) of at least three orders of magnitude. In order to further improve this expression level and, in addition, to use this system for overexpression of mutated lipase genes we constructed a novel host strain. A transposable element containing a *lac*UV5/*lac*I^q-regulated T7 RNA polymerase gene [18] was stably integrated into the chromosome of *P. aeruginosa* strain PABS which carries a deletion in the lipase structural gene *lipA* to give strain *P. aeruginosa* PABST7.1 (Fig. 2A). In addition, a 2.8 Kb fragment containing genes *lipA* and *H* was cloned into a pUC18-derived broad host range vector called pUCPKS which also contains the T7 gene10 promotor [19] giving pUCPL7. When *P. aeruginosa* strain PABST7.1/pUCPL7 was grown in batch cultures (10 ml tryptone phosphate medium in Erlenmeyer flasks shaken at 180 rpm and 30°C) the yield of extracellular lipase was 0.15 g per l of culture supernatant without any further optimization of media and growth conditions. At present, we are trying to set up a pilot-scale production protocol for extracellular *P. aeruginosa* lipase by high-cell density fermentation of this overexpressing strain.

2.2.2. 3D structural model

A 3D structural model for *P. aeruginosa* lipase had previously been constructed [20,21]. However, this model was based on the X-ray structure of *Burkholderia glumae* (formerly *P. glumae*) lipase [22] which was solved in the closed conformation and could therefore not provide detailed information on the nature of the active site and substrate binding domains. This was the reason to build a new 3D structural model (Fig. 3) by using the X-ray coordinates of the lipase from *B. cepacia* (formerly *P. cepacia*) the structure of which was solved in its open conformation [24]. Equivalent amino acid residues in this structure have been replaced using the program O [25] and some of the side chain positions were fitted manually to prevent collisions between adjacent amino acid residues. Subsequently, 40 cycles of conjugate gradient minimisation were executed using the program XPLOR [26] and the final coordinates were analyzed with PROCHECK [27]. The resulting

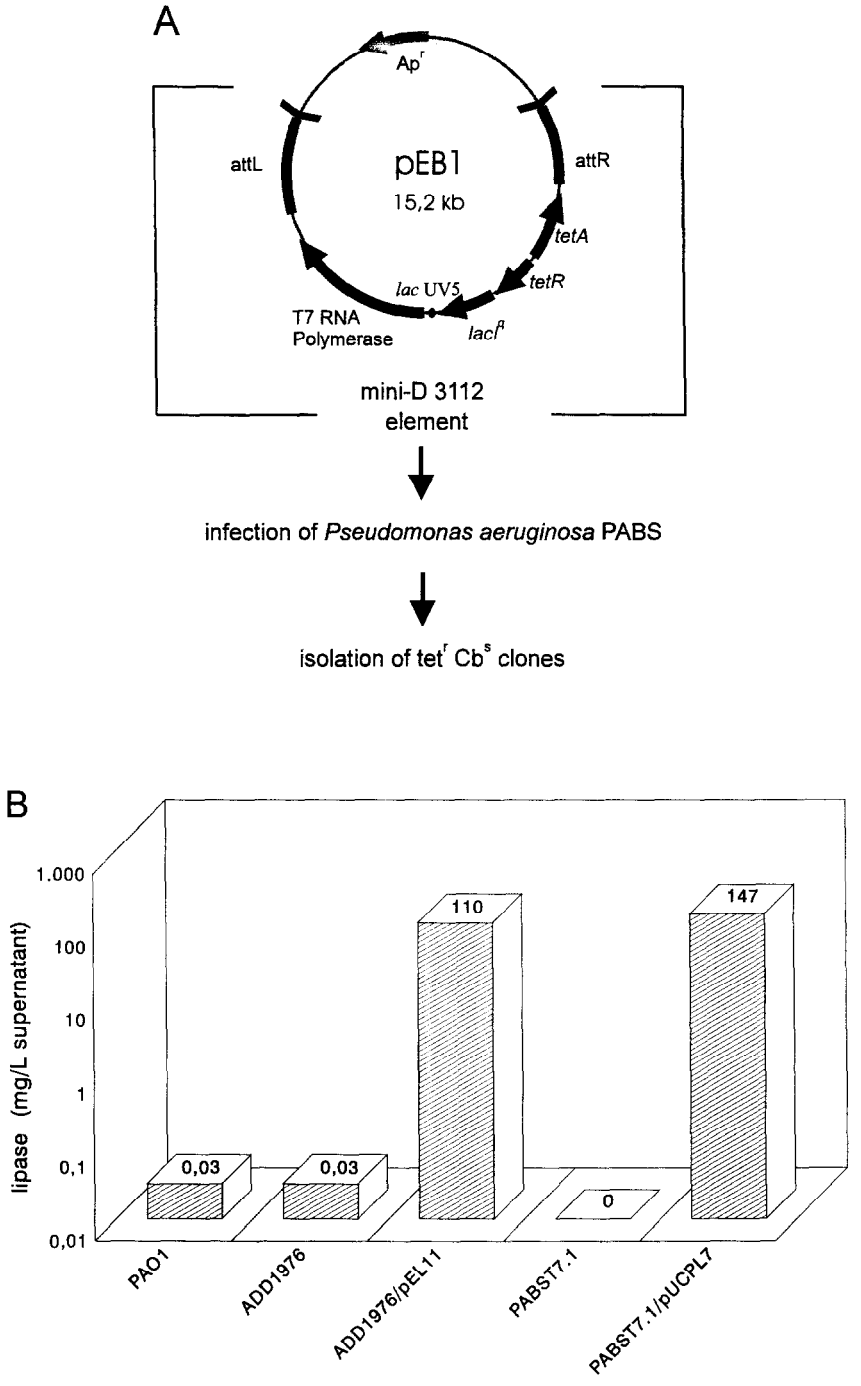


Fig. 2. Overexpression of lipase in *P. aeruginosa*. (A) Strategy to construct a host strain for overexpression of lipase by inserting the bacteriophage D3112-based transposable element mini-D3112 (shown in large brackets, see also [18]) containing the gene encoding RNA polymerase into the chromosome of the lipase-negative strain *P. aeruginosa* PABS giving expression strain *P. aeruginosa* PABST7.1. (B) Overexpression levels given in mg of enzymatically active lipase protein per l of *P. aeruginosa* culture supernatant obtained after cultivation of different strains as described in the text. Note that the ordinate is drawn to a logarithmic scale.

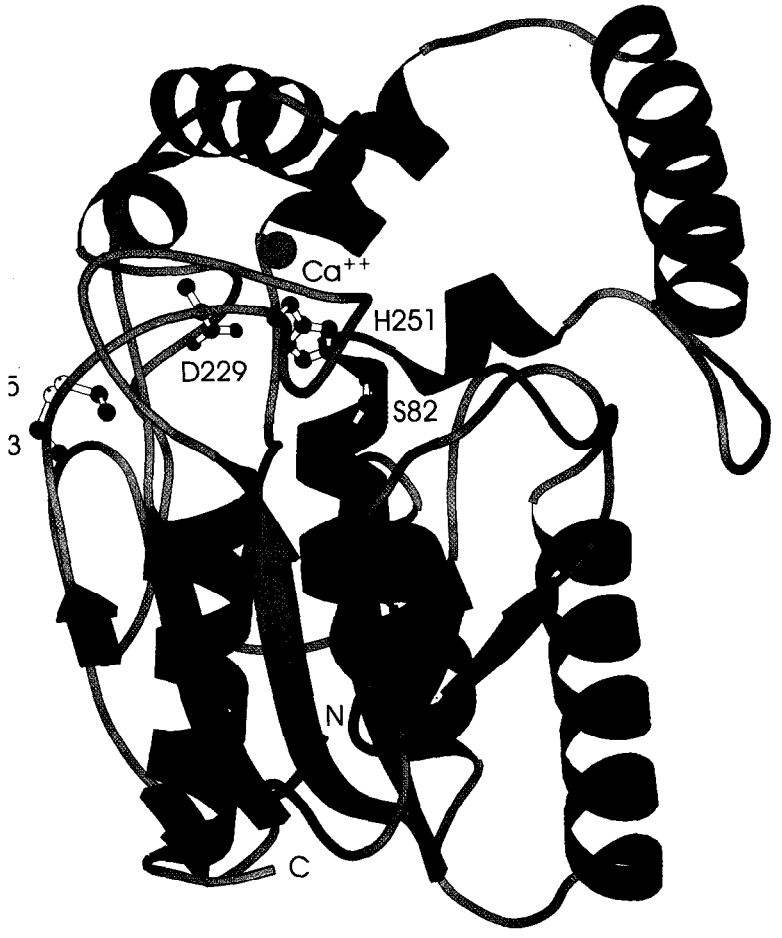


Fig. 3. 3D structural model of *P. aeruginosa* lipase shown as a ribbon plot drawn with MOLSCRIPT [23]. The catalytic triad residues S82, D229, and H251 and residues C183 and C235 forming a disulfide bond are numbered and drawn in a ball-and-stick presentation. The Ca^{2+}-ion is shown as a green ball. Amino-(N) and carboxy-(C) terminus of the lipase are indicated.

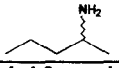
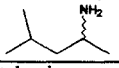
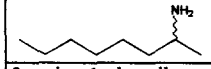
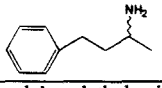
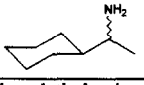
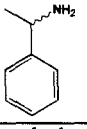
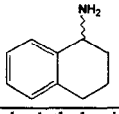
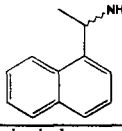
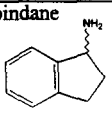
Ramachandran plot showed a phi/psi distribution of conformational angles in the allowed regions with the exception of the active site serine (Ser-82): this residue is located at the so-called nucleophilic elbow which is a β-ϵSer-α motif placing the nucleophilic serine into the active site [28]. This motif has also been found in structures of hydrolytic enzymes sharing the characteristic α/β hydrolase fold [29]. The features of the actual model further include a core structure of six central β-strands with one of them divided into a parallel and an antiparallel part and a catalytic triad which is now completely surface-accessible and consists of amino acid residues Ser-82, Asp-229, and His-251. A Ca^{2+}-ion (shown as a green ball in Fig. 3) is bound close to the active site and is stabilized by cooperative binding to residues Asp-209, Asp-253, and main chain oxygens O-257 and O-261. One disulfide bridge is formed between residues Cys-183 and Cys-235. A loop extending from Pro-122 to Leu-156 which contains an α-helix could serve as a possible lid covering the active site in the closed conformation. Based on this 3D structural model we have already introduced by site-directed mutagenesis ten different mutations into the lipase gene thereby exchanging amino acid residues involved in the

formation of the active site pocket in order to test their role in enzyme activity, substrate binding, and specificity of *P. aeruginosa* lipase.

2.2.3. Biotechnological applications

P. aeruginosa lipase hydrolyzed triglyceride substrates with a high stereoselectivity towards

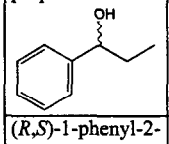
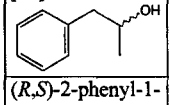
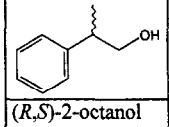
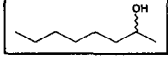
Table 1
Enantioselective conversion of amines by *P. aeruginosa* lipase

Substrate	conversion rate[b] [%]	enantiomeric excess of amide [%]
2-butylamine 	55	32
2-pentylamine 	47	96
4-methyl-2-pentylamine 	28	>98
2-octylamine 	43	81
3-amino-1-phenylbutane 	26	93
1-cyclohexylethylamine 	38	>99 [b]
1-phenylethylamine 	30	98 [b]
1,2,3,4-tetrahydro-1-naphtylamine 	32	99
1-naphtylethylamine 	20	>99 [b]
1-aminoindane 	49	96

[a] Values calculated from GC peaks represent area percent with the exception of *N*-acetyl-2-pentylamide which is mol percent.
[b] (*R*)-enantiomer determined by GC coinjection of amides obtained by amidation of the corresponding optically pure amines.

position *sn*-1 [30,31] which made it an interesting candidate for biotechnological applications. Recently, we have immobilized this lipase and used it for the kinetic resolution of two racemic model compounds, 1-phenylethanol and 2-pentylamine, respectively. Both compounds were enantioselectively acylated with high efficiency giving enantiomeric excesses of > 99% ee for the alcohol and 96% ee for the amine with average conversion rates of 50% [32]. These results led us to further investigate the potential of *P. aeruginosa* lipase for stereoselective conversion of a variety of amines, as well as primary and secondary alcohols. Culture supernatants obtained from lipase-overexpressing strains *P. aeruginosa* ADD1976/pEL11 and PABST7.1/pUCPL7 were sterile-filtered, concentrated by ultrafiltration, and freeze-dried giving crude lipase powder. This powder was suspended in tert-butyl methyl ether (for 2-pentylamine in diethyl ether), and amines were acylated with ethyl acetate or alcohols with propionic anhydride by shaking at room temperature. Periodically, aliquots were withdrawn and analyzed by gas chromatography as described previously [32]. Table 1 shows that all amines tested were acylated to give the corresponding amides, although at differing conversion rates when compared after 48 h of reaction time. High enantiomeric excess rates exceeding 90% ee were usually obtained. Interestingly, those amines possessing bulky cyclohexyl- or phenyl-groups (1-cyclohexylethylamine, 1-phenylethylamine, 1,2,3,4-tetrahydro-1-naphthylamine, 1-naphthylethylamine, and 1 aminoindane) were found to be efficiently acylated giving ee-values of ≥ 95%. An exception was 3-amino-1-phenylbutane which reacted slowly (conversion rate: 26%) and with a lower ee (93%) than the other amines. It is to early to speculate about the detailed interactions between substrate molecules and selected amino

Table 2
Enantioselective conversion of alcohols by *P. aeruginosa* lipase

Substrate	reaction time [h]	conversion rate [%]	alcohol recovered isomer[a]	alcohol recovered ee[%]	ester produced isomer[b]	ester produced ee[%]
(*R,S*)-1-phenyl-1-propanol 	6	49	*S*	73	R	97
(*R,S*)-1-phenyl-2-propanol 	6	53	*S*	22	*R*	25
(*R,S*)-2-phenyl-1-propanol 	2	60	*R*	7	*S*	15
(*R,S*)-2-octanol 	2	75	*S*	n.d.	*R*	32

[a] Determined by GC coinjection of the optically pure alcohol.
[b] Determined by GC coinjection of esters obtained by esterification of the optically pure alcohols.
n.d.: not determined.

acid residues forming the active site pocket in *P. aeruginosa* lipase, however, our results demonstrate that aromatic amines generally fit into the active site in *P. aeruginosa* lipase whereas the carbon side chain seems to be too short in butylamines and, on the contrary, too long in octylamines as reflected by low conversion rates and/or ee-values.

Racemic primary and secondary alcohols were stereoselectively esterified with *P. aeruginosa* lipase. The results presented in Table 2 demonstrate that these alcohols reacted much faster than did the amines (reaction time: 48 h); however, the overall stereoselectivity of the lipase as deduced from ee values was considerably lower towards alcohols as compared to amine substrates. It is interesting to compare the ee values obtained with racemic isomers (*R,S*)-1-phenyl-2-propanol and (*R,S*)-2-phenyl-1-propanol. The latter compound representing the primary alcohol reacted much faster, however, with inverse stereoselectivity preferentially forming the (*S*)-ester and with a somewhat lower stereoselectivity of 15% ee. Our results demonstrate that *P. aeruginosa* lipase (i) accepts a wide variety of stereochemically different substrates and (ii) yields in reasonable ee-values at acceptable conversion rates. In conclusion, *P. aeruginosa* lipase proved to be a promising enzyme for biotechnological applications, particularly in the field of chiral resolution to produce enantiomerically pure compounds for synthetic organic chemistry.

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