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Detection of a new enzyme for stereoselective hydrolysis of linalyl acetate using simple plate assays for the characterization of cloned esterases from *Burkholderia gladioli*

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Abstract

Plate assays were developed for the identification of specific hydrolytic activities of esterases from *Burkholderia gladioli*, cloned and expressed in *Escherichia coli*. Clones showing different substrate specificities were identified by fluorescence or azo-dye formation caused by the released alcohol moiety of the hydrolyzed substrates, or by colour change of pH indicators mediated by decreased pH. The use of 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS-) esters and linalyl acetate for these assays clearly allowed to discriminate substrate specificities for two different cloned esterases, EP6 and EP10. Long chain fatty acid HPTS-esters were only hydrolyzed by the EP10 clone. On the other hand, the EP6 clone showed significant activity in hydrolysis of the sterically hindered ester linalyl-acetate. Enantioselective hydrolysis of linalyl acetate could be verified with a crude EP6 preparation on a preparative scale.
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1. Introduction

Hydrolysis of racemic esters by means of hydrolytic enzymes such as proteases (Jones and Beck, 1976), lipases (Faber and Riva 1992; Ferraboschi et al., 1994) and esterases (Jones 1990; Barnier et al., 1993) has become a well established method for their resolution. Depending on the location of the chiral centre in the substrate ester—either in the acid or alcohol moiety—the production of a chiral carboxylic acid or an alcohol may be achieved by this means. Esterases represent a group of hydrolytic enzymes of broad

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natural variety concerning substrate and reaction type. Due to their diverse substrate specificities which differ from that of lipases, esterases may provide a substantial broadening of the currently available tool kit for biocatalytic applications in organic syntheses (Smith et al., 1992; Quax and Broekhuizen, 1994; Michor et al., 1996).

Screening of gene libraries of organisms regarded as potential esterase sources and designed for expression in *Escherichia coli* strains exhibiting low endogenous esterase activities offers several advantages over traditional screening programmes. A major advantage of this molecular approach is to get access to the diversity of esterases that are encoded in the genetic programme of a specific organism, as expression is made independent of the natural regulatory circuits. This also includes the immediate separation of a single enzyme from the background of other esterase activities expressed in that particular donor organism and thus, allows rapid identification of distinct specificities and selectivities. A further advantage of this strategy is that the respective genes are immediately available, which provides rapid access to enzyme improvement by protein engineering strategies such as sequential generations of random mutagenesis (Moore and Arnold, 1996).

Simple and quick plate assays that allow the characterization of the individual esterases with respect to substrate specificities or selectivities at an early stage of the screening-selection procedure are crucial for success. Several assays for the presence of esterase activity of bacterial cells grown to colonies on agar plates and based on chromogenic or fluorogenic substrates have been described previously (Freydiere and Gille 1991; Miles et al., 1992). The range of substrates and applications was very limited, however. Thus, plate assays for the characterization of specific esterase properties have not been described before. Results from selectivity analysis using fluorogenic or chromogenic substrates often are not directly transferable to substrates of technological interest because of the high influence of the chromogenic or fluorogenic part on substrate acceptance and selectivity of the esterases. Therefore, a detection method for hydrolysis of unmodified esters on agar plates is also desirable.

In this report we describe simple plate assays which indicate different substrate specificities of two different esterases (EP6 and EP10) of *Burkholderia gladioli* using chromogenic and fluorogenic substrates. In addition, we demonstrate the general possibility to apply a pH-shift based plate assay to detect hydrolytic activity on a technical substrate. The reliability of this strategy could be shown by detailed studies of the enzymatic hydrolysis of linalyl acetate using preparations of esterase EP6. Biocatalytic kinetic resolution of ($\pm$)-linalyl acetate was performed on a preparative scale using esterase EP6.

2. Materials and methods

2.1. Bacterial strains, plasmids and culture conditions

E. coli HB101 (ATCC 33649; F^- *hsd*S20 (r_B^- m_B^-) *sup*E44 *ara*A2 *rps*L20 (*str*R) xyl-5 *mtl*I *rec*A13) was used as a host for gene libraries and basic genetic work. *E. coli* BL21(DE3) (ATCC 10248; F^- *omp*T [*lon*] *hsd*S_B (r_B^- m_B^-) with DE3, a λ prophage carrying the T7 RNA polymerase) was used as a host for overexpression experiments. *E. coli* HB101(pGSP6Δ0) and HB101(pGSP10BΔ0) represent clones (Stubenrauch, 1991) containing genomic DNA fragments (3.9 and 2 kb) from *B. gladioli* ATCC 1024 (formerly *Pseudomonas marginata*) encoding esterases EP6 and EP10, respectively. These clones are based on general purpose cloning vectors. *E. coli* HB101 harbouring the plasmid pBluescript SK($-$) (Stratagene, La Jolla, CA) was used as an esterase negative control strain. The plasmids pEP6EX and pMSK16 are expression constructs containing the structural genes of EP6 and EP10, respectively under the control of an inducible *tac* promoter (Schlacher et al., 1997). Bacterial cells were routinely grown in LB medium (1% bacto tryptone, 0.5% bacto yeast extract, 0.5% NaCl). Media were solidified by the addition of 15 g l^{-1} agar. For plasmid selection, ampicillin (100 mg l^{-1}) was added. Cultures for enzyme preparation were generally grown at 30°C. For the plate assays, the cells were grown overnight at 37°C on LB agar plates containing 100 mg l^{-1} ampicillin.

2.2. Production of esterases EP6 and EP10

For a first preculture, 10 ml of LB medium were inoculated with bacterial cells grown over night on LB agar. After 12 h of growth, the first preculture was used to inoculate a second preculture (100 ml LB) which was grown to mid log phase. A total of 30 ml of this second preculture were used to inoculate 300 ml LB medium in shake flasks to grow the final overexpression culture. For induction of the *tac* promoter, the *E. coli* cultures were supplemented with 10 mM lactose after 3 h, which corresponds to the beginning of the exponential phase. For enzyme production, the cultures were incubated for a further 14 h (EP6), or 6 h (EP10), at 130 rpm.

For enzyme isolation, the cells were harvested by centrifugation for 10 min ($2500 \times g$) at 4°C. The cells were then washed once with 0.9% NaCl and resuspended in 0.05 M Tris–HCl buffer (pH 7) in the case of EP6, or a solubilisation buffer (0.05 M Tris–HCl, 0.01 M EDTA, 0.1 M NaCl, 1 mg l^{-1} lysozyme, 0.5% Triton X-100, pH 7) in the case of EP10. After applying two freeze thaw cycles, cells were disrupted by sonication (Braun Labsonic 2000, Branson Sonifier 250) for 3 min on ice with the duty cycle set to 50% and the output control set to 7. After the cell debris was removed by centrifugation for 30 min ($25000 \times g$) at 4°C, the supernatant was frozen with liquid nitrogen and lyophilized (B. Braun Biotech Christ α 1–4) to give a crude enzyme preparation for use in biocatalytic studies.

2.3. Protein determination and enzymatic assay

The protein concentration was measured by the method of Bradford using a commercial kit (Bio-Rad) as recommended by the supplier. Bovine serum albumin (Bio-Rad) was used as a standard.

All esterase activity assays were carried out photometrically in 0.1 M Tris–HCl buffer, pH 7 containing 4 mM *o*-nitrophenylbutyrate as a substrate. The specific absorption coefficient of 1.26 ml μmol^{-1} cm^{-1} at 420 nm for *o*-nitrophenol was used for quantification.

2.4. Enzymes and chemicals

The following crude lipase preparations were tested for hydrolysis of linalyl acetate: *Rhizopus oryzae* (Amano AP-15), *Geotrichum candidum* (Amano GC-20), *Rhizopus delemar* (Amano D), *Rhizopus niveus* (Amano N), *Pseudomonas* sp. (Amano P), *Mucor javanicus* (Amano M-10), *Penicillium camembertii* (Amano G-50), *Candida rugosa* (Amano AY-30, Sigma type VII), *Candida lipolytica* (Amano L-10), *Penicillium roquefortii* (Amano R), *Humicola lanuginosa* (Amano CE-5), *Thermomyces* sp. (Novo SP 523), *Candida antarctica* A (Novo SP 526), *Candida antarctica* B (Novo SP 525), porcine pancreas (Sigma type II), *Jatropha curcas* (Steiner A-3, A-12), *Aspergillus niger* (Steiner A-116), *Acremonium chrysogenum* (Steiner PC-190), *Rhizomucor miehei* (Steiner B-202), *Rhizopus oryzae* (Steiner B-29), *Rhizopus arrhizus* (Steiner B-81). Furthermore, we tested the protease subtilisin (Amano N) and penicillin G acylase (Recordati).

Stock solutions of 50 mM tetrazotized *o*-dianisidine (Sigma, St. Louis, MO) in H_2O and 60 mM naphtholesters (Sigma) in acetone were prepared. A total of 3 μM 4-methyl-umbelliferyl butyrate and 3 μM resorufin butyrate in ethanol and 3 μM acyl hydroxy pyrene trisulfonic acid (HPTS-ester) in H_2O were used as stock solutions of the fluorescent substrates which were all obtained from Lambda (Graz, Austria). ($\pm$)-Linalyl acetate and (*R*)-linalool were purchased from Fluka. The final substrate solutions were freshly prepared by diluting the stock solutions in 0.1 M phosphate buffer, pH 7.0. The final concentration of the naphthol substrate and *o*-dianisidine in the phosphate buffer was 3 and 1 mM, respectively. The fluorescence substrates were diluted to 30 nM in 0.1 M phosphate buffer, pH 7.0.

2.5. Esterase plate assays

The bacterial cultures were grown overnight on LB agar plates containing 100 mg l^{-1} ampicillin at 30°C. A total of 70 mm Whatman 541 hardened ashless filter circles were soaked with substrate solution. Excess fluid was allowed to drain. For detection of esterase activity, the bacterial

colonies were overlaid with the substrate-soaked filter discs. With naphthyl substrates, esterase activity was detected by development of a purple stain of the bacteria and the filter paper in their surrounding of the colonies within 15 min.

Visible fluorescence caused by the cleavage of the fluorescence ester substrates was observed within 5 min using a UVP TM20 transilluminator (maximum at 302 nm).

For the screening of esterase activity based on the detection of a shift to lower pH, the bacteria were grown on selective LB agar plates containing 30 mg l^{-1} bromothymol blue (Sigma) and 10 mM Tris–HCl, pH 7.5. A dry filter paper disc was placed on the agar plate to lift off the bacteria. By this way, the filter paper was also soaked with pH-indicator from the agar. After 10 min the filter paper discs with the fixed bacterial cells were transferred to a petri dish and soaked with 1 ml of substrate solution containing 5 μl of linalyl acetate in 0.02 M phosphate buffer, pH 7.5 for 15 min and then placed on filter paper to dry. Esterase activity was detected by change of the blue colour to yellow around positive colonies. For all plate assays, a control strain (*E. coli* HB101 harbouring an insert free vector) was used as a reference for background activity, which was usually low.

2.6. Thin layer chromatography analysis of enzymatic resolution of racemic linalyl acetate

A total of 15 mg of crude enzyme preparation or lyophilized cells was suspended in 1 ml phosphate buffer (0.1 M, pH 7.0) by gentle shaking ($\sim$30 min). Then, 15 mg of substrate racemic linalyl acetate (Fig. 1, *rac*-**1a**) was added and the mixture was agitated on a rotary shaker (200 rpm) at ambient temperature. Samples were periodically withdrawn and analyzed by TLC using Merck silica gel 60 F_{254} and petroleum ether/ethyl acetate (5:1) as eluent. Compounds were visualized by spraying with vanillin/H_2SO_4 conc. (5 g l^{-1}) and subsequent heat treatment.

2.7. Preparative biotransformation

A total of 500 mg of lyophilized crude esterase EP6 preparation (EP6 protein represented $\sim$30–40% of total proteins, as judged from SDS-PAGE analysis) were rehydrated in 25 ml phosphate buffer (0.1 M, pH 7.0) for $\sim$1 h with gentle shaking. After addition of 135 mg substrate (*rac*-**1a**), shaking was continued at room temperature. while the course of the reaction was monitored in time intervals by GLC using a calibration curve with menthol as internal standard. After $\sim$20 h, about 20% conversion was reached, and the reaction was stopped by addition of acetone (5 ml). After removal of the debris by centrifugation for 10 min at $3000 \times g$, the organic material was extracted with ethyl acetate. The organic phase was dried (Na_2SO_4) and evaporated to give a mixture of **1a** and **1b** (overall yield 85%), which were separated by column chromatography (silica gel, petroleum ether/ethyl acetate 5:1). The absolute configuration of **1b** was determined to be *S* via co-injection on chiral GLC with (*R*)-linalool as reference standard.

2.8. GLC analysis

The reaction (Fig. 1) was monitored via GLC analysis on a permethylsiloxane column (Biorad RSL 1701, 23 m $\times$ 0.25 μm, 0.25 μm film, N_2) using menthol as internal standard (100 μl of a 98.9 mM stock solution in ethanol). Program: 80°C, 10°/min to 160°C, 20°/min to 200°C (**1a** 7.3 min, menthol 6.7 min, **1b** 5.6 min). The optical purity of **1b** was determined on a permethyl-β-cy-

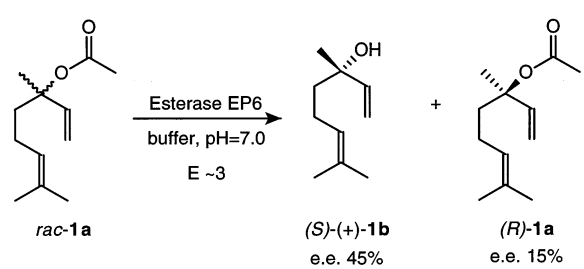

Fig. 1. Enzymatic resolution of (*rac*)-linalyl acetate catalyzed by esterase EP6. (a) and (b) denote linalyl acetate and linalool, respectively.

Table 1
Hydrolytic activity of *E. coli* esterase clones on naphthyl-, and 1-hydroxypyrene-3,6,8-trisulfonic acid (HPTS)-esters

Substrate	[a]	Activity[b]	
		HB101 (pGSP6Δ0)	HB101 (pGSP10BΔ0)
HPTS-acetate (C_2)	♦	−	−
HPTS-butyrate (C_4)	♦	−	−
HPTS-octanoate (C_8)	♦	−	+
HPTS-dodecanoate (C_{12})	♦	−	+
HPTS-hexadecanoate[a] (C_{16})	♦	−	++
HPTS-octadecanoate (C_{18})	♦	−	++
α-Naphthyl-acetate (C_2)	♣	++	++
α-Naphthyl-propionate (C_3)	♣	++	++
α-Naphthyl-butyrate (C_4)	♣	++	++
α-Naphthyl-hexanoate (C_6)	♣	+	+
α-Naphthyl-octanoate (C_8)	♣	−	+
α-Naphthyl-nonanoate[b] (C_9)	♣	−	+
α-Naphthyl-dodecanoate[c] C_{12})	♣	−	−
α-Naphthyl-hexadecanoate (C_{16})	♣	−	−
Linalyl acetate	⊕	+	−

[a] ♣, Purple colour or ♦, blue fluorescence of colonies; ⊕, yellow halo (pH-shift) of at least 2 mm around colonies.
[b] +, Weak activity; ++, good activity; −, no detectable activity.
Activity levels were visually judged in comparison to the control strain *E. coli* HB101 (pBSSK(−)). Good activity means that the colour or fluorescence signal appeared within a few seconds and was, at least for 2 min, much stronger than the control strain. Weak activity was classified when a clearly stronger signal was recognized within 1 min and the signal was at least ~30 s significantly stronger, as compared to the control strain.

clodextrin column (Chrompack Chirasil-Dex CB, 25 m × 0.25 mm, 0.25 μm film, 0.3 bar H_2). Program: 90°C isotherm ((*R*)-**1b** 14.2 min, (*S*)-**1b** 14.5 min). (*R*)-**1a** was chemically transformed into (*R*)-**1b** (K_2CO_3 in methanol at reflux) without racemization prior to chiral analysis.

3. Results and discussion

3.1. Influence of fatty acid chain length

In order to preliminary characterize the esterases EP6 and EP10 with respect to preference for chain length of the fatty acid moiety at an early stage, *E. coli* clones HB101 (pGSP6Δ0) and HB101 (pGSP10BΔ0) were analyzed by the filter overlay plate assays (Table 1). Using naphthol esters, the hydrolytic activity of EP6 and EP10 clones in dependence of the chain length of the carboxylic acid component clearly showed a preference for esters of short chain carboxylic acids. The EP6 clone showed highest activity on C_2–C_4 naphthol esters and almost no activity on esters of aliphatic carboxylic acids larger than C_6. On the contrary, the EP10 clone showed reasonable activity up to C_9. The analysis of hydrolytic activity on fluorogenic HPTS-carboxylic acid esters showed a different result. Both esterases could not hydrolyze HPTS-esters of short chain carboxylic acids (C_2, C_4). With increasing chain length, the EP10 clone showed clear hydrolytic activity on these substrates, whereas no reaction was observed with the EP6 clone and the control strain containing an insert free vector plasmid. Among the tested substrates with longer fatty acid chains (C_8, C_{12}, C_{16}, C_{18}), the strongest fluorescence was observed on hexadecanoyloxypyrene-3,6,8-trisulfonic acid (C_{16}) and oleoyloxypyrene-3,6,8-trisulfonic acid (C_{18}) (Fig. 2). These assays clearly showed that both components of the esterase substrate, i.e. alcohol and carboxylic acid moiety influence the acceptance as a substrate by EP6 and EP10. Although there was a preference for

esters of short chain carboxylic acids for both enzymes, at least EP10 was also able to hydrolyze esters of long chain fatty acids. We think this was caused by the high solubility of HPTS-fatty acid esters in water caused by the sulfonic acid substituents on the alcohol part. We also suggest that the inability of EP6 and EP10 to hydrolyze the short chain HPTS-esters (C_2, C_4) is due to the high polarity of the alcohol moiety. This is supported by the fact that short chain substrates with a less polar alcohol moiety such as resorufin butyrate and 4-methyl-umbelliferyl butyrate were efficiently hydrolyzed (Fig. 2). In addition, it is already known that HPTS-esters are only hydrolyzed by a few specific hydrolases (Wolfbeis and Koller, 1983).

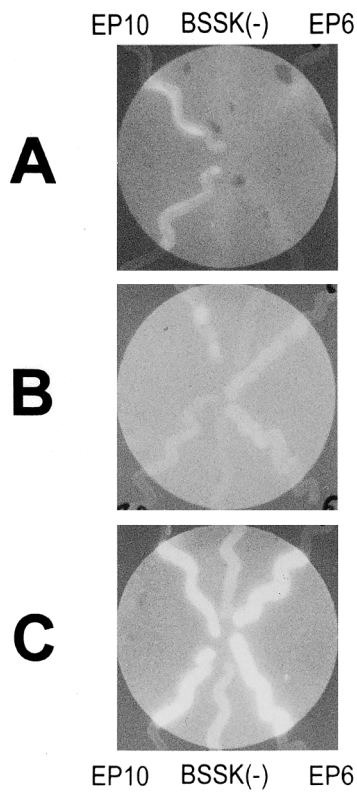

Fig. 2. Characterization of esterase clones by plate assays using fluorescent substrates. EP6, HB101 (pGSP6Δ0) encoding esterase EP6; EP10, HB101 (pGSP10BΔ0) encoding esterase EP10; BSSK(−), control strain HB101 (pBluescript II SK (−)); Substrates: (A) hexadecanoyloxypyrene-3,6,8-trisulfonic acid, (B) resorufin butyrate, (C) 4-methyl-umbelliferyl butyrate.

3.2. Detection of hydrolytic activity on a sterically hindered ester (Fig. 1)

For our search of novel carboxyl-ester hydrolases capable of hydrolyzing technologically interesting substrates (Osprian et al., 1996) we focused on the allylic tertiary alcohol linalool (Fig. 1) for the following reasons:

1. It constitutes one of the most important terpene alcohols used in the flavor and fragrance industry. Since both enantiomers differ in odor (Ohloff and Klein, 1962) their availability in optically pure form is desirable for flavor and fragrance composition. In natural sources the (*R*)-(−)-enantiomer ('licareol') is a major constituent (∼80%) of *Cinnamonium camphora* and *Cayenne linaloe* extracts, whereas the (*S*)-(+)-form primarily occurs in coriander oil ('coriandrol').
2. It is a bulky substrate and the search for novel exceptional hydrolytic enzymes such as lipases (Kallwass et al., 1994) and proteases (Schultz et al., 1992), which accept sterically hindered esters, has shown only limited success before.

The pH-shift assay, as described in Section 2, was used to study hydrolysis of linalyl acetate by EP6 and EP10 clones. A colour change from blue to yellow was observed around the colonies of HB101 (pGSP6Δ0) indicating acidification and thus, esterolysis. No reaction was observed with the EP10 clone and the control strain.

To support this finding, lyophilized EP6 and EP10 preparations, and (for comparison) a set of well studied hydrolytic enzymes, mainly lipases, (listed in Section 2) were qualitatively analysed by thin layer chromatography for the ability to hydrolyze racemic linalyl acetate. The only notable activity was observed with esterase EP6. All other tested enzyme preparations were found to be inactive.

To verify the hydrolysis of linalyl acetate by EP6 and in order to estimate the stereoselectivity, this reaction was studied in more detail by TLC and GLC analysis. Crude preparations of EP6, as described in Section 2, were used for preparative hydrolysis of racemic linalyl acetate. The (*S*)-enantiomer of linalyl acetate was preferentially hydrolysed to give (*S*)-(+)-linalool (**1b**) in 45%

enantiomeric excess at 20% conversion. The remaining (*R*)-acetate was present at an enantiomeric excess (e.e.) of 15%. The selectivity of the reaction-expressed as the enantiomeric ratio was calculated as $E \sim 3$. This relatively low *E*-value could be due to the steric similarity of the methyl- and the ethenyl-groups, which impedes the chiral recognition process.

Attempts to resolve linalool via biocatalytic methods have only scarcely been reported before. (*rac*)-Linalool was reported to be a non-substrate for several microbial lipases in esterification reactions (Okumura et al., 1979; Iwai et al., 1980). The corresponding acetate, however, could be hydrolyzed with varying success using whole microbial cells: Cleavage of the ester moiety (without detectable enantioselectivity) was observed during microbial hydroxylation of linalyl acetate using *Aspergillus niger* (Madyastha and Krishna-Murthy, 1988). Similarly, whole cells of *Bacillus subtilis* were able to hydrolyze linalyl acetate, but allylic rearrangement led to the formation of non-chiral geraniol and nerol, respectively, as by-products (Oritani and Yamashita, 1973). The optical purities of linalool and linalyl acetate thus obtained did not exceed 17%. Recently, novel bacterial isolates were described showing selectivities of up to $E \sim 5$ (Osprian et al., 1996).

In conclusion, this study demonstrated the usefulness and reliability of simple plate assays to evaluate specific traits of esterolytic enzymes. We were able to distinguish substrate preferences of different esterases. Furthermore, it was possible to detect rare activity on esters of tertiary alcohols. Further studies directed towards the selectivity-enhancement of reactions employing esterase EP6 for hydrolysis of linalyl acetate are in progress.

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References

Barnier, J.-P., Blanco, L., Rousseau, G., Guibe-Jampel, E., Fresse, I., 1993. Enzymatic resolution of cyclopropanols. An easy access to optically active cyclohexanones possessing an α-quarternary chiral carbon. J. Org. Chem. 58, 1570–1574.

Faber, K., Riva, S., 1992. Enzyme-catalyzed irreversible acyltransfer. Synthesis 895–910.

Ferraboschi, P., Grisenti, P., Manzocchi, A., Santaniello, E., 1994. Regio- and enantioselectivity of *Pseudomonas cepacia* lipase in the transesterification of 2-substituted-1,4-butanediols. Tetrahedron: Asymmetry 5, 691–698.

Freydiere, A.-M., Gille, Y., 1991. Detection of Salmonellae by using Rambach agar and by a C_8 esterase spot test. J. Clin. Microbiol. 29, 2357–2359.

Iwai, M., Okumura, S., Tsujisaka, Y., 1980. Synthesis of terpene alcohol esters by lipase. Agric. Biol. Chem. 44, 2731–2732.

Jones, J.B., 1990. Esterases in organic synthesis: Present and future. Pure Appl. Chem. 62, 1445–1448.

Jones, J.B., Beck, J.F., 1976. Asymmetric synthesis and resolutions using enzymes. In: Weissberger, A (Ed.), Application of Biochemical Systems in Organic Chemistry. Wiley, New York, pp. 114–231.

Kallwass, H.K.W., Yee, C., Blythe, T.A., McNabb, T.J., Rogers, E.E., Shames, S.L., 1994. Enzymes for the resolution of α-tertiary-substituted carboxylic acid esters. Bioorg. Med. Chem. 2, 557–566.

Madyastha, K.M., Krishna-Murthy, N.S.R., 1988. Transformations of acetates of citronellol, geraniol and linalool by *Aspergillus niger*: Regiospecific hydroxylation of citronellol by a cell-free system. Appl. Microbiol. Biotechnol. 28, 324–329.

Michor, H., Marr, R., Gamse, T., Schilling, T., Klingsbichel, E., Schwab, H., 1996. Enzymatic catalysis in supercritical carbon dioxide: comparison of different lipases and a novel esterase. Biotechnol. Lett. 18, 79–84.

Miles, R.J., Siu, E.L.T., Carrington, C., Richardson, A.C., Smith, B.V., Price, R.G., 1992. The detection of lipase activity in bacteria using novel chromogenic substrates. FEMS Microbiol. Lett. 90, 283–288.

Moore, J.C., Arnold, F.H., 1996. Directed evolution of a *para*-nitrobenzyl esterase for aqueous-organic solvents. Nat. Biotechnol. 14, 458–467.

Ohloff, G., Klein, E., 1962. Die absolute Konfiguration des Linalools durch Verknüpfung mit dem Pinansystem. Tetrahedron 18, 37–42.

Okumura, S., Iwai, M., Tsujisaka, Y., 1979. Synthesis of various kinds of esters by four microbial lipases. Biochim. Biophys. Acta 575, 156–165.

Oritani, T., Yamashita, K., 1973. Microbial resolution of (±)-acyclic alcohols. Agric. Biol. Chem. 37, 1923–1928.

Osprian, I., Steinreiber, A., Mischitz, M., Faber, K., 1996. Novel bacterial isolates for the resolution of esters of tertiary alcohols. Biotechnol. Lett. 18, 1331–1334.

Quax, W.J., Broekhuizen, C.P., 1994. Development of a new *Bacillus* carboxyl esterase for use in the resolution of chiral drugs. Appl. Microbiol. Biotechnol. 41, 425–431.

Schlacher, A., Stanzer, T., Soelkner, B., Klingsbichel, E., Petersen, E., Schmidt, M., Klempier, N., Schwab, H., 1997. New Pseudomonas esterases by genetic engineering. J. Mol. Catal. B: Enzymatic, 3, 25–27.

Schultz, M., Hermann, P., Kunz, H., 1992. Enzymatic cleavage of tert-butyl esters: Thermitase-catalyzed deprotection of peptides and *O*-glycopeptides, Synlett. 37–38.

Smith, M.R., van den Tweel, W.J.J., Kierkels, J.G.T., de Bont, J.A.M., 1992. Enantioselective resolution of methylesters of 3-chloro-2-methylpropionate by a carboxylesterase from *Rhodococcus* sp. ME6. Enzyme Microb. Technol. 14, 893–897.

Stubenrauch, G., 1991. Klonierung von Esterase-Genen aus *Pseudomonas marginata* und *Xanthomonas campestris*, sowie detaillierte Analyse des *Pseudomonas marginata* estA Gens und dessen Genprodukts (Esterase EP10). Ph.D. thesis. Technical University of Graz, Austria.

Wolfbeis, O.S., Koller, E., 1983. Fluorimetric assay of hydrolases at longwave excitation and emission wavelengths with new substrates possessing unique water solubility. Anal. Biochem. 129, 365–370.