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On the inactivity of *Candida antartica* lipase B towards strong acids

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ABSTRACT

Candida antarctica lipase B (CalB, Novozyme 435) was evaluated as catalyst for the conversion of so-called edible acids (e.g. malic and tartaric acid). While transesterification using these acyl donors proceeds smoothly, albeit with low regioselectivity, esterification is hardly catalyzed.

As major reason for CalB inactivation the high acidity of edible acids was identified leading to irreversible inactivation of the biocatalyst. Furthermore, indication exist that all acids exhibiting a pK_a value below 4.8 cause irreversible inactivation of CalB.

1. Introduction

Enzyme catalysis has become a standard technique within organic chemistry [1–4] with lipases representing the class of biocatalysts being most widely applied [5].

Advantages of enzyme-catalyzed reactions over 'classical' chemical routes not only derive from often high enantioselectivity [4] but also from the mild reaction conditions leading to purer products in more environmentally benign processes [6–8]. We became interested in using immobilized lipase B (CalB) from *Candida antarctica* (Novozyme 435) as catalyst for the preparation of esters of malic and tartaric acid. Such products are used for various purposes, for example as emulsifiers in food (e.g. di-acetyl tartaric ester of monoglyceride (DATEM) and corresponding citric acid analogs) as low-irritating humectants and exfoliants in cosmetics [9,10], as lubricants, and as building blocks for polymers. Compared to classical Lewis or Brønsted acid-catalyzed reactions we envisioned various advantages of enzyme catalysis. On the one hand, reaction temperatures well below those required for efficient chemocatalysis (140–180 °C) should circumvent undesired thermal side-reactions such has dehydratation and self-condensation of the acid. On the other hand, putative regioselectivity of the enzymatic reaction could give access to new products with unprecedented properties (Scheme 1).

To date, regioselective hydrolysis of the sterically less hindered carboxylate group in triethyl citrate [11] as well as regioselective esterification [12] and transesterification [13] of malic acid (esters) has been reported.

2. Experimental

Novozyme 435 was purchased from Novozymes (Bagsvaerd, Denmark), all other chemicals were purchased from Sigma–Aldrich (Munich, Germany) in the highest quality available and used without further purification.

Enzyme-free carrier material was prepared by using Novozyme 435 and desorption of CalB from the carrier: Novozyme 435 was incubated in a 10-fold excess (w/w) of a mixture water/acetonitrile (1:1, v/v) at 60 °C for 1 h, filtrated, washed with water, and dried. The resulting enzyme-free resin exhibited no significant esterification activity using lauric acid and 1-octanol.

2.1. Enzymatic reactions

In all cases mechanic stirring was performed using stirring blades instead of magnetic stirrer bars in order to circumvent enzyme grinding. At intervals, samples were withdrawn and analyzed.

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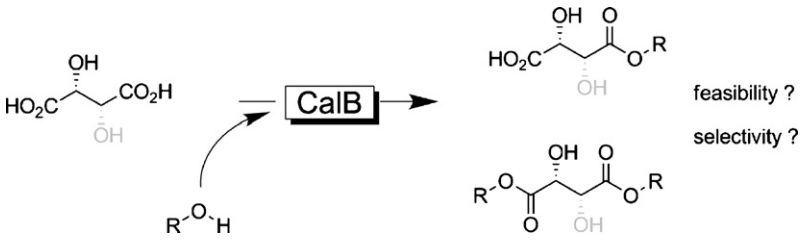

Scheme 1. Use of immobilized CalB (Novozyme 435) as selective catalyst for the conversion of edible acids (tartaric acid and malic acid).

Table 1
'CalB-catalyzed' condensative polymerization of tartaric acid with glycerol using Novozyme 435 and enzyme-free carrier compared to literature reported reactions.

Enzyme preparation	Activity (U g^{-1})	Yield (%)	Oligomerweight (Mn)
Novo 435	10,300	100	1045
Leached Novo 435	54	100	1296
Novo 435 [14,15]	–	100	1100

2.2. Esterification of tartaric acid with glycerol according to the literature [14,15] (Table 1)

Equimolar amounts of tartaric acid, glycerol, and ethanol were heated to 80 °C until a homogeneous solution was obtained. After addition of 5% (w/w) Novo 435 (native (entry 1), or enzyme-free carrier (entry 2)) the pressure was reduced to 250 mbar. After 24 h, pressure was further reduced to 50 mbar followed by an additional stirring for 24 h. Afterwards the reaction mixture was filtered and analyzed by GPC.

2.3. CalB-activity on substituted valeric acids (Fig. 1)

Equimolar mixtures of acid with 1-octanol were stirred at 60 °C in the presence of 0.5% (w/w) Novozyme 435 at ambient pressure and the initial activity was determined as described above.

2.4. CalB-catalyzed transesterifications (Fig. 2)

0.118 mol of acyl donor were dissolved in 90 mL 1-octanol (five-fold molar excess), supplemented with 5% (w/w$_{\text{total mass}}$) Novozyme 435, T = 60 °C, p = 50 mbar, and the initial activity was determined as described above.

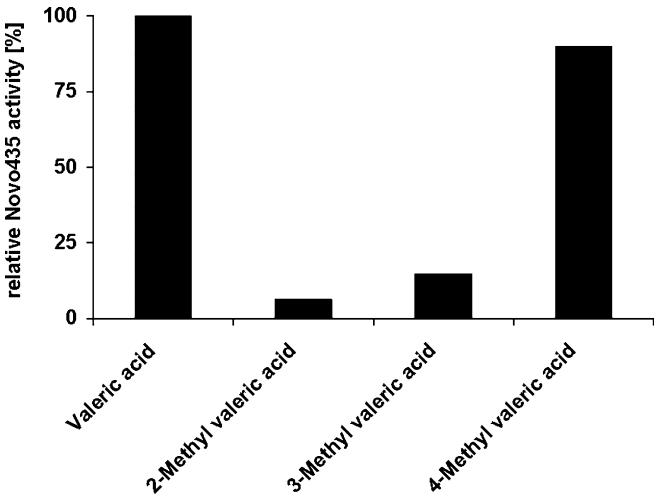

Fig. 1. Influence of the relative position of a methyl substituent in valeric acid on the rate of CalB-catalyzed esterification with 1-octanol. 100% corresponds to a specific Novozyme 435 activity of 4.34 U mg^{-1}.

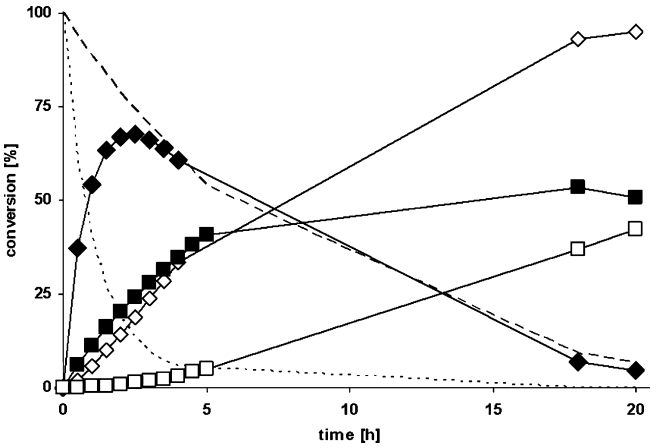

Fig. 2. Transesterification of diethylmalate (diamonds) and diethyltartrate (squares) with 1-octanol using Novozyme 435. Starting materials (– – –, . . .), mono-transesterification products (filled symbols), di-transesterification products (open symbols).

2.5. Influence of tartaric acid concentration on CalB-activity (Fig. 3)

Tartaric acid was dissolved in a mixture containing 288 mmol of lauric acid and 1-octanol each supplemented with 5% (w/w) DMSO at 80 °C. Reactions were started by the addition of 0.722 g Novozyme 435 and the initial activity was determined as described above.

2.6. CalB-activity with various acids (Fig. 4)

Equimolar solutions of the respective acid and 1-octanol were incubated at 65 °C in the presence of 5% (w/w) Novozyme 435 (exception: tartaric and malic acid which were dissolved 0.1 M in

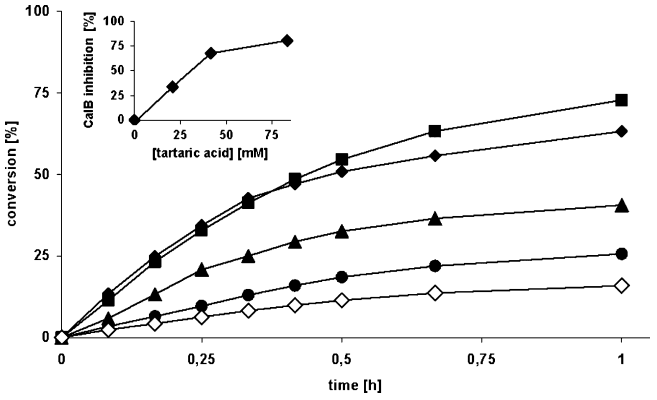

Fig. 3. Influence of tartaric acid (0 mM (♦), 0.4 mM (■), 20.8 mM (▲), 41.6 mM (●), and 83.3 mM (◊)) on the rate of Novozyme 435-catalyzed esterification of lauric acid with 1-octanol. Inset: Extend of CalB-inhibition as determined from initial rates.

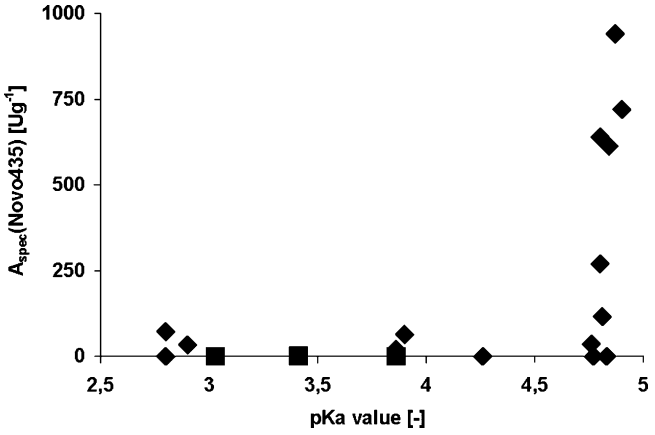

Fig. 4. CalB-activity with various acids. Shown as squares are lactic (pK_a = 3.86), malic (pK_{a1} = 3.41), and tartaric acid (pK_{a1} = 3.03). The activities are not corrected for the uncatalyzed background reaction. For the sake of clarity, unbranched fatty acids (all exhibiting a pK_a value of above 4.8 and a specific activity ranging from 2,000 to 10,000 U g^{-1} CalB) are not included in the graph.

1-octanol). Results in detail (uncorrected for background) [acid (pK_a, A(Novo 435) [U g^{-1}])]: malic acid (3.41, <20), tartaric acid (3.03, <20), lactic acid (3.76, <20), acetic acid (4.86, 36), propionic acid (4.87, 940), butyric acid (4.82, >5000), valeric acid (4.84, >5000), 2-methyl propionic acid (4.84, 613), 2-chloro propionic acid (2.9, <20), 2-bromo propionic acid (2.97, <20), acrylic acid (4.26, <20), 2-methyl butyric acid (4.82, 116), 2-oxo butyric acid (2.5, <20), 2-chloro butyric acid (2.8, <20), 2-bromo butyric acid (2.9, 72), 2-methyl valeric acid (4.9, 270), 3-methyl valeric acid (4.9, 640), 4-methyl valeric acid (4.9, 3900), hexanoic acid (4.9, >5000), 2-methyl hexanoic acid (4.9, 720), 2-bromo hexanoic acid (2.9, 34), and 2-hydroxy hexanoic acid (3.9, 64).

2.7. CalB-catalyzed oligomerization of edible acid esters (Table 2)

Conditions: equimolar mixtures of acyl donor and PEG-400, 1% (w/w) Novozyme 435, T = 80 °C, p = 50 mbar. Initial activities (2 h) and yields were determined based on hydroxyl values (methods according to DGF C-V 17 a (53) and Ph. Eur. 2.5.3 Method A) of the reaction mixtures. After 19 h, the reactions were stopped by removal of the biocatalyst and analyzed via GPC.

3. Analytical protocols

3.1. Esterification activity

Novozyme 435 activity in esterification reactions was determined by the titration of the residual carboxylic acid (similar to the official method described in DGF C-V 2, DIN EN ISO 2114 or ASTM D 974). Shortly, an aliquot of the reaction mixture was diluted in water-free ethanol and supplemented with a few drops of phenolphthalein solution (0.5% (w/w) in ethanol) and titrated with 0.1 M KOH (in ethanol). Novozyme 435 activity was calculated based on the decreasing amount of acid. One unit (1 U) is defined as the consumption of 1 μmol of acid/min.

Table 2
Novozyme 435-catalyzed oligomerization of diacids via transesterification with PEG-400.

Acyl donor	Activity (U g^{-1})	Yield (%)	Oligomerweight (Mn)
Diethyl succinate	478	81.5	1260
Diethyl malate	281	68.9	949
Diethyl tartrate	70	42.9	500
Diethyl fumarate	374	83.8	1204

3.2. Transesterification activity

Novozyme 435 activity in transesterification was determined via GC chromatography (Agilent 6890 GC, column: HP-5 (30 m), temperature profile: 80–300 °C at a heating rate of 5 °C min^{-1}). Unless stated otherwise, quantification was performed using authentic, chemically produced standards.

3.3. MW determination of polymers

MW determination of polymers was determined by GPC (Agilent 1100 RI detection, column: polystyrene-divinylbenzene resin, diameter: 0.8 cm, length: 60 cm, eluent: THF, flow rate 1 mL min^{-1}, T = 30 °C, MW standard: PS).

4. Results and discussion

To establish a CalB-platform for the preparation of edible acid esters, we first reproduced the literature-described esterification between tartaric acid and glycerol [14,15]. We were pleased to obtain essentially identical product properties as described in the literature. However, control experiments using enzyme-free carrier material (showing less than 1% of the original activity, obtained by desorption of the biocatalyst) also gave very similar results (Table 1).

Thus, we conclude that the observed condensative polymerization cannot or to a very minor extent be attributed to the biocatalyst immobilized on Novozyme 435.

One obvious explanation for the apparent inactivity of CalB with tartaric acid can be drawn from the steric demand of α- and β-substituents to the reacting carboxylic acid. In fact CalB-activity with methyl-substituted derivates of valeric acid (which itself is a good substrate for CalB) shows a dependence of enzyme activity with the position of substitution. Thus, α- and β-substituted valeric acid derivates were poorly converted, whereas γ-substitution hardly influenced CalB-activity (Fig. 1). Similar effects were also observed with a range of other substituted acids tested in our own lab. For example, 4-methyl hexanoic acid is converted at almost the same rate as unsubstituted hexanoic acid while α- and β-substituted hexanoic acid derivates are transformed at significantly decreased rates (data not shown) and are reported in the literature [16].

This suggests that substituents in α- and/or β-position in malic and tartaric acid severely impede proper binding of the substrate to the enzyme's active site which may result in either a significantly reduced v_{max} or increased K_M value for the substrate. In fact, attempts of Novozyme 435-catalyzed esterification of malic or tartaric acid with 1-octanol resulted in very poor conversions. A specific Novozyme 435 activity (without correction for the uncatalyzed esterification rate) of approximately 2 U g^{-1} for malic acid esterification was estimated while the activity for tartaric acid was too low to allow reliable quantification. This value corresponds well to the specific Novozyme 435 activity estimated from the literature (esterification of malic acid: 1.7 U g^{-1} [12], esterification of lactic acid: 18–56 U g^{-1} [17,18]) but falls back by up to three orders of magnitude behind Novozyme 435 activity with, e.g. lauric acid (>6000 U g^{-1}) under comparable conditions.

Interestingly, a different picture evolves from comparable transesterification reactions using diethyl malate or tartrate (Fig. 2).

Initial specific activities (determined after 1 h) of 303 U g^{-1} and 47 U g^{-1} for diethyl malate and tartrate were determined, respectively. No uncatalyzed background was observed for these reactions

under the reaction conditions applied. Thus, it seems that CalB-catalyzed transesterification of malic and tartaric acid is feasible (albeit at low reaction rates compared to 'optimal' acyl donors such as, e.g. lauric acid, which is most likely explained by the steric hindrance of the substrates used) while corresponding esterifications are not catalyzed. The very poor rate of diethyl tartrate conversion seems to be the consequence of coeval α- and β-substitution. It is noteworthy mentioning that no apparent decrease of enzyme activity was observed under the conditions of transesterification. Similar results were obtained for the esterification and transesterification with di-functional alcohols such as PEG-400 with edible acids. With the exception of succinic acid, where slight product formation was observed, all CalB-catalyzed polycondensations failed to give any product (data not shown) while corresponding transesterifications were conducted successfully (Table 2).

This striking difference in conversion rate between esterification and transesterification can only partially be explained by the fact that instead of the acid an activated acyl donor was used. Therefore, we assumed further factors to cause the dramatic difference between esterification and transesterification activity. Clearly, steric hindrance is not the only cause for the apparent CalB inactivity with tartaric and malic acid.

We suspected acidity of the substrates to influence the reactivity with CalB as recently suggested for lactic acid [19]. To test this hypothesis, we determined the CalB-activity for esterification of the very good substrate lauric acid in the presence of varying concentrations of tartaric acid (Fig. 3).

A clear correlation between CalB-inhibition and tartaric acid concentration can be observed. Similar results were also achieved with malic acid (data not shown). The inactivating effect could be circumvented upon the addition of two equivalents (to the edible acid) of triethylamine to neutralize the acid. Here at least 85% of the original activity was recovered.

Moreover, the extent of CalB-inhibition was time-dependent; for example pre-incubation of Novozyme 435 in 1-octanol/DMSO (95:5, w/w) in the presence of 41.6 mM tartaric acid followed by filtration and washing to remove tartaric acid resulted in an activity loss of approximately 50% and 90% after incubation for 20 min and 60 min, respectively (data not shown). These results also suggest that the tartaric acid irreversibly inactivates the biocatalyst.

Next, we decided to evaluate a broader range of acids as acyl donors for CalB-catalyzed esterification. Therefore, a selection of different acids was evaluated (Fig. 4). The acids were chosen to cover a broad range of size, steric hindrance, and acid strength (as estimated by the aqueous pK_a value). To our surprise, a very sharp distinction between acids exhibiting a pK_a value below and above approximately 4.8 can be made. None of the acids tested with a pK_a value of less than 4.8 was converted at significant rates (including small, steric unhindered acids such as acrylic acid). For acids with a pK_a above 4.8, their activity depended largely on the size and distance of the substituent from the carboxylic acid. It should be noted here that in these experiments the water thermodynamic activity was not controlled. This parameter may influence the enzyme activity and stability [20,21]. But as we use solvent-free systems and always the same alcohol it may also be assumed however, that variations in thermodynamic water activity between the single experiments are statistical and do not significantly account for the very sharp activity drop below pK_a 4.8.

To further demonstrate the different influences of sterical demand and acidity, we also compared the relative CalB-activity with some substituted C_3-acids in esterification and transesterification (Fig. 5).

While no esterification activity is observed with acids exhibiting a pK_a of less than 4.8, transesterification activity does not seem to follow the same trend. When adding an α-bromo group to propionic acid (causing a drop of the pK_a from 4.86 to 3.87 as well as adding additional sterical demand) transesterification activity is only slightly affected, whereas the esterification activity is almost zero. On the other hand, attaching a methyl group to α-position, thus causing a similar steric effect but almost no effect on the pK_a value, shows only marginal effects on esterification activity and a transeserification activity in the same area as for the α-bromo propionate. The observable minor differences in transesterification activity might be due to the electron-withdrawing effect of the α-brom.

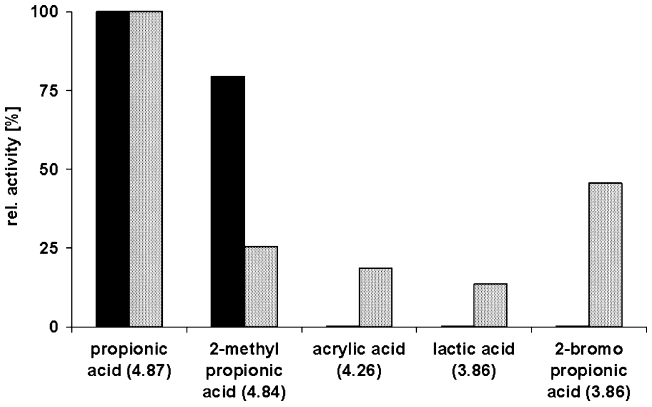

Fig. 5. Comparison of the relative conversion rates of some C_3-acids in esterification (solid) and transesterification (dotted). *Note*: 100% correspond to the rate determined with propionic acid (1.05 U mg^{-1} and 2.49 U mg^{-1} for the esterification and the transesterification, respectively). Note: as no authentic standards were available for 2-bromo propionic acid esters, the rate is an estimate from the peak-areas in the GC-chromatogram. Thus, the real rate may vary by approximately 5%.

5. Conclusions

Overall, we conclude that steric hindrance is an important factor governing CalB-activity with carboxylic acids leading to reduced conversion rates. But, more importantly for edible acids, their acidity leads to irreversible inactivation of the biocatalyst, limiting the CalB-catalyzed conversion of edible acids to the transesterification of their short-chain esters. The very sharp pK_a-transition at 4.8 may suggest protonation of a catalytically or structurally relevant glutamate or aspartate residue within CalB. Assuming protonation of Asp187 would readily explain the observed pK_a-dependency. Upon protonation, Asp187 cannot fulfill its function in the catalytic triade of CalB to activate Ser105 (via His224) for a nucleophilic attack on the acyl donor thereby explaining CalB's inactivity towards strong acids. Furthermore, the resulting carboxylate in the enzyme's active site would be significantly inactivated towards nucleophilic attack by Ser105. If true, modulation of Asp187's pK_a value within the active site by enzyme engineering may be an interesting approach to enable CalB-catalyzed esterification reactions of edible acids. Further titratable amino acid residues within CalB's active site are Asp134 and 223 as well as Glu188. Protonation of these may significantly change the electrostatic potential within the active site leading to inactive enzymes. Alternatively, it may also be assumed that strong acids protonate surface-exposed glutamates or aspartates which are involved in structurally essential hydrogen or salt bridges. Thus, protonation may be the first step in unfolding and irreversible denaturation.

Further investigations will be necessary to identify the amino acid residue(s) obviously being protonated by strong acids. Based on this, a suitable enzyme engineering strategy to develop CalB variants capable of converting edible acids can be devised.

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References

[1] K. Drauz, H. Waldmann, Enzyme Catalysis in Organic Synthesis, 2nd edition, Wiley-VCH, Weinheim, 2002.
[2] B.G. Davis, V. Boyer, Nat. Prod. Rep. 18 (2001) 618–640.
[3] A. Schmid, F. Hollmann, J.B. Park, B. Bühler, Curr. Opin. Biotechnol. 13 (2002) 359–366.
[4] U. Bornscheuer, R. Kazlauskas, Hydrolases in Organic Synthesis, 2nd edition, Wiley-VCH, Weinheim, 2006.
[5] A. Liese, K. Seelbach, C. Wandrey, Industrial Biotransformations, 2nd edition, Wiley-VCH, Weinheim, 2006.
[6] O. Thum, Tens. Surfact. Det. 41 (2004) 287–290.
[7] O. Thum, K.M. Oxenbøll, SOFW J. 134 (2008) 44.
[8] O. Thum, K.M. Oxenbøll, IFSCC Congress 2006, Osaka, Japan, 2006.
[9] R. Datta, S.-P. Tsai, P. Bonsignore, S.-H. Moon, J.R. Frank, FEMS Microbiol. Rev. 16 (1995) 221–231.
[10] A. Bailey, A. De Lucca, J. Moreau, J. Am. Oil Chem. Soc. 66 (1989) 932–934.
[11] R. Chênevert, B.T. Ngatcha, Y.S. Rose, D. Goupil, Tetrahedron: Asymmetr. 9 (1998) 4325–4329.
[12] C. Torres, M. Bernabé, C. Otero, Enzyme Microb. Technol. 25 (1999) 753–761.
[13] A. Liljeblad, L. Kanerva, Tetrahedron: Asymmetr. 10 (1999) 4405–4415.
[14] A. von der Hagen PCT Patent 0,034,501 (2000), to Cognis Deutschland GmbH.
[15] G.E. Jeromin, M. Bertau, Bioorganicum, Wiley-VCH, Weinheim, 2005.
[16] D. Klomp, J.A. Peters, U. Hanefeld, Tetrahedron: Asymmetr. 16 (2005) 3892–3896.
[17] M. From, P. Adlercreutz, B. Mattiasson, Biotechnol. Lett. 19 (1997) 315–318.
[18] C. Torres, C. Otero, Enzyme Microb. Technol. 25 (1999) 745–752.
[19] D. Pirozzi, G. Greco, Biotechnol. Prog. 22 (2006) 444–448.
[20] P.J. Halling, Enzyme Microb. Technol. 16 (1994) 178–205.
[21] N.A. Turner, B.B. Duchateau, E.N. Vulfson, Biotechnol. Lett. 17 (1995) 371–376.