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Increased stability of an esterase from *Bacillus stearothermophilus* in ionic liquids as compared to organic solvents

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Abstract

Three different ionic liquids, 1-butyl-3-methyl imidazolium bis[(trifluoromethyl)sulfonyl]amide [BMIM][BTA], 1-butyl-3-methyl imidazolium hexafluorophosphate [BMIM][PF_6] and 1-butyl-3-methyl imidazolium tetrafluoroborate [BMIM][BF_4] were used as reaction media for the transesterification of 1-phenylethanol catalysed by esterases from *Bacillus subtilis* and *Bacillus stearothermophilus*. No transesterification activity could be detected in the ionic liquids when the lyophilised powder of the esterases was used as biocatalysts. By immobilising the esterases onto Celite it was possible to obtain activity in the ionic liquids. The specific activity and enantioselectivity for the Celite-immobilised enzyme was compared to conventional organic solvents (*n*-hexane, MTBE and vinyl acetate). Highest specific activity was obtained in *n*-hexane for both enzymes while the specific activity was similar in MTBE, vinyl acetate and in the ionic liquids. The enantioselectivity ($E \sim 7.0$ and 3.0 for *B. subtilis* and *B. stearothermophilus* esterase, respectively) was however independent of the solvent. The kinetic resolution was also performed with two lipases, *Candida antarctica* lipase B and *Pseudomonas* sp. lipase (PS-D). Also for these two enzymes, the enantioselectivity was not affected by the solvent and high enantioselectivity $E > 100$ was obtained in all solvents investigated.

The stability of the esterase from *B. stearothermophilus* at 40 °C was considerably increased in the ionic liquids [BMIM][BF_4] and [BMIM][PF_6] as compared to *n*-hexane and MTBE. A half-life of >240 h was obtained in [BMIM][PF_6], which was >30- and >3-fold higher as compared to *n*-hexane and MTBE.

Keywords: Ionic liquid; Esterase; Water activity; Stability; Immobilisation

1. Introduction

Ionic liquids have emerged as alternative reaction media for biotransformations [1–3]. They consist of an organic cation and an inorganic anion where the ions do not pack well and therefore remain liquid at room temperature. By changing the nature of the anion or cation, it is possible to drastically change the properties (polarity, viscosity and density) of the ionic liquids [4,5]. They are therefore often referred to as designer solvents. Another advantage of ionic liquids is that they have no detectable vapour pressure and can therefore be considered as environmentally benign solvents as compared to volatile organic solvents. Furthermore they have good chemical and thermal stability and can therefore be used at elevated temperatures [6].

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It is of great interest to compare ionic liquids with other frequently used media for biocatalysis such as organic solvents and supercritical fluids. It has been proven that ionic liquids have some advantages as compared to organic solvents. The activity of α-chymotrypsin was higher at lower water activities in 1-butyl-3-methyl imidazolium bis[(trifluoromethyl)sulfonyl]amide [BMIM][BTA] than in MTBE and ethylacetate [7]. The regioselectivity of the CALB catalysed acetylation of β-D-glucose in 1-(2-methoxyethyl)-3-methylimidazolium tetrafluoroborate [MOEMIM][BF_4] was increased as compared to organic solvents [8]. Acetylation of glucose in organic solvents yielded a mixture of 6-*O*-acetylglucose and 3,6-*O*-diacetylglucose (2–3:1 mixture) while the acetylation in the ionic liquid yielded only the 6-*O*-acetylglucose [8].

It is however very important when comparing enzyme selectivity and activity in different solvents to separate 'true' solvent properties from other effects. To distinguish direct solvent effects from differences in enzyme hydration it is necessary to control the thermodynamic water activity of the reaction [9,10]. The solvent can also affect the activity and selectivity by affecting the enzyme and the substrate to a different extent. A higher solubility of the substrate in the solvent leads to a stabilisation of the ground state resulting in higher activation energy [11]. To separate effects due to differences in solubility of the substrate in different solvents the activity coefficients of the substrate in the solvents should be estimated. It is however often difficult to determine activity coefficients.

Here, we describe for the first time the use of esterases (from *Bacillus subtilis* and *Bacillus stearothermophilus*) in ionic liquids. A comparison of the stability, activity and enantioselectivity of the esterases in ionic liquids and organic solvents is performed.

2. Materials and methods

2.1. Materials and enzymes

Celite® 560, (±)-1-phenylethanol (>98%), vinyl acetate (>99%), *n*-hexanol (99%), *tert*-butyl-methylether (MTBE) (>99.8%), L-rhamnose (>99%) and *n*-hexane (>99%), were purchased from Fluka.

The ionic liquids 1-butyl-3-methyl imidazolium hexafluorophosphate (>98%) [BMIM][PF_6] and 1-butyl-3-methyl imidazolium tetraflouroborate (>98%) [BMIM][BF_4] were gifts from Solvent Innovation (Cologne, Germany) and 1-butyl-3-methyl imidazolium bis[(trifluoromethyl)sulfonyl]amide [BMIM]-[BTA] (>97%) was kindly donated from Prof. Udo Kragl (Fachbereich Chemie, Rostock University, Rostock, Germany).

Lipases PS-D from *Pseudomonas* sp. came from Amano Pharmaceutical Co. (Nagoya, Japan) and immobilised lipase B from *Candida antarctica* (Chirazyme L2) was donated by Roche Diagnostics (Penzberg, Germany). Other chemicals used were of analytical grade.

2.2. Cultivation of recombinant esterases

The two esterases from *B. subtilis* (BsubE) and *B. stearothermophilus* (BsteE) were previously cloned and overexpressed in *E. coli* and produced according to previously published protocols [12]. The protein contents of the lyophilised enzyme powders obtained after cultivation were determined with the Bradford method [13] and the activity in a standard esterase assay using *p*-nitrophenylacetate as a substrate [12]. The specific activity was 5 U/mg protein for BsubE and 8 U/mg protein for BsteE.

2.3. Immobilisation of esterases by deposition

The lyophilised esterases (4–23 mg corresponding to 2–11.5 mg protein), BsubE and BsteE, were dissolved in sodium phosphate buffer (50 mM, pH 7.5). The resulting enzyme solution (2 ml) was then added to Celite (1 g). The preparation was thereafter dried for 12 h under vacuum.

2.4. Measurement of enzyme activity

Substrate solution (10 mM alcohol, 200 mM vinyl acetate in solvent) and enzyme preparation (150 mg) were incubated over a saturated salt solution of LiCl to obtain a defined initial water activity ($a_w = 0.11$). The equilibration was performed overnight for the organic solvents and for the enzyme preparations. For the ionic liquids longer equilibration times are necessary

[14]. The equilibration was therefore performed for 7 days when [BMIM][PF_6] and [BMIM][BTA] were used as solvents. For the ionic liquid [BMIM][BF_4], the water activity was not controlled by the use of saturated salt solutions since it is a water miscible solvent [15]. This solvent was used as received from the manufacturer. The reactions were performed at 40 °C in 2 ml screw-capped vials with Teflon-lined septa and started by adding enzyme preparation to the substrate solution (10 mM alcohol, 200 mM vinyl acetate, 1 ml solvent). Reaction vials were shaken (1400 rpm) on a thermoshaker. Samples (50 μl) were withdrawn at different conversions and analysed by gas chromatography. In the case when [BMIM][BF_4] and [BMIM][PF_6] were used as solvents, the samples were extracted with MTBE (100 μl) and for [BMIM][BTA] the extraction was performed with *n*-hexane (100 μl) prior to the GC-analysis. The esterase activity was defined as the amount of ester produced (micromole) per minute and gram protein used in the reaction. The errors in the activity determinations were ±5%.

2.5. Determination of enzyme stability

The residual activity was measured by following the formation of hexylacetate in the transesterification of vinyl acetate with *n*-hexanol.

Esterase immobilised onto Celite (150 mg) was incubated in solvent (1 ml) at 40 °C. At different incubation times *n*-hexanol (1.2 μl) and vinyl acetate (18.5 μl) were added to the solution to obtain a final concentration of 10 and 200 mM, respectively. The initial activity was thereafter measured as described above. Half-lives were calculated from a first order exponential decay of the activity.

$$A = A_0 e^{-kt} \quad (1)$$

where k is the first order deactivation rate constant. A_0 and A are the initial and residual enzyme activity. When $A = A_0/2$ the half-life can be defined as $t_{1/2} = \ln(2/k)$.

2.6. GC-analysis

The alcohol substrate 1-phenylethanol and the ester product were analysed by a Shimadzu gas chromatograph (GC-14 A) equipped with a flame ionisation detector. The column used was a hydrodex-β-3P (25 m × 0.25 mm, Macherey-Nagel, Düren, Germany). Hydrogen was used as a carrier gas and the temperature of the injector and detector was 200 °C. The compounds were eluted isocratically at a column temperature of 110 °C. The retention times were 7.0 and 9.1 min for the (*S*)- and (*R*)-1-phenylacetate and 10.2, 11.2 min for the (*R*)- and (*S*)-1-phenylethanol, respectively.

In the cases when *n*-hexanol was used as nucleophile in the transesterification, the column temperature was 90 °C to separate the alcohol from the ester product. The retention times were 3.3 min for 1-hexanol and 5.1 min for hexylacetate. Standard curves of substrate and product were obtained to calculate response factors. The conversion was calculated from the amounts of the ester and alcohol peaks.

2.7. Calculation of enantioselectivity

The enantiomers of the (±)-1-phenylethanol and of the product (±)-1-phenylacetate were baseline separated in the GC-analysis. The conversion (C) was calculated by applying the equation

$$C = \frac{\text{ees}}{\text{ees} + \text{eep}} \quad (2)$$

The enantioselectivity for each reaction was determined by plotting C versus ees for all the points of each reaction and fitting the curve by non-linear regression.

$$C = 1 - \left[\frac{(1+\text{ees})^E}{1-\text{ees}}\right]^{1/1-E} \quad (3)$$

which gives the best E-value.

The Eq. (2) was derived from the enantioselectivity equation [16] for irreversible reactions.

$$E = \frac{\ln[(1-C)(1-\text{ees})]}{\ln[(1-C)(1+\text{ees})]} \quad (4)$$

The fit was performed with help of Kaleidagraph™ (Version 3.08d for Macintosh computers). The errors in the determination of enantioselectivity were below 5%.

3. Results and discussion

3.1. Effect of immobilisation

The specific activity for the esterase from *B. stearothermophilus* was measured in the transesterification of 1-phenylethanol with vinyl acetate in [BMIM][PF_6]. It is interesting to notice, that the lyophilised powder was active in *n*-hexane and not in [BMIM][PF_6] under the conditions used (Table 1). The fact that the enzyme activity in organic solvents can be increased by immobilisation onto a solid support is well established [17–19]. The esterase was therefore immobilised onto Celite and transesterification activity could thereafter be detected in [BMIM][PF_6] (Table 1).

The activity in [BMIM][PF_6] was highly dependent on the amount of protein that was loaded onto Celite (Fig. 1). Highest specific activity was obtained at a protein loading of 5 mg protein/g Celite. The results are in agreement with what has been previously reported when enzymes have been immobilised by deposition onto Celite for use in organic solvents [20]. At low protein loadings, the enzyme is directly inactivated by the support and at too high loading the onset of internal mass transfer limitations reduces the catalytic activity [20,21].

It has previously been reported that the protein amount on the carrier can significantly influence the enantioselectivity due to mass transfer limitations [22,23]. The enantioselectivity was, however not significantly influenced by the protein loading (Fig. 2) in [BMIM][PF_6]. Similar results were also observed in *n*-hexane. Highest specific activity (0.59 U/g protein) was obtained at a protein loading of 5 mg protein/g Celite and the enantioselectivity was independent of the protein loading.

3.2. Influence of solvent

The investigation was expanded to include four additional solvents. Two organic solvents, MTBE and vinyl acetate, which are commonly employed for biocatalysis and two ionic liquids [BMIM][BTA] and [BMIM][BF_4]. For both enzymes (BsubE, BsteE) the highest specific activity was obtained in hexane (Tables 1–3). The specific activity in *n*-hexane was 2.0–5.9-fold higher as compared to the more polar organic solvents (MTBE and vinyl acetate). This is in agreement with previous investigations showing that enzyme activity is promoted as the polarity of the organic solvent is decreased [24].

Of the three ionic liquids investigated, [BMIM]-[BTA] exhibited the highest specific activity for both

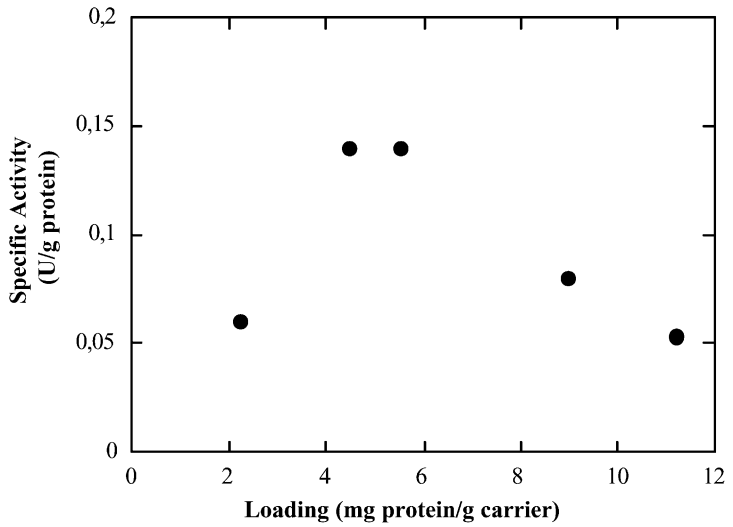

Fig. 1. Influence of protein loading on the specific activity of BsteE immobilised by deposition onto Celite in the transesterification of 1-phenylethanol (10 mM) with vinyl acetate (200 mM) in [BMIM][PF_6] at $a_w = 0.11$, 40 °C.

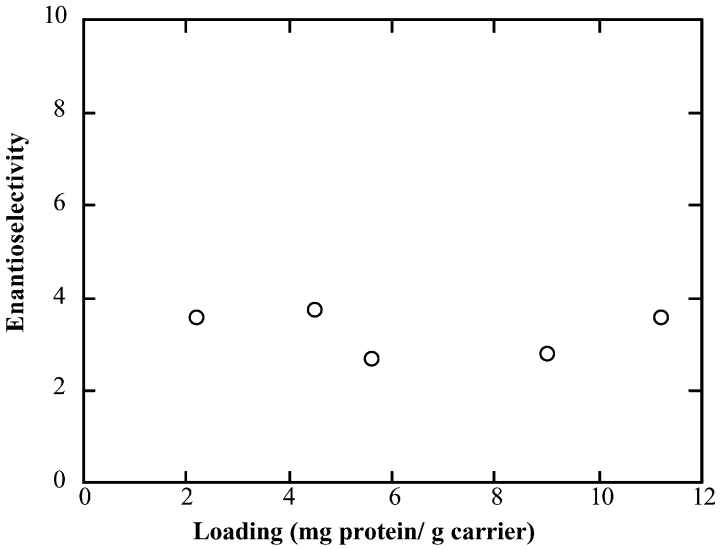

Fig. 2. Influence of protein loading on the enantioselectivity of BsteE in the transesterification of 1-phenylethanol (10 mM) with vinyl acetate (200 mM) in [BMIM][PF_6] at $a_w = 0.11$, 40 °C.

esterases investigated. An activity level that was either higher or similar to the ones obtained in vinyl acetate and MTBE was observed (Tables 1–3).

Highest enantioselectivity was achieved in [BMIM][BTA] ($E = 7.5$) for BsubE and in hexane ($E = 4.5$) for BsteE (Tables 1–3).

We tried to correlate the activity and enantioselectivity to the polarity of the different solvents employed with Reichardt's dye index ($E_T = 30$) obtained from [25–27]. No correlation between solvent polarity and enzyme activity and enantioselectivity was however obtained for both enzymes investigated.

The kinetic resolution was also performed with two commercial lipases *C. antarctica* lipase B (Chirazyme L2) and *Pseudomonas* sp. lipase (PS-D). The enantioselectivity was in these cases not significantly influenced by the solvent and the reactions progressed with high enantioselectivity ($E > 100$) in all solvents (results not shown). It has previously been reported that the enantioselectivity can be increased by the use of ionic liquids [14,28,29], while in other cases the enantioselectivity was similar in ionic liquids and in organic solvents [8]. Hence, so far there seems to be no straightforward correlation between

Table 1
Influence of enzyme preparation on specific activity and enantioselectivity of the esterase from *Bacillus stearothermophilus* in the transesterification of 1-phenylethanol (10 mM) with vinyl acetate (200 mM) in [BMIM][PF_6] and in *n*-hexane at $a_w = 0.11$, 40 °C

Preparation	Solvent	Specific activity (U/g protein)	*E*-value
Celite	[BMIM][PF_6]	0.14	3.5
Lyophilised	[BMIM][PF_6]	0	n.d.
Celite	Hexane	0.59	4.7
Lyophilised	Hexane	0.15	3.9

The enzyme loading was 5 mg protein/g Celite. n.d.: not determined.

Table 2
Enantioselectivity and specific activity of BsteE in the kinetic resolution of 1-phenylethanol (10 mM) with vinyl acetate (200 mM) at 40 °C, $a_w = 0.11$

Solvent	Specific activity (U/g protein)	*E*-value
MTBE	0.29	2.8
Vinyl acetate	0.10	2.0
[BMIM][BF_4][a]	0.33	3.5
[BMIM][BTA]	0.36	2.9

The enzyme was in all cases immobilised onto Celite 5 mg protein/g carrier.

[a] The water activity was not controlled for the solvent [BMIM][BF_4]. See Section 2.

Table 3
Enantioselectivity and specific activity for BsubE in the kinetic resolution of 1-phenylethanol (10 mM) with vinyl acetate (200 mM) at 40 °C, $a_w = 0.11$

Solvent	Specific activity (U/g protein)	*E*-value
Hexane	1.0	6.8
MTBE	0.44	7.2
Vinyl acetate	0.22	6.9
[BMIM][PF_6]	0.30	7.5
[BMIM][BF_4][a]	0.18	7.5
[BMIM][BTA]	0.42	7.2

The enzyme was in all cases immobilised by deposition onto Celite 5 mg protein/g carrier.
[a] The water activity was not controlled for the solvent [BMIM][BF_4]. See Section 2.

the enantioselectivity obtained in ionic liquids and in organic solvents.

3.3. Enzyme stability

The stability of the esterase from *B. stearothermophilus* was investigated in hexane, MTBE, [BMIM][BF_4] and [BMIM][PF_6] (Table 4). To shorten the reaction time when measuring the residual activity, a primary alcohol (*n*-hexanol) was used as nucleophile. Half-lives of >240 and 220 h were obtained in [BMIM][PF_6] and [BMIM][BF_4], respectively, which was >3.0- and 2.8-fold higher as compared to the stability in MTBE, and >30- and 27-fold higher as found for *n*-hexane, respectively (Table 4).

Increased stability of α-chymotrypsin in [BMIM][PF_6], [BMIM][BF_4], methyl trioctylammonium bis[(trifluoromethyl)sulfonyl]amide [MTOA][Tf_2N] and 1-ethyl-3-methylimidazolium 1,1,1-trifluoro-*N*-[(trifluoromethyl)sulfonyl]methanesulfonamide [EMIM][Tf_2N] as compared to 1-propanol has previously been reported [30]. Higher stability of *C. antarctica* lipase B in several ionic liquids as compared to 1-butanol and *n*-hexane was also reported recently [31]. Part of the explanation for the increased stability of *C. antarctica* lipase B and α-chymotrypsin was attributed to the differences in polarity of the different solvents which resulted in different hydration levels of the enzyme preparation. It has been reported that a higher water activity of the system results in a lower stability of the enzyme [32,33]. In the present paper, the water activity of the enzyme and the solvents [BMIM][PF_6], MTBE and *n*-hexane was controlled. The observed increase in stability in [BMIM][PF_6] can therefore not be attributed to differences in hydration of the enzyme in the different solvents and must therefore reflect a direct solvent effect. It can be speculated that electrostatic interactions occur between the ionic liquids and the protein, resulting in a more rigid protein, which needs to overcome a higher kinetic barrier to unfold as compared to when the enzyme is suspended in a non-ionic organic solvent.

Table 4
Stability of BsteE immobilised by deposition onto Celite in ionic liquids and in organic solvents at 40 °C, $a_w = 0.11$

Solvent	Half-life
[BMIM][PF_6][a]	>240
[BMIM][BF_4][b]	220
MTBE	80
Hexane	8.0

The residual activity was measured in the transesterification of *n*-hexanol (10 mM) with vinyl acetate (200 mM). The enzyme loading was in all cases 5 mg protein/g carrier.
[a] The residual activity was 100% after 240 h.
[b] The water activity was not controlled for the solvent [BMIM][BF_4]. See Section 2.

4. Conclusions

In conclusion, the two esterases studied (BsubE and BsteE) were active in the three ionic liquids investigated and exhibited similar activity and enantioselectivity as in organic solvents. Furthermore, the enzymes exhibited a higher stability in the ionic liquids as compared to organic solvents. In addition, ionic liquids also posses' several interesting physicochemical properties such as negligible vapour pressure, high chemical and thermal stability. It is therefore likely that these solvents will find several applications as alternative reaction media for biotransformations.

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References

[1] M. Erbeldinger, A. Mesiano, J. Russel, Biotechnol. Prog. 16 (2000) 1129.
[2] R. Madeira Lau, F. Rantwijk, K. Seddon, R. Sheldon, Org. Lett. 2 (2000) 4189.
[3] S.G. Cull, J.D. Holbrey, V. Vargas-Mora, K. Seddon, G.J. Lye, Biotechnol. Bioeng. 69 (2000) 227.
[4] J.G. Huddleston, A. Visser, M. Reichert, H. Willauer, G. Broker, R. Rogers, Green Chem. 3 (2001) 156.
[5] S. Dzyuba, A. Bartsch, Chem. Phys. Chem. 3 (2002) 161.
[6] R. Sheldon, Chem. Commun. 7 (2001) 2399.
[7] M. Eckstein, M. Sesing, U. Kragl, P. Adlercreutz, Biotechnol. Lett. 24 (2002) 867.
[8] S. Park, R. Kazlauskas, J. Org. Chem. 66 (2001) 8395.
[9] R. Valivety, P. Halling, A. Macrae, Biochim. Biophys. Acta 1118 (1992) 218.
[10] P. Halling, Enzyme Microb. Technol. 16 (1994) 178.
[11] K. Ryu, J.S. Dordick, Biochemistry 31 (1992) 2588.
[12] E. Henke, U.T. Bornscheuer, Appl. Microbiol. Biotechnol. 60 (2002) 320.
[13] M. Bradford, Anal. Biochem. 72 (1976) 248.
[14] M. Eckstein, P. Wasserscheid, U. Kragl, Biotechnol. Lett. 24 (2002) 763.
[15] G. Bell, A. Janssen, P. Halling, Enzyme Microb. Technol. 20 (1997) 471.
[16] C.S. Chen, Y. Fujimoto, G. Girdaukas, C.J. Sih, J. Am. Chem. Soc. 104 (1982) 7294.
[17] M. Persson, E. Wehtje, P. Adlercreutz, Chem. Biol. Chem. 3 (2002) 566.
[18] G. Ottolina, G. Carrea, S. Riva, F. Veronese, Biotechnol. Lett. 14 (1992) 947.
[19] G. Pencreac'h, J.C. Baratti, Appl. Microb. Biotechnol. 47 (1997) 630.
[20] R. Barros, E. Wehtje, P. Adlercreutz, Biotechnol. Bioeng. 59 (1998) 364.
[21] J. Bosley, A. Peilow, J. Am. Oil Chem. Soc. 74 (1997) 107.
[22] M.J. Litjens, K.Q. Le, A.J. Straathof, J.A. Jongejan, J.J. Heijnen, Biocatal. Biotransform. 19 (2001) 1.
[23] D. Rotticci, T. Norin, K. Hult, Org. Lett. 2 (2000) 1373.
[24] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechnol. Bioeng. 30 (1987) 81.
[25] S. Aki, J. Brennecke, A. Samanta, Chem. Commun. (2001) 413.
[26] C. Reichardt, Chem. Rev. 94 (1994) 2319.
[27] S. Dzyuba, A. Bartsch, Tetrahedron Lett. 43 (2002) 4657.
[28] P. Kielbasinski, M. Albrycht, J. Luczak, M. Mikolajczyk, Tetrahedron Asymm. 13 (2002) 735.
[29] K. Kim, B. Song, M. Choi, M. Kim, Org. Lett. 3 (2001) 1507.
[30] P. Lozano, T.D. Diego, J. Guegan, M. Vaultier, J. Iborra, Biotechnol. Bioeng. 75 (2001) 564.
[31] P. Lozano, T.D. Diego, D. Carrie, M. Vaultier, J. Iborra, Biotechnol. Lett. 23 (2001) 1529.
[32] E. Wehtje, H. de Wit, P. Adlercreutz, Biotechnol. Tech. 10 (1996) 947.
[33] D. Costes, G. Rotcenkovs, E. Wehtje, P. Adlercreutz, Biocatal. Biotransform. 19 (2001) 119.