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Catalysis by amine oxidases in nonaqueous media

Jennifer A. Chaplin, Cheryl L. Budde, Yuri L. Khmelnitsky*

EnzyMed, A Division of Albany Molecular Research, Inc., 2501 Crosspark Road, Suite C-150, Iowa City, IA 52242, USA

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Abstract

The synthetic potential of amine oxidases was examined in different reaction systems, ranging from aqueous solutions to organic solvents with low water content. Substantial conversion was achieved in biphasic systems, which eliminated the product inhibition observed in the aqueous system. The conversion was particularly high in the more hydrophobic solvents. The use of low water systems was studied using amine oxidase immobilized on celite and pre-equilibrated in a salt hydrate environment to reach a constant water activity. Addition of water in the solvent was shown to be unnecessary, with significant conversion being attained through the water supplied by pre-equilibration of the immobilized enzyme at $a_w = 0.55$. The use of organic solvent-containing reaction systems thus presents a convenient method for oxidising poorly water-soluble amines using amine oxidases. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Amine oxidase; Organic solvent; Biphasic system; Immobilized enzyme; Water activity

1. Introduction

Amine oxidases catalyze the oxidation of amines, producing an aldehyde, ammonia and hydrogen peroxide, according to Scheme 1.

The enzymatic oxidation results in a Schiff base, which is nonenzymatically hydrolysed to the aldehyde product [1].

Amine oxidases, which are all dimers of 70–95 kDa per subunit [2], can be grouped according to their cofactors. One of these groups, the copper-containing amine oxidases, possess either a topaquinone or 6-hydroxydopa cofactor. Members of this group include the plasma amine oxidases, diamine oxidases and various bacterial, yeast and plant monoamine oxidases [3]. Another group, the mitochondrial amine oxidases (including monoamine oxidase), contain a covalently bound flavin cofactor. There are two known forms of monoamine oxidase, designated monoamine oxidase A (MAO-A) and monoamine oxidase B (MAO-B), which differ in their substrate specificity and their sensitivity to inhibitors [4].

Amine oxidases have significant synthetic potential, as illustrated by a number of reported examples. The oxidation of amines to Schiff bases in organic solvent has been investigated [5], as has the transformation of the alkaloid nazlinin and its analogs, which was performed by porcine kidney diamine oxidase [6]. Pea seedling amine oxidase catalyzed the stereoselective oxidation of alkylamines, benzylamine and arylethylamines [7] and has been used for preparation of phenacyl intermediates for the chemical synthesis of certain alkaloids [8]. The synthesis of vanillin from vanillylamine by amine oxidase from *Aspergillus niger* has also been described [9]. With a few exceptions [5,10], these synthetic studies have

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* Corresponding author. Tel.: +1-319-626-5412;
fax: +1-319-626-5410.
E-mail address: ykhmelnitsky@enzymed.com (Y.L. Khmelnitsky).

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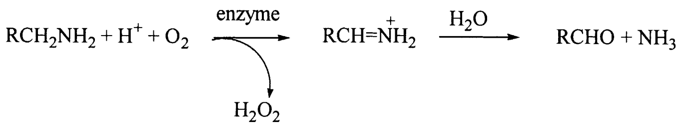

Scheme 1. Reaction catalyzed by amine oxidases.

been performed in aqueous medium, and very little is known about the effect of organic solvents on amine oxidases. However, many potential substrates of these enzymes are poorly soluble in water, thus requiring the presence of organic solvents in the reaction system. Therefore, the insufficient knowledge regarding the behavior of amine oxidases in nonaqueous environment limits their practical use.

The objective of this work was a detailed investigation of the tolerance of different amine oxidases to nonaqueous solvents and the feasibility of using various types of systems containing organic solvents as reaction media for these enzymes.

2. Experimental

2.1. Enzymes

The following enzymes were used: plasma amine oxidase (90 U/g protein; SAO), diamine oxidase from porcine kidney (0.04 U/mg solid; DAO), catalase from bovine liver (30,000 U/mg protein), all from Sigma; plasma amine oxidase from bovine plasma (27.8 U/mg dry weight; WAO) (Worthington). In the case of enzymes received from the manufacturer in the form of a suspension, the liquid was removed from the suspension by centrifugation and precipitated enzyme dissolved in the appropriate buffer.

2.2. Chemicals

All chemicals were obtained from Sigma-Aldrich. All solvents were HPLC grade and were obtained from Sigma-Aldrich or Fischer Scientific. The solvents used were ($\log P$ values in brackets) [11]: ethanol (−0.24), acetonitrile (−0.33), ethyl acetate (0.68), diethyl ether (0.85), methyl *tert*-butyl ether (MTBE) (1.15), methylene chloride (1.19), butyl acetate (1.7), diisopropyl ether (DIPE) (1.9), chloroform (2.0), benzene (2.0), toluene (2.5), carbon tetrachloride (3.0), cyclohexane (3.2) and heptane (4.0). All solvent concentrations are in volume percent.

2.3. Enzyme immobilization

Coimmobilization of amine oxidases and catalase by adsorption was carried out by dissolving amine oxidase (45.3 mg SAO or 31.0 mg WAO) and catalase (16 mg) in 950 μl of 0.2 M sodium phosphate buffer pH 7.2. The enzyme solution was added to 500 mg of celite (acid washed but not calcined). The mixture was thoroughly stirred, dried under reduced pressure to a free-flowing powder. It was then equilibrated over an appropriate salt hydrate, according to the modified published procedure [12]. Specifically, the salt was added to about one-third of the height of a screw cap sealable bottle and then saturated with water. Pre-weighed immobilized enzyme in a smaller container was then placed inside the bottle. The enzyme-containing vial was removed and weighed daily until a constant weight was achieved (approximately 4 days). The following salts were used: LiCl ($a_w = 0.11$), $ZnSO_4{\cdot}7H_2O$ ($a_w = 0.58$) and KCl ($a_w = 0.85$) [13].

2.4. Reactions in monophasic systems

Reaction mixtures were prepared by dissolving either SAO (0.15 mg) or DAO (3.3 mg), and catalase (0.15 mg) in 230 μl of 0.2 M sodium phosphate buffer pH 7.2. Catalase was added for removal of H_2O_2 formed during the reaction, in order to prevent inactivation of the enzyme. The reaction was started by addition of 20 μl of a 100 mM benzylamine solution in the phosphate buffer. The reactions were incubated at room temperature for 24 h. The effect of water-miscible organic solvents on enzyme activity was investigated by addition of 25% (v/v) solvent to the reaction mixture. All reactions were carried out in 2 ml glass vials, sealed with PTFE septa in the caps.

2.5. Reactions in biphasic systems

The reactions were carried out by addition of 250 μl of water-immiscible organic solvent to 250 μl of the aqueous phase containing 10 mM of the amine substrate, amine oxidase and 0.6 mg catalase in 0.2 M sodium phosphate buffer pH 7.2. The amounts of amine oxidases used were as follows (per 250 μl of the aqueous phase): SAO, 0.3 mg; DAO, 10 mg. The reactions were shaken at room temperature on an orbital shaker at 180 rpm for 48 h. All reactions were carried out in 2 ml glass vials, sealed with PTFE septa in the caps. The organic phase and the aqueous phase (where necessary) were analyzed by gas chromatography (GC).

2.6. Immobilized enzymes in low water systems

Reaction mixtures were prepared by adding 15 mg of the immobilized enzyme, pre-equilibrated over appropriate salt hydrate, to organic solvent containing 0, 1 or 5% (v/v) 0.2 M sodium phosphate buffer, pH 7.2. No separate aqueous phase was formed in all cases. The solvents had been dried over molecular sieves and contained 6.5 mM of the amine substrate. The final reaction volume was 200 μl. The reaction mixtures were incubated at room temperature on an orbital shaker for 24 h at 200 rpm before analysis.

2.7. Analytical procedures

GC analysis was carried out on a Shimadzu 17-A gas chromatograph, with a flame ionization detector, on an AT-5 column (Alltech; 30 m × 0.25 mm × 0.25 μm film thickness). The initial temperature of 70°C was held for 5 min before increasing to 270°C at a rate of 35°C/min. The final temperature was held for 4 min in the case of reactions with benzylamine, and for 10 min for all other amines (in order to ensure full elution of the Schiff base). Samples for analysis were withdrawn directly from the reaction mixture; if precipitate was present, this was removed by centrifugation prior to injection. The results were reported as conversion, i.e. the percentage of benzylamine converted to benzaldehyde.

Mass spectroscopy (MS) analysis was performed by flow injection analysis on a PE-Sciex API 2000 atmospheric pressure chemical ionization (APCI) mass spectrometer. The solvent phase was a 50:50 mixture of water and acetonitrile, both containing 0.4% acetic acid.

3. Results and discussion

3.1. Reactions in monophasic systems

The effect of several commonly used organic solvents on the conversion of benzylamine to benzaldehyde catalyzed by SAO and DAO is shown in Fig. 1.

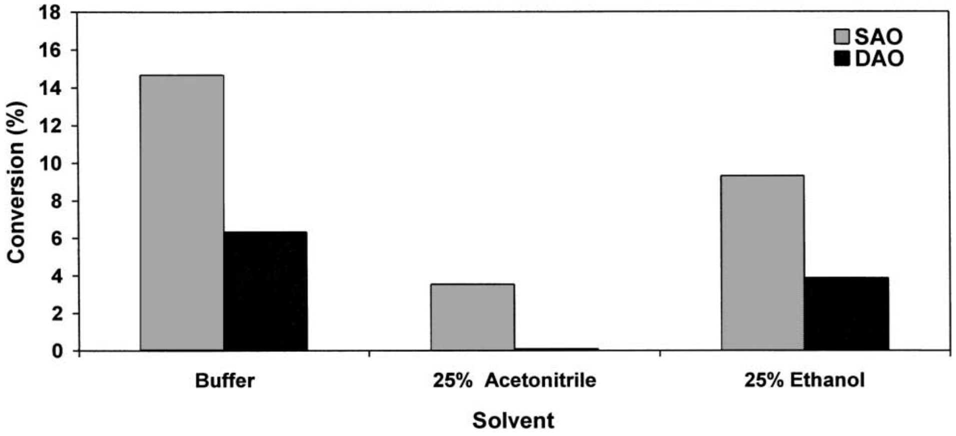

Fig. 1. Effect of organic cosolvent on oxidation of benzylamine by SAO and DAO in monophasic systems.

The relatively low conversions observed in monophasic systems may be the result of product inhibition by benzaldehyde, which has been shown to be an uncompetitive inhibitor of amine oxidases [10]. The possibility of impairment of the enzyme by the H_2O_2 released during the reaction was eliminated by the addition of catalase, which acts as a scavenger for this product. The addition of acetonitrile or ethanol decreased the conversion compared to the purely aqueous system, suggesting that the presence of cosolvents unfavorably affected the enzyme activity. The adverse effect of organic cosolvents on amine oxidases and potential problems with product inhibition considerably limit the utility of monophasic systems as reaction media for synthetic applications using these enzymes. These complications can be avoided in biphasic reaction systems, that are widely used as a medium of choice for biocatalytic transformations [14].

3.2. Reactions in biphasic systems

In biphasic systems, which are composed of water and water-immiscible organic solvent, the enzyme resides exclusively in the aqueous phase and therefore largely avoids the unfavorable direct contact with the nonaqueous environment. In contrast, substrates and products of the enzymatic reaction partition between water and organic solvent. If the reaction product is sufficiently hydrophobic, it will be extracted from water into the organic phase. In the case of an inhibitory reaction product, the partitioning will therefore prevent or decrease the loss of enzyme activity caused by product inhibition [14,15].

To verify these advantages of biphasic systems for catalysis by amine oxidases, we tested different biphasic system as reaction media for SAO and DAO. The results of these experiments are shown in Fig. 2. Obligingly, the oxidation of benzylamine to benzaldehyde was in general significantly more efficient than in monophasic systems, and practically complete conversion was achieved in many cases. Although the effect of several solvents, such as ethyl acetate, diethyl ether, butyl acetate and chloroform, was quite different on SAO and DAO, the conversion generally tended to increase with increasing hydrophobicity of the solvent, as determined by $\log P$. This trend could be explained by more efficient extraction of the hydrophobic inhibitory product, benzaldehyde, into more hydrophobic solvents, thus resulting in higher enzyme activity and better conversions. In addition, organic solvents with high $\log P$ are known to be less harmful to biocatalysts in biphasic systems [16].

Analysis of the solvent phase of the biphasic reaction mixtures by GC revealed the presence of an extra peak on chromatograms, presumably corresponding to a side product. MS analysis of the compound in this peak showed the molecular mass corresponding to the Schiff base formed by the interaction between

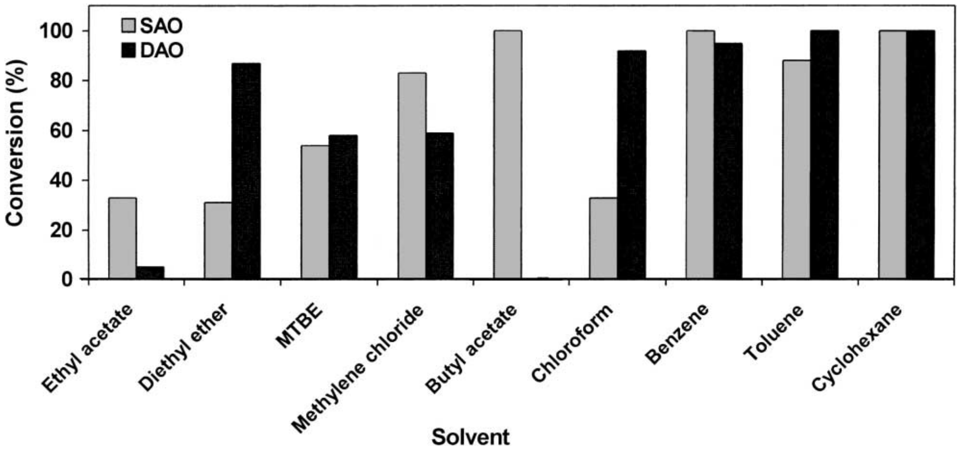

Fig. 2. Effect of solvent type on oxidation of benzylamine by SAO and DAO in biphasic systems. The solvents are arranged in order of increasing $\log P$.

benzaldehyde (reaction product) and benzylamine (substrate). The identity of the side product was further confirmed by GC analysis of an equimolar mixture of benzylamine and benzaldehyde incubated overnight in organic solvent (cyclohexane) at room temperature. The retention time of the authentic Schiff base prepared in this way was identical to the retention time of the side product found in the amine oxidase reactions. Quantitative estimates showed that significantly less than 10% of the benzaldehyde formed enzymatically in the biphasic system reacted with the residual benzylamine to form the Schiff base. This side product was also detected in monophasic systems. However, since the conversion in those systems was low, relatively small amounts of benzaldehyde were available for Schiff base formation, and therefore only traces of the side product were formed.

3.3. Immobilized amine oxidases in low water systems

Enzymatic reactions in biphasic systems generally involve the mass transfer of substrate and/or reaction products between phases, which can be the rate-limiting step in the overall process, especially at larger scale. In principle, mass transfer limitations can be reduced by increasing the stirring speed of the heterogeneous reaction system. However, the more vigorous stirring may result in emulsion formation, leading to difficulties in product recovery and loss of enzyme activity [17,18]. These problems can be avoided by using enzymes immobilized by adsorption on hydrated solid supports [17–19]. Immobilized enzyme catalysts provide easier handling and product recovery, potentially higher enzyme stability and better dispersion in the solvent.

In reaction systems based on immobilized enzymes dispersed in organic solvents, part of the available water is dissolved in the organic solvent, while the remaining water is bound to the support and/or adsorbed enzyme and usually does not form a visually detectable separate phase. In other words, all components of the reaction system, including solvent, support and enzyme, compete for available water. If the overall concentration of water in the system is high enough, it may form a continuous aqueous microphase within pores of the solid support, actually resulting in a biphasic system with supported aqueous phase. In this case, the immobilized enzyme experiences essentially aqueous microenvironment and may possess catalytic properties similar to those observed in bulk aqueous solution. However, at lower water concentrations the amount of available water may not be sufficient to form the aqueous microphase, leading to significant changes in the hydration state of immobilized enzyme and, as a result, strongly affecting the catalytic activity. The transition between the high and low hydration states is difficult to detect, and therefore, thorough control of water content is very important for achieving optimal performance of immobilized enzymes suspended in organic solvents.

The feasibility of the immobilization strategy for catalysis by amine oxidases in organic solvents was tested using SAO and WAO immobilized by adsorption on celite. The effect of water-immiscible organic solvents and concentration of added water on oxidation of benzylamine by immobilized plasma amine oxidases is shown in Fig. 3. In this experiment, the enzymes were pre-equilibrated at $a_{\mathrm{w}} = 0.85$ to eliminate possible variation of the water content in the system caused by the hydration water introduced with the immobilized enzyme. The results in Fig. 3 show that at the highest water content (5%), both SAO and WAO gave high conversions of benzylamine to benzaldehyde in all solvents tested, virtually regardless of the solvent nature. Similar to biphasic systems, a small amount (significantly less than 10%) of the Schiff base was also formed. Presumably, under these highly hydrated conditions the enzyme resides in essentially aqueous microenvironment within the support-bound water microphase and therefore is not significantly affected by the surrounding solvent. However, the behavior of SAO and WAO started to diverge with decreasing water content in the reaction system. At lower concentrations of added aqueous buffer conversions observed for SAO remained practically unchanged in all solvents. In contrast, WAO showed consistently decreasing conversions, especially in solvents with lower $\log P$, where conversions in some cases dropped to zero in the absence of added water (Fig. 3). These findings suggest that WAO and SAO strongly differ in their hydration requirements needed for catalytic activity.

In the case of water-miscible solvents, including ethanol, acetonitrile, tetrahydrofuran, dioxane and *N*,*N*-dimethylformamide, very low or no conversion

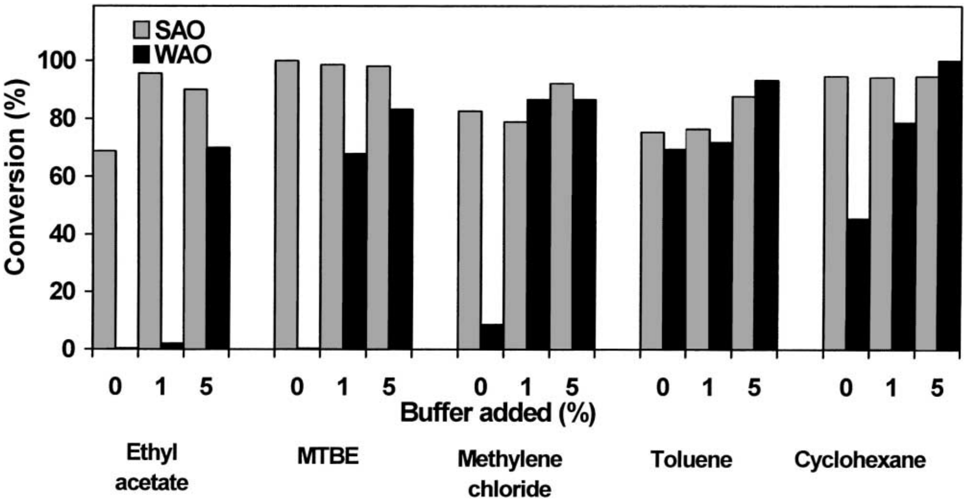

Fig. 3. Effect of solvent type and water content on oxidation of benzylamine by plasma amine oxidases (SAO and WAO) in organic solvent. The enzymes were immobilized onto celite and pre-equilibrated to $a_w = 0.85$. The solvents are arranged in order of increasing $\log P$; the water was added to the organic solvent.

was observed for celite-immobilized WAO and SAO at 0, 1 or 5% of added water (data not shown). The loss of activity is probably caused by the removal of the essential hydration water from the enzyme by polar organic solvents, known as water stripping [12,20]. The detrimental effect of polar organic solvents on the activity of amine oxidases is in general agreement with the results obtained for monophasic water–cosolvent systems (Fig. 1).

In a similar study, activity by a different amine oxidase, MAO-B, was reported in a range of water-immiscible organic solvents with low water content (1% v/v) [5]. Likewise, the enzyme, which was used in the freely suspended form, was inactive in

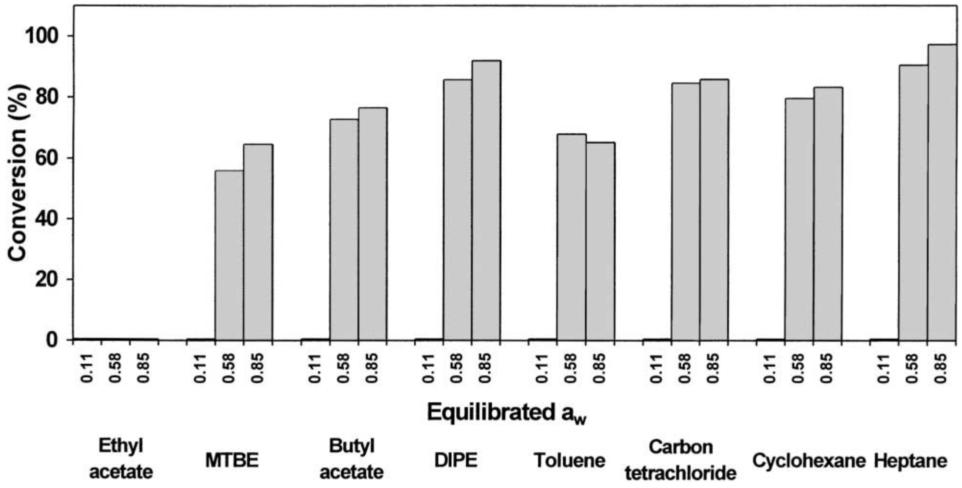

Fig. 4. Effect of solvent and water activity of the immobilized enzyme preparation on oxidation of benzylamine by plasma amine oxidase (SAO) in organic solvent. The enzyme was immobilized onto celite and pre-equilibrated to water activities of 0.11, 0.58 and 0.85 before addition to the solvent. The solvents are arranged in order of increasing $\log P$.

water-miscible solvents. Investigation of a range of water concentrations, which was performed in only one solvent, octane, revealed that a water concentration as low as 0.1% (v/v) was sufficient for enzymatic activity [5].

In order to further probe into the apparent ability of immobilized SAO to function under conditions of low hydration (Fig. 3), samples of this enzyme were prepared by equilibration at lower levels of a_w and tested in a series of anhydrous solvents. The results of this experiment (Fig. 4) revealed that while the catalytic activity was only marginally affected by decreasing a_w from 0.85 to 0.58, the enzyme was completely inactivated by further decrease to $a_w = 0.11$. Comparison of Figs. 3 and 4 shows that the general trend for the dependence of catalytic activity on hydration conditions is similar for SAO and WAO: both enzymes are highly active at high water contents and eventually lose activity at lower hydration levels. However, SAO seems to be considerably more tolerant to low water conditions and therefore can successfully function in much drier systems compared to WAO.

Similar to the results in Fig. 3, the effect of solvent is not clearly evident from the data in Fig. 4, except that in the latter experiment no activity was found in ethyl acetate, which has the lowest log P among the solvents shown in Fig. 4. Evidently, at the low hydration levels this relatively polar solvent plays a more substantial role in the competition for available water, resulting in the stripping of essential water from the enzyme with concomitant loss of activity, as described above for more polar solvents.

In conclusion, we have demonstrated that amine oxidases can be used as efficient synthetic catalysts in reaction systems with high contents of organic solvents. In preliminary studies, we also have found that these enzymes can accept a wide range of structurally diverse amines [21] that extends far beyond the limits of substrate specificity determined from the studies related to the role of amine oxidases in the development of pathological conditions such as Parkinson's disease [5–7]. Good solvent tolerance and broad substrate specificity highlight the great synthetic potential of amine oxidases and make these enzyme a valuable addition to the arsenal of practical biocatalysts.

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References

[1] A.S. Kalgutkar, K. Castagnoli, A. Hall, N. Castagnoli Jr., J. Med. Chem. 37 (1994) 944–949.
[2] F. Vianello, A. Malek-Mirzayans, M.L. Di Paolo, R. Stevanato, A. Rigo, Protein Express. Purif. 15 (1999) 196–201.
[3] A. Holt, G. Alton, H. Scaman, G.R. Loppnow, A. Szpacenko, I. Svendsen, M.M. Palcic, Biochemistry 37 (1998) 4946–4957.
[4] C.H. Williams, J. Lawson, F.R.C. Backwell, Biochem. J. 256 (1988) 911–915.
[5] J.C.G. Woo, X. Wang, R.B. Silverman, J. Org. Chem. 60 (1995) 6235–6236.
[6] E. Cheng, J. Botzem, M.J. Wanner, B.E.A. Burm, G.-J. Koomen, Tetrahedron 19 (1996) 6725–6732.
[7] A.R. Battersby, J. Staunton, M.C. Summers, J. Chem. Soc., Chem. Commun. 465 (1974) 548–549.
[8] J.E. Cragg, R.B. Herbert, M.M. Kgaphola, Tetrahedron Lett. 31 (1990) 6907–6910.
[9] A. Yoshida, Y. Takenaka, H. Tamaki, I. Frebort, O. Adachi, H. Kumagai, J. Ferm. Bioeng. 84 (1997) 603–605.
[10] J.C.G. Woo, R.B. Silverman, J. Am. Chem. Soc. 117 (1995) 1663–1664.
[11] C. Laane, S. Boeren, K. Vos, C. Veeger, Biotechol. Bioeng. 30 (1987) 81–88.
[12] R.H. Valivety, P.J. Halling, A.R. Macrae, Biochim. Biophys. Acta 1118 (1992) 218–222.
[13] P.J. Halling, Biotechol. Tech. 6 (1992) 271–276.
[14] G. Carrea, TibTechnology 2 (1984) 102–106.
[15] A.E.M. Janssen, A. van der Padt, K. van't Riet, Biocatal. Biotransform. 12 (1995) 223–240.
[16] C. Laane, S. Boeren, K. Vos, Trends Biotechnol. 3 (1985) 251–252.
[17] G. Carrea, S. Riva, R. Bovara, P. Pasta, Enzyme Microb. Technol. 10 (1988) 333–340.
[18] R.M. Blanco, P.J. Halling, A. Bastida, C. Cuesta, J.M. Guisan, Biotechnol. Bioeng. 39 (1992) 75–84.
[19] J.M. Guisan, R.M. Blanco, A. Bastida, R. Fernandez-Lafuente, C. Cuesta, P.J. Halling, Ann. New York Acad. Sci. 672 (1992) 158–166.
[20] A. Zaks, A.M. Klibanov, J. Biol. Chem. 17 (1988) 8017–8021.
[21] J.A. Chaplin, C.L. Budde, Y.L. Khmelnitsky, 1999, unpublished results.