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Lipase-catalyzed reaction in the packed-bed reactor with continuous extraction column to overcome a product inhibition

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Abstract

The resolution of *rac*-α-methyl-β-propiothiolactone (*rac*-MPTL) was performed in a packed-bed reactor (PBR) using *Pseudomonas cepacia* lipase (PCL) in organic media to produce enantiopure (*R*)-MPTL. By comparing enzyme stability of three enzyme forms, i.e. commercial PCL powder, Celite-immobilized PCL, and cross-linked enzyme crystals of PCL (CLECs-PCL), Celite-immobilized PCL was chosen for the construction of PBR because of its comparable stability to that of CLEC and easy handling. Owing to the severe product inhibition by 3-mercapto-α-methylpropionic acid (MMPA), the batch reaction system was inappropriate for the hydrolysis with PCL at high concentration of *rac*-MPTL. To overcome these problems, PBR with a continuous extraction column was used. The product inhibition was successfully overcome by incorporating an aqueous extraction unit. However, the yield of (*R*)-MPTL (enantiomeric excess, ee > 99%) was only about 20% based upon the initial concentration of *rac*-MPTL, due to the concomitant partitioning of *rac*-MPTL to the aqueous phase in the extraction column as well as the subsequent auto-hydrolysis of *rac*-MPTL to *rac*-MMPA during extraction. To reduce the undesired partitioning of *rac*-MPTL to aqueous phase, various salts were screened to modulate the partitioning coefficient of the reaction components. Using 1 M ammonium sulfate solution as the aqueous phase of the extraction column, the yield of (*R*)-MPTL (ee > 99%) was enhanced to 40%.

Keywords: (*R*)-α-methyl-β-propiothiolactone; Kinetic resolution; Lipase; Product inhibition; Extractive process

1. Introduction

The use of biotransformation to improve or simplify synthetic chemical process is becoming a fashion these days. One of the major examples is the use of enzyme-catalyzed reactions. Enzymatic reaction can be favored over chemical catalysis under such circumstances where thermal degradation of labile compounds is minimized [1] and use of chemicals with a potential for pollution can be avoided [1,2]. Furthermore, enzymes sometimes show excellent enantio- and regio-selectivity [3,4]. However, common drawbacks associated with the enzymatic process are the inhibition of enzyme at high concentration of substrate or product [5,6], and instability of

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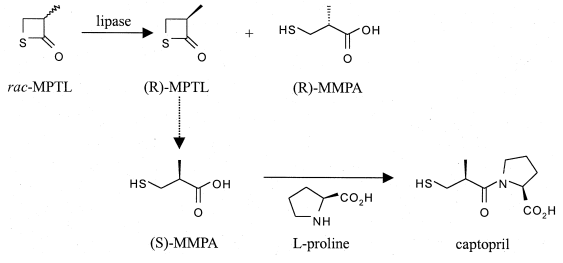
enzyme under the reaction condition [7] as well as in repetitive usage [8]. The substrate inhibition is usually lessened by a slow addition of the substrate, and a simple solution to overcome the product inhibition is either product removal from the reaction system or in situ product transformation to a noninhibitory material [9]. Therefore, the continuous removal of the product in the enzyme reaction is a key factor to proceed the reaction at high concentrations of substrate especially when the accumulation of product is inhibitory or promotes enzyme deactivation. Such an integration of the enzyme reaction and product separation steps [10,11] becomes very important in industrial process.

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) hydrolyzes triglycerides as well as catalyzes ester synthesis and transesterification under micro-aqueous condition. Lipases have been used to resolve racemic compounds to optically pure ones through stereoselective hydrolysis or esterification. Recently, we have found that the lipase from *Pseudomonas cepacia* (PCL) displayed unusual enantioselective hydrolysis activity to a (*S*)-thiolactone of four-membered ring, i.e. (*S*)-α-methyl-β-propiothiolactone ((*S*)-MPTL) compared with the corresponding (*R*)-thiolactone [18]. This hydrolysis of *rac*-MPTL by PCL was a novel process to produce (*R*)-MPTL, a key intermediate for synthesizing captopril (Scheme 1). However, a severe inhibitory effect of the hydrolyzed product, i.e. (*R*)-enriched 3-mercapto-α-methylpropionic acid (MMPA), on the hydrolysis activity of PCL was observed in this reaction, and this inhibition was the main problem against carrying out this reaction at high concentration of MPTL and its scaling up. To overcome this product inhibition at high concentration of the substrate, we have developed an integrated system with in situ product removal using continuous extraction against aqueous salt solution. In addition, to make the integrated system efficient, packed-bed reactors (PBR) using different forms of enzyme were compared and strategies to operate the integrated reaction system were investigated.



Scheme 1.

2. Materials and methods

2.1. Enzymes and chemicals

Lipases from PCL was purchased from Amano Pharmaceutical (Nagoya, Japan) and used without further purification. Thiolacetic acid, methacrylic acid, and methyl chloroformate were purchased from Aldrich (Milwaukee, WI). MPTL was synthesized according to the procedure described elsewhere [12]. All the other reagents were of analytical grade.

2.2. Enzyme immobilization and preparation of cross-linked enzyme crystals (CLECs)

Enzyme was concentrated by ultra-filtration membrane (molecular cut-off: 10,000) to 6.6 mg/ml of protein concentration and immobilized on Celite 545 and dried by lyophilization [13]. Protein concentration was measured by Bradford assay using bovine serum albumin as the standard protein. Lipase crystal was obtained by the method of Bornscheuer et al. [14] with slight modification. A total of 20 g of crude lipase was dissolved in 5 mM sodium phosphate buffer (pH 7.0), and centrifuged twice at 8000 rpm for 20 min at 4°C. The supernatant was applied onto a phenyl-Sepharose column (4.9 $cm^2 \times 20$ cm) equilibrated with 10 mM piperazine buffer (pH 5.0). Elution with a linear gradient of isopropanol from 0% to 75% resulted in purified lipase solution and the enzyme was crystallized at −20°C from the active frac-

tions of the phenyl-Sepharose chromatography. The concentration of the isopropanol in the crystallizing solution was analyzed to be 58% (v/v). Cross-linking of the enzyme was carried out using 5% glutaraldehyde solution at room temperature for 1 h. CLECs were sequentially washed with distilled water, ethanol, isopropanol and *n*-hexane and were stored at room temperature.

2.3. Enzyme reactions and reactor operation

All batch reactions were performed in 5-ml glass vials with shaking at 37°C. Cyclohexane displayed the highest enantioselectivity as well as the reaction rate for this resolution reaction [18]. Therefore, in a typical experiment, 6 mg (0.05 mmol) *rac*-MPTL, 1 ml cyclohexane, and 10 μl distilled water were mixed, then the reactions were started by adding 10 mg of lipase. In an experiment with CLECs-PCL, 1 mg of CLECs-PCL was added to the same reaction mixture to start the reaction.

Fig. 1 shows schematic diagram of PBR system coupled with continuous extraction column. To the cylindrical glass column (I.D. 10×50 mm), maintained at 37°C using water jacket, substrate solution was fed upwardly from the bottom of the column by metering pump (FMI Lab Pump Model QV, Fluid Metering Inc., New York). A total of 2 g of the lipase (crude or immobilized) were used for one-time PBR operation. The flow rate of the recycling reactor was set to 2.8 ml/min. At this flow rate, the whole reactor could be considered as a differential reactor, where no external diffusional limitations were observed. Likewise, the flow rate did not affect the reaction rate under this condition. For the operation with extraction unit, the outlet from the reactor was introduced into the extraction column (height, 20 cm; volume, 50 ml) through sparger. During contacting with water in the extraction column, the cyclohexane was saturated with water and all MMPA was extracted into water phase. The water-saturated cyclohexane was reintroduced into the substrate bottle from the clearly separated upper phase of extraction unit.

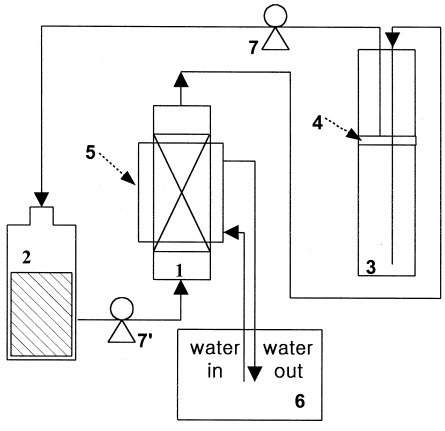


Fig. 1. Schematic diagram of apparatus for PBR system integrated with extraction column. (1) Glass column reactor filled with enzyme, (2) substrate reservoir, (3) aqueous phase of extraction column, (4) cyclohexane phase of extraction column, (5) water jacket, (6) water bath, (7) and (7′) metering pumps.

2.4. Analytical methods

The progress of reaction was monitored by HPLC using chiral column (Chiralpak, Daicel, Tokyo, Japan). The yield and enantiomeric excess (ee) were calculated based on the concentration of substrate, i.e. (*R*)- and (*S*)-MPTL. Analysis of each isomer of MPTL was done with the UV detector at 210 nm at room temperature. A total of 0.5 ml/min of *n*-hexane was used as the mobile phase. Water content was measured by Karl-Fisher water titrator (Mettler, Switzerland). Partitioning coefficient of *rac*-MPTL (K_{MPTL}) between cyclohexane and different salt solutions was measured by adding equal volume of salt solution to the cyclohexane containing 50 mM *rac*-MPTL, and comparing the concentrations of thiolactones in each phase until no change in concentration was observed.

3. Results and discussion

3.1. Selection of enzyme preparation

The stability of the enzyme is one of the critical factors for the successful operation of

PBR. In nonaqueous media enzymology, the stability of enzyme was often affected by the solvent-induced inactivation. For lipase, such inactivation was usually effectively overcome by the use of appropriate immobilization technique. It is well known that using immobilized enzyme can change the optimal conditions for enzyme reaction, such as water contents of the nonaqueous reaction medium, pH, reaction temperature, etc. [15]. In addition, immobilized enzyme is usually superior to crude enzyme for repeated usage of enzyme due to the easy recovery of the immobilized form. Among various immobilization methods, one of the simple but effective methods is the adsorption of enzyme on appropriate support matrix, and subsequent lyophilization. Removal of water by lyophilization can still retain essential water molecules for enzyme activity and can greatly enhance activity of enzyme in organic solvent. Several support materials were examined for the immobilization of the lipase. Although some ion exchange resins showed better protein retention, Celite 545 displayed the best property for the kinetic resolution of MPTL (data not shown). In recent decades, CLECs have been developed to guarantee the stability, activity, and regio- and stereo-selectivity of biocatalysts [16,17]. CLECs-PCL was prepared from purified protein, using crystallization and cross-linking with glutaraldehyde. The stability of these enzyme preparations were compared with that of crude enzyme (Fig. 2) by measuring their activities in batch glass vial after incubating in cyclohexane for several days.

The crude enzyme showed relatively large fluctuations (Fig. 2a) that were not correlated with the incubation period. This appeared to be caused by the irregular aggregation of lipase in the organic solvent. Celite-immobilized PCL was well dispersed in the reaction media and showed a stable reaction profile for 7 days (Fig. 2b). However, both preparations (crude and Celite-immobilized lipases) showed no significant differences in the stability for 7 days, in terms of the final conversion. The Celite-immobilized enzyme is quite competent compared with CLECs (Fig. 2c). Because of the difficulties in handling and high cost in the preparation of micro-crystals, *rac*-MPTL resolution reaction using CLECs appeared not to be economical and efficient. As a result, the Celite-immobilized enzyme was used for further study.

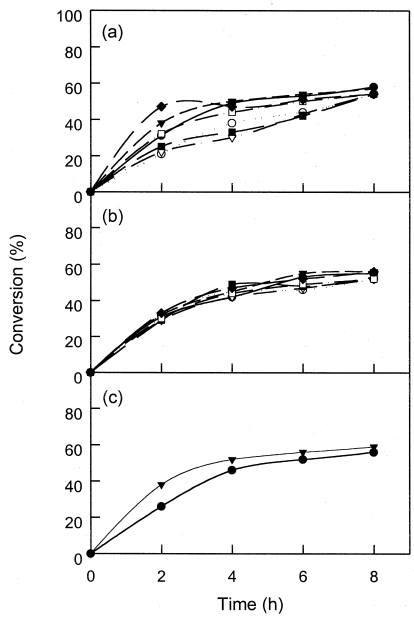


Fig. 2. Stability of various types of PCL in cyclohexane. Reaction profiles of hydrolysis of *rac*-MTPL of the PCL in a batch system was plotted. A total of 50 mM *rac*-MTPL and 10 mg of each enzyme after pre-incubation in cyclohexane were added to 1 ml of cyclohexane and incubated at 37°C with shaking. (a) Crude enzyme □: no incubation; ♦: 1 day; ■: 3 days; ▽: 4 days; ▼: 5 days; ○: 6 days; ●: 7 days. (b) Immobilized enzyme ♦: no incubation; ♦: 1 day; ■: 3 days; ▽: 4 days; ▼: 5 days; ○: 6 days; ●: 7 days. (c) CLECs ▼: no incubation; ●: 7 days.

3.2. Construction of PBR

To make the enzyme reaction successful at high substrate concentration, the substrate inhibition and product inhibition had been investigated. While no substrate inhibition had been observed for this reaction, severe competitive product inhibition had been identified by kinetic study [18]. This result suggests that continuous removal of product from the reaction system

might be useful for the enzyme reaction when high concentration of the substrate is used.

In general, PBR has some advantages for large-scale process, such as obtaining a high yield with low cost, and ease of design, operation and maintenance [19]. Additionally, in the case of enzyme reaction in PBR with serious product inhibition, integration of in situ product removal step is relatively easier than any other step such as coupled enzyme reactions. The optimal water content for the batch reaction was revealed as 5% in the preliminary kinetic study examined elsewhere [18]. In batch reaction, as the water contents of reaction medium increased, PCL's reaction rate increased, whereas the enantioselectivity of the reaction decreased due to the auto-hydrolysis of thiolactone. However, the use of water over 0.1% was impractical for the operation of PBR due to the separation of aqueous phase. Therefore, 0.1% of water was used for PBR reactions. Crude and immobilized enzymes showed nearly the same reaction profile when using 50 mM substrate (Fig. 3) in PBR runs.

3.3. High substrate concentration reaction in PBR

The product inhibition of the enzyme reaction by *rac*-MMPA in batch reaction is a major problem for the operation of PBR at high concentration of *rac*-MPTL. Although the initial reaction rates of PCL increased in proportion to the concentrations of *rac*-MPTL up to 300 mM, the generation of (*R*)-enriched MMPA above 50 mM significantly inhibited the further reaction progress. A kinetic study showed that the resolution of *rac*-MPTL virtually did not proceed after the formation of 50 mM of *rac*-MMPA in the reactions at high substrate concentrations.

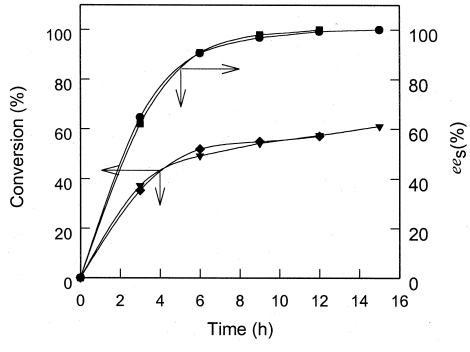


Fig. 3. Reaction profiles of low concentration reaction at PBR system for crude enzyme and immobilized enzyme. Concentration of *rac*-MTPL was 50 mM and total reaction volume was 20 ml. Other conditions are noted in the text. ▼: Conversion for crude enzyme; ●: ee_s for crude enzyme; ◆: conversion for immobilized enzyme; ■: ee_s for immobilized enzyme.

To overcome the product inhibition, the enzyme reaction with in situ continuous removal of the acid through an extraction column was carried out. The yields of (*R*)-MPTL were 22% for the crude enzyme and 24% for the Celite-immobilized enzyme, respectively, and the final ee_s of the (*R*)-MPTL were all above 99%. Continuous removal of the product (i.e. (*R*)-enriched MMPA) by the extraction column would be plausible by the wide difference in the solubility of the MMPA between the organic media and water. The partitioning coefficient of the *rac*-MMPA (K_{MMPA}) between cyclohexane and water was 0.05, suggesting that the most acid produced during the hydrolysis reaction diffuses into the aqueous phase of the extraction column. This removal of *rac*-MMPA from the cyclohexane enables the reaction to proceed. However, the final yields were much lower than the yields from the batch reaction at 50 mM of substrate concentration due to the undesirable partitioning of substrate MPTL into aqueous phase and its auto-hydrolysis in extraction unit.

3.4. Salt solution as aqueous phase of the extraction column

Using an extraction column for the continuous removal of the product, the product inhibition in the reaction of PBR could be successfully overcome to a great extent. However, relatively low yields of (*R*)-MPTL in cyclohexane phase were an unexpected result. The main cause of low yield was ascribed to the undesirable diffusion of the substrate (MPTL) to the aqueous phase of the extraction column and the

instability of MPTL in water. We had found that *rac*-MPTL is unstable in water and more rapidly auto-hydrolyzed by the pH change in the reaction medium caused by its acid forms (i.e. (*R*)- or (*S*)-MMPA). Then the partitioning of *rac*-MPTL between cyclohexane and aqueous phase is subsequently changed and the concentration of *rac*-MPTL in cyclohexane becomes lower than that without the auto-hydrolysis.

To avoid these undesirable effects on the MPTL partitioning and stability associated with the extraction unit, aqueous phase solution in the extraction column should meet two requirements, i.e. low partitioning coefficient of *rac*-MPTL in the aqueous phase and better stabilization of *rac*-MPTL. One simple but effective method for changing the properties of aqueous solution is adding inorganic salt to water. The partitioning coefficients of *rac*-MPTL (K_{MPTL}) between cyclohexane and aqueous solution were measured from 14 kinds of salt solutions. Among them, four showed the relatively high K_{MPTL} value and some stabilizing effect on the MPTL (Fig. 4). Because *rac*-MPTL was very labile and was easily hydrolyzed by contacting with water, we carefully measured its stability using various salt solutions at various concentrations. Defining the stability of *rac*-MPTL as the remaining concentration of *rac*-MPTL in cyclohexane phase ($C_{MPTL,org}$), ammonium bicarbonate solution showed the lowest $C_{MPTL,org}$, with which the highest K_{MPTL} value was observed. This indicates that the *rac*-MPTL was auto-hydrolyzed very rapidly in the aqueous phase in spite of its low partitioning into the ammonium bicarbonate solution The highest $C_{MPTL,org}$ was observed with 1 M ammonium sulfate solution, and this solution seemed to have desirable properties to reduce the partitioning of *rac*-MPTL into the aqueous phase and to lessen the auto-hydrolysis of *rac*-MPTL in the salt solutions. Using 1 M ammonium sulfate solution as aqueous phase of the extraction column, the final yields of MPTL were 38% and 37% with crude and Celite-immobilized enzyme, respectively, and their ee of the remaining (*R*)-MPTL was ca. $>99\%$ ((*S*)-MPTL was not detected at all in HPLC analysis) (Fig. 5).

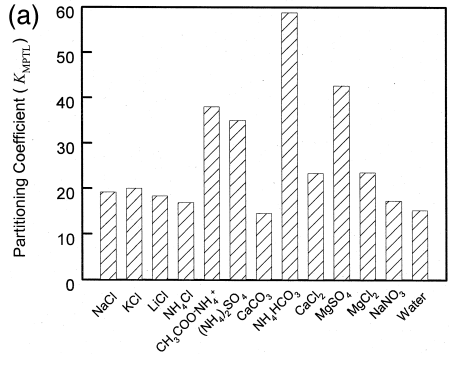


Fig. 4. Partitioning coefficients of α-methyl-β-thiolactone between cyclohexane and various salt solutions. The concentration of each salt in salt solution was 1 M and the concentration of *rac*-MTPL in cyclohexane was 50 mM.

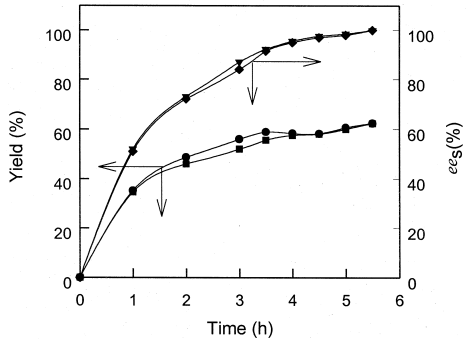


Fig. 5. Reaction profiles of high concentration reaction at PBR system with extraction column for crude enzyme and immobilized enzyme. Concentration of *rac*-MTPL was 500 mM and total reaction volume was 20 ml. A total of 1 M ammonium sulfate solution was used for aqueous phase solution of the extraction column and volume of aqueous phase was 50 ml. Other conditions are noted in the text. ●: Conversion using crude enzyme; ▼: ee_s using crude enzyme; ■: conversion using immobilized enzyme; ◆: ee_s using immobilized enzyme.

4. Conclusion

Two main objects of this study have been examined and partially answered. The first one was the demonstration of scale-up of lipase hydrolysis reaction for the production of chiral thiolactone, which exhibits high product inhibi-

tion, with good yields, and the other one was the comparison of stability of different enzyme forms for the reactor operation.

Product inhibition involved in the lipase hydrolysis reaction is one of the major obstacles that make the enzyme reactor incompetent compared with the chemical process. When this product or by-product is an acid compound such as in lipase reaction, substrate hydrolysis and enzyme stability become more complicated, since the reaction as well as enzyme stability are largely affected by the resulting products. There are many ways of overcoming this inhibitory effect of product in the enzyme reactor. Removal of the product from the reaction medium is one of the most efficient and commonly used methods. Here, we studied a model system of an enzyme reaction with serious product inhibition, and developed a PBR coupled with an extraction column for the product removal. Using this extraction column, we overcame the product inhibition successfully. Its capacity to remove the inhibiting product was controlled through the addition of unreactive additives, i.e. salt, in the aqueous phase of the extraction unit. Hence, partitioning coefficients of the product and stability of the substrate in the reaction media were changed at the same time. This kind of improvement in the product removal can be further enhanced by using membrane extraction units, since different surface properties of the membrane can be fully utilized for the effective separation of the products.

To improve enzyme stability in the enzyme reactor, which is one of the most important characteristics of the enzyme reactor, crude PCL powder, Celite-immobilized PCL and CLECs-PCL were compared. Recently, CLECs have been suggested as the most stable form of enzyme in organic solvent. But, surprisingly, we did not observe any significant difference in the improvement of stability within our time scale by using CLECs. In this thiolactone hydrolysis system, the stability of Celite-immobilized enzyme was almost the same as that of CLECs up to 7 days. Although we could not prove that the stability of immobilized PCL is similar to that of CLECs-PCL in cyclohexane for the longer period of time, easier preparation method using adsorption followed by drying or lyophilization, practical handling and the use of bead form appear to justify its usage and to compensate for the possible low stability of Celite-immobilized enzymes. The operation of PBR was also quite stable up to three times with Celite-immobilized PCL. However, superiority of CLECs stability under more stringent conditions such as long period of time at higher temperature and higher substrate concentration remains to be seen.

When a selective hydrolysis or synthesis reaction is used for racemate resolution, asymmetric synthesis is usually preferred, if there is any, owing to the high, i.e. 100%, theoretical maximum yield. Therefore, to improve the yield of any resolution reaction, inversion of one form of unwanted product is always needed using various means. If the recycling of the products, mainly (*R*)-MMPA, from the aqueous phase is possible to convert it into *rac*-MPTL, this resolution reaction can become more economical. One drawback of the aqueous extraction of (*R*)-enriched MMPA from cyclohexane is the difficulty in its back-extraction from the aqueous solutions through practical means, unless the concentration of (*R*)-enriched MMPA in the aqueous extractant is very high. Therefore, efficient adsorption and desorption of (*R*)-enriched MMPA with ion exchange column would be a possible candidate for further study to replace the extraction column.

Acknowledgements

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References

[1] R.A. Sheldon, Chirotechnology: Industrial Synthesis of Optically Active Compounds vol. 205 Marcel Dekker, New York, 1993.

[2] R.A. Sheldon, in: A.M.P. Koskinen, A.M. Klibanov (Eds.), Enzymatic Reactions in Organic Media, Blackie A&P, London, 1996, p. 266.
[3] K. Faber, Biotransformations in Organic Chemistry, 3rd edn., Springer, Berlin, 1997, p. 27.
[4] R.J. Kazlaukas, U.T. Bornscheuer, in: H.-J. Rehm, G. Reed (Eds.), Biotechnology vol. 8a Wiley-VCH, Weinheim, 1998, p. 37, Chap. 3.
[5] M. Markweghanke, S. Lang, F. Wagner, Enzyme Microb. Technol. 17 (1995) 512.
[6] R. Perraud, F. Laboret, Appl. Microbiol. Biotechnol. 44 (1995) 321.
[7] V.V. Mozhaev, Y.L. Khmelnitsky, M.V. Sergeeva, A.B. Belova, N.L. Klyachko, A.V. Levashov, K. Martinek, Eur. J. Biochem. 184 (1989) 597.
[8] L. Giorno, E. Molinari, E. Drioli, B.P. Cesti, J. Chem. Technol. Biotechnol. 64 (1995) 345.
[9] M. Ichikawa, R.L. Schnaar, Y. Ichikawa, Tetrahedron Lett. 35 (1995) 8731.
[10] A. Liese, M. Karutz, J. Kampuis, C. Wandrey, U. Kragl, Biotechnol. Bioeng. 51 (1996) 544.
[11] A.L. Paiva, X. Malcata, J. Mol. Catal. B: Enzym. 3 (1997) 99.
[12] H.B. Lee, H.Y. Park, B.S. Lee, Y.G. Kim, Magn. Reson. Chem., in press.
[13] J. Kim, B.G. Kim, Biotechnol. Bioeng. 50 (1996) 687.
[14] U. Bornscheuer, O. Reif, R. Lausch, R. Freitag, T. Scheper, F.N. Kolisis, U. Menge, Biochim. Biophys. Acta 1201 (1994) 55.
[15] M.Y. Arica, V. Hasirci, J. Chem. Technol. Biotechnol. 58 (1993) 287.
[16] J.J. Lalonde, C. Govardhan, N. Khalaf, A.G. Martibez, K. Visuri, A.L. Margolin, J. Am. Chem. Soc. 117 (1995) 6845.
[17] N. Khalaf, C.P. Govaedham, J.J. Lalonde, R.A. Persichetti, Y.F. Wang, A.L. Margolin, J. Am. Chem. Soc. 118 (1996) 4594.
[18] B.-Y. Hwang, H.B. Lee, Y.G. Kim, B.-G. Kim, Biotechnol. Prog., submitted for publication.
[19] E. Roca, N. Meinander, B. Hahn-Hägerdal, Biotechnol. Bioeng. 51 (1996) 317–326.