Available online at www.sciencedirect.com
ScienceDirect
Journal of Molecular Catalysis B: Enzymatic 42 (2006) 39–44
JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC
www.elsevier.com/locate/molcatb

Enantioselectivity modulation through immobilization of *Arthrobacter* sp. lipase: Kinetic resolution of fluoxetine intermediate

Asha Chaubey, Rajinder Parshad*, Surrinder Koul, Subhash C. Taneja, Ghulam N. Qazi

Biotechnology Division, Regional Research Laboratory (CSIR), Jammu Tawi-180001, India

Received 29 March 2006; received in revised form 31 May 2006; accepted 15 June 2006
Available online 24 July 2006

Abstract

Arthrobacter sp. lipase (ABL, MTCC no. 5125) has been identified for its excellent performance in kinetic resolution of a number of drug intermediates. ABL free enzyme provided product **II** and **V** (ee < 95%) from racemic fluoxetine intermediate (**I** and **IV**) compared to its cell biomass in naturally immobilized state (ee < 98%). To overcome this problem and obtaining high enantioselectivity (*R* isomer ee 99%), ABL enzyme was modulated by immobilization using various methods vis-à-vis substrate modification (Scheme 2). Immobilized enzyme obtained by hydrophobic binding provided 6710–7720 U/g, covalent binding 200 U/g, and sol–gel entrapment 65–110 U/g activity. Substantial improvement in enantioselectivity was obtained using acylates of ethyl 3-hydroxy-3-phenylpropanoate a fluoxetine drug intermediate (*R* isomer ee from 93 to 99% and *E* from 43 to 127–473) at 29–45% conversion in fixed time period of 21 h, indicating thereby some change in conformation of ABL immobilized enzyme. The ABL immobilized by covalent binding and sol–gel entrapment has demonstrated reasonable superiority over the free ABL in enantioselectivity as well as over all rate of hydrolysis. Immobilized enzymes prepared by covalent and entrapment methods have shown excellent operational stability and used for 10 cycles without loss in activity and the technique can be upscaled for process development.

Keywords: Immobilization; Enantioselectivity; Kinetic resolution; *Arthrobacter*

1. Introduction

Lipases, i.e. glycerol ester hydrolase EC 3.1.1.3 are enzymes that catalyse hydrolysis of fatty acid ester bonds in triacylglycerol to give fatty acids, diacylglycerols, monoacylglycerols and glycerol. Lipases are well known for their specificity and selectivity [1–4]. Therefore they are commonly used for resolution of racemic compounds and drug intermediates [5–8]. Thus through stereoselective enzymatic ester hydrolysis, direct potential access to one of the enantiomers within the racemates can be exploited. However, exploitation of common commercial lipases viz. *Porcine pancreatic* lipase (PPL), *Candida cylindracea* lipase (CCL), *Pseudomonas* sp. lipase (PSL), etc. has been limited due to economical constraints such as high cost of commercially available enzymes, instability, non reusability and high processing costs involved [9,10]. Therefore, attempts to develop or improve indigenous enzyme for better activity, selectivity, stability, easy processing and reusability with high enantioselectivity becomes necessary for industrial applications.

Applications of lipases in organic chemistry depend on the catalysis involving conformational changes of the enzyme molecule [11,12]. It is well known that the lipases exist in two structural forms viz. open and closed conformations. Equilibrium between these two forms could be achieved via immobilization involving different areas of enzyme, conferring different rigidity to the enzyme structure or generating certain microenvironment around the enzyme [13]. This may result in the shape of final open form of lipase.

Since lipases are considered to be one of the unstable enzymes as compared to other enzymes, sometimes they do not show optimal activity or enantioselectivity. Moreover lipases have a complex mechanism of action, therefore it is very much essential that lipases may be modified in such a way to give best of their activity for a particular application. Immobilization may be considered as a possible tool for improving and optimizing the activity and stability of lipases [14–16]. Immobilized lipases may be proved useful for scaling up of the process, e.g. simple performance, and design of the bioreactor, easy recovery of prod-

* Corresponding author. Tel.: +91 191 2572002x237.
E-mail address: Parshad_R@yahoo.com (R. Parshad).

1381-1177/$ – see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.molcatb.2006.06.011

ucts, multiple use of biocatalyst and better operational stability. Design of suitable immobilization procedure for lipases may result in different biotransformation properties through different mechanism of action on a specific substrate [17,18]. Binding of enzymes on a pre-existing support by covalent binding may improve the performance of lipases to a great extent by providing operational stability of the enzyme [19,20]. Hydrophobic binding on the sepharose derivatives may result in hyper activation of lipase through selective adsorption [21–23]. Entrapment of lipases within a porous support by sol–gel method may also provide excellent stability [24–26] though the entrapped enzyme can act only on the fraction of substrate soluble in the reaction medium and is able to penetrate the porous structure of immobilized biocatalyst.

A strain of *Arthrobacter* sp. (ABL) isolated at Regional Research Laboratory, Jammu (India) (MTCC no. 5125), used for various lipase/esterase applications was reported [27–29] and now target is to use the immobilized enzyme for newer substrates [30,31]. Keeping in view, commercial viability of the enzyme, an attempt has been made to immobilize ABL by covalent, hydrophobic binding and sol–gel entrapment methods. Proficiency of immobilized ABL in terms of activity, selectivity and reusability is studied so as to reduce the cost of overall process for resolution of ethyl 3-hydroxy-3-phenyl propanoate (fluoxetine intermediate) and its acylates vis-à-vis with native enzyme.

2. Experimental

2.1. Preparation of cell biomass for enzyme preparation

The *Arthrobacter* sp. cell biomass was prepared in shake flasks and in 10 L fermentor containing medium (1% peptone, and 0.5% NaCl and 0.5% beef extract, pH 7.0). The medium was inoculated with an overnight preculture prepared in the same broth. The culture was grown at 30 °C for 16–18 h at 200 rpm. The cell pellet was separated from the broth by centrifugation at 10,000 × *g* for 15 min at 4 °C. The cell pellet was preserved at −20 °C till further use for enzyme isolation. *Arthrobacter* sp. microbial culture (ABL, MTCC no. 5125), isolated at RRL Jammu has been deposited in MTCC culture collection under Budapest Treaty (2004).

2.2. ABL enzyme preparation

2.2.1. Isolation

RRL lipase from *Arthrobacter* sp. was obtained by ultrasonication of cells (1 U/mg wet biomass) in phosphate buffer, pH 7.0 using MSE Manor Roya Crawley RH 10 2QQ cell disrupter at 16 kHz. Cell free extract obtained was then partially purified before use.

2.2.2. Partial purification

The cell free extract obtained from the above method was partially purified by 60% ammonium sulphate precipitation. The precipitates were then dissolved in phosphate buffer (0.1 M, pH 7.0) and dialysed against phosphate buffer (10 mM, pH 7.0). The partially purified enzyme was lyophilised (specific activity 40 units per mg protein) and stored at −20 °C till use.

2.3. Immobilization of enzyme

2.3.1. Covalent immobilization

Coupling solution prepared by dissolving lyophilised ABL (150 mg) in 10 ml coupling buffer, 0.1 M $NaHCO_3$ pH 8.3 was added to the activated gel and left for coupling with gentle shaking. After 2 h the coupling gel was filtered and washed with 15 ml of coupling buffer to remove the excess enzyme adhered to the surface of matrix. In order to block any remaining active groups, gel was kept in Tris–HCl buffer, pH 8.0 for about 2 h. Immobilized enzyme was filtered followed by drying under vacuum.

2.3.2. Hydrophobic binding

Enzyme preparation (200 mg dissolved in 10 ml phosphate buffer) was added to phenyl agarose and octyl sepharose gels pre-equilibrated with phosphate buffer. The immobilization reaction was continued for 1 h. Thereafter the supernatant and immobilized enzyme, were separated by filtration under vacuum followed by washing with phosphate buffer, pH 7.0 and drying under vacuum.

2.3.3. Sol–gel entrapment of ABL

Tetraethylorthosilicate (TEOS) was hydrolysed in acidic medium (0.04N HCl) in a container under magnetic stirring to form sol at room temperature. The partially purified enzyme (180 mg dissolved in 10 ml phosphate buffer) was added to the sol and kept for stirring at 10 °C for gelation. After 24 h the gel was kept for aging for another 24 h followed by drying at room temperature to obtain white powder doped with ABL. The dry powder was washed well with phosphate buffer to elute loosely bound/entrapped enzyme and dried again at room temperature. The dry gel enzyme activity was evaluated using tributyrin as substrate.

2.4. Lipase activity measurements of free and immobilized enzyme

Activity of free and immobilized enzymes was measured in an emulsion containing 1% (v/v) tributyrin and 1% (w/v) gum acacia in the presence of NaCl and $CaCl_2$. pH of the emulsion was maintained 7.0 using pH stat for 5 min. One unit of enzyme releases 1 μM of fatty acid per minute from triacylglycerols.

2.5. Protein estimation

Protein estimations were carried out using standard Bradford's method [32]. Bovine serum albumin was used as standard protein.

2.6. Resolution of fluoxetine intermediate

Racemic ethyl 3-hydroxy-3-phenylpropanoate and its alkyl acylates (fluoxetine intermediate: substrate **I** and **IVa**, **IVb**, **IVc**: 25 mg/ml) in 0.1 M phosphate buffer, were continuously

stirred at room temperature in the presence of free and immobilized enzyme (6–7 units/ml) maintaining the pH 7.0 using pH stats. The progress of the enzymatic reaction both in free and immobilized state was monitored by drawing samples 100 μl periodically. The samples were extracted with ethyl acetate (3 × 100 μl). The progress of enzymatic reaction was monitored initially by thin layer chromatography followed by HPLC using a Whelk (SS)-chiral column (Lichro CART250-401, 5 μm); mobile phase (hexane:isopropanol:acetic acid in the ratio of 95:4.9:0.1), flow rate 1.0 ml/min, at 254 nm, with diode array detector.

2.7. Determination of enantiomeric excess (ee) and enantioselectivity (E)

Enantiomeric excess (ee) of the product was analysed by HPLC using chiral column and enantioselectivity (E) of product was determined according to Chen et al. [33]:

$$\text{ee}(P) = \frac{A - B}{A + B}$$

where A and B are the area under the curve for R and S isomers, respectively:

$$E(P) = \frac{\ln[1 - c\{1 + \text{ee}(P)\}]}{\ln[1 - c\{1 - \text{ee}(P)\}]}$$

where c is the extent of conversion and ee(P) is the enantiomeric excess of product.

3. Results and discussion

Immobilized enzyme properties mostly depend on microenvironment of proteins on support surface, i.e. presence of hydrophobic (hydrophobic binding), hydrophilic groups (covalent bonding) and within sol–gel derived materials (entrapment). The accessible active protein, accessible denatured protein, inaccessible active protein, and inaccessible denatured protein together are responsible for the catalytic properties of the immobilized enzyme [34]. ABL enzyme has been immobilized following various immobilization strategies (Section 2.3) obtaining seven immobilized lipase (A–G, Table 1). Fermentation experiments were performed five times obtaining wet cell biomass (1 U/mg) and enzyme isolation/partial purification was performed for each experiment. Immobilization on each support was carried out under controlled standardised conditions obtaining similar results. Results presented here are the average of five such immobilization experiments (five replicates). Hydrophobic binding of ABL in presence of large hydrophobic groups on the support (phenyl agarose) A and (octyl sepharose) B for the lipase to get adsorbed very strongly in an open and hyper activated manner [23] resulted in 6710 U/g binding on phenyl agarose (A) and 7720 U/g on octyl sepharose (B). ABL covalent immobilization by CNBr activation method [20] on sepharose led to highly stable immobilized enzyme with 200 U/g activity (C).

Similarly for ABL immobilization by entrapment method, sol–gel (D) was prepared directly from acid catalysed hydrolysis of tetraethylorthosilicate (TEOS), obtaining 80 U/g activity. Sol–gel derivative prepared in presence of polyvinyl alcohol as additive, sol–gel/PVA (E) resulted in increase of activity to give 110 U/g of immobilized enzyme. Other sol–gel derivatives obtained with polyethylene glycol, i.e. sol–gel/PEG (F) and sol–gel/fructose (G) showed 75 U/g and 65 U/g activities, respectively. Apparent less unit activities per g in sol–gel entrapped ABL may be attributed to diffusion limitations, steric hindrance or inaccessibility of active sites of enzyme to the substrate. Conformational changes may also lead to changed kinetics of the entrapped enzyme in a constrained microenvironment due to the possible interactions of the protein molecules with the surrounding matrix pore walls [35].

3.1. Operational stability of immobilized enzyme on various matrices

In order to ascertain the reusability of immobilized enzymes, 1 g of each derivative in Table 1 was used for lipase activity analysis using tributyrin as substrate under standard assay conditions. After every cycle the product was washed well with buffer to remove traces of substrate and products. Results shown in Fig. 1 indicate that ABL immobilized by covalent bonding (C) retains about 87% activity after 10 times reuse. Entrapped ABL by sol–gel method (D) was also found to retain 78% and entrapped ABL with additives (E–G) retained 79–83% activity after 10 cycles indicating good operational stability of immobilized enzyme. However, the activities of immobilized ABL on phenyl and octyl sepharose (A and B) were reduced to 56 and 60% in the second cycle itself. Thereafter a drastic loss in activity was observed due to enzyme elution from the support on reuse.

Table 1
ABL enzyme immobilization on different supports[a]

Support	Type of binding	Enzyme loaded (mg) per g support	Activity (U) per g support
Phenyl agarose (A)	Hydrophobic	200	6710
Octyl sepharose (B)	Hydrophobic	200	7720
Sepharose (C)	Covalent	150	200
Sol–gel (D)	Entrapment	180	80
Sol–gel/PVA (E)	Entrapment	180	110
Sol–gel/PEG (F)	Entrapment	180	75
Sol–gel/fructose (G)	Entrapment	180	65

[a] All types of immobilizates prepared above are from the enzyme prepared from five fermentation experiments, results shown are the average values of five experiments.

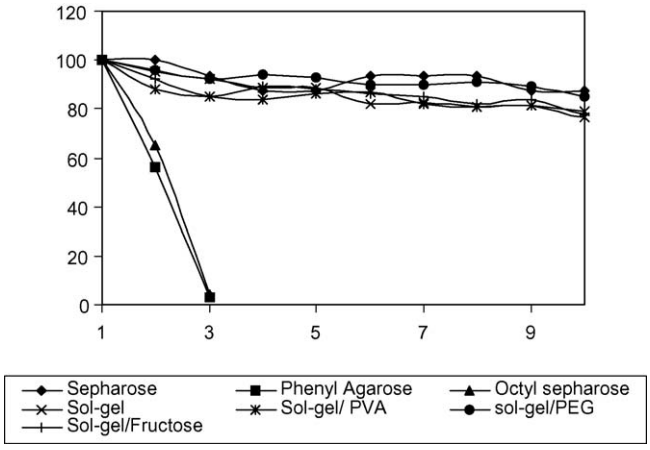


Fig. 1. Operational stability of immobilized ABL derivatives (A–G). Initial immobilized enzyme activity shown as 100% relative activity: phenyl agarose (A), 6710 U/g; octyl sepharose (B), 7720 U/g; sepharose (C), 200 U/g; sol–gel (D), 80 U/g; sol–gel/PVA (E), 110 U/g; sol–gel/PEG (F), 75 U/g; sol–gel/fructose (G), 65 U/g.

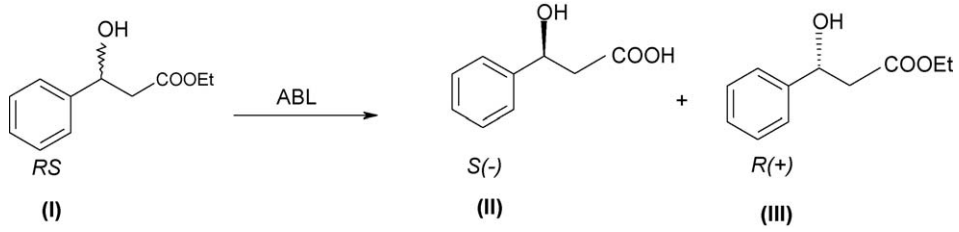
The immobilized derivatives prepared by covalent binding and entrapment methods were used continuously for several reaction cycles (Fig. 1). The immobilized enzymes were also tested for their pH stability, temperature stability and stability in organic solvents. The covalently bound (C) and sol–gel entrapped ABL (D–G) have shown excellent pH (5.0–9.0) and temperature (up to 80 °C) stability, therefore it was expected that ABL immobilized by these methods will be useful for multiple reaction cycles. In the following experiments we demonstrate the efficacy of the immobilized ABL in the resolution of acyl derivatives of ethyl 3-hydroxy-3-phenyl propanoate, an intermediate of antidepressant drug—fluoxetine.

3.2. Kinetic resolution of ethyl 3-hydroxy-3-phenylpropanoate fluoxetine intermediate using free and immobilized enzyme (ABL)

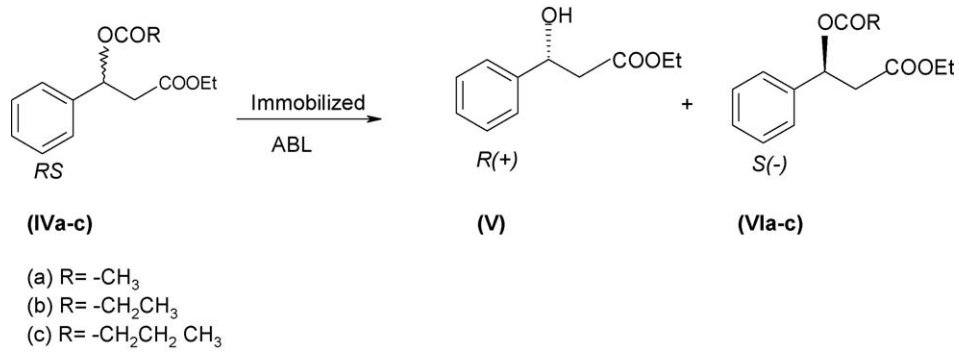
Hydrolytic resolution reaction (Scheme 1) was performed by using ethyl 3-hydroxy-3-phenylpropanoate (fluoxetine intermediate **I**), as substrate (25 g/l) with enzyme 6–7 K units (free and immobilized ABL: A–G) under stirred conditions at 20–25 °C for 21 h. The results obtained using cell free extract and immobilized ABL (A–G) in ester hydrolytic reaction provided only 13% conversion, with a very slow reaction rate and very less enantioselectivity (ee) 38% and *E*-factor only 2.36.

Based on results obtained (Scheme 1), substrate modification in **I**, alkyl acylates of ethyl 3-hydroxy-3-phenylpropanoate fluoxetine intermediate (compound **IVa–c**) were prepared and the hydrolytic resolution (Scheme 2) was carried out with cell free extract (CFE), cell biomass (in naturally immobilized state) and immobilized ABL (A–G). Results presented in Table 2 are the average values of five resolution experiments carried out under same conditions using immobilizates prepared from enzyme produced in five fermentaion experiments. In each experiment similar results were obtained with enantioselectivity (ee 99%).

The results observed (Table 2) in hydrolytic resolution of acylates (substrate **IVa–c**) were quite different from those observed with substrate **I**. All the parameters like conversion rate, ee and enantioselectivity improved significantly in the immobilized enzyme catalysed reactions affording *R* ester (**V**) with ee 98–99% at 21–45% conversion ($E \sim 127$–473). The cell free extract/cell biomass of ABL effectively hydrolysed all the acyl derivatives with a conversion of 29–30, 33–36 and 35–37% for acetate, propionate and butyrate derivatives respectively and ee of 93–98% to give (*R*) isomer as the hydrolysed product ($E \sim 43$–150). ABL immobilized on phenyl agarose (A) and



Scheme 1. ABL enzyme catalysed hydrolysis of ethyl 3-hydroxy-3-phenylpropanoate (**I**).



Scheme 2. Free and immobilized ABL enzyme catalysed hydrolysis of ethyl 3-hydroxy-3-phenylpropanoate acylates (**IVa–c**).

Table 2
Kinetic resolution of alkyl acylates of ethyl 3-hydroxy-3-phenylpropanoate (fluoxetine intermediate) using free and immobilized ABL enzyme

Enzyme source	Substrate	% Conversion	% ee[a] (configuration of product)	*E*-value[b]
	IVa	29	94 (*R*)	47
ABL-cell free enzyme	**IVb**	33	93 (*R*)	43
	IVc	35	93 (*R*)	45
ABL	**IVa**	30	98 (*R*)	150
Cells	**IVb**	36	95 (*R*)	67
	IVc	37	95 (*R*)	69
(A) Phenyl agarose	**IVa**	22	98 (*R*)	129
Immobilized	**IVb**	45	98 (*R*)	245
ABL	**IVc**	45	98 (*R*)	245
(B) Octyl sepharose	**IVa**	21	98 (*R*)	127
Immobilized	**IVb**	38	98 (*R*)	183
ABL	**IVc**	39	98 (*R*)	189
(C) Sepharose	**IVa**	27	99 (*R*)	285
Immobilized	**IVb**	40	99 (*R*)	397
ABL	**IVc**	43	99 (*R*)	450
(D) Sol–gel	**IVa**	29	99 (*R*)	296
Immobilized	**IVb**	44	99 (*R*)	473
ABL	**IVc**	44	99 (*R*)	473
(E) Sol–gel/PVA	**IVa**	33	99 (*R*)	319
Immobilized	**IVb**	44	99 (*R*)	473
ABL	**IVc**	40	99 (*R*)	397
(F) Sol–gel/PEG	**IVa**	26	99 (*R*)	280
Immobilized	**IVb**	42	99 (*R*)	430
ABL	**IVc**	41	99 (*R*)	412
(G) Sol–gel/fructose	**IVa**	17	99 (*R*)	242
Immobilized	**IVb**	36	99 (*R*)	350
ABL	**IVc**	43	99 (*R*)	449

Each % conversion represents average values of data in five experiments. Significance of data represented by increased ee from 93 to 99% and *E* from 43 to 473.
[a] Enantiomeric excess (ee) as determined by Chiral HPLC, Whelk (SS)-chiral column (Lichro CART250-401, 5 μm); hexane:isopropanol:acetic acid ratio, 95:4.9:0.1 as mobile phase; time, 21 h; concentration of substrate, 25 g/L; enzyme units, 6–7 K/L; pH 7.0; temperature, 20–25 °C.
[b] Enantioselectivity (*E*) as calculated by Chen et al. [33].

octyl sepharose (B) also showed preference for (*R*) isomer with ee 98% and *E* 127–245 in all the acylates but depicted better conversion for propionate and butyrate (38–45%) as compared to acetate (21–22%) (Table 2). The rate of reaction was found to increase by about two fold on using the propionate derivative (38–45%) compared to acetate derivative (21–22%). *E*-factor increased from 129 to 245 in phenyl agarose (A) and from 127 to 183 in octyl sepharose (B), respectively. Since for pharmaceutical applications 93–98% ee is not acceptable, therefore 99% ee with high *E*-value is mandatory for such applications. On using covalently immobilized ABL (C) the rate of hydrolysis increased from 27 to 40% simply by changing acetate derivative to propionate with retention of high ee (99%) and greater selectivity (*E*-value 285–397). On further increase in alkyl chain slight increase in rate of reaction to 43% resulted in increase in *E*-factor to 450.

The immobilized ABL by sol–gel entrapment method (D–G) also provided product (**V**) with high ee, 99% and greater selectivity (*E* 242–473) at 17–44% conversion. The sol–gel entrapped ABL (D–F) showed better conversion (26–33%) and 99% ee as compared to sol–gel entrapped ABL with fructose additive (G) which showed lower conversion (17%) for acetate but retention of high ee (99%). On increasing the alkyl chain of acylate, conversion rates raised with a factor of ∼1.5 in the entrapped enzyme catalysed reactions with increase in *E*-factor (350–473). All the supports represented even high enantioselectivity (*E*-factor up to 473) with butyrate derivatives. This can be attributed to the increase in hydrophobicity of the substrate with increasing alkyl chain length, due to which the affinity (formation of substrate–enzyme complex) of the immobilized enzyme is better and faster resulting in higher rate of reactions and high enantioselectivity. Among all the immobilized ABL derivatives, sol–gel entrapped ABL and sol–gel/PVA entrapped ABL represented fastest rate of reactions and highest enantioselectivity (*E* 473). *E*-factor of all the reactions indicate that hydrophobic groups present on the support as well as hydrophobicity of the substrate are responsible for faster reaction rates and improved ee and *E*-factor in propionate and butyrate derivatives. Immobilized enzyme, on further reuse for resolution reactions resulted in no loss in activity or selectivity.

4. Conclusions

Present work demonstrates immobilization of ABL enzyme as a tool for changing the catalytic and enantioselectivity

properties of ABL in a significant manner. Immobilization of ABL resulted in significant improvement in enzyme stability and enantioselectivity (99%) that is acceptable for pharmaceutical applications. Hydrolytic resolution results of racemic ethyl 3-hydroxy-3-phenylpropanoate acylates (**IVa–c**, fluoxetine intermediate) using immobilized ABL by different methods like covalent bonding and sol–gel entrapment has demonstrated significant increase in the enriched product *R*(+) ee 99%, compared with free enzyme under similar conditions obtaining maximum ee, i.e. 93–95%. Similarly the enantioselectivity in free ABL/ABL cells catalysed reaction was ($E \sim 43$–150) and increased when the reaction was carried out in the presence of covalently immobilized and sol gel entrapped ABL ($E \sim 242$–473). Therefore, immobilized enzyme demonstrates preferential accessibility to the acylates of *R*(+) ethyl 3-hydroxy-3-phenylpropanoate isomer in a racemic substrate (**IVa–c**) resulting in enriched product *R*(+) (compound **V**: ee 99%, $E \sim 242$–473). The ABL enzyme immobilized preparations like covalent and sol–gel entrapment methods were used for 10 consecutive cycles without loss in activity and enantioselectivity.

References

[1] K.E. Jaeger, M.T. Reetz, TIBTECH 16 (1998) 396–403.
[2] P. Berglund, Biomol. Eng. 18 (2001) 13–22.
[3] Y.X. Wu, S. Jaaskelainen, Y.Y. Linko, Enzyme Microbial Technol. 19 (1996) 226–230.
[4] J.B.A. Van Tol, J.A. Jongejan, J.A. Dunine, Biocatal. Biotransform. 12 (1995) 99–117.
[5] A. Zaks, D.R. Dodds, Drugs Discovery Today 2 (1997) 513–531.
[6] S. Zhu, Y. Wu, Y. Ziniu, J. Biotechnol. 116 (2005) 397–401.
[7] P.J. Hayball, Drugs 52 (1996) 47–58.
[8] T.A. Savidge, in: E.J. Vandame (Ed.), Biotechnology of Industrial Antibiotics, Marcel Decker, New York, 1984, pp. 177–224.
[9] H.Y. Wu, J.H. Xu, Y.Y. Liu, Synth. Commun. 31 (2001) 349.
[10] H.S. Wu, M.J. Tsai, Enzyme Microbial Technol. 35 (2004) 488–493.
[11] G. Fernandez-Lorente, M. Terreni, C. Mateo, A. Bastida, R. Fernandez-Lafuente, P. Dalmases, J. Huguet, J.M. Guisan, Enzyme Microbial Technol. 28 (2001) 389–396.
[12] J.M. Palomo, G. Fernandez-Lorente, C. Mateo, M. Fuentes, R. Fernandez-Lorente, J.M. Guisan, Tetrahedron: Asymmetry 13 (2002) 1337–1345.
[13] J.M. Palomo, G. Fernandez-Lorente, C. Mateo, O. Claudia, R. Fernandez-Lafuente, J.M. Guisam, Enzyme Microbial Technol. 31 (2002) 775–783.
[14] E. Katchalski-Katzir, Trends Biotechnol. 11 (1993) 471.
[15] R.J. Kazlauskas, U.T. Bornscheuer, in: H.J. Rehm, G. Reed, A. Puhler, P.J.W. Stadler, D.R. Kelly (Eds.), Biotechnology: Biotransformation with Lipases, vol. 8, VCH, 1998, pp. 37–191.
[16] M.T. Reetz, K.E. Jaeger, Chem. Phys. Lipids 93 (1998) 3–14.
[17] A.M. Brzozowski, U. Derewenda, Z.S. Derewenda, G.G. Dodson, D.M. Lawson, J.P. Turkemburg, F. Bjorkling, B. Huge-Jensen, S.S. Patkar, L. Thim, Nature 351 (1991) 491–494.
[18] G. Penzol, P. Armisen, R. Fernandez-Lafuente, L. Rodes, J.M. Guisan, Enzyme Biotechnol. Bioeng. 60 (1998) 518–523.
[19] Z.W. Bai, Y.K. Zhou, React. Funct. Polym. 59 (2004) 93–98.
[20] R. Axen, J. Porath, S. Ernback, Nature 215 (1967) 1491–1492.
[21] I. Nieto, S. Rocchietti, D. Ubiali, G. Speranza, C.F. Morelli, I.E. Fuentes, A.R. Alcantara, M. Terreni, Enzyme Microbial Technol. 37 (2005) 514–520.
[22] M.M. Diogo, S. Silva, J.M.S. Cabral, J.A. Queiroz, J. Chromatogr. A 849 (1999) 413–419.
[23] R. Fernandez-Lafuente, P. Armisen, P. Sabuquillo, G. Fernanzez-Lorente, J.M. Guisan, Chem. Phys. Lipids 93 (1998) 185–197.
[24] M.T. Reetz, A. Zonta, J. Simpelkamp, Biotechnol. Bioeng. 49 (1996) 527–534.
[25] A. Ghanem, V. Schurig, Tetrahedron: Asymmetry 14 (2003) 2547–2555.
[26] H.E. Rassy, A. Perrard, A.C. Pierre, J. Mol. Catal. B: Enzymatic 30 (2004) 137–150.
[27] S.K. Koul, S.C. Taneja, R. Parshad, G.N. Qazi, Tetrahedron: Asymmetry 9 (1998) 3395–3399.
[28] S. Johri, V. Verma, R. Parshad, S. Koul, S.C. Taneja, G.N. Qazi, Bioorg. Med. Chem. 9 (2001) 269–273.
[29] N.A Anand, M. Kapoor, S.C. Taneja, S. Koul, R. Parshad, K.S. Manhas, R.L Sharma, G.N. Qazi, Steroselective enzymatic process for the preparation of optically pure (+)-*cis*-(3*R*, 4*S*)-3 acetoxy-1-(4-methoxy phenyl)-4-phenylazetidin-2 one as precursor of taxol side chain, Indian Patent Application NF.0266/NF/2005, 2005.
[30] N. Anand, S. Koul, S.C. Taneja, R. Parshad, K.S. Manhas, R.L Sharma, G.N. Qazi, Stereoselective enzymatic process for the resolution of (±)-*cis*-3-acetoxy-1-(4-methoxyphenyl)-4-(2-furanyl)-2-azetidinone, (±)-*cis*-3-acetoxy-1-(4-methoxyphenyl)-4-(2-thiophenyl)-2-azetidinone and (±)-*cis*-3-acetoxy-4-(1,1-dimethylethyl)-2-azetidonone, Indian Patent Application (Filed, May 2006).
[31] A. Chaubey, R. Parshad, S. Koul, S.C. Taneja, G.N. Qazi, Appl. Microbiol. Biotechnol. 2006, in press.
[32] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[33] C.S. Chen, Y. Fujimoto, G. Girdaukas, C.J. Sih, J. Am. Chem. Soc. 104 (1982) 7294–7299.
[34] W. Jin, Brennan, Anal. Chim. Acta 461 (2002) 1–36.
[35] L.L. Hench, Sol–Gel Silica: Properties, Processing and Technology Transfer, Noyes Publications, Westwood, NJ, 1998, pp. 101–116.