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

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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-

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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