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Short communication

Esterification in organic solvents by lipase immobilized in polymer of PVA–alginate–boric acid

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Abstract

Candida rugosa lipase was immobilized in the polymer of polyvinyl alcohol (PVA), alginate and boric acid. Calcium alginate improved the surface properties whereas PVA contributed strength. A percentage ratio of 12.5:0.05 PVA:sodium alginate not only prevented agglomeration, but produced beads of high gel strength. The performance of the immobilized biocatalyst was evaluated for the synthesis of ethyl hexanoate in isooctane. The thermal stability of the enzyme increased ten times upon immobilization. The beads showed nearly complete retention of activity in reuse upto 10 cycles and possessed shelf life of 10 months. The immobilized lipase had higher esterification ability as compared to its free counterpart.

Keywords: Boric acid; Calcium alginate; *Candida rugosa*; Esterification; Immobilization; Lipase; Polyvinyl alcohol

1. Introduction

Immobilization of lipases has proved to be a useful technique for improving enzyme activity in organic solvents. Several methods have been reported, such as deposition on solid supports [1–3], covalent binding [4,5] and entrapment within a hydrophobic sol–gel material [6,7] or within a polymer matrix. The latter method has been more widely used to variety of lipases [8,9].

Recently, a synthetic polymer, polyvinyl alcohol (PVA), which is cheap and non-toxic to microorganisms, has been used for cell and enzyme immobilization [4,9,10]. A new method of immobilization of activated sludge using PVA cross-linked with boric acid was developed [11]. This method for the immobilization of activated sludge was used for wastewater treatment and for immobilization of cells for ferrous sulphate oxidation [12]. PVA–boric acid method of cell immobilization was used in treatment of heavy metals [13,14]. To the best of our knowledge this system has not been exploited for enzyme immobilization. Therefore, attempts were made for the immobilization of lipase.

It is desirable that the carrier used is amphiphilic during immobilization of lipase especially, if it has to be used in water poor system, so that it is compatible with both water and organic media and be able to keep some amount of water molecules tightly attached even in the dehydrative solvents [15]. PVA serves the purpose well. Here, we report the application of PVA–alginate–boric acid procedure for immobilization of *Candida rugosa* lipase. The immobilization procedure was optimized and immobilized enzyme was employed in the synthesis of ethyl hexanoate. The immobilized lipase was also tested for its thermostability. Attempts were also made to study the re-usability and shelf life of the immobilized biocatalyst.

2. Materials and methods

2.1. Materials

Candida rugosa lipase obtained from Sigma was used for immobilization. Polyvinyl alcohol (PVA) (100% hydrolyzed, average MW 14,000) and sodium alginate was purchased from CDH, Mumbai, India. All organic solvents were of HPLC grade. Milli Q water was used throughout the experiments. All other reagents used were of analytical reagent grade.

2.2. Immobilization of C. rugosa lipase

Immobilization was carried out by addition of water to 11.25 g PVA to obtain 90 ml of the 12.5% (w/v) solution. The solution was then heated to a temperature of around 60 °C to dissolve PVA. 10 ml, 0.5% (w/v) sodium alginate solution in water was prepared by gently stirring for 45 min and then added to the PVA solution. The PVA–alginate solution was then cooled to a

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temperature of around 35 °C, followed by addition of 10 ml of lipase solution (35 mg ml^{-1}) and mixed thoroughly.

The mixture was extruded as drops into a solution of saturated boric acid containing 2% (w/v) calcium chloride. The beads were stirred gently in this solution for 24 h at 4 °C to complete solidification and then washed with distilled water to remove any excess boric acid. The beads were washed with acetone and then air-dried at room temperature until constant weight (generally overnight) for the ester synthesis in organic media.

2.3. Experimental set-up and procedure

The experiments were performed in 250 ml glass stopper flask. The reaction mixture consisted each of suitable amount of alcohol and acid diluted upto 10 ml with isooctane. The reaction was started by adding 0.5 g of lipase immobilized PVA beads to the reaction mixture consisting of hexanoic acid and ethanol in the ratio of 2:1 (v/v) and enzyme concentration at 35 mg ml^{-1} at 37 °C (on rotary shaker) (150 rpm). Samples withdrawn were analyzed using gas chromatography.

2.4. Product analysis

The substrates and the ester products were analyzed by gas chromatography (Sigma, Baroda, India) equipped with a flame ionization detector and SS column, 10% DEGA (80/100 mesh). Nitrogen served as a carrier gas at a flow rate of 30 ml min^{-1}. The column temperature was programmed from 70 to 180 °C with the increment of 10 °C min^{-1}. The injector and detector were monitored at 250 °C. Ester identification and percentage production was based on comparison of retention time and peak area of the sample with standard.

2.5. Protein determinations

Protein was determined according to Lowry et al. [16].

3. Results and discussion

C. rugosa lipase was immobilized by the PVA–alginate–boric acid method. The cross-linking mechanism between PVA and boric acid is monodiol-type [17]. The enzyme gets entrapped in monodiol-type PVA–boric acid gel lattice. After the enzyme–polymer mixture were dropped in the treatment mixture (saturated boric acid solution containing 2% calcium chloride), spherical gel beads (3 mm in diameter) were formed without agglomeration, which exhibited rubber like elastic properties. PVA contributed strength and durability to the beads, whereas calcium alginate improved the surface properties, reducing the tendency to agglomerate. The percentage of PVA in the beads was kept in the range of 10–12.5% (w/v) as recommended, to get high bead strength [11]. However, concentrations higher than 12.5% were also tried which resulted into viscous solution. Various percentage ratio of PVA to sodium alginate were attempted for the immobilization procedure (Table 1). Ratio of 12.5:0.05 not only prevented agglomeration, but also produced gel of high strength. Beads of this combination did not breakdown during the esterification reaction. The total concentration of sodium alginate in the beads was approximately 0.05% (w/v), concluded to be the lowest concentration of alginate that would prevent bead agglomeration. It is believed that calcium alginate would be formed almost instantaneously when the sodium alginate comes in contact with calcium chloride solution, and the resulting polymeric structure is sufficient to keep the beads from agglomeration during the PVA cross-linking process [18]. The resulting beads were strong and highly elastic, and of spherical shape.

Table 1
Relationship between the percentage ratios of PVA:sodium alginate on formation of beads and relative gel strength

PVA:sodium alginate	Agglomeration	Formation of beads
6:0.05	Prevented	Not good
8:0.05	Prevented	Good
10:0.05	Prevented	Good
12.5:0.05	Prevented	Good

In the preparation of the lipase immobilized PVA beads, contact time with the boric acid affects gel strength greatly. Boric acid is consumed during the PVA-gelling reaction. Thus, an excess amount of the boric acid is required for a rapid progression of PVA polymerization. In general, one gram of boric acid per 18 ml of water was saturated at 25 °C. First, the gelling reaction occurred immediately on the surface of the immobilized beads. Subsequent gelling reaction inside the beads was accomplished with the further diffusion of the boric acid into the beads. Complete gelation in the beads occurred within 24 h.

Water has to be removed from this carrier, before application of the beads for synthesis in organic solvents [9]. Washing the beads with acetone appeared to be superior to other methods for removal of water, such as drying with ethanol or drying at room temperature.

The problem of agglomeration is solved by addition of calcium alginate. The other reason why this method was not investigated so far is the toxicity of the boric acid. It was thought that enzyme activity would be lost during the immobilization [18]. Since, lipase immobilized by this method could show appreciable product conversion, attempts were made to investigate the effect of immobilization procedure on lipase catalyzed esterification. The results are depicted in Fig. 1. There was around 56.4% loss in esterification in case of lipase exposed to treatment mixture. Total loss was observed when exposed to 2% calcium chloride solution. Furthermore, lipase when incubated only in boric acid retained 56.6% esterification. The results very well suggest that it is both the toxicity of the boric acid and calcium chloride that plays role in activity loss. Interestingly, enzyme gained 43% higher esterification compared to free lipase upon immobilization. The result clearly indicates that the enzyme entrapped suffers some loss when exposed to the treatment mixture but the remaining enzyme that is active shows higher esterification ability than the free lipase. Therefore, the entrapment improves the catalytic activity of the lipase, most probably by acting on its conformation and dispersing it more in the gel than directly in the solvent of the reaction. Indeed, when the lipase loaded directly in the organic medium, agglomerates form, which could also explain the low activity of the enzyme.

Fig. 2 exhibits the effect of enzyme loading on protein concentration in the immobilized beads. Protein loading increased as the lipase concentration is increased and levels off at about 30 mg ml^{-1} lipase concentration. The correspond-