Entrapment of lipase in polymer of polyvinyl alcohol-boric acid for esterification in organic media

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Polyvinyl alcohol (PVA)-boric acid method has been utilized for entrapment of Candida rugosa lipase. Immobilized lipase was used to produce ethyl butyrate, a flavour ester showing 80.2% conversion in 72 h. Lipase in PVA-boric acid beads possessed the ability to synthesize variety of esters and was stable in various organic solvents with varying log P value from 2 to 8 under incubation at 50°C for 1 h. The immobilized lipase showed nearly full retention of activity even after 8 cycles of use, the activity then gradually decreased reaching to 56% conversion efficiency after 20 cycles and possesses a shelf-life of 10 months. The thermostability of the lipase increased three times upon immobilization. The immobilized enzyme possessed 40% higher activity compared to its free counterpart.

Keywords: boric acid, entrapment, ethyl butyrate, lipase, polyvinyl alcohol

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Introduction
Efficient functioning of enzymes in organic solvents, by any means, opens up new possibilities of applications in biocatalysis. Enzymes in non-conventional media may exhibit new catalytic properties, such as synthetic activity (by hydrolytic enzymes), stereo- and regioselectivity and enhanced stability. This has resulted in growing interest in using enzymes, especially lipases, in apolar organic solvents. Lipases have been used successfully for the synthesis of esters, both on small and industrial scale.

Different methods have been proposed for retaining catalytic power of enzymes and making them functional in organic solvents. One such promising approach is immobilization of enzymes by entrapment. Recently, studies have been conducted on the use of amphiphilic compounds as an immobilization matrix. It is desirable that the carrier should be amphiphilic for to be used in water poor systems, especially during immobilization of lipases, so that it should be compatible with both water and organic media and be able to keep some amount of water molecules tightly attached even in the dehydrate solvents.

Polyvinyl alcohol (PVA) serves this purpose well. Immobilization using PVA is being done by various methods. Imai et al entrapped glucoamylase, invertase and cellulase onto PVA membrane using cross-linking by UV irradiation and reported high stability even on repeated use of immobilized enzyme. Immobilization using freeze-thaw technique produced rubber-like elastic gel beads with high strength. A method of immobilization of activated sludge using cross-linked PVA with boric acid has been reported. This method was also used for the treatment of heavy metals. PVA has been used in immobilizing lipases and many other enzymes. However, there is no report on immobilization of lipase by the PVA-boric acid method. Here, we report immobilization of lipase using PVA in combination with boric acid for esterification.

Materials and Methods
Materials
Candida rugosa lipase obtained from Sigma was used for immobilization. PVA (100% hydrolyzed, average MW 14,000) and sodium alginate were 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.

Immobilization of Lipase
Immobilization was carried out by addition of water to 11.25 g of PVA to obtain 90 mL of 12.5% w/v solution. The solution was then heated to a temperature of around 60°C to dissolve PVA. 10 mL sodium alginate solution (0.5%, w/v) 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 around 35°C. To this solution, 10 mL of concentrated lipase solution was added and mixed thoroughly.

Beads containing lipase were formed by cross-linking PVA with the boric acid. The beads 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 rinsed with distilled water to remove any excess boric acid. These beads containing biocatalyst were then dried with acetone for the ester synthesis in organic media.

Experimental Set-up and Procedure

The experimental set-up was in 250 mL glass stopper flask. The reaction mixture contained each of 0.1 M ethanol and n-butyric acid, diluted upto 10 mL with isooctane as a solvent. The reaction was started by adding 0.5 g of lipase immobilized PVA beads to the reaction mixture at 37°C under shaking condition (150 rpm). Samples withdrawn were analyzed using gas-chromatograph (GC).

Product Analysis

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

Stability of Lipase Immobilized Beads in Various Organic Solvents

Lipase immobilized beads (0.5 g) were incubated in various organic solvents ranging from log P=2 to 8 and were incubated at 50°C for 1 h. The beads were washed thoroughly with isooctane and added in the reaction mixture to check the enzyme activity.

Reusability of Lipase Immobilized Beads

Lipase immobilized beads of 0.5 g were used for the reaction. The reaction system used contained each of 0.1 M ethanol and n-butyric acid in isooctane at 37°C, under shaking condition (150 rpm). After each 12 h, the beads were washed thoroughly with isooctane and fresh reaction mixture was added to the beads. The beads were then used for the next cycle in similar manner.

Results and Discussion

Lipase from *C. rugosa* was immobilized by the PVA-boric acid method. The enzyme gets entrapped in monodiol-type PVA-boric acid gel lattice (Fig. 1). After the enzyme-polymer mixture were dropped in the treatment mixture (saturated boric acid and 2% calcium chloride solution), spherical gel beads (3 mm in diam) were formed without agglomeration, which exhibited rubber like elastic properties. PVA contributed strength and durability to the beads, whereas calcium alginate improved their surface properties, reducing the tendency to agglomerate. The percentage of PVA in the beads was kept in the range of 10 to 12.5% (w/v) as recommended for maximum bead strength[9]. 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). It was observed that no agglomeration of the beads occurred in the ratio of 6:0.05, 8:0.05, 10:0.05, and 12.5:0.05. Further, 12.5:0.05 ratio not only prevented agglomeration, but

![Fig. 1—PVA-boric acid gel lattice](image)
also produced gel of high strength. The total concentration of sodium alginate in the beads was approximately 0.05% (w/v), which was concluded to be the lowest concentration of alginate that would prevent bead agglomeration. It is believed that calcium alginate would almost be formed 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. The resulting beads were strong and highly elastic, and of spherical shape.

In the preparation of the PVA-immobilized lipase beads, contact time with the boric acid greatly affects gel strength. 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, 1 g 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. The 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. The washing the beads with acetone appeared to be superior to other methods of water removal, such as drying with ethanol or drying at room temperature.

The time-course of ethyl butyrate synthesis is shown in Fig. 2. The lipase immobilized beads showed 80.2% esterification in 72 h of incubation. Enzyme free beads did not show any conversion. Effect of the ethanol:butyric acid ratio on esterification was also investigated in the range of 0.25:1 to 2:1. As shown in Fig. 3, the maximum esterification was obtained when the acid:alcohol was in equal concentration ratio.

The results in Table 2 depict the stability of the enzyme-immobilized beads when incubated in different organic solvents at 50°C for 1 h. Apart from that, lipase entrapped in PVA-boric acid beads showed ability to synthesize a variety of esters (Table 3). For any application based on immobilized enzymes, the feasibility of regeneration of the enzyme activity and the consequent reuse of the support is beneficial for its industrial uses. A continuous assay of residual activity of the immobilized lipase was also performed to find out the retention of lipase over 20 enzyme cycles.

The immobilized lipase showed nearly full retention of activity of repeated use for 8 cycles. Even after 20 cycles the entrapped enzyme retained around 90% activity.
56% activity (Fig. 4). These results suggest that the PVA-boric acid method could be successfully used for the immobilization of enzymes. The activity of the immobilized enzyme was also compared to its free counterpart (Fig. 2), which shows that the immobilized enzyme was capable of 40% more esterification as compared to the free enzyme. The result clearly suggests that 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.

**Thermostability of PVA Immobilized Lipase**

In order to test the thermal stability of the immobilized lipase, residual activity of the free and immobilized enzyme in isooctane was measured. The heat stability of the lipase entrapped in PVA matrices was much better than that of the free enzyme (Fig. 5). The immobilized enzyme could be heated at 50°C for 2 h with full retention of its activity. In contrast, the free enzyme lost more than 50% of its original activity, starting from 30°C under the same conditions. For enzyme activity decay with time at 50°C in isooctane, the lipase in immobilized forms was found much more stable than the free form. It is likely that the lipase is not only physically entrapped, but also forms additional multiple interaction with PVA-boric acid lattice through hydrogen, ionic or hydrophobic interaction, and enzyme stabilization through multi-point attachment to the support can be postulated.

The storage stability data for *C. rugosa* lipase immobilized in PVA-boric acid beads are presented in Table 4. The immobilized lipase exhibited good stability with no significant decrease in activity during storage periods of 10 months at 4°C. Thus, *C. rugosa* lipase can efficiently be immobilized by this method and produce beads of high strength and stability, showing appreciable product conversion. The beads can also be used repeatedly.

One of the most important aims of enzyme engineering is to enhance the conformational stability of the enzymes. Immobilization enhances the conformational stability of lipase. It is well known that proteins with highly cross-linked structure are more resistant to denaturation. The present method of immobilization has not been exploited much since it had two problems, firstly the beads formed during the polymerization between PVA and boric acid had the tendency to agglomerate and the other was the

![Fig. 4](image)

**Fig. 4—Re-use of the immobilized lipase beads, 0.5 g reaction mixture had 0.1 M of substrates at 37°C for 12 h.**

![Fig. 5](image)

**Fig. 5—Thermal stability of lipase immobilized in a polymer of PVA-boric acid. Both free and immobilized enzyme was preincubated for 2 h at various temperatures and residual enzyme activity was assayed by standard method.**

<table>
<thead>
<tr>
<th>Esters</th>
<th>Esterification%</th>
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<tbody>
<tr>
<td>Ethyl butyrate</td>
<td>83</td>
</tr>
<tr>
<td>Isoamyl acetate</td>
<td>69</td>
</tr>
<tr>
<td>Isoamyl butyrate</td>
<td>60</td>
</tr>
<tr>
<td>Ethyl hexanoate</td>
<td>72</td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>80</td>
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<table>
<thead>
<tr>
<th>Storage duration (in months)</th>
<th>Conversion (%)</th>
</tr>
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<tr>
<td>0</td>
<td>80.2</td>
</tr>
<tr>
<td>2</td>
<td>80.1</td>
</tr>
<tr>
<td>4</td>
<td>80.2</td>
</tr>
<tr>
<td>6</td>
<td>80.0</td>
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<tr>
<td>8</td>
<td>79.1</td>
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<tr>
<td>10</td>
<td>79.1</td>
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Immobilized beads (0.5 g) were immersed into the reaction mixture containing each of 0.1 M acid and alcohol and the ester formed were measured.
toxicity of the saturated boric acid. However, the first problem could be solved by addition of calcium alginate. The present work clearly demonstrates that the lipase shows appreciable product conversion when immobilized in polymer of PVA-boric acid. Therefore, the future studies should be targeted on the effect of treatment solutions on enzyme integrity and also to minimize the detrimental effect of the treatment solutions, so that still higher activity could be obtained.

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References