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RESEARCH ARTICLE

Immobilization and stability studies of a lipase from thermophilic *Bacillus* sp: The effect of process parameters on immobilization of enzyme

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A thermostable lipase was partially purified from the culture supernatant of a thermophilic *Bacillus* sp. The enzyme is optimally active at 60ºC and pH 8.0. The enzyme showed enhancement in activity in presence of benzene or hexane (30% v/v each). The activity (assayed by determining the release of pNP from pNP laurate) was stimulated up to 60% of these solvents in enzyme reaction mixture. The catalytic properties of this thermostable enzyme can be further improved via the use of different immobilization techniques and reaction conditions. Enzyme was immobilized on different solid supports and their enzyme activity and stability was compared. The enzyme was adsorbed on silica and HP-20 beads followed by cross-linking with gluteraldehyde on HP-20, which improved the thermostability of enzyme. The optimum pH (pH 8.5) was nearly same for aqueous and immobilized enzyme while optimum temperature was nearly 5ºC higher in case of immobilized enzyme. The immobilized/cross linked enzyme was more thermostable at 70 and 80ºC in comparison to aqueous and surface adsorbed lipase on silica and HP-20. The optimum temperature for esterification reactions was determined to be 60-65ºC. Half-life of immobilized lipase was nearly 2.5 x higher than the aqueous enzyme at 70ºC. Esterification of methanol and oleic acid to methyl oleate by immobilized enzyme was studied in detail.

Lipases, as a class of enzymes, are stable and extremely valuable catalysts for many practical/industrial applications (Bjorkling et al. 1991). They have been used to generate chiral entities from alcohols, carboxylic acid esters, cyanohydrins, chlorohydrins, diols, amines, diamines and amino alcohols (Jaeger et al. 1999), which are used as building blocks for a variety of pharmaceuticals and other fine chemicals. Many lipases are only moderately stable at high temperature and pHs, that can influence their usefulness in some interesting reactions. Using lipases from thermophilic microorganisms, whose resistance to drastic conditions has been developed by nature, can solve this problem. At present, the majority of the thermophilic lipases that have been purified and characterized are obtained from *Bacillus* sp (Kim et al. 1994). To use these enzymes for industrial purposes, the most desirable step is to improve the features of biological catalyst to suit the industrial demand. The majority of current biocatalytic approaches rely on either using free enzyme in solution or living cells, which complicates product separation from the catalyst. The lipase immobilization may improve the stability and ease of product separation/reuse of enzyme. Although a variety of approaches exist for the immobilization of enzymes, the "science" of enzyme immobilization is still in its infancy. Several solid supports have been used for the purpose till now (Aucoin et al. 2004; Hwang et al. 2004; Palomo et al. 2004).

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Table 1. Immobilization of Lipase on different solid supports.

S No.	Solid supports	Immobilized lipase	
		Enzyme activity bound (% of total enzyme activity added)	Units g^{-1} of support
1	HP-20 (CL)	79	180.96
2	Silica	86	163.63
3	HP-20	78	180.00

Control = 68.48 Unit ml^{-1}.

A novel thermophilic *Bacillus* sp has been isolated that produced a thermostable lipase from hot springs of Himachal Pradesh, India (Nawani et al. 2006). Hexane is the preferred solvent in most of the trans-esterification reactions. This enzyme is quite stable in hexane. In the present investigation attempts have been made to further improve the catalytic properties of the enzyme via the use of different immobilization techniques and reaction conditions. Dosanjh and Kaur (2002) earlier reported that HP-20 acts as a good support for immobilization of a lipase In the present investigation HP-20 was used as an immobilization support for this thermostable lipase and immobilized lipase was tested for advantageous catalytic property and stability.

MATERIALS AND METHODS

The culture (isolated *Bacillus sp*) was grown under optimal conditions for lipase production. A series of 500 ml, Erlenmeyer flasks containing 100 ml wheat bran and nutrient broth medium, (1% w/v each) pH 8.0 were seeded with 2% inoculum and incubated at 60ºC for 48 hrs (Nawani et al. 2006). The culture was then centrifuged at 10,000 rpm to remove the cells. The clear supernatant containing the lipase was used for further studies.

Enzyme assay

The activity was determined according to the method of Sigurgisladottir et al. (1993) with slight modification. To 0.8 ml of 0.05 M phosphate buffer (pH 8.0), 0.1 ml enzyme and 0.1 ml 0.01 M pNP-laurate (Sigma, USA) was added. The reaction was carried out at 60ºC for 30 min, after which 0.25 ml 0.1 M Na_2CO_3 was added. The mixture was centrifuged and the activity was determined at 420 nm. One unit of lipase activity is defined as the amount of enzyme, which liberates 1 µg of p-Nitrophenol from pNP laurate as substrate in 30 min under standard assay conditions.

Partial purification of enzyme

Ammonium sulphate precipitation. To 900 ml of the culture supernatant, ammonium sulphate was added (70% saturation) at 4ºC. The precipitate was collected by centrifugation at 12000 x g at 4ºC for 20 min and dissolved in 0.05 M phosphate buffer (pH 8.0). The lipase activity and the protein concentration (Lowry et al. 1951) were determined.

Hydrophobic interaction chromatography. The precipitated enzyme (276 ml) was loaded on Phenyl Sepharose (Sigma) column (4.0 x 4.5 cm) pre-equilibrated with 0.05 M phosphate buffer (pH 8.0). It was washed with

Table 2. Retention of immobilized lipase activity on solid supports after different cycles.

S. No.	Solid	Residual enzyme activity (%) after				
	Supports	5 cycles	10 cycles	15 cycles	20 cycles	25 cycles
1	HP 20	100	93	86	79	71
2	Silica	100	89	78	58	46

0.05 M phosphate buffer (pH 8.0) followed by 1 mM phosphate buffer (pH 8.0). The enzyme was eluted with 40% ethylene glycol (80 ml) in 1 mM phosphate buffer (pH 8.0). Eight-ml fractions were collected and analyzed for lipase activity. Fractions with high lipase activity were pooled. The enzyme was concentrated by filtration through 10 kDa cut off membrane filters.

Effect of organic solvents on enzyme activity

To study the effect of organic solvents on aqueous enzyme activity, organic solvents such as methanol, ethanol, acetone, Dimethylsulphoxide (DMSO), glycerol, polyethyleneglycol, hexane, benzene (10%, 30% v/v) were added to the reaction mixture and the lipase activity was determined under standard assay conditions. The sample without any additive was taken as control (100%). The reaction mix with respective additives but without enzyme served as control. In addition enzyme was assayed in the presence of 60% of hexane or benzene in reaction mixture.

Immobilization

Adsorption kinetics of protein and lipase activity on solid support. Silica (Merck) and HP-20 beads (Diaion) were used for immobilization. HP-20 is aromatic in chemical structure (surface area ca. 600 m^2/g, pore radius 200-300 A), widely used for adsorption.

Silica. 5 ml of partially purified enzyme was mixed with 1 g of silica in duplicate. These were left at room temperature for 90 min with intermittent mixing. After different time points (0-90 min) fractions were taken out and centrifuged at 1000 g for 5 min. Supernatant was tested for unbound protein and enzyme activity. For esterification studies the silica was washed three times with 50 mM phosphate (pH 8.0) buffer before use.

HP-20 beads. 1g of HP-20 was first washed with distilled water, then washed for 30 min with isopropyl alcohol and further washed three times with distilled water to remove isopropyl alcohol traces. The half of the washed beads were used as such and rest half was treated with 2.5% gluteraldehyde for 1 hr. The resin was washed 3 times with distilled water to remove residual gluteraldehyde. 5 ml of enzyme was added to the resin and left on shaker for 90 min. After different time points (0-90 min) fractions were taken out and centrifuged at 1000 g for 5 min. Supernatant was tested for unbound protein and enzyme activity. For esterification studies HP-20 beads were washed thrice in 50 mM phosphate buffer and used for the experiment.

$$\text{Amount of lipase bound to solid support} = \text{Total lipase present in the binding medium} - \text{Remaining lipase present in the binding medium after removal of solid support}$$

Adsorbed lipases on different solid supports were assayed to define the adsorption capacity. Hundred mg of silica and HP-20 support (with and without cross-linking) with immobilized lipase was added into assay mixture and assayed with continuous shaking for 30 min as per standard method. The enzyme activity bound on each support was determined.

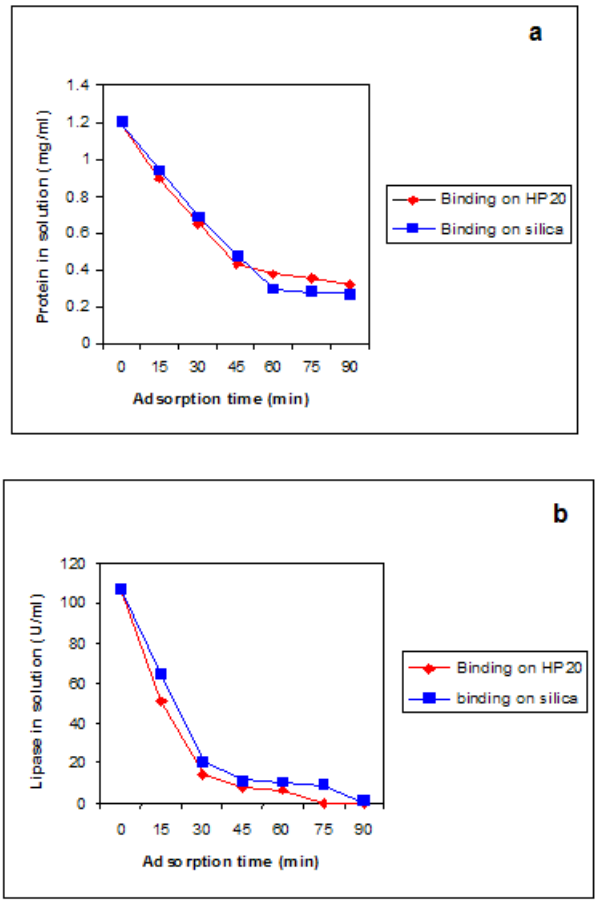

Figure 1. Adsorption kinetics on silica and HP-20.
(a) Time course of protein.
(b) Time course of lipase.

Retention of activity by immobilized lipase in continuous cycles. The immobilized enzymes were assayed by the method of Sigurgisladottir et al. (1993) for 25 cycles of 30 min each. For each cycle two ml of reaction mixture containing substrate was added to the immobilized support and incubated for 30 min with continuous shaking at 60ºC. The contents were then centrifuged and the supernatant was used for measuring absorption at 420 nm. The pellet washed thrice with the 0.05 M phosphate buffer (pH 8.0) and then used for the next cycle in similar manner.

Thermo-inactivation of free and immobilized lipase. To study the effect of temperature on free, immobilized and immobilized cross-linked lipase, different forms of enzymes were incubated at different temperatures (60, 70 and 80ºC) for 1 hr in 50 mM Tris-HCl pH 8.0. Residual lipase activity was determined under standard assay conditions. Residual activity in the samples without incubation (incubated at 4ºC) was taken as 100%.

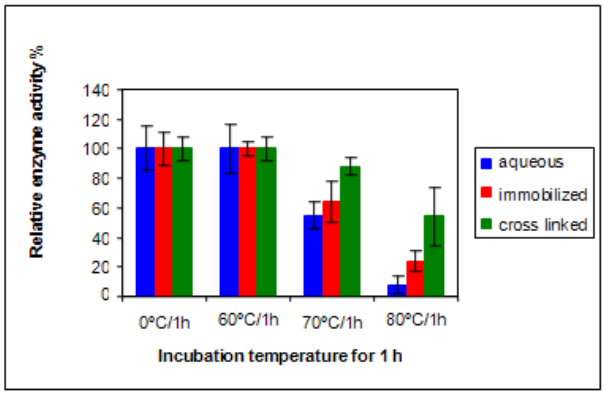

Figure 2. Effect of temperature on aqueous, immobilized and immobilized cross linked lipase.

To determine the half life of lipase, free (80 U/ml) and immobilized (180 U/g) lipase was incubated at 70ºC for 8 hrs. After different time periods, fractions of free (100 µl) and immobilized enzymes (100 µl sample having 50 mg of HP-20) were withdrawn and residual lipase activity was determined under standard assay conditions. Residual activity in the samples without incubation (incubated at 40ºC) was taken as 100%.

Effects of pH and temperature on enzyme activity. Lipase activity was assayed in the buffers of different pH (4.5-11.0) at 60ºC. The residual lipase activity was determined by standard assay method.

The optimum temperature of lipase activity was determined by carrying out the enzyme reactions at different temperatures (30-80ºC) and pH 8.0. The enzyme activity at the start of the experiment was taken as 100% and the residual lipase activity after incubation was determined.

Esterification studies by aqueous and immobilized lipase

For comparative study, aqueous and immobilized enzymes were used as biocatalysts for the esterification of oleic acid (0.25 M) and methanol (0.4 M) in hexane. The reaction was carried out at particular temperature with shaking for particular time with inactivated enzyme as a control. To 10 ml reaction mixture, 20 ml acetone: methanol 1:1(v/v) was added. The ester content was quantified by using alkalimetric method of titrating unreacted acid with 0.1 N NaOH using phenolphthalein as an indicator. The conversion (%) in ester synthesis was based on acid consumed (Bovora et al. 1993).

Time course of esterification. To determine the optimum time for the formation of methyl oleate from methanol and oleic acid by immobilized enzyme, the esterification reaction was carried out for different time points. The ester synthesis was analyzed as above.

Effect of temperature on esterification reaction. The optimum temperature for the formation of methyl oleate from methanol and oleic acid by immobilized enzyme, the esterification reaction was carried out at different temperatures (50-85ºC). The ester synthesis was analyzed as above

RESULTS AND DISCUSSION

Basic characteristics of free lipase

The strain was identified as *Bacillus* sp. based on characteristics such as aerobic growth, gram positive, rod shaped, motile, spore forming and catalase positive (Clans and Berkley, 1986). The organism could hydrolyze starch, casein and gelatin. It did not require sodium chloride and potassium chloride for growth. It was oxidase negative in nature and it did not produce any gas with glucose. On comparison of this *Bacillus* sp isolate with *Bacillus stearothermophilus* and other thermophilic *Bacillus sp.* (according to Bergey's manual), it showed the following different characteristics *i.e.* nitrate reduction, acid formation with arabinose, xylose and mannitol, casein hydrolysis, and no growth below 50ºC.

The lipase was partially purified by ammonium sulphate precipitation followed by Phenyl-Sepharose column chromatography (specific activity 160 U/mg protein). This partially purified enzyme was used for immobilization. As to the effect of organic solvents on enzyme activity, benzene and hexane had a highly stimulatory effect on the lipase activity (195 and 180% respectively). The activity of enzyme was stimulated up to 60% of hexane/benzene in

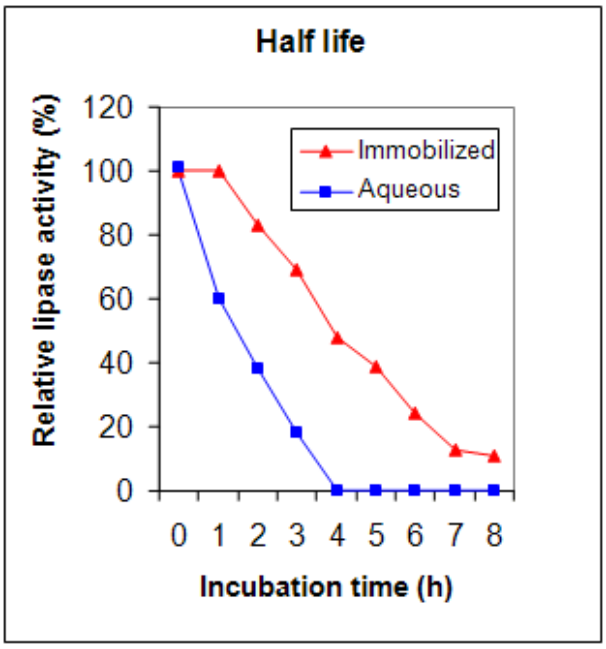

Figure 3. Determination of half-life of aqueous and immobilized lipase (HP-20).

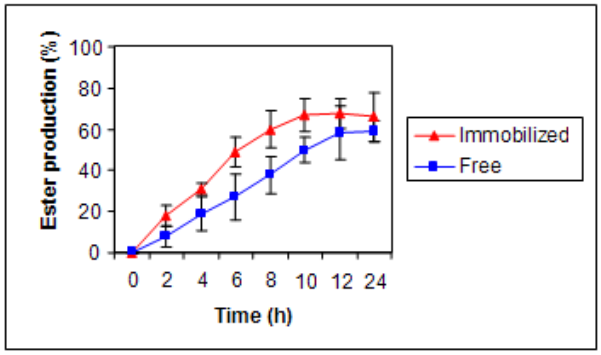

Figure 4. Time course of esterification of methanol and oleic acid by lipase at 60ºC. The amount of immobilized enzyme used was 180 U/mg solid support. The reaction was carried out for 24 hrs.

reaction mixture. The enzyme activity was slightly stimulated up to 30% in presence of acetone. DMSO (Dimethylsulphoxide) and ethylene glycol had almost no effect, while ethanol, methanol and propanol inhibited the enzyme activity. The effect of organic solvents might be attributed to the water content in reaction mix. Water plays a significant role in the activity of the enzymes (Klibanov, 1989). The inhibition of lipase in presence of ethanol, methanol while stimulation in water immiscible solvents confirms that polar water miscible solvents are more destabilizing than the water immiscible solvents (Fernández-Lorente et al. 2001). On the other hand effect of acetone on enzyme activity could not be explained by this theory.

Immobilization of lipase

The immobilization by ionic or physical adsorption was one of the simple and cheap methods to prepare an immobilized enzyme, although adsorbed enzymes were rather susceptible to desorption from the carrier (Rua and Ballesteros, 1994). Immobilization of isolated *Bacillus sp* lipase was carried out on HP-20 and Silica. The adsorption of lipase on various supports was determined by estimating the amount of lipase bound to the solid supports (Table 1). It was found that good adsorption was seen in HP 20 (with and without cross linking) and silica, where attachment to lipase was up to the extent of 78, 79 and 86% respectively. The kinetics of protein and lipase adsorption to silica and HP-20 indicate that the amount of protein and lipase was well below saturation (Figure 1a) while no unbound lipase could be traced (Figure 1b). The protein and lipase adsorption was fast and maximum binding was observed after 45 min for protein and 30 min for lipase. This suggests that lipase is fast adsorbing protein amongst other proteins. The hydrophobic nature of solid substrate suggests that adsorption of proteins are governed by hydrophobic interactions. Rua and Ballesteros (1994) used moderately hydrophobic support (Phenyl Agarose) in the purification of lipase from *C. rugosa*. Similar hydrophobic adsorption supports were used by other workers for immobilization (Aucoin et al. 2004; Palomo et al. 2004). Continuous assay of residual enzyme activity in the lipase immobilized to HP-20 and silica was performed to find out the retention of lipase activity by each support over 25 cycles of enzyme reaction. Less desorption or leaching of the bound enzyme in case of HP-20 cross-linked might be due to very tight binding of the lipase on the hydrophobic supports, (Table 2). The lipase immobilized on HP-20 without cross-linking behaved like silica (data not shown) and in both the cases the leaching of enzyme activity was observed. Lipase immobilized on silica might be lost from the system because of de-sorption, severing of chemical bonds or erosion of the support material. For clear presentation of results only enzyme immobilized on silica is compared with HP-20 cross-linked. Similar to our observations, Fernández-Lorente et al. (2001) could use the immobilized lipase using hydrophobic moieties for 10 esterification cycles without any significant decrease in enzyme biocatalyst.

Physico-chemical characterization and thermal stability of immobilized lipase

No change in optimum pH for lipase activity was observed in the case of aqueous and immobilized form. Optimum temperature of immobilized (CL) enzyme shifted from 60ºC to 65ºC. Yang and Rhee (1992) reported big shift in optimum reaction temperature from 37ºC for the free lipase to 50ºC for immobilized lipase. Effect of temperature on aqueous, immobilized and immobilized cross-linked lipase was further analyzed. Figure 2 demonstrates that the thermal stability of immobilized (CL) is higher in comparison to aqueous and immobilized lipase at 70 and 80ºC. Because immobilization and cross linking provided more rigid external backbone for lipase molecules, the effect of higher temperatures in breaking the interactions that were responsible for the proper globular, catalytic active structure, became less prominent, thus increasing the

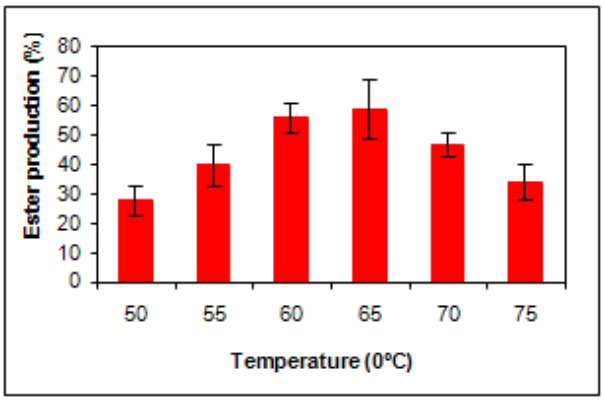

Figure 5. Effect of temperature on esterification of methanol and oleic acid by lipase after 10 hrs of incubation.

thermal stability of the immobilized lipase. Due to higher thermostability of immobilized cross-linked lipase (CL), HP-20 was used for further studies. It is reported that BTL2 adsorbed on a hydrophobic support exhibited a hyperactivation with respect to the soluble enzyme, whereas the other immobilized preparations suffered a slight decrease in the expressed activity (Palomo et al. 2004). Similar hyperactivation of *Rhizomucor miehei* lipase was observed by Aucoin et al. by hydrophobic xerogels (Aucoin et al. 2004).

Half-life of aqueous and immobilized lipase (HP-20) was calculated to be 85-90 min and 4 hrs respectively at 70ºC (Figure 3). A specific site, where the unfolding process begins during inactivation, characterizes an enzyme. Immobilization protects this unfolding nucleus only in one fraction of enzyme molecule, which could block the native unfolding pathway, resulting in enhanced stability. The second fraction with no protection behaved like a soluble enzyme.

Esterification reaction

The biosynthesis of esters is currently of much commercial interest because of the increasing popularity and demand for natural products amongst consumer. Bio-transformations and enzymatic methods of ester synthesis are more effective when performed in non-aqueous media (Chand et al. 1997). Because of the fact that the present lipases showed much activity and stability in hexane,the esterification of oleic acids and methanol in hexane was carried out.

The effect of incubation time on esterification reaction indicates that ester synthesis was essentially complete by 9-10 hrs with immobilized enzyme while it took more than 12 hrs with aqueous enzyme (Figure 4). No enhancement in ester production was observed after 10 hrs. Fig 5 illustrates the effect of temperature on esterification reaction. During the esterification reaction, water is released as a byproduct and was to be inhibitory to the esterification reaction at a particular concentration (Chand et al. 1997). The higher conversion rate observed in the case isolated lipases here may be explained by the reduction of the water content due to the higher incubation temperature thus decreasing the inhibitory effect of water on esterification reaction. In esterification conditions, one molecule of water is produced when one molecule of acid is esterified. In order to favor this reaction, the produced water must be removed from the medium. Selmi et al. (1997) described temperature increase with air ventilation as one of the possibility. The degree of esterification increased with temperature in the range of (50-70ºC). The reaction declined above 70ºC. Only 18% conversion was observed at 85ºC. This may be due to the thermal denaturation of enzyme.

For any application based on immobilized lipases, the feasibility of regeneration of the lipase activity (and consequent reuse of the support) provided clear economic benefits for its industrial use. HP-20 beads were comparatively cheap and quite rigid in nature. It had been demonstrated that the immobilization and cross linking of the lipase on HP-20 was advantageous for catalytic property and stability.

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