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PROPERTIES OF AN IMMOBILIZED LIPASE OF *BACILLUS COAGULANS* BTS-1

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Lipase (EC 3.1.1.3) is a tri-acylglycerol ester hydrolase, catalysing the hydrolysis of tri-, di-, and mono-acylglycerols to glycerol and fatty acids. To study the effect of adsorption of a lipase obtained from *Bacillus coagulans* BTS-1, its lipase was immobilized on native and activated (alkylated) matrices, i.e. silica and celite. The effect of pH, temperature, detergents, substrates, alcohols, organic solvent etc. on the stability of the immobilized enzyme was evaluated. The gluteraldahyde or formaldehyde (at 1% and 2% concentration, v/v) activated matrix was exposed to the Tris buffered lipase. The enzyme was adsorbed/entrapped more rapidly on to the activated silica than on the activated celite. The immobilized lipase showed optimal activity at 50ºC following one-hour incubation. The lipase was specifically more hydrolytic to the medium C-length ester (p-nitro phenyl caprylate than p-nitro phenyl laurate). The immobilization/entrapment enhanced the stability of the lipase at a relatively higher temperature (50ºC) and also promoted enzyme activity at an acidic pH (pH 5.5). Moreover, the immobilized lipase was quite resistant to the denaturing effect of SDS.

Keywords: *Bacillus coagulans* BTS-1 lipase, silica/celite and immobilization

Introduction

Lipases (tri-acylglycerol ester hydrolase, EC 3.1.1.3) are enzymes whose biological function is to catalyze the hydrolysis of tri-acylglycerols. These reactions usually proceed with high regio- and or enantio-selectivity, making lipases an important group of biocatalysts in organic chemistry. Microbial lipases have enormous biotechnological potential because they are stable in organic solvents,

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do not require cofactors, possess broad substrate specificity and exhibit also a high enantio-selectivity. The industrial use of lipases as enantio-selective catalysts depends on their efficient immobilization. Immobilization of lipase has been previously performed using different methods viz. covalent attachment, adsorption, entrapment and cross-linking on various matrices [1]. An easy and efficient method of immobilization of lipase is adsorption on more or less hydrophobic supports [2–9]. Immobilization of lipases on solid supports offers several advantages including simple recovery, allowing repeated use of the catalyst, easy separation of the enzyme from the product, possibility of continuous operation and improvement of enzyme stability. These advantages are well known for enzyme utilization in aqueous media [10]. However, it is currently difficult to assess the relative merits of different approaches [11, 12].

A promising approach to enzyme entrapment is to use inorganic matrices such as silica or celite [13]. In entrapped form many enzymes tend to display enhanced activities relative to those of the natural non-entrapped state [13]. Unfortunately, most of the attempts to extend this methodology to lipase have been intriguingly disappointing [14]. It is known that lipase is active at the lipid-water interface [15]. We observed that silica or celite modified by alkylation (exposure to gluteraldahyde or formaldehyde) resulted in a rapid adsorption of lipase of *B. coagulans* BTS-1. The present study showed that the immobilization/adsorption provided a modified microenvironment to the lipase.

Materials and methods

Maintenance of the microorganism and lipase production

A *B. coagulans* BTS-1 isolate was obtained from the Department of Biotechnology, Himachal Pradesh University, Summer-Hill, Shimla-171 005, India. The culture was maintained on a mineral based (MB) medium supplemented with 1% (v/v) cotton-seed-oil as the sole carbon-source. The MB-broth contained (gl^{-1}); $NaNO_3$ 3.0, K_2HPO_4 0.1, $MgSO_4.7H_2O$ 0.5, KCl 0.5 and $FeSO_4.7H_2O$ 0.01. The oil initially emulsified with gum arabic (0.5%, w/v) was added to the MB-broth. The pH of the medium was adjusted to 7.5. The medium was autoclaved at 1.2 bar for 20 min at 121ºC. The bacteria produced a maximum lipase activity at 48 h post-inoculation in MB-broth. The inoculated MB-broth (1 l) was harvested free of cells after 48 h by centrifugation (10,000 × g for 20 min at 4ºC). The supernatant filtered through a Millipore membrane of 0.22 mm porosity was

concentrated by freeze-drying (FTS Freeze Dryer, U.S.A.) and the protein concentration was estimated [16].

The concentrated/lyophilized cell-free culture broth was henceforth referred to as *crude lipase*.

Assay of lipase activity

The lipase activity was assayed in the cell-free culture broth and celite- or silica-immobilized lipase by a colorimetric method [17]. The stock solutions of p-nitro phenyl palmitate (pNPP; 20 mM), p-nitro phenyl caprylate (pNPC; 20 mM) and p-nitro phenyl acetate (pNPA; 20 mM) were prepared in iso-propanol. For the free-lipase assay, 75 µl of p-NPP stock-solution and 5 to 50 µl of crude enzyme (cell-free broth) were put in a glass tube. However, for immobilized lipase, 50 mg of celite- or silica-entrapped preparation was used. The final volume of this reaction mixture was made to 3 ml with Tris-HCl buffer (0.05 M) with pH adjusted to 8.5 for free lipase or to pH 5.5 for immobilized lipase. The test tubes were incubated at 45ºC for 20 min in a water-bath. Chilling the test tubes in ice for 10 min stopped further enzyme reaction. Appropriate control (activated matrix without enzyme) was also incubated with each assay. The absorbance of this control was subtracted from the absorbance of the corresponding test sample. The absorbance of p-nitro phenol released was measured at 410 nm (Shimadzu UV/Visible Spectrophotometer, Japan). The unknown concentration of p-nitro phenol released was determined from a reference curve of p-nitro phenol. Each of the assays was performed in triplicate unless otherwise stated and mean values were presented. The lipase activity associated with the immobilized matrix was represented as Ug^{-1} of matrix.

One unit of lipase activity was defined as microgram(s) of p-nitro phenol released by 1 ml of free-enzyme (or 1 g of immobilized matrix) at 45ºC under assay conditions.

Immobilization of bacterial lipase on matrices

The bacterial lipase was immobilized on native and activated (alkylated) matrices, i.e. silica and celite. Each of the matrices was activated by exposure to either gluteraldahyde or formaldehyde (1% or 2% in distilled water, v/v). The suspension-containing matrix (1 g of celite or silica) and 4 ml of activating agent were

incubated for 1 h at 37ºC. Each of the activated matrices was then given five washings with Tris-HCl buffer (0.05 M, pH 8.5) to get rid of the unbound activating agent.

Approximately, 2 ml (~340 U) of the enzyme was then added to each of the activated and non-activated matrices (1 g) and the suspension was incubated for another one hour at 37ºC. The unbound lipase was removed by four washings with Tris-HCl buffer (0.05 M, pH 8.5). Finally, the immobilized matrices were kept suspended in Tris-HCl buffer at 4ºC till further use. In each of the assays 50 mg of immobilized enzyme preparation (matrix) was used.

Immobilization kinetics

A study was conducted to find out the rate of immobilization of lipase on both activated and non-activated matrices. The concentration of the free lipase in the supernatant was estimated at intervals for a 1 h period.

Effect of temperature on the activity of immobilized enzyme

The immobilized enzyme was incubated at 30, 50, 60 and 70ºC for 20 minutes. The lipase activity was assayed thereafter at 45ºC and A_{410} values were recorded.

Effect of pH on the activity of immobilized enzyme

To evaluate the effect of pH (5.5, 7.0 and 8.5) on the activity of immobilized lipase, different immobilized lipase preparations were incubated with each of the buffers listed above for 20 minutes at 45ºC. Thereafter, the lipase activity was assayed and recorded.

Effect of acyl moiety of p-nitro phenyl ester on immobilized lipase

By varying the acyl moiety of the p-nitro phenyl ester substrates, the effect of immobilization on fatty acid specificity of the lipase was evaluated. The effect of exposure of these substrates of different C-length upon the immobilized lipase

was studied using pNPP, pNPA and pNPC. Each of these p-nitro phenyl esters was incubated with the matrix for 20 minutes and their lipase activity was assayed.

Effect of detergents on the activity of immobilized lipase

The denaturing effect of different detergents (0.5%, w/v) on the activity of immobilized lipase was evaluated. The different immobilized lipase preparations were separately incubated with different detergents such as sodium dodecyl sulfate (SDS), sodium lauryl sarcosine (SLS), Tween-20 (T-20), Tween-80 (T-80) and Triton X-100 (TX-100) for 20 minutes at 45ºC. The lipase activity was registered.

Effect of different solvents on the activity of immobilized lipase

In order to study the effect of different solvents on the activity of immobilized lipase, each of the matrices (50 mg) was suspended in 0.05 M Tris-HCl, pH 8.5 (1 ml). The suspension was thoroughly vortexed to achieve homogeneity. Twenty microliters of this homogenous suspension (matrix) were incubated with each of the selected alcohols (methanol, ethanol, iso-propanol and iso-butanol) or organic-solvents (chloroform, acetone, n-hexane, n-octane and n-nonane) in a volumetric ratio of 1:2 (i.e. 20 ml of matrix in Tris-HCl + 40 ml of solvent/alcohol) for 20 minutes at 45ºC in paraffin-wax paper-sealed Eppendorf tubes (1.5 ml capacity). After incubation, the solvent/alcohol was completely evaporated at 37ºC and the lipase activity was assayed.

Effect of temperature on free lipase

To optimize the incubation temperature for assay of free lipase, 5 ml of crude lipase was added to the reaction mixture containing 1 ml of p-NPP (16.5 mM) and 1.995 ml of Tris-HCl (pH 7.4, 0.05 M) in a test tube. The tubes were incubated at 25, 35, 45 and 55ºC for 20 min. Further enzyme reaction was stopped and lipase activity was assayed.

Effect of pH on free lipase

The effect of pH of the reaction mixture on free lipase was studied. The pH of the Tris-HCl buffer was adjusted to 7, 7.5, 8, 8.5 or 9. To the reaction mixture containing p-NPP and Tris-buffer of each of the specified pH, 5 µl of crude lipase was added and the reaction was allowed to proceed at 45°C for 20 min. On the completion of the incubation period the reaction was stopped and the lipase activity was recorded.

Results

Immobilization kinetics

The immobilization kinetics revealed that the lipase was more rapidly immobilized on the activated silica (within 5 minutes of incubation, results not shown) than on the activated celite (Figure 1). However, native celite was more efficient in immobilizing lipase than the modified celite. After 20 min incubation, most of the lipase was adsorbed and only little could be detected in the supernatant.

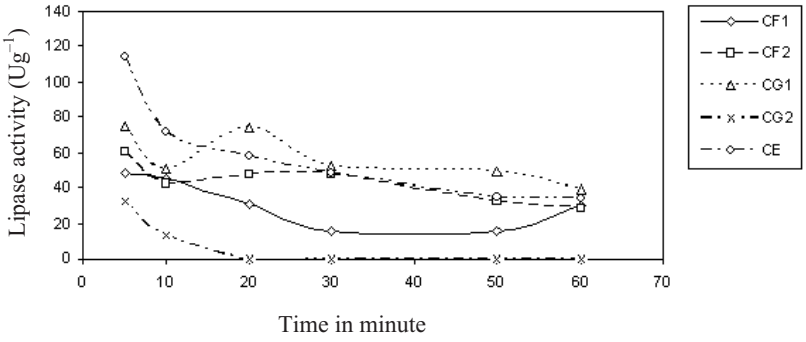

Figure 1. Immobilization kinetics of lipase

Abbreviations: The celite activated with 1 or 2 % gluteraldahyde was denoted by the abbreviation CG1 and CG2; and when activated with 1 or 2% formaldehyde was abbreviated as CF1 and CF2, respectively. Similarly, silica activated with gluteraldahyde was denoted as SG1 and SG2, and when activated with formaldehyde was abbreviated as SF1 and SF2, respectively. CE and SE are unactivated matrices with immobilized lipase, respectively

Effect of temperature on the activity of immobilized lipase

A gradual increase in the activity of immobilized lipase was observed when the incubation temperature was raised from 30 to 50ºC. Further increase in the temperature caused a rapid decrease in activity of silica-immobilized lipase (Figure 2A). In contrast, the lipase activity of not activated celite-immobilized lipase was markedly higher than the other celite-immobilized preparations (Figure 2B).

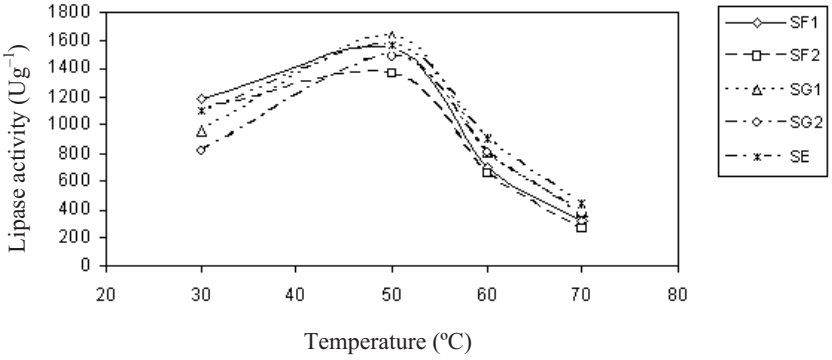

Figure 2A. Stability of silica-immobilized lipase at different temperatures
Abbreviations: see Figure 1

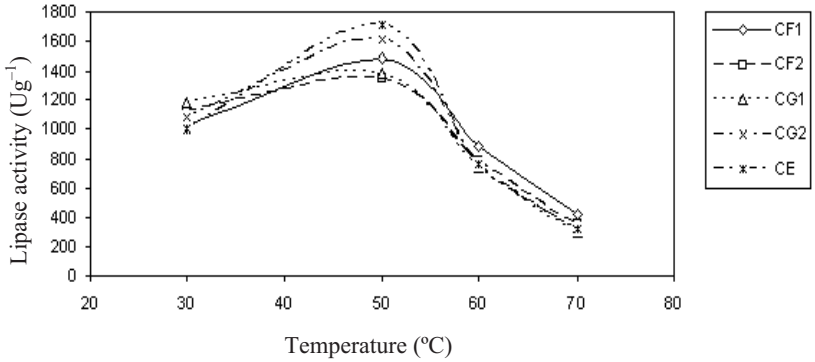

Figure 2B. Stability of celite-immobilized lipase at different temperatures
Abbreviations: see Figure 1

Effect of pH on the activity of immobilized lipase

The lipase activity was higher at acid pH (pH 5.5) and a change in pH from acid to alkaline (pH 8.5) resulted in a gradual decrease in activity of silica-immobilized lipase (Figure 3A). A similar pattern was observed in case of celite-immobilized lipase preparation. However, CG2 lipase exhibited an initial gradual decline in the enzyme activity between pH 5.5 to pH 7.0, and thereafter the decline was very rapid (Figure 3B).

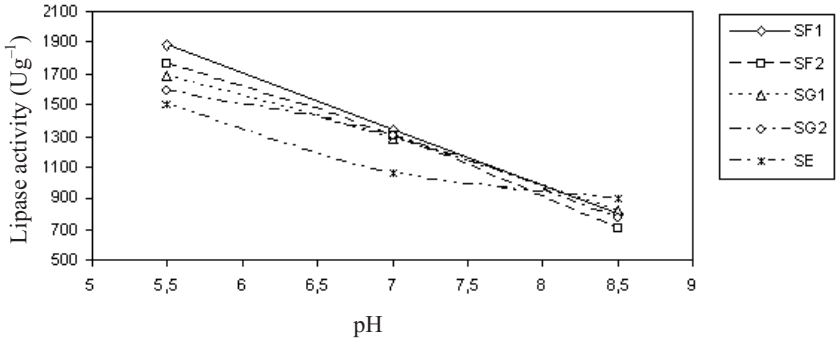

Figure 3A. Stability of silica-immobilized lipase at different pH
Abbreviations: see Figure 1

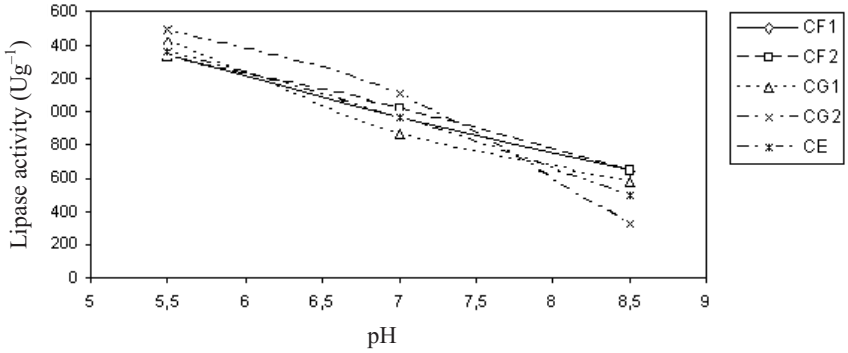

Figure 3B. Stability of celite-immobilized lipase at different pH
Abbreviations: see Figure 1

Substrate (C-chain length) specificity of immobilized lipase

Silica-immobilized lipase matrices exhibited more or less similar specificity for pNPP and pNPA. However, a marked increase in the esterase activity on medium C-chain length ester (pNPC) was noticed in all the modified silica-immobilized lipase matrices. SG1 brought about approximately 79% increase over the native silica-immobilized lipase (Figure 4A). SF1 and SF2 had more or less similar specificity for pNPC.

All modified celite preparations with immobilized lipase behaved similar to the native-celite with immobilized lipase. However, the modified celite preparations with immobilized lipase showed a marked decline in their specificity for pNPC (Figure 4B).

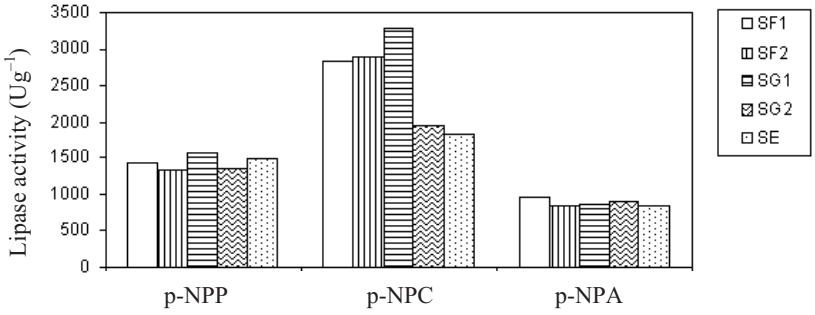

Figure 4A. Substrate specificity of silica-immobilized lipase
Abbreviations: see Figure 1

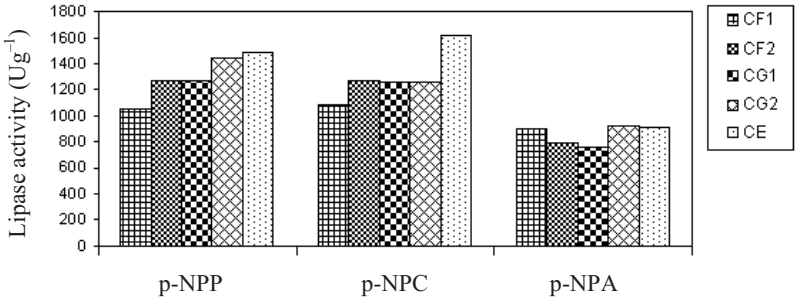

Figure 4B. Substrate specificity of celite-immobilized lipase
Abbreviations: see Figure 1

Effect of detergents on immobilized lipase

Most of the denaturing agents (detergents such as Tween-20, Tween-80 and Triton X-100) brought about a partial loss of esterase activity of immobilized matrices (Figures 5A and 5B). However, a complete loss of lipase activity was noticed in all the matrices following exposure to SLS. Interestingly, all the silica matrices and two of the celite preparations, i.e. CF1 and CF2 showed an increase in their catalytic activity after exposure to SDS (0.5%, w/v). It appeared that formaldehyde treated silica or celite with immobilized lipase developed greater catalytic potential when exposed to a strong anionic detergent, i.e. SDS.

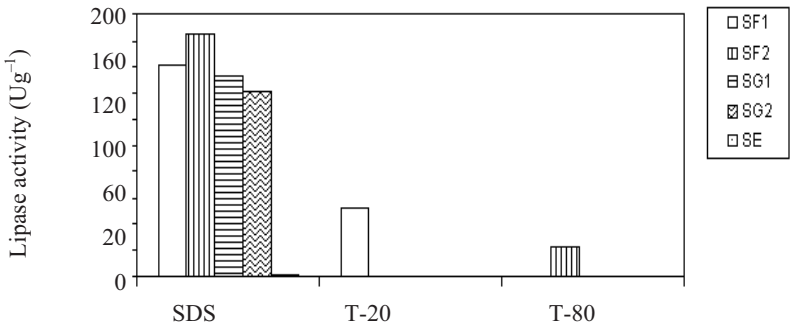

Figure 5A. Effect of detergents on silica-immobilized lipase
Abbreviations: see Figure 1

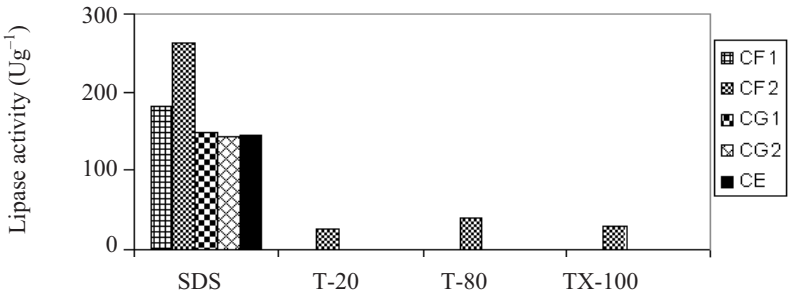

Figure 5B. Effect of detergents on celite-immobilized lipase
Abbreviations: see Figure 1

Effect of solvents on the activity of immobilized lipase

Most of the immobilized lipase preparations showed a decrease in their catalytic activity in the presence of organic solvents. The lipase activity severely declined following exposure to chloroform, acetone and n-hexane in comparison to the native matrices (SE or CE; Figures 6A and 6B). Interestingly, an increase in C-chain length of alkanes from C: 6 to C: 9 caused a corresponding increase in lipase activity relative to native matrices, i.e. SE or CE.

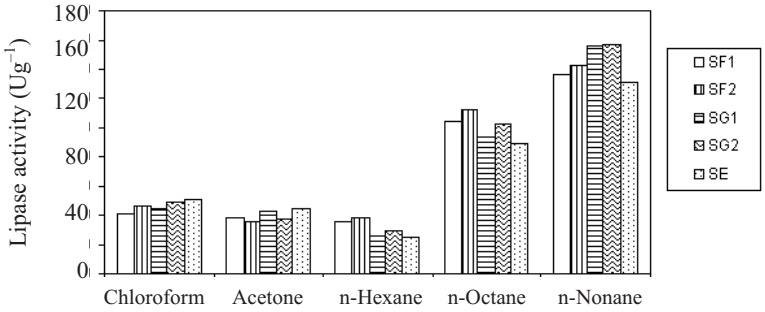

Figure 6A. Effect of organic solvents on the activity of silica-immobilized lipase
Abbreviations: see Figure 1

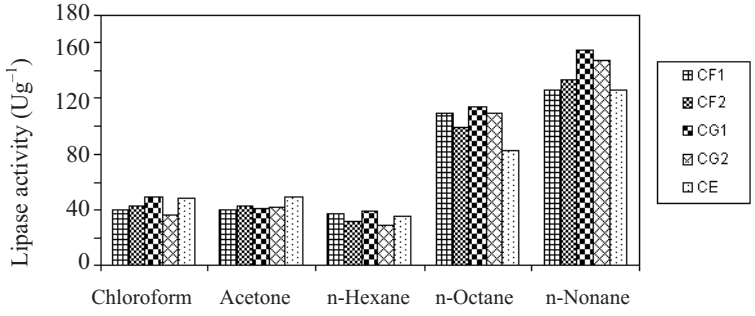

Figure 6B. Effect of organic solvents on the activity of celite-immobilized lipase
Abbreviations: see Figure 1

Amongst alcohols, an exposure to iso-propanol effected retention of lipase activity in all the immobilized preparations but the enzyme activity was more or

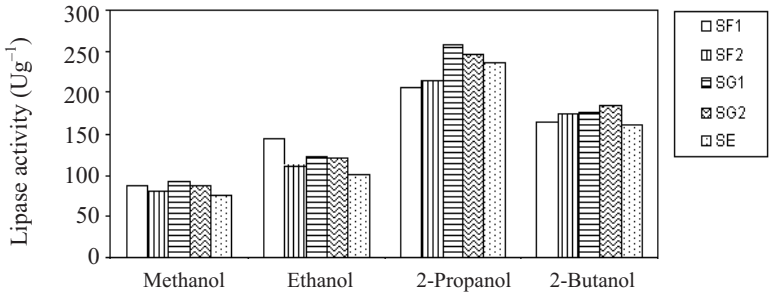

Figure 7A. Effect of alcohols on silica-immobilized lipase
Abbreviations: see Figure 1

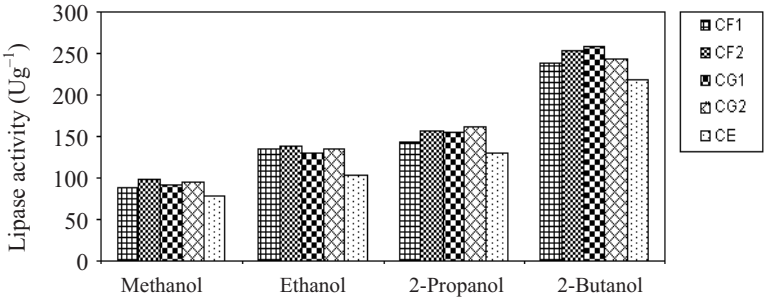

Figure 7B. Effect of alcohols on celite-immobilized lipase
Abbreviations: see Figure 1

less the same in both modified celite and silica preparations in comparison to the unmodified celite or silica matrix. The esterase activity was markedly higher when reaction was carried out in iso-propanol (Figures 7A and 7B).

Effect of pH on activity of free lipase

The optimal pH for free lipase enzyme was observed at pH 8.5. At pH 7.0, the lipase activity decreased to approximately one third of the value observed at pH 8.5. Broadly, the crude lipase retained its biological activity over a wide pH range (i.e. pH 7.0 to 9.0; Figure 8).

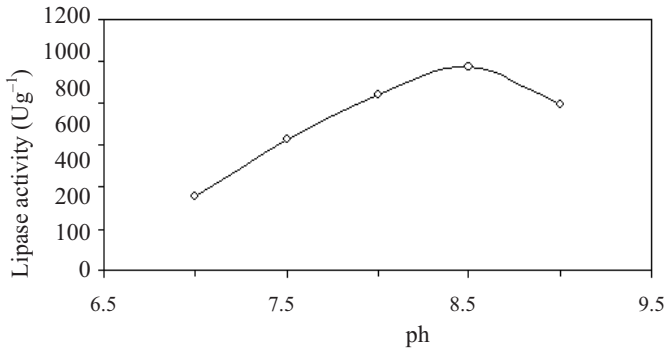

Figure 8. Effect of pH on activity of free lipase

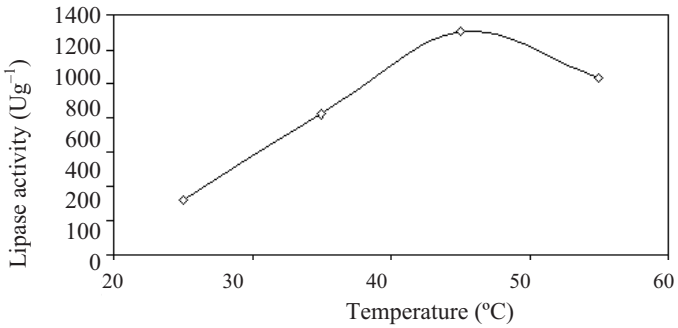

Figure 9. Effect of temperature on free lipase

Effect of temperature on free lipase

The free lipase exhibited a maximum enzyme activity at 45ºC at pH 8.5 (Figure 9).

Discussion

Immobilized enzymes are more useful for industrial purposes and in organic synthesis when immobilized on suitable support(s). A promising approach to enzyme entrapment makes use of inorganic matrices such as silica and celite. Quantification of catalytic activity was performed for lipase immobilized onto silica and porous glass [3, 18–20], celite [19, 21], nylon [22], polypropylene [23, 24]

and ion-exchange resins [23–25]. The widely different substrates, supports and enzymes employed led to an enormous amount of quantitative data, which, however, is difficult to compare.

In the present study, celite and silica initially activated by formaldehyde or gluteraldahyde were used for immobilization/entrapment of a lipase obtained from *Bacillus coagulans* BTS-1 using simple incubation steps. The *B. coagulans* (isolated from kitchen waste of a sweetshop) has been used in the present study because of its thermo-tolerant nature, easy maintenance and its thermostable lipase. The immobilization of the bacterial lipase was very rapid (< 5 min) on silica both activated and native (inactivated). In contrast, the immobilization was relatively gradual on activated celite. Exposure of celite to 2% (v/v) gluteraldahyde considerably increased its efficiency to adsorb/entrap lipase and the available lipase could be completely immobilized within a short period of 20 min. Both silica (SG1) and celite (CG2) matrices retained their catalytic activities within a wide range of temperature (30 to 70ºC) and the maximum being at 50ºC. Also, activated celite- and silica-immobilized lipase matrices, were stable at both acid and alkaline pH. Interestingly, an alkaline bacterial lipase, however, exhibited greater catalytic activity/stability at acidic pH (pH 5.5). Both formaldehyde-activated silica matrices (SF1 & SF2) and a gluteraldahyde-activated silica (SG1) matrix showed higher efficient/enhanced esterase activity than the native silica (SE) towards medium C-length ester (caprylate).

An industrially important lipase must be able to retain its activity in the presence of common detergents; sodium dodecyl sulphate, sodium sarcosine etc. Interestingly, the denaturing agent such as SDS modulated the catalytic potential of all silica-immobilized lipase preparations. A purified lipase from *Pseudomonas cepasia* immobilized on a commercially available microporous polypropylene support showed a high activity (100%) with pNPA and very low with pNPP [26]. Also, as in our study, immobilization (on polypropylene) increased enzyme stability by decreasing its sensitivity to temperature.

The most popular methods of lipase immobilization are based on their adsorption on poorly defined supports (polymeric resins, inorganic materials etc.). The common mechanism of immobilization is adsorption of lipase on more or less hydrophobic supports [2]. Lipases are adsorbed through a combination of hydrophobic, Vander Waals, electrostatic forces etc. Such immobilized derivatives show maintain their catalytic activity in aqueous/organic media. In the present study, it was observed that with increased hydrophobicity (chain length) of alkanes, esterase activity increased. Obviously, hydrophobicity of the environment modulated the activity of the immobilized bacterial lipase. Moreover, when work-

ing in very low water activity systems, solvent interfaces may penetrate into the porous structure of the support thus immobilized lipase molecules became activated on solvent interfaces. It appears that because of this reason a relatively higher enzyme activity could be observed in case of lipase-entrapped matrices in the present study.

Lipases may be very useful to carry out exciting industrial processes in high water activity systems [27]. Previously, adsorption of lipase on tailor made (octyl-agarose) strongly hydrophobic supports was found to have very high esterase activity [28]. The enzyme molecules in an immobilized state are more dispersed on the support than in a precipitate. Consequently, diffusion limitations inside the biocatalyst are much lower with the immobilized preparation [29].

Lipases, retaining their catalytic potential in water restricted organic solvents, could have applications in esterification and trans-esterification reactions. In the present study the lipase activity was severely damaged following exposure to chloroform, n-hexane, and acetone. However, exposure to iso-propanol did not affect the lipase activity in any of the immobilized matrices the enzyme activity remaining more or less the same in both celite and silica preparations in comparison to the unmodified celite or silica matrices. It appeared that exposure of celite/silica to gluteraldahyde or formaldehyde resulted in a change in the behavior of bacterial lipase as compared to free lipase (Figures 8 and 9). The changes are: (a) a shift in optimum temperature of lipase from 45 to 50ºC observed in case of immobilized lipase (Figure 8); (b) the bacterial lipase, which possesses a relatively high catalytic (esterase) activity in the free form at an alkaline pH (Figure 9) tends to show a shift in its optimal activity to an acidic pH (pH 5.5) in an immobilized state, and (c) the exposure of silica or celite to gluteraldahyde/formaldehyde and immobilization of lipase thereof increases/promotes the stability of lipase in the presence of a strong anionic detergent, such as SDS.

Further studies are required to evaluate the potential efficiency of free and immobilized bacterial lipase to mediate reverse esterification reaction(s) in organic or partially aqueous–organic phases in the presence of a variety of fatty acids and alcohols.

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