Thermostability and Esterification of a Polyethylene-Immobilized Lipase from *Bacillus coagulans* BTS-3

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Received 9 June 2005; accepted 9 November 2005
DOI 10.1002/app.24154
Published online in Wiley InterScience (www.interscience.wiley.com).

ABSTRACT: Extracellular lipase from *Bacillus coagulans* BTS-3 was immobilized on activated (alkylated, 2.5% glutaraldehyde) and native (nonactivated) polyethylene powder, and its thermostability and esterification efficiency were studied. Immobilization on activated support was found to enhance thermostability as well as esterification efficiency. The optimum time for immobilization on activated (AS) and nonactivated (NS) polyethylene support was found to be 10 min, and the binding of the lipase was markedly higher on AS. Lipase was more efficiently bound to AS (64%) than to NS (30%) at an optimum temperature of 37°C. The pH and temperature optima for AS- and NS-bound lipase were 9.0 and 55°C and 8.5 and 55°C respectively. At 55°C the free lipase, which had a half-life of 2 h, lost most of its activity at elevated temperatures. In contrast, AS-bound lipase retained 60%–80% of its original activity at 55°C, 60°C, 65°C, and 70°C for 2 h. Exposure to organic solvents resulted in enhanced lipase activity in *n*-hexane (45%) and ethanol (30%). Both AS- and NS-bound biocatalysts were recyclable and retained more than 85% of their initial activity up to the fourth cycle of hydrolysis of *p*-nitrophenyl palmitate. The AS-bound lipase efficiently performed maximum esterification (98%) of ethanol and propionic acid (300 m*M* each, 1 : 1) in *n*-hexane at 55°C. With free or NS-bound lipase in similar conditions, the conversion of reactants into ester was relatively low (40%). © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 102: 3986–3993, 2006

Key words: enzyme; esterification; immobilization; polyethylene; thermal properties

INTRODUCTION

Lipases (triacylglycerol ester hydrolase; EC 3.1.1.3) catalyze a wide range of reactions including hydrolysis, alcoholysis, esterification, and interesterification.[1] Several methods of protein stabilization have been reported in the literature, including enzyme immobilization. Many methods have previously been used to immobilize lipases, including adsorption or precipitation onto hydrophobic materials,[2] covalent attachment to functional groups,[3] entrapment in polymer gels,[4] adsorption in macroporous anion-exchange resins,[5] microencapsulation in lipid vesicles,[6] and use of supported liquid membrane encapsulation[7] and sol–gel entrapment.[8,9]

A promising approach is to use polyethylene powder containing micropores in its native (structure) and alkylated (activated) forms for immobilization. Reports from a number of groups[10,11] have suggested that lipases may be sufficiently hydrophobic in character to be strongly absorbed onto polypropylene or polyethylene. Solid supports such as these have been considered in lipases in particular because they are compatible with the nonpolar solvents that are appropriate if these enzymes are to be used for preparation of esters.

Here we report the immobilization of lipase from *Bacillus coagulans* BTS-3 previously purified[12] on activated and nonactivated polyethylene powder and then a comparison of these immobilized forms. The immobilized enzyme previously has been studied for its advantageous catalytic properties such as thermostability, effect of solvents, and esterification efficiency. The formation of ethyl propionate is important as it has a fruity rum odor and is used in perfumery, fragrances, and in the manufacture of various propionates used in the reduction of pharmaceuticals, antifungal agents, agrochemicals, plasticizers, rubber chemicals, dyes, and others.

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Contract grant sponsors: Department of Biotechnology, Ministry of Science and Technology, Government of India; Department of Biotechnology, Himachal Pradesh University, Shimla, India.

Journal of Applied Polymer Science, Vol. 102, 3986–3993 (2006)

EXPERIMENTAL

Preparation of lipase from *Bacillus coagulans* BTS-3

Bacillus coagulans BTS-3 was grown in 250-mL Erlenmeyer flasks containing 50 mL of the production medium {yeast extract [0.5% (w/v)], peptone [0.5% (w/v)], NaCl [0.05% (w/v)], $CaCl_2$ [0.005% (w/v)],

refined mustard oil [0.1% (v/v) with 0.5% (w/v) gum acacia]} at a temperature of 55°C and a pH of 8.5 for 48 h. The cells were removed from culture broth by centrifugation at 10,000 *g* for 20 min at 4°C. Ammonium sulfate was added to 500 mL of cold culture supernatant, with constant stirring to achieve 70% saturation. It was subsequently centrifuged at 10,000 *g* for 40 min. The precipitates reconstituted in 3 mL of Tris HCl buffer 0.1*M*, pH 8.5) were dialyzed against the same buffer overnight in order to remove traces of ammonium sulfate. The dialysate was loaded on a DEAE-sepharose column, which resulted in a single peak whose lipase activity[13] and protein[14] were determined. The DEAE-sepharose column purified fraction was referred as purified lipase (PL).

Enzyme assay

The activity of free and immobilized lipase was assayed by a colorimetric method,[13] as previously described,[15] by measuring the micromoles of 4-nitrophenol released from 4-nitrophenyl palmitate. In brief, a stock solution (20 m*M*) of 4-nitrophenyl palmitate (4-NPP) was prepared in HPLC-grade isopropanol. The reaction mixture contained 75 μL of 4-NPP stock solution, 5 μL of enzyme, and enough Tris buffer (0.1*M*, pH 8.5) to make final volume of 3 mL. The reaction mixture was incubated at 55°C for 10 min in a water bath. Chilling at −20°C for 8 min was employed to stop the reaction. A control containing heat-inactivated enzyme was also incubated with each assay. Absorbance of the 4-nitrophenol released was measured at 410 nm (Perkin Elmer UV/Vis Spectrophotometer Lambda 12, Norwalk, CT, USA). The concentration of 4-nitrophenol released was determined from a reference curve of 4-nitrophenol (2–20 μg/mL in 0.1*M* Tris HCl buffer, pH 8.5).

Each of the assays was performed in triplicate unless otherwise stated, and mean values are presented.

One unit (U) of lipase activity was defined as micromoles of 4-nitrophenol released by 1 mL of free enzyme or 1 g of immobilized enzyme per minute under standard assay conditions.

Activation of polyethylene powder

The solid support was washed three times with distilled water, treated with 0.6*N* HCl (30 min), and further washed three times with distilled water to remove traces of HCl. This matrix was further treated with 2.5% glutaraldehyde for 1 h, followed by extensive washing in distilled water to remove the residual glutaraldehyde. Therefore, this support is referred to as activated support (AS).

Nonactivated polyethylene powder

Similar samples were prepared without the addition of glutaraldehyde. The solid support was successively exposed to an excess of 0.6*N* HCl (30 min) and distilled water. This acid-washed support is referred to as nonactivated support (NS).

Immobilization of purified lipase on supports

The AS and NS were separately incubated with 2 mL of PL (2.32 U) at 37°C for 18 h. The unbound lipase was removed by washing the support with Tris buffer (0.1*M*, pH 8.5). The NS- and AS-bound lipases were assayed for bound lipase activity and protein and were stored at 4°C until further use. In each assay, 10 mg of immobilized enzyme and 5 μL of free enzyme were used.

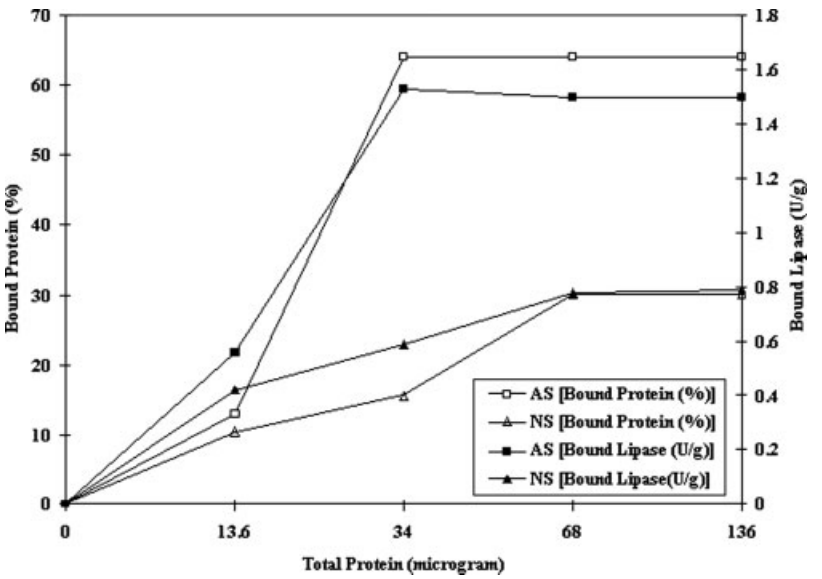

Figure 1 Effect of protein concentration on immobilization of lipase from *Bacillus coagulans* BTS-3.

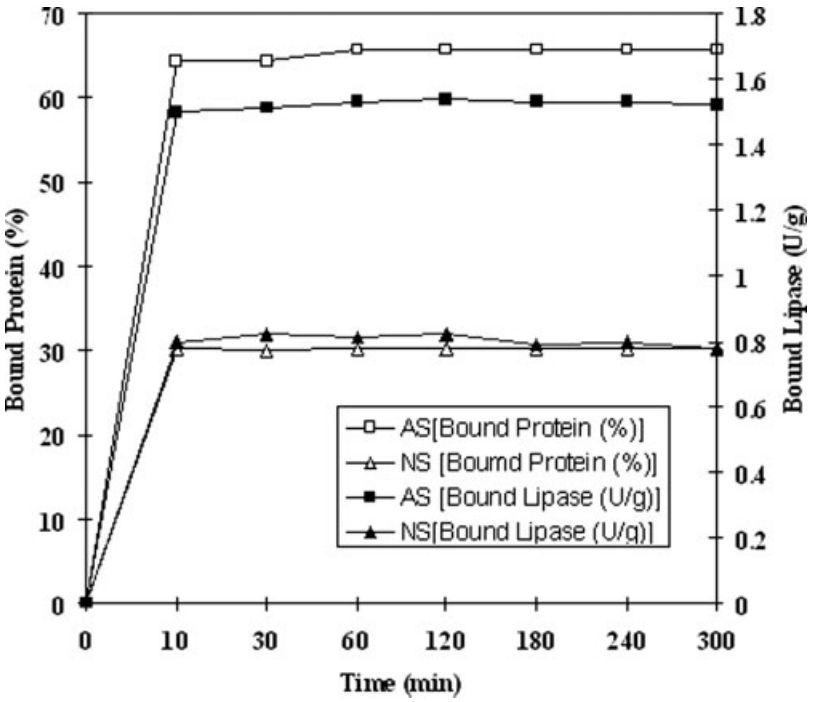

Figure 2 Immobilization kinetics of lipase from *Bacillus coagulans* BTS-3.

Immobilization (amount and activity) kinetics of enzyme

PL was incubated with AS and NS separately at 37°C for 5 h. Supernatant was withdrawn periodically and assayed for unbound protein and lipase activity.

Effect of pH on hydrolytic activity of immobilized enzyme

The optimum pH of the AS- and NS-bound lipases was determined by studying the hydrolytic activity toward 4-NPP prepared in 0.1*M* Tris HCl buffer (pH 7.0–10.0) at 55°C.

Effect of temperature on hydrolytic activity of immobilized enzyme

To determine the optimum temperature for lipase immobilized on AS and NS matrices, the support-bound enzyme was assayed in 0.1*M* Tris HCl buffer (pH 8.5) at preselected temperatures (30°C, 37°C, 45°C, 50°C, 55°C, and 60°C).

Reusability of immobilized lipase for hydrolytic activity toward 4-NPP

To determine reusability, after hydrolysis of 4-NPP support-bound lipases were washed extensively with 0.1*M* Tris buffer (pH 8.5). The washed support-bound biocatalysts were recovered and used to hydrolyze the substrate in the fresh reaction mixture. Activity was measured at 55°C for 10 min repeatedly until it was 25% of the original.

Effect of various organic solvents on immobilized enzyme

To study the effect of different solvents on the hydrolytic activity of immobilized lipases, each type of matrix (AS and NS) was separately suspended in 1 mL of 0.1*M* Tris buffer (pH 8.5), and the contents were thoroughly vortexed to achieve homogeneity. This suspension (20 μL) was separately incubated

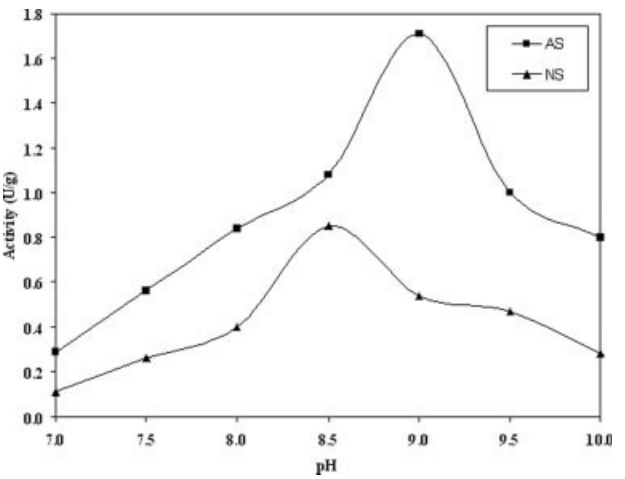

Figure 3 Effect of pH on the activity of lipase immobilized on activated (AS) and nonactivated (NS) support.

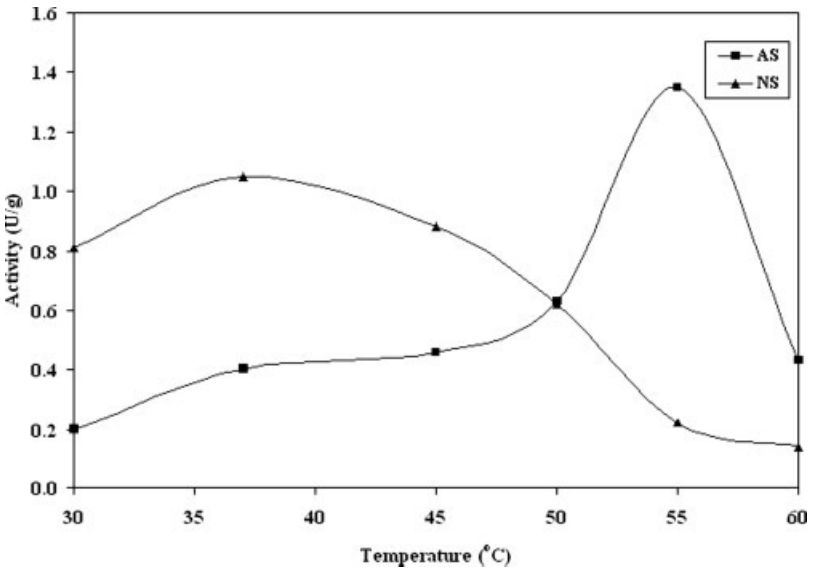

Figure 4 Effect of temperature on the activity of lipase immobilized on activated (AS) and nonactivated (NS) support.

with each of the selected alcohols (methanol, ethanol, isopropanol, and isobutanol) and organic solvents (chloroform, acetone, *n*-pentane, *n*-hexane, *n*-octane, and *n*-nonane) in a volumetric ratio of 1 : 2 for 30 min at 37°C. Residual support-bound lipase activity was measured as described previously.[15]

Thermostability of free and immobilized enzymes

Thermostability was studied by incubating free and immobilized enzymes separately at 55°C, 60°C, 65°C, and 70°C for 2 h in a water bath shaker. Thereafter, the residual activity of the support-bound biocatalyst was measured and compared with that of the free enzyme.

Esterification efficiency at different concentrations for different durations

For a comparative study, immobilized (10 mg of AS and NS) or free lipase was used as biocatalyst for the esterification of ethanol (100–400 m*M*) and propionic acid (100–400 m*M*) in a molar ratio of 1 : 1 in *n*-hexane. The reaction was carried out at 55°C under shaking (160 rpm) for 40 h. Heat-inactivated free

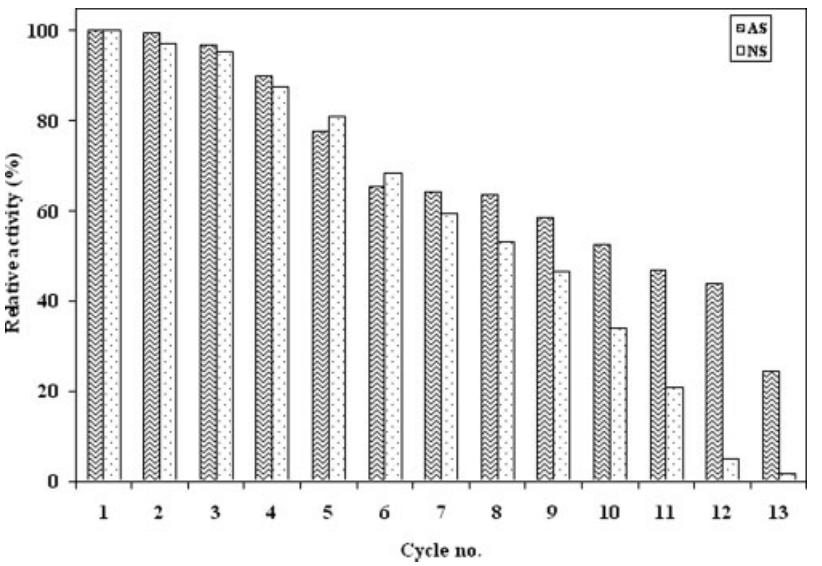

Figure 5 Reusability of immobilized enzyme for hydrolytic activity toward 4-NPP.

TABLE I
Effect of Organic Solvents on Catalytic Activity of Lipase Immobilized on Activated Support (AS) and Nonactivated Support (NS)

	Activity (U/g)	
Solvent	AS	NS
Methanol	0.90	0.50
Ethanol	1.00	0.25
Isopropanol	0.70	0.56
Isobutanol	0.70	0.58
Chloroform	0.56	0.50
Acetone	0.74	0.62
n-Pentane	0.75	0.66
n-Hexane	1.17	0.91
n-Octane	0.72	0.32
n-Nonane	0.56	0.26

enzyme (incubated at 75°C for 1 h) and matrix without enzyme were used as controls.

Analysis of ester by gas chromatography

The solvent phase (2 μL) was analyzed with gas chromatography (GC) using a packed column (10% SE-30 Chrom WHP, 2 × 1/8 m, 80–100 mesh size) and a flame ionization detector with nitrogen as the carrier gas (30 mL/min). The temperatures of the oven, the detector and the injector were all 200°C.

RESULTS AND DISCUSSION

Immobilization on support

In the present study, a purified lipase of *Bacillus coagulans* BTS-3 was immobilized on AS (alkylated) and NS (native) polyethylene powder as support for possible applications in hydrolysis and esterification reactions. The activation of the support was done by exposure to 2.5% glutaraldehyde (v/v), which helped it in crosslinking with enzyme.[15] Previously, polyethylene-immobilized lipase was used for the effective hydrolysis of perilla oil to produce α-linolenic acid.[16] When incubated with support (AS and NS) in a ratio of 1 : 3, the enzyme had a maximum binding efficiency of 64% for the activated support and 30.5% for the nonactivated support (Fig. 1).

Immobilization kinetics of enzyme

The time of incubation for immobilization on activated as well as nonactivated supports was found to be 10 min (Fig. 2). An efficient immobilization in such a short period min was of great significance in preparing the immobilized biocatalyst. Previously the lipase of *B. coagulans* BTS-1 was rapidly immobilized on activated silica.[15]

Effect of pH on activity of immobilized enzyme

A gradual increase in lipolytic activity of immobilized enzyme was seen when the pH of the assay buffer was increased from 7.0 to 9.0 for the AS and from 7.0 to 8.5 for the NS. Any further increase in pH markedly decreased the activity of the immobilized enzyme (Fig. 3). Enhanced activity in an alkaline pH range provided an edge over the free and nonactivated enzymes. Recently, lipase from a mutant strain of *Corynebacterium* sp. was immobilized, and a comparison of immobilized and free enzymes

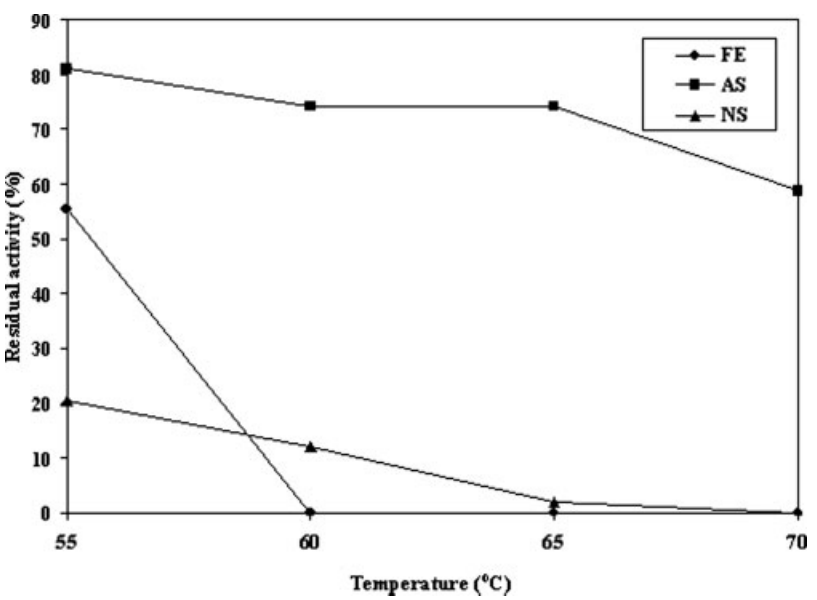

Figure 6 Thermostability of free lipase and enzyme immobilized on activated (AS) and nonactivated (NS) support.

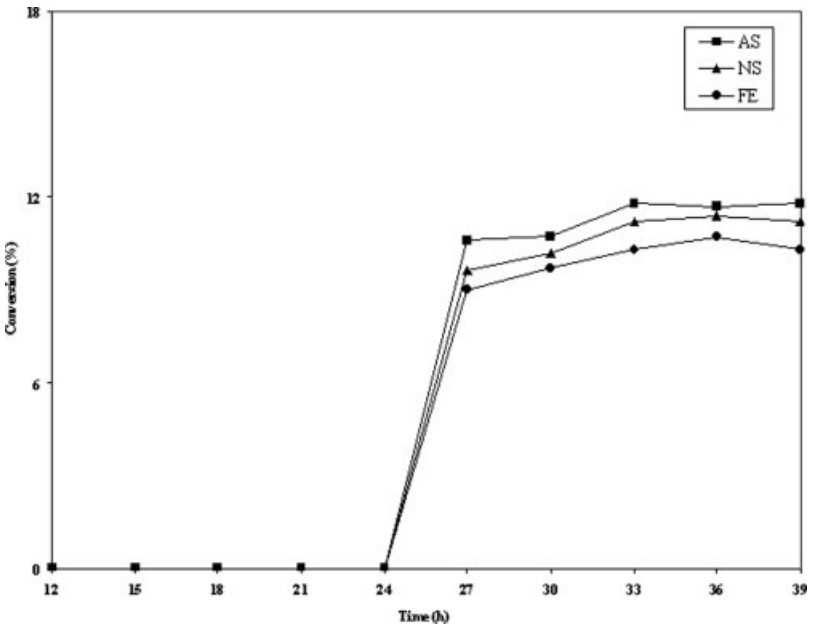

Figure 7 Esterification efficiency of free and immobilized enzymes at a 100 m*M* concentration of the reactants (1 : 1).

showed the optimum pH to be 8.0 for both.[17] Most lipases can act in a wide range of pHs and temperatures, although bacterial alkaline lipases are more common.[18–20]

Effect of temperature on the activity of immobilized enzyme

An increase in the activity of immobilized lipase was recorded when the temperature was increased from 30°C to 55°C for the AS. A temperature of 55°C was also optimum for the free enzyme.[12] For NS an increase in hydrolytic activity was recorded up to 37°C; further increases in temperature resulted in a marked decrease in activity (Fig. 4). In a recent study, the optimum temperatures for purified and immobilized enzymes were found to be 65°C and 50°C, respectively, for the lipase of a mutant strain of *Corynebacterium* sp.[17]

Reusability of immobilized enzyme for hydrolytic activity toward 4-NPP

A continuous assay of residual hydrolytic activity of the lipase immobilized on AS and NS was performed to find out how much lipase activity would

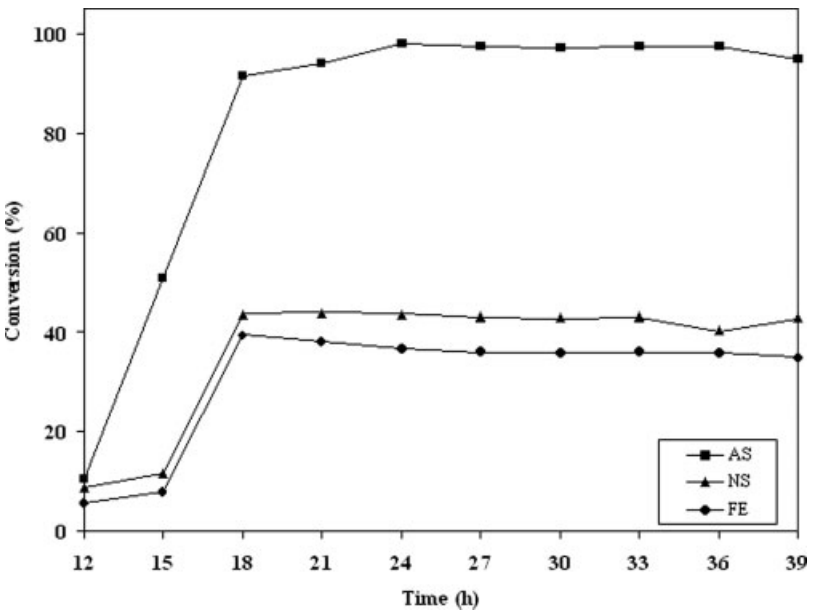

Figure 8 Esterification efficiency of free and immobilized enzymes at a 300 m*M* concentration of the reactants (1 : 1).

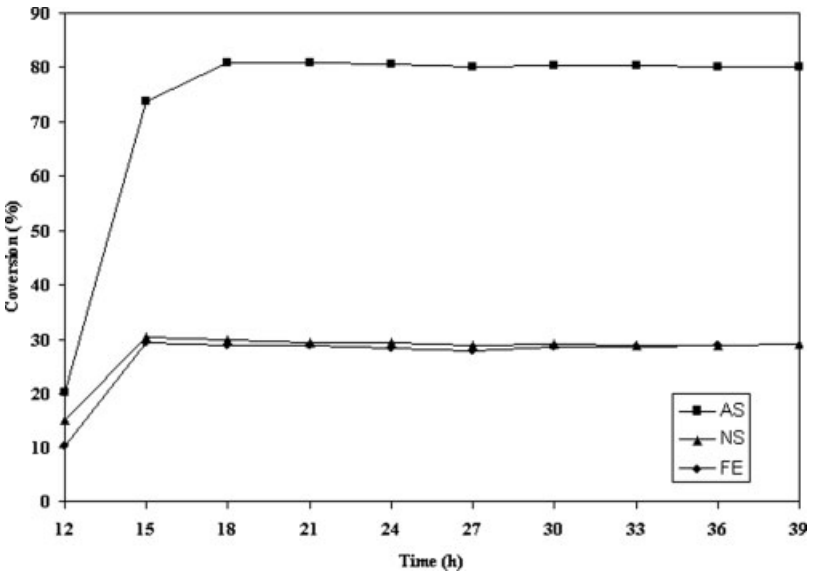

Figure 9 Esterification efficiency of free and immobilized enzymes at a 400 m*M* concentration of the reactants (1 : 1).

be retained by each support up to the 13th reaction cycle. AS-bound lipase retained more than 50% of its activity even after the 10th cycle, whereas nonactivated support showed 50% activity after the eighth cycle (Fig. 5). Previously, a lipase of *Bacillus* sp. immobilized on CNBr-activated Sepharose 4B retained full activity even after the 13th cycle of hydrolysis.[21]

Effect of organic solvents on immobilized enzyme

The AS showed an increase in catalytic activity in the presence of various organic solvents compared with that with the NS (Table I). The lipase activity of AS as well as NS was maximum in the presence of *n*-hexane; hence *Bacillus coagulans* BTS-3 lipase was subsequently used for esterification studies using *n*-hexane as a solvent system. Chloroform had more or less the same effect on enzyme immobilized on both AS and NS. Among various alcohols, exposure to ethanol markedly increased the activity of AS-bound biocatalyst relative to that in NS-bound enzyme (Table I). Previously a lipase from *Bacillus coagulans* BTS-1 immobilized on silica and celite showed decreased activity in the presence of *n*-hexane.[15]

Thermostability of immobilized enzyme

At 70°C the AS showed 60% residual lipase activity and the NS showed little residual lipase activity, whereas the free enzyme retained 55% residual activity at 55°C. Complete loss of enzyme activity was observed for free enzyme preincubated at higher temperatures (Fig. 6). Previously it was observed that immobilized enzymes were much more stable than the soluble enzyme when lipase from *Bacillus thermocatenulatus* was immobilized on hydrophobic supports, maintaining 100% of activity at 65°C.[22] *Bacillus* GK8 lipase immobilized on HP-20 beads retained total activity at 60°C.[21]

Ester synthesis by immobilized enzyme

When the concentrations of ethanol and propionic acid were increased in equal proportions from 100 to 300 m*M* for esterification, the esterification efficiency increased from 11.8% to 98% for AS. Any further increase in the concentration of any of the reactants led to a decrease in the conversion (Figs. 7–9).

The present study has shown that polyethylene was a suitable support for rapid, efficient, and stable binding of lipase of *Bacillus coagulans* BTS-3. The support-bound lipase also efficiently catalyzed esterification of ethanol and propionic acid into ethyl propionate at a temperature of 55°C; activation of the support with glutaraldehyde changed its optimum pH toward an alkaline range and thus enhanced thermostability as well as esterification efficiency.

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