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ORIGINAL PAPER

Synthesis of 4-nitrophenyl acetate using molecular sieve-immobilized lipase from *Bacillus coagulans*

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Abstract Extracellular lipase from *Bacillus coagulans* BTS-3 was immobilized on (3 Å × 1.5 mm) molecular sieve. The molecular sieve showed approximately 68.48% binding efficiency for lipase (specific activity 55 IU mg^{-1}). The immobilized enzyme achieved approx 90% conversion of acetic acid and 4-nitrophenol (100 mM each) into 4-nitrophenyl acetate in *n*-heptane at 65°C in 3 h. When alkane of C-chain length other than *n*-heptane was used as the organic solvent, the conversion of 4-nitrophenol and acetic acid was found to decrease. About 88.6% conversion of the reactants into ester was achieved when reactants were used at molar ratio of 1:1. The immobilized lipase brought about conversion of approximately 58% for esterification of 4-nitrophenol and acetic acid into 4-nitrophenyl acetate at a temperature of 65°C after reuse for 5 cycles.

Keywords *Bacillus coagulans* BTS-3 · Lipase · Molecular sieve · Immobilization · Ester synthesis

Introduction

Lipase-catalyzed esterification reactions have received extensive importance due to enzymatic properties and substrate specificities and numerous products that can be obtained viz., monoacylglycerols, esters and biosurfactants [4, 14–16, 18, 19, 27]. A new irreversible esterification method for carboxylic acids catalyzed by a lipase from *Candida antarctica* (Novozyme 435) in organic solvents yields range from 82 to 92% [12]. Practical applications prefer immobilized enzymes because they offer easy catalyst recycling, feasible continuous operations and simple product purification. Furthermore, immobilizing enzymes onto various insoluble or solid supports is a useful tool to increase their thermal and operational stabilities. In these cases, the structure of the support has a great impact on the behavior of the immobilized enzymes. Concerning a practical application of the immobilized enzyme, large surface area-to-volume ratio from the support is always desirable, since in this way the enzyme loading per unit volume of support and the catalytic efficiency of the enzyme can be effectively improved.

Molecular sieves involve such a series of materials that can discriminate between molecules, particularly on the basis of size. As support materials, they offer interesting properties, such as high surface areas, hydrophobic or hydrophilic behavior, and electrostatic interaction, as well as mechanical and chemical resistance, making them attractive for enzyme immobilization [42]. Molecular sieves help in continuous synthesis of ester. In this study, molecular sieve is used as matrix to immobilize lipase but earlier it has been used to remove water to enhance esterification. Addition of molecular sieve (4 Å) enhanced both initial rate and net conversion of ascorbic acid to oleoyl ascorbate [23]. Water content is a crucial parameter because it modifies the thermodynamic equilibrium of the reaction and then directs the reaction towards hydrolysis or synthesis. Addition of molecular sieve eliminated hydrolysis of propyl-glycoside lactate and allowed esterification of propyl-glycoside to rise from 66 to 75% [37].

A molecular sieve has a selective adsorption property capable of separating components of a mixture on the basis of a difference in molecular size and shape. Immobilization

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can help in providing high performance biocatalyst in biotechnological processing. Recently, the thermostability of the immobilized enzyme, whose half life was 2,500 min was greatly improved and was found to be significantly higher than that of free enzyme (80 min). [45]. Immobilized lipase from *Rhizomucor meihi* (Lipozyme IM-20) was used to catalyze esterification reaction between isovaleric acid and ethanol to synthesize ethyl isovalerate in *n*-hexane [8].

In this paper we describe immobilization of lipase on molecular sieve in combination with synthesis of 4-nitrophenyl acetate. 4-nitrophenyl acetate is used as a substrate for lipase assay. It is approximately fifty times more expensive than the alcohol from which it is synthesized during esterification, 4-nitrophenol.

Materials and methods

Organism and growth conditions

Bacillus coagulans BTS-3 strain was obtained from Department of Biotechnology, Himachal Pradesh University, Summer Hill, Shimla (India). The lipase sourced from *B. coagulans* BTS-3 was purified by successive salting out and DEAE-Sepharose anion exchange chromatography. Its molecular weight was 31 kDa on SDS-PAGE [21]. The purified lipase was immobilized on molecular sieve (Rankem, New Delhi).

Purification of enzyme

Lipase produced from thermophilic isolate *B. coagulans* BTS-3 was 61-fold purified to homogeneity by ammonium sulfate precipitation and DEAE-Sepharose column chromatography as discussed in our previous article [21].

Enzyme immobilization and esterification

Molecular sieve particles were rinsed with distilled water and contacted with buffered 2.5% glutaraldehyde solution at pH 7.0 for one hour. Finally, the glutaraldehyde treated molecular sieve particles were rinsed with buffer and contacted with a purified enzyme at 4°C for overnight. Increasing amount of protein (8.8–22 μg) was added to a fixed amount of matrix.

Immobilized enzyme assay was carried out by the method of Winkler and Stuckmann (1979) [40] by measuring the micromoles of 4-nitrophenol released from 4-nitrophenyl palmitate. One unit (IU) of hydrolytic activity of immobilized lipase was expressed as micromoles of 4-nitrophenol released per minute by hydrolysis of 4-nitrophenyl palmitate (≥98.0%) (Lancaster Synthesis, UK prepared as 20 mM stock in 2-propanol) at 65°C by 1 g of immobilized enzyme (including weight of the matrix) under assay conditions. Chilling was used to stop esterification reaction. Protein concentration was estimated as described by Lowry using bovine serum albumin as a standard [24]. The 4-nitrophenyl acetate formed in each of the combinations of the reactants was quantified by using alkalimetric method of titrating unreacted acid with 0.1 N NaOH using phenolphthalein as an indicator [5]. The conversion (%) in ester synthesis was based on acid consumed.

Optimization of reaction conditions for esterification

The effect of molar concentration and molar ratio of 4-nitrophenol and acetic acid (water free) on synthesis of 4-nitrophenyl acetate was determined by keeping the concentration of one of the reactants (4-nitrophenol/acetic acid) at 100 mM and varying the concentration of second reactant (50–300 mM) in a reaction volume of 1 ml in *n*-heptane. Kinetics of the immobilized lipase catalyzed synthesis of 4-nitrophenyl acetate was studied up to 9 h under continuous shaking. Temperature for the reaction was optimized by carrying the reaction at 45, 50, 55, 60, 65 and 70°C for 3 h in *n*-heptane. Both the above experiments were carried out using 10 mg immobilized enzyme with 100 mM each of the reactants. In order to choose an appropriate solvent system that is inert, non volatile at the catalytic site and keeps the reactants dissolved, esterification in similar reaction mixture was employed with different n-alkanes of varying C-chain length, i.e., *n*-pentane, *n*-hexane, *n*-heptane, and *n*-pentadecane. To see the effect of water on lipase catalyzed esterification, the reaction system was pre-equilibrated through the gas phase of saturated salt solution and pure water to generate low and high water activity conditions, respectively [1, 3, 9, 10, 35]. All the reaction mixtures were sampled in triplicates and synthesis of 4-nitrophenyl acetate was determined by taking reading at 410 nm.

The conversion of 4-nitrophenol and acetic acid to 4-nitrophenyl acetate was carried at varying concentration and ratio of reactants in a series of different solvents and the reaction mixture was incubated at different temperatures for different time periods. The 4-nitrophenyl acetate formed in each of the combinations of the reactants was quantified by using alkalimetric method. The conversion of 4-nitrophenol and palmitic acid to 4-nitrophenyl palmitate and 4-nitophenol and stearic acid to 4-nitrophenyl stearate was also quantified using alkalimetric method.

Reusability of immobilized lipase in continuous cycles of esterification for synthesis of 4-nitrophenyl acetate

The formation of 4-nitrophenyl acetate from 4-nitrophenol and acetic acid (100 mM each) catalyzed by immobilized

lipase in *n*-heptane was used to check the reusability of molecular sieve-immobilized enzyme. The immobilized lipase was used for 5 cycles of 3 h each, for 4-nitrophenyl acetate formation. After each cycle of esterification, the immobilized enzyme was washed twice for 5 min each in 1.0 ml *n*-heptane at room temperature. Thereafter *n*-heptane was decanted and matrix was reused for fresh cycle of ester synthesis under similar conditions.

Results and discussion

Purification of bacterial lipase

The cell free broth (500 ml) had a lipase specific activity of 0.9 U/mg protein. The protein was optimally precipitated at 70% (w/v) ammonium sulfate saturation. The precipitate was reconstituted in 3.0 ml of Tris–HCl, pH 8.5 buffer overnight to remove the ammonium sulfate [21]. The dialyzed enzyme showed specific activity of 6.4 U/mg protein. The chromatography of the dialyzed enzyme on DEAE-Sepharose column resulted in a single peak (Fig. 1). The fractions showing lipase activity were pooled in 9 ml, specific activity 55 IU mg^{-1}. The DEAE column purified lipase showed 61-fold purification with yield of 2.3%.

An extra-cellular lipase produced by *Bacillus licheniformis* MTCC 6824 was purified to homogeneity by ammonium sulphate fractionation, ethanol/ether precipitation, dialysis, followed by anion-exchange chromatography on Amberlite IRA 410 (Cl^- form) and gel exclusion chromatography on Sephadex G 100 using Tris–HCl buffer (pH 8.0) [6].

Protein binding efficiency and hydrolytic activity of the molecular sieve

Unlike in previous studies, where molecular sieve had been used to remove water to enhance rate of esterification, in the present study it has been used to immobilize alkaline lipase from a thermophilic strain, *B. coagulans* BTS-3.

The purified lipase of *B. coagulans* BTS-3 was optimally immobilized onto the molecular sieve that retained 68.48% of the total protein which was 13.2 µg used for immobilization (Fig. 2). A variety of fatty acid esters are now being produced commercially using immobilized lipase in non-aqueous solvent [13, 25, 30, 43]. Usually several methods have been used to immobilize lipases including adsorption or precipitation onto hydrophobic materials [41] covalent attachment to functional groups [33], entrapment in polymer gels [34], adsorption on macro porous anionic-exchange resins [32], micro encapsulation in lipid vesicles [2], and sol–gel entrapment [11, 20].

Effect of incubation time on synthesis of 4-nitrophenyl acetate

In the present study, the kinetics of the immobilized lipase catalyzed synthesis of 4-nitrophenyl acetate was studied up to 9 h under continuous shaking. The 4-nitrophenyl acetate was formed with the conversion rate of approximately 74.3% in 3 h (Fig. 3). Thus in the subsequent experiments a reaction time of 3 h at 65°C for immobilized lipase was considered optimum for synthesis of 4-nitrophenyl acetate. Advantage of the immobilized enzyme over free enzyme has been observed in one of the studies where rate of

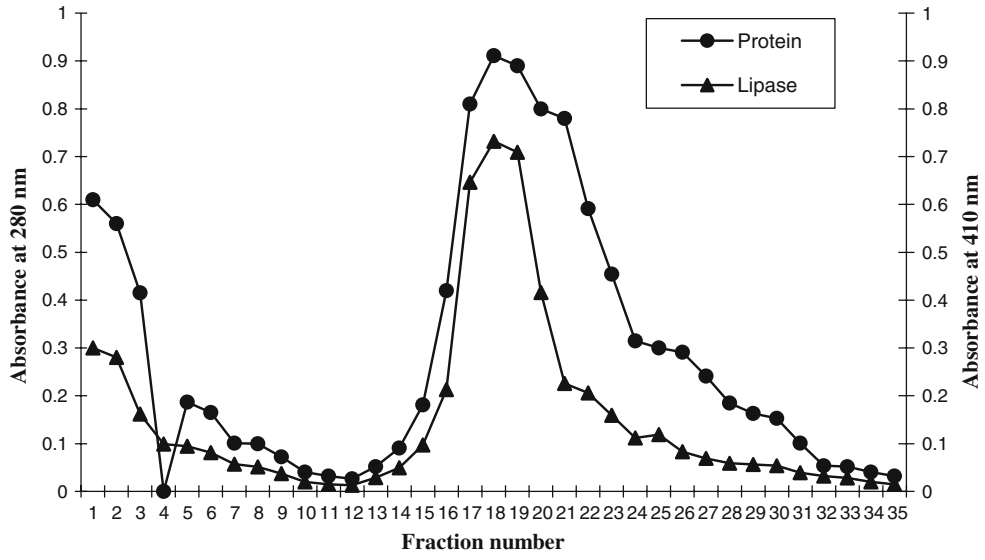

Fig. 1 Purification of lipase from *Bacillus coagulans* BTS-3 on DEAE-Sepharose column

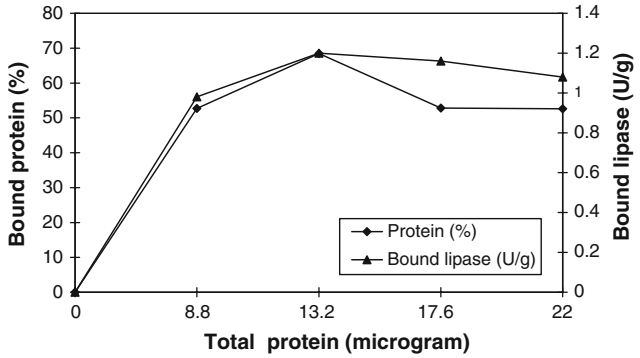

Fig. 2 Effect of protein concentration on immobilization of lipase from *Bacillus coagulans* BTS-3 on molecular sieve

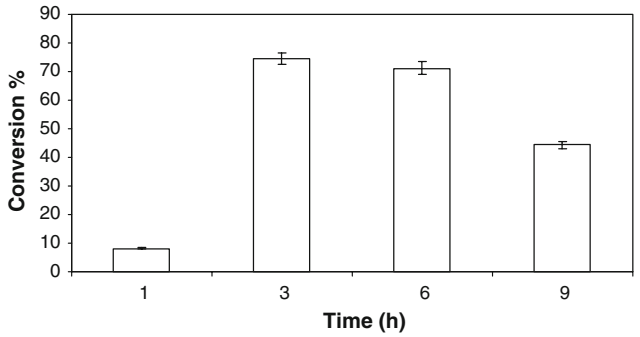

Fig. 3 Effect of incubation time on conversion of acetic acid and 4-nitrophenol (100 mM each) into 4-nitrophenyl acetate in *n*-heptane at 65°C

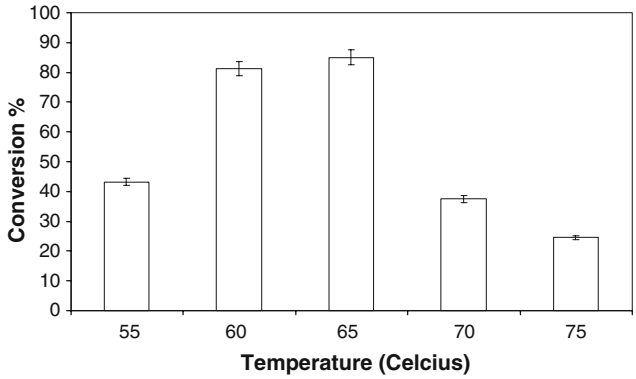

Fig. 4 Effect of incubation temperature on conversion of acetic acid and 4-nitrophenol (100 mM each) into 4-nitrophenyl acetate in *n*-heptane in 3 h

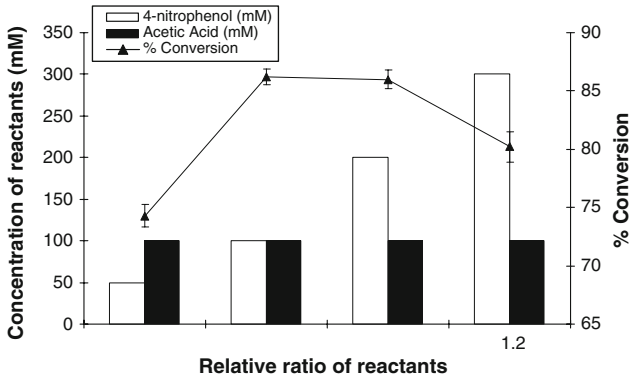

Fig. 5 Effect of varying concentration of 4-nitrophenol on conversion of acetic acid (100 mM) and 4-nitrophenol into 4-nitrophenyl acetate in *n*-heptane at 65°C in 3 h

reaction catalyzed by immobilized lipase was 6.9 times higher than that of the free enzyme [36].

Effect of incubation temperature on esterification

Formation of 4-nitrophenyl acetate accelerated with increase in temperature from 55 to 65°C with conversion of about 85% at 65°C, after which there was a decline in conversion rate with only 24.5% at 75°C (Fig. 4). The molecular sieve bound lipase efficiently catalyzed the esterification of 4-nitrophenol and acetic acid to 4-nitrophenyl acetate in short period of 3 h at 65°C. The bound lipase retained its stability/activity at enhanced temperature than free lipase. Higher temperature and liquefaction tend to make substrate more diffusible and hence easily acceptable to the enzyme [17]. Lipase QL from *Alcaligenes* sp. is a quite thermostable enzyme whose optimal temperature increased from 50°C (soluble enzyme) upto 70°C (hydrophobic support enzyme immobilized preparations) [39].

In the present study, such an effect might have increased diffusion of acetic acid and 4-nitrophenol onto the catalytic site and diffusion of 4-nitrophenyl acetate away from the catalytic site of the lipase.

Effect of molar concentration and relative ratio of reactants on synthesis of 4-nitrophenyl acetate

Molecular sieve immobilized lipase achieved 86.2% conversion of the reactants into the ester when used at a molar ratio of 1:1 (Figs. 5, 6). However, any increase in the concentration of the acetic acid had an inhibiting effect on 4-nitrophenyl acetate synthesis, with only 80.19% conversion at molar ratio of 1:3 of 4-nitrophenol:acetic acid.

It appeared that such a decrease in the ester synthesis might be because of change brought about by excessive concentration of either component at the catalytic site that comprised of the triad of serine, aspartic acid (or glutamic acid) and histidine; serine being highly conserved residue in various lipases including that from *B. coagulans*.

Effect of C-chain length of solvent (alkane) on synthesis of 4-nitrophenyl acetate

An ideal solvent system should be non volatile, inert and keep reactants dissolved at the temperature of catalysis so

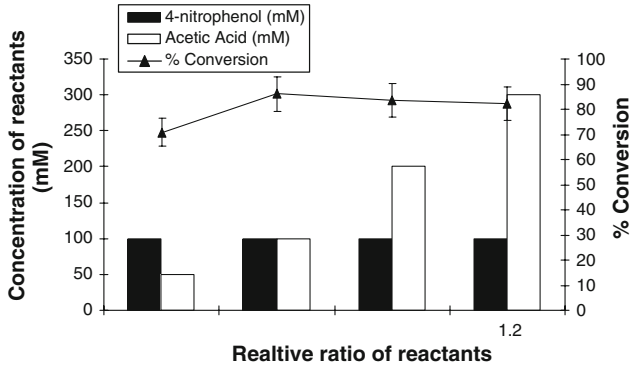

Fig. 6 Effect of varying concentration of acetic acid on conversion of acetic acid (100 mM) and 4-nitrophenol into 4-nitrophenyl acetate in *n*-heptane at 65°C in 3 h

as to carry out efficient esterification. In present study, the use of alkanes of C-chain different from that of *n*-heptane decreased the rate of esterification (Table 1). Thus *n*-heptane proved to be the best solvent for performing synthesis of 4-nitrophenyl acetate using molecular sieve bound lipase. In our recent study, the same enzyme when immobilized on glutaraldehyde activated Nylon-6 showed enhanced activity in *n*-heptane [29]. In another study, effect of a series of solvents such as ethanol, tetrahydrofuran, pyridine, 4-nitrophenol, tertiary amyl alcohol, hexanol, octanol and hexane was investigated on the synthesis of oleoyl ester and t-amyl alcohol was found to be most suitable [34]. In our earlier study, the same enzyme when immobilized on glutaraldehyde-activated-polyethylene showed enhanced synthesis of ethyl propionate in presence of *n*-hexane [22].

Reusability of the immobilized lipase in continuous cycles of esterification for synthesis of 4-nitrophenyl acetate

The immobilized lipase was used for five cycles of 3 h each at 65°C for synthesis of 4-nitrophenyl acetate from acetic acid and 4-nitrophenol (100 mM each) in *n*-heptane. The rate of conversion of acetic acid and 4-nitrophenol was found to be 58.5% after fifth cycle (Table 2). Earlier, the recombinant fungal cells were immobilized on Biomass Support Particles (BSPs) to facilitate the reusability of whole-cell biocatalyst. Whole-cell biocatalyst maintained its activity for more than 15 batch reaction cycles [38]. In our recent study, during repetitive transesterification using nylon-bound lipase from *B. coagulans,* 58.2% conversion of vinyl acetate and butanol into butyl acetate occurred after third cycle of reuse [28].

Table 1 Effect of carbon chain length of solvents on esterification

Organic solvents	% conversion
n-Pentane	27
n-Hexane	84
***n*-Heptane**	**93**
n-Pentadecane	15

The bold characters signify the reaction condition in which maximum % conversion has been achieved

Table 2 Reusability of immobilized lipase for synthesis of 4-nitro phenyl acetate

Cycle no.	% conversion
1	**88.65**
2	86.60
3	83.50
4	63.20
5	58.50

The bold characters signify the reaction condition in which maximum % conversion has been achieved

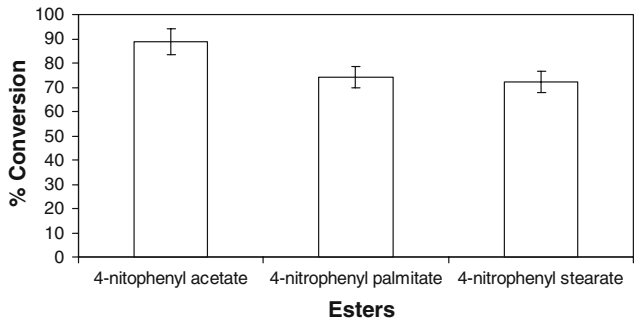

Fig. 7 Effect of varying acyl donors on conversion of acyl donor and 4-nitrophenol (100 mM each) into 4-nitrophenyl ester in *n*-heptane at 65°C in 3 h

Synthesis of other 4-nitrophenyl esters using molecular sieve immobilized lipase

Under optimized conditions for synthesis of 4-nitrophenyl acetate, the conversion of 4-nitrophenol and palmitic acid to 4-nitrophenyl palmitate and 4-nitrophenol and stearic acid to 4-nitrophenyl stearate was carried out. Lower % conversion was observed for 4-nitrophenyl palmitate and 4-nitrophenyl stearate as compared to 4-nitrophenyl acetate (Fig. 7).

When the diffusion parameter is small then the rate of chemical reaction at the polymer sites will be slow and the substrate will diffuse through most of the polymer film before it undergoes depletion due to chemical reaction at the sites [26]. Since stearic acid and palmitic acid are bulkier than acetic acid, diffusion and hence the accessibility of the substrate to the catalytic site of the enzyme probably reduced the rate of reaction and therefore % conversion.

Effect of water on lipase catalyzed esterification

Under previously optimized conditions, % conversion of acetic acid and 4-nitrophenol to 4-nitrophenyl acetate was

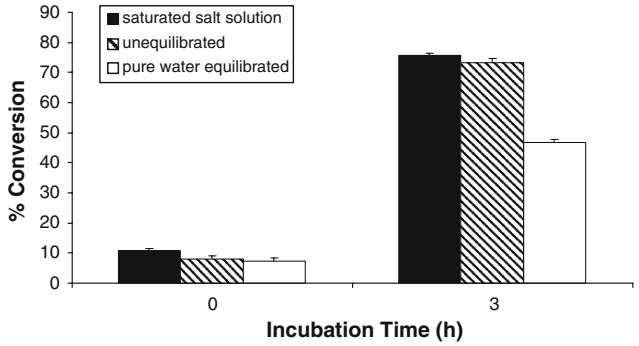

Fig. 8 Effect of water on lipase catalyzed conversion of acyl donor and 4-nitrophenol (100 mM each) into 4-nitrophenyl ester in *n*-heptane at 65°C in 0 h and 3 h

compared at three different conditions of water activity. Reaction system which was equilibrated using pure water had 68.6 and 61.6% of esterification as compared to systems equilibrated with saturated salt solution at 0 and 3 h, respectively (Fig. 8). However, there was not significant difference in % conversion of unequilibrated and salt solution equilibrated reaction system since water activity might have come to a similar level after 3 h of incubation as significant amount of stoichiometric water is released during esterification. Later, in the reaction possibly high water activity due to stoichiometric water released reversed the synthesis reaction to hydrolysis reaction without affecting the activity of immobilized lipase activity. The direct participation of water has been invoked to account for the decline in initial rate of esterification reactions above the optimal water content [44] or aw [35]. Lipase from *C. antarctica* foster better enantioselectivity and maximum reaction rate at aw = 0.1 [31]. *C. antarctica* lipase depends strongly on initial water activity. Conversion yield of fructose and initial rate of fructose monopalmitate synthesis were raised, respectively, to 73.4% (32 g l^{-1}) and 10.1 g l^{-1} h^{-1} from 28.5% and 4.9 g l^{-1} h^{-1} on reducing water activity <0.07 [7].

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