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Article

Covalent immobilization of *Candida antarctica* lipase B on nanopolystyrene and its application to microwave-assisted esterification

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ABSTRACT

Nanopolystyrene was used as a solid support for the covalent immobilization of *Candida antarctica* lipase B (CalB) using the photoreactive reagent 1-fluoro-2-nitro-4-azido benzene (FNAB) as a coupling reagent. The obtained derivative was then used as a biocatalyst in a microwave assisted esterification experiment. Factors such as contact time, pH, and enzyme concentration were investigated during immobilization. The hydrolytic activity, thermal, and operational stability of immobilized-CalB were determined. The maximum immobilized yield (218 µg/mg support) obtained at pH 6.8 exhibited optimum hydrolytic activity (4.42×10^3 mU *p*-nitrophenol/min). The thermal stability of CalB improved significantly when it was immobilized at pH 10, however, the immobilized yield was very low (93.6 µg/mg support). The immobilized-CalB prepared at pH 6.8 and pH 10 retained 50% of its initial activity after incubation periods of 14 and 16 h, respectively, at 60 °C. The operational stability was investigated for the microwave assisted esterification of oleic acid with methanol. Immobilized-CalB retained 50% of its initial activity after 15 batch cycles in the microwave-assisted esterification. The esterification time was notably reduced under microwave irradiation. The combined use of a biocatalyst and microwave heating is thus an alternative total green synthesis process.

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1. Introduction

The use of enzymes as a substitute for metal catalysts is rapidly gaining momentum because of their ability to work under mild conditions such as neutral pH, low temperature and pressure [1]. Lipases (E.C. 3.1.1.3) constitute a versatile group of enzymes that can hydrolyze and synthesize the esters of fatty acids. *Candida antarctica* lipase B is a well-known biocatalyst because of its widely used application in esterification, hydrolysis, aminolysis, and transesterification [2]. The use of enzymes in a non-natural environment requires immobilization to im-

prove their stability, activity, and reusability.

Several methods are available for the immobilization of proteins onto solid supports and include adsorption, and covalent attachment cross-linking, adsorption followed by cross-linking, and physical entrapment using commercial carriers [3–5]. In a commercially available preparation (Novozym 453), CalB is immobilized onto the resin (Lewatit OC 1600) and this is a macroporous, divinylbenzene-crosslinked polymer based on methacrylic ester. The leaching of enzymes and potential acyl donors from Novozym 453 has been confirmed in organic solvents and in ionic liquid media [6,7]. High costs and diffusion

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limitations are also constraints in the application of Novozym 435 [6]. These issues can be circumvented by the covalent immobilization of CalB onto solid supports [4].

The covalent attachment of enzymes onto an active support can take place through amino acid residues such as amino groups from lysine or arginine, carboxyl groups from aspartic acid and glutamic acid, hydroxyl groups from serine and threonine and sulfhydryl groups from cysteine [8]. However, the amine groups of proteins are the most commonly used moieties for covalent immobilization. These residues are present in most proteins. Amine groups are usually found on the surfaces of proteins and are very reactive, without any previous activation, toward most reactive groups on supports [4,9,10]. Generally, the covalent immobilization of enzymes is accomplished in three successive steps: the initial activation of supports followed by activated support modification and finally the coupling of the support to enzymes.

Unlike other lipases, most of the CalB surface is hydrophobic in nature. Retaining the active conformation is very important for enzyme activity. Although free CalB is active in a pH range from 6–10, it retains its active conformation at its isoelectric point (pH 6.8). Among the various functional groups available for CalB immobilization, the primary amine on the side chain of lysine is readily available for chemical attachment. At the isoelectric point (pH 6.8) this terminal amine is located at the hydrophobic outer surface of the tertiary structure of the protein, opposite to the active site and this is an easy target for immobilization. Therefore, immobilizing at pH 6.8 may favor the immobilization of CalB in its active conformation. The higher pK_a value (~ 10) of the lysine residue offers an opportune pH for the promotion of covalent bonding between CalB and the support. As the lysine residue is more active at pH 10, covalent immobilization can be strongly influenced. Several authors have reported that these two pH values can be used for covalent immobilization [11–13]. Furthermore, FNAB mediated nucleophilic substitution reactions are much quicker in alkaline media. Therefore, pH 6.8 and pH 10 were selected for the covalent immobilization of CALB onto FNAB activated nanopolystyrene.

In the last decade, nanomaterials such as nanofibers, nanotubes, and nanoparticles have been recognized as potential supports for the development of nanobiocatalytic systems [14–16]. Interest is increasing in the use of polymeric nanoparticles as solid supports because of their very large surface areas, as well as good mechanical and physical properties for enzyme immobilization [17,18]. FNAB activated nanopolystyrene prepared by nanoprecipitation might be an economical and potential candidate for the covalent immobilization of CalB.

The application of microwave heating in biocatalysis is rapidly gaining attention and it is an environmentally friendly alternative to convection heating [19,20]. Methyl oleate (fatty acid methyl ester) is biodegradable, renewable and non-toxic and is used in numerous applications including detergents, wetting agents, intermediate stabilizers, food, cosmetics, pharmaceuticals, emulsifiers and more importantly as biofuel [21,22]. Several by-products, pollution, product contamination, separation of esters, formation of soap because of the presence of free fatty acid, and equipment corrosion are the main con-

straints of the acid and alkali catalysts used in the synthesis of methyl oleate [21,23]. Therefore, the combination of green biocatalysts and microwave heating in methyl oleate synthesis can offer advantages in terms of recovery and reuse of the catalyst, shorter reaction times, efficient downstream processes and minimizing product contamination.

To the best of our knowledge, nanopolystyrene has not been investigated for the covalent immobilization of CalB. FNAB was thus used in this study to activate the polymeric support for the covalent immobilization of CalB. For this purpose nanopolystyrene was prepared by nanoprecipitation. The influence of contact time, lipase concentration, and pH of the lipase solution during the immobilization step on biocatalyst activity and stability were investigated. The photolinker (FNAB), nanosupport and derived biocatalyst were characterized by field emission scanning electron microscope (FESEM), Fourier transform infrared (FTIR), bovine serum albumin (BSA), protein assay, and hydrolytic activity. The immobilized CalB was used to synthesize methyl oleate under microwave heating.

2. Experimental

2.1. Materials

Polystyrene (M_w , 920000), *Candida antarctica* lipase B (CAS NO. 9001-62-1) in powder form, Novozym 435 (L4777 SIGMA), *p*-nitrophenyl acetate (*p*-NPA) and oleic acid were purchased from Sigma-Aldrich. Analytical grade methanol, tween-40, 4-fluoro-3-nitroaniline, NaNO_2 , NaN_3 were purchased from Merck. BSA and Coomassie Brilliant Blue reagent were purchased from Thermo Scientific. All solvents and reagents used were of analytical grade. Each experiment was performed in triplicate and mean values are reported.

2.2. Preparation of polystyrene nanoparticles (nanopolystyrene)

The enzyme support, nanopolystyrene, was prepared by nanoprecipitation of the preformed polymer using the method reported by Bukhari et al. [24]. The polymer phase was prepared in tetrahydrofuran by dissolving polystyrene 0.0045 g/mL in the dispersion phase via a syringe pump with continuous stirring at 600 rpm. Once the nanoprecipitation was complete, nanopolystyrene was recovered and purified using a Sepharose Cl-4B gel packed column. The nanoparticles were lyophilized at -40°C for 48 h. The size and shape of the nanoparticles were observed using FESEM (Supra 35 VP). Samples were prepared on a metal slab and sputtered with gold.

2.3. Synthesis of the coupling agent (FNAB)

FNAB was freshly prepared by the diazotization of 4-fluoro-3-nitroaniline according to the literature [25]. Typically, 5.0 g of 4-fluoro-3-nitroaniline was dissolved in a warm mixture of 30 mL concentrated HCl (37%, w/v), and 5 mL distilled water. NaNO_2 (2.4 g) was dissolved in 5 mL water and the solution was introduced slowly into the mixture. NaNO_2 (2.4 g) was

added slowly into the solution at -20 to -15 °C. Subsequently, NaN_3 (2.2 g) was dissolved in 8 mL distilled water and added into the solution at -20 °C. The orange FNAB precipitate was recovered and recrystallized. FNAB was characterized by FTIR and stored at -4 °C in a vial wrapped with aluminum foil.

2.4. FNAB coating and UV activation of nanopolystyrene

A concentrated solution (15 $\mu\text{mol/mL}$) of FNAB was prepared in methanol. FNAB and nanopolystyrene were mixed in a 1:1 (w/w) ratio and the methanol was allowed to evaporate in the dark at room temperature for 24 h. The FNAB coated nanopolystyrene was then subjected to UV irradiation for 4 min in a UVACUBE-400 and subsequently washed 4 times with methanol to remove the FNAB physically adsorbed on the nanoparticles, vacuum dried and stored at -4 °C until further use. FNAB activated nanoparticles were characterized by FTIR.

2.5. Covalent immobilization of *Candida antarctica* lipase B on activated nanopolystyrene

CalB was immobilized by covalent coupling onto activated nanopolystyrene at room temperature. FNAB coated particles were wetted with ethanol just before mixing. For covalent immobilization, we investigated the more effective pH values of 6.8 (isoelectric point) and 10 [4]. The effect of enzyme concentration on immobilized yield and hydrolytic activity were investigated using various support to enzyme ratios (4:1, 4:1.5, 4:2, 4:2.5) in phosphate buffer (2.5 mg CalB/mL in 100 mmol phosphate buffer) at pH 6.8 or pH 10. The effect of contact time on the enzyme yield and the activity between enzyme and support were investigated between 1 and 4 h. Upon the completion of the immobilization reaction the suspension was centrifuged at 3000 rpm for 15 min to separate the biocatalyst. The derivative was rinsed with the buffer (10 mL). The immobilized enzyme particles were lyophilized for 24 h and stored at -4 °C for further use. FESEM, FTIR and BSA assays were used to characterize the support and the immobilized CalB.

2.6. Determination of enzyme loading

Enzyme loading was determined using the method reported in literature [26]. The supernatant and washings were collected and subjected to protein assays according to Bradford's method [27] using BSA as the standard and Coomassie Brilliant Blue reagent. A BSA standard curve was constructed by plotting various concentrations of BSA against the absorbance data collected at 595 nm using a spectrophotometer (Shimadzu Mini UV 1241). The percentage of immobilized yield (immCalB) was determined using Eq. (1).

$$\text{immCalB} = \frac{\text{CalB}_{\text{int}} - \text{CalB}_{\text{rem}}}{\text{CalB}_{\text{int}}} \times 100 \quad (1)$$

Where CalB_{int} represents the amount of lipase added initially for immobilization and CalB_{rem} represents the leftover lipase in the washings and the supernatant in μg .

2.7. Hydrolytic activity assay

The activities of the free or immobilized CalB were determined by the hydrolytic rate of *p*-NPA in toluene at 35 °C and 250 rpm in a water bath [28]. The free or immobilized CalB (1.5 mg) was added to a 5 mL vial containing 3 mL toluene, 30 mg *p*-NPA and 10.5 mg methanol. An activity assay reaction was performed over 50 min while the vials were agitated horizontally during the reaction. The reaction was terminated by the removal of the immobilized biocatalyst using centrifugation. Samples (20 μL) were withdrawn and were diluted with 3 mL toluene. A 3.5 mL quartz cuvette was used for absorbance measurements in the spectrophotometer. The amount of *p*-nitrophenol (*p*-NP) produced was determined using the spectrophotometer (λ_{max} at 304 nm of *p*-NP). The concentration of *p*-NP produced was calculated using a *p*-NP standard curve. The activity of the free and immobilized CalB is given in milli international units (mU) (1 mU is the amount of free or immobilized CalB required to convert 1 millimole of substrate per unit time). All the experiments were performed in triplicate to ensure the reproducibility of the results.

2.8. Determination of the thermal stability and half-life of the immobilized CalB

The thermal stability and half-life are important parameters for the evaluation of enzyme immobilization. The half-life of the prepared lipase is defined as the time at which the activity of the enzyme is reduced to half its initial activity. The thermal stability of the free or immobilized CalB was determined by incubating in 0.1 mol sodium phosphate buffer at pH 7.0 and 60 °C. The samples were withdrawn at regular intervals and the residual activity was determined by the hydrolysis of *p*-NPA, as described earlier. Residual activity is given as a percentage of initial activity, which is 100% of the hydrolytic activity of the immobilized enzyme before incubation. The residual activity was determined by the hydrolysis of *p*-NPA as described in the hydrolytic activity assay section.

2.9. Immobilized CalB catalyzed esterification under microwave and conventional heating: methyl oleate synthesis

The esterification potential of the derivative was investigated under microwave and conventional heating for methyl oleate synthesis. Experiments were performed under the same conditions but with different modes of heating.

Conventional heating: oleic acid and methanol were mixed in a specific molar ratio (1:3) in a 125-mL Erlenmeyer flask, cotton plugged [29] and heated in a water bath. The amount of immobilized CalB was 6% of the oleic acid mass. Methanol was used in excess (3 moles) to drive the reaction forward. To minimize enzyme inactivation risks because of excess methanol, it was added in stages using three equal feeds [29,30].

Microwave reaction: for microwave esterification, a three-necked 100 mL flask was used in a MAS-II synthesis system. In the tunable wattage microwave system, the MAS-II synthesis was carried out at 200 W, and the temperature was measured directly using an infrared (IR) sensor. Oleic acid and methanol were mixed in a specific molar ratio (1:3) in the

three-necked 100 mL flask and heated in the MAS-II synthesis system. Similarly, the amount of immobilized CalB was 6% of the oleic acid's mass and methanol was used in excess (3 moles) to drive the reaction forward. Methanol was again added in three equal feed stages to minimize enzyme inactivation risks.

Both the esterification reactions were performed at various temperatures ranging from 30 to 60 °C at 200 rpm to study the influence of temperature on the reaction. The effect of time on the synthesis of methyl oleate was also investigated and the reaction time for conventional heating was varied from 10–80 min. The consumption of oleic acid was measured using a 0.1 mol/L alcoholic KOH titration and phenolphthalein was the indicator in accordance with the standard method described in ISO 1242 (International standard, Essential oils Determination of acid value ISO 1242 (1999) E). The total acid content of the blank sample was determined before the reaction began by alcoholic titration. The esterification yield and percentage conversion were calculated from the decrease in oleic acid concentration after a predetermined reaction time.

2.10. Operational stability

The operational stability of immobilized CalB was determined using successive batches of methyl oleate synthesis under the same conditions as those used for the synthesis of methyl oleate. At the end of each batch reaction, immobilized CalB was separated from the reaction medium by centrifugation (3000 rpm for 20 min), washed with *n*-hexane and dried at room temperature. After 1 h it was reused for the next batch. The residual activity of immobilized CalB was calculated as a percentage of the activity of the immobilized enzymes measured after each cycle, and this was compared with the activity of the immobilized enzymes before the first cycle (considered to be 100%).

3. Results and discussion

3.1. FTIR and FESEM characterization of the nanosupport

Figure 1 shows that nanopolystyrene bead supports for the immobilization of CalB were successfully prepared by nanoprecipitation. The FESEM image shows that most of the parti-

Fig. 1. FESEM micrograph of nanopolystyrene prepared by nanoprecipitation. The sample was prepared by sputtering with gold.

Fig. 2. FTIR spectrum of FNAB functionalized nanopolystyrene from 370–4000 cm^{-1} .

cles are in the 100–200 nm size range. The physical and chemical structure of the support determines the immobilized yield and activity of the enzymes. FNAB-activated nanopolystyrene contains fluoride aryl groups that are suitable for the immobilization of CalB.

Figure 2 gives a characteristic FTIR spectrum of FNAB-activated nanopolystyrene. Most of the peaks can be assigned to the stretching and bending vibration modes of aromatic rings in FNAB and polystyrene, and they overlap. However, two dominant new peaks at 1540 and 3081 cm^{-1} are present in the spectrum shown in Fig. 2. These peaks originate from a combination of C–N stretching and N–H bending vibrations, which establishes that photoactive linkages exist between FNAB and nanopolystyrene.

The FTIR spectrum of free CalB and immobilized CalB on activated nanopolystyrene are given in Figs. 3 and 4, respectively. In Fig. 3 the broad peak at 3410 cm^{-1} indicates the presence of a number of amine ($-\text{NH}_2$) groups in free CalB. By comparing the spectrum of activated polystyrene (Fig. 2) with immobilized CalB (Fig. 4), it is clear that the immobilized derivative can be distinguished by its three characteristic peaks at 1651, 3420, and 3150 cm^{-1} , and these come from an amine bond (between the support and the lipase) and intermolecular bonding in the protein [31,32]. An activation peak at 3081 cm^{-1} is present after immobilization. Because of the amine linkage between the support and CalB, the characteristic free CalB peak at 3410 cm^{-1} shifts to 3420 cm^{-1} . The $-\text{NH}_2$ group from the lysine of CalB likely attacked the aromatic carbon attached to fluorine, re-

Fig. 3. FTIR spectrum of free CalB.

Fig. 4. FTIR spectrum of immobilized CalB on FNAB-activated nanopolystyrene.

sulting in a nucleophilic substitution reaction. Therefore, HF was displaced and the amine linkage developed as shown in Fig. 4. From the FTIR results, FNAB activation and CalB covalent immobilization took place successfully.

Scheme 1 is a schematic of the activation of polystyrene and the chemical attachment of CalB onto the active support. It shows the mechanism involved in the photoactivation of nanopolystyrene surface and its covalent binding with CalB. Upon exposure to UV irradiations, FNAB coating of nanopolystyrene generates the highly reactive nitrene group, which subsequently reacts with nanopolystyrene and creates an amine linkage with release of nitrogen. The functional group (F-aryl) remained intact in the FNAB coated nanopolystyrene which later can create the amine linkage. The FNAB mediated photoactivation (UV irradiation) of polystyrene is simple and permits the activation within few minute unlike the thermochemical or nitration of aromatic ring activation techniques which will take several hours.

Scheme 1 also explains the mechanism involves in the covalent immobilization of CalB on activated support. The proposed type of reaction involved in covalent immobilization of CalB was the nucleophilic substitution reaction. Due to higher electronegativity of fluorine, the carbon attached to it was partly electron deficient and highly susceptible to nucleophile attack.

Scheme 1. Schematic of the immobilization of CalB onto FNAB-activated nanopolystyrene.

Nucleophilic substitution reaction occurred during the incubation of the mixture of CalB and activated support. In this reaction, the amine (-NH_2) group of CalB most likely from lysine attacked the aromatic carbon attached to the fluorine of activated support. As a result amine linkage (covalent bonding) between the CalB and active support was established with removal of hydrogen fluoride (HF) as depicted schematically in Scheme 1. It is a simple and straightforward technique for the covalent attachment of CalB on activated nanopolystyrene to enhance the stability and circumvent the enzyme leaking issues.

3.2. Effect of pH, contact time and enzyme concentration on the immobilized yield

The pH undoubtedly affected the enzyme activity and thus influenced the enzyme protocol (in terms of immobilization yields and enzyme stability). For the evaluation of various concentrations of enzyme relative to the support (w/w) that were incubated for 3 h, we found that the immobilized yield increased as the enzyme concentration increased. Figure 5 shows the influence of pH and enzyme concentration on the immobilization yield. Increasing the support:enzyme ratio from 4:1 to 4:2 (w/w) enhanced the immobilized yield at both pH values. The maximum immobilized yield was obtained at a support:enzyme ratio of 4:2 (w/w). A further increase beyond 4:2 did not improve the immobilized yield. However, the maximum immobilized yield was obtained at pH 6.8 after 3 h.

Figure 6 shows the immobilized yield as a function of contact time at a support:enzyme ratio of 4:2, which was found to be the optimum concentration. The maximum enzyme yield was achieved after 1 and 2 h for pH 6.8 and 10, respectively. Increasing the contact time further did not improve the immobilized yield.

The activated nanopolystyrene was expected to accommodate more enzyme molecules. The active group density on the support was almost constant, however, the yield of immobilized enzymes was higher at pH 6.8. The lower immobilized yield at pH 10 can be explained by the multipoint attachment of an enzyme molecule to the support surface. Although pH 6.8 was found to impart maximum activity on CalB, the experiment

Fig. 5. Effect of support:enzyme ratios (w/w) on the immobilized yield at pH 6.8 and pH 10 at constant contact time (3 h), agitation speed (200 rpm) and at 30 °C.

Fig. 6. Effect of contact time on the immobilization yield at pH 6.8 and pH 10 at a constant agitation speed (200 rpm), 30 °C, and at a support: enzyme ratio of 4:2 (w/w).

was also performed at pH 10 because it has been hypothesized that the activity of lysine increases at an alkaline pH, which improves the immobilized yield. However, the immobilization yield was found to decrease at pH 10. This was because the nucleophilic substitution reaction was probably more efficient at higher pH [33], which subsequently resulted in multiple attachments to 1 molecule of CalB. These multiple attachments resulted in low immobilization yields. However, at pH 6.8 the FNAB activated supports only produced 1–2 attachments in CalB. It has been reported that a higher intensity of covalent linkages between the support and the enzyme allows only a small number of molecules to bind with the support [4]. The immobilization of CalB at pH 6.8 was not due to a single attachment as the enzyme only immobilizes on the support when at least two simultaneous enzyme-support attachments are produced [34].

3.3. Hydrolytic activity of free and immobilized CalB

The activity of free and immobilized CalB was investigated by the hydrolysis of *p*-NPA and calculated in mU under the conditions mentioned above. Table 1 shows that as the enzyme yield increases, its derivatives exhibit higher hydrolytic activity. The derivatives prepared at pH 6.8 show higher hydrolytic activities than those prepared at pH 10. The pH undoubtedly affected enzyme activity and thus influenced the enzyme pro-

Table 1

Effect of immobilized pH and yield on the hydrolytic activity of CalB immobilized on nanopolystyrene determined by the hydrolysis of *p*-NPA in toluene at 35 °C and 250 rpm in a water bath.

Immobilization pH	Immobilized yield (μg/mg)	Hydrolytic activity (mU/min/mg) ($\mu\text{mol} \times 10^{-3}$)
6.8	124	2.453
	173	3.523
	219	4.420
	220	4.396
10	63	1.237
	83	1.603
	93	1.856
	94	1.796

ocols (in terms of immobilization yield and enzyme stability). Many reports [4,10,26] have revealed that enzyme (CalB) activity increases at pH 6.8 or near the isoelectric point, and we found that the immobilized yield and stability was significantly influenced under alkaline pH conditions. At pH 6.8 CalB likely retained its active conformation during immobilization. However, when immobilizing at the higher pH of 10, immobilization did occur but the catalytic efficiency was low because of structural changes. Therefore, we conclude that the lower pH of 6.8 has a positive influence on the catalytic activity of immobilized CalB on FNAB-activated nanopolystyrene. Similar pH effects for buffer solutions during covalent immobilization on various supports have been described in the literature [4,35]. However, activity is not the only criteria when designing industrial biocatalysts because thermal and operational stabilities are equally important.

3.4. Thermal stability and reusability

The thermal stability of free and immobilized derivatives was determined at different time intervals upon incubation at 60 °C in 0.1 mol phosphate buffer at pH 7.0 for 20 h. Figure 7 shows that free CalB loses its activity after 2 h of incubation. The immobilized CalB obtained at pH 6.8 and 10 retained 92% and 95% of its initial activity after 2 h, respectively. After 10 h of incubation the activity of CalB at pH 6.8 and 10 decreased to 65% and 80%, respectively. The CalB obtained at pH 6.8 and 10 retained 50% of its initial activity over incubation periods of 14 and 18 h, respectively. Because the covalent attachment of enzymes to the support directly influences the thermal stability of the derivative [36], the difference in the thermal stability profile comes from the different degrees of derivative attachment. These results indicate that the CalB immobilized on nanopolystyrene is more stable at high temperature than free CalB. Therefore, nanopolystyrene is a compatible support that improves the thermal resistance of conjugated CalB. The covalent attachment of CalB to the FNAB-activated surface of nanopolystyrene restricts the conformational transition of the enzyme, resulting in the reduction of mobility and in the prevention of enzyme distortion at high temperature. Therefore, immobiliza-

Fig. 7. Thermal stability of free and immobilized CalB on nanopolystyrene, incubated at 60 °C for 20 h. Activity was determined by the hydrolysis of *p*-NPA in toluene at 35 °C and at 250 rpm in a water bath.

tion protects CalB from thermal deactivation [4]. The enzyme yield and hydrolytic activity of the derivative prepared at pH 10 was too low for practical applications. Therefore, CalB immobilized at pH 6.8 was selected for esterification experiments.

3.5. Operational stability

The reusability of the immobilized enzymes is important in practical applications because of the costs associated with enzymes. The residual activity was measured after each successive batch of esterification experiments and is presented in terms of percentage activity compared with the activity of the immobilized enzyme before the first cycle. Figure 8 shows the effect of the repeated use of immobilized CalB on its residual activity. It is clear that the immobilized CalB prepared at pH 6.8 retained 90% of its esterification activity after 5 cycles. After 10 consecutive batches, the immobilized CalB retained 64% of its initial activity. We found that the activity of the preparation decreased to 50% after 15 consecutive cycles. The decrease in catalytic efficiency of immobilized CalB can be explained by the denaturation of protein upon repeated use. The elution of the minor amount of residual surface adsorbed enzymes can also result in a decrease in activity over the first few cycles [37]. The retention of 50% activity after 15 cycles is of great interest and is advantageous in practical applications.

4. Synthetic activity of immobilized CalB

4.1. Effect of enzyme concentration on esterification

The effect of immobilized CalB concentration on ester synthesis is important considering the cost of production. The immobilized CalB and Novozym 435 concentrations were varied from 4%–10% (by weight of oleic acid) in a methanolysis process. Figure 9 shows the observed effect of lipase concentration on the methanolysis. An increase in the amount of immobilized CalB from 4% to 6% increased the ester yield. We found that more than 6% immobilized CalB had no effect on oleic acid conversion. The maximum conversion of oleic acid was found for 8% Novozym 435 and this was equal to that of 6% immobilized CalB. Unlike Novozym 435, the increased degree of con-

version is likely due to the improved yield of immobilized CalB. The methyl ester yield increased upon increasing the lipase concentration but only up to a certain optimum range. Increasing the immobilized CalB concentration beyond 6% does not promote the esterification of oleic acid and will only result in wastage and higher costs.

4.2. Effect of temperature and mode of heating on immobilized CalB and Novozym 435 catalyzed esterification

The synthetic activity of immobilized CalB was investigated for the preparation of fatty acid methyl ester at different temperatures (30–60 °C). The mode of heating strongly influenced the stability and activity of the biocatalysts. Figure 10 shows the effect of temperature and mode of heating (conventional or microwave heating) on the esterification of oleic acid as a standard reaction for ester production and this was catalyzed by immobilized CalB and Novozym 435. The conversion rate of oleic acid into methyl oleate increased with an increase in temperature up to an optimum conversion at 45 °C for both heating methods.

The optimum stability and activity of enzymes are normally affected by the applied temperature, type of immobilization and support, solvents, and mass transfer rate. The optimum conversion of oleic acid by immobilized CalB on nanopolystyrene was found to be 68% and 66% at 45 °C under microwave and conventional heating, respectively. The reaction time was reduced significantly under microwave heating. By comparison, 65% and 63% conversions were achieved by Novozym 435 using dielectric and conventional heating, respectively. The difference is in the optimum biocatalysts concentration because 6% immobilized CalB achieved a higher conversion than the 8% Novozym 435. Unlike Novozym 435, CalB immobilized on nanopolystyrene is more exposed to microwave irradiation resulting in activity differences. The activities of both biocatalyst systems are comparable. Furthermore, the effect of the prevailing microenvironment on the stability and activity of the biocatalysts cannot be ignored. Moreover, the mechanism of microwave heating involves a polar interaction between microwave irradiation and the reactants and this can increase the

Fig. 8. Residual activity of immobilized CalB relative to its initial activity (100%) determined by the esterification of oleic acid with methanol in a mole ratio of 1:3 at 45 °C with an enzyme concentration of 6% (w/w) in oleic acid.

Fig. 9. Effect of immobilized CalB and Novozym 435 concentration on the degree of oleic acid conversion upon esterification at 45 °C in a water bath at 250 rpm for 80 min.

Fig. 10. Effect of heating modes and temperatures on the degree of oleic acid conversion. (a) 6% (w/w) immobilized CalB in oleic acid for 1 h, oleic acid:methanol (1:3); (b) 8% (w/w) Novozym 435 in oleic acid for 1 h, oleic acid:methanol (1:3).

number of collisions between the enzymes and the substrate. These collisions might help in enzyme-substrate complex formation and thus increase enzyme activity. The enzymes can oscillate more upon interacting with microwave irradiation to relieve steric repulsion and hence contribute to the rate acceleration.

Unlike conduction heating, microwave irradiation can result in the rearrangement of protein moieties, which can cause the loss of enzyme activity [38]. This effect is more prominent when enzyme immobilization interactions are weaker. In addition to the stabilization of CalB under conventional heating, we found that the covalent immobilization of CalB on nanopoly-styrene also provided stability under dielectric heating. The optimum temperature for the esterification of oleic acid with methanol using covalently immobilized CalB was found to be 45–50 °C. For most free lipases, the optimum working temperature has been reported to range from 30–37 °C [39], and in our work the immobilized CalB was stable beyond 50 °C. This stability of the biocatalyst is a result of the immobilization of CalB on activated nanopolystyrene.

In a recent study, it was reported that dielectric heating enhanced the speed of enzymatic reactions and allowed uniform heating [40] without affecting enzyme operating conditions such as temperature, stability, and specificity toward its substrate. Rejasa et al. [41] reported that immobilized CalB was

stable under microwave irradiation and that it exhibited enhanced stability and activity. The stability of immobilized CalB under dielectric heating is a step towards a green synthesis process.

4.3. Effect of heating time on esterification

The esterification reaction was performed over 70 min at the optimum temperature (45 °C) and the optimum enzyme concentration (6% immobilized CalB and 8% Novozym 435) under a constant stirring rate and with both microwave and conventional heating. The acid value index and percentage ester conversion for both these methods are shown in Fig. 11. Samples were taken at regular intervals and analyzed for the degree of conversion. We found that the ester conversion increased with the reaction time. It is clear from Fig. 11 that the highest ester conversions were achieved at 60 and 30 min with values of 67% and 70% under conventional and microwave heating, respectively. A further increase in the reaction time did not improve ester production. It is interesting to note that the optimum time for the microwave assisted reaction using immobilized CalB was only half that of the conventional optimum time. By comparison, an optimum conversion of 68% was found for Novozym 435 at 40 min. Under microwave heating the time taken by Novozym 435 for esterification was longer

Fig. 11. Effect of time and mode of heating on esterification at 45 °C, at an immobilized CalB content of 6% (w/w) in oleic acid at a mole ratio of oleic acid: methanol of 1:3. (a) Under microwave heating; (b) under conventional heating.

than that of immobilized CalB. This possibly occurred because Novozym 435 is deeply embedded in the matrix. Therefore, only a portion of the Novozym 435 was exposed to microwaves thus affecting the esterification time significantly. In 2012, Di et al. [42] reported 75% oleic acid conversion in 4 h when using a $P_2O_5/CaCl_2/Fe_3O_4$ composite as the catalyst. However, the recovery and reusability of the catalysts were not reported. Recently, Li et al. [43] claimed that a 2-h reaction time was required for oleic acid esterification when using sulfonated rice husk char as a solid acid catalyst. The authors reported 110 °C as the optimum operating temperature and 5% solid catalysts as the optimum amount of catalyst. Unlike previous findings, the reaction time was significantly reduced to 30 min for oleic acid esterification under microwave heating. In addition, the immobilized CalB esterification temperature decreased remarkably to 45 °C. Thus, in terms of energy consumption, reaction time and environmental issues, the enzyme mediated esterification of FFA under microwave heating is advantageous. Immobilized CalB can be recovered easily upon the termination of the reaction and it can be used repeatedly. We found that upon using the prepared biocatalysts for 10 successive batches they retained 64% of their residual activity. Thus, the combined use of immobilized enzymes and microwave heating in esterification is an efficient and environmentally friendly alternative to conventional heating and catalysts.

5. Conclusions

We conclude that fluoride aryl activated nanopolystyrene is a suitable support for CalB immobilization. This protocol has advantages such as a straightforward immobilization proce-

dure, improved enzyme yield, enhanced enzyme activity and stability for multiple reactions. Using this technique, enzyme rigidity was improved and the conformational changes induced by heat and microwave interactions were minimized. This approach can generally be applied to the immobilization of other enzymes. In particular, immobilized CalB can be used for the rapid esterification of oleic acid when using microwave heating. The rate of esterification is faster than that found in conventional heating. Biocatalysis under microwave irradiation offers a new opportunity for green manufacturing. Additionally, this nanobiocatalytic system can be employed in a batch reactor and also in a continuous reactor in addition to other types of reactors.

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Graphical Abstract

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Covalent immobilization of *Candida antarctica* lipase B on nanopolystyrene and its application to microwave-assisted esterification

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CalB was covalently immobilized on FNAB-activated nanopolystyrene to improve its stability, activity and reusability in applications such as esterification. The esterification time was significantly reduced and the yield was increased when using immobilized CalB and microwave heating.

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