Research Article

Activity and Stability of Lipases Immobilized onto Acetylated Bacterial Cellulose

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Abstract
Bacterial cellulose (BC) materials were used for lipase immobilization to improve enzyme activity and stability. BC films produced by *Komagataeibacter xylinus* were first acetylated in an acetic anhydride/iodine system to convert their OH groups to more hydrophobic acetyl groups. Activity yield (44.4%) and maximum specific activity (12.44 μmol mg^{-1} min^{-1}) were achieved when 400 mg of BC was acetylated in 20 mL of acetic anhydride containing 0.275 mM of iodine. Studies on the catalytic activity of lipase also show that the immobilization of lipase on acetylated BC (ABC) films significantly enhanced its tolerance to temperature and pH. Immobilized lipases retained 89% and 56% of their catalytic activities after being incubated at 60 °C and 80 °C for 1 h, respectively; while those of free lipases significantly decreased to 24% (60 °C) and only 11% (80 °C). Immobilized lipases incubated at pH 5.0 and pH 10.0 for 24 h also retained high catalytic activities (70% and 82%, respectively), considerably higher than those of free lipases (19% - pH 5.0 and 63% - pH 10.0). Tolerance to organic solvents, such as *n*-hexane, acetone, ethanol, isopropanol of ABC-immobilized lipase was also improved. The immobilization of lipase on ABC films significantly improved its reusability and storage stability: ABC-immobilized lipase still could be reused for 30 cycles with residual activities of more than 90%, and still retained 95% of its early activity after 15-day storage at 4 °C. This implies that ABC-immobilized lipase is potentially applied in food, medicine, biodiesel and detergent industries.

Keywords: *Komagataeibacter xylinus*, Bacterial cellulose, Acetylation, Lipase, Immobilization

1 Introduction

Enzymes, the powerful biocatalysts, have been found numerous applications in food production, biomedical assays, detection technology and industrial processes [1]. Among enzymes used in industries, lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) stand out due to their broad substrate specificity, high selectivity, commercial accessibility, and cofactor independence [2]. Especially, lipases are interfacial enzymes, performing catalytic activity at the interface between lipid and water. This unique feature of many lipases is attributed to the presence of a flexible subdomain called a lid located over the active site. In the closed conformation, this lid blocks the access of substrates to the active site. In the open form, substrates can freely access to lipase active site and be converted [3]. In addition to natural hydrolytic activity, lipases also catalyze many reactions in organic solvents such as esterification, transesterification and aminolysis [4]. Therefore, lipases have been widely used in various food sectors, such as fats and oils industry, dairy industry, meat processing, egg processing or baking [5]. Owing to the outstanding characteristics and

diverse applications, lipases have become a model for enzyme immobilization to seek for a cost-effective solution for the improvement of operational stability and the enhancement of enzyme recovery and reuse. A frequent immobilization method used for enzymes with catalytic mechanisms similar to lipase is to immobilize them on hydrophobic materials via an interfacial activation mechanism [6]. The hydrophobic interaction between the surface of supports and the lid of lipases could induce conformational change in lipases necessary for the accessibility of substrates to their active sites [6].

Bacterial cellulose (BC) is a bio-nanomaterial that has attracted considerable interest in recent years [7]. BC is mainly produced by bacteria from the genera *Acetobacter* and *Komobacter*, and has been long used in food industry as the raw material for the production of Nata de Coco and similar products [8]. Besides, BC can be applied as a fat replacer in meatballs and surimi [9], or as a stabilizing, thickening, or gelling agent in foods [8], [10]. Especially, due to its high purity as compared to plant-based cellulose, high degree of crystallinity, high mechanical strength and biocompatibility [8], BC materials have been employed as carriers for the immobilization of different types of enzymes such as laccase [11], glucoamylase [12], peroxidase and glucose oxidase [13], and lysozyme [14]. The success in enzyme immobilization could be ascribed to the great porosity and surface area of nanostructured BC materials, which could ease the entrapment of enzyme molecules, resulting in high protein loading [11]. However, due to the hydrophilic nature of bio-cellulose, BC cannot be uniformly dispersed in hydrophobic nonpolar media, and thus its applicability in immobilization of interfacial enzymes like lipases could be limited. To impart its hydrophobicity, chemical modifications, such as aldehyde-oxidation, acetylation and silylation have been applied [15]. Cai *et al.*, performed aldehyde modification on spherical BC (not BC films) to enhance its hydrophobicity, and lipase-immobilized aldehyde-modified BC beads expressed high catalytic activity and stability [16]. Among aforementioned chemical modification methods, surface acetylation, where hydroxyl groups in the BC structure are replaced by more hydrophobic acetyl groups, displays more advantages due to its ability to increase hydrophobicity while maintaining good mechanical properties [17], morphology [15], optical transparency and thermal stability of BC [18]. Hu *et al.*, reported that the solvent-free acetylation of BC conducted by using the acetic anhydride with iodine as a catalyst resulted in higher hydrophobicity and acceptable mechanical properties of acetylated BC membranes [17].

To the best of our knowledge, research on characteristics of lipases immobilized on acetylated BC films has not been reported. This current study hence aimed to obtain BC membranes from *Komagataeibacter xylinus* and investigate the effects of iodine concentration during acetylation process on the extent of acetylation. Subsequently, catalytic activity and stability of ABC film-immobilized lipases were thoroughly examined. The reusability and stability of ABC-immobilized lipase over storage were also evaluated and benchmarked to those of their free form.

2 Materials and Methods

2.1 *Enzyme, microorganisms and culture conditions*

Commercial lipase was obtained from Novaco Pharmaceutical Co.Ltd., Hanoi, Vietnam. *Komagataeibacter xylinus* (JCM 9730) was provided by the Department of Biotechnology, HCMC University of Technology (HCMC, Vietnam). *K. xylinus* was maintained on M1 agar plates at 4 °C. M1 medium was prepared using coconut water with the chemical composition as follows: 1.0% (w/v) sucrose, 0.1% (w/v) yeast extract, 0.05% (w/v) KH_2PO_4, 0.05% (w/v) K_2HPO_4, 0.8% (w/v) $(NH_4)_2SO_4$; 0.2% (w/v) $(NH_4)_2HPO_4$; and 0.1% (w/v) $MgSO_4$. The pH of the medium was adjusted to pH 5.0 with 100% (v/v) acetic acid. The preculture was prepared by transferring bacterial colonies on an agar plate to 100 mL of M1 medium. The preculture was agitated at 200 rpm with a magnetic stirrer (MS-H0810, DLab, USA) at room temperature for 72 h.

For cellulosic biomass production, cell stock from preculture was harvested and inoculated at a concentration of 0.04 g L^{-1} into 100 mL of M2 medium. M2 was prepared using coconut water with the chemical composition as follows: 1.0% (w/v) sucrose, 0.1% (w/v) yeast extract, 0.8% (w/v) $(NH_4)_2SO_4$, 0.2% (w/v) $(NH_4)_2HPO_4$, and 0.5% (v/v) concentrated acetic acid. The pH of the M2 medium was adjusted to

pH 4.0 using concentrated acetic acid. The cell cultures were kept at 30 °C under static condition for 7 days in an incubated shaker (IST-3075R, JeioTech, Korea).

All the chemicals used in this research were of analytical grade. All media for bacteria and apparatus, such as conical flasks, pipette tips, and magnetic bars were autoclaved (ALP CL-40M, Japan) at 121 °C for 15 min before use.

2.2 *Acetylation of BC and immobilization of lipase onto acetylated BC (ABC)*

2.2.1. Acetylation of BC

After 7 days of incubation, film-like cellulosic biomass floating on the surface of the liquid medium (Figure 1) with a thickness of 1.80 ± 0.08 mm (Mitutoyo caliper, Japan) was collected and then cut into round shape pieces (1.7 cm diameter). These BC pieces were cleaned twice with distilled water and treated with 0.25 M NaOH at 80 °C for 1 h to lyse the cells. BC pieces were washed again with distilled water three times and then oven-dried (Memmert UF260, Germany) at 80 °C for 2 h [17].

BC pieces of 400 mg were then immersed to 20 mL acetic anhydride containing different iodine concentrations (0.175 mM, 0.225 mM, 0.275 mM, 0.325 mM, and 0.375 mM). The mixture was heated at 80 °C for 1 hour using an incubated shaker (BS-21, Lab companion, Korea), and then was cooled to room temperature. The $Na_2S_2O_3$ solution of 50% (w/v) was subsequently dropped in, until the color of the mixture transformed from dark brown to colorless, meaning that all iodine was converted to iodide [17]. After acetylation, acetylated BC (ABC) samples were first cleaned with 75% (v/v) ethanol and then with distilled water for 5 times to completely detach the remaining by-products and unreacted chemicals. ABC films were then dried at 60 °C for 12 h [17]. The dried ABC films were used for lipase immobilization.

2.2.2. Immobilization of lipase onto ABC

Lipase solution (8 mg/mL) was prepared by dissolving lipase powder in 0.05 M sodium phosphate buffer at pH 7.0. Five mg of dried ABC films were submerged into 3 mL of lipase solution, and the mixture was gently shaken at 100 rpm at 25 °C for 2 h in an incubated shaker (IST-3075R, JeioTech, Korea). ABC films containing immobilized lipase were then removed and cleaned three times with sodium phosphate buffer. After the immobilization step, the reaction solution and washing solutions were collected to determine the residual protein concentrations [19], [20].

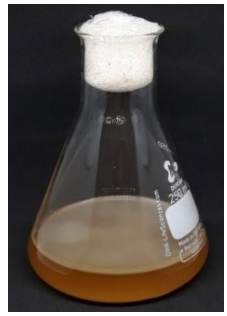

Figure 1: Film-like cellulosic biomass floating on the surface of liquid medium.

2.3 *Characterization of ABC-immobilized lipase*

2.3.1 Effects of pH and temperature on the hydrolytic activities of ABC-immobilized lipase and free lipase

The hydrolytic activities of the immobilized and free lipases (with olive oil as substrate) were studied in the pH range of 4.0–11.0 at 37 °C or in the temperature range of 20 °C–80 °C at pH 7.0. The following 0.05 M buffer systems were used in the experiment: acetate buffer, pH 4.0–5.0; sodium phosphate buffer, pH 6.0–8.0; glycine/NaOH, pH 9.0–11.0. Relative activities of immobilized and free lipases were computed as the ratio of the enzyme activity at different pHs or temperatures to the one at the standard condition (pH 7.0, 37 °C) [16], [21].

2.3.2 pH and thermal stabilities of immobilized and free lipases

The pH stability assays were conducted by incubating immobilized and free lipases at different pH values (from pH 4.0 to 11.0) for 24 h at 4 °C using different 0.05 M buffer systems as described in Section 2.3.1. On the other hand, their thermal stability was studied by incubating two lipase forms in sodium phosphate buffer (pH 7.0) at different temperatures (20 °C–80 °C) for 1 h, and then cooling the mixture down to room

temperature [21]. Their residual activities were then measured.

2.3.3 Organic solvent stability of immobilized and free lipases

Different organic solvent solutions were prepared by mixing pure solvent (ethanol, methanol, isopropyl alcohol, *n*-hexane, or acetone) with sodium phosphate buffer at the ratio of 2 : 8 (v/v). Organic solvent stability assays were done by incubating lipase-containing ABC or free lipase in different organic solvent solutions of 5 mg/mL for 1 h at 30 °C [22]. After the incubation, lipase-containing ABC samples were removed and dried to determine the hydrolytic activity. Similarly, 1 mL of free lipase-containing solvent solution was used to determine the hydrolytic activity. The hydrolytic activities of the immobilized and free lipases before and after incubation were used to calculate the residual activities. The enzyme activities were assayed at the standard condition (pH 7.0, 37 °C).

2.3.4 Storage stability and reusability of the immobilized lipase and free lipase

The storage stability of immobilized and free lipase was determined after storing the enzymes in sodium phosphate buffer at 4 °C and 30 °C for 2, 5, 10, and 15 days [23]. Their residual activities were then measured.

The reusability of ABC-immobilized lipase was investigated as follows: after each olive oil hydrolysis reaction (at 37 °C and pH 7.0), lipase-containing ABC films were removed, washed three times with 3 mL sodium phosphate buffer to detach any residual substrates and then reintroduced into fresh reaction medium to perform another olive oil hydrolysis. This procedure was repeated for 30 times, and hydrolytic activity of ABC-immobilized lipase at each run was measured and compared with the first run to calculate its residual activity [21], [23].

2.4 *Analytical methods*

Hydrolytic activity of lipase was determined using the olive oil emulsion method. The substrate of enzyme reaction was formulated by mixing 50 mL olive oil (45.87 g olive oil) with 50 mL emulsifier, and the mixture was homogenized for 10 min at 4000 rpm (T 18 Digital ULTRA-TURRAX®, IKA, Germany). The emulsifier mixture contained 17.9 g/L NaCl, 0.41 g/L KH_2PO_4, 540 g/L glycerol; 10 g/L gum arabic [24]. The hydrolysis reaction medium consisted of 5 mL of the substrate, 2 mL of sodium phosphate buffer, and 1 mL of lipase solution (containing 5 mg lipase/mL). To perform a hydrolytic reaction with ABC-immobilized lipase, 5 mg of lipase-containing ABC and 1 mL of corresponding incubation buffer were added to 5 mL of substrate and 2 mL of sodium phosphate buffer. The reaction was performed at 37 °C and pH 7.0 for 30 min, and was then stopped by adding 10 mL of ethanol-acetone mixture (1:1, v/v). The blank assays were similarly conducted without lipase. The catalytic activity of lipase was determined by the titration of the fatty acid released during hydrolysis with 0.025 M NaOH solution [20], [24]. One unit (U) of lipase activity corresponds to the amount of enzyme required to produce 1 μmol of free fatty acid per minute at 37 °C and pH 7.0 [20] [Equation (1)]:

$$y = \frac{(V_a - V_b) \times N \times 1000}{V \times t} \quad (1)$$

where y (μmol mL^{-1} min^{-1}) is the hydrolytic activity of lipase; V_a, and V_b (mL) are the volumes of NaOH used to titrate lipase and blank samples, respectively; N is the normality of the NaOH titrant used (0.025 M); V is the total reaction volume; and t is the reaction time (30 mins) [25].

The specific activity (μmol mg^{-1} min^{-1}) of lipase was calculated according to the following Equation (2) [25]:

$$Specific\ activity = \frac{(V_a - V_b) \times N \times 1000}{a \times t} \quad (2)$$

where a (mg) is the amount of protein enzyme added to the reaction medium.

The activity yield was calculated as Equation (3) [21]:

$$Activity\ yield\ (\%) = \frac{B}{A} \times 100 \quad (3)$$

where A (U) is the total activity of lipase used for the immobilization; and B (U) is the obtained activity of ABC-immobilized lipase.

The protein concentration in the solution was determined by Lowry method using egg albumin as standard [26]. The protein loading percentage was calculated as the following Equation (4) [21]:

$$Protein\ loading\ (\%) = \left(\frac{Amount\ of\ adsorbed\ proteins}{Initial\ amount\ of\ proteins}\right) \times 100 \quad (4)$$

Fourier transform infrared (FTIR) spectra of BC, ABC, and lipase-containing ABC were obtained with a FT/IR-4700 spectrometer (Jasco, Japan) using the attenuated total reflection attachment (ATR PRO ONE, Jasco, Japan). The wavenumber of 4000 cm^{-1} to 400 cm^{-1} was used with the resolution of 1 cm^{-1}.

The morphologies of BC films before and after acetylation were observed using Field Emission Scanning Electron Microscope (FE-SEM S4800, Hitachi, Japan) [17]. The BC films were dried at 80 °C for 1 day and coated with platinum by ion sputter coater to improve conductivity prior to observation [27].

2.5 *Statistical analysis*

Triplicate experiments were done and results were expressed as mean value with standard deviation. Statistical analyses were conducted by Minitab (version 19) using one-way ANOVA and Tukey posthoc tests with 95% confidence.

3 Results and Discussion

3.1 *Effects of iodine concentration on efficiencies of BC acetylation and lipase immobilization*

3.1.1 Chemical structure of BC films

The acetylation of BC in acetic anhydride with iodine as a catalyst was reported to be the highly efficient, cost-effective and environment-friendly method [17]. This experiment was hence conducted to investigate the effects of different iodine concentrations (0.175 mM, 0.225 mM, 0.275 mM, 0.325 mM, and 0.375 mM) on the acetylation of the BC films. Figure 2(a) shows the FT-IR spectra of BC and ABC films. It can be seen that both native BC and acetylated BC films present characteristic bands of cellulose, such as 3700–3000 cm^{-1} (the O-H stretching), 2980–2800 cm^{-1} (the C-H stretching

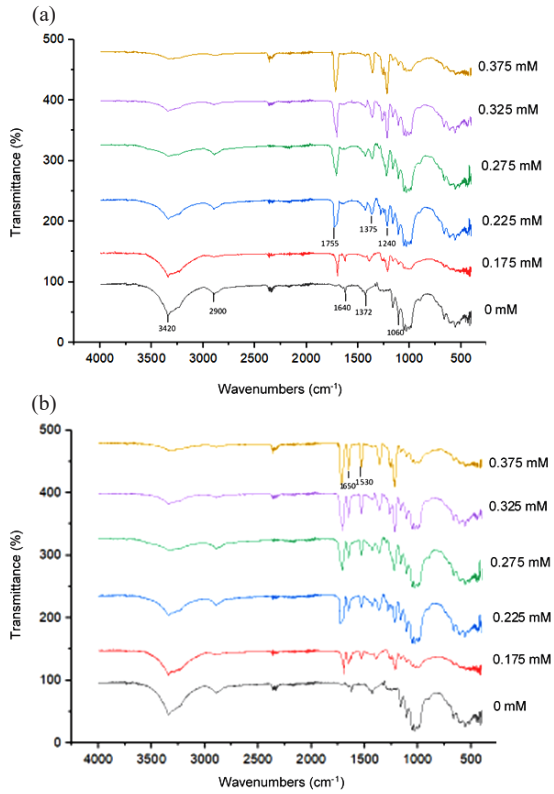

Figure 2: FT-IR spectra of BC films acetylated at different iodine concentrations (a) before and (b) after immobilization.

of CH_2 and CH_3 groups), and 1500–800 cm^{-1} (the fingerprint region of cellulose, corresponding to the C-H, O-H, C-O and C-O-C vibrations within the glycoside ring) [28]. It should be noted that the absorption peak at 1640 cm^{-1}, assigned to deformation vibration of water molecules, can be observed on FT-IR spectra of native BC and BC acetylated in iodine concentration of 0.175 mM, but gradually disappears from spectra of other ABC samples. Especially, all acetylated BC samples present characteristic absorption bands of acetyl groups, such as 1755–1720 cm^{-1} assigned to the C=O stretching vibration of carbonyl in the ester bonds, 1372–1369 cm^{-1} corresponded to the C-CH_3 band, and 1240–1229 cm^{-1} attributed to the stretching of C-O of the acetyl groups [17], [27]–[29]. As the iodine concentration used in the acetylation reaction increased, the absorption intensity of the absorption bands located at around 3700–3000 cm^{-1} decreased, indicating that the hydroxyl groups of native BC had been

substituted by acetyl groups during acetylation [17], [29]. The absorption intensity of the abovementioned characteristic absorption bands (i.e. 1755–1720 cm^{-1}, 1372–1369 cm^{-1}, 1240–1229 cm^{-1}) also increased when increasing iodine concentrations. These results were in line with those obtained by Hu *et al.* [17]. Besides, the absorption bands at wavenumbers of 1840–1760 cm^{-1} and 1700 cm^{-1} were not found, indicating that acetic anhydride and acetic acid (byproduct) were completely removed from ABC samples [17], [28], [30].

When ABC samples were used for lipase immobilization, the infrared spectra of ABC films present two new absorption bands at 1650 cm^{-1} and 1530 cm^{-1} [Figure 2(b)], which are attributed to amide I and amide II [28]. This result verified the existence of protein molecules (lipase) on the surface of ABC supports [28]. Moreover, the absorption intensity of these two absorption bands increased when increasing iodine concentration. These results indicated that as the iodine concentration increased, the extent of acetylation increased, leading to higher enzyme immobilization efficiency. On the contrary, the two new characteristic bands at 1650 cm^{-1} and 1530 cm^{-1} were not detected on the spectrum of BC [Figure 2(b)], indicating that lipase can not be immobilized on non-acetylated BC films.

3.1.2 BC films morphology

The morphologies of BC surfaces before and after acetylation at different iodine concentrations were observed using FE-SEM as illustrated in Figure 3. The structure of all films consisted of continuously arranged nanofibers where the cellulose fibers of the untreated BC and acetylated BC at iodine concentrations of 0.275 mM were quite smooth and intact [Figures 3(a) and (b)]. However, at the highest iodine concentration of 0.375 mM, the ABC fibers seemed deformed, became rougher and swollen in some regions [red arrows in Figure 3(c)]. This indicates that an excess degree of acetylation negatively affected the structure of BC [17]. The deformed and swollen morphology of acetylated BC nanofibers could be attributed to the penetration of the acetic acid and anhydride acetic deep into the film and fibers, resulting in the acetylation of hydroxyl groups on the surface of BC samples and also the rupture of nanofiber structure [30]. Thus, the increase in iodine concentration resulted in not only a higher degree of acetyl substitution but also the enhanced deformation of ABC samples.

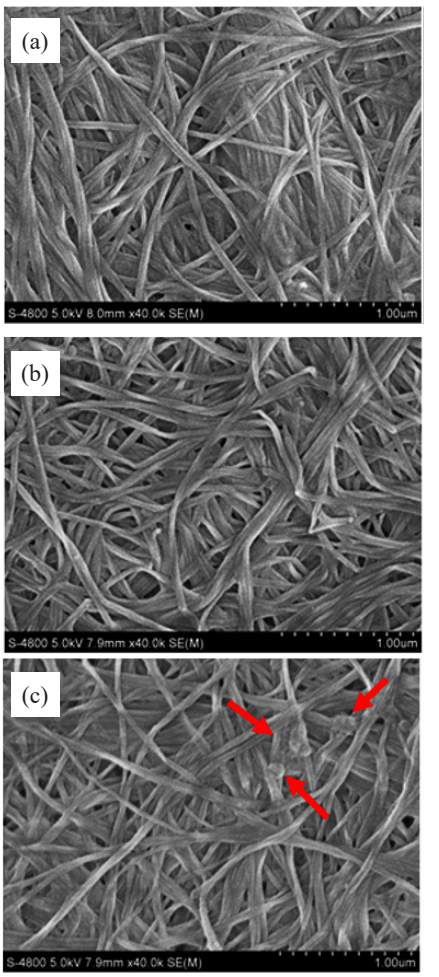

Figure 3: FE-SEM images of (a) native BC, and ABC treated at iodine concentrations of (b) 0.275 mM, and (c) 0.375 mM.

Results in Figure 3(a) and (b) also show that the acetylated nanofibers were more parted from each other as compared to those of native BC, hence forming wider interstitial cavities than the untreated ones. This could be attributed to the increased hydrophobicity of the ABC surfaces [17], [27]. According to Nogi *et al.*, the substitution of the hydrophilic OH groups by hydrophobic acetyl groups may impede the mutual interaction between BC nanofibers, thus separating the acetylated BC nanofibers [18]. It should be noted

that the increase in interstitial cavities could result in a higher absorption capacity of lipase molecules during immobilization. To confirm the presence of immobilized enzymes on ABC films, lipid hydrolytic activity of ABC-immobilized lipase was then investigated.

3.1.3 Lipid hydrolytic activity of ABC-immobilized lipase

In this experiment, lipase-containing ABC films were used to investigate their olive oil hydrolytic activity. Results clearly confirmed that lipase molecules were successfully immobilized on ABC supports without losing their catalytic activity. As shown in Table 1, protein loading, activity yield and specific activity significantly increased as the iodine concentration used during acetylation increased from 0.175 mM to 0.275 mM. This was due to enhanced hydrophobicity resulted from enhanced acetylation; ABC films hence more easily interact with hydrophobic substrates (olive oil), leading to a significant improvement in lipid hydrolytic activity. However, as the iodine concentration increased from 0.275 mM to 0.375 mM, activity yield and specific activity of immobilized lipase were significantly decreased ($p < 0.05$). This could be due to the steric hindrance generated by increased polymerization level in the ABC matrix, hindering the entry of lipids to the active site of the immobilized lipases [21]. Noticeably, the specific activity of lipase immobilized on the BC acetylated at the iodine concentration of 0.275 mM was similar to that of free lipase ($p > 0.05$). Hence, it can be concluded that the iodine concentration of 0.275 mM was suitable for the acetylation of BC to obtain the highest activity yield, protein loading and specific activity. This iodine concentration was used for BC acetylation in subsequent experiments.

Table 1: Protein loadings, activity yields and specific activities of lipase immobilized on BC films acetylated at different iodine concentrations

Iodine Concentration	Protein Loading (%)	Activity Yield (%)	Specific Activity (μmol mg^{-1} min^{-1})
0.175 mM	14.74 ± 2.25^{c}	10.52 ± 1.52^{d}	$10.34\pm1.93^{b,c}$
0.225 mM	45.20 ± 1.02^{b}	$29.60\pm1.52^{b,c}$	9.25 ± 0.32^{c}
0.275 mM	50.72 ± 1.26^{a}	44.43 ± 3.40^{a}	$12.44\pm0.14^{a,b}$
0.325 mM	51.62 ± 0.69^{a}	30.66 ± 0.58^{b}	$8.31\pm0.29^{c,d}$
0.375 mM	52.21 ± 1.03^{a}	25.10 ± 0.58^{c}	6.87 ± 0.32^{d}
Free enzyme			13.78 ± 0.55^{a}

Note: Different superscript letters in the same column indicate significant differences ($p < 0.05$).

3.2 *Effects of pH and temperature on the hydrolytic activities of ABC-immobilized and free lipases*

The effect of pH on olive oil hydrolytic activity of lipases was investigated in the pH range of 4.0–11.0. As shown in Figure 4(a), the highest activity was obtained at pH 7.0 for both immobilized and free lipases. At pH 6.0 and pH 8.0, the relative activities of the two lipase forms remained above 70%. However, both forms of lipase exhibited low catalytic activity (< 50%) at acidic pH values (pH < 5.0) and alkaline pH values (pH > 10.0). Cai *et al.*, also reported that free lipase (extracted from *Rhizopus chinensis*) showed low activities at pH < 6.0 and pH 10.0 (optimal activity at pH 9.0), however lipase immobilized onto aldehyde-modified BC beads exhibited optimal hydrolytic activities at two pH values of 5.0 and 8.0 [16].

The temperature dependence of hydrolytic activities of lipases was studied in the temperature range of 20–80 °C. As shown in Figure 4(b), both free and immobilized lipases had their highest catalytic activity at 37 °C. This result trend was different from those obtained in previous studies where the optimal reaction temperatures shifted from 40 °C (for free lipase) to 30 °C (for lipase immobilized onto aldehyde-modified spherical BC, [16]), or from 50 °C (for free *Talaromyces thermophilus* lipase) to 60 °C (for chitosan-immobilized lipase, [21]). Results in Figure 4(b) also indicate that at higher temperatures of 50 °C, 60 °C and 70 °C, the relative activities of ABC-immobilized lipases were lower than those of free lipases ($p < 0.05$). The multipoint attachment of lipase molecules on ABC supports could limit the conformational changes in lipase movements under higher temperatures, and hence lower the catalytic activity [31], [32].

Results in Figure 4 indicate that ABC supports did not affect the optimal pH and temperature of the hydrolytic reaction of lipase. Thus, ABC-immobilized lipase could be considered an easy-to-use enzyme because of its good hydrolytic activity at neutral pH and relatively low temperature (37 °C). The optimal pH 7.0 and temperature 37 °C for the hydrolytic reaction of free and immobilized lipases were then used for subsequent experiments.

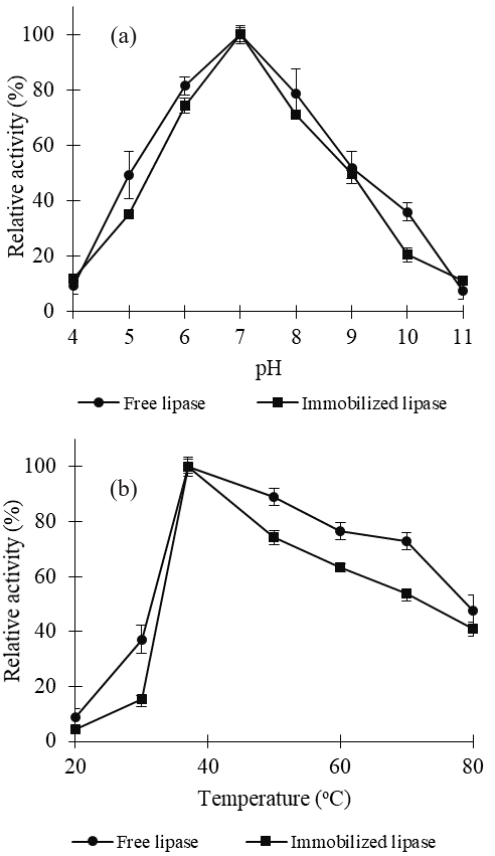

Figure 4: Effects of different (a) pH values and (b) temperatures on the relative activities of (●) free lipase and (■) immobilized lipase.

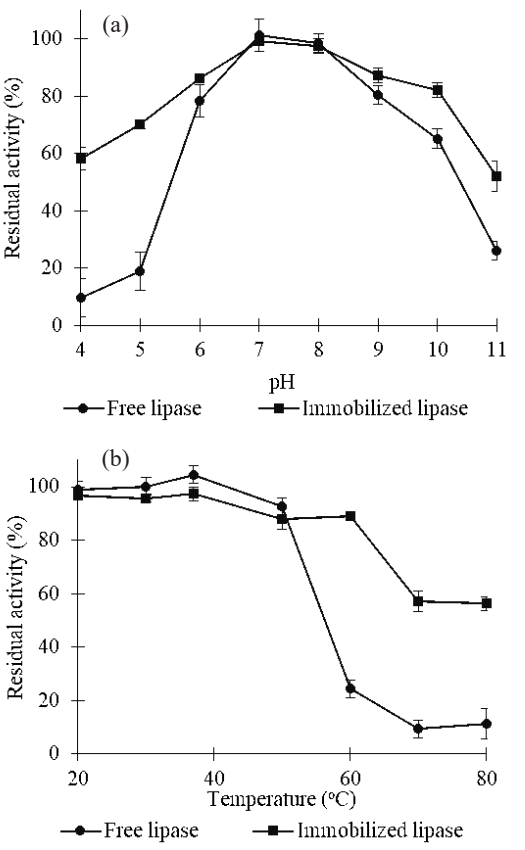

Figure 5: Effects of (a) pH and (b) temperature on stability of (●) free and (■) immobilized lipases.

3.3 *pH and thermal stabilities of immobilized and free lipases*

Lipases are proteins in nature, hence they are easily denatured and inactivated at extreme pH and temperature conditions. This would limit the applicability of lipases in large-scale production. Therefore, the immobilization of lipase is a potential approach to improve its stability. Results in Figure 5(a) show that both forms of enzyme retained high catalytic activity (more than 70%) after being incubated at pH 6.0 and pH 8.0 for 24 h at 4 °C. However, the stability of ABC-immobilized lipase was much higher at the incubated pH < 6.0 or pH > 9.0. In particular, ABC-immobilized lipase still retained high catalytic activities after the incubation at pH 5.0 (70%) and pH 10.0 (82%), which were 3.7 and 1.3 times higher than those of free lipase, respectively. The differences were even larger at the more extreme pH values of 4.0 and 11. Similar trends in pH stability enhancement were reported for lipases immobilized on oxidized cellulose fibers [32], silica aerogels [33] and alginate-based composite electrospun nanofibers [34].

It is obvious that the stability of the enzyme largely depends on pH. The incubation of free lipases at extreme pH conditions could change the chemical nature of amino acids on the protein chains and hence the active sites could be disrupted and enzymes could be easily denatured [34]. ABC materials, on the other hand, played an important role in protecting immobilized lipase molecules from being severely denatured after 24 h incubation at extreme pHs. This could be explained by the formation of secondary interactions, such as hydrogen bonding, ionic bonding and polar interactions between ABC films and lipase molecules during

immobilization [34]. Results also show that there were no residual proteins in the incubation buffers after incubating the lipase-containing ABC. Hence, it can be concluded that the interaction between ABC films and lipases not only prevented their leaching but also protected them from extreme reaction conditions. The pH stability of ABC-immobilized lipase renders it applicable in food, medicine, biodiesel and detergent industries [35].

Figure 5(b) shows the effects of incubation temperature on stability of free and ABC-immobilized lipases. Both free and immobilized lipases retained more than 88% of their catalytic activities at incubation temperature ranging from 20–50 °C. However, at higher incubation temperatures (60 °C and above), residual activity of free lipase significantly decreased. As shown in Figure 5b, only 24% of catalytic activity of the free lipase was retained after incubating at 60 °C for 1 h, which was 3.7 times lower than that of immobilized lipase (89%). It should be noted that after the incubation at 80 °C for 1 h, the catalytic activity of immobilized lipase was still as high as 56%, whereas free lipase was almost inactive (11%). This result clearly proved that ABC material had effectively protected lipase molecules from heat inactivation. The improvement in heat stability of ABC immobilized lipase could be due to 1) the immobilization of lipase molecules inside the micro-pores of ABC microfiber network, 2) the interactions between enzyme and ABC nanofibers, both resulting in better protection against conformation alteration of lipase molecules. The enhanced thermal stability of immobilized lipases was also observed in other studies [32]–[34]. The broad temperature stability implies that the ABC-immobilized lipases are potentially applied in brewing and wine industry [36], pharmaceuticals [35], [37], in production of human milk fat substitutes [38], structured triacylglycerol [39], aromatic esters [40], biodiesel and detergent [35].

3.4 *Organic solvent stability of immobilized and free lipases*

Lipases that have good performance in organic solvents can be used in esterification reactions, increasing their industrial applicability. However, the use of unsuitable organic solvent could induce enzyme denaturation, and hence their catalytic activity could be lost [21], [32]. The stability of ABC-immobilized lipase was investigated using different organic solvents including methanol, isopropanol, ethanol, acetone (polar solvents) and *n*-hexane (non-polar solvent).

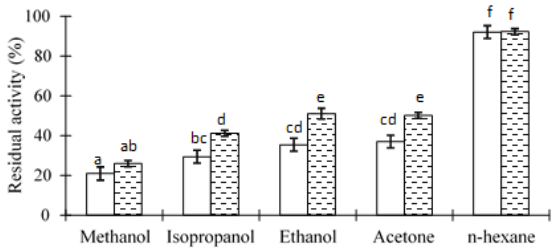

Figure 6: Effects of organic solvents on the activity of free (□) and immobilized (▒) lipases. Note: Different letters indicate significant differences among samples ($p < 0.05$).

Results in Figure 6 show that after the immersion in polar solvents such as methanol, isopropanol, ethanol and acetone, hydrolytic activities of both lipase forms significantly decreased. The lower hydrolytic activities were attributed to the removal of the water layer around lipase molecules by polar solvents, hence distorting the catalytic conformation of the lipase [21], [22], [32], [41]. These results were in accordance with a previous study investigating the stability of lipases in these solvents [22]. However, results show that the remaining activities of ABC-immobilized lipase were significantly higher than those of free lipase ($p < 0.05$). This clearly demonstrated that ABC materials could protect the enzymes from environmental inactivation factors by keeping them inside their microporous structure.

Results in Figure 6 also indicate that among five tested solvents, both free and ABC-immobilized lipases were more tolerable against *n*-hexane. Both types of lipase retained more than 80% of their catalytic activities. This was because non-polar solvents did not remove the crucial bound water on the enzyme surface during the incubation step, hence the catalytic conformation of enzyme was not distorted [21]. In addition, *n*-hexane could keep the lid of the active site in an open conformation, and hence the active site crevice was not blocked, resulting in remaining their catalytic activity [22]. Similar trends were also obtained in previous studies [22], [32], [33].

Results in this experiment display that lipase immobilized on ABC films can be considered a suitable catalyst to perform hydrolysis in industrial

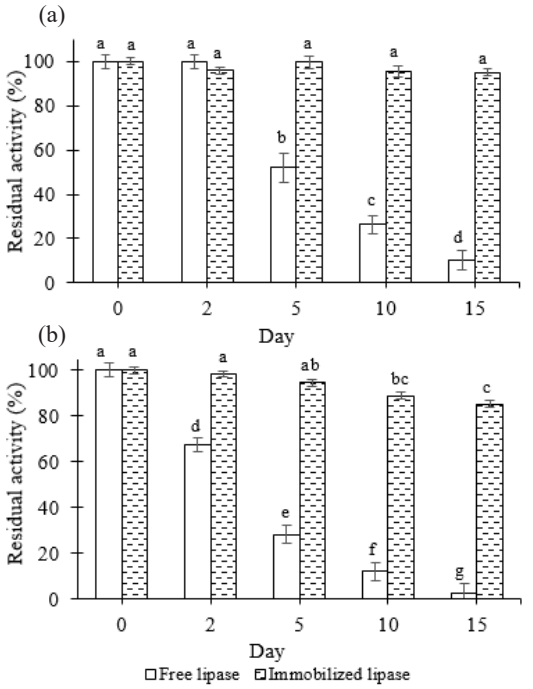

Figure 7: Storage stability of free (□) and immobilized (▒) lipases at (a) 4 °C, and (b) 30 °C. Note: Different small letters indicate significant differences among samples ($p < 0.05$).

applications involving organic solvents. ABC did not interfere the effect of *n*-hexane on the catalytic activity of lipase, whereas protecting lipase from severe structural changes caused by polar solvents.

3.5 *Storage stability and reusability of ABC-immobilized lipase and free lipase*

Storage stability and reusability of immobilized lipase are important factors determining its commercial application [16], [33]. In this study, free lipase and ABC-immobilized lipase were stored at 4 °C and 30 °C for 15 days, and their residual activities were periodically determined to investigate the storage stability. Results in Figure 7 show that after 15 day storage at 4 °C, free lipase only reserved 10% of its initial activity, and was almost inactive after 15 day storage at 30 °C. On the contrary, ABC-immobilized lipase still retained 95% and 85% of its initial hydrolytic activity after 15 days of storage at 4 °C and 30 °C, respectively. These results demonstrated

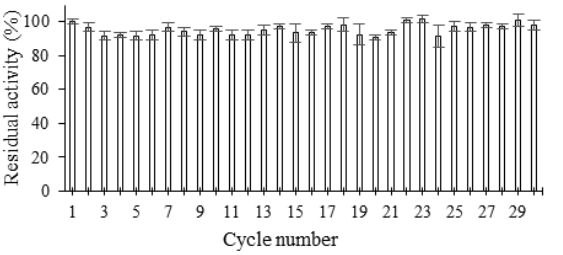

Figure 8: Residual activity of ABC-immobilized lipase after 30 cycles of usage.

that ABC supporters could limit severe changes in lipase structure during the storage period, and hence preventing significant denaturation of enzyme molecules. It could be concluded that ABC is a suitable and biocompatible material, contributing to improve the stability of immobilized lipase. Some studies had shown similar results on enhanced storage stability of lipases immobilized on cellulose nanofiber membrane [19] and chitosan nanofibrous electrospun membrane [42].

To investigate reusability of ABC-immobilized lipase, olive oil hydrolysis reaction was repeated for 30 cycles. Results obtained in Figure 8 show that after 30 consecutive cycles of usage, the ABC-immobilized lipase still retained more than 90% of its initial catalytic activity. The recycling efficiency of ABC-immobilized lipase obtained in this study was higher than those reported in other studies (Table 2). This demonstrated that lipase molecules and ABC materials still maintained good interaction after 30 reaction cycles, and ABC material was perfectly suitable for lipase immobilization. It should be emphasized that the high operational stability and high reusability of ABC-immobilized lipase could significantly reduce the production cost in practice [23].

4 Conclusions

In this study, the acetylated BC films obtained in an acetic anhydride/iodine system were used as carriers for lipase immobilization. Results show that lipase immobilized on ABC films exhibited high activity yield and specific activity. The immobilization of lipase on ABC films also enhanced its tolerance to pH, temperature and polar solvents. Especially, ABC-immobilized lipases showed considerably high storage

Table 2: Operational stability of lipases immobilized on different carriers

Lipase Carriers	Number of Use Cycles	Residual Activity (%)*	References
ABC film	30	> 90%	This work
Aldehyde-modified spherelike BC (∅ = 6.1 ± 0.5 mm)	10	70%	[16]
BC membrane	15	60%	[43]
Electrospun cellulose nanofiber membrane	8	~ 30%	[19]
Magnetic poly(GMA-MMA) beads **	10	62%	[23]
Chitosan	10	> 80%	[21]
Chitosan nanofibrous membrane	10	~ 46%	[42]

* residual activity of immobilized lipases after tested cycles.
**GMA-MMA: glycidylmethacrylate–methylmethacrylate.

stability and reusability, thereby the operational cost in practical applications could be reduced. To conclude, acetylated BC films could be used as potential carrier to prepare lipase-immobilization system for fat and oil hydrolysis with high operational stability.

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Author Contributions

L.T.K.V.: conceptualization, research design, data curation, data analysis, writing—reviewing and editing, supervision, project administration; A.T.K.N.: experimental design, investigation, data analysis, data curation, writing an original draft; N.L.L.: data analysis, reviewing and editing. All authors have read and agreed to the published version of the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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