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Immobilization of a thermophilic solvent-stable lipase from *Acinetobacter baylyi* and its potential for use in biodiesel production

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ABSTRACT: Lipase transesterification of triglycerides is an environmentally safe alternative to chemical processing during biodiesel production. However, the cost and low stability of this enzyme remains problematic for commercial production. In this study, a thermophilic-solvent stable lipase from *Acinetobacter baylyi* (ABL) immobilized on Sepabeads EC-OD showed improved solvent stability. The optimal reaction conditions of immobilized ABL were comparable with those of the suspended lipase. Immobilization of ABL resulted in a broader pH activity range and enhanced storage stability. Optimal conditions for transesterification of palm oil were 6-step methanol feeding, 1:4 oil/methanol molar ratios, 20% enzyme loading, and 4% water content for 24 h at 40 °C. Conversions of oil feedstocks to biodiesel of between 13 and 93% were obtained. Reusability for transesterification of immobilized ABL was comparable to that of commercial lipases. This study found that immobilized ABL is one of the biocatalyst candidates for further development and application in enzyme-catalysed biodiesel synthesis.

KEYWORDS: physical adsorption, solvent stability, thermostability, transesterification

INTRODUCTION

Biodiesel is becoming an increasingly important alternative fuel due to its domestic production thereby reducing petroleum imports and harm to the environment [1,2]. Several biodiesel production processes have been developed, among which lipase transesterification of triglycerides has become particularly attractive [3,4]. However, the main impediment to commercialize this process is the high cost and low stability of the lipase. In addition, inactivation of lipases by methanol hampers further applicability of enzymatic transesterification. Most attempts to overcome these problems use commercial immobilized lipases [5–8] but the enzyme cost preclude industrial implementation.

Acinetobacter baylyi lipase (ABL) is a novel thermophilic and solvent-stable enzyme capable of catalysing the transesterification of palm oil to fatty acid methyl esters [9]. *Acinetobacter* are well represented among fermenting bacteria to produce a number of extra- and intracellular economic products, including lipases [10,11]. Biotechnological contributions by *Acinetobacter* seem to mirror those of *Pseudomonas* sp. in vigour and versatility. The genome of *Acinetobacter* however contains few traits that might be associated with pathogenesis in contrast to *Pseudomonas* [11]. Most lipases produced by *Acinetobacter* sp. have biochemical properties similar to those produced by *Pseudomonas* sp. and *Burkholderia* sp., including stability and maximum activity under alkaline conditions at high temperature [10,12]. A suspended form of the enzyme usually exhibits lower stability and greater resistance to enzyme recovery and re-use, restricting its practicality [13]. Immobilized enzymes may overcome many of these issues within industrial applications, as they can lead to enhanced enzyme stability, activity and recovery, and ultimately to a significant production economic benefits [14]. In this study, ABL was immobilized onto commercial hydrophobic supports by simple adsorption. Lipolytic

activity and stability of the immobilized lipase were investigated in comparison to reported values for the suspended ABL[9] and commercial lipases. Finally, the transesterification of oils in methanol by immobilized ABL was assessed.

MATERIALS AND METHODS

Materials

Non-edible oils (cotton, papaya, physic nut, pomelo, pumpkin, rambutan, and rubber) were extracted from seeds[15]. Edible oils such as canola, coconut, corn, olive, palm, rice bran, safflower, sesame, soya bean, and sunflower were purchased in a supermarket. Commercial polymer supports (Amberlite XAD7HP, Amberlite XAD761, Amberlite XAD16, and Sepabeads EC-OD) were purchased from Rohm & Haas Co., Ltd. (USA) and Resindion Srl (Mitsubishi Chemical Corporation, Milan, Italy). Commercial immobilized lipase from *Rhizomucor miehei* (Lipozyme RM IM) was bought from Novo Nordisk (Denmark). The enzyme is immobilized on macroporous anionic resin beads (Duolite A568) (approximately 14% proteins are adsorbed onto the resin g/g). The yeast, *Candida antartica* lipase B immobilized on macroporous acrylic resin (Novozyme 435) was purchased from Sigma-Aldrich (Tokyo) and classified as belonging to the triacylglycerol hydrolases (EC 3.1.3.3), with a declared activity of $\geqslant$ 10 000 U/g (propyl laurate units per gram). All other chemicals were purchased from Sigma and were of analytical grade.

Bacterial strain and culture condition

The bacterial strain used in this study, *A. baylyi*, was isolated from marine sludge in Thailand[9]. Lipase was produced in 1-l of 5-fold dilution of Luria-Bertani medium[16] containing 0.8% (v/v) of tween80 as an inducer[17] at 25 °C and 150 rpm for 15 h. When cell growth reached the late exponential phase, the culture broth was centrifuged for 20 min at 4 °C, 10 000*g*. The supernatant containing extracellular lipase was filtered through a 0.2 µm nylon membrane filter and harvested for lipase assay. Purification was performed as previously described[9].

Lipase activity assay and protein determination

Hydrolytic activities of suspended and immobilized lipase were related to spectrophotometric absorption (410 nm) promoted by the hydrolysis of *p*-nitrophenol[18] (*p*-NPP). One unit (U) of enzyme was defined as the amount of enzyme releasing 1 µmol of *p*-NP per minute under assay conditions. Amount of *p*-NP was calculated from a standard curve. In addition, hydrolysis of palm oil was determined as described previously using 50 mM NaOH and phenolphthalein as titrant and indicator, respectively[19]. One unit of lipase activity was defined as the amount of enzyme associated with the release of 1 µmol of free fatty acid per minute under test condition. Enzyme activity of immobilized lipase is expressed as enzyme units/g of polymer support.

In transesterification, the stepwise methanolysis of palm oil was conducted as follows: 1 g of palm oil was added to lipase and later mixed with 1:3 mole ratio of methanol[20]. Reactions were carried out by stirring the mixtures for 24 h at 40 °C. Samples were taken from the reaction mixture and further analysed for the products by high performance liquid chromatography[15]. Conversion of the transesterification reaction was equated to the ratio of fatty acid methyl esters and glycerides produced in the reaction.

Total protein concentration was determined spectrophotometrically according to Bradford[21] using Bio-Rad assay reagent (Hercules, USA). The protein standard was bovine serum albumin. Lipase loading efficiency was defined as the percentage of enzymes on the supporter based on protein concentration before and after immobilization.

Preparation of immobilized lipase from *A. baylyi*

Hydrophobic supports (1 g) were washed with 3 ml of methanol and stirred (350 rpm) for 30 min at room temperature. Methanol was removed by filtration and the supports were washed 3 times with an equal volume of 20 mM phosphate buffer (pH 7.5) for 30 min. The supports were dried at 45 °C for 12 h. A specified amount of support (1 g) was suspended in 500 ml of ABL solution (3 mg/ml) prepared in 20 mM phosphate buffer (pH 7.5) and stirred (350 rpm) for 6 h at room temperature. Immobilized supports were separated by filtration and washed thoroughly with 20 mM phosphate buffer (pH 7.5) and distilled water (100 ml each). Supernatants and washings were assayed for lipase activity and protein content. Protein bound on supports was calculated as the difference between that loaded and suspended in supernatants and washings. Immobilized supports designated as immobilized ABL were dried in a desiccator at room temperature and finally activities were assayed from hydrolysis and transesterification of palm oil. During immobilization, parameters were optimized: pH 4.0–10.0, ionic strength 10–50 mM, enzyme loading 1–9 mg/ml, time 1–5 h, temperature 10–50 °C, and adjuvants (1–30% v/v of methanol, ethanol, 2-propanol, 1-butanol, t-butanol, SDS, ethylene glycol, tween80, and triton X-100).

Characterization of immobilized lipase

Lipase activity of immobilized ABL was analysed using *p*-NPP as a substrate over pH and temperature ranges of 3.0–12.0 (37 °C) and 20–80 °C (pH 8.0), respectively, to identify favourable conditions for lipase reaction. For pH stability, immobilized ABL was incubated in 50 mM buffer at the specific pH for 6 h at 37 °C and then the residual activity was determined at pH 8.0. Buffer systems were acetate (pH 3.0–6.0), phosphate (pH 6.0–8.0), Tris-HCl (pH 7.0–9.0) and carbonate (pH 10.0–12.0). Thermal stability of immobilized ABL was determined by incubating enzyme at different temperatures for 6 h in a water bath and then measuring the residual activity using the activity at 60 °C as control.

Immobilized ABL was mixed with an equal volume of each selected organic solvent to prepare the 50% organic solution. Mixtures were shaken and incubated at 37 °C for 12 h at 150 rpm. The solvent contained in the mixture was partially eliminated by evaporation at 37 °C for 5 min. Residual lipase activity was measured at 37 °C and pH 8.0 and compared to that of the control (no solvent).

Reusability and storage stability of immobilized lipase

Immobilized ABL was added to the transesterification reaction described above to determine lipase activity. After 24 h reaction, the immobilized ABL was collected by filtration, washed 3 times with cold-acetone and dried in desiccators at room temperature prior to being added to the next reaction. Each of five reactions was performed for 24 h (40 °C) and conversion efficiency of each was expressed relative to the original activity. Results were compared with that mediated by commercial lipases (Novozyme 435 and Lipozyme RM IM).

Immobilized ABL (suspended in 20 mM phosphate buffer, pH 7.5) was stored at 4 °C and its stability evaluated from *p*-NPP hydrolytic activity at regular time intervals of 30 days. Hydrolytic activity of fresh purified enzyme was taken to be 100%.

Parameter study for biodiesel production

Factors affecting biodiesel production catalysed by immobilized ABL were examined under optimum reaction conditions, namely substrate ratio (1:3 to 1:9 mole of palm oil per methanol), amount of catalyst (10–30% w/w), addition mode of methanol (1–7 steps), water content (0–5%), reaction temperature (30–50 °C), and reaction time (3–48 h). Immobilized ABL was used for biodiesel production from palm oil at 250 rpm, 40 °C for 24 h. Specificities towards various oil feedstocks were also tested. All experiments were conducted in triplicate, with standard deviations within 5%. Data were analysed by EXCEL build-in functions.

RESULTS AND DISCUSSION

Support selection

Adsorption on hydrophobic supports is an effective, easy and inexpensive method for lipase immobilization[22]. In addition, regeneration is feasible from the reversible adsorption of enzyme on the supports[23]. Most lipases display a large increase in activity when adsorbed on hydrophobic supports, which is ascribed to both conformational micro-tuning and selective adsorption during immobilization[23]. Lipases recognize such supports as natural substrates and undergo interfacial activation during immobilization[24]. In the present study, four commercial inorganic materials were selected as supports based on their properties such as high stability against physical, chemical, and microbial degradation. Highly porous polymer matrices with spherical beads and high hydrophobicity were especially appropriate for application in lipase-catalysed biodiesel production, in terms of greater capacity for enzyme loading and less negative effect from the by-product, glycerol adsorbed on the surface of the enzymes[13]. The physical properties of various supports such as functional groups, specific surface area, pores, and particle sizes have been shown to significantly affect the accessibility and portioning of substrates, products, and water within the reaction mixture[13,25,26] and consequently the catalytic efficiency of immobilized enzyme. High enzyme loading efficiency ($> 89\%$) was found for all supports. Immobilized ABL on Sepabeads EC-OD and Amberlite XAD16 showed high hydrolytic activity and that on Sepabeads EC-OD provided the highest transesterification activity (Table 1). This result was consistent with those of *C. rugosa* lipase[20].When compared with Amberlite XAD16, Sepabeads EC-OD has larger pore diameters, increasing enzyme particle adsorption rate[27]. Smaller particles of EC-OD make the external surface of the matrices accessible to enzyme molecules during assay procedures increasing frequency of collisions between substrates and ABL with catalytic activity. In addition, large porous structures facilitate a catalytic expression of immobilized enzyme by reducing limitations on substrate diffusion and product accumulation. Further, improved diffusion enhances biocatalytic reactions by inhibiting substrate or product accumulation inside pores[26,27].

Table 1 Activity and efficiency of *A. baylyi* lipase immobilized on various supports.

Supports	Hydrolytic activity of pNPP (unit/g support)	Hydrolytic activity of palm oil (unit/g support)	Transesterification of palm oil (% conversion)	Enzyme loading efficiency (%)
EC-OD	4.36 ± 0.02	3.24 ± 0.21	22.0	96.67
XAD7HP	0.41 ± 0.07	1.25 ± 0.11	9.20	95.98
XAD16	3.92 ± 0.05	3.83 ± 0.09	13.24	89.07
XAD761	0.98 ± 0.10	1.80 ± 0.05	13.26	92.01

Hence Sepabeads EC-OD were selected as a basal support for the following experiment.

Optimization of immobilization parameters

Immobilization of ABL on Sepabeads EC-OD between pH 4 and 8 increased lipase activity from 1.60–2.35 μmol/min/g-support but activity decreased at higher pH. It is suggested that low enzyme activity at pH extremes resulted from either change in enzyme conformation or denaturation. Lipase activity was highest at pH 8.0, close to the isoelectric point of ABL, 7.75 (data not shown) and consistent with its expected maximum adsorption[28]. An increase in lipase activity of immobilized ABL with phosphate buffer (pH 8.0) from 1.80–2.45 μmol/min/g-support correlates with a corresponding increase in ionic strength between 10 and 30 mM. High ionic strength however can interfere with enzyme molecules binding to the supports[29]. Hence, beyond an ionic concentration of 30 mM, lipase activity of immobilized ABL gradually decreased.

Lipase activity of Sepabeads EC-OD supports loaded with enzyme solution increased (from 1.02–3.98 μmol/min/g-support) with protein concentration from 1–9 mg/ml, above which it was relatively constant. The lipase activity of immobilized ABL however was steady between 7 and 9 mg/ml (from 3.85–3.98 μmol/min/g-support). Possibly this was a result of embedding active sites of lipase molecules during immobilization by increased stacking and diffusion limitations[28].

Relative residual activity of immobilized ABL optimized for Sepabeads EC-OD reached an equilibrium in 120 min at 25 °C after which it remained comparatively unchanged. Time to reach equilibrium varied inversely with temperature and directly with the rate at which lipase activity is reduced. Addition of 10% (v/v) methanol as an adjuvant improved lipase activity by approximately 20% whereas it was reduced by other adjuvants. This is consistent with the suggestion by Fernandez-Lafuente et al[24] that short-chain alcohols promote the interfacial activation of lipase and create an open, substrate-accessible active site.

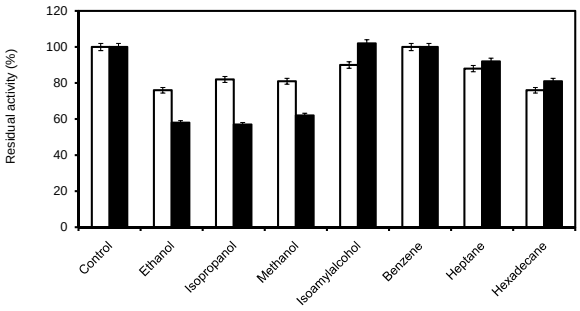

Fig. 1 Effect of organic solvent on suspended (dark solid bars) and immobilized (open bars) *A. baylyi* lipase. Enzyme was incubated at 37 °C and shaken at 150 rpm in the presence of 50% organic solvent for 12 h and the remaining activity measured in comparison to control (without solvent).

Stability of immobilized ABL

Knowledge of optimal conditions and stability of the immobilized enzyme are useful in exploring its potential applications. Both suspended and immobilized ABL exhibited optimal lipolytic activity at 60 °C with optimum pH of the former and latter, 8.0 and 9.0, respectively. Immobilized ABL seems more stable in alkaline pH (pH 10.0–12.0) than suspended enzyme as half of the activity remained. Our results demonstrate that both suspended and immobilized ABL are stable between 60 and 80 °C. Hydrolytic activity of immobilized ABL enzymes displayed enhanced stability in comparison to that of suspended enzyme in the presence of short-chain alcohols (Fig. 1). Minor conformational changes in enzyme structure after immobilization may take place, increasing stability of the immobilized enzyme[30].

Reusability and storage stability

Reuse of immobilized ABL enzyme for transesterification activity was examined since little is used in the chemical process (Fig. 2). Immobilized ABL retained less than 50% of its initial activity after the

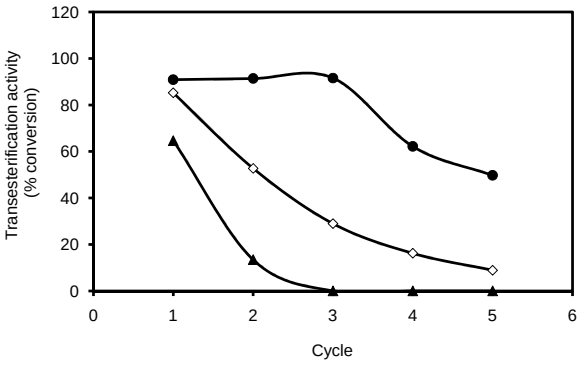

Fig. 2 Reusability of immobilized *A. baylyi* lipase (open diamonds) Novozyme 435 (closed circles) and Lipozyme RM IM (closed triangles) in subsequent cycles of biodiesel production from palm oil.

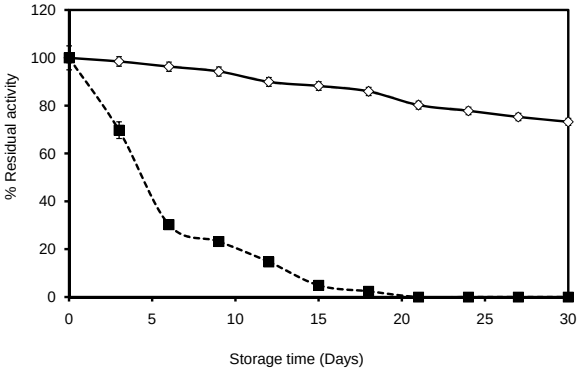

Fig. 3 Storage stability of suspended (closed squares) and immobilized (open diamonds) lipase from *A. baylyi*.

second cycle of transesterification of palm oil whereas the half-life of Novozyme 435 and Lipozyme RM IM under the same conditions was 5 and 1 cycle, respectively. Activity loss of immobilized ABL in the reaction progress was mainly attributed to leaching of lipase from the supporting surface and conformational changes from repeated use. Because most enzymes are very sensitive to environmental changes, treating them frequently would denature their molecular structure.

Storage stability is one of the most important criteria for the commercial application of an enzyme. Immobilized and suspended ABLs were stored at 4 °C and activities were measured periodically over 30 days. Immobilized lipase was more stable than suspended enzyme although some leakage from supports occurred for both forms (Fig. 3). Suspended lipase retained only 15% of its original specific activity after 12 days of storage at 4 °C while immobilized ABL remained at almost full activity for 30 days. From an economic perspective, the immobilized ABL distinguished itself with acceptable operational and good storage stability appropriate for biodiesel production.

Optimization of the transesterification reaction

The molar excess of alcohol over fatty acid in oil feedstock often increases the yield of transesterification but it can also inactivate the enzyme. At least three molar equivalents of methanol have been suggested for the complete conversion of oil to fatty acid methyl ester[31]. In this study, the amount of methanol used in the transesterification reaction catalysed by immobilized ABL was varied from 1:3 to 1:9 molar ratio equivalents, based on the mole of palm oil. Optimum methanol concentration was 4 molar ratio of methanol to palm oil at which conversion of methyl esters was about 73% (Fig. 4a). The production was constant with further increases in the methanol concentrations. Methanol was fed into the reaction every 3 h to reduce its effect on immobilized ABL. Addition of methanol in this manner lead to a 5-fold production over that when all was added at the beginning. Production of biodiesel gradually increased in each additional step and reached a maximum at the sixth step (74% conversion, Fig. 4b). Slight reductions in conversion efficiency occurred with further additions of methanol. Thus the recommended condition for biodiesel production of palm oil using immobilized ABL catalyst is the 6-step addition of methanol at 1:4 oil/methanol substrate ratios.

Biodiesel production from the transesterification of palm oil with methanol was enhanced with an increasing amount (10–30% (w/w)) of immobilized ABL. Based on the concentration of palm oil, methanol concentration was tested at 4 molar equivalents. Conversion efficiencies of palm oil to methyl ester differed little between 20 and 30% enzyme (80 and 85%, respectively, Fig. 4c). Thus on the basis of cost saving, the recommended optimum concentration of immobilized ABL is 20%.

Almost all lipases require water for their catalysis either hydrolysis or synthesis[32]. Conversion efficiency from immobilized ABL changed little from 78% with water increase to 1% (v/v) oil but reached a maximum of 92% at 4% (v/v) oil (Fig. 4d). Water is required for enzyme activation by increasing the available oil-water interfacial area. However, since lipases usually catalyse hydrolysis in aqueous media, excess water may also stimulate competing hydrolytic reactions.

Conversion of palm oil to biodiesel proceeded quickly during the initial 12 h after which it slowed,

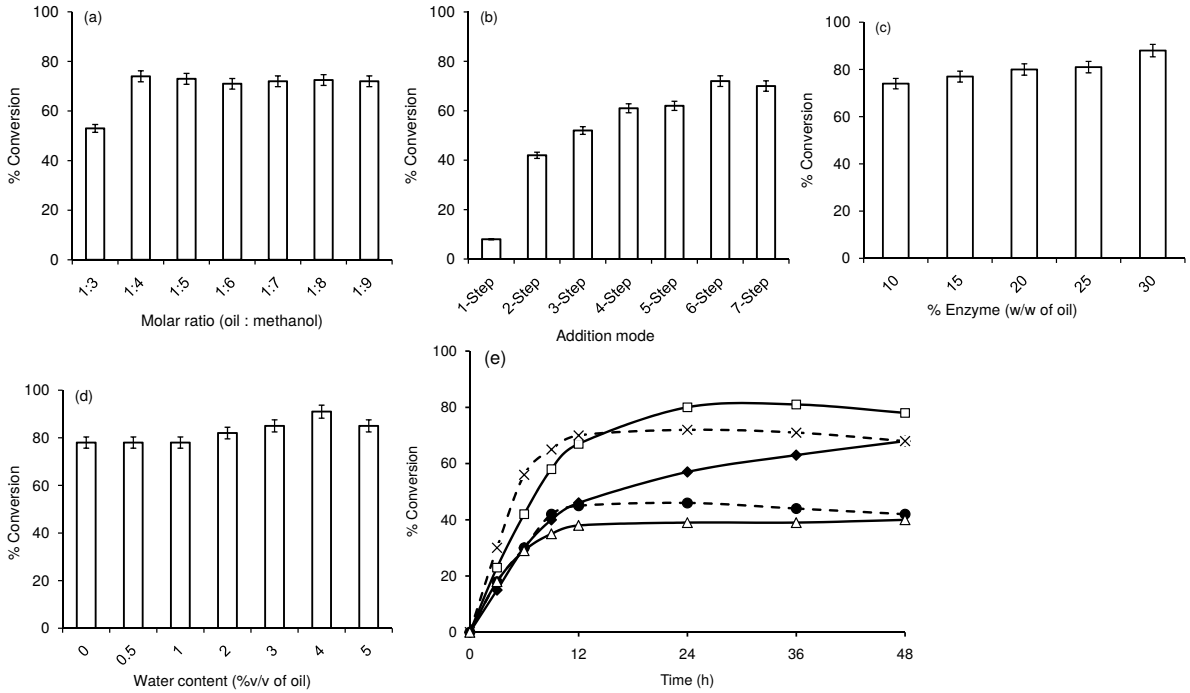

Fig. 4 Effect of (a) methanol concentration, (b) addition mode of methanol, (c) enzyme loading, (d) water concentration, and (e) time course on immobilized *A. baylyi* lipase catalysed transesterification of palm oil at different temperatures (♦: 30 °C, □: 35 °C, ×: 40 °C, ●: 45 °C, and △: 50 °C).

reaching an equilibrium after 24 h (Fig. 4e). Transesterification activity of the immobilized ABL at 40 °C was optimum, above which it decreased to 50% at 50 °C. This is attributed to temperature and activation energy being directly related and, the latter, inversely to enzyme inactivation rate. Production of methyl esters was practically identical when incubation time was longer than 24 h.

These results are similar to those reported by Noureddini et al, which were obtained via the methanolysis of soya bean oil using *Pseudomonas cepacia* lipase immobilized within a sol-gel support[33]. Furthermore, Jegannathan et al documented the complete conversion of palm oil was possible at 30 °C of reaction temperature with 7:1 of methanol to oil molar ratio and 1% water using *Burkholderia cepacia* lipase encapsulated in κ-carrageenan[34]. But, a higher amount of methanol was required for both reactions.

Production yield of different oil feedstocks

Edible and non-edible oils were used as substrates for transesterification catalysed by immobilized ABL. Immobilized ABL produced methyl ester from various oils. Biodiesel conversion from palm oil ($93 \pm 2\%$) catalysed by immobilized ABL was three times higher than that of suspended lipase[9]. Production yields $> 75\%$ were found with sesame oil ($77 \pm 5\%$), corn oil ($76 \pm 5\%$), and physic nut seed oil ($75 \pm 2\%$). Conversion efficiencies between 50% and 70% were obtained with safflower oil ($68 \pm 4\%$), canola oil ($62 \pm 2\%$), soya bean oil ($59 \pm 4\%$), rubber ($57 \pm 4\%$), olive oil ($55 \pm 4\%$), papaya seed oil ($55 \pm 3\%$), rambutan seed oil ($55 \pm 2\%$), and rice bran oil ($55 \pm 2\%$). Low yields of biodiesel production (10–40%) were shown with sunflower oil ($39 \pm 5\%$), pomelo seed oil ($38 \pm 2\%$), cotton seed oil ($32 \pm 4\%$), pumpkin seed oil ($18 \pm 3\%$), and coconut oil ($14 \pm 2\%$).

Comparative studies of biodiesel production from palm oil with immobilized ABL and commercial immobilized lipases (Novozyme 435 and Lipozyme RM IM) are summarized in Table 2. Immobilized ABL gave the higher conversion percentage in contrast to lower methanol dosages and reusability frequency. Low reaction temperatures are desirable, as they are closely related to the energy cost inherent to the process of biodiesel production[35]. Immobilized ABL reacted at lower temperatures than those of commercial lipases. This might be interesting. Moreover, high yield of biodiesel produced in the presence of water might be useful for domestic operation because waste cooking oil containing water can be used as biodiesel

Table 2 Comparison of transesterification from palm oil catalysed by immobilized *A. baylyi* and commercial lipases.

Parameters	Immobilized lipase used in the reaction		
	Immobilized ABL	Novozyme 435	Lipozyme RM IM
Biodiesel production (% conversion)	93	72	79
Reaction temperature (°C)	40	55	55
Reaction time (h)	24	24	24
Methanol addition	6	3	3
Molar ratio (methanol: palm oil)	4:1	3:1	3:1
Enzyme concentration (% v/v)	20	20	20
Water content (% v/w)	4	0	0
Repeated usage (half-life)	2	5	1

feedstock[36,37]. However, the data for this study were not based on a commercial application and further examination in recognition of scale could provide helpful information for future large operations.

Conclusions

Thermophilic-solvent stable lipase from *A. baylyi* was immobilized on hydrophobic support, Sepabeads EC-OD. Optimal lipase activity and stability occurred at similar pH and temperature for both suspended and immobilized lipase. Nevertheless, immobilized lipase displayed slightly better stability at alkaline pH and in the presence of short-chain alcohols and improved storage stability. This immobilized lipase showed comparable efficiency on transesterification with commercial immobilized lipases (Novozyme 435 and Lipozyme RM IM) which could be applied to transesterify and/or hydrolyse a wide variety of oils contained within waste agricultural plant material, resulting in the efficient reuse of these waste products.

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REFERENCES

1. Demirbas A (2007) Progress and recent trends in biofuels. *Progr Energ Combust Sci* **33**, 1–18.
2. Meher LC, Vidya Sagar D, Naik SN (2006) Technical aspects of biodiesel production by transesterification—a review. *Renew Sustain Energ Rev* **10**, 248–68.
3. Du W, Li W, Sun T, Chen X, Liu DH (2008) Perspectives for biotechnological production of biodiesel and impacts. *Appl Microbiol Biotechnol* **19**, 331–7.
4. Ranganathan SV, Narasimhan SL, Muthukumar K (2007) An overview of enzymatic production of biodiesel. *Bioresour Tech* **99**, 3975–81.
5. Du W, Xu Y, Liu D, Li Z (2005) Study on acyl migration in immobilized lipozyme TL-catalyzed transesterification of soybean oil for biodiesel production. *J Mol Catal B* **37**, 68–71.
6. Hsu A, Jones KC, Foglia TA, Marmer WN (2002) Immobilized lipase-catalyzed production of alkyl esters of restaurant grease as biodiesel. *Biotechnol Appl Biochem* **36**, 181–6.
7. Nie K, Xie F, Wang F, Tan T (2006) Lipase catalyzed methanolysis to produce biodiesel: optimization of the biodiesel production. *J Mol Catal B* **43**, 142–7.
8. Shimada Y, Watanabe Y, Samukawa T, Sugihara A, Noda H, Fukuda H, Tominaga Y (1999) Conversion of vegetable oil to biodiesel using immobilized Candida Antarctica lipase. *J Am Oil Chem Soc* **76**, 789–93.
9. Uttatree S, Winayanuwattikun P, Charoenpanich J (2010) Isolation and characterization of a novel thermophilic-organic solvent stable lipase from *Acinetobacter baylyi*. *Appl Biochem Biotechnol* **162**, 1362–76.
10. Snellman EA, Colwell RR (2004) *Acinetobacter* lipases: molecular biology, biochemical properties and biotechnological potential. *J Ind Microbiol Biotechnol* **31**, 391–400.
11. Young DM, Parke D, Ornston LN (2005) Opportunities for genetic investigation afforded by *Acinetobacter baylyi*, a nutritionally versatile bacterial species that is highly competent for natural transformation. *Annu Rev Microbiol* **59**, 519–51.
12. Bornscheuer UT, Bessler C, Srinivas R, Krishna SH (2002) Optimizing lipase and related enzymes for efficient application. *Trends Biotechnol* **20**, 433–7.
13. Wang L, Du W, Liu D, Li L, Dai N (2006) Lipase-catalyzed biodiesel production from soybean oil deodorizer distillate with adsorbent present in tert-butanol system. *J Mol Catal B* **43**, 29–32.
14. Salis A, Sanjust E, Solinas V, Monduzzi M (2003) Characterization of Accurel MP1004 polypropylene powder and its use as support for lipase immobilization. *J Mol Catal B* **24-25**, 75–82.
15. Winayanuwattikun P, Kaewpiboon C, Piriyakananon K, Tantong S, Thakernkarnkit W, Chulalaksananukul W, Yongvanich T (2008) Potential plant oil feedstock for lipase-catalyzed biodiesel production in Thailand.

Biomass Bioenerg **32**, 1279–86.
16. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular Cloning. A Laboratory Manual*, 2nd edn, Cold Spring Harbor Laboratory Press, Plainview, NY.
17. Uttatree S, Charoenpanich J (2011) Nutritional requirements and physical factors affecting the production of organic solvent-stable lipase by *Acinetobacter baylyi*. *CMU J Nat Sci* **10**, 115–31.
18. Pencreac'h G, Baratii JC (1996) Hydrolysis of *p*-nitrophenyl palmitate in *n*-heptane by the *Pseudomonas cepacia* lipase: A simple test for the determination of lipase activity in organic media. *Enzym Microb Tech* **18**, 417–22.
19. Yan G, Yang G, Xu L, Yan Y (2007) Gene cloning, overexpression and characterization of a novel organic solvent tolerant and thermostable lipase from *Galactomyces geotrichum* Y05. *J Mol Catal B* **49**, 28–35.
20. Winayanuwattikun P, Kaewpiboon C, Piriyakananon K, Chulalaksananukul W, Yongvanich T, Svasti J (2011) Immobilized lipase from potential lipolytic microbes for catalyzing biodiesel production using palm oil as feedstock. *Afr J Biotechnol* **10**, 1666–73.
21. Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dry binding. *Anal Biochem* **72**, 248–54.
22. Petkar M, Lali A, Caimi P, Daminati M (2006) Immobilization of lipases for non-aqueous synthesis. *J Mol Catal B* **39**, 83–90.
23. Palomo JM, Mateo C, Fernández-Lorente G, Solares LF, Diaz M, Sánchez VM, Bayod M, Gotor V, Guisan JM, Fernandez-Lafuente R (2003) Resolution of (±)-5-substituted-6-(5-choloropyridin-2-yl)-7-oxo-5,6-dihydropyrrolo[3,4b]pyrazine derivatives-precursors of (*S*)-(+)-Zopiclone, catalyzed by immobilized *Candida antarctica B* lipase in aqueous media. *Tetrahedron* **14**, 429–38.
24. Fernandez-Lafuente R, Armisén P, Sabuquillo P, Fernández-Lorente G, Guisán JM (1998) Immobilization of lipase by selective adsorption on hydrophobic supports. *Chem Phys Lipids* **93**, 185–97.
25. Lei J, Fan J, Yu C, Zhang L, Jiang S, Tu B, Zhao D (2004) Immobilization of enzymes in mesoporous materials: controlling the entrance to nanospace. *Micropor Mesopor Mater* **73**, 121–8.
26. Palomo JM, Muñoz G, Fernández-Lorente G, Mateo C, Fernández-Lafuente R, Guisán JM (2002) Interfacial adsorption of lipases on very hydrophobic support (octadecyl-Sepabeads): immobilization, hyperactivation and stabilization of the open form of lipases. *J Mol Catal B* **19**, 279–86.
27. Bosley JA, Clayton JC (1994) Blueprint for a lipase support use of hydrophobic controlled-pore glasses as model systems. *Biotechnol Bioeng* **43**, 934–8.
28. Villeneuve P, Muderhwa JM, Graille J, Haas MJ (2000) Customising lipases for biocatalysis: a survey of chemical, physical and molecular biological approaches. *J Mol Catal B* **9**, 113–48.
29. Bastida A, Sabuquillo P, Armisen P, Fernández-Lafuente R, Huguet J, Guisán JM (1998) A single step purification, immobilization and hyperactivation of lipases via interfacial adsorption on strongly hydrophobic supports. *Biotechnol Bioeng* **58**, 486–93.
30. Chaubey A, Parshad R, Koul S, Taneja SC, Qazi GN (2006) *Arthrobacter* sp. lipase immobilization for improvement in stability and enantioselectivity. *Appl Microbiol Biotechnol* **73**, 598–606.
31. Antczak MS, Kubiak A, Antczak T, Bielecki S (2009) Enzymatic biodiesel synthesis-key factors affecting efficiency of the process. *Renew Energ* **34**, 1185–94.
32. Manjón A, Iborra JL, Arocas A (1991) Short-chain flavour ester synthesis by immobilized lipase in organic media. *Biotechnol Lett* **13**, 339–44.
33. Noureddini H, Gao X, Philkana RS (2005) Immobilized *Pseudomonas cepacia* lipase for biodiesel fuel production from soybean oil. *Bioresour Tech* **96**, 769–77.
34. Jegannathan KR, Jun-Yee L, Chan E-S, Ravindra P (2010) Production of biodiesel from palm oil using liquid core lipase encapsulated in κ-carrageenan. *Fuel* **89**, 2272–7.
35. Jeong G-T, et al (2004) Production of biodiesel fuel by transesterification of rapeseed oil. *Appl Biochem Biotechnol* **114**, 747–58.
36. Talebian-Kiakalaieh A, Amin NAS, Mazaheri H (2013) A review on novel processes of biodiesel production from waste cooking oil. *Appl Energ* **104**, 683–710.
37. Atadashi IM, Aroua MK, Aziz ARA, Sulaiman NMN (2012) The effects of water on biodiesel production and refining technologies: A review. *Renew Sustain Energ Rev* **16**, 3456–70.