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Purification and characterization of lipase from *Burkholderia* sp. EQ3 isolated from wastewater from a canned fish factory and its application for the synthesis of wax esters

Pakpimol Ungcharoenwiwat, Aran H-Kittikun*

Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, Hat Yai 90112, Thailand

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ABSTRACT

The aim of this study was to purify and characterize the lipase produced by *Burkholderia* sp. EQ3, a bacterium isolated from the wastewater pond of a canned fish factory. The lipase was purified to homogeneity with a 70-fold increase in its activity by chilled acetone precipitation, Q-Sepharose Fast Flow and Sephadex G-50 column chromatography, respectively. The molecular mass of the purified lipase was 30.7 kDa. The purified lipase showed optimal activity at pH 7.0–7.5 and 30 °C. The enzyme was stable over the pH range of 5.0–8.0 and temperatures between 30 and 55 °C for 1 h. Its half-life was 2 h at 55 °C. The lipase activity was enhanced in the presence of Ca^{2+}, Mg^{2+} and K^{+} while it was inactivated by Cu^{2+}, Fe^{2+} and Zn^{2+}. It hydrolyzed the natural triglycerides with its highest activity on coconut oil (120%), olive oil (101%) and palm olein (100%). Gum arabic (1%) significantly increased the lipase activity whereas 1.0 mM phenyl methyl sulphonyl fluoride strongly reduced the activity. The purified lipase EQ3 retained 80% activity in iso-propanol, but was inactivated by ethanol and iso-octane as well as other hydrophobic solvents. The lipase EQ3 was immobilized by adsorption with Accurel MP-100 and used for wax esters synthesis and compared with five commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM, Novozyme 435, Lipase PS and Lipase AK). The transesterification reaction was carried out between coconut oil, palm olein and jatropha oil with oleyl alcohol by using 1 U of enzyme, a substrate molar ratio of oil and oleyl alcohol of 1:3 in hexane at 37 °C and 150 rpm for 72 h. The immobilized lipase EQ3 was most efficient in the synthesis of wax esters with 60.3, 49.6 and 50.1% wax esters from coconut oil, palm olein and jatropha oil, respectively. For the five commercial immobilized lipases, Lipozyme RM IM exhibited the highest wax esters synthesis with 49.2, 41.4 and 48.1% wax esters, respectively.

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1. Introduction

Lipases (triacylglycerol acyl hydrolase, E.C. 3.1.1.3) are found widely in animals, plants, and microorganisms. Microbial lipases have become increasingly important in the last two decades for industrial applications [1]. Some novel biotechnological applications have been successfully established using lipases for the synthesis of biopolymers and biodiesel, the production of enantiopure pharmaceuticals, agrochemicals, flavor compounds and the conversion of natural fats and oils into high value products such as cocoa butter equivalent and oils enriched with omega-3 fatty acids [2]. Lipases from different sources display variations in properties in terms of their regioselectivity, fatty acid specificity, thermostability, optimum pH and kinetics in solvent systems [3]. Their characteristics have recently accounted for a marked increase in the industrial usage of lipases. The lipases from *Burkholderia* species are among the most widely used enzymes in biotechnology [4] especially from *Burkholderia cepacia* that shows very high transesterification activity in organic solvents [5,6].

Wax esters can be extracted from a variety of natural sources, including honeycomb, jojoba seeds, carnauba, sperm whale, skin lipids, sheep wool and seafowl feathers. They are used in lubricants, polishes, plasticizers and coating materials [7]. With the steadily growing demand for wax esters in the food, pharmaceutical and cosmetic industries, methods for the chemical or enzymatic synthesis of wax esters have been developed. The crude lipase from *Burkholderia* sp. EQ3 could synthesize 95% wax esters from crude fish fat and cetyl alcohol in 6 h [8]. In addition, the wax esters from palm kernel oil and oleyl alcohol were synthesized by Lipozyme RM IM and produced 84% wax esters after 10 h [9] and 90% oleyl oleate was synthesized by Lipozyme IM-20 in 2 h [10].

* Corresponding author. Tel.: +66 74286363; fax: +66 74558866.
E-mail address: aran.h@psu.ac.th (A. H-Kittikun).

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In previous work, we isolated a mesophilic lipolytic bacterium from the wastewater pond of a tuna canning factory in southern Thailand. It was identified and named *Burkholderia* sp. EQ3 using 16S rDNA analysis. It synthesized high amounts of wax esters from crude fish fat and cetyl alcohol [8]. In this study the purification and characterization of the lipase of *Burkholderia* sp. EQ3 has been described. The application of the lipase EQ3 for the synthesis of wax esters from different oils in comparison with commercial lipases was also investigated.

2. Materials and methods

2.1. Bacterial strain and immobilized lipases

Burkholderia sp. EQ3 producing an extracellular lipase was isolated from a wastewater pond of the tuna canning factory in southern Thailand and was cultivated in the basal medium in g/L of distilled water (tryptone 1.0, yeast extract 1.0, K_2HPO_4·2.0, KH_2PO_4·1.0, $(NH_4)_2SO_4$·1.0, $MgSO_4$·0.2 and $CaCl_2$·0.15) containing 1% fish oil [8]. This strain was maintained at −20 °C in a medium containing 25% glycerol. The nucleotide sequence data for *Burkholderia* sp. EQ3 appeared in the DDBJ/EMBL/GenBank nucleotide sequence database with the accession number AB563172.1. Immobilized lipases, Novozyme 435 (from *Candida antarctica*), Lipozyme RM IM (from *Rhizopus miehei*) and Lipozyme TL IM (from *Thermomyces lanuginosus*) were obtained from Novozymes (Bagsvaerd, Denmark). Lipase AK (from *Pseudomonas fluorescens*) and lipase PS (from *Burkholderia cepacia*) were provided by Amano (Nagoya, Japan) and were immobilized in the laboratory according to Pawongrat et al. [11] by adsorption onto Accurel MP-100 (size 200–400 μm). The immobilized lipases were stored at 4 °C until used.

2.2. Substrates and chemicals

Coconut oil, soybean oil, olive oil, palm stearin, sunflower oil and palm olein (saponification values; 226, 165, 170, 176, 168 and 173, molecular weights; 735, 1011, 982, 947, 987 and 952 g/mole, respectively) were from a local market (Hat Yai, Thailand). Fish oil and jatropha oil (saponification value; 127 and 558, molecular weight; 302 and 1200 g/mole, respectively) were purified in the laboratory.

Q-Sepharose Fast Flow and Sephadex G-50 were from Amersham Pharmacia Biotech (Uppsala, Sweden). Chromatein Prestained Protein Ladder® (10.5–175 kDa) was from Vivantis (Selangor Darul Ehsan, Malaysia). The Silver Stain Plus kit was from Bio-Rad Laboratories, Inc. (Hercules, California, USA). Celite 545 (200 μm) was from Fluka (Buchs, Switzerland) and Accurel MP-100 (size 200–400 μm) was from Akzo Nobel Membrana (Obernburg, Germany). Oleyl alcohol was supplied by Sigma–Aldrich (St. Louis, Missouri, USA). Other analytical reagent grade chemicals were from local commercial sources.

2.3. Analyses

2.3.1. Lipase activity

The lipase activity was assayed in a two-phase system according to Lee and Rhee [12] using palm olein as a substrate in isooctane. The reaction was incubated at 37 °C by shaking at 300 rpm for 30 min. The lipase activity was determined by measuring the amount of fatty acids liberated as palmitic acid. One unit of enzyme activity was defined as the enzyme necessary to release 1 μmol of palmitic acid per minute at the specified conditions [8].

2.3.2. Determination of protein

The protein in the supernatants and samples from the purification steps as well as the immobilized enzyme was determined using Folin–Ciocalteu reagent according to Lowry et al. [13]. Bovine serum albumin was used as a standard. During chromatographic purification steps, the protein concentration was monitored by measuring the absorbance of fractions at 280 nm (A_{280}) [14].

2.3.3. Determination of wax esters

The percentage of wax esters was determined by thin-layer chromatography and a flame ionization detector (TLC-FID) (IATROSCAN MK5, Iatron Laboratories Inc. (Tokyo, Japan)). The sample diluted in chloroform (1:2, v/v) was spotted onto the chromarod S III (coated with silica gel powder) from Mitsubishi Chemical Medicine Corporation (Tokyo, Japan) and developed for 30 min in a mixture of hexane/diethyl ether/formic acid (64:6.4:0.4, v/v/v) [15]. After development and drying, the rods were subjected to scanning with FID. The percentage of the peak area was assumed to be the percentage content of the corresponding compound [8].

3. Methods

3.1. Lipase production

Burkholderia sp. EQ3 was cultivated in the basal medium containing 1% fish oil [8] as described above. The pH of the medium was adjusted to 6.0 with 0.1 M HCl. Fifty milliliter of the medium in a 250 mL Erlenmeyer flask was seeded with a 10% inoculum and incubated on a rotary shaker at 200 rpm and 37 °C. After 12 h of incubation, the culture supernatant containing extracellular lipase was harvested by centrifugation at 6000 × *g* at 4 °C for 15 min. The cell-free culture supernatant was used for lipase purification.

3.2. Lipase purification

3.2.1. Acetone precipitation

The cell free supernatant was precipitated with 70% chilled acetone overnight [8,16]. The precipitate was collected by centrifugation at 6000 × *g* at 4 °C for 15 min and dissolved in a small volume of 20 mM Tris–HCl buffer pH 7.5. The crude enzyme suspension was dialyzed against the same buffer at 4 °C overnight and concentrated by absorption on carboxymethyl cellulose.

3.2.2. Ion exchange chromatography

The enzyme solution (2.0 mL) after dialysis was applied to the Q-Sepharose Fast Flow (10 mL, 2.2 cm × 3.2 cm) anion exchange column equilibrated with 20 mM Tris–HCl buffer pH 7.5. The column was washed with the same buffer, and the unbound and bound proteins were eluted with a linear gradient from 0 to 2.0 M NaCl. The flow rate was 1.0 mL/min. The collected fractions were assayed for lipase and protein (A_{280}). The lipase active fractions were pooled, dialyzed against 20 mM Tris–HCl buffer pH 7.5 and concentrated by absorption on carboxymethyl cellulose and used for Sephadex G-50 gel filtration chromatography.

3.2.3. Gel column chromatography

The enzyme obtained from the Q-Sepharose Fast Flow step was applied to the Sephadex G-50 column (0.8 cm × 50 cm) previously equilibrated with 20 mM Tris–HCl buffer (pH 7.5). The elution was done using the same buffer at a flow rate of 0.2 mL/min. Fractions of 1.0 mL were collected and assayed for both lipase activity and protein (A_{280}). The lipase active fractions were pooled and concentrated. The specific activity of the purified enzyme of each purification step was compared with that of the crude enzyme and the purification fold was calculated. The samples from the purification process were used for sodium dodecyl sulfate-polyacrylamide

gel electrophoresis (SDS-PAGE). The purified lipase EQ3 from the final purification process was used for enzyme characterization studies.

3.3. Polyacrylamide gel electrophoresis

The purity and molecular weight of lipase were determined by SDS-PAGE carried out according to the method described by Laemmli [17]. The SDS-PAGE was performed using a 12% separating gel and a 5% stacking gel. The gel was stained using the Silver Stain Plus kit for protein detection. The protein molecular weight markers used for SDS-PAGE was a Chromatein Prestained Protein Ladder® (10.5–175 kDa) (Vivantis).

3.4. Staining for lipase activity

The Native-PAGE gel of 12% polyacrylamide was prepared according to Laemmli [17]. After protein separation, the gel was washed by soaking at room temperature with gentle mixing once in 20 mM Tris–HCl buffer (pH 7.5) with 2.5% (v/v) Triton X-100 for 30 min and twice in the same buffer for 30 min [18]. The gel was transferred onto a 1.5% agar plate containing an emulsion of 1% (w/v) palm olein, 0.1% (w/v) gum arabic and 0.001% (w/v) rhodamine B in 20 mM Tris–HCl buffer, pH 7.5. After incubation for 6 h at 37 °C, the lipase activity was visualized as a band of orange fluorescent under a UV light on the palm olein plate [19].

3.5. Characterization of purified lipase from Burkholderia sp. EQ3

3.5.1. Effect of temperature and thermostability

The optimum temperature for the activity of the purified lipase EQ3 was determined over the temperature range of 30–65 °C in 20 mM Tris–HCl buffer, pH 7.5. For determination of its thermostability, the purified lipase was incubated at different temperatures from 30–65 °C for 1 h in 20 mM Tris–HCl buffer (pH 7.5) before assaying the residual activity at 30 °C.

3.5.2. Effect of pH and pH stability

To study the effect of pH on the lipase EQ3 activity, the purified lipase activity was assayed at 30 °C between pH 4.0 and 9.0 using 20 mM of different buffers; acetate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0–7.0), Tris–HCl buffer (pH 7.0–9.0). For the pH stability study, one volume of the purified lipase was mixed with three volumes of the above buffers and the mixture was incubated at 30 °C for 2 h. The lipase activity was then assayed at pH 7.5.

3.5.3. Effect of metal ions

For determining the effect of metal ions on the lipase EQ3 activity, the purified lipase was pre-incubated in the reaction mixture with a final concentration of 1.0 mM of the following metal ions $CuCl_2$, $CaCl_2$, $MgCl_2$, KCl, LiCl, NaCl, $CoCl_2$, $ZnCl_2$ and $HgCl_2$ at 30 °C for 30 min in 20 mM Tris–HCl buffer (pH 7.5). The residual activity was then determined.

3.5.4. Effect of inhibitors and surfactants

The effect of various inhibitors and surfactants on the lipase EQ3 activity was investigated by incubating the purified enzyme for 30 min at 30 °C in a 20 mM Tris–HCl buffer (pH 7.5) containing the following chemical agents; inhibitors (1.0 mM): ethylene diamine tetra-acetic acid (EDTA), β-mercaptoethanol, phenyl methyl sulphonyl fluoride (PMSF) and surfactants (1.0%, w/v): Tween 20, Tween 80, Triton X-100, gum arabic and SDS. The lipase activity was then measured and compared relative to the control (without chemical agents).

3.5.5. Effect of organic solvents

The effect of various organic solvents at a concentration of 25% (v/v) on the lipase EQ3 activity was investigated as follows: 0.15 mL of organic solvent was added to 0.45 mL of lipase solution in a screw-cap test tube. The mixture was incubated for 1 h at 30 °C with shaking at 150 rpm. The solvents were selected based on their different polarity index value as follows: ethanol (5.2), methanol (5.1), acetone (5.1), ethyl acetate (4.4), isopropanol (3.9), xylene (2.5), toluene (2.4), isooctane (0.1) and hexane (0.1) [20]. The lipase activity was then measured and compared relative to the control (enzyme solution in the pH 7.5 buffer without solvent).

3.5.6. Substrate specificity

To determine the substrate specificity of the lipase EQ3 toward natural substrates; olive oil, corn oil, sunflower oil, coconut oil, soybean oil, rice bran oil, palm olein and jatropha oil were prepared as substrates instead of palm olein for lipase activity determination by the cupric acetate method [12].

3.6. Immobilization of lipase

After precipitation with 70% chilled acetone followed by dialysis, the crude enzyme was immobilized with Celite 545 or Accurel MP-100. 0.5 g of carrier was pre-wetted with 1.5 mL isopropanol and added to 5 mL (75 U) of the crude lipase. The mixture was stirred using a magnetic stirrer at room temperature for 1 h. Then, the mixture was filtered, washed twice with 10 mL of 20 mM Tris–HCl buffer pH 7.5. The immobilized lipase EQ3 was collected and dried in a desiccator overnight and kept at 4 °C [2,11]. The hydrolytic activity of un-adsorbed lipase and immobilized lipase was determined by the cupric acetate method [12]. The activity yields were calculated as follows:

$$\text{Activity yield}\,(\%) = \frac{\text{Total immobilized lipase activity}\,(\text{U}) \times 100}{\text{Total initial soluble lipase activity}\,(\text{U})}$$

3.7. Application of immobilized lipase EQ3 for synthesis of the wax esters

Commercial oils (fish oil, coconut oil, jatropha oil, olive oil, palm olein, palm stearin, sunflower oil and soybean oil) were used for the synthesis of wax esters by the alcoholysis reaction with oleyl alcohol using the immobilized lipase EQ3 in comparison with five commercial immobilized lipases (Lipozyme RM IM, Lipozyme TL IM, Novozyme 435, Lipase PS and Lipase AK). An amount of 150 μmole of oil was dissolved in 1.0 mL of hexane and mixed with 450 μmole of oleyl alcohol (the molar ratio of 1:3) in a 10 mL screwed cap tube. The immobilized lipase (1 U) was added and mixed by shaking at 150 rpm and 37 °C for 72 h. A 10 μL aliquot was withdrawn and diluted with chloroform 20 μL. The percentage of wax esters synthesis was determined using TLC-FID [8].

4. Results and discussion

4.1. Lipase production

The lipase production from *Burkholderia* sp. EQ3 in the basal medium containing fish oil was carried out by incubation at 37 °C on the rotary shaker at 200 rpm and the highest growth was at 36 h while the highest lipase activity was obtained at 12 h [8]. The cell-free supernatant from the 12 h culture broth contained lipase 2.3 U/mL and 2.2 U/mg protein. This supernatant was used for enzyme purification.

Table 1
Summary of lipase purification from *Burkholderia* sp. EQ3.

Purification step	Total protein (×10^2 mg)	Total activity (×10^2 U)	Specific activity (U/mg)	Yield (%)	Purification (fold)
Supernatant	10.9	23.4	2.2	100	1.0
Acetone ppt	0.42	11.9	28.4	51.1	13.2
Q-Sepharose FF	0.32	10.1	31.5	43.2	14.6
Sephadex G-50	0.01	1.78	153	7.6	70.9

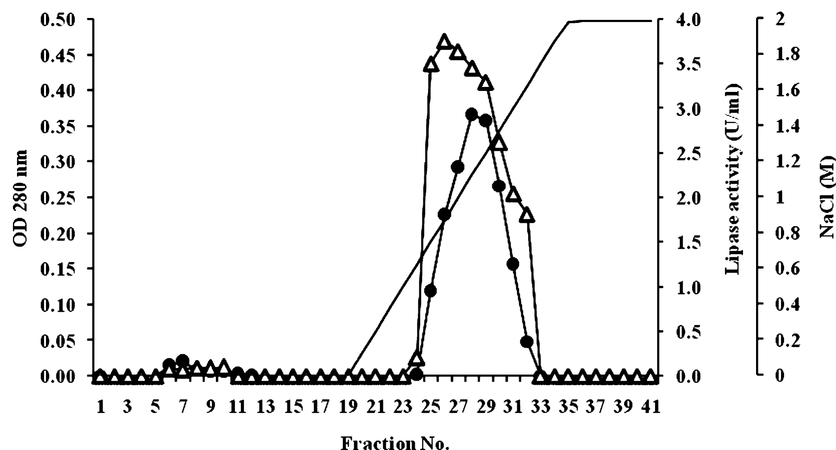

Fig. 1. Elution profile of *Burkholderia* sp. EQ3 lipase from a Q-Sepharose Fast Flow ion-exchange column. (–●–) Protein concentration by absorbance at 280 nm, (–△–) lipase activity in the fractions and (▬) NaCl gradient (0–2 M).

4.2. Purification of lipase from *Burkholderia sp. EQ3*

The lipase EQ3 was purified by a three-step sequential method consisting of 70% chilled acetone precipitation, Q-Sepharose Fast Flow anion-exchange chromatography and Sephadex G-50 gel filtration chromatography. The results of the purification profile of the lipase EQ3 are summarized in Table 1. The crude lipase was precipitated with 70% chilled acetone and then the precipitate was reconstituted in a minimum volume of 20 mM Tris–HCl buffer, pH 7.5, and dialyzed against this buffer. The dialyzed lipase was obtained with an overall yield of 51.1% and a 13.2-fold increase of activity.

The partially purified lipase from the previous step was further purified by anion exchange chromatography using Q-Sepharose Fast Flow. The enzyme was eluted at 1.0–1.5 M NaCl and showed a single peak when the absorbance was taken at 280 nm (Fig. 1). After that, the concentrated lipase EQ3 was loaded onto the Sephadex G-50 gel filtration column and showed two peaks (Fig. 2). Only the first peak showed lipase activity with a total activity of 178 U. After purification by Sephadex G-50, the lipase had a 70.9-fold increase in activity with a 7.6% yield. This recovery of the lipase activity was marginally lower than those reported for the purification of other *Burkholderia* lipases (4–12%) [14,19]. The lipase from *Burkholderia multivorans* PSU-AH130 was purified by a three-step sequential method consisting of precipitation with 70% saturated $(NH_4)_2SO_4$, DEAE-Toyopearl ion-exchange chromatography and Sephadex G-150 gel filtration chromatography with a 21.6-fold increase of activity and a yield of 12.1% [14]. However, the lipase from *B. cepacia* ATCC 25416 was purified by three steps consisting of $(NH_4)_2SO_4$ precipitation, phenyl-sepharose column and a DEAE Sepharose column, with a 4.9-fold increase of activity and a low yield of 4.8% [19].

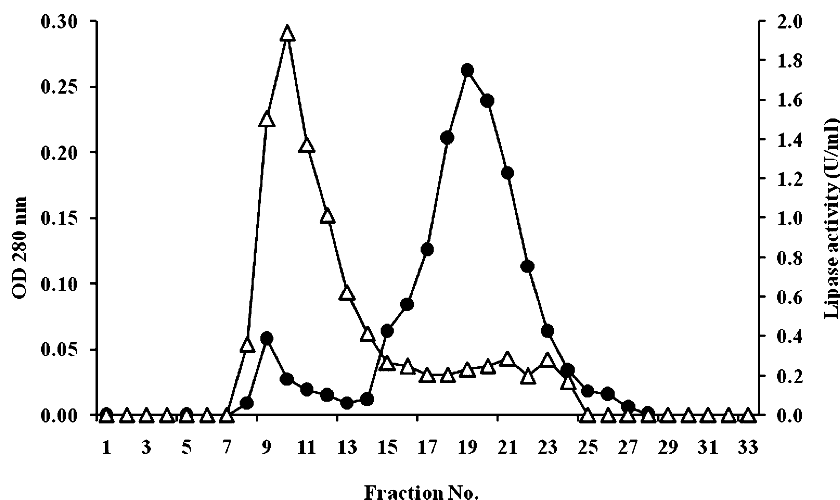

Fig. 2. Elution profile of *Burkholderia* sp. EQ3 lipase from a Sephadex G-50 gel filtration column. (–●–) Protein concentration by absorbance at 280 nm and (–△–) lipase activity in the fractions.

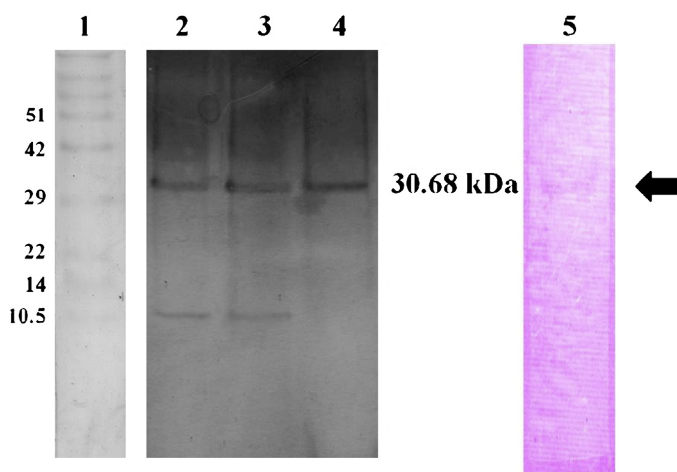

Fig. 3. SDS-PAGE of lipase from *Burkholderia* sp. EQ3. Lane 1: standard protein markers; lane 2: acetone precipitation step; lane 3: Q-Sepharose fast flow and land 4: Sephadex G-50 filtration; lane 5: activity staining of lipase from the acetone precipitation step.

4.3. Determination of molecular weight by SDS-PAGE

The purity of the lipase EQ3 was confirmed as it produced a single band with a relative molecular mass of 30.7 kDa as determined by SDS-PAGE analysis (Fig. 3). Moreover, staining of the lipase activity in Native-PAGE gel of the protein obtained from acetone precipitation step confirmed its actual size and the lipase from *Burkholderia* sp. EQ3 was a single protein (Fig. 3). Most of the known *Burkholderia* sp. lipases have been reported to have a molecular mass in the range of 29–39 kDa, e.g., 33 kDa for *B. multivorans* [21], *B. cepacia* strain G63 [22] and *B. cepacia* ATCC 25416 [23], and 39 kDa for *B. cepacia* LP08 [24].

4.4. Characterization of lipase from Burkholderia sp. EQ3

4.4.1. Effect of temperature on lipase activity and stability

The optimum temperature for the purified lipase activity from *Burkholderia* sp. EQ3 was determined. The lipase EQ3 had an activity over a broad range of temperatures between 30 and 55 °C with a maximum activity at 30 °C and showed a slightly decreased activity at 37, 45 and 55 °C (Fig. 4A). The enzyme activity at 55 °C was still higher than 80% of the activity at 30 °C but it decreased dramatically at 65 °C. According to Chakraborty and Paulraj [25], the extracellular lipase from *Pseudomonas fluorescens* MTCC 2421 was active in the temperature range of 35–50 °C with the maximal activity at 40 °C and the enzyme lost 44% of the activity at a higher temperature (50 °C). Moreover, the optimal temperature of lipases from *B. cepacia* ST 200 [26] and *P. pseudoalcaligenes* F-111 [27] was 45 °C and 40 °C, respectively whereas the optimal temperature of lipase from *B. multivorans* AH-130 was 55 °C [14].

The thermal stability profile of the purified lipase EQ3 was studied by measuring the activity after incubating the enzyme at various temperatures for 2 h (Fig. 4B). The enzyme was stable and exhibited more than 60% relative activity over the range of 30–55 °C with maximum stability at 30 °C. This lipase showed a high stability after 1 h at 37, 45 and 55 °C which retained 72, 63 and 64% activity compared to 30 °C, respectively. The half-life of this lipase at 45 and 55 °C was 2 h. The lipase EQ3 showed similar stability to other bacterial lipases. The lipase from *P. fluorescence* MTCC 2421 was stable at 35–45 °C with more than 75% residual activity after 30 min of incubation [25]. Moreover, the lipase from *Burkholderia* sp. HY-10 exhibited 50% activity at 60 °C for 30 min [28].

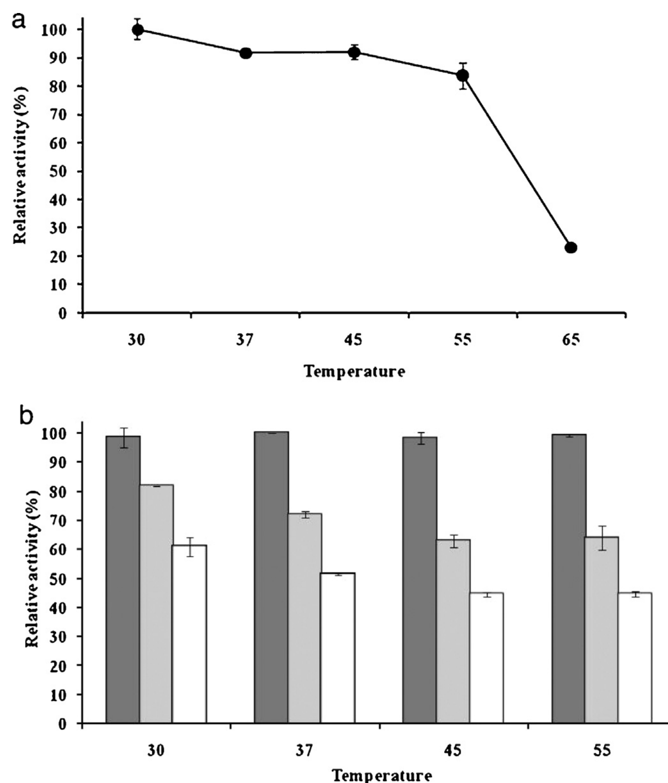

Fig. 4. Effect of temperature on the activity (A) and stability (B) of lipase from *Burkholderia* sp. EQ3. (—●—) lipase activity, (■) lipase stability at 0 h, (■) at 1 h and (□) at 2 h.

4.4.2. Effect of pH on lipase activity and stability

The purified *Burkholderia* sp. EQ3 lipase was active over a wide range of pH values 6.0–8.0 with more than 80% activity and had an optimum pH value at 7.0–7.5 (Fig. 5). The activity of the enzyme increased from pH 4.0–7.5. Its activity dropped drastically below a pH of 5.0 and above 8.0. It has been reported that most lipases produced by *Burkholderia* species had optimal activity in neutral and alkaline conditions. The lipase from *B. cepacia* LP08 showed an activity over a very wide pH range (5.0–10.6) and the maximal activity was observed at pH 9.0 [24]. Moreover, the lipase from *B. multivorans* PSU-AH130 had more than 80% relative activity in the pH range of 7.5–10.0 [14]. From the pH profile in this study the lipase EQ3 was active over a wide pH range and belonged to the neutral lipase family.

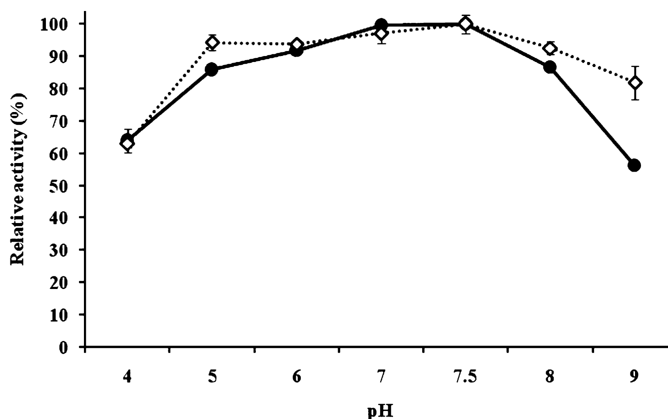

Fig. 5. Effect of pH on the activity and stability of lipase from *Burkholderia* sp. EQ3. (—●—) lipase activity, (-◇-) lipase stability.

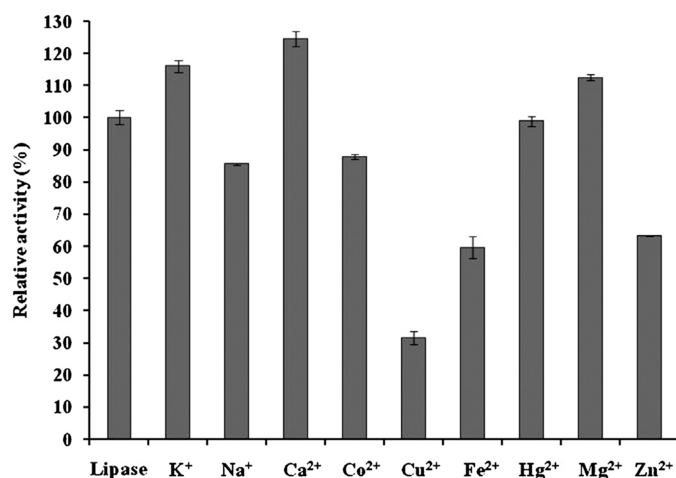

Fig. 6. Effect of metal ions on the activity of lipase from *Burkholderia* sp. EQ3.

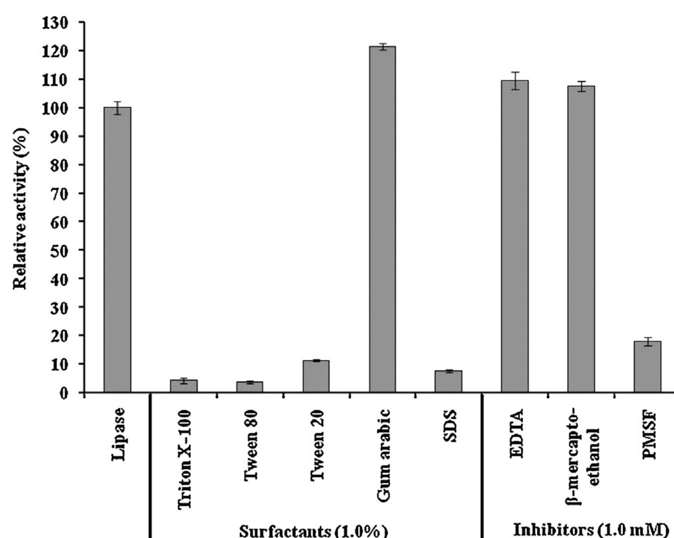

Fig. 7. Effect of surfactants and chemicals on the activity of lipase from *Burkholderia* sp. EQ3.

The pH stability of the lipase EQ3 was investigated after incubating at 30 °C for 1 h in buffers of pH 4.0–9.0 (Fig. 5). *Burkholderia* sp. EQ3 lipase retained more than 80% relative activity over a wide range of pH 5.0–9.0. The relative activity of lipase increased when the pH value increased but it decreased to 63% relative activity in the pH 4.0 buffer. In comparison, the lipase from *Pseudomonas mendocina* PK-12CS was very stable at a broad pH range of 5.6–9.0 [29]. The *Pseudomonas* sp. AG-8 lipase showed high stability at pH 7.0–10.0 [30] and *Pseudomonas aeruginosa* LST-03 lipase was very stable at pH 5.0–8.0 [31]. The stability of *B. cepacia* DSM 3401 lipase was nearly constant in the pH range 5.0–9.0 [32]. Moreover, the purified lipase from *B. multivorans* PSU AH130 showed good stability in the range of pH 7.0–9.0 at 45 °C for 3 h [14].

4.4.3. Effect of metal ions on lipase activity

Various metal ions were studied for their effects on the activity of the purified lipase EQ3 (Fig. 6). The lipase EQ3 activity was strongly enhanced in the presence of Ca^{2+} (124% relative activity). Calcium ion has been reported to form complexes with ionized fatty acids, changing their solubility and behaviors at interfaces [33]. Moreover, the calcium-induced increase on lipase activity could be attributed to the complex action of calcium ion on the released fatty acids. In addition, the enzyme structure was stabilized due to the binding of calcium ions to the lipase, bridging the active region to a second subdomain of the protein and hence stabilizing the enzyme tertiary structure [34]. The K^{+} and Mg^{2+} also had a stimulatory effect on the lipase activity whereas other metal ions (Na^{+} and Co^{2+}) inhibited the lipase activity. Many lipases from *Pseudomonas* [35,36] were strongly inhibited by Hg^{2+} but it had no effect on the lipase EQ3. However, in the present of Zn^{2+}, Fe^{2+} and Cu^{2+}, the lipase activity was reduced to 63, 60 and 31%, respectively. In contrast, Zn^{2+} decreased the lipolytic activity of the lipase of *B. multivorans* PSU AH130 by 18% and Cu^{2+} increased its activity [14]. The inhibitory nature of metals has been thought to be due to interaction of ions with charged side chain groups of surface amino acids, thus influencing the conformation and stability of the enzyme [37].

4.4.4. Effect of inhibitors and surfactants on lipase activity

The effect of inhibitors (1.0 mM) on the lipase EQ3 activity was studied after 30 min duration at 30 °C and a pH of 7.5 (Fig. 7). For the chelating agent, EDTA, it had no effect on the enzyme activity. Similar results were obtained by Dharmsthiti and Kuhasuntisuk [38], the lipase from *P. aeruginosa* LP602 was insensitive to EDTA. Moreover, the results showed that the addition of β-mercaptoethanol also had no effect on the lipase activity (107%). Hence the lipase enzyme produced by *Burkholderia* sp. EQ3 is a non-metallo monomeric protein with no disulfide bond in the structure. Dandavate et al. [16] found that the lipase activity of *B. multivorans* V2 was highly stimulated (about 4-fold higher) in the presence of β-mercaptoethanol.

PMSF is a serine specific inhibitor that displayed strong inhibition on lipase EQ3 activity with 18.2% relative activity. This may be caused by modification of an essential serine residue that plays a key role in the catalytic mechanism [39]. In addition, *B. multivorans* MV2 lipase was inhibited by PMSF to indicate that it was a serine hydrolase [16]. The study of Wang et al. [23] showed PMSF inhibitory effect on the lipase activity of *B. cepacia* ATCC 25416 since the catalytic center of the lipase contained the Ser-His-Asp triad.

Surfactants are known to increase the lipid water interfacial area, which in turn enhanced the rate of lipase catalyzed reactions [40]. Thus, an addition of surfactants provided a simple method to improve the reaction efficiency of lipases. The effect of surfactants (1.0%) on the lipase activity is shown in Fig. 7. Surfactants reduced the interfacial tension between oil and water and increased the lipid–water interfacial area [31]. However different surfactants had different effects on lipase activity. For the lipase EQ3, non-ionic surfactants such as Triton X-100, Tween 20 and Tween 80 inhibited the lipase activity. The lipase from *Pseudomonas* strain was also inactivated in the presence of Tween-20 and Tween-80 [41], and the lipase of *P. cepacia* had a loss of activity in 0.4% Triton X-100 [42]. In this study, only gum arabic enhanced the lipase EQ3 activity. Peng et al. [43] found that 1.2% gum arabic increased the lipase activity of *P. aeruginosa* CS-2 with a 130% relative activity. In addition, an anionic surfactant such as SDS inactivated the lipase EQ3 activity. However, the ionic surfactants (SDS, CTAB) only slightly inhibited the enzyme activity of *B. cepacia* ATCC 2541 [23].

4.4.5. Effect of organic solvents on lipase activity

Lipase catalyzes the hydrolysis reaction of water-insoluble substrates as the reaction must occur at an interface. The use of a mixture of water and organic solvents can facilitate the hydrolysis reaction of water-insoluble substrates by the lipase. The high activity and stability of lipases in organic solvents are desirable for biotransformation. The effect of organic solvents with various polarity indexes at 25% (v/v) concentration on the lipase activity of *Burkholderia* sp. EQ3 was studied (Table 2). Among nine different polar and non-polar organic solvents (polarity index 0.0–5.2) tested, the lipase EQ3 activity in the presence of organic solvents decreased after 1 h incubation. The lipase EQ3 showed the highest

Table 2
Effect of organic solvents on lipase activity of *Burkholderia* sp. EQ3.

Organic solvents (25%)	Polarity index[a]	Relative activity (%)
Ethanol	5.2	1.9 ± 0.9
Methanol	5.1	7.9 ± 0.9
Acetone	5.1	21.1 ± 2.0
Ethyl acetate	4.4	33.9 ± 0.7
Isopropanol	3.9	80.2 ± 0.8
Xylene	2.5	17.6 ± 0.5
Toluene	2.4	15.9 ± 0.4
Isooctane	0.1	1.6 ± 0.5
Hexane	0.1	10.4 ± 0.9
None	–	100 ± 0.8

[a] The polarity index was referred from Sadek [20].

stability in the presence of isopropanol with 80% relative activity. Moreover, hydrophilic solvents such as ethanol and methanol reduced the enzyme activity to 2–8% relative activity while with acetone and ethyl acetate there was 21 and 34% relative activity, respectively. The enzyme was not miscible with low polarity index solvents such as xylene, toluene, isooctane and hexane so they reduced lipase activity.

According to the polarity index value, highly polar organic solvents such as the various alcohols inhibited the lipase activity by reducing the water activity around the protein molecules and then promoted structural denaturation [26]. Other research had also found that the lipase of *B. cepacia* was not tolerant to any alcohols [44]. In addition, Chaiyaso et al. [14] found that the lipase of *B. multivorans* PSU-AH130 was deactivated by ethanol, acetone and methanol. However, the lipase from *Pseudomonas monteilii* TKU009 retained 50–75% activity in isopropanol, acetone, and ethanol and 80% of its activity was retained in methanol [45]. Hence the water miscibility was not the only critical factor of the solvents that affected enzyme stability. Other factors such as the solvent molecular structures and their functional groups as well as the enzyme structure and the type of surface amino acids may also play their roles [46]. Liu et al. [47] described the influence of different organic solvents on lipase structure and activity; the hydrophobicity, functional groups and molecular constitution of the organic solvents affected the microenvironment moisture around the catalytic active site leading to effects on activity as well as the protein's secondary structure. Moreover, the conformation change of *B. cepacia* lipase in various organic solvents analyzed by far UV-CD spectroscopy showed that there was decreased of the α-helix content with an increase of the *B. cepacia* lipase activity in the reaction medium [48].

4.4.6. Substrate specificity

To determine the substrate specificity, the purified lipase activity toward different natural oils was measured and the results are shown in Fig. 8. The hydrolytic activity toward palm olein was set as a control with 100% relative activity measured at pH 7.5 and 30 °C. The purified lipase from *Burkholderia* sp. EQ3 efficiently hydrolyzed a variety of vegetable oils and showed a high affinity for medium chain-length substrates. The lipase EQ3 showed the highest hydrolytic activity toward coconut oil with 120% relative activity. Moreover, the activity of lipase EQ3 toward olive oil and palm olein was similar. The use of corn oil and rice bran oil as a substrate showed 88% relative activity while the sunflower oil was less hydrolyzed by this lipase. The hydrolytic activity of jatropha oil and fish oil by lipase EQ3 was similar with 40% relative activity. The lipase from *B. cepacia* [5] showed high activity toward olive oil, coconut oil and jatropha oil.

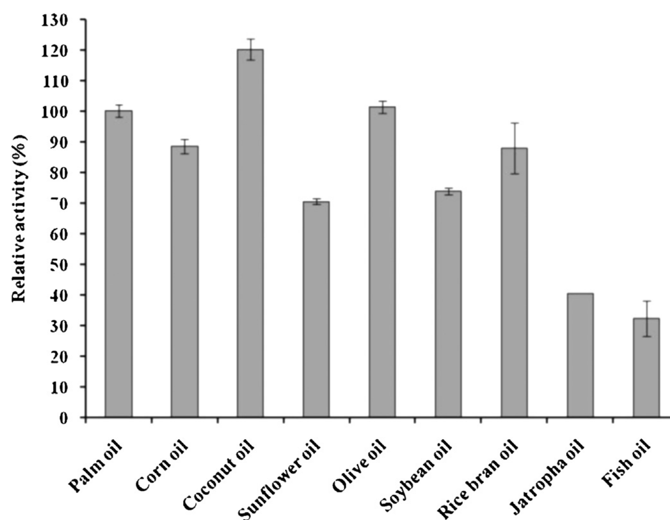

Fig. 8. Substrate specificity of lipase from *Burkholderia* sp. EQ3.

4.5. Immobilization of lipase from Burkholderia sp. EQ3

The lipase from *Burkholderia* sp. EQ3 was immobilized onto two types of carriers, Accurel MP-100 and Celite 545 by the adsorption method. Accurel MP-100 is polypropylene which is hydrophobic in nature while Celite 545 is a diatomaceous earth which is hydrophilic. The lipase EQ3 immobilized on Accurel MP-100 demonstrated very high lipolytic activity at 0.16 U/mg support and gave a 97% activity yield. The immobilized lipase on Celite 545 showed low activity (0.01 U/mg support) and had a 6% activity yield. In this investigation, when the lipase EQ3 was immobilized on Celite 545, it lost over 90% of its activity. Lipase immobilized on silica might be lost from the system because of desorption, severing of chemical bonds or erosion of the support material [49]. Nevertheless, the adsorption may predominantly occur on the external surface of the support due to the high molecular dimensions of the enzyme or also due to the hydrophobic interactions of the enzyme and the matrix, that caused a conformational change in the secondary and tertiary structures of the protein molecule, and led to a lower yield after immobilization [50]. This is why immobilization on Celite caused such a substantial lowering of activity. The better activity with Accurel MP-100 might be due to the stronger adsorption of the hydrophobic enzyme onto the polypropylene surface. Enzymes with a large lipophilic surface area will interact well with a hydrophobic carrier [51]. Kaewthong and H-Kittikun [2] reported that immobilization of lipase PS on Accurel MP-100 displayed the best immobilized activity of 0.37 U/mg support. Thus, the immobilized lipase EQ3 on Accurel MP-100 was selected for the synthesis of wax esters.

4.6. Application of immobilized lipase EQ3 for wax esters synthesis

Eight kinds of commercial oils were screened for wax esters synthesis by the transesterification reaction with oleyl alcohol using lipase EQ3 immobilized on Accurel MP-100. The TLC chromatogram of the wax esters synthesized and the results are shown in Figs. 9 and 10. The immobilized lipase EQ3 synthesized the highest amount of wax esters with coconut oil, palm olein and jatropha oil with the percentage of wax esters of 60.3, 49.6 and 50.1% at 72 h, respectively. Moreover, the crude lipase from *Burkholderia* sp. EQ3 had been reported to synthesize wax esters from crude fish fat and cetyl alcohol with 95% wax esters after 6 h [8]. Five commercial immobilized lipases; Lipozyme RM IM (11.5 U/mg protein),

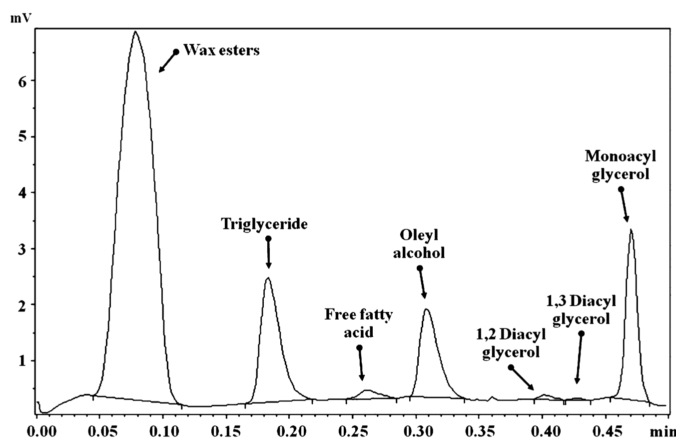

Fig. 9. The TLC chromatogram of wax ester synthesis.

Lipozyme TL IM (10.6 U/mg protein), Novozyme 435 (6.2 U/mg protein), Lipase PS (44.2 U/mg protein) and Lipase AK (41.7 U/mg protein) were used for the alcoholysis of coconut oil, palm olein and jatropha oil with oleyl alcohol in comparison with the immobilized lipase EQ3 (15.3 U/mg protein). The reaction was carried out with 1 U of lipase for 72 h and the results are shown in Fig. 11. The commercial immobilized lipases, Lipozyme RM IM exhibited the highest percentage of wax esters in all oils after 72 h (49.2, 41.4 and 48.1% of coconut oil, palm olein and jatropha oil esters) whereas Lipase AK gave a slightly lower wax ester. However, Lipozyme TL IM, Novozyme 435 and Lipase PS synthesized a much lower amount of wax esters. These results revealed that the hydrolytic activities did not corresponded to their wax esters synthesis because the highest specific activity of Lipase PS had a low synthesis of wax esters. So, it might be concluded that the wax esters synthesis depended on the capability of each lipase to catalyze the synthetic reaction. Salis et al. [52] reported that the sn-1, 3-specific lipase, Lipozyme RM IM was more active than the non-specific lipase, Novozyme 435 toward wax ester production via transesterification. Their work showed that the production of wax esters from sheep milk fat by Lipozyme RM IM produced a higher yield (90 mol%) than Novozyme 435 (70 mol%). In our study, the immobilized lipase EQ3 synthesized a higher percentage of wax esters than five of the commercial immobilized lipases tested.

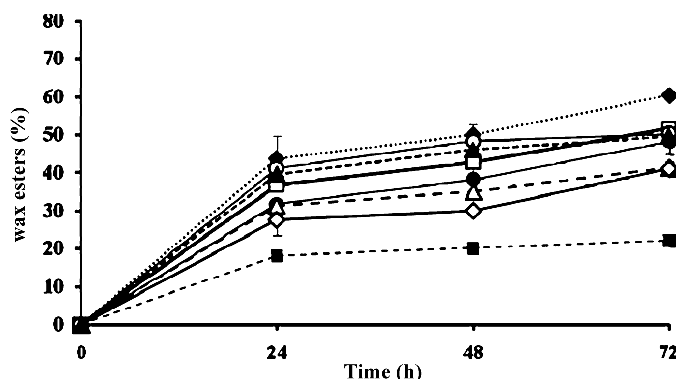

Fig. 10. Effect of oils on the wax esters synthesis by the immobilized lipase of *Burkholderia* sp. EQ3. (···◆···) coconut oil, (—●—) soybean oil, (– △ –) olive oil, (—◇—) palm oil, (—□—) sunflower oil, (– ■ –) fish oil, (—○—) jatropha oil and (--▲--) palm stearin based wax esters synthesis.

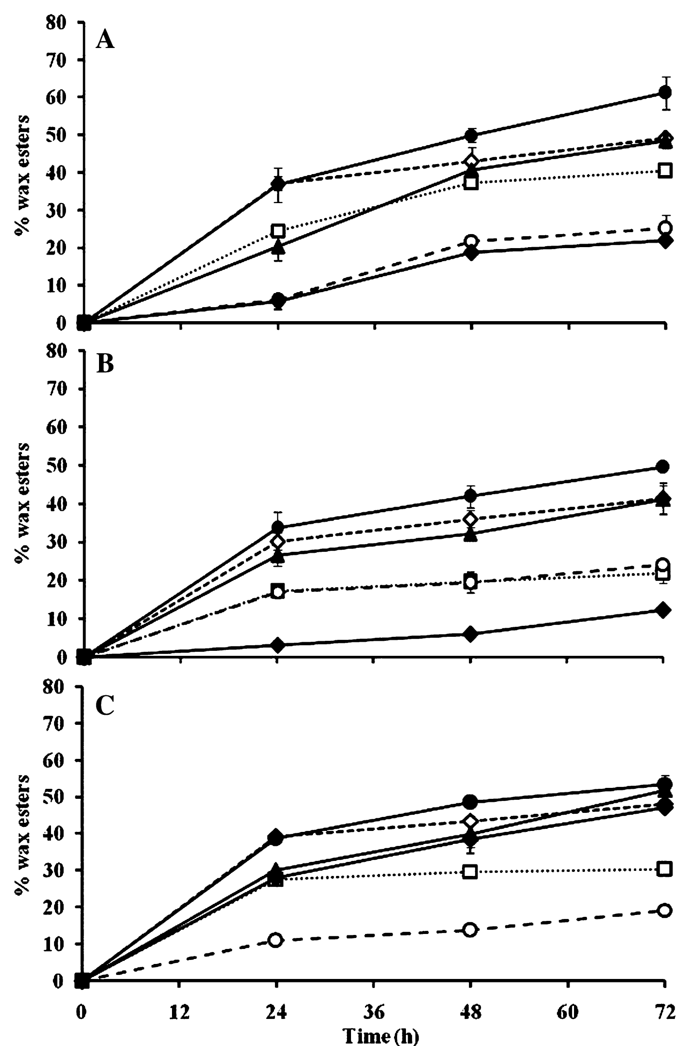

Fig. 11. Comparison of wax esters synthesis from coconut oil (A), palm oil (B) and jatropha oil (C) with oleyl alcohol by the immobilized lipase of *Burkholderia* sp. EQ3 and five commercial immobilized lipases. (-◇--) Lipozyme RM IM, (···□···) Lipozyme TL IM, (-○--) Novozyme 435, (—◆—) Lipase PS, (—▲—) Lipase AK and (—●—) Lipase EQ3.

5. Conclusions

The lipase from *Burkholderia* sp. EQ3 was purified by a combination of precipitation and chromatography. The purified lipase had an optimum activity at 30 °C and neutral pH (pH 7.5). The enzyme retained activity of more than 60% at 55 °C for 2 h and operated over a broad range of pH values. The lipase activity increased in the presence of K^+, Ca^{2+} and Mg^{2+}. The lipase activity was also increased by adding gum arabic while Tween 80, Tween 20 and Triton X-100 had negative effects on its activity. In the presence of 25% of the tested organic solvents, the enzyme activity decreased but it retained activity of more than 80% in iso-propanol. The enzyme exhibited a high preference for the lipid in coconut oil and olive oil in addition to palm olein. For wax esters synthesis, the immobilized lipase EQ3 synthesized a higher amount of wax esters from coconut oil, palm olein and jatropha oil than the commercial immobilized lipases. This enzyme is therefore promising for its use in the synthesis of food grade wax esters.

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