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Isolation and Characterization of a Novel Thermophilic-Organic Solvent Stable Lipase From *Acinetobacter baylyi*

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Abstract The benzene tolerant *Acinetobacter baylyi* isolated from marine sludge in Angsila, Thailand could constitutively secrete lipolytic enzymes. The enzyme was successfully purified 21.89-fold to homogeneity by ammonium sulfate precipitation and gel-permeable column chromatography with a relative molecular mass as 30 kDa. The enzyme expressed maximum activity at 60°C and pH 8.0 with *p*-nitrophenyl palmitate as a substrate and found to be stable in pH and temperature ranging from 6.0-9.0 to 60-80°C, respectively. A study on solvent stability revealed that the enzyme was highly resisted to many organic solvents especially benzene and isoamyl alcohol, but 40% inhibited by decane, hexane, acetonitrile, and short-chain alcohols. Lipase activity was completely inhibited in the presence of Fe^{2+}, Mn^{2+}, EDTA, SDS, and Triton X-100 while it was suffered detrimentally by Tween 80. The activity was enhanced by phenylmethylsulfonyl fluoride (PMSF), Na^{+}, and Mg^{2+} and no significant effect was found in the presence of Ca^{2+} and Li^{+}. Half of an activity was retained by Ba^{2+}, Ag^{+}, Hg^{+}, Ni^{2+}, Zn^{2+}, and DTT.

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The enzyme could hydrolyze a wide range of *p*-nitrophenyl esters, but preferentially medium length acyl chains (C_8-C_{12}). Among natural oils and fats, the enzyme 11-folds favorably catalyzed the hydrolysis of rice bran oil, corn oil, sesame oil, and coconut oil in comparison to palm oil. Moreover, the transesterification activity of palm oil to fatty acid methyl esters (FAMEs) revealed 31.64±1.58% after 48 h. The characteristics of novel *A. baylyi* lipase, as high temperature stability, organic solvent tolerance, and transesterification capacity from palm oil to FAMEs, indicate that it could be a vigorous biocatalyzer in the prospective fields as bioenergy industry or even in organic synthesis and pharmaceutical industry.

Keywords Solvent-stable lipase · Thermophilic lipase · *Acinetobacter baylyi* · Marine sludge · Biodiesel production

Introduction

Lipases or triacylglycerol hydrolases (EC 3.1.1.3) are some of the most important enzymes used as industrial biocatalysts for a variety of biotechnological applications [1]. Although their preferential catalytic reaction is hydrolysis of triglycerides at the water-lipid interface, organic solvents or organic-aqueous two phase media are favorable for some reactions, especially the prospective fields as pharmaceutical industry and biodiesel production [2, 3]. Uses of organic solvents provide main advantages to improve the solubility of substrates and ease of product recovery in organic phase in equilibrium two-phase systems [4, 5]. Nevertheless, as we know that enzymes are generally very labile catalysts and easy to lose their activities in organic solvent, several techniques such as medium engineering, substrate engineering, and protein engineering have been employed so far to improve the stability of enzymes [6–9]. However, if enzymes were naturally stable and active in hostile environments, they would be excellent biocatalyst for application. Thus, the search for natural enzyme that shows high stability and organic solvent tolerance is more straightforward.

Recently, solvent-tolerant bacteria as a relatively novel group of extremophilic microorganisms with unique ability to live in the presence of organic solvents have attracted a great attention among scientists and industrialists [5, 9–11]. Since extracellular enzymes secreted by organic-solvent tolerant microorganisms were possibly stable in the presence of organic solvents. Some of these microbes have been found to be sources of solvent-stable enzymes, some of which have already been commercialized [5–11]. Therefore, the aim of this study is to isolate and identify a novel organic solvent-tolerant bacterium. The catalytic activity, substrate specificity and the stability of the lipolytic enzyme produced by the isolated strain was investigated. The enzyme obtained from the new productive source was found to be thermophilic-organic solvent stable.

Materials and Methods

Isolation of Organic Solvent Tolerant Strain

Marine sludge samples were collected from ten different sites located near fish market in Angsila, Thailand during down tide period. Ten grams of each sample were mixed with 100 ml of sterile water by brief vortex. Then, the mixture was placed for sedimentation

before the upper phase was directly used (10% inoculation) for screening. The organic solvent tolerant strains were screened by 50% (*v/v*) of benzene enrichment in 0.2× Luria-Bertani medium [12]. The cultivation tube was closed with a chloroprene-rubber cock to prevent evaporation of benzene. Cultivations were maintained at 25°C with shaking at 250 rpm for 24 h. Cultures were then seven times repeatedly transferred in the same culture conditions. Growth was determined by the optical density at 600 nm. A pure culture was isolated by spreading the turbid culture (OD_{600} ranging from 0.6 to 1.0) onto nutrient agar plate and incubated in a box with equilibrium of benzene vapor. Growing colonies were further purified to be a single colony by repeated streaking under the same conditions.

Screening of the Most Productive Lipase

Preliminary screening of lipolytic bacteria were carried out on agar plates containing 1% (*v/v*) of palm oil as substrate, 0.4% (*w/v*) of NaCl, 0.8% (*w/v*) of nutrient broth, 1% (*w/v*) of gum arabic, and 0.001% (*w/v*) of rhodamine B [13]. Subsequently, hydrolysis activity using *p*-nitrophenyl palmitate (Sigma, Germany) as substrate was confirmed according to the method of Pencreac'h and Baratii [14]. One unit (U) of enzyme was defined as the amount of enzyme releasing 1 μmol of *p*-nitrophenol per minute under the assay conditions. The amount of *p*-nitrophenol was calculated from the *p*-nitrophenol (Sigma, Germany) standard curve. Protein concentration was determined spectrophotometrically according to the method of Bradford [15] using the Bio-Rad assay reagent (Hercules, USA) and bovine serum albumin as the standard. The strain that showed the highest lipase activity after 24 h cultivation was selected for further experiment.

Bacterial Identification

The isolated strain was identified based on "API Skills Bacterial Identification Method" and also via 16S rRNA sequence [16]. Polymerase chain reaction (PCR) amplification of 16S rRNA gene was done with primers designed from the conserve regions at positions 22-41 and 1,066-1,085 in 16S rRNA gene of *Escherichia coli*, respectively [17]. Chromosomal DNA (100 ng) prepared by GF-1 nucleic acid extraction kit (Vivantis, Malaysia) was used as DNA template for PCR reaction. The PCR was carried out with initial step at 95°C for 10 min and 30 cycles of 95°C for 30 s, 60°C for 45 s, and 72°C for 90 s. Final extension was performed at 72°C for 7 min. The expected PCR product (about 1.1 kb) was purified by GF-1 Gel DNA recovery kit (Vivantis, Malaysia). The obtained 16S rRNA fragment was then ligated into T/A cloning vector (RBC, Taiwan) according to manufacturer's instructions. After transformation into *E. coli* DH5α, plasmids were extracted by GF-1 Plasmid DNA extraction kit (Vivantis, Malaysia) and used as template for sequencing by the dideoxynucleotide method [18]. The similarity of nucleotide sequence was determined using BLAST (National Center for Biotechnology Information databases). The phylogenetic analysis was performed using CLUSTAL W-multiple sequence alignment software package at site http://align.genome.jp/. These sequence data have been submitted to the GenBank databases under accession no.1173320.

Effect of Oils Supplement on Lipase Production

To test the effect of oils on the lipase production, the nutrient medium supplement with different kinds of oils (0.5% (*v/v*), emulsified with gum arabic (1% *w/v*) was used for cultivation at 25°C, 250 rpm for 24 h. Then, the culture broth was centrifuged at 10,000×*g*

and 4°C for 20 min. The supernatant obtained was filtered through a 0.2 μm nylon membrane filter to collect the cell-free supernatant and used for enzymatic assay as mentioned above. Nutrient medium without the addition of oils was used as control. The oils added in the nutrient medium were olive oil, corn oil, sesame oil, organic camellia tea oil, safflower oil, canola oil, sunflower oil, coconut oil, soybean oil, refined rice bran oil, palm oil, and tributyrin. All experiment was done in triplicate.

Lipase Purification

A single colony of the obtained strain was grown in 500 ml of nutrient medium at 25°C, 250 rpm for 24 h. Cells from the cultures were removed by centrifugation (10,000×*g*, 20 min, 4°C). Then, ammonium sulfate was added to cell-free supernatant (crude enzyme) to a final concentration between 10% and 70% saturation with 10% interval. After that, the suspension was kept at 0°C for 30 min with gentle stirring. The precipitate was collected by centrifugation at 10,000×*g* and 4°C for 30 min and further dissolved in 50 mM Tris-HCl buffer (pH 8.0). The enzyme solution (60-70% ammonium sulfate precipitation) was dialyzed extensively against the same buffer. Then, the dialysate was loaded on Sephadex G-75 gel permeable column chromatography (1×65 cm; GE Healthcare Bio-Sciences AB, Sweden) equilibrated with 50 mM Tris-HCl buffer (pH 8.0). All fractions were assayed both for lipase activity as well as total protein (A_{280}). The fractions showing lipase activity were pooled and assayed for protein content as described above. The specific activity of the purified enzyme was compared with that of crude enzyme and purification fold was calculated. Finally, the insoluble materials found in the purified enzyme were discarded by filtration with 0.2 μm pore size of nylon membrane filter (Whatman, England) before being used as enzyme solution for characterization in triplicate.

Determination of Molecular Mass

Relative molecular mass of lipase was analyzed by 12.5% acrylamide gel of sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) under the conditions developed by Laemmli [19]. The molecular mass of the denatured lipase was estimated by using a Pierce Blue Prestained protein molecular weight marker mix (Thermo scientific, USA) as the standard protein markers. Also, the relative molecular mass of the native enzyme was estimated by gel permeable column chromatography as mentioned above.

Effect of pH on Lipase Activity and Stability

The effect of pH on lipase activity and stability were measured at 37°C at pH ranging from 3.0 to 12.0 using *p*-nitrophenyl palmitate (pNPP) as the substrate. For optimum pH determination, reaction mixture was incubated at 37°C for 15 min. The effect of pH on lipase stability was determined by the same method after pre-incubation of crude enzyme in 50 mM buffer at the specific pH for 6 h at 37°C. The pH stability was studied by measuring the residual activities using pH 8.0 as control (100% of relative activity). Buffer systems used 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).

Effect of Temperature on Lipase Activity and Stability

The optimum temperature for lipase activity was measured by incubating enzyme in the temperature range of 20-90°C in a thermostatically controlled water bath. The thermal

stability of lipase was measured by incubating lipase solution at different temperature for 6 h, after which the residual activity was determined. The 100% of relative activity at 60°C was used as control.

Effect of Organic Solvents on the Stability of Lipase

The lipase solution was mixed with equal volume of organic solvent to prepare the 50% organic solution, and then the mixtures were shaken and incubated at 37°C for 12 h at 150 rpm. Then, solvents contain in the mixtures were eliminated by evaporation at 40°C for 5 min and the remaining activity was measured at 37°C, pH 8.0. The stability of lipase was calculated by comparing the residual activity with the absence (control, 0% *v/v*) of organic solvent.

Effects of Chemicals on Lipase Activity

The effect of detergent, metal ions and inhibitors on lipase activity were analyzed by incubating an enzyme for 1 h at 37°C in 50 mM Tris-HCl buffer (pH 8.0) containing such chemicals. Activity was measured after pre-incubation. The lipase activity of the enzyme solution without any chemical compounds was used as control (100% of relative activity).

Substrate Specificity

To determine the substrate specificities of lipase towards *p*-nitrophenyl esters, *p*-nitrophenyl acetate (C_2), butyrate (C_4), caprylate (C_8), caprate (C_{10}), laurate (C_{12}), myristate (C_{14}), palmitate (C_{16}), and stearate (C_{18}) were determined by hydrolysis activity as mentioned above.

Specificity of lipase towards natural substrates; olive oil, corn oil, sesame oil, organic camellia tea oil, safflower oil, canola oil, sunflower oil, coconut oil, soybean oil, refined rice bran oil, palm oil, palm wasting oil, and lard was analyzed by titrimetrical activity assay as described previously using 50 mM NaOH as titrant [2]. One unit of lipase activity was defined as the amount of enzyme that caused the release of 1 μmol of free fatty acid per minute under test condition.

Lipase-catalyzed Transesterification

One gram of crude palm oil was added with 10% by weight of the lyophilized enzyme and later mixed with 1:3 mol ratios (3% *v/v*) of methanol. The stepwise reaction of crude palm oil was conducted as previously described [20]. The reaction was carried out by stirring the mixtures with magnetic stirrer (250 rpm) for 48 h at 40°C. Samples were taken from the reaction mixture and later analyzed for the products by high-performance liquid chromatography (HPLC) as described previously [21]. The LC-20A HPLC apparatus (Shimadzu Corp., Kyoto) was equipped with Apollo Silica 5U column (250mm×4.6 mm×5 μm) from Alltech (Deerfield, IL, USA) and ELSD-LT Evaporative Light Scattering Detector (Shimadzu Corp., Kyoto). Two mobile phases were employed: phase A consisted of hexane, 2-propanol, ethyl acetate, and formic acid (80:10:10:0.1 *v/v*) and phase B consisted of hexane and formic acid (100:0.05 *v/v*). The flow rate was 1.5 ml/min and the injection volume was 20 ml. The protocol employed for the mobile phase involved a linear elution gradient of 1% (*v/v*) A increasing to 98% (*v/v*) in 20 min. The final mixture (A:B, 98:2 *v/v*) was employed for 3 min. Next, the system was restored to its initial condition by passing the A:B, 1:99 (*v/v*), mixture through the column for 15 min. Conversion of the transesterification reaction, defined as the ratio between the produces FAME and the glycerides present in the reaction was evaluated.

Results and Discussion

Isolation and Identification of Organic Solvent-tolerant Lipase-producing Strain

To obtain organic solvent tolerant bacteria which produce lipolytic enzyme, a medium supplemented with the 50% (*v/v*) concentration of benzene was preliminary used in an enrichment culture. Among 150 isolates, there were 20 strains showing lipase activity. One isolate which had good production of the enzyme (4.41±0.02 U/ml) and could be tolerated to various kinds of organic solvent especially in acetone, butanol, and hexadecane (data not shown) was selected as the most potent producer of organic solvent-tolerant lipolytic enzyme. The strain was found to be a rod-shape Gram-negative bacterium and gave negative results in the reactions of cytochrome-oxidase, arginine dihydrolase, urease, and β-galactosidase and reduction of nitrate to nitrites, indol production, esculin hydrolysis, gelatin hydrolysis, and glucose fermentation. The strain could not assimilate arabinose, manose, maltose, and N-acetyl-glucosamine while it gave positive results with the assimilation of glucose, potassium gluconate, capric acid, adipic acid, malic acid, trisodium citrate, and phenylacetic acid. The 16S rRNA gene sequence revealed a consistently 99% similarity with *Acinetobacter baylyi* including *A. baylyi* 3R22 (accession no. EF178435.1), *A. baylyi* H2 (accession no. FJ009376.1), *A. baylyi* H8 (accession no. FJ009373.1), *A. baylyi* 723 (accession no. EU604245.1), and *A. baylyi* 1477 (accession no. EU604244.1). Construction of a phylogenetic tree using neighbor-joining method (Fig. 1) revealed a close relation between the isolate and *A. baylyi*. The result matches well with the conclusion from biochemical properties. Hence, this strain was identified as *A. baylyi*, a widespread robust and versatile microorganism contributing in medical, environmental, and biotechnological point of views [22, 23].

Effect of Oils Supplement on Lipase Production

Different kinds of oils were tested to determine their effect on the production of lipase. As shown in Table 1, no significance production level was found in the medium containing oil

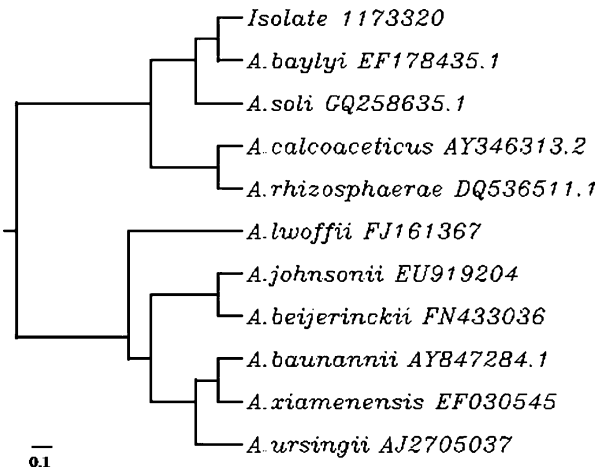

Fig. 1 Phylgenetic tree of *Acinetobacter* sp. homologues to the isolate. The phylogenetic tree was constructed by the neighbor-joining method with CLUSTAL W-multiple sequence alignment software package at site http://align.genome.jp/. The *scale* represents the number of nucleotide substitutions per site

Table 1 Effect of oils supplement of lipase production by *A. baylyi.*

Oils (0.5%, v/v)	Lipase activity (U/ml) ± SD[a]
Control	4.41 ± 0.02
Olive oil	4.35 ± 0.16
Corn oil	4.29 ± 0.21
Sesame oil	4.44 ± 0.05
Camellia tea oil	4.38 ± 0.03
Safflower oil	4.22 ± 0.08
Canola oil	4.38 ± 0.02
Sunflower oil	4.57 ± 0.09
Coconut oil	4.45 ± 0.03
Soybean oil	4.32 ± 0.23
Refined rice bran oil	4.54 ± 0.06
Palm oil	4.40 ± 0.12
Tributyrin	4.37 ± 0.07

[a] The lipase activity was expressed as the mean of three determinations with standard derivation.

substrate. This can be noted that this strain produce lipase constitutively. Earlier, bacterial lipases are generally produced using lipidic carbon source such as oils or fatty acids in the presence of an organic nitrogen source [24]. This phenomenon is also explained in the lipases from genus *Acinetobacter* [25, 26]. However, there were some reports suggesting the repression by long-chain fatty acids [27–29]. In the case of *A. baylyi*, the constitutive production of lipase seems to be a unique characteristic of the strain suitable for application and required more investigation of the secretion mechanism.

Enzyme Purification

Since *A. baylyi* secreted few native proteins at low level to the medium, the lipase was approximately free from contaminating proteins. The cell-free crude preparation from culture broth containing 5.62 U/mg of lipase specific activity have been successfully purified with an overall yield of 13.5% and a purification fold of 21.89 after Sephadex G75-column chromatography (Table 2), which resulted in a single peak (Fig. 2) when absorbance was taken at 280 nm. The purity was confirmed to be a single band with a relative molecular mass of 30 kDa determined by SDS-PAGE analysis (Fig. 3). The molecular mass of *A. baylyi* lipase showed a relatively smaller than other lipases previously reported from the same genus which found to posses between 32 and 62 kDa [30–35].

Table 2 Summary of lipase purification from *A. baylyi.*

Purification step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Purification fold
Crude lipase	2,205.13	392.49	5.62	100	1
Ammonium sulfate precipitation (60-70%)	617.44	19.84	31.12	28	5.54
Sephadex G 75-column chromatography	297.69	2.42	123.01	13.5	21.89

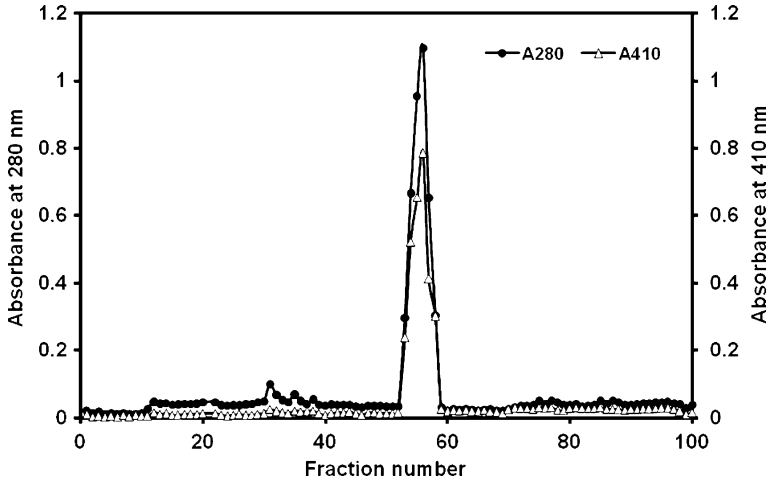

Fig. 2 Purification of lipase from *A. baylyi* on Sephadex G75-column chromatography

Effect of pH and Temperature on Lipase Activity and Stability

The optimum activity of the purified lipase was shown in Fig. 4a. The enzyme exhibited the highest activity at pH 8.0. The relative activity increased gradually from pH 5.0 to 7.0. The residual activity increased steeply and reached the maximum at pH 8.0 as well as decreased steeply after maximum. No lipase activity was observed after pH 10.0. The profile indicated that the lipase is active in a narrow pH range and belong to alkaline lipase. The alkaliphilic characteristic is very similar to the lipase from *Acinetobacter* [31–34, 36], which is inactive at acidic pH since it is losing coordinating metal ion, Ca^{2+} from the active site of enzyme [36, 37]. The stability of the lipase was determined kinetically after 6 h incubation at

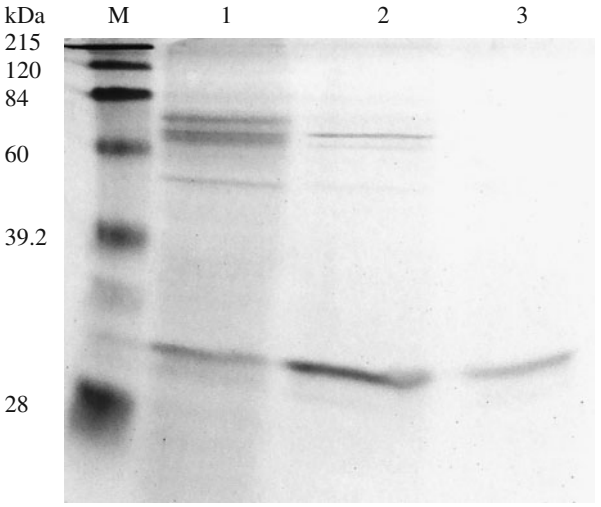

Fig. 3 SDS-PAGE analysis of the purified *A. baylyi* lipase. *Lane M* protein marker; *lane 1* cell-free supernatant; *lane 2* pooled lipase fraction from 60-70% ammonium sulfate precipitation; *lane 3* pooled lipase fraction from Sephadex G75-column chromatography

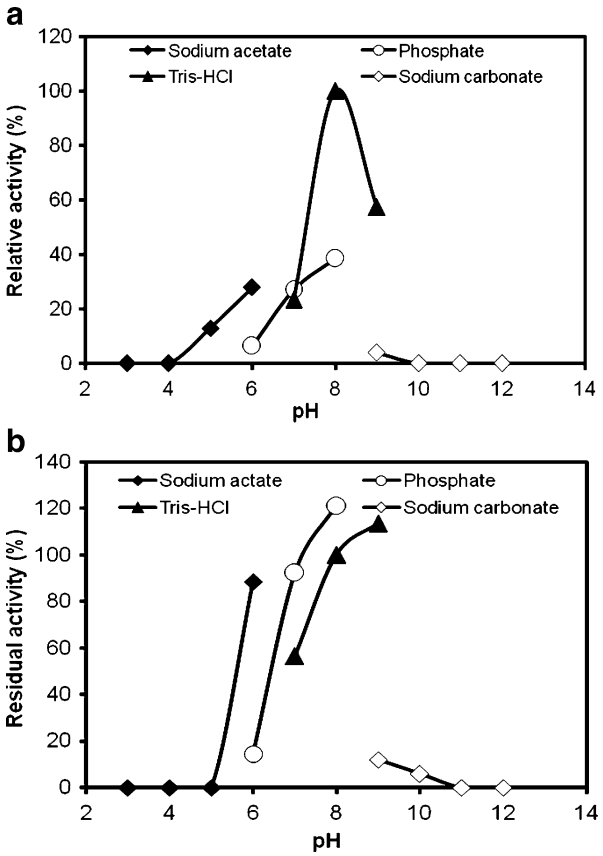

Fig. 4 Effect of pH on lipase activity (**a**) and stability (**b**). The activity was determined by incubating the reaction mixture in 50 mM buffer of a specific pH at 37°C using *p*-nitrophenyl palmitate as the substrate. Buffer systems used were acetate (pH 3.0-6.0, *closed diamonds*), phosphate (pH 6.0-8.0, *opened circles*), Tris-HCl (pH 7.0-9.0, *closed triangles*), and carbonate (pH 10.0-12.0, *opened diamonds*). The pH stability was studied by measuring the residual activities after 6 h incubation compares with control (100% relative activity) at pH 8.0

different pH (Fig. 4b). The result clearly indicated that *A. baylyi* lipase was stable in the pH range of 6.0-9.0, which similar to lipase from *Acinetobacter* sp. RAG-1 [33].

One important characteristic required for the application of biotransformation is the active enzyme at high temperature. Above 40°C was generally used to increase the miscibility and diffusion between different phases and increase the conversion ratio to oil. In this study, optimum temperature was determined by checking the activities of enzymes incubated at different temperature. The relative activities at various temperatures were shown in Fig. 5a. The enzyme exhibited the highest activity at 60°C. The lipase activity was raised gradually from 20 to 50°C and then increased steeply and reached to the maximum at 60°C as well as decreased steeply after maximum. From 70 to 90°C, the lipase activities reduce very slightly and it lost all activity at 100°C. Heat stability of *A. baylyi* lipase was studied by measuring the residual activities after incubation with various temperatures for 6 h (Fig. 5b). The enzyme appeared to be stable and retained more than 80% of initial activity at temperature ranging from 60 to 80°C. This result indicates that *A. baylyi* lipase is a thermal stable lipase. In comparison with other lipases from the same

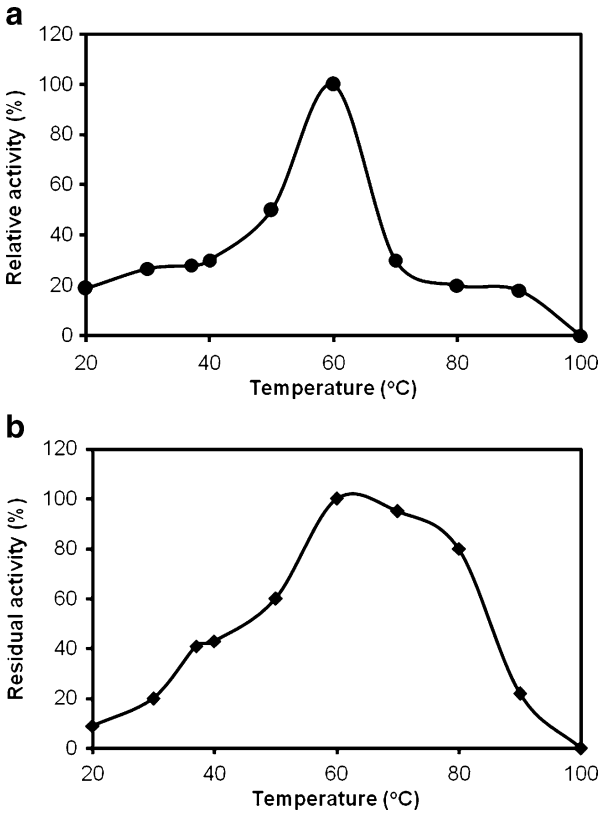

Fig. 5 Effect of temperature on lipase activity (**a**) and stability (**b**). The activity (*closed circles*) was measured by incubating the purified enzyme in the temperature range of 20-90°C in a thermostatically controlled water bath. The thermal stability of lipase was measured by pre-incubating lipase solution at different temperature for 6 h, after which the residual activity (*closed diamonds*) was calculated comparing with control (100% of relative activity) at 60°C

genus, this lipase has higher optimum temperature and stability that would be favorable for industrial use [31, 33–36].

Effect of Organic Solvents on the Stability of Lipase

A number of bacterium-producing organic solvent-tolerant lipase is limited and most of them are screened from deep sea or marine mud samples (ten). Since *A. baylyi* was isolated from marine sludge by enrichment in 50% concentration of benzene, production of solvent stable enzyme is possible. In this study, the stability of *A. baylyi* lipase was investigated in various polar and non-polar organic solvents. As described in Table 3, after 12-h incubation, the lipase from *A. baylyi* was stable in the presence of isoamyl alcohol (log$P_{o/w}$ 1.3) and benzene (log$P_{o/w}$ 2.0) while the activity was slightly reduced in the presence of n-hexane (log$P_{o/w}$ 3.5), n-heptane (log$P_{o/w}$ 4.0), decane (log$P_{o/w}$ 5.6), and hexadecane (log$P_{o/w}$ 8.8) by 72%, 92%, 75%, and 81%, respectively. These results are similar to the facts that solvents with high log$P_{o/w}$ values (hydrophobic solvents) cause hinder efficient interaction between enzymes and substrates [38]. The similar results of benzene tolerant enzyme were observed in lipase from *Staphylococcus saprophyticus* M36 [1] and protease from *Pseudomonas*

Table 3 Effect of organic solvents on lipase activity.

Organic solvents (50%)	log $P_{o/w}$	Relative activity (%)[a]
Control	-	100
DMSO	−1.22	92
Ethanol	−0.24	60
Acetonitrile	−0.15	66
Isopropanol	0.05	59
Methanol	0.8	64
Butanol	0.8	60
Isoamyl alcohol	1.3	108
Benzene	2.0	100
Hexane	3.5	72
Heptane	4.0	92
Decane	5.6	75
Hexadecane	8.8	81

[a] The purified enzyme was incubated at 37°C with shaking at 150 rpm in the presence of 50% organic solvent for 12 h. The remaining lipolytic activity was measured and expressed as the mean of three determinations comparing to control.

aeroginosa [39, 40]. Thus, it could be noted that tolerance to benzene seems to be the unique property of this enzyme and need more investigation. Hydrophilic solvent such as DMSO (log$P_{o/w}$ −1.22) slightly reduced the activity as 92% residual activity while 34% from initial activity was inhibited in the presence of acetonitrile (log$P_{o/w}$ −0.15). This might be from the reason that hydrophilic solvents (low log$P_{o/w}$ value) are able to dissolve enzyme resulting in invariable inactivation [41]. This is similar to the results of *Acinetobacter calcoaceticus* LP009 and *Bacillus sphaericus* lipases described previously [26, 42, 43]. The residual activity was 59% and 64%, respectively, when isopropanol (log$P_{o/w}$ 0.05) and methanol (log$P_{o/w}$ 0.8) were added to the crude enzyme 50% (*v/v*) for 12 h at 37°C, but there is 40% loss in the presence of ethanol (log$P_{o/w}$ −0.24) and butanol (log$P_{o/w}$ 0.8). These might be because the short-chain alcohols have low solubility in oils; therefore, a new liquid phase appears in the system at moderate concentrations leading to an inactivation of the enzyme [44]. Since short-chain alcohols such as methanol and ethanol are the suitable substrates to apply for biodiesel production and the stability of lipase was retained more than half at the concentration of 50%. Also, the activity was stable when incubating the enzyme in short-chain alcohols at ratio 1:3 found to be the most optimal for biodiesel production (data not shown). Moreover, it has been reported that using butanol as co-solvent for biodiesel production could improve the ester yield [45]. Thus, the stability in alcohols makes *A. baylyi* lipase attractive for further application in production of biodiesel.

Effects of Chemicals on Lipase Activity

The effect of various metal ions on lipase activity was tested with 1 mM of chemicals or 1% in the case of detergents. As summarized in Table 4, *A. baylyi* lipase was completely inhibited by Fe^{2+}, Mn^{2+}, EDTA, SDS, and Triton X-100 while it was enhanced by PMSF, Na^{+}, and Mg^{2+}. The complete inhibition by EDTA implies the involvement of metal ion at its active site believed to be Ca^{2+} located at the substrate-binding pocket and stabilizes the

Table 4 Effect of chemicals on lipase activity.

Chemicals (1 mM) and detergents (1%)	Relative activity (%)[a]
Control	100
Na^+	214
K^+	39
Li^+	98
Ag^+	56
Ba^{2+}	69
Ca^{2+}	98
Fe^{2+}	0
Mg^{2+}	265
Mn^{2+}	0
Zn^{2+}	67
Ni^{2+}	60
Hg^{2+}	77
Triton X-100	0
Tween-80	4
SDS	0
EDTA	0
DTT	86
PMSF	163

[a] The purified enzyme was incubated at 37°C with shaking at 150 rpm in the presence of chemicals for 1 h. The remaining lipolytic activity was measured and expressed as the mean of three determinations comparing to control.

structure of lipase [36, 37]. This hypothesis was confirmed by restored activity (90%) found with the addition of Ca^{2+} (data not shown). Although low amount of Triton X-100 increase the release of lipase from cells, as detergent, higher concentration might detrimentally effect. Action of the detergents on the enzyme can well be correlated to their hydrophilic/lipophilic balance (HLB), which is defined as the way a detergent distributes between polar and nonpolar phases [46]. Triton X-100 and SDS with HLB of 13.5 and 40, respectively, have the detrimental effect on the enzyme. These results are similar to the cases of *Acinetobacter baumannii* BD5 and *Acinetobacter venetianus* RAG-1 lipases [31, 33]. An enhancement in lipase activity after the addition of PMSF may be a result of nature of the active site serine imparts resistance to serine hydrolase inhibitor [42], while Na^+ may reflect the ability of the salts to react with free fatty acids adhering to the oil droplets and increasing their surface area to react with enzyme [47]. Activation effect of Mg^{2+} is similar to the results of lipase from *A. baumannii* BD5 [31] and protease from *Bacillus sp.* PS719 described before [48]. This might be because Mg^{2+} acts as a salt or ion bridge with conserve histidine in active site and thereby maintains the rigid conformation of the enzyme molecule [42, 49]. The presence of Li^+ and Ca^{2+} had no significant effect on the activity while Ag^+ inhibited the activity by nearly 50%. Among metal ions, Ba^{2+}, Hg^+, Ni^{2+}, and Zn^{2+} significantly reduced the activity up to 40% whereas K^+ ion had dramatic inhibitory effect. It has been reported that the activation of enzyme activity was increased with an increase in the ionic radii of the cation [50]. Ca^{2+} has the largest ionic radius (0.099 nm) among the divalent metal ions studied, whereas ionic radius for Zn^{2+} is 0.075 nm. This can be similarly explained in the case of monovalent cation. Another reason for the stability of

enzyme in the presence of Ca^{2+} is possibly due to stabilization of enzyme in its active conformation or involving in the catalytic reaction [36, 37]. Cysteine appears to play an important role for activity and structural integrity of the enzyme. This was inferred from the marginal inhibition by the metal ions Zn^{2+}, Hg^{2+}, and Ag^{+}. The obtained result is similar to the previous report of alkaline lipases from *A. radioresistens* CMC-1 [51, 52], *A. calcoaceticus* 69 V [23] and *A.venetianus* RAG-1 [31]. Incubation of purified lipase in the presence of reducing agent, DTT resulted in activity that was not dependent upon intact disulfide linkage. This implies the interaction between cognate lipase and specific fold are required during folding and secretion [33, 42, 52].

Substrate Specificity

To determine the substrate specificity, the lipase activity towards *p*-NP esters of different carbon chain length was measured spectrophotometrically at 410 nm, pH 8.0, and 60°C. Among *p*-NP esters, the lipase showed the highest activity towards *p*-NP caprate (C_{10}) and fixed as 100%. The typical profile of chain length specificity was shown in Fig. 6a. The relative activity towards caprylate (C_8) and laurate (C_{12}) was around 90%. The activity of the lipase towards butyrate (C_4), myristate (C_{14}), palmitate (C_{16}), stearate (C_{18}), and acetate

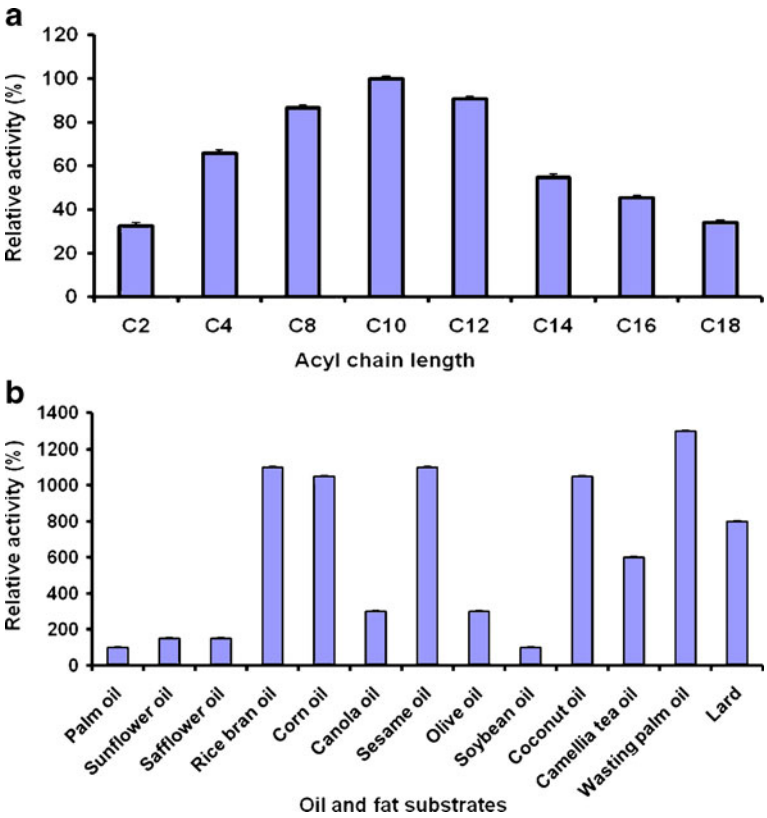

Fig. 6 Substrate specificity of *A. baylyi* lipase towards esters of *p*-nitrophenyl (**a**) and oil and fat substrates (**b**). Percentages shown are relative to maximum activity (C_{10}) and activity towards palm oil, respectively

(C_2) were 66%, 55%, 45%, 34%, and 33%, respectively. The results showed that this lipase has an optimum activity at medium-chain length substrates.

Among natural oils and fats, the activity towards palm oil was fixed as 100%. The specificities of this lipase towards natural oils and fats were shown in Fig. 6b. The lipase showed highest activity towards waste palm oil with about 13-folds compared with palm oil. The relative activity of rice bran oil, corn oil, sesame oil, and coconut oil was high as approximately 11-folds while three to eightfold upper were found in the case of lard, camellia tea oil, canola oil, and olive oil. The relative activity of sunflower oil, safflower oil, and soybean oil in comparison to palm oil seem to be similar. From the results, *A. baylyi* lipase hydrolyzed preferentially waste palm oil, rice bran oil, corn oil, sesame oil, and coconut oil that contain higher contents of medium length fatty acids [21, 53].

Transesterification Capacity for Biodiesel Production

To evaluate the potential of *A. baylyi* lipase for biodiesel production, the lipase was conducted under preliminary set of reaction condition, which may not have been the optimum condition. The conversion of palm oil to FAMEs reached 31.64±1.58% after 48 h of reaction. Results of this study suggested that thermophilic-organic solvent tolerant *A. baylyi* lipase is perspective catalyzer for biodiesel production.

It has been known that *Acinetobacter sp.* is easily isolated strain and many of them have been found to secrete esterolytic enzymes [35, 36]. Since many *Acinetobacter* lipases show stability and maximum activity at alkaline pH, however, thermostable and solvent tolerant lipase from this strain has not been reported [36]. Hence, this is believed to be the first report on the secretion of thermophlilic-organic solvent stable lipase by *A. baylyi*. In addition, the characteristics of novel *A. baylyi* lipase, as high temperature stability, organic solution tolerance, and transesterification capacity from palm oil to FAMEs, indicate that it could be a vigorous biocatalyzer in the prospective fields as bioenergy industry, or even in organic synthesis and pharmaceutical industry.

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