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Isolation and characterization of a thermophilic lipase from *Bacillus thermoleovorans* ID-1

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Abstract

A thermophilic microorganism, *Bacillus thermoleovorans* ID-1, isolated from hot springs in Indonesia, showed extracellular lipase activity and high growth rates on lipid substrates at elevated temperatures. On olive oil (1.5%, w/v) as the sole carbon source, the isolate ID-1 grew very rapidly at 65°C with its specific growth rate (2.50 h^{-1}) and its lipase activity reached the maximum value of 520 U l^{-1} during the late exponential phase and then decreased. In addition to this, isolate ID-1 could grow on a variety of lipid substrates such as oils (olive oil, soybean oil and mineral oil), triglycerides (triolein, tributyrin) and emulsifiers (Tween 20, 40). The excreted lipase of ID-1 was purified 223-fold to homogeneity by ammonium sulfate precipitation, DEAE-Sephacel ion-exchange chromatography and Sephacryl S-200 gel filtration chromatography. As a result, the relative molecular mass of the lipase was determined to be 34 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The enzyme showed optimal activity at 70–75°C and pH 7.5 and exhibited 50% of its original activity after 1 h incubation at 60°C and 30 min at 70°C and its catalytic function was activated in the presence of Ca^{2+} or Zn^{2+}. © 1999 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Lipase; Thermophile; *Bacillus thermoleovorans*

1. Introduction

Lipases (triacylglycerol acylhydrolases; EC 3.1.1.3) catalyze the hydrolysis of triglycerides at the oil-water interface [1]. Microbial lipases have currently received considerable attention with regard to biotechnological applications for detergents, transesterification and chiral compound synthesis [2]. In particular, lipases from thermophiles are expected to play a significant role in industrial processes, since they are thermostable and resistant to chemical denaturation.

There are some kinds of fats having melting points over room temperature. These often inhibited the enzymatic catalysis, so it is not efficient to use en-

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zymes from mesophilic sources. With respect to the enzymatic processing of lipids and oil-rich industrial effluents at high temperatures, thermostable lipases from thermophilic bacterial strains are required and lipase producing thermophilic bacterial strains can also be used to develop the process of lipid hydrolysis. Recently, several lipases have been purified and characterized from thermophilic isolates, mainly *Bacillus* [3–5]. The growth rates and lipase activities of thermophilic *Bacillus* strains on media containing lipid substrates, however, are relatively low in comparison with those of mesophilic bacteria. So, we screened and isolated a novel thermophilic *Bacillus* strain producing thermostable lipase from hot springs in Indonesia. Our new isolate shows a rapid growth rate on olive oil at 65°C ($\mu_{max} = 2.50$ h^{-1}) and a good enzyme production. The properties of the new isolate with the purification and the characterization of its lipase are also described here.

2. Materials and methods

2.1. Screening of lipase producing thermophiles

Enrichment cultures of the samples collected from hot springs in Indonesia were performed at 60°C and an initial pH 6.8 with shaking (180 rpm) in EM-1 medium of the following composition (per liter): yeast extract, 1.0 g; olive oil, 5.0 ml; NaCl, 2.0 g; $MgSO_4·7H_2O$, 0.4 g; $MgCl_2·6H_2O$, 0.7 g; $CaCl_2·2H_2O$, 0.5 g; KH_2PO_4, 0.3 g; K_2HPO_4, 0.3 g; $(NH_4)_2SO_4$, 0.5 g; vitamin solution, 1.0 ml (DSM catalogue no. 141); trace element solution, 1.0 ml (DSM catalogue no. 141). After enrichment, lipase producing thermophiles were screened on rhodamine B (Sigma) agar plates at 60°C [6]. Lipase production was monitored by irradiating plates with UV light at 350 nm.

2.2. Characterization of the new isolate

The morphological and physiological characterization of the strict aerobic thermophilic isolate ID-1 was performed according to the methods described by Slepecky and Hemphill [7]. Membrane fatty acids from the isolate ID-1 were extracted, separated and analyzed as described by Paisley [8]. Genomic DNA extraction, PCR-mediated amplification of the 16S rRNA and sequencing of the PCR products were performed as described by Rainey et al. [9].

2.3. Culture conditions for lipase production

Batch cultures of the isolate ID-1 were carried out in a 2-l bioreactor (Bioengineering AG, Wald, Switzerland) with a working volume of 1.5 l. The following modified TYEM medium was used for lipase production (per liter): tryptone, 6.0 g; yeast extract, 2.0 g; olive oil, 15 ml; $CaCl_2·2H_2O$, 0.2 g; $MgSO_4·7H_2O$, 0.1 g; $FeCl_3·6H_2O$ (1% stock solution.), 0.4 ml. The inoculum size was 1.0%. The growth temperature was 65°C and the pH was maintained at 6.0. Throughout the cultivations, the DO value was maintained above 10% air saturation in order to prevent oxygen limitation.

2.4. Lipase activity assay

Lipase activity was estimated using a spectrophotometric assay with *p*-nitrophenyl butyrate (PNPB) as a substrate, which was dissolved in acetonitrile at a concentration of 10 mM. Subsequently, ethanol and 50 mM potassium phosphate buffer (pH 7.5) were added to a final composition of 1:4:95 (v/v/v) of acetonitrile/ethanol/buffer, respectively [10]. The cell-free supernatant (0.3 ml) was added to the substrate solution (0.9 ml) and then, the mixture was incubated at 60°C. After 15 min, enzyme activity was measured by monitoring the change in absorbance at 405 nm that represents the amount of released *p*-nitrophenol (PNP). All measurements were carried out under first-order reaction conditions, i.e. with the catalyst in excess of ester. One unit of lipase activity is defined as the amount of enzyme releasing 1 μmol PNP per min under the assay conditions. The molar absorption coefficient of PNP at 405 nm was determined to be 1.457×10^5 cm^2 mol^{-1}.

The relative hydrolytic activity of the lipase towards different triacylglycerols was determined by a spectrophotometric assay using the formation of copper soaps for the detection of free fatty acids [3]. The substrate solution consisted of triglycerides (100 mM) emulsified with homogenizer in distilled water with gum arabic (0.2 mM) at maximum speed

for 2 min. Copper(II)-acetate-1-hydrate aqueous solution (90 mM), adjusted to pH 6.1 with pyridine, was used as copper reagent. The chromogenic reagent contained diethyldithio carbamic acid (Sigma) (5.8 mM) dissolved in absolute ethanol. The reaction was started by addition of 0.1 ml substrate solution (see above) to 0.9 ml of enzyme solution in 50 mM potassium phosphate buffer (pH 7.5). The enzyme reaction was carried out for 20 min at 60°C. Immediately after incubation, 0.45 ml of the reaction mixture was transferred to a test tube containing 0.25 ml of 3 M HCl. Fatty acids were subsequently extracted by addition of 3 ml *n*-hexane and vigorous vortexing for 2 min. The organic phase (2.5 ml) was transferred to a fresh test tube filled with 0.5 ml copper reagent. The mixture was vortexed for 1.5 min and phase separation was achieved by centrifugation. Then, the organic phase (2 ml) was mixed with 0.4 ml of the chromogenic reagent and the absorption was measured at 430 nm.

2.5. Lipase purification

The cell-free supernatant was prepared by centrifugation (10 000 × *g*, 15 min) of culture broth and it was passed through a 0.45-μm pore size membrane filter at 4°C. To the filtered supernatant was slowly ammonium sulfate added under stirring to achieve 30% saturation. The suspension was centrifuged (10 000 × *g*, 20 min) and then, ammonium sulfate was added to the supernatant to reach 80% saturation at 4°C. The final precipitate was collected by centrifugation (10 000 × *g*, 20 min, 4°C) and redissolved in a minimal volume of 20 mM Tris-HCl buffer (pH 7.5) at 4°C. The dissolved enzyme solution was dialyzed against the same buffer for 16 h at 4°C to remove the residual ammonium sulfate. The dialyzed solution was applied to a DEAE-Sephacel column (Pharmacia, 2.5 × 13.0 cm) previously equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and eluted with a linear gradient of sodium chloride (0–250 mM) at a flow rate of 0.2 ml min^{-1}. Protein fractions containing lipase activity were pooled and passed through a column of Sephacryl S-200 (Pharmacia, 1.5 × 90 cm) pre-equilibrated with 20 mM Tris-HCl buffer (pH 7.5) at a flow rate of 0.1 ml min^{-1}. Fractions containing lipase activity were then separated and viewed by sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fractions showing a single band on the gel were pooled and stored at 4°C.

2.6. Characterization of lipase

The temperature optimum for the enzyme was evaluated by using the lipase activity assay with PNPB at different temperatures. The activity assay was performed at pH 7.5.

The effect of temperature on the lipase stability was determined by measuring the residual activity after 30 min of pre-incubation in 50 mM potassium phosphate buffer (pH 7.5) at various temperatures.

The pH optimum of the enzyme was measured at 65°C and 405 nm by using the buffer solutions of different pH values. The following buffers (50 mM) were used: potassium phosphate (pH 4.0–6.0), Tris-HCl (pH 6.0–8.0) and glycine (pH 8.0–10.0), adjusted at the enzyme reaction temperature.

Substrate specificity towards different *p*-nitrophenyl esters (PNPEs) was analyzed by the spectrophotometric method as described above. The PNPE substrates (C_4–C_{18}) were dissolved in acetonitrile at a concentration of 10 mM.

For determining the effect of metal ions on lipase activity, the enzyme solution was pre-incubated with 1 mM of metal ions such as $ZnCl_2$, $LiCl_2$, $MnCl_2$, KCl, $MgCl_2$ or $CaCl_2$ at 65°C for 10 min and then, the residual activity was determined as described above.

To determine the effect of inhibitors such as EDTA, β-mercaptoethanol, sodium dodecyl sulfate, phenylmethyl-sulfonyl fluoride (PMSF) and dithiothreitol at a concentration of 1 mM, lipase activity was measured by the same procedure as for the effect of metal ions.

2.7. Molecular mass measurement

The relative molecular mass of the purified lipase was estimated by SDS-PAGE using a 15% (w/v) acrylamide gel according to the method of Laemmli [11]. Proteins were stained with Coomassie brilliant blue R-250(Sigma).

3. Results and discussion

3.1. Isolation and characterization of a new isolate ID-1

Twelve aerobic strains were primarily isolated from 128 samples by enrichment cultures in EM-1 medium containing olive oil (0.5%, v/v) at 60°C. Among them, the isolate ID-1 was chosen for a subsequent experiment due to its high activity and broad substrate specificity towards various triacylglycerides (C_4–C_{18}, data not shown). The new isolate ID-1 was an aerobic, rod-shaped and spore-forming thermophile which could grow within a temperature range of 40–73°C with an optimum at 65°C. Based on its morphological and physiological characteristics, the isolate ID-1 was assigned to *Bacillus* rRNA group 5. Further classification was carried out at the German collection of microorganisms and cell culture (DSMZ). The profile of membrane fatty acids was typical for the thermophilic bacilli. The partial 16S rDNA sequence showed a similarity of 99.8% to *Bacillus thermoleovorans* and *Bacillus kaustophilus*. DSMZ concluded that even under consideration of all data, it is not possible to identify one of the above mentioned two species reliable. However, Sunna et al. reported that *B. thermoleovorans, B. kaustophilus* and *Bacillus thermocatenulatus* should be combined into one species, namely *B. thermoleovorans* [12].

3.2. Culture conditions and enzyme production

The isolate ID-1 was a novel thermophilic strain, which grew optimally at pH 6.0 and 65°C, as determined by a series of batch cultures on olive oil as the sole carbon source. Its specific growth rate during the exponential growth phase on olive oil at 65°C was estimated to be 2.50 h^{-1}, which is the highest growth rate on lipid substrates among thermophilic bacilli reported so far (Fig. 1A). The batch data for a thermoalkalophilic lipase producing *Bacillus* indicate a maximum specific growth rate of about 0.5 h^{-1} in a Tween 80-supplemented medium [13]. Becker et al. reported a growth rate of 1.0 h^{-1} for *Bacillus* sp. IHI-91 grown on olive oil as the sole carbon source and claimed that their strain showed the highest growth rate [14]. However, the growth rate of our

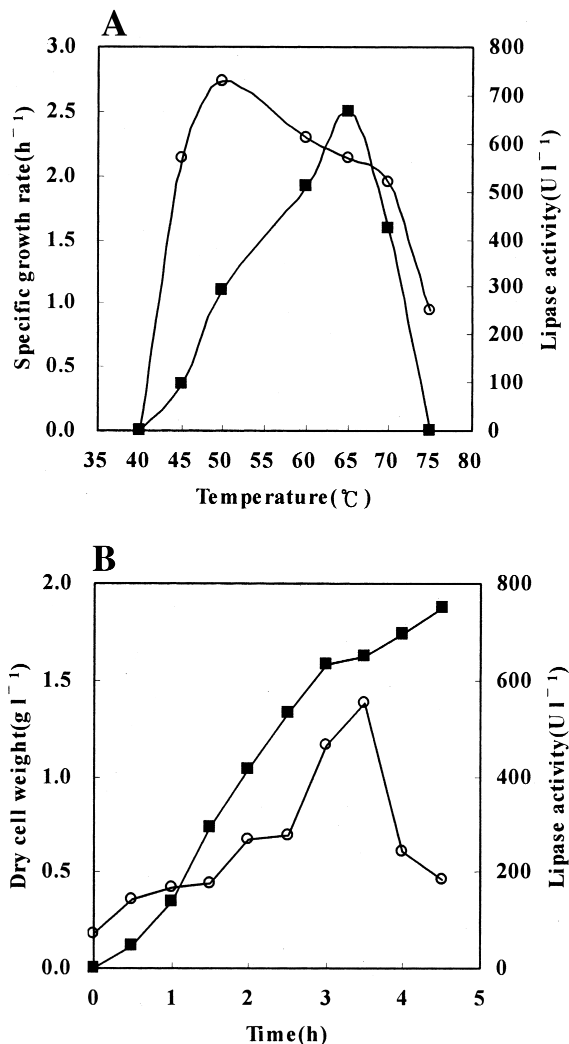

Fig. 1. Effect of the temperature on the specific growth rate and lipase production grown in batch culture on 1.5% (v/v) olive oil in modified TYEM medium at different temperatures and pH 6.0 (A) and the time course of a typical batch fermentation by the isolate ID-1 grown in batch culture on 1.5% (v/v) olive oil in modified TYEM medium at 65°C and pH 6.0 (B): dry cell weight (■), lipase activity (○).

new isolate ID-1 is much greater than that of *Bacillus* sp. IHI-91.

In contrast to the specific growth rate, the highest lipase production was obtained at 50°C, as shown in Fig. 1A. This activity (700 U l^{-1}) is higher than that of other thermophilic bacilli even though direct comparison is difficult. Schmidt-Dannert et al. [3] and Handelsmann and Shoham [15] reported that lipase

activities in the supernatant of batch cultures of thermophilic *Bacillus* sp. were in the range of 0.3–0.45 U ml^{-1} and maximum lipase activities were obtained after 14.5 and 36 h, respectively. Recently, Becker et al. reported a maximum activity of soluble lipase of 0.3 U l^{-1} after a 7.8-h batch culture of thermophilic lipolytic strain *Bacillus* sp. IHI-91 on olive oil at 65°C [14].

The time course of a typical batch culture on olive oil as the sole carbon source is shown in Fig. 1B. As other *Bacillus* strains, lipase production is associated with its growth. So, the isolate ID-1 showed maximum lipase production (520 U l^{-1}) after only 3.5 h cultivation due to its rapid specific growth rate. Commonly, a dramatic decrease in lipase activity was observed during the early stationary phase. This phenomenon was likely to be caused by thermal instability and proteolysis. Pereira-Meirelles et al. reported that the maximum level of lipase activity was maintained by adding a serine protease inhibitor (PMSF) to the culture medium [16]. The protease activity of the isolate ID-1 was also observed by a protease activity assay using azocasein as substrate (data not shown).

Table 1 shows the effect of carbon sources on cell growth and lipase production. The isolate ID-1 could grow very rapidly on various lipid substrates even at a high temperature of 65°C. The isolate ID-1 grew more rapidly on soybean oil and the level of lipase production was about double in comparison with that on olive oil. The highest growth rate (3.63 h^{-1}) was observed on glycerol, but the lipase activity was not detected. The isolate ID-1 was able to grow equally on tributyrin and triolein. However, the enzyme production on triolein was significantly higher than that on tributyrin. The results shown in Table 1 indicate that triolein is the best carbon source for lipase production by the isolate ID-1. Although the isolate ID-1 showed a relatively high growth rate on glucose, the basal level of enzyme activity was observed even in the presence of olive oil. Freire et al. reported that glucose repressed the lipase synthesis by *Penicillium restrictum* [17]. It was concluded from this work that lipase production by the isolate ID-1 was strongly induced by long chain triacylglyceride and repressed by glucose.

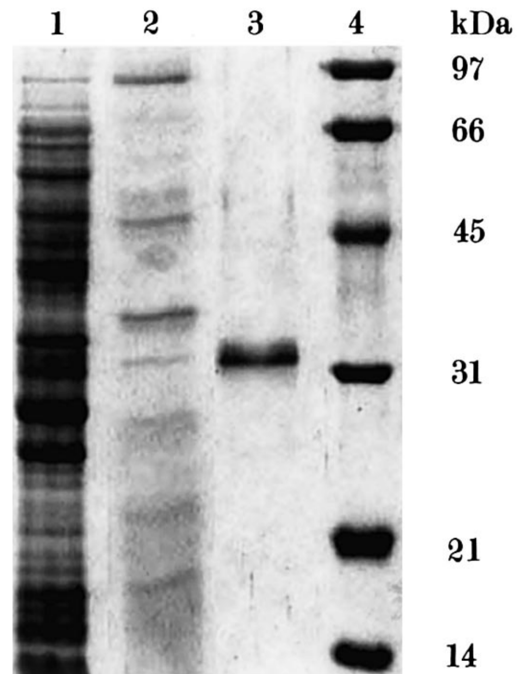

Fig. 2. SDS-PAGE of the purified lipase from the isolate ID-1. Lanes: 1, crude extract; 2, DEAE-Sephacel; 3, Sephacryl S-200; 4, standard proteins including phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (21 kDa), lysozyme (14 kDa).

Table 1
Effect of carbon sources on the specific growth rate and maximum lipase activities of the isolate ID-1 grown in batch culture at 65°C and initial pH 6.8 on a modified TYEM medium supplemented with various carbon sources (0.5%, w/v)

Carbon source	Specific growth rate (h^{-1})	Lipase activity (%)
Control	1.74	100
Glucose	2.22	88
Olive oil	2.14	345
Glucose+olive oil	2.28	112
Mineral oil	3.42	430
Soybean oil	3.31	605
Glycerol	3.63	N.A.
Tributyrin	1.69	60
Triolein	1.68	845
n-Butyric acid	N.G.	N.A.
Oleic acid	N.D.	120
Tween 20	1.36	770
Tween 40	0.86	140
Tween 80	N.G.	40
Triton X-100	N.G.	N.A.

N.A.: no activity; N.G.: no growth; N.D.: not determined due to high turbidity.

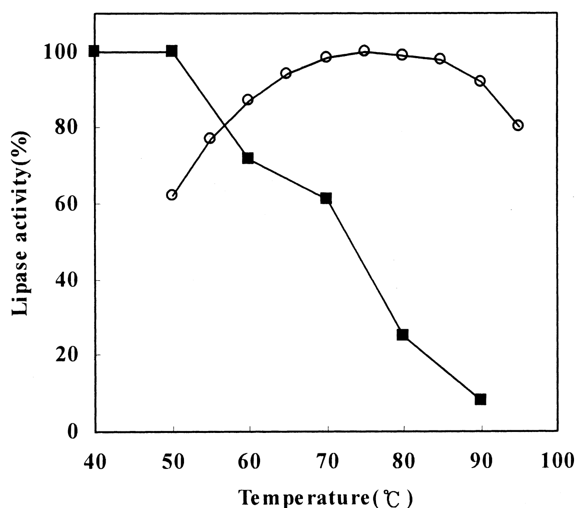

Fig. 3. Effect of the temperature on lipase activity (○) and stability (■). For the stability, the lipase was assayed after incubation at various temperatures for 30 min at pH 7.5.

3.3. Enzyme purification

Cell-free supernatant was prepared by centrifugation (10 000 × g, 15 min) of culture broth and passed through a 0.45-μm membrane filter for the removal of oil instead of the use of organic solvent such as n-hexane. The crude enzyme solution was precipitated by 30–80% ammonium sulfate fractionation and then dialyzed for 16 h at 4°C. This solution was loaded on a DEAE-Sephacel column equilibrated with 20 mM Tris-HCl buffer (pH 7.5) and the bound proteins were eluted with a linear gradient of sodium chloride at a flow rate of 0.2 ml min^{-1}. In the middle of the gradient, about 150 mM sodium chloride, the fractions containing lipase activity were recovered. The active fractions were collected and passed through a column of Sephacryl S-200 equilibrated with the same buffer at a flow rate of 0.1 ml min^{-1}. After the final gel filtration step, lipase was confirmed to be homogeneous by a single band on SDS-PAGE and its relative molecular mass was estimated to be about 34 kDa (Fig. 2). Molecular sizes of lipases from thermophilic bacteria are variable to some extent. The relative molecular masses of each lipase from *B. thermocatenulatus* and *Bacillus stearothermophilus* L1 were 16 and 43 kDa, respectively [3,18]. On the other hand, the molecular sizes of mesophilic enzymes exhibited much more diverse and somewhat higher, from 19.4 to 76 kDa [19–21]. It can probably be speculated that small and compact proteins may confer a higher thermostability than the bulky proteins. The specific activity of the 223-fold-purified lipase was 116 U mg^{-1} and the yield was 19%.

3.4. Enzyme characterization

Although the enzyme activity was insensitive to

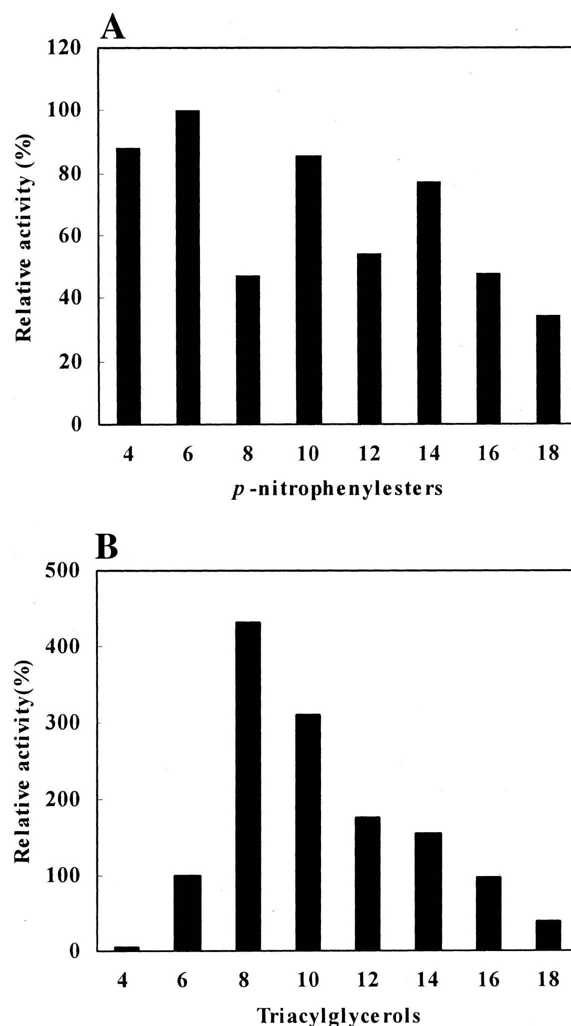

Fig. 4. Relative activities of the lipase on various PNPEs (A) and triacylglycerols (B). Lipase activities on each substrate are expressed as the percentage of those of p-nitrophenyl caproate (C_6) and tricaproin (C_6), which were taken as 100%, respectively.

Table 2
Effect of metal ions, enzyme inhibitors and detergents on the purified lipase

Reagent	Relative activity(%)
None	100
$LiCl_2$	95
$MnCl_2$	102
KCl	102
NaCl	95
$MgCl_2$	95
$CaCl_2$	118
$ZnCl_2$	126
EDTA	92
SDS	30
PMSF	64
β-Mercaptoethanol	84
DTT	88

The lipase solution was pre-incubated in various metals, inhibitors and detergents at 65°C for 10 min and the residual activity was assayed.

the reaction temperature at 65–85°C, the lipase of the isolate ID-1 showed the highest activity at 75°C (Fig. 3). The enzyme was stable up to 50°C and relatively thermostable since it retained 73 and 50% of its original activity at 60 and 70°C, respectively, when incubated for 30 min at pH 7.5. The pH optimum of the lipase activity was near neutral pH (pH 7.5) (data not shown).

The relative hydrolytic activity of the lipase towards different PNPEs (Fig. 4A) and triacylglycerols (Fig. 4B) was determined at reaction temperature. The lipase demonstrated a wide substrate specificity depending on the chain length of the fatty acids. The lipase showed the highest activity towards *p*-nitrophenyl caproate (C_6 acyl group) among PNPEs tested. However, it had a high activity towards tricaprylin (C_8), tricaproin (C_{10}) and trilaurin (C_{12}) in case of triacylglycerols.

In the presence of Ca^{2+} and Zn^{2+} (1 mM), the catalytic activity was enhanced to about 120% and inhibited by SDS and PMSF (Table 2). Partial inactivation of the lipase by PMSF may be caused by modification of an essential serine residue that plays a key role in the catalytic mechanism [22].

In conclusion, we isolated *B. thermoleovorans* ID-1 from hot springs in Indonesia and purified extracellular lipase from it. Due to its very high growth rate on variable lipid substrates and wide substrate specificity at elevated temperatures, this isolated strain could be used for industrial and environmental applications.

Acknowledgements

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