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Purification and Properties of Thermostable Lipase from a Thermophilic Bacterium, *Bacillus licheniformis* IBRL-CHS2

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Recently, properties of thermostable lipases receive a very special interest as the enzymes are suited for harsh industrial processes. This research was aimed to purify a thermostable lipase produced by *Bacillus licheniformis* IBRL-CHS2 and to determine the properties that could give advantages to the industries. The enzyme was purified to 6.76-fold with 1.81% recovery by double steps of gel filtration chromatography. The molecular weight was shown to be 37.3 kDa. The purified lipase showed the maximum activity at 70°C and pH 8.0. However, the lipase was more stable at 65°C with the half-life of 29 hours. The activity was improved in the presence of Ca^{2+}, K^{+}, Zn^{2+}, Mg^{2+}, potassium iodide and 2-mercaptoethanol. Besides that, the activity also retained in all organic solvents tested. For the effect of surfactants, the activity was enhanced by Tween 20 and Span 40. The lipase showed highest activity towards olive oil and trimyristin ($C_{14:0}$) as compared to other substrates. The K_m value obtained was 0.60 mM and the V_{max} value was 101 mM min^{-1} when the substrate used was trimyristin. Properties showed by this lipase make it suitable for a wide range of application such as food industry, sewage treatment and organic synthesis.

Key words: Thermostable lipase, *Bacillus licheniformis* IBRL-CHS2, Enzyme purification, kinetic parameters.

Lipases are useful in catalyzing the hydrolysis of triacylglycerides, to produce diacylglyceride, monoacylglyceride, glycerol and free fatty acids[1]. However, in non-aqueous media, the reaction could be reversible where the lipases can also catalyze esterification and transesterification[2]. Lipases have become an important group of biotechnologically relevant enzymes and have been used for a wide range of industrial applications especially in the area of detergents and food industry[3].

In industry, most of the lipase-catalyzed processes functions at 45°C or above[4]. Thus, lipase enzymes which can exhibit the optimum activity at higher temperatures are required. The enzymes must be thermostable in order to survive in elevated temperatures. Thermostable enzymes (including lipases) are found to be produced mostly by thermophilic bacteria[5,6]. They are reported to be more rigid where the enzymes are normally resistant to chemical denaturation and drastic conditions as compared to their mesophilic counterparts[7].

In conjunction with the significant role of thermostable lipases in industry nowadays, two aspects regarding the purification protocols and properties of the enzymes are extremely studied. These are the most important elements prior to the determination of three-dimensional structure and the structure-function relationships of the enzyme[8]. The information on the enzyme purification protocol and their properties will

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provide new lipases with better quality and could be applied in various lipase-catalyzed processes.

MATERIALS AND METHODS

Microorganism and growth conditions

Bacillus licheniformis IBRL-CHS2 used in this research was obtained from the culture collection of Industrial Biotechnology Research Laboratory (IBRL), Universiti Sains Malaysia, Penang, Malaysia. This isolate was previously isolated from Cangar Hot Spring in Malang, Indonesia. *B. licheniformis* IBRL-CHS2 was grown in 100 ml optimum medium (pH 8.0) in a 250 ml conical flask. The medium contained 1.00% (w/v) glucose, 0.25% (w/v) NaCl, 2.00% (w/v) peptone and 0.20% (v/v) olive oil as an inducer. The cultivation was carried out at 50ºC with the agitation rate of 200 rpm for 24 hours. After that, the cultivation broth was filtered through a Whatman no. 1 filter paper and centrifuged at 9000 rpm for 15 minutes. The cell-free supernatant was then used as the enzyme source.

Lipase assay

Lipase assay was carried out using a modified colorimetric method of Kwon and Rhee[9]. One milliliter of culture filtrate was added to the reaction mixture containing 1.48 ml Tris-HCl (100 mM, pH 8.0), 20 µl $CaCl_2.2H_2O$ (20 mM) and 2.5 ml oil emulsion as substrate. The emulsion was prepared earlier by mixing together 1% polyvinyl alcohol and olive oil (3:1; v/v). The reaction was carried out at 70ºC for 30 minutes with the agitation rate of 200 rpm. The reaction was terminated by the addition of 1.0 ml 6 M HCl. Then, 5 ml isooctane was added. After a slight shake, the mixture was allowed to stand still at room temperature (27ºC±2) for 10 minutes until it was well separated. Four milliliters of the upper layer was transferred to a new tube containing 200 µl of cupric reagent and mixed vigorously. Lipase activity was determined spectrophotometrically after 30 minutes at 715 nm based on the oleic acid as standard. One unit of enzyme activity is defined as the amount of enzyme that liberated 1.0 µmol of free fatty acids per minute at the specific reaction conditions (70ºC and pH 8.0). All experiments were carried out in triplicates.

$$\text{Lipase activity (U/ml)} = \frac{\mu\text{mol/ml}}{\text{min}}$$

Determination of protein amount

Amount of protein was determined according to Bradford method[10]. Bovine serum albumin (BSA) was used as standard.

Purification of lipase

Ultra filtration

Amicon Ultra 15 ml devices incorporate Ultracel ultra filtration membrane with molecular weight cut-offs 3000 Da was used to concentrate the crude lipase enzyme. One hundred and fifty milliliters of crude enzyme was concentrated for 10 times by centrifugation at 3500 rpm at 4ºC approximately. The final volume of the concentrated crude enzyme was 15 ml.

Gel filtration chromatography

Lipase from *B. licheniformis* IBRL-CHS2 was purified to apparent homogeneity by double steps of Sephadex G-100 gel filtration. Three percent of concentrated crude enzyme was applied to the Sephadex G-100 column (size 30 x 1.0 cm) which was previously equilibrated with 10 mM Tris-HCl buffer (pH 8.0) and then eluted with the same buffer. Fractions of 4 ml were collected at a flow rate of 33 ml/hr and assayed for protein and lipase activity. The active fraction obtained was then applied again to the same column and the same procedure was repeated.

Electrophoresis

Samples of crude enzyme, concentrated enzyme (ultra filtration) and fractions from gel filtration chromatography were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis[11] by using 12.5% acrylamide gels. The gels were stained with silver stain[12]. For determination of relative molecular weight, SDS-PAGE was run along with Unstained Protein Molecular Weight Marker (Fermentas) containing β-galactosidase (116.0 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), lactate dehydrogenase (35.0 kDa) and REase Bsp981 (25 kDa).

Effect of temperature on lipase activity and stability

The optimum temperature for lipase activity was determined by carrying out the enzyme reaction at different temperatures in the range of 40-90ºC for 30 minutes. Tris-HCl (pH 8.0) was used as buffer. Thermostability of lipase was studied by pre-incubating the enzyme at various temperatures ranging from 65-75ºC for different time intervals from 0-24 hours. After that, the residual lipase

activity was assayed under the standard assay conditions.

Effect of pH on lipase activity and stability

The optimum pH was measured by conducting the enzyme reaction at 70°C in different pH (pH 4.0-10.0) using different buffers at 0.1M concentration: citrate-phosphate buffer (pH 4.0-5.0), phosphate buffer (pH 6.0-7.0), Tris-HCl buffer (pH 8.0-9.0) and carbonate-bicarbonate buffer (pH 10.0). For pH stability studies, 1 volume of enzyme was mixed with 4 volumes of buffer and pre-incubated at different pH (pH 7.0-9.0) for different time intervals from 0-24 hours. The residual lipase activity was assayed under the standard assay conditions.

Effect of effector molecules on lipase activity

One volume of enzyme was mixed with 1 volume of various effector molecules (metal ions, oxidizing agents, reducing agents and chelating agents at a concentration of 1mM). The mixture was then pre-incubated at 70°C, pH 8.0 and 200 rpm of agitation rate for 30 minutes. Lipase activity was assayed under the standard assay conditions. Lipase activity without the addition of effector molecules was defined as 100%.

Effect of organic solvents on lipase activity

Three volume of enzyme was mixed with 1 volume of 25% (v/v) organic solvents. The mixture was then pre-incubated at 70°C, pH 8.0 and 200 rpm of agitation rate for 30 minutes. Lipase activity was assayed under the standard assay conditions. Lipase activity without the addition of organic solvents was defined as 100%.

Effect of surfactants on lipase activity

The effect of surfactants on enzyme activity was determined by pre-incubating 1 volume of enzyme with 1 volume of 10 mM surfactants for 30 minutes at 70°C, pH 8.0 and with the agitation rate of 200 rpm. The activity was assayed under the standard assay conditions. Lipase activity without the addition of surfactants was defined as 100%.

Substrate specificity

Substrate specificity was determined using various natural oils (sunflower oil, rice bran oil, palm oil, soy bean oil and corn oil) and triacylglycerols ($C_{2:0}$-$C_{18:0}$) with the concentration of 100 mM. These substrates were tested as the substitute to olive oil emulsion. The lipase activity was assayed under the standard assay conditions.

Determination of kinetic parameters

Lipase from *B. licheniformis* IBRL-CHS2 showed high preference towards trimyristin ($C_{14:0}$). Thus, trimyristin was used as substrate to determine the values of kinetic parameters, K_m and $V_{max.}$ Five hundred microliters of enzyme was added to the set of different concentrations (0.25 to 2.0 mM) of trimyristin. Enzyme reaction was carried out under the standard assay conditions and the velocity of the reaction was determined. Lineweaver-Burk plot was generated to determine the K_m and V_{max} values of the enzyme.

RESULTS AND DISCUSSION

B. licheniformis IBRL-CHS2 was identified as Gram-positive with rod-shaped bacteria. The colony was observed to be 3.0 mm in size, irregular shape, flat elevation, lobate margin, opaque, moist with shiny surface texture and beige in color (Fig. 1).

Purification of lipase

The extracellular crude enzyme produced by *B. licheniformis* IBRL-CHS2 was concentrated by ultra filtration and subsequently subjected to double steps of gel filtration chromatography for

Table 1. Purification of thermostable lipase from *B. licheniformis* IBRL-CHS2 started with the precipitation step by ultra filtration and subsequently followed by double steps of gel filtration chromatography using Sephadex G-100 column

Step	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Yield (%)	Fold
Crude enzyme	7375.00	504.55	14.62	100	1
Ultra filtration	1050.00	33.95	30.92	14.24	2.11
Gel filtration I	166.67	3.42	48.76	2.26	3.34
Gel filtration II	133.32	1.35	98.76	1.81	6.76

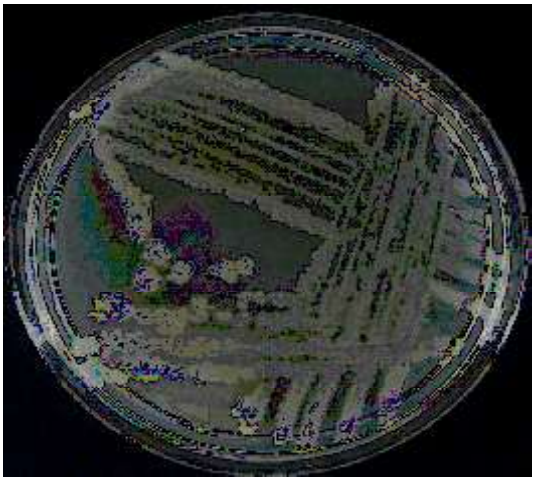

Fig. 1. Colony of *B. licheniformis* IBRL-CHS2 on nutrient agar plate

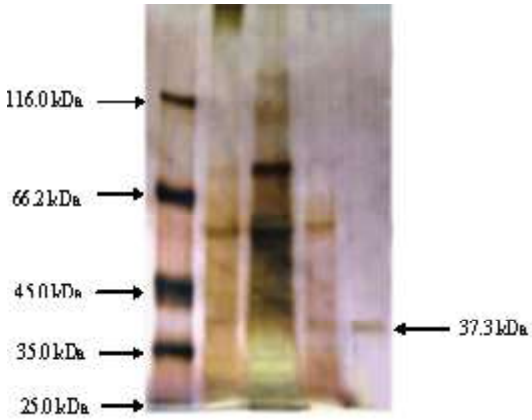

Fig. 2. SDS-PAGE analysis of thermostable lipase from *B. licheniformis* IBRL-CHS2. The analysis was conducted using 12.5% of acrylamide gel and stained with silver stain. Lane M: molecular weight marker, Lane 1: crude enzyme, Lane 2: ultra filtration, Lane 3: gel filtration I (PI), Lane 4: gel filtration II (PII)

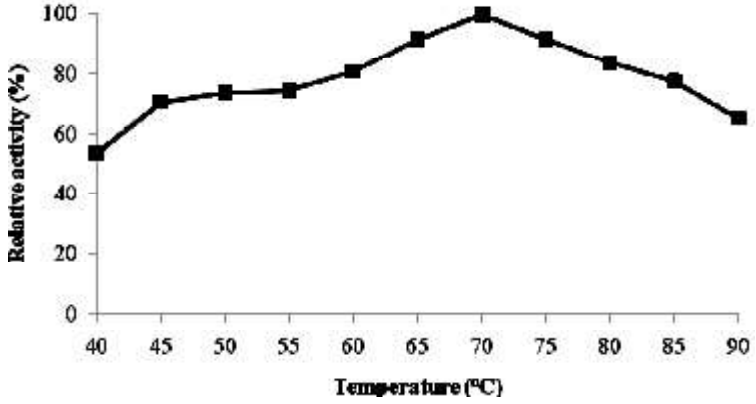

Fig. 3. Effect of temperature on the activity of purified thermostable lipase from *B. licheniformis* IBRL-CHS2

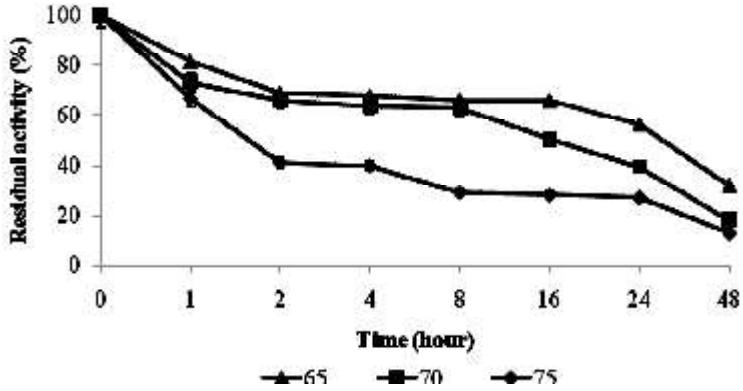

Fig. 4. Effect of temperature on the stability of purified lipase from *B. licheniformis* IBRL-CHS2

purification. This lipase was purified to 6.76-fold with 1.81% recovery (Table 1). It was confirmed to be homogeneous as a single band observed on SDS-PAGE with a molecular weight of 37.3 kDa (Figure 2). Previously, thermostable lipases have been reported to be produced by several thermophilic bacterial strains such as *Pseudomonas*[13], *Serratia*[14] and *Lactobacillus*[15], but lipases from *Bacillus* spp. are still of interest especially for food industry because of their non-pathogeneceity[16]. It was found that molecular weights of lipases from thermophilic *Bacillus* usually ranging from 11 kDa in *B. thermoleovorans* CCR11 [17] up to 69 kDa in *Bacillus* sp. THL027 [18] depending on the amino acids compositions. However, most of the thermostable lipases produced by *Bacillus* have the molecular weights of about 34 to 45 kDa. For instance, the molecular weight of lipase from *B. thermoleovorans* ID-1 was 34 kDa [19]. Both lipases from *Bacillus* sp. J33 [20] and *B. subtilis* NS 8 [21] have the molecular weights of 45 kDa. In this range, lipase from *B. licheniformis* IBRL-CHS2 was considered small since the molecular weight was 37.3 kDa. Small enzymes are important due to the smaller changes (unfolding) in tertiary structure which will lead to a higher thermostability than the bulky proteins[19].

Properties of lipase

It has been reported previously that optimum temperature of lipases from thermophilic bacteria to be in the range of 50°C 80°C (4; 16). The optimum temperature of the purified lipase from thermophilic *B. licheniformis* IBRL-CHS2 was observed at 70°C. This was followed by 65°C and

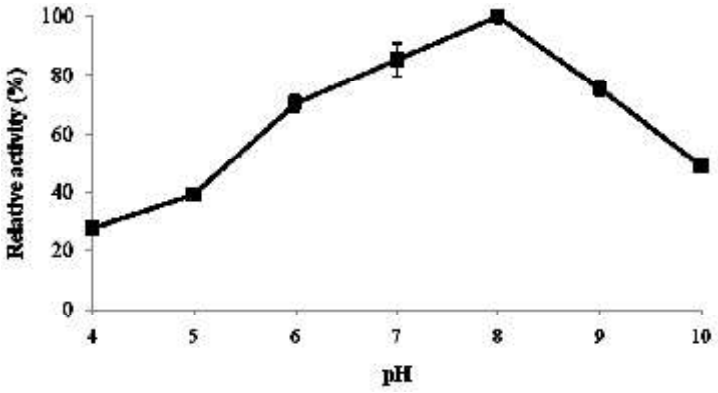

Fig. 5. Effect of pH on the activity of purified thermostable lipase from *B. licheniformis* IBRL-CHS2

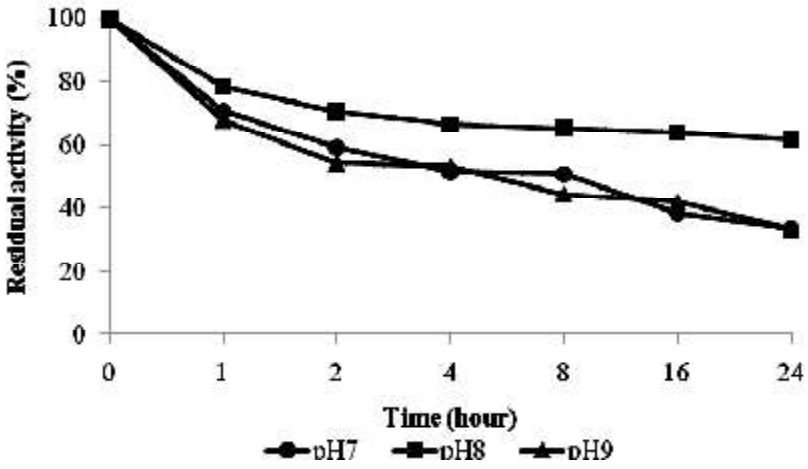

Fig. 6. Effect of pH on the stability of purified lipase from *B. licheniformis* IBRL-CHS2

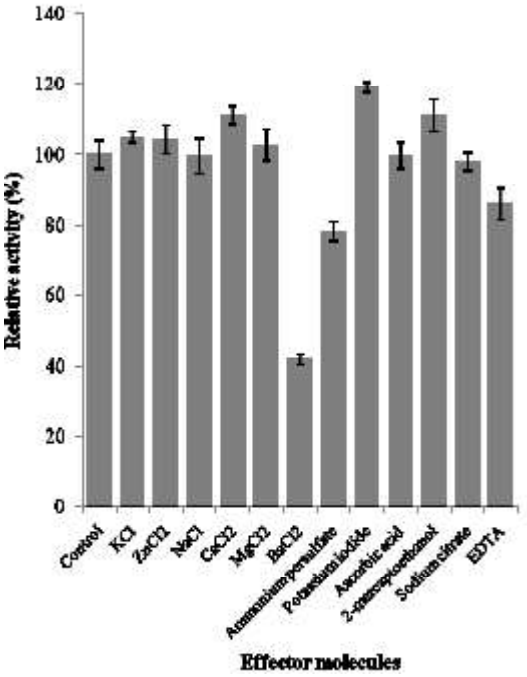

Fig. 7. Effect of metal ions, oxidizing agents, reducing agents and chelating agents on the purified thermostable lipase activity from *B. licheniformis* IBRL-CHS2 after 30 minutes of preincubation

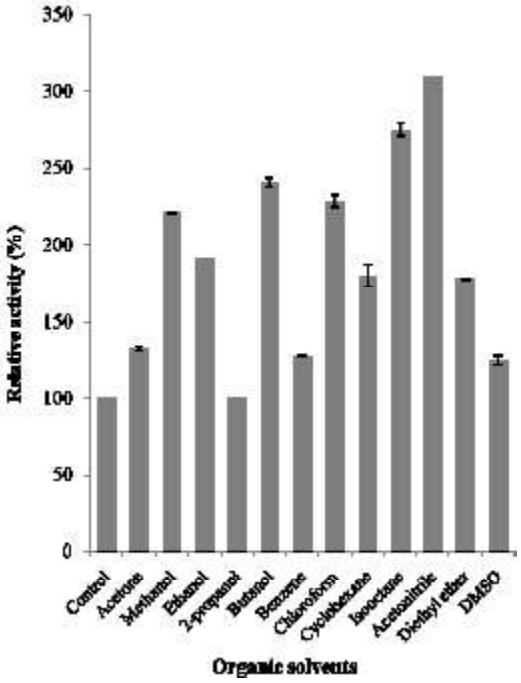

Fig. 8. Effect of organic solvents on the purified thermostable lipase activity from *B. licheniformis* IBRL-CHS2 after 30 minutes preincubation

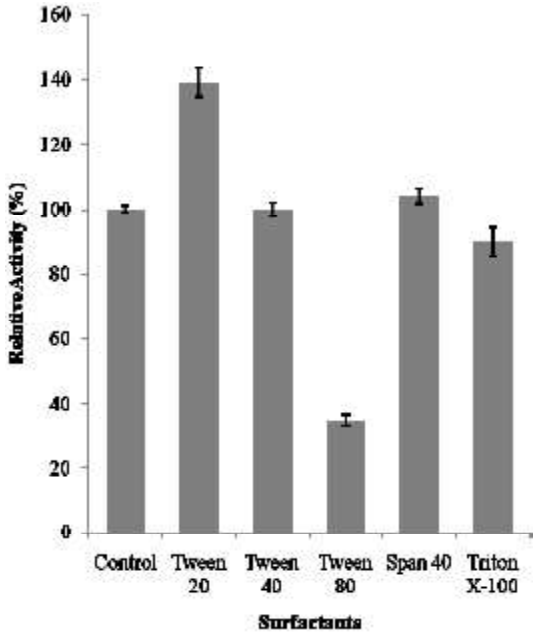

Fig. 9. Effect of surfactants on the activity of purified thermostable lipase from *B. licheniformis* IBRL-CHS2 after 30 minutes of preincubation

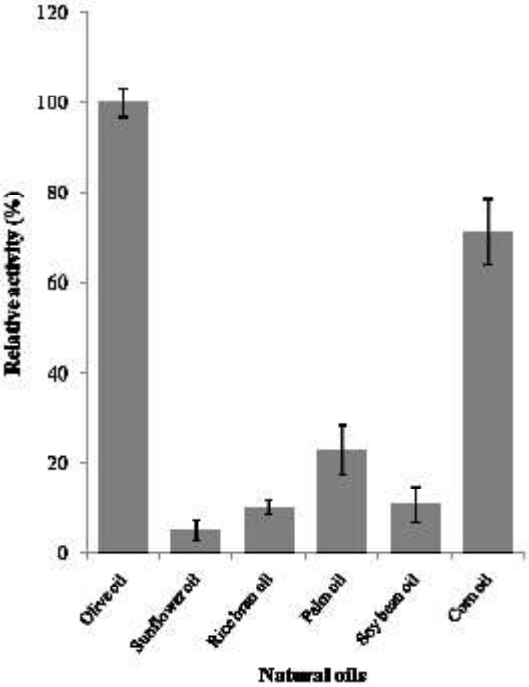

Fig. 10. Lipase activity towards different natural oils

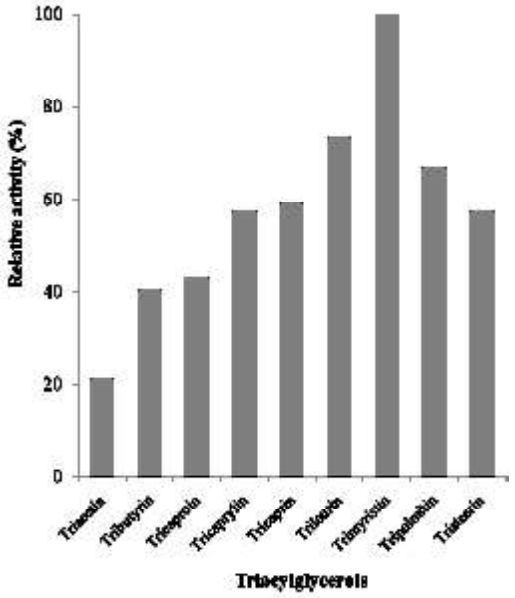

Fig. 11. Lipase activity towards different triacylglycerols

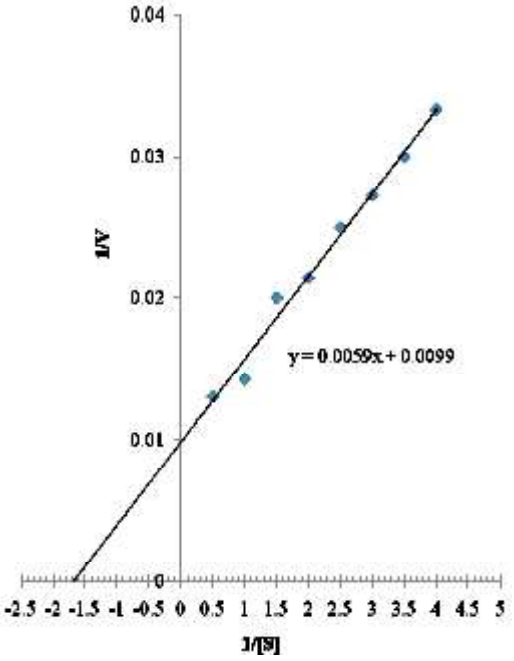

Fig. 12. Lineweaver-Burk plot of the purified lipase from *B. licheniformis* IBRL-CHS2

75°C where the relative activity was 91.7% for both (Fig. 3). For thermostability study, the lipase retained 81%, 73% and 66% of its original activity after 1 hour of incubation at 65°C, 70°C and 75°C, respectively. Meanwhile, the lipase retained 56%, 39% and 27% of its original activity after 24 hours of incubation at 65°C, 70°C and 75°C, respectively. This particular lipase was considered stable at high temperature as the half-life of the lipase was 29 hours at 65°C (Fig. 4). The result showed that the lipase has the longer half-life as compared to the previous reports of most of the thermostable lipases. For instance, the optimum temperature of lipase from *Bacillus* sp. which was also observed at 70°C has the half-life of 2 hours at 60°C [22]. The optimum temperature of lipase from *B. coagulans* BTS-3 was observed at 55°C, also with the half-life of 2 hours at the similar temperature[23]. However, the half-life of lipase studied in this research was found lower than the lipase produced by *Bacillus* sp.[24] where the optimum temperature was observed at 70°C with the half-life of 50 hours at 60°C. The longer half-life showed by the lipase from *B. licheniformis* IBRL-CHS2 most probably due to the high level of hydrophobic amino acids contained in this lipase as compared to other thermostable lipases. As it is well known, the higher the level of hydrophobic amino acids, the enzyme would be more thermostable[25].

Apart from thermostability, lipases which are found useful mainly in detergents industry required to be stable in alkaline condition. This situation shows that among the desirable properties that should be exhibited by commercial lipases, thermostability and alkali tolerant are the most on demand[26]. Lipase from *B. licheniformis* IBRL-CHS2 showed its activity in a wide range of pH. However, optimum activity (100%) was obtained at pH 8.0 (Fig. 5). It was closely followed by pH 7.0 and pH 9.0 with 85% and 75% of relative lipase activity, respectively. pH could affect the ionization state of the amino acids which determine the primary and secondary structure of the enzyme[27]. This shows that the lipase activity is crucially depends on its conformation. Thus, low lipase activity obtained at certain pH is most probably due to the disrupted conformation caused by the altered ionization state of the amino acids. Besides that, pH could also affect the ionization characteristics of substrate which can change its shape and charge properties. This would involve overall enzyme activity when the changed

substrate cannot bind to the active site of the enzyme. Fig. 6 shows the effect of pH on the stability of the lipase. The enzyme exhibited remarkable pH stability in alkaline condition since it retained 62% of its original activity even after 24 hours at pH 8.0. At pH 7.0 and pH 9.0, both retained 33% of its original activity after 24 hours of incubation.

Metal ions are one of the important factors involve in stabilization of thermostable enzymes from thermophilic microorganisms. Stabilization of enzymes at high temperatures involves the metal ion complexation, which is a process with a favorable entropy factor. As shown in Figure 7, lipase activity was not affected by Na^+ whereas the activity was decreased to 42% in Ba^{2+} after 30 minutes of incubation. In the presence of Ca^{2+}, K^+, Zn^{2+} and Mg^{2+}, the activity was increased to 111, 105, 104 and 103% respectively.

Among all, the presence of Ca^{2+} showed the highest activity of lipase. This suggested a very strong binding between the lipase enzyme and the calcium ions in bridging the active region to a second sub domain of the protein and finally leads to the enzyme tertiary structure stabilization[17]. The results also emphasizing that the effects took place even at low concentrations of calcium[4]. Previously, a number of reports show similar results where the lipase activity was enhanced by Ca^{2+} [28], K^+ [23], Zn^{2+} [19] and Mg^{2+} [4].

Reduction-oxidation (redox) reaction occurs when the oxidizing agents oxidize the –SH groups between two cysteine residues to form strong covalent disulfide (S–S) bonds[29]. Among the oxidizing agents tested in this research, lipase activity was increased to 119% in the presence of potassium iodide. The increment appeared to obey the theory involving redox reaction where reduced agents (potassium iodide) can improve the enzymatic activity[30]. However, the activity was decreased to 78% in the presence of ammonium persulfate. This is might be because of the inhibition by the accumulation of its products (fatty acids) which can cause competition for the active site of the enzyme[31]. Lipase from *Pseudomonas* sp. strain S5 also showed similar result when the activity decreased to 48% in the presence of ammonium persulfate as oxidizing agent[32]. Lipase activity was not affected by the presence of ascorbic acid whereas increased to 111% in the presence of 2-mercaptoethanol as reducing agents. The results proposed either[1] there is no disulfide bond formed between cysteine residues, or (2) there is a covalent bond formed but it does not affect the protein structure[33]. Lipase BTID-B produced by *B. thermoleovorans* ID-1 also showed similar result when the activity increased to 135% in the presence of 2-mercaptoethanol as reducing agent[34].

The activity of lipase from *B. licheniformis* IBRL-CHS2 was decreased to 98% and 86% with sodium citrate and EDTA respectively. Both are used as chelating agents. The results indicated that this lipase is a metalloenzyme that contains a bound metal ion as part of its structure. The metal ion can form a complex molecule with each of the chelating agent and subsequently disrupt the enzyme conformation.

Normally, substrates for lipase are insoluble in aqueous solutions. To overcome this problem, organic solvents and organic-aqueous two phase media can be used. Although most of the enzymes are not stable in the presence of organic solvents and are apt to denature[35], it is also well known that the effect of organic solvents on lipase activity vary from lipase to lipase. Therefore, many efforts were carried out to discover a new organic solvent tolerant lipase which can be applied in numerous industrial applications. In this experiment, exposure of the lipase from *B. licheniformis* IBRL-CHS2 towards various organic solvents for 30 minutes showed that the activity of this enzyme did not follow the log *P* trends and retained in all polar and non polar organic solvents tested (Fig. 8). The results suggested that the lipase is able to resist denaturation caused by organic solvents and also able to optimize the catalytic activity by forming hydrogen bonds for the structural flexibility and conformational mobility[36]. The highest relative activity was achieved at 310% in acetonitrile. Similarly, acetonitrile also gives the stimulating effect to the activity of lipases from *Pseudomonas aeroginosa* CS-2 [37] and *Acinetobactor* sp.[38]. On the contrary, acetonitrile showed the inhibiting effect on activity of lipases from *Bacillus* sp. RN2[39] and *Pseudomonas aeruginosa* strain PseA[40].

Fig. 9 shows the effect of surfactants on the activity of lipase from *B. licheniformis* IBRL-CHS2. The highest relative activity was achieved

in the presence of Tween 20 with 139%. This is most probably because of the improvement in substrate solubility and emulsion stability when the interfacial tension was decreased. Besides that, Tween 20 might also be used to stabilize the lipase conformation. The purpose is to prevent aggregation which can lead to the great improvement of catalytic performance[41]. The lowest activity was observed in the presence of Tween 80 with only 36%. The strong inhibitory effect is most likely due to the[1] competition for the catalytic centre of the lipase[37] and[2] denaturation of the enzyme might be involved[42]. The result was similar with the study reported earlier where the activity of lipase from *Pseudomonas aeruginosa* CS-2 was inhibited by Triton X-100 and Tween 80[37]. On the contrary, the presence of Triton X-100 and Tween 80 gave stimulation effect to the lipase from *Bacillus* sp[43].

Fig. 10 shows the activity of lipase against various natural oils (as relative to the olive oil, 100%). Subsequently after olive oil (contains 86% unsaturated fatty acids), corn oil (contains 83% unsaturated fatty acids) was observed as the second highest of lipase activity with 71%. The results imply high preference of this lipase against unsaturated fatty acids[21], respectively. Similarly, lipase isolated from *Bacillus* sp. strain 42 showed the highest affinity towards olive oil (100%) and subsequently followed by corn oil with 88% [44]. On the contrary, lipase from *B. subtilis* NS 8 showed that the activity against corn oil is relatively higher than olive oil with 102 and 100%, respectively[21].

Among all the triacylglycerols tested, lipase from *B. licheniformis* IBRL-CHS2 was found to have highest activity (100%) towards trimyristin ($C_{14:0}$). It was followed by trilaurin ($C_{12:0}$) with 73% and tripalmitin ($C_{16:0}$) with 67% (Figure 11). The result reveals that more than 50% of relative activity was achieved when the substrates used were triacylglycerols of middle (C_8-C_{12}) and long (C_{14}-C_{18}) chain fatty acids. The enzyme's preference towards medium to long long-chain fatty acids indicated that it is a true lipase, in contrast to esterase that could only hydrolyzes short chain fatty acids[39].

Nowadays, most of the industrial enzymes were reported to have the K_m values in the range of 10^{-1} to 10^{-5} M when react towards biotechnologically important substrates[45]. As calculated from the Lineweaver-Burk plot, K_m value of the lipase from *B. licheniformis* IBRL-CHS2 was 0.60 mM and the V_{max} value was 101 mM min^{-1} (Fig. 12) when the substrate used was trimyristin. As it is well known, trimyristin can be found naturally in many types of vegetable fats and oils. Thus, kinetic parameters obtained in this research are very well recommended to be used as a guideline in food processing industry especially in the area of modification of fats and oils. Kinetic parameters determination of *Bacillus* lipase was also performed by other researchers using different substrates. One of the examples is the study of lipase from *Bacillus* sp. J33 by using p-nitrophenyl laurate (pNPL) as substrate. The K_m value obtained was 2.5 mM and the V_{max} value was 0.4 µM min^{-1} [20]. Besides that, kinetic parameters of a lipase from *B. pumilus* RK31 was determined when the substrate used was p-nitrophenyl palmitate (pNPP). The K_m value obtained was 1.83 mM while the V_{max} value was 10 mM min^{-1} [46]. Basically, low K_m value represents high affinity which means the lower the K_m values, the greater the affinity of the enzymes towards the substrates. It is also explains that if the K_m value is low, then a low concentration of substrate is needed to achieve the maximal catalytic efficiency of the enzyme[47].

CONCLUSIONS

In conclusions, lipase enzyme from *B. licheniformis* IBRL-CHS2 which was isolated from Cangar Hot Spring, Malang, Indonesia was successfully purified. The properties showed by the lipase, suggested that this particular enzyme is a very good candidate of biocatalyst for various biochemical reactions in industrial biotechnological processes.

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Statement of ethical standard

The authors confirm that the manuscript meets the highest ethical standards.

Declaration of interest

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

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