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Research article

Novel thermostable lipase produced by a thermo-halophilic bacterium that catalyses hydrolytic and transesterification reactions

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ABSTRACT

Lipase belongs to the class of hydrolytic enzymes that are widely used in the biotechnology industries. The goal of this research was to purify and characterize lipase produced from a thermo-halophilic bacterium, namely Pria Laot Sabang 80 (PLS 80). Purification was performed using ammonium sulphate fractionation, followed by gel filtration chromatography using Sepharose Cl-6B. After purification, the enzyme had a specific activity of 326.6 U/mg with a purity of 6.02 higher than the crude extract; with a molecular weight of around 50 kDa. The optimum activity was observed at 70 °C and pH 9. The activity increased in the presence of 10 mM Mn^{2+}, K^{+} and Ca^{2+} ions, while Hg^{2+} only slightly increased the enzyme activity. In contrast, the activity decreased in 10 mM Mg^{2+}, Zn^{2+}, Co^{2+}, EDTA, and PMSF. The enzyme showed good hydrolytic activity on long fatty acids substrates (*p*-nitrophenyl palmitate) with a value of 35.5 U/mL. It was also able to catalyze a transesterification reaction. GC-MS result showed that the biodiesel consisted of methyl octanoate (5.3%), methyl caprate (12.4%), methyl laurate (34.1%), methyl myristate (10.7%), methyl palmitate (3.9%), and methyl stearate (1.2%) when using coconut oil as the substrate. The results suggested that the lipase from PLS 80 had unique attributes that could be useful in various industrial applications.

1. Introduction

The rapid development in the biotechnology field during the last few decades has made many industries moving towards biobased-processes using enzymes as the catalyst (Krüger et al., 2018; Pellis et al., 2018). One of the important enzymes used in an industrial process is lipase (Krüger et al., 2018). Lipase (triacylglycerol acyl hydrolases (E.C.3.1.1.3) catalyses the hydrolysis of long-chain triacylglycerides (with acyl chain lengths of >10 carbon atoms) to produce monoacylglyceride, diacylglyceride, glycerol and free fatty acids. Lipases can also catalyse esterification, transesterification, inter-esterification, and alcoholysis reactions (Arpigny and Jeager, 1999; Salleh et al., 2006; Wang et al., 2010; Febriani et al., 2013). Lipase can also catalyses substrate based on stereo and regio-selectivity (Li et al., 2009). The ability of lipase to catalyse various reactions made it widely used in industrial applications.

The primary applications of the lipase are in the detergent, additives (modification of taste/flavour), pharmaceuticals, cosmetics, oleochemicals, and fat processing industries, as well as for wastewater treatment purposes (Adrio and Demain, 2014). Lipase is also used to catalyse transesterification reactions to produce biodiesel (Sharma et al., 2001; Li and Zhang, 2005; Moecke et al., 2016). The biodiesel has several advantages such as non-toxic, biodegradable, good lubrication, low sulphur content, high flash point and environment-friendly (Sajjadi et al., 2016). The use of lipase in biodiesel industry offers several benefits, such as easy product separation, minimum wastewater treatment, and minimum side reactions, which make it a desirable biocatalyst (Wancura et al., 2019).

Effectiveness of lipase is determined from a high level of purity and its catalytic attributes. Isolation and purification of lipase from extremophilic bacteria have become one of the growing bioresource technology researches (Sanchez and Demain, 2017; Krüger et al., 2018). As the enzyme from thermophiles exhibits activity at high temperature, it has better thermodynamic stability than its mesophiles counterparts (Li and Zhang, 2005).

A thermo-halophilic bacterium has been isolated from underwater hot spring area in Pria Laot Sabang, Indonesia, namely Pria Laot Sabang strain 80 (PLS 80). In this study, we reported a lipase produced by the PLS 80, which was able to catalyse the hydrolysis of triglyceride as well as transesterification of triglyceride. The lipase from the PLS 80 had reasonable hydrolytic activity in the presence of Hg^{2+}, which is rarely

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reported in the previous studies. In this study, we report the purification and biochemical characterisation of lipase from PLS 80 that was able to catalyse multi-reactions, as the basis to use the enzyme further.

2. Materials and methods

2.1. Regeneration of PLS A

The microorganism in this study was isolated from underwater fumaroles in Pria Laot Sabang area, Aceh Province, Indonesia, named as PLS 80. The number indicates the sequence it was purified as a single colony. PLS 80 from glycerol stock was initially incubated on a solid medium of ½ Thermus (½ T) containing 0.2% NaCl, 0.5% glucose, 0.4% yeast extract, 0.8% peptone, 2.5 % agar bacto at 70 °C for 18 h. The pH was adjusted to 9.0 by a glycine-NaOH buffer.

2.2. Production and isolation of lipase

A single colony from the solid medium was transferred into a fresh ½ T liquid medium. The pH was adjusted to 9.0 by a glycine-NaOH buffer. The medium was then incubated at 70 °C in a shaker water bath at 150 rpm for 22 h. About 1.4 mL of the inoculums were then transferred to 100 mL of ½ T liquid medium containing 1% olive oil and 0.5% tween 80. The culture supernatant after centrifugation (10000×*g*, 10 min) was used to determine lipase activity and concentration (Febriani et al., 2019).

2.3. Purification of lipase

The crude lipase was purified from the supernatant using two-step purification, i.e. precipitation by ammonium sulphate and gel filtration chromatography. Ammonium sulphate precipitation was done using various saturation level of 0–20%, 20–40%, 40–60% and 60–80%. The salt was gradually added and stirred at low speed. Once the desired saturation level was reached, the solution was further stirred for 10 min, then allowed to settle overnight at 4 °C to ensure optimum precipitation. Centrifugation was then conducted at 10000×*g*, 4 °C for 10 min. The crude enzyme was further dialyzed in a cellophane membrane using 20 mM buffer Tris-HCl pH 8 that was changed regularly until no salt was detected in the buffer.

The fraction showing the highest lipase specific activity from ammonium sulfate precipitation was placed in dextran sepharose Cl-6B column that had been equilibrated with 20 mM tris HCL buffer pH 7.0. The fraction was then eluted at a rate of 0.3 mL/min using 150 mM tris-HCl buffer pH 7.0. Every 2 mL of eluent was collected as a fraction and subjected to lipase activity and protein concentration assays.

2.4. Determination of molecular weight

The lipase molecular weight was determined by Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and zymography methods. SDS-PAGE was carried out based on the Laemmli method (Laemmli, 1970). The concentrations of the separating gel and stacking gels were 10% and 5%, respectively. Electrophoresis was conducted using a running buffer containing 25 mM Tris-Cl and 192 mM glycine pH 8.3 at 120 V for 2 h. The gel was later stained using Silver Staining Kit (Fermentas).

Before conducting the zymography process, the gel was initially washed with distilled water for 10 min. The gel was then immersed into 150 mM Tris-HCl buffer pH 7.0 containing 0.5% Triton-X at 4 °C for 4 h. Lipase activity on the denatured gel was checked by incubating it for 6 h at 70 °C in a mixture containing 3 mM alpha-naphthyl acetate, 1 mM fast red TR (Sigma) and 150 mM Tris-HCl buffer pH 7.0 (1: 1: 98 v/v/v) (Soliman et al., 2007).

2.5. Biochemical characterization

The biochemical characterization of the purified lipase was conducted by determining the lipase activity and concentration at various temperature (50, 60, 70, 80 °C) and pH (5, 7, 9, 10), as well as in the addition of metal ions (10 mM $CaCl_2$, $HgCl_2$, KCl, $ZnCl_2$, $CoCl_2$) and organic compounds (EDTA and PMSF).

2.6. Lipase activity and protein assays

Lipase activity was determined using the spectrophotometry method as described by Lee et al. (1999) using *p*-nitrophenyl palmitate (*p*-NP) as the substrate. *p*-NP palmitate was dissolved in a mixture of acetonitrile, ethanol and sodium phosphate buffer (pH 8) with a composition of 1: 4: 95 (v/v/v). A total of 0.3 mL of enzyme solution was added to 1 ml of the substrate. The mixture was then incubated at 60 °C for 15 min. The enzyme reaction was stopped by the addition of phenyl methyl sulfonyl fluoride (PMSF) and incubated on dry ice for 10 min. The absorbance of the mixture was then measured at 400 nm. The control was prepared similarly as the sample but without the addition of the enzyme. One unit of lipase activity was defined as the amount of enzyme that releases one µmol *p*-nitrophenol per minute under experimental conditions.

$$Activity\ (U) = \frac{[pnitrofenol]\ (\mu mol)}{Reaction\ time\ (minute)} \times \frac{1000\ nmol}{\mu mol} \times \frac{V_{tot}\ (mL)}{V_{enzyme}(mL)}$$

The protein concentration was determined according to Bradford method using Bovine Serum Albumin (BSA) of various concentration 0; 1.25; 2.5; 5; 10; 15; 20; 25 µg/mL as the standard (Bradford, 1976). The absorbance of the mixture was measured at 595 nm.

2.7. Preparation of lipase for transesterification reaction

The purified lipase was modified using decanoyl chloride, as described by Zhao et al. (2007). A total of 30 mg of lipase was dissolved in 3 mL of 0.2 M phosphate buffer at pH 8.0. A mixture of 6 mL chloroform containing 15% (v/v) decanoyl chloride was then added. The pH of the mixture was maintained at pH 10 with the addition of 10% (w/v) NaOH. The mixture was then stirred for 8 h at room temperature, followed by centrifugation at 8000×*g* at 4 °C for 30 min. After centrifugation, three separate layers were visible where the modified enzyme was present in the middle layer in the form of solid-like cotton. The amount of lipase was determined from its absorbance at λ 280 nm.

2.8. Transesterification reaction

Transesterification reaction was carried out based on the method described by Karimi (2016). In this study, coconut oil (containing 0.8 % caproic acid, 8.93 % caprylic acid, 6.74 % decanoic acid, 40.91% lauric acid, 22.02 % myristic acid, 13.62 % palmitic acid, 7.37% oleic acid, 2.58 % stearate acid, and 1.35 % linoleic acid) was mixed with methanol in a molar ratio of 6:1 (Karimi, 2016; Arumugam and Ponnusami, 2017). The modified lipase with a concentration of 25% and 10% n-hexane were then added to the mixture. The mixture was incubated in a water bath shaker at 150 rpm and 70 °C for 24 h. The result was separated and analysed by GC-MS.

2.9. Characterization of transesterification product

The transesterification product was characterized by its density and viscosity. Pycnometer was used to determine the density. The mass of the product and the control of precisely similar volume were then compared. Ostwald viscometer was used to determine the viscosity. The viscosity of the product was measured at 37 °C. The ratio of the flow time required by the product and the control of travelling through the capillary tube was

Table 1. Purification of lipase from PLS 80.

Purification	Volume (mL)	Protein		Enzyme			Purity (fold)	Yield (%)
		Concen-tration (mg/mL)	Total (mg)	Activity (U/mL)	Specific Activity (U/mg)	Total Activity (U)		
Crude enzyme	1200	0.651	781.2	35.3	54.2	4236	1	100
Ammonium Sulphate (40–60%)	8.4	0.166	1.39	48.1	289.8	404.4	5.34	0.95
Sepharose CL-6B	10	0.101	1.01	33.0	326.6	329.9	6.02	0.78

compared. Ethanol was used as a control. The viscosity was calculated using the following formulae:

$$\frac{\mu_{biodiesel}}{\mu_{ethanol}} = \frac{t_{biodiesel} \times \rho_{biodiesel}}{t_{ethanol} \times \rho_{ethanol}}$$

$$\text{Kinematic viscosity} = \frac{\mu_{Biodiesel}}{\rho_{Biodiesel}}$$

2.10. Statistical analysis

Data in this study was presented as an average of duplicate (error bars = ±SD). One-way ANOVA, using SPSS version 18, was performed to determine the difference between the means in the specific activity of temperature and pH, as well as the addition of metal ions and inhibitors. Least Significant Different test was performed to make a comparison within groups. Significant differences were defined at $p < 0.05$.

3. Results and discussion

3.1. Purification of lipase

Optimum extracellular lipase was obtained at the end of the exponential phase (16 h) when grown in ½ T media containing olive oil and tween 80 at 70 °C and pH 9 (Data not shown). The specific activity of the lipase in the crude extract was 54.2 U/mg (Table 1). The crude enzyme was purified using ammonium sulphate fractionation, followed by gel filtration chromatography using Sepharose CL-6B.

Amongst the five fractions of ammonium sulphate saturation level (0–20%, 20–40%, 40–60%, 60–80% and 80–100%), the lipase specific activity was optimum in the 40–60% fraction with a value of 289.8 U/mg, 5.34 purification fold and 0.95% yield (Table 1). The result was consistent with several previous studies. Lipase from *Thermus aquaticus* purified by fractionation of ammonium sulphate showed the highest activity in the 40–60% fraction with a specific activity of 79.6 U/mg and a purity level 1.44 times higher than the crude extract (Febriani et al., 2013). Lipase from *Thermosyntropha lipolytica* also purified by ammonium sulphate fractionation shows the highest specific activity at 40–75% fraction and purity of 40-fold higher than the crude extract (Salameh and Wiegel, 2010).

The 40–60% fraction was further purified using Sepharose CL-6B gel filtration chromatography, resulting in a specific activity of 326.6 U/mg with 6.02 purification fold and 0.78% yield compared to the crude enzyme (Table 1). The purification result of the lipase using Sepharose CL-6B in this study is comparable with those using other gel filtration matrices. For example, purification of lipase from *Geobacillus thermodenitrificans* using Sephadex G-100 is 34-fold higher than the crude extract (Balan et al., 2012), while purification of lipase from *Aspergillus japonicus* using Superose 12 H is 4-fold higher than the crude extract (Souza et al., 2014).

3.2. Molecular weight of lipase

The lipase in the fraction 40–60% that was purified by gel filtration chromatography had a molecular weight of about 50 kDa after SDS-PAGE, which was confirmed by zymography result (Figure 1).

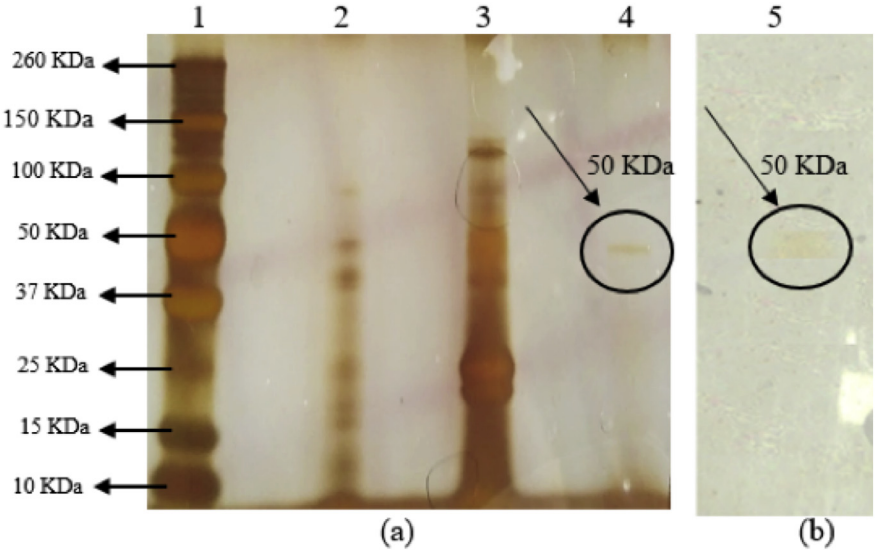

Figure 1. (a) SDS-PAGE result of lipase from PLS 80, 1 = Protein Marker, 2 = Crude Enzyme, 3 = Ammonium sulphate fraction 40–60% and 4 = Sepharose Cl-6B chromatography. (b) Zymography result, where gel was added with 3 mM alpha naphthyl acetate as the substrate and 1 mM 4-chloro-2-methylbenzendiazonium (Fast Red TR). See also supplementary material Figure 1a SDS PAGE result and Figure 1b Zymography Result.

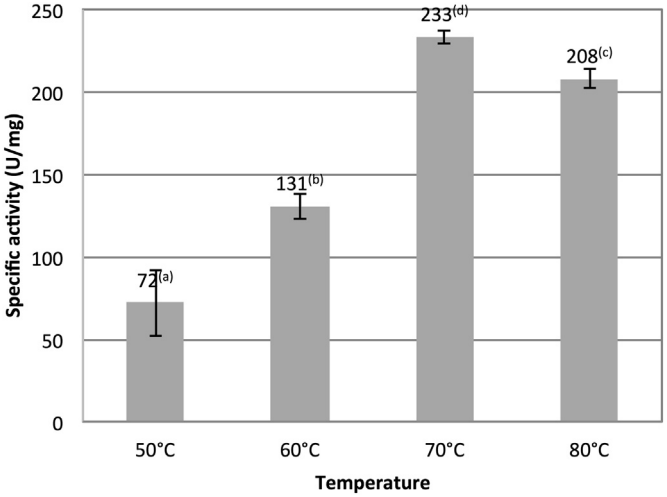

Figure 2. Effect of temperature on lipase specific activity from PLS 80. The experiment was conducted at pH 8 and 15 min of incubation time. The value was an average of duplicate (error bars = ±SD). ANOVA result shows a significant difference for all temperature ($p < 0.05$). Different notation (a, b, c, d) indicates that there is a difference within the group after the LSD test ($p < 0.05$).

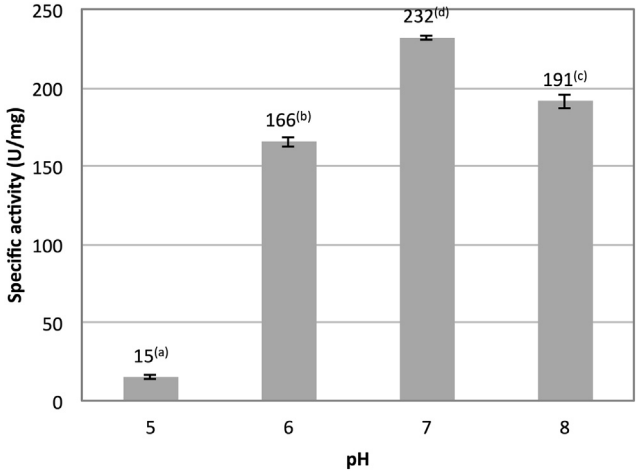

Figure 3. Effect of pH on lipase specific activity from PLS 80. The experiment was conducted at 70 °C and 15 min of incubation time. The value was an average of duplicate (error bars = ±SD). ANOVA result shows a significant difference for all pH ($p < 0.05$). Different notation (a, b, c, d) indicates that there is a difference within the group after the LSD test ($p < 0.05$).

The molecular weight of lipase in this study is comparable with those reported previously. For example, a lipase of *Thermus thermophilus HB8* purified by hydrophobic chromatography has a molecular weight of 56 kDa (Kretza et al., 2012). A lipase from *Geobacillus thermoleovorans* purified by gel filtration gel chromatography PD-10 has a molecular weight of 43 kDa (Soliman et al., 2007).

3.3. Effect of temperature on lipase activity

The effect of temperature on lipase activity was examined using *p*-nitrophenyl palmitate as the substrate at pH 8 and 15 min incubation time. The temperature was varied from 50 to 80 °C, with an interval of 10 °C. PLS 80 produced a thermostable lipase, showing a notable specific activity at a temperature of 50–80 °C. The highest specific activity of 233.4 U/mg was observed at 70 °C. The specific activities at 50 °C and 60 °C were about 31% and 89% of that at 70 °C, respectively (Figure 2).

It has been reported that lipase from *Geobacillus thermodenitrificans* shows optimum activity at 65 °C (Balan et al., 2012), while lipase from Janibacter sp. R02 has optimum activity at 90 °C (Castilla et al., 2017). A lipase from Gram-negative pleomorphic bacterium *Shewanella putrefaciens* also has an optimum activity at 80 °C (Khan et al., 2017).

3.4. Effect of pH on lipase activity

The effect of pH on the lipase activity of PLS 80 was shown in Figure 3. The lipase showed good activity in neutral to basic pH. It had a low specific activity at acidic pH. The highest specific activity was at pH 9, with a value of 231.9 U/mg. The specific activities at pH 7 and 10 were 165.5 U/mg and 191.4 U/mg, respectively.

Most of the thermostable lipases work well on alkaline pH. For example, lipases from *Geobacillus* sp T1 F16L mutant (Ali et al., 2012) and *Bacillus sp.* RSJ-1 (Sharma et al., 2002) show optimum activity pH 8.0–9.0 (Sharma et al., 2002). However, several thermostable lipases also work well at acidic pH. For example, a lipase with activity at acidic pH was obtained from *Pseudomonas gessardii* and *Spirulina platensis* (Javed et al., 2018). Also, lipase *Bacillus pumilus RK31* shows optimum activity at pH 6 and 60 °C (Kumar et al., 2012). Thus far, this study suggested that lipase from PLS 80 isolate was an alkaline thermostable lipase.

3.5. Effect of metal ions and inhibitors on lipase activity

The addition of Ca^{2+}, Mn^{2+} and K^{+}, Hg^{2+} ions increased the activity, and the values were 129%, 119%, 118%, 101% higher than the control, respectively (Figure 4). The highest relative activity was shown in the presence of Ca^{2+} as it might promote a more stable conformation when bound to the active site of the enzyme (Rahman et al., 2005). In contrast, the addition of Mg^{2+}, Zn^{2+} and Co^{2+} ions decreased the enzyme activity to 67.3%, 79,0%, 70.2%, respectively, compared to the control (Figure 4). The addition of EDTA and PMSF almost diminished the lipase activity. EDTA has a strong affinity to divalent metal ions. EDTA binds to Ca^{2+} ions that are already attached to the centre of the active side of lipase. The enzyme conformation is changed, thus significantly reduce its catalytic activity. Meanwhile, PMSF is a serine inhibitor that inhibits the serine hydrolase group of lipase that had an active side of Ser-His-Asp/Glu (Gupta et al., 2004; Febriani et al., 2013).

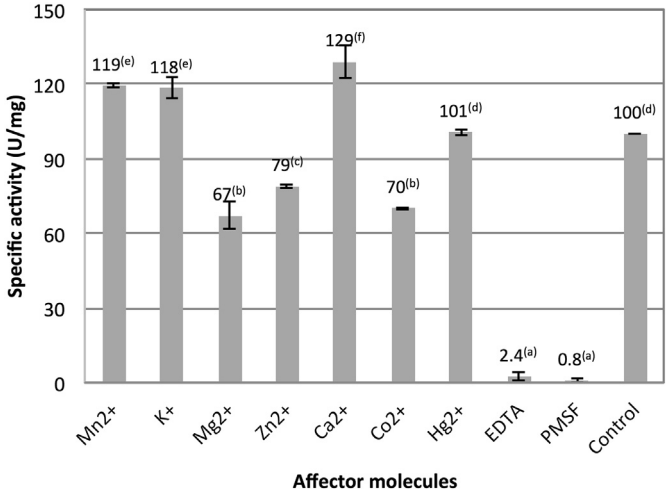

Figure 4. Effect of metal ions and inhibitors on lipase activity from PLS 80. The experiment was conducted at 70 °C, pH 9.0 and 15 min incubation time. The control was prepared without the addition of metal ions and set as 100%. ANOVA result shows a significant difference for all metals and inhibitors ($p < 0.05$). Different notation (a, b, c, d) indicates that there is a difference within the group after the LSD test ($p < 0.05$).

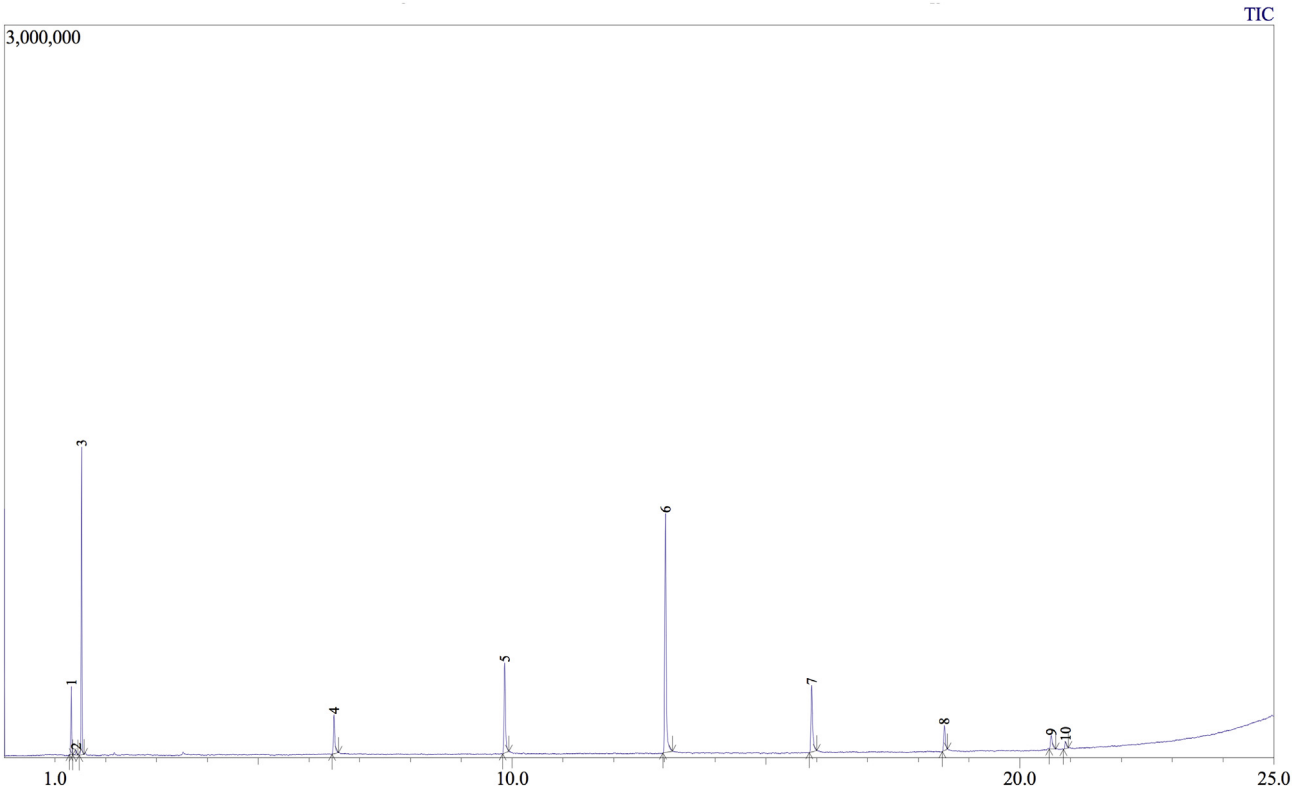

Figure 5. The chromatogram of GC-MS of the transesterification of coconut oil by the lipase from PLS 80. See also supplementary material Figure 5 Mass Spectrum.

Table 2. Composition of transesterification products from coconut oil catalysed by the lipase from PLS 80. The composition was recalculated based on methyl-esters.

Compound	Composition (%)
Methyl octanoate	7.6%
Methyl caprate	17.7%
Methyl laurate	48.6%
Methyl myristate	15.3%
Methyl palmitate	5.6%
Methyl cis-octadenate	3.6%
Methyl stearate	1.7%

High lipase activity in the presence of Hg^{2+} ions is rarely reported. On example is lipase from *Thermus thermophiles HB8* that has an increased activity, apart from Ca^{2+}, in the presence of Hg^{2+} ions (Kretza et al., 2012). Another lipase that has been reported to have an increased activity in the presence of Hg^{2+} is Lip906 produced by microorganisms isolated from mangroves. The lipase has a molecular weight of 33.2 kDa (Tang et al., 2017). Lipase from *Bacillus coagulans* MTCC-6375 also has a high activity the presence of Hg^{2+} ions, although the molecular weight of 103 kDa is considerably high (Kanwar et al., 2006).

Table 3. Density and viscosity of the methyl esters mixture from transesterification reaction catalysed by lipase PLS 80 using coconut oil as the substrate at 37 °C.

Standard	Standard value	Sample value
Density ASTM D-1298	0.85–0.90 g/cm^3	0.90 g/cm^3
Viscosity ASTM D445	1.9–6.0 cSt	9.25 cSt

3.6. Transesterification reaction

The ability of lipase from PLS 80 to catalyse the transesterification reaction was tested using coconut oil as the substrate. The enzyme was initially modified by decanoil chloride to promote its ability to catalyse the reaction in non-aqueous solution. The lipase showed an excellent activity on long fatty acids substrates (*p*-nitrophenyl palmitate) as it has a reasonably high transesterification activity (Table 1). The transesterification reaction produced seven methyl ester compounds (Figure 5 and Table 2).

Methyl laurate (48.6%), methyl caprate (17.7%) and methyl myristate (15.3%) were the three main products of the reaction. Lauric acid is indeed the primary fatty acid found in coconut oil, comprises nearly half of the total (Khrisna et al., 2010). The result of this study was relevant to the result of Brilliantoro (Brilliantoro et al., 2015), where methyl laurate is the main component of the transesterification product of coconut oil.

The quality of the methyl-esters produced after the transesterification reaction was determined based on the density and viscosity parameters and the results were compared to American Standard Testing and Material (ASTM) D-129 and D445, respectively (Table 3). The density of the transesterification product was 0.9 g/cm^3, which was within the range of ASTMD-1298 standard. However, the viscosity of the methyl esters mixture was 9.25 cSt which was higher than the ASTM D445 standard. Full characterization of the biodiesel is however still needed.

4. Conclusion

Lipase from PLS 80, with a molecular weight of 50 kDa, could catalyse hydrolysis and transesterification reaction. The enzyme showed a thermozyme attribute as it had an optimum activity at 70 °C. It was also an alkaline enzyme as the optimum pH for its activity was 9.0. The lipase was a metalloenzyme containing serine hydrolase group, as proven by the addition of EDTA and PSMF. The presence of 10 mM Ca^{2+} ions, as predicted, increased the enzyme activity. Its high activity in the presence

of Hg^{2+} could be further utilised in bioremediation study. The enzyme arguably catalysed the transesterification reaction efficiently, as the methyl esters composition was similar to the fatty acids content in coconut oil. The viscosity of the product was nevertheless beyond the standard range value. The ability of lipase from PLS 80 to catalyse the transesterification reaction may be further utilised to produce commercial biodiesel.

Declarations

Author contribution statement

Febriani, T. M. Iqbalsyah: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Naiwatul Aura: Performed the experiments; Wrote the paper.

Pati Kemala: Performed the experiments.

Nurdin Saidi: Analyzed and interpreted the data.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

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