Hydrolytic and Transesterification Activities of Thermostable Lipase ITB1.1

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Heterologous expression of local thermostable lipase (Lipase ITB1.1) has been carried out by using pET-30a(+) vector in *Escherichia coli* BL21(DE3). SDS-PAGE analysis showed that the protein size is around 50 kDa. Hydrolytic activity was determined at 70 °C and pH 8 by using *p*-Nitrophenyl palmitate (pNPP) as substrate. The activity of partial purified enzyme was significantly increased (0.56 U/mg) compared to that the crude extract (0.25 U/mg). Lipase ITB 1.1 has highest specific activity (1.23 U/mg) at 85 °C and pH 9.5. Further, characterization of the enzyme suggested that the enzyme exhibited transesterification activity. GC-MS spectra of the reaction product indicated that the coconut oil (substrate) was converted into methyl esters. The results suggesting that Lipase ITB1.1 is potential enzyme for biodiesel production.

Key words: Thermostable enzyme, Lipase, Hydrolytic, Transesterification.

Nowadays, lipases (EC 3.1.1.3) are widely used as biocatalyst in biotechnological and synthetic organic processes. Lipases are triacylglycerol ester hydrolazes catalyzing a broad range of novel and important reactions in aqueous and non-aqueous media. It is used to hydrolyze and to synthesize ester bonds from glycerol and long-chain fatty acids. Many lipases are active in organic solvents and catalyze a number of useful reactions, such as hydrolysis, transesterification, esterification reactions[1,2].

The application of the enzymes are selected based on its activity, stability and selectivity. Lipases become industrially important, especially in detergents, food processing, pharmaceuticals, synthesis of fine chemicals, cosmetics, oleochemical, paper manufacture and medical products[3]. Moreover, lipases have been found as important catalyst on application of biodiesel industry[4].

Biodiesel is mixture of fatty acid methyl esters (FAMEs) which are produced from a broad range of crude oil materials, such as vegetable oil, animal fats and waste oil, via transesterification of triacylglycerols (TAGs) with methanol or ethanol. Biodiesel regarded as a promising fuel that partially substitute the conventional fossil energy. As renewable, biodegradable and nontoxic fuel, biodiesel is suitable for use in vehicles, stoves or heaters[5].

Application of lipase for biodiesel production is environmentally friendly since mild reaction condition and easily product recovery.

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The enzymes that mostly used in biodiesel production is lipase B from *Candida antarctica* (CALB), which has been commercialized by Novozymes as an immobilized form (Novozym 435)[6]. Moreover, biodiesel synthesis from Jatropha oil has been reported by using immobilized lipase from *Enterobacter aerogenes*. The maximum yield of biodiesel was 94%[7].

Thermostability is one of desirable characteristic for commercial lipase in the biotechnological industry. Exploration of thermostable lipase-producing microbes have been widely carried out[8-12]. Most thermostable lipases exhibit higher thermodynamic stability, both at elevated temperatures and in organic solvents, as a consequence of adaptation of the corresponding microorganisms to higher growth temperatures[13]. Stability of lipases at high temperatures and organic solvents is an advantage to be applied in variety of ester bond synthesis reactions[14].

In previous studies, gene encoding thermostable lipase from Manuk hot spring, has been isolated and cloned[15]. The gene was inserted into pET-30a(+) expression vector. Further studies have also been conducted for heterologous expression of the gene by using *E. coli* BL21(DE3) as host cells. This paper describes the production and characterization of thermostable Lipase ITB1.1 by its hydrolytic and transesterification activities.

MATERIALS AND METHODS

Production of Lipase ITB1.1

Single colony of *E. coli* BL21 (DE3)-pITBlip1.2 was inoculated in 5 mL Luria Bertani (LB)-kanamycin liquid medium (1% (w/v) NaCl, 1% (w/v) tryptone, 0.5% (w/v) yeast extract and 0.1% (v/v) kanamycin (30 µg/mL)), and then incubated at 37 °C for 16 h with agitation at 150 rpm. Total of 1 mL of cell culture was transferred into 100 mL of LB-kanamycin liquid medium. Cell culture was induced with 0.1 mM IPTG after OD_{600}reached α 0.6. Induction was carried out for 4 h at 25 °C with agitation at 200 rpm. Cells pellet were harvested by centrifugation at 6.000 x g, 4 °C for 30 min. Subsequently, the pellet was added to the lysis buffer (5 mg lysozyme in 3 mL of phosphate buffer (pH 7.5) per gram of wetcells pellet), and then incubated at 25 °C for 4 h.

Crude extract was obtained by lysis of the cells in cold conditions (0 - 4 °C) using ultrasonicator for 15 min (interval time at every 30 seconds). There is due (cell debris) were separated from the supernatant (crude extract Lipase ITB1.1) by centrifugation at 6.000 x g, 4 °C for 30 min. Partial purification was carried out by heating the crude extract in water bath at 55 °C for 15 min and then centrifuged at 6.000 × g for 30 min. Lipase ITB1.1 was analyzed by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and determined its total protein concentration by Bradford method[16].

HydrolyticActivityAssay

Hydrolytic activity of Lipase ITB1.1 was measured based on the spectrophotometric techniques[17]. *p*-Nitrophenyl palmitate (pNPP) dissolved in acetonitrile to obtain concentration of 10 mM (solution A). Substrate emulsion was prepared by mixing the solution A with phosphate buffer (pH 7.5) and ethanol in the ratio of 1:95:4 (v/v/v). A total of 300 µL of sample solution (Lipase ITB1.1) were incubated at 70 °C for 1-2 min, then added 900 µL of substrate emulsion. The mixture was incubated at 70 °C for 15 min. The reaction was stopped by addition of 50 µL phenylmethyl sulfonyl fluoride (PMSF) solution (50 mM).

Lipase activity was measured by spectrophotometer at λ = 405 nm, based on the release of *p*-Nitrophenol (pNP) from pNPP substrate. Standard curve was constructed by ploting absorbance (λ = 405 nm) with the pNP concentration in the range of 0-8 µg/mL. Lipase activity expressed in units, where 1 unit is defined as 1 µmol PNP product that released per minute based on the assay conditions. Furthermore, effect of pH and temperature on the enzyme activity was determined by measuring its hydrolytic activity at various pH (6 - 10.5) and temperature (25.5-90 °C).

TransesterificationActivityAssay

Lipase ITB1.1 (1.5 U based on hydrolytic activity) was mixed to cold acetone ratio of 1: 4. The mixture was incubated at -20 °C for 1 h, and then separated by centrifugation at 6.000 x g for 30 min to obtain precipitate of the enzyme. Transesterification reaction is carried out by using reflux instrumentation. Coconutoil and methanol in the ratio of 1:4 (v/v) as substrate and *tert*-butanol (0.8 mL per 1 mLcoconutoil), mixed into there fluxflask[7]. Furthermore, precipitated enzyme was

added to the mixture, and then incubated at 70 °C for 24 h. Negative controls were made by incubating coconut oil, methanol and *tert*-butanol with the same composition, but without the addition of the enzyme.

The results of the transesterification reaction was transferred into tubes, then centrifuged at 6.000 x g for 30 min. Top layer of the supernatant were collected, and thenanalyzed by using Gas Chromatography–Mass Spectrometry (GC-MS). GC-MS analysis was carried out by using RTX 5 MS column, FID detector and Helium as carrier gas. The column temperature was hold at 150°C for 5 min, and raised at 15°C per minutet o 250 °C, where it was then maintained for 20 min. The temperatures of the injector and the detector were 245 and 300°C, respectively.

RESULTS AND DISCUSSION

Heterologous Expression of Gene Encoding Thermostable LipasefromManuk Hot Spring Isolate

Gene encoding thermostable lipase from Manuk hot spring isolate was inserted into the pET-30a(+) expression vector (pITBlip1.2). The recombinant vector was expressed as soluble form of protein in *E. coli* BL21(DE3) by addition of IPTG. The recombinant protein has succesfully been expressed with molecular weight at around 50 kDa based on SDS-PAGE electrophoregram, which was in line with molecular weight calculated by deduced amino acid sequence. Lipase ITB1.1 has 465 amino acid polypeptide consisting of 413 amino acid mature lipase and 52 amino acid derived from the pET-30a(+). Densitometer analysis on the SDS-PAGE electrophoregram showed that the Lipase ITB1.1 was expressed at around 32% of total protein (Figure 1).

Table 1. Total protein concentration and specific activity of Lipase ITB1.1

Lipase ITB1.1	[Protein] (mg/mL)	Specific activity (U/mg)
Crude extract	3.43	0.25
Partial purified	1.70	0.56

Partial purification of lipase was carried out by heating the crude extract at 55 °C for 15 min. The partially purification process was based on utilizing the properties of a thermostable lipase among other thermolabile protein from *E. coli*BL21(DE3). In addition, heating is degrading the protease enzyme[18].

Characterization of Lipase ITB1.1 on Hydrolytic Activity

Specific activity of Lipase ITB1.1 at 70 °C and pH 8 from partial purification showed significantly increased compared to that the crude extract (Table 1). Hidrolytic activity of Lipase ITB1.1 was influence by pH and temperature. Specific activity at pH 6-8, showed no significant increase, however at pH 8.5, the specific activity increased sharply to achieve highest activity at pH 9.5 (0.56 U/mg). Furthermore, at higher pH (10-10.5) the specific activity decreased significantly (Figure 2). The activity of the enzyme showed significant increase started at 75 °C until reach the highest activity at 85 °C. However, at 90 °C the specific activity was decreased (Figure 3).

Gene sequence of Lipase ITB1.1 has the highest homology with the lipase of *Geobacillus stearothermophilus* L1[15]. Most lipase from Geobacillus showed the maximum hydrolytic activity in the temperature range of 55-65°C at neutral to alkaline pH 7-8[19-22].The highest activity of the enzyme at 85 °C and pH 9.5 suggesting that

Table 2. Chemical compounds of Lipase ITB1.1 transesterification products

Peak to-	Retention times (minutes)	Name of compounds	Broad area	Product percentage (%)
1	3.601	Methyl caprate	33306	15.96
2	6.500	Methyl laurate	98820	47.36
3	9.033	Methyl myristate	41465	19.87
4	10.952	Methyl palmitate	14842	7.13
5	12.391	Methyl oleate	14552	6.97
6	12.584	Methyl heptacosanoate	5657	2.71

the enzyme differ from other known lipase of Geobacillus.

Transesterification Activity of Lipase ITB1.1

Transesterification reaction of Lipase ITB1.1 was carried out by using coconut oil and methanol as substrates. Methyl ester reaction products were analyzed by using GC-MS apparatus. Based on the GC-MS spectra, 6 peaks were produced as reaction products (Figure 4). Identification of chemical compounds in the reaction product was carried out by comparing the retention time in the MS Library WILEY7.LIB (Table 2).

The identification of the spectra showed same formation of methyl esters products, such asmethyl laurate, methyl myristate, methyl caprate, methyl palmitate, methyl oleate and methyl heptacosanoate (Figure 4a). This is probably due to coconut oil contains variation of fatty acid compounds. Coconut oil is composed of various fatty acid composition, both saturated fatty acids and unsaturated. Saturated fatty acids in coconut oil, such as: caprylic, capric, lauric, myristic, palmitic and stearic, whereas oleic and linoleic as unsaturated fatty acids. Lauric acid is known as the largest component with percentages at around 48.90%of total fatty acids in coconut oil[23]. The results suggested that the largest percentage of methyl ester product is methyl laurate at around 47.36%. Lipase ITB1.1 was potential catalyst for

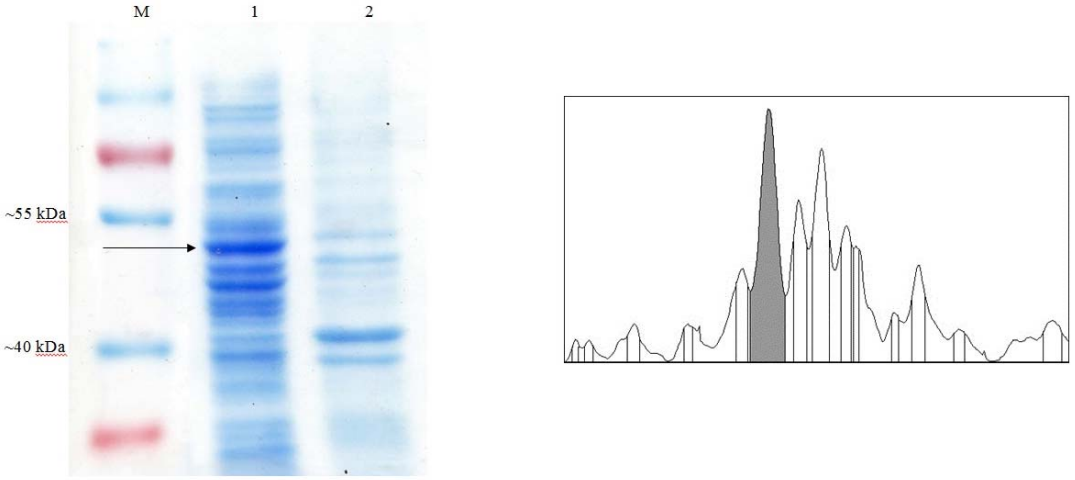

Fig. 1. (a) Electrophoregram of heterologousexpressionfrom*E.coli*BL21(DE3)-pITBlip1.2; proteinmarker (M); crudeextract of *E.coli* BL21(DE3)-pITBlip1.2 (1); debriscell of *E. coli* BL21 (DE3)-pITBlip1.2 (2). (b) Densitometer spectra of crudeextract of *E.coli* BL21(DE3)-pITBlip1.2 basedonImageJ software program.

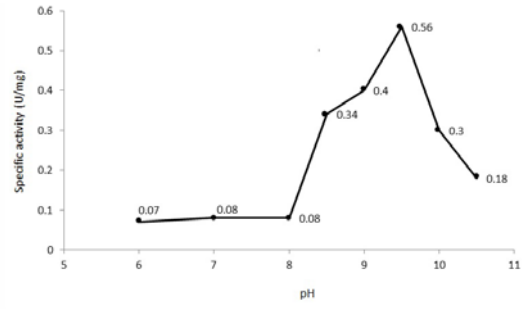

Fig. 2. Specific activity of crudeextract of *E.coli* BL21 (DE3)-pITBlip1.2 in various pH. Maximumactivity at pH 9.5.

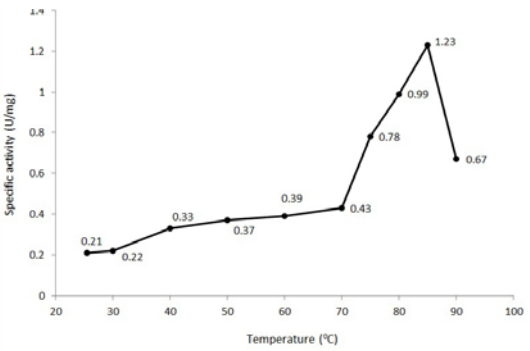

Fig. 3. Specific activity of crudeextract of *E.coli* BL21(DE3)-pITBlip1.2 in various temperatura. Maximumactivity at 85 °C.

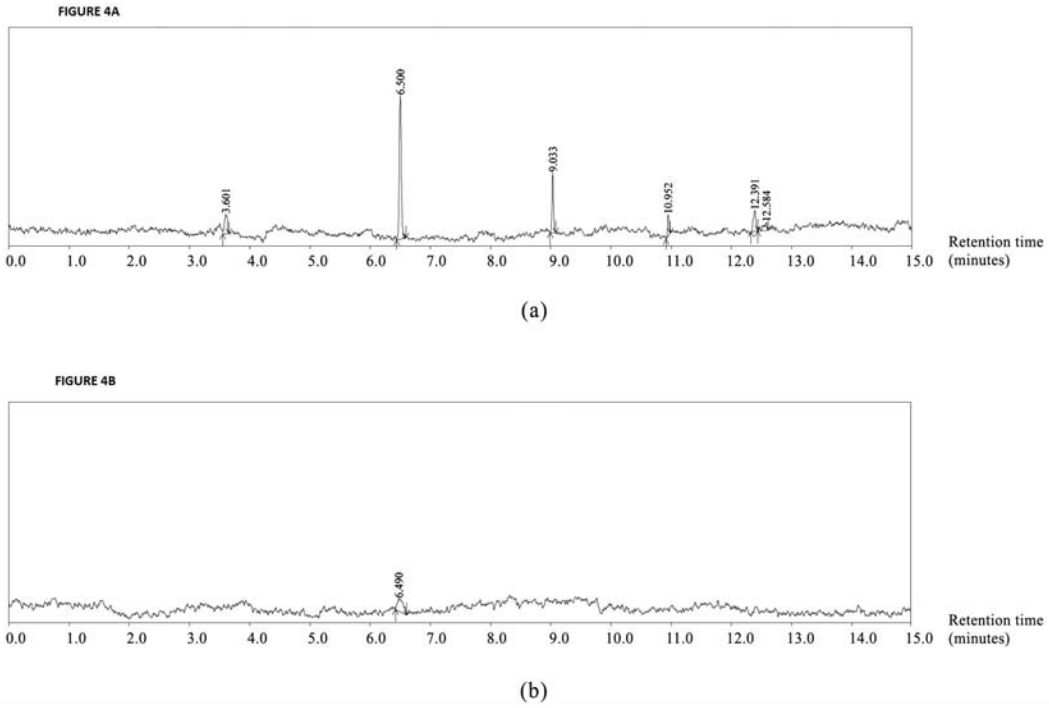

Fig. 4. GC-MS spectra of transesterification reaction product
(a) by using Lipase ITB1.1 (b) without Lipase ITB1.1

methanolysis of coconut oil to produce methyl ester, which is the main component of biodiesel.

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