

Identification and Characterization of Bacterial Lipase from Plateau Soil in West Java

Vivitri Dewi Prasasty^{1,*}, Vinella Winata¹, Muhammad Hanafi²

¹Faculty of Biotechnology, Atma Jaya Catholic University of Indonesia, Jakarta 12930

²Research Center for Chemistry, Indonesian Institute of Sciences, Puspiptek, Serpong, 15314

*Corresponding author: vivitri.dewi@atmajaya.ac.id

ARTICLE INFO	Abstract
<p>Article history Received date : 21 November 2016 Revised date : 6 December 2016 Accepted date : 5 January 2017 Available online at http://kimia.lipi.go.id/inajac/index.php</p>	<p>Lipases are known as glycerol ester hydrolases that catalyze the hydrolysis of triglycerides into free fatty acids and glycerols. Lipases are found in humans, animals, plants, and microorganisms. The aim of this research is to identify lipase producers and characterize bacterial lipases from West Java plateau soil. Bacteria from plateau soil samples were isolated on lipase screening medium containing Rhodamine B. Olive oil was used as a substrate in the production medium to screen for bacterial lipases. From 16 isolated lipase-producing bacterias, 14 were identified as <i>Bacillus</i> sp. while the others were identified as <i>Pseudomonas alcaligenes</i>. All isolates were taken into production step to determine their lipase activities. Three lipases with the highest activities were chosen to find optimum pH and temperature. Both characterizations showed optimum pH and temperature of each lipase. These optimum condition were used in heat stability characterization for each lipase sample. The results suggested that lipase from isolate COK 2 showed optimum activity at pH 4 and 50°C. Incubation for 1 to 5 hours resulted in the most stable lipase activity. Isolate COK 2 produced lipase with good efficiency at high temperature in acidic environment. These findings can be potentially used to improve efficiency of lipase production in larger scale.</p>
<p>Keywords: Lipase, identification, characterization, bacteria, plateau soil</p>	

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1. INTRODUCTION

Lipase (EC 3.1.1.3) represents a group of hydrolytic enzymes that are capable to catalyze the hydrolysis of long chain triacylglycerols into short chains at the interphase between water and lipid [1,2]. Triacylglycerols are formed from binding reaction between glycerol and three fatty acids. A few examples of the fatty acids are oleic, palmitic, and stearic acid. Lipase activity does not depend on the presence of co-factors. This enzyme does not catalyze side reactions because it belongs to hydrolase group [3].

Lipase has been known as enzyme with the third largest total sales volume after protease and carbohydrase [4]. It has been widely applied in various fields, particularly industry [5]. It is used in production of detergents, textiles, foods, dairy products, cosmetics, environmental, oil processing, biodiesel, surfactant production, and synthesis of chiral pharmaceuticals [3]. Lipase

can be produced by microorganisms, such as bacteria and fungi. 1R PAC *P. aeruginosa*, *Serratia marcescens*, *S. aureus*, *B. subtilis* [6], *B. pumilus*, *B. licheniformis*, and *B. alcalophilus* [3], *S. saprophyticus*, *S. haemolyticus*, *S. hyicus*, *S. warneri*, *S. xylosus*, *S. Epidermidis* [1] are bacteria which can produce lipase. Meanwhile, *Candida* sp. is a fungus that capable of producing similar enzymes. However, some common bacteria could produce lipase such as *B. subtilis*, *B. pumilus*, *B. licheniformis*, and *B. alcalophilus*. Lipase-producing bacteria using lipid as a substrate to maintain its viability. Lipid substrate can be found in olive oil, canola oil, grape seed oil, sunflower oil [7], sesame oil, soybean oil, and corn oil.

Olive oil is a vegetable oil extracted from *Olea europaea*. The composition of the fatty acid in olive oil are 77.2% oleic acid, 11.2% palmitic acid, 10.1% linoleic acid and 1.5% stearic acid

[8]. Olive oil is a source of lipids for the lipase-producing bacteria from plateau soil samples on selective media Rhodamine B and also in production media as well as the analysis of lipase activity.

The aim of this research is to isolate the bacteria producing lipase and to extract bacterial lipase that has unique character from plateau soil samples of West Java. Rhodamine B media is used to selectively screens lipase-producing bacteria. It is used to select the lipase activity in the agar dish with olive oil substrate. Production of lipase is observed under UV light irradiation. Positive colonies will show luminescence orange color [6].

Lipase has various properties. Some lipases can work well in a buffer with alkalophilic pH [9]. In the study, lipase alkalophilic was found in hyperthermostable of mesophilic bacteria, *Pseudomonas sp.* This enzyme is stable at pH 8-12 and active at pH 9-12. These enzymes work optimum on pH 11 and stable at high temperature, 90°C for over 13 hours. Previous research showed that lipase is stable at pH 7-10 and unstable at acidic pH¹¹. Lipase is produced by *B. coagulans* ZJU 318 works optimally at pH 9 and 45°C. One lipase unit (U) is defined as the amount of enzyme needed to liberate 1 mol of fatty acid per minute at specific conditions [11].

2. EXPERIMENTAL SECTION

2.1. Materials

Soil samples from the highlands of West Java, mineral salts, bacterial growth media, dH₂O, olive oil, BSA, citric buffer.

2.2. Methods

2.2.1. Sample Collection

Soil samples were obtained from three different plateau area with a depth of about 10 cm from the surface of the plateau area in West Java, they are: Protected Forest Anyer Carita, Cipelang, and Mount Gede. Sampling point for each site is as much as 2-3 points. Samples were stored at 4°C until further usage.

2.2.2. Sample Screening in Rhodamine B media

Each sample is dissolved in H₂O at a ratio of 1:1 and then diluted 100x and 1000x. The sample solution deployed in Rhodamine B media (2.5 mL olive oil, glycerol 0.2 g, 0.002 g Rhodamine B, polypeptone 1.5 g, Bacto agar 3 mM, 0.2 mM KH₂PO₄, MgSO₄·7H₂O 0:01 M, 0.5 M NaCl, biotin 0:01 M, extracts yeast 0.5 M, and 0.2 g of beef extract, all in% w / v dissolved in dH₂O, pH 7.2 media) and incubated at 37 °C for 24 hours and observed under UV light. Positive results indicate bacterial colonies have lipase activities on glowed orange fluorescence under UV light while a negative result lipase activity was shown by the absence of bacterial colonies luminescence under UV light. The number of positive colonies of each sample are counted. Some isolates with the brightest fluorescent are chosen from Rhodamine B medium selection. Further positive isolates are performed three section streaks (T streaks) on Rodhamine B media without biotin to obtain a single colony. Single colony from each positive isolate is stroke to ensure the purity of the isolate. At this stage, isolates colony morphology observation is also conducted.

2.2.3. Isolate Identification

Positive isolates Rhodamine B are prepared as T streaks on media to determine biochemical and gram staining assays. Biochemical assays are conducted on positive isolates using TSIA, KIA, Indol, oxidase, catalase, and Simmon's Citrate media. The results of gram staining and biochemical assays will be used to identify isolate species.

2.2.4. Lipase Production

Positive isolates are recultured on rhodamine B media. The isolates were inoculated one loop into a 50 ml pre-culture Tryptone Soy Broth media and incubated at 37 °C for one night. 5 ml pre-culture media are taken and inoculated into erlemeyer 500 ml flask containing 100 ml of production media. (1% olive oil, CaCl₂·2H₂O of 0.02%, a 0.01% MgSO₄·7H₂O, and 0.04% FeCl₃·6H₂O (1% in stock)). Production media is incubated at 37°C,

150 rpm for 72 hours using a water bath shaker. Samples are taken and placed in centrifuge tube and then added with sodium azide as much as 1/100 of sample volume. Furthermore, samples are centrifuged at 8500 rpm for 15 minutes. The supernatant is separated from pellet. Pellet sample is added with 10 ml citrate buffer pH 6 then followed by sonication for 10 minutes at 60 volts. Pellet is filtered and the supernatant is separated. The obtained supernatant is a lipase extract solution.

2.2.5. Lipase Activity Analysis

A total of 2 g olive oil is mixed with 4 g of citrate buffer pH 6 and 1 ml lipase extract. Lipase is incubated in a shaker water bath for 60 minutes at 37°C, 150 rpm. The mixture reaction is added with 10 ml acetone:alcohol (ratio 1:1) and 2-3 drops of phenolphthalein 1%. The reaction is titrated with NaOH calibrated until the solution becomes pink. Lipase activity unit is calculated using the following equation:

<p>Lipase Activity Unit (U):</p> $\frac{(A-B) \times N. \text{ NaOH} \times 1000 \times \frac{1}{P} \times \frac{1}{T}}{V}$

A: amount of calibrated NaOH for titrating the sample (ml)

B: amount of NaOH to titrate blank calibrated (ml)

N. NaOH: calibrated NaOH normality

V: volume of olive oil used in the solution (ml)

Q: Dilution Factor (enzyme volume / total volume)

One lipase activity unit (U) is defined as the amount of enzyme needed to liberate 1 mol of fatty acid in ml/min at specific condition [12].

2.2.6. Protein Concentration Determination

Measurement of protein concentration using Bradford method with Bovine Serum Albumin (BSA) as standard curve [13].

2.2.7. Specific Lipase activity unit

Furthermore, the specific lipase activity unit is determined. The specific activity is defined as activity units per milligram of total protein.

2.2.8. Characterization of buffer pH and temperature incubation

Three samples with the best enzyme activity is determined within each sample with various pH buffer at pH 4, 5, 6, 7, and 8, respectively. In addition, each sample is determined the optimum temperature by performing the assay at 20°C, 37°C, 50°C and 70°C, respectively. The optimum incubation temperature and pH buffer are used for heat stability assay of lipase activities.

2.2.9. Heat stability of lipase activity analysis

Three samples with the best enzyme activity is taken to check the activity of each sample within temperature and pH optimum incubation. The incubation time is increased from 1 hour to 2, 3, 4, and 5 hours to see the variations in the stability of lipase activity.

3. RESULT AND DISCUSSION

Soil samples from three different plateau area are identified their bacterial diversities by performing bacterial isolate screening according to their ability to produce lipase. Isolates showing positive results producing lipase, are selected 16 isolates with the brightest glowed orange fluorescence when the colonies are incubated on Rhodamine B media and observed under UV light (Figure 1). The observation of colony form, Gram staining, and several biochemical assays were conducted and analysed as listed on Table 1. The results showed that 14 out of 16 positive isolates have similar properties with *Bacillus* sp. and two other isolates have similar properties with *Pseudomonas Alcaligenes*. Isolates were prepared and grown as lipase production stage. Each sample was analyzed its lipase-producing bacterial isolate. Three samples with the highest lipase activity are selected for lipase characterizations.

Three isolates with higher lipase activities are isolate COK 2, DOC 5, and COK 6. These isolates then selected for further investigation. These three isolates are assumed as *Bacillus* sp. based on Gram staining and biochemical assays (Table 2). Determination of optimum temperature and pH were performed on all three isolates, COK 2, DOC 5 and 6 COK. According

to previous study, *Bacillus* sp. also found on soil samples, characterized similar biochemical assays as *Bacillus subtilis*, following purification result showed lipase with molecular weight 31 kDa [14]. Lipase extracts from those three isolates were characterized according to temperature and pH variations.

The results of temperature and pH characterizations were performed on three

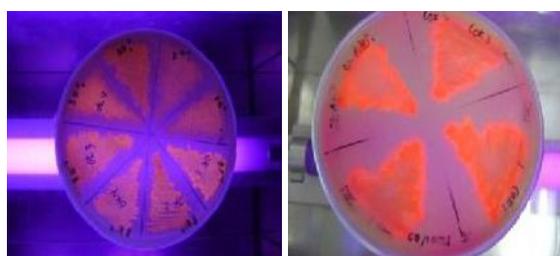
isolates showed various lipase activities at the optimum temperature and optimum pH. The analysis based on the characterization of pH effect lipase activities are depicted on Figure 2. Meanwhile, lipase activities which characterized based on temperature effect are depicted on Figure 3.

Table 1. Type of lipase-producing bacteria isolates

Isolates	Isolate similarity based on microscopic and biochemical assays	Isolates	Isolate similarity based on microscopic and biochemical assays
COK 1	<i>Bacillus</i> sp.	CX	<i>Bacillus</i> sp.
FOK 1	<i>Bacillus</i> sp.	DOK 5	<i>Bacillus</i> sp.
OK1 A10	<i>Bacillus</i> sp.	COK 2	<i>Bacillus</i> sp.
COK 3	<i>P. alcaligenes</i>	COK 5	<i>Bacillus</i> sp.
COK 6	<i>Bacillus</i> sp.	EOK 2	<i>Bacillus</i> sp.
FOK3	<i>Bacillus</i> sp.	COK BGT	<i>Bacillus</i> sp.
OK1 B10	<i>Bacillus</i> sp.	DOK 2	<i>Bacillus</i> sp.
EOK 1	<i>P. alcaligenes</i>	FOK 2	<i>Bacillus</i> sp.

Table 2. Results of microscopic and biochemical assays of three isolates

Biochemical assays	COK 2	DOK 5	COK 6
Gram staining	positive	positive	positive
Form	bacilli	bacilli	bacilli
Spore	formed	formed	formed
Oxidase	negative	negative	negative
Catalase	negative	negative	negative
Motility	positive	positive	positive



a.

b.

Fig. 1. Visualization of glowed orange fluorescence lipase-producing bacterial colonies in Rhodamine B media, a. lipase positive within eight bacterial zones; b. lipase positive within four bacterial zones

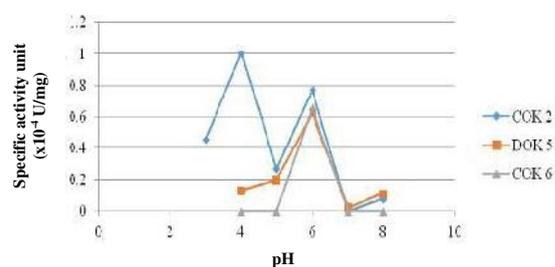


Fig. 2. The activity of lipase based on pH variation

Lipase from isolate COK 2, DOC 5 and 6 COK could work well on a slightly acidic pH at pH 6 (Figure 2). Additionally, COK 2 also can work well at pH 4. It showed that the lipase can also work in acidic conditions. This results show the uniqueness of lipase properties from three different isolates. In contrast, previous research on lipase-producing *B. coagulans* ZJU 318 conducted by Tang and Xia (2005) showed that *B. coagulans* ZJU 318 works stable within pH 7-10 but cannot work at acidic pH.

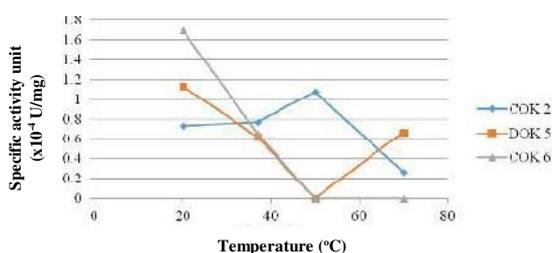


Fig. 3. The activity of lipase based on temperature variation

Figure 3 shows the difference between the optimum temperature in all three samples lipase. In comparison, both lipase of COK 2 and lipase of *B. coagulans* ZJU 318 worked optimally at high temperatures although in different optimum pH circumstances. COK 2 worked optimally at 50°C and *B. coagulans* 318 ZJU active at 45°C¹⁰. From Figure 3 we can see that the lipase COK 6 worked optimally at room temperature is about 20°C. It is probably because the sample taken from the mountains that has a temperature within 16-23°C. Therefore, bacterial lipase worked at temperatures similar to temperatures its natural bacterial habitat. bacterial lipase DOC 5 activity decreased after incubated at temperature from 20°C to 50°C but increased at a temperature of 70°C. Further research is needed to ensure the activity of lipase DOC 5 at higher temperature.

Thermal stability characterization is performed at pH and temperature optimum used on each lipase sample. The heat stability of characterization results showed lipase activity in these conditions can be kept stable for up to 5 hours (Figure 4). Lipase COK 2 activity is stable at high temperature for few hours, however the highest activity can be observed of all lipases at

3 hours incubation. Lipase activity increased at an incubation of 60 minutes up to 180 minutes of incubation then the activity decreased after 300 minutes incubation time.

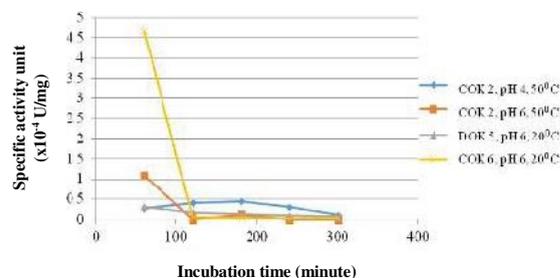


Fig. 4. The activity of lipase based on heat stability variation

The results of lipase COK 2 activity at pH 6 and temperature of 50°C during the incubation period of 1-5 hours indicates that although these enzymes can work at pH 6, the lipase activity is unstable. In Figure 3 shows lipase COK 6 decrease significantly after 1 hour incubation. Lipase from COK 2 worked within the range acidic pH 4-6. This condition is more efficient for lipase application in various industries that allow lipases work at low pH condition.

Lipase DOC 5 showed that its activity slightly decreased after one hour incubation. However, lipase DOC 5 activity is fairly stable at 1-5 hours incubation time. This condition also gives industrial application benefits for lipase DOC 5 as well as lipase COK 2.

Lipase COK 6 activity decreased significantly from the first hour to the second hour of incubation time. It showed that lipase DOK6 is unstable for a long incubation time by showing the highest activity in the first hour of incubation time.

4. CONCLUSION

This study has been successfully screened 16 bacterial isolates, most of which were identified as *Bacillus* sp. Lipase bacteria have been analyzed by the characterization based on pH, temperature, and heat stability properties. Lipases produced from COK 2, COK 6 and DOK 5 were further investigated. lipase COK 2 showed the most stable at pH 4 and 50°C.

Moreover, COK 2 can work at high temperature and acidic pH. On the other hand, DOC 5 is also quite stable for 1-5 hours incubation time. Meanwhile, COK 6 is not stable after 1 hour incubation. The usage of lipase COK 2 at pH 4 would provide higher efficiency for lipase productivity in the acidic conditions within several hours.

In conclusion, bacterial lipase from plateau soil bacteria can be used as a thermostable and resistant to severe pH. However, to obtain a better result, it is necessary to optimize the purification and concentrating stages.

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