Characterization of Organic Solvent Tolerance Lipase from Compost Indigenous Bacteria

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Abstract. Lipases are a group of hydrolase enzymes that play an important role in industry and biotechnology. In addition, lipase has the ability as a catalyst in ester hydrolysis, transesterifications, alcoholysis, and acydolysis reactions. This study aims to obtains the character of the tolerant lipase of organic solvents from the compost indigenous bacteria. The method used in determining the optimum conditions for lipase production, purification, and characterization of tolerant lipase in organic solvent. Lipase activity was tested by a spectrophotometric method using p-nitrophenyl palmitate as a substrate and protein content was determined using the Lowry method. The result showed that the compost indigenous bacteria could produce lipase tolerant of organic solvents at the optimum growth conditions of 48 h incubations time, pH 6, inducer concentrations of 2% with the addition of 3% hexane. The specific activity of lipase produces a value of 1455.68 U/mg. Purifications by fractionation of ammonium sulfate and dialysis increased the purity of the lipase 2.37 times with a specific activity of 3449.65 U/mg. Lipase activity increased with the addition of metal ions Ba^{+2} and Al^{+3} and n-hexane as solvent. Meanwhile, the presence of metal ions Na^{+}, K^{+}, Mg^{2+} Fe^{+3} as well as methanol and benzene solvents affected decreasing lipase activity.

Keywords: lipase · organic solvent · compost · indigenous bacteria

1 Introduction

Lipase is a hydrolase enzyme that catalyzes the hydrolysis of triacylglycerol into glycerol and free fatty acids. Lipases are also able to catalyze the reverse reaction, namely the synthesis of esters from free fatty acids and glycerol or other substrates [1]. Due to its wide use, lipase is widely used in various industries such as the food, detergent, pharmaceutical, leather, textile, cosmetic and paper industries [2].

Currently, the lipase needed by the industry is one that has good stability, so it can reduce production costs. The stability of the lipase enzyme is strongly influenced by pH, temperature, frequency of use the enzyme and the presence of organic solvents [3]. The stability of the lipase enzyme in organic solvents is very important, because in industrial

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reactions most of the non-polar compounds used are easily soluble in organic solvents [4]. For example, in the manufacture of biodiesel, fatty acids from triglycerides will be esterified with alcohol so that alkyl esters or biodiesel can be formed [3]. Likewise, reactions in the presence of other organic solvents such as esterification, alcoholysis, acidolysis, and aminolysis certainly require lipase enzymes that can survive in the presence of organic solvents [5, 6].

To obtain lipase-producing bacteria with good stability against organic solvents, many sources were explored, from extreme natural environments to artificial environmental sources. Several research results have been reported such as Bacillus sp. isolated from deep sea water is known to have good stability in the solvents benzene, butanol, hexanol, and toluene [7]. Lipase from bacteria isolated from oil contaminated soil at Pasar Anyar, Singaraja, Bali showed good stability in polar organic solvents (methanol and ethanol) and non-polar solvents (n-hexane) [8]. In fact, bacteria from residual oil sources in the kitchen are also known to have lipolytic activity in various organic solvents such as methanol, ethanol, butanol and acetonitrile up to a concentration of 4% [9]. Lip479 isolate from Taptani hot spring were found which produced lipase enzymes with good stability againts organic solvent methanol, DMF, DMSO, acetone and dichloromethane [10].

A study of five mesophilic isolates that were isolated from compost for their resistance to organic solvents methanol, benzene and n-hexane was carried out. The results showed that one of the five isolates had relatively better resistance to organic solvents, and it was suspected that this isolate was able to produce lipase enzymes which also had the ability to act in the presence of organic solvents. Based on this, this research was focused on characterizing organic solvent tolerant lipase enzymes from compost indigenous bacteria.

2 Method

A. *Lipase production from compost indigenous bacteria*

Lipase production was carried out at known optimum production conditions. Bacterial culture was put into 20 mL starter containing nutrient broth, tween 80, olive oil and 3% n hexane, then incubated at 110 rpm for 24 h. A total of 2% starter was transferred to a production medium containing 3% n-hexane, palm oil 2%, pH 6 and growth time of 48 h. The production medium was centrifuged to separate the filtrate. The filtrate is a crude extract of the enzyme, then tested for lipase activity and analyzed for protein content using the Lowry method.

B. *Partial Purification of lipase*

The purification process of the crude extract of the lipase enzyme was initiated by graded fractionation using ammonium sulfate salt with saturation levels of 0–20%, 20–40%, 40%–60%, 60–80%, and 80%–100%. Enzyme protein deposits obtained from each fraction were separated by centrifugation at a speed of 5000 rpm for 15 min then dissolved in phosphate buffer solution pH 7; 0.25 M. Each fraction was tested for lipase activity

using colorimetric method with p-NPP substrate and protein content with Lowry method. The enzyme from the ammonium sulfate fraction was purified again using cellophane membrane in phosphate buffer pH 7; 0.01 M.

C. *Characterization of Lipase*

a. Effect of pH on enzyme activity

The partially purified enzyme was reacted with the respective substrates in the pH range of 5, 6, 7 and 8 and incubated for 15 min. Lipase activity was determined by colorimetric method using p-NPP substrate.

b. Effect of temperature on enzyme activity

The partially purified enzyme was added to p-NPP and then incubated at a temperature range of 30, 35, 40, and 45ºC at its optimum pH for 15 min. Lipase activity was determined by colorimetric method using p-NPP substrate.

c. Effect of metal ion on enzyme activity

The metal ions used are K^+, Na^+, Ba^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+}. The enzyme was added to a metal solution with a concentration of 1 mM in a ratio of 1:1, incubated for 30 min at optimum pH and temperature, and added p-NPP substrate and then incubated for 15 min. Lipase activity was determined by colorimetric method using p-NPP substrate.

d. Effect of organic solvents on enzyme activity

The organic solvents used were hexane, benzene and methanol. The enzyme was added with an organic solvent ratio of 1:1 and then incubated for 30 min at its optimum pH and temperature, then added p-NPP substrate and incubated for 15 min. Then the lipase activity was determined.

3 Results and Discussion

A. *The fraction of the purified lipase enzyme*

Production of crude lipase extract was carried out in nutrient broth with the addition of 3% n-hexane, 2% palm oil inducer, pH 6 and incubation time of 48 h. The results obtained were the enzyme unit activity of 383 U/mL and protein content of 0.2636 mg/mL so that the specific activity was 1455.68 U/mg. The crude extract obtained from the production is further purified in 2 stages, namely fractionation with ammonium sulfate salt and dialysis.

The first stage, saturation levels of ammonium sulfate salt used are 0–20%, 20–40%, 40–60%, 60–80% and 80–100%. The relationship between the level of saturation of the ammonium sulfate salt and the specific activity of the lipase enzyme is shown in Fig. 1.

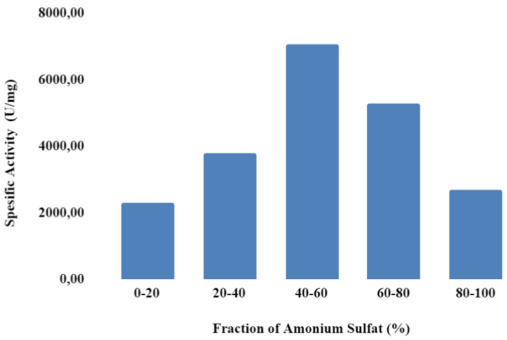

Fig. 1. The relationship between the level of saturation of ammonium sulfate salt with the specific activity of the lipase enzyme.

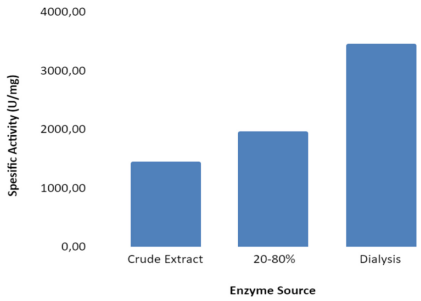

Fig. 2. Comparison of specific lipase activity between crude extract of the enzyme, purified with ammonium sulfate salt, and dialysis.

Figure 1 shows that the lipase enzyme was mostly precipitated at saturation levels of 20–40%, 40–60% and 60–80%, with the highest specific activity obtained at a saturation level of 40–60%, which was 7041.14 U/mg. Therefore, a saturation level of 20–80% was chosen to purify the crude extract of the enzyme that has been produced. Based on the measurement results, the crude extract of the enzyme purified with a saturation level of 20–80% had a specific activity of 1971.89 U/mg, greater than the specific activity of the crude extract which was only 1455.68 U/mg.

The second stage of purification was carried out by dialysis to remove residual salt or impurity ions that could affect enzyme activity. Comparison of specific activities between crude extract, enzyme after purification with ammonium sulfate, and enzyme after purification by dialysis is shown in Fig. 2.

Based on the graph in Fig. 2, it is known that the specific activity of the enzyme increased along with the purification steps carried out. The value of lipase enzyme activity from the steps carried out along with the level of purity is presented in Table 1.

Based on Fig. 2 and Table 1, there is an increase in specific activity along with the purification steps carried out. Lipase crude extract which has a specific activity of 1455.69 U/mg, after fractionation using ammonium sulfate increased to 1971.89 U/mg and at the dialysis stage, increased twofold compared to the crude extract, which was

Table 1. Lipase Purification from Compost Indigenous Bacteria

Step	Volume of enzim	Unit Activity (U/mL)	Protein Content	Specific Activity	Total Activity	Purity Level	Recovery
Crude Ekstrak	600	383.6700	0.263600	1455.50	230202.00	1.00	100.00
Fractionation	30	360.0000	0.182600	1971.52	10800.00	1.35	4.69
Dialysis	42.5	293.6700	0.085100	3450.88	12480.98	2.37	5.42

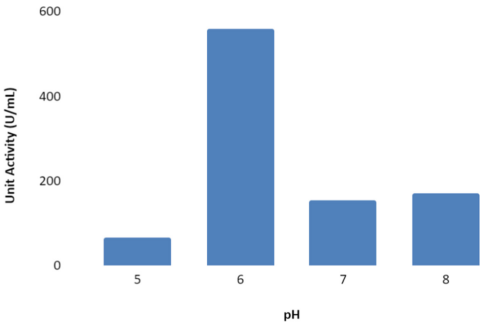

Fig. 3. Effect of pH on lipase activity

3449.60 U/mg. This result is similar to the study of Popoola and Olateru [11] which showed an increase in the specific activity of the lipase enzyme from *Pseudomonas fluorescens* along with the purification steps carried out, the crude extract obtained a specific activity of 0.397 U/mg, after fractionation it became 0.410 U/mg, and doubled after dialysis to 0.734 U/mg.

B. *Characteristics of Lipase Enzymes*

a. Optimum pH

The pH characteristics of the purified enzymes were carried out by varying the pH of the substrate at pH 5, 6, and 7 using phosphate buffer and pH 8 using tris-HCl buffer. The results of the measurement of enzyme activity can be seen in Fig. 3.

Based on the graph in Fig. 3, it can be seen that the lipase enzyme from dialysis gave the highest activity at pH 6. Changes in pH affected the changes in the structure and charge of the amino acid residues that were responsible for binding the substrate. Extreme pH can also cause denaturation of enzyme proteins due to interference with non-covalent interactions which are in charge of maintaining enzyme stability, so that enzyme activity decreases. Similar research results have been reported by Rasmey et al. [12] where the lipase enzyme derived from *P. monteilli* has an optimum pH of 6. Lipase produced by mesophilic isolates has an optimum pH of the enzyme at pH 5 and 7, which is the same as the pH of enzyme production, namely at pH 5 [13].

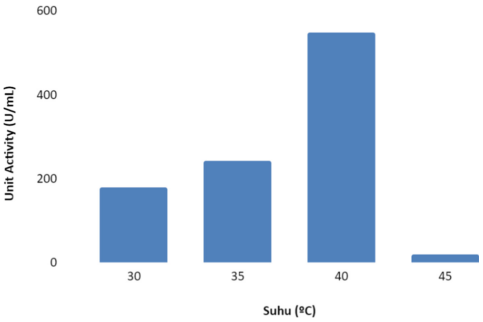

Fig. 4. Effect of temperature on lipase activity

b. Optimum temperature

Determination of the optimum temperature is done by varying the incubation temperature at 30; 35; 40; and 45 ℃ at optimum pH conditions. The results of the measurement of enzyme activity can be seen in Fig. 4.

Figure 4 shows that the lipase produced by the compost indigenous bacteria had the optimum incubation temperature at 40 ℃, and drastically decreased at temperatures greater than that temperature. This may be because these bacteria originate from the mesophilic compost environment so that the optimum temperature obtained is in the mesophilic temperature range. In general, in chemical reactions, increasing temperature can increase the rate of reaction, but in enzymatic reactions, temperatures that are too high can cause enzymes to denature or change the structure of enzymes. Denaturation of enzyme proteins can reduce the rate of reactions catalyzed by these enzymes [14]. This denaturation is caused by the breaking of several hydrogen bonds which are in charge of stabilizing the helical structure of the enzyme protein [15]. The highest lipase activity produced by the mesophilic isolate P. aeuroginosa SRT9 was also obtained at 37 ℃ [16]. Likewise in the research of Rasmey et al. [12] where the lipase enzyme derived from *P. monteilli* has the highest activity at 40 ℃.

c. Metal Ion Effect

Metal ions are very important in the catalytic activity of several enzymes. Metal ions play a role in the catalytic process of enzymes with their ability to attract or donate electrons. Some metal ions can also bind in coordination with the [17] The metal ions tested for their effect on lipase enzyme activity were K^+, Na^+, Ba^{2+}, Mg^{2+}, Al^{3+} and Fe^{3+}. The test results of the effect of metal ions on lipase activity are shown in Fig. 5.

Based on Fig. 5, it can be seen that the activity of the lipase enzyme increased by 32% after the addition of Ba^{2+} metal ions and increased by 44% with the addition of Al^{3+} metal ions. Meanwhile, metal ions Na^+ and Mg^{2+} decreased lipase activity by 5% and 12%, and metal ions K^+ and Fe^{3+} decreased lipase activity by 61% and 76%, respectively. Metal ions can associate with enzyme proteins and can form complexes with other molecules that bind to enzymes. Metal ions can also act as electron donors and acceptors, or as structural regulators of enzymes. These ions can activate or inhibit enzyme activity by

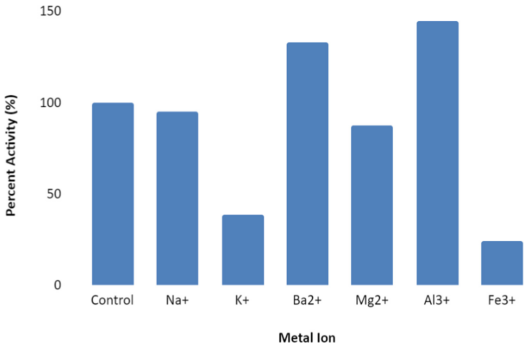

Fig. 5. Effect of metal ions on lipase activity

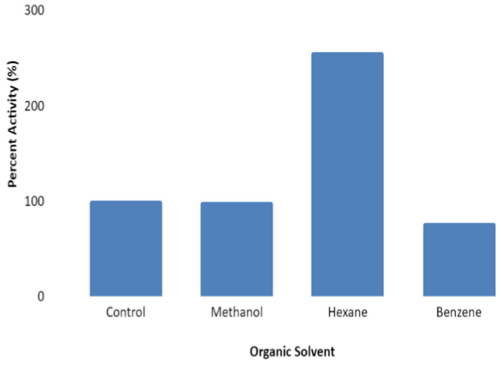

Fig. 6. Effect of organic solvents on enzyme activity

interacting with amines or carboxylic acid groups of amino acid residues [18] This is similar to the research of [19] metal ion Na^+ decreased lipase activity obtained from Bacillus sp. Likewise in the research of Rasmey et al. [12], namely the lipase enzyme produced by *P. monteilli* increased its activity by 28% after the addition of Ba^{2+} metal ions and decreased its activity with the addition of Fe^{3+} metal ions. The lipase enzyme produced by *P. aeruginosa* increased its activity with the addition of metal ions Al^{3+} [20].

d. Effect of Organic Solvent

To determine the effect of organic solvents on lipase activity used organic solvents methanol, n-hexane, benzene with a ratio of 1:1. The test was carried out at the optimum pH and temperature that had been obtained at the characterization stage. The test results of the effect of organic solvents on lipase activity are shown in Fig. 6.

Based on the graph in Fig. 6, it can be seen that methanol decreased lipase activity by 2%, n-hexane increased lipase activity by 155%, while benzene decreased lipase activity by 23%. Lipase activity in organic solvents is also influenced by the cumulative effect

of Log P solvent, denaturation capacity, hydrophobicity, and polarity index [4]. Log P is a measure of the hydrophobicity of organic solvents, the greater the value of log P, the more hydrophobic a solvent. Enzymes tend to have high activity at large log P values (hydrophobic) [21].

The increase in lipase activity by n-hexane may be due to the lipase production environment using the organic solvent n-hexane in its growth medium. In addition, n-hexane is also a hydrophobic solvent (log P 3.9). Organic solvents with hydrophobic properties (high log P) can stimulate the opening of the lid covering the active site of the lipase so that there is an interaction between the solvent and the hydrophobic lipase residue in the lid area which increases the activity of the lipase enzyme [22]. Meanwhile, organic solvents that have a lower log P will attract water to the enzyme so that the enzyme will denature and lose its activity [23]. It can be seen that the enzymes given benzene (log P 2.0) and methanol (log P $<$ 1) decreased their activity by 24% and 2%, respectively.

This is similar to that reported by Liu et al. [7] who found that *B. subtilis* DS9 can grow in the presence of organic solvents with Log P $\geq$ 2.5, even the lipase it produces increases its activity by 25% in the presence of xylene, heptane, n-hexane, isooctane and n-decane. In a study conducted by [24], Streptomyces sp. CS133 which was tested for lipase activity with the addition of organic solvent with a log P $\geq$ 0.87 increased its lipase activity in the presence of n-hexane (log P 3.9) and octane (log P 4.9). Lipase produced by *Stenotrophomonas maltophilia* CGMCC 4254 did not change its activity for 7 days with the addition of non-polar organic solvents (log P 2.0) at concentrations of 20% and 50% but lost its activity by 4% after 24 h of interaction with more polar organic solvents (log P 0) [25].

4 Conclusions

Based on the research it can be concluded that:

1. Indigenous compost bacteria can produce lipase tolerant of organic solvents with a specific activity of 1455.68 U/mg at growth conditions with the addition of 3% n-hexane, 2% palm oil inducer concentration, pH 6 with an incubation time of 48 h.
2. The specific activity of the lipase enzyme purified by dialysis increased two times greater than the crude extract of 3449.60 U/mg.
3. The lipase enzyme from the compost indigenous bacteria characteristic pH 6, temperature 40 C; addition of metal ions Ba^{2+} and Al^{3+} increased their activity while Na^{+}, K^{+}, Mg^{2+} and Fe^{3+} decreased their activity; the addition of organic solvent n-hexane increased the activity twofold, while methanol and benzene decreased the activity.

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