ISOLATION OF LIPOLYTIC BACTERIA FROM DOMESTIC WASTE COMPOST AND ITS APPLICATION TO BIODIESEL PRODUCTION

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ABSTRACT
The exploration of lipolytic bacteria presents its challenges in being able to supply lipase as a catalyst in the production of biodiesel. The compost is chosen to get the bacteria because of the lipid and fatty acids component contained in it. The research was developed to isolate the lipase-producing bacteria and determine the enzyme ability in bioconversion of lipid to biodiesel. The bacterial screening was conducted in Luria Bertani media with additional olive oil 1% (v/v) as an inducer. The colonies surrounded by bright areas were separated from others because they indicated having lipolytic activity. Identification of selected isolate was carried out according to the 16srRNA fragment DNA analysis. The fragment showed a score of nucleotide identity as 98% with the Proteus sp. The isolated bacteria grow well in LB media forming an exponential phase from 2 to 11 h, and have a double-time of cell growth at the 4 h. It produced lipase optimally at the 19 hours with its activity of 1.771 U/mL. The enzyme showed optimum activity at 45°C and pH 8. The lipase showed high activity in t-butanol then followed by sequentially in a mixed solvent (methanol and t-butanol), and a single solvent of isopropanol, methanol, and n-hexane. It exhibited good performance for the biodiesel production from microalgal oil, yielded a total fatty acid methyl esters (FAME) as 2.75% (v/v). The fatty acid composition of the methyl esters was composed of 9 octadecanoic acid and hexadecanoic acid.

Keywords: Lipolytic Bacteria, Lipase, Biodiesel, 16srRNA.

INTRODUCTION
Along with population growth, energy needs for fuel are estimated to continue to increase by 50 % or more by 2030¹. In a developing country such as Indonesia, fuel is obtained by processing various kinds of fossil resources. The continual use of fossil fuel as a source of energy has an impact on the depletion of fossil fuel reserves and an increase in the amount of CO₂ in the atmosphere. Today, research for environmentally friendly and renewable energy is an important challenge. The main alternative to replace fossil fuels that are currently being developed is biodiesel. Biodiesel is generally defined as methyl (ethyl) esters of fatty acids produced through transesterification (alcoholysis) of triglycerides³⁶. Biodiesel is a product of alkyl ester fatty acids (C14-C22 long-chain) with a short chain of primary alcohols, methanol, or ethanol². Biodiesel is a renewable energy source that is environmentally friendly because it can be easily degraded, has a low toxic effect, produces little waste and does not increase carbon dioxide, aromatic components, or other chemical substances that are harmful to the environment³.

One of the factors that influence the efficiency of biodiesel production is the choice of the type of catalyst used in transesterification. One of the catalysts that can be used in biodiesel production is lipase. Lipase in biodiesel production has functions to convert triglycerides to methyl (ethyl) esters through a
transesterification (alcoholysis) reaction process. The change of Triacylglycerol (TAG) into biodiesel occurs through several stages; the initial reaction of an alcohol with TAG as an alkoxide anion to produce FAME and diacylglycerol (DAG), alcohol (alkoxide) then release another FAME molecule and produce monoacylglycerol (MAG), and the last stage MAG undergoes alcoholysis and produce FAME and glycerol. The enzyme is easily obtained from many sources both animals, plants, and microbes. However, the choice of microbes as a source of lipase production is considered the most realistic due to its rapid growth factor, ease of handling, relatively low production costs, and control in fermentation, so that it can produce enzymes with large amounts with high activity. Lipolytic bacteria having their ability to decompose lipids are potentially used as sources for lipase production. They usually exist in various habitats containing lipids such as domestic waste, oil-contaminated soil, and compost heap. Several genera of bacteria are known to produce lipase enzymes including Staphylococcus sp., Pseudomonas sp., Bacillus sp., and Acinetobacter sp. The lipases of these microorganisms are generally inducible enzyme, with the fatty acid ester and in some instances free fatty acids serving as inducers. These microorganisms utilize the fatty acids produced from lipid hydrolyzing by their lipases. A compost heap is a good habitat for the growth of lipolytic bacteria because it contains various types of oil. Some oil from various composts are reported containing fatty acid of linoleic (C18: 2), oleic (C18: 1), and stearic (C18: 0). There are also palmitoleic (C16: 1), palmitic (C16: 0) and myristic (C14: 0) fatty acids. Lipase in the composting process serves to degradation of animal and plant fats. Lipases from the compost bacteria have good stability at high temperatures because fat degradation by the enzyme in the composting process occurs at over 60°C. Thermophilic enzymes compared to mesophilic enzymes generally show higher resistance against the unfavorable environmental conditions, where high temperatures are positively correlated with the safety of enzymes with organic solvents. The production of fatty acid methyl ester (FAME) with lipase biocatalyst has been carried out using various raw materials, one of which is microalgal oil. Microalgae as a biodiesel feedstock are considered to be the most profitable alternative raw material source because it can be obtained easily and does not require extensive land for cultivation. A novel lipolytic bacterial strain of Proteus sp. was isolated from the compost habitat from Jambangan composting house, Surabaya, Indonesia. The effects of various temperatures, pHs and organic solvents on this bacterial lipase were investigated in the study. Also, it was applied to biodiesel production. Because this finding is still a preliminary study of lipase from isolated Proteus, so the further study is needed to explore widely on the enzyme prospects for various applications as well as its biochemical properties in the future.

EXPERIMENTAL

Material
The material that has been used consists of compost, yeast extract, NaCl, commercial olive oil, agar, peptone, methanol, tert-butanol, isopropanol, n-hexane, Tween 20, p-NP, pNPP, isopropanol, NaOH, Tris, HCl, Na2CO3, citric acid, Na2HPO4. The instruments were used consisting of laminar thermo scientific 1300 Series A2, shaking incubator IKA-KS30001, magnetic stirrer, centrifuge Biobase BK-THR16K, autoclave daihan scientific polymax 1040, spectrophotometer visible shimadzu 1800, Incubator memmert expert in thermostatic, GC-MS Agilent 7000A. column GC Agilent 19091S-433UI, 30m x 250μm x 0,25μm.

Screening of Lipolytic Bacteria
A compost sample of 10 g was taken using a sterile spatula and put in a bottle, added with 100 mL sterile distilled water, then incubated at shaking incubator 120 rpm for 20 min. As much as 0.1 mL of culture was spread on the LB agar media consisting of 1% (w/v) tryptone, 0.5 % (w/v) yeast extract, 1% (w/v) NaCl, and 2% (w/v) agar with the addition of 3% (v/v) olive oil, then incubated at 37 °C for 24-48 h. The lipolytic bacteria were harvested from a cell colony that was surrounded by a clear area, then it was used for rejuvenating isolate. The culture was stored at 4 °C after sub-culture every week.

Identification of Bacteria
Identification was carried out according to the homology analysis of the sequence of 16srRNA fragment DNA from a selected isolate. The 16srRNA fragment was prepared by PCR method using genome DNA
from a selected isolate as a template. The genome DNA was isolated from *Proteus* cells by a genomic extraction kit from Promega. Lipolytic bacterial cell pellets were re-suspended with EDTA 50 mM and then lysed with lysozyme 20 mg/ml. After that centrifuged for 2 minutes and the supernatant removed. The cell pellets added with nuclei lysis solution to secrete the DNA, then added with protein precipitation solution. The DNA separated from debris by centrifuge at 10,000 rpm for 3 minutes. The supernatant was taken, then added with isopropanol to precipitate DNA. The deposited DNA washed twice with ethanol 70% (v/v), then followed by centrifugation at 12,000 rpm for 2 minutes. The ethanol is removed, while the existing DNA pellets are dissolved with a DNA rehydration solution containing RNAse. The results of genome DNA were analyzed by agarose gel electrophoresis.

The 16srRNA fragment DNA is prepared by PCR using the pair of primers UniB1 and BactF1. The sequence of UniB1 primer was 5'-ggttac(g/c)ttgttacgactt-3' and 5'-agagtttgatc(a/c)tggctcagc-3' for BactF1. The PCR reaction mixture was setted at 25 μl, consisting of a DNA template (100-300 ng); 2.5 μl PCR10X buffer; 10 mM dNTP mix, 1.0 Unit Ex Taq DNA polymerase; 1.0 pmol for each UniB1 and BactF1 primers and the rest ddH2O up to volume 25μl.

The PCR process was carried out by Gene Cycler in 25 cycles. Each cycle was run under conditions: denaturation at 94°C for 1 min; annealing at 53°C for 1 min; and polymerization at 72°C for 2 mins. This process was completed with the pre-denaturation conditions at 94°C for 5 mins and 72°C extension for 5 mins. The results of the PCR were then analyzed by agarose gel electrophoresis, then sequenced by the Sanger dideoxy method through Macrogen, using UniB1 and BactF1 primers. The sequence of 16srRNA fragments was analyzed for microbial identification.

**Growth Curve**

A loop of fresh planted media was transferred into 250 mL of Erlenmeyer flask containing 50 mL of Luria Bertani (LB) media. LB media is made with a composition of 10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, in 1L distilled water. It can be incubated at 37°C in a rocking incubator of 180 rpm for 24 h. Every hour the sample was taken and measured its optical density by spectrophotometer at 600 nm to determine the growth curve of cells.

**Lipase Production**

Ten grams of olive oil was suspended in 90 mL of LB media in 250 mL Erlenmeyer flask. Then it is autoclaved at a pressure of 1.5 atm with a temperature of 120°C for 20 min. About 1% of inoculum culture was added to the prepared media. After being mixed in Erlenmeyer flask, it was incubated at 37°C using a shaking incubator 150 rpm. The culture was sampled every hour to measure lipase activity for the determination of the optimum time on lipase production. The culture was centrifuged at 5000 rpm for 10 min at 4°C to remove the cell pellets. The supernatant that contains lipase enzyme was further for the detection of enzyme activity.

**Lipase Activity Assay**

The substrate used for the assay was p-nitrophenylpalmitate (pNPP). As much as 30 mg of pNPP dissolved in 10 mL isopropanol, followed by an addition 86.4 mL buffer of 50 mM Tris–HCl pH 8 and 0.4 mL tween 20. The 1800 μL of substrate solution was reacted with 100 μL crude enzyme and 100 μL Tris-HCl buffer, then incubated at 45°C for 15 min. The reaction was stopped by using 250 μL 0.1 M Na2CO3. The absorbance of yellow color yielded from the reaction was determined at λ = 410 nm. The unit activity of the enzyme (U) is expressed as the amount of enzyme needed to generate 1 μM p-nitrophenol (p-NP) product which is released every minute under test conditions.

**Detection of Optimum Temperature and pH**

It was done an assay of lipase activity at various temperature and pH, which were at range 30-70°C for temperature, and 6-9 for pH. The 0.05 M citrate-phosphate buffer was used to adjust the test condition at pH 6-7, then the 0.05 M tris-HCl buffer for pH 8-9.

**Detection of Organic Solvents Effect**

The work was done to observe the lipase stability in various organic solvents such as methanol, t-butanol, and n-hexane. Firstly, it was prepared a mixture of the crude enzyme with 30 % (v/v) of the solvents in the
ratio 1:1 for volume comparison. The mixed product then was reacted with p-NPP substrate like in the enzyme activity test. The assay was done at optimum pH and temperature but run at various times. The control used in the test was the enzyme without organic solvents.

Production of Fatty Acid Methyl Ester From Microalgal Oil
As much as 2 mL of oil was mixed with 0.1 g of lipase in two neck flasks, then 500 μL of t-butanol as co-solvent and 500 μL of methanol as a solvent were added. The mixture was refluxed at the optimum temperature and stirred with a magnetic stirrer at a speed of 200 rpm for 5 h. The reflux results were allowed to stand for one day to form two layers. The top layer of methyl ester fatty acid is dissolved in n-hexane for GC-MS analysis.

Fatty Acids Methyl Ester Analysis
The FAME produced from microalgal oil was detected by GC-MS Agilent 7000A with HP-5ms column, 30 m, 250 μm, 0.25 μm. The helium gas pressure for this analysis was set at 10,523 psi with a flow rate of 10:1. Mass spectrometers were operated with an injection sample volume of 1.0 μL with Run Time: 650 min. The data was processed by Mass Hunter (Agilent) software with reference from the database of Wiley and NIST libraries.

RESULTS AND DISCUSSION
Screening and Identification of Lipolytic Bacteria
Isolation of lipolytic bacteria was carried out on compost samples with a storage period of 6 days. Screening results showed clear zones in the solid LB media. Clear zone indicates the degradation of olive oil added to solid LB media by lipolytic bacteria. As shown in Fig.-1, two potential isolates could be selected as the lipase-producing bacteria.

![Fig.-1: Result of Screening Bacteria Lipolytic. Clear zone in around colony indicates the hydrolytic activity from lipase.](image)

The selected bacterial isolates were then identified by 16srRNA analysis. The Fragmen DNA of 16srRNA in genome DNA of the selected isolate was amplified by PCR resulting 1.5 kb fragment in agarose gel electrophoresis (Fig.-2). The fragment then was sequenced to determine the bacterial similarity identification by using the blast-x method (http://ncbi.nlm.nih.gov). The analysis showed the 16srRNA fragment DNA of the sample has a high identity score as 98% with the *Proteus* sp, likely shown in the result of phylogenetic analysis in Fig.-3. *Proteus* bacteria are spread in many places as saprophytes such as in rotten organic matter, animal or human feces, garbage, compost, and mammalian intestines. The genus *Proteus* is also known as a potential bacteria producing lipase enzyme such as *Bacillus* sp.

Growth Curve and Lipase Production of *Proteus* sp.
Bacterial growth is specifically studied by observing a growth curve using the optical density. The use of optical density (OD) is a method that has been widely used for cell measurement because it can directly correlate with the number of cells in the media and easily adapt to the automatic measurement system. In Fig.-4, the growth of *Proteus* showed four phases, namely: lag, exponential, stationary and death phase.
The lag phase occurs at 0 - 1 h where the cell growth does not start quickly, and an increase in population has also not been observed. The length of the lag phase depends mainly on the type of media used and the size of the inoculum. The second phase is the exponential phase (log phase). In this phase, the curve shows a logarithmic growth. The exponential phase of *Proteus sp.* occurs at 2 h up to 11 h. The third phase is the stationary phase. The growth rate at this phase was indicated by a straight line on the curve. The stationary phase occurs at the 11 h to 19 h, and is followed by the death phase.

![Electrophoregram of 16srRNA DNA Fragment resulted From PCR.](image)

**Fig.-2:** Electrophoregram of 16srRNA DNA Fragment resulted From PCR. Lane 1, The marker DNA 1 kb Leadder, Lane 2, Fragment 1.5 kb represent to 16srRNA Fragment

![Phylogenetic Tree of the Selected Isolate.](image)

**Fig.-3:** Phylogenetic Tree of the Selected Isolate. The isolate has high identity with *Proteus sp.*

There are some reasons why a closed culture system can reach the stationary phase. One common reason is that carbon and energy sources or important nutrients become fully consumed. When the source of carbon is used up, it does not mean all growth stops. This is because dying cells should be lysed and used as a source of nutrition. Also, when the waste products would be accumulated, it causes cells to become poisoned and thus inhibits the cell growth.\(^ {25,26}\) The last phase is the death phase. As mentioned above, the death phase occurs after the stationary phase, where the number of cells was decreased slightly from 20 h to 24 h.

Doubling time \((t_d)\) was also determined for the dynamics of cell development. For *Proteus sp.*, cell mass has doubled within 4 h. The doubling time is not the same between various microbes, depending on the speed of growth.\(^ {27}\) The production of lipase enzyme from *Proteus sp.* was shown in Fig.-5. The production media was LB with the addition of 3% inducer in the form of olive oil. Lipase activity was gradually increased at the culture time of 1 h to 19 h. The highest activity of lipase (1,770 U/ml) was obtained at 19 h.

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After that, lipase activity continued to decline when *Proteus sp.* reached the death phase. The decrease in lipase activity might be due to the nutrient depletion, accumulation of toxic waste from cells, and the presence of proteases in the culture media.\(^{28,29}\)

**Fig.-4: Growth Curve isolate *Proteus* sp.** In the growth of *Proteus* sp., the lag phase initially occurs at 0 - 1 h, a logarithmic growth at 2 h up to 11 h, a stationary phase at the 11 h to 19 h, then followed by the death phase. The cell mass of *Proteus* sp. has doubled within 4 h.

**Fig.-5: Lipase Activity *Proteus* sp.** Lipase activity was gradually increased at the culture time of 1 h to 19 h. The highest activity of lipase was obtained at 19 h.

**Optimum Temperature of Lipase**

Determination of the optimum temperature for the enzyme is very important because enzymes at low temperatures could become inactive while at high temperatures it could cause denaturation in the molecular structure. The optimum temperature of lipolytic activity from *Proteus sp.* was investigated in the range of 30-70 °C. The highest activity occurred at 45°C which was 1,758 U/mL (Fig.-6). The same temperature has also belonged to the lipase of *B. amyloliquefaciens*\(^{32}\), *B. subtilis*\(^{32}\), and *A. niger*\(^{33}\) respectively. Enzyme activity was generally increased up to 45°C and then decreased with increasing temperature. The activity of the enzymes concerning temperature is influenced by the kinetic energy of enzymes and substrate to collide with each other. Increasing temperature causes kinetic energy to increase. The activity at low temperatures (30°C and 40°C), was 5.7 and 0.4% lower than that at optimum temperature. This is because the collisions that occur between the enzyme and the substrate intensity are still low so that the enzyme-substrate complex formed is small and the product produced is also small. When the temperature was increased to the optimum temperature, collisions between enzymes and substrate would be increased, thus increasing the enzyme activity. At the optimum temperature, the collision between the enzyme and the substrate is very effective so that the enzyme-substrate complex is more easily formed and the product produced also increases. Above the optimal temperature, the enzyme can decrease its activity. This can be triggered by protein denaturation factors that damage the structure and function of enzymes\(^{31}\). The activity at 50°C, 60°C, 70°C were 1.7, 4.5, and 5.7% decreased respectively.
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Optimum pH of Lipase

Another factor that influences the lipase activity is pH. Enzymes generally have an optimum pH that is identical to the environment around normal intracells. Optimum pH for Proteus sp. was investigated in the pH range 6-9, and the highest activity was obtained at pH 8 with an activity of 1,745 U/mL (Fig.-7). The optimum pH was similarly found on lipase from Bacillus sp. and B. Licheniformis.

At optimum pH conditions, the important proton donor and acceptor groups on the catalytic site of the enzyme have the complicated three-dimensional structures that fit the substrate to bind the substrate appropriately, form an enzyme-substrate complex and produce the product. Lipase activity at other pHs was low because the enzyme was denatured. At low pH, the protonated enzyme loses its negative charge, whereas, at high pH, the substrate is ionized so it loses its positive charge. The activities at pH 6, 7, 7.5, 8.5, 9 were 6.9, 4.6, 3.5, 3.4 and 4.6% decreased respectively.

Effect of Organic Solvent on Lipase Activity

Enzymatic reactions in organic solvents provide many advantages in the field of industry. Many lipases are known to remain active against organic solvents because of conformation and interfacial activation characteristics. Figure-8 shows the effect of organic solvents on lipase activity. This lipase was found to have the highest activity (1,798 U/mL) in t-butanol solvent, followed by a mixture of t-butanol and methanol which has an activity of 1,784 U/mL. Isopropanol also showed a high activity of 1,777 U/mL. In the case of methanol, lipase activity was similar to the control. The highest activities for methanol and control were 1,749 U/mL and 1,744 U/mL, respectively. Lipase activity with the addition of n-hexane
showed the lowest value compared to other solvents, where the highest activity was 1,713 U/mL. Overall, lipase activities were still maintained up to 300 min in organic solvents despite decrease inactivity.

One of the factors that need to be considered on the influence of organic solvents is the solubility of substrates and products in the media. Substrates that are more soluble in nonpolar solvents would be difficult to diffuse into enzymes in aqueous media, causing the slow reaction and lower enzyme activity. The addition of less polar solvents such as t-butanol (log P = 0.35), or isopropanol (log P = 0.05) increases the solubility of the substrate in aqueous media. On the other hand, non-polar organic solvents such as n-hexane (Log P = 3.5) were added, the substrate becomes more insoluble in aqueous media because it is more soluble in the organic solvent. This is closely related to the lipase enzyme confirmation which also showed that nonpolar solvents can penetrate and block the active site of the enzyme, causing enzyme activity to decrease as the substrate diffusion rate decreases into the enzyme.

These results indicate that lipase is more active in the presence of less polar solvents than polar and nonpolar solvents. Different kinds of lipases could be reacted in different organic solvents with different levels of resistance in the reaction system.

Fig.-8: Lipase Activity at Various Organic Solvents. The highest activity was found in t-butanol, then followed by a mixed solvent of t-butanol and methanol (1:1). isopropanol, methanol, and n-hexane as the last solvents.

Production of Biodiesel and Its Analysis

A freeze-dried sample of lipase produced by *Proteus sp.* was used for the production of fatty acid methyl ester. The crude extract of the lipase enzyme was prepared to eliminate the water content by freeze-drying. This is because lipase leads to the hydrolysis reaction of triglycerides if there is water content around it. Therefore, the transesterification reaction can be performed in the absence of water and biodiesel would be produced properly. Freeze-dried lipase showed an activity of 234 U/g. After the transesterification reaction, two layers were formed. The top layer was the fatty acid methyl esters and the bottom layer was glycerol. The crude extract of lipase from *Proteus sp.* showed good performance for the biodiesel production from microalgal oil, yielded a total fatty acid methyl esters (FAME) as 2.75% (v/v). The methyl ester components observed in the chromatogram were hexadecanoic and 9-octadecanoic acid methyl ester (Table-1). The largest portion of methyl esters was 9-octadecanoate methyl esters of 2.365%. This result is related to the previous work that the most common biodiesel components obtained from microalgae were palmitate (hexadecanoate - C16: 0), stearate (octadecanoate -C18:0), oleate (9-octadecanoate - C18:1), linoleate (9, 12-octadeциnoate -C18:2) and linolenic acid (9,12,15-octekatrienoate - C18:3). The methyl ester component was not found in the negative control chromatogram obtained from microalgal oil without treatment. This confirms that the lipase enzyme from *Proteus sp.* has catalytic activity in the transesterification reaction. This catalytic activity can still be improved by optimizing the transesterification conditions such as oil and solvent volume ratio, incubation time, and enzyme purity. Transesterification is an equilibrium reaction where large amounts of alcohol (methanol or ethanol) are needed to promote the forward reaction to...
proceed. In molar stoichiometry the ratio of alcohol: triglycerides are 3: 1, but the excess of methanol which is insoluble in the media is well known to cause enzymes inactive, thus reducing the yield\(^2\). Another thing is that the lipase enzyme used in this study is still a crude extract that has not been purified. The purity of the enzyme greatly influences the activity of the enzyme. The disruption by the impure proteins or other impurities would affect the catalytic activity of the lipase enzyme.

This result is only the first step performed by the isolate of \emph{Proteus sp}. The next step should be continued to develop a more efficient process as a source of biocatalyst in biodiesel production. The use of this biocatalyst is expected to be an alternative to the use of chemical catalysts that are less favorable for the environment. However, optimization of the transesterification conditions with lipase from \emph{Proteus sp} still needs to be performed for the efficient production of biodiesel. This optimization includes the ratio of solvent and oil, and reaction time for transesterification. Further studies would be related to the purification of lipase from \emph{Proteus sp} for various applications.

### Table-1: Result of Analysis GC-MS

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### CONCLUSION

\emph{Proteus sp}. is one of the potential bacterial species producing lipase enzyme that was successfully isolated from compost samples made from household waste. The bacteria could significantly produce lipase. The highest lipase activity was obtained at 45°C and pH 8 which was 1,758 U/mL. The addition of slightly polar t-butanol and isopropyl alcohol increases the solubility of substrate in aqueous media, thereby increasing lipase.
activity. However, the enzyme activity decreases in n-hexane which is nonpolar. The crude extract of lipase showed good performance for fatty acid methyl esters production. This is indicated by the formation of 9-octadecanoate methyl esters from microalgal oil through transesterification.

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