Production and Purification of Lipase Enzyme from Bacillus licheniformis F11.4 as Biodetergent

Ramadhan Iskandar¹, Muhamad Sahlan¹, Siswa Setyahadi²

1. Chemical Engineering Department, Faculty of Engineering, University of Indonesia, Depok, 16424, Indonesia
2. Agency for Assessment and Application of Technology, Republic of Indonesia, Jakarta, Indonesia

*e-mail: ramadhan.iskandar@ui.ac.id

Abstract—Lipase enzyme can use in many industries, such as food, detergent, textile, pharmaceutical, biodiesel and agrochemical. In this research, lipase enzyme tested its ability as an additive in detergent. Lipase enzyme produced by Bacillus licheniformis F11.4 purified two times, by stirred-cell ultrafiltration and dialysis. Purification with stirred-cell ultrafiltration conduct at optimum operation condition; 50 kDa membrane size, 30 psi nitrogen gas pressure, and maximal concentrate. Washing efficiency of purified lipase enzyme on cloth with artificial dirt, olive oil, is 16.07%, if combine with detergent is 34.88%, and can enhance washing efficiency by detergent up to 6.05%.

Keywords—Bacillus licheniformis F11.4; dialysis; lipase enzyme; stirred-cell ultrafiltration

I. INTRODUCTION

Enzyme play important role in our life. Enzyme have many applications in industries, such as dairy, food, detergent, textile, pharmaceutical, cosmetics and biodiesel industry, agrochemical, and polymeric material [1]. Based on regulation from European Commission (EC) 648/2004, detergent industry is the biggest user of enzyme. Biotechnology-based cleaning agent, was cheaper and safer for the environment [7]. Detergents with a cleaning agent in the form of the enzyme has better cleaning properties when compared with the raw material of synthetic detergents. One of the types of enzymes that play a role in the growth of biotechnology is lipase. Lipase have been isolated from many species of plants, animals, bacteria, fungi, and yeast. Lipase is also one of the enzymes used in the manufacture of industrial detergents. The enzymes used in the manufacture of detergents, among others, protease, amylase, and cellulose. Lipase was used to remove stains in the form of fat such as butter, salad oils, sauces, and so forth. Enzyme in the detergent formulation is stable at high pH and temperature.

The use of enzymes in industry depends on the level of purity of the enzyme. The higher the purity of an enzyme, then the higher the activity generated. Therefore, various methods have been performed to purify the enzyme. Purification of lipase enzyme from a microbe commonly used methods of centrifugation or filtration as a pre-purification method. Then the results pre-purification concentrated by ultrafiltration, precipitation with ammonium sulfate or by using extraction with organic solvents [10]. After concentrating, then the next stage is the stage of polishing. At this stage, the enzyme was purified further by chromatography.

In this study, the lipase enzyme produced from Bacillus licheniformis F11.4 strain. Crude enzymes produced will be separated from the cells by using a centrifuge, and then purified by the ultrafiltration using a stirred-cell ultrafiltration membrane size variation, the pressure of nitrogen gas, and maximal concentrate. Washing efficiency of purified lipase enzyme on cloth with artificial dirt, olive oil, is 16.07%, if combine with detergent is 34.88%, and can enhance washing efficiency by detergent up to 6.05%.

II. MATERIALS AND METHOD

A. Materials

Bacillus licheniformis F11.4 was isolated from shrimp waste in Palembang, Indonesia [Waldeck, 2006] [8]. Bacillus licheniformis F11.4 was isolated from shrimp waste in Palembang, Indonesia. Bacillus licheniformis F11.4 is one of mutant from Bacillus licheniformis F11 which lack chitinase gene ChiA and ChiB [2]. Bacillus licheniformis F11.4 that used in this research was from Agency for Assessment and Application of Technology’s culture collection. For starter medium, pepton, yeast extract, NaCl, Agar are needed. For crude enzyme production, fish meal, olive oil, CPO (crude palm oil), KH₂PO₄, NaNO₃ are needed. For enzyme assay, pNPP (p-nitrophenyl palmitate), 2-propanol, gum arabic, triton x-100, tris HCl buffer are needed. For crude enzyme purification, PEG-20000 is needed. For lipase activity staining, CaCl₂, phenol red indicator are needed.
B. Tools

Shaking incubator is needed for microbial culture and crude enzyme production. Stirred-cell ultrafiltration is needed for prepurification and purification of crude enzyme. Dialysis tube is needed for purification of crude enzyme.

C. Preparation of Microorganism

The seed inoculum was prepared by inoculating a loop full of culture from nutrient agar into Luria-Bertani broth and after 3 h of growth at 50ºC, seed inoculum introduced to the production medium as much as 10% of production medium volume.

D. Preparation of the Crude Enzyme Extract

The production medium in this research was modified from research by Tirtarasa (2015)[3]. The production medium contained 2% (w/v) fish meal, 0.07% (v/v) olive oil, 0.5% (w/v) CPO, 0.3% (w/v) NaNO₃, and 0.1% (w/v) KH₂PO₄. pH of the medium was adjusted to 8. The production medium was incubated at 37ºC and kept shaking at 150 rpm. After 18 h incubation, enzyme was extracted. The culture broth was harvested by centrifuging at 3,800 rpm for 30 min at 4ºC.

E. Ultrafiltration Experiments

Ultrafiltration was use for both prepurification and purification. Prepurification step conducted to determine optimal operation condition for purification step using stirred-cell ultrafiltration. A dead-end stirred cell ultrafiltration system connected with a nitrogen gas cylinder and solution reservoir was designed to concentrate and purify crude enzyme. The system consisted of a ultrafiltration cell (model 8400, Millipore™) with a volume capacity of 400 mL. In this research, polyethersulfone membrane was used with inner diameter was 76 mm and effective membrane area of the membrane was 41.8 cm². All the ultrafiltration experiments were carried out at a stirring speed of 100 rpm and temperature kept at ±10ºC by placing the ultrafiltration column in a reservoir contained ice cube. Each membrane was initially pressurized at 5 psi for 10 min.

In prepurification step, ultrafiltration was conduct with three kinds of variation. First, the use of size of membrane are 10, 30, and 50 kDa. Second, the use of nitrogen gas pressure are 10, 20, and 30 psi. Last, crude enzyme was concentrate with different concentration factors, 2X, 5X, and 10X. From these kinds of variations, there are 27 kinds of sample conditions. The feed volume was initially 150 mL for each variations. A 600µL of retentate and permeate volume for each sample conditions was taken for enzyme assay.

In purification step, the operation condition of ultrafiltration was used from the best result of prepurification step. Some filtration properties was measured adapted by research from Zhao (2008)[4]. The flux (L/(m² h)) was calculated by the following equation :

\[ J = \frac{V}{A\Delta t} \]  \hspace{1cm} (1)

where V (L) was the volume of permeated water or protein solution, A (m²) was the membrane area, and Δt was the permeation time. There are 3 kinds of flux measured, water flux (Jw₁), protein flux (Jp), and water flux after protein filtration (Jw₂). The protein rejection ratio was calculated by the following equation :

\[ \text{Rejection ratio} = \left( 1 - \frac{C_p}{C_f} \right) \times 100\% \]  \hspace{1cm} (2)

where \( C_p \) and \( C_f \) (mg/mL) was the protein concentration of permeate and feed. Flux recovery ratio was calculated using the following equation :

\[ \text{FRR} = \left( \frac{Jw_2}{Jw_1} \right) \times 100\% \]  \hspace{1cm} (3)

In purification step, ultrafiltration was conduct with three kinds of variation. First, the use of size of membrane are 10, 30, and 50 kDa. Second, the use of nitrogen gas pressure are 10, 20, and 30 psi. Last, crude enzyme was concentrate with different concentration factors, 2X, 5X, and 10X. From these kinds of variations, there are 27 kinds of sample conditions. The feed volume was initially 150 mL for each variations. A 600µL of retentate and permeate volume for each sample conditions was taken for enzyme assay.

F. Purification with Dialysis-PEG 20000

After purified by ultrafiltration, crude enzyme filled into dialysis tube with membrane size was 12 kDa. Dialysis tube placed into plate and poured by polyethylene glycol with molecular weight was 20000 (Merck). Dialysis process was conduct at 4ºC to keep enzyme stability. Purified crude enzyme was concentrate until desired retentate volume was reached. Purification properties of each step of purification, purification factor and yield was calculated by using the following equations :

\[ \text{Specific Activity (U/mg) =} \]
Enzyme Activity (U/mL)

Protein Concentration (mg/mL)

\[
\text{Purification factor} = \frac{\text{Specific Activity of Fraction}}{\text{Specific Activity of Feed}}
\]

\[
\% \text{Yield} = \frac{\text{Total Enzyme Activity of Fraction} \times (U)}{\text{Total Enzyme Activity of Feed} \times (U)}
\]

G. Enzyme Assay and Protein Estimation

Crude and purified enzyme was assayed using \(p\)-nitrophenyl palmitate (Sigma) as a substrate. This assay was adapted by research from Silva (2004) [5]. Samples (0.1 mL) were mixed with 0.9 mL of substrate solution containing 3 mg of pNPP dissolved in 1 mL propanol-2-ol diluted in 9 mL of 50 mM Tris-HCl pH 8.0 containing 40 mg of Triton X-100 and 10 mg of gum arabic. After 30 min of incubation at 37ºC the absorbance was measured using spectrophotometer at 410 nm against an enzyme-free control. One lipase unit (U) was defined as the amount of enzyme that liberates 1 µmol \(p\)-nitrophenol/min. All enzyme assays were carried out in triplicate and the average values were calculated. Protein content of the appropriately diluted samples was estimated by Bradford’ method [9].

H. Preparation of Lipase Chromogenic Plates

A method from Amara (2009) [6], was used in this step. The pH of aquadest was adjusted to 7.3-7.4 by using 0.1 N NaOH, where 2% olive oil, 4% gum arabic, and 20 mM CaCl\(_2\) were added to the same volume of 4% melted agar. The pH indicator such as phenol red or congo red was added in final concentration 0.01% to the mixture. After mixing well, 25 mL were distributed in each agar plate. After complete solidification of the agar on plates, wells were punched out of the agar, by using a clean sterile cork borer. The base of each hole was sealed with a drop of melted sterile water agar (15 g agar per liter H\(_2\)O) using sterile pipette.

I. Detection of Lipase Activity on Plate

A method from Amara (2009) [6] was used in this research, where 50 µL of the purified enzyme expected to contain lipases were impregnated to each well while sterile media (without any growth) was served as control. The plates were incubated at 37ºC and 25ºC as control for 30 min. The changes in the color around the wells indicate the presence of lipase activity.

J. Determination of Washing Properties of Crude Enzymes

A method from Amara (2009) [6] was used in this research, where artificial dirt represented in olive oil on 4X4 cm white cotton fabrics. The weight of the fabrics with or without the dirt were determined. The dirt is dried at 38ºC for 12 h. The washing properties of crude enzymes were determined in presence or absence of detergent. 2 mg/mL of the detergent (contain 20% anionic surfactant and 15% phosphate). In this research, detergent brand “Rinso” was used, where the content of anionic surfactant was 21%. Four types of treatments were used: the first contained a tap water and 500 µL of the crude enzymes; the second contained tap water and 2 mg/mL detergent; the third contained 500 µL and 2 mg/mL detergent; the last contained only tap water and used as blank. Each treatment was added to tube and allowed to be shackled in shaker incubators (Kuhner) at 150 rpm at 38ºC for 50 min. After the process is completed, each fabrics was dried overnight at 40ºC and reweight. The lost in the weight due to each treatment has been calculated. The dirt removal was calculated by the following equation:

\[
\% \text{Oil Dirt Removal} = \left[ \frac{\text{weight of olive oil before washing} - \text{weight of oil after washing}}{\text{weight of olive oil before washing}} \right] \times 100
\]

The improvement in the dirt-removing percentage after mixing each enzyme with detergent was calculated using the following formula:

\[
\% \text{Improvement} = (\% \text{Removing of enzyme –detergent mixture}) - (\% \text{removing of detergent alone + } \% \text{removing of crude enzyme alone})
\]

III. RESULTS AND DISCUSSION

A. Prepurification

The crude enzyme was concentrated by stirred-cell ultrafiltration at different membrane sizes (10, 30, and 50 kDa), three nitrogen gas pressures (10,20, and 30 psi), and three different concentration factor (2, 5, and 10). The retentate crude enzyme at each conditions was assayed and measured the protein concentration. Then, purification properties, purification factor and %yield, was measured by using equation (8) and (9), respectively.
To determine which conditions was the most optimal operation condition for purification with stirred-cell ultrafiltration, first point that considered was purification factor, and then %yield. Based on Figure 1, sample condition which have highest purification factor was 5C and 9C with the value of purification factor were 2.57 and 2.59, respectively. But, sample 9C has higher %yield than 5C. Sample 9C has %yield of 40%, meanwhile sample 5C was 33%. Thus, the most optimal operation condition for purification with stirred-cell ultrafiltration was 9C condition; 50 kDa membrane size, 30 psi nitrogen gas pressure, and 10 concentration factor.

B. Purification

Purification process was conducted with selected operation condition. Flux and fouling properties was measured. Based on Table 1, the value of rejection ratio was quite large, up to 98.47%, indicating that the pore size of the outer surface 50 kDa PES membrane was smaller than the size of most of the molecules contained in the crude enzyme sample. According to Souza (2000) [11], rejection occurs due to transfer through convective pores and rejection decreases with the increase in pressure that occurs due to increased concentration in the membrane layer. When fouling occur, rejection will begin to increase the flux or pressure.

PES 50 kDa membrane used in this experiment has a value of the flux recovery ratio (FRR) by 25.63%. Where the higher value of the FRR, the higher value hydrophilic properties and antifouling membranes [4]. This indicates that the hydrophilic properties and antifouling at 50 kDa PES membrane used in these experiments is still low. PES membrane has mechanical strength, thermal resistance and pH stability was good, but the material is hydrophobic, makes it very easy to form a protein fouling [4].

In this experiment, the total flux is lost due to fouling was 97.78%, with 74.37% of it was due to irreversible fouling. Irreversible fouling is fouling trapped within the pores of the membrane. In ultrafiltration, adsorption and deposits of non-specific protein on the membrane surface or in the pores lead to fouling of the membrane. In general, an effective approach for reducing fouling of the membranes, especially in irreversible fouling is by increasing the hydrophobicity on the surface of the membrane [4].

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Symbol</th>
<th>Result</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water Flux</td>
<td>$J_w$</td>
<td>771.263</td>
<td>L/(m² h)</td>
</tr>
<tr>
<td>Protein Flux</td>
<td>$J_p$</td>
<td>17,088</td>
<td>L/(m² h)</td>
</tr>
<tr>
<td>Cleaning Water Flux</td>
<td>$J_{w2}$</td>
<td>197,663</td>
<td>L/(m² h)</td>
</tr>
<tr>
<td>Rejection Ratio</td>
<td>-</td>
<td>98.47%</td>
<td>-</td>
</tr>
<tr>
<td>Flux Recovery Ratio</td>
<td>FRR</td>
<td>25.63%</td>
<td>-</td>
</tr>
<tr>
<td>Total Flux Loss Caused by Total Fouling</td>
<td>$r_t$</td>
<td>97.78%</td>
<td>-</td>
</tr>
<tr>
<td>Total Flux Loss Caused by Reversible Fouling</td>
<td>$r_r$</td>
<td>23.41%</td>
<td>-</td>
</tr>
<tr>
<td>Total Flux Loss Caused by Irreversible Fouling</td>
<td>$r_{ir}$</td>
<td>74.37%</td>
<td>-</td>
</tr>
</tbody>
</table>

After two-step purification, by using ultrafiltration and dialysis bag, then the concentration of protein and enzyme activity was calculated to determine purification properties of crude enzyme from B. licheniformis F11.4

<table>
<thead>
<tr>
<th>Sample</th>
<th>Volume (mL)</th>
<th>Total Activity (U)</th>
<th>Specific Activity (U/mg)</th>
<th>Purification Factor</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Feed</td>
<td>200</td>
<td>480,478</td>
<td>38,346</td>
<td>1</td>
<td>100</td>
</tr>
<tr>
<td>Retentate UF</td>
<td>34</td>
<td>354,214</td>
<td>43,902</td>
<td>1.145</td>
<td>73.72</td>
</tr>
<tr>
<td>Retentate Dialysis-PEG</td>
<td>1.5</td>
<td>71,849</td>
<td>195,245</td>
<td>5.092</td>
<td>14.95</td>
</tr>
</tbody>
</table>

C. Staining Lipase Activity

Based on Figure 2, there is a difference between staining lipase activity in optimum conditions (temperature 37°C) shown in figure A and staining at room-temperature conditions.
Fig. 2. Staining lipase activity on plate. A) Staining lipase activity at 37ºC. B) Staining lipase activity at room temperature.

The difference can be seen on the 'halo' around the hole sample was formed. Figure A has a 'halo', which is broader than the image B. This indicates that the activity of lipase at a temperature of 37ºC is better than at room temperature. The difference in the two images can also prove that the 'halo' that is formed is not due to leaks in order.

D. Washing Test

In this experiment, the detergent used is based on the criteria of Amara (2009) [6], the detergent containing 20% anionic surfactant and 15% phosphate. Detergents used was ‘Rinso’ detergent, containing surfactant by 21%, while the phosphate content is unknown.

TABLE III. WASHING TEST RESULT OF PURIFIED LIPOASE ENZYME FROM BACILLUS LICHENIFORMIS F11.4

<table>
<thead>
<tr>
<th>Sample</th>
<th>Removing</th>
<th>Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tap water+Enzyme</td>
<td>16.07%</td>
<td></td>
</tr>
<tr>
<td>Tap water+Detergent</td>
<td>12.77%</td>
<td>6.05%</td>
</tr>
<tr>
<td>Tap water+Enzyme+Detergent</td>
<td>34.88%</td>
<td></td>
</tr>
</tbody>
</table>

From Table 3 it can be seen that the ability to wash the stains in the form of olive oil by the enzyme is higher than by detergent. However, if the enzyme and detergent are combined, there is an increased washing efficiency of 6.05%. This suggests that the concentrated enzyme lipase from Bacillus licheniformis F11.4 quite compatible with commercial detergents.

IV. SUMMARY

The most optimal operation condition of purification of lipase enzyme from Bacillus licheniformis F11.4 with stirred-cell ultrafiltration was using membrane 50 kDa size, 30 psi nitrogen gas pressure, and concentration factor up to 10.

Lipase enzyme from Bacillus licheniformis F11.4 can enhance washing ability of the detergent up to 6,05%.

REFERENCES