

PRODUCTION OF DRY EXTRACT LIPASE FROM *PSEUDOMONAS AERUGINOSA* BY THE SUBMERGED FERMENTATION METHOD IN PALM OIL MILL EFFLUENT

Heri Hermansyah^{1*}, Ambar Maresya¹, Dwini Normayulisa Putri¹, Muhamad Sahlan¹, Michel Meyer²

¹Department of Chemical Engineering, Faculty of Engineering, Universitas Indonesia, Depok, West Java, 16424, Indonesia

²Laboratoire de Génie Chimique, UMR CNRS/INPT/UPS, Université de Toulouse, 31432, France

(Received: January 2018 / Revised: February 2018 / Accepted: February 2018)

ABSTRACT

Palm Oil Mill Effluent (POME) is an agro-industrial waste product with high availability and which contains high quantities of organic compounds that are necessary for microbial growth. Cultures of *Pseudomonas aeruginosa* were grown in POME to produce lipase using the submerged fermentation method. The objective of this study is to obtain the optimum value of lipase activity produced by the cultures of *Pseudomonas aeruginosa* using POME as the substrate through the submerged fermentation method and to obtain the dry extract of lipase. In the study, the one factor at a time (OFAT) method was applied, which allowed observation of the effect of inoculum and additional nutrient concentrations, such as Ca²⁺ ion, olive oil, peptone and Tween 80, on the activity of lipase. These factors were investigated in shake flask fermentation at 30°C over 96 hours. The activity unit of lipase was determined by the titrimetric reaction of olive oil hydrolysis using crude lipase. The optimum value of the lipase activity unit (1.327 U/mL) was gained when 3% (v/v) of inoculum, 4 mM of Ca²⁺ ion, 0.4% (v/v) of olive oil, 0.9% (m/v) of peptone, and 0.9% of Tween 80 were added into the medium. Crude lipase was then dried using a spray dryer. Subsequently, 15.643 g of dry extract lipase was obtained from 500 mL of cell free supernatant. In further research, the lipase activity assay would be better achieved using the p-nitrophenyl palmitate hydrolysis method and examined by a spectrophotometer.

Keywords: Activity unit; Lipase; Palm oil mill effluent; Submerged fermentation

1. INTRODUCTION

Lipase is an enzyme which catalyzes the hydrolysis reaction of triglycerides into fatty acids and several types of glycerol, including di-acylglycerol and mono-acylglycerol (Mahadik et al., 2004). Recently, lipase has attracted significant interest for commercial production, as it has several advantages, including the fact that no cofactor is required, it is active in the interfaces of organic compounds, and facilitates a wide range of substrates (Hernández-Rodríguez et al., 2009). Therefore, lipase has many practical applications in industry, including as a biocatalyst for biodiesel synthesis, in bio-detergents, fine chemicals, waste water treatment, food additives, pharmaceuticals, leather processing and biomedical assays (Salihu et al., 2012).

*Corresponding author's email: heri.hermansyah@ui.ac.id, Tel: +62-8129-3196-666, Fax: +62-21-7863-515
Permalink/DOI: <https://doi.org/10.14716/ijtech.v9i2.1511>

At present, the utilization of extracellular enzymatic activity in several microorganisms has become a preferred approach because of its potential as a biological source of industrially economic enzymes (Abd-Elhalem et al., 2015). There are a number of lipases produced by microorganisms, such as fungi including *Candida*, *Geotrichum* and *Rhizopus*, and bacteria including *Bacillus*, *Pseudomonas*, *Burkholderia*, *Staphylococcus* and *Streptomyces* (Treichel et al., 2010). Among these strains, the *Pseudomonas* lipase is the most important, since it has better stability, selectivity, and a wide range of substrate specificity (Bose & Keharia, 2013). Furthermore, lipase from *Pseudomonas aeruginosa* has been extensively manufactured and used in organosynthetic reactions (Karadzic et al., 2006).

Previous lipase production from *Pseudomonas aeruginosa* has been studied using *Pseudomonas aeruginosa san-ai* (Karadzic et al., 2006), *Pseudomonas aeruginosa* AAU2 (Bose & Keharia, 2013), and *Pseudomonas aeruginosa* LX1 (Ji et al., 2010). Lipase from *Pseudomonas aeruginosa san-ai* has been isolated and purified from the medium containing mineral cutting oil with pH 10, obtaining excellent properties of lipase, including stability and activity in organic solvents (Karadzic et al., 2006). Besides, lipase from *Pseudomonas aeruginosa* AAU2 has also been produced by the submerged fermentation method using *Jatropha SeedCake* (JSC) as the carbon source, obtaining solvent tolerant lipase with a 11.4-fold higher enzyme yield in the optimum condition (Bose & Keharia, 2013). Another organic solvent tolerant lipase has also been produced from *Pseudomonas aeruginosa* LX1 (Ji et al., 2010). From its activity in transesterification reactions, lipase from *Pseudomonas aeruginosa* LX1 is considered to have potential for biodiesel production.

However, in the production of microbial lipase, production costs are a problem due to the high cost of the substrates. In order to lower these costs, agro-industrial waste can be used as the fermentation substrates. The utilization of agro-industrial waste would be a solution to the problem of disposal (Bose & Keharia, 2013). Palm Oil Mill Effluent (POME) is an agro-industrial waste product with high availability in Indonesia. Organic content, such as carbohydrates, protein, nitrogenous compounds, lipids and minerals, are present in POME (Wu et al., 2009). These contents are suitable for microbial growth and lipase production. Since POME is available in liquid form, application of submerged fermentation is a suitable method. The utilization of POME as a medium has been made by Salihu using *Candida cylindracea* with the submerged fermentation method, giving satisfactory results (Salihu et al., 2011). In addition, POME was also used as the basal medium by Wu et al. (2006) for protease production from wild type *Aspergillus niger* with the submerged fermentation method.

Submerged fermentation is one of the fermentation methods that have been used by industry for example in enzyme production, due to the better control of the parameters that affect the yield of the product (Hansen et al., 2015); temperature, pH and agitation are well-established for scaling the processes for industrial production capacity (Hansen et al., 2015). Lipase produced from submerged fermentation can be dried to obtain dry extract lipase. In a previous study, dry extract lipase was obtained from submerged fermentation by Suci et al. (2018) using *Bacillus subtilis* as the source and waste cooking oil as the basal medium. Dry extract lipase is more durable than liquid lipase and can be stored for a long time (Utami et al., 2017).

In this study, cultures of *Pseudomonas aeruginosa* were grown in POME to produce lipase using the submerged fermentation method, and the cell free supernatant was then dried to obtain dry extract lipase. In order to improve lipase production, the one factor at a time (OFAT) method was applied to investigate the influencing factors in lipase production. OFAT is a method in which the experimental factors are varied one at a time, with the remaining factors held constant (Bose & Keharia, 2013).

2. METHODOLOGY

2.1. Sample Collection

POME was collected in clean containers from a palm oil company at Bengkulu, Indonesia, and then stored at 4°C.

2.2. Inoculum Preparation and Microbial Culture

Pseudomonas aeruginosa B2290 was purchased from Indonesian Culture Collection (InaCC). The strain was then grown on Nutrient Agar (NA) plates at 30–35°C for 1 day. The isolate was maintained and preserved at 4°C. Preparation of the inoculum was made by suspending *Pseudomonas aeruginosa* from nutrient agar in 50 ml of sterile Luria Bertani broth using a sterile inoculating loop, and incubating it using a water shaker bath at 170 rpm and 30°C for 24 hours. The mixture was then used as the inoculum in further experiments.

2.3. Profile of Lipase Production

Inoculum of *Pseudomonas aeruginosa* was inoculated to an Erlenmeyer flask containing 100 ml of sterilized POME as the basal medium, and other nutrients including olive oil (0.2%, v/v), peptone (0.5%, w/v), CaCl₂·2H₂O (10mM) and Tween 80 (0.7%, v/v). The mixture was then incubated in a water shaker bath at 30°C. 5 ml aliquots were taken after 0, 12, 24, 36, 48, 60, 72, 84, 96 and 108 h of incubation in order to analyze the bacterial growth using a spectrophotometer (660 nm) and the lipase activity using lipase activity assay.

2.4. Optimization of the Medium for Lipase Production

The optimization of the medium was made using the one factor at a time (OFAT) method. In this way, the experimental factors were varied one at a time, while the other factors were kept constant. The medium for the lipase production was prepared using sterilized POME as the basal medium, which contained olive oil (0.1–0.2%, v/v), peptone (0.3–1.1%, w/v), CaCl₂·2H₂O (4.0–12.0 mM) and Tween 80 (0.3–1.1%, v/v). The inoculum (1.0–5.0%, v/v) was then added to the medium and incubated using a water shaker bath at 170 rpm and 30°C for 96 hours. After 96 hours of incubation, the culture was then centrifuged at room temperature, at 4000 rpm for 45 minutes, to obtain the cell-free supernatant. This was then filtered and assayed for the lipase activity calculation.

2.5. Lipase Activity Assay

The lipase activity was assayed by alkali titration using olive oil as the substrate according to work done by Pinheiro et al (2008) with several modifications in the titration method. Olive oil (10% m/v) was prepared and emulsified with PVA (5% m/v) in a 50 mM sodium phosphate buffer with a pH of 7.5. A crude enzyme sample of 2 mL was added into 18 mL of the emulsion and then incubated in a shaker for 15 minutes at 37°C and 150 rpm. After the reaction was stopped, 20 mL of 95% ethanol solution (1:1 v/v) was added to extract the fatty acids. Thereafter, three drops of 1% phenolphthalein were added as the titration indicator. Titration with 0.05 M NaOH then occurred, until the colour of the sample solution turned light violet, in order to calculate the amount of fatty acids liberated. The blanks for titration were run in similar steps, but the sample was added after the addition of ethanol. The lipase activity assay was performed in duplicate.

A unit activity of lipase was defined as the amount of enzyme which liberated 1 µmol of fatty acids per minute under the assay conditions. The protein concentration was estimated by following the Lowry method with BSA (fraction V) as the standard.

2.6. Production of Dry Extract Lipase

Scale-up production was conducted in a 30-L bioreactor with two six-flat-blade impellers. The bioreactor was filled with 20-L of POME as the basal medium, containing the optimum concentrations of olive oil, peptone, CaCl₂·2H₂O and Tween 80 obtained from the OFAT

method. The reactor was sterilized in situ and inoculated with the optimum concentration of prepared *Pseudomonas aeruginosa* inoculum, as described above. The fermentation was run at 30°C for 96 hours. The fermentation temperature was maintained using a digital control system attached to the bioreactor.

The aliquots were taken after 96 hours and then centrifuged at room temperature, at 4000 rpm for 45 minutes. The cell-free supernatant was filtered and assayed for lipase activity. It was then collected and dried with a spray dryer at Center for Postharvest Research and Development (*Balai Besar Penelitian dan Pengembangan Pascapanen*) Bogor, with an inlet temperature of 150°C and outlet temperature of 80°C. Before being dried, skimmed milk powder (12%, w/v) was added to the cell-free supernatant. The dry extract lipase was then assayed for the lipase activity calculation.

3. RESULTS AND DISCUSSION

3.1. Profile of Lipase Production

In this experiment, growth of *Pseudomonas aeruginosa* and lipase activity was observed (Figure 1). The culture grew rapidly up until 36 hours of fermentation. After 36 hours, the culture started to grow more slowly and then continue to become stationary. After 96 hours the culture growth decreased.

The cells grew rapidly at the beginning because the nutrients in the medium were still at a high level. The cultures then reached the stationary phase because the nutrients in the medium were limited, which caused cell growth restriction (Yates & Smotzer, 2007). After 96 hours above, culture growth decreased and reached the death phase because the environment no longer supported any growth (Yates & Smotzer, 2007), such as the lack of nutrients and oxygen in the medium. In submerged fermentation, consumption of oxygen is proportional to the conversion of the substrate into the product (Gullo et al., 2014).

The level of lipase activity fluctuated and reached a maximum value of 0.618 U/ml after 96 hours of incubation, when the culture growth reached the stationary phase. This fluctuation occurred because the lipase produced by the cultures had been used to degrade lipids into free fatty acids, as the lipids could not passively cross cell membranes as the cell cultures consumed them (Papagora et al., 2013). After 108 hours, the lipase activity decreased because cell growth had reached the death phase.

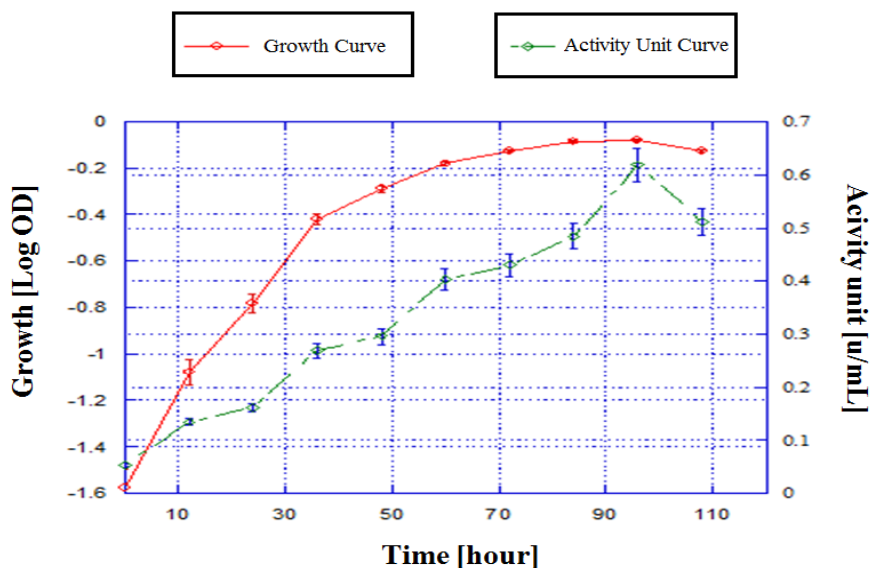


Figure 1 *Pseudomonas aeruginosa* growth (log OD) and lipase activity (u/mL) in POME

This pattern also occurred in *Pseudomonas aeruginosa* AAU2 cultured in Jatropha Seed Cake (JSC) with the submerged fermentation method (Bose & Keharia, 2013). Lipase production in JSC started after 6 hours of inoculation with a maximum lipase activity value (0.433 U/ml) at the stationary phase, which occurred after 60 hours of inoculation, after which production decreased (Bose & Keharia, 2013).

3.2. Optimization of Process Parameters for Lipase Production

Determination of the fermentation medium formulation will affect the profile of production, rate and yield of the lipase produced. The composition of the medium should be provided with inducer, carbon, nitrogen and trace elements (Salihu et al., 2011). Different concentrations of certain elements will have different effects. Therefore, in this research different concentrations of inoculum, Ca^{2+} ion, olive oil, peptone and Tween 80 were used to obtain the highest value of the lipase activity unit.

3.2.1. Effect of different inoculum concentration

To investigate the influence of inoculum concentration, 1.0–5.0 % (v/v) of inoculum was tested. The highest activity of 0.813 U/ml was obtained from 3.0% (v/v) of inoculum concentration (Figure 2). The limited supply of nutrients in the medium could cause the inoculum to compete, meaning enzyme production will be affected (Salihu et al., 2011). In a similar way, a low concentration of inoculum could reduce enzyme secretion because of the small number of cells (Reddy et al., 2008).

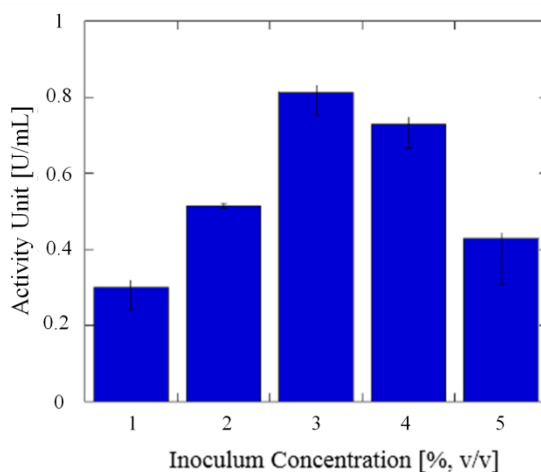


Figure 2 Effect of different concentrations of inoculum on lipase activity unit

The concentration of inoculum used for each production might differ, depending on the type of bacteria and fermentation medium. In the production of *Pseudomonas aeruginosa* FW_SH-1 lipase for 3 days with yeast extract as the medium, the inoculum used was 5% (v/v). The effect of inoculum concentration has also been investigated in lipase production from *Candida cylindracea* using POME as the basal medium. The maximum activity of 6.2 U/ml was obtained at about 2% (v/v) of inoculum concentration (Salihu et al., 2011).

3.2.2. Effect of different Ca^{2+} ion concentration

Ca^{2+} ions from $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ were also added at a concentration of 4 mM–6 mM to investigate their influence. The highest activity of 0.813 U/ml was obtained from 6 mM $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (Figure 3). Calcium is an essential element in cell signalling and is required as a cofactor for many enzymes (Guragain et al., 2016). However, a high concentration of calcium ions could be toxic to the cells and decrease growth (Guragain et al., 2016). Other research conducted by Vasiee et al. (2016) reported that optimal enzyme production conditions from *Bacillus cereus*

incubated at 37°C for 48 h were achieved with 24.23 mM MgCl₂ together with a coriander seed extract (CSE)/yeast extract ratio of 16.9 (m/m) and a concentration of 2.37 g/L of olive oil.

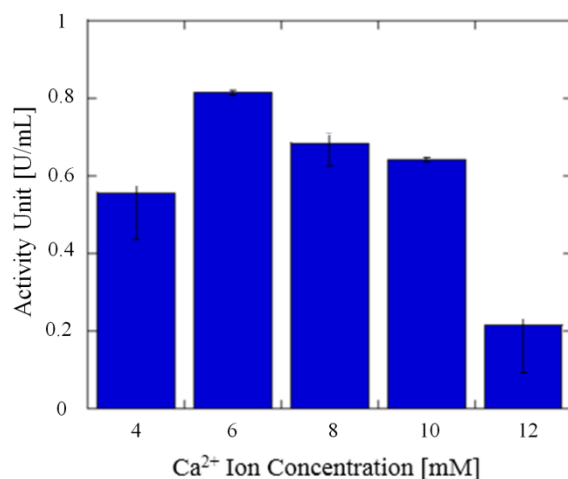


Figure 3 Effect of different concentrations of Ca²⁺ ions on lipase activity unit

3.2.3. Effect of different olive oil concentrations

The biosynthesis of enzymes may be affected by the varying sources and the different concentrations of carbon and nitrogen (Sharma et al., 2001). In this study, concentrations of 0.2–0.6 % (v/v) olive oil were investigated. *Pseudomonas aeruginosa* was shown to have the best lipase activity of 0.899 U/ml at 0.4 % (v/v) olive oil (Figure 4). Carbohydrates were mainly used as the carbon source in the carbon metabolism of microorganisms, but the microorganisms can utilize lipids as the carbon source in the presence of lipases (Papagora et al., 2013).

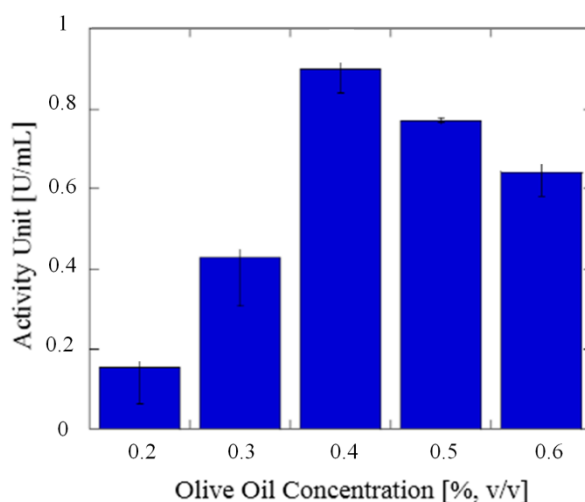


Figure 4 Effect of different concentrations of olive oil on lipase activity unit

In many other researches, lipase production depends on the presence of an inducer, and oil commonly acts as a good lipase inducer (Sharma et al., 2001). Lipase produced in this medium is hydrolyzed, and subsequently glycerol and fatty acids are released. The glycerol is consumed by microorganisms as the carbon source and the free fatty acids are consumed as inducers to form lipase (Goncalves et al., 2014). The addition of olive oil to the fermentation medium gave a positive result due to the oleic acid composition in the oil. (Dalmau et al., 2001). However, a high concentration of olive oil might decrease the oxygen transfer in the medium and have a negative effect on lipase production (Goncalves et al., 2014). Based on research conducted by

Papagora et al. (2013), the concentration of olive oil which influenced lipase production over 72 hours of fermentation time at 30°C was 19 g/L or 1.9% (m/v), with an activity value of 7.44 ± 0.28 U/mL.

According to a study of lipase enzyme production from *Candida cylindracea* conducted by Salihi et al (2011), the highest lipase activity unit with a value of 5.92–5.96 U/mL was reached when 0.2% (m/v) olive oil was added to the POME medium for 6-day fermentation.

3.2.4. Effect of different peptone concentrations

In order to investigate the influence of peptone, concentrations of 0.3–1.1 % (w/v) were tested. The highest activity of 1.327 U/ml was obtained at 0.9% (v/v) of peptone (Figure 5). The addition of peptone was found to have a positive effect on cell growth, as it is an important nutrient for growth (Rajendran & Thangavelu, 2012). However, a high concentration of peptone was found to have a negative effect on lipase production as an increase in protease production might cause proteolysis within it (Rajendran & Thangavelu, 2012). The protease production occurred along with the bacterial growth and accumulated in the stationary phase (Wu et al., 2006), as in lipase production.

Based on research by Cadirci & Yasa (2010) in their study of *Pseudomonas fluorescens* P21 lipase production in the nutrient medium for 12 hours, the addition of 0.3% (m/v) peptone gave the highest activity of 15 U/L. This value was higher than those of tripton and casein.

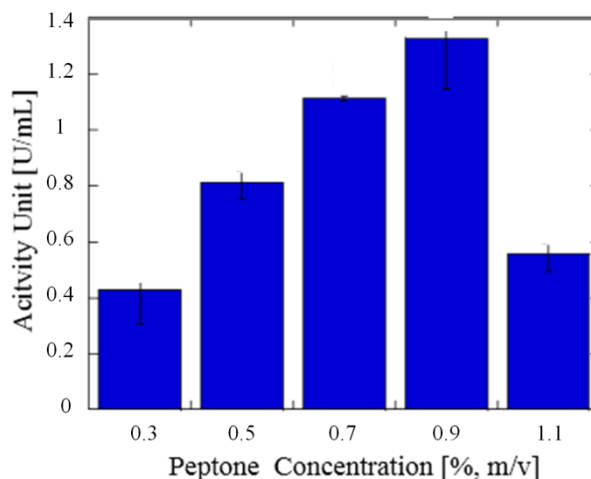


Figure 5 Effect of different concentrations of peptone on lipase activity unit

3.2.5. Effect of different tween 80 concentration

The presence of surfactant was found to have a positive impact on increasing the yield (Sharma et al., 2001). In this study, concentrations of 0.3–1.1 % (v/v) Tween 80 were investigated. *Pseudomonas aeruginosa* was shown to have the best lipase activity of 1.588 U/ml at 0.9% (v/v) of Tween 80 (Figure 6). Tween 80 is a surfactant which could induce the production of lipase as it enhances cell permeability and facilitates lipase transport through the cell membrane (Dalmau et al., 2001). In addition, it contains oleic acid, which is an intrinsic carbon source for bacterial growth (Li et al., 2004). It has a positive effect on lipase activity, but at higher concentrations it might inhibit it.

According to research by Bose & Keharia (2013) on the production of *Pseudomonas aeruginosa* AAU2 lipase in jatropha seed cake substrate for 60 hours, the addition of 0.05% (v/v) Tween 80 yielded the highest value of the lipase activity unit, at 4.0–5.0 U/mL. This value is higher than the addition of Triton X, Tween 20, Tween 40 or Tween 60.

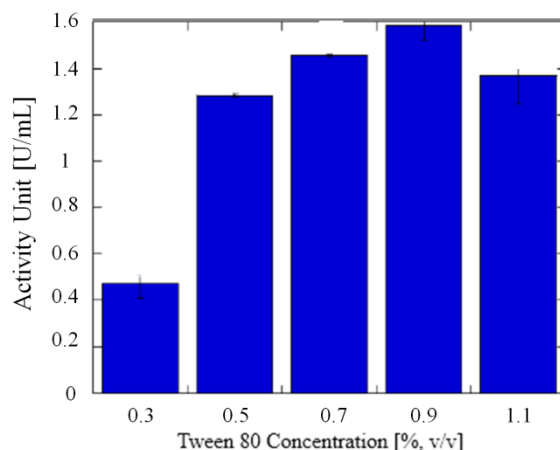


Figure 6 Effect of different concentrations of Tween 80 on lipase activity unit

3.3. Dry Extract Lipase Production

Scale-up fermentation was conducted in submerged fermentation for 96 hours using POME as the basal medium and with the addition of 3% (v/v) of *Pseudomonas aeruginosa* inoculum, 0.9% (m/v) of peptone, 0.4% (v/v) of olive oil, 4 mM of Ca^{2+} ions and 0.9% of Tween 80. These compositions were obtained from the OFAT method. The aliquots from fermentation were centrifuged to obtain cell free supernatant. The supernatant was then dried with a spray dryer. From 500 mL of cell free supernatant, 15.643 g of dry extract lipase was obtained. This had a lipase activity unit of 28.5 U/g and specific lipase activity of 2.417 U/g proteins. These results were higher than those from the experiment conducted by Suci et al (2018), with a lipase activity unit of 28 U/g solid and specific lipase activity of 1.44 U/g proteins. The dry extract lipase obtained by Suci was from *Bacillus subtilis* inoculated in submerged fermentation using waste cooking oil as the basal medium for 84 hours. The differing results could be due to the differences in bacteria, medium and fermentation time.

A spray drying process was needed to remove the water content in the cell free supernatant to obtain stable lipase; this process was more efficient and economical compared to the freeze drying process (Utami et al., 2017). In addition, commercial lipases are usually sold in dry form to simplify distribution and ease usage.

4. CONCLUSION

The activity unit of lipase from *Pseudomonas aeruginosa* using POME as the basal medium reached a maximum value of 1.327 U/mL from the crude form. The optimum values of the lipase activity unit were gained when 3% (v/v) of inoculum, 0.9% (m/v) of peptone, 0.4% (v/v) of olive oil, 4 mM of Ca^{2+} ions, and 0.9% of Tween 80 were added into the medium and fermented for 96 hours. The dry extracellular lipase obtained had an activity unit of 28.5 U/g, with a specific activity of 2.417 U/g proteins. The utilization of POME as an alternative medium would result in a considerable reduction in the cost of lipase production. The values of the lipase activities would achieve better results if the experiment used the hydrolysis reaction of p-nitrophenyl palmitate and examination by spectrophotometer.

5. ACKNOWLEDGEMENT

The authors are grateful for the research support provided by Ministry of Research, Technology, and Higher Education through International Research Collaboration Grant Program and for the publication support provided by the United States Agency for International Development (USAID) through the Sustainable Higher Education Research

Alliance (SHERA) Program for Universitas Indonesia's Scientific Modeling, Application, Research and Training for City-centered Innovation and Technology (SMART CITY) Project, Grant #AID-497-A-1600004, Sub Grant #IIE-00000078-UI-1.

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