Utilization of Waste Frying Oil as A Source of Carbon in The Production of Biosurfactant using *Exiguobacterium profundum*

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**ABSTRACT**

Biosurfactant is a secondary metabolite produced by microorganisms that can be used as an alternative to environmentally friendly surfactants. *Exiguobacterium profundum* is one of the biosurfactants producers that potentially to be used in the pharmaceutical field. The use of waste frying oil as a carbon source can be used as a solution in overcoming the high cost of producing biosurfactants. The purpose of this study was to obtain optimum conditions in the production of biosurfactants by utilizing waste frying oil as a carbon source. In this study, variations in the optimized production conditions included the concentration of waste frying oil, labeled 2%, 3%, 4%, and 5%, and the medium pH at 6, 7, and 8. The study was using Mineral Salt Medium as production medium, the amount of inoculum concentration was 10% v/v, agitation speed 160 rpm, and incubation at room temperature. The optimum conditions for biosurfactant production were determined based on the best emulsification index. The biosurfactant extraction was carried out using a combination of chloroform and methanol (2:1) solvents. The best concentrations of waste frying oil for *Exiguobacterium profundum* was 5%, and the best medium pH was 7. Biosurfactants produced from *Exiguobacterium profundum* amounted to 8.2 g/L with an emulsification index 63.2%.

Keywords: Mineral salt medium, emulsification index, pH

**Introduction**

Biosurfactants are surfactants obtained from microorganisms that have acted as a surface tension reducer. The use of surfactants produced by these microorganisms has more advantages than synthetic surfactants because they are non-toxic, effective, more easily degraded, and more environmentally friendly (Makkar et al., 2011). In the pharmaceutical field, biosurfactants can be used as foaming agents, emulsion stabilizers, antibacterial, antiviral and anticancer agents (Fakruddin, 2012). Research has been carried out to screen bacteria that produce biosurfactants, one of which is *Exiguobacterium profundum* with the type of biosurfactant being from the lipopeptide group (Setiani et al., 2019). Biosurfactants from these bacteria can also be used as antibacterial agents (Setiani et al., 2019). It is necessary to research to optimize the production of biosurfactants from these bacteria.

The use of biosurfactants has not been able to compete with synthetic surfactants. This is due to limitations in terms of the costs required for raw materials and the capacity of the manufacturing process (Banat et al., 2010). One of the efforts that can be done in reducing the cost of the biosurfactant production process is by selecting an affordable substrate (Nitschke et al., 2004). The best carbon source at a low price that can be used for biosurfactant production is waste oil that is no longer used. Research by Ghazal et al., (2017) stated that the use of 2% waste frying oil as a carbon source is able to produce biosurfactant using *Exiguobacterium profundum*.
oil as a carbon source produced biosurfactants with the highest emulsification index, which was 68% when compared to waste diesel oil and kerosene which respectively produced biosurfactants with an emulsification index of 13% and 22% on *Bacillus* sp.

The type, quality, and quantity of biosurfactants produced are influenced by the carbon source in the media and production conditions such as the pH of the media (Fakruddin, 2012). Optimization of biosurfactant production is the most important thing to maximize the production of biosurfactants produced by microorganisms. Therefore, this study aims to obtain the optimum conditions for the production of biosurfactants from *Exiguobacterium profundum* bacteria using waste frying oil as a carbon source.

**Material and Methods**

**Organoleptic identification**

The test was carried out organoleptically. 2 mL of waste frying oil was taken and placed on a clean and dry watch glass. The oil was observed for its color and smell (Badan Standarisasi Nasional, 2013).

**Determination of waste frying oil fatty acid levels**

10 grams of the waste frying oil was measured than placed into a 250 mL Erlenmeyer. 50 mL of warm 95% ethanol was added followed by 5 drops of phenolphthalein indicator (PP), titrated with 0.1 N NaOH until the color changed to pink (Badan Standarisasi Nasional, 2013).

\[
\% \text{ Free Fatty Acid} = \frac{V_{NaOH} \times N_{NaOH} \times MW_{Oil}}{W_{oil} \times 1000} \times 100
\]

**Production of Mineral Salt Medium (MSM)**

In 2 L Erlenmeyer, filled with 1 L of Mineral Salt Medium (MSM) with the composition (g/L): NaNO\(_3\) (7.0 grams), KH\(_2\)PO\(_4\) (0.5 grams), K\(_2\)HPO\(_4\) (1.0 grams), KCl (0.1 grams), MgSO\(_4\)\(_7\)H\(_2\)O (0.5 grams), CaCl\(_2\)\(_2\)H\(_2\)O (0.01 grams), FeSO\(_4\)\(_7\)H\(_2\)O (0.01 grams), yeast extract (0.1 grams) and distilled water up to 1 L. The medium was then sterilized at 121°C for 15 minutes (Hisham et al., 2019).

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**Bacterial growth curve**

Bacterial growth curves were made by inoculating 1-2 oses of bacteria into 100 mL of MSM medium in a 250 mL Erlenmeyer. The culture was incubated using a shaker at 160 rpm at room temperature. Observations were made on the value of Optical Density (OD) using a UV-Vis spectrophotometer at a wavelength of 600 nm every 24 hours until the bacteria entered the death phase. The absorbance value obtained is used to create a bacterial growth curve (Hamida, 2010).

**Optimization of biosurfactant production**

**Carbon Concentration Variations (Waste Frying Oil)**

A total of 25 mL of MSM media in a 50 mL falcon tube was added with waste frying oil with various concentrations of 2, 3, 4, and 5% (v/v). Then the media was adjusted in pH with 0.1 N NaOH to pH 7. After that, each bacterial suspension was inoculated into the media as much as 2.5 ml (10% v/v) then incubated with a shaker at 160 rpm for 6 days at room temperature. Cultures of samples were taken every 24 hours. The product was separated from the cells by centrifugation at 4,000 rpm for 30 minutes to obtain the supernatant.
pH Variations

A total of 25 mL of MSM media in a 50 mL falcon tube was added with waste frying oil with the best concentration result, then the media was adjusted for pH with 0.1 N NaOH or 0.1 N HCl to pH 6, 7, and 8. Each bacterial suspension was inoculated into the media as much as 2.5 ml (10% v/v) then incubated with a shaker at 160 rpm for 6 days at room temperature. Cultures of samples were taken every 24 hours. The product was separated from the cells by centrifugation at 4,000 rpm for 30 minutes to obtain the supernatant.

Emulsification index measurement (IE24)

The emulsification index was carried out on each supernatant, measured by inserting 2 mL of the supernatant and 2 mL of palm oil in a test tube. This mixture was stirred using a vortex mixer for 1 minute and then left for 24 hours at room temperature. After 24 hours the height of the emulsion layer formed was measured.

Emulsification Index (EI24) = \left(\frac{\text{emulsification layer height}}{\text{total solvent height}}\right) \times 100

Biosurfactant productions and extractions

The production of biosurfactants was carried out using the concentration of waste frying oil and pH which gave the best emulsification index. The production medium was made in a volume of 100 mL. Bacterial culture was inoculated into production media as much as 10% (v/v) and incubated using a shaker at 160 rpm and harvested on day 3. The product was separated from the cells by centrifugation at 4000 rpm for 30 minutes to obtain the supernatant.

The obtained supernatant was then acidified with 2 N HCl to pH 2 and allowed to stand for 12 hours at 4°C. The supernatant was then extracted with an equal volume of chloroform:methanol (2:1) solvent mixture using the stirring method for 2 hours. The solvent was evaporated using a water bath to obtain a crude biosurfactant. Crude biosurfactants were dried in an oven at 60°C, then measured to a constant weight (Hisham et al., 2019; Hamida, 2010).

Results and Discussion

Waste frying oil analysis

The results of the analysis of the color and smell of waste frying oil can be seen in table 1 and figure 1.

From the test results, it was found that waste frying oil had a dark yellow color and a slightly rancid smell. The color of used cooking oil is darker when compared to fresh palm oil, this is because in used cooking oil an oxidation reaction has occurred between oxygen and the double bonds contained in the oil (Suroso, 2013).

Determination of free fatty acid levels in waste frying oil was carried out using an alkalimetric titration method using NaOH reagent, where the principle of this method is the occurrence of a neutralization reaction due to a reaction between hydrogen ions from acids derived from oil and hydroxide ions from bases that used in the titrant. The addition of warm 96% ethanol in the determination of free fatty acid levels aims to dissolve fat or oil in the sample so that it can react with alkaline bases and so that the reaction takes place more quickly (Suroso, 2013). From the research results, it is known that the free fatty acid content in the waste frying oil samples is 0.31%. The formation of free fatty acids in waste frying oil is caused by the hydrolysis process that occurs during the frying process caused by heating at high temperatures (Kalapathy & Proctor, 2000). The presence of free fatty acids in waste frying oil can be utilized by bacteria as a carbon source to produce biosurfactants (Rengga et al., 2016).

Bacteria growth curve

The growth pattern of *Exiguobacterium profundum* which experienced a short lag phase that only took less than 24 hours. The length or shortness of the lag phase is largely determined by the
number of cells inoculated, the physiological and morphological conditions of the bacteria, and the appropriate cultivation medium (Setyati et al., 2015). Furthermore, the bacteria began to enter the exponential phase which was marked by a significant growth until the 48th hour. Furthermore, at the 48th hour to the 168th hour, the bacteria entered a relatively constant growth phase, namely entering the stationary phase. Biosurfactants are secondary metabolites so the production of biosurfactants will occur in the stationary phase (Gozan et al., 2014). Based on the results of the bacterial growth curve in MSM media, the length of time for biosurfactant production was carried out for 6 days, during which time the bacteria were still in the stationary phase and had not yet entered the death phase.

**Optimization of Biosurfactant Production**

**Carbon Concentration Variations (Waste Frying Oil)**

Waste frying oil can be easily used by bacteria in the production of biosurfactants because the hydrocarbon chains in waste frying oil are more dominantly composed of long branched but not double-carbon chains (Prastikasari, 2000). Biosurfactants are produced by microorganisms extracellularly to break down hydrophobic substrates such as waste frying oil so that the waste frying oil will be emulsified and more easily enter the bacterial cell membrane. The concentrations of waste frying oil in the production of biosurfactants were 2%, 3%, 4%, and 5% with a pH of 7. The fermentation conditions used were room temperature and agitation speed of 160 rpm. The fermentation process was carried out for 6 days according to the results of the growth curve that both bacteria were still in the stationary phase after the 6th day of fermentation. The culture was harvested every day starting from day 0 to day 6 of the fermentation process, then the biosurfactant activity was observed through a 24-hour emulsification index (IE24) test. Emulsification test is used to determine the ability of biosurfactants to emulsify liquids of different polarities. Emulsification test results are expressed as emulsification index. The emulsification index value is said to be good if the value is more than 50% (Purnomohadi, 2010).

In Figure 2 it can be seen that the four concentrations of waste frying oil produced varied IE24 values. *E. profundum* bacteria produced a biosurfactant with the best IE24 at 5% waste frying oil concentration on day 3, which was 73.2%. The results obtained were better than the production of biosurfactants from *Exiguobacterium sp.* with a carbon source of 2% coconut oil which produces a biosurfactant with an IE24 value of 35.71% (Tambekar et al., 2013). The concentration of waste frying oil that produces the best IE24 value is then used to optimize the production of biosurfactants with variations in pH.

**pH Variations**

Optimization of biosurfactant production with pH variation was carried out at 5% cooking oil concentration. The selection of this concentration is based on the results of the best IE24 value in the previous concentration determination. Bacterial growth media were treated with pH under acidic, neutral, and alkaline conditions. The pH treatments tested were pH 6, 7, and 8.

In Figure 3 it can be seen that the variation of pH used produces various IE24 values. The best IE24 value was obtained on the use of media with a pH of 7 on the 3rd day of 73.2%. Research conducted by Crapart et al. (2007) stated that the bacteria *Exiguobacterium sp.* can grow optimally at pH 7. pH is one of the important factors in bacterial growth. If the pH conditions are not suitable, then the growth of bacteria will not be optimal (Ferdaus et al., 2008). The pH of the media that produces the best IE24 value is then used in the biosurfactant production and extraction process.

**Biosurfactant productions and extractions**

Biosurfactant production is carried out using the best conditions resulting from the optimization process. The concentration of used cooking oil was 5% with a pH of 7. Bacteria were incubated using a shaker at 160 rpm and the culture was harvested after 3 days of incubation. The
selection of harvest time is based on optimization results where on the 3rd day with the conditions used, it produces biosurfactants with the best emulsification index values.

The harvested culture was then centrifuged at 4,000 rpm for 30 minutes to separate the product from bacterial cells. The supernatant portion resulting from the centrifugation process was then taken for the extraction process. Extraction was carried out using a solvent extraction method. The bacterial cell-free supernatant was acidified using 2 N HCl until it reached pH 2, then stored at 4°C for overnight (±12 hours). The purpose of this acidification process is to precipitate the dissolved biosurfactant in the supernatant (Hisham et al., 2019). After being allowed to settle overnight, the supernatant was then extracted using a mixture of chloroform:methanol (2:1) solvents in a ratio of 1:1 for 2 hours using the stirring method. Extraction using a combination of chloroform and methanol solvents is a method that is easy, affordable, and can be used to extract low molecular weight biosurfactants such as lipopeptides (Hisham et al., 2019). The purpose of using a combination of chloroform and methanol solvents in the extraction process is to facilitate polarity adjustment between the solvent as an extracting agent and the biosurfactant to be extracted. The hydrophilic molecules of the biosurfactants will be bound by methanol (polar) while the hydrophobic molecules of the biosurfactants will be bound by chloroform (non-polar) (Kuyukina et al., 2001).

The solvent was then evaporated using a water bath at a temperature of 60°C until a thick yellow liquid remained. The residue was then dried using an oven to obtain a constant weight. From the extraction results obtained crude biosurfactant with a yellow powder appearance (Figure 4). The yield value obtained is 8.2 g/L. The yield value produced in this study is greater than the research conducted by Zhu et al. (2007) in the production of biosurfactants from the Pseudomonas aeruginosa with a carbon source of 4% waste frying oil which produces a yield of 5.45 g/L and research conducted by Oliveira and Gracia-Cruz (2013) where the production of biosurfactant from Bacillus pumilus bacteria with 5% cooking oil concentration resulted in a yield of 5.21 g/L.

The bacteria-free supernatant and crude biosurfactant produced were then tested for their emulsification index. The concentration of crude biosurfactant used in the emulsification index test was 5% and dissolved in distilled water. The emulsification index test was carried out using a hydrocarbon in the form of palm oil. As a positive control used SLS with a concentration of 1% and as a negative control used MSM media.

From the emulsification index test, the IE24 value in the supernatant from Exiguobacterium profundum was 63.2%. MSM media as a negative control had an IE24 value of 6.25%. The results of the IE24 value on the supernatant produced by bacteria showed a value that was not much different from the positive control used, namely 1% SLS which resulted in an IE24 value of 66.7%.

Table 1 Results of organoleptic identification of waste frying oil

<table>
<thead>
<tr>
<th>Test</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Color</td>
<td>Dark yellow</td>
</tr>
<tr>
<td>Smell</td>
<td>Distinctive smell of used oil (slightly rancid)</td>
</tr>
</tbody>
</table>

Figure 1. Comparison of the oil used color (a) waste frying oil.; and (b) palm oil
Figure 2. Graph of Emulsification Index of Waste Frying Oil Concentration Variations

Figure 3. Graph of Emulsification Index of Waste Frying Oil pH Variations

Figure 4. Biosurfactant crude
Conclusion

The concentration of used cooking oil as the optimum carbon source in the production of biosurfactants from the bacterium Exiguobacterium profundum was 5% with a pH of 7. The emulsification index value of the biosurfactant was 63.2%.

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References


