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# Co-Utilization of Molasse and Glycerol as Carbon Sources on the Production of Biosurfactant by Isolate Bacteria LII61

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## ABSTRACT

Biosurfactants are amphiphilic compounds which has the ability to reduce surface and interfacial tension of the liquids. It can be produced by various types of microorganisms such as bacteria, mold, and yeast. Biosurfactants are not only produced from inexpensive and renewable substrates, but also industrial wastes. Molasse and glycerol were combined as carbon sources for producing biosurfactant by isolate bacteria LII61. The biosurfactants were characterized by measuring the surface tension and emulsification index of supernatant, determining the critical micelle concentration (CMC) value, and analyzing stability of biosurfactant. The results showed that isolate bacteria LII61 could produce the biosurfactant from molasse and glycerol with CMC value was about 5 g/l. Biosurfactant of these isolate could reduce the surface tention of medium from 72 to 52.70 mN/m and emulsify kerosene around 71.01%. The biosurfactant showed good surface activity (50-60 mN/m) in acidic or alkaline condition and high temperature, while emulsification index showed good on acidic condition and temperatures below 70ºC.
**KEYWORDS:** biosurfactant, molasse, glycerol, surface tension

## INTRODUCTION

The oil spill issues in land or ocean are always become a concern for researcher to resolved. There is so many ways to solve this problem using chemical, physical, and biological strategies[1]. The chemical remediation strategies could be using actinide chelators, chemical immobilization, critical fluid extraction, oxidation and many more methods that using synthetic chemical compounds which is giving potentially environmental and toxicology problems [2,3]. The physical remediation strategies could be using capping, electrokinetic remediation, incineration technologies and many more methods that not easy to apply and expensive[1]. The biological remediation strategies are mostly more environmental friendly because the methods are using potential microbe or indigenous microbe itself to solved the problems which is uses the ability of microbe to produce remediation agent such as biosurfactant. Not only can be utilized for biodegradation of environment contaminated hydrocarbon agent[2], biosurfactants are usually used for agricultural[4], foods[5], cosmetics and pharmaceutical application[6].

Biosurfactants are amphiphilic compounds which has the ability to reduce surface and interfacial tension of the liquids and form microsolubilization (emulsification)[7,8] which is important in oil bioremediation. It can be produced naturally by various types of microorganisms such as bacteria, mold, and yeast [9,10,11,12]. Biosurfactant-producing bacteria are commonly found in contamninated oil and fat areas [13]. In the oil bioremediation process, surface active of biosurfactant will emulsify the oil (hydrocarbon) and subsequently decomposed by microorganisms. Biosurfactants are natural surfactants with many advantages such as biodegradablility, low toxicity, also can be applied on extreme environmental conditions [12,14,15]. Those advantages have made biosurfactants advisable to replace the synthetic surface active agents [15,16]. In the other hand, biosurfactants are useful for emulsition polymerization, emulsification, de-emulsification, foaming, phase dispersion, and wetting [10].

Based on the chemical structure, there are various types of biosurfactants such as fatty acids, glycolipids, lipopeptides, peptides, phospholipids, etc. The best known lipopeptides is surfactin[17], while glycolipids are rhamnolipids, sophorolipids and trehalolipids[9]. The type of carbon sources strongly determine the quality and quantity of bioburfactant production. It is an important limiting factor in biosurfactant production[18]. Biosurfactants can be produced using inexpensive and renewable substrates, such as glycerol [19,20]. In the other hand, industrial wastes can also be a carbon source for biosurfactant production by microorganisms [21]. Joshi et al. 2008[22] reported that molasses is a low-cost material and alternative carbon source for biosurfactant production. Many studies have reported the production of biosurfactant with substrate combinations [23,24,25], but mostly in the yeast group. In this study was focused on the ability of isolate bacteria LII61 in producing

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biosurfactant from a combination of the industrial waste (molasse) and renewable substrate (glycerol). This combination is a sound strategy to reduce the industrial waste and cost production.

## MATERIALS AND METHODS

### Microorganism

Isolate bacteria LII61 was the culture collection of Microbiology Laboratory, Department of Biology, Faculty of Science and Technology, Airlangga University, Indonesia. The isolate was collected from traditional poultry slaughterhouse in Pegirian, Surabaya, East Java, Indonesia by Fatimah et al. 2011[26]. The isolate was cultured on nutrient agar (Oxoid) slants at 4°C. Every month it was re-cultured on fresh agar slants to maintain viability.

### Inoculum preparation

The inoculum preparation of bacterial LII61 was prepared by transferring a loop of cells from a slant culture to 50 ml of nutrient broth (Oxoid). The culture was incubated at room temperature in rotary shaker (120-150 rpm) for 24 h.

### Production medium preparation

The production medium was prepared by making minerals salt medium (MSM) that contained $CaCl_2$, 0.01 g/l; $CoCl_2.6H_2O$, 0.005 g/l; $CuSO_4.5H_2O$, 0.001 g/l; $FeSO_4.7H_2O$, 0.0012 g/l; $H_3BO_3$, 0.001 g/l; $KH_2PO_4$, 10 g/l; $K_2HPO_4$, 4 g/l; $MgSO_4.7H_2O$, 0.2 g/l; $MnSO_4.H_2O$, 0.001 g/l; NaCl, 10 g/l; $NaMoO_4.2H_2O$, 0.001 g/l; $(NH_4)_2SO_4$, 3 g/l; $ZnSO_4.7H_2O$, 0.001 g/l. This solution was arrenged to pH 7.0 with adding NaOH 1 N or HCl 1 N. 470.4 ml of MSM was transferred to 1000 ml Erlenmeyer flasks and added by 4.8 ml (1%) of molasses and 4.8 ml (1%) of glycerol. The medium was sterilized with autoclave for 15 min at 121°C and 1 atm.

### Biosurfactant production process

20 ml (4%) of inoculum ($OD_{650\ nm}$ = 0.5) was added to production medium. The cultures were incubated at room temperature in rotary shaker (120-150 rpm) for 24 h, 48 h, 72 h and 96 h. The value of biomass, pH, surface tension and emulsification index ($E_{24}$) were measured every 24 h.

### Biomass analysis

Biomass was collected by culture centrifugation at 6000 rpm for 10 min and the supernatant was separated from biomass. The biomass was dried at 65°C for 24 h then determined the bacterial dry weight.

### Determination of surface tension value

Surface tension was measured by a Du Nouy's Tensiometer (Ogawa Seiki Co., Ltd) using the ring method at room temperature. 20 ml of supernatant was poured into a clean glass container (30 ml) and placed on sample table of tensiometer. The platinum ring was submerged right on the surface of supernatant and then pulled back slowly. The surface tension was calculated from the equation.

$$\gamma = \gamma_o \text{ x } \frac{\theta}{\theta o}$$

$\gamma$ is surface tension of sample, $\gamma_o$ is the standard of surface tension water (72 mN/m), $\theta$ is the value of surface tension sample on Tensiometer and $\theta o$ is the value of surface tension water on Tensiometer. All experiments were carried out in triplicates.

### Determination of emulsification index ($E_{24}$)

The emulsification index was determined as explained in Petrikov et al. 2013[27]. Sampel and hydrocarbon (kerosene) (v/v) were placed into a measuring test tube and tighly closed. Those solution was vortexed vigorously for 2 min and then kept at room temperature for 24 h. The emulsification index ($E_{24}$) was calculated by dividing the total height of the emulsion layer by the total height of the solution and multiplying by 100[28]. All experiments were carried out in triplicates.

$$E_{24} = \frac{\text{total height of the emulsion layer}}{\text{total height of the solution}} \text{x } 100\%$$

### Crude biosurfactant extraction

Crude biosurfactant was extracted by acid precipitation method[27]. The pH of supernatant was decreased to 2.0 by adding HCl 6 N and stored at 4°C overnight. Extraction was done by centrifuging the solution at 6000 rpm. The pellet was collected by separating the supernatant and measured the wet weight of crude extract of biosurfactant.

### Lipopeptides of biosurfactant extraction

Lipopeptides of biosurfactant was extracted as described in Xia et al. 2011[29]. Crude extract of biosurfactant was dilluted with double-destillated water and adjusted dichlorometane (v/v). Those mixture was vortexed vigorously for 5 min and then incubated for 30 min at room temperature. Extraction was continued by centrifuging the mixture for 10 min at 6000 rpm. The solvent layer was collected and evaporated.

**Glycolipids of biosurfactant extraction**

Glycolipids of biosurfactant was extracted as describes in Petrikov et al. 2013[27]. Crude extract of biosurfactant was dilluted with double-destillated water and adjusted a mixture of chloroform and methanol (5:2, v/v). Those mixture was vortexed vigorously for 5 min and incubated at room temperature for 30 min. Extraction was continued by centrifuging the mixture for 10 min at 6000 rpm. The solvent layer was collected and evaporated.

**Determination of critical micelle concentration (CMC)**

Determination of critical micelle concentration (CMC) was begun by making the biosurfactant solution at different concentrations. The surface tension value of each solution was measured until a constant value was reached. The CMC was determined by making the plot of surface tension value versus biosurfactant concentrations. The unit of CMC value was grams per liter of biosurfactant.

**Biosurfactant stability analysis**

Biosurfactant stability was analyzed by determining the effect of pH and temperature on biosurfactant activity. Determination of pH effect was done by making the crude biosurfactant solution at CMC point with various of pH value from 2.5 until10.5. The surface tension and emulsification index ($E_{24}$) of each solution were measured. To determine the temperature effect, the crude biosurfactant solution at CMC point were heated at 30°C until 80°C for 60 min. Then the surface tension and emulsification index ($E_{24}$) were measured. All experiments were carried out in triplicates.

## RESULTS AND DISCUSSION

**Growth and biosurfactant production**

The biomass concentration, surface tension value and emulsification index of isolate bacteria LII61 grown in MSM with 1% molasse and 1% glycerol as carbon sources, have been presented in **Figure 1**. Biosurfactants started to be produced during the exponential phase, and reached the maximum after 48 h incubation time. Beside of that, the surface tension value reduced rapidly from 72 to 44.22 mN/m on the exponential phase, and emulsification index ($E_{24}$) significantly increased to 86.80% in 48 h. This showed that the isolate used carbon sources (molasse and glycerol) to grow and produce the biosurfactants. Mulligans and Gibbs 1993[30] stated the molasses contained around 20-30% of sucroses and 10-30% of reducing sugars, so it could be utilized by microorganisms as carbon source for producing biosurfactant. The sugars in molasse and glycerol will be transformed to glucose compounds in cell metabolism of microorgasims. In glycolysis pathway the glucose was modified into glucose-6-phosphate as a raw material of hydrophylic moiety. The glycolysis product of piruvic acid was transformed to acetyl CoA and entered into anabolism cycle became fatty acid compounds a raw material of hydrophobic moiety. Both of hydrophylic and hydrophobic moieties will be merged by an intermediate enzyme[8]. Decreasing of pH medium also explained the microorganisms released organic acid compounds from metabolic activity into the medium[31]. After 48 h of incubation, constant decreasing of surface tention was obtained. This might be the biosurfactants reaching its CMC[23]. However, the biosurfactant production decreased and impacted to increase of surface tension and decrease of emulsification index. This might be due to the decreasing of carbon source and acidic condition of medium inhibited microorganism growth[19].

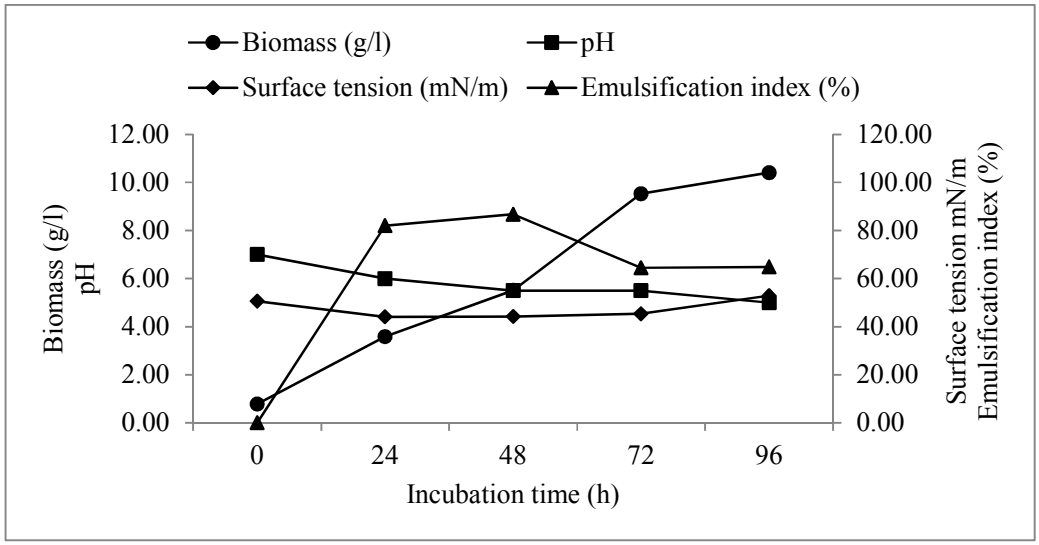


**Figure 1.** Growth, surface activity and emulsification index profiles of isolate bacteria LII61 grown in MSM with 1% molasse and 1% glycerol as carbon sources

**Characterization of biosurfactant extract**

The products of biosurfactant extraction have been presented in **Table 1**. After acid precipitation method, the crude biosurfactant was obtained around 1.61 g/l, whereas lypopeptides and glycolypids of biosurfactant were obtained 5.60 and 5.10 mg/l. This indicated the isolate bacteria LII61 has the potential in producing biosurfactants. The characteristic of biosurfactants was commonly determined from the CMC value and stability activity of biosurfactant in various environmental conditions. Based on **Figure 2**, the CMC of the biosurfactant product of bacteria LII61 was around 5 g/l which the surface tension reduced to 52.70 mN/m. Beside of that, this study also determined the emulsification index ($E_{24}$) of biosurfactant at CMC point. In **Table 1 and Figure 2**, the isolate bacteria LII61 was able to emulsify the kerosene around 71.01% at CMC and this result was better than biosurfactant product from yeast ($E_{24}$< 60%) [23]. This indicated the bacteria LII61 has better emulsification capabilities that could be applied in bioremediation technique, especially microbial hydrocarbon recovery.

**Table 1.** Profiles of biosurfactant extraction of isolate bacteria LII61

| Extraction product | Surface tension at CMC | Emulsification index at CMC | Weight of yield |
|---|---|---|---|
| Crude biosurfactant | 52.70 ± 0.44 mN/m | 71.01 ± 1.66 % | 1.61 ± 0.02 g/l |
| Lipopeptides of biosurfactant | ND | ND | 5.60 ± 0.01 mg/l |
| Glycolipids of biosurfactant | ND | ND | 5.10 ± 0.02 mg/l |
| ND: not determinated | | | |

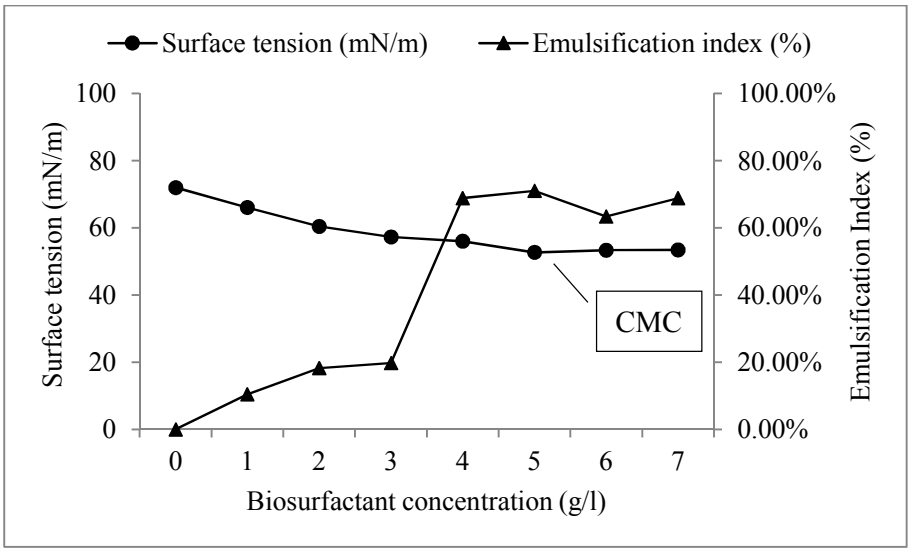


**Figure 2.** Surface tension and emulsification index versus biosurfactant concentration of isolate bacteria LII61 grown in MSM with 1% molasse and 1% glycerol as carbon sources

**Effect of pH and temperature on biosurfactant activity**

The effect of pH on biosurfactant activity at CMC was shown in **Figure 3a**. It showed that the surface tensions stabled in ranged of pH 3.5 to 6.5 and increased when in alkaline conditions. Whereas the emulsification index decreased when pH of biosurfactants decreased, even decreased significantly in alkaline conditions. It might be the biosurfactants was precipitated in alkaline conditions. This result was in contrast to the yeast biosurfactant which the emulsification index increased in alkaline conditions[23]. However, although the surface tensions increased in alkaline condition, but the values were ranged 50 to 65 mN/m (below the standard value). It meant the biosurfactants of isolate LII61 could reduce the surface tensions in acidic or alkaline conditions, but emulsifying activity was much better at low pH. In the other hand, the biosurfactants activity at CMC was more stable when the temperature increased, although there was a slight increased on surface tension values (**Figure 3b**). As well as the emulsification index, $E_{24}$ were ranged 70 to 80% at temperature condition reached 60ºC. It indicated that the biosurfactants of isolate bacteria LII61 has potential to be applied under high temperature conditions.

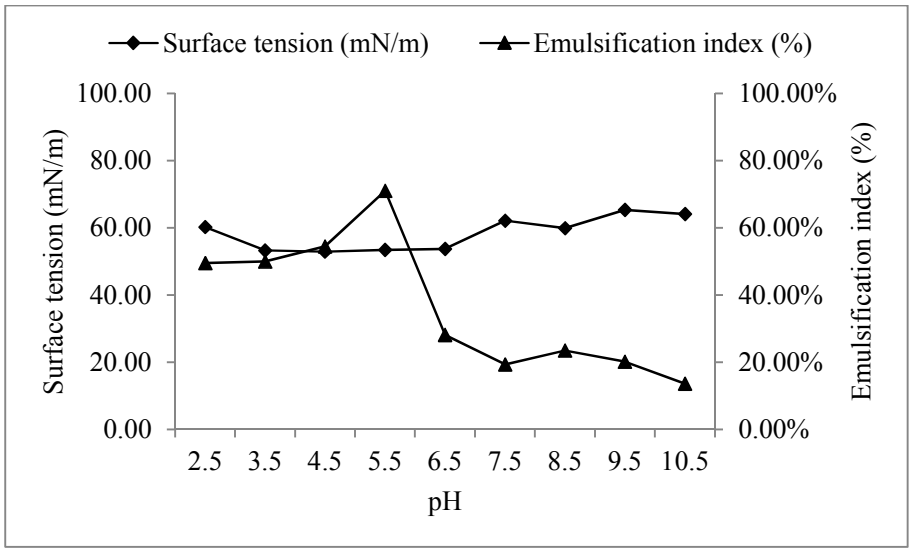


**Figure 3a.** Effect of pH on surface tension and emulsification index of biosurfactant of isolate bacteria LII61 grown in MSM with 1% molasse and 1% glycerol as carbon sources

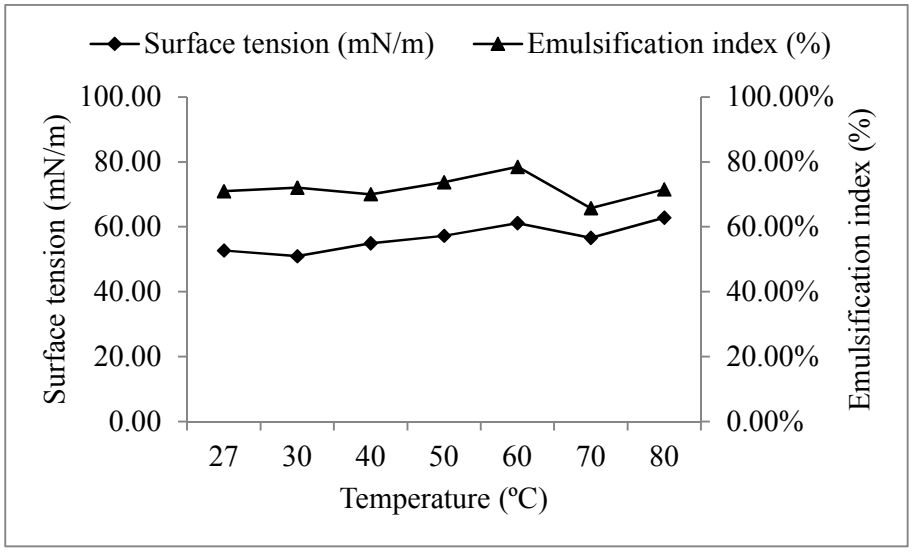


**Figure 3b.** Effect of temperature on surface tension and emulsification index of biosurfactant of isolate bacteria LII61 grown in MSM with 1% molasse and 1% glycerol as carbon sources

## CONCLUSION

This work demonstrated the potential of isolate bacteria LII61 in producing biosurfactant effectively with molasse and glycerol as carbon sources. The biosurfactants has a CMC value about 5 g/l and indicated a good surface active agent with the ability to reduce the surface tension of medium from 72 to 52.70 mN/m. This biosurfactants also has a high emulsification index of kerosene (71.01%). The biosurfactant stability experiments reported that the biosurfactant showed good surface activity (50-60 mN/m) in acidic or alkaline condition and high termpetature, while emulsification index showed good on acidic condition and temperatures below 70ºC.

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