Biosurfactants production using glucose and molasses as carbon sources by *Azotobacter vinelandii* and soil washing application in hydrocarbon-contaminated soil

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Abstract. The purpose of this research was to understand the influence of carbon source variation in the biosurfactant production by *Azotobacter vinelandii* as well as its potential application on remediation of hydrocarbon-contaminated soil by soil washing. The growth microorganism, biosurfactant quantity in exopolysaccharide and fatty acid fraction were examined. The quality of biosurfactant was analysed by emulsification index. The results show that biggest production was occurred at the 48th hour with μ\textsubscript{max1} = 0.416/hour, μ\textsubscript{max2} = 0.093/hour and K\textsubscript{s1} = 6.55 g/L, K\textsubscript{s2} = 4.69 g/L for glucose and molasses substrate respectively. Biosurfactant product of *Azotobacter vinelandii* in glucose 1.5%, 2%, 2.5%, and 3% showed emulsification index as much as 100%. While, emulsification index of the biosurfactant produced with molasses reached 100% within 1%, 1.5%, and 2% molasses concentration. These results showed that molasses has potential utilization as carbon source in biosurfactant production. In soil washing application, Biosurfactant and Tween 80 were used as emulsifier to removed Total Petroleum Hydrocarbons (TPH) by contacting in conical flask at rotary shaker. The results showed that highest removal achieved by using biosurfactant up to 48.89% which occurred at 100 rpm for 40 minutes.

1. Introduction

Industrial activity related with petroleum exploitation, exploration, transportation, consumption, potentially lead to release of hydrocarbon pollutants into the environment that caused serious problems [1]. Petroleum based pollutants are not only toxic to biological components of the environment but also carcinogenic. Even though, mechanical and chemical methods can reduce hydrocarbon pollution. However, they are often expensive, time consuming and not environment friendly [2]. Thus, bioremediation remains the method of choice for complete removal of hydrocarbon pollutants. The low solubility and high hydrophobicity of many hydrocarbon compounds had made them highly unavailable to microorganisms. In general, biosurfactants addition is one of alternative to accelerate PAH as well as hydrophobic compound degradation which has several advantages such as low or no environmental impact, low cost technology rather than conventional method, detoxifying hazardous substance instead of transporting material to another medium [1, 3-9].

Surfactants and emulsifiers are widely used for various industries such as cosmetic, pharmaceutical, petrochemical, food and beverages, textiles, soap, mining and manufacturing.
Surfactants also potentially was used in crude oil recovery and bioremediation of contaminated soil [3 - 9]. The use of surfactants usually able to enhance degradation of petroleum. But synthetic surfactant has been reported to reduce the level of degradation of organic compounds, which is probably due to the resulting toxic effects [10]. So, the use of biosurfactant which has relatively lower toxicity than synthetic surfactant [4], and easier to break down now become a very attractive option [8,10].

In the last ten to twenty years, the advantage of surfactants on improving bioavailability hydrophobic pollutants has been realized [6-10]. Addition of biosurfactant proved to increase the efficiency of degradation of hydrocarbons, because surfactants can enhance the oil solubility in water [9]. At this research we try to determine the pattern of biosurfactants production utilizing glucose and molasses as carbon source, as well as studied of soil washing for application of bioremediation activity.

2. Materials and Method

2.1. Materials
Crude oil that used in emulsification test was come from the Bula, Maluku. The petrofilic consortia were isolated from Java and Sumatra oil-contaminated soil. While, the soil sample which used in soil washing study was come from Coal Industry in Kalimantan. Ashby medium was used to acclimatize Azotobacter vinelandii with mannitol as the main carbon source as selective medium. Ashby medium contains (g/L) mannitol 15, K$_2$HPO$_4$ 0.5, MgSO$_4$.7H$_2$O 0.2, CaSO$_4$ 0.1, NaCl 0.2, and CaCO$_3$ 5.

Basal medium was used for production and study of bacterial growth [11]. The concentration variation of carbon sources included glucose: 2%, 2.5%, 3%, 3.5% (w/v), and molasses: 0.25, 0.5%, 1%, 1.5%, 2% (w/v). Basal medium was containing (in g/L) K$_2$HPO$_4$ 1.5, KH$_2$PO$_4$ 0.5, (NH$_4$)$_2$SO$_4$ 0.5, MgSO$_4$.7H$_2$O 0.2 g, and 10 mL of trace element which was containing (g/L) Na$_2$EDTA2.2H$_2$O 12, FeSO$_4$.7H$_2$O 2, CaCl$_2$ 1, ZnSO$_4$.7H$_2$O 0.4, NaSO$_4$ 10, MnSO$_4$.4H$_2$O 0.4, CuSO$_4$.5H$_2$O 0.1, Na$_3$MoO$_4$.2H$_2$O 0.5. Medium sterilized using autoclave at a temperature of 120 °C in 20 minutes with a pressure of 15 psi.

2.2. Methods

2.2.1. Biosurfactant isolation and biomass determination
A 30 mL sample of fermentation broth was centrifuged at 13,000 rpm for 30 minutes to obtain a cell free broth. After centrifugation, the supernatant was then dissolved in a 4 N hydrochloric solution and allowed to stand overnight at 4 °C, followed with the biosurfactant extraction step with a chloroform solvent at a room temperature. The organic layer was transferred to around-bottom flask and the aqueous layer was re-extracted twice for complete recovery of biosurfactant. The organic phases were combined yielding a viscous brown-colored crude biosurfactant product and evaporated to remove the solvent. The residue was collected and weighted. Vermani’s method was used to determine the exopolysaccharides fraction of biosurfactant [12]. A mixture of 1:1 (v/v) biosurfactant and chilled acetone were agitated and stand overnight to precipitate. Formed were filtered and were analyzed gravimetrically.

Growth of microorganisms tested by indirect method called spectrophotometric analysis. Increased turbidity a culture is an indication growth of bacteria. Turbidity measured using spectrophotometer at wavelength ($\lambda$) 420 nm for measuring the optical density (OD). Wavelength of 420 nm was used because it is the optimal wavelength to measure turbidity medium on yellow color.

2.2.2. Emulsification index (E-24)
Emulsification analysis was done by adding 5 ml cell free broth derived from the centrifugation for 20 minutes at 13,000 rpm in 5 mL of crude oil. Solution mixture of oil with biosurfactants then shuffled using vortex in high speed for 3-4 minutes until evenly mixed. Mixed of the solution was then left for 24 hours [13].
2.2.3. Soil washing

The soil washing study was conducted to observe crude oil removal with various surfactant solutions. The washing method from Urum [14] was adopted for this study. The initial and remaining oil was extracted using n-hexane. Ten mL of n-hexane were added to rinse the soil, shaken laterally for 5 min and the n-hexane-crude oil extract was removed. This process was repeated for times, as the fourth of n-hexane-crude oil extract gave the same absorbance reading as a pure n-hexane (zero absorbance). All the n-hexane-crude oil extract was collected into one volumetric flask and made up to 50 ml with n-hexane. A sample from 50 mL extract was centrifuged for about 20 minutes at 3000 rpm. This was to separate any suspended particle.

Crude oil remaining in soil, crude oil removed from the contaminated soil were determined using this equation (1):

\[
\text{Crude oil removed, } (\%) = \frac{(O_i - O_r)}{O_i} \times 100%
\]

Where \(O_i\) is the initial oil in the soil (g) before washing. \(O_r\) is the oil remaining in the soil (g) after washing.

3. Results and Discussion

3.1. Microbial enrichment for biosurfactant production

Specific microorganism requires very selective conditions for enrichment. Ashby medium, a selective medium for the growth of bacteria of the genus Azotobacter vinelandii with an absence of nitrogen in composition, was used for bacteria selection. Nutrients without the nitrogen was added in organic carbon and energy source then incubated in the aerobic condition with no light. It is suitable for growing of Azotobacter. Azotobacter vinelandii enrichment condition in this study was maintained at 4 °C on manitol enrichment. Subculture were made to fresh agar media every 2 month to maintain its viability [7].

3.2. Kinetic growth of Azotobacter vinelandii on glucose and molasses as carbon sources

A linear growth curve of Azotobacter vinelandii produces the exponential phase of the regression line with a slope which the specific growth rate. \(K_s\) values and \(\mu_{\text{max}}\) obtained by mapping the relationship between \(1/\mu\) with \(1/S\) with linearization Monod equation in Figure 1.

![Figure 1. Monod linearization of: a). glucose, b). molasses](image)

The result of the plot between \(1/\mu\) with \(1/S\) for glucose substrate was obtained from linear equation of the slope \(K_s/\mu_{\text{max}}\). Intercept of the x-axis shows the value of \(-1/K_s\) while the y-axis shows the value \(1/\mu_{\text{max}}\). From the linear equation \(1/\mu_{\text{max}} = 2.403\) note that \(\mu_{\text{max}} = 0.416/\text{hour}\), and \(K_s\) obtained by 0.655% or 6.55 g/L. \(K_s\) value of 0.655% indicates that Azotobacter vinelandii can grow with the specific
growth rate of $\frac{1}{2}$ of the maximum growth rate in glucose concentration as much as 0.655%.
If the concentration of glucose less than 0.655% the maximum growth rate of *Azotobacter vinelandii* will
not be achieved. While, the results by using molasses as substrate show that $\mu_{\text{max}} = 0.093$/hour, and
$K_s = 4.69$ g/L.

Value of Monod saturation constant ($K_s$) and maximum specific growth rate ($\mu_{\text{max}}$) is the specific
kinetic parameters for each of microorganisms and substrates. $K_s$ cells showed affinity toward the
substrate where the value of $K_s$ is the substrate concentration. If the bacteria were grown at a
concentration below $K_s$ value, rate of the maximum growth the microbes will not be achieved. On the
contrary, if microbes at concentrations above grow on his $K_s$ value and then any of the added substrate
will not increase the maximum rate of growth of these microbes. Therefore, the substrate is given in
the microbes to grow should be equal to or little greater than $K_s$ value.

3.3. Biosurfactants production

Biosurfactants, extracellular product by *Azotobacter vinelandii* bacteria, is affected by type of
substrate. The biosurfactants production started to increase during exponential phase. At glucose
concentration 2% (v/v), The highest production of biosurfactant occurs at 48 hours with total
biosurfactant as EPS and fatty acid as much as 9.19 g/L. At the same time, biosurfactant that was
produced using molasses only as much as 4.31 g/L. These results indicate that the maximum
biosurfactant synthesis from glucose and molasses were occurred at the end of the exponential growth
phase. Increasing the substrate concentration could increase the quantity of EPS and biomass of
bacteria, while the quantity of fatty acid tends to stagnant [15].

According to Desai and Banat [3], there are three patterns on kinetics of biosurfactant production.
They are growth associated, non-growth associated, and production of resting cell. According to
Figure 2, biosurfactant production pattern that produced by *Azotobacter vinelandii* follows growth
associated kinetic model.

![Figure 2](image)

**Figure 2.** Amount of biosurfactant and biomass that produced using glucose and molasses 2%
concentration

3.4. Emulsification Index (E-24)

To assess the emulsification capacity of biosurfactant produced by *Azotobacter vinelandii*, we
examined the emulsification index (E-24) test. Emulsification index is one of the parameters
determining the concentration of dissolved biosurfactant production, because the expected biosurfactants which were produced should not only a lot in terms of quantity, but also excellent in terms of quality.

Based on the results of research conducted, biosurfactants produced with carbon sources of glucose 2%, 2.5%, and 3% give an emulsification index value of 100%. Similar results were found in biosurfactant production using molasses at concentrations of 1%, 1.5%, and 2% giving an emulsification index of 100%. But, emulsification index by molasses at 0.25% and 0.5% concentration only provide 82.4% and 87.24% emulsification respectively. Total of biosurfactant production in EPS and fatty acid form as well as the emulsification index can be seen at Table 1.

Table 1. The quantity and quality of biosurfactants in various substrate concentration

| Variation | EPS (g/L) | Fatty Acid (g/L) | Total (g/L) | E-24 (%) |
|-----------|-----------|-----------------|-------------|----------|
| M 0.25%   | 3.26      | 0.35            | 3.61        | 82.4     |
| M 0.5%    | 2.65      | 0.66            | 3.31        | 87.24    |
| M 1%      | 3.55      | 0.61            | 4.16        | 100      |
| M 1.5%    | 2.56      | 0.8             | 3.36        | 100      |
| M 2%      | 3.51      | 0.8             | 4.31        | 100      |
| G 2%      | 7.05      | 2.14            | 9.191       | 100      |
| G 2.5%    | 9.73      | 2.71            | 12.44       | 100      |
| G 3%      | 10.78     | 2.02            | 12.80       | 100      |

Note: M: Molasses, G: Glucose

3.5. Soil washing

Several soil washing tests using surfactants which compared with biosurfactants in various concentration were conducted to understand the solubility of crude oil in contaminated-soil with several type of surfactants. The soil washing of hydrocarbon-contaminated soil were tested by using biosurfactant and Tween 80 at 5% (v/v) concentration with 10 mL volume. Distilled water with equal volume was used as a control. Stirring was done using a rotary shaker with speed variation of 80 and 100 rpm, and time variations of 20, 30, and 40 minutes. The results of soil washing using several agents can be seen at Figure 3.

![Figure 3](image_url)

Figure 3. The TPH of contaminated soil after soil washing with: (a) rotating at 100 rpm (b) rotating at 80 rpm

Crude oil removed by distilled water was about 5.8%. As the Tween 80 was 22.6%. While biosurfactant was 48.89%, all the surfactant solution showed improve crude oil removal. This trend was expected, since the force of attraction between soil and crude oil would be reduced due to the
increase in contact angle and wettability of the system in the presence of the surfactant. In comparison with distilled water washing, crude oil removal was not effective. The results show that surfactant and biosurfactant could remove the TPH. Similar result reported by Urum [14] show that the SDS and rhamnolipid biosurfactant show an oil removal of 96% and 80% in 15 minutes at 160 and 200 rpm.

4. Conclusions
In this research, biosurfactant production using glucose and molasses has been evaluated. The highest production was occurred at 48 hours during exponential phase by using glucose as carbon source. The biosurfactant production was following growth associated model. Although biosurfactants production by using molasses show that the total biosurfactant was less than total biosurfactant that produced by using glucose. However, the quality of biosurfactant that represent by emulsification index is equal with biosurfactant that produced by using glucose. It shows that, molasses has potential because it is low-cost carbon source on biosurfactant production. Application of biosurfactant in soil washing results the highest TPH removal up to 48.89% was occur in 100 rpm for 40 minutes using biosurfactants. It shows that biosurfactant produced by *Azotobacter vinelandii* has potential usage for soil washing.

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