Properties of biosurfactant produced by \textit{Pseudomonas putida} grown in Crude Palm Oil (CPO)

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Abstract. Biosurfactants produced by \textit{Pseudomonas putida} grown on crude palm oil (CPO) as carbon source have been characterized. Biosurfactant was produced in nutrient broth medium containing NaCl and 10\% v/v of crude palm oil (CPO) with 5 days of incubation time. Biosurfactants are able to reduce water surface tension from 72 to 54 mN/m and has a CCM value of 798 mg/L. Biosurfactant has a water in oil (w/o) emulsion system. Biosurfactants are able to form emulsions between water and several hydrocarbons, including benzene, lubricating oil, palm oil, soybean oil, sunflower oil and olive oil. The largest emulsion index of 83\% is obtained from the water emulsion with lubricating oil and can last up to 30 days.

1. Introduction

Surfactants are active compounds that have two functional groups in their molecules, namely hydrophilic groups and hydrophobic groups. Surfactants are classified into two, i.e. synthetic surfactants and biosurfactants. Synthetic surfactants are conventionally produced from chemical reactions in the form of petroleum derivatives, while biosurfactants are produced from cellular activity of microorganisms, such as bacteria and fungi [1-2]. Biosurfactants have several advantages compared to synthetic surfactants, including biodegradable, low toxicity and non-irritating. Biosurfactants have wide applications such as for bioremediation of oil-contaminated soils/pollutants and emulsifier in food, cosmetic, pharmaceutical and agricultural industries [3-5]. Biosurfactants have broad molecular coverage such as lipopeptides, glycolipids, phospholipids and polymeric biosurfactants. The molecular structures of biosurfactants are resulting from cellular activity of the microorganism which is influenced by the type of microorganisms and nutrients in the fermentation medium, such as carbon and nitrogen sources [6-7].

Genus Pseudomonas, especially the \textit{Pseudomonas aeruginosa} are known to have great potential as biosurfactant producers [8-14]. Other species of Pseudomonas, such as \textit{P. putida} have the potential to produce biosurfactants. These bacteria have been reported to produce a glycipipid class biosurfactant [15-17]. \textit{P. putida} is a cylindrical gram-negative bacteria that are non-pathogenic. Another advantage of this species is its high resistance to extreme environmental conditions, such as extreme temperatures, extreme pH and high salinity [18].

Microorganism-based surfactants can be produced from renewable materials such as fatty acids, carbohydrates, proteins and vegetable oils. Crude palm oil (CPO) which contains fatty acids and its abundant presence makes CPO potentially as a carbon source in biosurfactant production. Fatty acid compounds in CPO are palmitic acid (45\%), stearic acid (3\%), myristic acid (1\%), oleic acid (40\%) and linoleic acid (10\%) [19]. Palm oil has been used as a carbon source in the biosurfactant biosynthesis of glycolipids by \textit{Pseudomonas} [20-21]. We have described the production of biosurfactants by \textit{P. putida} using CPO as substrate on our previous work [14]. In this study, the properties of the biosurfactant produced by \textit{P. putida} grown on CPO are discussed.

2. Experimental

2.1. Materials
All chemical were used are analytical grade from e-Merck. The crude palm oil (CPO) was obtained from Centre for Chemical and Packaging, Jakarta, Indonesia. The strain used in this work, *P. putida* FNCC 0071, was purchased from IUC Food and Nutrition, Universitas Gadjah Mada, Indonesia.

2.2. Media Used and Growth Condition
Cultures of bacteria were maintained on nutrient agar. The media were used for biosurfactants production composed of nutrient broth (8 g/L), NaCl (5 g/L) and CPO (5%v/v). Media mixture was sterilized prior used. Fermentation was carried out at 150 rpm for 5 days at room temperature.

2.3. Biosurfactant recovery
Culture liquid of *P. putida* was filtered by Buchner filtrations using Whatman filtration paper grade 42. The supernatant was then acidified to pH 2.0 with HCl 6 N and lefted overnight at 4 °C. The supernatant was extracted twice with *n*-hexane for removing the remaining CPO. The aqueous solution was then extracted twice using ethyl acetate. Sodium sulphate anhydrous was then added to the organic layer. The organic layer which was free form water was then evaporated.

2.4. Biosurfactant Characterization
The surface tension and interfacial tensions were evaluated by capillary rise method. The CMC value was achieved by dissolving of biosurfactant in distilled water and the surface tension was calculated for various concentrations of the biosurfactant. The CMC was shown by sudden changes in the surface tension, which was obtained by plotting the surface tension as a function of biosurfactant concentration.

The conductivity test was applied for investigating the emulsion type of biosurfactants. Sodium chloride as an electrolyte was added to the emulsion and the conductivity was measured. If the conductance increases with increasing of concentration of sodium chloride, the emulsion is oil in water type. On the other hand, it is water in oil type if there is no significant changes in its conductance.

The E24 of the biosurfactants were examined by addition of 0.1 mg of biosurfactant to a crew-capped tube containing 1 mL of distilled water and 1 mL of hydrocarbons. The mixtures were then vortexed for 2 mins and lefted to stand for 24 h. The emulsification index (E24) is known as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm). The E24 of the formed emulsions were observed for 30 days.

3. Results and Discussion
The FT-IR spectrum of the produced biosurfactant suggested that rhamnolipids, glycolipids type biosurfactants, were produced by *P. putida* as reported previously [14]. Increased biosurfactant concentration in the medium resulted in lower surface tension of the medium. The occurrence of a sudden change in the value of the surface value of the medium or surface tension which tends to be constant at the addition of certain concentrations of biosurfactants, indicates that the CMC is achieved [10]. The CMC value of biosurfactant produced by *P. putida* is obtained for 798 mg/L. Janek *et al.* (2013) reported that biosurfactant from *P. putida* with glucose as carbon has a CMC value of 130 mg/L [7]. Martinez-Toledo and Rodriguez-Vazquez (2011) obtained a CMC value of 430 mg/L [15]. Although the biosurfactant produced in this study had a higher CMC value compared to previous studies, the CMC value of biosurfactant was lower when compared to SDS which had a CMC value of 2,100 mg/L [2]. The biosurfactant produced by *P. putida* was able to reduce the water surface tension by 18 mN/m (72 mN/m to 54 mN/m) at the CMC value. These results are not much different from the decrease in surface tension by biosurfactants produced in the study of Martinez-Toledo & Rodriguez-Vazquez (2011), which is 18.70 mN/m [15].

The emulsion system consists of two substances with different polarity which when mixed, one of these substances will be dispersed into other substances. This allows an o/w or w/o emulsion system. Conductivity measurement of oil and sample mixture before and after sodium chloride addition can be used to determine the emulsion system. Conductivity measurement of water emulsion with palm
oil which has been added with biosurfactant before and after the addition of sodium chloride was performed to determine the biosurfactant emulsion system (Table 1).

As presented in Table 1, the addition of sodium chloride to distilled water increased the conductivity value. In the water, sodium chloride will be ionized so that the more sodium chloride added the more the amount of ions moves in the water causes more increase in the conductivity value. Palm oil has a conductivity value of 0 (zero), both before and after the addition of sodium chloride. Sodium chloride is not soluble in oil so the value of conductivity remains zero. Conductivity measurements of the oil mixture and biosurfactant solution produced a constant conductivity value of 0.10 μs both before and after the addition of sodium chloride. The constant value of conductivity after adding sodium chloride indicates that biosurfactant forms an emulsion between water and palm oil with a w/o system.

Table 1. Biosurfactant emulsion type investigation by addition of NaCl.

| NaCl (mg) | Conductivity (µs) |   |   |
|-----------|-------------------|---|---|
|           | A         | B    | C    |
| 0         | 10.00     | 0    | 0.10 |
| 5         | 0.61 x10³  | 0    | 0.10 |
| 10        | 1.03 x10³  | 0    | 0.10 |
| 15        | 1.34 x10³  | 0    | 0.10 |
| 25        | 1.90 x10³  | 0    | 0.10 |
| 50        | 2.94 x10³  | 0    | 0.10 |
| 100       | 4.05 x10³  | 0    | 0.10 |

A : aquadest; B : palm oil; C : aquadest, palm oil and biosurfactants (800 mg/L)

The produced biosurfactant was characterized for main surfactant properties which is its ability to lower the surface tension of water and immiscible compounds mixtures (Table 2). The immiscible compounds which were used in this study were olive oil, sunflower oil, soybean oil, palm oil, lubricant oil, benzene, hexane, diesel oil, petroleum oil and kerosene. The emulsion stabilities were observed for 30 days by monitoring the E24.

One characteristic of biosurfactants is as an emulsifying agent. Biosurfactants produced in this study were analyzed for their ability as emulsifying agents and their stability using several hydrocarbons both with vegetable oil (such as: olive oil, sunflower oil, soybean oil, palm oil) and with petroleum derivatives (such as: lubricant oil, benzene, hexane, diesel oil, petroleum oil and kerosene). The ability of biosurfactants in emulsifying water with several hydrocarbons is summarized in Table 2.

The biosurfactants produced by *P. putida* have emulsification capabilities for both water emulsions with vegetable oils and petroleum-derived hydrocarbons. The highest emulsion index was obtained between water and lubricating oil with an emulsion index value of 83% and stable for 30 days. The water emulsion index with vegetable oil used in this study provides an emulsion index of about 42-50% and with benzene an emulsion index obtained was 26%. Water emulsions with olive oil can last up to 25 days with an emulsion index value of 30%. Water emulsions with sunflower oil and soybean oil are stable up to 20 days. These results indicated that the biosurfactant could be useful as an emulsifying and emulsion-stabilizing agent for vegetable oils. Water emulsions with other hydrocarbons such as hexane, diesel oil, petroleum ether and kerosene have a low emulsion index which is around 4-11%.

The ability of commercial surfactant such as SDS (Sodium Dodecyl Sulphate) in emulsifying water with several hydrocarbons was performed for positive control (Table 3). SDS have emulsion index values between 55-76% for water emulsions with all hydrocarbons used, and stable for 30 day. Based on these results, the average emulsification ability of biosurfactants produced by *P. putida* is still lower when compared to SDS. Nevertheless, the water emulsion index with lubricating oil produced by
biosurfactants is better when compared to SDS, it shows that the biosurfactant produced by *P. putida* using CPO as a carbon source has the potential to be applied in the environment.

**Table 2.** The emulsification index of emulsion between water and immiscible compounds with the presence of biosurfactants.

| Day | Olive oil (E24) (%) | Sunflower oil (E24) (%) | Soybean oil (E24) (%) | Palm oil (E24) (%) | Lubricant oil (E24) (%) | Benzene (E24) (%) | Hexane (E24) (%) | Diesel oil (E24) (%) | Petroleum oil (E24) (%) | Kerosene (E24) (%) |
|-----|-------------------|------------------------|----------------------|-------------------|------------------------|------------------|------------------|---------------------|-----------------------|-----------------|
| 1   | 48                | 50                     | 42                   | 83                | 26                     | 7                | 4                | 11                  | 11                    |                 |
| 5   | 44                | 44                     | 37                   | 79                | 22                     | 7                | 4                | 7                   | 7                     |                 |
| 10  | 42                | 41                     | 31                   | 75                | 11                     | 0                | 0                | 0                   | 6                     |                 |
| 15  | 33                | 39                     | 27                   | 75                | 4                      | 0                | 0                | 0                   | 0                     |                 |
| 20  | 30                | 29                     | 19                   | 71                | 4                      | 0                | 0                | 0                   | 0                     |                 |
| 25  | 30                | 14                     | 15                   | 71                | 4                      | 0                | 0                | 0                   | 0                     |                 |
| 30  | 11                | 11                     | 7                    | 67                | 4                      | 0                | 0                | 0                   | 0                     |                 |

**Table 3.** The emulsification index of emulsion between water and immiscible compounds with the presence of SDS (Sodium Dodecyl Sulphate).

| Day | Olive oil (E24) (%) | Sunflower oil (E24) (%) | Soybean oil (E24) (%) | Palm oil (E24) (%) | Lubricant oil (E24) (%) | Benzene (E24) (%) | Hexane (E24) (%) | Diesel oil (E24) (%) | Petroleum oil (E24) (%) | Kerosene (E24) (%) |
|-----|-------------------|------------------------|----------------------|-------------------|------------------------|------------------|------------------|---------------------|-----------------------|-----------------|
| 1   | 67                | 67                     | 67                   | 63                | 55                     | 76               | 72               | 65                  | 60                    | 67              |
| 5   | 57                | 54                     | 58                   | 54                | 48                     | 61               | 64               | 60                  | 59                    | 60              |
| 10  | 56                | 54                     | 56                   | 54                | 46                     | 59               | 62               | 58                  | 57                    | 60              |
| 15  | 56                | 54                     | 54                   | 54                | 45                     | 58               | 55               | 58                  | 53                    | 60              |
| 20  | 56                | 54                     | 56                   | 54                | 45                     | 58               | 53               | 58                  | 53                    | 59              |
| 25  | 56                | 54                     | 56                   | 51                | 45                     | 56               | 52               | 58                  | 47                    | 59              |
| 30  | 56                | 54                     | 56                   | 51                | 45                     | 48               | 50               | 58                  | 47                    | 59              |

4. Conclusions

Biosurfactant produced by *P. putida* has a CCM value of 798.46 mg/L, capable of reducing water surface tension from 72 to 54 mN/m. It has an w/o (water in oil) emulsion system. The produced biosurfactants capable of stabilizing the emulsion between water and benzene, lubricating oil, palm oil, soybean oil, sunflower oil and olive oil.

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