Optimization of biosurfactant production in soybean oil by *rhodococcus rhodochrous* and its utilization in remediation of cadmium-contaminated solution

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Abstract. Biosurfactant production by *Rhodococcus rhodochrous* in soybean oil was developed, where the effect of medium composition and fermentation time were evaluated. The optimum condition for biosurfactant production was achieved when a medium containing 30 g/L TSB (tryptic soy broth) and 20% v/v soybean oil was used as media with 7 days of fermentation. Biosurfactant was identified as glycolipids type biosurfactant which had critical micelle concentration (CMC) value of 896 mg/L. The biosurfactant had oil in water emulsion type and was able to reduce the surface tension of palm oil about 52% which could stabilize the emulsion up to 12 days. The batch removal of cadmium metal ion by crude and partially purified biosurfactants have been examined from synthetic aqueous solution at pH 6. The results exhibited that the crude biosurfactant had a much better adsorption ability of Cd(II) than that of partially purified biosurfactant. However, it was found that there was no significant difference in the adsorption of Cd(II) with 5 and 10 minutes of contact time. The results indicated that the biosurfactant could be used in remediation of heavy metals from contaminated aqueous solution.

1. Introduction
Biosurfactants are surface-active compounds which are produced by a variety of bacteria, fungi, and yeast [1-2]. They have better environmental compatibility than that of the chemical surfactants, e.g., biodegradable and lower toxicity [3]. In recent years, research has expanded a lot in developing the method of biosurfactant production and the application of biosurfactants [4-5].

Fermentation conditions and nutrient availability affects the production of biosurfactant. Different substrates have been used to produce biosurfactants [6-7]. We have reported that vegetable oil (soybean oil) could be used for rhamnolipids biosurfactants production by *P. aeruginosin* [8]. We also demonstrated that industrial waste (cassava flour wastewater) could be applied to the production of rhamnolipids biosurfactants by *P. fluorescence* [9].

Biosurfactants have potential use in numerous areas, such as in pharmaceutics, agriculture, food industry, the oil industry and the paper and pulp industry [5,10]. Application of biosurfactants in remediation technologies for organic and inorganic pollutants, such as hydrocarbons and metals, from contaminated soil and solutions, has shown an increasing interest [11-14]. The removal of metals from contaminated soil and solutions is mostly based on the ability of biosurfactants to form complexes with metals [15-17].

It is paramount importance in optimizing factors that influence growth in biosurfactant producing organisms for commercial application. As an extension of our work, we are interested in the
production of biosurfactants by *Rhodococcus rhodochrous* using soybean oil as a substrate and the use of these biosurfactants in the removal of cadmium from contaminated aqueous solution.

2. Materials and Methods

2.1. Materials

All chemical were used are analytical grade from e-Merck, whereas soybean oil from Sunbeam and palm oil from Bimoli. The strain used in this work, *R. rhodochrous* FNCC 0066, was purchased from Pusat Antar Universitas Gadjah Mada, Indonesia.

2.2. Media Used and Growth Condition

Cultures of bacteria were maintained on tryptic soy agar. Biosurfactant production optimization were performed using media composed of tryptic soy broth (30 g/l), and soybean oil (0, 5, 10 and 20% v/v). Fermentation was carried out on a rotary shaker (150 rpm) at room temperature for 12 days. The optical density of the cultures was monitored by UV-VIS Spectrophotometer, Shimadzu UV-160 IPC.

2.3. Biosurfactant recovery

Culture liquid of *R. rhodochrous* was centrifuged at 12,500 g for 20 minutes to obtain a crude preparation of biosurfactant (CPB). Partially purified biosurfactant (PPB) was obtained by extraction of culture supernatant with n-hexane and subsequently with chloroform. The extract was then evaporated to get biosurfactants free of the solvent. The PPB was analyzed by FT-IR spectrophotometer using Shimadzu FTIR-8201 PC spectrometer.

2.4. Biosurfactants Characterization

Emulsification index (E24) was determined by adding 2 mL of oil and the same amount of PPB, mixed by a vortex for 2 mins and left to stand for 24 h. The E24 is given as a percentage of the height of emulsified layer (mm) divided by the total height of the liquid column (mm). The surface tension and interfacial tensions were measured by the capillary rise method. The CMC value was obtained by dissolving of PPB in distilled water, and the surface tension was measured with various concentrations of the PPB. The sudden change in the surface tension was represented as the CMC, which was determined by plotting the surface tension as a function of PPB concentration.

2.5. Emulsion Type of Biosurfactants

Conductivity test was used to identify the emulsion type of PPB. Sodium chloride (1% w/w) as an electrolyte was added to the emulsion and the conductivity was measured. If the conductance increases, the emulsion is oil in water type. On the contrary, if there is no significant change, it is water in oil type.

2.6. Emulsification Properties of Biosurfactants

The E24 of the PPB were tested by addition of 0.1 mg of PPB to a crew-capped tube containing 1 mL of distilled water and 1 mL of palm oil. The E24 of the formed emulsions were monitored for 15 days.

2.7. Removal of Cd(II) Ion by Biosurfactants

The protocol of samples were prepared by dissolving cadmium (Cd(NO_{3})_{2}, 4H_{2}O in 1 mL of 0.1 M HNO_{3} to produce a metal solution (1000 ppm). The ph solution was adjusted to 6.0 before the biosurfactants were added. Batch adsorption technique was performed, where 10 mL of 2.0 ppm Cd(II) solution was mixed with either CPB (2 mL) or PPB (10 g) in a flask and stirred. The samples were kept at room temperature in a shaker at 150 rpm for 5 and 10 minutes. The solution was then filtered through Whatman filter paper No. 42. Residual metal ion concentration in the filtered samples was measured by Atomic Absorption Spectroscopy (AAS), Shimadzu type AA-6650. Experiments were made in triplicate, and the average value was calculated and presented as a percentage of metal removed.
2.8. Calculation
The adsorption capacity \( q_e \) of Cd(II) ion was calculated using the following equation

\[
q_e = \frac{(C_i - C_f) V}{m}
\]

(1)

where \( q_e \) is the amount of Cd(II) adsorbed per mass unit of biosurfactants (mg/g), \( C_i \) is the Cd(II) ion initial concentration (mg/L) and \( C_f \) is the Cd(II) ion final concentration (after treatment with PPB or CPB) (mg/L), \( V \) is the volume of the aqueous phase (L) and \( m \) is the amount of the CPB, or PPB used (g). The results were statistically examined using one-way analysis of variance (ANOVA), and significant differences were set at \( p<0.05 \).

3. Results and Discussion

3.1. Biosurfactants Production Optimization
Optimization of biosurfactant production was carried out in media containing 5, 10 and 20% v/v of soybean oil which was incubated for 12 days. The media with no addition of soybean oil was also performed as a negative control. The optical density, surface tension, and E24 were monitored during fermentation.

As presented in Figures 1(a)-(c), the addition of soybean oil in all concentrations significantly reduce the surface tension and enhance the optical density and E24 (\( p<0.05 \)). Statistically, the addition of 20% v/v soybean oil at 7 days of fermentation gave the highest optical density (\( p<0.05 \)). The addition of 20% v/v soybean oil provided notable significant different from each other where the highest E24 and the lowest surface tension were obtained. Although the optimum fermentation time could not be evaluated statistically based on the E24 and surface tension data (\( p>0.05 \)), the figures showed that the optimum fermentation time was achieved at 7 days of incubation. Overall, the addition of 20% v/v of soybean oil and 7 days of fermentation time were elected for the optimum condition for the biosurfactant production.

3.2. Biosurfactants Characterization
The obtained FT-IR spectra revealed a strong and broadband at 3400 cm\(^{-1}\) which might be related to the O-H stretching vibration. The \(-CH\) stretch was confirmed by the band at 2923 and 2854 cm\(^{-1}\). The asymmetric stretching vibration of \( C=O \) was identified by the band at 1747 cm\(^{-1}\) for the carboxylate and 1651 cm\(^{-1}\) for the ketone. The weak band at 1461 and 1377 cm\(^{-1}\) were obtained as a result of deformation and bending vibrations of aliphatic chains (\(-CH_3\) and \(-CH_2\)), reflecting the presence of alkyl chains in the compounds. The stretching of C-O group was found by the presence of the intense and strong band at 1242 cm\(^{-1}\). The obtained peaks of biosurfactants suggested that glycolipids biosurfactants were produced by \( R. rhodochrous \) as reported by other researchers that glycolipids (mostly trehalolipids) biosurfactants are produced by \( Rhodococcus \) sp. Trehalose tetra ester biosurfactants have been produced by \( R. erythropolis \) 51T7 [18], and a surface-active complex of acidic polysaccharides and lipids has been produced by \( R. rhodochrous \) CF222 [19]. Trehalolipids from rhodococci occur as complex mixtures with high structural diversities where their compositions vary depending on strain physiology and growth condition [20].

The produced biosurfactants presented the CMC value of 896 mg/L and the surface tension value of 37 mN/m. The produced biosurfactants were possibly ionic surfactant types as indicated by the higher value of CMC and the presence of carboxylate and hydroxyl groups. The addition of sodium chloride increased the conductance significantly, indicating that the emulsion type of the biosurfactants was oil in water (o/w). The biosurfactants were found to be effective emulsifiers. When palm oil was used as an immiscible compound, reducing the surface tension of water of about 52% from 62 mN/m to 32 mN/m. Furthermore, the biosurfactant enhanced the E24 of about 55% from 43% to 96% and the stable emulsion was reached up to 12 days.

3.3. Cadmium Removal from Synthetic Solutions by Biosurfactants
The cadmium removal from aqueous solution was examined for crude preparation biosurfactants (CPB) and partially purified biosurfactant (PPB). In this study, the pH value of 6.0 was chosen. At higher pH, the precipitation of the metal was observed whereas at lower pH, the excess of hydrogen
ions competed with the metal ion for binding with the active sites available in the sorbent [21]. Since the removal of cadmium was not correlated with the metabolism process, the absorption experiments were conducted with short contact time, e.g. 5 and 10 minutes.

The adsorption capacities of Cd(II) by CPB with 5 and 10 minutes of contact time were noted to be 1.659 and 1.766 mg/g, respectively. The adsorption capacities of Cd(II) by PPB with 5 and 10 minutes of contact time were found to be 0.344 and 0.558 mg/g, respectively (Figure 2). The results confirmed that the CPB has a much better absorption ability of Cd(II) removals than that of the PPB. Reduction in the number of functional groups of adsorbent due to purification occurred [15]. Accordingly, the amount of Cd(II) bound to the active adsorbent sites decreased, resulting in the adsorption capacity reduction. However, the results established that there was no significantly different in the removal of Cd(II) by CPB with the contact time of 5 and 10 minutes. Biosurfactants have been reported to be employed in the remediation of heavy metals, however, this potential for Rhodococcus biosurfactants still needs to be determined [20] and these results demonstrated that the biosurfactant produced by R. rhodochrous could be used for heavy metals removal from contaminated aqueous solution.

**Figure 1.** Optimization of biosurfactants production by *R. rhodochrous* grown in different amounts of soybean oil: (a) cell growth (optical density), (b) emulsification index and (c) surface tension.
4. Conclusion

Extracellular glycolipids biosurfactants could be produced by *R. rhodochrous* with soybean oil as a substrate. The optimum condition for biosurfactant production was reached at 7 days of incubation in a medium containing of 20% v/v soybean oil. The produced biosurfactants showed effective emulsifier properties. Biosurfactant exhibited CMC value of 896 mg/L and reduced the surface tension of water of about 52% when palm oil was used as an immiscible compound. Additionally, the biosurfactant enhanced the E24 of about 55%, and the emulsion was stable up to 12 days. The results suggested that the biosurfactant could be applied in remediation of heavy metals from aqueous solution. The crude biosurfactants performed a much better adsorption ability of Cd(II) than that of partially purified biosurfactants. Nevertheless, there was no considerable difference in the adsorption of Cd(II) by crude biosurfactants with 5 and 10 minutes of contact time.

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