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Physicochemical Properties of Biosurfactant Produced by *Pseudomonas fluorescens* Grown on Whey Tofu

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Abstract. The research aims to examine the physicochemical properties of biosurfactant produced by *Pseudomonas fluorescens*. Biosurfactant was produced in whey tofu media containing 8 g/L nutrient broth and 5 g/L NaCl which was fermented for 2 days at room temperature. Biosurfactant was identified as rhamnolipids which had critical micelle concentration (CMC) value of 638 mg/L and surface tension of 54 mN/m. The biosurfactant had water in oil (w/o) emulsion type. The biosurfactant was able to decrease the interfacial tension more than 40% for emulsion of water with hexane, pentane, benzene, lubricants or kerosene. The stable emulsions were reached up to 30 days with the E24 value of about 50% when paraffin, toluene, lubricants or palm oil was used as an immiscible compound. Commercial surfactants, such as Triton X-100 and Tween-80 were investigated to compare their emulsification activities and emulsion stabilities with the produced biosurfactant.

1. Introduction

Due to the limitation of surfactants derived from petroleum such as non-biodegradability, ability to accumulate and toxicity, there has been increasing interest in finding replacement of chemically surfactants with biological products. Biosurfactants are amphiphilic surface active compounds produced by microorganisms [1-3]. Biosurfactants have potential in the various application fields such as in bioremediation, biodegradation, oil recovery, food and pharmaceuticals. However, they have not being widely used yet due to high production cost [4].

Optimizing growth factors in the fermentation technology for biosurfactant production is of paramount importance. One possible strategy for reducing production costs is the use of cheaper and renewable substrates from agricultural waste, or that produced by the food industries, and oil waste industries. Several studies have reported the biosurfactants production using agricultural industrial waste, such as molasses, starchy substances, lactic whey, distillery wastes which are known for containing high levels of carbohydrates and of lipids which are necessary for substrates in the production of biosurfactants [5-9].

We have reported the production of biosurfactants using vegetable oils (such as soybean oil and corn oil) as well as agroindustrial wastes (such as cassava flour wastewater and molasses). We also have studied the application of biosurfactants for environmental remediation, such as removal of heavy metals and enhancement degradation of phenol [10-14]. The production of biosurfactants by *Pseudomonas fluorescens* has been studies using various substrates. However, whey tofu has not been used yet. Therefore, as extension of our work, we are interested in the production of biosurfactant by



P. fluorescens using whey tofu as a substrate. The physicochemical properties of the biosurfactant are discussed in this work.

2. Experimental

2.1. Materials

All chemical were used are analytical grade from e-Merck. The whey tofu was obtained from a local tofu industry. The strain used in this work, *P. fluorescens* FNCC 0063, 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 whey tofu containing 8 g/L nutrient broth and 5 g/L NaCl. Whey tofu was heated to boil and then filtered. Nutrient broth and NaCl were then added to the filtered whey tofu and the mixture was sterilized prior used. Fermentation was carried out at 150 rpm for 2 days at room temperature.

2.3. Biosurfactant recovery

Culture liquid of *P. fluorescens* was centrifuged at 10,000g for 10 mins. The supernatant was acidified to pH 2.0 with HCl 6N and leaved overnight at 4°C. The supernatant then was extracted using chloroform:methanol = 2:1. 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 capillary rise method was used for measuring the surface tension and interfacial tensions. The CMC value was obtained by dissolving of biosurfactant in distilled water and the surface tension was measured with various concentrations of the biosurfactant. The CMC was indicated by sudden changes in the surface tension, which was determined by plotting the surface tension as a function of biosurfactant concentration.

The emulsion type of biosurfactant was investigated by conductivity test. Sodium chloride (1, 2, 3 and 4% w/w) 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 dilution test was also applied for the emulsion type investigation [15].

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

Biosurfactant was dissolved at the CMC value in 4 mL distilled water and the immiscible compounds (1 mL) were added. The mixtures were shaken vigorously for 2 mins at room temperature. The resulting emulsion was allowed to stand for 10 min, and its absorbance was measured at 540 nm every 10 mins for 1 h. One unit of emulsification activity was defined as the amount of biosurfactant that affected an emulsion with an absorbance at 540 nm of 1.0. The log of the absorbance was plotted versus time, and its slope was taken as the decay constant, K_d [16-20].

3. Results and Discussion

The FT-IR spectrum of the produced biosurfactant is shown in Fig. 1. A strong and broad band at 3410 cm^{-1} due to the O-H stretching vibration has been observed. The -CH stretch of methylene was confirmed by the band at 2931 cm^{-1} . The asymmetric stretching vibration of C=O was characterised by the band at 1720 cm^{-1} for the carboxylate and 1651 cm^{-1} for the ketone. The band at 1404 were observed as a result of deformation and bending vibrations of aliphatic chains (-CH₃- and CH₂-),

suggesting the presence of alkyl chains in the compounds. The stretching of C-O ester was found by the presence of the intense and strong band at 1126 cm^{-1} . The obtained peaks of biosurfactant suggested that rhamnolipids, glycolipids type biosurfactants, were produced by *P. fluorescens* as reported by other researchers that *Pseudomonas* sp produces rhamnolipids [21-23].

The produced biosurfactant presented the CMC value of 638 mg/L and the surface tension value of 54 mN/m . The higher value of CMC and the presence of carboxylate and hydroxyl groups suggested that the produced biosurfactant was ionic surfactant types.

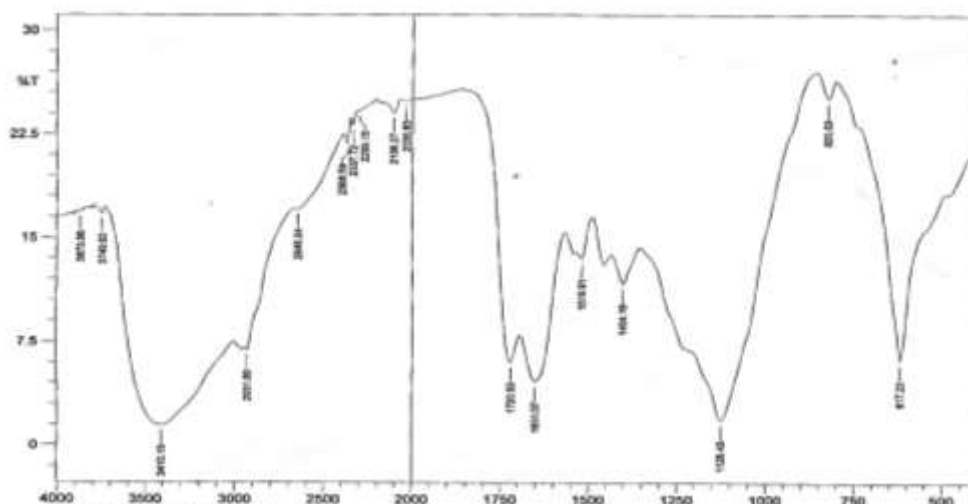


Figure 1. FT-IR spectrum of the produced biosurfactant.

Table 1 shows the conductivity of the solution for investigation of biosurfactant emulsion type. The conductivity of water as a positive control increased upon increasing addition of sodium chloride concentration. For the negative controls, the conductivity of the mixture of palm oil and water as well as water itself were measured. The conductivity of these negative controls were remained zero upon increasing addition of sodium chloride concentration. The addition of sodium chloride did not affect the conductance of the emulsion of water and palm oil with addition of biosurfactant, indicating that the emulsion type of the biosurfactant was water in oil (w/o).

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

Samples	Conductivity (μS)				
	Before addition of NaCl	After addition of NaCl (w/w)			
		1 %	2 %	3 %	4 %
Water	3.60	5.50	7.30	13.10	24.80
Palm Oil	0	0	0	0	0
Water and Palm Oil	0	0	0	0	0
Biosurfactant in water (650 ppm) and palm oil	0	0	0	0	0
Biosurfactant in water (1000 ppm) and palm oil	0	0	0	0	0

The emulsion type of biosurfactant was also determined by dilution test. The dilution test is based on the principle that an emulsion can be diluted with its continuous phase. The emulsion was not

diluted upon addition of palm oil. On the other hand, separation of the emulsion was observed upon addition of water. These results confirmed that the emulsion type was w/o emulsion. As controls, the dilution test was also performed for commercially available surfactants, such as Triton X-100 and Span 60 which have w/o and o/w emulsion type, respectively.

The produced biosurfactant was characterized for main surfactant properties, such as their ability to lower the surface tension of water and immiscible compounds mixtures (Table 2). The immiscible compounds which were used in this study were palm oil, soybean oil, olive oil, lubricant oil, kerosene, solar, paraffin, pentane, hexane, benzene, benzaldehyde, benzyl chloride, aniline, anisaldehyde, and toluene. The emulsion stabilities were observed for 30 days by monitoring the E24 (Table 3).

The biosurfactant could decreased the surface tension of water and benzene emulsion about 60.6 %. The decreased surface tension of emulsion water and lubricant oil, pentane or hexane were about 40-50%, whereas the decreased surface tension of emulsion water and soybean oil, kerosene or toluene were about 30-40%. These results suggested that the produced biosurfactant was a good emulsifier. The biosurfactant could emulsified the mixture of water and palm oil, soybean oil, olive oil, lubricant oil, solar, paraffin, benzaldehyde or toluene with the E24 value more than 50%. The stable emulsions were reached up to 30 days with the E24 value of about 50% when palm oil, lubricant oil, paraffin or toluene was used as an immiscible compound.

The emulsification activities and decay constant of the biosurfactant have been investigated (Table 4). The immiscible compounds used in these investigations were palm oil, paraffin and toluene because these compounds had the highest emulsion stabilities (Table 3). The emulsion activities and decay constants of biosurfactant have been compared with commercially available surfactants, such as Triton X-100 and Tween 80. These two surfactants were used as comparison because these surfactants have the same emulsion type with the produced biosurfactant which is water in oil emulsion (w/o).

Table 2. The surface tension of emulsion between water and immiscible compounds.

No.	Samples	Surface Tension		Decreased surface tension (%)
		Without Biosurfactant	With Biosurfactant	
1.	Palm oil	64.3	59.1	8.0
2.	Soybean oil	32.8	22.1	32.5
3.	Olive oil	54.1	44.0	18.8
4.	Lubricant oil	54.1	27.3	49.6
5.	Kerosene	50.7	30.5	39.9
6.	Solar	26.5	21.4	19.2
7.	Paraffin	58.3	43.2	25.9
8.	Pentane	54.0	30.6	43.2
9.	Hexane	52.5	27.3	48.6
10.	Benzene	42.4	16.7	60.6
11.	Benzaldehyde	56.0	45.6	18.6
12.	Benzyl chloride	58.6	46.9	20.0
13.	Aniline	56.7	39.8	29.9
14.	Anisaldehyde	65.0	53.3	18.1
15.	Toluene	64.1	43.0	32.8

The emulsification activities of biosurfactant for palm oil, paraffin and toluene was higher than that of Tween 80 but they are lower than that of Triton X-100. The best emulsification activities of biosurfactant was achieved at 0.60 for toluene. Decay constants reflect the stability of the emulsion,

the higher the decay constants, the better the stability of the emulsion [18-20]. Biosurfactant had the highest decay constant for palm oil among other surfactants studied. However, biosurfactant had higher decay constants than that of Tween 80 and lower decay constant than that of Triton X-100 for paraffin and toluene. These results indicated that the biosurfactant could be useful as an emulsifying and emulsion-stabilizing agent.

Table 3. The emulsification index of emulsion between water and immiscible compounds.

No.	Samples	Emulsification Index (E24) (%)															
		Day															
		1	2	4	6	8	10	12	14	16	18	20	22	24	26	28	30
1.	Palm oil	63	60	60	60	60	58	58	56	56	56	56	56	56	56	56	56
2.	Soybean oil	50	48	44	44	44	0	0	0	0	0	0	0	0	0	0	0
3.	Olive oil	52	52	48	46	44	44	0	0	0	0	0	0	0	0	0	0
4.	Lubricant oil	61	57	56	56	56	52	52	48	48	48	48	48	48	48	48	48
5.	Kerosene	19	14	14	14	14	14	14	11	0	0	0	0	0	0	0	0
6.	Solar	52	52	42	42	0	0	0	0	0	0	0	0	0	0	0	0
7.	Paraffin	65	65	65	54	54	54	54	54	54	54	54	54	54	54	54	54
8.	Pentane	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
9.	Hexane	64	63	55	50	44	35	29	15	8	0	0	0	0	0	0	0
10.	Benzene	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
11.	Benzaldehyde	52	48	44	41	41	0	0	0	0	0	0	0	0	0	0	0
12.	Benzyl chloride	48	41	41	41	41	0	0	0	0	0	0	0	0	0	0	0
13.	Aniline	8	8	8	8	8	8	0	0	0	0	0	0	0	0	0	0
14.	Anisaldehyde	48	48	39	37	37	37	37	0	0	0	0	0	0	0	0	0
15.	Toluene	62	62	61	61	61	59	59	59	59	59	59	59	59	59	59	59

Table 4. The emulsification activities and decay constants of biosurfactant and commercially available surfactants.

No.	Surfactant	Immiscible Compound	Emulsification	Decay Constant
			Activities (U)	$K_d (10^{-3})$
1.	Biosurfactants	Palm oil	0.43	-1.22
2.	Triton X-100	Palm oil	0.81	-12.44
3.	Tween-80	Palm oil	0.05	-5.69
4.	Biosurfactants	Paraffin	0.39	-2.45
5.	Triton X-100	Paraffin	3.41	-1.22
6.	Tween-80	Paraffin	0.07	-12.08
7.	Biosurfactants	Toluene	0.63	-2.94
8.	Triton X-100	Toluene	2.21	-1.53
9.	Tween-80	Toluene	0.06	-7.29

4. Conclusions

The rhamnolipids biosurfactant has been produced by *P. fluorescens* with whey tofu as a substrate. The physicochemical properties of the produced biosurfactant were evaluated and the results showed that it has effective emulsifier properties. The emulsion type of the biosurfactant was water in oil

emulsion type (w/o). The biosurfactant could lower the surface tension of emulsion with various immiscible compounds and the obtained emulsification index was relatively high. The emulsification activities and the decay constants are comparable to the commercially available surfactants, such as Triton X-100 and Tween 80.

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