BIOSURFACTANTS PRODUCTION BY *Pseudomonas aeruginosa* USING SOYBEAN OIL AS SUBSTRATE

Venty Suryanti [1]*, Sri Hastuti[1], Tutik Dwi Wahyuningsih[2], Mudasir[2], and Dina Ika Muliawati[1]

[1] *Department of Chemistry, Faculty of Mathematics and Natural Sciences, Sebelas Maret University Jl. Ir. Sutami 36A Surakarta 57126 Indonesia*

[2] *Department of Chemistry, Faculty of Mathematics and Natural Sciences, Gadjah Mada University Sekip Utara, P.O Box Bls. 21 Yogyakarta 55281 Indonesia*

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ABSTRACT

Optimization condition of the biosurfactants production by P. aeruginosa using soybean oil as substrate has been examined. The media containing 10% v/v of the soybean oil and 6 days of the fermentation time was the optimum condition for the biosurfactants production. The extraction technique using different solvent polarity (n-hexane, chloroform, ethyl acetate and buthanol, respectively) was applied for the isolation of the biosurfactants. The biosurfactant was found in the extract chloroform of the crude biospasoy (biosurfactants obtained from soybean oil as substrate) which then is called chlo-biospasoy. The chlo-biospasoy was identified as rhamnolipids which had oil in water (o/w) emulsion type, had the CMC of 860 mg/L and could reduced the surface tension of the water from 72 mN/m to 52 mN/m. The chlo-biospasoy could be used as an emulsifier to form emulsion between water and hydrocarbon such as palm oil, benzene, premium or toluene with various stability. The results indicated that chlo-biospasoy could be used as an emulsifying and emulsion-stabilizing agent.

Keywords: *Biosurfactants, P. aeruginosa, Soybean Oil, Emulsifier*

INTRODUCTION

Surfactants are a wide class of amphypatic molecules that are capable to reduce surface and/or interfacial tension between gases, liquids and solids, thus, these substances have been used in an extremely variety of products and processes in chemical, food and pharmaceutical industries. The toxicity, low biodegradability and effectiveness in a narrow pH and temperature range of synthetic surface-active compounds increased the interesting on the biosurfactants. These substances present ecological acceptance and effectiveness in a wide range of pH and temperature. Besides, the surface and interfacial activities, some of the biosurfactants presented anti-fungal, anti-viral and metal sorption capacities and are used in the petroleum industry to increase oil recovery processes [1].

Most biosurfactants are complex molecules, comprising different structures that include peptides, glycolipids, fatty acids and phospholipids [2]. Biosurfactants are produced by a wide variety of diverse microorganisms and have very different chemical structures and surface properties depending on the physical and chemical conditions under which they are grown [4]. Apart from the influence of different microorganisms, biosurfactant can be produced from a variety of renewable substrates, such as carbohydrates, lipids and proteins [3]. The structure of biosurfactant reflects the structure of the substrate provided in the culture and changing the substrate often alters the structure, and hence the properties of the product. Vegetable oils have been used as substrates in producing biosurfactants. Glucose and/or palm oil have been used for the production of sophorolipids by *Candida bombicola* [5]. Soybean olive, castor or sunflower oil as substrate has been used for the production of biosurfactants by *Serratia marcescens* [6].

Microbiological conversion of oleic acid to hydroxy fatty acid by the *Pseudomonas* was reported in the 60s [7-9]. The conversion of oleic acid to 10-hydroxystearic acid (10-HSA) was observed by Wallen *et al*. to reach a 14% yield [9]. 10-HAS produced by *Pseudomonas sp.* was found to be stereospecific, the hydroxy group having D-configuration [8]. *Pseudomonas sp*. 42A2, when cultivated in a mineral salt medium with olive oil as a sole carbon source; produced dihydroxyoctadecenoic acid [10]. The bioconversion of another *Pseudomonas sp.* afforded 7,10 dihydroxy-8(E)-octadecenoic acid (DOD) from oleic acid. The maximal yield of DOD was 72% [11, 12].

Soybean oil contain 65-90% of unsaturated fatty acids, which are oleic acid (42-62%), linoleic acid (21-34%) and linolenic acid (0-1%) [13]. The objective of this research is to produce biosurfactant and characterize it based on microbial production by *P. aeruginosa* using soybean oil as substrate.

* Corresponding author. Tel/Fax : +62-271-663375
Email address : venty_s@yahoo.com

EXPERIMENTAL SECTION

Material

All chemical were used are analytical grade from E-Merck, which are nutrient agar, nutrient broth, NaCl, n-hexane, chloroform, ethyl acetate, buthanol, benzene and toluene, whereas soybean oil from Sunbeam and palm oil from Bimoli. The strain used throughout this work, *P.aeruginosa* FNCC 0063, was purchased from Pusat Antar Universitas Gadjah Mada University, Indonesia.

Instruments

Optical density were measured by Shimadzu UV-160 1PC spectrometer and infrared spectra were obtained by a Shimadzhu FTIR-8201 PC spectrometer.

Procedure

Media Used and Growth Conditions

Cultures of bacteria were maintained on nutrient agar media. Experiments on growth optimization of biosurfactant production were performed using media composed of nutrient broth (8 g/l), NaCl (5.0 g/l) and soybean oil (0, 5, 10, or 20% v/v). The cultures were incubated at room temperature on reciprocal rotary shaker (150 rpm) for 12 days.

Surface Tension

The surface tension of samples were evaluated by capillary rising method. The surface tension could be calculated using the following equation:

$$\frac{\gamma_{aquadest}}{\gamma_x} = \frac{h_{aquadest} d_{aquadest}}{h_x d_x}$$

Whereas:

$\gamma_{aquadest}$: aquadest surface tension = 72.13 mN/m
γ_x : surface tension of *x* (dyne/cm)
$h_{aquadest}$: aquadest height
h_x : height of *x*
$d_{aquadest}$: aquadest density = 0.997 gr/cm^3
d_x : density of *x* (gr/cm^3)

Emulsification Index (E24)

E24 was determined by adding 1mL of hydrocarbon to the same amount of culture or aquades containing biosurfactants, mixing with a vortex for 2 minutes, and leaving to stand for 24 hours. The E24 index is given as percentage of height of emulsified layer divided by total heigth of the liquid column.

Isolation of Biosurfactants

The culture supernatant containing biosurfactants was separated from the cells by centrifugation at 12.000 *g* for 20 minutes. The extraction technique using different solvent polarity (n-hexane, chloroform, ethyl acetate and buthanol, respectively) were applied to the cell-free culture. The extract was evaporated to get biosurfactants free of the solvent.

CMC Value of Biosurfactants

The biosurfactants concentrations were determined by diluting the purified biosurfactants until reaching the CMC, which was determined by plotting the surface tension as a function of the biosurfactants concentration, and then the surface tension at that point was designated as ∂cmc.

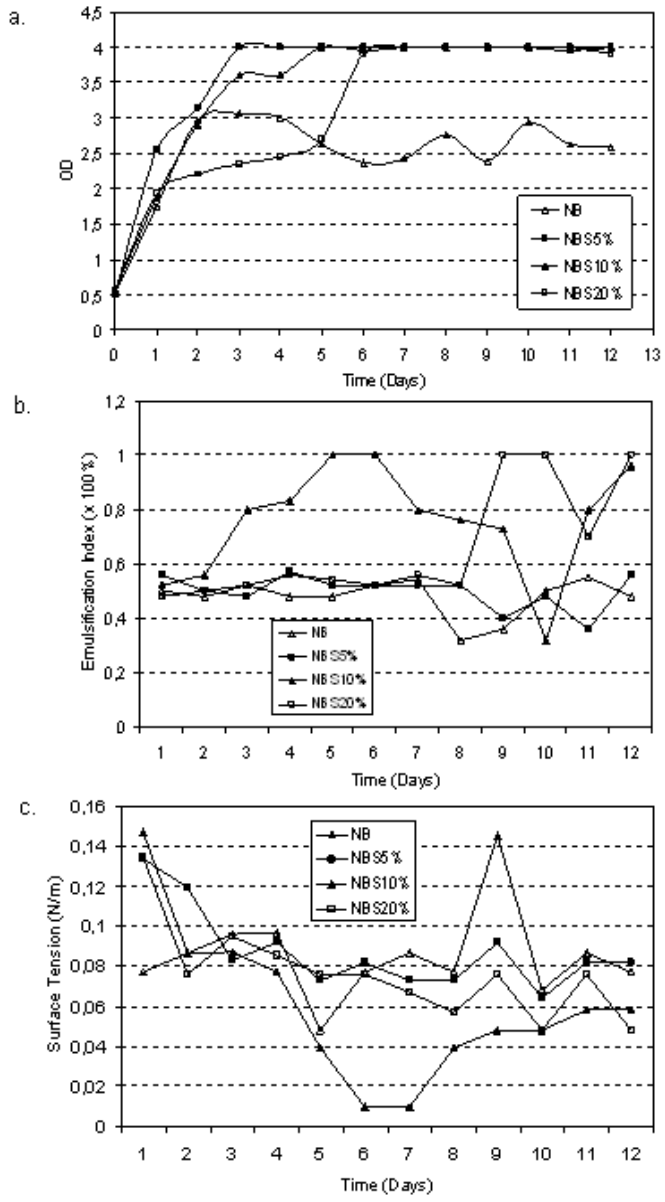

Figure 1. Growth and biosurfactants production of *P. aeruginosa*: (a) optical density, (b) emulsification index and (c). surface tension (NB: nutrient broth; NBS5%: NB + 5% v/v soybean oil; NBS10%: NB + 10% v/v soybean oil; NBS20%: NB + 20% v/v soybean oil).

Emulsion Type of Biosurfactants

Test used to identify the emulsion type of biosurfactants was conductivity test. 1% w/w of NaCl was added to the emulsion and the conductivity was measured.

RESULT AND DISCUSSION

Optimization of Biosurfactants Production

The variation amount of soybean oil (5%, 10% and 20% v/v) on the fermentation media were applied for the optimization of the biosurfactants production. The fermentation media contain only nutrient broth without soybean oil was also applied as control. The experiment on growth optimation of biosurfactants were monitored through optical density, surface activity and E24 for 12 days. The condition that had the lowest surface tension and the highest E24 is the optimum condition for the biosurfactant production.

Experiments on growth optimization of biosurfactants production using soybean oil as substrate is shown in Figure 1. Duncan statistics analysis based on data shown on Figure 1 shows that the amount of soybean oil had effect on the optical density, emulsification index and surface tension on the biosurfactants production. The optical density and the emulsificstion index of the media fermentation containing soybean oil were higher than that of without soybean oil, whereas the surface tension of the media fermentation containing soybean oil were lower than that of without soybean oil. Duncan statistics analysis also shows that the media containing 10% v/v of soybean oil and 6 days of the fermentation time were the optimum condition for the biosurfactants production.

Isolation of Biosurfactants

Since the polarity of the biosurfactants that were produced was not known, the extraction technique using different solvent polarity for the partial purification of the biosurfactants was applied. The biosurfactant was on the extract that had the highest emulsification index and the lowest surface tension. Partial purification of biosurfactants obtained from soybean oil as substrate, called crude biospasoy is shown in Table 1. Chloroform extract of the crude biospahosoy (is then called chlo-biospasoy) had the lowest surface tension (0.0116 N/m) and the highest E24 (100%). The chlo-biospasoys then was characterized.

Table 1. Isolation of the 150 ml crude biospasoy using different solvent polarity.

Solvent	Colour	Amount	Surface Tension (mN/m)	E24 (%)
Hexana	Yellow	5.6 g	66.7	53
Choloform	Brown	2.4 g	48.9	100
Ethyl acetate	Golden Brown	3.2 g	90.1	10
Buthanol	Dark Brown	0.48 g	83.4	36
Aquades	Light Yellow	0.68g	95.0	45

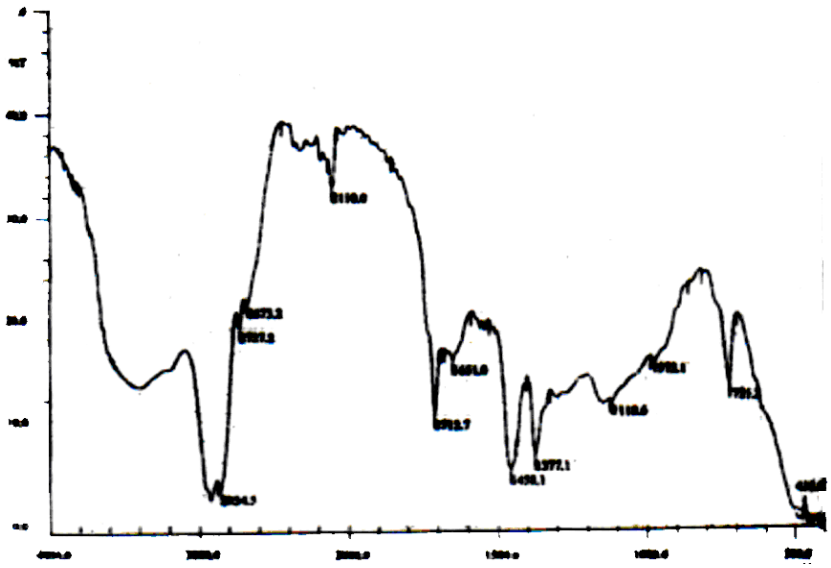

Figure 2. The infrared spectrum of the chlo-biospasoy with Shimadzhu FTIR-8201 PC spectrometer

The functional group identification of the chlo-biospasoy

FT-IR spectrum of chlo-biospasoy (Fig. 2) showed the hydroxyl (-OH) stretching as a broad absorption at 3400.0 cm^{-1}. Absorption band at 3000 – 2800 cm^{-1} together with absorbtion at 1377.1 cm^{-1} expressed the existence of methyl group. The absorption of methylene group appeared at 1458.1 cm^{-1}. Carbonyl stretching band was found at 1712.7 cm^{-1} which was characteristic for ester compounds. The ester carbonyl group was also prooved from the band at 1118.6 cm^{-1} which corresponds to C-O deformation vibrations. FT-IR spectra analyses of the chlo-biospasoy were identical to the rhamnolipids which was produced by *P. fluorescens* using olive oil as substrate and to the rhamnolipids which was produced by *P. putida* using hexadecana or glucose as substrate [14]. Thus, the chlo-biospasoy could be identified as rhamnolipids, the amphiphilic surface-active glycolipids usually secreted by *Pseudomonas* spp.

The CMC value of the chlo-biospasoy

The chlo-biospasoy was dissolved in distilled water, and the surface tension of the water was measured with various concentrations of the biosurfactants. As shown in Fig. 3, the CMC for the chlo-biospasoy was approximately 860 mg/L. The CMC value of the chlo-biospasoy is quite high compared to those of other biosurfactants. For examples, CMC value of flavolipids is 300 mg/L [15], that of rhamnolipids range from 27 to 40 mg/L [16, 17] and that of trehalose lipid range from 4 to 15 mg/L [18, 19].

At the CMC, the chlo-biospasoy reduced the surface tension of the water from 72 mN/m to 52 mN/m. Generally, the surface tension at the CMC for various purified biosurfactants has been reported to range from about 27 to 35 mN/m [20, 21, 22].

For practical purposes, it is important to distinguish between an efficient surfactants and an effective surfactants, where the efficiency is measured by the surfactants concentration required to produce a significant reduction in the surface tension of water, whereas the effectiveness is measured as the minimum value to which the surface tension can be reduced [20, 23]. Therefore, the important characteristics of a potent biosurfactants are its ability to lower the surface tension of aqueous solution and a low CMC [20].

The emulsion type of the chlo-biospasoy

To determine the emulsion type of chlo-biospasoy, the conductivity test was applied. This test is based on the basic principle that NaCl is a good electrolit. 1% w/w of NaCl was added to the emulsion and the conductivity was measured (Table 3). In the oil in water (o/w) emulsion, oil is dispersed phase and water is dispersion medium, whereas water in oil emulsion (w/o) emulsion, water is dispersed phase or internal phase and oil is the dispersion medium or the external phase. For those reason, the increased of conductivity by addition of NaCl indicates that the emulsion type of chlo-biospasoy is oil in water (o/w). In this type of emulsion, polar group of water bind to NaCl, so the conductivity is increased.

The capability of chlo-biospasoy to emulsify hydrocarbons

Table 2 summarises results obtained for chlo-biospasoy emulsifying several hydrocarbons. The results demostrated that the chlo-biospasoy could emulsify palm oil, premium, benzene and toluene with various stability. It indicated that chlo-biospasoy coud be used as an emulsifying and emulsion-stabilizing agent.

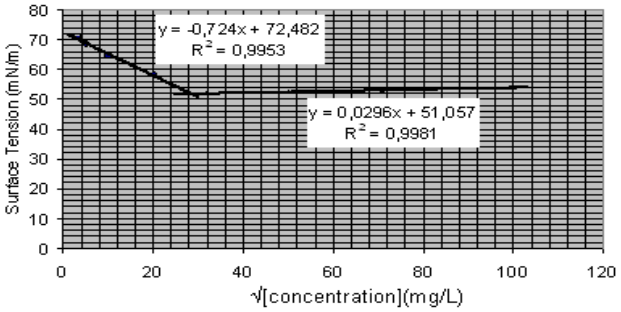

Figure 3. Determination of CMC value of chlo-biospasoy

Table 2. The capability of chlo-biospasoy to emulsify hydrocarbons

Hydrocarbons	Emulsification Index (%)							
	Without addition of *chlo-biospasoy*	with addition of chlo-biospasoy						
		Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Palm oil	30	50	40	30	30	20	15	10
Premium	0	22	11	0	0	0	0	0
Benzene	0	50	50	30	30	20	10	0
Toluene	0	10	10	10	0	0	0	0

Table 3. Conductivity data on emulsion test of chlo-biospasoy.

Sample Weight (g)		Conductivity (μs)
Emulsion	NaCl	
1	0	16.56
1	0.01	27.5
2	0	28.5
2	0.02	58.4

CONCLUSION

1. The biosurfactants could be produced based on microbial production by *P. aeruginosa* using soybean oil as substrate. The media containing 10% v/v of the soybean oil and 6 days of the fermentation time were the best condition for the biosurfactants production.
2. The extraction technique using different solvent polarity (n-hexane, chloroform, ethyl acetate and buthanol, respectively) were applied for the partial purification of the biosurfactants. The biosurfactant was in the extract chloroform of the crude biospasoy (chlo-biospasoy).
3. The chlo-biospasoy was identified as rhamnolipids.
4. The chlo-biospasoy had oil in water (o/w) emulsion type, the CMC of 860 mg/L and reduced the surface tension of the water from 72 mN/m to 52 mN/m.
5. The chlo-biospasoy could be used as an emulsifier to form emulsion between water and hydrocarbon such as palm oil, benzene, premium or toluene with various stability.

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