PRODUCTION AND CHARACTERIZATION OF BIOSURFACTANT BY *Pseudomonas fluorescens* USING CASSAVA FLOUR WASTEWATER AS MEDIA

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ABSTRACT

Biosurfactant with efficient emulsification properties could be produced by Pseudomonas flourescens using cassava flour wastewater (manipueira) as media. The ability of P. flourescens to produce biosurfactant could suggest potential use in industrial and environmental applications. Media containing a mixture of natural manipueira and nutrient broth with 48 h fermentation was the optimum condition for the biosurfactant production. Based on UV-Vis and FT-IR spectra, the biosurfactant was indicated as rhamnolipids containing hydroxyl, ester, carboxylic and aliphatic carbon chain functional groups. Biosurfactant exhibited critical micelle concentration (CMC) value of 715 mg/L and reduced the surface tension of the water from 80 mN/m to 59 mN/m. The biosurfactant was able to decrease the interfacial tension about 51-70% when benzyl chloride, palm oil and kerosene were used as water-immiscible compounds. The biosurfactant was able to form stable emulsion until 30 days when paraffin, soybean oil, lubricant oil and kerosene were used as water-immiscible compounds.

Keywords: *biosurfactant; manipueira; Pseudomonas flourescens; rhamnolipids*

ABSTRAK

Biosurfaktan dengan sifat emulsifikasi yang baik dapat diproduksi oleh P. fluorescens menggunakan limbah industri tepung tapioka (manipueira) sebagai media. Kemampuan produksi biosurfaktan oleh P. fluorescens bisa diaplikasikan di industri dan untuk penyelesaian masalah lingkungan. Penggunaan campuran media nutrient broth dan manipueira tanpa perlakuan awal dengan lama fermentasi 48 jam merupakan kondisi optimum untuk produksi biosurfaktan. Analisa spektrofotometer UV-Vis dan FT-IR menunjukkan bahwa biosurfaktan merupakan rhamnolipida yang mempunyai gugus hidroksi, ester, karboksilat dan rantai alifatik. Biosurfaktan mempunyai nilai konsentrasi kritis misel (KKM) 715 mg/L dan mampu menurunkan tegangan permukaan air dari 80 mN/m ke 59 mN/m. Biosurfaktan ini dapat menurunkan tegangan muka sebesar 51-70% untuk senyawa hidrokarbon benzil klorida, minyak sawit dan kerosene. Biosurfaktan mampu membentuk emulsi yang stabil sampai 30 hari untuk senyawa hidrokarbon parafin, minyak kedelai, minyak pelumas dan kerosene.

Kata Kunci: *biosurfaktan; manipueira; Pseudomonas flourescens; rhamnolipida*

INTRODUCTION

Biosurfactants are a group of secondary metabolites with surface active properties and are synthesized by a great variety micro-organism. These metabolites are complex amphiphilic molecules whose hydrophobic and polar domains depend on the carbon substrate and bacterial strain. Biosurfactants have several advantages over the chemical surfactants, such as lower toxicity, higher biodegradability [1], better environmental compatibility [2], higher foaming [3], higher selectivity and specific activity at extreme temperatures, pH and salinity [4], and the ability to be synthesized from renewable feedstock [5].

In previous studies, we found that biosurfactants could be produced by *P. aeruginosa* using soybean oil as media [6]. The biosurfactant was identified as rhamnolipids and had the CMC of 860 mg/L and reduced the surface tension of the water from 72 mN/m to 52 mN/m. The biosurfactant 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 biosurfactant could be used as an emulsifying and emulsion-stabilizing agent.

Although productions of biosurfactants by several micro-organisms and their application have been reported [7-35], these compounds have not yet been employed extensively in industry because of their high production cost. The uses of low cost raw materials appear as a natural choice to process overall economy. Biosurfactant can be produced by microbial

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fermentation processes using inexpensive agro-based substrates and waste materials, such as peat hydrolysate [36], olive oil mill effluent [36], lactic whey [37], soybean curd residue [38], potato process effluent [39-40] and molasses [41]. Cassava flour wastewater (manipueira) is an example of agroindustrial waste or by products that have high contents of carbohydrates for the use of substrate for biosurfactants production. This paper describes the biosurfactant production by *P. flourescens* using manipueira as substrates and the characterization of emulsifying properties of the biosurfactants produced.

EXPERIMENTAL SECTION

Materials

All chemical were used are analytical grade from Merck. *P. flourescens* was obtained from Food and Nutrition Culture Collection (FNCC) 007 Inter University Centre of Food and Nutrition Universitas Gadjah Mada, Indonesia.

Instrumentation

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

Procedure

Growth optimization conditions

Natural manipueira (NM), with the presence of insoluble solids, was simply thawed, homogenized and distributed in conical flasks. Decanted manipueira (DM), with the absence of insoluble solids, was prepared by heating the thawed waste until boiling to facilitate solids removing. After cooling, the substrate was centrifuged for 20 min and the supernatant was distributed in conical flasks.

Experiments on growth optimization and biosurfactant production were performed using five different media which all supplemented with 5 $g.L^{-1}$ NaCl. There were: NB, containing 8 $g.L^{-1}$ nutrient broth; M, containing NM; NBM containing NM and 8 $g.L^{-1}$ nutrient broth; MS containing DM; NBMS containing DM and 8 $g.L^{-1}$ nutrient broth. The pH of all media was adjusted to 7.0.

The cultures were incubated at room temperature on reciprocal rotary shaker (100 rpm at 30 °C) and were monitored through bacterial growth, surface tension and emulsification index (E24) for 12 days. Bacterial growth was monitored by measuring the optical density at 365 nm by Shimadzu UV-160 1PC spectrometer.

Production medium and culture conditions

The bacterial isolates were streaked on a nutrient agar slant and incubated for 24 h at 30 °C. A loop of culture was inoculated in 5 mL of NB in a test tube and incubated in a rotary shaker at 100 rpm at 30 °C for 21 h. An aliquot of 5 mL of inoculum was transferred to 250 mL of NBM media in a flask of 500 mL and incubated in a rotary shaker of 100 rpm at 30 °C for 48 h.

Crude biosurfactant purification

For purification of biosurfactant, the pH of the cultures supernatant fluid, obtained after removal of cells by centrifugation for 10 min, was adjusted to pH 2.0 using 6 N HCl and allowed to stand overnight at 4 °C. This was followed by extraction with a mixture of $CHCl_3$ and CH_3OH (2:1 v/v). The biosurfactant was dried by $NaSO_4$ anhydrous and evaporated on a rotary evaporator [42-43].

Emulsification index (E24)

E24 of culture samples was determined by adding 2 mL of hydrocarbons and the same amount of culture/distilled water, mixed by a vortex for 2 min, then leaved to stand for 24 h. The E24 is given as percentage of height of emulsified layer (mm) divided by total height of the liquid column (mm) [44].

Surface tensions, interfacial tensions and CMC

The surface tension and interfacial tensions of the biosurfactant were measured by the capillary rise method at room temperature [45]. The purified biosurfactant was dissolved in distilled water, and the surface tension of the water was measured with various concentrations of the biosurfactant. The concentration at which micelles began to form was represented as the CMC. At the CMC, sudden change in the surface tension was observed. The CMC was determined by plotting the surface tension as a function of biosurfactant concentration.

Detection of rhamnolipids

A modified orcinol method was used for analyzing the rhamnolipids using rhamnose as the standard [46]. A culture supernatant (3.3 mL) was extracted twice with diethyl ether (10 mL). The ether fractions were evaporated to dryness and 0.5 mL of water was added. After heating for 30 min at 80 °C the samples were cooled at room temperature for 15 min and the optical density readings by UV-Vis Spectrophotometer were taken at 421 nm.

IR spectrometric analyses

Infrared (IR) absorption spectra were obtained by a Shimadzu FTIR-8201 PC spectrometer in the range

of 4000 to 400 cm^{-1} spectral region at a resolution of 1 cm^{-1}.

Emulsification properties of the purified biosurfactant

The interfacial surface tension and E24 of the purified biosurfactant using different hydrocarbons (pen tane, hexane, benzene, anisaldehyde, benzaldehyde, toluene, benzyl chloride, aniline, paraffin, diesel, palm oil, soybean oil, olive oil, lubricant oil and kerosene) were tested. The purified biosurfactant (0.1 mg) was added to a crew-capped tube containing distilled water (1 mL) and the desired hydrocarbon (1 mL). The interfacial tensions of the emulsions with and without addition of purified biosurfactant were measured by the capillary rise method at room temperature. The E24 of the formed emulsions was monitored for 30 days.

Assay of emulsification activity and stability

Purified biosurfactant (3.3 mg) was diluted with 4 mL distilled water and 1 mL of hydrocarbons was added. The mixture was shaken vigorously in a vortex mixer for 2 min. The emulsion was allowed to sit for 10 min at room temperature, after which its absorbance was measured at 540 nm. The absorbance was used to express the emulsification activity. One unit of emulsification activity was defined as that amount of emulsifier that affected an emulsion with an absorbance at 540 nm of 1.0.

The emulsion stability was analyzed based on the emulsification activity [47]. The emulsified solutions were allowed to stand for 10 min at room temperature and then absorbance readings were also taken every 10 min for 60 min. The log of the absorbance was then plotted versus time. The slope (decay constant, K_d) of the line was calculated, then expressed as emulsion stability.

RESULT AND DISCUSSION

Optimization of Biosurfactants Production

Experiments on growth optimization and biosurfactant production were performed using five different media: NB, M, NBM, MS and NBMS as described in experimental sections. The experiment was monitored through optical density, surface tension and E24 for 12 days. Fig. 1 shows the time course of biosurfactant production from *P. fluorescens* during growth on manipueira as substrate. The surface tension reached a minimum of 60.5 mN/m and the E24 reached a maximal of 58% when the bacterial growth was at late log phase which was about 2 days (48 h) after inoculation (Fig. 1b and 1c). The bacterial growth was continued to its stationary phase till 5 days (60 h) of inoculation (Fig. 1a).

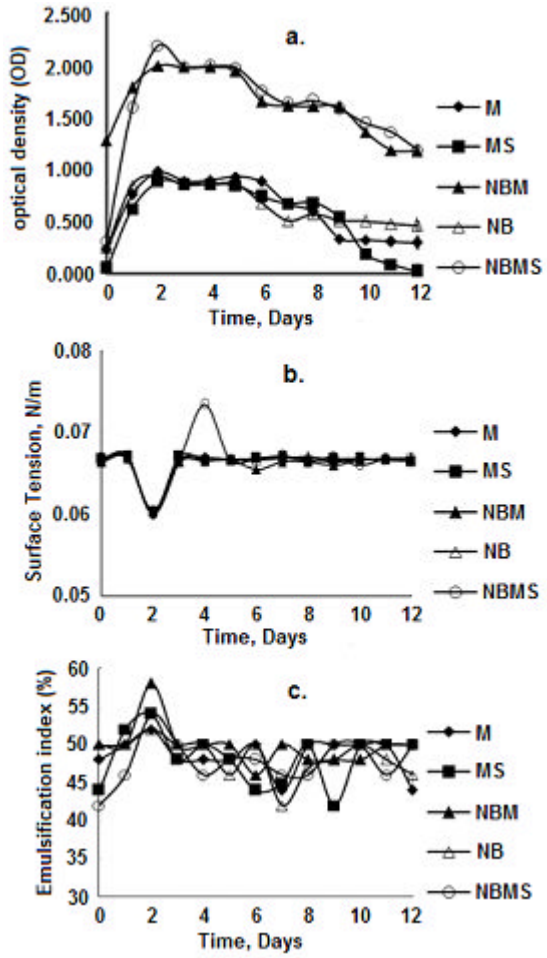

Fig 1. Growth of *P. fluorescens* in various media: (a) optical density, (b) surface tension and (c). emulsification index

The *P. fluorescens* were submitted to biosurfactant production in two different manipueira media: natural manipueira and decanted manipueira. The purpose of this experiment was to evaluate the viability of the use of manipueira waste in its natural form what could reduce costs and facilitate medium preparation. The condition that had the highest microbial growth, surface tension reduction and E24 is the best condition of biosurfactant production. Duncan statistics analysis based on data shown on Fig. 1 shows that bacterial growth using natural manipueira with the presence of nutrient broth as media with 2 days (48 h) of the fermentation time was the optimum condition for the biosurfactant production.

As comparisons, biosurfactant production by *P. aeruginosa* RB 28 (expressed as rhamnose equivalents) reached the maximum level at the stationary phase after 60 h of inuculation [48]. Production of biosurfactant from *P. aeruginosa* 44TI started after 14 h of inoculation and reached its

maximal level after 58 h [49]. Rhamnolipid production from *P. aeruginosa* GS3 started by 12 h and reached its maximum at 72 h [50]. A reduction in the surface tension of media as a result of biosurfactant production and accumulation during the period between the expontial growth and stationary phases has already been reported for several other microorganisms. Since biosurfactants are secondary metabolites, maximal glycolipid production was found to accumulate at the stationary stage of cell growth [51-52].

Detection of the Rhamnolipids

The rhamnose gave an absorption maximal at 417 nm. The UV-Vis spectra of the purified biosurfactant also possessed an absorption maximal at 417 nm which indicated that biosurfactant has rhamnose in its structure. The FT-IR spectra of purified biosurfactant showed the hydroxyl (-OH) stretching as a broad absorption at 3400 cm^{-1}. In the region 3000-2700 cm^{-1} were obtained several C-H stretching bands of CH_2 and CH_3 groups. The deformation vibrations at 1458 and 1381 cm^{-1} also confirmed the presence of alkyl groups. Carbonyl stretching band was found at 1720 cm^{-1} which is characteristic for ester compounds. The ester carbonyl group was also proved from the band at 1095 cm^{-1} which corresponds to C-O deformation vibrations. FT-IR spectra analyses of the biosurfactant were identical to the rhamnolipids which was produced by *P. fluorescens* using olive oil as a substrate and to the rhamnolipids which was produced by *P. putida* using hexadecana or glucose as a substrate [53]. FT-IR spectra analyses and the result of modified orcinol method indicated that *P. fluorescens* FNCC 007 produced a mixture of rhamnolipids, the amphiphilic surface-active glycolipids usually secreted by *Pseudomonas* spp.

CMC and the Surface Tension of the Biosurfactant

At the CMC, sudden changes in the surface tension were observed. The CMC was determined by plotting the surface tension as a function of biosurfactant concentration. The CMC for the purified biosurfactant was 715 mg/L. At the CMC, the purified biosurfactant reduced the surface tension of the water from 80 mN/m to 59 mN/m.

The CMC value of the biosurfactant is quite high compared to those of other biosurfactants. For examples, CMC value of rhamnolipids range from 27 to 40 mg/L [35-36] and that of trehalose lipid range from 4 to 15 mg/L [53-54]. At the CMC, the biosurfactant reduced the surface tension of the water from 80 mN/m to 59 mN/m. A good surfactant can lower surface tension of water from 72 to 35 mN/m [42]. The surface tension for other biosurfactants also has been reported to range from about 27 to 35 mN/m [55-57].

Table 1. The interfacial surface tension of water-immiscible compounds by biosurfactant.

Compounds	The decreased of interfacial surface tension (%)
Pentane	33
Hexane	20
Benzene	20
Anisaldehyde	21
Benzaldehyde	22
Toluene	25
Aniline	25
Paraffin	12
Diesel	33
Palm oil	66
Soybean oil	33
Olive oil	47
Lubricant oil	33
Benzyl chloride	51
Kerosene	62

Capability of the Biosurfactants to Emulsify Hydrocarbons

Addition of the biosurfactant decreased the interfacial surface tension of the emulsions tested (Table 1). About 51-70% decreased of interfacial tension was showed when using benzyl chloride, palm oil and kerosene as water-immiscible compounds; 31-50% decreased of interfacial tension was showed when using pentane, diesel, olive oil, soybean oil and lubricant oil as water-immiscible compounds; and 10-30% decreased of interfacial tension was showed when using hexane, benzene, anisaldehyde, benzaldehyde, toluene, aniline and paraffin as water-immiscible compounds. The ability of the produced biosurfactants to emulsify kerosene and crude oil is an important feature for bioremediation applications.

On day one, more than 40% of emulsification index were obtained when benzene, anisaldehyde, toluene, palm oil, soybean oil used as water-immiscible compounds (Table 2). It also showed that paraffin, soybean oil, lubricant oil and kerosene formed stable emulsion up to 30 days. The soybean oil, lubricant oil and kerosene were then used as water-immiscible compounds for emulsification activity and stability test.

As comparisons, biosurfactant produced from *P. aeruginosa* RB 28 emulsified hydrocarbons, hydrocarbons mixtures and vegetable oils and formed stable emulsions. Emulsions formed with vegetable oils are more stable [48]. It was reported that rhamnolipids had the ability to emulsify efficiently hydrocarbons and vegetable oils [43]. The stability of emulsions was controlled and held stable for 21 days. The results obtained with *i*-propyl palmitate (73%), castor oil (67%)

Table 2. The emulsification index of the water-immiscible compounds by biosurfactant.

Day	Emulsification index (%)													
	a	b	c	d	e	f	g	h	i	j	k	l	m	n
1	75	44	62	7	12	8	42	48	36	52	12	36	13	4
5	60	8	33	4	8	4	12	15	16	52	0	14	6	0
10	55	0	0	0	8	4	8	15	8	52	0	14	6	0
15	30	0	0	0	8	0	4	7	8	36	0	11	0	0
20	20	0	0	0	8	0	4	7	8	36	0	11	0	0
25	0	0	0	0	8	0	0	7	4	36	0	11	0	0
30	0	0	0	0	8	0	0	7	0	36	0	11	0	0

a = benzene; b = anisaldehyde; c = toluene; d = aniline; e = paraffin; f = diesel; g = palm oil; h = soybean oil; i = olive oil; j = lubricant oil; k = benzyl chloride; l = kerosene; m = pentane; n = hexane

Table 3. Emulsification and stabilization properties of soybean oil, lubricant oil and kerosene by biosurfactant and synthetic surfactants.

Compounds	Surfactants	Emulsification activity (OD_{540nm})[a]	Decay constant (K_d, 10^{-3})[b]
Soybean oil	Biosurfactant	0.134	-1.477
	Tween-80	0.835	-9.211
	Triton X-100	1.176	-4.286
Lubricant oil	Biosurfactant	0.380	-0.806
	Tween-80	1.986	-1.170
	Triton X-100	2.430	-0.181
Kerosene	Biosurfactant	0.482	-2.439
	Tween-80	2.060	-0.789
	Triton X-100	0.717	-5.962

or almond oil (83%) suggests a potential application in the pharmaceutical and cosmetic industries [5].

Emulsifying Activity and Stability of the Biosurfactant

The emulsification activity and stability of the biosurfactants and synthetic surfactants (Tween 80 and Triton X-100) were examined with soybean oil, lubricant oil and kerosene as water-immiscible substrates (Table 3). The stabilization ability of the biosurfactants was described by the decay constant, K_d (the slope of the emulsion decay plot) and the respective K_d values were then calculated. The emulsification activity of the biosurfactant was the lowest to that of the synthetic surfactants tested, although the emulsion stability of the biosurfactant was the greatest to that of the synthetic surfactants tested when using soybean oil as water-immiscible substrate. Emulsion stability of the purified biosurfactant was among that of the synthetic surfactants tested when using soybean oil or lubricant oil as water-immiscible substrate.

The lower K_d shows the better stability of a microemulsion. The biosurfactant exhibited good emulsion stability indicating having high surface-active properties. As a comparison, liposan from *C. lipolytica* grown on YNB medium supplemented with 1% hexadecane has emulsification activity of 0.75 and K_d of -6.0 x 10^{-3} for *n*-hexadecane as water-immiscible compounds [48].

The ability of *P. flourescens* FNCC 007 to produce biosurfactant using manipueira as media with efficient emulsification properties, could suggest potential use in industrial and environmental applications.

CONCLUSION

Biosurfactant could be produced based on microbial production by *P. flourescens* using cassava flour wastewater (manipueira) as media. The optimum condition for the biosurfactant production was obtained using media containing a mixture of natural manipueira and nutrient broth with 48 h fermentation. UV-Vis and FT-IR spectra indicated that the biosurfactant was a rhamnolipid containing hydroxyl, ester, carboxylic and aliphatic carbon chain functional groups. Biosurfactant exhibited CMC value of 715 mg/L. At the CMC, the biosurfactant reduced the surface tension of the water from 80 mN/m to 59 mN/m. The biosurfactant was able to decrease the interfacial tension about 51-70% when benzyl chloride, palm oil and kerosene were used as water-immiscible compounds. The biosurfactant formed stable emulsion until 30 days when paraffin, soybean oil, lubricant oil and kerosene were used as water-immiscible compounds.

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