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Production and characterization of biosurfactant produced by *Lactobacillus lactis* grown in media containing Crude Palm Oil (CPO)

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Abstract. *Suryanti V, Masykur A, Setyono HA, Ramadani S. 2021. Production and characterization of biosurfactant produced by Lactobacillus lactis grown in media containing Crude Palm Oil (CPO). Biodiversitas 22: 5501-5506.* Biosurfactants are surface-active compounds produced by a wide variety of diverse microorganisms. The structural diversity of biosurfactants depends on microorganisms and growth conditions (such as substrate and pH). The unique structure of biosurfactants provides unique properties for specific applications. Biosurfactant production still needs to be explored using a variety of microorganisms and growth conditions for producing the diversity of biosurfactant structures. Production and characterization of biosurfactant by *Lactobacillus lactis* using Crude Palm Oil (CPO) as an additional carbon source has been conducted. The optimization condition of biosurfactant production by *L. lactis* using media containing various concentrations of CPO was evaluated. Optical density, surface tension, and emulsification index were observed daily for 12 days. Optimal biosurfactant production condition was achieved when *L. lactis* was grown on media Nutrient Broth containing 10% (v/v) of CPO and incubated for 8 days. Biosurfactants were obtained for 80 mg/L media. Based on Thin Layer Chromatography (TLC) analysis and Fourier Transform Infra-Red (FT-IR) Spectroscopy, produced biosurfactant was identified as a glycolipid biosurfactant containing functional groups of hydroxyl, ester, carboxylic, methyl, and methylene. Produced biosurfactants exhibited the Critical Micelle Concentration (CMC) value of 1 g/L with a surface tension value of 49.46 mN/m. The emulsion system of produced biosurfactant was water in oil (w/o). The produced biosurfactant showed an emulsification index of 41-71% with soybean oil, palm oil, sunflower oil, olive oil, and lubricant oil. The stable emulsion was reached up to 30 days when soybean oil, olive oil, and lubricant oil were used as the water-immiscible compound.

Keywords: Biosurfactant, critical micelles concentration, crude palm oil, glycolipid, *Lactobacillus lactis*

INTRODUCTION

Surfactants are amphiphilic molecules consisting of hydrophobic and hydrophilic parts. Surfactants lower the surface tension of liquid so that they can emulsify two immiscible liquids. Therefore, surfactants could be applied in the cosmetic, food, textile, petroleum, and pharmaceutical industries (Reningtyas and Mahreni 2015). Most of the surfactants are derived from petroleum which is not environmentally friendly. Therefore, in recent years much interest and attention have been shifted to biosurfactants which are surface-active substances produced by a wide variety of diverse microorganisms. Various microorganisms such as bacteria, yeast, and fungi are capable of producing biosurfactants.

Biosurfactants have structural diversity since different microorganisms produce different structures of biosurfactants. Based on their chemical structures, biosurfactants can be classified into glycolipids, lipopeptides, fatty acids, polysaccharide-lipid complexes, and polymeric surfactants. The structural diversity of biosurfactants causes diversity in biosurfactant properties. Therefore, biosurfactants have remarkable applications in various industries and environmental restoration. Biosurfactants have various advantages over synthetic surfactants, such as being environmentally friendly, lower toxicity, easily biodegradable, better environmental biocompatibility, and higher selectivity and specific activity in the range of temperature, pH, and salinity (Desai and Banat 1997; Qazi et al. 2013).

The production of biosurfactants requires specific growth conditions which also result in the diversity of biosurfactants produced. Biosurfactant production is influenced by several factors, such as media composition (carbon source and nitrogen source), temperature, pH, agitation, oxygen availability, and incubation time (Gudina et al. 2012; Hamzah et al. 2013; Mohanty and Das 2018). Biosurfactants can be made from various renewable substrates which are low-cost raw materials. Glucose, fructose, sucrose, and glycerol have been used as carbon sources in the production of biosurfactants. Various vegetable oils, such as palm oil, corn oil, soybean oil, also have been used as carbon sources for biosurfactants production.

Lactobacillus spp. are capable of producing various metabolic by-products, including biosurfactants. Lactic Acid Bacteria (LAB) can produce cell-bound and excreted biosurfactants. Biosurfactants with diverse chemical structures such as glycolipids, glycolipopeptides, and glycoprotein biosurfactants have been reported to be

produced by *Lactobacillus spp*. Thavasi et al. (2011) succeeded in producing glycolipid biosurfactant by *Lactobacillus delbrueckii* which has emulsification activity and has potential for application in biodegradation of petroleum pollution. Sharma and Saharan (2014) reported the production of glycolipid biosurfactant by *Lactobacillus casei* which has antibacterial activity. Sharma et al. (2014) reported that *Lactobacillus helveticus* (Orla-Jensen 1919) Bergey et al. 1925 produce a glycolipid biosurfactant that has a xylolipid-like structure. Biosurfactant has also been produced by *Lactobacillus paracasei* which has a surface tension of 41.8 mN/m, a critical micelle concentration (CMC) of 2.5 mg/mL, and has antimicrobial activity (Gudina et al. 2010).

Crude Palm Oil (CPO) contains 46.4% unsaturated fatty acids and 53.6% saturated fatty acids, which are suitable for carbon sources in biosurfactant production. Indonesia is one of the biggest palm oil producers in the world. To increase its economic value, it is necessary to convert CPO into other compounds/products which are useful for various applications in industry or the environment. In our previous work, we reported the production, characterization, and application of biosurfactants produced by *Pseudomonas putida* grown in CPO (Suryanti et al. 2017; Suryanti et al. 2018; Suryanti et al. 2019). In this research, *Lactobacillus lactis* (Lister 1873) Schleifer et al. 1986 was used for the production of biosurfactants using CPO as a carbon source to generate a novel biosurfactant with diverse properties.

MATERIALS AND METHODS

Materials and instrument

All chemicals are analytical grade from E Merck. CPO was obtained from the Center for Chemical and Packaging, Jakarta, Indonesia. *Lactobacillus lactis* used in this study was bacteria collection of Center for Food and Nutrition Studies, Universitas Gadjah Mada, Indonesia.

Stock culture

Lactobacillus lactis was grown on a Nutrient Agar (NA) medium and stored at 4°C as a stock culture.

Optimizing of biosurfactant production

Fermentation medium

The fermentation medium was made with the composition of 8 g/L Nutrient Broth (NB) and 5 g/L NaCl. A single colony of *L. lactis* was taken from stock culture, and placed in a 3 mL fermentation medium then agitated at 70 rpm for 24 h at room temperature. After incubation, 1 mL of culture media was transferred into a 25 mL fermentation medium and agitated for a further 24 h. Three mL of the resulting culture media was transferred into a 200 mL fermentation medium.

Optimization biosurfactant production

The optimization of biosurfactant production was conducted according to our previous works (Suryanti et al. 2016a; Suryanti et al. 2016b). *Lactobacillus lactis* was grown in a 200 mL fermentation medium with and without the addition of CPO and incubated for 12 days. The concentrations of CPO added in the fermentation medium were 0, 5, 10, and 20% (v/v). During optimization of biosurfactant production, the fermentation media was monitored for cell growth, surface tension, and emulsification index (E24). Cell growth of bacteria was measured by UV-Vis Spectroscopy at the wavelength of 600 nm using Perkin Elmer Precisely Lambda 25 UV-Vis. Surface tension was calculated using the capillary method. The capillary tube was inserted into the fermentation medium and the increased solution in the capillary tube was measured. The E24 was calculated by the addition of 1 mL of fermentation media into 1 mL of palm oil and the mixture was homogenized using a vortex for 2 minutes. The emulsion was left for 24 hours. The E24 was calculated as the percentage of the height of emulsified layer (mm) divided by the total height of the liquid column (mm).

Production and purification of biosurfactants

The media used for biosurfactants production was composed of NB (8 g/L), NaCl (5 g/L), and CPO (10% v/v). Media was sterilized before use. The flasks containing 125 mL media were inoculated with 3 mL of an overnight *L. lactis* culture and incubated at room temperature on a reciprocal rotary shaker at 150 rpm for 8 days.

The liquid growth culture of *L. lactis* was filtered by Buchner using Whatman Grade 42 filter paper. The supernatant was then acidified to pH 2.0 with HCl 6 N and left overnight at 4°C. The supernatant was extracted twice with hexane to remove the remaining CPO. The aqueous solution was then extracted twice using ethyl acetate. Sodium sulfate anhydrous was added to the organic layer. The water-free organic layer was evaporated to obtain biosurfactants.

Structure identification of biosurfactant

Isolated biosurfactant (5 mg) was mixed with KBr and then analyzed for its functional groups by Shimadzu Prestige-21 FT-IR Spectroscopy at the wavelength of 400-4000 cm^{-1}. The TLC analysis was also performed on the isolated biosurfactants. The isolated biosurfactant was dissolved in chloroform and then spotted on a silica gel plate. The silica gel plate was inserted into a chamber containing the mobile phase of chloroform: methanol: glacial acetic acid (65:15:2, v/v). If the elution did not produce a good separation, it was replaced with a mixed eluent of chloroform: methanol: glacial acetic acid (95:4:1, v/v). After the elution was completed, the detection of sugars, lipids, glycolipids, and lipopeptides was carried out. Sugar detection was carried out by spraying anisaldehyde: sulfuric acid: glacial acetic acid (1:2:100) reagent (Antoniou et al. 2015). Lipid detection was performed with iodine vapor (Li et al. 2016). Glycolipid detection refers to Kaskatepe et al. (2015). The TLC plate was sprayed with 15% (v/v) H_2SO_4 reagent in ethanol, then the plate was heated at 100°C. Lipopeptide detection was carried out using ninhydrin reagent (0.5 g ninhydrin in 100 mL acetone) (Sivasubramani and Selvaraj 2017).

Biosurfactant characterization

The surface tension was evaluated by the capillary rise method. The CMC value was achieved by dissolving the biosurfactant in distilled water and the surface tension was calculated at various concentrations of the biosurfactant. The CMC was indicated by sudden changes in the surface tension, and it was obtained by plotting the surface tension as a function of biosurfactant concentration.

A conductivity test was applied to determine 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 the increasing concentration of NaCl, then the emulsion is oil in water type. On the other hand, if the conductance did not change significantly with the increasing NaCl concentration, so the emulsion is water in oil type

The emulsification index (E24) of the biosurfactants was performed by the addition of 0.1 mg of biosurfactant into a screw-capped tube containing 1 mL of distilled water and 1 mL of hydrocarbons. The mixture was vortexed for 2 mins and allowed to stand for 24 h. The E24 of the emulsion was observed for 30 days.

Data analysis

Optimization conditions of biosurfactant production were determined based on the parameters of cell growth (optical density), surface tension, and emulsification index. Experiments were performed in triplicate and Duncan statistical analysis was applied. A difference was considered statistically significant if $p \leq 0.05$. The type of biosurfactant was determined using TLC analysis by spraying a specific reagent that gives a certain color. FT-IR spectrum indicates a specific absorbance for a certain functional group.

RESULTS AND DISCUSSION

The growth media for the production of biosurfactants using *L. lactis* contained NB and NaCl, with or without the addition of CPO at various concentrations (5, 10, and 20% v/v). Bacterial growth was observed daily using a UV-Vis spectrophotometer at 600 nm for 12 days. The optimum conditions for biosurfactants were determined by measuring surface tension and emulsification index (Fig. 1). The optimum condition for biosurfactant production was obtained in media containing 10% v/v CPO (NBCPO 10% v/v) at 8 days of fermentation.

Extraction of biosurfactants was performed using a solvent mixture of chloroform: methanol (13:3). The bottom layer was chloroform containing biosurfactant while the top layer was methanol containing fermentation medium. The organic phase containing the biosurfactant was evaporated to remove the solvent. The result showed that the production of brown biosurfactant was 80 mg from one Liter of fermentation media.

Spraying a mixture of anisaldehyde: sulfuric acid: glacial acetic acid (1:2:100, v/v) on the TLC plate showed the presence of sugar moiety indicated by a purple spot. Spraying iodine vapor on the TLC plate showed the presence of a yellow spot as an indication of lipid moiety. Spraying 15% (v/v) H_2SO_4 in ethanol on the TLC plate showed the presence of glycolipids as indicated by a brown spot. Spraying of ninhydrin reagent on the TLC plate did not produce any color. The functional groups of biosurfactants were analyzed by FT-IR spectrophotometer. The FT-IR data was presented in Fig. 2 and Table 1.

The CMC value of biosurfactants was determined by measuring the surface tension of the emulsion with varying amounts of biosurfactant addition. The emulsion system was determined by measuring the electrical conductivity of the emulsion before and after the addition of an electrolyte, NaCl (Table 2). The electrical conductivity of aquadest increases as the amount of NaCl increased. In contrast, the electrical conductivity of palm oil was zero, since NaCl is not dissolved in palm oil. The electrical conductivity of the emulsion remained at 0.1 µS even though the amount of NaCl added into the water and palm oil emulsion increased (Table 2). The emulsification index and emulsification stability using various hydrocarbons, such as soybean oil, palm oil, sunflower oil, olive oil, hexane, benzene, petroleum ether, lubricating oil, diesel oil, and kerosene were determined (Table 3). The emulsification index of water and hydrocarbons with the addition of Sodium Dodecyl Sulfate (SDS) as a synthetic commercial surfactant was determined and used as control.

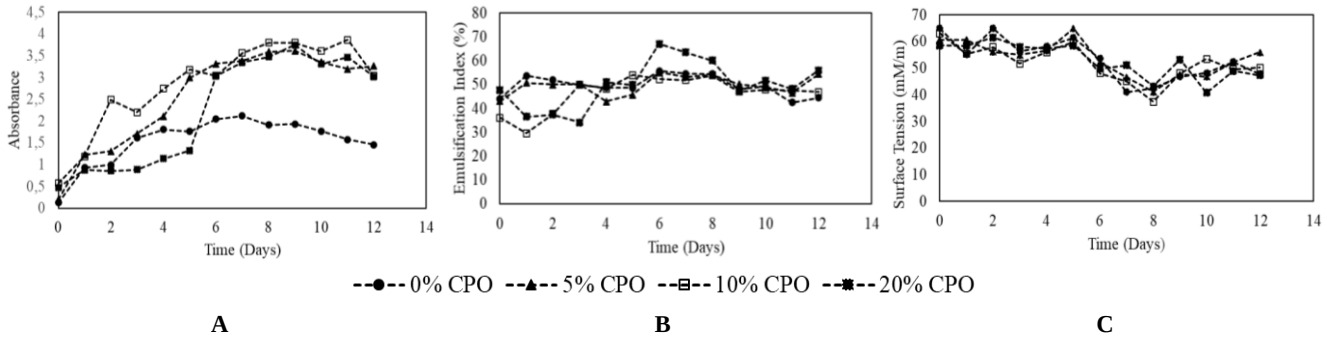

Figure 1. Optimization of biosurfactants production by *Lactobacillus lactis* grown in media with and without the addition of CPO: A. Cell growth (optical density), B. Emulsification index, C. Surface tension

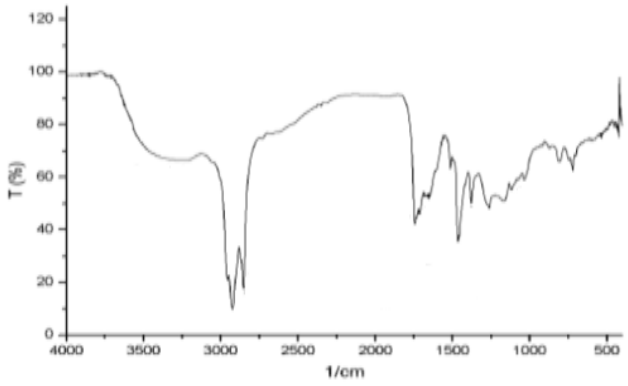

Figure 2. FT-IR Spectra of produced biosurfactant

Table 1. FT-IR peaks of produced biosurfactant

Wavelength (cm^{-1})	Functional groups
3211-3135	-OH
2923	C-H, methylene (-CH2-)
2851	
1743	C=O ester
1710	C=O carboxylate
1463	C-H,methyl (-CH3)
1377	
1261	C-O carboxylate
1118	C-O ester

Discussion

Optimization conditions for biosurfactant production

The growth of bacteria in four different media containing different amounts of CPO showed that the bacteria immediately reached an exponential stage and followed by a stationary stage. Overall, the bacterial growth was higher in media containing CPO. The addition of 10% v/v CPO resulted in the highest bacterial growth. At eight days of incubation, the bacterial reached a stationary stage. Therefore, the best time for harvesting biosurfactants was at eight days of incubation. The stationary stage in *L. paracasei* was characterized by a decrease in surface tension (Gudina et al. 2010). Statistical analysis revealed that the NBCPO 5% v/v and NBCPO 10% v/v media were in the same subset, indicating that those media had the same effect. However, NBCPO 10% v/v media had the highest cell density, therefore NBCPO 10% v/v media was chosen as an optimum medium.

The decrease in surface tension is one of the criteria for producing biosurfactants. The lowest surface tension was selected as the optimum condition for biosurfactant production. According to Francy et al. (1991), a decrease in surface tension of more than 10 mN/m indicates the potential of bacteria to produce biosurfactants. In this study, there was a decrease in surface tension of more than 10 mN/m on day eight of incubation, indicating that *L. lactis* produced biosurfactants. Figure 1 showed the lowest surface tension of NBCPO 10% (v/v) was obtained on day eight of incubation.

Table 2. Electrical conductivity of samples for emulsion type determination

Sample	Electrical Conductivity (µS)						
	Without NaCl	With the addition of NaCl (mg)					
		5	10	15	25	50	100
A. Aquadest	9.95	681	1033	1449	1897	3053	4036
B. Palm oil	0	0	0	0	0	0	0
C. Produced biosurfactant	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table 3. Emulsification index of emulsion with various immiscible liquids in the presence of produced biosurfactant

Immiscible liquids	Emulsification Index (%)					
	Days					
	1	6	11	20	25	30
Soybean oil	52	36	36	32	32	32
Palm oil	41	18	16	14	14	0
Sunflower oil	43	21	0	0	0	0
Olive oil	49	33	11	11	11	7
Hexane	13	7	0	0	0	0
Petroleum ether	19	7	0	0	0	0
Lubricant oil	71	63	60	60	60	58
Diesel oil	7	7	0	0	0	0
Benzene	24	21	14	14	14	0
Kerosene	23	7	0	0	0	0

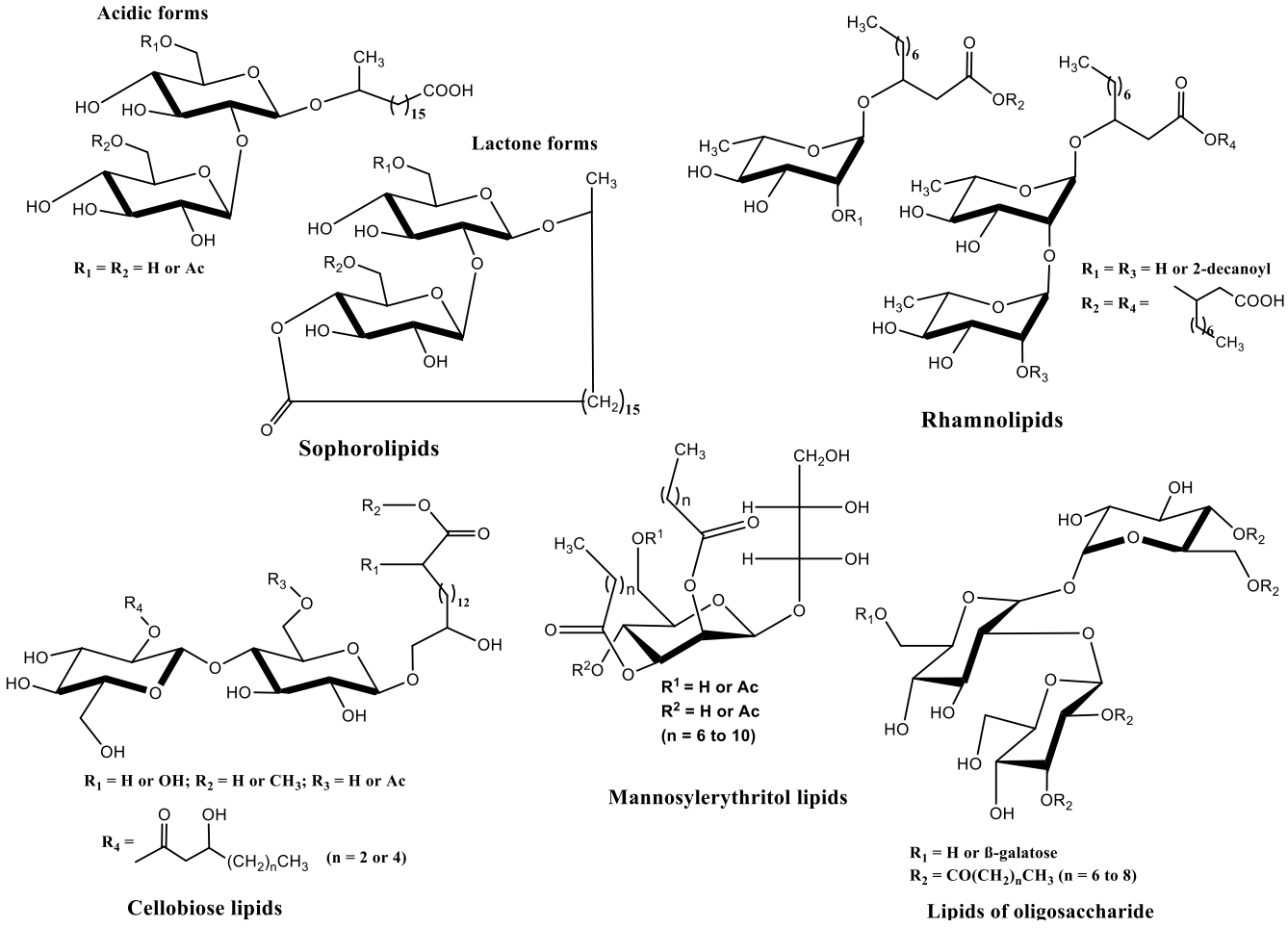

Figure 3. Types of known glycolipid biosurfactants (Ines and Dhouha 2015)

Two immiscible substances can form an emulsion with the addition of a surfactant. A good biosurfactant produces a relatively high emulsification index. In this study, palm oil was used as a hydrocarbon source. On day eight, the NBCPO 10% v/v media had an emulsification index similar to the other media (Fig. 1). Based on Duncan's statistical test, all media were in one subset, demonstrating they had the same effect on the emulsification index. However, on day eight of incubation, the NBCPO 10% (v/v) media had the highest average emulsification index. Media containing 10% CPO (v/v) that was incubated for eight days resulted in the best parameters for biosurfactants, such as bacterial growth, surface tension, and emulsification index. Brown biosurfactant was produced by *L. lactis* cultivated in media containing 10% v/v CPO with eight days of incubation.

Identification of biosurfactant structure

TLC analysis showed that the produced biosurfactant was glycolipid biosurfactant which consists of sugars and lipids. Fig. 3 shows types of known glycolipid biosurfactants (Ines and Dhouha 2015). The FTIR spectra of produced biosurfactant showed a band at 3211-3135 cm^{-1} for -OH absorption. There was a band at 1743 cm^{-1} for C=O ester absorption which was supported by a C-O absorption at 1118 cm^{-1}. A band at 1710 cm^{-1} was for carboxylate absorption which was supported by C-O absorption at 1261 cm^{-1}. Absorption at 2923 and 2851 cm^{-1} are typical absorptions of aliphatic hydrocarbons which are reinforced with wavenumbers of 1463 and 1377 cm^{-1} for methyl absorptions. The FT-IR analysis showed that the produced biosurfactant has functional groups of hydroxy, carboxylate, ester, methyl, and methylene. The functional group of produced biosurfactants had similarities with the functional group of glycolipid-type biosurfactants produced by *L. casei*. The hydrophobic part of biosurfactants generally contains fatty acids, whereas the hydrophilic part consists of phosphates, carboxylate, hydroxyl, carbohydrates, peptides, or proteins (Sharma 2016). In this case, the produced biosurfactant had a carboxylate group as a hydrophilic part and an aliphatic as a hydrophobic part.

Characterization of biosurfactants

In CMC determination, the initial surface tension was 66.30 mN/m, then decreased gradually and became constant at 49.46 mN/m. The obtained CMC value was 1

g/L. Biosurfactants produced by LAB have a CMC value in the range of 1.0 to 20 g/L (Sharma et al. 2016). The produced biosurfactant has a CMC value that was in the CMC range of biosurfactants produced by LAB. Biosurfactants produced by *Pseudomonas aeruginosa* (Schröter 1872) Migula 1900 and *Bacillus pseudomycoides* Nakamura 1998 have CMC values of 87.47 and 56 mg/L, respectively (Li et al. 2016; Gamez et al. 2017). Some of the CMC values of biosurfactants produced by LAB are not as efficient as those of the genera *Bacillus* and *Pseudomonas* (Mulligan et al. 2001). The CMC value of biosurfactants is different due to structural differences such as the presence of unsaturated bonds, aliphatic and branching chains, and hydrophilic size (Haba et al. 2002; Rahman et al. 2010).

The electrical conductivity of the emulsion before and after the addition of the electrolyte remained unchanged. It indicated that the emulsion system was water in oil (w/o) type. This system is similar to the emulsion system of a glycolipid type biosurfactant, Mannosylerythritol Lipid-A, from *Pseudozyma antarctica* which also forms water in oil (w/o) emulsions (Worakitkanchanakul et al. 2008). Water in oil (w/o) type emulsions of surfactants can be used in various applications such as cosmetic formulations, foods such as salad dressings, food texture improvement, and low-fat creams (Sharma 2016).

The initial emulsification indexes for the emulsion of water and hydrocarbons, such as soybean oil, palm oil, sunflower oil, olive oil, and lubricant oil were in the range of 41-71%. On day 25th, the emulsification index of biosurfactants of the water emulsion with those five hydrocarbons was 32, 14, 0, 11, and 60%, respectively. The emulsion between water with lubricant oil was stable for 30 days with an E24 value of 58%. Meanwhile, the addition of biosurfactant into a mixture of water and hexane, petroleum ether, diesel, or kerosene had a relatively low emulsification index and was stable only up to 6 days. A previous study showed that the addition of biosurfactant by *Lactobacillus jensenii* Falsen et al. 1999 resulted in an emulsification index between water and hydrocarbons, such as kerosene and hexane of 62%, diesel of 28.3%, and olive oil and sunflower oil between 61-70%. The biosurfactant produced by *Lactobacillus gasseri* Lauer and Kandler 1980 forms a high emulsification index value between water with olive oil and sunflower oil, while the emulsification index for kerosene was 28% (Morais et al. 2017). In terms of emulsion stability, the addition of biosurfactant was able to form a stable emulsion up to 30 days for the emulsion of water and soybean oil, olive oil, and lubricant oil. These results were similar to the research conducted by Sifour et al. (2007). The addition of biosurfactants to water emulsion with vegetable oils (olive oil, corn oil, and sunflower oil) were more stable than water emulsions with hydrocarbons (petroleum, kerosene, paraffin, hexane, octane, dodecane, and heptadecane). Radhakrishnan et al. (2011) reported that the addition of biosurfactants to the emulsion of water with vegetable oil formed an emulsion up to 90 days which was more stable than kerosene (30 days).

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