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Whole Cell Immobilization of Antibacterial Biosurfactant Producing Bacteria in Oil Palm Empty Fruit Bunch (OPEFB)

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Abstract

Whole cells immobilization is one of the methods to produced biosurfactant economically because no need to add the new pre-culture. This method has many advantages such as more stable in bioreactor production, could prevent cell release, and reduced dead cells replacement cost. Otherwise it was rapid, nontoxic, cheap, and versatile. PS109 isolate identified has the closest relation to Pseudomonas aeruginosa NBRC 12689. This isolate could produce biosurfactant using OPEFB medium and has antibacterial effect to Staphylococcus aureus. Immobilization of PS109 isolate in OPEFB showed the biosurfactant activity had the best activity until the second culture which was characterized by widened droplet shapes and clear zones produced in the oil spreading test, as well as decreased of surface tension, increased in emulsification activity, and also stability in cell numbers after washed by 0.85% NaCl. In conclusion, PS109 isolate which capable of producing biosurfactant can be immobilized in OPEFB and started to be stable on third until fourth culture cycle.

Keywords: *Antibacterial, immobilization, OPEFB, Pseudomonas aeruginosa, rhamnolipid*

INTRODUCTION

Biosurfactants are comprised by a hydrophobic group mainly constituted by fatty acids of alcohols, and hydrophilic component of polysaccharides, proteins or peptides, which make them have amphipathic structure [22]. The amphipathic moieties could partition at physical interfaces to reduce surface and interfacial tensions and form microemulsions. Some of biosurfactants applications are in the petroleum as EOR (Enhanced Oil Recovery), food (as emulsifiers), pharmaceutical (formulation of moisturizers, creams, and medicines), medical (antimicrobial agents), agricultural (fertilizers), and civil (waste and sewage treatment) industries [24]. Biosurfactants groups, such as glycolipids and lipopeptides, can damage the cell membrane, leading to lysis and consequent apoptosis, thus inhibiting the proliferation of cancer cells [24]. Production of biosurfactants in large scales still not widely done since the minimum yields. New methods have to be developed to minimize the production costs.

Whole cell immobilization is one of the techniques for economical production due to no need to restock bacterial cells [18]. The advantages of the whole cell immobilization are more stable in the bioreactor than the free cells. Immobilization can prevent the release of bacterial cells from matrix so the cell concentration is higher, reduce the cost of dead cells replacement, and increase higher metabolic activity for longer time in bioreactor [16]. Immobilization also an effective way to separate the product formation phases while the bacterial growth and it has big advantages in the continuous production of biosurfactant [12]. Immobilization of Nocardiopsis VITSISB (KC958579) in soybean meal were potential to be used as hydrocarbons biodegradation, because it could degrade the engine oil in laboratory test. The use of immobilized biosurfactant producing organism is a rapid, nontoxic, cheap, versatile method [23].

The carrier has some characteristics to be used for immobilization on biosurfactant production. Several studies have used biomass carriers for immobilization, such as sponge fiber, sugar cane, rice husks, corn cobs, cocopeat, sunflower seed shells, and cotton fibers [9]. Bacterial consortium that consists of Pseudomonas sp., Serratia sp., Flavobacterium sp., Chryseomonas sp., Xanthomonas sp., Agrobacterium sp., Arthrobacter sp., Bacillus sp., and Micrococcus sp. has the ability to attached on oil palm empty fruit bunches and rice husks [13]. Chemical analysis of OPEFB nutritional contents rich of minerals which consist of nitrogen (3.06%), potassium (4.74%), calcium (3.32%), magnesium (0.79%), and phosphorus (0.37%) [14]. As the nutritional content description, OPEFB has potential to be carrier for bacterial cell immobilization and also as a substrate for bacterial growth. However, until now immobilization of one bacterial species using OPEFB as a carrier has not been carried out. Therefore, this research aims to immobilize bacteria that have the potential to produce biosurfactants with antibacterial activity in OPEFB.

RESEARCH METHODS

Strain and Culture Medium

Isolate PS109 was used to produced biosurfactants. Bacteria was prepared on LB (Luria Bertani) agar medium, meanwhile LB broth was used to prepared seed culture. The medium that used for biosurfactant production contatined 11.25 g/L glucose, 11.25 g/L NaCl, 1.47 g/L $CaCO_3$, and 13.00 g/L OPEFB (modif. Chandrakar and Gupta 2019). In antibacterial experiments, the tested bacteria were *Escherichia coli* and *Staphylococcus aureus* that received from Indonesian Culture Collection (InaCC). Mueller Hinton Agar (MHA) was used for antibacterial test. Blood agar was used for hemolytic assay.

Biosurfactant Production

Seed culture of isolate PS109 on Luria Bertani (LB) incubated with culture conditions 30 °C and 150 rpm for 24 hours. Bacteria were grown in 100 mL of LB broth. The bacterial culture was used as a preculture 10% (v/v) of the TKKS culture medium. Bacteria were cultured under similar conditions for 7 days. After 7 days, the bacterial culture was centrifuged at 10,000 rpm for 10 minutes at room temperature.

Biosurfactant Activity Testing

Hemolysis test, drop-collapse test, oil-spreading assay, and surface tension measurements were carried out to determine biosurfactant activity. The isolate was streaked onto blood agar media then incubated for 24 – 48 hours to observe the clear zone produced by the bacteria [2]. The drop-collapse test was carried out by observing the shape of the droplets spreading on a plate coated with parafilm. If the droplet shape is spread out, then positively has biosurfactant activity, while the droplet shape is convex, then there is no biosurfactant activity. The positive control used was soap solution, while distilled water was used as a negative control. The oil spreading test uses 20 mL of distilled water in a petri dish with 2 mL of used oil added to the top, then 2 mL of bacterial cell-free supernatant is dropped and the spreading zone formed is observed [19]. Surface tension measurements were carried

out using Plate Method, Tensiometer K20 Kruss. Emulsification index measured by vortexed 1:1 (v/v) palm oil and supernatant free cells for 2 minutes then left for 24 hours.

Antibacterial Activity Test

The bacterial cell-free supernatant was extracted using two different solvent ethyl acetate and methanol at ratio of 1:1 (v/v), then the mixture was shaken for 15 minutes in a separating funnel. The mixture was allowed to separate after that the organic phase evaporated with water bath with temperature 40 °C, cooling temperature of 6 °C, and rotation speed of 70 rpm. The extract obtained was then freeze-dried at -80 °C. The dried extract was dissolved in 50% (v/v) methanol to obtained concentrations of 100 mg/mL and 50 mg/mL. Antibacterial activity tests were carried out on Staphylococcus aureus and Escherichia coli using the disk method on MHA media. The positive control is Chloramphenicol 1 mg/mL, while the negative control is the extract solvent.

Identification of Bacterial 16S rRNA

DNA multiplication was carried out using PCR mix with composition 5µL MyTaq, 0.5µL primer 1492R, 0.5 µL primer 27F, and ddH2O added to a volume of 2.5 µL. The PCR mix was added into a 1.5 mL microtube contained bacterial inoculum. The PCR cycles consisted of a pre-denaturation step of 95°C 1 minute, denaturation 95°C 30 seconds, annealing 53°C 30 seconds, extension 72°C a minute, final extension 72°C 5 minutes, and cooling at 4°C. The PCR cycle was carried out 30 times. DNA quality testing uses electrophoresis and continues with 16S rRNA analysis. The sequencing results obtained were then blasted into the NCBI Gene Bank and a phylogenetic tree was constructed using MEGA 11.

Characterization of Biosurfactant Compounds

Thin Layer Chromatography (TLC) was used for initial characterization of biosurfactant compounds contained in the extract. Rhamnolipid and dry extract were dissolved in absolute methanol to a concentration of 1 mg/mL. The mobile phases used were chloroform, methanol, and water with a volume ratio of 65: 25: 4 respectively. After drying the plate was sprayed using DAP (diaminopimelic acid) and heated in an oven at 90 °C [20]. Next, functional group analysis was carried out using FTIR (Fourrier Transform-Infrared) at a wavelength of 4000 cm-1 to 400 cm-1 [27].

Immobilization in OPEFB

Isolate PS109 precultured in 100 mL of LB media for 72 hours at 30 °C using a rotary incubator shaker with a speed of 150 rpm. The precultured was transferred 10% (v/v) to larger volume of immobilization medium and incubated under similar conditions for seven days. Every 7 days the culture was harvested and replaced with new substrate without adding new biomass. Before adding liquid media, the biomass was rinsed using physiological NaCl (0.85% w/v). The harvested culture was centrifuged and the bacterial cell-free supernatant was tested for biosurfactant activity using drop-collapse test, oil dispersion test, emulsification index, and surface tension measurements. The number of colonies produced by each culture was calculated with the Total Plate Count (TPC) method.

$$E24 = \frac{Emulsion\ height}{Total\ height}\ x\ 100\%$$

RESULTS AND DISCUSSION

Morphology and biosurfactant characteristics

Isolate PS109 belongs to Gram-negative bacteria group. It has form red rods from the Gram staining results (Figure 1). This isolate has biosurfactant activity which is characterized by the formation of a clear zone on the blood agar medium in the form of β-hemolysis, a clear zone on the oil spreading test, and widened droplet shape on the parafilm when the droplet shape is tested (Figure 1). These tests are commonly used as preliminary screening method of biosurfactant.

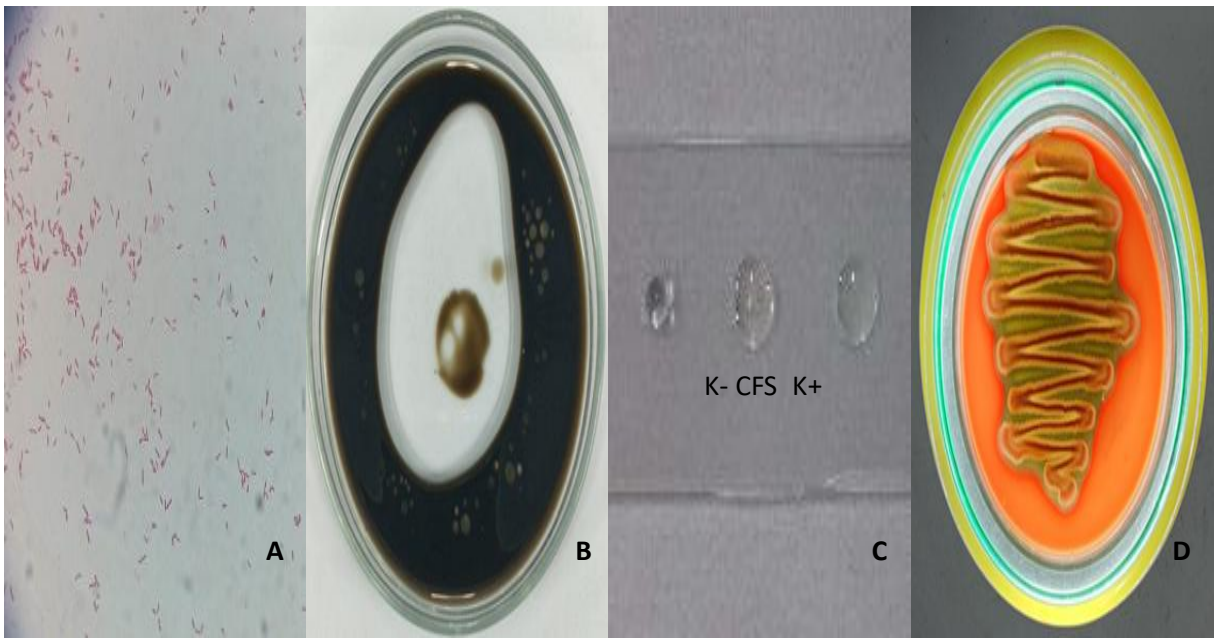

Figure 1: Morphology and biosurfactant characteristics of PS109 isolate. Bacterial microscopy morphology (A), clear zone on oil-spreading assay (B), shapes of drop-collapse test (C), and β-hemolytic activity (D)

Antibacterial Activity

The PS109 bacterial supernatant was extracted using methanol and ethyl acetate and then tested for antibacterial activity. The extract was dissolved in 50% methanol (v/v) and the best antibacterial activity observed could inhibit Staphylococcus aureus growth was from OPEFB-EtOAc extract in 100 mg/mL concentration. Clear zone diameter observed was 0.77 ± 0.01 cm, this equivalent to the positive control 10 mg/mL chloramphenicol with a clear zone produced 0.77 ± 0.12 cm (Figure 2, Table 1). Scanning Electron Microscopy (SEM) observations of rhamnolipid derived from *Pseudomonas gessardii* M15 against Multiresistant *Staphylococcus aureus* (MRSA) [5]. Pathogenic cells that are treated at the MIC level experience more changes in cell morphology, loss of cell integrity, and followed by leakage cytoplasmic components of the cell.

Table 1: Antibacterial activity from PS109 on OPEFB and LB culture extracted with MeOH and EtOAc against *Staphylococcus aureus* and *Escherichia coli*

Extract name	Concentration (mg/mL)	*S. aureus*		*E. coli*	
		Clear zone (cm)	Inhibition Index	Clear zone (cm)	Inhibition Index
OPEFB-EtOAc	100	0.77±0.01	0.54	-	-
	50	0.73±0.12	0.46	-	-
OPEFB-MeOH	100	-	-	-	-
	50	-	-	-	-
LB-EtOAc	100	0.77±0.02	0.54	-	-
	50	-	-	-	-
LB-MeOH	100	-	-	-	-

	50	-	-	-	-
Chloramphenicol	10	0.77±0.12	0.54	2.18±0.18	3.36
MeOH 50%		-	-	-	-

(-) no inhibition activity (no clear zone)

The antibacterial test against E. coli did not show clear zone because Gram-negative bacteria have a selective outer membrane without bacteriocidal activity. Glycolipid groups such as rhamnolipids have better antibacterial activity in Gram-positive group [25]. That was influenced by the composition of Gram-negative bacteria cell membrane have cell envelope on the outer membrane composed of lipopolysaccharides and phospholipids, then peptidoglycan, and inner plasma membrane. These structures are more complex and protective compared to Gram-positive bacteria [25]. Rhamnolipid concentration at MIC (Minimum Inhibitory Concentration) can also have an influence on antibacterial activity. Increasing the rhamnolipid concentration affects the release of LPS (lipopolysaccharide) from E. coli so that it can increase the permeability of the outer membrane which results in cell lysis [26].

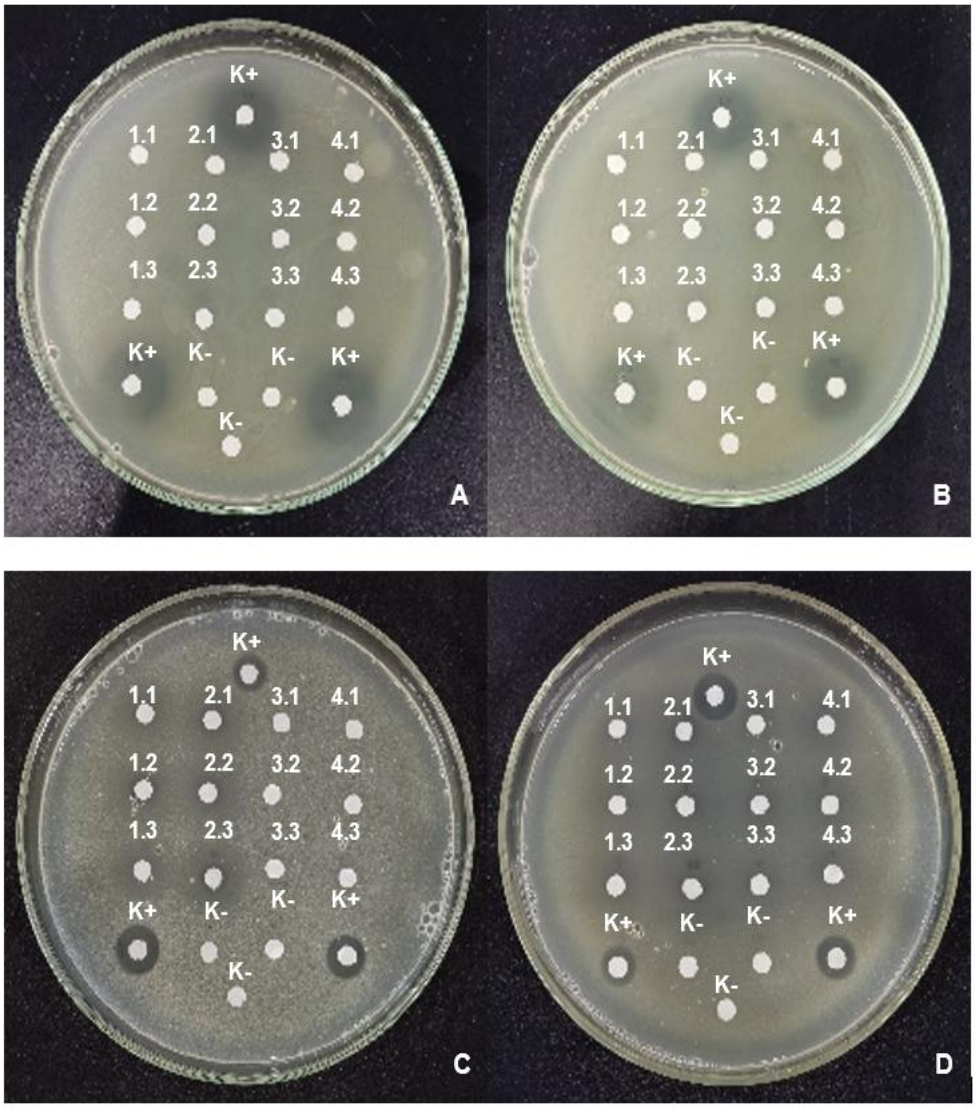

Figure 2: Antibacterial test results against E. coli and S. aureus using PS109 extracts (1 mg/mL in 50% MeOH (v/v)) from LB and OPEFB culture medium and different extraction solvents MeOH and EtOAC. Description: (A) OPEFB/E. coli, (B) LB/ E. coli, (C) OPEFB/S.aureus, and (D) LB/S.aureus. Extract name:1 EtOAc-100mg/mL, 2 EtOAc-50mg/mL, 3 MeOh-100mg/mL, 4 MeOH-50mg/mL, K+ Chloramphenicol 1mg/mL, and K- MeOH 50%

Bacterial Identification Using 16s rRNA

Isolate PS109 discovered have the ability to produced biosurfactants with antibacterial activity, 16S rRNA analysis was carried out with 27F and 1492R primers. The results of 16S rRNA gene electrophoresis shows a band measured approximately 1500 base pairs (Figure 3). Based on the phylogenetic tree, PS109 belongs to *Pseudomonas aeruginosa* with the closest relationship to *Pseudomonas aeruginosa* NBRC 12689 (NR_113599.1). The result described percentage identity value of 99.66%, E-value 0.0, and number of base pairs of 1461 bp (Figure 3). *P. aeruginosa* already known to have ability for biosurfactant compounds production from rhamnolipid group. Some applications of rhamnolipids in the industrial sector includes bioremediation and EOR (Enhanced Oil Recovery), pharmaceuticals and therapeutics, especially for Gram-positive bacteria such as *Bacillus cereus*, *Micrococcus luteus*, *S. aureus*, and *Listeria monocytogenes*. Rhamnolipid also also known to have roles in cosmetics, detergent and cleaner industries, as well as in agriculture to improve soil quality by increased the absorption of fertilizer and nutrients [21].

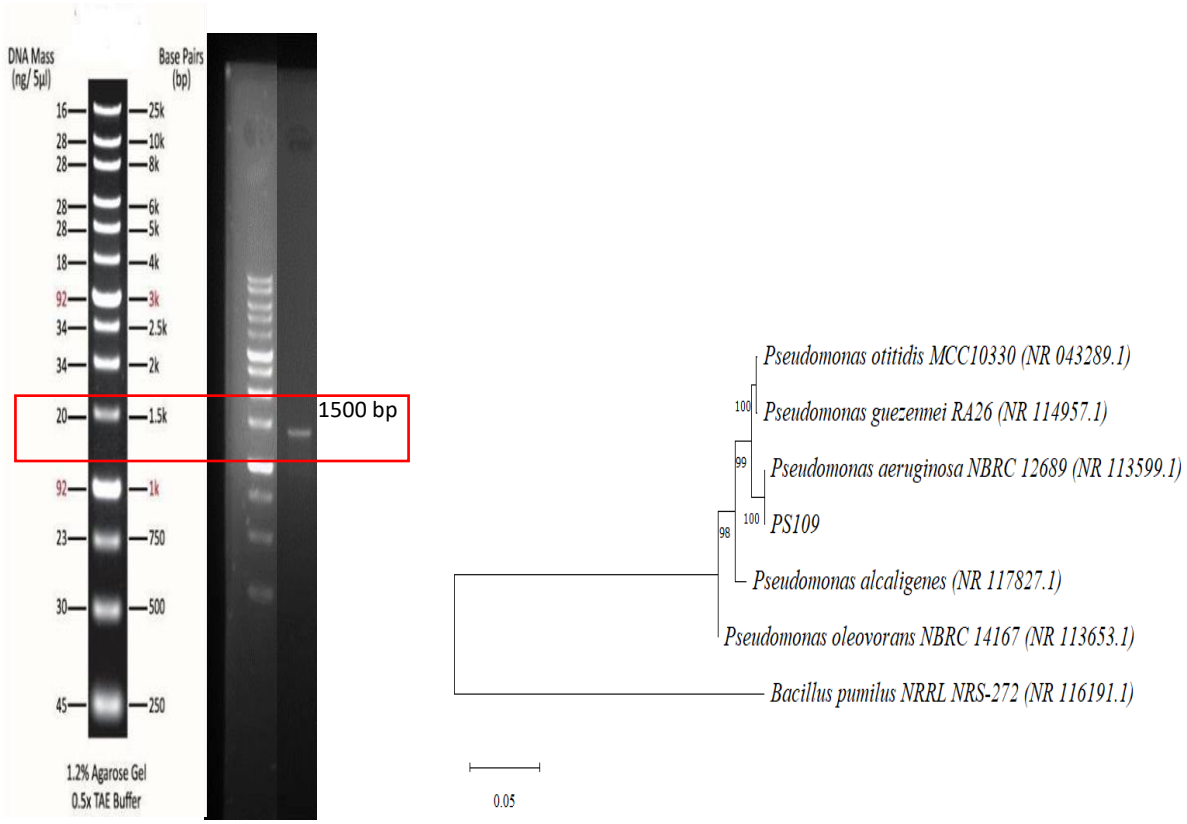

Figure 3: Electrophoresis on 1.2% agarose and 0.5x TAE (w/v) (left) and phylogenetic tree construction using outgroup *Bacillus pumilus* NRRL NRS-272 with 1000x bootstrap and Maximum Likelihood method on Mega11 (right)

Characterization of Biosurfactant

Characterization of biosurfactant compounds using TLC with 95% rhamnolipid standard (AGAE) showed there were bluish stain produced with Rf value of 0.36 and 0.58 which was parallel to OPEFB with EtOAc solvent (Figure 4). That proved the presence of monorhamnolipids and dirhamnolipids in the extract, not like supernatant that extracted by methanol solvent. The result shows a purpleish stain with Rf 0.17 [3] with the same mobile phase, had Rf values for dirhamnolipids and monorhmanolipids of Rf 0.35 and Rf 0.78, respectively. Mono-rhamnolipids detected at Rf 0.62 and di-rhamnolipids Rf 0.59 which have antimicrobial potential against *S. aureus*, *B. cereus*, and *E. coli*, as well as against several fungal species, such as *Saccharomyces cerevisiae*, *Aspergillus flavus*, and *Aspergillus niger* [20].

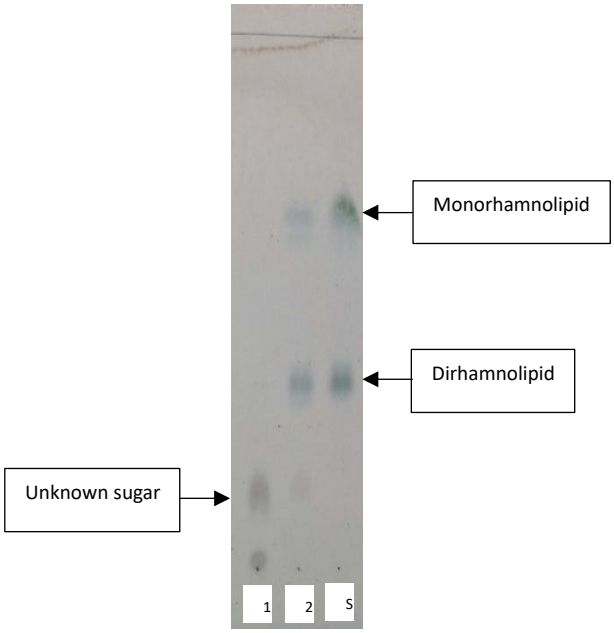

Figure 4. Thin Layer Chromatography (TLC) stain of biosurfactant extract with different growth media and extraction solvent TKKS/ MeOH (1), TKKS/ EtOAc (2), and Rhamnolipid standard 95% (AGAE) (S)

The FTIR spectrum of rhamnolipid produced from PS019 isolate OPEFB culture shows 27 peaks (Figure 5). Identification of functional groups in OPEFB culture extracts without immobilization using FTIR obtained O-H groups at absorption band 3330.84 cm^{-1} which are the characteristic of alcohol and carboxylic acid groups [10]. The absorption bands around 2954.90 cm^{-1}, 2926.66 cm^{-1}, and 2856.36 cm^{-1} were caused by stretching vibrations of C-H aliphatic groups [27]. The absorption band at 1731.44 cm^{-1} was caused by ester group [11]. Another research also described C=O groups was found at 1731 cm^{-1} [27]. Glycosidic bounds also found at wavelength 1041 cm^{-1} that described by C-O-C bonds. The structure of rhamnolipids can influence the biosurfactant activity carried out [8]. The TLC and FTIR analysis results confirmed that PS109 isolate has the ability to produce rhamnolipids using OPEFB.

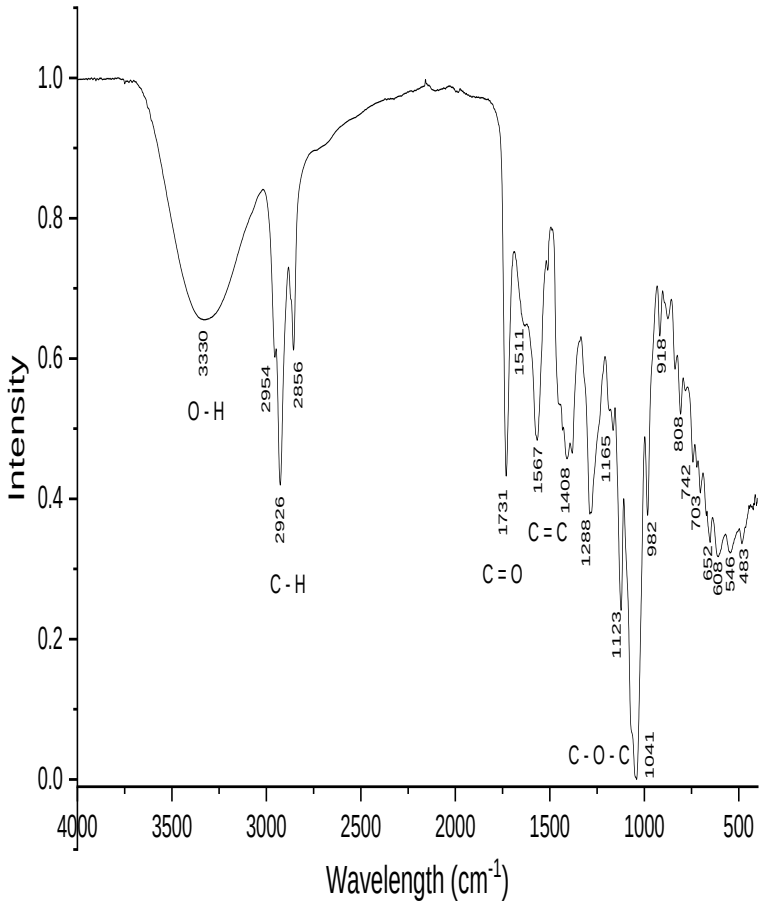

Figure 5. FTIR spectrum of rhamnolipid extract from PS109 isolate OPEFB seven days culture and EtOAc extraction

Bacterial Immobilization in OPEFB

Immobilization is the movement restriction through attachment to a solid structure [3]. The results of biosurfactant activity test after immobilization showed that the surface tension produced by supernatant culture was low but increased again after the third and fourth cycles (Table 2). *Pseudomonas aeruginosa* CGA1 has the ability to reduce the surface tension of water from 72.1 mN/m to 35.0 mN/m [1]. Based on surface tension activity (Table 3), it was known that bacteria immobilized in OPEFB are able to produce biosurfactants up to the 4th culture stage. Observation of biosurfactant activity after immobilization showed a decrease in biosurfactant activity after the second culture, due to an increase in surface tension values respectively 29.0 ± 0.00 mN/m, 27.8 ± 0.01 mN /m, 36.7 ± 0.03 mN/m, and 41.0 ± 0.05 mN/m. In contrast to surface tension activity, the emulsification index increased from 36.09% to 72.31%, even more than the emulsion ability of the positive control 61.04%.

Table 2. Biosurfactant activity after four cycles immobilization on OPEFB

Cycle[a]	Drop-collapse test	Oil-spreading assay	Emulsification index (%)	Surface tension (mN/m)	Number of viable cells (cfu/ mL)
1	++	++	36.0 ± 0.01	29.0 ± 0.00	1.7×10^9
2	++	++	40.4 ± 0.05	27.8 ± 0.01	5.7×10^7
3	+	+	48.9 ± 0.02	36.7 ± 0.03	6.5×10^6
4	+	+	72.3 ± 0.10	41.0 ± 0.05	3.8×10^6
K+	++	++	61.0 ± 0.01	28.4 ± 0.00	-
K-	-	-	0.0 ± 0.00	66.4 ± 0.00	-

[a]Every cycles contained seven days culture and the biomass washed by NaCl 0.85% after the culture harvested; (++) very good, (+) good, (-) no activity; K+: 10% soap solution, K-: water; cfu: colony forming unit.

The number of bacterial cells decreased over time in cultures. Stability began to be achieved on the third and fourth cycles, while on the first and second cycles number of colonies significantly decreased from 1.7×10^9 cfu/mL to 5.7×10^7 cfu/mL (Table 2). This happened because on the first culture, bacterial cells have been multiplied from the preculture stage so the number is very large. After the first harvested cycle, the number of colonies decreased due to the biomass already washed out by 0.85% NaCl. Cells number have not been stable because after the second washing, cells number were still high. The third cycle shows, number of the cells started to be stable around 6.5×10^6 cfu/mL and followed by cells number of 3.8×10^6 cfu/mL in the fourth cycle. Figure 6 shows attachment of the bacteria on OPEFB after 4th culture cycles meanwhile before immobilization the fiber didn't shows bacterial attachment. Immobilized consortium of bacteria in OPEFB and sawdust showed the decreased of free cells number. The number of free cells contained in the seed culture of OPEFB and sawdust 3.0×10^7 and 2.9×10^7 cfu/mL respectively, then increased by 18.9% and 23.7% in the second cycle (second week). When it reached the 4th cycle (eighth week), the number of consortium experienced a decrease in the number of colonies on both OPEFB and sawdust 3.9×10^6 and 4.4×10^6 cfu/mL respectively [13].

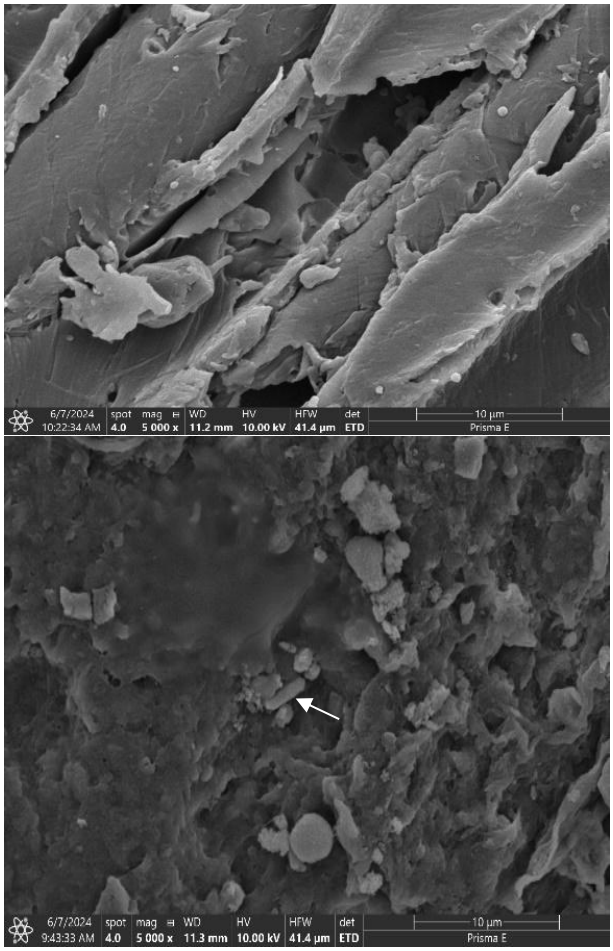

Figure 6. OPEFB without immobilization (left) and with immobilization (right) observed using 5000x magnification and *High Vacuum* 10.000 kV SEM method. The arrow shows PS109 attachment on OPEFB.

Whole cells immobilization using OPEFB shows that bacterial attachment activity occurs because there are pores on the surface of OPEFB which allow bacteria to form biofilms [17]. Empty palm oil bunches have pores (0.15 µm) with a layer of silica on the surface. Several studies used OPEFB treated with NaOH to open the pores and remove the silica layer from OPEFB [15]. Exopolysaccharide (EPS) secretion will increase when the organic compound content in the carrier is higher and this is related to the formation of biofilms in bacteria [17]. Biofilm formation is carried out as a strategy for bacteria to survive inappropriate conditions. Bacteria which lived in biofilms are protected by a matrix and supported by extracellular polymeric substances (EPS) [7]. Whole cells immobilization using OPEFB able to be applied for PS109 until fourth culture cycle because it found stable as the result of surface tension, index emulsification, and bacterial number.

CONCLUSION

PS109 isolate has the highest similarity with *Pseudomonas aeruginosa* NBRC 12689 (NR_113599.1). This isolate has ability to produce biosurfactant with antibacterial activity ainst Staphylococcus aureus. Biosurfactant characterization has found rhamnolipid type of biosurfactant by TLC and FTIR analysis. Immobilization of PS109 on OPEFB has found stable, as the bacterial number started to decreased the emulsification activity turns out increased. On the other side, the surface tension has the best activity on the second culture 27.8 ± 0.01 mN/m then increased on the third and fourth culture.

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