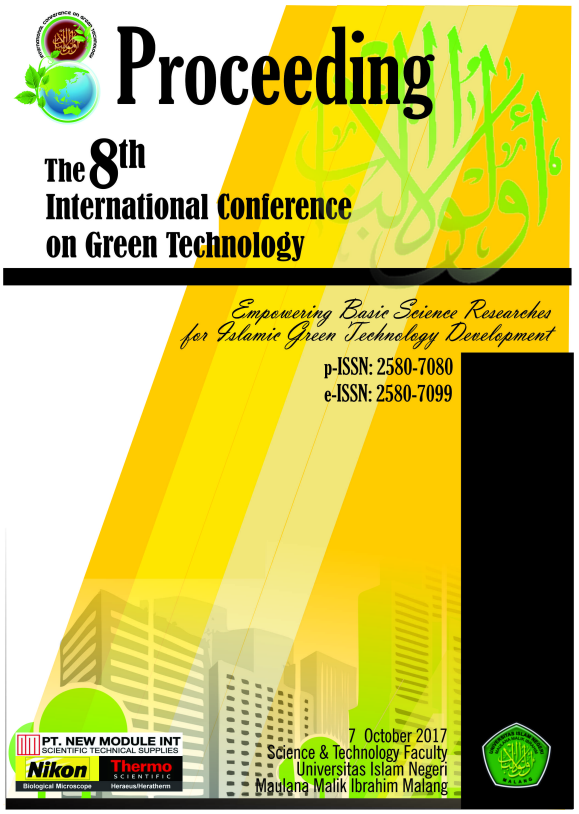

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POTENCY OF OIL SLUDGE INDIGENOUS BACTERIA FROM DUMAI-RIAU IN PRODUCING BIOSURFACTANT ON VARIATION OF SACCHARIDE SUBSTRATES

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ABSTRACT

This study aims to detect the ability of four species of Dumai-Riau oil sludge indigenous bacteria to produce biosurfactants using 3 types of substrate (D-glucose, sucrose and molasses) and to determine the relationship between the five biosurfactant detection methods. The bacteria were grown on liquid mineral medium with addition of 2% saccharide substrates. The culture was incubated for 4 days at 30^0C. Production of biosurfactants by bacteria is detected in various ways by indirectly evaluating the presence of biosurfactant in the culture supernatant by measuring the surface tension (mN / m), emulsification (%), oil spreading, drop collapse and knowing the bacteria's ability to lysis blood agar media. The study used a completely randomized design with 4 treatments and 3 replications. The data were analyzed descriptively and statistically using Anova and continued with Duncan test and Pearson correlation test was performed to test the correlation between methods. The results showed the four bacteria gave different growth responses when grown on different substrates. The ability of bacteria to produce biosurfactants also varies depending on the type of substrates. The result of surface tension method has correlation with result of oil spreading method, blood agar test and emulsification activity.

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1. INTRODUCTION

Oil sludge is one of the waste or by products from petroleum exploration and processing. Oil sludge is a hazardous and toxic substance, because of its nature, concentration and quantity that can harm the environment, and the survival of humans and other living things (Katz and Dawson, 1997). The oil sludge componentis generally toxic, mutagenic and carcinogenic (Liu, et al., 2010).

Oil mining and processing industries such as PT. Chevron Indonesia and PT. Pertamina contained in Dumai-Riau will produce sludge waste with oil pollutants in considerable amounts, where sludge is obtained from oil drilling and deposits contained in temporary oil storage tanks (Kurniasari, 2005). Oil sludge in oil storage tanks and oil processing pipelines creates mud and crust deposits which can lead to pipe blockage and affect the total volume of oil tanks.

In the oil sludge, there are indigenous microorganisms that can adapt to extreme conditions and lack of oxygen. Indigenous bacteria came from soil and sea water which able to live by utilizing petroleum hydrocarbon compounds as their carbon sources. The capabilities of these bacteria, making indigenous bacteria have an influence in the effort to clean up the oil sludge deposits on processing pipes and oil storage tanks. One of the efforts isutilize biosurfactant as cleaner of tank and oil processing pipe.

Biosurfactant is an amphiphilic compound produced from extra cells and parts of bacterial cell membranes with various substrates including sugar, oil, alkane and waste substrate which are highly potential to be produced in various industrial scales (Banat, 1999; Lin, 1996; Mulligan, 2005). A microorganism that capable to producing biosurfactant compound and a substrate are required to produce biosurfactant. Substrate as a source of nutrients for the growth of microorganisms. The saccharide substrate for the production of biosurfactants may utilize a group of water-soluble monosaccharides and disaccharides such as glucose, sucrose (Desai and Banat, 1997). Molasses is also a water-soluble complex compound containing several compounds of the saccharide group such as glucose, sucrose, fructose and other compounds (Paturau, 1982 in Suastuti, 1998).

To know the potency of indigenous oil sludge bacteria from Dumai-Riau to produce biosurfactant on variation saccharide substrate (D-glucose, sucrose and molasses), product biosurfactant is evaluated through several method, there are hemolytic activity, drop collapse, oil spreading, measurement of surface tension, and emulsification activity. This study is expected to provide information about potency of indigenous oil sludge bacteria from Dumai-Riau to produce biosurfactant on saccharide substrate for the future prospect as an oil tank cleaner.

2. RESEARCH METHOD

Materials

Microorganisms that involved in this study are the oil sludge indigenous bacteria from Dumai-Riau, those are: D1, D2, D3 and D4. Isolates are collection of Microbiology Laboratory of Airlangga University. The bacterial growth medium consisted of Nutrient Agar (NA) (Oxoid), NB (Oxoid) and Mineral Synthetic (MS) Water (Putrhi and Cameotra, 1997) that modified with (g/L): $(NH_4)_2SO_4$ 3g; $MgSO_4.7H_2O$ 0,2g; NaCl 10g; $CaCl_2$ 0.01g; $MnSO_4.H_2O$ 0.001g; H_3BO_3 0.001g; $ZnSO_4.7H_2O$ 0.001g; $CuSO_4.5H_2O$ 0.001g; $CoCl_2.6H_2O$ 0.005g; $Na_2MoO_4.2H_2O$ 0.001g. The buffer material (g/50mL) are KH_2PO_4 1g; K_2HPO_4 1g and $FeSO_4.7H_2O$ 0,0006g. The growth substrate were molasses, D-glucose (Phyto Technology Laboratories), sucrose (HIMEDIA). Then, the ingredients used for the detection of biosurfactants were agar (Oxoid), crude oil, and kerosene.

Methods

Production of Biosurfactants

Each bacterial culture of the inclined NA aged 24-48 hours was inoculated into 50 mL of NB medium. Then, it was incubated in the incubator shaker for 24 hours at a temperature of 27-30ºC to obtain $OD_{\lambda 650nm} = 0.5$. Bacterial culture is then referred to as a bacterial starter.

Biosurfactant production was performed by 4% (v/v) bacterial starter inoculated on 50 mL sterile MS medium with 2% substrate addition of D-glucose (w/v), sucrose (w/v) and molasses (v/v) on a 100 mL culture bottle separately. The culture was incubated in the incubator shaker with 120 rpm at 30°C for four days (Ni'matuzahroh et al., 2015).

Growth Assay

Quantification of bacteria was carried out from day zero to day four of incubation time using theTotal Plate Count (TPC) method with a series of dilutions.The growth test is accompanied by monitoring bacterial daily pH until the end of the incubation time. The pH measurements were performed using pH indicator and performed three replications in each bacterial culture.

Biosurfactant Detection Method

Hemolytic activity

Hemolytic activity can be done by scraping each strain of pure bacteria on blood agar medium then incubated for 48 hours at 37ºC (Youssef et al., 2004). Positive test of the method of hemolytic activity is the formation of inhibitory zone around bacterial colonies. Inhibitory zone classified as α hemolysis can be observed when around the colonies of bacteria formed greenish zone, zone β haemolysis can be observed to form a zone of white or clear around the bacterial colony. While the inhibitory zone classified as γ hemolysis has a noticeable feature there is no change in other colors around the bacterial colony (Shah et al., 2016).

Drop collapse

Drop collapse test is used to determine the ability of oil distribution by biosurfactant activity. The drop collapse test was performed on the fourth day of biosurfactant production by dripping 135 µLbiosurfactant supernatant over 100 µL of oil on a plate having a diameter of 8 mm and a height of 0.25 mm. The presence of biosurfactant is characterized by the formation of clear zone after 1-2 minutes (Jain et al., 1991).

Oil spreading

The method of oil spreading follows the procedure of Morikawa et al. (2000). Measurement of spreading oil activity was done by 40 mL of distilled water into a Petri dish (150 mm), then inserting 10 µL of crude oil on the surface of the aquades. 10µL of biosurfactant culture was added to the center of the surface of the crude oil. The diameter of the clear zone on the oil surface is measured using a sliding range.

Measurement of surface tension

Measurements surface tension (TP) were performed using a Du-Nouytensiometer. The surface tension value is expressed in units of mN/m or dyne/cm. Surface tension measurements were made on the fourth day of incubation time (Walter, 2010).

Calculation of surface tension using the formula:

$r = r_0 \frac{\theta}{\theta^0}$

Information:

r = sample surface tension

r_0 = surface tension aquades at t^0C

θ = scale of sample measurement results

θ_0 = scale of the result of aquatic size

Emulsification activity test

Emulsification activity test was done according to Suryatmana et al. (2006) by mixing 1 mL of supernatant with 1 mL of test oil (kerosene) into the test tube. The mixture was mixed for 2 minutes, then emulsification activity (AE) (%) was masured after 1 hour and 24 hours. Emulsification activity was observed on the fourth day of incubation time.

Here is the formula for calculating % emulsification activity:

$\%\text{emulsification} = \frac{height\ of\ emulsion}{total\ fluid\ volume}$ x100%

3. RESULTS AND DISCUSSION

The relationship of growth response and pH of indigenous oil sludge isolate on D-glucose, sucrose, and molasses substrate is presented in Figures 1, 2 and 3.

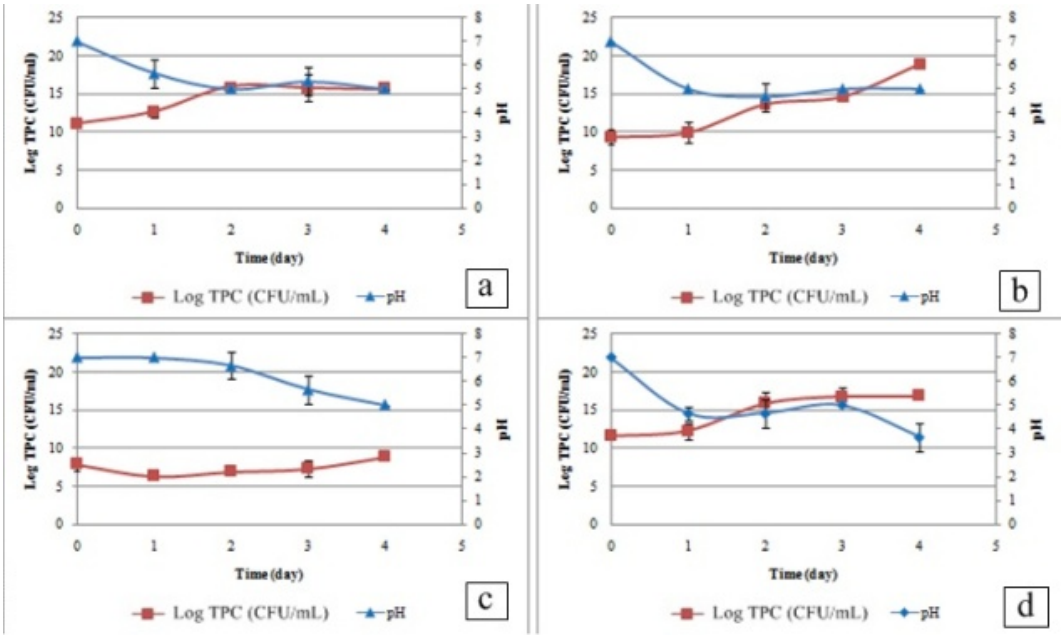

Figure 1. The growth response curve and pH of bacterial isolates (a) D1, (b) D2, (c) D3, and (d) D4 on D-glucose substrate .

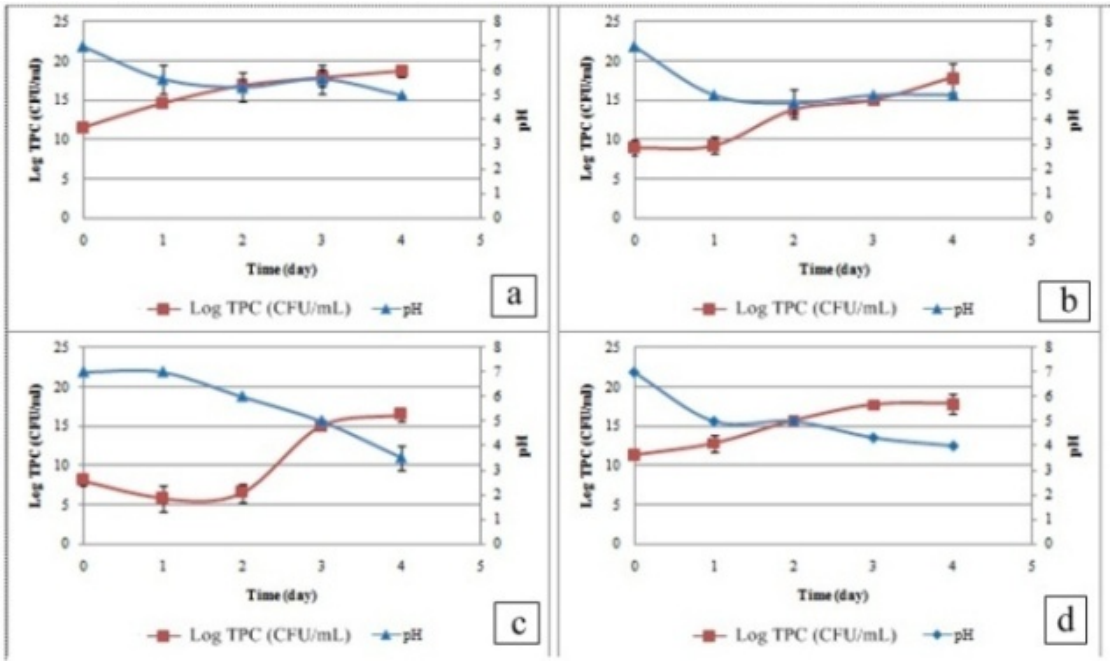

Figure 2. The growth response curve and pH of bacterial isolates (a) D1, (b) D2, (c) D3, and (d) D4 on the sucrose substrate

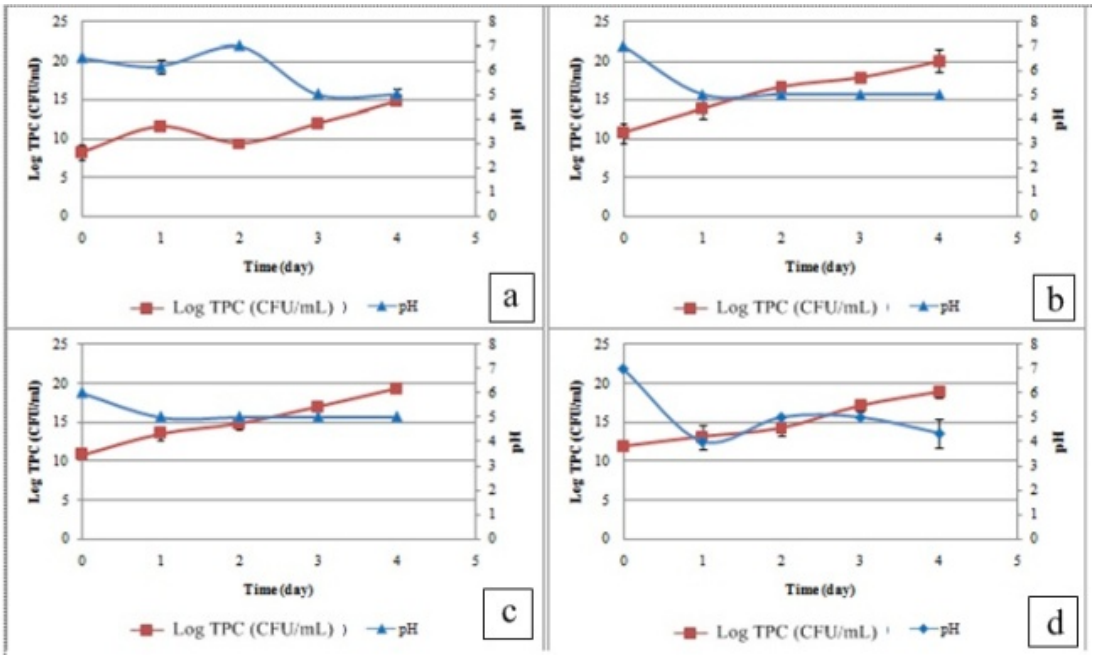

Figure 3. The growth and growth response curves of bacterial pH (a) D1, (b) D2, (c) D3, and (d) D4 on the molasses substrate

Test of hemolytic activity

The results of the hemolytic activity test of D1, D2, D3 and D4 bacteria in blood are listed in table 1.

Table 1. Results of hemolytic bacteria indigenous oil sludge Dumai-Riau bacterial activity test

No.	Bacterial Isolat	Clear Zone (cm)	Indeks of Clear Zone	Type of Clear Zone
1.	D1	1,97	1,26	α
2.	D2	0,74	0,49	ɤ-α
3.	D3	0	0	ɤ
4.	D4	**2,41**	**1,29**	**β**

D3 bacterial isolates did not have the ability to lyse blood cells as showed from the measurements of hemolytic zones as listed in table 1. Isolate D3 is classified in hemolysis zone γ because no hemolytic zone (clear zone) is formed around the bacterial colony. D1 produces a zone of α haemolysis, whereas D4 produces a zone of β hemolysis.

Drop collapse test

Drop collapse test results from D1, D2, D3 and D4 bacteria on D-glucose, sucrose, and molasses substrate are presented in Table 2.

Table 2. Drop collapse supernatant cultures of the fourth day of bacteria D1, D2, D3 and D4 on D-glucose, sucrose, and molasses substrate

Substrates	Clear Zone (cm)			
	D1	D2	D3	D4
D-glucose	**0,34 ±0,03**	0,35 ±0,09	0,26[a] ±0,02	0,22[a] ±0,04
Sucrose	0,31 ±0,01	0,28 ±0,06	0,20[a] ±0,03	0,24[ab] ±0,03
Molasse	0,21 ±0,08	0,39 ±0,03	0,36[b] ±0,06	**0,30[c] ±0,04**

The highest drop collapse value produced by D3 bacterial supernatant is 0.37 ± 0.05 cm, D1 bacteria on D-glucose substrate of 0.34 ± 0.03 cm, D2 bacteria on molasses substrate of 0.41 ± 0.05 cm and D4 bacteria on the molasses substrate of 0.30 ± 0.04 cm.

Oil spreading test

Tests of spreading oil supernatant bacteria D1, D2, D3 and D4 on sucrose substrate, D-glucose, molasses are presented in table 3.

Table 3. Result of oil spreading supernatant test of fourth day culture from bacteria D1, D2, D3 and D4 on D-glucose, sucrose, and molasses substrate

Substrates	Clear Zone (cm)			
	D1	D2	D3	D4
D-glucose	0,24 ±0,07	**0,55 ±0,15**	0,18[a] ±0,04	0,23 ±0,03
Sucrose	**0,41 ±0,13**	0,37±0,19	0,34[a] ±0,08	**0,28 ±0,09**
Molasse	0,29 ±0,03	0,26 ±0,05	**0,71[b] ±0,04**	0,22 ±0,00

The largest oil spreading value among the three other bacteria came from D3 bacteria on the molasses substrate of 0.71 ± 0.04 cm. Meanwhile, the largest oil distribution value in D1 and D4 bacteria occurred on sucrose substrate with value of 0.41 ± 0.13 cm and 0.28 ± 0.09 cm respectively, while the largest oil distribution value of D2 bacteria occurred at D-glucose substrate of 0.55 ± 0.15 cm.

Surface tension

Values and graphs of surface tension of bacteria D1, D2, D3 and D4 are shown in Fig. 4**.**

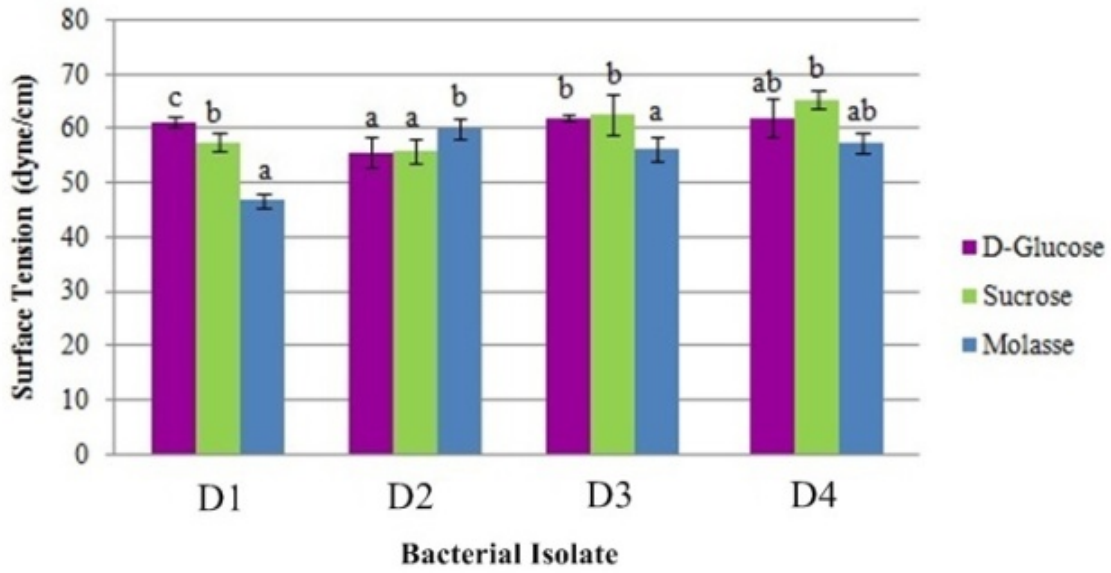

Figure 4. Diagrams of supernatant tissue surface degradation value of the fourth day of bacteria D1, D2, D3 and D4 on D-glucose, sucrose, and molasses substrate

In Figure 4, it is known that D1, D2, D3 and D4 bacteria grown on the D-glucose, sucrose, and molasses substrate have the ability to reduce surface tension to media and aquades. The average of surface tension reduction of fourthisolates on the variationsaccharide substrate is more than 10 dyne/cm.

Emulsification activity

Diagram of the value of 1-hour emulsification activity (E1) of bacteria D1, D2, D3 and D4 on D-glucose, sucrose, molasses is presented in figure 5.

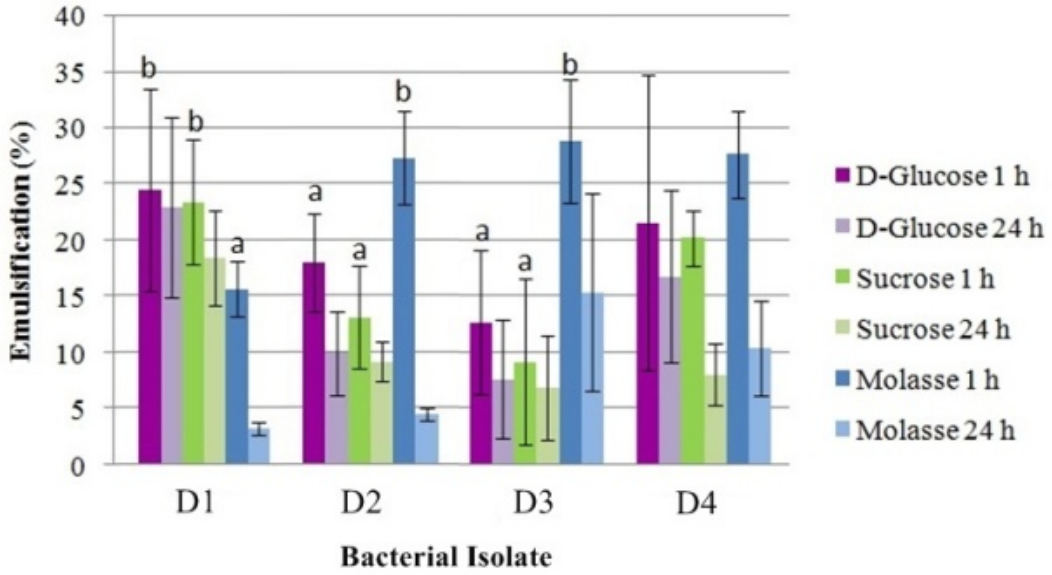

Figure 5. Diagram of 1 (E1) and 24 hour (E24) emulsification activity values of D1, D2, D3 and D4 bacteria on D-glucose,sucrose, molasses substrate with kerosene test compound.

In the D-glucose and sucrose substrate, the highest emulsification activity value was achieved by the same D1 and D4 bacteria with 24.53± 9.0% and 23.43±5.52% respectively. Meanwhile, the highest emulsification

activity value among the three other bacteria of 28.85± 5.55% occurred in D3 bacteria with molasses growth substrate. The average decrease in value of 1-hour emulsification activity to the value of the 24 hours emulsification activity is 8.56%.

The statistical test used to show the relationship between biosurfactant detection methods is Pearson correlation. Statistical test using SPSS software (version 21.0) Pearson correlation coefficient (ρ) can show the value between -1 (strong negative relationship) and 1 (strong positive relationship). Table 4 shows that on each substrate yields correlation coefficient values between different methods. In the glucose substrate, there is a relationship between oil spreading and surface tension with Pearson correlation coefficient ρ = -0,893. In the sucrose substrate, the strongest relationship (Pearson correlation coefficient ρ = 0.736) occurs between the emulsification activity and the blood agar. In the molasses substrate, several biosurfactant detection methods that show a relationship between oil spreading and blood agar (ρ = -0,776), surface tension and drop collapse (ρ = 0.738), and between surface tension and emulsification activity (ρ = 0.836) .

Comparative statistics of five screening methods

Table 4. Pearson's correlation results from five screening methods

		Pearson Correlation Coefficient (ρ)				
		OS	DC	ST	EA	BA
Glucose	OS	1	0,565	-0,893**	0,223	-0,179
	DC	0,565	1	-0,538	-0,318	-0,115
	ST	-0,893**	-0,538	1	-0,390	0,253
	EA	0,223	-0,318	-0,390	1	0,194
	BA	-0,179	-0,115	0,253	0,194	1
Sucrose	OS	1	0,043	-0,421	-0,272	-0,48
	DC	0,043	1	-0,485	0,509	0,415
	ST	-0,421	-0,485	1	-0,130	0,182
	EA	0,509	0,509	-0,130	1	0,736**
	BA	-0,48	0,415	0,182	0,736**	1
Molasse	OS	1	0,232	0,029	0,274	-0,776**
	DC	0,232	1	0,738**	0,547	-0,590*
	ST	0,029	0,738**	1	0,856**	-0,395
	EA	0,274	0,547	0,856**	1	-0,421
	BA	-0,776**	-0,590*	-0,395	-0,421	1

DISCUSSION

Biosurfactant production conducted until the fourth day aims to ensure the viability condition of bacterial cells at the time of biosurfactant production test on the fourth day of incubation time. The growth curve of D1, D2, D3 and D4 bacteria on the fourth day (figure 1) in cultures of variation of the saccharide substrate mostly occurs at the end of the exponential phase to the stationary phase. The exponential phase occurring on the fourth day of incubation time is quite possible because the growth medium is still sufficient in culture so that bacteria can still utilize the medium well for its growth. This condition is in accordance with that of Ni'matuzahroh et al. (2010), the optimum biosurfactant activity with 2% molasses substrate occurs on the fourth day as the exponential phase ends or at the beginning of the stationary phase.

Figure 1 shows that when bacterial growth in the exponential phase, the culture pH decreases. The larger the number of bacterial cells that grow in a culture the lower the pH culture. The condition is indicated that bacteria D1, D2, D3 and D4 can utilize well the saccharide substrate as carbon source in metabolism process of its growth which is accompanied by decrease of pH culture. Hoog (2005) in his research also mentions that the bacteria can

perform their metabolism by lowering the pH of the media environment due to the growth of bacteria in the growth medium. According to Pratiwi (2014), an increase in the number of bacteria along with a decrease in pH value due to the production of metabolites that can reduce the pH of the growth media environment.

Tests on biosurfactant products by indigenous oil sludge bacteria Dumai-Riau was first performed by hemolytic activity using blood. The positive test is characterized by the presence of a hemolysis zone formed. The inhibitory zone formed in the hemolytic activity test indicates the production of biosurfactant. This is consistent with that reported by Singh (2012) in his study, that the larger the diameter of lysis in the blood in order to result from the increasing concentration of biosurfactant. According to Zaragoza et al. (2010) the formation of a hemolytic zone (clear zone) in blood hemolysis tests is caused by two different mechanisms ie the dissolution of cell membranes that normally occur at high biosurfactant concentrations or due to increased membrane permeability to small soluble substances that normally occur when biosurfactant concentrations low, thus causing osmotic lysis.

After testing the hemolytic activity using blood agar, a stabilization test of surfactant droplets in an oil is coll drop and oil spreading test. According to Walter et al. (2010), drop collapse testing is associated with drip stability that depends on surfactant concentration and is related to surface and interface stresses. A supernatant containing biosurfactant will spread because of the interceptor force or tension between the supernatant and the reduced (hydrophobic) layer of the oil layer. The diameter of the clear zone produced by the oil spreading test indicates the presence of surfactant activity and there is a linear relationship between the surfactant and the resulting clear zone diameter.

Further biosurfactant detection is to use the principle of oil stabilization and water emulsion. In Figure 5, the value of D1, D2, D3 and D4 D1, D2, and D4 emulsification activity on D-glucose, sucrose, and molasses decreased from 1-hour emulsification activity (E1). This is due to the unstable emulsion properties due to the tendency of the emulsion particles to join the other particles, so the amount of emulsion produced tends to decrease at 24 hours. According to Karthik (2010), emulsification activity shows the stability of biosurfactant when incubated at room temperature.

A hydrophilic bacterial culture in contact with hydrophobic hydrocarbons (kerosene) is likely to have the ability to form emulsions that separate two distinct phases due to the high interface surface area. The spread and size of the emulsion grains may change over time. The result showed that the decrease of emulsification activity between the measurement time 1 hour and 24 hours. This is due to the unstable emulsion properties due to the tendency of the emulsion particles to join the other particles, so the amount of emulsion produced tends to decrease at 24 hours. The stability of the emulsion is proportional to the stability of the biosurfactant (emulgator) produced. As Karthik (2010) points out, the emulsification activity shows the stability of biosurfactant when incubated at room temperature.

The ability to stabilize emulsions indicates that bacteria can produce biosurfactants (Batista et al., 2006). Emulsification activity is also a mechanism used by bacteria to reach its growth substrate with the help of biosurfactants as emulsifiers produced by the bacteria itself. The value of different emulsification activities on different substrates indicates the effect of carbon sources on bacterial culture in generating emulsification. As reported by Hamed (2012), *S. aureus, Micrococcus spp., Pseudomonas sp. P. aeruginosa, Photobacter damsel* and *Chrysomonnasluteola* strains are more efficient in emulsifying mineral oil than crude oil.

The average value of the surface tension of four bacterial isolates yields a value of more than 10 dyne / cm. This indicates that four bacterial isolates are capable of producing biosurfactant as a surface active agent when grown on a variety of saccharide substrate. As reported by Ni'matuzahroh et al. (2013) that the reduction of the surface value of the bacterial supernatant of ≥10 dyne / cm indicates that biosurfactant is potential as a surface active agent. The occurrence of surface tension decline is caused by the production of surface active compounds by microorganisms (Batista et al., 2006).

According to some biosurfactant detection results, the four indigenous oil sludge bacteria Dumai-Riau are known to produce biosurfactants grown on D-glucose, sucrose and molasses substrate. The best sequence of bacteria to produce biosurfactants based on the high average values in the biosurfactant production test on all variations of the saccharide substrate were bacteria D1, D2, D3 and D4. However, the bacteria that have the most potential ability to produce biosurfactant in this study is bacteria D1. The most potential growth substrate for producing biosurfactants is the molasses substrate, evidenced by the superior values in the growth response test and biosurfactant detection of several bacterial cultures compared to D-glucose and sucrose substrate.

Correlation test results show the relationship between methods of each substrate. This suggests that growth substrate may affect the biosurfactant product, so the type of biosurfactant produced may also influence which detection method is most suitable for use. For biosurfactant detection, it is recommended to use several methods in order to obtain accurate data results by linking some results from other methods used.

CONCLUSION

The four indigenous oil sludge bacteria Dumai-Riau (D1, D2, D3 and D4) are able to produce biosurfactant on variations of saccharide substrate (molasses, sucrose, D-glucose) with clear zones of hemolytic activity, drop collapse value of 0.20-0.41 cm, the value of oil spreading ranges from 0.18-0.71 cm, the emulsification activity decrease value of 8.56% and the value of surface tension ≥ 10 dyne / cm. The average of the results of each biosurfactant production test on some bacteria, the molasses substrate has the best ability to produce biosurfactants. The most potent bacterial indigenous species produce bacterial biosurfactantA (4).

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