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Antibacterial activity of dialkyl-alginate biosurfactant cream against *Staphylococcus aureus* and *Pseudomonas aerugynosa*

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ABSTRACT

Dialkyl-alginate biosurfactant is an amphifilic rhamnolipid biosurfactant which has the potential to be developed into an antibacterial agent. The purpose of this study was to prove the biosurfactant of dialkyl alginate and it's cream formula as antibacterial activity especially against *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The antibacterial activity assay of biosurfactant dialkyl alginate at concentrations of 5%, 10% and 20% againts *Staphylococcus aureus* and *Pseudomonas aeruginosa* using modified quantitative method. Enbatic® 1% is used as a positive control and sterile aquadest as a negative control. Determination of antibacterial activity of dialkyl alginate biosurfactant followed by analysis of leakage of protein and nucleic acids using UV-Vis Spectrophotometry and leakage of Ca^{2+} and K^{+} metal ions using Atomic Absorption Spetrophotometry (AAS). The most active concentration was formulated into cream and tested antibacterial activity using the well method. The test results showed that the biosurfactants of dialkyl-alginate and it's cream formula has antibacterial activity. The concentration of 10% was the most active concentration having activity which did not differ significantly to positive control with p value of 0,05.

Keywords: antibacterial activity, alginate, biosurfactant, cell leakage, *Sargassum sp*

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INTRODUCTION

Biosurfactant is an amphiperic compound obtained from living organisms. Biosurfactants contain hydrophilic and hydrophobic groups that can reduce surface tension or interface in liquids (Irfan, 2015). Some biosurfactants in addition to having activity as an emulsifier or stabilizer, biosurfactants are also known to exhibit activity as anti-fungal, anti-bacterial and anti-viral. Biosurfactants that have been shown to have antibacterial activity include rhamnolipid produced from *Pseudomonas aeruginosa*, surfactin and iturin from *Bacillus substilis*, and sophorolipid from *Candida bombicola* (Ndlovu *et al.*, 2017).

According to Vasileva *et al.*, (2010) and Arino *et al.*, (1998) that rhamnolipid is known to be active against Gram-negative bacteria *Pseudomonas aerugynosa, Enterobacter aerogenes, Serratia marcescens* and *Klebsiella pneumonia*, as well as to Gram positive *Micrococcus sp, Streptococcus sp, Staphylococcus sp, Bacillus sp*. While the Sotirova *et al.*, (2009) study showed that rhamnolipid from *Pseudomonas sp* causes decreased levels of Lipopolysaccharide (LPS) and causes protein changes in the outer membrane of *Pseudomonas aerugynosa*.

Mahreni and Reningtyas (2015) reported the synthesizing of, a renewable biosurfactant of the rhamnolipid group, biosurfactant dialkyl alginate. This dialkyl-alginate biosurfactant is obtained from the esterification reaction of sodium alginate and stearic acid. It has the highest emulsion power of 3 macroalgae under study. The emulsion formed is stable at a concentration of 0.25% of the total volume with an interfacial stress test (IFT) of 0.8 dyne / cm. Dialkyl-alginate biosurfactant has the potential to be developed into a preparation for treating infectious disease because its properties and molecular structure are similar to rhamnolipid biosurfactants from *Pseudomonas aerugynosa*. One common and frequent infection is a topical infection of the skin that is sometimes difficult to overcome due to microorganism resistance (Handayani, 2016). One of the most positive and negative gram bacteria that causes infection and is most resistant to antibiotics is *Staphylococcus aureus* and *Pseudomonas aeruginosa*. The results of Ahmad *et al*., (2014) study show that *Staphylococcus aureus* is resistant to cafepime antibiotics (93%) and ciprofloxacin (50%), and intermediates to neomycin (28.6%), streptomycin (85.7%) and amikacin (50%). Meanwhile, based on the results of the research He *et al.*, (2012) *Pseudomonas aerugynosa* resistant to carbapenem class with 67% (doripenem-amikacin), 31% (doripenem-colistin), 23% (doripenem-levofloxacin).

The purpose of this research is to test the activity of bisorfactant cream against *Staphylococcus aureus* and *Psedomonas aerugynosa* bacteria and to conduct physical evaluation of biosurfactant cream that has been made.

MATERIAL AND RESEARCH METHODS

Materials

The materials used are Dialkyl-alginate Biosurfactant obtained from Faculty of Chemistry UPN Veteran Yogyakarta with specification of cream-colored powder, *Staphylococcus aureus* (FNCC 0047) and *Pseudomonas aerugynosa* (FNCC 0063).

Research methods

Standard Solution Turbidity Creation (Mc Farland Solution)

H_2SO_4 1% (0,36 N) solution was prepared as much as 99.5 mL / 9.95 mL and $BaCl_2.2H_2O$ 1.175% (0,048 mol/L) solution was 0.5 mL / 0.05 mL. Both solutions are mixed and stirred to form a cloudy solution. The turbidity of the test bacterial suspension to be used should be equal to the turbidity of Mc' Farland solution (equivalent to $1.5x10^8$ CFU / mL) made (EUCAST, 2015).

Antibacterial activity test by using the modified quantitative method Hammond, *et al*., (2011) and Determination of Minimum inhibitory concentrations (MIC) Value biosurfactant dialkyl-alginate.

The bacteria that has been rejuvenated for 1 x 24 hours on Nutrient Broth medium, in the pipette as much as 1 mL and diluted to 10^{-5} concentration. Five sterile blank discs are placed on the surface of the Nutrient agar plate and as much as 10 µl of the bacterial suspension from the 10^{-5} dilution are dripped onto the blank disc and allowed to seep.

The biosurfactant of dialkyl-alginate was dissolved with sterile aquadest to obtain concentrations of 5% (w / v), 10% (w / v), and 20% (w / v). For positive control, a solution of Enbatic® powder with a concentration of 1% (w /v) and negative control was used sterile aquadest. Each solution was dripped onto a 1 x 1 cm sterile gauze evenly and then the disc was covered with the screen. All petri dishes were incubated at 37 ° C for 8, 12 and 24 hours.

The bacterial inhibition effect was observed after 8, 12 and 24 h of treatment. The disc is separated from the screen and washed with Phosphat Buffer Saline (PBS) 3 times while in the vortex for 2-3 minutes for each washing until the bacterial cells are detached from the disc. The disk washings were diluted with PBS to obtain a small amount of bacteria, then 10 µl of the solution was inoculated on the Nutrient agar plate and incubated for 24 hours.

The number of growing colonies was calculated for each treatment, then analyzed and determined the minimum inhibitory concentration of the number of colonies obtained. The lowest concentration that can inhibit bacterial growth is defined as the minimum inhibitory concentration (MIC). The number of CFUs / disks is calculated using the formula: (CFU counts x dilution factor x 100) (Hammond *et al.*, 2011; Zakaria *et al*., 2016).

In vitro mechanism study inhibition of dialkyl-alginate biosurfactant in cell membranes

a. Analysis of nucleic acid leakage and protein

Suspension of 24-hour bacteria for *Staphylococcus aureus* and *Pseudomonas aerugynosa*, 10 ml centrifuged at 3500 rpm for 40 min. The supernatant was discarded and the cell deposits added phosphate buffer saline (PBS) and dialkyl-alginate biosurfactant with concentration of 0 (control), 1 and 2 x MIC. The test solution was incubated at 37 ° C for 24 hours, then centrifuged at 3500 rpm for 40 min, and separated supernatant from cell precipitate. Supernatants measured their absorbance with UV-Vis spectrophotometers at wavelengths of 260 and 280 nm (Azrifitria *et al.*, 2010; Bunduki, 1995; Agusta *et al.*, 2013).

b. Leakage analysis of metal ions

The analysis of ion leakage measured is in the form of Ca^{2+} and K^{+} ions which exit from bacterial cells due to treatment with dialkyl alginate biosurfactant suspension. Samples for leakage analysis of metal ions in the form of supernatants derived from the treatment of nucleic acid leakage and protein analysis. The supernatant was analyzed using Atomic Absorption Spectroscopy (AAS) (Azrifitria *et al.*, 2010; Harsini, 2017; Jamal *et al.*, 2013).

Formulation of cream

The aqueous phase consists of aquadest, methyl paraben, xanthan gum, biosurfactant dialkyl-alginate and triethanolamine. The water phase is heated to 75° - 80°C until all the water soluble materials. While the oil phase consists of Vaseline, stearic acid, glycerol monostearate, isopropyl myristate, propyl paraben and paraffin. All ingredients are mixed together and then heated to 75 ° C

until melted. When the water and oil phases are at the same temperature, the water phase is poured little by little into the oil phase while stirring slowly until homogeneous creams form.

antibacterial activity test of dialkyl-alginate biosurfactant cream

Dialkyl-alginate biosurfactant cream activity test was conducted by using diffusion method for the wells. In this method Petri dish containing agar and has been inoculated with bacteria, made well by using cork borer with diameter 6 mm. Each hole will be filled with dalkyl-alginate biosurfactant cream, Enbatic® ointment (positive control) and cream base (negative control) each triplo made each sample made 3 holes in 1 petri dish. Then stand for ± 2 hours and incubated at 37°C for 24 hours. The inhibitory parameter of the cream is seen from the clear zone formed around the wellbore (Syarifuddin, 2017).

Data Analysis

The analysis was performed with three replications and averaged. The data homogeneity test was performed by using Levene's test and for data normality test using Shapiro-Wilk test. If the data obtained homogeneous and normally distributed then proceed with parametric test that is one way ANOVA test with a significant level of 0.05. The software used was IBM SPSS Statistics 20. The results were reported as mean ± standard deviation (SD).

RESULTS AND DISCUSSION

Antibacterial activity test of biosurfactant dialkyl alginate used the concentration series of 5,10 and 20%, for positive control used Enbatic 1% powder and for negative control used sterile aquadest. The method used is a method of quantitative analysis that has been modified by Hammond *et al.,* (2011). The principle of this method is to conditioning a blank disc such as an open wound that is directly contacted with the antibacterial agent. The activity is measured by counting the number of colonies grown after the treatment and comparing it with the control.

Biosurfactants of 5, 10, 20% dialkyl-alginate concentrations and Enbatic® 1% powder were dissolved using a sterile aquadest. Dialkyl-alginate biosurfactant consists of large molecules which can only be completely dispersed when using a water solvent and to speed up the solubility process it needs to be assisted by heating up to 60°C. Biosurfactant has a very thick texture so it is very difficult to diffuse in the surrounding area, therefore standard methods such as disk diffusion, wells and dilution for biosurfactant antibacterial activity tests cannot be used. The modified quantitative modification method of Hammond *et al.,* (2011) is the preferred method of choice that can make biosurfactants direct contact with bacteria without having to diffuse inside agar.

The number of colonies obtained from this test varies greatly and there is a lot of unreadable data especially on the data with 8 and 12 hour treatments, so that can not be deduced. However, the data obtained in the 24-hour treatment for both *Staphylococcus aureus* and *Pseudomonas aerugynosa* is data that can represent the antibacterial activity of each treatment The results from antibacterial activity test indicated that biosurfactant dialkyl alginate positively inhibit the growth of *Staphylococcus aureus* and *Pseudomonas aeruginosa*. Different concentration biosurfactan dialky-alginate and different contact times gave different inhibition activities. The most active biosurfactant concentration in both test bacteria was 10% concentration with significance p=0,05. however, between these two bacteria, biosurfactant dialkyl-alginate is most active in inhibiting *Staphylococcus aureus*. This is probably due to the fact that gram-negative bacteria (*Pseudomonas aeruginosa*) have more complex cell wall constituent components and have more selective permeability. Test data are presented more clearly in Table I.

The mechanism of action of mass rhamnolipid is not yet known with certainty, but cell membranes are thought to be the action targets of rhamnolipid, because rhamnolipid constituents are able to increase cell permeability (Sotirova *et al*., 2009). Rhamnolipid has hydrophilic and lipophilic

properties which are able to form bonds with the constituent components of the membrane so that the membrane structure is stretched. Stretching that occurs causes the components of rhamnolipid fatty acids to enter the cell membrane and cause changes in cell ultrastructures such as the cell's ability to regenerate plasma membranes (El-Sheshtawy and Doheim, 2014).

Table I. The results of antibacterial activity test of biosurfactant dialkyl-alginate using modified quantitative method Hammond, *et al.*, (2011)

Variable's	Colony Count (CFU/ml)					
	Staphylococcus aureus			*Pseudomonas aeruginosa*		
	8-Hours	12-Hours	24-Hours	8-Hours	12-Hours	24-Hours
Positive Control	$13,86x10^8$	$1,757\ x10^8$	$3,033\ x10^8$	Countless	$7,613\ x10^8$	$1,003\ x10^8$
Negative Control	Countless	Countless	$19,40\ x10^8$	Countless	$10,20\ x10^8$	$7,907\ x10^8$
Concentration 20%	$26,45\ x10^8$	$7,593\ x10^8$	$15,36\ x10^8$	Countless	$8,293\ x10^8$	$6,313\ x10^8$
Concentration 10%	$15,79\ x10^8$	$7,757\ x10^8$	$9,020\ x10^8$	Countless	$4,520\ x10^8$	$2,407\ x10^8$
Concentration 5%	$72,80\ x10^8$	$1,413\ x10^8$	$11,81\ x10^8$	Countless	$7,027\ x10^8$	$4,993\ x10^8$

The results of nucleic acid leakage test and protein showed that there was a significant difference between the absorbance value of 0% concentration with concentrations of 5 and 10%. This proves that there is an increase of nucleic acid and protein levels in bacterial suspension along with increasing concentration of dialkyl-alginate biosurfactant against *Staphylococcus aureus* and *Pseudomonas aerugynosa* bacteria. The effect of biosurfactant concentration on nucleic acid leakage and cell protein can be seen in Table II.

Table II. Effect of Biosurfactant Concentration on Nucleic Acid Leakage (OD 260 nm) and Leakage of Cell Protein (OD 280 nm)

Bacteria	Wave length	Absorbance's		
		0%	5%	10%
Staphylococcus aureus	260 nm	0.075 ± 0.020	1.822 ± 0.152	3.305 ± 0.167
	280 nm	0.232 ± 0.021	1.803 ± 0.130	3.090 ± 0.168
Pseudomonas aerugynosa	260 nm	0.108 ± 0.006	1.861 ± 0.088	3.508 ± 0.474
	280 nm	0.259 ± 0.007	1.829 ±0.090	3.204 ± 0.314

The result of leakage test of metal ion to bacterium *Staphylococcus aureus* and *Pseudomonas aerugynosa*, showed that K^+ ion obtained by different result significantly with p value <0,05 while Ca^{2+} ion level vice versa that is not significantly different with P> 0,05. Low levels of Ca^{2+} ions are most likely to be affected by the nature of the active substance when the alginate meets the Ca^{2+} ion it will form a crosslink like the egg box to form a gel network (Estiasih and Ahmad, 2009), which is many Ca^{2+} ions also settle during the centrifugation process . The leakage of Ca^{2+} and K^+ ion were presented in Table III.

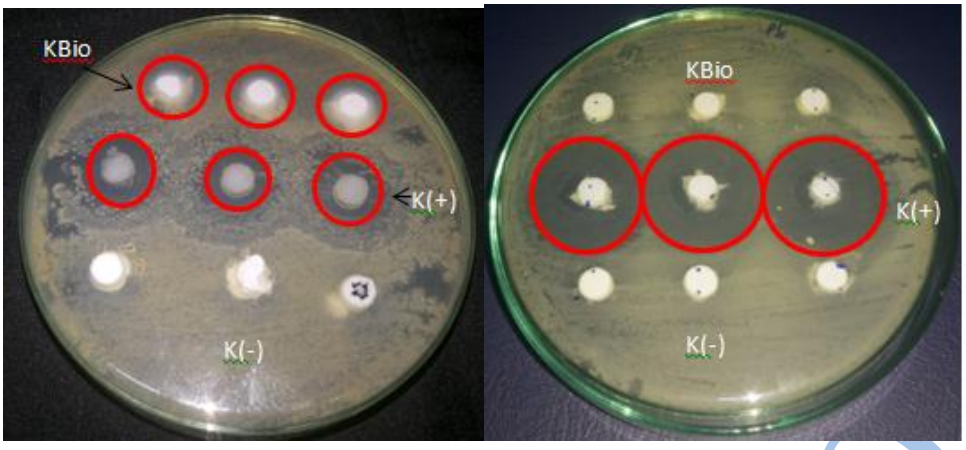
Table III. The Leakage of Ca^{2+} and K^{+} Ion of *Staphylococcus aureus* and *Pseudomonas aerugynosa* Leakage Analysis After Dialkyl-alginate Treatment

Bacteria	Parameter's	Concentration (ppm)		
		0%	5%	10%
Staphylococcus aureus	Ca^{2+}	2.111 ± 1.123	0.823 ± 0.334	0,911 ± 0.200
	K^{+}	19.002 ± 8.766	100.393 ± 6.263	160.496 ± 9.111
Pseudomonas aerugynosa	Ca^{2+}	2.639 ± 0.361	0.419 ± 0.139	0.695 ± 0.105
	K^{+}	27.206 ± 20.100	97.039 ± 0.566	156.960 ± 9.562

The most active concentration of dialkyl alginate biosurfactants based on quantitative assay results and leakage of bacterial cell test was 10%. The concentration of 10% is then formulated into an emulsion preparation that is Cream. Cream is the preferred choice because of its soft consistency, not greasy and also easy to wash. Biosurfactant cream is expected to inhibit the activity of test bacteria and has good physical properties, so it can be used as an alternative to topical antibiotic therapy of choice. In the test of biosurfactant dialkyl-alginate cream activity, the method used is the method of wells. The activity of the cream is seen from how big the clear zone formed around the disc on the agar medium that has been previously inoculated with the test bacteria *Staphylococcus aureus* and Pseudomonas aerugynosa. To determine the strength of the preparations required comparative Enbatic® ointment as a positive control and cream base as a negative control.

The results of the dialkyl alginate biosurfactant test against *Staphylococcus aureus* were clear zones formed around the disk, with an average of 6.83 mm. While the clear zone for positive control averaged at 8.83 mm and the clear zone on the negative control was completely invisible. Based on ANOVA (Post Hoc-Turkey) comparative test showed that the clear zone formed on biosurfactant cream and positive control did not differ significantly with P> 0,05. Biosurfactant cream is able to inhibit the growth of *Staphylococcus aureus* with inhibitory strength is almost the same as Enbatic® ointment which is a combination of active substance between B-polymyxin and bacitrasin sulphate. Combination antimicrobial polymyxin-B and bacitrasin sulphate is one of the main options for topical infection therapy because it is able to inhibit the activity of various pathogenic bacteria especially on the skin, with the mechanism of inhibition of membrane and bacterial cell wall. From this result biosurfactant cream has the potential to be one of the alternative therapy for skin infection considering *Staphylococcus aureus* is one of the most common bacteria found in skin infections that occur in general.

In contrast to the results of the dialkyl alginate biosurfactant activity obtained in *Staphylococcus aureus* bacteria, the dialki-alginate biosurfactant cream does not inhibit the growth of *Pseudomonas aerugynosa*. For positive control the clear zone formed is much larger than that formed in *Staphylococcus aureus*, which is an average of 14.5 mm. where as for negative control the result is the same as biosurfactant cream that there is no clear zone. However, these results differ from the results of the dialkyl alginate biosurfactant extract test and leak test, although the activity of the dialkyl alginate biosurfactant extract is greater with *Staphylococcus aureus* but the dialkyl alginate biosurfactant remains active against *Pseudomonas aerugynosa*. Observation results of biosurfactant inhibitory zone can be seen in Figure 1.



(A) (B)

Figure 1. Antibacterial Activity Test of Cream Dialkyl-Alginate Biosurfactant against (A) *Staphylococcus aureus*, and (B) *Pseudomonas aerugynosa*. KBio : Cream Dialkyl-Alginate Biosurfactant, K(+) : Enbatic® Oint, K(-) : Cream Base

CONCLUSION

The most active concentration of dialkyl-alginate biosurfactant inhibits the activity of *Staphylococcus aureus* and *Pseudomonas aerugynosa* bacteria is 10% concentration. The cell leakage test results using UV-Vis spectrophotometry at wavelengths of 260 nm and 280 nm and atomic absorption spectrophotometry showed a leakage occurring on the membrane and second cell wall of test bacteria. Dialkyl-alginate biosurfactant activity after formulation into cream still actively inhibits the growth of *Staphylococcus aureus* but is not active against *Pseudomonas aerugynosa*.

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