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Research Article

Potential of Biosurfactant as an Alternative Biocide to Control Biofilm Associated Biocorrosion

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Abstract

Background and Objective: The aim of this research was to screen biosurfactants that were potential to be used as an alternative biocide for biofilm associated with biocorrosion. Eight type of biosurfactants extracted from indigenous bacterial isolates were investigated for their ability to eradicate or inhibit biofilm growth. **Materials and Method:** Biofilm assays were performed using broth microdilution method in microtiter plate 96 wells. The parameters used were Minimum Inhibitory Concentration (MIC), Minimum Biofilm Inhibitory Concentration (MBIC) and Minimum Biofilm Eradication Concentration (MBEC). MIC and MBIC were tested on biofilm consortium liquid culture, while MBEC was tested on preformed biofilm. **Results:** Biosurfactant of isolate F3 exhibited the highest eradication level (68.3%) at $100 \mu\text{g mL}^{-1}$. The MIC, MBIC₅₀, MBIC₉₀ and MBEC₅₀ were observed at 50, 25, 25 and $25 \mu\text{g mL}^{-1}$, respectively. Based on these results of this study, biosurfactants produced by isolate F3 was potential to be an alternative biocide that can eliminate biofilm-forming microbial consortium. **Conclusion:** This study affirms that biosurfactant is not only useful for enhancing oil recovery in petroleum industry. It is also potential to be an alternative biocide to eliminate biofilm associated with biocorrosion.

Key words: Biosurfactant, biocide, biocorrosion, MIC, MBIC, MBEC, biofilm eradication

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

One of the main problems in various industries including oil and gas industry is corrosion¹. Corrosion that is triggered or accelerated by microbial metabolic activities is termed biocorrosion². These biocorrosion-inducing microorganisms are mainly present in biofilm form which is an assemblage of bacterial cells that attach permanently to a surface, form aggregates and secrete extracellular polymeric substances³. Biofilm activities can fluctuate the oxygen concentration, pH, the type and concentration of ions in the solution and other micro environment parameters. This will affect interface characteristics such as wettability and electrostatic charges and may lead to the occurrence of undesirable corrosion reaction⁴.

To prevent biocorrosion from damaging important infrastructures, the use of biocide to inhibit or eradicate biofilm in industry has become inevitable. Synthetic biocides have been widely used by various industries. However, they are toxic and environmentally destructive. Therefore, it is important to find an alternative biocide that is more environmentally friendly. One of the potential candidates is biosurfactant. Many studies show that biosurfactants have antimicrobial and antibiofilm activity. One of its mode of action is forming a layer of biosurfactants on the surface thus preventing hydrophobic interactions between planktonic cells and surfaces at the early stages of biofilm formation⁵. Biosurfactants are environmentally friendly because they have low toxicity and are biodegradable⁶. In oil industries, biosurfactant was commonly used for enhanced oil recovery yet there is no study related to its utilization as biocide for combating biofilm associated biocorrosion.

Therefore, this study was conducted to determine the potential of biosurfactants as an alternative biocide to eliminate biofilm, particularly in oil and gas industry. These biosurfactants were obtained from indigenous biosurfactant-producing bacteria isolated from oil reservoir in previous studies⁷. Through this research, the biosurfactant can be used in oil industry not only for enhanced oil recovery but also as an alternative biocide. This study was conducted by screening biosurfactants for antibiofilm activity against biofilm associated with biocorrosion.

MATERIALS AND METHODS

Microorganisms: Biosurfactants used in this study were produced by 8 isolates that had been isolated from one of South Sumatran oil reservoir. Biofilm used in this study

was formed from the consortium of 3 bacterial species isolated from oil reservoir as well⁸.

Biosurfactant extraction: Biosurfactant producing bacteria were grown on SMSS (Stone Mineral Salt Solution) medium consisted of (g L⁻¹ distilled water) 2.5 NH₄NO₃; 0.5 MgSO₄·7H₂O; 0.2 MnCl₂·4H₂O; 0.5 CaCO₃; 1 Na₂HPO₄·7H₂O; 0.5 KH₂PO₄⁹; added with 0.1% of yeast extract and 2% of crude oil at 50°C for 48 h. Extraction of biosurfactant was carried out by separating cell biomass and supernatant through centrifugation at 7500 rpm and 4°C for 30 min. Then the supernatant was precipitated with acid precipitation (pH 2) using HCl 6 N and then incubated overnight at 4°C. After precipitation, the solution was centrifuged at 7500 rpm and 4°C for 30 min. Biosurfactants obtained on the pellet was separated and the remaining biosurfactants in the supernatant was extracted by adding methanol:chloroform (2:1) solution. The mixture would separate and the lower phase was taken, dried and then combined with the previous biosurfactants extract. The dry weight of biosurfactant extract was determined⁷. Biosurfactant extract obtained was dissolved in sterile deion before use¹⁰. Biofilm forming bacteria were grown in nutrient broth medium (NB). The optimum culture age was based on previous studies⁸.

Screening of biosurfactants based on antibiofilm activity:

The screening of biofilm eradication activity was carried out on 8 types of biosurfactants produced by different isolates⁷ using broth microdilution method¹¹. Biofilm was prepared in microtiter well by growing 200 µL of bacterial consortium in each well at 50°C for 48 h⁸. After incubation, microtiter plate was washed twice with 300 µL of sterile PBS solution, added with 200 µL of biosurfactant solution (100 µg mL⁻¹) into each wells and incubated at 50°C for 24 h. After incubation, microtiter plate was washed again PBS solution and stained using 200 µL of 0.1% crystal violet. Crystal violet that attached to biofilm was dissolved with 200 µL of 10% glacial acetic acid and the absorbance was measured at a wavelength of 595 nm using ELISA reader^{11,12}.

Emulsification index test: Crude oil was added to cell free supernatant in 1:1. The mixture was vortexed for 2 min and let idle for 24 h. The height of emulsion formed was measured and stated as percentage to calculate Ei/E24¹³.

Biosurfactant antibiofilm assay: The assay was performed on 3 types of biosurfactants with the best antibiofilm activity

from previous screening results. Further screening was conducted by determining the values of MIC, MBIC and MBEC of biosurfactants using microdilution method on microtiter plate 96 wells.

Determination of minimum inhibitory concentration (MIC) of biosurfactants: The method used was adapted from Fu *et al.*¹⁴. Assay began by adding 20 μL of bacterial consortium (10^6 CFU mL^{-1}) on microtiter plate 96 wells. Then an aliquot of 180 μL of biosurfactant with concentration variation of 25; 50; 75 and 100 $\mu\text{g mL}^{-1}$ were added into the wells. OD (Optical Density) at 595 nm was measured using ELISA Reader before and after incubation of microtiter plate 96 wells at 50°C for 24 h.

The negative control of biofilm inhibition was 20 μL of bacterial consortium and 180 μL of deionized water without any addition of biosurfactant. The MIC was determined as the lowest biosurfactant concentration that inhibited of bacterial growth in planktonic state. The value was determined by calculating the difference of turbidity values between before and after incubation¹⁴.

Determination of minimum biofilm inhibitory concentration (MBIC) of biosurfactants: Determination of Biosurfactant MBIC was performed using broth microdilution method¹⁵. Each well in the microtiter was added with 100 μL of biofilm-forming consortium grown in NB medium. Then, 100 μL of biosurfactant was added using 4 variation of concentrations (25; 50; 75; 100 $\mu\text{g mL}^{-1}$). Negative control used for the assay was 100 μL of bacterial culture and 100 μL of deionized water without any addition of biosurfactant. The culture was incubated for 48 h at 50°C¹¹. Each well were washed using PBS solution and stained with crystal violet as described in the first screening stage. The absorbance of crystal violet was measured at 595 nm wavelengths^{11,13}. MBIC value was determined when the percentage of inhibition reach $\geq 50\%$ for MBIC₅₀ and $\geq 90\%$ for MBIC₉₀¹⁶.

Determination of minimum biofilm eradication concentration of biosurfactant: MBEC test was performed using broth microdilution method^{11,15}. Each well in microtiter was added with 200 μL of biofilm-forming consortium and incubated for 48 h at 50°C to form the optimum biofilm¹². Microtiter that had been incubated was rinsed with 200 μL of PBS (Phosphate Buffer Saline) solution twice for each well and added with 200 μL biosurfactant with 4 concentration variations (25; 50; 75; 100 $\mu\text{g mL}^{-1}$). Negative control used was 200 μL of sterile deionized water. The incubation process was continued for 24 h at 50°C. Then the microtiter was

washed with PBS solution, stained using 0.1% crystal violet and measured at 595 nm wavelengths as described in the first screening stage^{11,13}. MBEC value was determined when the percentage of elimination reached $\geq 50\%$ (MBEC₅₀)¹⁶.

Identification of potential biosurfactant-producing bacteria: The selected biosurfactant-producing bacteria were identified with 16S ribosomal DNA analysis. DNA sequencing was carried out at MacroGen™ Korea with universal primer of 785F and 907R. The sequencing results were cross-checked manually using Bioedit and the similarity was determined using BLASTN. Multiple sequence alignment was conducted using CLUSTAL X and phylogenetic tree was constructed using MEGA6 software.

Statistic analysis: The research used ANOVA single factor for comparing the significant differences of the data.

RESULT

Screening of biosurfactants based on antibiofilm activity:

The screening results of 8 biosurfactants based on biofilm eradication activity were shown in Fig. 1. The highest eradication percentages were exhibited by biosurfactant produced by isolate F3, F7, N2 and D1 with eradication value of 68.3, 69.7, 62.3 and 61.4% respectively. These four biosurfactants showed higher eradication results compared to glutaraldehyde as the positive control (44.6%).

Isolate F3 and F7 had been phylogenetically analyzed to determine the species in previous studies⁷. The results showed that isolate F3 were closely related to *Pseudoxanthomonas mexicana* strain NBRC 101034 and *Pseudoxanthomonas japonensis* strain NBRC 101033. Therefore isolate F3 was identified as *Pseudoxanthomonas* sp. as shown in Fig. 2. Isolate F7 isolate showed close relation with

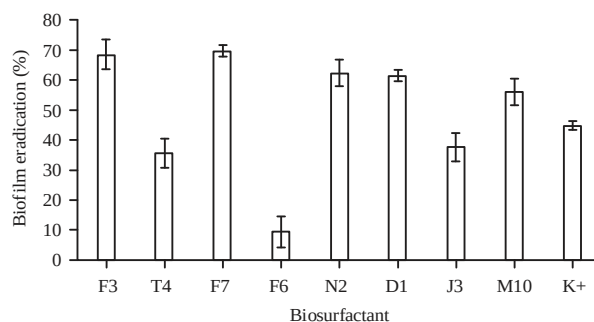


Fig. 1: Biofilm eradication of 8 types of biosurfactants; F3, T4, F7, F6, N2, D1, J3 and M10. K+ signifies glutaraldehyde as the positive control

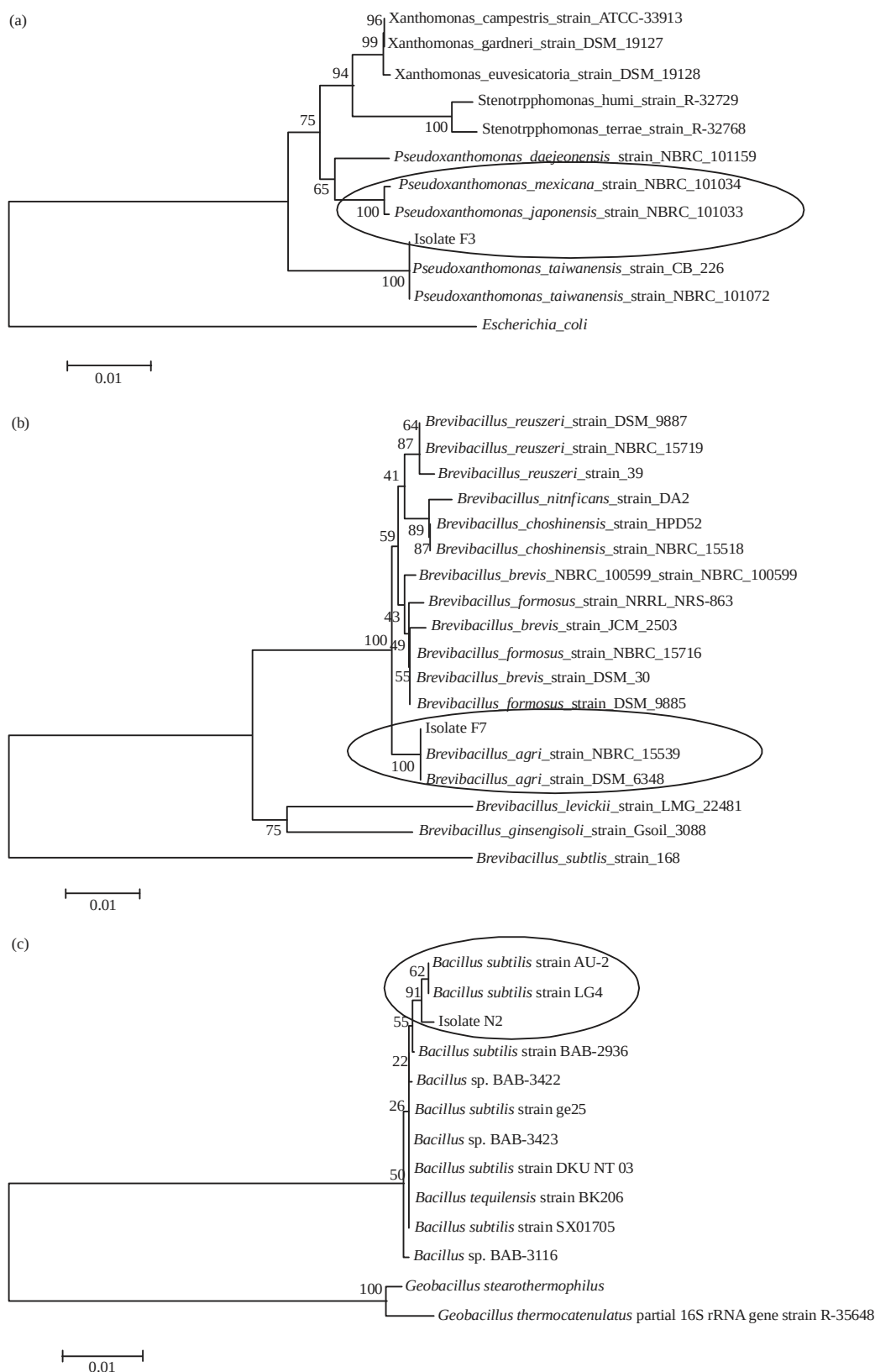


Fig. 2(a-c): Phylogenetic tree of the potential biosurfactant producing bacteria, (a) F3, (b) F7 and (c) N2

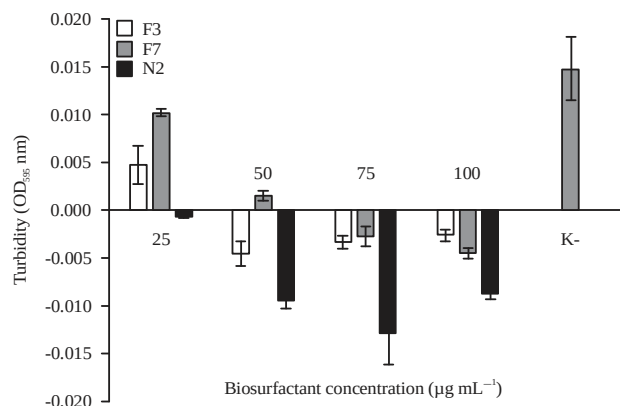


Fig. 3: Minimum inhibitory concentration (MIC) assay result of biosurfactants from F3, F7 and N2 isolates on biofilm-forming consortium. K(-) signifies bacterial consortium without biosurfactant treatment as the negative control

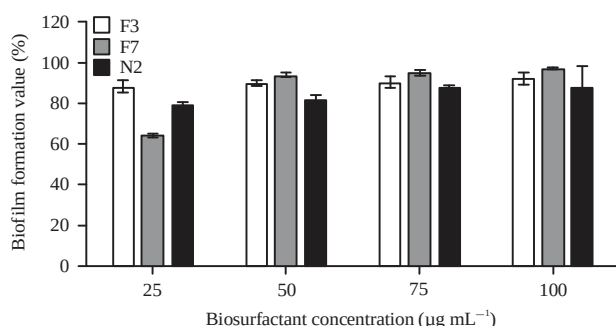


Fig. 4: Minimum biofilm inhibitory concentration (MBIC) assay result of biosurfactants from F3, F7 and N2 isolates on biofilm-forming consortium

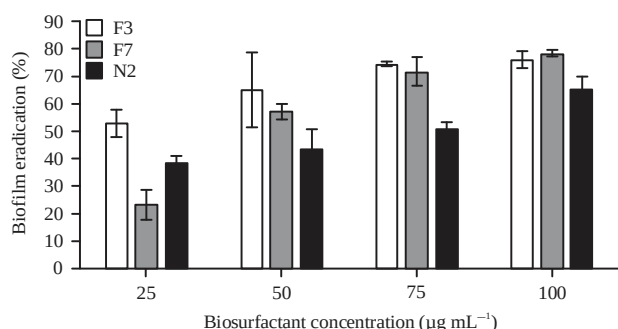


Fig. 5: Minimum biofilm eradication concentration (MBEC) assay result of biosurfactants from F3, F7 and N2 isolates on biofilm-forming consortium

Brevibacillus agri strain NBRC 15538 and *Brevibacillus agri* strain DSM 6348. Therefore, isolate F7 was identified as

Table 1: Results of screening stage

Results of screening stage	Biosurfactants scoring
Biofilm eradication results (100 µg mL ⁻¹)	F3>F7>N2>D1
Emulsification results	F3>F7>N2>D1
Biosurfactant extraction results	F3>F7>N2>D1
Selected biosurfactants	F3, F7, dan N2

Brevibacillus sp. The result of phylogenetic analysis of isolate F7 was shown in Fig. 2. The phylogenetic analysis was also performed on isolate N2 and the result showed that it was closely related to *Bacillus subtilis* of AU-2 strain and *Bacillus subtilis* of LG4 strain (Fig. 2). Therefore isolate N2 was identified as *Bacillus* sp.

Emulsification index test: Emulsification index was determined to analyze surfactant ability to emulsify oil. Emulsification index and biosurfactant yield was determined as additional parameters to select the best biosurfactant. The emulsification index for F3, F7, N2 and D1 were 72.9, 68.57, 58.10 and 53.65% while the yields of biosurfactant (mg mL⁻¹) were 0.283, 0.187, 0.167 and 0.153, respectively. Based on biofilm eradication test, emulsification index and biosurfactant yield, F3, F7 and N2 were selected for further investigation (Table 1). Molecular identification results showed that isolate F3, F7 and N2 were *Pseudoxanthomonas taiwanensis*, *Brevibacillus agri* and *Bacillus subtilis* (Fig. 2).

Biosurfactants antibiofilm assays: Based on MIC assay, the three biosurfactants tested showed the ability to inhibit planktonic cell growth (Fig. 3). This was indicated by the turbidity that was lower compared to negative control. The decreased turbidity indicated that the cells underwent lysis, or cell growth was inhibited³. The highest MIC value was obtained from biosurfactant N2 treatment at 25 µg mL⁻¹. The second and third highest MIC values were obtained from biosurfactants F3 and F7 treatment at concentrations of 50 and 75 µg mL⁻¹, respectively.

The result of MBIC values from three biosurfactant is shown in Fig. 4. MBIC₅₀ was observed at 25 µg mL⁻¹ for all three biosurfactants tested. The highest to lowest inhibition percentage was shown by biosurfactant F3 (88%), N2 (78.9%) and F7 (63.7%), respectively. MBIC₅₀ value in biosurfactant F3 can also be determined as MBIC₉₀ value because the inhibition percentage was approaching 90%. MBIC₉₀ values can also be obtained from biosurfactant of *Brevibacillus* sp. F7 at 50 µg mL⁻¹ with 93,6% biofilm-forming inhibition. As for biosurfactant N2, MBIC₉₀ value was obtained at 75 µg mL⁻¹ with inhibition percentage of 87,6%.

The results of biofilm eradication by biosurfactants F3, N2 and F7 are shown in Fig. 5. MBEC₅₀ value of biosurfactant F3

Table 2: Compilation of MIC, MBIC and MBEC results of biosurfactants F3, F7, N2

Biosurfactant	F3	F7	N2
MIC ($\mu\text{g mL}^{-1}$)	50.0	75.0	25.0
MBIC ₅₀ ($\mu\text{g mL}^{-1}$)	25.0	25.0	25.0
Inhibition values (%)	88.0	63.7	78.9
MBIC ₉₀ ($\mu\text{g mL}^{-1}$)	25.0	50.0	75.0
Inhibition values (%)	89.6	93.6	-
MBEC ($\mu\text{g mL}^{-1}$)	25.0	50.0	75.0

was obtained at $25 \mu\text{g mL}^{-1}$, whereas MBEC₅₀ values of biosurfactant F7 and N2 were obtained at 50 and $75 \mu\text{g mL}^{-1}$ respectively. The results of MIC, MBIC and MBEC assay were compiled in Table 2.

DISCUSSION

The four biosurfactants selected from the first screening stage showed higher eradication activity compared to glutaraldehyde as one of the most widely used biocides in oil industry. Glutaraldehyde's main mode of action was attacking microorganism's protein system¹⁷. MIC assay showed that biosurfactant N2 had the highest inhibitory activity against planktonic cells as indicated by MIC results.

Brevibacillus sp. and *Bacillus* sp. can produce lipopeptide biosurfactants^{18,19}. Lipopeptides are biosurfactants with hydrophilic groups composed of amino acids. Biosurfactants that belong to lipopeptide are surfactin, fengycin and polymyxin¹⁸. Fengycin has the ability to eradicate 90% biofilm of both Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli*). Surfactin has also exhibited antibiofilm activity, particularly to *Salmonella* sp. biofilm. One of the mechanisms is increasing the channels in biofilm so as to ease antibiofilm penetration. The mode of action of polymyxin against planktonic cells is related to their high affinity for lipopolysaccharide (LPS). Polymyxin induces aggregation of LPS and increases the surface charge of LPS thus causing the internalization and binding to phosphatidyl membrane. Polymyxin is also known to reduce *Pseudomonas aeruginosa* biofilm up to 99%. These can lead to the leakage of the intracellular contents. Another types of lipopeptide is complex lipopeptides composed of polymyxin, fusaricidin and a little surfactin. One of bacteria that produces this compound is *Paenibacillus polymixa*. Lipopeptide complex has the ability to inhibit biofilm formation of Gram-positive bacteria such as *S. aureus*, *S. bovis*, *Micrococcus luteus* and Gram-negative bacteria such as *P. aeruginosa*¹⁹. Previous study revealed that biosurfactant F3 of *Pseudoxanthomonas taiwanensis* belongs to the glycolipid group of rhamnolipid⁷. Rhamnolipid is one type of biosurfactant with a hydrophilic group composed of rhamnose sugar²⁰.

Rhamnolipid biosurfactant demonstrated its ability as an antibiofilm by decreasing the adhesion of bacteria to the surface and accelerating biofilm dispersion through the removal of lipopolysaccharide protein complex. This protein complex is from the outermost membrane of the cell as one of EPS component in the biofilm. Other tests were carried out on the planktonic cells of *Bacillus subtilis*, *Staphylococcus epidermidis* and *Propionibacterium acnes* and rhamnolipid biosurfactant was shown to exhibit antimicrobial activity against those isolates⁵. It was suspected this complex inhibit bacterial growth by affecting the permeability of planktonic cells²⁰. Biosurfactant is also able to interfere adhesion process of bacterial cells to the surface of silicone rubber through non-covalent interactions (van der Waals and hydrophobic). Hydrophobic interactions between biosurfactant and the surface cause the surface to be no longer hydrophobic. Bacterial attachment to the surface that usually uses hydrophobic interactions thus become inhibited. Rhamnolipid ability to eradicate biofilm was also observed in the study. One possible mode of action was reducing the interfacial tension between biofilm and surfaces. Consequently, interactions between surfaces with biofilms become decreased thus causing damage to the biofilm²¹.

The differences of MIC values observed on the biosurfactants might occur due to differences of the type of biosurfactant and different inhibitory mode of action²¹. The results showed that biosurfactant N2 had a higher influence on bacterial consortium compared to other biosurfactants. This might occurred because the mode of action of this biosurfactants was more prominent against planktonic cells compared with other biosurfactants tested²².

Based on the data, 50 and 90% inhibition of biofilm can be achieved by all three biosurfactants as indicated by MBIC₅₀ and MBIC₉₀ assays. The highest MBIC₅₀ and MBIC₉₀ values can be achieved by biosurfactants F3 at the same concentration of $25 \mu\text{g mL}^{-1}$. In general, the percentage of biofilm formation inhibition by all biosurfactants tested were relatively high. This suggested the possibility that the biosurfactants' mode of actions were potent to interfere with planktonic cell attachment to the surface thus inhibiting the formation of biofilm²⁰. One possible mode of action is the formation of surface films by biosurfactants on the surface of the material thus preventing hydrophobic interactions between planktonic cells and surfaces as the early stages of biofilm formation⁵.

MBEC assay result showed lower eradication values compared to the inhibitory values of biofilm formation. This might happen because mature biofilm's resistance towards antimicrobial compounds³ such as biosurfactants are higher than planktonic cells. Consequently, the same concentration

Table 3: Characteristics of isolate F3 and biosurfactant F3⁷

Isolate F3 characteristics ⁷		Presumptive biosurfactant characteristics ⁷	
Gram staining	Negative	CTAB characterization	Glycolipid biosurfactant
Cell morphology	Rod		
Colony morphology	Round, pale yellow		
Genus	<i>Pseudoxanthomonas</i> sp.	FTIR characterization	Rhamnolipid
Closest relatives	<i>Pseudoxanthomonas mexicana</i> strain NBRC 101034 <i>Pseudoxanthomonas japonensis</i> strain NBRC 101033		
Isolate name	<i>Pseudoxanthomonas</i> sp. F3		

of biosurfactants could perform better to inhibit biofilm formation than to eradicate preformed biofilm. Some possible mechanisms of biosurfactant in eradicating established biofilms are interfering biofilm interactions with the surface²³ and increasing the number of channels in the biofilm that leads to increased penetration of antibiofilm compound¹⁹.

Based on MIC, MBIC and MBEC results as compiled in Table 2, biosurfactant F3 showed the most promising activity as antibiofilm. The characteristics of *Pseudoxanthomonas* sp. F3 and its biosurfactant are listed in Table 3.

CONCLUSION

Biosurfactant F3 showed the best antibiofilm activity with MIC, MBIC₅₀, MBIC₉₀ and MBEC₅₀ values of 50; 25; 25 and 25 µg mL⁻¹, respectively. Based on these results, biosurfactants F3 was potential to become an alternative biocide for eliminating microorganisms associated with biocorrosion.

SIGNIFICANCE STATEMENT

This study aimed to study the potential of biosurfactant to mitigate biofilm-biocorrosion. This study demonstrated biosurfactant ability to inhibit bacterial growth, prevent initial biofilm formation and eradicate biofilm consortium which can be beneficial to improve the process of biocorrosion mitigation. This study will serve as a preliminary study for another researchers to reveal further findings regarding biofilm, biocorrosion and ways to overcome it.

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REFERENCES

1. Turkiewicz, A., J. Brzeszcz and P. Kapusta, 2013. The application of biocides in the oil and gas industry. *Nafta-Gaz*, 69: 103-111.
2. Skohvus, T.L. and C. Whitby, 2011. *Applied Microbiology and Molecular Biology in Oilfield Systems*. Springer, London, pp: 20-23, 126.
3. Clark, D.P., M.T. Madigan, J.M. Martinko and D.A. Stahl, 2012. *Brock-Biology of Microorganisms*. 13th Edn., Pearson Education, San Fransisco, pp: 674-676.
4. Videla, H.A. and L.K. Herrera, 2005. Microbiologically influenced corrosion: Looking to the future. *Int. Microbiol.*, 8: 169-180.
5. Dusane, D.H., V.S. Pawar, Y.V. Nancharaiah, V.P. Venugopalan, A.R. Kumar and S.S. Zinjarde, 2011. Anti-biofilm potential of a glycolipid surfactant produced by a tropical marine strain of *Serratia marcescens*. *Biofouling*, 27: 645-654.
6. Mouafi, F.E., M.M.A. Elsoud and M.E. Moharam, 2016. Optimization of biosurfactant production by *Bacillus brevis* using response surface methodology. *Biotechnol. Rep.*, 9: 31-37.
7. Astuti, D.I. and R.E. Putri, 2016. *Pseudoxanthomonas* sp. G3, isolat bakteri penghasil biosurfaktan yang potensial untuk aplikasi MEOR. *Repository Tugas Akhir SITH-ITB.*, 2: 1-8.
8. Taufik, I., D.I. Astuti, I.A. Purwasena and W.N. Rodiana, 2018. Effect of lemongrass (*Cymbopogon citratus*) essential oil on biofilm-biocorrosion in formation water environment. *Asian J. Sci. Res.*, (In Press).
9. Rudjito, R.C., 2014. Isolasi bakteri pendegradasi minyak bumi dari sumur minyak mangunjaya untuk aplikasi MEOR. *Proceedings of the International Symposium on Earth Science and Technology*, December 4-5, 2014, Japan.
10. Mendes, A.N., L.A. Filgueiras, J.C. Pinto and M. Nele, 2015. Physicochemical properties of rhamnolipid biosurfactant from *Pseudomonas aeruginosa* PA1 to applications in microemulsions. *J. Biomater. Nanobiotechnol.*, 6: 64-79.
11. Adukwu, E.C., S.C.H. Allen and C.A. Phillips, 2012. The anti-biofilm activity of lemongrass (*Cymbopogon flexuosus*) and grapefruit (*Citrus paradisi*) essential oils against five strains of *Staphylococcus aureus*. *J. Applied Microbiol.*, 113: 1217-1227.

12. O'Toole, G.A., 2011. Microtiter dish biofilm formation assay. *J. Vis. Exp.*, Vol. 47. 10.3791/2437
13. Satpute, S.K., S.S. Bhuyan, K.R. Pardhesi, S.S. Mujumdar, P.K. Dhakephalkar, A.M. Shete and B.A. Chopade, 2010. Molecular Genetics of Biosurfactant Synthesis in Microorganisms. In: *Biosurfactants*, Ramkrishna, S. (Ed.). *Advances in Experimental Medicine and Biology*, Vol. 672. Springer, New York, pp: 14-41.
14. Fu, Y., Y. Zu, L. Chen, X. Shi, Z. Wang, S. Sun and T. Efferth, 2007. Antimicrobial activity of clove and rosemary essential oils alone and in combination. *Phytother. Res.*, 21: 989-994.
15. Soumya, E.A., I.K. Saad, L. Hassan, Z. Ghizlane, M. Hind and R. Adnane, 2011. Carvacrol and thymol components inhibiting *Pseudomonas aeruginosa* adherence and biofilm formation. *Afr. J. Microbiol. Res.*, 5: 3229-3232.
16. Tetz, G., V. Tetz and D. Vikina, 2015. Antimicrobial activity of mul-1867, a novel antimicrobial compound, against multidrug-resistant *Pseudomonas aeruginosa*. *Ann. Clin. Microbiol. Antimicrob.*, Vol. 15, No. 19. 10.1186/s12941-016-0134-4.
17. EON., 2013. Eonbac BC 3190 J Biocide. <http://www.eonchemicals.com/wp-content/uploads/2014/12/Bac-BC-3190-J-170713-Logo-N.pdf>
18. Rautela, R., A.K. Singh, A. Shukla and S.S. Cameotra, 2014. Lipopeptides from *Bacillus* strain AR2 inhibits biofilm formation by *Candida albicans*. *Antonie Van Leeuwenhoek*, 105: 809-821.
19. Haddad, N.I., J. Wang and B. Mu, 2008. Isolation and characterization of a biosurfactant producing strain, *Brevibacillus brevis* HOB₁. *J. Ind. Microbiol. Biotechnol.*, 35: 1597-1604.
20. Dusane, D.H., Y.V. Nanchaiah, S.S. Zinjarde and V.P. Venugopalan, 2010. Rhamnolipid mediated disruption of marine *Bacillus pumilus* biofilm. *Colloids Surf. B: Biointerfaces*, 81: 242-248.
21. Gudina, E.J., V. Rocha, J.A. Teixeira and L.R. Rodrigues, 2010. Antimicrobial and antiadhesive properties of a biosurfactant isolated from *Lactobacillus paracasei* ssp. paracasei A20. *Lett. Applied Microbiol.*, 50: 419-424.
22. Banat, I.M., M.A.D. De Rienzo and G.A. Quinn, 2014. Microbial biofilms: Biosurfactants as antibiofilm agents. *Applied Microbiol. Biotechnol.*, 98: 9915-9929.
23. Gomes, M.Z.D.V. and M. Nitschke, 2012. Evaluation of rhamnolipid and surfactin to reduce the adhesion and remove biofilms of individual and mixed cultures of food pathogenic bacteria. *Food Control*, 25: 441-447.