RESEARCH ARTICLE

Biofilm disruption potential of a glycolipid biosurfactant from marine *Brevibacterium casei*

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biofilm disruption; glycolipid biosurfactant; antimicrobial activity; sponge bacteria; *Actinobacteria*.

Abstract

The antibiofilm activity of a glycolipid biosurfactant isolated from the marine actinobacterium *Brevibacterium casei* MSA19 was evaluated against pathogenic biofilms *in vitro*. The isolate *B. casei* MSA19 was a potential biosurfactant producer among the 57 stable strains isolated from the marine sponge *Dendrilla nigra*. The biosurfactant production was optimized under submerged fermentation. The purified glycolipid showed a broad spectrum of antimicrobial activity. Based on the minimum inhibitory concentration/minimum bactericidal concentration ratio, the glycolipid was determined as bacteriostatic. The glycolipid biosurfactant disrupted the biofilm formation under dynamic conditions. The disruption of the biofilm by the MSA19 glycolipid was consistent against mixed pathogenic biofilm bacteria. Therefore, the glycolipid biosurfactant can be used as a lead compound for the development of novel antibiofilm agents.

Introduction

Biofilms are complex aggregation of microorganisms growing on a solid substrate (Davis *et al*., 1989). They are mainly formed on biotic or abiotic surfaces as single- or multiple-species communities. Biofilm formation is an important mechanism for microbial survival in the environment. Biofilm-forming microorganisms are less susceptible to many antimicrobial agents as well as other biocides. Biofilm formation on medical devices plays an important role in the problem of many nosocomial and health-associated diseases. Based on the above reasons, novel antibiofilm compounds are required for the prevention/control of pathogenic microbial biofilms on the surfaces and hosts. It was established that most of the marine organisms have evolved efficient strategies to combat epibiosis. Especially, marine sponges produce specific deterrents to ward off biofilm-forming microorganisms (Selvin *et al*., 2010). However, it has been hypothesized that these toxic deterrents might be produced by the associated microorganisms instead of the host sponge.

Biosurfactants are a heterogeneous group of bioactive amphiphilic molecules produced mostly on microbial cell surfaces or extracellularly (Karanth *et al*., 1999; Soumen *et al*., 2006). The most significant advantages of microbial surfactants are biodegradability and nontoxicity to natural environments (Banat, 1993). The biomedical importance of biosurfactants was established due to their antibacterial, antifungal and antiviral properties; inhibition of fibrin clot formation; and their antiadhesive action against several pathogenic microorganisms (Meylheuc *et al*., 2001, 2006; Singh & Cameotra, 2004; Rodrigues *et al*., 2006). Sponge-associated marine bacteria are emerging as a potential source of novel biosurfactants (Gandhimathi *et al*., 2009; Kiran *et al*., 2009, 2010). It has been hypothesized that the antimicrobial fouling process represents a chemical defense of host sponges mediated by the associated bacteria. Therefore, a glycolipid biosurfactant produced by the sponge-associated marine actinobacterium was evaluated for the control of pathogenic biofilms. In the present study, we report the biofilm disruption potential of *Brevibacterium casei* MSA19, a glycolipid biosurfactant producer, isolated from the marine sponge *Dendrilla nigra*.

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Materials and methods

Isolation and screening of a biosurfactant producer

The host sponge *D. nigra* was collected from the Vizhinjam coast (southwest coast of India) by SCUBA diving at 12 m depth. Only unbroken samples were used for microbiological analysis to avoid cross contamination. The collected specimens were kept for 2 h in sterilized aged seawater (SAS) to remove loosely associated microorganisms from the inner and outer sponge surfaces. One cubic centimeter of sponge tissue was excised from the middle of the entire sponge using sterile scissors. The excised portion was washed three times with SAS to remove any bacteria within the current canals and then the tissue was homogenated using a tissue homogenizer (Omni). The resultant homogenate was serially diluted with SAS and preincubated at 40 °C for 1 h for the activation of dormant and inactive cells. The resultant aliquot was plated on various isolation media and incubated at 25 °C under dark aerobic conditions until visible colonies appeared. Standard bacteriological media (Himedia) such as Zobell marine agar, seawater agar, nutrient agar supplemented with 2% NaCl, actinomycetes agar supplemented with 2% NaCl and marine sponge agar (Selvin *et al*., 2004) were used for the isolation of sponge-associated bacteria. Only consistent morphotypes in subsequent subcultures were selected for screening. The biosurfactant activity was determined based on the hemolytic activity (Carillo *et al*., 1996), drop-collapsing test (Youssef *et al*., 2004), oil displacement test (Morikawa *et al*., 1993), emulsification index (Paraszkiewicz *et al*., 1992) and lipase activity (Kiran *et al*., 2009). The isolate MSA19 was optimized under submerged fermentation conditions (Gandhimathi *et al*., 2009) for enhanced biosurfactant production.

Chemical characterization of glycolipid

The strain MSA19 was grown in a 250-mL Erlenmeyer flask containing 100 mL of actinomycetes isolation broth supplemented with 2% NaCl and incubated at 26 °C for 7 days at 200 r.p.m. The culture was centrifuged (Eppendorf) at 12 000 ***g*** for 10 min at 4 °C and the supernatant was filtered through a 0.2-μm filter. The cell-free supernatant and the pellet were acidified with concentrated HCl to obtain pH 2.0 and extracted with an equal volume of solvents such as ethyl acetate, diethyl ether and dichloromethane. The resultant aliquot was evaporated in a rotary vacuum evaporator (Yamato) and tested for emulsification activity as described above. The orcinol assay (Chandrasekaran & Bemiller, 1980) was used to determine the amount of glycolipids in the sample. Glycolipid production was confirmed by thin-layer chromatography (TLC) using chloroform–methanol–water (65 : 25 : 4) as a solvent system (Zhang & Miller, 1994). The TLC-purified fraction was analyzed in preparative HPLC, GC-MS (Shimadzu 2014) and Fourier transform infrared spectroscopy (FT-IR) to confirm the purity and structural moieties of the surface-active molecule. The antimicrobial activity of the TLC-purified compound was evaluated using the double-layer cylinder plate method (Gandhimathi *et al*., 2008). The list of pathogenic and nonpathogenic bacteria used in the antimicrobial assay is presented in Table 1. The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) were determined using the microdilution method (Selvin *et al*., 2009).

Evaluation of biofilm disruption potential by a microtiter plate assay

The biofilm strains were cultured overnight in Zobell marine broth (Himedia) and their microtiter plate adherence was determined as per Stepanovic *et al*. (2000). Cells were washed and resuspended in phosphate-buffered saline (pH 7.2) to a turbidity equivalent to a 0.5 M McFarland standard. Wells of sterile 96-well U-bottomed microtiter plates were filled with 80 μL of Zobell marine broth, 10 μL of each cell suspension and 10 μL each of the test concentration/controls, in triplicate. The control was set with a newly developed biofilm, negative control wells were filled with culture broth and 10 $\mu g\,mL^{-1}$ $CuSO_4$ was used as a positive control. All the triplicate wells were set with TLC-purified glycolipid of 5, 10 and 30 $\mu g\,mL^{-1}$, respectively. The control and treated wells were set in a Latin-square arrangement and the experiment was repeated six times to validate the results statistically. Plates were placed on a platform shaker to obtain dynamic culture conditions. Planktonic cells and spent media were discarded, and adherent cells were gently

Table 1. Panel of pathogens used for antimicrobial activity, biofilm formation and biofilm disruption assay

Pathogens used	Accession number	Nature of the strains
Candida albicans	FC1	Clinical isolate
Escherichia coli	MTCC 2939	Human pathogen
Proteus mirabilis	PC1	Clinical isolate
Hemolytic *Streptococcus*	PC2	Clinical isolate
Pseudomonas aeruginosa	MTCC 2453	Human pathogen
Klebsiella pneumoniae	PC3	Clinical isolate
Vibrio parahaemolyticus	MTCC 451	Fish pathogen
Vibrio harveyi	MTCC 3438	Fish pathogen
Vibrio alginolyticus	MTCC 4439	Fish pathogen
Vibrio alcaligenes	MTCC 4442	Fish pathogen
Vibrio vulnificus	MTCC 1145	Fish pathogen
Thalassomonas sp.	MMD12	Marine biofilm bacterium
Alteromonas sp.	MMD16	Marine biofilm bacterium
Pseudoalteromonas sp.	MMD18	Marine biofilm bacterium
Pseudoalteromonas sp.	MMD19	Marine biofilm bacterium
Ruegeria sp.	MMD27	Marine biofilm bacterium

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rinsed twice with deionized water and allowed to air dry before being stained. The biofilms were stained by 200 μL of a 0.4% crystal violet solution (w/v) for 10 min, after which the dye was discarded and the wells were rinsed twice with deionized water. The wells were allowed to air dry before solubilization of the crystal violet with 200 μL of dimethyl sulfoxide. The OD was determined at 595 nm in an enzyme-linked immunosorbent assay reader (Labnics). The plate was observed under a phase-contrast microscope with × 40 magnification (Optica) to evaluate the disruption of biofilms.

The results of the plate assay were confirmed by the *in vitro* biofilm-forming assay. The biofilm was developed in a cover glass immersed in a 50-mL Erlenmeyer flask containing Zobell marine broth and incubated at 28 °C for 24 h. To study the biofilm disruption, the mature biofilm was exposed to a 0.5% glycolipid biosurfactant for 30 min at 28 °C. The exposed cover slip was stained with a 0.4% crystal violet solution (w/v) and observed under a phase-contrast microscope (Optica) at × 40 magnification.

Results and discussion

Glycolipid biosurfactant from MSA19

The heterotrophic bacteria (MSA01–MSA57) isolated from the marine sponge *D. nigra* were screened for biosurfactant activity. Among the five potential biosurfactant producers, based on the antimicrobial activity of the biosurfactants, the isolate MSA19 was chosen for the present study. Biosurfactant production of the isolate MSA19 was confirmed using conventional screening methods including hemolytic activity (8 mm), oil displacement (8 mm), lipase activity (9 mm), positive drop-collapsing test and emulsification activity (20%). The isolate MSA19 was Gram-positive, motile and showed unique spores in Ziehl Nielsen staining. The isolate was positive for the MR-VP test and hydrolyzed starch, gelatin, cellulose and tributyrin. The isolate utilized glucose, but not mannitol. The strain MSA19 was sensitive to ciprofloxacin and chloramphenicol and resistant to ampicillin. Based on the morphological, biochemical characteristics, the results of the RDPII SEQMATCH program (http://rdp8.cme.msu.edu/html/) and phylogenetic analysis (http://www.megasoftware.net/), the isolate MSA19 was identified as *Brevibacterium casei* MSA19. The sequence data of the strain MSA19 were deposited in GenBank with an accession number GQ153944.

The optimum production (65% of the emulsification index) was attained with pH 7, temperature 30 °C, 2% salinity and glucose as carbon source and peptone as nitrogen source, respectively. The effective factors optimized for the production of glycolipid biosurfactant by MSA19 are presented in Table 2. Glycolipids are typically synthesized during the late-exponential or the stationary phase of growth when the cell density is high, and this production is considerably enhanced by nutrient limitation (Guerra-santos *et al.*, 1986). However, it has been shown previously that growth in a nitrogen-limited medium increases the production of the glycolipid biosurfactant (Desai & Banat, 1997). The active compound was extracted with three solvents including ethyl acetate, diethyl ether and dichloromethane. The ethyl acetate extracts showed the highest activity compared with the other solvents. The active compound was characterized by TLC, HPLC, FT-IR and GC-MS analyses. In TLC, a single discrete spot was obtained with an R_f value of 0.81 and it was found to be glycolipid. The GC-MS (Table 3) and FT-IR (Fig. 1) analyses revealed the compound produced by MSA19 to be a glycolipid with a hydrophobic nonpolar hydrocarbon chain (9,12,15-octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl) oxy]methyl]ethyl ester, (Z,Z,Z)) and the hydrophilic sugar part as 1H-cyclopenta[c]furan-3(3aH)-one, 6,6a-dihydro-1-(1,3-dioxolan-2-yl)-, (3aR,1-trans,6a-cis). The retention times, relative intensities (%) and electron impact mass spectrometry (EIMS) of the relevant peaks are as follows: Peak A: 17.13, 60, EIMS *m/z* (% rel. intensity), 108 (30), 95 (40), 79 (80), 67 (75), 55 (60), 41 (100), 29 (30) and Peak B: 15.38, 40, EIMS *m/z* (% rel. intensity), 69 (100), 40 (100), 29 (50). Based on the FT-IR and GC-MS data, the surface-active compound was characterized as a glycolipid. The compound showed 30% higher activity over the synthetic surfactants such as sodium dodecyl sulfate and Tween 80.

Table 2. Factors optimized for the production of a biosurfactant by *Brevibacterium casei* MSA19 under submerged fermentation

Factors	Ranges	Effective factor
pH	5–9 (with increments of 1)	7
Temperature	10–50 °C (with increments of 10 °C)	30 °C
Salinity	1–3% (with increments of 0.5%)	2%
Carbon sources	Glucose, olive oil, kerosene, vegetable oil (1%)	Glucose
Nitrogen sources	Yeast extract, beef extract, $(NH_4)_2NO_3$, Peptone (1%)	Peptone
Metals	$FeSO_4$, $CuSO_4$, $MnCl_2$, $MgCl_2$ (1%)	$FeSO_4$
Aminoacids	Asparagine, valine, leucine, glycine, glutamic acid (1%)	Asparagine

Antibiogram of MSA19

The antibiogram of MSA19 showed that the TLC-purified glycolipid was a broad-spectrum antimicrobial agent (Fig. 2). The MIC determined against biofilm bacteria such as *Escherichia coli* and *Vibrio alginolyticus* were 68 and 72 μg glycolipid mL^{-1}, respectively, and the concomitant MBC values were 38 and 41 glycolipid mL^{-1}, respectively.

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Table 3. Predicted compounds and major fractions of GC-MS data of *Brevibacterium casei* MSA19

Sl. no.	Peak name	Retention time	Peak area	%Peak area
1	Name: cyclopropyl carbinol Formula: C_4H_8O MW: 72	3.59	567 738	0.2203
2	Name: hydroxyurea Formula: $CH_4N_2O_2$ MW: 76	10.04	67 540	0.0262
3	Name: propanamide Formula: C_3H_7NO MW: 73	11.15	86 907	0.0337
4	Name: 2-aminononadecane Formula: $C_{19}H_{41}N$ MW: 283	14.59	560 945	0.2177
5	Name: 1H-cyclopenta[c]furan-3(3aH)-one, 6,6a-dihydro-1-(1,3-dioxolan-2-yl)-, (3aR,1-trans,6a-cis)- Formula: $C_{10}H_{12}O_4$ MW: 196	15.38	102 623	0.0398
6	Name: benzeneethanamine, 2,5-difluoro-á,3,4-trihydroxy-*N*-methyl- Formula: $C_9H_{11}F_2NO_3$ MW: 219	15.99	81 144	0.0315
7	Name: imidazole, 2-amino-5-[(2-carboxy)vinyl]- Formula: $C_6H_7N_3O_2$ MW: 153	16.17	115 737	0.0449
8	Name: 9,12,15-octadecatrienoic acid, 2-[(trimethylsilyl)oxy]-1-[[(trimethylsilyl)oxy]methyl]ethyl ester, (Z,Z,Z)- Formula: $C_{27}H_{52}O_4Si_2$ MW: 496	17.13	93 022	0.0361
9	Name: 1,2-benzenedicarboxylic acid, butyl octyl ester Formula: $C_{20}H_{30}O_4$ MW: 334	17.57	467 975	0.1816
10	Name: propanoic acid, 3,3′-thiobis-, didodecyl ester Formula: $C_{30}H_{58}O_4S$ MW: 514	29.87	255 571 296	99.1682
				100.0000

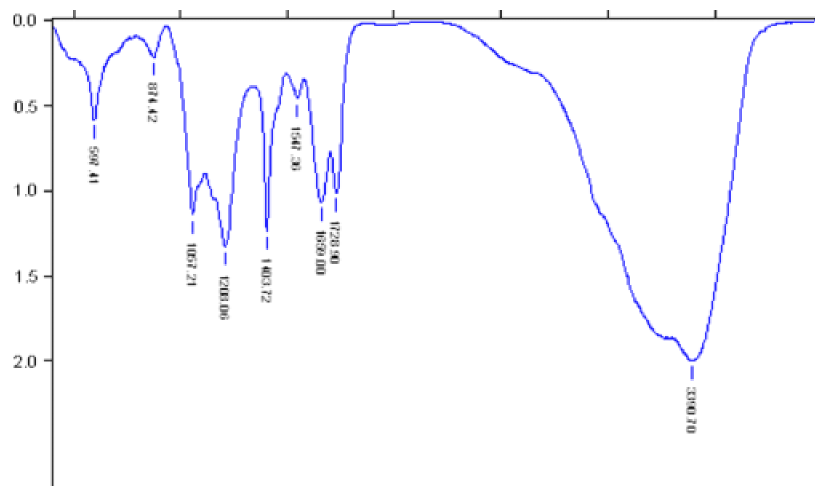

Fig. 1. FT-IR spectra of the surfactive fraction separated by column chromatography.

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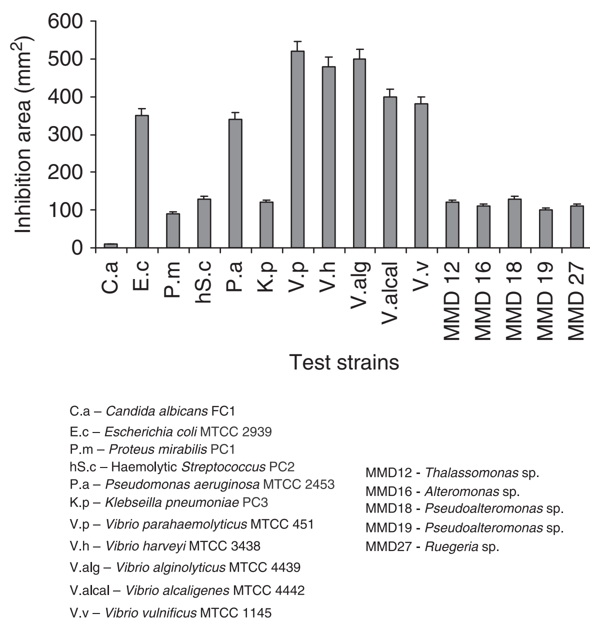

Fig. 2. Antimicrobial potential of *Brevibacterium casei* MSA19. The activities are presented in the inhibition area of the halo produced by *B. casei* MSA19. The test was conducted using 100 μg TLC-purified glycolipid in 100 μL of phosphate buffer (pH 7.0).

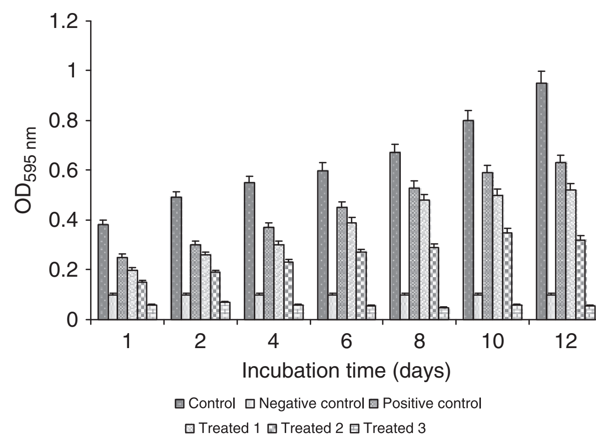

Fig. 3. Efficacy of MSA19 glycolipid in the biofilm-forming potential of mixed bacteria. The values are presented as the mean and SD of six trials. The inocula used for the mixed biofilm formation include *Vibrio parahaemolyticus* MTCC 451, *Vibrio harveyi* MTCC 3438, *Vibrio alginolyticus* MTCC 4439, *Vibrio alcaligenes* MTCC 4442, *Vibrio vulnificus* MTCC 1145, *Pseudomonas aeruginosa* MTCC 2453 and *Escherichia coli* MTCC 2339. The biofilm formation capacity was determined under dynamic conditions. Treated 1, 5 $\mu g\,mL^{-1}$; Treated 2, 10 $\mu g\,mL^{-1}$; Treated 3, 30 $\mu g\,mL^{-1}$.

Based on the MIC/MBC ratio, the MSA19 glycolipid was determined to be bacteriostatic. Considering the bacteriostatic potential of the MSA19 glycolipid, the efficacy in biofilm-forming capacity was determined using the lower concentrations of 5, 15 and 30 μg glycolipid mL^{-1}, respectively.

Biofilm disruption potential of the MSA19 glycolipid

Based on their surface-active properties, we hypothesized that glycolipids could affect biofilm formation. To test the hypothesis, we examined the effects of the MSA19 glycolipid on the biofilm-forming capacity of biofilm infection-causing pathogenic bacteria. The MSA19 glycolipid potentially disrupted biofilm formation under dynamic conditions. The biofilm disruption potential of the MSA19 glycolipid was consistent against mixed biofilm bacteria (Fig. 3) and individual biofilm bacterium (Fig. 4). The biofilm-forming capacity of both mixed culture and individual strains was significantly inhibited at 30 μg glycolipid mL^{-1}. In the cover slip assay, the biofilm disruption was evident and showed a disrupted biofilm under phase-contrast microscopic observation (Fig. 5). The microtiter plate assay also showed biofilm disruption on microtiter plates under Stereozoom microscopic observation. The present study showed that the MSA19 glycolipid could inhibit the beginning of the attachment mediated by flagella and pili (Shi & Sun, 2002). In a previous report, lauroyl glucose effectively disrupted both fungal and bacterial biofilms (Dusane *et al.*, 2008). However, its MIC was $> 500\,\mu g\,mL^{-1}$. Considering the lowest MIC/MBC values and biofilm disruption potential, the glycolipid biosurfactant would be an ideal lead

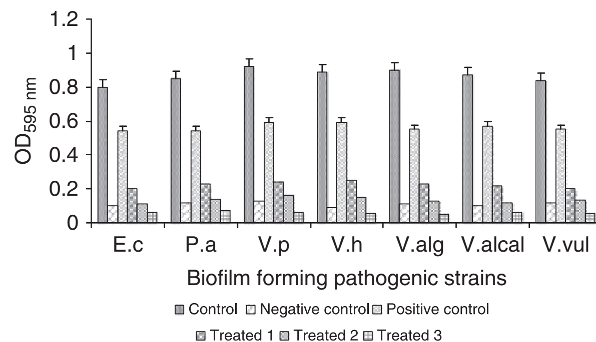

Fig. 4. Efficacy of the MSA19 glycolipid in the biofilm-forming potential of biofilm bacteria. The values are presented as the mean and SD of six trials. The efficacy of the MSA19 glycolipid in the relative biofilm formation capacity of biofilm-forming pathogenic strains including *Vibrio parahaemolyticus* MTCC 451 (V.p), *Vibrio harveyi* MTCC 3438 (V.h), *Vibrio alginolyticus* MTCC 4439 (V.alg), *Vibrio alcaligenes* MTCC 4442 (V.alcal), *Vibrio vulnificus* MTCC 1145 (V.vul), *Pseudomonas aeruginosa* MTCC 2453 (P.a) and *Escherichia coli* MTCC 2339 (E.c) was determined after 24 h under dynamic conditions. Treated 1, 5 $\mu g\,mL^{-1}$; Treated 2, 10 $\mu g\,mL^{-1}$; Treated 3, 30 $\mu g\,mL^{-1}$.

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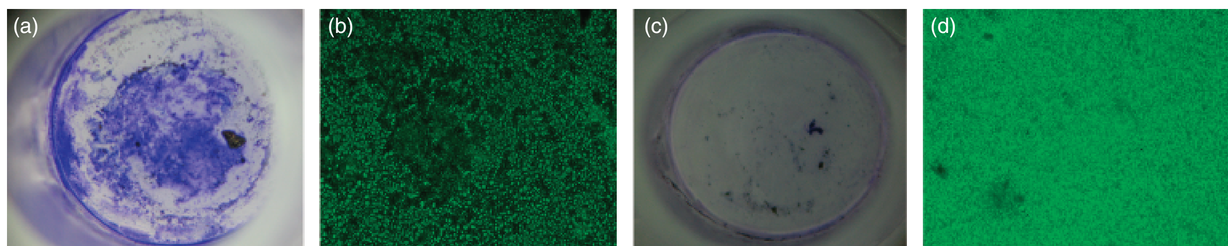

Fig. 5. Biofilm disruption potential of the MSA19 glycolipid against biofilm-forming pathogenic bacteria under microscopic views. (a) Biofilm formation in a microtiter plate under Stereozoom microscopic view. (b) Biofilm formation in a glass coverslip under a phase-contrast microscopic view. (c) Disrupted biofilm in a microtiter plate under Stereozoom microscopic view. (d) Disrupted biofilm in a glass coverslip under a phase-contrast microscopic view.

compound for the development of a novel antibiofilm drug. This natural biosurfactant can be produced on a large scale under submerged fermentation for commercial applications.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Biosurfactant producers screened from marine actinobacterial isolates (57).

Fig. S1. Growth and production curve of *Brevibacterium casei* MSA19.

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