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ORIGINAL ARTICLE

Inhibition of bacterial adhesion and anti-biofilm effects of *Bacillus cereus* and *Serratia nematodiphila* biosurfactants against *Staphylococcus aureus* and *Pseudomonas aeruginosa*

Alireza Keyhanian[1], Mojgan Mohammadimehr[1*], Farshad Nojoomi[1], Hamed Naghoosi[1], Mehdi Shakouri Khomartash[2], Mohsen Chamanara[3]

[1]Infectious Diseases Research Center, Aja University of Medical Sciences, Tehran, Iran
[2]Medical Biotechnology Research Center, Aja University of Medical Sciences, Tehran, Iran
[3]Toxicology Research Center, Aja University of Medical Sciences, Tehran, Iran

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ABSTRACT

Background and Objectives: Biosurfactants are amphiphilic surface-active agents that mainly produced by various microorganisms. In this study, the anti-biofilm and inhibition of bacterial adhesion activities of two bacterial biosurfactants were investigated.
Materials and Methods: After extraction and evaluation of *Bacillus cereus* and *Serratia nematodiphila* biosurfctants, inhibition of bacterial adhesion and anti-biofilm effects of them on *Staphylococcus aureus* and *Pseudomonas aeruginosa* were determined.
Results: On average, the synergistic effect of two bacterial biosurfactants, caused about 60% decrease in adhesion and about 80% decrease in biofilm formation of *S. aureus* and *P. aeruginosa*.
Conclusion: The results of this study showed that combination of *B. cereus* and *S. nematodiphila* biosurfactants would increase the potential of attachment inhibition and biofilm eradication with very low toxicity.

Keywords: Bacterial adhesion; Biofilms; *Bacillus cereus*; Surface-active agents; *Staphylococcus aureus*; *Pseudomonas aeruginosa*

INTRODUCTION

A biofilm is an assemblage of surface-associated microbial cells that is enclosed in an extracellular polymeric substance (1, 2). In addition, biofilms are communities of various microorganisms including bacteria, yeasts or a few filamentous fungi that are common causes of hospital infections. Biofilms are composed primarily of microbial cells and extracellular polymeric substance (EPS) matrix (3). Biofilms formed by various pathogens cause persistent and recurrent chronic infections. The challenge of treating biofilm infections is related to the increasing resistance of bacteria within the biofilm due to low metabolic activity and slow growth and a decrease in the intensity of the cell division rate, which lead to the inefficiency of common methods in eradicating biofilm infections, and the management of these diseases from treatment is very difficult (4). Complex structure of Staphylococcal biofilm decreases the

*Corresponding author: Mojgan Mohammadimehr, PhD, Infectious Diseases Research Center, Aja University of Medical Sciences, Tehran, Iran. Tel: +98-2143822995 Fax: +98-2143822992 Email: mojganmehr20@yahoo.com

penetration of antibiotics and leads to drug resistance (5). In *P. aeruginosa*, also, there is a strong barrier in the structure of biofilm (Alginate) that makes it difficult for antimicrobial agents to penetrate. The formation of this biofilm in pulmonary infectious patients such as cystic fibrosis (CF) makes treatment difficult (4). Biofilms can be formed on both biotic and abiotic surfaces, including on living tissues, indwelling medical devices, industrial or portable water system piping, and natural aquatic (6). Conventionally, biofilms are controlled via combination of antibiotics, chemical techniques and using detergents, in addition to physical strategies and chemical reagents. However, many biofilms have become resistant to conventional methods that were designed for planktonic cell models (7). Biosurfactants are safe and effective metabolites, that have potential to replace with chemical substances and mechanical methods. Biosurfactants can change the characteristics of the bacterial adhesions and the formation of bacterial biofilm. This property will prevent bacteria from attaching to solid surfaces and inhibit biofilm formation. In addition, biosurfactants have high biodegradability and low toxicity compared with chemical substances (8). The aim of current study was to investigate the anti-adhesive and anti-biofilm activities of biosurfactants of *B. cereus* and *S. nematodiphila* against *S. aureus* (ATCC 25923) and *P. aeruginosa* (ATCC 27853) and compare the results with vancomycin and imipenem.

MATERIALS AND METHODS

Bacterial strains. *B. cereus* and *S. nematodiphila* have been isolated from Oil-contaminated area (Amirabad port of Caspian Sea, Iran), identified by using 16S rRNA PCR and bacteriologic methods. These strains were stored in the glycerol-containing medium at -80°C for long-term maintenance. Biofilm producing strains; *Staphylococcus aureus* ATCC 25923 and *Pseudomonas aeruginosa* ATCC 27853 were purchased from Bahar Azma (Bahar Azma, Iran) (3).

Preparation of bacterial suspension. Biofilm producing bacteria were cultured on Trypticase soy agar (TSB) (Himedia, India) and incubated for 24 hours. Subsequently, bacterial suspension was prepared in TSB containing 1% glucose 0.5 McFarland concentration (10^8 cfu/mL) and used for antimicrobial and anti-biofilm tests (9).

Preparation of biosurfactant solution. For the initial assessment of biosurfactant producing bacteria and to perform two subsequent experiments, biosurfactant producing bacteria were cultivated in different media consisted of 100 mL nutrient broth (Himedia, India) medium containing crude oil, gasoline, and olive oil in amounts of two, one and one ml, respectively, and incubated for one week at 30°C and 150 rpm shaking. The media were centrifuged at 6,500 rpm for 10 minutes and supernatant containing biosurfactant was separated from the sediment and used in Oil dispersion and Interfacial tension (IFT) tests (10).

Oil dispersion test. A 30 mL of distilled water was poured into a petri dish and 20 µL of crude oil was added to it. After a minute, 10 µL of the supernatant dripped in center of petri dish and after two minutes, if the solution contains biosurfactant, it would cause the crude oil to move to the edge of the petri dish and a transparent layer would be formed on it. The diameter of the created layer is in accordance with the efficacy of biosurfactant (11).

IFT test. Based on the method of Barry et al. (2015), IFT test was performed with the IFT400 device at oil productivity improvement Co., Shiraz, Iran. In this experiment, the reduction of interfacial tension of crude oil was evaluated using biosurfactant solution (12).

Extraction and purification of the Biosurfactant. A colony of *B. cereus* and *S. nematodiphila* was separately inoculated to 500 mL of nutrient broth (NB) (Himedia, India) containing 10 mL of n-Hexane as hydrocarbon source, placed in shaker incubator at 30°C and 150 rpm shaking for one week. After that, centrifugation was performed at 5,000 rpm at 4°C for 20 minutes and the supernatant was harvested. The pH was adjusted to be 2.0 by addition of 2N sulfuric acid. Then, equal volume of chloroform and methanol (2:1) mixture was added. Subsequently, it was placed in a 60°C oven to evaporate the solvent. The final product was used as pure biosurfactant.

Antimicrobial assay. To determine the minimum inhibitory concentration (MIC) in planktonic bacteria, 96-well flat-bottom polystyrene plate was used. After determining the dry weight of the extracted

biosurfactant, it was diluted with deionized water and serial dilutions were prepared from it. 100 µL of Mueller Hinton broth (Himedia, India) were placed into each of the wells. 100 µL of the prepared biosurfactant dilutions were placed in the wells.). 20 µL of prepared microbial suspension with concentration about 0.5 McFarland (10^8 cfu/mL) was added to each of the wells. Also, biosurfactant of *B. cereus* (BBC1) and biosurfactant of *S. nematidophila* (BSN8) were tested in combination with each other to evaluate the synergistic effect in separate wells for planktonic cells. The same values were tested for vancomycin and imipenem. To determine minimum bactericidal concentration (MBC), the content of the wells that have visual inhibition were inoculated in Mueller Hinton Agar (Himedia, India) and incubated at 37°C for 24 hours. Each experiment was repeated three time (13).

Inhibition of biofilm formation. A 180 µL of TSB medium with 1% glucose was poured into the microplate wells. A 100 µL of each serial concentrations of BBC1 and BSN8 individually and synergistically were poured into the wells, and 20 µL of the bacterial suspension were added to each well. The microplate was placed in the incubator at 37°C for 24 hours. *P. aeruginosa* in TSB without biosurfactant treatment was used as control. The supernatant was removed after incubation, and the wells were washed with sterile water. The biofilm at the bottom of the wells was stained by crystal violet. After a few minutes, the optical density (OD) of the wells were measured using microplate reader (Mikura, UK) at 570 nm. OD > 1 considered as High biofilm formation, 1 > OD > 0.1, Low biofilm formation and OD < 0.1 considered as no biofilm formation (14, 15).

Destruction of mature biofilm. A180µL of TSB with 1% glucose was poured into each microplate wells and 20 µL of bacterial suspension was added to it. The microplate was incubated at 37°C for 72 hours. Then, 200 µL of fresh TSB medium with 1% glucose was replaced every 24 hours to continue the biofilm growth in the wells. The supernatant was removed after 3 days and the wells were washed three times with sterile water to remove the planktonic cells. After that, 200 µl of biosurfactant of *B. cereus* 1(BBC1) and biosurfactant of *S. nematidophila* 8(BSN8) individually and synergistically (100:100 µL) with different dilutions were added to the wells and incubated for 150 minutes at 37°C. 200 µL of sterile water were used as control solution. Wells were washed three times and the remaining biofilms were stained using crystal violet. To measure the amount of residual biofilm at the bottom of the wells, the optical density was measured at 570 nm (10, 16).

Anti-adhesion effects of BBC1 and BSN8. To check the adhesion of bacteria on the solid surface, 200 µL of bacterial suspension was poured into the wells and serial dilutions of BBC1 and BSN8 individually and synergistically were added to it. Bacterial suspension without biosurfactant was used as control negative. The microplate was incubated at 37°C for 4 hours. The wells were washed to remove planktonic cells after incubation. Afterwards, the optical density at 600 nm was measured to check the adhesion of bacteria. All of the tests were repeated three times for each sample.

Statistical analysis. The Graph pad Prism software v8.4.0 was used to draw the graphs statistical calculations were performed by One-Way ANOVA and $P < 0.0001$ was considered as significant P. value.

Ethical approval. This study was confirmed by the Ethics committee of Aja university of medical sciences. Confirmation code: IR.AJAUMS.REC.1401.054 (2022 July).

RESULTS

Oil dispersion test. The supernatant solution of *B. cereus* and *S. nematodiphila* formed a transparent layer with 3 and 3.5 cm diameter, respectively.

IFT test. IFT of *S. nematodiphila* has reduced from 20 to 10 mN/m (about 50% reduction). Also, *B. cereus* was able to reduce the IFT from 20 to 12 mN/m.

Antimicrobial effects of biosurfactant against *S. aureus* and *P. aeruginosa*. Antimicrobial results of biosurfactants individually showed that only BBC1 had an admissible antibacterial effect against standard strains of *S. aureus* and *P. aeruginosa*. As shown in Table 1 after the combination of two biosurfactants (BBC1 + BSN8), the MIC and MBC concentrations were reduced.

Anti-biofilm activity of biosurfactants and antibiotics. As shown in Fig. 1A, BBC1 and BSN8 caused up to 85% and 65% decrease in biofilm formation of *S. aureus*, respectively. While, high concentrations of BBC1+BSN8 caused almost complete inhibition of *S. aureus* biofilm formation. The effect of vancomycin and imipenem in preventing the biofilm formation of *S. aureus* were significantly higher than BBC1 and BSN8, but the inhibitory effect of BBC1+BSN8 was higher than two mentioned antibiotics. As shown in Fig. 1B, imipenem caused significant decrease in biofilm formation of *P. aeruginosa* (up to 95%), compared with vancomycin and BSN8. Effect of lower concentrations (10-40 µg/mL) of BBC1+BSN8 on *P. aeruginosa* biofilm formation was significant higher than BBC1, BSN8 and two mentioned antibiotics, but

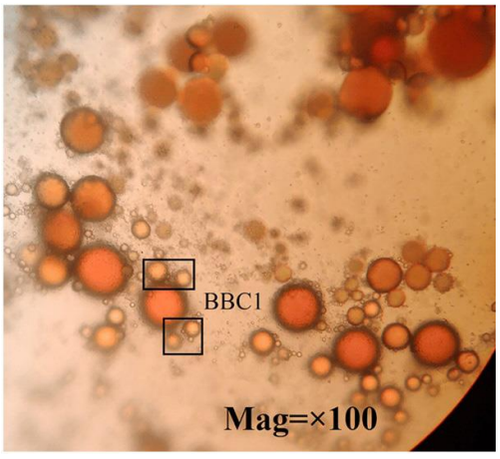

Fig. 2. Optical microscopic image of *S. aureus* ATCC 25923 biofilm after exposure to BBC1

Table 1. Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) of BBC1and BSN8 compared with vancomycin and imipenem

	MIC (µl/mL)					MBC (µl/mL)				
	BBC1	**BSN8**	**BBC1+ BSN8**	**VAN**	**IMP**	**BBC1**	**BSN8**	**BBC1+ BSN8**	**VAN**	**IMP**
S. aureus 25923	40	320	5	160	0.63	160	>5120	20	160	0.63
P. aeruginosa 27853	20	2560	5	160	0.63	80	>5120	10	320	1.25

BBC1 and BSN8 were incubated with a regulated inoculum of *S. aureus* and *P. aeruginosa* compared with vancomycin (VAN) and imipenem (IMP) after 24 h at 37°C.

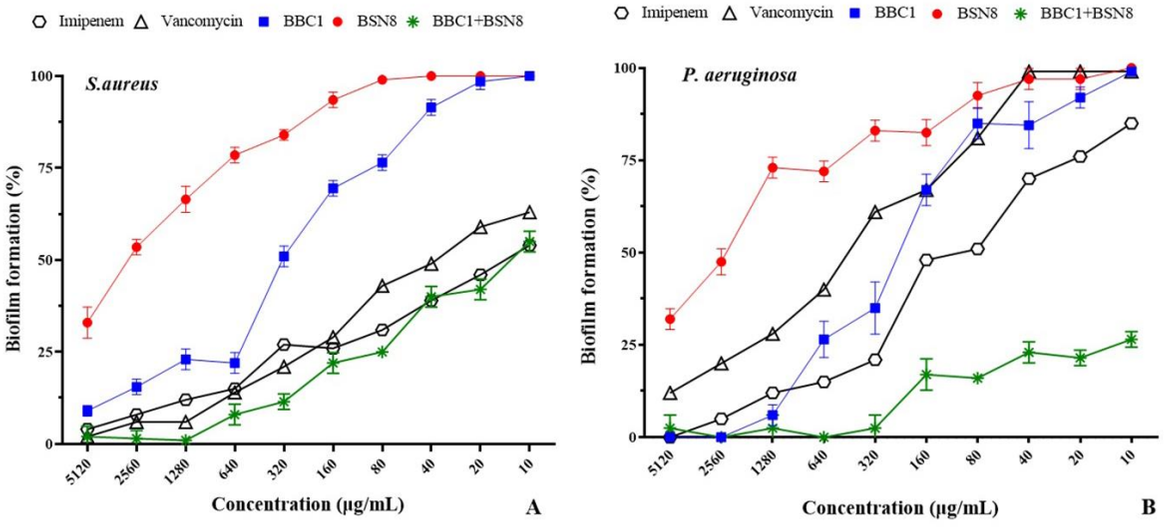

Fig. 1. Inhibitory activity of biosurfactant of *B. cereus* (BBC1) and biosurfactant of *S. nematodiphila* (BSN8) individually and synergistically compared with broad-spectrum antimicrobial agents. (A) *S. aureus* (B) *P. aeruginosa*. Biofilm without treatment was used as positive control. Optical density of the biofilm was measured at 570 nm. Each experiment was repeated three times, and the average percentage of the data was recorded. Error bars represent standard deviation from the mean. ($P < 0.0001$).

at higher concentrations (1280-5120 µg/mL), there was no significant difference between BBC1+BSN8, imipenem and BBC1 alone.

Disruption of mature *S. aureus* and *P. aeruginosa* biofilms by biosurfactants. According to Fig. 2, biosurfactant particles gather around the biofilm and biologically surround it. Reducing the surface tension is able to destroy the biofilm structure. Results show that high concentrated BBC1 disrupts the biofilm formation by *S. aureus* up to 40% and combined by BSN8, the synergistic effect caused the destruction of both biofilms up to 80% and 60% in *P. aeruginosa* and *S. aureus*, respectively (Fig. 3). The antibiotic agents; imipenem and vancomycin at 5120 µg/mL could not lead to significant disrupt the formation of biofilm. Results showed that at 10 to 640 µg/mL, biosurfactants are able to disrupt the mature biofilm with their synergistic effect.

Anti-adhesive activity of biosurfactants and antibiotics. As shown in Fig. 4, BBC1, BSN8 and BBC1+BSN8, caused up to 70%, 58% and 90% decrease in adhesion of *S. aureus*, respectively. The effect of vancomycin and imipenem in preventing the attachment of *S. aureus* were significant lower than biosurfactants. In the presence of BBC1, BSN8 and BBC1+BSN8, Adhesion of *P. aeruginosa* decreased up to 47%, 53% and 85%, respectively. The inhibitory effect of vancomycin and imipenem on adhesion of *P. aeruginosa* were significant lower than BBC1 and BBC1+BSN8.

DISCUSSION

Biofilm formation in medical devices such as contact lenses, catheters, prostheses, heart valves also occurs on different surfaces of the body, including the mucous surfaces of the respiratory and digestive systems (17). Bacterial biofilms strongly resist against antibacterial agents and play an important role in chronic and nosocomial infections (18). In the present study, we tried to assay the anti-adhesion and anti-biofilm effects of biosurfactants produced by *B. cereus* and *S. nematodiphila*. According to MIC and MBC results, both BBC1 and BSN8 mainly led to cell lysis and killing by deforming the cytoplasmic membrane of *S. aureus* and *P. aeruginosa*. Such lysis mechanism indicates that biosurfactants kill a wide range of microorganisms. BBC1 had higher antibacterial effect than BSN8. However, after combining, a great synergistic effect was seen. The MIC and MBC decreased about 8 times after the combination of BBC1 and BSN8. The MBC of *P. aeruginosa*, decreased from 80 µg/mL BBC1 to 10 µg/mL BBC1+BSN8. Such phenomenon was seen research by Sana et al. that synergistic effect of BS15 biosurfactant and rhamnolipid had good results in destroying the cell wall of *S. aureus* and *E. coli* (19). The anti-

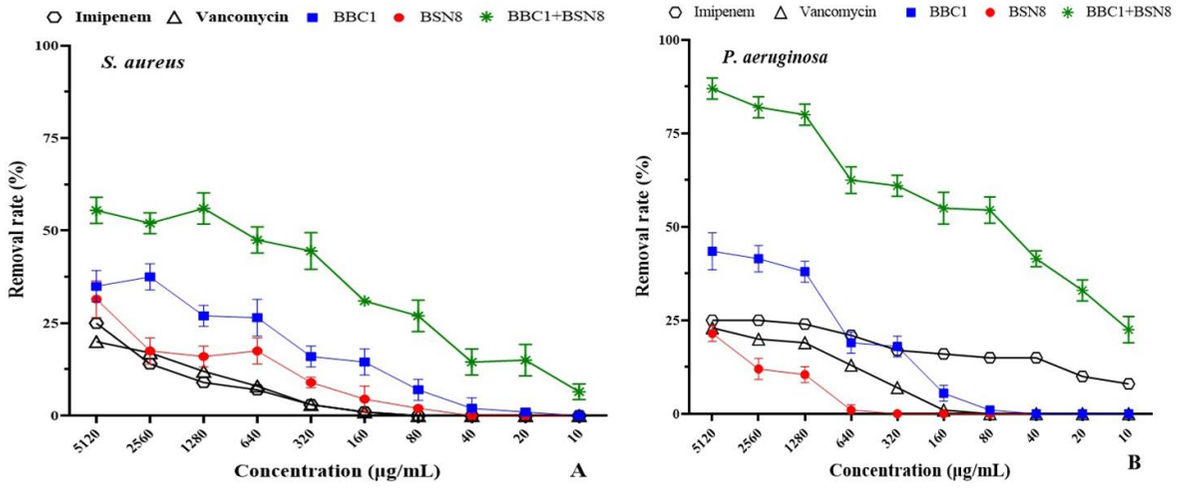

Fig. 3. Inhibitory effect of BBC1 and BSN8 compared to antibiotic agents on mature biofilm in polystyrene surface. (A) *S. aureus* (B) *P. aeruginosa*. The biofilm was exposed to different concentrations of biosurfactant and antimicrobial agents and measured by the crystal violet method. The amount of biofilm biomass compared to the control was described as an average ($P < 0.0001$).

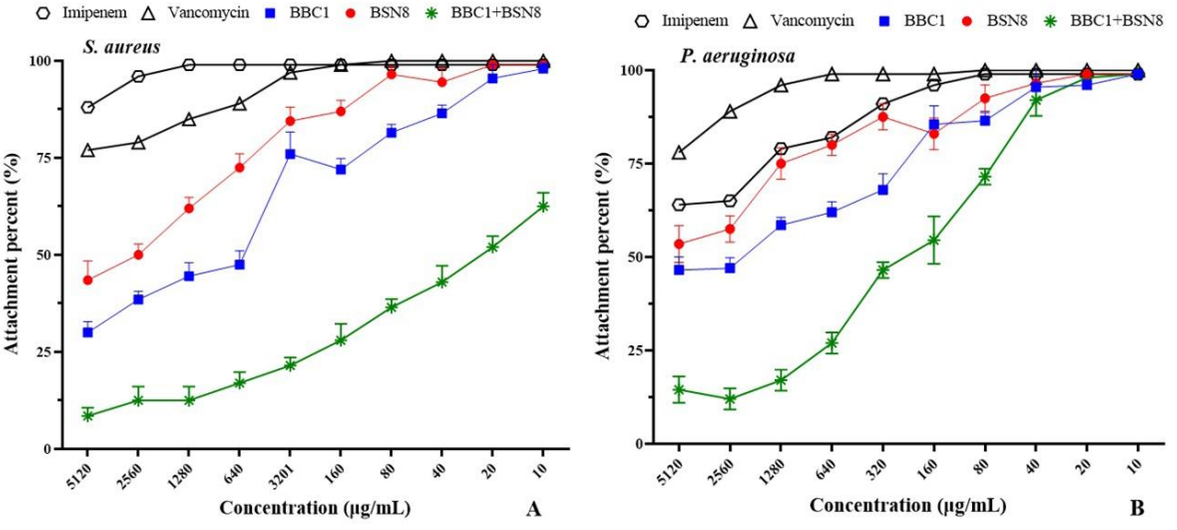

Fig. 4. Measuring the adhesion of (A) *S. aureus* (B) *P. aeruginosa* to the surface of polystyrene plate in 600 nm. Results of this test showed that the adhesion of planktonic bacteria to the microplate surfaces after exposure to biosurfactant, especially in the synergistic mode, was greatly reduced. The experiments were repeated three times and compared with the control ($P < 0.0001$)

biofilm activity of biosurfactant were also reported in other studies (20, 21). Karlapudi et al. reported the anti-bacterial and anti-biofilm effect of biosurfactant extracted from *Acinetobacter* against *S. aureus* biofilm (21). A basic strategy to overcome the biofilm structure is to reduce its surface tension. In this study, BBC1 and BSN8 were able to move the oil to edge of the plate and create transparent diameter up to 3.5 cm in oil replacement technique. Also, IFT test results showed that BBC1 and BSN8 had high reduction in the surface tension up to 50%. This result indicates an influence on the rheological behavior of the surface for biofilm formation. Biosurfactant compounds contain a hydrophobic and a hydrophilic moiety, and have the ability to reduce interfacial and surface tension between different fluid phases. Interfacial tension and Critical Micelle Concentration (CMC) of biosurfactants were also comparable with studies of Karlapudi, Makkar and Pardhi (21-23). Since one of the best candidates for destroying and disrupting biofilm formation is biosurfactants, in the present study, we noticed increase in the concentration of biosurfactants, results in an increase in their antibacterial, anti-biofilm and anti-adhesion activities. Interruption of connection between the solid surface and the bacterial membrane may be prevented the formation of biofilm (24-26). This feature has an effect on the adhesion of *S. aureus*, as it is the first stage of biofilm formation on solid surfaces in *S. aureus* strains (27, 28). Our results showed that BBC1 alone and BBC1+BSN8 have inhibitory effects on adhesion and biofilm formation of *S. aureus* and *P. aeruginosa*. This activity was observed even in lower concentrations of above mentioned biosurfactants. After combination of BBC1 and BSN8, about 90-95% reduction in biofilm formation and adult biofilm destruction of *S. aureus* and *P. aeruginosa* was observed. Combination of biosurfactants has reduced the dosage for antimicrobial and anti-biofilm usage. Therefore, it will reduce the cost and also provide more safety of administration.

CONCLUSION

The present study shows that BBC1 and BSN8 biosurfactants produced by *B. cereus* and *S. nematodiphila* have effective anti-biofilm and anti-adhesion properties and can be used in the disinfection of medical devices. These biosurfactants with their anti-adhesion properties have opened new way to prevent the formation of wide range of biofilms. However, there are still many uncertainties for the use of such valuable bio-products, that requires extensive studies and there may be many limitations for the further use of biosurfactants.

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