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Antimicrobial and antibiofilm activity of rhamnolipid and sophorolactone on some pathogenic bacteria

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ABSTRACT: Biosurfactants are varieties of surfactants, usually produced by microorganisms. These substances are used in various fields such as pharmaceutical industry, cosmetics production, food, agriculture, animal husbandry applications and waste treatment. These substances also have antibacterial, antiviral and antibiofilm activities. In our study, the antimicrobial, antibiofilm and mature biofilm eradicating effects of rhamnolipid and sophorolactone biosurfactants on standard and clinical isolates of *Staphylococcus aureus*, *Enterococcus faecalis*, *Acinetobacter baumannii*, *Escherichia coli* and *Pseudomonas aeruginosa* were investigated. The antimicrobial activity was determined by agar well diffusion method, minimum inhibitory concentration and minimum bactericidal concentration were determined by microdilution method and antibiofilm activity was determined by crystal violet staining method in microplate. As a result of the study; rhamnolipid and sophorolactone were found to have antimicrobial effect on standard and clinical isolates of *S. aureus*, *E. faecalis*, *A. baumannii*, and *P. aeruginosa*, which are important human pathogens, and also inhibited the biofilm development ability of these pathogens.

KEYWORDS: Rhamnolipid; sophorolactone; antibacterial activity; antibiofilm activity; pathogens.

1. INTRODUCTION

Surfactants are compounds with hydrophobic and hydrophilic properties and have a wide range of applications. Biosurfactants, a type of surfactants, are mainly surfactants produced by some microorganisms. These compounds are mainly divided into groups such as glycolipids, lipopeptides, lipopolysaccharides, phospholipids and fatty acids. These substances differ from traditional surfactants because they are of biological origin and are produced without any chemical treatment. Biosurfactants are increasingly used in various fields such as pharmaceutical industry, cosmetics production, food, agriculture, animal husbandry applications and waste treatment. They have been extensively studied in last years since they also have advantages over synthetic surfactants, such as being less toxic, less harmful to the environment and having higher activity in some areas. Some of the biosurfactants also have microbiological activities. Rhamnolipids and sophorolactone used in our study are biosurfactants. Rhamnolipids are included in glycolipid surfactants. Rhamnolipids are mainly produced by *Pseudomonas aeruginosa*. Rhamnolipids have known antibacterial and anticancer properties. Sophorolactone is a sophorolipid and is mainly produced by non-pathogenic yeasts such as *Starmerella bombicol* [1-8]. Sophorololipids are mainly known for their antimicrobial, antiviral, skin and hair protective activities [6, 9]. In addition, the ability of biosurfactants as immunomodulators [10] and to enhance the antimicrobial and antibiofilm activity of various substances has been tested in many nanoparticle formulations and in combination studies with antimicrobial agents [11-15].

Pathogenic microorganisms cause infections with virulence factors. Biofilm is one of these virulence factors. Biofilm is a complex structure formed by one or more microorganisms coated by an extrapolymeric substance secreted by microorganisms. It is known that, microorganisms cover themselves with these substances. Biofilm can protect microorganisms from the immune system and antimicrobial compounds.

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With the protective feature of biofilm, it prevents antimicrobial substances to reach sufficient concentration and contributes to the development of antimicrobial resistance [16, 17]. Antimicrobial resistance is one of the most important problems that adversely affect world health today. Although there are various ways to solve the resistance problem, the main ones are the discovery of new antimicrobial effective compounds or preventing the development of infection by inhibiting the virulence factors of microorganisms [18, 19]. Although there are antimicrobial activity studies on many pathogenic microorganisms related with rhamnolipids and sophorolipids, studies on biofilm development and efficacy on mature biofilms, especially for microorganisms that are serious infection agents for humans, are limited. That is why, we investigated the antimicrobial and antibiofilm activity of rhamnolipid and sophorolactone on some pathogenic microorganisms, including microorganisms identified by WHO as the main pathogenic microorganisms to be combated [20].

2. RESULTS

2.1. Antimicrobial activity

Rhamnolipid and sophorolactone were found to have varying amounts of antimicrobial activity against all microorganisms used except *E. coli* ATCC 25922. *E. coli* CI and *P. aeruginosa* CI. MIC values ranged between 5.2-20.83 mg/ml for rhamnolipid and 2.08-4.16 mg/ml for sophorolactone. MBC values ranged between 10.4-41.66 mg/ml for rhamnolipid and 2.08-8.32 mg/ml for sophorolactone. When these data are analyzed, it is seen that sophorolactone was effective against bacteria at lower concentrations than rhamnolipid (Table 1).

Table 1. Antimicrobial activity of rhamnolipid and sophorolactone

Bacteria	Rhamnolipid			Sophorolactone		
	ZD (mm)	MIC (mg/ml)	MBC (mg/ml)	ZD (mm)	MIC (mg/ml)	MBC (mg/ml)
S. aureus ATCC 29213 (MSSA)	21.07±0.21	20.83	41.66	16.45±0.26	2.08	4.16
MSSA CI	20.95±0.10	10.4	20.83	15.15±0.26	2.08	4.16
S. aureus ATCC 43300 (MRSA)	23.61±0.14	5.2	10.4	15.60±0.25	1.04	2.08
MRSA CI	19.01±0.20	20.83	41.66	14.90±0.22	4.16	8.32
E. faecalis ATCC 29212	8.32±0.09	20.83	41.66	13.95±0.11	2.08	4.16
E. faecalis CI	8.55±0.11	10.4	20.83	12.17±0.13	4.16	8.32
A. baumannii ATCC 19606	7.23±0.11	10.4	20.83	12.88±0.23	4.16	8.32
A. baumannii CI	8.29±0.22	20.83	41.66	14.75±0.37	2.08	4.16
E. coli ATCC 25922	0	-	-	0	-	-
E. coli CI	0	-	-	0	-	-
P. aeruginosa ATCC 27853	9.58±0.11	20.83	41.66	1.88±0.14	4.16	8.32
P. aeruginosa PAO1	11.11±0.28	10.4	20.83	7.67±0.18	4.16	8.32
P. aeruginosa CI	0	-	-	0	-	-

ZD: Zone diameter, MIC: Minimum inhibitory concentration, MBC: Minimum bactericidal concentration, CI: Clinical isolates, MSSA: Methicillin susceptible *S. aureus*, MRSA: Methicillin resistant *S. aureus*, -: Not tested

2.2. Investigation of the effectiveness of the biosurfactants on biofilm development

The inhibitory effect of rhamnolipid and sophorolactone on biofilm development of the bacteria used in our study was found to be quite high. This activity was dose-dependent in many microorganisms. Rhamnolipid and sophorolactone showed similar levels of activity on these microorganisms. Results with increased biofilm formation or biofilm inhibitory effect of 5% or less are shown as "0" in table 2. The

inhibitory effect of both substances on biofilm formation was lower in *E. coli* and *P. aeruginosa* bacteria than that in the other bacteria (Table 2).

Table 2. Efficacy of rhamnolipid and sophorolactone on biofilm development

	Biofilm inhibition (%)							
	Rhamnolipid				Sophorolactone			
Bacteria	**MIC***	**1/2 MIC****	**1/4 MIC**	**1/8 MIC**	**MIC***	**1/2 MIC****	**1/4 MIC**	**1/8 MIC**
S. aureus ATCC 29213 (MSSA)	88±2.32	78±1.35	78±2.11	53±0.83	90±1.19	81±2.48	76±2.97	5±1.18
MSSA CI	90±1.88	89±0.80	89±1.46	86±1.26	91±2.42	84±2.06	78±2.12	23±1.81
S. aureus ATCC 43300 (MRSA)	87±3.21	88±1.48	90±1.80	85±1.1	89±2.80	85±1.09	68±1.29	0
MRSA CI	88±2.55	89±1.66	69±2.37	73±1.35	90±2.14	89±1.84	59±1.44	54±0.88
E. faecalis ATCC 29212	89±1.45	90±2.24	90±1.24	84±2.59	91±3.12	89±1.28	50±1.15	11±2.11
E. faecalis CI	88±1.23	88±1.17	87±1.04	71±2.41	92±1.45	48±1.99	50±1.54	0
A. baumannii ATCC 19606	90±2.33	87±1.57	80±1.58	17±0.87	89±1.78	87±2.53	71±2.55	29±1.11
A. baumannii CI	68±2.88	61±2.17	60±2.90	0	82±2.15	81±2.68	75±1.16	0
E. coli ATCC 25922	48±1.22*	30±1.75**	0	0	40±1.20*	31±1.88**	0	0
E. coli CI	35±1.89*	10±0.58**	0	0	32±1.43*	8±0.88**	0	0
P. aeruginosa ATCC 27853	64±1.54	62±1.86	62±1.65	36±1.09	55±2.76	48±1.45	26±2.71	0
P. aeruginosa PAO1	55±2.01	52±1.92	32±1.05	0	51±3.01	45±1.33	0	0
P. aeruginosa CI	23±2.22	10±0.39	0	0	32±2.11	18±1.18	9±1.04	0

MIC: Minimum inhibitory concentration, CI: Clinical isolates, MSSA: Methicillin susceptible *S. aureus*, MRSA: Methicillin resistant *S. aureus*, *: Since no antimicrobial activity was detected, a concentration of 10 mg/ml was tried. **: Since no antimicrobial activity was detected, a concentration of 5 mg/ml was tested in this well.

2.3. Investigation of the minimum biofilm eradication concentration

Biofilm eradicating concentrations of rhamnolipid and sophorolactone were generally above 166.66 mg/ml for rhamnolipid and 33.33 mg/ml for sophorolactone (Table 3).

Table 3. Minimum biofilm eradication concentration of rhamnolipid and sophorolactone

Bacteria	**Rhamnolipid (mg/ml)**	**Sophorolactone (mg/ml)**
S. aureus ATCC 29213 (MSSA)	>166.66	33.33
MSSA CI	166.66	33.33
S. aureus ATCC 43300 (MRSA)	>166.66	33.33
MRSA CI	>166.66	33.33
E. faecalis ATCC 29212	>166.66	>33.33
E. faecalis CI	>166.66	>33.33
A. baumannii ATCC 19606	166.66	>33.33
A. baumannii CI	>166.66	33.33
E. coli ATCC 25922	>166.66	>33.33

E. coli CI	>166.66	>33.33
P. aeruginosa ATCC 27853	>166.66	>33.33
P. aeruginosa PAO1	>166.66	>33.33
P. aeruginosa CI	>166.66	>33.33

MSSA: Methicillin susceptible *S. aureus*, MRSA: Methicillin resistant *S. aureus* , CI: Clinical isolates

3. DISCUSSION

Biosurfactants, a type of surfactant, have been found to have many activities, especially antibacterial, antifungal, antiviral, antibiofilm, immunomodulatory and anticancer properties. In addition, many studies have reported increased antimicrobial and antibiofilm activities when combined with some compounds with antimicrobial activity [1-15]. Due to these properties, in our study, the antimicrobial and antibiofilm properties of rhamnolipid, a glycolipid group biosurfactant, and sophorolactone, a sphorolipid biosurfactant, on some pathogenic bacteria were investigated as biosurfactants with the aim of determining new antimicrobial effective compounds for major pathogens, which is the main option necessary to prevent or slow down antimicrobial resistance.

In our study, firstly, the antimicrobial activity of rhamnolipid and sophorolactone against various pathogenic bacteria was investigated by agar well diffusion method, and then the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) were determined for microorganisms. Then biofilm development of microorganisms and the minimum biofilm eradicating concentrations (MBEC) of rhamnolipid and sophorolactone on mature biofilm were also determined.

Antimicrobial activity results (Table 1) showed that rhamnolipid and sophorolactone showed antimicrobial activity against all microorganisms except *E. coli* ATCC 25922, *E. coli* CI and *P. aeruginosa* CI. In addition, antimicrobial activity was generally stronger against Gram positive bacteria. These findings are consistent with similar studies [21-25]. It has also been found by other researchers that biosurfactants generally do not show antimicrobial activity against Gram-negative bacteria or this activity is less [21-25].

Antimicrobial activity of biosurfactants has been investigated for some pathogenic microorganisms. In terms of antimicrobial activity in studies involving the microorganisms used in our study, rhamolipid was found to have antimicrobial activity against *A. baumannii* [7, 23], *S. aureus* ATCC 9144 [24, 26], *S. aureus* ATCC 6538 [21, 23, 27], *P. aeruginosa* [23], *Enterobacter* spp. [23], while sophorolipid has antimicrobial effect on *S. aureus* ATCC 9144 [24], *S. aureus* [28], *P. aeruginosa* PAO1 [24, 26], *E. coli* NCTC 10418 [26], *E. coli* [29]. Considering the existing studies, it is seen that there are few studies with rhamnolipids and soforolipids, especially for the bacteria used in our study, and clinical microorganisms have not been sufficiently investigated about their microbiological effects of biosurfactants. Some researchers have obtained the biosurfactants used in their studies in vitro through various microorganisms. [23, 26, 27]. Due to the differences in the methods used and the substances tested in similar studies, there are also differences in the antimicrobial activity results obtained.

There are few studies that determine the antimicrobial activity by the method we used in our study. In these studies, rhamnolipid extracted from *P. aeruginosa* UKMP14T [23] was found to be antimicrobial against *S. aureus*, *A. baumannii* and *P. aeruginosa*, and sophorolipid was found to be antimicrobial against *S. aureus* [28]. Habibah et al [21] reported that rhamnolipid showed antimicrobial activity against *S. aureus* ATCC 6538 and *Bacillus subtilis* ATCC 6633, but no antimicrobial activity against *E. coli* ATCC 6539 and *Salmonella typhi* ATCC 8939 in their study done on rhamnolipid with copper obtained by green synthesis. Hashim et al. [30] found that rhamnolipid from *Lactobacillus plantarum* showed antimicrobial activity against *S. aureus* NCTC 8325. In other studies, antimicrobial activity was determined by using different methods. Therefore, there is variability in the presence of antimicrobial activity or in the indicated effective doses [7, 26].

When the MIC and MBC results obtained for rhamnolipid and sophorolactone in our study were analyzed (Table 1), it was found that the values varied in a wide range according to the microorganism type, the MIC range for rhamnolipid was between 5.2-20.83 mg/ml and the MBC range was between 10.4-41.66 mg/ml, while the MIC range for sophorolactone was between 1.04-4.16 mg/ml and the MBC range was between 2.08-8.32 mg/ml. In addition, our results showed that the MIC and MBC values of sophorolactone were lower than rhamnolipid (Table 1).

In the studies conducted with the microorganisms used in our study, MIC and MBC values obtained for rhamnolipid, the MIC value against *S. aureus* ATCC 9144 was 0.5 % (v/v) [24], against *S. aureus* ATCC 6538 was 19 µg/mL [21], a rhamnolipid extracted from *P. aeruginosa* UKMP14T had MIC and MBC values of 7.81-62.5 µg mL/ml and 31.25-1000 µg /ml against *S. aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *P. aeruginosa*, respectively [23], a rhamnolipid extracted from *L. plantarum* 50 mg/ml against the endodontic pathogen *E. faecalis* RB17 and 0.097 mg/ml against *S. aureus* NCTC 8325 [30], rhamnolipid and sophorolipid against *S. aureus* at 50 µg/mL [31], 1 % (v/v) [24] against *P. aeruginosa* PAO1, *E. coli* NCTC 10418, *S. aureus* ATCC 9144 for sophorolipid. When the evaluated the data obtained in our study (Table 1), it is seen that the MIC and MBC values are similar to some studies in the literature and some of them are very different. The differences in the results may be due to the differences in rhamlolipids, sophorolipids and the microorganisms used.

In our study, the efficacy of rhamnolipid and sophorolactone at 1-1/8 MIC concentration range on the biofilm development of bacteria and mature biofilm was investigated for each microorganism. The results showed that rhamnolipid and sophorolactone were able to inhibit biofilm growth of all microorganisms very strongly at varying rates, especially at MIC and close values (Table 2). It was realized that as the concentration increased the biofilm inhibition rate increased. This indicates the effectiveness of rhamnolipid and sophorolactone on biofilm growth. The biofilm inhibition efficiency of rhamnolipid at different concentrations was 32-90%, whereas that of sophorolactone was 5-92% (Table 2). When the effect of rhamnolipid and sophorolactone on mature biofilm was examined, it was observed that concentrations above the maximum concentration of rhamnolipid and sophorolactone were generally required for the eradication of microorganisms in biofilm (Table 3). Regarding the minimum biofilm eradication concentration (MBEC) results, rhamnolipid was effective against MSSA CI and *A. baumannii* ATCC 19606 at a concentration of 166.66 mg/ml, while sophorolactone was effective against *S. aureus* ATCC 29213, MSSA CI, *S. aureus* ATCC 43300, MRSA CI and *A. baumannii* CI at a concentration of 33.13 mg/ml. In this respect, sophorolactone was shown to be more effective (Table 3). Overall, the results show that rhamnolipid and sophorolactone have a strong effect on the inhibition of biofilm growth, whereas very high concentrations are required for the destruction of microorganisms in mature biofilms.

Studies on rhamnolipids and sohorolipids have shown that rhamnolipids and sohorolipids alone inhibit biofilm growth and in combination may increase the ability of some substances to inhibit biofilm growth [7, 11, 24, 26-28, 32-38]. It has also been reported that especially soforolipids have high potential as antimicrobial and antibiofilm agents [26].

Rhamnolipids and sophorolipids have been used in studies on biofilm development and the destruction of microorganisms in mature biofilms with the bacteria used in our study. For instance rhamnolipid derived from *P. aeruginosa* UKMP14T showed antibiofilm activity against *A. baumannii* at a concentration of 1000 µg/ml [7]. In a study investigating the effectiveness of biosurfactants in combination with caprylic acid, sophorolipid (1%) inhibited the surface adhesion and biofilm formation of *P. aeruginosa* PAO1, *E. coli* NCTC 10418 [24]. Moreover rhamnolipid isolated from *P. aeruginosa* MA01 inhibited the biofilm growth of *S. aureus* ATCC 6538 at a concentration of 60mg/ml and decreased the activity of some biofilm formation and quorum-sensing genes [27]. Sophorolipid was also shown to have inhibitory effect on *S. aureus* biofilm formation at concentrations of 50-200 µg/ml [28].

In some studies, the effectiveness of sophorolipid mixtures on biofilm was investigated. It was found that the mixture of different sophorolipids had no effect on the growth of *P. aeruginosa* PAO1, this mixture had a destructive effect on biofilm and reduced the number of microorganisms in the biofilm [36]. Some researchers have examined the effectiveness of biosurfactant mixtures on biofilms formed by two bacteria together. For example, the combination of sophorolipid (0.01%) and rhamnolipid (0.04%) on biofilm formed by *P. aeruginosa* ATCC 15442 and *S. aureus* ATCC 9144 caused the death of microorganisms in the biofilm. In addition, researchers have reported that Gram positive bacteria were more sensitive to sophorolipids than Gram negative bacteria [37]. According to the results of the existing studies, antibiofilm activity has been observed against *A. baumannii*, *P. aeruginosa*, *E. coli*, and *S. aureus* strains, which were similar to the results of our study. Considering the concentrations at which the inhibitory effect on biofilm was obtained, a system based on MIC was used in our study to determine the efficacy on biofilm. It is seen that the effective doses obtained in our study are concentrations above the doses in other studies [7, 24, 27, 28, 36, 37]. We believe that this may be due to differences in the biosurfactants or microorganisms used. There are few studies involving *E. faecalis* ATCC 29212 and *E. faecalis* clinical isolates. On this topic, rhamnolipid extracted from *L. plantarum* has been reported to reduce the ability of the endodontic infection isolate *E. faecalis* to adhere to surfaces [30]. In another study with this microorganism, it was reported that sophorolipid showed

synergistic activity in terms of antimicrobial and antibiofilm activity against *E. faecalis* ATCC 29212 and *P. aeruginosa* PAO1 when used in combination with kanamycin and cefotexime [38]. In our study, it was found that rhamnolipid showed a high biofilm inhibition rate of 71-90% in the MIC-1/8 MIC concentration range for these microorganisms, and sophorolactone showed a high biofilm inhibition rate of 48-92% in the MIC-1/2 MIC concentration range (Table 2).

4. CONCLUSION

Rhamnolipid and sophorolactone, which we used as biosurfactants in our study, have had antimicrobial effects on standard and clinical isolates of *S. aureus*, *E. faecalis*, *A. baumannii*, and *P. aeruginosa*, which are important human pathogens. They also greatly inhibited the biofilm development ability of these pathogens. Although the data obtained in our study are consistent with the literature, the antimicrobial and antibiofilm activities revealed in our study were generally obtained at higher concentrations compared to other studies. As seen in our study, biosurfactants have a significant potential in terms of antimicrobial and antibiofilm activities.

5. MATERIALS AND METHODS

5.1. Microorganisms

In our study; *S. aureus* ATCC 29213, *S. aureus* ATCC 43300, *E. faecalis* ATCC 29212, *A. baumannii* ATCC 19606, *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, *P. aeruginosa* PAO1 standard bacteria and methicillin resistant *S. aureus* (MRSA), methicillin susceptible *S. aureus* (MSSA), *E. faecalis*, *A. baumannii*, *E. coli* and *P. aeruginosa* clinical isolates (Indicated by CI: Clinical Isolate) were used. Clinical isolates were isolated from different clinical samples and identified by MALDI-TOF MS device (Biomerieux). Microorganisms were grown by passaging on tryptic soy agar medium and incubated at 37 °C for 18-24 hours and stored at -20 °C in tryptic soy broth medium containing 20% glycerol. Clinical microorganisms were obtained from Haydarpaşa Numune Training and Research Hospital Microbiology Laboratory with the decision of Marmara University, Institute of Health Sciences Ethics Committee dated 18.02.2019 and numbered 51.

5.2. Biosurfactants

Rhamnolipid (AGAE R90) and sophorolactone (Cayman 14718) were purchased commercially and used after being dissolved in ethyl alcohol. Soforolactone used in our study is 1',4"-Sophorolactone 6',6"-diacetate and is a standard compound used for sophorolipids.

5.3. Determination of antimicrobial activity

5.3.1. Agar well diffusion method

Agar well diffusion method was used to determine antimicrobial activity. Microorganisms were grown by passaging on tryptic soy agar medium and incubated at 37 °C for 24 hours. Microorganism suspensions were prepared from colonies on solid media in 0.85% physiological saline solution (PSS) and adjusted to Mc Farland 0.5 standard turbidity with a concentration of 10^8 CFU/ml. These suspensions were spread on the surface of the Mueller Hinton Agar (MHA) medium by using a sterile swab. Wells with a diameter of 5 mm were made on the medium using a sterile punch at certain intervals and 50 µl of the samples (rhamnolipid 333 mg/ml, sophorolactone 66.6 mg/ml) then these samples dissolved in ethyl alcohol were placed in the wells. In addition, meropenem (10 µg/well), ethyl alcohol and physiological saline were used as controls. The experiments were conducted with triplicate and arithmetic averages were calculated [39, 40].

5.3.2. Detection of minimum inhibitory and minimum bactericidal concentration

Minimum inhibitory concentration (MIC) was determined by broth microdilution method according to European Committee of Antmicrobial Susceptibility Testing (EUCAST) standards. Bacterial suspensions were taken from 24 hours bacterial culture colonies, prepared according to Mc Farland 0.5 standard turbidity and diluted to 5 x 10^5 CFU/ml. Serial dilutions (Concentration range 166,5-0,16 mg/ml for rhamnolipid, 33,33-0,03 mg/ml for sophorolactone) of the samples were made in sterile U-bottom microdilution plates by using Mueller Hinton broth (MHB) medium. Then, 5µl of bacterial suspension was added to the wells containing the biosurfactants and incubated at 37 °C for 24 hours and the lowest sample concentrations without growth at the end of incubation were determined as MIC [41]. MHB, ethyl alcohol, and meropenem as control. In the determination of minimum bactericidal concentration (MBC), 10 µl was taken from the

wells where no growth was observed and inoculation to tryptic soy agar medium and incubated at 37 °C for 24 hours. After incubation, the lowest concentration at which no growth was observed was determined as the MBC. The experiments were conducted in triplicate.

5.4. Detection of antibiofilm activity

In order to determine the antibiofilm activity two separate studies were conducted. The effectiveness of the samples on biofilm development and minimum biofilm eradication concentration were investigated.

5.4.1. Investigation of the effectiveness of the biosurfactants on biofilm development

Microorganisms were inoculated in 5 ml tryptic soy broth with 1% glucose (TSB-G) and was incubated at 37 °C for 24 hours. Bacterial suspensions equivalent to McFarland 0.5 standard in TSB-G were prepared from the cultures. The prepared suspensions were diluted with 1% TSB-G to a final concentration of 5×10^5 CFU/ml. 180 µl of the prepared bacterial suspensions of were dispensed into the wells of the flat bottom microplate. According to the MIC values of the samples against each strain tested, the final concentrations of the samples in the wells were prepared in TSB-G as 1, 1/2, 1/4 and 1/8 times of the MIC value. In addition, the biosurfactant concentration was used as 10 mg/ml for bacteria which showed no antimicrobial activity in our study. 20 µl biosurfactant sample was added as to the respective wells containing bacteria in the plate. The plates were incubated at 37 °C for 24 h. After incubation, the wells were carefully emptied with an automatic micropipette and washed twice with 250 µl phosphate buffered saline (PBS). After washing, 200 µl of 99% methanol was added to the wells and left for 15 minutes and the wells were emptied and the microplate was left to dry. After the microplate was dried, 1% crystal violet (200 µl) was added to the wells and kept for 5 minutes and the excess dye was removed by washing with water and the microplate was left to dry. Then 200 µl of 95% ethanol was added to the wells and kept for 30 minutes and optical density values were measured at 595 nm in a microplate reader. The experiments were carried out in 3 repeats and the arithmetic averages of the optical density (OD) values of each strain incubated with samples of different concentrations were taken. The OD values of the microorganism wells containing only TSB-G and biosurfactant-free used as control were measured according to the applied method and the mean values were calculated. The OD value of the biosurfactant-free control wells of each strain was taken as 100%. According to this value, the percentage values corresponding to the OD values of the wells containing samples at different concentrations were determined. The absorbance values of the wells containing the sample were subtracted from the absorbance value of the negative control. The change in biofilm formation rates of strains in the presence of different concentrations of sample was calculated by the formula below. The experiments were performed as tripilicate and the changes in biofilm formation rates were given as percentages rounded to the nearest whole number [42-44].

Biofilm change rate = [OD]D/[OD]C × 100
[OD]D= OD value of wells containing different concentrations of samples
[OD]C = OD value of the positive control well

5.4.2. Investigation of the minimum biofilm eradication concentration

Microorganisms were inoculated in 5 ml of sterile TSB-G and incubated at 37 °C for 24 hours. After incubation, the cultures were diluted with sterile TSB-G to a final concentration of 5×10^7 cfu/mL in sterile TSB-G and 200 µL was distributed to each well of 96-well flat bottom plates. The plates were incubated at 37 °C for 24 hours. After incubation, the wells were carefully drained with an automatic pipette and washed twice with 250 µl PBS. The aliquots of the samples were prepared (Concentration range 166,5-20,81 mg/ml for rhamnolipid, 33,33-4,17 mg/ml for sophorolactone) with TSB-G in tubes and 200 µl was added to the wells and incubated at 37 °C for 24 hours. After incubation, the wells were washed twice with 250 µl PBS. Then washing, 250 µl PBS was added to the wells and the biofilm at the bottom of the wells was scraped with a micropipette tip. Then 5 µl of each well was added to tryptic soy agar and incubated at 37°C for 24 hours. After incubation, the lowest concentration without growth was considered as the minimum biofilm eradication concentration (MBEC). The experiments were done in triplicate [43-45].

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