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Screening the Antimicrobial Activity of Biosurfactants Produced by Microorganisms Isolated from Refinery Wastewaters

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ABSTRACT

In this study, isolation and identification of microorganisms from petroleum refinery wastewater with biosurfactant producing ability and characterization of biosurfactants were investigated. Biosurfactant production ability of microorganisms isolated from wastewater was determined by drop collapse method. The initial characterization of biosurfactants was investigated by biochemical tests and FTIR analysis. Biosurfactants were investigated for potential antimicrobial activity as inhibition zone, minimum inhibitory concentration, and minimum bactericidal concentration against medically important bacterial strains. We found that the biosurfactants showed profoundly distinct antimicrobial activity toward test organisms. Maximum antibacterial activity was shown by BS-III against *K. pneomoniae* followed by BS-IV against *E.coli*. Also biosurfactants displayed a antifungal activity against tested yeasts with the diameters of zone inhibition ranging between 12 and 17 mm. The maximum antifungal activity was obtained with BS-III against *C. krusei*. The MIC values obtained in this study were lower than the MBC values for all tested biosurfactants. Maximum MBC values were recorded with *E. coli* and were 64mg/ml for BS-I. The main commercial use of biosurfactants is oil industry, foods and environmental technology because of their ability to stabilize emulsions. The antimicrobial activity of biosurfactants has not been reviewed extensively. From preliminary characterization results it can be said that biosurfactants are a suitable alternative in potential applications of medical fields.

Key Words: Biosurfactant, antimicrobial activity, FTIR spectrum, refinery wastewater

INTRODUCTION

The microbial surfactants called as biosurfactants are microbial compounds with a distinct surface activity that exhibit a broad diversity of chemical structures such as glycolipids, lipopeptides and lipoproteins, lipopolysaccharides, phospholipids, fatty acids and polimeric lipids [1,2]. Therefore, it is reasonable to expect diverse properties and physiological functions of biosurfactants such as increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, heavy metal binding, bacterial pathogenesis, quorum sensing and biofilm formation [3]. A host of interesting features of biosurfactants have led to a wide range of potential applications in the medical field. They are useful as antibacterial, antifungal and antiviral agents, and they also have the potential for use as major immunomodulatory molecules and adhesive agents (4).

The antimicrobial activity of several biosurfactants has been reported in the literature for many different applications. Biosurfactants appears to be great potential and suitable alternative to synthetic medicines and antimicrobial agents and may be used as safe and effective therapeutic agents. There has been increasing interest in the apllications of biosurfactants on human and animal cells and cell lines [3,5]. The aim of this study was to isolate and identify the biosurfactants produced by different microorganisms isolated from petroleum refinery wastewaters. Moreover, the partial functional characterization was established by the determination of the antimicrobial and anti-fungal activity, biochemical analysis and Fourier transform infrared spectroscopy

MATERIAL AND METHODS

Isolation, selection and identification of the microorganism

The microorganisms were isolated from water samples collected from coasts of Kızılırmak contaminated with Kırıkkale Petroleum-Rafinery wastes. The method of serial dilutions of the sample (0.1 ml) was inoculated on nutrient agar plates and incubated at 37 ^{o}C for 24 h. After this period the selected colonies with different properties were purified by repeated inoculation on Mac. Conkey Agar (MCA) at 30^{o}C for 48 h. Some biochemical tests such as Gram staining, oxidase activity, indol, lactose and growth at 42^{o}C tests were applied to bacterial strains obtained from different colonies.

To determine the biosurfactant production ability, bacterial strains obtained from different colonies were inoculated with mineral salt medium (MSM) proposed by Zhang and Miller (6). Biosurfactant production ability was determined by drop collapse method of Jain et al. [7]. For the drop collapse method, 2.0 µl of mineral oil was added to each well of a 96-well microtiter plate lid. The lid was equilibrated for 1 h at room temperature. MSM culture of bacterial isolates was santrifuged and then 5 µl of the supernatant was added to the surface of oil. The shape of the drop on the surface of oil was evaluated after 1 min. After determination of biosurfactant producing-isolates identification of microorganisms were performed using VITEK 2 analyzer (Biomérieux SA, France).

Biosurfactant production and isolation

Isolates were grown in 500 ml Erlenmeyer flasks, each containing 100 ml medium of wastewater (5% ,v/v) and MSM. The flasks were incubated at 37^{o}C on a shaker incubator for 96 h. To isolate the biosurfactant, the bacteria were removed by centrifugation and the remaining supernatant liquid was filtered through a 0.22µm pore-size filter (Millipore). Biosurfactant was obtained by adjusting the supernatant pH to 2.0 using 6 N HCl and keeping it at 4^{o}C overnight. The precipitate thus obtained was pelleted at 8000g for 20min, dried and weighted. For further purification, the crude surfactant was dissolved in distilled water at pH 7.0 and dried at 60 ^{o}C. The dry product was extracted with chloroform:methanol (65:15), filtered and the solvent evaporated.

Biochemical analysis and Fourier transform infrared spectroscopy (FTIR)

The protein content of surfactant was estimated using the Biuret and ninhydrin method [8] and the lipid content estimated by the method of Folch et al. [9]. Total carbohydrates were estimated using the molish test [10]. Fourier transform infrared spectra of the biosurfactant samples was obtained by using a FTIR spectrophotometer (Perkin Elmer Paragon 1000).

Antimicrobial activity

The microbial strains are identified strains and were obtained from Kırıkkale Medicine Faculty, Microbiology Laboratory, Turkey. The studied bacterial strains were *Escherichia col i* DM, *Pseudomonas aeruginosa* DSM 50071, *Staphylococcus aureus* COWAN 1, *Klebsiella pneumonia* FMC 5 and *Bacillus cer eus* ATCC 7064. Antifungal activity of biosurfactants were determine with *Candidia albica ns* FMC 17 and *Candida krusei* ATCC 6258. The antibacterial activity of biosurfactants was evaluated by agar disc diffusion method (11). A 0.2 ml of inoculum (inoculum size was 10^{7}-10^{8} ml as per McFarland standard) was inoculated into the Muller Hinton agar media. Sterile discs (0.6 cm) were introduced to medium and 20 µl of each biosurfactant at a concentration of 20 mg/mL was loaded on the disc. After incubation period at 37±0.1 °C for 18-24±2 h microbial growth was determined by measuring the diameter of zone of inhibition. For antifungal activity investigations, yeasts (0.5-2.5x10^{6}/ml) were cultivated on Sabouraud 2%-dextrose agar. Biosurfactant introduction on disks were apllied same as mentioned above. After cultivation for 24-37 h at 25 ± 2 °C (12) the growth was determined by measuring the diameter of zone of inhibition. Controls were maintained in which pure solvents were used instead of the test substances. The experiment was done three times and the mean values are presented.

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

MIC determinations were performed in Tryptic Soy Broth and the strains were cultured overnight at 37°C. Bacterial suspensions were diluted to match the 0.5 Mac Farland standards (approximately 1.5×10^{8} CFU/ml). Dilutions of the biosurfactant solutions were prepared with a range of 64-0.5 mg/ml, then added to tryptic soy broth plates. The plates were incubated under normal atmospheric conditions for 24 h at 37°C. The MIC value was defined as the lowest concentration of biosurfactants at which no visible growth could be observed.

MBC was determined by subculturing the test dilution on to a fresh drug-free solid medium and incubating further for 18-24 h. The highest dilution that yielded no single bacterial colony on a solid medium was taken as MBC.

RESULTS

Microbial Isolates

Twelve morphologically distinct microbial colonies were isolated, including 8 bacteria, 3 filamentous fungi and 1 yeast. Seventy-eight percent of the bacterial isolates were Gram-negative.

Four isolates were identified as biosurfactant producing bacteria using the drop-collapse method (Table 1). If the culture broth contained biosurfactant, the droplets of the broth in the oil-coated wells collapsed. If not, there was no change in the shape of the droplets. Water and cell free culture broth samples remained beaded, meaning they did not contain any type of microbial surfactant. However, culture supernatants of isolate 4, 7, 9 and 10 showed spreading movement, meaning that they produced biosurfactants (Figure 1). After colonial, biochemical examinations and identification process, it has been observed that four of the biosurfactant-producing microorganisms are of the *Pseudomonas* genus. Isolate 4, 7, 9 and 10 strains were identified as *Pseudomonas aeruginosa* RWI, *Pseudomonas putida* RWII, *Pseudomonas fluorescens* RWIII and *Burkholderia cepacia* RWIV.

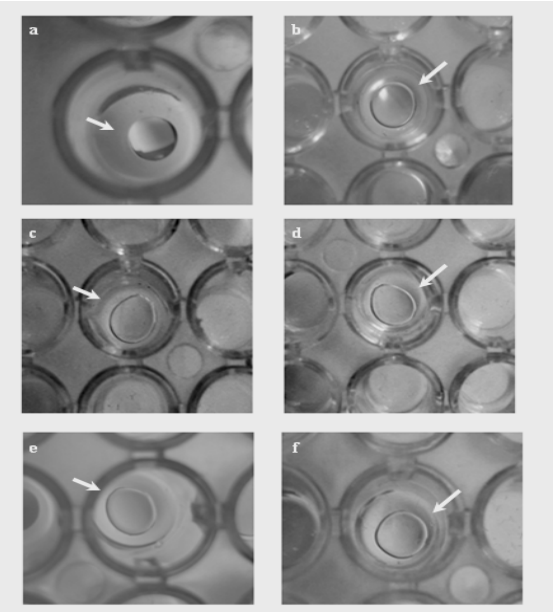

Figure 1. Drop collapse test of samples; a. water, b. Cell free culture broth, c. culture broth of isolate 4, d. culture broth of isolate 7, e. culture broth of isolate 9, f. culture broth of isolate 10

Biosurfactant characterization

Biosurfactants produced by *Pseudomonas aeruginosa* RWI, *Pseudomonas putida* RWII, *Pseudomonas fluorescens* RWIII and *Burkholderia cepacia* RWIV strains were coded as BS-I, BS-II, BS-III and BS-IV, respectively. The structural analysis of this biosurfactants were performed by lipid, protein and carbohydrate contents and also by FTIR analysis. Ninhydrin and Biuret tests were used to detect the presence of amino acids and protein contents of biosurfactants, respectively. BS-I and BS-II gave the negative reaction with biüret and ninhydrin test however BS-IV showed positive reaction. BS-III gave a negative result with biüret but positive result in ninhydrin test. Molisch's test is a sensitive chemical test for the presence of carbohydrates, based on the dehydration of the carbohydrate by sulfuric acid to produce an aldehyde, which condenses with two molecules of phenol resulting in a red- or purple-colored compound. From the results of Molisch's test, it was shown that all biosurfactants except BS-IV, contains carbohyrate residues. The sulfo-phospho-vanillin reaction tested for lipid content of biosurfactants has significant advantages over traditional methods. All biosurfactants gave the positive results with sulfo-phospho-vanillin reaction. This means all biosurfactants obtained in this study have a lipid content.

The functional groups of biosurfactants were confirmed by FT-IR spectra (Figure 2). FT-IR-spectra of BS-I, BS-II, BS-III and BS-IV revealed that a heterogeneity evidenced by different characteristic peaks, was in agreement with the possible presence of amino, carboxylic, hydroxyl and carbonyl groups. The FTIR spectra of BS-I have the characteristic stretching vibration band of –OH, around 3400 cm^{-1}. The strong peaks at 3000 cm^{-1} and 1650 cm cm^{-1} were caused by the bending and stretching of –CH and –C=O groups, respectively. Similiar, the FT-IR spectra of the BS-II had an intense peak at a frequency level of 3200-3500 cm^{-1} representing –CH and –OH groups. The strong peaks at 2500-2900 cm^{-1} and 1650 cm^{-1} were caused by the bending and stretching of –CH3 and –C=O groups, respectively. In BS-III spectra the peaks at around 3400, 2960 and 1650 cm^{-1} representing –OH, –CH3 and –C=O stretching vibrations, respectively. The FTIR spectra of BS-IV have absorption band different from BS-I, BS-II and BS-III at 1537 cm^{-1} which is the characteristic of -CN stretching vibration band arising from protein residues. And also BS-IV have the characteristic stretching vibration band of -NH, around 3320 cm^{-1} which confirmed the protein residues in the structure of BS-IV. When a preliminary characterization of the biosurfactants was performed for BS-I, BS-II, BS-III and BS-IV, it was found that these substances contain high amounts of proteins, carbohydrate and lipids. The results obtained from biochemical analysis were confirmed by FTIR analysis.

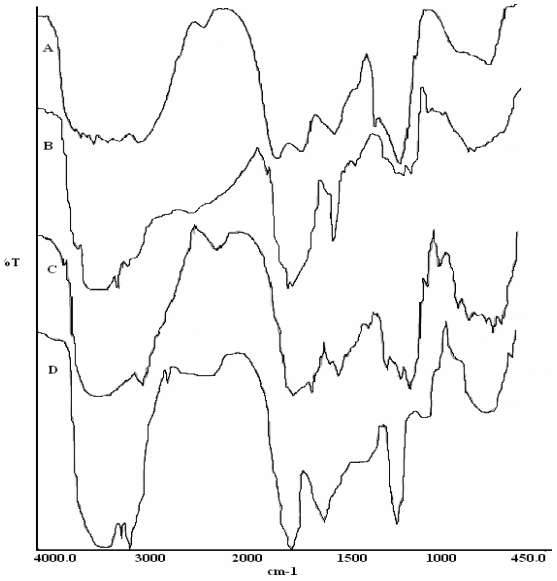

Figure 2. FTIR spectra of A. BS-I, B. BS-II, C. BS-III and D. BS-IV

Antimicrobial activity

The antimicrobial activity of the biosurfactants and their potency were quantitatively assessed by determining the MIC and MBC as given in Table 2 and 3. In our earlier studies we found that the biosurfactants showed profoundly distinct antibacterial activity toward test organisms. BS-I showed low activity against *S. aureus,* high activity against *E.coli.* Similiar, minimum antibacterial activity was shown by BS-II against *S. aureus* with a inhibition zone of 9 mm. Maximum antibacterial activity against *K. pneomoniae* was shown by BS-I followed by BS-IV, while minimum activity was shown by BS-II and BS-III. The biosurfactants displayed antifungal activity against tested yeasts with the diameters of zone inhibition ranging between 12 and 17 mm. The MIC values obtained in this study were lower than the MBC values for all tested biosurfactants. Maximum MBC values were recorded with *E. coli* and were 64mg/ml for BS-I.

DISCUSSION

It is well known that microorganisms growing on hydrocarbons frequently produce biosurfactants with emulsifying activity. This property is considered as a biological strategy to facilitate the availability of hydrophobic substrates (13,14). In this study, a higher percent of the bacterial strains isolated from petroleum-refinery wastewaters were Gram-negative. It has previously been reported that most bacteria isolated from sites with a history of contamination by hydrocarbon and derivatives are Gram-negative, and this may be a characteristic that contributes to survival of these populations in such harsh environments (15). Four isolates were identified as biosurfactant producing bacteria using the drop-collapse method (Table 1). Jain et al. (7) suggested the use of drop collapse method as a sensitive and easy method to test for biosurfactant production. After colonial, biochemical examinations and identification process, biosurfactant-producing microorganisms are identified as *Pseudomonas aeruginosa* RWI, *Pseudomonas putida* RWII, *Pseudomonas fluorescens* RWIII and *Burkholderia cepacia* RWIV. Growth of the mentioned strains in refinery wastewater riched medium showed that these strains can tolerate and degrade different polycyclic aromatic hydrocarbons.

From the biochemical and FTIR analysis, it was assumed that BS-I, BS-II and BS-III have a glycolipid structure, however BS-IV have a lipopeptide structure. Owing to the structure of biosurfactants, a range of interactions are involved between charged biosurfactants and cell surfaces. Most natural cell surfaces have an overall negative charge (16). From this phenomenon it was concluded that positively charged groups in biosurfactants exert an adhering effect on cell surfaces of microorganisms [17]. When a preliminary characterization of the biosurfactants was performed for BS-I, BS-II, BS-III and BS-IV by FTIR analysis, it was found that these substances contain high level of charged groups such as hydroxyl, carboxyl and amine groups. We assume that the amine groups are responsible to adhere negative charged surfaces.

From results, it was shown that BS-IV showed high antibacterial activity against Gram-negative strains compared to Gram-positive. The FT-IR spectra of BS-IV have absorption band different from other biosurfactants at around 3320 cm^{-1} which confirmed the amine bonds. The positively charge amine bonds can be explained the higher antibacterial activity of BS-IV against Gram-negative strains. Also the MIC and MBC values support the findings of the diffusion method. The area of concern is that MIC values of the biosurfactants obtained in this study were lower than the MBC values, suggesting that the biosurfactants were bacteriostatic at lower concentration and bactericidal at higher concentration. The antimicrobial activity of several biosurfactants has been reported in the literature for many different activities. Nielsen et al. [18] reported a cyclic biosurfactant, to be a new antifungal surface-active agent produced by *Pseudomonas fluorescens*. Gerard et al. [19] isolated a biosurfactant from the *Pseudomonas* species and found to exhibit in vitro antimicrobial activity against *Mycobacterium tuberculosis* and *Mycobacterium avium* . Kitamoto et al. [20] showed that a glycolipid biosurfactant produced by *Candida* strains exhibits antimicrobial activity, particularly against Gram-positive bacteria. Gottenbos et al. [21] demonstrated that positively charged biosurfactant surfaces exert an antimicrobial effect on adhering Gram-negative bacteria, but not on Gram-positive bacteria. Also the biosurfactants showed a antifungal activity against *Candida albicans* and *Candida krusei*. Similiarly, Thimon et al. [22] reported the biosurfactant produced by *B. subtilis* have a antifungal effect on yeast cells.

The antimicrobial effects of biosurfactants can be expalined by the structures of biosurfactants resembled to cell membrane. Biosurfactants are amphipathic molecules with hydrophilic moiety consisting of amino acids or peptides anions or cations; mono-, di-, or polysaccharides; and a hydrophobic moiety consisting of unsaturated, saturated, or fatty acids. Insertion of fatty acids components of biosurfactants into a cell membrane caused significant ultrastructural changes in the cells such as ability of the cell to interiorize plasma membrane. Also, antimicrobial effects of biosurfactants could be that the bleb formation represented an increase in the size of the membrane due to insertion of lipid material. However, this would imply that the biosurfactant is undergoing a phospholipid bilayer flip-flop, which is doubtful because of its hydrophilic sugar head. Alternatively, it is possible that insertion of the shorter acyl tails into the cell membrane causes a disruption between cytoskeletal elements and the plasma membrane, allowing the membrane to lift away from the cytoplasmic contents [23]. Hingley et al. [24] reported the rhamnolipids were removed the dynein arms from the microtubules caused a direct breakdown of interactions between microtubules and granules. One explanation of the antimicrobial effect of biosurfactants is the adhering property of biosurfactants to cell surfaces caused a deterioration in the integrity of cell membrane and also breakdown in the nutrition cyle. All these cumulative effects can be explained the antimicrobial effects of biosurfactants.

Table 1. Biochemical characteristics of isolates

Isolates	Gram Stain	Oxidase	Indol	Lactose	Growth at 42°C	Biosurfactant production
1	-	-	-	+	+/-	-
2	+/-	+	+	-	-	-
3	+	-	-	-	-	-
4	-	+	-	-	+	+
5	+/-	+	+	+	-	-
6	+/-	+	+	+	-	-
7	-	+	-	-	-	+
8	+	-	-	+	-	-
9	-	+	-	-	-	+
10	-	+	-	+/-	+/-	+
11	+/-	-	+	-	-	-
12	-	-	-	+/-	-	-

Table 2. Antimicrobial activities of biosurfactants against several bacterial and yeast strains

	BS-I	BS-II	BS-III	BS-IV
Microorganisms	Inhibition zone (mm)			
E. coli	14	15	15	19
P. aeruginosa	12	13	15	14
S. aureus	10	9	15	12
B. cereus	11	8	9	10
K. pneomoniae	13	16	20	19
C. albicans	13	14	12	13
C. krusei	15	14	17	14

Table 3. MIC and MBC values of biosurfactants

Microorganisms	BS-I		BS-II		BS-III		BS-IV	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
E. coli	12	64	12	24	16	48	24	48
P. aeruginosa	8	32	8	12	12	24	16	48
S. aureus	16	32	24	32	12	32	8	24
B. cereus	12	16	16	48	16	32	12	32
K. pneomoniae	16	48	12	32	24	48	16	28
C. albicans	8	16	8	12	12	32	16	24
C. krusei	12	32	16	48	16	32	8	24

*MIC and MBC values given as mg/ml

CONCLUSIONS

Biosurfactants are surfactants that are produced extracellulary or as part of the cell membrane by bacteria, yeast and fungi. The main commercial use of biosurfactants is oil industry, foods, cosmetics, pharmacology and environmental technology because of their ability to stabilize emulsions. The antimicrobial activity of biosurfactants has not been reviewed extensively. Many studies have shown that many biosurfactants possess antimicrobial activity, but this is the first report of antimicrobial activity of the biosurfactants produced by microorganisms isolated from refinery wastewaters. The initial chemical characterization of the obtained biosurfactans are also presented with biochemical and FTIR analysis. Finally, biosurfactants are a suitable alternative to synthetic medicines and antimicrobial agents and may be used as safe and effective therapeutic agents.

REFERENCES

[1] Ahimou F, Jacques P, Deleu M. 2000. Surfactin and iturin A effects on Bacillus subtilis surface hydrophobicity. Enzyme Microbiol Techno, 27:749-754.

[2] Maier R, Soberon-Chavez G. 2000. Pseudomonas aeruginosa rhamnolipids: biosynthesis and potential applications. Appl Microbiol Biotechnol, 54:625-633.

[3] Singh P, Cameotra S. 2004. Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol, 22:142–146.

[4] Rodrigues L, Ibrahim M, Banat J. 2006. Biosurfactants: potential applications in medicine. Journal of Antimicrobial Chemotherapy, 57:609–618.

[5] Banat IM, Makkar R, Cameotra S. 2000. Potential commercial applications of microbial surfactants. Appl Microbiol Biotechnol, 53:495–508.

[6] Zhang Y, Miller RM. 1992. Enhanced octadecane dispersion and biodegradation by a Pseudomonas rhamnolipid surfactant (biosurfactant). Appl Environ Microbiol, 58: 3276-1279.

[7] Jain DK, Collins-Thompson DL, Lee H. 1991. A drop-collapsing test for screening biosurfactant-producing microorganisms. J Microbiol Methods, 13:271– 279.

[8] Gornall AG, Bardawill CS, David MM. 1949. Determination of serum protein by means of the biuret reaction. J Biol Chem, 177:751-756.

[9] Folch J, Lees M, Sloane-Stanley GH. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J Biol Chem, 226:497-509.

[10] Spiro RG 1996. Analysis of sugars found in glycoproteins. Methods in Enzymology, 8:3-26.

[11] Bauer AW, Kirby WMM, Sheriss JC. 1966. Antibiotic susceptibility testing by a standardized single disk method. Ame J Clin Pathol, 45:493-496.

[12] Bradshaw LJ 1992. Laboratory Microbiology. Fourth Edition. U.S.A. 13-55.

[13] Desai AJ, Patel KM, Desai JD. 1988. Emulsifier production by Pseudomonas fluorescens during the growth on hydrocarbons. Curr Sci, 57:500–501.

[14] Tonkova E, Galabova D, Stoimenova E. 2008. Characterization of bacterial isolates from industrial wastewater according to probable modes of hexadecane uptake. Microbiological Research, 4:481-486.

[15] Nitschke M, Pastore G. 2003. Cassava flour wastewater as a substrate for biosurfactant production. Appl Biochem Biotechnol, 108:295-301.

[16] Bicca CF, Fleck LC, Ayub MAZ. 1999. Production of biosurfactant by hydrocarbon degrading Rhodococcus rubber and Rhodococcus erythropolis. Braz J Microbiol, 30: 231–236.

[17] Craig VSJ, Ninham BW, Pashley RM. 1993. Effect of electrolytes on bubble coalescence. Nature, 364:317–319.

[18] Nielsen T, Christophersen C, Anthoni U. 1999. Viscosinamide, a new cyclic depsipeptide with surfactant and antifungal properties produced by Pseudomonas fluorescens DR54. J Appl Microbiol, 86:80–90.

[19] Gerard J, Lloyd R, Barsby T. 1997. Antimycobacterial cyclic depsipeptides produced by two Pseudomonas isolated from marine habitats. J Nat Prod, 60:223–229.

[20] Kitamoto D, Yanagishita H, Shinbo T. 1993. Surface active properties and antimicrobial activities of mannosylerythritol lipids as biosurfactants produced by Candida antarctica. J Biotechnol, 29:91–96.

[21] Gottenbos B, Grijpma D, Vander M. 2001. Antimicrobial effects of positively charged surfaces on adhering Gram-positive and Gram-negative bacteria. J Antimicrob Chemother, 48:7–13.

[22] Thimon L, Peypoux F, Wallach J. 1995. Effect of lipopeptide antibiotic, iturin A, on morphology and membrane ultrastructure of yeast cells. FEMS Microbiol Lett, 128:101–106.

[23] Desai JD, Banat IM. 1997. Microbial production of surfactants and their commercial potential. Microbiol Mol Biol Rev, 61:47-56.

[24] Hingley ST, Hastie AT, Kueppers F. 1986. Effect ofciliostatic factors from Pseudomonas aeruginosa on rabbit respiratory cilia. Infect Immun, 51:254-258.