Antibacterial Activity of the Lipopeptide Biosurfactant Produced by *Bacillus mojavensis* PTCC 1696

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ABSTRACT: *Bacillus mojavensis PTCC 1696 (a member of Bacillus subtilis group) has been isolated form Iranian oil field (Masjed-I Soleyman), in order to examine its ability to produce biosurfactant (lipopeptide type) [28]. The present study was designed to characterize the antibacterial activity of the isolated biosurfactant. The antibacterial activity towards several bacteria including clinical isolates and type strains species was examined. For detecting the extent of antibacterial activity, the agar disc diffusion method was used, where the measured diameter of the zone of inhibition was used as an index for determining the antibacterial activity. Among the test microorganisms, the antibacterial activity was highest in Pseudomonas aeruginosa ATCC 27853. At concentration as low as 16 µg/ml, the inhibitory effect of the lipopeptide biosurfactant was detectable. The stability of the test biosurfactant also was examined over a wide range of temperatures (40–100 ℃) and pH values (2-11). The stability was further tested using protease and lipase, where the substance showed clear sensitivity towards lipase. The potentiality of this antibacterial agent in clinical applications is of interest and needs to be further recognized.*

KEYWORDS: *Antibacterial activity; Lipopeptide, biosurfactant; Surface activity; Bacillus mojavensis PTCC 1696.*

INTRODUCTION

Biosurfactants are amphiphilic compounds that tend to partition preferentially at the interfaces of fluid/fluid or solid/fluid based on the electrical charge or polarity of head of their molecules. These molecules have surprising properties because of their structural characteristics and their ability to reduce surface and interfacial tensions to very small values [1]. These are attractive surface active agents for potential use in a variety of applications including environmental [2-4], industrial [5, 6], biological [7, 8] and biomedical [9-11] fields. In recent years, many researchers have focused on the use of these compounds as antimicrobial substances [9, 12-17]. The tendency to this area is on the increase due to the emergence of the new infectious diseases and their resistance to current antibiotics.

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Among the biosurfactants with the various structures, lipopeptides, mainly produced by members of the *Bacillus*, can reduce surface tension of water to very a low value [18]. Despite the wide variety of lipopeptide biosurfactants [19] and their potential application as therapeutic drug compounds, only few studies have been conducted in this regard as compared with other applications. These lipopeptide compounds can act as antimicrobial, antiviral and antitumour agents [18, 19]. The antimicrobial properties of the lipopeptide biosurfactants against gram positive and gram negative bacteria have been investigated [20, 21]. Lipopeptides from *Bacillus* sp. have been shown to have anti-bacterial activity against *Listeria monocytogenes* and *Bacillus cereus* [22-24]. Daptomycin is a well known antibacterial lipopeptide which exhibits activity against Gram-positive bacteria that cause serious illness in hospital [17, 25]. In addition to therapeutic properties, these compounds have been used as biological control agents [26, 27]. The antibiotic properties of cyclic lipopeptides produced by *Pseudomonas* strains to obtain new species for biological control of agricultural and plant pests have been investigated [27]. Thus, it was shown that the *P. fluorescens* strains with the ability to produce cyclic lipopeptides can be used as biological control agents.

Therefore, the present study was conducted to demonstrate the inhibitory activity of a lipopeptide biosurfactant produced by *Bacillus mojavensis* strain PTCC 1696 against a range of bacteria. The nature of this biosurfactant has been characterized in previous study [28]. To achieve the goal of the present study, the antibacterial substance was first prepared by culture of strain PTCC 1696 during batch fermentation into a bioreactor and investigates its inhibitory activity against several clinical bacteria. Thereafter, the effects of heat, pH and enzymatic hydrolyses on antibacterial activity were evaluated.

EXPERIMENTAL SECTION

Microorganisms and culture media preparation

B. mojavensis, as a test bacterial producer of lipopeptide, was obtained through our previous study [28], deposited in the Persian Type Culture Collection, Tehran, Iran (accession number as PTCC 1696). The test microorganisms sensitive to antibacterial activity were obtained from clinical isolates: Yersinia, Acinetobacter baummanii, Enterobacter cloacae, Serratia marcescens, Stenotrophomonas maltophilia. Some other test microorganisms were type strains: Enterococcus faecalis ATCC 29212, Pseudomonas aeruginosa ATCC 27853, and Enterobacter aerogenes ATCC 13048. The test microorganisms were grown aerobically using Muller Hilton agar medium. A defined mineral salt medium (the medium E) was used for aerobic growth of the *B. mojavensis* PTCC 1696 (g/L): 1 $(NH_4)_2SO4$, 0.25 $MgSO_4$, 10 Sucrose, 50 NaCl, 13.9 K_2HPO_4, 2.7 KH_2PO_4. For completion of the medium E, it was necessary to add 10 ml of the trace salt solution (to the later solution) which contains the following items (g/L): 1 EDTA, 3 $MnSO_4.H_2O$, 0.1 $FeSO_4.7H_2O$, 0.1 $CaCl_2.2H_2O$, 0.1 $CoCl_2.6H_2O$, 0.1 $ZnSO_4.7H_2O$, 0.01 $CuSO_4.5H_2O$, 0.01 $AlK(SO_4)_2.12H_2O$, 0.01 H_3BO_3, 0.01 $Na_2MoO_4.2H_2O$. With 1M NaOH, the medium E was adjusted to pH 7.

Preparation of the lipopeptide biosurfactant

In the present study, a 5 lit laboratory fermenter (New Brunswick Scientific, Discovery série 100) with a working volume of 2.5 lit was used for biosurfactant production in a batch mode at 42 °C. Two 4-bladed Rushton turbine impellers were fixed in the fermenter. The foam formed during aeration process was controlled using a mechanical foam breaker. An adequate amount of the inoculum solution required to be added to the bioreactor was prepared through flask experiments. A loop of *B. mojavensis* PTCC 1696 from its fresh culture was transferred into the 200 ml flask containing 100 mL nutrient broth medium, and then the culture was incubated overnight using a rotary shaker incubator (250 rpm) at 42°C. The cell suspension was centrifuged (10000×g at 4°C for 10 min), followed by cell separation using a 0.5% peptone solution. The optical density of the prepared solution was adjusted to 0.5 at 600 nm. The inoculum was ready to be added to the fermenter (5% v/v). The fermentation condition used in this study included: 42°C, 1.5 vvm and 250 rpm. Upon completion of the fermentation process, the cells were separated from the broth by centrifugation (10000×g at 4°C for 10 min). Thereafter, the supernatant was acidified to pH 2.0 using 12 N HCl. The formed precipitate was allowed to stand overnight (4°C). The precipitate was centrifuged (10000×g for 15 min) and a suspension of the sediment

was prepared using small amount of distilled water. The pH of the solution was adjusted to 7.0 using 1 N NaOH. The solution was lyophilized. With the use of methanol, crude biosurfactant was extracted, followed by filtration and solvent removal with the help of a rotary evaporator (under reduced pressure). The crude biosurfactant sample was weighted and the dried sample was dissolved in distilled water and adjusted to pH 7.0.

Antibacterial activity assay

To determine the antibacterial activity of the produced biosurfactant, a disc diffusion method was used [23]. In this regard, a cell suspension from each test microorganism was prepared. The turbidity of the suspension was adjusted to an equivalent 0.5 McFarland standard solution. The prepared test cell suspension was inoculated on petri plate containing Muller Hilton agar and appropriate volume of the crude surfactant (20 µL) was aseptically applied on the paper discs (6 mm diameter) on the agar medium. Thereafter, the plates were incubated using a laboratory incubator (37°C and for 24 h). The appearance of clear zones on the agar surface during the test indicated a positive results and the diameter of this zones was measured according to the relevant procedure.

The minimum inhibitory concentration of the antibacterial substance was determined against *P. aeruginosa* ATCC 27853 as follows: The paper discs were saturated to different concentrations of the produced biosurfactant and were placed on the surface of Muller Hilton agar plates, where *P. aeruginosa* ATCC 27853 was the bacterium used for the agar inoculation. The diameter of clear zones, which appeared around each paper discs, were determined accordingly [23].

Surface tension measurements

The surface tension of the produced biosurfactant possessing antibacterial activity was measured using Digital tensiometer (model K10ST, Krüss, Germany). The measurement was carried out at room temperature and it was based on the use of De Nouy ring detachment method.

Antibacterial stability tests

The relevant tests were performed against heat, pH and enzymatic hydrolyses [29]. For thermal stability tests, the aqueous sample solution was incubated in a water bath equilibrated to a range of temperatures (40 to 100°C) for specified time between 30 to150 min. An autoclave (121°C, 4 atm for 15 min) was used for measuring the antibacterial stability for the higher temperature. The stability test in terms of antibacterial response to pH change was carried out by incubating the samples at different pHs (pH 2-11) for 30-150 min (at 25°C), followed by neutralization of the samples to pH 7.0. The effects of two hydrolytic enzymes (lipase and protease, provided by Novozyme Co.) on the stability of antibacterial substance were evaluated. The water bath was used for the enzymes tests (37°C for 60 min, followed by 3 min at 100°C) [29].

RESULTS AND DISCUSSION

Growth kinetics of B. mojavensis and production of antibacterial substance

Growth kinetics of *B. mojavensis* and lipopeptide biosurfactant (antibacterial substance) production were monitored in a stirred tank bioreactor with 2.5 lit working volumes. The biosurfactant producer has the ability to reduce the surface and interfacial tensions of the culture system by secreting surface active materials. In the present study, the extent of biosurfactant production was determined by measuring the changes of culture surface tension during the fermentation period. As shown in Fig. 1, the test bacterium entered the exponential phase of growth apparently without any delay time (no lag phase was observed), the culture surface tension changed from 62 to 55 mN/m within 3.5 h (less than 11% decrease in surface tension).

As the *B. mojavensis* bacterium reached the stationary phase at about 16 h, the surface tension of the culture decreased by 55% and the value equaled 27 mN/m (Fig.). Thus, based on the findings shown in the figure, biosurfactant can be categorized as a growth associated metabolite and the aeration appears to be essential for its growth and production by *B. mojavensis*. In previous study, surfactants produced by microbes, such as *B. subtilis*, *B. subtilis* C9, *B. licheniformis* JF-2, were characterized as the secondary metabolites and these are in agreement with the results obtained in the present study [30-33]. Moreover, the surface tension reduction properties of the biosurfactants positively affect many different processes such as enhanced oil recovery,

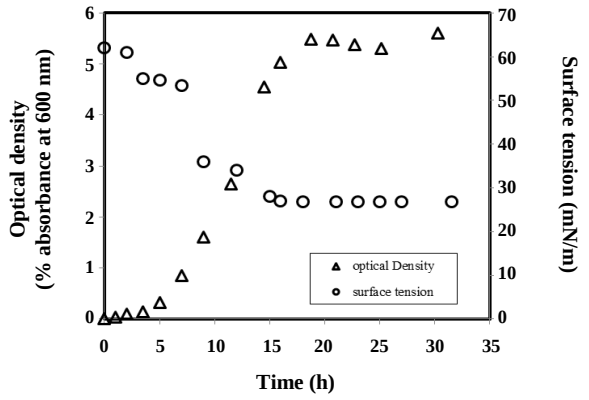


Fig. 1: Growth rate and surface tension reduction of medium during the cultivation of B. mojavensis PTCC 1696 in batch aerobically fermentation into a stirred bioreactor at 42 ℃, 300 rpm and 1.5 vvm.

progression of the two-phase reactions, emulsification, and etc. [19]. Thus, the extents of surface tension reduction in different areas differ and the microbes' responses in this category vary. For instance, the surfactin, a cyclic lipopeptide, can reduce the surface tension of aqueous media from 72 to 27.9 mN/m, while sophorolipids, as glycolipids, are capable of reducing surface tension from 72 to 30-40 mN/m [34]. In a study, the biosurfactant producing bacterium isolated from sea water in the region of Sfax, Tunisia was reported to be *B. mojavensis* AZ1 and based on characterization, its application in bioremediation processes was evaluated (that is, to enhance the solubility of hydrophobic compounds) [35]. The effectiveness of the biosurfactant in diesel solubilization under the influence of several environmental factors (such as pH, temperature and NaCl concentration) was determined and the results are in agreement with the findings of the present study. The findings mentioned here in this study show novelty in observing the antibacterial effect of the biosurfactant and this type of activity may be present in other biosurfactants.

Inhibitory spectrum of the antibacterial substance

To examine the antibacterial activity of the test biosurfactant, the response of several bacterial species (*E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 27853 and *E. aerogenes* ATCC 13048 along with some other G+ and G- as the clinical isolates including a strain *Yersinia*, *Acinetobacter baumanii*, *E. cloacae*, *S. marcescens*, *S. maltophilia*) was recorded and the results are shown in Table 1.

A major barrier in materials exchange between the cell and its internal and external environments is the cell membrane, and an established balance between hydrophilic and hydrophobic portions of this cell surface structure is necessary for the integrity of the cell membrane. The entrance of the lipid portion of the lipopeptide biosurfactant into the target cell disturbs the membrane integrity and cell functionalization. According literature, the sensivity of G+ bacteria to biosurfactants is much higher than the G- responses to these agents [14]. Cell barrier to antimicrobial agents in G- bacteria may relate to their cell wall architecture consisting of two layers with different composition and thickness. On this basis, the decreasing trend of the inhibition zone for the G- bacteria examined in the present study is as follows (Table 1): *P. aeruginosa* ATCC 27853 > *Yersinia* > *A. baumanii* > *S. maltophilia* > *E. aerogenes* ATCC 13048 > *E. cloacae* > *S. marcescens.* Therefore, the antibacterial substance may represent a relevant alternative against several important pathogenic bacteria. Table 2 shows several bacterial strains responsible for the production of lipopeptide. The biosurfactants produced by these strains could affect the growth of *B. cereus* [21, 24], *K. pneumonia* [36], *S. aureus*, *B. subtilis* [21, 36, 37], *L. monocytogenes* [22, 37], *P. aeruginosa* [36] and *E. coli* [21, 36].

The results of the present study (Table 1) showed considerable similarity in the chemical nature of the test biosurfactants. Lipopeptides with their lipidic nature interfere with the cell membrane andlack of structural integration affects the materials exchange between the cell and its environment (that is, antibacterial activity). In addition to this intrinsic property of the microbes, living cell actually uses several mechanisms to survive in the extreme environments; for instance, cell adhesion to the solid surface leads to cells accumulation and finally to the biofilm formation (that is, surface-attached microbial communities) [38]. Biosurfactant adsorption on the solid surface also changes the hydrophobicity character of the attached microbial cell. These adsorption/desorption processes thus, are under the influence of the cell structural modification [38]. In the present study, it was observed that the lipopeptide produced by the *B. nato* TK-1 possessed antiadhesive property through which

Table 1: Antibacterial activity of the biosurfactant produced by B. mojavensis PTCC 1696 against test microorganisms.

Indicator Organism	Temperature (°C)	Inhibition zone (mm)	Gram reaction
Yersinia (clinical isolate 1)	37	15.9	-
Yersinia (clinical isolate 2)	37	19.4	-
Acinetobacter baumanii (clinical isolate 1)	37	9.8	-
Acinetobacter baumanii (clinical isolate 2)	37	16.2	-
Enterobacter cloacae (clinical isolate)	37	11.3	-
Enterobacter aerogenes (clinical isolate)	37	-	-
Enterococcus faecalis ATCC 29212	37	12.4	+
pseudomonas aeruginosa ATCC 27853	37	25.9	-
Enterobacter aerogenes ATCC 13048	37	11.6	-
Klebsiella pneumoniae (clinical isolates 1 and 2)	37	-	-
Klebsiella oxytoca (clinical isolate)	37	-	-
Escherichia coli (clinical isolates 1, 2 and 3)	37	-	-
Staph aureus (clinical isolates 1 and 2)	37	-	+
Staph epidermidis (clinical isolate)	37	-	+
Serratia marcescens (clinical isolate 1)	37	10.78	-
Citrobacter freundii (clinical isolate)	37	-	-
Staphylococcus aureus (clinical isolate)	37	-	+
Stenotrophomonas maltophilia (clinical isolate 1)	37	12.6	-
Stenotrophomonas maltophilia (clinical isolate 2)	37	10.5	-

Table 1: Antibacterial activity of the lipopeptide biosurfactants against test microorganisms.

Producer microorganism	Biosurfactant/structure	Effect on	Reference
Bacillus licheniformis BAS50	Lipopeptid	*B. cereus, B. subtilis, S. aureus, E. coli*	[21]
Bacillus subtilis	Lipopeptid	*L. monocytogenes*	[22]
Bacillus licheniformis M104	Lipopeptid	*B. cereus, B. subtilis, S. aureus, P. aeruginosa, E. coli, K. pneumoniae*	[36]
Bacillus natto TK-1	Lipopeptid	*S. aureus*	[39]
Bacillus subtilis	Lipopeptid	*B. subtilis, S. aureus, L. monocytogenes*	[37]

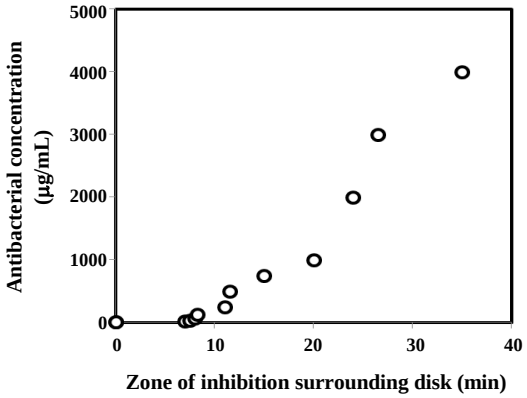


Fig. 2: Inhibitory activities of antibacterial substance produced by B. mojavensis PTCC 1696 at various concentrations against P. aeruginosa ATCC 27853.

the growth of *E. coli* and *S. aureus* were significantly inhibited, while *E. coli* bacterium response to the antibacterial activity of the test biosurfactants was negative [39]. The result of the *E. coli* response to the antibacterial activity shown in Table is in agreement with the findings reported in previous study [39]. While the antimicrobial effect of the test biosurfactant was not effective against *S. aureus*. Also, the result of the growth of *K. pneumoniae* obtained in the present study is in agreement with the findings of previous study on *B. licheniformis* performance reported elsewhere [38]. Since the maximum inhibitory zone was related to the effect of antibacterial substance on *P. aeruginosa* ATCC 27853, this strain was used to investigate the effect of biosurfactant concentration on its inhibitory activity. In literature, this test microorganism was used to test antimicrobial activity [40, 41]. The dependency of antimicrobial effect of lipopeptide on the biosurfactant concentration has been addressed in literature [38]. In the present study, the level of Minimum Inhibitory Concentration (MIC) for the antibacterial substance was 16 µg/ml, while the MIC values for the lipopeptide and rhamnolipid produced by *B. licheniformis* and *P. aeroginosa*, respectively were 6 and 32 µg/mL [38]. The relationship between the inhibitory concentrations of antibacterial substance and inhibition zones around *P. aeruginosa* ATCC 27853 after overnight incubation on Muller Hilton agar plates are shown in Fig. 2. The inhibition zone at concentration of 16 mg/lit was about 7 mm and the zone was increased to 35 mm by increasing the solution concentration to about 4000 mg/L.

Fig. 3 shows the responses of the *P. aeroginosa* to the test antibiotic agents which are generally used in clinical practices (the details are given in the figure).

Chemical stability of antibacterial substance

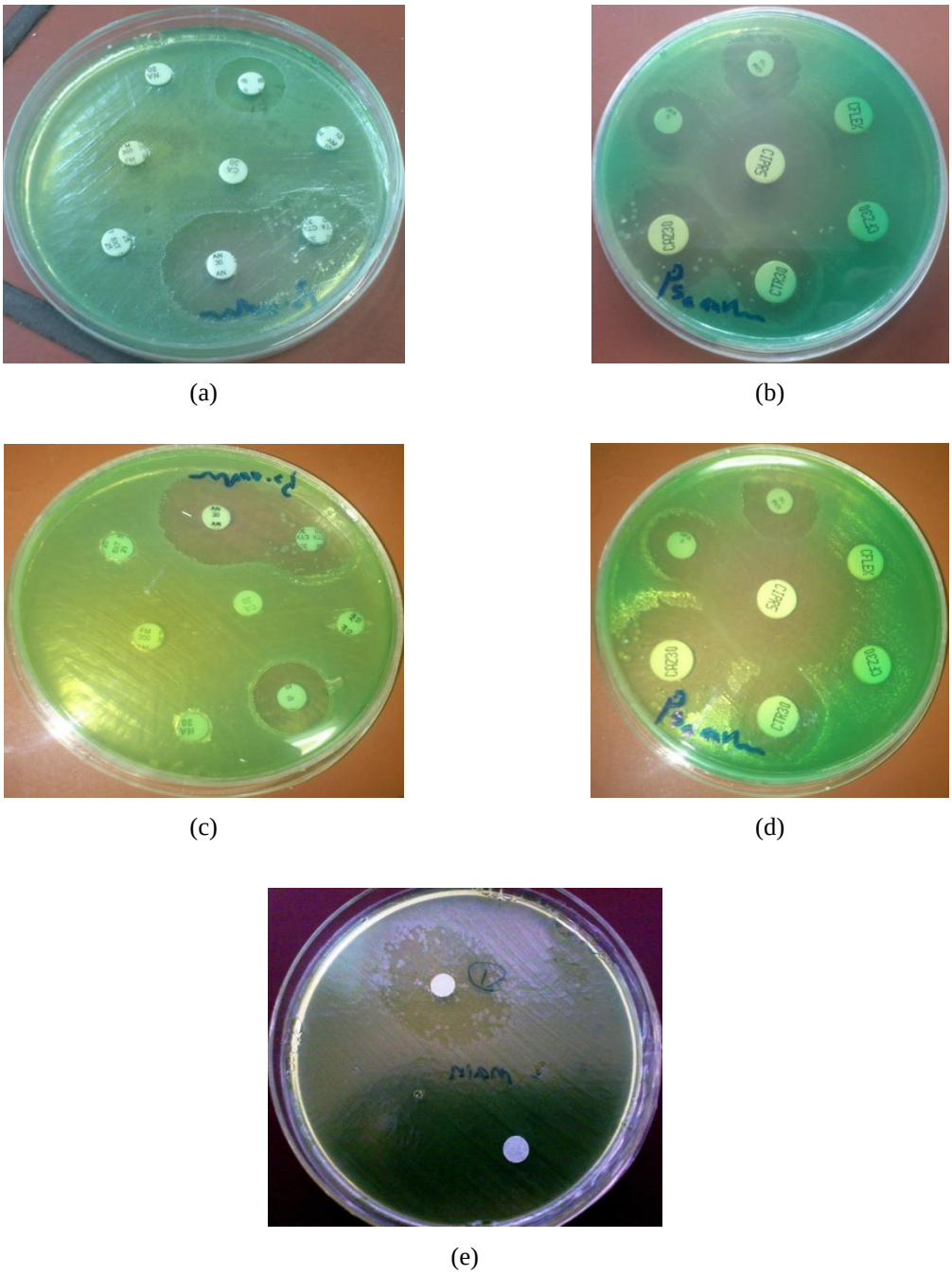
The sensitivities of antimicrobial substance to heat, pH and enzyme hydrolyses treatments were evaluated by disc diffusion tests against *P. aeruginosa* ATCC 27853. The sensitivity analyses results are shown in Table 3.

The results showed that the inhibitory properties of lipopeptide were retained when the antibacterial substance was incubated up to 100°C for 150 min, whereas it reduced after increasing the temperature and heat treating times. In addition, the activity was almost retained when the substance was treated in all tested pH in this study (2-11). The stability of antibacterial substance against enzyme hydrolyses, lipase and protease, was also tested.

The results showed that the substance was resistant to protease but affected by lipases at concentrations of 1 mg/lit (Table 3). These results are in agreement with the findings of other studies conducted on the antibacterial substance produced by *Bacillus subtilis* sp. strain B38 [42, 43]. However, *Motta et al.* [23] reported residual activities to be equal to zero and 15% for a lipopeptide substance produced by a *Bacillus* species treated with protease and lipase, respectively at concentrations of about 2 mg/lit. D'Costa et al. [44] showed that hydrolyzing enzymes play a key role in the inactivation of a lipopeptide (daptomycin), resulting in one or both of the following structural modifications: ring hydrolyzing which resulted in linearization or deacylation of the lipid tail.

CONCLUSIONS

In our previous study, the lipopeptide nature of the biosurfactant produced by the *B. mojavensis* PTCC 1696 was identified [28]. Since biosurfactant also expresses antibacterial activity, detection of this microbe's behavior was done in the present study using the well described disc tests. The largest inhibition was found to be expressed against *P. aeruginosa*. This compound showed antibacterial activity at low concentrations and its activity was maintained over a long period of time. Considering a wide range of pH and temperature, the stability of the antibacterial agent was also demonstrated. The use of this substance in medical fields heavily depends on repetitions



(a) (b)

(c) (d)

(e)

Fig.3: Inhibitory of growth of P. aeruginosa ATCC 27853 by standard antibiotics (plates (a) – (d)) and antibacterial substance produced by B. mojavensis PTCC 1696 (plate (e)). The zones (mm) around the standard antibiotic disks (µg per disc) used in this test (plates (a) – (d)) were: 26 mm for 30 µg Amikacin; 19 mm for 30 µg Ceftrixone; 0 mm for 30 µg Cephalexin; 0 mm for 30 µg Cephalothin; 31 mm for 5 µg Ciprofloxacin; 0 mm for 25 µg Co-Trimoxazole; 0 mm for 300 µg Nitrofurantoin; 0 mm for 30 µg Cefazolin; 25 mm for 30 µg Ceftazidime; 0 mm for 10 µg Ampicilin; 23 mm for 30 µg Cefotaxime; 0 mm for 30 µg Nalidixic Acid; 21 mm for 10 µg Gentamycin; 18 mm for 5 µg Ofloxacin; 19 mm for 10 µg Impenem + 10 µg Cilastatin.

Table 2: Effect of heat, pH and enzyme hydrolyses treatments on stability of the lipopeptide biosurfactant at concentration of 1000 g/l on P. aeruginosa ATCC 27853.

Treatment			Treatment time (min)	Inhibition zone (mm)
Temperature (°C)		40	30	21
		40	150	21
		60	30	21
		60	150	19
		100	30	15
		100	150	12.75
		120	15	0
pH		2	30	19.5
		2	150	20.5
		5	30	19.25
		5	150	18.5
		9	30	20
		9	150	19
		11	30	19.25
		11	150	19.75
Enzymes (mg/ml)	Lipase	0	60 min at 37 °C and then 3 min at 100 °C	20.5
		1		15.5
		2		15
	Protease	0		20.5
		1		20

of the evaluation methods by central laboratories and provides response to some uncertainties that may be associated with the obtained results.

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