ANTIMICROBIAL ACTIVITY OF *BACILLUS* SP. STRAIN FAS_1 ISOLATED FROM SOIL

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ABSTRACT

During screening for antibiotic producing microorganisms from environmental soil samples, the supernatant of a bacterial isolate was found to have antibacterial and antifungal activity on the standard indicator species. The standard cylinder-plate method was used to determine the inhibitory effect of the crude supernatant of each isolate on 6 bacterial and 3 fungal standard strains by measuring the diameter of inhibition zone. The highest inhibition zone on *Aspergillus niger* belonged to culture broth of isolate FAS_1 by 25 mm, and this isolate was the most efficient microorganism to inhibit standard bacterial and fungal species. Based on morphological and biochemical properties as well as 16S rDNA gene analysis, the selected isolate (isolate FAS_1) belonged to *Bacillus* genus. Investigation on the ability of different culture media for antibiotic production led to select Luria-Bertani media for further studies. Treatment of the culture broth of the isolate FAS_1 using typical protease didn't decrease the antimicrobial activity of the supernatant. After extracting of culture broth of the selected isolate by ethyl acetate as an organic solvent, the inhibitory effect was mainly increased. More investigation was done by bioautography method where the ethyl acetate fraction of the broth culture was separated on TLC by chloroform:methanol, 60:40 as mobile phase and R_f were calculated for inhibition spots.

Keywords: Antimicrobial activity, *Bacillus*, bioautography, cylinder-plate method, screening.

INTRODUCTION

Treatment of infectious diseases caused by pathogenic bacterial and fungal strains was one of the most traditional problems in the clinical field (Mehrgan *et al*., 2008; Fazly Bazzaz *et al*., 2005; Calvin, 1993). This necessity encouraged the investigators to synthesize novel and more potent inhibitory compounds (like azoles and quinolones derivatives) (Emami *et al*., 2008; Shafiee *et al*., 2008) to fight them. However, the adverse effects and also appearance of bacterial or fungal resistances persuaded the investigators to study on natural products from microorganisms or herbal extracts to discover novel and safe lead compounds (Mehrgan *et al*., 2008; Fazly Bazzaz *et al*., 2005). To reach this approach, screening of fungal and bacterial strains able to produce inhibitory compounds is the first step in the discovery of novel antibiotic compounds (Imada *et al*., 2007). Marine microorganisms and also cyanobacteria were found to produce such inhibitory compounds (Imada *et al*., 2007; El-Sheekh *et al*., 2006), but most of the available antibiotics are originally produced by terrestrial fungal and bacterial strains (Mannanov and Sattarova, 2001).

The genus of *Bacillus*, one of the most abundant bacterial strains found in soil, is able to produce two dozens of antibiotic compounds with various chemical properties (Stein, 2005), among which peptide derivatives are more studied (Mannanov and Sattarova, 2001; Stein, 2005). Furthermore, other groups of antibiotics, like phospholipid derivatives (i.e., Bacilysocin) were also found as antibacterial agents produced by this earthborn genus (Tamehiro *et al*., 2002). Many investigations have been carried out to isolate different strains of terrestrial *Bacillus* and identify their inhibitory compounds (Mannanov and Sattarova, 2001; Tamehiro *et al*., 2002; Lisboa *et al*., 2006).

The present study is the first attempt to isolate terrestrial bacterial strains with antibiotic production ability and focused on the screening for microorganisms able to inhibit growth of both standard bacterial and fungal strains followed by identification of the most efficient isolate by 16S rDNA technique. Beside antimicrobial tests for culture supernatants, bioautography was performed to determine the R_f of inhibitory spots on the TLC sheets.

MATERIALS AND METHODS

Chemicals and equipments

Mueller-Hinton agar, Sabouraud-2%-dextrose broth (SDB), Nutrient broth, agar and 3-(4-iodophenyl)-2-(4-nitrophenyl)-5-phenyl-2H-tetrazolium chloride (INT) were supplied from Merck (Darmstadt, Germany). Standard bacterial (2 Gram-positive and 4 Gram-negative) and fungal species (one yeast and two fungal strains) were

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purchased from Persian Type Culture Collection, Iranian Research Organization for Science and Technology (PTCC, Tehran, Iran).

Preparation of standard indicator microorganisms for antimicrobial screening

The lyophilized powder of 9 indicator microorganisms which are listed in table 1 were suspended in nutrient broth (for bacterial strains) or SDB (for fungal species) and transferred to nutrient agar and Sabouraud-2%-dextrose agar (SDA) plates, respectively. After 18-24 h incubation at 37°C (for bacterial and yeast species) and 30°C (for fungal species), a loopful of each test strains was added to nutrient broth (for bacterial strains) or SDB (the fungal strains) and incubated at optimum temperature to reach the turbidity of 0.5 McFarland standard suspension. 1 mL of such suspension, containing 1.5×10^8 microorganisms, was added to 200 mL of molten Muller-Hinton agar (42-45°C) for bacterial indicator species or SDA (for yeast and fungal strains) followed by pouring 15 mL of such pre-inoculated media (as seed layer) into sterile Petri dishes containing a thin layer of solidified medium (Awais *et al*., 2007).

Screening

The soil samples were collected from different green places and parks in Kerman (57° 01' E and 30° 15' N), Iran. After adding 100 mL of sterile normal saline to 2 grams of each sample and filtering, 1 mL of the filtrates spread on the surfaces of pre-inoculated bacterial or fungal plates which prepared as described above. Incubation at optimum temperature was followed to find bacterial colonies which produced inhibition zone around their colonies. These active isolates were transferred to newly prepared nutrient plate and subcultured to acquire an axenic isolate.

Antimicrobial assay

Each bacterial isolate from the previous step was cultivated into Luria-Bertani medium for 48 h at 30°C and its supernatant (after removing cell by centrifugation at 5000 g for 10 min) was subjected to antimicrobial test. The standard cylinder-plate method was used to investigate the antimicrobial activity of the supernatant of each soil bacterial isolate. Briefly, the stainless steel cylinder, (inner diameter 6 mm, outer diameter 7 mm and height of 10 mm) were applied onto pre-inoculated plates (as said above) followed by introducing of each supernatant. After 24-48 h incubation, the inhibitory zones (in mm) were measured (Moshafi *et al*., 2006). In a separate trial, the inhibition zone of ciprofloxacin (a broad range antibacterial agent) and ketoconazole (a typical antifungal compound) were determined.

Identification of the selected microorganism

The phenotypic properties and typical biochemical tests were performed by the methods described in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994). 16S rDNA technique was used to amplify rDNA gene and compare it to sequences available in GenBank of NCBI. After overnight cultivation of the isolate in Luria-Bertani media, the cells were harvested by centrifugation (11000 g for 5 min) followed by three times washing cells by apyrogen sterile water. Genomic DNA was acquired by boiling the cell suspension at 94°C for 10 min and consequent centrifugation (11000 g for 5 min). The primer pair 27F (5'-AGAGTTTGATCCTGGCTCAG-3') as forward primer and 1492R (5'-

Table 1: Antimicrobial activity (inhibition zone in mm) of cultural broth of five bacterial isolates on 6 bacterial and 3 fungal indicator strains

	Isolate names						
	FAS_1		FAS_2	FAS_3	FAS_4	FAS_5	Cip[a]
	Test fraction						
Indicator strains (PTCC No.)	S[b]	E[c]	S	S	S	S	
E. coli (1330)	17	19	-	14	-	-	21
Klebsiella pneumoniae (1053)	18	21	-	17	18	16	25
Salmonella typhi (1609)	18	20	-	-	-	14	21
Staphylococcus aureus (1112)	21	25	-	-	-	-	23
Staphylococcus epidermidis (1114)	22	27	18	-	-	-	38
Pseudomonas aeruginosa (1074)	20	23	-	17	-	16	27
							Ket[d]
Candida albicans (5027)	22	21	-	-	-	-	20
Aspergillus flavus (5004)	22	24	12	-	30	23	26
Aspergillus niger (5012)	2	28	-	-	-	23	17

[a] Ciprofloxacin was used as standard antibiotic at concentration of 6 µg/ml
[b] S: Supernatant of each isolate culture after 48 h incubation in LB medium at 30°C
[c] E: Ethyl acetate fraction of 48-h culture broth of isolate FAS_1
[d] Ketoconazole was used as standard antifungal at concentration of 500 µg/ml

TACGGTTACCTTGTTACGACTT-3') as reverse primer (Faramarzi *et al*., 2009) were used to amplify 16S rDNA fragment by thermal cycler (PEQLAB, Erlangen, Germany) which was programmed as follows: a) initial denaturation at 94°C for 3 min. b) 30 cycles of denaturation at 94°C for 1 min, annealing at 60°C for 45 sec and synthesis at 72°C for 90 sec. and, c) final extinction at 72°C for 90 sec (Faramarzi *et al*., 2009). Amplified DNA was separated on 1% agarose gel in TAE buffer. The BLAST program was used to compare the acquired sequence to GeneBank of NCBI.

Selection of the best medium for antimicrobial production

The capability of different media containing Mueller-Hinton broth (MHB), Trypticase Soy broth (TSB), nutrient broth (NB), Caso broth (CB), Luria-Bertani (LB), peptone water (PW, peptone 1g/L; NaCl 8 g/L) for antimicrobial production were determined by cultivation of the selected bacterial strain in described media followed by standard cylinder-plate antimicrobial test of acquired supernatants. The best medium was used for further investigation.

Growth curve and antimicrobial activity of isolate FAS_1

Determination of OD_{600} (inoculated culture compares to non-inoculated one) was performed to obtain the growth curve of the selected isolate. The changes in pH of supernatant were also measured. Culture broth samples were taken every 12 h and antibacterial activities were investigated as said previously.

Enzymatic treatment effects on antimicrobial activity

To study the effect of proteolytic treatments on antimicrobial activity of the supernatant, trypsin and proteinase K in 0.1 M phosphate buffer pH 7 were added to culture broth to reach the final concentration of 0.1 mg/mL. After incubation of supernatant (2 h at 37°C) the enzyme reaction was stopped by heating at 100°C for 5 min (Batdorj *et al*., 2006). Untreated samples were used as control and change in antimicrobial activity of treated supernatant were compared to untreated sample.

Antimicrobial activity of ethyl acetate fraction

In order to study the antibacterial activity of the ethyl acetate fraction of the selected isolate, its supernatant was mixed to ethyl acetate and ethyl acetate fraction was decanted. The ethyl acetate was evaporated and the residue was dissolved in 1:1 methanol:DMSO solvent. Inhibitory effect of this fraction was tested as said previously. The central cylinder in each plate was designed to investigate antibacterial activity of solvent (1:1 methanol:DMSO) used to solve ethyl acetate fraction.

Bioautography experiments

After cultivation of the isolate in LB medium, the bacterial cells were separated by centrifugation (9000 g for 10 min), ethyl acetate was added to the supernatant and the ethyl acetate fraction was decanted and concentrated. The ability of different mobile phase for separation of ethyl acetate fraction on TLC sheets was determined and the chloroform:methanol 60:40 was found to be the best for separation. The TLC sheets were mounted on Petri dish plates containing a thin layer of Mueller-Hinton agar (for bacterial test strains) or SDA (for fungal test strains) and then introducing a thin layer of pre-inoculated medium (as said in previous section) on TLC sheets. The Petri dishes were incubated at optimum temperature for 18-36 h followed by spraying dehydrogenase indicator (INT, 0.2%) on the plate surfaces to appear inhibition zones and then R_f of such inhibition zones were measured.

RESULTS

Screening of antibiotic producing microorganisms

Among 35 various soil samples, five bacterial isolates were found to produce inhibitory zones around their colonies on plates pre-inoculated by the indicator bacterial and fungal strains. The diameter of inhibition zones of crude supernatant of each isolate, supernatant and ethyl acetate fraction of isolate FAS_1 and also ciprofloxacin and ketoconazole (as antibacterial and antifungal agents, respectively) are summarized in table 1. As it can be seen, isolate FAS_1 was the most efficient isolate to inhibit 6 bacterial and 3 fungal strains. Isolate FAS_5 was also inhibit three bacterial (*Klebsiella pneumoniae*, *Salmonella typhi* and *Pseudomonas aeruginosa* by 16, 14 and 16 mm inhibition zones, respectively) and 2 fungal strains (*Aspergillus flavus, A. flavus* by 23 and 23 mm inhibition zones, respectively).

Identification of the selected isolate

Morphological studies showed that the isolate FAS_1 was a Gram-positive, rod-shape strain capable of forming endospore which produces a yellowish white colony on nutrient agar plate. Comparison of 16S rDNA amplified gene (fig. 1) to sequences of GenBank, showed 99% of identity to the genus of *Bacillus*. Comparison of biochemical properties (table 2) by strains of *Bacillus* categorized in Bergey's Manual of Determinative Bacteriology (Holt *et al*., 1994) didn't reveal which strain was the isolate FAS_1 belonged to. According to these findings, the selected isolate was determined to be *Bacillus* sp. and its 16S rDNA (1396 bp) gene was deposited in GenBank by accession number of HM369111.

Selection of the best medium

Based on inhibition zones diameter measured for different culture media (fig. 2), the LB medium was found to be the best for consequence investigations. The growth curve of isolate FAS_1 and also its antibacterial activity were determined in LB medium as shown in figs. 3 and 4, revealed that the inhibitory effect of isolate FAS_1 culture broth was achieved after steady state of growth.

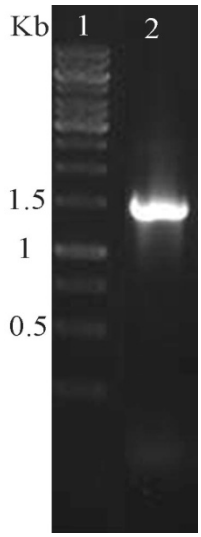

Fig. 1: Agarose gel electrophoresis of 16S rDNA amplified gene of isolate FAS_1. Lane 1, DNA ladder; Lane 2, amplified fragment.

Table 2: Biochemical characterization of the selected isolate FAS_1.

Characteristics	Results
Catalase production	+
Oxidase activity	-
Voges-Proskauer test	-
Methyl red test	-
Acid from	
D-Glucose	+
L-Arabinose	-
D-Xylose	-
D-Mannitol	-
Hydrolysis of	
Casein	+
Gelatin	+
Starch	+
Utilization of citrate	+
Nitrate reduced to nitrite	+
Formation of	
Indole	-
Dihydroxyacetone	-
Growth in NaCl	
2%	+
5%	+
7%	-
10%	-
Growth at	
5°C	-
30°C	+
40°C	+
50°C	-

Treatment of culture broth by typical protease

The inhibitory activity of culture broth wasn't affected by protease activity and no loss of activity revealed after and before proteolytic treatment.

Determination of R_f by Bioautography

To identify which fraction of ethyl acetate extract was responsible for antimicrobial activity and also to determine its corresponded R_f, the *Bioautography* method was performed. After comparison of different solvent systems as mobile phase, the chloroform:methanol 60:40 was found to be efficient (data not shown) to separate ethyl acetate fraction on TLC. The inhibition zones were illustrated at fig. 5 and the corresponded R_f were summarized in table 3.

Table 3. R_f of inhibition zone after bioautography of ethyl acetate fraction of isolate FAS_1.

Indicator strains	Determined R_f
E. coli	0.42–0.92
Klebsiella pneumoniae	0.58–0.92
Salmonella typhi	0.58–0.92
Staphylococcus aureus	0.75–0.92
Staphylococcus epidermidis	0.58–0.83
Pseudomonas aeruginosa	0.58–0.92
Candida albicans	0.66–0.83
Aspergillus flavus	0.66–0.83
Aspergillus niger	0.75–0.83

DISCUSSION

Sensitivity reactions and side effects of chemical derivative antimicrobial agents persuaded investigators to study on natural products from herbal origin and screen terrestrial and marine microorganisms to find out new lead compounds to fight with infectious diseases (Mehrgan *et al.*, 2008). Among terrestrial bacterial strains, the genus of *Bacillus* has been studied due to its ability to produce different structure of inhibitory compounds (Stein, 2005). Most of these inhibitory agents have been categorized in peptide derivative family (Stein, 2005; Tamehiro *et al.*, 2002). For example, Zheng and Slavik (1999) reported isolation and characterization of a bacteriocin produced by a newly isolate of *Bacillus subtilis*. The structures and biosynthetic pathways of ribosomal antibiotics (like subtilin, ericin and sublancin) and non-ribozomal antibiotics (such as iturin and fengycin) were reviewed by Stein (2005). In addition to peptide derivatives, lipopeptide antibiotics like surfactin have been also isolated and characterized from this earn born genus (Mannanov and Sattarova, 2001). Biologically active inhibitory agents with hydrophobic properties were characterized by Mannanov and Sattarova (2001) after extracting of broth culture of *Bacillus* sp. by chloroform. Bacilysocin, a novel and broad spectrum phospholipid

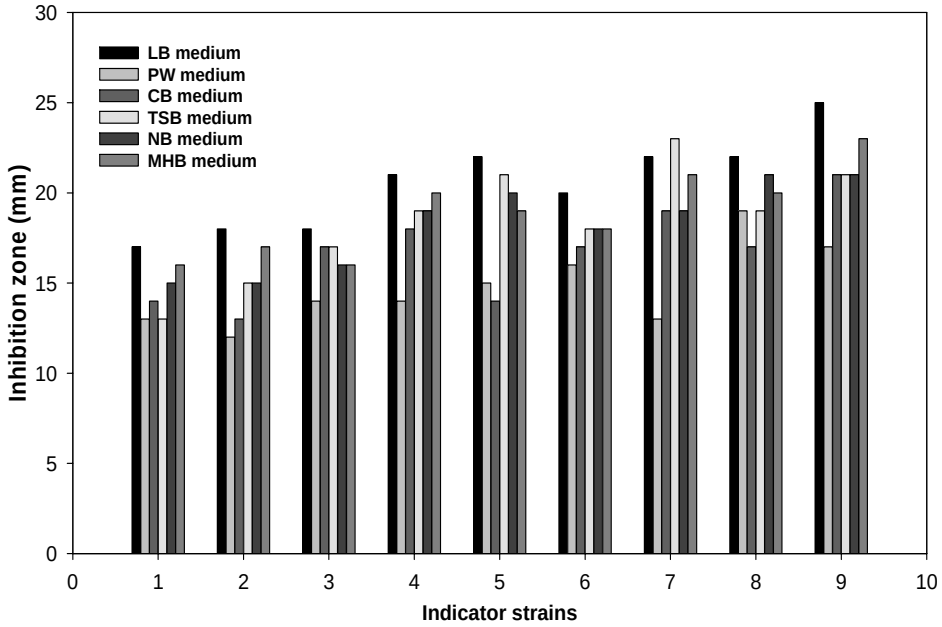

Fig. 2: Comparison of antimicrobial activity (inhibition zone in mm) of isolate FAS_1 cultivated in different media (abbreviations are presented in section 2). Indicator strains containing 1, *E .coli*; 2, *Klebsiella pneumoniae*; 3, *Salmonella typhi*; 4, *Staphylococcus aureus*; 5, *Staphylococcus epidermidis*; 6, *Pseudomonas aeruginosa*; 7, *Candida albicans*; 8, *Aspergillus flavus*; 9, *Aspergillus niger*.

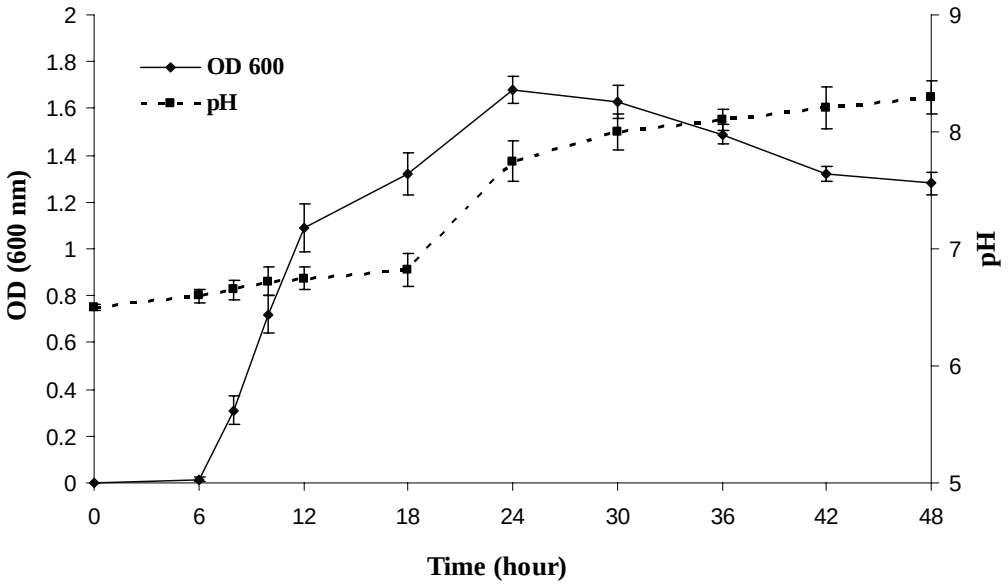

Fig. 3: Growth curve of isolate FAS_1 in LB medium.

antibiotic, was purified and characterized from butanol extract fraction of *Bacillus subtilis* 168 (Tamehiro *et al*., 2002). These two latest reports indicated that *Bacillus* spp. can produce agents with chemically different properties from peptide group.

In the present study, the culture broth of a soil bacterial isolate, identified as *Bacillus* sp., was subjected to antimicrobial studies due to its ability to inhibit 6 bacterial and 3 fungal indicator strains. In presence of culture broth of isolate FAS_1, an inhibition zone of 25 mm was detected on *Aspergillus niger* and isolate FAS_1 was the efficient one to inhibit both bacterial and fungal indicator strains. Awais *et al*., (2007) studied inhibitory effects of a *Bacillus* sp. isolate on 2 pathogenic strains of *Micrococcus luteus* and *Staphylococcus aureus* and

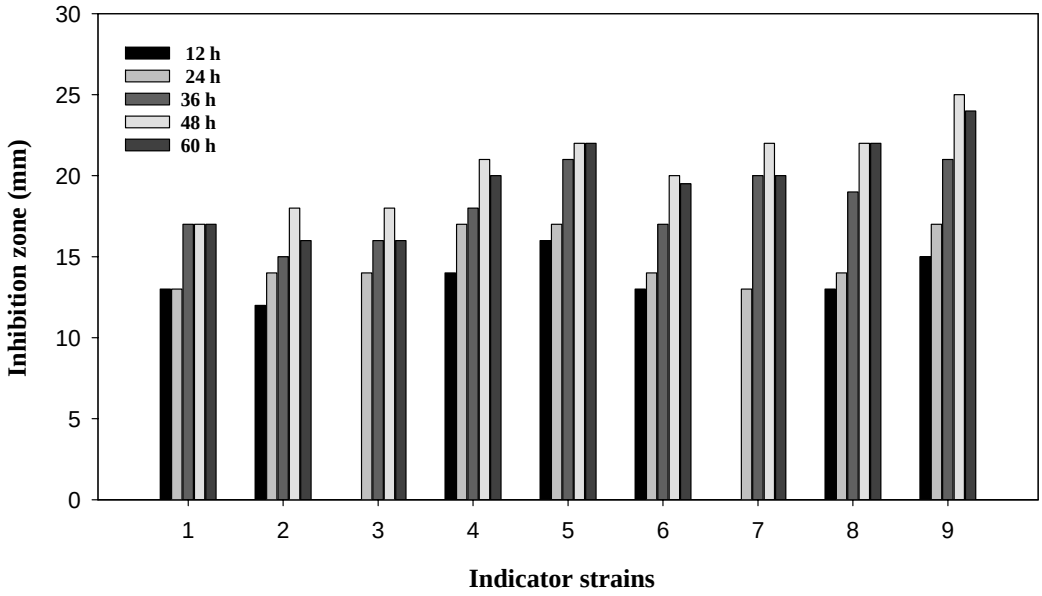

Fig. 4: Antimicrobial activity of isolate FAS_1 broth culture after 12, 24, 36, 48 and 60 h incubation in LB medium at 30°C. Indicator strains containing 1, *E .coli*; 2, *Klebsiella pneumoniae*; 3, *Salmonella typhi*; 4, *Staphylococcus aureus*; 5, *Staphylococcus epidermidis*; 6, *Pseudomonas aeruginosa*; 7, *Candida albicans*; 8, *Aspergillus flavus*; 9, *Aspergillus niger*.

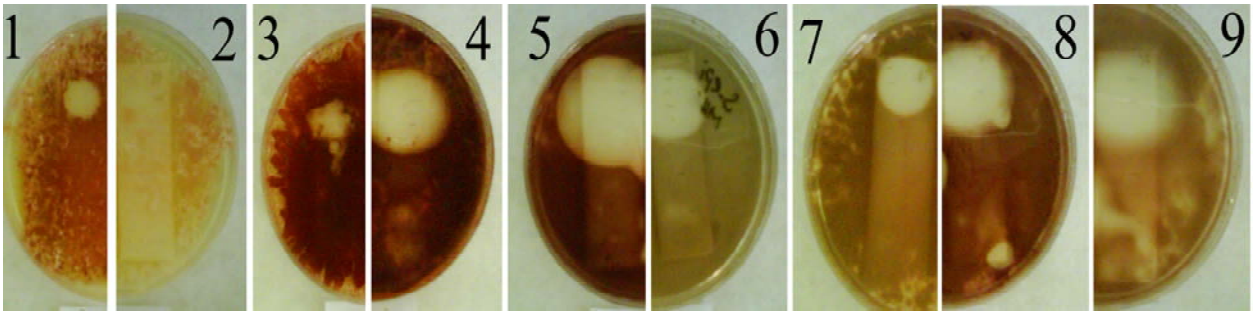

Fig. 5: Bioautography of ethyl acetate fraction of isolate FAS_1. Indicator strains containing 1, *Aspergillus flavus*; 2, *Aspergillus niger*; 3, *Candida albicans*; 4, *Pseudomonas aeruginosa*; 5, *E .coli*; 6, *Staphylococcus epidermidis*; 7, *Staphylococcus aureus*; 8, *Klebsiella pneumoniae*; 9, *Salmonella typhi*.

determined 18 mm inhibition zone for this two indicator strains.

After early investigation which confirmed the ability of the selected isolate to show antibacterial and antifungal effects, the best media for antimicrobial agent productivity of this strain was found to be the LB medium. This result was in agreement with the result of Lisboa *et al*., (2006), indicated the more rich culture media (brain heart infusion), the higher antibiotic productivity.

The growth curve and inhibitory activity revealed that the most antimicrobial activity obtained after steady state of growth curve. Most antimicrobial agents which categorize as secondary metabolites are produced in this stage of growth (Lisboa *et al*., 2006; Tamehiro *et al*., 2002).

Proteolytic studies using various proteases didn't show any decrease on the inhibitory ability of culture broth, so consequence studies focused on ethyl acetate fraction of the supernatant. TLC analysis of this fraction showed four spots when chloroform:methanol was used as mobile phase. Three of these spots were close together and had R_f value of 0.5–0.9 and another spot showed R_f 0.3. Further analysis of ethyl acetate fraction by bioautography method showed that the most efficient zone had R_f value of 0.5–0.9. Mannanov and Sattarova (2001) indicated that a fraction with R_f of 0.8 was corresponded to antimicrobial activity when the chloroform extract of *Bacillus* sp. supernatant was separated on TLC plates.

CONCLUSION

We reported here a soil bacterial isolate, identified as *Bacillus* sp., capable of inhibiting growth of standard bacterial and fungal strains. We also determined the R_f of inhibition spots on TLC plate by bioautography technique and now, we are investigating on isolation and characterizing of corresponded spots.

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REFERENCES

Awais M, Ali Shah A, Hameed A and Hasan F (2007). Isolation, identification and optimization of bacitracin produced by *Bacillus* sp. *Pak. J. Bot*., **39**: 1303-1312.

Batdorj B, Dalgalarrondo M, Choiset Y, Pedroche J, Metro F, Prevost H, Chobert JM and Haertle T (2006). Purification and characterization of two bacteriocins produced by lactic acid bacteria isolated from Mongolian airag. *J. Appl. Microbiol*., **101**: 837-848.

Calvin M and Kunin MD (1993). Resistance to antimicrobial drugs a worldwide calamity. *Ann. Intern. Med*. **118**: 557-561.

El-Sheekh MM, Osman MEH, Dyab MA and Amer MS (2006). Production and characterization of antimicrobial active substance from the cyanobacterium *Nostoc muscorum*. *Environ. Toxicol. Pharmacol*., **21**: 42-50.

Emami S, Foroumadi A, Falahati M, Lotfali E, Rajabalian S, Ebrahimi S, Farahyar S, and Shafiee A (2008). 2-Hydroxyphenacyl azoles and related azolium derivatives as antifungal agents. *Bioorg. Med. Chem. Lett*., **18**: 141-146.

Faramarzi MA, Fazeli M, Tabatabaei Yazdi M, Adrangi S, Jami Al Ahmadi K, Tasharrofi N and Aziz Mohseni F (2009). Optimization of cultural conditions for production of chitinase by a soil isolate of *Massilia timonae*. *Biotechnol*., **8**: 93-99.

Fazly Bazzaz BS, Khajehkaramadin M and Shokooheizadeh HR (2005). In vitro antibacterial activity of *Rheum ribes* extract obtained from various plant parts against clinical isolates of Gram-negative pathogens. *Ir. J. Pharm. Res*., **2**: 87-91.

Holt JG, Krieg RN, Sneath PHA, Staley JT and Williams ST (1994). Bergey's Manual of Determinative Bacteriology, 9th edn. Williams and Wilkins, Baltimore.

Imada C, Koseki N, Kamata M, Kobayashi T and Hamada-Sato N (2007). Isolation and characterization of antibacterial substances produced by marine actinomycetes in the presence of seawater. *Actinomycetologica*., **21**: 27-31.

Lisboa MP, Bonatto D, Bizani D, Henriques JAP and Brandelli A (2006). Characterization of a bacteriocin-like substance produced by *Bacillus amyloliquefaciens* isolated from the Brazilian Atlantic forest. *Int. Microbiol*., **9**: 111-118.

Mannanov RN and Sattarova RK (2001). Antibiotics produced by Bacillus bacteria. *Chem. Nat. Comp*., **37**: 117-123.

Mehrgan H, Mojab F, Pakdaman S and Poursaeed M (2008). Antibacterial activity of *Thymus pubescens* methanolic extract. *Ir. J. Pharm. Res*., **7**: 291-295.

Moshafi MH, Forutan H and Mehrabani M (2006). Investigation of antibacterial effects of *Hibiscus Sabdariffa* L. dried calyx by agar diffusion and bioautographic methods. *J. Kerman. University Med. Sci*., **13**: 103-110.

Shafiee A, Haddad Zahmatkesh M, Mohammadhosseini N, Khalafy J, Emami S, Moshafi MH, Sorkhi M and Foroumadi A (2008). Synthesis and in-vitro antibacterial activity of N-piperazinyl quinolone derivatives with 5-chloro-2-thienyl group. *DARU*., **16**: 189-195.

Stein T (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Mol. Microbiol*., **56**: 845-857.

Tamehiro N, Okamoto-Hosoya Y, Okamoto S, Ubukata M, Masa Hamada M, Naganawa H and Ochi K (2002). Bacilysocin, a novel phospholipid antibiotic produced by *Bacillus subtilis* 168. *Antimicrob. Agents Chemother*., **46**: 315-320.

Zheng G and Slavik MF (1999). Isolation, partial purification and characterization of a bacteriocin produced by a newly isolated Bacillus subtilis strain. *Lett. Appl. Microbiol*., **28**: 363-367.