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Antibacterial activity of some actinomycetes isolated from soils of Alborz province, Iran

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ABSTRACT

Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years by scientists. Actinomycetes have the capability to synthesize many different antibiotics. A total of 69 actinomycete isolates were recovered from soil samples collected from Alborz Province. Selected colonies (rough, chalky) of actinomycetes were purified. All screened isolates were identified morphologically and physiologically, all belonging to *Streptomyces*. These were then assessed for their antibacterial activity against pathogenic bacteria. Four pathogenic test strains were used in this study including *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATTC 6633, *Klebsiella pneumoniae* ATTC 10031, and *Escherichia coli* ATCC 29998. Determination of antibacterial activities of isolated actinomycetes performed by using modified spektra-plak method and Mueller Hinton agar (Oxoid) plates. Antagonism was detected by formation of inhibition zone. Results of the study indicated that 12 isolates were active against *S. aureus,* 15 isolates against *B. subtilis,* six isolates against *K. pneumonia,* and four isolates were active against *E. coli*.

Keywords: antibacterial activity, bioactive isolate, pathogenic bacteria, screening, *Streptomyces*.

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Introduction

Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years by scientists. Actinomycetes are Gram-positive bacteria with high guanine and cytosine (G+C) content. Their morphology is highly pleomorphic; growing as filaments that branch into radiate or star-like shape. They are primarily saprophytic and are known to contribute in nutrient turnover, using many available nutrient sources for their development (1). They play an important ecological role in soil cycles. Many are also well known for their economic importance as producers of biologically active substances, such as antibiotics, pesticides, vitamins and enzymes (2-5). Antibiotics have been used in many fields that are agriculture, veterinary and pharmaceutical industry (6).

In the present investigation, soil samples from Karaj, Iran, were examined for the presence of actinomycetes and the 69 obtained isolates were tested for the production of antibacterial metabolite against four pathogenic bacteria.

Materials and Methods

Samples selection and test micoorganisms

Soil samples were collected from different parts of Alborz province, Iran. Samples were collected from 5-15 cm depth into sterile plastic bags. The target strains used for antimicrobial activity were obtained from PTCC (Persian Type Culture Collection) including *Staphylococcus aureus* ATCC 6538, *Bacillus subtilis* ATTC 6633, *Klebsiella pneumoniae* ATTC 10031, and *Escherichia coli* ATCC 29998.

Isolation and maintenance of actinomycete strains

For each collected sample, one g of preheated soil at 50°C for 1 h were suspended in 10 ml of physiological water (NaCl 9 gl^{-1}) and serial dilutions up to 10^{-6} were prepared. The strains were isolated by diluting the samples (an aliquot of 0.1 ml of each dilution) in physiological water, plating on starch casein agar and ISP2 agar. The amphotericin B 75 mg ml^{-1} was added to media to inhibit fungal contamination. The inoculated plates were incubated for 7-14 days at 28°C. After this initial cultivation, rough and chalky colonies successively grew on both agar plates to obtain pure colonies. Plates containing pure cultures were stored at 4°C until further examinations.

Characterization of isolates

Actinomycetes colonies were recognized on the basis of morphological characteristics by light microscopy. According to the recommendations of International *Streptomyces* Project (ISP), actinomycete isolates were characterized based on morphological and physiological features (7). The isolates were identified as species belonging to the genus *Streptomyces* by biochemical tests (8), and also their morphological characteristics were analyzed, and the morphology of spore bearing hyphae with entire spore chain compared as described in Bergey's Manual (9) using cover slip method (10).

Determination of antibacterial activity by modified spektra-plak method

Antimicrobial activities of isolates were tested preliminarily by modified spektra-plak method (11). Mueller Hinton agar plates were inoculated with actinomycetes cultures by a single streak of inoculums in the center of the

petri dish and incubated at 28°C for 4 days. 24 hrs cultures of *S. aureus, B. subtilis, E. coli* and *K. pneumoniae* were streaked by a single streak at a 90° angle to actinomycetes strains. Antagonism was detected by formation of inhibition zone and measured by the determination of the size of the inhibition zone.

Antibacterial activity resulting from liquid production media

Well-agar diffusion method was employed for antibacterial activity test of the four selected strains. A loopful spore of each actinomycete isolate was inoculated into a 100 ml flask containing 20 ml of ISP2 broth. The flasks were incubated at 220 rpm, 28°C, for 96 hours. The fermentation broths were then centrifuged at 4000 rpm for 15 minutes. Antimicrobial activity of the supernatant was examined by using well (6 mm in diameter) agar diffusion assay against pathogenic test strains on Muller-Hinton agar plates. After incubation for 24 hrs at 37°C, the diameter of inhibition zone was measured. The activity was assessed in triplicates.

Results

Isolation of actinomycetes

This research was aimed to highlight the presence of actinomycetes in this province and to select the strains with antibacterial activity. A total of 69 different actinomycete strains were recovered from 9 soil samples collected from 2012 to 2013 in Alborz Province, Iran. Also, in the present study, starch casein agar and ISP2 agar supplemented with amphotericin B 75, as an antifungal agent, were used.

The majority of the strains were collected from the ISP2 agar medium. The soils of Andisheh show the higher number of actinomycetes isolates (14 isolates) with respect to others soils (Table 1). The recovery of actinomycetes in Golshahr was lower (three isolates) than the other zones (Table 1).

Table 1. Initial screening of actinomycete isolates

Origin	Soil pH	Total strains isolated	Number of active isolates against bacteria (%)
Mehrshahr	7.2 ± 0.2	6	2 (33.33)
Gohardasht	6.2 ± 0.2	7	4 (57.14)
Mohammadshahr	6.8 ± 0.2	8	3 (37.5)
Kharazmi Uni	7.5 ± 0.2	7	2 (28.57)
Golshahr	6.7 ± 0.2	3	2 (66.66)
Shahriar	5.8 ± 0.2	8	2 (25)
Andisheh	7.1 ± 0.2	14	5 (35.71)
Karaj	6.1 ± 0.2	6	3 (50)
Ferdowsieh	7.0 ± 0.2	9	2 (22.22)
Total		68	25 (36.76)

Numbers in parentheses represent the percentage out of the total isolates in each origin

Morphological, physiological and biochemical characteristics of isolates

All isolates grew on a range of agar media showing morphology typical of *Streptomycetes* (8, 12-14) since the colonies were slow growing, aerobic, glabrous or chalky, folded and with aerial and substrate mycelia of different colors. In addition, all colonies possessed an earthy odor. To confirm identification of isolates to genus Gram-stain, acid-fastness and degradation of casein, tyrosine and xanthine were done. All the strains were Gram positive and acid-fast negative. The morphological characteristics and pigment production of the *Streptomyces* isolates are shown in Table 2. All of these isolates fitted the genus description as reported by several investigators (7, 14). *Streptomyces* strains had different mycelium colors. They were categorized into five color series according to the color of their mature sporulated aerial mycelium. The most abundant mycelium color was white (Table 2). 16 isolates (23.18%) produced Melanin. Diffusible pigments were produced by 21 strains (30.43%). 32 (30.43%) strains showed distinctive reverse side pigment (Table 2).

Table 2. Morphological and cultural characteristics of the *Streptomyces* isolates

Number of isolate	Color series					
	White	**Gray**	**Yellow**	**Orang**	**Pink**	**Total (%)**
Pigment production	42	16	7	3	1	69
Melanin	11	4	1	0	0	16 (23.18)
Soluble	13	5	2	1	0	1121(30.43)
Reverse side	18	8	3	2	1	32 (46.38)
Sporophore morphology						
Recti flexible (RF)	28	11	5	2	1	47 (68.12)
Spirales (S)	13	5	2	1	0	21 (30.43)
Retinaculiaperti (RA)	1	0	0	0	0	1 (1.45)

Numbers in parentheses represent the percentage out of the total isolates

According to the shape of the spore chains observed under light microscopy, the isolates were categorized as Rectus-Flexibilis (68.12%), Spira (30.43%) and Retinaculiaperti (1.45%) (8).

Determination of antibacterial activity by modified spektra-plak method

The antibacterial activity of the test isolates was varied. 25 of 69 actinomycetes isolates were shown to have antibacterial activity against *S. aureus, E. coli, K. pneumoniae* and *B. subtilis.* Antibacterial activity was observed in 36% of the strains and appeared promising. The antibacterial activity of *Streptomyces* strains against *E. coli* was 5.8%. 21.7% of isolated strains were active against *B. subtilis*, 17.4% against *S. aureus*, and 8.7% against *K. pneumoniae* (Table 3).

Table 3. Antibacterial activities of active isolates (inhibition zone diameter- mm)

Isolates	*S. aureus*	*B. subtilis*	*K. pneumoniae*	*E. coli*
KD12	12	9	-	10
KD30	-	10	-	-
KH4	3	5	-	-
KH67	-	11	-	-
KH5	22	-	-	-
KH16	-	-	31	5
AK52	7	-	-	-
AK23	12	-	-	-
AK11	2	4	-	-
MD31	13	-	-	-
MD19	-	11	5	-
MK9	-	-	-	28
MK27	-	21	-	-
BK33	9	13	-	-
BK17	-	16	-	-
A25	-	-	13	6
A66	-	-	9	-
A10	-	3	-	-
A48	16	12	18	-
A44	-	5	-	-
GM54	31	-	-	-
GM1	6	11	-	-
GM41	-	15	-	-
HM16	11	-	2	-
HM56	-	8	-	-
Number of active isolates	12	15	6	4
Total percentage (%)	17.4	21.7	8.7	5.8

The isolates GM54, MK9, KH16 and MK27 exhibited a good activity against *S. aureus* (31 mm), *E. coli* (28 mm), *K. pneumoniae* (31 mm) and *B. subtilis* (21 mm), respectively (Table 3, Fig. 1). 40% (10) of bioactive isolates were active against more than one test strains such as KD12, KH4 and KH16 (Table 3).

Approximately 36% (25) of the isolates were active against pathogenic bacteria. 68% (17) of bioactive isolates produced antibacterial against only Gram positive bacteria, 16% (4) of them only against Gram negative bacteria, and 16% (4) of isolates against both. The most broad spectrum antibacterial activity on test pathogen bacteria were shown by isolate A48 (16 mm against *S. aureus*, 12 mm against *B. subtilis* and 18 mm against *K. pneumoniae*). But the most bioactive producing isolates against Gram positive and Gram negative pathogenic bacteria were GM54 (31 mm against *S. aureus*), and KH16 (31 mm, against *K. pneumoniae*) (Table 3) (Fig. 1).

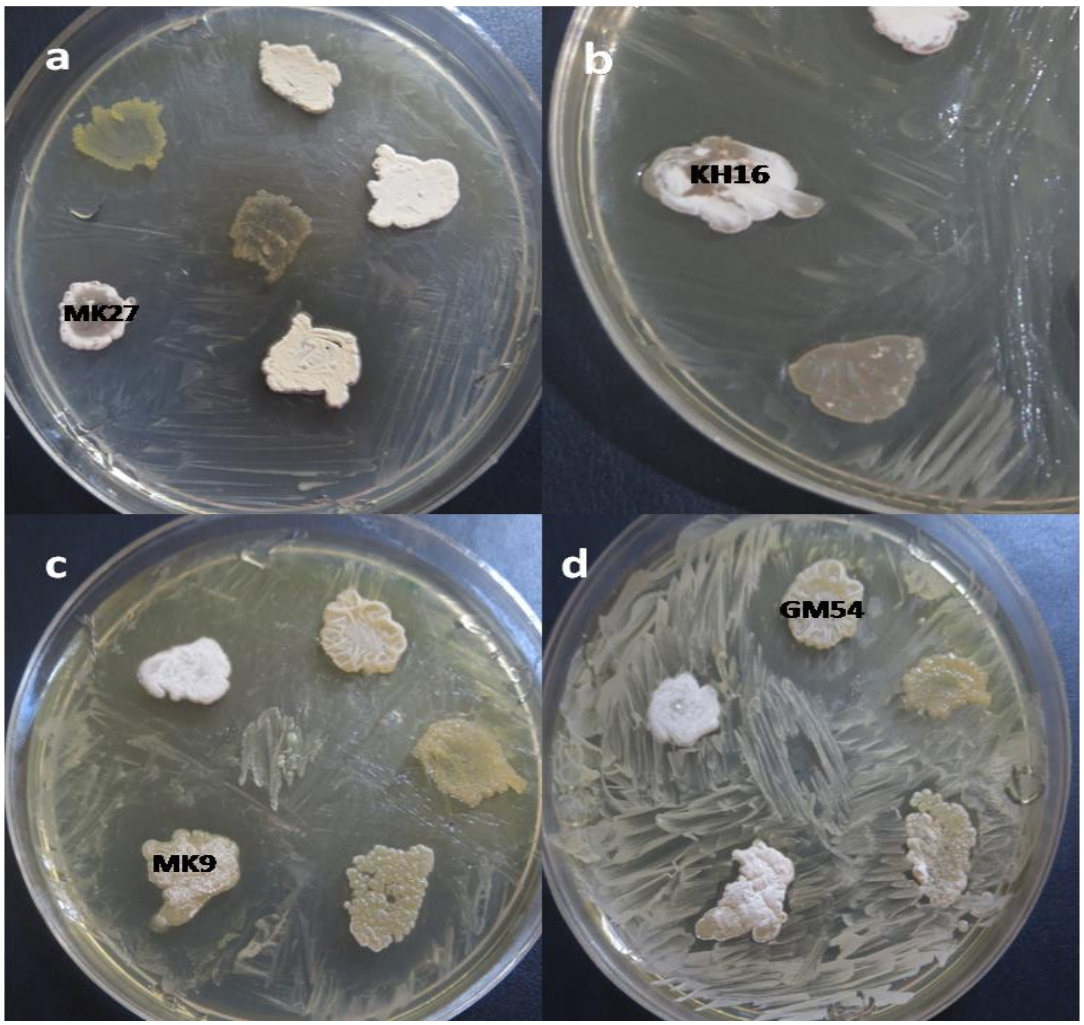

Figure 1. The isolates MK27, KH16, MK9, and GM54 exhibited good activity against a) *B. subtilis* (21 mm), b) *K. pneumoniae* (31 mm), c) *E. coli* (28 mm) and d) *S. aureus* (31 mm), respectively

Antibacterial activity resulting from liquid production media.

The antibacterial activity of four selected isolates GM54, MK9, KH16 and MK27 were evaluated finally by Well-agar Diffusion Method. The crude extracts of all the four bioactive strains, GM54, MK9, KH16 and MK27 exhibited a good activity against *S. aureus* (21 mm), *E. coli* (19 mm), *K. pneumoniae* (24 mm), and *B. subtilis* (16 mm), respectively.

Discussion

Over 6000 of antibiotics and other useful secondary industrial metabolites are produced by *Streptomyces* species. Of these compounds, antibiotics predominate in therapeutic and commercial importance (15-18).

In spite of the tremendous success of the past secondary metabolite research, the number of terrestrial antibiotics currently seems approached a saturation curve with an apparent limit in the near future. The urgent demand for new leading structures in pharmacology have enforced the research for new metabolites. Iran has diverse climates and located in one of the biodiversity hotspots of the world, but Iran's natural resources are a little screened for its microorganisms. Our

interest focused on microorganisms belongs to the Actinomycetaceae and specifically to *Streptomyces* genus: members of which have demonstrated interesting antimicrobial activity. In this work, a screening program on isolation and selection of active actinomycetes against some pathogenic bacteria is carried out.

Out of 9 farming soil samples collected in Alborz Province, 69 isolates of actinomycetes were isolated. The majority of the strains were collected from the ISP2 agar medium. This medium seems to be considerably specific and sensitive for actinomycetes since it contains glycerol that most actinomycetes use as a carbon source. These reported results were anticipated because earlier studies have shown the importance of the ingredients of the cultures under which the producing microorganisms were cultivated (19).

As it is shown in Table 2, actinomycetes mycelium's colors, melanin production, soluble pigments and reverse side colors in this work are more diverse than the other reports (20-22). Such a diversity has rarely been reported.

In the present study, 36% of the isolates produced active compounds against *S. aureus*, *B. subtilis*, *K. pneumoniae* and *E. coli*. The isolates GM54, MK9, KH16 and MK27 were found to have greater potency in inhibition of our pathogenic test strains. Assessment used for well diffusion method was confirmed primary antibacterial test for the selected actinomycetes.

As indicated in results, the isolated actinomycetes were more active against gram positive strains than the gram negative pathogenic test strains. Similar results were obtained from other antibacterial studies (23-24). Perhaps, it is because of the outer membrane of gram negative strains which does not permit bioactive metabolite penetration.

All screened isolates were identified morphologically and physiologically. Examination of the isolates clearly indicates that these belong to the genus *Streptomyces* (8, 14, 18, 25- 27).

As soils in Iran are not completely screened, we hope microbial strains that isolated from various parts of the provide us with rare and novel industrial antibiotic or metabolites, which might be more effective than the existing ones in order to cure diseases.

REFERENCES

1. Solano, G., Rojas-Jiménez, K., Jaspars, M. and Tamayo-Castillo, G. (2009) Study of the diversity of culturable actinomycetes in the North Pacific and Caribbean coasts of Costa Rica. *Antonie van Leeuwenhoek,* 96, 71–78.
2. Horan, A.C. (1999) Secondary metabolite production actinomycetes other than *Streptomyces*. In: Flickinger, M.C. and Drew, S.W. (ed.) *Encyclopedia of Bioprocess Technology: Fermentation, Biocatalysis and Bioseparation*, New York: Wiley and Sons. pp. 2333–2348.
3. Lazzarini, A., Caveletti, L., Toppo, G. and Marinelli, F. (2000) Rare genera of actinomycetes as potential producers of new antibiotics. *Antonie van Leeuwenhoek*, 78, 399–405.
4. McCarthy, A.J. and Williams, S.T. (1992) Actinomycetes as agents of biodegradation in the enviroment– a review. *Gene*, 115, 189–192.
5. Sanglier, J.J., Haag, H., Huck, J.A. and Fehr, T. (1996) Review of actinomycetes compounds 1990–1995. *Expert Opin Investig Drugs*, 5, 207–223.
6. Oskay, M, Üsame, T.A. and Azeri, C. (2004) Antibacterial activity of some actinomycetes isolated from farming soils of Turkey, *Afr. J. Biotechnol*., 3, 441-446.
7. Shirling, E.B. and Göttlieb, D. (1966) Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.*, 16, 313-340.
8. Korn-Wendisch, F. and Kutzner, H.J. The family Streptomycetaceae. The Prokaryotes. A handbook on the biology of bacteria: ecophysiology, isolation, identification, applications, Chapter 41.
9. Thakur, D., Yadav, A., Gogoi, B.K. and Bora, T.C. (2007) Isolation and screening of Streptomyces in soil of protected forest areas from the states of Assam and Tripura, India, for antimicrobial metabolites. *J. Mycol. Med.*, 17, 242-249.
10. Locci, R. (1989) Streptomycetes and related genera. In: Williams, S.T., Sharpe, M.E., Holt, J.G., (ed). *Bergey's Manual of Systematic Bacteriology*. Baltimore: Williams and Wilkins, pp. 2451-2493.
11. Madigan, M.T., Martiko, J.M. and Parker, J. (1997) *Antibiotics: Isolation and characterization.* In: Brook Biology of Microorganisms, 8th edn. Prentice-Hall International Inc. New Jersey, pp. 440-442.
12. Anderson, A.S. and Wellington, M.H.E. (2001) The taxonomy of *Streptomyces* and related genera. *Int. J. of Syst. Evol. Microbiol*., 51, 797-814.
13. Wendisch, F.K. and Kutzner, H.J. (1991) *The family Streptomycetaceae*. In: Ballows, A. (Ed.) Prokaryotes, Springer Verlag. Second edition. Vol. 1. pp. 922-995.
14. Williams, S.T., Goodfellow, M. and Alderson, G. (1989) Genus *Streptomyces* Waksman and Henrici 1943.339AL. In: Bergey' s Manual of Systematic Bacteriology. Williams & Wilkins Company, Baltimore. Vol: 4. pp. 2452-2492.
15. Lacey, J. (1973) *Actinomycetales: Characteristics and Practical Importance*. Edited by Sykes, G. and Skinner, F., The Society for Applied Bacteriology Symposium Series. Academic Press London-New York No. 2.
16. Ouhdouch, Y., Barakate, M., and Finance, C. (2001) Actinomycetes of Moroccan habitats: isolation and screening for antifungal activities. *Eur. J. Soil. Biol.*, 37, 69-74.

17. Saadoun, I. and Gharaibeh, R. (2003) The *Streptomyces* flora of Badia region of Jordan and its potential as a source of antibiotic-resistant bacteria. *J. Arid. Environ*., 53, 365-371.
18. Waksman, S.A. (1961) *The Actinomycetes, Classification, Identification and Description of Genera and Species*. Baltimore: The Williams and Wilkins Company. Vol. 2, 61-292.
19. Iwai, Y. and Omura, S. (1992) Cultural conditions for screening of new antibiotics. *J. Antibiot*., 34, 123-141.
20. Passari, A.K., Mishra, V.K., Saikia, R., GuptaV.K., and Singh. B.P. (2015) Isolation abundance and phylogenetic affiliation of endophytic actinomycetes associated with medicinal plants and screening for their invitro antimicrobial biosynthetic potential. *Frontiers in Microbiol*., 6, 273
21. Mrinalini, J.S. and Padmavathy, S. (2014) Isolation screening and characterization of endophytic PGRP actinomycetes present commonly Innerm & Tulsi leaves invitro study (Tomato). *Int. J. Rec. Sci. Research*, 5 (3), 574-579.
22. Shilpa, A.J., Soni, R., Patel, H., Prajapati B. and Patel, G. (2014) Screening, isolation and characterization of keratin degrading actinomycetes: *Streptomyces* sp. and *Saccharothrix xinjiangensi* and analyzing their significance for production of keratinolytic protease and feed grade aminoacids. *Int. J. Curr. Microbiol. App. Sci*., 3(9), 940-955.
23. Walsh, S.E., Maillard, J.Y., Russell, A.D., Catrenich, C.E., Charbonneau, D.L. and Bartolo, R.G. (2003) Activity and mechanisms of action of selected biocidal agents on Gram-positive and negative bacteria. *J. App. Microbiol*. 94 (2) 240–247.
24. Goljanian Tabrizi, S., Hamedi, J. and Mohammadipanah, F. (2014) Screening of actinomyectes against *Salmonella* serovar *Typhi* NCTC 5761 and characterization of the prominent active strains. *I. J. M.*, 5 (4) 356-365.
25. Cross, T. (1989) Growth and examination of actinomycetes, some guidelines. In *Bergey' s Manual of Systematic Bacteriol*. Williams & Wilkins Company, Baltimore, Vol. 4., pp.2340-2343.
26. Lechevalier, H.A. (1989) The Actinomycetes III, A Practical Guide to Generic Identification of Actinomycetes. *Bergey's Manual of Systematic Bacteriology*. Williams & Wilkins Company, Baltimore. Vol. 4., pp.2344-2347.
27. Goodfellow, M. (1989) Suprageneric Classification of Actinomycetes. *In Bergey's Manual of Systematic Bacteriology*. Williams & Wilkins Company, Baltimore, Vol. 4, pp. 2333-2339.