Isolation and Characterization of Aerobic Actinomycetes from Soil in Northern Iran and Evaluation of their Antimicrobial Potential

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ABSTRACT

Background: Aerobic actinomycetes can be detected in soil, worldwide. But, their diversity can differ depending on ecological and environmental factors including, temperature, humidity and vegetation, etc. The aim of this study was antimicrobial activities of aerobic actinomycetes Isolated from soil in Northern Iran.

***Methods*:** Fifty soil samples throughout Northern Iran provinces, including Guilan, Mazandran and Golestan, have been collected and cultured in selective medium, Starch Casein Agar (SCA). In the first step, isolates were assayed by pointing inoculation in solid medium, agar spot, for antimicrobial activity. Then, for antibiotic production, International Streptomyces Projects 2 (ISP2) and Glucose Yeast Extract Malt extract (GYM) media by submerge technique were used. Well diffusion agar method was used for detection of antimicrobial activity and antibiotic sensitivity, and finally metabolites of most active specious detected by GC/MS and GC techniques.

***Results*:** In this study eighty strains were isolated from soil samples. In primary screening, 12 strains (15%) recognized as active actinomycetes, among them strain SA3 showed the highest antimicrobial potential. In the secondary screening in the liquid ISP2 medium, 3 (25%) isolates (SA7, SA3, SA16) and in GYM medium 7 (58.33%) isolates (SA28, SA27, SA7, SA26, SA16, SA2, SA3) have shown the highest antimicrobial potentials; also it was found that there is a significant relation between humidity and pH of soil with the number of isolated colonies. According to results of primary and secondary screening, strains SA3 and SA7 were selected as active actinomycetes and biochemical test revealed that these two active strains isolates belong to the genus Streptomyces. Finally, produced metabolites by strain SA3 were analyzed by GC/MS and GC methods and Oleic acid was revealed as the highest peak.

***Conclusion*:** The findings of the present research show that actinomycetes from Northern Iran soils have considerable antimicrobial activities against different microbial pathogens.

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Introduction

Actinomycetes are gram-positive and filamentous bacteria, which have free-living, saprophytic and occasionally plants colonizing forms. These bacteria can be isolated from all ecosystems, including soil, water, marine sediments and hot waters (hot springs). The most important roles of actinomycetes in soil can be decomposition of organic materials, causing plant disease, microbial balance and antibiotic production. Actinomycetes have a high capacity to synthesize secondary metabolites such as antibiotics, enzymes, herbicides, anticancer drugs and other useful compounds; they produce more than 75% of antibiotics and different antimicrobial compounds. These compounds have known as active new sources (1, 2). Actinomycetes frequency depends on physical characteristic, amount of organic matters and pH of the environment; also the number of them in grasslands and prairie is more than infields (agricultural lands). Actinomycetes may not be drought resistance but their spores can be drought resistant. Totally, actinomycetes are the main source of clinically important antibiotics, are applicable in human and animal treatment, in industry and agriculture (3, 4). Ecology of soil actinomycetes has been surveyed in different countries such as India, Turkey, Egypt, Malaysia, Spain, Nigeria, Kuwait and Nepal (5, 6, 7, 8, 9, 10, 11, 17). Different studies have been done in Iran (12, 13, 14). The aim of this study is isolation and characterization of aerobic actinomycetes from Northern Iran soils and determination of their antimicrobial activity.

Materials and Methods

Sampling

Fifty soil samples (300 gr), including agricultural, forest and coastal soils from the Northern provinces of Iran (Guilan, Mazandaran and Golestan) were collected (summer, autumn and winter 2012-2013) in Sterile containers and were transported to the laboratory in the Up to 4 hours; then humidity and pH of soil samples were recorded.

Isolation and purification of Actinomycetes

One milligram of soil was dissolved in 100 milliliters of distilled water and then was cultured in the Starch Casein Agar (SCA) in 10-1 – 10-4 dilutions. For prevention of the growth of bacterial and fungal contaminants, 25 mg/lit Kanamycin and 500 mg/lit Cycloheximide were added to the medium and incubated in 28°C for 10 days. The dry and powdery Colonies of actinomycete were isolated from the agar surface. Primary identification of actinomycetes performed based on morphological characteristics and then cultured in SCA medium and preserved at 4°C temperature.

Providing and preparation of pathogenic factors

Five well-known pathogens including, *Bacillus subtilis* PTCC1365, *Klebsiella pneumonia* PTCC1402, *Staphylococcus aureus* PTCC111, *Salmonella typhi* PTCC 1609 were provided by the Persian Type Culture Collection, Tehran, Iran and were used in nutrient broth media with McFarland 0.5 turbidity standards.

Screening and survey of antimicrobial activity

For primary screening of actinomycetes with antimicrobial potential, the Agar spot method was used. In this method, the plates were incubated at 28°C for 6 days and then plates reversely placed in the proximity of chloroform for 40 min. The antibacterial activity was tested on TSA medium and antifungal activity was tested on potato dextrose agar (PDA) medium. Bacterial plates were incubated at 37°C for 24 hours and fungal plates were incubated 28°C for 5 days, then Inhibition zones measured, and the most active strains of actinomycete selected for the next stage. The actinomycetes that were active in the first

detection and have antimicrobial potential, were inoculated in ISP2 (International *Streptomyces* Projects) and GYM (Glucose Yeast Malt extract) media. These two media were used for biomass production as submerge cultures. Inoculated media were placed in a shaker incubator at 150 rpm and 28°C for 7 days; after that samples centrifuged at 4000 rpm for 10 min and the supernatant were assayed for in vitro antimicrobial activity with Standard Well Diffusion method.

GC and GC/MS

Metabolites of most active strain were identified by Gas chromatography and Gas chromatography–mass spectrometry methods. Different picks obtained from GC that was used for identification and determination; by GC-MS different metabolites, that produced by active isolate, were separated and identified. In the GC-MS method, the length of the tube was 30 mm and detector type was MS, also helium gas was used as mobile phase.

Results

From fifty soil samples (19 forest soil samples, 17 agricultural samples and 14 coastal samples) collected from 22 areas from the Northern provinces of Iran. Collectively, 80 actinomycete colonies were purified. Most of the actinomycetes were isolated from soils with 15% humidity and pH values around 6 – 7.5., there was a detectable association between the humidity and the pH of soil samples and the number of isolated colonies. In the primary screening procedure 12 strains (15%) were determined as actively antimicrobial producing actinomycetes among which. Five isolates were collected from agricultural soil, 5 isolates from forest soil and 2 cases from coastal soils. Also, 5 isolates (41.66 %) showed antifungal activity and all of the active isolates, in this stage, manifested antibacterial activity, including 9 isolates against gram positive bacteria, and 8 isolates (66.66%) against gram negative bacteria (Table 1).

When ISP2 medium was used for production of antimicrobial compounds, from 12 active isolates in primary screening, 3 (25%) isolates (SF16, SA3, SA7) in secondary screening has shown antimicrobial activity; all of these 3 isolates showed both antifungal and antibacterial activity. The two isolates (66.66%) showed antibacterial activity against gram positive bacteria and one isolate (33.33%) against gram negative bacteria. According the results, 33.33% of isolates were effective against *Staphylococcus aureus*, 66.66% against bacillus subtilis and 33.33% against S*almonella typhi*, while none of them was effective against *Klebsiella pneumoniae*. Among this isolates, SA3 has the best antimicrobial activity.

In GYM medium from this 7 isolates (SA28 ،SA27، SA7، SA26، SF16، SF2، SA3) 3 of them (42.85%) had antifungal activity and 100% of isolates showed antibacterial activity. From these isolates, 42.85% were effective against *Staphylococcus aureus*, 57.14% against *Bacillus subtilis*, 28.57% against *Klebsiella pneumoniae* and 42.85% against B*acillus subtilis* (Table 2).

According to results of primary and secondary screenings, isolates SA3 and SA7 were selected as active. According to Bergey's Manual and using biochemical tests (fermentation of sugars, ureas test, nitrate reduction, casein and starch hydrolysis test, methyl red and Voges-Proskauer test) Morphological tests (gram stain, colony shape, Acid-fast stain, secondary mycelium stain and spore characters) and physiological tests (salt tolerance and antibiotic resistance tests), isolates were identified at the genus level and according to these results, isolates SA3 and SA7 belong to the genus *Streptomyces* (Table3).

Finally, produced metabolites by SA3 active strain were analyzed using GC, GC/MS. In the compound analysis by GC, 4 pikes were manifested with retention time at 21/21, 21.31, 23.06 23.25 (Figure 1).

Based on the, GC/MS analyses the molecular weights of the metabolites was as 502, 298, 282 and 444 gr/mol, respectively. After that, using the available data bank, the metabolites were identified. The metabolites according to the above

mentioned molecular weight were characterized as Phthalic acid (benzene-1, 2-dicarboxylic acid), Isopropyl palmitate (Propan-2-yl hexadecanoate) and Oleic acid (cis-9-Octadecenoic acid), respectively. Among these metabolites the highest pick belonged to Oleic acid and the lowest belonged to Phthalic acid (Figure 2).

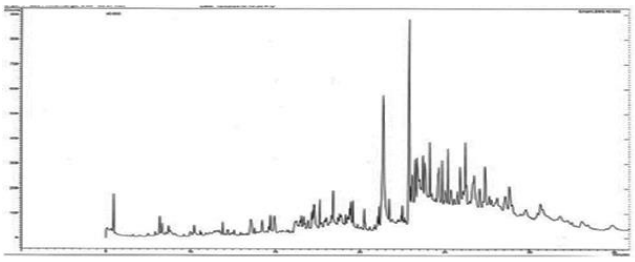

Figure 1. GC/MS chromatogram of the extracted culture. .

Discussion

Antibiotic resistance due to the widespread use of antibiotics is one of the major causes of failure in antibiotic treatment. Therefore, secondary metabolites of microorganisms for discovery of new antibiotics has been an interesting ground for applicable research (15). In this study, most of the actinomycetes (19 forest soil sample, 17 agricultural samples and 14 coastal samples) were isolated from soil samples with pH of 6-7.5 and 15% humidity. The low number of isolated actinomycete from coastal area may be due to more humidity in these soils; also coastal soils in contrast to agricultural and forest soils was less alkaline. During this survey, ISP2 and GYM media were used for antibiotic production. SA3 active strain in Agar spot method against all microbial agents, showed antimicrobial activity; but in well diffusion agar method only *Staphylococcus aureus*, *Bacillus subtilis* and *Candida albicans* had antimicrobial activity. Also, using GYM antibacterial activity was observed only against gram negative bacteria, *Klebsiella pneumoniae* and *Salmonella typhi.* In as much as carbon source is the only difference between these two media, therefore it can be inferred that the difference in carbon source can affect the type and amount of different metabolites produced by active isolates of actinomycetes. Some of the active isolates in primary screening, showed no activity in the secondary screening, while some showed less and some showed more activity. In this study, using Agar spot methods as primary screening 12 isolates showed antimicrobial activity, but in well diffusion agar method only 7 active isolates were identified and this is because of the types of media used in these two screening methods for antibiotic production. Also, sensitivity and accuracy of Agar spot are much less than well diffusion agar method. In the primary screening, biologically inactive metabolites and poisonous compounds produced that cause elimination of microbes, but in well diffusion agar method we centrifuged the produced metabolites and then inject them into the wells that may some of the compounds that used in Agar spot method, during centrifuge, precipitated and were not used in diffusion method. Also, in well diffusion method, extraction of the produced metabolites of active isolates was done by ethyl acetate that maybe many of biologically inactive metabolites that showed antimicrobial activity in primary screening, cannot be extracted with the used method, therefore at this stage antimicrobial activity may be reduced.

In a study in India soils, using the Agar spot method all of the actinomycete colonies, in this screening stage, had antimicrobial activity, 59.09% of isolates had antibacterial activity, 42.72% of isolates had antifungal activity and 30% of isolates showed both activity (5). While in our study from 80 isolated colonies, 12 (15%) isolates in primary screening by Agar spot method showed antimicrobial activity, from this number, 100% of isolates showed antibacterial activity, 41.66% showed antifungal activity and also 41.66% of isolates showed both antibacterial and antifungal activity.

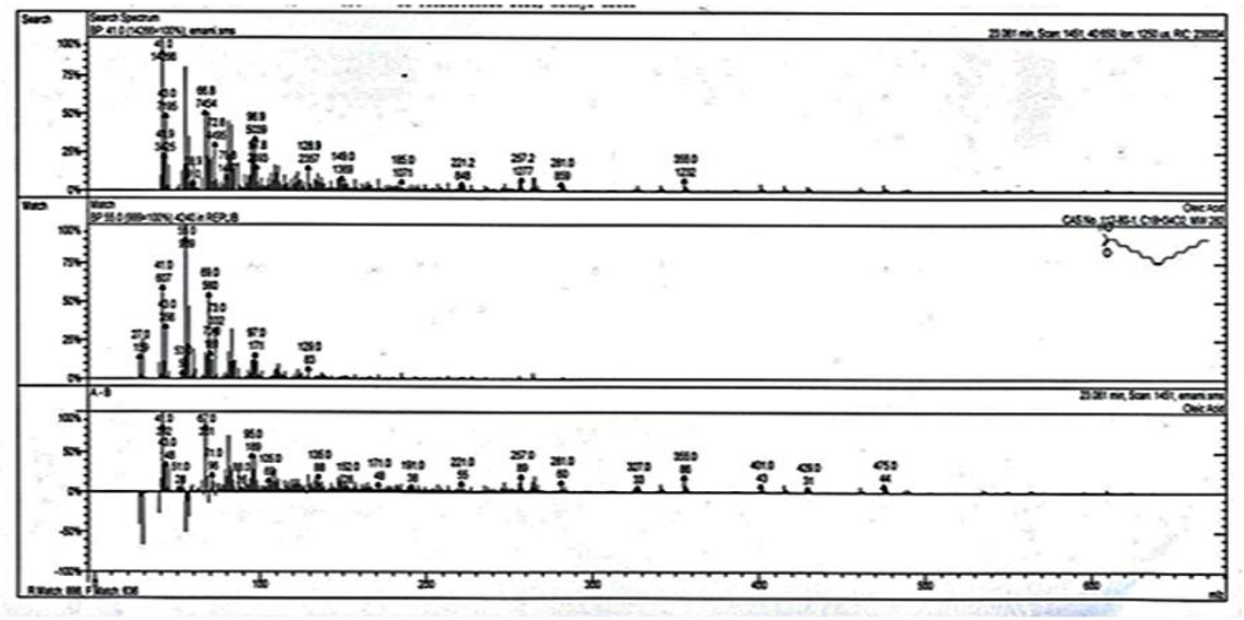

Figure 2. Oleic acid has been identified with GC/MS.

Table 1. Initial screening of actinomycetes using spot inoculation method.

Halo size	Minimum inhibitory concentration using spot inoculation method (mm)				
Standard strain / Isolates	1	2	3	4	5
SF1	-	-	-	24	-
SA3	33	35	28	24	15
SF13	-	-	-	34	-
SF2	-	15	24	27	-
SF16	14	-	-	15	17
SF18	-	-	-	14	12
SA7	-	15	13	17	-
SC1	-	17	13	13	-
SC8	21	-	-	-	-
SA26	10	-	-	-	-
SA27	13	12	-	-	30
SA28	-	19	-	-	28
Frequency	%41.66	%50	%33.33	%66.66	%33.33

1) *Staphylococcus aureus* PTCC 111; 2) *Bacillus subtilis* PTCC 1365; 3) *Salmonella typhi* PTCC 1609; 4) *Klebsiella pneumoniae* PTCC1402; 5) *Candida albicans* PTCC 1215.

Table 2. Antibacterial activities of actives isolates using well diffusion method.

Halo size	Minimum inhibitory concentration against standard strains using well diffusion method (mm)									
Production medium	The antibiotics produced in submerged culture(ISP2)					The antibiotics produced in submerged culture(GYM)				
Microbial strains / **Isolates**	1	2	3	4	5	1	2	3	4	5
SA3	30	29	-	-	10	-	-	13	12	-
SF2	-	-	-	-	-	-	-	15	12	12
SF16	-	11	-	-	11	-	11	-	12	-
SA7	-	-	-	11	10	15	14	-	-	-
SA26	-	-	-	-	-	10	-	-	-	-
SA27	-	-	-	-	-	12	11	-	-	29
SA28	-	-	-	-	-	-	10	-	-	25
Frequency	%33.33	%66.66	%0	%33.33	%100	%42.85	%57.14	%28.57	%42.85	%42.85

1) *Staphylococcus aureus* PTCC 111; 2) *Bacillus subtilis* PTCC 1365; 3) *Salmonella typhi* PTCC 1609; 4) *Klebsiella pneumoniae* PTCC1402; 5) *Candida albicans* PTCC 1215.

Table 3. Morphology, biochemical and physiological characteristics of tow active selected isolates.

Active Isolates / Characteristics	SF17	SF25
Primary mycelium color on CAS	Opalescent colonies	Weak yellow
Secondary mycelium color on CAS	white	Cream
Spore chain morphology	RF	S
Acid-fast Staining	_	_
Fermentation of Glucose	++	++
Fermentation of L-Arabinose	++	++
Fermentation of D-Mannitol	_	_
Fermentation of Galactose	++	_
Fermentation of D-Fructose	_	_
Fermentation of sucrose	_	_

Fermentation of Maltose	_	_
Nitrate Reduction	_	_
Urease	++	_
Casein hydrolysis test	++	++
Starch hydrolysis test	++	++
TSI	Alk/alk	Alk/alk
SIM	_	_
Methyl Red	_	_
Voges Proskauer Test	_	_
Utilisitation of Citrate	++	++
Growth with NaCI %3	++	++
Growth with NaCI %7	+	_
Growth with NaCI %10	_	_
Resistance to Penicillin	+	+
Resistance to Ampicillin	+	+

In another study in India, on costal soils isolated actinomycetes, from 64 isolated colonies with actinomycete morphological characters, 21 (32.8%) of isolates had antimicrobial activity that 12 (18.8%) showed antibacterial and 13 (20.3%) of isolates antifungal activity and 9 (14.1%) showed both activity (16). In most of such researches, antibacterial activity against gram positive bacteria has been reported to be more than gram negative bacteria. In a study concerning the antibacterial activity of actinomycetes in Nepal, manifested that 36 (33.96%) of isolates were active in secondary screening (well diffusion method) which 2 (5.55%) isolates were effective against gram negative bacteria and 8 (22.22%) isolates against gram positive bacteria (6). Also, in study accomplished in Turkey by well diffusion method. From 50 isolated actinomycetes, 17 (34%) isolate had antibacterial activity, 8 (16%) isolate against gram positive bacteria and 3 (6%) isolates against gram negative bacteria (9). In our study using well diffusion method and ISP2 medium, 33.33% of isolates had acted against either gram positive and gram negative bacteria while using GYM medium, 71.42% of isolates against gram positive bacteria and 42.85% of isolates against gram negative bacteria showed activity. This difference in sensitivity to different metabolites by gram positive and gram negative bacteria, could be associated to the phenotypic differences between these microorganisms (9). In our study produced metabolite by active *Streptomyces* strain SA3 strain, were identified by GC and GC/MS techniques. Produced metabolites by this strain are oleic acid, Phetaleic acid, Isopropil palmitate and 9-octadecenoic acid; Oleic acid had the highest

pick, therefore it can be said that Oleic acid has the most important role in antimicrobial activity of this strain. In a survey by Davidson et al., Oleic acid showed activity against some gram positive bacteria and *Candida albicans*. This activity can be because of fatty acid uptake and disturbing of a proton gradient across a membrane and effect on ATPase activity and cause antimicrobial activity (18). In a study conducted in Korea on antimicrobial activity of fatty acid, especially Oleic acid, it has been manifested that Oleic acid can act as GTPase inhibitor and do antimicrobial activity (19). Also in an another study antimicrobial activity of oleic acid and other fatty acids revealed that the antimicrobial activity of oleic acid could be associated to the cellular membrane properties and free radical production. Also, showed that antimicrobial activity of unsaturated fatty acids is more than saturated fatty acids (20).

Conclusion

In conclusion, most isolates of actinomycetes sp. showed good antimicrobial activity against different microbial pathogens and could be used in the development of new antibiotics for pharmaceutical or agricultural purposes. GC/MS results showed Oleic acid have the highest peak.

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Conflict of interest

None declared conflicts of interest.

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