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Full Length Research Paper

Investigation antibacterial activity of *Streptomycetes* isolates from soil samples, West of Iran

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In this research, our goal is to determine *Streptomyces* species with antimicrobial activity from some regions of Northwest of Iran. The future studies will be performed to investigate the type of antimicrobial agents. In order to achieve to this aim, soil sample collected were diluted and cultured in SCA medium. The *Actinomycetes* were isolated considering morphological characteristics in macroscopic and microscopic levels and examined for the microbial activity. The antimicrobial positive bacteria were selected for further biochemical and molecular studies. Through the molecular studies, 16srDNA gene of the each bacterium was amplified and digested using TaqI endonuclease. The handling of RFLP pattern of 16srDNAs was done using genedoc bioinformatics software to determine the strains of the bacteria. 150 isolated *Actinomycete* colonies, 12 different strains showed antimicrobial activity in which 11 strains belonged to the *Streptomycetes* and were known as different strains of the *Streptomyces* genus, we identified 11 *Streptomyces* strains from the mentioned regions of the northwest of Iran with high antimicrobial activity. To determine the antimicrobial agents further studies are needed. In this research, we tried to study the ability of the production of anti-microbial agents by *Streptomyces* species from some regions of Northwest of Iran. Our results led to the 12 isolates with the different ability to produce antibiotics.

Key words: *Actinomycetes*, antibiotics, antimicrobial activity, Iran, *Streptomycetes.*

INTRODUCTION

The *Actinomycetes* are gram positive bacteria having high G+C (> 55%) content in their DNA. The majority of *Actinomycetes* are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. *Actinomycetes* population has been identified as one of the major group of soil population which may vary with the soil type (Agrawal, 2002; Lacey, 1973). *Streptomycetes* are the most well known genus of *Actinomycetes* family which has been notified because of their ability to produce and secrete a large variety of industrial, medical, biotechnological and agricultural secondary metabolites (Peczynska et al., 1988).

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Streptomyces is the largest antibiotic genus, producing both antibacterial and antifungal and also a wide range of other compounds such as immunosuppressant (Okami et al., 1978). They produce over two thirds of the clinically useful antibiotics of natural origin (e.g. Neomycin and chloramphenicol). Nevertheless, periodic replace of the existing antibiotics is necessary to prevent transmissible resistant among microorganisms to the available anti-biotics that are already in the market. Some new screening programs have been already developed for discovering of new species or unknown bioactive substances. One of the modern approaches is isolation and screening of microorganisms from relatively unknown or unstudied areas. Basilio and colleagues (2002) studied patterns of antimicrobial activities from soil *Actinomycetes*. Particular protocol for isolation of anti-biotic-producing organisms from soils was developed by Ensign and Emeritus (2002). The protocol included

3 parts such as (i) determination of the number of colony forming units (CFU's) of the genus *Streptomyces* per gram of soil (ii) identification and pure culture of *Streptomyces* and (iii) assays. In this study, the isolation and characterization, as well as the anti-microbial activity of local *Streptomyces* isolates from northwest of Iran, were studied. The main aim of the study was identification of the native *Streptomyces* species with antimicrobial activity. The future studies will be performed to investigate the type of antimicrobial agents.

MATERIALS AND METHODS

Bacterial strain and growth condition

The soil samples (400 g) were collected from various locations northwest of Iran. Several habitats in different areas were selected for the isolation of *Streptomyces* strains. These habitats included the rhizosphere of plants, agricultural soil, preserved areas and forest soils. The samples were taken unto a depth of 15 cm from the soil surface. The samples were placed in polyethylene bags to avoid external contamination and kept in 4°C. *Actinomycetes* from the soil were isolated by spreeding technique on starch-casein agar plates after serial dilutions in distilled water. Based on macroscopic and microscopic observations, desired colonies were selected and isolated. Thus isolated colonies were preserved in glycerol based media and stored at - 70°C (Ozgur et al., 2008).

Test microorganisms

Staphylococcus aureus, *Proteus vulgaris*, *Escherichia coli*, *Salmonella* spp., *Proteus* spp. and *Pseudomonas* spp. were used to determine the anti-microbial activity of isolated *Streptomyces* strains.

Screening of *Actinomycetes* for antimicrobial activity

The screening method consisted of steps as primary screening and secondary screening. In primary screening the antimicrobial activity of pure isolates were determined by perpendicular streak method on nutrient agar (NA). The test organisms were *S. aureus*, E. *coli*, *Salmonella* spp., *Proteus* spp., *Pseudomonas* spp.

Secondary screening was done through the agar well method against the standard test organisms *E. coli*, *S. aureus*, *P. vulgaris* (Dhanasekaran et al., 2005; Lorain, 1991).

Primary screening of the antimicrobial activity

The primary antimicrobial activity was done by perpendicular streak method. In this method bacterial colonies were streaked on center of nutrient agar plates as a linear culture and incubated at 28°C for 7 days. After 7 days, the test microorganisms were inoculated perpendicularly to the linear cultures and incubated at 37°C for 48 h. The antimicrobial producer isolates inhibited the growth of test microorganisms and were selected for further experiments (Dhanasekaran et al., 2005).

Isolation of antibacterial metabolites

The selected isolates were culture in starch casein broth and incubated at 28°C for 7 days. After 7 days bacterial cultures were filtrated using 0.2 µm filter. Antibacterial compounds were recovered from the filtrate by solvent extraction with ethyl acetate. Ethyl acetate was added to the filtrate in the ratio 1:1 (v/v) and shacked for 1 h for complete extraction. The ethyl acetate phase that contains an antibiotic agent was separated from the aqueous phase. It was evaporated to dryness in a water bath at 80 - 90°C (Athlete et al., 1981).

The obtained compound was used to determine the anti-microbial activity. 100 µl of obtained compound solution was placed onto Muller Hinton agar plates that previously seeded with different pathogen bacteria. The plates were incubated at 37°C for 48 h and examined for zones of inhibition (Pandey et al., 2002).

Characterization of the isolates

The selected *Actinomycetes* via antibacterial tests were characterized through morphological and biochemical tests. Morphological methods consisted of macroscopic and microscopic methods. The mycelium structure, color and arrangement of spores on the mycelium and other properties such as the color of colonies, soil pH and etc. were observed. The observed structures were compared with Bergey`s manual of determinative bacteriology, Ninth edition (2000) and the organisms were identified. Moreover several biochemical tests such as casein hydrolysis, starch hydrolysis and urea hydrolysis, acid production from various sugars, NaCl resistance and Temperature tolerance were done.

Molecular assays

In order to identify the strains of the isolated *Actinomycetes* and isolation of *Streptomyces* species among them, several molecular and bioinformatical assays were performed.

Genomic DNA extraction

Genomic DNA extraction was conducted according to the protocol described by Corbin method with some modifications. Briefly, a single colony was cultured in 50 ml liquid ISP2 medium for 18 - 24 h in shaker incubator at 26°C. Then the culture was centrifuged for 3 min at 5000 rpm and supernatant was discarded. The bacterial cells were pulverized in liquid nitrogen, suspended in a solution I containing 10 mM Tris (pH: 7.4), 1 mM EDTA, 0.5% SDS and 0.1 mg/ml of proteinase K, and lysed by incubation at 37°C for 1 h, then the solution II containing 0.8 M NaCl and 1% CTAB was added to the lysates, and incubated at 65°C for 20 min and extracted with equal volume of chloroform isoamylalcohol (24:1). Nucleic acid was precipitated from the aqueous phase with 0.6 volume of isopropanol and finally purified using ethanol 70% (Corbin et al., 2001).

Amplification of 16srDNA of bacteria

To amplify the fragment of 16srDNA gene 2 primers called StF:5´-AAGCCCTGGAAACGGGGT-3´ and StR:5´-CGTGTGCAGCCCAAGACA-3´ as forward and reverse primers were designed. PCR amplification was performed in the presence of 2 primers and genomic DNA of bacterial cells. The PCR reaction mixtures (50 µl) contained 50 pmol each of StF/StR primers, 4 dNTPs at 0.2 mM each, 2.0 mM $MgCl_2$, 0.5 ng/µl bacterial genomic DNA as the template DNA and 1.5 U Taq DNA polymerase (Cinagen, Iran). The PCR amplification was achieved with 94°C for 5 min as primary denaturing temperature, then 94ºC for 1 min as denaturing temperature, 57ºC for 60 s as annealing temperature, 72°C for 105 s as extension time, in 35 cycles, and 72ºC for 10 min as final extension time. The PCR products were analyzed using

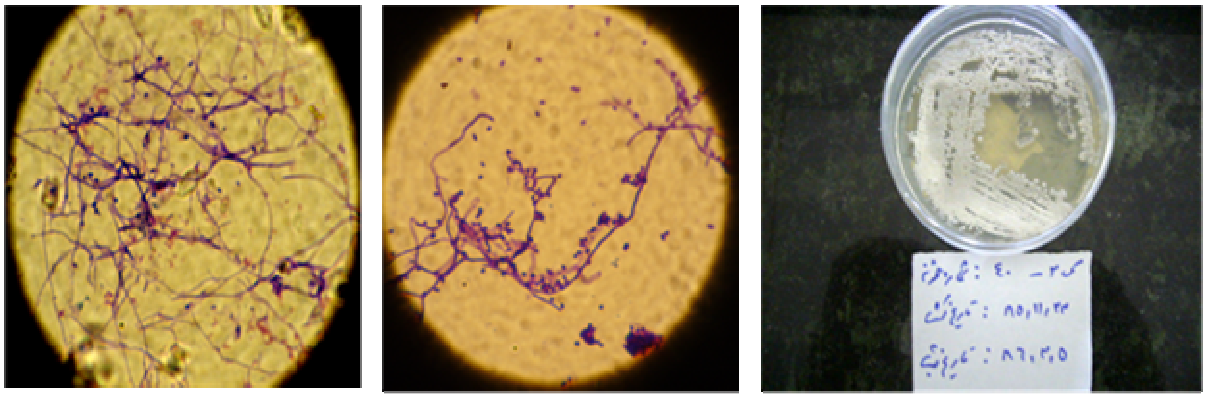


Figure 1. The samples colony, mycelium shapes were observed through the microscopic and macroscopic studies.

Table 1. Table that indicated soil and positive strains colony properties.

Soil pH	Strain	P. Surface	P. behind	Sampling temperature (°C)	Soil dilution	Colony count
67r[3]	8.73	Albus	Cinneacoloris	4	10^{-2}	10
67r[1]	8.73	Albus	Flavus	4	10^{-2}	7
70r[3]	8.98	Cinneacoloris	Cinneacoloris	4	10^{-2}	5
39r[3]	8.36	Albidus	Cinneacoloris	4	10^{-2}	6
107r[1]	8.26	Cinneacoloris	Cinneacoloris	4	10^{-2}	8
108r[1]	8.10	Cinneacoloris	Cinneacoloris	4	10^{-2}	9
32r[4]	8.50	Albidus	Cinneacoloris	4	10^{-2}	10
31r[1]	8.39	Albus	Fradia	4	10^{-2}	4
72r[3]	8.21	Cinneacoloris	Albidus	4	10^{-2}	5
94r[2]	8.42	Albus	Albidus	4	10^{-2}	8
Gr3.[2]	8.2	Albus	Flavus	4	10^{-2}	15
Aj5.[1]	8.49	Albidus	Albidus	4	10^{-2}	8

electrophoresis on 1% agarose gel and marked using 1 kb DNA ladder (Fermentas Co.) as the size marker.

Restriction pattern analysis (RFLP)

In order to compare the bioinformatics analysis of band patterns of amplified 16srDNA gene, the amplified fragments were digested with TaqI endonuclease.

The band patterns of digestion were comprised with RFLP pattern of 16srDNA gene with TaqI enzyme from *Streptomyces* species. The 16srDNA gene sequences were obtained from National Center for Biotechnology Information (NCBI) using Genedoc software.

RESULTS

Colony isolation

Following dilution and culture of 100 soil samples, about 150 *Actinomycetes* colonies were selected. This selection was done based on morphological characterization of bacterial colonies. The gram stain of selected bacterial cells showed a filamentous structure of *Actinomycetes*. The results of morphological observations were given in Figure 1 and Table 1.

Antimicrobial activity assays

Out of 150 actinomycetes subjected for primary screening process, only 20 isolates showed the activity against test organisms. From the 20 isolates, 8 isolates were active against only gram negative organism, 8 isolates against gram positive organisms and 4 isolates against both gram positive and gram negative organisms. Also among these 20 isolates, 12 of the isolates were active against *E. coli*, 17 against *S. aureus*, 11 against *Salmonella typhi* and 14 against *Proteus* spp.

Out of the 20 isolates that were subjected for the secondary screening, only 12 isolate have activity with an inhibition zone at ≥ 10 mm which 5 isolates were active against both *S. aureus* and *E. coli*, 3 isolates were active

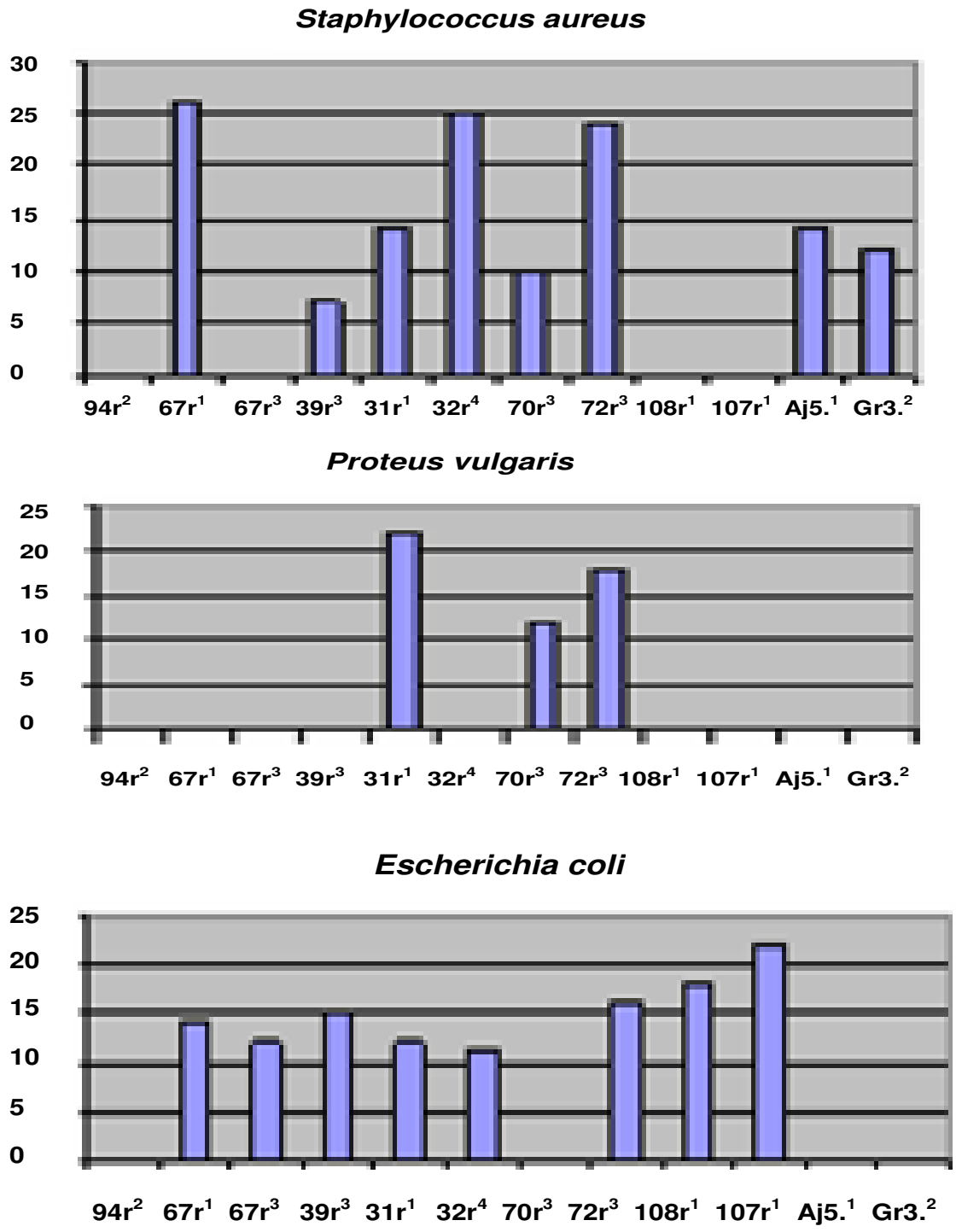


Figure 2. The activity rates of the isolated bacteria to test pathogens in secondary screening.

against *P. vulgaris* (Figure 2 and Table 2)

Biochemical experiments

A great variety biochemical features of 12 strains which coded as 67R1-67R3-39R3-31RR-70R3-108R1-107R1-5/1AJ-GR3/2-72R3-94R2-32R4 were studied. The results of this section have been shown in Table 3. The strains showed similarity in their ability to assimilate 11 carbon sources. Some differences occurred in assimilation of mannitol, sucrose and inositol. Degradation of 4 sub-strates by the strains was tested. They differed in ability to decompose casein, starch and gelatin. Acid formation

Table 2. High activity of samples to test bacteria.

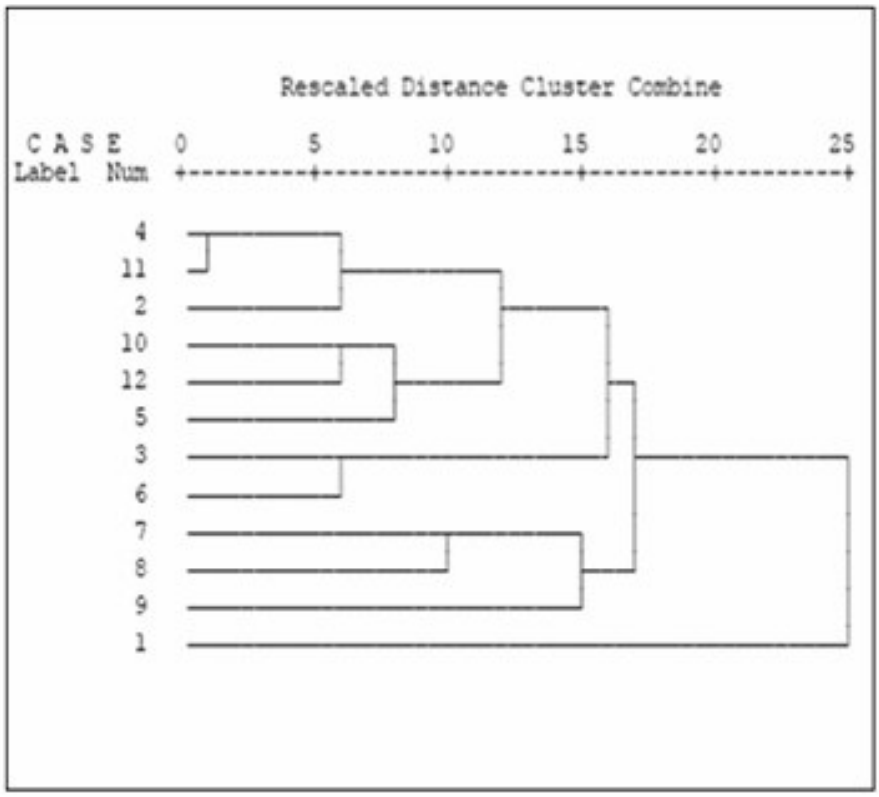
Number	Bacterial code	*E. coli* (mm)	*S. aureus* (mm)	*P. Vulgaris* (mm)
1	94r[2]	0	0 mm	0 mm
2	67r[1]	14	26 mm	0 mm
3	67r[3]	12	0 mm	0 mm
4	39r[3]	15	7 mm	0 mm
5	31r[1]	12	14 mm	22 mm
6	32r[4]	12	25 mm	0 mm
7	70r[3]	0	10 mm	12 mm
8	72r[3]	16	24 mm	18 mm
9	108r[1]	18	0 mm	0 mm
10	107r[1]	22	0 mm	0 mm
11	Aj5.1	0	14 mm	0 mm
12	Gr3.2	0	12 mm	0 mm

Table 3. Biochemical characteristics of isolated bacterial strains.

Strain code	Starch de compounds	Catalase	Fructose	Galactose	Raffinose	Rhamnose	Casein	Oxidase	45°C
94r[2]	+	+	+	-/+	+	+	+	--	+
67r[1]	+	+	+	+	+	+	+	--	--
67r[3]	+	+	+	+	+	+	+	--	--
39r[3]	+	+	+	+	+	+	+	--	--
31r[1]	+	+	+	+	+	+	+	--	--
32r[4]	+	+	+	+	+	+	+	--	--
70r[3]	+	+	+	+	+	+	+	--	--
108r[1]	+	+	+	+	+	+	+	--	+
107r[1]	+	+	+	+	+	+	+	--	--
Aj5.1	+	+	+	-/+	+	+	+	--	--
Gr3.2	+	+	+	+	+	+	+	--	--
72r[3]	+	+	+	+	+	+	+	--	--

Strain code	Inositol	Sucrose	Xylose	Manitol	Glucose	Arabinose	NaCl 1.5%	NaCl 13%	NaCl 5%
94r[2]	+	+	+	+	+	+	--	--	--
67r[1]	+	+	+	+	+	-/+	+	+	--
67r[3]	+	+	+	-	+	-/+	+	+	+
39r[3]	-/+	--	+	+	+	+	+	+	--
31r[1]	--	--	-/+	--	+	+	+	+	+
32r[4]	+	+	+	--	+	+	+	+	--
70r[3]	+	+	+	+	+	+	+	--	+
108r[1]	+	+	+	+	+	+	+	--	+
107r[1]	--	+	+	+	+	+	+	+	--
Aj5.[1]	+	--	+	+	+	+	+	+	+
Gr3.2	+	--	+	+	+	+	+	+	--
72r[3]	+	--	+	--	+	+	+	+	+

from 11 different sugars (mannitol, sucrose, glucose, xylose, ramnose, galactose, arabinose, raffinose,) was studied. The strains showed different ability to produce acid. The growth of *Streptomycetes* at 45°C and 3 different concentrations of NaCl (1/5, 3 and 5%) in the media were thoroughly studied. One of strains (94r[2])



1	94r2
2	67r1
3	67r3
4	39r3
5	31rr
6	32r4
7	70r3
8	108r1
9	107r1
10	5.1aj
11	gr3.2
12	72r3

Figure 3. Dendrogram that indicated grouping of samples.

grew at 45°C and all of strains grew at 1/5% NaCl which most of them were tolerant to 3% NaCl in the media, only 5 strains ($94r^{2}$-$39r^{3}$-31rr-$32r^{4}$-$107r^{1}$-$gr^{3/2}$) didn't grow at 5% NaCl.

Classification of the bacteria

In order to classify the bacteria based on their biochemical traits, the strains were subjected on jacard cluster analysis. The result of clustering has been shown as dendrogram in Figure 3. Three clusters were formed at 30% similarity. The first cluster included 8 strains (4, 11, 2, 10, 12, 5, 3, 6) the second cluster included 3 strains (7, 8 and 9) and the third cluster included only one strain (1).

On the basis of determined characteristics of the strains it could be concluded that they are close to the genera *Streptomyces* and *Nokardia* with different species.

Molecular assays

PCR reactions resulted in amplification of a desired fragment about 1500 bp (Figure 4). This result referred that the isolated bacteria probably are some species of *Streptomyces* genus.

The digestion reaction showed high polymorphisms between the bacteria and RFLP pattern comparison results displayed that the isolated bacteria are *Streptomyces* species (Figure 5). The RFLP patterns of the bacteria were completely correspond to the RFLP patterns of submitted 16srDNA sequences from *Streptomyces* species such as *anthocyanicus, armeniacus, ataratus, bobil, erythrogriseus, gobitricini and violaceoruber* in NCBI. These species were announced previously as results of biochemical assays in this experiment.

So the results of molecular study confirmed the results of morphological and biochemical studies in this investigation.

Conclusion

The gram positive, filamentous *Streptomycetes* are the most studied and well known group of *Actinomycetes* which have a great ability to produce most important secondary metabolites such as antibiotics, anti tumors, anti viral, anti fungal and etc. since some of the most important antibiotics that used in medicine obtain from

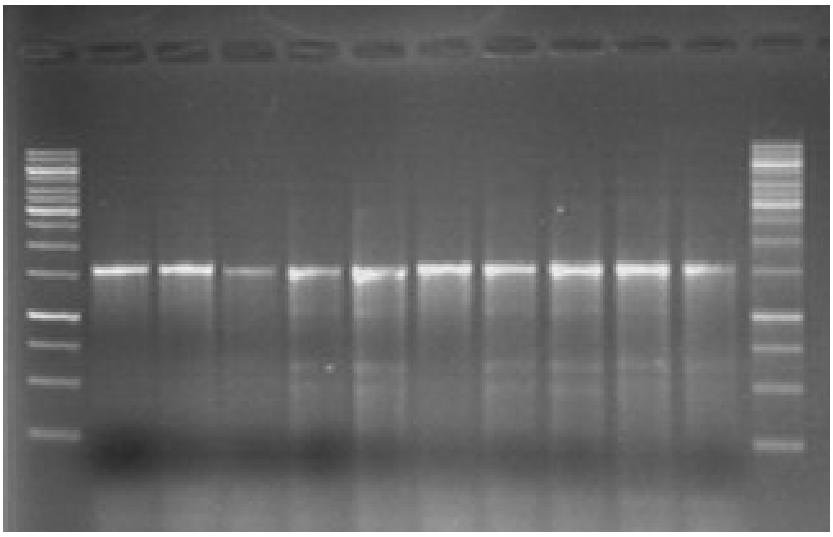


Figure 4. PCR amplification resulted in a fragment about 500 bp in length referring to *Streptomycetes* 16srDNA gene.

Streptomyces resources, the investigation on the production of this kind of secondary metabolites from *Streptomyces* species is already outspread. So in this research we tried to study the ability of the production of antimicrobial agents by *Streptomyces* species from some regions of northwest of Iran. Our results led to the 12 isolates with the different ability to produce antibiotics.

The investigation of putative isolates using primary screening and secondary screening revealed different results: some of the active isolates didn't show the activity in the secondary screening while some showed little activity and some showed improved activity (Pandey et al., 2002). According to Bushell (1993), during the screening of the novel secondary metabolite, *Streptomycetes* isolates are often encountered which show antibiotic activity on agar but not in liquid culture. The result of primary and secondary screening revealed that most of the active isolates were active against gram positive bacteria (*S. aureus*) rather than gram negative bacteria. The reason for different sensitivity between gram positive and gram negative bacteria could be described to the morphological differences between these microorganisms, gram negative bacteria having an outer polysaccharide membrane carrying the structural lipopolysaccharide components (Pandey et al., 2002). Morphological examination of the 12 isolates clearly indicates that these belong to the *Streptomyces* genera and *Streptomycetaceae* family (spore chain with coiling and branching, spore surface color, colony shapes and gram staining) (Cross, 1989; Lechevalier, 1989; Locci, 1989).

Although various biochemical tests were performed, it was unable to identify the *Streptomyces* up to species level due to the lack of other tests. According to Kutzner (1972) for proper identification of genera and species of *Actinomycetes* as well as morphological and physiological properties, various other biochemical properties such as cell wall chemo type, whole-cell sugar pattern, peptidoglycan type, phospholipids type and G+C% of DNA should be done. Moreover molecular studies are one of the strangest and easiest methods to identification of microorganisms such as bacteria. So in this study we used the molecular tests to determine the species of the isolated bacteria. Molecular assays resulted in confirming biochemical results. PCR amplification using 2 specific designed primers for 16srDNA gene from *Streptomycetes* was led to amplify the desired fragment of the gene.

The PCR products were digested using TaqI enzyme and bioinformatics analysis confirmed that the isolated bacteria are *Streptomyces* species. By comparing the RFLP results with RFLP pattern of 16srDNA of *Streptomyces* species using genedoc software the species of the bacteria could be known. The known strains somehow corresponded with the potentially announced strains through morphological studies. Although obtaining the mentioned consequences led us to be sure that our biochemical outcomes were correct and reliable, but also in order to identify the exact species of the bacteria sequencing of the amplified 16srDNA is suggested. Finally, as the result of the present study, we identified 11 *Streptomyces* strains from mentioned regions of the Northwest of Iran with high antimicrobial activity. To determine the antimicrobial agents further studies are needed.

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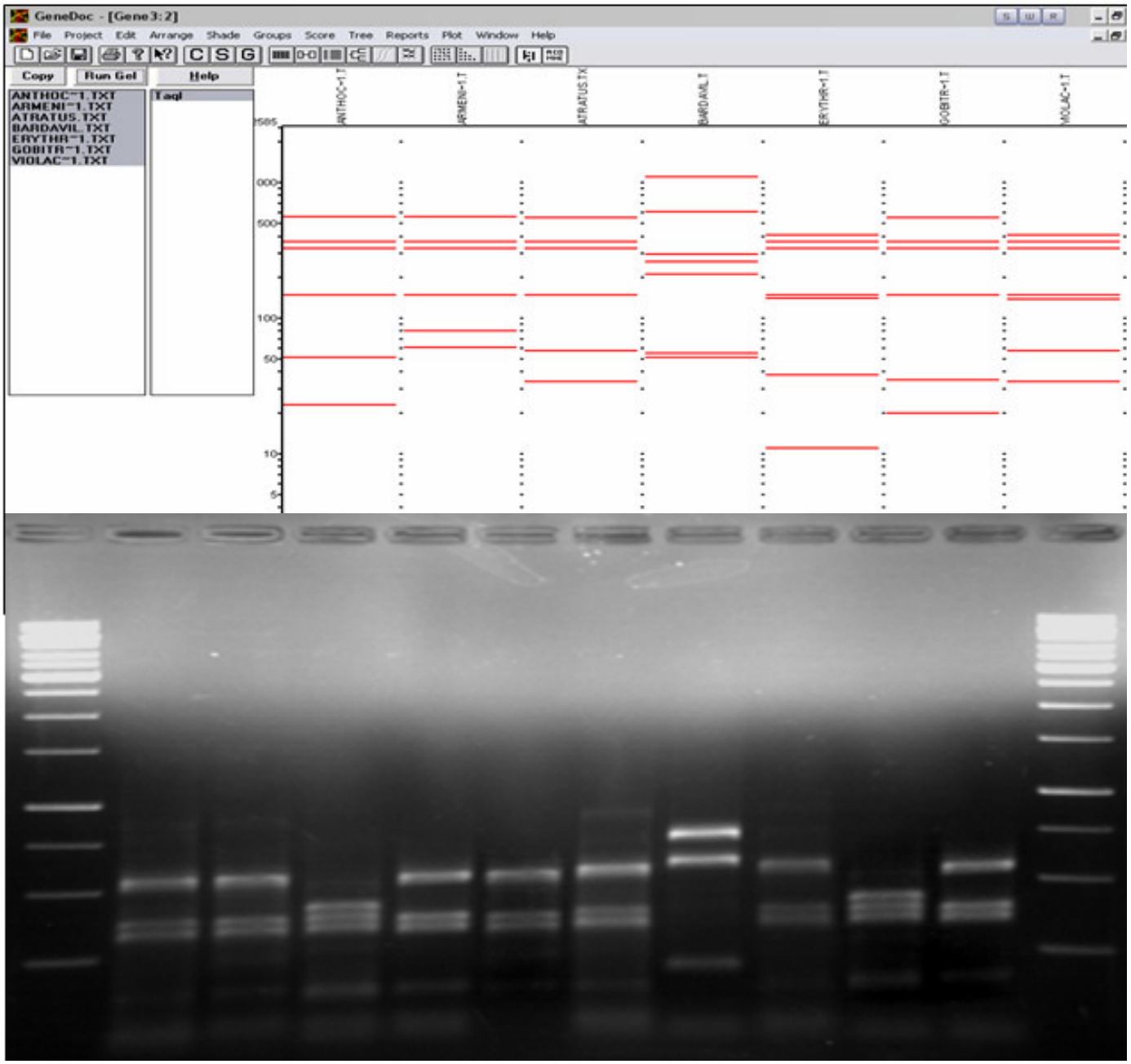

Figure 5. the comparison results of RFLP pattern of the 10 bacteria using TaqI endonuclease with other *Streptomyces* species in NCBI.

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