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Identification of Antibiotic-Producing Streptomyces Species in Iran's soil by Phenotypic and Genotypic Methods

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Abstract

Introduction: Actinomycetes are gram-positive and filamentous bacteria which include most microorganisms in the soil. According to the researches, three-quarters of all known antibiotics are produced by Actinomycetes. In this regard, screening for antibacterial activities as well as identifying them, 16S rRNA gene and phenotyping methods were used.
The aim of this study was to evaluate actinomycetes in soils of different parts of Iran for their antimicrobial properties and also characterization of active strain using 16S rRNA gene and phenotypic methods.
Methods: Actinomycetes isolates were separated from the gathered soil samples; initial screening have done using cross streak method in agar culture and second screening have done using disk diffusion method against the studied microorganisms: S. aureus, ATCC 25923 and E.coli ATCC 25922. Final confirmation of the produced antibiotic has been conducted by HPLC and identification of strains was done by PCR and DNA sequencing.
Results: From 100 gathered soil samples, 52 actinomycete isolates were separated; 30 isolates of primary screening and 3 isolates of secondary screening were selected. Strain 28 had a peak (RF) similar to gentamicin and isolates 34 and 4 strains had similar peak (RF) to streptomycin in HPLC. 16S rRNA genes of isolates were sequenced, in which isolate 28 had 99.93 percent similarity to Streptomyces youssoufiensis and isolate 4 had 99.93 percent similarity to Streptomyces cyaneofuscatus.
Conclusion: The results showed that there are new isolates in the soil samples of Iran that have the ability to produce antibacterial substances.

Keywords: Streptomyces, Antibacterial material, separation from soil, 16 S rRNA genes

Introduction

Antibiotics are among the most prescribed drugs in modern medicine that by killing or damaging the bacteria, they improve diseases. More than 30 percent or more of all patients admitted to hospitals are treated with one or several periods of antimicrobial agents; that means the usage of antibiotics in hospitals and even in society and arbitrarily is high(1).

Streptomyces genus is the most known genus of actinomycetes that most studies have been conducted on it (2-3).

Actinomycetes have the ability to produce a variety of secondary metabolites which antibiotics are among these metabolites (4); and research has shown that the most important source for the production of antibiotics in actinomycetes specifically belongs to Streptomyces. Only in 1984 about 3,500 of antibiotics have reported to be produced by Streptomyces (5).

Currently, the most common methods for the identification and classification of these bacteria are the use of morphological and biochemical characteristics as well as comparing their various genes sequence; among these genes, 16S rDNA gene due to its preserved sequences during evolution is very convenient to identify the bacteria (6-7).

According to these features, in current study the isolation and identification of antibiotic-producing stroptomyces of Iran's soil samples were examined and the types of antibiotics in the isolated strains were determined.

Methods

1-Pretreatment of soil samples

In the current study, 100 soil samples from different regions of Iran were prepared and transferred to biotechnology laboratory of Qazvin University of Medical Sciences. Serial dilution of soil samples were done(1).

2-Screening and isolation of actinomycetes from soil samples

For the isolation of actinomycetes, S.C.A (Starch Casein Agar) culture medium in sterilized Petri dish was used.

One mililitre of the obtained diluted specimens were placed in each plate and paned so that the suspension of soil distributed perfectly on the surface of the culture medium. These plates were incubated at 28 ° c; after 7-10 days of incubation, colonies of actinomycetes, as well as some fungi and bacteria, appeared in the environment (1).

Also, for comparing the strain with the standard strains, Streptomyces bacteria including Streptomyces aureofaciens PTCC (1119) and Streptomyces griseus PTCC (1127) were purchased from Iranian Research Organization for Science and Technology (IROST).

3-Morphologic Identifications

a-Macroscopic obeservations: morphological studies of colonies were performed after 7 to 10 days from planting of Streptomyces. The growth duration, the color of colony in the back and front of the disk and other characteristics were studied (8).

b- Microscopic observations: Gram staining of colonies under an optical microscope at x40 and x100 were done for evaluation of aerial mycelia and chains of spores (9).

4-Biochemical tests:

biochemical tests such as pigment production, starch hydrolysis, production of hydrogen sulfide, catalase, oxidase, citrate, MR, VP tests were performed (10-11).

5-Isolation of antibacterial-producing actinomycetes

To isolate the active actinomycetes that produced antibacterial substances, screening were performed in two separate stages. For initial screening, cross streak method on Mueller Hinton agar medium were used. In this method, first, actinomycetes isolates were cultured linearly and then test strains were cultured by crossing on them . Test strains such as Staphylococcus aureus (ATCC 25923) and Escherichia coli (ATCC 25922) were obtained from the collection center of fungi and bacteria of Iranian Research Organization for Science and Technology (IROST).

For the second stage of screening, positive strains tested with anti bacterial activity of first stage, were transferred to Mueller Hinton Broth for further studies. After 14 days of incubation in 28 °C, the liquid culture were collected and centrifuged. Equal amounts of the surface liquid were mixed with ethyl acetate and placed in shaking incubator at 180 rpm and 28 °C for 1 hour. Then the solvent phase were separated from the aqueous phase and those solvents that contained metabolites were condensed by evaporator and brought to a volume of 10cc and maintained at a temperature of 4 °C. Extracted metabolites were studied by disk diffusion method using 6 mm blank disks against standard strains and halos of inhibition were recorded. Therefore, antimicrobial activity of the metabolites were confirmed (1, 9, 12).

6-Final identification of antibacterial substances

For final identification of antibacterial substances , gold standard method of HPLC with the following characteristics were used.

Column characteristics: XDB - C18 (4.6 × 250mm, 5µm),

Detector : (UV) (270 nm),

Solvents: methanol / acetonitrile / water (25:35:40, by v / v / v).

To determine the identity of antibacterials, these materials along with standard drugs such as streptomycin, gentamicin and tetracycline were injected into the HPLC device (13-15) and their Rfs were compared.

7-Genotypic identification and sequencing of 16S rRNA gene

Genomic DNA extraction from bacterial cultures were performed using Gram positive extraction Cinnapure DNA kit (Containing lysosozym) of Sinaclone Company. DNA concentration was measured at a wavelength of 260 nm.

PCR for 16S rRNA gene was performed using primers mentioned on table 1.

PCR program were conducted based on the following table in 33 cycles:

Table 1. Primers sequences for PCR of 16S rRNA gene

Primer	Sequencing
4f	5'- TATCGGAGAGTTTGATCCTGG -3'
1505r	5'- GATACGGCTACCTTGTTACGA -3'

Table 2. PCR program for 16S rRNA gene

PCR Protocol		
Initial Denaturation	95°C	3′
Denaturation	93°C	45″
Annealing	58°C	60″
Extension	72°C	90″

Purification of PCR product was conducted with the use of GF1 kit produced by Vivantis Company. After purification of PCR products, gel electrophoresis was performed and with the use of gel documentor, photos were taken.

Finally, for genotypic identification of certain strains, purified products of PCR were sent to Bioneer Company of Korea for sequencing. Sequencings were performed with the following primers (16-18) presented on table 3.

Table 3. Primers used for 16S rRNA sequencing

Primer	Sequencing
4f	5'- TATCGGAGAGTTTGATCCTGG -3'
16r339	5'- ACTGCTGCCTCCCGTAGGAG -3'
16f358	5'- CTCCTACGGGAGGCAGCAG-3'
704f	5'- GTAGCGGTGAAATGCGTAGA- 3'
1505r	5'- GATACGCTACCTTGTTACGA- 3'

Then, obtained sequences were compared with nucleotide sequences found in NCBI database, Ribosomal Database Project and Eztaxon.

Results:

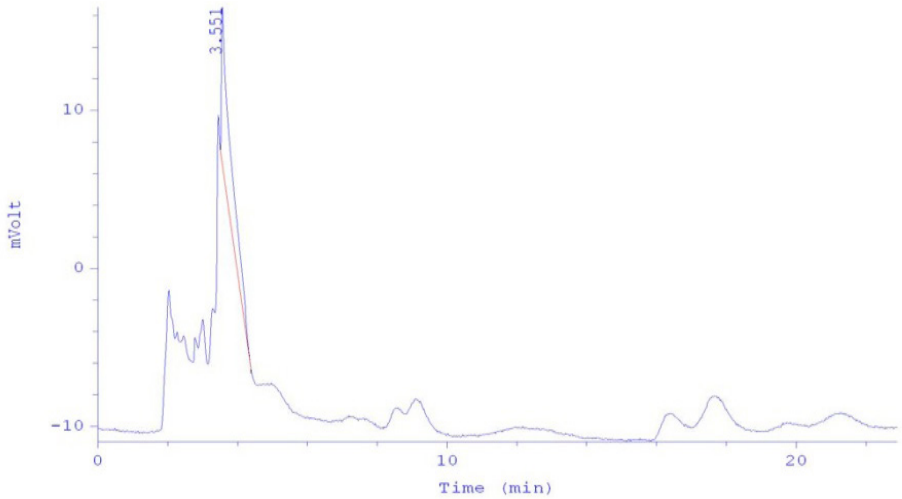
From 30 different places in the country, 100 samples were selected. 52 isolates were isolated from soils. Thirty of isolates were positive in the initial screening which 29 isolates had antibacterial activity against S. aureus and 1 isolate had such activity against E. coli. In the second screening, ethyl acetate extractions of isolates of 4, 28, 34 as well as the standard strain of Streptomyces aureofaciens PTCC (1119) had the best antibacterial activity against S.aureus.

Table 4. Summary of soil samples, isolation

Samples and isolates	No:
Total sample	100
Total isolates	52
Initial screening	30
Secondary screening	3

Results from the identification of antibacterial substances by HPLC

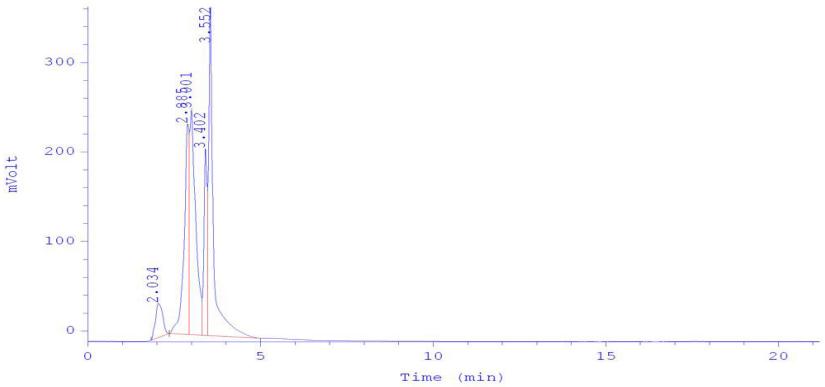
Ethyl acetate extractions of fermentation broths of isolates number of 4, 28, 34 and standard strain of Streptomyces aureofacien were injected into HPLC system; isolates 34 and 4 had similar peaks (RF) to the standard peak of Streptomycin (Figs: 1,2,3) , isolate 28 has similar peak (RF) to the Gentamicin and the standard strain of Streptomyces aureofaciens had a similar peak (RF) to the Tetracycline.



	Ret. Time [min]	start [min]	End [min]	compound	amount	Units	Area mV/min	Hight m.Volt	Width [min]	Type
1	3.551	3.50	3.49		0		3.02556	9.77526	0.354	BB

	Time min	Follow ml/min	Valve A%	Valve B%	Valve C%	Valve D%	D.In
1	0.00	1.00	0.00	100	0	0	1
2	50.00	1.00	0.00	100	0	0	--

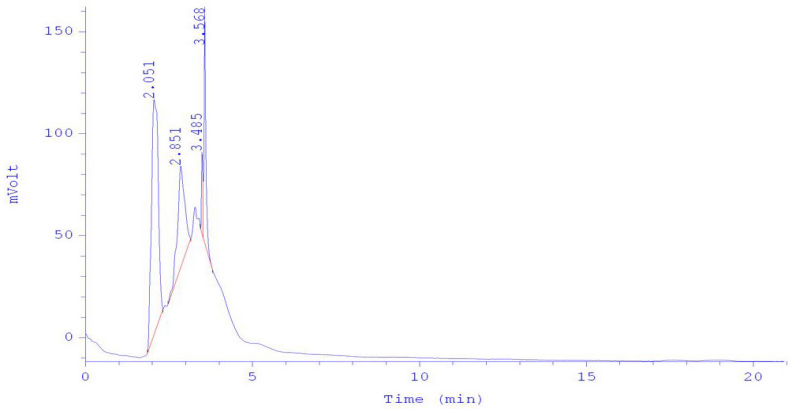
Figure 1. HPLC chromatogram with streptomycin peak of RF: 3.5



	Ret. Time [min]	start [min]	End [min]	compound	amount	Units	Area mV/min	Hight m.Volt	Width [min]	Type
1	2.034	1.83	2.35		0		9.1638	38.0997	0.229	BB
2	2.885	2.35	2.93		0		38.7049	235.367	0.109	BP
3	3.001	2.93	3.30		0		51.356	252.575	0.129	PP
4	3.420	3.30	3.47		0		22.7975	208.48	0.066	PP
5	3.552	3.47	5.00		0		58.3574	376.664	0.087	PB

	Time min	Follow ml/min	Valve A%	Valve B%	Valve C%	Valve D%	D.In
1	0.00	1.00	0.00	100	0	0	1
2	50.00	1.00	0.00	100	0	0	--

Figure 2. HPLC chromatograms of antibacterial substances of isolate 34 that have an RF of about 3.5



	Ret. Time [min]	start [min]	End [min]	compound	amount	Units	Area mV/min	Hight m.Volt	Width [min]	Type
1	2.051	1.85	2.32		0		26.8878	115.281	0.222	BB
2	2.851	2.47	3.15		0		12.2367	49.6702	0.226	BB
3	3.485	3.43	3.52		0		1.84798	40.0486	0.083	BP
4	3.568	3.52	3.80		0		7.23286	115.904	0.082	PB

	Time min	Follow ml/min	Valve A%	Valve B%	Valve C%	Valve D%	D.In
1	0.00	1.00	0.00	100	0	0	1
2	50.00	1.00	0.00	100	0	0	--

Figure 3. HPLC chromatogram of antibacterial substances of isolate 4 that has an RF peak of about 3.5

The results of genotypic identification of two best strains which their antibiotic activities confirmed by HPLC):

The obtained results of DNA concentrations of extraction of strains are shown in table 2:

Table 5. DNA Concentration at wavelength of 260 nm

	Strain	Strain Code	DNA concentration (ng/μl)
1	28	1426-01	53.2
2	4	1426-02	71.4

Electrophoresis of purified PCR products of isolates 4 and 28 are shown in Figure 4.

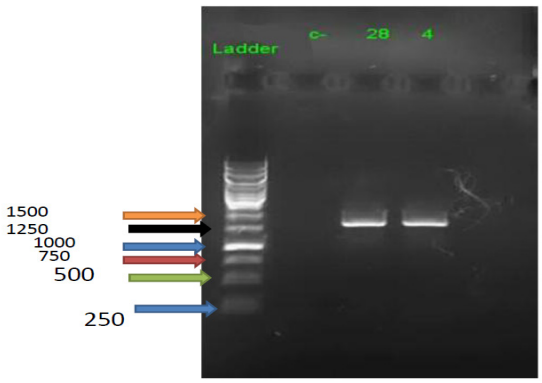


Figure 4. Electrophoresis of PCR products confirming 1500 nucleotides fragments of the isolates 4 and 28

Identification of active strains using 16S rRNA gene sequences

By comparing the obtained sequences with nucleotide sequences in, Eztaxon and Ribosomal Database Project and specially databases of NCBI, the following results have been obtained:

1- Strain: 28

The similarity of 16S rRNA gene of this strain with 16S rRNA gene of Streptomyces youssoufiensis DSM 41920T (which have been registered with FN421338 in NCBI) was 99.93%.

2- Strain: 4

The similarity of 16S rRNA gene of this strain with 16S rRNA gene of Streptomyces cyaneofuscatus NRRL B-2570T (its whole genome has been registered by JOEM01000050 in NCBI) was 99.93%.

Discussion

In recent years, sporadic studies have been conducted on the isolation and identification of actinomycetes from different regions of Iran (19-20). Studying these resources and data shows that so far no comprehensive study has been done in this field.

Actinomycetes bacteria are the largest group of bacteria that produce secondary metabolites, in particular anti-bacterial and anti-fungal substances (21); so in order to produce these substance, hundreds of centers and research groups studied these organisms (19,20, 22,23). On the other hand, this issue becomes more important when we know pathogenic bacteria develop resistance to the available antibiotics over time (19) and virtually, these substances being out of the treatment field. Therefore, with the aim of isolating and screening active actinomycetes, in this reserch soil samples were collected from different regions of Iran and studied.

Initial screening was performed by cross streak method which is a convenient method for examining antagonistic activities between microorganisms. Our result are consistent with predictions made previously by Bushell et al (24).

For secondary screening, disk diffusion method were used. In this method, the release of antibiotics from a source to a solid medium causes a creation of an inhibiting growth halo; the diameter of this halo is proportional to the logarithm of the antibiotic concentration (19).

Although the size of halo alone in disk diffusion method , does not determine the strength of the effect of antibacterial or the susceptibility of bacteria (25), but according to the results that were obtained from the secondary screening in this study , it seems that S.aureus test strains have been inhibited more than other test isolates .

Dhanaskaran et al. have studied Actinomycetes in soils of different regions of India in 2005. In order to extract the active metabolites of antibacterial liquid medium for secondary screening, they used solvents such as ethyl acetate, aniline, chloroform and Pyridine. They showed that ethyl acetate is the best solvent for extracting these substances (26) which the current study also confirms this.

In the field of identifying Streptomyces, molecular methods such as DNA-DNA hybridization, Complete DNA-cutting enzyme of bacteria, RAPD-PCR and comparison of 16S rDNA gene sequences are used. Among the above methods, determining 16S rDNA gene sequenc is more common in recent years due to its high precision (1). The well-documented case has been reported in 1991 by Stackebrandt et al.; in which they used this gene for the classification of actinomycetes that also have been confirmed by other classification methods such as biochemical tests and numerical taxonomy (27).

One of the reasons for the superiority of 16S rDNA gene is that during evolution, it had the lowest mutation and change; on the other hand, three areas of α, β, and γ have been identified in its nucleotide sequence that has the sufficient variety to identify actinomycetes; especially γ area which is suggested for classification in strain level (4, 16, 28) the importance of γ region among these areas is due to its closer position to 5' end form nucleotides 158-203; in sequence reading, therefore, by having its full nucleotide sequence, the desired isolate can be identified and recorded with high accuracy (4). In the current

study, the amplification was done using previously listed primers which covered all three regions of α, β, and γ. Also, with the use of previously listed primers, sequencing for gamma area was done.

Streptomyces youssoufiensis initially were discovered in a phosphate mine in Morocco called Youssoufia. It was studied with the use of molecular methods and by genotyping of this strain it was named X4 strains. Examining of 16S r DNA sequence of this strain showed that it had similarities to such Streptomyces as ramulosus and kasugaensis but it was different in some features of DNA and biochemical properties from the above strains .Therefore, it has been named Streptomyces youssoufiensis (29).

Streptomyces cyaneofuscatus has been discovered for the first time in Dagestan of Russia and it has been used for biosynthesis of valinomycin (30).

In a study by Brana and colleagues in 2015 on the soil samples from beaches and deep sea areas, a strain of Streptomyces have been identified with the help of phylogenetic and 16S rRNA methods which was 99.9% similar to Streptomyces cyaneofuscatus. This strain produces antibacterial, antifungal, anti-inflammatory and anti-tumor substances. Additionally it has anti-tuberculous activity (31). Therefore, considering an almost hundred percent similarity of isolated strains in the current study with the above strains, it seems that they can be used in the production of other secondary metabolites. It is one of the most recommendations of current research.

The difference in strains of bacteria can lead to the identification of new strains which in itself could be considered as a new source of new secondary metabolites.

Conclusion

The results of this study indicate that new isolates of productive Streptomyces in soil samples of Iran have the ability to produce antibacterial materials which may be used for industrial production of new antibiotics.

CONFLICT OF INTEREST

None declared.

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Ethics approval : Ethics committee of QUMS was approved this research according to ethics code No. QUMS.1394.247

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