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Isolation and Molecular Identification of *Streptomyces spp.* with Antibacterial Activity from Northwest of Iran

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ABSTRACT

Introduction: *Streptomyces* are a group of prokaryotes that are usually found in all types of ecosystems including water and soil. This group of bacteria is noteworthy as antibiotic producers; so the isolation and characterization of new species seemed to be crucial in introduction of markedly favorable antibiotics. Therefore, in this study we aim to isolate and characterize novel strains of *Streptomyces* with high antibiotic production capability. ***Methods:*** To achieve this goal, from 140 isolates collected throughout northwest of Iran, 12 selected *Streptomyces* isolates which exhibited high antibacterial activity against pathogenic bacteria were subjected to PCR reaction for identification via 16S rDNA gene and random amplified polymorphic DNA (RAPD) pattern analysis. ***Results:*** Analysis of morphological and biochemical characteristics and the 16S rDNA gene sequence indicated that all 12 selected isolates belonged to the genus *Streptomyces*. Moreover, screening of the isolates with regard to their antimicrobial activity against indicator bacteria as well as their classification using RAPD analysis revealed that G614C1 and K36C5 isolates have considerable antimicrobial activity and high similarity to *Streptomyces coelicolor* and *Sreptomyces albogriseolus*, respectively. ***Conclusion:*** Since many isolates in this study showed inhibitory effects against pathogenic bacteria, soil of northwest of Iran could be used as a rich source to be explored for novel *Streptomyces* strains with high potency of antibiotic production.

Introduction

Actinomycetes, particularly *Streptomyces spp.* have been a widely exploited group of microorganisms in the production of secondary metabolites and enzymes of commercial importance in medical and agricultural applications.[1] *Actinomycetes* are found in all types of natural resources and profoundly in soil.[2] The genus *Streptomyces* seems to provide wide variety of new antibiotics more than any other genus; hence, it is of the foremost importance for both industrial application and human health care.[3] They produce a large number of secondary metabolites and particularly antibiotics that are in favor of pharmaceutical companies nowadays, resulting in conduction of widespread investigation towards discovery of new antibiotics. Additionally, *Streptomyces* is one of the model systems for the evaluation of various bacterial characteristics.[4]

In terms of number and variety of identified species, *Streptomyces* represents one of the largest taxonomic units of identified *Actinomycetes*. They were distinguished as gram-positive bacteria with a high GC content in their DNA to more than 70%.[5] Soil as their main natural habitat is nutritionally, biologically and physically complex and variable; as a result, they are able to perform a broad range of metabolic processes and to produce an immense diversity of bioactive secondary metabolites.[3] Furthermore, sequencing the genome of *Streptomyces* which is in connection with human health care, veterinary medicine, biotechnology, and ecology, revealed that the morphological, biochemical and genomic heterogeneity are a correct reflection of their biodiversity.[5,6]

Although various studies have been reported on screening and identification of *Streptomyces* from all around Iran,[7-9] reviewing provided data has demonstrated that not yet comprehensive survey on this issue has been conducted. Therefore, in this study different soil samples considering their pH value and altitude of locations were obtained from all around northwest of Iran and subjected to molecular marker-based identification and clustering of promising isolates in terms of antibiotic production yield.

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Materials and methods

Isolation of bacterial strain

Soil samples were collected from all regions in northwest of Iran from 10-15 cm depth and kept at 4°C until microbial assays be performed on them.[10] While sampling, region's specific features such as pH value and altitude was recorded.[11] For preparation of the soil suspension, 5 g of soil sample was transferred into a sterile bottle then 45 ml of sterile distilled water was added and shaken for 30 min. Five sets of ten-fold serial dilutions were prepared from original supernatant, then 100 µl of third concentration was used for inoculation of Starch Casein Agar (SCA) (Sigma-Aldrich, Germany) and the plates incubated for 7 days at 28°C. Morphological features of colonies such as colony pigmentation were used for preliminary classification of the bacterial population. One hundred forty disparate isolates with different morphological characteristics were kept at -80°C and tagged as a distinct isolate based on their sampling location and the order of colony isolated from same soil sample.[12,13]

Assay of Antimicrobial activity

In this experiment, a series of indicator bacteria have been used including: *Escherichia coli* (ATCC 1399), *Klebsiella pneumoniae* (ATCC 1290), *Shigella flexneri* (ATCC 1234), *Listeria monocytogenes* (ATCC 19115), *Bacillus cereus* (ATCC 1431), *Yersinia enterocolitica* (ATCC 35669), and *Staphylococcus aureus* (ATCC 29213). Antimicrobial assay of *Streptomyces* isolates were tested by overlay as well as disk diffusion methods.

The overlay method was used for the examination of intact *Streptomyces* inhibitory effect against indicator bacteria.[14] In this method, the plate containing the overnight culture of *Streptomyces* strain was overlaid with top agar (Sigma-Aldrich, Germany) which contains the indicator strain (e.g., *E. coli*). The inhibitory effect was evaluated after 7 days of incubation at 28°C and the results were evaluated by the measurement of inhibition zone diameter.

The disc diffusion method was used for testing the antimicrobial activity of extracted secondary metabolites[15] by different polar and non-polar solvents including diethyl ether, dichloromethane, ethyl acetate, n-Hexane, chloroform, methanol (Merck, Germany) and water. For extracts' preparation, the isolates were inoculated into 70 ml of Muller-Hinton broth (Difco, Germany) and incubated for 26 h at 28°C. Then the culture medium was centrifuged (Sigma, United Kingdom) in 18625 ×g for 20 min and the pellets were suspended in the various solvents. The solution was shaken for about 1 h at 28°C in 180 rpm, concentrated to 10 ml using rotary evaporator (Hiedolph, Germany) and kept in sterile tubes at 4°C.[2] The extract of all *Streptomyces* isolates were filter sterilized (0.24 µm) and the blank discs of 6 mm diameter were immersed in cell free supernatant. Then, the discs containing secondary metabolite of each isolates were placed on inoculated Muller-Hinton agar (Difco, Germany) with approximately McFarland turbidity standard No. 0.5 of indicator bacteria. All plates were maintained at 4°C for 30 min allowing perfusion of disk content and then incubated at 37°C for 24 h. Positive results were measured as clear zones of inhibition around the discs after overnight incubation at dark and room temperature (~27°C).[13] Negative control for evaluation of the possible inhibitory effect of the solvent against indicator bacteria was also included in this study.

PCR amplification and sequencing of 16S rDNA gene

The isolates which showed strong antibacterial activity were subjected to further evaluation by molecular methods. The extraction of genomic DNA was carried out using a previously published protocol[16] with slight modifications. A single colony of each isolate was cultured in 50 ml of International Streptomyces Project Medium 2 (ISP2) for 18-24 h at 26ºC. Then the culture was centrifuged for 3 min at 2375 ×g and supernatant was discarded. Eventually the genomic DNA of lysed bacterial cells was precipitated with 0.6 volume of isopropanol and purified using ethanol 70%.[17]

To amplify the fragment of 16S rDNA gene, a set of primers (i.e., forward (St-F): 5´-AAGCCCTGGAAACGGGGT-3´ and reverse (St-R): 5´-CGTGTGCAGCCCAAGACA-3´) was used.[8] PCR amplification was performed using Master Mix (Ampliqon, Denmark), 0.4 µM primer, 40 ng chromosomal DNA, and the final volume was reached to 25 µl. The PCR amplification was performed using the thermal cycler (Veriti, Applied Biosystems, USA) program as follows: 94ºC for 5 min as a primary denaturation step, 35 cycles of 94ºC for 1 min, 57ºC for 1 min, 72ºC for 105 sec and final extension was 72ºC for 10 min. The PCR products were visualized using gel electrophoresis on 1% agarose (Qiagen, Germany) and compared with 1 kb DNA ladder (Fermentas, Germany).

PCR products of high yield isolates were purified using PCR purification kit (Qiagen, Germany) according to the manufacturer's instructions. The purified products were sequenced by MacroGen Co. (Seoul, Korea). Furthermore, using BLAST software, the determined sequences were compared with the sequences deposited in NCBI GenBank as 16S rDNA gene of different *Streptomyces* species.

RAPD-PCR and data analysis

The samples with positive result in amplification of 16S rDNA for further discrimination were subjected to RAPD analysis with five primer pairs as follow: P1-P2-P3-P4-P5.[18] The amplification reaction was carried out with the final volume of 25 µl with the following conditions: 94°C for 4 min as a primary denaturation, 40 cycles of 94°C for 50 sec, 40°C for 50 sec, 72°C for 1 min, and final extension for 10 min at 72°C. Electrophoresis of PCR products were performed on 1.5% agarose gels with molecular marker of 1Kb DNA ladder. After RAPD-PCR the polymorphic DNA band patterns were analyzed and genetic relationships among the *Streptomyces* isolates

were calculated using NTSYS-PC™ (Exeter software, USA). A Dendrogram and a distance matrix were calculated by a UPGMA (Unweight Pair-Group Method by Arithmetic Average) program in order to determine genetic relationships among the isolates.

Results

The result of preliminary screening based on the effect of intact bacteria revealed 30 potent isolates in terms of antimicrobial activity against indicator bacteria, however considering the secreted metabolite of these isolates, only 12 isolate showed to be active in two types of screenings (Table 1). Based on the results presented in Table 1, it seems that *S. aureus* and *B. cereus* are much more inhibited which supports the findings of Scherrer and Gerhardt (1971) who stated that gram positive bacteria are more sensitive to metabolites produced by *Streptomyces*.[19] Furthermore almost 50% of the selected isolates exhibit antibacterial activity against same indicator bacteria, when the diethyl ether, dichloromethane, ethyl acetate, n-Hexane, chloroform, methanol, and water extract of each isolate was exploited for antibacterial activity assay using disc diffusion method. It should be noted that the significant result was obtained only from ethyl acetate extract.

As shown in Table 2, among all data (sampling location, altitude, colony pigmentation and pH value) for the 12 highly active isolates, the isolates with high antimicrobial activity originate from the soil with pH value above 8, and altitude of higher than 1500 meters, where higher microbial diversity is also detected. Given such fact which is in agreement with the results achieved by Oksay et al 2004,[13] it can be deduced that soil with high altitude (>1500) and pH value more than 8 is the best candidate for exploration studies on *Streptomyces* regarding their antibacterial activity.

Performing PCR reactions using primer pair StF/StR on DNA from the 12 soil isolates produced a single ~1500 bp band in all tested isolates (Fig. 1). Sequencing of the isolates K36C5 and G614C1, which displayed high antimicrobial activity in preliminary evaluation, revealed high similarity with *Streptomyces albogriseolus* and *Streptomyces coelicolor*, respectively. The G614C1 presented new *Streptomyces*-specific sequence which was deposited in Gene Bank (Accession number: JF431461).

Twelve *Streptomyces* isolates with high antimicrobial activity were further characterized using RAPD-PCR (Fig. 2) and the relationship between *Streptomyces* strains were evaluated by UPGMA Dendrogram to compare the banding patterns generated by each primer. The organisms were also compared with appropriate marker to establish the position of undefined strain within the evolutionary radiation encompassed by *Streptomyces*. Based upon these findings, the isolates can be classified into four clusters (Fig. 2). The isolates K11C3 and K47C1 generated the identical RAPD patterns, so they were possibly categorized as the same strain. The first cluster was composed of 5 isolates from the genus *Streptomyces*, and among them the isolate

Table 1. Inhibitory effect of intact bacteria and ethyl acetate extract of putative isolates against some selective indicator bacteria.

Sample Code	*E. coli*	*S. aureus*	*Y. enterocolitica*	*B. cereus*
G614 C1	R*(+)	I(+)	R(+)	I(+)
K36 C5	R(+)	S(+)	I(+)	S(+)
M00 C1	R(+)	S(+)	I(+)	S(+)
K11 C3	S(+)	S(+)	S(+)	R(+)
K22 C2	R(-)	R(+)	R(+)	R(+)
G151 C1	R(+)	S(-)	R(-)	S(-)
K47 C1	R(-)	R(-)	R(+)	R(-)
M41 C1	R(+)	I(+)	R(+)	R(+)
G1111 1	R(-)	R(-)	S(-)	S(+)
K35 C1	I*(-)	R(+)	R(+)	R(+)
M42 C1	I(-)	S(+)	I(-)	R(-)
GB C1	S*(+)	R(-)	I(+)	R(+)

The + and – symbols show positive and negative inhibitory effect of intact bacteria; R: resistance; S: sensitive; I: intermediate inhibition of ethyl acetate extract. * R≤16, I= 17-19, S ≥ 20.

Table 2. Morphological characterization of selective *streptomyces* isolates with high antibacterial activity collected from soil with different pH value and altitude.

Samples code	Soil pH	Location altitude (m)	Colonies pigmentation	
			Surface	Back
G614 C1	8.9	1618	*Griseus*	*Flavus*
K36 C5	7.6	3080	*Lavendulae*	*Rubro-aurantiacus*
M00 C1	7.7	2450	*Griseus*	*Globisporus*
K11 C3	7.7	2450	*Albus*	*Rubro-aurantiacus*
K22 C2	8.5	1800	*Albidus*	*Cinecolor*
G151 C1	8.7	1340	*Griseus*	*Globisporus*
K47 C1	8.2	1720	*Globisporus*	*Globisporus*
G41 C1	8.3	1650	*Glaucus*	*Albidus*
G1111 1	8.5	1530	*Chromogenes*	*Albidus*
K35 C1	7.3	1606	*Griseus*	*Griseus*
M42 C1	8.7	630	*Griseus*	*Globisporus*
GB C1	8.5	1459	*Griseus*	*Globisporus*

G614C1, due to its high antibiotic production yield, was subjected to the sequencing analysis. The isolate K36C5 is the promising component of group three with high antibiotic production capacity which was subjected to sequencing analysis. Thus, RAPD could be a useful tool to identify *Streptomyces* with high sensitivity and specificity to cluster and also to decrease identification time.

Discussion

Soil is an ecological treasury with many organisms living together and some of them producing useful natural products, including antibiotics. In this study, we tried to identify the active strains of *Streptomyces* strains with high yield of antibiotic production from various samples of soil in northwest of Iran using various biochemical and molecular techniques. Distinguishing factors such as soil pH and altitude of sampling location together with morphological features of colonies such as color and pigmentation were used for preliminary classification of bacterial population.

According to the result of antimicrobial assay, the antibacterial activity of putative isolates showed arbitrary result in primary and secondary screening; it means, there was no correlation between the activity of intact bacteria and their extracts. This arbitrary result in the case of extract superiority may arouse from the inability of intact bacteria to get in touch with pathogen as well as a loss of competitive ability in intact bacteria (Table 1).[20,21] Furthermore, in some cases the extraction of secondary metabolite from liquid culture affects the antimicrobial activity of active substance. For example, isolates K22C2, M41C1 with acceptable result in the primary selection showed no antibacterial activity when their extract was applied against indicator bacteria.[22]

Considering all the complications facing with biochemical and morphological test which are laborious, time

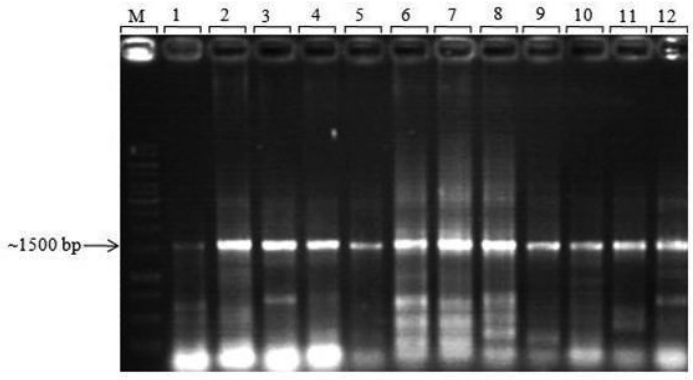

Fig. 1. Amplification of the 16S rDNA gene, using PCR for 12 highly active bacteria. The symbols in PCR lanes represent M: Marker; 1: M00 C1; 2: K36 C5; 3: K11 C3; 4: K22 C2; 5: G151 C1; 6: K47 C1; 7: G614 C1; 8: M41 C1; 9: G1111 1; 10: K35 C1; 11: M42 C1; 12: GB C1

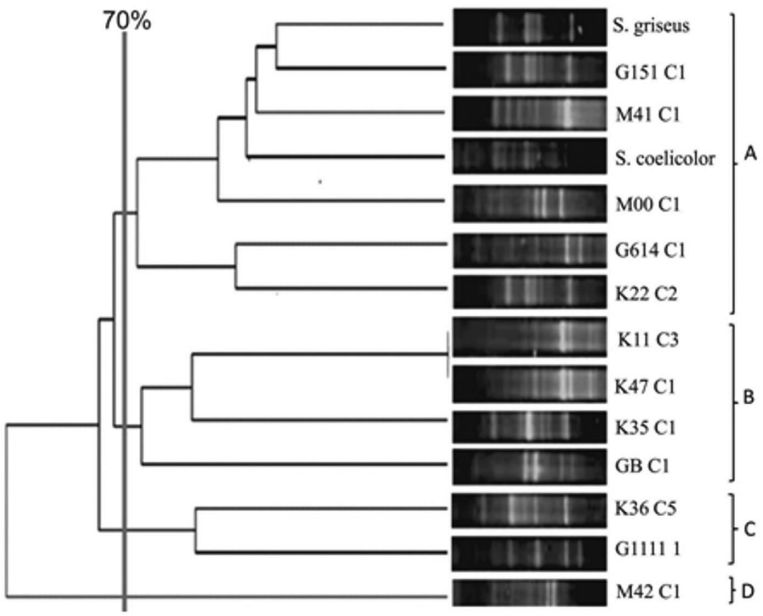

Fig. 2. RAPD-PCR and dendrogram clustering pattern. Data represents grouping of samples into 4 clusters including A, B, C, D. Isolates "G614C1" and "K36C5" represent promising antibacterial activity.

consuming, and sometimes imprecise because of the subjectivity of interpreting the results, currently there exist molecular methods available for the isolation and characterization of new strain from different soil samples. Among them, application of 16S rDNA gene is more simple, yet efficient, in identification of new *Streptomyces* strains.[2] It is worth noting that although 16S rDNA gene has less changes and transformation through evolution, it is deemed to be a superior candidate for taxonomic studies because of 5' variable areas including α, β, δ, ε, and particularly variable γ part which shows relatively high polymorphism at the 5′ end of its structure[23-25] which could be exploited for studying the genetic diversity of various *Streptomyces* species.[22,26] Identification of new strains of *Streptomyces* have been frequently described in the literature using amplification of hyper variable regions that can provide strain specific signature.[27,28] Oh et al in 2005, identified a new strain of *Streptomyces* with high antibiotic production capacity and higher homology to *Streptomyces echinatus* using the assessment of cultural, morphological and phylogenetic evaluation provided by 16S rDNA sequence analysis.[29] In the similar work by the Higginbotham et al in 2010, the 16S rDNA sequence of the new strain exhibited higher homology with *Streptomyces lavendulae* and *Streptomyces globosus*.[30]

In conclusion, we identified some of the isolates with high antibacterial activity; however, further investigations are needed in order to determine the active metabolite of these isolates. It should be noted that, by designing appropriate strategies for isolating and identification of various *Streptomyces* genus in the future, introduction of new and much more effective antibiotics from native species could be conceivable.

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Ethical issues

The authors declare no ethical issues.

Competing interests

The authors declared no competing interests.

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