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Isolation and Molecular Identification of *Streptomyces* spp. Producing Antibacterial Compounds from Iranian Soil

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ABSTRACT

Backgrounds: This study was conducted with the aim of isolation and molecular identification of *Streptomyces* spp. producing antibacterial compounds from Iranian soil.

Materials & Methods: In this study, 50 soil samples were collected from different areas of Sanandaj city. Soil samples were cultured on starch casein media. *Streptomyces* species were characterized using morphological and biochemical assays. Molecular identification was performed by 16S rRNA sequencing. Antimicrobial activity was evaluated using perpendicular streak and agar well diffusion methods.

Findings: To identify active *Streptomyces* strains in terms of producing antibacterial agents, screening was performed in two stages. Among 20 *Streptomyces* strains isolated from soil samples, six isolates were selected in the primary screening stage based on their ability to limit the growth of pathogens. Of the two solvents used in the secondary screening stage, ethyl acetate was the most suitable solvent for extracting effective metabolites of *Streptomyces*. Among the six isolates selected based on their antimicrobial activity, two isolates with the highest antibacterial activity were selected for the sequencing process. By analyzing the dendrogram and the data obtained from the NCBI database, it was found that one isolate (Yellow 4A) was 98% similar to *S. fradiae*, and the other isolate (Green 4A) was 98% similar to *S. coelicolor.*

Conclusion: The use of proper strategies to identify potential new *Streptomyces* species with antibacterial properties may bring a bright future in the treatment of resistant pathogens. However, more studies are required to detect active metabolites of the mentioned isolates.

Keywords: Streptomyces coelicolor, Streptomyces fradiae, Soil, PCR, Sequencing.

CITATION LINKS

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Introduction

Identification and development of novel antibacterial products for use in future therapeutic strategies is an indispensable necessity [1, 2]. Soil is a natural ecological habitat where many organisms live together, some of soil inhabitants produce a wide range of natural beneficial active products such as clinical antibiotics [3]. Over the last century, the emergence and spread of antibiotic-resistant pathogens has become a global public health concern that threatens advances in treatment and the achievement of sustainable development goals [4]. Therefore, to address the challenges ahead, conducting numerous studies to identify unknown active microbial strains is a practical approach to manage and prevent the spread of resistant bacteria [5, 6]. Microbial molecules are rich substrates for the discovery of potential therapeutic agents. More than 5000 antibiotics have been reported so far. However, approximately 100 antibiotics have been commercially applied in therapeutic fields [7, 8]. In recent years, several studies have been performed on the isolation and screening of new antimicrobial-producing organisms as a tool for bio-control [9]. *Actinomycetes*, especially *Streptomyces* spp., are a group of microorganisms that have been widely exploited for the production of antibiotics and bioactive molecules as well as for medical and agricultural purposes [10, 11]. Previous studies have shown that over 74% of all reported antibiotics are produced by the genus *Streptomyces*; therefore, they have unique applications in various fields like agriculture, food industry, and human health [12, 13]. Although *Streptomyces* spp. could produce many antibiotics, they are only a tiny part of the bioactive agents. Therefore, identification of unknown active microbial agents is necessary to discover new antibiotics and control the problem of antimicrobial resistance [14]. Various studies have been conducted in the field of screening and identification of *Streptomyces* in Iran, but based on the findings, no comprehensive information has been provided in this regard yet. For this reason, the isolation and characterization of novel and efficient *Streptomyces* strains from natural resources is an important achievement [15]. In this study, 50 soil samples were collected from four different areas of Sanandaj city by considering their pH values, and then 20 *Streptomyces* strains were isolated from these soil samples. **Objectives:** This study aimed to analyze *Streptomyces* populations and discover its different species according to 16S rRNA gene sequences. Therefore, the results of this study are expected to be helpful in providing information about the distribution and antibacterial activity of microorganisms collected from different sample sites.

Materials and Methods

Sample collection: Soil samples were collected from four different areas of Sanandaj from a depth of 10-15 cm. The collected samples were transferred to the laboratory in a package containing ice. Soil pH and sampling site temperature were measured, and the place and date of sampling were recorded. In order to prepare a suspension from the soil sample, 10 g of soil was mixed with 90 mL of sterile distilled water and shaken for 15 min. To reduce the number of microorganisms per unit volume, serial dilutions (10^{-1} to 10^{-7}) were prepared and cultured on a nutrient agar medium and incubated at 35°C for 24-48 hrs.

***Streptomyces* isolation**: Bacterial serial dilutions were cultured linearly on starch casein agar plates. All plates were kept at 28 °C for 7 days. *Streptomyces* colonies were then isolated based on their characteristics and transferred to another culture medium.

Characterization of *Streptomyces*: Morphological and biochemical assays were per-

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formed to characterize *Streptomyces* isolates.

Table 1) Response of isolates against standard pathogens in the initial selection stage

Information of Bacterial Isolates	Number of Bacterial Isolates
Positive isolates (in terms of antibacterial activity) against at least one pathogen	1
Positive isolates against Gram-positive bacteria	3
Positive isolates against Gram-negative bacteria	2
Positive isolates against both Gram-positive and -negative bacteria	6
Positive plates	6
Positive plates for *E. coli*	3
Positive plates for *S. aureus*	3
Positive plates for *B. cereus*	4
Positive plates for *B. subtilis*	5
Positive plates for *P. aeruginosa*	2

Antimicrobial activity: In order to identify active *Streptomyces* strains producing antibacterial agents, screening was performed in two separate stages. The following standard pathogens were prepared from the microbial collection available in Iran (*Bacillus cereus* PTCC 1247, *Staphylococcus aureus* PTCC 1112, *B. subtilis* PTCC 1023, *Escherichia coli* PTCC 1330, and *Pseudomonas aeruginosa* PTCC 1707). Perpendicular streak method was used in the primary screening stage, the isolated strains were cultured on Mueller Hinton agar plates and incubated at 28 °C for 7 days. After growing the bacteria in the middle of the plates, common pathogenic bacteria were cultured with the primary bacterial samples and incubated in an incubator at 37 °C. Well diffusion method was used in the secondary screening stage. The positive isolates limiting the growth of pathogenic bacteria in the primary screening stage were selected and inoculated in yeast extract-malt

Table 2) Inhibitory effects of *Streptomyces* isolates against standard pathogens in the primary screening stage

Standard Pathogens	Bacterial Isolates					
	Yellow2D	Yellow 4A	Green 4A	1B	3A	3C
S. aureus	Positive	Positive	Negative	Positive	Negative	Negative
B. cereus	Positive	Positive	Negative	Positive	Negative	Negative
B. subtilis	Positive	Positive	-	Positive	Positive	Negative
P.aeruginosa	Negative	Negative	-	Negative	-	-
E. coli	Negative	-	Positive	Negative	Positive	Positive

Table 3) The results of growth inhibition areas against standard pathogens in the secondary screening stage

Standard Pathogens	Bacterial Isolates and Solvents											
	Yellow 2D		Yellow 4A		Green 4A		1B		3A		3C	
	E A[1]	M[2]	E A	M	E A	M	E A	M	E A	M	E A	M
S. aureus	16[3]	12	18	-	20	-	16	-	14	-	12	-
B. cereus	12	-	13	-	12	-	-	-	-	-	12	-
B. subtilis	15	-	20	10	18	-	10	-	10	-	12	-
P.aeruginosa	17	10	17	-	20	-	12	9	17	12	16	-
E. coli	20	-	18	-	20	10	12	-	13	-	22	15

1: Ethyl acetate, 2: Methanol, 3: Diameter of clear zone inhibition (mm)

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Table 4) Characterization of *Streptomyces* isolates by biochemical tests

Bacterial Isolates	Biochemical Tests									
	Catalase	Oxidase	Simmon Citrate	Endol	Movement Test	H_2S	MR	VP	TSI	Bile Esculin Test
Yellow 2D	+	-	+	-	-	+	-	-	AK/AK	-
Yellow 4A	+	-	+	-	+	-	+	-	A/A	+
Green 4A	+	-	+	-	+	+	+	-	A/A	+
3A	+	-	+	-	-	+	-	-	AK/AK	-
1B	+	-	+	-	-	-	-	-	AK/AK	-
3C	+	-	+	-	-	-	-	-	AK/AK	-

extract broth and incubated at 28 °C for 10 days. Then the medium was centrifuged. The supernatant was mixed with an equal ratio of ethyl acetate or methyl acetate, and the supernatant was separated. Then according to the 0.5 McFarland turbidity standard, indicator bacteria were cultured on Mueller Hinton agar medium, and wells were made. The extract of metabolites was injected into the wells, and the media were incubated at 37 °C for 24 hrs. Antimicrobial properties were determined by measuring the inhibition zone diameter around the colonies

DNA extraction and PCR amplification of 16S rRNA: DNA extraction was performed using Axygen genomic DNA extraction kit (AxyPrep Bacterial Genomic DNA Miniprep, USA). A primer pair was used to identify the conserved region of the S16 rRNA gene (5´-AGAGTTTGATCCTGGCTCAG-3´ as forward primer and 5´-TACCTTGTTACGACTT-3´ as reverse primer). This primer pair was taken from previous studies [16]. For PCR amplification, 23 µL of PCR master mix, containing 2 µL of sample DNA, 0.2 µL of Taq polymerase (5 U/µL), 2.5 µL of PCR buffer, 0.6 µL of $MgCl_2$ (25 mM), 0.5 µL of dNTP (5 mM), 0.5 µL of each primer (10 p.mol), and 18.2 µL of distilled water, was used for each sample. PCR amplification was performed using the following program: an initial denaturation step at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 2 min, and a final extension step at 72 °C for 10 min.

Sequence analysis: ABI Capillary System (Macrogen Research, Seoul, Korea) was used for DNA sequencing, and the sequencing results were observed and evaluated through MEGA software (Ver. 6). The BLAST network service was used to search for similar sequences in EMBL - GenBank databases (www.ncbi. nlm.nih.gov/BLAST/).

Statistical analysis: SPSS software Ver. 21.0 (SPSS Inc., Chicago, Illinois, USA) was used to perform statistical analyses.

Findings

Bacterial count in each region: The total count of bacteria based on sampling location is shown in Figure 1.

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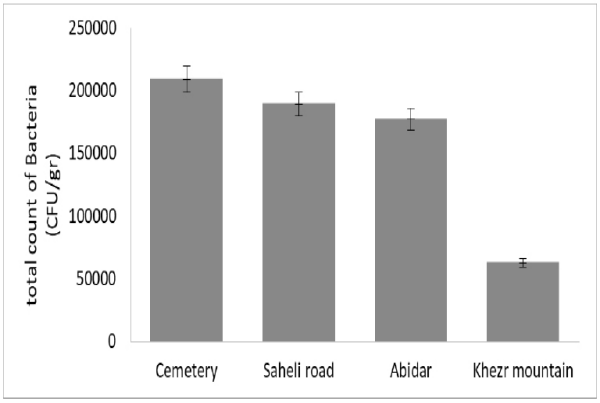

Figure 1) Sampling area and bacterial count

Characterization of *Streptomyces:* Both Gram staining and biochemical tests were used to identify *Streptomyces* species. Figure 2 shows Mueller Hinton broth media, cultured colonies, and Gram staining of branched bacterial forms.

To identify active *Streptomyces* strains in terms of producing antibacterial agents, screening was performed in two stages. In both stages, the standard pathogens were used. After 24 hours of bacterial growth, the growth line of the pathogens was restricted, which indicated the spread of antimicrobial agents into the environment, restricting the growth of pathogens. At this stage, among the 20 *Streptomyces* bacterial strains that were isolated from the soil samples, six isolates were selected in the primary screening stage based on their ability to limit the growth line of pathogens (Figure 3). Table 2 compares the intensity of pathogen growth inhibition by *Streptomyces* isolates. The positive strains showed more ability to inhibit the growth of pathogens, the negative isolates had no inhibitory effect, and the other isolates showed a very weak inhibitory effect. Regarding the growth inhibition zone diameter, the secondary screening stage results showed that the largest inhibition zone diameters were related to Yellow 4A and Green 4A isolates.

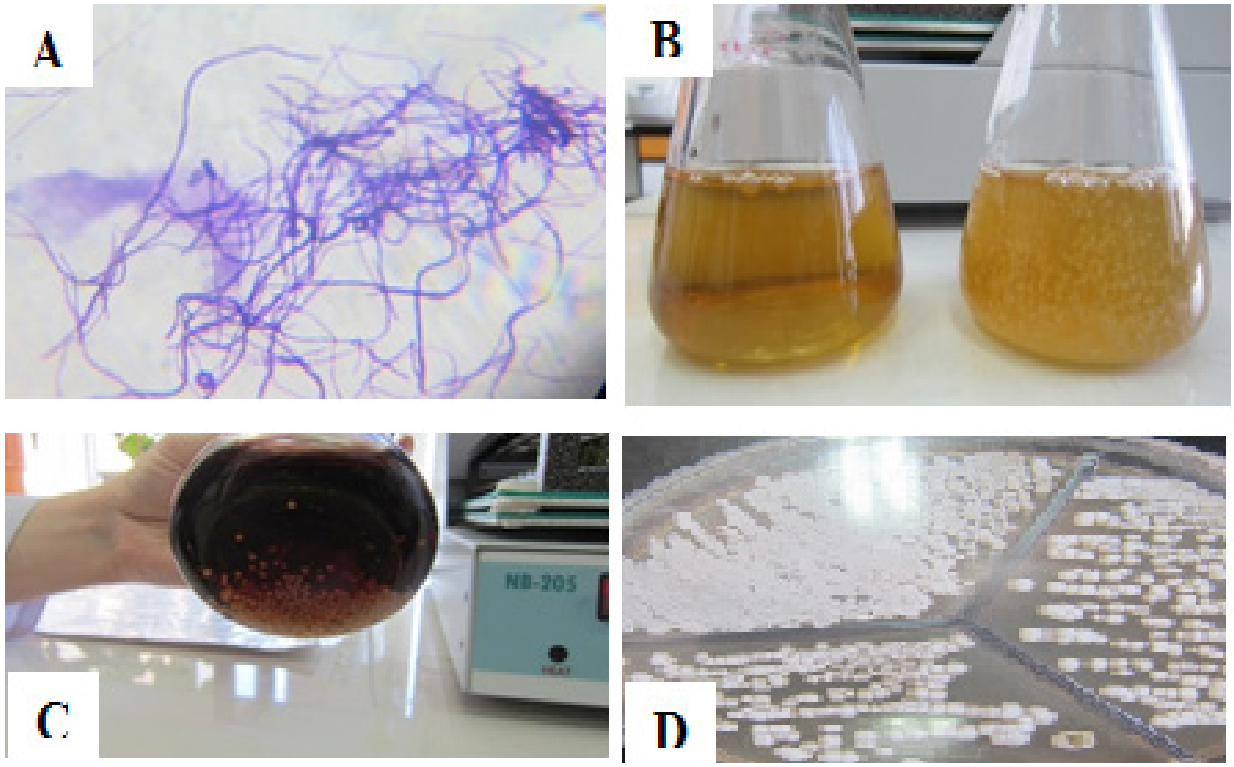

Figure 2) A) Gram staining of *Streptomyces* isolates with branched forms. B and C) Mueller Hinton broth media and cultured colonies, and D) colonies on starch casein agar media

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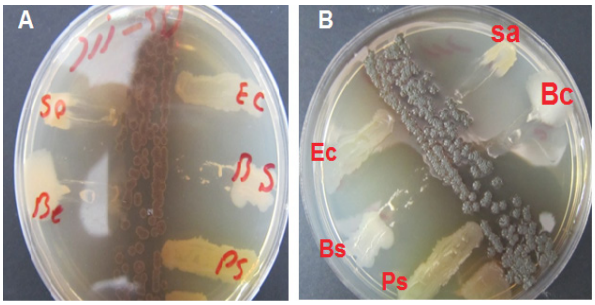

Figure 3) Streak plating technique used for primary screening of antibacterial activity of isolated *Streptomyces* strains. A and B) back and front of the culture of Yellow 4D isolate, respectively; the vertical line is related to *Streptomyces* isolate, and the perpendicular lines are marked with the abbreviated names of pathogenic strains; Sa: *S. aureus*, Bc: *B. cereus*, Ec: *E. Coli*, Bs: *B. subtilis*, and Ps: *Ps. aeruginosa.*

Biochemical Tests: The results of all biochemical tests performed are given in Table 4.

DNA extraction and PCR: Among the six isolates selected based on their antimicrobial activity, two isolates with the highest antibacterial activity were selected for the sequencing process. These two strains had the highest growth inhibition zone diameters against standard pathogens. After DNA extraction, 2 µL of the extracted DNA was used for gel electrophoresis to confirm the accuracy and quality of DNA content. Figure 4 presents the 16s rRNA region.

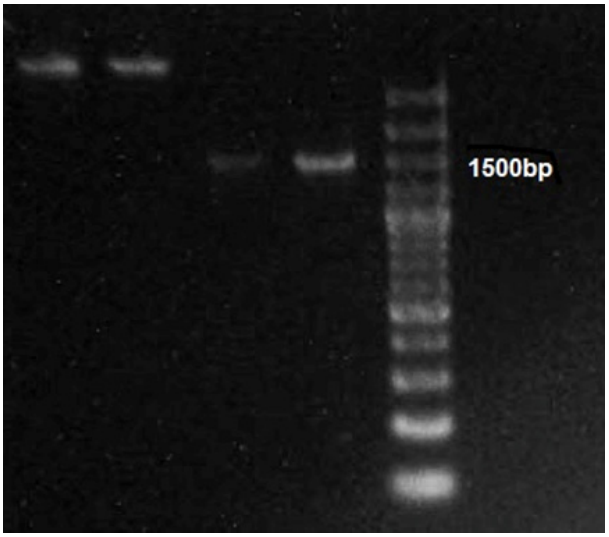

Figure 4) 16s rRNA region of isolated and selected bacteria on agarose gel electrophoresis

Sequence searches in the NCBI database and sequencing results: Two selected isolates were superior to other isolates in terms of producing antimicrobial substances and were selected for molecular identification. The chromatogram and sequence of each sample were presented on a CD (compact disc) in ab1 format by Gene Fan Avran. Then the necessary analysis was done by MEGA software (Ver.6).

Figures 5 and 6 show the molecular identification results of the isolates. Based on the dendrogram analysis and NCBI database, isolates Yellow 4A and Green 4A with 98% similarity were identified as *S. faradia* and *S. coelicolor*, respectively.

Discussion

Streptomyces species are widely used to produce secondary metabolites with diverse biological activities, including antibiotics [17]. Isolation of new microbial species from undiscovered environments is essential to generate potentially valuable metabolites [18]. In this study, an attempt was made to identify the active strains of *Streptomyces* with high antibiotic production efficiency in different soil samples collected from Sanandaj city in Iran using various biochemical and molecular techniques. Distinguishing factors such as soil pH, sampling site temperature, and morphological features of colonies such as color and pigmentation were used to classify the bacterial population. According to the results of the antimicrobial assay, the antibacterial activity of putative isolates was desirable in both primary and secondary screening stages.

The isolates that showed antimicrobial activity in the initial screening stage, such as isolate 1B, were inactive during extraction and analysis with experimental microorganisms. One of the reasons for justifying this finding may be the lack of proximity of pathogens to these isolates and the loss of competitive

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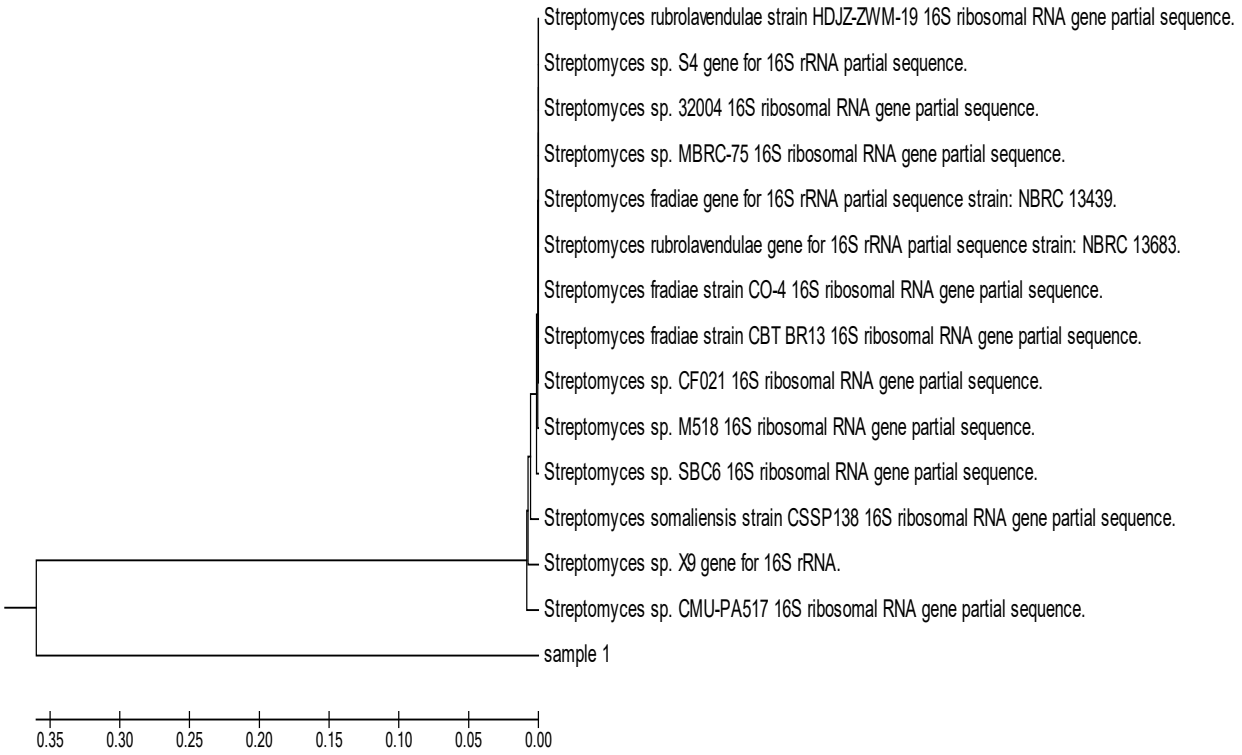

Figure 5) 16S rRNA tree showing the phylogenetic relationship for Yellow 4D isolate

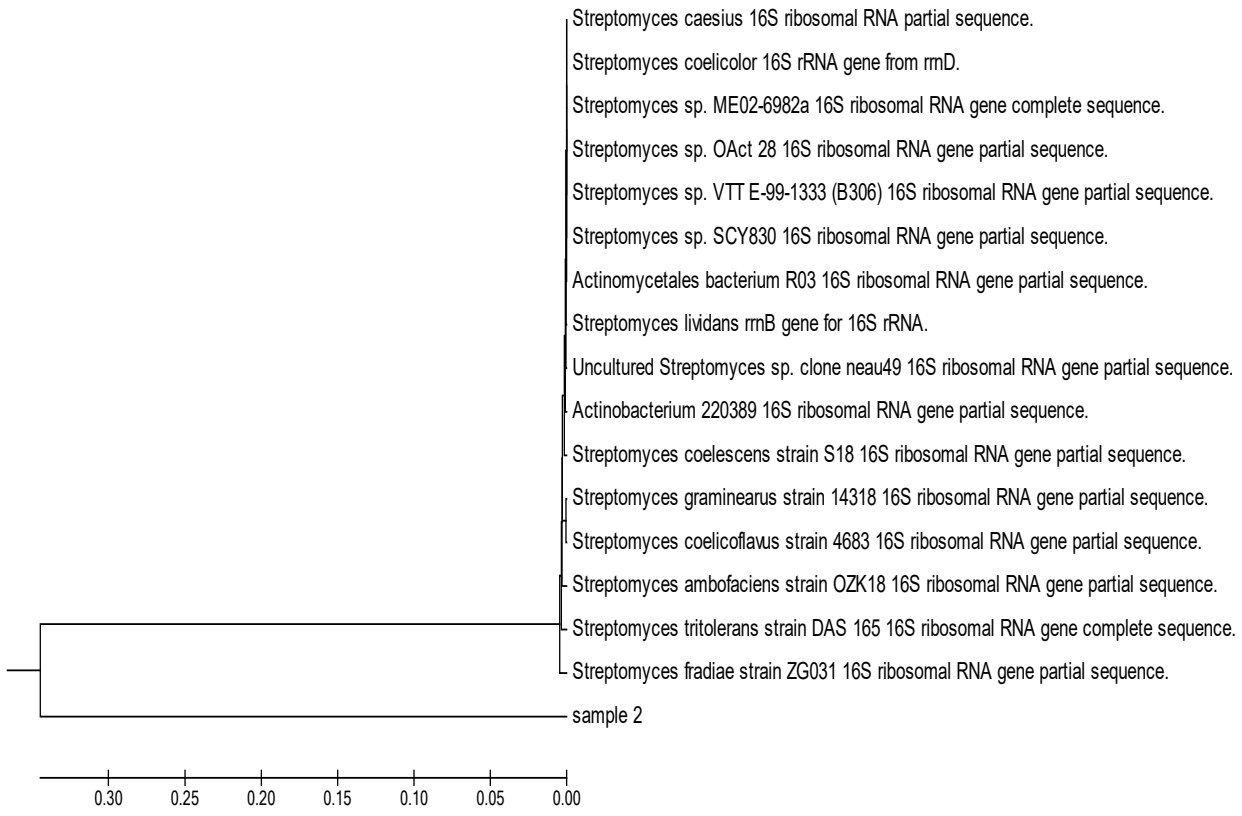

Figure 6) 16S rRNA tree showing the phylogenetic relationship for Green 4A isolate

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space. On the contrary, the results showed that isolates 3C and Green 4A had no significant activity in the primary screening stage, but among the isolates tested in the secondary screening stage, they showed an acceptable activity, so that they could powerfully limit all five pathogens. Consistent with this study results, Asgharzadeh and Manda (2021) reported the presence of new isolates of *Streptomyces* in the soil samples of West Azerbaijan province, which produced new types of antimicrobial therapeutic agents [19]. In this study, well diffusion method was carried out to screen antibacterial activities. In this method, the release of antibiotics from a well into the solid culture medium inhibits the growth of bacteria. According to Gao and Cranston (2008), halo formation is a qualitative method and alone is not sufficient to accurately evaluate the potency of bacterial activity [20].

However, according to this study results, the growth of *B. subtilis* seemed to be more limited than the growth of other bacteria. This finding is in line with the findings of previous studies reporting that Gram-positive bacteria are more sensitive to metabolites produced by *Streptomyces*. The reason for the difference in the sensitivity of bacteria could be ascribed to their unique cell wall structure [18]. According to Shirling and Gottlieb (1966), this difference is due to the fact that Gram-negative bacteria are resistant to a wide range of lipophilic compounds due to their impermeable outer membrane compared to Gram-positive bacteria. In contrast, Gram-positive bacteria are surrounded only by layers of peptidoglycan, which is not an effective permeability barrier [21]. In the present study, it was found that in soil samples with pH values above 7, *Streptomyces* species had more bacterial population than other genera. This result is also in agreement with the findings of Dehnad et al. (2010) [22]. Moreover, this study results showed that the population diversity of *Streptomyces* spp. was higher in the soils of agricultural areas than in other soils. In agreement with this result, Burke et al. (1989) also showed that *Streptomyces* communities increased in areas under agriculture [23]. Also, Zhang et al. (2016) reported that *Streptomyces* populations increased after land use conversion from forest to agricultural soil [24]. Based on this study findings, among the solvents (ethyl acetate and methanol) used to extract metabolites, ethyl acetate was the most suitable solvent, this solvent probably removes non-polar metabolites from the culture medium. These results confirm the findings of Soofiani et al. (2011), which indicated that among the different solvents (ethyl acetate, chloroform, hexane, dichloromethane, and diethyl ether) used to extract metabolites, ethyl acetate was the best solvent for extracting metabolites [25]. Maleki et al. (2013) conducted a study to isolate and characterize new strains of *Streptomyces* with high antibiotic production ability. Of the 140 isolates collected, 12 *Streptomyces* isolates were identified with high antibacterial activity against pathogenic bacteria. Using RAPD analysis, they showed that the two isolated strains G614C1 and K36C5 had significant antimicrobial activity and were very similar to *S. coelicolor* and *S. albogriseolus*. In the present study, one of the two isolates with high antimicrobial activity was similar to *S. coelicolor* [26]. Sapkota et al. (2020) conducted a study to isolate various strains of *Actinomycetes* with antimicrobial activity from soils of different areas of Nepal. In their study, 41 strains were isolated from 11 soil samples, and then the strains with different morphological characteristics were tested for antimicrobial activity. During the secondary stage of screening active isolates using ethyl acetate extract, 70.7% were identified as *Streptomyces* with antibiotic activity [27].

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Conclusion

In conclusion, in the current research, 50 soil samples were collected from four different areas of Sanandaj city, and 20 *Streptomyces* strains were isolated from these soil samples. In order to identify *Streptomyces* strains producing antimicrobial agents, screening was performed in two primary and secondary stages. Two isolates with 98% similarity were identified as *S. faradia* and *S. coelicolor*, respectively. However, more studies are required to detect active metabolites of the mentioned isolates. It is worth noting that the use of proper strategies to identify potential new *Streptomyces* species with antibacterial properties may bring a bright future in treatment of resistant pathogens.

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Ethical permissions: This study desing was evaluated and approved by the ethics committee of the Islamic Azad University.

Conflicts of interests: The authors declare that they have no conflict of interest.

Authors' contributions: Moravej R. (First author), Original researcher/ Discussion author (60%); Honarmand Jahromi S. (Second author), Original researcher/ Methodologist/ Discussion author (20%);Azad P. (therth author), original researcher/ Methodologist (20%).

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