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RESEARCH ARTICLE

Molecular Identification and Antibacterial Activity of *Streptomyces* spp. Isolated from Sulaymaniyah Governorate Soil, Iraqi Kurdistan

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ABSTRACT

Soil is play an important role for reserve abundant groups of microorganisms, especially *Streptomyces*. *Streptomyces* are recognized as prokaryotes, aerobic and Gram-positive bacteria with high Guanine + Cytosine contents in their DNA. These groups of bacteria show filamentous growth from a single spore and they are normally found in all kinds of ecosystems, including water, soil, and plants. A total of three *Streptomyces* strains were isolated from soil of the sides of Darband Ranya in Sulaimani governorate. Different approaches were followed for the identification of the isolated stains. Morphological and cultural properties of these isolates have shown that the isolates are belonging to the genus *Streptomyces*. Desired colonies of the isolates were distinguished and separated from other bacteria on the basis of colony morphology, pigmentation, ability to produce a different color of aerial hyphae, and bottom mycelium on raffinose-histidine agar and starch-casein agar media. In addition, analysis of phylogenetic tree based on 16S rRNA gene sequences, the strains related the genus. KS010 isolates had the highest identity (99.32%) with the type strain of *Streptomyces atrovirens*, while KS005 and KS007 isolates were most closely related to *Streptomyces lateritius* by identity 99.32%. The isolated test strains were also active against Gram-negative and Gram-positive pathogenic bacteria following co-cultivation technique. However, further study should be done to identify the types of these antibacterial compounds.

Keywords: 16S rRNA; Antimicrobial activity; Genomic DNA; *Streptomyces*; Tyndallization technique

INTRODUCTION

Streptomyces species are Gram-positive, aerobic, slow-grower bacteria that have filamentous morphology extensively branched substrate and aerial mycelia (Jüttner and Watson, 2007; Dyson, 2011). They produce and release spore after the segmentation of aerial hyphae (Glauert, 1961). The spore produces a wide range of pigments that they responsible for the color of the aerial mycelia and vegetative (Flärdh and Buttner, 2009).

Rather than the morphological characterization such color of spore or hyphae, *Streptomyces* bacteria can be identified by genomic analysis of 16S rRNA, DNA-DNA hybridization, and the types of cell wall composition such as fatty acids, phospholipids, and peptidoglycan (Korn-Wendisch and Kutzner, 1992). *Streptomyces* contains high guanine and cytosine contents (69–73.0%) in their DNA sequence (Dastager and Sunil, 2016) while, interestingly, the origin of chromosomal replication is high in adenine and thymine contents (Ventura et al., 2007).

For the 1st time, genus *Streptomyces* was introduced by Waksman and his colleague in 1943 (Waksman and Henrici, 1943). *Streptomyces* are able to live and grow in various habitats, soil is one of the best environment for its surviving and reproduction (Maleki et al., 2013; Mokrane et al., 2013; Hasani et al., 2014). This bacterium is defined as the largest diverse microorganism in soil that covers 40% of soil bacteria, while some biological, physical, and chemical factors are an effect on the distribution of *Streptomyces* in soil, including temperatures, pH, salinity, moisture, soil texture, food stress, and climate. This genus has great economic and industrial importance since they produce valuable enzymes and about two-thirds of all-natural origin useful antibiotics (Hopwood, 2007; Belghit et al., 2016; Bunyapaiboonsri et al., 2016). Moreover, anti-parasite, immunosuppressant, antibacterial, and antifungal compounds have been identified as secondary metabolism products of *Streptomyces* (Sanghvi et al., 2014; Balachandran et al., 2015; Rambabu et al., 2015). High ratios (75%) of bioactive medicinal and commercial beneficial molecules are produced by this type microorganism. Thus, researchers have focused on *Streptomyces* (Bérdy, 2005).

Some species of *Streptomyces* have the ability to produce melanin that is important for protection against broken by chemical stress, high temperature, and biochemical threats (Madhusudhan et al., 2014).

The present study was aimed to isolate and characterize antibiotic producer *Streptomyces* from different soil samples collected at various unique environments of Darband Ranya in Sulaimani governorate because that place has not been investigated for isolation of *Streptomyces* and other Actinobacteria genera. Hence, it is really necessary to isolate *Streptomyces* from these soil samples so as to recover new *Streptomyces* species which produce antimicrobial compounds.

MATERIALS AND METHODS

Soil Sampling and Isolation of *Streptomyces* spp.

The sites of soil sample collection were in specific habitats of lakesides of Darband Ranya in Sulaimani governorate. Twelve soil samples were collected from the lakeside. To collect these samples, a sterile tubular was used from a depth of 15 cm after removing nearly 5 cm of the topsoil and placed the materials in sterile plastic bags. The samples were kept in a refrigerator (4°C) until microbial assays were performed in Microbiology Laboratory for bacterial isolation. They were subjected for serial dilution up to 10^{-4} dilution.

Soil serial dilution plating technique (0.1 ml of dilution 10^{-3} and 10^{-4}) was followed to isolate *Streptomyces* spp. starch casein agar medium supplemented with nystatin (50 μg/ml), cycloheximide (50 μg/ml), and novobiocin (25 μg/ml), and raffinose-histidine agar medium supplemented with nystatin (50 μg/ml) and cycloheximide (50 μg/ml) (Vickers and Williams, 1987). Culture plates were incubated at 28°C for 7–10 days (Tatar and Sahin, 2015; Balachandran et al., 2016). *Streptomyces* strains were identified on the basis of their ability to utilize carbon and nitrogen sources, biochemical reactions, and amplification of the whole 16S rRNA gene. After incubation, colonies of *Streptomyces* were identified based on the pigmentation of the colonies and ability to produce a different color of aerial hyphae and substrate mycelium on each of Starch-casein agar and Raffinose–Histidine agar culture media. Desired colonies were distinguished and chosen for analyzing in detail.

Chemotaxonomy and Morphology

Morphology of the bacterial colonies was used as one of the crucial characters to identify the genus of *Streptomyces*. Among the similar color of colonies, only one colony was selected to study on. The bacterial colonies were streaked on Oatmeal agar to detect spore aerial hyphae, substrate mycelium color, and pigmentation of the diffusible pigments. Modified Bennett's agar was also used to obtain pure colonies from mix cultures. A pure colony was suspended in 20% glycerol by scrapping the aerial and substrate mycelium from the modified Bennett's agar plates. The suspension was finally stocked at −80°C. Standard procedures were followed to detect isomers of diaminopimelic acid and sugar analysis (Staneck and Roberts, 1974).

Nutritional tests on the isolated strains

Nitrogen source

It has been observed that the amount of antibiotic synthesis by the bacteria relates to each concentration and type of nitrogen source in their culture media (Rafieenia, 2013; Sánchez et al., 2010). *Streptomyces* species were checked for their ability to utilize nitrogen compounds such as potassium nitrate, L-tyrosine, and L-histidine. These compounds were sterilized by following Tyndallization technique (Atalan, 1993). The isolated strains were added to the sterilized nitrogen medium to give 0.1 concentrations (W/V). To test nitrogen utilization, the following media were used; basal medium contained L-Proline was used as a positive control and free nitrogen basal medium was used as a negative control. These media were incubated at 28°C for 4–7 days after inoculating the media with the bacterial strains and supplementing with desired nitrogen sources.

Carbone source

Glucose substrate becomes slow down the rate of antibiotic production growth (Lounes et al., 1996; Sánchez et al., 2010). The isolated strains were tested for their capability to use the following carbon sources; Dextran, D-Fructose, D-Mannitol, D-Raffinose, Lactose, Sucrose, Maltose, D-Mannose, Sodium acetate, and sodium citrate. These carbon sources were added to carbon utilization agar (ISP9). In addition to ISP9 medium, a basal medium contained D-glucose was used as a positive control and free basal medium used as a negative control. These media were inoculated with the desired isolates, incubated for 4–7 days at 28°C.

Antimicrobial activity assay

The following wild bacteria were used to check of antibacterial activity of the tested strains: *Klebsiella pneumoniae, Staphylococcus aureus*, *Pseudomonas fluorescens*, *Escherichia coli,* and *Bacillus subtilis* (El-Naggar, 2015). Perpendiculars technique was followed to study the antibacterial activity of the isolates. In this method, the isolates were inoculated on Muller–Hinton agar and incubated at 28°C for 7–10 days. The wild pathogenic strains were then streaked around the growth area of the isolates. The culture medium was incubated again at 37°C for up to 48 h. Antibacterial producer isolates were able to inhibit the growth of the pathogenic strains and made a clear zone.

Tolerance Tests

The isolates were studied to investigate their ability to grow at 28°C in the modified Bennett's agar medium

supplemented with the following chemical inhibitors; crystal violate (0.0001%, w/v) and sodium azide (0.01%, w/v). The culture medium was incubated for 7–10 days at 28°C and 50°C (Atalan, 1993).

Molecular Characterization and Phylogenic Analysis of *Streptomyces* spp.

Pure isolates were streaked on glucose yeast malt extract agar and incubated at 28°C for 7–10 days. A loopful of an isolate was inoculated into glucose yeast malt extract broth and incubated at 28°C for more 7–10 days under shaking condition (190 rpm). After the incubation period, 1 ml of the liquid culture was added into a sterile 1.5 ml Eppendorf tube and centrifuged at 13,000 rpm for 5 min to precipitate the pellet at the bottom of the tube. The pellets were collected to make genomic DNA as described by Pitcher (Pitcher et al., 1989), following Guanidine thiocyanate DNA isolation method and using DNA isolation kit (Invitrogen, Pure Link (R) Genomic DNA Kit).

Purified genomic DNA (3 µl) was run in an agarose gel electrophoresis (60 ml of ×1 Tris Borate EDTA buffer, 0.6 g agarose) contained ethidium bromide (4 µl). The following specific universal primers, 27f (5'-AGAGTTTGATCMTGGCTCAG-3') and 1525R (5'-AAGGAGGTGWTCCARCC-3') (Lane, 1991), were used to amplify 1500 base pairs the whole 16S rRNA gene (Chun and Goodfellow, 1995). Polymerase chain reaction (PCR) mixtures of 50 µl were prepared. This stock was contained 1 µl of the genomic DNA, 1 µl of each primer, 25 µl of GoTaq® Hot Start Colorless Master Mix, and 22 µl of ddH_2O. Thermal cycler (MyGenie-96 Gradient Thermal Cycler, Korea) was used to amplify the 16S rRNA gene under the following PCR condition (Shen et al., 2016); denaturation at 95°C for 15 min/1 cycle, denaturation at 95°C for 1 min/35 cycle, annealing at 55°C for 1 min/35 cycle, extension at 72°C for 3 min/35 cycle, extension at 72°C for 10 min/35 cycle, and holding at 25°C for 1 min/1 cycle. The PCR product (3 µl) was run in 1% agarose gel electrophoresis (60 ml of ×1 Tris Borate EDTA buffer, 0.6g agarose) contained ethidium bromide (4 µl) to confirm the quality of the DNA [Figure 1]. The PCR product was then purified and sequenced with these sequencing primers; 518F (5'-CCAGCAGCCGCGGTAAT-3'), 800R (5'-TACCAGGGTATCTAATCC-3'), and Mg5f (5'-AAACTCAAAGGAATTGACGG-3'). Sequencing of the 16S rRNA was carried out by Macrogen Company in Netherland. The sequenced 16S rRNA were compared with out-group *Kitasatospora nipponensis* HKI 0315T (AY442263) using ChromasPro (V2.1.3) (Kim et al., 2012). Phylogenetic dendrograms were drawn using the neighborhood-joining algorithm and the evolutionary distance matrix (Jukes and Cantor, 1969). EzTaxon server was used to find the closest strains to the tested strains using 1000 replicates and MEGA6 package program used for the phylogenetic analyzes (Tamura et al., 2013), while bootstrap analysis of the phylogenetic trees was done using Felsenstein (Felsenstein, 1985).

RESULTS

Morphological and Physiological Characterization of *Streptomyces spp.*

Melanin pigment formation on peptone-yeast extract iron agar, ability to produce diffusible pigment colors, substrate mycelial, and aerial spore mass on oatmeal agar was followed as some characters to identify the selected *Streptomyces* strains. Most of the researchers agree with that morphology is a suitable method to differentiate taxes (Gauze et al., 1957). Pigmentation was one of the primitive approaches to identify *Streptomyces* isolates (Pridham, 1958). Colony colors of the isolates were compared with the direct matching of color charts tables ISSC-NBS [Table 1 and Figure 1] (Shirling and Gottlieb, 1970). Based on this chart, gray, olive, and pink color groups were counted as *Streptomyces* genus. Only one isolate from each color group was selected to further study. The selected isolates were also studied to show their ability to utilize different carbon and nitrogen compounds [Table 2]. These data show that the strains utilize some or all of the nutritional sources. For example, both KS005 and KS010 utilized L-Histidine,

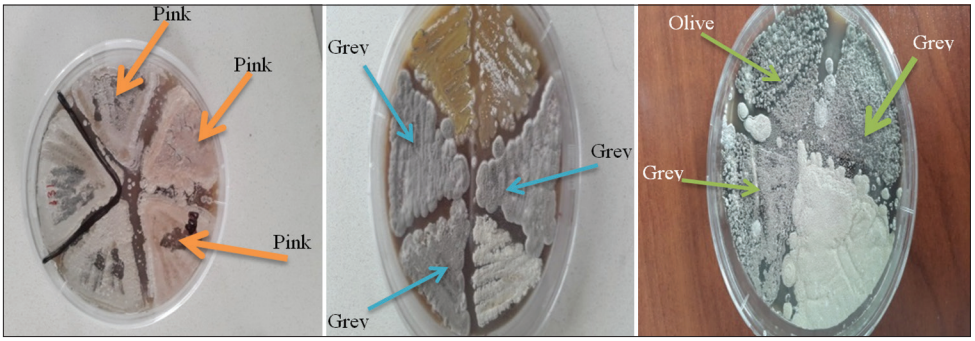

Figure 1: Selected isolated bacteria that were grown on oatmeal agar. These show three different colors on the basis of aerial and substrate mycelium. The samples were incubated for 7–10 days at 28°C, KS010 (pink), KS005 (Gray), and KS007 (Olive)

Table 1: Color grouping of the isolated bacteria

Bacterial isolates	Aerial spore mass	Colony reverse	Melanin pigmentation on peptone yeast extract iron agar
KS003, KS001, KS012, and KS005	Gray	Pale orange-yellow	None
KS007, KS002, KS008, and KS011	Olive	Olive green	Yes
KS010, KS009, KS006, and KS004	Pink	Light yellow	None

They were grown on peptone yeast extracts iron agar and oatmeal agar at 28ºC for 7–10 days. KS designates the source of the soil samples (Darband Ranya in Sulaimani governorate), 003 to 012 designate to the 12 sources of the soil collection areas

Table 2: Characteristics of Streptomyces isolates

Test strain microorganism	KS005	KS007	KS010
Growth in sole nitrogen source			
Potassium nitrate	Positive	Negative	Positive
L-Tyrosine	Negative	Positive	Negative
L-Histidine	Positive	Negative	Positive
Growth in sole carbon source			
Dextran	Negative	Positive	Negative
D-Fructose	Positive	Positive	Positive
Lactose	Negative	Negative	Negative
D-Mannitol	Negative	Positive	Positive
D Raffinose	Positive	Positive	Positive
Sucrose	Negative	Negative	Negative
Maltose	Positive	Positive	Positive
D-Mannose	Negative	Positive	Negative
Sodium acetate	Positive	Positive	Positive
Sodium citrate	Positive	Negative	Positive
Pathogenic bacteria used to check antibacterial activity of the isolates			
Klebsiella pneumoniae	Negative	Positive	Positive
Staphylococcus aureus	Positive	Positive	Positive
Pseudomonas fluorescens	Negative	Negative	Negative
Escherichia coli	Positive	Negative	Positive
Bacillus subtilis	Negative	Positive	Positive
Thermophilic bacteria			
50ºC	Negative	Negative	Negative

Growing of the isolates in carbon and nitrogen sources, in high-temperature environment and growing with pathogenic bacteria

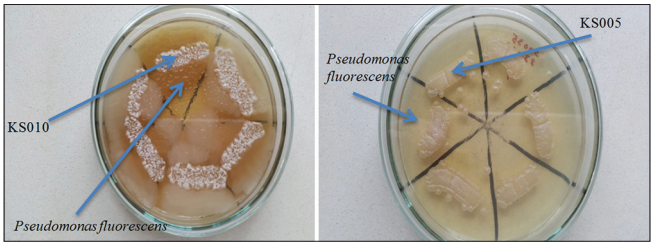

Figure 2: Antimicrobial activity of the isolates against pathogenic bacteria. KS005 and KS010 strains were grown on Muller–Hinton agar media incubated at 28°C for 4–7 days and the pathogenic bacteria were then streaked to around test strains, incubated for more 48 h at 37°C

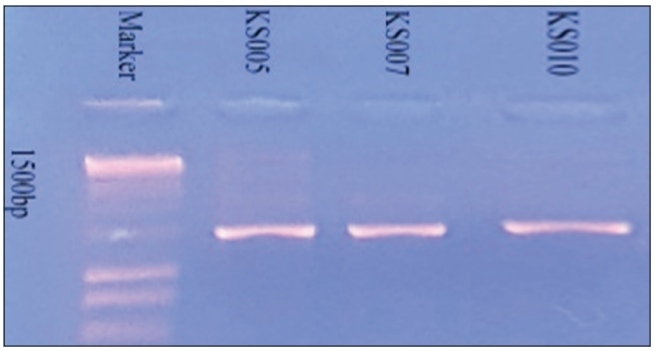

Figure 3: Agarose gel electrophoresis (1%) of PCR products. The product was from amplified 16S rRNA gene marker

but KS007 strain was not able to utilize it, while KS007 used L-tyrosine instead. In addition, the behavior of the strains to inhibit the growth of *S. aureus* and *B. subtilis* Gram-positive and *E. coli*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae* Gram-negative bacteria were studied [Table 2 and Figure 2]. The isolates produced active antibacterial substances against all of the pathogens apart from *P. fluorescens*. Further study can be done to identify these antibacterial molecules. As another characterization, the strains were grown in high temperature (50°C) so as to isolate thermophilic *Streptomyces* bacteria because most of the antimicrobial producer *Streptomyces* are life in high temperature (Place and Station, 1987). This result shows that none of the isolates can grow in this environment.

Streptomyces spp. Identification using Conserved 16S rRNA

The 16S rRNA gene region was sequenced using sequenced primers 27f, 800r, and MG5f [Figure 3]. The sequenced data were aligned with the sequence data of the closest related species which are in international databases using EzTaxon-e server to determine (%) similarities. Phylogenetic analysis showed that the new strains were cluster with reference *Streptomyces* spp. The 16S rRNA gene of the tested KS005 and KS007 strains had the highest sequence similarities (99.32%) with their type strain *Streptomyces lateritius*, while KS010 was closely related to *Streptomyces atrovirens* with a high identity of 99.32% [Figures 4 and 5]. Along with the physiological and morphological properties, the phylogenetic analyses have also provided crucial evidence that the tested strains are belong to *Streptomyces* genus.

DISCUSSION

There are some antibiotic-resistant microbes that have become universal health problems, especially in developing countries (Mazel and Davies, 1999). This study worked toward to isolate and characterize *Streptomyces* from distinct habitats in Sulaimani governorate so as to identify *Streptomyces*

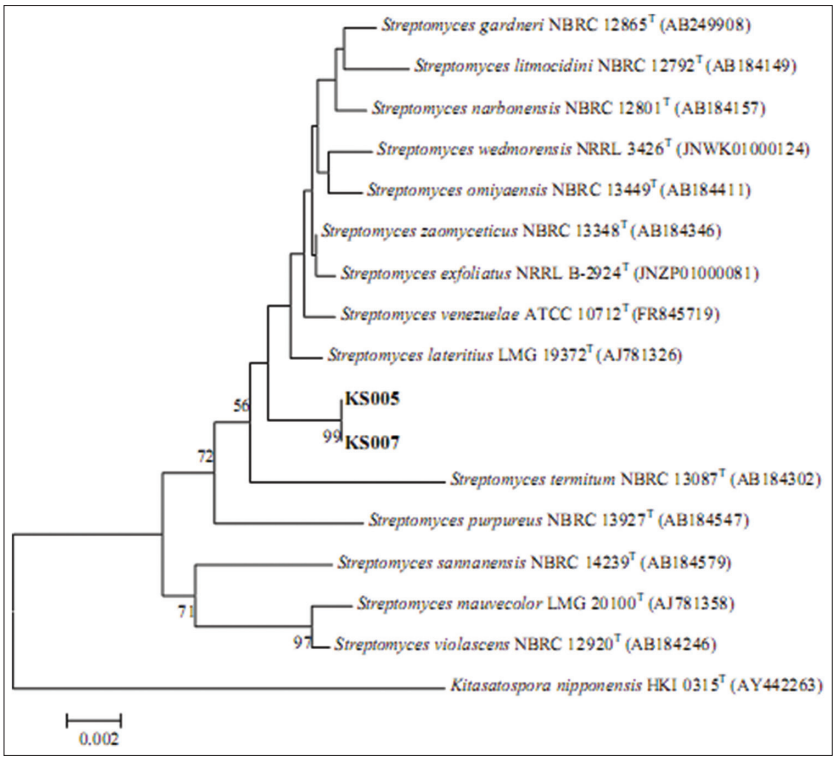

Figure 4: Phylogenetic dendrogram comparison of KS005 and KS007 tested strains with references strains. Complete sequence 16S rRNA gene of reference strains was downloaded from GenBank to align with the new strains using dendrogram neighbor-joining algorithms. The dendrogram and numbers at nodes detected the level of bootstrap support, only value ≥56% is shown. The 16S rDNA sequence of *Kitasatospora nipponensis* HKI 0315^T (GenBank accession number AY442263) was used as an out-group. Numbers on the branches indicate Bootstrap percentage after 1000 replications during constructing the phylogenetic tree. Scale bar refers to a phylogenetic distance of 0.002 nucleotide substitutions per site

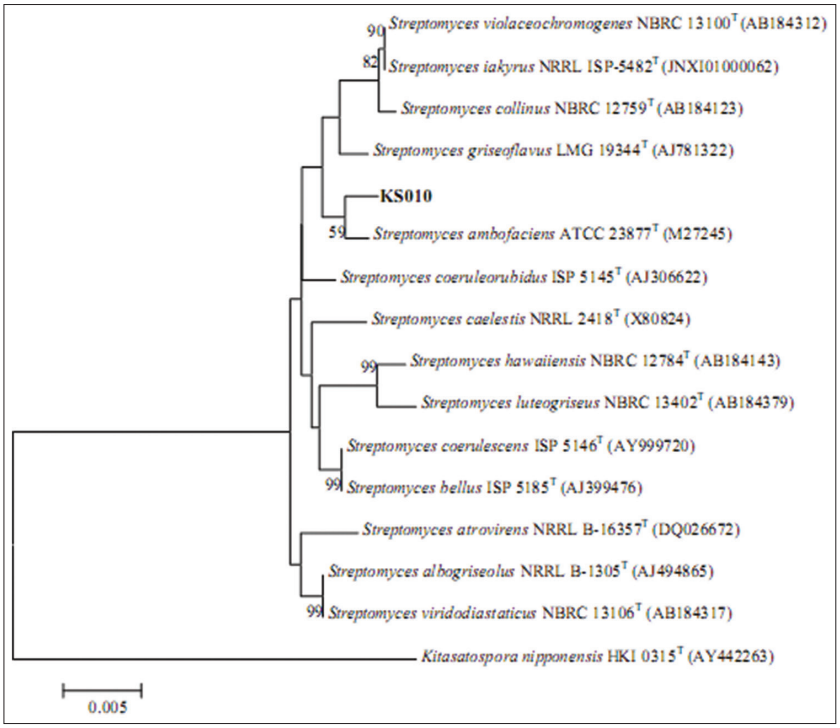

Figure 5: Phylogenetic dendrogram comparison of KS010 isolate with references strains. Dendrogram produced neighbor-joining algorithms and numbers at nodes detect the level of bootstrap support (%), only value ≥59% is shown. The 16S rDNA sequence of *Kitasatospora nipponensis* HKI 0315^T (GenBank accession numbers AY442263) was used as an out-group. Numbers on the branches indicate Bootstrap percentage after 1000 replications during constructing the phylogenetic tree. Scale bar refers to a phylogenetic distance of 0.005 nucleotide substitutions per site

strains. As two crucial properties, morphological characters of colonies such as pigmentation and antimicrobial activity were followed for initial identification of the selected strains. In addition, it is reported that 16S rRNA gene is stable, ubiquitous, and conserved subject to horizontal gene transfer (Stackebrandt et al., 2002). Therefore, it is an effective molecular marker for the primary key for phylogeny-based identification of archaea and bacteria (Kim et al., 2012; Olsen and Woese, 1993; Stackebrandt et al., 2002; Tindall et al., 2010). The phylogenetic analysis revealed that KS010 strain is closely related to *S. atrovirens* based on the highest identity (99.32%), while KS005 and KS007 strains were close to *S. lateritius*. These phenotypic and genotypic properties have provided evidence that all of these three strains are belonging to *Streptomyces* genus. Furthermore, KS010 isolate can make an inhibition zone against all the pathogenic Gram-negative and Gram-positive bacteria except *P. fluorescens*, and KS005 and KS007 strains have shown antibacterial activity against *S. aureus*. This might relate to these isolates can make a useful type of bioactive compounds against pathogenic organisms (Mao et al., 2011; Balachandran et al., 2015).

CONCLUSION

This study has shown several *Streptomyces* strains which were isolated from specific environmental habitats of Darband Ranya in Sulaimani governorate. All of the physiological and phylogenetic analysis have provided evidence that KS005, KS007, and KS010 strains are belong to the genus *Streptomyces*. The isolates also have made antibacterial compound against most of the tested pathogens. This behavior plus biochemical properties suggesting that this microorganism might be adapted to survive in a specific environment. Further characterizations of the three *Streptomyces* are necessary to confirm that they are novel strains. Additional studies can be done to isolate and identify more *Streptomyces* genera from the studied areas following the DNA-DNA hybridization technique.

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