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Abstract

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Keywords

Actinobacteria, Streptomyces, Antibiotic, 16Sr DNA and Diaminopimelic acid, Soil

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RESEARCH ARTICLE

Phylogenetic Analysis of *Streptomyces* spp. Isolated from Soil Samples in Sulaimani Governorate

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ABSTRACT

Streptomyces species have been an important source of bioactive secondary metabolites and clinically useful for antibiotic production, including fosfomycin, daptomycin, oxytetracycline, streptomycin, and chloramphenicol. The main objective of this study was to isolate actinomycetes, especially *Streptomyces* from 8 soil samples that collected from two different places, which are districts in Sulaimani governorate, Kurdistan Region of Iraq. Totally, 30 diagnostic tests were carried out for representative of isolated *Streptomyces* depending on the color groups. The 16Sr DNA gene was sequenced, for ten isolated test strains were amplified with 2 universal primers after extraction of genomic DNA, only 8 of them were successfully sequenced. Phylogenetic analysis of 8 test strains carried out using base sequences of 16Sr DNA genes in the core genome. Four of isolated test strains were identified as a *Streptomyces fulvissimus*, and others were nominated as a *Streptomyces anulatus*. The obtained sequence data were compared with the sequence data of the closest related species in the international databases using EzTaxon Server program and (%) similarities were determined. Finally, phylogenetic analysis indicated that isolated test strains were nominated as (H001, H002, H003, and H004), which recognized members of the *S. anulatus* and the similarity with their type strain is 99.93%. Whereas, other four isolated test strains, including D001, D002, D003, and D004 recognized as members of the *S. fulvissimus* and similarity with their type strain is 100%.

Keywords: Actinobacteria; *Streptomyces*; Antibiotic; 16Sr DNA and Diaminopimelic acid; Soil

INTRODUCTION

Actinobacteria are one of the most widely distributed groups of microorganisms in the world, particularly in the soil. *Streptomyces* species are belonging to the Actinobacteria class. They are recognized as gram-positive and aerobic soil bacteria that show filamentous growth from a single spore. As their filaments grow through tip extension and branching, they ultimately make a network of branched filaments called a substrate mycelium (Dyson, 2011). Biochemical analysis data were carried out to study *Streptomyces* (Williams et al., 1983; Kampfer et al., 1991) and also rRNA/DNA similarities were used by Witt and Stackebrandt (Witt and Stackebrandt, 1990). The member of the genus *Streptomyces* is filamentous bacteria, which protect soil from erosion during wind; cause remains the texture of the soil and ability to survive during alkaline and dry condition (Vetsigian et al., 2011). In general, *Streptomyces* is described as the largest genus of Actinobacteria (Yousif et al., 2015). More than 770 species of *Streptomyces* have been recognized (Bunyapaiboonsri et al., 2016). Back to year (1943), species of *Streptomyces* for the 1st time were introduced by Waksman and Henrici (Waksman and Henrici, 1943). *Streptomyces* are able to grow in various environments (Maleki et al., 2013). Soil is the best habitat for reproduction and surviving of *Streptomyces* (Mokrane et al., 2013). Species of *Streptomyces* greatly dispersed in soil, water, and other environments. They are the largest diversity bacteria in the soil, including 40% of soil bacteria. Some biological, physical, and chemical factors are effects on the distribution of *Streptomyces* in an ecosystem, including temperatures, pH, salinity, moisture, soil texture, food stress, and climate (Hasani et al., 2014). Synthesizing of bioactive secondary metabolites and complex developmental cycle are common characters of *Streptomyces*. This genus normally produces 1/3 of commercial antibiotics that are available nowadays. Moreover, antiparasitic, immunosuppressant, antibacterial, and antifungal compounds have been selected as secondary metabolism products of *Streptomyces* (Omura, 1992; Hopwood, 2007; Newman and Cragg, 2007; Goodfellow and Fiedler, 2010; Mao et al., 2011). *Streptomyces* species have a dual role in the source of bioactive secondary metabolites, especially antibiotics that have a contribution to human health, as an antibiotic producer microorganism, *Streptomyces* accounting for perhaps 70% of the antibiotic compounds so far discovered (Lewin et al., 2016; Bérdy,

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2005). Renewing recognition, the value of this genus is an attempt to understanding the ecology of *Streptomyces* for drug discovery (Asamizu et al., 2015).

MATERIALS AND METHODS

Collectıon of Soil Samples

Different locations such as mountains, valleys, and lakesides were chosen to obtain soil samples. They were obtained from different sites of Pshdar and Ranya/Sulaimani governorate. They were collected in a depth of 20 cm after removing nearly 5 cm of the surface soil and put in a sterile container. The samples were stored in sterile plastic bags before being transported to the lab, then they kept in the refrigerator at 4°C till the microbial assays performed.

Physiochemıcal Characterıstics of Soil Samples and Isolation Process

Physicochemical characters of soil samples (pH, moisture, and organic matter content) were measured quickly after samples brought to the laboratory (Reed and Cumming, 1945). The bacterium was maintained by cultured at approximately weekly intervals on starch-casein agar (Küster, 1964) and raffinose-histidine agar (Vickers et al., 1984). The plates were inoculated inside of the laminar flow cabinet and they were allowed to dry, then they inverted position and incubated at 28°C for 2 weeks. After incubation, the desired colonies were distinguished and separated from other bacteria on the basis of colony morphology, pigmentation, and ability to produce a different color of aerial hyphae and substrate mycelium on starch-casein agar and raffinose-histidine agar plates [Table 1]. Then, they were isolated and coded according to locality. Furthermore, modified Bennett's agar was used to get a pure colony of desired strains; after that, they were stored in a sterile 2 ml Eppendorf tube containing 20% glycerol and stored at −80°C.

Table 1: List of isolated *Streptomyces* species, type of isolation medium, and source of test strains

Strains	Source	Isolation medium
D001	Delo	Starch casein agar
D002	Nuraddin	Starch casein agar
D003	Darband	Raffinose-histidine agar
D004	Piran	Raffinose-histidine agar
H001	Halsho	Starch casein agar
H002	Darwina	Raffinose-histidine agar
H003	Qamtaran	Starch casein agar
H004	Dukan	Starch casein agar

Table 2: List of the diagnostic tests used for identification of isolated microorganisms

No	Tests	No	Tests
1.	Casein	16.	Potassium nitrate
2.	Raffinose	17.	Tyrosine
3.	Gelatin	18.	Sodium chloride 7%
4.	Dextran	19.	Crystal violet 0.0001%
5.	Fructose	20.	Ampicillin (20 mg)
6.	Maltose	21.	Vancomycin (30 mg)
7.	Mannose	22.	Penicillin (30 mg)
8.	Starch	23.	Gentamycin (10 mg)
9.	Sucrose	24.	Rifampin (5 mg)
10.	Lactose	25.	Erythromycin (15 mg)
11.	Mannitol	26.	*Escherichia coli*
12.	Histidine	27.	*Pseudomonas fluorescens*
13.	Sodium citrate	28.	*Bacillus subtilis*
14.	Sodium acetate	29.	*Candida* sp.
15.	S. propionate	30.	*Staphylococcus aureus*

Biochemical Analysis of Desired Test Strains

The isolated test strains were examined for these tests including (Nutritional test: Capability of test strains to use ten different nitrogen carbon sources for growth and energy, degradation of sterilized: Casein, starch, and gelatin, capability of isolates to grow in different chemical inhibitors: Antibiosis, antimicrobial activity, and test strains were evaluated for their antimicrobial action against Gram-positive bacteria [*Staphylococcus aureus* and *Bacillus subtilis*] and Gram-negative bacteria [*Escherichia coli, Pseudomonas fluorescens*] and fungi including *Candida* sp.) [Table 2].

Molecular Characterization of the Isolated Test Strains

Isolation of genomic DNA

Genomic DNA test was examined very accurately, and carry out on eight test strains representatives of color grouping were subject to DNA isolation. The method for DNA isolation of the isolated test strains is described by Pitcher (Pitcher et al., 1989). DNA isolation method with DNA isolation kit (Invitrogen, Pure Link (R) Genomic DNA Kit) was used.

DNA isolation control

About 1% agarose gel (60 ml 1X TBE buffer, 0.6 g agarose) supplemented with ethidium bromide 4 µl was prepared and used to control the presence of extracted DNA. The prepared gel was loaded by adding a mixture of 3 µl total genomic DNA and 2 µl of ethidium bromide. After DNA loaded, agarose gel placed in the electrophoresis tank and run at 100 volts for 45 min. The DNA band was checked under UV transilluminator (Vilber Lourmat, UV) and captured a photograph.

Polymerase chain reaction (PCR) amplification of 16s rDNA gene

After isolation of genomic DNA, the16S rRNA gene was amplified by two universal primers 27f, (5'-AGA GTT TGA TCM TGG CTC AG-3) and 1525R, (5'- AAG GAG GTG WTC CAR CC-3'). Stock solutions were prepared for the PCR reaction by sterile ddH_2O. They were separated in sterile Eppendorf tubes in small quantities 25–100 µl

to remove the risk of contamination and stored at −20°C until use. PCR procedures for the 16S rDNA genes were carried out at a Thermal Cycler (MyGenie-96 Gradient Thermal Cycler, Korea) in a 0.2 ml PCR tube.

Phylogenetic analysis of 16s DNA sequence

The 16S rDNA gene was subjected to sequencing with methods supplied by Chun and Goodfellow (Chun and Goodfellow, 1995). The obtained PCR product were sequenced with three primers, including 518F, (5'-CCAGCAGCCGCGGTAAT-3), 800R (5'-TACCAGGGTATCTAATCC-3'), and Mg5f (5'-AAACTCAAAGGAATTGACGG-3). DNA sequencing of 16S rDNA of eight isolated test strains was carried out by MacroGen Company (Netherland). The complete 16S rDNA sequences of all isolates strain were analyzed with Chromas version 1.7.5 (McCarthy, School of Health Sciences, and Griffith University, Queensland, Australia) program. The DNA sequencing was calculated aligned with the previously deposited sequences, the similarities and most closely related with their type of strains using EzTaxon server (Kim et al., 2012). The sequences of isolates type of strains were bring from GenBank and aligned using (CLUSTAL W in MEGA6) program (Tamura et al., 2013). Construction of the phylogenetic trees was achieved using the neighbor-joining (Saitou and Nei, 1987), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Fitch, 1971), and algorithms in the MEGA6 program (Tamura et al., 2013). The topology of the phylogenetic trees was analyzed by the bootstrap resampling method of Felsenstein (Felsenstein, 1985) with 1000 replicates.

Chemotaxonomic Characterization

The strains were subjected for morphological and chemotaxonomic properties of the typical for genus *Streptomyces* (Trujillo, 2012). Chemotaxonomy is a biological method for studying the chemical difference in microbial cell and the use of chemical features in the classification and identification of bacteria, including *Streptomyces* and it is a crucial technique. One of the isolated test strains was inoculated in glucose yeast malt extract broth (ISP6) in flask with shaken 180 rpm at 28°C for 1 week to determine the chemotaxonomic study. Cells were collected by centrifugation and washed 2 times in sterile distilled water and re-centrifuged and freeze at −80°C, after that lyophilized at lyophilization for 16 h to getting dried bacterial cell mass. Then, chemotaxonomic studies, including sugar analysis and diaminopimelic acid (DAP), have been done.

RESULTS

Physiochemical Parameter of Soil Samples

The physiochemical results of the soil samples were investigated, including organic pH matter content and moisture contents of the soil samples. The highest pH (8.63) was recorded in soil sample number D001 in Piran location. The highest amount of organic matter content (10.2%) was recorded in soil sample number H003 that collected from eSewa. The highest amount of moisture content (9.13) was recorded in the soil sample number H002 that collected from Darband Table 3.

Distribution and Numbers of *Streptomyces*

Starch casein agar and raffinose-histidine agar were used to isolate *Streptomyces* bacteria (Atalan, 1993). The soil sample was inoculated after doing serial dilution and the Petri plates prepared for the isolation process. After inoculation, they incubated at 28°C for 14 days. Eight pure strains were isolated by streak plate method and some of the isolation Petri plates are shown in Figure 1.

Phenotypic Characterization

Traditional identification tests such as biochemical, carbon source, nitrogen source, chemical inhibitor, temperature, antibiotics, morphology, pigmentation, and growth tests were used for both identification and numerical analysis. Total eight selected test strains were examined for 30 tests [Table 4 and Figures 2 and 3].

Genomic DNA Extraction

The genomic DNA of eight test strains was extracted using a specific method that was described by (Pitcher et al., 1989) and DNA isolation kit (Invitrogen, Pure Link (R) Genomic DNA). The extracted total genomic DNA was loaded on agarose gel electrophoresis and shown in Figure 4.

Figure 1: Colony appearance of *Streptomyces* on plates of starch-casein agar and Bennett's agar

Table 3: pH, moisture, and organic matter content of collected soil samples

Number	Isolated strain	pH	Organic content	Moisture content
1.	D001	8.63	8.21	2.60
2.	D002	7.84	7.27	5.23
3.	D003	7.33	8.37	5.50
4.	D004	7.27	6.43	5.27
5.	H001	8.44	5.53	3.57
6.	H002	7.35	2.77	9.13
7.	H003	8.55	10.20	5.45
8.	H004	7.97	5.83	5.67

PCR Product of 16S rDNA Genes

The 16S rDNA gene region for eight test strains was amplified using the gradient PCR with the universal primers 27f and 1525r. The size of 16S rDNA region was average 1500 base pairs. The 16S rDNA bands that amplified by PCR are seen on agarose gel electrophoresis [Figure 5].

Phylogenetic Tree and Analysis of 16S rDNA Sequence

The 16S rDNA genes region were amplified by QIA quick PCR Purification Kit; then, they sequenced with primers 27f, 800r, and MG5f. EzTaxon server and (%) similarities were used for comparison of obtained sequence data with the sequence data of the closest related species in the international databases. The dendrogram was generated to determine phylogenetic positions of test isolated strains and their relations with 16S rDNA sequence data [Figure 6].

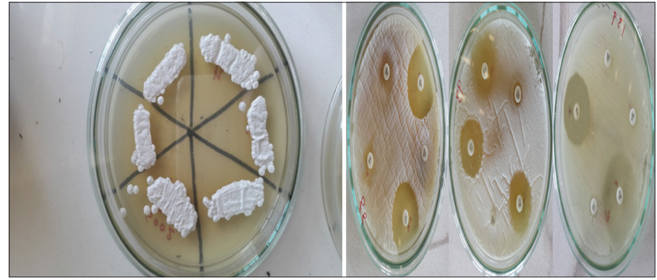

Figure 2: Antimicrobial activity of isolated strain against pathogenic bacteria and fungi with Antibiotic resistance tests

Table 4: Data obtained for biochemical analysis of representative of *Streptomyces* strains

No. of strain tests	D001	D002	D003	D004	H001	H002	H003	H004
Nutritional tests								
Growth on sole carbon source (1%, w/v)								
Dextran	+	−	−	+	−	+	−	−
Fructose	+	+	+	+	+	+	+	−
Lactose	−	−	−	−	−	−	−	−
Mannose	−	−	+	−	+	+	+	+
Raffinose	+	+	−	+	+	−	−	−
Sucrose	−	−	+	−	−	−	−	+
Maltose	+	−	+	−	+	+	+	−
Mannitol	−	+	−	+	+	+	−	+
Growth on sole carbon source (0.1%, w/v)								
Sodium acetate	+	−	−	+	−	−	−	−
Sodium citrate	+	−	−	−	+	−	+	−
Sodium propionate	+	+	+	+	+	+	+	−
Growth on sole nitrogen source (0.1% w/v)								
Histidine	+	+	+	+	+	+	+	+
KNO3	+	+	−	+	+	−	+	+
Tyrosine	-	+	+	−	+	−	+	+
Degradation tests								
Casein	+	−	+	−	−	+	−	+
Starch	+	−	−	−	+	−	+	−
Gelatin	+	−	+	−	−	+	−	+
Tolerance tests								
Resistance to chemical inhibitors								
Sodium chloride 7%	+	−	+	−	−	−	+	−
Crystal violet 0.0001%	−	+	−	−	+	+	−	−
Resistance to antibiotics								
Amoxicillin AMC (30 mg)	+	+	+	+	+	+	+	+
Rifampicin RA (5 mg)	−	+	+	+	−	−	−	+
Ampicillin SAM (20 mg)	+	−	+	−	+	+	+	+
Gentamycin CN (10 mg)	−	+	−	−	−	−	−	−
Erythromycin E (15 mg)	+	+	+	−	+	+	+	+
Amoxicillin AMC (30 mg)	+	−	+	+	+	+	+	+
Antimicrobial activity tests								
Escherichia coli	+	+	+	−	+	+	+	+
Staphylococcus aureus	+	−	+	+	+	+	+	+
Candida sp.	−	+	+	−	+	+	+	−
Pseudomonas fluorescens	+	−	+	+	+	−	+	+
Bacillus subtilis	+	+	+	−	−	−	−	−

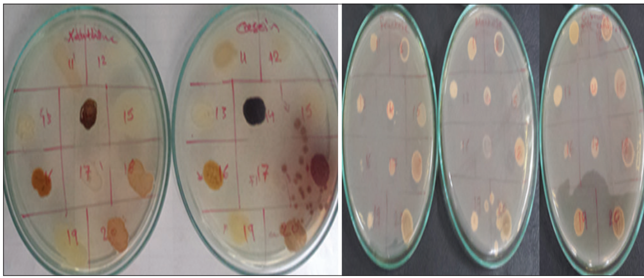

Figure 3: Nutritional source for isolated strain including carbon and nitrogen source

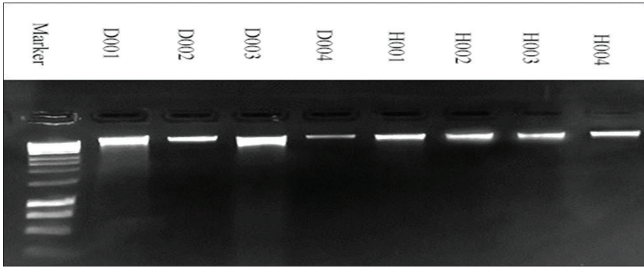

Figure 4: Whole genomic DNA bands of 8 test strains on 1% agarose gel electrophoresis image (left side is marker 1500 bp DNA ladder)

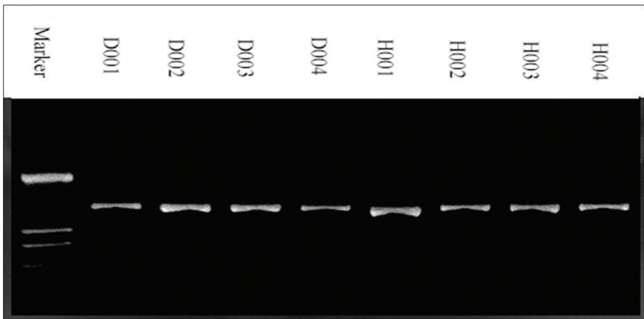

Figure 5: Polymerase chain reaction amplified product of the 16S rDNA gene region was loaded using 1.5% agarose GEL electrophoresis (left side is marker 1500 bp DNA ladder)

The dendrogram was drawn using the neighborhood-joining algorithm and the evolutionary distance matrix (Jukes and Cantor, 1969). MEGA6 package program was used for phylogenetic analyzes (Tamura et al., 2013). The phylogenetic trees bootstrap analysis created according to Felsenstein (Felsenstein, 1985) for 1000 replicates. *Kitasatospora nipponensis* HKI 0315T (AY442263) used as an outgroup for all strains of *Streptomyces* dendrograms as a result of phylogenetic analysis of sequence data, 8 isolate strains were identified as *Streptomyces* spp.

Chemotaxonomic Analysis

Chemotaxonomic analyses were performed to determine the characteristic chemical properties of the isolates. These analyzes include DAP and sugar analyzes. The one-dimensional thin-layer chromatography was used to determine DAP type in the cell wall of the test strain and

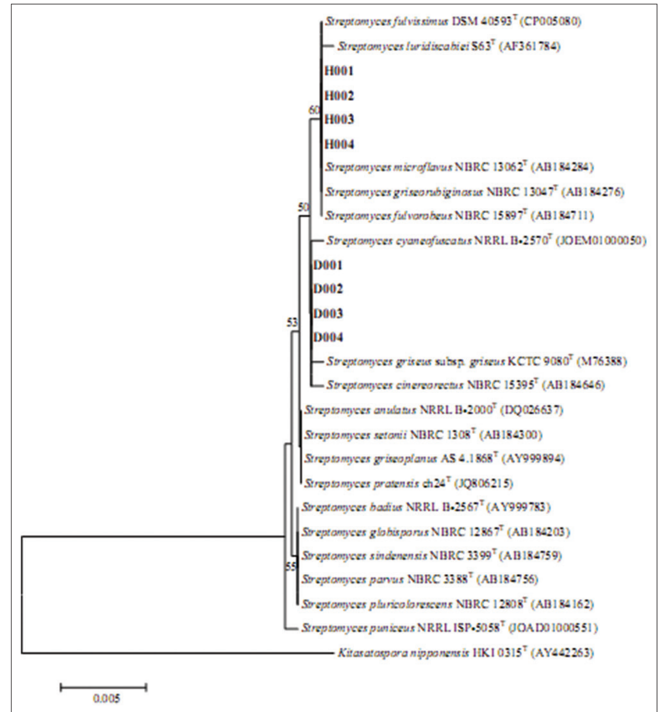

Figure 6: Phylogenetic dendrogram shows the relation of 8 test strains of *Streptomyces* regarding base sequence of 16S rDNA gene. Dendrogram produced neighbor-joining algorithms and numbers at nodes indicate the level of bootstrap support (%), only value ≥50% is shown. GenBank accession numbers are given in parentheses. Bar 0.005 substitutions per nucleotide position

compared with the standard A2pm solution. The result shows that test strain *Streptomyces* contains the LL-A2pm content and whole-cell sugar profile contains glucose.

DISCUSSION

Soil is an ecological treasury with many organisms living together and some of them producing useful natural products, including antibiotics. The isolated strains showed arrange of phylogenetic, morphological properties, and chemotaxonomic content. They classified that are belong to the genus *Streptomyces* (X-L, 2012). Pshdar and Ranya are two of the places that have not been investigated for isolation of *Streptomyces* and other Actinobacteria genera. In this study, we tried to identify the active strains of *Streptomyces* species. Ranya is the place that has not been investigated the soils for isolation of *Streptomyces* and other Actinobacteria genera. Hence, it was really necessary to isolate *Streptomyces* from the soil sample there because of recovering new *Streptomyces* species producing a new antimicrobial compound. The physicochemical characters of soil samples affected the recovery of *Streptomyces* and other bacteria on selective media. However, the number of soil *Streptomyces* were detected and different based on soil use and also parallel with soil pH. Watkins reported that

organic matter content of the soil correlates with land use and pH, but does not correlate with recovery of *Streptomyces* (Watkins, 2013). Antony-Babu et al. suggested that the numerical analysis result of the color group supported the visual display of data as dendrograms posed and show the recognition of taxa regards to similar color characteristics (Antony-Babu et al., 2010). Some researchers suggested that the ability to separate unknown *Streptomycetes* to color groups, which can be equated with species-groups (Atalan et al., 2000; Sembiring et al*.,* 2000) (Tan et al., 2006). Identification of bacterial strains is still a difficult subject for microbiologist despite the development of molecular biology techniques and the development of kits that are commercially available phenotype-based identification tests. Since the 16S rRNA gene is ubiquitous, stable, conserved, and poorly subject to horizontal gene transfer, it is an effective molecular marker for the identification of bacteria. It is well known that analyses of 16S rRNA gene sequence of the strains are a fundamental technique for archaea and bacteria and are being used for the identification with the classification of the prokaryotes (Olsen and Woese, 1993) (Stackebrandt et al*.,* 2002). Hence, sequencing and phylogenetic analysis of the 16S rDNA genes is easy and reliable method to classify and identify of bacterial isolates at various taxonomic levels. Moreover, the almost complete 16S rRNA gene sequence was compared with the corresponding sequences of representative members of color groups using MEGA (Altschul et al*.,* 1997) and the results revealed the highest similarity 99.93%. D001, D002, D003, and D004 were identified *Streptomyces anulatus*. While, (H001, H002, H003, and H004) strains were identified with *Streptomyces fulvissimus* at 100% similarity. In conclusion, the phylogenetic analysis based on the sequencing of 16S rRNA gene revealed that 8 test strains belong to genus *Streptomyces* and have high sequence similarity to *Streptomyces*.

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