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Halotolerant streptomycetes isolated from soil at Taif region, Kingdom of Saudi Arabia (KSA) I: Purification, salt tolerance range, biological and molecular identification

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This study focuses on isolation and purification of some halotolerant streptomycetes from soil and sea water of western region, KSA as a source of salt tolerance gene(s). A few numbers (32) of streptomycetes-like colonies (SLC) were isolated and purified from two regions. From Jeddah, a number of 22 out of the 32 SLC were obtained, distributed between the sea water 12 (54.55%) and sea sediment soil 6 (27.27%).From Taif-soil, only 10 SLC were isolated. Results show that 31 SLC were grown on 3.5% salt, while, in the presence of 7.0% salt, 2, 3, 18, and 4 showed abundant (+++), moderate (++), weak (+), and in doubt (±) growth, respectively. Only five SLC were not able to grow at 7% salt. At the level of 10.5% salt, the number of SLC was decreased up to 4 (2 (+++), 1 (++) and 1(+)). In either 14% or 21% salt, four isolates were varied in their ability in growth as moderate or weak growth was recorded. These isolates were considered as halotolerant as they were able to grow in either the presence or normal growth medium. The four isolates which tolerate 14% salt (isolates 4 and 6) and 21% salt (isolates 2 and 8) were completely identified. The streptomycete isolate 2 appeared to be related to *Streptomyces cirratus.* Comparing the cultural, morphological and physiological characteristics of *Streptomyces* isolate 4 and 6, they were very likely to be strains of *S. rishiriensis* and *S. alboflavus*, respectively. *Streptomyces* isolate 8 was identified as a strain of *S. luteogriseus*. The nucleotide sequences of 16S rRNA gene was partially determined using the DNA template of the *Streptomyces* isolate 8. Results show that a final sequence of about 1462 nts was obtained and compared with eight universal *Streptomyces* and bacterial strains. This isolate could be classified as a new species of gray *Streptomyces*, and it was suggested to be named a new halotolerant *Streptomyces* sp. TSA-KSA strain (GenBank AB731746).

Key words: Key words: *Streptomyces,* halotolerant, identification, 16S rRNA, Taif, Kingdom of Saudi Arabia (KSA).

INTRODUCTION

Streptomycetes are usually considered to be strict aerobes and they can grow in sterile soil at low oxygen concentration and in dry soil, their counts decrease (Wong and Griffin, 1974). Several recent investigations reported the presence and importance of actinomycetes belonging to the genus *Streptomyces* in different soil types (El-Abyad et al., 1996 and Altalhi and Mohamed, 2010). Mohamed et al. (2000) reported the presence of halotolerant actinomycete in soils collected from Damietta, Ismailia, Port Said and Sinai Governorates, Egypt. Mohamed et al. (2001) isolated five streptomycetes obtained from Sinai sandy soil in Egypt, having the ability to tolerate 9 to 12% NaCl in the growth medium. Mohammad and Galal (2005) isolated four halotolerant *Streptomyces* (QS01, QS02, QS03 and QS04) from Qaroon Lake, which had the ability to grow on 7% NaCl concentration in the starch nitrate agar medium. Ai et al. (2009) revealed that the novel salt-tolerant strain DUT_AHX, that was identified as *Streptomyces albidoflavus* was able to grow in the presence of NaCl up to 12 (w/v).

At Taif, KSA, Mohamed et al. (2012) isolated and purified some actinomycetes. The isolates were identified as streptomycetes and their color groups were determined. The salt tolerance range of the purified isolates was determined by growing them on starch nitrate agar medium supplemented with different NaCl concentrations ranging from 3.5 to 10.5%. The DNAs of the identified isolates were extracted and used for determination of DNA fingerprinting of the isolates using the random amplified polymorphic of DNA-polymerase chain reaction (RAPD-PCR). They also, amplified the 16S rRNA gene of a *Streptomyces* isolate using PCR from its DNA followed by determination of its nucleotide sequences and used as a classified molecular tool. Shori et al. (2012) showed that seven streptomycete isolates of KSA-soil samples were able to grow in the presence of 7% NaCl in the starch nitrate agar medium. At concentration of 10.5% NaCl, four isolates grew with weak growth (+) and three isolates showed in-doubt growth (±).

Song et al. (2004) classified scab-causing *Streptomyces* spp. and relatives isolated from potato scab lesions collected in Jeju, Korea, and they determined the 16S rRNA gene sequences for 34 strains, including 11 isolates. On the basis of phylogenetic analysis of 16S rRNA gene sequences, most of the isolates were classified as *Streptomyces scabiei* and *Streptomyces acidiscabies*. The importance of halotolerant *Streptomyces* isolated from soil and/or marine as sources of new compounds from their metabolites were recently reported by several studies (Alvarez-Mico et al., 2013; Bhave et al., 2013 and Su et al., 2013). In the course of a screening program for bioactive compounds from a marine natural product library, a newly isolated Actinomycetes strain, designated as MS100061, exhibited strong anti-*Mycobacterium bovis* Bacillus Calmette-Guérin (BCG) activity. The strain belongs to the genus *Streptomyces* according to its morphological and 16S rDNA phylogenetic analysis (Chen et al., 2013).

This investigation was designed to isolate and purify some halotolerant streptomycetes from soil and sea water at Taif (western region, KSA) and determine the salt tolerance range of the purified isolates followed by biological and molecular identification of the selected isolate(s) using the 16S rRNA gene.

MATERIALS AND METHODS

Collection of samples

Sea water and soil samples were collected from different locations of two areas (Taif and Jeddah) of western region of Kingdom of Saudi Arabia.

Isolation, purification and maintenance of halotolerant streptomycetes

Starch nitrate agar medium (Waksman and Lechevalier, 1961) prepared using sea water was used for isolation, purification and maintenance of streptomycetes existing in all tested samples. Plate technique (Mohamed, 1998) was applied for streptomycetes isolation. The inoculated plates were incubated at 30 ± 2°C for 7 to 15 days. Thereafter, streptomycetes-like colonies appearing on the plates were picked up, purified by streaking technique (Mohamed, 1998) and maintained on starch nitrate agar slants at 4 to 5°C until used.

Determination of salt tolerance range of purified streptomcetes

All purified streptomycetes were tested for their ability to grow on increasing salt (NaCl) concentrations, that is, 0.5 (normal NaCl concentration of the medium), 3.5, 7.0, 10.5, 14.0 and 21% in the growth medium. The growth of streptomycetes on media with and without NaCl was recorded as described by Mohamed et al. (2000).

Identification of selected halotolerant streptomycetes up to species

Identification of selected streptomycetes which showed abundant or

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moderate growth on high concentration of NaCl up to species was done according to the proposed key of Pridham and Tresner (1974) in Bergey's Manual of Determinative Bacteriology (1974). Media as well as methods used in these keys were described by Shirling and Gottlieb (1966). Identification was based on cultural, morphological and physiological characters.

Cultural characters

The selected halotolerant streptomycetes were determined as described by Shirling and Gottlieb (1966) using three media, that is, inorganic salt-starch agar medium, glycerol asparagine agar medium and yeast extract-malt extract agar medium.

Morphological characters

The morphology of spore chain was determined by direct microscopic method (Kawato and Shinobum, 1959) using starch nitrate agar medium. The spore chain of the selected streptomycetes was defined based on the morphological groups described by Pridham et al. (1958). In addition micromorphology of spore surface was determined by the technique described by Tresner et al. (1961) using transmission electron microscope (Jeol JEM-1008 electron microscope) at electron Microscope Unit, National Cancer Institute, Cairo University, Egypt.

Physiological characters

The ability to produce melanoid pigment was detected using the media, that is, tyrosine agar medium, peptone-yeast extract iron agar medium (and tryptone-yeast extract broth medium. Observation was made after two and four days from inoculation. The formation of diffusible grayish brown to brown black pigments or a distinct brown pigment modified by other colors were recorded as positive reaction, while the absence of these colors was recorded as negative reaction.

Ability to utilize different carbon sources as sole sources of carbon was achieved using basal mineral salt medium as described by Shiring and Gottlieb, (1966). In this test, eight carbon sources, namely, D-glucose, D-xylose, L-arabinose, L-rhamnose, D-fructose, D-mannitol, i-inositol and sucrose were separately added at the rate of 10 gL^{-1}. The inoculums of halotolerant *Streptomyces* isolates were streaked on the surface of the Petri dish (containing 20 ml of solid medium). After incubation for 14 days at 30ºC±2 the growth was recorded and compared with the positive as well as negative controls (Mohamed, 1998). Medium containing D-glucose served as positive control, while medium without carbon source served as negative control.

Growth on Czapek-Dox agar medium

The ability of halotolerant *Streptomyces* isolates to grow on Czapek-Dox agar medium (Waksman, 1967) was tested. The slants were inoculated and incubated at 30 ± 2°C up to 15 days.

Sensitivity to streptomycin

Sensitivity of selected streptomycetes to the streptomycin was checked on starch nitrate agar medium as described by Mohamed et al. (2000).

Antibiosis activities of the selected halotolerant *Streptomyces*

Antimicrobial activities of selected streptomycetes against bacteria (*Salmonella sp., Staphylococcus* sp*., Escherichia coli* and *Bacillus subtilis*) and three fungi (*Aspergillus* sp., *Rhizoctonia* sp*.* and *Fusarium* sp.) were carried out. These test organisms were kindly provided by Department of Biology (Girls Branch, Qarwah), Faculty of Science, and Taif University. Standard inoculums for each tested *Streptomyces* strain were prepared as described by Mohamed *et al*. (2000).

Bacterial strains were cultivated on nutrient agar medium (Jacobs and Gerstein, 1960) and fungal strains on potato agar medium (Waksman and Lechevalir, 1961). Using sterile discs of filter-paper Whatman of about 10 mm diameter were made, 3-4 discs were saturated with 0.1 ml of the supernatant. Petri-dishes were further kept in a refrigerator for one hour to permit diffusion of the supernatant. Inoculated plates were incubated at 30ºC±2 for 24-48 hours. The inhibition zones (mm) were determined as described by British Pharmacopoeia (1968). To serve as control, 0.1 ml of un-inoculated starch nitrate broth was used as a control.

Molecular identification of a halotolerant *Streptomyces* strain using 16S rRNA gene

The DNA of the selected halotolerant *Streptomyces* isolate was used as a template for PCR-isolation of 16S rRNA gene using two universal primers (518F: CCA GCA GCC GCG GTA ATA CG; 800R: TAC CAG GGT ATC TAA TCC) (Mohamed et al., 2012). PCR amplification was performed in a GeneAmp 2400 PCR machine using the following program: Denaturation (5 min at 95°C, 1 cycle; 35 cycles, each of denaturation at 95°C for 1 min, Annealing for 1 min at 56°C and extension for 2 min at 72°C. The primer extension segment was extended to 5 min at 72°C in the final cycle. The PCR products were resolved by electrophoresis in a 1.2% agarose gel at 80 V for 1 h and then stained with ethidium bromide solution for around 10 to 15 min. Amplified fragments were visually examined under UV transilluminator.

The PCR product of 16S rRNA amplified from the DNA extract of the selected *Streptomyces* strain was sent to Macrogen® (908 world meridian venture center, #60-24, Gasan-dong, Geumchun-gu, Seoul 153-781, Korea). PCR tube was firmly packed and was delivered to Lab-Technology® (Macrogen agent in Egypt) on crushed ice. Sequencing was performed using the ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits, ABI PRISM 3730XL Analyzer (96 capillary type) sequencer (Applied Biosystems), MJ Research PTC-225 Peltier Thermal Cycler, DNA polymerase (FS enzyme) (Applied Biosystems). Following the protocol supplied by the manufacturer, Single-pass sequencing was performed on each template using the same primers pairs described in the PCR step. The fluorescent-labeled fragments were purified from the unincorporated terminators with an ethanol precipitation protocol. The samples were re-suspended in distilled water and subjected to electrophoresis in an ABI 3730xl sequencer. DNA sequence was analyzed using BLASTN 2.2.23+ software (http://www.ncbi.nlm.nih.gov/blast/) against the isolates collected from the database for genotyping. The sequence that showed the lowest e-value and maximum identity was considered as the genotype of the sample analyzed.

Table 1. Total number of SLC isolated from two regions of western region.

City	Sea		Soil	Total
	Water	Sediment soil		
Jeddah	12	6	4	22
Taif	0	0	10	10
Total				32

RESULTS AND DISCUSSION

Isolation, purification and maintenance of halotolerant streptomycetes

The genus *Streptomyces* is represented in nature by the largest number of species and varieties among the family Streptomycetaceae (Taddei et al., 2006). The occurrence of actinomycetes in the sea and lakes revealed their widespread distribution (Johnston and Cross, 1976; Mohamed, 1998; Mohammad and Galal, 2005; Saha et al., 2006). The genera *Micromonospora*, *Streptomyces* and nocardioform actinomycetes were isolated from lakes (Johnston and Cross, 1976).

Data in Table 1 shows that a few numbers (32) of streptomycetes-like colonies (SLC) were isolated from two regions of western region of KSA. From Jeddah, 22 out of the 32 SLC were obtained; distributed between the sea water, 12 (54.55%) and sea sediment soil, 6 (27.27%). On the contrary, only 4 SLC were obtained from agricultural soil, which represent 18.2%. From Taif-soil, only 10 SLC were isolated. At the level of color of SLC, results could be summarized as the presence of white color series (12, 37.5%) followed by gray color series (11, 34.38%), red color series (4, 12.5%), yellow color series (3, 9.37%), and green and brown color series (1 for each, 3.13%). It was also noticed that both white and gray color series were found in the sea water and sea sediment. This result agrees with that of Mohamed (1998), Mohamed et al. (2012) and Shori et al. (2012).

Determination of salt tolerance range of purified SLC

In this experiment, the ability of the purified SLC to grow in the presence of different salt (NaCl) concentrations in the growth medium was tested. Results show that all SLC grew on 3.5% salt, while, in the presence of 7.0% salt, 2, 3, 18 and 4 showed abundant (+++), moderate (++), week (+), and in-doublet (±) growth, respectively. Only five isolates were not able to grow at 7% salt. At the level of 10.5% salt, the number of LSC was decreased up to 4 [2 (+++), 1 (++) and 1(+)]. In either 14 or 21% salt, four isolates varied in their growth ability as moderate or week growth was recorded. The SLC were considered as halotolerant, as they grew in the normal growth medium that contain 0.5% NaCl as well as in increased salt concentrations up to 14% salt. Results are shown in Figure 1.

Several studies reported the ability of actinomycetes as well as streptomycetes to grow in high concentrations of salt (Tresner et al., 1968; Mohamed et al., 2000; Mohamed et al., 2001; Saha et al., 2006; Thumar and Singh, 2007; Ai et al., 2009; Cai, 2009; Ameur et al., 2011; Thumar and Kumar, 2012).

Identification of the selected halotolerant streptomycetes up to species

It was important to find out the taxonomical variations which might exist between the actinomycete isolates that are able to tolerate salt up to 14 or 21%. Therefore, halotolerant streptomycetes-like isolates which tolerate 14% salt (isolates 4 and 6) and 21% salt (isolates 2 and 8) were completely identified. The cultural and the morphological characteristics of these isolates revealed that all of them belong to the genus *Streptomyces*, since they formed well developed branching non-septate aerial mycelia carrying long spore chains. The non-motile spores were not borne on verticillate sporophores (Pridham and Tresner, 1974). These four streptomycetes were completely identified according to keys proposed by Pridham and Tresner (1974).

Streptomyces isolate 2

It was found that the Streptomyces isolate 2 belonged to the gray colour series, while the vegetative mycelium was pigmented with gray. This isolate had spiral spore chain with smooth/warty surface (Figure 2). Melanoid pigments were produced on tyrosine agar medium (Shinobu, 1958), peptone-yeast extract iron agar medium (Tresner and Danga, 1958) and tryptone-yeast extract broth medium (Pridham and Gottlieb, 1948). It gave

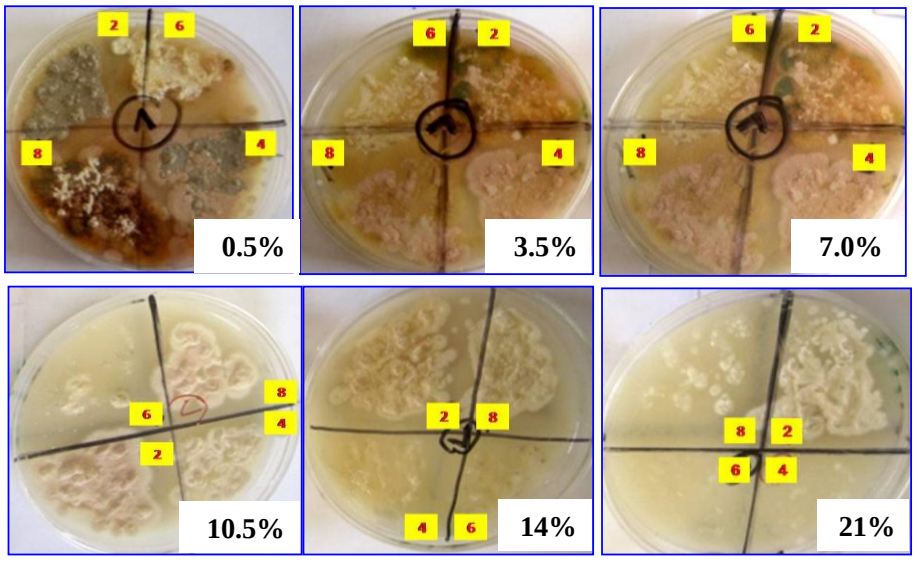

Figure 1. Growth of four selected halotolerant streptomycete-like isolates (2, 4, 6, and 8) on starch nitrate agar medium contains different concentration of NaCl.

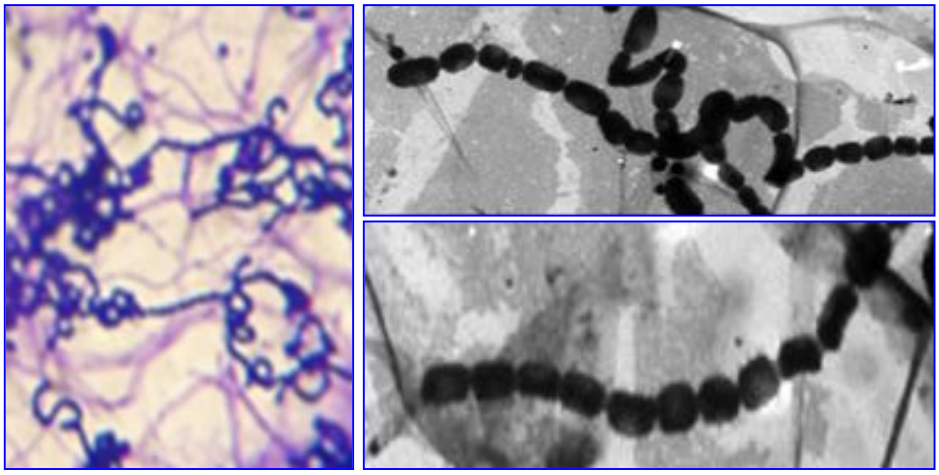

Figure 2. Microphotograph and electron micrograph of *Streptomyces* isolate No. 2 showing spiral spore chain (X-1000) and warty/smooth spore surface (X-10000).

also an excellent growth on Cazpek's agar medium and actively utilized D-glucose, D-xylose, L-arabinose, D-fructose and sucrose as carbon sources for growth. It was not able to use D-mannitol and i-inositol as sole carbon sources. This isolate showed antibacterial and antifungal activities. However, no growth was observed in the presence of 4 μg ml^{-1} streptomycin antibiotic in the medium. This isolate was tolerant to NaCl up to concentration of 21%. According to the key proposed by Pridham and Tresner (1974), the experimental isolate 2 appeared to be related to *Streptomyces cirratus* although there was slight difference in the melanoid pigment production, use of L-Rhamnose as a sole source of carbon and NaCl tolerance. Therefore, isolate 2 could be considered a strain of *S. cirratus.*

Streptomyces isolate 4

Results illustrated in Figure 3 clearly indicate that the *Streptomyces* isolate 4 belonged to the gray colour

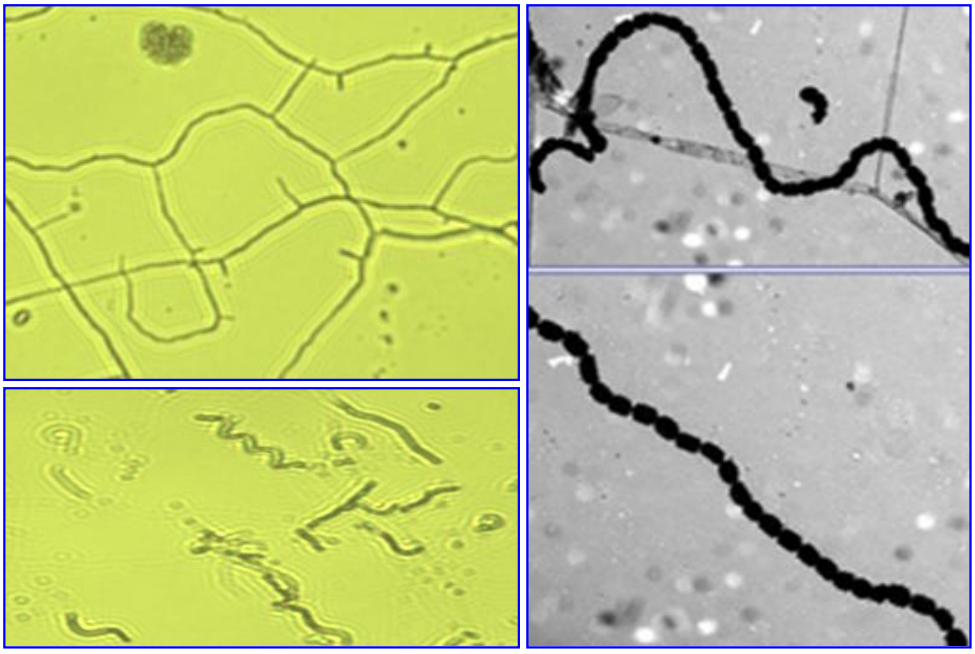

Figure 3. Microphotograph and electron micrograph of *Streptomyces* isolate No. 4 show RF/S spore chain (X-1000) and warty/smooth spore surface (X-10000).

series. Aerial spore chains belonged to section RF and sometimes spiral; the spores were characterized as smooth surface with ornamentations (warty). Melanoid pigments were detected on the standard media used. This isolate was characterized by excellent growth on Cazpek's agar medium. The physiological characteristics showed that D-glucose, D-xylose, L-arabinose, L-rhamnose, D-mannitol, D-fructose and sucrose were used as carbon sources for growth, while i-inositol did not support any growth. In addition, this isolate showed antimicrobial activities and sensitivity to streptomycin (4 μg ml^{-1}) was observed. However, it was able to grow in the presence of 14% NaCl in the growth medium. When comparing the cultural, morphological and physiological characteristics of the *Streptomyces* spp. in Pridham and Tresner (1974) with those of *Streptomyces* isolate 4, this isolate is very likely to be a strain of *S. rishiriensis*.

Streptomyces isolate 6

Results of *Streptomyces* isolate 6 show that, this isolate has yellow aerial mycelium (yellow colour series), while the vegetative mycelium was pigmented with creamy colour. It had straight and long spore chains (section RF) (Figure 4) and the spores were characterized by smooth surface without any ornamentations. This isolate was also characterized by good growth on Cazpek's agar medium; actively utilized all used sugar, tolerant to NaCl concentration up to 14%, sensitive to streptomycin (4 μg ml^{-1}) and antagonized some of bacterial and fungal used. Considering the description key proposed by Pridham and Tresner (1974), the tested isolate 6 was closely related *to S. alboflavus*.

Streptomyces isolate 8

Results show that the isolate 8 was characterized by gray aerial mycelium (gray colour series) and the reverse side of substrate mycelium was dark gray. Spore chains belonged to section RF or spiral with hairy surface (Figure 5). This isolate was also found to produce melanoid, did not produce soluble pigments and had a good growth on Cazpek's medium. Concerning the utilization of carbon sources, the isolate was able to give a good growth in the presence of all sugars as sole carbon source. The isolate also showed antimicrobial activities, was not inhibited with streptomycin (4 μg ml^{-1}) and grew on NaCl concentrations up to 21%. Considering the description of *Streptomyces* spp. present in Pridham and Tresner (1974), the *Streptomyces* isolate 8 was closely related to *S. luteogriseus* and could be identified as a strain of *S. luteogriseus* although there were slight differences in utilization of sucrose for growth as sole carbon source and in tolerance to NaCl concentrations.

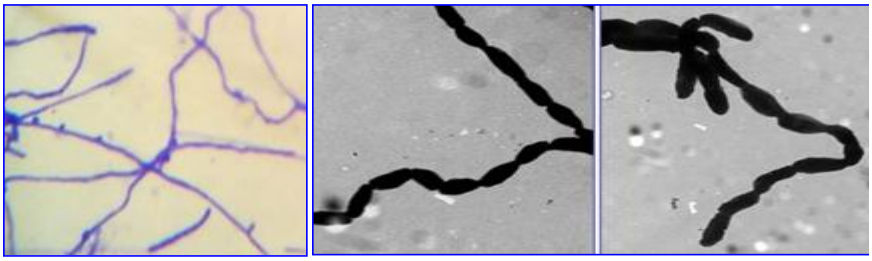

Figure 4. Microphotograph and electron micrograph of *Streptomyces* isolate No. 6 show RF spore chain (X-1000) and smooth spore surface (X-10000).

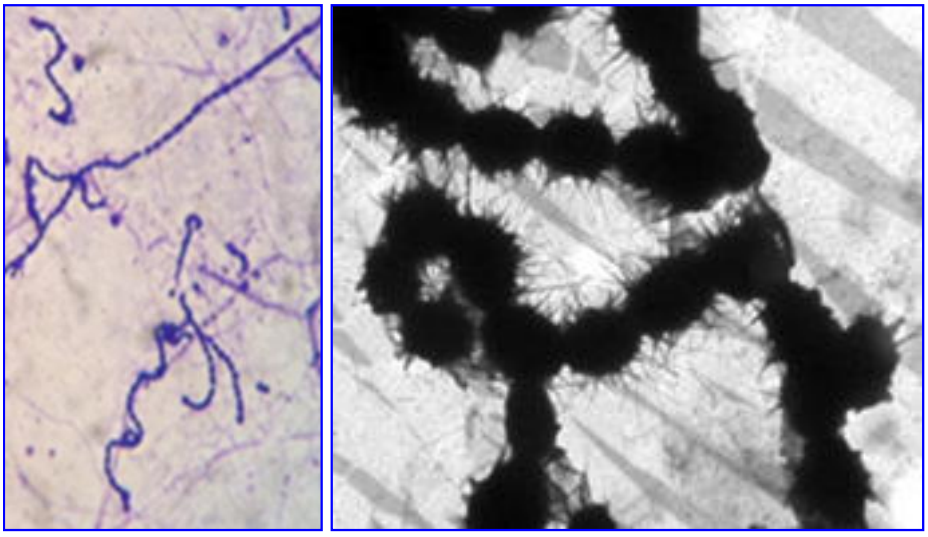

Figure 5. Microphotograph and electron micrograph of *Streptomyces* isolate No. 8 show RF spore chain (X-1000) and hairy spore surface (X-10000).

Molecular identification of a halotolerant *Streptomyces* strain using 16S rRNA gene

In this study, the nucleotide sequences of 16S rRNA gene were partially determined using the DNA template of the *Streptomyces* sp. (isolate 8). Results show that the two sequences of both forward and reverse direction were matched to each other, and the final sequence of about 1462 nts was obtained and compared with 8 universal bacterial isolates. These bacteria are *Streptomyces luteogriseus*, strain ISP 5483 (AJ399490.1), *S. luteogriseus*, strain NBRC 13402(NR_041128.1), *S. luteogriseus*, strain ZG728 (GQ985454.1), *S. luteogriseus*, strain ES_62con (EU934092.1), *S. luteogriseus*, strain NBRC 13402 (AB184379.1), Bacterium enrichment culture clone DT1-3 (HM593859.1), Bacterium enrichment culture clone DT3-5 (HM593864.1) and Bacterium enrichment culture clone DT3-1 (HM593863.1).

Results in Table 2 shows that the percent identities between the isolate of this study and the compared *S. luteogriseus* strains ranged from 80 to 82 as compared to the other enrichment bacterial clones (clone DT1-3; HM593859.1; clone DT3-5, HM593864.1 and clone DT3-1, HM593863.1) as a 99% identity was recorded between the *S. luteogriseus* isolate 8 of this study and theirs.

The phylogenetic tree in Figure 6 shows that the *S. luteogriseus*-isolate 8 lied in the same cluster with the bacterium enrichment culture clones. This isolate could be classified as a new species of gray *Streptomyces*, and it was suggested to be named a new halotolerant *Streptomyces* sp. TSA-KSA strain (GenBank AB731746). These results are in harmony with that of some investigators, who identified some actinomycetes using the 16S rRNA gene (Moran et al., 1995; Cook and Meyers, 2003; Song et al., 2004; Jose et al., 2011; Al-

Table 2. Maximum identities between *S. luteogriseus*-08 and the related overseas bacterial strains based on 16S rRNA gene.

Accession	Description	Query coverage (%)	Max identity (%)
AJ399490.1	*Streptomyces luteogriseus*, strain ISP 5483	83	82
NR_041128.1	*Streptomyces luteogriseus*, strain NBRC 13402	83	82
GQ985454.1	*Streptomyces luteogriseus*, strain ZG728	96	80
EU934092.1	*Streptomyces luteogriseus*, strain ES_62con	77	81
AB184379.1	*Streptomyces luteogriseus*, strain NBRC 13402	83	82
HM593859.1	Bacterium enrichment culture clone DT1-3	100	99
HM593864.1	Bacterium enrichment culture clone DT3-5	100	99
HM593863.1	Bacterium enrichment culture clone DT3-1	100	99

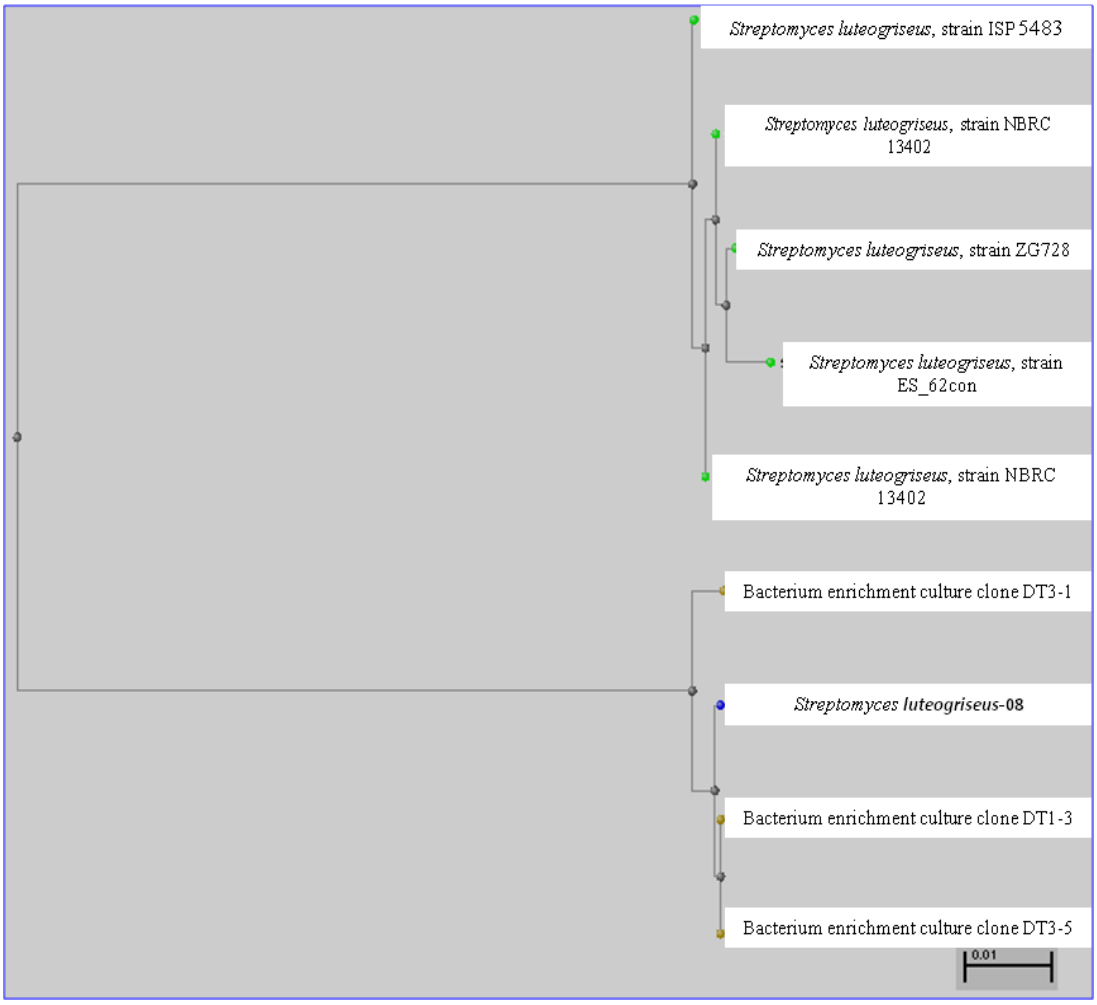

Figure 6. Phylogenetic tree of nucleotide sequence of the PCR product of 16S rRNA gene amplified from the DNA of S. *luteogriseus*-08 and the related universal bacterial strains.

Askar et al., 2011; Mahasneh et al., 2011; Mohamed et al., 2012; Shori et al., 2012). A moderately halophilic actinomycetes strain, designated as WH26, was isolated from Weihai Solar Saltern in China. The identification of the strain WH26 was performed by its morphological characteristics, physiological and biochemical tests as

well as phylogenetic analysis based on 16S rRNA sequence comparison. The results showed that the nucleotide sequence of the 16S rRNA gene (1,677 bp) of the strain WH26 exhibited close similarity (97 to 99 %) with other Streptomyces 16S rRNA genes and the strain WH26 was identified to belong to the genus *Streptomyces* (Liu et al., 2013).

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