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BIOCHEMICAL & MOLECULAR IDENTIFICATION AND ANTIMICROBIAL ACTIVITY OF ANTIBIOTIC-PRODUCING *STREPTOMYCES* SP. ISOLATED FROM SAUDI ARABIA

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ABSTRACT

In the present study we carried out a survey for searching about antibiotic producing bacterial strains in the soil of Northern Border Region, Kingdom of Saudi Arabia; especially in Rafhaa City. During our survey we isolated an *Actinomycetales* strain. The Identification of this strain was performed Biochemically according to spore morphology, cell wall chemo-type, cultural and physiological characteristics. In addition, we carried out a molecular characterization by sequencing of 16S rRNA gene. Both of the biochemical and molecular characterizations suggested that this strain is a *Streptomyces* sp. Further studies were performed on this isolate such as antimicrobial activity against some bacterial species from Gram-positive and Gram-negative groups, as well as some fungi. These studies showed that this strain exhibits a strong antimicrobial activity.

Keywords: *Actinomycete*, identification, 16S rRNA gene, antimicrobial activity

INTRODUCTION

Antibiotics are the most important natural secondary metabolites produced by the soil microorganisms such as bacteria and fungi and are important in medicine as well as in economy. One of the most widely distributed soil microbes in the earth are that belong to the Order *Actinomycetales*. Actinomycetes belong to the order *Actinomycetales* and produce wide range of secondary metabolites. Among these microorganisms, the actinomycetes are the largest source of antibiotics, anti-tumor and immune suppressant agents **(1)**. Also, this Order has the most well-known genus *Streptomyces* which includes the largest number of species and strains in the order *Actinomycetales*. The genus *Streptomyces* is an aerobic Gram-positive, spore-forming actinomycetes belonging to the family *Streptomycetaceae* **(2)**. Approximately 80% of the naturally derived antibiotics, which are in clinical use, were derived from *Streptomyces* genus alone **(3,4)**.The specific/intra-specific relationships in the streptomycetes and the way they are reflected in the biosynthetic potential to produce bioactive compounds could significantly influence strategies for search and discovery, screening and bioprocess development.

To overcome the problem of spread resistant microorganisms, there is an interest to discover novel antibiotics and new therapeutic agents by continuous screening of secondary microbial products produced from potential bacterial taxa **(5)**. Therefore searching for new *Actinomycetales* strains in a new habitat is a promising way to overcome the problem of spread of the antibiotic resistant microorganisms. In addition; to our knowledge, no study on the investigation and survey of the antibiotic producing bacteria were carried out in the Northern part of Saudi Arabia. As far as we know, only the isolation and characterization of several streptomycetes strains from western region soil in Saudi Arabia were carried out by Malibari **(6)** and Atta *et al*.**(7)**. Therefore, the current study was designed to describe the isolation of an antibiotic producing *Actinomycetales* strain isolated from soil samples collected from different localities at Northern part of Saudi Arabia. Isolation, biochemical and molecular characterization and identification of the *Actinomycetales* strain were reported. Also, the antimicrobial activity of the *Actinomycetales* strain was tested against several Gram negative and Gram positive bacteria and Fungi.

MATERIALS AND METHODS

2.1. Sampling

In the present study five different soil samples were collected from different localities at Rafha Governorate in the Northern Border Region, Kingdom of Saudi Arabia (KSA). Soil sample from each site was taken from the top layer of the soil surface (0-30 cm depth) and directly transferred into polyethylene bags to minimize moisture losses during transportation and then subjected to bacteriological analyses.

2.2. Pretreatment of soil samples

Soil samples were subjected to three physical and chemical pretreatment methods in order to facilitate the better isolation of actinomycetes populations **(8)**. These soil samples were air-dried and subjected to heat treatment up to 40-45°C for 15 h to kill the Gram negative bacteria, 1.4% of the phenol solution was mixed with one gram of soil sample and incubated at room temperature for 10 min to kill the normal bacteria, then an equal amounts of soil sample and $CaCO_3$ were mixed with sufficient amount of water and incubated at room temperature for one week to enrich the soil for better isolation of actinomycetes.

2.3. Isolation of streptomycetesstrains

Soil samples were serially diluted by diluting one gram of soil sample in 9 ml of sterile distilled water and shaken vigorously to make a stock solution. From this stock solution, 1ml was used to prepare the final volume of 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} by serial dilution method. Finally, 0.1ml of soil sample suspension from 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4} and 10^{-5} were used to spread on sterilized starch nitrate agar medium (g/L): Soluble starch 20.0; $NaNO_3$ 2.0;K_2HPO_4 (anhydrous) 1.0; KCl 0.5; $MgSO_4.7H_2O$ 0.5; $CaCO_3.2H_2O$ 2.0; Agar 15. The medium was adjusted to the initial pH 7.0 prior to sterilization using 0.1 N NaOH or 0.1 N HCl solutions **(9)**and incubated at 37°C for 7 days; triplicates were maintained for each dilution. After 7 days, the plates were observed for actinomycetes colonies. The grown actinomycete colonies were purified using the dilution plate technique. The obtained purified cultures were sub-cultured on starch casein agar slants and stored at 4°C for further studies. For every 30 days the cultures were sub-cultured freshly for better bioactive production.

2.4. Screening for antimicrobial activity of the isolated streptomycete

Testing antimicrobial activity of the isolated actinomycete cultures against the microbial test strains (**Table: 1**) were

performed by diffusion plate methods **(10)**, based on the observation of inhibition zone of bacterial growth on agar media.

2.5. Taxonomic characterization of the most active streptomycete NBR isolate

2.5.1. Conventional taxonomy

The characterization of isolated streptomycetewas carried out according to the guidelines adopted by International *Streptomyces* Project **(11)**. The cultural characteristics were studied according to the guidelines established by the ISP **(11)**,colours were assessed on the scale indicated by Kutzner *et al.* **(12)**. Micro-morphological studies were carried out using light and scanning electron microscope (JEOL JSM 5300, JEOL Technics Ltd., Japan). Diaminopimelic acid isomers in the cell-wall and whole cell sugar pattern were analyzed using the method of Becker *et al.***(13)**. The physiological and biochemical characteristics; melanin pigment production, utilization of carbon and nitrogen sources, enzymatic activities and other physiological characters were also studied **(11, 12)**.

2.5.2. Molecular characterization

2.4.2.1. DNA Extraction

The culture of the streptomycete strain NBR was inoculated into 50 ml of starch nitrate broth and incubated at 200 rpm and 28 °C for 72 hours. The total genomic DNA was extracted according to the method of Kumar *et al.***(14)**. The extracted total genomic DNA was used as a template for PCR reactions.

2.4.2.2. PCR Amplification & Sequencing

The 16S rRNA of the isolate was amplified by the following primers 10F; 5'-AGTTTGATCCTGGCTC-3'and 1525R; 5'-AAGGAGGTGATCCAGCC-, using the TopTaq Master Mix Kit (Qiagen) following the manufacturer's instruction. The PCR was carried out by Gene AMP PCR System 9700 from PE Applied Biosystems (Perkin Elmer, Ohio, USA). PCR mixture conditions were performed according to El-Naggar **(15)**. The PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and were detected using a gel documentation system, (Alpha-Imager 2200, CA, USA) then the purified PCR product was sequenced with BigDye Terminator using an ABI PRISM 377 DNA sequencer and ABI PRISM Cycle Sequencing (Perkin Elmer, Ohio, U.S.). The nucleotide sequence data was aligned using the ClustalW. A multiple sequence alignment, molecular phylogenetic analyses and the phylogenetic tree were carried out by the DNASTAR Lasergene (V.7.1) program.

RESULTS AND DISCUSSION

2.6. Taxonomic characterization of streptomycete isolate NBR

2.6.1. Conventional taxonomy

Actinomycetes are filamentous Gram-positive bacteria. During our survey on the antibiotic producing bacteria in the Northern Border Region; Rafha Governorate - KSA, we isolated a new streptomycete NBR strain. This strain was grown on different ISP media (**Fig.1**) with the following cultural characteristics, such as; the aerial hyphae of this isolate were grey, therefore it was assigned to the gray color series. Diffusible pigments were ranged from gray red purple on yeast-malt extract agar (ISP–2) to very deep red purple on each of oat meal agar medium (ISP–3), inorganic-trace salt starch agar (ISP–4) and glycerol-asparagine agar (ISP–5). Also the organism was found to produce deep olive brown melanin pigment on peptone yeast extract iron agar (ISP–6). It was obvious that this NBR isolate could produce wide variety of pigments responsible for the color of the vegetative and aerial mycelia depending on the type of media. In addition, the color of the substrate mycelia had wide range and variable colors as shown in (**Table: 2**). Goodfellow *et al.* **(2)** mentioned that the color of the substrate mycelium has been used in a preliminary approach to group streptomycetes. Detailed results of the culture characteristics of streptomycete isolate NBR are recorded in (**Table: 2**).

The micro-morphological characteristics of NBR strain that grown on inorganic salts-starch agar (ISP–4); under light microscopy (**Fig. 2a**), exhibited spiral shaped mycelium (x600) and each spore was characterized under scanning electron microscope (x7500) by a smooth spore surface (**Fig. 2b**). In addition the whole cell hydrolysate of this strain contained LL-diaminopimelic acid (LL-DAP) and glycine indicating that the strain had a chemo-type I cell wall but no characteristic sugars could be detected. Cell-wall composition analysis is one of the main methods that can be employed to identify the chemotaxonomic characteristics of *Streptomyces*; the presence of LL-DAP in the cell wall also showed that this strain is *Streptomyces* **(16)**.The chemotaxonomic results indicated that this strain had the characters of the *Streptomyces* genus. So that according to the identification methods described in the Bergey's Manual of Determinative Bacteriology, 9thedition **(17)**, we called our strain by *Streptomyces* sp. strain NBR. Streptomycetes have many differential colonial features, such as pigmentation of spores, substrate mycelium and diffusible exopigments, together with the

morphology of colonies and the texture of the aerial mycelium. The production of different pigments has been widely used in classification and identification, but it is important to mention that colony morphology is too variable for use as a taxonomic character **(2)**.

2.6.2. Physiological and biochemical properties

The physiological and biochemical characteristics of the *Streptomyces* strain NBR are indicated in (**Table: 3**). The carbon sources utilization by this *Streptomyces* strain NBR were found positive for all carbon sources except for sucrose, D-galactose and L-rhamnose that showed no growth. However, in the case of nitrogen sources utilization, the growth of this isolate was abundant and much better than the presence of the carbon source (**Table: 3**). So we can conclude that the growth of the NBR isolate can be influenced by the type of carbon and nitrogen sources **(7, 18-20)**.

The enzymatic activities of this isolate were positive with all tested enzymes except for lipase, pectinase and H_2S production as shown in (**Table: 3**). Generally, it is known that very few strains from streptomycetes are able to produce lipase, pectinase and hydrogen sulfide. Goodfellow listed in the latest edition of the Bergy's Manual of Systematic Bacteriology more than 500 streptomycete strains; from these strains only 5, 15 and 14 streptomycete strains produce lipase, pectinase and H_2S respectively. This means that the production of lipase, pectinase as well as hydrogen sulfide might need special environmental conditions or might be in extreme environments. So we suggest that our strain was free from lipase, pectinase and hydrogen sulfide production because it was isolated from a normal habitat.

The optimum temperature for the growth of the NBR strain was 30^oC-40^oC, but no growth was found at the high temperature 50^oC, as well as, lower temperatures 10-25^oC. These results suggesting that our strain belongs to the group of mesophilic bacteria. It is known that the optimum growth temperature for most actinomycetes is 23-37°C **(21, 22)**. However there are also thermotolerant and thermophilic actinomycetes **(23)**.

The NBR strain was tolerant to the NaCl concentration till 7% with good growth, however there was a weak and no growth observed at 8% and 9% NaCl concentration respectively. Atta *et al.***(7)** and Das *et al.***(24)** reported the inhibition in growth of some streptomycetes strains at NaCl concentration higher than 7%. The

growth pH was best at pH 7, but no growth was observed in both of the highly acidic and highly alkaline media. These results indicated that our strain is a neutrophil **(2, 25)**. Streptomycetes are known to prefer neutral to alkaline environmental pH, the optimal growth pH range being 6.5 to 8.0. Kumar *et al.***(26)** reported an isolate that has a pH range from 4 -12. In addition, acidophilic and alkalophilic streptomycetes have also been found **(20, 24, 27)**.

In addition the NBR isolate was sensitive to the growth inhibitors sodium azide (0.02%) and thallus acetate (0.001%). Previous studies were carried out by Atta *et al.***(7)** on Actinomycete isolate and showed the inhibition of the bacterial growth at (0.001%) thallus acetate. However a normal growth was observed in case of other growth inhibitors such as phenol, crystal violet as well as low concentration from sodium azide (0.01%). Finally, this strain was resistant to the antibiotics norfloxacin (30 µg/ml) and rifampicin (50 µg/ml), however, it was sensitive to erythromycin, penicillin and others as shown in (**Table: 3**).

2.6.3. Molecular and phylogenetic identification

The molecular techniques give better and accurate identification at species-level. One of these important methods is the using of the 16S rRNA gene which is highly conserved among species, it is easily to use for discrimination at species level. We were able to reconfirm the identity of the streptomycete strain NBR. So the 16S rRNA gene of the NBR isolate was amplified by PCR method with total size of approximately 1.5kpb (**Fig. 3**). Then a partial sequencing of the 16S rRNA gene was sequenced and then sent to DNA database for homology search and FASTA analysis. Multiple sequence alignment was done using sequences of the 16S rRNA genes of 25 *Streptomyces* and *actinomyces* strains from the DNA Data Bank in addition to our NBR isolate. Computer assisted DNA similarly searches against bacterial database revealed that 16S rRNA sequence was 97% similar to *Streptomyces coeruleorubidus* strain NSWG-20 (accession number JX905302). A phylogenetic tree was constructed by the DNASTAR Lasergene (V.7.1) program using the nucleotide sequence of the 16S rRNA gene of NBR strain as well as another 25 *Streptomyces*and *Actinomyces* strains and showed that NBR strain is grouped with *Streptomyces coeruleorubidus* strain NSWG-20 in the same clade which indicate that our strain might be a new *Streptomyces coeruleorubidus*strain (**Fig. 4**).

The phylogenetic analysis based on 16S rDNA gene sequences of the NBR strain isolated from the soil of Rafha, KSA confirmed that this isolate belongs to the *Streptomyces* genus and might phylogenetically related to the known species *Streptomyces coeruleorubidus*. Despite of the NBR isolate had high similarity to *Streptomyces coeruleorubidus*but it differed in some physiological and biochemical characteristics such as the production of melanin pigment in yeast-malt agar, salts-starch agar and glycerol-asparagine agar, in addition to, the utilization of sucrose and rhamnose**(2)**.

3.2 Antimicrobial activity of streptomycete isolate NBR

The culture filtrate of this streptomycete isolate NBR exhibited strong antibacterial activity mainly against Gram-positive bacteria, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538 with mean diameter of inhibition zones 27.0 and 18.0 mm respectively, and moderate antibacterial activity against Gram-negative bacteria, *Escherichia coli* ATCC 7839 with mean diameter of inhibition zone 19.0 and 14.0 mm respectively. In addition, a strong antifungal activity against *Candida albicans* ATCC 10231 with inhibition zone 22.0 mm. Data of antimicrobial activity of streptomycetesp. strain NBR are recorded in (**Table: 4**). On the other hand, two filamentous fungi, *Aspergillus niger* ATCC 16404 and *Aspergillus flavus* ATCC16883 gave normal growth after the treatment with the NBR filtrates (data not shown). So we conclude that our *Streptomyces*NBR strain had broad spectrum characteristics of its active metabolites.

Previous studies showed the advantage of broad spectrum characteristic of the metabolites from *Streptomyces* species against Gram-positive bacteria, Gram-negative bacteria and *Candida* species (6, 7, 18, 20, 24, 26).

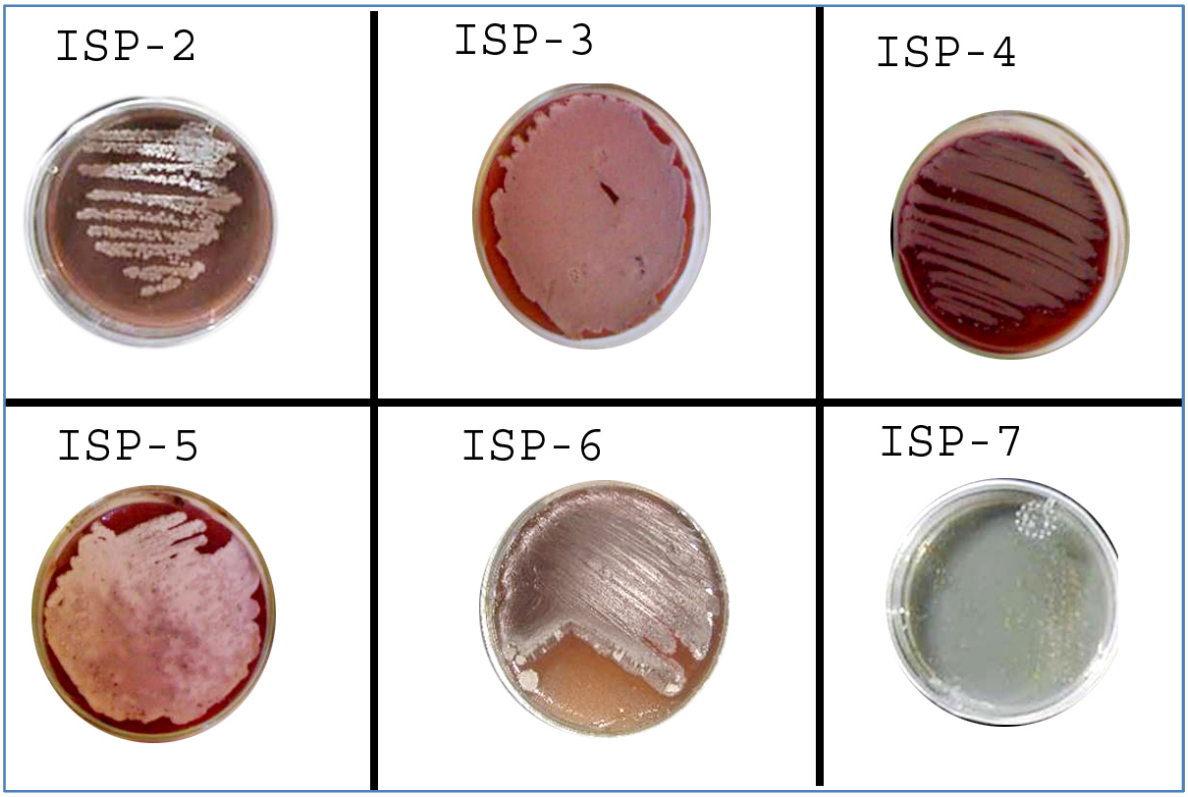

Figure (1): Growth of actinomycete isolateNBR grown on different ISP-media

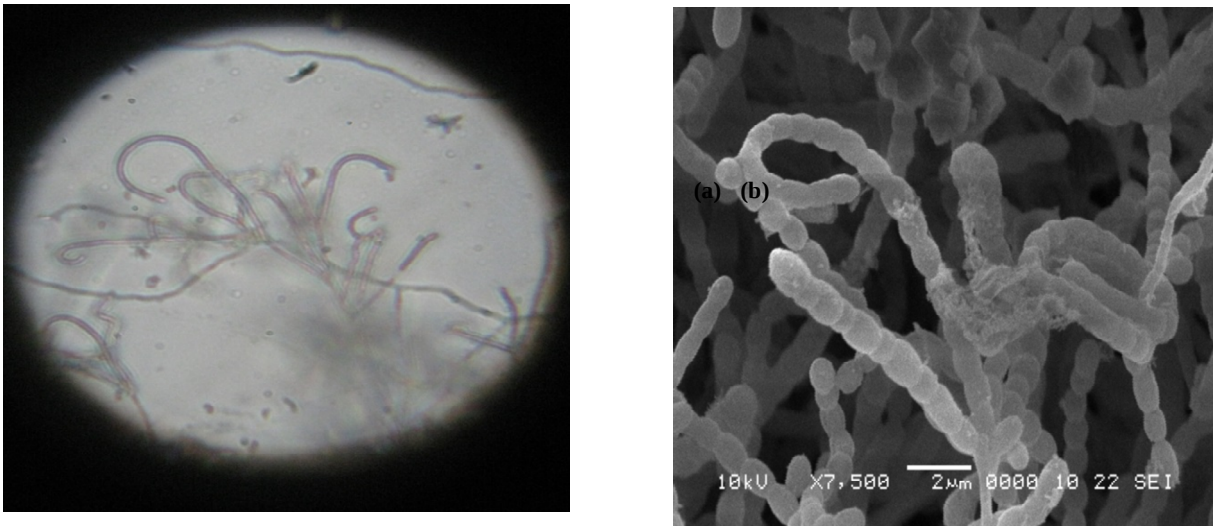

Figure (2): a; Phase-contrast micrograph of the aerial mycelium showing spiral shaped mycelium (x600) b; Scanning electron microscopy (SEM) showing a smooth spore surface (x7500) of actinomycete isolate, NBR grown on inorganic salts-starch agar (ISP-4) for 21 days.

Table (1): Bacterial & Fungal strains used in this study:

Strain Name	Type
Bacillus subtilis (ATCC 6633)	Gram-positive bacteria
Staphylococcus aureus (ATCC 6538)	Gram-positive bacteria
Staphylococcus aureus (MNBR)	Gram-positive bacteria
Escherichia coli (ATCC 7839)	Gram-negative bacteria
Pseudomonas aeruginosa (ATCC 9027)	Gram-negative bacteria
Candida albicans (ATCC 10231)	Unicellular fungi
Aspergillus niger (ATCC 16404)	Filamentous fungi
Aspergillus flavus(ATCC16883)	Filamentous fungi

Table (2): Cultural characteristics of *Streptomyces* sp. strainNBR on ISP-media

Medium	Growth	Substrate mycelium	Aerial mycelium	Diffusible pigments
Tryptone-yeast extract broth (ISP–1)	Weak	Dark yellow (ISCC-NBS 88)	White (ISCC-NBS 263)	None
Yeast -malt extract agar (ISP–2)	Good	Pinkish Gray (ISCC-NBS 10)	Moderate gray (ISCC-NBS 265)	Gray red purple (SCC-NBS 245)
Oatmeal agar (ISP–3)	Good	Slightly purple (ISCC-NBS 218)	Pinkish Gray (ISCC-NBS 10)	Very deep red purple (SCC-NBS 243)
Inorganic-trace salt- starch agar (ISP–4)	Good	Pinkish gray (ISCC-NBS 10)	Pinkish gray (ISCC-NBS 10	Very deep red purple (ISCC-NBS 243)
Glycerol-asparagine agar (ISP–5)	Good	Pinkish gray (ISCC-NBS 10)	White (ISCC-NBS 263)	Very deep red purple (ISCC-NBS 243)
Peptone-yeast extract iron agar (ISP–6)	Moderate	Dark yellow ISCC-NBS 88)	Deep gray (ISCC-NBS 266)	Deep olive brown (ISCC-NBS 96)
Tyrosine agar (ISP–7)	No growth	-	-	-

Table (3): Physiological and biochemical characteristics of *Streptomyces* sp. strainNBR

Character	Results	Character	Results
Melanin pigment		Gelatin liquefaction	+
Tryptone-yeast extract broth	–[a]	Nitrate reduction	+
Peptone-yeast extract iron agar	Deep olive brown	H_2S production	–
Tyrosine agar	–	Tolerance to NaCl concentrations	
Carbon sources utilization		1.0 - 6.0 %	+++
Starch	+[b]	7.0 %	+
Maltose	+	8.0 %	Wg[e]
D–Glucose	++[c]	9.0 %	–
L–Arabinose	+	Growth temperature ^oC	
D–XyLose	+	10.0 – 25.0 ^oC	–
Lactose	+	30.0 – 40.0 ^oC	+++
Mannitol	+	45.0 ^oC	++
Sucrose	–	50.0 ^oC	–
D–Galactose	–	Growth pH	
L–Rhamnose	–	4.0 – 6.0	–
Nitrogen sources utilization		7.0	+++
L-asparagine	+++[d]	8.0 – 9.0	++
Leucine	+++	10.0	–

L-tryptophan	+++	Tolerance to growth inhibitors	
L-glutamic acid	+++	Sodium azide (0.01%)	+
L-arginine	+++	Sodium azide (0.02%)	–
L-histidine	++	Phenol (0.1%)	+
L-serine	++	Crystal violet (0.0001%)	+
Lysine	++	Thallus acetate (0.001%)	-
L-methionine	+	Resistance to antibiotics	
L-tyrosine	–	Erythromycin (15 µg/ml)	–
Enzymatic activities		Penicillin (25 µg/ml)	–
Protease	+	Ciprofloxacin (30 µg/ml)	–
Lecithinase	+	Tetracycline (15 µg/ml)	–
Lipase	–	Bacitracin (50 µg/ml)	–
Pectinase	–	Chloramphenicol (30 µg/ml)	–
Catalase	+	Norfloxacin (30 µg/ml)	+
Urease	+	Rifampicin (50 µg/ml)	+
Xanthine degradation	+		

[a](-) = Negative or no growth, [b](+) = Positive or moderate growth, [c](+++) = Abundant (Very good growth), [d](++) = Good growth, [e](wg) = Weak growth.

Table (4): Antimicrobial activities of the culture filtrate from *Streptomyces*sp strain NBR

Test organism	Mean diameter of inhibition zone (mm)
Bacteria	
Bacillus subtilis (ATCC 6633)	27.00
Staphylococcus aureus (ATCC 6538)	18.00
Escherichia coli (ATCC 7839)	19.00
Pseudomonas aeruginosa (ATCC 9027)	14.00
Yeasts	
Candida albicans (ATCC 10231)	22.0

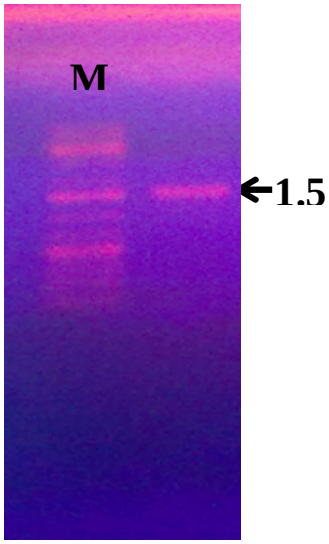

Figure (3): Amplified fragment of 16S rRNA gene, (M): 100 bp DNA Ladder

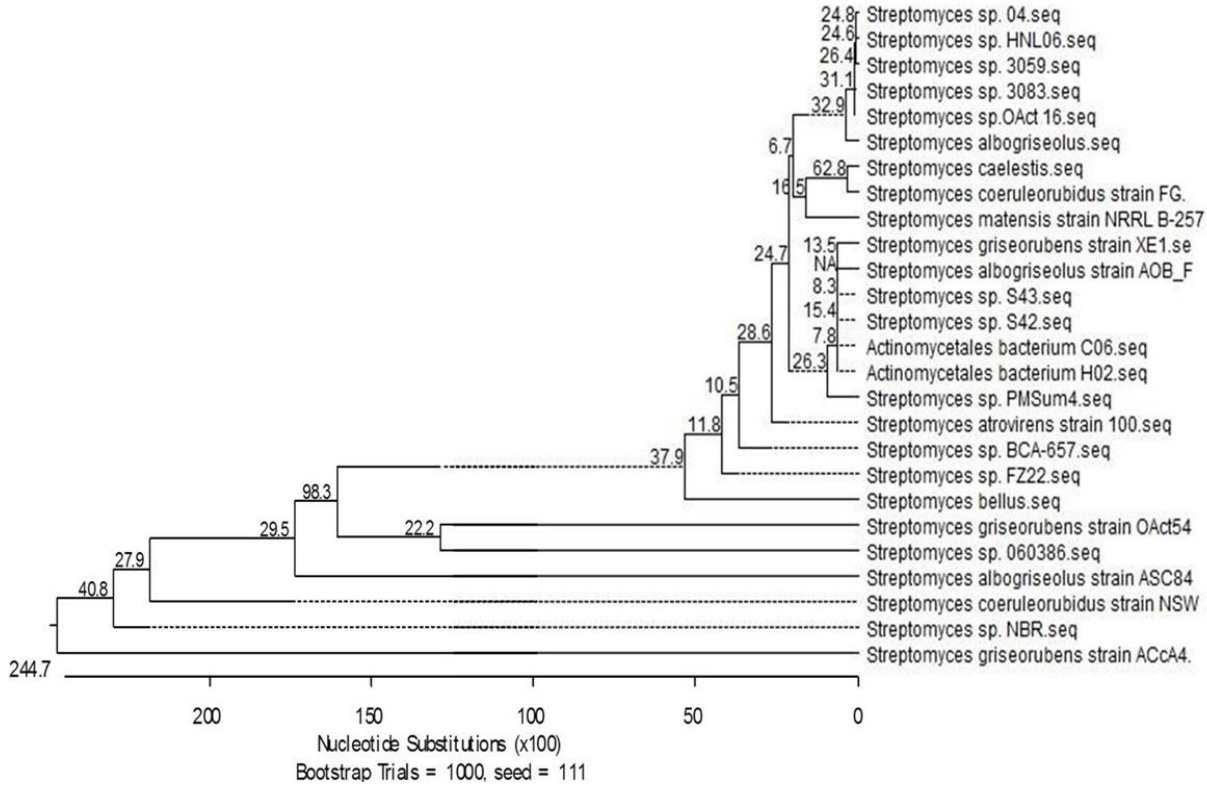

Figure (4): The phylogenetic tree of *Streptomyces* sp.strain NBR was constructed using the neighbor-joining method with aid of DNASTAR Lasergene (V.7.1).

CONCLUSION

From the results of the present study, we can conclude that the streptomycetesp. NBR isolate may represent a new species and might produce one or more active metabolites with broad spectrum against Gram-positive, Gram-negative bacteria and *Candida*. However, further studiesand investigations on structural characterization on the metabolites as well as clinical studies in rats may lead to the development of a novel drug with a high broad spectrum.

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