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Isolation, Screening and Identification of Newly Isolated Soil Streptomyces (*Streptomyces* sp. NRC-35) for β-Lactamase Inhibitor Production

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Abstract: During the screening study, a total of 60 different Streptomyces species were isolated from soil samples collected from different areas in Egypt. *Streptomyces* sp. NRC-35 was selected for its high β-lactamase inhibitor activity mediated by the production of clavulanic acid. This newly isolated strain was able to produce up to 65 mg L^{-1} clavulanic acid. The taxonomical properties of the strain were examined according to International Streptomyces Project (ISP) scheme. The primary identification of this isolate was based on spore morphology and cell wall chemo-type. Furthermore, culture physiological characteristics of the isolated strain, *Str.* NRC-35 were compared to other reference strains belong to Streptomyces species. The analysis of nucleotide sequence of the 16S rRNA indicated similarity binary level of 91% with *Streptomyces* species.

Key words: Streptomyces isolation · Streptomyces taxonomy · Phylogenetic analysis · Clavulanic acid · *Streptomyces sp* · NRC-35

INTRODUCTION

Screening of microorganisms for the production of novel antibiotics has been intensively pursued for many years. Antibiotic has been used in many fields including agriculture, veterinary and pharmaceutical industry. Actinomycets have the capability to synthesize many different bioactive secondary metabolites such as antibiotics, herbicides, pesticides, antiparastic and enzymes [1]. The discovery of antibiotics had a major impact on the control of infectious diseases and the development of pharmaceutical industry. During the last few decades, the pharmaceutical industry has not only continued to screen microbial metabolites of antimicrobial activity but has successfully extended to the isolation of new compounds for many other medical applications [2].

Isolation media containing starch or glycerol as the carbon source and nitrate, casein or arginine as the nitrogen source have proven to be the most effective growth media for selective isolation of *Streptomycetes* [3]. During the course of strain isolation, the addition of antifungal agents to the isolation medium suppresses the fungal growth. Cycloheximide (50-100 μg mL^{-1}), as well as pimaricin and nystatin (10-50 μg mL^{-1}) have been used for this purpose [3].

Actinomycetes are major source of bioactive secondary metabolites and representing about 70-80% of the all isolated compounds [4]. The Streptomycetes form a distinct clad within the radiation encompassed by the high GC Gram-positive bacteria in the 16S rDNA tree. There is evidence that specific metabolites, such as clavulanic acid, may be synthesized by strains in a specific clad. For example: streptomycin and related metabolites appear to be randomly distributed across the whole genus [5]. The 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 [6].

A probabilistic identification matrix for *Streptomycetes*, based on 41 characters like spore chains, spore morphology, pigmentation, physiological characteristics, antibiosis and resistance to antibiotics, was developed [7]. Kämpfer *et al.* revised the data using more characters and testing more strains and comparing the data with published genetic and chemotaxonomic data

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[8]. Cultivable microbes can be identified to the genus or species level using standard microbiological methods. Under standardized growth conditions, many isolates could be identified to the genus level with this approach. However, species identification often requires more specific methods, including physiological testing, analysis of biochemical markers and DNA sequencing [9].

Identification of *Streptomycetes* by morphological examination is difficult; thus molecular methods could represent an improvement. Numerous studies dealing with *Streptomycetes* identification showed the possibility for complete strain identification based on morphology and physiology, whereas in other cases these data were not sufficient to achieve the proper classification of the microorganism [10]. On the other hand, DNA-DNA re-association has shown to be useful method in the identification of *Streptomyces* species. However, because of the genomic instability of this type of microorganisms, this method should not be used alone but in combination with other tests as well [11].

The PCR method was more specific than the culture method. The culture method identified the colonies simply as *Actinomycetes*, which are not routinely identified to the genus level, whereas the PCR method indicated the presence of the genus *Streptomyces*. Although, there was no correlation found between culture and PCR results, there may be several reasons for this including the different error sources inherent in both methods [12]. The modern *Streptomyces* identification approach is based on 16S rDNA sequence data. This method provides invaluable information about *Streptomyces* systematic and has been used to identify several newly isolated *Streptomyces* [13].

However, with the increased prevalence of β-Lactamase producing pathogenic bacteria, penicillin's and cephalosporins have become less effective. Thus, the discovery of microbial strain with capability for β-Lactamase inhibitor producing is one of the most interesting topics of research in antibiotic field. Higgens and Kastner isolated a new species of *Streptomyces* named *Str. clavuligerus* which produce two new cephalosporin antibiotics [14]. One of them was the novel β-lactamase inhibitor, clavulanic acid (CA), [15]. This study was undertaken to isolate new soil Streptomyces strains with β-lactamase inhibitor activity from different locations in Egypt. The isolated strains were identified using different techniques and their potential for CA production was determined.

MATERALS AND METHODS

Sampling Procedure: From 2003 to 2004, 10 farming soil samples, obtained from 20-25cm depth, were collected in sterile containers from different area in Egypt. The collected soil samples were air dried at 50°C for 10 min.

Isolation of Streptomyces Colonies from the Farming Soil Samples: Isolation and enumeration of actinomycetes colonies were performed by soil dilution plate technique using two different media: the first medium; actinomycetes isolation agar medium (Difco, New Jersey (NJ), USA), composed of (g L^{-1}): glycerol, 5; sodium propionate, 4; sodium caseinate, 2;KH_2PO_4, 2.0; asparagine, 0.1; $MgSO_4.7H_2O$, 0.1 and $FeSO_4.7H_2O$, 1 mg; Agar, 15 and pH 7.0. The second medium, Streptomyces medium consists of (g L^{-1}): glucose, 5; L-glutamic, 4; KH_2PO_4, 1.0; $MgSO_4.7H_2O$, 0.7; NaCl, 1; $FeSO_4.7H_2O$, 3 mg and Agar, 25. This medium was supplemented with 25 μg mL^{-1} penicillin G (Sigma, USA). One gram of dried soil was suspended into test tube containing 9 ml sterile NaCl (0.85 %) solution and one drop of Tween 80 and 0.05 % lauryl sulfate (Sodium dodecyle sulfate, SDS) and heated at 50°C for 10 min [16]. Different dilutions, 10^{-3}, 10^{-5} and 10^{-7} of the suspension were plated onto agar medium. The plates were incubated for 7 to 10 days at 28°C. Selected colonies were transferred from mixed culture of the plates onto respective agar plates and incubated onto at 28°C for other 7 days. Plates containing pure cultures were stored at 4°C until further examination.

Isolation of β-Lactamase Inhibitor Producer: Sixty *Streptomyces* species have been isolated from different Egyptian soil samples. Isolates were tested for their ability of β-lactamase inhibitor (CA) by the specific synergistic bioassay using a resistant test strain of *E. coli* at 25 μg mL^{-1} Penicillin G [17]. The *Streptomyces* isolates were cultured on the Bennett's agar medium (g L^{-1}): beef extract, 1; yeast extract, 1; Glucose, 10; N-Z Amine, 2; Agar 15 and pH 7.3, [20]. After 7 days incubation at 28°C, 0.9 mm agar disk of each isolates were used for bioassay [14]. Clavulanic acid production was detected by thin layer chromatography (TLC) and confirmed by HPLC assay. The promising isolates, which showed the maximum CA production, were selected for further identifications.

Submerged Culture: Preliminary screening for antibiotic production was done by conventional spot inoculation method on agar medium. Subsequent screening of promising isolates was done using submerged culture. Pure streptomycetes isolates were spot inoculated on actinomycetes isolation agar medium (Difco, NJ, USA). A piece of agar from each seven-day-old culture grown on actinomycetes isolation agar medium was used to inoculate the flasks. These cultures were grown in 250 ml Erlenmeyer flasks containing 50 ml of yeast malt extract medium (YME) consists of (g L^{-1}): yeast extract, 3; malt extract, 3; Glucose, 10 and peptone, 5 and pH 7.3, [20] on a rotary shaker (New Brunswick, NJ, USA) at 200 rpm and 28°C for 144 h. The culture broth was separated from the mycelium by centrifugation at 5000 rpm for 10 min. The supernatant was sterilized by filtration and used for the evaluation of the inhibitory activity by agar well diffusion method against *Escherichia coli* as test microorganism [17].

Bioassay Determination Methods: The bioassay was carried out by agar well diffusion method with some modification using Penicillin G resistant strain of *E. coli* as an indicator strain to quantity CA production with Muller Hinton assay medium supplemented with 25 μg mL^{-1} Penicillin G. By using a sterile cork borer, wells were punctured in appropriate agar medium plates previously seeded with the test organism [17]. One hundred microliter of supernatant of each isolate was administrated in each well. The agar plates were kept at 4°C for at least 1h to allow the diffusion of produced antimicrobial metabolites to agar medium. The plates were then incubated at 37°C and the diameters of inhibition zone were determined after 24 h. Each experiment was repeated three times and the mean values of inhibition zones were calculated. Pure CA, kindly provided by Glaxo Smith Kline Co. (England), was used as standard. The inhibition zone diameter was measured and converted to antibiotic concentration using standard curve.

Thin Layer Chromatography (TLC): Clavulanic acid was evaluated on TLC at 20°C, using a solvent system consisting of n-butanol-ethanol-water (4:1:5, v/v/v, top phase) [15]. It has an R_f value of 0.44 when appears as a dark red spot on the glass coated chromatogram after spraying with 2, 3, 5-triphenyltetrazolium chloride (TTC) reagent.

HPLC Method: Clavulanic acid concentration was determined by HPLC after derivatization with imidazole [18] with some modification according to the optimum pH of imidazole, using a Polaris C-18 reverse phase (4.6 mm x 250mm, 5um) column. The mobile phase was composed of methanol and 0.1M K_2HPO_4 (6:94) adjusted to pH 3.2 with H_3PO_4 at flow rate 1.5 ml/min, at 28°C. The peak was detected at 312 nm using HPLC (SIKIM, Germany) and UV detector of Jasko (Japan) while standard pure CA was prepared daily.

Determination of Total Dry Weight (TDW): The dry cell weight was determined as follows: mycelia were harvested by centrifugation at 8000g for 10 min and 4°C. After separation, the cells were resuspended in distilled water, washed twice with distilled water and dried at 105°C for 24h.

Taxonomic Grouping of Active Actinomycete Isolates: *Actinomycete* colonies were characterized morphologically and physiologically following the directions given by the International *Streptomyces* project (ISP) [19] and Bergey's Manual of Systematic Bacteriology [20]. Cultural characteristics of pure isolates in various media were recorded after incubation for 7 to 14 days at 27°C. Morphological observations were made with a light microscope (Model SE; Nicon) by using the method of [19]. Active purified isolates of actinomycetes were identified up to the species level by comparing their morphology of spore bearing hyphae with entire spore chain and structure of spore chain with the actinomycetes morphologies, as described in Bergey's manual [24-20] This was done by using cover-slip method [21] in which individual cultures were transferred to the base of cover slips buried in ISP 4 medium, Bennett's and ISP-5 for photomicrographs. Colors of spores (aerial and substrate mycelia) were visually estimated by using a Stamp Color Key based on the computer color wheels of [25]. Carbon utilization was determined on plates containing ISP basal medium 9 [26] to which separately-sterilized carbon sources were added to a final concentration of 1.0%. The plates were incubated at 28°C and growth was noticed after 7, 14 and 21 days using glucose as positive control [19]. Cell wall analysis of DAP isomers in the cell wall composition was analyzed by paper chromatography by Lechevalier and Lechevalier [27].

Total DNA Isolation: *Str.* sp. NRC-35 was inoculated in 25 ml of the YME broth medium and incubated at 28°C with agitation speed 200 rpm overnight. After that genomic DNA of the strain was isolated as described by Pospiech and Neumann [28] The collected pellets were left to dry and dissolved in a suitable volume (100 µl) of TE buffer (100 mM NaCl, 1 mM EDTA, 100mM tris-HCl, pH 8.00) or deionized water and stored at -20°C.

PCR Amplifications and Detection of DNA: Amplifications using *Streptomyces*-specific Polymerase chain reaction (PCR) primers were performed on Gene AMP, PCR system 9700, PE Applied Biosystemes, (Perkin Elmer, USA). *Streptomyces*-specific PCR primers StrepB, 5'- ACA AGC CCT GGA AAC GGG T-3' (forward) and SterF 5'-ACG TGT GCA GCC CAA GACA -3' (reveres) using Biolego BV software (Biolegio, Nijmegen, The Netherlands). This primer set is used for the amplification of 16S rRNA fragments from DNA isolated [29]. The primer pairs StrpB/strpF amplified 520 bp and 1070 bp fragments, nucleotides 139-1212 (*Str. ambofaciens* numbering [30]. The PCR reaction mixture (25µl) contained PCR beads 0.5µl from each primer StrepB and SterF and 2 µl of template DNA up to finale volume 25 µl reached by distilled water. Amplification was performed with an initial denaturation step of 3 min at 94°C and then 35 cycles of (60 sec denaturation at 94°C, 30 sec. at 59°C for primer annealing and 60 sec at 72°C for primer extension) and kept at 72°C for 7 min to complete extension. Electrophoresis of the PCR products was carried out on 1 % agarose gel containing ethidium bromide (0.5 µg mL^{-1}), to ensure that a fragment of the correct size had been amplified [31] and detected by Gel documentation system, (Alpha-Imager 2200, CA, USA). PCR products were purified using Qia quick PCR purification kit (Qiagen, Hilden, Germany) and outsourced for sequencing to the Gene Analysis Unit (Macrogene Inc., Seuol, Korea) Sequencer AB-13730.

Phylogenetic Analysis of Streptomyces: Nucleotide sequences were compared with those maintained in the GenBank Database through NCBI Blast [32]. For phylogenetic analysis, sequences were aligned with those of reference strains with the program BioEdit version 7.0.4.01 [33]. The phylogenic tree was derived from the distance matrices using neighbor-joining method [34]. All analyses were performed on a bootstrapped dataset containing 100 replicates (generated by the program).

RESULTS AND DISCUSSION

Soils Sampling: This study was undertaken with an aim of highlighting the selecting of the strains with β-lactamase inhibitory activity (CA). Using the selective media and cultivation conditions described previously a total of 60 different Streptomyces isolates were obtained from 10 soil samples were collected from different locations in Egypt during the period of Jan. - Dec. 2004. Compared to other samples, Cairo farm soil from Ezbet El Nakhl area give the highest number of actinomycetes isolates (25 isolates) followed by New valley area (15 isolates). Samples from other locations have less actinomycetes number (Table 1). All streptomycetes were isolated at mesophilic temperatures (25-37 °C). These results were in agreement with other authors [3, 23] who found that; most species of actinomycetes were isolated at mesophilic temperatures. With exception of some actinmycetes who can adapt to wide pH range [35], *Streptomycetes* are usually neutrophiles and cultivated in medium of pH 7.0-7.5 at temperatures from 25 to 37°C for 14 days. All of these strains were isolated on two different culture media, the actinomycetes isolation agar medium and Streptomyces medium. Actinomycetes isolation agar medium contained sodium propionate, which acts as an antifungal agent to inhibit the fungal contamination and glycerol which most actinomycetes use as a carbon source. The addition of antifungal agents to the isolation media suppresses the growth of fungal species on the plates. On the other hand, penicillin G was added to the medium to inhibit bacterial contamination. The addition of antifungal agents to the isolation media suppresses the growth of fungal species on the plates. For this purpose either Cycloheximide (50-100 µg mL^{-1}), or nystatin (10-50 µg mL^{-1}) are used [3]. The selectively of isolation method toward actinomycetes was improved by drying the soil samples at elevated temperature to kill unwanted bacteria.

Detection of CA Producers: In this study, the activities of the isolates toward CA production have been tested using the biological assay method against the indicator organism *E. coli* by using a piece of agar from each seven-day-old culture grown on actinomycetes isolation agar medium. Sixteen out of sixty isolates of *Streptomyces* sp. obtained showed noticeable β-lactamase inhibitor activity against *E.Coli* resistant to penicillin G (Table 2). The active isolates were cultivated in submerged culture for further antimicrobial production investigation.

Table 1: Origin of samples for initial screening of *Streptomyces* on actinomycetes isolation agar media

Origin of soil samples	Total number of isolates	Number of isolates reported positive inhibitory activity
1- New valley	15	4
2- Dakahlia	5	1
3- Aswan	4	1
4- Elkaluobia	5	1
5- Cairo	26	8
9-Raas Ghareb	5	1
Total	60	16

Table 2: Isolates checked for CA production and some morphological characteristics

			Color		
Isolate Number	Activity [mg L^{-1}]	Growth degree	AM	SM	SP
1.1	12	Abundant	Grey	Grey	Brown
1.2	6	Poor	White	White	None
1.4	6	Abundant	White	White	None
1.5	8	Moderate	White	White	None
1.7	8	Abundant	White	Beige	None
2.1	12	Moderate	White	Ivory	Brown
3.2	6	Moderate	White	Ivory	Brown
4.1	6	Abundant	White	White	Brown
5.4	13	Abundant	White	White	None
5.5	28	Abundant	White	White	None
5.6	10	Abundant	Grey	Ivory	None
5.13	20	Moderate	White	Ivory	None
5.14	40	Moderate	White	Ivory	None
5.15	65	Abundant	Grey	Ivory	None
5.19	50	Moderate	White	Ivory	None
5.20	13	Abundant	White	White	None
9.3	55	Abundant	White	Grey	None

The aerial mycelium (AM), Substrate mycelium (SM) and Soluble Pigment (SP)

After 144 h cultivation, the supernatant was sterilized by filtration and used to determine the inhibitory activity by bioassay and HPLC methods. Three isolates numbers 35, 44 and 56 were characterized with high activity of β-lactamase inhibitor. The most active isolate (No.35), which isolated from Ezbet El Nakhl plantation area, Cairo, was submitted further identified by conventional (based on biochemical and morphological characterization) and molecular identification methods.

Morphological and Culture Characteristics: The characterization of *Streptomyces* species is mainly based on the aerial, substrate mycelia color, soluble pigment production, the shape and ornamentation of spore surface. Other additional testes are also considered to ascertain species classification of new isolates strains as recommended [36]. *Str.* sp. NRC-35 grew on all of the used media. The abundance and the color of aerial mycelium depended on the medium composition and the age of the culture. The results in Table 3 indicated that the aerial mass color varied from grayish to gray with the exception of neutral agar medium, therefore it could be assigned to the gray serious. The substrate mycelium color varied depending on the medium composition. No soluble pigment produced on all of the used media. The classification of streptomycetes was originally based on morphological and biochemical characterization, later on physiological tests [35]. For *Str.* sp. NRC-35, it was observed that the aerial hypae bears spores of spiral type as shown in Fig.1. According to the shape of the spore surface under electron microscope, the spore wall ornamentation were grouped as smooth (SM), warty (WTY), spiny (SPY) and hairy (H). The individual spores of this strain was grouped as cylindrical with smooth surface as observed under the transmission electron microscope (Fig. 2).

Physiological and Biochemical Characteristics: Physiological characters such as degradation of starch, gelatin, inositol, rhamnose and reduction of

Table 3: Cultural characteristics of *Streptomyces* sp. NRC-35

		Color		
Type of medium	Growth	AM	SM	SP
Yeast extract- malt extract (ISP-2)	Moderate	Ivory	Ivory	None
Oatmeal (ISP-3)	Moderate	Grey	Grey	None
Inorganic salts- starch (ISP-4)	Abundant	Grey	Grey	None
Glycerol- asparagine (ISP-5)	Abundant	Grey	Pale Yellow	None
Bennets agar	Moderate	Grey	Pale Yellow	None
Czapeks agar	Fair	Grey	Yellow	None
Glucose-asparagine agar	Moderate	Grey	Ivory	None
Tyrosine (ISP-7)	Fair	Grey	Yellow	None
Nutrient agar	Fair	Colorless	Colorless	None

The aerial mycelium (AM) was classified according to the Bergey's manual of systematic bacteriology in the following color series, Substrate mycelium (SM) Soluble Pigment (SP)

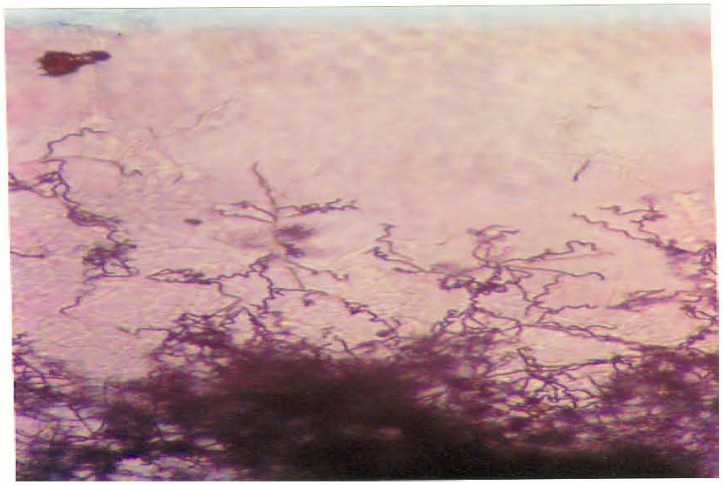

Fig. 1: Morphology of spore-bearing aerial hypaae of *Streptomyces* sp. NRC-35 after 14 days cultivation on Bennett's agar medium at 28°C showing sporechain spiral-(2000x)

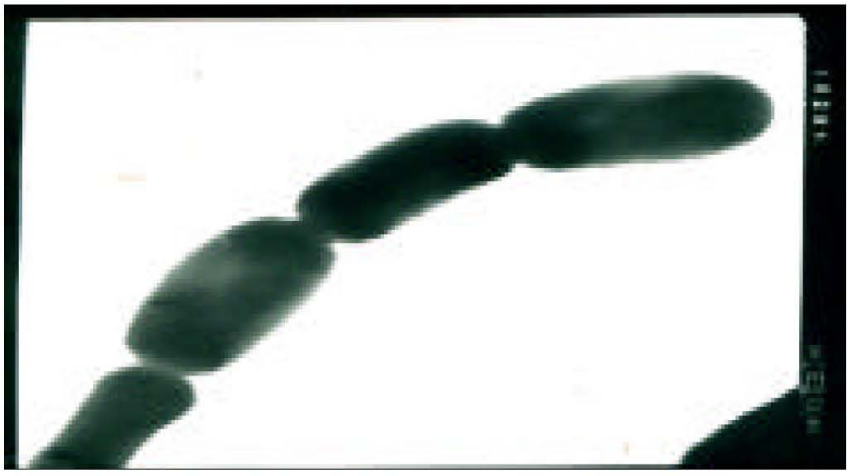

Fig. 2: Transmission electron micrograph of *Streptomyces* sp. NRC-35 spores after 21 days cultivation on Bennett's agar medium at 28 °C. (X 40,000) showing spore surface smooth and cylindrical

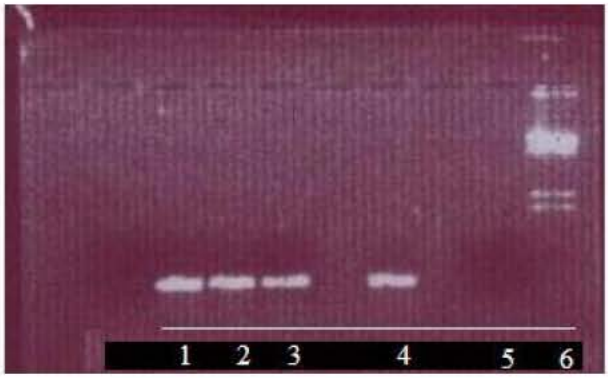

Fig. 3: Agarose gel electrophoresis of PCR products obtained by amplification of DNA mixtures isolated from 3 *Streptomyces* species with the primer pairs StrepB/StrepF. Lane 1 Isolate No.44 ; Lane 2, positive control (*Str. clavuligerus* NRRL-3585); Lane 3 and 4 Isolate No.35; Lane 5 negative control (without template DNA); Lane 6 molecular weight marker 50bp ladar

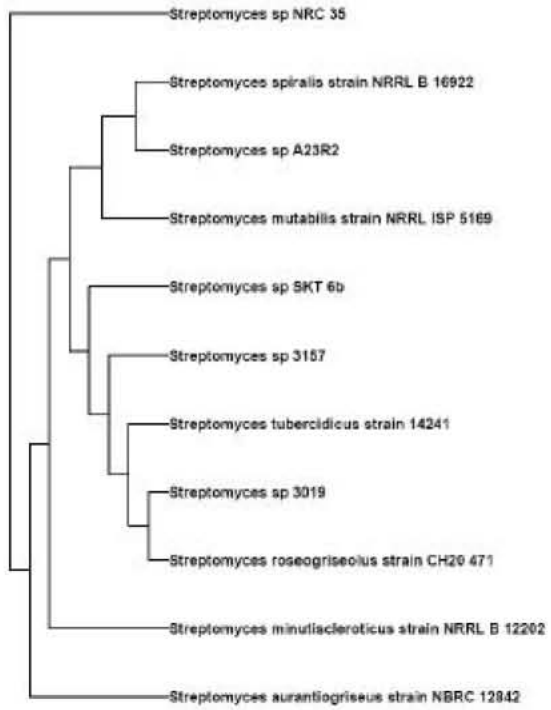

Fig. 4: The phylogenetic position of *Streptomyces* sp. NRC-35 among neighboring method showing 16S rDNA tree of the phylogenic similarity (%) comparing with the sequences of other known Streptomyces species

nitrates, some additional testes relative to the use of arabinose, glycerol, galactose and mannitol are also considered to ascertain species classification of new isolates strains as recommended by different authors [37-39].

Table 4: Primary physiological properties of *Streptomyces* sp. NRC-35.

Properties	Results
Hydrolysis of starch	Positive
Milk coagulation	No coagulation in 14 days
Nitrate reduction	Negative
Gelatin liquefaction	Negative
Melanin production	Negative
Cellulose decomposition	Positive

Table 5: Carbon source utilization by *Streptomyces* sp. NRC-35

Carbon sources	Degree of utilization
D-glucose	+
D-xylose	+++
L-arabinose	++
L-rhamnose	+++
D-fructose	+
D-galactose	++
D-raffinose	±
D-mainnitol	++
Meso-inositol	+
D-Salicin	+
Sucrose	±
Cellobiose	++
Maltose	+++

+++ = Abundant growth, ++ = Moderate growth, + = Faire growth, ± = Doubtful

The results in Table 4 Indicated that, the *Str.* sp. NRC-35 was characterized by its inability to reduce nitrate to nitrite. Also, melanin pigment production was not observed on tyrosine agar medium (ISP-7) and it grew on gelatin without liquefying it. This strain was able to grow on skim milk but did not cause coagulation and peptonization. On the other hand, it hydrolyzed starch

and cellulose. Furthermore, the ability to utilize different C-sources using basal medium ISP9 [26] was illustrated in Table 5. The strain showed abundant mycelium growth on the media supplemented with D-xylose, L-rhamnose and maltose followed by L-arabinose, D-galactose, D-mannitol and cellulose. While, it grew poorly in medium containing D-glucose, D-fructose, meso-inositol and D-salicin. However, the growth was doubtful on the media with sucrose and raffinose. Cell wall determination is one of the main characteristics of Streptomycetes identification [27]. The cell wall composition of this strain was found to the L-type.

Comparison with the Known Strain: Based on the taxonomic properties described above, strain NRC-35 belongs to the genus *Streptomyces*. The characteristics of this strain were compared with published descriptions of various *Streptomyces* species [40, 41]. It was considered *Str.* sp. NRC-35 to be nearly related to *Str. Parvullus* and *Str. humidus* which are differed for strain *Str.* sp. NRC-35 for using sucrose, raffinose and galactose. Therefore, *Str. Parvullus* produced actinomycin and *Str. humidus* produced dehydrostreptomycin. In particular, *Str.* sp. NRC-35 produced clavulanic acid no resembles completely of any known strains till *Str. clavuligerus* did not utilize tested sugars [14]. In general, the taxonomic classification and identification of streptomycetes is based on morphological and biochemical characterization later on physiological test [37], thus molecular methods (16S rRNA gene sequence analysis) could represent an improvement [10,29]. Nowadays, the detection and classification of Streptomyces in both environmental and pure culture samples are now most commonly performed by molecular approaches based on selective PCR amplification [42]. Many approaches have been tried to aid in the classification of Streptomyces isolates to the genus, species and strain levels. Genetic methods are more rapid and convenient than classification methods based on phenotypic characteristics [43].

PCR Amplification: Experimental analysis of the PCR amplification performance the forward primer Strep B in conjunction with the reverse primer Strep F was conducted under the reaction conditions described. The primer pairs Strep B/Strep F amplified a fragment of the expected size from the DNA isolated from positive control strain *Str. clavuligerus* NRRL-3585. The specificity of the primers was further examined by PCR amplification using DNA isolated from three Streptomyces strains. Primer pairs Strep B/StrepF, produced fragments of the expected size only in the presence of *Str. clavuligerus* NRRL-3585 (Fig. 3). The specificity of the PCR is affected by multiple factors, such as the primers, the properties of the gene regions flanking the target site, the annealing temperature in the PCR reaction and the reaction conditions [44].

Phylogenetic Taxonomy of Streptomyces: The alignment of the nucleotide sequences (749bp) of *Str.* sp. NRC-35 was done through matching with the 16S rRNA reported genes sequences in the gene bank. The database of NCBI Blast available at (www.ncbi-nlm-nih.gov) was used to compare the isolated strains with sequence of the reference species of *Streptomyces* contained in genomic database bank. The results exhibited similarity level 91% with 10 strains from the uncultured known *Streptomyces* species. The 16S rDNA nucleotide sequence of *Str.* sp. NRC-35 consisted of 749 bp and the GC-content was 70%. These results were in accordance to many authors who mentioned that, the GC-content of the Streptomyces DNA is 69-78 % [11, 23, 45]. Modern *Streptomyces* identification systems are based on 16S rDNA sequence data, which have provided invaluable information about *Streptomyces* systematic and then have been used to identify several newly isolated *Streptomyces* [13]. For data analysis, the phylogenetic tree in Fig. 4 was derived from the distance matrices using neighbor-joining method [34]. The majority of sequences clustered into groups in the phylogenetic analysis. However, the results indicate the presence of several different types of *Streptomycete* 16S rDNA sequences in buildings, suggesting higher diversity with several species. These strains together with sequences from uncultured known *Streptomyces* species which were differed for them in the some morphological characters; carbon utilization and active secondary metabolites production, where the different detection limits of the methods make presence/absence comparisons difficult [12]. On the other hand, the binary similarity between this isolate and the known producers of clavulanic acid from *Streptomyces* strains was low. Therefore, *Str.* sp. NRC-35 is suggested to be new *Streptomyces* species. Recently, the Streptomyces identification is based on 16S rDNA sequence data, which have provided invaluable information about Streptomyces systematic and then have been used to identify several newly isolated Streptomyces, [13]. The 16S rRNA gene sequence of *Str.* sp. NRC-35 was compared to other *Streptomyces* species. It has been shown that the highest binary similarity value of 91 % with the different *Streptomyces* species Fig: (4) which was differed for them in the morphological characters and carbon utilization where the different

detection limits of the methods make presence/ absence comparisons difficult [12]. Therefore, *Str.* sp. NRC-35 was suggesting to be new genomic *Streptomyces* species. The sequence data were submitted in the GenBank database under accession number GU198170.

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