MOLECULAR AND MORPHOLOGICAL IDENTIFICATION OF STREPTOMYCES SP. NRC-88 NOVA SPECIES AS β-LACTAMASE INHIBITOR FOR PHARMACEUTICAL APPLICATION

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ABSTRACT

Objective: Clavulanic acid (CA) is a vital agent in the treatment of bacterial infections since it is a potent inhibitor of an extensive variety of β-lactamase enzymes. Its production from Streptomyces strains is fact of expanding clinical significance. This study aimed to isolate and characterize a promising Streptomyces (S) species strain which produced an effective β-lactamase inhibitor.

Methods: Streptomyces sp. NRC-88 was isolated from an Egyptian soil sample. The phenotypic and phylogenetic examinations of 16S rRNA gene were investigated. The active metabolite of this strain (CA) was determined by particular synergistic bioassay, spectrophotometric assay, recognized by thin layer chromatography, and structure of the CA affirmed by high-performance liquid chromatography (HPLC) method.

Results: A phylogenetic examination of the 16S rRNA gene sequence of the NRC-88 strain consistent with conventional taxonomy was carried out, and confirmed that the strain NRC-88 was most similar to S. aburavensis S-66 (99%). The active metabolite of this strain (CA) was determined by different methods and confirmed the structure of the CA by the HPLC method. It produced up to 87 mg/l in a specific CA production medium.

Conclusion: A new species of Streptomyces sp. NRC-88 isolated and recognized by phenotypic and genotypic proof. This strain suggested the name, Streptomyces sp. NRC-88 (accession number KM014489). It was able to produce CA as the β-lactamase inhibitor.

Keywords: Isolation, Streptomyces sp. NRC-88, Phenotypic and phylogenetic identification, Clavulanic acid production.

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INTRODUCTION

The diseases caused by microorganisms are considered to be serious threats against people's health worldwide and a great deal of money is spent to treat them. Genuine contaminations brought about by microorganisms that have turned out to be impervious to normally utilize antibiotics have turned into a noteworthy worldwide social insurance issue in the 21st century [1].

The β-lactam antibiotics are a standout among the most prevalent classes of antibacterial operators, whose system of activity is depicted by the means of the hindrance of bacterial cell divider union [2]. Not long after the use of the β-lactam antibiotics, the resistance of β-lactam antibiotics is associated with inactivation of the β-lactam structure due to the opening of the β-lactam ring by β-lactamase produced by bacteria [3]. Microorganisms which are impervious to specific antibiotics are progressively causing difficult issues in the treatment of irresistible maladies [4].

One of the techniques utilized by this gathering of bacteria to resist against β-lactam antibiotics is the generation of a β-lactam hydrolyzing enzyme β-lactamase, which has the capacity kill these anti-infective agents by severing the β-lactam ring [5]. Along these lines, to defeat this resistance, β-lactamase inhibitors are frequently utilized as a part of conjunction with β-lactam antibiotics as these mixes maintain the debasement of these antibiotics and increment the adequacy of these medications [6].

CA is a powerful inhibitor of an extensive variety of β-lactamase from pathogenic living beings which are in charge of the defense of microorganisms against β-lactam antibiotics [3]. CA or clavulanate, is commercially used along with amoxicillin or ticarcillin (augmentin), which is β-lactam antibiotics with high levels of antibacterial activity and this combination has been listed as an important and very successful antibacterial agents in preventing infections due to Gram-positive (Staphylococcus sp.) and Gram-negative (Klebsiella sp., Hemophilus sp., Proteus, Shigella, Pseudomonas). β-lactamase-producing pathogens in the WHO list of essential medicines [2015]. Amoxicillin + CA is most commonly prescribed out by fixed dose combinations of all antimicrobials and can be of a very effective alternative treatment against the deadly multidrug resistant Gram-negative bacteria [7]. In Brazil, medicines containing amoxicillin and potassium clavulanate are mainly produced by Glaxo-SmithKline Beecham Farmacêutica Laboratories under the name of clavulin or Augmentin with reported global sales of >$2.1 billion [5].

Microorganisms fill in as alluring assets, attributable to their capacity to coordinate significant items with novel structures and activities [8]. Soil, specifically, is a seriously abused biological specialty of the inhabitants of the bacteria domain. Actinomycetes demonstrated an uncommon capacity to create possibly novel, clinically valuable, secondary metabolites, for example, antitumor, anti-infection agents, cell reinforcement, antiviral, antimicrobial, and enzymes [9-11].

CA was first produced by S. clavuligerus [12]. Jensen and Paradkar [13] mentioned that CA itself is even more restricted, with only four producing Streptomyces species reported to date. The strain is S. clavuligerus [12]. S. jumoninensis [14], S. katsuraharanaus [15] Streptomyces sp. [16] More recently, Streptomyces sp. NRC-35 [17], Streptomyces sp. NRC-77 [18] and Streptomyces sp. MuNRC-77 [19]. This study presents the isolation and characterization of a promising strain of Streptomyces species producing a β-lactamase-inhibitor.

METHODS

Microorganisms
Strain NRC-88 was isolated from an Egyptian soil sample by screening program. The new identified Streptomyces species-produced CA. This
Strain was deposited in the Actinomycetes Culture Collection, National Research Centre, Cairo, Egypt. A resistant strain of *Escherichia coli*, at 25 µg/ml Penicillin-G (Sigma, St. Louis, USA), was used as a test strain for CA production. These strains were stored at ~80°C in 50% glycerol for further studies.

**Streptomycyes isolation and cultivation conditions**

Thirty *Streptomycyes* isolates have been isolated from different Egyptian soil samples. Strain NRC-88 was isolated using the Streptomyces plate technique on two different media. The first medium is actinomycetes isolation agar medium (Difco, NJ, USA) which composed of (g/l): glucose 5; sodium propionate 4; sodium caseinate 2; KH₂PO₄ 2; asparagine 0.1; MgSO₄.7H₂O 0.1 and FeSO₄.7H₂O 1 mg; agar 15 at pH 7.0. The second medium is *Streptomycyes* medium which consists of (g/l): glucose 5; L-glutamic acid 4; KH₂PO₄ 1; MgSO₄.7H₂O 0.7; NaCl 1; FeSO₄.7H₂O 1 mg; agar 25. The isolation medium supplemented with 25 µg/ml of penicillin G and cycloheximide (50 µg/ml) to minimize the bacterial and fungal contamination, respectively, and then, incubated at 28°C for 7-10 days [17]. The isolates were initially tested for their ability of β-lactamase inhibitor by the specific synergistic bioassay [20] using a resistant test strain of *E. coli*. CA was detected by thin-layer chromatography (TLC) [21]. It was determined by spectrophotometric method at 312 nm after derivatization with imidazole according to Bird et al. [22], and confirmed the structure of the CA by high performance liquid chromatography (HPLC) assay [23]. The promising isolates, which showed the maximum CA production, were selected for further identification.

**Production medium and cultivation conditions**

Strain NRC-88 was cultivated in Erlenmeyer flask 250 ml containing 50 ml (production medium) on rotary shaker Innova 4080 (New Brunswick, NJ, USA) at 200 rpm and 28°C for 6 days. The production medium composed of (g/l): Soy bean meal - 30; soluble starch - 47; KH₂PO₄ - 0.1; FeSO₄.7H₂O - 0.1; pH 6.5±0.2. The inoculation was carried out using a spore suspension of NRC-88 strain slanted on International Streptomycyes Project (ISP) 2 medium. The culture broth was separated from the mycelium by centrifugation at 8000 rpm for 10 minutes. The supernatant was sterilized by filtration and used for the evaluation of the inhibitory activity, which was carried out using the above mentioned three methods.

**Composition of the culture media used for identification studies**

The media composition and the cultivation conditions were performed according to Shirling and Gottlieb [24].

**Cultural, morphological, physiological, and biochemical analysis of strain NRC-88**

Cultural characteristics describe the growth, the color of the aerial mass, and the substrate mycelium, and the soluble pigment of the strain on different ISP media. These characteristics of strain NRC-88 were observed on the 7th, the 14th, and the 21st day for mature cultures grown on various media followed the guidelines adopted by ISP [24]. The color of the aerial mass, the substrate mycelium, and the soluble pigment were visually estimated using Stamp Color Key based on the computer color wheels [25].

Morphological properties include both spore chains and spore surface of the isolated strain. The spore chains of strain NRC-88 were determined by a light microscope (Model SE. Nikon Inc., NY, USA). This was done using the cover slip technique in which individual cultures (14 days age) were transferred to the base of cover slips buried in Bennett’s medium for photographs. The spore surface of strain NRC-88 was observed under transmission electron microscope (TEM) Zeiss EM 10 (Zeiss, Oberkochen, Germany) from the culture on 21 days of incubation [24]. These characters and photos of the Streptomyces isolate were compared to the similar *Streptomyces* species in Bergey’s Manual of Determinative Bacteriology Ninth edition [26] and Bergey’s Manual of Systematic Bacteriology [27] to identify the isolate to the species level.

The physiological tests included starch hydrolysis, gelatin liquefaction, skim milk coagulation, melamin pigment production, and nitrate reduction. The starch hydrolysis, the growth on gelatin and skim milk were tested [24] using special media described above. The production of a melanin pigment was observed on media ISP 1, 6, and 7 after 7-10 days and nitrate reduction was tested on nitrate broth medium (Fluka, NY, USA) following to the directions given by ISP [24].

The chemotaxonomy included the cell wall analysis and carbon sources utilization. Diaminopimelic acid (LL-DAP) isomers in the cell wall were analyzed by paper chromatography using the method of Lechevalier and Lechevalier [28]. The ability of the strain to utilize different carbon sources was examined on basal medium ISP 9 to which separately-sterilized carbon sources were added to a final concentration of 1.0% using glucose as positive control. The plates were incubated at 28°C and the growth was noticed after 7, 14, and 21 days.

**Molecular identification and phylogenetic analysis of strain NRC-88**

**Extraction of Streptomyces genomic DNA**

The strain was grown on slant of the actinomyces isolates agar medium for 7-10 days at 28°C. Two ml of spore suspension were inoculated in the ISP 2 broth medium and incubated on incubator shaker Innova 4080 (New Brunswick, NJ, USA) at 200 rpm on 28°C for 24 h to form pellets of vegetative cells. Total genomic DNA preparation was performed using the method of Lee et al. [29]. The collected pellets were left to dry and dissolved in a suitable volume (100 µl) of TE buffer (100 mmol NaCl 1 mmol ethylenediaminetetraacetic acid, 100 mmol tris-HCl, pH 8.00), or deionized water and stored at ~20°C. An aliquot was analyzed by agarose gel electrophoresis (1.5%) to assess the DNA concentration.

**Polymerase chain reaction (PCR) amplification and sequencing**

The PCR reactions were carried out in 0.2 ml Eppendorf tubes in total volume of 25 µl. The amplifications were performed in a Gene AMP PCR system 9700, PE Applied Biosystems, (Perkin Elmer, Ohio, USA). The sequencing *Streptomyces*-specific PCR primers used were StrepB, 5'-ACA AGC CCT GGA AAC GGG T-3' (forward) and StrepF 5'-ACG TGT GCA GCC CAA GACA-3' (reverse) using Biologet BV software (Biologet, Nijmegen, the Netherlands). These primers were used for the amplification of 16S rRNA fragments from genomic DNA isolated Streptomyces [30].

The PCR reaction mixture (25 µl) contained PCR beads (Amersham Bioscience Europe GmbH, Freiburg, Germany), 0.5 µl from each primer StrepB and StrepF and 2 µl of template genomic DNA up to final volume 25 µl reached by deionized water. Amplification was performed with an initial denaturation step of 3 minutes at 94°C and then 35 cycles of (60 seconds denaturation at 94°C, 30 seconds at 59°C for primer annealing and 60 seconds at 72°C for primer extension) and kept at 72°C for 7 minutes to complete extension.

Electrophoresis of the PCR products was carried out on 1% agarose gel containing ethidium bromide (0.5 µg/ml) to ensure that a fragment of the correct size had been amplified [31] and detected by a Gel documentation system (Alpha-Imager 2200, CA, USA). Amplified 16S rRNA fragments were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany).

Sequencing of the purified PCR product was carried out by ABI Prism 377 DNA Sequencer (Perkin Elmer, Ohio, USA) with the reaction kit Big Dye® Terminator v3.1. Cycle sequencing ready reaction (Applied Biosystems, Foster City, USA) and the universal primers listed above in gene’s analysis unit (Cornell University, NY, USA sequencing facility center).

**Phylogenetic analysis**

Phylogenetic data were obtained by aligning the nucleotides of different 16S rRNA retrieved from the Basic Local Alignment Search
The main goal of this study was to isolate and characterize a new Streptomyces strain with β-lactamase inhibitory activity of (New Valley locality) Egyptian soil. Isolation of Streptomyces sp. NRC-88 was carried out using different selective Streptomyces media such as an Actinomycetes isolation agar medium (Difco, NJ, USA) and Streptomyces medium according to our previous study by Awad et al. [17] and Awad and El-Shahed [18]. The enlargement of antifungal agents to the confinement media stifles the development of fungal species on the plates. For this purpose, either cycloheximide (50-100 µg/ml) or nystatin (1-10 µg/ml) was used [34]. On the other hand, Penicillin G was added to the medium to minimize the bacterial contamination.

In this work, five out of thirty isolates of Streptomyces sp. obtained showed noticeable inhibitory activity against E. coli resistant to Penicillin G. This inhibitory activity may be due to any substance inhibiting or degrading the β-lactam antibiotic. We selected the most active isolate (based on inhibition zone diameter) for further study. The selected strain was named NRC-88. The inhibitory activity of strain NRC-88 was tested using a specific synergistic biological assay against a resistant Gram-negative bacterium E. coli by utilizing a small amount of agar from 7-day-old culture developed on actinomycetes isolation agar medium as primary screening. The results of the primary screening showed a noticeable β-lactamase inhibitory activity against E. coli resistance bacterium. For the secondary screening, Streptomyces sp. NRC-88 was cultivated on submerged culture using a specific CA production medium for further investigation. After 6-day cultivation, the supernatant was sterilized by filtration and used to determine the inhibitory activity. The CA yield was determined by specific synergistic bioassay, spectrophotometric assay, detected by TLC, and confirmed by HPLC assay of which the CA peak was at retention time of 3.22 minutes from a culture broth of strain NRC-88 and 3.525 minutes for standard pure CA (Fig. 1).

The maximal CA yield of 87 mg/ml was obtained. These obtained results were close to those quoted in the literature using the complex medium. For example, Chen et al. [35] obtained 115 mg/l CA from the medium containing soy flour without any addition of amino acids. Neto et al. [36] obtained about 200 mg/l using the mixture of complex medium components of soy bean hydrolyzed protein, malt extract, and yeast extract in addition to other minerals. However, all these studies were conducted in a stirred tank bioreactor level with well-controlled conditions. Therefore, strain NRC-88 was submitted for identification.

The production medium that was used contains soy bean protein in the form of an extract. Several studies have shown the advantages of soy bean meal extract that is used in the production of both antibiotics generally and especially CA. One of these studies is Chen et al. [35], who observed that the highest CA production was obtained when soybean

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**RESULTS AND DISCUSSION**

**Soil sample and isolation**

The main goal of this study was to isolate and characterize a new Streptomyces strain with β-lactamase inhibitory activity of (New Valley locality) Egyptian soil. Isolation of Streptomyces sp.NRC-88 was carried out using different selective Streptomyces media such as an Actinomycetes isolation agar medium (Difco, NJ, USA) and Streptomyces medium according to our previous study by Awad et al. [17] and Awad and El-Shahed [18]. The enlargement of antifungal agents to the confinement media stifles the development of fungal species on the plates. For this purpose, either cycloheximide (50-100 µg/ml) or nystatin (1-10 µg/ml) was used [34]. On the other hand, Penicillin G was added to the medium to minimize the bacterial contamination.

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flour was utilized. Another study was done by Rosa et al. [37], who mentioned that soy bean protein is the most important nutrient for the CA biosynthesis. The soybean meal used in the production medium seems to be effective for production of secondary metabolites because of the slow breakdown during the fermentation process. It provides proper cellular growth and contains arginine, the precursor of the CA molecule [38] and has been proven to be the safest high protein cereal grain to use in antibiotic production.

**Taxonomy of Streptomyces strain NRC-88**

**Conventional taxonomy**

Cultural and morphological characteristics

Strain NRC-88 propagated on a series of agar media with the different degrees in the growth, displaying morphology typical of Streptomyces [27], since the colony was slow growing, aerobic, white and layered, with an earthy odor, and aerial substrate mycelia of altered colors. The abundance and the color of aerial mycelium depended on the medium composition and the age of the culture. The growth was abundant on most of the used media but was moderate on ISP 2 and ISP7 media, but it is fair using an ISP 5 medium (Table 1). The color of aerial mycelium was white. Therefore, strain NRC-88 was assigned in the white series. The strain did not produce any diffusible pigment on most of the media used. While strain NRC-88 produced a soluble pigment, whose color was brown on ISP 5, Bennett’s, ISP 7, and nutrient agar media, the color was yellowish on ISP 2 medium (Table 1). Culturing method and morphological characteristics were used as a method to identify Streptomyces species using the selective plating technique [39].

The morphology spore chains’ of the strain NRC-88 was an open spiral type (Fig. 2a), and can be assigned in a spiral group. As indicated by the state of the spore chains seen under light microscopy, the isolates were gathered as rectus-flexibilities, spiral (S), and retinaculapierti [27]. The spore surface ornamentation of strain NRC-88 was observed by TEM showing the smooth spore surface (Fig. 2b). Adopting from Shirling and Gottlieb [24] research facilities having admittance to an electron microscope must incorporate electron micrographs of the spore surface as one of the unmistakable portrayals for each type of culture.

Physiological and biochemical properties

Strain NRC-88 was tested on melanin pigment production media such as ISP 1, 6, and 7. It grew on these media, but no melanin pigment was observed on all the used media. The strain grew in the gelatin medium but could not liquefy the gelatin. Moreover, it grew on skimmed milk medium but did not cause coagulation and peptonization. Therefore, the NRC-88 strain did not harbor the respective degrading enzyme. The strain did not reduce nitrate to nitrite, and it hydrolyzed the starch (Table 2). With auxiliary identification, some physiological appeals, such as degradation of starch, gelatin, and decline of nitrate, are also well-thought-out to determine the species’ taxonomy of novel isolates strains as commended by Rosselló-Mora and Amann [40].

Examination of the whole-cell hydrolyzate of strain NRC-88 exposed the occurrence of chemo type, L cell wall LL-DAP acid (Table 2). The incidence of chemo Type I, LL-DAP in the cell wall indicates that this strain is Streptomyces as identified by Lechevalier and Lechevalier [28], who recognized that cell wall composition investigation is one of the key chemotaxonomic appeals of Streptomyces identification. Strain NRC-88 was capable of consuming different C-sources (Table 2).

The strain NRC-88 made abundant mycelium on the basal medium with, L-arabinose, L-ribose, D-galactose, rafafinosi, D-mannitool, myo-inositol and maltose, while the strain grew moderately in the occurrence of D-xylose, fructose, salicin, sucrose, and cellulose. The strain cultivates poorly on a medium having D-glucose.

**Comparison between strain NRC-88 and the references Streptomyces strains**

Based on the phenotypic assets, strain NRC-88 was classified in the genus Streptomyces. The classification of a Streptomyces species is predominantly based on the color of aerial and substrate mycelium, and soluble pigment, the shape, and ornamentation of the spore surface because of its constancy. The characteristics of this strain were compared to the known Streptomyces species in Bergey’s Manual of Determinative Bacteriology 9th edition [26] and Bergey’s Manual of Systematic Bacteriology [27]. Strain NRC-88 does not resemble any known strain, but has similarity to Streptomyces species (Streptomyces ochraceisceroticus, Streptomyces herbescens, and Streptomyces flocculus). These strains have the same aerial, and substrate mycelia colors, spore shapes, and physiological characters with some differences between them.

Strain NRC-88 differs from all the previous Streptomyces species in its characteristics, such as its inability to utilize of most tested sugars, and produces CA, unlike other species. As supported by Rosselló-Mora and Amann [40], carbon source usage, as an extra test was likewise.

![Fig. 2: Morphology of spore-bearing aerial mycelium of NRC-88 isolate, spore chains of the spiral type are shown, ×40.000 (a) and Spore surface is shown as smooth, ×1.000 (b) after 14 and 21 days of the incubation on Bennett’s agar medium at 28°C](image)

| Table 1: Cultural characteristics of NRC-88 strain |
|---------------------------------------------------|
| **Agar medium** | **Amount of growth** | **Color of Arial mycelium** | **Color of Substrate mycelium** | **Soluble pigment** |
| Yeast extract-malt extract (ISP 2) | Moderate | Beige and white from edge | White cottony | Yellowish |
| Oatmeal (ISP 3) | Abundant | White cottony | White-to-brownish | Brownish |
| Inorganic salts-starch (ISP 4) | Abundant | White cottony | Beige-light yellow | None |
| Glycerol-sparagine (ISP 5) | Fair velvety | White cottony | Beige-light yellow | None |
| Bennett’s agar | Abundant (velvety) | White cottony | Beige-light yellow | None |
| Czapek’s agar | Abundant | White cottony | Beige-light yellow | None |
| Glucose-asparagine agar | Abundant | White cottony | Beige-light yellow | None |
| Tyrosine agar (ISP 7) | Moderate | White cottony | Beige-light yellow | None |
| Nutrient agar | Abundant | White cottony | Beige-light yellow | None |

ISP: International Streptomyces Project
considered to discover species arrangement of a new isolates strain. The ordered grouping, and recognizable proof of a *Streptomyces* species in light of morphological, physiological, and biochemical portrayals is troublesome and not adequate [41]. In this manner, genotypic approaches illustrate an enhancement and have been utilized to distinguish a few recently isolated *Streptomyces* [42].

Molecular identification and phylogenetic analysis of strain NRC-88

PCR amplification and primer specificity

The experimental analysis of the PCR amplification performance of the forward primer StrepB in combination with the reverse primer StrepF was accompanied by the reaction conditions pronounced in materials and methods. The specificity of the PCR is affected by numerous factors, particularly the primer pairs. In this study, the (StrepB/StrepF) primer pair was used as a specific primer for *Streptomyces*. The primer was studied by PCR amplification using genomic DNA, isolated from strain NRC-88. The primers were positively used to amplify genomic DNA from the isolated samples. These results are in agreement with Rintala et al. [30], who showed that these primers are specific for *Streptomyces*.

Sequencing and phylogenetic analysis

The nucleotide sequence (466 bp) of strain NRC-88 was subjected to match with the 16S rRNA reported gene sequences in the gene bank database. The database of NCBI BLAST available at (www.ncbi.nlm.nih.gov/BLAST) was used to compare the strain NRC-88 with those of member *Streptomyces* species strains. Due to the high sequence similarity of the species (99%) with *Streptomyces* strains, strain NRC-88 is most closely related to S. aburaviensis S-66. However, they have the highest similarity on a genetic level, show differences on some phenotypic level. Furthermore, they are not different in their CA productivity, but in their ability to produce CA.

A taxonomy that has been founded on restricted phenotypic and hereditary criteria are changing, regularly and fundamentally, since new phylogenetic in view of 16S rRNA sequence information give significant data about *Streptomyces*. Systematic techniques have turned out to be accessible at lower taxonomic levels as well as at the division and order levels utilized in identifying a few recently isolated *Streptomyces* [43,44].

In the alignment of the phylogenetic tree, all the unclassified and classified *Streptomyces* strains, which were most different from strain NRC-88 in terms of phenotypic characteristics, were discarded. A phylogenetic tree resulted from the distance matrices using a neighbor-joining method (Fig. 3).

| Character                  | Results            | Character                  | Utilization     |
|---------------------------|--------------------|---------------------------|-----------------|
| **Morphological characteristic** |                    | **Utilization**            |                 |
| Spore chains              | Spirals            | DL-methionine             | Poor growth     |
| Spore surface             | Smooth             | DL-iso-leucine            | Weak growth     |
| Color of aerial mycelium  | White              | L-arginine                | Weak growth     |
| **Physiological characteristics** |                    | L-lysine                  | Abundant growth |
| Hydrolysis of starch      | Positive           | L-glutamic                | No growth       |
| Action of milk            | No coagulation in 14 days | L-histidine               | Poor growth     |
| Nitrate reduction         | Negative           | Ph-alanine                | Abundant growth |
| Gelatin liquefaction      | None               | L-asparagine              | Weak growth     |
| Melanin production        | None               | L-valine                  | Weak growth     |
| Cell wall hydrolysis      |                    | L-cystaine                | No growth       |
| LL DAP                    | Positive           | Glycine                   | Good growth     |
| Sugar pattern             | ND                 | Proline                   | Weak growth     |
| Utilization of C-source   |                    | Ornithine                 | Weak growth     |
|                          |                    | Tyrosine                  | Good growth     |
|                          | D-glucose          | +                         | Moderate growth |
|                          | D-xylose           | ++                        | Antibiotics susceptibility (mm) |
|                          | L-arabinose        | +++                       | (RD 5 µg)       | 00 |
|                          | L-rhamnose         | +++                       | (VA 30 µg)      | 20 |
|                          | D-fructose         | ++                        | (S 10 µg)       | 15 |
|                          | D-galactose        | +++                       | (N 30 µg)       | 12 |
|                          | Raffinose          | +++                       | (TE 5 µg)       | 18 |
|                          | D-mannitol         | +++                       | (NA 30 µg)      | 06 |
|                          | Mes-o-inositol     | +++                       | (NV 30 µg)      | 00 |
|                          | Salicin            | ++                        | (CDX 30 µg)     | 22 |
|                          | Sucrose            | +                         | Resistance toward sodium chloride (%) |
|                          | Cellobiose         | ++                        | 0               | Weak |
|                          | Maltose            | +++                       | 2               | Abundant |
|                          | Temperature tolerance (°C) | 4              | Moderate |
|                          | 20-37              | Abundant                  | 7               | Weak |

*: Weak growth, ++: Moderate growth, +++: Abundant growth, DAP: Diaminopimelic acid, RD: Rifamycin, VA: Vancomycin, S: Streptomycin, N: Neomycin, TE: Tetracycline, NA: Nalidixic acid, NV: Novobiocin, CDZ: Cefodizime

Fig. 3: Neighbor-joining phylogenetic representation of the strains and their closest National Center for Biotechnology Information relatives based on 16S rRNA gene sequences of *Streptomyces* sp. NRC-88 and some known sequences of *Streptomyces*. 

Table 2: Physiological, morphological, biochemical properties and amino acids utilization of NRC-88 strain
A good similarity was found between the 16S rRNA sequence of *S. aburaviensis* S-66 and strain NRC-88. In contrast, variations were found between the binary similarity of the 16S rRNA gene sequence of strain NRC-88 and the 16S rRNA gene sequences of all other *Streptomyces* strains matching on the GenBank database that had a similarity of 99% as shown in Table 3.

**Polysporic approach**

There exist some differences in the morphological, biochemical and physiological characters of strain NRC-88 and the neighbors *S. ochraceiscleroticus*, *S. herbescens*, *S. flocculus* and *S. aburaviensis* S-66, especially in the utilization of most sugar used as shown in Table 4.

Owing to these differences, *S. aburaviensis* S-66 produces other metabolites like antifungal antibiotics and an enzyme inhibitor [45]. On the other hand, *S. ochraceiscleroticus* produces xylanase, cellulases and antitumor antibiotic. While, *S. flocculus* produces streptonigrin, the benzoxazine one antibiotic shows immunosupressant, anti-HIV and antileukaemic activity. The lowest binary similarity between strain NRC-88 and the CA producer strains such as *S. clavuligerus*, *S. jumonjinensis*, and *S. katsurahamanus* was recorded. Antibiotic production by actinomycetes, therefore, may not be species-specific but strain-specific [46]. However, CA is no antibiotic, but a secondary metabolite acting as a potent β-lactamase inhibitor. Furthermore, the *Streptomyces* sp. NRC-88 strain is known to produce CA but is a different strain from *S. clavuligerus* as the reference strain of CA.

It is clear from phylogenetic investigation that, strain NRC-88 speaks to an unmistakable phyletic line recommending another genomic species. It is obvious from Table 4 that strain NRC-88 can be recognized from the type strains of its most prompt phylogenetic neighbors of the family *Streptomyces* by its phenotypic and phylogenetic characterization.

The use of genotypic and phenotypic systems (polysporic approach) gives a superior resolution in the species level identification proof (Mizui *et al.*). [47]. It is clear from the genotypic and phenotypic information that strain NRC-88 should be perceived as the type strain of a novel species in the genus *Streptomyces*. The name proposed for this taxon is *Streptomyces* sp. NRC-88 nova species. It is a potential source of active compounds.

**Secondary structure prediction and Restriction site analysis**

The RNA secondary structure was predicted for 16S rRNA of *Streptomyces* sp. NRC-88 (Fig. 4). It showed that the free energy of structure is −100.6 kcal/mol, threshold energy is −4.0 with cluster factor, conserved factor 2 and compensated factor 4 and conservativity is 0.8. The prediction of restriction sites of the strain NRC-88 showed the restriction sites for various enzymes such as BsaI, SmalI, Eco53KlIse1, PspGI and BsaHI, etc. (Fig. 5).

**CONCLUSION**

The novel *Streptomyces*, NRC-88 strain, was confined from an Egyptian soil sample that was equipped for creating CA. It had biochemical,

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**Table 3: Streptomyces sp. NRC-88 and its closest phylogenetic neighbors as representatives of *Streptomyces* species**

| S.No. | Strain name | Accession number | Similarity % |
|-------|-------------|-----------------|-------------|
| 1     | *Streptomyces* sp. 35/02 | AY571804 | 99 |
| 2     | *Streptomyces* spiroverticillatus sj33 | JX013967 | 99 |
| 3     | *Streptomyces* chrestomyceticus HA11055 | JQ799044 | 99 |
| 4     | *Streptomyces* candidus NBRIC 12184 | NR_112302.1 | 99 |
| 5     | *Streptomyces* claviger f-1-5 | KJ571024 | 99 |
| 6     | *Streptomyces* claviger M3A-2009 | KJ14249956 | 99 |
| 7     | *Streptomyces* spiroverticillatus A3E | JX570583 | 99 |
| 8     | *Streptomyces* candidus CA-261520 | JX849062 | 99 |
| 9     | *Streptomyces* beijiangensis A6 | JX122145 | 99 |
| 10    | *Streptomyces* finlay PM409 | JQ422174 | 99 |
| 11    | *Streptomyces* finlayi PM341 DR045 | JQ422171 | 99 |
| 12    | *Streptomyces* finlayi CGMCC 4.1436 | JQ906133 | 99 |
| 13    | *Streptomyces* bottropensis CS-4 16S | JN603591 | 99 |
| 14    | *Streptomyces* avellaneus NRRL B-3447 | NZ_J0PK00000000 | 99 |
| 15    | *Streptomyces* aburaviensis S-66 | NR_043375 | 99 |

**Table 4: Characteristics that separate strain NRC-88 from the type strains of phenotypically and phylogenetically closely related *Streptomyces* species**

| Bergey's taxonomy | *Streptomyces* sp. NRC-88 | *Streptomyces ochraceiscleroticus* | *Streptomyces herbescens* | *Streptomyces flocculus* |
|-------------------|--------------------------|----------------------------------|--------------------------|-------------------------|
| Similarity from phenotypic characterization [W; S; C; SM] | | | | |
| Sugar utilization | No sugar | - | - | - |
| D-glucose | + | + | + | + |
| D-xylase | + | + | + | ± |
| L-arabinose | + | + | + | + |
| L-rhamnose | + | + | + | - |
| D-fructose | + | + | + | - |
| D-galactose | + | + | + | - |
| Raffinose | - | - | - | - |
| D-mannitol | + | + | + | + |
| Myoinositol | + | + | + | - |
| Salicin | + | + | + | + |
| Sucrose | + | + | + | + |

*: Positive utilization, ±: Doubtful utilization, -: Negative utilization, ?: Not detected, W: White series by light microscope, S: Spiral spore surface, RF: Rectiflexibles, C: Melanin negative, SM: Smooth spore chains by transmission electron microscope
Fig. 4: Secondary structure prediction of 16s rRNA of the strain Streptomyces sp. NRC-88 was done using GeneBee online software.

Fig. 5: Restriction sites of the strain Streptomyces sp. NRC-88 were predicted using NEB cutter.

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