African Journal of Microbiology Research Vol. 5(29), pp. 5308-5315, 9 December, 2011
Available online http://www.academicjournals.org/ajmr
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.346

Full Length Research Paper

Streptomyces aegyptia NEAE 102, a novel cellulolytic streptomycete isolated from soil in Egypt

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Accepted 20 September, 2011

In the course of our screening programme for cellulolytic activities, a novel actinomycete, strain NEAE 102, which produced high cellulolytic activities, was isolated from a soil sample collected from Dakahliyah governorate, Egypt. It developed ivory aerial mycelium and faint yellow substrate mycelium on yeast extract - malt extract agar. The colour of the substrate mycelium was not sensitive to changes in pH. The organism produced long, straight spore-chains with elongated, irregular and smooth-surfaced spores. Strain NEAE 102 did not produce melanin or any diffusible pigments. Phylogenetic analysis based on 16S rRNA gene sequence indicated that the strain NEAE 102 consistently falls into a clade together with *Streptomyces venezuelae* NBRC 12595(AB184836) and *Streptomyces zaomyceticus* cfcc3116 (FJ792553). Strain NEAE 102 is clearly distinct from *S. venezuelae* NBRC 12595(AB184836), *S. zaomyceticus* cfcc3116 (FJ792553) and other related *Streptomyces* species described in the literature by using a combination of morphological, cultural, and physiological characteristics. On the basis of its morphological, physiological and biochemical characteristics, as well as 16S rRNA sequence, it is evident that strain NEAE 102 represents a novel species of the genus *Streptomyces*, for which the name *Streptomyces aegyptia* NEAE 102 is proposed.

Key words: *Streptomyces aegyptia* NEAE 102, 16S rRNA sequences analysis, morphological, characteristics, physiological characteristics.

INTRODUCTION

The actinomycetes are well known for their ability to decompose complex molecules, particularly lingo-cellulose components, which make them important agents in decomposition processes (Lacey, 1997). In addition, *Streptomyces* species are the most industrially important actinomycetes, due to their capacity to produce numerous secondary metabolites (Berdy, 1995). The genus *Streptomyces* was proposed by Waksman and Henrici (1943) and classified in the family streptomycetaceae on the basis of morphology and cell-wall chemotype. As with the other actinobacteria, streptomycetes are Gram-positive, aerobic micro-organisms and have genomes with high GC-content; they produce extensive branching substrate and aerial mycelia that develops into chains of spores by the formation of cross-walls in the multinucleate aerial filaments (Anderson and Wellington, 2001).The presence of LL-diaminopimelic acid (LL-DAP) and the absence of characteristic sugars in the cell wall is one of the most important cell-wall properties of the strains belonging to the *Streptomyces* (Lechevalier and Lechevalier, 1970b). The ability of many species of *Streptomyces* to produce antimicrobial substances was considered by some taxonomists (Gauze et al., 1957; Krasilnikov, 1960; Baldacci, 1962) as one of the important criteria in species differentiation. More than 500 *Streptomyces* species and subspecies have been described, the largest number of any bacterial genus (Hain et al., 1997). During the course of our study on isolation methods of cellulolytic actinomycetes, we isolated a variety of actinomycete strains that have cellulolytic activities. Among them, strain *Streptomyces* sp. strain NEAE 102 produced high cellulolytic activities.

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The aim of the present study was to characterize the taxonomic position of this strain, designated NEAE 102, using a combination of genotypic and phenotypic procedures. It is apparent from the results that the organism should be recognized as representing a novel species of the genus *Streptomyces*.

MATERIALS AND METHODS

Microorganisms and cultural conditions

Strain NEAE 102 was kindly provided by Dr. Noura El-Ahmady El-Naggar (Department of Bioprocess Development, Genetic Engineering and Biotechnology Research Institute, City for Scientific Research and Technology Applications, Alexandria, Egypt).

This isolate was maintained on slopes containing starch-nitrate agar medium (Waksman, 1959) of the following composition (g/L): Starch 20; KNO_3 2; K_2HPO_4 1; $MgSO_4.7H_2O$ 0.5; NaCl 0.5; $CaCO_3$ 3; $FeSO_4.7H_2O$ 0.01; agar 20 and distilled water up to 1L. Slopes were incubated for a period of 7 days at 30°C. Nystatin (50 µg/mL) was incorporated as an antifungal agent to minimize fungal contamination. The isolate was stored as spore suspensions in 20% (v/v) glycerol at –20°C. Biomass for chemotaxonomic and molecular systematic studies was obtained by growing the strain in shake flasks (at 200 rpm) using International *Streptomyces* Project Medium 2 broth at 30°C for 2 days. Mycelia and cells were harvested by centrifugation, washed with distilled water and then freeze-dried.

Morphology and cultural characteristics

The morphology of the spore chain and the spore surface ornamentation of strain NEAE 102 were examined on inorganic salt/starch agar (ISP medium 4) after 14 days at 30°C using the coverslip technique of Kawato and Shinobu (1959). The gold-coated dehydrated specimen can be examined at different magnifycations with Analytical Scanning Electron Microscope Jeol JSM-6360 LA operating at 20 Kv at the Central Laboratory, Mubarak City for Scientific Research and Technology Applications, Alexandria, Egypt. Aerial spore-mass colour, substrate mycelial pigmentation and the production of diffusible pigments were observed on tryptone-yeast extract agar (ISP medium 1), yeast extract-malt extract agar (ISP medium 2), oatmeal agar (ISP medium 3), inorganic salt starch agar (ISP medium 4), glycerol- asparagine agar (ISP medium 5) peptone-yeast extract iron agar (ISP medium 6) and tyrosine agar (ISP medium 7) as described by Shirling and Gottlieb (1966), and on starch-ammonium sulphate agar and Czapek's solution-agar ; all plates were incubated at 30°C for 14 days.

Chemotaxonomy

Sugars and diaminopimelic acid (DAP) isomers were identified by thin-layer chromatography by the method described by Staneck and Roberts (1974).

Physiological characteristics

Carbon source utilization was tested on plates containing ISP basal medium 9 (Shirling and Gottlieb, 1966) supplemented with a final concentration of 1% of the tested carbon sources. The plates were incubated at 30°C and read after 14 days. Melanoid pigment production was examined on peptone-yeast extract-iron agar (ISP medium 6), on tyrosine agar (ISP medium 7), and in tryptone-yeast extract broth (ISP medium 1) (Shirling and Gottlieb, 1966). Growth in the presence of sodium chloride was determined according to Tresner et al. (1968). Degradation of casein was tested following the method of Gordon et al. (1974) and reduction of nitrates to nitrites (Williams et al., 1983) was examined.

Liquefaction of gelatin was evaluated by using the method of Waksman (1961). The ability to coagulate or to peptonize milk and hydrogen sulphide production was determined as described by Cowan and Steel (1974). Lecithinase activity was conducted on egg–yolk medium according to the method of Nitsch and Kützner (1969) and the capacity to decompose cellulose was tested following the method of Ariffin et al. (2006). The ability of strain to produce α-amylase was determined; the isolate was streaked onto starch nitrate medium plates containing 2% soluble starch and incubated at 30°C for 7 days. After incubation, the plate is flooded with Gram's iodine solution and zone of clearance was observed (Mishra and Behera, 2008). The ability of the organism to inhibit the growth of four bacterial (*Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Escherichia coli*, or *Klebsiella*), and five fungal strains (*Rhizoctonia solani*, *Alternaria solani*, *Bipolaris oryzae*, *Fusarium oxysporum*, *Fusarium solani*) was determined. Some additional tests can be considered to be useful in completing the description of a strain or species, even if they are not very significant or indicative on their own, The ability of strain NEAE 102 to produce uricase (Azab et al., 2005); asparaginase (Gulati et al., 1997) and chitosanase activity (Choi et al., 2004) were tested.

16S rRNA sequencing

The preparation of genomic DNA of the strain was conducted in accordance with the methods described by Sambrook et al. (1989). The PCR amplification reaction was performed in a total volume of 100 µl, which contained 1 µl DNA, 10 µl of 250 mM deoxyribonucleotide 5'-triphosphate (dNTP's); 10 µl PCR buffer, 3.5 µl 25 mM $MgCl_2$ and 0.5 µl Taq polymerase, 4 µl of 10 pmol (each) forward 16s rRNA primer 27f (5'-AGAGTTTGATCMTGCCTCAG-3') and reverse 16s rRNA primer 1492 r (5'-TACGGYTACCTTGTTACGACTT-3') and water was added up to 100 µl. The PCR-apparatus was programmed as follows: 5 min denaturation at 94°C, followed by 35 amplification cycles of 1 min at 94°C, 1 min of annealing at 55°C, and 2 min of extension at 72°C, followed by a 10 min final extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The purified PCR product of approximately 1400 bp was sequenced by using two primers, 518F; 5'-CCA GCA GCC GCG GTA ATA CG-3' and 800R; 5'-TAC CAG GGT ATC TAA TCC-3'. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing product was resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA) and deposited in the GenBank database under accession number HQ677021.

Sequence alignment and phylogenetic analysis

The nearly complete 16S rRNA gene sequence of strain NEAE 102 was aligned with the corresponding 16S rRNA sequences of the type strains of representative members of the genus *Streptomyces* retrieved from the GenBank, EMBL, DDBJ and PDB databases by using BLAST program (www.ncbi.nlm.nih. gov/blst) (Altschul et al., 1997) and the software package MEGA4 version 2.1(Tamura et al., 2007) was used for multiple alignment and phylogenetic analysis.

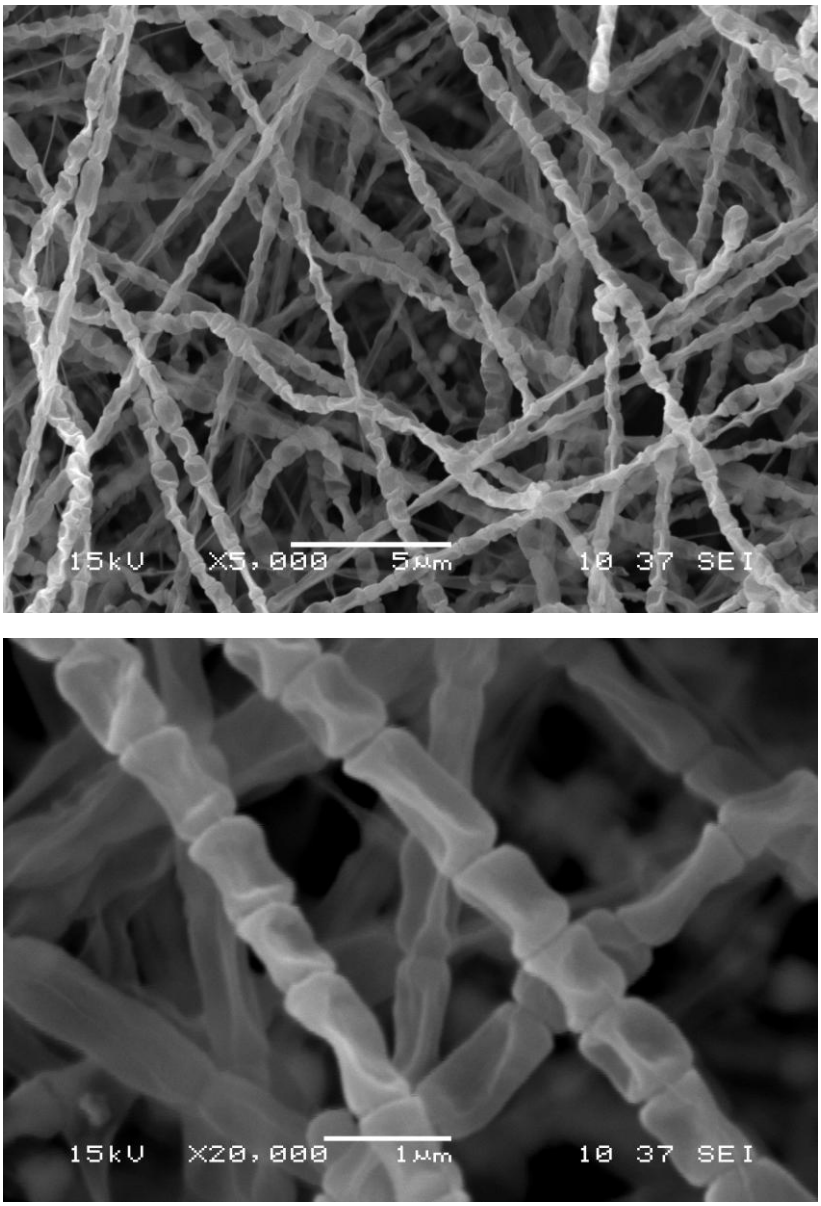

Figure 1. Scanning electron micrograph showing the spore-chain morphology and spore-surface ornamentation of strain NEAE 102 grown on inorganic salts/starch agar for 14 days at 30 °C.

The phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987) based on the 16S rRNA gene sequences of strain NEAE 102 and related organisms.

RESULTS AND DISCUSSION

Morphology

The colonial morphology of strain NEAE 102 was consistent with its assignment to the genus *Streptomyces* (Williams et al.1989).It formed an extensively branched substrate mycelium and aerial hyphae which differentiated into long straight chains (Rectiflexibiles type) carrying more than 50 elongated (0.55 – 0.60 x 0.85 –1.39 µm in diameter) smooth-surfaced spores (Figure 1).

Cultural characteristics

Cultural characteristics of strain NEAE 102 are shown in Table 1. The colour of the substrate mycelium was varied from faint yellow to light yellowish brown. Diffusible pigments were not produced on all test media. Aerial mycelium of the strain was abundant, well-developed and

Table 1. Culture properties of the *Streptomyces* isolate NEAE 102.

Medium	Color of			Growth
	Aerial mycelium	Substrate mycelium	Diffusible pigment	
Starch-ammonium sulphate agar	Grey	Yellowish brown	Non-pigmented	Good
Czapek's solution-agar	Ivory	Faint yellow	Non-pigmented	Very good
ISP medium1 (Tryptone-yeast extract agar)	Beige	Faint yellow	Non-pigmented	Good
ISP medium 2 (Yeast extract -malt extract agar)	Ivory	Faint yellow	Non-pigmented	Excellent
ISP medium 3 (Oatmeal agar)	Beige	Faint yellow	Non-pigmented	Good
ISP medium 4 (Inorganic salt-starch agar)	Ivory	Faint yellow	Non-pigmented	Excellent
ISP medium 5 (Glycerol asparagines agar)	Ivory	Faint yellow	Non-pigmented	Very good
ISP medium 6 (Peptone-yeast extract iron agar)	No-sporulation	No-sporulation	Non-pigmented	Weak
ISP medium 7 (Tyrosine agar)	Beige	Yellowish brown	Non-pigmented	Excellent

Figure 2. Color of the aerial mycelium of *Streptomyces* strain grown on starch - nitrate agar medium for 14 days of incubation at 30°C.

varied from ivory to beige (Figure 2) on different test media, grey on starch-ammonium sulphate agar medium only.

Physiological characteristics

The physiological and biochemical reactions of strain NEAE 102 are shown in Table 2. The optimal growth temperature of strain NEAE 102 was 30°C and optimal pH was 7.0.

Chemotaxonomy

Chemotaxonomic tests showed that the cell wall contained LL-diaminopimelic acid in whole-organism hydrolysates, indicating that it was of cell-wall type I (Lechevalier and Lechevalier, 1970a, b). The whole-cell hydrolysates contained mainly xylose and galactose.

16S rRNA gene sequence comparisons and phylogenetic analysis

The nearly complete 16S r RNA gene sequence (1450 nucleotides) was determined for strain NEAE 102. A BLAST search (Altschul et al., 1997) of the GenBank database using this sequence showed its similarity to that of many species of the genus *Streptomyces*. A phylogenetic tree (Figure 3) based on 16S rRNA gene sequences of members of the genus *Streptomyces* was constructed according to the neighbour-joining method of Saitou and Nei (1987) with MEGA4 (Tamura et al., 2007).This tree shows the close phylogenetic association of strain NEAE 102 with certain other *Streptomyces* species. Phylogenetic analysis indicated that the strain NEAE 102 consistently falls into a clade together with *S. venezuelae* NBRC 12595(AB184836) and *S. zaomyceticus* cfcc3116 (FJ792553). Strain NEAE 102 can also be distinguished from these and other closely related species by using a combination of morphological, cultural, and physiological characteristics. It is evident; therefore, that strain NEAE 102 represents a new centre of taxonomic variation within the genus *Streptomyces*.

Taxonomic conclusions

On the basis of morphological, cultural and chemotaxonomic properties above, together with the physiological properties of strain NEAE 102 shown in Table 2, it is evident that strain NEAE 102 belongs to the genus *Streptomyces.* Strain NEAE 102 produced ivory aerial mycelium and faint yellow substrate mycelium on yeast extract-malt extract agar. The colour of the

Table 2. Phenotypic properties that separate strain *Streptomyces* NEAE 102 from related *Streptomyces* species.

Characteristics	1	2	3	4	5	6
Aerial mycelium on ISP medium 2	Ivory	Grey or red	Red or grey	Grey-white	Grey	Grey
Substrate mycelium on ISP medium 2	Faint yellow	Brown	Brown	Reddish brown	Grey	Grayish yellow
Production of diffusible pigment	None	Brown	None	Violet-blue	Green	Yellow to pinkish brown
Spore surface	Smooth	Smooth	Smooth	Smooth	Smooth	Smooth
Spore shape	Elongated	Oval to oblong		Cylindrical	Oval or long round	
Spore chain morphology	RF	RF	RF	RF	RF	RF
Coagulation of milk	+ (slow)	–	–	+		
Peptonization of milk	±	+ (slow)	+	+		
Starch Hydrolysis	+	–	+	+		+
Melanoid pigment	–	+	±	+		±
Nitrate reduction	+	+	+			+
Lecithinase activity	+	+	+			+
Gelatin liquefaction	+	+	+		+	+
H_2S Production	–	+	+	+	+	
NaCl tolarance	3%	7%	4%		2.5%	4%
Growth on sole carbon sources (1.0 %, w/v)						
D(-) Fructose	+	+	+	+	+	±
D(+) Xylose	+	–	+	+	+	+
D(+) Glucose	+	+	+	+	+	+
Sucrose	+	±	+	+	+	±
Cellulose	+	–	–		+	
Antagonistic activity						
Escherichia coli	–	–	–	–		–
Pseudomonas aeruginosa	+			–		
B. subtilis	–	±	±	–		±
C. albicans	–	–	–	–		–
Pseudomonas fluorescens	–	–	–			–
Sacchromyces cerevisiae	–	–	–			–
Aspergillus niger	–	–	–			–

Abbreviations: RF, Rectiflexibiles; +, Positive; –, Negative; ±, Doubtful; Blank cells, no data available

Strains: 1, *Streptomyces* NEAE 102; 2, *Streptomyces venezuelae* NBRC 12595 (AB184836); 3, *Streptomyces zaomyceticus* cfcc3116 (FJ792553); 4, *Streptomyces vietnamensis* GIMV4 (DQ311081); 5, *Streptomyces viridobrunneus* SCPE-09 (HM459435); 6, *Streptomyces violaceorectus* 173442(EU570641). Data for reference species were taken from Shirling and Gottlieb (1969), Williams et al. (1989) and from previous studies (Dastager et al., 2006; Zhu et al., 2007).

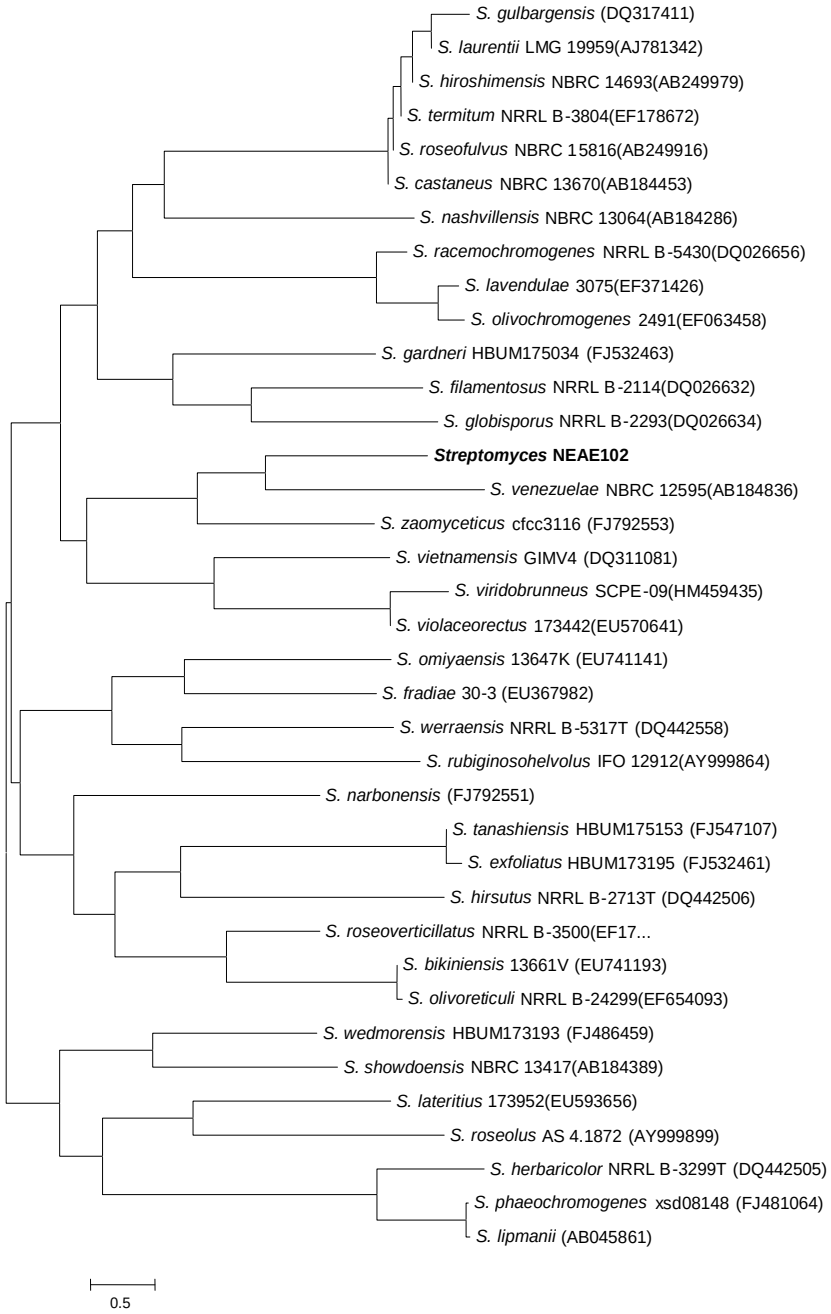

Figure 3. Neighbour-joining tree (Saitou and Nei, 1987) based on 16S rRNA gene sequences, showing the phylogenetic relationship between strain NEAE 102 and 36 related species of the genus *Streptomyces*. GenBank sequence accession numbers are indicated in parentheses after the strain names. Phylogenetic analysis was conducted in MEGA4 (Tamura et al., 2007).

substrate mycelium was not sensitive to changes in pH.

The organism produced long, straight spore-chains with elongated, irregular and smooth spores. Melanin or any diffusible pigments was not produced.

A comparative study between strain NEAE 102 and its closest phylogenetic neighbours, however, revealed significant differences from those *Streptomyces* species showing 97 to 99% 16S rRNA gene sequence similarity in morphological, cultural, and physiological characteristics as summarized in Table 2. Strain NEAE

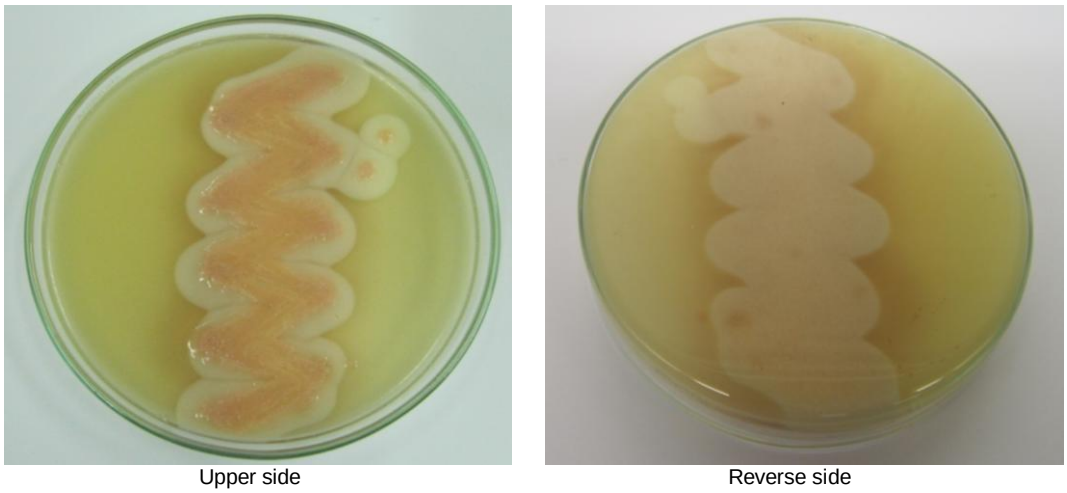

Figure 4. Reaction on egg yolk agar is one of the characteristics used to identify *Streptomyces* sp. In the positive reaction: Lecithinase degrades the lecithin present in the egg yolks and releases insoluble diglycerides, resulting in the formation of a white opaque zone of precipitation (zone of opalescence) in the medium surrounding growth extending away from the bacterial growth, as shown on the left.

102 differed from those *Streptomyces* species in that it produced an ivory aerial mycelium, faint yellow substrate mycelial pigment and did not produce a diffusible pigment on ISP media while *S. venezuelae* produced grey or red aerial mycelium, no distinctive substrate mycelium pigment (greyish yellow) and a dark brown diffusible pigment (Ehrlich et al., 1948); *S. zaomyceticus* produced a brownish grey aerial mycelium, no distinctive substrate mycelial pigment and yellow diffusible pigment (Hinuma, 1954); *S. vietnamensis* produced a grey-white aerial mycelium, reddish-brown substrate mycelial pigment and violet-blue diffusible pigment (Zhu et al., 2007); *S. viridobrunneus* produced a grey aerial mycelium, grey substrate mycelial pigment and green diffusible pigment (Krasilnikov, 1970) and *S. violaceorectus* produced a grey or red aerial mycelium, greyish yellow brown or greyish reddish brown substrate mycelial pigment and pink or pinkish brown diffusible pigment (Pridham et al.,1958).

In conclusion, the cellulolytic strain NEAE 102, isolated from soil in a mentha field in Dakahliyah governorate, Egypt, is clearly distinct from other phylogenetically and phenotypically related *Streptomyces* species described in the literature, and thus it should be classified as a novel status in the genus *Streptomyces*. Therefore, we proposed this organism should be a new species, for which we propose the name *S. aegyptia* NEAE 102 and sequencing product was deposited in the GenBank database under accession number HQ677021.

Description of *S. aegyptia* NEAE 102 sp. nov.

S. aegyptia (ae. gyp'ti.a. L. fem. adj. *aegyptia* pertaining to Egypt, the geographical location from which the type strain was isolated).

Aerobic, mesophilic, Gram-positive actinomycete that develops abundant and well-developed substrate and aerial mycelium. It developed ivory aerial mycelium, faint yellow substrate mycelium on yeast extract -malt extract agar. The colour of the substrate mycelium was not sensitive to changes in pH. Verticils are not present. The mycelium does not fragment. Strain NEAE 102 grew well on yeast extract -malt extract agar (ISP medium 2), inorganic salt-starch agar (ISP medium 4), glycerol–asparagine agar (ISP medium 5), tyrosine agar (ISP medium 7) and Czapek's solution-agar. It exhibited moderate growth on tryptone-yeast extract agar (ISP medium1), oatmeal agar (ISP medium 3) and starch-ammonium sulphate agar medium, but poor growth on peptone-yeast extract iron agar (ISP medium 6). The organism produced long, straight spore-chains with more than 50 elongated, irregular and smooth-surfaced spores.

Diffusible pigments are not produced on any medium tested. Gelatin liquification, milk coagulation, lecithinase activity (Figure 4), nitrate reduction, growth on cellulose and starch hydrolysis (Figure 5) were positive. Melanin production and hydrogen sulphide production were negative. Milk peptonization was doubtful. Protease, cellulase, amylase and chitosanase are produced while asparaginase and uricase are not produced. Glucose, xylose, fructose, galactose, mannose, maltose, sucrose and cellulose are utilized but not ribose and gluconic acid. The cell wall peptidoglycan contains LL-diaminopimelic acid (chemotype I cell wall).

The whole-cell hydrolysates contain galactose and xylose. It exhibited weak antibacterial activity against *Pseudomonas aeruginosa*, but no antimicrobial activity against *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella*, *Rhizoctonia solani*, *Alternaria solani*, *Bipolaris*

Figure 5. Plate assay showing zone of hydrolysis of starch by strain NEAE 102. All the starch in the medium near the microbe has been hydrolyzed by extra-cellular amylases.

oryzae, *Fusarium oxysporum* or *Fusarium solani*.

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