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Nocardiopsis synnemasporogenes sp. nov., NEAE-85, a Novel L-Asparaginase Producing Actinomycete Isolated from Soil in Egypt

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A novel actinomycete, strain NEAE 85, was isolated from a soil sample collected from Alwaraq, Egypt. This strain shows high L-asparaginase activity and subjected to taxonomic analyses. It showed a range of chemical and morphological properties consistent with its classification in the genus *Nocardiopsis*. The strain formed well developed substrate mycelium, aerial hyphae are long, moderately branched, straight to flexuous, or irregularly zigzagged or spiral forms, different spirals are wrapped together to form synnemata. The mycelia of a synnema fragment in later stages to form bacillary spores of various lengths. Phylogenetic analysis using 16S rRNA gene sequences showed that the isolate was closely related to *Nocardiopsis baichengensis* strain YIM 90130 (87% 16S rRNA gene sequence similarity) and *Nocardiopsis trehalosi* strain VKM Ac-942 (88%). However, a comparative study between strain NEAE-85 and its closest phylogenetic neighbours of the genus *Nocardiopsis* revealed significant differences between them in morphological, cultural, and physiological characteristics. It is evident that strain NEAE-85 clearly represents a novel species of the genus *Nocardiopsis*, for which the name *Nocardiopsis synnemasporogenes* NEAE-85 sp. nov. is proposed and sequencing product was deposited in the GenBank database under accession number KJ200340.

Key words: *Nocardiopsis* sp. NEAE-85, 16S rRNA sequences analysis, Scanning electron microscope, phenotypic properties.

The demand for L-asparaginase is expected to increase several fold in coming years due to its potential industrial applications as food processing aid besides its clinical applications[1]. Use of L-asparaginase has revolutionised the anti-leukaemia therapy in acute lymphoblastic leukemia[2]. Its antitumor effect results from the depletion of asparagine, an amino acid essential to leukemia cells, and subsequent inhibition of protein synthesis leading to cytotoxicity. However, its use has been limited by a high rate of hypersensitivity in the long-term used[3] and development of anti-asparaginase antibodies, which causes an anaphylactic shock or neutralization of the drug effect. Therefore there is a continuing need to screen soil samples from various sources for isolation of potential microbes in order to obtain strains capable of producing new and high yield of L-asparaginase with less adverse effects.

Actinomycetes are also a good source for the production of L-asparaginase[4]. The genus *Nocardiopsis* is a member of the family Nocardiopsaceae including actinomycetes showing fragmenting mycelium and a cell wall containing *meso*-diaminopimelic acid, but no diagnostically important carbohydrates[5]. The genera classified in the family Nocardiopsaceae can be distinguished by using a combination of

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chemotaxonomic, morphological and physiological criteria, by 16S rRNA gene signature nucleotides[6] and by comparisons of 16S rRNA gene sequenced data. Standard chemotaxonomic procedures can be used for the detection of diagnostic amino acids and sugars in whole-cell hydrolysates[7].

Nocardiopsis strains are Gram positive, aerobic, chemo-organotrophic, nonacid fast, nonmotile filamentous actinomycetes. Growth temperature range is 10–45°C. Widely distributed in saline and alkaline soils, and found in compost, vegetable matter, indoor environments, and clinical material of animal and human origin[8]. Substrate mycelium is well developed and hyphae are long and densely branched. Fragmentation into coccoid and bacillary elements may occur. Aerial mycelium is well developed and sparse to abundant; aerial hyphae are either long and moderately branched, straight to flexuous, or irregularly zigzagged, completely fragmenting into oval to elongated, rod-shaped smooth-surfaced spores[9; 10]. Initiation of sporulation is often characterized by twisted hyphae, which by examination at higher magnification, reveal a zigzag arrangement of the developing spores. The elongated spores are smooth and can divide subsequently into smaller spores of irregular size by cross-wall formation. Spores are enclosed within a fibrillar sheath and have thickened polar walls[11]. *Nocardiopsis synnemataformans* is the only species known to form synnemata from spiral aerial hyphae that wrap together to form long ropes that subsequently fragment into small rod-shaped elements[12]. *Nocardiopsis* strains do not produce sporangia, sclerotia, or motile elements .

The aim of the present study was to identify the strain NEAE-85 by using a combination of chemotaxonomic, morphological, physiological criteria and 16S rRNA gene sequence.

MATERIALS AND METHODS

Microorganisms and cultural conditions

Actinomycetes from the soil had been isolated using standard dilution plate method procedure on Petri plates containing starch nitrate agar medium 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 1 L; then plates were incubated for a period of 7 days at 30°C. Actinomycete isolates were purified and maintained as spore suspensions in 20 % (v/v) glycerol at -20 °C for subsequent investigation.

Morphology and cultural characteristics

The morphology of the spore chain and the spore surface ornamentation of strain NEAE-85 were examined on starch nitrate agar medium after 14 days at 30°C. The gold-coated dehydrated specimen was examined at different magnifications with Analytical Scanning Electron Microscope Jeol JSM-6360 LA operating at 20 Kv at the Central Laboratory, City of Scientific Research and Technological Applications, Alexandria, Egypt. Aerial spore-mass color, 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 [13]; all plates were incubated at 30°C for 14 days.

Chemotaxonomy

Sugars and diaminopimelic acid (DAP) isomers were identified by the method described by Staneck and Roberts[7].

Physiological characteristics

Carbon source utilization was tested on plates containing ISP basal medium 9 [13] 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)[13]. Growth in the presence of sodium chloride was determined according to Tresner *et al*[14]. Degradation of casein was tested following the method of Gordon *et al*.[15] and reduction of nitrates to nitrites[16] was examined. Liquefaction of gelatin was evaluated by using the method of Waksman[17]. The ability to coagulate or to peptonize milk and hydrogen sulphide production was determined as described by Cowan and Steel[18]. Lecithinase activity was conducted on egg–yolk medium according to the method of Nitsch and Kützner[19] and the capacity to

decompose cellulose was tested following the method of Ariffin *et al*.,[20]. 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[21]. The ability of the organism to inhibit the growth of five bacterial strains (*Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Klebsiella*), five fungal strains (*Rhizoctonia solani, Fusarium oxysporum, Alternaria solani, Bipolaris oryzae, Aspergillus niger*) and two yeast (*Sacchromyces cerevisiae, Candida albicans*) 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-85 to produce uricase[22]; asparaginase[23] and chitosanase activity[24] 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.*[25]. 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'-TACGGYTACCTT GTTACGACTT-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 KJ200340.

Sequence alignment and phylogenetic analysis

The 16S rRNA gene sequence of strain NEAE-85 was aligned with the corresponding 16S rRNA sequences of the type strains of representative members of the genus *Nocardiopsis* retrieved from the GenBank, EMBL, DDBJ and PDB databases by using BLAST program (www.ncbi.nlm.nih. gov/blst)[26] and the software package MEGA4 version 2.1 [27] was used for multiple alignment and phylogenetic analysis. The phylogenetic tree was constructed via the bootstrap test of neighbor-joining algorithm[28] based on the 16S rRNA gene sequences of strain NEAE-85 and related organisms.

RESULTS AND DISCUSSION

Morphology and cultural characteristics of the isolate no. NEAE-85

Substrate mycelium is well developed. Aerial mycelium is well developed and abundant. Cultural characteristics of strain NEAE-85 are shown in Table 1. Aerial mycelium is white, beige, grey to olive grey (Fig. 1) and the substrate mycelium is brown, white to brownish grey. Faint brown diffusible pigments are produced on ISP 2 medium (Yeast extract -malt extract agar) and ISP 6 medium (Peptone-yeast extract iron agar). This pigment is not pH-sensitive when tested with 0.05 M NaOH or HCl. Vegetative hyphae are well developed and fragmented (Fig. 2). Strain NEAE-85 grew well on all tested media (Table 1). Aerial hyphae are long, moderately branched, straight to flexuous, or irregularly zigzagged (Fig. 2) or spiral forms, different spirals are wrapped together to form synnemata. The mycelia of a synnema fragment in later stages to form bacillary spores (0.58-0.0.82 µm in width and 1.83-2.43 µm in length) of various lengths. Initiation of sporulation is often characterized by twisted hyphae, which by examination at higher magnification; reveal a zigzag arrangement of the developing spores. The elongated spore surface is smooth and can divide subsequently into smaller spores of irregular size by cross-wall formation. Spores are enclosed within a fibrillar sheath and have thickened walls.

Physiological characteristics

The physiological and biochemical reactions of strain NEAE-85 are shown in Table 2. Milk coagulation, milk peptonization, starch hydrolysis, gelatin liquefaction, protease, cellulase, uricase, chitosanase and asparaginase are positive, but H_2S production, nitrate reduction and lecithinase activity are negative. Optimum growth is at 30–37°C and pH 7 with NaCl tolerance 4% (w/v) NaCl. D-fructose, D-xylose, D-galactose, D-glucose, L-arabinose, rhamnose, ribose, D-mannose and cellulose are utilized for growth as carbon sources but not sucrose, trehalose and raffinose. Only trace of growth on maltose. It exhibited no antimicrobial activity against *Sacchromyces cerevisiae, Candida albicans, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Rhizoctonia solani, Fusarium oxysporum, Alternaria solani, Bipolaris oryzae, Aspergillus niger, Klebsiella pneumoniae.*

Chemotaxonomy

Chemotaxonomic tests showed that the cell wall is chemotype III (meso isomer of diaminopimelic acid and no characteristic sugars in whole-cell hydrolysates)[29]

16S rRNA gene sequence comparisons and phylogenetic analysis

The 16S r RNA gene sequence (1538bp) was determined for strain NEAE-85. A BLAST search[26] of the GenBank database using this sequence showed its similarity to that of many species of the genus *Nocardiopsis*. A phylogenetic tree (Fig. 3) based on 16S rRNA gene sequences of members of the genus *Nocardiopsis* was constructed according to the neighbour-joining method of Saitou and Nei[28] with MEGA4[27]. Phylogenetic analysis indicated that the strain NEAE-85 falls into a clade together with *Nocardiopsis trehalosi* strain VKM Ac-942 (GenBank/EMBL/ DDBJ accession No. NR_024958.1) and *Nocardiopsis baichengensis* strain YIM 90130(GenBank/EMBL/ DDBJ accession No. NR_043033.1). A combination of morphological, cultural, and physiological characteristics showed that strain NEAE-85 could be differentiated from its closest phylogenetic relatives. It is evident; therefore, that strain NEAE-85 be classified as a representative of a novel species of the genus *Nocardiopsis.*

Taxonomic conclusions

Strain NEAE-85 was grown on standard ISP media[13] for 14 days at 30 °C, and was examined for pigmentation, aerial mycelium and other morphological features. The organism exhibited phenotypic properties typical of members of the genus *Nocardiopsis*[30]. It was an aerobic, non-motile, Gram-positive actinomycete and formed long, well-developed and branched substrate mycelium. The colour of the substrate mycelium was not sensitive to changes in pH. Aerial mycelium is well developed and abundant aerial hyphae are

Table 1. Culture properties of the *Nocardiopsis* sp. strain NEAE- 85

Medium	Color of			Growth
	Aerial mycelium	Substrate mycelium	Diffusible pigment	
Tryptone-yeast extract agar (ISP medium 1)	Whitish grey	brownish grey	Non-pigmented	Excellent
Yeast extract -malt extract agar (ISP medium 2)	Olive grey	Dark brownish	Faint brown	Excellent
Oatmeal agar(ISP medium 3)	Beige	Faint brown	Non-pigmented	Excellent
Inorganic salt-starch agar (ISP medium 4)	Beige	Faint brown	Non-pigmented	Excellent
Glycerol asparagines agar (ISP medium 5)	White	White	Non-pigmented	Good
Peptone-yeast extract iron agar (ISP medium 6)	Creamy beige	Brown	Faint brown	Excellent
Tyrosine agar(ISP medium 7)	Grey	Brown	Non-pigmented	Good

The substrate mycelium pigment was not pH sensitive when tested with 0.05 N NaOH or 0.05 N HCl.
The diffusible pigment was not pH sensitive when tested with 0.05 N NaOH or 0.05 N HCl.

Table 2. Phenotypic properties that separate *Nocardiopsis* sp. strain NEAE- 85 from its closest phylogenetic neighbours.

Characteristic	*Nocardiopsis* sp.NEAE-85	*Nocardiopsis baichengensis*	*Nocardiopsis trehalosi*
Aerial mycelium on ISP mdeium 2	Olive grey	White to yellow-white	White to cream or yellowish gray
Synnemata	+	-	-
Substrate mycelium on ISP medium 2	Dark brownish	Light yellow to deep orange-yellow	Pale olive-brownish to pale orange-yellow
Vegetative hyphae	Well developed and abundant	Well developed and fragmented	Zigzag or twisted-ribbon-like at the beginning of sporulation
Production of diffusible pigment	Faint brownw	No diffusible pigments	A light yellow-brownish or light orange-yellow
Spore chain morphology	The mycelia of a synnema fragment to form bacillary spores of various lengths	Long spore chains	
Spore surface	Smooth	Smooth	Smooth
Spore shape	Cylindrical, elongated		Irregularly sized (mostly elongated)
Sensitivity of diffusible pigment to pH	This pigment is not pH-sensitive	No diffusible pigments are produced	
Melanin production on ISP medium 6	±	+	-
Degradation of			
Lecithin	-		
Casein	+		+
Starch	+	+	
Max. NaCl tolerance (%, w/v)	4	18	5
Optimum growth temperature range (°C)	30–37°C	37–40°C	28–37°C
Growth on sole carbon source (1%, w/v)			
D(-) Fructose	+	+	+
D(+) Xylose	+	+	+
D(+) Galactose	+	+	+
D(+) Glucose	+	+	+
L-arabinose	+	+	+
Ribose	+	+	
D(+) Mannose	+	+	+
Sucrose	-	+	-
Maltose	±	+	+
Rhamnose	+	+	+
Cellulose	+		+
Trehalose	-		
D-lactose	+	-	+
Raffinose	-	-	-
Growth of pH	5–9		6–9
Enzymes			
α –amylase (Starch hydrolysis)	+	-	
Gelatinase (Gelatin liquification)	+	+	-
Reduction of nitrates to nitrite	-	-	-
H_2S production	+	+	+
Coaggulation of milk	+	-	
Peptonization of milk	+	-	
Antimicrobial activities	-		+[1]

Data for reference species were taken from Bergey's Manual® of Systematic Bacteriology -volume five the actinobacteria [8].
Abbreviations: RF, Rectiflexibiles; +, Positive; –, Negative; ±, Doubtful; Blank cells, no data available. Melanin production on ISP1, ISP7 media is negative.
Protease, cellulase, uricase, chitosanase and asparaginase of strain NEAE-85 were produced while lecithinase was not produced. The optimal growth temperature was 30 °C and optimal pH was 7.0.
Antimicrobial activities are negative against *Sacchromyces cerevisiae, Candida albicans, Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa, Rhizoctonia solani, Fusarium oxysporum, Alternaria solani, Bipolaris oryzae, Aspergillus niger* and *Klebsiella pneumoniae.*
[1]*Nocardiopsis trehalosi* (previously *N. trehalosei*) has been reported to produce 3-trehalosamine, a disaccharidewith activity against *Bacillus subtilis*

Fig. 1. Color of the aerial mycelium of *Nocardiopsis* sp. NEAE -85 grown on starch -nitrate agar medium for 7-14 days of incubation at 30°C

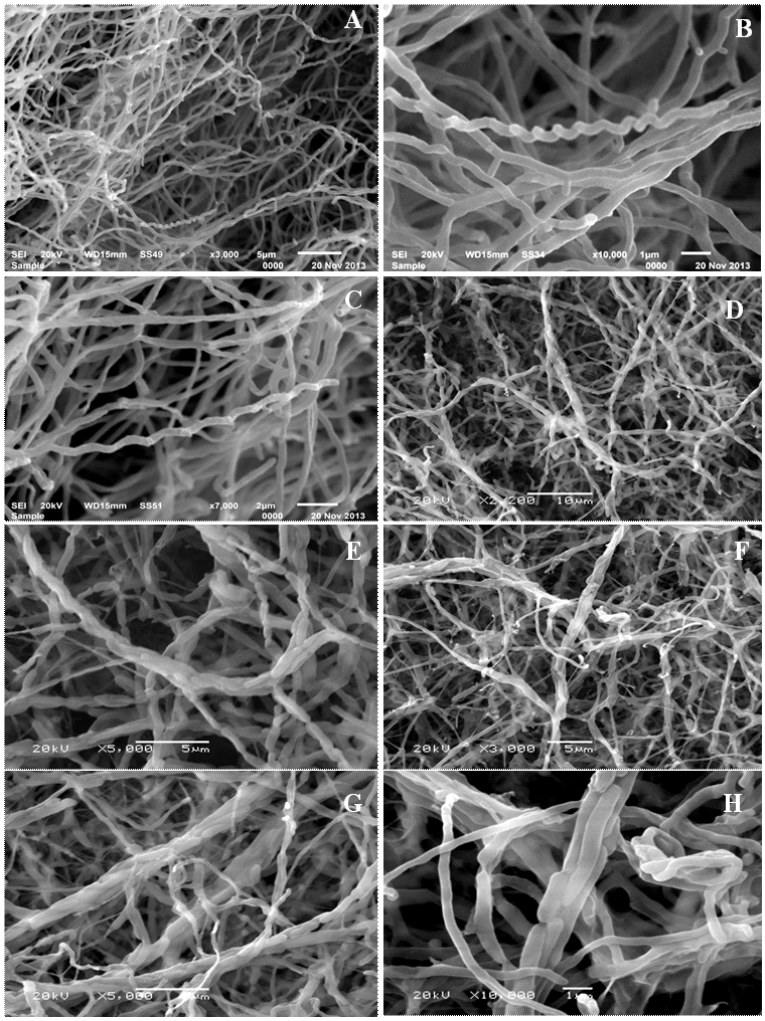

Fig. 2. Scanning electron micrograph of strain *Nocardiopsis* sp.NEAE-85, showing zigzag hyphae(A, B), initiation of sporulation (C), spores enclosed within a fibrillar sheath and spore chains with a smooth surface (E, H). The culture was grown on on starch nitrate agar medium for 14 days at 30 °C

long, moderately branched, straight to flexuous, or irregularly zigzagged or spiral forms, different spirals are wrapped together to form synnemata. Phylogenetic analysis using 16S rRNA gene sequences showed that the isolate was closely related to *Nocardiopsis baichengensis* strain YIM 90130 (87% 16S rRNA gene sequence similarity) and *Nocardiopsis trehalosi* strain VKM Ac-942 (88 %). A comparative study between strain NEAE-85 and its closest phylogenetic neighbours of the genus *Nocardiopsis* revealed significant differences in morphological, cultural, and physiological characteristics as summarized in Table 2. Strain NEAE-85 could be differentiated from its closest phylogenetic neighbour, *Nocardiopsis baichengensis* and *Nocardiopsis trehalosi* in that its aerial hyphae form synnemata, the mycelia of a synnema fragment to form bacillary spores of various lengths while the two its closest phylogenetic neighbor don't form synnemata.

The isolate also could be differentiated from its closest phylogenetic neighbours by its olive grey aerial mycelium, dark brown substrate mycelium and faint brown diffusible pigment on yeast extract -malt extract agar while the two phylogenetic neighbours produced white to

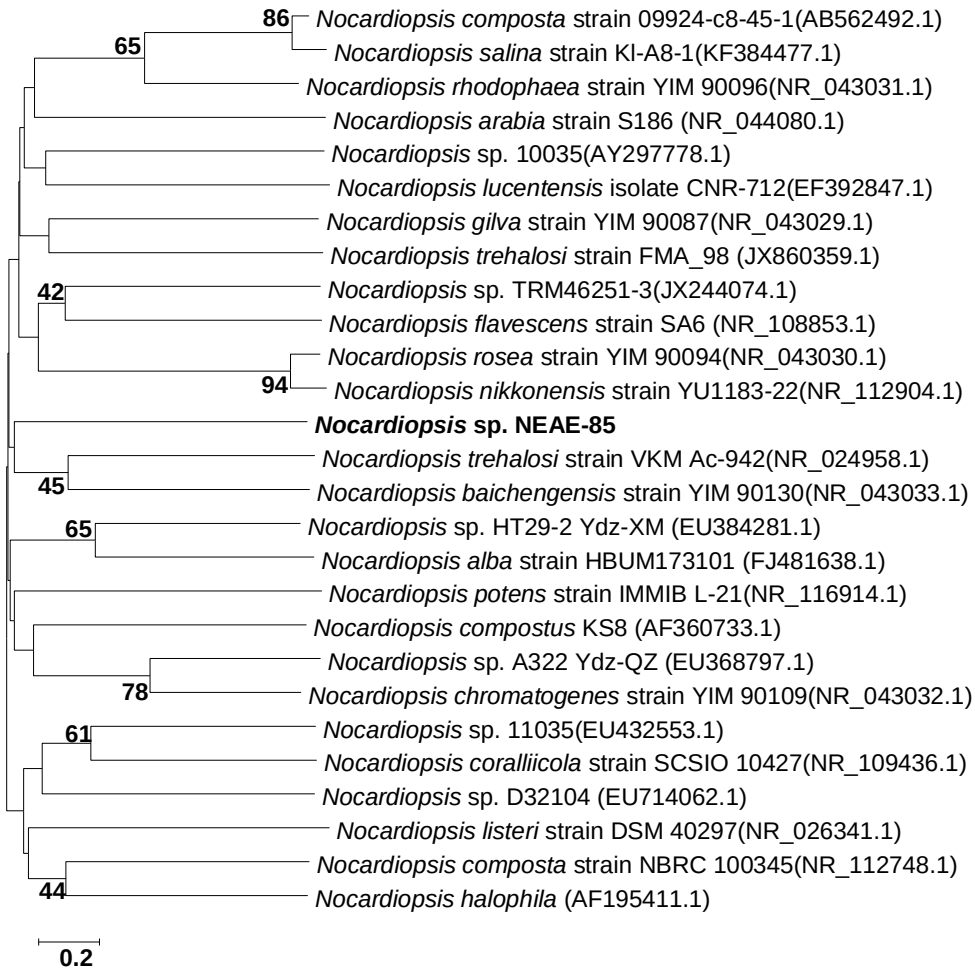

Fig. 3. Bootstrap neighbour-joining phylogenetic tree based on 16S rRNA gene sequences, showing the relationships between strain NEAE-85 and related species of the genus *Nocardiopsis*. Only bootstrap values above 40 %, expressed as percentages of 1000 replications, are shown at the branch points. GenBank sequence accession numbers are indicated in parentheses after the strain names. Phylogenetic analyses were conducted in the software package MEGA4. Bar, 0.2 substitution per nucleotide position.

yellow-white(*N. baichengensis*) or white to cream or yellowish gray(*N. trehalosi*) aerial mycelium . *N. baichengensis* produced light yellow to deep orange-yellow and no diffusible pigments are produced while *N. trehalosi* produced pale olive-brownish to pale orange-yellow and a light yellow-brownish or light orange-yellow is produced on some media. The isolate also could be differentiated from its closest phylogenetic neighbours by its ability to to coaggulate and peptonize milk.

In conclusion, It is evident from the genotypic and phenotypic data that the asparaginase producing strain NEAE-85, isolated from a soil sample collected from Alwaraq, Egypt, represents a novel species of the genus *Nocardiopsis*, for which the name *Nocardiopsis synnemasporogenes* NEAE-85 sp. nov. is proposed and sequencing product was deposited in the GenBank database under accession number KJ200340.

Description of *Nocardiopsis* sp. NEAE-85 sp. nov

Syn.nema. sporo. genes. synnema (pL synnemata; Gr. adv. *syn* together ; *nema* thread; N.Gr. n. *synnema* threads wrapping together; *spora* a spore; N.L. suff. *-genes* (from Gr. v. *gennaô* to produce) producing. *synnemasporogenes*, referring to the ability of the organism to form synnemata producing spore.

Aerobic, nonmotile filamentous actinomycete. Substrate mycelium is well developed. Aerial mycelium is well developed and abundant aerial hyphae are long, moderately branched, straight to flexuous, or irregularly zigzagged or spiral forms, different spirals are wrapped together to form synnemata. The mycelia of a synnema fragment in later stages to form bacillary spores of various lengths. Initiation of sporulation is often characterized by twisted hyphae, which by examination at higher magnification; reveal a zigzag arrangement of the developing spores. The elongated spore surface is smooth and can divide subsequently into smaller spores of irregular size by cross-wall formation. Spores are enclosed within a fibrillar sheath and have thickened polar walls. No diagnostic sugars are found in whole-organism hydrolysates.

No soluble pigment is produced except faint brown pigment found in medium in yeast-malt agar and peptone-yeast extract iron agar. Melanoid pigments are not produced on either ISP media 1 or 7 while it is produced in peptone-yeast extract iron agar (ISP 6 medium). D-fructose, D-xylose, D-galactose, D-glucose, L-arabinose, rhamnose, ribose, D-mannose and cellulose are utilized for growth as carbon sources but not sucrose, trehalose and raffinose. Only trace of growth on maltose. Maximum NaCl tolerance for growth was 4% (w/v). Optimum growth occurs at 30- 37°C.

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