World Applied Sciences Journal 21 (3): 360-370, 2013
ISSN 1818-4952

DOI: 10.5829/idosi.wasj.2013.21.3.2857

A Novel *Actinomycete* sp. Isolated from Egyptian Soil has $-Lactamase Inhibitor Activity and Belongs to the *Streptomyces rochei* Phylogenetic Cluster

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Abstract: Many pathogenic bacteria secrete $-lactamase enzymes as a mechanism of defence against $-lactam antibiotics. A new *Streptomyces sp.*, NRC-77, was isolated from an Egyptian soil sample. The NRC-77 strain has biochemical, chemotaxonomic, morphological and physiological properties consistent with classification in the genus *Streptomyces*, with the nearest species being *Streptomyces rochei.* Furthermore, a phylogenetic analysis of the 16S rRNA gene sequence and ribosomal database project (RDP Classifier) consistent with conventional taxonomy confirmed that strain NRC-77 was most similar to *Streptomyces rochei* (99%). Therefore, the name *Streptomyces rochei* NRC-77 was proposed for this strain. The nucleotide sequence of the 16S rRNA gene has been deposited in GenBank under the ID: HM237347. The active metabolite of this strain (clavulanic acid) was determined by a specific synergistic bioassay and a spectrophotometric assay. The clavulanic acid was detected by TLC and the structure of was confirmed by HPLC. The *Streptomyces* sp. NRC-77 produced 88 mg/l of clavulanic acid in a specific clavulanic acid production medium.

Key words: *Streptomyces rochei* NRC-77 % Clavulanic acid % Conventional taxonomy % Phylogenetic analysis

INTRODUCTION

Certain pathogens are resistant to $-lactam antibiotics and are associated with the inactivation of the $-lactam structure through the opening of the $-lactam ring by bacterial $-lactamases [1]. Pathogenic microorganisms that are resistant to certain antibiotics pose increasingly serious obstacles to the treatment of infectious diseases and have become a major global healthcare problem in the 21st century [2]. The growing global resistance of pathogenic bacteria to existing antibiotics poses a threat to public health. Research efforts are currently pursuing the discovery of novel and efficient antibacterial compounds [3]. Oral and injectable formulations of potassium clavulanate with amoxicillin (approved name co-amoxiclave) and ticarcillin are marketed as Augmentin and Timentin, respectively. These antibiotics are prescribed in more than 150 countries and have attained yearly sales in excess of 2 billion dollars [4]. Pathogenic bacteria that are resistant to certain antibiotics are posing increasingly serious problems for the treatment of infectious diseases. One modern strategy to address these problems is the discovery and development of potent and selective enzyme inhibitors lacking intrinsic antimicrobial properties such that the antibiotics are protected from the hydrolytic activity of diverse $-lactamases [5]. Until 1970, only two classes of naturally occurring $-lactam antibiotics were known: penicillins and cephalosporins. However, with the advent of new screening and isolation techniques, a variety of molecules containing a $-lactam ring have been identified [6]. The clavulanic acid (CA) is a natural inhibitor of $-lactamases that was first isolated from *S. clavuligerus* [7] and that is produced industrially by the same species. Other natural CA producers include *S. jumonjinensis*, *S. katsurahamanus*, *Streptomyces* sp. FERM-P 2804 [8] and *Streptomyces* sp. NRC-35 [9]. Certain *Streptomyces* isolates produce more than 180 different secondary metabolites [10]. The *Streptomycetes* produce more than half of the 10,000 documented bioactive compounds and have been investigated for over 50 years by industrial and academic researchers [11].

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In this study, a new *Streptomyces* strain that produces CA, NRC-77, was isolated from an Egyptian soil sample, characterised and identified phenotypically and phylogenetically.

MATERIALS AND METHODS

Microorganisms: The strain NRC-77 was isolated from an Egyptian soil sample by a screening programme. This newly identified *Streptomyces* species produced CA. This strain was deposited in the *Actinomycetes* Culture Collection, National Research Centre, Dokki, Giza, Egypt. A strain of *E. coli* capable of resistanting 25 µg/ml of 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.

Streptomyces Isolation and CA Detection: Fifty *Streptomyces* isolates were obtained from different Egyptian soil samples. Strain NRC-77 was isolated on two different media using the serial dilution plate technique. These media were Actinomycetes isolation agar medium (Difco, NJ, USA) and *Streptomyces* medium. The isolation media were supplemented with cycloheximide (50 µg/ml) to minimise the fungal contamination and were incubated at 28°C for 7-10 days [9]. The isolates were primarily tested for their abilities as $-lactamase inhibitors through the specific synergistic bioassay using a penicillin-resistant test strain of *E. coli* [12]. The CA was detected by thin layer chromatography (TLC) [13]. The measurement was made by the spectrophotometric method at 312 nm after derivatisation with imidazole according to Bird *et al.* [14] and the structure of the CA was confirmed by HPLC [15]. The isolates that showed the greatest CA production were selected for further identification.

Production Medium and Cultivation Conditions: Strain NRC-77 was cultivated in an Erlenmeyer flask (250 ml) containing 50 ml production medium on an Innova 4080 rotary shaker (New Brunswick, NJ, USA) at 200 rpm and 28°C for six days. The production medium was composed of (in g/l) soybean meal, 30; soluble starch, 47; $FeSO_4.7H_2O$, 0.1; and KH_2PO_4, 0.1, with pH 6.5 ± 0.2. The inoculation was conducted using a spore suspension of the NRC-77 strain slanted on ISP 2 medium. The culture broth was separated from the mycelium by centrifugation at 5000 rpm for 10 min. The supernatant was sterilised by filtration and used for the evaluation of the inhibitory activity, which was conducted using the three methods [12, 14, 15].

Conventional Taxonomy: The cultural, morphological, physiological and biochemical characteristics of strain NRC-77 were assessed following the guidelines adopted by the International *Streptomyces* Project (ISP) [16]. The diaminopimelic acid (LL-DAP) isomers (chemotaxonomy character) in the cell wall were analysed as described by Lechevalier and Lechevalier [17]. The media composition and the cultivation conditions were implemented as described by Shriling and Gottlieb [16].

Molecular Characterisation

***Streptomyces* Genomic DNA Isolation, PCR Amplification and Sequencing:** The total genomic DNA preparation was conducted using the method of Awad *et al*. [9] and Lee *et al.* [18]. The amplifications were performed in a Gene AMP PCR System 9700 (PE Applied Biosystems, Perkin Elmer, Ohio, USA). The universal 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 the Biolegio BV software (Biolegio, Nijmegen, the Netherlands) [19]. The PCR reaction mixture conditions were as described in Awad *et al*. [9]. The PCR products were photographed using a gel documentation system (Alpha-Imager 2200, CA, USA) and were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany). The sequencing of the purified PCR products was conducted using an ABI Prism 377 DNA Sequencer (Perkin Elmer, Ohio, USA) with the Big Dye® Terminator v3.1. Cycle Sequencing Ready reaction kit (Applied Biosystems, Foster City, USA) and the universal primers listed above obtained from the Gene Analysis Unit (Cornell University, NY, USA, Sequencing Facility Center).

Phylogenetic Analysis and Tree Construction: The phylogenetic data were obtained by aligning the nucleotides of various 16S RNAs retrieved by the BLAST algorithm (www.ncbi.nlm.nih.gov/BLAST) using the CLUSTALW programme with standard parameters. The classifier was trained on the new, phylogenetically consistent higher-order bacterial taxonomy (Ribosomal Database Project, RDP Classifier) that was proposed by Wang *et al*. [20] (http:// rdp.cme.msu.edu/ classifier/classifier.jsp). The phylogenetic and molecular evolutionary analyses were conducted using BioEdit Programme. A rooted phylogram was derived from the distance matrices using the neighbour-joining method. All of the analyses were performed on a bootstrapped data set consisting of 1000 replicates (generated by the programme).

Nucleotide Sequence ID: The nucleotide sequence of the 16S rRNA gene of strain NRC-77 has been deposited in GenBank under the ID: HM237347.

Secondary Structure Prediction and Restriction Site Analysis: The RNA secondary structure of the isolate NRC-77 was predicted, as described by Brodsky *et al*. [21], using the GeneBee online software (http://www.genebee.msu.su/services/rna2_reduced.html) with the greedy method and the restriction sites of the NRC-77 DNA were analysed by NEBcutter V2.0 (http://tools.neb.com/NEBcutter2/), as described by Vincze *et al*. [22].

Bioassay Determination Method of CA: The production of CA was assessed by a specific synergistic bioassay, as described by Romero *et al.* [12], with several modifications, as described by Awad *et al*. [9]. Pure CA that was generously provided by Glaxo Smith Kline (Glaxo Wellcome UK Ltd., Middlesex, UK) was used as a standard.

Spectrophotometric Determination of CA: A derivative of CA with imidazole was measured by the spectrophotometer at 312 nm, as described by Bird *et al.* [14].

CA Detection by TLC: CA was detected in the TLC at 20°C using a solvent system consisting of n-butanol, ethanol and water (4:1:5, v/v/v, top phase) [13]. A dark red spot at R_f value 0.44 appeared on the glass-coated chromatogram after spraying with 2, 3, 5-triphenyltetrazolium chloride (TTC) reagent.

CA Assay by HPLC: The CA concentration was determined by HPLC after derivatisation with imidazole [15] with certain modifications according to the optimum pH of imidazole (6.8±0.05) using a Polaris column C-18 (4.6 mm x 250 mm, 5 µm). The mobile phase was composed of methanol and 0.1 M KH_2PO_4 (6:94) adjusted to pH 3.2 with H_3PO_4 at a flow rate of 1.5 ml/min at 28°C. The assay was carried out using HPLC (SIKIM, Ammerbuch, Germany) and the peak was detected at 312 nm using the UV/Visible detector model UV-2070 Plus (Jasco, Tokyo, Japan). Standard pure CA was prepared daily.

RESULTS AND DISCUSSION

***Streptomyces* Isolation and Detection of $-Lactamase Inhibitory Activity:** The main objective of this study was to isolate and characterise a new *Streptomyces* strain with $-lactamase inhibitory activity from New Valley locality Egyptian soil. The isolation of *S. rochei* NRC-77 was carried out using different selective *Streptomyces* media, including Actinomycetes isolation agar medium and *Streptomyces* medium, as described in the previous study by Awad *et al.* [9]. The addition of antifungal agents to the isolation media suppressed the growth of fungal species on the plates. Cycloheximide at 50-100 µg/ml was used for this purpose [23]. In this study, five out of 50 isolates of *Streptomyces* sp. that were obtained showed noticeable inhibitory activity against *E. coli* that was resistant to penicillin-G. This inhibitory activity may be due to any substance that inhibits or degrades the $-lactam antibiotic. We selected the most active isolate (based on the inhibition zone diameter) for further study. The selected strain was named NRC-77. The inhibitory activity of strain NRC-77 was tested using a specific synergistic biological assay against a penicillin-resistant strain of *E. coli* using a piece of agar from each seven-day-old culture grown on Actinomycetes isolation agar medium as a primary screening. The results of the primary screening showed a noticeable $-lactamase inhibitory activity against penicillin-resistant *E. coli*. For the secondary screening, *S. rochei* NRC-77 was cultivated on a submerged culture using a specific CA production medium for further investigation. After the 6^{th} day of cultivation, the supernatant was sterilised by filtration and used to determine the inhibitory activity.

CA Determination: The CA yield was determined by a specific synergistic bioassay and a spectrophotometric assay; the compound was detected by TLC and the structure was confirmed by the HPLC assay. The chromatogram from the HPLC shows a retention time of 3.22 min for an unknown sample (NRC 77-culture broth) compared with the standard pure CA at a retention time of 3.375 min (Fig. 1). A maximum CA yield of 88 mg/L was obtained. This result was close to those described in the literature using complex media. For example, Chen *et al.* [24] obtained 115 mg/L CA from a medium containing soy flour without the addition of any amino acids. Furthermore, Neto *et al*. [7] obtained approximately 200 mg/L using a complex medium. However, all of these studies were conducted in stirred-tank bioreactors with well-controlled conditions. Therefore, strain NRC-77 was submitted for identification. The production medium that was used contained soybean protein in the form of an extract. Several studies have shown the advantages of soybean meal extract, which is used in the production of both antibiotics in general and CA in particular. One of these studies was by Rosa *et al*. [25], who mentioned that

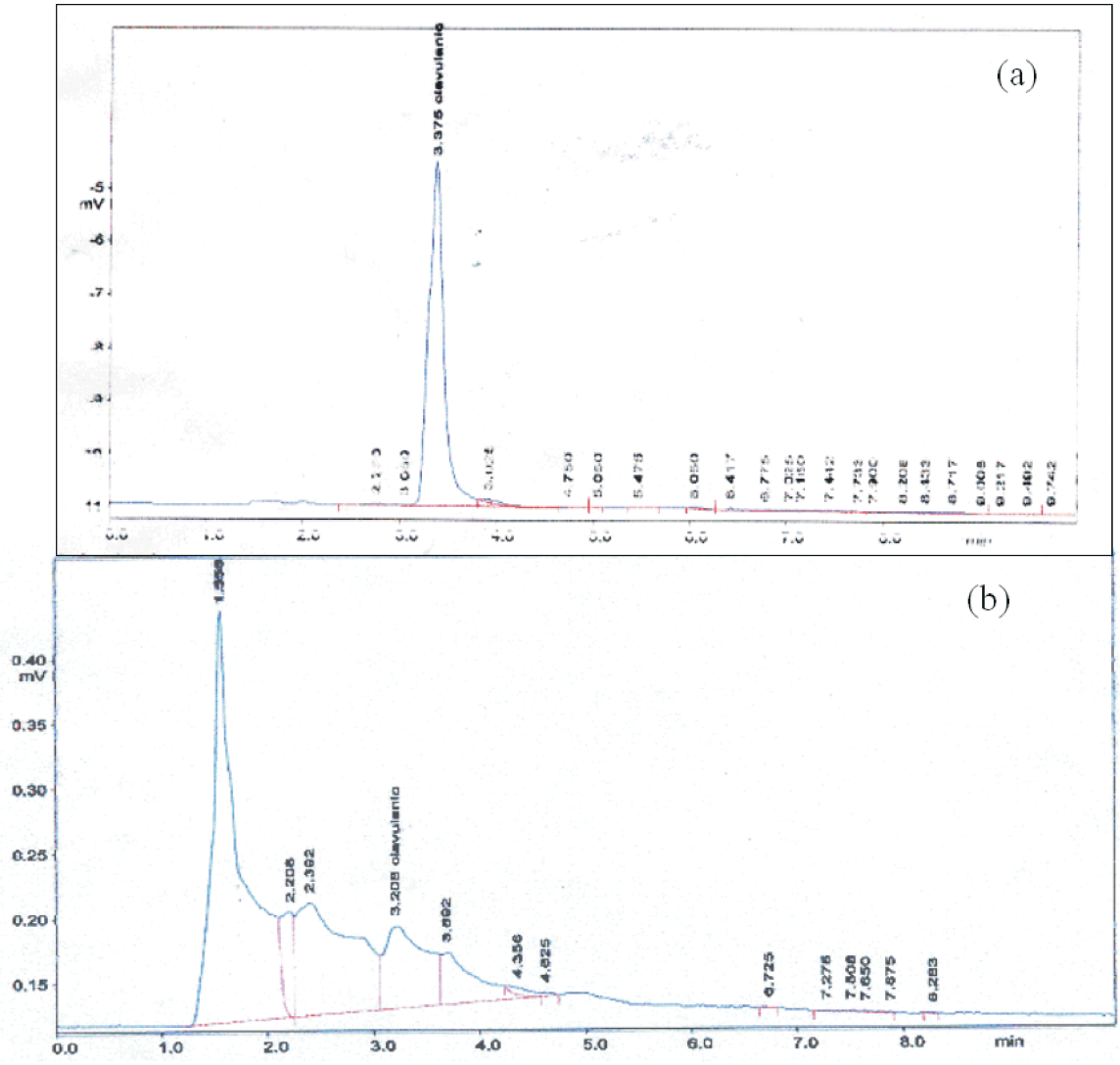


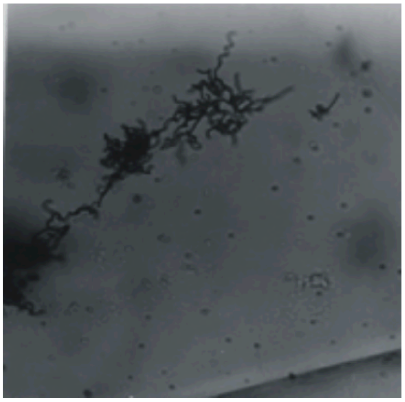
Fig. 1: An analytical HPLC chromatogram showing (a) pure clavulanic standard at 3.375 min. and (b) clavulanic acid in the fermented broth from strain NRC-77 at a retention time of 3.22 min.

Table 1: Cultural characteristics of *Streptomyces rochei* NRC-77

		Colour of		
Agar medium	Amount of growth	Aerial mycelium	Substrate mycelium	Soluble pigment
Yeast extract-malt extract (ISP-2)	Abundant	Grey	Greyish	-
Oatmeal (ISP-3)	Abundant	Greyish	Gray to ivory	yellowish
Inorganic salts-starch (ISP-4)	Abundant	Greyish	Greyish	brownish
Glycerol-asparagine (ISP-5)	Abundant	Gray	Greyish-yellowish	-
Bennett's agar	Moderate	Gray	Greyish-yellowish	-
Czapek·s agar	Moderate	Gray	Greyish	-
Glucose-asparagine agar	Fair	Gray	Greyish	-
Tyrosine agar (ISP-7)	Fair	Greyish	White to gray	-
Nutrient agar	Abundant	Gray	Greyish	Brown

ISP= International *Streptomyces* Project

(a)



(b)

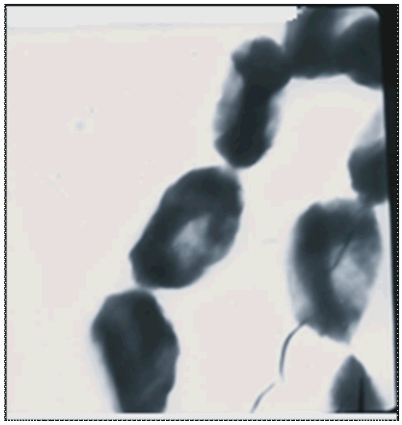


Fig. 2: The morphology of *Streptomyces rochei* NRC-77. Spore chains of the spiral type are shown at 2,000 X (a) and the spore surface is shown as smooth to warty by TEM at 40,000 X (b) using Bennett's agar medium at 28°C.

soybean protein is the most important nutrient for the biosynthesis of CA. Another study was conducted by Chen *et al*. [26], who observed that the highest CA production was obtained when soybean flour was utilised. The soybean flour provides nutrients for proper cellular growth and contains arginine, which is the precursor of the CA molecule [26]; soybean has been proven to be the safest high-protein cereal grain for use in antibiotic production.

Conventional Taxonomy: Cultural and Morphological Characteristics: Strain NRC-77 exhibited different degrees of growth on a range of agar media and showed the morphology typical of *Streptomyces* [27]. The growth was abundant on most of the media tested but was moderate on Bennett's and Czapek's media and was fair using glucose, asparagine and tyrosine media (Table 1). The colour of the aerial mycelium varied from grey to greyish. Therefore, strain NRC-77 was assigned to the grey series. The culturing method and morphological characteristics have been developed to identify *Streptomyces* species using the selective plating technique [28]. The morphology of the spore chains of strain NRC-77 was of an open spiral type (Fig. 2a); therefore, this strain can be assigned to a spiral group according to the categorisation of spore chain shapes devised by Locci [27]. The spore surface ornamentation of strain NRC-77 was observed by TEM, revealing a smooth to warty spore surface (Fig. 2b). According to the work of Shriling and Gottlieb [16], laboratories with access to an electron microscope should include electron micrographs of the spore surface as one of the descriptive characterisations for each type of culture.

Physiological and Biochemical Properties: Strain NRC-77 was tested on a melanin pigment production, gelatine and skimmed milk media. The results showed that no melanin pigment was observed; the strain could not liquefy the gelatine and it did not cause coagulation and peptonisation of the milk. Therefore, the strain does not harbour the respective degrading enzymes. The strain reduced nitrate to nitrite and it hydrolysed starch (Table 2). With further identification, certain physiological characteristics, such as the degradation of starch or gelatine and the reduction of nitrate, are also considered to ascertain the species classification of newly isolated strains, as recommended by Rossello-Mora and Amann [29]. Analysis of the whole-cell hydrolysate of strain NRC-77 showed the presence of a chemotype I cell wall containing LL-DAP (Table 2). The presence of LL-DAP in the cell wall indicates that this strain is *Streptomyces*, as identified by Lechevalier and Lechevalier [17], who established that cell-wall composition analysis is one of the main chemotaxonomic characteristics of *Streptomyces* identification. Strain NRC-77 was able to utilise different C-sources (Table 2). Taken from the work by Rossello-Mora and Amann [29], several additional tests relating to the use of arabinose, inositol, rhamnose, galactose and mannitol were used to identify new strains.

Comparison with *Streptomyces* Members: Based on its phenotypic properties, strain NRC-77 was classified in the genus *Streptomyces*. The characteristics of this strain were compared with the known *Streptomyces* species in Bergey's 'Manual of Determinative Bacteriology, 9th edition' [30] and Bergey's 'Manual of Systematic Bacteriology [27]. Strain NRC-77 does not resemble any known strain, but has a similarity to

Table 2: Phenotypic features of *Streptomyces rochei* NRC-77

Characteristics	Results
Morphological characteristics	
Spore chain	Open spiral
Spore mass colour	Gray
Spore surface	Smooth
Chemotaxonomic analysis:	
Diaminopimelic acid (DAP)	LL-DAP
Nitrate reduction	Positive
Sugar pattern	ND
Physiological characteristics:	
Melanin pigment on media:	
Peptone-yeast extract iron agar (ISP 6)	-[a]
Tyrosine agar (ISP-7)	-[a]
Tryptone-yeast extract broth (ISP-1)	-[a]
Action of milk	No coagulation in 14 days
Gelatin liquefaction	-[a]
Hydrolysis of starch	positive
Utilisation as a carbon source	
D-Glucose	+[b]
D-Xylose	++[c]
L(+)Arabinose	++
L-Rhamnose	++
D-Fructose	++
D-Galactose	++
D (+) Raffinose	++
D-Mannitol	++
Meso-Inositol	++
Cellobiose	+
Salicine	++
Sucrose	++
Cellulose	-[a]
Starch	++

ND= not detected, [a] negative, [b] weak growth, [c] good growth

S. rochei, *S. albogriseolus* and *S. vastus*. These strains have the same aerial mycelium colours, spore shapes and physiological characteristics, although with certain differences between them. Strain NRC-77 differs from all of the previous *Streptomyces* species in its characteristics, such as its utilisation of D (+) raffinose and sucrose and the fact that it produces CA, unlike other species. The taxonomic classification and identification of *Streptomycetes* species based on conventional methods is difficult and not sufficient [11]. Thus, molecular methods represent an improvement over previous approaches and have been used to identify several newly isolated *Streptomyces* [31].

Molecular Identification

PCR Amplification and Primer Specificity: Several experiments were carried out to select the optimum PCR reaction conditions. The experimental analysis of the PCR amplification performance using the forward primer StrepB in conjunction with the reverse primer StrepF was conducted under the reaction conditions described in the Materials and Methods. The primer pair StrepB/StrepF amplified the fragments of the expected size from the genomic DNA isolated from the positive control strain *S. clavuligerus* NRRL-3585.The primer StrpB/StrepF located at position 139-158 and 1194-1212, respectively [19]. This primer pair was able to amplify 1074 bp fragment. The primer was tested by PCR amplification using genomic DNA isolated from strain NRC-77 and strain NRRL-3585 as a positive control. The primers were successfully used to amplify genomic DNA from the isolated samples. These results are in agreement with those of Rintala *et al.* [19], who found that these primers are specific for *Streptomyces*.

Sequencing and Phylogenetic Analysis: The nucleotide sequence (998 bp) of strain NRC-77 was compared with the 16S rRNA gene sequences that are reported in the GenBank database. Due to the high sequence similarity (99%) with *S. rochei* strains, strain NRC-77 is most closely related to *S. rochei*. However, these bacteria have the highest similarity on a genetic level but show differences in several phenotypic levels. Furthermore, these strains are different not merely in their CA productivity but in the ability to produce CA. The G+C content of the genomic DNA was 75%. These results were in accordance with those of Anderson and Wellington [11], who mentioned that the G+C content of the *Streptomyces* genomic DNA ranges from 69 to78%.

Phylogenetic Tree Construction: A phylogenetic tree was derived from the distance matrices using a neighbour-joining method (Fig. 3). A good congruence was found between the 16S rRNA sequences of the *S. rochei* strain cfcc 3115 and of strain NRC-77. In contrast, variations were found between the binary similarity in the 16S rRNA gene sequence of strain NRC-77 and the 16S rRNA gene sequences of all of the other *S. rochei* strains identified in the GenBank database that have similarity of 99%, or of certain *Streptomyces* species selected based on their morphological and chemo-physiological traits.

Polysporic Approach: The use of genotypic and phenotypic techniques gives a better resolution in the species-level identification [32]. It is clear from the phylogenetic analysis that strain NRC-77 clustered neither with *S. rochei* nor with any other *Streptomyces* species and thus this strain represents a distinct phyletic line,

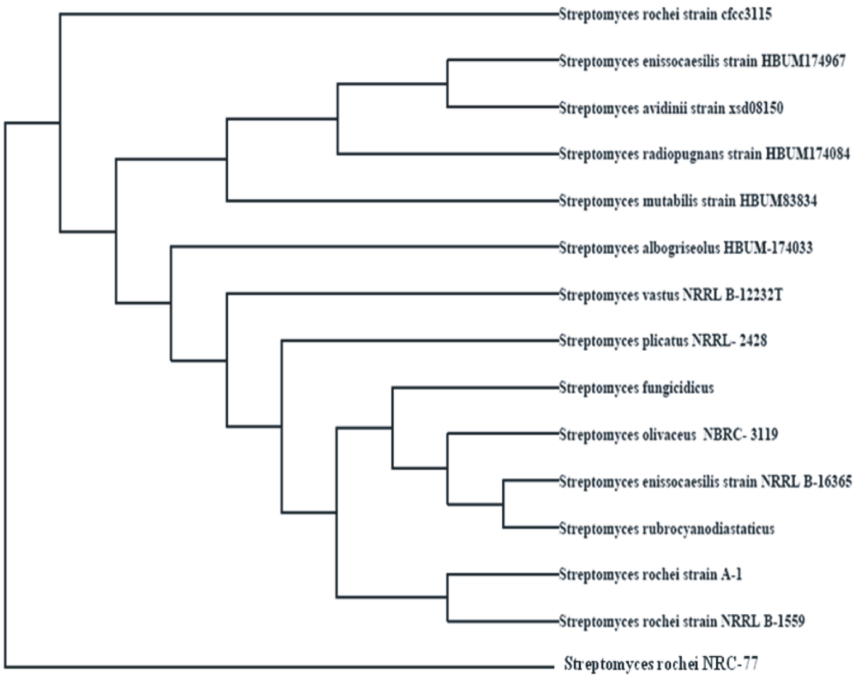


Fig. 3: A phylogenetic tree showing the position of *Streptomyces rochei* NRC-77 constructed using the neighbour-joining method

Table 3: Gene products of the clavulanic acid biosynthesis gene cluster

Gene cluster	Protein code in GenBank	Start bases	Similarity to cephamycin
ORF2	*Acetohydroxy acid synthase*	--	High
ORF6	*Ornithine transacetylase*	--	High
ORF7	*Oligopeptide permease*	--	Medium
ORF9	*Clavulanic acid dehydrogenase*	GGCGG	
ORF10 (*cyp*)	*Cytochrome* P_{450}	GGTGG	High
ORF11 (*cla*)	*Proclavaminate amidinohydrolase*	GGTGA	--
ORF12 (*tsr*)	*Induction CA biosynthesis*	(Encoded)	--

suggesting a new genomic species. However, strain NRC-77 is suggested to be a new variety of *S. rochei*. Thus, the newly isolated strain NRC-77 is designated *S. rochei* NRC-77. The isolated strain is a potential source of active compounds. It has long been known that certain Actinomycete strains of the same species produce different antibiotics, whereas various other strains belonging to different species produce the same antibiotics. Antibiotic production by Actinomycetes, therefore, may not be species-specific but strain-specific [33]. However, CA is not an antibiotic but is a secondary metabolite acting as a potent $-lactamase inhibitor. Furthermore, to date, no *S. rochei* strain is known that is able to produce CA, though this strain produces other metabolites, such as antibiotics and antifungal substances [34].

Clavulanic Acid Gene Cluster: The CA biosynthetic cluster of *S. rochei* NRC-77 is similar to the CA gene cluster of *S. clavuligerus*, which is composed of open reading frames (ORFs). Furthermore, the CA biosynthetic cluster (Table 3) has the same biosynthetic gene cluster as the cephamycin of *S. jumonjinensis* and *S. Katsurahamanus* [35]. The nucleotide sequence of *S. rochei* NRC-77 (Fig. 4) showed the genomic regions corresponding to several ORFs and to ORF12, where the gene encompassing ORF2 through ORF9 was capable of producing CA and ORF10 and ORF11 are also involved in CA production. ORF10 is located 286 bp downstream of ORF9 (*cad*) and is preceded by the sequence (GGXGG), whereas ORF11 consists of 207 nucleotides, starts from a GTG and is preceded by GGTGA. Moreover, the ORF12 start codons, ATG and GTG, are separated by 21 and 39 nucleotides [36].

GCGTCACCTTCCTCGAGTTGACCCCTGGCGGTCTCCCGTGGAGTCCCTAGCACCACAAGGGCCTGCTGGCAACAC
GGGACAAGGGTTGCGCTCGTTGCGGGACTTAACCCAACATCTCACGACACGAGCTGACGACAGCCATGCACCA
CCTGTACACCGACCACAAGGGGGACCCTGTCTCCAGGGTTTTCCGGTGTATGTCAAGCCTTGGTAAGGTTCTTC
GCGTTGCGTCGAATTAAGCCACATGCTCCGCCGCTTGTGCGGGCCCCCGTCAATTCCTTTGAGTTTTAGCCTTGC
GGCCGTACTCCCCAGGCGGGGCACTTAATGCGTTAGCTGCGGCACGGACAACGTGGAATGTTGCCCACACCTAG
TGCCCACCGTTTACGGCGTGGACTACCAGGGTATCTAATCCTGTTCGCTCCCCACGCTTTCGCTCCTCAGCGTCA
GTATCGGCCCAGAGATCCGCCTTCGCCACCGGTGTTCCTCCTGATATCTGCGCATTTCACCGCTACACCAGGAA
TTCCGATCTCCCCTACCGAACTCTAGCCTGCCCGTATCGACTGCAGACCCGGGGTTAAGCCCCGGGCTTTCACA
ACCGACGTGACAAGCCGCCTACGAGCTCTTTACGCCCAATAATTCCGGACAACGCTTGCGCCCTACGTATTAC
CGCGGCTGCTGGCACGTAGTTAGCCGGCGCTTCTTCTGCAGGTACCGTCACTTTCGCTTCTTCCCTGCTGAAAGA
GGTTTACAACCCGAAGGCCGTCATCCCTCACGCGGCGTCGCTGCATCAGGCTTTCGCCCATTGTGCAATATTCC
CCACTGCTGCCTCCCGTAGGAGTCTGGGCCGTGTCTCAGTCCCAGTGTGGCCGGTCGCCCTCTCAGGCCGGCTACC
CGTCGTCGCCTTGGTGAGCCGTTACCTCACCCAACTAGCTGATAGGCGCGGGCTCATCCTGCACCGCGGGAGCTT
TCGAACCTCGCAGATGCCTGCGAGGATCAGTATCCGGTA

Fig. 4: The nucleotide sequence of 16S rRNA from *Streptomyces rochei* NRC-77

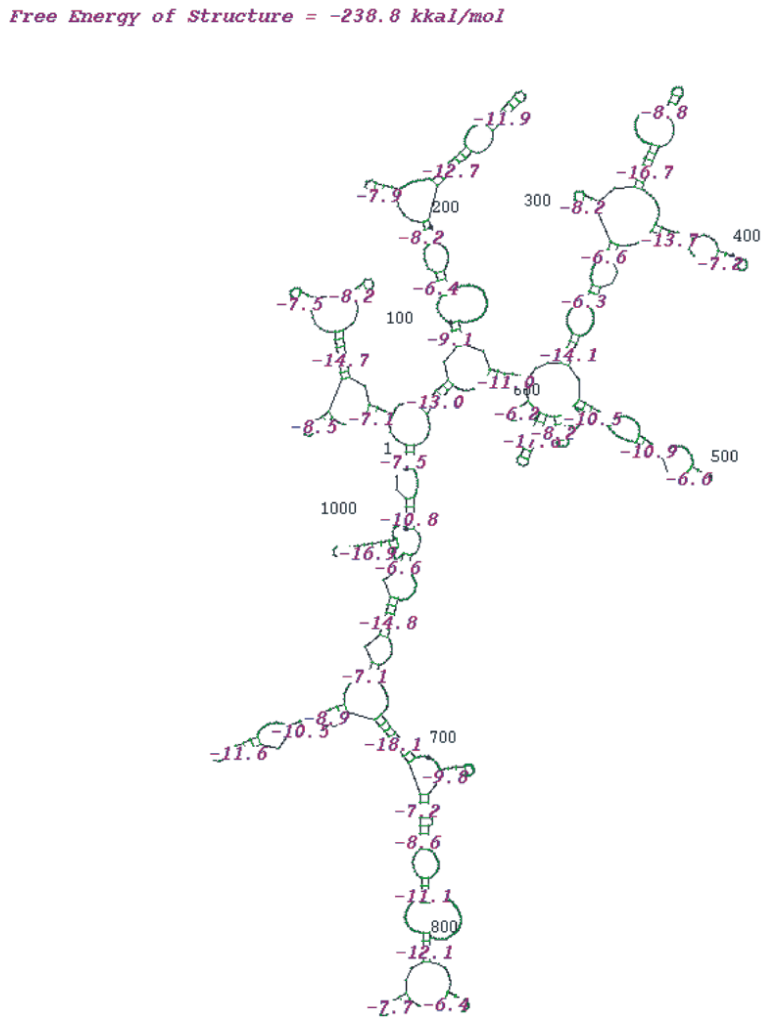


Fig. 5: Secondary structure prediction for the 16s rRNA of the strain *Streptomyces rochie* NRC-*77* was performed using GeneBee online software.

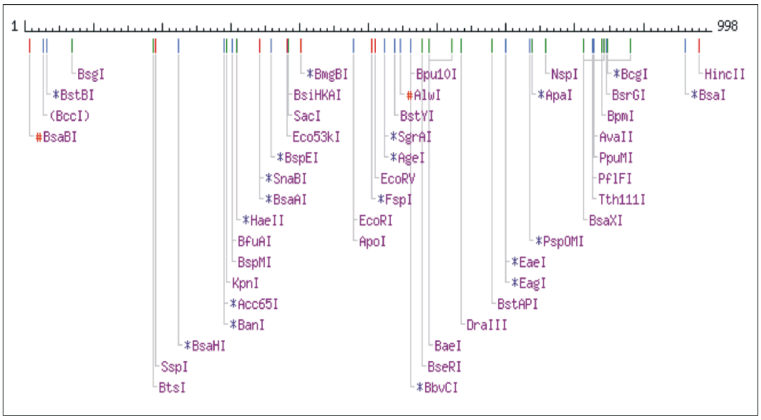


Fig. 6: Restriction sites of the strain *Streptomyces rochei* NRC-77 were predicted using NEBcutter

Secondary Structure Prediction and Restriction Site Analysis: The RNA secondary structure was predicted for the 16S rRNA of *Streptomyces* sp. NRC-77 (Fig. 5). This prediction showed that the free energy of the structure is -238.8 kcal/mol; the threshold energy is -4.0 with cluster factor, conserved factor 2 and compensated factor 4; and the conservativity is 0.8. The prediction of restriction sites in the strain NRC-77 showed the restriction sites for various enzymes, such as BsaB l, SnaBl, EcoR l, Agel and Bsal (Fig. 6).

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