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Identification and Antibiosis of a Novel Actinomycete Strain RAF-11 Isolated From Iraqi Soil.

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Abstract

A total of 35 actinomycetes strains were isolated from and around Baghdad, Iraq, at a depth of 5-10 m, by serial dilution agar plating method. Nineteen out of them showed noticeable antimicrobial activities against at least, to one of the target pathogens. Five among the nineteen were active against both Gram positive and Gram negative bacteria, yeasts and moulds. The most active isolate, strain RAF-11, based on its largest zone of inhibition and strong antifungal activity, especially against *Candida albicans* and *Aspergillus niger*, the causative of candidiasis and aspergillosis respectively, was selected for identification. Morphological and chemical studies indicated that this isolate belongs to the genus *Streptomyces*. Analysis of the 16S rDNA sequence showed a high similarity, 98 %, with the most closely related species, *Streptomyces labedae* NBRC 15864T/AB184704, *S. erythrogriseus* LMG 19406T/AJ781328, *S. griseoincarnatus* LMG 19316T/AJ781321 and *S. variabilis* NBRC 12825T/AB184884, having the closest match. From the taxonomic features, strain RAF-11 matched with *S. labedae*, in the morphological, physiological and biochemical characters, however it showed significant differences in morphological characteristics with this nearest species, *S. labedae,* which encourage us to consider our starin as a novel isolate and was given the suggested name, *Streptomyces labedae* strain RAF-11. ISP-4 broth medium supplemented with glucose and soybean powder at concentrations of 1g % and 0.1g % as carbon and nitrogen sources respectively, for 120h incubation at 28 °C, increased the active compounds production, where we recorded a strong activity against yeasts, 42mm inhibition zone against *Candida albicans*, 41mm against *C. pseudotropicalis*, 40mm against *C. tropicalis*, followed by 38mm against *Rhodotorula minota and Aspergillus niger* then, 35mm against both *Aspergillus flavus* and *Bacillus subtilis.* N-butanol was best solvent for antibiotic extraction compared to the other tried solvents.

Keywords: Actinomycetes, identification, antimicrbial activity, strain RAF-11, Streptomyces.

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1. Introduction

Antimicrobial resistance is one of our most serious health threats. Infections from resistant bacteria are now too common, and some pathogens have even become resistant to multiple types or classes of antibiotics (antimicrobials used to treat bacterial infections).The loss of effective antibiotics will undermine our ability to fight infectious diseases and manage the infectious complications common in vulnerable patients undergoing chemotherapy for cancer, dialysis for renal failure, and surgery, especially organ transplantation, for which the ability to treat secondary infections is crucial [1]. Actinomycetes, one of the most important groups of microbes, exhibit many interesting activities such as degradation and transformation of organic and metal substrates together with production of antibiotics [2].

Species of the genus Streptomyces, which constitute the vast majority of taxa within the family Streptomycetaceae, are a predominant component of the microbial population in soils throughout the world and have been the subject of extensive isolation and screening efforts over the years because they are a major source of commercially and medically important secondary metabolites [3].

The present work involved the isolation and characterization of new actinomycetes strains able to produce natural bioactive compounds effective against Gram positive bacteria including methecilin resistance Staphylococcus aureus and Micrococcus luteus in addition to the causative agents of Candidiasis and aspergillosis, Candida albicans and Aspergillus species, respectively. Suitable broth, carbon and nitrogen sources influence the production of active compounds were also studied and best solvent for effective extraction was determined.

2. Materials and Methods

2.1 Collection of Soil samples and Processing

Several soil samples were randomly collected from and around Baghdad – Iraq, using an open-end soil borer (20 cm depth and 2.5 cm diameter) as described by [4]. Soil samples were taken from depths of 05 - 20 cm bellow the soil surface, the top region (5 cm from the surface) was excluded. Samples w ere air-dried at room temperature for 14 days and then passed through a 0.8 mm mesh sieve to remove various contaminant materials, mixed thoroughly with CaCO3 (10 % w/w), saturated with water and incubated at 28 °C for 10 days before use, [5, 6]. Samples (10 g) of treated soil were aseptically added to 90 ml sterile distilled water. The mixtures were shaken vigorously for about an hour and then allowed to settle for 30 minutes. Portions (1 ml) of soil suspensions (diluted 10^{-1}) were transferred to 9 ml of sterile distilled water and subsequently diluted to 10^1, 10^2, 10^3….. 106.

2.2 Isolation and maintenance of actinomycetes

Actinomycetes strains were isolated by using the dilution agar plating technique [7], on a basal salts agar medium [8]. The plates were incubated at 28 °C for two weeks. From day 5 on, the developed colonies of actinomycetes, which were characterized by their sharp round edges, were picked up by a sterile needle and then re-inoculated on the same medium. Pure cultures were maintained on (ISP-2) slants (International Streptomyces Project) at 4 ˚C and as spore suspensions as well. Spores suspensions were prepared according to the method of [9] by adding 20 % (v/v) glycerol onto a well-sporulated plates and scraping off the spores from the surface of each plate one by one. The

suspensions were then filtrated through sterile non-absorbent cotton wool to remove mycelial remnants and stored at -20 ˚C before use.

2.3 Antimicrobial activity

To determine the antimicrobial activity in solid and liquid medium as well, pure isolated strains were grown on ISP-4 and starch-nitrate agar medium for 7 days at 28 °C and then, 6mm diameter discs were cut out from these cultures under aseptic conditions using sterile cork borer and aseptically transferred to the surface of agar plates seeded with different test organisms. In case of liquid cultures, Erlenmeyer flasks (250 ml capacity) having 50 ml of ISP-4 and starch nitrate broth were inoculated with the experimented strains and incubated at 28 °C on a rotary shaker at 200 rpm, for 5 days. Antimicrobial potentialities were assayed according to the method of [10], 0.2 ml of the filtrate broth were transferred to 6mm diameter wells cut in nutrient agar and Czapek's-Dox agar plates, which were previously seeded with the same test organisms. The plates were then kept in a refrigerator for 2 – 4h to permit diffusion of the metabolites before the growth of the test organisms take place. Inhibition zones were expressed as diameter and measured after incubation at 37 °C for 24 h for bacteria and yeasts, and at 28 °C for 48 - 72 h for filamentous fungi. The used target germs were: Bacillus subtilis (ATTC 6633), Micrococcus luteus (ATCC 9314) Staphylococcus aureus (ATCC 33591) and E. coli (ATCC 25922) in addition to yeasts and moulds from the culture collection of the Microbial Chemistry Laboratory, National Research Centre, Dokki - Cairo, Egypt, which were (Candida albicans NRRLY-12983, C. tropicalis NRRLY-12968, C. pseudotropicalis NRRLY-8281, Rhodotorula minuta NRRLY-1589, Aspergillus niger NRRLA-326, A. flavus NRRLA-1957, A. terreus NRRLA-571, Botrytis allii NRRLA-2502, Fusarium oxysporum NRRLA-2018, Helmenthosporium turcicum NRRLA-1081, Machrophomina phaseoli NRRLA-62743, Trichoderma viride NRRLA-63065. Bacteria and yeasts were cultivated on nutrient agar whereas; filamentous fungi were grown on Czapek's Dox agar.

2.4 Identification of the most active isolate

The isolate which showed large antimicrobial activity, especially against yeasts and moulds, and looked promising, was designated RAF-11 and selected for identification by conventional and molecular methods.

. 2.4.1 Morphological and cultural characteristics:

The morphological and cultural characteristics of the selected isolate were determined using various International Streptomyces Project (ISP) media. (ISP-2) [11], (ISP-3 and ISP-4) [12], (ISP-5) [13] and starch nitrate agar medium [14]. All of these media were sterilized at 1.5 atmospheres for 15 minutes. Media were cooled to about 50 ˚C and dispensed aseptically into sterile Petri dishes. The poured plates were left for a minimum of 24 hours at 37 ˚C to promote moderate drying and check sterility before inoculation. Six plates for each medium were used to be inoculated by the bacterium. Incubation was made at 28 ˚C, 2 plates were observed after7 days, 2 at the end of 14

days and the two others at the end of 21 days. The colour of mature sporulating aerial mycelium, the substrate mycelium (reverse side pigments) and the diffusible (soluble) pigments other than melanin were recorded. The plates that were prepared for morphological studies were also used for colours determination. The observations and records were performed according to the ISCC-NBS centroid colour charts [15]. The spores chains and spore surface ornamentation were examined according to the method of [16], using EM10 Karl-Zeiss electron microscope. Cell wall analysis was carried out using the methods described by [17, 18, and 19].

2.4.2 Physiological characteristics:

Numerous tests were considered for this investigation, including The production of melanin pigment on (ISP-6) and (ISP-7) media [20]. The utilization of carbohydrate compounds evaluated on C1 medium, according to [21, 22]. The degradation of many organic compounds such as: coagulation and peptonisation of skimmed milk [23], hydrogen sulphide production [8], nitrate reduction and indole production [24], starch degradation [20], gelatine liquefaction [23], Enzymes and related biochemical activities were also performed, including protease, lecithinase and lipolytic activities, using egg-yolk medium according to the method of [25], pectinase and chitin decomposition [26, 27], catalase production [28] and urease production on SSR [28] urea medium, according to [24]. Sensitivity to potassium cyanide (KCN) [24], sodium azide 0.01 %; crystal violet 0.001, sodium chloride 1 %, to 9 %; [24, 29 and 30], and the growth at pH range 5 to 9 and at temperature 42˚C were also investigated. Finally, the sensitivity to antibiotics was also studied on medium supplemented with 5 different antibiotics, Chloramphenicol (25 mg/l), Erythromycin (10 mg/l), Gentamicin (5 mg/l), Oxytetracycline (25 mg/l) and Penicillin (25 mg/l) [30].

2.4.3 Molecular characterization

2.4.3.1 Genomic DNA extraction and PCR amplification

Chromosomal DNAs were isolated from mycelia of the studied stain according to the protocol of [31], with some modifications. Mycelia (5 ml) grown in a LB broth shake culture were centrifuged, rinsed with TE and re-suspended in 0.4 ml SET buffer (75 mM NaCl, 25 mM EDTA, 20 mM Tris, pH 7.5). Lysozyme was added to a concentration of 1 mg ml -1 and incubated at 37 °C for 0.5_1 h. Then 0.1 vol. 10 % SDS and 0.5 mg Proteinase K mg ml -1 were added and incubated at 55 °C with occasional inversion for 2 h. One-third volume 5 M NaCl and 1 vol. chloroform were added and incubated at room temperature for 0.5 h with frequent inversion. The mixture was centrifuged at 4500 g for 15min and the aqueous phase was transferred to a new tube using a blunt-ended pipette tip. Chromosomal DNA was precipitated by the addition of 1 vol. 2-propanol with gentle inversion. The DNA was transferred to a new tube, rinsed with 70 % ethanol, dried under vacuum and dissolved in a suitable volume (about 100 µl) of distilled water. The dissolved DNA was treated with 20 µg RNase A ml -1 at 37 °C for 1 h. Samples were extracted in the same volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and precipitated with 2.5 vols cold ethanol and 0.1 vols 3 M sodium acetate. The pellets were washed with 70 % ethanol, dried and dissolved in TE or distilled water.

Whereas, E. coli DH5alpha was cultivated at 37 °C on Luria bacteria (LB) plates supplemented with ampicillin (50 µg/ml) and 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (40 µg/ml) when appropriate [32], Cultures were stored at -20 °C.

The 16S rDNA gene was amplified using primers fD1 (AGAGTTTGATCCTGGCTCAG) and rP2 (ACGGCTACCTTGTTACGACTT) [33]. It was performed in iCycler PCR BIORAD, in a total volume of 50 mL containing 30-50 ng DNA, 100 mM each primer, 10 mM dNTP , 10Xbuffer (100 mM Tris/HCL, pH8.0; 500 mM KCl, 20 mM mgcl2, 0.1% gelatine) and 1.5 U Taq DNA polymerase (Promega). PCR was performed under the following conditions: 3 min at 94 °C, followed by 35 cycles of 1 min 94 °C, 1 min at 58 °C, 2 min at 72 °C, and then final extension at 72 °C for 7 min, The PCR reaction mix was analyzed by agarose gel electrophoresis and the DNA of the expected size was purified then cloned into pGEM_T Easy vector (Promega).

2.4.3.2 Preparation and transformation of competent E. coli DH5α cells

10 ml of LB was inoculated with a single E. coli DH5α colony from a 24 hr culture, and was incubated at 30°C for 24 hrs. 200 µl of this culture was used to inoculate 50 ml of LB which was incubated at 30°C for 3 hrs until OD600 = 0.4. After incubation the cells were placed on ice for 15 mins and then centrifuged for 15 mins at 3,000 g and the supernatant was discarded. The pellet was re-suspended in 1 ml of ice-cold 0.1 M $MgCl_2$ and centrifuged at 13,000 rpm for 1 min. The supernatant was discarded and the pellet re-suspended in 1 ml of ice-cold 0.1 M $CaCl_2$ and centrifuged at 13,000 rpm for 1 min. The pellet was again re-suspended in 1 ml of ice-cold 0.1 M $CaCl_2$ and left on ice for at least 3 hrs prior to use. 50µl of competent cells were added to 2µl of ligation mixture in a 1 ml Eppendorf and incubated on ice for 2-5 mins. The cells were heat shocked at 42 °C for 45 sec and immediately transferred to ice for 5 mins. 950µl of LB was added to the cells and incubated at 37°C for 1 h 30 mins. LB plates containing 100 µg/ml were pre-warmed and spread with 40µl of 2x X-gal and 10µl of 100mM IPTG. 100µl of cells were incubated on the pre-prepared LB plates and incubated overnight at 37°C.

2.4.3.3 Plasmid extraction

10 ml of LB containing 100 µg/ml ampicillin was inoculated with the transformed E. coli cells and incubated for 24 hrs at 30 °C. Plasmid DNA was extracted using the QIAGEN Mini-prep kit as per manufacturers' instructions.

2.4.3.4 Cloning and nucleotide sequence determination

PCR products of the 16S rDNAs of the selected strain were sub-cloned into pGEM-T Easy Vector for nucleotide sequence determination using an automated laser fluorescence sequencer (3100 genetic analyzer ABI PRISM, Applied Biosystem, HITCHI, USA). Sequencing reactions were carried out with the Big Dye termination kit (Applied Biosystems) according to the supplier's instructions. Nucleotide sequence of the 16S rDNAs of strain

RAF-11 was determined and the level of similarity was compared to the reference species of bacteria contained in genomic database banks, using the ‘‘NCBI Blast’’ available at http://www.ncbi-nlm-nih.gov (website).

2.4.3.5 Phylogenetic analysis

Phylogenetic and molecular evolutionary analysis were conducted using software included in MEGA version 3.0 [34], package. The 16S rDNAs sequence of RAF-11 strain was aligned using the CLUSTAL W program [35], against corresponding nucleotide sequences of representatives of the genus Streptomyces retrieved from GenBank. Evolutionary distance matrices were generated as described by [36], and phylogenetic tree was inferred by the neighbour joining method [37]. Tree topologies were evaluated by bootstrap analysis [38], based on 1000 resembling of the neighbour joining data set.

2.5 Selection of suitable broth and correct culture conditions

For optimum formation of active compounds, a number of broth media such as ISP2, ISP-4, tryptone-yeast extract-glucose (TYG), tryptic soy broth (TSB) and starch nitrate broth were tried. After incubation at 28 ˚C for 144 h in New Brunswick Scientific Shaker at 200 rpm, antibacterial activities were assayed for each culture supernatant. After determination of the better culture broth, effect of carbon and nitrogen sources on the active compound formation were also investigated in the same culture conditions described above. Finally, and based on the obtained results, the effect of incubation periods (up to 144 hours) was also determined by well diffusion method, in order to determine the suitable period for high active compound formation.

2.5.2 Selection of suitable solvent for active compounds extraction

To extract the active compounds, four different solvents were screened for effectiveness which were: petroleum either, n-hexane, ethyl acetate and n-butanol. The organic extracts were evaporated to dryness then recuperated in 1 ml of methanol and tested for their antimicrobial activities using discs of 6mm against Bacillus cereus and Candida albicans. The solvent which gave maximum inhibition diameter, using respective solvents as control, was then kept for the extraction of active compounds, while mycelium was extracted with ethanol.

3 Results

3.1 Isolation and screening of actinomycetes

In this investigation, 35 actinomycetes strains were isolated. Nineteen out of them showed noticeable antimicrobial activities against at least to one of the target pathogens. Five among the nineteen were active against both Gram positive and Gram negative bacteria, yeasts and moulds Table 3. 1.

Table 3.1: Antimicrobial activity of thirty five isolates of Streptomyces spp. against G+ve and G-ve bacteria, yeasts and fungi

Isolate no.	Staphylococcus aureus	Bacillus subtilis	Escherichia Coli	Candida albicans	Aspergillus Niger
BS1	-	+	+	-	-
BS2	+	+	-	-	-
BS3	-	-	+	-	-
BS4	-	+	+	-	-
BS5	+	+	+	-	-
BS6	-	-	-	-	-
BS7	-	+	+	-	-
BS8	+	+	+	+	+
BS9	-	-	+	-	+
BS10	+	+	+	+	+
BS11	-	-	-	-	-
BS12	-	-	-	-	-
BS13	-	-	-	-	-
BS14	+	+	+	-	-
BS15	-	-	+	-	-
BS16	+	+	-	-	-
BS17	-	-	-	-	-
BS18	-	-	-	-	-
BS19	+	+	+	-	-
BS20	-	+	+	-	-
BS21	+	-	-	-	-
BS22	+	+	+	+	+
BS23	-	+	+	-	-
BS24	-	-	-	-	-
BS25	-	-	-	-	-
BS26	-	-	-	-	-
BS27	+	+	+	-	+
BS28	+	-	+	-	-
BS29	+	+	+	+	+
BS30	-	-	-	-	-
BS31	-	+	+	-	-
BS32	-	-	-	-	-
BS33	+	-	-	-	-
BS34	-	-	+	-	+
BS35	+	+	+	+	+

+ Antibiosis - no effect BS = Baghdad Strain

The most active strain (MS29), based on its largest zone of inhibition was selected for identification and designated strain RAF11, results are given in Table.3.2

Table. 3. 2: Antimicrobial activity of the five most active strains.

Isolate no.	Staphylococcus aureus	Bacillus subtillis	Escherichia coli	Candida albicans	Aspergillus niger
BS2	20*	25	23	18	16
BS10	22	26	23	18	21
BS22	19	22	20	18	17
BS29	28	30	28	30	29
BS35	18	20	20	17	15

*Antimicrobial activity was measured as growth inhibition zone diameter (mm)

3.2 Taxonomy

Examination of strain RAF-11 grown on ISP2 medium at 28 C for 7 days revealed that sporophores are spiral. Transmission electron micrograph showed that spores were numerous, oval with spiny membranes Fig 1.

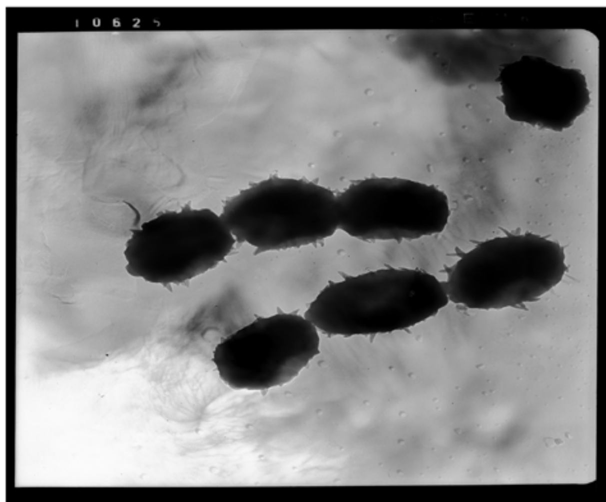

Figure 1. Transmission electron micrograph of strain RAF-11, showing spiny spores.

The substrate mycelium (SM) had no distinctive colour. It varied depending on the type of the used media. Strain RAF-11 grew well to moderate on the tested organic and synthetic media. The colour of areal mycelium was yellow gray to yellow white or medium gray; it varied depending on the type of used media, as it is recorded in Table 3.3.

Table 3.3: Cultural characteristics of the selected *Streptomyces* strain RAF-11.

Media	Growth	Arial Mycelium	Substrate mycelium	Diffusible pigment
Oat meal agar medium	Moderate	Y.gray. 93	gy.y 90	gy.y 90
Yeast -malt extract agar medium	Well	y.gray 93	gy.y 90	m.y 87
Glycerol asparagine agar medium	Well	l .01 Br 94	m .01 Br 94	gy.y 90
Fish meal extract agar	Moderate	y.white 92	gy.y 90	m.y 87
Soybean agar	Well	l .01 Br 94	l .01 Br 94	—
Czapeck-dox agar	Moderate	l .01 Br 94	l .01 Br 94	l .01 Br 94
Starch nitrate agar	Well	Med.gray 264	d.gray 93	1-01gy112

Y = Yellow, Br = Brown, M = Medium, G = Gray, D = Dark, GY = Gray yellowish. Numbers (see code number in colours charts of Kneeth, 1958)

This bacterium hydrolysed starch, produced H2S, liquefied gelatin, peptonised milk but did not reduce nitrate nor produce melanin pigments. It utilised most used carbohydrates and nitrogen sources as well. It was not able to grow on glucose-yeast extract agar (GYEA) medium supplemented with 5 different antibiotics, Chloramphenicol (25 mg/l), Erythromycin (10 mg/l), Gentamicin (5 mg/l), Oxytetracycline (25 mg/l) and Penicillin (25 mg/l). Strain RAF-11 did not grow in the presence of sodium azide at 0.001 %, crystal violet at the same concentration and at 0.8 % sodium chloride. On the other hand, well growth was recorded at a temperature range of 15 to 42 °C and at pH range 6 to 9. It utilized citrate and tolerated 7% NaCl, as it is given in (Table 3.4).

Table 3.4: Physiological and biochemical characteristics of strain RAF-11

Physiological and biochemical tests	Activity
Degradation activity	
Starch hydrolysis	+
Arbutin	+
Casein	+
Gelatine liquefaction	+
Nitrate reduction	-
H2S production	+
KCN	+
Citrate	+
Nacl tolerance	7 %
Effect of inhibitory compounds	
Sodium azide 0.001 % (w/v)	-
Crystal violet 0.001 % (w/v)	-
Growth temperature	15 – 42
Melanin production	
Tyrosine agar medium	-
Peptone yeast extract iron agar medium	-
PH range	6 – 9
Enzyme activity	
Amylase	+
Gelatinase	+
Pectinase	+

Protease	+
Lipase	+
Lecithinase	+
Urease	+
Cellulase	+
Resistance to antibiotics	
Rifampicin (5 mg/l)	-
Penicillin (25 mg/l)	-
Streptomycin (10 mg/l)	-
Erythromycin (10 mg/l)	-
Gentamicin (5 mg/l)	-

Utilization and fermentation of carbon sources

Carbon source 1% (w/v)	Growth	Fermentation
No carbon (control)	-	-
Glucose	+	+
Raffinose	+	-
D-xylose	+	+
I-inositol	+	-
D-mannitpl	+	-
Fructose	+	+
L-rhamnose	+	-
Sucrose	+	-
L-arabinose	-	-
Cellubiose	-	-

+ Well utilized - Not utilized or not producrd

The chemotaxonomic study showed the presence of a chemotype I cell wall characterized by (LLDAP) as mentioned by [19] no diagnostic sugars were detected. The characteristics of RAF-11 such as obtained cultural and morphological properties and enzyme activities were compared with those of known species of actinomycetes described in Bergey's Manual of Systematic Bacteriology [39], suggested strongly that strain RAF-11 belongs to genus *Streptomyces.*

3.2.1 16S rRNA sequence comparison

The alignment of the nucleotide sequence of strain RAF-11, through matching with 16S rRNA reported genes sequences in the gene bank using the ‘‘NCBI Blast’’ available at http://www.ncbi-nlm-nih.gov (website), and compared to sequences of the reference species of bacteria contained in genomic database banks exhibited a high similarity, 98 %, with *Streptomyces labedae* NBRC 15864T/AB184704, *Streptomyces erythrogriseus* LMG 19406T/AJ781328, *Streptomyces griseoincarnatus* LMG 19316T/AJ781321 and *Streptomyces variabilis* NBRC 12825T/AB184884, having the closest match. The phylogenetic tree obtained by applying the neighbour-joining method using BioEdit software [40], is illustrated in Fig 3.2.

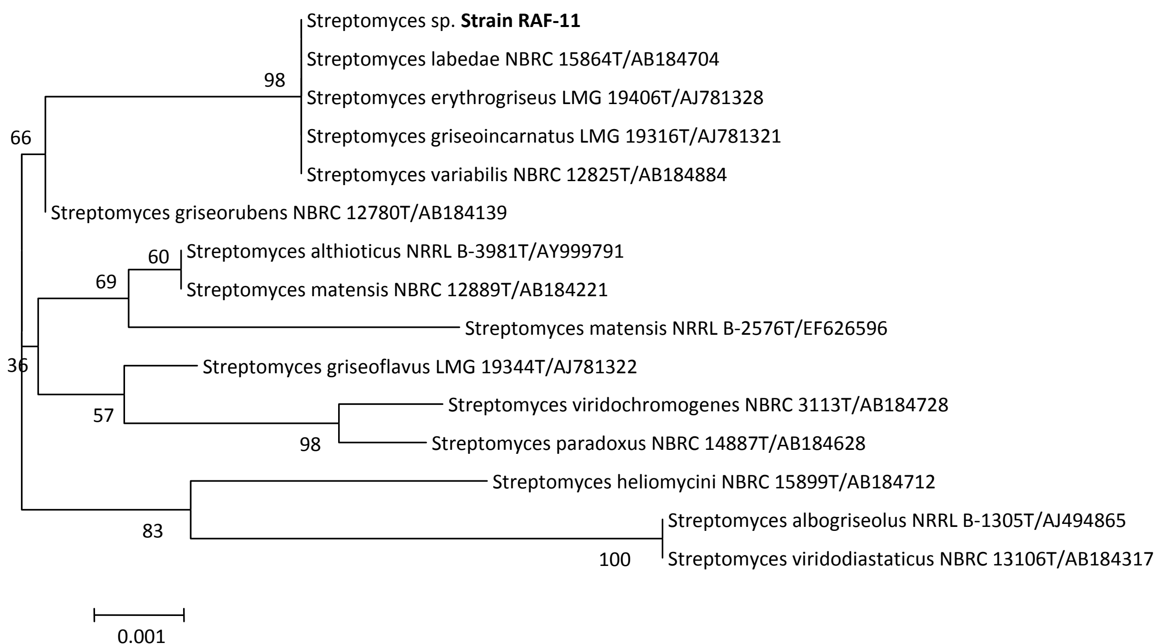

Figure 3.2: 16S rDNA tree showing the phylogenitic relationship neighbour-joining method between strain RAF-11 and other known sequences of Streptomyces sp.

3.3 Antimicrobial activity

Results in Table 3.4, show the broad antimicrobial spectrum of strain RAF-11 against various pathogen microorganisms using ISP-4 broth after the determination of optimum conditions for antibiotic production. It exhibited a strong activity against yeasts, where we recorded inhibition zone of 42mm against *Candida albicans*,

41mm against *Candida pseudotropicalis*, 40mm against *Candida tropicalis*, followed by 38mm against *Rhodotorula minota and Aspergillus niger,* then, 35mm against both *Aspergillus flavus* and *Bacillus subtilis.*

Table 3.5: Antibiosis of *Streptomyces labedae* strain RAF-11 towards various pathogens (modified ISP-4 medium)

Microorganisms	Inhibition zone diameter (mm)
Bacillus subtilis	35
Micrococcus luteus	32
Staphylococcus aureus	31
Escherichia. Coli	33
Kleibsella pneumonia	30
Aspergillus niger	38
Asprgillus flavus	35
Aspergillus terreus	33
Botrytis allii	30
Diplodia oryzae	25
Fusarium oxysporium	29
Helmenthosporum turcicum	32
Machrophomina phaseoli	26
Trichoderma viride	27
Candida albicans	42
Candida tropicalis	40
Candida pseudotropicalis	41
Rhodotorula minuta	38

3.4 Optimisation and suitable solvent for antibiotic extraction

3.4.1 Optimum conditions for antibiotic production

Different broth media, carbon and nitrogen sources and incubation periods were tested for maximum active compounds production. It was found that, ISP-4 broth using, starch and glucose at concentrations of 2.0 and 1.0 % (w/v) as carbon sources with ammonium sulphate and soybean powder at concentrations of 0.2 and 0.1 % (w/v) as nitrogen sources

respectively, for 120 h at 28 °C in orbital incubator with shacking at 180 rpm were the most suitable for active compounds formation by this bacterium.

3.4.2 Suitable solvent for extraction

The ethanol extract of the biomass had no antimicrobial activity. N-hexane and petroleum either were negative for antibiotic extraction, while ethyl acetate and n-butanol were positive for extraction. Because, n-butanol was the best one, it was then kept for antibiotic extraction.

4- Discussion

Need for novel, safe and more efficient antibiotics is a key challenge to the pharmaceutical industry today. The ever increasing knowledge in the area of pathogen`s drug resistance has evoked the discovery of new antibiotics by the screening of microbes. Last few decades has witnessed the production of novel antibiotics from different microorganisms. At present, aerobic actinomycetes have attracted considerable attention of bacteriologist, geneticist and ecologist because of the production of novel antibiotics [41].

On the basis of its morphological and chemical properties strain RAF-11 was classified in the genus *Streptomyces*. The characterization of *Streptomyces* species is mainly based on, morphological and cultural characteristics such as ,colour of substrate and aerial mycelia, soluble pigments, shape and ornamentation of spore surface because of their stability [42, 39]. Modern *Streptomyces* identification systems are based on 16S rRNA sequence data, which have provided invaluable information about streptomycetes systematic, and then have been used to identify several newly isolated *Streptomyces* [43, 44,45, 46, 47and 48]

The 16S rRNA nucleotide sequence of strain RAF-11 was compared to those of other *Streptomyces* species of the reference species of bacteria contained in genomic database banks; it exhibited a high similarity, 98 %, with *Streptomyces* labedae NBRC 15864T/AB184704, *Streptomyces erythrogriseus* LMG 19406T/AJ781328, *Streptomyces griseoincarnatus* LMG 19316T/AJ781321 and *Streptomyces variabilis* NBRC 12825T/AB184884, having the closest match. Due to our knowledge, there are no publications about the nearest *Streptomyces* species mentioned above, to compare with our strain, except few information about *Streptomyces labedae* the most closely one, which was a novel species called *Streptomyces labedae* sp. nov. *Streptomyces labedae* (la'bed'ae.; L. gen. n. *labedae,* named after first described by, D. P. Labeda, who first recorded the difference between the type strain and second reference strain of *Streptomyces erythraeus* designated by Waksman, [49].

Furthermore and for adequate identification, some physiological characters such as degradation of starch, gelatine, inositol, rhamnose and reduction of nitrates, some additional testes relative to the use of arabinose, glycerol, galactose and mannitol are also considered to ascertain species classification of new isolates strains as recommended by [50]. Thus, cultural and physiological characteristics of Strain RAF-11 was compared to *Streptomyces labedae* characteristics as published by [49], where we recorded some significant differences such as; substrate mycelium of

S. labedae was olive gray to dark reddish brown, whereas, in case of strain RAF-11, it was gray yellowish, or medium brown to dark gray. The aerial mycelium was white to pinkish, especially on glycerol-asparagine or Czapek agars for *S. Labedae*. While it was yellow gray to yellow white or medium gray for strain RAF-11. Spores of *S. Labedae* were moderate gray on most media and reddish gray on glycerol-asparagine or Czapek agars, where they were moderate gray on all used media for RAF-11strain. Spore chains were open spirals *(Spirales)* and spore surface was spiny for both of them and they do not produce melanin pigments on ISP-6 and ISP-7 medium. Which concern the utilisation of carbon sources, raffinose was poorly assimilated by *S. Labedae* and not utilised at all by strain RAF-11, while, arabinose and sucrose were assimilated moderately well by *S. labedae*; whereas, RAF-11 strain assimilated sucrose well but, it could not use arabinose. These differences let us consider our isolate as a novel strain of *Streptomyces labedae*, as it is very obvious in Table 3.

Table. 3.6: Description of strain RAF-11 and *Streptomyces labedea*

Characteristics	Strain RAF-11	Streptomyces labedea
Substrate mycelium	Gray yellowish or med- brown to dark gray	olive gray to dark reddish brown
Aerial mycelium	yellow gray to yellow white or medium gray	white to pinkish
Spores	gray on most media.	moderate gray on most media and reddish gray on glycerol-asparagine or Czapek agars
Spore chains	open spirals	long, open spirals.
The spore surface	Spiny	Spiny
Melanin pigments	not produced	not produced
Carbon source utilization		
Glucose	++ +	++ +
Xylose	++ +	++ +
Rhamnose	+++	++ +
Fructose	+++	++ +
Galactose	++	++ +
Mannitol	++	++ +
Inositol	++	++ +
Arabinose	_	++
Salicin	ND	++
Sucrose	++ +	++
Raffinose	+	+
Wall chemotype	I, with LLDAP and no diagnostic sugars were detected.	I, with LLDAP and glycine

+++ well assimilated ++ assimilated moderately well + poorly assimilated – not assimilated

It is well known that, many factors affect antibiotic production from different micro-organisms, especially the three independent variables namely concentration of carbon and nitrogen sources and temperature of incubation [44, 51, 52 and 35], The antimicrobial activity of strain RAF-11, was increased when using ISP-4 broth with the new obtained data, to be supplemented with 1g % glucose and 0.1g % soybean powder as carbon and nitrogen sources respectively, for 120h incubation period instead of 144h when using the basal ISP-4 broth medium. Suitable solvent was n-butanol, thus, it was kept for antibiotic extraction.

In conclusion, in view of all the previous characteristics of strain RAF-11 and recorded distinctions, compared to *S. Labedae*, it could be stated that, strain RAF-11, is suggested of being a novel strain of *Streptomyces labedae*. Thus, it was designated as *Streptomyces labedae* strain RAF-11. It is a potential source of active substances. Findings obtained from this investigation are promising and hence merit further studies concerning optimisation, extraction and purification of active secondary metabolites.

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