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RESEARCH ARTICLE

Phylogenetic analysis of heavy metal resistant *Streptomyces* spp. isolated from soil in Mosul / Iraq

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Manuscript Info *Abstract*

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Biosurfactants are heterogeneous surface active compounds and can be potentially deployed for bioremediation hydrocarbons. In present study 7 strains of *Streptomyces* spp. from 21 hydrocarbons contaminated soil have been isolated and identified through morphological cultural and biochemical tests. Biosurfactant production was confirmed byconventional screening methods including hemolytic, lipase production, modified drop collapsing and oil spreading methods. Antibiotic & heavy metal resistance phenotypes were determined.

The results showed that most of strains were resistance to penicillin10 IU, methicillin 5 µg, erythromycin 10 µg, ceftriaxone 30 µg, tetracycline 30 µg, ampicillin 10 µg, cephalothin 30µg, trimethoprim and sulfaminoxazole 25 µg.The isolates showed resistant to 1000µg/ml of Nickel sulphate , Zinc sulphate , Lead acetate& nitrate, Silver acetate& chloride, Titanium dioxide , Cobalt nitrate & chloride. Also all isolates showed sensitive to Mercuric sulphate & chloride.

The results of polymerase chain reaction (PCR)showed that bands of 16s rDNA using universal primers 27f & 1392r were found at 1350 bp compare with the 100bp ladder.The nucleotide sequence of the 16s rDNA gene identified two strains that similar to *Strepto.flavogriseus*ATCC 33331 &five strains belongs to*Strepto.albus*J1074.

Thefive strains belongs to*Strepto.albus*J1074 grouped in cluster A at similarityof 97.5%& the two strains that belongs to *Strepto.flavogriseus*ATCC 33331 accumulate in cluster B at similarityof 99%. The phylogenetic tree depending on the nucleotide sequence using Clustal W program and the UPGMA method.The evolutionary distances were computed using the Maximum Composite Likelihood method based on the Tamura model distance coefficient with neighbor joining (NJ), using Mega 0.5

Introduction

Streptomycetes are Gram-positive aerobic members of the order Actinomycetales within the class Actinobacteriaand have a DNA G-C content of 69±78 mol% (Garrity, et al., 2004). Streptomyces is a large genus; there are around 150 species. Members of the genus are strict aerobes and form chains of nonmotile spores. Streptomycetes are very important both ecologically and medically (Madigan, et al.,2013) .The natural habitat of most Streptomycetes is the soil, where they may constitute from 1 to 20% of the culturable population. In fact, the odor of moist earth is largely the result of Streptomycetes production of volatile substances such as geosmin. Streptomycetes play a major role in mineralization. They are flexible nutritionally and can degrade resistant substances such as pectin, lignin, chitin, keratin, latex, agar, and aromatic compounds. Streptomycetes are best known for their synthesis of a vast array of antibiotics(Willey, etal., 2009; Chater ,et al., 2010).

The genus Streptomyces remains a focus of systematics research, not only because Streptomycetes are still a promising source of commercially significant compounds, but also because of taxonomic difficulties within the genus caused by

the large number of isolates and insufficient species definition. The classification of Streptomycetes is strongly influenced by polyphasic taxonomy, taking into account genetic characteristics as well as phenotypic characteristics. Nevertheless, closely related Streptomyces species within species groups are incompletely circumscribed (Rong and Huang., 2010).

Actinomycetes are unsurpassed as producers of bioactive metabolites, primarily those with antimicrobial, anticancer properties; however, their capacity for producing other natural products such as biosurfactants, has been less explored. Biosurfactants are heterogeneous surface active compounds and can be potentially deployed for bioremediation of hydrocarbons and as emulsifying agents in agriculture, pharmaceutical and food industries(Karanth, etal., 2005).Hydrophobic pollutants present in petroleum hydrocarbons, soil and waterenvironment require solubilization before being degraded by microbial cells.Surfactants canincrease the surface area of hydrophobic materials, such as pesticides in soil andwater environment, thereby increasing their water solubility.Hence, the presence ofsurfactants may increase microbial degradation of pollutants(Hamzah ,et al.,2013).

Environmental contamination caused byindustrial activity is due to accidental or deliberate releaseof organic and/or inorganic compounds into the environment.Such compounds pose problems for remediation, asthey become easily bound to soil particles(Lakshmipathy, 2010). The applicationof biosurfactants in the remediation of organic compounds,such as hydrocarbons, aims at increasing their bioavailability(biosurfactant-enhanced bioremediation) or mobilizing and removing the contaminants by pseudosolubilisation andemulsification in a washing treatment. The application ofbiosurfactants in the remediation of inorganic compoundssuch as heavy metals, on the other hand, is targeted atchelating and removal of such ions during a washing stepfacilitated by the chemical interactions between the amphiphilesand the metal ions(Banat, etal., 2010). Biosurfactants play a number of roles including increasing the surface area and bioavailability of hydrophobic water-insoluble substrates, binding of heavy metals, quorum sensing and biofilm formation.Compared with synthetic surfactants, biosurfactants have higher surface activity, lower toxicity, higher biodegradability and better environmental compatibility (Hamzah , et al.,2013).

This article emphasizes in the heavy metal resistance & the production of biosurfactants from *Streptomyces*spp. phylogenetic analyses in which 16s rDNA sequencing are used may need to be included in Streptomycetes classification systems, as demonstrated in this study.

So to determine the level of antibiotic resistance patterns and distribution of heavy metal resistance of *Streptomyces* spp. isolated from hydrocarbon contaminated soil , and to confirm if there is a relationship between antibiotic and heavy metal resistance.

Materials and Methods

Bacterial isolates & identification

Twenty sample were collected from uncontaminated &contaminated soil with organics (petroleum hydrocarbon,oil , diesel). Soil samples were serially diluted and 1ml from 10^{-3}dilution sample was plated on Actinomycetes agar by pour plate technique. The plates were incubated at 28°C for 7-10 days. Seven isolates from 21 isolates were collected and identified by morphological characters using slid culture technique. The ability to growth in deferent concentration of (1,3,5,7%) NaCl. Sensitivity to antibiotic using modified Kirby-Bauer method in Mueller-Hinton agar. Sensitivity to mineral salts using well diffusing method, and the ability to produced antibiotics also cared out.

Biosurfactant production by*Streptomyces* spp.

1- Hemolytic Activity: All the strains were streaked on blood agar plate and incubated for 7 days at 28°C.The plates were visually observed for the zone of clearness around the colony. The concentration of biosurfactant is depends on the diameter of the clear zone(Karthik, et al .,2010).
2- Lipase production: Lipase production by the actinobacteria was determined usingTributyrin agar plates, incubated at 28°C for 7 days, and examined for clear zone around the colonies(Karthik, et al.,2010).

Seven isolates givepositive results for both hemolysis and lipase were culture for 5 days inmaltose yeast extract broth (Maniyar , et al., 2011) the culture medium was centrifuged at 3000 rpm at 4°C for 30min. The supernatant was collected and used for screening for biosurfactant present using a drop-collapse test, oil spreading technique:

3-Drop Collapsing Test: A modified drop collapse method was carried out using microscope slidescoated with crude oil. 10 µl of the sample tested were placed on the slides.Biosurfactant productionwas considered positive when the drop diameter was larger than those produced by distilled water and also by culture medium as negative controls(Čipinytė, et al., 2011).

4-Oil Spreading Method : 50ml of distilled water was added to the large petriplate followed by 20 µl of crude oil on the surface of the water. Ten microliters of culture were then added to the surface of oil. The diameter of the lysis was measured compared with unculture media. (Thampayak,et al., 2008,Hamzah, et al ,2013).

Heavy Metal Resistance:

Screening for heavy metal resistance wascarried out using standard heavy metal salt solutions of Nickelsulphate, Zinc sulphate, Mercuric sulphate,Mercuric chloride, Lead acetate, Lead nitrate, Silver acetate, Silverchloride, Titanium dioxide, Cobaltnitrate and Cobalt chloride. The concentration of thestandard heavy metal solutions was 1000µg/ml. The salt solutions were prepared with phosphatebuffer saline, PBS (pH 6.8). The standard and saltsolutions were sterilized separately for 15min at 120°C.

Agar Diffusion Method:Lawn culture of the isolates, grown for 7days in broth was prepared on Mueller Hinton agar. Using a sterile well borer wells were made on the surface of the media seeded with the *Streptomyces*spp. To each well 100µl of eachsalt solution were added and incubated at 28°C for 7 days. The area of inhibition (mm) was measured. An inhibition zone of 10mm on the agar surface was considered as resistant to metal salt solution (Lakshmipathy, et al.,2010).

Antimicrobial Susceptibility Testing:

Antimicrobial susceptibility testing was carried out by Kirby-Bauerdisk diffusion method following National Committee for Clinical Laboratory Standards (NCCLS,2011). As recommended by the NCCLS, Mueller-Hinton agar were used as the culture medium. The antimicrobial agent disks used in this study were erythromycin10 µg, ciprofloxacin10 µg,ceftriaxone 30 µg, gentamicin10µg, cephalothin 30µg,methicillin5µg, tetracycline 30 µg, chloramphenicol 30 µg, trimethoprim and sulfaminoxazole 25µg, vancomycin 30 µg, amikacin 10 µg, imipenem 10 µg, tobramycin 10 µg, norfloxacin 10 µg, ampicillin 10 µg, penicillin 10 IU. The zone diameters around all disks were interpreted by using the recommendations of the NCCLS.

DNA preparation and PCR:

A PCR reaction with specific primers as in the Table 1 were performed to identified genotypes of each 7 isolates. DNA template was prepared using colony PCR by tack one milliliter of bacterial culture was centrifuged at 3,000 rpm for 2 min, poured, and resuspended in 1 ml of distilled water. One hundred microliters of resuspended cells was heat lysed at 95°C for 15 min in a eppendorf PCR (Germany) thermo cycler&centrifuged at 13.000 rpm for 15 min.

The PCR mixture (total volume, 50 µl): Green master mix 25 µl, forward & reverse universal primers (10 pmol)1.5 µl for each one (promega). Primersnucleotide sequences as in the Table 1, DNA template 8µl (50ng/µl),nuclease free water 14 µl in eppendorf tube.

DNA amplification was carried out in a eppendorf PCR (Germany) by using the following conditions:

1 cycle 94 5 min
35 cycle 94 35 sec
58 1.35 min
72 1.35 min
1 cycle 72 10 min

A 5 µl of the PCR product was electrophoresis in 2% agarose using 50 volt at 75 min., the 1350 bp band position of 16s rDNA were compared with 100bp ladder from promega. Modified from (Bodour, et al.,2003).

DNA sequence analysis:

Sequencing of 16 s rDNA gene in 7 strains was performed by Macro gen company, USA. Homology search was conducted using Basic Local Alignment Search Tool (BLAST) program which is available at the National Center Biotechnology Information (NCBI) online.

Construction of the phylogenetic tree

The phylogenetic analysis of nucleotide composition of each aligned sequence were conductedin Mega 0.5 by Clustal Wusing UPGMA methodwith Maximum Likelihood method and the distance coefficient with neighbor joining (NJ), based on the Tamuraet al 2004 model.

Results andDiscussion:

Seven isolates from twenty one isolates were have the ability to hydrolyze both blood and lipid were selected for production of biosurfactant by drop collapsing and oil spreading methods as in the Table 2: it indicate that not all the strain that hydrolyze the blood and lipid give positive results to drop collapse that depending on the quantity of biosurfactant as well as oil spreading method this depending on culture condition, although the type and amount of the microbial surfactants produced depend primarily on the producer organism, factors like carbon and nitrogen, trace elements, temperature, and aeration also affect their production by the organism (Karanth, etal., 2005) . These

results suggested that the oil-spreading technique is more sensitive and detecting low levels of biosurfactant production than the other methods for biosurfactant detection in the supernatant from a culture medium (Hamzah ,et al.,2013).

Table 1: Nucleotide sequences of PCR primers

Primer	Sequence 5-3	Amplicon 16s rDNA size (bp)	Reference
27f **1392r**	**5' AGAGTTTGATCCTGGCTCAG 3'** **5' GACGGGCGGTGTGTAC 3'**	1350	(Lane, 1991)

Table2:The ability of *Streptomyces* spp. to produce biosurfactant

methods **strains**	**Blood hydrolysis**	**Lipid hydrolysis**	**Drop collapse**	**Oil spreading**
(1)	+	+	_	_
(2)	+	+	+	++
(3)	+	+	_	++
(4)	+	+	_	++
(5)	+	+	_	++
(6)	+	+	_	+
(7)	+	+	+	++

Seven isolates that have the ability to produced biosurfactant, characterized by formation of substrate and aerial mycelium using slide culture technique, production of earthy odor and pigments, that is belongs to Streptomyces as in the Table 3 .

Table 3: Identification characteristics of *Streptomyces*spp.

Characters	Colony colors in Mueller hinton agar		Soluble Pigments	Shape of Aerial mycelium	Blood hydrolysis		Colonycolors in actinomycetes agar		Growth in NaCl % & production of Soluble Pigments			
	substrate mycelium	Aerial mycelium			24 h	72 h	substrate mycelium	Aerial mycelium	1	3	5	7
(1)	orang	gray	brown	Rectalflex	+	+	Light yellow	Light gray	+	+ Orang to brown	+ Light pink	_
(2)	brown	Gray &white	-----	branched open spiral lop	+	+	Light yellow	Light gray& white	+	+	+	+

(3)	brown	gray	------	branched open spiral lop	+	+	yellow	Light gray& white	+	+	+	+
(4)	Light brown	gray	------	branched condense spiral lop	_	+	Light yellow	Light gray& white	+	+	+	_
(5)	Light brown	Light blue	brown	branched condense spiral lop	_	+	Light yellow	white	+	+ Light brown	+ Light brown (w)	+
(6)	Light brown	Gray & white	------	Pranged condense spiral lop	_	+	Light yellow	Light gray& white	+	+	+	+
(7)	orang	Light gray &white	brown	Rectalflex	+	+	Light yellow	Gray	+	+ Orang to brown	+ Light pink	_

- **All produces earthy odor, (w) weak**

The isolates show resistant to penicillin 10 IU, methicillin 5 µg, erythromycin 10 µg, ceftriaxone 30 µg, tetracycline 30 µg, ampicillin 10 µg, cephalothin 30µg, trimethoprim and sulfaminoxazole 25 µg. most of them resistant to erythromycin & tetracycline but this antibiotics inhabit the formation of aerial mycelium of the strain number 1&7 as in the Table 4 .

Table 4: Susceptibility of *Streptomyces* spp. toantibiotics

Strains	**penicillin**	**chloramphenicol**	**methicillin**	**erythromycin**	**ceftriaxone**	**tetracycline**	**ampicillin**	**cephalothin**	**trimethoprim and sulfaminoxazole**	**amikacin**	**vancomycin**	**norfloxacin**	**ciprofloxacin**	**tobramycin**	**gentamicin**	**imipenem**
1	0	1-0	0	0*	0	0*	0	0	0	1	1	1-0	1	1	1	1
2	0	0	0	0	0	0	0	0	0	1	1	1-0	1	1	1	1
3	0	0	0	0	0	0	0	0	0	1	1	0	1	1	1	1
4	0	1-0	0	1	0	1	0	0	0	1	1	0	1	1	1	1
5	0	1	0	0	0	1	0	0	0	1	1	1	1	1	1	1
6	0	1-0	0	0	0	0	0	0	0	1	1	1	1	1	1	1
7	0	1	0	0*	0	0*	0	0	0	1	1	0	1	1	1	1

0= resistance 1= sensitive 1-0= moderate sensitive *inhabit formation of aerial mycelium

With deference to heavy metal-containing soils, resistance is a requirement for survival and contribution to biogeochemical processes. All the isolates show resistant to Nickel sulphate , Zinc sulphate , Lead acetate, Lead nitrate, Silver acetate, Titanium dioxide , Cobalt nitrate and Cobalt chloride, some of them sensitive to Silver chloride and all of them sensitive to Mercuric sulphate & Mercuric chloride as in the Figure 1. In the study of (Lakshmipathy, et al.,2010) the isolates belong to *Streptomyces* spp. Show resistant to cadmium and lead. Will Cobalt nitrate and Cobalt chloride increase the production of melanin pigments as in the Figure 2.

Figure 1:Heavy metal resistantof seven strains belong to *Streptomyces* spp.

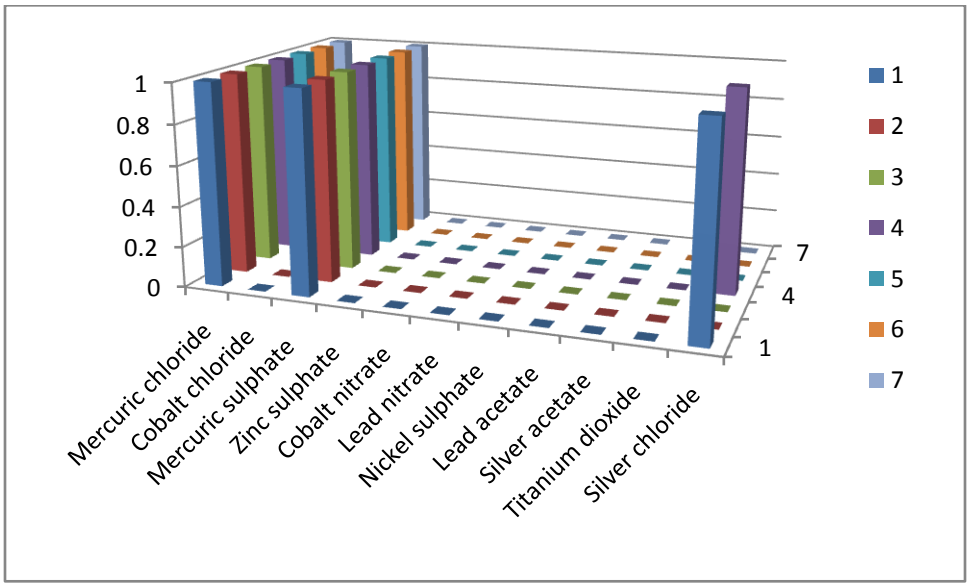
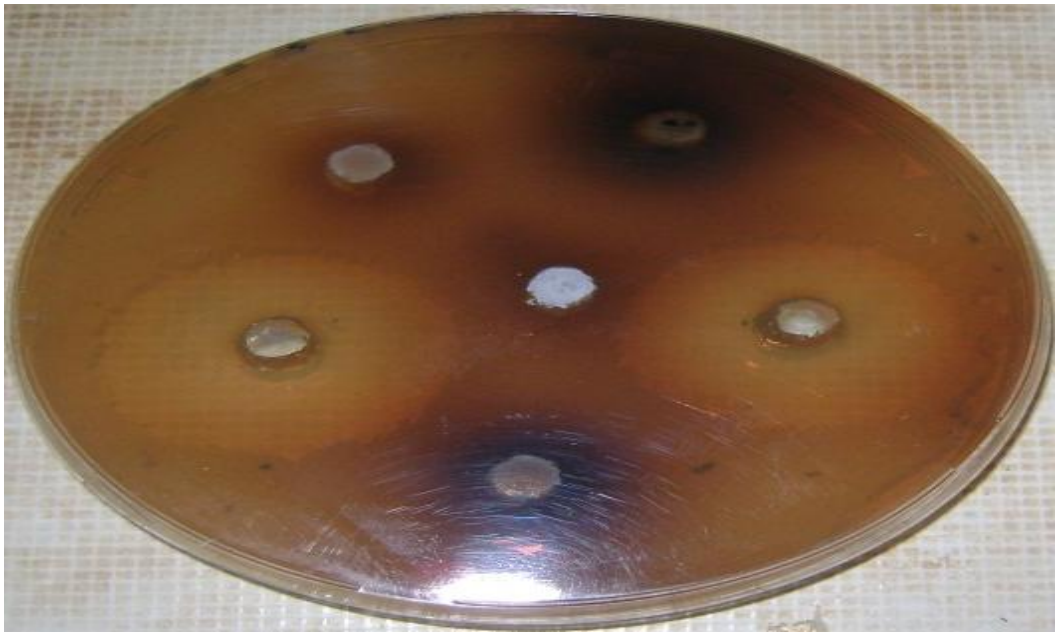
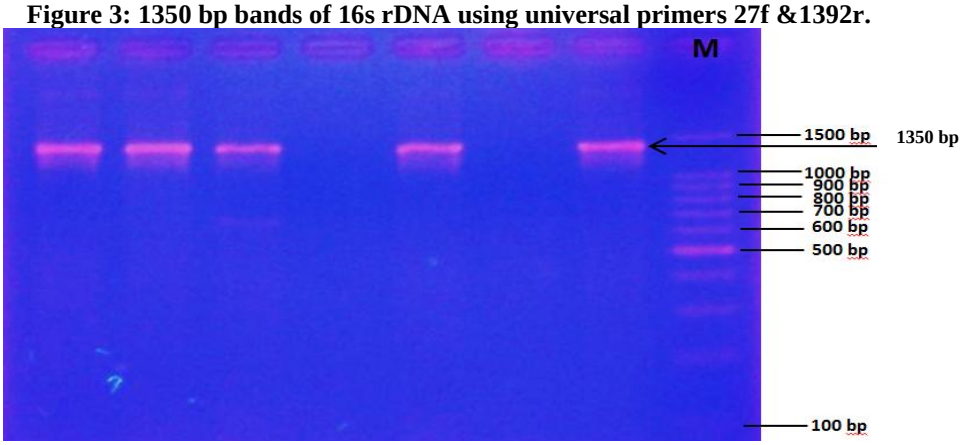


Figure 2 : Sensitivity of strain number 5 to Mercuric sulphate & Mercuric chloride and increasing of melanin pigments production byCobalt nitrate and Cobalt chloride



Phylogenetic analysis.
The PCR products bands of 16s rDNA were 1350 bp compare with the 100bp ladder as in the Figure 3

Figure 3: 1350 bp bands of 16s rDNA using universal primers 27f &1392r.



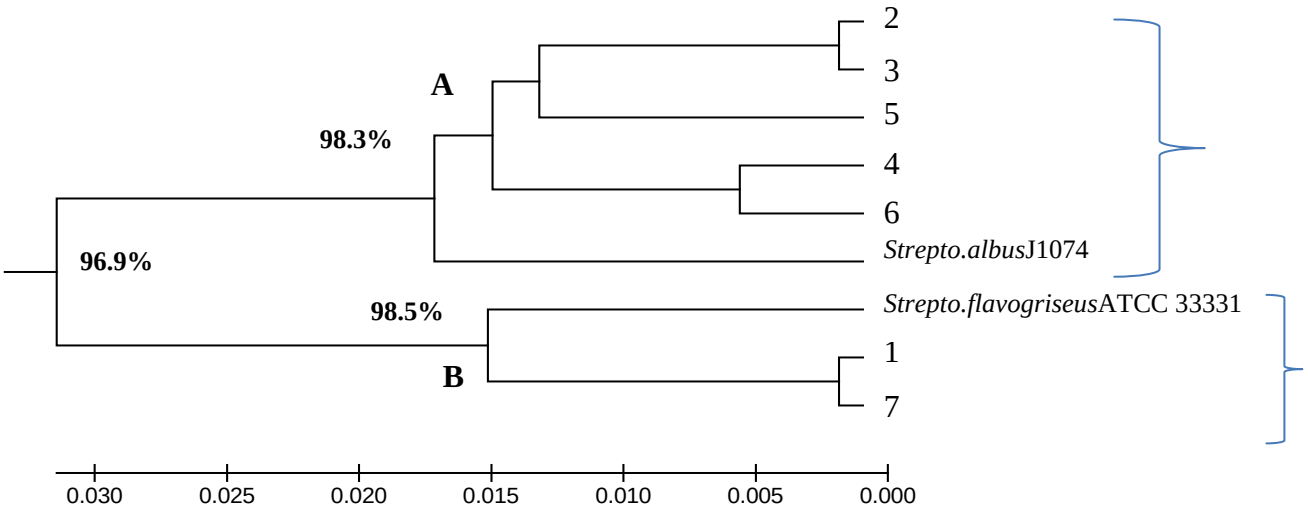
The 16S rDNA gene sequence obtained was analyzed by an NCBI Blast search which clearly showed that the organism belongs to the genus Streptomyces and that the closest matches to sequences from dependably described species *Sterpto.albus*J1074and*Strepto.flavogriseus* ATCC 33331as in the Table5.

Table 5:The sequencing matches of strains under the study

strains	Scientific name	Percentage of similarity%	Numberof 16s rDNA gene bp
1	*Strepto.flavogriseus* ATCC 33331	95	1249
2	*Strepto.albus* J1074	96	1255
3	*Strepto.albus*J1074	98	1313
4	*Strepto.albus*J1074	98	1296
5	*Strepto.albus*J1074	98	1295
6	*Strepto.albus*J1074	97	1204
7	*Strepto.flavogriseus*ATCC 33331	96	1172

The genetic distances between sequences were estimated by using MEGA 0.5 . A phylogenetic tree was constructed using the UPGMA method as in the Figure 4.,The strains grouped into two major clusters A&B. Within each of clusters A and B strains shared identical 16S rDNA gene sequences compared with the sequencing of the species *Strepto.albus*J1074&*Strepto.flavogriseus*ATCC 33331 respectively taken from NCBI.

Figure 5: phylogenetic tree of *Streptomyces* spp. depending on 16s rDNA by Clustal W using UPGMA method .The evolutionary distances were computed using the Maximum Likelihood method based on the (Tamura, et al 2004) model distance coefficient with neighbor joining (NJ), Nucleotide composition of each aligned sequence was carried out using Mega 0.5. the cluster A have 5 strains(2, 3,4,5,6,) belong to *Strepto.albus* J1074& the cluster B have 2 strains (1,7) belong to*Strepto.flavogriseus* ATCC 33331

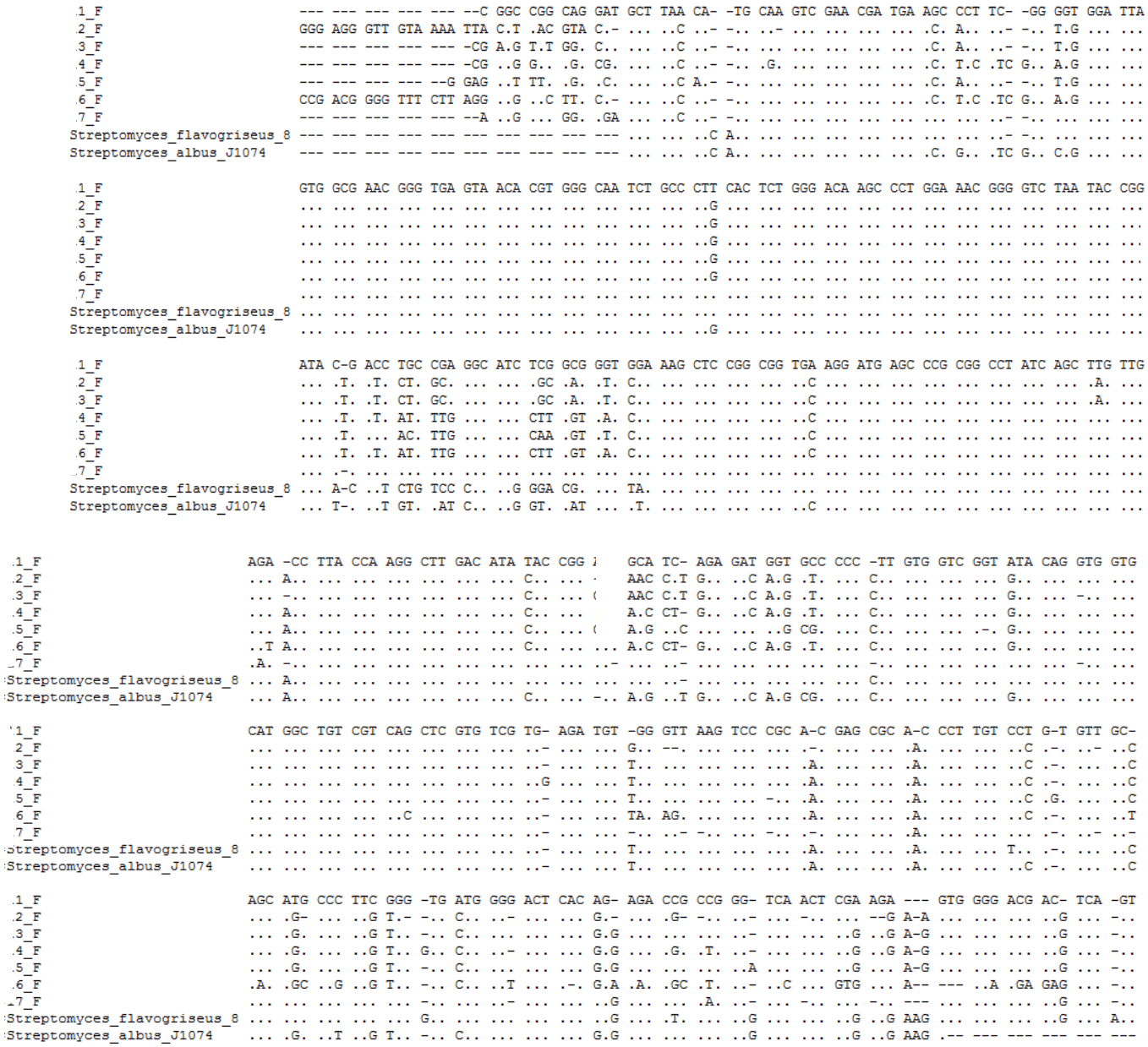


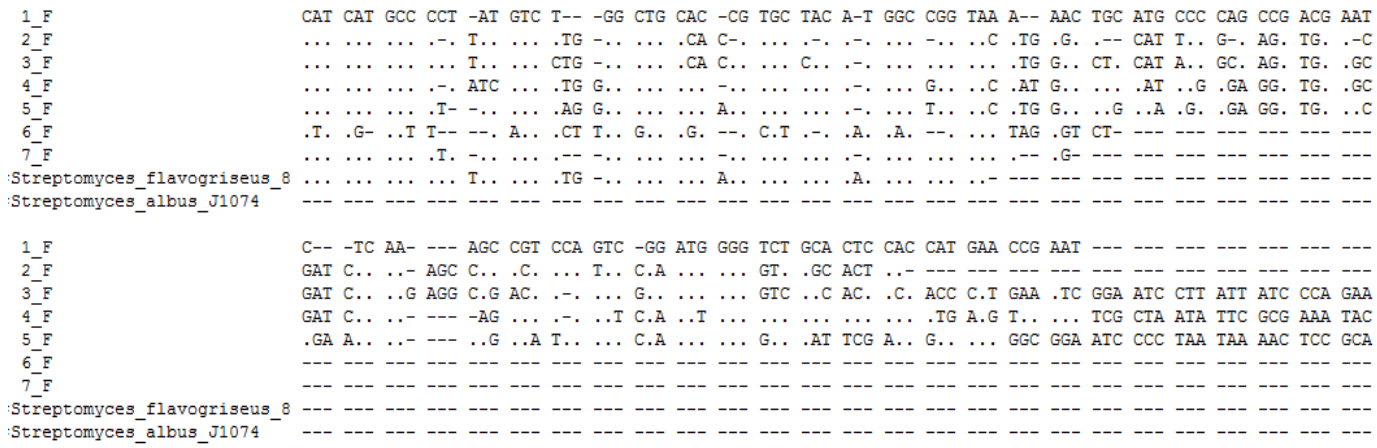
The strains 2,3,4,5,6, in cluster A which belongto *Strepto.albus*grouped in 98.3%, that have branched open spiral lop, growth in the presence of 7% NaCI.The two strains 1&7 in cluster B that correlated in 98.5% belongs to the species *Strepto.flavogriseus*. This species is characterized by rectalflex grey spores, with melanin production, no growth in the presence of 7% NaCI, resistant to erythromycin & tetracycline but inhabit the formation of aerial mycelium only.The cluster A and B correlated in 96.9%these results suggest that strains of each cluster phylogenetically related and confirmed the lack of close relationships among the *Streptomyces*spp.

All the strains have the ability to produce biosurfactant and resistance to same antibiotics and heavy metals in spite it is belong to deferent species. This important features made it so important to use it in the remediation of organic or inorganic compounds, such as hydrocarbons orheavy metals, in polluted area.

The sequence alignment of 16s rDNAs of the 7 strains using MEGA O.5 shows that the*Streptomyces*spp. Have a highly variable region between the 7 strains under the study in the position 30-48 bp&159-252bp&938-1300 bp.this regions may be valuable for rapid identification of *Streptomyces*spp.as in the Figure 5:

Figure 5: Variable regionsof multiple sequence alignments (partial presentation).sequence alignments of the four strains belongs to *Strepto.albus* with two strain of *Strepto.flavogriseus*were compared by Clustal W sequence alignment in MEGA 0.5.The identical regionshow indots and the insertion- deletions (indels) are indicated by dashes. The most highly variable regions in the alignments sequence of the 7 strains of *Streptomyces* spp.in position 30-48bp &159-252bp& 938-1300bp





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