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Research article / Araştırma makalesi

Biodiversity of Streptomyces of from Soil Samples of Halapja, Iraq

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Abstract: The aim of this study is to isolate actinomycetes, particularly Streptomyces, species from soil samples were collected from Halabja at Iraq. For this aim, total 30 soil samples were collected from the different part of Halabja and physicochemical parameters of them were measured after brought the laboratory. First of all, a conventional isolation method used to isolate Streptomyces. Ten-fold dilution of soil samples were inoculated onto strach casein agar plates supplemented cycloheximide, nystatin and novobiocin and raffinose-histidine agar plates supplemented with cycloheximide and nystatin and 105 colonies having substrate and aerial mycelium was selected and purified. All isolates presumably were colour grouped according to aerial spore mass, colony reverse and diffusible pigment colours formed on oatmeal agar and on their capacity to produce melanin pigments on peptone-yeast extraction agar and isolates were assigned to 10 colour group.20 representatives strains of coluor groups were tested for 40 diagnostic identification test and then the results were analyzed by computer-assisted program generating a dendrogram. These 20 test strains were assigned 10 clusters and the cluster of dendrogram showed to match those obtained by manual colour-grouping of the isolates more or less. Also, the diaminopimelic amino acid (DAP) content of the cell wall of 4 test strains was determined and the sugars of the whole cell hydrolysates were analyzed. 3 test strains had LL-DAP (diaminopimelic) acid as the diagnostic cell wall diamino acid for Streptomyces genus, and glucose, ribose and mannose were detected in the whole cell hydrolysates while one of test strains contained meso-A2pm DAP as diagnostic for Nocardiosis species, and galactose and ribose in the whole cell hydrolysates. The results of chemotaxonomic studies supported the phenotypic analysis findings. Streptomyces colonies number were too low in general and there was no growth of Streptomyces colonies in 18 soil samples. These results may be owing to chemical bombing of Halapja in 1988.

Halepçe (Irak) Toprak Numunelerinden *Streptomyces* Bakterilerinin Biyoçeşitliliği

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Öz: Bu çalışmanın amacı özelde Streptomyces ve genelde actinomycetes bakterilerini Irak Halabja'sinde toplanan topraklardan izole etmektir. Bu amaçla, Halepçenin farklı 30 bölgesinde toprak numuneleri toplanarak laboratuvara getirildi ve fizikokimyasal paramatreleri ölçüldü. İlk olarak klasık izolasyon yöntemi kullanıldı. Toprak numunelerinden hazırlanan dilüsyonlar içerisine cycloheximide, nystatine ve novobiocin eklenmiş nişasta-kazein agar ve raffinoz-histidine agar petri kutularına ekim yapıldı ve

Anahtar Kelimeler
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Halabja,
Actinobacteria

toplamda substrat ve havasal miselyomlara sahip 105 koloni seçildi ve saflaştırıldı. Bütün izolatlar oatmeal agar besi ortamında havasal spor, substrat miselyum renkleri ve diffüziye pigment renkleri ve ayrıca pepton-yeast agar besi ortamında melanin üretimi dikkate alınarak renk gruplandırılması yapıldı ve sonuçta 105 izolat 10 renk grubuna ayrıldı. Renk gruplarını temsilen seçilen 20 izolat 40 diagnostik teşhis testleri yapıldı ve sonrasında bilgisayara dayalı programla sonuçlar analiz edildi ve dendrogram oluşturuldu. Bu 20 test organizması 10 gruba ayrıldı ve sonuçlar renk gruplandırması ile yaklaşık benzerlik gösterdi. Teşhisi yapılan bu 20 test suşu 10 kümeye ayrıldı ve bu kümeler renk gruplandırması sonucunda elde edilenlerle hemen hemen aynı suşları içerdi. 4 test suşunun hücre duvar yapısındaki hem diamino pimelik asit çeşidi hem de içerdiği şeker tipi tayin edildi. Bu suşlardan 3'ü Streptomyces bakterileri için belirleyici olan LL-diaminopimelik asit (DAP) ve yine tüm hücre hidrlizatlarında belirleyici olan şeker tiplerini (riboz, glukoz ve mannoz) içerdiği belirlendi. Bir suş ise Nocardiopsis bakterileri için belirleyici olan meso-A2pm DAP ve tüm hücre hidrolizatlarında galaktoz ve riboz şeker tipini içerdiği belirlendi. Kemotaksonomik çalışma sonuçları fenotipik analiz sonuçlarını destekledi. Streptomyces koloni sayısı genelde düşük seviyede olduğu belirlendi ve 30 toprak numunesinden 18'inde Streptomyces kolonisi gözetlenmedi. Bunun muhtemel nedeni 1988'de Halepçe'nin Kimyasal bombalarla bombalanması olabilir.

1. Introduction

Actinobacteria have important ecological roles plus their important influence on human health. Latest studies reported that *Actinobacteria* make contribution as widespread symbionts of eukaryotes, helping herbivores gain access to plant biomass as nutritional mutualists and producing natural products as defensive mutualists. Members of *Streptomyces* genus are known for their large genomes and capacity for producing a vast array of secondary metabolites. Additionally, several studies have indicated that the distribution of *Streptomyces* spp. is influenced by soil properties (Atalan, 1995, 1997, Atalan *et al.,* 2000; Schlatter *et al.,* 2008; Asamizu *et al.,* 2015). They produce many important drugs, including most antibiotics that have contribution to human health (Lewin *et al.,* 2016). Halabja was exposed to mustard gas at 1988 and making it an ideal location to assess differences in soil *Streptomyces* community structure across land that may be influenced by the chemical weapons, combination of at least three gases mustard, organic phosphate (sarin, tabun, cyanide or derivatives), etc. The province is located at north of Iraq and are determined based on terrain texture, brown colur of soil, geologic structure and natural history. While many studies have carried out to isolate actinobacteria genera in soil on the World. No studies of this sort have been performed specifically on communities of *Streptomyces* in soil of Halabja or around cities. This study aimed to isolate *Streptomyces* communities within soil from different land soils of province of Halabja. For this aim, we isolated *Streptomyces* strains and examined the distribution of cultivable *Streptomyces* using selective media. Also, these isolates were subject to identification and characterization methods such as colour grouping, numerical analysis and chemotaxonomic techniques those were DAP analysis and sugar analysis of cell-wall content. The conclusions provide an aspect of abundance of some *Streptomyces* or other genera of actinobacteria. The diversity of *Streptomyces* communities in this environment may be explored and scan for bioactive compounds.

2. Material and Methods

2.1. Soils and isolation

Halabja is a city in Iraqi Kurdistan and the capital of Halabja governorate that located about 240 km (150 mi) at north-east of Baghdad and 14 km from the Iranian border. The total area of Halabja is 1,599 km^2 and the elevation of Halabja 721 m asl (2.365 fit). Thirty soil samples were collected from three different places of province such as Eneb, Golan, and Biyawela. These locations were contaminated by mustard chemical gas in 1988.

Physicochemical characters of soil samples (pH, moisture and organic matter content) were measured as soon as samples brought to laboratory (Reed and Cummings, 1945). Starch casein agar (Küster and Williams 1964) and Raffinose Histidine agar plates (Vickers *et al.*, 1984) were used for isolation of *Streptomyces* and total actinomycetes. Both selective media were supplemented with cycloheximide and nystatine to inhibit fungi and unwanted growth of other bacteria.

The colonies of an isolation plates were examined by both eye and binocular microscope after 14 days incubation. The desired colonies were distinguished from other bacteria on the basis of colony morphology, pigmentation and ability to produce different color of aerial hyphae, and substrate mycelium on selective plates. Representatives of actinomycetes were taken from selective isolation medium were transferred and streaked on to modified Bennett's agar (Jones 1949) in order to get pure colonies. Isolates were coded according to locality and stored in sterile 2 ml Eppendorf tube containing 20% glycerol and were stored again at -80 ºC after use.

Total 105 isolates of *Streptomyces* were inoculated on Oatmeal agar (Küster 1959) and Peptone-iron agar plates (Shirling and Gottlieb, 1966) and incubated at 28 °C for 2 weeks. After incubation, colonies growing on plates were observed by eye to detect spore color of aerial hyphae, substrate mycelium color and pigmentation of the diffusible pigments. Colors were determined by direct matching of the strains examined against color charts reference tables from the ISSC-NBS Color-Name charts Illustrated with Centroid color. The peptone-iron agar (ISP6) was checked to detect ability of test strain to produce dark-colored melanin pigmentation. Total 20 test microorganisms (Table 1) selected as representative of color groups were examined for 40 test including nutritional test, biochemical, degradation, temperature susceptibility chemical inhibitor, antibiosis, antimicrobial activity and growth tests (Wiliams *et al.*, 1983a, b; Table 2).

Table 1. Test microorganisms, type of isolation medium and source of test strains

Strains	Source	Isolation medium
S0035	Halabja / Biyawela	Raffinose histidine agar
S0155	Halabja / Eneb	Starch casein agar
S0107	Halabja / Golan	Starch casein agar
S0100	Halabja / Biyawela	Starch casein agar
S0029	Halabja / Eneb	Raffinose histidine agar
S0037	Halabja / Eneb	Raffinose histidine agar
S0139	Halabja / Golan	Starch casein agar
S0017	Halabja / Biyawela	Raffinose histidine agar
S0016	Halabja / Eneb	Raffinose histidine agar
S0043	Halabja / Biyawela	Raffinose histidine agar
S0002	Halabja / Golan	Raffinose histidine agar
S0055	Halabja / Golan	Raffinose histidine agar
S0010	Halabja / Biyawela	Raffinose histidine agar
S0143	Halabja / Biyawela	Starch casein agar
S0014	Halabja / Biyawela	Raffinose histidine agar
S0154	Halabja / Golan	Starch casein agar
S0115	Halabja / Eneb	Starch casein agar
S0131	Halabja / Eneb	Starch casein agar
S0072	Halabja / Eneb	Raffinose histidine agar
S0133	Halabja / Biyawela	Raffinose histidine agar

2.2. Identification of test organisms

Most of test were scored as two state characters and coded "–" for negative, and "+" for positive result. 20 test strains were examined for 40 diagnostic properties tests (Table 2). Resultant data were typed in TAXON program. Later is a program for data input and analysis of binary data and is run on a computer. After numerical analysis, a dendrogram has been generated by the program and test strains were grouped. S_{SM} (Simple matching coefficients; Sokal and Michener 1958) coefficient used to analyse similarity of organisms. Test strains showing 85 S_{SM} level may be belong to same species.

Table 2. List of diagnostic tests used for identification of isolates

No	Tests	No	Tests
1.	Dextran	2.	H2S production
3.	Fructose	4.	Nitrate reduction
5.	Lactose	6.	Urea hydrolysis
7.	Mannose	8.	Phenol 0.1%
9.	Raffinose	10.	Sodium azide 0.01%
11.	Sucrose	12.	Sodium chloride 7%
13.	Maltose	14.	Crystal violet 0.0001%
15.	Mannitol	16.	Ampicillin (20 mg)
17.	Sodium acetate	18.	Vancomycin (30 mg)
19.	Sodium citrate	20.	Penicillin (30 mg)
21.	S. propionate	22.	Gentamycin (10 mg)
23.	Histidine	24.	Rifampin (5 mg)
25.	Potassium nitrate	26.	Erythromycin (15 mg)
27.	Tyrosine	28.	Growth at 50Ċ
29.	Xanthine	30.	*Escherichia coli*
31.	Casein	32.	*Pseudomonas fluorescens*
33.	Starch	34.	*Bacillus subtitous*
35.	Gelatin	36.	*Klebsiella pneumoniae*
37.	Lecithinase activity	38.	*Staphylococcus aureus*
39.	Lipolysis activity	40.	*Candida sp.*

2.3. Chemotaxonomic study

Four isolates strains (S0107, S0017, S0014 and S0133) were inoculated in glucose yeast malt extract broth (ISP6) in shaken flask 180 rpm at 280C for 1 week for chemotaxonomic study. Thin layer chromatography was used to characterized Diaminopimelic acid (DAP) and determined isomers (*LL, meso* and hydroxy) of four isolates (S0107, S0017, S0014 and S0133) using Becker *et al.* (1965) Staneck and Roberts (1974) method.

Total 4 test microorganisms (S0107, S0017, S0014 and S0133; Table 7) were selected as representative cluster of phylogenetic dendrogram for whole cell sugar analysis. The protocol followed to analysis whole sugar of cell wall is given bellow. Then analysis of sugar type for these test strains carried out by thin layer chromatography.

3. Results

3.1. Physiochemical parameter of soil samples

Physiochemical result including amount of organic matter, moisture contents and pH of the soil sample are shown on Table 3. The amount of organic matter from all soil samples ranged from 9.3% to 20.4% and the higher amount of organic matter was recorded in soil sample S09 collected

from Eneb location. The amount of moisture content was ranged from 6% (S14) to 9.2% (S21). The range of pH of all soil sample recorded from 7.4 (S21) to 8.0 (S03 and S04).

3.2. Distribution and numbers of *Streptomyces*

Streptomyces colonies were isolated from petri plates after incubation at 28 °C for 14 days. Total 105 pure strains were isolated by streak plate method. The number of total *Streptomyces* were recorded and counted as a colony forming unit (C.F.U. Table 4.). Photographs of some isolation petri plates are shown on Figure 1. The highest numbers of colony were recorded from soil sample S10 which was 20 X 10^4 cfu. No colony of *Streptomyces* were found on some soil samples those are S03, S05, S06, S08, S11, S12, S13, S15, S17, S21, S22, 23, S25, S26, S27, S28, S29 and S30. Also, *Streptomyces* colony appeared on only either SCA such as S24 or RH agar plates such as S02, S04 and S14.

3.3. Color grouping

In much early, morphology is the only characters were used for description of taxa. The 105 isolates presumptively classified as *Streptomyces* were assigned to 10 color according to the color of aerial hyphae and substrate mycelium and black melanin pigmentation (Table 5). The appearance of representative strains of some isolates growing on oatmeal agar and peptone-iron agar are shown on Figure 2.

3.4. Phenotypic characterization

Identification test scores for each 20 test strains are shown on Table 6. Data analyzed using TAXON program and produced a dendrogram (Figure 4). It can be seen on dendrogram that 20 test strains were assigned 10 cluster based on 80 percent Simple matching coefficient. 3 out of 10 cluster were single membered group while other 7 cluster were contained 2 or more test strains. Traditional identification tests such as biochemical, carbon source, nitrogen source, chemical inhibitor, temperature, antibiotics, morphology, pigmentation, growth tests are used for both identification and numerical analysis.

The ability of 20 test microorganisms were examined for their ability to inhibit the development which are 6 pathogenic microorganisms as gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtilius*), gram negative bacteria (*Escherichia coli*, *Pseudomonas fluorescens* and *Klebsiella pneumoniae*), and fungi (*Candida sp*.). The result was recorded as positive during formation of zone around the test strains and negative when pathogenic microorganisms were grown around the test strains. As a result of observation, strains S0035, S0055, S0014 and S0133 were inhibited growth of all pathogenic microorganisms, but S0143 and S0115 were unable to inhibit growth of any pathogenic microorganisms. Some results are given in Figure 3.

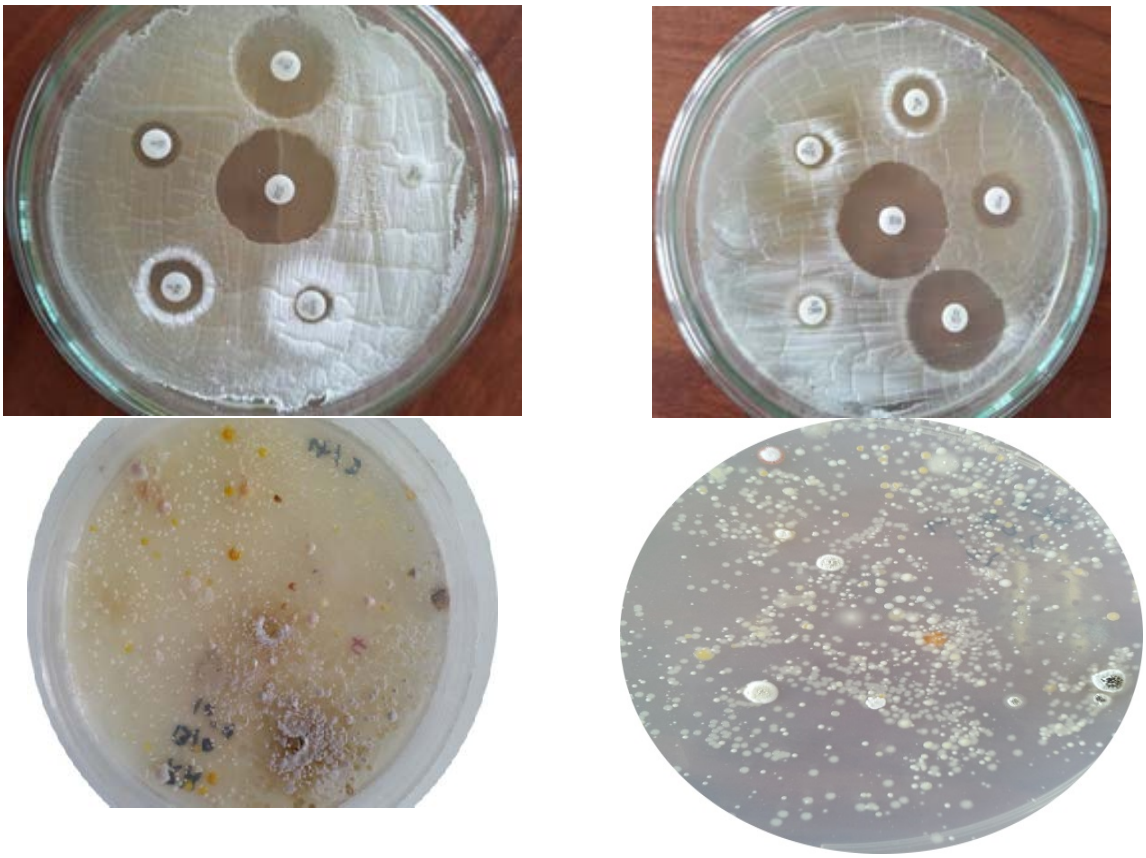
3.5. Chemotaxonomic analysis

Chemotaxonomic analyzes were performed to determine the characteristic chemical properties of cell wall of *Streptomyces* isolates. The spots of DAP type in the cell wall of test strains are seen on Figure 5 which the position of the bands formed in the one-dimensional thin layer chromatography (TLC) compared with the standard A2pm solution. A one-dimensional TLC chromatogram show that 3 test strains contain the *LL*-A2 pm while one strain (S0014) contained *meso*-A2pm which is belong to another genus of Actimycetes, probably *Nocardiopsis* genus.

The whole cell sugar profile in the cell wall chemotype was determined by comparing two standards that contains seven sugars for test microorganisms in one dimensional thin layer chromatography. It is seen on Figure 6 isolates of S0133 and S0017 belonging to the genus *Streptomyces* were found to contain glucose, ribose and mannose. But strain S0107 (*Streptomyces*) contains ribose and mannose and S0014 isolate belong to *Nocardiopsis* caontains galactose and ribose (Figure 6).

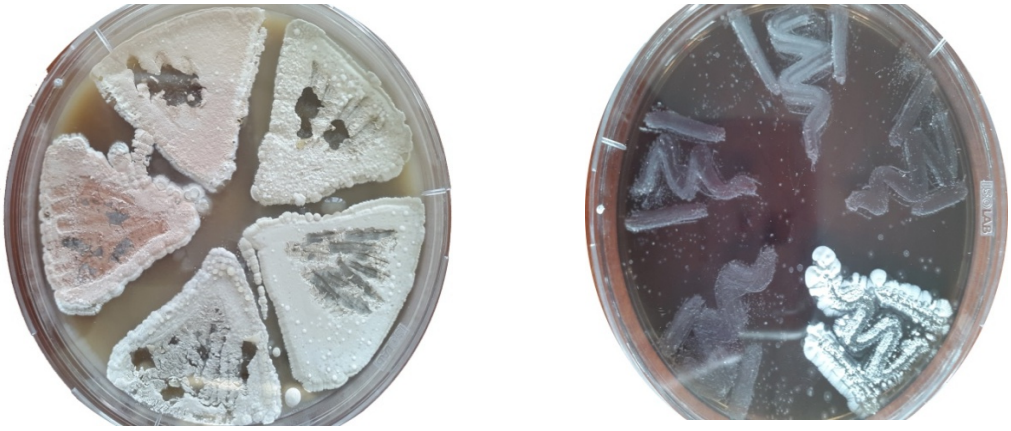
Table 3. Lists of collected of soil sample from Halabja/Iraq and physiochemical character of soils

Soil sample	Place	Location	Date of collection	pH	Moisture content (%)	Organic matter content (%)
S01	Halabja	Eneb	22/8/2015	7.9	8	15.5
S02	Halabja	Eneb	22/8/2015	7.5	7	15.3
S03	Halabja	Eneb	22/8/2015	8	9	13.3
S04	Halabja	Eneb	22/8/2015	8	7	13.3
S05	Halabja	Eneb	22/8/2015	7.8	8	12.2
S06	Halabja	Eneb	22/8/2015	7.7	9	18.4
S07	Halabja	Eneb	22/8/2015	7.9	9	15.2
S08	Halabja	Eneb	22/8/2015	8	7	9.3
S09	Halabja	Eneb	22/8/2015	7.8	9	18.6
S10	Halabja	Eneb	22/8/2015	7.6	8	14.7
S11	Halabja	Golan	22/8/2015	7.8	8	16.5
S12	Halabja	Golan	22/8/2015	7.9	7	13.4
S13	Halabja	Golan	22/8/2015	7.8	7	12.4
S14	Halabja	Golan	22/8/2015	7.7	9.2	15.6
S15	Halabja	Golan	22/8/2015	7.9	7	12.4
S16	Halabja	Golan	22/8/2015	7.7	8	10.3
S17	Halabja	Golan	22/8/2015	8	8	13.5
S18	Halabja	Golan	22/8/2015	7.7	9	12.4
S19	Halabja	Golan	22/8/2015	7.7	7	14.6
S20	Halabja	Golan	22/8/2015	7.6	9	10.3
S21	Halabja	Biyawela	22/8/2015	7.4	6	17.5
S22	Halabja	Biyawela	22/8/2015	7.7	7	15.5
S23	Halabja	Biyawela	22/8/2015	7.6	9	14.4
S24	Halabja	Biyawela	22/8/2015	7.7	8	12.5
S25	Halabja	Biyawela	22/8/2015	7.7	7	12.4
S26	Halabja	Biyawela	22/8/2015	7.6	8	11.3
S27	Halabja	Biyawela	22/8/2015	7.9	9	14.4
S28	Halabja	Biyawela	22/8/2015	7.6	9	16.5
S29	Halabja	Biyawela	22/8/2015	7.7	9	20.4
S30	Halabja	Biyawela	22/8/2015	7.8	8	19.4



S0133 (Biyawela) on RHA S0115 (Eneb) on SCA

Figure 1. Colony appearance of *Streptomyces* on plates of starch-casein agar & raffinose histidine agar.



Oatmeal agar (SO139 Peptone-irone agar (SOO55, S0010, S0037)

Figure 2. Representatives strains of *Streptomyces* growing on oatmeal agar and peptone iron agar plates at 25 °C after 10 days growth.

Bennets agar Bennets agar

Figure 3. Antibiotic resistance tests.

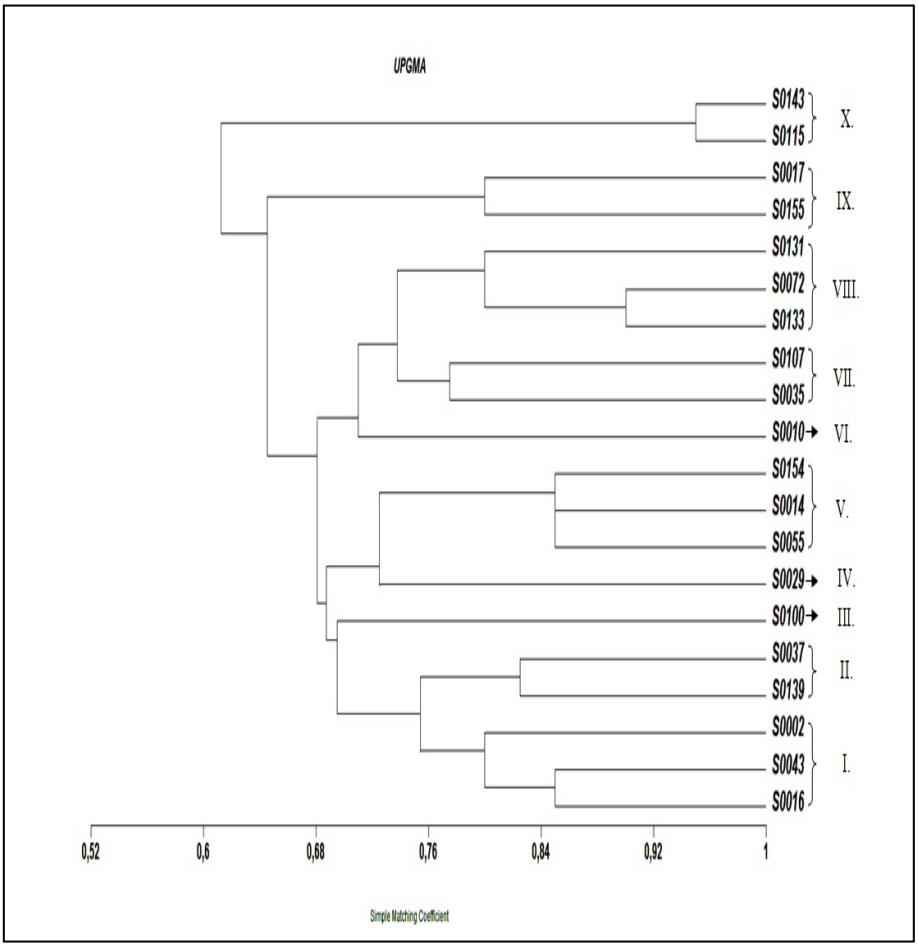


Figure 4. Dendrogram showing relationships between representatives of *Streptomyces* groups analyzed data using S_{SM} (Simple matching coefficient) UPGMA algorithm.

Table 4. Total number of *Streptomyces* (c f u/g/dry weight soil) growing on starch casein agar supplemented with nystatin (50 µg/ml), Cycloheximide (50 µg/ml) and novobiocin (25 µg/ml) and raffinose histidine agar supplemented with Cycloheximide (50 µg/ml) and nystatin (50 µg/ml) seeded with soil suspension and incubated for 14 days at 28 °C

Soil sample No	Total Streptomyces on RHA 1g soil sample $X10^4$ CFU	Total Streptomyces on SCA 1g soil sample $X10^4$ CFU	Soil sample No	Total Streptomyces on RHA 1g soil sample $X10^4$ CFU	Total Streptomyces on SCA 1g soil sample $X10^4$ CFU
S01	2.5	6.2	S16	4.8	1.2
S02	2.3	0.0	S17	0.0	0.0
S03	0.0	0.0	S18	2.5	1.1
S04	2.2	0.0	S19	2.8	1.2
S05	0.0	0.0	S20	4.8	6.7
S06	0.0	0.0	S21	0.0	0.0
S07	6.4	8.1	S22	0.0	0.0
S08	0.0	0.0	S23	0.0	0.0
S09	3.7	2.6	S24	0.0	4.3
S10	20	9.8	S25	0.0	0.0
S11	0.0	0.0	S26	0.0	0.0
S12	0.0	0.0	S27	0.0	0.0
S13	0.0	0.0	S28	0.0	0.0
S14	4.5	0.0	S29	0.0	0.0
S15	0.0	0.0	S30	0.0	0.0

Table 5. Colour grouping of isolates microorganisms on oatmeal agar and peptone-iron agar

Group No.	Strain group	Color on oatmeal agar		Melanin pigmentation
		Aerial spor mass	Colony reverse	
1.	**S0010**, S0021, S0053	Grey	Violet	Yes
2.	**S0037**, S0038, S0039, S0041, S0156, S0157, **S0139**	Misty rose	Wheat	Yes
3.	S0052, **S0017,** S0042, S0024, **S0155**	Snow	Coral	Yes
4.	**S0014, S0055**	Beige	Olive	Yes
5.	**S0100**, S0103	Beige	Light brown	No
6.	S0105, S0106, **S0107**, S0112, S0116, S0150, S0026, **S0035**, S0145	Pink	Orange	No
7.	S0063, S0065, S0066, S0067, **S0115,** S0068, S0071, S0108, S0110, **S0143**, S0148, S0152, **S0154**	Olive	Dark olive green	No
8.	S0006, **S0131** S0007, S0008, S0009, S0040, S0069, S0073, S0075, S0078, S0080, S0094, S0096, SO119, S0121, **S0133**, S0122, S0123, S0125, S0137, S0138, S0140, S0142, S0145, S0151, S0153, S0148, **S0072**	Grey	Dark green	No
9.	**S0029**, S0032, S0033, S0034, S0036, S0082	Grey	Saddle brown	No
10.	S0012, S0013, S0015, **S0016**, S0025, **S0043**, S0045, S0058, S0059, S0079, S0093, S0097, S0113, S0118, S0120, S0124, S0129, S0132, S0135, S0136, S0144, S0130, S0001, **S0002**, S0003, S0004, S0005, S0031, S0044, S0061,	Ivory	Olive	No

Note: Bold strains were selected for phenotipic characterization and chemotaxonomic analysis.

Table 6. Data obtained from traditional identification tests for representative of *Streptomyces* selected from isolates

No. of strain Tests	S0035	S0155	S0107	S0100	S0029	S0037	S0139	S0017	S0016	S0043	S0002	S0055	S0010	S0143	S0014	S0154	S0115	S0131	S0072	S0133
Biochemical tests																				
1 H_2S production	+	-	-	+	+	+	+	+	+	-	-	+	-	-	-	+	-	+	-	-
2 Nitrate reduction	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3 Urea hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Nutritional tests																				
Growth on sole carbon source (1%, w/v)																				
4 Dextran	+	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-
5 Fructose	-	-	+	-	+	+	+	-	+	-	-	-	-	-	+	+	+	+	-	-
6 Lactose	+	-	+	+	-	+	+	-	+	+	-	-	-	-	-	-	-	-	-	-
7 Mannose	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	+
8 Raffinose	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-
9 Sucrose	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
10 Maltose	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	+	-	+	-	-
11 Mannitol	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	-	+	-	+
Growth on sole carbon source (0.1%, w/v)																				
12 Sodium acetate	+	-	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-
13 Sodium citrate	+	-	+	-	-	+	+	-	-	-	-	+	+	-	-	-	-	-	-	-
14 Sodium propionate	-	+	-	+	-	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-
Growth on sole nitrogen source (0.1% w/v)																				
15 Histidine	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16 KNO_3	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+
17 Tyrosine	+	+	-	-	+	+	+	+	+	+	+	+	-	-	-	-	-	-	+	+
Degradation tests																				
18 Xanthine	-	+	-	-	-	-	-	+	-	-	+	+	-	-	+	-	-	-	-	-
19 Casein	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	+	-	-	-
20 Starch	-	-	-	+	+	-	-	+	-	-	-	+	-	-	+	+	-	-	-	-
21 Gelatin	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	-	-	-	-
22 Lecithinase activity	+	+	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	+	+	+
23 Lipolysis activity	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	-	+	+	+
Tolerance tests																				
Resistance to chemical inhibitors																				
24 Phenol 0.1%	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25 Sodium azide 0.01%	+	-	+	+	-	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
26 Sodium chloride 7%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27 Crystal violet 0.0001%	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+
Resistance to antibiotics																				
28 Ampicillin (20 mg)	+	+	+	+	+	+	-	+	-	-	-	-	+	-	-	-	-	+	+	+
29 Vancomycin (30 mg)	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	+	+
30 Penicillin (30 mg)	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-	-	+	+	+
31 Gentamycin (10 mg)	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-

Table 6. Continued

32	Rifampin (5 mg)	+	-	+	-	+	-	-	-	+	+	-	+	+	-	+	+	-	+	+	+
33	Erythromycin (15mg)	+	+	+	-	+	+	+	-	+	+	+	-	+	-	-	-	-	+	+	+
Resistance to temperature																					
34	50°C	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
35	30°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
36	25°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Antimicrobial activity tests																					
37	*Escherichia coli*	+	+	+	-	-	-	+	+	-	-	-	+	+	-	+	+	-	-	+	+
38	*Pseudomonas fluorescens*	+	-	-	-	+	-	-	-	-	-	-	+	+	-	+	+	-	-	-	+
39	*Bacillus subtitous*	+	+	-	-	+	+	+	+	-	+	-	+	-	-	+	-	-	+	-	+
40	*Klebsiella pneumoniae*	+	-	+	+	+	-	-	+	-	-	+	+	+	-	+	+	-	+	+	+
41	*Staphylococcus aureus*	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	+	+	+
42	*Candida* sp.	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	+	-	+	+	+

Note: cfu, colony forming unit

Table 7. Analysis of some chemotaxonomic feature of 4 test microorganisms

Isolated strains	Code Number	DAP	Sugar
Streptomyces sp.	S0107	LL-A2pm	glucose, ribose, mannose
Streptomyces sp.	S0017	LL-A2pm	glucose, ribose, mannose
Streptomyces sp.	S0133	LL-A2pm	Ribose, mannose
Nocardiopsis sp.	S0014	mezo-A2pm	Galactose, ribose

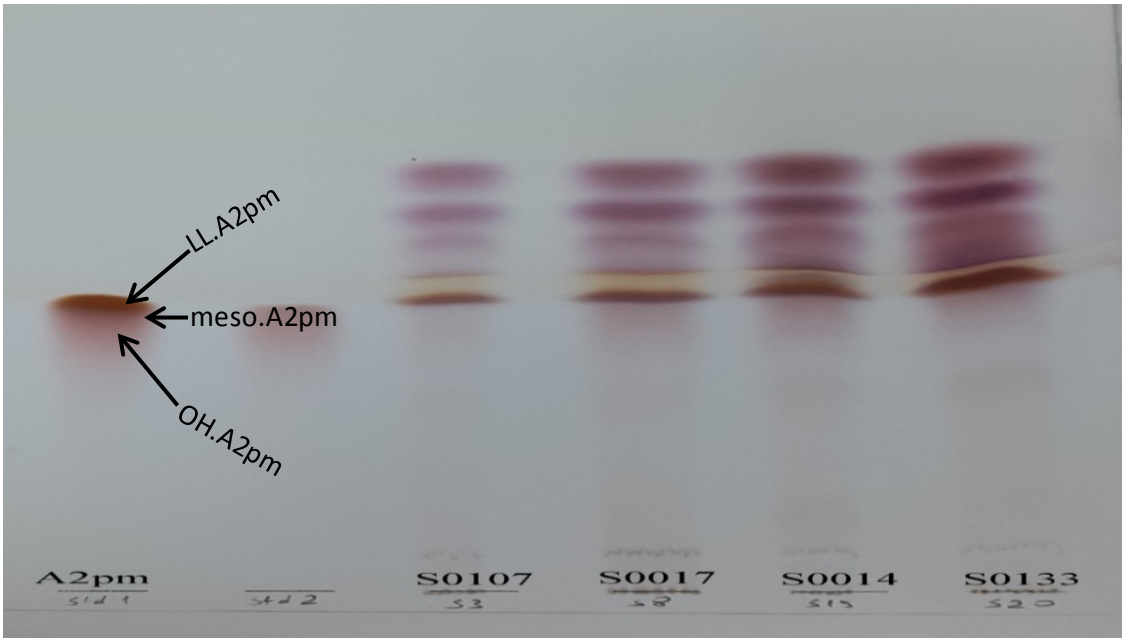


Figure 5. One-dimensional thin film chromatogram of A2pm isomers of 4 isolates. Two Standard: A2pm (Diaminopimelic acid DAP) – Sigma.

Figure 6. One-dimensional thin-layer chromatogram of the sugar profile of 4 test isolates. Std1, standard 1: Gal, galactose; Ara, arabinose and Xyl; xylose. Std 2, standard 2: Glu; glucose; Man, mannose; Rib, ribose and Rham, rhamnnose.

4. Discussion and Conclusion

Halabja is unusual and underexplored habitats exposed chemical bomb 1988 and the city is in North of Iraq. Such places may be a ecosystem that is sources of novel filamentous actinomycetes some of which have a capacity to produce interesting new natural products, notably antibiotics. *Actinobacteria* grow in a wide range of environments and they can grow on naturally occurring substrates (Goodfellow and Fiedler 2010; Mao et al., 2011; Antony-Babu *et al.*, 2010). Especially *Streptomyces* are abundant in most soil samples (Atalan 1993). Organic matter content, moisture content and pH were comparable to other soil samples. Organic matter content was ranged from 9.3 to 20.4% while moisture content of samples was changed between 6.0 and 9.2%. It is surprise low number of *Streptomyces* bacteria were isolated from 30 soil samples and no colony observed from 18 soil samples. Generally, high number of *Streptomyces* bacteria live in worldwide soils. Probably chemical bombing of these land changes the ecology of soils. Classical dilution method was used for isolation of *Streptomyces* using SCA and RHA plates. Total 130 presumptively *Streptomyces* strains were able to isolate from 30 soil samples and the number of bacteria on plates were ranged from 11000 to 84000 cfu per gram. Several reasons can cause low number of bacteria in the samples such as difficulties in achieving a representative sample of microorganisms from heterogenic substrates since high content of organic matter of samples. Secondly, unsuitable isolation technique and lack of universal criteria for microbial species (Goodfellow and O'Donnell 1989). One reason of low number of *Streptomyces* can be chemical bombing of these lands. The highest number of *Streptomyces* were counted 2 X 10^5 cfu/g dry soil on raffinose histidine agar plates from soil sample S10 while 98000 cfu/g dry soil on starch-casein agar plates. Jia *et al.* (2015) isolated low number of *Streptomyces* from soil samples of volcanic sediment collected from Longwan, Jilin province, north China. Reason for low count of bacteria from isolation study may be lack of information on their ecological and geographical distribution or on their activities and interactions in natural habitats and may be related to contamination of the location sample by mustard gas. Both environmental studies and the formulation of objective procedures for the selection of representative *streptomycetes* for pharmacological screening programs is related to basic ecological information on *Streptomyces* species. Okoro *et al.* (2009) reported that many novel streptomycetes with the capacity to produce commercially significant, natural products are present in natural ecosystems, notably in understudied and neglected habitats such as Halabja soil.

It is well known that the assignment of streptomycetes to colour groups is based on their ability to produce diagnostic pigments on oatmeal and peptone-yeast extract-iron agars and it has been

used to gain an insight into the taxonomic richness of these organisms in marine sediments and other soil samples (Atalan *et al.*, 2000; Sembiring *et al.*, 2000). Total 105 isolates of *Streptomyces* were assigned 10 colour group. 20 representatives' stains from colour grouping were selected for further study such as numerical analysis and chemotaxonomic studies. Representatives selected strains are shown on Table 5 are bold characters.

Cluster of numerical analysis were more or less congruence found in the present study between the composition of the manual and computer-assisted numerically defined colour-groups. That means colour grouping approach can be used to generate a colourgroup database that overcomes the limitations of the procedure. Antony-Babu *et al.* (2010) suggested that there was a linear correlation between the similarity percentage values obtained by colour-group and rep-PCR fingerprint analyses. The rep-PCR fingerprint analyses require a PCR while the colour-group protocol can be prepared using basic facilities available in any microbiology laboratory.

The numerical analysis of test strains data also allows the visual display of data as dendrograms and highlights the recognition of taxa based on similar identification characteristics. In our study, 20 test strains were assigned into 10 cluster that 3 cluster one membered while other cluster contained 2 or 3 test strains (see Figure 4). As it has been mentioned above most of test strains in clusters were separated regarding to colour groups. Our results show that the computer-assisted numerical analysis method provides a cheap and reliable alternative to molecular and chemical methods. This technique promise that offers a valid minimal taxon description method, especially when working with large numbers of isolates, and it does not require users to obtain detailed taxonomic information of all their isolates. For ecological study, a cumulative database can be generated and be used to objectively select representative strains. There was a consequence between the colour groups and cluster of numerical analysis. 19 out 20 test strains were assigned in same group of both colour group and cluster generated by numerical analysis except S16 (S0154). Manfio *et. al.* (2003) used probability matrix to identify unknown *Streptomyces* colonies. Also, Ertaş *et al.* (2013) has isolated and identify *Micromonospora* strains isolated from soil samples of Van lake basin.

Chemotaxonomic profiles of 4 test strains are shown in Table 6. It is seen on Figure 5 that DAP type of cell wall of S0107, S0017, S0133 strains are LL- type, but S0014 strains is meso- type. Also, cell wall of S0107, S0017 and S0133 test strains contain glucose, ribose and mannose sugars (Figure 6). Cells of 3 strains were observed to contain LL-diaminopimelic acid as the diamino acid, indicating these strains is of cell wall chemotype I (Lechevalier and Lechevalier 1970a, b). Whole-cell hydrolysates were found to contain ribose and glucose as these sugars are indication of *Streptomyces* but S0014 strain contain galactose and ribose. The comparation of polyphasic taxonomic which used three different technique those are color grouping, numerical analysis and chemotaxonomic identification (DAP test and sugar analysis) are showed on Table 8. Lan et al. (2019) Streptomyces strain isolated from desert sample was found to contain LL-diaminopimelic acid and the whole cell sugars were identified as glucose and fucose and our finding is in good agreement with their results. Manita et al. (2019) identified new species of Streptomyces spp. based on the genotypic, phenotypic and chemotypic data analysis. Most of the results from different technique are mostly agree each other.

The aim of this study is to isolate different actinomycetes bacteria from Halabja soil samples using selective media starch-casein agar and raffinose histidine agar. Total 105 *Streptomyces* strains were isolated, stocked in 20% glycerol and colour grouped growing on oatmeal and pepton iron agar. 20 strains selected from colour groups and phenotipic and chemotaxonomic study carried out to identify. New colonies of *Streptomyces* were isolated from various types of soils Halapja for the first time. Polyphasic taxonomy which are chemotaxonomic and phenotypic method was carried out to identify and characterize strains isolated from soil samples of Halabja, Iraq (Coenye *et al.*, 2005). In conclusion, it is evident from chemotaxonomic and phenotypic data that following deduction can be say briefly from this study. Halabja soil is poor source for actinomycetes. This may be owing to mustard bomb at 1988.

Table 8. Comparation of polyphasic taxonomy of three different technique for identification of test microorganisms

Soil and Strain Code	Color grouping	Numerical analysis	DAP type	Sugar type
S01 (S0035)	6	VII		
S03 (S0107)	6	VII	LL-A2pm	glucose, ribose and mannose
S02 (S0155)	3	IX		
S08 (S0017)	3	IX	LL-A2pm	glucose, ribose and mannose
S04 (S0100)	5	III		
S05 (S0029)	9	IV		
S06 (S0037)	2	II		
S07 (S0139)	2	II		
S09 (S0016)	10	I		
S11 (S0002)	10	I		
S21 (S0043)	10	I		
S12 (S0055)	4	V		
S15 (S0014)	4	V	meso-A2pm	galactose and ribose
S13 (S0010)	1	VI		
S14 (S0143)	7	X		
S17 (S0115)	7	X		
S16 (S0154)	7	V		
S18 (S0131)	8	VIII		
S19 (S0072)	8	VIII		
S20 (S0133)	8	VIII	LL-A2pm	ribose and mannose

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