ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *STREPTOMYCES* FROM SOIL IN HALABJA/IRAQ

Syamand Ahmed QADIR

MASTER THESIS

Biology Department

Supervisor: Prof. Dr. Ekrem ATALAN

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This thesis was approved unanimously by the following jury in the date of 23/01/2017

Prof. Dr. Ekrem ATALAN	Assist. Prof. Dr. Bülent KAYA	Assist. Prof. Dr. Fethi Ahmet ÖZDEMİR
President of the Jury	**Member**	**Member**

The above result have been approved by

Prof. Dr. İbrahim Y. ERDOĞAN
Institute Director

ACKNOWLEDGEMENTS

As always, all praises and thanks to Allah almighty for all strengths and blessings in completing this thesis. I am very pleased to have the opportunity to thank those who have guided and supported me during my postgraduate career and the writing of this thesis.

I would like to extend my gratitude and sincere thanks to my honorable supervisor Prof. Dr. Ekrem ATALAN. He is not only a great lecturer with deep vision but also most importantly a kind person. I can't thank him enough for encouraging me to continue in the right direction. My honest thanks are going to the manager of the Institute of science Prof. Dr. Ibrahim ERDOĞAN for his support and making things easier.And Special thanks to the crew of central laboratory of Bingol University for their support and department of molecular biology and genetics and biology staffs especially Assist. Prof. Dr. Bulent KAYA, Assist. Prof. Dr. Fethi Ahmed OZDEMIR, Assist. Prof. Dr. Can Ali AGCA and Mr. Musa TARTIK which, they helped and supported me. Also, I would like to express my thanks to Prof. Dr. Nevzad Sahin and Ars. Gor. Hayrettin SAYGIN which helped me in the laboratory especially during molecular and phylogenetic analysis.

I would like to thank my mother who has supported me by doing everything they could for the whole my life of education; they are the source of my strength and success. Many thanks also go to my supportive brothers and sisters who encouraged me to achieve my dreams.

Finally, I would like to thank Bingol University Research Project Department (BÜBAP) for financial support for the research, project number: BAP-889-301-2015.And my colleagues and friends fortheir help during my study especially Mr. Hardi Rafat Baqi and Mr. Marwan Y. Ghafour

Syamand Ahmed QADIR

2017 Bingol

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LIST OF SYMBOLES

DNA	: Deoxyribonucleic acid
DAP	: Diaminopimelic acid
Km	: Kilometer
cm	: Centimeter
mi	: Mile
°C	: Centigrade
KNO_3	: Potassium nitrate
NaCl	: Sodium chloride
$MgSO_4.7H_2O$	: Magnesium sulphate heptahydrate
$CaCO_3$	: Calcium carbonate
$FeSO_4.7H_2O$	: Iron sulphate heptahydrate
K_2HPO_4	: Dipotassium monohydrogen phosphate
KH_2PO_4	: Monopotasium dihydrogen phosphate
Na_2HPO_4	: Disodium hydrogen phosphate
Mg	: Microgram
Zn^{++}	: Zinc ion
Mg	: Milligram
S_{SM}	: Simple matching
mM	: Mili-molar
EDTA	: Ethylene diaminetetra acetic acid
Rpm	: Rotation per minute
F	: Forward
R	: Reverse
DdH_2O	: Deionized distilled water
PCR	: Polymerase chain reaction
TLC	: Thin layer chromatography

G+C	: Guanine+Cytosine
C.F.U	: Colony forming unites
RHA	: Raffinose-histidine agar
SCA	: Starch casein agar
Ml	: Milliliter
H_2S	: Hydrogen sulphide
Kb	: Kilo base
Bp	: Base pair
G	: Gram
$CuSO_4.5H_2O$	: Copper sulphate pentahydrate
$MnCl_2.4H_2O$	: Manganese chloride tetrahydrate
$ZnSO_4.7H_2O$	: Zinc sulphate heptahydrate
PFGE	: Pulse-field gel electrophoresis
TIRs	: Terminal inverted repeats
UPGMA	: Unweighted pair group method with arithmetic means
ITS	: Internally transcribed spacer
ISP	: International *Streptomyces* Project
µl	: Micro liter
TB	: Tetris/ Borate
Sj	: Jaccard coefficients
UV	: ultra-violent
DDC	: dispersion differential centrifugation

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HALABJA (IRAQ) TOPRAK NUMUNELERİNDEN *STREPTOMYCES* BAKTERİLERİNİN İZOLASYONU, TEŞHİSİ, MOLEKÜLER KARAKATERİSAZYONU

ÖZET

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 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 dikate 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 organisması 10 gruba ayrıldı ve sonuçlar renk gruplandırması ile yaklaşık benzerlik gösterdi.

Filogenetik analiz için, 20 test suşunun 16S rDNA genleri genomik DNA ekstraksiyonundan sonra polimeraz zincir rekasiyonunda (PZR) evrensel primerler kullanılarak çoğaltıldı ve akabinde nükleotid zinciri belirlendi, fakat 17 test suşunun nükleotid sıralaması belirlendi. Bu 17 test suşunun 16S rDNA genlerinin nükleotidlerinin filogenetik analizi sonucu 10 suşun *Streptomyces* türü, ki bunlar *Streptomyces* badius NRRL B-2567, 5 *Streptomyces* cinereoruber subsp. cinereoruber NBRC 12756 and 2 *Streptomyces* enissocaesilis NRRL B-16365, olarak teşhis edildi. 3 test suşuda Mesorhizobium huakuii IAM 14158 olarak teşhis edilirken 3'üde Nocardiopsis synnemataformans DSM 44143 olarak teşhis edildi. Fakat 1 suştaPseudomonas taiwanensis BCRC 17751Ts olarak teşhis edildi, fakat bu muhtemelen kontaminasyon suştur.

Filogenetik analiz sonuçlarımıza göre Mesorhizobium huakuii IAM 14158 olarak teşhis edilen suş %98,45 benzerlikten dolayı ve tip türden yüksek oranda farklılık gösterdiğinden yeni tür olabilir. Yeni tür olarak yayınlamak için gelecekte DNA-DNA

hibridisazyonu uygulanmalıdır ve bakteriyoloji biliminde mecburidir.Ayrıca tüm hücrede ve duvarında 4 test suşunun diaminopimelik amino asitd (DAP) ve şeker tipleri belirlenerek analzi edildi. 3 suşun *Streptomyces* türlerine has olan LL-DAP içerdiği ve glukoz, riboz and mannoz şeker tipi içerdiği belirlenirken 1 test suşuda Nocardipsis türlere özgü olan mezo-A2pm DAP ve galaktoz and riboziçerdiği belirlendi. Kemotaksonomik çalışmanın sonuçları filogenetik analiz sonuçları ile uyum gösterdi.

Anahtar Kelimeler: *Streptomyces,* Halabja, 16S rDNA, *Actinobacteria*.

ISOLATION, IDENTIFICATION AND MOLECULAR CHARACTERIZATION OF *STREPTOMYCES* FROM SOIL IN HALABJA, IRAQ

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 extractiron 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.
For phylogenetic analysis, 16S rDNA genes of 20 test strains were amplified with universal primers after extraction of genomic DNA and then sequenced, but 17 of them were successfully sequenced. Phylogenetic analysis of 17 test strains carried out using base sequences of 16S rDNA genes in the core genome resulted that 10 test strains were identified as *Streptomyces* species those were 3 *Streptomyces badius* NRRL B-2567, 5 *Streptomyces cinereoruber* subsp. cinereoruber NBRC 12756 and 2 *Streptomyces enissocaesilis* NRRL B-16365. In addition, 3 test strains were identified as *Mesorhizobium huakuii* IAM 14158 while 3 of them were *Nocardiopsis synnemataformans* DSM 44143, one of them was identified as *Pseudomonas taiwanensis* BCRC 17751^{T}species that may be a contaminated strains. Results of phylogenetic analysis showed that test strains identified as *Mesorhizobium huakuii* IAM 14158 may be a new species due to 98,45% similarity level, that is differences is to high with type species. For this reason, DNA-DNA hybridization should be carried out in future to accept new species due to essential technique for description a new species.

Also thediaminopimelic 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-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 mezo-A2pm DAP as diagnostic for *Nocardiopsis* species, and galactose and ribose in the whole cell hydrolysates. The results of chemotaxonomic studies supported the phylogenetic analysis findings.

Keywords: *Streptomyces*, Halabja, 16S rDNA, *Actinobacteria*.

1. INTRODUCTION

Soils offer a variety of basic ecosystem services, including providing structure and nutrients to crops and forests, water filtration, and regulation of the earth's temperature. Additionally, soils are the habitat for billions of organisms, many of which are actively involved in the decomposition of organic matter and nutrient cycling. Soils help to provide clean air and water, which in turn support wildlife and yield a variety of landscapes. The wildlife and landscapes are utilized anthropogenically and soils are often amended in order to better support crops or animals. Actinobacteria are present high number in soil and contain different genera. They have role in decompoisng of organic compounds and have interaction with other microorganisms since some genera are rich source of bioactive compounds. They can grow in different condition ranging from anaerobic, unicellular organisms to aerobic, filamentous, and spore-forming lineages. Almost ~16,000 of bacterial species of actinobacteria (http://www.bacterio.net/) were described and 5% of them are belong to the genus *Streptomyces* alone.

In addition, 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 (Schlatter et al. 2008), but few studies have focused on the distribution of metabolites produced by these organisms. Studies relate to *Actinobacteria* have led to the discovery of new antibiotics with potential pharmaceutical applications as defensive mutualists (Scott et al. 2008), renewing recognition of the value in understanding the ecology of *Streptomyces* for drug

discovery (Asamizu et al. 2015). They produce many important drugs, including most antibiotics that have contribution to human health (Lewin et al. 2016).

Relative to undeveloped soil, human use of lands alters soil properties and the resident actinobacterial community, with cultivation leading to decreased soil carbon content and microbial biomass and increased actinobacterial abundance (Bossio et al. 2005). With urbanization, nitrogen inputs due to agricultural run-off and fossil fuel combustion, as well as increased soil carbon resulted in increased microbial biomass, with microbial responses dependent upon the relative availabilities of C and N (McCrackin et al. 2008). Greater abundance of *Actinobacteria* in urban soils has also been observed (Hill et al. 2010).

The genus of *Streptomyces* will lead to discover new drugs. It well known *Streptomyces* are implicated as important decomposers of plant material in nature, their cellulolytic enzymes and antibiotics can be used to more efficiently biologic control of plant disease instead of using herbicide or pesticide. 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). 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 soilsof 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 characterisation methods such as colour grouping, numerical analysis and pylogenetic analysis of 16S rDNA genes and chemotaxonomic techniques those were DAP nalayis and sugar analysis of cel-wall content. The conclusions provide some new species of *Mesorhizobium* and known *Streptomyces* or

other genera of actinobacteria. The diversity of *Streptomyces* communities in this environment may be explored and scan for bioactive compounds.

2. LITERATURE REVEIW

2.1. Phylum Actinobacteria

Most of Actinobacteria are live alone and abundant in terrestrial and aquatic ecosystems such as plant symbionts, plant or animal pathogens, or gastrointestinal commensals. The actinobacterial genomes sequencing show these organisms are heterogen and biodiversity. G+C rate of genomes of Actinobacteria are high and Gram-positive filamentousand produce mycelim compose of branched hyphea. Their name is combination of aktis or aktin and fungi-mukes. At early time, it was thought as transistent form between fungi and bacteria and reproduce spores. Later it has been accepted as bacteria since their cells contain single and a peptidoglycan cell wall andtheir cells are susceptible to antibacterial agents. Most of Actinobacteria are aerobic, heterotrophic orchemoautotrophic that can degrade various complexpolysaccharides (Gao and Gupta 2012).

One of the largest taxa in theBacteria domain is the phylum Actinobacteria is (Ludwig et al. 2012) and circumscribed data based on analyses of the 16S and 23S rRNA genes (Zhi et al. 2009; Stackebrandt et al. 1997). The phlum include the classes of Actinobacteria Acidimicrobidae, Coriobacteridae, Nitriliruptoridae, Rubrobacteridae and Thermoleophilia. By the way, Thermophilia encompases some genera of Rubrobacteria. The order Actinomycetales now restricted to the family Actinomycetaceae and some of suborders are excluded but still can be used. These changes have been revised after using molecular technique in phylogenetic studies and many previously terminology is inappropriate. For instance, Bacillus term refered previously only the genera but now it is accomodate all aerobic, endospore-forming rods. The phylum Actinobacteria contain 6 classes those are Actinobacteria, Nitriliruptoria, Acidimicrobiia, Coriobacteriia, Thermophilia and Rubrobacteria, 50 families and 251 genera (Euzéby 2016; Kämpfer

2012) and 800 valid species published (http://www.bacterio.net/*Streptomyces*a.html; Li et al. 2016) according to the latest studies

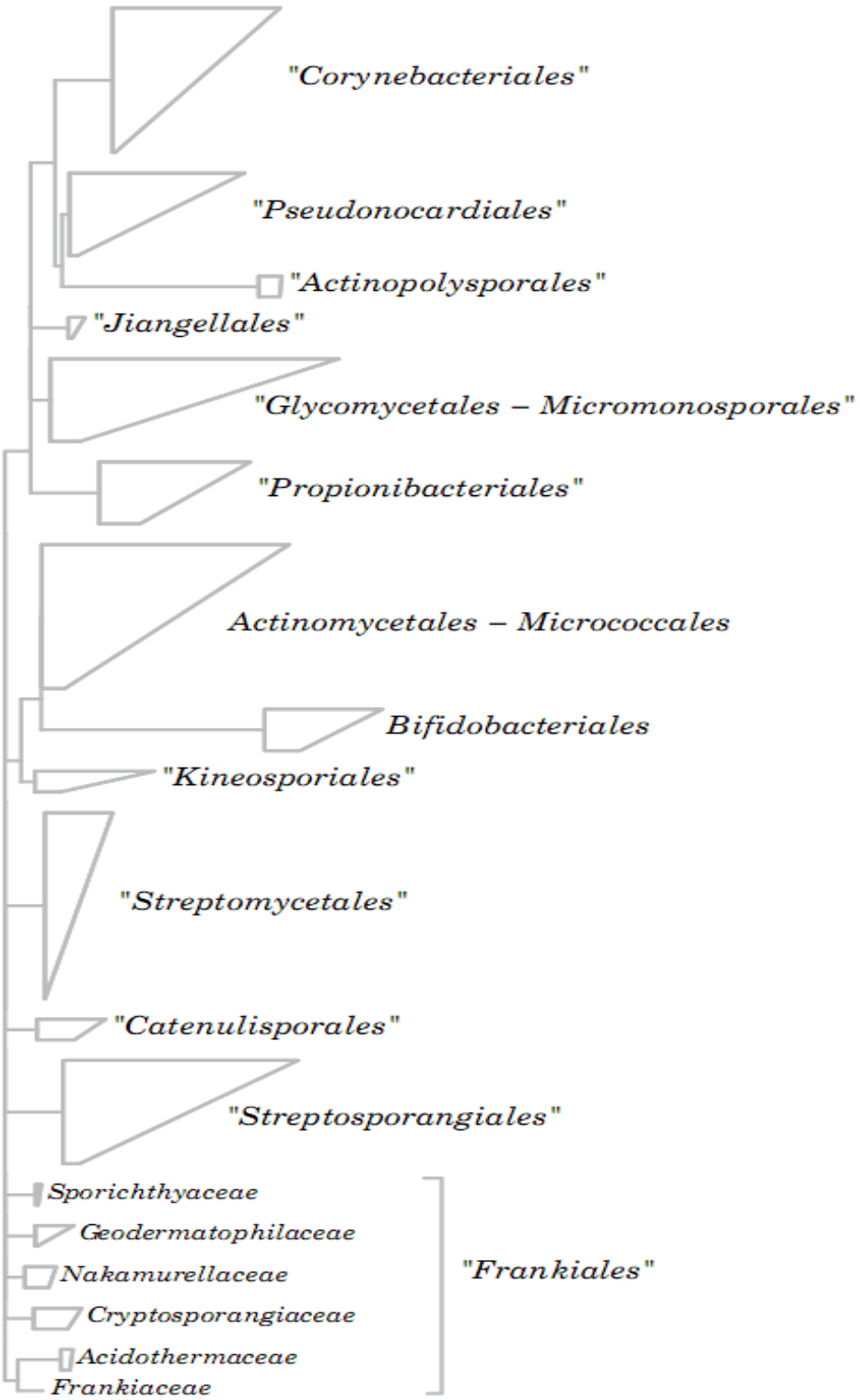


Figure 2.1. Orders of the class Actinobacteria (from Bergeys Manual of Bacteriology)

2.2. Class Actinobacteria

As mentioned above, Actinobacteria class include all suborders of the order Actinomycetales (Figure 2.1.), Bifidobacteriales and the order Jiangellales (Tang et al. 2011). A type order of the class Actinobacteria was not determined upto now (Euzéby and Tindall 2001). At the moment Actinomycetales order seems to be type order owing to conventional use. The class encompose two large clade according to data based on rRNA gene analyses. First clade contain the orders Actinopolysporales, Corynebacteriales, Glycomycetales, Jiangellales, Micromonosporales, Propionibacteriales and Pseudonocardiales while the second clade encompase the orders Actinomycetales, Bifidobacteriales, Kineosporiales, and Micrococcales. The orders Catenulisporales, Streptomycetales, and Streptosporangiales are assigned together in the phylogenetic dendrogram. Frankiales orders assigned alone in the dendrogram (Zhi et al. 2009).

Streptomycetales orders elevation of suborder of Streptomycineae compose of only the family Streptomycetaceae. Kitasatospora and Streptoverticillium genera grouped within Streptomycetales (Garrity et al. 2005). Girard et al. (2014) reported Kitasatospora should be regarded as a separate genus according to analysis of 16S rDNAgene analysis whereas Streptoverticillium species were accomodated to the genus *Streptomyces* on (Kämpfer 2012a). Also Streptacidiphilus has been transfered to the family of Streptomycetaceae (Kim et al. 2003; Zhi et al. 2009). Over600 species of *Streptomyces* have been published and identification of *Streptomyces* and other genera of the family is impossible just using phenotipic features owing to their wide diversity (Kämpfer 2012b). Also other two genera, Kitasatospora and Streptacidiphilus, assigne different cluster in dendrogram produced by seunece analysis of rRNA gene. *Streptomyces* species are stored in culture collection due to producing many kind of bioactive compounds such as antibiotics etc. and undermine other genera species.

Many Nocardiopsis species are included in the family Streptomycetaceae and they should be studied in detail for reclasification. Two related groups include 4 species Nocardiopsis and the last group includes 4 species of Nocardiopsis. Haloactinospora genus contain some Nocardiopsis species while Nocardiopsis arabia seemsin the Streptomonospora. The remaining genera in the family Nocardiopsaceae include the monospecific genus

Marinactinospora and the genus Thermobifida. All classes, orders and families recognized within actinobacteria are listed on Table 2.1.

2.3. Family Streptomycetaceae

Firstly Waksman and Henrici (1943) discovered and then amended by (Ward-rainey and Stackebrandt 1997; Kim et al. 2003; Zhi and and Stackebrandt 2009). They generate many spores or artrspore (more than 3 to 50), aerobic, gram-positive, non-acid–alcohol-fast and produce aerial and substrate hyphea of mycelium at maturity. While, on the substrate mycelium some species bear shortchains of spores. Aerial and substrate mycelium colour is important features that are produce pigments. They are chemoorganotrophic and aerobic organisms that live different pH ranges of acid and basic. Cell wall of substrate mycelium contain either LL- or meso-diaminopimelic acid (A2pm) while their aerial or spores include LL-A2pm.Galactose and rhamnose are sugar type of cell wall.

Hexa- and octa-hydrogenated menaquinones with nine isoprene units are tipical lipid and their fatty acids are compose of saturated, iso- and anteiso-fatty acids but no mycolic acids. The family contain G+C rate in genomic DNA ranged from 66 to 74%.

Labeda et al. (2012) reported that the family contain diverse species and observed 130 clades and single member cluster when they investigated result of 16S rRNA gene sequence of 615 taxa of the family Streptomycetaceae. They tried to clarify between *Streptomyces* type strains and related species and relive statue of the genera Kitasatospora and Streptacidiphilus. They reported that coluld not clarified the status of the genera Kitasatospora orStreptacidiphilus and genus Streptoverticillium. They suggested that these genera are polyphyletic of *Streptomyces*. Also it has been suggested that utilizing sequencing of multiple house-keeping genes is important technique to deliniate species of Streptomycetaceae (Rong andHuang 2010; Labeda2011). Streptoverticillium, Sporichthya, and Microellobosporia, Actinopycnidium, Actinosporangium, Chainia, Elytrosporangium, Kitasatoa, Streptoverticillium and Microellobosporia have been listed as *Streptomyces* synonims (Pridham and Tresner 1974; Witt and Stackebrandt 1990).

Table 2.1. All taxa recognised in Actinobacteria

Phylum XXVI. *Actinobacteria*	Order VII. *Glycomycetales*
Class I. *Actinobacteria*	Family I. *Glycomycetaceae*
Order I. *ActinomycetalesA*	Order VIII. *Jiangellales*
Family I. *Actinomycetaceae*	Family I. *Jiangellaceae*
Order II. *Actinopolysporales*	Order IX. *Kineosporiales*
Family I. *Actinopolysporaceae*	Family I. *Kineosporiaceae*
Order III. *Bifidobacteriales*	Order X. *Micrococcales*
Family I. *Bifidobacteriaceae*	Family I. *Micrococcaceae*
Order IV. *Catenulisporales*	Family XII. *Promicromonosporaceae*
Family I. *Catenulisporaceae*	Family XIII. *Rarobacteraceae*
Family II. *Actinospicaceae*	Family XIV. *Ruaniaceae*
Order V. *Corynebacteriales*	Family XV. *Sanguibacteraceae*
Family I. *Corynebacteriaceae*	Order XI. *Micromonosporales*
Family II. *Dietziaceae*	Family I. *Micromonosporaceae*
Family III. *Mycobacteriaceae*	Family II. *Nocardiopsaceae*
Family IV. *Nocardiaceae*	Family III. *Thermomonosporaceae*
Family V. *Segniliparaceae*	Order *Incertae sedis*
Family II. *Beutenbergiaceae*	Class II. *Acidimicrobiia*
Family III. *Bogoriellaceae*	Order I. *Acidimicrobiales*
Family IV. *Brevibacteriaceae*	Family I. *Acidimicrobiaceae*
Family V. *Cellulomonadaceae*	Family II. *Iamiaceae*
Family VI. *Dermabacteraceae*	Class III. *Coriobacteriia*
Family VII. *Dermacoccaceae*	Order I. *Coriobacteriales*
Family VIII. *Dermatophilaceae*	Family I. *Coriobacteriaceae*
Family IX. *Intrasporangiaceae*	Class IV. *Nitriliruptoria*
Family X. *Jonesiaceae*	Order I. *Nitriliruptorales*
Family XI. *Microbacteriaceae*	Family I. *Nitriliruptoraceae*
Family I. *Propionibacteriaceae*	Order II. *Euzebyales*
Family II. *Nocardioidaceae*	Family I. *Euzebyaceae*
Order XIII. *Pseudonocardiales*	Class V. *Rubrobacteria*
Family I. *Pseudonocardiaceae*	Order I. *Rubrobacterales*
Order XIV. *Streptomycetales*	Family I. *Rubrobacteraceae*
Family I. *Streptomycetaceae*	Class VI. *Thermoleophilia*
Order XV. *Streptosporangiales*	Order I. *Thermoleophilales*
Family I. *Streptosporangiaceae*	Family I. *Thermoleophilaceae*
Family VI. *Tsukamurellaceae*	Order II. *Solirubrobacterales*
Order VI. *Frankiales*	Family I. *Solirubrobacteraceae*
Family I. *Frankiaceae*	Family II. *Conexibacteraceae*
Family II. *Acidothermaceae*	Family III. *Patulibacteraceae*
Family III. *Cryptosporangiaceae*	
Family IV. *Geodermatophilaceae*	
Family V. *Nakamurellaceae*	
Family VI. *Sporichthyaceae*	

2.4. Genus *Streptomyces*

Aerobic, Gram-positive, non-acid-fast bacteria that having extensively branched aerial and substrate mycelia. The size of vegetative hyphae are 0.5–2.0 μm in diameter and rarely fragment. During maturity the aerial mycelium form three too many spore designed in a chain also some species on the substrate mycelium form short spore chain and other show sporangia, pycnidial, sclerotia and synnemata like structures. Spores are nonmotile and Colonies are lichenoid and discrete, leathery or butyrous. At the beginning colonies show a smooth surface, but later grow a weft of aerial mycelium that may appear floccose, granular, velvety, or powdery. They can produce a wide range of pigment that responsible for the color of aerial and vegetative mycelia. some strains produce a diffusible pigments. Many species can produce one or more antibiotic compounds. Many species of *Streptomyces* can use a wide range of organic compounds as a sources of carbon for energy and growth. Chemoorganotrophic, and having an oxidative type of metabolism. In general, most represents can reduce nitrates to nitrites and degrade polymeric substrates such as gelatin, casein, hypoxanthine, cellulose and starch, catalase-positive.

The optimum temperature for most species is in the range 25–35°C; some species, while, can grow at temperatures within the thermophilic and psychrophilic; and for growth the optimum pH range is 6.5–8.0. The cell-wall peptidoglycan contains major amounts of LL-A2pm. In some cases, low amounts of meso-A2pm can be detected and no mycolic acid, contain major amounts of saturated, iso- and anteiso-fatty acids, and lipid profile possess either hexa- or octahydrogenated menaquinones with nine isoprene units. Tipic diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides are polar lipid molecules. Member of genus *Streptomyces* are widely distributed and abundant in soil, such as composts. Some species are pathogenic for animalsand manwhilea few are phytopathogens. DNA G+C content (mol%) ranged from 66 to 78 and type species is *Streptomyces* albus (Rossi Doria 1891; Waksman and Henrici 1943, 339^{AL}).

Although *Streptomyces*, *Thermobifida*, and *Frankia* produce mycelia, *Streptomyces* has of great interest owing to abundant in the soil, showing wide phylogenetic distrubition

(Aderem 2005) and producing a various bioactive secondary metabolites used in industry and pharmacy (Hoopwood 2007).

Genetics and genomics: The genetics of *streptomycetes* is a topic that has improved swiftly and a great amount of information about them has been published recently (Hopwood 2007; Dyson 2010). They can produce various hydrolytic exoenzymes, such as chitinases and cellulases (Schrempf 2006). Especially, one strain, *Streptomycescoelicolor* A3(2), is of prime importance since it presents a fundamental type of organism to study developmental complexity. Furthermore, *streptomycetes* are significant as they are natural sources of antibiotic compounds as well as bioactive metabolities. The *Streptomyces* investigated until now include a large genome that can be either circular or linear. With a high degree of probability the chromosome of *Streptomyces* strains come off in circular and linear forms (Redenbach et al. 1996). Pulse-field gel electrophoresis (PFGE) studies have shown that a linear chromosome exists (Pang et al. 2002) and a *Streptoverticillium* sp. (Redenbach et al. 1998).

The *Streptomyces coelicolor* A3(2) chromosome include around 8667 Mbp, which is equal to 7825 genes (Bentley et al. 2002). The *Streptomyces avermitilis* MA-4680T genome was contained about 9025 Mbp, which corresponds to 7574 genes. Whole-genome synteny plots revealed a high preservation of the overall position and orientation of mutual genes in the chromosomes of *Streptomyces coelicolor* A3(2) and *Streptomyces avermitilis* MA-4680T. Ventura et al. (2007) indicated that the genes conjuct to the four *Streptomyces* genomes only consisted about 17% of the 3566 that were seen in *Escherichia coli* K-12 and *Bacillus subtilis* 168.

In order to compare the genome coverage of 5 *Streptomyces*, Hsiao and Kirby (2008) used DNA–DNA microarray hybridization About 93% similarity was found between the genome sequence of *Streptomyces avermitilis* ATCC 31267T and the genome of closely related *Kitasatospora* species. The prime region was high degree well preserved in comparison to the terminal areas of the linear chromosome seen throughout all four strains. The relaps eof linear *Streptomyces* chromosomes and plasmids is triggered from an origin in which DNA box sequences are abundant and it advances dually to the telomeres (Schrempf 2006). It has been seen in the chromosomal ends of some

Strptomyces species that they contain terminal inverted repeats (TIRs). The lengths of TIR and the telomeres are replicated by a special mechanism.

There are several metabolic genes in the genomes of *streptomycetes*. Carbon storage tansactions are the function of metabolic genes. Genes comprising enzymes of the pentose phosphate pathway, genes specific to different hyphal cell types, and multiple fabH-like genes are related to the first step in fatty acid biosynthesis (Ventura et al. 2007). Similarly the conserved ftsA gens are involved in the complex cell division process, also absent from actinobacteria but they are present in the domain Bacteria.

Streptomyces colonies demonstrated extempore variability in antibiotic biosynthesis, pigmentation and sporulation. Unstable genes encode the various antibiotic resistances, A-factor formation, and synthesis of tyrosinase or arginosuccinate are encoded by unstable genes (Schrempf 2006). The chromosomal DNA's variability is mostly enhanced by its interaction with linear and circular plasmids, phagestransposons, and insertion elements. *Streptomyces* strains contain extrachromosomal elements, plasmids (Kieser et al. 2000). Althuogh plasmids are prevalent and various among streptomycetes, circular plasmids can be found too (Ducote et al. 2000). Linear plasmids on the other hand are seen commonly in streptomycetes that encode antibiotic production and mercury resistance. Actinomycetales harbor genes on linear plasmids, among them those required for isopropylbenzene and trichlorethylene catabolism, biphenyl degradation (Kosono et al. 1997), hydrogen autotrophy and fasciation in plants. Relatively, not much is known about *Streptomyces*transposons (Schrempf2006). Several bacteriophages that have been used to classify strains can be obtained from soil and they usually have broad or narrow host ranges. Furthermore they have countless larger islands of species-specific DNA (Ventura et al. 2007). Almost half of these islands were shared with *Streptomycesambofaciens*. Thereof it is obvious that in pairwise synteny plots the genes in the subtelomeric arms of *Streptomyces* chromosomes are so far from being conserved between species than those in the core (Choulet et al. 2006).

2.5. Cell-envelope composition

Peptidoglycan: The ultrastructure and chemical compound of the cell walls of streptomycetes are ordinary for Gram-stain- positive bacteria. They seem as homogeneous electrondense layers around 16-35 nm with a electron microscope. Multilayered peptidoglycan strands form the cell walls. The sugar backbone is composed of alternating b-1,4-linked units of the sugar derivatives N-acetylglucosamine and N-acetylmuramic acid. In *Streptomyces*, the cell envelope is formed by the resulting macromolecular structure. This LL-A2pm–Gly5 is also called the A3g peptidoglycan type (Schleifer and Kandler 1972) and is diagnostic for streptomycetes as well as some other combined-wall chemotype I actinomycetes (Lechevalier and Lechevalier 1970a). Lechevalier and coworkers grouped actinomycetes into 4 group of Wall Chemotypes depending molecule in the cell wall. Cell walls with meso-A2pm and LL-A2pm were the first to be detected. Cell-wall composition can diversify with the progressional stage of streptomycetes (Takahashi et al. 1984).

Just like in all other actinomycetes N-acetylmuramic acid is known to be present in the glycolyl type in the cell walls of *Streptomyces* (Uchida and Aida 1977). Muramic acid phosphate residues are essential as attachment points to teichoic acids; the latter are polymeric substances containing repeating phosphodiester groups. They consist of polyols or N-acetylamino sugars or both and are valuable for the identification of Gram-stain-positive bacteria. The structure does not differ between streptomycetes and other Gram-stain-positive bacteria. The polymers of teichoic acids consist either of ribitol phosphate or glycerol phosphate (Naumova et al. 1980). The sugars or amino sugars are linked to glycerol or ribitol via glycosidic bonds. Cell-wall polysaccharides seem to be of no diagnostic value (Lechevalier et al. 1971) for strains that have LL-A2pm in their cell wall. Occasionally, diagnostic sugars found in have been reported in *streptomycetes*.

The lipids of *streptomycetes* consist mainly of diphosphatidylglycerol, phosphatidylethanolamine, phosphatidylinositol, and phosphatidylinositol mannosides. To identify *streptomycetes* glycolipids cannot be used since they do not consistently and their qualitative and quantitative lipid compositions are determined by culture conditions on a large scale. Polar lipids have a significant taxonomic value in *actinomycetes*

(Lechevalier et al. 1977). The absence or presence of certain nitrogenous phospholipids characterize the phospholipid groups. Members of the family Streptomycetaceae have phospholipid type II. The marker lipids of this type are phosphatidyletha-nolamine, methyl-phosphatidylethanolamine, hydroxyphos-phatidylethanolamine, and lysophosphatidylethanolamine, however diversification can be made using extra lipids and the existence or the non-existence of phophatidylglycerol and phosphatidylinositol.
Streptomycetes include solely menaquinones. The synthesized quinones have a partly saturated isoprenoid side chain at position 3 of the naphthoquinone ring. Menaquinone composition has a great taxonomic value for the differentiation of actinomycetes. The following three variations are useful for classification and identification: a) the different numbers of isoprene units; b) the different degree of hydrogenation; c) and the position of hydrogenated isoprene units. The menaquinones of streptomycetes have a highly hydrogenated isoprenoid chain and three to four saturated iso-prene units. The actinomycetes, which belong to this type, can be differentiated by a different degree of saturation.

Streptomyces species synthesize terminally branched fatty acids. 2-methylbutyrate as a starting compound results in anteiso-branched fatty acids with an odd number of carbon atoms. In contrast, isovalerate and isobutyrate as starting compounds lead to the formation of iso-branched fatty acids with even and odd numbers of carbon atoms, respectively. For this reason, iso- and anteiso-branched fatty acids appear in pairs with odd numbers of carbon atoms only.

2.6. Numerical taxonomic

Methods were developed in the 1960s for both the classification and identification of bacteria, including streptomycetes. Silvestri et al. (1962) carried out the first numerical taxonomic studies on streptomycetes and found considerable diversity within the genus, as well as groups which corresponded to initial morphological descriptions. A large-scale numerical taxonomic study was undertaken by Williams et al. (1983a) who analyzed 475 strains for 139 unit characters using the simple matching (S_{SM}) and Jaccard coefficients (Sj), and the mean linkage algorithm UPGMA (unweighted pair group method with arithmetic means). The 394 type strains were assigned to 19 major (6–71 strains), 40

minor (2–5 strains), and 18 single clusters recovered at the 77±5% S_{SM} level. Cluster 1 included strains which showed considerable phenotypic diversity. Nearly 40% of the strains produced compounds with anti-fungal activity, 32% of the compounds were active against Gram-stain-positive microorganisms, and 10% inhibited Gram-stain-negative micro-organisms (Williams et al. 1983b). This example highlights the extensive diversity found within a single cluster and exemplifies problems associated with streptomycete systematics (Anderson and Wellington 2001).

The comprehensive survey of Williams et al. (1983a) led to a reduction in the number of described *Streptomyces* species (Williams et al. 1989), although the problem of overspeciation remained. The problem was addressed by the generation of probability matrices for the identification of streptomycetes (Langham et al. 1989; Williams et al. 1983b). Kämpfer et al. (1991) carried out an extensive numerical taxonomic analysis of the genus *Streptomyces* and, where possible, included more than one strain of each species. These workers examined 821 strains for 329 physiological properties and compared their data with those of Williams et al. (1983a). They also examined their strains for genetic and chemotaxonomic properties and compared them with the numerical data. They recognized many of the clusters defined by Williams et al. (1983a) and found that some previous classifications were reliable. However, exceptions were also observed for *Streptomyces* hygroscopicus strains. Data generated in this study were used to construct a probability matrix for the identification of streptomycetes (Kämpfer and Kroppenstedt 1991). Numerical taxonomic data also helped resolve intergeneric relationships within the family Streptomycetaceae (Witt and Stackebrandt 1990). On the other hand, the relationships between the genera Kitasatospora and Streptacidiphilus remains contentious (Kämpfer 2011).

Chemotaxonomy: Chemotaxonomy were used in parallel with the application of numerical taxonomic procedures for the classification of streptomycetes. The additional phenotypic methods used to study streptomycetes included cell-wall analysis, fatty acid profiling (Saddler et al. 1987), rapid biochemical tests based on the use of 4-methyl-umbelliferone-linked substrates, serological assays, phage typing (Korn-Wendisch and Schneider 1992), Curie-point pyrolysis MS of whole cells (Sanglier et al. 1992), whole

organism protein profiling (Lanoot et al. 2002), and comparison of ribosomal protein patterns (Ochi 1995).

Genotypic methods: Many different technique are used both identification and characterisation of bacteria(Vandamme et al. 1996) while clasification of bacteria based on phenetic characters are insufficient the relationships between actinomycetes and other organisms (Stackebrandt and Schumann 2006). The rRNA gene sequence analysis is important for phylogenetic analysis of bacteria. In this respect, 16S rRNA gene, interspace gene of 16 and 23S rRNA genes and 23S rRNA gene and 5S rRNA gene sequencing play role in differentiation of *Streptomyces* (Huddleston et al. 1997) whereas 16S rRNA is not enough to identify a unknown strain (Mehling et al. 1995; Song et al. 2003; Ochi 1995). Wenner et al. (2002) reported that a high level of ITS variability is common characteristic amongst *Streptomyces* species and frequent recombination ocur between rDNA.

DNA hybridization: The technique is used to confirm pylogenetic dendrogram produced after analysis of 16S rRNA gene sequencing (Wayne et al. 1987). The method was used to define species using whole chromosamal DNA (Stackebrandt et al. 2002; Mordarski et al. 1986). Labeda and his collegues (1998) used technique to see corelation between phenetic group of *Streptomyces* of Williams et al. (1983a) study but little agreement found while corelation with phenetic group of Kämpfer et al. (1991) was higher. One thing should be taken into account that chromosaomal DNA is stable and can be affected by plasmids.

2.7. Ecology

Streptomyces live in various habitats, particularly ins soil. They can degrade many kind of organic and complex compounds such as pectin, kitin, lignin (Gottschalk et al. 2003; Kaneko et al. 2003). Streptomycetes are involved in lignin decomposition (McCarthy et al. 1986) and degradation of common hazardous mycotoxin including aflatoxin B1 (AFB1) and zearalenone (ZON) (Harkaiet al. 2016).*Streptomyces* use nitogen source compounds and addition of nfertilizers containing N (nitrogen) can change bacterial community as well *Streptomyces* in the soil (Schlatter et al. 2013). Some of

*Streptomyces*species produce exoglucanases and endoglucanases (Wirth and Ulrich 2002). They produce many various type antibiotics and known source of them. Antibiotic production is result of interaction of microorgansism in the habiatats and use it for competetion (Davelos et al. 2004). Also they produce xylanases, particularly by thermophilic actinomycetes and esophilic streptomycetes (Morosoli et al. 1999). Also, Other polymeric including pectinolytic complexes and chitinolytic complexes have been isolated from some *Streptomyces* species (Schrempf 2006). Cotton, plant fibers, wool hydrocarbons in jet fuel, emulsions, rubber can be degraded by streptomycetes (Schrempf 2006). The number of *Streptomyces* in habitats is related to resource avability, microbial or plant–microbe interactions, or general life history strategies (Fierer et al. 2007).

Streptomycetes produce aerial and substrate mycelium and they can live to various changing physical conditions. These factors are aeration, drought, frost, hydrostatic pressure and anaerobic conditions, moisture tension, pH, semi-dormant stages in the life cycle and can survive in soil for long periods (Mayfield et al. 1972). Spore forms can stay for long period and viable for 70-year-old soil samples. Streptomycetes are almost always present as inactive spores in soil and they can compete with other microorganisms. Spores can grow for a short time and resporulate and some factors those are the presence of exogenous nutrients, water, and Ca^{2+} may be responsible for the germination of spores (Ensign 1978).

Some of the many saprophytic *Streptomyces* species are plant pathogens including potato scab. Main causes of scab are some streptomycetes species, especially *Streptomyces*scabiei and others (Park et al. 2003). It has been isolated from peanut, beets, radish and carrot amongst other crops (Loria et al. 2006). All of these species confirmed by 16S rRNA gene sequence analyses that they are same (Loria et al. 2006). It can be attributed to the polyphyletic nature of scab causing species and the existence of a transmissible pathogenicity island, which seems to confer the pathogenic phenotype on some otherwise non-pathogenic species, as reviewed by Loria et al. (2006, 2008). *Streptomyces* use conjugation system to transfer the gene of infection to other bacteria, thereby accounting for the emergence of new pathogens in agricultural systems.

Quintana et al. (2008) reported that a few streptomycetes pathologic in human and edemic disease of actinomycetoma is an infection caused by actinomycetes bacteria those are Actinomadura and *S. somaliensis*.

Streptomyces are growing in various environments (Mokraneet al. 2013). Soil is the best habitat for reproduction and surviving of *Streptomyces* (Yousif et al. 2015). The character and community composition of any habitat, including the microbial community are affected by abiotic and biotic factors, especially vegetation, content and kind of organic matter, soil type, season and climate, temperature, circulation of water and air, and pH (Kieser et al. 2000). In most soils, streptomycetes constitute about 1–20% of the total viable count and the detection and localization of different *Streptomyces* species in their natural habitat are based mainly on cultivation-dependent techniques. Color-grouping method has been used for intrageneric classification of the genus. This classification into color-groups was then used as a tool by other researchers to study the diversity of streptomycetes in natural habitats (Atalan et al. 2000; Sembiring et al. 2000).

Most attention has been focused on neutrophilic streptomycetes although acidophilic streptomycetes, alkaliphilic streptomycetes are common in acidic and alkaline soils, respectively and can be isolated using starch-casein agar adjusted to pH 5.0 supplemented with anti-fungal agents (Antony-Babu and Goodfellow 2008). Also Streptomycetes have been isolated from the intestinal tract of earthworms, the gut of arthropods and pellets produced by millipedes and woodlice. It is known that antibiotic-producing organisms have a competitive advantage over nonproducing organisms. However, there is no clear evidence for the in situ production of antibiotics in soil or it is difficult to detect in soil owing to low concentrations and unstable (Williams 1982). *Streptomyces* bacteria have potential of biologic control of pathogenic plant microorganisms and have important role (Goodfellow et al. 2007; Bonaldi et al.2014)in competition with other rhizosphere bacteria. Zhang et al. (2014) studied phylogenetic analysis for isolated new species of *Streptomyces* from soil of province, south China. Depending their growing temparature, they can divide into pyscrophilic, mezophilic, thermophilic and hyper thermophilic type. Also, they are named either acidophilic, notrophlic and alkalophilic bacteria depending pH rate (Millner 1982; Kim and Goodfellow 2002). Ningthoujam et al. (2013) isolated of novel species of *Streptomyces* from Limestone quarry, India.

Actinomycetes genera can live in aquatic habitats such as seawater, fresh water and rivers or lake, but mostly washed from soil by rain into water reserviors (Zhang et al. 2014). Marine actinomycetes, especially the members of *Streptomyces*, represent a major source for chemical and actinobacterial diversity (Veyisoglu and Sahin 2015). Streptomycetes have been found in the littoral and inshore zone and in deep-sea sediments. Although streptomycetes can be isolated from both localities, they are not necessarily part of the autochthonous microflora, but are probably derived from terrestrial habitats (Chandramohan et al. 1972). Sharma et al. (2016) isolated of novel species of *Streptomyces* from salt water of Lonar Lake, India. The different ratios of actinomycetes in sediments are dependent on the location and depth of the site of samplings (Weyland and Helmke 1988). Streptomycetes are mainly found in sediments of shallow seas (70–520 m deep) with 300–1270 colonies per cm^3 (Okami and Okazaki 1978), but not depths of 2800 and 5000 m in the Pacific Ocean. (Pathom-aree et al. 2006) isolated actinomycetes, particularly streptomycetes; from the Mariana Trench in the Pacific at a depth of 10,898 m. isolated of more antibiotic-producing streptomycetes from marine habitats (Goodfellow and Fiedler 2010). Novel species of *Streptomyces* were isolated from hot water spring, Pakistan (Amin et al. 2016).

2.8. Isolation and cultivation

The number of individuals relative to the number of other microorganisms within the habitat and the nature of the microorganism can affect the isolation procedures of microorganisms (Kieser et al. 2000; Stolp and Starr 1981). The dispersion and differential centrifugation (DDC) technique can be used to increase the number and diversity of actinobacteria from natural habitats, which is a multistage procedure, a combination of several physicochemical treatments (Goodfellow and Fiedler 2010). Subsequent treatment of samples differs little from usual bacteriological practice. Another possibility is the use of the soil particles for the incubation of soil plates; this method is also used to isolate fungi. Another highly recommended procedure is to mix the soil suspension with the molten agar (Korn-Wendisch and Kutzner 1992), A 100-fold increase in streptomycete colonies on isolation plates can be achieved by the addition of $CaCO^3$ to air-dried soil samples (10:1, w/w).

Lingappa and Lockwood (1962) described a chitin medium for selective isolation due to degrataion of chitin by streptomycetes. Later, a useful medium for the isolation of actinomycetes from water samples was formulated by Hsu and Lockwood (1975), who added mineral salts to the chitin medium, but it had little effect when isolating actinomycetes from soil. Starch is a suitable selective carbon source for streptomycetes, as it is degraded by the vast majority of streptomycetes. The combination of starch with nitrate is used by many streptomycetes in contrast to other bacteria (Flaig and Kutzner 1960a) and Küster and Williams (1964a, b), added starch or glycerol as the carbon source with arginine, casein, or nitrate as a source of nitrogen.

pH is one of factor that affecting growth of *Streptomyces* in habitats and as we mentioned they can live in range of 2-11 pH (Khan and Williams 1975; Mikami et al. 1982). Temperature also affecting distrubition and growth of *Streptomyces* in habitats and during isolation. Most of streptomycetes are grow at 22-37°C. Addition of antibiotics such as cyloheximide, nystatin, rifampisin into selective media support *Streptomyces* but ingibite the growth of undesirable organisms such as fungi (Kim et al. 2003). The members of genus are great source for new antibiotics, alkaline enzymes and enzyme inhibitor (Sharma et al. 2016).

3. MATERIAL AND METHODS

3.1. Description of sampling side

Thirty soil samples were collected from three different places of Halabja. Halabja is a city in Iraqi Kurdistan and the capital of Halabja governorate, located about 240 km (150 mi) north-east of Baghdad and 14 km (9 mi) from the Iranian border. The total area of Halabja is 1,599 km^2 and the elevation of Halabja 721 m (2.365 fit).the place of collection sample area and elevation of Halabja city are given on Figure 3.1

Figure 3.1. Map of Halabja city

3.2. Collection of soil sample:

Total thirty soil sample were collected from three different places in Halabja (Eneb, Golan, Biyawela, Table 3.1). These locations were contaminated by mustard chemical gas in 1988.

The samples were taken at 5 cm depth after removing nearly 3 cm of soil that externally appears. The soil samples were put in a sterile container closed firmly that labeled the date of collection and transferred to the laboratory, stored in a refrigerator.

3.3. Physical characterization of the soil samples

3.3.1. Measurement of pH

The method of Reed and Cummings (1945) were used for measuring the pH of soil samples. About 20-25 g of soil samples were placed in beakers and slowly distilled water added until wetted the soil samples in a beaker with shaking. On the surface of the soil sample, a flimsy layer was appeared. A glass electrode pH meter was used to measure pH of soil samples. pH of soil samples were measured after dissolving of soil in distilled water

3.3.2. Measurement of moisture

The moisture content of the samples was measured by placed one gram of soil samples in pre-weighted crucible and samples accurately weighted. The weighted soil samples in a crucible were put in hot air oven, then the temperature adjusted at 105°C for up to three days. The soil samples were dried weighted until taken a constant weight at 105°C and re-weighted. The average percentage of the loss weight of each sample were recorded as moisture content.

3.3.3. Measurement of organic matter

After measurement of moisture content for each soil samples, organic matters contents of samples were recorded. The crucible that contains the soil samples without moisture were placed in a muffle furnace (ashing) and heated at 700°C for 90 minutes and then measured. Losses of weight were recorded as organic matters content. The average percentage of the organic matter were recorded when the crucible had cooled. The results of the physicochemical characterization of soil sample are given on Table 3.1.

3.4. Media used for isolation and phenotypic identification

Following medium were used for isolation, preservation and identification of *Streptomyces*

1. Starch casein agar (Starch 10 g, Casein 0.3 g, KNO3 2 g, NaCl 2 g, MgSO4.7H2O 0.05 g, CaCO3 0.02 g, FeSO4.7H2O 0.01 g, 20% K2HPO4 10 ml, Agar 12 g and Distilled water 1000 ml) complemented with Nystatin and Cyclohexamide (50 µg/ml) and Novobiocin (25 µg/ml), pH adjusted to 7.00-7.20.
2. Raffinose Histidine agar (Raffinose 10 g, L-Histidine 1 g, K2HPO4 1 g, MgSO4.7H2O 0.5 g, FeSO4.7H2O 0.01 g, Agar 15 g and Distilled water 1000 ml), pH adjusted to 7.00-7.20 and complemented with Nystatin and Cyclohexamide (50 µg/ml), Bennett's agar (yeast extract 1 g, Lab Lemco 0.8 g, Bacto Casitone 2 g, Glycerol 8 ml, Agar 18 g, Distilled water 1000 ml.
3. Peptone-iron agar.
4. Oatmeal agar and Muller Hintone agar.
5. Carbon source utilization agar (ammonium sulphate 2.64 g, KH2PO4 2.38 g, K2HPO4 5.65 g, MgSO4 1 g, Pridham and Gottiebs trace salt solution 1ml, Bacto-agar 18 g, Distilled water 1000 ml) and pH was adjusted to 7.2.
6. Nitrogen source utilization agar (Glucose 10 g, MgSO4.7H2O 0.5 g, FeSO4.7H2O 0.01 g, K2HPO4 1 g, NaCl 0.5 g, Bacto-agar 6 g, Distilled water 1000 ml with adjusted pH to 7.2).
7. Egg yolk agar (Bacteriological Peptone 10 g, Glucose 1 g, NaCl 10 g, Yeast extract 5 g, Bacto-agar 15 g, Egg-yolk emulsion 5%, v/v and pH adjusted at 7.2).
8. Nitrate agar (KNO3 2 g, Nutrient broth 1 L, Bacto-agar 6 g and pH adjusted to 7.2) and Urea reduction medium (KH2PO4 9.1 g, Na2HPO4 9.5 g, Phenol red 0.01 g, Yeast extract 0.1 g, distilled water 1000 ml, Sterilized urea solution 133 ml and pH adjusted to 7.2). The lists of medium used are given at Appendix I.

3.4.1. Selective isolation of *Streptomyces*

One gram of soil sample was accurately weighted and aseptically taken in 9 ml of sterile Ringers solution ¼ strength (Sigma), agitated by vortex and the test tube that contain 10^{-1}

were pretreated by putting in a water bath at 55°C for 10 minutes. After pretreatment, the dilution re-agitated by vortex for a few second. Tenfold of serial dilution were prepared by pipetted 1ml of pretreated sample aseptically up to 10^{-4} dilution. And the test tube that containing 9 ml of Ringers solution ¼ Strength and 1 ml of diluted sample were mixed by using a vortex mixer for each dilution.

Generally, 0.1 ml of each dilution were taken aseptically by a sterile pipet that fitted with sterile tips and inoculated onto the selective medium containing starch casein agar (Küster and Williams 1964) supplemented with nystatin (50 μg ml^{-1}), cycloheximide (50 μg ml^{-1}) and novobiocin (25 μg ml^{-1}). Also, it inoculated on raffinose histidine agar (Vickers et al. 1984) plates supplemented with nystatin (50 μg ml^{-1}) and cycloheximide (50 μg ml). Two plates were inoculated per dilution and inoculum were spread on the surface of the agar plates (Vickers & Williams 1987) using L-shape spreader. Plates inoculated in the laminar flow cabinet and were allowed to dry, then in an inverted position were incubated at 28°C for two weeks.

3.4.2. Selection and purification of *Streptomyces*

The colonies of an isolation plates were examined by both eye and binocular microscope at 100X and 400X magnifications 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 Starch-casein agar and Raffinose-histidine agar. 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. To purity colonies, isolates subcultured until getting pure colonies.

3.4.3. Culturing and stocking of isolates

Isolates were coded according to locality and stored in sterile 2 ml eppendorf tube containing 20% glycerol. Suspension of desired pure colony on modified bennett's agar plates transferred and stock at -80ºC. The suspensions of glycerol were prepared by scrapping aerial and substrate mycelium on modified bennetts agar plates. The frozen

glycerol culture served as a mean of long term storage and also uses as a convenient source of the test strains. Inoculate were taken from by thawing in the room temperature for about 10 minutes and inoculated into broth culture. The glycerol suspensions were stored again at -80ºC after use.

3.5. Color grouping of test microorganisms

Morphological observation is an important character for classification of actinomycetes, but it is not adequate for differentiate between many genera. In fact, at first it was the only way to chosen different strains. 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.

3.6. Numerical analysis of test microorganisms

Total 20 test microorganisms 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 (Table 3.2).

3.6.1. Nutritional test

3.6.1.1. Carbone source

The strains capability to use eleven different carbon sources for growth and energy (Table 3.2) were examined (Wiliams et al. 1983a, b). The carbon compounds were added to carbon utilization agar (Difco ISP9; Shirling and Gottlieb 1966) to give a final concentration either (0.1% or 1%, w/v). Tyndallization technique was used for

Table 3.1. 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

sterilization of each carbon sources. The sterilized basal medium complemented with (1%, w/v) of D-glucose used as a positive control and the free basal medium as a negative control. The strains were inoculated on free basal medium with D-glucose and medium supplemented with tests carbon source for 4-7 days at 28°C.Growths of the strains were read for comparing of medium with carbon source alone against control plate. Result registered as a positive when growth on positive control less than test plate and result

recorded as negative when growth on positive control equal or better than negative control. The experiment was repeated in case of ingrowing of strains of positive control.

3.6.1.2. Nitrogen source

The strains capability to utilize three nitrogen compounds (Table 3.2) was examined by Wiliams et al. (1983a, b). The test compounds were added to nitrogen utilization medium to give final concentration (0.1, w/v). Tyndallization technique was used for sterilization of the nitrogen compounds. The sterilized basal medium supplemented with L-Proline (1%, w/v) was used as a positive control and the free nitrogen basal medium as a negative control. The strains were inoculated on the positive control medium and the basal medium supplemented with test nitrogen sources at 30°C for 4-7 days. Growths of the strains were read for comparing the positive control with negative plates. Result recorded as a positive when growth on control less than test plate and result registered as negative when growth on control equal or better than negative plates. Tests were repeated in case of ingrowing of strains of positive control.

3.6.2. Biochemical test

3.6.2.1. Urea hydrolysis

Urea reductions in liquid medium were used for described hydrolysis of urea by test strains (Table 3.2). Alkaline response were produced during production of urease enzymes by used an indicator. The resultant was change of pH to alkaline by altering color of the indicator recorded as a positive result.

3.6.2.2. Nitrate production and H_2S production

Nitrate agar (Skerman 1967) was used for determine nitrate reductase activity (Table 3.2). Nearly four ml of medium were put on test tube and set it to form slant, test strains were inoculated on each test tube and incubated for 6 days at 30ºC. When three drops of (0.8 ml of sulphanilic acid in 100 ml of 5N acetic acid) with 3 drop of (0.6 ml of dimethyl-α-naphthylamine in 100 ml of 5N acetic acid) supplemented to all tubes and the

result recorded. Positive result was registered during formed red pigment after supplementation of second reagent. Powders of Zn^{++} were added when color not formed which acted as nitrate reductase enzyme for catalysis nitrate in a medium to nitrite. The result detected as negative when red pigment was formed and positive result was developed for not color change after added Zn^{++} powders to test tubes.

Also for H_2S production, nitrate agar were used in additional test tube. Filter paper saturated with lead acetate hanged over edge of test tube inoculated with strains. The results were examined after incubation of inoculated test tube with lead acetate paper for 5 days at 30°C. Produced of H_2S were described as a positive result by formation black color on the filter paper.

3.6.2.3. Degradation tests

Degradation of sterilized xanthine, casein, starch and gelatin (Table 3.2) by Tyndallization were performed to detect enzymes production by test strains on modified Bennett's agar. Positive results were recorded when under or around the inoculated strain clear zone produced. All compounds were added modified Bennet's agar as 1% concentration. Degradation of lecithinase and lipolysis test: egg yolk agar medium (Nitsch and Kutzner 1969) were used for inoculation of strains, 5 strains per plate. The inoculated plates were incubated for 3 and 5 days at 30°C for enzyme activity and the result examined by eyes. Lecithinase action was examined when around the inoculated strains 6-10 mm clear zone were investigated during degradation of lecithin exist in the medium and dissolution of fat in the medium. Lipolysis of free fat in the egg yolk agar were indicated by production of pearly layer clear zone around the inoculated strain 10-16 mm and below reflected light zones were investigated.

3.7. Tolerance tests

3.7.1. Resistance to chemical inhibitors

To determine the capability of isolates to grow in different chemical inhibitors, modified Bennett's agar were supplemented with some chemical inhibitor (Table 3.2) including

sodium azide (0.01%, w/v), sodium chloride (7%, w/v), phenol (0.1%, w/v) and crystal violate (0.0001%, w/v). Inoculated test strains were examined after incubated at 28°C for 2 weeks. modified Bennett's agar alone was used as a positive control. The growth of test strain on the test plates was recorded as a positive result.

3.7.2. Antibiotics susceptibility

Resistance and susceptibility of isolated strains were examined by disk diffusion method (Table 3.2) including Ampicillin (20 mg), Vancomycin (30 mg), Penicillin (30 mg), Gentamycin (10 mg), Rifampin (5 mg) and Erythromycin (15 mg). Tests were inoculated on Muller Hinton agar and results were detected by the formation of clear zone around the disc. If zone formed around the antibiotic disk it mean the isolates strain was sensitive to this antibiotic, if the isolates strain could grow around the disk it mean the strain was resistance to that antibiotic.

3.7.3. Growth at 50°C

The test strains were examined by their ability to grow on modified Bennett's agar at 50°C, 30°C and 25°C (Table 3.2). Tests strains were examined for growth after incubation for 2 weeks. A positive result was recorded by visible growth by the naked eye.

3.8. Antimicrobial activity tests

Twenty isolates were evaluated for their antimicrobial action against six different pathogenic bacteria (Table 3.2) including Gram negative bacteria (*Escherichia coli*, *Pseudomonas fluorescens, and Klebsiella pneumoniae*), Gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtitous*) and fungi (*Candida sp.*) by perpendiculars technique. After inoculation of the test isolates on Muller Hinton agar at 30°C for nearly 7 days, total grow of isolates pathogenic strain were streaked around the isolated strains 5 per plate and incubated at 37°C for 24 hr. The positive result were recorded when isolated strain inhibited growth of pathogenic strains by formed clear zone

around test isolates and negative result were recorded when pathogenic strains able to grow around the test strains.

3.9. Numerical analysis of phenotypic characters

Most of test were scored as two state characters and coded – for negative + for positive result. 20 test strains were examined for 40 diagnostic properties tests. 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. SSM (Simple matching coefficients; Sokal ve Michener 1958) koeficient used to analyse similarity of organisms. Test strains showing 85 S_{SM} level may be belong to same species.

3.10. Molecular characterization of test microorganisms

3.10.1. Isolation of genomic DNA

Total 20 test strains representatives of color grouping were subject to DNA isolation (Table 3.2). The method for DNA isolation of test strains is described by Pitcher et al. (1989). Guanidine thiocyanate DNA isolation method and the DNA isolation kit (Invitrogen, Pure Link (R) Genomic DNA Kit) were used.

3.10.2. Obtaining cell pellet for DNA isolation

The test organisms developed in pure cultures on glucose yeast extract agar were incubated at 28ºC for 10 days, and then inoculated into liquid medium under aseptic conditions at 180 rpm for 10 days at 28°C in a shaking incubator (Excella, New Brunswick Scientific Co., Inc, NJ). 1 ml of each liquid culture was transferred to sterile 1.5 ml eppendorf tube by sterile automatic pipettes and the cell pellet was precipitated by centrifugation at 13000 rpm for 5 min. The remaining liquid phase was removed from the top of the cell pellet. The same procedure was repeated until sufficient cell pellet. Subsequently, the cells were washed at least 2 times with sterile 300 µl TE buffer (10

mM Tris, 1 mM EDTA, pH 8) and the cell pellets were stored at -20°C until DNA isolation.

Protocol of DNA isolation

1. Cell pellets were taken out from the storage at -20°C and kept at room temperature until the ice was dissolved.
2. 180µl of lysozyme added to the cell pellets and homogenizing with an automatic pipettor.
3. Incubated at 37°C overnight in dry incubator.
4. Added 2 µl of Triton-X 100 to the lysozyme supplemented pellet and incubate at 37°C for 30 minutes.
5. Added 20µl Proteinase K and mix well by pipetting slowly.
6. Added 20µl RNase and mix by pipetting slowly.
7. Added 200µl of Pure Link Genomic Lysis / Binding Buffer and mixed well.
8. Wait for 30 minutes at 55°C in dry incubator.
9. 200 µl 96% ethanol is added to Lysate and shaken thoroughly to obtain a homogenous solution.
10. Remove the Spin Column from a package in a PureLink Collection Tube for each test strain.
11. Column tube placed on 2 ml collection tube.
12. Mixed cell transferred from eppendorf tube to PureLink Spin Column and centrifuged at room temperature for 1 minute at 13000 rpm.
13. After centrifugation the collection tube is discarded and the spin column is placed in a clean PureLink Collection Tube.
14. Added 500 µl of genomic washing buffer 1 (previously ethanol was added).
15. Wash buffer 1 is centrifuged for 1 minute at 13000 rpm in the attached column at room temperature.
16. After centrifugation the collection tube is discarded and the spin column is placed in a clean collection tube.
17. 500 µl of genomic Wash buffer 2 added to spin column (previously ethanol was added to washing buffer).

Table 3.2.List of tests and 20 test microorganisms, type of isolation medium and source of test strains

No	Tests		Strains	Source	Isolation medium
1.	Dextran		S0035	Halabja / Biyawela	Raffinose histidine agar
2.	Fructose		S0155	Halabja / Eneb	Starch casein agar
3.	Lactose		S0107	Halabja / Golan	Starch casein agar
4.	Mannose		S0100	Halabja / Biyawela	Starch casein agar
5.	Raffinose		S0029	Halabja / Eneb	Raffinose histidine agar
6.	Sucrose		S0037	Halabja / Eneb	Raffinose histidine agar
7.	Maltose		S0139	Halabja / Golan	Starch casein agar
8.	Mannitol		S0017	Halabja / Biyawela	Raffinose histidine agar
9.	Sodium acetate		S0016	Halabja / Eneb	Raffinose histidine agar
10.	Sodium citrate		S0043	Halabja / Biyawela	Raffinose histidine agar
11.	S. propionate		S0002	Halabja / Golan	Raffinose histidine agar
12.	Histidine		S0055	Halabja / Golan	Raffinose histidine agar
13.	Potassium nitrate		S0010	Halabja / Biyawela	Raffinose histidine agar
14.	Tyrosine		S0143	Halabja / Biyawela	Starch casein agar
15.	Xanthine		S0014	Halabja / Biyawela	Raffinose histidine agar
16.	Casein		S0154	Halabja / Golan	Starch casein agar
17.	Starch		S0115	Halabja / Eneb	Starch casein agar
18.	Gelatin		S0131	Halabja / Eneb	Starch casein agar
19.	Lecithinase activity		S0072	Halabja / Eneb	Raffinose histidine agar
20.	Lipolysis activity		S0133	Halabja / Biyawela	Raffinose histidine agar
21.	H2S production				
22.	Nitrate reduction				
23.	Urea hydrolysis				
24.	Phenol 0.1%				
25.	Sodium azide 0.01%				
26.	Sodium chloride 7%				
27.	Crystal violet 0.0001%				
28.	Ampicillin (20 mg)				
29.	Vancomycin (30 mg)				
30.	Penicillin (30 mg)				
31.	Gentamycin (10 mg)				
32.	Rifampin (5 mg)				
33.	Erythromycin (15 mg)				
34.	Growth at 50Ċ				
35.	*Escherichia coli*				
36.	*Pseudomonas fluorescens*				
37.	*Bacillus subtitous*				
38.	*Klebsiella pneumoniae*				
39.	*Staphylococcus aureus*				
40.	*Candida sp.*				

18. Centrifuged the column at room temperature for 3 minutes at 13000 rpm and discard the collection tube.
19. The spin column tube placed in new collection tube and centrifuged at 13000 rpm for 3 minute without adding anything until dry the matrix.
20. Place the spin column in a clean 1.5 ml microcentrifuge tube.
21. 50 µl of elution buffer were added to the center of column matrix and let it for 3 minute for absorption elution buffer by matrix.
22. Centrifuged at 13000 rpm for 60 second to elute the purified DNA.
23. Finally the 1.5 ml microcentrifuge tube contains pure DNA.
24. The purified DNA should be stored at -20°C.

Preparation agarose gel

Agarose gel electrophoreses were used for adopt total genomic DNA. 1% agarose gel (60 ml 1XTBE buffer, 0.6 g agarose) supplemented with ethidium bromide 4 µl was prepared. Then the bands of total genomic DNA were visualized under illuminator.

1. A glass form surrounded by tape across the end was prepared for gel electrophoresis tank and to make a pore for loading reactions a special comb was placed in the form.
2. 1 g of agarose were dissolved in 100 ml of 1X TBE, boil and heat continuously in microoven until the agarose is dissolved, left dissolved agarose cool until you can touch the flask comfortably.
3. Cooled formula was poured in to the form gently, and left it to solidified, remove tape and immersed the gel to 1X TBE in the gel electrophoresis tank and carefully removed the comb to form a pores.
4. Loaded 3 µl of DNA sample were mixed with 2 µl of dye buffer to the wells in the gel.
5. Loaded DNA run upto end of gel.
6. The band of total genomic DNA gene was checked in a UV transilluminator.

3.10.3. DNA isolation control

1% agarose gel (60 ml 1xTBE buffer, 0.6 g agarose) supplemented with ethidium bromide 4 µl was prepared to control the presence of DNA for resulting DNA isolation. The prepared gel was loaded with 3 µl total genomic DNA mixed with 2 µl of dye. DNA loaded agarose gel placed in the electrophoresis tank and run at 100 volts for 30 minutes and the DNA presence was checked under UV transilluminator (Vilber Lourmat, UV) and captured a photograph.

3.10.4. PCR amplification of 16S rDNA gene

Pure DNA were obtained from the organisms and the region encoding 16S rRNA gene amplified by two universal primers 27f, (5'-AGA GTT TGA TCM TGG CTC AG-3) and 1525R, (5'- AAG GAG GTG WTC CAR CC-3 ') (Lane 1991). Stock solutions prepared for the PCR reaction were prepared with sterile ddH_2O. Stock solutions were separated in sterile eppendorf tubes in small quantities 25-100 µl to remove the risk of contamination and stored at -20°C until use. Polymerase chain reaction procedures of 16S rDNA were performed in a Thermal Cycler (MyGenie-96 Gradient Thermal Cycler), (Korea) in a 0.2 ml PCR tube.

3.10.5. Preparation of mixed reaction for 16S rDNA gene

50 µl of reaction cocktail were prepared for 16s rDNA gene amplification. Contents of cocktail reaction are given on Table 3.3.

Protocol of PCR amplification

1. Separately 1 µl of each DNA samples were transferred into sterile 0.2 ml PCR tubes.
2. The reaction mixture was prepared to give a total volume of 49 µl for each sample in 1.5 ml of PCR tube.
3. 49 µl of reaction mixture were transferred to each PCR tube that contain 1 µl of pure DNA

4. Immediately after transfer, the PCR reaction (MyGenie-96 Gradient Thermal Cycler, Korea) was started working**.**

3.10.6. PCR Program

After preparation of 50 µl PCR mix seen above, 3 different PCR program including denaturation, annealing and extension, respectively with arranged temperatures and time are given on Table 3.4 (MyGenie-96 Gradient Thermal Cycler), (Korea) were used for study.

Table 3.3.Cocktail reaction for amplification of 16S rDNA

Cocktail	**Concantration**	**volume (µl)**
DNA	50-100 ng	1
27F	10 µM	1
1525R	10 µM	1
GoTaq® Hot Start Colorless Master Mix		25
DdH2O		22
Total		50

3.10.7. Purification of PCR product of 16S rDNA

1.5% agarose gel (60 ml 1xTBE buffer, 0.9 g agarose) supplemented with ethidium bromide 4 µl was prepared to control the amplified 16S rDNA . The prepared gel was loaded with 16S rDNA and placed in the electrophoresis tank and run at 100 volts for 30 minutes. Bands of amplified 16S rDNA were checked under UV transilluminator (Vilber Lourmat, UV) and photographs were captured.

3.10.8. Analysis of gene sequence for 16S rDNA

Pure PCR products whose bands were proven by agarose gel electrophoresis were sent to sequence using the ABI PRISM 3730XLGenetic Analyzer (PE Applied Biosystems) automated sequencing instrument with the three primers. Three primers (Table 3.5) were

sent to the company. Sequencing of 16S rDNA gene region were carried out by MacroGen Inc., Netherland.

Table 3.4. PCR reaction conditions for amplification of 16S rDNA gene region

Denaturation	Amplification			Final	Hold
	Denaturation	Annealing	Extinction	Extinction	
95 °C	95 °C	55 °C	72 °C	72 °C	25 °C
15 min	1 min	1 min	3 min	10 min	1 min
1 Cycle	35 Cycle				1 Cycle

Table 3.5.Sequence primers for 16S rRNA gene region amplification

Primer name	**Sequence row (5'-3 ')**	**Size**	**Reference**
518F	CCAGCAGCCGCGGTAAT	17	Buchholz-Cleven et al., 1998
800R	TACCAGGGTATCTAATCC	18	Chun, 1995
Mg5f	AAACTCAAAGGAATTGACGG	20	Chun, 1995

3.10.9. Phylogenetic analysis of 16S rDNA sequence

Genomic DNA was extracted from isolates strain and the complete 16S rDNA sequences of all isolates strain were analyzed with chromas version 1.7.5 (C.McCarthy, School of Health Sciences, Griffith University, Queensland, Australia) program. Seqence were calculate the similarities and most closely related with their type strains using EzTaxon server (http://www.ezbiocloud.net/eztaxon;Kim et al. 2012). The sequence of type strains were regained from GenBank and aligned using CLUSTAL W in MEGA6 (Tamura et al.

2013). Phylogenetic trees were constructed using the neighbor-joining (Saitou and Nei 1987), maximum-likelihood (Felsenstein 1981) and maximum-parsimony (Fitch 1971) algorithms in the MEGA6 program (Tamura et al. 2013). The phylogenetic trees topology was evaluated by the bootstrap with 1000 replicates. (Felsenstein 1985).

3.11. 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 28^0C for 1 week for chemotaxonomic study. The cells were harvested by centrifugation in a large falcon tube at 5000 rpm for 5 minute in macrocentrifuge and repeated until gotten enough amount of pellet. Pellets washed three times with sterilized distilled water and got a pure pellet of isolates strain. Test strains were lyophilized with lyophilizater. For pellets of test strains were transferred in to falcon tube and covered with paraffin. Late, four or more pore have been opened on paraffin.

3.11.1. Protocol Diaminopimelic acid (DAP) analysis

Thin layer chromatography was used to characterized 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.

1. Lyophilized isolates were put in room temperatures for 24 hours.
2. Approximately 10 mg of lyophilized cell sample were transferred to 2 ml viols resistance temperature.
3. Added 200 µl of 6NHCL to cell sample and shaken very well.
4. Kept it in oven at 100°C for 16 hours for hydrolysis.
5. The hydrolysates were cooled at room temperature.
6. Transferred the dissolved part of hydrolysate cell to sterilized Eppendorf tube.
7. Centrifuged at 13000 rpm for 10minute.
8. Removed the participated part and transferred supernatant to new Eppendorf tube.
9. Supernatant in a new Eppendorf tube were kept at 120°C for 2-3 hours, until the liquid was dried.

10. 100 µl of sterilized distilled water were added to dissolve hydrolysates and shaken very well by hand.
11. Loaded 3 µl of isolates and standard of DAP to TLC sheet plate.
12. After the sheet was thoroughly dried, it was run for 3 hours in the running phase.
13. Thin-layer chromatography running phase (JT Baker) were prepared: ddH2O: Methanol: 6NHCL: Pyridine (26 ml: 80 ml: 4 ml: 10 ml).
14. The aluminum sheet from the solvent was dried in a drawer for 15 minutes.
15. After thoroughly dried, it was sprayed with ninhydrin (0.2% w / v) and waited for 2 minute.
16. Dried by heat in oven at 100°C for 5 min, and photographed.

3.11.2. Whole cell sugar analysis

Total 4 test microorganisms (S0107, S0017, S0014 and S0133) were selected as representative cluster of phylogenetic dendrogram. 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.

1. Approximately 50 mg lyophilized dry cells were transferred to 2 ml viols.
2. Added 1.5 ml of 1N H2SO4 to cell sample, shaken very well and hydrolyzed at 100°C for 3 hours in hot air oven.
3. After hydrolyzed the pH was adjusted pH 5-5.5 with saturated Ba (OH) 2 in a small beaker.
4. The pH adjusted cell sample was transferred to large falcon tube.
5. The cell sample was centrifuged at 6000 rpm for 25 minutes in macrocentrifuge and the temperature of centrifuge adjusted at 4°C.
6. The supernatant was transferred to new falcon tube and precipitated part was removed.
7. The supernatant was carefully evaporated until the liquid was completely removed.
8. The residue part were dissolved by added 600 µl of ddH2O to each cell sample.
9. Loaded each sample and standard mixture to the TLC sheet plate (7 µl and 5 µl), respectively.

10. After the aluminum sheet plate thoroughly dried it was carried out for 3 hours in the running phase.
11. TLC executive phase (JT Baker) was prepared: ddH2O: Propanol: ethyl acetate (5 ml: 25 ml: 70 ml).
12. The sheet plate from the solvent was dried in a drawer for 10 minutes.
13. TLC sheet plate then it was run for 1.5-2 hours in the executive phase.
14. TLC plate was then dried for 20 minute in the drawer.
15. After totally dried, it was sprayed with aniline-phthalate and waited for 3 minute.
16. Dried sheet plate at 100ºC for 4 minutes in hot air oven and captured a photograph.

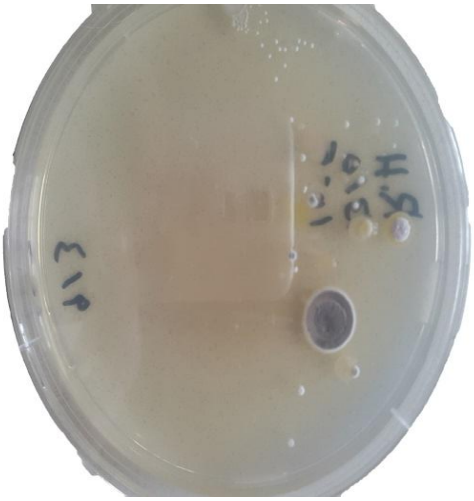
4. RESULT

4.1. Physiochemical parameter of soil samples

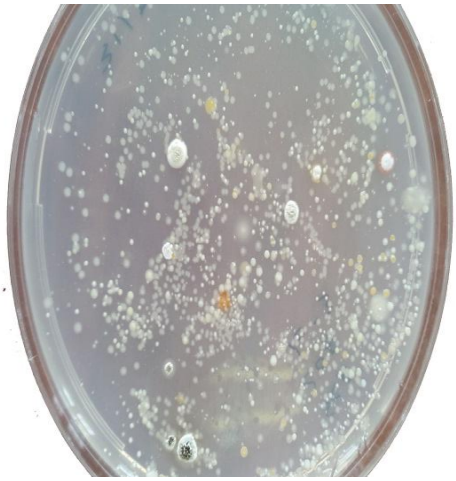
Physiochemical result including amount of organic matter, moisture contents and pH of the soil sample. The amount of organic matter from all soil samples ranged from 9.3% to 20.4% and the higher amount of organic matter was recorded in soil sample number 9 collected from Eneb location and the lowest amount found in soil sample number 8 collected in Biyawela location. The amount of moisture content was ranged from 6% to 9.2% and the highest amount was recorded in sample number four collected Golan location and the lowest amount was recorded in soil sample number one collected from Eneb location. The range of pH of all soil sample recorded from 7.4-8 and the lowest pH was recorded in soil sample number one in Eneb location and the highest pH were recorded in four different soil sample in Biyawela and Golan location. The list of physiochemical parameters are shown on Table 4.1.

4.2. Distribution and numbers of Actinomycetes microorganisms

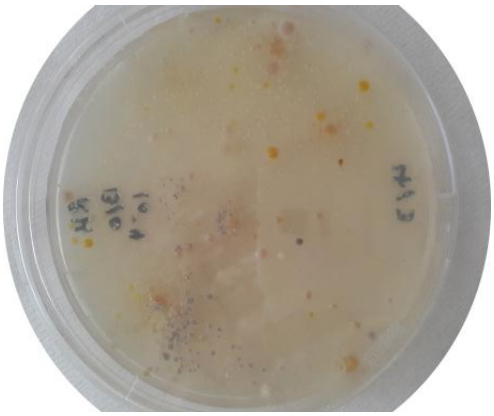
Isolation was performed by using two different medium starch casein agar and raffinose histidine agar. Soil sample was inoculated by serial dilution and petri plates prepared for the isolation were allowed to incubate at 28°C for 14 days. The 105 pure strains were isolated by streak plate method. The number of total *Streptomyces* were recorded and counted as a colony forming unit (C.F.U. Table 4.2.). Photographs of some isolation petri plates are shown on Figure 4.1.



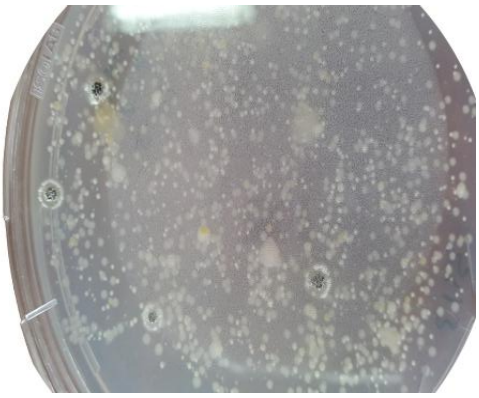
S0002 (Golan) on RHA



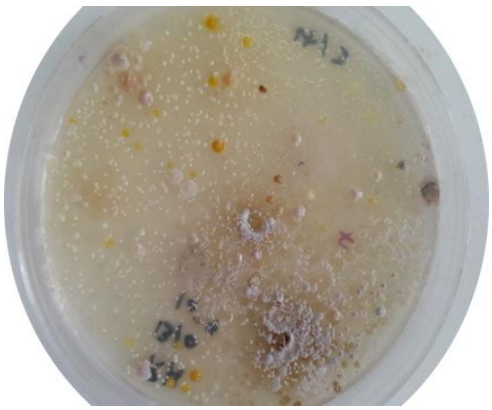
S0155 (Eneb) on SCA



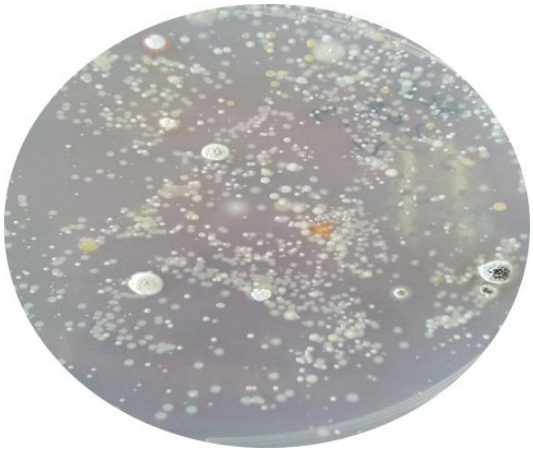
S0016 (Eneb) on RHA



S0143 (Biyawela)) on SCA



S0133 (Biyawela) on RHA



S0115 (Eneb) on SCA

Figure 4.1. Colony appearance of Actinomycetes on plates of starch-casein agar & raffinose histidine agar

Table 4.1.pH, moisture content and organic matter content of soil samples collected from agricultures place in Halabja

Soil sample	***pH***	***Moisture content %***	***Organic matter content %***	***Isolation date***
S01	7.9	8	15.5	23/03/2016
S02	7.5	7	15.3	23/03/2016
S03	8	9	13.3	23/03/2016
S04	8	7	13.3	23/03/2016
S05	7.8	8	12.2	23/03/2016
S06	7.7	9	18.4	23/03/2016
S07	7.9	9	15.2	23/03/2016
S08	8	7	9.3	23/03/2016
S09	7.8	9	18.6	23/03/2016
S10	7.6	8	14.7	23/03/2016
S11	7.8	8	16.5	22/03/2016
S12	7.9	7	13.4	22/03/2016
S13	7.8	7	12.4	22/03/2016
S14	7.7	9.2	15.6	22/03/2016
S15	7.9	7	12.4	22/03/2016
S16	7.7	8	10.3	22/03/2016
S17	8	8	13.5	22/03/2016
S18	7.7	9	12.4	22/03/2016
S19	7.7	7	14.6	22/03/2016
S20	7.6	9	10.3	22/03/2016
S21	7.4	6	17.5	23/03/2016
S22	7.7	7	15.5	23/03/2016
S23	7.6	9	14.4	23/03/2016
S24	7.7	8	12.5	23/03/2016
S25	7.7	7	12.4	23/03/2016
S26	7.6	8	11.3	23/03/2016
S27	7.9	9	14.4	23/03/2016
S28	7.6	9	16.5	23/03/2016
S29	7.7	9	20.4	23/03/2016
S30	7.8	8	19.4	23/03/2016

The highest numbers of colony were recorded from soil sample S10 which was 20 X 10^4 cfu. No *Streptomyces* colony 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.

Table 4.2. Total number of *Streptomyces* (c f u/g/dry weight soil) growing on starch casein agar supplemented with nystatin (50 µg/ml), cyclohexamide (50 µg/ml) and novobiocin (25 µg/ml) and raffinose histidine agar supplemented with cyclohexamide (50 µg/ml) and nystatin (50 µg/ml) seeded with soil suspension and incubated for 14 days at 28°C

Soil sample	**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
S02	2.3	0.0
S03	0.0	0.0
S04	2.2	0.0
S05	0.0	0.0
S06	0.0	0.0
S07	6.4	8.1
S08	0.0	0.0
S09	3.7	2.6
S10	20	9.8
S11	0.0	0.0
S12	0.0	0.0
S13	0.0	0.0
S14	4.5	0.0
S15	0.0	0.0
S16	4.8	1.2
S17	0.0	0.0
S18	2.5	1.1
S19	2.8	1.2
S20	4.8	6.7
S21	0.0	0.0
S22	0.0	0.0
S23	0.0	0.0
S24	0.0	4.3
S25	0.0	0.0
S26	0.0	0.0
S27	0.0	0.0
S28	0.0	0.0
S29	0.0	0.0
S30	0.0	0.0

Note: cfu, colony forming unit

4.3. 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. The color of aerial hyphae and substrate mycelium was used to group isolates. So isolates were inoculated on oat- meal agar to examine aerial and mycelium colour. In addition isolates were inoculated on peptone iron agar to observe melanin pigmentation. Inoculated plates were incubated for 2 weeks at 28ºC. Melanin production was positive in case of distinctive black formation in the reverse side of plate (Table 4.3.). The appearance of representative strains of some isolates growing on oatmeal agar, peptone-iron agar, modified bennets agar and glucose yeast malt extract agar are shown on Figure 4.2.

4.4. Phenotypic characterization

Numerical and identification test scores for each 20 test strains are shown on Table 4.4. Data analysed using TAXON program and produced a dendrogram (Figure 4.3.). 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 bochemical, carbon source, nitorgen source, chemical inhibitor, tempreature, antibiotics, morphology, pigmentation, growth tests are used for both identification and numerical analysis. Photographs of some tests are shown on Figure 4.4.

Table 4.3. 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

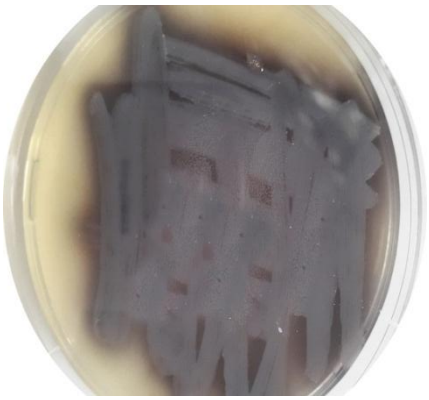
Note: Bold strains were selected for numerical, chemotaxonomic and molecular analysis

Oatmeal agar (S0014)

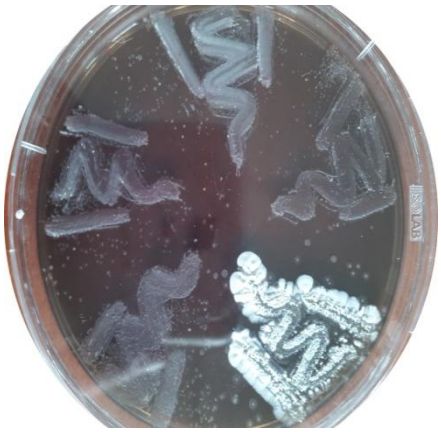
Oatmeal agar (S0010)

Oatmeal agar (SO139)

Oatmeal agar (S0155)



Peptone-irone agar (S0155)



Peptone-irone agar (SOO55, S0010, S0037)

Figure 4.2. Representatives strains of *Streptomyces* growing on oat meal agar, peptone iron agar, bennets agar and glucose yeast malt agar plates at 25C after 10 days growth

Modified bennetts agar (S0131)

Glucose yeast malt agar (S0155)

Modified bennetts agar (S0010)

Glucose yeast malt agar (S0035)

Modified bennetts agar (S0037)

Glucose yeast malt agar (S0133)

Figure 4.2. (continued)

Ability of test microorganism to utilizing eleven different carbon compounds for energy and growth was done after compared growth of test microorganisms in test compounds to positive control and negative control (Figure 4.4a). The result was showed mannitol was most assimilated carbon as a source of energy it used by sixteen test strains and sucrose was least assimilated carbon source it used only by two test strain. Among all test microorganisms S0107^T, S0035^T, S0107^T, S0100^T, S0037^T and S0139^T had most ability to use different carbon compounds. The capacity of 20 test microorganisms to utilizing three different nitrogen compounds as a source of energy and growth was done. After compared growth of test strains in test compounds and compared to negative control and positive control (Figure 4.4b). The result was showed histidine and potassium nitrate were more assimilated nitrogen as a source of energy and was used by all test microorganisms and tyrosine was least source compared to each nitrogen source and used by 12 test microorganisms. According to all test microorganisms S0107^T, S0100^T, S0010^T, S0143^T, S0014^T, S0154^T, S0115^T, S0131^T had not ability to use all nitrogen as a source of energy.

The ability of twenty test strains for resistance to six different antibiotics in a different concentration on modified Bennett's agar (Figure 4.4c). The result was showed among all test microorganisms S0010 strain was resistance for all antibiotics, S0107, S0029, S0131, S0072 and S0133 at least resistance to five antibiotics and test strains S0143 and S0115 were sensitive to all antibiotics, strains S0002, S0055 and S0154 were sensitive at least to five antibiotics. The capacity of 20 test strain to grow on modified Bennett's agar supplemented with chemical inhibitor (Figure4.4d). The result showed that all 20 test microorganisms were grown on medium supplemented with sodium chloride and any test strain couldn't grow on medium supplemented with phenol.

The ability of 20 test microorganisms were examined for their ability to inhibit the development of 6 pathogenic microorganisms gram positive bacteria (*Staphylococcus aureus* and *Bacillus subtitous*), 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 observed, strains S0035, S0055, S0014 and S0133 were inhibited growth of all pathogenic

microorganisms and S0143 and S0115 were unable to inhibit growth of any pathogenic microorganisms. Some results are given in (Figure 4.4e). The 20 test microorganisms were tested for their ability to production of enzyme by degradation of some different compounds (Figure 4.4f). The ability of 20 test microorganism to produce H_2S (Figure 4.4g) and their capacity to hydrolysis of urea by production of urease enzyme. (Figure 4.4h).

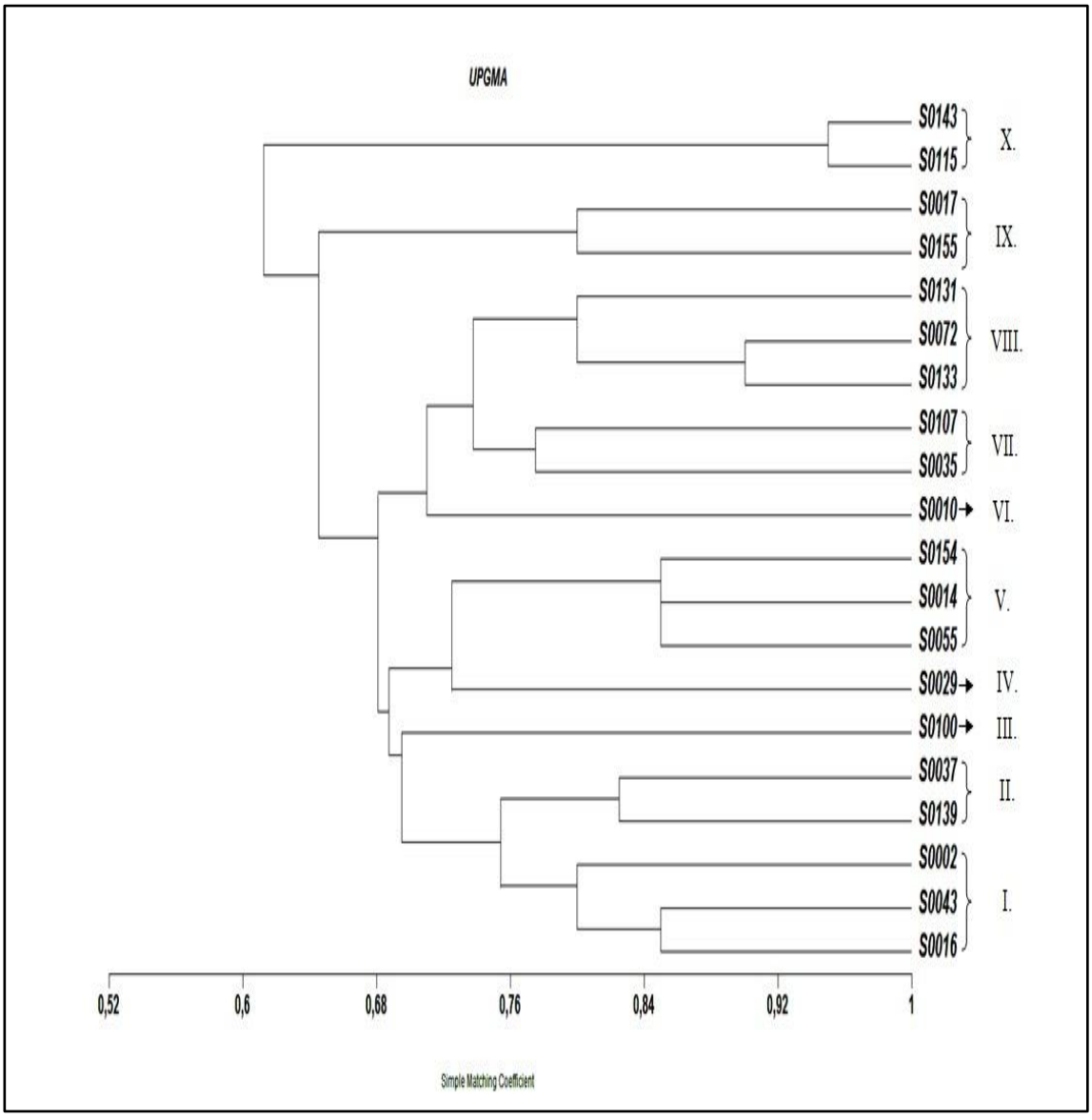


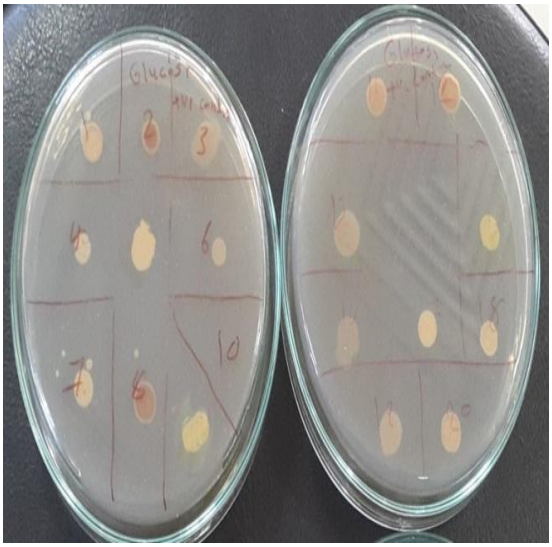
Figure 4.3. Dendrogram showing relationships between representatives of *Streptomyces* groups analyzed data using S_{SM} (Simple matching coefficient) UPGMA algorithm

Table 4.4. Data obtained from traditional identification tests for representative of *Streptomyces* selected from isolates

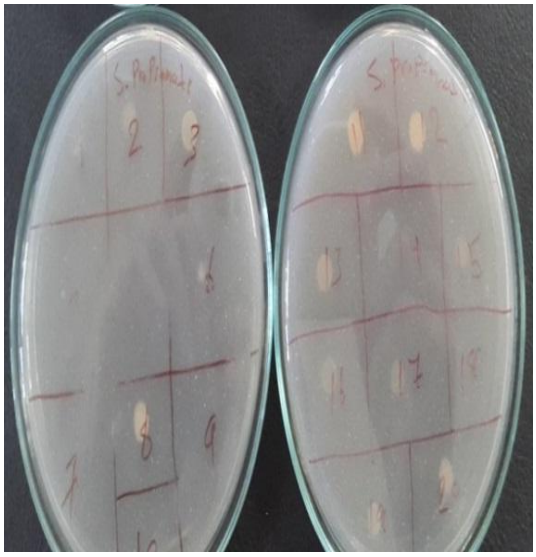
No. of strain Tests	S0035	S0155	S0107	S0100	S0029	S0037	S0139	S0017	S0016	S0043	S0002	S0055	S0010	S0143	S0014	S0154	S0115	S0131	S0072	S0133
Biochemical tests																				
1. H2S production	+	-	-	+	+	+	+	+	+	-	-	+	-	-	-	+	-	+	-	-
2. Nitrate redu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
3. Urea hydrolysis	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
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 viol 0.0001%	+	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+

Table 4.4. (continued)

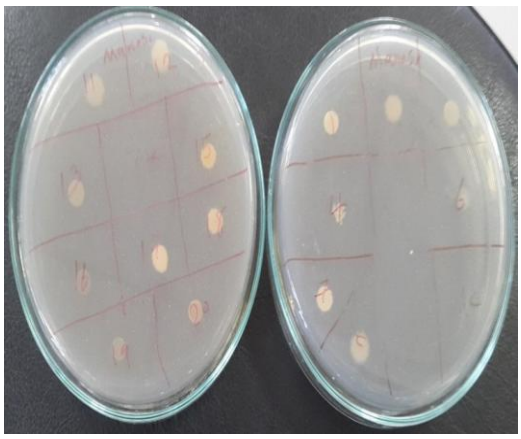
Resistance to antibiotics																				
28. Ampicillin (20 mg)	+	+	+	+	+	+	-	+	-	-	-	-	+	-	-	-	-	+	+	+
29. Vancomycin (30 mg)	-	-	+	-	-	-	-	-	-	+	-	-	+	-	-	-	-	+	+	+
30. Penicillin (30 mg)	+	+	+	+	+	+	+	+	-	-	-	-	+	-	+	-	-	+	+	+
31. Gentamycin (10 mg)	-	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-
32. Rifampin (5 mg)	+	-	+	-	+	-	-	-	+	+	-	+	+	-	+	+	-	+	+	+
33. Erythromycin (15mg)	+	+	+	-	+	+	+	-	+	+	+	-	+	-	-	-	-	+	+	+
Resistance to temreature																				
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.	+	+	+	+	-	-	+	+	-	-	-	+	+	-	+	+	-	+	+	+



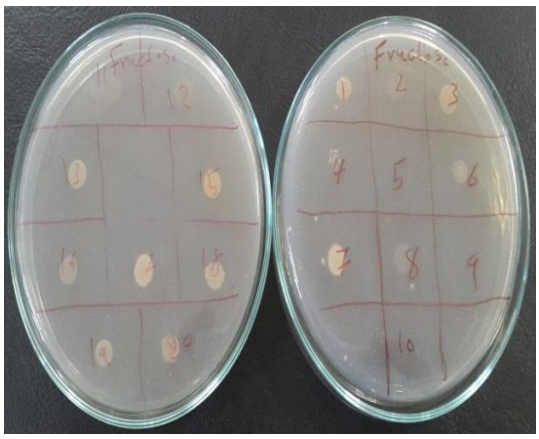
Glucose test



Sodium propionate



Mannose

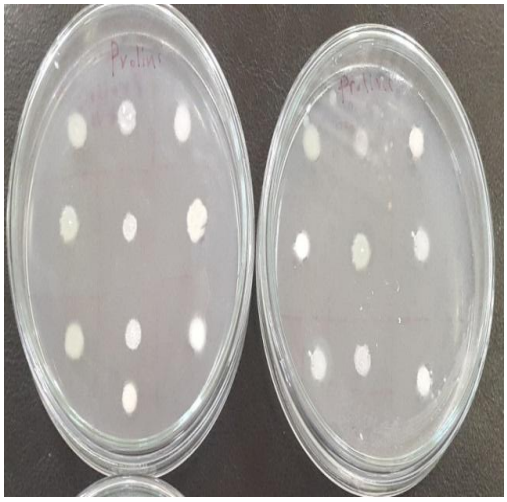


Fructose

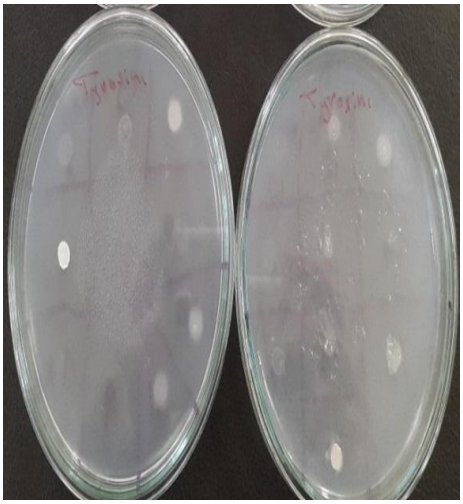
Raffinose

Mannitol

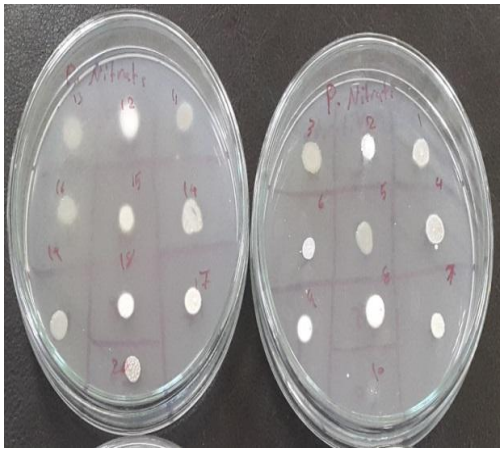
Figure 4.4a. Carbon source tests



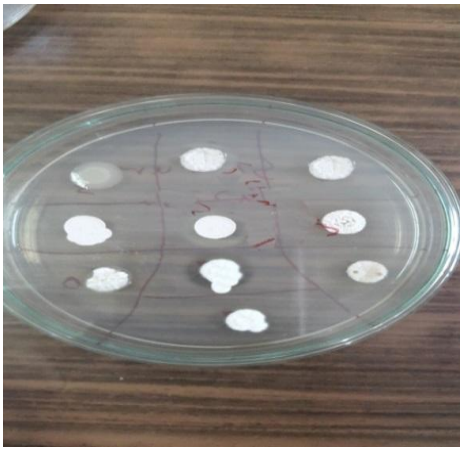
Proline



Tyrosine



Potasium nitrate



Histidine

Figure 4.4b. Nitrogen source tests

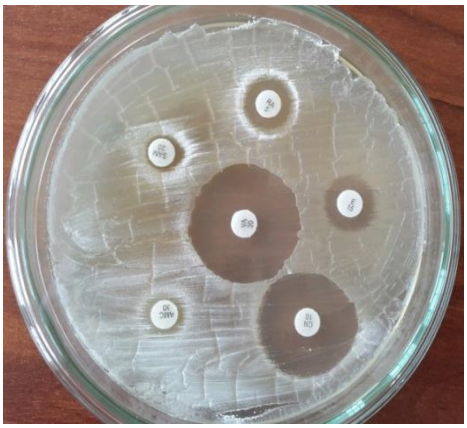


Figure 4.4c. Antibiyotic resistance tests

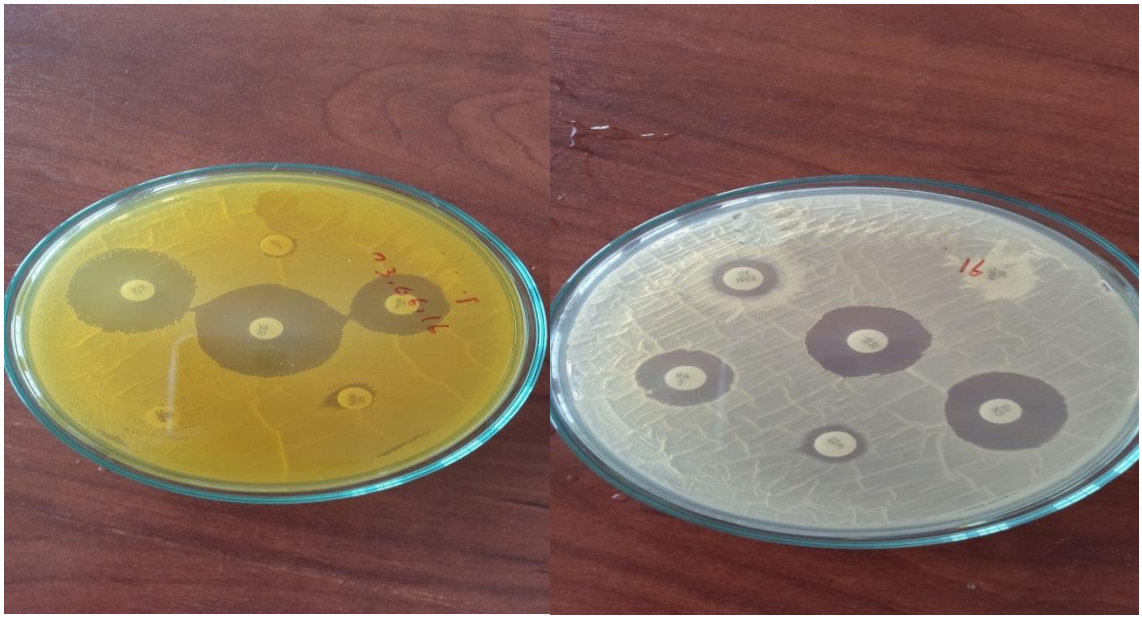


Figure 4.4c. Antibiotic resistance tests (continued)

Figure 4.4d. Chemical inhibitor tests

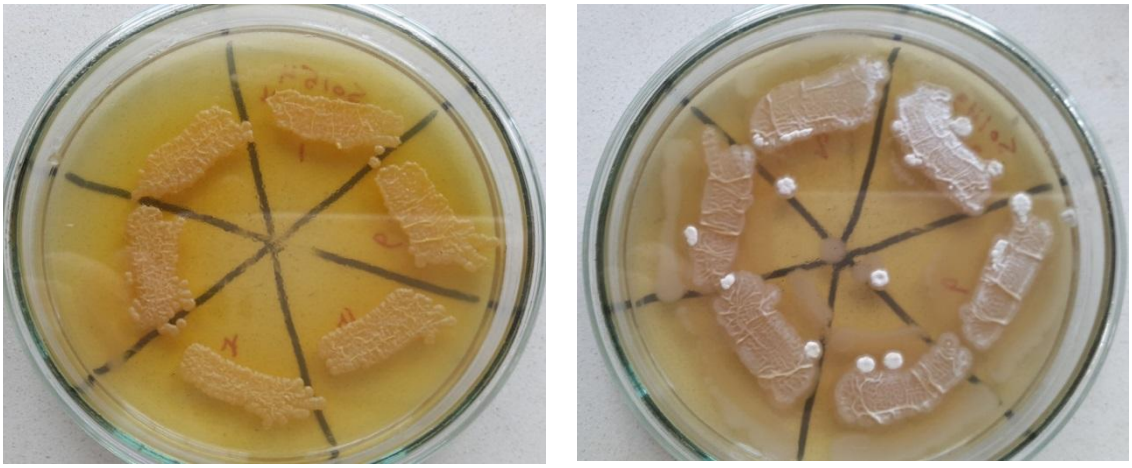
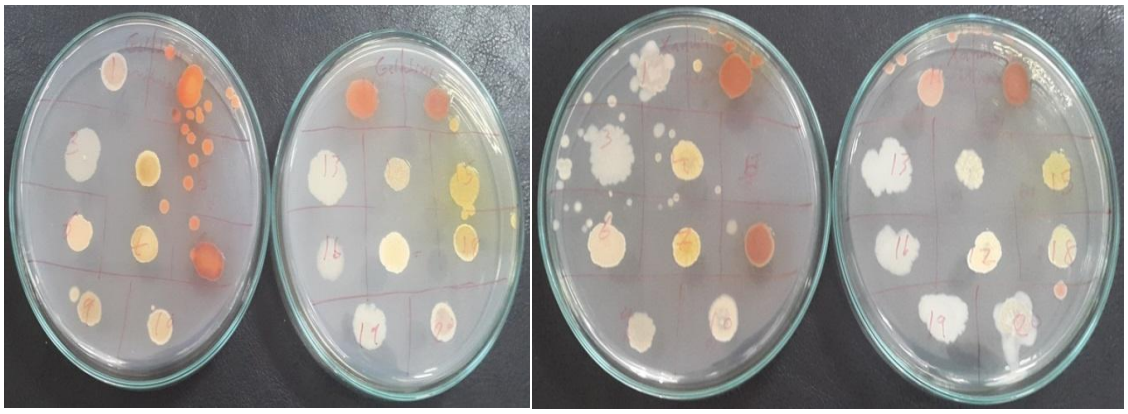
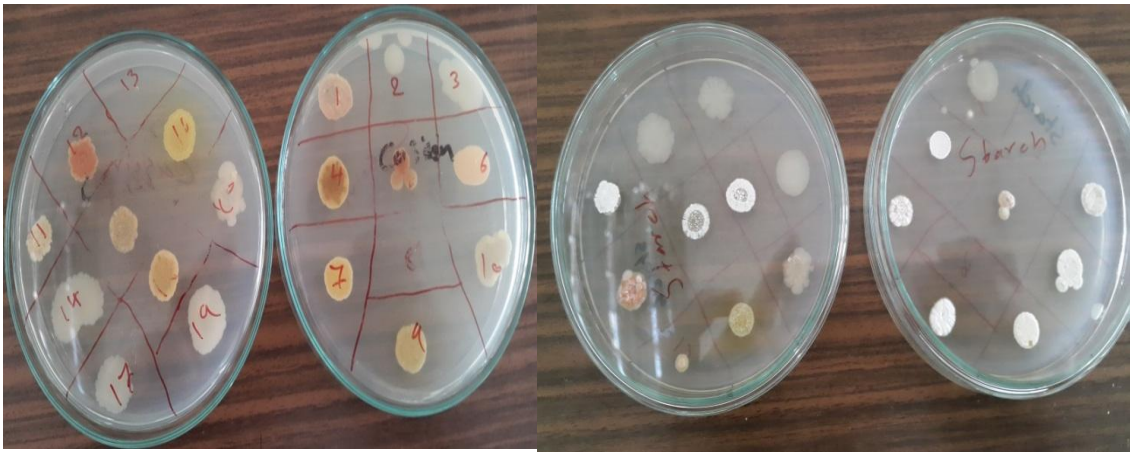


Figure 4.4e. Antimicrobial activity test strains against pathogenic bacteria and fungi



Gelatine Xanthine



Casein Starch

Figure 4.4f. Degradation tests

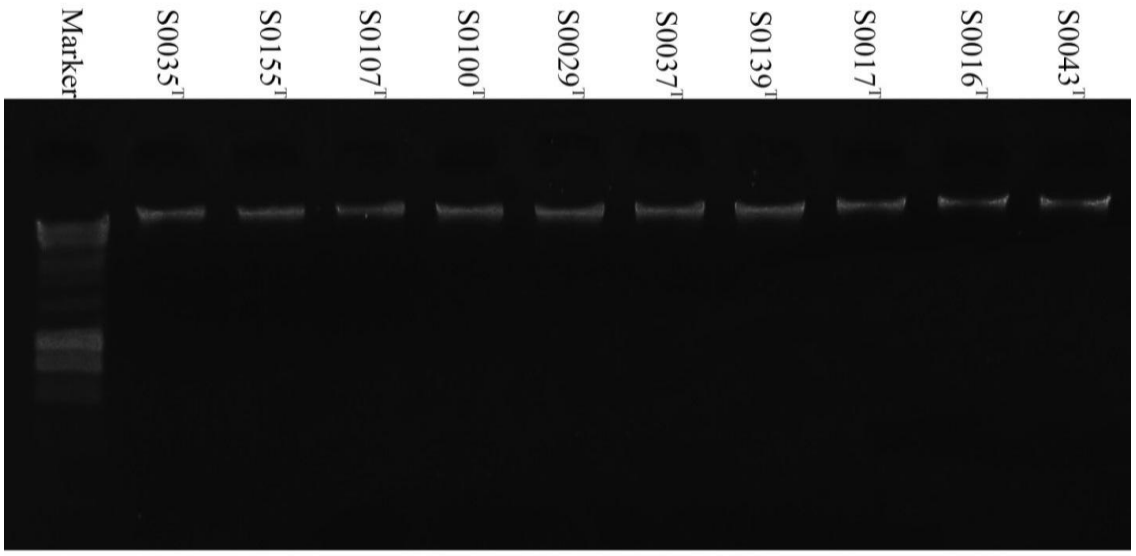
Figure 4.4g. H_2S production by test strains

Figure 4.4h. Urease production by test strains

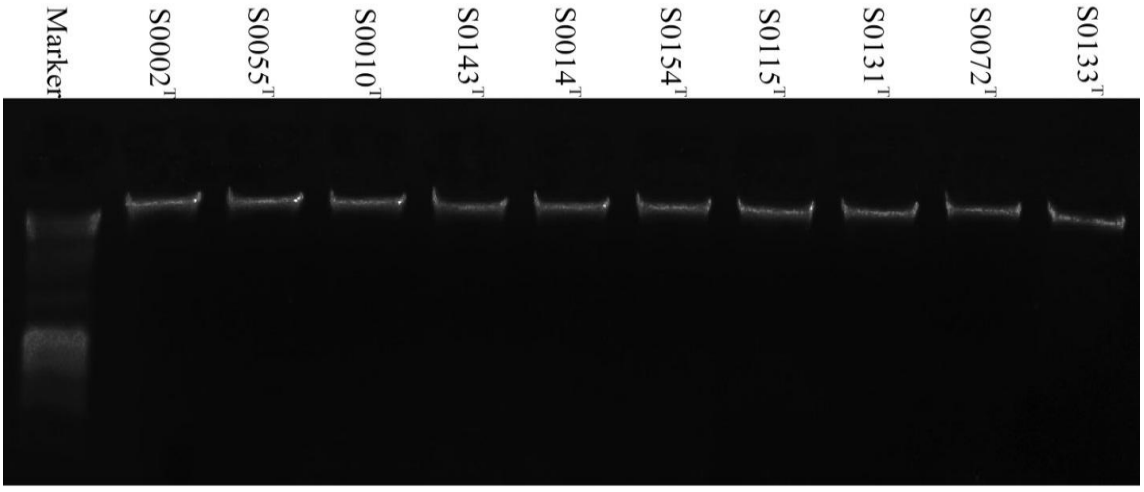
Figure 4.4. Photographs show growth of test strains (a, b, c, d, e, f, g and h)Molecular characterization

4.4.1. Result of genomic DNA

The genomic DNA of 20 test strains were extracted using method described by Pitcher et al. (1989) and DNA isolation kit (Invitrogen, Pure Link (R) Genomic DNA Kit). The total genomic DNA of test strains on agarose gel electrophoresis are shown in Figure 4.5.



A

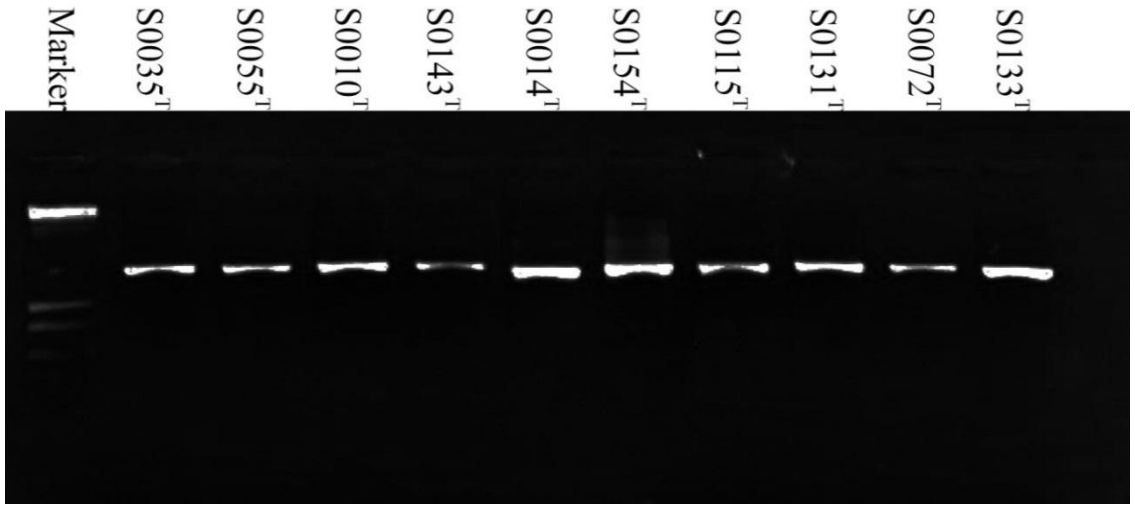


B

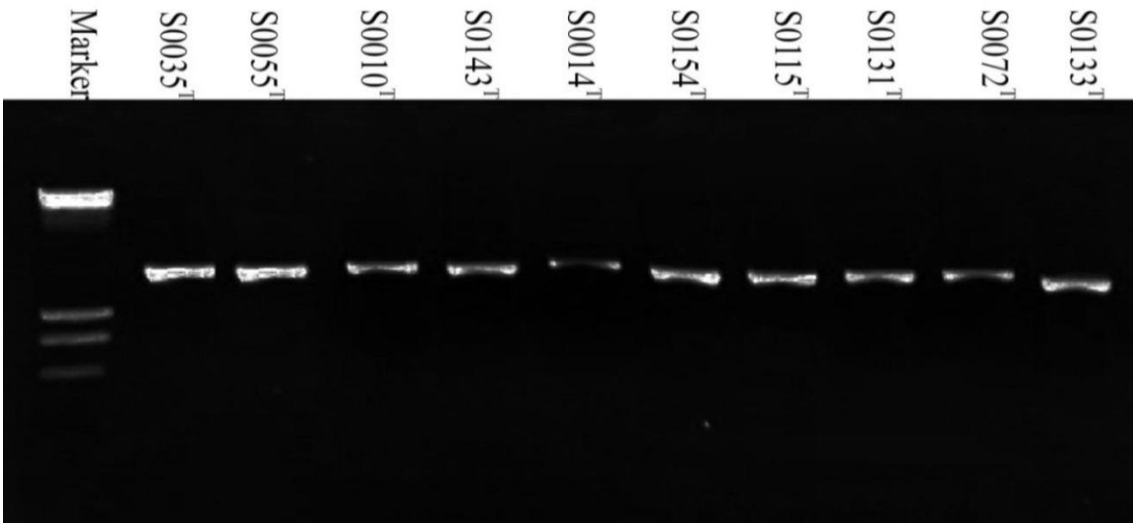
Figure 4.5. Whole genomic DNA bands of 20 test strains on 1% agarose gel eelectrophoresis image (Left side is marker 1kb DNA Ladder)

4.4.2. Result of PCR

16S rDNA gene region was amplified with the Gradient PCR using universal primers 27f and 1525r from genomic DNA of 20 test strains. The sizes of 16S rDNA region were average 1500 base pairs. The results of PCR amplified gene on agarose gel electrophoresis are shown on Figure 4.6.



A



B

Figure 4.6. PCR amplified of the 16S rDNA gene region was performed using 1.5% agarose gel electrophoresis (Marker; Sigma, 1500 bp DNA Ladder)

4.4.3. Analysis of 16S rDNA sequence and phylogenetic tree

Sequencing of 16S rDNA gene region amplified by PCR were performed by primers 27f, 800r and MG5f after purification with theQIA quick PCR Purification Kit. The obtained sequence data was compared with the sequence data of the closest related species in the international databases using EzTaxon Server and % similarities were determined. Base sequences of 16S rDNA of 16 test strains are given at Appendix I. A graph showing the distribution of isolates identified to type strains are given on Figure 4.7 according to sequence analysis of base sequences of 16S rDNA. Dendrogram were generated to determine phylogenetic positions of test isolates 16S rDNA taking in to account sequence data (Figures 4.8., 9, 10, 11 and 12). The dendrograms were drawn using the neighborhood-joining algorithm and the evolutionary distance matrix(Jukes and Cantor 1969). MEGA6 package program was used for phylogenetic analyzes (Tamura et al. 2013). The bootstrap analysis of the phylogenetic trees created (Felsenstein 1985) was done in 1000 replicates. *Aquamicrobium terrae* hun6^{T} (KC840671) used as an out group for *Mesorhizobium*dendrogram, *Kitasatospora nipponensis* HKI 0315^{T} (AY442263) was used as an out group for *Streptomyces* phylogenetic tree and *Murinocardiopsis flavida* 14-Be-013^{T} (FN393755) used as an out group for of Nocardiopsis dendrogram. As a result of phylogenetic analysis of sequence data, 10 isolates were identified as *Streptomyces* while, 3 isolates were *Nocardiopsis* genus. Also 3 isolates were *Mesorhizobium* genus while 1 isolate of *Pseudomonas* genus that may contaminate strain. Phylogenetic similarities to the closest species of isolates according to 16S rDNA sequence results are given in Tables 4.5.

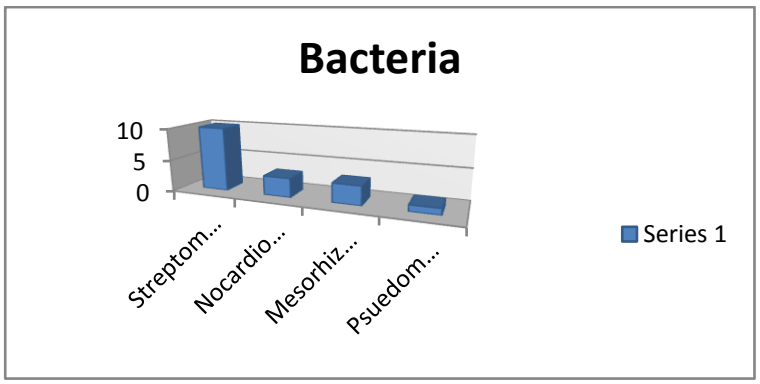


Figure 4.7. Distribution genus of test isolates according to the results of 16S rDNA sequence analysis

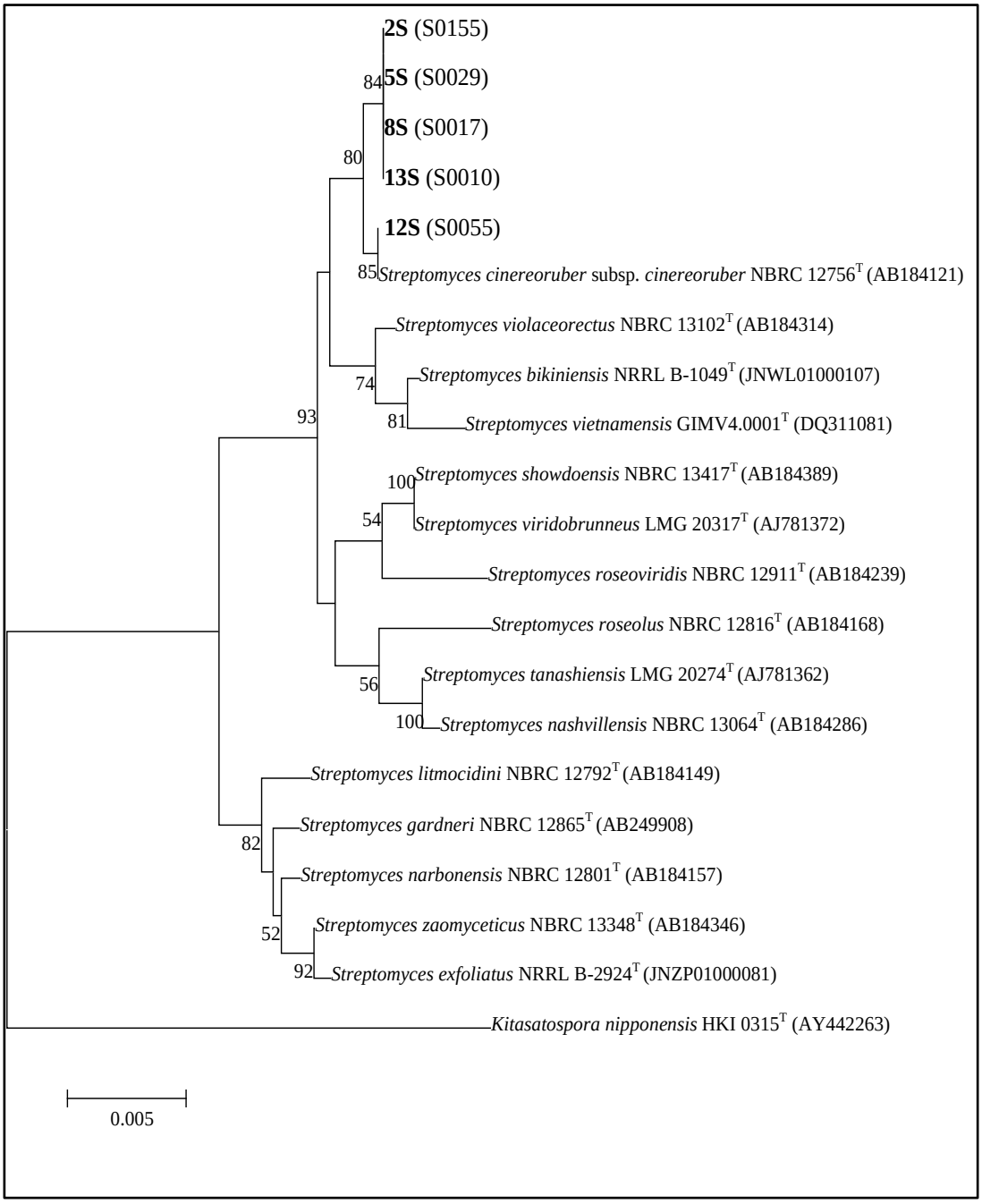


Figure 4.8. Phylogenetic dendrogram showing the relation of 5test strains of *Streptomyces* regarding base sequence of 16S rDNA gene. Dendrogram produced neighboor joining algorithms and numbers at nodes indicate the level of bootstrap support (%), only value ≥52% are shown. Genbank accesion number are given in parantheses. Bar 0.005 substitions per site

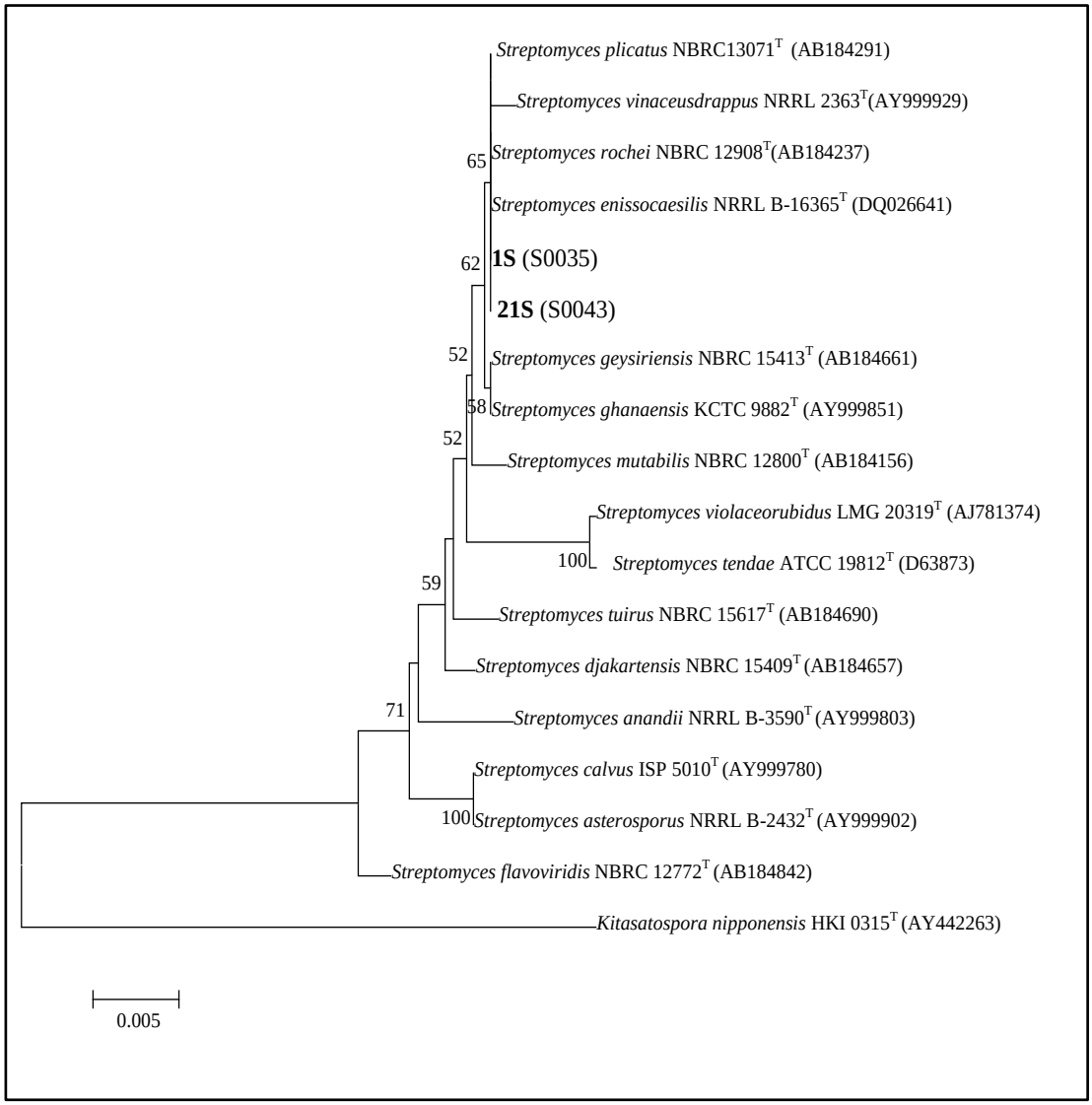


Figure 4.9. Phylogenetic dendrogram showing the relation of 2 test strains of *Streptomyces* regarding base sequence of 16S rDNA gene. Dendrogram produced neighboor joining algorithms and numbers at nodes indicate the level of bootstrap support (%), only value ≥52% are shown. Genbank accesion number are given in parantheses. Bar 0.005 substitions per site

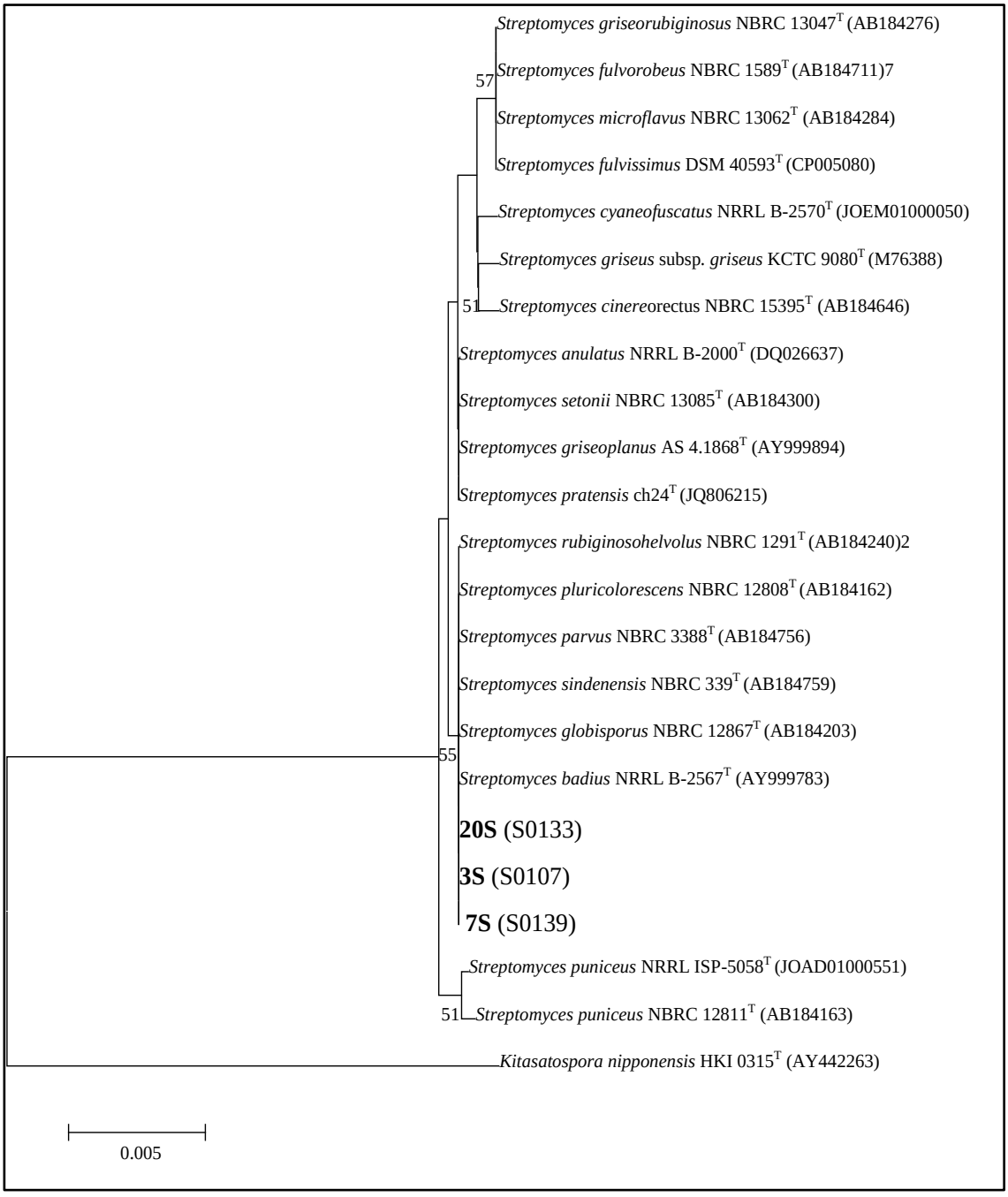


Figure 4.10. Phylogenetic dendrogram showing the relation of 3test strains of *Streptomyces* regarding base sequence of 16S rDNA gene. Dendrogram produced neighboor joining algorithms and numbers at nodes indicate the level of bootstrap support (%), only value ≥51% are shown. Genbank accesion number are given in parantheses. Bar 0.005 substitions per site

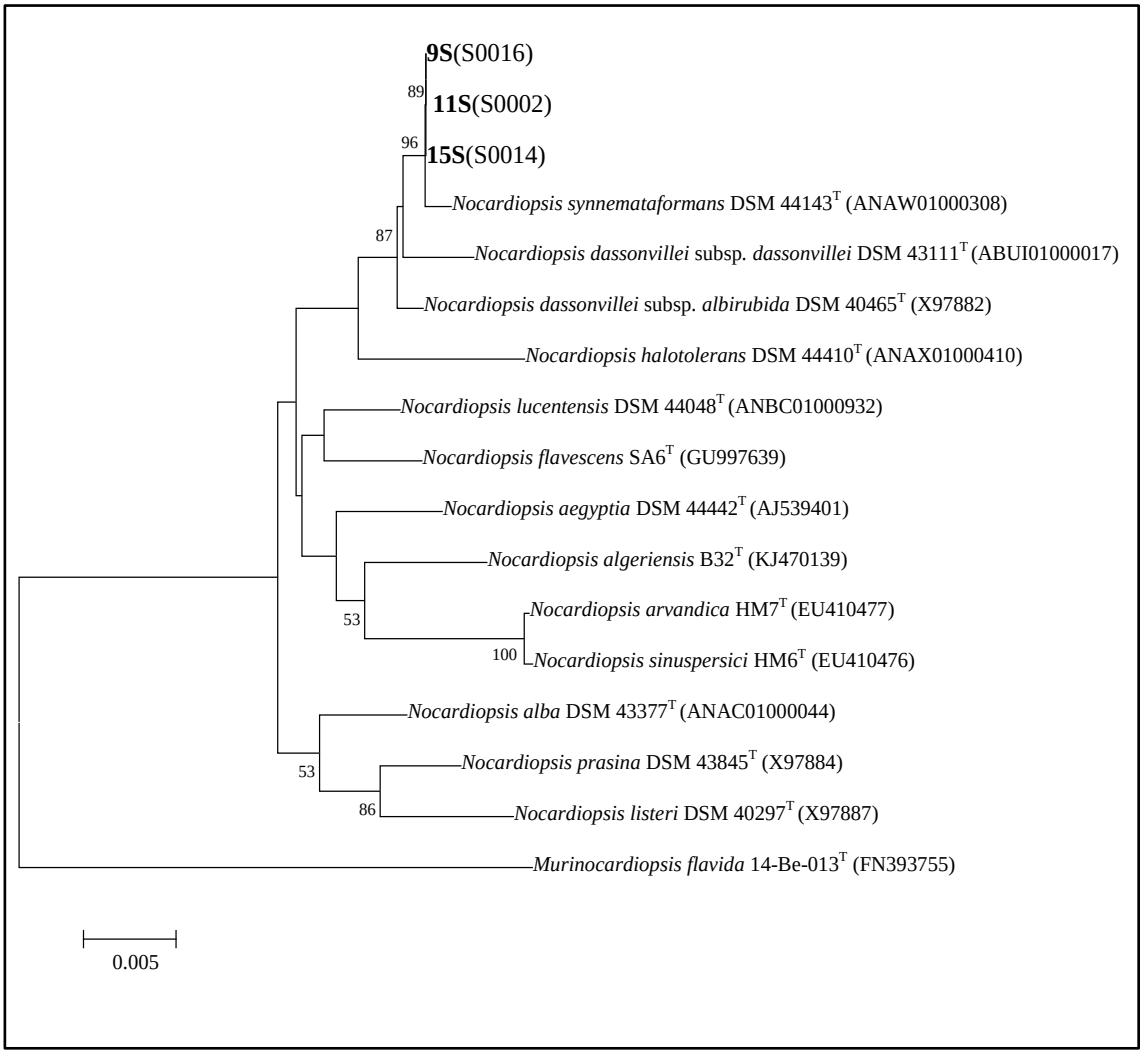


Figure 4.11. Phylogenetic dendrogram showing the relation of 3 test strains of Nocardiopsis regarding base sequence of 16S rDNA gene. Dendrogram produced neighboor joining algorithms and numbers at nodes indicate the level of bootstrap support (%), only value ≥53% are shown. Genbank accesion numbers are given in parantheses. Bar 0.005 substitions per site

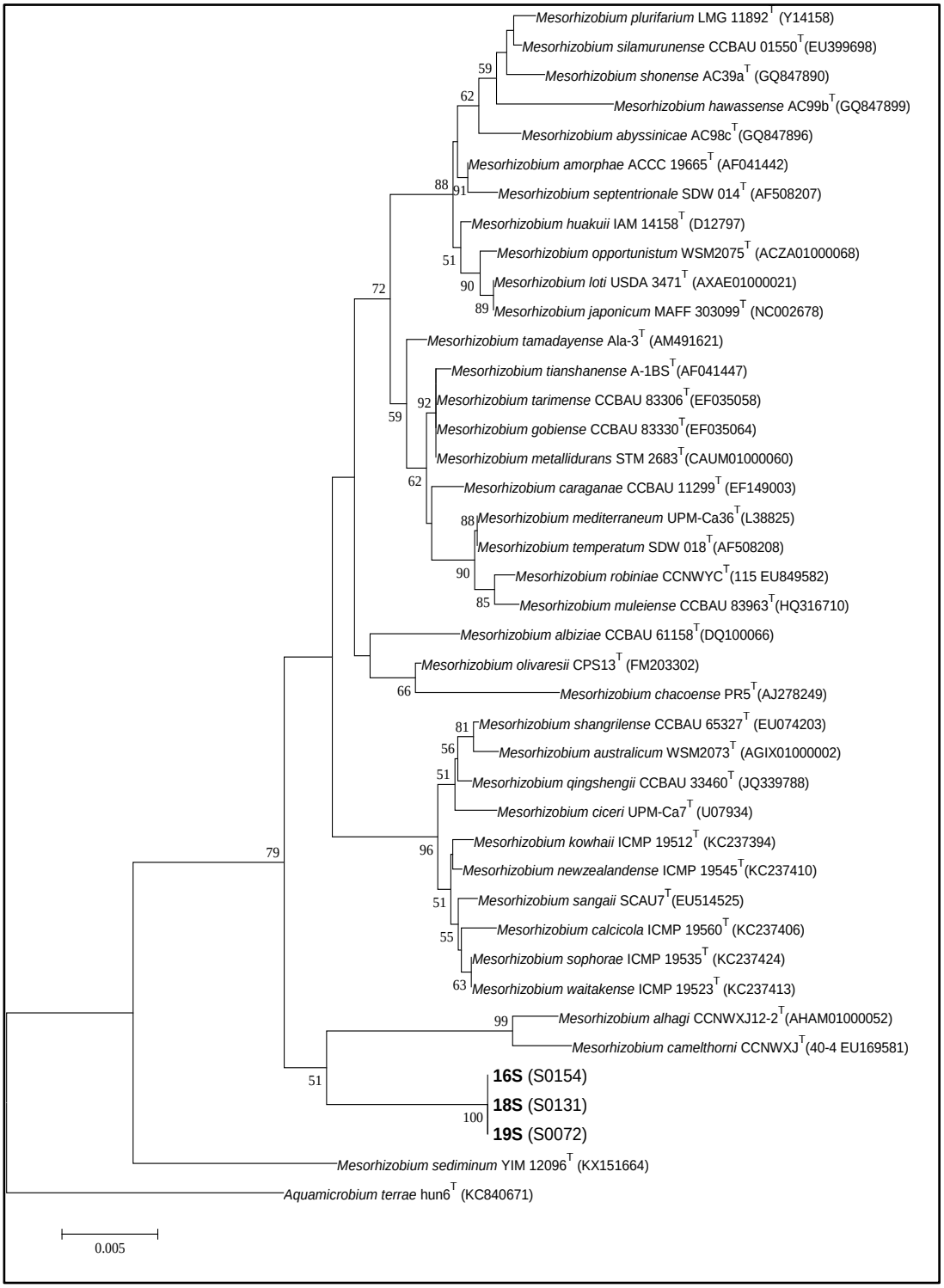


Figure 4.12. Phylogenetic dendrogram showing the relation of 3 test strains of Mesorhizobium regarding base sequence of 16S rDNA gene. Dendrogram produced neighboor joining algorithms and numbers at nodes indicate the level of bootstrap support (%), only value ≥51% are shown. Genbank accesion numbers are given in parantheses. Bar 0.005 substitions per site

Table 4.5. Phylogenetic similarity with closely related species

No	Strains code (Lab Code)	The nearest strains that test stains has been identified	similarity
1.	20S (S0133)	*Streptomyces* badius NRRL B-2567(T)	100.00
2.	7S (S0139)	*Streptomyces* badius NRRL B-2567(T)	100.00
3.	3S (S0107)	*Streptomyces* badius NRRL B-2567(T)	100.00
4.	13S (S0010)	*Streptomyces* cinereoruber subsp. cinereoruber NBRC 12756(T)	99.86
5.	12S (S0055)	*Streptomyces* cinereoruber subsp. cinereoruber NBRC 12756(T)	100.00
6.	8S (S0017)	*Streptomyces* cinereoruber subsp. cinereoruber NBRC 12756(T)	99.86
7.	5S (S0029)	*Streptomyces* cinereoruber subsp. cinereoruber NBRC 12756(T)	99.86
8.	2S (SO155)	*Streptomyces* cinereoruber subsp. cinereoruber NBRC 12756(T)	99.86
9.	21S (S0043)	*Streptomyces* enissocaesilis NRRL B-16365(T)	100.00
10.	1S (S0035)	*Streptomyces* enissocaesilis NRRL B-16365(T)	100.00
11.	19S (S0072)	Mesorhizobium huakuii IAM 14158(T)	98.45
12.	16S (S0154)	Mesorhizobium huakuii IAM 14158(T)	98.45
13.	18S (S0131)	Mesorhizobium huakuii IAM 14158(T)	98.45
14.	15S (S0014)	Nocardiopsis synnemataformans DSM 44143(T)	99.86
15.	11S (S0002)	Nocardiopsis synnemataformans DSM 44143(T)	99.86
16.	9S (S0016)	Nocardiopsis synnemataformans DSM 44143(T)	99.86
17.	14S (S0143)	Pseudomonas taiwanensis BCRC 17751(T)	99.79

4.5. Chemotaxonomic analysis

Chemotaxonomic analyzes were performed to determine the characteristic chemical properties of cell wall of the isolates. For this aim diaminopimelic acid and sugar analyzes were carried out Table 4.6.

Table 4.6. 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

4.5.1. Diaminopimelic acid test

The aim this study is to determine DAP type in the cell wall of test strains. The spots were determined when 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-A2pm content of isolates of the genus *Streptomyces* while one strain (S0014) contained mezo-A2pm which was identified as Nocardiopsis genus from 16 S rDNA analysis (Figure 4.10).

4.5.2. Sugar analysis

The whole cell sugar profile in the cell wall chemo-type was determined by comparing two standards that contains seven sugars for test microorganisms in one dimensional thin layer chromatography. It is seen on Figure 4.11 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 4.11).

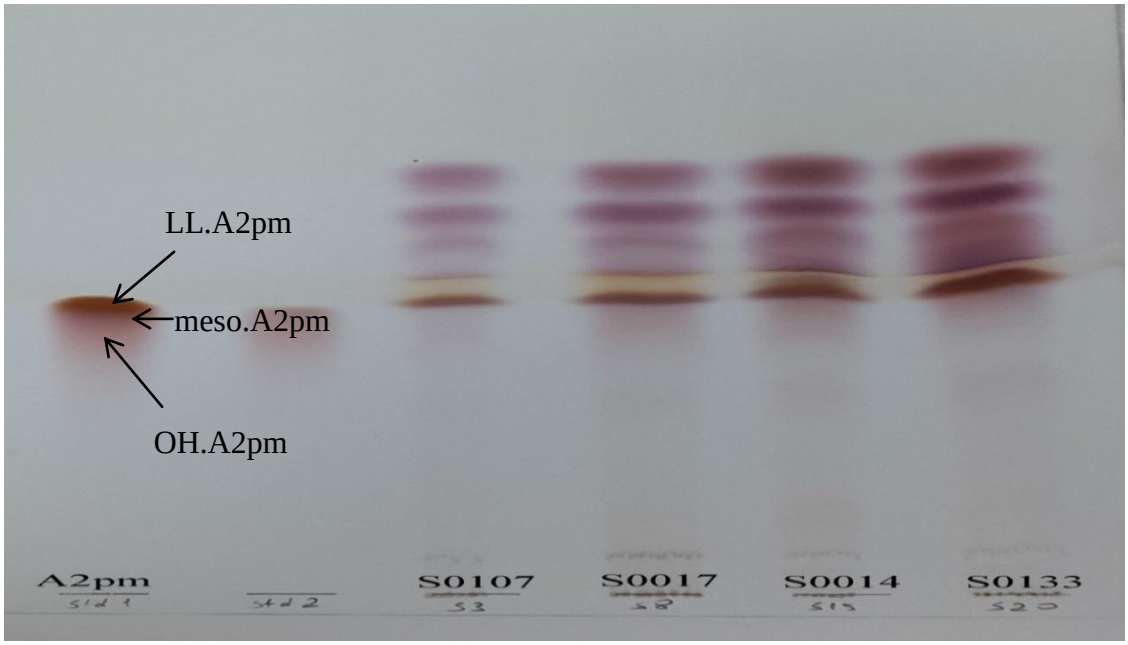


Figure 4.13. One-dimensional thin film chromatogram of A2pm isomers of 4 isolates. Two Standard: A2pm (Diaminopimelic acid DAP) – Sigma

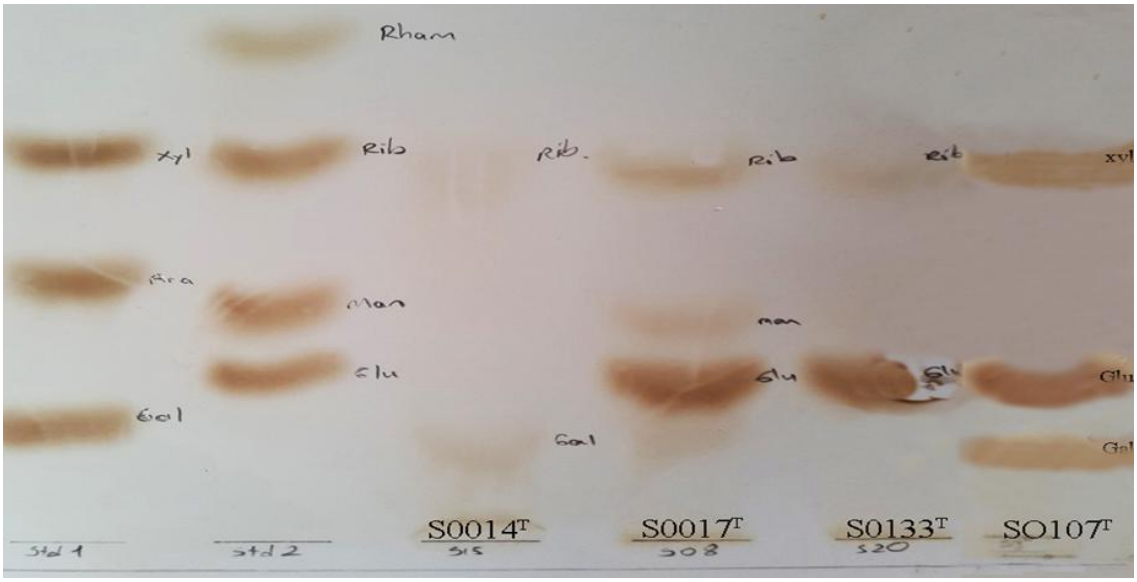


Figure 4.14. One-dimensional thin-layer chromatogram of the sugar profile of 4 test isolates. Std1, standard 1: Gal, galactose; Ara, arabinose and Xyl; xylose. Std 2, standard 2: Glu; glucose; Man, mannose; Rib, ribose and Rham, rhamnnose

5. DISCUSSION

Halabja is unusual and underexplored habitats exposed chemical bomb 1988 and the city is located in North of Iraq. Such places may be a ecosystems 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 have the ability to 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. Physicochemical character of soil samples were normal level comparing to other places such as European or Turkey countries (Atalan 1993). Organic matter content, moisture content and pH were comparable to other soil samples. Organic matter content were ranged from 9.3 to 20,4% while moisture content of samples were changed between 6 and 9.2%. It is suprise to isolate actinobacteria genera but low number of *Streptomyces* bacteria were only isolated from 30 soil samples and no colony observed from 18 soil samples. Normaly, high number of *Streptomyces* bacteria live in soil worldwide. Classical dilution method were 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 heterogen 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 low level of moisture content and high pH of sampels. pH of isolation media were adjusted to 7 but most of soil samples were about 8.

In our study, *Streptomyces* colony were isolated from 12 soil samples out of 30 and low number of colonies were appeared on selective isolation plates. The highest number of *Streptomyces* were counted 200000 cfu/g dry soil on raffinose histidine agar from soil sample S10 while 98000 cfu/g dry soil on starch-casein agar plates. It is suprise to isolate low number of *Streptomyces* owing to high count expectation. 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 programmes 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 that Halabja soil is one of this kind geography. So, it is need to dereplicate and rapidly characterise streptomycetes isolated from natural habitats in order to select representative strains for ecological and screening purposes.

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 4thand 5th colour group contained two isolates S0014, S0055 and S0100, S0103 and 8th and 10th colour group contained 28 and 30 strains. 20 representatives stains from colour grouping were selected for further study such as numerical analysis, molecular identification and chemotaxonomic studies. Representatives selected strains are shown on Table 4.3 are bold characters. Test strains were selected according to number of strains contained in the colour groups that higher number of groups higher strains.

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 4.3). 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).

Last 2 decades, use of PCR and DNA sequencing, 16S rDNA sequencing is widespread technique to identfy and characterise microorganisms. Particularly, 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria in microbiology laboratories. The technique help microbiologist to identify accurately test organisms. Meantime it help clinician for chossing antibiotics and infection control procedures (Woo et al 2008). 16S rDNAgene is a gene that is stabile, conserved and impossibility of gene transfer. Nevertheless it is being used in taxonomic study and identification of bacteria (Ramasamy et al. 2014). In this study, total genomic DNA of 20 test strains were extracted (Figure 4.5) and 16S rDNA gene of these stains were amplified (Figure 4.6). DNA sequencing of 16S rDNA of 20 test strains were carried out by Macrogen Company (Netherland) but 3 of them were

not sequenced for some reason. So base sequence of them are given in Appendix II. The almost-complete 16S rDNA gene sequence of 17 test strains were compared with the 16S rDNA gene sequences of all members of the genus *Streptomyces*. The highest level of sequence similarity of 21S (S0043) and 1S (S0035) were found to be 100% with S. enissocaesilis (Figure 4.9), while 12S (S0055) was 100% similar to type strain S. cinereoruber subsp. cinereoruber (Figure 4.8). Test strains of 20S (S0133), 7S (S0139) and 3S (S0107)were 100% similar to type strain of S. badius (Figure 4.10), while strain 8S (S0017), 5S (S0029) 13S (S0010) and 2S (S0155) were 99.86% similar to type straıns *S. cinereoruber subsp. cinereoruber* (Figure 4.8). strains were grouped together and seems are new species. Coenye at al. (2005) suggested that a new species should be at least 3% nucleotid diferences of 16S rDNA gene and less than 70% DNA-DNA homology similarity. To intruduce these test strains as new species, DNA-DNA homology test should be carried for these strains with nearest type strains. 16 (S0154), 18S (S0131) and 19S (S0072) strains are 98,45% similar to type strain of Mesorhizobium huakuii (Figure 4.12) probably new species, it make a new clade in phylogenitic tree. Test strains of 9S (S0016), 11S (S0002) and 15S (S0014) were grouped together and 99.86% similar to their type strain *Nocardiopsis synnemataformans* (Figure 4.11). Similarity of test strains based on DNA sequencing of 16S rDNAare given on Table 4.5. The sequences were used to construct a neighbour-joining phylogenetic tree test strains which demonstrated the relationship of strain 20S, 7S and 3S to closely related *Streptomyces* species. Furthermore, DNA–DNA hybridization should be done between test strains and type strains of *Streptomyces*. One of test strain 14S (S0143) was identified 99,79% *Pseudomonas taiwanensis,* but it is probably contaminated test strain during purification.

Chemotaxonomic profiles of 4 test strains are shown in Table 4.6. It is seen on Figure 4.13 that DAP type of cell wall of S0107, S0017, S0133 strains are LL- type, but S0014 strains is meso- type. HV38T can be distinguished from previously described species of the genus *Streptomyces*. Also, cell Wall of S0107, S0017 and S0133 test strains contain glucose, ribose and mannose sugars (Figure 4.14). 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), molecular identification and chemotaxonomic identification (DAP test and sugar analysis) are showen on Table 5.1. Most of the results from different technique are mostly agree each other.

Table 5.1. Comparation of polyphasic taxonomy of three different technique for identification of test microorganisms

Strain No.	Color grouping	Numerical analysis	16S rDNA study	DAP type	Sugar type
S01 (S0035)	6	VII	*Streptomyces enissocaesilis*		
S03 (S0107)	6	VII	*Streptomyces badius*	LL-A2pm	glucose, ribose and mannose
S02 (S0155)	3	IX	*Streptomyces cinereoruber subsp. cinereoruber*		
S08 (S0017)	3	IX	*Streptomyces cinereoruber subsp. cinereoruber*	LL-A2pm	glucose, ribose and mannose
S04 (S0100)	5	III	Bad sequencing result		
S05 (S0029)	9	IV	*Streptomyces cinereoruber subsp. cinereoruber*		
S06 (S0037)	2	II	Bad sequencing result		
S07 (S0139)	2	II	*Streptomyces badius*		
S09 (S0016)	10	I	*Nocardiopsis synnemataformans*		
S11 (S0002)	10	I	*Nocardiopsis synnemataformans*		
S21 (S0043)	10	I	*Streptomyces enissocaesilis*		
S12 (S0055)	4	V	*Streptomyces cinereoruber subsp. cinereoruber*		
S15 (S0014)	4	V	*Nocardiopsis synnemataformans*	mezo-A2pm	galactose and ribose
S13 (S0010)	1	VI	*Streptomyces cinereoruber subsp. cinereoruber*		
S14 (S0143)	7	X	*Pseudomonas taiwanensis*		
S17 (S0115)	7	X	Bad sequencing result		
S16 (S0154)	7	V			
S18 (S0131)	8	VIII			
S19 (S0072)	8	VIII	*Mesorhizobium huakuii*		
S20 (S0133)	8	VIII	*Streptomyces badius*	LL-A2pm	ribose and mannose

CONCLUSION

In this study, polyphasic taxonomy which are genomic, chemotaxonomic and phenotypic method was carried out to identfy and characterise strains isolated from soil samples of Halabja, Iraq. Polyphasic taxonomy is important approach to identfy a bacteria at species level (category) owing to based on 3 different method (Coenye at al. 2005).

Species descirebed at genus level according to base sequences of 16S rDNA gene can be identify as species by applying DNA-DNA homology test which having maximum 3% nucleotid diferences of test strains with the nearest type strain. Also chemotaxonomic method such as sugar analysis and DAP type of cell wall can be used to characterise the test strain further.

The aim of this thesis 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 oat meal and pepton iron agar. 20 strains selected from colour groups and phenotipic, molecular and chemotaxonomic study carried out to identfy.

In conclusion, it is evident from the genotypic, chemotaxonomic and phenotypic data that following deduction can be say briefly from this study.

1. 105 *Streptomyces* strains were isolated from Halabja soil is poor source for actinomycetes. This may be owing to mustard bomb at 1988.
2. Test strains of 8S, 5S 13S and 2S may be a new species of *Streptomyces*. To say that, DNA-DNA homology should be carried out with *S. cinereoruber subsp. cinereoruber*. Our isolater may have potential for biotechnological product such as antibiotics and ezymes of in to new isolate of virgin soil. they can be scan for new antibiotics or other biotechnologic in industrial compounds

3. 16S, 18S and 19S strains were identified as type strain of Mesorhizobium huakuii and probably new species because similarity with their type strain 98.45% , 22 neucliotide differ from their type strain and form a new clade on phylogenetic dendrogram. To say that, DNA-DNA homology should be carried out with thier type strain.
4. Test strains of 15S, 11S and 9S were identified as *Nocardiopsis synnemataformans.*

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APPENDIX

APPENDIX I:

Media and Reagents

1. MEDIA

Bennetts agar (modified after Jones, 1949)	
Yeast extract	1 g
Lab Lemco	0.8 g
Bacto Casitone	2.0 g
Glycerol	10.0 g
Agar	18 g
Distilled water	1000 ml
Autoclaved at 121°C for 20 minutes pH adjusted to 7.2	

Carbone Source Utilisation Medium (Shirling & Gottlieb, 1966)	
Amonium sulphate	2.64 g
KH_2PO_4	2.38 g
K_2HPO_4	5.65 g
$MgSO_4$	1 g
Pridham and Gottliebs trace salts solution	1 ml
Bacto-agar	18 g
Distilled water	1000 ml
Autoclaved at 121°Cfor 20 minutes pH adjusted to 7.2	

Carbon source: solution of each carbon source were sterilized by Tyndallization, then aseptically added to sterile basal medium to give a final concentration of 1.0 g l^{-1} or 10.0 g l^{-1}

Chemical inhibitors tolerance tests: Modified Bennetts agar for each individual tests was supplemented with a sterile solution of the following compounds.	
Sodium chloride	7%, w/v
Sodium azide	0.01%, w/v
Phenol	0.1%, w/v
Cristal violet	0.0001%, w/v

Nitrogen Source Utilisation Medium (Williams et al., 1983a)	
Glucose	10.0 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g
K_2HPO_4	1.0 g
NaCl	0.5 g
Bacto-agar	6.0 g
Distilled water	1000 ml
Autoclaved at 121°C for 20 minutes pH adjusted to 7.2	

Raffinose-histidine agar (Vickers et al., 1984)	
Raffinose	10.0 g
L-Histidine	1.0 g
$MgSO_4.7H_2O$	0.5 g
$FeSO_4.7H_2O$	0.01 g
K_2HPO_4	1.0 g
Bacto-agar	15.0 g
Distilled water	1000 ml
Raffinose and histidine were added after steaming the basal medium . Autoclaved at 121°Cfor 20 minutes pH adjusted to 7.2	

Starch Casien Agar *(Küster & Williams, 1964)*	
Starch (dissolved separately)	10.0 g
Casien	0.3 g
KNO_3	2.0 g
NaCl	2.0 g
K_2HPO_4 (Autoclaved separately)	2.0 g
$MgSO_4.7H_2O$	0.05 g
$FeSO_4.7H_2O$	0.01 g
$CaCO_3$	0.02 g
Agar	18 g
Distilled water	1000 ml
Autoclaved at 121°Cfor 15 minutes pH adjusted to 7.2	

Nitrate agar (Skerman, 1967)	
KNO_3	2.0 g
Nutrient broth	1000 ml
Bacto-agar	6.0 g
Autoclaved at 121°Cfor 15 minutes pH adjusted to (7.2). Hydrogen sulphide production was detected by inserting sterile lead acetate filter paper strips in to the necks of the test tubes	

Lecithinase and Lypolysis reactions: Egg Yolk Agar (Nitsch & Kutzner, 1969)	
Bacteriological peptone	10.0 g
Glucose	1.0 g
NaCl	10.0 g
Yeast extract	5.0 g
Bacto-agar	15.0 g
Egg-yolk emulsion	5%, v/v
Autoclaved at 121°Cfor 15 minutes pH adjusted to 7.2	

Urea reduction	
KH_2PO_4	9.1 g
Na_2HPO_4	9.5 g
Phenol red	0.01 g
Yeast extract	0.1 g
Distilled water	1000 ml
pH	7.2
Autoclaved at 121°Cfor 15 minutes pH adjusted to 7.2 Urea solution (15%, w/v) (supplement after autoclaved)	133 ml

Degradation test: Modified Bennetts agar for each tests was supplemented with sterile solution of one of the following compounds	
Xanthine	1%, w/v
Gelatine	1%, w/v
Starch	1%, w/v
Casien	1%, w/v

Pridham and Gottliebs trace salts solution (Shirling & Gottlieb, 1966)	
$CuSO_4.5H_2O$	0.64 g
$FeSO_4.7H_2O$	0.11 g
$MnCl_2.4H_2O$	0.79 g
$ZnSO_4.7H_2O$	0.15 g
Distilled water	100 ml

Oatmeal agar, Peptone yeast extract iron agar and Muller Hinton agar

2. Reagents

Nitrate reduction tests

Reagent A: 0.8 ml sulphanilic acid in 100 ml of 5 molar acetic acid

Reagent B: 0.6 ml of dimethyl-naphthylamine in 100 ml of 5 molar acetic acid

APPENDIX II: Base sequences of 16S rDNA of 17 test strains

>1S (S0035)

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GGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTG
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CGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGAGGAAGGT
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>14S (S0143)

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TCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCT
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TCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATC
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GCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTT
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CTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCGGTGACAA
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GCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATAC
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>15S (S0014)

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GCCACCTCATGGTGGAGGGTGGAAAGTTTTTCGGTCAGGGATGGGCTCGCGG
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>16S (S0154)

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>18S (SO131)

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>19S(S0072)

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CCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTTGGTTTTACCCGAA
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>20S (S0133)

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GTCCCGCATGGGACGGGGTTGAAAGCTCCGGCGGTGAAGGATGAGCCCGCG
GCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGC
CGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGCGAAAGCCTGATGC
AGCGACGCCGCGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCA
GGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACG
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GCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCT
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>21S (S0043)

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CCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTCGGAGGAAG
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TACAATGGCCGGTACAATGAGCTGCGATACCGCGAGGTGGAGCGAATCTCAA
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GCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGGTGGGACTGGCGATTGGGAC
GAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGA

Note: 1st code is from anaysis program and code in bracket is lab code of test strains

CURRUCULAM VITAE

I was born on January 11th1986 in Gamesh Tappa village / sayid sadiq-sulaimani of Kurdistan region of Iraq. I completed my primary school in Nasir, secondary and high school in sayid sadiq. I started studying biology science at University of Sulaimani, collage of science/ biology department in 2007 and graduated in 2011, holding a bachelor's degree in biology science. I work as an assist biology in the Medical laboratory techniques department at Halabja Technical Institute/ Sulaimani Polytechnic University from 2013 to present. I started my masters study in February 2015 at Bingol University/ Turkey and awarded the master's degree in Microbiology in January 2017.

Mobile: 009647507175249
Email:syamandbio@gmail.com