Journal of Biology and Medical Sciences - JBMS
An Open Access International Journal published by University of Babylon, Iraq
Vol.1 2013

Isolation and Identification of Streptomyces from Different Sample of Soils

Ali. Al-Saadi, NooraMajidHameed, Eman Mohammad Jaralla
University of Babylon, College of Science
noora_majid_89@yahoo.com

Abstract
A total of 36 actinomycetes were isolated and purified from soil samples collected from agricultural soils in Hilla. The isolates were morphologically distinct on the basis of spore mass color, reverse slide color, aerial and substrate mycelia formation and production of diffusible pigment. Only two isolates which were S.A.2 and S.S.10 was selected for further investigation due to its strong antibacterial activity against six pathogenic bacteria which were *(Staphylococcus aureus*, *Escherichia coli, Pseudomonas aeroginosa,Serratiamarcescens, Klebsiella pneumonia, Aeromonashydrophila).*These two isolates was identified as*Streptomycesorientalis*and *Streptomyces humidus* respectively based on its morphological, cultural, physiological, microscopic features, utilization of carbon sources, biochemical characteristics and molecular analysis of the 16S rRNA gene primers.
Keywords :actinomycetes, spore mass color, 16S rRNA gene primers.

Introduction

Actinomycetes are a group of prokaryotic organisms phylogenetically grouped as gram-positive bacteria with high guanine + cytosine in their DNA. Most of them are in subclass Actinobacteridae*,* order actinomycetales comprising of 14 suborders, 49 families, and over 140 genera (Adegboye and Babalola, 2012).They are filamentous bacteria which produce two kinds of branching mycelium, aerial mycelium and substrate mycelium. Actinomycetes constitute a significant component of the microbial population in most soils and *Streptomyces* a count for 90% of the total Actinomycetes population (Poopal and Laxman, 2009). They produce a wide range of secondary metabolites and more than 70% of the naturally derived antibiotics that are currently in clinical use are derived from soil actinomycetes (Elardo*et al*., 2009).In particular, the genus *Streptomyces* accounts for about 80% of the actinomycete natural products reported to date (Bull and Stach, 2007)**.** The genus *Streptomyces* was proposed by Waksman &Henrici for aerobic and spore formingActinomycetes (Williams *et al*. 1989).They are well known by a linear chromosome, complex morphological differentiation(El-Gendy*et al.,* 2008b). The most interesting property of *Streptomyces* is the ability to produce bioactive secondary metabolites such as antibacterials, antifungals, antivirals, antitumoral, anti-hypertensives and mainly antibiotics and immunosuppressives (Patzer and Volkmar, 2010). Many species belonging to the genus *Streptomyces* are well known as biocontrol agents that inhibit or lyse several soil borne and air borne plant pathogenic fungi (Sousa *et al*., 2008).

Materials and methods

Samples collection

Agricultural soil samples was collected from diffirent sites in Hilla at various depth of surface, ranging from layers of 15 to 20 cm depth. The samples were collected in sterile small plastic containers by using a trowel and properly labeled indicating the date of collection and the depth and transferred to the laboratory for the study.

Isolation of *Streptomyces* from soil

Soil samples were mixed thoroughly and passed through 2 mm sieve filter to remove gravel and debris. The samples were kept at 55 °C for 5 min, for pre - treatment. In conventional dilution plate technique, 1 gm of soil sample was suspended in 9 ml of sterile water and successive dilution was made upto 10^{-4}. An aliquot (0.5 ml) of suspension from the last dilution test tube was spread on yeast-malt extract agar medium (ISP-2 according to (Pridham*et al*., 1957)and incubated for 7-9 days at 28-30°C. After incubation period, the plates were examined for typical colonies of *Streptomyces*. The typical round, small, opaque, compact, frequently pigmented colonies were examined under a light microscope (100X). The colonies that bear typical *Streptomyces* morphology were purified and sub-cultured on Yeast extract-Malt extract agar plates and stored for further assay (Bernard, 2007).

Initial screening of the pure isolates

Preliminary screening for inhibitory metaboliteproducing ability of the isolate was tested by Cross streakmethod against one Gram positive bacteria(*Staphylococcus aureus*), five Gram negative bacteria *(Escherichia coli, Pseudomonas aeroginosa, Serratiamarcescens, Klebsiellapneumoniae,* and*Aeromonashydrophila*). The isolate was inoculated as a single streak in the centre of the petridish containing Mueller Hinton medium and incubated at 28°C for 3-4 days to permit growth and antibiotic production. Later the test bacteria were inoculated by streaking perpendicular to the growth of isolate. The plates were incubated for 24-48 hours at 37°C. After

incubation, inhibition of test bacteria around the growth of isolate was taken as positive for inhibitory activity (Nanjwade*et al*., 2010).

Secondary screening

The two active isolates were inoculated on fermentation broth medium according to (Ahmed, 2007), after incubation for 10-15 days at 28°C, the cultures were filtered by Waksman No.1 filter and the antimicrobial agent extracted using organic solvent Ethyl acetate (V:V), then tested for its inhibitory activity by agar well diffusion method (Atta, 2010).

Identification of S.A.2 and S.S.10 isolates:

The isolates S.A.2 and S.S.10 was further characterized based on morphological, biochemical, cultural, physiological features and microscopic characterization. Cultural characteristics were tested in yeast extract-malt extract agar (ISP-2), oatmeal agar (ISP-3according to (Kuster, 1959a), inorganic salt-starch agar (ISP-4 according to (Kuster, 1959a.), glycerol-asparagine agar (ISP-5according to(Pridham and Lyons, 1961), peptone-yeast-extractiron agar (ISP-6 according to (Tresner and Danga, 1958), tyrosine agar (ISP-7 according to (Shinobu, 1958) and carbone utilization medium (ISP-9 according to (Modified from Pridham and Gottlieb, 1948).Biochemical tests including (starch and gelatin hydrolysis, voges-proskauer, citrate utilization, Indole, methyl red, oxidase,H_2S production and blood hemolysis according to (MacFaddin, 2000), lecithinase production according to (Janda and Bottone, 1981), catalase according to (Colleet *al*., 1996).. In addition to genomic DNA extraction according to method recommended by SolGnet kit for DNA extraction and molecular amplification of 16S rRNA gene primersF, (5'ACGTGTGCAGCCCAAGACA3) and R,(5'-ACAAGCCCTGGAAACGGGGT-3) (Atta *et al*., 2011).

Results and Discussion

Antimicrobial activity assay

The two active isolates showed inhibitory activity against six test pathogenic bacteria as shown in table (1 and 2) and which were chosen for further investigation. S.A.2 isolate showed inhibitory activity against (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeroginosae and Klepsiellapneumoniae*) but negative for (*Serratiamarcescens and Aeromonashydrophila),* while S.S.10 isolate showed inhibitory activity against all test bacteria but with less ability to inhibit growth of test pathogenic bacteria than S.A.2 isolate.

Table (1) Primary screening of *Streptomyces* isolates for antimicrobial activity

Test bacteria	Results	
	S.A.2	S.S.10
Staph. aureus	+	+
E. coli	++	+
Pseudo.aeroginosae	+	+
Serr. marcescens	-	+
Kleb. pneumonia	+	+
Aero. hydrophila	-	+

Table (2) Inhibition zone of culturefiltrate extract of the two isolatesagainst test organisms on MuellerHinton agar medium measured bymm., (R: Resistant).

Test bacteria	Results	
	S.A.2	S.S.10
Staph. aureus	**18**	**9**
E. coli	**20**	**9**
Pseud. aeroginosae	**15**	**7**
Serr. marcsesenc	**R**	**13**
Kle. pneumonia	**8**	**14**
Aer. hydrophila	**R**	**11**

Identification of Actinomycete isolates

Morphology, as previously mentioned, has alwaysbeen an important characteristic used to identifyactinomycete strains, and, in fact, it was theonly characteristic used in many early descriptions,particularly of *Streptomyces* species in the first feweditions of *Bergey's manual.* Morphological observationsare best made on a variety of standard cultivationmedia. Several of the media suggested forthe International *Streptomyces* Project(Shirlingand Gottlieb, 1966) and by(Pridham*et al.,* 1956) have proven to be useful in ourhands for the characterization of

strains accessionedinto the Actinomycetales Culture Collection.Most clinical microbiologylaboratories offer presumptive identification of *Streptomyces*species, ie, long filamentous gram-positive bacteria thatgrow aerobically and are negative for partial acid-fast stain.These features distinguish *Streptomyces* species from othermorphologically similar genera within Actinomycetales, ie,anaerobic *Actinomycetes*, and aerobic *Nocardia*and*Rhodococcus*that are usually partially acid-fast. The actinomycete isolates was identified on the basis of microscopic, cultural, biochemical and molecular characteristics.Scanning electron microscopy can providefar more detailed information concerning the sporulationmicromorphology of actinomycetes, particularlythose whose spore structures are associatedwith the vegetative mycelium. Both of two isolates spore chain arrangement was examined under light microscope and have spore chains fexibiles and smooth spore surface under scanning electron microscope.

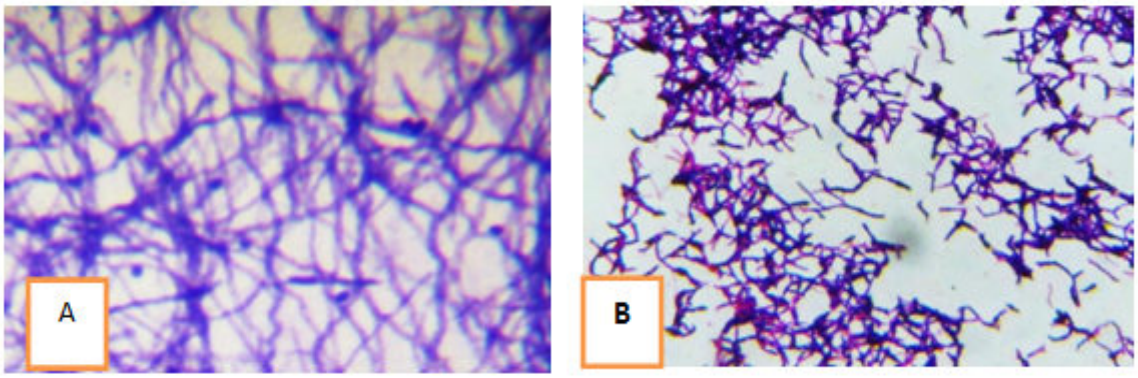

Figure (1) Mycelium morphology of (A) S.A.2 isolate and (B) S.S.10 isolate

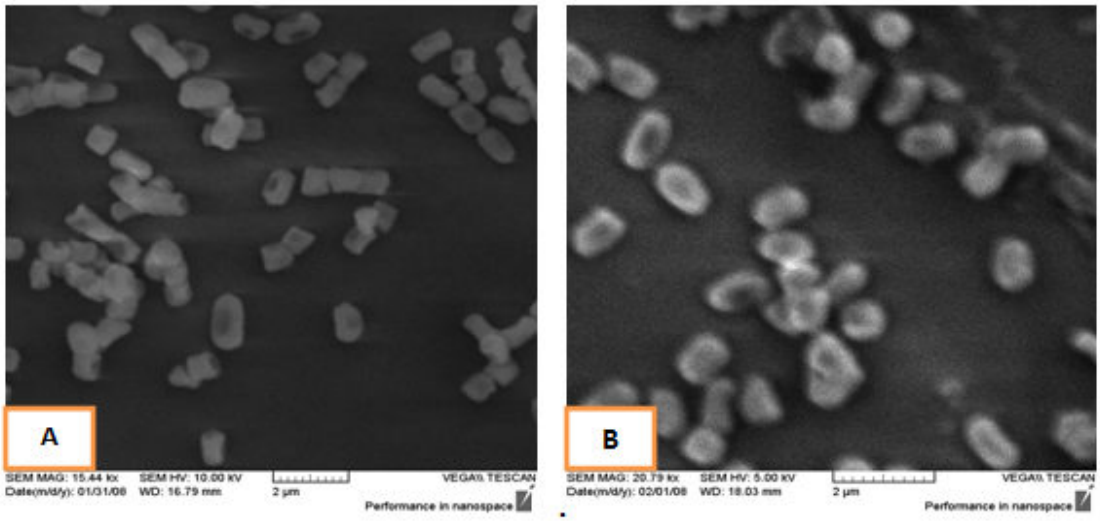

Figure (2) Spores morphology for (A) S.A.2 isolate and (B) S.S.10 isolate

The colonies was white and Grey on ISP-3 medium respectively, with no melanin production and no diffusible pigments in ISP-6 and ISP-7. Both of isolates could utilize Arabinose, Fructose, Xylose and Mannitole but negative forRaffinose, Sucrose and cellulose, In case of S.A.2 could utilize Inositol while S.S.10 negative for Inositol.

Table (3) Morphological and physiological characteristics of *Streptomyces* isolates on ISP media

ISP media	S.A.2		S.S.10	
	Aerial mycelium	Substrate mycelium	Aerial mycelium	Substrate mycelium
ISP - 2 / Yeast – Malt extract agar	White	Yellow	White	Yellow
ISP - 3 / Oatmel agar	White	White	Gray	Yellow
ISP - 4 / Inorganic salts agar	Gray	Gray	Gray	yellow
ISP - 5 / Glycerol Asparagine agar	Yellow	Yellow	Yellow	Yellow
ISP - 6 / Peptone-yeast extract iron agar (melanin production)	-		-	
ISP – 7/ Tyrosine agar (melanin production)	White / -	White / -	White / -	White / -
ISP – 9/ Utilization of Carbone sources				
Positive control (D-Glucose)	+		+	
Negative control (no Carbone source)	-		-	
Arabinose	+		+	
Raffinose	-		-	
Fructose	+		+	
Sucrose	-		-	
Xylose	+		+	
Mannitole	+		+	
Cellulose	-		-	
Inositole	+		-	
Diffusible pigments	-		-	

The two isolates was positive for Voges-proskauer, citrate, H_2S and starch hydrolysis but negative for methyl red, indole and catalase, first isolate was positive for Gelatine liquefaction and oxidase while the second isolate was negative, blood hemolysis test was positive in second isolate but negative in first isolate. Theseresults emphasized that the actinomycetes isolate relatedto a group of *Streptomyces* (Williams, 1989 and Hensyl,1994). In view of all the previously recorded data of the two isolates, it could be stated that the isolates (S.A.2 and S.S.10) belonging to *Streptomycesorientalis*that agreed with(Antonova et al., 2005)and*Streptomyces humidus*respectively (Kuster, 1972). Regarding the spore chain type of *Streptomyces humidus*, (Pridham and Tresner, 1975) apportioned itto the Spiralesbut later, in the description only referred to it as atypical Retinaculum Apertum. (Pridham and Tresner, 1975) also apportioned the spore mass of this actinomycete to the Grey colour series. (Hnamura*et al.,*1956 ; Waksman, 1961 and Gauze *et al.,*1983) regarded S. *humidus*to be closely related toS. *hygroscopicus*and couldProducedihydrostreptomycin, humidin and cobalamines (Hnamura*et al.* 1956 and Pridham and Tresner, 1975).

Table (4) Biochemical tests for *Streptomyces* isolates

Test	S.A.2	S.S.10
Gram stain	+	+
Indole	-	-
Methyl red	-	-
VogesProskauer	+	+
Citrate Utilization	+	+
Lecithinase	+	-
Starch Hydrolysis	+	+
Gelatineliquifaction	+	-
Catalase	-	-
Blood Hemolysis	-	+Alpha
H_2S Production	Acidic – Acidic No gas No H2S	Acidic – acidic No gas No H2S
Oxidase	+	-

This result was supported by molecular amplification of 16S rRNA gene primers thatshowed 1.5kbp fragments for both isolates which indicates that the two isolates belonged to the genus *Streptomyces*.

DNA ladder S.A.2 S.S.10 S.A.2 S.S.10

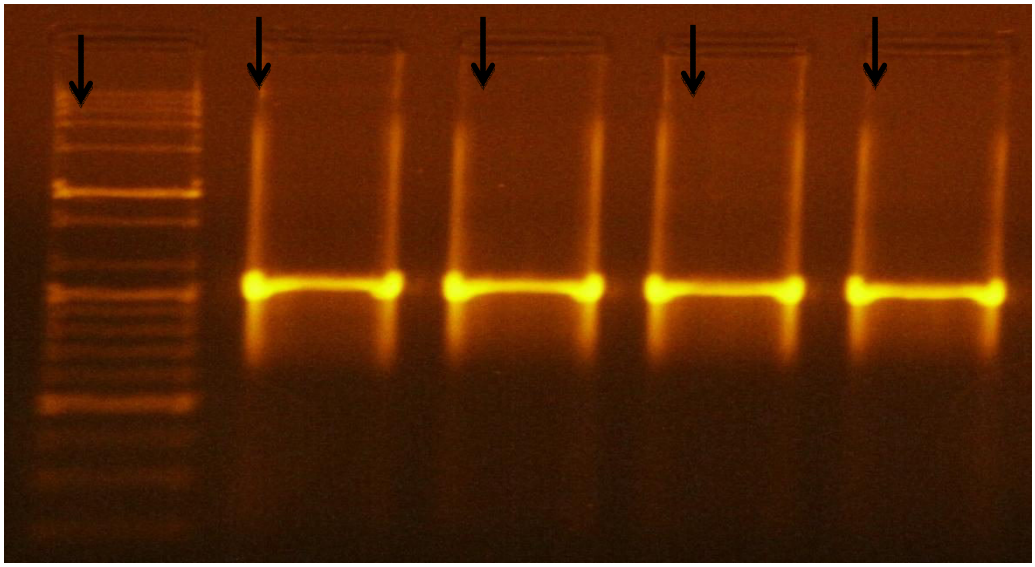

Figure (3) Amplification of 16S rRNA gene primers for the two isolates

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