# Extraction and Purification of Antimicrobial agent Produced from Actinomycetes Isolated from Agriculture Soils

**Eman mohammd jarallah , noor haidar Rahman**

## Abstract:

One hundard and twenty agricltuer soil samples were collected from different sites of Hilla city during the period from October/2011 to Dcenmber /2012. Only fifteen Actinomycetes isolate were obtained .After purification of these isolate ,the antimicrobial activity was tested for all of them .One isolate have been produce antimicrobial activity against three type of bacteria *Escherichia coli* (18mm), *Pseudomonas aeruginosa* (8mm) and *Staphylococcus aureus* (8mm) and one fungus which was *Mucor sp*.(22mm).Morphological and agricultural properties of this isolate were studies .So it grown on starch casein agar (ISP1), Yeast extract-malt extract (ISP2), Oatmeal (ISP 3), Inorganic salt-starch (ISP 4), Glycerol asparagines agar (ISP 5), Nutrient agar, Tyrosine agar (ISP 7) and clucose aspargin agar. For characterization of the isolate Actimicrobial agent was purified using thin layar chromatography(TLC) method with n-butanol-ethanol-water(4:1:5) as asolvant .After 45min Rf value was 0.42.

## الخلاصة :

تم جمع ١٢٠ عينة تربة زراعية من مناطق مختلفة من مدينة الحلة من فترة تشرين الاول وحتى كانون الاول /٢٠١٢ . وقد وجد ان خمسة عشر عزلة تعود الى جنس الاكتينومايسيس . وبعد تنقية العزلات درست فعاليتها المضادة للميكروبات . وقد اظهرت النتائج ان عزلة واحدة فقط تمتلك هذه الفعالية ضد ثلاث انواع بكتريا وهي اشريشيا القالونية (١٨) ملم والزوائف الزنجارية (٨ )ملم والعنقوديات الذهبية (٨ )ملم بالاضافة الى نوع واحد من الفطريات تابع لجنس الميوكر( ٢٢) ملم .وقد درست الصفات المظهرية والزرعية لهذه العزلات وذلك بزراعتها على اوساط غذائية مختلفة وسط النشأ والكازئين الصلب ، وسط خلاصة الخميرة والشعير ،وسط الشوفان ،وسط النشأ والاملاح اللاعضوية،وسط الكليسيرول والاسبارجين الصلب ،وسط الاكار المغذي ،وسط التايروسين ووسط الكلوكوز والاسبارجين الصلب . تم تنقية العامل المضاد للميكروبات باستعمال كروماتوكرافية الطبقة الرقيقة باستخدام محلول التشرب الذي يتكون من البيوتانول –ايثانول–ماء :٤:١:٥ وقد وجد ان قيمة معدل الجريان هي ٠.٤٢ بعد ٤٥ دقيقة.

## Introduction

*Actinomycetes* are gram positive bacteria ,free living ,saprophytic bacteria widely distributed in natural and manmade environments, and play an important role in the degradation of organic matter. They are also well known as a rich source of antibiotics and bioactive molecules, and are of considerable importance in industry. Tissue culture microtiter-plate based screens were developed for the cytotoxic agents *Streptomyces* are the most well known genus of *Actinomycetes* family (Kanzaki *et al.*, 2000). It is represented in nature by the largest number of species and varieties among the family Actinomycetaceae. They differ greatly in their morphology, physiology and biochemical activities, (Suneetha and Zaved, 2011). They have ability to produce and secrete a large variety of industrial ,medical,biotechnological and aricultural secondary metabolites. *Streptomyces* is the largest antibiotic genus producing both antibacterial and antifungal ,and also a wide range of other compounds such as immunosuppressant.They produce over two thirds of the clinically useful antibiotic of natural origin (e.g neomycin and chloroamphenicol). Serious infections caused by bacteria that have become resistant to commonly used antibiotics has become a major global healthcare problem in the 21st century (Alanis,2005).

Nevertheless,aperiodic replace of the existing antibiotic is necessary to prevent transmissible resistant among microorganism to aviable antibiotics already marketed . Antibiotics are antimicrobial compounds produced by living microorganisms .These compounds were used therapeutically and some times prophlactically in the control of

infections diseases . Over 4000 antibiotic have been isolated befor ,there are only 50 have achieved wide usage .The other antibiotic compounds failed to achieve commercial importance for some reason such as toxicity to human and animal ,ineffectiveness or high production costs (Smith ,1996). Many antibiotic were produced by microorganism as secondary metabolites the isolation of antibiotics from microorganism is relatively easy as compared to chemical synthesis of antimicrobial agent .(Kulkarin and Aynihorjri ,1995).

## MATERIALS AND METHODS

### Soil sampling

One hundarnd and twenty soil samples were randomly collected from different sites of Hilla city using an openend soil borer (10 cm depth and 2.5 cm diameter) then air dried, by incubation at 30°C for 10 days in order to reduce the incidence of bacteria and molds (Lee *et al*., 2005).

### Isolation of *Streptomyces spp*

Plates containing basal salts agar medium adjusted to pH 7.0 were used for isolation of the isolates using the serial soil dilution technique .The medium composed of the following components(g/l): 20.0, starch; 2.0, potassium nitrate; 1.0, dipotassium hydrogen phosphate; 0.5, magnesium sulphate; 0.5, sodium chloride; 3.0 calcium carbonate and 0.01, ferrous sulphate. It was inoculated with a soil suspention (0.1% w/v) and incubated at 28°C for 7 days(You and Park,1996).

Purification and identification of isolates various *Streptomyces* isolates, based on their special morphological characteristics as different colored aerial mycelium with sitting colonies, were selected and purified by streaking (5-7times) on agar plates. Long term preservation of isolates was achieved in nutrient agar slant (Collins *et al*., 1995).

### Microscopic Examination

Microscopic morphology of the actinomycete isolates were examined by slide culture method. A small peripheral portion of well-grown mature parts of the colony was picked using a sterile inoculation loop. (Kavitha and Vijayalakshmi*.,* 2007).

### Cultural Characteristics:

The morphological and cultural characteristics of isolates were made according to the guidelines of the International *Streptomyces* Project(ISP). The cultural aspect of the pure isolates was studied on different ISP medium which include(Inorganic salt-starch (ISP 4), Oatmeal (ISP3), Glycerol asparagines agar (ISP 5), Tyrosine agar (ISP 7), Starch casein medium(ISP1), Yeast extract-malt extract (ISP2)and Nutrient agar, after 14 days incubation at 28°C. Colours were determined according to color symbolism (Morton, 1997).

### Physiological and biochemical characteristics

The physiological and biochemical characteristics were determined according to the methods of Shirling and Gottlieb (1966), Waksman (1954) and Holt *et al.,* (1994). Cultures were incubated at 30°C and examined after 7-14 days, except for gelatin liquefaction as it was tested during growth after 2, 4 and 7 days.Utilization of carbon sources was investigated according to(MacFaddin, 2000). Carbon sources were added to the basal salt medium at 1.0% (w/v). Growth and gas production were recorded after 7, 14 and 21 days.

**Isolation of ß-Lactamase Inhibitoer Producer:**

Streptomyces species have been isolated from different soil samples .Isolates were tested for their ability of ß-lactamase inhibitoer clavulanic acid by specific synergistic bioassay using a resistant test strain of *E.coli* at 25µg $ml^{-1}$ penicillin G (Romero and Martin; 1984) .The streptomyces isolate was cultured on the Bennetts agar medium which composed of the following:- (g $L^{-1}$) : beef extract ,1;yeast extract ,1; glucose,10;N-Z amine,2;agar15 and pH 7.3 (Locci;1989). After 7days incubation at $28C^0$ ,0.9mm agar disk of isolate used for bioassay(Higgens and Kastner,1971) .Clavulanic acid production was detected by thin layer chromatography (TLC).

**Fermentation**

A well grown agar slant of *Streptomyces spp,* was inoculated into one liter flask containing 500 ml of the fermentation medium consisting of (g/l): soluble starch, 20.0; $(NH_4)_2SO_4$, 2.0; $K_2HPO_4$, 1.0; NaCl, 1.0; $MgSO_4$, 1.0; $CaCO_3$, 2.0; trace salt solution ($FeSO_4.7H_2O$, 0.1; $ZnSO_4.7H2O$, 0.1 and 1000 ml distilled water) ,pH value of the medium was adjusted at 7.2 before sterilization. The flask was incubated at 25ºC for 5 days in incubator. Total volume was filtered through Whatman No.1 filter paper, followed by centrifugation at 5000 r.p.m for 20 minutes. The clear filtrates were tested for their activities against the test organisms (Sathi *et al.,* 2001).

**Extraction of the antimicrobial agent**

Equal volumes of the filtrate and ethyl acetate solvent was mixed thoroughly by shaking them in 250 ml capacity separating funnel and allowed to stand for 30 min. Two layers were separated; the aqueous layer and the organic layer, which contained the solvent and the antimicrobial agent. The organic layer was concentrated by evaporation under vacuum to the least volume, after the dehydration with anhydrous $Na_2SO_4$. The aqueous layer re-extracted and the organic layer added to the above organic layer. Antimicrobial activity was monitored by the agar diffusion method inoculated with the *E.coli and Psedomonas aeruginosa* and *Staphylococcus auerus* as test organism. (A control test for each solvent was also preformed). The plates were incubated at $37C^0$ for 16-24 hour(Atta, 2010).

**Purification of antimicrobial agent**

The extracted antimicrobial agent was purified by thin layer chromatography on silica gel plate in (2cm wide and 5cm length)size was used for loaded antimicrobial agent on silica gel slide a capillary tube was dipped into the extracted antimicrobial agent syrup. Then the end of a capillary tube was touched with one side of the coated slide about 1 cm from the bottom end (not allowing large drop to flow) and then allowed to air dry. After the drop dried it was then ready to be developed. These slides are kept in screwed bottles containing 5 ml of n-butanol-ethanol - water(4:1:5).Solvent in a vertical position. The solvent was allowed to run through silica gel layer until the solvent front reached about 1 cm of the top of the slides then they were removed from the bottles. The solvents were allowed to evaporate from the slides and then they were observed by examing them under the visible spectrophotmeter UV light model Camspec M330 UK as shown in figuer(Brewster *et. al.,* 1977).

# Results

**Colony isolation**

One hundarnd and twenty soil samples collected from several sites of Hilla city as shown in table-1 were15 *Actinomycetes* colonies were selected. This selection was done based on morphological characterization of bacterial colonies. The gram stain of selected bacterial cells showed a gram positive filamentous structure of *Actinomycetes.* (Figure .1),(Table-2).

Table(1):-No. of soil samples collected from several sites of Hill city

| Collection site | No. isolate | Precentag |
|---|---|---|
| AL- rowashid | 30 | 25% |
| AL-bu abe alla | 35 | 29.1% |
| Emskamesh | 2٥ | 20.8% |
| AL-bu derbush | 3٠ | 25% |
| collectivity | ١٢٠ | 100% |

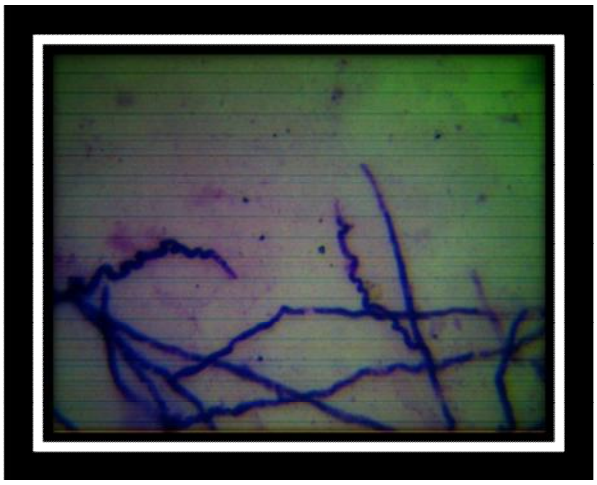

Fig (1) Morphology of spore bearing aerial hyphae of Streptomyces spp after 14 day cultivation on the yeast malt extract agar at $28c^0$showing spore chain spiral.

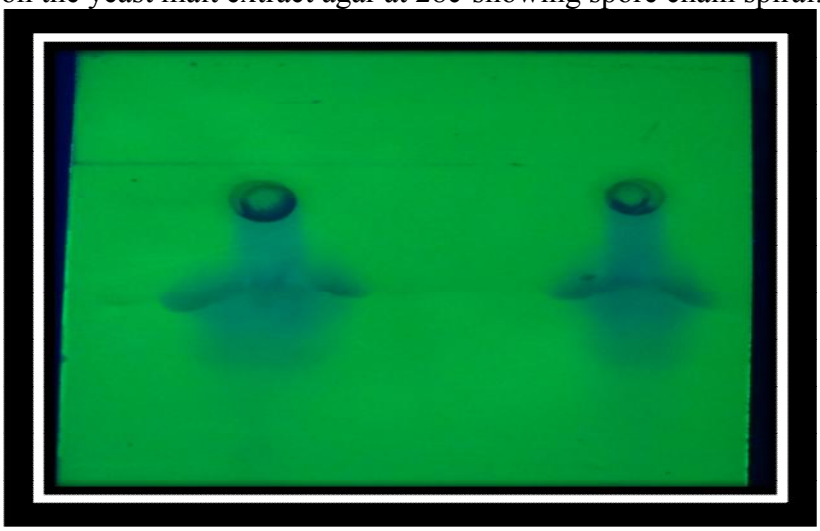

Fig (2) Thin layar chromatography of antimicrobial agent

**Morphological properties and biochemical tests of *Streptomycess* spp isolated from soil samples** Micromorphology of the actinomycete isolates were examined by slide culture method. During observation with light microscopy on the yeast malt extract agar, 4 spore-bearing hyphae(sporophore) of the isolate was spiral chain (Figure 1 ). On the other hand, the vegetative hyphae were branched but not fragmented. Verticils, synnemata, sclerotia, or sporangia were not detected. The physiological and biochemical characteristics of the isolate (Table 3) indicated that

these isolates have ability to utilize all tested carbohydrates as a carbon sources without gas production , have ability to growth in the presence of nitrogen sources and have no ability to growth in the presence of growth inhibitor such as crystal violet , sodium azide and phenol the strain was able to hydrolysis gelatin but not starch and it catalase enzyme and use citrates as a sole carbone source .Beside it have ability to hydrolysis lecithin.The antibiotics resistance profile showed that this isolate have a resistance to Carbenicilin, Rifampicin, Ampicillin ,Ciprofloxacin, Rifampin and Ceftriaxone.

Table(2) Morphological properties of *Streptomyces* sp. isolated from soil samples

| Characteristics | Results |
|---|---|
| Gram staining | positive |
| Spore chain morphology-(Sporophore) | simple (spiral) none segmented |
| Color of aerial mycelium | white |
| Melanin production | present |
| soluble pigment | absent |
| Substrate mycelium color (reverse color) | yellow-brown |
| Color of colony | white |
| Texture of colony | Powdery |
| Type of branch | straight |
| Earthy odor | + |
| Growth on :<br>-yeast extract medium<br>-starch casein medium<br>-potato dextrose agar | +<br>+<br>+<br>+ |

Table(3) :- Biochemical tests of *Streptomyces* sp. isolated from soil samples

| Test | Result |
|---|---|
| **Sugar fermentation**: | |
| 1-sucrose | + |
| 2-maniose | + |
| 3- D-xylose | + |
| 4-lactose | + |
| 5-fructose | + |
| 6-arabinose | + |
| 7- calactose | + |
| 8- rabinose | + |
| 9-Glucose | + |
| **Nitrogen sources** | |
| Valin | ++ |
| Asparagin | +++ |
| Serine | ++ |
| Ammonium sulphate | + |
| Alanine | +++ |
| **Resistant to antibiotics** | |
| Carbenicilin | R |
| Rifampicin | R |
| Ampicillin | R |
| Ciprofloxacin | R |
| Rifampin | R |
| Ceftriaxone | R |
| **Growth in the presence of inhibitors** | |
| Crystal violet(0.01) | – |
| Sodium azide (0.01) | – |
| Phenol (0.001) | – |

R=resistant, --=Negtive , += Positive,+++ very good growth,++good growth,+weekgrowth

**Cultural Characteristics of the *Streptomyces* Isolate:**

Growth properties, the aerial and substrate mycelium color and formation of soluble pigments were observed in different media after 14 days of incubation. The bacterial isolate was grow on inorganic salt-starch (ISP 4), Oatmeal (ISP 3), Glycerol asparagine (ISP 5) Yeast extract-malt extract (ISP2) Tyrosine agar (ISP 7) and the colour was detected according to (Morton ,1997). The isolates produce melanoid pigment .Table(4)

Table (4): Culture characteristics of *Streptomyces* sp. isolate on different culture media:-

| Media | Growth density | Color of | | Soluble pigment |
|---|---|---|---|---|
| | | Aerial Mycelium | Substrate Mycelium | |
| Inorganic salt-starch (ISP 4) | ++ | White | Yellow-brown- | absent |
| Oatmeal (ISP 3) | +++ | White | Yellow-brown- green | absent |
| Yeast extract-malt extract (ISP2) | +++ | White | Dark- avocado green | absent |
| Glycerol asparagines agar (ISP5) | ++ | White | Yellow-brown- green | absent |
| Tyrosine agar (ISP 7) | +++ | White | Brown-Oranig | present |
| Nutrient agar | ++ | White | Bright-yellow green | absent |
| Glucose asprgin agar | ++ | White | Bright-yellow | absent |
| Starch casein medium(ISP1) | +++ | White | Yellow-brown- green | absent |

,+++ very good growth,++good growth,+week growth

**Antimicrobial activity assays**

Out of 15 *Actinomycetes* isolates subjected for primary screening process, only one isolates showed the antibactrial activity against test organisms against both gram positive and gram negative organisms. *S. aureus, E.coli* and *Psedomonus.*

## Discussion

The actinomycete isolate was isolated from soil sample collected from Different sites of Hilla city The isolate was growing on starch casein agar medium for investigating its potency to produce antimicrobial agents. The actinomycete isolate exhibited a wide spectrum antibacterial agent (Dharumaduari *et la.,* 2008). This study was undertaken with an aim of highlighting the selecting of the strains with ß-lactamase inhibitory activity (CA)using the selective media and cultivation conditions .These results were in agreement with other authors (Kutzner,1986;Williams *et al*.,1989) .Which isolated actinomyces from different location in Egypt . Identification process has been carried out according (Holt *et al*., 1994) and numerical taxonomy of *Streptomyces* species program (Numerical taxonomy program, 1989). For the purpose of identification of actinomycete isolate, the morphological characteristics and microscopic examination were observed under the oil immersion lence (100X). The observed structure was characterize due to Bergey's manual of determinative bacteriology, 9th edition 2000 (Holt *et al*., 1994). The spore chain is spiral of the isolate.Spore mass is dark green, substrate mycelium is white and no diffusible pigment was produced. Growth properties, the aerial and substrate mycelium color and formation of soluble pigments were observed on different media after 14 days of incubation. The bacterial isolate was grow on inorganic salt-starch (ISP 4), Oatmeal (ISP 3), Glycerol asparagine (ISP 5) Yeast extract-malt extract (ISP2) Tyrosine agar

(ISP 7) and the colour was detected according to (Morton ,1997). The isolates produced melanoid pigment .

The results of physiological, biochemical characteristics of actinomycetes isolate,such as degradation of gelatin and starch , sucrose maniose, D-xylose, D-xylose, fructose, arabinose, calactose,
rabinose clucose considered to classification of isolates strain as recommended by different authors (Buchanan and Gibbson, 1974; Holt *et al*., 1994).The results shown in table(3) indicated that the streptomyces sp.was distinguished by its inability to hydrolyzed starch , $H_2S$ production, Lecithinase, (Bergey's manual 2000). Also melanin production was obaerved on tyrosin agar medium (ISP-7) .and liqueflying gelatin .On other hand, it have ability to utilize different carbone sources using basal medium ISP9 (Pridham and Gottlieb;1948). These results emphasized that the actinomycetes isolate is related to a group of *Streptomyces* (Williams *et al*., 1989; Holt *et al*., 1994). In view of all the previously recorded data, the identification of actinomycete isolate was suggestive of being belonging to *Streptomyces* .The result in table (3) indicated that the streptomyces sp. was distinguished by its ability to resistance to Carbenicilin, Rifampicin, Ampicillin ,Ciprofloxacin, Rifampin and Ceftriaxone. Our investigation also resulted in a similar type of finding which is comparable to that of Ibrhaim(Ibrhaim,2006). The active metabolites were extracted by etheyal acetate at pH 7 at the level of 1:1 (v/v) The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The crude extracts were dissolved in a small amount of methanol .Its color is brown the similar result were obtained by(Augustine, *et al*.,2005; Dharumaduari, *et al*., 2008).Separation of antibacterial agent into individual components was carried out by thin-layer chromatography using a solvent system composed of n-butanol-ethanol - water(4:1:5). After ascending the plate was taken out and dried .It has an $R_f$ value of 0.42 when appears as a dark red spot on the plate by UV light as shown in figuer 2. showed antibecterial activity the similar result were obtained by (Dharumaduari *et al*., 2008).

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