Original Research Article

Study the Inhibitory Effect of *Streptomyces* spp against the Growth some Pathogenic Bacterial

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### Abstract

The aimed of the study is screening of antibiotic producing *Streptomyces* isolates. Thirty soil samples, collected from different areas in the city of Baghdad, were screened for *Streptomyces* effectiveness as a source for active antibacterial, 26 (86.6%) samples were suspected to contain *Streptomyces*, out of them, 24 (80%) isolates were obtained with different morphological characteristics. Suspected Actinomycetes colonies were sub-cultured in ISP2 agar media carefully to obtain a pure isolate which was characterized as colored in aerial and substrate mycelium, dried, rough/smooth, with an irregular/regular margin; generally convex colony. The isolates were identified as *Streptomyces* sp. based on their morphological, physiological and biochemical characteristics. Most *Streptomyces* isolates were screened for their antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* on malt extract yeast extract agar medium (ISP2) using a cross-streak technique.

**Keywords**

Bacteria, actinomycetes, *Streptomyces*, antibacterial, Iraq

**Article Info**

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### Introduction

Actinomycetes are a group of Gram-positive bacteria with high guanine and cytosine content in their DNA (Kumari *et al.*, 2006; Khucharoenphaisan *et al.*, 2012; Al-Rubaye *et al.*, 2018 a, Risan *et al.*, 2019). The major group of Actinomycetes, *Streptomyces* spp. can produce an array of secondary metabolites having antibacterial or antifungal properties which applied for the human pharmaceutical use (Hughes *et al.*, 2008). It has been reported that most of the actinomycetes are widely used in industries due to their ability to produce numerous antibiotics (Raja and Prabakarana, 2011; Al-Rubaye *et al.*, 2018b), enzymes, vitamins, growth hormones and anti-cancerous agents (Berdy, 1995).

*Streptomyces* genus can also produce valuable metabolites, enzyme inhibitors commercially valuable enzymes like lipases, cellulases,
amylase and proteases (Ravel et al., 2000). Over 600 species of *Streptomyces* bacteria have been described (Euzeby, 2008). As with the other Actinomycetes, *Streptomyces* are Gram-positive, and have genomes with high guanine and cytosine content. *Streptomyces* are found predominantly in soil and this results in decaying vegetation (Amin et al., 2016; Risan et al., 2016; Qasim and Risan 2017). Most *Streptomyces* produces spores, and are noted for their distinct "earthy" odor that results from the production of a volatile metabolite, geosmin (Madigan and Martinko 2005). *Streptomyces* are a unique collection of prokaryotes microorganisms having diverse morphological, biochemical, cultural and physiological characters (Chavan Dilip et al., 2013).

### Materials and Methods

#### Soil samples collection

Thirty soil samples were collected from December 2018- January 2019. Samples were collected from different areas in the city of Baghdad. The total number of soil samples and the areas for sampling selected for this study are shown in table 1.

Different areas were used for the isolation of *Streptomyces* spp. from each area. The samples were taken up to a depth of 10-15 cm after removing approximately 3 cm of the soil surface. The samples were placed in polyethylene bags, closed tightly and stored in a refrigerator. Soil samples were incubated at 70°C for 2 hours to kill other microorganisms, followed by a screening procedure for the *Streptomyces* isolation (Korn and Kutzner, 1992; Risan et al., 2017).

#### Isolation and identification of *Streptomyces* spp. from soil samples

About one gram of dried soil samples were used to make suspension, by adding it to 99 ml of sterile distilled water (stock suspension). The samples were shaking in a shaker at 120 rpm for 30 minutes at room temperature. Serial dilutions from $10^{-1}$ to $10^{-3}$ were made from the stock suspension and left for 10 minutes.

After shaking, 0.1 ml of each dilution was pipetted and put on supplemented Yeast extract-malt extract agar (YEME) with Tetracycline 50 μg/L and Nystatin 50 μg/L, then spread by a sterile swab to make a uniform distribution of the suspension on the surface of the media. The inoculated plates were incubated at 28°C for 7 to 10 days.

Based on cultural characteristics, suspected colonies of actinomycetes were selected for their identification by types of Gram’s stain, aerial and substrate mycelium color, pigment production and pigment color. The colonies were transferred from the first screening step (mixed culture) into separate agar plates and incubated at 28±1°C for 7 days to obtain a pure growth of actinomycetes species, the last steps were repeated several times. The pure culture was kept at 4°C for a further study (Oskay et al., 2004; Risan et al., 2016).

#### Pathogenic bacteria used for antimicrobial activities

Two isolates, including Gram positive bacteria (*Staphylococcus aureus*) and Gram negative bacteria (*Escherichia coli*) were used to determine the antibacterial activity of *Streptomyces* isolates (both of them isolated from urine).

These microorganisms were obtained from the College of Biotechnology/Al-Nahrain University, and activated by culturing in a
Nutrient Broth at 37±0.1°C for 24 hrs using 4-5 colonies. The inoculum was standardized by a turbidity standard (McFarland standard), for example 0.5 McFarland = 1.5 x 10^8 CFU/ml adjusted by the naked eye (Cockerill et al., 2012) (Table 2).

**Morphological characterization**

Morphological characterization was done according to the directions given for the International *Streptomyces* Project (ISP2). The morphological characterization of each isolate was first performed by:

**Colony characteristics**

Suspected *Streptomyces* isolates which grew on ISP 2 and GYE medium were characterized morphologically according to the colony characteristics as follows:

Mass color or mature, sporulating aerial surface growth.

The color of substrate mycelium as viewed from the reverse side.

Diffusible soluble pigments other than melanin.

Mature cultures spore mass surface was observed after 7-14 days of incubation and the color of aerial mycelium of *Streptomyces* was determined by a code collected by Prauser (1964) for color tabs of Baumann Farbtonkarte Atlas.

**Gram's stain**

A single colony was transferred by a loop to a clean glass slide. The smear was stained with crystal violet, treated with iodine, decolorized by the ethanol (95%), and stained with safranine, then examined by a microscope (Aghighi et al., 2004).

**Physiological and biochemical Characterization**

The physiological and biochemical tests are important in the characterization of *Streptomyces* spp. following the directions given for the International *Streptomyces* project (ISP) (Shirling and Gottlieb, 1966; Macfadden, 2000), and some the biochemical tests described by Bergey's Manual of Systematic Bacteriology 2nd Edition Volume 2 (2005)

**Melanin production**

It was investigated as follows: ISP2 or ISP4 agar slants were streaked by *Streptomyces* spp. and incubated at 28°C for 7 to 10 days to detect a deep brown to black diffusible pigment (+). The absence of the color was recorded as negative (-).

**Carbon utilization test**

It was done by using Starch, Glycerol or dextrose as a carbon source. The preparation was done as described in the ISP2 and ISP4. ISP2 or ISP4 agar slants supplemented with indicator were streaked by *Streptomyces* spp. and incubated at 28°C for 7 to 10 days. The positive result was detected by growing the bacteria in this media and changing the color of media to pink.

**Citrate utilization test**

Simmon's citrate agar slants were streaked by *Streptomyces* spp. and incubated at 28°C for 7 to 10 days. The positive result was detected by changing the medium color from green to blue which indicated the *Streptomyces* ability to utilize citrate.

**Indole production test**

A loopful of *Streptomyces* spp. culture was inoculated in test tubes containing indole broth
and incubated at 28°C for 7 to 10 days. The production of indole derivatives by the isolates was determined by the addition of Kovac's reagent. The formation of a red colored ring in the tubes indicates a positive reaction.

**Catalase test**

A drop of 3% hydrogen peroxide solution was added immediately on loopful with *Streptomyces* culture on a sterile glass slide to observe the bubbles formation which indicated the production of catalase.

**Antibacterial activity of Streptomyces spp.**

**Pathogenic bacteria used for antibacterial activity**

The pathogenic microorganisms used as reference isolates for testing the antibacterial activity. Two isolates, including Gram positive (*S. aureus*) and Gram negative (*E. coli*) were used to determine the antibacterial activity. The routine inoculum prepared by activation of the mentioned bacteria in a Nutrient Broth (NB) at 37±0.1°C for 24 hours using 4-5 colonies. The inoculum was standardized by a turbidity standard (McFarland standard), for example 0.5 McFarland = 1.5 x 108 CFU/ml adjusted by naked eye (Cockerill *et al*., 2012; Risan *et al*., 2018).

**Primary screening for antimicrobial activities of Streptomyces**

Initial screen (primary screen) for antimicrobial activities were done by the cross-streak method according to Oskay, (2009) and Kumar *et al*., (2010), in which the isolated *Streptomyces* used against two different microbial pathogens. The *Streptomyces* were streaked as across lines in the middle of plates poured with Muller-Hinton agar and inoculated plates were incubated at 28°C for 7 days, after the *Streptomyces* were completely cultivated, the tested bacterial pathogens were streaked perpendicular to the *Streptomyces*, then plates were reincubated at 37°C for 24 hrs. The antimicrobial activities were observed by the naked eye in which the reference strains failed to grow near the *Streptomyces* line.

**Results and Discussion**

**Isolation, purification and identification of Streptomyces isolates**

Thirty soil samples, collected from different areas in the city of Baghdad, were screened for *Streptomyces* effectiveness as a source for active antibacterial. *Actinomycetes* were observed in addition to other microorganisms as mixed colonies after culturing the diluted soil sample (10⁻¹ to 10⁻⁶) for 7-10 days on ISP2 agar. Figure 1 shows white to gray small powdery colonies suspected to be *Actinomycetes* isolates. In this figure, a single *Actinomycete* colony is shown among the mixed colonies. The single colony of *Actinomycetes* isolate was clearly observed in figure 2. Colonies other than *Actinomycetes* found within the culture may be due to the presence of their spores in the soil or they were not killed by heating. The suspected colonies were grown on ISP2 agar and selected in accordance to their color (either gray or creamy or white) with colony diameter sized ranged from to 10 mm) and their morphology (which have smooth surface at the beginning then became powdery, soft and granular by forming the aerial mycelium), the same results were reported by Risan *et al*., (2017). From 30 soil samples, 26 (86.6%) samples were suspected to contain *Actinomycetes* out of them, 24 (80%) isolates were obtained with different morphological characteristics. Suspected *Actinomycetes* colonies were sub-cultured in ISP2 agar media carefully to obtain a pure isolate which was
characterized as colored in aerial and substrate mycelium, dried, rough\ smooth, with irregular/regular margin; generally convex colony. Most colonies that were isolated possess earthy odors as described by Williams et al., (1983).

**Selection by streaking a plate for single colonies**

As observed in figure 3, a single colony was formed by the streak plate method, to purify cultures of actinomycetes. This plating technique serially dilutes the number of bacteria in each streak, the first streak probably has a very high concentration of bacteria since it comes from a concentrated stock. By dragging a new (or freshly sterilized) tool across only one small part of the initial line, we spread a small part of the initial line over a much larger area (the second line). This second line has less bacteria, and therefore increases the chances of seeing individual colonies. The dilution was repeated many times by streaking the entire plate from the initial concentrated streak, so somewhere on the plate a single isolated colony was picked as reported by Williams et al., (1993) and Singh and Agrawal (2003).

**Identification and characterization of *Streptomyces* spp.**

**Morphological characterization**

The isolates of *Streptomyces* were identified according to the variability in their colony morphology and microscopic characteristics like the aerial and substrate mycelium, soluble pigment, spore chain arrangement (Table 3). Some *Streptomyces* isolates produced diffusible pigment in the surrounding media in accordance with the aerial mycelium colour. Soluble pigment was also observed in 15 isolate. Figure 4 shows distinctive yellowish (isolate 30) series established in the Bergey’s manual of determinatives bacteriology (Buchanan and Gibbons, 1974) and in the the Bergey’s manual of systemic Bacteriology\ category 4. As shown in figure 5a, a colony morphology showed different *Streptomyces* isolates with regular edge and irregular edge. The mycelium surface is shown in some species with rough surface and smooth surface in others. The aerial mycelium colour either white, dark, pale gray or greenish gray. Substrate mycelium was either dark brown or light brown while one isolate showed a dark green figure 5b.

**Table.1** Distribution of soil samples according to the selected areas at Baghdad city

| No  | Type            | Areas of study |
|-----|-----------------|----------------|
| 15  | Soil samples    | Al- Jadria     |
| 10  | Soil samples    | Al- Qadesia Qr |
| 5   | Soil samples    | Al- Aamerya    |

**Table.2** The source of pathogenic bacteria used for detection the antibacterail activity of *Streptomyces* isolates

| Source of samples          | Type               | Site of isolation |
|----------------------------|--------------------|-------------------|
| Biotechnology College\ Al-Nahrain University | *Staphylococcus aureus* | Urine             |
|                            | *Escherichia coli*  | Urine             |
### Table 3: Morphological characteristics of *Streptomyces* isolates

| Isolate No. | Isolates name | Colony morphology | Arial mycelium | Substrate Mycelium | Reverse side pigments | Mycelium surface | Soluble pigment | Spore chain morphology |
|-------------|---------------|-------------------|----------------|---------------------|------------------------|-----------------|-------------------|-----------------------|
| 1.          | B3-2          | Irregular edge-circular | Light gray | Light brown | smooth | brown | straight |
| 2.          | B12           | Regular edge-circular | Light gray | Light brown | smooth | Light brown | straight |
| 3.          | B1-3          | Irregular edge-circular | Light gray | Light brown | smooth | Light brown | straight |
| 4.          | B3-4          | Regular edge-circular | Light gray | Yellowish | Smooth | Yellow | straight |
| 5.          | B1-4          | Regular edge-circular | gray | Dark brown | Rough | No pigment | straight |
| 6.          | B18           | Irregular edge-circular | Light gray | Dark brown | smooth | Light yellow | straight |
| 7.          | B76           | Regular edge-circular | gray | Brown | smooth | Light brown | straight |
| 8.          | B25           | Irregular edge-circular | White gray | Light brown | smooth | No pigment | spiral |
| 9.          | BT5           | Irregular edge-circular | gray | Darck brown | rough | light yellow | straight |
| 10.         | BH14          | Regular edge-circular | Light gray | Light brown | smooth | dark | straight |
| 11.         | B1            | Regular edge-circular | Light gray | Light brown | smooth | No pigment | spiral |
| 12.         | 1-3C          | Regular circular | White gray | Brown | smooth | No pigment | spiral |
| 13.         | 4-3C          | Regular edge-circular | Light gray | Light brown | smooth | Dark yellow | straight |
| 14.         | B2-4          | Irregular edge-circular | gray | Light brown | smooth | No pigment | straight |
| 15.         | B3            | Irregular edge-circular | White gray | brown | rough | Light yellow | straight |
| 16.         | B21           | Regular edge-circular | gray | Light brown | smooth | No pigment | spiral |
| 17.         | B4-4          | Regular edge-circular | gray | Brown | smooth | Light yellow | straight |
| 18.         | B1-4          | Regular circular | gray | Brown | smooth | No pigment | rectiflexible |
| 19.         | B3-3          | Irregular circular | Light gray | Light brown | smooth | Dark brown | straight |
| 20.         | B5            | Irregular circular | gray | Brown | smooth | Light yellow | Straight |
| 21.         | B5-5          | Irregular circular | White | Light yellow | rough | Light yellow | straight |
| 22.         | BM3           | Regular | Gray | Brown | smooth | No pigment | straight |
| 23.         | B23           | Irregular circular | White gray | Light brown | rough | Light yellow | rectiflexible |
| 24.         | B5-2          | regular | gray | Light brown | smooth | Light brown | straight |

### Table 4: Biochemical tests of *Streptomyces* spp

| No | Test                  | Reaction        | Result   |
|----|-----------------------|-----------------|----------|
| 1. | Melanin               | Black to brown  | Negative |
| 2. | Catalase              | Bubbles         | Positive |
| 3. | Citrate Utilization   | Deep blue color | Positive |
| 4. | Indole production     | No color zone   | Negative |
| 5. | Sugar utilization     | Growth          | Positive |
Fig.1 Actinomycetes first screening in ISP2 agar from soil samples dilution $10^{-3}$ at 28°C for 7-10 days

Fig.2 Colorful chalky/dusty appearance of the single Actinomycete colony
Table 5 Primary screening of antibacterial activities of *Streptomyces* isolated from sediment soils against *S. aureus* and *E. coli* by cross streaking method

| Isolates | S. aureus | E. coli | Notes     |
|----------|-----------|---------|-----------|
| B3-2     | +         | +       |           |
| B12      | +         | +       | Selected  |
| B1-3     | +         | +       | Selected  |
| B3-4     | +         | +       | Selected  |
| B1-4     | -         | -       |           |
| B18      | +         | +       | Selected  |
| BT6      | -         | -       |           |
| B25      | +         | -       | Selected  |
| BT5c     | +         | +       | Selected  |
| BH14     | +         | +       | Selected  |
| Be1      | +         | +       | Selected  |
| 1-3C     | -         | -       |           |
| 4-3C     | -         | -       |           |
| B2-4     | +         | +       | Selected  |
| B3       | +         | +       |           |
| B21      | +         | +       | Selected  |
| B4-4     | -         | -       |           |
| B1-4     | +         | +       |           |
| B3-3     | +         | +       | Selected  |
| B5       | +         | -       |           |
| B4-3     | +         | +       | Selected  |
| BM3      | -         | -       |           |
| B23      | -         | -       |           |
| B5-2     | +         | -       |           |

Fig. 3 Single colony formation of *Streptomyces* spp. cultured on ISP2 formed by streak plate method
**Fig. 4** *Streptomyces* spp. cultured on glycerol yeast extract media at 28°C for 7-10 days. Left isolate without pigment, Right isolate with yellow pigment.

**Fig. 5a** Arial mycelium of *Streptomyces* grown in ISP2 media at 28°C for 7-10 days.
**Fig. 5b** Substrate mycelium of *Streptomyces* grown in ISP2 media at 28°C for 7-10 days

**Fig. 6a** Antimicrobial activity of 13 *Streptomyces* isolates against *S. aureus* (*Staph*) and *E. coli* (*E coli*), using cross streaking method with positive result
Twenty four isolates that grew on ISP2 media belong to the genus *Streptomyces* since colonies were slow growing, aerobic, glabrous or chalky, folded. Most colonies produce an earthy odour and they possessed aerial and substrate mycelia with different colors.

All the isolates were examined under a microscope after 7-10 days of incubation to see the hyphae. The spore chain morphology was observed after 2 weeks of incubation, showing various arrangements either straight, spiral or flexuous depending on the *Streptomyces* species. Most strains were with straight chain arrangement, except three strains with spiral chain arrangement and two with rectiflexible arrangement. The same results were reported by Sakiyama *et al.*, (2014).

*Streptomyces* are chemoheteroorganotrophs. They make a large class of Gram positive bacteria, forming hyphae like that in fungi with a growing temperature and pH at 28°C and 8, respectively. They produce a characteristic “earthy” smell of soil by the production volatile low molecular weight compounds called geosmins. They can utilize complex organic materials in the soil and use them as sources for carbon and energy making these bacteria essential for the production of fertile soil.

*Streptomyces* belong to the order Actinomycetales, characterized by the formation of substrate and aerial mycelium on solid media, presence of spores. The majority of soil actinomycetes form a very important class of bacteria since they produce numerous natural products such as antibiotics and enzymes. More than 70% of the known natural antibiotics produced are from Actinomycetes (Berdy, 2005).

**Biochemical test**

Biochemical results of *Streptomyces* spp are shown in table 4. The *Streptomyces* have the ability to produce enzymes like catalase, gelatinase and urease. Simmon’s citrate utilization was positive while indole production was negative. Sugar utilization was represented by growing of *Streptomyces* in media supplemented with Dextrose or starch or Glycerol as a carbon source, using the biochemical test to analyze was reported by Vijayalakshmi *et al.*, (2011).

**Primary screened of *Streptomyces* for antibacterial activity**

About 24 *Streptomyces* isolates were obtained from 3 regions as a source of soil samples and tested for their antibacterial activities against...
E. coli and S. aureus using the cross streaking method. Table 5 shows a summary of the antibacterial activity of all Streptomyces isolates including the positive (+ve) result which indicates the ability of Streptomyces products to stop the growth of pathogenic bacteria, while the negative (-ve) result indicates no antibacterial activities which was neglected and was not selected for further analysis. Risan et al., (2017) showed similar results for their isolates regarding antibacterial activities. Out of 24 isolates, 16 (66.6%) isolates showed high antibacterial activities, 13 (43.3 %) had antibacterial activity against both S. aureus and E. coli, while only 3 (10%) isolates showed activity against S.aureus. The same results were represented by Parungao et al., (2007) who showed that the antibacterial activity of Streptomyces secondary metabolites against Gram positive bacteria are more active than Gram negative bacteria. The isolates which showed the highest antibacterial activity are represented in figure 6a, highlighted and summarized in table (5), and all were subjected to a secondary screening. While 8(1.9%) isolates showed no antibacterial activity figure 6b.

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