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

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A B S T R A C T

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.

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 Actinomyces, *Streptomyces* are Gram-positive, and have genomes with high guanine and cytosine content The *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 being characterized as small, white, and pin-point, rough, chalky and a clear zone of inhibition around them.

The suspected colonies were subjected 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 28ºC 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 28ºC 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 28ºC 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 28ºC 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 size 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 *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 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 out 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	Darck brown	Rough	No pigment	straight
6.	B18	Irregular edge-circular	Light gray	Darck brown	smooth	Light yellow	straight
7.	BT6	Regular edge-circular	gray	brown	rough	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	No pigment	straight
10.	BH14	Regular edge-circular	Light gray	Light brown	rough	light yellow	straight
11.	B1	Regular edge-circular	Light gray	Light brown	smooth	dark	straight
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	Iregular 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

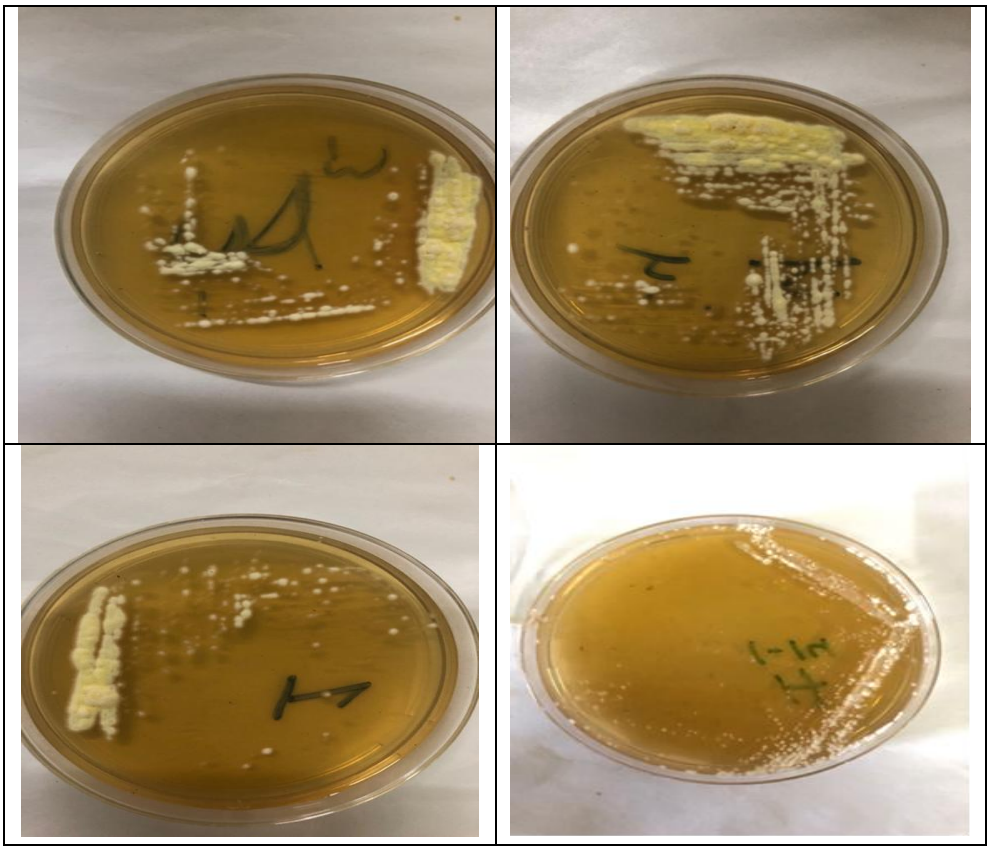


Fig.2 Colorful chalky/dusty appearance of the single Actinomycete colony

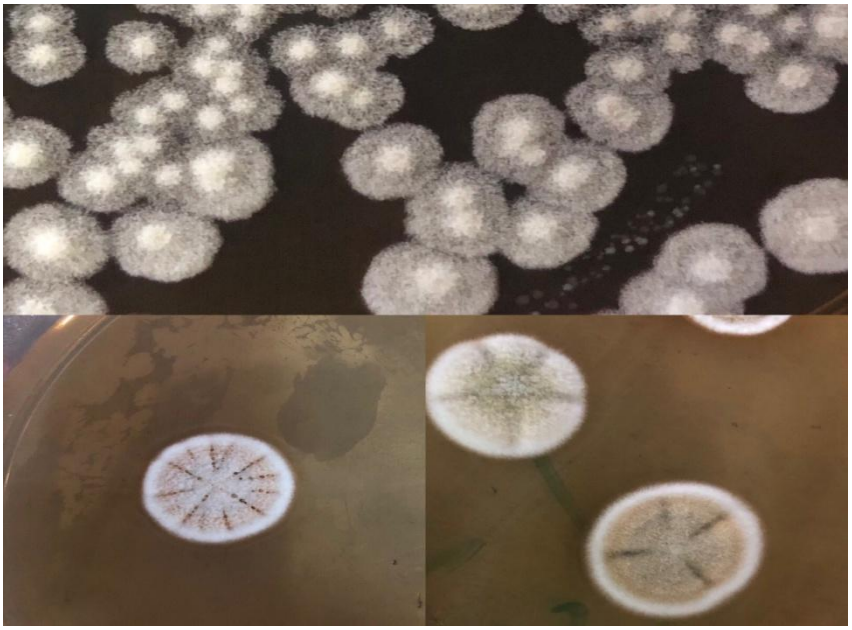


Table.5 Primary screening of antibacterial activities of *Streptomyces* isolated from sediment soils against *S. aureus* and *E. coli* by cross streaking method

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

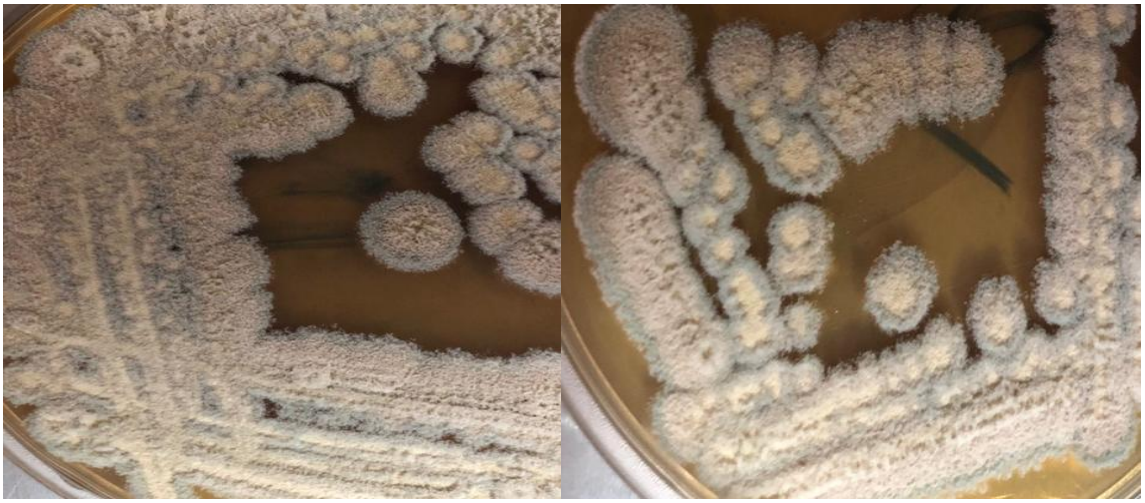


Fig.4 *Streptomyces* spp. cultured on glycerol yeast extract media at 28°C for 7-10 days. Left isolate without pigment, Right isolate with yellow pigment

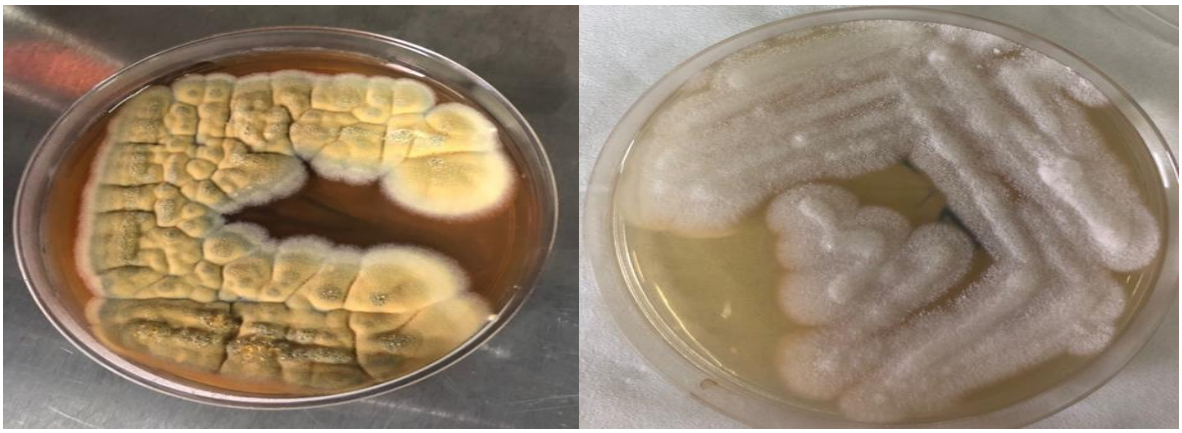


Fig.5a Arial mycelium of *Streptomyces* grown in ISP2 media at 28°C for 7-10 days

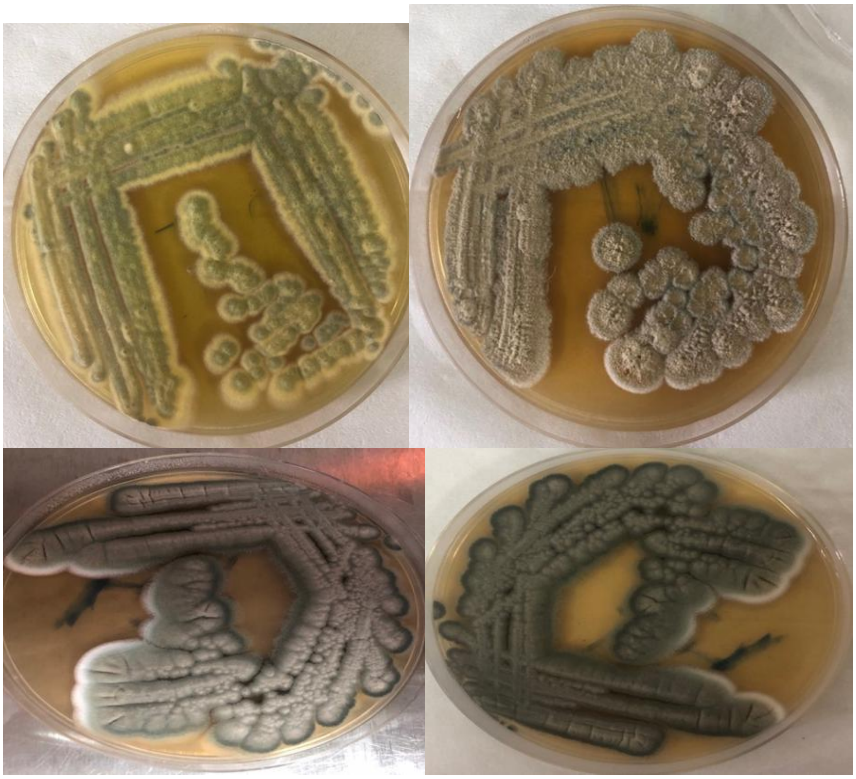


Fig.5b Substrate mycelium of *Streptomyces* grown in ISP2 media at 28°C for 7-10 days

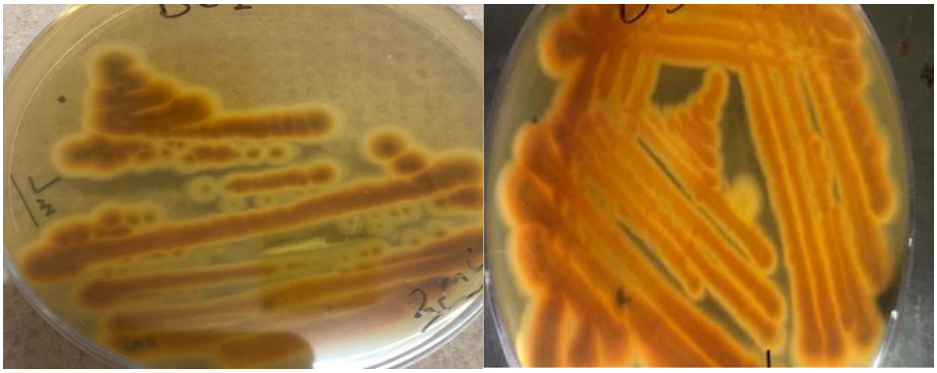


Fig.6a Antimicrobial activity of 13 *Streptomyces* isolates against *S. aureus* (*Staph*) and *E.coli* (*E coli*), using cross streaking method with positive result

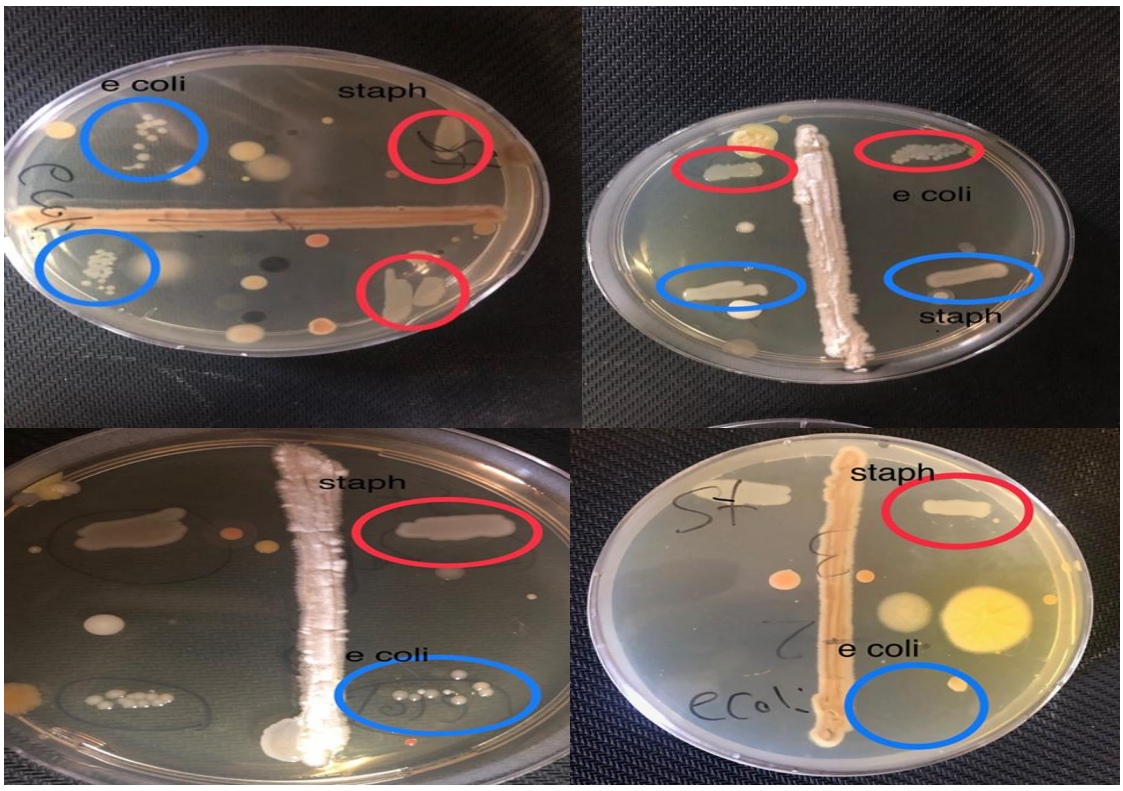
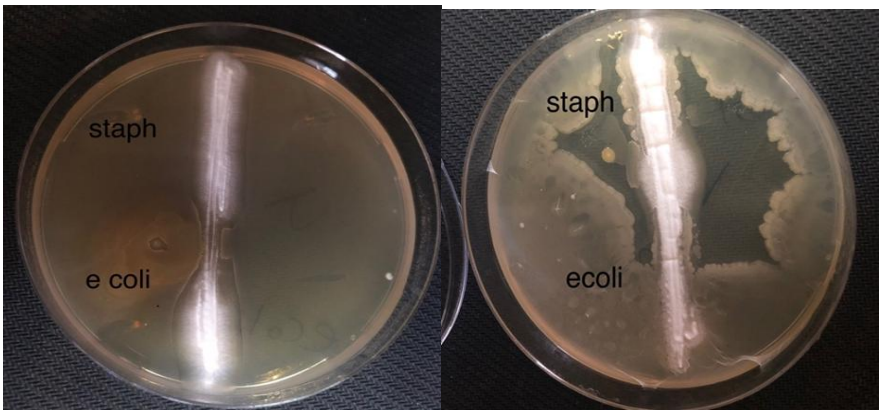


Fig.6b Antimicrobial activity of 8 *Streptomyces* isolates against *S. aureus* and *E. coli*, using cross streaking method with negative result



Twenty four isolates that grew on ISP2 media belong to the genus *Streptomyces* since colonies were slow growing, aerobic, glabours or chalky, folded. Most colonies produce D 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 anti-bacterial 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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