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# IDENTIFICATION AND IN VITRO ANTIMICROBIAL ACTIVITY OF MARINE *STREPTOMYCES* SPP. BACTERIA FROM TIGRIS RIVER SEDIMENTS IN BAGHDAD CITY

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## ABSTRACT

The aim of this study is to isolate different *Streptomyces* spp. from Tigris river sediment soil samples and characterization of their bioactive compounds. About 44 (88%) samples of soil were suspected to contain *Streptomyces*. About 42 (84%) *Streptomyces* spp. have been isolated which showed different morphological characteristic. Primary screening for antimicrobial activities to these isolates against *E. coli* and *S. aureus* by cross streaking technique, indicated that 22 (52.38%) isolates have high antibacterial activities. Out of them 17 (40.47%) have antibacterial activities against *S. aureus* and *E. coli*, which obtained from the selected area (Abn 1, Abn 4, Abn 8, Abn 13, Abn 17, Abn 19, Abn 21, Abn 23, Abn 24, Jad 26, Jad 27, Jad 29, Jad 32, Kar 36, Kar 39, Kar 41, Kar 42, while only 5 (11.9%) isolates showed activities against *S. aureus*, from the selected area (Abn 3, Abn 7, Abn 11, Abn 16 and Jad 34). Secondary screening for bioactive compounds production by well diffusion method showed that 14 (63.63%) isolates have a very good zone of inhibition against *S. aureus* and *E. coli*. Samples with the highest antibacterial activities (21, M5, $N^-$ and $D^-$) were chosen for optimization and characterization. The optimization of these isolates revealed that yeast extract and malt extract (ISP2) is more optimum than GYE with a highest OD (0.58) at day 4, at pH 7.8, NaCl tolerance 1% and temperature 30°C.

**KEYWORDS:** Tigris river; Actinomycetes; Antimicrobial; *Streptomyces*.

## INTRODUCTION

The importance of Actinobacteria in research is related to their diversity and complexity of life cycles.[1] Actinomycetes found as free, in the soil, water and roots of tree.[2] Actinomycetes constitute a significant component of the microbial population in most soils and the most important member of the actinomycetes is the genus *Streptomyces* whichaccount for 80% of the total Actinomycetes population, which recognized as highly producing of useful bioactive metabolites with broad spectrum activities, as antibacterial, antifungal, antibiotic, antiparasitic, antitumor and antiviral, immunomodulators agents.[3,4,5] They form approximately 80% of total antibiotic products as compared to other actinomycetes genera and considered to be the major source of bioactive secondary metabolites and antibiotics producer, forming more than half of the naturally produced antibiotics.[5,6] The needs for new and novel antibiotic is related to increase the antimicrobial resistance worldwide in an alarming rate and the emergency of drug resistant pathogens which causing life threatening infections especially in immunodeficient patients, increase toxicity of currently used compounds and the evolution of new diseases.[7,8] Primarily Actinomycetes were recognized according to their morphological criteria, moreover, in the past the Actinomycetes taxonomy was associated with their morphology to differentiate between different related species and among a lot of genera.[9,10] Therefore, the aim of the study is isolation and identification of *Streptomyces* spp. from Tigris river sediment that have the ability to produce antimicrobial agent activity.

## MATERIALS AND METHODS

### Soil samples collection

A study area of this project has covered sediment discharge of the Tigris river at Baghdad \ Iraq, about (4 Km2) from 3 zones (Al-Jadria region, Karada and Abu-Nous), from each zone 10, 15 and 25 respectively through December 2017 to January 2018. 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. Incubated soil samples at 70°C for 2 hours to kill other microorganisms. Followed by screening procedure for the *Streptomyces* isolation.[11]

**Tested microorganism used for antimicrobial activities**

Gram positive (*Staphylococcus aureus*) and Gram negative (*Escherichia coli*) were used to determine the antimicrobial activity of *Streptomyces* isolates. These microorganisms were obtained from the Biotechnology College\ Al-Nahrain University, which activated by culturing in a Nutrient Broth at 37°C for 24 hours.

**Isolation and identification *Streptomyces* spp.**

One gram of dried Tigris river sediment soil samples was used to make suspension, by adding it to 99 ml of sterile distilled water (stock suspension), The samples were shacking 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 starch casein agar (ISP4) with Tetracycline 50 mg/L and Nystatin 50 mg/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 14 days.[12,13]

**Cultural and biochemical Characterization of *Streptomyces* isolates**

Bacterial isolates grew on ISP 2 and GYE medium were characterized morphologically according to the colony characteristics,[14] Gram's Stain[15] and Physiological and biochemical Characterization following the directions given for the International *Streptomyces* project (ISP).[16,17]

The physiological and biochemical test are important parameters in characterization the *Streptomyces* spp. isolates, following the directions given for the International *Streptomyces* project (ISP).[16,17]

**Primary screening (Giant Colony/ cross plate technique)**

Nutrient agar plates were inoculated with *Streptomyces* isolate by a single streak of inoculum in the middle of plates. After 7 days of incubation at 28ºC, the *Streptomyces* were completely cultivated, then the tested bacterial pathogens were streaked perpendicular to the *Streptomyces* (a single streak at a 90° angle to the *Streptomyces* isolates), then plates were re incubated at 37°C for 24 hours. The antimicrobial activities were observed by naked eye in which the reference strains failed to grow near the *Streptomyces* line.[18,19]

**Fermentation Condition for secondary screening**

Shake flask fermentation was carried out by the inoculation of 1.5ml of prepared stock suspension cultures with 150ml of Antibiotic production medium (ISP2 broth and GYE broth), pH 7.5 and incubated at 28°C, 170rpm for 7days in a shaking incubator.[20,21]

**Secondary screening (well plate method)**

The *Streptomyces* isolates which showed higher production of bioactive compounds during primary screening, were submitted to secondary screening for antimicrobial activity. The most active isolate was chosen for identification and characterization of antimicrobial metabolites. Supernatant that prepared in last step were collected and separated from the crude precipitation from each isolate by centrifugation at 10,000 rpm\2 min. After solidification of 20 ml of sterilized muller-Hinton agar, spread 100 microliters of pathogenic activated bacteria by L shape spreader. Wells (6 mm in diameter) were prepared in each seeded agar plate and each well was filled with 100 microliters of filtered supernatant (0.45µm) and screened via agar well diffusion procedure mentioned previously against the reference strains, the same was done to the crude precipitation by separation the supernatant and leaving a little amount of the broth to mix them very well, then each well was filled with 100 microliters of each crude precipitation, the plates incubated at 37 °C and the zone of inhibition was determined after 24 hours overnight.[22]

**Estimation of the growth requirements for optimization**

The *Streptomyces* isolates were optimized by using two different media (ISP2 broth and GYE broth), different temperature (26, 28, 30 and 32°C), different pH (6.5, 7.3, 7.5, 7.8 and 8) and with sodium chloride (1, 2, 4, and 6%).

## RESULTS AND DISCUSSION

**Isolation of *Streptomyces* from the Tigris river soil sediments**

The 50 sediment soil samples, collected from different locations of the Tigris river, 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$10^{-3}$) for 7-14 days on casein salt starch agar. Figure (1) shows white to grey small powdery colonies suspected to be Actinomycetes isolates in addition to other microorganisms (like yeast, other bacteria and fungus). The single colony of Actinomycetes isolate clearly observed in figure (2). Colonies other than Actinomycetes found within the culture may be due to presences of their spores in the soil or they were not killed by heating. The suspected colonies were grown on ISP4 agar and selected in accordance to their color (either gray or creamy or white) with colony diameter size ranged from 1 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 and his colleagues.[23]

From 50 sediment soil samples, 44 (88%) samples were suspected to contain *Streptomyces*, out of them 42 (84%) isolates obtained with different morphological characteristics. Suspected Actinomycetes colonies were sub-cultured in ISP2, ISP4 and GYE agar media carefully to obtain a pure isolate which characterized as

colored in aerial and substrate, dried, rough\ smooth, with irregular/regular margin; generally convex colony, most colonies that were isolated possess earthy odors as described by Williams and Cross.[24]

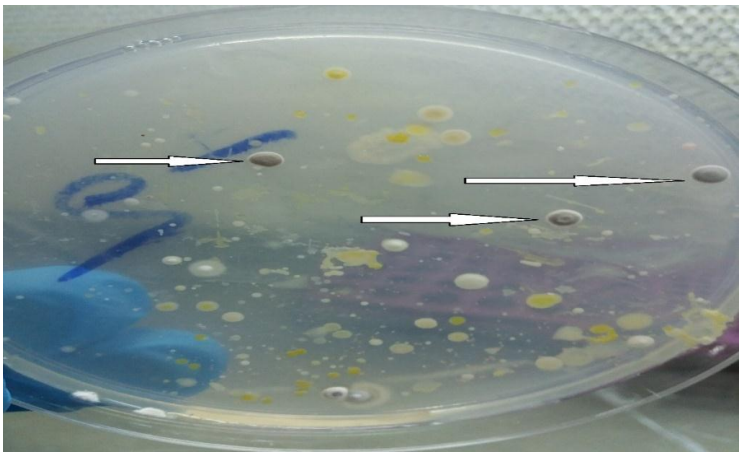

**Figure 1: Actinomycetes first screening in casein salt starch agar from soil samples dilution 10 $^{-3}$at 30°C for 7-14 days. Arrow shows a single Actinomycete colony among the mixed colonies.**

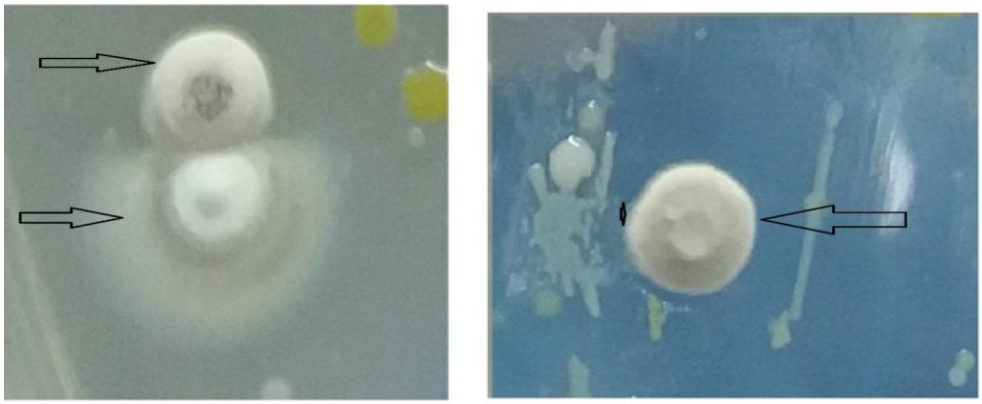

**Figure 2: Note the colorful chalky/dusty appearance (arrows show the single Actinomycete colony).**

**Selectionby streaking a plate for single colonies**

As observed in figure (3), a single colony was formed by streak plate method (ABCD streaking), to purify cultures of actinomycetes. This plating technique is serially dilute 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 that to see individual colonies. The dilution repeated many times by streaking the entire plate from the initial concentrated streak, so somewhere on the plate a single isolated colony picked as reported by Williams and Cross[24] and Singh and Agrawal.[25]

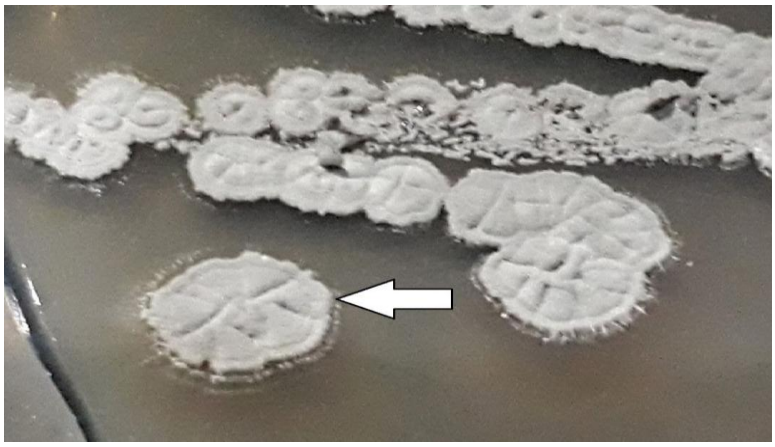

**Figure 3: Single colony formation.**

**Identification and Characterization of *Streptomyces* spp.**

**Morphological characterization**

The isolates were identified according to the variability in their colony morphology and microscopic characteristics like the aerial and substrate mycelium, soluble pigment, spore chain arrangement (table1). Some isolates produced diffusible pigment in the surrounding media in accordance with the aerial mycelium color, soluble pigment was also observed in 15 isolates, figure (4) showed distinctive yellowish color (isolate 30) series established in the Bergey's manual of determinative bacteriology by Buchanan and Gibbons.[26]

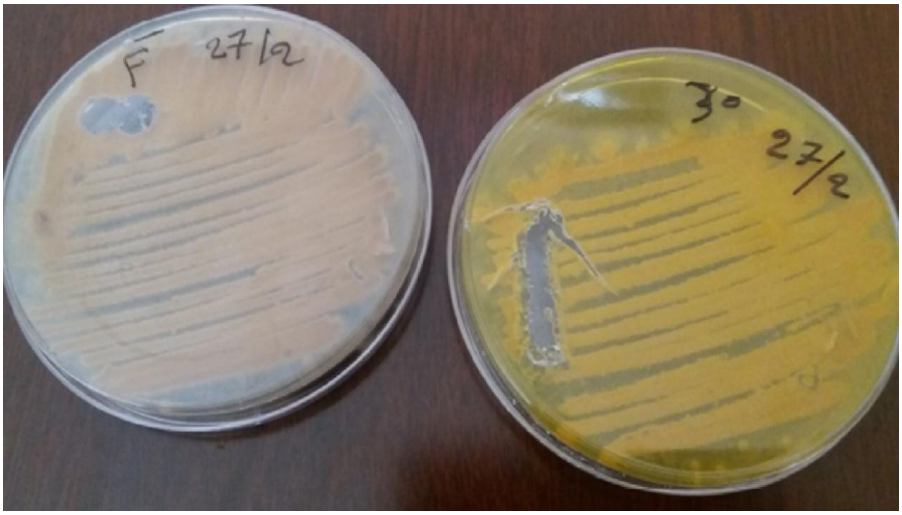


**Figure 4: Left isolate without pigment, Right isolate with yellow pigment.**

**Table 1: Morphological characteristics of 42 *Streptomyces* isolates.**

| Isolate No. | Isolates name | Colony morphology | Arial mycelium | Substrate Mycelium Reverse side pigments | Mycelium surface | Soluble pigment | Spore chain morphology |
|---|---|---|---|---|---|---|---|
| 1. | 2 | Irregular edge-circular | Light grey | Light brown | smooth | brown | straight |
| 2. | 33 | Regular edge-circular | Light grey | Light brown | smooth | Light brown | straight |
| 3. | F⁻ | Irregular edge-circular | Light grey | Light brown | smooth | Light brown | straight |
| 4. | 30 | Regular edge-circular | Light grey | Yellowish | Smooth | Yellow | straight |
| 5. | B⁻ | Regular edge-circular | grey | Darck brown | Rough | No pigment | straight |
| 6. | m2 | Irregular edge-circular | Light grey | Darck brown | smooth | No pigment | straight |
| 7. | 21 | Regular edge-circular | grey | brown | rough | Light brown | straight |
| 8. | 20 | Irregular edge-circular | White grey | Light brown | smooth | No pigment | straight |
| 9. | T5c | Irregular edge-circular | grey | Darck brown | rough | No pigment | straight |
| 10. | N⁻ | Regular edge-circular | Light grey | Light brown | rough | light yellow | straight |
| 11. | B | Regular edge-circular | Light grey | Light brown | smooth | dark | straight |
| 12. | 3 | Regular circular | White grey | Brown | smooth | No pigment | straight |
| 13. | M3 | Regular edge-circular | Light grey | Light brown | smooth | Dark yellow | straight |
| 14. | M4 | Irregular edge-circular | grey | Light brown | smooth | No pigment | straight |
| 15. | t8c | Irregular edge-circular | White grey | brown | rough | No pigment | straight |
| 16. | likeI | Regular edge-circular | grey | Light brown | smooth | No pigment | straight |
| 17. | M7 | Regular edge-circular | grey | brown | smooth | No pigment | straight |
| 18. | 21 | Regular circular | grey | brown | smooth | No pigment | rectiflexible |
| 19. | D⁻ | Irregular circular | Light grey | Light brown | smooth | Dark brown | straight |
| 20. | M5 | Iregular circular | grey | Brown | smooth | No pigment | Straight |
| 21. | I | Irregular circular | White | Light yellow | rough | No pigment | straight |
| 22. | M1 | Regular | Grey | brown | smooth | No pigment | straight |
| 23. | 23 | Irregular circular | White grey | Light brown | rough | Light yellow | straight |
| 24. | E⁻ | regular | grey | Light brown | smooth | Light brown | straight |
| 25. | m6 | Irregular | grey | orange | rough | brown | Spiral |
| 26. | F⁻ | Irregular | grey | brown | rough | No pigment | straight |
| 27. | C⁻ | Irregular | White grey | Light brown | rough | Dark brown | straight |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| 28. | W- | Irregular | White grey | Light brown | rough | No pigment | straight |
| 29. | E | regular edge-circular | Light grey | brown | smooth | No pigment | straight |
| 30. | I- | Irregular edge-circular | White grey | Darck rown | rough | No pigment | straight |
| 31. | A- | Irregular edge-circular | grey | Light brown | rough | No pigment | straight |
| 32. | H- | Irregular edge-circular | White grey | darck brown | rough | No pigment | straight |
| 33. | T4c | Irregular edge-circular | White grey | Light brown | rough | No pigment | straight |
| 34. | 10 | Irregular edge-circular | White grey | Light brown | rough | No pigment | straight |
| 35. | 4 | Irregular edge-circular | pink grey | pink | rough | No pigment | straight |
| 36. | T6c | Irregular edge-circular | grey | Darck brown | rough | No pigment | straight |
| 37. | 17 | Irregular edge-circular | White grey | brown | smooth | No pigment | straight |
| 38. | 25 | Irregular edge-circular | White grey | black | rough | Darck pink | straight |
| 39. | 18 | Irregular edge-circular | White grey | Light brown | rough | No pigment | straight |
| 40. | 3 | Irregular edge-circular | White | Light brown | rough | No pigment | straight |
| 41. | 11 | Irregular edge-circular | White grey | brown | rough | No | straight |
| 42. | 9 | Irregular edge-circular | grey | brown | smooth | brown | straight |

As shown in figure (5a), colony morphology showed 6isolates with regular edge and irregular edge. The mycelium surface showed in some species with rough surface and smooth surface in others. The aerial mycelium color either white, dark, pale grey or greenish grey.Substrate mycelium either dark brown or light brown while one strain showed dark green figure (5b).About 42 isolates that grew on SC media belong to the genus *Streptomyces* since colonies were slow growing, aerobic, glabours or chalky, folded (figure 6), most colonies produce an earthy odor and they possess aerial and substrate mycelia with different colors.

**Figure 5(a): Arial mycelium of *Streptomyces* grown in ISP4.**

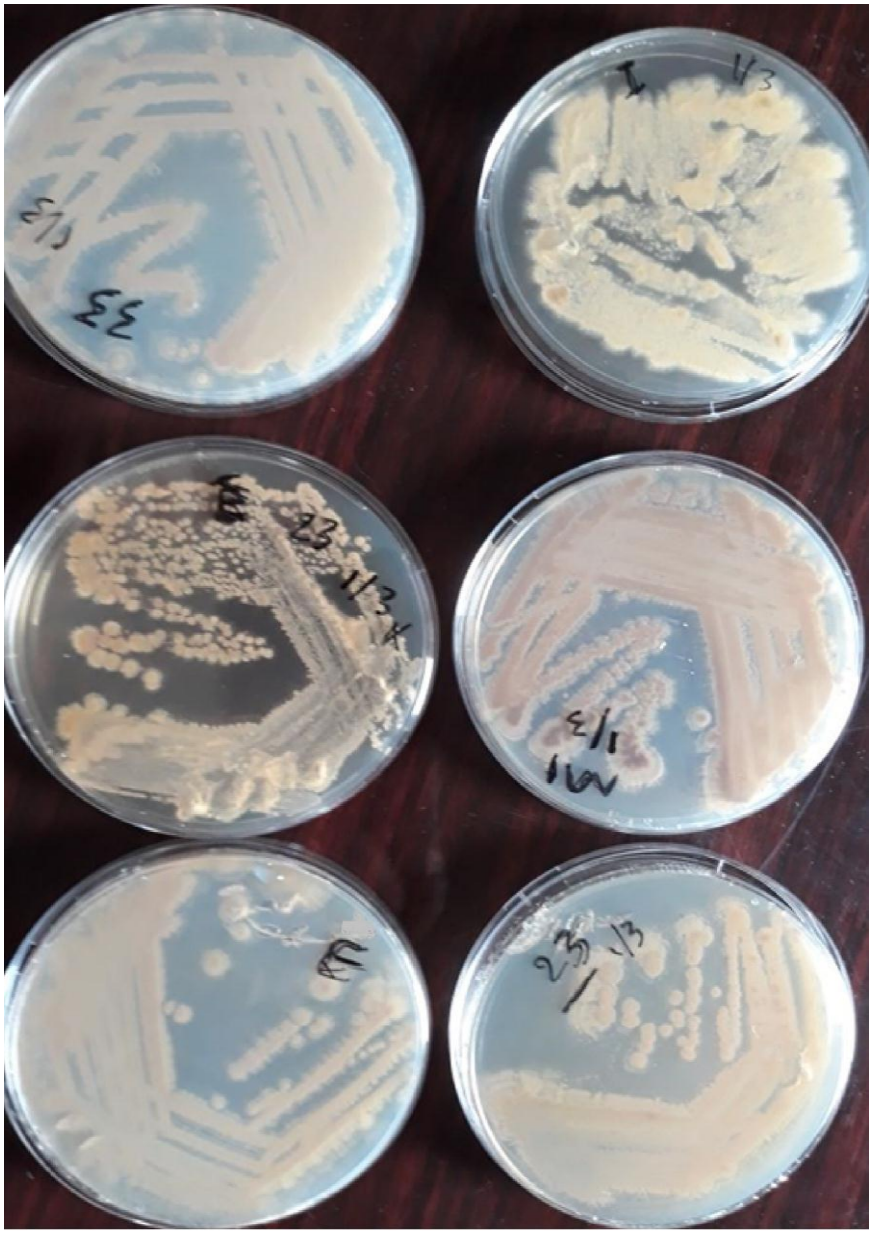

**Figure 5(b): Substrate mycelium of *Streptomyces* grown in ISP4.**

**Figure 6: *Streptomyces* isolate, folded aerial mycelia.**

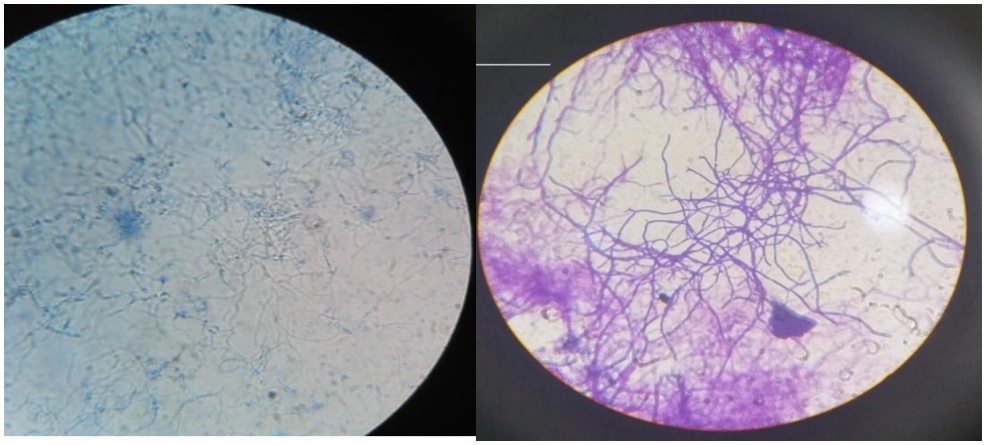

**Figure 7: Slide of a *Streptomyces* spp. hyphae, grown on ISP2 agar (left) and on ISP2 broth (right). Branching filaments, abundant aerial mycelia, and long chains of small spores are visible, all of which are characteristic of *Streptomyces* spp.100X.**

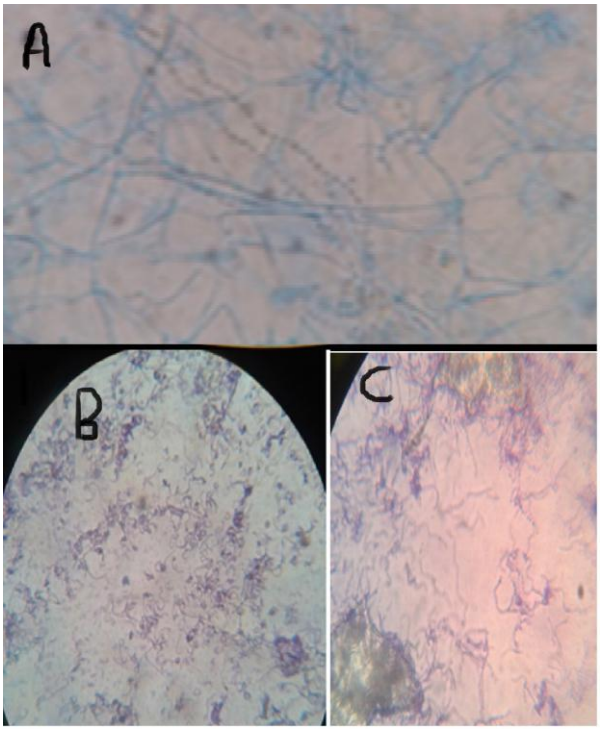


**Figure 8: Spores chain arrangement: Spores were arranged in long straight to chains (A). Spore were arranged in spiral shape chains (B). Spores were arranged in long straight to flexuous chains (C). The isolatesare Gram positive.**

All the isolates were examined under microscope after 7-14 days of incubation to see the hyphae as shown in figure (7). The spore chain morphology was observed after 2 weeks of incubation, showing various arrangement either straight, spiral or flexuous depends on the *Streptomyces* species. Most strains with straight chain arrangement, except two strains with spiral chain arrangement and one with rectiflexible arrangement, which observed in table (1) and figure (8).

The *Streptomyces* are chemoheteroorganotrophs. They make a large class of Gram positive bacteria, forming hyphae like that in fungi with growing temperature and pH at 28°C and 8 respectively. Theyproduce a characteristic "earthy" smell of soil by the production a 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. Actinomycetes are belonging 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.[27]

**Biochemical tests**

Biochemical results of *Streptomyces* spp are shown in table (2). 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 represented by growing of *Streptomyces* in media supplemented with Dextrose, starch or Glycerol as a carbon source, using the biochemical test to analyze marine strains was reported by Vijayalakshmi and his colleagues.[28]

**Table 2: The results of *Streptomyces* spp. biochemical tests.**

| No | Test | Reaction | Result |
|---|---|---|---|
| 1. | Sugar utilization | Growth | Positive |
| 2. | Melanin | Black to brown | Negative |
| 3. | Catalase | Bubbles | Positive |
| 4. | Citrate Utilization | Deep blue color | Positive |
| 5. | Gelatinase | Narrow zone | Positive |
| 6. | Indole production | No color zone | Negative |
| 7 | Urease | Red to deep pink | Positive |

**Primary screened of *Streptomyces* for anti-bacterial activity**

About 42 *Streptomyces* isolates were obtained from 3 regions as a source for sediments soil samples and tested for their antibacterial activities against *E. coli* and *S. aureus* using cross streaking method. Table (3), showed summarization 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 and highlighted for secondary antibacterial screening as a highly producer (1) and moderate producer (2), while the negative (-ve) result indicates no antibacterial activities which neglected and not selected for further analysis. Risan *et al.*, (2017) showed similar results for their isolates regarding antibacterial activities.[23] The antimicrobial activity of all potential isolates was interpreted when *E. coli* and *S. aureus* showed no growth near the wells which filled with the biomass having the antibacterial compounds produced by *Streptomyces* isolates. Out of 42 isolates, 22 (52.38%) isolates showed high antibacterial activities, 17 (40.47%) have antibacterial activities against both *S. aureus* and *E. coli*, while only 5 (11.9%) isolates showed activities against *S. aureus*, the same results represented by Parungao *et al*., (2007), they showed that the antibacterial activities of *Streptomyces* secondary metabolites against Gram positive bacteria is more active than Gram negative bacteria.[29] The isolates which showed the highest antibacterial activities represented in figure (9a, b, c) were highlighted and summarized in table (3), all were subjected to a secondary screening.

**Table 3: The primary screening of antibacterial activities of *Streptomyces* isolated from sediment soils against *S. aureus* and *E. coli* by cross streaking method.**

| Sample No. and abbreviated | *S.aureus* | *E. coli* | Notes |
|---|---|---|---|
| Abn 1 | +ve | +ve | Selected-1 |
| Abn 2 | -ve | -ve | Ignored |
| Abn 3 | +ve | -ve | Selected-2 |
| Abn 4 | +ve | +ve | Selected-1 |
| Abn 5 | -ve | -ve | Ignored |
| Abn 6 | -ve | -ve | Ignored |
| Abn 7 | +ve | -ve | Selected-2 |
| Abn 8 | +ve | +ve | Selected-1 |
| Abn 9 | -ve | -ve | Ignored |
| Abn 10 | -ve | -ve | Ignored |
| Abn 11 | +ve | -ve | Selected-2 |
| Abn 12 | -ve | -ve | Ignored |
| Abn 13 | +ve | +ve | Selected-1 |
| Abn 14 | -ve | -ve | Ignored |
| Abn 15 | -ve | -ve | Ignored |
| Abn16 | +ve | -ve | Selected-2 |
| Abn17 | +ve | +ve | Selected-1 |
| Abn18 | -ve | -ve | Ignored |
| Abn19 | +ve | +ve | Selected-1 |
| Abn20 | -ve | -ve | Ignored |
| Abn21 | +ve | +ve | Selected-1 |
| Abn22 | -ve | -ve | Ignored |
| Abn23 | +ve | +ve | Selected-1 |
| Abn24 | +ve | +ve | Selected-1 |
| Abn25 | -ve | -ve | Ignored |
| Jad 26 | +ve | +ve | Selected-1 |
| Jad 27 | +ve | +ve | Selected-1 |
| Jad 28 | -ve | -ve | Ignored |
| Jad 29 | +ve | +ve | Selected 1 |
| Jad 30 | -ve | -ve | Ignored |
| Jad 31 | -ve | -ve | Ignored |
| Jad 32 | +ve | +ve | Selected-1 |
| Jad 33 | -ve | -ve | Ignored |
| Jad 34 | +ve | -ve | Selected-2 |
| Jad 35 | -ve | -ve | Ignored |
| Kar 36 | +ve | +ve | Selected-1 |
| Kar37 | -ve | -ve | Ignored |
| Kar38 | -ve | -ve | Ignored |
| Kar39 | +ve | +ve | Selected-1 |
| Kar40 | -ve | -ve | Ignored |
| Kar 41 | +ve | +ve | Selected-1 |
| Kar 42 | +ve | +ve | Selected-1 |

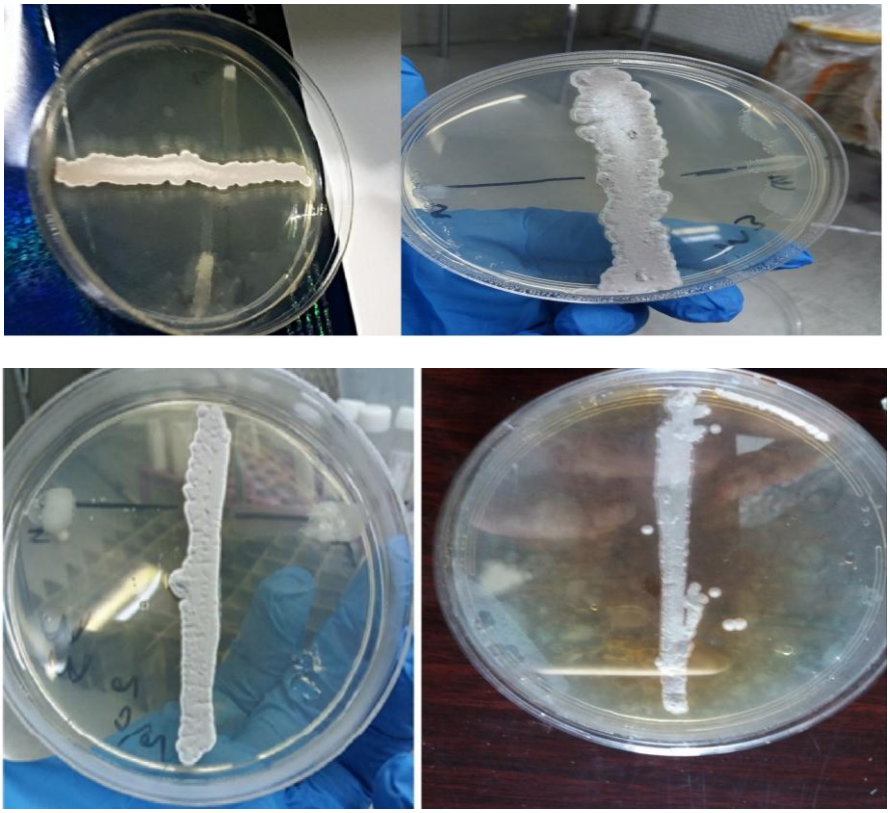

**Figure 9(a): Antimicrobial activity of 17 *Streptomyces* isolates against *S. aureus* and *E. coli*, using Giant colony technique.**

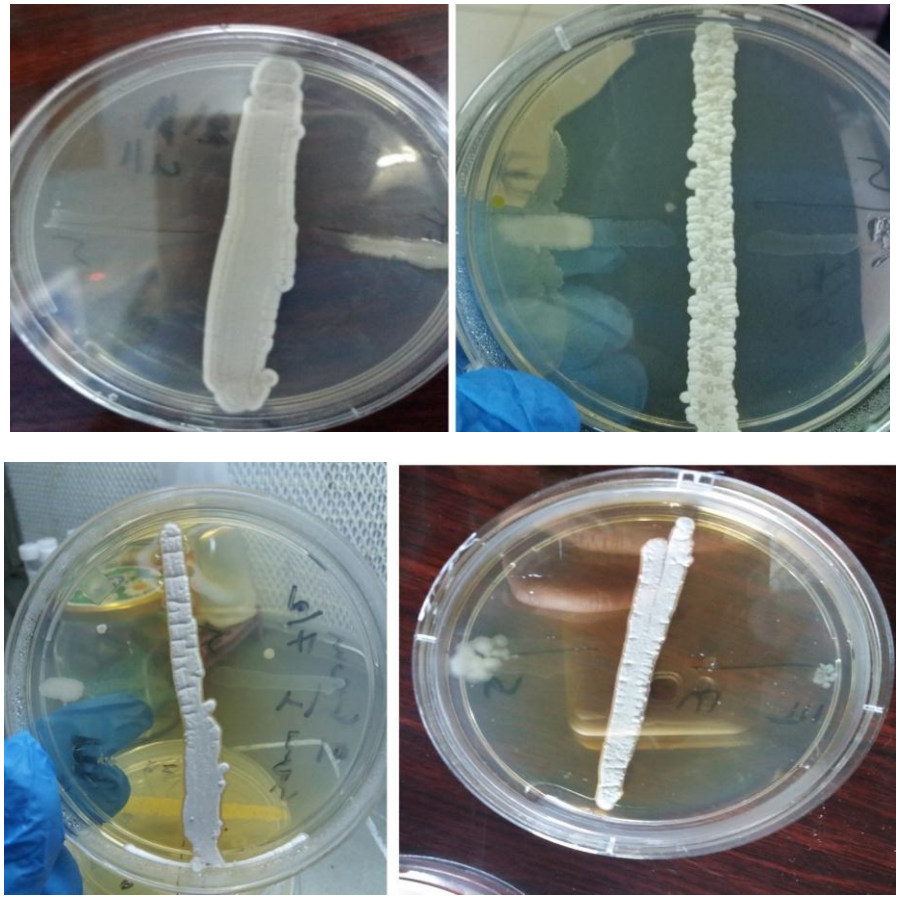

**Figure 9(b): Antimicrobial activity of 17*Streptomyces* isolates against *S. aureus* and *E. coli*, using Giant colony technique.**

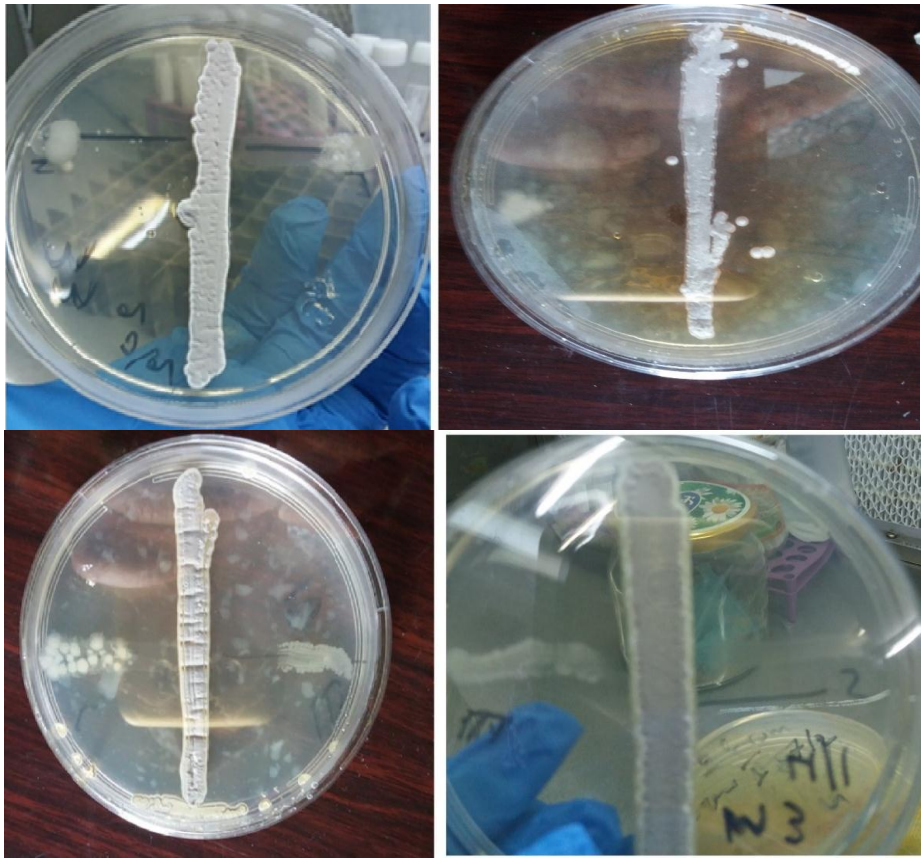

**Figure 9(c): Antimicrobial activity of 17*Streptomyces* isolates against *S. aureus* and *E. coli*, using Giant colony technique.**

**Secondary screening of *Streptomyces* isolates for bioactive compounds production**

Twenty two *Streptomyces* isolates whichselected from primary screening were grown on ISP2 broth for 7-14 days at 28 °C in shaking incubator 170 rpm.[41] The flasks fermentation of the selected *Streptomyces* (figure 10) were subjected to plate well method by using the filtrate and the crude precipitate. The bioactive compound production were screened by comparing the activities of the biomass crude and the supernatant filterate (filtered by 0.45µm) against the pathogenic Gram negative and Gram positive (*E.coli* and *S. aureus*). Surprisingly, the antibacterial activity of the supernatant was very weak, as represented in isolates $D^-$ and $I_s$ (figure 11), and less than the antibacterial activity of the biomass crude (figure 12), as observed by a study reported by Khan and Patel.[20] A very good zone of inhibition was obseved in 14 isolates, however only 4 isolates (21, M5, $N^-$, $D^-$) have the highest antibacterial activities (table 4).

**Figure 10: Shake flask fermentation for most active *Streptomyces* isolates, selected from primary screening, was carried out in 500 ml flasks containing 150 ml of production medium (ISP2) broth, shaken at 180 rpm for 7 days.**

**Figure 11: Antibacterial activity of supernatant crude of *Streptomyces* against *S. aureus* and *E.coli.***

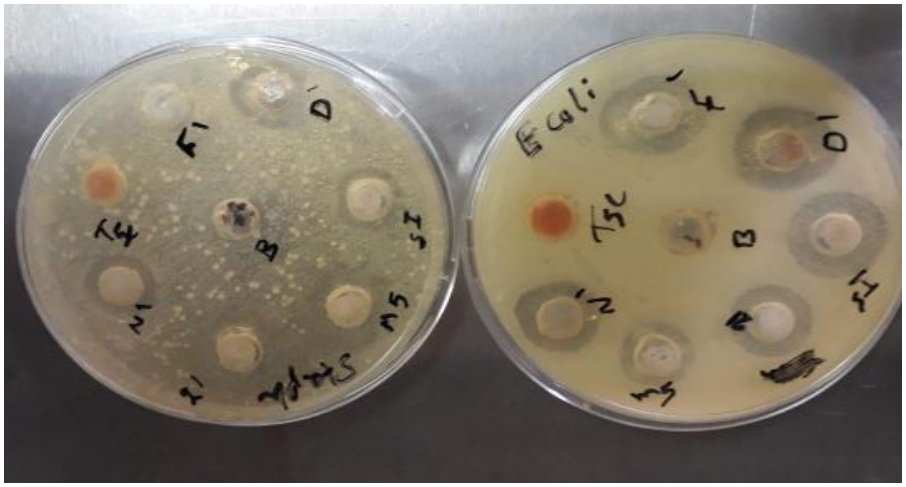

**Figure 12: Antibacterial activity of Biomass crude of *Streptomyces* against *S. aureus* and *E .coli.***

**Table 4: The *Streptomyces* antibacterial activities against *S. aureus* and *E. coli,* showing the zone of inhibition for each isolate with highlighted the highest active ioslates.**

| Isolate NO | Isolate name | *S. aureus* | *E. coli* |
|---|---|---|---|
| 1. | $D^-$ | 14 | 20 |
| 2. | $N^-$ | 12 | 17 |
| 3. | M5 | 16 | 12 |
| 4. | 21 | 17 | 11 |
| 5. | M3 | 15 | 8 |
| 6. | 2 | 12 | 13 |
| 7. | Is | 7 | 16 |
| 8. | T8C | 6 | 9 |
| 9. | M6 | 11 | 12 |
| 10. | 30 | 15 | 10 |
| 11. | 33 | 13 | 10 |
| 12. | M7 | 12 | 9 |
| 13. | M4 | 8 | 6 |
| 14. | M2 | 10 | 8 |

**Estimating Cultural Growth Requirement for *Streptomyces***

The *Streptomyces* isolates, ($D^-$, $N^-$, M5, 21) which have the highest antibacterial activities at primary and secondary selection were submitted for optimization using many parameters, which include two different media (ISP2 and GYE), four different temperature (26°C, 28°C, 30°C and 32°C), five different pH value (6.5, 7.3, 7.5, 7.8 and 8), Growth Curve (1-10 days) and NaCl -Tolerance (1%, 2%, 4% and 6%).

The *Streptomyces* considered the best source for producing a large number of important bioactive compounds.[30,31] The *Streptomyces* are unique bacteria exist in natural and artificial environments, they vary in their physiological, morphological and biochemical characteristics.

The cultural conditions and nutritional requirements play a critical and economical role in secondary metabolites production, so optimization of these conditions by using efficient and appropriate medium composition and optimum cultural conditions can increase the secondary metabolites production, for example changing the sources of carbon and nitrogen have been reported to affect antibiotic biosynthesis in *Streptomyces*, in addition to other cultivation factors like incubation period, pH, NaCl tolerance and temperature which have an important role in production of secondary metabolites.[32,33]

**Estimating incubation period for *Streptomyces* at two different growth media**

The flask fermentation of 4 isolated *Streptomyces* which selected from secondary screening ($D^-$, $N^-$, M5, 21) were grown on GYE and ISP2 at 28°C to estimate the growth absorbance by spectrophotometer with optical density at 600 nm, from day 1 to day 9, which starting after 24 hours of incubation. Figure (13) shows the differences in the mean of the OD between the isolates grew on ISP2 broth and GYE broth. Stationary phase of isolates grew on ISP2 started at day 4 with highest OD (0.58), while the stationary phase of these isolates grew on GYE started at day 7 with lower OD values (0.49). This indicated that the ISP2 is more perfect for the secondary metabolites production because on it all strains accomplished growth curve (lag and log phase) in 4 days, while in GYE broth they needs to more time for reaching the maximum, Risan *et al*., were reported the same results of ISP2 as a more perfect medium for *Streptomyces* production secondary metabolites with a stationary phase started at day 4.[23] In *Streptomyces*, the stationary phase of growth in liquid culture is characterized by the shift to secondary metabolism and production of a wide variety of secondary metabolites.[34]

**Estimating the best temperature for isolated Streptomyces**

**Temperature Optimization for *Streptomyces* isolates**

The selected *Streptomyces* (4) isolates incubated at various temperatures (26°C, 28°C, 30°C and 32°C) in ISP2 broth to estimate the best temperature for the growth. Figure (14) illustrate the differences in the for OD along the incubation with different temperature, as shown the best growth temperature was at 30 °C with the highest OD (0.6) value which refers to the mean growth activity of 4 isolates after three days of incubation, 32°C and 26°C showed the lowest growth rate and 28°C moderate growth rate.

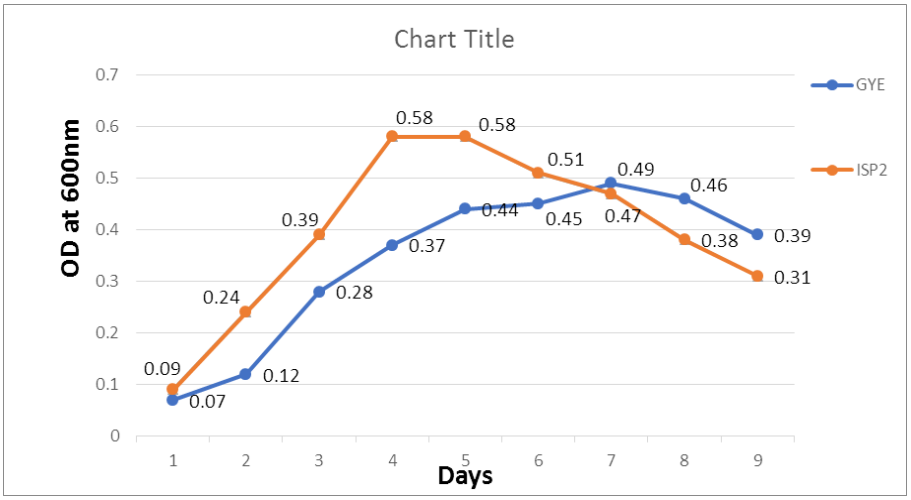


**Figure 13: Estimating the best growth medium and incubation peroid of the *Streptomyces* isolates using GYE broth and ISP2 broth incubated 1-10 days by spectrophotometer absorbance at 600 nm.**

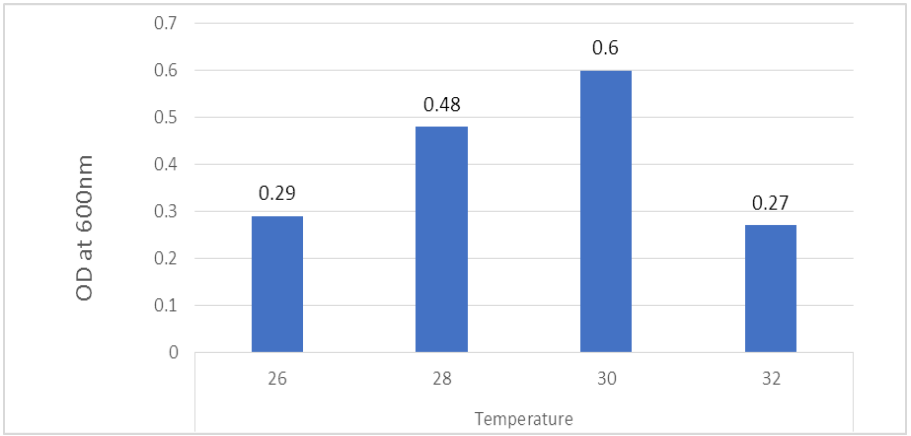


**Figure 14: Estimating the best temperature for growing *Streptomyces* isolates. Highest *Streptomyces* growth measured by the spectrophtometer absorbance at 600 nm, after four days of incubation at different temperature (26°C, 28°C, 30°C and 32°C) on ISP2 broth.**

**Estimating the best pH for isolated *Streptomyces***

The preferred pH for Actinomycetesis the neutral and alkaline, with optimum pH for *Streptomyces* spp. Between 7-8. The *Streptomyces* also exist in an alkalophilic and acidophilic condition.[35] Selected *Streptomyces* isolates (21, M5, N, D) were growing on

ISP2 adjusted with pH value as follows: (6.5, 7.3, 7.5, 7.8 and 8) to determine the optimum pH by measuring the growth rate with spectrophotometer at 600 nm. The highest absorbance with (0.521) was observed at 7.8 on day 4 of incubation (figure 15), with little differences ranged between 0.03 -0.05 between the tested isolates. The results show the same with that found by Palanichamy *et al.*[36] who found that the optimum pH for isolated marine *Streptomyces* was between 7.8 and 8.

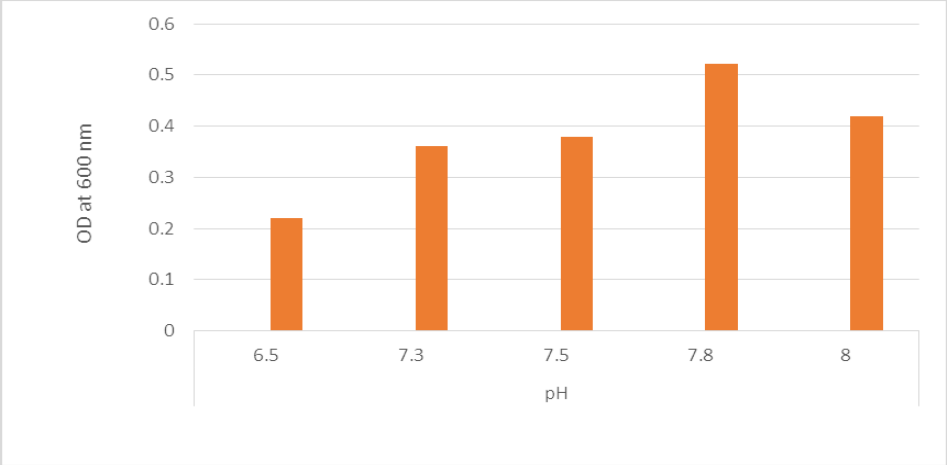


**Figure 15: Estimating the best pH value for growing *Streptomyces* isolates. Highest *Streptomyces* growth measured by the spectrophtometer absorbance at 600 nm, after four days of incubation at different pH (6.5, 7.3, 7.5 ,7.8 and 8) on ISP2 broth.**

**Estimating the NaCl tolerance for *Streptomyces* isolates**

The four selected *Streptomyces* isolates cultured on ISP2 broth supplemented with different concentration of NaCl rangedas follows: (1%, 2%, 4% and 6%) (w/v) incubated for 4 days at 30°C with OD at 600nm to determine the growth rate. Figure (16) shows the good salt concentration for all isolates growth was 2%, while the NaCl tolerance at 6% which indicated that the isolates can grow till 6%, while above this concentration may not grow, this results in aggrement with many studies,[23,37-40] who showed that the optimum NaCl percentage for marine *Streptomyces* growth was 3.5%, so the absence of salt or high concentration are affect the growth rate, this explained by the importance of salt for bacterial growth because the role of slat in maintaining the osmotic pressure to facilitate the molecule movement across bacterial cell, while the high salt concentration makes the $Na^+$ concentration highly toxic to the cell.

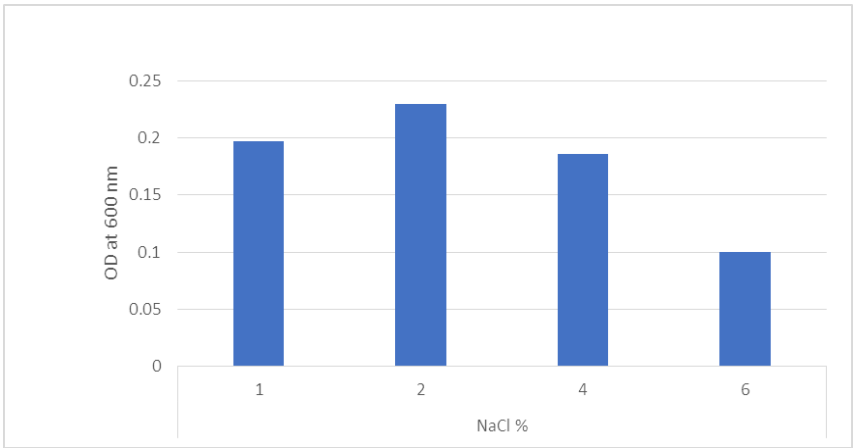


**Figure 16: Estimating the NaCl tolerance for *Streptomyces* isolates. Highest *Streptomyces* growth measured by the spectrophtometer absorbance at 600 nm, after four days of incubation at different pH (1%, 2% , 4% and 6%) on ISP2 broth.**

## CONCLUSION

The Actinomycete is a bacterium which produces more than 70% of the naturally occurring antibiotics that are instantly inclinical use. An increasing in the resistance ofpathogens is become a major public health problem all over the world, so it will be interesting to see the bioactive compounds derived from *Streptomyces* spp., for this reason we focused on our study to isolate *Streptomyces* spp. fromTigris river sediment in Baghdad city and testing their ability to produce bioactive

compounds. The results showed high activities at secondary screening against Gram- positive and Gram - negative bacteria.

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## CONFLICT OF INTEREST

No conflict of interest.

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