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Original Article

Screening of bacteria *Streptomyces* Waksman and Henrici 1943 (Streptomycetaceae) Isolates from Soil Samples in Iraq

Bassam Qasim[1], Mohsen Hashim Risan[2]*

[1]Department of Biology, College of Alsalam, Al-Nahrain University, Baghdad, Iraq
[2]Department of Biotechnology, College of Biotechnology, Al-Nahrain University, Baghdad, Iraq

*Corresponding Author E-mail: M_risan@yahoo.com

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ABSTRACT

Background: The genus *Streptomyces* Waksman & Henrici 1943 includes aerobic, gram-positive, and filamentous bacteria which produce well developed vegetative hyphae with branches. The wall consists of a large mixture of different compounds, including peptidoglycan, teichuronic acid, teichoic, and polysaccharides. The peptidoglycan components consist of glycan as a chains of irregular N-acetyl- d-muramic acid (NAM), diaminopimelic acid, and N-acetyl-d-glucosamine (NAG) and DAP, which is unique in the cell walls of prokaryotic microorganisms. The teichoic and teichuronic acid are chemically bonded to peptidoglycan.

Methods: One gram of soil samples was used to make suspension, by adding 99 mL of sterile distilled water (stock suspension) into it and shaking it in a shaker at 160 rpm for 30 minutes at room temperature. Serial dilutions from 0.1-0.001 were made from the stock suspension, and left for 10 minutes. After shaking, 0.1 mL of each dilution was cultured on Yeast Extract and Malt Extract agar (YEME) with Streptomycin 50 ug/mL. The inoculated plates were incubated at 28 °C for 7 to 10 days. Based on cultural characteristics, suspected colonies of *Streptomyces* were selected, which are characterized as small, white, pin-point, rough, chalky, and a clear zone of inhibition around them. These colonies were confirmed their identification by types of Gram's stain, aerial and substrate mycelium color, pigment production, and pigment color. *Streptomyces* were re-streaked on International *Streptomyces* project (ISP) to obtain pure colonies used for identification.

Results: The current study aimed to screen bacteria *Streptomyces* isolates. Only 21 samples of soil were suspected to contain *Streptomyces*, and 45 isolates were obtained with different morphology types per samples of soil. The colonies suspects were selected basis on color that ranged from gray, white and creamy. The microscopic examination of local *Streptomyces* spp. after Gram-staining method was conducted. The observations revealed that local *Streptomyces* is gram positive and rod shaped similar to

features of fungal in possessing branched mycelium. The *Streptomyces* produced extra cellular enzymes like amylase, urease, catalase, protease, Gelatinase, cellulase and phosphatase. Utilization of citrate was positive, with no Melanine reaction production and soluble pigmented, and negative for indole production.

Conclusion: The identification of the *Streptomyces* is a very complex process. Morphological and biochemical characteristics are two important aspects for the classification in the Streptomycetaceae family. By studying the morphological, cultural, and biochemical characteristics, it is observed that the local isolates are belonging to the genus of *Streptomyces*.

Keywords: Kidney Stones, Vitamin D, Hypercalciuria*.*

1. Introduction

Streptomyces Waksman & Henrici 1943 is the type genus of the family Streptomycetaceae [1] and currently covers close to 576 species with the number increasing every year [2, 3]. *Streptomyces* includes aerobic, Gram-positive, and filamentous bacteria which produce well developed vegetative hyphae (between 0.5-2.0 μm in diameter) with branches. They form a complex substrate mycelium that aids in scavenging organic compounds from their substrates [4]. Although the mycelium and the aerial hyphae that arise from them are amotile, mobility is achieved by dispersion of spores [4]. Spore surfaces may be hairy, rugose, smooth, spiny or warty [5].

The actinomycetes cell wall is an inflexible structure that maintains the cell wall of actinomycetes shape through cell wall which prevents bursting of the cell high osmotic pressure [6]. The wall consists of a large mixture of different compounds, including peptidoglycan, teichuronic acid, teichoic, and polysaccharides. The peptidoglycan components consists of glycan as a chains of irregular N-acetyl-d-muramic acid (NAM), diaminopimelic acid, and N-acetyl-d-glucosamine (NAG) and DAP, which is unique in the cell walls of prokaryotic microorganisms; the teichoic and teichuronic acid are chemically bonded to peptidoglycan [7]. The chemical composition of their cell wall is similar to that of gram-positive bacteria, but because of their well morphological developed and cultural characteristics, actinomycetes have been considered as a group, which separate from the other ordinary bacterial group [8]. Therefore, the current study aimed to screen bacteria Streptomyces isolates in Iraq.

2. Materials and Methods

2.1. Sample collection

Thirty-five samples were collected from different regions of Iraq (Baghdad, Najaf and Babylon), summarized in Table 1. 250 grams of soil samples were collected from regions that were mentioned previously at a depth ranging from 5 to 15 cm, and were kept in polyethylene bags (20 * 40 cm). The soil samples were exposed to the air for a week, also they were pretreated with CaCO3 (with a ratio of 10:1 soil: CaCO3) and kept at ambient temperature for a week, to enrich actinomycetes which usually prefer alkaline conditions and also to reduce the contamination with molds and fungi [9].

Table 1. Sites and numbers of soil samples were collected for isolation of locally *Streptomyces* in Iraq

No.	Site	Sample Abbreviated no.	No.	Site	Sample Abbreviated no.
1	Baghdad	Bag1	19	Najaf	Naj19
2	Baghdad	Bag2	20	Najaf	Naj20
3	Baghdad	Bag3	21	Najaf	Naj21
4	Baghdad	Bag4	22	Najaf	Naj22
5	Baghdad	Bag5	23	Babylon	Bab23
6	Baghdad	Bag6	24	Babylon	Bab24
7	Baghdad	Bag7	25	Babylon	Bab25
8	Baghdad	Bag8	26	Babylon	Bab26
9	Baghdad	Bag9	27	Babylon	Bab27
10	Baghdad	Bag10	28	Babylon	Bab28
11	Baghdad	Bag11	29	Babylon	Bab29
12	Baghdad	Bag12	30	Babylon	Bab30
13	Najaf	Naj13	31	Babylon	Bab31
14	Najaf	Naj14	32	Babylon	Bab32
15	Najaf	Naj15	33	Babylon	Bab33
16	Najaf	Naj16	34	Babylon	Bab34
17	Najaf	Naj17	35	Babylon	Bab35
18	Najaf	Naj18			

2.2. Isolation and Identification of Streptomyces from Soil

One gram of dried and treated soil samples was used to make suspension, by adding 99 mL of sterile distilled water (stock suspension) and shaking it in a shaker at 160 rpm for 30 minutes at room temperature. Serial dilutions from 0.1-0.001 were made from the stock suspension, and left for 10 minutes. After shaking, 0.1 mL of each dilution was cultured on Yeast Extract and Malt Extract agar (YEME) with Streptomycin 50 ug/mL, then spread by sterile swab for making 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 *Streptomyces* were selected which are characterized as small, white, pin-point, rough, chalky and a clear zone of inhibition around them. These colonies were confirmed for their identification by types of Gram's stain, aerial and substrate mycelium color, pigment production, and pigment color. These colonies were transferred from the mixed culture into separate agar plates and incubated at 28±1°C for 7 days. In order to obtain a pure growth of *Streptomyces* were re-streaked on International *Streptomyces* project (ISP) to obtain pure colonies used for identification[10-13].

2.3. Streptomyces isolation and identification media

International *Streptomyces* Project (ISP2) Medium of *Streptomyces* isolation (Table2) was used [14]. This medium was prepared by dissolving amount of each component in 1000 mL distilled water and sterilized by autoclaving at 121 C, (15 Ib\ inch^2) for 15 minutes.

Table 2. International *Streptomyces* Project (ISP2) Medium [17]

Component	Quantity (g/l)
Yeast extract	4
Malt extract	10
Dextrose	4
	20

2.4. The Best Media Composition for Antimicrobial Production

According to international *Streptomyces* projects (ISP) [14, 15], different media were used for isolation and identification of *Streptomyces* spp. furthermore, to achieving the best types of media composition for production of antimicrobial metabolites, different media with different composition were used (Table 3). After seven days of incubation, antimicrobial metabolites extraction was carried.

Table 3. International *Streptomyces* projects (ISP) used for isolation and identification of *Streptomyces* [16]

No.	Medial name	Abbreviation
1	Tryptone-yeast extract broth	ISP1
2	Yeast extract-malt extract broth	ISP2
3	Inorganic salts-starch broth	ISP4
4	Glycerol-asparagine broth	ISP5
5	Peptone-Yeast Extract Iron agar	ISP6
6	Tyrosine Agar	ISP7
7	Glycerol yeast extract broth	GYE

2.5. Identification of Bacterial Isolates

Suspected bacterial isolates were primarily identified by Microscopic and cultural examinations, then by the biochemical tests for final identification as follows:

2.5.1. Morphological Characteristics

The morphological characterization of each isolate was first performed by:

2.5.1.1. Colony Characteristics [19]

Bacterial isolates grew on ISP medium were characterized morphologically and physiologically according to the International *Streptomyces* project (ISP).

2.5.1.2. Gram's Stain [20]

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.

2.5.2. Biochemical properties

Streptomyces spp. isolates were selected for biochemical characterization, and various biochemical tests were studied. Many characteristics were studied including czapeck medium [16], sugars utilization medium [17], organic acids formation medium [17], urease test, amylase test, Protease test, cellulase test, Indol production test, Kovae's reagent, phosphatase test, citrate utilization test, gelatinase test and catalase test [18, 19].

2.5.3. Identification onn,..., f bacteria by VITEK 2 system

The VITEK 2 which is recently installed at the Central Health Laboratories/Ministry of Health is an automated microbiology system utilizing growth-based technology. A sterile swab sample was used to transfer a sufficient number of colonies of a pure culture and to suspend them into 3 mL of normal saline (NaCl 0.45%, pH 5-7). Then, turbidity was adjusted by a turbidity meter called the DensiCheck to match 0.5– 0.6 McFarland, which is the proper inoculum density for Gram-negative and gram-positive bacteria as stated by the manufacturer.

3. Results

3.1. Isolation of *Streptomyces* bacteria from the soil

The technique of serial dilution was used to isolate *Streptomyces* bacteria from 35 different samples of soil. Then, the plates were inoculated with soil suspension on media Yeast Extract Malt Extract and the plates were incubated for 7 days with a range of dilution between 0.1-0.001. From the above soil samples on the basis of forming colonies with inhibitory or clear zone of inhibition around them, the suspected *Streptomyces* were obtained, which were small white, chalky and rough.

From 35 soil samples, only 21 samples of soil were suspected to contain *Streptomyces*, mean 60% of them, and 45 strains were obtained with different morphology types per samples of soil. The colonies suspects were selected based on color varied between gray, white and creamy, which were grown on yeast extract malt extract agar.

Also, nine different types of media were tested for their efficiency of *Streptomyces* to support larger number of colonies from the soil (Table 4). These media were used to isolate the Local *Streptomyces* spp. Several types of *Streptomyces* bacteria were isolated on these media, but in this study only one type was used. This bacteria Local *Streptomyces* spp. was isolated from soil collected from Baghdad under depth of 20 cm. The Local *Streptomyces* spp. isolation and purification are shown in Figures 1 and 2.

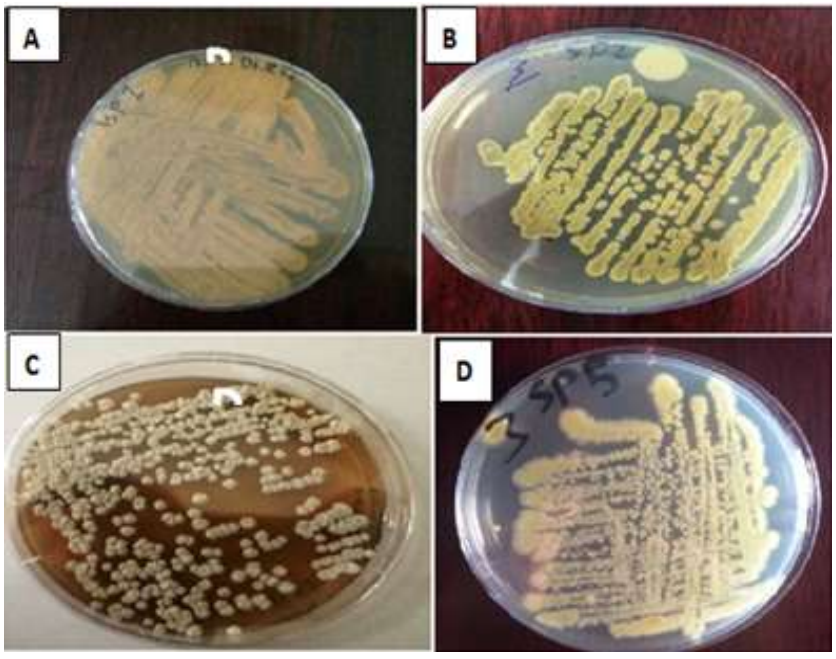

Figure 1. Local *Streptomyces* spp. Isolates grow on ISP1 (A), ISP2 (B), ISP4 (C), and ISP5

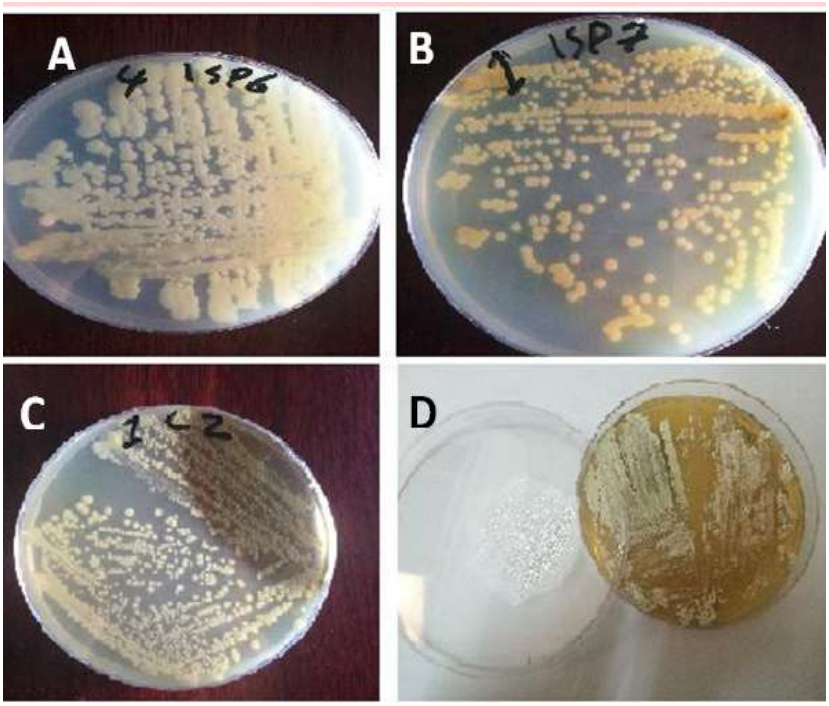

Figure 2. Local *Streptomyces* spp. Isolates grow on ISP6 (A), ISP7 (B), czapeck agar (C), and potato dextrose agar (D)

Table 4. The morphological characteristics of colony on special medium (ISP) and other workers

Medium	Growth	Aerial mycelium
ISP1	++	Pale yellow
ISP2	++++	Creamy
ISP4	++	Pale white
ISP5	+++	Pale yellow
ISP6	+++	Pale white
ISP7	+++	Creamy
CZ	++	Pale white
PDA	+++	Light white – yellow
N.A.(nutrient agar)	++	Pale - white

+ : less, ++: moderate , +++ : good , ++++: very good

3.2. Microscopic examination

The microscopic examination of local *Streptomyces* spp. after Gram- staining method was conducted and the observations revealed that local *Streptomyces* is gram positive and rod shaped similar to features of fungal in possessing branched mycelium.

3.3. Biochemical properties

The results of biochemical testes of *Streptomyces* spp. are shown in Table 5. The *Streptomyces* produced extra cellular enzymes like amylase, urease, catalase, protease, Gelatinase, cellulase and phosphatase. Utilization of citrate was positive, with no Melanine reaction production (Medium ISP 2 and Medium ISP6) and Soluble Pigmented (ISP4 and PDA), and negative for indole production.

Table 5. The biochemical tests properties of *Streptomyces* spp

Reaction	Response	Result
1. Melanine reaction Medium ISP.2	Brownish of medium	Negative
Medium ISP.6	Brownish of medium	Negative
2. Soluble Pigmented	NO Brown	Negative
ISP4 PDA	Dark Brown pigment	Positive
3. Urease	Red to deep pink	Positive
4. Catalase	Bubbles	Positive
5. Amylase	Clear zone	Positive
6.Protease	Clear zone	Positive
7. Gelatinase	Narrow zone	positive
9. Cellulase	Clear zone	Positive
10. Phosphatase	Clear zone	positive
11. Indole production	No color zone	Negative
12. Citrate Utilization	Deep blue color	Positive

3.4. Identification of *Streptomyces* by VITEK2 system:

The *Streptomyces* was identified by VITEK 2. By this system of identification, we were able to identify *Streptomyces* accurate. The results of VITEK 2 system agreed with the obtained those of the biochemical tests that were applied for the bacterial isolates (Table 5).

4. Discussion

The technique of serial dilution was used to isolate *Streptomyces* bacteria from 35 different samples of soil. After the plates were inoculated with soil suspension on media Yeast Extract Malt Extract, the plate were incubated for 7 days with a range of dilution between 0.01-0.001, from the above soil samples on the basis of forming colonies with inhibitory or clear zone of inhibition around them the suspected *Streptomyces* were obtained as small white, chalky and rough [20-22].

The results were in agreement with the past study [23]. The colonies size and morphology were in the range from 1 to 10 mm in diameter with relatively smooth surface at the growth beginning, whereas developed to an UN aerial mycelium that appeared as powdery, soft and granular. Isolated *Streptomyces* colonies were chalky, slowly growing, aerobic, piled, as well as with different color of aerial and substrate mycelium. Also, all colonies that were isolated possessed earthy odors. The colonies were stored in refrigerator at 4°C for further study. The results agreed with those of [24] and [25]. In association with similar habitats, the *Streptomyces* diversity exhibited with few different colony types, and process of isolation, where each plate often contained one or few colony types ranging from 2-4 colonies.

Depending on result of [26], they described *Streptomyces* existent in more than one soil types and surface layer of soils are more abundant besides the favoring of alkaline soils, river's mud, compost and riverbeds. On the other hand, the study on *Streptomyces* isolation in the soil [10] showed that physical properties pH, moisture, soil texture, organic matter content and soil reactions were considered as the important factors affecting *Streptomyces* distribution.

A study showed [27] the sample was not collected from surface of soil because of the fact that *Streptomyces* was found on lower soil surface than 11-15 cm depth into the soil, which may be attributed to the favorable combination of suitable pH and water content. The number of *Streptomyces* in black-alkaline sandy soil was very high and the second cause of selecting one isolate was the isolation of *Streptomyces* from the soil in nature complicated by their characteristic slow growth relative to that of other bacteria. This has resulted in the development of selective isolation [28].

Local *Streptomyces* is gram positive and rod shaped similar to features of fungal in possessing branched mycelium in their morphology of cell [29]. The Gram-staining response of bacteria is an important criterion; it is different in the ultra-structure and chemical composition of the two main kinds of prokaryotic cells that are found in nature, which is gram positive and gram negative. These two types of cells are different from each other depending on the absence or presence of an outer lipid membrane that is more fundamental and reliable features for the cell of bacteria [30]. All bacteria of gram- positive are bounded by only a single unit lipid membrane and they contain, in general, a thick layer (20-80 nm) of peptidoglycan responsible for the gram- stain positive (purple) retaining [31]. We further characterized the strains *Streptomyces* spp for biochemical properties, nutritional uptake and all the isolates yielded similar results obtained by several investigators [4].

The identification of the *Streptomyces* is a very complex process. The *Streptomyces* classification system is mainly dependent on characteristics like the form of spores, melanoma and use of carbon [32]. Morphological and biochemical characteristics are two important aspects for the classification in the Streptomycetaceae family [33]. By studying the morphological, cultural and biochemical characteristics, it is observed that the local *Streptomyces* spp. is belonged to the genus of *Streptomyces* [34], referred to the probability and confidence of identification of Vitek 2 system as the accuracy of the VITEK2 system. It has been pointed out [35] that the VITEK2 system is efficient to handle systems that provide faster results during 4 to 15 h and have reasonably accurate means for the identification of the species of bacterial. One of the mean advantages of the VITEK2 system is the reducing the handling time significantly, having a positive impact on the work flow of the laboratory of clinical microbiology.

5. Conclusions

The identification of the *Streptomyces* is a very complex process. Morphological and biochemical characteristics are two important aspects for the classification in the Streptomycetaceae family. By studying the morphological, cultural and biochemical characteristics, it is observed that the local *Streptomyces* spp. is belonged to the genus of *Streptomyces*.

Authors' contributions

M.H.R., designed this study, B.Q. collected and analyzed the data. B.Q. supervised the collection of samples and the identification. M.H.R. and B.Q., proceeded to the data quality control and the manuscript drafting. . M.H.R. revised the final version.

Consent for publications

All authors agree to have read the manuscript and authorize the publication of the final version of the manuscript.

Conflict declaration

The authors declare that there is no conflict.

Conflict of interest

None of the authors has any conflict of interest to declare.

Availability of data and material

Data are available on request from the authors.

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Ethics approval and consent to participate

Samples collection were obtained from soil.

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