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Biochemical and Molecular identification of bacteria Streptomyces fradiae in Iraq

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ABSTRACT

Background: Streptomyces Waksman & Henrici 1943 is the type genus of the family Streptomycetaceae, it is a group of bacteria that are classified as aerobic, Gram-positive, and filamentous. These bacteria are capable of producing well-developed vegetative hyphae that include branches.

Methods: The period of time from December 2023 to January 2024 saw the collection of fifty soil samples. Several locations inside the city of Baghdad were chosen for the collection of samples. After sample dilution it put on ISP2 agar for Streptomyces isolation. Several biochemical tests were done for the isolates.

Results: Thirty Streptomyces isolate were obtained depended on morphological, cultural and biochemical characteristic. The colonies under suspicion were cultivated on ISP2 agar and chosen based on their color (gray, creamy, or white) and colony diameter (ranging from 1 to 10 mm). Additionally, their morphology was considered, with the colonies initially having a smooth surface and later becoming powdery, soft, and granular as they formed aerial mycelium. The isolates were positive to catalase, Citrate Utilization, Sugar utilization, Nitrate reduction, Casein hydrolysis and starch hydrolysis biochemical tests, Whole genome DNA from overnight cultures of isolates of *Streptomyces fradiae* were extracted.

Conclusion Streptomyces identification is a complex procedure. Classification within the Streptomycetaceae family relies heavily on morphological and biochemical characteristics. The local Streptomyces spp. is classified as belonging to the genus Streptomyces based on morphological, cultural, and biochemical trait and genomic DNA analysis.

Keywords: *Biochemical, Molecular, bacteria, Streptomyces fradiae*

1. INTRODUCTION

Streptomyces Waksman & Henrici 1943 is the type genus of the family Streptomycetaceae [1]. It is currently home to close to 576 species, and the number of species is growing at an alarming rate [2, 3]. Streptomyces is a group of bacteria that are classified as aerobic, Gram-positive, and filamentous. These bacteria are capable of producing well-developed vegetative hyphae that include branches and range in diameter from 0.5 to 2.0 µm. They do this by forming a complex mycelium that is attached to their substrates and helps them remove organic molecules from those substrates [4]. The mycelium and the aerial hyphae that emerge from it are both amotile; nonetheless, movement is achieved by the dispersion of spores [4]. It's possible for the surface of a spore to be hairy, rugose, smooth, spiny, or warty [5]. An inflexible structure known as the actinomycetes cell wall is responsible for maintaining the shape of the actinomycetes cell wall through the cell wall. This structure also prevents the strong osmotic pressure from bursting the cell [6]. The wall is composed of a complex web of substances, including peptididoglycan, teichuronic acid, teichoic acid, and polysaccharides. A large variety of substances make it up. A characteristic of prokaryotic microbial cell walls is the presence of peptidoglycan, which is composed of glycan linked to irregular chains of N-acetyl-d-muramic acid (NAM), diaminopimelic acid, Nacetyl-d-glucosamine (NAG), and DAP [7]. Actinomycetes are culturally and morphologically distinct from the other group of common bacteria, which has led to their classification as a separate category [8]. Reliable descriptions of type strains of 458 Streptomyces species were published by the International Streptomyces Project (ISP) using the standard criteria for species determination. Subsequently, chemotaxonomy, physiological and biochemical traits, and DNA-DNA hybridization (DDH) of total chromosomal DNA

were utilized as additional fundamental phenotypic markers for classification [9]. They are chemically similar to gram-positive bacteria in terms of the composition of their cell wall. So, biochemical and molecular characterization of Streptomyces fradiae bacteria was the goal of the study.

Methods

The period from December 2023 to January 2024 was used to collect fifty soil samples. Samples were collected from various spots all throughout Baghdad. Streptomyces spp. were isolated from each location using specific areas. After excavating about 3 cm of dirt, the depth to which samples were collected was 10-15 cm. After being carefully packed in polyethylene bags, the samples were placed in the refrigerator for storage. After a 2-hour incubation period at 70°C to kill any remaining microorganisms, the soil samples were screened to isolate Streptomyces.

Streptomyces spp. Isolation and Identification from Soil Sources

The solution was prepared by mixing about one gram of dried soil samples to 99 ml of sterile distilled water, which is known as the stock suspension. For 30 minutes at room temperature, the samples were shaken in a shaker set to 120 revolutions per minute. Serial dilutions ranging from 10^-1 to 10^-9 were prepared using the stock suspension. Following this, they were incubated for ten minutes. The supplemented yeast extract-malt extract agar (ISP2) was pipetted with 0.1 milliliters of each dilution after stirring. The agar contained 50 µg/L of tetracycline and Fifty µg/L of nystatin. The mixture was then evenly distributed throughout the surface of the media using a sterile brush. Seven to ten days of incubation at 28°C was used for the inoculation plates. Small, white, pinpoint, rough, chalky, and surrounded by a distinct zone of inhibition colonies of actinomycetes were chosen for characterisation purposes according to cultural criteria. Pigment synthesis, coloration of pigments, Gram staining, and the color of aerial and substrate mycelium were used to identify the colonies that were suspected. Additionally, some biochemical tests are used for identification. The procedure was reiterated multiple times until the colonies were transitioned from the mixed culture phase to separate agar plates, where they were incubated at 28±1°C for 7 days to achieve pure growth of actinomycete species on ISP2 agar. The pure culture was stored at 4°C until it could be utilized for further investigation [10].

Biochemical analysis

Melanin, Catalase, Citrate Utilization, Indole production, Sugar utilization, Hydrogen sulfide production, Nitrate reduction, Oxidase production, Casein hydrolysis, Melanine reaction and Starch hydrolysis tests were conducted on *Streptomyces* isolates.

2. MOLECULAR STUDY

DNA Extraction

Following the manufacturer's directions, genomic DNA from bacterial isolates was extracted using the ABIOpureTM Total DNA Bacteria Kit.

Agarose Gel Electrophoresis

The existence of amplification was confirmed by using agarose gel electrophoresis after PCR amplification. The criteria for the extracted DNA were absolutely reliable for PCR. Direct loading of PCR products was performed. Wells were directly loaded with 5µl of PCR product. After 60 minutes, the power was switched on at 100 volts per milliampere. DNA travels from the negative cathode to the positive anode. The Gel imaging system was used to visualize the bands in the gel that were stained with Ethidium bromide.

3. RESULTS AND DISCUSSIONS

Purification and identification of Streptomyces sp isolates

Streptomyces spp. isolation from dehydrated soil specimens

A total of fifty soil samples obtained from various locations inside the Baghdad city, were examined to assess the efficacy of *Streptomyces* as a potential source of active antibacterial agents.

Streptomyces sp. were detected alongside other microorganisms in the form of mixed colonies following the cultivation of a diluted soil sample (10^{-6}) on ISP2 agar for a period of 7-10 days.

Figure (1) displays little powdery colonies ranging from white to grey, which are likely to represent isolates of *Streptomyces*. This picture displays a solitary Actinomycete colony within a collection of mixed colonies.

Colonies originating from sources other than *Streptomyces* within the culture may be attributed to the existence of their spores in the soil or their resistance to heat treatment. The colonies under suspicion were cultivated on ISP2 agar and chosen based on their color (gray, creamy, or white) and colony diameter (ranging from 1 to 10 mm). Additionally, their morphology was considered, with the colonies initially having a smooth surface and later becoming powdery, soft, and granular as they

formed aerial mycelium. Similar findings were reported by [11].

Out of the 50 soil samples analyzed, 35 (86.6%) were found to potentially contain *Streptomyces*. Among these samples, 33 (80%) of *Streptomyces* were successfully isolated, each exhibiting distinct morphological traits. Using a polyphasic method that included morphological and biochemical analysis, Streptomyces isolates were identified in the study by Antido et al. [12]. From varied soil habitats, 103 distinct Streptomyces species were found, each with its own unique morphology and biochemistry. A pure isolation was achieved by delicately transferring the likely Streptomyces colonies to ISP2 agar medium[13]. The isolate was subsequently characterized by its vibrant aerial and substrate mycelium, dry and coarse or smooth texture, and irregular or regular margin. The colony had a convex morphology. The predominant solitary colonies display earthy fragrances, as noted by [14,15]. Streptomyces is the predominant genus of actinomycetes found in soil. The aerial and substrate mycelium displays a tan hue when grown in ISP-2 and AIA medium. It does not produce any diffused pigment when grown in the stated solid medium; but, it can develop a deep brown pigment under submerged conditions in AIA liquid medium. The Streptomyces colony exhibits a filamentous, dull, umbonate, rough, and translucent morphology [16].

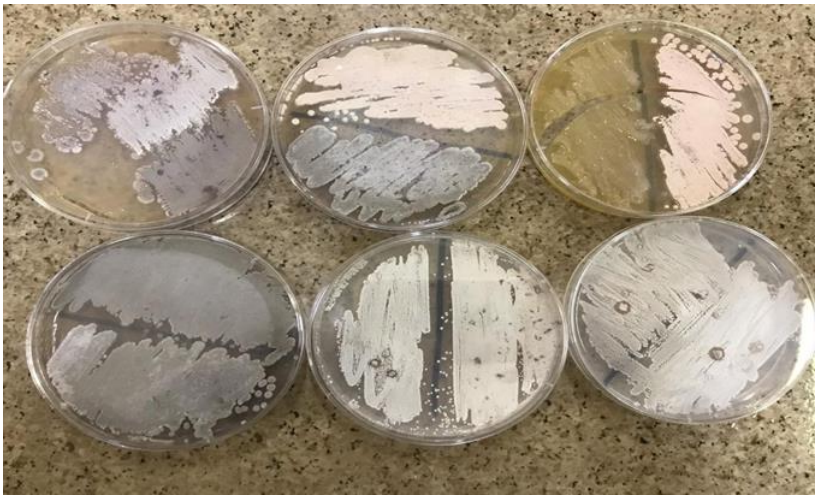


Figure (1): Single colonies of *Streptomyces* sp on ISP2 agar

Isolation by streak plating for individual colonies

The streak plate approach was employed to establish a solitary colony for the purification of actinomycete cultures, as illustrated in figure (2). This plating method utilizes sequential dilution to diminish the bacterial count in each streak. The initial streak is anticipated to have a significantly high bacterial concentration because it originates from a concentrated supply. By employing a new or sterilizing equipment on a specific section of the original line, we allocate a segment of the original line over a little expanded surface area, thereby creating a second line. The reduced bacterial count in this second line enhances the likelihood of identifying distinct colonies. The dilution method was performed multiple times by dispersing the entire plate, starting from the initial concentrated stripe. This was performed to obtain a unique isolated colony[17,18].

Figure (2): Streptomyces fradiae, when grown on ISP2 agar at 28°C for 7-10 days using the streak plate method, forms a single colony.

Morphological characterization

Streptomyces samples have been identified based on alterations in colonies shape and microscopic features, comprising the development of substrate and aerial mycelium, together with the existence of soluble pigments, and the configuration of spore chains. Multiple Streptomyces sp. isolates demonstrated the capacity to produce a colored compound that permeated the surrounding medium, corresponding to the hue of the aerial mycelium. Furthermore, a soluble pigment was detected in 20 isolates.

All of the isolates were microscopically inspected after seven to ten days of incubation to observe the hyphae, as illustrated in figure (3). Following a 7-10 day incubation period, the morphology of the spore chains was analyzed and revealed several shapes, including, a spiral, flexuous or straight, contingent upon the specific species of Streptomyces. The majority of strains demonstrated a linear chain configuration, except for three strains that revealed a spiral chain structure and two strains that displayed a rectiflexible arrangement. References (19, 20) documented congruent results, as illustrated in Table 1.

Table (1): Morphology attributes of Streptomyces isolates.

No of isolation	Colony feature	Mycelium surface	Spore chain morphology	Arial Mycelium	Soluble pigment	Substrate Mycelium reveres side pigments
1	Irregular edge/circular	smooth	straight	Light grey	brown	Light brown
2	Regular edge/circular	smooth	straight	Light grey	Light brown	Light brown
3	Irregular edge/circular	smooth	straight	Light grey	Light brown	Light brown
4	Regular edge/circular	smooth	straight	Light grey	Yellow	Yellowish
5	Regular edge/circular	Rough	straight	grey	No pigment	Dark brown
6	Irregular edge/circular	smooth	straight	Light grey	Yellow	Dark brown
7	Regular edge/circular	Rough	straight	grey	Light brown	brown
8	Irregular edge/circular	smooth	straight	white grey	No pigment	Light brown
9	Irregular edge/circular	Rough	straight	grey	No pigment	
10	Regular edge/circular	Rough	straight	Light grey	Light yellow	Light brown
11	Regular edge/circular	smooth	straight	Light grey	Dark	Light brown
12	Regular edge/circular	smooth	straight	White grey	No pigment	brown
13	Regular edge/circular	smooth	straight	Light grey	Dark yellow	Light brown
14	Irregular edge/circular	smooth	straight	grey	No pigment	Light brown
15	Irregular edge/circular	Rough	straight	White grey	No pigment	brown

16	Regular edge/circular	smooth	straight	grey	No pigment	Light brown
17	Regular edge/circular	smooth	straight	grey	No pigment	brown
18	Regular edge/circular	smooth	rectiflexible	grey	No pigment	brown
19	Irregular edge/circular	smooth	straight	Light grey	Dark brown	Light brown
20	Irregular edge/circular	smooth	straight	grey	No pigment	brown

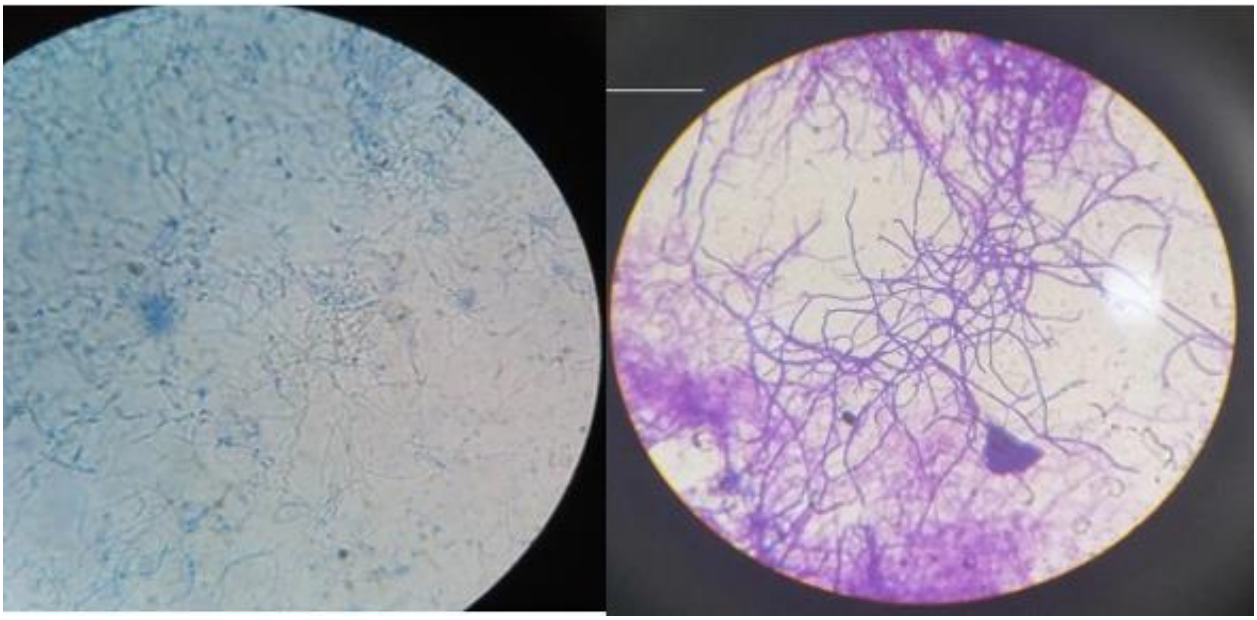


Figure (3): *Streptomyces* sp. hyphae, grown on ISP2 agar (left) and on ISP2 broth (right). Branching filaments, prevalent aerial mycelia, and elongated chains of diminutive spores are seen, all of which are indicative of Streptomyces spp. at 100X magnification.

Biochemical test

The biochemical findings of *Streptomyces* sp. are presented in table (2). Streptomyces possess the capacity to synthesize enzymes such as catalase. Simmon's citrate usage was confirmed, indicating a positive result, but indole synthesis was not detected, indicating a negative result. The use of sugar was investigated by cultivating *Streptomyces* in media containing Dextrose, starch, or Glycerol as carbon sources. The analysis was conducted using a biochemical test [21,22].

Table (2): Biochemical results of *Streptomyces fradiae*

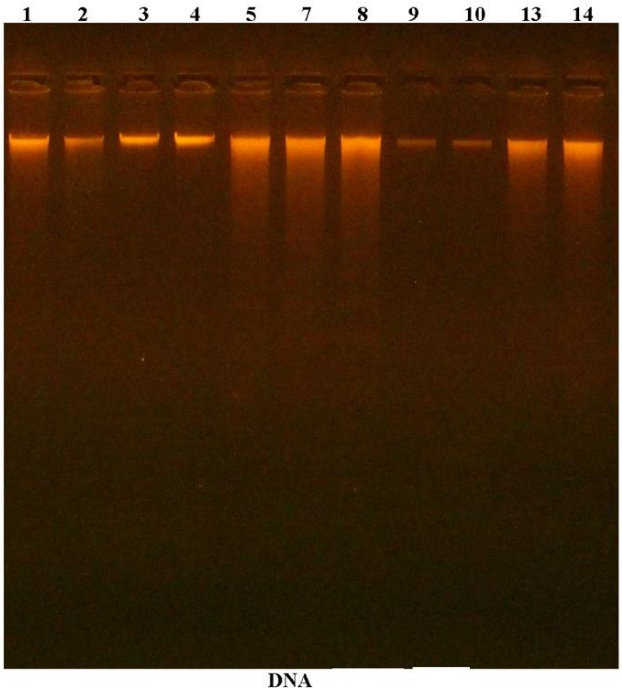
Test	Result
Hydrogen sulfide production	+ve
Nitrate reduction	+ve
Melanin	-ve
Oxidase production	+ve
Utilization of Citrate	+ve

Casein hydrolysis	+ve
Catalase	+ve
Melanin	-ve
Indole production	-ve
Sugar utilization	+ve
Starch hydrolysis	+ve

4. MOLECULAR ASSAY

Extraction of genomic DNA

Whole genome DNA was easily recovered from overnight cultures of 13 isolates of Streptomyces fradiae using the ABIOpure TM Whole DNA extraction kit, adhering to the procedure outlined in chapter two. The concentration and purity of the isolated DNA were assessed using Nanodrop. Moreover, Figure (3) depicts the presence of a singular band of extracted DNA, signifying the efficacy of the used extraction method[23].



Figure(3): Agarose gel imaging of the DNA extracted for the bacterial isolates

5. CONCLUSIONS

Streptomyces identification is difficult. Morphological and biochemical traits are used to classify Streptomycetaceae. Morphological, cultural, biochemical, and genomic DNA study classify the local Streptomyces spp. as Streptomyces.We have demonstrated in this study the utilization of a molecular approach for enhanced isolation and identification of *Streptomyces* from soil habitats**.**

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