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Identification of Antibiotic Producing Actinomycetes Isolated from Sediment in Basra, Iraq

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Abstract : The increasing prevalence of commercial drug-resistant microbes is a worrisome issue that calls for the search for new antibiotics by isolating microbes that produces active secondary metabolites from diverse environments to obtain new strains and products. In the present study, 25 samples were obtained from marine and marshes sediments in Basrah city, Iraq. The screening revealed that 13 isolates possess antimicrobial activity. Three of these strains (W2, W5, and W11) were found to produce antibacterial and antifungal metabolites, W5 has maximum inhibition zone (28 and 22 mm) was selected against *E. coli* and *Staphylococcus aureus* respectively, W2 recorded 14 mm of inhibition zone against *Aspergillus niger*, while W11 has 13 mm of inhibition zone against *Candida albicans*. These isolates were identi? ed as *Streptomyces* sp. TRM46619 (W2), *Micromonospora auratinigra* SB29 (W5) and *Streptomyces carpaticus* PES-A23 (W11) depended on amplified 16S rDNA gene by using universal primers. All strains have broad antimicrobial activity against pathogenic bacteria gram-positive and gram-negative, as well as against fungi, and this indicates a promising potential for new antibiotics.

Keywords: *Antimicrobial activity, Streptomyces, Micromonospora, Sediment*

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1. INTRODUCTION

The appearance of antibiotics resistant infectious disease-caused by microbial pathogens was highly spread because of the broad application of the antimicrobial agents. Accordingly, the destruction of the microbial resistance was of huge demand. Several secondary metabolites against those pathogenic microbes such as antibiotics were produced by Actinomycetes.[1] Actinomycetes are filamentous gram-positive bacteria, primarily saprophytic microorganisms of the soil, where they contribute significantly to the turnover of complex biopolymers, such as lignocellulose, hemicellulose, pectin, keratin, and chitin.[2-3] The *Streptomyces* species are widely spread in different ecological and ability to produce antibacterial and antifungal agent, marine environments are a largely untapped source of isolation of new microorganisms with the possibility of producing active secondary metabolites today.[4-5].[6] The high salinity of marine environment, as well as other environmental factors, have a direct effect on the production of antibacterial by Streptomyces.[6] Some Actinomycetes have been isolated from various ecosystems including river sediments and areas of super salinity.[7-8] *Streptomyces* and *Micromonospora* are important groups of bacteria that are used for antibiotic production, that about 80% of all antibiotic products by *Streptomyces* and less than 10% by *Micromonospora*.[9-10] The *Streptomyces* genus was proposed by Waksman and Henricii (1943). In the past, Streptomycetaceae family was identified based on phenotypic features. Recently, molecular tools are used for accurate diagnosis at the species level. *Streptomyces* are gram-positive aerobic bacteria that belong to the Actinobacteria, it has a high G + C content (69-78%) in DNA.[12] Members of the *Micromonospora* genus are filamentous bacteria possess colonies that range from yellowish orange to reddish-orange, turns into dark brown or black through spores formation, characterized as a major source for biologically active compounds producer that medicinal importance, at least 7,000 different byproducts of the *Streptomyces* genus were discovered.[13] Cho et al. [14] reported *Streptomyces* spp. can produce a difference of secondary metabolites that interact with the environment, including volatile organic compounds that have an inhibitory effect against pathogenic fungi. The molecular weight of these metabolites are small 100 - 3000 dalton and have significant activity against fungal cell wall formation.[15] The present study was conducted to search for isolation of novel Actinomycetes strains from salinity environments and production of bioactive substances.

2. MATERIALS AND METHODS

2.1 Sample collection

Twenty five sediment samples were collected from Al-Hartha, Abu Al-Khaseeb, and Coral Reef areas in Iraqi marine waters. Samples were placed in sterile plastic bags and transferred immediately to the laboratory for Actinomycetes isolation. The pathogenic bacteria were isolated from urinary tract infections in general Basrah hospital.

2.2 Actinomycetes isolation

The sediment samples after collection left at room temperature to dry under air under sterile condition. One gram of sediment sample was added to screw cap container having 10 ml sterile distilled water, shaken well a few minutes and serial dilution was made upto 10^{-8}. To isolate Actinobacteria, three media were used, Actinomyces Isolation Agar and International Streptomyces Project (ISP-4 and ISP-5). These media are supplied with antibiotics solution (75 mg/l of Nalidixic acid and 80mg/l Cycloheximide) to prevent the growth of other bacteria and fungi. 0.1 ml of each dilution and placing it on the surface of the isolation medium and spreading by sterile L-shape glass rod. The media left to dry and incubated at 28 ◦C for 7-14 days, the Actinomycetes colonies were selected and preserve at 4◦C until subsequent tests. [16]

2.3 Purification of Actinomycetes

Thirteen Actinomycetes isolates were selected based on their colony morphology on the ISP-5 culture medium by using streak plate method. Pure cultures of actinomycetes isolates were named as, W1, W2 to W13.[17]

2.4 Test bacteria and fungi

The pathological bacteria which isolated from urinary tract infections (*Staphylococcus aureus*, *E.coli*) obtained from the Applied Microbiology Research Laboratory / Department of Biology / College of Science / University of Basra, while fungal isolates (*Aspergillus niger* and *Candida albicans*) were obtained from the Fungi Research laboratory in the same department.

2.5 Screening for antimicrobial activity

Thirteen isolates of Actinomycetes were carried out according to the phenotypic characteristics. To detect the ability of Actinomycetes isolates for antimicrobial agents production, carried out by using plug method on the plate agar, actinomycetes isolates were cultivated on the ISP-5 medium for 7 days, then Mueller Hinton agar was prepared and sterilized at 121 °C for 20 minutes and poured into the sterile Petri plates, laved to solidify before use. Pathogenic bacteria was activated in 5 ml of nutrient broth overnight at 37 °C, while fungi activation in 5 ml of potato dextrose agar for 3 days at 25 °C. After incubation time, bacterial and fungi inoculums were diluted with a sterile physiological solution to obtain 10^8 CFU/mL (turbidity = McFarland standard 0.5). [17] Activated isolates inoculum uniformly were spread on the MH agar and leaves a few minutes, Cork borer plug of each actinomycetes isolates was made on the medium by use metal borer after sterilized with a burn, about 1cm in diameter plugs were carefully transfer to the MHA surface and push with forceps, 20 mm apart from one another then inoculated at 37 °C for 24 hr. for bacteria and 30 °C for 72 hr. for fungi. The media were daily monitoring for the formation of clearing zone around the plugs after incubation that indicated positive result, inhibition zone (IZ) was measured in mm by a standard ruler.[18]

2.6 Identification of Actinomycetes

Three Actinomycetes isolates were diagnosed depending on the screening for antimicrobial activity test, these isolates labelled as W2 isolated from Al-Hartha sediment, W5 isolated from the coral reef area of Iraqi marine waters, and

W11 isolated from Abu Al-Khaseeb. Which appears the highest activity for antimicrobial.

2.7 Phenotypic and Biochemical characteristics

The isolates were examined after cultivated on ISP-5 for seven days, the first examined using morphological and cultural characteristics was carried out under a dissecting microscope where the phenotypic characteristics of the colonies were diagnosed depending on the shape, size, colour, sporulation and morphologic of the colony. The shape of the cells was examined under the light microscope [19] Biochemical tests for Actinomycetes isolates include production of catalase enzyme, oxidase production and gram staining. [20-22]

2.8 Molecular Identification of isolates

Cells of each isolate harvest in Eppendorf tubes (1.5 ml) for DNA extraction by using The Presto ™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd, Taiwan) According to the steps followed by the manufacturer's manual that description in protocol procedure. The 16s rDNA genes about (1500 bp) were amplified using universal 27 forward primers and 1525 reverse primer in 25 µl mixtures (Master mix, Bioneer, Korea) by a polymerase chain reaction.[23] PCR amplification was achieved in a thermal cycler (Bioneer, Korea), the PCR product at 4 °C.[24] The amplification products were examined by 1.5% agarose gel electrophoresis with 100 bp DNA ladder of nucleic acid markers (Bioneer, Korea) PCR of bacterial 16S rDNA gene products was sequenced by (Macrogen, Korea) company and comparing to 16S rDNA sequences available in the nucleotide databases that founded in the GenBank by using (BLAST) program.[25] to identify bacteria at online service (<http://www.ncbi.nlm.nih.gov/>)in (NCBI).

3. STATISTICAL ANALYSIS

For statistically significant between data at $p < 0.05$ using one-way analysis of variance (ANOVA) tests which were performed using statistical package for version 23.0 (SPSS).

4. RESULTS

The results of the physical and chemical properties of the sediment samples from Al-Hartha, Abu Al-Khaseeb, and the coral reef area of Iraqi marine waters as shown in Table 1. The samples were showed similarities in the environmental characteristics, neutral pH (7.3, 7.5 and 7.4) respectively and moderate salinity (1.7, 1.6 and 1.8) respectively.

Table 1.Environmental characteristics of sample collection site

Samples location	Mean salinity ppm±SD	Mean P^H ±SD
Al-Hartha	1700±10*	7.3±0.07*
Abu Al-Khaseeb	1600±7.9*	7.5±0.1*
Coral reef area	1800±38*	7.4±0.07*

Values are mean ±SD; (n=12); * P>0.05.

4.1 Isolation of Actinomycetes

The isolation of Actinomycetes by using selective media supplied with antibiotic solution and incubating at 30 ° C showed high efficiency, three strains (W2, W5 and W11) were obtained.

4.2 Cultivation and morphological of Actinomycetes:

The results of phenotypic characteristics of isolates on the culture media showed all three isolates appear as dry colonies on ISP-5 medium after 7 days incubation, the colonies of (W2 and W11) were distinguished by a white, cotton colour, with disc growth formed around the colonies, formed aerial spores, white to whitish-grey in colour, produced dyes that changed the colour of the medium to dark. The (W5) strain was a grey colony converted to black when spores formed, also track pigment production as shown in figure 1.

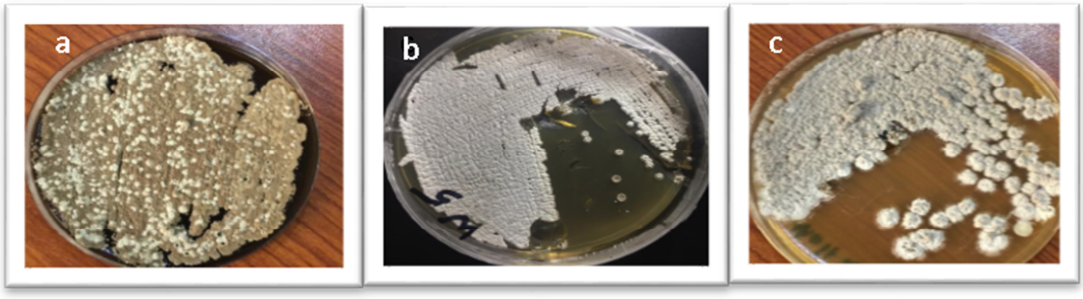

Fig 1. Morphological colonies of Actinomycetes isolate on plate agar after 7 days incubation on Isp-5 medium (a) W2 strain, (b) W5 strain and (c) W11 strain.

4.3. Antibacterial and antifungal screening assay

The ability of isolates to produce antimicrobial substances evaluated by the size of an inhibition zone around a plug containing the growth of isolation. The results showed the

ability of isolates to produce antibiotics against the positive and negative bacteria by all three isolates. Also, the results showed antifungal activity against fungi are negative as shown in Figure 2 and Table 2

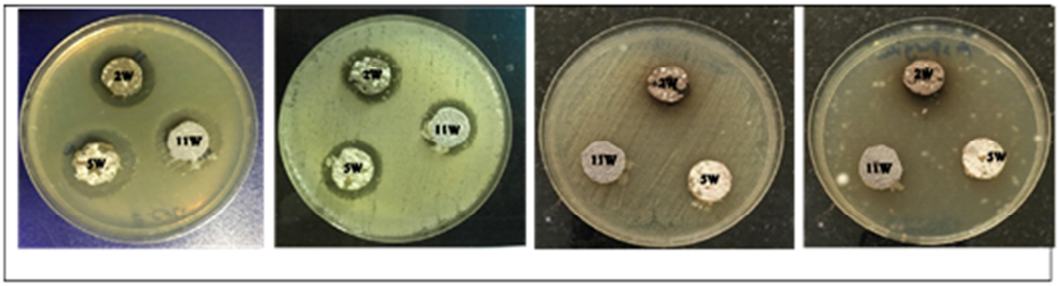

Fig 2. Antibiotic production by actinomycetes strains against the positive, negative bacteria and fungi.

Table 2. Inhibition zone value appeared around the plug growth

No. Isolates	*Mean inhibition zones (mm)* ±SD			
	E.coli	*S. aureus*	*C. albicans*	*A. niger*
W2	22±0.5	20±0.8	12±1.4	14±0.8
W5	28±0.2	22±1.6	0	12±1.0
W11	24±1.4	20±1.2	13±2.4	12±1.6

Values are mean ±SD; (n=3); * P>0.05.

4.4 Identification of Actinomycetes

The results included biochemical tests that included, the Oxidase, Catalase and Gram stain as shown in table (2).

Table 3. Results of the Oxidase, Catalase and Gram stain tests

Isolates	Gram stain	Oxidase test	Catalase test
W2	+	-	+
W5	+	-	+
W11	+	-	+

4.5 Molecular identification

The molecular identification by amplification of the 16SrRNA gene of the actinomycetes strains, showed the present bands of the gene on the agarose gel about 1500 pb as shown in figure 4 that determined depending on the DNA ladder. By using the BLAST program to find parity sequences of the 16SrRNA gene for the isolates that match with the information in the NCBI Genbank, showed the similarity of the isolates to the antibacterial actinomycetes group. The isolate W2 showed 95.9% similarity with *Streptomyces* sp., the isolate W5 shared 95.9% to *Micromonospora auratinigra* strain SB29 and W11 similarity 98% with *Streptomyces carpaticus* strain PES-A23 (Table 2).

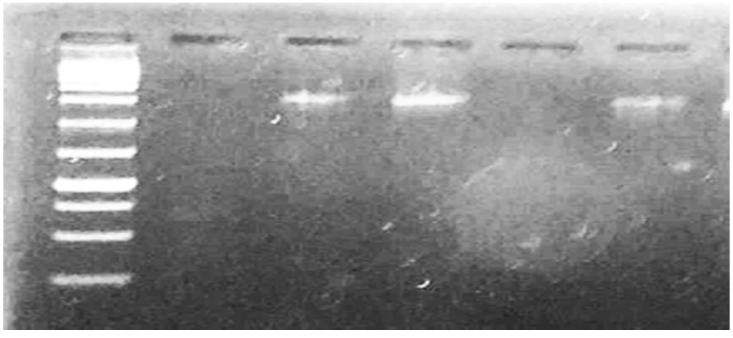

Fig 4. 16S rDNA amplicon of actinomycetes isolates

Table 4. Molecular identification of 3 actinomycetes isolates partial 16S rDNA gene sequence.

Isolates No.	Strain	Accession No.	Identity
W2	*Streptomyces* sp. TRM46619	JX244133.1	95.9%
W5	*Micromonospora auratinigra* SB29	MG407702.1	97.8%
W11	*Streptomyces carpaticus* PES-A23	MH712039.1	98%

5. DISCUSSION

Many competent professionals are making unremitting efforts in the field of discovering and developing effective drugs against microbes, but infectious diseases especially caused by bacteria are still a major cause of death worldwide annually due to weak immune system, such as for the elderly and children.[26-27] The results of isolating actinomycetes from the sediments of different geographical regions in Basrah Governorate showed that 3 strains were obtained from 25 sediment samples, and this indicates that the Iraqi marine and soil environment is a rich source for the isolation of actinomycetes, especially *Streptomyces*, this is consistent with the study carried by Burghal et al.[28] who were able to isolate six strains of the genus *Streptomyces* from Iraqi soil, so in this study, expansion to the orientation of marine environments was made to uncover new sources for isolating actinomycetes. Competition between microorganisms is one of the most important relationships that take place in the environment, so some microorganisms produce antibiotics to determine the species, such as *Streptomyces*. These substances may help in creating a symbiotic relationship between *Streptomyces* and plants so that one benefits from the other.[29] In this study, using universal primers in phylogenetic analysis for gene 16S rDNA amplification, confirmed that the isolated W11 as *Streptomyces carpaticus*, the genetic identification was performed by researchers to determine of different types of actinomycetes.[30] In the study from Subramanian et al.[31] who isolated of *Streptomyces carpaticus* from Seawater, it produced antimicrobial activity against pathogenic bacteria that infect fish, genetic identified by amplification and sequencing the gene 16S rDNA of the actinomycetes isolates. Therefore the molecular identification plays an important tool in Actinobacteria identification by introducing the common application of genetic criteria including 16S rDNA gene sequence nucleotide similarity.[32] The results of screening antimicrobial activity showed that this material is a broad-spectrum activity against gram positive and gram negative bacterial and fungi human pathogens, and this result is in consistent with Schneider et al.[33] In another study from Djinni et al. [7] which shows several strains of Actinobacteria can produce antibacterial and antifungal molecules, some of these shown interesting antitumor activities. In a review by Talukdar et al.[34] they observed that the development of novel antibiotics against pathogenic microbes requires the development of new biological materials similar to pharmaceutical drugs, this occurs through isolation strains of rare actinomycetes bacteria such as *Micromonospora* spp. which is an important source for the production of modern antibiotics with specific isolation media from various environments and uses screening techniques to reveal its ability to produce effective secondary metabolites.

6. CONCLUSION

The present study concluded that the isolation of three strains of actinomycetes was diagnosed based on the genetic characteristics and proved by screening its ability to produce secondary metabolites that have efficacy against (gram-positive and gram-negative) pathogenic bacteria and therefore fungi.

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8. AUTHORS CONTRIBUTION STATEMENT

Walaa made sample collection, cultivation, recovered growth isolation, bacterial identification based on molecular techniques. Besides, she conducted the primary screening, production of antibiotics, and photographing the results. Dr Ahmed supervised the laboratory work, analyzed the results of genetic diagnosis, statistical analysis, reviewing, and editing the results.

9. CONFLICT OF INTEREST

Conflict of interest declared none.

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