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Screening of Bioactive Secondary Metabolites of *Streptomyces* spp. Isolated from the Sediments

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Abstract: The purpose of this study was to assess the activity of *Streptomyces* spp., isolate and identify them from samples of silt soil and Tigris river water in Mosul, Iraq. Variations in the isolates' microscopic and biochemical properties were used to identify them., such as soluble pigment, aerobic mycelium, and substrate mycelium. Using the perpendicular streak method, eight of the twelve actinomycete isolates that were isolated from the water and sediments of the Tigris River exhibited antibacterial activity against specific pathogenic bacteria. *Staphylococcus aureus* (MTCC 3260), *Vibrio parahaemolyticus*, *Klasiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *E. coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424), and *Aeromonas hydrophila* bacteria were all susceptible to the broad-spectrum activity of isolate Sediment Lake Iraq E2. According to the findings of phylogenetic analysis, isolate E2 shared the closest kinship with *Streptomyces* sp. (EU257231). Using high-performance liquid chromatography (HPLC), the *Streptomyces* isolate E2's secondary metabolite was further examined. There is evidence that these secondary metabolites are harmless and may have antibacterial properties against human pathogens.

Keywords: Tigris river, Secondary metabolites, Antimicrobial, *Streptomyces*.

Introduction

Marine environment is largely untapped source for the isolation of new microorganisms with potentiality to produce active secondary metabolites. Among such microorganisms, actinomycetes are of special interest, since they are known to produce chemically diverse compounds with a wide range of biological activities (Bredholt et al., 2008).

Actinomycetes are filamentous bacteria that produce antibiotics. It has long been recognized that *Streptomyces* species exhibit two distinct growth phases, referred to as the primary and secondary mycelium, respectively. These phases are the substratum mycelium and the aerial mycelium, they are found in freshwater and marine water habitats (Fenical & Jensen, 2006; Weinstein et al., 2005). The dominant actinomycetes *Micromonospora* can be isolated from aquatic habitats such as streams, rivers, lake mud, river sediments, beach sands, sponge and marine sediments (Rifaat, 2003; Eccleston et al., 2008).

A multitude of novel bioactive compounds have been identified from aquatic actinomycetes, such as rifamycin originating from *Micromonospora* (Huang et al., 2008), salinosporamide-A, an anticancer metabolite derived from a *Salinispora* strain (Fehling et al., 2003), marinomycins sourced from *Marinophilus* sp. (Jensen et al., 2005); abyssomicin-c extracted from *Verrucospora* sp. and Marino pyrroles obtained from *Streptomyces* sp. (Riedlinger et al., 2004; Hughes et al., 2008).

Out of a total of 22,500 biologically active compounds derived from microbial sources, 45% are attributed to actinomycetes, 38% to fungi, and 17% to other bacterial taxa (Berdy, 2005; Hayakawa et al., 2007). The genus

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Streptomyces is responsible for over 70% of the overall antibiotic production, while Micromonospora represents less than one-tenth of the quantity produced by Streptomyces (Lam, 2006; Gurung, et al., 2009).

Valli et al. (2012) meticulously conducted a comprehensive investigation wherein they successfully isolated a total of 21 potential actinomycetes derived from a diverse range of marine environmental samples, and subsequently documented that each and every one of the isolates exhibited promising antimicrobial activity against at least one of the tested pathogenic organisms, as reported in their pivotal study (Valli, et al., 2012). In a similar vein, Kalyani et al. (2012) undertook an extensive isolation process that yielded 20 distinct species from marine soil samples, among which three species demonstrated a significant level of antimicrobial efficacy against the notorious pathogens *S. aureus* and *E. coli*, thus contributing valuable insights to the field of microbiological research (Kalyani, et al., 2012).

Multiple drug-resistant pathogenic strains caused significant morbidity and mortality, particularly among elderly and immunocompromised patients. Antimicrobial drugs used for prophylactic or therapeutic purposes in human, veterinary, and agricultural settings were favoring the survival and spread of resistant organisms (Barsby et al., 2001; Parungao et al., 2007). The goal is to improve or discover a new class of active compounds that function differently from antibiotics in order to solve this problem. It was essential to continually screen secondary microbial products derived from potential bacterial taxa in order to identify novel chemicals for the development of new medical treatments (Lazzarini et al., 2000). Many researchers are now looking for novel antibiotics in many unexplored ecosystems to determine whether they produce antibiotics (Oskay et al., 2004). Consequently, the purpose of this work was to evaluate the activities of Streptomyces spp. And extract and identify them from samples of Tigris River water and sediments in Mosul, Iraq.

Method

Sampling Area

The samples of water and sediments were collected from Tigris river in Mosul, Iraq.

Sample Collection

Totally 10 water and 10 sediment samples were collected. The water samples were collected. In 500 ml sterile screw capped bottles and sufficient space was provided for aeration and thorough mixing. The sediments were collected by sterilized spatula transferred to wide mouth sterilized bottle. All samples were labeled and transported to the Microbiology Laboratory/ College of science/University of Mosul.

Isolation and Identification Streptomyces spp.

Actinomycetes agar served as the medium for the marine actinomycetes' isolation and culture. After autoclaving, 50 and 20 $\mu\text{g mL}^{-1}$ of tetracycline and nystatin, respectively, were added to the medium as antibacterial and antifungal agents to prevent bacterial and fungal contamination. One gram of dried Tigris river silt soil samples was combined with 99 milliliters of sterile distilled water to make the stock suspension. Then, for 30 minutes at room temperature, the materials were agitated in a shaker that was set to 120 rpm. After being serially diluted from 10^{-1} to 10^{-3} , the stock suspension was let to sit for ten minutes. 0.1 ml of each dilution—water and sediments—was pipetted and put on actinomycetes agar after shaking.

The suspension was thereafter uniformly applied throughout the media's surface with a sterile brush. The inoculation plates were cultured at 28°C for 7 to 14 days, and chosen isolates were aseptically streaked on actinomycetes and nutritional agar, followed by incubation at 28°C for 7 days. Pure culture was inoculated onto slants and stored at 4°C for further analysis. Morphological traits, including colony features, pigment synthesis, and the presence or absence of aerial and substrate mycelium were examined (Oskay et al., 2004).

Morphological and Physiological Characterization

Following inoculation on specific medium (SCA, MA, NA, PDA, and ISP2), the obtained actinomycete isolates were grown for seven days at 30°C and observed daily. Actinomycete isolates were analyzed for

micromorphology, including gram staining, morphology, and dimensions under a light microscope, alongside culture characteristics such as colony morphology, comprising surface elevation, texture, density, coloration of aerial and substrate mycelium, and pigment synthesis.

Biochemical Characterization

The *Streptomyces* isolates were characterized by conventional biochemical tests. The tests encompass the following: cytochrome oxidase, catalase, tween-40, tween-60, tween-80, esculin, glucose gas production, casein hydrolysis, urea hydrolysis, nitrate reduction, hydrogen sulfide production, methyl red test, indole test, Voges-Proskauer test, citrate utilization test, and glucose gas production. The isolates were inoculated in actinomycetes broth enriched with specific sugars and incubated for seven days at 28°C to evaluate acid production from various carbohydrate sources, including adonitol, fructose, sorbitol, dextrose, lactose, inositol, maltose, sucrose, raffinose, and xylose.

Select Bacteria for Antibacterial Activity

The selected human microbiological pathogens for the assessment of antibacterial activity included *Staphylococcus aureus* (MTCC 3260), *Vibrio parahaemolyticus*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Proteus mirabilis*, *Escherichia coli* (MTCC 443), *Pseudomonas aeruginosa* (MTCC 424), and *Aeromonas hydrophila*. The bacteria were obtained from the Microbiology Laboratory at the College of Science and were activated by culture in Nutrient Broth at 37°C for 24 hours.

Primary Screening (Cross Plate Technique)

Streptomyces isolates were inoculated into nutrient agar plates by streaking the middle of each plate. The examined bacterial pathogens were inoculated perpendicularly to the *Streptomyces* (a single streak at a 90° angle to the *Streptomyces* isolates) once the *Streptomyces* had fully developed after 7 days of incubation at 28°C. The plates were subsequently re-incubated at 37°C following a 24-hour period. When the reference strains did not proliferate near the *Streptomyces* line, the antibacterial action was observable to the naked eye.

Secondary Evaluation (Antagonistic Activity)

The most active *Streptomyces* isolates were cultured in 250 mL flasks using 50 mL of actinomycetes broth. Following seven days of incubation at 28°C, the cultures underwent centrifugation for fifteen minutes at 10,000 rpm. The antibacterial activity of the clear supernatant broth was assessed against a range of human pathogenic pathogens using the agar well diffusion method. Fifty microliters of crude culture supernatant was dispensed into each well, and following a 24-hour incubation of the bacterial species at 37 °C, the diameters of the inhibitory zones were measured. The most potent active streptomyces isolate was chosen for the next testing.

Extraction of Streptomyces Isolates Secondary Metabolites

The cultivation conditions, incubation duration, and fermentation medium were refined for the identification of secondary metabolite chemicals. These include cultivating *Streptomyces* isolates in 1 liter of ISP2 medium (comprising 3 g of yeast extract, 3 g of malt extract, 10 g of glucose, and supplemented with 0.5 ml of glycerol) in 2-liter flasks, incubated for 9 days at 29°C with shaking at 200 rpm. An adapted approach from Jensen et al. (2007). The bioactive metabolite compounds were isolated by an extraction procedure. The bacterial culture of each isolate was centrifuged for 15 minutes at 10,000 rpm, after which the cell-free supernatant was collected, mixed with an equivalent volume of ethyl acetate, and incubated on a rotary shaker at 200 rpm overnight. The solvent layer was collected and subsequently evaporated using a rotary evaporator to get the crude extracts. Pure dimethyl sulfoxide (DMSO) was subsequently included into the crude extract to formulate a 50 mg/mL (w/v) stock, which was then preserved at 4 °C for use in the agar diffusion experiment.

Agar Diffusion Assay

Cultured overnight twenty milliliters of nourishing broth medium were inoculated with 500 microliters of the selected human pathogenic bacteria, followed by incubation for two to three hours at 30°C. An optical density of 0.7 at 600 nm was achieved by shaking at 170 rpm. The agar overlay test employed semi-solid nutritional agar (0.8% agar), maintained at 45°C. Ten milliliters of culture (OD 600nm = 0.7) were included before plating the supernatants onto the medium. Wells in the plate were created with a sterile cork borer with a diameter of 6 mm. Each well was then filled with 30 µL of streptomyces supernatants. Antibacterial activity was detected in a zone of clearing at the center. The fresh nutrient broth served as the negative control. Three duplicates of the bioassay were performed, and the mean value was applied. The following tests were conducted using the isolate that was the most active.

DNA Extraction

The bead beater-phenol extraction method was used to prepare the DNAs (Ko et al., 2002). A 2.0 ml screw-cap microcentrifuge tube was filled with 100 ml (packed volume) of glass beads (diameter, 0.1 mm; Biospec Products, Bartlesville, Okla., U.S.A.) and 100 ml of phenol-chloroform-isopropyl alcohol (50:49:1), and a loopful of each isolate's culture was suspended in 200 ml of TEN buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl; pH 8.0). After one minute of oscillating the tube on a Mini-Bead Beater (Biospec Products) to disturb the bacteria, the phases were separated by centrifugation (12,000 ×g, 5 min). Following the transfer of the aqueous phase to a different tube, 250 ml of ice-cold ethanol and 10 ml of 3 M sodium acetate were added. The mixture was then maintained at 20 °C for 10 minutes in order to precipitate the DNA. Thereafter, the DNA pellet was washed with 70% ethanol, dissolved in 60 ml of TE buffer (10 mM Tris-HCl, 1 mM EDTA; pH 8.0), and used as the template for PCR.

Polymerase Chain Reaction (PCR) Amplification of the 16S rRNA Gene

The 16S rRNA gene was sequenced to identify the selected streptomyces isolate. PCR products obtained from the total genomic DNA were used to amplify the 16S rRNA using the forward primer and reverse primer 8F: (5' AGA GTT TGA TCC TGG CTC AG 3') 1492R: (5' ACG GCT ACC TTG TTA CGA CTT 3') (Deepa et al., 2013).

PCR was performed in a 50 L reaction mixture that contained 10 µL of 5X GoTaq Flexi buffer, 1 µL of 0.2 mM PCR-grade of deoxynucleoside triphosphate (dNTP), 8 µL of 4mM MgCL₂, 1 µL of 1 µM reverse primer, 1 µL of 1 µM forward primer, 1 µL of 0.5 µg DNA template, 0.25 µL of 1.25 u Go Taq DNA polymerase, and 27.75 L of sterile Milli-Q water. A Bio-Rad MyCycler thermal cycler (Bio-Rad, USA) was used to carry out PCR at an initial denaturation step with 95°C temperature for 2 min, 35 cycles of 95°C for 1 min, at 50°C for 1 min, at 72°C for 1 min, and a final extension step at 72°C for 5 min. The amplification DNA products were separated by electrophoresis technique on 1.5 % (w/v) agarose gel in 1X TAE buffer (40 mM Tris acetate, 1 mM EDTA, pH 8.0) at 80 V for 45 min, following PCR amplification. The gel was pre-stained with FloroSafe DNA stain while 1 kb DNA ladder was used as DNA size marker. Finally, all gels were viewed and captured by UV trans illuminator Gel Documentation System (Syngene, UK). A comparison between the sequences available online and the obtained DNA sequences was done using the gen bank database (<http://www.ncbi.nlm.nih.gov>). utilizing the online bioinformatics resources, such as BLAST (www.ncbi.nlm.nih.gov/BLAST). A phylogenetic tree comprising a chosen isolate and additional related genera that are available in the NCBI database was created using a homology search using Clustal X 1.8. The evolutionary history was inferred using MEGA6's neighbor-joining approach. 1000 bootstrap replicates of the original sequence data were used to assess each branch's confidence value.

High Performance Liquid Chromatography (HPLC) Chromatography

Separation of the bioactive components from the secondary metabolites of the active isolate was performed using HPLC on a C₁₈, 3µm column with acetonitrile: methanol: propanol (40:50:10). The flow rate was 0.8 ml/min.

Results and Discussion

The physical characteristics of the identified *Streptomyces* isolates are displayed in figure (1). The suspected colonies were cultivated on agar and chosen based on their morphology (they had a smooth surface initially, but as the aerial mycelium formed, they became soft, granular, and powdery) and color (either gray, creamy, or white), with colony diameter sizes ranging from 1 to 10 mm. The identification, characterization, and classification of Actinomycetes rely on many features that should stay constant when the microbe is isolated under similar growth conditions (Reddy et al., 2011). Actinomycetes sourced from marine environments are well-documented to thrive on starch casein agar (SCA) (Laidi et al., 2006; Hemashenpagam, 2011; Reddy et al., 2011). Consequently, SCA augmented with 50% old natural seawater, aged for 30 days, was employed to extract streptomycete strains, which were noted to flourish abundantly on this medium, the colony morphologies and the surface and aerial mycelium, as delineated in this study, are characteristic of *Streptomyces* spp. Colony morphology has often been employed to identify Actinomycetes (Oskay et al., 2004; Hemashenpagam, 2011; Reddy et al., 2011)

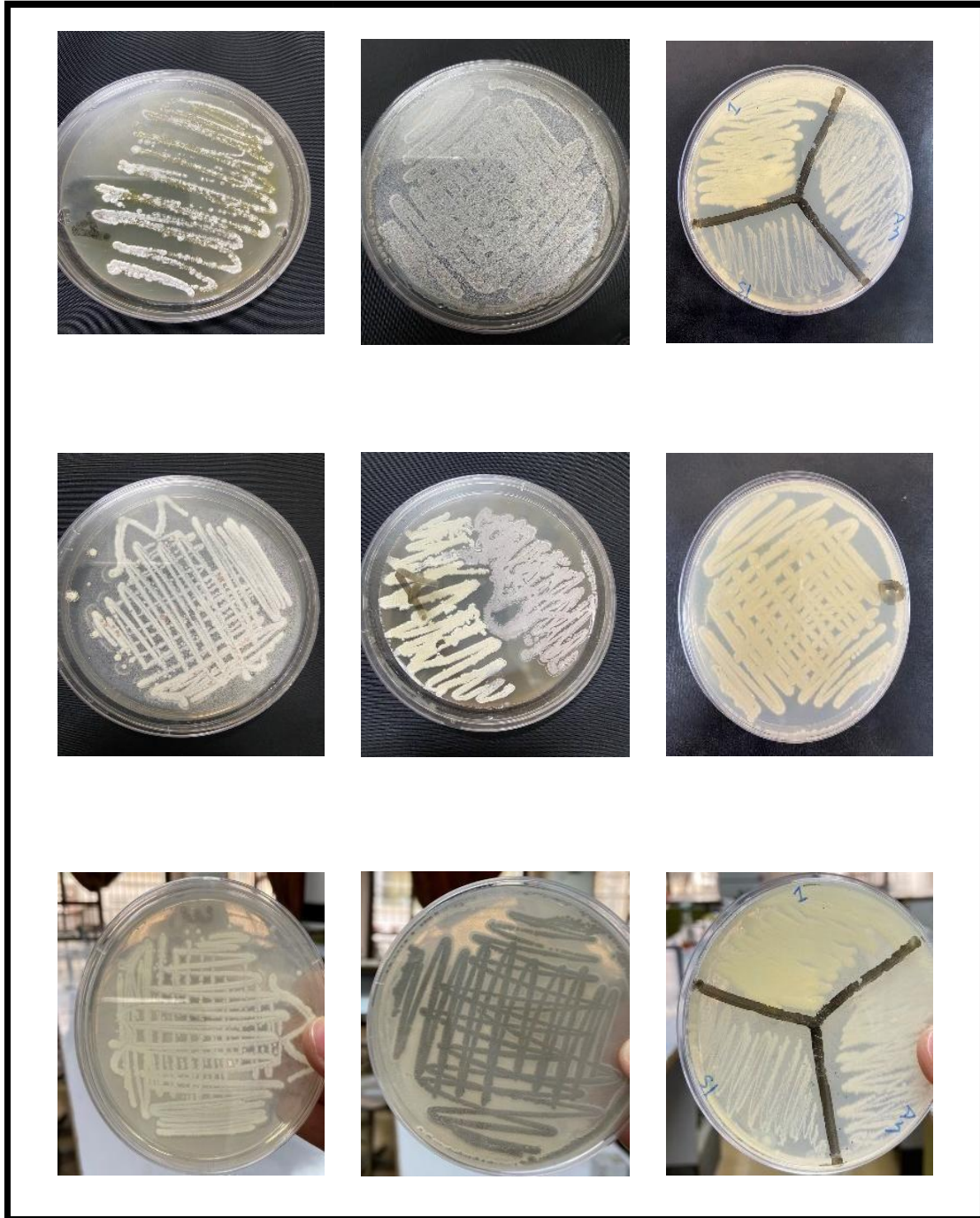


Figure 1. Aerial mycelium of streptomyces isolates grown on actinomycetes agar.

The isolates were examined under microscope after 7-14 days of incubation to see the Gram positive bacteria hyphae as shown in Figure 2.

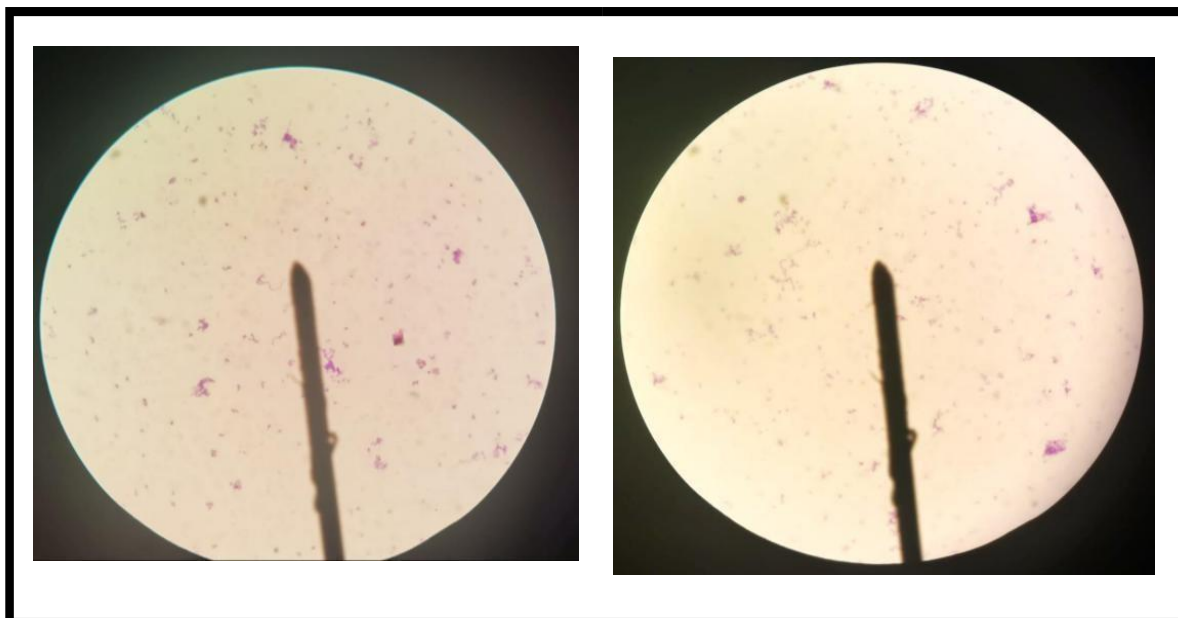


Figure 2. Microscopic examination: Gram positive bacteria, forming hyphae

Twelve isolate designated E1-E12, showed varied morphological characteristics (Table 1). The growth and colony morphology of streptomyces isolates were observed to grow very well on all the 6 selected media (SCA, MA, NA, PDA, ISP2 and Actinomycetes agar). Diffusible pigment on the other hand was absent in all the media. Chemotaxonomy is the examination of chemical variations among species and the application of chemical characteristics for classification and identification. This is one of the effective methods for identifying the genera of actinomycetes (Remya & Vijayakumar, 2008).

The actinomycetes isolated in this investigation were identified as Streptomyces based on their morphological, physiological, pigment production and biochemical characteristics (Suneetha et al., 2011; Bundale et al., 2015; Singh et al., 2016). Their compact, chalk-like, dry colonies, which ranged in hue from pink to white, demonstrated strong sporulation. According to Suneetha et al. (2011), the isolates were determined to be gram-positive organisms with branching mycelium in their cell shape. Gelatin, casein, and starch were all hydrolyzed effectively by the isolates (Manteca & Sanchez, 2009). Catalase was positive in every isolation, although indole synthesis was completely negative.

Table 1. Morphological characterization of streptomyces isolates

Media types	Growth	Aerial mycelium	Substrate mycelium	Diffusible pigment
SCA	+++	White	Beige	Absent
MA	+++	White	Beige	Absent
NA	+++	White	Beige	Absent
PDA	+++	Grey	Brown	Absent
ISP 2	+++	White	Pale yellow	Absent
Actinomycetes agar	+++	White Grey	Beige	Absent

Physiological and Biochemical Characteristics of Streptomyces Isolates

Streptomycete isolates' physiological and biochemical traits were displayed in Table 2. Oxidase, starch agar, urea hydrolysis, skim milk agar, mannitol, xylose, D-galactose, D-Fructose, L- Arabinose, and citrate utilization were all detected in the long filamentous, Gram-positive isolates. Additionally, the isolates tested negative for non-motile, Rhamnose VP gelatin hydrolysis, and xanthine agar

Table 2. Physiological and biochemical characteristics of the *Streptomyces* isolates

Characteristics	<i>Streptomyces</i> spp
Gram stain	Positive
Shape and growth	Long filamentous
Motility	Non-motile
Starch hydrolysis	+
Oxidase	+
Casein hydrolysis	+
Urea hydrolysis	+
Gelatin Hydrolysis	+
Skim milk agar	+
Xanthine Agar	-
Mannitol	+
Indole	-
Catalase	+
Xylose	+
Rhamnose	-
D-Galactose	+
D-Fructose	+
L-Arabinose	+
Citrate utilisation	+
VP	-
TSI	Alk/Alk

Primary Screening of Isolates

During the primary screening, isolates were screened against selected pathogenic bacteria strains by using perpendicular streak. The zone of inhibition near the colonies were observed, (Figure 3)

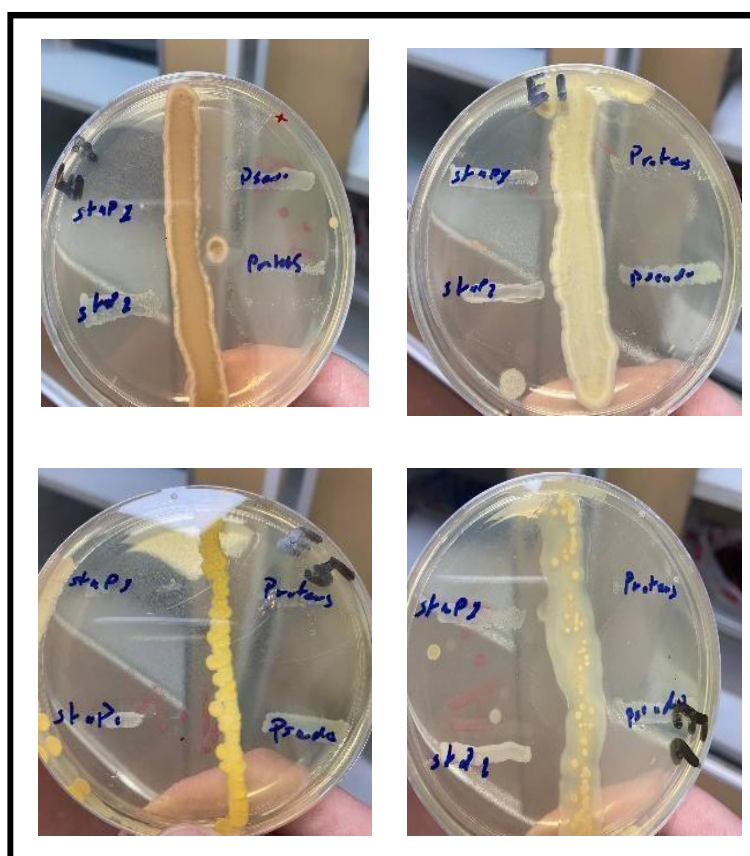


Figure 3. Antimicrobial activity of *Streptomyces* isolates against selected pathogenic bacteria using perpendicular streak.

Among the 12 streptomycetes isolate from water sediments of Tigris river, 8 isolates showed antibacterial activities against at least bacteria, therefor were chosen for the following experiments.

Antibacterial Activities of *Streptomyces* Secondary Metabolites

Figure 4 displays the E2 isolate's antibacterial properties against a subset of human pathogenic microorganisms. When tested against the chosen bacterium, the isolate showed varied levels of antibiotic activity. (*A. hydrophilia*, *S. aureus*, *K. pneumoniae*, *E. coli*, *P. aeruginosa*, *S. typhi*, and *V. parahaemolyticus*). The results showed the maximum activity against *V. parahaemolyticus*, *S. aureus*, *A. hydrophilia* and *S. typhi* while the least activity was found against *E. coli*.

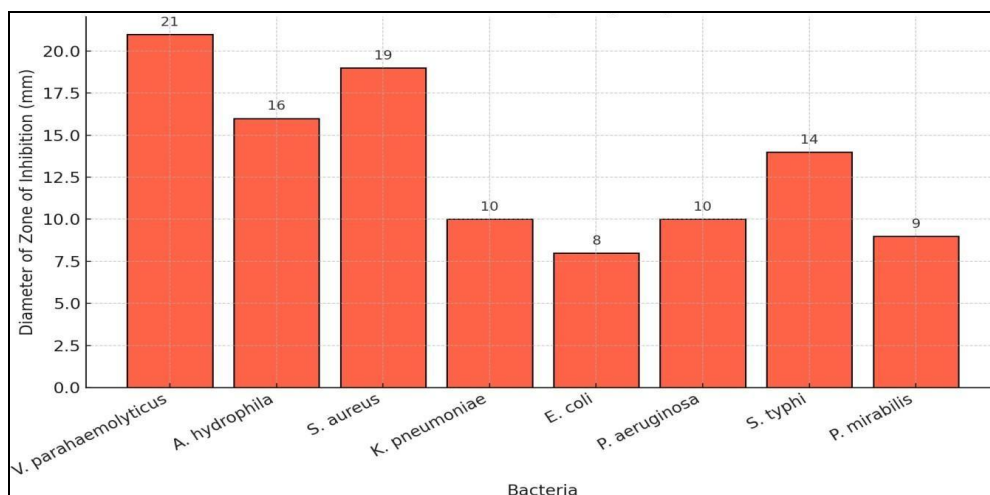


Figure 4. Antibacterial activities of *Streptomyces* strain E2 against selected pathogenic bacteria

Diverse microorganisms possess distinct needs for culture conditions and optimal parameters for antibiotic manufacture; these elements affect bacterial development, subsequently influencing antibiotic synthesis (Yoshida et al., 1972; Akhurst, 1982; Geetha & Vinoth, 2012). Actinomycetes are recognized for their ability to produce antibiotics (Geetha & Vinoth, 2012). Among the genera of actinomycetes, *Streptomyces* is a notably prolific source of natural products, generating two-thirds of commercially available antibiotics (Bentley et al., 2002). The selected E2 isolate in this study exhibited significant antagonistic activity against seven human pathogenic pathogens, with the most pronounced effect shown against *S. aureus* and *V. parahaemolyticus*. These findings align with those seen in prior pertinent studies (Remya & Vijayakumar, 2008; Geetha and Vinoth, 2012; Singh et al., 2014; Singh et al., 2016)

PCR and Gel Electrophoresis

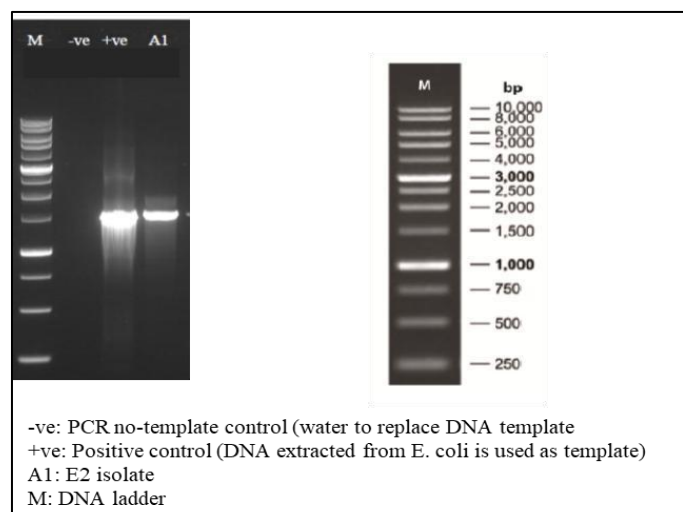


Figure 5. Gel electrophoresis of PCR products from *Streptomyces* strain E2

of roughly 1500 bp of the rRNA gene. The findings revealed that the selected E2 isolate is tightly associated with *Streptomyces* and *Nocardia* species, respectively.

High Performance Liquid Chromatography

The chemical composition of bioactive extract of *Streptomyces* strain E2 was investigated using HPLC analysis (Maleki & Mashinchian, 2011; Kumar et al., 2014). And the result is shown in Figure 7. Twenty two peaks were observed following the analysis. The study's findings indicate that the E2 isolate has active compounds that absorb most efficiently throughout the range of 380 to 450 nm. According to comparable studies (Ezra et al., 2004; Kumar et al., 2009), the majority of peptide antibiotics exhibit peak absorbance in the ranges of 210 to 230 nm and 270 to 280 nm. The antibiotic activities demonstrated by the isolate may result from these peptide antibiotics.

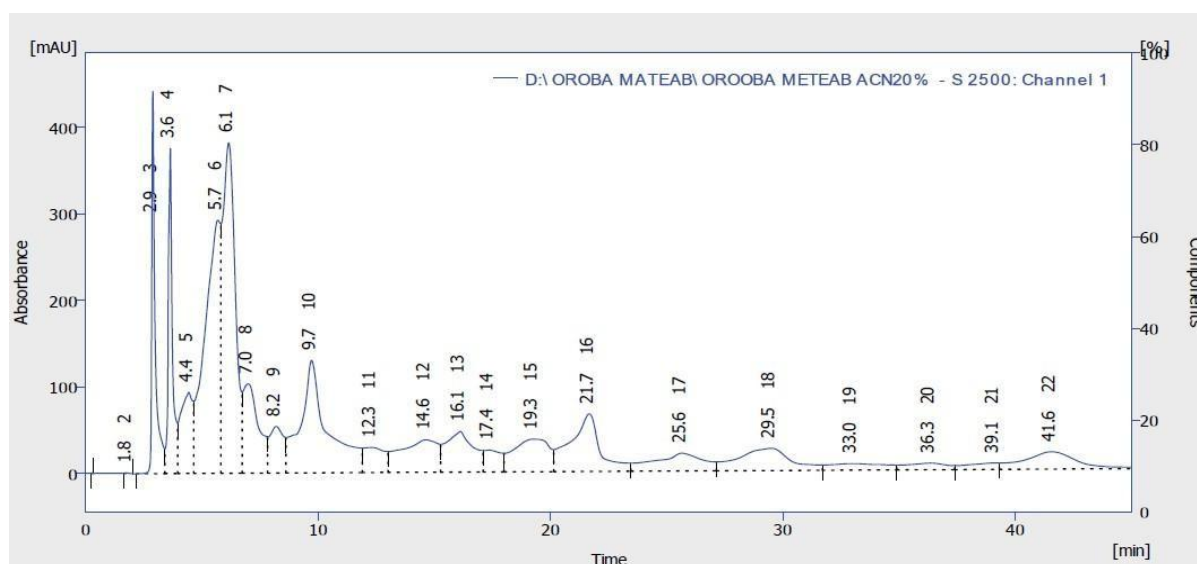


Figure 7. Analysis HPLC chromatogram of *Streptomyces* strain E2 secondary metabolites

Conclusions

Based on the findings of this study, the sediments of the Tigris river in Mosul/Iraq is a fertile land of isolation *Streptomyces* which have varying cultural, morphological, biochemical and carbon source requirements. Some of the actinomycetes could be having antibiotics of medical important thus there is need to further screen the isolates for antibiotics production. The streptomycete isolates obtained could be subjected to further analysis for the production of potent antibiotics that could mitigate the issues of antibiotic resistance. The confirmation of novelty of these compounds need further investigations in order to determine the functional groups, clarify the structure and physicochemical nature of the antibiotics

Scientific Ethics Declaration

* The author declares that the scientific ethical and legal responsibility of this article published in EPHELS journal belongs to the author.

Conflicts of Interest

* The author declares no conflict of interest.

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