

Screening for Antimicrobial Agents Producing Actinomycetes Isolated from Agricultural Soils in Hilla/Iraq

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Abstract: Twelve agricultural soil samples were collected from different sites of Hilla city in Iraq . Out of these samples, 30 Actinomycetes isolates were isolated and purified in order to be tested for their antibiotics production ability . Primary screening exhibited there was one active isolate that posses antibacterial activity against four pathogenic bacterial species : *Pseudomonas aeruginosa* , *Escherichia coli* , *Morganella* sp. and *Staphylococcus aureus* , in addition to antifungal activity towards two fungal species : *Candida albicans* and *Candida glabrata* . This active isolate was encoded as *Streptomyces KGL-13* ,then it was identified by culturing on different International Streptomyces Project media (ISP-1 to ISP-7) and characterizing by several biochemical tests.partial purification of antimicrobial substances was done using ethyl acetate (at ratio 1:1) and evaporated at 45°C to get pasty mass. The zone of inhibition (ZOI) diameter in mm of extract was determined on Muller Hinton agar .The results showed the highest ZOI happened towards *E.coli* and *P. aeruginosa* when gave 22 mm and 21 mm ,respectively. While it show a good activity against *C.albicans* ,but it had less activity towards *C.glabrata*.

Key Words: *Streptomyces* , antimicrobial agents , primary screening

I. Introduction

Actinomycetes are gram positive , spore forming bacteria present in soil. They are characterized by their tough powdery , pigmented colonies. They are free living, saprophytic bacteria it considered as an intermediate group between bacteria and fungi. They are consisting of a major group of antibiotic producer and it play a role in recycling of organic matter[1]. A bout 80% of the total antibiotic production has been produced by streptomyces [2].

The members of streptomyces are chemoorganotrophic, non fastidious microorganisms, so more simple synthetic media containing organic carbon source such as glucose, starch, sucrose, or lactate an inorganic nitrogen source (NH_4^+ , NO_3^-) with some minerals can be used for their isolation. But to obtain a good growth can be cultivate on complex media containing oat meal, Yeast extract and malt extract [3]. The member of streptomyces species consider as the most important medicines. They are the sources of antibiotics against bacterial and fungal infections, anticancer, transplant rejection and others [4].

Secondary metabolite production can be describe to symbiotic microorganisms only when synthesis has been distributed to the culture media, but the ability of streptomyces cultures to form antibiotics is not a fixed feature , so it can be greatly increased or completely lost under different conditions of nutrition and cultivation [5]. Therefore, the culture media composition together with the metabolic capacity of the producing organism greatly affects antibiotic biosynthesis [6].

The present study was done to screen biological activity of crude extract of *Streptomyces* species isolated from local soils in Hilla, Iraq.

II. Materials & Methods

- Soil samples:

Twelve soil samples were collected from different sites in Hilla city, Iraq .All samples were taken from depth of the earth surface at 5-15cm. It was transferred to a laboratory by sterile small plastic containers. These soil samples were dried in electric oven at 60-65°C for 3hr., then stored at room temperature till examined.

- Isolation of Actinomycetes from soil samples:

Actinomycetes strains were isolated and obtained as pure cultures by using yeast –malt extract agar medium supplemented with 30 $\mu\text{g/ml}$ chloramphenicol. In dilution plate technique, 1 g of soil sample was suspended in 9ml of sterile water and serial dilution was made up to 10^{-5} . An aliquot (0.5ml) of suspension spread from the last dilution over the surface of starch casein agar medium and incubated at 30°C for 7-10 days [7]. After period of incubation, the plates were examined for colonies of Actinomycetes, the typical colonies were purified by sub- culturing on Yeast extract – glucose agar plates and stored for further study[8].

The pure cultures of Actinomycetes isolates were screened for their antimicrobial activity against three types of pathogenic test bacterial species *pseudomonas aeruginosa*, *Staphylococcus aureus*, *Morganella*

sp. and Escherichia coli in addition to two human pathogenic fungi:- Candida albicans and Candida glabrata. Perpendicular streaking technique was used for this purpose. The plates were incubated for 24 hr at 37°C for bacteria, and 72hr at 25 °C for fungi. After incubation period the clear zone growth of test bacteria and fungi beside the growth of isolate was considered as positive results for inhibitory activity [9].

Identification of Actinomycetes isolates:-

Cultural features such as color of aerial and substrate mycelium, pigmentation of selected active strains were examined on International Streptomyces Project (ISP) media [10]. The morphology of cellular shape of Streptomyces strains were tested by slide culture method[2] .The utilization of the mode of carbon sources was carried out due to methods of Gottlieb (1961)[11]. The ability of isolates to produce different enzymes was examined by using standard methods [12]. In addition to their ability to produce H₂S, indole and melanin pigments.

Fermentation broth for Antibiotics production:

Loop-full culture of the Actinomycetes isolate was inoculated into conical flasks sterile starch casein broth medium under aseptic conditions. The culture flasks incubated at 28 °C for 7 days aerobically. After incubation period, the fermentation culture broth was filtered through sterile whatman filter paper No.1 aseptically. Then the filtrate broth was mixed with equal volume of ethyl acetate solvent . The organic layer was evaporated at 45°C till get pasty mass [13].

Antimicrobial assay:

The antibacterial and antifungal activity of the eight isolates was estimated in vitro by standard disc diffusion technique [14]. The discs (50 μg/disc) of extract were applied on the sterilized filter paper discs (5 mm in diameter) in an a septic condition and allowed to evaporate the solvent in a septic hood for a few minutes . Erythromycin (30 μg/disc) and nystatin (30 μg/disc) were used as standard antibiotics for bacteria and fungi, respectively. The Petri dishes were incubated at 28°C for 24 hr. for bacterial growth and 72 hr. for fungi. The antibacterial and antifungal activities of the crude compounds were evaluated by measuring the respective zone of inhibition (ZOI) diameter in mm.

Melanin production:

The color of the aerial and substrate mycelium of the isolates were examined on yeast extract-glucose agar plate after 7 days of incubation at 30°C . The pigment production was determined according to the national bureau of standards color chart [15].

III. Results & Discussion

A total of 30 isolates of Actinomycetes were isolated from twelve local agricultural soil samples in Hilla city / Iraq. The colony forming units (CFU/g) were calculated by spreading method. The results showed the maximum number of colonies was (8.6 x 10⁷ CF/g) of soil which obtained from the cultivated sites (Table-1). This high number may be return to richness of these soils with natural composts that loaded by nutrients and mineral salts needed for growing of these microorganisms.

Table (1): Numbers of Actinomycetes colonies per gram growing on ISP-2 of soil sites

Collection site	No. of colonies*	No. of Actinomycetes (CFU/g of soil)
Alkarama (garden)	80	8.6x10 ⁷
Babylon university garden	65	7.5 x10 ⁷
AL- jamiae park	47	60 x10 ⁸
AL- Shawi City	20	4.5 x10 ⁷
Hilla City center	0	0
AL- Muhandisin	0	0
AL- Muharbeen	51	1.5 x10 ⁷
Forty street garden	22	2.8 x10 ⁷
Sixty street garden	38	3.9 x10 ⁷
AL- Iskan City	42	4.8 x10 ⁷

* Each value represents three replicates

Microscopic examination of Streptomyces KGL-13 showed typical filamentous structure with observed spore chains (Fig.1). Furthermore, colonies morphological characteristics showed green color of aerial mycelium ,while substrate mycelium was yellow to brown in color when grown on ISP-4 (Fig.2 A and B).

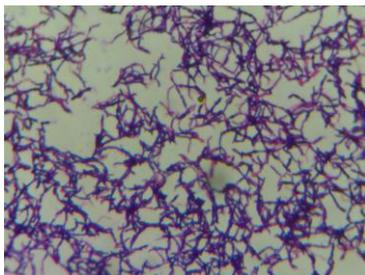


Fig.1. Microscopic features (1000X) of *Streptomyces* KGL-13

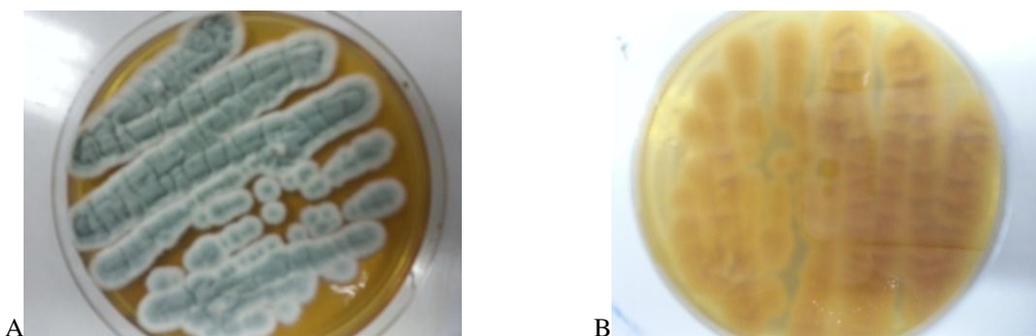


Fig.2. Morphological characteristics of *Streptomyces* KGL-13 grown on inorganic salts -starch agar (ISP-4): A: aerial mycelium , B:substrate mycelium

The biochemical results of this isolate revealed it can hydrolyze starch and liquefy gelatin ,but it gave negative result for catalase, indole, oxidase, and noH_2S production (Table . 2).Furthermore, it was able to utilize all the used carbohydrate listed in table 3.

Table (2) : Biochemical tests of the strain *Streptomyces* KGL-13

Biochemical test	Result
Gram stain	+
Starch hydrolysis	+
Gelatin liquefaction	+
Methyl red	-
Citrate utilization	-
Catalase	-
Licithinase	-
Indol	-
Voges proskaur	-
Oxidase	-
Blood hemolysis	+ (α -hemolysis)
Nitrate reduction	-

Table-3: Utilization of carbon sources by *Streptomyces* KGL-13 isolate

Carbon source	Result	Production* of yellow pigment
D- glucose	+	++
D- fructose	+	+++
Sucrose	+	++
Mannitol	+	++
Inositol	+	+
D-arabinose	+	+++
D- xylose	+	+++
Sorbitol	+	+

The sign (“+++”) mean strong production (“++”) mean good production, (“+”) weak production and (“-”) indicate no change.

The preliminary antibacterial assay of the isolates by perpendicular streak method exhibited that KGL13 isolate caused inhibition of test bacterial growth especially *E.coli* and *P.aeruginosa* ,but it was less active against *Morganella* sp. and *S.ureus* . On the other hand it caused disappearing of *C.albicans* growth ,while it caused little inhibition of *C.glabrata* growth (Fig.3. A and B).

This result similar to that obtained by Prashith et al., (2012)[16,17] who isolated *Streptomyces* isolate from Agumbe soils in India that able to depletion the growth of several bacterial and fungal species .

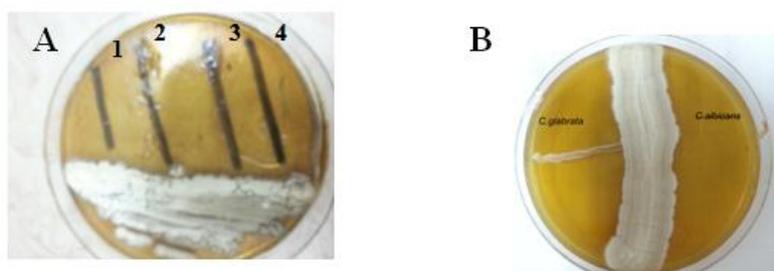


Fig.3.Primary screening of *Streptomyces* KGL-13 : A-against bacterial test organisms (1- *E. coli*, 2- *P. aeruginosa*, *S. aureus*, *M. sp.*). B : against two fungal species ; *C. albicans* and *C. glabrata*

The active isolate was cultured on different types of ISP media in order to be identified (Table 4) . The results exhibited that this isolate belonged to *Streptomyces* sp. so it encoded as *Streptomyces* KGL-13.

Table (4): Cultural properties of *Streptomyces* KGL-13 strain (incubation period: 7 days, Temp. 28°C)

Medium	Growth	Aerial mycelium	Substrate mycelium
Yeast-extract-malt extract agar (ISP2)	+++	White	Yellow
Oatmeal agar(ISP-3)	++	White	Light Gray
Inorganic salts starch agar (ISP-4)	++	green	Yellowish brown
Glycerol asparagine agar (ISP-5)	++	Yellow	Yellow
Peptone-yeast extract iron agar (ISP-6)	-	-	-
Tyrosine agar (ISP-7)	+	Brownish	Brown

The FT-IR spectrum of antimicrobial substances produced from *Streptomyces* KGL-13 isolate was showed absorbance at 3529-3468 cm⁻¹ which refer to amine group, furthermore, there is sharp bond at 1687 cm⁻¹ which attributed to (C= N) bond (fig .4). On the other hand, maximum absorption of uv spectrum was exhibited at 270-280 nm (Fig. 5) .This result refer to peptide antibiotic which exhibited maximum absorbance at wave length 210-230nm or 270-280nm[18] .

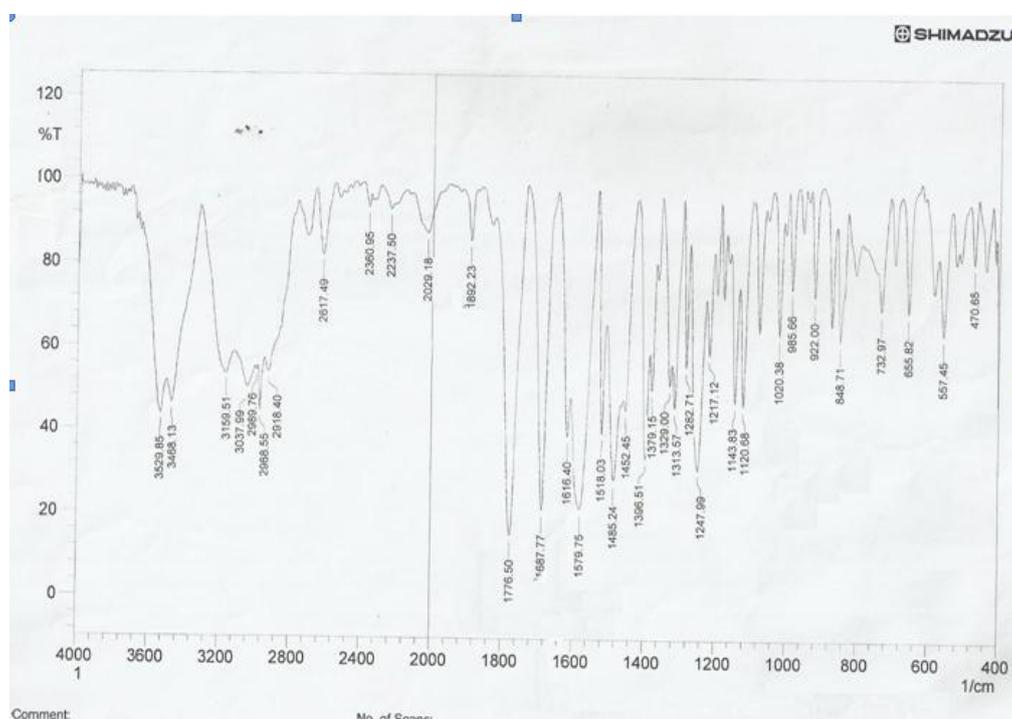
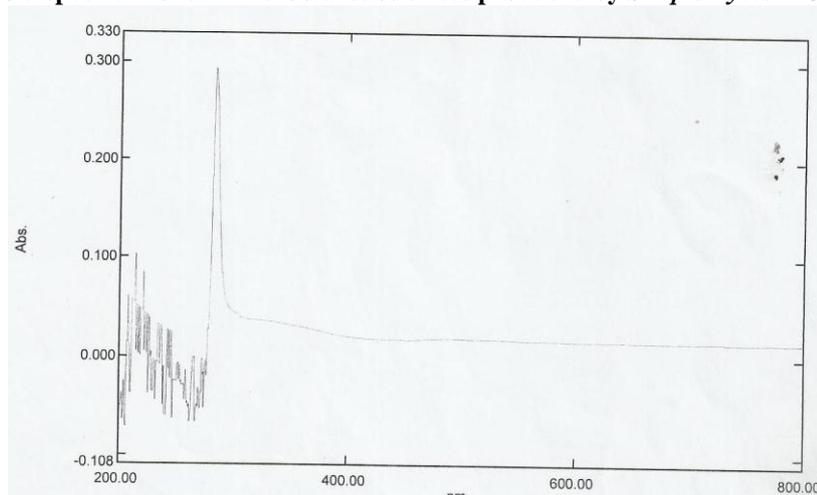


Fig.4.FT-IR spectrum antimicrobial substances produced by *Streptomyces* KGL-13 isolate

Fig.5.UV spectrum of antimicrobial substances produced by *Streptomyces* KGL-13 isolate



The antimicrobial activity of extract in terms of zone of inhibition (ZOI) were tested by disc diffusion method . The results exhibited that all the tested bacterial species (gram positive and gram negative species) showed a good sensitivity towards (50 µg/disc) but the highest activity was against E.coli and p. aeruginosa that gave 22 mm and 21mm, respectively compared to 25 mm of erythromycin (30 g/disc) as shown in(Table -5).

Table.5.Antimicrobial activity of *Streptomyces* KGL-13extract

Test organism	extract ±SE*	Inhibition zone diameter(mm)
Bacterial species		Erythromycin (30µg/disc)
E. coli	22 ± 0.1	25
p. aeruginosa	21 ± 0.2	25
Morganella sp.	15 ± 0.2	21
S.aureus	17 ± 0.3	20
Fungal species		Nystatin (30 µg/disc)
C.albicans	19 ± 0.2	18
C.glabarta	6 ± 0.1	17

*SE=Standard Error

These results were resemble to many previous studies that included isolation of *Streptomyces* species isolated from locally soils that potent significant antimicrobial activity towards several pathogenic microorganisms[19; 20 ;21].

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