Effect of Hygromycin B Antibiotic Produced by *Streptomyces* Isolates on some Pathogenic Bacteria

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Abstract

To select the most efficient isolate in production of the antimicrobial agent Hygomycin B, 53 *Streptomyces* soil isolates were examined. DNA of these isolates was extracted and screened by Polymerase Chain Reaction (PCR) technique for the presence of Hygomycin B gene which is responsible for production of such agent. Among the 53 isolates, only three gave positive results for the existence of this gene when the three isolates were evaluated for their antimicrobial activity, an isolate symbolled *Streptomyces* "10cm/35pigment" was the superior by giving the highest activity against four of the seven test microorganisms (*Proteus mirabilis, Staph. aureus, Sal. typhi and Sacch. cerevisiae*) used in the study. Their recorded inhibition zone diameters were 70, 55, 33 and 38 mm, respectively. After subjection of the superior isolate to the cultural, microscopic and biochemical characterization, it was identified as *Streptomyces hygroscopicus*.

Keywords: *Streptomyces*, Hygomycin B gene, *Streptomyces hygroscopicus*.

Introduction

Streptomyces genus includes Gram positive, high GC-content, sporulation bacteria found predominantly found in soil. *Streptomyces* is characterized by its complex secondary metabolism for producing antibiotic compounds and other metabolites with medicinal properties. Genomic studies, genomic mining and biotechnological approaches have been employed in the search for new antibiotics and other drugs [1].

Hygromycin B is an unusual antibiotic in that it is active against both prokaryotic and eukaryotic cells and specifically inhibits protein synthesis at the translocation step. It is produced by *Streptomyces hygroscopicus* which possesses a unique hygromycin B phosphotransferase (HPH) that fully inactivates the antibiotic by phosphorylation of the 7"-hydroxyl group of the destomic acid moiety thus being a new type of aminocyclitol-0-phosphotransferase. *S. hygroscopicus* apparently requires only the HPH activity to provide resistance to hygromycin B [2].

Hygromycin B is a trisaccharide aminoglycoside with a terminal cyclitol, reported by researchers at Eli Lily in the early 1950s. It belongs to the destomycin family of aminoglycosides which contain a unique cyclic structure linking the terminal saccharide. Hygromycin B exhibits broad spectrum antibiotic and anthelmintic activity, and is used as a feed additive for some animal meats like poultry as anti-worming agent in a product name: Hygromix. Hygromycin B inhibits polypeptide synthesis by binding peptidyl-tRNA and preventing translocation by Elongation Factor 2 [3]. The project aimed to identify and characterize the species of *Streptomyces* producing hygromycin B antibiotic.

Materials and Methods

***Streptomyces* isolates:** Fifty three ***Streptomyces* isolates** were obtained from Al–Nahrain Biotechnology Research Center, Baghdad, Iraq. The isolates were maintained by growing them in Inorganic Salt-Starch broth (Isp 4)[4] for 5 days at 30°C first, then by preserving on agar of this medium in the refrigerator with continuous weekly reactivation. The most efficient *Streptomyces* isolate was identified microscopically[5], then by cultural examinations through growing on diagnostic media ISP2 (international streptomyces project) medium, Melanin production medium, Czapeck-Dox medium, Sabaroud maltose agar, Asparagine- dextrose–meat extract agar, Bennet's Agar, Isp1, Isp 4, 2 Glycerol–asparagines agar ISP5, [4] and finally by biochemical tests.

Test organisms: Six species of pathogenic bacteria; 1 Gram +ve (*Staphylococcus aureus*) and 5 Gram –ve (*Pseudomonas aeruginosa, Proteus mirabilis, Escherichia coli, Klebsiella pneumoniae, Salmonella typhi*) in addition to one yeast (*Saccharomyces cerevisiae)* were treated by the *Streptomyces* isolates to test the antimicrobial activity of Hygromycin B produced by the later isolates. The test isolates were obtained from the same center above.

DNA extraction: It was extracted according to the manufacturing company protocol and recommendations (favorgen/USA). All DNA samples, were analyzed by Polymerase Chain Reaction (PCR) **technique** for Hygromycin B gene detection through using specific primers designed according to basic information that obtained from database of DNA sequences in NCBI **Hygro1** Forward '5-TCGTATCCGCCAATGAGTCG -3' Reverse 5'- TTGCG TT CGGAGACGAAGAA -3', **Hygro2** Forward '5-GATCGTGATCCTCTGCCAGG -3' Reverse '5- TGCAGTAT CAG GACCCCGTA -3 and **Hygro3** Forward '5-TGTG CGTC GGTCATCAAGAA-3' Reverse '5-GTAGATGGTGATCAGCCGGG-3'. PCR technique conditions were; reaction mixture (25 µl): DNA sample 10 pmol, forward and reverse primers (1pmol) and Master mix (1X). Amplification procedure was performed according the following program:

Primers / *Step*	*Hygro1*			*Hygro2*			*Hygro3*		
	Temp. (oC)	*Time (min)*	*No. of cycles*	*Temp. (oC)*	*Time (min)*	*No. of cycles*	*Temp. (oC)*	*Time (min)*	*No. of cycles*
Initial denaturation	94	5	1	94	5	1	94	5	1
Denaturation	95	2	35	95	2	35	95	2	35
Annealing	64	30Sec	35	60	30Sec	35	58	30Sec	35
Extension	72	1	35	72	45sec	35	72	45sec	35
Final extension	72	7	1	72	7	1	72	7	1

The products of PCR were analyzed on agarose gel (1.5%) by using horizontal electrophoresis unit.

Detection of antimicrobial activity:

Antimicrobial activity of *Streptomyces* isolates against the seven test organisms was performed by using well agar diffusion method by using Moller-Hinton medium.

Results and Discussion

Ability of *Sreptomyces* isolates and the gene responsible for production of hygromycin B were tested using sets of primers that amplify the gene. Only 3 of the 53 isolates tested showed positive results for gene presence after analyzing on agarose gel as illustrated in Fig.(1).

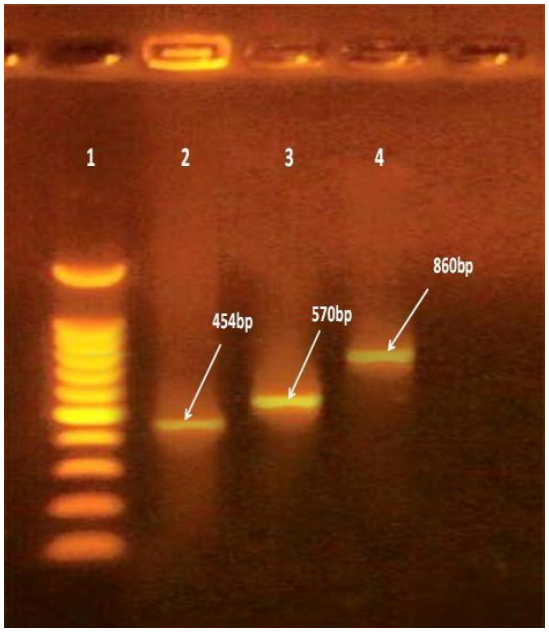


Fig.(1): Agarose gel (1.5%) electrophoresis of PCR products of hygromycin B gene in Streptomyces isolates for 1.5 h at 5v/cm.

Lane 1:- 100bp DNA ladder marker.
Lane 2:- product of Hygro1 primer.
Lane 3:- product of Hygro2 primer.
Lane 4:- product of Hygro3 primer.

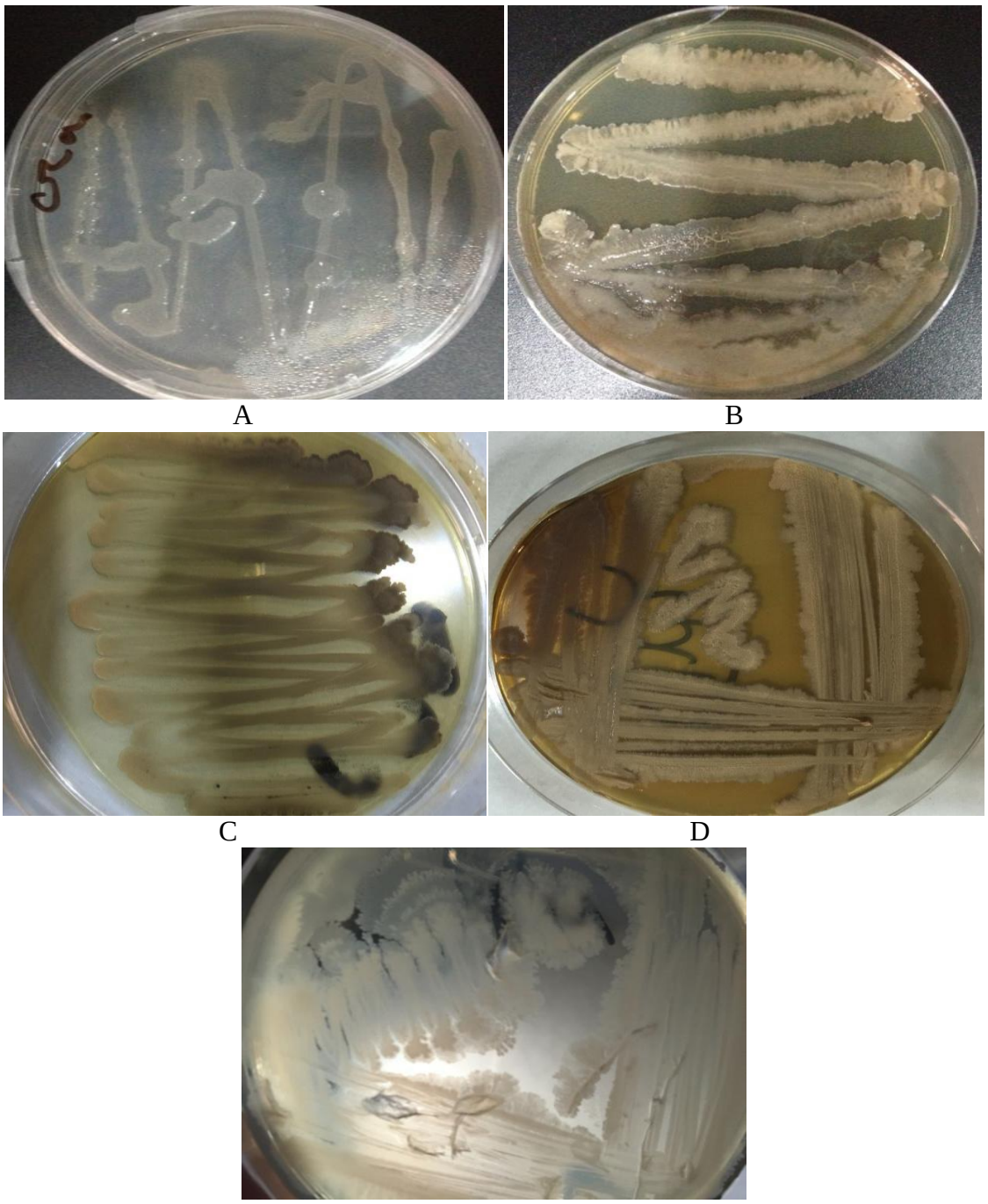
Isolate symbol	primerInhibition zone diameter (mm)						
	Staph. aureus	Ps. aeruginosa	Proteus mirabilis	E. coli	K.pneumoniae	Sal. typhi	Sacch. cerevisiae
1/2/34	10	10	30	60	12	30	31
12/34m	22	18	25	33	9	18	23
10cm/35pigment	55	17	70	30	10	33	38

The superior *Streptomyces* isolate "10cm/35pigment" was first identified by cultural examination through growing on several diagnostic standard identification media proposed by the International *Streptomyces* Project ISP [4,6] in order to characterize their color of aerial mass and substrate mycelium, reverse-side pigment, melanin pigments, spore chain morphology and spore morphology.

Cultural characteristics were also investigated on Czapeck dox medium, Sabaroud maltose agar, Asparagine-dextrose–meat extract agar, Bennetts agar, PDA and nutrient agar after incubation for 14 days at 13˚C [9]. The cultural characteristics together with certain physiologic reactions were examined and recorded. The recorded growth was moderate to abundant on some of the media used as shown in figure (2/A,B,C,D.E). Cultural results of this isolate indicate that they belong to the *Streptomyces* genus [4,12,13]. Results in Table (2) which represent the cultural properties of the superior *Streptomyces* isolate on different media show that the isolates grew well on both yeast extract-malt extract agar ISP2 and inorganic salt-starch agar ISP4 after incubation for 10 days at 30˚C. The color of aerial mycelium appeared light grayish, while the substrate mycelium appeared white. The reverse side of colonies was ranged from gray to colorless. These results came in accordance with those of the ISP by Shirling and Gottlieb, 1972 [7] as referred to *Streptomyces hygroscopicus* which described the mycelium as brownish grey or light greyish reddish brown (Grey color series) on ISP media 1,2, 4, and 5. While the reverse side colors of the colonies were either colorless to greyish yellow, pale yellow, light olive brown or grey on ISP media 1, 2,4 and 5.

Color series of the aerial mycelium established in the Bergey's manual of determinatives bacteriology [8] and in the category 4 of the Bergeys manual of systemic bacteriology, could be grouped in grey and white the series. Table (2) Cultural characteristics of *Streptomyces* isolate 10cm/35pigment grown on different identification media.

medium	growth	Aerial mycilum	Sustrate mycilum	reverse side of colony	Diffusible pigment
ISP1	abundant	Whitish	Pale grayish	colorless	Yellow to light brown
ISP2	abundant	Whitish to brown	brown	Light yellow	non
ISP4	moderate	non	Light brown	Light yellow	non
ISP5	abundant	Whitish	Light gray	colorless	non
Czapeck dox medium	moderate	non	white	colorless	non
Sabaroud maltose agar	abundant	Whitish to brown	white	Colorless to gray	non
Asparagine-dextrose– meat extract agar	abundant	Whitish	White to gray	Colorless to gray	non
Bennetts Agar	abundant	gray	Brown to green	Light green	Light green



A B C D E

Fig.(2): Growth of Streptomyces isolate 10cm/35pigment on: A- Czapeck dox agar, B- Bennetts agar, C- PDA medium, D- ISP2 mediun and E- Nutrient agar.

The *Streptomyces* isolate was *also* characterized microscopically after staining by Gram staining method as G (+), while the spore chain appeared as spirals. Morphology of the spore chains varied depending on the species, showing that they were straight and flexuous forms, like hooks with open loops and coils, which are usually used, among other features, to establish the differences between the isolates.

Biochemical characteristics which are shown in Table (3) declare that *Streptomyces* isolate 10cm/35pigment gave positive results for catalase, amylase (which hydrolyze the starch as shown in Fig.(3)), gelatinase, urease and utilization of citrate as the sole carbon source. Adversely, this isolate was unable to: produce indole, to lyse red blood cells and to produce melanin pigment.

Results of carbohydrates fermentation Table (3) indicate that the isolate was able to ferment (glucose, Inositol, sucrose, fructose, Mannitol, and D-xylose) but unable to ferment L-arabinose**.**

Such findings are similar to those biochemical characteristics described by

researchers about *Streptomyces hygroscopicus* [12,13].

It can be concluded from the above results and findings that some species of *Streptomyces*, especially *Streptomyces hygroscopicus*, have the ability to produce hygromycin B antibiotic which possesses the ability to inhibite growth of several pathogenic bacteria.

Table (3)
Biochemical characterization of Streptomyces isolate 10cm/35pigment.

Characteristic	***Result***
Catalase	positive
Indole	negative
Urease	positive
Starch hydrolysis	positive
Gelatin liquification	positive
Blood hemolysis	negative
Citrate utilization	positive
Melanin production	negative
Carbohydrates fermentation	
D-glucose	positive
Inositol	positive
L-arabinose	negative
Sucrose	positive
D-xylose	positive
Fructose	positive
Mannitol	positive

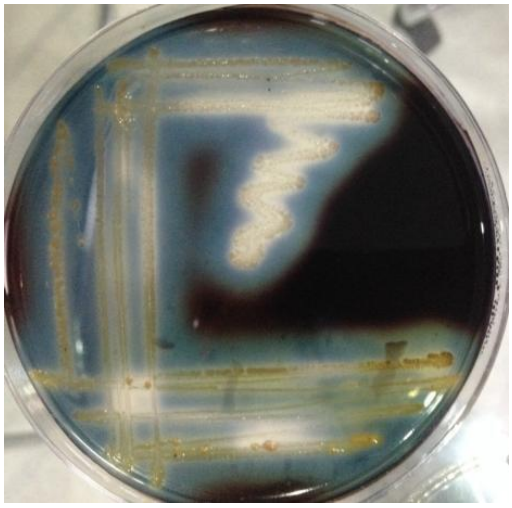


Fig.(3): Hydrolysis of starch by Streptomyces isolate10cm/35pigment grown on starch agar.

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الخلاصة

لأختيار اكثر عزله منتجه لمضاد الـ Hygomycin B، تم اختبار 21 عزله وذلك بعزل الحامض النووي الـ DNA والبحث عن المورث المسؤل عن انتاج المضاد بأستخدام بادءات متخصصه في تقنية الـ PCR من هذه الـ 21 عزله، فقط ثلاث عزلات اثبتت وجود الجين وتم قياس الفعاليه الحيويه لهذه العزلات، العزله بالرمز "10cm/ 35pigment" تفوقت على باقي العزلات باعطاء اعلة فعاليه حيويه ضد اربعه من سبع احياء مجهريه تحت الاختبار (*Proteus mirabilis, Staph. aureus, Sal. typhi and Sacch. Cerevisiae)* استخدمت في هذه الدراسه، حيث اعطت منطقة تثبيط ,70, 55, 33 and 38 mm بالتعاقب بعد اخضاع العزله المتفوقه لفحوصات مظهريه ومجهريه وكيموحيويه شخصت على انها *Streptomyces hygroscopicus*.

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