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Ss9Screening and Identification of melanin producing *Actinomycetes*

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Abstract:
Twenty-five soil samples were collected from Hilla city. Ten isolates of *Actinomycetes* were identified. These isolates were screened for melanin production. Out of these two isolates (*Actinomycetes* .2, *Actinomycetes* .5) were produce brown pigment on peptone- yeast extract agar. These isolates were cultured on tyrosine broth and found *Actinomycetes* .2 were able to produce melanin by formation red color. *Actinomycetes* .2 was grey aerial mycelium, yellow – brown substrate mycelium and able to ferment glucose, sucrose, fructose and xylose. *Actinomycetes* .2 isolate showed antibacterial activity against *S.aureus, E.coli* with inhibition zone (15,20)mm subsequently. Melanin was extract and showed antibacterial activity against *S.aureus* (18mm) and *E.coli* (19mm) and antifungal activity against *C.albicns* with 15mm inhibition zone.

Introduction:

***Actinomycetes* are free living, saprophytic bacteria found widely distributed in soil, water and colonizing plants. *Actinomycetes* population has been identified as one of the major group of soil population which may vary with the soil type [9].*Streptomycetes* are filamentous bacteria having high G+C content about 60-70 mol%. There are most of 500 *Streptomyces* species characterized by formation of aerial mycelium and spores on solid mediums, they were easily identified with their different colors, dry and wrinkle mature colony. *Streptomycetes* are Actinomycetes with cell wall type I, belonging to the family *Streptomycestaceae* a member of the order Actinomycetales with complex life cycle [26].**

Actinomycetes constitute a significant component of the microbial population in most soils and *Streptomyces* a count for 90% of the total Actinomycetes population (Poopal It play a significant role in the pharmaceutical industry for their capacity to produce secondary metabolites with diverse chemical structures and biological activities. Tens of thousands of such compounds have been isolated and characterized, many of which have been developed into drugs for treatment of wide range of diseases in human, veterinary and agriculture sectors [4, 23]. Melanin compounds are irregular, dark brown polymers that are produced by various microorganisms by the fermentative oxidation, and have the radioprotective and antioxidant properties that can effectively protect the living organisms from ultraviolet radiation [10]. Melanins are frequently used in medicine, pharmacology, and cosmetics preparations and its enigmatic pigments that are produced by a wide variety of microorganisms including several species of bacteria and fungi [22]. Melanin is a common substance produced by animals, plants and microorganisms. These pigment of high molecular weight formed by oxidative polymerization of phenolic or indolic compounds and usually are dark brown or black [13]. Several types of melanins have been described in bacteria, plants, animals, and fungi: eumelanins, phaeomelanins, allomelanins and

pyomelanins. Eumelanins are formed from quinines and free radicals. Phaeomelanins are derived from tyrosine and cysteine. Allomelanins are synthesized from nitrogen-free precursors, and pyomelanins are derived from the catabolism of tyrosine via *p*-hydroxyphenylpyruvate and homogentisic acid (HGA) [12].It has been shown to protect micro-organisms against UVradiation, enzymatic lysis, oxidants and killing by alveolar macrophages [6]. It has also been shown to chelate metal ions, to function as a physiological redox buffer, to provide structural rigidity to cell walls and to help to store water and ions [13,10].

Materials and Methods

Isolation of Actinomycetes from soil samples:

Soil samples were collected from Hilla city. Samples were placed in a Petri dish and tightly sealed. The samples were then pretreated with $CaCO_3$ (10:1 w/w) and incubated at 37°C for 4 days and subjected to serial dilution (up to 10^{-6}dilution. About 1.0 ml of diluted samples were plated on starch casein agar prepared as (10 gram of starch, 1 gram of casein, 0.5 gram of K_2HPO_4) in one liter of distilled water) the cycloheximide (25 µg/ml), nalidixic acid (25 µg/ml) was added to avoid bacterial and fungal contamination by spread plate technique and incubated at 28°C for 7 to10 days [7].

Characterization and biochemical tests:

Culture characterizations were determined according to the International *Streptomyces* project (ISP) [23]. The general criteria used for *Streptomyces* identifications are morphology, aerial mycelium, substrate mycelium,spore morphology, production of diffusible pigments, production of melanin pigment, and utilization of various carbon and nitrogen sources [1] Carbon sources like,glucose , fructose, xylose, sucrose, were tested on phenol red broth supplemented with 1% carbon source [1].

Melanin formation:

Melanin formation was tested on peptone-yeast extract iron agar prepared by adding (36 gram from Bacto-Peptone Iron Agar, dehydrated (Difco) , 1 gram from Bacto-Yeast Extract (Difco), 1 liter of distilled water, pH was adjusted to 7.0-7.2 before autoclaving. The synthetic tyrosine agar was prepared by added of (15 gram of tyrosine , 0.5 gram of glycerol and 0.5 gram from L- asparagines and mixed with0.5 gram for each of $MgSO_4.7H_2O$, K_2HPO_4 and $FeSO_4.7H_2O$ and 1ml of trace salt solution which contain on (mixing 0.1 gram from $FeSO_4.7H_2O$, MnCl2 4H20 and ZnS04.7H2O and 100 ml from Distilled water [23]. 10 µl of suitable liquid media were dispensed in test tubes and inoculated with one loop full of the spores of the *Streptomyces* and subjected to stationary stage at 27ºC for seven days. Melanin formation was tested using 10µl of suitable liquid media, dispensed in test tubes and inoculated with one loop full of the spores of the *Streptomyces* and subjected to stationary stage at 37°C for seven days. Melanin pigment was estimated by taking 2 ml of the culture and 1 ml of 0.4% substrate solution (L-tyrosine). The reaction mixture was incubated at 37°C for 30 min and a red coloration was observed [20,23].

Antibacterial activity of *actinomycetes*:

The purified actinomycetes colonies were screened for antibacterial activity against *Escherichia coli Stapyllococous aureus,* by well diffusion method. The isolates were grown in a production broth until adequate turbidity was achieved. 100 µl of the actinomycetes broth culture was placed in wells made on Muller Hinton agar plates seeded with the test bacterial pathogen cultures. The plates were incubated at 37°C and observed for inhibition zone after 24 h. [14].

Extraction of melanin pigment:

After incubation cultures were centrifuged at 3,000 rpm for 30 min, equal volume of chloroform, ethyl acetate and methanol were added with cell free supernatant and mixed well. This step was repeated 2 to 3 times. The solvents were then evaporated and powdered while the pigment residues were collected [19].

Antibacterial and antifungal activity of melanin crude pigment extract:

Antibacterial activity was tested by well diffusion method. *E. coli* and *S. aereus* and were swabbed on a Muller Hinton agar against 100 µl of pigment extract and incubated 37°C for 24 h.. The antifungal activity was tested against *C. albicans*. The plate were incubated at 37ºC for 48 - 72 h and the diameter of the inhibition zones of the test fungi around each well was measured [25,7].

Results and Discussion:

Isolation of *Actinomycetes*:

A total Twenty-five soil samples were collected, ten isolates of *actinomycetes* were isolated on starch casein agar and identified as *Actinomycetes* according to [23] and [5].

Screening of *Actinomycetes* isolates for melanin production:

Ten isolates of actinomycetes were screened for melanin production on peptone-yeast extract iron agar. Out of these two actinomycetes(*Actinomycetes* .2 and *Actinomycetes* .5) isolates were produce brown pigment on peptone-yeast extract iron agar (Table 1) .*Strepetomyces* isolates produce a diffusble dark brown pigment on both peptone-yeast extract agar and synthetic tyrosine agar, the pigment has been referred to be as dark brown pigment, as melanoid or melanin [8].The formation of brown color for *Streptomyces* isolates on peptone-yeast extract iron agar was observed by [25].

Table(1):Screening of *Actinomycetes* isolates for melanin production on peptone-yeast extract iron agar

Actinomycetes isolates	Production of pigment
Actinomycetes .1	-
Actinomycetes .2	(+) brown
Actinomycetes .3	-
Actinomycetes .4	-
Actinomycetes .5	(+)brown
Actinomycetes .6	-
Actinomycetes .7	-
Actinomycetes .8	-
Actinomycetes .9	-
Actinomycetes .10	-

Detection of melanin production isolate:

Our results for production of melanin was confirmed by using tyrosine as substrate and after mixing of 2ml of culture broth with tyrosine. The formation of red color indication for melanin production. The formation of melanin observed by *Actinomycetes* .2 by formation of red color. It is difficult to determine, whether the diffusible pigments produced are melanoid (dark brown) or merely a brown substance, especially when complex organic media is employed. The method of testing melanin production by L-tyrosine as a substrate may be the good criterion for the identification and classification of *Streptomyces*. [8]. Our results agreed with result obtained by [25] who found that melanin producing actinomycetes produce red color on liquid media.The formation of diffusible pigment dark or brown by *Streptomyces* was observed by many reports ,when colonies of *Streptomyces* was melanin negative [15].*Streptomyces albidoflavus*-143 was moderate growth on peptone yeast extract-malt extract iron agar medium (ISP-6), the diffusible pigment is moderate yellowish brown with no production of melanin [3].

Characterization of *Actinomycetes*. 2:

***Actinomycetes.*2 was gram positive, grey aerial mycelium, with yellow brown substrate mycelium. These isolate was produce brown pigment on peptone yeast extract agar. These isolate are able to ferment glucose, sucrose, fructose. *Actinomycetes.*2 was able to produce melanin (Table 2).**

Table (2):Characterization and biochemical test of *Actinomycetes.*2

character	results
Gram stain	+
Aerial mycelium color	grey
Substrate mycelium color	Yellow-brown
Melanin production	+
Fermentation: glucose	+
sucrose	+
fructose	+
xylose	+

Antibacterial activity of *Actinomycetes*.2 isolate:

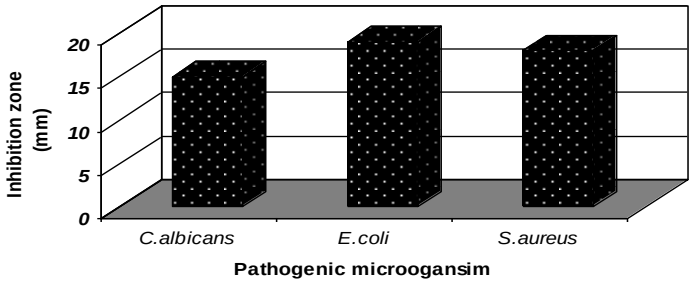
The antibacterial activity of actinomycetes. 2 isolate was tested against *S.aureus* and *E.coli*. Actinomycetes. 2 showed lower activity against *S.aureus* with inhibition zone 15mm while 20mm inhibition against *E.coli*.. Our results agreed with results obtained by [5], who found that *actinomycetes* isolated from soil samples were active against *S.areus* and *E.coli* with inhibition zone 16,15 mm subsequently. *Streptomyces* are able to produce wide range of molecules with board spectrum activities that is antibacterial, antifungal, and antiviral [2].

The antibacterial activity of *Streptomyces* isolates were obtained from soils in the Aegean and East Black Sea regions of Turkey and were found to be active against tested microorganisms active against *S. aureus* and, *E. coli* . [18].

Antimicrobial activity of melanin extract:

The melanin extract was tested for antimicrobial activity against *S.aereus, E.coli,* and *Candida albicans*. The crude of melanin showed antibacterial activity against *S.aereus* with inhibition zone 18 mm ,while the extract showed 19mm against

E.coli **.The results showed that the extract have antifungal activity against *Candida albicans* with inhibition zone 15 mm. (Figure 1).Our results agreed with results obtained by [25] who found that the melanin extract with antibacterial activity against *S.aereus* and *E.coli* with inhibition zone 18and 19mm subsequently.Our results agreed with the results obtained by [11] who found that the *Streptomyces* spp. was able to inhibition growth of *C. albicans* .**



Figure(1):
Antimicrobial activity of melanin extract against pathogenic microorganisms.

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عنوان البحث:

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العنوان: جامعة بابل /كلية العلوم/قسم علوم الحياة

الخلاصة:

جمعت خمس وعشرون عينة تربة من مدينة الحلة.شخصت عشر عزلات تعود الى الاكتينومايستات.فحصت هذه العزلات لانتاج الميلانين ووجد ان العزلتين2,5 تنتج صبغة بنية على وسط مستخلص الخميرة والببتون. زرعت هاتين العزلتين على وسط التايروسين السائل واظهرت العزلة 2 القدرة على تكوين الميلانين بتكوين اللون الاحمر .تمتلك العزلة2 مايسليم رصاصي ومايسليم اساسي اصفر-بني اللون.وكانت لها القدرة على تخمير الكلوكوز والسكروز والفركتوز والزايلوز . أظهرت هذه العزلة فعالية مضادة لبكتريا المكورات العنقودية وبكتريا الايشريشيا كولاي باقطار تثبيط 15 و20 ملم بالتتابع.استخلص الميلانين من العزلة رقم 2 واظهرفعالية ضد المكورات العنقودية مع قطر تثبيط 18ملم وفعالية ضد الايشريشيا كولاي مع قطر تثبيط 19 ملم وايضا اظهرت الميلانين فعالية ضد الكانديدا بقطر تثبيط 15ملم.