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Survey for Tyrosinase production by *Streptomyces species*

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ABSTRACT

The present study was focused on isolation and screening of tyrosinase enzyme produced by actinomycetes. The isolates were primarily identified to genus level on the basis of their microscopic and cultural characteristics. Biochemical and other characters were used to identify isolates to species level. A total five soil samples were collected from different sites at Damietta EL-Gededa (Egypt) and Meslatah (Libya). The total 36actinomycete isolates were obtained from soil by performing serial dilution technique and using starch-nitrate agar and primary screened for tyrosinase production by using tyrosine agar medium. Based on the result of primary screening,8 isolates 2D, 4D, 5L, 6L, 12D, 13D, 14D and 26L showed blackish brown pigment development due to melanin production, was selected. These isolates were taken for secondary screening(tyrosinase assay) by culturing on tyrosine broth at 30^{O}C for 5 days. Greenish black color was reported in tyrosine broth indicated the positive tyrosinase activity. Out of them, isolates13D,14D and 26Lwhich showed the highest tyrosinase activities (1.2 U/ml, 4.8 U/mL and 8.4 U/mL, respectively) were identified on the basis ofcultural, morphological, physiological, biochemical as *Streptomyces malachitorectus*, *Streptomycesiakyrus* and *Streptomycesechinatus*, respectively.

Key words: Actinomycetes, *Streptomyces*, Tyrosinase, Screening.

INTRODUCTION

A wide variety species of *Streptomyces* are known of producers of bioactive molecules like antibiotics, pigments and many extracellular enzymes as glucose isomerase, amylase, cellulase, and protease. Their capacity to produce tyrosinase was studied in a lesser extent. In addition, this group of actinomycetes is also able, when are cultivation on organic media, to synthesize and excrete dark pigments, melanin or melanoid, which are considered as useful criteria in taxonomic studies (Zonova, 1965; Arai and Mikami, 1972). *Streptomyces* tyrosinasesare the most thoroughly characterized enzymes of bacterial origin.(Della *et al*., 1998; Matoba *et al*., 2006). The first bacterial tyrosinase have been purified from cell extracts of *Streptomycesnigrifaciens*(Nambudiri and Bhat, 1972) and *Streptomyces glaucescens* (Lerch and Ettlinger,1972).

Tyrosinase is a type 3 copper-containing enzyme that has been found widely distributed in microorganisms, plants and animals (Mukherjee *et al*. 2013). Tyrosinase catalyzes the hydroxylation of monophenols to o-diphenols and the oxidation of diphenols to o-quinones followed by a series of nonenzymatic and enzymatic reactions steps resulting in the formation of melanin (Claus and Decker 2006; Valipour and Arikan, 2015; Balakrishnan and Kalirajan 2015). The mechanism by which an oxygen atom is transferred to the phenolic substrate is suggested to begin with either an oxodicopper [III] intermediate or a peroxodicopper [II]

intermediate (Zaidi *et al*., 2014).Tyrosinase plays an important role in wound healing and the primary immune response of plant life, sponges and many invertebrate (Danialand Al-Bishri, 2018; Decker and Tuczek 2000).

Tyrosinaseis one of the most important industrial enzymes. They have attracted interest because of the diversity of their applications. Major industrial applications of tyrosinase in the field of the medical science, food industry, and industrial biotechnology such as, phenols and dyes, waste water management(Durán and Esposito 2000; Girelli *et al*., 2006; Duarte., *et al*. 2012),protein cross linking in food technology(Lantto, 2007) and synthesis and bioconversion of diphenol drugs, like L-DOPA(Durán *et al*., 2002; Haq *et al*., 2002).

The present work aims to isolate and identify some tyrosinase enzyme producing streptomycetes species.

MATERIALS AND METHODS

Samples collection

Soil samples were collected from different sites of Libya (Mesallata) and Egypt (Damietta EL-Gededa). Soil samples were collected from various depths of the earth surface up to meter depth and transferred into clean plastic sterile bags and then stored for 1 day in a cold condition after adding calcium carbonate (2 g /10 g sample).

Isolation of Actinomycetes

Actinomycetes used in this study were isolated from various soil samples using standard dilution plate method(Johnson *et al*.,1959).The plates were incubated for a period of seven days at 30°C. Colonies of streptomycetes were selected, isolated, purified and maintained as spore suspensions in 20% (v/v) glycerol at -20°C for subsequent investigation (Hopkins *et al*., 1985). The medium used for isolation, cultivation and stock maintenance of isolated strains was starch nitrate agar medium (Waksman, 1959). That contained (g/L): soluble starch, 20; KNO_3, 2; K_2HPO_4, 1; NaCl, 0.5; $MgSO_4.7H_2O$, 0.5; $FeSO_4.7H_2O$, 0.01; $CaCO_3$, 3; agar, 20; and distilled water up to 1L.The isolated colonies were further separated and purified using streak plate technique and screened for tyrosinase production.

Primary screening method

The primary screening for tyrosinase enzyme activity was conducted using streaked isolates on tyrosine agar (pH 7.2) (Shinobu, 1958); containing (g/L): Ca $(NO_3)_2$, 2.5; K_2HPO_4, 1; $MgSO_4.7H_2O$, 0.3; NaCl, 0.1; $FeSO_4.7H_2O$, 0.01; $CaCL_2$, 0.1 and distilled water up to 1L, and all the plates were incubated at 30°C for 5days. After incubation, the occurrence of brown pigmented colonies that gradually changed its color to black (melanin formation) was indication of tyrosinase positive organism (Raval *et al*., 2012).

Secondary screening method

Tyrosinase producing actinomycetes were further secondary screened by tyrosine broth. Survey for tyrosinase activity was carried out by inoculated of the each of the above isolates in conical flasks (250 ml) containing 50 ml tyrosine medium (Shinobu, 1958). The pH of the medium was adjusted to 7.2- 7.4 using NaOH and HCl (0.1M) prior to sterilization. The activated culture of the organism after cell growth at 30^OC for 5days on starch-nitrate agar medium (Waksman, 1959), 2 discs of grown bacteria, were used to inoculate flasks. The flasks were incubated statically at 30^OC for 5 days. After the incubation period, cultures were harvested by

centrifugation at 5000 rpm for 15 minutes. Cultures supernatants were used in assaying for tyrosinase activity. If tyrosinase is produced broth colour changes from colourless to brown and ultimately greenish black. The activity of tyrosinase was assayed periodically after different time intervals.

Tyrosinase assay

In order to carry out the tyrosinase assay the reaction was carried out as follows: 1.0 ml of 0.5 M phosphate buffer, pH 6.5 at 25°C, 1.0 ml of 0.001 M L-tyrosine solution (Prepared in 100 ml in deionized water using L-tyrosine, Free Base, Sigma Prod. No. T-3754.), 0.1 ml crude enzyme and 0.9 ml of reagent grade water were added into test tube. The reaction mixture was oxygenated by bubbling 99.9% pure O_2 through a capillary tube for 3 to 5 minutes to reach temperature equilibration and absorbance was recorded at 280 nm by using UV-Vis spectrophotometer (Raval *et al*., 2012).

The enzyme activity was calculated by using the following Formula:

$$\text{Units/ml enzyme} = \frac{(\Delta A280\text{nm/min Test} - \Delta A280\text{nm/min Blank})\ (\text{df})}{(0.001)\ (0.1)}$$

Where, df = Dilution factor

0.001 = The change in A280 nm/minute per unit of tyrosinase at pH 6.5 at 25°C in a 3 ml reaction mix.

0.1 = Volume (in milliliters) of enzyme used.

One unit will cause an increase in A280nm of 0.001 per minute at pH 6.5 at 25°C in a 3 ml reaction mix containing L-tyrosine.

Identification of the selected actinomycetes

The streptomycetes isolates used in this investigation was identified according to International *Streptomyces* Project (Shirling and Gottlieb, 1968a; 1968b; 1969; 1972; Pridham and Tresner, 1974a; 1974b; Buchanan and Gibbons, 1974).

Electron microscopy studies

Electron microscopy was performed using the cover slip technique. The cover slip was cut with a glass file and a suitable fragment with growth on surfaces of starch nitrate agar cultures was chosen. It was mounted on a specimen-tube, coated with gold-palladium under vacuum and examined with electron microscopy(scanning, transmission electron microscopy) (SEM, JEOL, JSM-5300 electron microscope at Alexandria University, Egypt) operating at 10KV.

RESULTS

Isolation of the actinomycetes

A total 36 actinomycetes were isolated from soil samples collected from Egypt and Libya. The colour of the colonies on starch nitrate agar medium were also indicated, were actinomycetes related to grey series dominated (23 isolate out of 36) followed by pink series (5 isolates), 4 isolates were related to blue and 4 isolates belong to white series (Table 1).

Screening of tyrosinase producers

Screening of actinomycetes was conducted on tyrosine agar plate as a preliminary study for choosing tyrosinase producers. After 5 days of incubation, 8isolates (coded as 2D, 12D, 6L, 13D, 4D, 14D, 5L& 26L) out of 36actinomycetes showed appearance of brown pigmented colonies that gradually changed its color to black (melanin formation) (indication of tyrosinase

positive organisms) were selected. 3 isolates namely, 13D, 14D and 26L exhibited the highest brown colored pigmentation that gradually changed its color to black (melanin formation) on tyrosine agar.

On the basis of the results obtained on tyrosine agar plates, the potent isolates were inoculated in tyrosine broth(eight isolates). The activities of the enzymes were assessed after 5 days. All eight isolates (2D, 12D, 14D, 4D, 6L, 13D, 5L and 26L) were able to utilize tyrosine but produced different levels of tyrosinase activity after the 5th day (Fig. 1). Isolate 26L was found to produce the greatest tyrosinase activity (8.4 U ml^{-1}), followed by isolate 14D (4.8 Uml^{-1}) followed by isolate 13D (1.2 U ml^{-1}).

Isolates identification

The selected three actinomycetes isolates (13D, 14Dand 26L) were identified according to their morphological cultural, and biochemical characteristics which represented (Table 2). Morphological studies indicated the presence of aerial and substrate mycelia with spiral spore chains arrangement and spiny spore surfaces showed in (Figs. 3, 4 & 5). Biochemical characters of tyrosinase producing isolates 13D, 14Dand 26L were reported (Table 2). Isolate 13D, 14D and 26L was gram positive and showed good growth on several media, including Starch-nitrate agar, Glycerol-nitrate agar, Glycerol asparagine agar, Glucose-nitrate agar, Yeast-malt agar, Dox agar, Oat meal agar and Starch-ammonium sulphate agar. Varying colours (brown, yellow, grey &white) of substrate and aerial mycelium were observed on these media. It was also observed that some of the strains produced diffusible pigments in the surrounding medium, some of them melanoid were produced on tyrosine agar medium. The cell wall of isolates 13D, 14D and 26L contained L-diaminopemilic acid (L-DAP) (Fig. 2) and there was no characteristic sugar pattern indicating that isolates 13D, 14D and 26L are a chemotype, cell wall type I (Lechevalier and Lechevalier, 1980).The scanning and transmission electron microscopic observation showed that the mycelia were well branched and 20-30 spores usually included in the chains of flexuous to spiral. Ornamentation of spores was confirmed as spiny (Figs. 3, 4 & 5). Thus, the identification of actinomycete isolates 13D, 14D and 26L were performed based on the morphological cultural, biochemical characteristics and the cell wall analysis and they are designated as *Streptomyces malachitorectus*, *Streptomyces iakyrus* and *Streptomyces echinatus*, respectively.

DISCUSSION

The products from *Streptomyces* have immense importance in different sectors; have always been a source of thousands of bioactive compounds. Enzymes are one of the important products of this unusual group of bacteria. In present study, *Streptomyces malachitorectus*, *Streptomyces iakyrus* and *Streptomyces echinatus* were capable of producing the tyrosinase in the medium "Tyrosine". Likewise, several other organisms were reported earlier to produce the tyrosinase enzyme, Tyrosinase enzymes and their genes have previously been characterized from bacteria, fungi, plants and mammals. Bacterial tyrosinases have been reported, of which *Streptomyces* tyrosinases are the most thoroughly characterized enzymes of bacterial origin (Della-Cioppa *et al*., 1998; Matoba *et al*., 2006). The first bacterial tyrosinases have been purified from cell extracts of *Streptomycesnigrifaciens* (Nambudiri & Bhat, 1972) and *Streptomyces glaucescens* (Lerch & Ettlinger, 1972).

Soil is considered the main source for large number of extremely diverse organisms; Streptomyces is one of a large number of groups of organisms that can be isolated from the soil. In this present study, 36 actinomycetes isolates were isolated from different soil samples,in accordance with the aerial mycelium color series established in the Bergey's manual of

determinative bacteriology (Buchanan and Gibbons, 1974), and in the category IV of the Bergey's manual of systematic bacteriology (Locci, 1989), the isolates could be grouped in the following series: gray (23), pink (5), and four each in the blue and white. Various colors of substrate mycelia were also observed, the shades beige, yellow, and green being the most predominant. This agreement with other many works (Taddei *et al*., 2006) from total of 71 actinomycetes isolates were isolated from Venezuelan soil samples, (42) isolates gray, (12) white, (10) red, (4) yellow and one each in the green, blue and black. The isolates were primarily screened for tyrosinase production by using tyrosine agar. 36 isolates were isolated from soil samples. All bacterial isolates were screened for tyrosinase production using medium containing tyrosine and only 3 isolates namely, 13D, 14D and 26Lexhibited the highest ratio of brown colored pigmentation that gradually changed its color to black (melanin formation) on tyrosine agar compared with the isolates. The production of brown/ black color pigment indicates the tyrosinase enzyme production (Jones *et al*., 2007). The production of brown color was due to the production of melanin by oxidative polymerization of phenolic compound by tyrosinase. Normally, observation of the intensity of brown/black pigment is a conventional method being followed for the qualitative screening of tyrosinase producing microorganisms. Formation of brown/black of the isolates on tyrosine agar (Shivaveerakumar *et al*., 2014), in addition to the intensity of pigment, significantly reveals the degree of synthesis of tyrosinase and can be an important criterion to select efficient isolates for the production of tyrosinase. Likewise, Gare & Kulkarni (2013) studied tyrosinase producing actinomycetes from soil of Shirala region. Out of sixty isolates 14 isolates were screened for tyrosinase activity by using tyrosine agar. Two isolates were showing change in color from colorless to pink to brown and finally turned into black at 30^{o}C for 24 to 48 hrs. Raval *et al*., (2012) studied on Biotransformation of a single amino-acid L-tyrosine into a bioactive molecule L-DOPA. From the soil sample only 8 isolates showed positive result on Tyrosine agar plate. The occurrence of a distinct brown spot which gradually changed its color to black (melanin formation) was indicative of the fact that the above isolates were tyrosinase positive.

On the basis of the results obtained on tyrosine agar plates, the potent isolates (3 positive isolates) were inoculated in tyrosine broth to quantify the amount of tyrosinase production, after incubation, all isolates showed positive result for tyrosinase. The color of the inoculated tyrosine broth changed from brown to greenish brown with further incubation at medium containing L-tyrosine. The production of blacked-brown color indicates the secretion of tyrosinase enzyme. The color intensity of isolates 13D, 14D and 26L were much higher than rest of isolates with maximum tyrosinase activity (1.28Uml^{-1}, 4.8Uml^{-1} and 8.4 Uml^{-1} respectivily). The isolates 13D, 14D and 26L produced brown coloration, due to melanin production, in both tyrosine agar plate and tyrosine broth revealing the ability of the isolates to produce tyrosinase. This result showed similarity with the observation by Dalfard *et al*., (2006); Le Roes-Hill *et al*., (2015). The formation of brown color was mainly due to the melanin production by oxidative polymerization of phenolic compound by tyrosinase.

Isolates 13D, 14D and 26Lpossessed LL-Diaminopimelic acid and it contains glycine in its cell wall. Presence of L,L Diaminopimelic acid along with glycine indicate the cell wall chemotype-I, which is the characteristic of the genera *Streptomyces*, *Streptoverticillium*, *Chainia*, *Actinopycnidium*, *Actinosporangium*, *Elyptrosporangium* and *Microellobosporia*. The morphological characters of the isolates 13D, 14D and 26L are similar to the genus *Streptomyces*. The morphological and biochemical characteristics of tyrosinase producing

isolates 13D, 14D and 26Lwere tested. The results are compared with those of *Streptomyces* species given in the key of Nonomura and also with the species described in the Bergey's Manual of Determinative Bacteriology. The isolates 13D, 14D and 26L showed similar characters to *Streptomyces malachitorectus*, *Streptomyces iakyrus* and *Streptomyces echinatus*. Hence the 13D, 14D and 26L has been tentatively identified as *Streptomyces malachitorectus*, *Streptomycesiakyrus* and *Streptomyces echinatus* respectively. Gare and Kulkarni (2015) reported *Streptomyces luteogriseus* as maximum tyrosinase producer. Extracellular tyrosinases were previously reported bacteria, there are several reports on tyrosinase from *Bacillus thuringiensis* strains (Dalfard *et al*., 2006). Also, some strains of *Streptomyces* have extracellular tyrosinases (Claus and Decker 2006).

In conclusion, Samples collected from all soil environments showed maximum percentage of *Streptomyces* genera. The actinomycetes isolated from soil sample were found to be the potential producer of tyrosinase.

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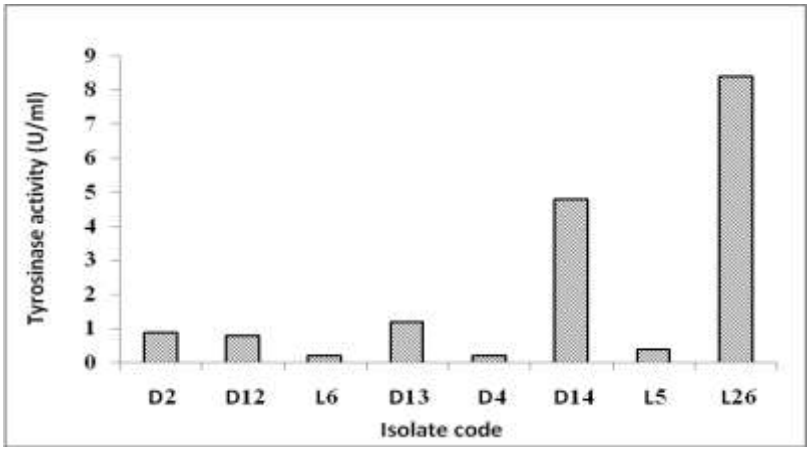

Fig, 1. Determination of tyrosinase activities in culture supernatants of selected isolates after 5 days.

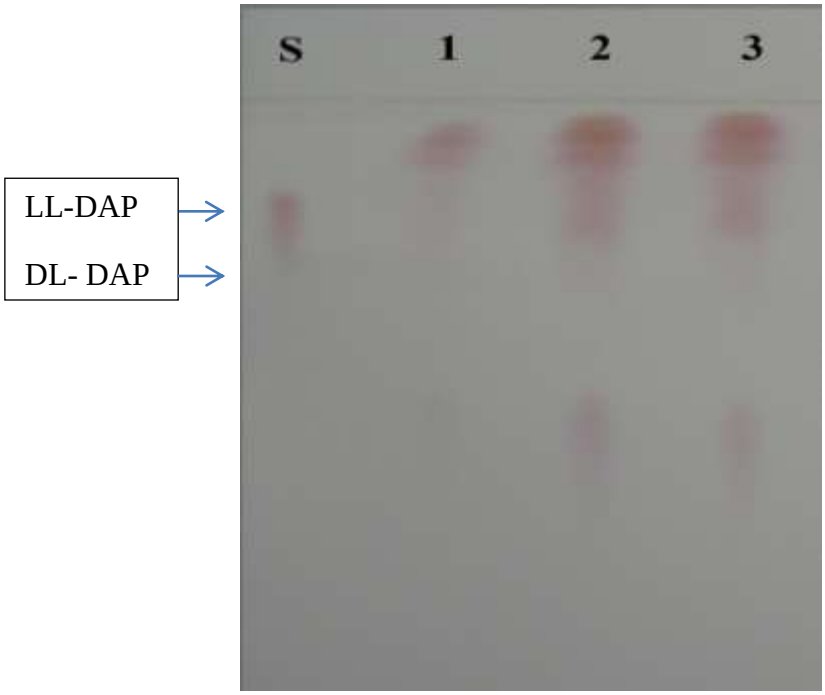

Fig. 2.Thain layer chromatography of Diaminopimelic acid (DAP) in the enzymatic streptomycetes (S) Standard DAP containing LL-DAP and DL-DAP. (1)strain 13D, (2) strain 14D and (3) strain 26L.

Table 1: Actinomycetes isolated from soil samples.

Location							
Mesallata		**Damietta El-Gededa**		**Mesallata**		**Damietta El-Gededa**	
Soil sample No.1		**Soil Sample No.2**		**Soil Sample No.3**		**Soil Sample No.4**	
Isolate No.	Color colonies	Isolate No.	Color colonies	Isolate No.	Color colonies	Isolate No.	Color colonies
1	Grey	10D	Grey	19L	Grey	28L	Pink
2D	Grey	11D	Grey	20L	Blue	29L	Grey
3D	Grey	12D	White	21L	Grey	30L	Grey
4D	Blue	13D	Grey	22L	Pink	31L	Pink
5L	Grey	14D	Pink	23L	White	32L	Grey
6L	Grey	15D	Pink	24L	Grey	33L	Grey
7D	Grey	16D	Blue	25L	White	34L	Grey
8D	Blue	17D	Grey	26L	Grey	35L	Grey
9D	Grey	18D	Grey	27L	White	36L	Grey

Table 2. Cultural, morphological and physiological characteristics of the selected isolates

Characters		13D	14D	26L
Colour and pigmentation	Aerial mass color	grey	grey	grey
	Melanoid pigment on: tyrosine, peptone yeast and synthetic media	+	+	+
	Reverse side pigment	Brownish	Greenish	Greenish
	Soluble pigment	+	-	-
Spore morphology	Spore chain	Flexuous	Flexuous to spiral	spiral
	Spore surface	Spiny	Spiny	Spiny
Carbon source utilization	Arabinose	+	+	+
	Xylose	+	+	+
	Inositol	+	+	+
	Mannitol	+	+	+
	Fructose	+	+	+
	Rhamnose	+	+	+
	Sucrose	+	+	+
	Raffinose	+	+	+
Nitrogen source utilization	Potassium nitrate	+	+	+
	L-valine	+	+	+
	L-threonine	+	+++	++
	L-serine	++	++	++
	L-Methionine	+	+	+
	L-histidine	+	+	+
	Hydroxy proline	+	+	+
	L-proline	++	++	++
	L-cysteine	+	+	+
	L-phenylalanine	+	+	+
Physiological properties	Milk coagulation	+	+	+
	Milk peptonization	+	+	+
	Starch hydrolysis	+	+	+
	Urea utilization	+	+	+
	Nitrate reduction	-	-	-
	Gelatin liquification	+	+	+
	Melanin/L-tyrosine	+	+	+
	Cellulose degradation	±	++	+
	Esculin degradation	+	+	+
	Hydrogen sulfide production	-	+	+

(+) good, (±) Little, (-) nil.

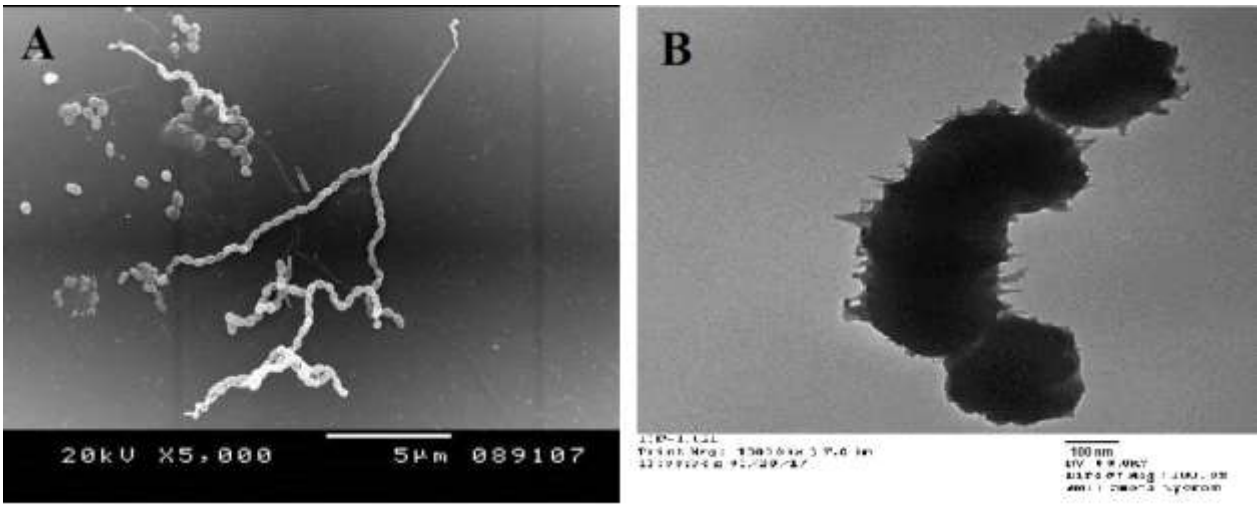

Fig. 3. (A) Scanning electron micrograph and (B) Transmission electron micrograph of spore surface ornamentation (10,000X) showing the isolated streptomycetes 13D growth after 7 days at 30°C.

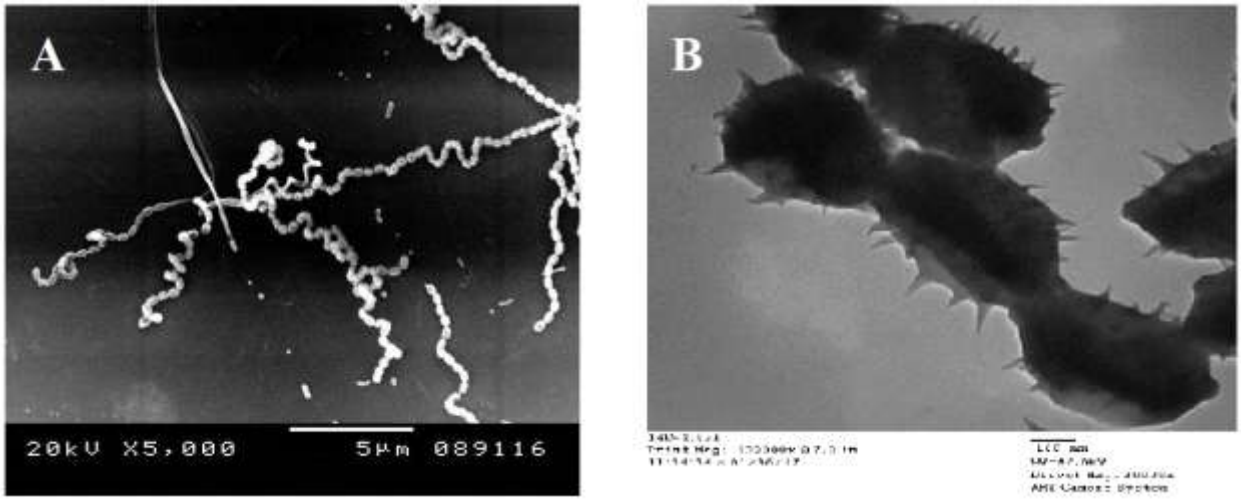

Fig. 4.(A) Scanning electron micrograph and (B) Transmission electron micrograph of spore surface ornamentation (10,000X) showing theisolated streptomycetes 14D growth after 7 days at 30°C.

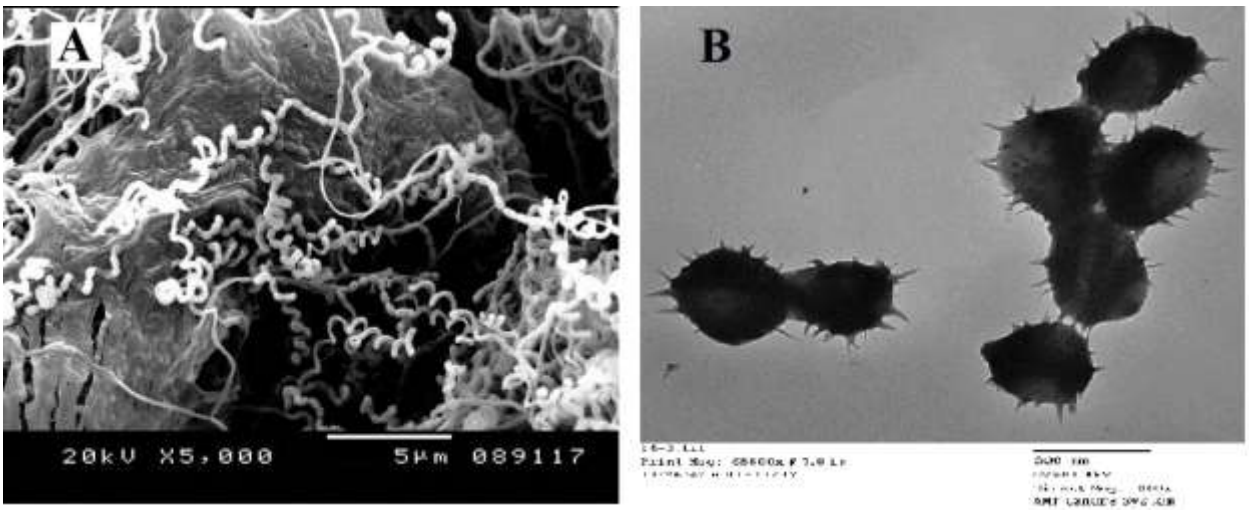

Fig. 5. (A) Scanning electron micrograph and (B) Transmission electron micrograph of spore surface ornamentation (10,000X) showing the isolated streptomycetes 26L growth after 7 days at 30°C.

مسح إنتاج التيروسينيز بواسطة سلالات إستربتوميسيس

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المستخلص

يهدف هذا البحث الى عزل بعض الأكتينوميسيتات التى تعيش فى أراضى مصر(محافظة دمياط)، والدولة الليبية- (مسلاته) والقادرة على إنتاج إنزيم التيروسينيز . وقد تمت عملية مسح للكائنات علي بيئة التيروسين الغذائية الصلبة وكان إنتاج المستعمرات للّون البني التي تغيرت تدريجيا لونه إلى الأسود (تشكيل الميلانين) يدل على أن العزلات كانت إيجابية لإنتاج التيروسينيز. وأسفرت نتائج عملية المسح الأولية للعينات على الوسط الغذائي الصلب عن وجود 8 عزلات فقط من هذه الكائنات والتى تعطى بقع بنيه داكنة أو سوداء عند نموها على نفس البيئة السابقة. تم اختيار هذه العزلات التي لها نشاط إنزيمي لتكسير التيروسين وتم تنميتها فى بيئة التيروسين سائلة (المسح الثانوي) لتقدير عدد ما تنتجه هذه العزلات من إنزيم التيروسينيز.

وقد أوضحت النتائج علي أن العزلات 13D و14Dو26L هي الأكثر نشاطا لإنتاج الإنزيم،فكانت (1.2 وحدة/مل)، (4.8 وحدة/مل)، (8.4 وحدة/مل) على التوالى. قد تم أيضا تعريف هذه العزلات من الكائنات المعزولة والأكثر نشاطا لإنتاج التيروسينيز من خلال دراسة الصفات التزريعية والمورفولوجية باستخدام الميكروسكوب الضوئي والميكروسكوب الإلكتروني وأيضا بدراسة بعض الصفات الفسيولوجية والكيموحيوية تبعا لأحدث الطرق العالمية المتبعة في هذا المجال وقد تم تسميتها كالتالي: *Streptomyces malachitorectus* (إستربتوميسيس مالاكاتوريكتس) ، *S. iakyrus* (إستربتوميسيس أيكيرس) *S. echinatus* (إستربتوميسيس إيكاناتس) .