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Identification, Characterization and Optimized Antimicrobial Production of *Streptomyces thinghirensis* Isolate

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ABSTRACT

The present study was conducted to isolate, identify and characterize the *Streptomyces* strain using morphological and biochemical analysis. This strain was molecular identified by sequencing the 16S rRNA gene as *Streptomyces thinghirensis* EGDA6S isolate. Results for scanning electron microscopy (SEM) revealed that the tested isolate is belonging to yellow series of *Streptomycetes* with smooth spore surface. Its cell wall contains LL-Diaminopimelic acid (LL-DAP) with non-characteristic sugar pattern and it does not produce melanin pigmentation. *S. thinghirensis* EGDA6S' metabolite revealed a considrable antimicrobial activity against some microorganisms namely: *Bacillus subtilis, Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Candida albicans*. Whereas, the maximum antimicrobial activity was observed on oat meal followed by starch nitrate media. Maximum antimicrobial activity being 7 days, at 30^{o}C and pH 7, for optimum incubation period, temperature and pH.

Keywords: *Streptomyces*, optimization, 16S rDNA sequencing, antimicrobial activity

INTRODUCTION

Streptomyces are gram-positive actinomyces dominated in soil representing 95% of soil actinomycetales populations. They are aerobic microorganisms with broad branching substrate and aerial mycelia, at a mature stage in life cycle, they forming long arthrospores chains within the aerial mycelia (Williams *et al.*, 1989). Most of the streptomycetes are soil saprophytes as they under nutrient limited conditions; they spend the most of their life cycles as semi dormant spores (Mayfield *et al*., 1972).

Streptomyces classification depends on both morphological and biochemical characteristics. The morphological ones include the substrate mycelium structure, the formation and nature of aerial mycelium, the structure and branching of hyphae, the surface of spore, growth on different media, color of either aerial or substrate mycelia, and soluble pigments formation. Biochemical characteristics include utilization of carbon and nitrogen sources, proteolysis features, ability to produce different enzymes in addition to their sensitivity to antimicrobial agents. Recently, modern molecular techniques are used such as genetic characters including 16S rRNA gene sequences and serological reactions to differentiate between closed related genera and species (Taddei, *et al*., 2006; Reddy *et al*., 2011; Baskaran, *et al*., 2014).

Different *Streptomyces* species have the ability to produce several bioactive metabolites. *Streptomyces* can produce approximately 7600 compounds such as antivirals, antibacterial, insecticide, antifungals, antithrombatic, herbicideanti-hypertensive, antitumor, and immunosuppressive agents (Bérdy, 2005, Baltz, 2008; Atta and Ahmad 2009; Patzer and Volkma 2010; Reddy *et al*., 2011; Rakshanya, *et al*., 2011).

Streptomyces has the ability to produce secondary metabolites depending on different environmental factors including light, nutrients, pH, temperature and moisture. When bacterial growth is limited, the secondary metabolites production is stimulated by the reduction of nitrogen, carbon, phosphate or other important sources of nutrient. Both concentration and composition of nutrients have a significant effect on complex mechanisms including in the regulation of global gene and affect conditions that activate the production of various secondary metabolites (Bibb, 2005; Sánchez *et al*., 2010; Van Wezel and Mc Dowail, 2011).

This study aimed to identify and characterize antimicrobial activity for producing activity for *Streptomyces* strain and optimizing its culturing conditions for maximum production of its antimicrobial compounds.

MATERIALS AND METHODS

Isolation of *Streptomyces* strain

The *Streptomyces* strain used in this study was isolated from rhizosphere of palm tree at Damietta El-Gededa city, on starch nitrate agar plates. The pure colonies were obtained by sub-culturing on the same medium till become free from any fungal or bacterial contamination. Then, it preserved in 20% glycerol at -80^{o}C for further study under EGDA6S code.

Screening for antimicrobial activity

Antimicrobial activity was tested against some Gram-positive bacteria (*Bacillus subtilis*, *Bacillus cereus* and *Staphylococcus aureus*), Gram-negative bacteria (*Klebsiella pneumoniae*, *Escherichia coli*, *Proteus mirabilis*, *Salmonella Sp*. *Pseudomonas aeruginosa* and Plant pathogenic *Pseudomonas* sp.), fungi (*Fusarium oxysporum, Rhizopus nigricans* and *Penicillium notatum)* and yeast (*Candida albicans)*.

The bacterial and yeast strains were obtained from the culture collection of microbiology lab, Botany and Microbiology Department, Faculty of Science, Damietta University. The fungal strains were kindly provided by Prof. Dr. Amira A. El-Fallal, Botany and Microbiology Department, Faculty of Science, Damietta University.

Classical identification of *Streptomyces* isolate:

The identification of *Streptomyces* isolate was carried out according to Nonomura (1974) and Bergey's Manual of Systematic Bacteriology (Williams *et al*., 1989). The micro-morphological properties including the size and shape of *Streptomyces* mycilia and spores were performed according to Nallamuthu *et al.,* (2015) using scanning electron microscopy (SEM) at 15 kV (GSM, EM unit, Mansoura University).

The occurrence of LL- or DL-hydroxyl-diaminopimelic acid (hydroxyl-DAP) and whole cell sugars were determined by thin layer chromatography (TLC) of whole cell hydrolysates according to Schon and Groth (2006) in microbiology lab, Faculty of science, Damiatta university.

Molecular identification of *Streptomyces* isolate

The genomic DNA of the *Streptomyces* isolate was extracted and purified as described by Kumar *et al.*

(2010). The 16S rRNA gene was amplified by using the universal prokaryotes primers, 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3') according to Lane (1991), the poly chain reactions (PCR) product was sequenced by an automated sequencer (Macrogen, South Korea) using the same previous primers.

Alignment and phylogenetic analyses:

Phylogenetic tree analysis was performed and analyzed using MEGA version 4 (Tamura and Nei, 1993).

Antimicrobial sensitivity of *Streptomyces* metabolite

Metabolites from liquid cultures were obtained by centrifugation at 10000 rpm for 10 minutes. The resulting filtrates were tested for antimicrobial sensitivity by adding 100 μL into 0.8 mm well in nutrient agar plate for bacteria and candida strains, while potato extract media agar was used for fungi strains. The resulting inhibition zones were measured in mm after the appropriate periods of incubation for each tested organism.

Optimization of antimicrobial production

Effect of incubation periods, pH, salinity, and different media for maximum antimicrobial production was studied by the method of Atta *et al*. (2015).

RESULTS AND DISCUSSION

Identification and characterization of the *Streptomyces* isolate

- Morphological and biochemical

Generally, identification and classification of streptomycetes are based on morphological, biochemical as well as physiological characteristics. On the basis of morphological ones, different growth intensity was observed when the *Streptomyces* isolate grow on different tested media .With respect to the substrate mycelia and aerial mycelia, different variable colors were observed in (Table 1). The yellow color was the most predominant color indicating that this isolate is belonging to yellow series of streptomycetes (Omura *et al*., 1977; Taddei *et al*., 2006). The shape of spore chains and hyphal branching were also examined by scanning electron microscope (SEM). Results revealed that there were a simple branching of hypha (Fig.1 A & B). The spore surface was smooth and arranged in straight chain (Fig.1 A and C).

Table 1. Cultural properties of *Streptomyces* isolate EGDA6S of 7 days old on different culture agar media.

Agar Medium	Growth	Color of		Soluble pigment
		Substrate mycelium	Aerial mycelium	
Starch-nitrate	++++	Yellow	Yellow	Yellow
Oat meal	+++	Yellow	Gray	Yellow
Starch ammonium sulphate	++	Off white	Off white	-
Glycerol-asparagine	++	Yellow	White	-
Glycerol-nitrate	++	Buff	pale pinkish	-
Glucose-nitrate	++	Off white	White	-
Czapek-dox	+	Pale green	White	Pale green
Yeast-malt extract	-	-	-	-

+ light growth, ++ moderate growth, +++ good growth, ++++ very good growth

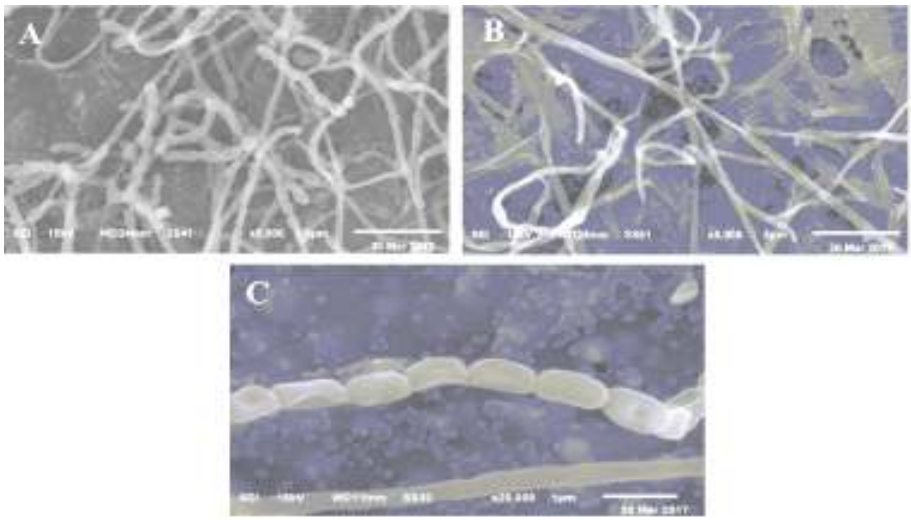

Fig. 1. Scanning electron microscope (SEM) showed the ultra-structural characterize of *Streptomyces* isolate EGDA6S.

The physiological and biochemical characteristics of tested *Streptomyces* isolate are represented in Table 2. It had the ability to utilize various either carbon or nitrogen sources with different variable growth intensity. The most pounced growth with respect to carbon source was observed on D-xylose while with respect to nitrogen source was observed on proline. The results revealed that this isolate could produce different enzymes such as amylase, lecithinase, cellulose, urease, caseinase and nitrate reductase. Furthermore, it was resistant to different wide range of antibiotics including rifampicin, lincomycin, levofloxacin and gentamycin. Also, it possessed an ability to resist both heat and salinity. Growth intensity was reduced by increasing the concentration of sodium chloride. Temperature is one of the most effective factors influenced on the growth of actinomyctes. In the present study, the growth intensity was ceased at 15^{o}C and above 45^{o}C with maximum growth at 30^{o}C. It could not produce melanin pigmentation on peptone-yeast iron agar or tyrosine agar media. The TLC revealed that its wall was type I which contains non-characteristic sugar and possessed LL-DAP.

Initially, according to the previous morphological and biochemical analysis based on Bergey's Manual of Systematic Bacteriology Williams *et al*., 1989 and Loqman *et al*., 2009, we conclude that this isolate might be belonging to genus *Streptomyces* and species *thinghirensis*.

Table 2. Physiological and biochemical characteristics of *Streptomyces* isolate EGDA6S.

Test	Result	Test	Result
Enzymes:		Melanin formation	
Amylase	+	on:	
Gelatinase	-	Peptone-yeast iron	-
Lecithinase	+	agar	
Cellulase	+	Tyrosine agar	-
Nitrate reductase	+	Carbon sources	
Urease	+	utilization:	++
H_2S production	-	L-arabinose	+++
Caseinase	+	D-fructose	+++
Esculin hydrolysis	-	D-glucose	+
Sensitivity to antibiotics:		D-lactose	+++
Penicillin-G (100µg)	-	D-mannitol	+++
Rifampicin (100µg)	+	Starch	+
Lincomycin (100µg)	+	Sucrose	++++
Cefotaxime (100µg)	-	D-xylose	+
Cefepime (100µg)	-	Meso-inositol	±
Levofloxacin(100µg)	+	Sodium acetate	±
Cephalexin (100µg)	-	Cellulose	+
Amoxillin & cluvanic	-	D-galactose	-
acid (100µg)	+	D-mannose	
Gentamycin(100µg)		Nitrogen source	+++
Growth temperature:	-	utilization:	+
15˚C	+	Proline	+
25˚C	+++	Valine	-
30˚C	++	Threonine	+
37˚C	+	Cysteine	+
40˚C	+	Hydroxy proline	+
45˚C	-	Phenyl alanine	++
50˚C		Serine	+
Cell wall chemical	glucose,	KNO_3	
structure:	mannose,	Heat resistance	+++
Sugar pattern	ribose	(100 c for 10 min)	+++
	(not	Growth in presence	+++
	characteristic	of NaCl:	++
	sugar)	5 gm/l	+
		10 gm/l	+
Type of Diaminopimelic	L-DAP	20 gm/l	-
acid (DAP)		40 gm/l	
		60 gm/l	-
		100 gm/l	
		120 gm/l	
		Inhibitor:	
		azide 0.01, crystal	
		violet 0.001	

(± low growth, + light growth, ++ moderate growth, +++ good growth, ++++ very good growth,- no growth).

- Molecular identification

The obtained partial sequence of 16S DNA sequence (1410 bp) was submitted to the GenBank with accession number (MH633729). According to obtained phylogenetic tree, the DNA sequence alignment of the 16S DNA partial sequence for the studied *Streptomyces* isolate EGDA6S showed highest identity (about 99.8%) with *Streptomyces* Sp. NEAE1 and S. *lienomycini* (KF991646) and *S. rubrogriseus* FG. B. 22, some other different *Streptomyces* strains showed less similarity which reached to 99%. The phylogenetic tree based on 16S DNA sequence as presented in (Fig. 2) clustered the studied *Streptomyces* isolate in a clade that possessing approximate dissimilarity distance reached to 0.045 with an out grouping strain *Amycolatopsis orientalis* IMSNU2. By comparing 16S rRNA gene sequence and different studied of morphological and biochemical characters, it can be concluded that the *Streptomyces* EGDA6S isolate is belonging to *thinghirensis* species (Loqman *et al*., 2009).

Regarding to, *S. thinghirensis* S10 clustered separately in the clade 102 which close to clade 103 that includes *S. lienomycini* and *S. rubrogriseus* (Labeda *et al*., 2012). Furthermore, *S. lienomycini* produces melanin pigment while *S. thinghirensis* does not produce it (Loqman *et al*., 2009). Also, *S. rubrogriseus* differs from *S. thinghirensis* by in many properties namely; producing red substrate mycelium, light grey aerial mycelium on oatmeal agar, while *S. thinghirensis* produce yellow substrate, white grey aerial mycelium and yellow pigment (Gause *et al*., 1983). Labeda *et al*. (2012) reported that morphological and biochemical identification is the base of identification of unknown *Streptomyces* strain and can confirmed by molecular identification through the 16S rDNA sequences.

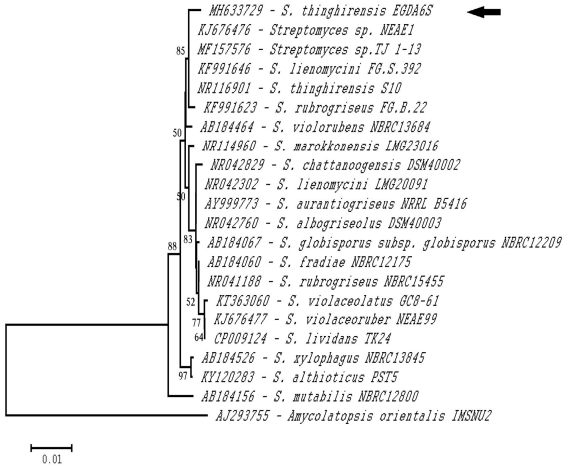

Fig. 2. Phylogenetic tree based on 16S rDNA sequence alignment for *S. thinghirensis* EGDA6S isolate (Accession. no. MH633729) with other related species possessed the highest identity at the database. The bootstrap values 20 or above were only considered and represented next to the phylogenetic tree branches with confidence levels estimated by 1000 bootstrap replicates. The scale represents the dissimilarity distance.

Antimicrobial activity of *Streptomyces* metabolite

- Effect of incubation period:

The time coarse for the production of antimicrobial agent for *S. thinghirensis* EGDA6S was studied on starch agar media. Result revealed that it gave a maximum antimicrobial effect against *B. subtilis, S. aureus*, *P. aeruginosa* and *C. albicans* at 7th day (Fig. 3). The lower activity at 2nd and 3rd days, and then started to increase afterwards. The antimicrobial effect almost became constant from the 5th day and slightly decreased after the 9th day of incubation period. Sujatha *et al*. (2005) showed that the streptomycete BT-408 gave a maximum activity at 4th day also, Narayana and Vijayalakshmi (2008) found that the antibiotic production by *Streptomyces albidoflavus* started after 3rd day and reached its maximum at 5th day

which represented as the stationary phase of incubation period. While Vijayakumar *et al*. (2012) reported that the 9th day was optimum for Streptomyces afghaniensis antimicrobial activity.

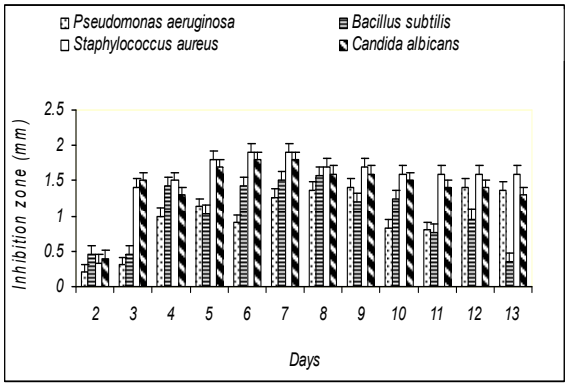

Fig. 3. Effect of incubation period on the antibacterial activity of *S. thinghirensis* EGDA6S metabolite against different bacterial strains.

- Effect of pH:

The effect of pH on antimicrobial activities for *S. thinghirensis* EGDA6S was studied at 7th day and 30°C on starch nitrate media. The antimicrobial activity gradually increased from pH 4 to pH 6, and then started to decline after pH8. Generally, the optimum pH for production maximum antimicrobial activity was being at pH 7 for *P. aeruginosa*, *B. subtilis* and *S. aureus* (Figure 4). Kumar *et al*. 2010 also isolated different *Streptomyces* strains from wasteland alkaline soil showed antimicrobial activity. Narayana and Vijayalakshmi (2008) founded that the optimum pH was 7 and highest pH is not favorable for antibiotics activity. The concentration of hydrogen ion plays a main role in the enzymes activity and secondary metabolites production (Guimaraes *et al*., 2004). Ripa et al. (2009) showed that the pH 8.0 was optimum for antibiotic production *Streptomyces* sp. This may be attributed to the presence of active enzymes for antimicrobial metabolites synthesis at pH 7-9; each *Stryptomyces* strain has an optimum, minimum and maximum pH at which it gave an optimum enzymes activity (Vijayakumar *et al* 2012).

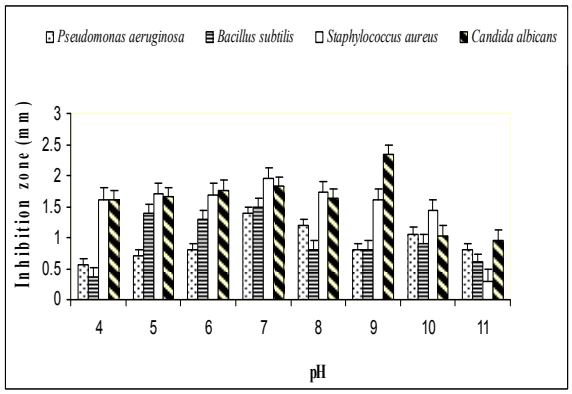

Fig. 4. Effect of pH on the antibacterial activity of *S. thinghirensis* EGDA6S metabolite against different bacterial strains.

- Effect of temperature:

The effect of different temperatures (20, 25, 30, 35, 40 and 45) was also studied on starch nitrate media at pH 7 for *S. thinghirensis* EGDA6S isolate as represented in Fig. 5. The maximum activity for microbial inhibition was 30°C against *P. aeruginosa*, *B. subtilis, S. aureus* and *C. albicans* respectively. This could be due to stable metabolizing enzymes, which enabled the production of the antimicrobial compounds at such temperature. Lower activity could be recorded at temperature 20, 25, 35 and 40°C and very low activity at 45°C. Vijayakumar *et al* (2012) found that the mesophilic *Streptomyces* isolated from Moderate soils produce an optimum antimicrobial production at 30°C. At high temperature microbial metabolism might be influenced (James *et al.*, 1991). Also Bhavana *et al*. (2014) have reported that optimum mycelial growth and antibiotic production by *Streptomyces carpaticus* occurred at pH 7.2 and at 30°C. On the other hand, Ripa *et al*. (2009) reported that isolated *Streptomyces* spp. produced high levels of antibiotic production as it cultured in medium and subsequently incubated at 39°C.

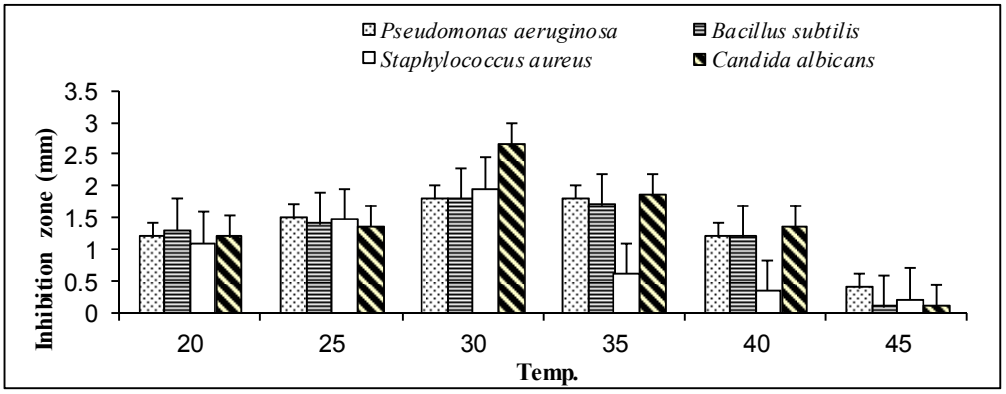

Fig. 5. Effect of temperature on the antibacterial activity of *S. thinghirensis* EGDA6S metabolite against different bacterial strains.

- Effect of different culturing media:

Eight different media were used for study the effect on the production on antimicrobial activity for *S. thinghirensis* isolate EGDA6S. This was performed at pH 7 and 30°C for 7 days. Results revealed that the oat meal medium was the best one for maximum antimicrobial activity followed by starch nitrate medium. Other tested media recorded low antimicrobial activity. The antimicrobial activity was totally inhibited by yeast extract malt extract medium (Fig. 6). Starch has been

found to be the best carbon source for antibiotic production (Gao *et al.,* 2009). Alaa-ElDin *et al*. (2015) reported that the maximum antibiotics production was on the starch casein medium *Streptomyces* sp. Reddy *et al*. (2011) stated that changes in the nature and types of nitrogen and carbon sources have been recorded to affect antibiotic productivity in *Streptomyces* (. Carbon sources affect directly on the secondary metabolites production and nitrogen sources is important precursors for antibiotic synthesis (D'mato and Pisano, 1975; Slininger and Shea-Wilbur, 1993; Vijayakuma *et al*., 2012).

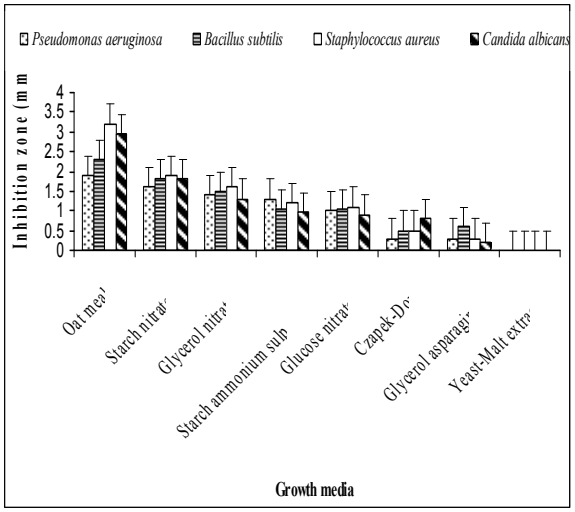

Fig. 6. Effect of different culturing media on the antibacterial activity of *S. thinghirensis* EGDA6S metabolite against different bacterial strains.

CONCLUSION

Based on both traditional and molecular methods, actinomycetes strain was identified as *Streptomyces thinghirensis* isolate EGDA6S. It exhibited maximum antimicrobial activity against *B. subtilis, S. aureus*, *P. aeruginosa* and *C. albicans* on oat meal and starch nitrate media after the 7th day at 30^{o}C and pH7. Further studies will be performed in order to establish an extraction, purification and characterization of its antimicrobial agent.

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تحديد وتوصيف وإنتاج مضادات الميكروبات المعززة لسلالة *Streptomyces thinghirensis*

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يهدف هذا البحث إلى عزل وتعريف وتوصيف الأكتينوميسيتات شكليا وكميائيا. ولقد تم تعريف السلالة المنتقاه بطرق التعريف التقليدية والجزيئية والتى أوضحت أن السلالة المختارة لها القدرة على إنتاج بعض الأصباغ الذائبة وبعض الإنزيمات كما أنها لها القدرة على مقاومة بعض المضادات الحيوية والحرارة والملوحة وأتضح ان السلالة المعزولة هي *Streptomyces thinghirensis*. وبدراسة الظروف المختلفة من درجات الحرارة والأوساط الغذائية والرقم الهيدروجينى وجد أن اليوم السابع , ودرجة حرارة٣٠ درجه مئوية, والرقم الهيدروجينى المتعادل (pH 7) والوسط الغذائي oatmeal يليه الوسط الغذائي starch nitrate هم الظروف المثلى لإنتاج المضاد الحيوي من السلالة المختارة.