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Production of Anti-Mycobacterial Agents by Egyptian *Streptomyces* Isolates

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Abstract: Seventy five streptomycete isolates were isolated from the soil at different localities of Egypt and assessed for their antibacterial activity against *Mycobacterium smegmatis* (ATCC 607). Results of this work showed that 8 % of the isolates (6 isolates) produced anti-mycobacterial metabolites. The most efficient isolate showing anti-mycobacterial activity was *Streptomyces* SM9 which was identified as *Streptomyces nigrifaciens*. In addition, the most suitable medium for production of anti-mycobacterial metabolites by the selected strain was studied. Fish meal extract was found to be the best, followed by oat meal extract, inorganic salts starch, starch nitrate and yeast-malt extract media.

Key words: *Mycobacterium smegmatis* · Antibacterial · Secondary metabolites

INTRODUCTION

Tuberculosis (TB) is an infectious deadly disease that kills about 3 million people per year worldwide [1]. It is estimated that by the year 2020, nearly 1 billion more people will be infected, 200 million people will become sick and 70 million will die from TB if control is not strengthened. Moreover, TB has also been recognized as one of the most frequent opportunistic infections in persons suffering from human immunodeficiency virus (HIV) in developing countries, particularly in Africa [2].

More than a dozen anti-mycobacterial drugs are currently available for treating tuberculosis and are indispensable for preventing progression of the disease, but uncontrolled usage is generating drug resistant strains [3]. The search for novel antibiotics has therefore become more urgent.

Actinomycetes produce about 2/3 of all known antibiotics of microbial origin. It was found that *Streptomyces* spp. are capable of producing approximately 60% of antibiotics developed for agricultural use [4]. Moreover, *Streptomyces* spp. provide a larger number and wider variety of new antibiotics than any other actinomycetes genera, suggesting that substantial numbers of *Streptomyces* species or strains with novel antibiotic productivity exist in nature [5]. Furthermore, over 6000 antibiotics are obtained by different species of *Streptomyces* and many of these compounds are commercially available as anti-infective (antibiotics, antifungal and antiparasitic), anticancer or immunosuppressant agents [6-9].

Recent studies have shown that certain antibiotics isolated from *Streptomyces* spp. have potent anti-mycobacterial activity. One example of such antibiotics is pamamycins which are active against *Mycobacterium tuberculosis*, *Mycobacterium bovis* and *Mycobacterium smegmatis* [10]. The present study was designed to screen different *Streptomyces* isolates from Egyptian soil for their anti-mycobacterial activity in an attempt to identify new agents for this deadly disease.

MATERIALS AND METHODS

Isolation of *Streptomyces* spp: Soil samples taken from 10-20 cm depth were collected from different locations in Egypt. Samples were air dried at room temperature for 7 days, passed through a 0.8 mm mesh sieve, mixed with $CaCO_3$ (10%) and incubated at 28±2°C for 7 days under saturation condition of humidity [11, 12]. Ten grams of pretreated soil were suspended in 100 ml sterile distilled water, shacked vigorously for 1 hour and then one ml of soil suspension was subsequently diluted with sterile distilled water up to 10^{-6} dilution. One ml of the finally diluted soil suspension was spread onto the surface of

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starch nitrate agar and incubated at 28±2°C for 7 days [13, 14]. The isolates were preliminary identified according to traditional morphology criteria, including characteristics of colonies on the plates and morphology of aerial and substrate mycelia [15]. *Streptomyces* colonies obtained after incubation were picked up and re-cultivated several times (by streaking technique) under the same previous conditions of isolation for purity. Sub-culturing was usually carried out every two months, using starch nitrate agar medium [16]. The purified *Streptomyces* isolates were identified up to the Genus level according to their cultural and morphological characteristics [17, 18].

Mycobacterial Strain and Growth Conditions: *Mycobacterium smegmatis* (ATCC 607) was purchased from the American Type Culture Collection. The strain was cultured on tryptic soy agar slants (Oxoid) and incubated at 37°C for 72 hours. The culture was mixed with a sufficient volume of tryptic soy broth (Oxoid) to reach a turbidity equivalent to that of McFarland's nephelometer No. 1 standard. To obtain the test inoculum, this suspension was further diluted 1:20 with the same culture medium immediately before use.

Antagonistic Activity of *Streptomyces* Isolates Against *M. smegmatis*: The anti-mycobacterial activities of different *Streptomyces* isolates were tested by the diffusion method [19]. The isolates were grown on starch nitrate agar for 7 days at 28°C. After growth, 6 mm in diameter of previous agar culture were transferred to the surface of tryptic soy agar plates, previously inoculated with *Mycobacterium smegmatis* (ATCC 607). The plates were then incubated at 37°C for 72 hours. The antagonistic activity was recorded by measuring the inhibition zone (mm) around *Streptomyces* agar discs.

Identification of *Streptomyces* Isolate SM9: *Streptomyces* isolate SM9 was completely identified, based on their cultural, morphological and physiological characteristics according to the standard methods adopted by Shirling and Gottlieb [20]. Also the keys proposed by Bergey's Manual [17] were consulted. In addition, the description of *Streptomyces* species of the International *Streptomyces* Project (ISP) introduced by Shirling and Gottlieb [21] were used.

Preparation of *S. nigrifaciens* Strain SM9 Inocula: Spores suspension of *S. nigrifaciens* strain SM9 was prepared by growing the organism for 15 days at 28±2°C on starch nitrate agar medium [13].The spores were resuspended in 50 ml of sterile saline by scraping the whole surface of agar plates and the spore suspension was adjusted to a concentration of $16x10^9$ spore ml^{-1}.

Detection of the Most Suitable Medium for Anti-Mycobacterial Activity Produced by *S. nigrifaciens* Strain SM9: In this test, 50 ml of oat meal, fish meal, yeast- malt extract, inorganic salts starch and starch nitrate medium [20] were transferred separately into 250 ml Erlenmeyer flask, sterilized, inoculated with 2 ml of previous spores suspension and incubated at 28 ±2°C for 5 days on rotary shaker incubator at 200 rpm [24, 25]. After growth, 0.3 ml of cultural filtrate from each previous culture was separately transferred into 6 mm wells pored in tryptic soy agar plates, previously inoculated with *Mycobacterium smegmatis* (ATCC 607). The plates were incubated at 37°C for 72 h and examined. The antagonistic activity was recorded by measuring the inhibition zone (mm) around wells.

RESULTS AND DISCUSSION

Seventy five streptomycete isolates were isolated from soil samples obtained from different localities of Egypt. Results revealed that they were belonging to the Genus *Streptomyces*. *Streptomyces* isolates were divided into brown, gray, red and white groups according to their color of aerial mycelium [26]. Data in table 1 showed that, 5 isolates were brownish, 5 whitish, 2 reddish and 64 grayish streptomyces. Out of 64 only 6 grayish *Streptomyces* isolates were anti-mycobacterial producers representing 8 % (6 isolates) of total isolates. Thus, the grayish group of *Streptomyces* isolates was the most distributed isolates in Egyptian's soil compared to other *Strepromyces* groups. Furthermore, the most efficient *Streptomyces* isolates possessing anti-mycobacterial activity was *Streptomyces* SM9 which was used in the subsequent studies.

The data in table 2 and figure 1 indicated that the soil isolate SM9 appeared to resemble *Streptomyces nigrifaciens* and *Streptomyces flavovirens* [17, 21]. However, the later species couldn't utilize inositol or salicin. Furthermore, the various morphological, cultural and physiological characteristics of the experimental isolate appeared to be similar to those of *Streptomyces nigrifaciens* with slight differences in utilization of salicin. Therefore, the experimental isolate SM9 was identified as a strain of *Streptomyces nigrifaciens*.

Table 1: Anti-mycobacterial activity of *Streptomyces* isolates

	Streptomyces isolates		Anti-mycobacterial activity		Total *Streptomyces* isolates showing anti-mycobacterial activity	
Streptomyces color	Count	%	No	Inhibition zones (mm)*	Total	%
Brown	5	6.7	-	-	-	-
Grey	64	85	MS3	19	6	9.38
			MS9	28		
			MS13	18		
			MS17	14		
			MS10	15		
			MS16	15		
Red	2	2.6	-	-	-	-
White	5	6.7	-	-	-	-
Total	75	100	6	-	6	8

* Inhibition zones including *Streptomyces* agar discs (6 mm) diameter

Table 2: Cultural, morphological and physiological characteristics of *Streptomyces* isolate SM9 as compared with those of similar species reported in different identification keys

Characteristics	SM9	S. flavovirens	S. nigrifaciens
(1) Cultural characteristics			
Color of aerial mycelium	Grey	Grey	Grey
Color of substrate mycelium	Colorless	Colorless	Colorless
Diffusible pigments	Colorless	Colorless	Colorless
(2) Morphological characteristics			
Spore surface ornamentation	Smooth	Smooth	Smooth
spore chain morphology	Flexuous	Flexuous	Flexuous
(3) Physiological characteristics			
Melanoid pigment produced	-	-	-
Growth on Czapek`s medium	+	+	+
Sodium chloride tolerance	≤10	≤10	≤10
Sensitivity to streptomycin	-	-	-
Antimicrobial activity	Antibacterial	Antibacterial	Antibacterial
(4) Utilization of different carbon sources			
No Carbon	-	-	-
D-Glucose	+	+	+
D-Xylose	±	+	+
L-Arabinose	+	+	+
L-Rhamnose	+	+	+
D-Fructose	+	+	+
Galactose	+	+	+
Raffinose	+	+	+
D-Mannitol	+	+	+
Inositol	+	-	+
Salicin	+	-	-
Sucrose	-	-	ND

ND: Not determined. +: positive; - : negative; ±: doubtful.

Fig. 1: Transmission electron micrograph of spore morphology of *Streptomyces* isolate SM9 (x 10 000)

Data in figure 2 showed that, *Streptomyces* strain MS9 could grow and produce the anti-mycobacterial metabolites in different media compositions with different C/N ratios and that may be referred to the ability of the strain to produce anti-mycobacterial agents using different raw materials and different industrial wastes. In addition, the most suitable medium for production of anti-mycobacterial metabolites by *Streptomyces* strain MS9 after 5 days of incubation at 28°C on rotary shaker 200rpm was fish meal extract, followed by oat meal extract, inorganic salts starch, starch nitrate and yeast-malt extract medium which gave 36, 33, 26, 22 and 17mm inhibition

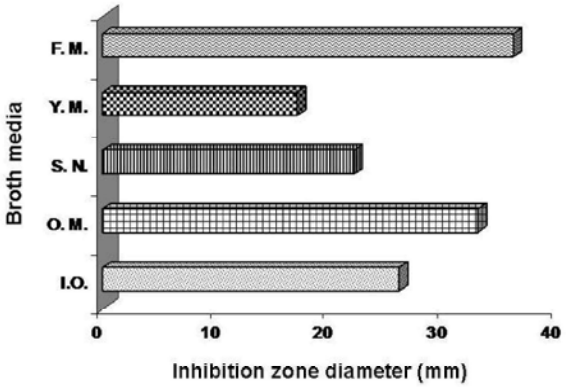

Fig. 2: Anti-mycobacterial activiy of *Streptomyces* isolate SM9 grown in:.
Inorganic salts starch (I.O.); fish meal extract (F.M.); oat meal extract (O.M.); starch nitrate (S.N.) and yeast- malt extract medium (Y.M.)

zone, respectively (Figure 2). The obtained data in this study are in agreement with a recent finding by Al-Zahrani [27], who reported that the ability of *Streptomyces* isolate to produce antibiotics is not consistent, but could be increased or decreased remarkably under different cultural compositions.

The local *Streptomyces* SM9 strain was isolated and identified as *Streptomyces nigrifaciens* from Egyptian soil and exhibited large anti-mycobacterial activity against *Mycobacterium smegmatis* ATCC 607. Fish meal extract was found to be the best suitable medium for production of anti-mycobacterial metabolites.

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