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Isolation of *Streptomyces* sp. producing antifungal agent

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ABSTRACT

Candida albicans is the most common cause of invasive fungal infection in immunocompromised patients causing high morbidity and mortality rate. Forty *Streptomyces* isolates were isolated from various soil samples in Egypt. These isolates screened for their antifungal activity against standard fungal strains. The most potent *Streptomyces* isolate coded 4 was evaluated against clinical *Candida* isolates. *Streptomyces* sp.4 was subjected for morphological, physiological and biochemical characterization.

Key words: *Streptomyces*, antifungal, *Candida albicans.*

Introduction

Among the genera of actinomycetes, the genus *Streptomyces* is represented in nature by the largest number of species and varieties, which differ greatly in their morphology, physiology, and biochemical activities. Interestingly, the majority of the antibiotic and antifungal-producing actinomycetes are found among these species, e.g. nystatin, amphotericin B, natamycin produced by *S. noursei*, *S. nodosus* and *S. natalensis* respectively (Raja and Prabakarana, 2011) which leads to a growing economic importance for this group of organisms.

Candida albicans is an important nosocomial pathogen infecting immunocompromised patients and causing a high morbidity and mortality rate. This is related to the increased development of fungal resistance (Kanafani and Perfect, 2008), toxicity of existing drugs, significant drug interactions and insufficient bioavailability of the conventional antifungals.

The present work was carried out as a screening program for antifungal agents produced by Genus *Streptomyces*. *Streptomyces* sp. 4 was isolated from rhizoshphere of citrus trees in Sidi Kerir (Alexandria) and showed potent activity against fungi.

Materials and methods

Isolation of Streptomyces

Streptomyces were isolated from different locations (Egypt) by a soil dilution-plate method using the starch-casein medium of Kuster and Williams (1964).

Isolation of clinical Candida

These were collected from biological fluids of patients including urine, sputum, pus, vaginal and pleural fluids (Mervat laboratory, Egypt). Specimens were directly inoculated on Saboraud Dextrose Agar (SDA) and all isolates were confirmed using CHROM agar *Candida* medium (Odds and Bernarets, 1994).

Standard test strains

Filamentous fungi (*Aspergillus niger* NRRL 363); unicellular fungi (*C. albicans* ATCC 10231).

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Screening of antifungal activity of the isolates

Primary screening

It was carried out against *C. albicans* (ATCC 10231) and *Aspergillus niger* (NRRL 363) using plug agar method (Pridham, *et al*., 1956). The results were reported by the presence or absence of inhibition zone.

Secondary screening

It was done for the active microbial isolates obtained from the primary screening against *C. albicans* (ATCC 10231) using agar well diffusion method (Kavanagh, 1972).

Evaluation of the antifungal activity of active strain on clinical Candida isolates

Colonies were taken from CHROM agar *Candida* medium and was adjusted to 0.5 McFarland Standard in saline solution. This *Candida* suspension was inoculated on Mueller-Hinton agar plates supplemented with 2 % dextrose (glucose) and 0.5 µg/ml methylene blue, using a sterile cotton-swab, removing excess fluid by pressing against the tube. Inoculate the agar by streaking while rotating the plate, swabbing over the entire agar surface.

The culture filtrates of active strain (1 mg of crude extract/disk) was tested against clinical *Candida* isolates following National Committee for Clinical Laboratory Standards (NCCLS, 2004). Positive and negative control antibiotic discs were involved. Positive control antifungal discs e.g. (ketoconazole 10 µg, fluconazloe 25 µg, amphoteracin B 20 µg and nystatin 100 units) and negative control non-antifungal discs e.g. (metronidazole 5 µg, polymyxin B 300 units and fusidic acid) (Oxoid) were also evaluated. Reading of the test should be made as soon as possible after not more than 18-24 hours.

Characterization of Streptomyces strain

Morphological characterization

The *Streptomyces* strain was inoculated on starch casein (SC) agar medium (Shirling and Gottlieb, 1966), incubated at 30^{o}C for 5 days and the macro-morphology of strains including colors of mature sporulating aerial mycelium and substrate mycelium was monitored. The micro-morphology of the strains including the form of the sporophores was examined under light microscope. The morphology of the spores and the spore chains was investigated by Scanning Electron Microscopy (SEM) (JEOL, JSM-5910, Japan). A plug of agar containing the culture was removed and fixed in glutaraldehyde vapor (2% v/v) at room temperature for 3 hours. Then, samples were dehydrated by exposure to increasing ethanol concentrations (50, 60, 70, 80 and 95%, 15 min each; twice with 100% ethanol, 30 min/time). Ethanol was substituted with acetone and subjected to critical-point dryer (CPD7510, Polaron, Rang).The samples were sputter coated with gold in a SPI-ModuleTM Sputter Coater (SPI Supplies, Division of Structure Probe Inc., USA).

Physiological characterization

A physiological characterization of *Streptomyces* that include melanin production and carbon sources utilization according to the ISP recommendation (Shirling and Gottlieb,1966); seven sugars were used as a carbon sources: D- glucose, D-sucrose, D-sorbitol, D-mannitol, L-arabinose, Meso-inositol, L-rhamnose, D-melibiose and D-amygdalin (Sigma).

Biochemical characterization

The active isolate was characterized by different biochemical tests as per Bergey's Manual of Systematic Bacteriology (Williams *et al*., 1989) which included indole production test, Voges Proskauer (acetoin) test, citrate test, hydrogen sulfide production, the ability to hydrolyze starch, gelatin, urea and casein.

Effect of different production media on antifungal production of active Streptomyces isolate

Inoculum preparation

A forty five ml of broth medium was prepared in 250 ml Erlenmeyer flasks, which were independently inoculated with 10 % (v/v) of an overnight preculture of the active isolate adjust the optical density to 1.0 A° at 600 nm. Then they were incubated in a reciprocating incubator shaker (New Brunswick Scientifics Co., New Brunswick, NJ, USA) at 30°C and 180 rpm. The antifungal activity was monitored using *C. albicans* ATCC 10231 as a test strain.

Production media

Seven different media were used to determine the optimal media for the growth and antimicrobial production, the media that were used the following compositions (g/l): ***Starch casein medium*** **(**Shirling and Gottlieb, 1966): soluble starch, 10.0; casein, 1.0; $CaCO_3.2H_2O$; K_2HPO_4, 0.5; the pH was adjusted to 7. ***Bennett's medium*** (Seong *et al*.; 2001): D-glucose, 10.0; beef extract, 1.0; yeast extract, 1.0; N-Z amine types A, 2.0 and tap water; the pH was adjusted to 7. ***Soybean medium*** (Nayera *et al*., 2012): dextrin, 15.0; soybean, 30.0; $CaCO_3.2H_2O$, 10.0; $MgSO_4.7H_2O$, 1.0 and tap water; the pH was adjusted to 7. ***C medium*** (Gastaldo and

Marinelli, 2003): D-glucose, 10.0; soluble starch, 35.0; casein hydrolysate, 5.0; yeast extract, 8.0; meat extract, 3.5; soybean meal, 3.5; $CaCO_3.2H_2O$, 2.0 and tap water; the pH was adjusted to 7. ***ISP-2 medium*** (Pridham *et al*.; 1957): glucose, 4.0; malt extract, 10.0; yeast extract, 4.0 and tap water; the pH was adjusted to 7. ***Modified C medium*** (unpublished data): soluble starch, 45.0; soybean meal, 3.5; $CaCO_3.2H_2O$, 2.0 and tap water; the pH was adjusted to 7. ***Starch nitrate medium*** (Küster and Williams,1964) starch nitrate medium of the following composition (g/l): Starch 20; KNO3 1; K2HPO4 0.5; MgSO47H2O 0.5; NaCl 0.5; Fe SO4 0.01.

Results and Discussion

Isolation and screening of the most potent strains producing antifungal agent

Forty *Streptomyces* were isolated from different soil samples. After primary and secondary screening (unpublished data), a potent *Streptomyces* isolate coded 4 that showed antifungal activity against *C. albicans* ATCC 10231 and *A. niger* NRRL 363 and was isolated from rhizosphere of citrus trees in SidiKerir (Alexandria).

Evaluation of the antifungal activity of Streptomyces sp. 4 against clinical Candida isolates

A total of fifteen *Candida* isolates were collected from various clinical samples (Table: 1). *Streptomyces* sp. 4 showed activity against 40% of the clinical *Candida* isolates. On the other hand, the clinical *Candida* isolates were sensitive to ketoconazole, amphotericin B, nystatin and fluconazole with 100, 60, 47 and 40% respectively.

Table 1: Antifungal activity by disk diffusion method of crude extracts of *Streptomyces* sp. 4 and reference antibiotics on clinical *Candida* isolates.

Candida **isolate no.**	**Source**	**Antifungal discs**				**Positive Control**			**Isolate 4**
		KET	**FCA**	**AB**	**NS**	**MTZ**	**PB**	**FD**	
Cand.01	urine	S	R	R	I	R	R	R	S
Cand.02	urine	S	I	R	S	R	R	R	S
Cand.03	urine	S	R	R	I	I	R	R	S
Cand.05	urine	S	R	S	I	R	R	R	R
Cand.06	urine	S	R	S	I	R	R	R	R
Cand.04	sputum	S	R	I	S	R	I	R	S
Cand.07	sputum	S	S	I	R	R	R	R	R
Cand.09	sputum	S	S	I	R	R	R	R	R
Cand.11	sputum	S	S	R	R	R	R	R	R
Cand.12	sputum	S	S	R	R	R	R	R	R
Cand.13	sputum	S	S	R	R	R	R	R	S
Cand.08	pus	S	R	S	R	R	R	R	R
Cand.10	vagina	S	R	S	I	R	R	R	S
Cand.14	vagina	S	R	S	R	R	R	R	R
Cand.15	pleura	S	R	S	R	R	I	R	R

Abbreviations: R, Resistant;I, Intermediate; S,Susceptible; KET, Ketoconazole; FCA, Fluconazloe; AB, Amphoteracin B; PB, Polymxin B; NS, Nystatin; MTZ, Metronidazole, FD: Fucidic acid.

Identification of Streptomyces strain

In general, the taxonomic classification and identification of Streptomycetes is based on morphological, biochemical and physiological characteristics. With respect to morphological features, *Streptomyces* sp. 4 had grey color of the aerial mycelium. The substrate mycelium was not pigmented and did not produce diffusible pigments in the surrounding medium. Microscopically, it had hook to open spiral spore chain shape. The Scanning Electron Microscope (SEM) images of spores and spore chain morphology are shown in figure 1.

The physiological test of *Streptomyces* sp. 4 revealed that the isolate was able to produce melanin. Sucrose, mannitol, rhamnose and inositol were utilized for growth by this isolate. Regarding the biochemical tests, *Streptomyces* sp. 4 had the capabilities to utilize citrate and produced acetoin (Voges- Proskaur) and produced different enzymes such as amylase, gelatinase and urease. The morphological, physiological and biochemical characteristics of the *Streptomyces* sp. 4 is presented in table 2.

Table 2: Morphological, physiological and biochemical characteristics of *Streptomyces* sp.4

Morphological characteristics	
Spore morphology	hook shaped- open spiral spore chain
Aerial mycelium presence	Present
Color of aerial mycelium	Grey
Color of substrate mycelium	un-pigmented
Diffusible pigments	-
Physiological characteristics	
Melanin production	-
Carbon sources fermentation/oxidation:	
Glucose	-
Mannitol	+
Inositol	+
Sorbitol	-
Rhamnose	+
Sucrose	+
Melibiose	-
Amygdalin	-
Arabinose	-
Biochemical characteristics	
Indole production	-
Voges Proskauer Test (acetoin)	+
Citrate test	+
Hydrogen sulfide production	-
Hydrolysis of starch	+
Hydrolysis of gelatin	+
Hydrolysis of urea	+
Hydrolysis of casein	-

The isolates' metabolic activities were tested. A plus sign (+) indicates that the isolate was positive for the test; a negative sign (-) indicates a negative reaction for the test.

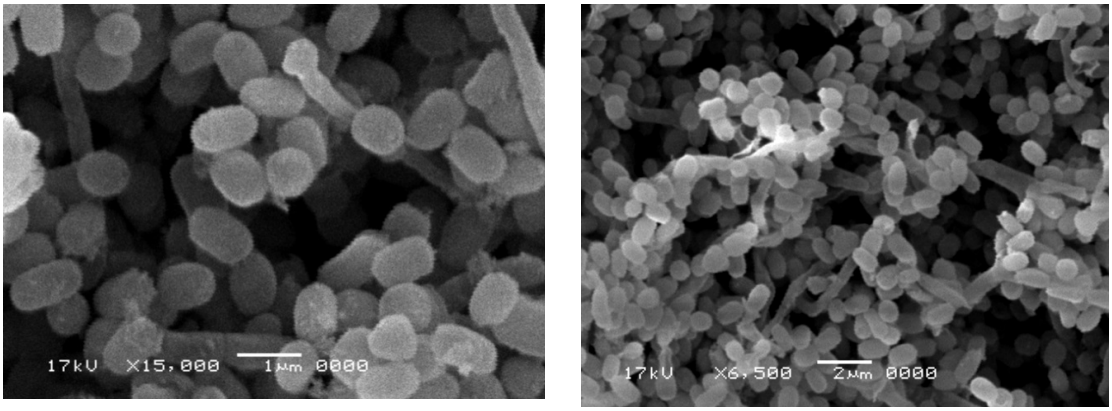

Fig. 1: Scanning electron microscope (SEM) of Stretomyces sp.4 showing hook- open spiral spore chains and spiny spore surface

***Effect of different production media on antifungal production of Streptomyces* sp.4**

The aim of this experiment was to find out the most favorable medium for the antifungal biosynthesis of *Streptomyces* sp.4 in production media. The results in table (3) showed that, the maximum yield of the antifungal secondary metabolites was produced in fermentation medium of starch casein (SC) where the diameter of inhibition zone reached 20 mm after 144 h incubation time. followed by bannet's medium whereas, the inhibition zone reached 18 mm after 144 h. On the other hand, no inhibition zone was detected against *C. albicans* upon using modified C medium.

Table 3: Effect of different media on the antifungal *produced by Streptomyces* sp.4

Time of Incubation/h / Media	*Streptomyces* sp.4					
	48 h		96 h		144 h	
	pH	I.Z. (mm)	pH	I.Z. (mm)	pH	I. Z. (mm)
1. SC	7.2	0	7.4	14	7.5	20
2. B	7.5	0	8	11	9.0	18
3. SB	7.5	0	8.2	0	8.7	12
4. C	7.5	0	7.9	11	9.0	15
5. ISP-2	7.5	0	7.0	12	6.0	18
6. Modified C	6.5	0	6.0	0	5.0	0
7. SN	7.5	0	7.7	12	8.0	16

Abbreviation: SC: Starch casein medium, B: Bennett's medium, SB: Soybean medium, C: C medium, ISP-2: International Streptomyces Project-2 medium, Modified C: Modified C medium, SN: Starch nitrate medium, IZ: Inhibition zone.

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