Available online freely at www.isisn.org

Bioscience Research

Print ISSN: 1811-9506 Online ISSN: 2218-3973

Journal by Innovative Scientific Information & Services Network

RESEARCH ARTICLE BIOSCIENCE RESEARCH, 2020 17(2): 590-605. **OPEN ACCESS**

Production and characterization of anti-candida compound produced by actinomycete isolate

Shimaa Maher Hawila, Mohamed Bedaiwy, Samia Abas Saad Shabana and Yehia A.-G. Mahmoud*

Botany and Microbiology Department, Faculty of Science, Tanta University, Tanta 31527, **Egypt**

***Correspondence:** Yehia.mahmoud@science.tanta.edu.eg **Received 16-03-2020, Revised: 25-04-2020, Accepted: 30-04-2020 e-Published: 02-05-2020**

In this study, forty actinomycete isolates were isolated on starch-nitrate agar medium from soil, seashore soil, sand, fresh water and marine water. The isolates were screened for their antifungal activity against (*Candida. lusitaniae, Candida. tropicalis, Candida parapsilosis, Candida. kefyr, Tricosporon mucoides, Candida albicans, Candida glabrata, Candida krusie, and Candida dublinesis*) using well diffusion method. The promising results were obtained by isolate NO.7. Therefore, it was selected for further work. Preliminary identification of the selected isolate by scanning electron microscopy in addition to morphological, physiological, biochemical characteristics indicated that the isolate belonging to the genus Streptomyces. 16S rRNA molecular identification revealed that the isolate is identified as *Streptomyces rochei*. Culture conditions of the selected isolate were optimized for maximizing growth and anti-candida active compound production. The results under the optimized conditions of incubation period, pH, temperature, carbon and nitrogen source recorded the maximum anti-candida activity against *C. kruseii* with inhibition zone 29.6 mm followed by *C. lusitaniae* with inhibition zone 29 mm. Bioactive compounds produced by the selected isolate were extracted using ethyl acetate solvent and characterized using UV spectrum, Fourier transformed infrared spectrophotometer (FT-IR) and gas chromatography-mass spectrophotometer (GC-MS). MIC test was carried out and found to be at concentration 6.25 mg/ml against *C. albicans*. *In vivo* study showed that the treatment of mics' skin wounds superficially infected with MDR strains *C. albicans* with the extract of *Streptomyces rochei* culture was led to 100% healing at 17th day whereas with miconaz cream was 50-85 % at 17th day and the results was confirmed by Histopathological examination.

Keywords: Actinomycetes, Candida, Antimicrobial compounds, Miconazole

INTRODUCTION

Fungal infections have dramatically increased in the past two decades as a result of improved diagnostics, high frequency of catheterization, instrumentation and an increasing number of immunosuppressed patients, particularly invasive fungal infections are showing extremely high mortality rate (Khan et al. 2010).

Compared to antibacterial agents, the number of antifungal compounds approved for human use is quite limited, a circumstance explained by the high toxicity of these substances to the hosts. However, it comes as no surprise that the use of antifungal drugs has led to resistant fungi populations.

Resistant strains find their way to hosts in three colonization and infection scenarios: (i) exposure to an initially susceptible strain that subsequently mutates and becomes resistant; (ii) exposure to a number of strains of which one is resistant and eventually is the only one to thrive, resisting the presence of antifungal drugs in the

host organism; and (iii) exposure to an inherently resistant strain (Spadari, et al.2013).

Actinomycetes are biotechnologically valuable bacteria which are well exploited for secondary metabolites (Muthu et al.2013). Screening, isolation and characterization of promising strains of actinomycetes producing potential secondary metabolites have been a major area of research by many research groups worldwide for many years (Laidi et al. 2006).

Actinomycetes especially, Streptomyces species are widely recognized as industrially important microorganisms as they are a rich source of several useful bioactive natural products with potential applications (Lakshmipathy and Kannabiran 2009). Of all known drugs, 70% have been isolated from Actinomycetes bacteria and out of which 75% and 60% are used in medicine and agriculture respectively (Kumar et al. 2012).

MATERIALS AND METHODS

In this study, actinomycetes used were isolated from water, soil and seashore soils samples collected from Alexandria, Hurghada, Seiwa, Sohage, Tanta, Marsa Matroh, El santa, Kafer Elzyate.

Isolation of actinomycetes from soil and sand

Samples were collected from different depths 0-5 cm in different localities in clean plastic bags. The samples transferred to the laboratory, air-dried and heated to 50°C for 15 min. One gram of each sample was stirred in 100 ml distilled water for 5 min in 250 ml Erlenmeyer flask and the suspension was allowed to stand for 30 min. Serial dilutions were prepared and 0.1 ml of each dilution was spread on the surface of starch-nitrate agar medium. The plates were incubated at 28°C for 7 days. The developed colonies were purified by subculture on fresh starch-nitrate agar plates.

Isolation of actinomycetes from water

Surface samples from shallow water have been collected in sterilized bottles or submerged just below the surface, generally shallow water sediments have been scooped by hand in sterilized container. About 200 ml of each water sample was placed in water bath at 40°C for one h, and then filtrated through sterilized membrane filter (Millipore 0.45 mm). The membrane filter was placed in about 10 ml sterilized saline solution and shaked well (3-5 min) before making serial dilution and 100µl was spread over starch nitrate medium for isolation of actinomycetes (Aly, 1997).

Preliminary Screening Of the actinomycetes isolates for their anti-candida activities

One ml of spore suspension of each actinomycete isolates was separately cultured in 250 Erlenmeyer flasks, each containing 50 ml of autoclaved starch-nitrate liquid medium. Three replicates were used. After incubation of culture flasks on a rotary shaker at 30°C for 7 days at 120 rpm, the cultures of each actinomycete isolates were centrifuged at 3000 rpm for 30 min and the supernatant was collected for testing its antimicrobial activity. 72hrs broth cultures of fungal strains (*Candida. lusitaniae, Candida. tropicalis, Candida parapsilosis, Candida. kefyr, Tricosporon mucoides, Candida albicans, Candida glabrata, Candida krusie, and Candida dublinesis*) were swabbed individually into freshly prepared sabrouad's dextrose agar (SDA) plates. Then the wells were made (about 5mm in diameter) in the inoculated plates using corkborer and each well was loaded with 100 µl of cell free culture supernatant. The plates containing fungal strains were incubated at room temperature for 4-5 days. After incubation, the zone of inhibition was measured and expressed as millimeter in diameter (Mohanraj et al.2011).

Characterization of the selected actinomycete isolate

Morphological identification

Different media (Starch-nitrate agar medium; Glucose-peptone agar medium; Inorganic salt-starch agar medium; Glucose-asparagine agar medium; Oatmeal agar medium; Yeast extract-malt extract agar medium and Nutrient agar) were prepared, then each medium was dispensed into sterilized plates and after solidification the plates were inoculated with the selected isolate. Incubation was carried out at 28°C for 7 days.

Scanning electron microscope

This was determined using a coverslip from the examined cultures of the selected isolate. The coverslip was cut with a glass file and a suitable fragment of growth was mounted into a specimen stub, coated with gold-palladium under vacuum, and examined at magnification of 10.000X (using scanning electron microscope. Spore surface ornamentation was described as smooth, warty, spiny, hairy or rugose according to categories (Williams and Davies, 1967, Dietz and Mathews, 1971).

16S rRNA Approach

To isolate genomic DNA from sample, a small sample amount (2–3 mm) from an isolated colony by using a 1 µL loop or the straight end of a 1 µL loop, the cells were suspended in 100 µL of PrepMan™ Ultra Sample Preparation Reagent in a 2-mL screw-cap microcentrifuge tube or any other microcentrifuge tube that can be tightly closed, then add PrepMan™ Ultra Sample Preparation Reagent, then vortex the sample for 10 to 30 seconds. Heat the sample for 10 minutes at 100°C in a heat block, then cool the sample to room temperature for 2 minutes, and then centrifuge the sample for 2 minutes in a microcentrifuge at maximum speed. Transfer 50 µL of the supernatant into a new microcentrifuge tube.

To dilute genomic DNA for PCR, pipet 495 µL of nuclease-free water into a 1.5-mL microcentrifuge tube, then add 5 µL of the PrepMan™ Ultra supernatant to obtain a 1:100 dilution. To perform the amplification run, purify PCR products for cycle sequencing by removing unused dNTPs and primers from each PCR product with ExoSAP-IT™. Perform the cycle sequencing run then Purify extension products. Configure the instrument for electrophoresis then prepare samples and perform electrophoresis. When the run is complete, review the data using the MicroSEQ™ ID Analysis Software. All the steps were performed at Colors Medical Labs in Cairo, Egypt.

Optimization of culture conditions of the selected actinomycete isolate for its anti-candida activities against the tested pathogenic Candida species

Factors like different carbon, nitrogen, pH, temperature and incubation period affecting the growth and the production of the antifungal materials were optimized by using 250 Erlenmeyer flasks, each containing 50 ml of starch-nitrate liquid medium, after autoclaving, each flask was inoculated with 100µl (10^6spore/ml) of spore suspensions of the selected actinomycete isolate. Each of the carbon sources like glucose, fructose, maltose, lactose, starch, glycerol, sucrose and arabinose was added at 2 % (w/v) concentration separately to the production media. Potassium nitrate, calcium nitrate, tryptone, sodium nitrate, peptone and ammonium sulfate at 0.2 % (w/v) concentration were supplemented to the production media as different nitrogen sources. The influence of various physiological conditions on the growth and the antifungal activity was evaluated by maintaining the production media at different temperature ranges (25, 30, 35 and 40°C), pH (5.0, 5.5, 6.0, 6.5, 7.0 and 8.0) and incubation period (48, 72, 96 and 120 h). After incubation, each culture was centrifuged at 3000 rpm for 30 min; the supernatant was taken to evaluate the anti-candida activity of the selected isolate against the tested pathogenic candida separately by using well diffusion agar method. The biomass of the selected isolate was transferred to a pre-weighed dry filter paper using a clean spatula and then placed in an oven at 50°C overnight to reach a constant weight. Mycelial dry weight was determined and expressed as (g/50 ml) (Singh et al. 2014).

Extraction of the antifungal materials produced by the selected isolate

The fermentation broth of the selected isolate was prepared and centrifuged at 3000 g for 20 min at 4°C. The supernatant was filtered, and the filtrate was subjected to solvent extraction to recover anti-candida metabolites. Organic solvents: chloroform, petroleum ether, ethyl acetate, acetone and diethyl ether were used for the extraction of the anti-candida metabolites from the selected isolate. The different solvents were added separately to the filtrate of the selected isolate in a ratio of 1:1 (v / v) and were shaken vigorously for 1 h to complete extraction. Ethyl acetate was standardized as the best solvent to extract the antibiotic; the organic ethyl acetate phase was collected and concentrated by evaporation to near dryness under reduced pressure by using a rotary evaporator (El-Nagar et al.2017).

Chemical characterization of the isolated antifungal materials from the selected isolates

UV spectrum of the produced antifungal extract

The UV spectra of the extracted materials were determined using quartz cuvette containing the solution of the tested material of the selected isolate. The spectra of each tested material were achieved using UV-visible, SHIMADZU-UV 220 spectrophotometer at wavelengths ranged from 190 to 550 nm (Dinya and Sztracicskia, 1986).

The infra-red spectra (IR)

The infra-red spectra of the ethyl acetate extract of each of the selected isolate was measured (as Potassium bromide discs) in the range of 400-4000 cm^{-1} on Fourier-transform infrared (FTIR) spectrophotometer (Shimadzu FTIR-8400 S, Kyoto, Japan). The important IR bands, such as (C-N), (O-H), (CH), (C=C). (NH),

(CO) and (CH) symmetric and asymmetric stretching, and stretching frequencies were studied to determine the presence of functional groups in the ethyl acetate crude extracts of the selected isolate (El-Naggar et al. 2017).

GC-MS Analysis

Ethyl acetate extract of the selected isolate was analyzed by GC-MS method sing the method described by Rana and Salam, (2014). GC-MS technique was performed by using GC Shimadzu QP2010 system and gas chromatograph interfaced to a Mass spectrometer (GC-MS) equipped with Elite-1 fused silica capillary column. Helium gas (99.99%) was used as the carrier gas at a constant flow rate of 1.51ml/min and an injection volume of 2µl was employed (split ratio: 20). Injector temperature was 200°C. The oven temperature was programmed from 70°C isothermal for 2 min, with an increase of 300°C for 10min. Mass spectra were taken at 70eV; a scan interval of 0.5 seconds with scan range of 40-1000m/z. Total GC running time was 35 min

Determination of the MIC of the antimicrobial materials produced by the selected actinomycetes isolates NO.7

The minimal inhibitory concentrations (MIC) of the antimicrobial material extracted from isolate NO. 7 were determined by agar well diffusion method according to Zamanian et al. (2005). Different concentrations of the ethyl acetate crude extracts produced by the selected isolates NO. 7 were prepared. Sterilized and solidifying Sabourad medium plates were seeded with 1ml of 10^7 CFUs/ml suspension of *C. albicans*. 100µl of each concentration of each concentration were transferred separately into wells in the tested plates. Then plates were incubated at 37°C for 24 hrs. The lowest concentration of the tested extract inhibited the growth of was considered as the MIC value. *C. albicans* strain used in the test isolated from skin infection and was recorded to be resistant to Fluconazole, Itraconazole, Metronidazole and Nystatin

In vivo effect of the active antimicrobial materials of the identified actinomycetes against infection of *C. albicans*

This experiment was carried out using the method described by (Nessma and Ali, 2016). Six groups of health albino mice (each contain four mice) were used for *in vivo* application of the antimicrobial materials produced by the selected isolates NO. 7. The mice were housed at room temperature (7days) in the animal unit of Zoology Department, Faculty of Science, Tanta University, Tanta, Egypt. The selected MDR strains *C. albicans* were used to cause superficial infection of the experimental animal models. The infected area covered with plastic film using leucoplast tape until a definite lesion was appeared. Some scales were collected from the infected mice's skin lesions and cultured on Sabourad agar media for reisolation of *C. albicans* colonies to assure the presence of infection caused by the tested pathogens. Group 1 of mice (each weight of 30 g) served as negative control in which mice were not burnt, did not get infected or receive any treatment. Group 2 served as positive control in which mice were burnt, infected with suspensions of *C. albicans*, but did not receive any treatment. Group 3 (mice were burnt, infected with suspensions of *C. albicans* and treated with extract of actinomycete isolate no.7). Group 4 in which mice were burnt, infected suspensions of *C. albicans* and treated with miconaz (miconazole mm)

RESULTS

Isolation of actinomycetes and Screening Of the isolates for their anti-candida activities

Forty actinomycete isolates were collected from cultivated soils, sand (sea shore), rhizosphere, fresh and marine waters of different locations (Alexandria, Hurghadra, Marsa Matroh, Sewa, Sohage, Tanta, Kafer Elzyat and Elsanta). Actinomycete isolates were identified according to their different colony morphology, colony color variations and the color of aerial and substrate mycelium on starch-nitrate medium based on Bergey's Manual of Systematic Bacteriology volume 5 of the second edition. The results listed in Table 1 showed that out of the 9 tested pathogenic candida species, 8 species were sensitive and showed zones of inhibition by the supernatant of the most potent actinomycetes isolate. Among the tested pathogenic candida species *C. albicans* and *C. parapsilosis* showed the highest sensitivity recorded the highest diameters of inhibition zones which were 18 and 17mm respectively. (Table (1)

Table.1. Screening of actinomycete isolate for its antifungal activity (Mean)

Fungal pathogens	*C. lusitaniae*	*C. tropicalis*	*C. parapsilosis*	*C. kefyr*	*C. galabrata*	*Tricosporon* sp.	*C. dublinesis*	*C. albicans*	*C. kruseii*
Inhibition zone(mm)	15.5	15.5	17	12	11	15.5	12	18	0.0

Table 2. Isolate no.7 culture characteristics on different growth media.

Media	Growth	Colour of aerial mycelium	Colour of substrate mycelium	Colour of soluble pigment
Starch- nitrate	Heavy	Grey white	Pale grey	Pale brown
Glucose peptone	Weak	Pale grey	Beige	--
Inorganic salts-starch iron	Moderate	Grey	Grey	Pale brown
Glucose-asparagine	Heavy	Grey	Beige	Pale brown
Oatmeal	Heavy	Grey	Grey	Brown
Yeast extract-malt extract	Heavy	Grey white	White	--
Nutrient agar	Weak	Grey	Grey	--

Identification of the selected actinomycete isolate

The highest antifungal activity was obtained by the isolate no. 7, so it was selected for further study and identified. The growth, color of aerial mycelium, color of substrate mycelium and color of soluble pigments were obtained by cultivation of the selected actinomycete isolate on different solid culture media as (Starch-nitrate agar medium; Glucose-peptone agar medium; Inorganic salt-starch agar medium; Glucose-asparagine agar medium; Oatmeal agar medium; Yeast extract-malt extract agar medium and Nutrient agar). The result in table 2 showed the nature of growth.

Morphological examination using scanning electron microscope of the selected actinomycete isolate grown on starch-nitrate medium was carried out. The results exhibited that the spores had smooth surface and spiral shape were observed (Figures. 1and 2).

BLAST analysis

The bioinformatics tool as BLAST result reported that the sequence of the identified culture was *Streptomyces rochei* with 100% similarity.

The gene sequence was submitted to NCBI GenBank with the accession number KX440952 (Figure. 3).

Optimization of culture conditions of the selected actinomycete isolate and antifungal activities against the tested pathogenic Candida species

Effect of incubation time shown in (Figure. 4) revealed that the mycelium growth gradually increasing up to 8 days, and then entering a stationary phase. Antimicrobial metabolite production was detected in the culture broth after 4 days of incubation and reached a maximum at eighth day.

Effect of temperature shown in (Figure. 5) clarified that 30˚C was the optimum temperature for both production of antimicrobial metabolite and mycelium growth. Higher temperatures had an adverse effect on both growth and metabolite production.

Effect of pH shown in (Figure.6) revealed that the pH 7.0 gave the highest zones of inhibition and mycelium growth while acidic and alkaline conditions supported neither growth nor metabolite production. Changing the carbon source of the culture broth was of a significant influence on both metabolite production and mycelium growth.

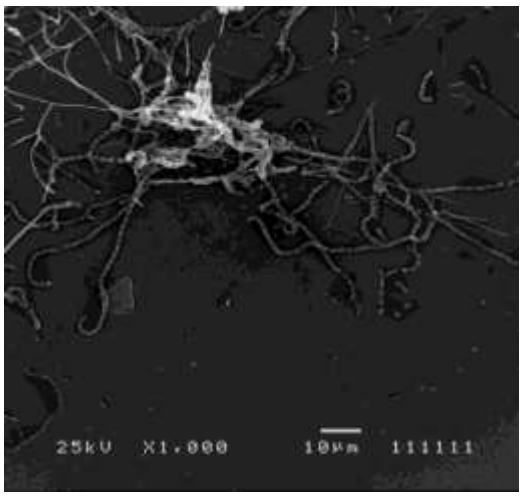

Figure 1: Scanning electron microscope showing the spore-chain morphology (1) of actinomycete isolate (2).

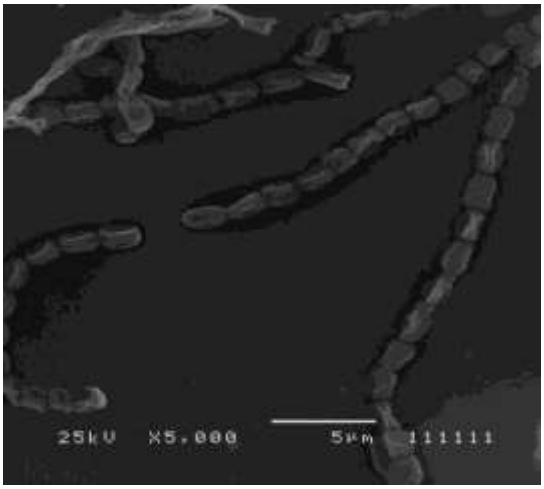

Figure 2: Scanning electron microscope showing spore-surface ornamentation of actinomycete isolate (2).

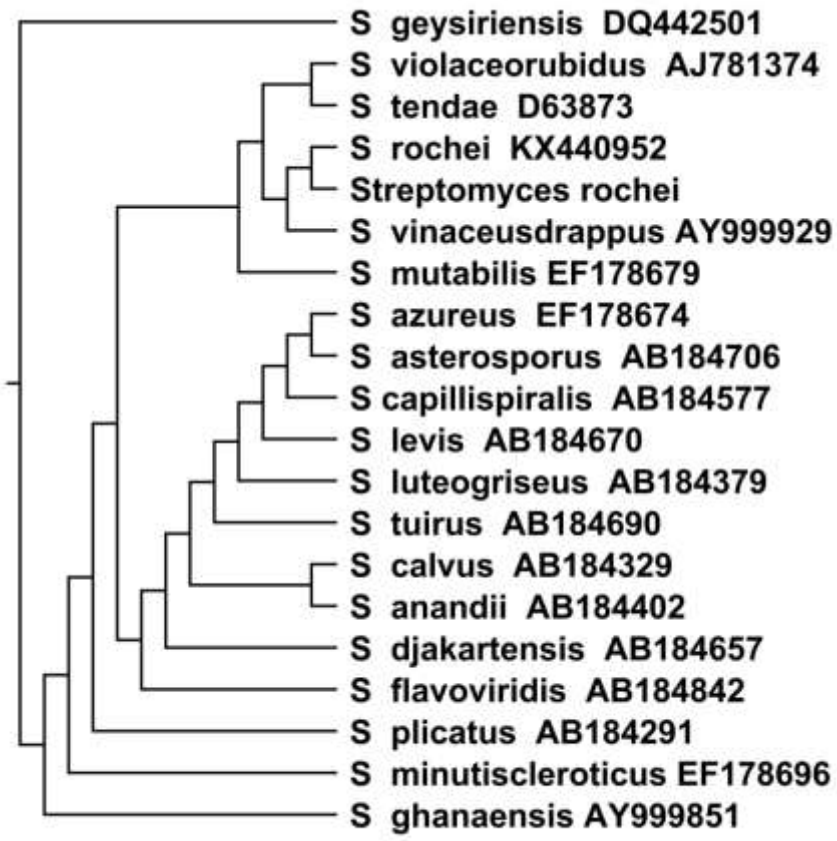

Figure 3; Phylogenetic analysis of *Streptomyces rochei* based on partial sequencing of 16rDNA

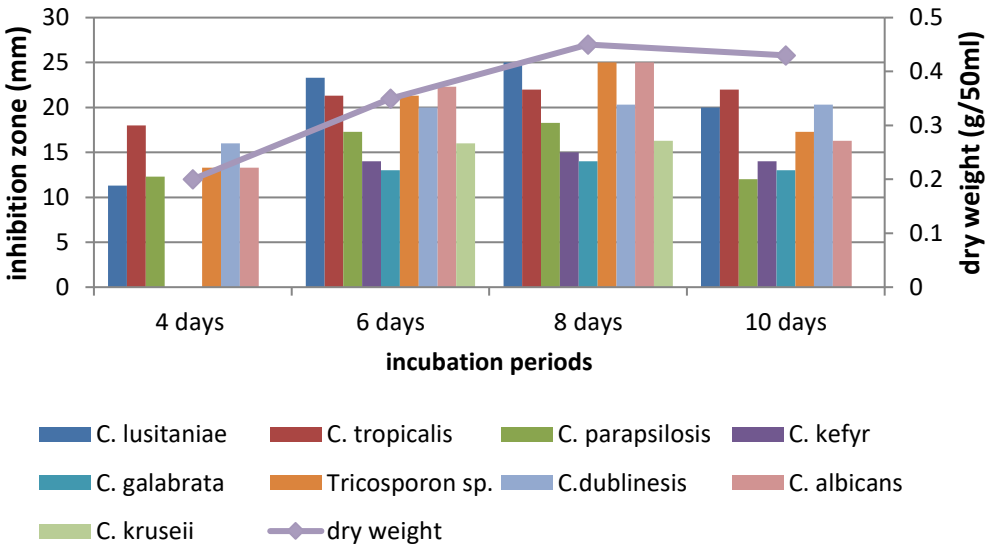

Figure 4; Effect of different incubation periods on the growth and the antifungal activity of *Streptomyces rochei*

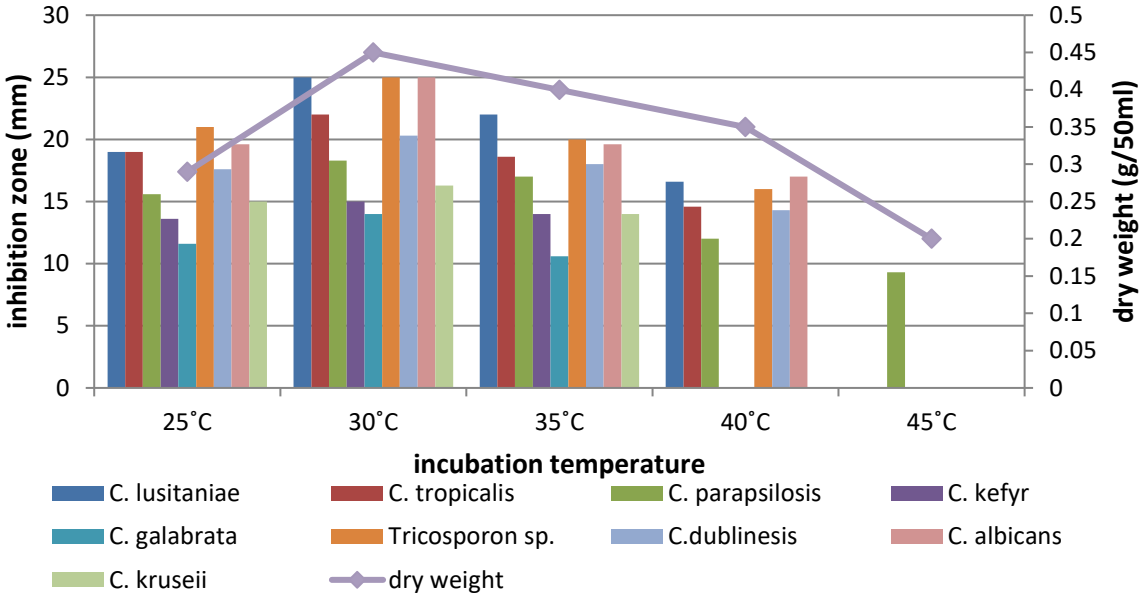

Figure 5; Effect of different incubation temperature on the growth and the activity of *Streptomyces rochei*

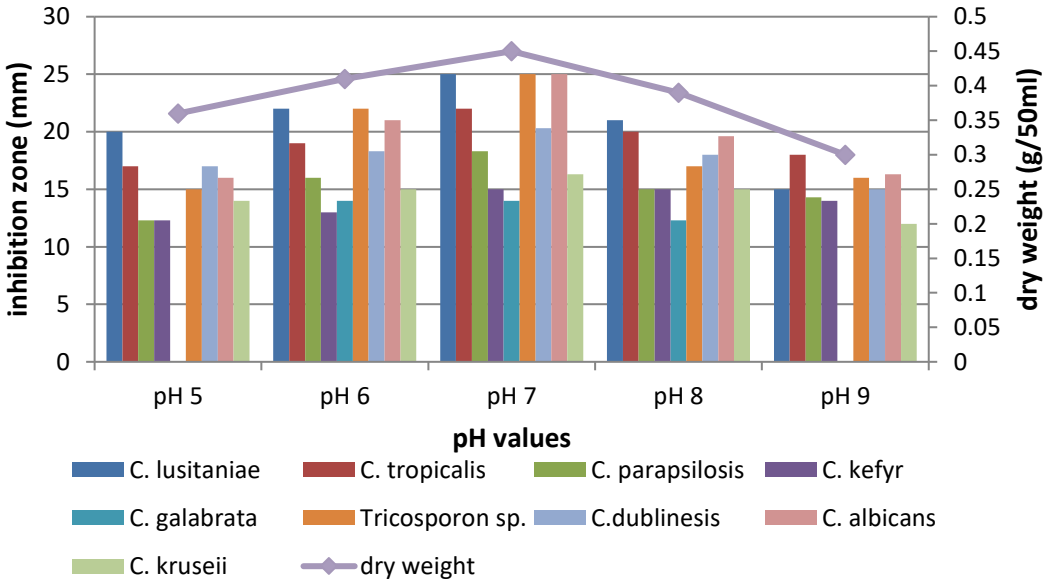

Figure 6: Effect of different initial pH values on the growth and the antifungal activity of *Streptomyces rochei*

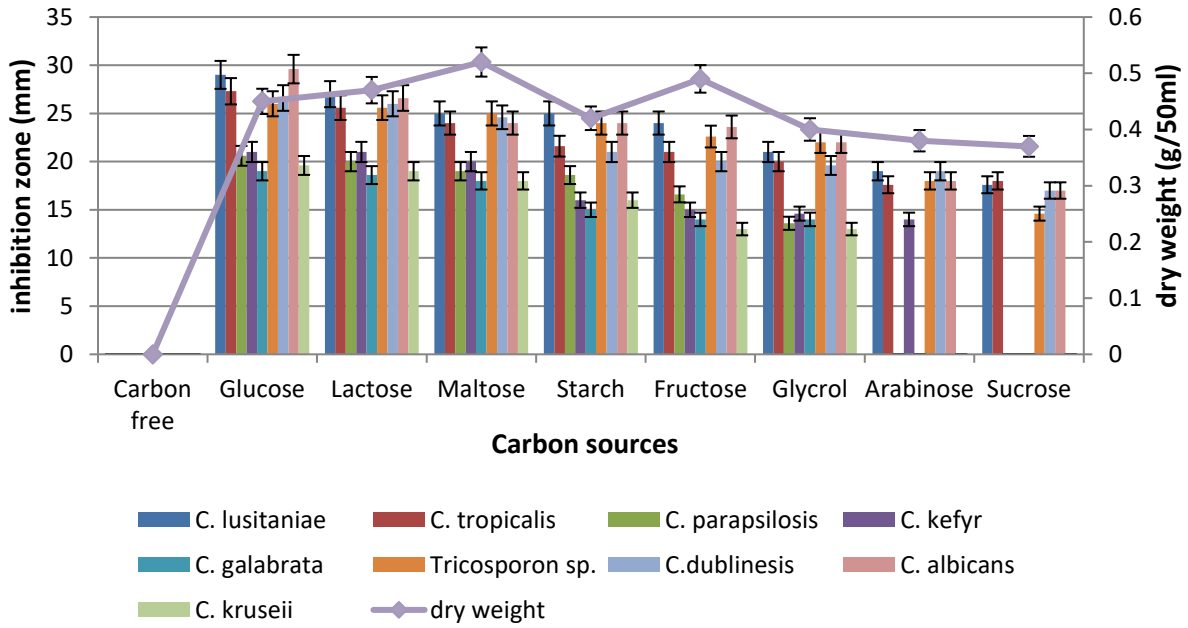

Figure 7: Effect of different carbon source on the growth and the antifungal activity of *Streptomyces rochei*

Glucose was the optimum carbon source for metabolite production, but maltose and fructose were better for mycelium growth (Figure.7).

By studying the effect of different nitrogen sources, the results in Figure. 8 showed that potassium nitrate was the optimum nitrogen source for both metabolite production and mycelium growth. On the other hand, sodium nitrate and peptone suppressed the production of the active metabolite. Figs :4-8

Characterization of the active metabolites produced by *Streptomyces rochei*

Different solvents like chloroform, petroleum ether, ethyl acetate, acetone and diethyl ether were used and tested for the extraction of the antimicrobial materials Preliminary detection revealed that out of the five solvents, ethyl acetate was the best solvent for maximum antifungal materials extraction of *Streptomyces rochei.*

UV spectrum of the produced antifungal extract

The UV spectrum of the antifungal materials of *Streptomyces rochei* was found to have a maximum absorption at 310 nm (Fig.9).

FT-IR spectroscopy

The IR spectrum showed several absorption beaks indicating the presence of many groups including hydroxyl group (3355 cm^{-1}), hydrocarbon group (2930cm^{-1}), alkene group (1638 cm-1) , COO^{-1}group (fatty acid ester)at 1409 cm^{-1} and the peaks appearing at 1000-1300 cm^{-1} indicating various C-O bounds like ethers, phenols and esters. Toxic cyano group (C≡N; 2220-2260 cm^{-1}) and acetylenic group (C≡C; 2100-2260 cm^{-1}) are absent that proved the safety of the extract of *Streptomyces rochei* **.**Fig. (10)

GC-Mass analysis of the antifungal materials produced by *Streptomyces rochei*

The extract of *Streptomyces rochei* was assessed by GC-MS analysis and the profile revealed the presence of a number of chemical compounds with different retention times, peak area, and abundance. GC-MS of the components of *Streptomyces rochei* was illustrated in Fig.11. The chromatogram showed the presence of three major peaks, the first peak appeared at retention time 25.21 with area % 48.21for Oleic Acid with 100% abundance, the second peak appeared at retention time 29.11, 9-Octadecenoic acid, (E)- with 16.62 % abundance and the third peak appeared with 14.13% abundance indicating the presence of Ethanol, 2-(9-octadecenyloxy)-, Other compounds were also recorded like n-hexadecanoic acid, Phthalic acid, bis(7-methyloctyl) ester, Squalene Fig. (11)

MIC determination for the extract of *Streptomyces rochei*

Serial dilution was carried out for a solution of the extract at concentration of 50 mg/ml concentration. The test was carried out against *C. albicans*. Table (29) showing the results which illustrate that the MIC was forth fold (6.25 mg/ml) in the serial dilution. The same concentration was used to make a cream for application in the treatment of the albino rat infected superficially with *C. albicans* for the in vivo study. Table. (3)

In vivo* effect of the active antimicrobial materials of *Streptomyces rochei* against microbial infection of MDR *C. albicans

The results in Table (4) showed significant inhibitory effect of the active antimicrobial materials in the extracts of *Streptomyces rochei* at MIC (6.25 mg/ml) and miconaz antibiotic against microbial MDR *C. albicans* infecting mice skin comparing with the untreated positive control. Out of the treatments, the treatment with the active antimicrobial materials of *Streptomyces rochei* recorded the highest inhibitory effect against the infected wound with microbial MDR *C. albicans* where the percentage of wound healing reached to 100% after 17 day. Whereas the percentage (%) of the wound healing of the treated skin with miconaz antibiotic was ranged from 50-85 from the 4th to the 17th day. The lowest redness of wound and moderate hair growth was observed after 7-14 days with the treatment by the active antimicrobial materials of *Streptomyces rochei***.** Furthermore, at the end of the experimental period after 21 days, complete disappearance of inflammation and considerable growth of hair was detected (table 5)**.** Tables (4 and 5)

Mics' skin wounds infected with MDR *C. albicans*

The histopathological investigation of healthy mice skin tissues (Negative control) showed normal epidermis as the keratinized fibers of stratum corneum were regularly arranged, appeared condensed without any disruption and the dermis appeared normal with normal fibroblasts. Whereas, the histological section of the positive control group infected and untreated showed round, short elongated cells and some thread swelling within the stratum corneum where its keratinized fibers appear loose and disrupted, and the dermis showed chronic inflammatory cellular infiltrate mainly formed of lymphocytes and plasma cells. In comparison with control, the histopathological investigation of mice skin tissues treated with the active antimicrobial materials in the extract of *Streptomyces rochei* possessed no thread swelling, no significant toxic effects; the skin tissue appeared with normal epidermis as keratinized fibers of stratum corneum were regularly arranged, appeared condensed without any disruption and the dermis appeared normal with minimal inflammatory cellular infiltrate. On the other hand, miconaz antibiotic treated skin possessed abnormal epidermis as the keratinized fibers of stratum corneum still showed some disruption and few hyphal swellings of candidiasis were remained and the dermis showed edema with some inflammatory cellular infiltrate of lymphocytes, figures 12: A, B, C and D.

Table 3: MIC of the crude extract of *Streptomyces rochei* against MDR *Candida albicans*

Concentration (mg/ml)	**50**	**25**	**12.5**	**6.25**	**3.12**	**1.5**	**0.78**
Inhibition zone (mm)	34.0	25.0	19.0	15.0	0.0	0.0	0.0

Table 4: Treatment of infected mice with microbial MDR *C. albicans* using the active antimicrobial materials of *Streptomyces rochei* and miconaz

Treatment groups	**Skin wound healing days**													
	1		4		7		10		14		17		21	
	mm	%	mm	%	mm	%	mm	%	mm	%	Mm	%	mm	%
Gp1	Not infected													
Gp2	0	0	3	15	4	20	6	30	7	35	9	45	10	50
Gp3	0	0	13	65	15	75	18	90	19	95	20	100	20	100
Gp4	0	0	10	50	12	60	15	75	16	80	17	85	19	90

mm = Diameter of wound healing, %= Percentage of wound healing Gp= group

Table 5: Signs of inflammation of microbial infection by MDR *C. albicans* and skin wound healing during 21 days

Treatment Group	1-7 days		7-14 days		14-21days	
	Redness	Hair growth	Redness	Hair growth	Redness	Hair growth
Gp1	Not infected					
Gp2	++++	-	+++	-	++	-
Gp3	+++	++	-	+++	-	++++
Gp4	+++	-	++	++	-	++

++++ High degree +++ Intermediate degree ++ Low degree - No growth Gp= group

Histopathological examination of the treated and untreated

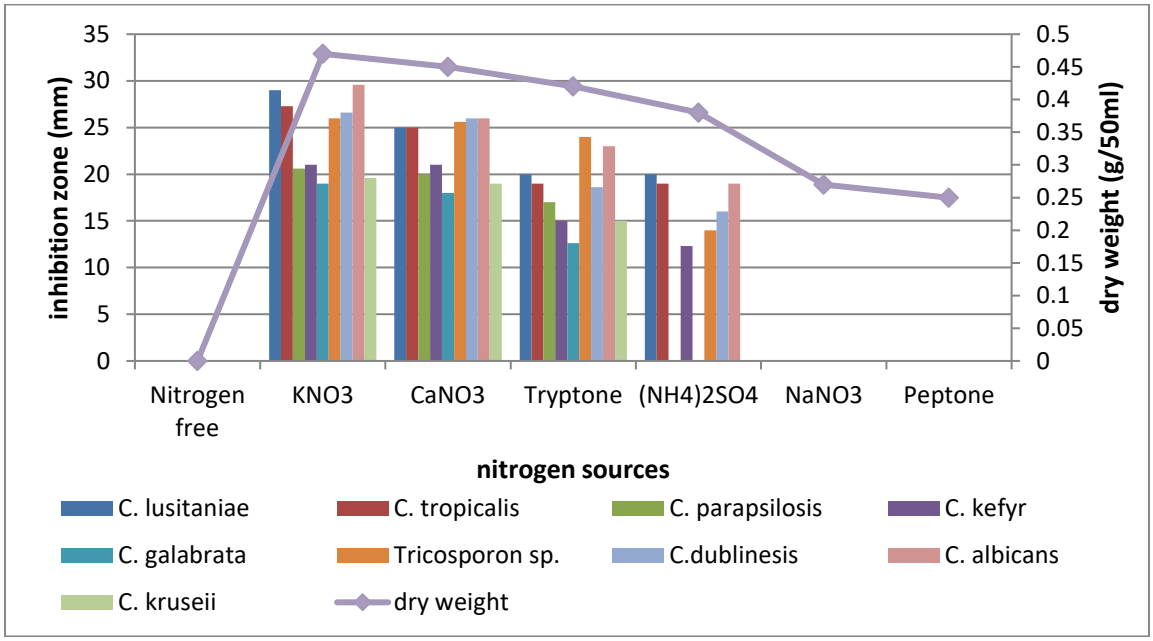

Figure 8: Effect of different nitrogen source on the growth and the antifungal activity of *Streptomyces rochei*

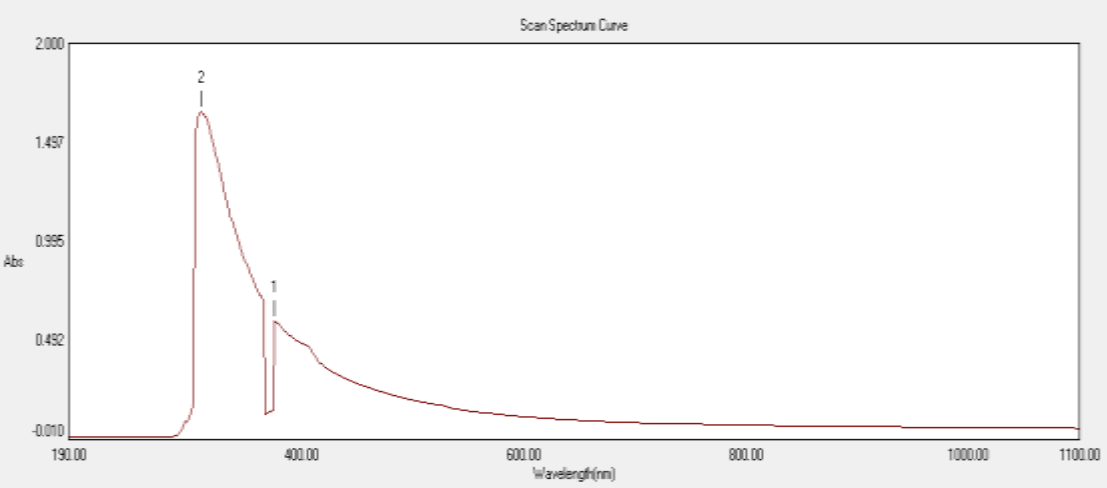

Figure 9: UV spectrum of the antifungal materials produced by *Streptomyces rochei*

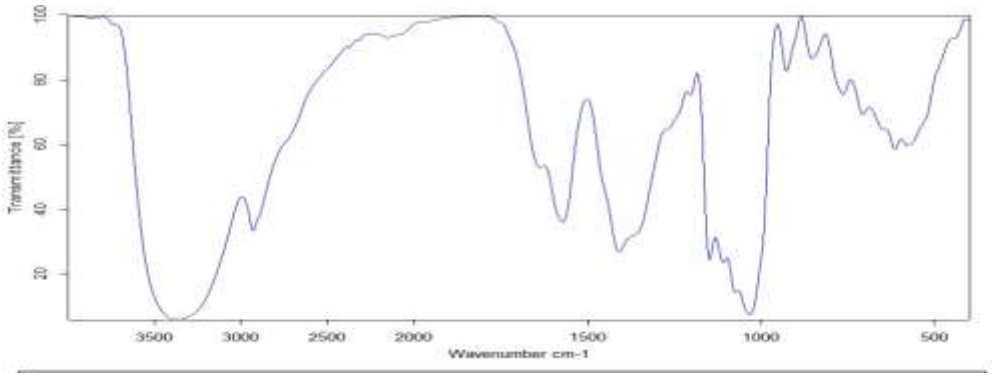

Figure 10: FT-IR chromatogram of the antifungal materials produced by *Streptomyces rochei*

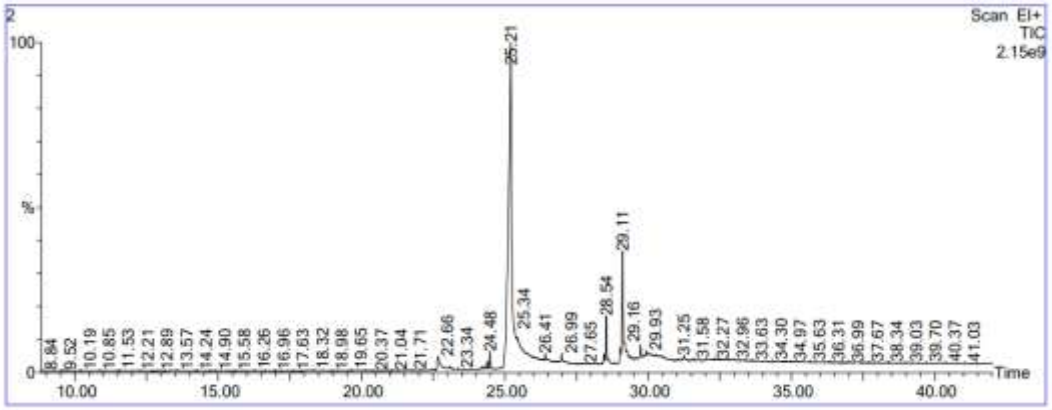

Figure 11: GC-Mass spectroscopy of the antifungal materials produced by *Streptomyces rochei*

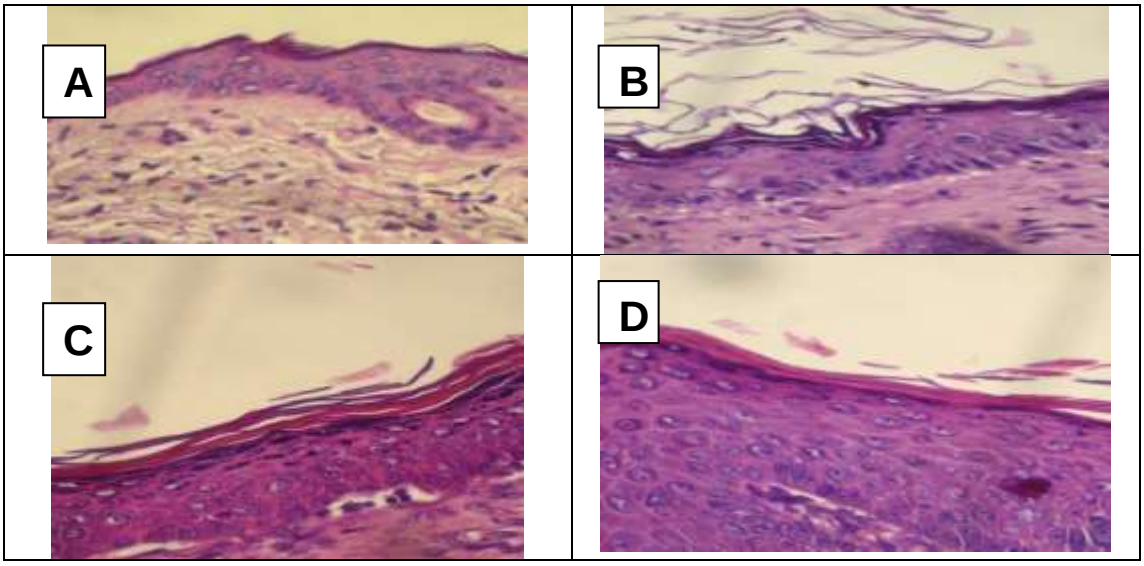

Figure. 12 : A-The histopathological investigation of healthy mice skin tissues after 21 days, B-The histological section of mice skin tissues infected with MDR Candida albicans (untreated) after 21 days, C-the histopathological investigation of mice skin tissues infected with MDR Candida albicans and treated with miconaz antibiotic after 21 days and D- the histopathological investigation of mice skin tissues infected with MDR Candida albicans and treated with actinomycetes .2 extract after 21 day (Complete healing)

DISCUSSION

Forty isolates were collected for the recent study the most active isolate identified morphologically, physiologically and by 16S rRNA as *Streptomyces rochei*, it was isolated from sand sample collected from Marsa Matrouh, specifically from Elgharam peach. The highest inhibition zones recorded against C. albicans and C. lusitaniae with values 29.6 mm and 29 mm respectively. Jain and Jain (2007) found that *Streptomyces sampsonii* possess antagonistic activity against four fungi of which Candida albicans and *Aspergillus niger*. Kim et al. (2006) stated that actinomycete strain VC-A46T, which exhibits antifungal activity, was isolated from a soil sample collected from Cheonan, Korea. The strain was determined to belong to the genus Streptomyces, based on its morphological and chemotaxonomic characteristics.

Parameters like initial pH, temperature, the nutritional sources of carbon and nitrogen, have a profound effect on the production of bioactive metabolites by actinomycetes (Himabindu and Jetty, 2006). In our study the optimum incubation time recorded at 8 days. Similar result was obtained by Ramani and Kumar (2010) who reported that the maximum antimicrobial activity of *Streptomyces* sp Sh7 was obtained at the 8th day of incubation. Also, Bundale et al., (2015) reported that maximum bioactive metabolite production by *Streptomyces purpurascens* was obtained at the 8th day.

Effect of temperature clarified that 30˚C was the optimum temperature for both production of antimicrobial metabolite and mycelium growth. Similar results stated by Tawfik and Ramadan (1991) studied in Saudi Arabia that stated the optimum temperature for growth and antibiotic production by two strains of Streptomyces was 30°C. The fact that maximum yield of the antimicrobial agent occurred at the end of an incubation temperature of 30˚ C was in complete accordance with those reported by (Managamuri, et al.2017 and Moghannem, 2018).

Effect of pH shown in (fig.6) revealed that the pH 7.0 gave the highest zones of inhibition and mycelium growth. The same results were obtained by Bundale et al. (2015) and Moghannem (2018) who reported that pH 7.0 was optimum for the maximum production of Cinnamycin by *Streptomyces cinnamyces*.

Ripa et al. (2009) found that supplementation of medium with glucose (2%) as sole carbon source produced high levels of antimicrobial metabolites by new Streptomyces species (RUPA-08PR) which was isolated from Bangladeshi soil**.** Luthra et al. (2019), also reported that media containing dextrose shows maximum yield whereas those with fructose shows very less yield. Dextrose supports the growth and product formation more as compared to the other carbon sources

By studying the effect of different nitrogen sources, potassium nitrate was the optimum nitrogen source for both metabolite production and mycelium growth. This result is similar to that obtained by Moussa (1999**)** who found that potassium nitrate at 0.2 g/ 100ml was the best for enhancing the production of antimicrobial substances produced by *Streptomyces aureofaciens, S. fradiae and S. roseolilacinus.* Jadon, et al. (2019) also stated that the suitable nitrogen sources was potassium nitrate with concentration of 2 %.

GC–MS analysis of the ethyl acetate extract was carried out and the profiles of the fractions indicated the presence of a different number of chemical compounds with different retention times and abundance. The highest peak recorded at retention time 25.21 indicating the presence of oleic acid. Emami et al. (2017) reported that the produced metabolites from Streptomyces SA3 were analyzed by GC-MS and oleic acid was revealed as the highest peak. In a study conducted in Korea on antimicrobial activity of fatty acids, especially oleic acid, has been manifested that oleic acid can act as GTPase inhibitor and do antimicrobial activity (Won et al. 2007). Balachandar et al. (2016) indicated that GC-MS of the extract produced by Streptomyces sp contained major bioactive compounds oleic acid and 9-octadecanic acid that have antimicrobial activity. It has been hypothesized that fatty acids act by destabilizing the lipid bilayers of the bacterial membrane. Fatty acids are known to act as anionic surfactants and to show antibacterial and antifungal activity (Abou-Elela et al. 2009). The second peak recorded at retention time 29.1 indicating the presence of 9-Octadecenoic acid, (E). Octadecanoic acid has antifungal, antitumour and antibacterial activity has been identified to be produced by a novel Streptomyces isolate Chy 2-3 from Chyulu National Park (Hsouna et al.2011 and Karanja., 2012). The third peak recorded at retention time 30.0 indicating the presence of Ethanol, 2-(9-octadecenyloxy)-, (Z), Selvi and Murugesh (2017) stated that this compound has antimicrobial properties.

There was many other peaks in the GC-MS

profile indicating the presence of compounds with different activity of which n-hexadecanoic acid: Antifungal, Antioxidant, hypocholesterolemic**,** nematicide, pesticide, haemolytic, 5-Alpha reductase inhibitor, potent antimicrobial agent, antimalarial and antifungal(Zito, 2010, Hsouna et al. 2011 and Hema, 2011), Phthalic acid, bis(7-methyloctyl) ester Antimicrobial, Antifouling (Santhi and Kannagi, 2016). Squalene has antibacterial, antioxidant, antitumor, cancer preventive and immunostimulant activity (Sermakkani and Thangapandian, 2012)

In the *in vivo* study, the wound healing percentage reached 100% at the 17th day using the active antimicrobial materials of *Streptomyces rochei*, while using miconaz antibiotic it was 85%, as well as in the histopathological investigation of mice skin tissues, the treatment with the extract of *Streptomyces rochei* broth was better than that with miconaz antibiotic.

CONCLUSION

It was concluded from the present study that the extract of *Streptomyces rochei* culture is a good candidate for treating skin candidiasis where it led to skin complete healing after seventeen days of infection with Candida albicans (MDR). The results were confirmed by Histopathological examination.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

ACKNOWLEGEMENT

We would like to thank Staff members at Central Laboratory, Tanta University, Egypt for valuable help during carrying out samples analysis.

AUTHOR CONTRIBUTIONS

MB, SASS, SMH and YA-GM designed and performed the experiments. YA-GM and MB wrote the manuscript. SMH performed animal treatments. YAGM reviewed the manuscript. All authors read and approved the final version.

REFERENCES

Abou-Elela GM; Abd-Elnaby H; Ibrahim HA; and Okbah MA, 2009. Marine natural products and their potential applications as anti-infective agents. World Applied Science Journal. *7*(7): 872-880.

Aly MM, 1997. Potency of selected actinomycetes for certain antibiotic production. (Doctoral dissertation, Ph D.-Thesis, Section Microbiology, cooperation system between Faculty of Science, Tanta University, Egypt and Faculty of Pharmacy, Nancy University, France).

Balachandar R; Kumar KA; and Karmegam N, 2016. GC-MS analysis of ethyl acetate extract of Streptomyces species isolated from Vermicast. International Journal of Pharma and BioScience. 7(4): (B) 416 – 419.

Bundale S; Begde D; Nashikkar N; KadamT; and Upadhyay A, 2015. Optimization of culture conditions for production of bioactive metabolites by Streptomyces spp. isolated from soil. Advances in Microbiology. *5* (06): 441.

Dietz A and Mathews J, 1971. Classification of Streptomyces spore surfaces into five groups. Applied and Environmental Microbiology. 21(3): 527-533.

Dinya ZM and Sztaricskai FJ, 1986. Ultraviolet and light absorption spectrometry, in "Modern Analysis of Antibiotics, A.Aszalos, ed, Marcel Dekker, New York.

El-Naggar NEA; El-Bindary AAA; Abdel-Mogib M and Nour NS, 2017. In vitro activity, extraction, separation and structure elucidation of antibiotic produced by *Streptomyces anulatus* NEAE-94 active against multidrug-resistant Staphylococcus aureus. Biotechnology and Biotechnology Equipment. 31(2): 418-430.

Emami E; Issazadeh K; Sepehi AA and Tajehmiri A, 2017. Isolation and Characterization of Aerobic Actinomycetes from Soil in Northern Iran and Evaluation of their Antimicrobial Potential. Journal of Medical Microbiology. 6 (3-4): 14-22.

Hema R; Kumaravel S and Alagusundaram K, 2011. GC/MS determination of bioactive components of *Murraya koenigii.* American Journal of Science. *7*(1): 80-83.

Himabindu M and Jetty A, 2006. Optimization of nutritional requirements for gentamicin

production by *Micromonospora echinospora*. Indian Journal of Experimental Biology. 44(10): 842.

Hsouna AB; Trigui M; Mansour RB; Jarraya RM; Damak M and Jaoua S, 2011. Chemical composition, cytotoxicity effect and antimicrobial activity of *Ceratonia siliqua* essential oil with preservative effects against Listeria inoculated in minced beef meat. Int. Journal of Food Microbiology. 148(1): 66-72.

Jadon P; Dwivedi A; Singh C and Kumar A, 2019. Optimization of various physiochemical parameters to enhance production of secondary metabolite from soil actinomycetes against dermatophytes. Environmental Conservation Journal.; 20(1&2): 35-40.

Jain PK and Jain PC, 2007. Isolation, characterization and antifungal activity of *Streptomyces sampsonii* GS 1322. Indian Journal of Experimental Biology.45(2):203-6.

Karanja EN; Boga HI; Muigai AW; Wamunyokoli F; Kinyua J and Nonoh JO, 2012**.** Growth characteristics and production of secondary metabolites from selected novel *Streptomyces* species isolated from selected Kenyan national parks. In Scientific Conference Proceedings.

Khan MSA; Ahmad I; Aqil F; Owais M; Shahid M and Musarrat J, 2010. Virulence and pathogenicity of fungal pathogens with special reference to Candida albicans. In *Combating Fungal Infections* (pp. 21-45). Springer, Berlin, Heidelberg.

Kim HJ; Lee SC and Hwang BK, 2006. *Streptomyces cheonanensis* sp. nov., a novel streptomycete with antifungal activity. International Journal of Systematic and Evolutionary Microbiology. 56(2): 471-475.

Kumar PS; Raj JPP; Duraipandiyan V and Ignacimuthu S, 2012. Antibacterial activity of some actinomycetes from Tamil Nadu, India. Asian pacific Journal of tropical biomedicine. 2(12): 936-943.

Laidi RF; Kansoh AL; Elshafei AM and Cheikh B, 2006. Taxonomy, identification and biological activities of a novel isolate of Streptomyces tendae. *Arab* Journal of *Biotechnol*ogy. *9*(3): 427-36.

Lakshmipathy DT and Kannabiran K, 2009. A morphological, biochemical and biological studies of halophilic Streptomyces sp. isolated from saltpan environment. American Journal of Infectectious Disease. 5(3): 200-6.

Luthra U; Trivedi A; Singh N; Bagwe V and Lade M, (2019): Studies to explore the impact of Carbon & Nitrogen source for Lincomycin production at shake flask level using *Streptomyces lincolnensis.*

Managamuri U; Vijayalakshmi M; Ganduri VRK; Rajulapati S B; Bonigala B; Kalyani BS and Poda S, (2017): Isolation, identification, optimization, and metabolite profiling of *Streptomyces sparsus* VSM-30. *3* Biotechnology. *7*(3):217.

Moghannem SA, (2018): Antibacterial Activity of Streptomyces Tunisiensis Mg520500 Isolated from Soil Samples. *The Asia Journal of Applied Microbiology*: *5*(1): 1-14.

Mohanraj D; Bharathi S; Radhakrishna M and Balagurunathan R, 2011. Bioprospecting of actinobacteria from Yelagiri hills with special reference to antibacterial activity. J. chem. pharm. res.; 3(3): 439-446

Moussa TAA, 1999. Towards the biological control of some root-rot fungal pathogens of sugar beet in Egypt (Doctoral dissertation, Ph. D. Thesis, Department of Botany, Faculty of Science, Cairo University, Egypt).

Muthu MR; Subbaiya R; Balasubramanian M; Ponmurugan P and Selvam M, 2013. Isolation and identification of Actinomycetes *Isoptericola variabilis* from Cauvery River soil sample. InternationalJournal of Current Micro biology and Applied Sciences. 2(6): 236-245.

Nessma A and Ali SS, 2016. Pyocyanin as anti-tyrosinase and anti-tinea corporis: A novel treatment study. Microbial Pathogenesis; 100:1e8.

Ramani DG and Kumar TV, 2010. Antibacterial activity of *streptomyces* sp sh7 isolated from Cardamom fields of Western Ghats in South India. International Journal of pharma and bio sciences. 3(4): 957-68.

Rana S and Salam MD, 2014. Antimicrobial potential *of* actinomycetes isolated from soil samples of Punjab. Indian Journal of Microbiology. *1*(2): 00010.

Ripa FA; Nikkon F; Zaman S and Khondkar P, 2009**.** Optimal conditions for antimicrobial metabolites production from a new *Streptomyces* sp. RUPA-08PR isolated from Bangladeshi soil. Mycobiology. 37(3): 211-214.

Santhi V and KannagiA, 2016. Bioactive potential of *Purpura* persica. Journal of Chemical and Pharmaceutical Research. 8(3):700-707.

Selvi P and Murugesh S, 2017. Identification of Phytoconstituents from the methanolic

extracts of *Cissus arnottiana* using GC-MS analysis. International Journal of Biology, pharmacy and applied science; 6(2): 253-262

Sermakkani M and Thangapandian V, 2012. GC-MS analysis of Cassia italica leaf methanol extract. Asian Journal of Pharmaceutical and Clinical Research.5 (2): 90-94.

Singh LS; Sharma H and Talukdar NC, 2014. Production of potent antimicrobial agent by actinomycete, S*treptomyces sannanensis* strain SU118 isolated from phoomdi in Loktak Lake of Manipur, India. BMC. Microbiology. 14(1): 278.

Spadari C; Antunes T; Teixeira R; Minotto E; Fuentefria AM; Sand D and Van S, 2013. Antifungal activity of actinobacteria against fungus isolates of clinical importance. Revista brasileira de biociências. 11(4): 439-443.

Tawfik KA and Ramadan E M, 1991. Factors Affecting the Biological Activity of *Streptomyces aureofaciens* MY18 and *Str. roseviolaceus* MR13. Journal of King Abdulaziz University-Science. 3(1):5-19.

Williams ST and Davies FL, 1967. Use of a scanning electron microscope for the examination of actinomycetes. Microbiology. *48*(2): 171-177.

Won SR; Hong MJ; Kim Y M; Li CY; Kim JW and Rhee HI, (2007): Oleic acid: an efficient inhibitor of glucosyltransferase. FEBS letters. *581*(25): 4999-5002.

Zamanian S; Shahidi GH and Saadoun I, 2005**.** First report of antibacterial properties of a new strain of *Streptomyces plicatus* (strain 101) against *Erwinia carotovora* subsp. *carotovora* from Iran. Biotechnology. 4(2):114-120.

Zito P; Sajeva M; Bruno M; Maggio A; Rosselli S; Formisano C and Senatore F, (2010): Essential oil composition of stems and fruits of *Caralluma europaea* NE Br.(Apocynaceae). Molecules*.15*(2):627-638.