Natamycin Antibiotic Produced By *Streptomyces* sp.: Fermentation, Purification and Biological Activities

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Abstract: This work was carried out for the biosynthesis of antifungal substance that demonstrated inhibitory effects against pathogenic fungi from *Streptomyces* sp. It is active in *vitro* against some fungal pathogenic viz: *S. cerevisiae* ATCC 9763; *Candida albicans*, IMRU 3669; *Asp. flavus*, , IMI 111023 ; *Aspergillus niger* IMI 31276; *Aspergillus fumigatus* ATCC 16424; *Aspergillus flavus* IMI 111023; *Fusarium oxysporum*; *Rhizoctonia solani*; *Alternaria alternata*; *Botrytis fabae* and *Penicillium chrysogenium*. The active metabolite was extracted using n-Butanol (1:1, v/v) at pH 7.0. The separation of the active ingredient of the antifungal agent and its purification was performed using both thin layer chromatography (TLC) and column chromatography (CC) techniques. The physico-chemical characteristics of the purified antibiotic viz. color, melting point, solubility, elemental analysis (C, H, N, O & S) and spectroscopic characteristics (UV absorbance and IR, Mass & NMR spectra) have been investigated. This analysis indicates a suggested empirical formula of $C_{33}H_{47}NO_{13}$. The minimum inhibition concentrations "MICs" of the purified antifungal agent were also determined. The purified antifungal agent was suggestive of being belonging to Natamycin "polyene" antibiotic produced by *Streptomyces* sp.

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Keywords: Natamycin; Antifungal polyene; *Streptomyces* sp.; Fermentation and Biological Activities

1. Introduction

The search for new antibiotics continues to be of utmost importance in research programs around the world because of the increase of resistant pathogens and toxicity of some used antibiotics. Among microorganisms, actinomycetes are one of the most investigated groups particularly members of the genus Streptomyces from which, a large number of antibiotics was obtained and studied [Okami and Hotta, 1988]. *Streptomycetes* especially the genus *Streptomyces* were very potent producers of secondary metabolites including antibacterial enzymes and toxins [Ren *et al*., 2009]. About of 10.000 known antibiotics, 45-55% was produced by *Streptomyces* [Lazzarini *et al*., 2000]. Natamycin, also known as pimaricin, is a polyene macrolide antibiotic, formula $C_{33}H_{47}NO_{13}$ produced in submerged cultures of certain *Streptomyces* strains such as *S. natalensis*, *S. chattanoogensis*, and *S. gilvosporeus* [Raab, 1972; Thomas, 1976 and Aparicio *et al.*, 2003]. The importance of this antibiotic lies in its broad-spectrum activity against yeasts and molds, with low toxicity against mammalian cells [Fisher *et al.*, 1978]. Natamycin is used to treat fungal keratitis [Malecha, 2004]. It is especially effective against *Candida*, *Aspergillus*, *Cephalosporium*, *Fusarium* and *Penicillium* [Prajna *et al.*, 2002]. Besides its medical applications, natamycin is also used as a food preservative and has been approved as a GRAS (Generally Regarded As Safe) product by FDA (Food and Drug Administration, USA) for use in food manufacturing. Therefore, natamycin is widely used in many food industries to increase the shelf life without any effect on flavor or appearance [Davidson and Doan 1993, Delves-Broughton *et al*., 2006]. Because natamycin shows no activity against bacteria, its preparations are especially suitable to be used as preservatives in foods fermented by bacteria, such as cheese and sausages, preventing mould growth but not affecting the bacterial fermentation or ripening of the food [Basilico *et al.*, 2001]. A stereoisomer of 22-(3-Amino-3,6-dideoxy-β-D-mannopyranosyloxy)1,3,26-trihydroxy-12-methyl-10-oxo-6,11,28 trioxatricyclo [22.3.1.O]octacosa-8,14,16,18,20-pentaene-25carbo-xylic acid (Fig. 1).

In the present study, the production of the bioactive substances that demonstrated inhibitory affects against fungal pathogenic from *Streptomyces* sp. were reported, along with some physico-chemical properties of secondary metabolites with high biological activities.

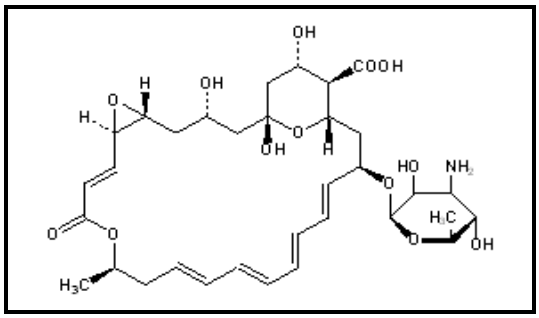

Fig. 1. Structure of Natamycin antibiotic

2. Material and Methods

2.2. Test organisms:

2.2.1. Bacteria:

2.2.1.1. Gram-positive Bacteria: *Staphylococcus aureus*, NCTC 7447; *Bacillus subtilis*, NCTC 1040; *Bacillus pumilus*, NCTC 8214 and *Micrococcus luteus*, ATCC 9341.

2.2.1.2. Gram-negative Bacteria: *Escherichia coli*, NCTC 10416; *Klebsiella pneumonia*, NCIMB 9111 and *Pseudomonas aeruginosa*, ATCC 10145

2.2.2. Fungi:

2.2.2.1. Unicellular fungi

Saccharomyces cerevisiae, ATCC 9763, *Candida albicans* IMRU 3669.

2.2.2.1. Filamentous fungi

Aspergillus niger, IMI 31276.*; Aspergillus flavus*, IMI 111023, *Aspergillus fumigatous*, ATCC 16424; *Aspergillus terreus; Fusarium solani; Fusarium oxysporum, Fusarium moniliform, Alternaria alternata, Botrytis cinerea, Penicillium chrysogenum* and *Rhizoctonia solani*.

2.2. Media

The solid medium for slants and isolation was composed of (g/l): glucose, 10.0; malt extract, 3.0; yeast extract, 3.0; peptone 5.0; and agar, 20.0. The pH of the medium was adjusted to 7.2-7.4 before sterilization. The seed medium was composed of (g/l): glucose, 20.0; malt extract, 6.0; peptone, 6.0; and NaCl, 0.2. The pH of the medium was adjusted to 7.0-7.2 before sterilization. The antibiotic production medium was composed of (g/l): soluble starch, 50; glucose, 20; soybean meal, 10.0; peptone, 5.0; yeast extract, 5.0; beef extract, 5.0; NaCl, 2.0; $CaCO_3$, 5.0; and $MgSO_4$, 1.0. The pH of the medium was adjusted to 7.4 before sterilization.

2.3. Screening for antimicrobial activity

The anti- microbial activity was determined according to [Kavanagh, 1972].

2.4. Fermentation

A loopful of the, *Streptomyces* sp. from the 5-day culture age was inoculated into 250 ml Erlenmeyer flasks containing 75 ml of antibiotic production medium (seven flasks). The flasks were incubated on a rotary shaker (200 rpm) at 30 ^{0}C for 5 days.

Twenty-liter total volume was filtered through Whatman No.1 filter paper, followed by centrifugation at 5000 r.p.m for 20 minutes. The clear filtrates were tested for their activities against the test organisms [Sathi *et al.*, 2001].

2.5. Extraction

The clear filtrate was adjusted at different pH values (4 to 9) and extraction process was carried out using different solvents separately at the level of 1:1 (v/v). The organic phase was concentrated to dryness under vacuum using a rotary evaporator [Atta, 2010].

2.6. Precipitation

The precipitation process of the crude compound dissolved in the least amount of the solvent carried out using petroleum ether (b.p 60-80 °C) followed by centrifugation at 5000 r.p.m for 15 min. The precipitate was tested for its antifungal activities [Atta *et al.*, 2009].

2.7. Separation

Separation of the antifungal agent(s) into its individual components was conducted by thin layer chromatography using chloroform and methanol (24:1, v/v) as a solvent system [Atta *et al.*, 2009].

2.8. Purification

The purification of the antimicrobial agent(s) was carried out using silica gel column (2 X 25) chromatography. Chloroform-methanol-water (2:3:1, v/v/v), was used as an eluting solvent. The column was left for overnight until the silica gel (Prolabo) was completely settled. One-ml crude precipitate to be fractionated was added on the silica gel column surface and the extract was adsorbed on top of silica gel. Fifty fractions were collected (each of 5 ml) and tested for their antimicrobial activities [Lu *et al.*, 2008].

2.9. Physico-chemical properties of the antifungal agent

2.9.1. Elemental analysis

The elemental analysis C, H, O, N, and S was carried out at the micro analytical center, Cairo University, Egypt.

2.9.2. Spectroscopic analysis

The IR, UV, Mass and NMR spectra were determined at the micro analytical center of Cairo University, Egypt.

2.9.3. Biological activity

The minimum inhibitory concentration (MIC) could be determined by the cup assay method [Kavanagh, 1972].

2.9.4. Characterization of the antifungal agent

The antifungal agent produced by *Streptomyces* sp. was identified according to the recommended international references of [Umezawa, 1977; Berdy, 1974; Berdy, 1980a b & c and Eric, 1999].

3. Results

3.1. Screening for the antimicrobial activities

The metabolites of the *Streptomyces* sp. exhibited various degrees of activities against unicellular and filamentous Fungi (Table 1).

3.2. Fermentation and Separation of the antifungal agent

The fermentation process was carried out for three days at 30°C using liquid starch nitrate medium as production medium. Filtration was conducted followed by centrifugation at 5000 r.p.m. for 15 minutes. The clear filtrates containing the active metabolite (20 liters), was adjusted to pH 7.0 then the extraction process was carried out using n-Butanol at the level of 1:1 (v/v). The organic phase was collected, and evaporated under reduced pressure using rotary evaporator. The residual material was dissolved in the least amount of DMSO and filtered. The filtrates were test for their antifungal activities. The antifungal agent was precipitated by petroleum ether (b.p. 60-80°C) and centrifuged at 5000 r.p.m for 15 minute where a yellowish brown oil precipitate could be obtained. Separation of the antifungal agent(s) into individual components was carried out by thin-layer chromatography using a solvent system composed of chloroform and methanol (24:1, v/v). Among three bands developed, only one band at R_f 0.7 showed antifungal activity. The purification process through column chromatography packed with silica gel indicated that the most active fractions against the tested organisms ranged 23 to 31.

3.3. Physicochemical characteristics of the antifungal agent

The purified antifungal agent produces characteristic odour, their melting point is 180°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in water, petroleum ether, hexane and benzene.

3.4. Elemental analysis

The elemental analytical data of the antifungal agent(s) revealed the following: C 59.44%, H 7.22%, N 2.10%, O 31.24% and S=0.0. This analysis indicates a suggested empirical formula of $C_{33}H_{47}NO_{13}$.

3.5. Spectroscopic characteristics

In the IR spectrum, the peaks at υ>3000 cm^1 indicated that there is a typical carboxyl-structure; the υ=1716 cm^{-1} peak revealed a conjugated ester; the υ=1570 cm^{-1} peak corresponded to a primary amine; and the υ=1294-1116 cm^{-1} peaks showed the existence of different C-O- (Fig. 2). The UV spectrum showed that the active compound dissolved in methanol-water presented four typical absorbance peaks at wavelengths 281 nm, 291 nm, 305 nm and 319 nm, which is the typical characteristic of conjugated polyene chemicals (Fig. 3). The mass spectroscopy revealed that the molecular weight is 665 (Fig. 4). The NMR- spectrum were also determined (Fig. 5).

3.6. Biological activities of the antifungal agent

Data of the antifungal agent spectrum indicated that the agent is active against unicellular and filamentous fungi (Table 2). The MIC of antifungal antibiotic was determined and the results showed that the minimum inhibitory concentration (MIC) of the compound against unicellular fungi *Saccharomyces cervisiae* ATCC 9763 (52.7 µg/ ml) and *Candida albicans*, IMRU 3669 (52.7 µg/ ml) and maximum inhibitory activity was observed against filamentous fungi *Aspergillus niger* IMI 31276 (46.9 µg/ ml), *Aspergillus flavus* (46.9), *Botrytis fabae* (31.25 µg/ ml), *Fusarium oxysporum* (31.25 µg/ ml), *Rhizoctonia solani* (52.7 µg/ ml), *Alternaria alternate* (62.5 µg/ ml), *Aspergillus fumigatus* ATCC 16424 (62.5 µg/ ml), and *Penicillium chrysogenium* (62.5 µg/ ml).

3.7. Identification of the antifungal agent

On the basis of the recommended keys for the identification of antibiotics and in view of the comparative study of the recorded properties of the antifungal agent, it could be stated that the antifungal agent is suggestive of being belonging to Natamycin "polyene" antibiotic (Table 3).

Table 1. Antimicrobial activities produced by *Streptomyces* sp.

Test organisms	Mean values of inhibition zones (in mm)
A- Bacteria	
a. Gram positive cocci	
Staph. aureus, NCTC 7447	0.0
Micrococcus luteus, ATCC 9341	0.0
b. Gram positive bacilli	
Bacillus subtilis, NCTC 10400	0.0
Bacillus pumilus, NCTC 8214	0.0
c. Gram negative bacteria	
Escherichia coli, NCTC 10416	0.0
Klebsiella pneumonia, NCIMB 9111	0.0
Pseudomonas aeruginosa, ATCC 10145	0.0
B- Fungi	
a- unicellular fungi	
Candida albicans, IMRU 3669	21.0
Saccharomyces cervisiae ATCC 9763	20. 5
b- filamentous fungi	
Aspergillus niger IMI 31276	24.0
Aspergillus fumigatus ATCC 16424	19.5
Aspergillus flavus IMI 111023	24.0
Fusarium oxysporum	25.0
Rhizoctonia solani.	21.0
Alternaria alternata	19.0
Botrytis fabae	25.0
P. chrysogenum	19.0

Table 2. Biological activities (MIC) of the antifungal agent by paper method assay.

Test organisms	MIC (µg/ml) concentration
1-Unicellular fungi:	
Candida albicans, IMRU 3669	52.7
Saccharomyces cervisiae, ATCC 9763	52.7
2-Filamentous fungi:	
Aspergillus niger, IMI 31276	46.9
Aspergillus fumigatus, ATCC 16424	62.5
Aspergillus flavus, IMI 111023	46.9
Fusarium oxysporum	31.25
Rhizoctonia solani	52.7
Alternaria alternata	62.5
Botrytis fabae	31.25
Penicillium chrysogenium	62.5

Table 3. A comparative study of the characteristic properties of the antifungal agent in relation to reference Natamycin (polyene) antibiotic

Characteristic	Purified antibiotic	Natamycin antibiotic
1- Melting point	180°C	ND
2- Molecular weight	665	665.72
3- Chemical analysis (%):		
C	59.44	59.54
H	7.22	7.12
N	2.10	2.10
O	31.24	31.24
S	0.0	0.0
Ultra violet	281, 291, 305 and 319 nm	281, 291, 305 and 319 nm
Formula	$C_{33}H_{47}NO_{13}$	$C_{33}H_{47}NO_{13}$
Active against	Unicellular and filamentous fungi	Unicellular and filamentous fungi

ND=No data

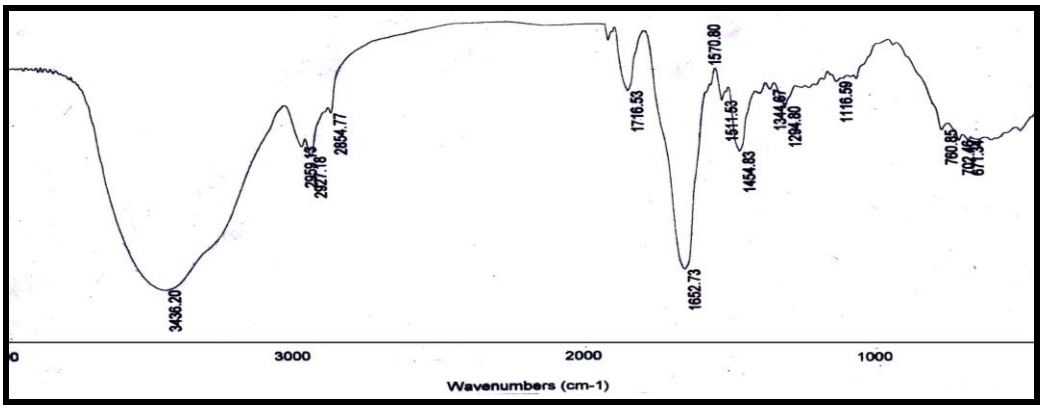

Figure 2. I.R spectrum of antifungal agent produced by *Streptomyces* sp.

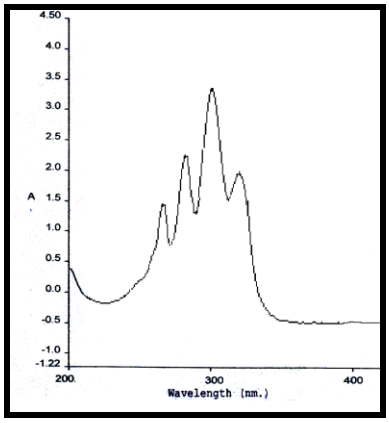

Figure 3. Ultraviolet absorbance of antifungal agent produced by *Streptomyces* sp.

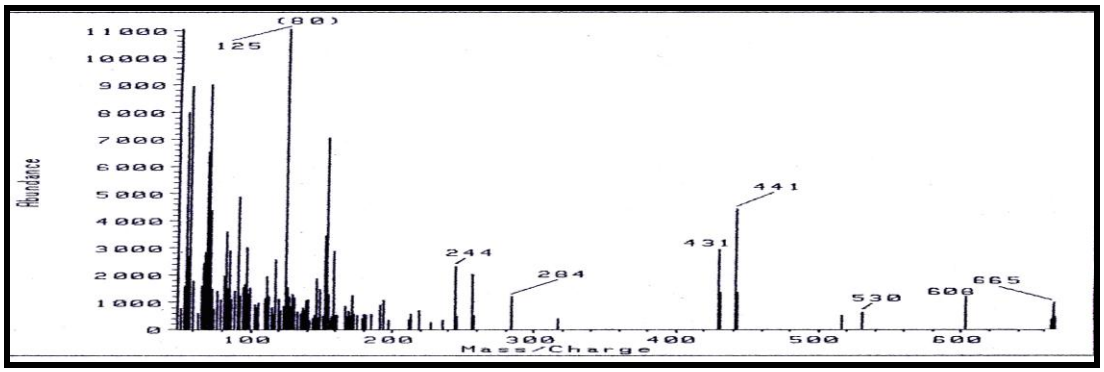

Figure 4. Mass-Spectrum of antifungal agent produced by *Streptomyces* sp.

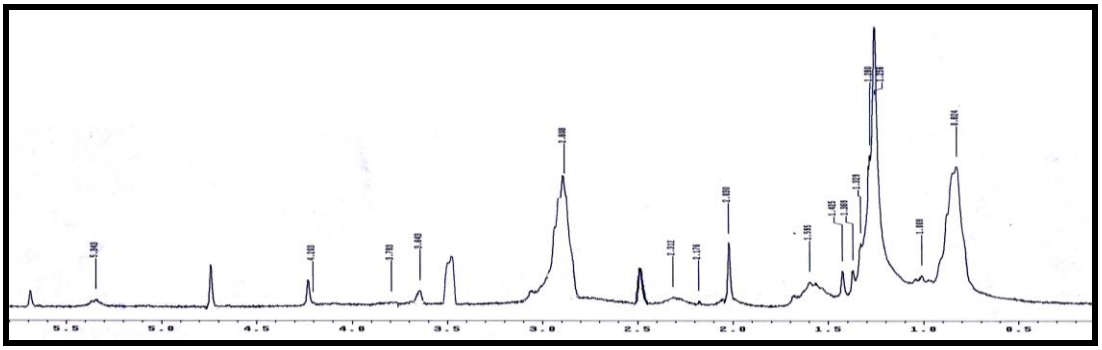

Figure 5. NMR-Spectrum of antifungal agent produced by *Streptomyces* sp.

4. Discussions

Natamycin is a macrolide polyene antifungal drug, which is widely used for the treatment of fungal keratitis and also in the food industry to prevent mold contamination of cheese and other non-sterile foods [Anton *et al.,* 2004]. The active metabolites were extracted by ethyl acetate at pH 7.0. Similar results were obtained by [Criswell *et al.,* 2006; Sekiguchi *et al.,* 2007 and Atta *et al.,* 2011]. The organic phase was collected and evaporated under reduced pressure using rotary evaporator. The extract was concentrated and treated with petroleum ether (b.p. 60-80°C) for precipitation process, where only one active fraction was obtained in the form of whitish yellow oil. The purification process through a column chromatography packed with silica gel and an eluting solvents composed of Chloroform-methanol-water (2:3:1, v/v/v), indicated that fractions activities was recorded from fraction Nos. 23 to 31. Many workers used a column chromatography packed with silica gel. Similar results were obtained by [Jois and Gurusiddaiah, 1986; Hitchens and Kell, 2003; El-Naggar, 2007 and Atta *et al.,* 2009]. The physico-chemical characteristics of the purified antibiotic revealed that, their melting point is 180°C. The compound is freely soluble in chloroform, ethyl acetate, n-butanol, acetone, ethyl alcohol, methanol and 10 % isopropyl alcohol, but insoluble in water, petroleum ether, hexane and benzene. Similar results were recorded by [Yoram *et al.,* 2006 and Wenli *et al.,* 2008]. A study of the elemental analysis of the antifungal agent C 59.44%, H 7.22%, N 2.10%, O 31.24% and S=0.0 lead to an empirical formula of $C_{33}H_{47}NO_{13}$. The spectroscopic characteristics of the antifungal agent under study revealed the IR spectrum, the peaks at υ>3000 cm^{-1} indicated that there is a typical carboxyl-structure; the υ=1716 cm^{-1} peak revealed a conjugated ester; the υ=1570 cm^{-1} peak corresponded to a primary amine; and the υ=1294-1116 cm^{-1} peaks showed the existence of different C-O-. The UV spectrum showed that the active compound dissolved in methanol-water presented four typical absorbance peaks at wavelengths 281 nm, 291 nm, 305 nm and 319 nm, which is the typical characteristic of conjugated polyene chemicals. The mass spectroscopy revealed that the molecular weight is 665. Similar results were recorded by [Eric, 1999 and Lu *et al.*, 2008].

The MIC of antifungal antibiotic was determined and the results showed that the minimum inhibitory concentration (MIC) of the compound against unicellular fungi *Saccharomyces cervisiae* ATCC 9763 (52.7 µg/ ml) and *Candida albicans*, IMRU 3669 (52.7 µg/ ml) and maximum inhibitory activity was observed against filamentous fungi *Aspergillus niger* IMI 31276 (46.9 µg/ ml), *Aspergillus flavus* (46.9), *Botrytis fabae* (31.25 µg/ ml), *Fusarium oxysporum* (31.25 µg/ ml), *Rhizoctonia solani* (52.7 µg/ ml), *Alternaria alternate* (62.5 µg/ ml), *Aspergillus fumigatus* ATCC 16424 (62.5 µg/ ml), and *Penicillium chrysogenium* (62.5 µg/ ml). Similar investigations and results were attained by [Kavitha and Vijayalakshmi, 2007 and Atta, 2010].

Identification of the antifungal agent according to recommended international keys indicated that the antibiotic is suggestive of being Natamycin antibiotic (polyene antibiotic) [Eric, 1999 and Lu *et al.,* 2008].

5. Conclusion

It could be concluded that: The Natamycin "polyene" antibiotic produced by *Streptomyces* sp. demonstrated obvious inhibitory affects against pathogenic fungi.

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