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# Isolation and screening of *Streptomyces* from local area in Sudan in the presence of amphotericin A and B

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### Abstract

Thirty five isolates of *Streptomyces* from soil sample of the regions of Aljazira and Khartoum States were characterized and screened for the production of amphotericin B. Two isolates: IS(1) and IS(5) out of the 35 (6%) have shown a promising result with higher levels of amphotericin B of 23mm and 34 mm diameter of inhibition zone respectively. Isolate IS(5), IS(2), IS(4) and IS(6) out of 35 (11%) gave activity of amphotericin A of 3.25 nm, 3.5nm, 1.06nm and 0.43nm absorbance ranged from 304-318nm respectively. While Is (5) excretes both amphotericin A and B at the same time.

## 1. Introduction

Different area in Sudan has been hosting a unique diversity of microorganisms known to have great potential for treating fungus ailments in humans and animals. Antibiotics are derived from special microorganisms or other living systems and are produced on an industrial scale using fermentation process. Antibiotics are either antibacterial, antifungal, antiviral, insecticidal molluscidal, herbicidal.....etc (1) (2). *Actinomycetes* produced the majority of natural antibiotics, 66% of them are produced by members of the genus *Streptomyces*. Fungi produce a large number, contributing approximately 18% of the total, and true bacteria in the family *Bacillaceae* are also important antibiotics producers yielding about 8% of the total (3).

The number and types of *Actinomycetes* present in a particular soil is greatly influenced by geographical location and soil conditions such as soil temperature, soil type, soil pH, organic matter content, cultivation, aeration and moisture content. *Actinomycetes* populations are relatively lower than other soil microbes and contain a predominance of *Streptomyces* which is a tolerant genus to acid conditions (4). Despite the long list of currently available antibiotics in the market, antifungal and antibiotics are a very small but significant group of drugs and have an important role in the control of mycotic diseases. Only a limited number of antifungal agents are currently available for the treatment of life-threatening fungal infections (5). The need for new, safe and more effective antifungal is a major challenge to the pharmaceutical industry today, especially with the increase in opportunistic infections in the immuno-compromised host. These antifungal agents show some limitations, such as the significant nephrotoxicity of amphotericin B (6) and emerging resistance to the azoles (7) despite several recent improvements, such as lipid formulations of pollens with lower toxicity and new triazoles (voriconazole, rovuconazole and pasaconazole) with a wider spectrum of action,

including activity against some azole-resistant isolates (8). The development of new antifungal agents, preferably naturally occurring with novel mechanisms of action, is an urgent medical need.

Amphotericin B is essentially a high molecular weight macrocyclic lactone, better known as amacrolide, possessing a chromophore of 7 conjugated double bonds. In addition to the large lactone nucleus, amphotericin B has other characteristic groups including an amino sugar (10).

*Streptomyces nodosus* is the only known high producer of amphotericin B, a widely used antifungal drug for chronic and systemic fungal infections. (11). In 1961 and 1962, two patents were granted to Ducher and his team (12). This has opened newer areas of research to improve the yield, reduce toxicities, explore new organisms for similar or better product which can result in a much more efficient and reliable product with antifungal agents compared to antibacterial, are few but have an important role in the control of plant and human mycotic diseases. The search for new, safe, broad-spectrum antifungal agents with greater potency has been progressing slowly in immune side effects (9). This has opened newer areas of research to improve the yield, reduce toxicities; explore newer organisms for similar or better. This study is suggested in response to the worldwide need for production of amphotericin B antifungal activity with for use in medicine.

## 2. Materials and Methods

### 2.1. Isolation of the Microorganism

Soil samples are collected from Algazira and Khartoum states, Sudan, for each collected sample, 1000 mg of the soil were suspended in 100 ml of physiological water ( $\text{NaCl } 9 \text{ g l}^{-1}$ ) then incubated in an orbital shaker incubator at 28 °C with shaking at 200 rpm for 30 min. Mixtures were allowed to settle, and serial dilutions up to  $10^{-4}$  were prepared using sterile physiological water and agitated with the vortex at maximum speed. An aliquot of 0.1 ml of each dilution was taken and spread evenly over the surface of on selective medium (ISP-2 medium). The isolated colonies were taken up for microscopic examination and further identification. Upon a primary identification to species level, they were taken up for screening for amphotericin B production. The isolates were maintained on glucose asparagine slants at 8 °C.

### 2.2. Growth Medium

The culture upon primary selection were inoculated into growing medium (yeast extract 1%, Dextrose 1%, Calcium carbonate 0.01%) for 48 hours at 30 °C in shaking conditions. 5% of the inoculum was transferred into production medium (production medium: Bactopeptone 1%, Dextrose 5%,  $\text{CaCO}_3$  1%,  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  0.001%, and  $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$  0.01% at pH of 7.4) and incubated at 30°C for 192 hours on orbital shaker incubator. 0.4 mg/ml of streptomycin sulphate solution was added to the medium at 24 and 96 hours. The

concentration of product was measured using spectrophotometer for amphotericin A and B (13).

### 2.3. Analysis

After 192 hrs of incubation, the production medium was analysed for the presence of amphotericin A and B. In two microfuge tubes, 1 ml of 20% production medium in Dimethyl sulphoxide was pipetted. The tubes were vigorously shaken for 60 minutes and centrifuged at 10,000 rpm for 10 minutes. 1 ml of 20% supernatant in methanol was transferred into fresh microfuge tubes, mixed well and centrifuged again. The supernatant solution was analyzed using spectrophotometer between 250 and 450 nm. The absorbance obtained were plotted on the standard graph for amphotericin B (13).

#### 2.3.1. Growth on ISP-2 Agar Medium

The isolates formed characteristic creamish –white colonies embedded into the medium which sporulated well upon longer incubation turning completely white in colour. The reverse of the colony showed clear demarcations and was pigmented to pinkish brown colour.

#### 2.3.2. Gram Staining

Gram positive stained thin mycelium with open and closed spirals typical of *Streptomyces* species. *Streptomyces nodosus* is a gram positive bacterium. Which can be identified by several methods, along with a positive gram stain, the mycelium are very distinct when viewed under 640X magnifications showing open and closed (14).

#### 2.3.3. Growth on Glucose

Asparagine Agar Medium showed grayish colored growth with yellow color water insoluble fluid deposit at the bottom of the slant. This proved the identity to belong to *Streptomyces* species. A yellow liquid was also found to be present surrounding the colonies on the slant which when analysed were found to Amphotericin B.

## 3. Results and Discussion

Microorganism plays a significant role in the pharmaceutical industry; about 23,000 bioactive secondary metabolites were produced by microorganisms and about 150 of them are being used in pharmacology, agriculture or other fields. *Actinomycetes* produce 10,000 bioactive metabolites which represent 45% of all bioactive microbial metabolites discovered so far (15),(16). Thirty five presumptive *Streptomyces* isolates were recovered from different soil samples collected from different locations in Sudan: 50% from Algazira State, 25% from Khartoum State (Soba & Tutti), 25% of the isolates from Sinar and were given a number prefixed with IS (Table 1). All of the isolates were considered as *Streptomyces* based on the morphology, microscopic observation growth on Asparagine Agar Medium and ISP-2 growth medium depending on mycelia growth nature and their abilities to grow on ISP2 supplemented with

50ug/ml Nystatin and with 1 ug/ml of penicillin (17). This medium is selected for *Streptomyces* since it contain glycerol that most *actinomycetes* use as a sole carbon source. Nystatin reduces fungal growth whereas penicillin reduces the development of non-filamentous bacteria and *actinomycetes* other than *Streptomyces* (18).

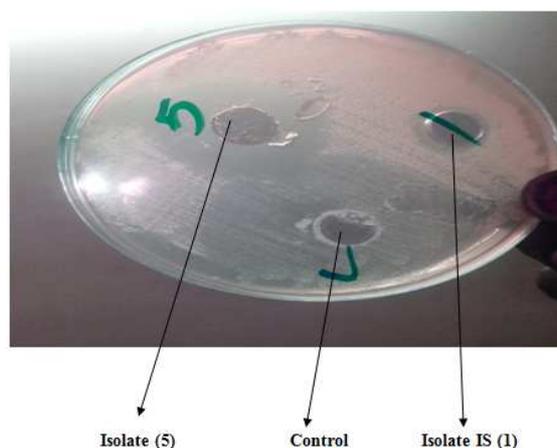
The obtained isolates were screened for their abilities to inhibit the growth of certain pathogenic fungi. Screening was performed by the well Diffusion Agar Method and the diameter of growth inhibition zones were measured in millimeters for each isolate. The results are shown in plate (1). As clear from Table 2, approximately 6 isolates (17.1% ) of the *Streptomyces* isolates have in vitro anti-fungal activities against *Candida albicans*, where as the rest 29 isolates (82.8%) failed to show any activity against any of the tested fungi. The highest activities were shown by the isolates IS (1) and IS(5) against *candida albican* with inhibition zone diameters of 23 and 34 millimeters, respectively. The entire exercise was repeated 3 times to check the consistency and stability of the yields obtained. Among more than 400 actinomycetes screened against *Magnaporthe grisea*, the strain BG2-53 showed 98% of the fungal control at 50 mg/ml. (19)

**Table 1.** *Streptomyces* Presumptive isolates and their sources

Isolate	Source of soil sample
IS (1)	
IS (2)	
IS 3	
IS 4	
IS 5	
IS 6	
IS 7	
IS 8	
IS 9	Aljazeera State
IS 10	
IS 11	
IS 12	
IS 13	
IS 14	
IS 15	
IS 16	
IS 17	
IS 18	
IS 19	
IS 20	
IS 21	
IS 22	Khartoum (Soba & Tutti )
IS 23	
IS 24	
IS 25	
IS 26	
IS 27	
IS 28	
IS 29	
IS 30	
IS 31	Sinar State
IS 32	
IS 33	
IS 34	
IS 35	

**Table 2.** Presence of *Streptomyces* in the samples

Isolates	Activity against <i>candida albican</i>
IS 1	+
IS 2	+
IS 3	-
IS 4	+
IS 5	+
IS 6	+
IS 7	+
IS 8	-
IS 9	-
IS 10	-
IS 11	-
IS 12	-
IS 13	-
IS 14	-
IS 15	-
IS 16	-
IS 17	-
IS 18	-
IS 19	-
IS 20	-
IS 21	-
IS 22	-
IS 23	-
IS 24	-
IS 25	-
IS 26	-
IS 27	-
IS 28	-
IS 29	-
IS 30	-
IS 31	-
IS 32	-
IS 33	-
IS 34	-
IS 35	-



**Plate 1.** Inhibition zones around *Streptomyces* colonies using Agar Diffusion method.

### 3.1. Characterization *Streptomyces*

*Streptomyces* which showed in vitro activities against any of the tested fungi were selected and characterized according to the directions given by the International *Streptomyces* Project (17) and Bergey's Manual of Systematic Bacteriology (20) and involved cultural, microscopic and biochemical characteristics.

### 3.2. Cultural Characteristics

The results of cultural characterization showed that the colonies of the tested isolates were opaque and the majority were round in shape. They were initially; relatively small and smooth -surfaced but later they developed a weft of aerial

mycelium that appears granular and powdery. Initially, all isolates have whitish creamish colonies but later they produced a variety of pigments that colored that vegetative. Aerial mycelia (table 3). All tested isolates grew on ISP2 agar showing morphology typical to *actinomycetes* (17), (21)

Table 3. Cultural characteristics of potential *Streptomyces* isolates

Isolates	Shape	Chromogenesis	Edge	Opacity	Elevation	Surface	Consistency
IS 1	filamentous	Creamish-White	Filamentous	Opaque	Flat	Powdery	Dry
IS 2	filamentous	White	Smooth	Opaque	Raised	Powdery	powdery
IS 4	filamentous	Yellow-pale	Smooth	Opaque	Flat	Powdery	Dry
IS 5	filamentous	Grayish-yellow	Filamentous	Opaque	Flat	Powdery	Smooth
IS 6	filamentous	White	Filamentous	Opaque	Raised	Powdery	Powdery
IS 7	filamentous	yellow	Smooth	Opaque	Flat	Powdery	Dry

### 3.3. Microscopical Characteristics

The results of microscopic characterization are shown in the Table 4. It is a clear that all of the tested isolates are gram-positive, acid-fast negative and filamentous.

All were branched but not fragmented (22). At maturity the aerial hyphae of all isolates differentiated into long spiral chain of cylindrical immotile spores of about 0.3µm in diameter (23) noticed similar spores of about 2-0.5µm in diameter upon *Streptomyces* hyphae differentiation.

Table 4. Microscopic characteristics of potential *Streptomyces* isolates

Isolates	Tests		
	Gram stain	Motility	Aerial mycelium
IS 1	+ve	+ve	present
IS 2	+ve	+ve	present
IS 4	+ve	+ve	present
IS 5	+ve	+ve	present
IS 6	+ve	-ve	present
IS 7	+ve	+ve	present

### 3.4. Production of Amphotericin A and B by *Streptomyces* in Submerged Culture

Table 5. Spectrophotometric characterization of amphotericin A and B

No	Sample	Absorbance in nm			
		304 nm	318 nm	382 nm	405 nm
		Amphotericin A		Amphotericin B	
1.	Control -1	-	3.3	-	2.96
2.	Control -1	-	3.22	2.9	-
3.	Isolate No 1	-	-	1.2	-
4.		-	-	1.11	-
5.	Isolate No 2	3.5	-	-	-
6.	Isolate No 4	3.02	1.06	-	-
7.		-	-	-	-
8.	Isolate No 5	-	3.26	2.83	-
9.	Isolate No 6	-	0.436	-	-
10.	Isolate No 7	3.3	-	-	-

After 192 hrs of the incubation, the production medium was analysed for the presence of amphotericin A and B. In Two microfuge tubes, 1 ml of 20% production medium in dimethyl sulphoxide was pipetted. The tubes were vigorously shaken for 60 minutes and centrifuged at 10.000 rpm for 10

minutes. 1 ml of 20% supernatant in methanol was transferred into fresh microfuge tubes, mixed well and centrifuged again. The supernatant solution was analyzed using spectrophotometer between 250 and 450 nm. The absorbances obtained were plotted on the standard graph for Amphotericin A and B (24). Table 5.

The greater advantage of these isolates is the reduction in the Amphotericin B levels which can help in the making a better potency Amphotericin A with less of Asparagine Agar Medium contamination. Iso (5) showed higher level of Amphotericin A and B of 3.26 and 2.83 at 318 nm and 382 nm respectively. Where as iso (2), (4) and (7) gave higher production of Amphotericin A of 3.5, 3.02 and 3.3 at 304 nm respectively.

Thus it can be well concluded from the above experiments that different area in Sudan are indeed a rich source of microbial biodiversity holding within it immense novelty and potentiality of identifying new isolates for production of life saving drugs. Further studies on the pharmacological and toxicological studies need to be carried out to see if these strains can also reduce the toxicity levels when delivered on a prolonged basis, thereby making them potential candidates for reduced nephrotoxicity. Mutation studies are also needed to be designed for yield improvement.

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### References

- [1] Vining, L.C. (1990). Functions of seconda metabolites, Annual Review of Micrbiology. 44: 3'J5—42
- [2] Berdy, J. (1995). Are *Actinomycetes* Exhausted As A source of Secondary Metabolites. In the proceedings of the 9th Symposium on *Actinomycetes*, pp. 13-34. Where it have been held.

- [3] Champness, W. (2000). *Actinomycetes* Development, Antibiotic Production and Phylogeny: Question and Challenges. In: Burn Y. V and Skmkets, L.J.(eds). Prokaryotic Development. American Society for Microbiology, Washinton D.C.
- [4] Davies F. L. and Williams,S.T. (1970). Studies on the ecology of *Actinomycetes* in a pine forest soil. *Soil Biochem.*2: 227-238.
- [5] Vicente MF, Basilio A, Cabello A, Pela'ez F. 2003. Microbial natural products as a source of antifungals. *Clin Microbiol Infect.* 9:15—32.
- [6] Georgopapadakou N.H, Walsh T.J. (1994). Human mycoses: drugs and targets for emerging pathogens. *Science*; 264: 371—3.
- [7] Carledge JD, Midgley J, Gazzard BG. (1997) Clinically significant azole cross-resistance in *Candida* isolates from HIV-positive patients with oral candidiasis. *AIDS*;11:1839—44.
- [8] Granier F. (2000) Invasive fungal infections. *Epidemiology and new therapies.* Presse Med; 29:2051—6.
- [9] Gupte M, Kulkarni P, Ganguli BN. (2002). Antifungal, antibiotics. *Appl Microbiol. Biotechnol.* 58:46—57.
- [10] Metzger and Julio. 1976. Amphotericin complexes. U.S.pat.3965090
- [11] Gold, W., et al., (1956). Amphotericin A and B, antifungal antibiotics produced by a *Streptomyces*, *In vitro* Studies. *Antibiotics Ann.* 576-578.
- [12] Dutcher, J.D 1959. Amphotericin B, its production and its salts. U.S. patt. 2,908,611
- [13] Haraldm A.B. Linke. (1974). Production of Amphotericin B<sup>14</sup>-C *Streptomyces nodosus* fermentation and preparation of the Amphotericin B<sup>14</sup>-C ester. *The J. of Antibiotic.* 17: 155-160.
- [14] Janina B. 1990. Amphotericin B: Current understanding of mechanism of action. *Antimicro . Agents. Chemother.* 183-188.
- [15] Omura, S.; Ikeda .; Ishikawa, J.; Hanamoto, A.; Takahashi, C.; Shinose, M.; Takahashi, Y.; Horikawa, H.; Nakazwa, H.; Osonoe, T.; Kikuchi, H.; Shiba, T.; Sakaki, Y.; and Hattoori, M. (2001). Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. *Proc. Natl. Acad. Sci.USA* 98: 12215-12220.
- [16] Arasu, M.V.; Duraipandiyam, V.; Agastian, p. and Ignacimuthu, S. (2008). Antimicrobial activity of *Streptomyces* spp. ERI-26 recovered from Western Ghats Tamil Nadu. *J. Mycol. Med.*18: 147-153.
- [17] Porter, J. N.; Wilhelm, J. J. and Tresner, H.D. (1960). A method for the preferential isolation of *Actinomycetes* from soils. *Applied Microbiolog.* 8(3):74 178.
- [18] O'Grady, F.; Lambert, H.P. Finch, R.G.; and Greenwood, D.(1997). *Antibiotic and Chemotherapy* 7<sup>th</sup> ed., Churchill Livingstone, New York. 987pp.
- [19] Hoon, L.; Kim, B.; Choi, J. G.; Gho, Y. K.; Yang, H.; Shin, C.H.; Min, S.; and Lim, Y.(2002). *Streptomyces* with anti-fungal activity against rice blast causing fungus *magnaporthe grisea*. *J. Microbiol. Biotechnol.*12:1026-1028.
- [20] Shining, E. B. and Gottlib, D. (1972).Co-operative description of type species of *Streptomyces*. V. Additionnal descriptions *Int. J. Sys. Bacteriol.* 22: 265-394.
- [21] Locii, R. (1989). *Streptomyces* and Related Genera. In : *Bergey's Manual of Systematic Bacteriology* , William & Wilkins Company, Baltimore. 4: 2451-2508.
- [22] Williams, S. T.; Wellington, E. M. H.; Goodfellow, M.; Alderson, G.; Sackin, M. and Sneath, P .H. A .(1981). The genus *Streptomyces* a taxonomic enigma *Zentralblatt fi5r Bakterioiogie, Mikrobiologie and Hygiene.* i. Abteiinc. supplement. 11: 47-57
- [23] Anderson, A, S. and Welling ton, E. M. H. (2001). The taxonomy of *Streptomyces* and related genera. *Int .J. Syst. Evol. Microbial.* 51:797-814.
- [24] Good fellow, M. and Williams, S. T. (1983). Ecology of *Actinomycetes*. *Annu. Rev. Microbiol.*37: 189-216.
- [25] Harstel, S., and Bolard . 1996. Amphotericin B: new life for an old drug. *Trend. Pharmacol. SC.* 17: 445-449