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Research Paper

ISOLATION AND CHARACTERIZATION OF *Streptomyces* ISOLATES AS A SOURCE OF BIOACTIVE SECONDARY METABOLITES IN SUDAN

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Abstract

One of the greatest threats to global health is that of antibiotic resistance e.g. MRSA. The main purpose of this study was to discover biologically active secondary metabolites, particularly those with activity against resistant bacteria. Sudanese soil is an unexplored source of antibiotic- producing microorganisms , here we reported the screening of soil samples from Sudan for Streptomyces bacteria that have antimicrobial activity. 300 actinomycete strains were isolated from 50 soil samples collected from different geographical areas in Sudan. All these isolates were purified and screened for their antimicrobial activity against pathogenic microbes including Gram positive bacteria, Gram negative bacteria, yeasts and fungi. Out of these, 60 (20%) of the isolates strongly inhibit the growth of Gram positive, Gram negative bacteria, yeasts and fungi. Twenty one promising isolates with strong antimicrobial activity against pathogenic microbes were taxonomically characterized on the basis of morphological and physiological characteristics. Cultural characteristic studies strongly suggested that these isolates are members of the genus *Streptomyces*. The results indicated that the Sudanese soil is rich of Streptomyces having antimicrobial activity. The bioactive Streptomyces isolates will subjected for further identification using molecular genomic fingerprint and chromatographically analysis with the aim of identification of their bioactive metabolites.

Key words: *Streptomyces sp* , screening, antibiotic resistance, Sudanese soil.

INTRODUCTION

The emergence of antibiotic resistance among pathogenic bacteria has become a serious problem worldwide. MRSA (methicillin-resistant *Staphylococcus aureus*) for instance, causes an infection that is resistant to an entire class of penicillin-like antibiotics called beta-lactams. Another example the Vancomycin-resistant *S. aureus* (VRSA). In fact, vancomycin-resistant clinical isolates have been recently reported (Weigel *et al*, 2003; Tenover *et al*, 2004). The overuse of antibiotics in a number of settings is contributing to the increase in antibiotic-resistant microorganisms, so the need for the discovery and development of new and effective antibiotics is a priority so new approaches to antibiotic discovery are needed. Currently, Streptomycetes bacteria are the source of several useful antibiotics that are used not only in the

treatment of various human and animal diseases but also as biological control and biochemistry as metabolic poisons (Jones 2000). At least 70 of the approximately 100 marketed antibiotics used for the treatment of infections in humans are derived from substances produced by *Streptomyces* spp. Streptomycetes are known producers of industrial enzymes and medically important compounds, Indeed, different Streptomyces species produce about 75% of commercially and medically useful antibiotics e.g. polyketides, tetracyclines , antitumor agents and the best known are antibiotics currently used worldwide as pharmaceutical and agrochemical products (El-Naggar *et al*., 2003 Ben-Fguira *et al*., 2005., Abdelhalim *et al* ,2013). Streptomycetes are among the most numerous and ubiquitous soil bacteria, where they play a central role in the degradation of organic matter (Goodfellow and Williams 1983), carbon recycling, enhance soil fertility and have antagonistic activity against wide range of soil-borne pathogen **(**Anitha. and Rebeeth, 2009**).** Many investigators reported that different Strepomyctes have either inhibitory or stimulatory effects on other different microorganisms**.** **(**Hyo *et al* 2006,. Akhand *et al*, 2010). The main aim of this study is to isolate and characterize *Streptomyces* bacteria from soils collected from different region in the Sudan producing active secondary metabolites against microbial pathogens with especial of resistant to antibiotic currently used in hospital.

MATERIAL AND METHODS

Isolation and purification of *Streptomyces* isolates

Isolation of Streptomyces were performed by soil dilution plate technique using starch-casein nitrate agar (SCNA), the medium was supplemented with 10 µg/ml cyclohexamide (Singh and Agrawal, 2002 and 2003). One gram of dried soil was suspended in 100 ml sterile distilled water, vigorously agitated and preheated at 50^0 C for 0.5 hour. The mixture were allowed to settle then serially diluted up to 10^{-7}, from each dilution 100µl of the aliquot was taken and with sterile L- spread evenly shape glass rod over the surface of starch casein nitrate agar plates. The plates were incubated at 30^0C for 10 days under aerobic conditions (Goodfellow, 1987). Selected colonies (colours, rough, chalky) with irregular/regular margin of actinomycetes were transferred from mixed culture of the plates onto SCNA agar plates and incubated at 30^o C for 7 days. Streak plate method was used to purify cultures of actinomycetes (Srinvasan *et al*., 2008).

Target microorganisms

Several pathogenic microorganisms were used to screen the antimicrobial activity of the streptomyces isolates. Bacterial target strains were grow over night at 37^o C in nutrient agar. Yeast and fungi strains were grow in Sabauraud dextrose agar and malt extract agar at 25-30^o C for 48-72 hours. The test-microorganisms used for screening of antimicrobial activity were Gram-positive bacteria which included *Staphylococcus aureus* (MRSA) and vancomycin resistant *Enterococcus* sp. (VRE) *Staphylococcus aureus* (ATCC 25923), *Staphylococcus aureus* (ATCC 43306), *Enterococcus faecalis* (IMD 27) and *Bacillus subtilius* (NCTC 8263), and the Gram-negative bacteria included *Escherichia coli* (ATCC 25922), *Escherichia coli* (local isolate), *Proteus vulgaris* (ATCC 6380), *Pseudomonas aeruginosa* (ATCC 27853), *Salmonella typhie* (IMD 39), the yeast include *Candida albicans* (ATCC 7596), and the fungi *Asperigillus flavus* (local isolate), *Asperigillus niger* (ATCC 9763) and *Pencilium* sp (IMD 1089) were obtained from the culture collection of the School of Biomolecular and biomedical Sciences, University College Dublin.

Screening of *Streptomyces* isolates for antimicrobial activity

screening of pure isolates were determined on solid media by perpendicular streak method on Muller Hinton Agar (MHA). MHA plates were prepared and inoculated with *Streptomyces i*solate by a single streak of inoculums in the center of the petridish. After 4 days of incubation at 30^o C the plates were seeded with test organisms by a single streak at 90 angles to *Streptomyces* isolates as close as possible without touching them. The activity was assessed by measuring the proximity of the test organism's growth to the initial culture (Mustafa *et al*, 2004). Isolates with the broadest range of activity were selected for further study. Pure and active Streptomyces

isolates were stored for long term as lyophils and in glycerol deeps and for short term (up to 6 months) at 4o C on agar slopes.

Morphological and Cultural characteristics of the isolates

Streptomyces colonies were characterized morphologically and physiologically following the rules given by the International Streptomyces Project (ISP) (Shirling and Gottlieb, 1966) and Bergey's Manual of Systematic Bacteriology (1994). Cultural characteristics of pure isolates in various media were recorded after incubation for14 days at 30o C. Morphological observations were made by the light microscope (Model ML 8000) under oil immersion (1000X) by using the methods of Shirling and Gottlieb (1966). Growth, colouration of substrate mycelia, formation of soluble pigment were tested in eight different media including, nutrient agar, Bennett agar, yeast extract malt extract agar (ISP- 2), oat meal agar (ISP-3), inorganic salt starch agar (ISP-4), glycerol – asparagines agar (ISP-5), ISP 6 (Peptone-yeast extract iron agar) and tyrosin agar (ISP-7) following the procedures of ISP, all media agar plates were inoculated with the spores and the results were recorded in 14 days of incubation.

Physiological and biochemical characterization

Effect of temperature, pH, Growth in the presence of different concentrations of NaCl in TSB was determined according to the method described in John *et al*. (1994), Growth in the presence of inhibitors compounds Growth in the presence of inhibitors compounds (phenol 0.1%), crystal violet (0.05%). Sodium azide (0.01%) and lysozyme (0.005%) were performed in the TSA medium using modified Bennett's agar (Williams *et al*. 1983). Antibiotic resistance Ampicillin (20 mg/l), Kanamycin (25 mg/l), Vancomycin (5 mg/l, and Steptomycin (10 mg/l). Starch hydrolysis, Gelatin hydrolysis, Casein hydrolysis, Urease production, Triple Sugar Iron (TSI) and Hydrogen Sulfide production, Melanin pigment production, Soluble pigment production and Carbon utilization by the Streptomyces isolates were tested according to Shirling and Gottlieb (1966).

Secondary metabolite production

Several broth media have been examined to test the ability of the active Streptomyces isolates to produce inhibitory bioactive compounds against the different pathogenic microbes. The isolates that showed activity against several target organisms by single streak methods were grown in submerged cultures. 250 ml baffle flasks containing 50 ml of the following broth culture media: tryptone soya broth (TSB), Yeast Extract Malt Extract Broth (YEMEB), Starch Casein Nitrate Broth (SCNB), and Starch-Glycerol Nitrate Broth (SGNB), Nutrient Broth (NB) and Streptomyces Antibiotic Medium (SAM) all media were purchased from Oxoid and from fluka Chemie GmbH. Coiled springs (Shannon Coiled Springs Ltd., Ireland) were used as baffles to aerate liquid cultures. An aliquot of Streptomyces spores suspension was used to inoculate TSB; the inoculated flasks were kept on a rotary shaker (200 rpm) at 30° C for 48 hours. The culture were harvested, centrifuged at 6000 for 15 minutes. and the antibiotic bioassay was tested by agar well diffusion method using culture supernatant (Shahidi *et al* 2006).

RESULTS AND DISCUSSION

Isolation and characterization of biologically active *Streptomyces* sp.

During the screening of Streptomyces for bioactive natural products a total of 300 Streptomyces were isolated from 50 soil samples collected from different geographical areas in the Sudan (Figure 1). All of these isolates were selected based on their colony morphology, resembling that of Streptomyces species and matches the genus description as reported by Shirling and Gottlieb (1966) and in the Bergyes manual. The colony morphology of the Streptomyces isolates on starch casein nitrate agar plates (SCNA) after 10-14 days of incubation at 30° C indicated that they were small (1-10 mm diameter) discrete and leathery, initially relatively with smooth surface but later developed a weft of aerial mycelium that appeared granular, powder and velvety. All isolates were tested for their antibiotic production against a range of target organisms. Preliminary screening by the perpendicular streak method showed that 60 (20%) had antimicrobial activity against one or more of the target organisms.

Fig 1: Samples of the soil collected from different locations in the Sudan

Therefore, only 21 isolates were selected and identified as Streptomyces due to its typical morphology on International Streptomycetes Project medium 4. These isolates were showed broad spectrum activity against tested organisms. Based on the prominent antimicrobial activities, the morphological and cultural characteristics of the selected isolates were observed after 10 days incubation on the International Streptomyces Project (ISP) media and various other media and they showed considerable variations. All isolates produces well- spores on most media tested. They showed good growth on most media except ISP2 media and Bennett's Agar. According to the culture characteristics ISP medium 3 and ISP medium 4 were the best media for growth, spores formation and soluble pigment production. The spore chains were white grey to dark grey. yellowish, brown, orange, white ,pink and rose as listed in Tables (1) and figures (2, 3,4).

Fig 2. Morphological type of Colonies of *Streptomyces* spp. in ISP4 medium after 10 days incubation at 30°C.

It is evident that different physiological characteristics are influencing the growth rate of the Streptomyces (Shimizu *et al.,* 2000). Table 1 showed a range of biochemical and physiological characteristics of the isolates that were examined.

The antibiotic activities of the culture supernatant of the selected Streptomyces isolates were investigated against a broad spectrum of microorganisms using culture supernatant of the TSB medium and found to have significant activity against resistant bacterial strains as presented in table (2) and figure (5, 6). The bioactive Streptomyces isolates will be subjected for further identification using molecular genomic fingerprint and chromatographically analysis with the aim of identification of their bioactive metabolites.

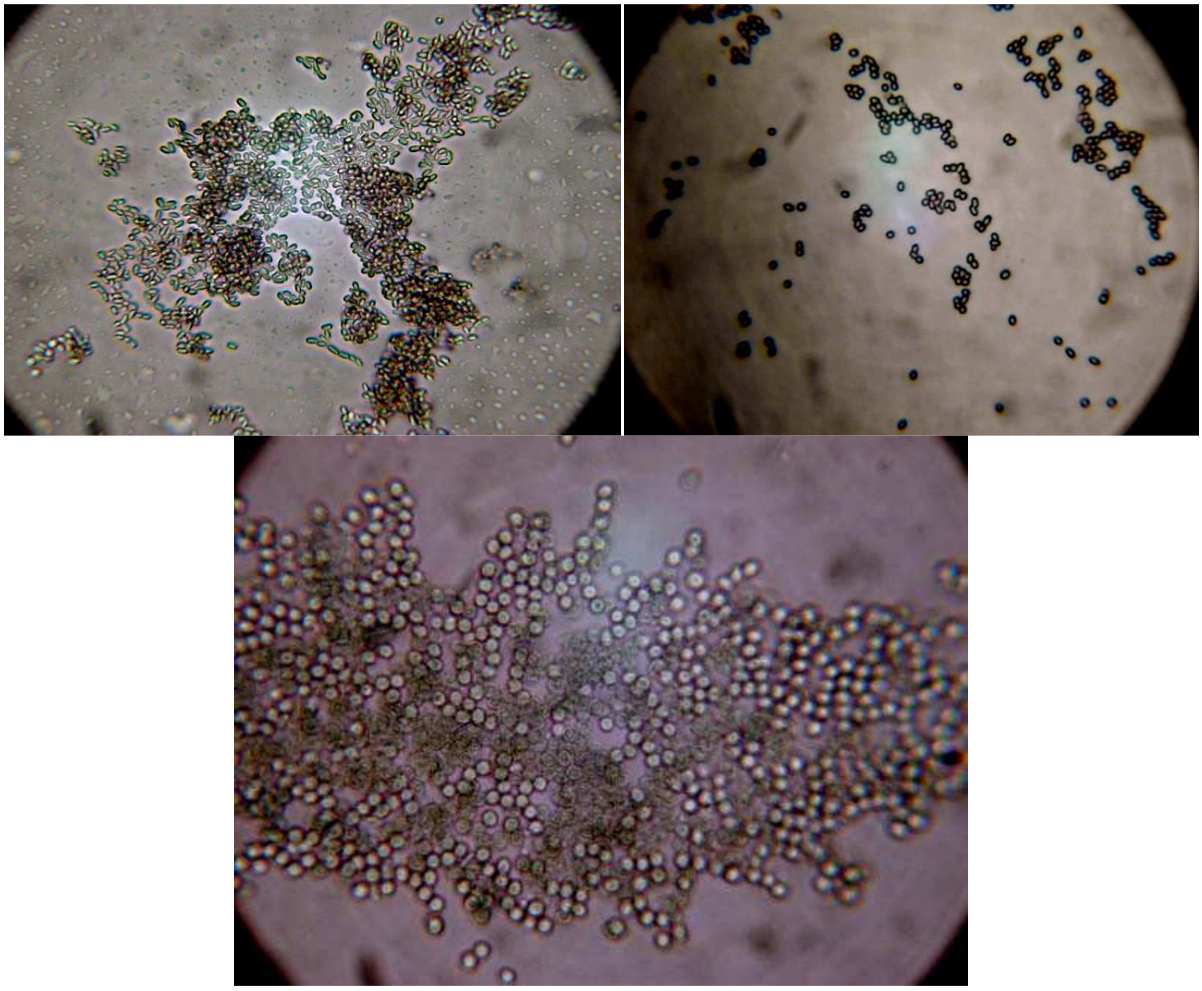

Fig 3. Spores of the selected Streptomyces spp. under light microscope

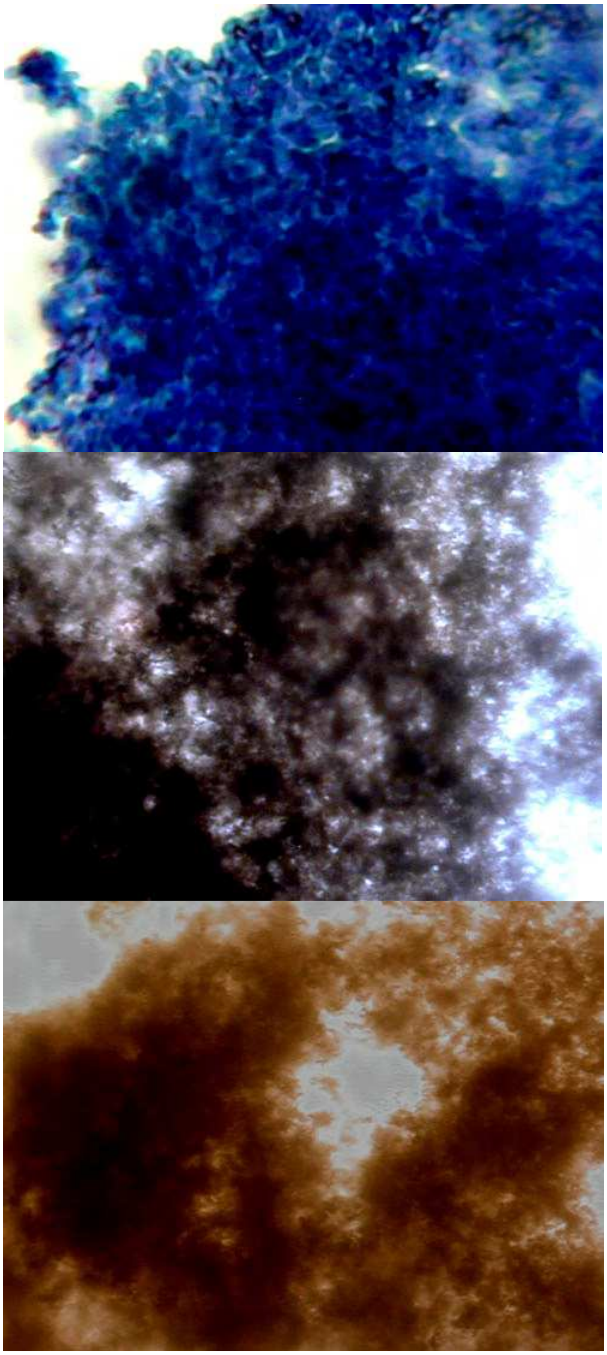

Fig 4. Mycelial Morphology as seen under Light Microscope

Fig (5) . Antibacterial activity of the culture supernatant of the bioactive Streptomyces isolates

Fig (6). Antifungal activity of the culture supernatant of the bioactive Streptomyces isolates

Table 1. Physiological and biochemical properties of the bioactive Streptomyces isolates

	Bioactive Streptomyces isolates																			
Property	**30**	**37**	**74**	**47**	**50**	**57**	**67**	**76**	**79**	**117**	**137**	**113**	**114**	**145**	**193**	**22**	**212**	**192**	**293**	**196**	**244**
Starch hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Casein hydrolysis	+	+	_	+	_	_	_	+	+	_	+	+	_	+	+	+	+	+	+	+	+
Unease production	+	+	_	+	+	+	+	+	+	_	+		+	+	+	_	_	+	_	+	+
Gelatin hydrolysis	+	+	+	_	+	+	+	+	+	_	+	+	_	_	_	+	+	+	+	+	_
pigment production	_	_	_	yellow	green	_	_	_	_	_	Broun	_	Yellow	yellow	Broun	_	_	_	_	Yellow	+
H_2S production	_	_	_	_	_	_	_	+	+	+	+	+	_	+	_	_	+	_	+	+	+
pH / growth	6-8	6-9	6-8	6-8	7-9	6-9	6-8	6-9	6-8	6-8	6-8	6-8	7-9	6-8	7-9	6-8	6-9	6-8	6-9	6-8	6-8
Temperature/ growth C°	25 - 45	25 - 45	25 - 45	25 -45	25 - 45	25 - 45	25 - 45	25 - 45	25-35	25 - 45	25 - 40	25 - 45	25-45	25 -45	25 - 45	25 - 40	25 - 45	25 - 35	25 - 45	25 -45	25 - 35
Antibiotic resistance																					
Ampicillin (20 mg/l)	+	+	+	+	+	+	_	_	+	_	+	+	+	_	_	+	+	_	+	+	_
Kanamycin (25 mg/l)	_	+	_	_	+	+	+	_	+	_	+	+	_	_	_	+	+	_	+	_	+
Vancomycin (5 mg/l)	+	+	+	_	+	+	_	+	_	+	+	_	_	+	+	+	+	+	_	+	_
Steptomycin (10 mg/l)	+	+	_	_	+	+	_	+	_	+	+	_	_	+	+	+	_	+	_	+	_
NaCl tolerance																					
NaCl 2%	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
NaCl 4%	+	+	+	+	+	+	+	+	+	_	+	+	+	_	_	_	_	_	_	+	+
NaCl 7%	_	_	_	_	_	_	_	_	_	_	_	+	_	_	_	_	_	_	_	_	_

Growth on inhibitory compounds																				
Phenol 0.1%	+	+	+	+	+	+	_	_	+	+	+	_	+	_	+	+	_	+	_	+	+
Lysozyme 0.005%	+	+	+	+	_	+	_	+	+	+	_	_	+	_	+	+	_	+	+	_	+
Sodium azide 0.01%	_	+	+	_	+	+	_	+	+	+	+	_	_	_	+	_	+	_	+	+	+
Crystal violet 0.05%	_	+	_	_	_	+	_	+	+	_	+	_	_	_	+	+	+	+	_	_	+

Table 2. Antimicrobial activity of the culture supernatant of the bioactive Streptomyces isolates

Inhibition zone (mm)																					
Target organisms	**Bioactive Streptomyces isolates**																				
Gram-positive bacteria	**30**	**37**	**74**	**47**	**50**	**57**	**67**	**76**	**79**	**117**	**137**	**113**	**114**	**145**	**193**	**22**	**212**	**192**	**293**	**196**	**244**
B. subtillus NCTC 8263	25	30	30	15	0	0	0	25	30	22	0	24	22	0	30	27	24	30	30	26	0
S.aureus ATCC 43306	26	22	28	15	0	0	0	22	25	25	0	30	20	0	20	25	30	25	30	30	0
S.aureus (MRSA)	0	30	20	0	0	0	0	26	28	20	0	0	0	25	22	23	0	0	0	0	0
Enterococcus sp (VRE)	16	20	0	0	0	24	0	0	0	0	17	16	18	29	0	0	0	0	0	0	15
E. feacalis ATCC 1054	28	25	26	18	0	0	0	24	30	25	0	0	0	0	0	0	0	23	26	24	0
Gram-negative bacteria																					
E. coli ATCC 25923	30	23	20	12	0	0	0	20	25	17	0	0	0	0	0	0	0	0	20	0	0
E. coli (local isolate)	30	30	25	20	0	0	0	25	26	0	0	0	0	0	0	0	0	0	0	0	0
Ps. aeruginosa ATCC 27853	25	25	30	12	0	0	0	26	20	20	0	0	0	0	0	22	0	0	0	0	0

P. vulgaris ATCC 6380	22	26	23	18	0	0	0	22	26	20	0	0	15	0	0	0	0	0	0	0	0
S. typhi ATCC 19106	29	22	27	20	0	0	0	25	30	21	0	0	16	0	0	19	0	0	0	0	0
Fungi																					
C. albicans ATCC 7596	30	20	30	0	0	0	25	0	28	30	20	0	0	28	0	0	0	0	0	0	25
As. niger ATCC 9763	22	30	0	0	25	25	0	26	31	30	0	0	0	15	0	0	0	0	0	0	0
As. flavous (local isolate)	35	30	0	0	25	24	20	0	15	32	36	0	0	30	0	20	0	0	0	0	0
Penecilum sp (local isolate)	33	26	0	0	20	20	30	0	20	35	32	0	0	30	0	0	0	0	0	0	26

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