

A Morphological, Biochemical and Biological Studies of Halophilic *Streptomyces* sp. Isolated from Saltpan Environment

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Abstract: Problem statement: Dermatophytes have developed resistance to the existing antifungal antibiotics. As a part of our continuous search we had isolated, identified and characterized actinomycetes from the halophilic environment having antagonistic activity against the dermatophytes namely *Trichophyton*, *Microsporum* and *Epidermophyton*. **Approach:** Actinomycetes were isolated from the soil sample collected from the Ennore saltpan region, Chennai, India and screened for antidermatophytic secondary metabolite production by well diffusion method. Four dermatophytes *Trichophyton rubrum* [MTCC 3272], *Trichophyton mentagrophytes* [MTCC 7687], *Microsporum gypseum* [MTCC 2819] and *Epidermophyton floccosum* [MTCC 7880] were used to study its susceptibility to the isolated actinomycetes. Actinomycetes which showed antidermatophytic activity were subjected to cultural characterization with respect to aerial and substrate mycelia color, diffusible and melanin pigment production and the growth of the organisms on different media. Further the micro morphological characteristics such as spore surface ornamentation and spore chain morphology determined by Scanning Electron Microscopic (SEM) analysis also suggested that the isolates belonged to the genus *Streptomyces*. The isolates were also tested for utilization of various carbon and nitrogen sources, degradation of complex compounds, sensitivity to antibiotics and inhibitory compounds. **Results:** All the 3 isolates exhibited different cultural and morphological characteristics. Based on the cultural characters and morphology they were assigned to the genus *Streptomyces*. The three isolates produced an inhibition zone of 30-31 mm on an average, utilized a wide range of carbon and nitrogen sources, degraded almost all the complex compounds and exhibited a broad spectrum of antibiotic resistance. They were designated as *Streptomyces* sp. *DKDVIT1*, *Streptomyces* sp. *DKDVIT2* and *Streptomyces* sp., *DKDVIT3*. **Conclusion:** The *Streptomyces* sp. isolated from the Ennore saltpan of Bay of Bengal exhibited potential antidermatophytic activity. The extraction and characterization of secondary metabolites from these *Streptomyces* may be used as a lead compound/therapeutic agent for dermatophytosis.

Key words: *Streptomyces*, halophilic, rectiflexibles, retinaculiaperti, dermatophytosis

INTRODUCTION

Actinomycetes are gram-positive bacteria, free living, saprophytic bacteria widely distributed in soil, water and colonizing plants showing marked chemical and morphological diversity but form a distinct evolutionary line of organisms^[1]. Actinomycetes are potential source of many bioactive compounds^[2-5] which have diverse clinical effects and important applications in human medicine^[6].

Members of the actinomycetes genus especially *Streptomyces* sp. have long been recognized as prolific producers of useful bioactive compounds, providing more than half of the naturally occurring antibiotics

discovered to date and continuing to be a major source of many types of antibiotics and other class of biologically active secondary metabolites that may ultimately find application as anti-infectives, anti-cancer agents or other pharmaceutically useful compounds^[7-9]. Some *Streptomyces* sp. produces antibiotics during sporulation^[10].

Most actinomycetes were believed to be terrestrial; however, some strains have also been found to occur in marine environments. Some actinomycetes were isolated from a marine environment which required seawater for growth and these strains were designated marine actinomycetes^[11]. Oceans account for more than 70% of the earth's surface and the microorganisms

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growing in marine environments are metabolically and physiologically diverse from terrestrial organisms^[12].

Dermatophytes are a group of closely related fungi that have the ability to invade keratinized tissue such as skin, hair and nail of humans and animals to produce an infection^[13]. The relation to a dermatophyte infection may range from mild to severe as a consequence of the hosts' reactions to the metabolic products of the fungus. These organisms are classified into genera of *Trichophyton*, *Microsporum* and *Epidermophyton*^[14]. Dermatophytes, like most filamentous fungi of ascomycetous affinity have a secondary metabolism characterized by the production of substantial quantities of distinctive metabolites^[15]. India is a large subcontinent with remarkably varied topography, located within the tropical and subtropical belts of the world. Its ambiance is conducive to the acquisition and maintenance of mycotic infections^[16].

The prevalence of antimicrobial resistance among pathogens is increasing at an alarming rate worldwide^[17]. Antibiotics such as imidazole compounds that are currently employed to treat dermatophytic infections possess a series of limitations such as undesirable side effects or rapid development of resistance^[18]. As a consequence, new antifungal agents preferably naturally occurring with novel mechanisms of action and free from any side effects are needed to improve the treatment of superficial fungal infections^[19].

The present study evaluates micro and macro morphological, cultural, biochemical and biological characteristics of *Streptomyces* sp. recovered from the soil sample collected at the Ennore saltpan of the Bay of Bengal, Chennai.

MATERIALS AND METHODS

Sample collection: Marine soil samples were collected at the Ennore saltpan region of the Bay of Bengal at a depth of 5-15 cm into sterile polyethylene bags, transported to the laboratory and stored in the refrigerator at 4°C until use.

Isolation of actinomycetes: Starch caesin agar supplemented with seawater, was used for the isolation of actinomycetes *in situ*. The samples were appropriately diluted with sterilized seawater and 0.1 mL was spread on the media. After incubation for 1 week at 30°C, the isolated actinomycetes colonies were selected and purified^[20].

Biological characterization of actinomycetes: The purified actinomycetes colonies were screened for

antidermatophytic activity against *Trichophyton rubrum*, *Trichophyton mentagrophytes*, *Microsporum gypseum* and *Epidermophyton floccosum* by well diffusion method^[21]. The isolates were grown in ISP1 broth until adequate turbidity was achieved. 100 µL of the actinomycetes broth culture was placed in the wells made on Sabouraud dextrose agar plates (pH 5.6) seeded with the test fungal cultures. The plates were incubated at 28°C and observed for inhibition zone after 3-4 days.

Macro and micro morphological characterization:

The potential isolates were inoculated on Starch caesin agar, incubated at 30°C for 7 days and were examined for cultural characteristics such as colony size, shape and margin; aerial and substrate mycelium color and diffusible pigment production. The optimum media for the growth of the isolates was determined by inoculating the cultures on solid media such as Starch caesin agar, ISP1 agar, ISP7 agar, Marine Zobell agar, Actinomycetes isolation agar and Seawater agar^[22-23].

The spore chain morphology and spore surface ornamentation of the isolates were evaluated by SEM analysis (S3400, 20 KV, 5.00 µm). The cultures were grown on Starch caesin agar for 7 days at 30°C.

Biochemical characterization: Utilization of various substrates as carbon and nitrogen source by inoculating the isolates in ISP1 broth supplemented with the respective substrates for carbon and nitrogen and incubating for 7 days at 30°C. The production of melanoid pigment was determined for all the 3 isolates by inoculating on tryptone yeast extract iron agar (ISP-6) and tyrosine agar (ISP-7) and incubating at 30°C for 7 days^[23].

The pectinolytic and chitinolytic activity of the isolates was determined by inoculating the isolates on Starch caesin agar supplemented with pectin and chitin respectively, incubating at 30°C and observing the plates for zone of clearance after 7 days^[23].

Degradation of starch, caesin, gelatin, esculin, tyrosine, adenine and guanine was evaluated using Bennett's agar supplemented with the respective compounds. The degradation of starch and gelatin was determined by the presence of zone of clearance around the colonies after flooding the plates with mercuric chloride and iodine respectively. Similarly the degradation of caesin, tyrosine, adenine and guanine was determined by observing the plates for clear zones around the colonies whereas the degradation of Esculin was determined by observing the plates for blackening around the colonies^[23].

The degradation of Tween 80 was determined using Sierra medium^[24]. The presence of white precipitate around the colonies after 7 days indicated positive results. Keratinolytic activity of the three isolates was determined by inoculating the cultures in Starch caesin agar containing the chicken feather and horse hair respectively and incubating at 30°C for 7 days^[25]. Cellulase activity was determined using Starch caesin agar supplemented with Carboxy Methyl Cellulose (CMC) and incubating for 7 days at 30°C^[26]. Lecithinase activity was tested using Egg yolk agar^[27].

The susceptibility of the isolates to various antibiotics was studied on Starch caesin agar plates by placing the various antibiotic discs on the agar surface and incubating at 30°C for 7 days whereas the susceptibility of the isolates to inhibitory compounds was evaluated using Starch caesin agar supplemented with the respective inhibitory compounds^[23].

RESULTS

On screening the 100 isolates of actinomycetes for antidermatophytic activity our isolates namely *DKDVIT1*, *DKDVIT2* and *DKDVIT3* produced a clear zone of 30-31 mm for *T. rubrum* and 18-35 mm for *M. gypseum* (100 µL per well). The inhibition zone produced was compared with that of the standard antifungal agent Fluconazole (10 mg/100 µL per well) (Table 1).

Cultural properties of the isolates are presented in the (Table 2). Actinomycetes isolates grew well at 28°C on Starch Caesin agar (optimal medium) producing pinpoint to medium sized, slow growing, powdery, irregular to regular, flat to raised colonies possessing an earthy odour characteristic of actinomycetes. On Starch caesin agar our isolates produced white coloured aerial spore mass and yellow coloured substrate mycelium with no distinct diffusible pigment production. Based on the cultural characters and morphology they were assigned to the genus *Streptomyces*. The isolates are designated as *Streptomyces* sp. *DKDVIT1*, *Streptomyces* sp. *DKDVIT2* and *Streptomyces* sp. *DKDVIT3*. The morphology of the aerial hyphae was determined by SEM analysis of the culture surface. The aerial mycelium of the isolates *DKDVIT1* and *DKDVIT2* formed unfragmented, branched, straight hyphae bearing non-motile spores with smooth surface (Fig. 1A and B) whereas the isolate *DKDVIT3* formed unfragmented, branched, looped hyphae showing 2 curves and bearing non-motile spores with smooth surface (Fig. 1C).

Table 1: Antidermatophytic activity of *Streptomyces* isolates *DKDVIT1*, *DKDVIT2* and *DKDVIT3* against dermatophytes

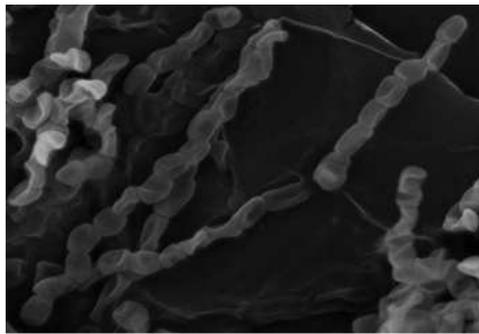
Test organism	Inhibition zone diameter (mm)			
	Culture supernatant			
	<i>DKDVIT1</i>	<i>DKDVIT2</i>	<i>DKDVIT3</i>	Control
<i>Trichophyton rubrum</i>	30	31	30	35
<i>Trichophyton mentagrophytes</i>	-	-	-	20
<i>Microsporum gypseum</i>	35	20	18	20
<i>Epidermophyton floccosum</i>	-	-	-	20

Table 2: Cultural characteristics of the *Streptomyces* isolates *DKDVIT1*, *DKDVIT2* and *DKDVIT3*

Tests	<i>DKDVIT1</i>	<i>DKDVIT2</i>	<i>DKDVIT3</i>
Form:			
Size	Medium	Medium	Pinpoint
Surface	Smooth	Smooth	Smooth
Texture	Powdery	Powdery	Powdery
Margin	Irregular	Irregular	Regular
Elevation	Raised	Flat	Flat
Color:			
Aerial spore mass	White	White	White
Substrate mycelium	Yellow	Yellow	Yellow
Odor:			
Pigmentation	Earthy odor	Earthy odor	Earthy odor
Diffusible pigment	-	-	-

The physiological characteristics of the isolates are summarized in the (Table 3). The melanin pigment producing ability was checked on tryptone yeast extract agar^[31] and none of the isolates produced melanin. The isolates *DKDVIT1* and *DKDVIT3* utilized a wide range of carbon source but did not utilize glycerol whereas isolate *DKDVIT2* utilized all the carbon sources. The isolate *DKDVIT1* grew utilizing a variety of organic and inorganic nitrogen source except ammonium sulphate, ammonium nitrate and potassium nitrate. Similarly isolate *DKDVIT2* and *DKDVIT3* utilized a range of nitrogen source except valine and ammonium nitrate. In addition, *DKDVIT2* also failed to use ammonium sulphate. All the 3 isolates degraded gelatin, starch, caesin, tyrosine, Esculin, adenine, guanine and Tween 80 but failed to degrade keratin, lecithin, pectin and cellulose.

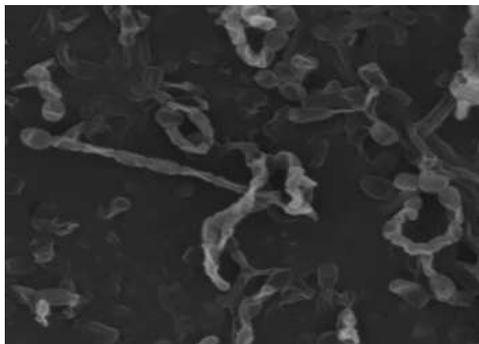
The sensitivity to various antibiotics varied among the 3 isolates (Table 3). The isolate *Streptomyces* sp. *DKDVIT1* was found to be sensitive to a variety of antibiotics but resistant to cephtazidime, chloramphenicol, penicillin, tetracycline and erythromycin. Similarly *Streptomyces* sp. *DKDVIT2* exhibited sensitivity to many antibiotics but resisted the inhibitory action of only ampicillin, cephotaxime, cephtazidime, streptomycin and rifampicin. While *Streptomyces* sp. *DKDVIT3* was found to be resistant to ampicillin, cephtazidime, ciprofloxacin, penicillin,



(A)



(B)



(C)

Fig. 1: SEM micro morphology of *Streptomyces* isolates *DKDVIT* (A) *DKDVIT2* (B) and *DKDVIT3* (C) showing the spore chain and spore surface ornamentation

rifampicin and tetracycline, it also exhibited sensitivity to many other antibiotics. Also the susceptibility of the isolates to the inhibitory compounds crystal violet indicated that the isolates *DKDVIT1* and *DKDVIT2* were resistant to crystal violet but failed grow in the presence of sodium azide, phenol and potassium tellurite whereas the isolate *DKDVIT3* was resistant to crystal violet and potassium tellurite but sensitive to sodium azide and phenol (Table 3).

Table 3: Physiological characteristics of *Streptomyces* isolates *DKDVIT1*, *DKDVIT2* and *DKDVIT3*

Tests	<i>DKDVIT1</i>	<i>DKDVIT2</i>	<i>DKDVIT3</i>
Carbon utilization (w/v)*:			
Glucose	+	+	+
Xylose	+	+	+
Sucrose	+	+	+
Raffinose	+	+	+
Starch	+	+	+
Galactose	+	+	+
Maltose	+	+	+
Arabinose	+	+	+
Fructose	+	+	+
Lactose	+	+	+
Inositol	+	+	+
Glycerol	-	+	-
Mannitol	+	+	+
Rhamnose	+	+	+
Nitrogen source (w/v)*:			
Yeast extract	+	+	+
Peptone	+	+	+
Caesin	+	+	+
Cysteine	+	+	+
Arginine	+	+	+
Histidine	+	+	+
Serine	+	+	+
Valine	+	-	-
Hydroxy proline	+	+	+
Methionine	+	+	+
Phenylalanine	+	+	+
Ammonium sulphate	-	-	+
Ammonium citrate	+	+	+
Ammonium nitrate	-	-	-
Sodium nitrate	+	+	+
Potassium nitrate	-	+	+
Enzymatic activity			
Pectin	-	-	-
Gelatin	+	+	+
Starch	+	+	+
Caesin	+	+	+
Tyrosine	+	+	+
Esculin	+	+	+
Adenine	+	+	+
Guanine	+	+	+
Keratin	-	-	-
Tween 80	+	-	+
Cellulose	-	-	-
Lecithin	-	-	-
Growth in the presence of antibiotics:			
Ampicillin	-	+	+
Cephotaxime	-	+	-
Cephtazidime	+	+	+
Streptomycin	-	+	-
Chloramphenicol	+	-	-
Ciprofloxacin	-	-	+
Gentamicin	-	-	+
Rifampicin	-	+	+
Penicillin	+	-	+
Neomycin	-	-	-
Vancomycin 10	-	-	-
Vancomycin 30	-	-	-
Tetracycline	+	-	+
Erythromycin	+	-	-
Growth in the presence of inhibitors:			
Crystal violet	+	+	+
Sodium azide	-	-	-
Phenol	-	-	-
Potassium tellurite	-	-	+

*: Growth of the strains was measured as dry weight of the mycelium

DISCUSSION

In our screening for novel actinomycetes exhibiting potential antidermatophytic activities, soil samples were collected from the Ennore saltpan region of Bay of Bengal, Chennai. In the course of our continuous search a total of 100 isolates were recovered from the collected soil samples.

Screening for the antidermatophytic activity by well diffusion method of the culture supernatant was carried out for all the 100 pure isolates against the dermatophytes, *T. rubrum*, *T. mentagrophytes*, *M. gypseum* and *E. floccosum*. Among them the 3 isolates DKDVIT1, DKDVIT2 and DKDVIT3 exhibited potential inhibitory activity against *T. rubrum* and *M. gypseum* whereas no activity was detected against *T. mentagrophytes* and *E. floccosum*. The antibacterial and enzymatic activities of *Streptomyces* sp. are well known, however its antifungal property has been reported only against common fungal pathogens. Similar antidermatophytic studies were carried out by Augustine *et al.*^[21]. Their studies confirmed antidermatophytic activity of *Streptomyces rochei* AK 39 isolated from soil and water samples collected at various locations of Pune, Maharashtra, India.

The colony morphology of our isolates were similar to that described by Locci in his earlier studies^[22] which clearly indicates that the isolates under investigation belonged to the genus *Streptomyces*. All the 3 potential isolates grew well on a range of agar media showing characteristics typical of *Streptomyces*^[28]. *Streptomyces* have been reported to grow well on Starch caesin agar by earlier workers in this field^[29]. Our studies on the micro morphology of the three isolates showed revealed two spore morphology types namely Rectiflexibles and Retinaculiaperti. Stefka *et al.*^[30] has done a similar study but reported a different type of spore morphology where the aerial mycelium formed monopodially branched spore-bearing hyphae with the shape of hooks, loops, open or compact spirals with 3-6 curves with smooth surface.

In our studies we observed that all the three isolates could degrade almost all available complex substances. Reports on the degradation and enzymatic activity of *Streptomyces* by Rabah *et al.* also revealed a much similar result^[29,32].

Studies on resistance to antibiotics by Ibrhaim^[32] indicated that *Streptomyces* isolates used in their studies were sensitive to gentamycin, tobramycin, rifampicin and penicillin G, tetracycline but resistant against oleandomycin, lincomycine, vancomycin, streptomycin and neomycin. Our investigation also

resulted in a similar type of finding which is comparable to that of Ibrhaim^[32]. Also the studies on the resistance to inhibitory substances can be compared to the results reported by Ibrhaim^[32].

CONCLUSION

From the above mentioned results, it may be concluded that all the 3 isolates *Streptomyces* sp. DKDVIT1, *Streptomyces* sp. DKDVIT2 and *Streptomyces* sp. DKDVIT3 belongs to the genus *Streptomyces* and showed strong antidermatophytic activity against *T. rubrum* and *M. gypseum* but no activity against *T. mentagrophytes* and *E. floccosum*. Further studies on the purification and characterization of the antidermatophytic secondary metabolites are currently in progress.

ACKNOWLEDGEMENT

The researchers thank the management of VIT University for providing the facilities to carry out this study. Financial support from the International Foundation for Science (IFS, F 4185-1), Sweden and the Organization for the Prohibition of Chemical Weapons (OPCW), The Hague to one of the author (KK) is gratefully acknowledged.

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