ANTICANDIDAL ACTIVITY OF *STREPTOMYCES PARAGUYENSIS* ISOLATED FROM MARINE SEDIMENT SAMPLES COLLECTED AT THE PUDUCHERRY COAST, BAY OF BENGAL, INDIA

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Summary

The marine actinomycetes isolated from sediment samples collected at the Puddcherry coast, Bay of Bengal, India was screened for anticandidal activity by well diffusion method against *Candida albicans* under *in vitro* conditions. The potential isolate was subjected to physiological, biochemical and cultural characterization and based on the key for classification and identification of 458 species of *Sreptomyces* included in the International Streptomyces Project (ISP), it was identified as *Streptomyces paraguyensis (*SP). The optimal media for SP was ISP 1 and maximal growth was seen at 28°C and pH 8.0. It was sensitive to antibiotics including erythromycin, tetracycline, vancomycin, streptomycin, carbenicillin and resistant to ceftazidime, co-trimoxazole, amphotericin–B, fluconazole and nalidixic acid. It hydrolyses starch, gelatin, casein and esculin. The culture broth (100 µl) of SP showed the zone of inhibition (20mm) against *Candida albicans* whereas amphotericin-B (25 µg/disc) showed the zone of inhibition of 8 mm. The acetone crude extract (1000 µg/ml) obtained from cells of SP showed the inhibition zone of 17 mm against *Candida albicans*. Based on the results of the study it can be concluded that SP produces an intracellular secondary metabolite having anticandidal activity against *Candida albicans* under in vitro conditions.

***Keywords**:* Actinomycetes, *Streptomyces paraguyensis, Candida albicans,* anticandidal activity.

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Introduction

Candidosis, caused by *Candida species* are the most common opportunistic fungal pathogen has intrigued clinicians and scientists for many decades (1). *Candida* spp. became the third most usually present isolates found in hemocultures in developed countries. Moreover, the death rate due to the infections with *Candida*, Aspergillus and Fusarium among patients under immunosuppressive chemotherapy or transplant patients is relatively high (2). In clinical settings, candidiasis is commonly treated with antimycotics and other antifungal drugs commonly used to treat candidiasis are topical clotrimazole, topical nystatin, fluconazole, and topical ketoconazole, amphotericin-B, caspofungin, or voriconazole may also be used (3). It was already been reported that use of large quantities antifungal drugs causes mouth and throat ulcerations in nursing babies, and has been linked to mouth cancer in humans and cancer in digestive tract of other animals (4). But recurring infections and repeated administration of antifungal drugs produce resistance and continuous use of other antifungal drugs may also produce resistant strains. *C. albicans* has been reported to develop resistance to antimycotic drugs (5). Many infections due to *Candida* species are refractory to antifungal therapy (6). Hence, the search for new drugs against fungal infections is a major challenge to current research in mycotic diseases (7). Despite the long list of currently available antibiotics only a limited number of antifungal agents are currently available for the treatment of life-threatening fungal infections (8). Further, the need for new, safe and more effective antifungals is a major challenge to the pharmaceutical industry today. New sources of antimycotic agent are very much needed, particularly in view of the opportunistic capabilities of yeast and moulds in patients afflicted with terminal diseases.

There are approximately 32, 500 natural products reported from microbial sources (Antibase databse), including about 1000 derived from marine microbes (9). Marine microorganisms are of current interest as a new and promising source of biologically active compounds. They produce a variety of metabolites, some of which can be used for drug development (10). Marine actinomycetes yielded long list of breakthrough medicines derived from secondary metabolites produced by these organisms. Each actinomycete strain has the genetic potential for producing 10-20 secondary metabolites (11.They are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific (12). *Streptomyces* are widely used in industries due to their ability to produce numerous chemical compounds including antibiotics and other therapeutic agents (13). The species belonging to the genus *Streptomyces* constitute 50% of the total population of actinomycetes and 75% of molecules with antibiotic activity are produced by this genus (14). *Streptomyces* constitute 75-80% of the commercially and medicinally useful antibiotics which have been derived from this genus (15). In the present investigation the actinomycete isolate was characterized and identified and the antifungal activity of the culture broth and acetone crude extract obtained from SP was tested against *C. albicans* under *in vitro* conditions.

Materials and methods

Sample collection and processing

Marine sediment samples were obtained from five different locations at the Pudduchery coast, Bay of Bengal, India. From each location, 15g of sample was collected at 50 to 100 cm depth from the surface. These samples were placed in small pre-labeled plastic bags and tightly sealed. Air dried for one week to avoid bacterial contamination and it was subjected to serial dilution (up to 10^{-6} dilution) by adding 1g of soil sample in 10 ml of distilled water (16)..

Isolation of actinomycetes

About 0.1ml of each dilution was pour plated on Starch Casein Agar (SCA) media and incubated for one week at room temperature. After incubation, actinomycetes isolates were distinguished from other microbial colonies by characteristic white powdery colonies (17). Based on the colony morphology, eight strains of actinomycetes were isolated. The isolates were then purified by streaking on ISP 1 agar plate (Tryptone yeast extract agar).

Screening of actinomycetes for antifungal activity

The isolates were inoculated on ISP 1 broth and incubated for 10 days at 28 ˚C in incubator shaker. Isolates were screened against six fungal strains (*Candida albicans* MTCC 183*, Aspergillus niger* MTCC 281*, Aspergillus flavus* MTCC 277*, Aspergillus fumigatus* MTCC 343*, Rhizopus* MTCC 262*, Mucor* MTCC 157*)*. The test organisms were inoculated in Sabouraud dextrose broth for one day before the test and swabbed over the Sabouraud dextrose agar media. Using a cork borer of width 7mm, wells were made without disturbing the solidified agar in the plate. To the wells, 10 days old broth culture of actinomycetes was added. Amphotericin-B (25 µg/disc) was used as standard antifungal antibiotic. After 24 hrs of incubation the diameter of the zone of inhibition (mm) was measured. The effective strain which showed greater inhibition over amphotericin-B was selected for further studies.

Cultural characterization

The effective isolate was characterized by morphological, physiological and biochemical properties (18). in International Streptomyces project (ISP) and Bergey's manual of systemic Bacteriology.Various broths such as Kuster's broth, Nutrient broth, Soybean casein digest broth, Nitrate broth, Glycerol yeast extract broth, Yeast extract dextrose broth, Tryptone yeast extract broth and Bennet's broth were used as the base to determine the optimal nutritional and cultural conditions for maximal growth and production of secondary metabolites was determined based on the dry weight (19). Various media including ISP media, Nitrate agar, Nutrient agar, Kuster's agar, Zobell Marine agar, Yeast Extract Dextrose agar, Kenknight and Munaier's agar, Czapek Dox agar, Potato Dextrose agar, Soybean casein digest agar, Muller Hinton agar, Starch Casein agar, Sabouraud Dextrose agar, Bennet's agar, MacConkey agar and Actinomycetes Isolation Agar were tested for maximal growth of the isolate.

Phenotypic Characterization

Aerial mass colour and reverse side pigments

The mature sporulating aerial mycelium colour was recorded in Oat meal agar (ISP 3), Yeast extract malt extract agar (ISP 2), Inorganic salt starch agar (ISP 4), Glycerol asparagine agar base (ISP 5), Tyrosine agar base (ISP 7), Starch casein agar and Czapek dox agar (13). The reverse side pigments of the colony, namely distinctive (+) and not distinctive (-) was tested using Peptone yeast extract iron agar (ISP 6) (13).

Spore chain and surface morphology

The arrangement of spores in the mycelium was observed under 1000x magnification by cover slip method using a well grown sporulated culture plate. The spore surface morphology of the mycelium was observed in 14 days old culture under scanning electron microscope (13)

Physiological and biochemical characterisation

The ability of the isolate to utilize various carbon and nitrogen sources were studied by the method recommended in International Streptomyces project. Carbon sources like mannitol, fructose, xylose, sucrose, raffinose, inositol, arabinose and rhamnose were tested on Carbon utilization agar (ISP 9) supplemented with 1% carbon sources (20). The ability of the isolate to utilize various nitrogen sources like leucine, histidine, tryptophan, serine, glutamic acid, lysine, arginine, methionine and tyrosine for growth were also tested.

Sodium chloride tolerance

Different concentrations of sodium chloride (0, 2, 5, 10, 12, 14, 16, 20, and 25%) was added to the ISP 1 media and plated. The plates were incubated at 28 ˚C for 7 -14 days and salt tolerance was tested

Effect of temperature and pH

The growth of the isolate on ISP 1 media incubated at different temperatures (20˚C, 28 ˚C, 37˚C and 45˚C) and pH (5, 6, 7, 8, 9, 10, 11 and 12) was tested to identify the optimal temperature and Ph.

Antibiotics sensitivity test

Standard antibiotic discs, streptomycin, ceftazidime, bacitracin, chloramphenicol, vancomycin, erythromycin, co-trimoxazole, ampicillin, tetracycline, amphotericin–B, fluconazole, nalidixic acid, carbenicillin and amoxycillin of different units were placed on the plates containing ISP 1 media swabbed with 7 – 14 days old actinomycetes broth culture. The plates were then incubated for 5-7 days and the zones of inhibition were measured (19)

Hydrolysis tests

The hydrolysis of starch by the isolate was tested on glucose starch agar plates. After streaking incubated for 4–5 days and amylase production in the plates (clear zone around the streaked line) were tested by adding iodine solution. Gelatin hydrolysis was tested on nutrient broth mixed with gelatin (12%), boiled and cooled then SP was inoculated. After 3 days of incubation at room temperature, it was refrigerated for 15 minutes at 4˚C and the formation of liquefied media indicates gelatin hydrolysis. Casein hydrolysis was tested on Skim milk agar plate streaked with the isolate. After incubation the degradation of casein is indicated by a clear transparent zone of inhibition around the streaked lines. Esculin hydrolysis was tested by streaking the isolate on Esculin agar and the appearance of black colour indicates esculin degradation.

Preparation of the crude extract

The isolate was inoculated on ISP 1 broth and incubated for six days. It was then centrifuged and the supernatant (cell free extract) collected was mixed with equal volume of ethyl acetate and kept in a shaker for 24 hrs. It was separated in a separating funnel and concentrated in rotary evaporator and air dried. It was dissolved in know volume distilled water. The pellet containing cells was extracted in acetone and both the crude extracts were tested for antifungal activity against *C. albicans*.

Results

Out of eight isolates only one isolate was active against *C. albicans*. The culture broth of the isolate (100 µl) produced the zone of inhibition of 20mm whereas amphotericin B (25 µg/disc) showed 8 mm (Figure 1) against *C.albicans.*

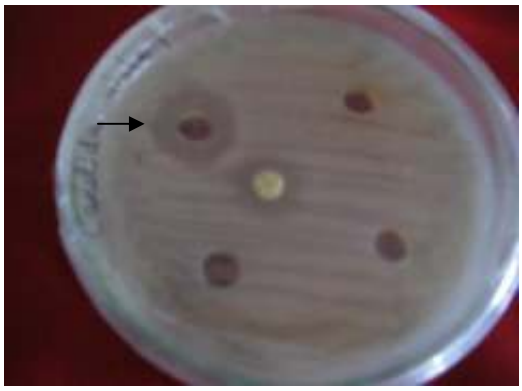


Figure 1. Anticandidal activity of *Streptomyces paraguyensis* by well diffusion method. The zone of inhibition by the isolate was indicated by arrow and compared with the standard, amphotericin-B (25 µg/disc).

The growth of the isolate in various agar media, aerial mass colour and reverse side pigment is given in Table 1. The isolate produced white powdery colonies in all agar

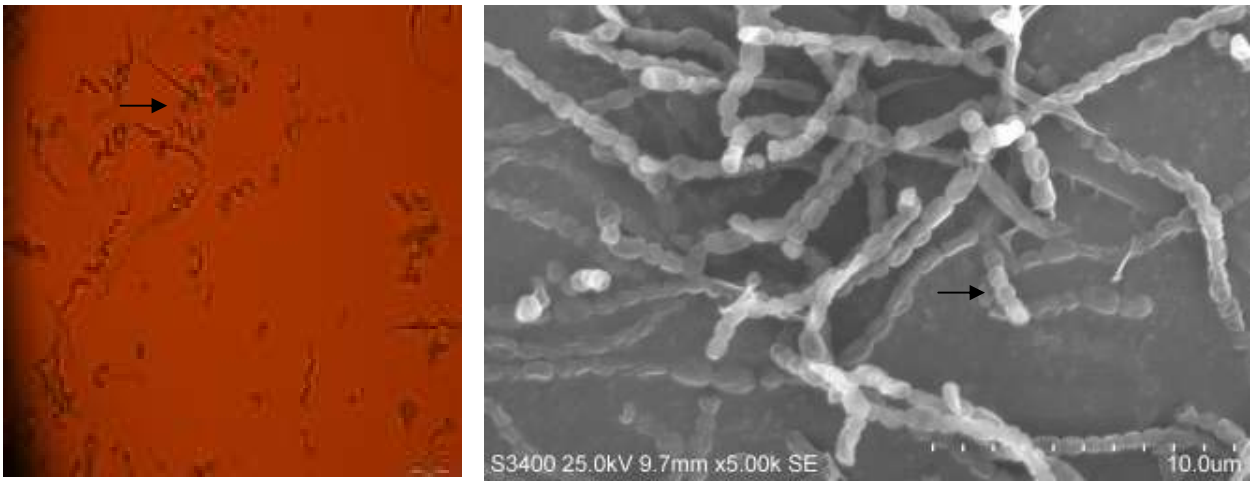
plates tested. The reverse side pigment, reddish orange was observed only in ISP 6 media.

Table 1. Growth characteristcs of the isolate on various culture media

Media	Growth	Mycelium colour		Reverse side pigment	Soluble pigment
		Aerial	Substrate		
Tryptone yeast extract agar (ISP 1)	++	White	Pale yellow	---	---
Yeast extract + malt extract agar (ISP 2)	++	White	Pale yellow	---	---
Oat meal agar (ISP 3)	+	White	Pale yellow	---	---
Inorganic salt starch agar (ISP 4)	±	White	Grey	---	---
Glycerol asparagine agar base (ISP 5)	±	White	White	---	---
Peptone yeast extract iron agar(ISP 6)	++	White	Orange red	Reddish orange	---
Tyrosine agar base (ISP 7)	±	White	White	---	---
Nitrate agar (ISP 8)	++	White	Pale yellow	---	---
Carbon utilization agar (ISP 9)	+	White	White	---	---
Kenknights agar	±	White	White	---	---
Czapek dox agar	+	White	Light yellow	---	---
Soybean casein digest agar	++	White	Pale yellow	---	---
Marine zobile agar	+	White	White	---	---
Yeast extract dextrose agar	±	White	White	---	---
Bennets agar	±	White	Dull white	---	---
Nutrient agar	+	White	Yellow	---	---
Kustar's agar	+	White	White	---	---
Muller hinton agar	±	White	White	---	---
Starch casein agar	±	White	Dirty white	---	---

(++) - Abundant Growth; (+)- Good Growth; (±)- Moderate Growth; (---) – No colour

The arrangement of the spores in the mycelium was found to be spiral under light microscope, 1000X magnification (Figure 2 A) by cover slip technique and the surface morphology of spores in the mycelium under scanning electron microscope (Figure 2 B) revealed that the spore surface is smooth.



A B

Figure 2. Spiral spore chain morphology (A) (arrow) under light microscopy (1000x magnification) and smooth spore surface morphology (B) (arrow) under scanning electron microscopy.

Media optimization based on the dry weight (w/v) of the culture grown on various broth cultures revealed that Kuster's agar (1.34 g) was found to be more efficient in promoting the growth of the isolate followed by Nutrient broth (1.09 g). Studies on the requirement of carbon and nitrogen sources for growth showed that inositol and arabinose are needed as carbon sources for abundant growth of the isolate and tyrosine was the best nitrogen source when compared to other amino acids. The salt tolerance for growth of the isolate was 1-12% Na Cl concentration with the optimal salt tolerance of 2%. The optimal temperature for maximal growth of the isolate was 28°C and the pH range of 7.0-10.0 with the optimal pH of 8.0. Biochemical characterization of the isolate indicates that the isolate is gram positive, hydrolyses starch, gelatin, casein and esculin.

The isolate was sensitive to antibiotics including erythromycin (45mm), tetracycline(40mm), vancomycin (38mm), streptomycin (30mm), carbenicillin (30mm), and chloramphenicol (20mm) and resistant to ceftazidime, co-trimoxazole, amphotericin–B, fluconazole and nalidixic acid.

The crude acetone extract obtained from the pellet (1000 µg/ml) showed the zone of inhibition of 17 mm whereas amphotericin-B (25 µg/disc) showed the zone of inhibition of 8 mm against *C. albicans* (Figure 3). The ethyl acetate crude extract prepared from supernatant (cell free extract) did not show any inhibition against *C. albicans*.

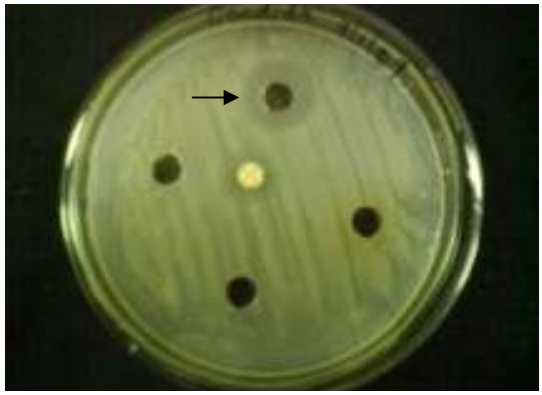


Figure 3. Antifungal activity of acetone crude extract (1000 µg/ml) of the isolate by well diffusion method against *Candida albicans* (indicated by arrow) and compared with the standard, amphotericin-B (25 µg/disc).

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Based on physiological, biochemical and cultural conditions the isolate was classified and identified by using the key for classification and identification of *Sreptomyces* species included in the International Streptomyces Project (ISP) (20) and identified as *Streptomyces paraguyensis* (Table 2).

Table.2 Classificaion and identification of the isolate by International Streptomyces Project (ISP) key

Characteristics	Test isolate	*S.paraguayensis*
Aerial mass colour	White	White
Melanoid pigment	0	0
Reverse side pigment	1	1
Soluble pigment	0	0
Spore chain	Spiral	Spiral
Spore surface	Smooth	Smooth
Carbon sources		
Arabinose	+	+
Xylose	-	-
Inositol	+	+
Mannitol	-	-
Fructose	+	+
Rhamnose	-	-
Sucrose	+	+
Raffinose	+	+

Discussion

The anticandidal activity of acetone crude extract of SP against *C. albicans* has been reported here. Several nucleoside antibiotics of microbial origin have been reported to be very wide ranging biological activities and effective against *C. albicans* (21). Two urauchimycins A and B, isolated from a fermentation broth of a *Streptomyces* sp.Ni-80, is the first antimycin antibiotic exhibited inhibitory activity against morphological differentiation of *C. albicans* (22). Potent anticandidal activity of Sirolimus, produced by a *Streptomyces hygroscopicus* isolated from Easter Island soil samples have already been reported (23). Anticandidal activity of pure cultures of soil actinomycetes isolated from Kerman Province was effective against C. *albicans* (24). Marine actinomycetes *Nocardiopsis dassonvillei* MAD08 have been shown to be very effective against *Candida* strains, MTCC 227 and other clinical isolates of *Candida* (25). Chitinase isolated from *Streptomyces* sp. DA11 associated with South China sponge *Craniella australiensis* has been shown to possess antifungal activity against *C. albicans*.

The potential strain isolated from marine sediments inhibits *C. albicans,* physiological, biochemical and cultural characterists indicates that ISP 1 media is the optimal media with pH 8.0 and 28 °C for maximal growth. It produces white powdery colonies on agar plates and reddish orange reverse side pigment on ISP 6 media. It requires inositol and arabinose as carbon sources and tyrosine and methionine as nitrogen sources. It is moderately halophylic in nature and the grown up strain produces white aerial mycelium and pale yellow substrate mycelium. It is a gram positive organism, alkaline in nature, positive to urea, capable of hydrolyzing starch, gelatin, casein and esculin. It is highly sensitive to erythromycin and resistant to amphotericin-B. The acetone crude extract of the potential strain is very effective in inhibiting the growth of *C. albicans* under *in vitro* conditions. Based on the physiological, biochemical and cultural characterization results the strain is classified and identified as *Streptomyces paraguyensis* using the key for classification and identification of 458 species of *Sreptomyces* included in the International Streptomyces Project (ISP).

From the results it is clear that the secondary metabolite produced by *Streptomyces paraguyensis* under optimized media and cultural conditions, inhibits the growth of *Candida albicans* under *in vitro* conditions. However, the anticandidal secondary metabolite produced by the *Streptomyces paraguyensis* needs to be studied further with respect to structural and molecular characterization apart from molecular taxonomic and chemotaxonomic characterization of the strain.

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