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ORIGINAL RESEARCH ARTICLE

Studies on Isolation and Characterization of Marine Actinomycete Strain NMR 2 from South East of India

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ABSTRACT

The present research work was aimed to were isolated actinomycetes from marine sediment samples and screened for antimycotic against human pathogenic fungi and optimize the most suitable medium with specific temperature and salinity. The strain NMR 2 showed significant antimycotic activity against *C. albicans* as well as *D. oryzae, C. gloeoporioide and C. lunata.* The isolate NMR 2 were subjected physiological and biochemical properties in different media. Maximal growth well in ISP-7 medium well supplemented with carbon source Sorbitol, Trehalose and incubation temperature of 28˚ C, salt tolerance of 2% and incubation time of 4-7 days designate. The result of this study suggests that the NMR 2 could be a promising source for potent antimycotic agents.

Key words: Actinomycetes, marine sediment, antimicrobial activity, human pathogen.

1. INTRODUCTION

Actinomycetes are aerobic, gram-positive bacteria. They are one of the major groups of soil population and are very widely distributed [1]. It has been estimated that approximately two-third of the thousands of naturally occurring antibiotics have been isolated from actinomycetes [2]. Due to the adaptation of marine actinomycetes it produces the novel bioactive metabolites which have found applications in human medicine.

Candida albicans is a diploid fungus that grows both as yeast and filamentous cells and a causal agent of opportunistic oral and genital infections in humans [3]. Virtually all humans play host to *C. albicans*. This unicellular fungus coexists in our body along with many species of bacteria in a competitive balance. Under normal health, the immune system keeps *Candida* sp. proliferation under control but when immune response is weakened, *Candida* sp. growth can precede unhindered. It is an opportunistic organism, when given the opportunity it will attempt to colonize all body tissues. Even though *Candida* is part of the ecological balance in the body since birth, it is still recognized by the immune system as a foreign body that needs to be controlled. So when over growth occurs, a chronic stimulation to the immune system results, in an attempt by the immune system to regain control. In time, it is believed that this can exhaust the immune system, predisposing one to more serious degenerative processes [4].

The treatment of human mycosis has been a great challenge before the clinicians and dermatologists. The opportunistic fungal infections are increasing with alarming rate. Concomitantly allergic reactions of the skin are increasing day by day. The latter is due to a higher rate of sensitization power of the present generation of antimycotic agents. The potential human pathogenic fungi in general and opportunistic fungal infections in particular are usually treated by the use of drugs are limited to polyenic antifungals of microbial origin, such as azole compounds, flucytosine, and other new antifungal agents. Amphotericin-B remains the drug of choice for treating most fungal diseases because it has a broad spectrum and potent fungicidal activity, in spite of well known side effects [5].

2. MATERIALS AND METHODS

Collection and Isolation of Actinomycetes:

Sediment sample was collected from Pamban bridge marine sediment located near mandapam,

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3 km west of the Rameswaram town. Collected samples were immediately transferred to a sterile polythene bottle sealed, labeled and transported to laboratory. In the laboratory the composite sediment sample was spread in a clean aluminium tray and dried in a hot air oven for seven days at 40°C. The dry sediment sample after removing large debris was transferred to a fresh clean polythene bag, secured, labeled and stored at 4°C until examined.

Actinomycetes from the sediment had been isolated by pour plate technique on Starch-casein agar and starch ammonium sulphate agar after serial dilution in distilled water. Dry colonies of actinomycetes were selected and isolated. Thus isolated colonies had been subcultured in Glycerol based media and stored at -20°C.

Assay of Antimycotic Activity:

NMR 2 strain was selected for the antimicotic activity against fungi pathogen namely,

Candida albicans, Rhizoctonia solani, Fusarium solani, Drechslera oryzae,

Colletotrichm gloeosporioides, Curvularia lunata, Pythium aphanidermatum by cross streak method [6].

In addition to Antimycotic also tested in dual culture as described by Dennis and Webster [7]. Small inoculum of NMR 2 strain was point inoculated on PDA near the periphery of the plates then incubated for ten days at 25± 2°C. After ten days the plates were inoculated with a mycelial plug (5 mm) of actively growing *R. solani, C. gloeospoioides, D.oryzae, F. oxysporium, C. lunata* and *P. aphanidrmatum* plate cultures. Separate plates were inoculated for each fungus. Plates were observed for growth inhibition after 3-8 eight days depending on the fungus. Reduction in radial growth was measured and recorded.

Phenotype characterization:

Characterization of the isolate NMR 2 was carried out according to the methods recommended by International Streptomyces Project (ISP) [8]. Spore chain and sporophore morphology of a mature colony were determined under light microscope.

Growth in different temperatures, NaCl tolerance

Growth in different temperatures was tested by incubating PDA slants inoculated with spore suspension of the test isolates at the following temperatures 20, 25, 30, 35, 40, 45 and 50°C in an incubator for 10days. For salt tolerance studies Glucose-yeast extract-malt extract medium was used as the basic medium. The various NaCl concentrations (w/v) used were: 0%, 2%, 4%, 6%, 8%, 10%, 12% and 15%. The slants were inoculated by streaking the agar surface with a loopful of spore suspension of the Isolates-MNR 2. The inoculated tubes were incubated at 25 ± 2°C for 20 days.

Utilization of different carbon sources

The ability of the test strain NMR2to use 20 different sugars as sole carbon sources for energy and growth was examined in carbon utilization medium suggested by Shirling and Gottlieb [9]. The various compounds were added to the liquid medium to the final concentration of 1%. The inoculated tubes were incubated at 25±2°C for 20 days [10].

Extraction and Purification of the active metabolites

Fermentation of the active isolate-NMR 2 first involved generation of a seed culture in PDB. The seed culture (20ml) was grown with shaking in a 40 ml glass tube for five days at 25 ± 2°C. The seed culture was used to inoculate five 250 ml conical flasks containing 50ml PDB. Each flask was inoculated with 1ml of the seed culture and incubated on a laboratory table (still culture) at 25 ± 2°C for 15 days. After fifteen days the contents from all the five flasks were filtered through a cotton pad to separate the culture broth and the mycelial mass. The mycelial mass was extracted in 50 ml methanol and combined with the culture broth. From the combined filtrate 100 ml broth was sequentially extracted with equal volumes of benzene and ethyl acetate. The aqueous fraction was also retained for bioassay. The solvent fractions were condensed by evaporation. The dry residue was reconstituted in 2ml of the respective solvents. The aqueous fraction was also dried at 35-40°C and reduced to 2 ml. All the three fractions were tested for anti yeast activity by disc diffusion method [11].

Disc diffusion assay

Disc diffusion assay was performed to determine the antimicotic activity of the different solvent fractions. The test organisms used in the bioassay was *C. albicans.* The test organism was grown in 10 ml potato dextrose broth for 24 hours. A 100 µl of the 24h old broth culture of organism was aseptically transferred to 50 ml molten (40°C) PDA in three different flasks. After through mixing the contents of the flask were dispensed into three sterilized 90 mm dia. Petri plates and allowed to cool. Four 6mm dia. sterile discs (Himedia) were loaded with 20µl of each of the

three fractions. Using a sterile forceps the four discs were placed at equidistance on PDA plates seeded with *Candida* sp., The plates were immediately kept at 4°C for at least 4h to allow the diffusion of the metabolites and were then incubated at 25 ± 2°C for 30h. Control plates contained discs dipped in the respective solvents. After 30h the plates were observed for the development of inhibition zones and recorded [12].

3. RESULT AND DISCUSSION

The increase in the variety of pathogens associated with serious fungal infections has not been matched by a corresponding increase in the number of antifungal agents available for their treatment. Amphotericin-B and Azole derivatives are the primary drugs used for treatment of serious fungal infections [13]. However, limitations in the efficacy and /or tolerability of these agents have prompted a search for new drugs that may be effective in the management of patients with mycoses due to a wide range of filamentous fungi and yeast pathogens. In the present investigation marine sediment actinomycetes collected from Pamban bridge, Mandapam post Ramananthapuram district, Tamilnadu, India [Latitude (N) 9° 17' N and Longitude (E) 79° 22 ' E]. A total of 49 actinomycetes of different colour series and morphology were obtained from marine sediment.

In this study on of the promising isolates actinomycets strain NMR 2 is characterized partially and screened for bioactive potential. As well, strain NMR 2 inhibited the unicellular fungus *C. albicans* filamentous fungi *C. gloeoporioides*, *C. lunata*. Antifungal activity against *C. albicans* as well as actinomycetes isolated from mangrove sediments of Pichavaram southeast coast of India exhibiting prominent antibiotic activity against *C. albicans* [14, 15].

In addition to anticandida and antibacterial activity the actinomycetes were also tested for antifungal activity towards filamentous fungi in dual culture [8]. Cultural characteristics of the antagonistic actinomycete strain NMR 2 was determined on eleven different types of media recommended by ISP and others. The data are summarized in Table 1. Strain NMR 2 grew well on ISP-4, ISP-5, ISP-7, potato dextrose agar (PDA) and Bennet's agar (BA). Moderate growth was noticed on ISP-2, ISP-3 and starch casein agar (SCA). No growth occurred on ISP-1 nutrient agar (NA) and Czapek's agar (CZ). Aerial mycelium was grey or grey mixed with rose or black in almost all the media tested. Basal mycelium was yellow to light yellow in all media except in ISP-2. Similarly reverse side of the colony was light yellow to dark yellow in all media tested. ISP-7 only produced melanin pigment. However, yellow colored diffusible pigment was observed in all media except ISP-2 and SCA.

Table 1: Biochemical and physiological characteristics of actinomycetes strain NMR 2

Reaction	Response	Result
Starch Hydrolysis	Zone appeared	Positive
Casein hydrolysis	Zone appeared	Positive
Urease	Phenol red to deep pink	Positive
Catalase	Brisk effervescence	Positive
Growth		
20˚C	+	Poor growth
25˚C	++	Moderate growth
28˚C	+++	Good growth
35˚C	++	Moderate growth
40˚C	-	No growth
50˚C	-	No growth
NaCl Tolerance (%)		
0%	+++	Good growth
2%	++	Moderate growth
4%	++	Moderate growth
6%	+	Less growth
8%	-	No growth
10%	-	No growth
12%	-	No growth
15%	-	No growth

Correspondingly, *Nocardiopsis* sp maximal with the use of ISP 1 medium supplemented with sea water, pH 7.4, and incubation temperature of 28 C, salt tolerance of 2% and incubation time of 4-7 days as well as actinomycete strain grown on ISP medium at 28°C for 7 days but moderate growth on the synthetic media [16, 17].

Physiological and biochemical properties of the isolates NMR 2are presented in Table 1. The colony well grew abundantly on the medium without NaCl (0%); moderate growth occurred at 2% and 4% NaCl concentrations. The antagonist grew between 20 ºC to 35 ºC with optimum at 28 ºC. The isolate was catalase positive and could hydrolyse starch, casein and lipid.

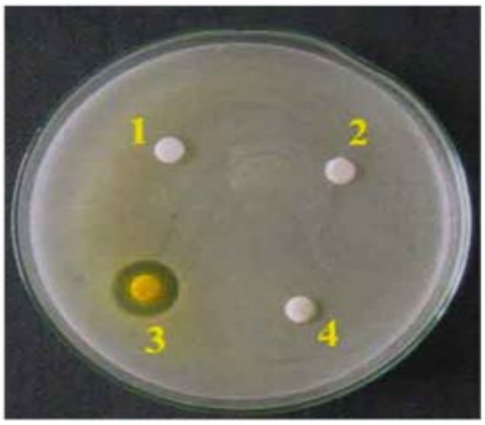
The results on the ability of the isolate NMR 2 to utilize 20 different sugars as sole source of carbon is presented in (**Table 2**). The actinomycete grew better in sorbitol and trehalose. Moderate growth was observed in cellobiose, dextrose, fructose, inositol, maltose, melibiose, rhamnose, sucrose, adonitol dulcitol, inulin and salicin. No growth in galactose and lactose.

Table 2: Carbon utilization pattern of the strain NMR2

Utilization	Carbon source
Positive	Sorbitol, Trehalose (+++)
Moderate	Cellobiose, Dextrose, Fructose, Inositol, Maltose, Melibiose Rhamnose, Sucrose (+++), Adonitol, Dulcitol, Inulin, Salicin (++)
Weak positive	Mannitol, Mannose, Raffinose, Xylose (+)
Doubtful	Galactose, Lactose

+ (Less growth), ++ (Less to moderate), +++ (moderate), ++++ (Good), +++++ (very good)

The antimicotic efficacy of the isolates was tested by using benzene, ethyl acetate and aqueous fraction produced the maximum inhibitory zones against *C. albicans*. Maximum zone of inhibition showed against ethyl acetate extract and there was no inhibition in benzene and aqueous extract illustrate in (**Fig 1**). Similarly, various solvents were used for the extraction of antibiotics from actinomycetes by many workers using ethyl acetate as the best solvent for the extraction of bioactive compounds from actinomycetes [18, 19]. The results of this study indicate that the marine sediments are a potential and new source of additional genera and species of actinomycetes in the search for novel metabolites that can be used in human health care and for other applications.



1 -Control; 2 - Benzene; 3-Ethyl acetate; 4 - Aqueous

Figure 1: Antimicotic activity of actinomycetes strain NMR2 against *C. albicans*

4. CONCLUSION

Based on result it can be concluded that the pigmented strain NMR 2 isolated from the Pamban bridge marine sediment has broad spectrum of antifungal activity. The isolate was designated as *Streptomyces* sp. based on the results of biochemical, cultural and morphological characterization. Further the isolate was found to be a promising source for producing antibacterial antibiotic which needs to be studied further. Molecular taxonomic identification of the isolate NMR 2, pigment extraction, purification, identification of chemical nature of the compound are under progress.

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