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Actinomycetes of Loktak Habitat: Isolation and Screening for Antimicrobial Activities

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Abstract: A total of 37 actinomycetes with distinct characteristics were isolated from the soil sample collected from the Phoomdi in Loktak lake of Manipur, India. These isolates were screened for their antimicrobial activity. Out of 37 isolates, only 21 showed antimicrobial activity against test microorganisms in primary screening process by spot inoculation technique on agar medium. These 21 putative isolates were then subjected to Submerge culture and their antimicrobial activity was evaluated. Of these 21 isolates, 12 were found to be active against the test microorganisms namely *Bacillus subtilis, Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, Micrococcus luteus, Mycobacterium phlei, Candida albicans, Fusarium moniliforme.* Most of these active isolates showed to possessed antifungal property. These 12 active isolates were identified to be of the genus *Streptomyces.* Isolate no. A2D was found to have a broad spectrum of activity against all the tested microorganisms. The antibiotic profile of this isolate underlined its potential as a source of novel antibiotic.

Key words: Phoomdi, Actinomycetes, *Streptomyces*, antimicrobial activity

INTRODUCTION

Actinomycetes are prokaryotes having high G+C content in their DNA, with extremely various metabolic possibilities. The metabolic diversity of the Actinomycete family is due to their extremely large genome, which has hundreds of transcription factors that control gene expression, allowing them to respond to specific needs (Goshi *et al*., 2002). They are noteworthy as antibiotic producers, making three quarters of all known products; the *Streptomyces* are especially prolific and can produce a great many antibiotics and other class of biologically active secondary metabolites. They cover around 80% of total antibiotic product, with other genera trailing numerically; *Micromonospora* is the runner up with less than one-tenth as many as *Streptomyces.* If we include secondary metabolites with biological activities other than antimicrobial, actinomycetes are still out in front, over 60%, *Streptomyces* spp. accounting for 80% of these (Kieser *et al*., 2000).

The majority of Actinomycetes are free living, saprophytes found widely distributed in soil, water and colonizing plants. Actinomycete population forms an important component of the soil microflora. According to the estimate of Alexander (1961) 70-90% of the Actinomycetes in virgin and cultivated soils are *Streptomyces* species. *Streptomyces* strains are also found to occur in fresh water and marine environments (Cross, 1981; Okami and Okazaki, 1978; Weyland, 1981). Actinomycetes hold a prominent position as targets in screening programme due to their diversity and their proven ability to produce novel antibiotic and other nonantibiotic lead molecules of pharmaceutical interest. Since the discovery of actinomycin, the first antibiotic from an Actinomycete, many commercially important bioactive compounds and antitumour agents have been produced using Actinomycetes (Tanaka and Omura, 1990; Waksman and Lechevalier, 1962). The list of novel Actinomycetes and products derived from poorly explored areas of the world, stresses the importance of investigating new habitats (Nolan and Cross, 1988).

The isolation and screening of Actinomycetes from diverse ecological niches of vast untapped Indo-Burma belt of North-eastern India deserves special attention to explore the potentialities of the diverse micro flora of this region, as North-eastern India being a part of the Indo-Burma biodiversity hot spots (Myers *et al*., 2000). In view of the above, soil *Streptomyces* endowed with novel antibiotic property has been isolated from tea garden soil of Assam, India (Bordoloi *et al*., 2001, 2002*).* One of the successful approaches to obtain new types of useful microbial metabolites is to carry out exploration on the untapped Phoomdi (a Manipuri word meaning floating mats of soil and vegetation) of the fresh water Loktak lake of Manipur, India, where a unique ecological condition prevails. Phoomdi is a habitat with heterogenous mass of

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soil, vegetation and organic matter in different stages of decay (Sanjit *et al*., 2005). The present study is a part of the unexplored biodiversity of Loktak lake of Manipur for isolation and characterization of the Actinomycetes, which can be a milestone in the discovery of novel antibiotic producing strains.

MATERIALS AND METHODS

Location and collection of soil sample: Soil samples were collected at a depth of 10-15 centimeters from Phoomdi in Loktak lake (24° 25' - 24° 42' N; 93° 46' - 93° 55' E), which is the largest Natural lake in eastern (Manipur) India (289 km^2). The samples were placed in a sterile bottle, which was brought to the laboratory and used for isolation purposes. The present study was carried out during 2004-2005 at Defence Research Laboratory, Tezpur, Assam (India).

Culture media. A synthetic medium, Starch Casein Agar (SCA), which is composed of soluble starch or glycerol, 10 g; casein, 0.3 g; KNO_3, 2 g; NaCl, 2 g; K_2HPO_4, 2 g; $MgSO_4$. $7H_2O$, 0.05 g; $CaCO_3$, 0.02 g; $FeSO_4$. $7H_2O$, 0.01 g; Agar, 18 g; distilled water, 1,000 mL.; pH 7.2 was used for the isolation of actinomycetes. Actinomycetes Isolation Agar Medium (HIMEDIA Laboratories Pvt. Limited Mumbai, India.) containing Sodium caseinate 0.2%, Asparagine 0.01%, Sodium propionate 0.4%, Dipotassium phosphate 0.05%, Magnesium sulphate 0.01%, Ferrous sulphate 0.0001% and Agar 2% was used for screening by spot inoculation technique. For submerge culture, Glucose Soyabean meal broth (GSB) containing Soyabean meal 1%, Glucose 1%, NaCl 1%, $CaCO_3$ 0.1% and pH adjusted to 7.5 was used as the production medium.

Test microorganisms: The following test microorganisms procured from Microbial Type Culture Collection and gene bank (IMTECH, Chandigarh, India), were used during the investigation: *Bacillus subtilis* (MTCC-736), *Staphylococcus aureus* (MTCC-96) *Escherichia coli* (MTCC-739), *Klebsiella pneumoniae* (MTCC-3040), *Micrococcus luteus* (MTCC-2987), *Mycobacterium phlei* (MTCC-1724), *Candida albicans* (MTCC-227) *and Fusarium moniliforme* (MTCC-2088). The fungi were grown at 28°C on Potato dextrose agar medium and the bacterial cultures were grown at 37°C on Nutrient agar medium. All cultures were stored at 4°C and sub-cultured as needed.

Isolation of Actinomycetes: One gram from the thoroughly mixed soil sample was suspended in 100 mL sterile distilled water and incubated in an orbital shaking incubator at 28°C with shaking at 140 rpm for 30 min. Mixtures were allowed to settle and serial dilutions upto 10^{-4} were prepared. 0.1 mL from 10^{-4} dilution was pour plated (in triplicates) on SCA medium and then incubated at 28°C for 10 days (El-Nakeeb and Lechevalier, 1963; Kuster and Williams, 1964). Individual colonies with characteristics of Actinomycete morphology were isolated and pure culture of the respective isolates were obtained by repeated streaking on SCA plates. The pure isolates were transferred to SCA slants and preserved at 4°C. These isolates were evaluated for their antimicrobial activity.

Screening procedure and *in vitro* antimicrobial bioassay
Spot inoculation on agar medium: The antimicrobial activity was studied primarily by spot inoculating the isolates on agar medium (Shomurat *et al*., 1979). Pure Actinomycete isolates were spot inoculated on actinomycetes isolation agar medium. The plates were incubated at 28°C for 6 days, then inverted for 40 min over chloroform in a fumehood. Colonies were then covered with a 0.6% agar layer of potato dextrose medium (for fungi) and Nutrient agar medium (for bacteria) previously seeded with the test microorganisms. The bacterial plates were incubated at 37°C for 24 h. and fungal plates were incubated at 28°C for 5 days. The zone of inhibition of the test microorganisms was observed after incubation.

Submerge culture: After preliminary testing of the isolates for their antimicrobial potentiality, further studies for the production of antibiotics in liquid medium were carried out in Shake flask condition. The Actinomycete isolates found to be active in the preliminary screening were inoculated into the flask containing GS broth as the production medium and were incubated at 28°C in an orbital shaker (220 rpm). To monitor the activity, aseptically small aliquots of culture broth were taken every 24 h. for 10 days and the activity was evaluated. The 7th day of post inoculation broth, in which the activity reached maximum was taken for the bioassay. The broth cultures were centrifuged at 10,000 rpm for 10 min and the supernatant was tested for extracellular antimicrobial activity by standard well diffusion method against test microorganisms (Gramer, 1976).

Well diffusion method: For evaluation of antimicrobial activity of broth culture, by using a sterile cork borer, wells (6x4 mm) were punctured in fresh test microbial lawn-cultures on Nutrient agar medium for bacteria and Potato Dextrose agar medium for fungi. The supernatant culture broths were then administered to fullness in each well. The bacterial plates were incubated at 37°C for 24 h.

and fungal plates were incubated at 28°C for 5 days. Bioactivity was determined by measuring the diameter of inhibitory zones (mm) of test microorganisms around the well after incubation. Each experiment was repeated three times and the mean value of inhibitory zones was calculated. Blank wells without the broth was taken as control.

Classification and characterization of the active isolates: The 12 active Actinomycete isolates were characterized morphologically to the genus level by comparing the morphology of spore bearing hyphae with entire spore chain as described in Bergey's manual (Locci, 1989). This was done by using cover slip method in which individual cultures were transferred to the base of cover slips buried in SCA medium (Williams and Cross, 1971). The cover slips were removed after 8 days of incubation and the morphology details were observed using 100 X magnification.

For physiological and biochemical characterization, their growth on media having four different concentrations of NaCl (i.e., 2, 5, 7 and 9%), three different pH (i.e., 5, 8 and 9), effect of different temperatures (i.e., 15, 25, 37, 42 and 50°C) on growth, utilization of eleven different sugars and ten biochemical properties were studied following standard methods (Holding and Collee, 1971; Locci, 1989).

RESULTS AND DISCUSSION

Isolation of Actinomycetes: From the soil sample collected from Phoomdi in Loktak lake of Manipur (India), 37 Actinomycete isolates were obtained in pure form and analysed for their antimicrobial activities. The pure cultures were maintained on the same medium that was used for isolation and preserved at 4°C.

Taxonomy of the Actinomycetes: The 12 active isolates were identified upto the genus level and found to be *Streptomyces* sp. (spiral spore chain with coiling and branching) based on the morphological, physiological and biochemical properties. The biochemical and physiological characters of the active *Streptomyces* sp. strain A2D is provided in Table 1 while the morphological features and utilization of different carbon sources is shown in Table 2.

Screening and bioassays: In screening for Actinomycetes having antimicrobial activity, out of 37 isolates, only 21 (57%) isolates showed the activity against test microorganisms while 16 (43%) isolates were found to be non active in primary screening on agar medium. Out of these 21 isolates that were subjected to submerge culture, 12 (57%) isolates were found to exhibit antimicrobial activity while the other 9 (43%) isolates did not exhibit activity in broth culture. The diameter of zone of inhibition of the respective test microorganisms showed by the culture broth of the 12 active isolates is represented in Table 3. It was observed that most of the active isolates possessed antifungal property. Of these, isolate No. A2D is found to exhibit a broad spectrum of activity against all the tested microorganisms.

The putative isolates when subjected to submerge culture showed different activity from that of primary screening. Some of the isolates did not show the activity in the liquid medium. These results were anticipated since similar findings had also been reported by Pandey *et al.* (unpublished data). During the screening of the novel secondary metabolite, Actinomycete isolates are often encountered which show antibiotic activity on agar but not in liquid culture (Personal Communication, Bushell).

Table 1: Biochemical and physiological characters of *Streptomyces* sp. strain A2D

H_2S production	-ve
Methyl red test	-ve
Vogues proskauer test	-ve
Indole	-ve
Citrate utilization	+ve
Starch	-ve
Casein	-ve
Gelatin	+ve
Nitrate reduction	+ve
Urease test	-ve
Growth in the presence of NaCl 2%	+ve
NaCl 5%	+ve
NaCl 7%	+ve
NaCl 9%	-ve
Growth at pH 5	+ve
pH 8	+ve
pH 9	+ve
Growth at temperature 15°C	-ve
Temperature 25°C	+ve
Temperature 37°C	+ve
Temperature 42°C	+ve
Temperature 50°C	-ve

+ ve : Positive, -ve: Negative

Table 2: Morphological features of *Streptomyces* sp. strain A2D and its utilization of different carbon sources

Morphological feature		Carbon source	Utilization
Cell shape	Mycelial	Glucose	+
Sporulation of aerial mycelia	Long chain	Arabinose	+
Spore chain	Spiral	Mannitol	+
Aerial mass color	Creamish white	Xylose	-
		Inositol	+
		Raffinose	+
		Rhamnose	-
		Salicin	+
		Sucrose	+
		Galactose	-
		Fructose	-

+ve: Positive, -ve: Negative, + : Utilization, - : No utilization.

Table 3: Antimicrobial activity of the active Actinomycete isolates by standard well diffusion method

	Test microorganisms							
Actinomycete isolates	a	b	c	d	e	f	g	h
Streptomyces sp. strain 103	20	-ve	-ve	-ve	-ve	-ve	20	-ve
Streptomyces sp. strain 106	20	20	-ve	18	10	-ve	40	-ve
Streptomyces sp. strain 110	25	25	25	20	-ve	10	34	-ve
Streptomyces sp. strain 112	-ve	-ve	-ve	-ve	-ve	-ve	20	-ve
Streptomyces sp. strain 115	-ve	-ve	-ve	-ve	-ve	-ve	34	-ve
Streptomyces sp. strain 118	20	32	-ve	-ve	-ve	12	-ve	-ve
Streptomyces sp. strain 124	-ve	-ve	-ve	-ve	-ve	-ve	40	-ve
Streptomyces sp. strain 146	-ve	-ve	-ve	-ve	-ve	-ve	34	20
Streptomyces sp. strain 149	-ve	-ve	-ve	-ve	-ve	-ve	32	11
Streptomyces sp. strain 160	-ve	-ve	-ve	-ve	-ve	-ve	22	-ve
Streptomyces sp. strain 161	14	20	-ve	-ve	27	15	24	24
Streptomyces sp. strain A2D	15	25	21	23	32	20	10	10

a: *Bacillus subtilis*; b: *Staphyllococcus aureus*; c: *Escherichia coli;* d: *Klebsiella pneumoniae*; *e*: *Micrococcus luteus;* f: *Mycobacterium phlei;* g: *Candida albicans*; h: *Fusarium moniliforme*; -ve: No activity; Numericals indicate mean value of the diameter zone of inhibition in mm

The present finding highlights the importance for further investigation towards the goal of obtaining novel antimicrobial agent out of the Streptomycetes from this untapped habitat. The land of Manipur, being an unexplored area in this field, with unique ecological niches and rich in biodiversity, the microbiology of the Phoomdi soil has to be further explored in order to get benefit out of the precious biowealth, because any new antibiotics and its producing organisms have been a great demand from the health care point of view to combat against the existing and emerging drug resistant pathogens. The emergence and dissemination of antibacterial resistance is well documented as a serious problem worldwide (Cohen, 2000; Gold and Moellering, 1996; WHO, 2001). The emergence of bacterial resistance threatens to return us to the era before the development of antibiotics (Smith *et al*., 1999). The perspective of rapid emergence of drug resistance among bacterial pathogens shows that the potencies of prevalent antibiotics are decreasing steadily, leading to reduced useful-period of drugs. This situation compounds the need for the investigation of new, safe and effective antimicrobials for replacement with invalidated antimicrobials or use in antibiotic rotation programs (Gerding *et al*., 1991; Niederman, 1997; Quale *et al*., 1996). The antimicrobial spectrum exhibited by isolate no. A2D highlights its potential and could be a candidate in this regard. Further study on the bioactive metabolites produced by isolate No. A2D, which exhibits a wide spectrum of activity is under progress. Moreover, this study gives the first hand information on the antimicrobial activity of Actinomycetes from the Phoomdi soil in Loktak lake of Manipur, India.

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