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Studies on Bioactive Actinomycetes in a Niche Biotope, Nambul River in Manipur, India

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Abstract

As part of our ongoing studies on actinomycete diversity in Manipur, an underexplored zone falling in the Indo-Burma biodiversity hotspot, this paper reports bioactivity screening and characterization of bioactive actinomycetes from Nambul River. Bioprospecting studies on actinobacteria have been largely focused on terrestrial and, more recently, on marine ecosystems but freshwater habitats have been largely neglected and studies on freshwater actinomycetes are very scanty in India. Hence we investigated the actinomycete diversity in one of the freshwater rivers of Manipur, Nambul River in Manipur, India. A total of 156 actinomycetes were isolated from three samples of Nambul River. Based on the results of primary screening, 23 isolates were selected for secondary screening. Nine strains showed significant antibacterial or broad spectrum antimicrobial (antibacterial and antifungal) activities in the secondary screening. Phylogenetic analyses indicated that a majority of them were *Streptomyces* species though some rare actinobacteria were also recovered. Seven strains were identified as *Streptomyces* spp. while one strain each was identified as *Nocardia* sp. and *Micromonospora* sp. Three strains showed promising antifungal activities against human and plant pathogens. This study highlights the potential for discovering bioactive actinomycetes in underexplored niche biotopes such as river sediments.

Keywords: Nambul river; Bioactive; Antifungal; Novel species; *Streptomyces*

Introduction

Actinomycetes are a group of physiologically versatile, high GC, gram-positive, filamentous bacteria found in most environments including terrestrial and aquatic habitats [1]. *Streptomyces* has been reported as the dominant genus in freshwater habitats whereas *Micromonospora* and related genera are predominant in freshwater and marine sediments [2].

There is increasing realization of the potential for wetlands as sources of actinomycetes that produce useful bioactive compounds. Cross [3] reported freshwater habitats as promising sources of bioactive actinomycetes. Okami [4] reported that actinomycetes of freshwater origin produce novel bioactive substances. There is an urgent need for screening of novel bioactive compounds from underexplored biotopes such as freshwater habitats. This is also dictated by the rise of emerging diseases and antibiotic-resistant human pathogenic bacteria such as multidrug resistant (MDR) strains of *M. tuberculosis*, vancomycin resistant enterococci (VRE), methicilin resistant *Staphylococus aureus* (MRSA), *Pseudomonas aeruginosa* and *Candida albicans* [5] etc. The focus is increasing towards novel biotopes, niche ecosystems and extreme environments for isolating novel bioactive strains [6] especially actinobacteria which produce nearly 80% of all known antibiotics [7]. Additionally the microbial profiles also serve as an indicator of freshwater ecological health [8].

Materials and Methods

Sampling and pretreatment

Sampling was done from three different sites of the Nambul River, which is one of the major rivers in Manipur (62.7 km in length), originating from Kangchup Hill range in the western side at an elevation of 1830 m above mean sea level. The river flows through the thickly populated area of the city and ultimately discharges into the Loktak Lake. The potentially polluted stretch of the river is within the Imphal Municipality area for a length of about 1.45 km and its tributary Naga Nala for a length of about 1 km. Soils and sediment samples were collected from the Nambul river bank, river bed and the rhizospheric sediments of river water vegetation in polyethylene bags, closed tightly, and stored in a refrigerator before processing.

Pretreatment of the soil samples were carried out by air-drying them at room temperature for about four weeks [9,10].

Enrichment and isolation

To further enrich the actinomycete population, 1.0 g air-dried sediment was mixed with 0.1 g of $CaCO_3$ and kept at ambient temperature for a week to enrich actinomycetes which usually prefer alkaline conditions and also to reduce the contamination of molds and fungi [11]. 1.0 g air-dried sediment was suspended in 99.0 ml of sterile distilled water and incubated in an orbital shaker at room temperature at 150 rpm for 30 minutes. The soil suspension was then serially diluted and 0.1 ml of 10^{-3} to 10^{-7} dilutions were spread plated in duplicates on Starch Casein Nitrate Agar (SCNA, pH 7.2) plates [12] supplemented with 50 $\mu g.mL^{-1}$ each of nystatin and cycloheximide [13] and finally incubated at 28°-30°C for up to 4 weeks.

Selected actinomycete colonies were further purified on SCNA plates and pure isolates were maintained on modified Bennett's agar

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[14] slants at 4°C and as spore suspensions on 20% (v/v) glycerol at -20-°C [15] for further studies.

Antimicrobial assay

Test organisms: The test bacteria used were the Gram positive organisms *Staphylococcus aureus* (MTCC 96), *Micrococcus luteus* (MTCC 106), and *Bacillus subtilis* (MTCC 121), and the Gram negative bacteria *Escherichia coli* (MTCC 739) and *Pseudomonas species* (DN1); and the test fungi used were *Candida albicans* (MTCC 227) and *Aspergillus niger* (MTCC 1344). All the reference strains were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India except for DN1 [16] which is a strain isolated in our laboratory.

Initial antimicrobial assay of the putative actinomycete isolates was carried out using the cross-streak technique [17,18]. Actinomycete isolates which showed inhibition of >50% against the test organisms in the primary screening were further subjected to secondary screening by Kirby Bauer method [19] against the above test organisms. The actinomycete strains showing positive antimicrobial activities were subjected to phenotypic and genotypic characterization.

Biocontrol assay of the bioactive strains

Fungal pathogens were procured from MTCC, Chandigarh except for LSMU1(procured from Life Science Department, Manipur University). The bioactive strains were tested for biocontrol activity by **dual culture method** [20] against the rice pathogens, *Fusarium oxysporum* MTCC 287, *Pyricularia oryzae* MTCC 1477, *Curvularia oryzae* MTCC 2605 and *Bipolaris oryzae* LSMU1.

Phenotypic and genotypic characterization

The various morphological, physiological and biochemical characterization tests were carried out using the standard procedures [21-24]. The micromorphologies of the spore chains and the spore surfaces of 14 days old culture grown on *Streptomyces* agar were determined using Carl Zeiss microscope (AxioScope A.1, Germany, magnification 600X). The cultural properties of the strains were evaluated according to the guidelines of the International Streptomyces Project (ISP) as described by Shirling & Gottlieb [24].

16S rDNA amplification and sequencing were carried out for the bioactive isolates (having an inhibition zone of more than 17 mm diameter against the test organisms) using the primers (8F, 5'-AGAGTTTGATCCTGGCTCAG-3'; 357F, 5'- CTCCTACGGGAGGCAGCAG-3'; 1100R, 5'-GGGTTGCGCTCGTTG-3'; 1492R, 5'-GGTTACCTTGTTACGACTT-3'). The 16S rDNA sequences were submitted to EzTaxon server version 2.1 [25], which contain manually curated databases of type strains of prokaryotes, for sequence analysis. Related strains were selected for alignment by CLUSTAL W program and phylogenetic analyses were done according to the neighbour-joining method [26] using the MEGA version 4.1 [27,28]. To determine the support of each clade, bootstrap analysis was performed with 1000 replications [29].

Results and Discussion

Isolation of actinomycetes

A total of 156 actinomycetes were isolated from the Nambul River, of which 47 (NRB1-1 to NRB1-47) were from the bank, 69 (NRS1-1 to NRS1-69) from the river bed, and 40 (NRP1-1 to NRP1-40) from the aquatic rhizospheric samples.

Test isolates	Test organisms						
	Gram positive bacteria			Gram negative bacteria		Yeast/fungi	
	MTCC 96	MTCC 106	MTCC 121	MTCC 739	DN1*	MTCC 227	MTCC 1344
	Inhibition zone (in mm diameter)						
Standard antibiotic discs	Erythromycin 16	Penicillin-G 18	Amikacin 18	Streptomycin 18	Rifampicin 12	Amphotericin-B 16	Nystatin 13
NRB1-1	-	13±0.29	16±0.76	-	-	-	-
NRB1-9	-	-	15±0.29	-	-	-	11±0.58
NRB1-19		17±0.29	18±0.76	19±1.0	-	-	15±0.76
NRB1-20	-	-	15±0.58	-	-	-	-
NRB1-25	-	-	16±1.0	-	-	-	-
NRB1-29	-	-	15±0.58	13±1.5	-	-	-
NRB1-33	-	-	13±0.29	16±0.58	-	-	-
NRB1-44	16±1.0	-	18±0.76	-	-	-	17±1.0
NRP1-5	-	13±0.58	14±0.5	11±0.76	-	-	-
NRP1-13	-	-	18±1.0	-	-	-	20±0.76
NRP1-14	-	-	-	-	-	20±1.0	21±0.58
NRP1-18	21±0.58	16±0.5	15±0.76	-	-	-	17±0.29
NRP1-20	-	-	15±1.0	-	-	-	-
NRP1-26	22±0.58	15±0.29	16±0.5	18±1.0	-	12±0.76	18±0.76
NRP1-28	-	-	16±0.29	-	-	-	-
NRP1-29	-	-	15±0.76	-	-	-	-
NRP1-35	-	-	18±0.5	16±1.0	-	-	18±0.76
NRP1-40	-	-	13±1.0	-	-	-	-
NRS1-1	-	12±1.5	15±0.29	-	-	-	-
NRS1-11b	18±0.58	20±0.29	17±1.0	18±0.76	-	-	-
NRS1-18	13±0.58	-	15±0.58	17±0.29	-	-	-
NRS1-30	-	-	14±1.0	-	-	-	-
NRS1-39	-	-	16±0.58	15±0.29	-	-	-

Table 1: Secondary Screening profile of the selected isolates exhibiting good antimicrobial activity in primary screening.

Antimicrobial assay

Based on the results of primary screening, 23 strains (11.1%) showed an inhibition zone of more than 50%, against one or more of the test pathogens These isolates were then shortlisted for secondary screening (Table 1). Of 23 strains subjected to secondary screening, 9 (39.1%) isolates (NRB1-19, NRB1-44, NRP1-13, NRP1-14, NRP1-18, NRP1-26, NRP1-35, NRS1-11b and NRS1-18) showed good antimicrobial activities with inhibition zone diameters of 17 mm or more against one or more of the test organisms. Among these bioactive isolates, 2 (NRS1-11b and NRS1-18) were found to be purely antibacterial and 6 (NRB1-19, NRB1-44, NRP1-13, NRP1-18, NRP1-26 and NRP1-35) had broad antimicrobial activities. Interestingly, the strain NRP1-14 specifically showed potent antifungal activity against *C. albicans* and *A. niger*. None of the isolates was found to be bioactive against *Pseudomonas aeruginosa*. Reports of antimicrobial actinomycetes from freshwater habitats are rare. Elliah et al. [30] obtained 30 actinomycete isolates from sediments of Krishna River in Andhra Pradesh, India, of which 16 (53.3%) exhibited excellent antagonistic properties in cross streak method. On detailed submerged fermentation studies, it was found that 12 isolates (40.0%) had antibacterial and 9 (30%) had antifungal activities. Five (16.6%) isolates showed both antibacterial and antifungal activities. Singh et al. [31] isolated 37 actinomycetes from *phoomdi* (floating putrefying vegetation) in Loktak Lake in Manipur, India. Twentyone (56.7%) isolates showed antimicrobial activities against test microorganisms in primary screening. Of these, 12 (32.4%) were found to have broad spectrum (antibacterial and antifungal) activities.

Biocontrol assay of the bioactive strains

Three of the bioactive isolates, i.e NRP1-14, NRP1-18 and NRP1-26, showed antagonistic activity against one or more rice fungal pathogens [32]. These strains also exhibited phosphate solubilizing, siderophore, ammonia production and chitinase activities, showing their potential

Test isolates	P solubilization	IAA	Siderophore	NH_3 Production	Chitinase activity
NRP1-14	+	-	+	+	+
NRP1-18	+	+	+	+	+
NRP1-26	+	+	+	+	+

Table 2: Plant growth promoting characteristics of the antagonistic actinomycete isolates.

Name of the test	NRB1-19	NRB1-44	NRP1-13	NRP1-14	NRP1-18	NRP1-26	NRP1-35	NRS1-11B	NRS1-18
Gram's staining	+	+	+	+	+	+	+	+	+
Production of diffusible pigment	-	-	-	-	-	-	-	-	-
Growth at									
4^{0}C	-	-	-	-	-	-	-	-	-
15^{0}C	-	-	-	+	-	-	-	-	-
25^{0}C	+	+	+	+	+	+	+	+	+
30^{0}C	+	+	+	+	+	+	+	+	+
37^{0}C	+	+	+	+	+	+	+	+	+
42^{0}C	-	+	+	+	+	+	+	-	-
60^{0}C	-	-	-	-	-	-	-	-	-
Growth at pH range									
5.2	+	+	+	+	+	+	+	+	-
7.0	+	+	+	+	+	+	+	+	+
8.0	+	+	+	+	+	+	+	+	+
9.0	+	+	+	+	+	+	W	+	+
10.0	+	+	+	+	+	+	-	+	+
Growth in the presence of									
2% NaCl	+	+	+	+	+	+	+	+	-
5% NaCl	+	+	+	+	+	+	+	+	-
7% NaCl	W	+	+	+	+	+	+	+	-
10% NaCl	-	-	-	+	-	-	W	-	-
Degradation of									
Adenine 0.5%	+	-	-	-	-	-	-	+	+
Guanine 0.05%	-	-	-	-	-	-	-	-	-
Tyrosine 0.5%	-	+	-	+	+	+	-	+	-
Xanthine 0.4%	-	-	-	-	-	-	-	+	-
Hydrolysis of									
Casein	+	+	+	-	+	+	-	+	+
Starch	+	+	+	-	+	+	+	+	+
Urea	-	-	+	-	+	+	-	+	-
Biochemical tests									
Catalase activity	+	+	+	-	-	-	-	+	-
Oxidase activity	+	-	-	-	-	-	-	-	-
Methyl Red (MR)	+	-	+	-	-	-	+	-	-
Voges Proskauer (VP)	-	-	-	-	+	+	-	-	-
Citrate utilization	-	+	-	+	-	-	-	+	-
Indole production	-	-	-	-	-	-	-	-	-
Nitrate reduction	+	-	+	+	+	+	+	+	-
Gelatin liquefaction	-	-	-	-	-	-	-	-	-
H_2S production	-	-	-	-	-	-	-	-	-

Table 3: Biochemical and physiological tests of the bioactive actinomycete isolates.

Media	Isolate →	NRB1-19	NRB1-44	NRP1-13	NRP1-14	NRP1-18	NRP1-26	NRP1-35	NRS1-11b	NRS1-18
ISP1	AM	Cream	Grey	Magenta	Off White	Cream	Cream	Grey	Sandal wood	Reddish
	SM	Cream	Light Grey	Magenta	Cream	Cream	Cream	Grey	Cream	Reddish
ISP2	AM	Pale cream	Cream	Magenta	Cream	Pale Cream	Pale Cream	Grey	Sandal wood	Reddish brown
	SM	Cream	Light Yellow	Magenta	Light Yellow	Cream	Cream	Brown	Light Yellow	Reddish Brown
ISP3	AM	Cream	Cream	P.G.	Pale Cream	Grey	Grey	Grey	Cream	Reddish
	SM	Cream	Brown	P.G.	Cream	Grey	Grey	Brown	Cream	Reddish Brown
ISP4	AM	Cream	Grey	P.G.	Off White	Grey	Grey	Grey	Sandalwood	P.G.
	SM	Cream	Light Grey	P.G.	Light Grey	Grey	Grey	Brown	Cream	P.G.
ISP5	AM	White	Off White	Light range	Off White	White	White	Grey	Cream	P.G.
	SM	White	White	Light Orange	White	Cream	Cream	Grey	Cream	P.G.
ISP6	AM	White	Pale Cream	Magenta	Cream	Pale Cream	Pale Cream	Grey	Cream	Reddish Brown
	SM	Pale Cream	Pale Cream	Cream	Brown	Pale Cream	Pale Cream	Dark Brown	Cream	Reddish Brown
ISP7	AM	Off White	Cream	P.G.	Light Grey	Grey	Grey	Grey	Sandal wood	P.G
	SM	Off White	Light Grey	P.G.	Grey	Grey	Grey	Light grey	Cream	P.G
SCNA	AM	Off White	Grey	Light Orange	Grey	Grey	Grey	Grey	Off White	Orange
	SM	Grey	Brown	Orange	White	Yellow	Yellow	Brown	Yellow	Brown
SA	AM	Cream	Cream	Magenta	Pale Cream	Cream	Cream	Grey	Sandal Wood	Reddish Brown
	SM	Cream	Light Brown	Cream	Cream	Light Brown	Light Yellow	Black	Cream	Brown
TSA	AM	Pale Cream	Pale Cream	Pale Cream	Pale Cream	Pale Cream	Pale Cream	Off White	Cream	P.G.
	SM	Pale Cream	Pale Cream	Pale Cream	Pale Cream	Pale Cream	Pale Cream	Light Grey	Pale Cream	P.G

ISP- International Streptomyces Project, SA – Streptomyces Agar, TSA- Tryptone Soya Agar
AM- Aerial mycelium, SM- Substrate mycelium
P.G. – Poor Growth

Table 4: Growth morphology on different ISP and other actinomycete specific media.

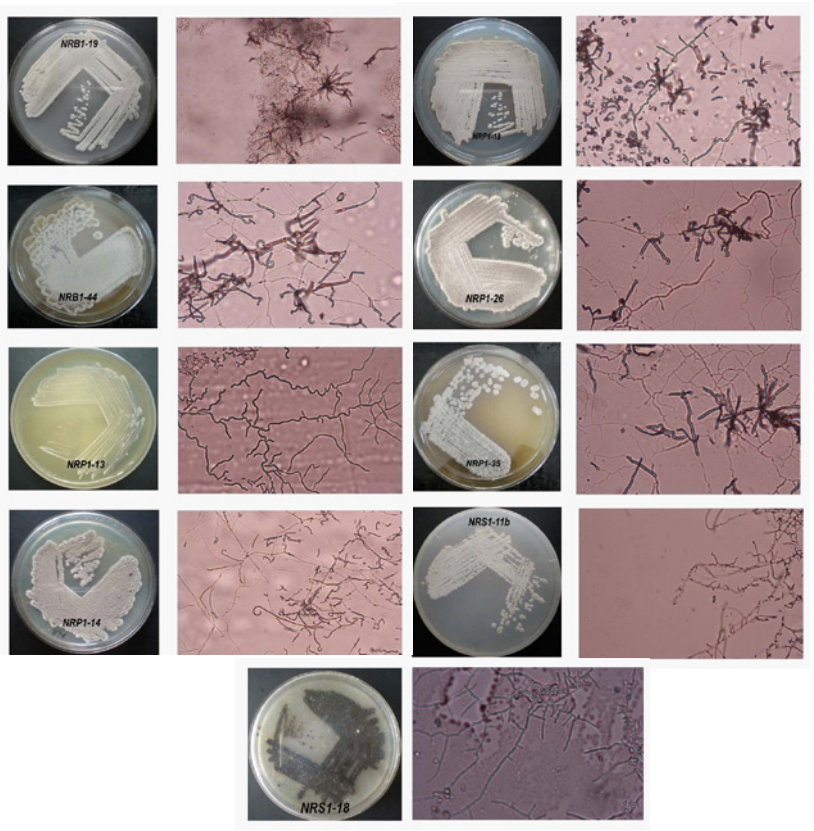

Figure 1: Morphological feature of the bioactive Nambul actinomycetes (NRB1-19, NRB1-44, NRP1-13, NRP1-14, NRP1-18, NRP1-26, NRP1-35, NRS1-11b, NRS1-18) and their micromorphologies.

for plant growth promotion and biocontrol of pathogens (Table 2). These strains also had IAA producing abilities, with the exception of NRP1-14.

Phenotypic and genotypic characterization

Phenotypic characteristics of the bioactive strains and their growth morphologies on different ISP and other actinomycete specific media are shown in (Tables 3, 4.) The gross morphologies of the bioactive strains grown on SCNA media and their micromorphologies are shown in Figure 1.

NRP1-13 grew at 25-42°C, pH 5.2-10, and tolerated up to 7% NaCl while NRS1-18 grew at 25-37°C, pH 7-10 and could tolerate < 2% NaCl. NRB1-19 and NRS1-11B grew well at 15-37° C, pH 5.2-10 and tolerated up to 7% NaCl. Four isolates (NRB1-44, NRP1-18, NRP1-26 and NRP1-35) grew at 25-42° C, pH 5.2-10 (though NRP1-35 grew poorly at pH 10) and tolerated 2-7% NaCl (NRP1-35 could grow even at 10% NaCl). NRP1-14 grew well at 15-42° C, pH 5.2-10 and tolerated 2-10% NaCl. Most isolates were positive for casein as well as starch hydrolysis, except for NRP1-35, which was negative for casein hydrolysis, and NRP1-14, which was negative for both casein and starch hydrolysis.

Results of phylogenetic analyses (Figure 2) of the bioactive actinomycetes revealed that *Streptomyces* was the predominant actinomycete genus among the Nambul river strains, though *Micromonospora* and *Nocardia* were also recovered. Seven strains were identified as *Streptomyces* species. NRB1-19 was most closely related to *Streptomyces parvus* (similarity index 100%), NRB1-44 to *Streptomyces thinghirensis* (similarity index 100%), NRP1-14 to *Streptomyces mutabilis* (similarity index 99.805 %), NRP1-18 to *Streptomyces subrutilis* (similarity index 100%), NRP1-26 to *Streptomyces enissocaesilis* (similarity index 99.728%), NRP1-35 to *Streptomyces drozdowiczii* (similarity index 99.428%) and NRS1-11b *Streptomyces fragilis* (similarity index 100%). NRP1-13 was found to be most closely related to *Nocardia asiatica* (similarity index 99.780%) and NRS1-18 to *Micromonospora chalcea* (similarity index 99.659%).

Rifaat [33] reported the predominance of *Streptomyces* in water sample and that of *Micromonospora* in sediments of the Nile

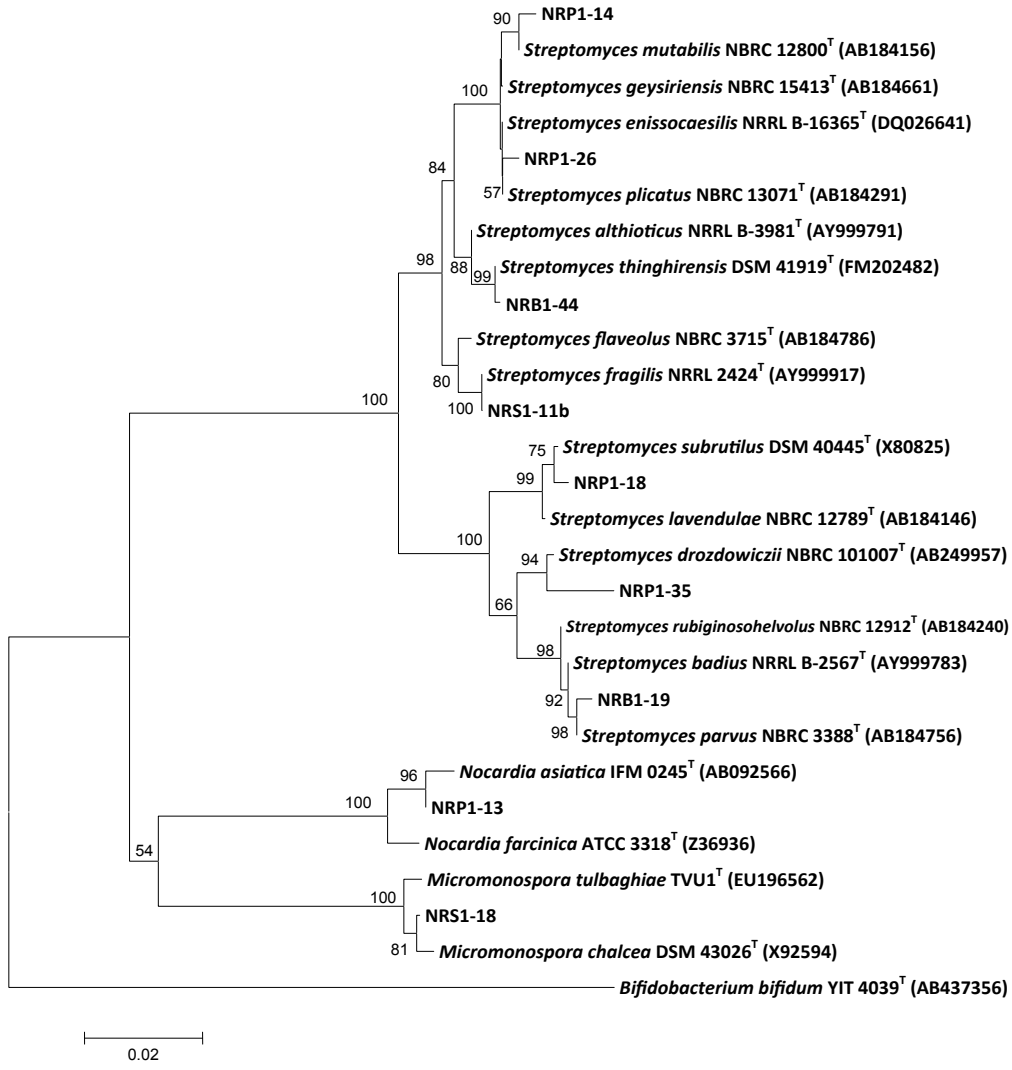

Figure 2: Phylogenetic relationship of the bioactive Nambul actinobacteria with its closest homolog.

River. These *Streptomyces* strains were reported to have significant antimycotic activity. Elliah et al. [30] observed that *Streptomyces* strains, from Krishna river sediments in India, had significant antibacterial and antifungal activities. Our group had earlier showed potential for obtaining bioactive actinomycetes from niche habitats in Manipur including Nambul River [34]. The present study reemphasizes the promise of Nambul as source of antimicrobial actinomycetes. Although, freshwater habitats have been long ignored for actinomycete exploration, several recent reports corroborate the importance of such ecosystems for the search of antibiotic producing actinomycetes. A *Streptomyces* sp. AZ-NIOFD1, with broad-spectrum antimicrobial activity, was isolated from water sample of the Nile River in Egypt by Atta et al. [35]. Cwala et al. [36] reported *Actinopolyspora* sp. TR008, from Tyume River in South Africa which was active against both Gram positive and Gram negative bacteria. Sibanda et al. [37] recently stressed the significance of freshwater habitats as source of bioactive actinomycetes. They obtained actinomycete species belonging to *Sachharopolyspora* and *Actinosynemma* from Tyume River, South Africa. Crude extracts of these strains were found to exhibit potent antibacterial activity against both Gram positive and Gram negative bacteria.

Our preliminary findings showed promise of obtaining bioactive (antibacterial and antifungal) actinomycetes in an underexplored habitat, Nambul River in Manipur, India. Further studies on actinomycete population in the plethora of wetlands in Manipur-lakes, rivers, ponds, and marshes etc.- hold promise for obtaining novel strains, or even species, of bioactive actinomycetes.

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