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ORIGINAL ARTICLE

Characterization and identification of actinomycetes isolated from 'fired plots' under shifting cultivation in northeast Himalaya, India

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Abstract A total of 35 actinomycetes was isolated from soil samples collected after fire operations at agricultural sites under shifting cultivation in northeast India. More than one-half of these isolates were observed in viable but nonculturable (VBNC) state. Five isolates were always seen embedded with slimy bacteria during subculture; 11 morphologically distinct and cultivable isolates were subjected to characterization and identification. The isolates developed circular to irregular colonies of between 3 and 6 mm on tryptone yeast extract agar plates at 28 °C following 7 days of incubation. The isolates could survive at temperatures between 4 and 50 °C (optimum 28 °C), and pH 5–11 (optimum 8). The isolates varied in cell morphology, utilization of carbon sources, sensitivity to antibiotics, and salt tolerance. Based on 16S rRNA sequencing, the isolates revealed maximum similarity to the genus *Streptomyces* (9), and to *Kitasatospora* and *Nocardia* (1 each). Several isolates were found to be positive for production of lytic (chitinase and glucanase) and industrially important (amylase, lipase, and protease) enzymes. The occurrence of actinomycetes in VBNC state and embedded with bacteria was attributed to coping mechanisms associated with these organisms under stress (high temperature) conditions. The cultivable cultures extend the opportunity for further investigations on ecological resilience during fire operations.

Keywords Actinomycetes · *Streptomyces* · Shifting cultivation · VBNC · Ecological resilience

Introduction

In northeast India, the biological diversity of ecosystems is used and conserved by local communities, following traditional ecological knowledge. Shifting cultivation, locally known as *jhum*, is a predominant form of agricultural practice in the hills of northeast India. It is an age-old practice, believed to have evolved several thousand years ago, and still a dominant form of land use in the northeastern states of India and some other parts of the country. Shifting cultivation is a cyclic process that involves (1) clearing land through the burning of natural vegetation, (2) clearing the burnt material, (3) sowing of crops, and (4) leaving the land fallow for several years after a period of crop cultivation (Paul and Paul 2009; Tangjang 2009; Rawat et al. 2010). The 'slash and burn' cycle offers a unique opportunity to study the effect of fire on soil microbial communities, with particular reference to their role in this age old agricultural practice. A recent study, based on physico-chemical and microbial analysis of soil samples collected after fire operations at agricultural sites, highlighted the microbiological merits of shifting cultivation. The study revealed the effect of fire operations on three groups of microorganisms, viz. bacteria, actinomycetes, and fungi (Pandey et al. 2011).

Actinomycetes—Gram-positive filamentous organisms—are thought to be a transition group between bacteria and fungi. These organisms are distributed widely in nature and are well known for their beneficial properties. Many of these

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filamentous soil bacteria, streptomycetes in particular, are known as a rich source of a large number of bioactive natural products, which are used extensively in pharmaceuticals and agrochemicals. Streptomycetes produce about 75 % of commercially and medically useful antibiotics (Berdy 2005). Several species of the genus *Streptomyces* have received attention due to their ability to produce a range of secondary metabolites, including antibiotics and extracellular enzymes (Chater et al. 2010). Production of antifungal compounds, and extracellular hydrolytic enzymes by various species of *Streptomyces* has been studied by several workers, under the major area of biocontrol of plant diseases (Joo 2005; Prapagdee et al. 2008).

Fire, either as a natural or anthropogenic activity, influences microbial dynamics, which, in turn, affects soil fertility. This is likely to lead to changes in responses of biological components of the ecosystem, due to their interdependencies. The overall effect of fire on ecosystems is complex, ranging from reduction of aboveground biomass to belowground physical, chemical, and microbial mediated processes (Neary et al. 1999). In the present study, 11 cultivable actinomycetes isolates, recovered from the soil samples collected after completion of fire operations under shifting cultivation, have been studied for their phenotypic and genotypic characters along with their possible role in biotechnological applications.

Materials and methods

Study site and isolation of actinomycetes

Soil samples were collected after the completion of fire operations (approximately 4 weeks; when the fire had subsided and the soil temperature had come down to ambient), from the village Lobi of Papumpare District, Itanagar, Arunachal Pradesh (latitude 27°06′50.2″N, longitude 93°36′ 40.8″E, altitude 392 m above mean sea level), under shifting cultivation. The fired plots were situated in the midhill, whereas the tophill was observed vegetated with predominant trees, and a variety of bamboos and herbs (Pandey et al. 2011). The composite samples, resulting in five replicates, were used for isolation of microorganisms. The soil was serially diluted and appropriate dilutions were plated on tryptone yeast extract agar (TYA) and actinomycetes isolation agar (AIA). Actinomycetes were isolated from these plates following 1–3 weeks of incubation at 28 °C. Following subculture, pure cultures were maintained on slants (4 °C), and in glycerol stocks (−20 °C).

Culture characteristics

The culture and growth characteristics of the isolates were studied on the following culture media: TYA (ISP medium no.1), tryptone soya agar (TSA), potato dextrose agar (PDA), and AIA, following incubation at 28 °C for 7 days. Observations on colony morphology (size, shape, configuration, elevation, margin, and pigmentation), were recorded on TYA. For cell morphology, Gram staining was performed, and slides were viewed using an Image analyzer microscope (Nikon H550S).

Biochemical and physiological characterization

Biochemical tests, viz. utilization of carbon sources, production of extracellular and intracellular enzymes, and antibiotic sensitivity, were performed following standard procedures. Catalase and oxidase activities were determined by formation of oxygen bubbles with 3 % hydrogen peroxide solution, and by the oxidation of TMPD (tetramethyl-p-phenylenediamine dihydrochloride, provided in the form of discs), respectively. Hydrolysis of starch was performed by flooding Gram's iodine, on 1-week-old actinomycete colonies grown on starch agar (2 % starch), and observing presence or absence of clearing around the colony. Lipase activity was determined by growing the isolates on tributyrin agar (1 % tributyrin as substrate) and observing the absence or presence of a zone around the colony. Citrate utilization was determined by the appearance of a change in color of citrate agar medium. Utilization of carbon sources was determined by a change in the color of Andrade peptone water containing 0.1 % Andrade indicator. The sensitivity to selected antibiotics was tested in TYA at concentrations of 10, 50, and 100 μg/mL.

For physiological growth characteristics, the isolates inoculated on TYA plates were incubated at different temperatures (4, 9, 14, 21, 28, 35, 42 and 50 and 55 °C) and pH levels, i.e., 4–12 (with an interval of 0.5 units) to determine minimum, optimum and maximum temperature and pH requirements. For determination of salt tolerance, the isolates were inoculated on TYA with different salt concentrations (0.5, 2.0, 5.0, 7.5 and 10.0) and incubated at 28 °C for 7 days.

Molecular characterization

DNA extraction and 16S gene amplification

Extraction and purification of DNA from stable enrichment cultures in TYA was carried out by the method of Yates et al. (1997). The 16S rDNA was amplified as described by Sambrook et al. (1989) with eubacterial specific primers 27F (5′-GAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR cycling conditions included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min, followed by a final extension for a

period of 10 min at 72 °C. The amplified PCR product was checked on 1 % agarose in 1 X TAE buffer, and purified with a mixture of 20 % polyethylene glycol (PEG) and 2.5 M NaCl. Sequencing was done using a 96-capillary 3730xl DNA analyzer (Hitachi). The identity of the isolates was determined through a BLAST search. Sequences were aligned using CLUSTAL_X, version 1.81 algorithm (Thompson et al. 1997). Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4.0. The phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987) using the distance matrix from the alignment.

Determination of biotechnological applications

Production of antimicrobial substances

Production of antimicrobial substances by actinomycete isolates was determined following dual culture assays on potato carrot agar (PCA), as described in Chaurasia et al. (2005). *Rhizoctonia solani* and *Cladosporium* sp., isolated from the same study location under shifting cultivation, were used as test fungi. Plates were observed for a zone of inhibition after 7 days of incubation at 28 °C. Production of antimicrobial compounds, viz. siderophore, ammonia, hydrogen cyanide and salicylic acid, was determined following

Table 1 Morphology and growth characters of actinomycete isolates. *Opt* Optimum, *Temp* temperature

Serial no.	Isolate no.	Description (colony morphology, microscopic features, growth characters)
1	NEA 5	Aerial mycelium white, powdery, circular, colony reverse yellow
		Filaments branched, spores oval in short chains
		Temp. range 4-50 °C (opt. 28 °C), pH range 5-12 (opt. 8), salt tolerance up to 7.5 %
2	NEA 15	Arial mycelium brown, smooth, irregular, colony reverse dark brown
		Filaments small, spores oval in single
		Temp. range 4-45 °C (opt. 28 °C), pH 5-12 (opt. 8), salt tolerance up to 7.5 %
3	NEA 19	Aerial mycelium gray, smooth, circular, colony reverse light brown
		Filaments branched, spores oval in single
		Temp. range 9-45 °C (opt. 28 °C), pH 6-11 (opt. 8), salt tolerance up to 7.5 %
4	NEA 21	Aerial mycelium yellow, rough, circular, colony reverse yellowish
		Filaments branched, spores oval in short chains
		Temp. range 4-50 °C (opt. 28 °C), pH 5-12 (opt. 8), salt tolerance up to 7.5 %
5	NEA 24	Aerial mycelium cream, smooth, circular, colony reverse off white
		Filaments branched, spores oval in short chains
		Temp. range 9-45 °C (opt. 28 °C), pH 5-11 (opt. 8), salt tolerance up to 7.5 %
6	NEA 25	Aerial mycelium gray, rough, circular, colony reverse brown
		Filaments branched, spores oval in short chains
		Temp. range 9-45 °C (opt. 28 °C), pH 5-11 (opt. 8), salt tolerance up to 7.5 %
7	NEA 30	Aerial mycelium pink white, smooth, circular, colony reverse pink
		Filaments branched, spores oval in short chains
		Temp. range 9-45 °C (opt. 28 °C), pH 5-11 (opt. 8), salt tolerance up to 7.5 %
8	NEA 32	Aerial mycelium light yellow, rough, irregular, reverse light brown
		Filaments branched, spores oval in short chain
		Temp. range 9-45 °C (opt. 28 °C), pH 5-11 (opt. 8), salt tolerance up to 7.5 %
9	NEA 33	Aerial mycelium gray, smooth, circular, colony reverse gray
		Filaments branched, spores oval in single
		Temp. 9-45 °C (opt. 28 °C), pH 6-11 (opt. 8)
10	NEA 55	Aerial mycelium white, powdery, circular, colony reverse brown
		Filaments branched, oval spores in long chains
		Temp. 4-50 °C (opt. 28 °C), pH 5-12 (opt. 8), salt tolerance up to 7.5 %
11	NEA 70	Aerial mycelium gray, smooth, rough, irregular, colony reverse gray
		Filaments branched, spores oval in single
		Temp. 5-50 °C (opt. 28 °C), pH 5-11 (opt. 8), Salt tolerance up to 7.5 %

procedures described in Trivedi et al. (2008) and Malviya et al. (2009).

Quantification of hydrolytic enzymes

Quantification of chitinase and glucanase were determined following procedures described in Nagarajkumar et al. (2004), using standard assay at 50 °C for 20 min by measuring released reducing sugars from 0.2 % appropriate substrate in 1 M phosphate buffer (pH 6). The amount of released reducing sugars was determined by the method of Nelson (1944). Amylase activity was determined by measuring released reducing sugars from 1 % appropriate substrate at 40 °C, pH 7, for 10 min; released reducing sugars were determined by the dinitrosalicylic acid method (Miller 1959). One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol reducing sugar in 1 min per milliliter. Substrates used in these estimations were: N-acetyl-D-glucosamine for chitinase, glucose for glucanase, and maltose for amylase. Protease activity was determined by a modified method described in Yang and Wang (1999). One unit of protease was defined as the amount of enzyme that produced an absorbance at 280 nm equivalent to 1 μmol tyrosine in 1 min under experimental conditions.

Results

A total of 35 isolates of actinomycetes was isolated from the soil dilution agar plates. More than one-half of these isolates, observed mostly as brown-to-black colored colonies exhibiting production of diffusible pigment, could not be revived during subculturing. Five of these isolates were always observed ‘embedded’ in slimy bacterial growth. Following several rounds of subculturing, only 11 isolates could be obtained as pure cultures. The colony and cell morphology, and growth characteristics of these 11 isolates

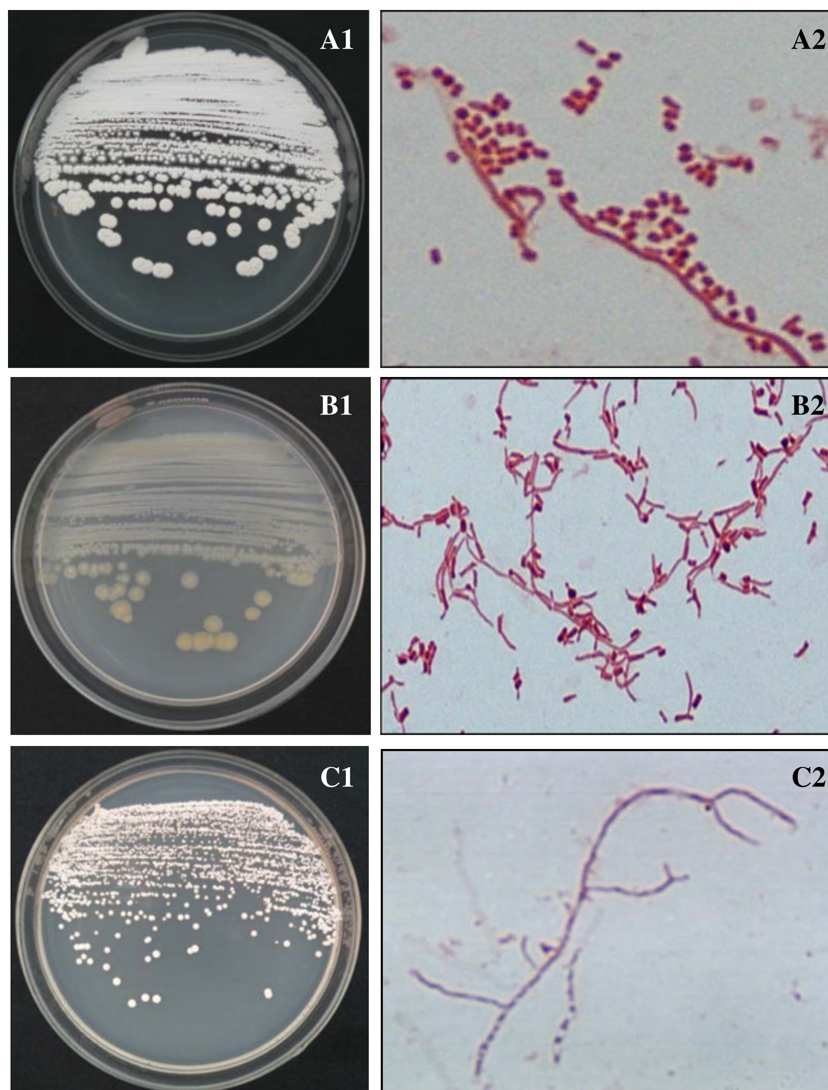

Fig. 1 **A1**–**C2** Colony morphology (*1*) and respective microscopic features (*2*) of three representative genera of actinomycetes isolates (NEA5, NEA15 and NEA30). *Bar* 2 μm

are presented in Table 1. All the isolates developed well grown colonies (3–8 mm), following 7 days incubation at 28 °C. The colonies developed were observed as smooth with aerial and substrate mycelia of varying colors usually with entire margins. All the isolates were observed as Gram positive with branched filaments, many producing oval spores arranged singly or in short to long chains. The colony growth characteristics observed on TYA, along with the respective microscopic features of the three representative genera, are illustrated in Fig. 1. The optimum temperature for growth of all the isolates was 28 °C; however, seven isolates could grow at 5 °C, and five at 50 °C. The optimum pH for growth of all the isolates was 8; none could grow at pH 4 or pH 12. Seven of the isolates tolerated salt concentration up to 7.5 %, while none could grow at 10 % concentration.

Results of biochemical characters (enzyme activity and sugar utilization) and production of antimicrobial substances are presented in Table 2. All the isolates were positive for oxidase and catalase. From 11 isolates, 4 hydrolyzed starch, 6 hydrolyzed casein, and 8 hydrolyzed lipid and gelatin. The isolates varied in terms of utilization of carbon sources such as arabinose, fructose, galactose, maltose, lactose, trehalose, sucrose and mannitol. The isolates varied in terms of production of hydrolytic enzymes, namely amylase, protease, lipase, chitinase and glucanase. The sensitivity of the actinomycete isolates to the concentration of different antibiotics varied. All the isolates showed resistance up to 50 μg/mL ampicillin, penicillin, streptomycin, chloroemphenicol and carbenicillin. Isolate nos. NEA5 and NEA55, tolerated ampicillin, penicillin, streptomycin and chloramphenicol up to 100 μg/mL, while NEA21 and NEA30 tolerated ampicillin and penicillin up to 100 μg/mL. Phylogenetic analysis revealed maximum similarity of nine isolates to genus *Streptomyces*, and one each to *Kitasatospora* sp. and *Nocardia*. The phylogenetic relationship and the similarity index of 11 isolates to the closest species are presented in Fig. 2 and Table 3.

Four of the actinomycetes isolates (NEA5, NEA21, NEA33, and NEA55) produced diffusible substances causing

Table 2 Biochemical characters of actinomycete isolates and production of antifungal substances

Biochemical characters	Isolate no.										
	NEA5	NEA15	NEA19	NEA21	NEA24	NEA25	NEA30	NEA32	NEA33	NEA55	NEA 70
Oxidase	+	+	+	+	+	+	+	+	+	+	+
Catalase	+	+	+	+	+	+	+	+	+	+	+
Gelatinase	+	+	-	+	-	+	+	-	+	+	+
Urease	-	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	+	+	-	-	+	-	-	-	+	-
Nitrate reduction	+	+	-	+	-	+	+	-	+	+	+
Utilization of carbon sources											
Arabinose	+	-	+	-	-	+	-	+	-	-	-
Fructose	+	-	-	-	-	+	+	-	-	+	+
Galactose	-	-	-	-	-	-	-	-	-	-	-
Lactose	-	+	-	-	-	-	-	+	-	-	-
Trehalose	+	-	+	-	-	+	-	+	-	+	+
Sucrose	+	-	-	+	-	-	-	-	-	-	-
Maltose	-	-	-	+	-	-	+	+	-	-	+
Mannitol	+	-	+	-	+	-	+	-	-	+	-
Antagonistic properties (production of antimicrobial substances and lytic enzymes)											
Ammonia	+	+	+	+	+	+	+	+	+	+	+
HCN	-	-	-	-	-	-	-	-	-	-	-
Siderophore	-	-	-	-	-	-	-	-	-	-	-
Salicylic acid	+	-	-	+	-	+	-	-	-	+	-
Amylase	+	+	-	+	-	-	-	-	-	+	-
Lipase	+	+	+	+	-	-	+	+	+	+	+
Protease	+	-	+	+	-	-	-	-	+	-	+
Chitinase	+	-	+	+	-	-	-	-	+	+	-
Glucanase	+	-	+	+	-	-	-	-	+	+	-

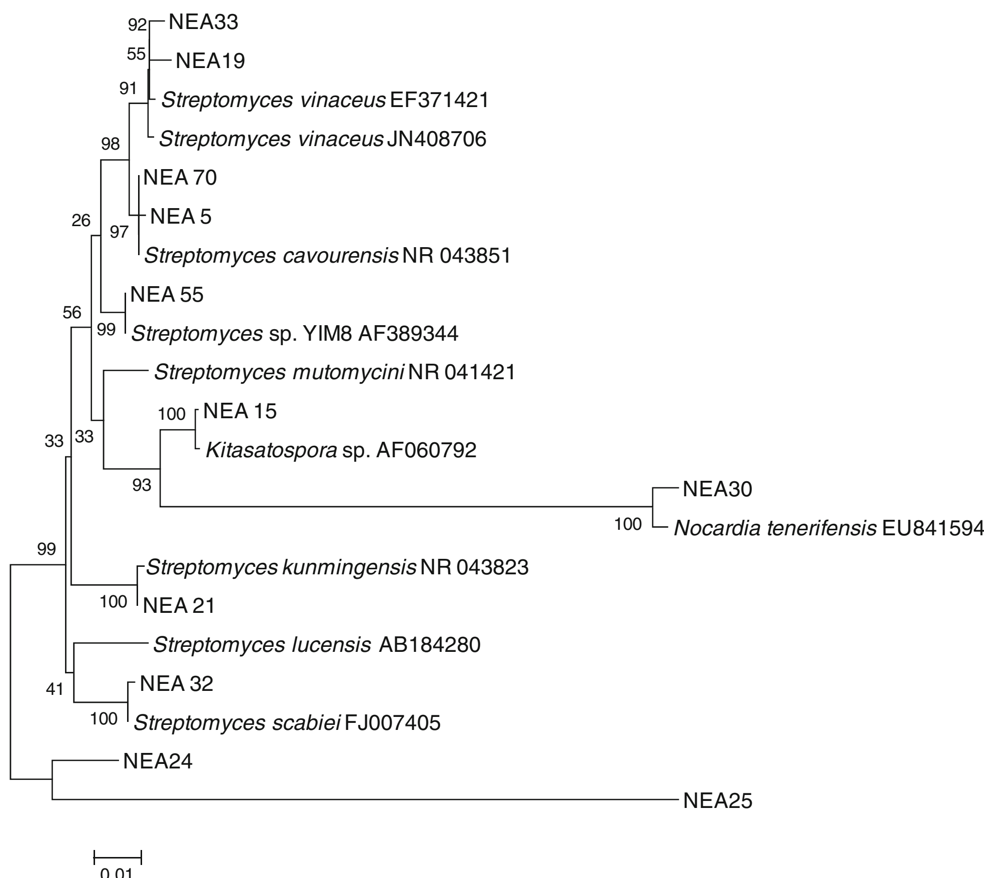

Fig. 2 Phylogenetic tree showing the similarity between 11 isolates of actinomycetes

reduction in radial growth of the test fungi, *R. solani* and *Cladosporium* sp.; NEA5 and NEA55 being the best antagonists. Four isolates were found to be positive for production of salicylic acid, while five were positive for chitinase and glucanase activity. All the isolates were positive for ammonia, while none produced siderophore and HCN. The range of activities measured for four important lytic enzymes are presented in Table 4. The isolates are being maintained in the Microbiology Laboratory of GB Pant Institute of Himalayan Environment and Development, Almora; the best antagonists, NEA5 and NEA55 have been deposited with National Centre for Cell Science, Pune, India (Accession nos.: MCC2003 and MCC2005, respectively). The nucleotide sequences have been accessioned by NCBI (Table 3).

Discussion

Three categories of actinomycetes recovered from soil after completion of fire operations were observed: (1) cultures

Table 3 Phylogenetic relationship of the actinomycetes isolates

Isolate code	Closest neighbor	Similarity (%)	Sequence accession no.[a]
NEA 5	*Streptomyces cavourensis* (NR_043851)	98	JQ627393
NEA 15	*Kitasatospora* sp. AF060792	99	JQ627385
NEA 19	*Streptomyces vinaceus* JN408706	97	JQ627386
NEA 21	*Streptomyces kunmingensis* NR_043823	99	JQ627394
NEA 24	*Streptomyces atrocyaneus* AB184449	99	JQ627395
NEA 25	*Streptomyces lucensis* AB184280	98	JQ627387
NEA 30	*Nocardia tenerifensis* EU841594	98	JQ627388
NEA 32	*Streptomyces scabiei* FJ007405	99	JQ627389
NEA 33	*Streptomyces vinaceus* EF371421	97	JQ627390
NEA 55	*Streptomyces* sp. YIM8 AF389344	99	JQ627391
NEA 70	*Streptomyces mutomycini* NR_041421	97	JQ627392

[a]NCBI http://www.ncbi.nlm.nih.gov

Table 4 Enzyme activity and total protein of actinomycete isolates

Enzyme	Enzyme activity (U/ml)	Total protein (mg)
Chitinase	0.058–0.152	6.00–7.50
Glucanase	0.112–0.231	6.00–8 .00
Amylase	0.251–0.311	7.00–12.00
Protease	0.099–0.193	7 .00–14.00

that probably entered a VBNC state, (2) cultures that were observed 'embedded' in a bacterial film, and (3) cultivable cultures that were obtained in 'pure' form. Absence of ability to grow upon subculturing indicated that a large number of actinomycetes entered the VBNC state. Entering a VBNC state is likely to be a coping mechanism adopted by these actinomycetes for survival against stress caused by high temperature during fire operations. Colwell and Grimes (2000) described the occurrence of VBNC microorganisms under various environmental conditions and its importance for the survival of microorganisms under extreme conditions. Microorganisms under VBNC state are metabolically active but incapable of undergoing propagation under laboratory conditions. These microorganisms can be resuscitated from VBNC state to an actively metabolizing and culturable form (Oliver 2005). Flocculability of a species of *Arthrobacter*—an actinomycete resuscitated from the VBNC state with reference to fermentation conditions—has been reported recently (Su et al. 2012).

Isolation of actinomycetes is, in general, a difficult task. Five of the actinomycetes, which were always obtained embedded with slimy bacterium even following several rounds of subculturing, were initially considered as contaminated. However, the possible occurrence of an ecological association cannot be ruled out; such observations deserve attention in future research. In these cultures, while it was possible to obtain the bacterial partner in 'pure' form, the actinomycete partner could not grow 'singly'. Use of a variety of media, providing a range of nutrients, is often suggested to obtain a large number of isolates. Of the four media used, TYA gave the best results for recording observations on colony morphology. This medium was also considered best for preservation of the isolates through subculturing. Colony and cell morphology, along with microscopic observations on mycelia, spore characteristics and growth requirements, provide useful clues to the group / genus level identification of actinomycetes (Locci 1989).

Physiological and biochemical characteristics, besides providing clues as to identification of the microorganisms for phenotypic characterization, also indicate important characters for biotechnological applications (Taddei et al. 2006; Chater et al. 2010). Utilization of carbon sources and production of antimicrobials and industrially important enzymes give promising clues for applied value for further research on these isolates. The importance of incorporation of different antibiotics into the medium in selective isolation of some genera of actinomycetes, such as *Micromonospora*, *Nocardia*, and *Streptomyces*, has been recognized (Goodfellow and Williams 1983). Inhibition of *Streptomyces* strains by a range of antibiotics has been reported recently (Hamid 2011).

Species level identification was performed following the well accepted polyphasic approach, including phenotypic and genotypic characters of the microorganisms. While nine of the isolates revealed species level similarity in the first instance, two of the isolates (NEA15 and NEA55) showed species level similarity at the third and fourth instances, respectively, in the NCBI blast search. NEA15 showed maximum similarity with *Kitasatospora* sp. C2 (AF060792), followed by *Streptomyces* sp. CS 133 (JN177516) and *Streptomyces aburaviensis* (AB184477). Similarly, isolate NEA 55 showed maximum similarity with *Streptomyces* sp. YIM8 (AF389344.1), followed by *Streptomyces* sp. VTT E-062980 (EU430554), *Streptomyces* sp. QZGY-A9 (JQ812066) and *Streptomyces flavogriseus* ATCC 33331 (CP002475). 16S rRNA sequencing has been used for the rapid identification of actinomycetes up to genus level (Cook and Meyers 2003).

Chater et al. (2010) reported excretion of a range of secondary metabolites by *Streptomyces*, including important antibiotics, often regulated by extracellular signaling molecules. Actinomycetes isolated from various environments, including extreme conditions, have been studied for their taxonomic and antagonistic properties (Ling et al. 2005; Meklat et al. 2011; Sait Byul et al. 2011). Antagonism based studies are important in defining the role of these organisms in the biocontrol of phytopathogenic fungi. A variety of microorganisms, including actinomycetes, has been studied for their plant growth promoting characters, through direct and indirect mechanisms, following biofertilizer preparations (Chang and Yang 2009; Hayat et al. 2010).

Documentation of microbial diversity with reference to their applications, such as formulation of bioinoculants specifically for use under mountain ecosystems, in various ecological niches of the Indian Himalayan Region (IHR) has received much attention in last two decades (Pandey and Palni 1998; Pandey et al. 1999, 2002, 2006; Kumar et al. 2007; Trivedi et al. 2012). The present study is important in view of the documentation of the diversity of microorganisms, actinomycetes in particular, as influenced by fire under shifting cultivation in northeast Himalaya, India. Slash burning—one of the steps under shifting cultivation—is used as a management tool in agriculture and forestry to improve soil fertility. The major findings of this study that need to be addressed in future research are (1) the occurrence of large number of actinomycetes in VBNC state, which can be attributed to the stress caused during fire operations; (2) the occurrence of a few actinomycetes that, under general

observation, could be considered as contaminants but nevertheless need attention from an ecological association viewpoint; and (3) the pure cultures obtained—an opportunity for taking up further advanced studies on their taxonomy, production of secondary metabolites, and ecological resilience. The selected isolates possessing beneficial properties related to plant growth and biocontrol extend the opportunities for formulation of bioinoculants that can be integrated into traditional ecological knowledge.

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