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Majority of Actinobacterial Strains Isolated from Kashmir Himalaya Soil Are Rich Source of Antimicrobials and Industrially Important Biomolecules

Pulak Kumar Maiti, Sukhendu Mandal*

Department of Microbiology, Laboratory of Molecular Bacteriology, University of Calcutta, Kolkata, India
Email: *sukhendu1@hotmail.com

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Abstract

Studies on actinobacterial diversity are immensely important research arenas as they are the major root of antimicrobial, anticancer and immunosuppressant agents. Here we have performed a community analysis and isolated about 135 morphologically different actinobacteria using five different Actinomyces-specific media from an unique unexplored site of Kashmir Himalaya, India, in order to screen different bioactive compounds specially the antimicrobials. Among these actinobacteria, 42 isolates show antimicrobial activity against a set of pathogenic organisms. Secretion of different biomolecules of industrial significance were assessed which resulted 53 xylanase producers, 57 IAA producers, and 63 isolates produce gelatinase. Pectinase, phosphatase, lipase, amylase, and cellulase producing ability have been shown by about 41, 32, 102, 92, and 83 isolates, respectively. Analysis of their 16S rRNA sequence followed by phylogenetic analysis has depicted that most of the isolates belong to the genus of *Streptomyces* and rests are *Streptosporangium*, *Lentzea*, *Gordonia*, *Amycolatopsis and Lechevalieria*. We have also screened the presence of secondary metabolite production pathways by PCR amplifying the biosynthetic gene of polyketide synthase (PKS-I) and non ribosomal peptide synthetase (NRPS) from the antibiotic producing strains. Antibiotic sensitivity profile of these isolates has also been studied. The potential antimicrobial compound producing strains have further been studied in order to find their efficacy to kill bacterial and fungal pathogens. Thus the study led to a promising field of antibiotic discovery and bioprospecting actinobacteria through their metabolites of industrial potential in near future.

Keywords

Phylogenetic Analysis, Community Analysis, Actinomycetes, Antibiotic Producer, Soil Microbes

1. Introduction

Actinomycetes belong to one of the major abundant class of bacteria, and present as about 10^7 among the 10^9 CFU in each gram soil [1]. The number and types of actinomycetes differs in various geographical locations depending on the nutrient, pH, aeration, moisture, temperature etc. [2]. Actinomycetes are Gram positive, filamentous bacteria that are most dynamic for the production of several forms of biomolecules, such as chitinase, xylanase, pectinase, asparaginase, lipase, cellulase, amylase, and tyrosinase which have immense industrial importance. Besides the industrial application of these biomolecules they have great significance on the environmental bioremediation, recalcitrant compound degradation and also to maintain the biogeochemical cycle [3] [4]. Moreover, actinomycetes induce production of numerous types of bioactive secondary metabolites that can be used as antitumor, antioxidant, anti-inflammatory, antihelminthic, antifungal and antibacterial compounds [5] [6], thereby imposing influence on medical science. Terrestrial samples are the abundant source of actinomycetes [7] but unfortunately the number of new isolates and compound extraction is decreasing rapidly. Isolation of novel secondary metabolites from actinomycetes of natural habitats diminished drastically mostly because of the redundancy of metabolites and increased rate of reisolation of known strain [8]. Isolation from the same type of habitat using the traditional culture method is among the major cause of such redundancy. Thus the assumptions that pick samples from widely diverse locations are more likely to yield novel microorganisms and therefore possible isolation of novel metabolites. Isolation of around 45% of bioactive compounds [8] has been carried out from actinomycetes but there are several limitations. Eradication of rapid emerging multidrug resistance pathogen is a tremendous challenge. It was reported that huge amount of antibiotics are used in crop land and farm land for plant pathogen and animal pathogen which in turn accumulates into the environment, particularly in soil an exerting selection pressure to generate antibiotic resistant strains. Consequently, most of the existing antibiotics are not effective against pathogen which forces researcher for screening of novel isolates to get of new generation antibiotics. The exhaustion of the usual terrestrial sources and the rise of resistant pathogens dictate the search for new antibiotics. To meet urgent clinical needs, screening for secondary metabolites from actinobacteria residing in unexplored habitats is warranted to generate possible novel compounds.

The present study is focused to isolate novel actinomycetes from unusual habitat of Kashmir, India followed by identification, characterization and extrac-

tion of diverse biomolecules, especially the antimicrobials, keeping it in mind that actinomycetes, especially *Streptomyces sp.* prefer to grow in neutral to alkaline. The sample has been collected from a low nutrient containing unexplored soil environment of Rangreth, a small place of Badgam district of Kashmir valley. The soil of the valley called as Karewas soil which is lacustrine in origin. The annual average temperature is 13.6˚C which fluctuates between 30˚C and 4˚C. The high altitude, low organic content, and temperature fluctuation creates a harsh environmental condition for the survival of microorganism that might force the microbial flora for aggravated competition, which in turn might select antibiotic producing strain to grow. We have chosen five different media for exploration of actinomyctes. In this work we have also characterized isolated actinobacteria for their possible prospects based on the important enzymes and antimicrobial production ability. The presence of genes for ployketide synthase (PKS-I) and non ribosomal peptide synthetase (NRPS) in the antibiotic producing strains has been envisaged. We have also screened the antibiotic sensitivity profile of these isolates against 30 standard antibiotics. The potential antimicrobial compound producing strains has further been studied in order to find their efficacy to kill bacterial and fungal pathogens. We further performed 16S rDNA based molecular systematic in order to group them. As strains of actinomycetes are the pivotal root in drug discovery, such studies has potential towards discovering new-generation antimicrobials and other metabolites of human benefit.

2. Materials and Method

2.1. Sample Collection, Pretreatment and Physicochemical Analysis of Soil

In this study soil samples were collected from five different spots (at least 10 yards apart) of Rangreth of Kashmir Himalaya, India (Latitude: 34˚-01'N; Longitude: 74˚-47'E; Altitude: 5328 ft; Annual rainfall: 743 mm, Average temperature; 13.6˚C) from the depth of 5 - 7 cm by the use of sterile spatula and kept in sterile container for further study (Figure 1). Soil samples were dried at room

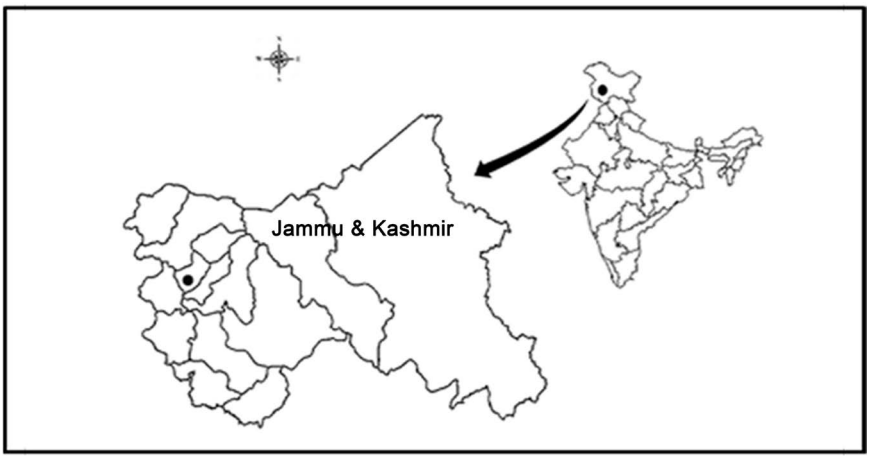


Figure 1. Sampling location of the study area, Rangreth, Jammu and Kashmir, India.

temperature and crushed with mortar pestle for further Physicochemical analysis. In this study soil pH, electrical conductivity [9], organic carbon, organic matter following tritration method [10], total available phosphorous as in Sarkar and Haldar (2005) were checked. Total available potassium was also studied by flame photometer [11]. CH_3COONH_4 was used for extraction of available potassium in soil; NH_4 replaces the potassium present in soil into the solution followed by filtration through Whatman filter paper. The sample then was analysed by flame photometer on the basis of emission spectrometry principle.

2.2. Screening of Actinomycetes

Soil samples were pre-treated with $CaCO_3$ for 7 days (El-Nakeeb MA, Lechevalier HA) (1963) Ningthoujam *et al.* (2011) [12], followed by heat treatment for 2 hr in hot air oven at 65˚C in order to enrich and selectively isolate Actinobacteria. 1 gm soil samples were dissolved in 1 ml of 0.9% NaCl followed by serial dilution to ten fold with 0.9% NaCl upto 10^{-5} dilution. From these dilutions, each 0.1 ml of sample was spread on different solid medium such as starch casein (for 1 L medium, starch 10 gm, KNO_3 2 gm, NaCl 2 gm, K_2HPO_4 2 gm, $MgSO_4$, $7H_2O$ 0.05 gm, $CaCO_3$ 0.02 gm, $FeSO_4$ 0.01 gm, casein 0.3 gm, agar 20 gm, pH 7.0); Bennett medium (for 1 L medium, dextrose 10 gm, yeast extract 1 gm, beef extract 1 gm, casein 2 gm, agar 20 gm, pH 7.3); Actinomycetes isolation agar (AIA) (for 1 L medium, glycerol 5 gm, sodium propionate 4 gm, sodium caseinate 2 gm, L-asparagine 0.1gm, $FeSO_4$, $7H_2O$ 0.001 gm, agar 15 gm, pH 8.1); *Streptomyces* agar (for 1 L medium, malt extract 10 gm, yeast extract 4 gm, dextrose 4 gm, $CaCO_3$ 2 gm, agar 20 gm, pH 7.2) and ISP-7 (ISP stands for International *Streptomyces* Project) (for 1 L medium, L-asparagine 1 gm, L-tyrosine 0.5 gm, K_2HPO_4 0.5 gm, glycerol 15 gm, $MgSO_4$, $7H_2O$ 0.5 gm, NaCl 0.5 gm, trace salt 1 ml [$FeSO_4$, H_2O 1.36 mg, copper chloride 0.027 mg, cobalt chloride 0.040 mg, sodium molybdate 0.025 mg, $ZnCl_2$ 0.02 mg, boric acid 2.85 mg, $MnCl_2$ 1.8 mg, sodium tartarate 1.7 mg] agar 20 gm, pH 7.3) along with 50 µg/ml of both cycloheximide and nystatin to inhibit the fungal growth. The inoculated plates were incubated for 3 - 4 days at 28˚C. After incubation individual colonies were picked based on their morphology and colour of mycelium using sterile toothpick and streaked on fresh plate to get pure cultures (**Table 1**).

2.3. Characterization of Actinomycetes

2.3.1. Morphological and Cultural Characteristics

The aerial and substrate mycelium and diffusible pigments of all isolates were observed after growing for 14 days on various medium like ISP-2 (for 1 L medium, malt extract 10 gm, yeast extract 4 gm, dextrose 4 gm, agar 20 gm) (**Figure 2(a)**); ISP-3 (for 1 L medium, oat meal 20 gm, Fe_3SO_4 0.001 gm, $MnSO_4$ 0.001 gm, $ZnSO_4$ 0.001 gm, agar 20 gm, pH 7.3); ISP-4 (for 1 L medium, starch 10 gm, K_2HPO_4 1 gm, $MgSO_4$ 1 gm, $(NH_4)_2SO_4$ 2 gm, $CaCO_3$ 2 gm, $FeSO_4$, $7H_2O$ 0.001 gm, $MnCl_2$ 0.001 gm, $ZnSO_4$ 0.001 gm, agar 20 gm, pH 7.2); ISP-5 (for 1 L medium, L-asparagine 1 gm, K_2HPO_4 1 gm, trace salt 1 ml [$FeSO_4$ 0.001 gm, $MnSO_4$

Table 1. Colony morphology of different actinomycetes.

Name of the isolates	Isolation media	Aerial mycellium	Substrate mycellium	Soluble pigment in isolation medium	Diffusible pigment in isolation medium
PSKA 01	AIA	Copper	Copper	-	-
PSKA 02	AIA	Marron	Marron	Deep brown	-
PSKA 13	AIA	White	Light yellow	Light brown	-
PSKA 09	AIA	White	White	-	-
PSKA 10	AIA	White	White	Light brown	Deep brown
PSKA 11	AIA	Transparent	Transparent	Deep brown	-
PSKA 14	AIA	Brown	Brown	Light brown	Deep brown
PSKA 18	AIA	Champagne	Light brown	Deep brown	-
PSKA 40	AIA	Red orange	Red orange	-	-
PSKA 48	AIA	White	Light brown	-	-
PSKA 49	AIA	White	Brown	-	Pinkish red
PSKA 22	ISP-7	Bronze	Light yellow	Light brown	-
PSKA 23	ISP-7	Sepia	Sepia	Deep brown	-
PSKA 24	ISP-7	Brown	Brown	Light brown	-
PSKA 26	ISP-7	Tan	Tan	Light brown	-
PSKA 28	ISP-7	Tan	Tan	-	-
PSKA 03	ISP-7	Black	Black	Light brown	Black
PSKA 50	ISP-7	Brown	Brown	-	-
PSKA 56	ISP-7	Bronze	Bronze	-	-
PSKA 33	Bennett	White	Yellow	-	-
PSKA 39	Bennett	Champagne	Copper	-	-
PSKA 54	Bennett	Golden yellow	Golden yellow	-	-
PSKA 55	Bennett	Champagne	Champagne	-	-
PSKA 47	Bennett	White	Beige	-	-
PSKA 38	Bennett	Light brown	Light brown	-	-
PSKA 42	Bennett	Beige	Beige	-	-
PSKA 43	Bennett	Beige	Beige	-	-
PSKA 52	Bennett	Ice blue	Dark brown	-	-
PSKA 07	Starch casein	Light red	Light red	-	-
PSKA 29	Starch casein	White	Black	-	-
PSKA 30	Starch casein	Green	Green	Light green	-
PSKA 37	Starch casein	Light brown	Light brown	-	-
PSKA 46	Starch casein	White	Brown	-	-
PSKA 53	Starch casein	Beige	Red	-	-
PSKA 36	Starch casein	White	Light brown	-	-
PSKA 45	Starch casein	Brown	Brown	-	-
PSKA 44	ISP-2	Champagne	White	-	-
PSKA 51	ISP-2	Cream	Bisque	-	-
PSKA 41	ISP-2	Champagne	Apricot	-	-
PSKA 31	ISP-2	White	White	Black	-
PSKA 32	ISP-2	White	White	Light brown	-

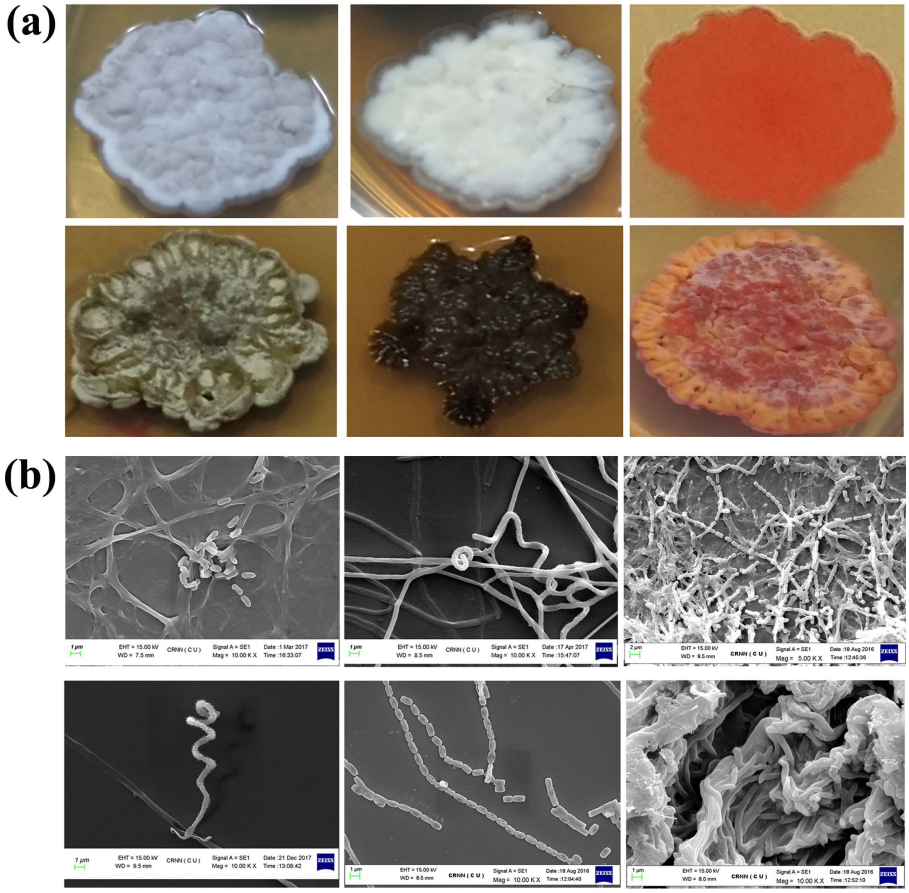


Figure 2. Morphological appearance of representative isolates (a) Actinomycetes grown in ISP-2 medium; (b) SEM image of isolated actinomyces.

0.001 gm, $ZnSO_4$ 0.001 gm], agar 20 gm, pH 7.4); ISP-6 (for 1 L medium, peptic digest of animal 15 gm, proteose peptone 5 gm, yeast extract 1 gm, ferric ammonium citrate 0.5 gm, K_2HPO_4 1 gm, sodium thiosulphate 0.080 gm, agar 15 gm, pH 6.7) and ISP-7 according to international *Streptomyces* project [13]. Colony morphology which includes size, shape, margin, texture, form, optical property of the colony, was noticed after 14 days of incubation on ISP-2 medium. Spore morphology was seen by the cover slip culture method [14] through scanning electron microscope (**Figure 2(b)**). Growth pattern of actinomycetes on different pH (4 to 10), temperature (10˚C to 40˚C), and NaCl (1% to 8%) were also observed.

2.3.2. Biochemical Characterization

Actinomycetes isolates were characterized for their ability of indole production, mixed acid fermentation (methyl red, Voges-Proskauer), citrate utilization, nitrate reduction, H_2S production, catalase production, various carbon source utilizations (pyruvate, ribose, D-xylose, glucose, D-fructose, D-galactose, D-mannose, D-maltose, D-arabinose, lactose, sucrose, D-melibiose, D-melizitose, dulcitol, adonitol, D-raffinose, L-rhamnose and inositol) following the international *Streptomyces* project [13]. Nitrogen source utilization (L-alanine, L-asparagines, and

L-arginine) of these isolates were also performed following Williams *et al.* (1983) [15]. Hypoxanthine, xanthine, tyrosine, and arbutin hydrolysis by these isolates were observed according to Williams *et al.* 1983 [15].

2.3.3. Assays for Biomolecule Production

All isolates were screened for qualitative exoenzyme production such as amylase, cellulase, pectinase, xylanase, and lipase. Detection of cellulose and xylan degradation has been done through congo red overlay method [16] by streaking the colonies on respective cellulose or xylan containing medium (1% CM cellulose for detection of cellulase and 1% xylan for detection of xylanase along with 0.3% beef extract and 2% agar). To execute the experiment, plates were flooded with 0.1% aqueous congo red for 10 min and washed with 1 M NaCl. Hankin medium was used for pectinase assay and plates were flooded with 1% CTAB [17]. Lipid degrading assay were done by tributyrin (10 ml/L) agar media [18]. Degradation of such component (cellulose, xylan, pectin, and lipid) was detected by clearing zone around the colony on respective medium. Gelatinase assay was done by nutrient gelatin agar (Himedia, India). Starch degradation has been performed in modified starch agar media (1% starch, 0.3% beef extract and 2% agar) and detection of amylase was confirmed with Gram's iodine solution. Urea broth and YM (yeast extract and malt extract) broth (along with 2 mg/ml L-trytophan) was used to detect presence of urease and indole acetic acid (IAA) production, respectively. Phosphate solubilizing actinomycetes has been screened by Pikovakaya's agar medium (Himedia). Caseinase secretion has been studied by skim-milk containing agar medium [15].

2.3.4. Antimicrobial Activity

All isolates were selected for preliminary antagonistic effect against Gram positive (*Staphylococcus epidermidis* MTCC 3086, *Bacillus cereus* MTCC 1272) and Gram negative (*Escherichia coli* MTCC 1687, *Pseudomonas aeruginosa)* bacteria along with yeast (*Saccharomyces cerevisiae*) using cork-borer method on the basis of Kirby-Baur difusion assay [19]. 100 µl of bacterial and yeast culture were spread independently ontonutrient agar (NA) and yeast extract potatoes dextrose (YEPD) agar medium, respectively. Actinobacteria like colony were cut out by sterile cork borer or toothpick and placed it on the surface of prepared above both agar plates. Plates were kept in 4˚C for 2 hrs for homogenous distribution of antimicrobial compound before the growth of test organism and followed by the incubation at 37˚C for 24 hrs for bacteria and 28˚C for 48 hrs for yeast culture. After incubation, zone of inhibition was observed and measured around the actinomycetes colonies.

2.3.5. Genomic DNA Isolation

Actinomycetes strains were grown in 5 - 6 ml respective broth with agitation at 30˚C for 3 - 4 days and purity of individual isolates checked by morphological similarity by naked eye examination on respective plate. Genomic DNA of these isolate were extracted by phenol-chloroform methods [20].

2.3.6. Amplification and Sequencing of 16S rDNA Gene, and Phylogenetic Analysis

The 16S rDNA gene was amplified by using universal primer 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-ACGGTTACCTTGTTACGACTT-3') according to [21]. Amplification were performed in thermocycler (Bio-Rad, USA) in a final volume of 50 µl [reaction mixture containing 1.25 µl of each forward and reverse primer (10 µM each), 1.25 µl of template DNA, 10 µl of 5x reaction buffer, 2.5 µl of $MgCl_2$ (25 mM), 0.25 µl (5unit) DNA polymerase and autoclave Mili-Q grade, water to makeup volume up to 50 µl]. The thermal cycle were set as initial denaturation at 95˚C for 5 min, followed by 35 cycles of denaturation at 95˚C for 30 sec, annealing at 55˚C for 45 sec, extension at 72˚C for 1.30 min, and a final extension at 72˚C for 7 min to allow for extension of any incomplete products. PCR product were analysed through agarose gel electrophoresis and visualized under UV-transilluminator by using a Bio-rad Gel Doc (ChemiDoc^{TM}XRS^{+}) system and purified by using commercial gel extraction kit (Promega corporation, USA). Purified 16S rDNA product was sequence through sanger method. Both stand of sequence nucleotide was aligned by using Bioedit software (http://bioedit.software.informer.com) and retrieved partial or complete sequence of 16S rDNA gene of respective isolates. The partial sequence of DNA was submitted at EZbiocloud server (https://www.ezbiocloud.net/) to identify the most closest strain. It was also compared by using NCBI BLASTn program (https://blast.ncbi.nlm.nih.gov). From the EZbioCloud server [22] all type strains were taken and multiple sequence alignment was obtained with clustal W program using Mega 7 [23]. Phylogenetic tree were constructed by neighbor joining (NJ) method [24] and maximum likelihood (ML) method [25]. Evolutionary distance was calculated by Kimura 2 parameter model [26] for NJ method and Tamura 3 parameter model [27] used for ML method. Bootstrap analysis has been performed based on resampling at 1000 times. In order to validate each clade of the phyllogentic tree, all sequences were used to know the phylogenetic classification on the basis of their 16S rDNA secondary structure by the use of Q-INS-I of MAFT web server (https://mafft.cbrc.jp/alignment/server) [28].

2.3.7. Detection of Biosynthetic (PKS-I and NRPS) Gene by PCR Amplification

Non-ribosomal polypeptide synthetase (NRPS) were amplified by primer A3F (5'-GCSTACSYSATSTACACSTCSGG-3'), and A7R (5'-SASGTCVCCSGTSGCGTAS-3') and polyketide synthase (PKS) type 1 were amplified by primer KIF (5'-TSAAGTCSAACATCGG BCA-3') and M6R (5'-CGCAGGTTSCSGTACCAGTA-3'). The reaction mixture was prepared similarly as prepared in case of 16S rDNA PCR but with additional 10% DMSO. The thermal cycle were: initial denaturation at 95˚C for 5 min, followed by 35 cycles of which denaturation at 95˚C for 30 sec, annealing at 55˚C for 2 min, extension

at 72˚C for 4 min and final extension at 72˚C for 10 min [29].

2.4. Antibiotic Sensitivity Test

Thirty standard antibiotic discs (Himedia, India) were used against all actinomycetes in order to negate redundancy, keeping it in mind that the resistant strain might be the producer. These antibiotics which includes in this study are cell wall inhibitior: Carbenicillin (10 µg) (CB), Penicillin G (10 U) (P), Cefazolin (30 µg) (CZ), Cefuroxime (30 µg) (CXM), Aztreonam (30 µg) (AT), Amoxocillin/Clavulanic acid (20/10 µg) (AMC/CL), Cefoperazone (75 µg) (CPZ), Vancomycin (30 µg) (VA), Mezlocillin (75 µg) (MZ), Cefoxitin(CX), and Cefepime (30 µg) (CPM); nuclic acid inhibitors: Norfloxacin (10 µg) (NX), Co-trimoxazole (25 µg) (COT), Ciprofloxacin (5 µg) (CIP), Levofloxacin (5 µg) (LE), Novobiocin (30 µg) (NV), Rifampicin (30 µg) (R), Ofloxacine (5 µg) (OF) and Nitrofurantoin (30 µg) (NIT); outer membrane inhibitor: Colistin (10 µg) (CL), Polymyxin B (30 µg) (PB); protein synthesis inhibitor: Oleandomycin (15 µg) (OL), Clindamycin (2 µg) (CD), Lincomycin (15 µg) (L), Tobramycin (10 µg) (TOB), Tetracyclin (30 µg) (TE), Chloramphenicol (30 µg) (C), Gentamycin (10 µg) (GEN), Streptomycin (25 µg) (S), Amikacin (30 µg) (AK). 100 µl of fresh cultures were spreaded with a sterile spreader on Muller Hinton agar plate. The antibiotic discs were placed on the plate and incubated at 28˚C for 2 - 3 days. Antibiotic sensitivity was observed by measuring inhibition zone diameter. Actinomycetes isolates were either considered as sensitive (S), intermediate (I), or resistant (R) to an antibiotic.

2.5. Statistical Analysis

To understand the biochemical parameter-based relatedness among the isolated actinomycetes, PAST (version 3.17) software [30] has been used to generate dendogram by UPGMA algorithm based on Jaccard similarity coefficient. All data of biochemical test were taken as binary digit which was 1 means positive and 0 means negative.

Accession no of isolates

16SrDNA of 27 isolates deposited in gene bank and their accession number respectively MG930084, MG930085, MG930086, MG930087, MG930088, MG930089, MG930090, MG930091, MG930092, MG930093, MG930094, MG930095 MG930096, MG930097, MG930098, MG930099, MG930100, MG930101, MG930102, MG930103, MG930104, MG930105, MG930106, MG930107, MG930108, MG930109, MG930110.

3. Results and Discussions

3.1. Description of Sampling Sites

To isolate actinobacteria selectively, five independent samples were collected from atleast 10 yards apart and used for further isolation. The sample collection sites included an non-agricultural abandoned site which does not have disturbances from anthropogenic activities and become worn out land that might

gradually loose soil physiochemical property. The soil color ranging from whitish to light grey and the moisture content is moderate which reflected from the granular forms *i.e.* neither dough nor muddy and commonly known as Karewas soil. The general characteristics of the samples used for isolation are alkaline in nature (pH 8.26). The soil conductivity is 3.63×10^3 ds/cm that means this is non saline soil. Organic carbon, total organic matter, total phosphorous and potassium were 0.39%, 0.672%, 1.35 kg ha^{-1}, 12.5 ppm, respectively. Thus it indicates that the soil sample has relatively low amount of organic carbon, potassium and phosphorus. Although Karewas soil is originally nutrition rich but it has been predicted that the chemical property loosens dramatically in time (ENVIS Newsletter; https://www.scribd.com/document/348306673/April-June-2015).
We observed that organic matter is too low which might affect the diversity of microbial community because of having higher competition for the limited carbon source. Therefore survival probability of fastidious organism as well as variation of microbial community is also under challenge. It is well known that the soil with low nutrient is good habitat for actinomycetes [31].

3.2. Culture Dependent Community Analyses of Actinobacteria

Demand of novel drugs against widespread pathogens is always high. Thrust towards the discovery of new antibiotics is even more promising when there is considerable resistance towards existing regimen of antibiotics. Hence search for novel actinobacterial isolates from unexplored area enhanced the probability to obtain new compound and potential bioactive molecules. We found that maximum actinomycetes can grow in autotrophically which also hypothesised that this type of actinomycetes are quite different from the large sets of established one. A total of about 135 actinomycetes were isolated from Kashmir soil sample using five different media. Among the five media used, the most versatile pigment-producing strains were found in starch casein and AIA media particularly based on their various colour of aerial and substrate mycelium. Based on 16S rDNA sequence analysis, it was found that among these isolates 3 genus (*Lechevalieria*, *Lentzea*, *Streptomyces*) obtained from Bennett medium, 2 genus (*Lechevalieria*, *Streptomyces*) from ISP-7, 2 genus (*Gordonia*, *Streptomyces*) from AIA, 2 genus (*Amycolaptosis*, *Streptomyces*) from *Streptomyces*-agar media andstarch casein media showed only *Streptomyces sp*. Even the medium compostion was different the *Streptomyces* is once again found to be the most abundant genera of this soil which corroborates with the previous reports by many researcher who concludes that among Actinomycetes, *Streptomyces* are most prevalent genus. Characteristics like colony morphologies (filamentous, leathery, powdery, rough, mostly dull umbonate. concave or convex), carbon and nitrogen utilization pattern, colour of their mycelium, pigmentation, ability of various enzyme secretion, and metabolite production of each isolates confer that they belong to different types of actinomycetes. Among the 135 isolates, 52 colonies displayed melanin pigment on their respective medium. The isolated colonies have showed potential secretion of exoenzymes (**Figure 3(a)**) and could es

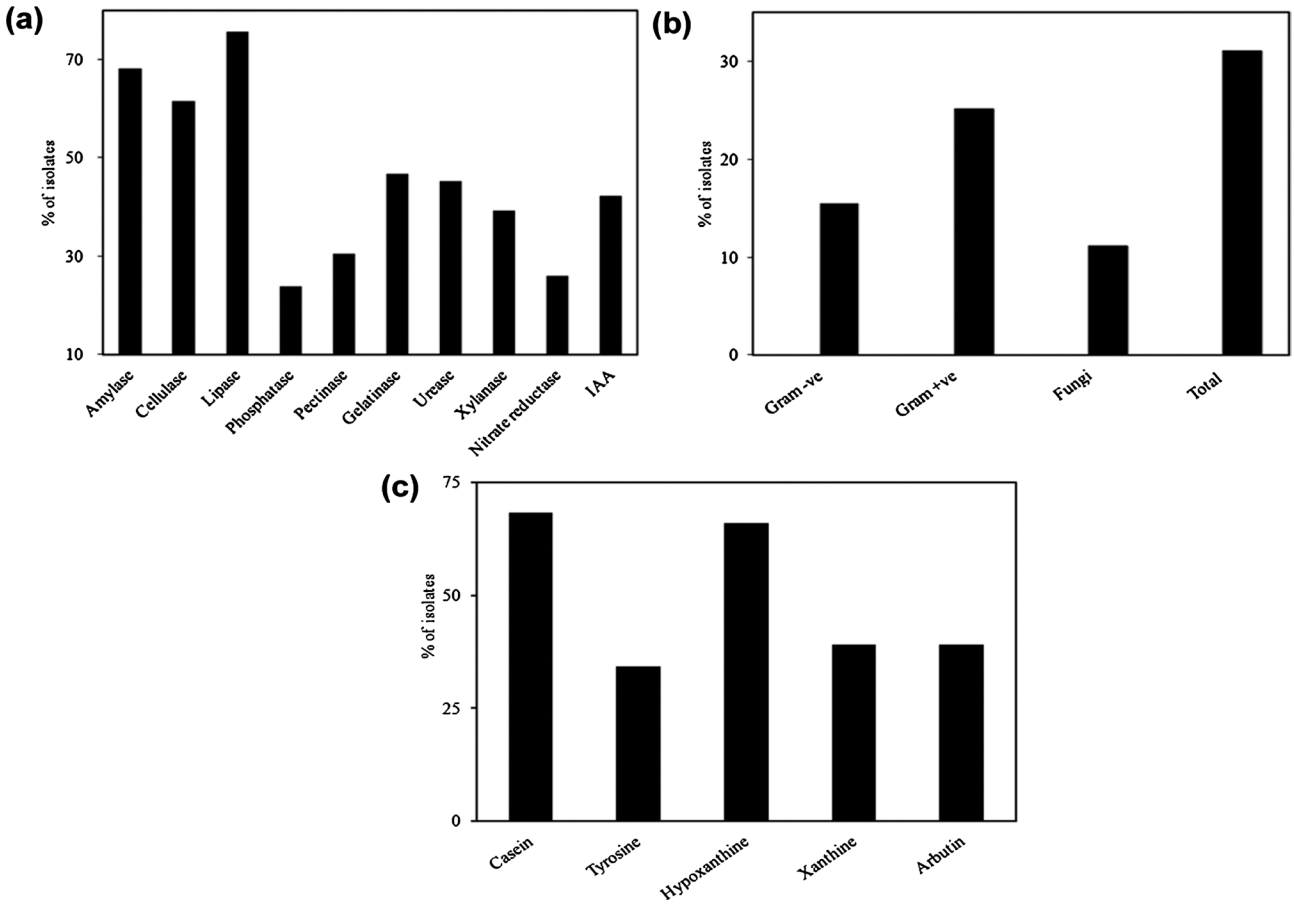


Figure 3. Potentiality of isolated actinomycetes for production of biomolecules (a) Production of different enzymes by135 isolated actinomycetes; (b) Antimicrobial activity against different test organisms, the bar graph showing the effect of all antimicrobials produced by different unique isolates on Gram positive bacteria, Gram negative bacteria, and fungi; (c) Degradation of different biomolecules by selected 41 actinomycetes.

sentially open significant biotechnological application. Cellulase, amylase and lipase secretion was shown by most of the isolates while few among them showed substantial xylanase and pectinase production. Primary screening method revealed that out of 135 isolates, 42 isolates showed antibacterial activity against at least one organism, 6 isolates exhibited both antibacterial and antifungal activity and 15 isolates with antifungal activity (**Figure 3(b)**). Among the 42 antibiotic producers it has been seen that maximum antibiotic producers *i.e.* 19 isolates obtained from cultivating on AIA medium and 10 isolates from ISP-7 medium. Glycerol was the common carbon source among the AIA and ISP-7 medium which may be reason for inducing the antibiotic production. The study revealed that most of the isolates were more effective against Gram positive organism compare to that of the Gram negative. In our study we have chosen 21 actinomycetes for further study which display more than 5 mm zone of inhibition in agar diffusion method, whereas, 20 non antibiotic producing actinomycetes were used for furher characterization capable of production of various enzyme. This total 41 isolates showed different colour of mycelium, 14 of which produce diffusible pigments on their respective medium. Out of these 41 iso-

lates, 28, 14, 27, 16, and 16 isolates able to degrade casein, tyrosine, hypoxanthine, xanthine, arbutin, respectively (**Figure 3(c)**). Within the 20 non-antibiotic producer, all isolates produce lipase enzyme; 18, 17, 16, 6, 5, 9, 14 and 13 isolates produce amylase, cellulase, xylanase, pectinase, phosphatase, IAA, gelatinase and urease, respectively. Furthermore, these 41 isolates showed diverse sensitivity against standard antibiotics and can utilize various carbon and nitrogen source differently (**Figure 4(a)**, **Figure 4(b)**). Among the 41 isolates, 15 isolates can grow in 10˚C, 2 isolates can withstand 40˚C, and all others can grow in 20˚C but their optimum temperature is 28˚C ± 2˚C. Out of 41 isolates, 2 isolates can survive 8% NaCl, 9 isolates can tolerate upto 7% NaCl, 26 isolates can grow upto 6% NaCl, and all can tolerate 4% NaCl (**Figure 4(c)**). All isolates in this study can even grow without NaCl, on the contrary most of them can withstand as much as 8% NaCl and justify themselves to be halotolerant actinomycetes. It was also noted that total of 41 isolates, 26 colony can grow in pH 5 to 10, all colony can grow in pH 6 to 10 but none of them can grow in pH 4. They prefer to grow in neutral to alkaline pH 7 to 8 (**Figure 4(c)**). 16S rDNA sequence homology of of 41 isolates was obtained from the BLASTn analysis from NCBI and mostly showing 98% - 100% similarity with the *Streptomyces*, while 2 among them are 99% similar to *Lechevaliveria*, one isolate closest to *Lentzea* which exhibit 98% similarity, 1 isolate is 99% similar to *Streptosporangia* and 2 isolates found to be 99% similar in their 16S rDNA sequence with *Gordonia* and *Amycolaptosis sp.* About 76% antibiotics are derived from *Streptomyces* while rests are derived from *Micromonospora*, *Nocardia*, *Streptosporangium*, *Actinomadura*, *Saccharopolyspora* etc. [8]. Among the total sequenced isolates, 4 isolates may be novel on the basis of their morphological, biochemical and molecular characterization because this 4 isolates show less than 98.7% similarity based on 16S rDNA sequence which is the modern trend to claim new species [32]. Whereas 5 isolates showed less than 99% but greater than 98.7% 16S rDNA sequence similarity which also have the potential to be new actinobacterial species. Subsequently their phylogenetic analysis elicits that most of them does not belong to the monophyletic group, (*i.e.* showing close homology with its recent ancestor) rather preferred to belong from the paraphyletic and polyphyletic group based upon their origin of recent ancestry. Both neighbor joining (NJ) (**Figure 5**) and maximum likelihood (ML) methods of phylogenetic analyses (data not shown) showed that most isolates (except PSKA01, PSKA32, PSKA18, PSKA07, PSKA23) belong to same cluster in phylogenetic tree. Secondary structure based phylogeny of 16S rRNA (data not shown) also showed that each cluster position were followed the NJ and ML linear tree. Although UPGMA based dendogram (**Figure 6**) does not correlates evolutionary time scale but it gave a graphical representation about chemical relationship among the isolates with the help of Jaccard similarity coefficient. The study also delineates the exploration of probable secondary metabolite synthesis pathway in 42 antimicrobial producing isolates by amplification of NRPS and PKS-I gene. The existence of these genes might not be confirmatory for presence of antibiotic synthetic genes but added

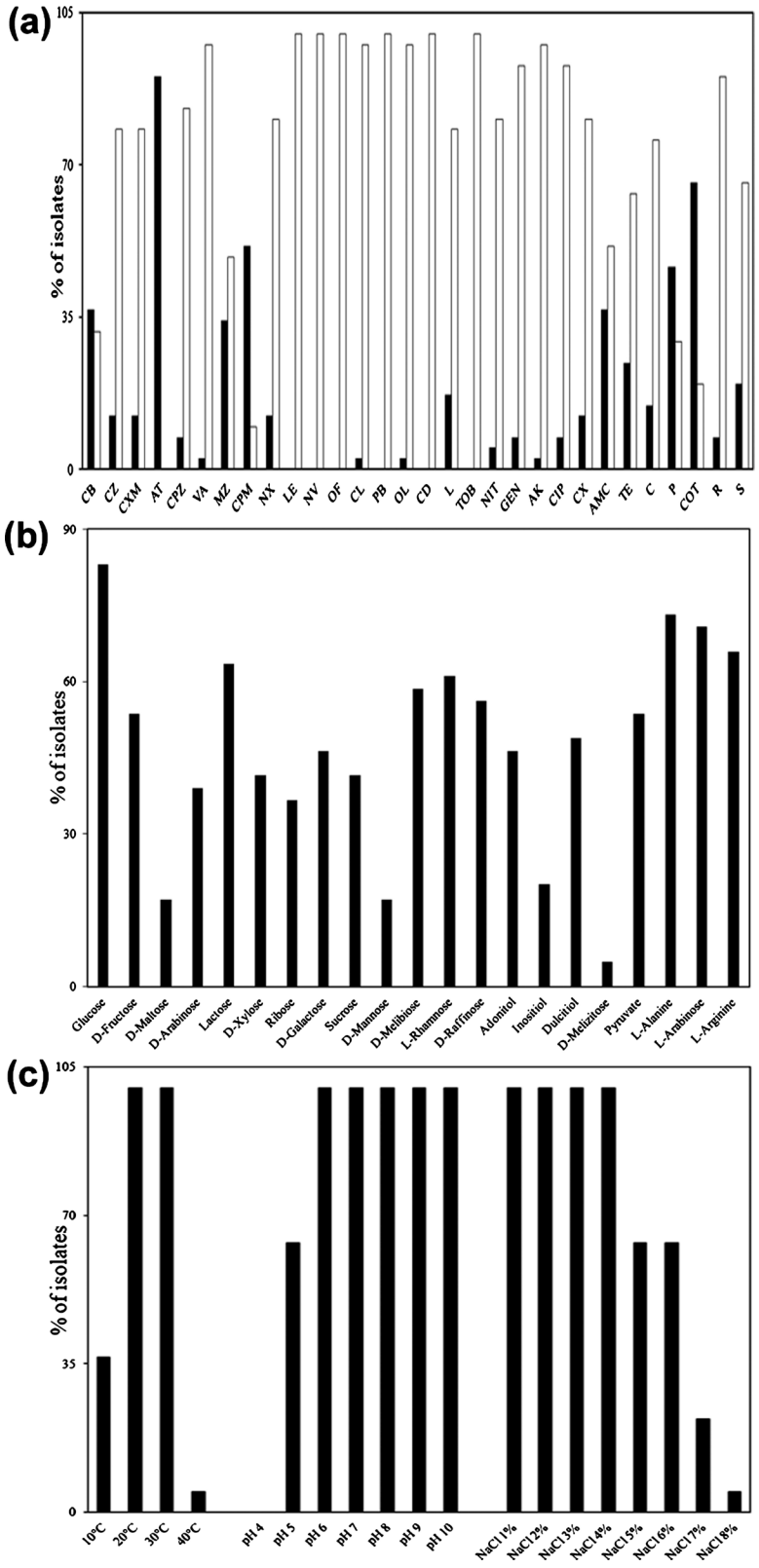
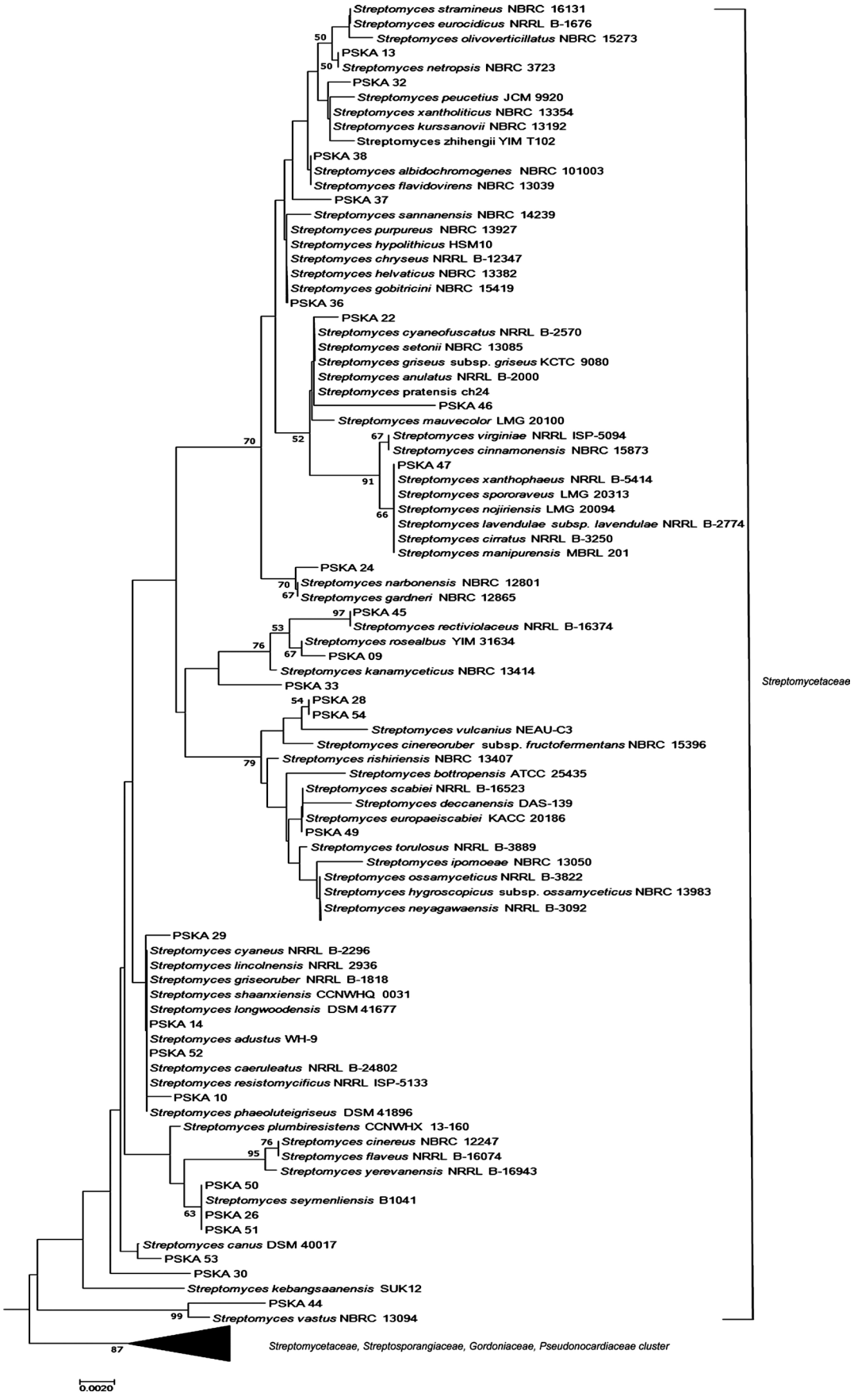


Figure 4. Growth parameters of isolated actinomycetes (a) Antibiotic sensitivity of 41 isolated actinomycetes, black bar represent resistant isolates and empty bars represent sensitive isolates (b) Carbon and nitrogen utilization pattern of 41 isolated actinomycetes; (c) Physiological parameter of 41 isolated actinomycetes.


Streptomyces stramineus NBRC 16131
Streptomyces eurocidicus NRRL B-1676
Streptomyces olivoverticillatus NBRC 15273
PSKA 13
Streptomyces netropsis NBRC 3723
PSKA 32
Streptomyces peucetius JCM 9920
Streptomyces xantholiticus NBRC 13354
Streptomyces kurssanovii NBRC 13192
Streptomyces zhihengii YIM T102
PSKA 38
Streptomyces albidochromogenes NBRC 101003
Streptomyces flavidovirens NBRC 13039
PSKA 37
Streptomyces sannanensis NBRC 14239
Streptomyces purpureus NBRC 13927
Streptomyces hypolithicus HSM10
Streptomyces chryseus NRRL B-12347
Streptomyces helvaticus NBRC 13382
Streptomyces gobitricini NBRC 15419
PSKA 36
PSKA 22
Streptomyces cyaneofuscatus NRRL B-2570
Streptomyces setonii NBRC 13085
Streptomyces griseus subsp. griseus KCTC 9080
Streptomyces anulatus NRRL B-2000
Streptomyces pratensis ch24
PSKA 46
Streptomyces mauvecolor LMG 20100
Streptomyces virginiae NRRL ISP-5094
Streptomyces cinnamonensis NBRC 15873
PSKA 47
Streptomyces xanthophaeus NRRL B-5414
Streptomyces spororaveus LMG 20313
Streptomyces nojiriensis LMG 20094
Streptomyces lavendulae subsp. lavendulae NRRL B-2774
Streptomyces cirratus NRRL B-3250
Streptomyces manipurensis MBRL 201
PSKA 24
Streptomyces narbonensis NBRC 12801
Streptomyces gardneri NBRC 12865
PSKA 45
Streptomyces rectiviolaceus NRRL B-16374
Streptomyces rosealbus YIM 31634
PSKA 09
Streptomyces kanamyceticus NBRC 13414
PSKA 33
PSKA 28
PSKA 54
Streptomyces vulcanius NEAU-C3
Streptomyces cinereoruber subsp. fructofermentans NBRC 15396
Streptomyces rishiriensis NBRC 13407
Streptomyces bottropensis ATCC 25435
Streptomyces scabiei NRRL B-16523
Streptomyces deccanensis DAS-139
Streptomyces europaeiscabiei KACC 20186
PSKA 49
Streptomyces torulosus NRRL B-3889
Streptomyces ipomoeae NBRC 13050
Streptomyces ossamyceticus NRRL B-3822
Streptomyces hygroscopicus subsp. ossamyceticus NBRC 13983
Streptomyces neyagawaensis NRRL B-3092
PSKA 29
Streptomyces cyaneus NRRL B-2296
Streptomyces lincolnensis NRRL 2936
Streptomyces griseoruber NRRL B-1818
Streptomyces shaanxiensis CCNWHQ 0031
Streptomyces longwoodensis DSM 41677
PSKA 14
Streptomyces adustus WH-9
PSKA 52
Streptomyces caeruleatus NRRL B-24802
Streptomyces resistomycificus NRRL ISP-5133
PSKA 10
Streptomyces phaeoluteigriseus DSM 41896
Streptomyces plumbiresistens CCNWHX 13-160
Streptomyces cinereus NBRC 12247
Streptomyces flaveus NRRL B-16074
Streptomyces yerevanensis NRRL B-16943
PSKA 50
Streptomyces seymenliensis B1041
PSKA 26
PSKA 51
Streptomyces canus DSM 40017
PSKA 53
PSKA 30
Streptomyces kebangsaanensis SUK12
PSKA 44
Streptomyces vastus NBRC 13094
Streptomycetaceae
Streptomycetaceae, Streptosporangiaceae, Gordoniaceae, Pseudonocardiaceae cluster
0.0020

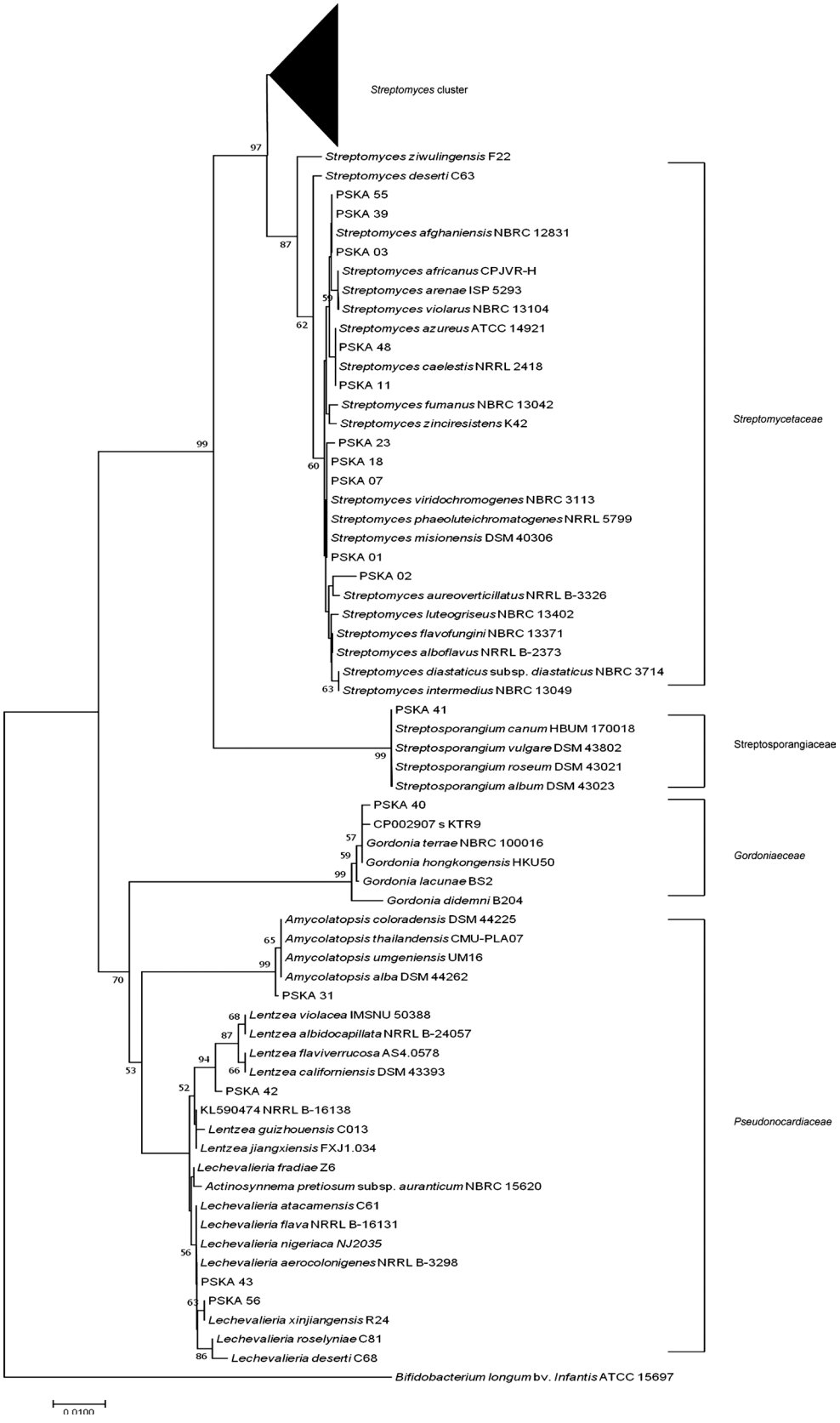


Figure 5. Neighbour joining tree based on 16S rDNA linear sequence. Only >50% bootstrap value are shown at branch node from 1000 replicants.

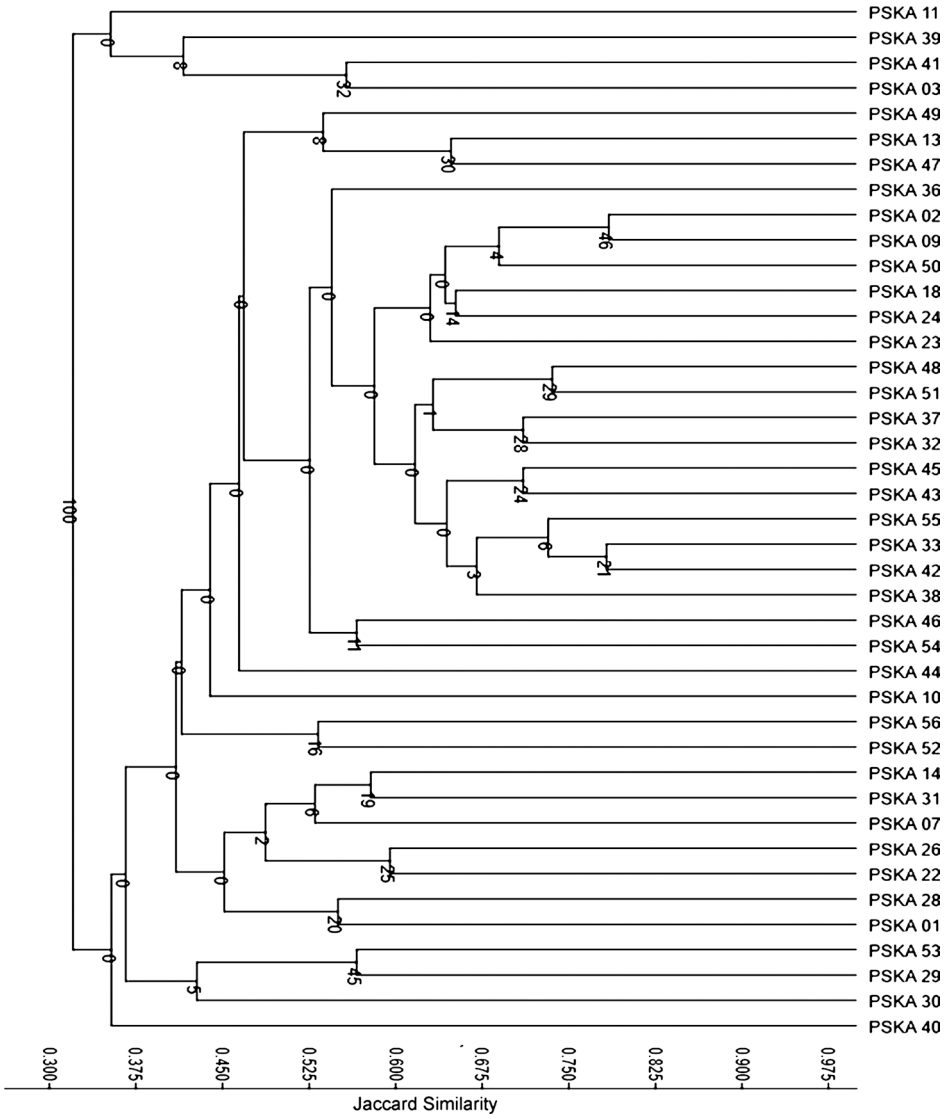


Figure 6. UPGMA based cluster analysis using Jaccard similarity on the basis of biochemical charecteristics of isolated actinomycetes.

extra features about drug synthetic mechanism. Only 13 isolates showed positive against PKS-I but none of them found positive result for having NRPS gene. The present study revealed that 28 isolates does not showed the appearance of either of these genes but showed antagonistic effect during *in vitro* screening. It was found that none of the isolates resistant against levofloxacin, novobiocin, ofloxacin, polymyxin B and tobramycin which might be the indication for these actinomycetes to not having the ability to produced such type of antibiotics. Also it was found that 37 isolates out of 41 were resistant against aztreonam (AT). Highest resistance showed against carbenicillin, mezicillin, cefepime, and penicillin. In this study we also observed that one or the other of this actinomycetes is resistance against at least one among all category of antibiotics (**Figure 4(a)**). We believe, such studies have potential towards discovering new-generation antimicrobials and other metabolites of human benefit.

4. Conclusion

The present study overviews the community analysis of potent actinomycetes to survey its antimicrobial biosynthesis potential. Various actinomycetes are isolated from unexplored sites of Rangreth, Kashmir Himalayas, using five different culture media optimal for their growth condition. The study emphasized that even if the soil quality is poor in organic content, it accommodates quite a rare and novel actinomycetes. Among the total isolates, 42 isolates are antibiotic producer and most of them produce exo-enzymes. Overall the above community analyses may predict that certain isolates are novel based on their 16S rDNA sequence as well as physiochemical charecteristics. Most of the identified isolates belong to *Streptomyces sp.*, from which major source of biomolecules are extracted for various industrial uses. Some isolates consist of PKS-I gene which may be revealed from their probable type of compound. Among the potent isolates further studies shall be conducted on antibiotic extraction, purification, structural analysis and potency of antibotic against multidrug resistant pathogen.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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