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Actinomycetes from Soil of Lachung, a Pristine High Altitude Region of Sikkim Himalaya, Their Antimicrobial Potentiality and Production of Industrially Important Enzymes

Laishram Shantikumar Singh[1*], Hemant Sharma[1], Dinabandhu Sahoo[2]

[1]Institute of Bioresources and Sustainable Development, Sikkim Centre, DBT, Gangtok, India
[2]Institute of Bioresources and Sustainable Development, DBT, Imphal, India

Email: *shantiks@rediffmail.com

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Abstract

Investigations on actinomycetes are profoundly significant area of research since they form major resource for bioactive compounds, antimicrobials, anticancer agents, immunosuppressants, and biological control agents. A total of 59 actinomycetes were isolated from the soil sample collected from Domang, Lachung, North Sikkim, Sikkim, India. Out of the total isolates, 26 isolates with unique and distinct characteristic features were selected and analysed for antimicrobial activity as well as extracellular enzyme production. Out of 26 isolates, 17 (66%) isolates exhibited different level of growth inhibition against the test microorganism. 12 (47%) isolates showed antifungal activity and six (23%) isolates showed antibacterial activity. Most of the isolates showed antifungal activity. Isolate RCS260 was found to exhibit maximum growth inhibition (60%) against *Colletotrichum gloeosporioides* MTCC 8021. Isolate RCS252 showed maximum growth inhibition (67%) against *Bacillus subtilis* MTCC 441. Out of 26 isolates, 14 (54%) isolates exhibited chitinase activity, 25 (96%) isolates showed cellulase production, 20 (77%) isolates produced amylase enzyme and 17 (65%) isolates showed positive for protease activity. Potential isolate RCS260 has been characterized and identified as *Streptomyces vinaceus* strain RCS260 while isolate RCS252 was identified as *Kitasatospora aburavienis* strain RCS252. The antagonistic profile of strain RCS260 highlights its potential as antifungal agent against phytopathogens.

Keywords

Actinomycetes, Antimicrobial Activity, Extracellular Enzyme Production

1. Introduction

Actinomycetes are filamentous bacteria possessing high G + C content (>55 mol%) in their nucleic acid. The metabolic diversity of actinomycetes is mainly because of large genome having many transcription factors which enable them to direct gene expression according to precise requirements [1]. They are ubiquitous organisms with wide physiological and morphological diversity and have been isolated from all kinds of terrestrial and aqueous habitats where they can exist as free-living bacteria as well as pathogens [2] or in symbiotic associations with plants [3] and insects [4] or as endophytes [5]. The genus *Streptomyces* is dominant among the actinomycetes. They are largely exploited for their capability to produce variety of secondary metabolites including antibacterials [6], antifungals [7], antivirals [8], antitumor [9], anti-hypertensives [10], immunosuppressives [11] and plant growth promoting factors [12]. *Streptomyces* is the most prominent among all the actinomycetes with regard to production of antibiotics which accounts to about 75% of commercially used and almost half of the antibiotic compounds identified so far [13]. Actinomycetes exhibiting antimicrobial activity have been reported from Phoomdi, Loktak Lake, Manipur, India [14]. *S. tanashiensis* strain A2D was reported to possess significant antimicrobial activity [15]. Our group has also reported the isolation of *S. sannanensis* strain SU118 and production of antimicrobial agent [16]. They are also exploited for various enzymes of commercial importance [17] [18] [19] [20]. They produce several enzymes including chitinase, pectinase, amylase etc. which are of significant values in industries. Apart from their values in industries, these enzymes play a major role in bioremediation of recalcitrant substances and to uphold the biogeochemical cycle [21] [22]. Several species of *Streptomyces* are also responsible for decomposing organic molecules like cellulose, lignin, hemicelluloses, chitin and other recalcitrant compounds [13]. Chitin is one of the most abundant polysaccharides found on this planet. Chitinolytic enzymes are responsible for degradation of chitins available in nature. Chitinases have been found to be responsible for the breakdown of chitin which is present in the cell wall of fungi. Their potential role as a biocontrol agent against fungal phytopathogens is of considerable interest in organic farming system. Chitinase from one of the strains of actinomycetes, *S. griseus* HUT6037, has been found to increase plant resistance to disease caused by *Trichoderma reesei* [23]. Our group has reported the chitinolytic activity of *Streptomyces* sp. MT7 and its antagonistic activity against wood-rotting fungi [24]. Keeping in view of the fact that in spite of massive exploration of actinomycetes for different functionalities in earlier times, the investigation for potent organism possessing distinctive characteristics still holds to be fascinating field of research. During the process of search for effective metabolites, many studies have been targeted in isolating novel actinomycetes from unique and virgin habitats. Even though terrestrial habitats have been explored by several researchers for many years, it is pertinent to highlight that the actinomycetes discovered so far is very meagre [25]. Studies on actinomycetes from

untapped environments have attracted noteworthy interest in the present time for the production of secondary metabolites and other functionalities. The growing inventory of novel actinomycetes and the outcomes obtained from lesser studied areas such as Antarctica [26] as well as some habitats of Manipur [14] [15] [16] imply that a vigilant investigation of new environment might lead to be valuable. Such work on screening of actinomycetes capable of producing bioactive metabolites is of immense importance that would lead to the development of effective antifungal as biocontrol agent towards the fight against phytopathogens causing diseases in crop plants. Biocontrol agents offer safer alternatives compared to the chemicals which are hazardous. This investigation is a component of our continuing effort for screening of actinomycetes from pristine locations of north east region of India within the Eastern Himalayan biodiversity hot-spot so as to comprehend the microbial resources and their worthiness to the society. This study focus on the exploration and screening the actinomycetes from rhizosphere soil of *Panax bipinnatifidus* from Lachung, a high altitude region of North Sikkim, Sikkim, India for their antimicrobial activity and their capability to produce some important industrial enzymes.

2. Materials and Method

2.1. Location and Sample Collection

Lachung, a mountain village close to the Tibetan border, in the northeastern Indian state of Sikkim in the North Sikkim district is located about 118 km from Gangtok. It is divided by the Lachung River. The picturesque valley, also known for its pine forest, waterfalls, hot springs, serene environment, pristine habitats is part of the Eastern Himalayan Biodiversity Hot Spot region. Soil samples were collected from a depth of 5 - 10 cm from the rhizosphere of *Panax bipinnetifidus* from Domang area, a pristine ecological niche of Lachung (Latitude: 27˚41'20.61"N; Longitude: 88˚44'34.7"E; Altitude: 3114 meters, Average temperature: 14.3˚C; Annual rainfall: 1094 mm). Three samples each of about 50 g were collected from the three sites approximately 5 meters apart. The samples were kept in sterile container after collection using sterile spatula and brought to the laboratory for study. The three samples were pooled together and mixed. **Figure 1** shows the sampling site of the study area.

2.2. Culture Media for Isolation

The following media with the mentioned components were used during the isolation of actinomycetes. Starch casein agar (SCA): Soluble starch—10.0 g; Casein—0.3 g; KNO_3—2.0 g; NaCl—2.0 g; K_2HPO_4—2.0 g; $MgSO_4 \cdot 7H_2O$—0.05 g; $CaCO_3$—0.02 g; $FeSO_4 \cdot 7H_2O$—0.01 g; Agar—20.0 g; distilled water—1000.0 ml; pH—7.0. Casein Starch Peptone Yeast extract Malt extract agar (CSPY-ME): K_2HPO_4—0.5 g; Casein—3.0 g; Soluble starch—10.0 g; Peptone—1.0 g; Yeast extract—1.0 g, Malt extract—10.0 g; Agar—20.0 g; distilled water—1000.0 ml, pH—7.5. Streptomyces Agar (SA) (HiMedia Laboratories Pvt. Limited, Mumbai, India);

Figure 1. Location of sampling site, Lachung, North Sikkim, Sikkim, India.

Actinomycete Isolation Agar (AIA) (HiMedia, Laboratories Pvt. Limited, Mumbai, India).

2.3. Test Microorganisms

Colletotrichum gloeosporioides MTCC8021, *C. capsici* MTCC3414, *C. acutatum* MTCC2213, *Bacillus subtilis* MTCC441 and *Escherichia coli* MTCC739 procured from Microbial Type Culture Collection Centre, CSIR-Institute of Microbial Technology (IMTECH), Chandigarh, India while *C. gloeosporioides* ITCC 5514 procured from Division of Plant Pathology, Indian Agriculture Research Institute, Pusa campus, New Delhi were used for detection of antimicrobial activity of the actinomycetes during the investigation.

2.4. Pretreatment of Soil Sample and Isolation of Actinomycetes

The pooled and mixed sample was air dried sufficiently for a week time. 1.0 g of the dried sample was taken and treated with 0.1 g of $CaCO_3$ and kept at room temperature for 7 days so as to enrich actinomycetes that generally prefer alkaline pH [27]. 1.0 g of the treated sample was then added to 99.0 ml of sterile distilled water and placed in a shaking incubator maintained at 28˚C with shaking speed of 140 rpm for one hour. The mixture was then allowed to settle down. 1.0 ml from the suspension was taken and then subjected to serial dilution up to 10^{-5} dilutions. 0.2 ml from 10^{-3}, 10^{-4} and 10^{-5} dilutions were taken and plated in duplicates on SCA, CSPY-ME agar, SA and AIA plates which were previously added with 100 µg/ml Cycloheximide and 50 µg/ml Nalidixic acid by adopting spread plate technique [28]. The plates were incubated for 10 days at 28˚C. Individual colonies having characteristics features of actinomycetes were isolated after incubation. The isolates were streaked repeatedly on the respective agar plates so to obtained pure cultures. The cultures of the selected pure isolates were transferred to agar slants and stored at 4˚C. The selected pure isolates were then studied for antimicrobial activity against test microorganisms and also evaluated for

production of extracellular enzymes. For long term preservation and future use the purified actinomycete colonies were scraped from agar plates by sterile blunt tips using a glycerol buffer solution and one ml of buffer-actinomycete suspension was transferred to sterile cryo-vials and stored at −80˚C.

2.5. *In-Vitro* Antimicrobial Activity of the Isolates

2.5.1. Antibacterial Activity

The selected actinomycete isolates were evaluated for antibacterial activity by adopting spot inoculation technique [14]. Each pure isolates were spot inoculated in the centre of the SCA plate and incubated for seven days at 28˚C. After incubation the colonies were covered with a 0.6% agar layer of nutrient agar (NA) earlier added with the test bacterial (*B. subtilis* MTCC441and *E. coli* MTCC739) suspensions having 1.5×10^8 colony forming units (CFU)/ml in normal saline [29]. The plates were then incubated for 24 h at 37˚C. After incubation the zone of growth inhibition of the test bacterial strain around the actinomycete isolates was examined. The evaluation was performed in triplicates. The percentage inhibition of growth of the test bacteria was calculated according to de Oliveria *et al.* [30] and recorded.

2.5.2. Antifungal Activity

The actinomycete isolates were evaluated for *in-vitro* antagonistic activity towards *C. gloeosporioides,* the blight pathogen of *Amomun subulatum* (Large cardamom) as well as other phytopathogens by adopting dual culture assay method [31] with little modification. A loopful of spore suspension (10^6 spores/ml) of the actinomycete isolates was inoculated on one side of Potato Dextrose Agar (PDA) plate and incubated for 5 days at 28˚C. At this time the colonies had become distinctly visible and sporulated. Fungal mycelial plug of 5.0 mm diameter from 72 h old culture of *C. gloeosporioides* grown on PDA was cut and transferred to the other side of the PDA plate already grown with the actinomycete isolate. The fungal mycelial plug placed on PDA plates uninoculated with actinomycetes serve as control. The plates were further kept for incubation for 5 days at 28˚C. The radial fungal growth in the direction of the antagonist in the dual culture plates as well as in the control was measured after incubation. Growth inhibition was considered when the growth of the fungal mycelium in the direction of the actinomycete colony was hindered or prevented. All the evaluation was performed in triplicates. Similarly the antagonistic activity of the actinomycete isolates was also performed against other phytopathogens. The percentage growth inhibition of the pathogens was calculated according to the method reported by Zivkovic *et al.* [32].

2.6. Screening for Production of Extracellular Enzymes by the Isolates

2.6.1. Chitinase Detection

The chitinolytic property of the actinomycetes was detected by adopting the method of Nagpure *et al.* [24]. The isolates were spot inoculated by a loop at the

centre of the basal salt agar medium having the following composition (in g/L of distilled water): K_2HPO_4 0.7, KH_2PO_4 0.3, $MgSO_4$ 0.5, $FeSO_4$ 0.01, $ZnSO_4$ 0.001, $MnSO_4$ 0.001, $(NH_4)_2SO_4$ 0.25, Agar 20.0, yeast extract 1.0% and 1.0% colloidal chitin. To detect chitinase activity the plates were incubated at 28˚C for 7 days and then the agar plates were flooded with an aqueous solution of 0.1% (w/v) Congo red for 40 min. Congo red solution was then poured off and destained with 1 M NaCl for 20 min. Any clearance zone thus formed around the colony indicates positive for the production of chitinase enzyme was observed and recorded.

2.6.2. Cellulase Detection

The detection of cellulase enzyme production was carried out by adopting the method of Kasana *et al.* [33]. The isolates were inoculated in basal salt agar medium containing 0.5% Carboxy Methyl Cellulose (CMC). The plates were incubated at 28˚C for 7 days. Upon incubation the plates were then flooded with Gram's iodine and observed after 5 minutes. Gram's iodine forms a bluish black complex with cellulose but not with hydrolysed cellulose, thereby producing a distinct clear zone around the cellulase producing isolates. Plates without the CMC (non-substrate) were used as controls in all experiments. Formation of clear zone indicates positive for cellulase production by the isolates which were recorded.

2.6.3. Amylase Detection

Detection of amylase production was carried out according to Rengasamy & Thangaprakasam [34] using Starch agar medium (HiMedia Laboratories Pvt. Limited, Mumbai, India) containing 0.2% soluble starch. The actinomycete isolates were inoculated in starch agar plate and incubated at 28˚C for 7 days. After incubation 3 ml of 1% iodine was flooded in each plate and then development of a zone around the colonies indicated the production of amylase. Iodine forms a bluish black complex with starch but not with hydrolysed starch, thereby producing a clear zone around the amylase producing isolates. Development of clear zone indicates positive for amylase production and the results were recorded.

2.6.4. Protease Detection

The detection of protease enzyme production by the actinomycete isolates were performed according to the method described by Jeyadharsan [35] using Skim milk agar medium (HiMedia Laboratories Pvt. Limited, Mumbai, India) containing 2.8% skim milk powder. The actinomycete isolates were inoculated in the centre of Casein agar plate and incubated at 28˚C for 7 days. After incubation the plates were observed for the development of zone of clearance around the colony. The enzyme activity was visualized as a clear zone of substrate utilization and the results were recorded.

2.7. Siderophores Production

The production of siderophores by the actinomycete isolates were evaluated ac-

cording to the methods of Beneduzi *et al.* [36] Pure actinomycete isolates were spot inoculated on King's B medium supplemented with a complex chrome azurol S [CAS/Iron (III)/hexadecyltrimethyl ammonium bromide]. The plates were incubated at 28˚C for 7 - 10 days. The isolates that were able to produce siderophores grew and formed a yellow halo in the blue coloured medium after the iron chelation by siderophores. Thus the actinomycete isolates that showed yellow zone were recorded as positive for siderophores production.

2.8. Characterisation of the Actinomycetes

2.8.1. Morphological and Cultural Characteristics

The colony features, cultural characteristics and the diffusible pigments of all isolates were studied and observed by culturing for 14 days on medium like ISP-2 (in g/L of distilled water: Malt extract 10.0, Yeast extract 4.0, Dextrose 4.0, agar 20.0, pH 7.2); ISP-4 (in g/L of distilled water: Soluble starch 10.0, K_2HPO_4 1.0, $MgSO_4$ 1.0, $(NH_4)_2SO_4$ 2.0, $CaCO_3$ 2.0, $FeSO_4{\cdot}7H_2O$ 0.001, $MnCl_2$ 0.001, $ZnSO_4$ 0.001, agar 20.0, pH 7.2) according to International *Streptomyces* Project (ISP) [37]. The morphological characteristics were studied by using cover slip culture method in which the culture was transferred to the base of cover slips buried at an angle of 45˚ in SCA medium [16] and incubated at 28˚C for 10 days. The characters such as aerial mycelia, spore arrangements, morphology of spore bearing hyphae with entire spore chain was then studied as described in Bergey's manual [38] by observing under phase contrast microscope.

2.8.2. Identification of the Potential Actinomycete Strains

The two potential strains namely RCS252 and RCS260 were identified by adopting polyphasic approach including cultural characteristics, morphological, biochemical characteristics and molecular method by the 16S rRNA gene sequencing. Utilization of different carbon sources was carried out by growth on ISP9 [39] supplemented with 1% carbon source at 28˚C. Temperature range, pH range and NaCl tolerance for growth have been evaluated on ISP4 by growing the two isolates separately at different temperatures (20˚C - 42˚C), pH (5 - 10) and NaCl concentration (1% - 5% NaCl) respectively. Starch hydrolysis, Gelatine Liquefaction as well as other biochemical tests was performed according to the protocol described by Gordon *et al.* [40]. Nitrate reduction test were evaluated by adopting the procedure of ISP [37]. Evaluation for meso and LL-Diaminopimelic acid (LL-DAP) were performed by following the method of Becker *et al.* [41] and Lechevalier and Lechevalier [42]. The results of all the experiments were observed and noted down after incubation for seven days.

2.8.3. Genomic DNA Isolation and Amplification of 16S rDNA Gene

The two actinomycetes strains namely RCS252 and RCS260 were grown in 5 ml Soyabean Casein Digest medium by incubating in a shaking incubator at 28˚C for 7 days with agitation at 140 rpm. Genomic DNA of the strains was extracted separately by adopting phenol-chloroform method [43]. The 16S rDNA gene amplification of the two strains was carried out separately. Fragment of 16S rDNA gene of

the strain RCS252 was amplified by 27F and 1492R primers while amplification of 16S rDNA gene of the strain RCS260 was done by ACT283F and ACT1360R primers. The gene amplification were performed in thermal cycler (Bio-Rad, USA) in a final volume of 25 µl [reaction mixture containing 0.625 µl of each forward and reverse primer (10 µM each), 1.0 µl of template DNA, 2.5 µl of 10× reaction buffer, 1.0 µl of $MgCl_2$ (25 mM), 0.30 µl (5 unit/µl) DNA polymerase and sterilized Mili-Q grade water to makeup volume up to 25 µl]. In the PCR process the thermal cycle was set as initial denaturation at 94˚C for 10 min, followed by 36 cycles of denaturation at 94˚C for 1 min, annealing at 56˚C for 1 min, extension at 72˚C for 30 sec, and a final extension at 72˚C for 7 min to allow for extension of any incomplete products. The PCR product were observed and resolved on agarose gel electrophoresis and visualized under UV-transilluminator by using a Bio-Rad Gel Doc (ChemiDoc^{TM}XRS+) system. The PCR amplicon was purified by using commercial gel extraction kit (Promega Corporation, USA). Purified 16S rDNA genes of the two stains were subjected to sequencing separately by Sanger method through the courtesy of Eurofins Genomics India Pvt Ltd., Bangalore, India. The DNA sequencing reaction of PCR amplicon was carried out using BDT v3.1 Cycle sequencing kit on ABI 3730 ×l Genetic Analyzer. Consensus sequence of 16S rDNA gene was generated from forward and reverse sequence data using aligner software.

2.8.4. Construction of Phylogenetic Tree

The 16S rRNA gene sequences of the two strains RCS252 and RCS260 were aligned separately with the nucleotide sequences of *Streptomyces* and related genera in GenBank database using BLAST [44]. Sequences with more than 98% homology were taken for the analysis. Multiple alignments of 16S rRNA nucleotide sequences of the strains RCS252 and RCS260 and sequences from GenBank database were performed separately with CLUSTAL W program [45]. Phylogenetic trees of both the strains were generated separately using neighbor-joining method [46] with bootstrap testing [47] of 1000 replicates, in MEGA6 [48].

2.9. Statistical Analysis

It is significant that all the experiments were performed in triplicates and average values were considered. All data were studied and analysed by using Microsoft Office Excel and also to draw the figures.

3. Results and Discussion

3.1. Isolation of Actinomycetes

The soil sample collected from Domang, in Lachung, North Sikkim, Sikkim, India was used for isolation of actinomycetes. The average soil temperature and relative humidity were found to be 12.21˚C ± 0.24˚C and 52.07% respectively in October at the time of sample collection. The soil sample was found to have moisture content of 61.07% and pH of 5.65. Four media such as SCA, CSPY-ME, SA and AIA were used for the isolation. A total of 59 actinomycete isolates were

obtained from the soil sample. Out of the four media used, AIA was found to be most suitable medium for the isolation of actinomycetes as 36 colonies of actinomycetes were observed in this medium, followed by CSPY-ME agar medium with 10 colonies, while 8 and 5 colonies were observed in SA and SCA medium respectively as shown in Figure 2. From the total isolates, 26 isolates having unique and distinct morphological features were selected and analysed for antimicrobial activity as well as production of extracellular industrially important enzymes. Pure cultures were maintained in the same medium that was used for isolation and store at 4˚C. The isolates were also stored at −80˚C using glycerol buffer solution in sterile cryo-vials for long term preservation. Our finding highlighting the suitability of AIA for isolation of actinomycetes is also in conformity with the reports by Maiti and Mandal [49] wherein more numbers of actinomycetes were isolated using AIA medium. Similar findings of isolation of actinomycetes using AIA medium was reported by Ganesan *et al.* [50].

3.2. Morphological and Cultural Characteristics of the Actinomycetes

The cultural characteristic features of the actinomycete isolates were observed and noted down with respect to colour, substrate and aerial mycelium, diffusible pigments, colony texture. The results are provided in Table 1. Figure 3 shows cultural morphology of some of the isolates in agar plates as well as the microscopic observation by phase contrast microscopy after growing by cover slip culture.

A per the observation by phase contrast microscopy the spore bearing hyphae and the types of spores arrangement have been note down. Spore chains can be divided morphologically based on length and number of spore [51] and has been noted down in Table 1. Most of the isolates were found to belong to the rectiflexibiles type of spore arrangements, followed by retinaculiaperti type, which

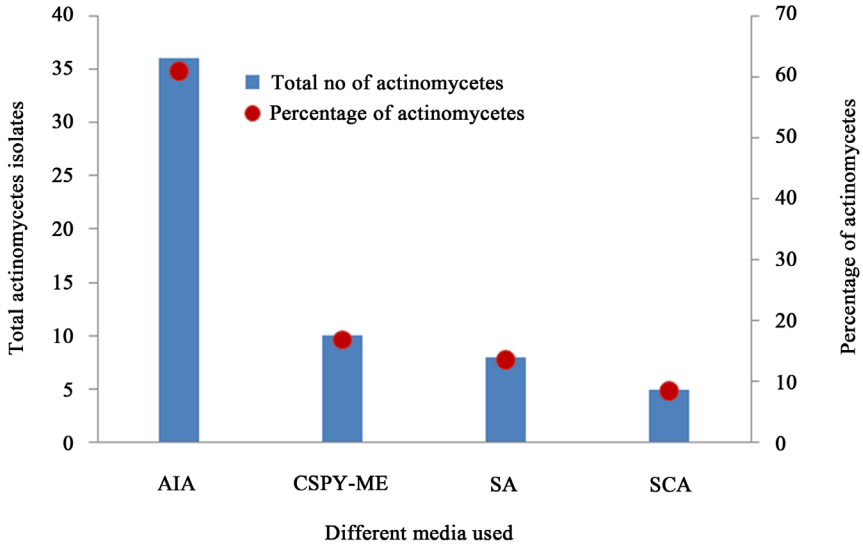
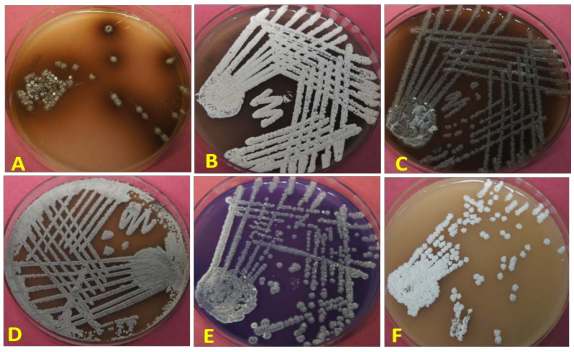


Figure 2. Different nutrient media used for isolation of actinomycetes.

Table 1. Cultural and colony characteristics of actinomycete isolates from soil of Domang, Lachung, North Sikkim.

Isolates	Colony texture	Substrate mycelium	Aerial mycelium	Soluble pigment in medium	Diffusible pigment in medium	Types of spore arrangement
RCS244	Dry, hard	Brown	Light grey	Brown	Blackish	Monosporous
RCS245	Dry powdery	Brown	White	Brown	Black	Disporous
RCS246	Dry shiny	Light brown	Dark grey	Light brown	No	Rectiflexibiles
RCS247	Powdery	Light grey	White	Light brown	No	Rectiflexibiles
RCS248	Dry	Light brown	White	Light brown	No	Rectiflexibiles
RCS249	Dry shiny	Brown	Whitish	Brown	Black	Rectiflexibiles
RCS250	Powdery	Light grey	Off white	Black	No	Rectiflexibiles
RCS251	Dry	Cream	Light brown	No	No	Retinaculiaperti
RCS252	Dry	Yellowish	Brown	No	Light brown	Long spore chain
RCS253	Powdery	Light grey	Off white	Black	No	Retinaculiaperti
RCS254	Dry	Pink	Cream	Brown	No	Rectiflexibiles
RCS255	Powdery	Dark grey	Grey	Brown	Light brown	Retinaculiaperti
RCS256	Dry, hard	Black	Grey	Dark brown	No	Retinaculiaperti
RCS257	Creamy	Yellow	Off white	Yellowish	No	Fragmenting branched mycelia
RCS258	Powdery	Dark grey	Grey	No	No	Rectiflexibiles
RCS259	Powdery	Dark grey	Grey	No	No	Rectiflexibiles
RCS260	Powdery	Grey	Whitish	Light grey	No	Rectiflexibiles
RCS261	Dry	Grey	Off white	Greyish	No	Fragmenting branched mycelia
RCS262	Powdery	Grey	Light Brown	No	No	Rectiflexibiles
RCS263	Powdery	Grey	Off White	Light brown	No	Rectiflexibiles
RCS264	Dry shiny	Grey	Light Grey	Purple	Purple	Fragmenting branched mycelia
RCS265	Dry	Grey	Off white	No	No	Retinaculiaperti
RCS266	Cottony	Brown	White	Light brown	No	Long spore chain
RCS273	Powdery	Brown	Grey	Light brown	No	Spira
RCS274	Dry shiny	Light brown	Cream	Light black	No	Monosporous
RCS276	Dry	Light brown	Cream	No	No	Monosporous

are the characteristic features of *Streptomycetes*. This reveals that most of the isolates belong to *Streptomyces* genus. These features of *Streptomyces* have also been reported by Shirling and Gottlieb [37]. The colour of the substrate mycelia, aerial mycelia, production of soluble pigments, spore arrangements provide important references in the determination of actinomycetes classification. Few isolates have exhibited production of diffusible pigments in the medium as shown in the **Figure 3(I)**.



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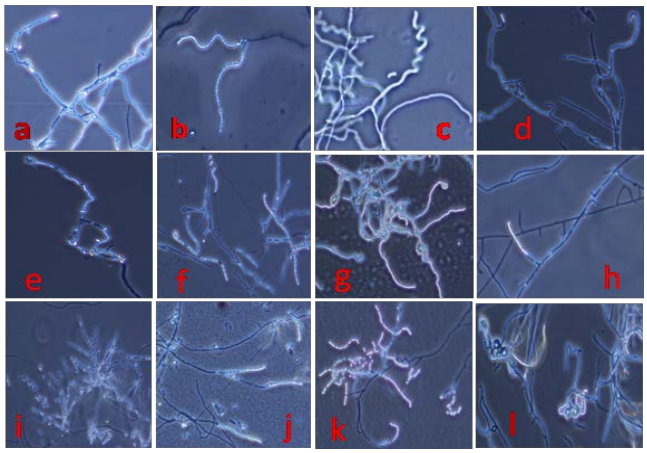

(II)

Figure 3. Morphological features of some of the isolates: (I) Actinomycete isolates [(A) RCS244; (B) RCS245; (C) RCS249; (D) RCS255; (E) RCS264 & (F) RCS266)] in agar plates (II). Microscopic observation by phase contrast microscopy showing the spore and mycelia of some isolates at 1000× magnification (10 × 100) [(a) RCS244; (b) RCS246; (c) RCS247; (d) RCS251; (e) RCS252; (f) RCS253; (g) RCS255; (h) RCS258; (i) RCS260; (j) RCS263; (k) RCS266; (l) RCS273].

3.3. Antimicrobial Activity of the Isolates

All the 26 selected actinomycete isolates were evaluated for antimicrobial potentiality against fungal phytopathogens and bacteria. Out of 26 isolates, 17 (66%) isolates were found to exhibit different level of growth inhibition against the test microorganism. Table 2 shows the antimicrobial activity of only the active isolates. 12 (47%) isolates showed antifungal activity and six (23%) isolates showed antibacterial activity. Most of the isolates exhibited antifungal activity. Isolate RCS260 was found to exhibit maximum growth inhibition (60%) against *C. gloeosporioides* MTCC 8021 followed by isolates RCS263 and RCS246 which showed 57% growth inhibition against *C. gloeosporioides* MTCC 8021 and *C. gloeosporioides* ITCC 5514 respectively. Isolate RCS252 showed maximum growth inhibition (67%) against *B. subtilis* MTCC 441 followed by RCS253 and RCS251. Figure 4(A) showed the *in-vitro* antagonistic activity of RCS260 against *C. gloeosporioides* MTCC 8021 and *C. capsici* MTCC 3414 while Figure 4(B) showed the antibacterial activity of RCS252 against *B. subtilis* MTCC 441

Table 2. Antimicrobial activity of active actinomycete isolates against test microorganisms expressed as percentage.

Isolate no.	*C. gloeosporioides* MTCC 8021	*C. gloeosporioides* ITCC 5514	*C. capsici* MTCC 3414	*C. acutatum* MTCC 2213	*E. coli* MTCC 739	*B. subtilis* MTCC 441
RCS246	55	57	38	43	−ve	−ve
RCS247	35	19	10	−ve	−ve	−ve
RCS249	25	15	−ve	−ve	−ve	−ve
RCS250	31	20	−ve	−ve	−ve	−ve
RCS251	32	25	−ve	10	−ve	35
RCS252	−ve	−ve	−ve	−ve	24	67
RCS253	−ve	−ve	−ve	−ve	−ve	36
RCS255	47	30	25	33	−ve	−ve
RCS258	46	28	23	31	−ve	24
RCS260	60	55	50	45	−ve	−ve
RCS262	−ve	−ve	−ve	−ve	−ve	35
RCS263	57	53	40	41	−ve	−ve
RCS265	51	48	35	41	−ve	−ve
RCS266	36	21	11	23	−ve	−ve
RCS273	44	24	19	30	−ve	−ve
RCS274	−ve	−ve	−ve	−ve	−ve	−ve
RCS276	−ve	−ve	−ve	−ve	−ve	6

Note: Numericals indicate Percentage Growth Inhibition; −ve = No growth Inhibition.

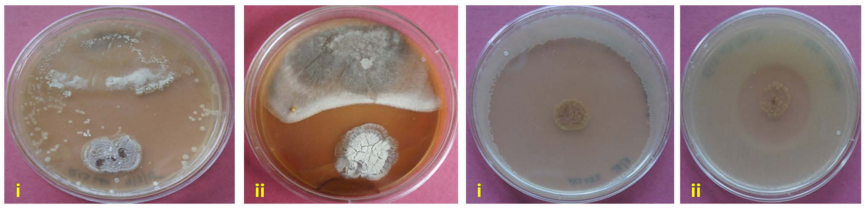

Figure 4. (A) *In-vitro* antagonistic activity of RCS260 against (i) *C. gloeosporioides* MTCC 8021 (ii) *C. capsici* MTCC 3414; (B) Antibacterial activity exhibited by RCS252 against (i) *B. subtilis* MTCC 441 (ii) *E. coli* MTCC 739.

and *E. coli* MTCC 739. The results signify that the isolates grown on agar plates produced extracellular diffusible metabolites (s) which inhibited the hyphae growth of the fungal pathogens. Similarly the diffusible metabolites produce in the agar plates brought about the growth inhibition of the bacterial test pathogens in the of agar overlaying assay. Similar findings of actinomycetes exhibiting antimicrobial activity in solid medium have been reported by Singh *et al.* [14]. Prapagdee *et al.* [31] reported the strong inhibition of fungal phytopathogen including *C. gloeosporioides* by *S. hygroscopicus* due to production of extracellular metabolites. Antagonistic potential of actinomycetes against phytopathogenic fungi were brought about due to the production of extracellular hydrolytic en-

zymes as well as secondary metabolites [52] [53] [54]. Potential isolates such as RCS252 and RCS260 which showed maximum activity were taken for further identification up to species level.

3.4. Production of Extracellular Enzyme by the Isolates

All the 26 isolates were tested qualitatively for production of extracellular enzymes such as chitinase, cellulase, amylase and protease in solid agar medium. Out of 26 isolates, 14 (54%) isolates showed positive for chitinase activity, 25 (96%) isolates tested positive for cellulase production, 20 (77%) isolates were found to produce amylase enzyme and 17 (65%) isolates showed positive for protease activity as shown in Figure 5. The qualitative assay for the enzyme activity of some of the isolates is shown in Figure 6. It was also observed that all the isolates produced at least one of the enzymes.

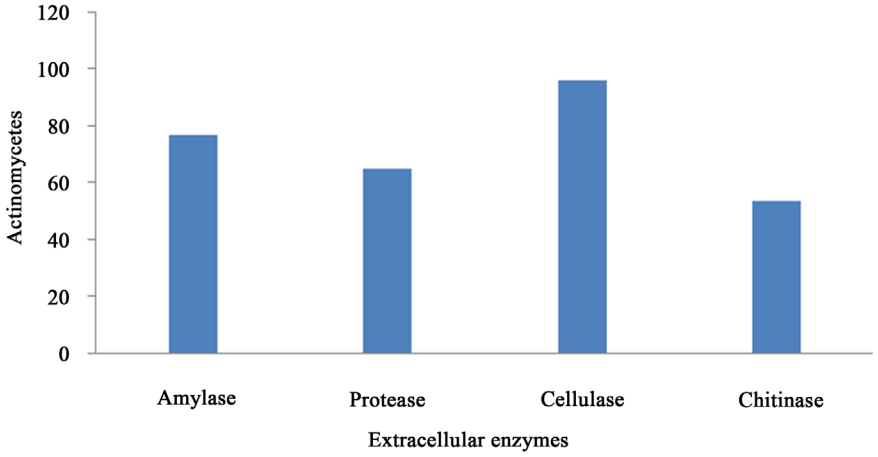

Figure 5. Actinomycete isolates showing production of different extracellular enzymes.

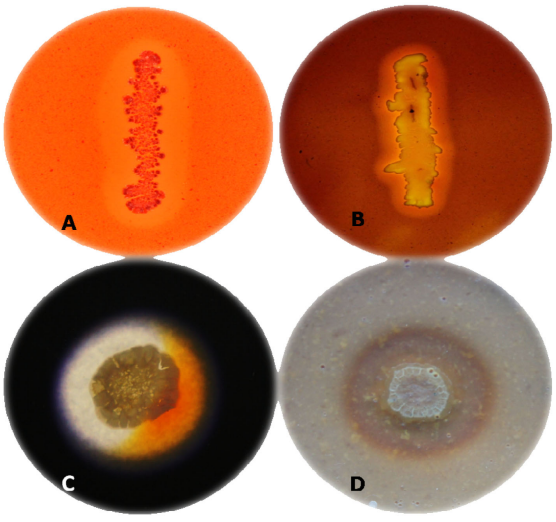

Figure 6. Screening for extracellular enzyme production by actinomycete isolates (A) Chitinase activity of RCS248 (B) Cellulase activity of RCS260 (C) Amylase activity of RCS260 and (D) Protease activity of RCS252.

Positive chitinase activity was assayed by observing the characteristic clear zone around the colony. Isolate RCS248 was found to exhibit notable chitinase activity as evident from the zone of clearance. The formation of clear zone was virtually due to the degradation of chitin in the growing medium. The larger zone formation indicates greater activity. Not all isolates provide good results. Isolates which does not produce clear zone might be because of the rate of reaction that did not hydrolyzed chitin well. Chitinase degrade chitin into monomers and brings about inhibiting the growth of fungal pathogens. The appearance of clear zone around the colony, caused the termination of the β-1, 4 N-acetylglucosamine bond by chitinase into monomer GlcNAc. More the number of monomer GlcNAc is produced; the larger clear zone was formed around the colonies. Addition of Congo red dye in the medium make the visibility of clear zone better. Positively charged Congo red detects the nonpolar hydrogen bonding in carbohydrates resulting in orange red color, whereas clear zone formed due to the reduction of chitin remains clear. Cellulase activity was observed in medium containing CMC. Formation of clear zone around the colonies after staining with Gram's iodine indicates production of cellulase by the isolates. Isolate RCS260 was found to exhibit significant cellulase activity as indicated from the zone of clearance. The isolates utilized cellulose from the medium. Qualitative screening for extracellular cellulase production by microorganisms is often carried out on agar medium containing CMC as substrate [55]. The method is easier as the results are obtained directly and quickly. In our study the actinomycete isolates bring about the hydrolytic activity on carboxymethyl cellulose due the production of enzyme cellulase in the medium. Amylase production by the isolates was detected on starch agar medium. The formation of clear zone around the colonies indicates positive for amylase activity by the isolates. The isolates displayed various zone of clearance. Isolate RCS260 displayed noteworthy amylase activity. Similar analysis of amylase production on starch agar has been carried out. Notable amylase production by S. *gulbargensis* DAS 131 and *S. avermitilis* have been reported [56] [57]. Amylase is one of the most significant industrial enzymes. Huge quantity of amylase is being used every year in the various industrial applications. Amylase finds various applications in food industry, fermentation, textile and paper industries and there is huge demand on a global market [58] [59]. Amylases are produce from various sources however microbial sources are the most preferred one for large scale production and have found successful applications in starch saccharification, brewing as well as distilling industries [60]. The proteolytic activity of the isolates was detected using skim milk agar. Formation of clear zone around the colonies indicated positive for the extracellular protease production in the medium by the isolates. The hydrolysis of casein in the medium due to the release of enzyme results in the development of zone around the colony. Isolate RCS252 was found to exhibit significant proteolytic activity. Production of protease by actinomycetes has been reported by Jeyadharsan [35].

3.5. Siderophores Production

Siderophores productions by the isolates were screened by chrome azurol S method. The isolates which produced siderophores were detected as the presence of iron chelator siderophores was indicated by decolourization of a blue coloured medium containing ferric CAS complex into yellow halo zone around the colonies. Out of 26 isolates screened, 19 (73%) isolates showed the production of siderophores in agar medium. Isolate RCS265 exhibited noteworthy siderophores production as evident from the yellow halo around the colony which is shown in Figure 7. Siderophores production by *Streptomyces* sp. MT7 has been reported by Nagpure *et al.* [24]. Chelation of Fe^{3+} by siderophores results in unavailability of iron to the phytopathogens. It has often been assumed that competition for Fe in the rhizosphere is controlled by the Fe affinity of the siderophores, whereby the ligands produced by the biocontrol agent have higher formation constants than those of the pathogen [36].

3.6. Identification and Characterization of the Potential Strains

The two potential isolates RCS260 and RCS252 have been characterized and identified. Both the strains are Gram-positive filamentous actinobacteria. The morphological, physiological and biochemical characteristics of the two strains is shown in Table 3. The substrate mycelium of strain RCS260 showed grey colour while the aerial mycelium showed whitish colour. The strain does not produce any diffusible pigments in the medium. The culture, when observed by phase contrast microscopy reveals rectiflexibiles type of sporophores arising from the aerial mycelium and may be placed in the Rectus-Flexibilis (RF) group of *Streptomyces* species [61]. LL-diaminopimelic acid (L-DAP) was present in the cell wall of strain RCS260 but no characteristic sugar. The 16S rRNA gene sequence of the strain RCS260 was compared with the nucleotide sequences of other *Streptomyces* strains from the NCBI GenBank database. The phylogenetic tree generated on the basis of 16S rRNA gene sequence of the strain RCS260 and the

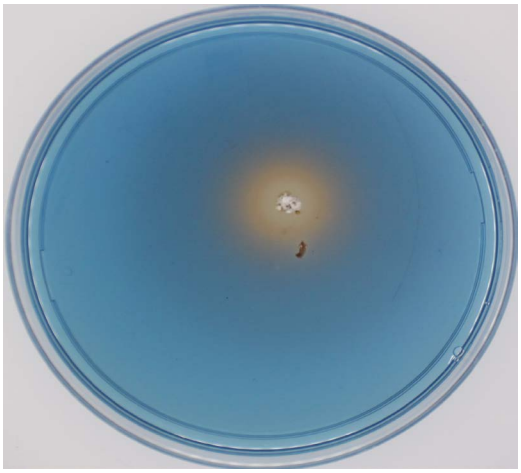

Figure 7. Actinomycete isolate RCS265 exhibiting siderophores production indicated by the yellow halo around the colony.

Table 3. Morphological, physiological and biochemical characteristics of strain RCS252 and RCS260.

Property	Result	
	Kitasatospora aburaviensis strain RCS252	*Streptomyces vinaceus* strain RCS260
Morphological characteristics		
Cell shape	Mycelial	Mycelial
Sporophore morphology	Long spore chain	Rectus-Flexibilis (RF)
Aerial mycelium colour	Brown	Whitish
Substrate mycelium colour	Yellowish	Grey
Physiological characteristics		
Growth under anaerobic condition	−ve	−ve
Acid-fast reaction	−ve	−ve
Temperature range for growth	25˚C - 40˚C	25˚C - 40˚C
Optimum temperature for growth	28˚C	28˚C
pH range for growth	5 - 8	5 - 8
Optimum pH for growth	7	7
Growth on Mc Conkey agar	−ve	−ve
NaCl tolerance	3%	4%
Biochemical characteristics		
Gram reaction	+ve	+ve
Catalase production	−ve	−ve
Oxidase production	−ve	−ve
Urease production	−ve	−ve
Hydrogen sulfide production	−ve	−ve
Nitrate reduction	−ve	−ve
Gelatin liquefaction	+ve	+ve
Methyl red test	−ve	−ve
Vogues proskauer test	−ve	−ve
Indole production test	−ve	−ve
Citrate utilization test	−ve	−ve
Starch hydrolysis	+ve	+ve
Casein hydrolysis	+ve	+ve
Acid production from		
Glucose	−ve	−ve
Arabinose	−ve	−ve
Mannitol	−ve	−ve
Xylose	−ve	−ve
Meso-inositol	−ve	−ve
Sucrose	−ve	−ve
Galactose	−ve	−ve
Fructose	−ve	−ve
Cell-wall amino acids	Both LL-Diaminopimelic acid and mes-DAP	LL-Diaminopimelic acid

nucleotide sequences from closely related *Streptomyces* strains using neighbor-joining method is presented in Figure 8. The strain has got highest 16S rRNA gene sequence similarity with *S. vinaceus* strain AS-65. Hence, on the basis of morphological, physiological, biochemical and analysis of the 16S rRNA gene sequence, the new isolate RCS260 has been identified and designated as *S. vinaceus* strain RCS260. 16S rRNA gene partial sequence of the isolate RCS260 has been deposited in the GenBank database under the accession number MK942687.

The substrate mycelium of strain RCS252 showed yellowish colour while the aerial mycelium showed brown colour. The strain produces light brown diffusible pigments in the medium. The culture exhibited long chain of spores arising from the aerial mycelium. Both LL-diaminopimelic acid (L-DAP) and meso-DAP was observed in the cell wall of strain RCS252. Presence of both L-DAP and meso-DAP is the characteristic features of the genus *Kitasatospora* as reported by Takahashi [62]. The 16S rRNA gene sequence of the strain RCS252 was compared with the nucleotide sequences of other *Kitasatospora* and *Streptomyces* strains from the NCBI GenBank database. Similarly the phylogenetic tree was generated based on the 16S rRNA gene sequence of the strain RCS252 and the nucleotide sequences from closely related strains using neighbor-joining method as shown in Figure 9. The strain RCS252 has got highest 16S rRNA gene sequence similarity with *K. aburaviensis* strain CSSP531. Thus on the basis of different characteristic features and analysis of the 16S rRNA gene sequence, the new isolate RCS252 has been identified and designated as *K. aburaviensis* strain RCS252. 16S rRNA gene partial sequence of the strain has been deposited in the NCBI GenBank database under the accession number MK942686.

The results of the present invetigation indicate that the antagonistic activities of the selected actinomycete isolates against the fungal test pathogen could be the result of production of chitinase, cellulase, protease along with other antimicrobial metabolites. Some of the actinomycete strains are also able to inhibit the

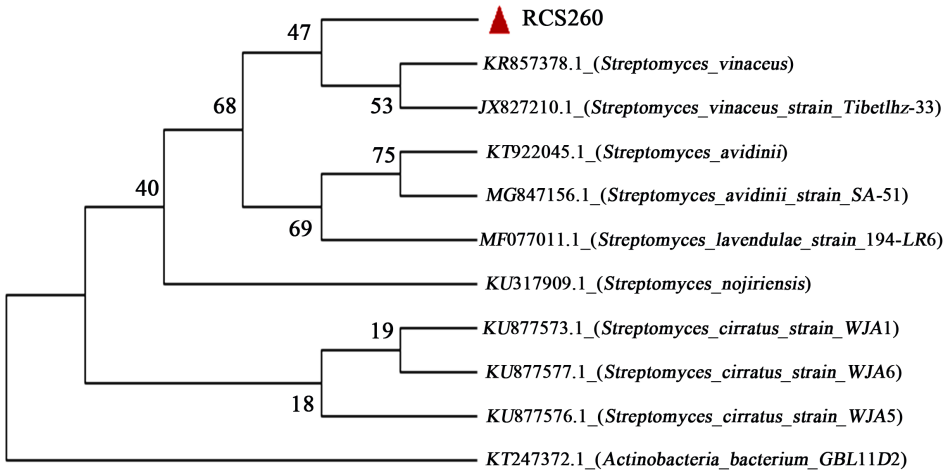

Figure 8. Phylogenetic tree based on 16S rRNA gene sequences from *Streptomyces vinaceus* strain RCS260 with other closely related *Streptomyces* species.

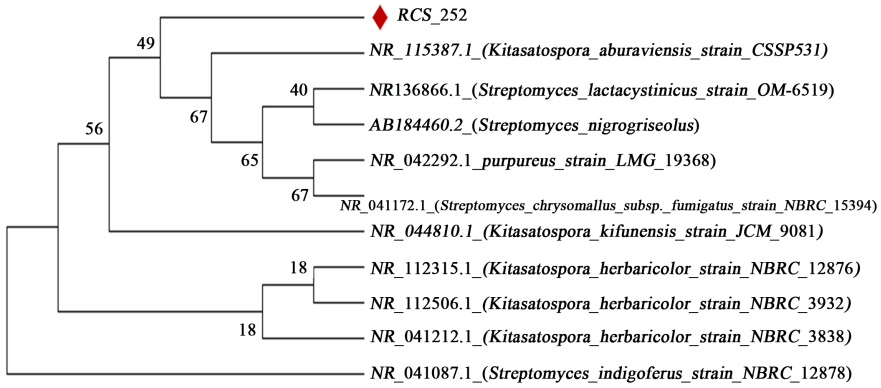

Figure 9. Phylogenetic tree based on 16S rRNA gene sequences from *Kitasatospora aburaviensis* strain RCS252 with other closely related species.

growth of gram positive and gram negative bacterial test pathogen with less activity against the gram negative pathogen. The antifungal activity of antagonists has been attributed to the action of hydrolytic enzymes including chitinase, alpha 1, 3-glucanase, chitosanase as well as protease [63] [64]. Therefore the production of extracellular chitinase and cellulase by the isolates in our study has significant relevance since most of the isolates which produce these enzymes also exhibited antifungal activity. It has been reported that the production of chitinase and alpha 1,3-glucanase enzymes by *Streptomyces* was related to fungal growth inhibition and the biological control of fungal pathogens was possible because of the ability of *Streptomyces* to degrade fungal cell walls [52] [63]. Microbial products as biological control agents are being examined as alternatives to synthetic chemicals in agriculture. Sikkim is one of the largest producers of large cardamom (*Amomum subulatum* Roxb.), which is also a major source of income for the farmers of this tiny Himalayan state. There has been a significant decline in the yield of large cardamom over the years. Among many factors for the decline, fungal blight of large cardamom cause by *C. gloeosporioides* is a major concern [65]. So far there is no effective control measure for this devastating disease of large cardamom. Sikkim being declared as organic state, natural products from microbial sources could be used as an alternative to chemical pesticides. Our study holds relevance in this aspect since most of actinomycete isolates screened exhibits noteworthy antifungal activity against the *C. gloeosporioides.* Many commercially important bioactive compounds, antibiotics, other bio agents have been discovered from actinomycetes isolated from pristine regions. *Streptomyces* sp. MT7 obtained from Manipur has been reported to exhibit antagonistic efficiency against fungal pathogen [24] Antifungal activity of a polyene metabolite obtained from *Streptomyces* sp. isolated from Kodachadri region of Western Ghats has been reported by Shobha and Onkarappa [66]. Thakur *et al.* [67] reported the isolation of *Streptomyces* sp. from soil samples collected from the forest of Assam and Tripura highlighting broad spectrum antifungal and antibacterial activity. Bharti *et al.* [68] reported the dominant antifungal nature of *Streptomyces* sp. compared to other genera of actinomycetes,

isolated from unexplored Garhwal region. Consequently, the taxonomic diversity even within the well-studied group of terrestrial actinomycetes is likely to be far from exhausted.

4. Conclusion

The present investigation outlined the potential of actinomycetes isolated from rhizosphere soil from Lachung, North Sikkim, India exhibiting antimicrobial activity. Out of the total isolates, 17 (66%) isolates showed antimicrobial activity and almost all the isolates produce at least one extracellular enzyme. The antifungal spectrum of antagonistic activity exhibited by the potential isolate RCS260 against the phytopathogens further testifies the prospective of the unexplored actinomycetes from this region. This screening highlights the potential of the strain RCS260 as a candidate towards the search of antifungal agent to mitigate the burden of phytopathogens causing crop losses. This result throws light on the merit for further study of the potential strain. Further studies on the extraction, analysis of the nature of the metabolite will be carried out to establish and confirm the importance of the isolate from this pristine ecological niche.

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Authors' Contribution

LSS: performed the entire experiment and prepared the manuscript. HS: assisted LSS in conducting the experiment and also in preparing the manuscript. DS: Coordinated the study. All authors have duly checked and approved the manuscript before submission.

Conflicts of Interest

The authors declare that there are no conflicts of interests.

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