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ORIGINAL RESEARCH ARTICLE

Isolation and Screening of Antibiotics Producing *Streptomyces* spp from the Soil Collected around the Root of *Alnus nepalensis* from Godawari

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Abstract

Actinomycetes are considered as the most invaluable prokaryotes whose genome mining show a great number of putative secondary metabolite biosynthesis pathways as well as gene clusters ranging from 20 to 50 per genome. The genus *Streptomyces* has been explored for its ability to produce 60% antibiotics worldwide. *Alnus nepalensis* (Alder) has been found to harbor diverse Eubacteria in its rhizosphere. To evaluate the antibiotic production potential from Actinomycetes, we collected soil samples from rhizosphere (5-7 cm deep) of Alder tree. Primary screening was done by cross-streak method against Multidrug Resistant (MDR) such as Methicillin resistant *Staphylococcus auereus* (MRSA), Vancomycin resistant *Enterococcus feacalis* (VRE), Imepenem resistant *Acinetobacterbaumannii*, Vancomycin resistant *Klebsiella pneumonia* and Imepenem resistant *E. coli* as well as Non-MDRs (*E. coli, Bacillus subtilis, Klebsiella pneumoniae, S. aeureu* and *Enterococcus feacalis*). Extraction of antibiotics was done using rota-vapour from extract obtained by solid-substrate fermentation technique followed by solvent extraction. Secondary screening was done using well diffusion assay against MDRs. Among total of 40 isolates of Actinomycetes recovered, 14 showed remarkable zone of inhibition (ZOI) to various MDRs. NASA 303 showed 26 mm of ZOI against VRE, NASA 101 had ZOI of 34 mm against MRSA, NASA 319 had 33.7 mm ZOI against Imepenem resistant *E. coli*, NASA 306 had 36 mm of ZOI against Vancomycin resistant *Klebsiella pneumoniae*, and NASA 108 showed ZOI of 29.6 mm against Imepenem resistant *E. faecalis*. This investigation revealed that the Actinomycetes found in Rhizosphere of Alder tree had MDR killing potent antibiotics, which needs to be further explored.

Keywords: Actinomycetes, *Alnus nepalensis,* Antibiotics

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Introduction

Nepal is a narrow rectangular country in the Himalayas. It is bounded by cold, arid Tibetan plateau in north and hot humid planes to the south. Being a transition zone between the two extremes of north and south, the vegetation and the microbes in this goldilocks zone has thrived spectacularly. The alder (*Alnus nepalensis*) of Nepal [1] is one of the examples for the diversified ecosystem, which houses amazing diversity of microbes within its rhizosphere region. Alder is semi-deciduous tree; present throughout the Himalaya at 500-3000m elevation from Pakistan through Nepal and Bhutan to Yunnan in southwest China. Moreover, the Alder houses microbes capable of producing antimicrobial metabolites that prevents it from being infected by different pathogenic fungi and bacteria. Its rhizosphere can be screened in-search of novel antibiotic producing microbes [2]. Actinomycetes are filamentous bacteria that belong to the phyla Actinobacteria and the order Actinomycetales. Actinomycetes are considered as the most invaluable prokaryotes in medicinal and biotechnology industries because of their ability to produce number of bioactive molecules, particularly of the antibiotic compounds. *Streptomyces* that belong to the genus Actinomycetes has been considered for the production of 60% of the antibiotics [3-5].

The rapidly decreasing costs of genome sequencing has made genome mining, an invaluable resource for drug discovery: A great number of putative secondary metabolite biosynthesis pathways have been discovered using these genome data [6, 7]. In Actinomycetes species, about 20-50 gene clusters per genome encoding pathways for secondary metabolite biosynthesis are present [6, 8]. Intriguingly, a

large number of these pathways are cryptic: they are not expressed under standard laboratory conditions, and their products are therefore unknown. Exciting proof-of-principle successes have already been achieved in awakening cryptic secondary metabolites [10].

It has been known for over a century that the overwhelming majority of microbial species do not grow on synthetic media (*in-vitro)* and remain unexplored [9]. The rRNA and meta-genomics approaches demonstrated a spectacular diversity of these uncultivated species. Accessing this "missing" microbial diversity is of significant interest for both basic and applied sciences and has been recognized as one of the principal challenges for microbiology today [10]. In recent years, technical advances in cultivation methodologies have recovered a diverse set of ecologically relevant species [11]. However, by and large, the gap between microbial diversity in nature and that in culture collections remains unchanged, and most microbial phyla still have no cultivable representatives [12]. One of the oldest unresolved microbiological phenomena is why only a small fraction of the microbiological population grows on artificial media. The "uncultivable" microbial majority arguably represents our planet's largest unexplored pool of biological and chemical novelty. Here we utilize this approach and develop a novel platform for parallel cultivation and isolation of previously uncultivated microbial species from a variety of environments. We followed the simple method of isolation of Actinomycetes by using the selective media starch casein agar. Microbial derived natural products are major sources of antibiotics and other medicines, but discovering new antibiotic scaffolds and increasing the chemical diversity of existing ones are formidable challenges.

Bacteria are known producers of metabolites used in pharmaceuticals and industries respectively. However the emergence of multidrug resistant pathogens has rekindled the need to discover new antimicrobials from the environment. Among many metabolites, antibiotics are one produced by *Streptomyces* spp. which is known to be found around the rhizosphere of *Alnus nepalensis*. The findings of this study will therefore shed light on isolating and screening of *Streptomyces* spp. that produces antibiotic from the soil around the root of *Alnus nepalensis.*

The world is facing an ever-increasing problem with antibiotic resistant bacteria and we are rapidly heading for a post-antibiotic era. There is an urgent need to investigate alternative treatment options while there are still a few antibiotics left. New resistance mechanisms emerge and spread globally threatening our ability to treat common infectious diseases, resulting in death and disability of individuals who, until recently, could continue a normal course of life.

Without effective anti-infective treatment, many standard medical treatments will fail or turn into very high risk procedures. Infections caused by resistant microorganisms often fail to respond to the standard treatment, resulting in prolonged illness, higher health care expenditures, and a greater risk of death. When infections become resistant to first-line drugs, more expensive therapies must be used. A longer duration of illness and treatment, often in hospitals, increases health care costs as well as the economic burden on families and societies. The achievements of modern medicine are put at risk by antimicrobial resistance. Without effective antimicrobials for prevention and treatment of infections, the success of organ transplantation, cancer chemotherapy and major surgery would be compromised. Thus, world urgently needs new and more than ever effective antimicrobials in order to treat the ever-increasing threat of antibiotics resistance.

Material and Methods

Sample collection and growth of Actinomycetes

One gram soil sample was collected from random rhizosphere region of Alder tree, situated at an altitude of 1580 msl and brought to laboratory for optimization by dry heat (100° C for 1 hour) and wet heat (80° C for 40 minutes) methods. Starch Casein Agar (SCA) media was taken as selective media and 10-fold serial dilution technique was followed using NaCl. Spread plate technique was performed and

isolated colonies of Actinomycetes were selected and revived for pure culture.

Isolation and characterization of Actinomycetes

Identification of Actinomycetes: It was done by morphological method. The morphological method consists of macroscopic examination and characterization. Microscopically the actinomycetes isolates were differentiated by their colony characters, e.g. size, shape, color, consistency etc. Colonies showing white powdery characteristics was identified. Isolated colonies were sub-cultured to obtain pure culture. Actinomycetes were named as NASA 101 to NASA 115, NASA 201 to NASA 203, NASA 301 to NASA 324, NASA 401 to NASA 404 respectively.

Primary screening: Perpendicular streak method was used to screen Actinomycetes from library against all collected MDR [Methicillin Resistant *Staphylococcus aureus* (MRSA), Vancomycin Resistant *Enterococcus feacalis* (VRE), Imepenem Resistant *Actinobacter baumannii*, Imepenem resistant *E. coli* and Vancomycin Resistant *Klebsiella pneumonia*] as well non-MDR (*Klebsiella pneumonia, S. aureus, Bacillus subtilis, E. coli, E. facealis*).

Extraction of antibiotics

Solid substrate fermentation: 100 gm of rice grain was selected for fermentation by pouring in jam bottle and mixed with 100 ml buffer solution and autoclaved. 40 such jam bottles were made. For each NASA culture inoculation of culture was done in each bottle and left for 10 days at 28°C in environment chamber. This was smashed and dried again in environmental chamber and then smashed rice was dissolved into 200 ml of methanol and ethylacetate on 1:1 ratio. Then this was kept on water bath shaker at 28°C for 24 hrs at 120 rpm. After 24 hrs the solution was filtered from Whatman paper.

Extraction using Rota vapor:

Filtrate was taken for extraction and crude antibiotic compounds were obtained after use of Rota vapor at 60° temperature in 100 rpm. This crude compound was in the form of liquid and obtained after color phase change.

Secondary screening: Well diffusion method was performed against similar MDR and non-MDR freshly grown in Trypton Soya broth for 3 hours. MHA media plates were made and 1-4 holes were punched; one hole contained solvent (methanol and ethylacetate) in ratio of 1:1. Other 3 holes contained different extract from Rota vapor method. All the plates with solvent and numbers of extract were left for nearly 1-2 hrs for diffusion. Then these plates were swabbed with MDR and Non-MDR microorganisms according to the requirements. Spread plate technique was used for it. One control plate with antibiotic disc placed on MHA media was also prepared. These spread plates with microorganisms were incubated at 37° C for 24 hrs. This plating was done for all the extract along with solvent. Then zone of inhibition was observed around extract holes.

Results

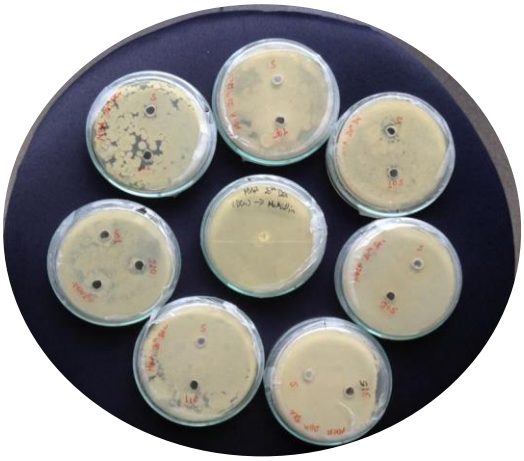

Figure 1: NASA 101 showing ZOI against MRSA

We obtain Actinomycetes isolates after primary screening from soil of rhizosphere of Alder tree found in different sites around Godawari (**Table 1**). These Actinomycetes showed antibacterial activities when cross-streaked with Non-MDR microorganisms and MDR microorganisms. Some Actinomycetes showed antibacterial activities after primary screening but not after secondary screening. NASA 103, NASA 104, NASA 105, NASA 106, NASA 107, NASA 109, NASA 110, NASA 111, NASA 114, NASA 115, NASA 201, NASA 301, NASA 302, NASA 305,

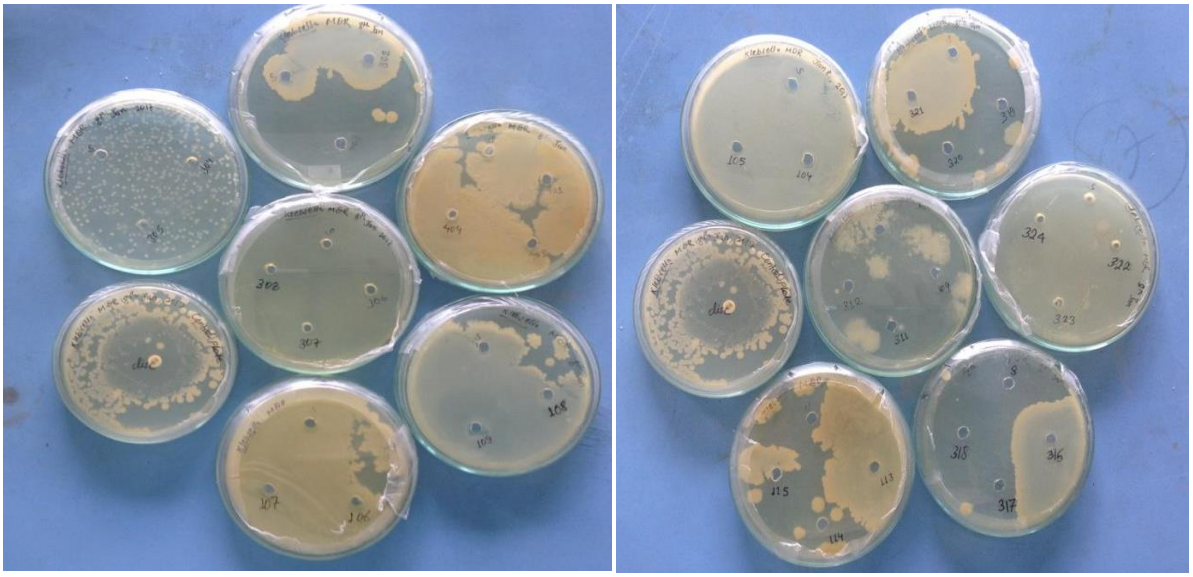

Figure 2: NASA 108, 113 showing ZOI against MDR *Enterecoccus feacelis*

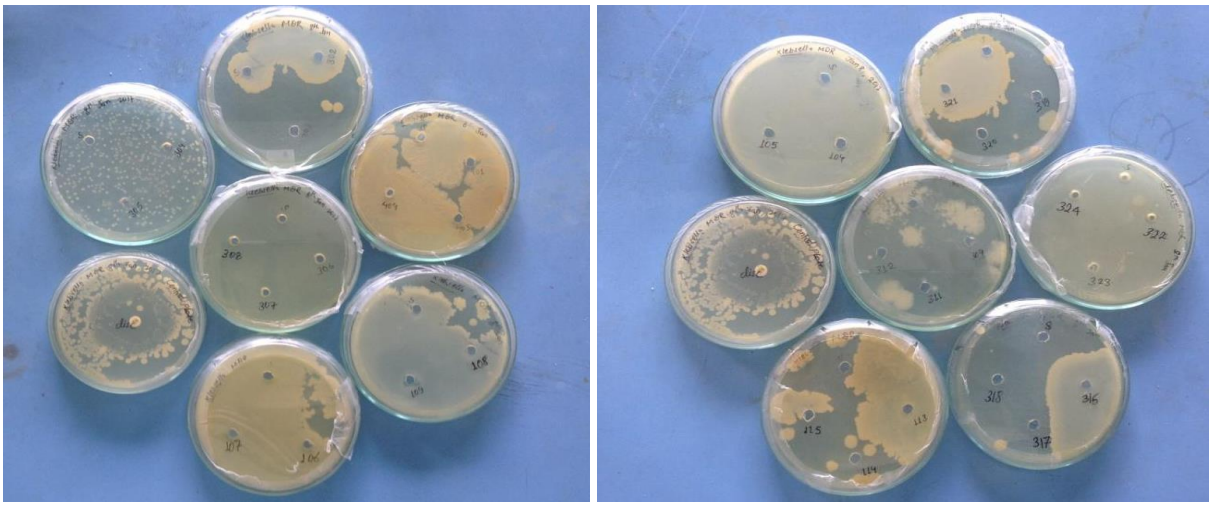

Figure 3: NASA 303, 304, 306, 307, 308, 318 showing ZOI against MDR *Klebsiella pneumoniae*

NASA 310, NASA 311, NASA 314, NASA 315, NASA 316, NASA 317, NASA 320, NASA 324 (**Table 2** and **Table 3**).

Among the 40 isolates of Actinomycetes from rhizosphere of alder tree, about 14 isolates showed antibacterial activity against different MDR bacteria. NASA 101 (Zone of Inhibition-ZOI-34mm) was found to inhibit growth of MRSA (**Figure 1**). NASA 108(ZOI-29.66mm) and NASA 113(ZOI-26.4mm) showed antibacterial activity against Imepenem resistant *E. faecalis* (**Figure 2**). NASA 303 inhibited both VRE and MDR *Klebsiella pneumoniae.* MDR *Klebsiella pneumoniae* (**Figure 3**) was inhibited by NASA 303 (ZOI-34mm), 304 (ZOI-29.7mm), 306 (ZOI-36mm), 307 (ZOI-32mm), 308 (ZOI-31mm) and

Table 1: Description of samples collected from different sites of Godawari and number of obtained Actinomycetes

S.N	Sample sites	Districts	Specific soil sample area	Soil depth (cm)	Numbers of isolates and their code
1.	J1	Lalitpur	Leech prominent area	5	NASA 306, 321
2.	E1	Lalitpur	Entrance to Phulchwoki	5	NASA 307, 308, 309
3.	E2	Lalitpur	Entrance to Phulchowki	5	NASA 108
4.	O1	Lalitpur	Slope area of Phulchowki	5	NASA 113, 304, 313, 312
5.	O11	Lalitpur	Slope area of Phulchwoki	5	NASA 303
6.	O3	Lalitpur	Slope area of Phulchwoki	5	NASA 318, 319
7.	PP	Lalitpur	Phulchwoki	5	NASA 101

Table 2: Primary screening of Actinomycetes for antibacterial activity by perpendicular streak method

Isolates	Non-MDR microorganisms					MDR microorganisms				
	B. subtilius	*E. coli*	*K. pneumoniae*	*E. faecalis*	*S. aureus*	MRSA	VRE	IpR	*E. coli*	*K. pneumonia*
NASA 101	+	+	-	-	+	+	-	-	+	+
NASA 103	+	-	-	-	-	-	-	-	-	+
NASA 104	-	-	-	-	-	-	-	-	-	+
NASA 105	-	-	-	-	-	+	-	-	-	+
NASA 106	+	-	-	-	-	-	-	-	-	-
NASA 107	+	-	-	-	-	-	-	-	-	+
NASA 108	+	-	-	+	+	-	-	+	-	-
NASA 109	+	-	-	-	-	-	-	-	-	+
NASA 110	+	+	-	-	+	-	-	-	-	+
NASA 111	-	-	-	-	+	-	-	-	-	-
NASA 113	+	-	-	-	-	-	-	+	-	-
NASA 114	-	-	-	-	-	-	-	-	-	-
NASA 115	+	-	-	+	+	+	+	-	-	+
NASA 201	+	+	-	+	+	+	+	+	-	+
NASA 301	-	-	-	-	-	+	+	+	-	-
NASA 302	+	-	-	-	+	-	-	-	-	+
NASA 303	-	-	-	-	-	+	+	-	-	-
NASA 304	+	-	-	-	+	-	-	-	-	+
NASA 305	+	-	-	-	+	-	-	-	-	+
NASA 306	-	-	-	-	-	-	-	-	-	-
NASA 307	-	-	-	+	-	+	-	-	+	+
NASA 308	+	-	-	-	+	+	-	-	-	+
NASA 309	-	-	-	-	+	+	-	-	-	-
NASA 311	-	-	-	-	-	-	-	-	-	-
NASA 312	+	-	-	-	+	-	-	-	-	+
NASA 313	-	-	-	-	-	+	-	+	-	-
NASA 314	+	-	-	-	+	-	-	-	-	+
NASA 315	+	-	-	-	-	-	-	-	-	+
NASA 316	+	-	-	-	+	-	-	-	-	-
NASA 317	-	-	-	-	-	-	-	-	-	-
NASA 318	-	-	-	-	+	-	-	-	+	+
NASA 319	+	-	-	-	+	-	-	-	+	-
NASA 320	+	-	-	-	-	+	-	-	-	+
NASA 321	+	-	-	-	+	-	+	-	-	-
NASA 324	-	-	-	-	-	+	-	+	-	-
NASA 401	-	-	-	-	-	+	+	+	-	-
NASA 403	-	-	-	-	-	+	-	-	-	-
NASA 404	-	-	-	-	-	+	+	-	-	-

= zone of inhibition absent; + = zone of inhibition present

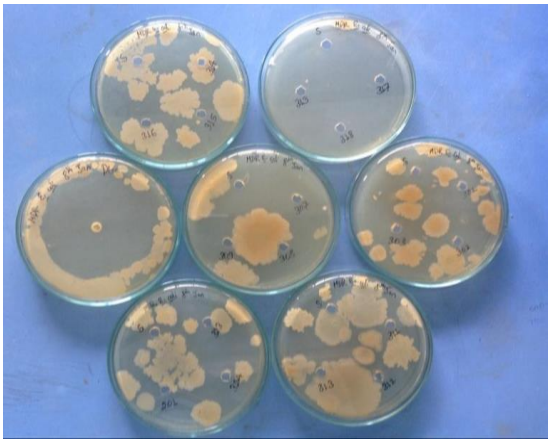

Figure 4: NASA 307, 318, 319 showing ZOI against Imipenem resistant *E. coli*

318 (ZOI-28.5mm). NASA 307 (ZOI-22.7mm) also showed antibacterial activity against MDR *E. coli* (**Figure 4**) so did NASA 318 (ZOI-31.7mm) and 319 (ZOI-33.7mm). Also, NASA 303 (ZOI-26mm), 309 (ZOI-25.6mm), 312 (ZOI-20mm), 313 (ZOI-22mm) and 321 (ZOI-24.6mm) showed antibacterial activity against VRE (**Figure 5**) Morphological features for example aerial mycelium, substrate mycelium, soluble pigment, reverse side and growth of selected 14 strains were studied (**Table 4**). NASA 101 showed ash gray aerial mycelium and yellow substrate mycelium; soluble pigment was absent and the reverse side was whitish gray showing good growth (**Figure 6**).

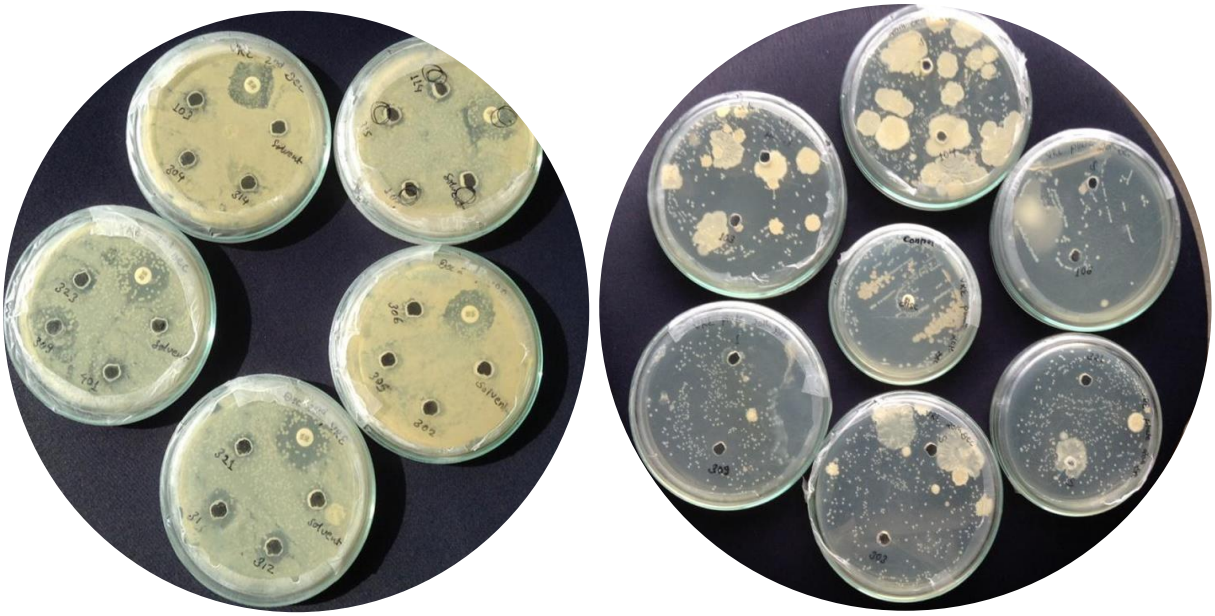

Figure 5: NASA 303, 309, 312, 313, 321 showing ZOI against VRE

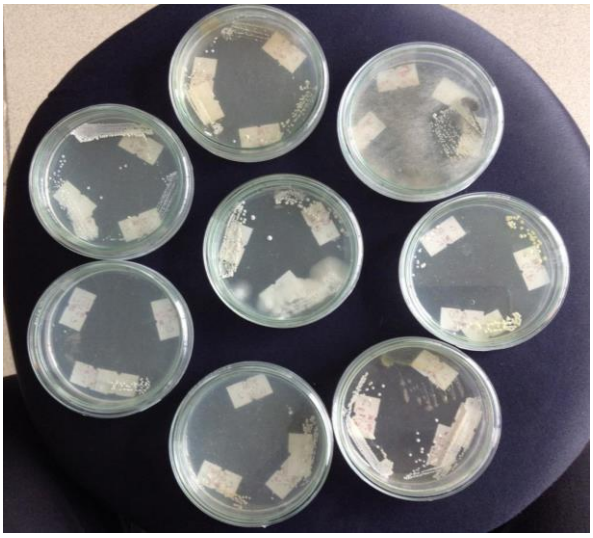

Figure 6: Growth of isolated Actinomycetes in SCA media

Discussion

The rising problem in the treatment of bacterial infections can be accounted to the presence of pathogens that are resistant to the currently available antibiotics and hence these pathogens are termed multidrug resistant (MDR) pathogens. MDR pathogens reported from Nepal includes *Escherichia coli* [13], *Salmonella* and *Shigella* species, *Escherichia coli, Salmonella* spp., *Klebsiella pneumoniae, Pseudomonas aeruginosa, Citrobacter freundii , Proteus* spp *[14]* , methicillin resistant *Staphylococcus aureus* (MRSA)[15], Vibrio cholera [16]. MDR pathogens reported from South Asia includes *Acinetobacter baumannii, Pseudomonas aeruginosa*, *Klebsiella pneumonia [17], Shigellasonnei [18] , Escherichia coli [19]*, *Salmonella enteric [20].* There is certain resemblance in MDR pathogens found in context of Nepal and South Asia yet there are differences in resistant levels of certain bacteria to different drugs. It shows MDR pathogens can be specific to its geographical location. Actinomycetes producing Vancomycin producing is *Streptomyces orientalis* [21], Methicillin is produced by *Penicillium sps.* [22] and *Imepenem by Streptomyces cattleya* [23]. Thus, Actinomycetes are found to produce drugs such as vancomycin, imepenem, etc., due to which we selected Actinomycetes in our project. Isolation of Actinomycetes from soil was done using a different method- pretreatment of soil for 1 week by air drying followed by isolation of Actinomycetes using selective media composed of starch yeast extract agar (SYE), yeast extract-malt extract agar (ISP2), oatmeal agar (ISP3), inorganic salt-starch agar (ISP4), starch-casein agar (SCA), glucose asparagine agar (GAA), Actinomycete isolation agar (AIA) and marine agar (MA)[24], whereas other authors used crowded plate method for isolation of Actinomycetes [25]. Microbially derived natural products are major sources of antibiotics and other medicines, but discovering new antibiotic scaffolds and increasing the chemical diversity of

Table 3: Secondary screening of antibacterial activity of the Actinomycetes isolated

Isolate No.	Name of the Test Organism (Inhibition zone diameter in mm)				
	VRE	MRSA	Imepenem Resistant *Escherichia coli*	VRE *Klebsiella pneumonia*	Imepenem Resistant *Enterococcus feacelis*
NASA 101	-	34	-	-	-
NASA 108	-	-	-	-	29.66
NASA 113	-	-	-	-	26.4
NASA 303	26	-	-	34	-
NASA 304	-	-	-	29.7	-
NASA 306	-	-	-	36	-
NASA 307	-	-	22.7	32	-
NASA 308	-	-	-	31	-
NASA 309	25.6	-	-	-	-
NASA 312	20	-	-	-	-
NASA 313	22	-	-	-	-
NASA 318	-	-	31.7	28.5	-
NASA 319	-	-	33.7	-	-
NASA 321	24.6	-	-	-	-

Table 4: Morphological feature of obtained Actinomycetes

Symbol of strain	Aerial mycelium	Substrate mycelium	Soluble pigment	Reverse side	Growth
NASA 101	ash gray	Yellow	-	whitish gray	+++
NASA 108	whitish gray	slimey yellow	-	whitish gray	+++
NASA 113	White	Gray	-	yellow gray	+++
NASA 303	ash gray	slimy yellow	-	dark gray	+++
NASA 304	White	White	-	dark gray	++
NASA 306	ash gray	Gray	-	dark gray	+++
NASA 307	whitish gray	Yellow	-	slimey yellow	+++
NASA 308	White	Yellow	-	slimey yellow	++
NASA 309	whitish gray	slimey yellow	-	slimey yellow	+++
NASA 312	ash gray	slimey yellow	-	slimey yellow	+++
NASA 313	dark gray	White	-	White	++
NASA 318	whitish gray	White	-	White	+++
NASA 319	dark gray	White	-	slimey yellow	+++
NASA 321	dark gray	White	-	slimey yellow	+++

- = absent; ++ = moderate growth; +++ = good growth

existing ones are formidable challenges. A total of 40 isolates of Actinomycetes were obtained, among which secondary screening NASA 101 was found to inhibit MRSA, and NASA 303, 309, 312, 313, 321 were found to inhibit VRE. MRSA is resistant to methicillin antibiotic and NASA 101 must produce antibiotic of different structure than that of methicillin [26]. Similarly, the structure of antibiotics produced by NASA isolates (**Table 3**) inhibiting VRE and Vancomycin resistant *Klebsiella pneumoniae*, should be of different structure than that of vancomycin [26]. Likewise, the structure of antibiotics produced by NASA isolates (**Table 3**) showing zone of inhibition to Imipenem resistant *E. coli* and *Enterococcus feacalis* should be different than that of imipenem [26]. From this we can say that the class of antibiotics produced by NASA isolates may be unique one in its structural and functional basis.

The results of primary and secondary screening did not match quite well in our project. Some of the strains showing zone of inhibition in primary screening did not show inhibition during secondary screening (well diffusion method). Example: NASA 101, NASA 105, NASA 115, NASA 201, NASA 401, NASA 404, NASA 301, NASA 313, NASA 321, NASA 324. This may be due to the protocol followed for extraction of antibiotics, effect of solvent used in extraction process. Extraction of secondary metabolites, such as antibiotics, are generally done by maintaining the compositions of the media containing yeast extract, dextrose, starch, casein hydrolysates, ammonium sulphate, calcium carbonate. This kind of composition leads to increased production of antibiotics [27]. Antibiotics are also extracted by using nutrient broth and the method of chromatography which helps in efficient extraction of minute amount of antibiotics that may be present in the broth [28]. But we extracted antibiotics using fermentation followed by Rota vapor technique using solvents such as methanol and ethyl- acetate (1:1). Since we used SCA media for the primary screening, the antibiotics could have expressed quite well. However, we used solid substrate fermentation method for extraction of antibiotics using rice as the media with other chemical supplements which may not allow antibiotics to be expressed on those isolates [29]. This might be due to the differences in morphology of Actinomycetes when grown in solid and liquid media as filamentous mycelia and fragmenting mycelia respectively [30]. In the secondary screening zone of inhibitions were observed in NASA 108, NASA 303, NASA 307, NASA 308, NASA 309, NASA 318, NASA 319 and NASA 321 strains but not in the primary screening. This might be due to the solid fermentation method of the NASA strains on rice medium, favoring more to these antibiotics expressing NASA strains on the secondary screening than that on SCA medium, not favoring them to be expressed during the primary screening. Many previous studies have reported a high degree of concordance in the zone of inhibition between the primary and the secondary screening extracts [30].

It was found that root soil of the analyzed trees such as pine, alder, birch contained more microorganisms than soil distal to the roots. Thus, we selected the rhizosphere of alder tree, the indigenous tree of Nepal. The presented analysis of the diversity of Actinomycetes in the isolation (different soils and rhizosphere of different trees) indicated a lack of significant differences between the microorganisms. The only sources of isolation which were relatively diverse were the root-free soil of birch and the birch rhizosphere. The lowest diversity of Actinomycetes was found in bulk soil and the action rhizal rhizoplane of alder [31]. Multidrug resistant bacteria are found in context of Nepal that are entirely native to this geographical location and thus differs from those found in other parts of the world. Hence, we need to screen and isolate Actinomycetes that are native to this environmental condition as well as novel in order combat those MDRs.

Conclusion

In this study, we isolated Actinomycetes from soil around the roots (rhizosphere) of Alder tree. We demonstrated that the rhizosphere of Alder tree houses clinically important microorganisms. Further study of these isolated strains can be done to determine exact molecules responsible

for anti-MDR property. Whereas the macroscopic morphology of the isolates could merely provide presumptive genus identification, 16S rRNA assay and subsequent sequencing analysis, in the future, may possibly resolve their species level identity. Since, MDRs are unique to certain geographical locations, Actinomycetes capable of combating such MDRs must be prevailed in that particular environmental condition. Thus, intensive research to isolate such clinically significant Actinomycetes must be encouraged using indigenous natural resources.

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