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Research Article

ISOLATION AND CHARACTERIZATION OF SOIL MYXOBACTERIA FROM NEPAL

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ABSTRACT

Realizing myxobacteria as a potential source of antimicrobial metabolites, we pursued research to isolate myxobacteria showing antimicrobial properties. We have successfully isolated three strains (NR-1, NR-2, NR-3) using the *Escherichia coli* baiting technique. These isolates showed typical myxobacterial growth characteristics. Phylogenetic analysis showed that all the strains (NR-1, NR-2, NR-3) belong to the family *Archangiaceae*, suborder *Cystobacterineae*, and order *Myxococcales*. Furthermore, 16S rRNA gene sequence similarity searched through BLAST revealed that strain NR-1 showed the closest similarity (91.8 %) to the type strain *Vitiosangium cumulatum* (NR-156939), NR-2 showed (98.8 %) to the type of *Cystobacter badius* (NR-043940), and NR-3 showed the closest similarity (83.5 %) to the type of strain *Cystobacter fuscus* (KP-306730). All isolates showed better growth in 0.5-1 % NaCl and pH around 7.0, whereas no growth was observed at pH 9.0 and below 5.0. All strains showed better growth at 32° C and hydrolyzed starch, whereas casein was efficiently hydrolyzed by NR-1 and NR-2. Besides, preliminary antimicrobial tests from crude extracts showed activities against Gram-positive, Gram-negative bacteria, and fungi. Our findings suggest that the arcane soil habitats of Nepal harbor myxobacteria with the capability to produce diverse antimicrobial activities that may be explored to overcome the rapidly rising global concern about antibiotic resistance.

Keywords: Antimicrobial, *Cystobacterineae*, Myxobacteria, Soil habitat of Nepal

INTRODUCTION

The emergence of public health-threatening multi-drug resistance (MDR) bacteria and pathogenic fungi caused a severe threat to human health and modern medicine (Butler, 2004). The heavy use of antibiotics and fungicides in agriculture, food industries, farm animals as well as unwanted prescriptions of drugs from health personnel create an environment for microorganisms, which are nowadays termed as "superbugs", to develop resistance against them, (Landers *et al.*, 2012). According to the US Center for Disease Control and Prevention (CDCP, 2016), superbugs kill 700,000 people annually. If this trend continues, then by 2050 A.D, 10 million people will die each year. The dramatic increment in resistance towards commercially available antibiotics and fungicides demands to explore new chemical entities (Weissman & Müller, 2009). However, the pipeline for the development of new antibiotics is limited (Fenical & Jensen, 2006). Though there has been increased research towards identifying novel chemical entities from both natural products and synthetic molecules, the development of new compounds as drugs are not satisfactory partly to re-isolation of already identified compounds from natural products and toxic side effects from the compound developed synthetically (Amin *et al.*, 2012; Lam, 2006; Ishida *et al.*, 2012). Therefore, research to identify new putative antibiotics has to be pursued and intensified (Schäberle & Hack, 2014). Natural products, especially microbe-derived compounds, have been proven to be a good source for antibiotics (Leeds *et al.*, 2006). Currently, anti-infective molecules produced from myxobacteria have come into focus (Schäberle *et al.*, 2014; Muller & Wink, 2014). They are excellent producers of secondary metabolites, which are known to exhibit antibacterial, antifungal, antiviral, antitumor, immunosuppressant, anti-tuberculosis, and antimalarial activities (Weissman & Muller, 2009).

Myxobacteria are Gram-negative rod-shaped, aerobic bacteria belonging to the delta subgroup of Proteobacteria. They can be distinguished from other bacteria for their unique gliding motility, swarming colonies on the agar surface, cooperative feeding behaviors, and produce fruiting bodies under starvation (Kaiser, 2004; Mauriello *et al.*, 2010; Shimkets, 1990; Dawid, 2000).

In search of myxobacteria, a soil sample from a tropical rain forest was considered to be the best source (Dawid, 2000). Furthermore, they have been isolated from substrate rich in cellulosic plant material (bark of trees, rotten wood materials) and from the dung of herbivorous animals such as rabbit, deer, moose, etc. Based on a biodiversity guided survey, the ecosystem of Nepal ranges from tropical to alpine and provides a remarkable ecological diversity. Thus, screening of myxobacteria has immense potential for isolating new bacteria that produce

novel bioactive metabolites that hold tremendous promise for human therapy against infectious diseases.

According to Reichenbach (1999), there is a possibility of finding novel myxobacteria choosing a unique sampling site, exhibiting significant biological complexity. The isolation methods and culture media could be designed to mimic the natural environment during cultivation. Myxobacteria are highly specialized in bio-macromolecules degradation, and they require various nutrients for their growth and survival (Reichenbach, 1999; Thaxter, 1904). Hence baiting with *E. coli* and inoculation of filter paper with soil are the favorable methods to isolate myxobacterial species (Reichenbach, 2005; Garcia *et al.*, 2009), predatory as well as cellulose-decomposing genera. This research was focused on the isolation of myxobacteria from Nepalese soil and characterized them based on morphological and molecular methods.

MATERIALS AND METHODS

Sample collection

From different geographical regions of Nepal, soil samples were taken from the upper part, digging a few centimeters down from the soil profile. Southern, Western, and Eastern provinces were chosen to isolate the myxobacterial strains having diverse biological characteristics. Samples were collected on a sterile plastic zip bag and transported to the laboratory in dried form Dawid (2000). Myxobacteria were screened from those soil samples collected from different parts of Nepal.

Culture media

CY Agar: Casitone (Difco), 0.3 %; Yeast extract (Difco), 0.1 %; $CaCl_2.2H_2O$, 0.1 %; Agar, 1.5 % (w/v); pH to 7.2. VY/2 Agar: Bakers' Yeast (Commercial yeast cake), 0.5 %; $CaCl_2.2H_2O$, 0.1 %; Cyanocobalamin 0.5mg/µL; Agar, 1.5 % (w/v) pH to 7.2. WCX: $CaCl_2.2H_2O$, 0.1 %; Agar, 1.5 %; HEPES (*N-2-*(hydroxyl-ethyl) piperazine-*N'-2-*ethane sulfonic acid), 20 mM, 25 µg/mL of cycloheximide was used for the isolation of myxobacteria. Chitin decomposition was tested using synthetic S agar (0.5 g $CaCl_2.2H_2O$, 0.5 g $MgSO_4.7H_2O$, 0.06 g K_2HPO_4, 8 mg Ferric EDTA, 50 mM HEPES, 1-liter distilled water; pH 7.2) with 0.7 % (w/v) chitin as the sole nutrient source.

E. coli baiting technique

Myxobacteria were isolated by the baiting technique from the collected soil samples. Alive *E. coli* and autoclaved baker's yeast were streaked, cross streaked, or dotted on cycloheximide (25 µg/mL) incorporated water agar as three streaks, cross streaks or dot. Cycloheximide (25 µg/mL) treated soil samples were placed on *E. coli*/autoclaved yeast and incubated at 30° C for 3-20 days (Reichenbach, 2005). Colony morphology and fruiting bodies were viewed under a stereomicroscope to confirm the myxobacteria according to the protocol previously developed by Reichenbach and Dworkin (1992). The organisms were stained by Gram's stain as well as (0.01 % w/v) Congo-red aqueous solution flooded on five days' culture plates (McCurdy, 1969). For cryopreservation of pure strains, pieces of agar taken from an actively growing swarm colony were placed in cryotube vial and stored at -70° C at National College, affiliated to Tribhuvan University, Nepal.

Growth response to temperature, pH, salt

Growth response at different temperatures; 37° C, 32° C, 30° C, 28° C, 25° C, 18° C and 4° C, at various pH; 5.5, 6.0, 6.5, 7.2, 8.4 and 9.0 and at salt concentration; 0 %, 0.2 %, 0.5 %, 1.0 %, 2.0 %, 3.0 %, 4.0 % and 5.0 % (w/v) was tested in VY/2 agar medium adjusting the pH, salt concentration accordingly Garcia *et al.* (2009). The relative growth was determined by measuring the diameter of swarms in successive 1, 4, 6, 10, and 12^{th} day (McDonald, 1967).

Physiological tests

Casein hydrolysis was determined by the agar well diffusion method on a medium containing 2 % (v/v) skim milk after incubating 24 hrs at 32° C (Isolauri *et al.*, 1995). Starch hydrolysis was detected on the medium containing 0.2 % (w/v) solution of potato starch, and the reaction was determined after flooding 4-week old culture with a 2 mL (0.01 M) iodine solution (Harley, 2004). Chitin decomposition was tested using synthetic S agar with 0.7 % (w/v) chitin as the sole nutrient source (Reichenbach, 2005).

Microbial predation tests

Overnight culture of *E. coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Staphylococcus aureus* ATCC 25923, *Salmonella typhimurium* ATCC 14028, and 36 hrs old culture of *Streptomyces coelicolor* A3(2) & *Saccharomyces cerevisiae* were spotted on water buffered agar. The growth of the myxobacteria and bacterial lysis, as indicated by swarm spreading on the spotted pellet of the above-mentioned culture, was observed on a phase-contrast microscope.

DNA Isolation and PCR Amplification

Genomic DNA was extracted from the actively growing eight days old culture as described by Wilson (2001). The ethanol precipitated DNA was rinsed twice with 70 % and 90 % (v/v) ethanol, air-dried, and dissolved in 20 µL TE buffer for further use. The 16S rRNA gene was amplified using specifically designed oligonucleotides (Macrogen Inc, Republic of Korea) MyxoF (5´-GAGTTTGATCCTGGCTCAGGA-3´), MyxoR (5´-AAGGAGGTGATCCAGCCGCA-3´). The PCR reaction mixture was adjusted to 20 µL, which consisted 10 µL PCR premix (*Taq* polymerase), 0.5 µL of each

oligonucleotides MyxoF (10 pM) and MyxoR (10 pM), 8 µL dH_2O and 1 µL of DNA template (150 ng). The PCR conditions were adjusted as follows: initial denaturation of 95° C for 5 min followed by 29 cycles of 95° C for 1 min denaturation, 60° C for 1min annealing, 72° C for 2 min extension; and final extension at 72° C for 10 min (Wu *et al*., 2005; Sood *et al.*, 2015). PCR products were stored at 4° C and visualized by electrophoresis. The gel was photographed in a gel documentation system (Syngene). The amplified product was purified with DNA Clean & Concentrator™-5 (Zymo Research, USA) following the manufacturer's instructions and triplicates DNA samples of NR-1, NR-2, NR-3 were sequenced from Macrogen Inc., Republic of Korea.

Phylogenetic tree analysis

The 16S rRNA gene sequence of the isolates was compared with those sequences available in public databases (GenBank/EMBL/DDBJ) using the Nucleotide Basic Local Alignment Search Tool (BLASTN version 2.2.29) (Zang *et al*., 2003; Morgulis *et al*., 2008). Sequence homology was compared with 16S rRNA gene sequences available in the database using the FASTA algorithm (http://www.ddbj.nig.ac.jp), and all reference sequences were obtained through the Ribosomal Database Project II (http://rdp.cme.msu.edu/). The 16S rRNA gene sequences of other myxobacteria, representing the type strains of the suborder *Cystobacterineae*, *Sorangiineae* and *Nannocystineae*, were retrieved from the GenBank. The sequence was aligned using CLUSTAL W ver.2.01 (http://clustalw.ddbj.nig.ac.jp/), and the phylogenetic tree was constructed using MEGA ver.6 by the neighbor-joining method with bootstrap values calculated from 1,000 replications. The assigned GenBank DNA sequence accession number for isolates NR-1, NR-2, and NR-3 are LC203133, LC203134, and LC203135, respectively.

Fermentation

Fermentation was carried out in 500 mL Erlenmeyer flask containing 250 mL of CY-H fermentation medium and incubated at 30° C for 14 days with shaking at 200 rpm. After 14 days of fermentation, the culture was harvested and treated according to the process described by Kunze *et al.* (1998).

Liquid phase extraction

The bacterial culture of 250 mL was extracted by adding 250 mL of ethyl acetate. The mixture was shaken for 60 min at room temperature on separating funnel using reciprocal shaker (150 strokes per min). It was allowed to separate for 45 min, and the organic phase was filtered through the filter paper (Whatman No.1). Filtrates were then concentrated to dryness at 40° C under vacuum and stored at 4° C for further use.

Antimicrobial tests

Fresh culture of four reference pathogenic bacteria *S. aureus* ATCC 25923, *E. coli* ATCC 25922, *K. pneumoniae* ATCC 700603, and *S. typhimurium* ATCC 14028 was taken for *in vitro* antibacterial assay. *Aspergillus niger* ATCC 16404, *Trichoderma harzianum* ATCC 52445, *Rhizopus stolonifer* ATCC 14037, *Aspergillus flavus* ATCC 204304 were chosen for *in vitro* antifungal tests. The *in vitro* antimicrobial studies were carried out by the agar well diffusion method against test organisms (Joshi *et al.*, 2009). For the antibacterial test, Muller Hilton Agar (MHA) plates were swabbed with 100 µL bacterial suspension matched with 0.5 McFarland standard turbidity of the test organism. Using a sterile cork borer well of 3mm diameter made in Petri plate. The myxobacterial crude extract was dissolved in DMSO (25 mg/mL), and 100 µg/well were added using sterile pipettes. Ampicillin 50 µg/mL was taken as a positive control & DMSO as a negative control. Likewise, for antifungal test *Aspergillus niger* ATCC 16404, *Trichoderma harzianum* ATCC 52445, *Rhizopus stolonifer* ATCC 14037, *Aspergillus flavus* ATCC 204304 were matched with 0.5 McFarland standard. Agar well diffusion methods were performed, taking 25 µg/mL cycloheximide as the positive control and DMSO (25 mg/mL) as the negative control, after 3-7 days of incubation, the zone of inhibition was observed.

RESULTS

Morphology and culture characteristics

Myxobacterial strains (NR-1, NR-2, NR-3) were isolated by collecting soil samples from different parts of Nepal (Annexure Resource 2). Isolates were transparent on agar medium (e.g., VY/2, WCX), showing films like colony, while a light pink colony was observed on VY/2 agar after 1-2 weeks of incubation in a bright environment. Radial patterns were recognized within the swarm area. The cells swarmed in a circular pattern but with unstructured of loose colony edges. NR-1, NR-2, NR-3 showed small flare-like swarms produced at the colony border, reminiscent of some members of *Myxococcales*, as shown in Fig. 1(a). The fruiting bodies were dense clusters commonly developed on agar surfaces, especially close to the center of the colony. The fruiting body of the sample (NR-1) appeared as yellowish-orange to golden in color, as shown in Fig. 1(b). Sample NR-2, NR-3 showed densely packed chestnut like brownish fruiting bodies, as depicted in Fig. 1(c). All the isolates were Gram-negative and absorbed Congo red stain, as indicated in Figs 1(d), 1(e) and 1(f).

Lysis of bacterial cells

Among cell suspensions of five selected species (*E. coli*, *K. pneumoniae*, *S. aureus*, *S. typhimurium*, *S. coelicolor*, *S. cerevisiae*) lysis was indicated by the appearance of

clear zones around the areas of myxobacterial growth, as shown in Figs 1(g), 1(h) and 1(i). NR-1, NR-2, NR-3 showed the lytic property against *E. coli*, *K. pneumoniae*, *S. aureus*, *S. typhimurium*, *S. cerevisiae*, and were unable to lyse the *S. coelicolor* (Table 1)

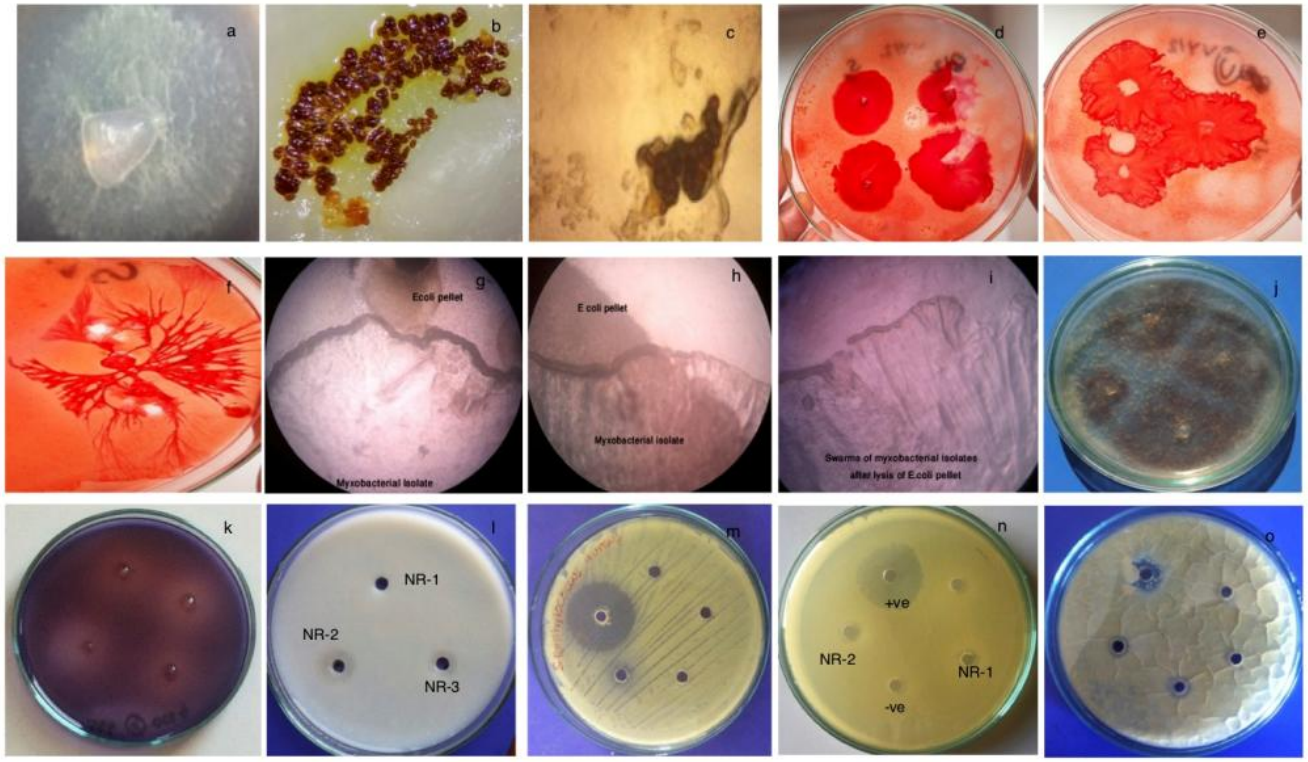

Fig. 1. (a) Flare-like swarming motility produced at the colony border by NR-1; (b) fruiting bodies arranged in clusters by NR-1 obtained by stereomicroscope; (c) fruiting bodies produced by NR-2 obtained on a phase-contrast microscope; (d), (e) & (f) congo red stain of NR-1, NR-2, NR-3, respectively; (g), (h) & (i) photograph of lytic activity of strain NR-1; (g) frontal attack strategy of myxobacteria on *E. coli* ATCC 25922 pallet; (h) & (i) swarming motility of myxobacteria over *E. coli* pellet; (j) chitinase activity; (k) starch hydrolysis of NR-1, NR-2, NR-3; (l) casein hydrolysis of NR-1, NR-2, NR-3; (m) screening of antimicrobial activity over *S. aureus* ATCC 25923; (n) screening of antimicrobial activity of ethyl acetate extract of NR-1, NR-2, NR-3 over *S. typhimurium* ATCC 14028 and (o) screening of antifungal activity of NR-1, NR-2, NR-3 ethyl acetate extract on *Rhizopus stolonifer* ATCC 14037

Table 1. Lytic property of isolates against different bacteria

Sample	Microorganisms					
	E. coli	***K. pneumonae***	***S. aureus***	***S. typhimurium***	***S. coelicolor***	***S. cereveciae***
NR-1	+	+	+	+	-	+
NR-2	+	+	+	+	-	+
NR-3	+	+	+	+	-	+

Note: (+) = Able to lyse microbial cell pellet. (-) = unable to lyse the microbial cell pellet

Growth response to temperature, pH & salt

All the isolates barely grew at 4° C, but better between 25°-32° C and decreased the growth at 37° C. All the isolates NR-1, NR-2, NR-3 grew better between pH 6-8.4, but showed weak growth at pH 5.5 and pH 9.0 (McDonald, 1967). All the isolates grew at 0.2-4.0 % NaCl and showed better growth at 1.0 % (Annexure Resource 1).

On the surface of the overlay agar, NR-1, NR-3, showed the dissolution of chitin around the areas of growth, as depicted in Fig. 1(j). NR-3 hydrolyzed starch producing clear areas in blue background flooded with iodine

solution in the medium containing 0.2 % starch (w/v) as shown in Fig. 1(k). NR-1, NR-2 efficiently hydrolyzed casein showing a clear zone around the agar well of the medium containing 2 % skim milk, as shown in Fig. 1(l).

Antimicrobial tests

The antimicrobial test was performed by the agar well diffusion method using ethyl acetate extract of isolates. The extract of sample NR-1 and NR-2 was active against most of the bacterial strains taken under study. NR-1 showed the highest ZOI (zone of inhibition) 9.33±0.881 mm, and NR-2 showed 7 ± 0.57 mm ZOI against *S. aureus* ATCC 25923. The zone of inhibition recorded was mentioned in Table 2 and shown in Figs 1(m) and 1(n).

Antifungal tests

Agar well diffusion method was performed on *Aspergillus niger* ATCC 16404, *Trichoderma harzianum* ATCC 52445, *Rhizopus stolonifer* ATCC 14037, *Aspergillus flavus* ATCC 204304, after 3-7 days of incubation zone of inhibition were observed as shown in Fig. 1(o). The ethyl acetate extract of NR-1 and NR-3 showed the highest zone of inhibition to *Aspergillus niger* ATCC 16404, 7.33 ± 0.34 mm, and 6.5 ± 0.28 mm, respectively (Table 3).

Table 2. The antimicrobial activity of ethyl acetate extract of the isolates

Sample	Zone of inhibition (mm)			
	S. aureus	***K. pneumoniae***	***S. typhimurium***	***E. coli***
NR-1	9.33±0.89	-	6.33±1.20	4.66±0.34
NR-2	7±0.57	4.33±0.89	-	5.66±1.45
NR-3	-	-	3.33±1.45	3.66±0.34
Ampicillin 1	12±0	14.66±0.34	14.66±0.34	17±0

Note: average diameter of zone of inhibition (ZOI) was given as the mean ±standard error

Table 3. Antifungal property of ethyl acetate extract of the isolates

Sample	Zone of inhibition (mm)			
	A. niger	***T. harzianum***	***R. stolonifer***	***A. flavus***
NR-1	**7.33**±0.34	-	4.66±1.34	4±0.57
NR-2	-	-	4.33±0.34	5±1.73
NR-3	6.5±0.28	3.66±0.34	3.66±0.34	4.33±0.89
Cycloheximide	11.66±0.34	14.33±0.34	14.33±0.34	16.33±0.67

**Average diameter of zone of inhibition (ZOI) was given as the mean ±standard error*

PCR amplification of 16S rRNA and sequence studies

The amplified PCR product ~1500 bp, as expected for 16S rRNA using the oligonucleotides MyxoF and MyxoR, were purified and sequenced (Fig. 2). Sequences (16S rRNA) of isolates NR-1, NR-2, and NR-3 were compared by BLAST analysis, as shown in (Annexure Resource 3), together with the morphologies, fruiting bodies and swarms to determine their genera and species. Phylogenetic analysis of 16S rRNA sequences of NR-1, NR-2, and NR-3 were identified to the species level as in (Fig. 3).

NR-1, NR-2, and NR-3 strains were firmly related to the type strains of *Vitiosangium cumulatum* (NR-156939), *Cystobacter badius* (NR-043940) and *Cystobacter fuscus* (KP-306730) with 16S rRNA gene sequence similarity values of 91.8 %, 98.8 %, and 83.5 %, respectively. It is presumed strain NR-1 could be a new species. As there is extensive documentary evidence that two strains sharing less than 97 % 16S rRNA gene sequence similarity are not members of the same species (Amann *et al.*, 1992; Collins *et al.*, 1991; Fox *et al.*, 1992; Martinez-Murcia & Collins 1990; Martinez-Muricia *et al.*, 1992).

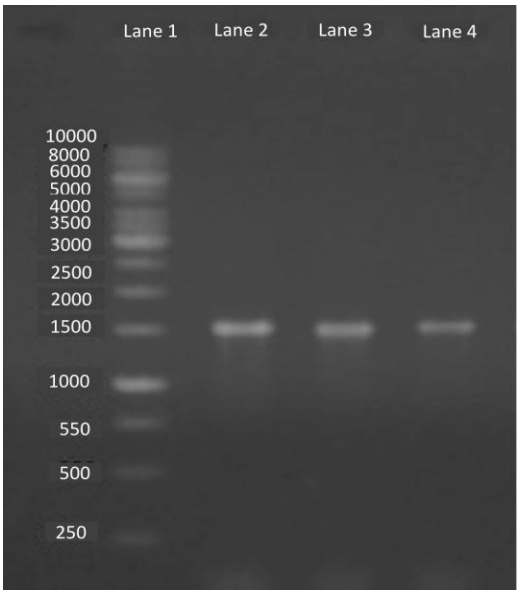

Fig. 2. Gel profile of amplified DNA product of NR-1, NR-2, NR-3; Lane 1: 1kb DNA Ladder (New England), Lane 2: NR1, Lane 3: NR2, Lane 4: NR3

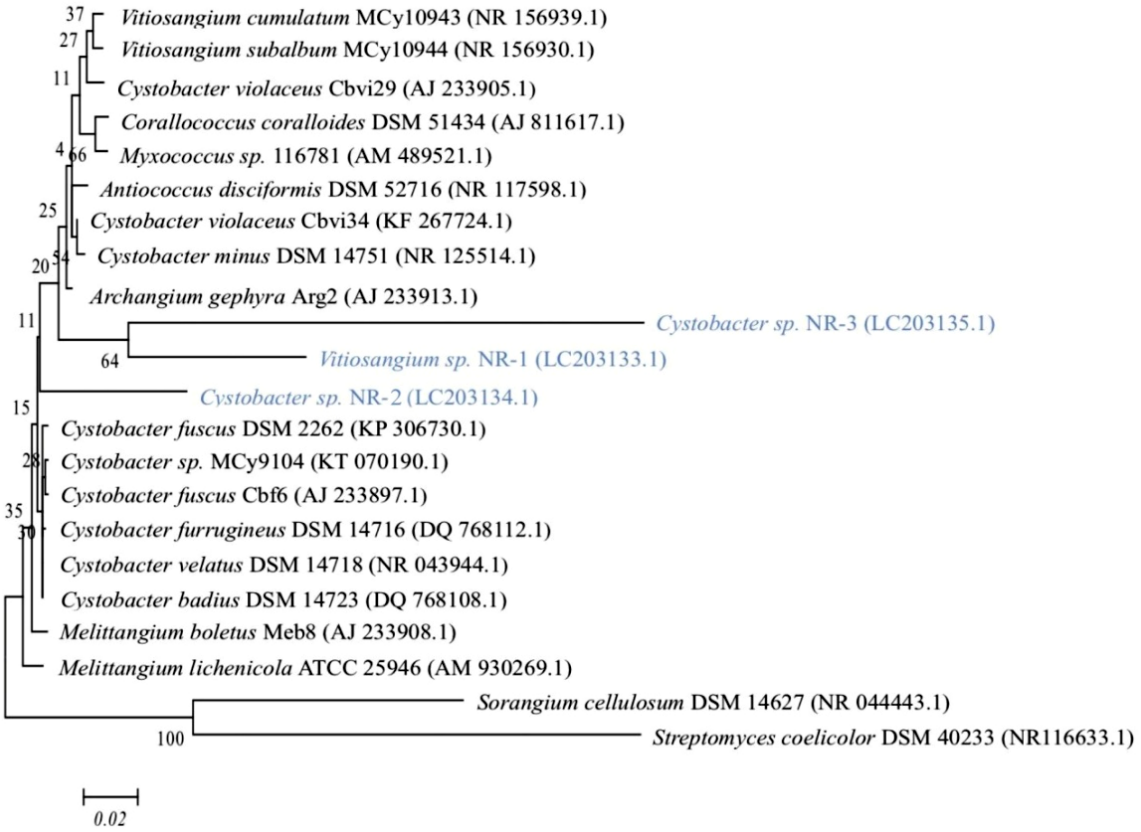

Fig. 3. Neighbor-joining tree of myxobacteria based on 16S rRNA gene sequences showing the position of the isolates NR-1, NR-2, NR-3 in the family *Archangiaceae*, suborder *Cystobacterineae* and order *Myxococcales*. GenBank accession numbers are shown in parentheses. Values at branch points indicate bootstrap support (%) based on 1000 resamplings. *S.coelicolor* DSM 40233 (GenBank accession no. NR_116633.1) & *Sorangium cellulosum* DSM 14627 (GenBank accession no. NR_044443.1) were used as an outgroup to root the tree. Bar 0.02 substitutions per nucleotide position

DISCUSSION

Myxobacteria are nonpathogenic predatory bacteria with most of them known to degrade complex biomolecule such as cellulose (*Sorangium* and *Byssovorax* species) and/or prey on other microorganisms. They are ubiquitous social bacteria present in deep-sea vent (Moyer *et al*., 1995), hydrothermal springs (Iizuka *et al*., 2006), marine samples (Iizuka *et al.*, 2003; Li *et al*., 2002), freshwater environments as well as found in varied climatic zones and ecological niches (Dawid, 2000; Jahn 1924; Hook, 1977; Mohr, 2018). Most of the myxobacteria are mesophilic and tend to grow at a neutral to slightly alkaline pH of 6.8-7.8. Nevertheless, they can also tolerate psychrophilic to halophilic environmental conditions. Isolation of myxobacteria from the environment exhibiting significant biological complexity and unique sampling sites yield the highest probability of isolating novel taxa (Garcia *et al.*, 2009; Dawid, 2000).

Myxobacteria are recently gaining interest in the researchers due to their ability to produce a wide spectrum of useful secondary metabolites (Gerth *et al.*, 2003). However, the isolation and identification of novel or new myxobacteria from environmental samples are limited and need to be explored. According to literature, less than 10 % of natural type myxobacteria have been isolated, which means isolation and characterization of myxobacteria are still crucial for the development of new metabolites (Zhang *et al*., 2012).

Nepal, a country of Himalayas which was once believed to be at the bottom of the ocean billion years ago, has remarkable ecological diversity. It is believed that more primitive myxobacteria could be isolated from Nepalese soil undoubtedly. The untouched Nepalese soil is a promising source for myxobacteria. Results indicated that NR-1, NR-2 & NR-3 produced multicellular fruiting bodies and showed swarming growth, which is essential and uniqueness to characterize myxobacteria.

Furthermore, in contrast to many *Sorangiineae*, isolates barely penetrate deep into the agar and exhibit shallow depressions. Again, they differ from *Nannocystis* through

the absence of deep agar holes (Spröer *et al.*, 1999). All strains belonged to the suborder *Cystobacterineae* as stained with Congo red solution, and they showed the usual effect of temperature and pH. Generally, terrestrial myxobacteria grow at NaCl concentration below 1 %. However, in this study, it grew at NaCl concentration between 0.2-4 %, and better growth was observed at 1 % concentration. A preliminary test showed quite remarkable antimicrobial, antifungal, chitinase activities, and starch hydrolysis.

This research illustrates the presence of myxobacterial strains in Nepalese soil. The clustering NR-1 and NR-3 were closely related to *Vitiosangium cumulatum* (MCy10943; NR-156939), *Cystobacter fuscus* (DSM 2262; KP-306730), with 16S rRNA gene sequence similarity values of 91.8 % and 83.5 % respectively. The phylogenetic tree suggests that they belong together in a common taxon, whereas their phylogenetic divergence to *Polyangium*, *Cystobacter*, and *Nannocystis* implies that they belong to at least a novel genus (NR-1) and a new family (NR-3). NR-2 belongs to the *Cystobacter sp.*, which is closely related to *Cystobacter badius* (DSM 14723; NR-043940), with a 16S rRNA gene sequence similarity of 98.8 %. We found that the isolated strains have a similarity score <97 %, so we urge to perform DNA-DNA hybridization studies shortly. DNA-DNA hybridization has traditionally been required to provide a definitive answer for identification and clustering questions (Janda & Abbott, 2007). While 16S rRNA sequence data can be used for a multiplicity of purposes, unlike DNA hybridization (>70 % reassociation) there are defined "threshold values" (98.5 % similarity) above which there is universal agreement of what constitutes definitive and conclusive identification to the rank of species (Stackebrandt & Goebal, 1994).

CONCLUSION

Overall, this work constitutes a primary description of myxobacteria isolated from Nepalese soil. Isolated myxobacterial strains could produce antibiotics and show antifungal activities. Apart from that, isolated strains can also hydrolyze chitin & casein. Moreover, the isolation of myxobacteria from the soil in Nepal is the upcoming area of research.

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ANNEXURES

Annexure Resource 1. ***Growth response to different temperature, pH & salt concentration***

Sample code																				
	5.5	**6**	**6.5**	**7.2**	**8.4**	**9.0**	**4°C**	**18°C**	**25°C**	**28°C**	**32°C**	**37°C**	**0.2**	**0.5**	**1.0**	**2.0**	**3.0**	**4.0**	**5.0**
Diameter of the colony in different pH (in mm)									**Diameter of isolates on different temperature (in mm)**				**Diameter of colony on different concentration of NaCl (in mm)**						
NR-1	-	4.0±0.57	9.67±0.89	10.6±0.33	9.8±89	-	-	13.66±0.58	15±1.52	20±0.57		31.67±0.88	12.33±1.45	9±10	9.33±0.88	15.66±1.85	8.66±2.84	8.33±0.33	4.33±0.88	-
NR-2	4±1.15	19.33±0.67	22.66±0.89	19±0.58	6.33±0.33	-	-	12±0.58	22.66±0.89	33±1.527		31.67±0.89	6.33±0.67	5±0.578	11±0.58	22.66±0.67	9±0.57	8.66±0.67	3.66±0.67	-
NR-3		2.66±1.20	15.33±0.33	18.66±1.85	10.33±0.33	-	-	13.66±58	17±0.577	23±0.577		46.33±0.33	11±0.577	5±0.578	8±0.578	13±0.5784	7.33±0.881	5.33±1.201	3.33±0.333	-

* Average diameter of colony were given as mean ±standard error

Annexure Resource 2. Name of the sample code, collection site, and its location

Sample code	Collection site	Type of sample	Latitude	Longitude
NR-1	Bardia National Park	Soil	28˚23'0''N,	81˚30'0''E
NR-2	Ghodaghodi Lake, Kailali	Soil	28˚41'00''N,	80˚56'45''E
NR-3	Bharatang	Soil	26˚59'36.7''N,	87˚04'53.7''E

Annexure Resource 3. Bioinformatics analysis of sequence data

Sample code	Highest similar hit	Match	Total	% Similarity	Accession no.
NR-1	*Vitiosangium cumulatum*	1526	1526	91.8	NR156939
	Cystobacteraceae bacterium (MCy10944)	1962	1962	91	KX430041
NR-2	*Cystobacter badius*	2261	2261	98.8	NR043940
	Cystobacter sp. MCy 9104	1136	1139	95	KT070190
	Cystobacter fuscus	2289	2289	95	AJ233879
NR-3	*Cystobacter fuscus*	944	1072	83.5	KP306730
	Cystobacter badius	1146	1146	84	NR043940
	Cystobacter sp. SDU-1	1146	1146	84	AY996786

NR-1, NR-2 and NR-3 sample shows less than 91.8 %, 98.8 % and 83.5 % similarity to its nearest hit Vitiosangium cumulatum (NR156939), Cystobacter badius (NR043940), Cystobacter fuscus (KP306730), respectively.