



Morphological, enzymatic screening, and phylogenetic analysis of thermophilic bacilli isolated from five hot springs of Myagdi, Nepal

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ABSTRACT

The present study was conducted to identify and characterize the thermophilic bacteria isolated from five hot springs, namely, Sinkosh, Singha, Bhurung, Ratopani, and Paudwar located in Myagdi district, Nepal, using phenotypic and genotypic methods. The hot spring has temperature 42–62°C and pH 6.5–6.8. Isolation of thermophiles was done using simple enriched nutrient broth media at 60°C. Selected strains were screened for thermostable enzymes; cellulase, hemicellulase, amylase, protease, gelatinase, and lipase using substrates carboxymethylcellulose, xylan, soluble starch, casein, gelatin, and Tween (20, 40, 60, or 80), respectively. The bacteria were grouped into 16 groups based on morphological and biochemical characteristics. 16S rRNA sequence analysis of 16 isolates and phylogenetic analysis showed a cluster of five distinct taxonomic groups. The groups were identified as genus *Anoxybacillus*, *Aeribacillus*, *Brevibacillus*, *Bacillus*, and *Geobacillus*, based on $\geq 95\%$ similarity with reference strains. This is the first study that reports *Anoxybacillus* sp., *Brevibacillus* sp., and *Aeribacillus* sp. from the hot springs of Nepal.

1. INTRODUCTION

Hot springs represent unique geothermal environments supporting extremophilic microorganisms that include members of all three domains of life, Archaea, Bacteria, and Eukarya [1,2]. Thermophiles can grow at higher temperatures (55–80°C) as they possess physically and chemically stable enzymes showing high metabolism also at the higher temperature. Their study has become a major domain of research due to their potential to produce industrially interesting thermostable enzymes (proteases, amylases, lipases, xylanases, and DNA polymerases) and exopolysaccharides that function under extreme conditions [3]. Thermophilic organisms grow in a limited number of sites, and exploration of such microorganisms from geothermal environments has not only provided greater insights into the origin and evolution of earliest life but also has provided access to significant bioresources with potential applications in food and biotechnology industry [4]. Therefore, thermophiles are of interest to enhance the thermostability of industrial enzymes [5].

Bioethanol, the most common renewable fuel today, is commonly derived from corn grain (starch) or sugarcane (sucrose) [6]. The production of bioethanol from starch and sugarcane is often criticized as it may affect the human food chain. Such bioresources are limited to be able to use for biofuel production and compete to replace or even partially supplement fossil fuels. Renewable lignocellulosic biomass from agricultural waste, weeds, and forest biomass, relatively abundant, may be used for large-scale production of alcohol-based fuels, and the process can be cost-efficient [7]. One of the limitations of this process is the complex and crystalline structure of lignocellulose that makes it difficult to hydrolyze to fermentable sugar [8]. Use of thermostable enzymes and thermophilic microorganisms for the degradation of the lignocellulosic material offers an advantage of minimizing the risk of contamination, and it may lead to the single-step process of enzymatic hydrolysis and fermentation [9].

In Nepal, there are more than 28 hot springs stretching right across a Southeast-Northwest elongated region. The distribution of these hot springs is mainly restricted to the Main Central Thrust area of Nepal that separates the higher Himalayan zone from the lesser Himalayan zone [10]. None of these hot springs have been explored in detail for the microbial community; accordingly, their biological diversity and biotechnological potential remain to be explored. Myagdi district of Nepal is endowed with several hot springs, which could be the potential source of thermophilic bacteria.

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Nepal is one of the countries with no any conventional fuel sources and is entirely dependent on imported fuel. A recent search for biofuel in Nepal has mainly focused on the diesel plant, *Jatropha* other than this; molasses has been the major feedstock for ethanol production. However, molasses cannot fulfill all the needs of alternative fuel. Therefore, other possible nonfood-based alternative such as lignocellulosic wastes from agro-based industries can be a potential source of bioethanol production, and Nepal is one of the agricultural economy-based countries, also exist many industries such as paper industry and textile industry, in which the industrial waste is primarily lignocellulosic waste [11]. Thus, through these lignocellulolytic bacteria isolated from the hot spring of Nepal and their enzyme system can act as the bioremediation tools.

2. MATERIALS AND METHODS

2.1. Sampling Site

Samples were collected from five different hot springs located in Myagdi district, namely, Sinkosh, Singha, Bhurung, Ratopani, and Paudwar [Figure 1] on October 2013. The location of sampling site is described in Table 1. Water samples were collected from streams, tributaries, and overflow channels as close to the center of flow as possible. Biomats were collected with a sterile spatula, kept in zipping locked plastic bags, and brought to the laboratory of Nepal Academy of Science and Technology, Kathmandu, Nepal.

2.2. Cultivation of Thermophilic Bacteria

One gram of biomats was suspended in 10 ml of sterile distilled water that served as a stock sample. 1 ml of biomats and 1 ml of sterile hot water samples were used for dilutions under sterile conditions. Spread plate and pour plate methods were used to obtain pure colonies. The diluted cultures (100 µL) were evenly spread on nutrient agar (NA) plates and the plates were incubated at 60°C for 24–48 h. Isolates were grouped based on differences in the shape, color, and size of the colony [12]. The pure cultures on NA medium were transferred to freshly prepared NA slant. Glycerol stocks were made and stored for further study.

2.3. Morphological and Biochemical Test

Colony morphology was determined after 2–4 days growth on NA plates incubated at 60°C. Each isolate was examined by Gram staining and endospore staining [13]. Most of the biochemical tests were performed according to MacFaddin [14]. Each isolated colony was streaked on NA slants and incubated at 60°C for up to 48 h to obtain abundant growth. The following tests were carried for potential strains: Oxidase, catalase, methyl red; Voges-Proskauer, and indole production. The temperature and pH range were determined by incubating the bacteria at different temperatures and pH, respectively.

2.4. Screening for Industrially Important Enzymes

The isolated pure cultures were used for screening of industrially important enzymes: Amylase, lipase, protease, cellulase, hemicellulase, and gelatinase. All screenings were done in bacterial cultures grown for 24–48 h at 60°C. Plates were also further incubated for slow-growing thermophiles.

2.4.1. Amylase

Screening of the amylase activity was performed on starch agar medium (Himedia, Bengaluru, India). The presence of amylase activity was confirmed by the appearance of a clear halo zone around the colonies after staining with Gram's iodine solution [15].

2.4.2. Protease

Screening of protease activity was performed on skimmed milk agar medium (Himedia, Bangalore, India) containing 0.5% tryptone, 0.3% yeast extract, 1.5% agar, and 25% skimmed milk. Protease activity was confirmed by the appearance of a clear zone around the colonies indicating degradation of milk casein [16].

2.4.3. Cellulase

Determination of the cellulase activity was performed on NA (Himedia, Bangalore, India) containing 0.2% carboxymethylcellulose (CMC). Cellulase activity was identified by the appearance of a clear halo around the tested strain after treatment with Gram's iodine [17].

2.4.4. Xylanase

Testing of xylanase activity was done on NA (Himedia, Bangalore, India) medium containing 0.5% beechwood xylan (Sigma Chemical Co., St Louis, USA). The presence of xylanase activity was confirmed by the appearance of a clear zone around the strain tested following the staining with Gram's iodine [18].

2.4.5. Lipolytic enzymes

For lipolytic activity nutrient, agar medium (Himedia, Bangalore, India) was supplemented with 1% of Tween 20, Tween 40, Tween 60, or Tween 80. An opaque halo around the colonies indicated the positive lipolytic activity [19].

2.4.6. Gelatinase

Screening of gelatinase activity was performed on gelatinase agar (Himedia, Bangalore, India) containing (g/l) gelatin 30.0, casein enzymatic hydrolysate 10.0, sodium chloride 10.0, agar 15.0, and final pH (at 25°C) 7.0±0.2. The appearance of a clear zone around the colony indicated gelatinase positive [20].

2.5. Production of Enzymes and Assessment of their Activity

Strains showing comparatively larger halo zone were selected for further assay of amylase, cellulase, and xylanase production.

2.5.1. Cellulase production

Cellulase activity in the strains was determined in cultures grown in minimal salt solution (MSS). MSS contained (g/l) NaNO₃ 0.5, K₂HPO₄ 1.0, MgSO₄·7H₂O 0.5, FeSO₄·7H₂O 0.01, and yeast extract 1.0 containing 0.5% CMC at pH 7. Cultures were incubated for 24 h at 60°C and 180 rpm [21]. Culture was centrifuged at ×12000 g for 20 min at 4°C. Enzyme extract, 500 µl, was added to 500 µl of 1% CMC solution prepared in 0.1 M sodium phosphate buffer pH 7. The mixture was incubated for 30 min at 60°C and 180 rpm. Then, 2 ml of 3,5-dinitrosalicylic acid (DNSA) reagent was added, boiled for 10 min, and cooled in water to stabilize the color formation. The absorbance was measured at 540 nm. 1 unit of activity was defined as the amount of enzyme required to produce 1 µmole of reducing sugar per minute [22,23].

Table 1: Location (coordinates) and physical characters of five hot springs of Myagdi district, Nepal

Hot Springs	GPS location		Elevation	pH	Temperature
	Northing	Easting			
Sinkosh	28°34.801'	083°23.517'	1903M	6.6	42.3
Singha	28°22.067'	083°30.149'	898M	6.9	50.2
Ratopani	28°28.702'	083°38.473'	1170M	7.2	57.4
Bhurung	28°29.742'	083°39.242'	1183M	6.7	60.7
Paudwar	28°30.013'	083°39.340'	1235M	6.8	65.3

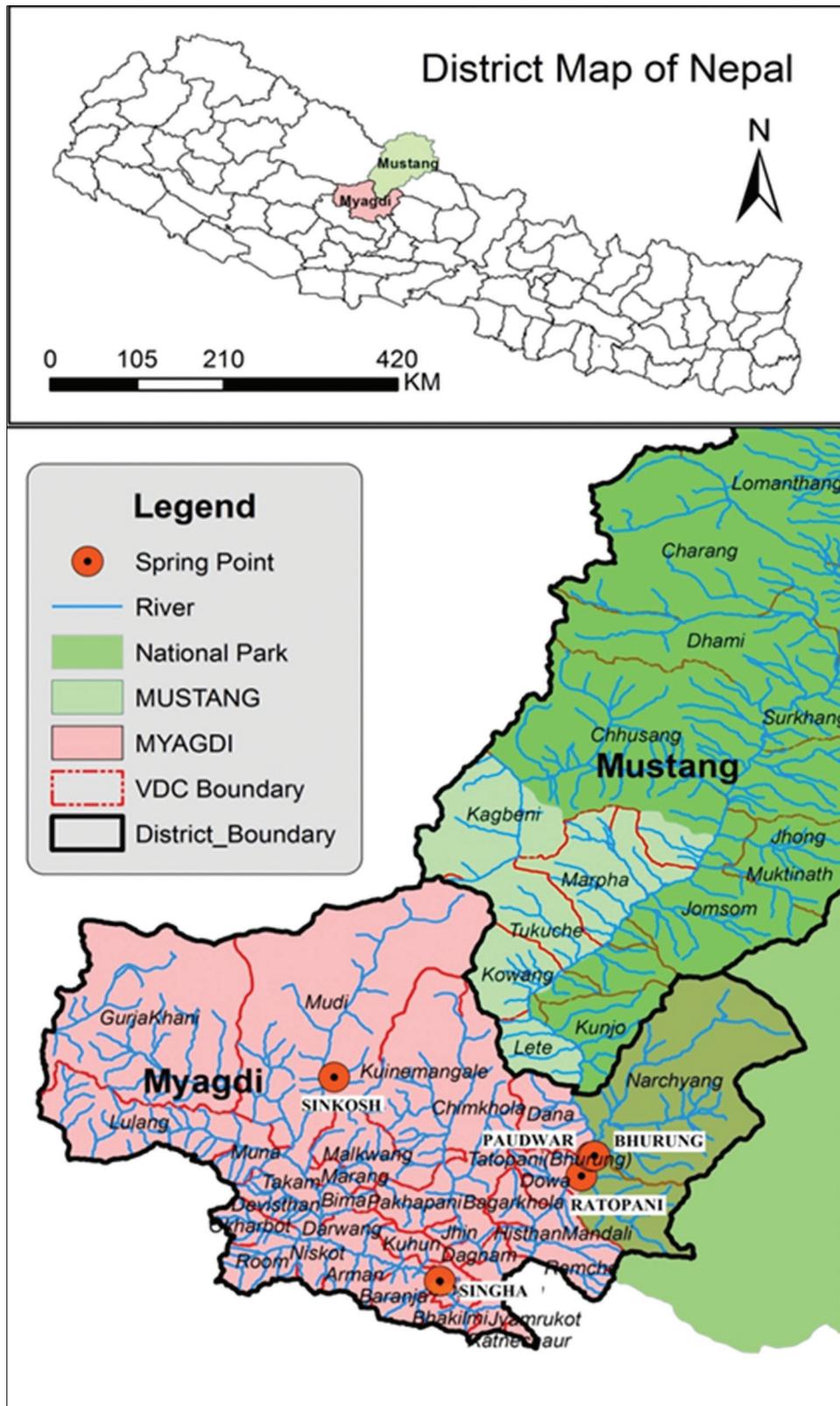


Figure 1: Site of sample collection located in Myagdi district, Nepal (Courtesy: Survey of Nepal).

2.5.2. Xylanase production

Xylanase activity was determined by testing the strains cultured in MSS described above containing 0.5% beechwood xylan at pH 7. Cell-free supernatant was used as xylanase enzyme source

and the xylanase activity was determined by DNSA method described as above. 1 unit of xylanase activity was defined as the amount of enzyme required to produce 1 μmol of xylose per minute [24].

2.5.3. Amylase production

Starch degrading isolates were subcultured in MSS containing 0.5% starch. Cell-free supernatant was estimated for amylase activity by DNSA method as mentioned above. 1 unit of amylase activity was defined as the amount of enzyme that reduces the absorbance of the iodine-starch complex at 620 nm by 1%/min [25].

2.5.4. Extracellular, cell-bound, and intracellular cellulase activity

Isolated microbial strains were grown in MSS medium supplemented with 1% CMC at 60°C. Culture broth was centrifuged at ×12000 g for 20 min, and the supernatant was used as extracellular cellulose enzyme source. The pellets were resuspended in 1 M sodium phosphate buffer at pH 7 and sonicated (Sonics-Vibracell ultrasonic processor, Newton, CT, USA) keeping Sonifier output at 70 amp (20 strokes of the 30 s each with 10 s interval each at 4°C). The cell homogenates were centrifuged at ×12000 g for 20 min and the supernatant was used as the intracellular enzyme. The pellet after centrifugation was suspended in buffer and used for the measurement of cell-bound enzyme activity [26].

2.6. DNA Extraction and 16S rRNA Gene Amplification

Chromosomal DNA was extracted using the DNA Purification Kit (Promega Inc., Madison, WI, USA). 16S rRNA gene was amplified using universal primers 27f (5'-AGAGTTTGTATCCTGGCTCAG-3') and 1492r (5'-GGTTACCTTGTACGACTT-3') [27]. The amplified product was purified, using a QIA quick PCR purification kit (Qiagen Inc., San Diego, CA, USA), and sequenced in an ABI Prism 3700 automatic DNA sequencer by the use of a BigDye Terminator Cycle Sequencing kit (Applied Biosystems, Inc., Palo Alto, CA, USA) [28].

2.7. Phylogenetic Analysis

The sequence of the 16S rRNA gene obtained was used for the initial BLAST search. The BLAST analysis was performed through the EZTaxon server [29]. Sequences closely related were retrieved manually from National Center for Biotechnology Information database (<http://www.ncbi.nlm.nih.gov>), and the sequences were aligned using the ClustalW program, and the results obtained were edited manually. Phylogenetic trees were reconstructed using the neighbor-joining (NJ) and maximum-likelihood (ML) methods available in the MEGA6 software. Evolutionary distances between the NJ and ML trees were calculated using Kimura's two parameters and Tamura-Nei models, respectively [30].

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3. RESULTS

3.1. Phenotypic Characteristics of Selected Strains

A total of 150 thermophilic (growing above 60°C) bacteria were isolated. All isolates were Gram-positive, rod-shaped, and endospore-forming. The strains showed different types of colonies (large, matt, or granular) with variable shapes (circular, regular edges, or jagged) or colonies with wavy contours and creamy consistency. Optimum growth was between 55°C and 65°C, while the growth was drastically reduced at a temperature above 70°C. Only eight isolates tolerate 75°C and none was able to grow at 80°C.

3.2. Isolates Producing Industrially Important Enzymes

All 150 isolates were screened for hydrolase activity. Out of 150 isolates, 135 isolates produced at least one extracellular hydrolytic enzyme, 100 produced amylases, 95 produced cellulase, 85 produced xylanase, and 15 produced lipases. Only one strain produces gelatinase and proteases. Based on morphological, biochemical, and enzymatic screening [Table 2], the isolates were divided into 16 groups.

3.3. 16S rRNA Gene Sequence and Phylogenetic Analysis

One isolate from each group was taken for further molecular characterization from 16S RNA gene sequence. DNA fragments with a size of about 1.5 kbs of 16 isolates were amplified. BLAST analysis of 16S rRNA gene showed high homology with *Bacillus*, *Brevibacillus*, *Geobacillus*, *Aeribacillus*, and *Anoxybacillus* genera. The closest relationship of each sequence was determined by constructing phylogenetic trees.

Table 2: Bacterial isolates grouped based on their temperature tolerance and hydrolase activity

Groups	Isolates	Temperature (°C)	Hydrolytic enzyme activity	Isolates identification*
I.	NAST PD13	30–70	C+; X+; A+; G-; P-; G-; T20-; T40-; T60-; 80-	<i>Anoxybacillus kamchatkensis</i>
II.	NAST PD17	42–75	C+; X+; A+; G-; P-; G-; T20-; T40-; T60-; 80-	<i>Anoxybacillus salavatliensis</i>
III.	NAST RP5	50–75	C-; X-; A(+); G-; P-; G-; T20-; T40-; T60-; 80-	<i>Geobacillus toebii</i>
IV.	NAST SK28	42–75	C-; X-; A-, G-; P-; G-; T20-; T40-; T60-; 80-	<i>Aeribacillus pallidus</i>
V.	NAST PD15	55–75	C(+); X(+); A(+); G+; P-; G-; T20-; T40-; T60-; 80-	<i>Aeribacillus pallidus</i>
VI.	NAST RP11	42–75	C-; X-; A-, G+; P-; G+; T-20-; T40-; T60-; 80-	<i>Aeribacillus pallidus</i>
VII.	NAST PD25	42–75	C+; X+; A(+), G-; P-; T-20-; T40+; T60-; 80-	<i>Geobacillus galactosidasius</i>
VIII.	NAST PD16	42–75	C-; X-; A+; P-; G-; T-20-; T40-; T60-; 80-	<i>Geobacillus toebii</i>
IX.	NAST SG19	30–70	C+; X-; A+; P-, G-, T-20-; T40-; T60-; 80-	<i>Anoxybacillus salavatliensis</i>
X.	NAST SG29	30–75	C+; X(+); A+; P-; G-; T-20-; T40-; T60-; 80-	<i>Anoxybacillus kamchatkensis</i>
XI.	NAST SG22	30–75	C+; X-; A+; P-, G-, T-20-; T40+; T60-; 80-	<i>Anoxybacillus flavithermus</i>
XII.	NAST BR26	42–75	C+; X+; A+; P-; G-; T-20+; T40+; T60+; 80-	<i>Anoxybacillus salavatliensis</i>
XIII.	NAST SK12	42–70	C+; X+; A+; P-; G-; T-20+; T40+; T60-; 80-	<i>Anoxybacillus flavithermus</i>
XIV.	NAST BR8	42–70	C+; X+; A+; P-; G-; T-20-; T40-; T60-; 80-	<i>Brevibacillus thermoruber</i>
XV.	NAST SG1	42–75	C+; X+; A+; P-; G-; T-20-; T40-; T60-; 80-	<i>Anoxybacillus flavithermus</i>
XVI.	NAST BR17	37–75	C+; X+; A+; P+; G-; T-20-; T40-; T60-; 80-	<i>Bacillus thuringiensis</i>

*Identified based on 16S RNA sequence comparison, C: Cellulase, X: Xylanase, A: Amylase, G: Gelatinase, P: Protease, T: Tween, +: Positive, -: Negative, (+): Weakly positive

Phylogenetic tree enables to claim that the isolates belong to five genera [Figure 2], i.e. *Brevibacillus*, *Aeribacillus*, *Geobacillus*, *Bacillus*, and *Anoxybacillus*. Analysis of microbial communities of Myagdi hot springs showed the presence of eight different species of Bacilli: *Anoxybacillus kamchatkensis*, *Anoxybacillus salavatliensis*, *Anoxybacillus flavithermus*, *Aeribacillus pallidus*, *Geobacillus toebii*, *Geobacillus galactosidasius*, *Brevibacillus thermoruber*, and *Bacillus thuringiensis*. *Anoxybacillus* dominated in all five hot springs.

The detailed phylogenetic analysis of the bacterial community is first time reported from the five hot springs located in Myagdi, Nepal. *Bacillus licheniformis*, *Bacillus subtilis*, and *Bacillus pumilus* had been reported earlier from Bhurung hot spring [31], but these bacteria were not found in the present study. *Anoxybacillus* sp., *Brevibacillus* sp., and *Aeribacillus* sp. isolated in this study are reported first time from Nepal.

3.4. Biochemical Characterization

Four isolates such as NAST SG1, NAST PD13, NAST BR26, and NAST SK12 were selected on the basis of their thermostability, and cellulase and xylanase activity for further biochemical analysis. The detail biochemical characters are given in Table 3. Fundamental reasons to choose thermostable enzymes are due to their unique characteristics of long shelf life, increased tolerance to organic solvents, reduced risk of microbial contamination, minimal loss of enzyme activity during processing at the elevated temperatures, and their possible benefit in pre-treatment of biomass [32]. Results show that NAST PD13, NAST BR26, NAST SK12, and NAST SG1 belonging to genus *Anoxybacillus* show variation in their biochemical characteristics such

Table 3: Phenotypic characteristics of the isolates selected by their potential as enzyme producers

Biochemical characters	NAST PD13	NAST BR26	NAST SK12	NAST SG1
Catalase	+	+	+	+
Oxidase	+	+	+	+
Methyl red	+	+	+	+
Voges-Proskauer	-	+	+	+
Indole test	-	-	-	-
Hydrolysis of				
Gelatin	-	-	-	-
Casein	-	-	-	-
Starch	+	+	+	+
Cellulose	+	+	+	+
Xylan	+	+	+	+
Tween 20	-	+	+	-
Tween 40	-	+	+	-
Tween 60	-	+	-	-
Tween 80	-	+	-	-
Esculin	+	-	-	-
Growth at 27°C	-	-	-	-
Growth at 37°C	-	-	-	-
Growth at 42–75°C	+	+	+	+
Optimum temperature	60°C	60°C	60°C	60°C
Optimum pH	7	7	6.5	6.5

as growth temperature, pH, hydrolysis of Tween, and Esculin. Further detail study can reveal some novel characteristics too.

3.5. Production of Enzymes and Assessment of their Activity

Quantitative analysis of extracellular amylase, cellulase, and xylanase production from NAST SG1, NAST PD13, NAST BR26, and NAST SK12 showed higher extracellular xylanase and amylase production compared to cellulase [Figure 3]. Among four isolates, NAST PD13 showed highest cellulase (0.31 IU/ml), xylanase (3.02 IU/ml), and amylase (5.15 IU/ml) activity. Among *Anoxybacillus* sp., only *A. flavithermus*, and *Anoxybacillus* sp., 527 are reported as cellulose degrader [33]. However, in the present result, *A. salavatliensis* (NAST BR26) and *A. kamchatkensis* (NAST PD13) also degraded cellulose. Multiple enzyme systems aid in creating an efficient degradation of the lignocellulosic materials composed of cellulose, hemicellulose, pectin, and lignin.

3.6. Extracellular, Cell-bound, and Intracellular Cellulase Activity

For microorganisms to hydrolyze and metabolize insoluble cellulose, extracellular cellulase must be produced that are either free or cell associated [34,35]. It was noticed that all four strains, namely, NAST-SG1, NAST-SK12, NAST-PD13, and NAST-BR26, produce all three types of cellulase (exoglucanases), i.e., they are present in extracellular, intracellular as well as cell-bound fractions. Result shows

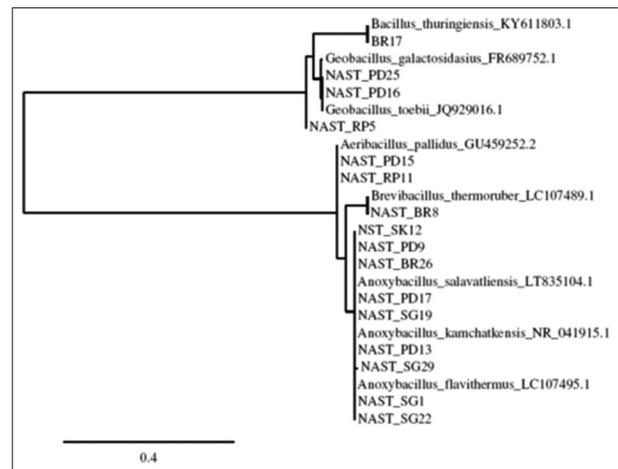


Figure 2: Phylogenetic tree based on 16S DNA sequences of thermophilic bacteria isolated from five hot springs of Myagdi, Nepal.

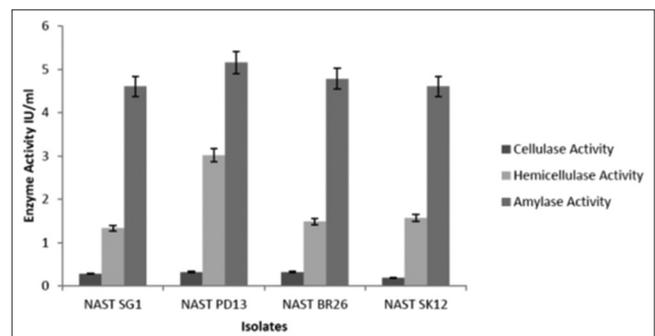


Figure 3: Cellulase, xylanase, and amylase activity of the four bacterial isolates such as NAST SG1, NAST PD13, NAST BR26, and NAST SK12.

endoglucanase concentrations are highest in cell-bound fractions [Figure 4] of NAST-SG1 (1.42 IU/mL), NAST-SK12 (0.91 IU/mL), NAST-PD13 (0.84 IU/mL), and NAST-BR26 (0.86 IU/mL).

4. DISCUSSION

Samples collected from five different hot springs were analyzed in NB medium as NA medium. NA is suitable for growing the Bacilli groups and the *Bacillus* species are of increasing importance in industry and medicine and that diagnosis to the species level is highly desirable in many instances [36]. The use of other enrichment media may likely to recover different and some novel microbes [37].

All the isolated strains were able to grow at 30–70°C, with optimal growth temperature at 60°C. Thus, all strains could be classified as thermophiles [38,39]. The temperature of hot springs was below 60°C except at Bhurung and Paudwar hot springs which show that any microbes do not necessarily have the same optimum temperature as their natural habitats [40].

Cultivated microbial community within the five hot springs of Myagdi belongs to genus *Brevibacillus*, *Aeribacillus*, *Geobacillus*, *Bacillus*, and *Anoxybacillus* and had a close relationship to *A. kamchatkensis*, *A. salvatiensis*, *A. flavithermus*, *A. pallidus*, *G. toebii*, *G. galactosidarius*, *B. thermoruber*, and *B. thuringiensis* predominated by *Anoxybacillus* in all five hot springs. All the isolated microbes belong to the domain *Bacteria*, phylum *Firmicutes*, class *Bacilli*, and order *Bacillales* (<http://rdp.cme.msu.edu/set> match). As NA medium is suitable for growing the bacilli group [33], therefore, there was great probability of isolating mostly thermophilic bacilli from the hot springs. The use of other enrichment media may likely to recover different and some novel microbes [37].

Genera, *Geobacillus*, *Anoxybacillus*, *Brevibacillus*, and *Bacillus*, are commonly reported in thermal environments including hot springs, oil reservoirs, mines, and geothermal aquifers. The genera *Bacillus* and *Brevibacillus* have also been isolated from mesobiotic environments.

In the context of Nepal, no such detailed phylogenetic analysis of the bacterial community is yet reported from the hot spring ecosystem. Till date, *B. licheniformis*, *B. subtilis*, and *B. pumilus* have been reported from Bhurung hot spring of Myagdi district, Nepal [31]. This is the first study on the microbial diversity of thermophiles in five different hot springs of Myagdi district. This study reports first time *Anoxybacillus* sp., *Brevibacillus* sp., and *Aeribacillus* sp. from the hot springs of Nepal.

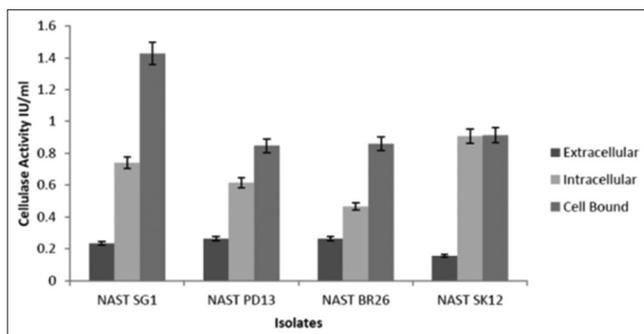


Figure 4: Extracellular, intracellular, and cell-bound endoglucanase activity of four bacterial isolates such as NAST SG1, NAST PD13, NAST BR26, and NAST SK12 grown in carboxymethyl cellulose medium.

Here, we report the microbial community analysis of Myagdi hot spring, and the analysis was based on culture-dependent strategies to get a first insight into the microbial communities in this ecosystem which may be the limitation of this study. Consequently, it has been reported that the microbial diversities analyzed by culture-independent methods show higher diversity than culture-dependent method [38]. Therefore, culture-independent methods can give more detail on the diversity of the thermophilic community of Myagdi hot springs.

Screening of thermostable enzymes cellulase, hemicellulase, lipase, amylase, gelatinase, and protease from the isolated strain showed at least one hydrolase activity. Fundamental reasons to choose thermostable enzymes is due to their certain properties such as intrinsic thermostability, which implies possibilities of long shelf life, increased tolerance to organic solvents, reduced risk of microbial contamination, as well as low activity losses during processing even at the elevated temperatures and their possible benefit in pre-treatments [32]. Such enzymes will have better commercial value. In addition, several microorganisms produce multiple individual enzymes that can act synergistically and hydrolyze the polymeric lignocellulose into shorter metabolizable intermediates. Therefore a cost-efficient process of the renewable resource (agricultural and forest biomass) available in large enough quantities can be considered for large-scale production of alcohol-based fuels [41]. The lignocellulosic materials are often subjected to thermal treatments to facilitate its degradation; accordingly, the thermostable strains producing thermostable enzymes will be of great advantage to it [42]. NAST SG1, NAST PD13, NAST BR26, and NAST SK12 apparently produce thermally stable cellulase, hemicellulase, and amylase enzymes. We believe these strains can be used to improve utilization of the lignocellulosic carbohydrate biomass to produce alcohol-based biofuels.

Enzyme production experiments in previous studies show that bacterium has both extracellular and cell-bound endoglucanase activities, of which up to 80% of all cellulose enzyme fraction is found in the extracellular fluid and it is consistent with other stationary-phase cellulose-grown cultures [35,43]. The location of cellulase has not been identified in *Anoxybacillus* sp. but extracellular endoglucanase has been reported in prolonged incubation. *Amylopullulanase* of the thermophilic *Anoxybacillus* sp. SK3-4 (ApuASK) was detected in cell-bound fraction [44]. The emerging picture is that association of fibrolytic enzymes within cell differs from one enzyme to another and with the age of the cell. From studies of cell-associated cellulase in *Ruminococcus albus* and *R. flavefaciens*, cellulase appears to vary from partially to complete cell associated depending on the strain and growth condition. *R. flavefaciens* was largely cell associated during exponential growth and was found in the extracellular fluid when cell aged [45]. Use of cell-free supernatants in the early stage for enzyme assay in this study, therefore, may have underrepresented cellulase activity. In thermophiles, the problem associated with enzyme production is also due to low cell yield associated with their growth. Special equipment, process configuration, medium compositions, and culture optimizations can increase cell yield in thermophilic bacteria and may also increase cellulase production [30,46].

Nepal has many geothermal areas with different physical and chemical properties. Detail study on microbial diversity will provide the opportunity to explore novel thermophilic microbes. Cultivation method is the limitation of this study because the distinctions are not based mainly on DNA relatedness studies, molecular probing, and chemotaxonomic analyses but also characteristic of phenotypic profiles and biochemical reactivity of the isolates [47].

Although this is not the first report of thermophilic *Bacillus* from hot spring of Myagdi, Nepal and could be a potential source of a thermostable enzyme of industrially important hydrolase that can have many industrial applications. Efforts are made to optimize lignocellulose-degrading enzymes production in different temperature and pH and to purify the enzyme.

5. ACKNOWLEDGMENT

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