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Exploration of thermophilic bacteria from hot springs of Garhwal Himalayas and their screening for industrially important enzyme production

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Abstract

The present study delineates thermophilic bacterial diversity of two hot springs, Soldhar and Suryakund of Uttarakhand Himalaya region and their exploration for production of industrially important enzymes. The predominant isolates belonged to Genus *Bacillus*. *Paenibacillus* spp. were common to both sites. *Pseudomonas* spp. from Soldhar, *Geobacillus* spp. and *Brevibacillus* spp. were recovered from Suryakund. The isolates when qualitatively checked for amylase and cellulase production exhibited maximum index for amylase activity of 1.51 from Soldhar and 1.28 from Suryakund while for cellulase maximum index was 0.87 from Soldahr and 3.00 from Suryakund. Bacterial population recovered from Suryakund comprised of more active amylase (55%) and cellulase (50%) producers as compared to Soldhar which has 34% amylolytic and 28% cellulolytic population. These isolates can be further exploited for various biotechnological applications which necessitate the conservation of these microbial resources as these hot springs are subjected to various anthropogenic disturbances.

Key Words: Amylase, Bacillus, Cellulase, Hot springs, Thermophiles

Introduction

The microbial world is the largest unexplored reservoir of the biodiversity on the earth. It is an important frontier in biology under intensive investigation. (Verma *et al.,* 2014). Microorganisms that thrive at extreme environment are referred as extremophiles (Macelroy, 1974). The Indian Himalayan Region (IHR) with inherent variations, particularly with respect of topographic, as well as geographic and climatic conditions, supports a wide range of extreme habitat like hot springs, glaciers etc (Pandey *et al.,* 2014a). Hot springs are characterized by moderate to high temperature environment, formed as a result of geothermal activity of earth and a habitat for thermophiles. Thermophiles can survive at 45^0C- 80^0C and an area of interest as when temperature approach towards boiling point of water only thermophiles can thrive.The cellular components of thermophiles are extremely thermostable and these together with their unique metabolic capabilities, offer considerable promise for biotechnological applications. So, high temperature environments are thus of great interest to microbiologists and biotechnologists, as the organisms isolated from these environments are a good source of thermostable enzymes (Sharma *et. al.,* 2013). North India which is also termed as a home of hot springs provides an oppurtunity to study their microbial ecology (Verma *et al.,* 2014; Kumar *et al.,* 2014). Manikaran and Soldhar are amongst the microbiologically explored hot springs from North India (Kumar *et al.,* 2004; Sharma *et al.,* 2008; Bhardwaj and Tiwari., 2010; Bhardwaj *et al.,* 2011; Pandey *et al.* 2014a; Pandey *et al.,* 2014b; Verma *et al.,* 2014; Kumar *et al.,* 2014; Sharma *et al.,* 2015). The other hot springs that are also well studied include: Taptapani hot spring Odhisha (Panda *et al.,* 2013b); TulasiShyam hot spring, Gujarat (Gehlani *et al.,* 2015), Unkeshwar hot spring, Maharashtra (Mehtre *et al.,* 2015), Deulajhari hot spring, Odhisha (Singh and Subudhi, 2016). The culturable bacterial diversity reported from various hot springs include *Acineto bacter spp., Brevi bacillus spp., Bacillus amylolique faciens*, *Bacillus, Paeni bacillus spp., Bacillus cereus, Geobacillus stearothermophilus, Bacillus megaterium, Bacillus subtilis,* (Bisht *et al.,* 2011; Ghati *et al.,* 2013, Panda *et al.,* 2013a; Sharma *et al.,* 2013; Verma *et al.,* 2014). Phyla Firmicutes, Aquificae,

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Deinococcus-Thermus, Hydrogenobacter, Actono bacteria, Verruco microbia, Bacteroides, Cloroflexi, Proteobacteria, Chlorobi, Acidobacteria, Spirochaetes are residents of hot springs reported by metagenomic (unculturable) technique (Bhatia *et al.,* 2015; Mehtre *et al.,* 2015; Singh and Subudhi*et al.,* 2016) Thermophiles are a potent source of thermozymes, which show utmost stability under conditions of high temperature. Thus, biocatalysis using thermophiles as well as thermozymes is rapidly being transformed from an academic science to an industrially viable technology. Each group of the thermophiles has unique features, which can be harnessed to provide enzymes with a wide range of application possibilities (Sellek and Chaudhari, 1999; Fujiwara, 2002; Haki and Rakshit., 2003).Among industrially important enzymes amylases and cellulases are getting more importance because these enzymes have wide applications in many fields. Amylases are used in food, detergent, paper, textile industries. Fungal and bacterial amylases could be potentially useful in the pharmaceutical and fine-chemical industries (Ghorai*et al.,* 2009). The application of enzyme increases more with the discovery of thermostable enzymes because thermophilic process is more stable, faster, needs lower costs (Rasooli*et al.,* 2008). Cellulases are industrially important enzymes that are sold in large volumes for use in different industrial applications *viz.* in starch processing, animal feed production, grain alcohol fermentation, malting and brewing, extraction of fruit and vegetable juices, pulp and paper industry and textile industry (Ogel*et al.,* 2001). There is a growing merit for cellulase in the field of detergents and saccharification of agriculture waste for bio ethanol technology.

In the Uttarakhand region, more than 50 individual occurrences of moderate to high temperature springs have been reported along the banks of the major rivers - Tons, Yamuna, Bhagirathi, Mandakini, Alaknanda, Dhauli Ganga, and Kali (GSI, 1991). Some of the well known hot springs are located in Soldhar, Suryakund (Yamnotri), Gangnani, Badrinath, GaramPani near Nainital. Several hot springs occur between Pala and Gangnani, north of Uttarkashi (Dimri, 2013). Most of the hot springs of Uttrakhand is associated with pious, spiritual values. It was almost a decade earlier Kumar *et al.* (2005) explored Soldhar and Ringigad for bacterial diversity. Major *Bacilli spp.* and *Geobacillus spp.* are reported from Soldhar and Ringigad (Kumar *et al.,* 2004, 2005; Trivedi*et al.,* 2006; Sharma *et al.,* 2008; Pandey *et al.,*2014a, Pandey *et al.,* 2014b). Thermophilic cyanobacteria are also reported from two hyperthermal springs (Soldhar and Ringigad). *Spirulina meghiniana* and *Chlorogloeopsis spp.* are the new record for thermal springs of Uttrakahand. *Lyngbya hieronyamusi, Pseudanabaenaglaeata* and *Chlorogloeopsis* spp. a new record for Indian thermal spring has been reported (Bhardwaj *et al.,* 2010, 2011). Besides, documentation of bacterial or cyanobacterial diversity, functionally active diversity from hot springs of Uttarakhand is still yet to be explored. Moreover, the hot springs are either facing natural disturbances like landslides or encountered by anthropogenic interventions so it is also important to preserve gene pool of native thermophilic population of these sites.

Keeping the above facts in mind there is a need to explore hot springs of Uttrakhand from biotechnological point of view. Present study was therefore undertaken to document thermophilic bacterial diversity from two high temperature hot springs located in different districts of Uttrakhand and to explore recovered isolates for production of industrially important enzymes amylase and cellulase. As both sites are anthropogenically disturbed this is an effort to preserve gene pool of functionally important thermophiles.

Material and Methods

Sampling

The samples were collected from Soldhar (Disrtict Chamoli) and Suryakund (Yamunotri, District Uttarkashi) situated at Garhwal Himalaya region. Water samples were collected in autoclaved bottles while for collection of soil sample autoclaved plastic bags were used. pH and temperature of water samples were recorded at sampling site.

Recovery of bacterial isolates

Purification, maintenance and preservation of cultures

Water samples were serially diluted and plated on nutrient agar medium(NAM) and dextrose tryptone (DT) medium. Plates were incubated for 36-48 hours at 55^0C. Soil sample was dried, serially diluted and plated on NAM and DT medium and

plates were incubated at 55^{0}C for 36-48 hours. The maintainance of cultures was done in glycerol stocks according to Aneja (2003). Glycerol stocks were maintained in cryovials and preserved at -20 °C.

Morphological characterization

Colony morphology (shape, size, form, elevation and margin) and cell morphology (Gram's reaction, cell shape and arrangement) of isolates were studied according to Cappucino and Sherman (2007).

Biochemical characterization

The various biochemical tests viz., Oxidase test, Indole-Methyl Red -Voges-Proskauer-Citrate Utilization test (IMViC), Triple Sugar Iron (TSI) test, Urease and Nitrate reduction tests were carried out according to Cappucino and Sherman (2007).

Functional characterization

The functional characteristics of recovered isolates viz., amylase (Cappucino and Sherman, 2007) and cellulase (Teather and Wood, 1982) were studied. The qualitative index of enzyme activity for amylase and cellulase was calculated according to Mangunwardoyo *et al.* (2011) using the following formulae:

Index of relative enzyme activity =

$$\frac{\text{Diameter of Clear Zone - Diameter of Bacterial Colony}}{\text{Diameter of Bacterial Colony}}$$

Garhwal region is a hillock area of Uttarakhand. For the present study, soil and water samples from Soldhar (Distt. Chamoli) and Suryakund (Yamunotri, Distt.Uttarkashi) were collected. Soldhar is an open hot spring mound situated at roadside near Tapovan at Joshimath- Malari road. It is frequently visited by tourists and pilgrims on the way to Badrinath, Auli and Malari. It has been earlier studied for bacterial and cyanobacterial diversity (Kumar *et al.,*2004; Kumar *et al.,* 2005; Trivedi *et.al.,* 2006; Sharma *et al.,* 2008; Bhardwaj *et al.,*2010, 2011; Pandey *et al.,* 2014a, Pandey *et al.,* 2014b). In Soldhar, hot water from source (origin) was collected. Hot water falls from mound on roadside, so sample of water from exit where it leaves the mound was also collected.Suryakund is located at Yamunotri, one of the famous *char dham* shrines of Uttrakhand. It has high spiritual value and visited by number of pilgrims during *yatra* season. Devotees cook rice in Suryakund and take it home as *prasad.* Suryakund scarcely been explored for microbial diversity earlier. Water samples were collected from source (origin) of the hot spring. Hot water form run off channel & falls in the pool, before entering to the pool it is mixed with normal water and used by pilgrims for spiritual bathing so water sample was also collected from exit(before entering to pool).

Physical characterization

Both springs have temperature near to 90^{0}C and pH lies between near neutral to alkaline (Table 1).

Results and Discussion

Table 1: Temperature and pH of sampling sites

Sample	Site	Temperature (in ^{0}C)		pH	
		Source	Exit	Source	Exit
Water	Soldhar	90	71	8.2	8.6
Soil	Soldhar	90	-	8.4	-
Water	Suryakund	86.5	57.4	7.4	7.9
Soil	Suryakund	86.5	-	7.4	-

Bacterial population profile

The population profile of both springs was not significantly variable (Table 2). In Soldhar, the population count ($\log_{10}$ cfu) was found to vary from 3.07 (source) to 3.00 (exit) and 3.12 for soil smaple on NAM while on DT the population count ($\log_{10}$ cfu) was 3.19 (Source), 3.93 (exit) and 4.04 (soil). In Suryakund, the population count ($\log_{10}$ cfu) on NAM was 4.34 (source), 4.44 (exit) and 4.34 (soil). On DT, the population count ($\log_{10}$ cfu) was 4.25 (source), 4.17 (exit) and 4.24 (soil) (table 2).So bacetrial count on DT was comparatively higher than NAM in case of Soldhar while in case of

Suryakund NAM has high bacterial count as compared to DT. But overall, there are minor differences in population count on NAM and DT so both media are equally capable to support growth of thermophilic bacteria.

Table 2: Microbial population count (Log_{10}CFU/gm or Log_{10}CFU/ml) in soil and water samples on nutrient agar medium (NAM) and dextrose tryptone medium (DT) at 55^0C

Sample	Soldhar		Suryakund	
	NAM	DT	NAM	DT
Water (source)	3.07	3.19	4.34	4.25
Water (exit)	3.00	3.93	4.44	4.17
Soil	3.12	4.04	4.35	4.24

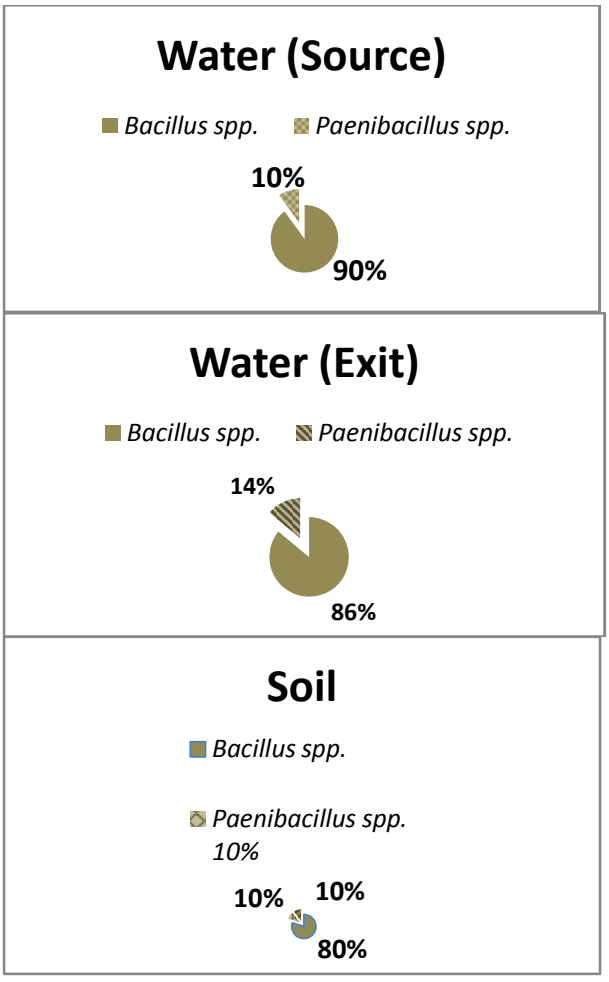

Fig 1: Percentage distribution of recovered isolates from Soldhar

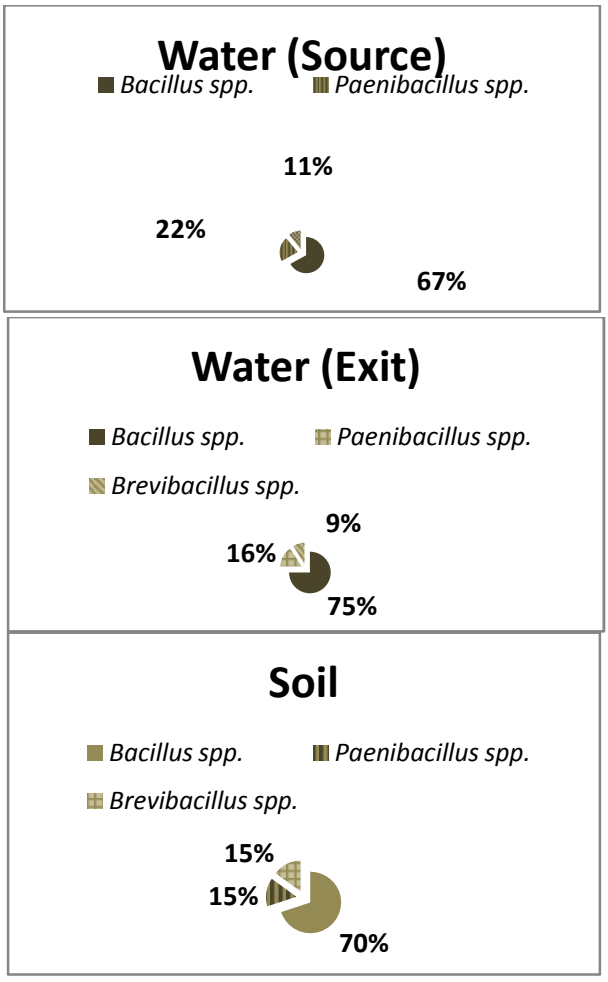

Fig 2: Percentage distribution of recovered isolates from Suryakund

A total of 35 isolates were recovered from Soldhar while a total of 34 isolates were recovered from Suryakund. These isolates were characterized morphologically as well as biochemically. Most of the isolates belonged to genus *Bacillus* from both the sites. *Paenibacillus* was also observed in both study sites. *Geobacillus* and *Brevibaillus* were observed in Suryakund while from Soldhar *Pseudomonas* was recovered (Figure 1; Figure 2)

Functional characterizaton of recovered bacterial isolates

The recovered isolates were also explored for their potential to produce amylase and cellulase enzyme. Qualitative enzyme activity was reported as the index of relative enzyme activity was calculated as follows-

Index of relative enzyme activity =

$$\frac{\text{Diameter of Clear Zone - Diameter of Bacterial Colony}}{\text{Diameter of Bacterial Colony}}$$

From Soldhar, out of 35 recovered isolates 12 were amylolytic and the highest index for amylase activity was found to be 1.51. As compared to amylase, celluase producing isolates were few and only 10 were found cellulolytic with highest index of 0.87 (Table 3).

Table 3: Index of relative enzymatic activity (Soldhar)

Name of Isolate	Amylase			Cellulase		
	Diameter of colony (mm)	Diameter of clear zone (mm)	Index	Diameter of colony (mm)	Diameter of clear zone (mm)	Index
SU1	-	-	-	-	-	-
SU2	24.00	31.00	0.29	53.00	65.00	0.22
SU3	-	-	-	-	-	-
SU4	-	-	-	-	-	-
SU5	21.00	29.00	0.38	13.33	24.00	0.80
SU6	-	-	-	-	-	-
SU8	20.66	29.00	0.40	11.33	15.66	0.38
SU9	-	-	-	-	-	-
SU10	-	-	-	-	-	-
SU11	-	-	-	-	-	-
SD1	-	-	-	-	-	-
SD2	-	-	-	-	-	-
SD3	18.33	20.00	0.09	-	-	-
SD5	-	-	-	-	-	-
SD6	-	-	-	-	-	-
SD7	-	-	-	-	-	-
SD8	-	-	-	-	-	-
SD10	22.66	25.00	0.10	-	-	-
SD11	-	-	-	-	-	-
SD12	-	-	-	13.33	25.00	0.87
SD13	10.00	20.33	1.03	-	-	-
SD14	-	-	-	-	-	-
SD15	-	-	-	-	-	-
SD18	-	-	-	-	-	-
SD19	20.66	27.00	0.30	26.66	41.00	0.53
SS6	9.33	16.66	0.78	-	-	-
SS7	9.66	24.33	1.51	-	-	-
SS8	24.00	31.00	0.29	56.33	64.00	0.13
SS9	18.00	21.00	0.27	18.00	21.00	0.27
SS10	-	-	-	18.00	25.30	0.45
SS11	-	-	-	43.33	50.66	0.16
SS13	-	-	-	-	-	-
SS14	-	-	-	26.00	33.00	0.26
SS19	9.33	16.66	0.78	-	-	-
SS23	-	-	-	-	-	-

- ***Indicates no enzymatic activity***

From Suryakund, maximum isolates were amylolytic as 19 showed hydolysis of starch on starch agar medium and highest index was 1.28. 17 isolates were cellulolytic and highest index for cellulase activity was calculated as 3.00 (Table 4)

Table 4: Index of relative enzymatic activity (Suryakund)

Name of Isolate	Amylase			Cellulase		
	Diameter of colony (mm)	Diameter of clear zone (mm)	Index	Diameter of colony (mm)	Diameter of clear zone (mm)	Index
YS1	16.00	21.00	0.31	19.00	29.00	0.52
YS2	37.00	40.00	0.89	8.60	20.00	1.32
YS3	57.00	70.00	0.23	7.60	25.00	2.28
YS4	8.30	19.00	1.28	-	-	-
YS5	15.00	21.00	0.40	8.60	19.00	1.20
YS6	16.00	21.00	0.31	56.0	18.00	2.21
YS7	8.30	17.00	1.04	-	-	-
YS8	-	-	-	-	-	-
YS9	21.00	32.00	0.52	76.00	18.00	1.36
YE1	29.00	35.00	0.18	5.00	7.00	0.04
YE2	-	-	-	-	-	-
YE3	-	-	-	-	-	-
YE4	-	-	-	7.00	9.00	0.02
YE5	25.00	29.00	0.15	10.00	15.00	0.51
YE6	-	-	-	18.00	28.00	0.54
YE7	-	-	-	-	-	-
YE8	-	-	-	-	-	-
YE9	-	-	-	-	-	-
YE10	34.00	38.00	0.13	-	-	-
YE11	-	-	-	-	-	-
YE12	-	-	-	-	-	-
Y1	-	-	-	-	-	-
Y2	7.00	9.00	0.02	-	-	-
Y3	3.00	6.00	0.10	8.60	24.00	2.00
Y4	7.00	9.00	0.02	-	-	-
Y5	-	-	-	-	-	-
Y6	-	-	-	-	-	-
Y7	-	-	-	-	-	-
Y8	5.00	7.00	0.04	52.00	61.00	0.17
Y9	54.00	62.00	0.15	48.00	51.00	0.06
Y10	7.00	9.00	0.02	11.00	30.00	1.61
Y11	-	-	-	6.00	24.00	3.00
Y12	7.00	9.00	0.02	35.00	50.00	0.42
Y13	5.00	7.00	0.04	61.00	78.00	0.27

From Soldhar 34% isolates were found to be amylolytic and 28% isolates were cellulolytic. While from Suryakund, 55% recovered isolates had amylolytic potential and 50% isolates were

cellulolytic (Fig 3). Few isolates from both hot springs produced amylase as well as cellulase. 17% isolates from Soldhar were recorded to produce amylase-cellulase while from Suryakund 41% isolates were amylase-cellulase producers (Fig 4).

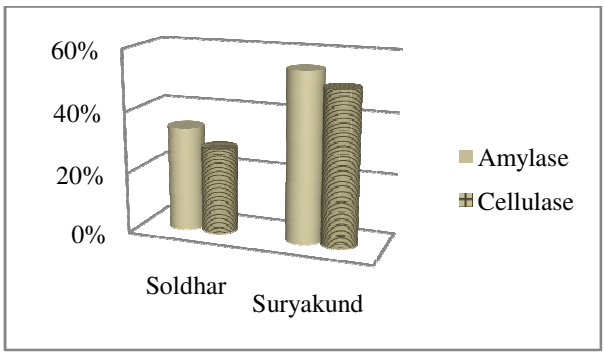

Fig 3: A comparative percentage distribution of enzymatic potential of recovered isolates from Soldhar and Suryakund.

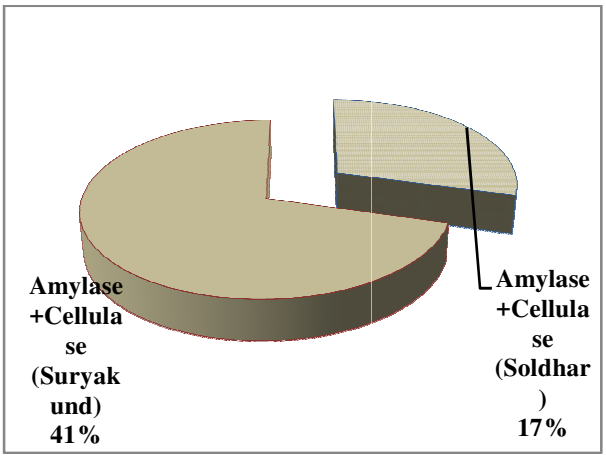

Fig 4: A percentage distribution of amylase+cellulase producing isolates from Soldhar and Suryakund

Microbial diversity under extreme conditions of temperature of Indian Himalayan region has acquired attention of researchers (Chaurasia *et al.*, 2005; Pandey and Palni., 2007; Sharma *et al.*, 2008; Sharma *et al.*, 2015). Thermophiles are an important tool for research as they have value in both basic and applied biology. In the present study an attempt was made to explore bacterial diversity and their enzymatic potential from two high temperatures hot springs of Garhwal Himalayas. From both the study area maximum recovered isolates belonged to Bacillus spp. Species of Bacillus, as in the present case, are found to be most competent colonizers of high temperature environment as compared to other species like Geobacillus or cyanobacteria that survive at comparatively low temperature and form a minor community at high temperature niche (Sharma et al., 2015). To the best of our knowledge there are no earlier studies on bacterial diversity and functional potential of bacteria from Suryakund. While Soldhar, is designated as 34 hotspots of biodiversity due to its physicochemical properties (Sharma *et al.*, 2015) and been extensively studied for microbial diversity. Previous studies on culturable diversity of Soldhar documented presence of Bacillus, Geobacillus and cyanobacteria (Kumar *et al.*, 2004; Sharma *et al.*, 2008; Bhardwaj *et al.*, 2010; Bhardwaj *et al.*, 2011; Pandey *et al.* (2014b). Recently, Sharma *et al.* (2015) has published 16S rRNA study of microbial diversity of Soldhar. According to this recent study, Proteobacteria is the most predominant phylum with one OTU related to Paenibacillus spp. has been reported. Genus level distribution of 16S rRNA bacterial clones also documented presence of Pseudomonas spp. Hence, the present study of Soldhar is in good agreement with earlier and recent studies. Several species of Geobacillus have been isolated from this spring during previous studies (Sharma *et al.*, 2008; Pandey *et al.*, 2014a). However, no sequences related to this genus neither has been reported by Sharma *et al.* (2015) nor we are reporting this genus from Soldhar in culturable diversity. It is thus possible that this group represents a minor community in the hot spring and that its dominance in culture-based studies may be attributable to some bias in the growth conditions (Sharma *et al.*, 2015). The picture of culturable diversity is very important in microbial ecology studies because cultivable population suggest the active fraction of bacterial communities (Ellis *et al.*, 2003; Frette *et al.*, 2004). There were earlier studies on culturable and unculturable diversity of Soldhar hot spring and conservation of its microflora was also recommended by Trivedi *et al.* (2006) while Suryakund (Yamunotri) is one of the important pilgrimage destinations and bacterial diversity of this hot spring is still not explored. So, this paper provides a picture of culturable bacterial diversity of important hot springs of Uttrakhand. These are biological assets of Garhwal region and possess functionally important microbial diversity hence

gene pool of microorganisms as well as these sites is needed to be conserved and protected. The use of enzymes, obtained from microorganisms as biotransformation catalysts for the formation of various products is well established and well documented (Eichler, 2001; Irwin and Baird, 2004). As industrial process conditions are harsh, there are demands for biocatalysts that can withstand the process conditions. The majority of the enzymes used originate from mesophilic organisms and, despite their many advantages; the application of these enzymes is restricted due to their limited stability at the extremes of temperature, pH and ionic strength. On the other hand, thermophiles are a potent source of thermozymes, which show utmost stability under conditions of high temperature. Thus, biocatalysis using thermophiles as well as thermozymes is rapidly being transformed from an academic science to an industrially viable technology. Each group of the thermophiles has unique features, which can be harnessed to provide enzymes with a wide range of application possibilities (Sellek and Chaudhuri, 1999; Fujiwara 2002; Haki and Rakshit, 2003). So, in present study recovered isolates were screened for two industrial important enzymes viz. amylase and cellulase. This helps us to first understand the functional role played by bacterial isolates in their niche and secondly to obtain best enzyme producing isolates. The preliminary screening showed promising isolates with relative index of amylase activity 1.51 and for cellulase it is 3.00.

The result is encouraging as bacteria with useful enzyme production potential are found in the hot springs of Garhwal region. The present study is a preliminary screening report of bacterial diversity and their enzyme producing potential. This study revealed a taxonomic diversity among the Bacilli. Other studies on Indian hot springs also revealed presence of different Bacillus spp. (Bisht *et al.*, 2011; Ghati *et al.*,2013, Panda *et al.*,2013; Sharma *et al.*, 2013; Verma *et al.*, 2014). It is known that Bacillus spp. has greater biotechnological potential compared to other group of bacteria (Oguntoyinbo, 2007; Bal *et al.*, 2009). The isolation of microbes from extreme environment would also provide ample scope to assess their biotechnological potential. So, attempt should be made for proper evaluation and exploration of these microbes for the biotechnological applications.

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