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Functional characterization of thermophilic bacteria from hot springs of Garhwal Himalayas

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Abstract

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 50^0C- 80^0C and an area of interest as when temperature approach towards boiling point of water only thermophiles can thrive. In the present study, bacterial diversity of one high temperature (Soldhar) and other moderate temperature (Tapovan kund) hot spring from Chamoli district, Uttarakhand was investigated as these sites are under anthropogenic interventions which therefore necessitate the conservation of gene pool of these microbial resources. The isolates were further explored for their functional potentiality to understand the role played by them in their niches. Based on culture dependent analysis, *Bacillus* was the predominant genus in both hot springs. Other dominant genus was *Paenibacillus* and *Pseudomonas.* The isolates recovered from Tapovan kund comprised of more active amylase (69%), lipase (65%) and protease (46%) producing bacterial population while Soldhar harbors 45% proteolytic, 42% lipolytic and 34% amylolytic population. These isolates can be further exploited for various biotechnological applications.

Keywords: Hot springs | Thermophilic bacteria | Soldhar | *Bacillus* | *Paenibacillus* | *Pseudomonas*

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Introduction

Extreme environments harbor microorganisms that represent the oldest inhabitants on earth, and whose high adaptability has continued to challenge the understanding of biochemistry, biology and evolution (Tekere *et al.,* 2015). Microorganisms that thrive at extreme environment are referred as extremophiles (Macelroy, 1974). The geothermal springs or hot springs represent one of the extreme environments. The geothermal springs are

substantially higher in temperature than the air temperature of the surrounding region. They are everywhere: different countries and areas, even some on the seafloor (Verma *et al.*, 2014). The most recent use of geothermal waters is for their biological wealth, among which are the thermophilic microorganisms. Thermophiles are a group of extremophilic organisms that live in hot environments and are a valuable genetic resource (Tekere *et al.*, 2015). It has been speculated that the thermophiles were among the first living organisms on this planet, developing and evolving during the primordial birthing days of the earth when surface temperatures were quite hot and thus, have been called as "Universal ancestor'' (Doolittle, 1999). Thermophiles are found in various region of the earth such as hot spring like those in Yellowstone national park and deep sea hydrothermal vents. Thermophiles are an important area of research because they are potent source of thermozymes, which show utmost stability under conditions of high temperature. The enzymes need to fulfill numerous requirements such as activity and stability, substrate specificity and enantioselectivity due to this the thermostable enzymes are often preferred for the desired biotechnological applications (Khiyami *et al.*, 2012). In recent years thermophilic proteases, lipases and polymer degrading enzymes such as cellulases, gelatinases and amylases have found their way into various industrial applications. The use of higher temperature in industrial processes reduces the risk of microbial contamination caused by mesophiles and simultaneously thermozymes are much more useful in the processing of lower viscosity fluids, as at higher temperature viscosity is usually reduced, that lowers shear consequently, the costs of pumping, filtration, and centrifugation (Panda *et al.*, 2013).

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, Garam Pani 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.* (2004) 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.*, 2009; 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 hieronyamusii* , *Pseudanabaena glaeata* 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.

The present study was undertaken to document structural and functional diversity of thermophilic bacterial from one high temperature hot spring (Soldhar) and other moderate temperature hot spring (Tapovan kund) .

Materials and Methods

Sampling site

The samples were collected from Soldhar (Disrtict Chamoli) and Suryakund (Yamunotri, District Uttarkashi) situated at Garhwal Himalaya region.

Collection of samples

Water samples were collected in autoclaved bottles while for collection of soil sample autoclaved plastic bags were used. The samples were immediately transported to laboratory for further processing.

Physical characterization

pH and temperature of water samples were recorded at sampling site.

Recovery of bacterial isolates

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 55^0C for 36-48 hours.

Purification, maintenance and preservation of cultures

Isolates were purified by streaking on nutrient agar and pure cultures were maintained. Glycerol stocks were prepared by adding 1.0 ml of autoclaved glycerol to1.0 ml of overnight grown culture in Nutrient Broth. 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.

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 (1992).

Functional characterization

The functional characteristics of recovered isolates viz., amylase (Chadha *et al.*, 1997), lipase (Cappucino and Sherman, 1992) and protease (Ladd and Butler, 1972) were studied.

Results

Garhwal region is a hillock area of Uttarakhand. For the present study, soil and water samples from Soldhar and Tapovan kund 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.*, 2009; 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.

Tapovan kund is located at Tapovan near Joshimath. This hot spring is devoted to Lord Shiva hence has got spiritual values and visited by pilgrims, tourists and local people. Soil and water samples were collected in sterile autoclaved bag and sterile autoclaved bottles. Water sample was collected from source (origin) of hot water spring. Hot water form run off channel and falls in the pool where it is mixed with normal water and used by local people and visitors for bathing**.** The exit sample was collected before water enters into the pool.

Physical characteristization

Soldhar has temperature 90^0C and pH was alkaline while Tapovan kund has temperature of 52^0C and pH was near neutral (Table 1)

Sample	Site	Temperature (in ^{0}C)		pH	
		Source	Exit	Source	Exit
Water	Soldhar	90	71	8.2	7.9
Soil*	Soldhar	90	-	8.4	-
Water	Tapovan kund	52.2	45.1	6.5	6.7
Soil*	Tapovan kund	52.2	-	7.4	-

*Soil sample was collected from the source

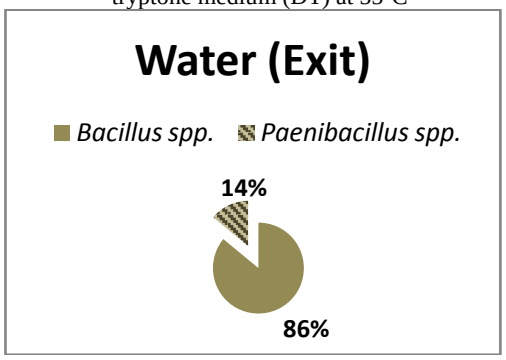
Table 1: Temperature and pH of sampling sites

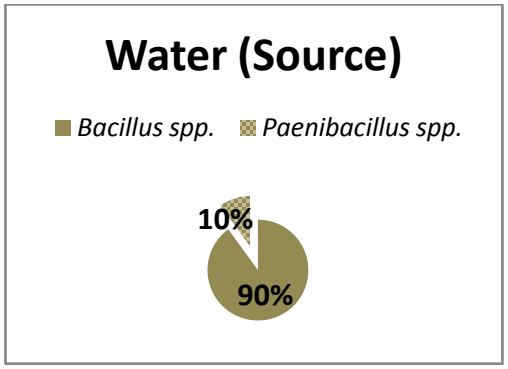
Bacterial population profile

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 Tapovan kund, the population count (log_{10} cfu) on NAM was 3.99 (source), 3.76 (exit) and 3.66 (soil). On DT, the population count (log_{10} cfu) was 3.79 (source), 3.79 (exit) and 3.91 (soil) (table 2).So bacetrial count on DT was comparatively higher than NAM in case of Soldhar while in case of Tapovan kund 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.

Sample	Soldhar		Tapovan kund	
	NAM	DT	NAM	DT
Water (source)	3.07	3.19	3.99	3.79
Water (exit)	3.00	3.93	3.76	3.79
Soil	3.12	4.04	3.66	3.91

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





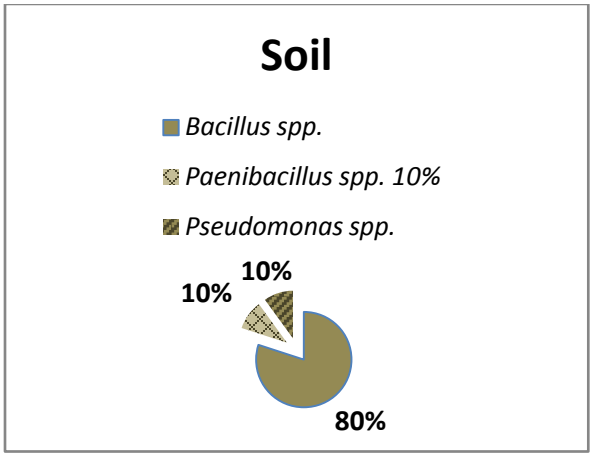
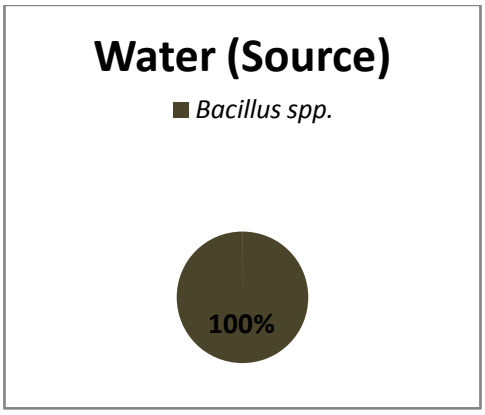
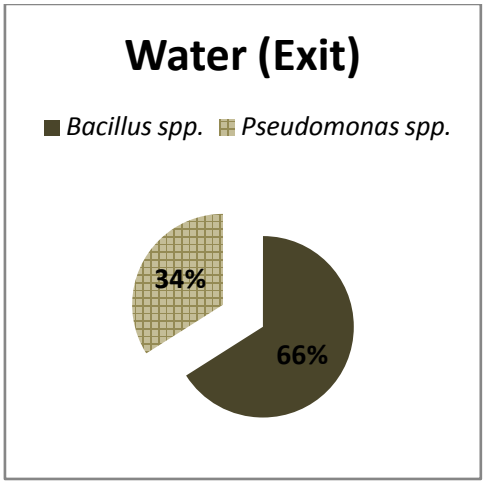


Figure 2: Percentage distribution of recovered isolates from Soldhar hot spring

A total of 35 isolates were recovered from Soldhar while a total of 26 isolates were recovered from Tapovan kund. These isolates were characterized morphologically as well as biochemically. Most of the isolates belonged to genus *Bacillus* from both the sites. *Paenibacillus* was also recovered from Soldhar while *Pseudomonas* was recovered from Soldhar (soil) and Tapovan kund (soil and exit water) (Figure 2; Figure 3)





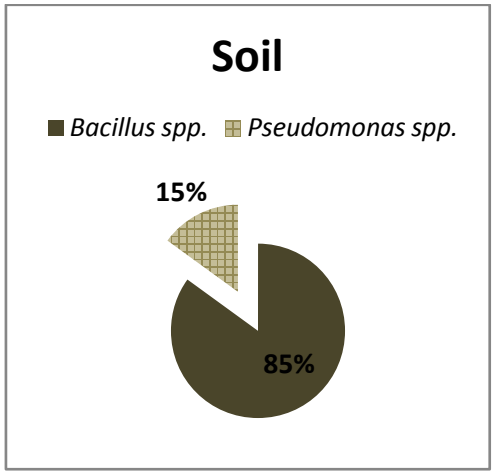


Figure 2: Percentage distribution of recovered isolates from Tapovan kund

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-

$$\text{Index of relative enzyme activity} = \frac{\text{Diameter (Dia.) of Clear Zone} - \text{Diameter (Dia.) of Bacterial Colony}}{\text{Diameter (Dia.) of Bacterial Colony}}$$

From Soldhar, out of 35 recovered isolates 12 were amylolytic and the highest index for amylase activity was found to be 1.51. Lipase producing isolates were 15 in number with highest index of 1.20 while protease producing isolates were 16 and highest index for protease activity was 2.81 (Table 3).

Isolate	Amylase			Lipase			Protease		
	Dia.of colony (mm)	Dia. of clear zone (mm)	Index	Dia. of colony (mm)	Dia. of clear zone (mm)	Index	Dia. of colony (mm)	Dia. of clear zone (mm)	Index
SU1	-	-	-	-	-	-	6.00	20.66	2.44
SU2	24.00	31.00	0.29	16.60	19.60	0.18	22.00	32.00	0.45
SU3	-	-	-	-	-	-	7.00	16.33	1.33
SU4	-	-	-	-	-	-	-	-	-
SU5	21.00	29.00	0.38	17.30	19.30	0.11	22.00	36.60	0.66
SU6	-	-	-	-	-	-	-	-	-
SU7	20.66	29.00	0.40	-	-	-	13.00	18.30	0.40
SU8	-	-	-	-	-	-	-	-	-
SU9	-	-	-	-	-	-	-	-	-
SU10	-	-	-	-	-	-	-	-	-
SD1	-	-	-	-	-	-	-	-	-
SD2	-	-	-	-	-	-	12.00	19.00	0.58
SD3	18.33	20.00	0.09	-	-	-	-	-	-
SD4	-	-	-	23.66	25.00	0.05	-	-	-
SD5	-	-	-	-	-	-	-	-	-
SD6	-	-	-	-	-	-	-	-	-
SD7	-	-	-	-	-	-	8.66	27.66	2.19
SD8	22.66	25.00	0.10	-	-	-	-	-	-
SD9	-	-	-	-	-	-	-	-	-
SD10	-	-	-	19.00	25.66	0.35	-	-	-
SD11	10.00	20.33	1.03	10.00	22.00	**1.20**	-	-	-
SD12	-	-	-	9.33	12.33	0.32	-	-	-
SD13	-	-	-	-	-	-	5.33	20.33	**2.81**
SD14	-	-	-	-	-	-	-	-	-
SD15	20.66	27.00	0.30	14.33	16.33	0.13	18.33	20.00	0.09
SS1	9.33	16.66	0.78	-	-	-	-	-	-
SS2	9.66	24.33	**1.51**	8.33	10.00	0.20	-	-	-
SS3	24.00	31.00	0.29	48.00	51.00	0.06	10.00	15.00	0.50
SS4	18.00	21.00	0.16	23.66	25.00	0.05	9.44	11.77	0.24
SS5	-	-	-	18.00	25.30	0.40	8.23	10.33	0.25
SS6	-	-	-	34.33	39.00	0.13	11.43	16.72	0.46
SS7	-	-	-	18.60	24.60	0.32	18.93	23.78	0.25
SS8	-	-	-	46.66	49.33	0.05	8.67	14.98	0.72
SS9	9.33	16.66	0.78	-	-	-	-	-	-
SS10	-	-	-	10.33	12.00	0.16	12.96	18.98	0.46

- Indicates no enzymatic activity

Table 3: Index of relative enzymatic activity of bacterial isolates recovered from Soldhar hot spring

From Taovan kund, 18 isolates were amylase producing and highest index for amylase activity was 2.44, 17 isolates were lipase producing and highest index was 1.08. Proteolytic isolates were 12 and highest index for protease activity was 3.00 (Table 4).

From Soldhar 45% were proteolytic, 42% isolates were lipolytic and 34% isolates were found to be amylolytic. While from Taovan kund, 69% recovered isolates had amylolytic potential, 65% isolates were lipolytic and 46% were proteolytic (Figure 4).

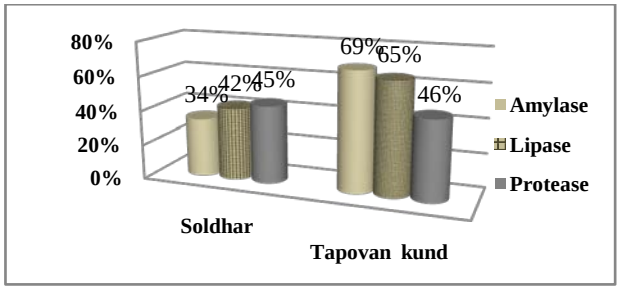


Figure 4: A comparative percentage distribution of enzymatic potential of recovered isolates from Soldhar hot spring and Tapovan kunad

Few isolates from both hot springs produced amylase + lipase; lipase + protease and amylase + protease. 20% isolates from Soldhar were recorded to produce amylase + lipase, 25% isolates were reported to produce lipase + protease and 17% isolates were amylase + protease producing (Figure 5). From Tapovan kund 57% isolates were amylase + lipase producers, 30% isolates were lipase + protease producers and 26% isolates were amylase + protease producing (Figure 6). 14% isolates from Soldhar and 26% isolates from Tapovan kund were amylase + lipase + protease producers (Figure 7).

Isolate	**Amylase**			**Lipase**			**Protease**		
	Dia.of colony (mm)	Dia. of clearzone (mm)	Index	Dia. of colony (mm)	Dia.of clear zone (mm)	Index	Dia. of colony (mm)	Dia. of clear zone (mm)	Index
TKU1	-	-	-	-	-	-	11.00	24.00	1.18
TKU2	71.00	77.00	0.08	22.00	26.00	0.18	-	-	-
TKU3	-	-	-	-	-	-	3.00	12.00	**3.00**
TKU4	7.00	9.00	0.28	38.00	41.00	0.07	-	-	-
TKU5	12.00	19.00	0.58	60.00	65.00	0.08	-	-	-
TKU6	28.00	38.00	0.35	10.00	19.00	0.90	-	-	-
TKU7	21.00	39.00	0.85	36.00	39.00	0.08	50.00	53.00	0.06
TKD1	-	-	-	9.30	11.00	0.18	9.30	21.00	1.25
TKD2	8.30	12.00	0.44	9.00	17.00	0.88	-	-	-
TKD3	23.00	35.00	0.52	-	-	-	-	-	-
TKD4	19.00	25.00	0.31	10.00	12.00	0.20	32.00	24.00	0.33
TKD5	65.00	83.00	0.28	-	-	-	-	-	-
TKD6	23.00	32.00	0.39	34.00	38.00	0.11	-	-	-
TKS1	44.00	51.00	0.15	30.00	35.00	0.16	33.00	37.00	0.12
TKS2	16.00	34.00	1.12	34.00	42.00	0.23	19.00	30.00	0.57
TKS3	41.00	47.00	0.14	21.30	30.00	0.40	-	-	-
TKS4	-	-	-	-	-	-	-	-	-
TKS5	48.00	58.00	0.20	70.00	80.00	0.14	32.30	42.00	0.03
TKS6	-	-	-	-	-	-	29.00	37.00	0.27
TKS7	13.00	30.00	1.30	19.00	24.00	0.26	47.00	51.00	0.08
TKS8	-	-	-	47.00	57.00	0.21	-	-	-
TKS9	59.00	75.00	0.27	54.00	66.00	0.22	10.00	18.00	0.80
TKS10	63.00	77.00	0.22	-	-	-	-	-	-
TKS11	9.00	31.00	**2.44**	12.00	25.00	**1.08**	-	-	-
TKS12	-	-	-	-	-	-	-	-	-
TKS13	-	-	-	-	-	-	14.00	27.00	0.92

- Indicates no enzymatic activity

Table 4: Index of relative enzymatic activity of bacterial isolates recovered from Tapovan kund

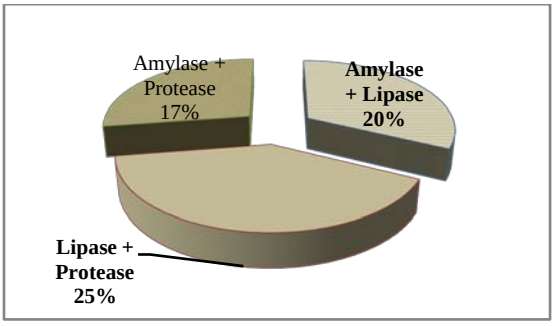


Figure 5: A percentage distribution of amylase + lipase, lipase + protease and amylase + protease producing isolates from Soldhar hot spring

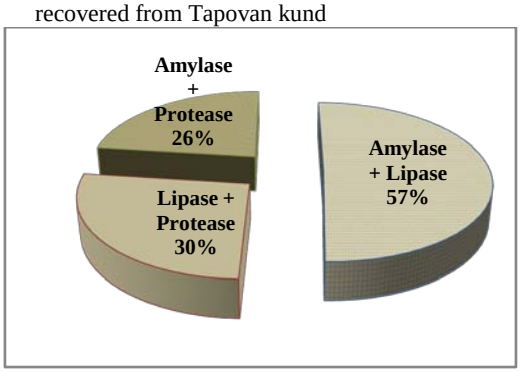


Figure 6: A percentage distribution of amylase + lipase, lipase + protease and amylase + protease producing isolates from Tapovan kund

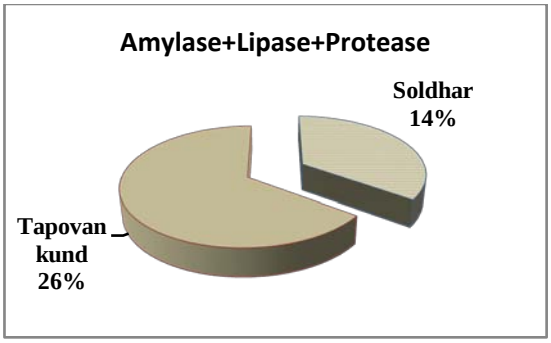


Figure 7: A percentage distribution of amylase + lipase + protease producing isolates from Soldhar hot spring and Tapovan kund

Discussion

Hot springs harbors a rich microbial diversity. Besides having structural diversity the springs have functionally diverse population and have always drawn the attention of researchers. The present study was done to characterize the structural and functional diversity of thermophilic bacterial population.

The predominant genus from both the hot springs was *Bacillus*. Species of *Bacillus*, 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.*, 2014). Previous studies on culturable diversity of Soldhar documented presence of *Bacillus*, *Geobacillus* and cyanobacteria (Kumar *et al.*, 2004; Sharma *et al.*, 2009; Bhardwaj *et al.*, 2010; Bhardwaj *et al.*, 2011; Pandey *et al.* (2014b). Recently, Sharma *et al.* (2014) 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., 2009*; Pandey *et al.*, 2014a). However, no sequences related to this genus has neither been reported by Sharma *et al.* (2014) nor this genus was recovered by us. It is thus possible that this group represents only a minor community in the hot spring and the dominance of this genus reported in previous studies may be attributable to some bias in the growth conditions (Sharma *et al.*, 2014).

The functional analysis of recovered isolates revealed that both the study sites posses active enzyme producers. The bacterial population profile of Tapovan kund (moderate temperature hot spring) was functionally more active as compared to Soldhar (high temperature hot spring) bacterial population (Figure 4). In most of the cases, hot springs represent an oligotrophic habitat. Physicochemical analysis of Soldhar by Sharma *et al.* (2014) documented that it is also an oligotrophic environment with very low sulfur content. From both the study sites majority of isolates were multi enzyme producers i.e. producing combination of any two or all the three enzymes. This shows that the bacterial community of both the geothermal spring has fully adapted themselves to survive in oligotrophic conditions.

Thus, the geothermal provinces of Uttrakhand have functionally important microbial resources. Besides surviving in nutrient depleted environment these are also facing the

natural phenomenon like landslides and anthropogenic interventions which are severely affecting this diversity.

Conclusions

The hot springs of Garhwal Himalaya are under various anthropogenic interventions which necessitate the conservation of bacterial gene pool otherwise there will be a loss of this diversity. The present study revealed the dominance of *Bacillus*, *Paenibacillus* and *Pseudomonas* in these hot springs. The recovered bacterial population is functionally dominant which can be further exploited in various industrial applications.

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