CHARACTERIZATION OF BACTERIAL DIVERSITY OF HOT SPRINGS OF CHAMOLI REGION OF GARHWAL HIMALAYA

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ABSTRACT

In the present study, bacterial diversity of a high temperature (Soldhar) and a 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. *Bacillus* was observed to be the dominant genus in both hot springs. Other dominant genera were *Aneurinibacillus* spp., *Brevibacillus* spp., *Deinococcus* spp., *Geobacillus* spp., *Lysinibacillus* spp., *Paenibacillus* spp. and *Pseudomonas* spp. The recovered isolates of Tapovan kund were observed to be more diverse as compared to isolates of Soldhar but Soldhar isolates were more functionally active. 73% lipase producers and 50% each amylase and protease producers were recovered from Soldhar while 71% lipase, 35% protease and 26% amylase producers were recovered from Tapovan kund. 13.6% isolates from Soldhar and 6.45% isolates from Tapovan kund were extremely active from functional view point as they were able to produce all the three investigated enzymes. Such isolates can be further exploited for various biotechnological applications in basic and applied biology.

Keywords: Hot springs, Thermophilic bacteria, *Bacillus, Paenibacillus, Pseudomonas.*

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). The geothermal springs or hot springs represent one of the extreme environments and these are substantially higher in temperature than the air temperature of the surrounding region. The hot springs can be found everywhere, different countries and areas, even some on the seafloor (Verma *et al.,* 2014). Geothermal waters are well known for their biological wealth and explored for thermophilic microorganisms (Ranawat *et al.,* 2017). 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 referred 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 enantio-selectivity 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).

According to Geological Survey of India (GSI), there are nearly 400 hot springs in India amongst them various hot

springs are present in Garhwal Himalaya region along the banks of river- Alakananda, Bhagirathi, Kali and Yamuna (GSI 1991; Dimri 2013; Poddar *et al.,* 2017). The hot springs of Garhwal (Soldhar, Ringigad and Suryakund) were also explored earlier revealing the presence of *Bacillus* spp., *Brevibacillus* spp., *Geobacillus* spp., *Paenibacillus* spp. and *Pseudomonas* sp. (*Kumar et al.,* 2004; 2005; Trivedi *et al.,* 2006; Sharma *et al.,* 2009; Arya *et al.,* 2015; Pandey *et al.,* 2014a, Pandey *et al.,* 2014b; Ranawat *et al.,* 2017). Besides, bacteria cyanobacterial diversity was also reported from Soldhar and Ringigad which include *Chlorogloeopsis* spp. *Chroococus turgidus*, *C. tenex*, *Gloeocapsalivida*, *Hydrococcus rivularis, Lyngbya hieronyamus, Myxosarcina* spp., *Oscillatoria animalis*, *O. pseudogeminata, O. simplicissima, O. cruenta, O. princeps, Phormidium bohaneri*, *P. cabennense*, *Pseudanabaena galeata*, *Spirulina meghiniana, Spirulina subsalsa*, and *Synechocystis sallensis* (Bhardwaj *et al.,* 2010; 2011). Amongst these reported cyanobacteria *Spirulina meghiniana* and *Chlorogloeopsis spp.* were the new record for thermal springs of Uttrakahand. *Lyngbya hieronyamusii*, *Pseudanabaena glaeata* and *Chlorogloeopsis* spp. were a new record for Indian thermal spring (Bhardwaj *et al.,* 2011). Besides, bacterial diversity the enzymatic profile of hot springs of Garhwal Himalayas need to be explored as these geothermal reservoirs stores microorganisms which have immense potential of producing thermostable enzymes. So, the present study document the structural and functional diversity of thermophilic bacteria of one high temperature (Soldhar) and other moderate temperature hot spring (Tapovan kund) of Garhwal Himalayas.

EXPERIMENTAL SITES

The samples were collected from Soldhar and Tapovan kund (Disrtict Chamoli) located at Garhwal Himalaya region. 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. 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. 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. Soil samples from both the sites were collected from source.

METHODOLOGY

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. pH and temperature of water samples were recorded at sampling site. 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 55ºC. Soil sample was air dried, serially diluted and plated on NAM and DT medium and plates were incubated at 55ºC for 36-48 hours. Isolates were purified by streaking on nutrient agar and pure cultures were maintained. Glycerol stocks were prepared by adding 1.0 ml of glycerol to 1.0 ml of overnight grown culture in Nutrient broth. Glycerol stocks were maintained in cryovials and preserved at -20ºC. Colony morphology (shape, size, form, elevation and margin) and cell morphology (Gram's reaction, cell shape and arrangement) of isolates were studied. The various biochemical tests *viz.,* catalase, 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). The functional characteristics of recovered isolates *viz.,* amylase lipase and protease were studied (Chadha *et al.,* 1997; Cappucino *et al.,* 2007; Ladd *et al.,* 1972).

Table 1. Temperature and pH of sampling sites

Sample	Site	Temperature (in ºC)		pH	
		Source	Exit	Source	Exit
Water	Soldhar	90.1	73.3	8.0	7.9
Soil*	Soldhar	90.5	-	8.5	-
Water	Tapovan kund	52.5	46.7	6.4	6.5
Soil*	Tapovan kund	54.2	-	7.4	-

*Soil samples were collected from source

RESULTS AND DISCUSSION

Physical characterization

Soldhar has temperature 90ºC and pH was alkaline while Tapovan kund has temperature of 52ºC and pH was near neutral (Table 1).

Bacterial population profile

The biodiversity of hot springs is always a thrust area of study which has attracted attention of researchers worldwide. Besides, structural diversity the springs also have functionally diverse population which has enormous applications in the various fields of science. The present study was done to characterize the structural and functional diversity of thermophilic bacterial population. The culture based approach has been used to study the bacterial population of the study area. With the advent of unculturable techniques the culture based approach sounds much tedious and time taking but culturable techniques have their own advantages in the microbial world as these techniques generate valuable germplasm and allow to preserve the microbial strains for future studies and to explore them for biotechnological applications (Akabani *et al.*, 2010; Acharya *et al.*, 2012).

In Soldhar, the population count (log_{10}cfu) was 2.93±0.089 (source water), 2.82±0.16 (exit water) and 4.07±0.11 (soil) on NAM while on DT the count 2.80±0.14 (source water), 2.92±0.11 (exit water) and 4.06±0.16 (soil). In Tapovan kund, the count on NAM was 3.15±0.10 (source water), 3.16±0.13 (exit water) and 4.25±0.06 (soil) while on DT the population count was 3.13±0.08 (source water), 3.03±0.17 (exit water) and 4.19±0.13 (soil). This suggests

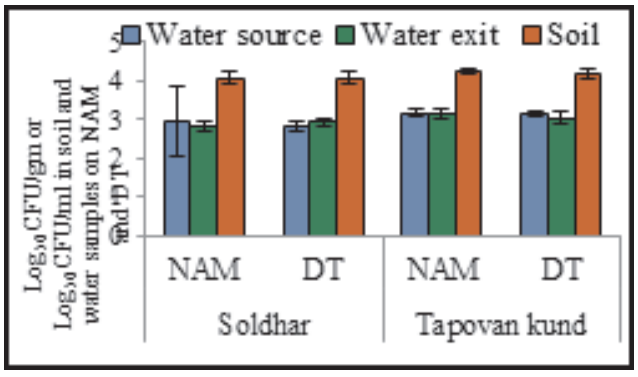

Fig. 1. Microbial population count (Log_{10}CFU/gm or Log_{10}CFU/ml) in soil and water samples on nutrient agar medium (NAM) and dextrose tryptone medium (DT) at 55ºC

that Tapovan kund has highest bacterial count on NAM and DT in comparison to Soldhar. The soil samples of both sites has highest population count as compared to water and among two different culture media *i.e.* NAM and DT there were minor differences in bacterial count and both culture media were good enough for growth of thermophilic bacteria. The population profile of both springs (each reading is a mean of three replicates) (Fig. 1).

A total of 22 isolates were recovered from Soldhar while a total of 31 isolates were recovered from Tapovan kund. These isolates were characterized morphologically as well as biochemically and tentative identification up to genus level was done by Bergey's Manual of Systemic Bacteriology (Holt *et al.,* 1994) and Advanced Bacterial Identification Software (ABIS) (Stoica *et al.,* 2017). Majority of the isolates belonged to genus *Bacillus* from both the sites. In Soldhar, *Bacillus* spp. (60%) and *Brevibacillus* spp. (40%) were reported in source water. The exit water also had similar bacterial count and composition with 60% *Bacillus* spp. and 40% *Brevibacillus* spp. While from soil 75% *Bacillus* spp., 9% *Brevibacillus* spp. and 8% each of *Lysinibacillus* spp. and *Pseudomonas* spp. were reported (Fig. 2). From Tapovan kund, in source water 44% isolates were *Bacillus* spp., 22% each were *Brevibacillus* spp. and *Paenibacillus* spp and 12% were *Lysinibacillus* spp. while in exit water 30% *Bacillus* spp., 20% each of *Paenibacillus* spp. and *Lysinibacillus* spp. and 10% each of *Aneurinibacillus* spp., *Brevibacillus* spp., and *Deinococcus* spp. In soil, the isolates belonged to 4 genera- *Bacillus* spp. (50%), 17% each of *Geobacillus* spp., *Paenibacillus* spp. and *Deinococcus* spp. (16%) (Fig 3).

In the present study, *Bacillus* was the dominant genus recorded in both the sites. *Bacillus* spp. are well known survivors of harsh environments and are well adapted to hot surroundings (Connor *et al.,* 2010; Kawasaki *et al.,* 2012; Cihan *et al.,* 2014). The culture-dependent techniques were applied for exploration of thermophilic bacteria from hot springs of Garhwal Himalaya by Kumar *et al.,* 2004 who had recovered 58 thermophilic bacilli from two hot springs in a single time sampling amongst which 53 were gram positive rods. Sharma *et al.,* 2009 reported characterization of 13 morphologically distinct bacteria from Garhwal hot springs and 11 of them were gram positive bacilli and characterized them as *Geobacillus* spp. Arya *et al.,* 2015 recovered 11 distinct thermophilic bacteria from geothermal spring of

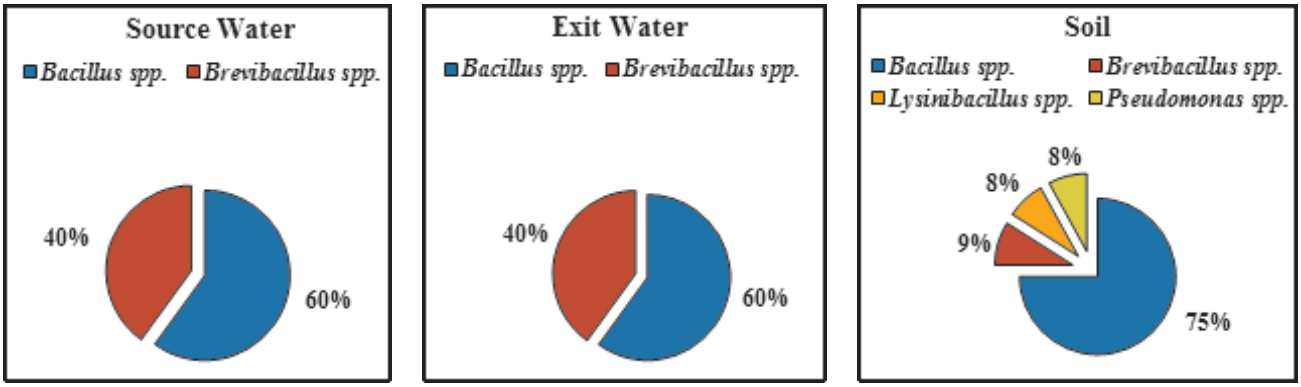

Fig. 2. Percentage distribution of recovered isolates from Soldhar hot spring

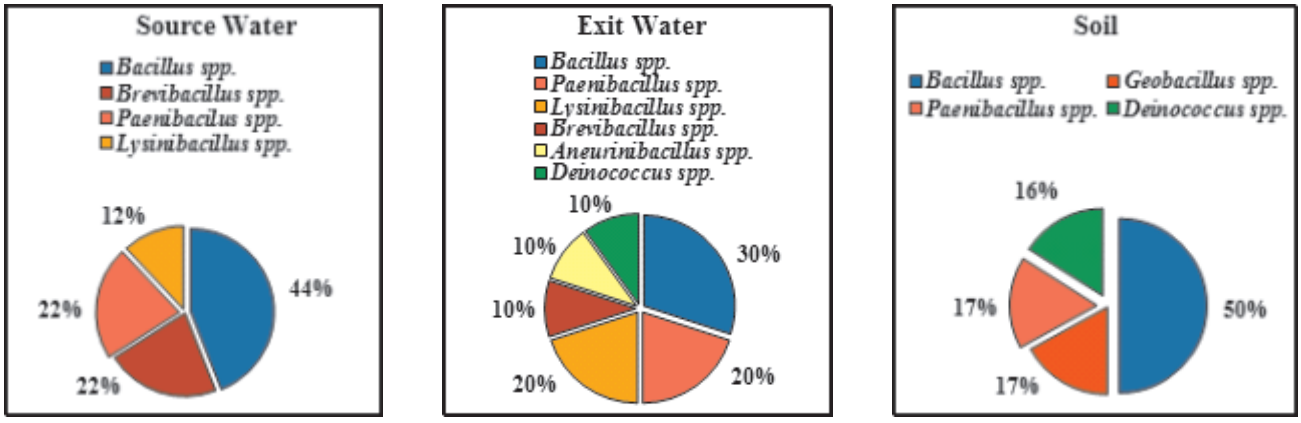

Fig. 3. Percentage distribution of recovered isolates from Tapovan kund

Garhwal and this study was also dominated by the presence of gram positive bacilli. *Bacillus* spp. was also reported as dominant genus in the bacterial diversity studies of Soldhar and Suryakund hot springs (Ranawat *et al.,* 2017).

The study included other genera *viz., Aneurinibacillus* spp., *Brevibacillus* spp., *Paenibacillus* spp. and *Lysinibacillus* spp. All the reported genera had also been reported from other geothermal areas. The species of *Brevibacillus* reported from hot springs are *Brevibacillus aydinogluensis, Brevibacillus agri, Brevibacillus brevis, Brevibacillus formosus, Brevibacillus gelatini, Brevibacillus levicki, Brevibacillus parabrevis, Brevibacillus sediminis, Brevibacillus thermoruber* (Allan *et al.,* 2005; Cihan *et al.,* 2012; Inan *et al.,* 2012; Pathak *et al.,* 2014; Verma *et al.,* 2014; Inan *et al.,* 2016; Xian *et al.,* 2016). Species of *Paenibacillus* reported from hot springs are *P. hashemite, P. lautus, P. marinum, P. rimawi, P. thermophilus, P. urinalis* (sp. strain TCA20), *P. zara,* (Bouraoui *et al.,* 2013; Akel *et al.,* 2008; Zhou *et al.,* 2012; Fujinami *et al.,* 2014; Mead *et al.,* 2012). *Lysinibacillus spahericus* has been reported from few hot springs like Unkeshwar India (Pathak *et al.,* 2014). *Geobacillus* spp. is the other genus in this study that has been reported form Tapovan kund however this genus was not recovered from Soldhar. Earlier, Sharma *et al.,* 2009 also reported species of *Geobacillus* from Soldhar. The species recovered were *G. thermoleovorans, G. kaustophilus, G. stearothermophilus* and *G. subterraneus* Arya *et al.,* 2015 had also reported *Geobacillus thermocatenulatus* and *G. thermoleovorans* form Soldhar. In this investigation, the possible reason for the absence of *Geobacillus* spp. from Soldhar could be the difference in sampling time and season or the anthropogenic activities that are continuously disturbing the site as Soldhar is located at road side and facing interventions like road constructions and landslides.

Pseudomonas spp., due to its ubiquitous nature was also detected in geothermal area. In the present investigation this genus was also detected, the observations are in good agreement with Sharma *et al.,* 2014 who reported genus *Pseudomonas* by studying unculturable diversity of Soldhar

using denaturing gradient gel electrophoresis (DGGE). Besides, *Bacillus* spp. one coccus form was also registered from Tapovan kund that was identified as *Deinococccus* spp. *Deinococcus* spp. like *D. geothermalis* and *D. murrayi* is thermophilic and also recovered from hot springs of Italy *(*Ferreira e*t al., 1997).*

Functional characterization of recovered bacterial isolates

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

$$\text{Index of relative enzyme activity} = \frac{\text{(Diameter of clear zone – Diameter of bacterial colony)}}{\text{Diameter of bacterial colony}}$$

Table 2. Index of relative enzymatic activity of bacterial isolates recovered from Soldhar hot spring

Bacterial isolates	AMYLASE			PROTEASE			LIPASE		
	Diameter (mm)			Diameter (mm)			Diameter (mm)		
	Bacterial colony	Clear Zone	Index	Bacterial colony	Clear Zone	Index	Bacterial colony	Clear Zone	Index
SU1	9.66	12.33	0.27	43.00	47.00	0.09	31.33	35.66	0.13
SU2	-	-	-	15.33	23.33	0.52	17.00	26.00	0.52
SU3	-	-	-	-	-	-	17.00	19.00	0.11
SU4	26.66	31.66	0.18	-	-	-	-	-	-
SU5	-	-	-	57.33	63.66	0.11	16.00	20.00	0.25
SD1	8.33	15.33	0.84	36.00	47.00	0.30	18.00	22.00	0.22
SD2	17.33	20.66	0.19	-	-	-	-	-	-
SD3	-	-	-	13.66	16.00	0.17	16.00	23.00	0.43
SD4	-	-	-	10.00	18.00	0.80	20.00	30.00	0.50
SD5	-	-	-	16.00	20.00	0.25	17.66	30.66	0.73
SS1	13.66	24.66	0.80	-	-	-	12.33	23.00	0.86
SS2	11.66	19.66	0.68	3.66	9.33	1.55	12.66	34.33	1.71
SS3	17.33	26.33	0.51	-	-	-	28.66	39.33	0.37
SS4	14.33	23.66	0.65	-	-	-	13.66	20.00	0.46
SS5	-	-	-	60.66	63.00	0.03	-	-	-
SS6	20.00	31.66	0.58	-	-	-	9.00	17.66	0.96
SS7	26.00	31.00	0.19	-	-	-	21.00	28.33	0.34
SS8	10.66	18.00	0.68	-	-	-	16.00	21.00	0.31
SS9	-	-	-	37.00	41.00	0.10	-	-	-
SS10	-	-	-	32.00	38.00	0.18	-	-	-
SS11	-	-	-	-	-	-	14.66	18.00	0.22
SS12	-	-	-	-	-	-	-	-	-

- *Indicates no enzymatic activity*

From Soldhar, out of 22 recovered isolates 11 were amylolytic and the highest index for amylase activity was found to be 0.84. Proteolytic isolates were 11 in number with highest index of 1.55 while lipolytic isolates were 16 in number with highest index of 1.71 (Table 2).

From Taovan kund, 8 isolates were amylase producing and highest index for amylase activity was 0.69, proteolytic isolates were 11 and highest index for protease activity was 1.41 and 22 isolates were lipase producing and highest index was 1.45 (Table 3).

From Soldhar, 50% isolates were proteolytic, 50% isolates were amylolytic and 73% isolates were found to be lipolytic. While from Taovan kund, 26% recovered isolates had amylolytic potential, 35% isolates were proteolytic and 71% were lipolytic (Fig. 4).

Table 3. Index of relative enzymatic activity of bacterial isolates recovered from Tapovan kund

Bacterial isolates	AMYLASE			PROTEASE			LIPASE		
	Diameter (mm)			Diameter (mm)			Diameter (mm)		
	Bacterial colony	Clear Zone	Index	Bacterial colony	Clear Zone	Index	Bacterial colony	Clear Zone	Index
TKU1	-	-	-	-	-	-	16.66	26.33	0.58
TKU2	17.66	25.00	0.41	12.00	29.00	1.41	12.33	16.33	0.32
TKU3	-	-	-	11.66	16.66	0.42	21.66	25.00	0.15
TKU4	-	-	-	13.00	15.00	0.15	-	-	-
TKU5	24.33	28.33	0.16	70.00	72.66	0.03	21.33	31.33	0.46
TKU6	70.00	73.00	0.04	-	-	-	31.33	35.00	0.11
TKU7	-	-	-	-	-	-	-	-	-
TKU8	-	-	-	-	-	-	19.00	30.00	0.57
TKU9	-	-	-	-	-	-	-	-	-
TKD1	-	-	-	13.66	28.00	1.04	12.00	16.00	0.33
TKD2	-	-	-	8.00	10.00	0.25	7.33	18.00	1.45
TKD3	-	-	-	9.00	12.33	0.37	12.00	16.00	0.33
TKD4	-	-	-	25.00	34.33	0.37	17.66	23.66	0.34
TKD5	-	-	-	7.66	18.33	1.39	18.66	20.00	0.74
TKD6	-	-	-	-	-	-	23.00	28.66	0.24
TKD7	-	-	-				16.66	24.66	0.48
TKD8	-	-	-	-	-	-	11.33	15.00	0.32
TKD9	-	-	-	-	-	-	-	-	-
TKD10	-	-	-	-	-	-	-	-	-
TKS1	-	-	-	-	-	-	-	-	-
TKS2	70.00	73.00	0.04	-	-	-	21.33	31.00	0.45
TKS3	27.33	36.33	0.34	-	-	-	14.00	17.66	0.26
TKS4	-	-	-	15.00	25.33	0.68	7.33	18.00	1.45
TKS5	15.33	26.00	0.69	-	-	-	-	-	-
TKS6	20.00	22.00	0.10	-	-	-	18.66	20.66	0.10
TKS7	-	-	-	-	-	-	-	-	-
TKS8	-	-	-	-	-	-	6.00	8.00	0.33
TKS9	-	-	-	-	-	-	5.00	7.33	0.46
TKS10	15.66	30.66	0.65	-	-	-	14.00	20.00	0.42
TKS11	-	-	-	-	-	-	9.00	13.00	0.44
TKS12	-	-	-	9.66	13.33	0.38	-	-	-

- *Indicates no enzymatic activity*

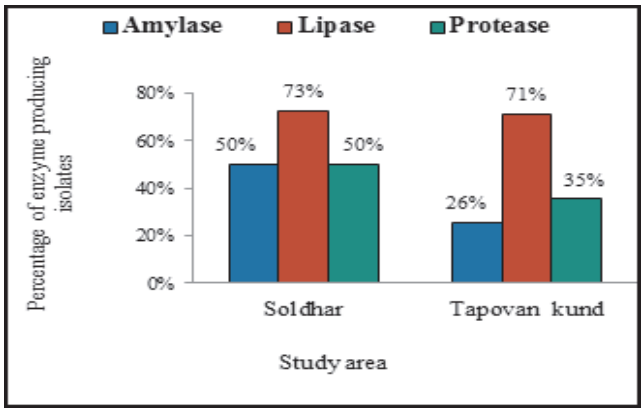

Fig. 4. A comparative percentage distribution of enzymatic potential of recovered isolates from Soldhar and Tapovan kund

Few isolates from both hot springs produced multiple enzymes or only single enzyme. From Soldhar, 3 isolates were able to produce amylase, protease and lipase while from Tapovan kund 2 isolates has potential of producing all the three investigated enzymes. Amylase and lipase was produced by 6 isolates from Soldhar and 5 isolates from Tapovan kund. Lipase and protease was produced by 5 isolates from Soldhar and 7 isolates from Tapovan kund. Only lipase was produced by 2 isolates from Soldhar and 8 isolates from Tapovan kund. 3 isolates from Soldhar and 2 isolates from Tapovan kund were capable of producing only protease while only amylase was produced by 2 isolates from Soldhar and 1 isolate from Tapovan kund. None of the isolates from both the study area were able to produce amyalse and protease. 1 isolate from Soldhar and 6 isolates

from Tapovan kund produced none of the investigated enzyme. The overall enzymatic profile of recovered isolates (fig. 5).

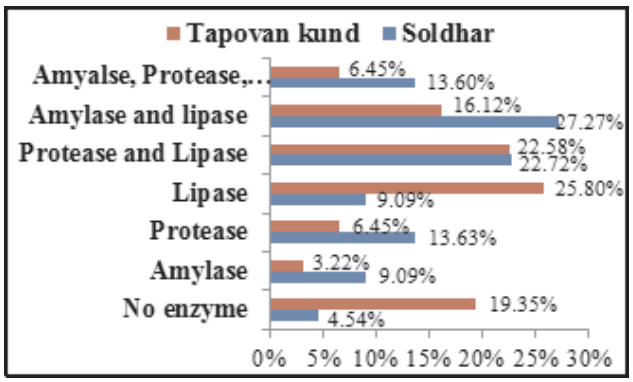

Fig. 5. Enzymatic profile of recovered isolates from Soldhar and Tapovan kund

Over all, the population of both the springs is active lipase producer. In Soldhar, the isolates were active lipase producer followed by equal number of protease and amylase producers while in Tapovan kund the population of lipase producers was followed by protease producers and amylase producers were lowest in number. This study was in concurrence with the study of Yavuz *et al.,* 2003 who reported 98% lipase producers, 95% amylase producers and 49% protease producers from Balçova geothermal region. A study from Manikaran hot spring (Himachal Pradesh) also reported presence of lipase producers in highest number (84.26%), followed by cellulase producers (74.07%), amylase producers (43.52%) and protease producers (42.59%) (Devi 2015). The **e**xtracellular amylase activity of mycelial yeast, *Saccharomycopsis fibuligera* was also reported by Kumar *et al.,* 2005 from hot spring of Garhwal Himalaya. The enzyme producing population of Soldhar was also reported earlier which revealed the presence of 34% amylase producers and 28% cellulase producers while other hot spring of Suryakund had 55% amylase producers and 55% cellulase producers (Ranawat *et al.,* 2017).

In this study, it was also observed that isolates were able to produce multiple enzymes. This suggests the highest functional activeness of investigated isolates. The property of production of two or more enzymes by microorganisms helps them to survive in hostile environments so that they can acquire nutrition by breakdown of variety of nutrients (Devi 2015). Thus, the present investigation on the bacterial diversity of two hot springs of Garhwal Himalaya and their functional potential, revealed the presence of diverse bacterial population in these geothermal springs dominated by *Bacillus* spp. Majority of isolates were lipase producers and also capable to produce amylase and protease which suggests that these isolates are storehouse of thermo stable enzymes and can be utilized for various biotechnological applications. This study is also an effort to preserve the gene pool of these important microorganisms as both of these sites are under anthropogenic interventions which continuously disturb the native microbial population of the hot water ecosystems of Himalaya region.

CONCLUSION

The present study revealed the dominance of *Bacillus* genus in the hot springs of Garhwal region. The recovered bacterial population is functionally dominant and can be further exploited in various industrial applications.

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