Research Paper

Cold-active hydrolases producing bacteria from two different sub-glacial Himalayan lakes

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Microorganisms, native to the cold environments have successfully acclimatized their physiological, metabolic and biological features, exhibiting uniqueness in their enzymes, proteins, and membrane structures. These cold-active enzymes have immense biotechnological potential. The diversity of culturable bacteria in two different water lakes (the sub-glacial freshwater and the brackish) of Himalayas was analyzed using SYBR green staining and cultural methods. A total of 140 bacteria were isolated and were grouped as psychrophiles, psychrotrophs and psychrotolerant organisms, based on their optimal temperature for growth. The amplified ribosomal DNA restriction analysis using three restriction enzymes facilitated the grouping of these isolates into 96 genotypes at ≥85% polymorphism. Phylogenetic analysis using 16S rRNA gene sequences revealed that the bacterial strains from both lakes belonged to Firmicutes, Proteobacteria (α, β, and γ) or Actinobacteria. Screening of the germplasm for the activity of different cold-active hydrolases such as protease, amylase, xylanase and cellulase, revealed that about sixteen isolates were positive, and exhibiting a wide range of stability at various temperature and pH. Our results suggest that the distinctly different ecosystems of sub-glacial freshwater and brackish water lakes have diverse groups of bacteria, which can be an excellent source of extracellular hydrolases with a wide range of thermal stability.

Keywords: Cold-active hydrolases / 16S rRNA gene / Psychrophiles / Himalayan lakes

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Introduction

Microorganisms living under the extreme environments provide vital information about the physical and chemical limits for physiological and biological adaptability. The extreme cold habitats and their microflora in different parts of the world have gained the attention of many researchers in the recent years [4, 18, 19]. In addition to survival, microbial communities in these cold environments were investigated for their diversity, biogeography, colonization, and/or biofilm formation [8, 32]. In contrast to the general perception, a greater degree of microbial diversity was observed in the extremely cold environments of Arctic and Antarctica [4, 5, 17, 19, 22, 37]. However, the microbial communities within the freshwater and brackish water psychrophilic lakes have been less explored compared to the marine psychrophilic microbial communities.

The Himalayas have received special attention because of their distinct psychrophilic habitats such as glacial ice, permafrost, tundra wetlands, tundra soil, sub-glacial, peri-glacial soil and lakes at high altitudes. Although the distribution and abundance of diverse bacterial species from sub-glacial and surface soil samples of certain parts of Himalayas have been reported [14, 18, 39], there are many unexplored cold freshwater and brackish water lake habitats. The sub-glacial cold lakes with structural differences in terms of physical and chemical properties may prove to be a source of

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novel cold-adapted bacteria [10]. The cold-adapted microorganisms with abilities to produce different cold-active enzymes are of economic significance in biotechnology, agriculture and medicine, especially for their enzyme and biochemical characteristics, such as protein folding [11, 26, 41–44]. Although the psychrophiles are known to synthesize very low amount of cold-active enzymes, they could be employed for developing transgenics and large scale exploration of recombinant psychrophilic enzymes [45–50]. In an effort to understand the diversity and distribution of culturable bacteria in the Himalayas, the microbial members of two snow-capped lakes were isolated and studied for their morphological, biochemical and molecular diversity and the cold-active hydrolases from these bacteria were characterized.

Materials and methods

Study area and sample collection

The sub-glacial freshwater and brackish water lakes of the Himalayas are the study sites for the present investigation (Fig. 1). The lake Gurudongmar (GDM) is situated at 5250 m above mean sea level at (28° N 88° E), which is at the footfall of Kanchenjunga peak in eastern part of the Himalayas, India. The annual mean temperature of this lake varies between –10 and 4 °C while the pH of water ranges from 7.2 to 8.0. The lake Pangong (PNG) is one of the largest brackish water (salinity 25 ppt) snow capped lake and is located at 4250 m above mean sea level at 33° N, 78° E. This endorheic lake of 134 km long and 5 km wide is completely frozen during winter.

The above two lakes were surveyed and samples (water/ice/sediment) were collected during September and December 2009 from GDM and PNG lakes respectively. From GDM lake, nine different samples, comprising three each of surface water (10–50 cm from the surface), sub-surface (100–150 cm from surface) water and deep sediments (10–50 cm from the bottom) were collected in sterilized bottles, as per the standard procedure. In case of PNG lake, a total of twelve different samples, comprising four each of deep sediments, sub surface water and surface water/ice samples were collected. A special care was taken during the sample collection from snow capped lake (PNG) as per the procedure described earlier [11, 26].

For direct enumeration of bacteria by light microscopy, water samples of 500 ml from each site were collected into sterile polypropylene tubes (Tarson Pvt. Ltd., India) and the samples were immediately fixed with formaldehyde at a final concentration of 0.74%, and stored at 4 °C in dark for further analysis. All the samples were kept at 4 °C and were transported to the laboratory with minimum time lapse, for further analyses.

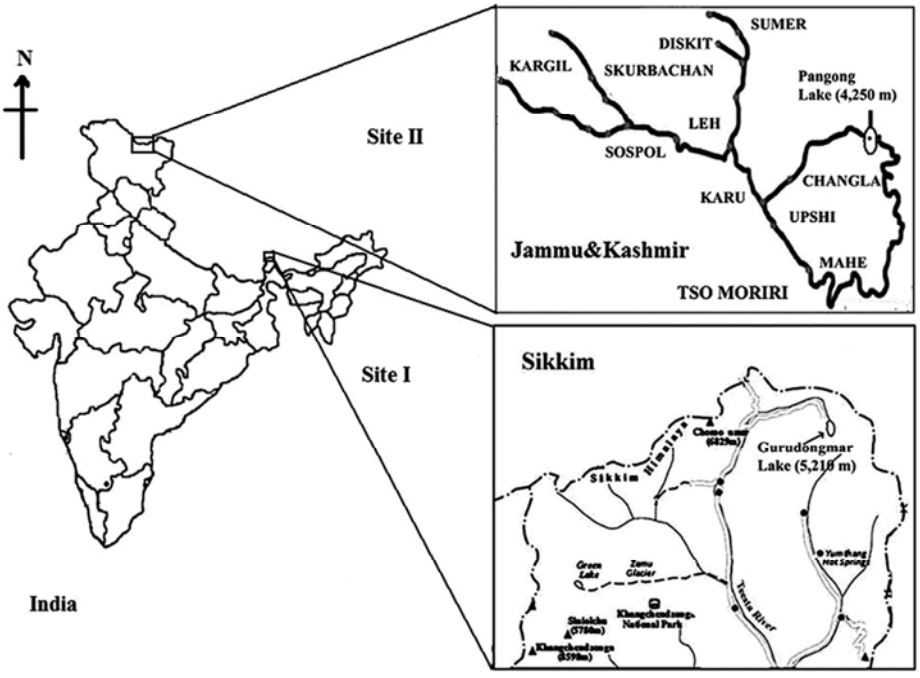

Figure 1. Geographical map of India locating the Gurudongmar (GDM) and Pangong (PNG) lakes.

Enumeration and isolation of bacteria

Enumeration of bacterial cells was carried out using the formaldehyde fixed samples with SYBR Green I (Invitrogen Corp.) stain, as per the protocol described earlier by Noble & Fuhrman [20]. In brief, 100 μl of appropriately diluted water samples were filtered through 0.02 μm anodisc filter (Whatman Int. Ltd.), then the filter was placed on a clean glass slide and stained with 2 X SYBR Gold (Invitrogen Corp.) and incubated for 15 min in dark conditions. After drying of each stained filter, 10 μl of anti-fade mounting reagent (50% glycerol in phosphate buffered saline with 0.1% *p*-phenylenediamine) was added and covered with a cover slip. Later, a drop of immersion oil was added onto the cover slip before examination under the epifluorescence microscope (IX70, Olympus, Tokyo) using blue-green light excitation at 480–495 nm. The bacterial cells were observed under 100 X magnification and counted based on their relative size and brightness to distinguish from viruses and virus like particles [20]. For each sample, a minimum of three replicate filters were prepared and in each filter, at least 4 randomly selected microscopic fields were examined for enumeration of bacteria. All the data was subjected for the analysis of variance (ANOVA), for evaluating significant differences. Standard deviations were also calculated.

The samples obtained from two different lakes were processed for colony forming units (CFUs) count, immediately after reaching the laboratory [18, 38]. In brief, for enrichment, 10 mL of each water sample was inoculated directly into 50 ml of 0.02 X nutrient broth (1 X stock contains the following ingredients:, in g/l: peptone, 5.0; yeast extract, 3.0; sodium chloride, 5.0; D(+) glucose, 1.0). For sediment samples, 5 g of soil/sediment was mixed with 10 ml of filter sterilized water of corresponding lake and inoculated to 50 ml of 0.02 X nutrient broth. All the samples were incubated at 4 °C for 2 h. Aliquots of 100 μl of each enriched samples were appropriately diluted and spread on to different agar media (Table 1) and CFUs were recorded after incubation at 5 °C for 6–15 d. Colonies having different morphology and color on the plates were isolated and purified. The agar plates were kept for extended incubation up to 35 d after inoculation (DAI), to isolate slow growing bacteria. All the purified cultures were transferred in to 20% glycerol and stored at –20 °C until further use.

In order to estimate the un-culturable bacterial population, total CFU of different samples were compared, with respective total bacterial cell count, as measured through microscopy. The percentage of un-culturable bacteria (UB%) in each sample was estimated by using the equation: UB (%) = $(T_{BC} - T_{CFU}/T_{BC}) \times 100$, Where T_{BC} is total no. of bacterial cells, and T_{CFU} is total no. of CFU.

Physiological characteristics of bacterial isolates

To test the growth pattern under different temperature (5, 20, 30 and 37 °C) and pH (4–10), all the isolates were inoculated into micro-well plates having 200 μl of 0.02X nutrient broth and incubated in a temperature-controlled Automated Microbiology Growth Analysis System (Oy Growth Curve Ab Ltd, Finland). In case of PNG brackish water lake samples, the nutrient broth (0.02X) was supplemented with NaCl (4%). The optical density at 600 nm was measured at regular intervals. All the isolates were analyzed in triplicates and depending on the temperature range for optimal growth, all the isolates were grouped as psychrophiles, psychrotrophs and/or psychrotolerant mesophiles.

Amplification of smaller subunit 16S and 16–23S rRNA genes

Genomic DNA from all the bacterial cultures was extracted using DNeasy Tissue kit (Qiagen GmbH, Hilden, Germany) as per the manufacturer's instruction. All the DNA samples were subjected to PCR amplification of partial 16S, and 16S–23S (~1500 bp each) regions using the universal primer sets PA (5′-AGAGTTTGATCC TGGCTCAG-3′), PH (5′-AAGGAGGTGATCCAGCCGCA-3′) and pHr (5′-TGCGGCTGGATCACCTCCTT-3′), p23SRO1 (5′-GGCTGCTTCTAAGCCAAC-3′) as per the standard procedures [2, 9].

Table 1. The different media used in this study for isolation of bacteria from two different lakes.

S. no.	Media and composition* per liter
1.	Tryptic soy agar: 17 g, tryptone; 3 g soya meal; 2.5 g dextrose; 5 g NaCl; 2.5 g K_2HPO_4, 20 g agar
2.	Antarctic Bacterial Medium (ABM): 5 g peptone; 2 g yeast extract; 20 g agar
3.	Starch yeast peptone agar: 2 g starch; 0.8 g yeast extract; 0.1 g peptone; 15 g agar
4.	Water agar: –15 g agar
5.	Sea water medium: 5 g tryptone; 10 g yeast extract; 5 g casamino acid; 3 g citrate solution; 2 g KCl; 20 g $MgSO_4 \cdot 7\ H_2O$; 20 g agar
6.	Nutrient agar: 5 g peptone; 5 g NaCl; 3 g beef extract; 20 g agar

* In case of PNG lake all media were supplemented with 4% NaCl.

The amplified ribosomal DNA restriction analysis (ARDRA) was performed to facilitate the genotypic grouping of these bacterial isolates. PCR products of 16S and 16S–23S rDNA regions from all different isolates were subjected for restriction fragment analysis using three different restriction endonucleases such as *Alu*I, *Hae*III and *Hha*I (Promega, USA) and reactions were carried out as described earlier by Sahay *et al.* [29]. Similarity coefficients were calculated using Jaccard's coefficient and a combined UPGMA dendrogram was constructed using NTSYS-pc 2.02e [27].

Sequencing and phylogenetic analysis

One representative strain from each ARDRA group was selected for phylogenetic analysis. PCR products of partial 16S rRNA gene were cloned into pGEM®T vector (Promega Corp. Madison, WI, USA) and sequenced as described earlier by Babu *et al.* [3]. The 16S rRNA gene sequences were aligned to those of closely related bacterial species available at GenBank database using BLASTn program. Bacterial isolates were identified based on percentage of sequence similarity (≥97%) with that of a prototype strain sequence in the GenBank [18, 30]. The phylogenetic relationships among the cold-active enzyme producing bacterial isolates of two lakes were estimated separately and the phylogenetic trees were constructed by neighbor-joining (NJ) method using MEGA 4.0.2 [31]. All the bootstrap replications (about 1,000) indicating more than 85% support were indicated at respective nodes in the phylogenetic tree.

Screening of bacterial strains for cold-active enzymes production

All the psychrophilic, psychrotrophic and psychrotolerant mesophilic bacterial isolates obtained from both lakes were investigated for the production of extracellular cold-active enzymes such as protease, amylase, xylanase and cellulase. In the primary screening, the utilization of different substrates by various isolates was studied as indication of enzyme production. Each isolate from the lake GDM was inoculated in to a basal medium (per liter), which contained yeast extract (1 g), KH_2PO_4 (1 g), $MgSO_4 \cdot 7\ H_2O$ (0.1 g), $CaCl_2 \cdot H_2O$ (0.05 g), NaCl (5 g), $NaCO_3$ (1 g) and agar (15 g), and for the isolates of lake PNG, the basal medium was substituted with 4% NaCl. Different substrates such as starch (2.5 g l^{-1}), xylan (10 g l^{-1}), carboxy methyl cellulose (CMC) (5 g l^{-1}) were added separately to the basal medium to study the production of amylase, xylanase and cellulose, respectively. For protease activity, 10% skimmed milk was added to the basal medium.

Each culture was further inoculated in to a 250 ml flask containing 100 ml basal medium with corresponding substrate and incubated at its respective optimum growth temperature and pH (determined through growth kinetics) for 3–5 d. The enzyme production and activity was determined by estimating the substrate utilization pattern of each isolate through standard protocols as described earlier for amylase [6, 24], xylanase [35], cellulase [40] and protease [16]. The stability of each enzyme in terms of activity was estimated using an aliquot of the crude enzyme (cell free extract of culture) after pre-incubation at different temperatures (5, 20 and 30 °C) for 30 min at pH 7.0, quickly chilled and assayed at its respective optimum temperature. The effect of pH on enzyme activity and optimum pH range of each enzyme was determined by carrying out the enzyme assay using buffers of different pH ranging from 4.0 to 10.0. Three different buffers (each at 0.05 M) viz. citrate buffer (pH 4.0–6.0), phosphate buffer (pH 6.0–8.0) and Tris-HCl (pH 8.0–10.0) were used. An aliquot of the crude enzyme was pre-incubated with the above mentioned buffers of different pH (4.0–10.0) for 30 min at 4 °C temperature followed by measurement of activity using sodium phosphate buffer (0.05 M, pH 7.0).

The stability of enzyme activity (SE) was inversely proportional to the reduced enzyme activity; therefore SE at variable temperatures was calculated as follows:

$$SE = 100 - R_A; \quad \text{where} \quad R_A\ (\%) = E_{TP} - E_{dTP}/E_{TP} \times 100$$

R_A represents the percentage of reduced enzyme activity, E_{TP} is the total enzyme activity at its optimum temperature and pH, E_{dTP} is enzyme activity after temperature treatment at its respective optimum pH. All the enzyme assays were conducted in triplicates and data were subjected to analysis of variance (ANOVA) using software SPSS ver. 10 and least significant difference (LSD) at $P < 0.01$ among means compared.

Results

Enumeration and isolation of bacteria

Total bacterial cell count and enumeration of culturable bacterial populations were performed for different samples obtained from both lakes (Fig. 1). The total bacterial cell count was expressed as a mean of three sample locations for each site, ranged from 118.4 to 193.3×10^4 and 95.2 to 125.5×10^4 ml^{-1} or g^{-1} (water or sediment), for the lakes GDM and PNG, respectively (Table 1). Significant variations were observed among the culturable bacterial population (CFU) of each sam-

Table 2. Distribution and abundance of bacterial populations in surface, sub-surface and sediment samples obtained from two different lake ecosystems in Himalayas.

Sl. no.	Name of the lake and sample location/type	Bacterial cells 10^4/ml	× 10^3 CFUs/ml[a]	Morphotypes[b]	ARDRA genotypes[c]	Un-culturable bacteria (%)
1. Gurudongmar lake						
A	Surface Water/Ice	118.4 (1.26)*	19.5	12	9	98.4
B	Sub-Surface Water	138.7 (0.76)*	20.1	18	16	98.6
C	Sediment Water	193.4 (0.45)*	25.4	30	27	99.9
2. Pangong lake						
A	Surface Water/Ice	95.3 (0.62)*	6.2	21	11	99.4
B	Sub-Surface Water	153.4 (1.06)*	25.5	20	9	98.3
C	Sediment Water	125.5 (0.37)*	15.2	39	24	98.8

[a] Average CFUs on six different media with triplicate plates and data expressed as one location. [b] Based on cultural and morphological characters. [c] Based on 16S and 16–23S restriction fragment length polymorphism. * Mean of average of the bacterial abundance from three to four sites for each location and for each site three filters with four Microscopic fields on each filter were given along with standard deviation in parenthesis.

ple on six different media, however no new colonies were observed on any of media during extended incubation period (i.e. 20 to 35 d) (Table 1). In GDM lake samples, the bacterial population density was high in sediments (i.e. 25.41×10^3 CFU g^{-1}) than that of sub-surface and surface water; while in PNG lake, the sub-surface water samples have higher CFUs than the surface water/snow or bottom sediments (Table 2). In the bottom sediment of the lake GDM, total cell count was more than the culturable bacterial density. In case of the lake PNG, both bacterial cell count and the density of culturable bacteria were high in the sub-surface water. The abundance of un-culturable bacteria was derived by using the data obtained from total cell count and culturable bacterial density (CFU) of each sample. In both lakes, more than ≥98% of un-culturable bacteria were observed in all three samples, i.e. sediment, sub-surface and surface water (Table 2). The pure colonies obtained from each sample on six different media were isolated based on colony morphology and cultural characteristics. A total of 60 and 80 distinct bacterial colonies were obtained from the lakes GDM and PNG, respectively.

Physiological characteristics and growth pattern of bacterial isolates

The effect of temperature and pH, on growth of each bacteria isolated from two different lakes were studied and all the bacterial cultures were placed into psychrophilic (5–20 °C), psychrotrophic (5–30 °C), and psychrotolerant mesophilic (5–37 °C) groups. Out of 52 isolates obtained from the lake GDM, 12 were psychrophilic, 15 psychrotrophic and 25 showed psychrotolerant mesophilic growth. In the growth kinetic studies, the generation time of the isolates belonging to psychrophilic group was about 8–12 h after inoculation, whereas those of psychrotrophic and psychrotolerant mesophilic groups ranged from 10–18 h (Table 3). NBGD19 and 54 recorded lowest generation time of 8 h, while NBE33 recorded the highest generation time of 18 h. Further, all the isolates were able to grow at a pH range of 4.0 to 9.0. Similarly, out of 44 isolates obtained from the lake PNG, 9, 16 and 19 isolates were categorized in to psychrophilic, psychrotrophic and psychrotolerant mesophilic groups, respectively. In case of PNG lake isolates, the tolerance of pH for 18 isolates ranged between 6 and 11; 17 isolates tolerated pH of 6–10. All other strains such as NBA1, NBB1, NBB20, NBD5, NBH3, NBB17, NBN2, NBC4 and NBG2 showed a narrow range of pH tolerance between 6 and 9 (Tables 2–3).

Amplified ribosomal DNA restriction analysis (ARDRA)

Genetic diversity of all culturable bacteria was studied by the amplified ribosomal DNA restriction analysis (ARDRA). Based on the restriction banding pattern obtained by three different restriction enzymes, separate UPGMA dendrograms were constructed using the pooled data of three restriction enzymes. The isolates having more than 85% polymorphism were grouped in to separate clusters; as a result all sixty isolates of the lake GDM were grouped in to fifty two clusters. Likewise, the eighty isolates from the lake PNG were grouped in to forty four clusters (data not shown).

16S rRNA gene sequencing and phylogenetic analysis

All the representative isolates, one from each ARDRA cluster (i.e. 52 from GDM and 44 from PNG) were subjected to 16S rRNA gene sequencing. All the sequences

Table 3. Growth characteristics and enzyme production by various bacteria isolated from the sediment and water samples of Gurudongmar and Pangong lake.

Isolate no.	Growth	Generation time at optimum temp (h)	pH	Stability of enzyme activity (SE)											
				Protease			Amylase			Cellulase			Xylanase		
				5 °C	20 °C	30 °C	5 °C	20 °C	30 °C	5 °C	20 °C	30 °C	5 °C	20 °C	30 °C
1. Gurudongmar Lake															
NBGD1	Psychrotrophic	11	5–7	++	+++	++	–	–	–	–	–	–	–	–	–
NBGD43	Psychrotrophic	10	4–7	–	–	–	+	++	++	+	+++	+++	+	+++	++
NBGD49	Psychrotrophic	10	4–9	–	–	–	–	–	–	+	++	++	++	++	+++
NBGD46	Psychrotrophic	12	5–9	++	+++	+	++	++	+++	–	–	–	–	–	–
NBGD24	Psychrotrophic	11	5–9	++	+++	++	–	–	–	–	–	–	–	–	–
NBGD57	Psychrotrophic	14	5–9	–	–	–	++	+++	++	–	–	–	–	–	–
NBGD27	Psychrotrophic	13	5–7	+++	+++	++++	–	–	–	–	–	–	–	–	–
NBGD21	Psychrotrophic	12	4–7	++	+++	++	–	–	–	–	–	–	–	–	–
NBGD30	Psychrotrophic	13	4–9	–	–	–	+	–	–	++	+++	++	–	–	–
NBGD11	Psychrotrophic	14	4–7	+++	+++	++	++	++++	++	+	+++	++	–	–	–
NBGD4	Psychrophilic	12	5–8	+++	+++	+++	+++	++	–	++	+++	+	+++	+++	++
NBGD41	Psychrophilic	11	5–7	+++	+++	++	++++	+++	++	–	–	–	–	–	–
NBGD19	Psychrophilic	8	5–8	++++	+++	+	+++	++	–	–	–	–	–	–	–
NBGD12	Psychrophilic	11	4–9	++	++	++	–	–	–	–	–	–	–	–	–
NBGD22	Psychrophilic	12	4–9	+++	++	–	+++	++	+	+++	+++	++	+++	++	++
NBGD50	Psychrophilic	9	5–9	++	++	–	–	–	–	–	–	–	++	+	–
NBGD54	Psychrophilic	8	5–8	–	–	–	+++	++	–	–	–	–	–	–	–
NBGD25	Psychrophilic	10	4–9	–	–	–	+++	++	–	+++	+++	+++	–	–	–
NBGD15	Psychrotolerant mesophilic	16	5–9	++	+++	++	–	–	–	–	–	–	–	–	–
NBGD39	Psychrotolerant mesophilic	15	4–7	++	+++	++++	+	+	+++	–	–	–	–	–	–
NBGD58	Psychrotolerant mesophilic	10	4–9	–	–	–	–	–	–	+	++	++	–	–	–
NBGD59	Psychrotolerant mesophilic	12	4–9	+	+	–	+	+	++	–	–	–	–	–	–
NBGD34	Psychrotolerant mesophilic	15	5–9	++	+++	++++	–	–	–	–	–	–	–	–	–
NBGD9	Psychrotolerant mesophilic	12	4–9	+	+	–	++	++++	+++	–	–	–	–	–	–
NBGD20	Psychrotolerant mesophilic	14	4–7	–	–	–	+++	+++	+++	–	–	–	–	–	–
NBGD29	Psychrotolerant mesophilic	14	5–9	+	+++	++	–	–	+++	+	++	+++	–	–	–
NBGD31	Psychrotolerant mesophilic	13	4–9	–	–	–	–	–	–	–	–	–	+	++	++
NBGD56	Psychrotolerant mesophilic	14	5–9	++	+++	+++	++	+++	++	++	++	+++	++	++	+++
NBGD40	Psychrotolerant mesophilic	15	5–9	+++	+++	++++	–	–	–	–	–	–	++	++	+++
NBGD5	Psychrotolerant mesophilic	16	5–9	+++	+++	++++	–	–	–	–	–	–	–	–	–
2. Pangong Lake															
NBA1	Psychrotrophic	14	6–9	–	–	–	++	++	++	–	–	–	–	–	–
NBF1	Psychrotrophic	12	6–11	++	+	+	++	+++	++	–	–	–	++	++	+++
NBJ5	Psychrotrophic	14	6–10	–	–	–	+	++	+++	++	+++	++	–	–	–
NBA3	Psychrotrophic	16	6–10	+++	++++	+++	++	++	++	–	–	–	–	–	–
NBJ7	Psychrotrophic	14	6–11	++	++	+++	–	–	–	–	–	–	++	+++	++
NBE31	Psychrotrophic	16	6–10	–	–	–	+	++	+++	–	–	–	–	–	–
NBE33	Psychrotrophic	18	6–10	++	+++	+++	+++	+++	++	–	–	–	+++	++	++
NBB1	Psychrotrophic	14	6–9	–	–	–	–	–	–	++	+++	++	++	+++	++
NBC7	Psychrotrophic	12	6–11	–	–	–	–	–	–	++	++	++	+++	+++	++
NBN2	Psychrotrophic	14	6–9	++	++	+++	–	–	–	++	+++	++	–	–	–
NBN1	Psychrotrophic	14	6–11	–	–	–	++	+++	++	–	–	–	++	++	+++
NBD7	Psychrotrophic	14	6–10	++	+++	+++	++	++	+++	++	++	+++	++	++++	+++

NBC3	Psychrotrophic	14	6–10	++	++	+++	–	–	++	+++	+++	–	–	–
NBL3	Psychrophilic	9	6–10	++	+++	+	–	–	–	–	–	+++	+++	++
NBB20	Psychrophilic	10	6–9	–	–	–	+++	++	+++	+	+	++	++	++
NBB12	Psychrophilic	10	6–11	–	–	–	++	+++	–	–	–	–	–	–
NBC6	Psychrophilic	9	6–11	+++	++	++	–	–	–	–	–	–	–	–
NBJ4	Psychrotolerant mesophilic	14	6–10	++	++++	++++	+	++	+++	–	–	–	+++	++
NBB7	Psychrotolerant mesophilic	14	6–10	–	–	–	+++	++++	+++	–	–	–	–	–
NB01	Psychrotolerant mesophilic	14	6–11	++	+++	+++	+++	+++	++++	–	–	–	–	–
NBI3	Psychrotolerant mesophilic	12	6–11	+++	+++	+++	++	+++	+++	–	–	–	–	–
NBC2	Psychrotolerant mesophilic	14	6–10	–	–	–	++	++	+++	++	+++	+++	–	–
NBD5	Psychrotolerant mesophilic	12	6–9	–	–	–	+++	++++	+++	–	–	–	–	–
NBB6	Psychrotolerant mesophilic	12	6–11	++	+++	+++	–	–	–	++	++	+++	–	–
NBG2	Psychrotolerant mesophilic	16	6–9	++	++	+++	++	+++	+++	–	–	–	–	–
NBH3	Psychrotolerant mesophilic	17	6–9	–	–	–	++	++	++	–	–	–	++	+++

Stability of enzyme activity (SE) was represented as per the scale (45 to 55% – +), (56 to 75– ++), (76 to 85 – +++), (86 to 100 – ++++).

were deposited in the GenBank and accession numbers such as HQ003403–HQ003454 and HQ256517–HQ256560 were obtained for GDM and PNG lakes respectively. The bacterial isolates which were negative in the assays for extracellular enzyme activity (Table 3) were excluded from the phylogenetic analysis. Phylogenetic trees were constructed separately for the isolates obtained from both lakes and the reference sequences having ≥97% similarity were used as taxonomic representatives. Out of 30 isolates from the lake GDM, 19, 8 and 3 cultures were affiliated with three different clusters of Proteobacteria (α, β and γ), Firmicutes and Actinobacteria, respectively (Fig. 2a). In case of the lake PNG, all 26 cold-active enzyme producers were selected for phylogenetic analysis, which distinguished the isolates in to three major clusters such as Firmicutes (19), Proteobacteria (α, β and γ) (6) and Actinobacteria (1) (Fig. 2b). Among GDM lake isolates, Proteobacteria were dominant group, whereas Firmicutes formed a major cluster among the isolates of PNG lake. Actinobacteria formed a minor cluster with three isolates from GDM lake; while a single isolate was obtained from lake PNG.

Characterization of bacterial strains

Extracellular enzyme activity at different temperature and pH was determined for all the isolates. Our results indicated that out of 52 bacterial cultures from lake GDM, 30 were showed activity for one or more enzymes at different temperatures (5, 20 and 30 °C). Among all the isolates from lake GDM, four strains such as NBGD11, NBGD4, NBGD22 and NBGD56 exhibited activity of all the four enzymes tested (Table 3). In case of lake PNG, about 26 isolates showed various levels of enzyme activity at different temperatures. All these 26 isolates produced more than one enzyme with ≥50% enzyme stability. In general, majority of the PNG isolates showed highest enzyme activity at 20 °C. The culturable bacterial isolates of both lakes showed stable enzyme activity at a wide range of pH (Table 3). Further, most of the GDM isolates (NBGD1, NBGD4, NBGD11, NBGD15, NBGD20, NBGD22, NBGD24, NBGD34, NBGD 39, NBGD40, NBGD41, NBGD58, and NBGD59) showed ≥75% of stable enzyme activity, while only four of the PNG isolates (i.e NBA3, NBB6, NBC3 and NBG2) showed stable enzyme activity at three different temperatures (5, 20 and 30 °C) (Table 3).

Discussion

Survival of microorganisms in the severely cold environments requires optimization of certain basic cell

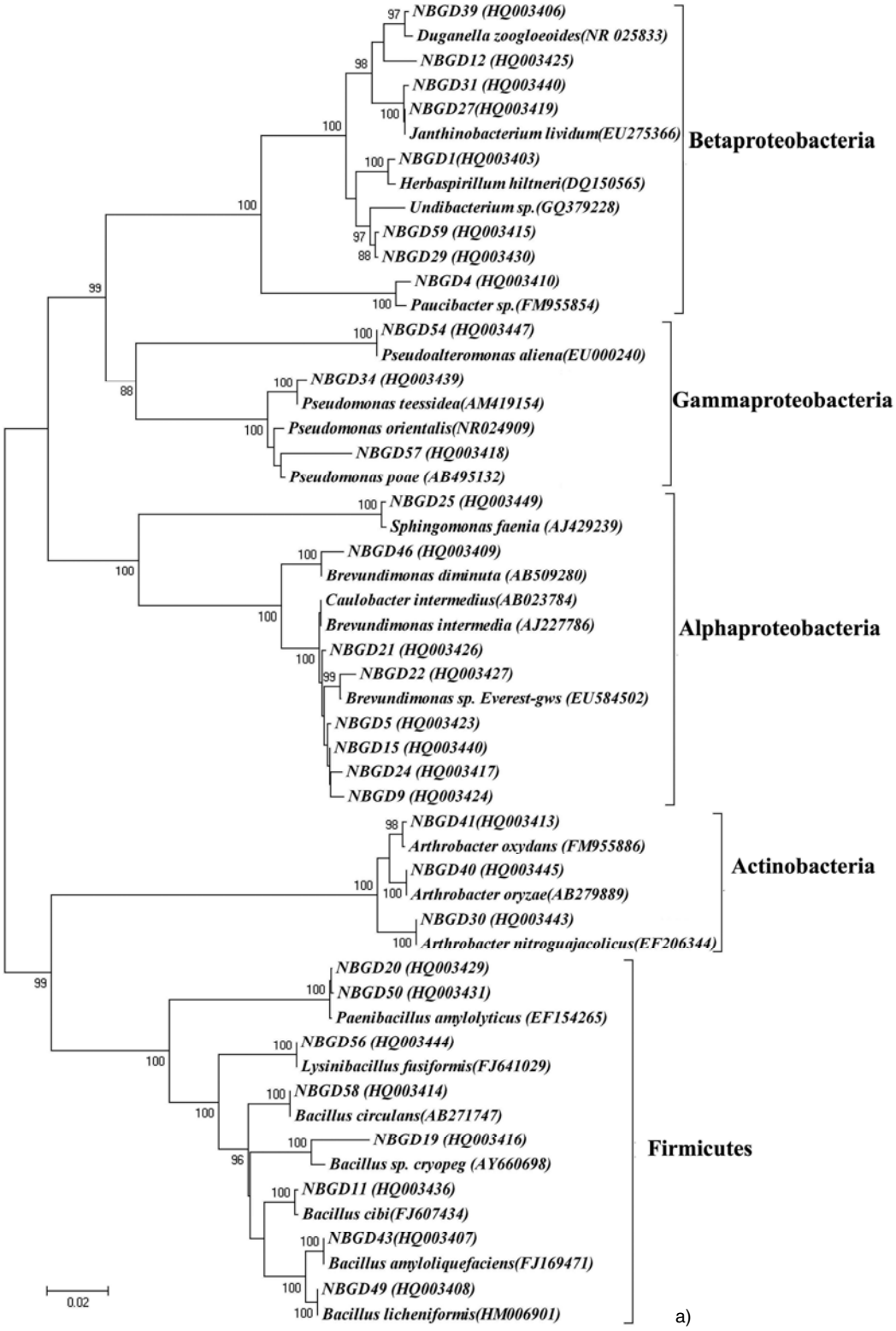

Figure 2. Phylogenetic analysis of the cold-active hydrolases producing bacteria obtained from GDM and PNG lakes. The dendrograms of the 16S rRNA gene sequences of the isolates representing a) the lake GMD and b) the lake PNG were constructed by neighbor-joining method. Accession numbers of each isolate was given in parenthesis, and reference sequences having ≥97% similarity were used as taxonomic representatives. The corresponding bacterial groups and sub-groups such as Firmicutes Proteobacteria (α, β and γ) and Actinobacteria were given at each cluster. The bootstrap values (1,000 replications) supporting ≥85% were indicated at the nodes.

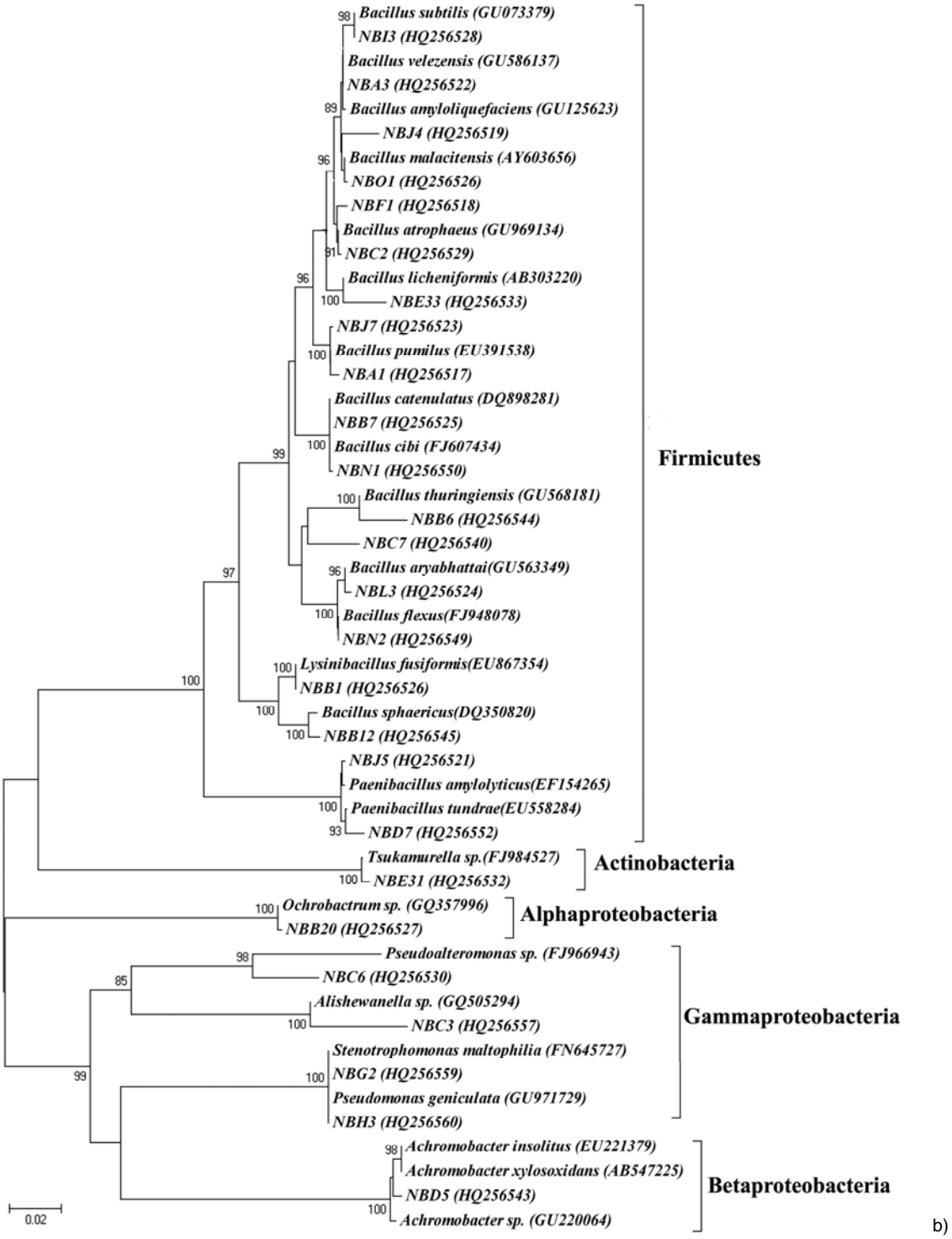

Figure 2. (Continued).

processes which include enzyme modulation at structural/biochemical level, nutrient transport and membrane function [28]. The diversity of culturable and non-culturable microorganisms living under cold environments is enormous, and these organisms can form an important gene pool for biotechnological applications. In the present study, we isolated several psychrophilic (5–20 °C), psychrotrophic (5–30 °C), and psychrotolerant mesophilic (5–37 °C) bacteria from subglacial freshwater and brackish water lakes of the Hi-

Table 4. List isolated bacterial cultures for which enzyme assays were negative obtained from Gurudongmar and Pangong lakes.

Sl. no. Type of bacteria	Isolate number and pH range for optimum growth
1. Gurudongmar Lake	
a. Psychrophilic	NBGD51(4–9), NBGD53(5–9), NBGD36(5–7), NBGD3(4–10)
b. Psychrotrophic	NBGD48(5–8), NBGD42(5–9), NBGD35(4–8), NBGD26(4–8), NBGD6(5–9)
c. Psychrotolerant mesophilic	NBGD18(5–9), NBGD13(4–8), NBGD28(5–9), NBGD16(5–8), NBGD32(5–7), NBGD14(5–10), NBGD44(5–10), NBGD23(6–8), NBGD33(5–9), NBGD45(5–8), NBGD47(5–10), NBGD60(6–9), NBGD17 (6–9)
2. Pangong Lake	
a. Psychrophilic	NBA4(6–11), NBA6(6–11), NBD8(6–11), NBB17(6–9), NBD2(6–11)
b. Psychrotrophic	NBC4(6–9), NBB19(6–10)
c. Psychrotolerant mesophilic	NBJ3(6–10), NBJ2(6–11), NBB13(6–11), NBD10(6–11), NBB11(6–10), NBB18(6–10), NBB14(6–10), NBP1(6–10), NBC5(6–11), NBE32(6–11), NBB3(6–10)

malayas which exhibited the activity of hydrolytic enzymes. Culturable bacterial diversity of these two lakes showed distinct morphology and colored colonies on different culture media. Interestingly, the density of culturable bacterial population was more at the subsurface than that of surface and sediment. These results corroborate with the earlier reports on the morphologically diverse microbial populations of the subglacial and surface soil samples from the Himalayas [14, 18, 23, 39]. Our results indicated that the sediment samples of both freshwater and brackish water lakes have highest bacterial count, than the surface and subsurface water samples. When assessed for distribution and abundance of un-culturable bacteria, the bottom sediments were found to have more number of culture-independent bacteria than the sub-surface water (Table 2). The snow-capped surface waters of PNG lake have unique culturable bacterial species such as *Tsukamurella* sp. *Alishewanella* sp. *Sphingomonas* sp. *Ochrobactrum* sp. and *Brevundimonas* sp. which did not appear either in sub-surface or bottom sediments. The distribution and occurrence of bacterial communities in different zones of the sub-glacial lake may be determined by several factors such as nutrient availability, photo-chemical properties of water, oxic-anoxic interface, and the presence of other life-forms [10, 23]. The spatial and temporal studies on the heterotrophic bacterial populations in extreme environments are often constrained due to the limitations imposed by the cultivation approaches (labor intensiveness and careful sampling required). On the other hand, culture independent approaches suffer from cost-intensiveness, which includes cloning, sequencing and oligonucleotide probing etc. [21].

The growth studies at different temperatures revealed that only 21 isolates were obligate psychrophiles. Majority of the culturable bacteria were either psychrotrophs or psychrotolerant mesophiles, suggesting their wider capabilities for physiological adaptation. Although different culture techniques (media/temperature) were employed in the present study, the total bacterial cell count using SYBR green staining method highlighted the fact that there are many yet-to-be cultured bacteria in these environments. Earlier reports also clearly suggest that most of the extreme environments harbor high density of un-culturable bacterial population [13, 34].

The cultural characteristics of bacterial isolates such as colony morphology, pigment production, temperature effect and different media provided interesting and basic information for screening of morphotypes, and the generation time of these isolates were in agreement with the earlier reports [14, 18, 39]. The DNA based studies such as ARDRA was also helpful in understanding the variations among the bacterial isolates, not only for primary discrimination among bacterial isolates but also in terms of genetic diversity [1, 36]. In the present study, the ARDRA facilitated to distinguish all the bacteria into 96 different genotypes at ≥85% polymorphism.

All the culturable bacteria from these two lakes were identified by comparing the phenotypic characteristics and sequence similarities. Based on the similarity of 16S rRNA gene sequences with the GenBank sequences at ≥97% [18, 30], these bacteria were placed into different genera. Two isolates such as NBGD4 (HQ003410) and NBGD39 (HQ003406) aligned 99% and 98% similarity with *Paucibacter* sp., and *Duganella zoogloeoides,* respectively. Interestingly, the activity of all four hydrolases at different temperature and pH isolate in NBGD4 makes it a promising strain for biotechnological interventions (Table 3). Although the 16S rRNA sequence of the isolate NBGD39 showed similarity (98%) with that of *Duganella zoogloeoides* strain IAM, however, their morphological and other colony characteristics were not comparable with the earlier report of Hiraishi *et al.* [15].

Our results are in agreement with the suggestions of Vandamme *et al.* [33] and Christensen *et al.* [7] that the morphological and biochemical similarities are as im-

portant as the 16S rRNA gene sequence homologies before assigning a taxonomic status to any bacteria. In contrast to the general perception, our results showed the presence of highly diverse bacterial populations, belonging to Proteobacteria (α, β, and γ), Firmicutes and Actinobacteria in these lakes. The presence of diverse subgroups within a single domain suggested that both lakes have high degree of inter-species diversity than inter-genus diversity. These results are in agreement with the report of Foght *et al.* [12] (Fig. 2a and b). All the members of Proteobacteria obtained from the sediments of brackish water lake in the present study were gram-negative. Similarly, Ravenschlag *et al.* [25] reported that the gram-negative proteobacteria are highly distributed in the sediments of brackish water and marine waters. When these isolates were screened for the production of different cold-active hydrolases such as protease, amylase, xylanase and cellulose, about 56 isolates exhibited the capacity to utilize different substrates at different temperatures (Table 3). The isolates of both lakes showed enzyme stability at wide range of pH variations. From this study, we obtained sixteen novel isolates, which were capable of producing hydrolases with greater stability at various temperature and pH (Table 3). The present work was an attempt to unravel the bacterial diversity in two distinctly different lakes of the Himalayas. The activity of hydrolases, produced by the bacterial cultures, especially their psychrophilic nature and ability to be stable under the wide range of pH (5–9) and temperatures can have potential uses in industrial processes, such as detergent formulations, sewage treatment, and leather processing. Future studies on optimization of enzyme production and purification under large scale production are in progress to exploit the native diversity for useful purposes.

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