Research Article

16S rRNA gene sequencing and MALDI-TOF mass spectrometry based comparative assessment and bioprospection of psychrotolerant bacteria isolated from high altitudes under mountain ecosystem

Anita Pandey[1] · Rahul Jain[1] · Avinash Sharma[2] · Kusum Dhakar[1] · Gaurav S. Kaira[1] · Praveen Rahi[2] · Ashish Dhyani[1] · Neha Pandey[1] · Priyanka Adhikari[1] · Yogesh S. Shouche[2]

Abstract

The Himalayan Mountains are placed among the globally recognized biodiversity hot spots. While the Indian Himalayan Region (IHR) has been subjected to extensive studies on plant and animal biodiversity, microbial diversity is now being studied for its bioprospection. The present paper deals with the evaluation of bacterial diversity in high-altitude soil samples from IHR following polyphasic approach including comparison between the MALDI-TOF mass spectrometry and 16S rRNA gene sequencing for species-level identification. Initially, a culture collection of large number of bacterial isolates was established in the laboratory. Performing morphological and biochemical screenings, sixty-one representative isolates were selected for mass spectrometry and gene sequencing. Both the methods emerged with bacterial identification showing maximum number of *Bacillus* followed by *Pseudomonas* species. The other frequently isolated strains belonged to the genera *Alcaligenes*, *Carnobacterium*, *Lysinibacillus*, *Microbacterium*, *Paenarthrobacter*, *Rhodococcus*, *Serratia* and *Stenotrophomonas*. Although the MALDI-TOF technique appeared to be advantageous as less time-consuming in comparison with 16S rRNA-based method, the discrepancies at species level indicated the limited database of MALDI Biotyper and species complexity in the genera. The remarkable characteristics of the bacterial isolates were their tolerance to wide range of pH and temperature. Their potential to produce industrially valuable enzymes indicated their importance in bioprospection. Accessioning of these bacterial isolates in microbial culture collections is a cautious effort for their availability to conduct advanced research on these cold-adapted bacteria in future.

Keywords Indian Himalaya · Bacterial diversity · Polyphasic approach · MALDI-TOF · 16S rRNA gene · Bioprospection

1 Introduction

Microorganisms can grow over a wide range of temperature. Accordingly, they have been divided into three broad categories—thermophiles, mesophiles, and psychrophiles. The last category is further subdivided into psychrophiles with optimal growth temperatures below 15 °C and an upper limit of 20 °C, and psychrotrophs (psychrotolerants) with the ability to grow at 0 °C or below but grow optimally at temperatures around 20–25 °C [27]. In the recent literature, the cold-adapted microorganisms have also been classified as stenopsychrophiles that grow in a narrow range of low temperature (T_{max} ~ 20 °C and T_{opt} 5–15 °C) and eurypsychrophiles that can grow at the broad range of temperature (psychrotrophs/psychrotolerants; T_{max} > 20 °C and T_{min} < 0 °C with T_{opt} > 20 °C) [35]. Both psychrophiles and psychrotrophs are important in global ecology as the majority of aquatic and terrestrial ecosystems of our planet are permanently or seasonally submitted to low temperatures [14]. These microorganisms, therefore, should be regarded as the most

✉ Anita Pandey, anita@gbpihed.nic.in; anitapandey333@gmail.com | [1]Centre for Environmental Assessment and Climate Change, G.B. Pant National Institute of Himalayan Environment and Sustainable Development, Kosi-Katarmal, Almora, Uttarakhand 263 643, India. [2]National Centre for Microbial Resource, National Centre for Cell Science, Pune, Maharashtra 411021, India.

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successful colonizers of the planet Earth [36]. Psychrotolerant bacteria, along with their ability to adapt to the temperatures close to or below freezing, require specific strategies for the continuance of their metabolic activities including maintenance of the membrane fluidity and the protein synthesis at low temperature. Furthermore, the psychrotolerant bacteria and their cold-active enzymes are being focused for their importance in biotechnological applications and physiological adaptations [1, 2, 26, 41].

Accurate species-level identification is critical in view of understanding the biodiversity of a particular ecological niche. Species-level identification of cultured bacterial isolates usually relies on the polyphasic approach consisting of phenotypic and genotypic characters. Most of the genotypic identification methods are mainly based on the polymorphism of the 16S rRNA gene sequences. Sequencing of the 16S rRNA gene has been accepted as the reference method for species-level identification of various groups of bacteria, for which the availability of an extensive and comprehensive quality-controlled database is a prerequisite [13]. In further evolution on the subject line, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS) emerged as a promising technology for species-level identification. It may be a useful tool if a detailed database is available. In particular, this technique is being considered useful for clinical isolates where databases are quite satisfying. However, for environmental bacteria (particularly for extreme environments) lot of database upgrading is needed [45]. The technology involves analysis of protein composition of a bacterial cell by measuring the exact size of peptides and small proteins, which is assumed to be characteristic of each bacterial species. This technology has the merit to identify the strains within few minutes, starting the analysis with the whole cells, cell lysates, or crude bacterial extracts [10, 15, 34].

Himalaya is one of the coldest regions on Earth and represents one of the 34 globally recognized biodiversity hot spots. Microbial diversity in high altitudes of Indian Himalayan Region (IHR) is increasingly receiving attention mainly for their bioprospection, conservation, and the associated survival strategies [7, 49]. The present study aims to assess the culturable bacterial diversity from high altitudes of IHR on the basis of their phenotypic characters, the 16S rRNA gene sequencing, and the MALDI-TOF MS. The importance of this bacterial diversity in bioprospection is also emphasized.

2 Materials and methods

2.1 Study sites

The study area covered a wide range of altitudes, including the temperate, sub-alpine, and alpine zones of Pindari Glacier region (33°5′–30°10′N to 79°48′–79°52′E) and cold desert (30°46′24.8″N; 79°29′33.4″), between 1800 and 3610 m above mean sea level in IHR. The area is identified with heavy rainfalls and snowfalls, maintaining low temperature up to sub-zero levels. The soil pH at the sampling sites ranged between 4.5 and 6.5. The mean monthly temperature is reported to be between 5.5 °C (January) and 21.5 °C (August) [30].

2.2 Isolation and characterization of bacteria

Bacteria, based on the distinct colony morphology, were initially isolated from the soil dilution agar plates following incubation at different temperatures (9, 14, 21 and 28 °C) on tryptone yeast extract (TY) and nutrient agar (NA) media using standard serial dilution method. A culture collection of pure bacterial isolates was established and maintained at 4 °C in a refrigerator and as glycerol stocks at − 20 °C, till further use. The large number of pure bacterial isolates (more than 500) obtained from the agar plates were de-replicated using multiple screening on the basis of growth characteristics, colony and cell morphology, and a number of biochemical characters. Finally, 61 bacterial isolates were selected for the present study.

Colony morphology of the bacterial isolates was recorded from the agar plates following incubation at 21 °C for 2–5 days. Cell morphology was studied using a Nikon Eclipse 50i microscope in oil emersion. The growth requirements of the bacterial isolates were performed subject to different temperatures (5–60 °C with an interval of 5°) and pH levels, i.e., 1.0–14.0 (with an interval of 1.0 pH), for their minimum, optimum, and maximum temperature and pH range.

2.3 Enzyme assays

Bioassays for three enzymes (amylase, lipase, and protease) were performed using starch (0.2%), tributyrin (0.1%), and skim milk (3.0%) in the medium, separately. The plates were incubated at 25 °C, and observations were recorded after 72 h.

2.4 MALDI-TOF MS

Loopful taken from the fresh cultures of bacteria was harvested in 1.5-ml vials containing 300 µl of deionized water and mixed thoroughly by vortexing for a minute. Absolute ethanol (900 µl) was added and mixed thoroughly. The suspended cells were centrifuged at 10,000 rpm for 2 min. Following centrifugation, supernatant was carefully discarded without disturbing the pellet. The pellet was air-dried to get rid of the residual ethanol. Dried pellet was then re-suspended in 70% formic acid (20 µl) by vigorous

mixing followed by the addition of acetonitrile (20 µl) and mixed thoroughly. This mixture was again centrifuged at maximum speed (13,000 rpm) so that all the material is collected neatly in a pellet. 1 µl of extract (clear supernatant) was placed on a MALDI standard target, air-dried, and overlaid with the 1 µl of alpha-cyano-4-hydroxycinnamic acid (HCCA) matrix; the target plate was allowed to dry at room temperature; and then it was loaded in the instrument AUTOFLEX TOF/TOF (Bruker Daltonik GmbH, Germany) [19].

Mass spectra were acquired in a linear positive ion extraction mode at a laser frequency of 200 Hz, within a mass range from 2000 to 20,000 Da. The ion source 1 voltage was 19.5 kV, ion source 2 voltage was maintained at 18.2 kV, lens voltage was 7 kV, and the extraction delay time was 240 ns. The spectra were calibrated externally using the standard calibrant mixture (*Escherichia coli* extracts including the additional proteins RNase A and myoglobin, Bruker Daltonics). The MALDI Biotyper software 3.0 (Bruker Daltonik) was used to identify the bacterial isolates and to visualize the mass spectra. Species-level identity was considered for the isolates with biotyper score value > 2.0, while the score value ranging from 1.7 to 1.99 was considered for genus-level identity.

2.5 16S rRNA gene sequencing

The DNA extraction was performed using single colony enriched in TY agar. The 16S rDNA was amplified with bacterial specific primers 27F (5′-GAGTTTGATCMTGGCTCAG-3′) and 1492R (5′-TACGGYTACCTTGTTACGACTT-3′). The PCR cycling conditions included initial denaturation at 94 °C (3 min), followed by 35 cycles of denaturation at 94 °C (1 min), annealing at 55 °C (1 min) and extension at 72 °C (1 min), followed by final extension for a period of 10 min at 72 °C. The amplified PCR product was purified with a mixture of 20% polyethylene glycol (PEG) and 2.5 M NaCl. Sequencing was conducted using 96-capillary 3730xl DNA Analyzer (Hitachi). The identity of the isolates was determined through 16S rRNA gene sequence analysis using EzTaxon database search. The sequences were aligned using MUSCLE algorithm [9]. Phylogenetic and molecular evolutionary analyses were conducted using MEGA, version 7.0 [20]. The phylogenetic tree was constructed by the neighbour joining method [37], using the distance matrix from the alignment.

2.6 Accessioning of bacterial cultures and the nucleotide sequences

The bacterial cultures and their nucleotide sequences have been accessioned at National Centre for Microbial Resource, National Centre for Cell Science, Pune, India [39] and at https://www.ncbi.nlm.nih.gov/, respectively; the accession numbers are given in Table 2.

3 Results and discussion

3.1 Characteristics of psychrotolerant bacteria

Almost all the bacterial isolates developed colonies on TY agar within 2–7 days of incubation at 25 °C. Compilation of the results on colony morphology, Gram staining, temperature and pH requirement, and enzyme activity with respect to amylase, lipase, and protease of 61 bacterial isolates is presented in Table 1. The bacterial isolates represented 38 Gram-positive and 23 Gram-negative species. The remarkable features of the bacteria were observed as their ability to grow at wide range of temperature and pH. All the bacterial isolates, except 2 (GBPI_Hb9 and GBPI_Hb34), could grow at 5 °C. The upper limit tolerance for temperature was recorded at 35, 45, and 55 °C in case of 24, 25, and 12 bacterial isolates, respectively. Two of the isolates could grow up to 15 °C and one up to 20 °C only. Similarly, the bacterial isolates could grow at wide range of pH (minimum 2–4 to maximum 12–14). The isolates grew luxuriantly between 5 and 9 pH.

Microorganisms from extreme environments are increasingly receiving recognition mainly due to their remarkable phenotypic characteristics, and one of such characteristics in the present study, is their ability to tolerate wide range of pH from extreme acidic to extreme alkaline. This phenomenon has recently been reviewed with reference to the bacteria and fungi that were isolated from extreme high-temperature (hot springs) as well as extreme low-temperature (cold deserts, glaciers, and temperate forests) environments. The majority of the case studies analysed in this review were from Indian Himalaya conducted over more than 20 years. Tolerance to wide pH range was considered as a hidden character in view of their isolation sites with neutral or slightly acidic/alkaline pH. It was considered that the genome of these organisms is a probable reservoir of the hidden variations that result in the expression of the associated specific genes in response to environmental stress [7]. On the similar lines, tolerance to wide range of temperature is a prerequisite for the microorganisms that survive in an environment with frequent fluctuations in temperature. These features can be attributed to the strategies evolved in these microorganisms to adapt to the extreme environments. Tolerance of microorganisms to multiple environmental stresses is referred to as polyextremophile that is now emerging as an interesting topic in extreme environments research [38].

Besides the 61 bacterial isolates under consideration, a number of bacterial cultures were observed in VBNC

Table 1 Characteristics of the bacterial isolates

S. No.	Isolate code	Colony morphology	Microscopy	Temperature (°C) and pH limit		Enzyme activity		
				Temp	pH	Amy	Lip	Pro
1	GBPI_Hb0	Watery, slimy, raised, medium	Gram –ve rods	5–45 (25)	3–14 (5–7)	+	+	+
2	GBPI_Hb1	Round, smooth, flat, large	Gram –ve rods	5–55 (25)	4–14 (5–7)	+	+	+
3	GBPI_Hb5	Round, smooth, raised, small	Gram –ve rods	5–45 (25)	2–14 (5–7)	–	+	+
4	GBPI_Hb6	Round, smooth, small	Gram –ve rods	5–45 (25)	3–14 (7–9)	–	+	+
5	GBPI_Hb7	Spread, undulated, flat, medium	Gram +ve rods	5–35 (25)	4–14 (5–7)	+	+	+
6	GBPI_Hb9	Spread, irregular, flat, medium	Gram +ve rods	9–55 (35)	4–12 (5–7)	+	+	+
7	GBPI_Hb12	Round, undulated, flat, medium	Gram +ve rods	5–55 (35)	3–14 (5–7)	+	+	–
8	GBPI_Hb14	Round, smooth, flat, small	Gram +ve rods	5–45 (25)	2–14 (5–9)	–	+	–
9	GBPI_Hb15	Concentric, convex, smooth, large	Gram +ve rods	5–55 (35)	2–14 5–9)	+	+	+
10	GBPI_Hb17	Wrinkled, smooth, raised, large	Gram +ve rods	5–55 (25)	2–14 (5–9)	+	+	–
11	GBPI_Hb18	Round, smooth, small drops	Gram –ve rods	5–45 (25)	2–15 (5–9)	–	+	–
12	GBPI_Hb19	Round, smooth, medium drops	Gram –ve rods	5–45 (25)	2–14 (5–9)	–	+	–
13	GBPI_Hb24	Round, smooth, convex, large	Gram +ve rods	5–55 (35)	2–12 (7–9)	+	+	+
14	GBPI_Hb34	Round, smooth, small drops	Gram –ve rods	9–35 (25)	2–14 (7–9)	–	+	–
15	GBPI_Hb37	Rhizoidal, irregular, flat, large	Gram +ve rods	5–35 (25)	3–14 (7–9)	+	+	–
16	GBPI_Hb41	Rhizoidal, irregular, flat, large	Gram +ve rods	5–35 (25)	3–14 (5–7)	–	+	+
17	GBPI_Hb52	Round, smooth, convex, small	Gram +ve rods	5–35 (25)	2–14 (7–9)	–	+	–
18	GBPI_Hb55	Rhizoidal, flat, irregular, large	Gram +ve rods	5–35 (25)	4–14 (7–9)	+	+	+
19	GBPI_Hb61	Round, smooth, flat, small	Gram –ve rods	5–35 (25)	4–14 (5–7)	–	+	+
20	GBPI_Hb120	Round, smooth, medium drops	Gram –ve rods	5–35 (25)	2–14 (5–9)	–	+	–
21	GBPI_Hb149	Wrinkled, raised, undulated, medium	Gram –ve rods	5–35 (25)	2–14 (5–7)	–	+	–
22	GBPI_Hb171	Round, smooth, small drops	Gram –ve rods	5–45 (25)	3–14 (7–9)	–	+	+
23	GBPI_Hb227	Round, smooth, small drops	Gram –ve rods	5–35 (25)	2–14 (5–7)	–	+	–
24	GBPI_Hb232	Rhizoidal, flat, irregular, large	Gram +ve rods	5–35 (25)	4–14 (7–9)	+	+	+
25	GBPI_Hb240	Spread, irregular, flat, small	Gram +ve rods	5–45 (25)	2–14 (5–9)	+	+	+
26	GBPI_Hb243	Round, smooth, flat, large	Gram +ve rods	5–45 (35)	3–14 (5–7)	+	+	+

Table 1 (continued)

S. No.	Isolate code	Colony morphology	Microscopy	Temperature (°C) and pH limit		Enzyme activity		
				Temp	pH	Amy	Lip	Pro
27	GBPI_Hb249	Round, undulated, raised, large	Gram +ve rods	5–55 (35)	3–12 (5–7)	+	+	+
28	GBPI_Hb256	Round, smooth, raised, medium	Gram +ve rods	5–45 (35)	2–14 (5–7)	+	+	+
29	GBPI_Hb308	Round, smooth, raised, large	Gram +ve rods	5–55 (35)	3–14 (5–7))	+	+	+
30	GBPI_Hb309	Round, smooth, small	Gram +ve rods	5–45 (15)	3–14 (5–9)	–	+	–
31	GBPI_Hb310	Round, smooth, umbonate, medium	Gram +ve rods	5–55 (35)	2–14 (5–7)	–	+	+
32	GBPI_Hb312	Rhizoidal, irregular, flat, large	Gram +ve rods	5–35 (25)	2–14 (7–9)	+	+	+
33	GBPI_Hb314	Round, smooth, convex, medium	Gram –ve rods	5–45 (35)	3–14 (7–9)	+	+	+
34	GBPI_Hb330	Spread, smooth, flat, large	Gram +ve rods	5–45 (35)	2–14 (5–9)	+	+	+
35	GBPI_Hb501	Round, smooth, small	Gram +ve rods	5–45 (15)	3–14 (5–9)	–	+	–
36	GBPI_CDB5	Round, smooth, flat, medium	Gram –ve rods	5–35 (25)	3–14 (5–7)	–	+	–
37	GBPI_CDB6	Round, smooth, raised, medium	Gram +ve rods	5–45 (25)	4–14 (7–9)	–	–	–
38	GBPI_CDB51	Round, smooth, small drops	Gram +ve rods	5–55 (25)	4–12 (7–9)	+	+	+
39	GBPI_CDB57	Round, undulated, raised, medium	Gram +ve rods	5–35 (25)	4–14 (7–9)	+	+	+
40	GBPI_CDB58	Round, smooth, raised, medium	Gram +ve rods	5–45 (25)	3–14 (5–7)	+	+	+
41	GBPI_CDB61	Round, smooth, medium drops	Gram +ve rods	5–45 (35)	3–14 (7–9)	+	+	+
42	GBPI_CDB65	Round, smooth, raised, medium	Gram +ve rods	5–35 (25)	3–14 (7–9)	+	+	+
43	GBPI_CDB76	Concentric, flat, smooth, large	Gram +ve rods	5–55 (35)	3–14 (5–7)	+	+	+
44	GBPI_CDB77	Round, smooth, drop, medium	Gram –ve rods	5–35 (25)	4–14 (7–9)	–	+	+
45	GBPI_CDB84	Round, smooth, medium drops	Gram –ve rods	5–35 (25)	2–14 (7–9)	–	+	+
46	GBPI_CDB86	Round, smooth, medium drops	Gram +ve rods	5–35 (25)	4–14 (7–9)	+	+	–
47	GBPI_CDB87	Round, smooth, small	Gram –ve rods	5–45 (25)	2–14 (5–7)	–	+	+
48	GBPI_CDB94	Round, smooth, medium drops	Gram –ve rods	5–35 (25)	3–14 (7–9)	–	+	–
49	GBPI_CDB96	Round, smooth, medium drops	Gram +ve rods	5–35 (25)	3–14 (5–7)	+	–	–
50	GBPI_CDB140	Round, smooth, raised, medium	Gram +ve rods	5–55 (35)	3–14 (5–9)	+	+	+
51	GBPI_CDB141	Wrinkled, raised undulated, medium	Gram +ve rods	5–35 (25)	3–12 (5–7)	+	+	+
52	GBPI_CDB142	Concentric, undulated, raised	Gram +ve rods	5–45 (25)	2–14 (5–9)	+	+	–

Table 1 (continued)

S. No.	Isolate code	Colony morphology	Microscopy	Temperature (°C) and pH limit		Enzyme activity		
				Temp	pH	Amy	Lip	Pro
53	GBPI_CDB143	Round, smooth, small drops	Gram –ve rods	5–35 (25)	2–14 (5–9)	–	+	–
54	GBPI_CDB144	Round, smooth, raised, medium	Gram +ve rods	5–45 (25)	4–12 (5–7)	–	+	–
55	GBPI_CDB145	Round, smooth, raised, medium	Gram +ve rods	5–45 (35)	2–14 (5–9)	–	+	–
56	GBPI_CDB146	Concentric, flat, undulated, large	Gram +ve rods	5–35 (25)	3–14 (7–9)	–	+	–
57	GBPI_CDB147	Round, smooth, medium drops	Gram –ve rods	5–45 (25)	2–14 (5–9)	–	–	–
58	GBPI_CDB149	Round, smooth, raised, small	Gram –ve rods	5–35 (20)	2–14 (5–8)	–	+	+
59	GBPI_506	Round, smooth, raised, medium	Gram –ve rods	5–45 (25)	2–14 (5–8)	–	+	–
60	GBPI_507	Round, smooth, raised, medium	Gram –ve rods	5–45 (25)	2–14 (5–8)	–	+	+
61	GBPI_508	Round, smooth, convex, medium	Gram –ve rods	5–45 (25)	2–14 (5–8)	–	+	+

Values in parentheses show the optimum temperature and pH requirement of the bacterial isolates; *Amy.* amylase; *Lip.* lipase; *Pro.* protease

(viable but not culturable) state. VBNC state has been attributed to the survival strategies possessed by the microorganisms against environmental stress such as low or high temperature [4]. Characteristics and resuscitation mechanisms of these microorganisms with respect to their survival under extreme conditions are now receiving attention in applied research [51].

3.2 Comparative assessment of psychrotolerant bacteria using 16S rRNA gene sequencing and MALDI-TOF-based biotyping

Quick and reliable methods are required to identify the unexplored diversity of any ecological niche. In this study, matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI-TOF MS)-based identification was included and the results were compared with 16S rRNA-based method of identification (Table 2). On the basis of 16S rRNA analysis, the bacteria represented 11 genera and 31 species. Among these *Bacillus* represented 13; *Pseudomonas* 7; *Serratia*, *Rhodococcus* and *Lysinibacillus* 2 each; and *Stenotrophomonas*, *Alcaligenes*, *Carnobacterium*, *Microbacterium* and *Paenarthrobacter* 1 each (Fig. 1). The phylogenetic tree of the bacterial isolates along with their closely related species is presented in Fig. 2. MALDI-TOF MS-based biotyping showed similar identification of 3 (4.92%) bacteria at species level and 36 (59.01%) bacteria at genus level with respect to 16S rRNA gene sequence analysis (Table 3). The discrepancies in identification were 54.10% and 13.11% at species and genus levels, respectively. Twelve (19.67%) bacterial isolates could not be identified due to non-reliability in identification (log score < 1.7). Although the technique was less time-consuming in comparison with 16S rRNA-based method, the discrepancies at species level indicated limited reference database (main spectra, MSPs) of MALDI Biotyper for identification of soil microorganisms using MALDI-TOF MS technique.

The recent advances of new molecular technologies in genomics and proteomics offer alternatives to conventional microbiological procedures with respect to the identification of microorganisms. Advantages of polyphasic taxonomic approach that includes molecular identification techniques have been recognized and reviewed [5]. Koubek and co-workers [19] compared the three methods of taxonomic identification including biochemical methods, MALDI-TOF MS, and sequencing of 16S rRNA genes. Strejcek et al. [44] also compared whole-cell MALDI-TOF MS with 16S rRNA gene analysis for identification of recurrent bacterial isolates. The authors confirmed the potential of MALDI-TOF MS as a rapid screening method in environmental microbiology for determining similarities between bacterial isolates at genus level with various limitations such as low number of peaks, availability of reference mass spectra of only well-studied and described strains in database, etc. Therefore, agreement between MALDI-TOF MS-based biotyping and 16S rRNA gene sequencing was observed to be limited. While the emerging MALDI

Table 2 Comparison of bacterial identification using 16S rRNA gene sequencing and MALDI-TOF MS-based biotyping

S No.	Isolate (culture accession)[a]	16S rRNA identification (% similarity) (nucleotide accession)[b]	Identification by MALDI (log score)
1	GBPI_Hb0 (MCC 3300)	*Pseudomonas granadensis* (99.79) (HG764746)	*Pseudomonas koreensis* (2.2)
2	GBPI_Hb1	*Serratia quinivorans* (99.87) (AJ233430)	NRI
3	GBPI_Hb5 (MCC 3295)	*Pseudomonas paralactis* (99.79) (AJ936933)	*Pseudomonas fluorescens* (2.1)
4	GBPI_Hb6	*Stenotrophomonas maltophilia* (100)	*Stenotrophomonas maltophilia* (2.2)
5	GBPI_Hb7	*Bacillus wiedmannii* (100) (AE016877)	*Bacillus thuringiensis* (1.8)[c]
6	GBPI_Hb9 (MCC 3123)	*Bacillus velezensis* (99.93) (JTKJ01000077)	NRI
7	GBPI_Hb12 (MCC 3148)	*Bacillus tequilensis* (99.93) (AMXN01000021)	*Bacillus subtilis* (2.1)
8	GBPI_Hb14 (MCC 3296)	*Pseudomonas azotoformans* (99.79) (AF074384)	*Pseudomonas cedrina* (2.0)
9	GBPI_Hb15	*Bacillus mobilis* (100) (ACNF01000156)	*Bacillus cereus* (2.2)
10	GBPI_Hb17 (MCC3124)	*Bacillus megaterium* (99.93) (JJMH01000057)	NRI
11	GBPI_Hb18	*Rhodococcus qingshengii* (100) (X79289)	*Rhodococcus erythropolis* (2.5)
12	GBPI_Hb19	*Serratia marcescens* subsp. *sakuensis* (100) (JMPQ01000005)	ND
13	GBPI_Hb24	*Bacillus aryabhattai* (100) (EF114313)	*Bacillus megaterium* (1.8)
14	GBPI_Hb34 (MCC3184)	*Alcaligenes pakistanensis* (99.48) (AJ242986)	*Alcaligenes faecalis* (1.8)
15	GBPI_Hb37	*Bacillus weihenstephanensis* (99.56) (AB049195)	*Bacillus mycoides* (2.1)
16	GBPI_Hb41	*Bacillus weihenstephanensis* (99.67) (ACMU01000002)	*Pseudomonas extremorientalis* (1.7)
17	GBPI_Hb52	*Bacillus wiedmannii* (100) (CP006863)	*Lactobacillus* sp (2.4)
18	GBPI_Hb55	*Bacillus weihenstephanensis* (99.90) (BAUY01000093)	*Bacillus mycoides* (2.0)
19	GBPI_Hb61 (MCC 3297)	*Pseudomonas proteolytica* (99.30) (AJ537603)	*Pseudomonas marginalis* (2.0)
20	GBPI_Hb120 (MCC3340)	*Rhodococcus globerulus* (100) (X80619)	*Rhodococcus erythropolis* (1.9)
21	GBPI_Hb149 (MCC 3293)	*Serratia marcescens* subsp. *sakuensis* (99.93) (JMPQ01000005)	NRI
22	GBPI_Hb171 (MCC 3143)	*Stenotrophomonas maltophilia* (100) (JALV01000036)	*Stenotrophomonas maltophilia* (2.3)
23	GBPI_Hb227	*Rhodococcus qingshengii* (100) (DQ185597)	*Rhodococcus erythropolis* (2.4)
24	GBPI_Hb232	*Bacillus weihenstephanensis* (100) (AB190217)	*Bacillus mycoides* (2.1)
25	GBPI_Hb240 (MCC3317)	*Bacillus mobilis* (99.69) (ACNF01000156)	*Bacillus thuringiensis* (1.9)
26	GBPI_Hb243	*Bacillus wiedmannii* (99.63) (AE016877)	*Bacillus cereus* (1.8)
27	GBPI_Hb249 (MCC3125)	*Bacillus velezensis* (99.79) (CP000560)	*Bacillus cereus* (2.1)
28	GBPI_Hb256	*Bacillus cereus* (100) (AE016877)	ND
29	GBPI_Hb308	*Bacillus velezensis* (99.37) (JTKJ01000077)	NRI
30	GBPI_Hb309	*Carnobacterium maltaromaticum* (99.92) (ND)	*Lactobacillus* sp (2.5)
31	GBPI_Hb310 (MCC3140)	*Bacillus safensis* (100) (ASJD01000027)	NRI
32	GBPI_Hb312 (MCC 3146)	*Bacillus weihenstephanensis* (100) (ACMU01000002)	NRI
33	GBPI_Hb314	*Serratia marcescens* subsp. *sakuensis* (99.93) (JPUX01000001)	*Serratia marcescens* (1.8)
34	GBPI_Hb330 (MCC 3147)	*Bacillus wiedmannii* (99.93) (AE016877)	NRI
35	GBPI_Hb501 (MCC3181)	*Carnobacterium maltaromaticum* (99.65) (JQMX01000001)	*Lactobacillus* sp (1.8)
36	GBPI_CDB5	*Microbacterium maritypicum* (99.76) (Y17227)	*Microbacterium luteolum* (1.9)
37	GBPI_CDB6	*Bacillus paramycoides* (99.88) (AB190217)	NRI
38	GBPI_CDB51 (MCC 3149)	*Bacillus atrophaeus* (99.93) (AB021181)	*Bacillus atrophaeus* (2.0)
39	GBPI_CDB57	*Bacillus atrophaeus* (99.86) (AB021181)	ND
40	GBPI_CDB58	*Brevibacterium frigoritolerans* (99.91) (AM747813)	*Bacillus atrophaeus* (1.8)
41	GBPI_CDB61	*Bacillus atrophaeus* (99.64) (AB021181)	*Bacillus atrophaeus* (1.9)
42	GBPI_CDB65	*Bacillus velezensis* (99.79) (JTKJ01000077)	NRI
43	GBPI_CDB76	*Bacillus siamensis* (99.41) (JTKJ01000077)	*Bacillus subtilis* (1.7)
44	GBPI_CDB77	*Rhodococcus qingshengii* (99.33) (AP008957)	ND
45	GBPI_CDB84 (MCC3341)	*Pseudomonas paralactis* (99.57) (AF074384)	*Pseudomonas tolaasii* (2.0)
46	GBPI_CDB86	*Paenarthrobacter nitroguajacolicus* (99.92) (AJ512504)	*Arthrobacter aurescens* (2.0)
47	GBPI_CDB87	*Pseudomonas azotoformans* (99.66) (ND)	*Pseudomonas libanensis* (2.1)
48	GBPI_CDB94	*Serratia marcescens* subsp. *sakuensis* (100) (JPUX01000001)	*Arthrobacter aurescens* (2.0)
49	GBPI_CDB96	*Paenarthrobacter nitroguajacolicus* (99.91) (AJ512504)	*Arthrobacter nicotinovorans* (1.9)
50	GBPI_CDB140	*Bacillus atrophaeus* (99.88) (AB021181)	*Bacillus atrophaeus* (2.0)

Table 2 (continued)

S No.	Isolate (culture accession)[a]	16S rRNA identification (% similarity) (nucleotide accession)[b]	Identification by MALDI (log score)
51	GBPI_CDB141 (MCC 3150)	*Lysinibacillus xylanilyticus* (99.66) (FJ477040)	NRI
52	GBPI_CDB142 (MCC 3292)	*Bacillus simplex* (99.92) (ACNF01000156)	ND
53	GBPI_CDB143 (MCC 3298)	*Pseudomonas paralactis* (99.93) (D84009)	*Pseudomonas marginalis* (2.0)
54	GBPI_CDB144	*Bacillus simplex* (99.92) (AB363738)	*Bacillus muralis* (1.8)
55	GBPI_CDB145 (MCC 3166)	*Lysinibacillus fusiformis* (99.71) (AB271743)	*Lysinibacillus fusiformis* (1.8)
56	GBPI_CDB146	*Bacillus weihenstephanensis* (99.88) (ACMU01000002)	*Bacillus mycoides* (2.0)
57	GBPI_CDB147	*Serratia marcescens* subsp. *sakuensis* (100) (JPUX01000001)	*Arthrobacter histidinolovorans* (1.9)
58	GBPI_CDB149 (MCC3294)	*Pseudomonas marginalis* (99.77) (ND)	NRI
59	GBPI_506 (MCC2694)	*Pseudomonas azotoformans* (99.86) (D84009)	*Pseudomonas extremorientalis* (1.8)
60	GBPI_507 (MCC2693)	*Pseudomonas chlororaphis* subsp. *aurantiaca* (100) (DQ682655)	*Pseudomonas chlororaphis* (2.1)
61	GBPI_508 (MCC2692)	*Pseudomonas palleroniana* (99.72) (AY091527)	*Pseudomonas veronii* (1.8)

NRI no reliable identification (log score < 1.7); *ND* not done

[a]Culture accession of bacteria (in parentheses) deposited in National Centre for Microbial Resource, National Centre for Cell Science, Pune, India

[b]Nucleotide accession of bacteria deposited in NCBI (second parentheses)

[c]Biotyper score values from 1.7 to 1.99 indicate genus-level identification

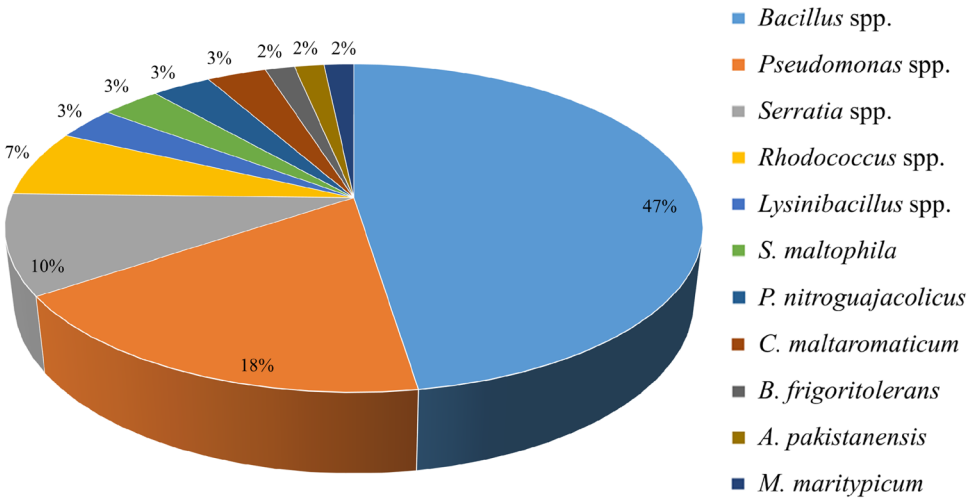

Fig. 1 Distribution of 61 bacterial isolates among 11 different bacterial genera

biotyping is being popular in different fields of microbiology that has partly replaced conventional biochemical identification methods [12], variability of this methodology for the identification of microbes has been attributed to factors such as sample processing methods, matrix solutions, spectral quality, and data analysis methods [6, 21]. The methodology is becoming more popular in clinical set-ups for speedy and low-cost microbial identification [28, 34] and also in the analysis of bioactive compounds [46].

3.3 Bioprospection of psychrotolerant bacteria

Diversity of psychrotolerants in alpine cold habitats has already become a topic of interest in research with respect to their adaptation strategies and bioprospection [11, 23, 40, 50]. The present study showed predominance of the genus *Bacillus* which can be attributed to their spore-forming characteristic that allows them to survive under varied environmental extremes such as temperature, pH, pressure [25]. Predominance of endospore formers is important due to their contribution in nutrient cycling and biodegradation. *Pseudomonas* was the second most dominating genus representing psychrotolerant culturable species. The genus *Pseudomonas* has been reported to inhabit the low-temperature environments [29, 33]. Species of *Bacillus* and *Pseudomonas* have also been found suitable for developing the cold-tolerant bioinoculants for field applications under colder regions in IHR and Tropical Andes due to their plant growth-promoting and biocontrol abilities [31, 32]. The other

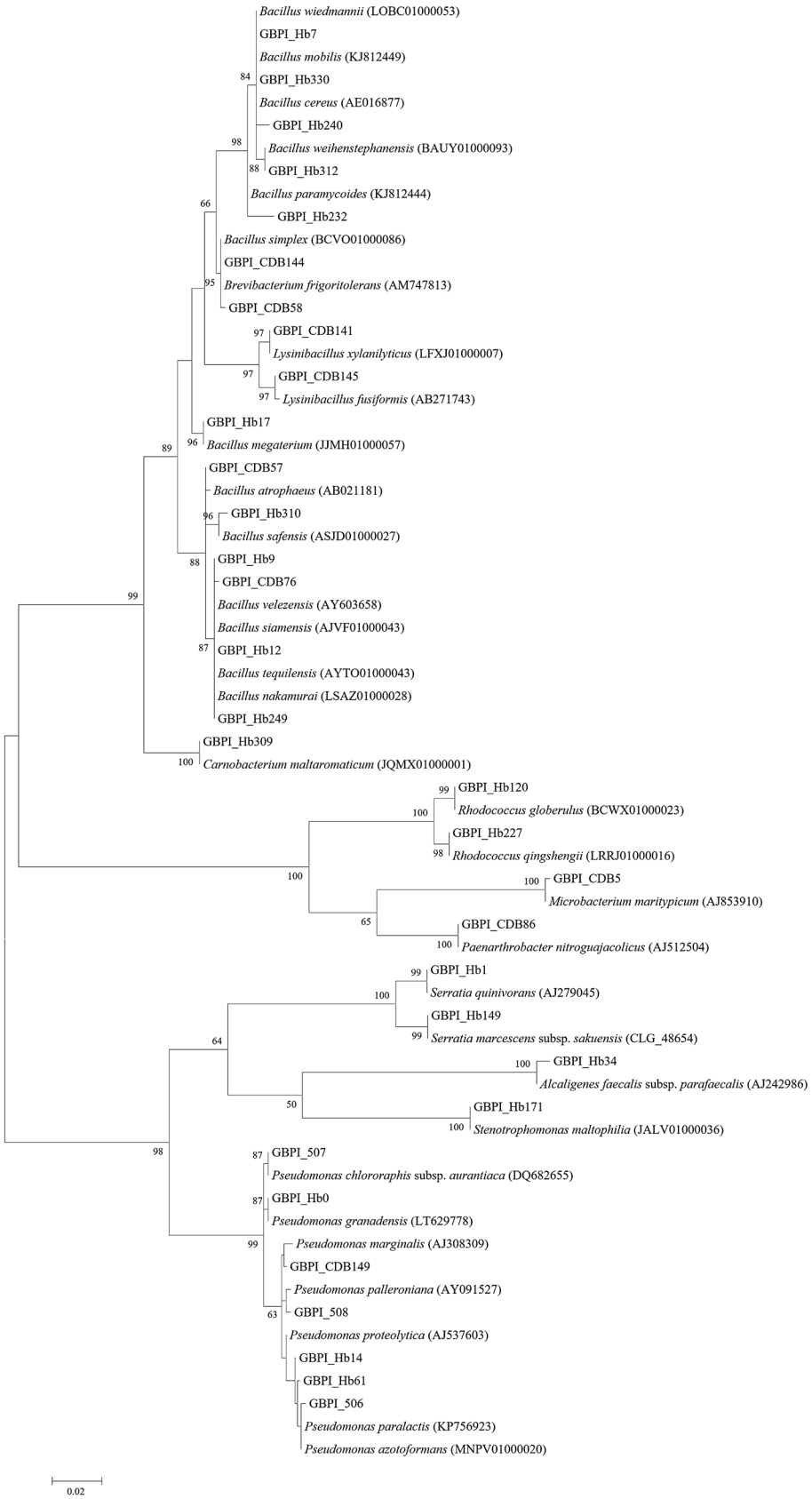

Fig. 2 Neighbour joining phylogenetic tree (bootstrap = 1000) of representative bacterial isolates. The evolutionary distances were computed using the maximum composite likelihood method

Table 3 Similarities and discrepancies in identification of 61 bacterial isolates identified by 16S rRNA gene sequencing and MALDI-TOF MS technique

Identification level	16S Identification by rRNA (no. of isolates)	No. of isolates (%) following MALDI-TOF MS-based identification			
		Similarities	Discrepancies	NRI	ND
Species level	61	3 (4.92)	33 (54.10)	12 (19.67)	5 (8.19)
Genus level	–	36 (59.01)	8 (13.11)		

bacterial species belonging to *Carnobacterium*, *Lysinibacillus*, *Microbacterium*, *Paenarthrobacter*, *Rhodococcus*, *Serratia* and *Stenotrophomonas* have been known for their influences in ecological processes like biodegradation and bioremediation [18, 22, 47]. Some of these have also been reported as pathogens and of clinical importance [3, 48].

Out of sixty-one bacterial isolates, fifty-eight were positive for lipase, thirty-six for protease, and thirty for amylase. Twenty-two isolates were positive for all the three enzymes under consideration. While the enzymes produced by cold-adapted microorganisms are considered to play an important role in the survival of these organisms under temperature stress, they are also making their way to biotechnological industries with economic advantages [2, 8]. Among the bacteria under study, *Pseudomonas proteolytica* has been reported to produce cold-active lipase [17]. Production of phenazine-1-carboxylic acid and laccase enzyme, from psychrotolerant and pigmented strains of *Pseudomonas chlororaphis* and *Serratia marcescens*, respectively, from this bacterial culture collection are few other examples in this line [16, 18]. The ability of cold-tolerant microorganisms to grow and multiply at low temperatures is subjected to the adaptive changes in cellular proteins and lipids. The role of bacterial and archaeal membranes in adapting to environmental extremes has been reviewed in recent years [42, 43]. Enzymes produced by the cold-adapted microorganisms are known to have higher activities at lower temperatures in comparison with their mesophilic and thermophilic counterparts [24]. Bioprospection of microorganisms from any ecological niche needs three major components including taxonomic as well as genetic diversity, the ability to produce biomolecules of biotechnological applications, and establishment of a facility for preservation/conservation of the microbial cultures. In bioprospection perspective of these psychrotolerant bacteria, their preservation in Culture Collection will help in advanced studies on these bacterial cultures in future.

4 Conclusion

The extreme cold environments restrict the normal life and are mainly inhabited by the cold-adapted microorganisms. Bioprospection of cold-adapted microorganisms, with their ability to produce cold-active biomolecules, is evolving as an interesting area in industrial biotechnology. The research groups are consistently trying to figure out the most abundant taxa of such microbial communities following various methods. With advancement in taxonomy, the phylogenetic methods based on the analysis of the 16S rDNA using DNA sequencing emerged and dominated as a reliable tool for species-level identification of bacteria.

New technologies for accurate and rapid identification of bacteria have always been desired. The emerging studies indicate that identification of microorganisms can now be achieved using protein 'fingerprints' measured by MALDI-TOF mass spectrometry. In this perspective, the polyphasic approach that has been considered as a combination of phenotypic and genotypic characters needs to be updated with inclusion of new technologies such as MALDI-TOF MS. However, due to difficulties with the reproducibility of results because of the use of different cultivation conditions and the limited availability of reference main spectra, MALDI-TOF MS-based biotyping has not yet been widely used for species identification. However, in view of the upcoming advanced and high-throughput methods along with the expansion of databases it is being recommended as a preferred tool for identification of microorganisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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