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DIVERSITY OF *BACILLUS* SPECIES FROM CHUMBU GLACIER

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ABSTRACT: The genus *Bacillus* encompassed debatably the most diverse and ecologically important group of bacteria on the earth. The members of *Bacillus* are abundantly found in the natural habitats. The universal distribution of genus *Bacillus* represents a notable degree of genetic and physiological flexibility. In the present study, for the first time different bacteria from the Phylum Firmicutes were isolated from the Glacier Chumbu Accumulation zone ice core sample, of North Sikkim, India. On the basis of morphology and biochemical characterization, 10 bacterial isolates were characterized from Chumbu glacier ice samples. These bacterial species were identified on the basis of 16S rRNA gene sequences. It was found that these 10 isolates belong to different members of Family Bacillaceae-*Bacillus wiedmannii*, *Bacillus velezensis*, *Paenibacillus odorifer* and *Lysinibacillus fusiformis*, based on 99% of similarity with other references sequences of those species included in the Gen Bank. This is the first study of culturable *Bacillus* species diversity from Himalayan Chumbu glacier accumulation zone ice sample.

KEYWORDS: Chumbu Glacier, Psychrotolerant, *Bacillus*, Accumulation zone, Sikkim

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1.INTRODUCTION

The genus *Bacillus* is a huge and heterogeneous group of rod-shaped, Gram-positive bacteria which are found in huge numbers in almost all the natural habitats [1, 2, 3, 4]. Because of their spore formation under adverse conditions and utilization of the diverse range of organic substrates, *Bacillus* is the most predominant group of bacteria in many ecosystems [5, 6, 7]. Also, aerosols are the main vehicles of spore distribution that ensures the occurrence of these bacteria and renders them as omnipresent [8, 9]. Since, the genus *Bacillus* includes species of industrial, environmental, biotechnological and clinical interest, diverse studies on its genetic variety from different ecosystems are been investigated [10]. However, within genus *Bacillus,* some species share similar morphological and biochemical properties and thus it is difficult to identify their species or strain without genetic analysis [11]. Microbiological classification on the basis of morphology, physiology and biochemical properties is used to group the different isolates cultivated through bacterial culture media. 16S rRNA gene sequencing is a rapid and gene based taxonomical approach for identification of bacteria [12]. In recent years, glacier studies have attracted a lot of attention as it is believed that glaciers and its microbiota can be the best indicators of climate change and also a source of several types of psychrophilic microorganisms. The aim of this study was to isolate and differentiate the *Bacillus* sp. diversity through biochemical characterization and tolerance to different pH, NaCl and temperature dependent profile of the isolates along with the screening of enzymes like protease, amylase, gelatinase and pectinase.

2. MATERIALS AND METHODS

2.1 Sample collection and pretreatment

The sample was taken by drilling 2 meter of ice core from Accumulation zone of Chumbu glacier, North Sikkim. The ice samples were immediately transported to the laboratory. The glacial ice core sample was processed and cut into small pieces about 6 inches and was kept for storage at -20ºC. The aseptic measures were taken and the glacial ice core samples were cut with a sterilized saw-tooth knife and around 5mm annulus was discarded. The remaining glacial inner core was rinsed with cold ethanol (4°C; 95%), and finally with cold autoclaved water (4°C). The glacial ice core samples were placed in the sterile containers and melted at 4°C cold incubator. These handling procedures were undertaken at temperatures below 20°C aseptically using positive pressure laminar flow hood as described by Xiang [13].

2.2 Isolation and Cultivation of *Bacillus* species

200µL thawed glacier melt water from each inner ice core sample was directly spread using Luria Bertani Agar (LBA) and Antarctic Bacterial Medium (ABM) agar. The cultures were incubated at 15°C for 3 weeks. Also, 200 µL thawed glacier melt water sample was enriched in a 200mL conical flask containing 50mL Luria Bertani broth and was then incubated at 15°C for 14 days in incubator

cum shaker at 120rpm. After enrichment, the culture was spread plate using LBA and ABM agar media and incubated for 3 weeks at 15°C. Morphologically different colonies were selected and were sub-cultured by streak plate method [14]. A total of 10 pure isolates were obtained from Chumbu glacier. The purified colonies were stored at -80°C in 40% glycerol.

2.3 Genomic DNA extraction and 16S rRNA gene amplification and sequencing

A total of 10 isolates were chosen for DNA extraction and sequencing. Bacterial genomic DNA was extracted by QIAamp kit according to the manufacturer protocol (QIAGEN, India). 16S rDNA genes were PCR amplified using the universal bacterial primer 27F (5'-AGAGTTTGATCCTGGC TCAG-3') and 1492R (CGGTTACCTTGTTACGACTT-3') [15]. PCR was carried out in a final volume of 50µL using 2µL template DNA, 2µL $MgCl_2$, 4µL dNTPs, 1µL forward and reverse primer, 1µL Taq DNA polymerase, and 33µL ddH_2O. Reactions were performed in the thermocycler (BioRAD PCR System) with the following reaction conditions; 94°C for 5 min for initial denaturation, followed by 30 cycle of 94°C for 30s, 55°C for 1 min, 72°C for 1 min and the final extension at 72°C for 10 min. The PCR products were purified with the QIAquick PCR clean up system kit (QIAGEN, India) and sequenced by ABI Applied Biosystems (3500 Genetic Analyzer) using each universal primer i.e., 27F and 1492R. The sequences were assembled and aligned using Codon Code Aligner software. The sequences were then identified using nucleotide blast (NCBI). The identified sequences were then submitted to NCBI gene bank in order to get accession numbers. The phylogenetic tree was created by using neighbor-joining method [16, 17].

2.4 Biochemical characterization

All the strains were phenotypically characterized based on their fermentative degradation of 13 different sugars under aerobic conditions was carried out in a fermentation tube. Fermentation tube is a culture tube that contains a Durham's tube for the detection of gas production as an end product of metabolism. The Fermentation broth contains the ingredients of nutrient broth, a specific carbohydrate and a pH indicator (Phenol red), which is red at neutral pH (7.0) and turns yellow at or below a pH of 6.8 due to the production of an organic acid.

2.5 Growth at different: Temperature, NaCl and pH

The temperature dependent growth profile of the isolated was checked on Luria Bertani broth at 5°C, 10°C, 15°C, 20°C, 30°C and 40°C in an incubator. Similarly, NaCl dependent growth profile was checked at different percentage of NaCl (0%, 1% 2%, 4%, 6%, 8% and 10%) incubated at 15°C incubator and pH dependent growth profile were also checked at different range pH (4, 6, 8 and 10) at 15°C in a cold incubator cum shaker at 120rpm [17].

2.6 Enzyme activity

Amylase, protease, gelatinase and pectinase enzyme activities were tested qualitatively using agar plates containing the specific substrate and incubating it at 15°C for 10-15 days. The starch agar plates were prepared on Starch Agar medium (HiMedia, Mumbai) and the amylolytic isolates were selected by flooding the Starch Agar plate with Gram's iodine (2g KI and 1g iodine in 300mL distilled water). Isolates with distinct clear halo zone around the colonies were identified as amylase producer [18]. For protease activity, plates were prepared with the screening medium containing casein and incubated at 15 °C for 10-15 days. The strains that produced a hydrolytic zone were identified as protease producing strains. Then, for each strain, the diameter of the hydrolytic zone was calculated [19]. For gelatinase and pectinase enzyme activity were tested qualitatively using gelatin agar and pectin agar respectively.

3. RESULTS AND DISCUSSION

The present work was aimed to check the *Bacillus* species diversity from Chumbu glacier accumulation zone ice core samples, North Sikkim, India, which was more or less pristine as they are far from human habitat and has very less anthropogenic activities. It has been reported that many *Bacillus* sp. were recovered from glacier ice samples from different parts of the world. Therefore, it was interesting to study the *Bacillus* species diversity isolated from this Himalayan glacier.

3.1 Morphological and biochemical characterization of the isolates

Using Luria Bertani Agar (LBA) and Antarctic Bacterial Media (ABM), a total of 10 isolates were obtained. The selection and grouping were done on the basis of their morphological and biochemical characterization. These 10 isolates were mostly of white color and circular in shape (Table 1). The endospore and Gram-staining results showed that all the isolates were Gram-positive and spore former. The biochemical test has shown that among 10 bacterial isolates all the isolate showed catalase positive, two had protease activity, one each isolate showed nitrate, gelatinase, amylase positive and none of the isolates had pectinase activity as shown in Fig 1. Carbohydrate fermentation test has shown that the maximum number of isolates were able to ferment sugars such as Galactose, Mannitol, Arabinose, Fructose, Maltose, and Cellobiose. However, the isolates were unable to ferment Dextrose, Rhamnose, Dulcitol and Xylose etc. (Table 2).

Table 1: Showing the morphology of the isolates from Chumbu glacier

Isolates	Gram Reaction	Shape	Spore
C1	+ve	Rod	√
C2	+ve	Rod	√
C3	+ve	Rod	√
C4	+ve	Rod	√
C5	+ve	Rod	√
C6	+ve	Rod	√
C7	+ve	Rod	√
C8	+ve	Rod	√
C9	+ve	Rod	√
C10	+ve	Rod	√

Morphological and microscopic characterization of study isolates (√ & +ve) the sign indicates that the isolates were positive, and (× &-ve) a sign indicates that the isolates were negative against the respective test.

Fig 1: Enzymatic activity test of Chumbu glacier isolates

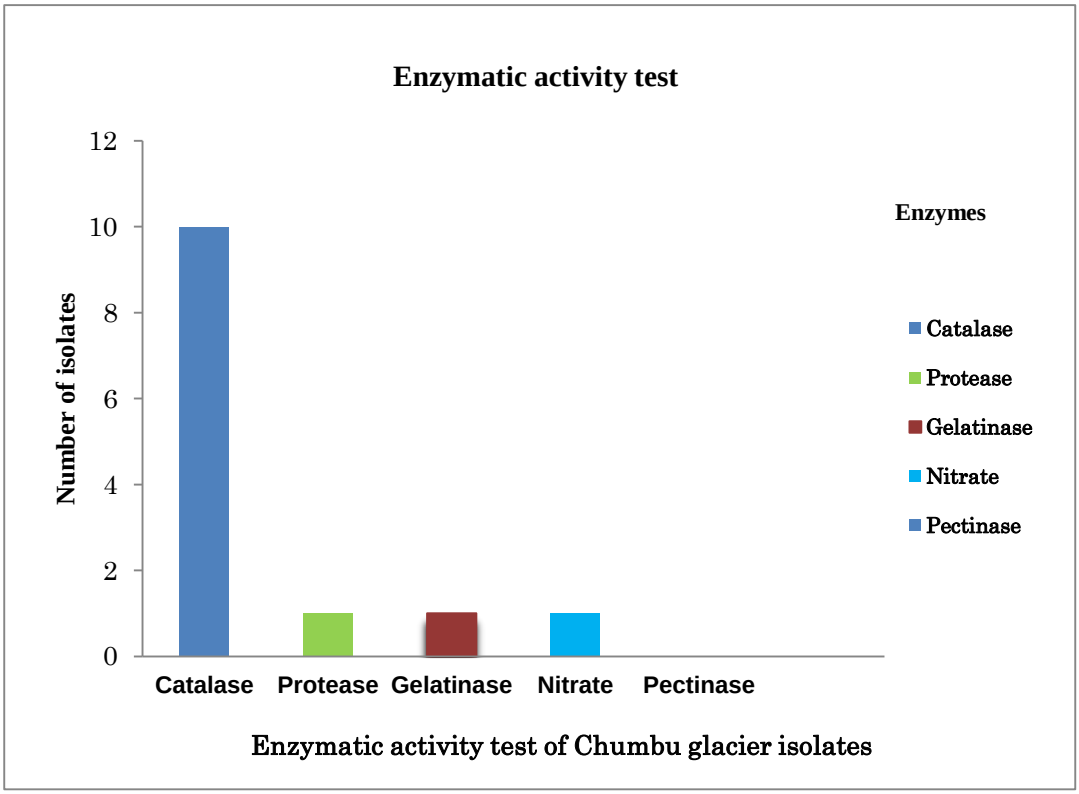

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Table 2: Carbohydrate fermentation results of the isolates

ISOLATES	Carbohydrate Fermentation												
	Arabinose	Cellobiose	Dextrose	Glucose	Galactose	Maltose	Mannitol	Fructose	Mannose	Rhamnose	Raffinose	Dulcitol	Xylose
C1	-	-	-	+	-	-	-	+	-	-	-	-	-
C2	+	+	-	+	+	+	+	-	-	-	-	-	-
C3	+	-	-	+	-	+	+	+	+	-	+	-	-
C4	+	-	-	+	-	+	+	+	+	-	+	-	-
C5	+	+	-	+	+	+	+	-		-	-	-	-
C6	-	-	-	+	-	-	-	+	-	-	-	-	-
C7	-	-	-	+	-	-	-	+	-	-	-	-	-
C8	+	+	-	+	+	+	+	-	-	-	-	-	-
C9	-	-	-	+	-	-	+	-	-	-	-	-	-
C10	+	+	-	+	+	+	+	-	-	-	-	-	-

Carbohydrate fermentation test of Chumbu glacier - The isolates were tested against various carbohydrates and the (+) sign indicates that the isolates were able to ferment that particular carbohydrate and (-) sign indicates that the isolates were unable to ferment that particular carbohydrate.

3.2 Growth at different: Temperature, pH and NaCl

Isolates were examined for their growth at different temperatures (0°C - 40°C) so as to find out the optimum temperature required for their growth. Maximum isolates showed good growth at 20°C and 30°C (Fig 2), thus it might be the optimum temperature for these bacteria. Also, it suggests that these bacteria are psychrotolerant rather than psychrophilic. Most of the isolates showed better growth at pH8, followed by pH6, pH10, and pH2, thus suggesting that these isolates are required neutral pH conditions (Fig 3). These isolates showed better growth below 1% NaCl which is not surprising as in glacier ice samples hypo-saline condition mainly prevails (Fig 4).

Fig. 2: Temperature dependent growth profile of Chumbu glacier isolates

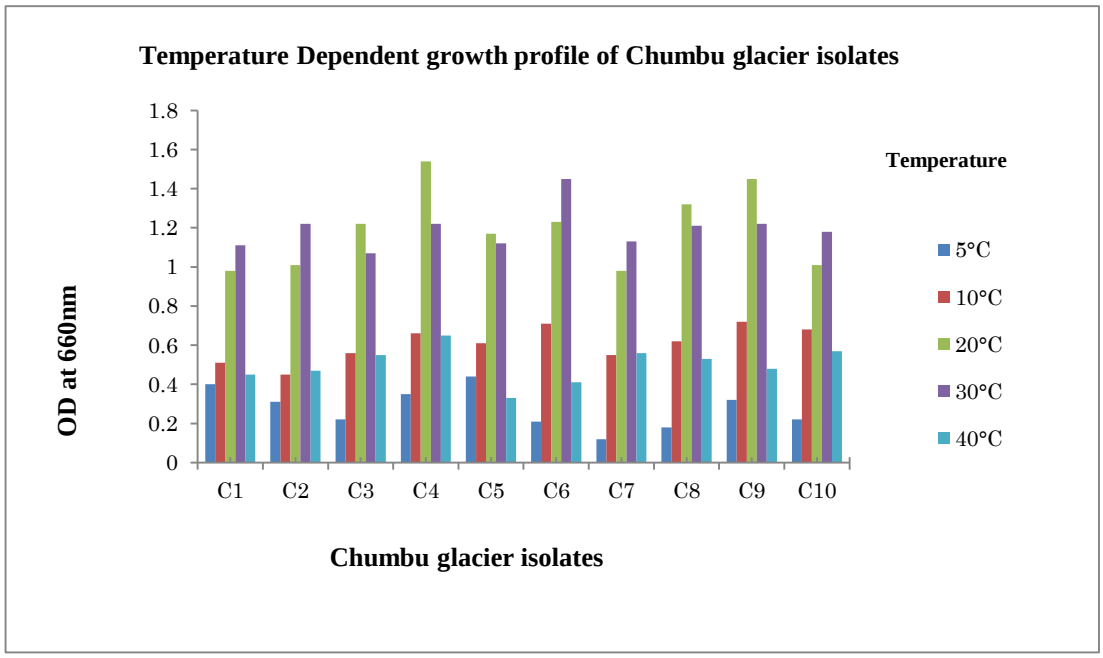


Fig. 3: pH dependent growth profile of Chumbu glacier isolates

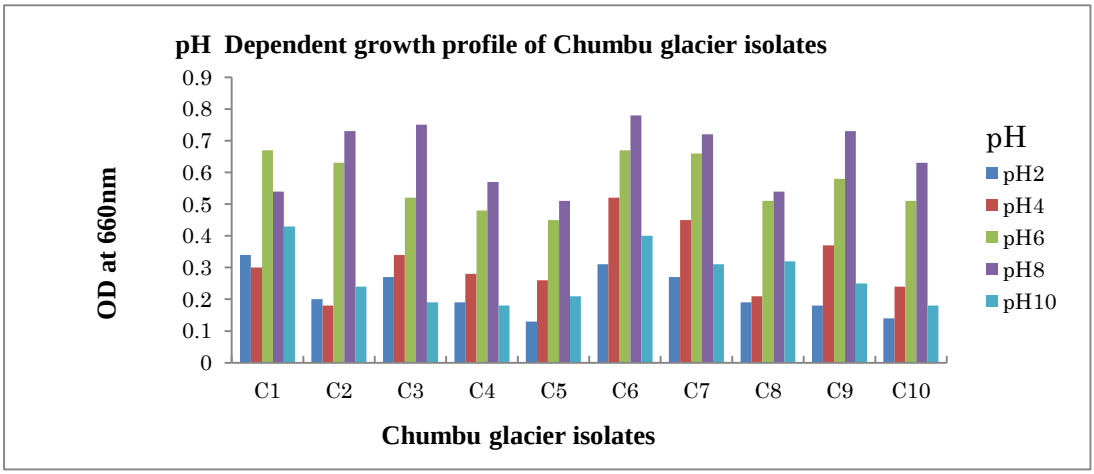
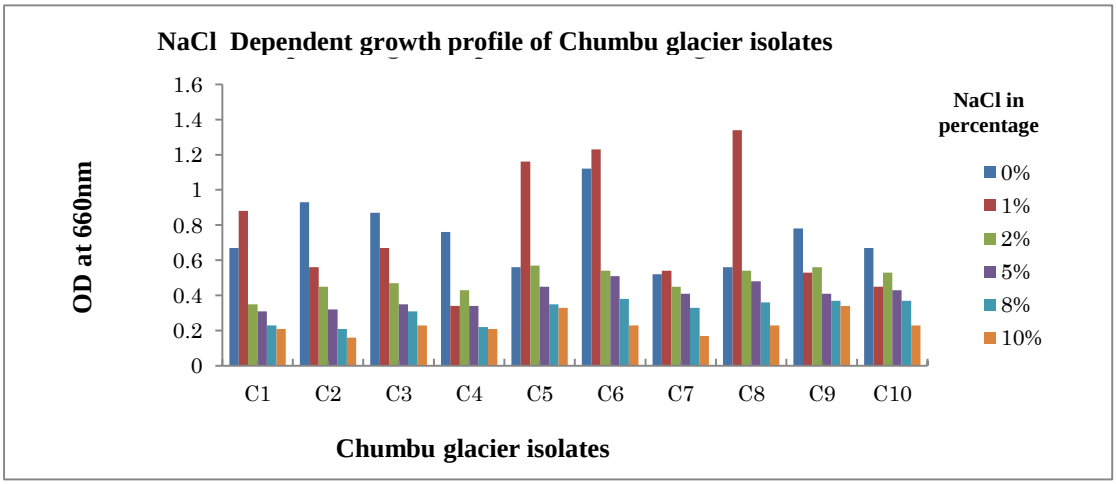


Fig. 4: NaCl dependent growth profile of Chumbu glacier isolates



3.3 Identification of bacterial isolates

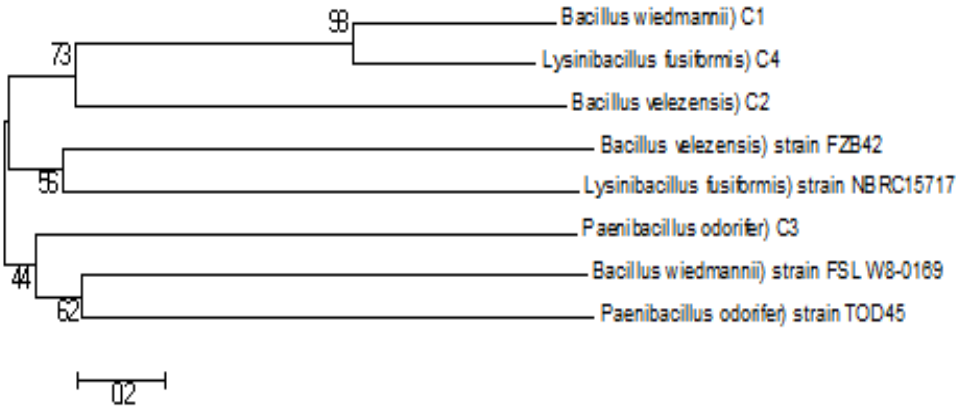
The selected isolates were identified by colony PCR amplification and sequencing of 16S rRNA by ABI Applied Biosystems (3500 Genetic Analyzer). Briefly, a single colony from an overnight grown culture of each isolate was suspended in 30µL milliQH_2O and the DNA was liberated by heating for 15-20 min at 98˚C using BioRad Thermo-Cycler. Crude DNA extract was used as the DNA template for PCR.2µL of template DNA was amplified by PCR using universal primer 27F and 1492R. On the basis of morphology and biochemical characterization, four bacterial isolates were further analyzed for 16S rRNA gene sequencing. On the basis of 16S rRNA gene sequences similarity, these four isolates were identified as: *Bacillus wiedmannii*, *Bacillus velezensis*, *Paenibacillus odorifer* and *Lysinibacillus fusiformis*, based on 99% of similarity with other references sequences of those species included in the Gen Bank (Table 3). The accession number, percent homology of all the isolates are given in Table 3. The phylogenetic tree was created using MEGA7 by the Neighbor-Joining method (Fig 5) [16].

Table 3: The accession number and percent homology of the isolates

Isolates	Identification	Percentage of Homology	Accession number
C1	*Bacillus wiedmannii*	99%	MH157240
C2	*Bacillus velezensis*	99%	MH157241
C3	*Paenibacillus odorifer*	99%	MH157242
C4	*Lysinibacillus fusiformis*	99%	MH157243

The result presented here, suggests that *Bacillus* species are commonly present in Chumbu glacier ice core, North Sikkim. For the first time, a plant-associated bacterium, *Bacillus velezensis* was identified from Chumbu glacier ice core. This bacterium has the functional properties to promote plant growth and can be also used as bio-control agent against plant pathogens [20, 21, 22, 23]. *Bacillus wiedmannii* [24] and *Paenibacillus odorifer* which are considered as psychrotolerant bacteria were also found and reported for the first time in glacier Chumbu ice core sample [25, 26]. *Lysinibacillus fusiformis* recovered from Chumbu glacier ice, was found in Siachen glacier, Pakistan by Rafiq and his group [17]. It has been reported as heavy metal resistant bacteria [27, 28].

Fig 5: Phylogenetic tree of the isolates from Chumbu Glacier



Phylogenetic tree of Chumbu glacier isolates: Neighbor-joining trees showing the phylogenetic relationships of bacterial 16S rRNA gene sequences from Chumbu glacier isolates closely related sequences from the GenBank database.

4. CONCLUSION

This is the first report of its kind on *Bacillus* species diversity of Chumbu glacier ecosystem of Sikkim, India. Interestingly, we found four different bacteria of Bacillaceae family among ten isolates from Chumbu glacier accumulation zone ice core sample, North Sikkim, India. All the identified bacterial showed the best growth at 20°C and 30°C, pH 6-8 and 0-1% NaCl. Their dominance in such harsh glacial condition might be due to the presence of their spore. We found high genotypic and phenotypic heterogeneity in the *Bacillus* isolates obtained from Chumbu glacier ice core.

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6. CONFLICT OF INTEREST

Authors have no conflict of interests to declare.

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