Studies on Microbial Diversity and Biotechnological Potential of Selected Polar Organisms

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CERTIFICATE

This is to certify that the thesis entitled **"Studies on Microbial Diversity and Biotechnological Potential of Selected Polar Organisms"** submitted by Purnima Singh, ID No. 2010PHXF811G for award of Ph.D. degree of the Institute embodies original work done by her under my supervision.

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Dedicated to
My Parents

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4. OBJECTIVE 2: *SCREENING AND CHARACTERIZATION OF A FEW SELECTED ORGANISMS FOR THEIR BIOTECHNOLOGICAL POTENTIALS.*

ABSTRACT

The characterization of the microbial communities within glaciers cryoconites, ice cores and permafrost is important for monitoring the effects of climate change on biodiversity within the environment. In addition the genetic materials of these communities are very valuable with respect to bioprospecting, and therefore study of these communities is immensely significant.

Glacier cryoconite holes being the repertoire of underexplored microbes have ecological, biogeochemical and biotechnological importance. The culturable bacterial diversity from cryoconite holes at Austre Brøggerbreen (AB), Midre Lovénbreen (ML) and Vestre Brøggerbreen (VB) glaciers has been characterised. The total bacterial numbers ranged from 5.07×10^5 to 1.50×10^6 cells/g while the culturable bacterial count ranged from 2.7×10^3 to 8.8×10^4 CFUs/g in these three glaciers. A total of 28 bacterial isolates were recovered, which were morphologically distinct. On the basis of 16S rRNA sequence data, the identified species were found belonging to eight genera: *Polaromonas*, *Pseudomonas*, *Micrococcus*, *Agreia*, *Subtercola*, *Leifsonia*, *Flavobacterium* and *Cryobacterium*. The isolates showed difference in their NaCl tolerance, growth temperature, pH, carbohydrate utilization, enzyme activities, and antibiotics sensitivity tests. The results of fatty acid profile indicate the predominance of branched fatty acids in the isolates. To our knowledge this is the first work on culturable bacterial communities and their characterization from glacier cryoconites, Svalbard Arctic. High protease and amylase activities expressed by *Micrococcus* sp. MLB-41 and protease, amylase and lipase activities expressed by *Cryobacterium* sp. MLB-32 provide evidence to the potential applications of these organisms for the prospect of biotechnology in Arctic.

Conventional and molecular techniques were used to characterize culturable psychrophilic yeast and filamentous fungi from AB, ML, and VB glaciers cryoconites. The parameters like effect of temperature, different salt

concentrations and microbiological media used on growth of the cultures were studied. Measurements on the bioavailability of trace metals and nutrients were recorded through different methods including inductively coupled plasma mass spectrometry (ICPMS). Colony forming unit (CFU) per gram of sediment sample was calculated to be about from 7×10^3 to 1.4×10^4 for yeast and from 4×10^3 to 1.2×10^4 for filamentous fungi from the Arctic. On the basis of morphotaxonomy and DNA sequencing data, these were identified as *Cryptococcus gilvescens*, *Rhodotorula* sp., *Mrakia* sp., *Thelebolus* sp., *Articulospora tetracladia* and *Phialophora alba*. Amongst all these, *Phialophora alba*, *Cryptococcus gilvescens* and *Mrakia* sp. zhenx-1 were reported for the first time from Svalbard Arctic. Besides this novel yeast, *Rhodotorula* sp., (95% gene similarity) was also recovered. *Cryptococcus gilvescens* showed high lipase activity whereas the novel species *Rhodotorula* sp. MLB-1 produced high amylase activity. *Mrakia* sp. expressed phosphate solubilization ability between 4–15°C which is reported as a first record. Chemical analysis of cryoconites showed the presence of nitrogen, organic carbon and phosphorus in substantial amounts. The role of yeasts and filamentous fungi in the cryoconites help in the degradation of organic macromolecule through cold-adapted enzyme secretion, thus assisting in nutrient cycling in subglacial environments. In addition, these cold-adapted enzyme-producing isolates recovered from the Arctic may provide an opportunity for more biotechnological exploration. This is also the first ever report involving on mycological investigation into glacier cryoconites from Svalbard, Arctic.

The psychrophilic yeast (*Rhodotorula* sp., 95% gene similarity) isolated from glacier cryoconite holes in Svalbard was characterized and established as a novel species. Nucleotide sequences of the strain were studied using D1/D2 domain, internal transcribed spacer (ITS) region and partial sequences of mitochondrial cytochrome *b* gene. The strain belonged to a clade of psychrophilic yeasts but differed from others in the D1/D2 domain. Biochemical characterization of the isolate showed marked differences from related species, with highest relatedness to *R. psychrophenolica.* Effects of different media, salt concentration and

temperatures on growth of the isolates were also studied. Cultures were screened for amylase, protease, cellulase, lipase, catalase and urease activities. *Rhodotorula* sp. showed positive antifreeze protein (AFP's) activity. With decrease in temperature, increased unsaturated fatty acid production was detected in cultures via fatty acid methyl ester (FAME) analysis. The major fatty acids observed were linoleic, oleic, linolenic, stearic, palmitic, pentadecanoic and myristic acids. On the basis of morphological, physiological and sequencing data of the strain, a novel species, *Rhodotorula svalbardensis* and designate strain MLB-I as its type strain was proposed.

Microbial communities of the glacier ice cores promise biotechnological and ecological importance. The current study was aimed at characterizing bacteria in the ice cores from ML glacier, Svalbard. The total bacterial numbers ranged from 7.20×10^4 to 2.59×10^7 cells/mL (mean 3.12×10^6 cells/mL) while the viable count was from 10 to 7000 CFU/mL (mean 803 CFU/mL). 14 morphologically distinct isolates were selected for molecular characterization. On the basis of 16S rRNA sequence data, the identified species belonged to three genera and seven species, namely *Pseudomonas orientalis, P. oryzihabitans, P. syncyanea, P. fluorescens, Sphingomonas phyllosphaerae, S. dokdonensis* and *Bacillus barbaricus,* with a range of sequence similarity between 93.5 and 99.9% with taxa present in the database. The isolates showed unique phenotypic properties and the three novel species isolated (MLB-2, MLB-5 and MLB-9) are yet to be described. To our knowledge, this is the first record of culturable bacterial communities and their characterization from glacier ice cores from Svalbard, Arctic. As most of the isolates produce high protease, lipase, cellulase, urease and amylase activities these could be used as a potential source for industrial applications.

From glacier ice cores of Svalbard a total of 10 strains of psychrophilic yeasts were studied. The ice melt water contained about $3x10^3$–$1x10^4$ CFUs/mL of yeasts. The 18S rDNA (Deoxy Ribonucleic Acid) sequence analysis using D1/D2 domain identified five yeast species, namely *Cryptococcus albidosimilis* (JX192656), *C. adeliensis* (JX192655), *C. saitoi* (JX192659), *Rhodotorula*

mucilaginosa (JX192664) and *Rhodosporidium* sp. (JX192657). A study was conducted on the effect of temperature on growth of these isolates. The strains were able to grow at temperature ranging between 1 and 20 ^{o}C. Screening of the cultures for cellulase, amylase, protease, urease, lipase, and catalase activity was carried out showing varied amounts of enzyme production at different temperatures. For the strain *Cryptococcus* sp. MLB-24 characterization of lipase activity was done. FAME analysis of the cultures grown at different temperatures (1, 4, 15 and 20^{o}C) was also performed which indicates that the unsaturated fatty acids increased with decrease in temperature and with the increase in temperature high amount of oleic acid were accumulated. The strains in glacial ice cores are thought to survive due to the presence of unsaturated fatty acids as the intra- and extracellular filtrate of the cultures showed negative antifreeze protein (AFP) activity. This observation leads to the conclusion that probably the isolates in the Ice core study adapt to low temperatures, by PUFA and enzyme secretion rather than by AFP production.

Permafrost is a conspicuous habitat in polar environment and has immense ecological and biotechnological significance. The present study is focused on characterization of bacterial communities from frozen sediments from pit profiles of Svalbard, Arctic. The culturable count of the isolates ranged from 1.50 x 10^3 to 2.22 x 10^5 CFUs/g while the total bacterial numbers ranged from 1.14 x 10^5 and 5.52 x 10^5 cells/g soil. Bacterial isolates were identified through 16S rRNA gene sequencing and results indicate that *Arthrobacter* and *Pseudomonas* were the most dominant genera with *A. sulfonivorans, A. bergeri, P. mandelii* and *P. jessenii* as the dominant species. Rest of the species belonged to the genera *Acinetobacter, Bacillus, Enterobacter, Nesterenkonia, Psychrobacter, Rhizobium, Rhodococcus, Sphingobacterium, Sphingopyxis, Stenotrophomonas* and *Virgibacillus*. Radiocarbon dates of the permafrost pit profile sediments were analysed in the present study. The bacteria preserved in the different sediments studied aged 12250 to 44800 years old. To the best of our knowledge this is the first record of culturable bacterial communities and their chronological

characterization from permafrost pit profile from Svalbard, Arctic. Physiological, biochemical, antibiotic susceptibility and enzyme screening tests were carried out. Enzyme production ability of the bacterial isolates provides a clue to their potential prospect in biotechnological research from ancient bacteria.

The biochemical adaptations of fungi to the extreme conditions of the Arctic indicate that these organisms possess useful properties. Puffball mushroom (*Lycoperdon molle* Pers.) samples collected at Ny-Ålesund, Svalbard, were examined *in vitro* for antioxidant potentials by investigating its free-radical scavenging (FRS) activity, Trolox equivalent antioxidant capacity (TEAC) and inhibition of lipid peroxidation (ILP) assays. The FRS activity of the samples in four organic solvents (methanol, ethanol, acetone and dimethyl sulfoxide (DMSO) were found to be in the range of 44.00 to 89.60%, while ILP activities were in the range from 32.00 to 54.41%. The methanol extract indicated the highest levels of FRS (89.60%) and ILP (54.41%) compared with standard antioxidants butylated hydroxytoluene (BHT) and butylated hydroxyanisol (BHA). The TEAC value was found to be greater in comparison with the standard vitamin E analogue Trolox (3.9 mM). Extract of *Lycoperdon molle* showed negative antimicrobial activity against the tested microorganisms. Electrospray ionization mass spectrometry (ESI-MS) indicates the presence of phosphoethanolamine, phosphatidyl glycerol, monomethyl arsenic acid, phosphoionositol, lysophosphatidyl choline and phosphoserine compounds. *L. molle* showed strong antioxidant abilities compared with the standards, suggesting it to be a valuable source of natural antioxidants for the pharmaceutical industry. So far, this is the first study of the antioxidant activity in any Arctic mushroom.

Six Arctic bacterial strains belonging to four species, *Pseudomonas ficuserectae* (two strains), *Cryobacterium psychrotolerans* (two strains), *Cryobacterium psychrophilum* and *Leifsonia* sp., isolated from cryoconite holes of Svalbard glaciers, were screened for antifreeze proteins (AFPs**)**. *Pseudomonas ficuserectae* (Cry-g) showed a high thermal hysteresis (TH) activity at upto 2ºC, depending on the concentration. The round shaped ice crystals produced by six

bacterial AFPs did not change their morphology and size within the TH window. Cry-g AFP failed to inhibit ice recrystallization indicated that the degree of TH activity did not relate to the IRI activity. Antifreeze protein analysis by SDS-PAGE suggested that their apparent molecular weights were ~22 kDa. Till date from the Arctic only one species of bacteria (*Pseudomonas putida*) was reported to produce AFPs. Therefore, this study is important as it screens different species of bacterial strains from the Arctic for AFP activity. Analysis of the N-terminal amino acid sequence indicated that the isolated bacterial AFPs belonged to the ice binding protein-1 (IBP-1) family of AFPs. The habitat from which the AFP was isolated is interesting and has been discussed. Arctic glaciers bacterial strains which have shown thermal hysteresis (TH) and ice recrystallization inhibition (IRI) activities were further characterized. The AFP from extremophile strain such as *Cryobacterium psychrotolerans* (Cry-c) MLB-29 (JX205196) and *Pseudomonas ficuserectae* Cry-g (JF790967) were analyzed by electrospray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) at a protein concentration of 10 µM. ESI-Q TOF analysis of the Cry-c antifreeze protein revealed its exact molecular mass to be 22.14 kDa corroborating its previous observation on SDS-PAGE analysis that was approximately 22 kDa.

Subseqeuntly, for the purified AFPs of Cry-c and Cry-g were subjected for multi-enzymatic limit digestion (MELD) and nano-Liquid Chromatography Electropsray Ionization Mass Spectrometry (LC-ESI-MS/MS) analysis; 21 (Cry-c) and 55 (Cry-g) peptides were sequenced with a high-level confidence. The entire 20 N-terminal amino acid sequence of Cry–c was obtained as AVPVGSVRAXVXXGAATTFX. However, this sequence did not find any significant homology in the NCBI-protein data with any known antifreeze or ice binding protein.The detection of relatively glycine-rich sequences such as GPSGEAGKMFVHQGPTGL, GPAGPHGPPGKDGR were in Cry-c and SPGAGPGGERGPWPD, $SP(G)_6LGSAKDR$ in Cry-g AFP. The presence of polyglycine motif in the AFP produced by *P. ficuserectae* (Cry-g) was not reported earlier anywhere else.

Asparagines and alanine-rich sequences DMALNPSKE, NMALNPSKE, LANPSKE, AVGLLQNNSGAAKHATLR, ASALTGNVGASPLTQALHL and QAAGSVAVGTTAHFE signify the essential roles of those amino acids in the antifreeze activity. The noticeable diversity existing amongst various polar organisms indicate that due to probable convergent evolution the AFP produced by any particular living system might be unique to that microbe or milieu. For antifreeze proteins, merely handful bacterial sources have been studied so far in any detail and less number of AFPs have been isolated and characterized at the protein structure level. The present study are able to shed light on compositional characteristics of some of the special features of a Cry-c and Cry-g AFPs.

Whole genome sequencing of two unique isolates one from cryoconite (*Cryobacterium* sp. MLB-32) and other from 44800 yrs old permafrost (*Nesterenkonia* sp. PF2B19) was performed. The *Cryobacterium* sp. MLB-32 genome analysis revealed the presence of genes encoding industrially important enzymes, such as alcohol dehydrogenases, alpha-amylase, pullulanase, esterase, protease, hydantoinase and chitinase. Also, several genes associated with biosynthesis of plant growth promoting hormones like auxin, cytokinin, abscisic acid, ethylene, salicylic acid and indole acetic acid were detected indicating its immense potential to be used as a Plant Growth Promoting *Rhizobacterium* (PGPR) in cold environments. Analysis of annotated genome sequence of *Nesterenkonia* sp. PF2B19 revealed the presence of genes involved in production of α-amylase and maltase showed its immense biotechnological potentials. In addition, the genes encoding proteins involved in resistance to heavy metals and toxic compounds, including copper homeostasis, cobalt-zinc-cadmium resistance, arsenic resistance, and β-lactamase, were identified. These results show the genetic potential of the *Nesterenkonia* sp. to adapt to extreme lifestyles.

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FIGURE No. **TITLE**

LIST OF SYMBOLS AND ABBREVIATIONS

AFP	-	Antifreeze protein
Bp	-	base pair
DNA	-	Deoxy Ribonucleic acid
dNTP	-	deoxy nucleotide triphosphate
EDTA	-	Ethylene Diamine Tetra Acetic acid
HCl	-	Hydrochloric acid
kDa	-	kilodalton
M	-	Molarity
$MgCl_2$	-	Magnesium Chloride
mL	-	nvestigat
mg	-	milligram
mg/L	-	milligram per litre
mM	-	millimolar
NaCl	-	Sodium Chloride
PCR	-	Polymerase Chain Reaction
pH	-	Negative logarithm of hydrogen ion concentration
rpm	-	revolutions per minute
sp.	-	Species
U/µL	-	Unit/mictroliter
v/v	-	volume/volume
w/v	-	weight by volume
°C	-	degree Celsius
µL	-	microlitre
DPPH	-	1,1-diphenyl-2-picryl-hydrazyl
H_2O_2	-	hydrogen peroxide
ABTS	-	2-,2'-azinobis 3-ethylbenzothiazoline-6-sulfonate
AAPH	-	2,2'-azobis (2-amidinopropane) dihydrochloride

1. INTRODUCTION

Microbes are both the primary producers of biomass and the principle recyclers of nutrients not only in glacier but everywhere, and are essential to the survival of the entire ecosystem. Baseline data on their current community composition and activity is an absolute prerequisite for being able to estimate the effects that climate change will have on the glacier ecosystem. The rapid loss of glacier thickness represents serious threat to microbiota inhabiting this extreme ecological niche, in addition to global warming. Due to the essential effect of microbes on the ecosystem as a whole, climate change influence on microbial diversity of such niches are extremely important for a better understanding of glacier-microbial interaction in the climate system.

Polar habitats are exciting places on Earth where psychrophiles and psychrotolerant microbes inhabit. These microbes through novel biochemical adaptations survive through the extreme environmental conditions such as low temperature, intermittent freezing, ultraviolet light and prolonged period of darkness or light. The microbial diversity existing in these realms, although scanty compared to the temperate or tropical regions are much more distinctive and chemically well-equipped.

The adaptation strategies employed by the psychrophilic/psychrotolerant microbes lead to the production of several metabolic compounds. These compounds such as cold tolerant enzymes, antimicrobials, antioxidants and antifreeze proteins, find immense applications in the field of pharmaceutical and biotechnological industries, and are therefore provide opportunities for bioprospecting in Antarctic and Arctic.

Considering the biotechnological value of these species in general, it is essential that the micro-organisms should be correctly identified before bioprospecting. Molecular taxonomic studies of polar microbial species are fragmentary. Although a modest number of microbial species have been reported

from the region, there may be some more novel species yet to be discovered. Rare species of microbes found in the region also needs to be documented.

Cryoconite holes are water filled pocket like structures that are distributed over the glaciers ablation zone and sea ice. They contain a soft, dark colored granular material, mainly consisting of organic and inorganic matter. The organic matter mainly includes algae, cyanobacteria, bacteria, fungi, and rotifers while, the inorganic matter is composed of minerals and trace elements. In the Antarctic, the cryoconites on glaciers remain in the frozen state at the surface by thick ice (~30 cm), giving an insulating effect of the contents from the influence of atmosphere and meltwater for a decade (Tranter *et al.* 2004; Hodson *et al.* 2008) barring a few of the holes getting exposed to the atmosphere only during the Antarctic summer (Wharton *et al.* 1985; Mueller *et al.* 2001). Contrarily, the Arctic and Alpine cryoconite holes normally stay exposed to the atmosphere during each summer, allowing gaseous exchange, along with nutrients and biological propagules (Hodson *et al.* 2007). Simultaneously Ice cores provide unique, natural culture collections for studying microbial diversity, ecology, and evolution (Nichols *et al.* 1999, Segawa *et al*. 2005; Vincent 2000). The microbes present in these ice cores are crucial since they display distinct physiological characteristics (Moore *et al.* 1998; Zhang *et al*. 2008). The existence of physiological and genomic heterogeneity in bacteria act as a pointer towards a wide diversity surviving in glacial ice, most of which are ubiquitous pscyhrotolerant/psychrophilic organisms and a few endemic ones.

Permafrost soils are permanently frozen polar soils that have remained pristine and relatively static for millions of years. It is a unique ecosystem in which long-term microbial lineages occur at sub-zero temperatures. Permafrost is a model habitat for understanding the ecological functioning and evolutionary study of microbes.

The proposed work was investigated on studying the microbial diversity in glacial cryoconites, ice cores, and permafrost samples collected from Svalbard, Arctic. Characterization was done using a combined approach of physiological,

biochemical and molecular analyses. These microhabitats are unique and have not been fully explored erstwhile for unearthing the microbial diversity. It is in these habitats that the true psychrophiles inhabit and was therefore highly desirable to focus the investigation on the microbial diversity and potentials from these climatically extreme microhabitats.

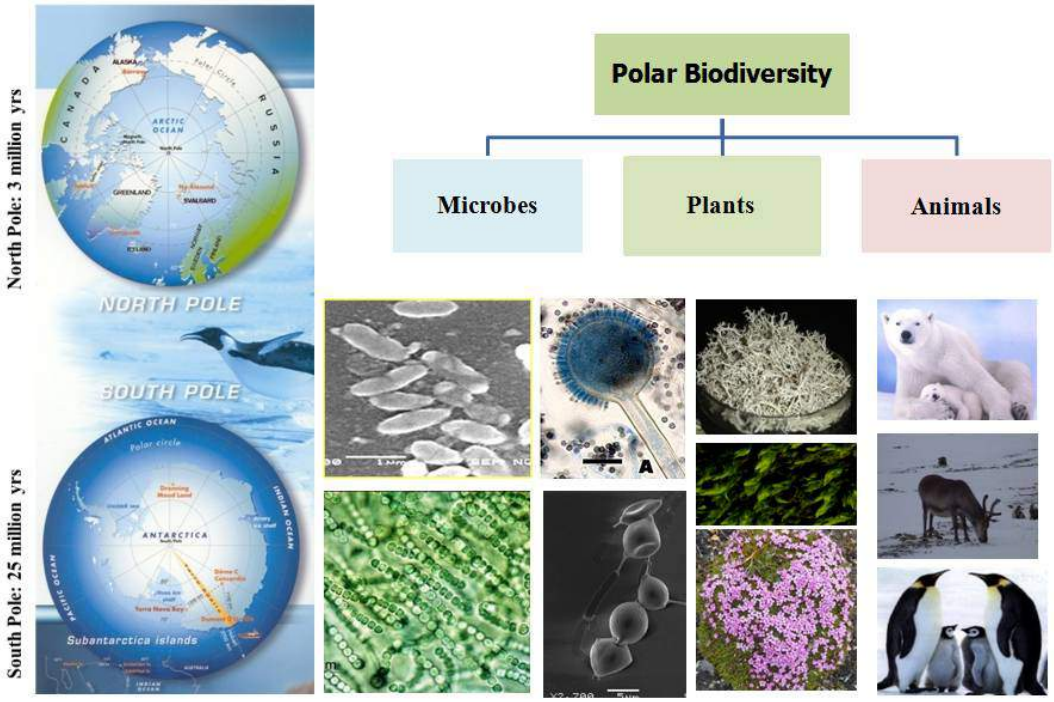

Fig. 1 Geographical locations of Poles and Polar Biodiversity in birds eye view.

1. REVIEW OF LITERATURE

Poles have lowest diversity on the Earth but not fully explored as yet. The studies on culturable diversity of glacier cryoconites and biotechnological potential of the polar organisms are fragmentary and still a thrust area of investigation.

Microscopy-based approaches towards cryoconite studies began with the study of brown polycellular algae by Leslie (1879). Later, algal and cyanobacterial communities from bipolar glaciers (Mueller *et al.* 2001, Mueller and Pollard 2004), cyanobacteria from Arctic glaciers (Kastovska *et al.* 2005, Stibal *et al.* 2006, Vonnahme *et al.* 2016), bacteria, cyanobacteria, protozoa and rotifers from Antarctic (Porazinska *et al.* 2004), viruses and virus-like particles from Arctic (Säwström *et al.* 2002, Anesio *et al.* 2007) and diatoms from Arctic glacier cryoconites (Yallop and Anesio 2010) were studied. Studies on microbial ecology of cryoconite holes have been limited even in the international scenario (Sawstrom *et al.* 2002, Anesio *et al.* 2009).

It is believed that the microbes in cryoconite granules play an important role in glacier ecosystems, but the information on their community structure is still limited, and their ecosystem functionality is poorly understood. Segawa *et al.* (2014) detected marker genes for nitrogen fixation, nitrification and denitrification from cryoconite granules and presented an evidence for the occurrence of nitrogen cycling in cryoconite granules on a glacier in Central Asia. Edwards *et al* (2014) assessed cryoconites community structure-function relationships from alpine regions through combined application of T-RFLP and FTIR. Ni *et al.* (2014) characterized the community composition and phylogenetic analysis of cyanobacteria from supraglacial cryoconite of Tianshan Mountains (China) by amplified 16S rRNA genes from the extracted cryoconite DNA. Webster-Brown *et al.* (2015) characterized Bacterial communities of Antarctic closed cryoconite holes (CCHs) by Automated Ribosomal Intergenic Spacer Analysis and high-throughput sequencing.

Pursuing the non-culturable approach, Edwards *et al.* (2011) deployed terminal restriction fragment length polymorphism (T-RFLP) analysis by sequencing of 16S rRNA gene clone libraries to examine the distributions of bacterial communities in cryoconite holes exisiting in the Austre Brøggerbreen (AB), Vestre Brøggerbreen (VB) and Midre Lovénbreen (ML). Cameron *et al.* (2011, 2016) and Stibal *et al.* (2015) endeavoured to reveal the bacterial and archaeal diversity of cryoconite microbial communities in several Arctic and Antarctic regions using terminal restriction fragment length polymorphism (T-RFLP), sequence analysis and quantatitative PCR of coextracted DNA and RNA genes. Grzesiak *et al.* (2015) investigated microbial community development on the surface of the Svalbard glaciers using denaturating gradient gel electrophoresis (DGGE) band-based clustering. Musilova *et al.* (2015) studied temporal, structural transformations of microbial communities and biogeochemical nutrient cycling on Greenland glaciers.

However, some researchers from this subcontinent have started nvestig their research and investigated bacterial diversity of sea habitat (Srinivas *et al.* 2009), streams (Reddy *et al.* 2009), antagonistic interaction networks in soil environment (Prasad *et al.* 2011), phosphate solubilising fungi (Singh *et al.* 2011b, Gawas-Sakhalkar *et al.* 2012), enzymes from soil fungi (Singh *et al.* 2012a), antioxidants of lichens and vascular plants (Singh *et al.* 2011a, Singh *et al.* 2012b) from the Svalbard Arctic.

Though in early endeavours, the culture-based techniques were used for cryoconite holes to examine the bacterial diversity in the Antarctic (Christner *et al.* 2003), and Alpine glacier (Lee *et al.* 2011). A novel species of bacteria (*Pedobacter cryoconitis*) has been recorded from alpine glacier cryoconites (Margesin *et al.* 2003). No work has been significantly carried out to comprehend the culturable bacterial diversity and biotechnological potentials of the cryoconites from Svalbard Arctic.

Similarly very limited work has been done on microbial diversity and biotechnological potential based studies using ice cores in the Polar Regions. Only recently, in order to evaluate the phylogenetic diversity of bacteria sealed in ice cores has been studied from Malan and Puruogangri glaciers of Tibetan Plateau (Zhang *et al*. 2002; 2003, Yao *et al.* 2006, Zhang *et al*. 2006, 2008), Muztag Ata glacier of Pamirs Plateau (Xiang *et al*. 2005) and Qinghan-Tibetan plateau of Western China (Christner *et al*. 2000, Christner *et al*. 2003). It is very important to note that very few studies have also been carried out on ice cores from Antarctica (Abyzov *et al.* 1998, Karl *et al*. 1999, Priscu *et al*. 1999, Abyzov *et al*. 2001, Christner *et al*. 2001, Brinkmeyer *et al*. 2003, Foreman *et al*. 2011, Shivaji *et al*. 2013). Though in some earlier studies Arctic glaciers populated by psychrophilic microbes had been recorded. Evidence of colonization of Arctic glaciers by psychrophilic microbes has also been recorded (Skidmore *et al*. 2000; 2005, Mindl *et al.* 2007). Only a few authors focused their investigations on microbial ecology and bacterial diversity of Svalbard surface glacial habitats (Mueller *et al*. 2001, Säwström *et al*. 2002; 2007, Kastovska *et al*. 2005; Anesio *et al*. 2007; 2009, Hodson *et al*. 2008; Edwards *et al*. 2011, Zeng *et al.* 2013). There has been a deep glacier ice core study from Greenland, the Arctic (Miteva *et al*. 2004; 2009; 2014, Knowlton *et al*. 2013) employing cultivation and culture-independent analyses. Microbial populations isolated from Arctic and Antarctic glaciers have also been correlated to past climate conditions (Priscu and Christner 2003). Miteva *et al.* (2004) studied phylogenetic and physiological diversity of microorganisms of Arctic (Greenland) glacier ice core while Butinar *et al.* (2007) isolated yeast from basal ice layers of high arctic glacier of the Svalbard archipelago. Besides this, no literature is available on the bacterial diversity and distribution patterns in ice cores from the northernmost area (Svalbard) of Arctic. Also no report is available on the investigation done on the screening of the ice core bacteria for the biotechnological potential (enzymes, AFPs, antioxidants). So far there is only one publication on AFPs of Arctic yeast (Amir *et al.* 2004).

Permafrost soils are chronological archives of past and present microorganisms (Friedmann 1994). The presence of bacteria in the permafrost was first reported at the end of 19th century with the discovery of mammoths in Siberia (Omelyansky 1911). Significant numbers of microbes have been isolated since then in both the Polar Regions from the cores several metres deep frozen grounds.

Though metagenomic efforts have shed light on the permafrost microbiome in some parts of polar regions, but on global perpective it represents a largely understudied genetic resource. Hu *et al.* (2014) investigated the fungal diversity and community structure in a 10-m-long permafrost core from the Qinghai-Tibet Plateau by culture-dependent technique combined with cloning-restriction fragment length polymorphism (RFLP) analysis. Subsequently, Hu *et al.* (2016) analysed prokaryotic diversity from the same permafrost core by restriction fragment length polymorphism analysis targeting the 16S rRNA gene. Hu *et al.* (2015) also studied the vertical distribution of bacterial communities in a Permafrost core from the Qinghai-Tibet Plateau. Wei *et al.* (2014) investigated the diversity and distribution of archaea community along a stratigraphic of an alpine permafrost profile by using 16S rRNA sequences analyses. Frey *et al.* (2016) applied a high-throughput sequencing of ribosomal markers to characterize the microbiota of 12000 yrs old alpine permafrost. Deng *et al.* (2015) investigated the shifts of bacterial and archaeal communities along a permafrost thaw gradient in Alaska by using Illumina MiSeq sequencing of 16S rRNA gene amplicons. Schostag *et al.* (2015) studied seasonal variations in the bacterial community of active layer of permafrost soil of Svalbard (78°N) by co-extracting DNA and RNA analyses. Coolen and Orsi (2015) studied metagenomics of Alaskan permafrost by using ultrahigh throughput Illumina HiSeq sequencing of reverse transcribed messenger RNA, and suggested a large metabolic potential of microbes for carbon processing, including pathways for fermentation and methanogenesis. Ganzert *et al.* (2014) investigated the bacterial communities of permafrost-affected soils of Greenland by using culture-independent and culture-

dependent methods and suggested that the shape of bacterial communities is significantly pH-dependent. Yun *et al.* (2014) studied the bacterial (methanotrophs) community structure in two permafrost of Tibetan Plateau (China) by using 16S rRNA-based quantitative PCR (qPCR) and 454 pyrosequencing. Yang *et al.* (2012) investigated community structure from permafrost soils along the China-Russia Crude Oil Pipeline and Steven *et al* (2013) studied the bacterial diversity from Canadian Arctic in permafrost employing pyrosequencing. Yergeau *et al.* (2010) studied functional potential of permafrost soil microbial communities by using metagenomic sequencing, quantative PCR (qPCR) and microarray analyses. Wilhelm*et al.* studied (2011) Microbial diversity of active layer and permafrost in an acidic wetland from the Canadian High Arctic. Hansen et al. (2007) investigated the viability, diversity and bacterial community composition in permafrost soil from Spitsbergen (78^{o}N) employing culture-dependent and culture independent methods.

Wagner *et al.* (2005) estimated the total microbial biomass in the Siberian permafrost to be comparable to that of communities of temperate soil ecosystems, descriptions of Canadian permafrost soils with respect to chemical properties and ecology were performed by Bölter *et al*. (2003a). Bacterial diversity of permafrost was studied from Canadian Arctic (James and Sutherland 1942, Steven *et al*. 2007), Alaska (Boyd and Boyd 1964), Russian Arctic (Gilichinsky *et al.* 1992, Shi *et al*. 1997, Vorobyova *et al.* 1997, Vishnivetskaya *et al.* 2000; 2006, Ponder *et al.* 2005, Zhang *et al.* 2013) and Antarctica (Cameron and Morelli 1974, Friedmann *et al.* 1996, Gilichinsky *et al*. 2007). The oldest cells from the Arctic dates back to ~3 million years while those from the Antarctic to ~5 million years (Gilichinsky *et al*. 2008). The microbes existing in the permafrost are crucial since they exhibit distinct physiological capabilities. A study on the reproduction and metabolism of the permafrost bacteria at subzero temperatures has been carried out (Rivkina *et al*. 2000, Bakermans *et al*. 2003). The presence of physiological and genomic heterogeneity in bacteria indicates that a wide diversity of bacteria survive in permafrost, most of which are ubiquitous

psychrotolerant/psychrophilic organisms and a few endemic ones. The co-existence of psychrophiles, psychrotolerants and mesophiles has also been shown by rRNA analyses in Siberian permafrost (Shi *et al*. 1997). A special ability of microorganism to withstand long phases of low nutrient concentrations has been observed (Bölter *et al.* 2003b). The studies on microbial activities in the Arctic soils have implications on soil ecology and global change (Bölter *et al*. 2004a, Oechel *et al*. 2000, Pears 1989). As cold-adapted enzymes have high thermo sensitivity, they have gained interest from biotechnology industry, in food preservation or bioremediation processes (Bej *et al*. 2010; Feller and Gerday 2003, Gerday *et al*. 1999). The work on microbial diversity of Arctic permafrost is also inadequet. There is no radiocarbon dating *vis-à-vis* bacterial diversity, antibiotic screening, carbon source utilization and enzymes screening data available that could suggest any information about microbial succession and characterizations over the years in the Svalbard (79^{o}N) Arctic.

Cold-active enzymes have ability to catalyze reactions at low temperatures, avoiding side reactions taking place at higher temperatures and preserving the integrity of products. Recently, De Santi *et al.* (2014, 2016) investigated the biotechnological potential of Arctic marine bacteria for their ability to produce a broad spectrum of cold-active enzymes. Park *et al.* (2014) identified proteolytic bacteria from the Arctic Chukchi seawater and subsequently Kim *et al.* (2015) studied the lipolytic enzyme in Chuckchi Sea bacterium, (*Psychrobacter* sp. ArcL13), by using statistical optimization and fed-batch fermentation. Han *et al.* (2015) investigated protease production by an Antarctic bacterium (*Pseudoalteromonas Arctica* PAMC 21717) *via* Statistical Optimization of Mineral Components and FED-Batch Fermentation. Han *et al.* (2015) nvestigation Protease production by an Antarctic bacterium (*Pseudoalteromonas arctica* PAMC 21717) via Statistical Optimization of Mineral Components and FED-Batch Fermentation. Qoura *et al.* (2014) purified and characterized a cold-adapted pullulanase from a psychrophilic bacterial isolate (*Shewanella arctica* 40-3), isolated from arctic sea ice. Kim *et al.* (2014) highlighted a new approach for discovering cold-active chitinase enzyme in a cell mixture of pure-cultured bacteria by colony-PCR with chi22718

III-specific primers. Kim *et al.* (2010) studied the diversity of cold-active protease-producing bacteria from arctic terrestrial and marine environments by using enrichment culture. Schmidt and Stougaard (2010) studied cloning and expression of a cold-active β-galactosidase from a novel Arctic bacterium *Alkalilactibacillus ikkense*. Similarly, Novototskaya-Vlasova *et al.* (2013) described cloning and expression of genes coding for lipase Lip2Pc and lipase-specific foldase LifPc from a psychrotolerant *Psychrobacter cryohalolentis* K5-T) isolated from a Siberian cryopeg (permafrost). Rasmussen *et al.* (2008) investigated the secretion of cold active β-1,3-glucanases by the arctic bacterial isolates *Flavobacterium* sp. strain 4221 and *Pedobacter* sp. strain 4236. Yu *et al.* (2011) isolated the novel cold-active esterases (EstM-N1 and EstM-N2), from a metagenomic DNA library of arctic soils. Bonugli-Santos *et al.* (2015) also highlighted diversity of fungal enzymes and their biotechnological applications from other than polar oceans.

Mushroom species could be used in food and pharmaceutical industries as natural antioxidants. Very oflate, antioxidant and anticholinesterase activities of mushroom species and their bioactive contents have been investigated (Tel-Cayan *et al.* 2016, Tel *et al*. 2015, Kamal *et al.* 2015, Sarikurkcu *et al.* 2015). Recently, Besse *et al.* (2015) investigated the ecological role or potential applications of antimicrobial peptides and proteins of Archaea from extreme environment.

Antifreeze proteins (AFPs) play important role in the survival of polar organisms and in health care applications. Lewis *et al.* (2016) highlighted the role of AFPs in the organs and other complex tissues banking. Olijve *et al.* (2016) studied thermal hysteresis (TH) and ice recrystallization inhibition (IRI) activity of all major classes of AFPs. Basu *et al.* (2016) reported AFP activity of midge (Chironomidae) due to a tyrosine-rich ice-binding site and atypical ice plane affinity. Davies (2016) investigated Antarctic moss epiphytic bacteria which secrete antifreeze proteins. Todde *et al.* (2015) studied the influence of antifreeze proteins of two families: hyperactive (snow flea-sfAFP) and moderately active (winter flounder-wfAFP) antifreeze proteins on the ice/water interface. Park *et al.*

(2012) characterized the ice-binding protein from Arctic yeast *Leucosporidium* sp. AY30, and subsequently Koh *et al.* (2015) studied the effect of the antifreeze protein from the arctic yeast *Leucosporidium* sp. AY30 on cryopreservation of the marine diatom *Phaeodactylum tricornutum.* Yoshida *et al*. (2016) investigated the structure and collective dynamics of hydrated anti-freeze protein type III from 180 K to 298 K by X-ray diffraction and inelastic X-ray scattering. Kar *et al.* (2016) studied structure and dynamics of Antifreeze Protein-Model membrane interactions by using a combined spectroscopic and molecular dynamics.

With the review of global (national and international) status, it becomes quite evident that cryoconites, ice-cores and permafrost are pristine habitats of Arctic constituting a major focus of research for bacterial and fungal diversity, enzymes, AFPs, PUFAs and therefore needed a systematic investigation.

Gap in Existing Research

Many organisms of Polar Regions have biotechnological value as sources of cryopeservatives, drugs and other pharmaceutical products. Biotechnological potentials of polar organisms have been very poorly explored so far.

Though, the non-culturable approaches namely T-RFLP, DGGE, qPCR were used for cryoconite holes to examine the bacterial diversity in the Antarctic, Arctic and Alpine glaciers, no significant work was carried out to comprehend the culturable bacterial diversity and biotechnological potentials of the Svalbard cryoconites. The systematic study has therefore been required to understand the adaptation strategies of bacteria and fungi from supraglacial cryoconite. The studies on adaptation strategies of bacteria and fungi from supraglacial cryoconite, were a gap area. No information on the characterization of the isolated bacterial and fungal strains for carbon utilization, and antibiotic resistance ability in the oligotrophic cryoconites, environment is available. Presence of heterotrophic organisms like filamentous fungi and yeasts in the cryoconite holes and their importance in driving the process of degradation of organic macromolecules

through the secretion of extracellular hydrolytic cold-adapted enzymes was poorly described. Although, the presence of organic carbon, nitrogen and phosphorus has been reported earlier, the lack of knowledge in detailed elemental composition of cryoconite sediments was still a gap area for investigation.

Similarly very limited work has been done on microbial diversity and biotechnological potential based studies using ice cores in the Polar Regions. No literature was available on the bacterial diversity and distribution patterns in ice cores from the northernmost area (Svalbard) of Arctic. No information has been available so far on the diversity of yeast distributed in the Ice cores of the Midre Love´nbreen glacier, their adaptation strategies and their biotechnological potential. Also no report was available on the screening of the ice core bacteria for carbon utilization, and antibiotic resistance ability.

The work on microbial diversity of Arctic permafrost is also inadequet. There is no radiocarbon dating *vis-à-vis* bacterial diversity data available that could suggest any information about microbial succession over the years in the Svalbard (79^{o}N) Arctic. No information on the characterization of the isolated bacterial and fungal strains for carbon utilization, enzyme production and antibiotic resistance ability in the oligotrophic environment permafrost is available.

Despite promising applications of enzymes in the fields of health, agriculture and industry, cold-active enzymes also play an important role in survival of bacteria and fungi in oligotrophic environment. Although a few studies on extracellular enzyme activities by marine Arctic bacteria are available; however no studies on the cold-active enzymes have been conducted so far on microbes of Arctic cryoconites, ice cores, and permafrost. Therefore, the present investigation has been the first ever enzymatic study on microbes of Arctic cryoconites, ice cores, and permafrost and is therefore has significance in terms of finding a potential for the future.

The study on antioxidants which play important roles in scavenging obnoxious free radicals produced during any physiological stress in Polar

environment is rather rare. No investigation on chemical composition of the antioxidant and its potential of any Arctic mushroom has been conducted so far.

As of now, the studies on antifreeze protein from the Arctic microorganisms are inadequate. Recently, AFP from a yeast strain *Leucosporidium* sp., isolated from a pond of Svalbard, and from *Pseudomonas putida*, isolated from plants in the high Canadian Arctic have been reported. However, there are no studies on AFPs purification and biochemical characterization from cryoconites, glacier ice cores, and permafrost.

Whole genome sequences of cryoconite bacteria (*Cryobacterium* sp. MLB-32), and 44800 yrs old permafrost bacteria (*Nesterenkonia* sp. PF2B19) were not available so far. The whole genome analyses of these species may unveil the mechanistic insights into the metabolic adaptation of bacteria to Arctic ecosystems. Keeping in background all the entire information endeavours were made to bridge the aforementioned gaps.

Objectives: The focus of the thesis work was therefore on the following objectives:

1. Assessment of microbial diversity of extreme habitats (glacial cryoconite holes, ice core and permafrost) and their identification up to the species level.

2. Screening and characterization of a few selected organisms for their biotechnological potentials.

3. Purification and Biochemical characterization of the most promising substance of biotechnological interest from the selected organism.

3. OBJECTIVE 1: *ASSESSMENT OF MICROBIAL DIVERSITY OF EXTREME HABITATS (GLACIAL CRYOCONITE HOLES, ICE CORE AND PERMAFROST) AND THEIR IDENTIFICATION UP TO THE SPECIES LEVEL.*

3a. Diversity of bacteria from Cryoconites, Ice Cores and Permafrost

3a.1 Introduction

Glaciers ice cover about 10% of the Earth's surface (Benn and Evans 1998). Despite the environmental stresses such as sub-zero temperatures, freeze–thaw cycles, irradiation, nutrient deficiency and long phase of darkness, microbial communities survive in the subglacial and supraglacial cold habitats. This number however is much lower compared to most of the ice-free environments.

Cryoconite holes are formed when dark material (e.g. soil, dust, microbes and mineral) is deposited onto the surface of the glacier. The low albedo of this material causes increased absorption of solar energy, relative to the surrounding ice, resulting in melting of ice below. As the ice melts, a waterfilled hole is created into which dark material sinks (Gajda 1958, McIntyre 1984, Cameron *et al.* 2011). Cryoconites are reported to cover up to 10% of the glacier surface ranging in diameter from a few centimeters to more than a metre (Anesio *et al.* 2009). The cryoconite holes in Svalbard average about twelve holes per meter square to represent ~6% of the glacier surface. Typically, the depth of cryoconite holes on the Svalbard glaciers were measured to be of ~8–30 cm and 5–50 cm in diameter containing debris of about 0.1–1,000 g (Gerdel and Drouet 1960, Säwstörm *et al.* 2002).

The cryoconite holes demonstrated a couple of distinct zones- the pelagic and the benthic. The pelagic zone consists of the meltwater whereas the benthic zone contains soppy, small, spherical dark granular material (0.1–3 mm in diameter). Microbial communities sheltering in the cryoconites thrive in the presence of nutrients leached from the mineral sediments and build a unique and biologically active microhabitats within these ice entombed environments (Säwström *et al.* 2002, Foreman *et al.* 2007).

The specific aim of the present study was, first, to explore whether cryoconites microbial communities could exhibit variability within the three

aforementioned glaciers of High Arctic. Secondly, the study was focussed to characterize the physiological characteristics of dominant bacterial strains and screen them for their biotechnological potentials. Taken together, these studies were aimed to ameliorate our understanding about the diversity, possible origins and functions of microbial communities in cryoconite holes.

Glacial ice cores are cylindrical cores drilled into the glacial ice from the surface to the deeper layers. These ice cores are natural archival of wind-transported microorganisms of past and present along chronology. Midre Lovénbreen is a well-investigated glacier and represents a mass balance record since 1960s (Hagen *et al.* 2003). Although several studies on this glacier were completed, no significant information on the microbial diversity, adaptation mechanism and biotechnological potential of cold tolerant bacteria and yeasts in the ice cores of Midre Lovénbreen glacier is available. In current investigation, the bacterial abundance and culturable bacterial diversity was studied in depth. Study on phenotypic characteristics was performed, and 16S rRNA gene sequences of these ice core bacterial isolates were carried out to comprehend the composition of bacterial isolates within the ice core. Additionally, these bacteria were tested for the production of fatty acids and cold-active enzymes.

Permafrost soils are defined by their subzero temperatures throughout two or more consecutive years. The layers of soils which lie above the permafrost and undergo annual freeze-thaw cycles are known as the active layer (Muller 1947, ACGR 1988). The northern hemisphere is covered by more than 25% permafrost of the land surface (Zhang *et al.* 1999). The importance of permafrost as a model of extraterrestrial habitats was reported by Gilichinski (2002).

The present study was conducted with an objective of chronological illustration of bacteria in the sub-surface and permafrost layers of five pit profiles at Svalbard, Arctic. Characterization of the isolated bacterial strains for carbon utilization, enzyme production and antibiotic resistance ability was also accomplished.

3a.2 Materials and methods

3a.2.1 Sampling procedures

3a.2.1.1 Cryoconites sampling

Two polythermal valley glaciers namely, Midre Lovénbreen (ML) and Vestre Brøggerbreen (VB), and a cold-valley glacier Austre Brøggerbreen (AB) (Hagen and Satrang 1991) located in the Kongsfjord region of Spitsbergen (78º53`N, 12º04` E) were selected for the present investigation (Fig. 2, a-c). The five cryoconite hole samples (Fig. 2, d-f) were collected from each of these three glaciers during Arctic summer. The temperature of water in the cryoconite holes was measured between 0.2–1.9 °C and pH 7.1–8.6. Cryoconite samples were collected by aspirating spherical dark granular material (debris) using a sterile 50 milliliter syringe, placed in sterile ampules and stored at -20ºC until analyses.

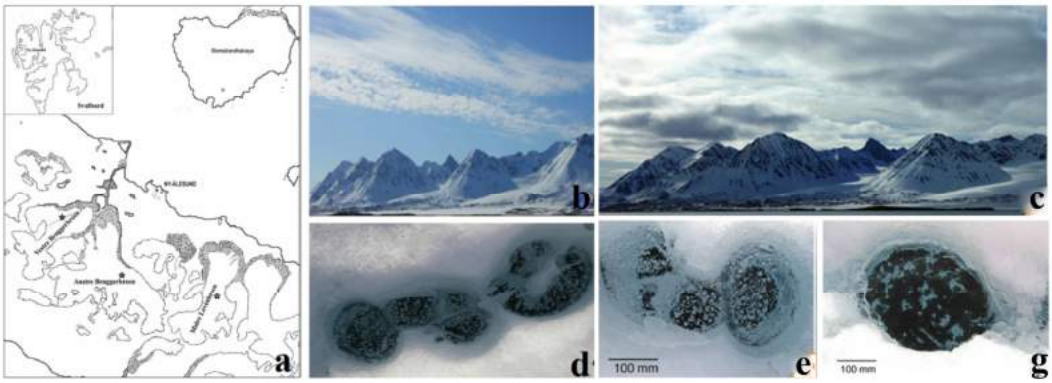

Fig. 2 a) An outline map of the coast of Spitsbergen, showing cryoconites sampling (star circle) over the glaciers (Midre Lovénbreen, Vestre Brøggerbreen and Austre Brøggerbreen), b-c) Landscape of Svalbard valley glaciers, d-e) 'Cryoconite holes' exposed after the melt of soft ice during Arctic summer, f) Manually opened a cryoconite hole prior to sampling.

3a.2.1.2 Ice cores sampling

For the current study, ice cores samples were collected from Midre Lovénbreen glacier, located in Ny-Ålesund (78°55`N, 11°56` E), Svalbard archipelago, the Arctic (Fig. 3, a-c). Using a cleaned hand-operated corer, up to 1 metre depth was

cored in the Ice cores (Fig. 3, d) across 4 different locations [**1**: 78°52′.815″ N 12°02′.404″ E, 273 m a.s.l.; **2**: 78°53′.006″ N 12°02′.610″ E, 242 m a.s.l.; **3**: 78°53′.197″ N 12°02′.940″ E, 205 m a.s.l.; **4**: 78°53′.670″ N 11°50′.770″ E, 164 m a.s.l.], on the glacier between 164 to 273 m above sea level, following strict contamination-free procedures (Veysseyre *et al*. 2001). Each of the core was subsequently sliced into three segments (~33 cm each), which were stored in the frozen state until analysis (Fig. 3, e, g). The inner part of the sub-samples were thawed and stored in sterile containers inside a laminar flow for avoiding any contamination.

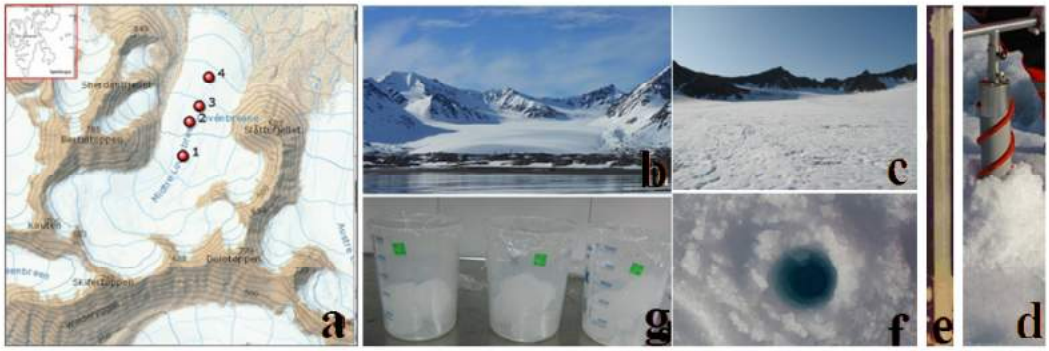

Fig. 3 a) Ice core sampling locations 1-4, b-c) Landscape of Midre Lovénbreen glacier, d) Ice coreing, e-g) Ice core sample f) Ice cored point.

3a.2.1.3 Permafrost sampling

Permafrost sampling was done on the west coast of Spitsbergen, Svalbard archipelago. Topographical features of this region include glaciers, moraines, glacial streams and rivers flowing northward to Kongsfjord. The surface soils are loose and poorly structured (Klimowicz and Uziak 1988). The samples were collected from five different locations: 78°55.082′ N, 11°51.527′ E; 78°55.165′ N, 11°52.660′ E; 78°55.254′ N, 11°54.256′ E; and 78°54.978′ N, 11°57.330′ E; 78°54.817′ N 11°58.378′ E (Fig. 4, a). Pits were dug by a mini excavator (and sterile shovel, digging bar, trowel) at these locations (Fig. 4, b-d). The depths of these five pits ranged from 1.5 to 1.8 m. Surface soils were collected from different depths, and from top layer as recent soil collected from just 5 cm below

from surface, middle layer from active zone and bottom layer of pit from the permafrost zone. As the study included microbiological observations, collections were made using sterile sample collectors (Himedia), to avoid contamination. Samples were stored at -20^{o}C until analyses.

Fig. 4 a) Map of Spitsbergen, Arctic showing sampling sites (•1 to •5), b) Landscape of permafrost, c) Permafrost sampling near Vestre Brøggerbreen glacier, d) Permafrost pit profile.

3a.2.2 Determination of Total counts (TC)

Total count was estimated following the method of Kuwae and Hosokawa, (1999). One g of sediment of cryoconite and permafrost were aseptically added to 5 ml of filter-sterilized saline each and vortexed thoroughly to separate the microbial cells from the sediment grains. One milliliter (mL) of ice core melt water was aseptically added in 5 ml of filter-sterilized saline and slowly vortexed to dislodge the microbial cells. One ml of the suspension was added to 20 µl DAPI (4,6-Diamino-2-phenylindole) solution (0.25% w/v in sterile distilled

water) taken in an autoclaved vial. After incubating in the dark for 30 minutes, the sample was filtered using a 0.2 µm Nuclepore polycarbonate track etched membrane filter. The unbound DAPI was washed off by rinsing with a few drops of sterile saline. The membrane filter was examined under epifluorescent microscope (BX-51, Olympus), using non-fluorescent immersion oil. Bacterial cells appeared as blue spots against a dark background were enumerated and the bacterial cell number per gm soil was calculated.

3a.2.3 Isolation and culturing of bacteria and determination of Colony Forming Units (CFU's)

One gram of sediment from cryconite and permafrost was suspended in 9.0 millilitre of autoclaved saline solution and serially diluted up to third dilution (10^{-1}, 10^{-2} and 10^{-3}) while ice core melt water (100 µl) was directly plated onto media plates. Isolation of culturable bacteria was performed by spread plate method (0.1 mL) on Nutrient Agar (NA), 1/10 NA, Zobell Marine Broth (MB), 1/10MB, Antarctic Bacterial Medium (ABM), 1/10 ABM and incubated at two different temperature 4°C, 15°C and 22°C for 14-30 days. The colonies appearing after incubation were counted subsequently and CFU number per gram soil/per mL (ice melt water) calculated. Isolates were subsequently picked up randomly based on unique morphotypes from each plate. The pure isolates obtained were stored in 20% glycerol stock at -70ºC.

3a.2.4 Phenotypic characterization of the bacterial isolates

The observations of colony characteristics were performed using stereo-zoom microscope (Nikon SMZ1500). Carbon source utilization tests were carried out using HiCarbo^{TM} kits (KB009, HiMedia, India) with little modification (incubated for 2-7 days at 15°C, the optimum growth temperature of isolated strains). Ortho-Nitrophenyl-β-D-galactopyranoside (ONPG) test is used to detect β-galactosidase activity in the organism. Galactosidase is required for lactose utilization and it hydrolyzes ONPG into galactose and nitrophenol. Esculin utilization test of

isolates detected esculin hydrolysis; citrate and malonate utilization tests detected capability of bacteria to utilize citrate and sodium malonate as sole carbon sources. Growth pattern at different temperatures (4, 15, 22 and 30°C), pH (4.0, 5.0, 6.0, 7.0, 8.0 and 9.0) and salt concentrations (2, 5 and 7%) was carried out on ABM plates following standard protocol.

3a.2.5 Antibiotic susceptibility Test

The disc diffusion method (Bauer *et al.* 1966) with slight modification was used to screen the susceptibility of the isolates towards antibiotics. The bacterial cultures were grown at optimum temperature 15 °C in nutrient broth up to turbidity of 0.08-0.13 and optical density (OD) measured at 620 nm. Using sterile spreaders, 100 µl of the bacterial suspensions were spread onto the surface of Mueller Hinton agar (MHA) medium. Antibiotic discs were carefully placed over the uniformly spread bacterial inoculum and the plates were kept for incubation at 15 °C for 3-4 days. The zone of inhibition (in millimeter) was inspected and recorded.

3a.2.6 Molecular characterization

3a.2.6.1) *Polymerase Chain Reaction (PCR) and DNA Sequencing*

Freshly grown pure bacterial isolates of cryoconite and Ice cores were used for total DNA extraction using DNA Extraction Kit (BIO-RAD Laboratories). Amplification of extracted DNA was performed by PCR method using DNA polymerase (Toyobo Co, Ltd, Japan). The 16S rRNA gene was amplified using the universal primers: 9F (GAGTTTGATCCTGGCTCAG) and 1541R (AAGGAGGTGATCCAGCC). The amplification cycle involved an initial denaturation step at 95 ºC for 2 min and then DNA was amplified in accordance with the following 35 cycles; 95 ºC for 1 min to denature double strand DNA, 52 ºC for 30 s for annealing, and 72 ºC for 1.0 min for elongation. A final elongation step was carried out at 72 ºC for 7 min. DNA sequences were obtained with ABI prism 3100 Sequencer (Applied Biosystems) following ABI standard protocol.

Genomic DNA from each permafrost culture was extracted by the method described by Pospiech and Neumann (1995). 16S rRNA was amplified using primers pA and pH (Edwards *et al*. 1989) from the extracted genomic DNA. PCR was performed under following conditions: initial denaturation at 94 °C for 5 min, followed by 40 cycles of 30 s each at 94°C (denaturation), 45s at 52°C (annealing) and 1 min 30 s at 72 °C (extension) and a final extension of 7 min at 72 °C. Amplification products were resolved in ethidium bromide stained 1.5% agarose gel, and gel images were captured using Alpha-Imager system.

Purification of amplified 16S rRNA was done using a Quaquick purification kit (Qiagen). Sequencing of both strands was carried out using the same PCR primers for di-deoxy fluorescent terminators (ABI Big Dye BDT v3.1).

3a.2.6.2) ***DNA Sequence alignment and Phylogenetic analysis***

Sequence alignment of 16S rRNA region of each bacterial isolate was performed using Clustal W option of the software Molecular Evolutionary Genetics Analysis (MEGA v4.0) (Tamura *et al*. 2007). The DNA sequences of bacterial isolates were subjected to a NCBI BLAST search, and the sequences were deposited in the DNA data bank (NCBI). The 16S rRNA region sequences were aligned using Clustal W together with the homologous Genbank sequences of closely related species.

To calculate the sequence similarity for 16S rRNA region the matrix was analyzed with the Neighbour joining method (Saitou and Nei 1987) using the Tamura-Nei model (Tamura and Nei 1993) and Maximum Parsimony method (Tamura *et al.* 2011). The bootstrap consensus tree (Felsenstein 1985) was inferred from 1000 replicates to represent the evolutionary history of the taxa analyzed.

3a.2.7 Fatty Acid Methyl Esters (FAME) Analysis

The freeze dried bacterial culture (100 mg) was used for whole-cell fatty acids extraction using acid methanolysis method. The analysis was performed by Gas Liquid Chromatography (GLC) equipped with flame ionization detector (model GC353, GL Science) at an initial temperature of 140°C for 15 min and then increased to 240°C at an increment of 4°C min^{-1} (Yumoto *et al.* 2001). Fatty acids were identified by comparison with FAME mix standards (Supelco 37 component FAME mix).

3a.2.8 Radiometric dating

^{14}C radiocarbon dates of the two depth (middle and bottom layer) samples from five pits were analyzed by the National Ocean Science Accelerator Mass Spectrometry (NOSAMS) in Woods Hole Oceanographic Institute, USA, by Accelerator Mass Spectrometeric analysis.

3a.2.9 Statistical analyses

PAST software ver. 2.01 (Hammer *et al.* 2001) were used for diversity and evenness indices.

3a.3 Results

The culture-based approach used in the present study has the advantage over culture-independent approaches in several perspectives. Further, the characterization of culturable microorganisms could provide valuable information of several taxa of the community, and thus strengthen our understanding regarding the community structure derived from molecular approaches (Jiang *et al.* 2006). Additionally, a culture-based approach could be used to compile a collection of microorganisms for conducting biochemical and genetic experiments, and to study the inter- and intra-species interactions (Jiang *et al.* 2006).

3a.3.1 Total count (TC) and culturable bacterial count

i) Cryoconites

The cultivable bacterial count (colony forming units, CFU) in the cryoconites varied in different samples. The count varied from 2.7 x 10^3 to 3.02 x 10^4 (mean 1.13 x 10^4 ± 0.84 x 10^4) CFU/g at ML, 2.3 x 10^4 to 8.8 x 10^4 (mean 4.97 x 10^4 ± 3.40 x 10^4) CFU/g at AB and 3.00 x 10^4 to 3.1 x 10^4 (mean 3.02 x 10^4 ± 0.04 x 10^4) CFU/g at VB glaciers.

Total bacterial count per gram through DAPI showed varying trends at ML [5.07 x 10^5 to 11.9 x 10^5, (mean 8.16 x 10^5 ± 3.07 x 10^5)], AB [1.24 x 10^6 to 1.50 x 10^6, (mean 1.34 x 10^6 ± 0.14 x 10^6)] and VB [1.17 x 10^6 to 1.33 x 10^6 (mean 1.24 x 10^6 ± 0.08 x 10^6)] glaciers.

ii) Ice cores

The viable bacterial count in ice cores ranged from 10-7000 CFU/ mL (mean 1105 CFU/ mL), 10-850 cells/ml (mean 349 CFU/mL) and 10-4950 CFU/mL (mean 725 CFU/mL) in the top, middle and bottom layers respectively. The total bacterial count in the ice core samples as determined by DAPI staining method ranged from 7.20 x 10^4 to 2.59 x 10^7 cells/mL (mean 5.3 x 10^6 cells/mL) in the top layer, 1.18 x 10^5 to 1.15 x 10^7 cells/mL (2.07 x 10^6 cells/mL) in the middle layer and 1.14 x 10^5 to 1.19 x 10^7 (mean 1.83 x 10^6 cells/mL) in bottom layer. The overall total count ranged from 7.20 x 10^4 to 2.59 x 10^7 cells/mL (mean 3.12 x 10^6 cells/mL).

iii) Permafrost

The top layers (sub-surface) of the pits had a total bacterial count ranging from 2.33 x 10^5 to 4.67 x 10^5 cells/g soil (mean 3.8 x 10^5 cells/g soil). For other soil depths the total count ranged between 1.14 x 10^5 and 5.52 x 10^5 cells/g soil as determined by DAPI staining technique (Table 1). The culturable bacterial counts through direct plating in the top layers (sub-surface) of pits ranged from 9.00 x

10^4 to 3.26 x 10^5 CFU/g (mean 1.82 x 10^5 CFU/g). In rest of the soil depths the culturable count ranged between 1.50 x 10^3 to 2.22 x 10^5 CFU/g (Table 1).

Table 1. Mean values of total and culturable counts in the permafrost pit profile soils.

Sampling location	Elevation m a.s.l	Pit Depth (In meter)	Sampling depth (in cm)	Sediment/soil age (yrs before present)	Direct count	Culturable count
					(Mean)Cells/g	(Mean)CFU/g
				Recent	3.80 x 10^5	1.82 x 10^5
PF-5 78°54.817′ N 11°58.378′ E	17	1.5m	75-85	12250	3.18 x 10^5	6.25 x 10^3
			140-150	14150	5.52 x 10^5	1.50 x 10^3
PF-1 78°55.082′N, 11°51.527′ E;	36	1.8m	80-90	13850	1.49 x 10^5	6.88 x 10^4
			170-180	22700	1.88 x 10^5	3.08 x 10^4
PF-4 78°54.978′ N, 11°57.330′ E	31	1.5m	75-85	14850	2.12 x 10^5	1.80 x 10^5
			140-150	24300	2.45 x 10^5	2.22 x 10^5
PF-3 78°55.254′ N, 11°54.256′ E	30	1.5m	75-85	32300	4.40 x 10^5	7.60 x 10^4
			140-150	37000	3.61 x 10^5	6.33 x 10^4
PF-2 78°55.165′ N, 11°52.660′ E	60	1.5m	75-85	38300	1.14 x 10^5	6.45 x 10^4
			140-150	44800	2.83 x 10^5	7.75 x 10^4

3a.3.2 Characteristics of the bacterial isolates

i) Cryoconites bacteria

Of the numerous colonies appearing on the culture media plates, 22 morphologically distinct isolates were purified for further studies (Table 2). Out of these, 4 isolates were from AB, 6 from ML, and 12 from VB glaciers. The bacterial colonies were of different colours - cream, grey, orange, yellow and white (Fig. 5, a). The colony characteristics such as entire margin; opaque and smooth texture; and the surface appeared convex along with pulvinate to raised elevation. The shape of bacterial cells were cocci and rods (Fig. 5, b-c). The rod-shaped bacteria ranged in size from 0.15 -0.5 μm x 0.07-0.17 μm while the cocci forms ranged from 0.22 to 0.25 μm in diameter. Majority of the isolates showed

growth temperature ranging from 1 to 22°C, except one isolate at 30°C. It is notable to observe that the optimum temperature for growth was 15°C. The representative strains were therefore psychrophilic in nature. The growth for the isolates were recorded at 2 and 5% salt (NaCl) concentration but no growth at 7% salt concentration (Fig 6) was observed. The optimum pH values recorded for the bacterial isolates were between 6.0 and 7.0.

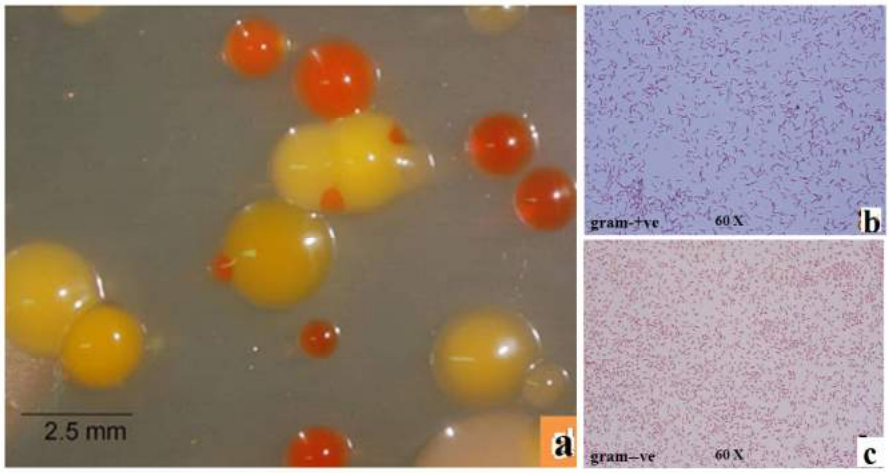

Fig. 5 a) Bacterial colonies grown on culture media at 4 °C for 30 days, b-c) Gram staining of cryoconite bacteria (60x).

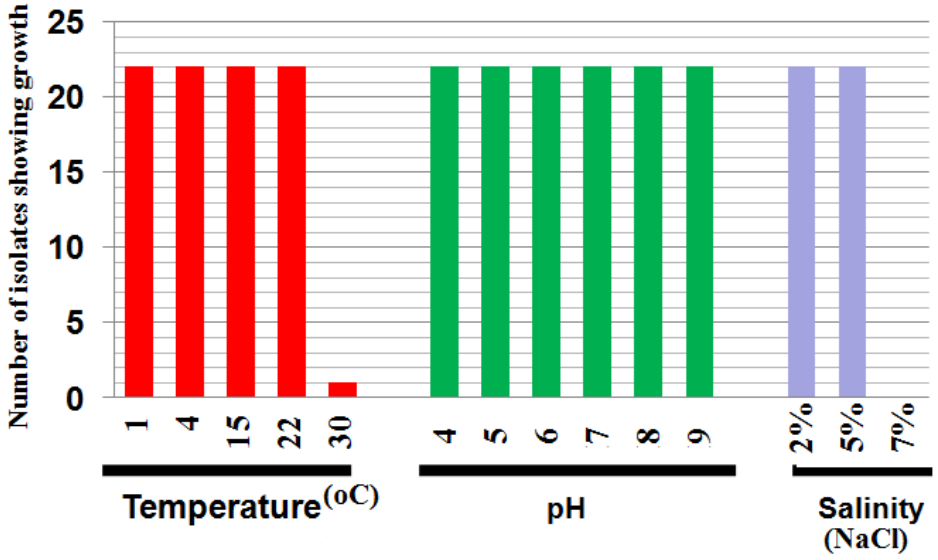

Fig. 6 Cryoconite isolates showing growth at different temperature, pH and salt tolerance.

Table 2. Distribution, morpho-taxonomic features and physiological characteristics of cryoconite bacterial isolates.

Isolate Code	Culture Characteristics											Physiological characters													
	ML	AB	VB	Colony colour	Colony Shape	Colony margin	Colony Elevation	Colony Texture	Colony opacity	Cell Shape	Cell Size (µm)	Growth Temp. Range(1-30^{0}C) 1	4	15	22	30	Growth at NaCl (%) 2	5	7	Growth at pH 4	5	6	7	8	9
MLB42	+			Orange	Round	Entire	Convex	Smooth	Opaque	Short rods	L(0.17to0.39) B(0.078to0.099)	+	+	++	+	+	++	+	-	+	+	++	++	+	+
MB-5	+			Cream	Round	Entire	Convex	Smooth	Opaque	Short rods	L(0.24to0.32) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-40	+			Orange	Round	Entire	Convex	Smooth	Opaque	Short rods	L(0.15to0.30) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-30	+			White	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.20to0.30) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-47		+		Cream	Round	Entire	Convex	Smooth	Opaque	Short rods	L(0.22to0.32) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
NA-9		+		White	Round	Entire	Convex	Smooth	Opaque	Short rods	L(0.27to0.32) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-34		+		Light	Round	Entire	Convex	Smooth	Opaque	Short rods	L(0.32to0.38) B(0.099to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-44			+	Cream	Round	Entire	Convex	Smooth	Opaque	Short rods	L(0.19to0.32) B(0.14to0.17)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-45			+	Greyish	Round	Entire	Raised	Smooth	Opaque	Short rods	L(0.25to0.34) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-36			+	Orange	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.15to0.36) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-41			+	Yellow	Round	Entire	Pulvinate	Smooth	Opaque	Cocci in pairs, tetrad & in group	L(0.22to0.24) B(0.22to0.25)	+	+	++	+	-	++	+	-	+	+	++	++	+	+

MLB-35			+	White	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.17to0.50) B(0.099to0.12)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-38			+	Orange	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.19to0.34) B(0.099to0.12)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-39			+	Orange	Round	Entire	Pulvinate	Smooth	Opaque	Very Short rods	L(0.12to0.17) B(0.078to0.12)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
NA-b	+			Yellow	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.17to0.39) B(0.070to0.078)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-29		+		Cream	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.21to0.32) B(0.070to0.078)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-43	+			Yellowish	Round	Entire	Convex	Smooth	Opaque	Long rods	L(0.14to0.29) B(0.070to0.078)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MB-g			+	Yellow	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.26to0.37) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-32			+	Orange	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.15to0.33) B(0.078to0.078)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-33			+	Orange	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.17to0.29) B(0.078to0.078)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-31			+	Red	Round	Entire	Pulvinate	Smooth	Opaque	Short rods	L(0.19to0.37) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+
MLB-46			+	Brownish	Round	Entire	Convex	Smooth	Opaque	Short rods	L(0.22o0.34) B(0.078to0.099)	+	+	++	+	-	++	+	-	+	+	++	++	+	+

ii) Ice cores bacteria

Of the numerous colonies appearing on the media agar plates 14 distinct isolates were purified for further studies. The colonies of different hues from cream, light brown, orange, yellow and white, were recorded. The colonies posessed erose to entire margin, texture smooth or rough, and the surface convex, pulvinate to umbonate elevation. The bacterial cells appeared short rods with size varying from 1.0-2.8 μm x 0.7-1.2 μm. The isolates were tested for their physiological characteristics and it was observed that the culture grew between -1 and 30 °C. In total, 10 bacterial isolates showed growth at 2% salt (NaCl) concentration whereas only eight isolates survived at 7% salt concentration (Fig 7). All the isolates grew at pH 7.0 to 9.0. While 12 cultures survived a lower pH range of 5.0 to 6.0, no culture survived at pH 4.0 (Table 3).

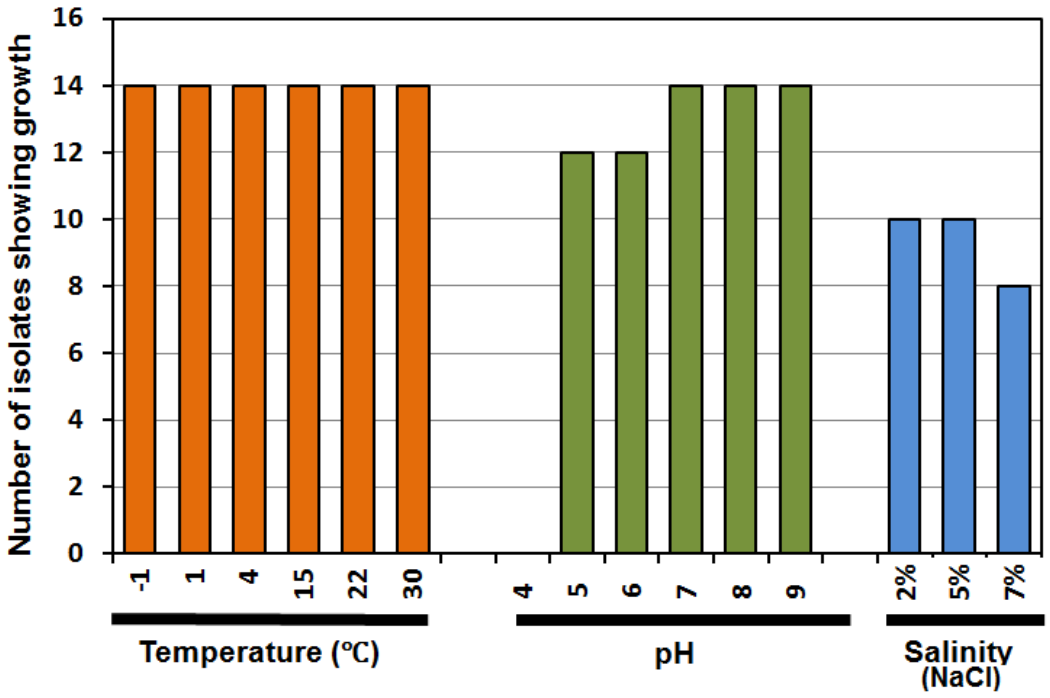

Fig. 7 Number of Ice cores bacterial isolates showing growth temperature, pH and salt tolerance.

Table 3. Growth of Ice cores bacterial isolates at different temperature, pH and salt concentrations

Sr. No.	Isolate detail	Temp^{0}C						pH						NaCl %		
		-1	1	4	15	22	30	4	5	6	7	8	9	2	5	7
1	MLB-2	+	+	++	++	++	++	-	++	++	++	++	++	++	+	+
2	MLB-3	+	+	++	++	++	+	-	+	++	++	++	++	++	+	+
3	MLB-4	+	+	+	+	+	++	-	+	+	+	+	+	+	+	-
4	MLB-5	+	+	+	+	+	++	-	+	+	+	+	+	+	+	-
5	MLB-6	+	+	++	++	++	++	-	++	++	++	++	++	++	+	+
6	MLB-7	+	+	+	++	++	+	-	-	-	+	W	+	-	-	-
7	MLB-8	+	+	++	++	++	++	-	+	++	++	++	++	++	+	+
8	MLB-9	+	+	+	++	++	+	-	+	+	+	+	+	-	-	-
9	MLB-11	+	+	+	++	++	+	-	-	+	+	+	W	-	-	-
10	MLB-12	+	+	++	++	++	++	-	+	++	++	++	++	++	+	+
11	MLB-14	+	+	++	++	++	++	-	++	++	++	++	++	++	+	+
12	MLB-15	+	+	+	+	+	+	-	++	-	+	+	+	-	-	-
13	MLB-16	+	+	++	++	++	++	-	+	+	+	++	+	+	+	+
14	MLB-17	+	+	++	++	++	++	-	+	++	++	++	++	++	+	+

iii) **Permafrost bacteria**

106 distinct isolates were purified out of the numerous colonies appearing of the culture media plates. These selected colonies were of different colours - white, cream, yellow, pale pink and pale brown; they were characterized by colony morphology which was irregular, erose, undulate to entire margin; surface

appeared smooth texture and varied from flat, raised, convex, pulvinate to umbonate elevation. The shape of the bacterial cells varied from curved rods and short rods to long rods with size ranging from 0.54-1.3 µm x 1.53-0.5 µm. As many as thirty three representative isolates were tested for their physiological characteristics. It was observed that five of the cultures PF1B3, PF2B4, PF4M4, PF1T3 and PF4T2 could grow between temperatures 1 to 22°C while rest of the isolates grew between temperatures of 1 to 30°C (Fig. 8). All 33 cultures showed salt tolerance to 2 to 7% NaCl and showed visible growth between the pH range of 4.0 to 9.0 (Table 4).

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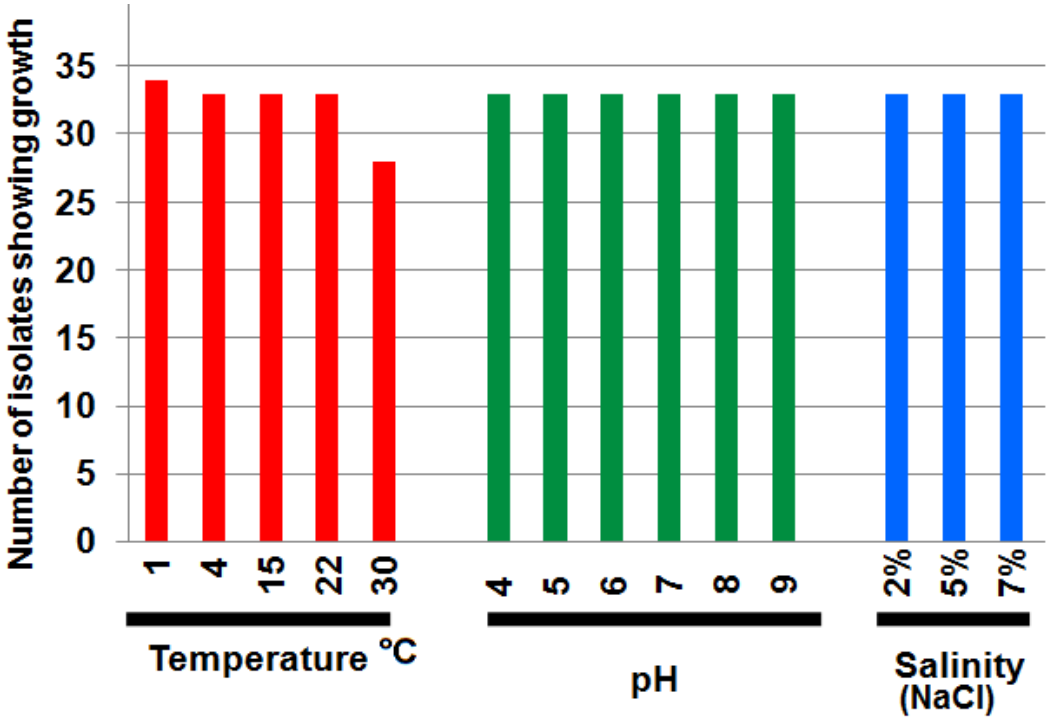


Fig. 8 Permafrost bacteria showing growth temperature, pH and salt tolerance.

Table 4. Physiological characteristics of the permafrost bacterial isolates.

Identification	Strain no.	Temp^{0}C					pH						NaCl%		
		1	4	15	22	30	4	5	6	7	8	9	2	5	7
Acinetobacter johnsonii	PF1B1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter bergerei	PF1T1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF3B3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF3B2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF2M1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF3B4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF4B2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF2M3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF4M4	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF1T4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF4T1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF4T2	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF5B1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF5M1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sulphonivorans	PF1T3	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF3T1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arthrobacter sp.	PF4M3	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Bacillus sp.	PF2B4	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Pseudomonas jessenii	PF2M8	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mandelii	PF2M12	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp.	PF2M7	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas mandelii	PF3M2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp.	PF1B3	+	+	+	+	-	+	+	+	+	+	+	+	+	+
Pseudomonas sp.	PF3B5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp.	PF2M9	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp.	PF3T4	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp.	PF3T5	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp.	PF3T2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pseudomonas sp.	PF5T1	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Psychrobacter sp.	PF5B2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sphingobacterium sp.	PF3T6	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Stenotrophomonas sp.	PF1M2	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Virgibacillus pantothenticus	PF1B6	+	+	+	+	+	+	+	+	+	+	+	+	+	+

3a.3.3 Antibiotic resistance patterns of the isolated strains

i) Cryoconites bacteria

The isolates were screened for antibiotics sensitivity and it was observed that all isolated were sensitive to antibiotics such as amikacin, cefotaxime, doxycyline hydrochloride, kanamycin, levofloxacin, lomefloxacin, polymyxin-B, piperacillin, and tetracycline (Table 5). Methicillin was the least effective antibiotics screened, susceptible to only two isolates (MLB-40 and NA-9). Of the various isolates tested, *Cryobacterium* sp. NA-9 was the most sensitive isolate having sensitivity towards all the antibiotics tested while *Cryobacterium* sp. MLB-31 was the most resistant showing resistance to 24 of the 45 antibiotics screened. The susceptibility to other antibiotics showed varied response from isolate to isolate (Fig. 9).

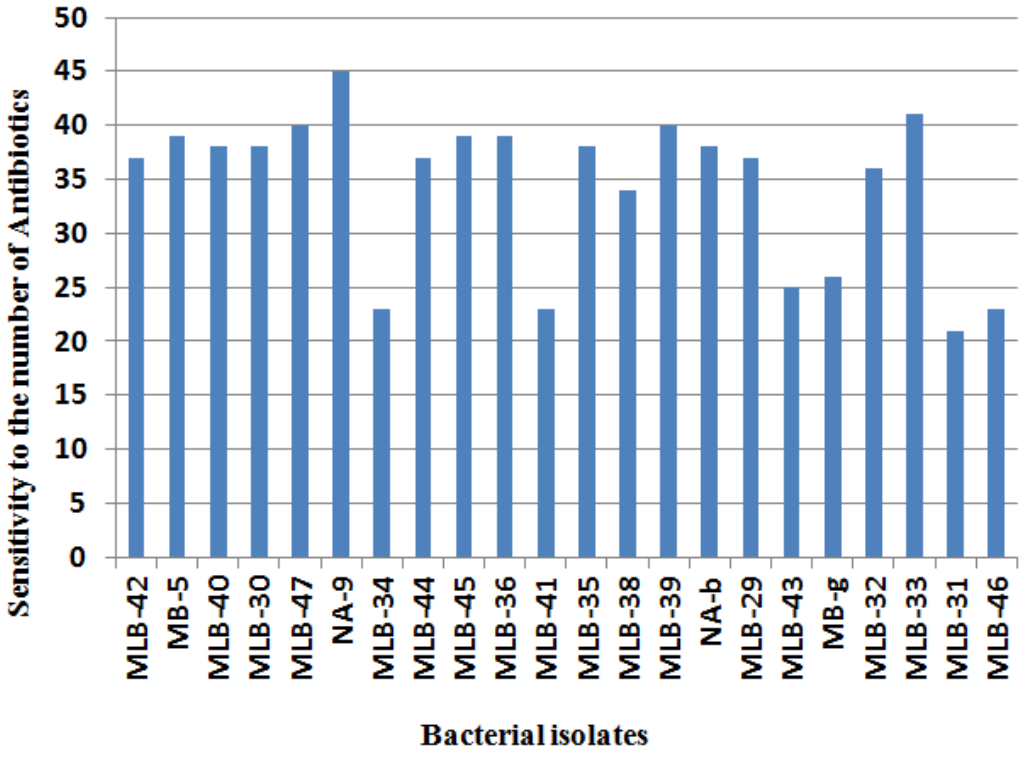


Fig. 9 Cryoconite bacterial isolates showing Antibiotic resistance.

Table 5. Antibiotic resistance patterns of the cryoconite bacterial isolates.

Sr.No.	Antibiotics	Isolate code																					
		MLB-42	**MB-5**	**MLB-40**	**MLB-30**	**MLB-47**	**NA-9**	**MLB-34**	**MLB-44**	**MLB-45**	**MLB-36**	**MLB-41**	**MLB-35**	**MLB-38**	**MLB-39**	**NA-b**	**MLB-29**	**MLB-43**	**MB-g**	**MLB-32**	**MLB-33**	**MLB-31**	**MLB-46**
1	**Amikacin AK30**	+	++	++	++	+	+	+	+	++	++	+	+	+	+	+	++	+	+	+	+	+	+
2	**Ampicillin AMP10**	+	++	+	+	+	+	NZ	+	+	++	+	+	NZ	+	+	++	NZ	+	+	++	NZ	NZ
3	**AzithromycinAT15**	+	++	++	++	++	++	NZ	+	++	++	+	+	+	++	+	++	+	+	++	++	NZ	NZ
4	**Carbencillin CB100**	++	++	+	+	+	+	+	++	+	++	+	++	NZ	++	++	++	+	+	+	++	+	+
5	**Cefaclor CF30**	+	++	+	+	+	+	NZ	+	+	++	+	+	NZ	+	+	+	NZ	+	+	++	NZ	NZ
6	**Cefazolin CZ30**	+	++	NZ	+	++	++	NZ	+	+	++	NZ	+	NZ	+	+	+	NZ	+	++	+	NZ	NZ
7	**Cefixime CFM5**	+	NZ	NZ	NZ	+	+	NZ	+	+	NZ	NZ	+	NZ	+	+	NZ	NZ	NZ	+	+	NZ	NZ
8	**Cefmetazole CMZ30**	NZ	+	NZ	NZ	+	+	NZ	NZ	+	+	NZ	+	NZ	+	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ
9	**Cefoperazone CPZ75**	NZ	++	+	+	+	++	+	NZ	+	++	+	NZ	+	NZ	NZ	NZ	+	+	+	++	+	+
10	**Cefotaxime CTX30**	++	++	+	+	+	+	+	++	++	++	+	+	+	+	+	++	+	+	+	+	+	+
11	**Cefoxitin CX30**	NZ	+	+	+	NZ	+	NZ	NZ	NZ	+	NZ	NZ	NZ	+	NZ	+	NZ	NZ	+	++	NZ	NZ
12	**Ceftazidine CAZ30**	NZ	NZ	+	NZ	NZ	+	+	NZ	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	+	NZ	++	+	NZ	+
13	**Ceftriaxone CTR30**	+	+	+	++	+	++	+	+	+	+	NZ	++	+	++	++	++	+	+	+	+	NZ	NZ
14	**Cefuroxime CXM30**	+	+	+	++	++	++	NZ	+	+	+	NZ	+	+	+	+	++	NZ	+	+	+	NZ	NZ
15	**Cephalothin CH30**	+	+	++	+	+	+	NZ	+	+	+	NZ	+	+	+	+	+	NZ	NZ	+	+	NZ	NZ
16	**Chloramphenicol C30**	+	+	++	+	+	+	NZ	+	+	+	NZ	+	++	++	+	++	NZ	NZ	+	+	NZ	NZ
17	**Ciprofloxacin CIP5**	+	+	+	++	+	+	++	+	+	+	+	+	++	++	+	++	+	NZ	+	++	+	++
18	**Clidamycin CD2**	+	+	+	+	++	++	NZ	+	+	+	NZ	+	+	+	+	+	NZ	NZ	NZ	+	NZ	+
19	**Doxycyline Hydrochloride DO30**	+	++	++	++	+	++	+	+	++	++	+	++	++	++	++	++	+	+	++	++	+	+
20	**Erythromycin E15**	++	++	++	+	+	+	NZ	++	+	++	NZ	+	+	++	+	+	NZ	+	+	+	NZ	NZ
21	**Gatifloxacin GAT5**	+	+	++	++	++	++	++	+	+	+	+	+	+	+	+	++	+	NZ	++	+	+	+
22	**Gentamicin GEN10**	+	++	+	+	+	+	+	+	+	++	+	+	+	+	+	+	+	NZ	++	+	+	+
23	**Kanamycin K30**	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	**Levofloxacin LE5**	+	+	+	++	++	++	++	+	+	+	+	+	+	+	+	++	+	+	++	++	+	+

25	**Linezolid LZ30**	++	++	+	++	+	+	NZ	++	++	++	NZ	++	++	++	++	+	NZ	NZ	+	+	NZ	NZ
26	**Lomefloxacin LO10**	+	+	++	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
27	**Meropenem MRP10**	+	+	++	++	++	++	++	NZ	+	+	+	+	+	+	+	++	+	+	+	++	+	+
28	**Methicillin MET 5**	NZ	NZ	+	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
29	**Nalidixic Acid NA30**	NZ	NZ	NZ	NZ	NZ	++	+	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	NZ	+	+
30	**Netillin NET30**	+	+	NZ	+	+	+	+	NZ	+	+	+	+	+	+	+	+	+	+	+	+	+	+
31	**Nitrofurantoin NIT300**	NZ	NZ	+	NZ	++	+	NZ	+	NZ	NZ	NZ	NZ	+	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ
32	**Norfloxacin NX10**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	NZ	+	+	+	+
33	**Ofloxacin OF5**	+	+	++	++	++	+	+	NZ	+	+	+	+	+	+	+	+	+	+	+	+	+	+
34	**Oxacillin OX1**	NZ	NZ	NZ	NZ	NZ	+	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	++	NZ	NZ
35	**Penicillin-G P10**	+	++	+	+	+	+	NZ	+	+	++	NZ	+	+	++	+	++	NZ	NZ	+	+	NZ	NZ
36	**Piperacillin P100**	+	+	+	+	++	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
37	**Polymyxin-B PB300**	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	++	+	+	+
38	**Rifampicin R5**	+	++	++	++	++	++	+	+	++	++	NZ	++	++	++	++	++	+	+	+	++	+	+
39	**Streptomycin S10**	+	++	+	++	++	++	NZ	+	+	++	NZ	+	+	+	+	++	+	+	+	++	NZ	NZ
40	**Teicoplanin TE30**	+	++	++	+	++	+	NZ	+	+	++	NZ	+	+	+	+	+	NZ	NZ	NZ	++	NZ	NZ
41	**Tetracycline T30**	+	++	+	++	+	+	+	+	+	++	+	++	++	++	++	++	+	+	+	+	+	+
42	**Ticarcillin T175**	++	++	+	+	+	++	NZ	++	+	++	NZ	++	+	+	+	+	NZ	+	+	++	NZ	NZ
43	**Tobramycin TOB10**	+	++	+	+	++	+	+	+	+	++	+	+	+	+	+	+	+	NZ	NZ	++	+	+
44	**Trimethoprim TR5**	++	+	NZ	+	+	++	NZ	++	+	+	NZ	+	+	+	+	++	NZ	+	+	++	NZ	NZ
45	**Vancomycin VA30**	+	++	++	++	+	+	NZ	+	+	++	NZ	++	+	++	+	++	+	NZ	NZ	+	NZ	NZ

Zone size (1mm-1.5cm) = + , Zone size (1.6cm-2 cm & above) = ++ NZ = No zone

ii) Ice cores bacteria

All 14 bacterial isolates were screened for antibiotic sensitivity tests (Table 6). All the isolates were sensitive to antibiotics such as amikacin, carbencillin, cefotaxime, doxycycline hydrochloride, gentamicin, gatifloxacin, kanamycin, lomefloxacin, levofloxacin, meropenem, norfloxacin, netillin, ofloxacin, polymyxin-B, and tobramycin. *Pseudomonas* sp. MLB-15 showed sensitivity towards all the antibiotics tested, while three *Pseudomonas* sp. (isolates MLB-12, MLB-14 and MLB-17) were the most resistant showing resistance to 24 of the 45 antibiotics screened. Other isolates indicated varying resistance pattern (Fig.10).

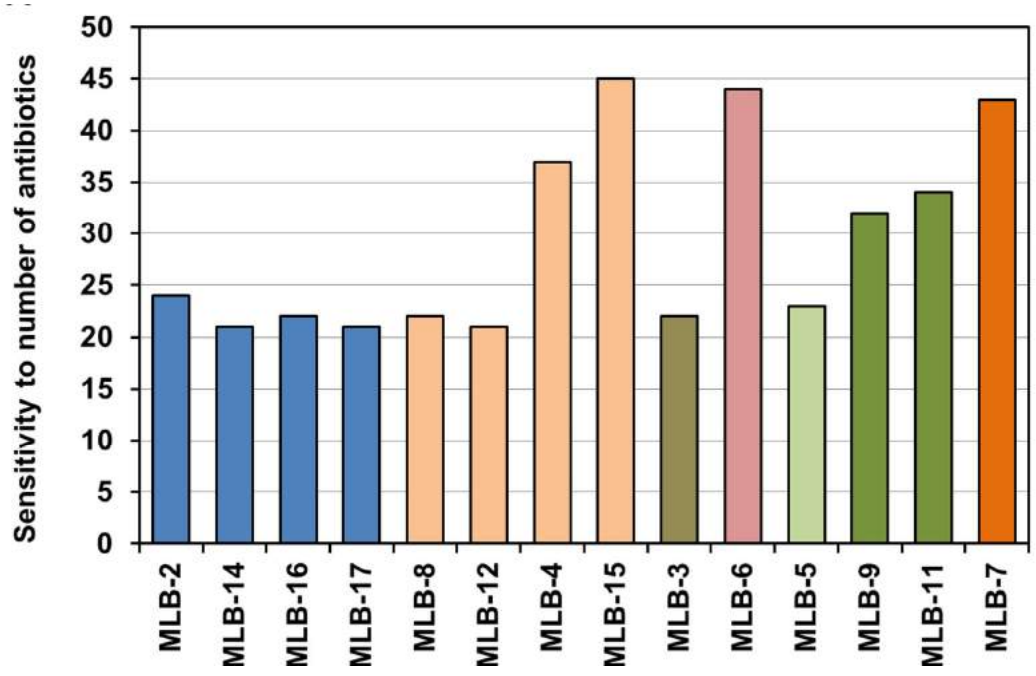


Fig. 10 Ice core bacterial isolates showing sensitivity against antibiotics.

Table 6. Screening for antibiotic sensitivity of Ice core bacterial isolate.

Sr. No.	Antibiotics	MLB-2	MLB-3	MLB-4	MLB-6	MLB-17	MLB-16	MLB-12	MLB-14	MLB-8	MLB-5	MLB-7	MLB-9	MLB-11	MLB-15
1	Amikacin AK30	+	+	++	+	+	+	+	+	+	+	+	+	++	+
2	Ampicillin AMP10	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	+	+	+
3	Azithromycin AT15	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	+	+	+
4	Carbencillin CB100	+	+	+	+	+	+	+	+	+	+	+	+	++	+
5	Cefaclor CF30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	++	+	+	+
6	Cefazolin CZ30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	+	+	+	+
7	Cefixime CFM5	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	+
8	Cefmetazole CMZ30	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	++	+	+	+
9	Cefoperazone CPZ75	+	+	++	+	+	+	+	+	+	+	+	NZ	NZ	+
10	Cefotaxime CTX30	+	+	++	+	+	+	+	+	+	+	+		+	+
11	Cefoxitin CX30	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+		+	+
12	Ceftazidine CAZ30	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	Ceftriaxone CTR30	+	NZ	++	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	+	+	+
14	Cefuroxime CXM30	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	+	+	+
15	Cephalothin CH30	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	+	+	+
16	Chloramphenicol C30	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	+	+	+
17	Ciprofloxacin CIP5	++	++	++	+	+	+	+	++	++	+	+	NZ	+	+
18	Clidamycin CD2	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	NZ	+
19	Doxycyline Hydrochloride DO30	+	+	+	+	+	+	+	+	+	+	+	+	++	+
20	Erythromycin E15	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	+	+
21	Gatifloxacin GAT5	+	++	++	+	+	+	+	+	+	+	+	+	+	+
22	Gentamicin GEN10	+	+	++	+	+	+	+	+	+	+	+	+	+	+
23	Kanamycin K30	+	+	++	+	+	+	+	+	+	+	+	+	+	+
24	Levofloxacin LE5	++	++	++	+	+	+	+	+	+	+	+	+	+	+
25	Linezolid LZ30	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	+	+	+
26	Lomefloxacin LO10	+	+	++	+	+	+	+	+	+	+	+	+	+	+
27	Meropenem MRP10	+	+	++	++	+	++	+	++	+	+	+	+	+	+
28	Methicillin MET 5	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	NZ	+
29	Nalidixic Acid NA30	+	+	++	+	++	+	+	+	+	+	+	+	NZ	+
30	Netillin NET30	+	+	++	+	+	+	+	+	+	+	+	+	+	+
31	Nitrofurantoin NIT300	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	NZ	+
32	Norfloxacin NX10	+	++	+	+	+	+	+	+	+	+	+	+	+	+
33	Ofloxacin OF5	+	+	++	+	+	+	+	+	+	+	+	+	+	+
34	Oxacillin OX1	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	NZ	+

35	Penicillin-G P10	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	NZ	+
36	Piperacillin P100	+	+	++	+	+	+	+	+	+	+	+	NZ	+	+
37	Polymyxin-B PB300	+	+	+	+	+	+	+	+	+	+	+	+	+	+
38	Rifampicin R5	+	NZ	+	NZ	NZ	+	NZ	NZ	+	NZ	++	+	+	+
39	Streptomycin S10	NZ	NZ	+	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	NZ	+
40	Teicoplanin TE30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	++	+	+	+
41	Tetracycline T30	+	+	++	NZ	+	+	+	+	+	+	+	+	+	++
42	Ticarcillin T175	+	NZ	++	++	NZ	NZ	NZ	NZ	NZ	+	+	+	NZ	++
43	Tobramycin TOB10	+	+	++	+	+	+	+	+	+	+	+	+	+	+
44	Trimethoprim TR5	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	NZ	NZ	+
45	Vancomycin VA30	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	NZ	+	+	+	+

Zone diameter (1mm-1.5cm) = +, Zone diameter (1.6-2 cm & above) = ++, NZ = No zone of inhibition

iii) Permafrost bacteria

Permafrost isolates were subjected to antibiotic screening tests. The antibiotic sensitivity and resistance profile of 33 isolates were recorded (Table 7). *Arthrobacter* sp. strains PF1T4 and PF4B2 were the most susceptible isolates, sensitive against 44 of the 45 antibiotics tested, while other *Arthrobacter* strains showed varied sensitivity. *Pseudomonas jessenii* strain PF2M8, *P. mandelii* strain PF2M12, *Stenotrophomonas* sp. strain PF1M2 and *Virgibacillus pantothenticus* strain PF1B6 were the least susceptible and were sensitive to only 23 antibiotics.

Antibiotics carbencillin, cefoperazone, cefotaxime, ciprofloxacin, doxycyline hydrochloride, meropenem, ofloxacin and tetracycline were highly effective in inhibiting the growth of all the cultures tested while methicillin and oxacillin were effective against only 11 bacterial cultures tested (Fig. 11 a,b) in the present study.

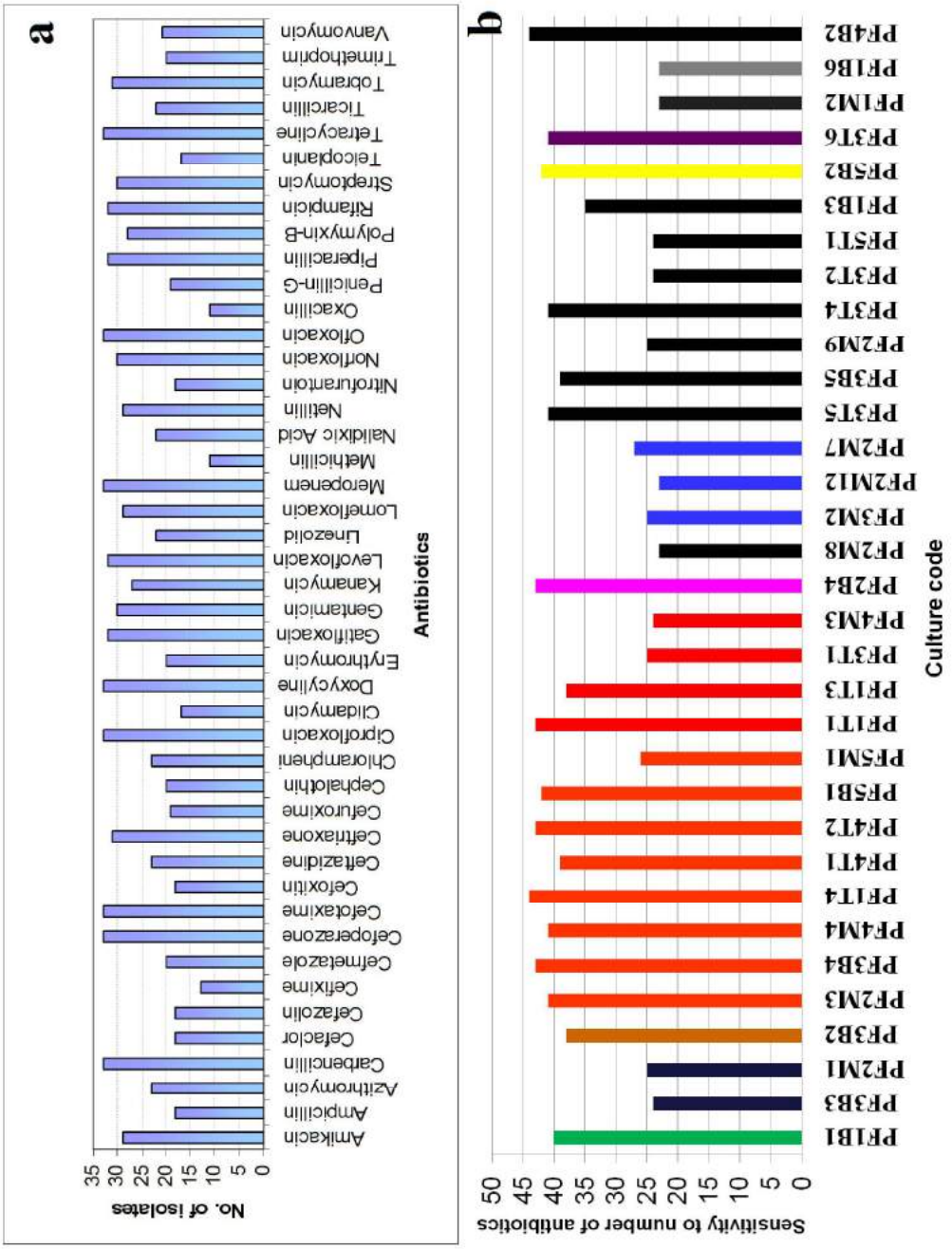


Fig. 11 Permafrost bacterial isolates showing sensitivity against antibiotics.

Table 7. Antibiotic sensitivity tests measured as zone of inhibition (in millimeters).

Identification	Strain No.	Amikacin AK30	Ampicillin AMP10	Azithromycin AT15	Carbencillin CB100	Cefaclor CF30	Cefazolin CZ30	Cefixime CFM5	Cefmetazole CMZ30	Cefoperazone CPZ75	Cefotaxime CTX30	Cefoxitin CX30	Ceftazidine CAZ30	Ceftriaxone CTR30	Cefuroxime CXM30	Cephalothin CH30	Chloramphenicol C30	Ciprofloxacin CIP5	Clidamycin CD2	Doxycyline Hydrochloride DO30	Erythromycin E15	Gatifloxacin GAT5	Gentamicin GEN10	Kanamycin K30
Acinetobacter johnsonii	PF1B1	10	10	12	10	1	2	5	5	9	10	2	5	7	8	4	2	8	0	12	5	7	8	12
Arthrobacter bergerei	PF1T1	10	10	10	10	10	10	10	10	10	10	0	10	10	10	10	10	10	10	10	10	10	10	5
Arthrobacter sp.	PF3B3	12	0	0	10	0	0	0	0	5	5	0	3	3	0	0	0	5	0	5	0	2	15	10
Arthrobacter sp.	PF3B2	15	15	10	10	0	18	13	12	15	20	15	15	15	15	15	10	20	10	15	10	5	10	0
Arthrobacter sp.	PF2M1	10	0	0	9	0	0	0	0	5	5	0	0	3	0	0	0	15	2	7	1	15	11	11
Arthrobacter sp.	PF3B4	15	15	15	15	15	15	2	7	20	15	15	2	13	20	20	15	15	15	23	17	20	12	15
Arthrobacter sp.	PF4B2	20	15	10	10	15	15	10	15	11	15	25	10	12	20	20	20	10	6	15	10	11	10	15
Arthrobacter sp.	PF2M3	10	10	10	10	8	7	0	1	10	10	9	0	10	3	15	10	9	10	10	10	13	10	3
Arthrobacter sp.	PF4M4	10	20	20	20	10	13	0	2	14	17	4	12	10	10	10	10	10	10	10	10	14	10	6
Arthrobacter sp.	PF1T4	20	20	20	20	20	14	10	16	7	15	15	3	16	20	7	20	15	5	15	16	15	10	6
Arthrobacter sp.	PF4T1	10	5	5	5	5	0	5	2	5	5	2	3	5	5	1	3	8	0	6	2	6	7	5
Arthrobacter sp.	PF4T2	10	10	10	10	8	10	0	3	10	12	5	0	10	6	10	10	10	10	10	10	10	10	5
Arthrobacter sp.	PF5B1	10	10	10	10	10	20	0	7	17	15	10	0	7	10	20	10	10	10	10	10	10	5	5
Arthrobacter sp.	PF5M1	10	0	2	10	0	0	0	0	5	8	0	8	5	0	0	4	12	0	7	0	10	10	13
Arthrobacter sulphonivorans	PF1T3	10	10	10	10	10	10	0	2	10	10	5	0	2	5	4	10	10	0	10	10	10	10	0
Arthrobacter sp.	PF3T1	12	0	0	8	0	0	0	0	7	7	0	8	6	0	0	3	15	0	7	0	13	11	15
Arthrobacter sp.	PF4M3	0	0	0	10	9	0	10	1	5	13	0	0	10	5	0	0	5	6	2	0	5	0	0
Bacillus sp.	PF2B4	15	15	15	15	15	16	0	6	20	20	10	4	17	16	15	15	15	15	15	15	15	10	10
Pseudomonas jessenii	PF2M8	10	0	0	10	0	0	0	0	5	6	0	5	0	0	0	0	12	0	10	0	12	10	10
Pseudomonas mandelii	PF2M12	11	0	0	8	0	0	0	0	5	5	0	3	0	0	0	0	12	0	7	0	13	10	12
Pseudomonas sp.	PF2M7	11	0	3	10	0	0	0	0	7	7	0	2	3	0	0	2	11	1	4	0	12	9	9
Pseudomonas mandelii	PF3M2	12	0	0	10	0	0	0	0	7	7	0	6	6	0	0	2	15	0	6	0	14	14	14
Pseudomonas sp.	PF1B3	0	0	2	2	0	5	0	10	10	12	8	0	10	8	10	10	10	0	10	10	10	10	0
Pseudomonas sp.	PF3B5	10	10	10	10	10	10	0	2	10	10	5	0	2	5	4	10	10	2	10	10	10	10	3
Pseudomonas sp.	PF2M9	11	0	0	10	0	0	1	0	5	6	0	2	3	0	0	0	13	0	9	0	12	11	11
Pseudomonas sp.	PF3T4	10	10	10	12	10	15	0	2	15	10	10	10	10	10	10	12	5	10	12	10	10	10	10
Pseudomonas sp.	PF3T5	8	5	8	3	3	1	5	2	5	5	2	5	5	5	2	3	10	0	10	3	5	8	5
Pseudomonas sp.	PF3T2	11	0	0	9	0	0	0	0	5	7	0	10	10	0	0	0	17	0	10	0	11	11	14
Pseudomonas sp.	PF5T1	11	0	0	10	0	0	0	0	8	7	0	10	10	0	0	0	17	0	10	0	11	11	14
Psychrobacter sp.	PF5B2	10	10	10	10	10	6	8	11	3	12	10	7	8	0	5	0	10	0	10	3	14	12	10
Sphingobacterium sp.	PF3T6	15	15	15	15	15	20	2	5	16	18	13	0	7	10	22	15	15	10	15	15	10	10	7
Stenotrophomonas sp.	PF1M2	0	0	1	9	0	0	0	0	5	5	0	1	4	0	2	2	4	0	3	0	8	0	0
Virgibacillus pantothenticus	PF1B6	0	0	2	10	0	0	15	0	5	15	0	0	6	0	0	0	3	10	3	0	0	0	0

Identification	Strain No.	Levofloxacin	Linezolid	Lomefloxacin	Meropenem	Methicillin	Nalidixic Acid	Netillin	Nitrofurantoin	Norfloxacin	Ofloxacin	Oxacillin	Penicillin	Piperacillin	Polymyxin-	Rifampicin	Streptomycin	Teicoplanin	Tetracycline	Ticarcillin	Tobramycin	Trimethoprim	Vancomycin
Acinetobacter johnsonii	PF1B1	12	6	11	11	0	10	11	2	10	10	0	5	0	3	5	10	0	11	10	10	3	5
Arthrobacter bergerei	PF1T1	10	10	5	10	10	0	10	5	8	8	10	10	10	3	10	8	5	10	10	3	10	8
Arthrobacter sp.	PF3B3	10	0	10	12	0	8	10	0	8	8	0	0	5	5	2	5	0	10	0	10	0	0
Arthrobacter sp.	PF3B2	0	5	10	10	20	0	15	0	0	15	10	10	15	10	0	10	10	15	15	15	15	15
Arthrobacter sp.	PF2M1	15	0	11	17	0	7	12	0	14	15	0	0	11	8	2	4	0	8	0	10	0	0
Arthrobacter sp.	PF3B4	20	18	18	15	0	0	10	23	10	21	4	20	25	10	15	15	25	20	25	10	20	25
Arthrobacter sp.	PF4B2	10	16	10	12	12	0	14	3	6	10	10	12	12	5	15	10	9	15	15	21	17	10
Arthrobacter sp.	PF2M3	11	15	3	10	1	0	7	10	3	10	0	15	18	3	20	8	10	20	15	5	8	14
Arthrobacter sp.	PF4M4	20	11	3	12	0	0	10	11	5	10	0	20	16	3	15	12	8	15	20	20	10	20
Arthrobacter sp.	PF1T4	15	15	6	14	2	0	6	6	13	13	2	13	17	10	15	10	5	12	20	20	6	10
Arthrobacter sp.	PF4T1	8	3	5	10	0	5	0	1	5	10	0	3	8	5	2	5	0	10	2	8	1	2
Arthrobacter sp.	PF4T2	10	10	6	9	3	6	9	5	5	10	3	10	10	3	10	10	10	10	10	5	10	10
Arthrobacter sp.	PF5B1	10	10	6	14	2	0	6	3	15	15	3	15	10	3	10	10	10	10	10	5	10	10
Arthrobacter sp.	PF5M1	12	0	8	12	0	5	12	0	9	10	0	0	10	7	3	10	0	10	0	11	0	0
Arthrobacter sulphonivorans	PF1T3	10	10	8	10	10	10	8	5	10	10	10	5	10	0	10	10	0	10	10	10	0	10
Arthrobacter sp.	PF3T1	14	0	10	12	0	6	12	0	8	10	0	0	11	6	2	4	0	8	0	12	0	0
Arthrobacter sp.	PF4M3	5	5	0	5	0	5	0	0	0	4	0	0	13	0	15	0	5	1	12	0	6	2
Bacillus sp.	PF2B4	15	15	15	20	3	5	11	12	11	20	0	15	12	17	15	12	17	20	15	10	20	20
Pseudomonas jessenii	PF2M8	15	0	11	15	0	8	11	0	12	12	0	0	9	8	2	4	0	8	0	10	0	0
Pseudomonas mandelii	PF2M12	12	0	7	13	0	3	10	0	10	8	0	0	10	8	2	2	0	10	0	10	0	0
Pseudomonas sp.	PF2M7	14	0	11	15	0	2	10	0	12	10	0	0	10	5	2	5	0	5	0	9	0	0
Pseudomonas mandelii	PF3M2	14	0	10	20	0	7	10	0	11	10	0	0	12	8	3	4	0	10	0	13	0	0
Pseudomonas sp.	PF1B3	10	10	8	10	10	10	8	5	10	10	10	5	10	0	10	10	0	10	10	10	0	10
Pseudomonas sp.	PF3B5	10	10	3	10	0	0	5	1	5	8	0	10	5	0	10	8	8	10	10	2	1	10
Pseudomonas sp.	PF2M9	16	0	10	15	0	4	11	0	15	10	0	0	13	10	3	2	0	10	0	10	0	0
Pseudomonas sp.	PF3T4	10	10	0	10	0	0	5	8	2	10	10	15	15	5	10	15	10	15	20	5	10	16
Pseudomonas sp.	PF3T5	10	5	5	8	0	2	8	2	5	10	0	5	10	5	5	5	0	10	8	8	2	3
Pseudomonas sp.	PF3T2	15	0	10	15	0	3	15	0	10	10	0	0	11	10	2	8	0	11	0	15	0	0
Pseudomonas sp.	PF5T1	15	0	10	15	0	3	15	0	10	10	0	0	11	10	2	8	0	11	0	15	0	0
Psychrobacter sp.	PF5B2	20	12	10	8	2	10	11	3	10	9	1	6	10	6	5	10	2	10	10	10	2	7
Sphingobacterium sp.	PF3T6	3	10	10	10	0	0	10	5	4	8	0	10	10	6	16	10	10	10	12	5	20	11
Stenotrophomonas sp.	PF1M2	9	5	0	8	0	3	0	0	3	8	0	0	15	0	15	0	2	5	10	0	5	0
Virgibacillus pantothenticus	PF1B6	5	5	0	10	0	5	0	0	0	3	0	0	11	3	15	0	5	10	10	10	3	5

3a.3.4 Carbohydrate utilization ability of the isolated strains

i) Cryoconite bacteria

Of the 35 carbon sources tested, 19 cryoconite isolates were able to assimilate mannose sugar, 18 could hydrolyse esculin while 17 each could hydrolyse rhamnose and xylose sugars (Table 8). None of the isolates were capable of hydrolysing compounds such as erythritol, melezitose, α – methyl-D-glucoside, sodium gluconate, α–methyl-D-mannoside or sorbose. Amongst the isolates tested, *Pseudomonas* sp. MLB-42 had the ability to hydrolyse as much as 27 of the 35 carbon sources while *Cryobacterium* sp. MLB-32 was the most specific utilizing only 3 carbohydrates (citrate, esculin and malonate). Carbon source utilization tests of isolates are given in Figure 12.

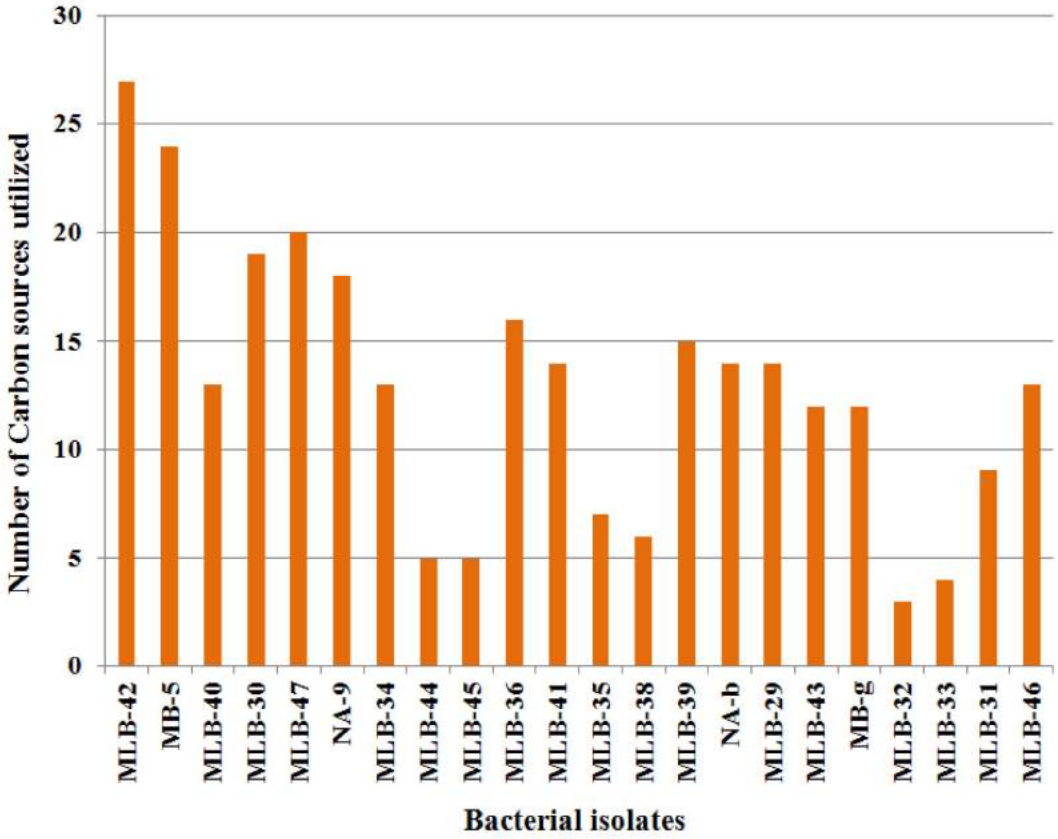


Fig. 12 Cryoconite bacterial isolates showing the pattern of carbon source utilization.

Table 8 Phenotypic characters of the cryoconite bacterial isolates (+ hydrolysis; - no hydrolysis)

Sr.No	Test	MLB 42	MB-5	MLB 40	MLB 30	MLB 47	NA-9	MLB 34	MLB 44	MLB 45	MLB 36	MLB 41	MLB 35	MLB 38	MLB 39	NA-b	MLB 29	MLB 43	MB-g	MLB 32	MLB 33	MLB 31	MLB 46
1	Lactose	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	+	-	-	-	+	+
2	Xylose	+	+	-	+	+	+	+	-	+	+	+	+	-	+	+	+	+	+	-	-	+	+
3	Maltose	+	+	+	+	+	+	-	-	-	+	+	-	+	+	+	-	-	-	-	-	+	-
4	Fructose	+	+	+	+	+	+	-	-	-	+	+	-	-	+	-	+	+	-	-	-	-	-
5	Dextrose	+	+	-	+	+	+	+	-	-	+	+	-	+	+	+	+	+	+	-	-	-	-
6	Galactose	+	+	+	+	+	+	+	-	-	+	+	-	-	+	+	-	+	+	-	-	-	+
7	Raffinose	+	+	+	+	+	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	+
8	Trehalose	+	+	+	+	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-	-	-	-
9	Melibiose	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	+	+	+	-	-	+	+
10	Sucrose	+	+	-	-	+	+	-	-	-	+	+	-	-	+	+	+	-	-	-	-	-	+
11	L-Arabinose	+	+	-	-	+	+	+	+	-	+	-	-	-	+	+	+	+	+	-	-	+	+
12	Mannose	+	+	-	-	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+
13	Inulin	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-
14	Sodium gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
15	Glycerol	+	+	-	-	+	-	-	+	-	+	+	+	+	-	+	-	-	-	-	-	-	+
16	Salicin	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
17	Dulcitol	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
18	Inositol	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
19	Sorbitol	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
20	Mannitol	+	+	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
21	Adonitol	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
22	Arabitol	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
23	Erythritol	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
24	α – Methyl- D - glucoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
25	Rhamnose	+	+	+	-	+	+	+	+	-	+	+	-	-	+	+	+	+	+	-	-	+	+
26	Cellobiose	+	+	+	-	+	+	+	+	-	-	-	-	-	+	+	+	-	-	-	-	-	-
27	Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
28	α – Methyl- D - mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
29	Xylitol	+	+	-	-	+	+	+	-	+	+	+	+	-	+	+	+	-	+	-	-	-	+
30	ONPG	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
31	Esculin hydrolysis	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-
32	D - Arabinose	+	+	+	-	+	+	+	-	-	+	-	-	+	+	+	-	-	+	-	-	-	-
33	Citrate utilization	+	+	+	-	-	-	+	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+
34	Malonate utilization	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+	+
35	Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

ii) Ice cores bacteria

Ice core isolates were also tested for their carbohydrate utilization ability (Table 9, Fig. 13). Of the various carbon sources tested 12 isolates were observed to utilize dextrose and sucrose, 11 isolates had ability to utilize arabinose, galactose, mannose, xylose and xylitol. None of the isolates had the capability to utilize compounds such as adonitol, dulcitol, melezitose, α–methyl-D–mannoside, raffinose, sodium gluconate, salicin, and sorbose. Amongst the isolates tested, *Pseudomonas* sp. MLB-8 showed the ability to hydrolyse as many as 20 of the 35 carbon source tested while isolate *Bacillus* MLB-7 had ability to utilize only two of the carbohydrates (esculin and sucrose).

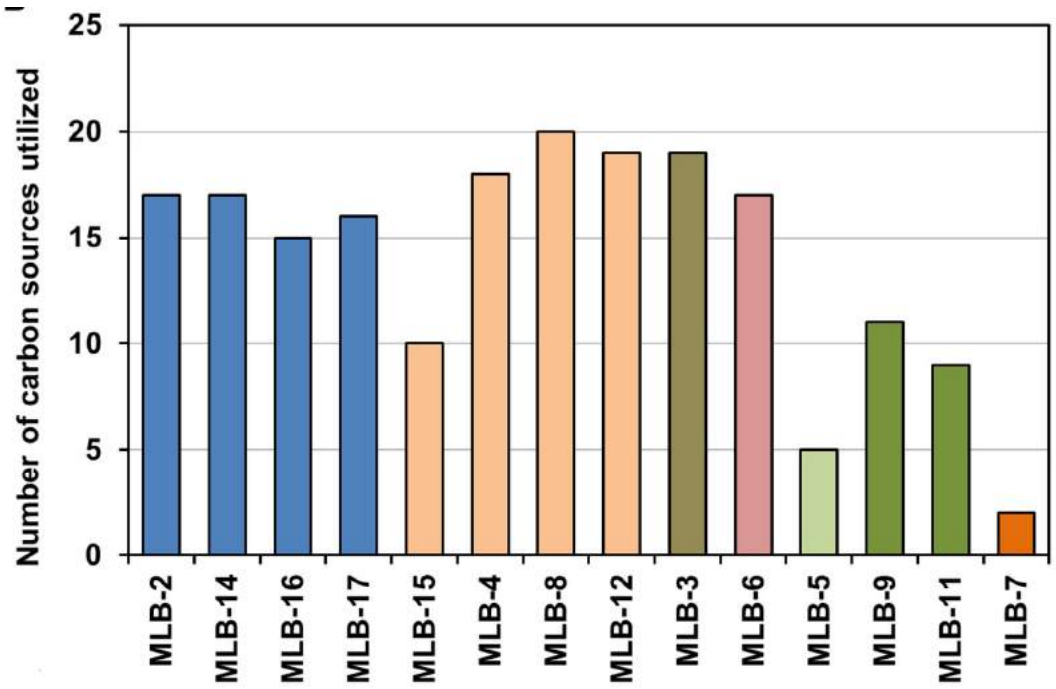


Fig. 13 Ice cores bacterial isolates showing the ability of carbon source utilization.

Table 9. Carbohydrate utilization of the Ice core bacterial isolates.

Test	MLB-2	MLB-3	MLB-4	MLB-6	MLB-17	MLB-16	MLB-12	MLB-14	MLB-8	MLB-5	MLB-7	MLB-9	MLB-11	MLB-15
Lactose	-	-	-	-	-	-	-	-	-	-	-	S	-	-
Xylose	+	+	+	+	+	-	+	+	+	-	-	S	+	S
Maltose	-	-	+	-	-	-	-	-	-	-	-	S	S	+
Fructose	+	S	S	S	S	S	S	S	S	-	-	-	-	+
Dextrose	+	S	+	S	+	+	+	+	S	-	-	S	S	+
Galactose	+	S	+	+	+	+	+	+	+	S	-	-	+	-
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Trehalose	+	S	+	S	+	S	S	S	S	-	-	-	-	+
Melibiose	+	+	+	+	+	+	+	+	+	S	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+	-	S	+	+	-
L-Arabinose	+	+	+	S	+	+	+	+	+	-	-	S	+	-
Mannose	+	+	+	+	+	+	+	+	+	-	-	+	-	+
Inulin	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Sodium gluconate	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glycerol	S	S	-	S	S	S	S	S	+	-	-	-	-	-
Salicin	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Inositol	-	S	S	S	-	-	-	S	S	-	-	-	-	-
Sorbitol	S	+	S	+	+	S	+	S	+	-	-	-	-	-
Mannitol	+	+	S	S	S	S	+	S	S	-	-	-	-	S
Adonitol	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arabitol	+	S	S	S	S	S	+	S	S	-	-	-	-	-
Erythritol	S	S	-	-	-	-	S	-	S	-	-	-	-	-
α-Methyl- D-glucoside	-	-	-	-	-	-	S	-	-	-	-	-	-	-
Rhamnose	-	S	-	-	-	-	S	-	S	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-	+
Melezitose	-	-	-	-	-	-	-	-	-	-	-	-	-	-
α-Methyl-D mannoside	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Xylitol	+	+	+	+	+	-	+	+	+	S	-	S	+	-
ONPG	-	-	-	-	-	-	-	-	-	-	-	+	-	-
Esculin hydrolysis	-	-	-	-	-	-	-	-	-	-	+	+	+	S
D-Arabinose	-	-	S	-	-	S	-	-	S	-	-	S	+	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Malonate utilization	+	+	+	+	+	+	+	+	+	+	-	-	-	-
Sorbose	-	-	-	-	-	-	-	-	-	-	-	-	-	-

+, hydrolysis activity, **S=** slightly positive hydrolysis, -, no activity

iii) Permafrost bacteria

The isolates tested for their carbon utilization ability are shown in Table 10. Of the various carbon sources tested 23 isolates utilized malonate while none could utilize cellobiose, inositol, α–Methyl-D-glucoside, sodium gluconate, α–Methyl-D-mannoside or sorbose.

Amongst the isolates screened, the isolate PF3B2 (*Arthrobacter* sp.) utilized the highest number (18) of carbon sources. *Acinetobacter johnsonii* (PF1B1) utilized citrate and malonate and *Arthrobacter* sp. (PF4M3) could utilize Ortho-Nitrophenyl-β-D-galactopyranoside (ONPG) and Xylitol as 2 carbon sources each. Within the 16 *Arthrobacter* isolates, 13 utilized ONPG as substrate while 10 utilized sucrose as carbon source. Amongst the 11 *Pseudomonas* species, most isolates (9) utilized glycerol, citrate and malonate as carbon sources (Fig. 14 a,b).

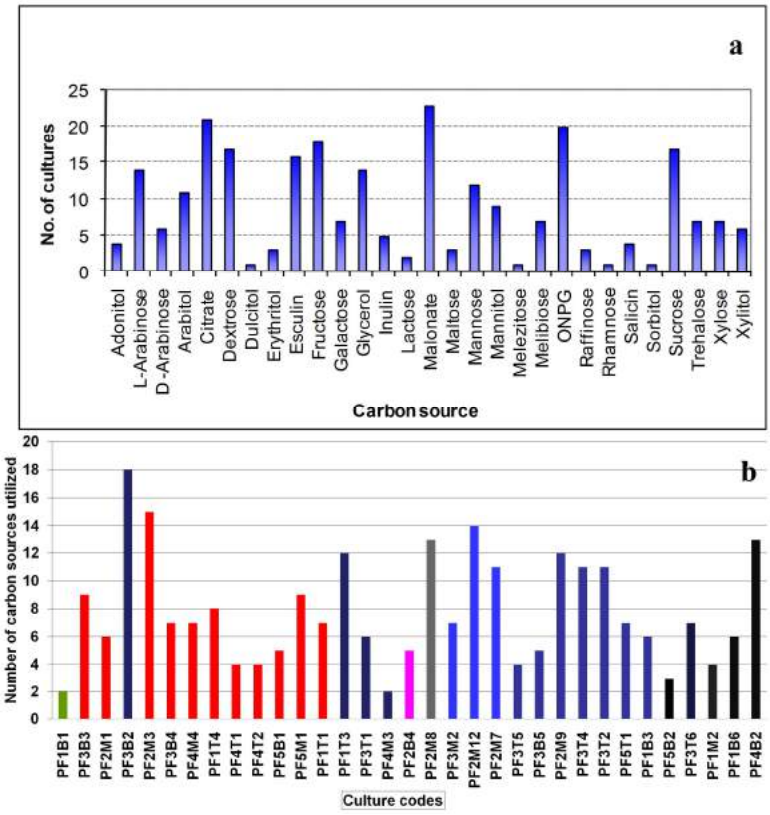


Fig. 14 Permafrost bacterial isolates showing the ability of carbon source utilization.

Table 10. Carbon utilization ability of the isolated strains of Permafrost.

Identification	Strain no.	Lactose	Xylose	Maltose	Fructose	Dextrose	Galactose	Raffinose	Trehalose	Melibiose	Sucrose	L-Arabinose	Mannose	Inulin	Sodium	Glycerol	Salicin	Dulcitol	Inositol	Sorbitol	Mannitol	Adonitol	Arabitol	Erythritol	α – Methyl-D - glucoside	Rhamnose	Cellobiose	Melezitose	α – Methyl- D - mannoside	Xylitol	ONPG	Esculin hydrolysis	D - Arabinose	Citrate utilization	Malonate	Sorbose
Acinetobacter johnsonii	PF1B1	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Arthrobacter bergerei	PF1T1	-	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-
Arthrobacter sp.	PF3B3	-	+	-	-	-	+	-	+	-	-	+	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	+	+	-
Arthrobacter sp.	PF3B2	+	-	+	+	+	+	-	+	-	+	+	+	-	-	+	-	-	-	-	+	+	+	+	-	+	-	-	-	-	+	+	+	-	-	-
Arthrobacter sp.	PF2M1	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-
Arthrobacter sp.	PF3B4	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-
Arthrobacter sp.	PF4B2	-	-	-	+	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-
Arthrobacter sp.	PF2M3	-	-	-	+	+	-	+	+	+	+	+	-	-	-	+	-	-	-	-	+	+	-	-	-	-	-	-	-	-	+	+	+	+	+	-
Arthrobacter sp.	PF4M4	-	-	-	+	+	-	-	-	-	-	+	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-
Arthrobacter sp.	PF1T4	-	-	-	+	-	-	+	-	-	+	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-
Arthrobacter sp.	PF4T1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-
Arthrobacter sp.	PF4T2	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Arthrobacter sp.	PF5B1	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	+	+	-	-	-	-
Arthrobacter sp.	PF5M1	-	-	-	+	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-
Arthrobacter sulphonivorans	PF1T3	-	-	-	-	+	-	-	-	-	+	-	+	+	-	+	-	-	-	+	+	-	+	-	-	-	-	+	-	-	+	-	-	+	+	-
Arthrobacter sp.	PF3T1	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	+	-	+	+	-
Arthrobacter sp.	PF4M3	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Bacillus sp.	PF2B4	-	-	-	+	+	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Pseudomonas jessenii	PF2M8	-	+	-	+	+	+	-	+	+	-	+	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-
Pseudomonas mandelii	PF2M12	-	+	-	+	+	+	-	+	+	+	+	+	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-
Pseudomonas sp.	PF2M7)	-	+	-	-	-	-	-	-	+	+	+	+	-	-	+	-	-	-	-	+	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-
Pseudomonas mandelii	PF3M2	-	-	-	-	-	-	-	-	-	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	-
Pseudomonas sp.	PF1B3	-	-	-	+	+	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-
Pseudomonas sp.	PF3B5	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+	+	-
Pseudomonas sp.	PF2M9	-	+	-	-	+	+	-	+	+	-	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	+	+	-
Pseudomonas sp.	PF3T4	-	-	-	+	+	-	-	-	-	-	+	-	+	-	+	-	-	-	-	+	+	-	+	-	-	-	-	-	-	+	-	-	+	+	-
Pseudomonas sp.	PF3T5	-	-	-	+	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-
Pseudomonas sp.	PF3T2	-	+	-	-	+	+	+	-	+	+	+	+	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Pseudomonas sp.	PF5T1	-	+	-	-	-	-	-	-	+	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	-	-	-	+	-	-	-	+	+	-
Psychrobacter sp.	PF5B2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-
Sphingobacterium sp.	PF3T6	-	-	-	+	+	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	-	+	-
Stenotrophomonas sp.	PF1M2	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-
Virgibacillus pantotheticus	PF1B6	+	-	+	+	+	+	-	+	-	+	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	+	+	-

3a.3.5 Fatty acid profiles

i) Cryoconite bacteria

Nine isolates (four belonging to the genera *Cryobacterium*, two each to *Micrococcus* and *Agreia* and one to *Leifsonia*) were subjected to fatty acid analysis. The fatty acids analysed were grouped as saturated ($C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$), unsaturated ($C_{17:1\omega9c}$, $C_{18:1\omega9c}$, $C_{18:3\omega6,9,12c}$) and branched (iso $C_{11:0}$ 3OH, anteiso-$C_{13:0}$, iso $C_{14:0}$, iso $C_{15:0}$, anteiso $C_{15:0}$, iso $C_{15:1}$ G, anteiso $C_{15:10}$ A, iso $C_{16:0}$, iso $C_{17:0}$, anteiso $C_{17:0}$, iso $C_{17:1\omega5c}$, iso $C_{18:1}$ H). It was observed that all the isolates had the higher concentration of branched chain fatty acids as compared to the saturated or unsaturated fatty acids. The most abundant fatty acids recorded in the isolates were anteiso-$C_{15:0,}$ anteiso-$C_{17:0}$ iso-$C_{15:0}$, iso-$C_{16:0}$ and anteiso-$C_{15:1}$ A.

ii) Ice core bacteria

Six isolates (four belonging to the genera *Pseudomonas* and two to *Sphingnomonas*) were subjected to fatty acid analysis (Table 11). Amongst the three fatty acids (palmitoleic, heptadecanoic and linolelaidic acids) analysed, it was observed that all the six cultures produced high amounts of heptadecanoic acid followed by linolelaidic acid. Palmitoleic acid was the least produced. No effect of temperature was observed on the production of fatty acid.

Table 11. Fatty acid profiles of representative isolates of three genera isolatedfrom Ice core

	Palmitoleic acid			Heptadecanoic acid			Linolelaidic acid			Myristoleic acid			Palmitic acid		
Bacterial isolates	1 ℃	4 ℃	20 ℃	1 ℃	4 ℃	20 ℃	1 ℃	4 ℃	20 ℃	1 ℃	4 ℃	20 ℃	1 ℃	4 ℃	20 ℃
Pseudomonas sp. MLB-4	11.17	11.05	11.94	41.91	48.98	35.94	30.03	30.52	31.85	ND	ND	ND	ND	ND	ND
Pseudomonas sp. MLB-12	9.80	10.89	12.77	36.99	47.71	-	31.70	29.25	12.51	ND	ND	ND	ND	ND	ND
Pseudomonas sp. MLB-3	8.75	10.05	ND	47.80	59.69	ND	35.39	30.26	ND	ND	ND	ND	ND	ND	ND
Pseudomonas sp. MLB-2	ND	50.98	6.25	ND	-	27.45	ND	-	21.42	ND	ND	ND	ND	ND	ND
Sphingnomonas sp. MLB-5	11.32	44.44	15.15	47.94	-	35.15	22.06	30.86	35.65	ND	ND	ND	ND	ND	ND
Sphingnomonas sp. MLB-9	10.18	23.18	19.98	44.57	21.97	-	25.54	24.15	-	ND	ND	ND	ND	ND	ND
Bacillus sp. MLB-7	ND	52.03	ND	ND	9.45	ND	ND	ND	ND	ND	22.66	ND	ND	5.89	ND

3a.3.6 Phylogenetic analyses

i) Cryoconite bacteria

The total sequence lengths after alignment, % sequence similarities, number of base changes at different positions, and the NCBI sequence deposition numbers are given in Table 12. Pairwise alignment was performed using EMBOSS Matcher-Pairwise Sequence Alignment tool (http://www.ebi. ac.uk/ Tools/ psa/ emboss_matcher/nucleotide.html). The sequence analysis of the 16S rRNA region of the isolates *Pseudomonas* sp. MB-g (JF790967), *Pseudomonas* sp. MLB- 42 (JX205209), *Pseudomonas* sp. MLB- 46 (JX205212) and *Pseudomonas* sp. MLB-43 (JX205210) showed their closest relationship with the species of *Pseudomonas oryzihabitans* IAM1568^T (AM262973) by 92.9%, 92.9%, 93.2%, 93.2% and with *Pseudomonas ficuserectae* JCM2400^T (AB021378) by 96.9%, 96.9%, 97.3%, 97.8%, respectively.

Polaromonas sp. MLB-45 (JX205214) showed very close phylogenetic relationship with the species of *Polaromonas naphthalenivorans* CJ2^T (AY166684) by 97.8% and to *Polaromonas jejuensis* NBRC106434^T (AB682445) by 98.3%. Sequence analysis of isolates *Micrococcus* sp. MB-18 (JF790969), and *Micrococcus* sp. MLB-41 (JX205208) resembled with *Micrococcus endophyticus* YIM56238^T (EU005372) by 99.1% and 99.8% respectively, with *Micrococcus yunnanensis* YIM65004^T (FJ214355) by 99.4% and 98.7% respectively, with *Micrococcus luteus* DSM20030^T (AJ536198) by 99.4% and 98.6% respectively, and with *Micrococcus lylae* DSM20315^T (X80750) by 98.1% and 97.6% respectively. Sequence analysis of isolate *Subtercola* sp. MLB-47 (JX205212) resembled *Subtercola frigoramans* DSM1305^T (AM410673) by 97.8% and with *Agreia pratensis* DSM 14226^T (AJ310412) by 95.2%.

Sequence analysis of *Agreia sp.* MLB-28 (JX205195) and *Agreia sp.* MB-6 (JF790970) indicated their closest relationship with *Agreia pratensis* DSM 14226^T (AJ310412) by 95.5%, 94.7%, respectively, and with *Subtercola*

frigoramans DSM1305^T (AM410673) by 95.2%, 94.5%, respectively. Sequence analysis of *Leifsonia* sp. MLB-40 (JX205207) and *Leifsonia* sp. NA-8 (JF790971) resembled *Leifsonia antarctica* SPC-20^T (AM931710) by 98.4%, 98.4% respectively and with *Leifsonia kafniensis* KFC-22^T (AM889135) by 99.3%, 96.6% respectively. The nucleotide sequence analysis of isolates *Cryobacterium* sp. MLB-32 (JX205199), *Cryobacterium* sp. MLB-31 (JX205198), *Cryobacterium* sp. MLB-33 (JX205200), and *Cryobacterium* sp. MB-l (JF790968) showed their closest relationship to the species of *Cryobacterium psychrophilum* DSM4854^T (AM410676) by 95.6%, 96.5%, 95.7% and 95.5% respectively.

Sequence analysis of isolates *Cryobacterium* sp. MLB-38 (JX205205), *Cryobacterium* sp. MLB-39 (JX205206), *Cryobacterium* sp. MLB-36 (JX205203) resembled *Cryobacterium psychrotolerans* hp36 (JN637331) by 95.5%, 98.4%, 98.2%, respectively, and with *Cryobacterium* sp. DR9 (FJ464984) by 96.1%, 99.0%, 98.9% respectively. *Cryobacterium* sp. MLB-35 (JX205202) showed resemblance with *Cryobacterium* sp. DR9 (FJ464984) by 99.7% and with *Cryobacterium psychrotolerans* hp36 (JN637331) by 98.9%. *Cryobacterium* sp. MLB-29 (JX205196), *Cryobacterium* sp. MLB-30 (JX205197), *Cryobacterium* sp. MLB-34 (JX205201) and *Cryobacterium* sp. MB-5 (JF790972) showed their closest relationship with *Cryobacterium* sp. DR9 (FJ464984) by 98.5%, 98.3%, 99.7%, 98.3% respectively and with *Cryobacterium psychrotolerans* hp36 (JN637331) by 98.2%, 98.0%, 99.5% and 98.3% respectively.

In the phylogenetic tree (Fig.14) constructed by the NJ method, strains *Pseudomonas* sp. MLB-46 had 99% while MLB-42 and MLB-g showed 67% bootstrap support respectively, whereas MLB-43 showed 65% boot strap support. The strain *Polaromonas* sp. MLB-45 showed 99% boot strap support whereas *Micrococcus sp.* MLB-41 and MLB-18 showed 86% boot strap support as compared to *Subtercola* sp. MLB-47, *Agreia* sp. MLB-28 and MB-6 that showed 100% boot strap support. Other polar strains *Leifsonia* sp. MLB-40 and NA-8 showed 99% boot strap support, *Cryobacterium* sp.MLB-31 showed 63% while

MLB-33 and MB-I had 98% boot strap support, however *Cryobacterium* sp. MLB-32 had 63% and MLB-38, MLB-39, MLB-36 had 89% boot strap support. *Cryobacterium* sp. MLB-35 had 63% while MLB-29, MLB-30 had 57% boot strap support, MLB-34 had 78% and MB-5 had 54% boot strap support respectively. A phylogenetic tree of cryoconite isolates used in this study was constructed with closely related reference strains (Fig. 15).

Table 12 Identification of cryoconites bacterial isolates by16S rRNA region sequences similarity (%) [*= Sequence not used in phylogenetic tree (>1000 bp)].

Isolate Code	Generic Identification	Sequence deposition no.	Mol G+C %	Total sequence length after alignment	No. of base changes	16S rRNA gene sequences similarity (%)
MB-g	*Pseudomonas* sp. MB-g	JF790967	61.33	1433	45	*Pseudomonas ficuserectae* JCM2400^T (AB021378) by 96.9%
MLB 42	*Pseudomonas* sp. MLB- 42	JX205209	59.42	1482	46	*Pseudomonas ficuserectae* JCM2400^T (AB021378) by 96.9%
MLB 46	*Pseudomonas* sp. MLB-46	JX205212	54.66	1377	37	*Pseudomonas ficuserectae* JCM2400^T (AB021378) by 97.3%
MLB 43	*Pseudomonas* sp. MLB-43	JX205210	50.89	1474	32	*Pseudomonas ficuserectae* JCM2400^T (AB021378) by 97.8%
MLB 45	*Polaromonas* sp. MLB-45	JX205214	62.02	1383	31	*Polaromonas naphthalenivorans* CJ2^T (AY166684) by 97.8%
*MLB 44	*Polaromonas* sp. MLB-44	JX205211	62.72	981	10	*Polaromonas naphthalenivorans* CJ2^T (AY166684) by 99.0%
MLB 41	*Micrococcus* sp. MLB-41	JX205208	ND	1415	3	*Micrococcus endophyticus* YIM56238^T (EU005372) by 99.8%
MB-18	*Micrococcus* sp. MB-18	JF790969	ND	1365	12	*Micrococcus endophyticus* YIM56238^T (EU005372) by 99.1%
MLB 47	*Subtercola* sp. MLB-47	JX205212	58.88	1444	32	*Subtercola frigoramans* DSM1305^T(AM410673) by 97.8%
MLB-28	*Agreia sp.* MLB-28	JX205195	ND	1436	65	*Agreia pratensis* DSM 14226^T (AJ310412) by 95.5%
MB-6	*Agreia sp.* MB-6	JF790970	ND	1421	75	*Agreia pratensis* DSM 14226^T (AJ310412) by 94.7%
MLB 40	*Leifsonia* sp. MLB 40	JX205207	60.41	1389	22	*Leifsonia antarctica* SPC 20^T (AM931710) by 98.4%
NA-8	*Leifsonia* sp. NA-8	JF790971	ND	1252	53	*Leifsonia antarctica* SPC 20^T (AM931710) by 95.8%
MB-l	*Cryobacterium* sp. MB-l	JF790968	ND	1414	64	*Cryobacterium psychrophilum* DSM4854^T(AM410676) by 95.5%
MLB 31	*Cryobacterium* sp. MLB-31	JX205198	59.88	1470	64	*Cryobacterium psychrophilum* DSM4854^T(AM410676) by 95.6%

MLB 32	*Cryobacterium* sp. MLB-32	JX205199	59.90	1473	52	*Cryobacterium psychrophilum* DSM4854^{T}(AM410676) by 96.5%
MLB 33	*Cryobacterium* sp. MLB-33	JX205200	57.96	1479	63	*Cryobacterium psychrophilum* DSM4854^{T}(AM410676) by 95.7%
MLB 36	*Cryobacterium* sp. MLB-36	JX205203	63.44	1418	26	*Cryobacterium psychrotolerans* hp36 (JN637331) by 98.2%
*MLB 37	*Cryobacterium* sp. MLB-37	JX205204	63.12	686	3	*Cryobacterium psychrotolerans* hp36 (JN637331) by 99.6%
MLB 38	*Cryobacterium* sp. MLB-38	JX205205	ND	1424	64	*Cryobacterium psychrotolerans* hp36 (JN637331) by 95.5%
MLB 39	*Cryobacterium* sp. MLB-39	JX205206	62.18	1424	23	*Cryobacterium psychrotolerans* hp36 (JN637331) by 98.4%
MLB 35	*Cryobacterium* sp. MLB-35	JX205202	63.73	1456	4	*Cryobacterium* sp.DR9 (FJ464984) by 99.7%
MB-5	*Cryobacterium* sp. MB-5	JF790972	60.12	1289	22	*Cryobacterium* sp.DR9 (FJ464984) by 98.3%
MLB 29	*Cryobacterium* sp. MLB-29	JX205196	55.72	1419	26	*Cryobacterium psychrotolerans* hp36 (JN637331) by 98.2%
MLB 30	*Cryobacterium* sp. MLB-30	JX205197	59.96	1383	27	*Cryobacterium psychrotolerans* hp36 (JN637331) by 98.0%
MLB 34	*Cryobacterium* sp. MLB-34	JX205201	60.36	1298	6	*Cryobacterium psychrotolerans* hp36 (JN637331) by 99.5%
*NA-9	*Cryobacterium* sp. NA-9	JF790966	63.22	799	16	*Cryobacterium* sp. DR9 16S ribosomal RNA gene (FJ464984) by 98.0%
*NA-b	*Leifsonia* sp. NA-b	JF790973	60.40	687	5	*Leifsonia* sp. 4-69 16S ribosomal RNA gene (GU213322) by 99.3%
*NA-i	*Cryobacterium* sp.NA-i	ND	63.09	600	ND	*Cryobacterium* sp. Lc30-4 16S ribosomal RNA gene, partial sequence (GU733465) by 97%
*NA-d	*Flavobacterium* sp. NA-d	ND	37.11	700	ND	*Flavobacterium degerlachei* strain LMG 21915 16S ribosomal RNA (AJ557886) by 97%
*NA-1	*Leifsonia* sp. NA-1	ND	60.53	650	ND	*Leifsonia* sp. 4-16 16S ribosomal RNA gene (GU213305) by 98%
*MB-3	*Leifsonia* sp. MB-3	ND	63.33	500	ND	*Leifsonia* sp. 4-69 16S ribosomal RNA gene (GU213322) by 94%
*MB-10	*Cryobacterium* sp. MB-10	ND	63.89	550	ND	*Cryobacterium* sp. 4-57 16S ribosomal RNA gene, partial sequence by 98%
*MB-12	*Leifsonia* sp. MB-12	ND	62.80	450	ND	*Leifsonia* sp. 3030 16S ribosomal RNA gene by 96%
*NA-k	*Leifsonia* sp NA-k	ND	63.65	500	ND	*Leifsonia* sp. 16S ribosomal RNA gene by 97%

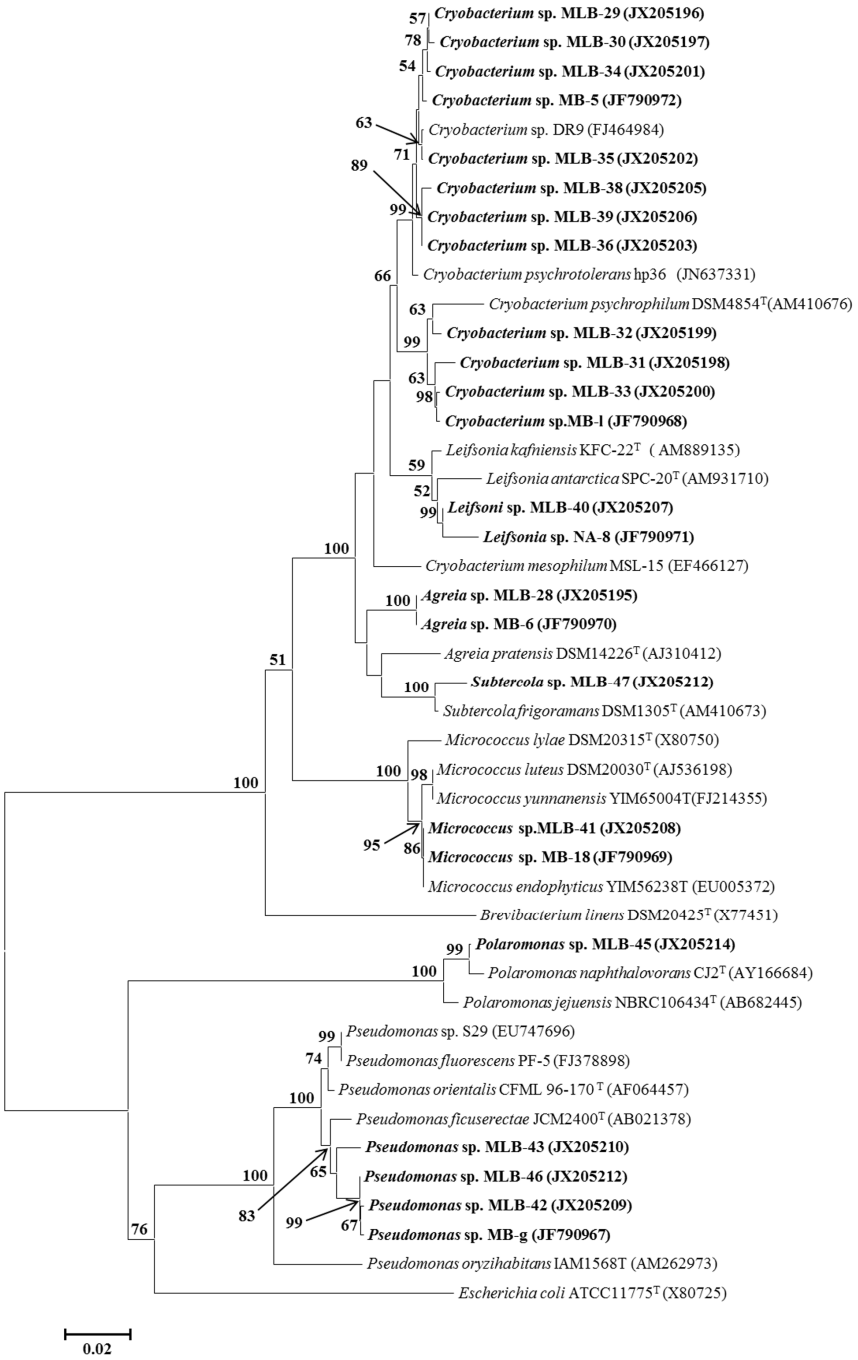


Fig. 15 Phylogenetic tree showing the cryoconite isolates with their closely related species based on the 16S rRNA gene sequences. The accession numbers of strains are given in parentheses. Neighbor-joining method was used for construction of phylogenetic tree by using the Jukes–Cantor distance estimation. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated as substitutions per nucleotide position.

ii) Ice cores bacteria

The total sequence lengths after alignment, % sequence similarities, number of base changes at different positions, and the NCBI sequence deposition numbers are given in Table 13. The pairwise alignment was performed using EMBOSS Matcher - Pairwise Sequence Alignment tool http://www. ebi.ac. uk/ Tools /psa /emboss_matcher/nucleotide.html). The sequence analysis of the 16S rRNA domain of isolate *Bacillus* sp. MLB-7 (JX192642) showed their closest relationship to species of *Bacillus barbaricus* DSM14730 (AJ422145) by 98.1%, *B. arsenicus* con a/3^T (AJ606700) by 96.5%, *B. niacini* DSM2923 (AB021194) by 93.0%, *B. lentus* IAM12466 (D16272) by 91.9%, *B. benzoevorans* DSM5391 (D78311) by 92.1%.

Sphingomonas sp. MLB-9 (JX192644) and *Sphingomonas* sp. MLB-11 (JX192646) resembled with *Sphingomonas phyllosphaerae* FA2 (AY453855) by 96.3%, *S. phyllosphaerae* FA1 (AY563441) by 96.3% sequence similarity in the 16S rRNA region, respectively. *Sphingomonas* sp. MLB-5 (JX192640) resembled with *S. dokdonensis* DS-4 (DQ178975) by 96.4%.

Pseudomonas sp. MLB-3 (JX192653) showed their closest relationship to species of *Pseudomonas oryzihabitans* IAM1568^T (AM262973) by 99.6%. *Pseudomonas* sp. MLB-2 (AB704759) resembled with *P. orientalis* CFML96-170^T (AF064457) by 93.5%, *P. putida* To15 (AB008001) by 92.2%, *P. ficuserectae* JCM2400T (AB021378) by 91.7%, *P. meliae* MAFF301463^T (AB021382) by 92.1%.

Sequence analysis of isolates *Pseudomonas* sp. MLB-12 (JX192647), *Pseudomonas* sp. MLB-14 (JX192649), *Pseudomonas* sp. MLB-16 (JX192651) and *Pseudomonas* sp. MLB-17 (JX192652) resembled *Pseudomonas orientalis* CFML96-170^T (AF064457) by 96.2%, 97.8%, 98.0% and 98.1% respectively whereas similarity to *P. putida* To15 (AB008001) was by 95.6%, 97.4%, 97.3% and 97.6% respectively. MLB-12, 14, 16 and 17 showed similarity to *P. ficuserectae* JCM2400T (AB021378) by 94.7%, 96.6%, 96.0% and 96.8%

respectively whereas the sequence similarity to *P. meliae* MAFF301463^{T} (AB021382) was by 95.0%, 96.8%, 96.3% and 97.1% respectively.

Table 13. Identification of Ice core bacterial isolates by 16S rRNA region sequence similarity (%).

Isolate Code	Genus Identification code	Sequence deposition no.	Total sequence length after alignment	No. of base changes	16S rRNA gene sequences similarity (%)
MLB-2	*Pseudomonas* sp. MLB-2	AB704759	1361	89	*Pseudomonas orientalis* CFML96-170^{T} (AF064457) by 93.5%
MLB-14	*Pseudomonas* sp. MLB-14	JX192649	1412	31	*Pseudomonas orientalis* CFML 96-170^{T} (AF064457) by 97.8%
MLB-16	*Pseudomonas* sp. MLB-16	JX192651	1425	28	*Pseudomonas orientalis* CFML 96-170^{T} (AF064457) by 98.0%
MLB-17	*Pseudomonas* sp. MLB-17	JX192652	1418	9	*Pseudomonas orientalis* CFML 96-170^{T} (AF064457) by 99.4%
MLB-4	*Pseudomonas* sp. MLB-4	JX192654	1451	2	*Pseudomonas fluorescens* FP-5 (FJ378898) by 99.9%
MLB-8	*Pseudomonas* sp. MLB-8	JX192643	1451	3	*Pseudomonas fluorescens* FP-5 (FJ378898) by 99.8%
MLB-12	*Pseudomonas* sp. MLB-12	JX192647	1417	36	*Pseudomonas fluorescens* FP-5 (FJ378898) by 97.5%
MLB-15	*Pseudomonas* sp. MLB-15	JX192650	1410	5	*Pseudomonas fluorescens* FP-5 (FJ378898) by 99.6%
MLB-3	*Pseudomonas* sp. MLB-3	JX192653	1466	6	*Pseudomonas oryzihabitans* IAM1568^{T} (AM262973) by 99.6%
MLB-6	*Pseudomonas* sp. MLB-6	JX192641	1462	6	*Pseudomonas syncyanea* NBRC3757 (AB680130) by 99.6%
MLB-5	*Sphingomonas* sp. MLB-5	JX192640	1387	50	*Sphingomonas dokdonensis* DS-4 (DQ178975) by 96.4%
MLB-9	*Sphingomonas* sp. MLB-9	JX192644	1436	53	*Sphingomonas phyllosphaerae* FA2 (AY453855) by 96.3%
MLB-11	*Sphingomonas* sp. MLB-11	JX192646	1383	23	*Sphingomonas phyllosphaerae* FA1 (AY563441) by 98.3%
MLB-7	*Bacillus* sp. MLB-7	JX192642	1361	26	*Bacillus barbaricus* DSM14730 (AJ422145) by 98.1%

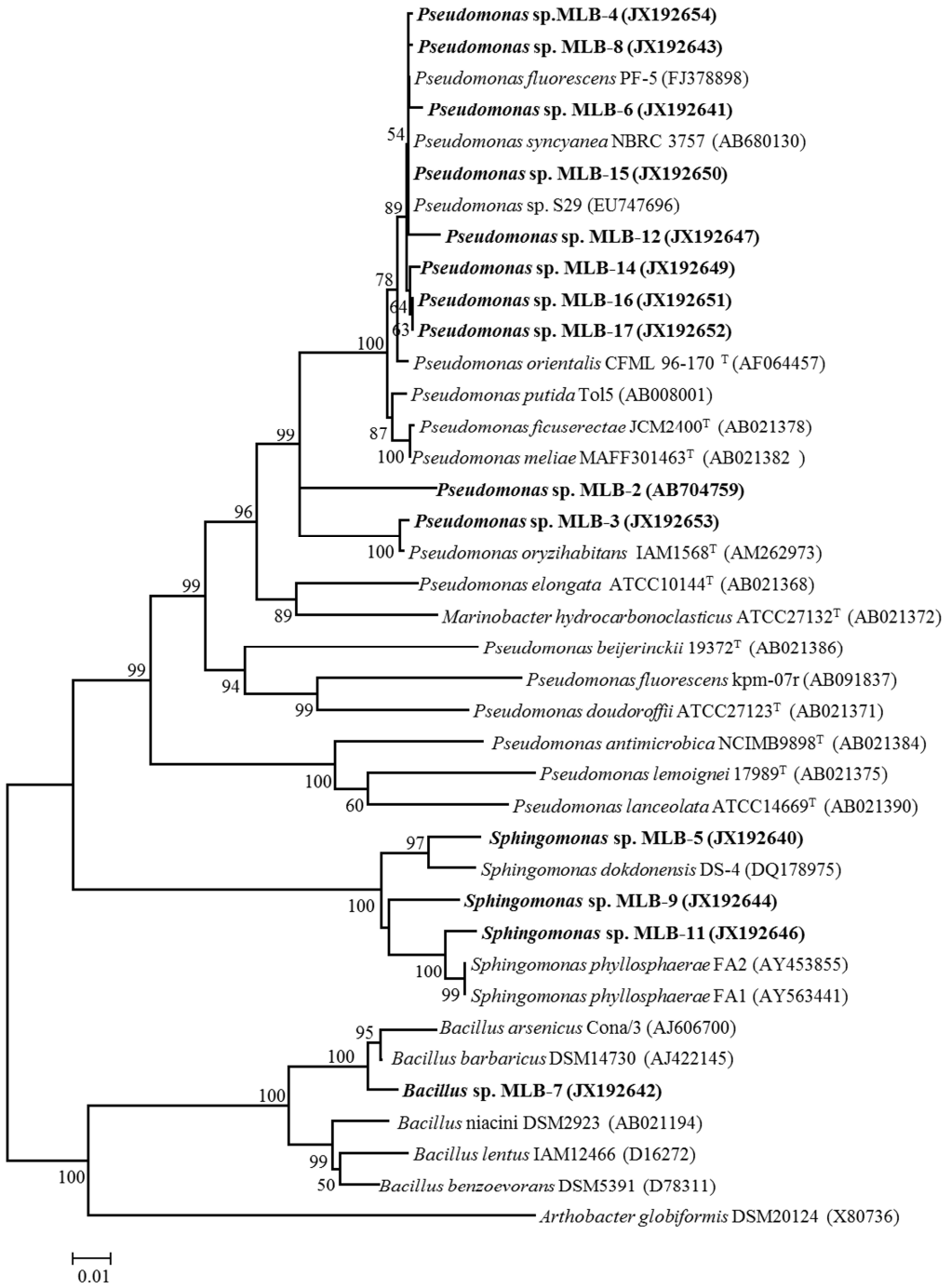


Fig. 16 Phylogenetic tree showing the Ice core bacterial isolates with their closely related species based on the 16S rRNA gene sequences. The accession numbers of strains are given in parentheses. Neighbor-joining (NJ) method was used for construction of phylogenetic tree. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated as substitutions per nucleotide position.

Sequence analysis of isolate *Pseudomonas* sp. MLB-15 (JX192650) indicated their closest relationship to the species of *Pseudomonas* sp. S29 (EU747696) by 99.6% sequence similarity. Sequence analysis of isolates *Pseudomonas* sp. MLB-6 (JX192641) showed their closest relationship to *P. syncyanea* NBRC3757 (AB680130) by 99.6% sequence similarity.

Sequence analysis of isolates *Pseudomonas* sp. MLB-4 (JX192654) and *Pseudomonas* sp. MLB-8 (JX192643) resembled *P. fluorescens* FP-5 (FJ378898) by 99.9% and 99.8%, and *P. syncyanea* NBRC3757 (AB680130) by 99.9% and 99.9% sequence similarity. In the neighbour-joining phylogenetic tree, strains *Bacillus* sp. MLB-7 showed 100% bootstrap support, *Sphingomonas* sp. MLB-11 showed 100% bootstrap support, whereas *Sphingomonas* sp. MLB-9 showed merely 45% bootstrap support as compared to *Sphingomonas* sp. MLB-5 that had 97% bootstrap support, *Pseudomonas* sp. MLB-3 had 100% bootstrap support, *Pseudomonas* sp. MLB-2 had 52% bootstrap support. *Pseudomonas* sp. MLB-12, 14, 16, 17, showed 53%, 89%, 64% and 63% bootstrap support, *Pseudomonas* sp. MLB-4, 8, 15 had 50% bootstrap support and *Pseudomonas* sp. MLB-6 had 18% bootstrap support. The phylogenetic tree of the representative strains is shown in Fig. 16.

iii) Permafrost bacteria

The total sequence lengths after alignment, % sequence similarities, and the NCBI sequence deposition numbers are given in Table 14. One hundred six bacterial strains were sequenced and the result showed 13 genera belonging to seven families namely, Bacillaceae (*Bacillus*, *Virgibacillus*), Micrococcaceae (*Arthrobacter, Nesterenkonia*) Nocardiaceae (*Rhodococcus*) belonging to group of Gram-positive bacteria, and Moraxellaceae (*Acinetobacter*, *Psychrobacter*), Rhizobiaceae (*Rhizobium*), Xanthomonadaceae (*Stenotrophomonas*), Sphingobacteriaceae (*Sphingobacterium*), Pseudomonadaceae (*Pseudomonas*), Enterobacteriaceae (*Enterobacter*), Sphingomonadaceae (*Sphingopyxis*)

belonging to group of Gram-negative bacteria. Phylogenetic trees of different taxa belonging to seven families were constructed based on 16S rRNA gene sequences using the NJ method (Fig. 17-19).

The sequence analysis of the 16S rRNA domain of isolates of *Acinetobacter* sp. showed their closest similarity with *Acinetobacter johnsonii* ATCC17909^T (Z93440). *Psychrobacter* sp. showed closest similarity with *Psychrobacter luti* NF11^T (AJ430828). *Virgibacillus* sp. showed closest similarity with *V. pantothenticus* IAM11061^T (D78477). *Bacillus* sp. showed closest similarity with *B. selenatarsenatis* SF-1^T (AB262082). *Rhizobium* sp. showed closest similarity with *R. giardinii* H152^T (U86344). *Stenotrophomonas* sp. showed closest similarity with *S. maltophilia* LMG20578 (AY040357). *Sphingobacterium* sp. showed similarity with *S. kitahiroshimaense* 10C^T (AB361248). *Pseudomonas* strains indicated their closest similarity with *P. jessenii* FB28 (AM933519), *P. meliae* MAFF301463^T (AB021382), *P. mandelii* CIP105273^T (AF058286) and *P. congelans* BZ27 (HQ588846). *Arthrobacter* sp. showed closest similarity with *A. bergerei* CIP108036^T (AJ609630), *A. scleromae* JCM12642^T (AF330692), *A. globiformis* DSM20124^T (M23411) and *A. sulfonivorans* DSM14002^T (AF235091). The details of the gene sequence similarity (%) of individual isolate/strain are given in Table 14.

Table 14. Identification of Permafrost bacterial isolates by 16S rRNA region sequences similarity (%) [*= Sequence not used in phylogenetic tree (>1000 bp)].

Culture/ Isolate number	Age	NCBI Accession No.	Total sequenc elength	16S rRNA gene sequence similarity (%)
PF1B1	13850	KC311564	1384	*Acinetobacter johnsonii* ATCC17909^T Z93440, (99.5%)
PF1M1	22700	KC311563	1430	*Acinetobacter johnsonii* ATCC17909^T Z93440, (99.2%)
PF3B1	37000	KC311587	1440	*Acinetobacter johnsonii* ATCC17909^T Z93440, (99.3%)
PF1B7*	13850	KC311631	727	*Acinetobacter johnsonii* KF049130, (99.3%)
PF5B2	12250	KC311625	1468	*Psychrobacter luti* NF11^T AJ430828, (98.8%)
				Psychrobacter glacinicola DSM12194^T AJ312213, (98.4%)
PF1T6	Recent	KC311570	1446	*Arthrobacter bergerei* CIP108036^T AJ609630, (98.9%)
PF1T1	Recent	KC311565	1448	*Arthrobacter bergerei* CIP108036^T AJ609630, (98.1%)
PF2T3	Recent	KC311574	1442	*Arthrobacter scleromae* JCM12642^T AF330692, (97.7%)
				Arthrobacter globiformis DSM20124^T M23411, (96.2%)
PF2T2	Recent	KC311573	1449	*Arthrobacter scleromae* JCM12642^T AF330692, (97.8%)
				Arthrobacter globiformis DSM20124^T M23411, (96.8%)
PF5M1	14150	KC311598	1450	*Arthrobacter scleromae* JCM12642^T AF330692, (97.8%)
				Arthrobacter globiformis DSM20124^T M23411, (96.8%)
PF1T4	Recent	KC311568	1390	*Arthrobacter scleromae* JCM12642^T AF330692, (98.4%)
				Arthrobacter globiformis DSM20124^T M23411, (97.6%)
PF4M1	14850	KC311593	1357	*Arthrobacter scleromae* JCM12642^T AF330692, (98.2%)
				Arthrobacter globiformis DSM20124^T M23411, (97.6%)
PF3B2	37000	KC311588	1429	*Arthrobacter scleromae* JCM12642^T AF330692, (98.2%)
				Arthrobacter globiformis DSM20124^T M23411, (97.3%)
PF4M3	14850	KC311595	1319	*Arthrobacter scleromae* JCM12642^T AF330692, (98.1%)
				Arthrobacter globiformis DSM20124^T M23411, (97.6%)
PF4M4	14850	KC311596	1416	*Arthrobacter scleromae* JCM12642^T AF330692, (98.0%)
				Arthrobacter globiformis DSM20124^T M23411, (96.6%)
PF2B3	44800	KC311584	1302	*Arthrobacter scleromae* JCM12642^T AF330692, (97.7%)
				Arthrobacter globiformis DSM20124^T M23411, (97.1%)
PF4M2	14850	KC311594	1430	*Arthrobacter scleromae* JCM12642^T AF330692, (97.2%)
				Arthrobacter globiformis DSM20124^T M23411, (98.0%)
PF3B3	37000	KC311589	1411	*Arthrobacter scleromae* JCM12642^T AF330692, (96.6%)
				Arthrobacter globiformis DSM20124^T M23411, (97.5%)

PF4T1	Recent	KC311591	1450	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (97.5%)
PF1T2	Recent	KC311566	1447	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (97.0%)
PF3M1	32300	KC311586	1412	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (98.1%)
PF4T2	Recent	KC311592	1452	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (97.4%)
PF3B4	37000	KC311590	1411	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (98.4%)
PF2T4	Recent	KC311575	1449	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (97.9%)
PF3T1	Recent	KC311585	1449	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (97.6%)
PF5B1	12250	KC311599	1450	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (97.8%)
PF1T5	Recent	KC311569	1446	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (97.5%)
PF1T7	Recent	KC311571	1452	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (98.2%)
PF1T3	Recent	KC311567	1447	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (97.9%)
PF2B1	44800	KC311582	1331	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (99.2%)
PF2M1	38300	KC311576	1335	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (99.0%)
PF2M6	38300	KC311581	1346	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (98.9%)
PF2M4	38300	KC311579	1423	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (98.2%)
PF4B1	24300	KC311597	1291	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (99.0%)
PF2M2	38300	KC311577	1426	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (98.7%)
PF2B2	44800	KC311583	1328	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (99.2%)
PF2M5	38300	KC311580	1408	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (98.2%)
PF2M3	38300	KC311578	1411	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (98.5%)
PF2T1	Recent	KC311572	1450	*Arthrobacter sulfonivorans* DSM14002^T AF235091, (97.9%)
PF2M13*	38300	KC311632	934	*Arthrobacter* sp. JF313071, (98.8%)
PF2M14*	38300	KC311633	893	*Arthrobacter* sp. JF313071, (98.8%)
PF2M15*	38300	KC311634	688	*Arthrobacter* sp. JF313091, (99.1%)
PF2M16*	38300	KC311635	911	*Arthrobacter* sp. AM491456, (99.7%)
PF2B5*	44800	KC311636	968	*Arthrobacter* sp. JX949854, (97.2%)
PF3M6*	32300		624	*Arthrobacter* sp. JF313064, (99%)
PF3B6*	37000	KC311637	590	*Arthrobacter* sp. GU321363, (99.3%)
PF3B7*	37000	KC311638	861	*Arthrobacter* sp. FJ517630, (98.4%)
PF3B8*	37000	KC311639	735	*Arthrobacter* sp. HQ202868, (100%)
PF3B9*	37000	KC311640	724	*Arthrobacter* sp. GU321363, (99.8%)
PF3B10*	37000	KC311641	774	*Arthrobacter* sp. (JX266381, (99.9%)
PF4M7*	14850	KC311642	702	*Arthrobacter* sp. AY332095, (98.9%)
PF4M8*	14850	KC311643	615	*Arthrobacter* sp. JF313071, (99.8%)
PF4M9*	14850	KC311644	546	*Arthrobacter* sp. GU321363, (99.6%)
PF4M10*	14850	KC311645	740	*Arthrobacter* sp. JN688165, (99.5%)
PF4M11*	14850	KC311646	769	*Arthrobacter* sp. JF313087, (99.0%)
PF4B3*	24300	KC311647	477	*Arthrobacter* sp. JX949860, (99.6%)
PF2B6*	44800	KC311650	880	*Nesterenkonia* sp. EF151510, (99.5%)
PF2B4	44800	KC311600	1408	*Bacillus selenatarsenatis* SF-1^T AB262082, (99.3%)
				Bacillus jeotgali YKJ-10^{T}AF221061, (99.5%)

PF1B6	13850	KC311630	1401	*Virgibacillus pantothenticus* IAM11061^{T} D78477, (98.4%)
PF1M3*	22700	KC311648	512	*Enterobacter* sp. JF681289, (99.8%)
PF1M4*	22700	KC311649	492	*Enterobacter* sp. EU816586 (99.0%)
PF2M8	38300	KC311608	1370	*Pseudomonas jessenii FB28* AM933519, (99.7%)
PF1B5	13850	KC311604	1218	*Pseudomonas jessenii* FB28 AM933519, (99.8%)
PF1B4	13850	KC311603	1307	*Pseudomonas jessenii* FB28 AM933519, (99.8%)
PF3B5	37000	KC311621	1427	*Pseudomonas meliae* MAFF301463T AB021382, (98.9%)
PF1B3	13850	KC311602	1437	*Pseudomonas meliae* MAFF301463T AB021382, (99.1%)
PF3M2	32300	KC311617	1262	*Pseudomonas mandelii* CIP105273T AF058286, (99.8%)
PF3M5	32300	KC311620	1330	*Pseudomonas mandelii* CIP105273T AF058286, (99.7%)
PF2M7	38300	KC311607	1382	*Pseudomonas mandelii* CIP105273T AF058286, (99.8%)
PF2M12	38300	KC311612	1443	*Pseudomonas mandelii* CIP105273T AF058286, (98.7%)
PF2M11	38300	KC311611	1337	*Pseudomonas mandelii* CIP105273T AF058286, (99.9%)
PF4M5	14850	KC311622	1443	*Pseudomonas mandelii* CIP105273T AF058286, (99.5%)
PF2M9	38300	KC311609	1432	*Pseudomonas mandelii* CIP105273T AF058286, (99.7%)
PF3M4	32300	KC311619	1398	*Pseudomonas mandelii* CIP105273T AF058286, (99.9%)
PF2M10	38300	KC311610	1430	*Pseudomonas mandelii* CIP105273T AF058286, (99.5%)
PF3M3	32300	KC311618	1387	*Pseudomonas mandelii* CIP105273T AF058286, (99.7%)
PF1B2	13850	KC311601	1434	*Pseudomonas congelans* BZ27 HQ588846, (98.2%)
PF3T3	Recent	KC311614	1459	*Pseudomonas congelans* BZ27 HQ588846, (97.0%)
PF3T2	Recent	KC311613	1461	*Pseudomonas congelans* BZ27 HQ588846, (97.2%)
PF3T4	Recent	KC311615	1397	*Pseudomonas congelans* BZ27 HQ588846, (97.9%)
PF5T2	Recent	KC311624	1457	*Pseudomonas congelans* BZ27 HQ588846, (97.5%)
PF5T1	Recent	KC311623	1457	*Pseudomonas congelans* BZ27 HQ588846, (97.8%)
PF2T5	Recent	KC311605	1455	*Pseudomonas congelans* BZ27 HQ588846, (98.0%)
PF2T6	Recent	KC311606	1456	*Pseudomonas congelans BZ27 HQ588846, (97.7%)*
PF3T5	Recent	KC311616	1461	*Pseudomonas congelans* BZ27 HQ588846, (97.8%)
PF1B8*	13850	KC311657	830	*Pseudomonas* sp. HF545330, (99.4%)
PF2M17*	38300	KC311656	631	*Pseudomonas* sp. JX949977, (99.7%)
PF3M7*	32300	KC311651	671	*Pseudomonas* sp. JX949980, (99.5%)
PF3M8*	32300	KC311652	780	*Pseudomonas* sp. AY864639, (98.9%)
PF3B11*	37000	KC311653	735	*Pseudomonas* sp. JQ800176, (98.5%)
PF3B12*	37000	KC311654	646	*Pseudomonas* sp. JX949980, (99.8%)
PF3B13*	37000	KC311655	792	*Pseudomonas* sp. JF313044, (100%)
PF4M12*	14850	KC311658	512	*Pseudomonas* sp. KF147104, (99.6%)
PF4B4*	24300	KC311659	469	*Pseudomonas* sp. DQ339611, (99.8%)
PF4B5*	24300	KC311660	664	*Pseudomonas* sp. JX949981, (99.7%)
PF4B6*	24300	KC311661	680	*Pseudomonas* sp. JF313066, (96.9%)
PF4B7*	24300	KC311662	435	*Pseudomonas mandelli* HF545330, (99.8%)
PF4M6	14850	KC311626	1038	*Rhizobium giardinii* H152T U86344, (98.4%)
PF1M5*	22700	KC311663	546	*Rhizobium* sp. EU256432, (99.3%)
PF2M18*	38300	KC311664	750	*Rhizobium* sp. JQ396566, (99.6%)
PF2M19*	38300	KC311665	542	*Rhizobium* sp. EU256432, (99.2%)
PF1M6*	22700	KC311666	731	*Rhodococcus* sp. JX949806, (99.6%)
PF2M20*	38300	KC311667	545	*Sphingopyxis sp.* FN821841, (98.9%)
PF3T6	Recent	KC311627	1458	*Sphingobacterium kitahiroshimaense* 10CT AB361248, (97.3%)
				Sphingobacterium faecium JCM21820T AJ438176, (97.1%)
PF1M2	22700	KC311628	1340	*Stenotrophomonas maltophilia* LMG20578 AY040357, (99.7%)
				Stenotrophomonas rhizophila DSM14405^{T} AJ293463, (99.5%)
PF1B9*	13850	KC311668	795	*Stenotrophomonas* sp. JF262559, (99.7%)

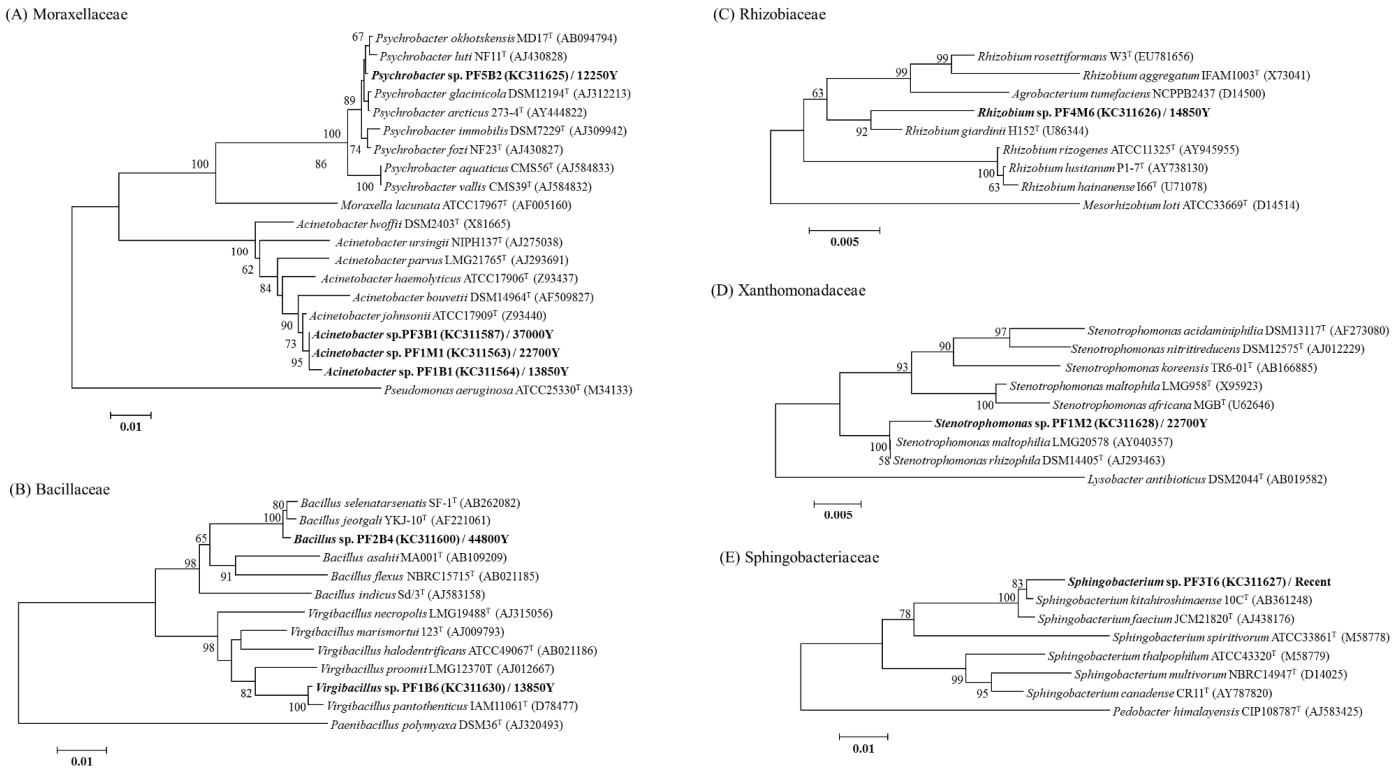


Fig.17 Phylogenetic trees show the permafrost bacterial strains of different genera (*Acinetobacter, Bacillus, Psychrobacter, Rhizobium, Stenotrophomonas, Sphingobacterium* and *Virgibacillus*) with closely related species based on 16S rRNA region sequences analyses. The accession numbers of strains with Age are shown in parentheses. Trees were constructed with neighbor-joining method. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated substitutions per nucleotide position.

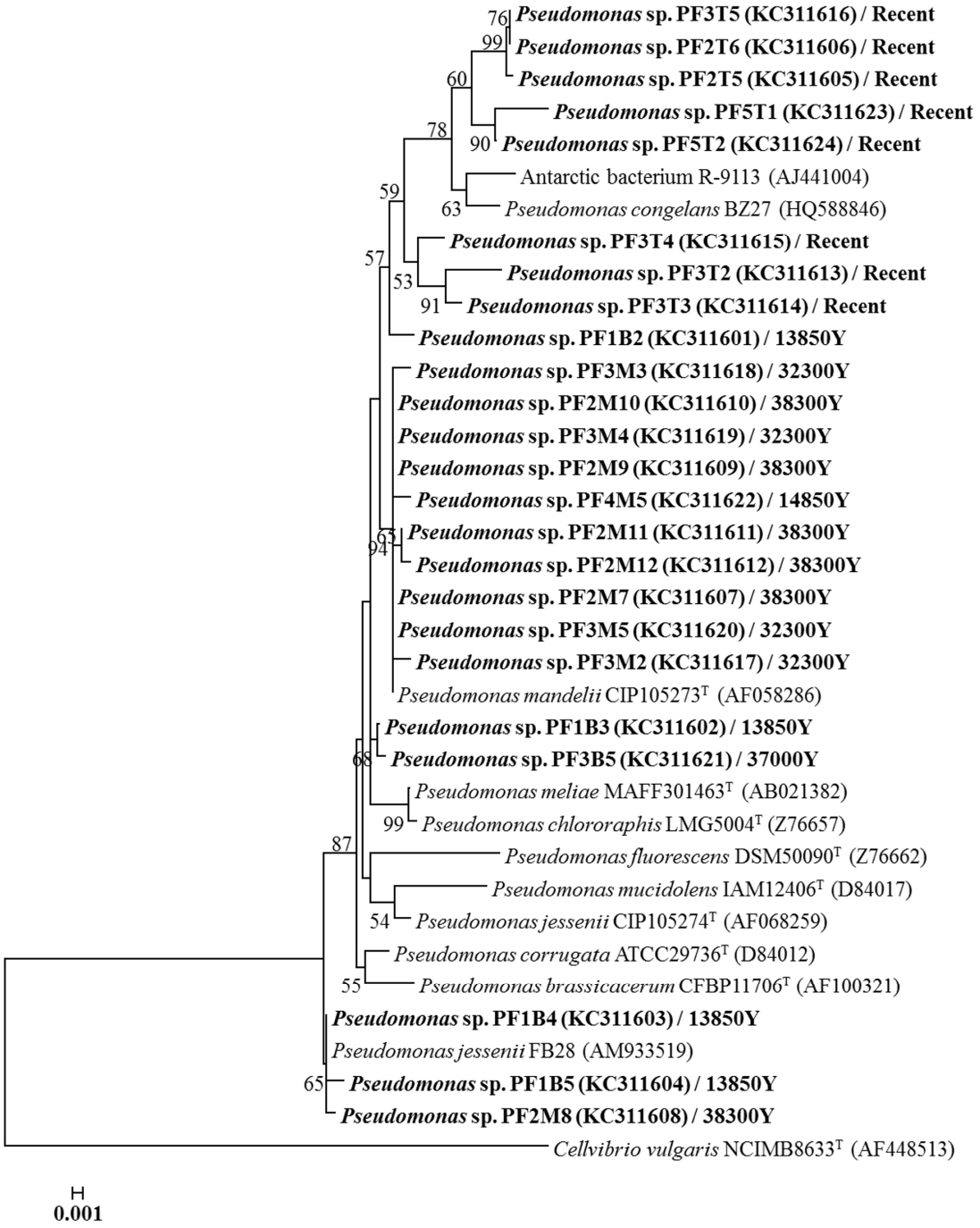


Fig.18 Phylogenetic tree shows the *Pseudomonas* strains of permafrost with closely related species based on 16S rRNA region sequence analyses. The accession numbers of strains with Age are shown in parentheses. Trees were constructed with neighbor-joining method.

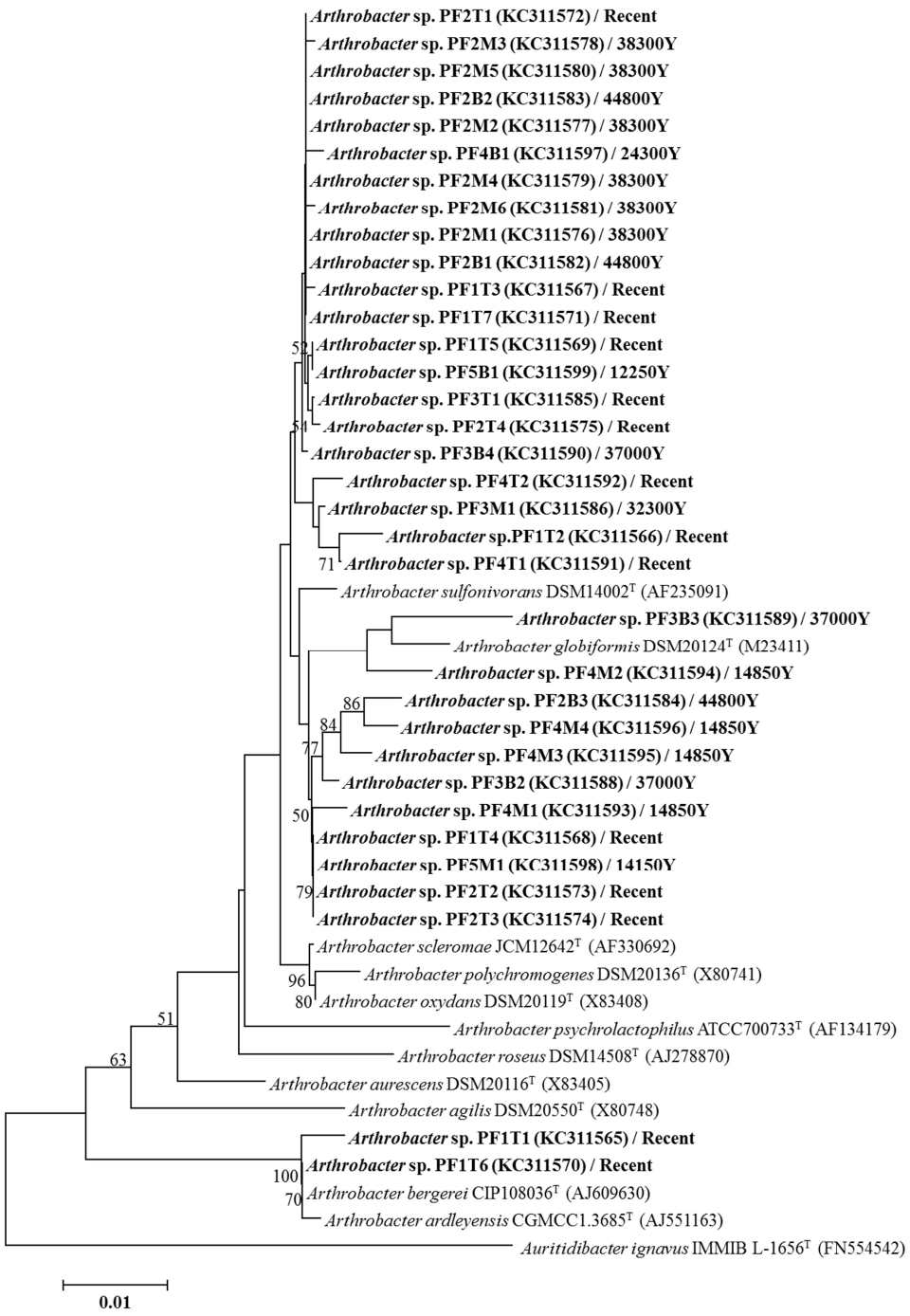


Fig. 19 Permafrost strains of *Arthobactor* sp. show close phylogenetic relatedness with other *Arthobactor* sp. based on 16S rRNA region sequence analyses. The accession numbers of strains with age are shown in parentheses.

3a.3.7 Distribution pattern of the bacteria in three pristine habitats (Cryoconites, Ice cores, and Permafrost)

i) Bacteria in the cryoconites of the three glaciers

The cryoconites bacterial isolates from three glaciers belonged to eight genera namely, *Agreia*, *Cryobacterium*, *Flavobacterium*, *Leifsonia*, *Micrococcus*, *Polaromonas*, *Pseudomonas*, and *Subtercola*. Number of taxa varied from 3 to 8 while number of isolates varied from 5 to 17 at each glacier. Evenness index ranged from 0.912 (VB) to 0.976 (ML); Simpson's diversity index (1-D) from 0.64 (AB) to 0.85 (VB); and Shannon's diversity index (H) from 1.05 (AB) to 1.99 (VB). Species *Pseudomonas ficuserectae* (GC 50.89-59.42%), *Agreia pratensis*, *Leifsonia antarctica* (GC 60.41%) and *Cryobacterium psychrotolerans* (GC 59.96%), were isolated from ML, *Cryobacterium psychrotolerans* (GC 55.72-60.36%) and *Subtercola frigoramans* (GC 58.88%) from AB and *Pseudomonas ficuserectae* (GC 54.66-61.33%), *Polaromonas naphthalenivorans* (GC 62.02-62.72%), *Micrococcus endophyticus*, *Cryobacterium psychrophilum* (GC 57.96-59.9%), *C. psychrotolerans* (GC 62.18-63.44 %) and *Flavobacterium degerlachei* (GC 37.11%), from VB. The most predominant species was *Cryobacterium psychrotolerans* followed by *C. psychrophilum* (Table 12). *C. psychrotolerans* was found to all glacier cryoconites. Isolates belonged to genus *Pseudomonas* were the next most abundant and taxon *Pseudomonas ficuserectae* exhibited irregular distribution along the different glacier cryoconites and were not found in the AB glacier cryoconite. Isolates belonged to taxon *Leifsonia antarctica* were present in ML glacier while absent in AB and VB glaciers. Genera *Agreia*, *Flavobacterium*, *Micrococcus*, *Polaromonas*, and *Subtercola* were least represented. *Polaromonas naphthalenivorans* and *Micrococcus endophyticus* were found to be present in only VB glacier; *Subtercola frigoramans* was present only in AB glacier. The presence of *Agreia pratensis* was recorded uniquely in ML glacier whereas *Flavobacterium degerlachei* was present particularly in VB glacier. Quite interestingly, amongst the three glaciers studied, Glacier VB showed maximum diversity as represented by eight different

species whereas AB glacier showed less diversity represented by merely three species.

ii) Bacteria in Ice cores Vertical Profile

Altogether fourteen isolates were isolated from three different parts (top, middle and bottom section) of the ice core. Seven isolates were obtained from the top section followed by five isolates from the bottom section and only two isolates were found from the middle section of the ice core. While *Pseudomonas orientalis* was isolated from the top and middle layers of the Ice core, *Pseudomonas fluorescens* and *Sphingomonas phyllosphaerae* were found at the top and bottom layers respectively. *Sphingomonas dokdonensis* and *Pseudomonas syncyanea* were exclusively isolated at the top layers. *Pseudomonas oryzihabitans* was found at the middle while *Bacillus barbaricus* was detected at the bottom layers.

iii) Bacteria in Permafrost Vertical Profile

In total hundred and six strains were isolated from different depths of permafrost pits. Radiocarbon dates of soil samples from each depth of these pits were recorded. Number of genera reported at each soil age is depicted in Fig 20.

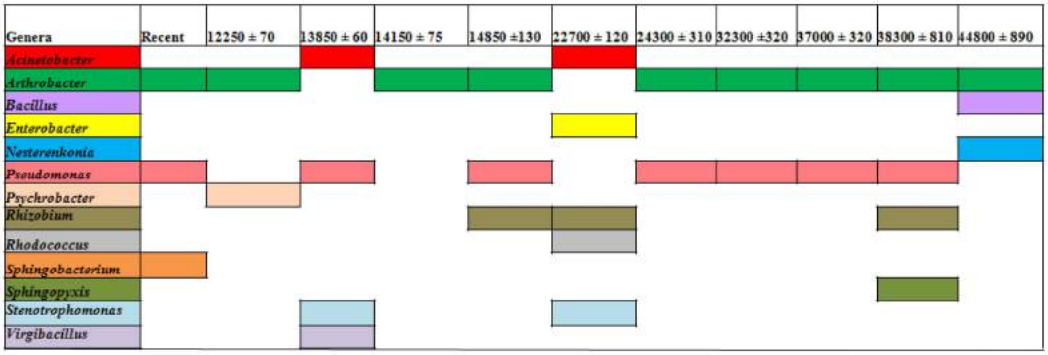


Fig. 20 Number of genera reported at each permafrost soil age.

The oldest soil studied was 44800 years and represented by three genera, *Arthrobacter, Bacillus* and *Nesterenkonia* (Fig.20). Species of genera *Arthrobacter, Pseudomonas, Rhizobium* and *Sphingopyxis* were present in the soils that aged between 38300 to 32300 years. During 24300 to 37000 year old soils only two genera *Arthrobacter* and *Pseudomonas* were present. Interestingly 22700 years old soils contained five genera namely, *Acinetobacter, Enterobacter, Rhizobium, Rhodococcus* and *Stenotrophomonas* species. Soils between ages of 14850 to 14150 contained *Arthrobacter, Pseudomonas* and *Rhizobium* species. About 13850 years old soils revealed the presence of four genera namely, *Acinetobacter, Pseudomonas, Stenotrophomonas* and *Virgibacillus.* Two different strains namely *Psychrobacter* and *Arthrobacter* were reported from soils of about 12250 years old. However, the soils of the recent past contained genera such as *Arthrobacter, Pseudomonas* and *Sphingobacterium.*

3a.4 Discussion

i) Cryoconites bacteria

The present investigation revealed the pH values of the cryoconite holes vary between 7.1 to 8.6. These data are unique as the pH value of each hole is distinct, differing from one glacier to another and often the pH are influenced by the distance amongst these cryoconite holes. The organic and mineral constituents of the cryoconite largely influence the pH values. Working on the crycoconite holes, Kastovska *et al.* (2005) measured the pH values of these ecological niches on Svalbard glaciers approximately 4.7 whereas the pH values of the cryoconite holes on the Canada glacier (Antarctica) and the White glacier (Arctic) was reported between 5.9-9.6 and 3.4-4.5, respectively (Mueller and Pollard 2004). The nitrate content in the Svalbard glaciers (AB,VB, ML) was measured between 44-50 µg/g while at White glacier, Canada it was recorded between 10-33 µg per litre (Mueller and Pollard 2004). It was reported earlier that cyanobacterial

community plays an important role in nitrogen fixation (Vincent and Howard-Williams 1994); however the phosphorus cycle is maintained by atmosphere, and mineral resources (Mueller *et al.* 2001).

The bacterial CFUs recorded ranged from 2.7 x 10^3 to 8.8 x 10^4/g and TC from 5.07 x 10^5 to 1.50 x 10^6 /g at the three different glaciers studied (ML, AB and VB). This observation is similar to that observed for cryoconite meltwater on glaciers in Antarctica where the maximum number of CFU was recorded to be 8.6 x 10^4 CFU/ ml (Christner *et al*. 2003). Foreman *et al.* (2007) reported the bacterial cell numbers to be between 1.27 x 10^4 to 7.94 x 10^4 CFU/ml in the cryoconite overlying ice from Hughes and Canada glaciers of Yaylor valley, Antarctica. This data is also in agreement with that observed by Säwström (2002) in which he reported the total bacterial concentration at the ML glacier to be typically between 2.5 and 5.70 x 10^4/ ml. In fact, the microbial concentration is always greater in the sediment-containing samples compared to the overlying ice (Foreman *et al.* 2007).

One of the earlier study has indicated that the TC ranged from 87.8 x 10^8 to 2802.3 x 10^8 cells per mg at ML, 183.6 x 10^8 to 1405.3 x 10^8 cells per mg at AB and 35.3 x 10^8 to 433.9 x 10^8 cells per mg at VB glaciers (Kaštovská *et al.* 2005). This indicates that the bacterial cells colonise depending on the nature of the glacier and surface area; the process of bacterial colonization are influenced by size, age and geochemistry of the cryoconite niches.

The growth profile of the isolates was recorded between 1 and 22°C, barring for one that grew even at 30°C. The optimum temperature of growth for all was 15°C which points out that the bacterial isolates are strictly cryophilic in nature. Working on a similar line with bacterial isolates from Antarctic cryoconite samples, Christner *et al.* (2003) observed profuse growth at 4 and 15°C as compared to 22°C. Conversely, the cryoconite study involving the alpine glaciers by Lee *et al.* (2011) revealed that the bacterial isolates were mostly psychrotolerant having an optimum growth temperature at 30°C.

Taxonomic analysis of the strains

The phylogenetic tree constructed to determine the affiliation of the representative strains of is shown in Fig. 15. The two Gram-negative taxa groups (*Pseudomonas* and *Polaromonas*) belonging to the phylum *Proteobacteria* constituted a separate clade. One of the isolates, *Flavobacterium,* belonging to the phylum *Bacteroidetes,* could not be considered during the construction of phylogenetic tree as the nucleotide sequence length obtained was less than 1000 bp (Table 12).

The 5 remaining Gram-positive groups were affiliated to the phylum *Actinobacteria.* The isolates under this group showed close resemblance with the cryophilic strains, reported elsehwhere. An isolate of *Cryobacterium* from Crater lake, Antarctica and from Dongekemadi glacier, Qinghai-Tibetan Pleateau showed close affinity with *Cryobacterium* isolates. *Leifsonia kafniensis,* isolated from Kafni glacier, India and *Leifsonia antarctica,* isolated from Larsemann Hills, Antarctica revealed close affinity with the *Leifsonia* isolate. The occurrence of related phylotypes in geographically diverse sub-zero climatic conditions suggests that they might be armed with comparable adaptation sytems that enabled them to withstand and subsist at low temperatures (Abyzov *et al.* 1998). In this regard, these adaptation strategies might include possession of pigments or PUFAs or enzymes that are active at low temperature (Chintalapati *et al.* 2004; Shivaji *et al.* 2007).

Distribution of the isolates in the three glaciers cryoconites

The cryoconite bacterial isolates belonging to eight genera namely, *Pseudomonas, Polaromonas, Micrococcus, Subtercola, Agreia, Leifsonia, Cryobacterium,* and *Flavobacterium.* Genera such as *Pseudomonas, Flavobacterium* and *Cryobacterium* are of common occurrence in cryoconite habitat and have been previously reported from the Antarctic cryoconites (Christner *et al.* 2003). Genera belonging to *Pseudomonas* and *Flavobacterium* were earlier been reported in the Alpine cryoconites (Lee *et al.* 2011). Besides this, genera like *Polaromonas, Micrococcus, Subtercola* and *Agreia* are cold-adapted and were reported earlier

from Arctic habitats such as snow, permafrost and sea ice (Bakermans *et al.* 2003; Groudieva *et al.* 2004, Steven *et al.* 2007, Harding *et al.* 2011). Most of the species reported during this study (*Cryobacterium psychrotolerans, C. psychrophilum, Leifsonia antarctica, Polaromonas naphthalenivorans, Pseudomonas ficuserectae, Micrococcus endophyticus* and *Subtercola frigoramans*) showed a GC content of >55% with the exception of a species *Flavobacterium degerlachei,* where the GC content was low. Bacterial isolates with differential GC content was also observed by Xiang *et al.* (2005) at Muztag Ata Glacier and by Zhang *et al.* (2008) in Puruogangri Ice Core. Xiang *et al.* (2005) described that the GC value discrepancies in all those isolates are due to the difference in origin and micro-niche besides the nutrient availability within the ice. The diversity indices of all the bacterial isolates from the three glaciers were compared; VB has a greater diversity of cryoconite bacteria followed by ML and then AB. The bacterial isolates identified from the ML glacier showed greater evenness index as compared to the bacteria from the other two glacier sites.

Fatty acid profiles

Isolates belonging to the genera *Cryobacterium* and *Micrococcus* were subjected to fatty acid analysis. The analysis shows that the concentration of branched fatty acids was much higher than the saturated fatty acids. The most dominant fatty acids recorded in the isolates were anteiso-$C_{15:0}$, anteiso-$C_{17:0,}$ iso-$C_{15:0,}$ iso-$C_{16:0}$ and anteiso-$C_{15:1}$ A. The erstwhile study corroborate these results which indicated that unsaturated or branched fatty acids are common in cold-adapted bacteria (Chintalapati *et al*. 2004, Reddy *et al*. 2009, Srinivas *et al*. 2009, Shivaji *et al*. 2013). It was cogently argued that in order to support growth at low temperatures, bacterial cells undergo two pronged adaptation in their fatty acid composition; they truncate the length of fatty acid chain and modify the branching pattern from iso to anteiso. Fatty acids play significant role in maintaining the bio-membrane fluidity of the microorganisms and therefore are crucial for their continued survival at low temperatures (Nishida and Murata 1996). Cryoconite habitats often remain shrouded with ice during the lengthy winter season. During the

short summers when the overlying ice undergoes thawing, the cryconites become exposed to the atmosphere; therefore in order to overcome the cold stress, the bacterial isolates growing in these cryconite holes, might be producing such branched forms of fatty acids.

ii) Ice cores bacteria

Investigation on bacterial diversity, distribution and biotechnological potential from the Arctic Glacier Ice cores is scarce. More recently, Sheridan *et al*. (2003) and Miteva *et al*. (2004) studied the distributions of bacterial communities in deep Greenland ice cores while Singh *et al*. (2013) explored yeast communities from Arctic (Svalbard). No information has been available so far on the culturable diversity of bacteria distributed in the ice cores of the Svalbard Arctic, their adaptation strategies or their biotechnological potential.

The bacterial colonies that emerged on the Petriplates ranged from 10 to 7000 CFU/mL. This is similar to the observations made by Zhang *et al*. (2008) in the Puruogangri ice core (Tibetan Plateau) melt water where the count ranged from 0 to 760 CFU/mL. Abyzov *et al*. (1982) also observed that in polar ice, sometimes, the CFU values may be less than one per ml.

Total bacterial count in the glacier ice melt water ranged from 7.20×10^4 to 3.65×10^7 cells/mL. The values are in range between that observed in the deep Greenland Glacier ice core by Yung *et al*. (2007) [$7.0 \times 10^3 \pm 6.7 \times 10^2$ cells/mL] and by Sheridan *et al*. (2003) [6.1×10^7 to 9.1×10^7 cells/mL]. The values are also similar to the surface snow bacterial counts of 2×10^4 cells per mL as recorded by Amato *et al*. (2007). The isolates were capable of growing between 1 and 30°C, with good growth rate at 4 to 22°C indicating their psychrotolerant nature. Similar observation of occurrence of psychrotolerant bacterial isolates in ice cores was made by Shivaji *et al*. (2013) while working on Antarctic ice core bacterial isolates.

Taxonomic analysis of the strains

The phylogenetic tree was constructed to determine the affiliation of the representative strains is shown in Fig. 15. The two Gram-negative genera (*Pseudomonas* and *Sphingomonas*) belong to the phylum *Proteobacteria* while the Gram-positive genus *Bacillus* was affiliated to the phylum Firmicutes. The three groups formed distinct clades. The bacterial isolates from the Ice core samples belonged to three genera namely, *Pseudomonas*, *Sphingomonas* and *Bacillus*. Genera such as *Pseudomonas* and *Sphingomonas* have been reported previously from tropospheric clouds (Amato *et al.* 2007). Precipitation could have resulted in the deposition of these bacterial cells on ice. Members of the three genera have been previously reported from Greenland ice cores (Miteva *et al*. 2004; Knowlton *et al*. 2013).

Fatty acid profiles

Fatty acids are crucial for their existence at low temperatures (Nishida and Murata 1996). Isolates of genera *Pseudomonas* and *Sphingnomonas* were subjected to fatty acid analysis. The most dominant fatty acids recorded in the isolates were Heptadecanoic acid followed by Linolelaidic acid. These results differ from the earlier observation that unsaturated or branched fatty acids are present in higher concentrations in cold-adapted bacteria (Chintalapati *et al.* 2004, Reddy *et al.* 2009, Srinivas *et al.* 2009, Shivaji *et al.* 2012).

iii) Permafrost bacteria

Arctic habitats such as the ice cores have been examined for their bacterial diversity (Sheridan *et al.* 2003, Miteva *et al.* 2004). The permafrost soils however, have received less attention in this regard. Steven *et al.* (2006) observed that the bacterial counts in the permafrost were equal if not greater than in other cryo-environments, including the cold, aquatic, osmotic stress-free environments.

Vishnivetskaya *et al.* (2000) while experimenting with various low temperature recovery strategies for isolation of bacteria from permafrost sediments observed that direct plating enabled the recovery of diverse genotypes, some of which could not be recovered by enrichment experiments. They also observed that diluted media were effective for the recovery of Siberian permafrost bacteria, indicating their oligotrophic characteristic. In the present study, both the strategies (employing of direct plating and use of diluted and full strength media) were used to maximize the recovery of permafrost bacteria.

The bacterial colonies that emerged on the Petriplates when counted ranged from 1.50×10^3 to 2.22×10^5 CFU/g (Table 14). While the CFU ranged from 10^1 to 10^3 CFU/g soil in the High Canadian Arctic permafrost (Steven *et al.* 2004), it was from 0 to 10^8 CFU/g soil in the Siberian permafrost (Rivkina *et al.* 1998, Gilichinsky 2002, Vishnivetskaya *et al.* 2000). In the Antarctic permafrost, the CFU/g soil value ranged from 0 to 10^5 (Horowitz *et al.* 1972, Cowan *et al.* 2002).

According to Steven *et al.* (2006), the viable cell recovery ability depends on the age rather than the temperature of the Permafrost. The soil samples in the present study range from 44800 years to recent times, in age. The highest value (2.22×10^5 CFU/g) was recorded in the soils of 24300 years of age, the time which marked the start of the stadial period (Singh *et al.* 2012). In a similar study on Siberian permafrost, Zhang *et al.* (2013) examined 3.5 million years old aged soil samples and observed that the CFU's range between 7.4×10^4 to 5.8×10^4/g dry soil.

There are apparently high numbers of bacteria in permafrost soils. Total bacterial count in the permafrost layers range between 1.14×10^5 and 5.52×10^5 cells/g soil in the present study. The total count of bacteria (measured by epiflorescence microscopy) in the Siberian permafrost soils range from 10^3 to 10^8 cells/g (Rivkina *et al.* 1998, Vishnivetskaya *et al.* 2000, Gilichinsky 2002), and about 10^7 cells/g at High Canadian Arctic (Steven *et al.* 2004) and between 10^5 to 10^6 cells/g in the Antarctic permafrost (Horowsitz *et al.* 1972, Cowan *et al.* 2002). The direct count in other than permafrost soils have shown a higher level from 10^6

to 10^8 cells/g in Antarctic (Bölter 1990, 1995) and between 10^8 and 10^9 cells/g in Arctic (Bölter and Pfeiffer1997, Gounot 1999, Schmidt and Bölter 2002).

Phenotypic characterization and adaptation strategies

The tundra and taiga environments covered with permafrost soils are the source of plenty of psychrophilic and psychrotolerant organisms (Bölter 2004b). In current study, Gram-positive and psychrotolerant bacterial isolates/strains are dominant, and the bacterial cells are mostly short rods and cocci. This is in agreement with the analyzed data of Arctic and Antarctic which show significant reduction in surface area to short rods-coccid forms, in Gram-positive organisms (Bölter 1990; 1995, Friedmann 1982, Psenner 1999). The fact that we find mostly size-reduced forms in permafrost could be attributed to a reduction of water and a higher ratio of surface and volume in oligotrophic environments (Taylor and Jones 1990, Cavicchioli *et al.* 2003, Ley *et al.* 2004, Bölter 2004b).

The non-spore forming bacterial cells and well preserved algal cell structures were observed, whereas very rare filamentous fungi and yeasts were recorded on fungal culture media plates during the current study from permafrost samples of Svalbard. Similar observations were also made by Soina and Vorobyova (1994) and Bölter (2004b). The presence of non-spore forming cells (vegetative form) also suggests an adaptation strategy in permafrost. The dominance of bacteria suggests its better strategy for metabolic activity at low temperatures than mycelial fungi and yeasts which are sensitive to permafrost environment.

The membrane-associated pigments play an important role in low temperature adaptation. The colonies isolated from permafrost soil of Svalbard showed different colours (yellow, pink and brown) due to presence of pigments (carotenoid) which has probably a role in buffering membrane fluidity in permafrost environment; it is also known that the modulation of membrane fluidity is brought about by changing the levels of polar and non-polar carotenoids (Jagannadham et al. 1991, 2000). Thus, it seems that the carotenoid pigments interact with cell membranes, increase its rigidity and help in cold-adaptation in

permafrost environment, it is known that the modulation of membrane fluidity is brought about by changing the levels of polar and non-polar carotenoids (Jagannadham *et al.* 1991, 2000). Thus, it seems that the carotenoid pigments interact with cell membranes, increase its rigidity and help in cold-adaptation in permafrost environment.

Bacteria from permafrost soils also show thickened cell wall which is possibly an another strategy of adaptation to the harsh conditions by the production of protective compounds outside or inside the cell. The multilayered sheets and mucilage are known from algae and cyanobacteria as protections against water loss (Hawes *et al.* 1992, Vincent *et al.* 1993). All these special ways of protection against extreme cold are necessary to withstand stresses such as temperature, light, water deficiency, low nutrients and freeze-thaw cycles (Convey 2000). The individual factors, or stressors, can act as signals. The signals need to be noticed, transduced into the cell, where special processes induce the production or activation of special enzymes so as to regulate the production of compatible solutes, the viscosity of the cell fluids, and ice-nucleation (Orser *et al.* 1985, Booth 1999, Bölter 2004b). Such stress responses at the sub-cellular level are common for many organisms at different taxonomic levels (Fuller *et al.* 2004).

Soil components and ecological conditions

The soil component measurements and biomass values recorded by Singh *et al.* (2012), suggest that during the time span between ~38 to 24 ka B.P the present study area, remained deglaciated and had marine influence. During this time the organic carbon, mineralizable nitrogen and biomass nitrogen values are high indicating the prosperity of lower forms of life. Between 25 and 12.5 ka B.P the region remained glaciated and most of the measured soil components remained minimum. Despite the combination of cold and poor nutrients during stadial period (glacial) environments, bacteria can survive in starved stage, which may probably have a higher demand for nutrients during interstadial (interglacial) warm period and due to fast growth increased the biomass (Singh *et al.* 2012).

Felip *et al.* (1996) also observed that increasing nutrient contents stimulate the bacterial growth rates. It has also been observed that even in cold desert soils from the Arctic as well as from Antarctic environments, microbial biomass and number are well related to soil organic matter content (Bölter 1993; 1994, Bölter *et al.* 1994; 1997; 2003a, Bölter 2001, Bölter and Pfeiffer 1997). The abundance of metabolic remnants, such as ammonium, nitrite and nitrate (Janssen and Bock 1994) was also documented for permafrost soils. Thus, microbes living in permafrost conditions exhibit all possible metabolic activities such as their ability to degrade high molecular weight carbohydrates (Baross and Morita 1978), produce and consume methane and are involved in the process of nitrification and nitrogen fixation through free living and associated cyanobacteria (Davey and Marchant 1983, Nakatsubo and Ino 1987, Bölter 1995). Thus, metabolic processes have contributed their remains in time and space. A comparison of the Svalbard permafrost soils (Singh *et al.* 2012) with the Siberian permafrost soils (Vishnivetskaya *et al.* 2000) reveals that the Siberian permafrost had higher organic carbon content and chlorine values than Svalbard. pH values and concentration of HCO_3^-, SO_4^-, Ca^{++}, Mg^{++}, K^+ and Na^+ was high in Svalbard as compared to the Siberian permafrost soils.

There has been a serious problem in RNA/DNA analysis from permafrost soils, as bacterial DNA has been reported to persist over geological time spans in those environments. Further, Gram-positive organisms' DNA show longer live times than those from Gram-negative ones (Willerslev *et al.* 2004). As the reasons behind this still remained unknown, some ideas arose that dormancy or extreme low basic metabolic levels might occur (Rivkina *et al.* 2000, Mulyukin *et al.* 2001). The abundance of Gram-negative bacteria in permafrost soils between 1.8 and 3 million years old was shown by Shi *et al.* (1997), actual temperatures at that site were -10 °C. They assumed that those organisms were in an active state, but probably in a kind of dormancy, which maintained them in a reversible state of "anabiosis" (Glichinsky 1994). Survival is closely related to a strategy of a kind of dormancy, not only for metabolic processes but also for replication (Panikov and Sizova 2007). Price and Sowden (2004) offered a three-way model to explain

such metabolic processes, a) a rate measured under laboratory conditions, b) a rate which maintains basic processes but no growth, and c) a rate which just keeps survival or repair mechanisms for DNA (Friedmann 1994). This might explain doubling time of 160 days at -20 °C (Rivkina *et al.* 2000). A most important feature to keep metabolism active is the availability of liquid water for the exchange of nutrients and metabolic products. Water availability in soils depends on the grain size of the mineral part and the content of particulate organic matter. In this phase the possibility of super-cooled water exist, which can act as a final medium for supporting life (Gilichinsky *et al.* 2003).

The present study resulted in the isolation of 106 distinct strains. A similar study on Siberian permafrost led to the isolation of 29 bacterial isolates (Shi *et al.* 1997). The 33 representative isolates selected for physiological and biochemical characterizations were able to grow at different pH and NaCl concentrations. Most of the isolates are capable of growing from 4 to 30°C, indicating their psychrotolerant nature. Psychrotolerant nature of permafrost isolated bacteria was also observed in Siberian permafrost which grew well at 30°C (Shi *et al.* 1997). Steven *et al.* (2006) also observed that the permafrost organisms are psychrotrophic in nature rather than psychrophilic, and further that the permafrost bacteria are more halotolerant in nature as compared to the ice bacteria. The ability of the isolates to grow at various temperatures, pH and in salinity suggests that the organisms are adaptable to changes in these conditions in nature, and are therefore referred to as “community of survivors” by Friedmann (1994).

Taxonomic analysis of the strains

The bacterial strains isolated during the study belong to 13 genera namely, *Acinetobacter, Arthrobacter, Bacillus, Enterobacter, Nesterenkonia, Pseudomonas, Psychrobacter, Rhizobium, Rhodococcus, Sphingobacterium, Sphingopyxis, Stenotrophomonas and Virgibacillus.* Amongst all these, *Arthrobacter* is the most dominant genus reported in almost every layer of the soil. *Pseudomonas* is the next most dominant genus. Genera such as *Arthrobacter,*

Bacillus, Pseudomonas and *Psychrobacter* were also reported by Shi *et al.* (1997) and Bakermans *et al.* (2003) from the Arctic permafrost. *Arthrobacter* and *Bacillus* were known to be predominant in the Antarctic permafrost (Vorobyova *et al.* 1997).

The isolates represent 4 bacterial phyla namely, Proteobacteria, Bacteriodoites, Firmicutes and Actinobacteria. Proteobacteria was the most dominant phylum representing 7 genera namely *Acinetobacter, Enterobacter, Pseudomonas, Psychrobacter, Rhizobium, Sphingopyxis* and *Stenotrophomonas.* Actinobacteria represented three genera namely *Arthrobacter, Nesterenkonia* and *Rhodococcus.* Firmicutes represent two genera, *Bacillus* and *Virgibacillus* while Bacteriodoites represented one genus, *Sphingobacterium*. Presence of members belonging to Proteobacteria, Actinobacteria and Firmicutes was also reported by Steven *et al.* (2007) from Canadian high Arctic permafrost samples.

Carbon utilization ability of Cryoconites, Ice cores and Permafrost bacterial isolates

The carbon sources are limited in oligotrophic environment eventhough it is an essential requirement for microbial metabolism in the cryoconite holes. From the results of carbohydrate utilization tests it is evident that several of the bacterial isolates from the cryoconite holes prefer monosaccharides, the simpler forms of carbohydrates, such as mannose, rhamnose and xylose, as carbon sources. Marine beach bacterial isolates on Southern Baltic coast,also indicated a high percentage of mannose utilization (Mudryk *et al.* 2005). These isolates however failed to utilize xylose. Carbohydrate utilization study in the Gdansk Deep region of Baltic Sea also indicated that amongst the simple sugars tested mannose was very efficiently utilized followed by xylose (Donderski *et al.* 1998).

Ice has extremely limited source of carbon. Carbohydrate utilisation ability by these isolates therefore has special relevance. The results of the carbohydrate utilization tests showed that several of the bacterial isolates from the Glacier Ice cores preferred mono- and disaccharides, the simpler forms of carbohydrates, such as dextrose, sucrose, xylose and galactose, as carbon sources. In similar

studies on carbon utilization by bacteria isolated from the surface sea waters of the Arctic Ocean it was observed that the isolates utilized D-cellobiose and n-acetyl-D-glucosamine, which is a common source of carbon in the marine environment (Sala *et al*. 2010).

Carbon utilization ability by the Arctic permafrost soils is of special relevance as the soils have limited source of carbon. From the present study it is observed that the isolates differ in their preference for carbon sources probably to avoid competition for available resources. Ponder *et al.* (2005) observed that temperature plays an important role in the utilization of carbon substrates by the Arctic permafrost bacteria and attributed this difference to the changes in membrane transport properties. *Pseudomonas mandelii* strain PF2M12 and *Pseudomonas* sp. strain PF2M7, *P. jessenii* strain PF2M8, *Pseudomonas* sp. strain PF2M9 and *Arthrobacter* sp. strain PF3B3, that recovered from the permafrost soils between to 38300 years ago utilize xylose, L-arabinose, mannose, glycerol, citrate and malonate as carbon sources. *Pseudomonas* sp. strains PF1B3, PF3T4 and PF3T5 and *Arthrobacter* sp. strain PF5B1 that recovered from the permafrost soils between 13850 years B.P. to modern times showed fructose utilization. The isolates are restricted in their carbon utilization abilities for lactose, maltose, galactose, raffinose, trehalose, melibiose, mannose, salicin, dulcitol, sorbitol, α–methyl-D-glucoside, rhamnose, melezitose, xylitol, D-arabinose. None of the isolates is able to utilize cellobiose, inositol, sodium gluconate, α–methyl-D-mannoside, and sorbose. According to Ponder *et al.* (2005), certain carbon sources may not be able to enter the bacterial cells at low temperature due to changes in transport-associated proteins which are influenced by membrane composition. Also, as suggested by Vermersch *et al.* (1990) the substrate may get bound when membrane and protein flexibility alter with temperature. Bacterial unsaturated fatty acids maintain the required fluidity of the membrane and thus allow nutrient exchange and enzyme activity, and most important the prevention of intracellular ice nucleation (Wynn-Williams 1990, McGrath *et al.* 1994, Schinner and Sonnleitner 1996) in cold environment. Further, Bölter (2001,

2004b) opined that though temperature can be regarded as a key factor, there are several other stress factors, which act on cellular processes and regulate the use of nutrients, and also coordinate influence of both free water and temperature on the survival of bacteria in permafrost. During extreme environmental conditions, enzymes regulate the osmotic status of the cell by producing sugars, sugar alcohols, polyols or antifreeze proteins. High amounts of e.g., sucrose, trehalose, various polyols and sugar alcohols have been reported from polar soils, and considered as products from cell lyses or exudation of over-productions of cryoprotectives (Melick and Seppelt 1992, Montiel and Cowan 1993, Tearle 1987) or as carbon sources (Melick *et al.* 1994).

Antibiotic resistance patterns of of Cryoconites, Ice cores and Permafrost bacterial isolates

The transmission of antibiotic resistance genes across bacterial species in any specific micro-environments are a common phenomenon where dense microbial communities are often exposed to intensive use of antibiotics. The first record of antibiotic resistance plasmids in the pristine environments was recorded by Gardner *et al.* (1969). However, until recently such studies on antibiotic resistance in natural ecosystem has been fragmentary. Only a few studies of antibiotic resistant activities were reported from the Arctic (Allen *et al.* 2008, Sjolund *et al.* 2008). Several studies detected a few antibiotic-resistant bacteria or antibiotic resistance genes in the wild-type isolates of geographically isolated natural environments (Dib *et al.* 2009, Allen *et al.* 2010, Ushida *et al.* 2010). In the present study it was observed that the susceptibility of isolates towards antibiotics varied. Using the antibiotic susceptibility assay, it was observed that wild-type microbial isolates from the Arctic showed higher sensitivity towards antibiotics as compared to the microorganism belonging to the tropical and temperate regions. The results obtained here is in agreement with that of Segawa *et al.* (2013) wherein they reported least antibiotic resistance genes in the microbial isolates from the Antarctic region than found in the Arctic microbes. In the cryoconite

isolates it was observed that the most sensitive isolate (NA-9) and the most resistant isolate (MLB-31) belonged to the genus *Cryobacterium*. This clearly indicates that despite the restricted microbial diversity in these microecohabitats, the strains differ widely in terms of antibiotic sensitivity.

In the Ice core study, it was observed that *Pseudomonas* sp. MLB-15 was the most sensitive strain having sensitivity towards all the antibiotics screened while *Pseudomonas* sp. isolates MLB-17, MLB-12 and MLB-14 were the most resistant. This result is similar to the cryoconites isolates. Working on a similar line, on bacteria isolated from the bottom sediments in the Wijdefjorden, Spitsbergen, Konieczna *et al*. (2012) observed that most of the bacterial isolates were sensitive to antibiotics such as cefoperazone, piperacillin, gentamycin, tobramycin, ciprofloxacin and ofloxacin.

In the permafrost study, it is observed that *Arthrobacter* sp. strain PF1T4 and *Arthrobacter* sp. strain PF4B2 are the most susceptible isolates sensitive against 44 of the 45 antibiotics tested while *Pseudomonas jessenii* strain PF2M8, *P. mandelii* strain PF2M12, *Stenotrophomonas* sp. strain PF1M2 and *Virgibacillus pantothenticus* strain PF1B6 are the most resistant isolates. This indicates that the Arctic permafrost soil bacterial strains differ widely in their antibiotic sensitivity characters. Ponder *et al.* (2005) observed that increase in temperature might, to some extent, increase the resistance power of the Arctic bacterial strains to antibiotics. They assigned this ability to the membrane flexibility of the microbes. Together it is observable that in terms of antibiotic susceptibility, the Arctic bacterial strains show variability and this might be the part of the adaptation strategy enabling the bacterial strains to overcome the antagonistic stresses and help them thriving in extreme environment. Martinez (2012) on this content opined that the antibiotic resistance genes have been present for billions of years and have evolved over the years for detoxifying the original host from the effect of antibiotic it produces. Sjolund *et al.* (2008) and Segawa *et al.* (2013) attributed horizontal transfer of these antibiotic resistance traits in the Antarctic and the Arctic, by the airborne bacteria and migratory birds.

3a.5 Conclusion

So far no systematic investigation on the culturable bacterial communities and their characterization from glacier cryoconites of Svalbard, Arctic was reported. 16S rRNA sequence analysis of bacterial isolates identified them to belong to eight genera namely *Pseudomonas, Polaromonas, Micrococcus, Subtercola, Agreia, Leifsonia, Cryobacterium,* and *Flavobacterium.* Fatty acid analysis indicated that the cryoconite bacterial isolates produce high amounts of branched chain fatty acids which have ecological adaptive strategies in cryoconite environment.

The report of culturable bacterial communities and their characterization from glacier ice cores of Svalbard, Arctic are made available for the first time. 16S rRNA sequence analysis of bacterial isolates identified seven species, and *Pseudomonas* was the dominant genus in the glacier habitats. Fatty acid analysis indicated that the ice cores bacterial isolates produce high amounts of saturated fatty acids. Probably the organisms have evolved these acclimatization mechanisms for carrying out metabolic activities for growth, multiplication and protection at low and even at sub-zero temperatures.

It is very important to note that for the first time the diversity of culturable bacterial communities and their chronological characterization from the permafrost pits in Svalbard (79^{o}N), Arctic are recorded by virtue of the present study. 16S rRNA sequence analysis of bacterial isolates identified them as belonging to 13 genera. *Pseudomonas* and *Arthrobacter* are the dominant genera. Carbon utilization abilities of the isolates indicate that the bacterial isolates prefer varied forms of carbon sources. The isolates also show varying susceptibilities to antibiotics indicating that although the bacterial diversity in these microhabitats is restricted, the strains differ widely in their antibiotic sensitivity. This adaptation strategy probably enables the bacterial strains to overcome the antagonistic stresses and survive in all possible unique environments.

3b) Diversity of yeasts and filamentous fungi from Cryoconites, and Ice Cores

3b.1 Introduction

Glacier habitats include one of the largest unexplored and extreme biospheres of Earth (Buzzini *et al.* 2012). Arctic harbors forty six species of yeasts (Buzzini *et al.* 2012) which have been isolated from various habitats such as Siberian sands, sediments, and permafrost layers (Dmitriev *et al.* 1997, Golubev 1998, Vishniac 1999, Gilichinsky *et al.* 2005, Margesin *et al.* 2007, Vishniac and Takashima 2010), Iceland soils (Vishniac 2002, Birgisson *et al.* 2003), ancient Greenland ice cores (Ma *et al.* 1999), Svalbard glacier associated habitats (Butinar *et al.* 2007, Lee *et al.* 2010, Pathan *et al.* 2010, and glaciers in Alaska (Turchetti *et al.* 2008, Uetake *et al.* 2012). Evidence of colonization and succession of Arctic glaciers by psychrophilic microbes has been reported (Skidmore *et al.* 2000; 2005, Mindl *et al.* 2007). Isolates obtained from deep ice cores have been shown to be metabolically active even under subzero temperature (Amato *et al.* 2009). Recently, Hoshino and Matsumoto (2012) coined a new category, 'cryophilic', for cold-adapted yeast and filamentous fungi and this term has been adopted in the present study.

A novel species of *Rhodotorula* has also been addressed. The genus *Rhodotorula* Harrison is a member of the basidiomycetous yeasts, occurring naturally in air, soil and other habitats comprising of 47 species (Sampaio 2011). According to the latest records of Mycobank (www.mycobank.org), the genus holds sixtyeight species. The genus is polyphyletic in nature and is known to have subglobose, ovoid, ellipsoidal to elongate cells which reproduce through multilateral and polar budding (Sampaio 2011). Species of the genus *Rhodotorula* colonize extreme environments (Sampaio 2004) and psychrophilic yeasts grow at temperatures below 5^{o}C and exhibit no growth above 20^{o}C (Vishniac 1999). Margesin *et al.* (2007) described three phenol-degrading novel yeast species, namely *Rhodotorula glacialis*, *R. psychrophila* and *R. psychrophenolica* from the

alpine habitats. Recently, a few novel species of yeasts (*Rhodotorula glacialis*, *Mrakiella cryoconiti*) have been recorded from alpine glacier cryoconites (Margesin *et al*. 2007, Margesin and Fell 2008).

A study on the occurrence of filamentous fungi in supraglacial cryoconite holes and their characterization for extracellular enzymes was poorly described. In this study, the results on the isolation, identification, adaptation strategies and biotechnological potential of culturable yeasts and filamentous fungi from supraglacial Arctic cryoconite, and ice cores are presented. The present study also focused on Morphological, physiological and molecular parameters were taken into consideration to delineate a new species of *Rhodotorula*. D1/D2, Internal transcribed spacer (ITS) and mitochondrial DNA (mtDNA) regions were investigated for presenting molecular phylogeny. To understand the adaptation strategies of this novel species whole cell fatty acids composition and antifreeze protein (AFP) activity was studied. The isolate has been screened for enzyme production to understand its biotechnological potentials.

3b.1 Materials and methods

3b.2.1 Sampling site

3b.2.1.1 Cryoconites sampling

For the present investigation, sampling sites were selected over the ablation zone of Midre Lovénbreen, Austre Brøggerbreen, and Vestre Brøggerbreen glaciers in Svalbard (Fig. 21b,c). Samples were collected from cryoconite holes, from locations designated as G1, G2, G3 and G4 at ML (Fig. 21b) and at AB and VB (Fig. 21c) glaciers. The cryoconites samples were collected by using a sterile syringe, kept in sterile ampules (Himedia) and stored at low temperature (-20^{o}C) until analyses.

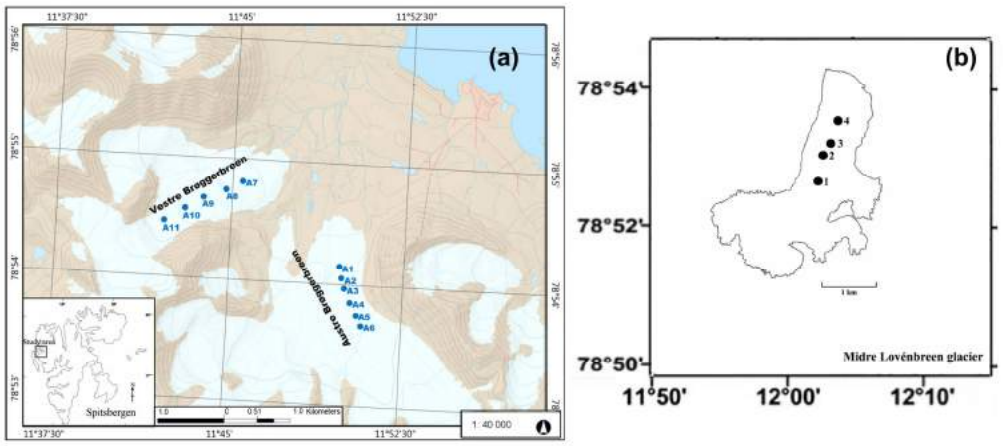


Fig. 21 a) Sampling locations on Austre Brøggerbreen (A1-A6) and Vestre Brøggerbreen glaciers (A7-A11), b) Sampling locations (G1-G4) over Midre Love´nbreen glacier.

3c.2.1.2 Ice cores sampling

Ice cores samples were cored out from four different locations [G1(78°52′.815″ N 12°02′.404″ E, 273 m a.s,l.); G2 (78°53′.006″ N 12°02′.610″ E, 242 m a.s,l.); G3 (78°53′.197″ N 12°02′.940″ E, 205 m a.s,l.) and G4 (78°53′.670″ N 11°50′.770″ E; 164 m a.s,l.)] on Midre Lovénbreen glacier, (Svalbard) (Fig. 21b), following procedures as described earlier.

3b.2.2 Sediment analysis

The cryoconite sediments were air dried and crushed using a wooden pestle and mortar. Organic carbon content was analysed by wet digestion method (Walkley and Black 1934), available N by the procedures of Subbiah and Asija (1956) using VAP30 distillation apparatus (Gerhardt), available P by method of Bray and Kurtz (1945) by extracting $NaHCO_3$ and estimated by spectrophotometer. For elemental analyses, the cryoconite sediments were oven dried at 40^{o}C, sediment samples were finely grind with pestle and mortar and passed through 0.2 mm size mesh, 0.5 gm of each sample was weighed into PTFE TFM vessels for microwave digestion (Ethos 1, M/s. Milestone, s.r.l, Italy) alongwith addition of 3 ml of 69 % Subpure HNO_3 obtained using a sub boiling system (SubPUR M/s. Milestone,

s.r.l, Italy). One mL suprapure 30 % HCl (Merck, Darmstadt, Germany) and one mL of 30% H_2O_2 (Merck specialities Pvt. Ltd, India), after addition of acid vessels were closed, raised the temperature upto 180^0 C within 20 min and hold it for 15 min at 180^0 C. With completion of digestion programme cool the vessels and volume was make up with deionized water. Blank samples were also prepared by same procedure of samples and those values obtained were substracted from samples. Elemental concentrations present in the samples were determined through inductively coupled plasma mass spectrometry (ICPMS, Thermofisher Scientific, Bermen, Germany, model ICPMS-X series II). Elemental concentrations were measured in triplicates and were recorded in ppm and ppb.

3b.2.3 Isolation of fungi from cryoconites

One gram of cryoconite sample was the serially diluted following procedure of Waksman (1916), and plated on six different media (Table 15) by pour plate as well as spread plate techniques.

Table 15. Details of fungal Media and its composition.

S. No.	Name of Media	Composition of Media
1	YPD	Yeast extract, 5 g/l; mycological peptone, 5 g/l; dextrose, 10 g/l; pH 6.5; agar, 20 g/l; chloramphenicol, 100 mg/l
2	MYP	Malt extract, 7 g/l; yeast extract, 0.5 g/l; soytone, 2.5 g/l; agar,15 g/l; pH 4; chloramphenicol, 100 mg/l
3	MEA	Malt extract 30.0g/l, Mycological peptone 5. 0g/l, Agar 15.0g/l, Final pH (at 25°C) 5.4±0.2
4	PDA	Potatoes infusion 200g/l; Dextrose 20.0g/l; 15.0g/l; Final pH (at 25°C) 5.6±0.2
5	SDA	Dextrose 40.0g/l; Mycological, peptone 10.0 g/l; 15.0g/l; Final pH (at 25°C) 5.6±0.2
6	PCA	Potato Carrot Agar (Himedia, Mumbai)

Fungal viable counts were measured following the procedure of Turchetti *et al.* (2008), using Rose Bengal agar (RB) with tetracycline. Plates were incubated at 1, 4, 15, and 20 ^{o}C for the period of 2–4 weeks in triplicates. Plates were periodically monitored; emerging fungal colonies counted, purified and stored at 4^{o}C in PDA agar slants. Morphological, physiological and biochemical characterisation of the isolates were determined as described by Yarrow (1998). All assimilation tests were performed in duplicates, and results were recorded after 1 and 3 weeks. Morphology of yeast cells and morphotaxonomic characters of filamentous fungi (Fig. 22a–e) were studied using light microscopy (Olympus BX51 and IX71).

3b.2.4 Isolation of yeasts and filamentous fungi from Ice cores

Ice melt water (100 µl) was plated onto six different media (Table. 15), by pour plate as well as spread plate methods. Plates were incubated at 1, 4, 15, and 20 ^{o}C for 2–4 weeks in triplicates. Plates were periodically monitored and emerging yeast colonies were counted, purified and stored at 4 ^{o}C on PDA agar slants. Appearance of filamentous fungi was not observed in media plates. Procedure of isolation and identification of yeasts were followed as suggested by Kurtzman *et al.* (2011). Morphology of yeasts cells was observed using light microscopy. Pure cultures were deposited at the Microbial Type Culture Collection and Gene Bank (MTCC), India.

3b.2.5 Identification of yeast and filamentous fungi

Identification of yeast was carried out by polyphasic approach (conventional and molecular techniques). Morphological characteristics and physiological tests were taken in account for initial identification of yeasts by following standard procedures of Yarrow (1998) and Kurtzman *et al.* (2011). Initial identification of filamentous fungi was achieved on the basis of morphotaxonomy with the help of standard literatures (Barnett 1960, Barron 1977, Carmichael *et al.* 1980, Ellis

1971; 1976, Kirk *et al.* 2008). Strains with similar morphological and physiological characteristics were grouped together, and a representative strain of each group was used for sequence analysis of D1/D2 domain of the rDNA gene. BLAST search was carried out with NCBI database (http:// www. ncbi. nlm. nih. gov/BLAST/). Pure cultures of yeasts and filamentous fungi were deposited at the National Fungal Culture Collection of India (NFCCI-WDCM 932) Pune and JCM Japan.

3b.2.6 Molecular characterization

3b.2.6.1 Polymerase Chain Reaction (PCR) and Sequencing

Total DNA was extracted from cultures (grown on PDA for 3 weeks at 4ºC) using the ISOPLANT Ⅱ kit (Wako pure chemical industries Ltd., Japan). Yeast cells were homogenized and the extracted DNA was amplified by PCR method using KOD-plus DNA polymerase (Toyobo Co. Ltd, Japan). ITS region was amplified using primers: ITS1F (5'-GTA ACA AGG TTT CCG T) and ITS4 (5´-TCC TCC GCT TAT TGA TAT GC). Following primers were used to amplify the D1/D2 domain: NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG) and NL4 (5'-GGT CCG TGT TTC AAG ACG G). Mitochondrial cytochrome *b* gene was amplified using primers: forward primer E1M4: 5'-TGRGG WGCW ACWG TTATTACTA, reverse primer M2mr3: 5'-GGWA TAGCA CGTA RAAY WGC RTA. The amplification cycle involved an initial denaturation step at 95 ºC for 2 min and then DNA was amplified in accordance with the following 35 cycles; 95 ºC for 1 min to denature double strand DNA, 52 ºC for 30 sec for annealing, and 72 ºC for 1.0 min for elongation. A final elongation step was carried out at 72 ºC for 7 min. Sequences were obtained with ABI prism 3100 Sequencer (Applied Biosystems) using ABI standard protocol.

3b.2.6.2 Phylogenetic Analysis

The sequences of isolates were deposited in the DNA data bank and were subjected to a NCBI BLAST search. The sequence alignment of ITS and D1/D2

regions, together with the homologous sequences of closely related species of Genbank, was performed using Clustal W option of MEGA software v4.0 (Tamura *et al.* 2007).

To calculate the sequence divergence, the matrix was analyzed with Neighbour joining method (Saitou and Nei 1987) using the Tamura-Nei model (Tamura and Nei 1993) and Maximum Parsimony method (Tamura *et al.* 2011). To represent the evolutionary history of the taxa, the bootstrap consensus tree was inferred from 1000 replicates (Felsenstein 1985).

3b.2.7 Growth characteristics of yeast and filamentous fungi

An experiment was performed to assess the influence of temperature on growth of the isolates. In order to determine maximum and minimum growth temperatures, the isolates were tested for their ability to grow at different temperatures (4, 10, 15 and 20 ^{o}C) on MYP agar, PDA, YPD agar for yeast and PCA, PDA, MEA and SDA for filamentous fungi. The plates were inoculated with a suspension of yeast cells grown for 2–4 days and well sporulated cultures of filamentous fungi. Growth was observed visually for 20 days. Salt tolerance of the isolates was measured by growing the cultures at different concentrations of sodium chloride ranging from 1.0 to 5.0 M.

3b.2.8 Biolog identification method

Biolog (Microlog System TM 4.2) was used for the biochemical identification of the yeasts. Strain MLB-I was grown on Biolog Universal Yeast (BUY) agar, inoculated on a yeast (YT) microplate (96-well plate) and incubated at 15°C for 24, 48 and 72 h to generate metabolic fingerprints. This system, on the basis of oxidation and utilization of 94 carbon sources, generates the metabolic fingerprint of test strain. This metabolic pattern was interpreted by MICRO LOG software TM3, which searches through its database and identifies species.

3b.2.9 Fatty Acid Methyl Esters (FAME) analysis

Acid methanolysis method and Gas Liquid Chromatography (GLC) equipped with flame ionization detector (GC353, GL Science) was used for whole-cell fatty acid analyses (Yumoto *et al.* 2001). Fatty acids were identified comparing with FAME mix standards (Supelco 37 component FAME mix).

3b.2.10 Statistical analysis

In order to compare fungal diversity within the different localities were compared by using online software's: Shannon-Wiener Diversity Index/Shannon Entropy Calculator (http://www.changbio science.com/genetics/shannon.html), Online Calculation (http:// www2. biology. ualberta. ca/jbrzusto /rarefact.php) and Past software (Hammer *et al.* 2001).

3b.3 Results

3b.3.1 Yeasts and filamentus fungi from Cryoconites of Midre Lovénbreen glacier

The average water content in the cryoconite sediments ranged from 67.16 to79.01% and organic carbon content ranged between 1.07 and 1.86% per dry weight of sediment. Some important trace nutrients such as calcium, magnesium, nitrogen, and phosphate, were recorded in the samples in varying amounts. The physico-chemical characteristics of cryoconite sediments are shown in Table 16.

Table 16. Physico-chemical properties of Cryoconite sediments.

Locality No.	**G-1**	**G-2**	**G-3**	**G-4**
GPS Location and elevation	78 52.815 N 12 02.404 E 273m a.s.l.	78 53.006 N 12 02.610 E 242 m a.s.l.	78 53.197 N 12 02.940 E 205m a.s.l	78 53.670 N 11 50.770 E 164 m a.s.l.
Temperature (^{o}C)	0.8	0.8	1	1.9
pH	8.6	8.6	8.5	7.1
Organic carbon %	1.07	1.86	1.77	1.32
NO_3-N (ppm)	44.13	44.83	44.48	50.43
Mineralisable N (ppm)	270.67	284.12	327.27	308.22
P (ppm)	436	348	349	392
Ca (ppm)	510	560	560	460
Mg (ppm)	9,930	9,680	10,055	9,430
Li (ppm)	30.68	33.23	32.83	32.89
Be (ppm)	1.96	2.16	2.44	2.07
Al (ppm)	38,599.75	42,629.75	41,759.75	42,869.75
V (ppm)	79.99	90.45	84.23	87.84
Cr (ppm)	52.37	58.76	60.68	59.57
Mn (ppm)	320.39	362.09	373.89	380.79
Fe (ppm)	28,182.89	30,440.00	32,412.89	31,492.89
Co (ppm)	15.68	16.65	17.86	16.74
Ni (ppm)	38.56	68.56	42.17	40.89
Cu (ppm)	38.99	42.83	43.98	40.06
Zn (ppm)	155.47	142.07	150.97	132.67
Ga (ppm)	19.49	21.03	19.07	24.1
As (ppm)	14.22	13.73	12.05	11.01
Se (ppb)	2,557.00	2,943.00	2,346.00	1,866.00
Rb (ppm)	86.43	92.67	92.72	91.36
Sr (ppm)	32.09	28.27	27.16	26.45
Cd (ppb)	65.04	84.75	1,919.55	48.04
In (ppb)	97.29	120.19	73.03	58.73
Cs (ppb)	6.03	6.45	6.5	6.42
Pb (ppm)	73.87	85.07	57.17	49.86
Bi (ppb)	1,031.85	1,224.85	777.75	548.35
U (ppb)	2,823.45	2,882.45	2,895.45	2,672.45

A total of 43 yeast and 40 filamentous fungal isolates were isolated and purified from different locations on ML glacier (G1, G2, G3 and G4). The viable counts ranged between 7 x 10^3–1.4 x 10^4 and 4 x 10^3–1.2 x 10^4 cells/gm (Table 17).

Table17. Fungal count (CFU/g) in cryoconites samples.

Sampling localion	Mycelial fungi		Yeast			Isolate code
	CFU.	No. of isolates	CFU.	No. of isolates	Total No. of isolates	
G-1	4×10^3	4	1.1×10^4	11	15	CCP-I, CCPOY
G-2	1.2×10^4	12	1.4×10^4	13	25	CCP-I, CCPWY, CCP-II
G-3	1.2×10^4	12	1.2×10^4	12	24	CCP-I, CCPII,CCPWY
G-4	1.2×10^4	12	7×10^3	7	19	CCP-I, CCP-WY, CCP-V, CCP-OY

Based on polyphasic approach (morphological, physiological and biochemical characteristics), the isolates were grouped into three yeasts and two filamentous fungi (Table 18).

Table 18. Distribution, Phenotypic and enzymatic characteristics of mycelial fungi and Yeast isolated from Arctic Cryoconite holes.

Strains No.	Location				Colony colour	Radial grooves	Colony margin	Reverse	Temp. Range	NaCl (M)
	G-1	G-2	G-3	G-4						
Phialophora alba J.F.H. Beyma (CCP-I)	+	+	+	+	White, pale regular to irregular	Defined	1-2mm, defined	Light yellowish	4-20	1
Articulospora tetracladia Ingold (CCP-V)	+	+	-	+	White, floccose, grayish	Not defined	1-3 mm, regular floccose	Yellow-brown	4-20	2
Mrakia sp. (CCP-IWY)	-	+	+	-	Milky white	Shining	Regular	-	4-15	3.5
Cryptococcus gilvescens Chernov (CCP-IOY)	-	-	+	+	Orangish	-	Regular	-	4-18	3
Rhodotorula sp.(CCP-II)	-	-	-	+	Radish to Orange	-	Regular	-	4-20	1.5

G-1 Sampling location 1, G-2 Sampling location 2, G-3 Sampling location 3, G-4 Sampling location 4

Based on phylogenetic analyses, three yeast species viz *Cryptococcus gilvescens* (99% AF181547.1), *Mrakia* sp. zhenx-1 (99% EU680778.1), *Rhodotorula* sp. (95% FN400942.1), and two species of filamentous fungi such as *Phialophora alba* (99% AB100618.1) and *Articulospora tetracladia* (100% EU998921.1) were identified (Fig. 22). The details of identification of isolates are shown in Table 19.

Table 19. Identification of Midre Lovénbreen glacier isolates based on morphotaxonomical features ITS and D1/D2 region of 28S rDNA sequences similarity (%).

Isolate code	Morphotaxonomical and 18S rDNA gene sequences similarity (%)
*CCP-I	*Phialophora alba* IFM 51363, (AB100618.1), 99%
*CCP-OY	*Cryptococcus gilvescens* (AF18157.1), 99%
*CCP-WY	*Mrakia* sp. zhenx-1 (EU680778.1), 99%
**CCP-II	*Rhodotorula* sp. KBP 3844 (FN4009421), 95%
CCP-V	*Articulospora tetracladia* CCM-F-113 (EU998921), 1100%

*New to the Svalbard; ** Novel finding

Strains differed from the closest related type strain by two or fewer nucleotides in the D1/D2 region and were considered to be the same species. Among the identified representative strains, two are presumably new species; viz. *Rhodotorula* sp. (CCP-II) showed 20 nucleotide substitutions compared to the closest neighbor *Rhodotorula* sp. (FN400942.1), and *Mrakia* sp. (CCP-III-WY) showed three nucleotide substitutions when compared to the closest sequence *Mrakia* sp. (EU680778.1) which has not yet been validly described. The isolate CCP-I showed the closest identity to *Phialophora alba* (AB 100618.1) with three nucleotide substitutions. Phylogenetic trees of yeasts and filamentous fungi are shown in Fig. 23 and 24.

Phialophora alba occurred in all four studied sampling sites. Simpson's and Shannon's diversity index (H`) showed the diversity of G-4 to be the highest and that of G-1 to be the lowest as compared to the rest of sampling points (Table 20).

Table 20. Diversity indices for the isolates of Midre Lovénbreen glacier.

	G-1	**G-2**	**G-3**	**G-4**
Distinct Taxa	2	3	3	4
Individuals	15	25	24	19
Dominance_D	0.6089	0.3664	0.375	0.2909
Shannon_H	0.5799	1.051	1.04	1.312
Simpson_1-D	0.3911	0.6336	0.625	0.7091
Evenness_e^H/S	0.8929	0.9537	0.9428	0.9281

Effect of temperature variation on growth of cultures showed that all filamentous fungal strains were able to grow at temperatures below 20^{o}C but not at or above 25^{o}C. Growth rate of cultures at 20^{o}C was slow as compared to the lower temperatures. However yeast strains grew luxuriantly at 10 and 15^{o}C but did not grow above 20^{o}C. The optimum temperature for growth of *Cryptococcus gilvescens* (dry biomass wt. 1.2 g/l) and *Rhodotorula* sp. (dry bio mass wt. 2.3 g/l) was 15^{o}C while that of *Mrakia* sp. (dry bio mass wt.1.5 g/l) was 10^{o}C, after 30 days of incubation. The effect of growth medium and temperatures are shown in Fig. 22.

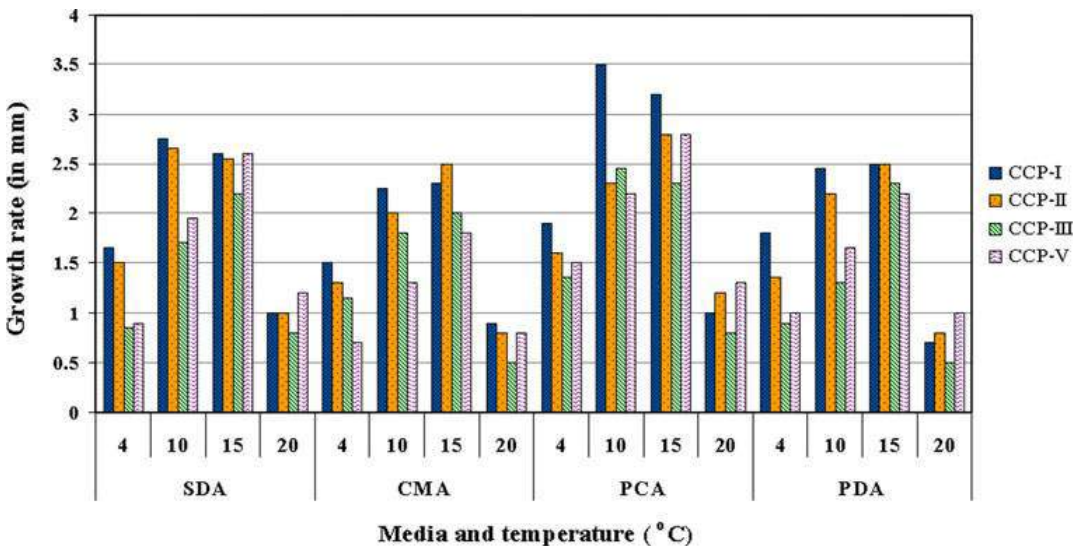


Fig.22 Growth of filamentous fungi at different temperature on different media after 30 days. (SDA, Sabouraud dextrose agar; CMA, corn carrot agar; PCA, potato carrot agar; PDA, potato dextrose agar; CCP-I, *Phialophora alba*; CCP-II, *Rhodotorula* sp.; CCP-III, *Cryptococcus gilvescens* and *Mrakia* sp.; CCP-V, *Articulospora tetracladia*).

The optimum temperature for growth of filamentous fungi *Phialophora alba* was 20°C (colony dia. 3.0 mm) while that of *Articulospora tetracladia* was 15°C (colony dia. 3.5 mm) on PCA medium. Of the four different media tested all showed good growth, but the luxuriant growth and sporulation were observed on potato carrot agar medium. Salt tolerance studies showed that the cultures showed varying response. *Mrakia* sp. was the highest salt-tolerant culture (3.5 M), while *Phialophora alba* (1 M) was the least tolerant.

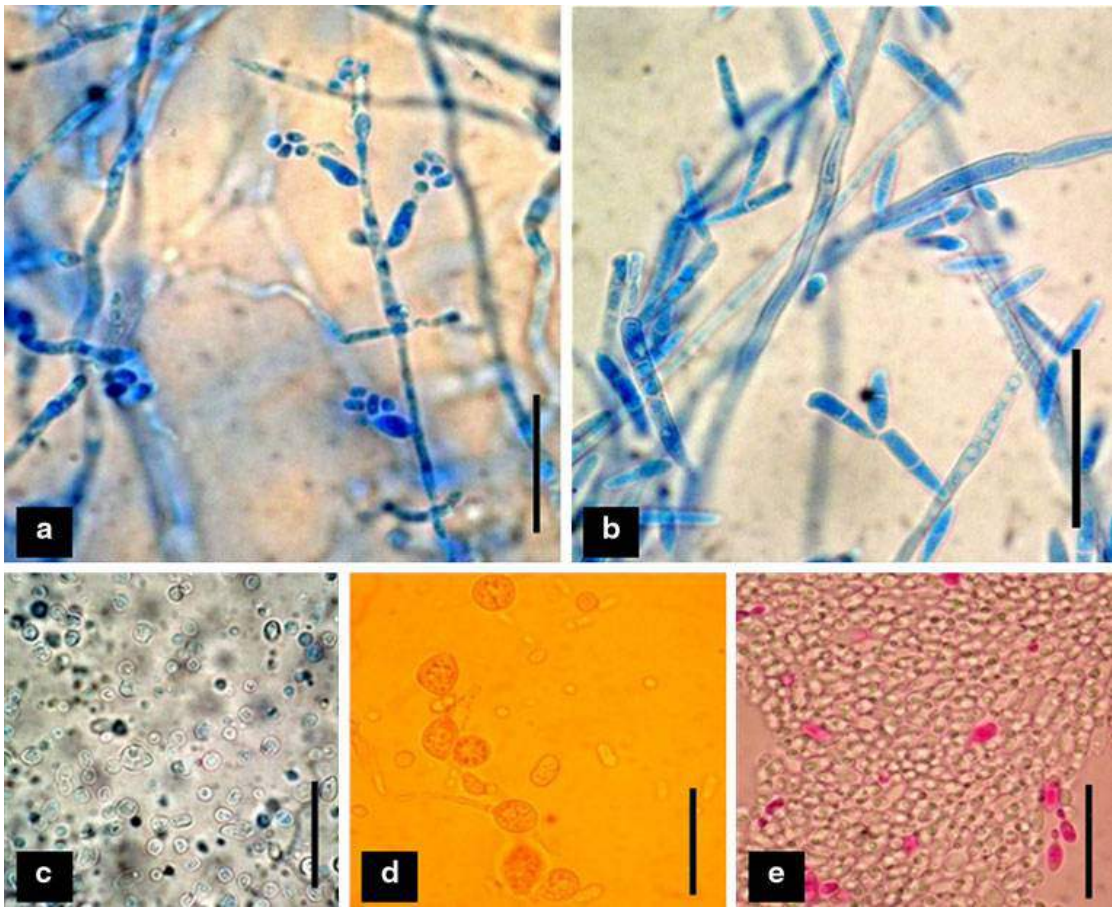


Fig. 23 a) *Phialophora alba* conidia and conidiophores, b) *Articulospora tetracladia* conidia and conidiophores, c) *Cryptococcus gilvescens* budding cells, d) *Rhodotorula* sp. budding cells and Chlamydospores, e) *Mrakia* sp. budding cells. Scale bar = 100 µm.

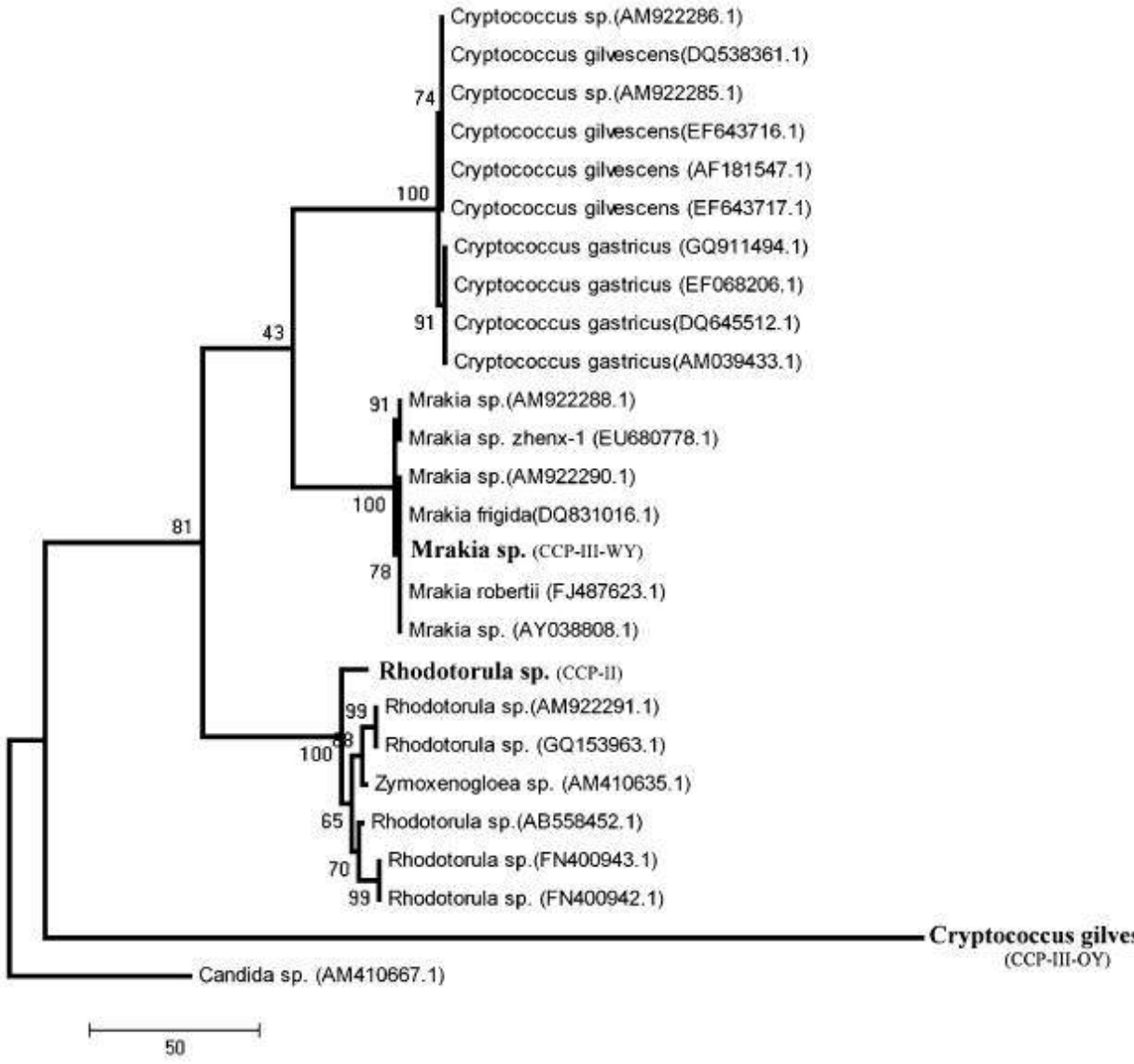


Fig. 24 Phylogenetic tree constructed according to maximum parsimony method using D1/D2 domain of 26S rRNA gene sequence showing relationship of *Cryptococcus, Mrakia* and *Rhodotorula* yeast strains from Arctic. *Candida* sp. was used as an out group. Bootstrap value for 1,000 replication is given in the branch nodes. The figures in parenthesis refer to the accession numbers of the D1/D2 domin of the 26S rRNA gene of the various yeasts obtained from GeneBank.

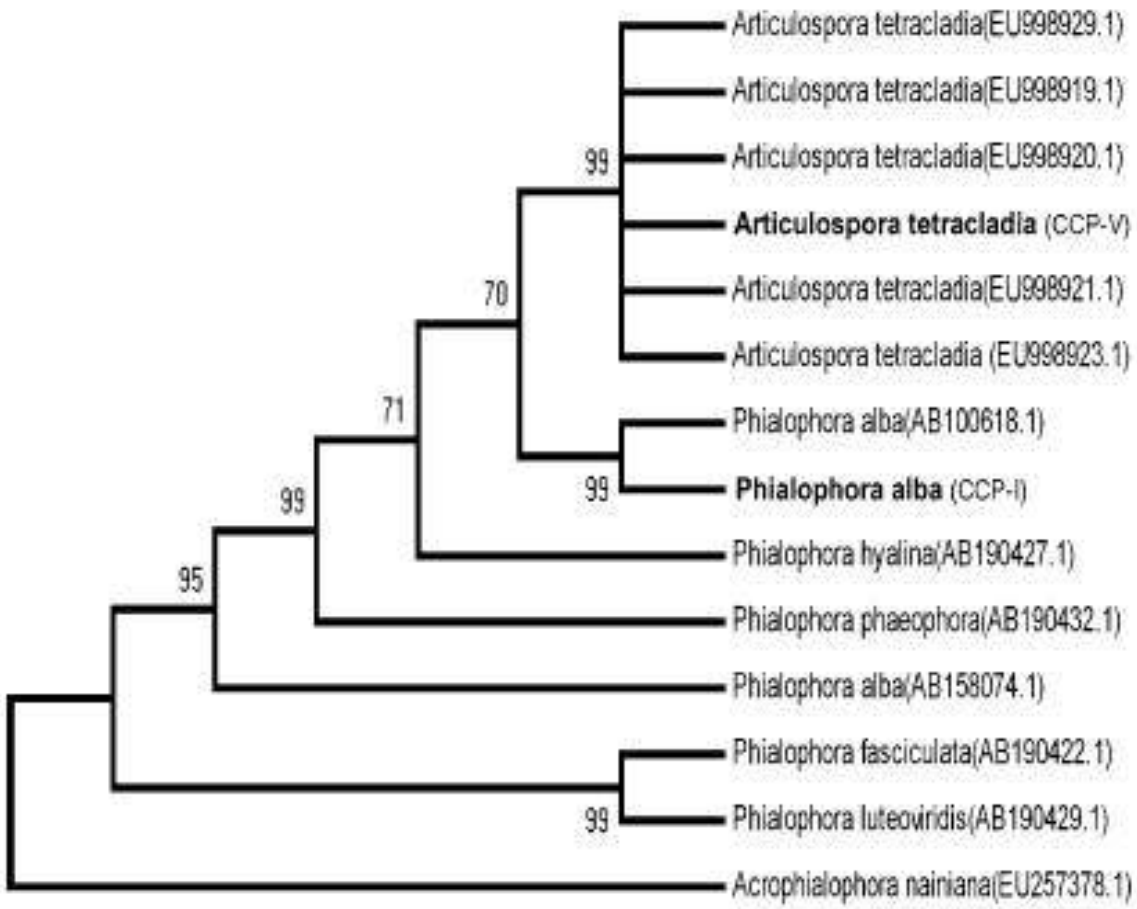


Fig. 25 Phylogenetic tree constructed according to maximum parsimony method using D1/D2 domain of 26S rRNA gene sequence showing relationship in filamentous fungi *Phialophora* and *Articulospora* strains from Arctic. *Acrophialophora nainana* was used as an out group. Bootstrap value for 1,000 replications is given in the branch nodes. The figures in parenthesis refer to the accession numbers of the D1/D2 domain of the 26S rRNA gene of the various strains obtained from GeneBank.

3b.3.2 Yeasts and filamentous fungi from cryoconites of Brøggerbreen glaciers

A total of 20 isolates were obtained which were representing three genera of yeasts and filamentous fungi belonging to two classes: Ascomycota (*Articulospora* sp., *Thelebolus* sp.) and Basidiomycota (*Rhodotorula* sp.).

Sequencing of ITS region and subsequent BLAST search showed that the isolates of *Articulospora* sp. Cry-FB1 and Cry-FB2 closely resembled with *Articulospora tetracladia* M-Lob-30 (JN569103) by 94.2%., *A. tetracladia* UMB-320.07 (GQ411292) by 94.4%., *A. tetracladia* UMB-333.07 (GQ411290) by 94.6%, *A. tetracladia* UMB-343.07 (GQ411293) by 94.6%, *A. tetracladia* (GQ152144) by 94.2%, *A. tetracladia* (GQ152145) by 94.0% and with *Articulospora* sp. AU_CRYP06 (JN995644) by 98.5% (Fig. 26a, Table 21a).

To confirm the novelty of *Articulospora* sp. Cry-FB1 and Cry-FB2 strains, the sequences of a more stable region (5.8S rRNA) which has slower evolutionary change rates were further analyzed. 5.8S rRNA region showed closest sequences similarity (%) with *Articulospora tetracladia* M-Lob-30 (JN569103) by 88.7%, *A. tetracladia* UMB-320.07 (GQ411292) by 89.4%, *A. tetracladia* UMB-333.07 (GQ411290) by 89.4%, *A. tetracladia* UMB-343.07 (GQ411293) by 90.1%, *A. tetracladia* (GQ152144) by 89.4% and *A. tetracladia* (GQ152145) by 88.7% (Table 1b). These analyses confirmed that *Articulospora* sp. Cry-FB1 (AB703291) andCry-FB2 (AB703292) are novel strains. In phylogenetic tree the isolates *Articulospora* sp. Cry-FB1 and Cry-FB2 are presented as novel species, are yet to be established. The total sequence lengths after alignment, % sequence similarities and number of positions with base changes are summarized in Table 21a and 21b.

Table 21a. Sequence similarities of putative novel species *Articulospora* sp. using ITS region.

Culture Code	Sequence deposition no.	Total sequence length	No. of base changes	Bootstrap support %	ITS region gene sequences similarity (%)
***Articulospora* sp. Cry-FB1**	***AB703291***	553	9	99	Uncultured Ascomycota clone 6_m10 (HQ211861) by 98.4%.
		543	8	99	*Articulospora sp.* AU_CRYP06 (JN995644) by 98.5%.
		553	11	99	Uncultured Ascomycota clone 8_f21 (HQ212240) by 98.0%.
		553	11	99	Uncultured Gyoerffyella clone 8_d20 (HQ212213) by 98.0%.
		553	25	88	*Helotiales sp.* SM12-2 (EF093150) by 95.5%.
		514	30	94	*Articulospora tetracladia* M-Lob-30 (JN569103) by 94.2%.
		514	29	94	*Articulospora tetracladia* UMB-320.07 (GQ411292) by 94.4%.
		514	28	94	*Articulospora tetracladia* UMB-333.07 (GQ411290) by 94.6%.
		514	28	94	*Articulospora tetracladia* UMB-343.07 (GQ411293) by 94.6%.
		554	32	94	*Articulospora tetracladia* (GQ152144) by 94.2%.
		554	33	94	*Articulospora tetracladia* (GQ152145) by 94.0%.
		561	111	100	*Articulospora atra* CCM F-00684 (FJ000402) by 80.2%.
		551	111	100	*Articulospora atra* CCM F-01384 (FJ000396) by 79.9%.
		516	68	14	Uncultured fungus clone 162 (HM044618) by 86.8%.
		545	117	93	*Articulospora proliferata* (FJ000395) by78.5%.
		420	119	-	*Exophiala* sp. SDH-2005 (AY957553) by71.7%.
		553	6	100	***Articulospora* sp. Cry-FB2 (AB703292)** by 98.9%.

Table 21b. Identification of putative novel species *Articulospora* sp. (using more conserved region 5.8S rDNA region), total sequence lengths after alignment, % sequence similarities, and number of positions with base changes.

Culture Code	Sequence deposition no.	Total sequence length	No. of base changes	Bootstrap support %	5.8S rDNA region gene sequences similarity (%)
***Articulospora* sp. Cry-FB1**	***AB703291***	151	0	99	Uncultured Ascomycota clone 6_m10 (HQ211861) by 100%.
		151	0	99	*Articulospora sp.* AU_CRYP06 (JN995644) by 100%.
		151	0	99	Uncultured Ascomycota clone 8_f21 (HQ212240) by 100%.
		151	0	99	Uncultured Gyoerffyella clone 8_d20 (HQ212213) by 100%.
		151	15	30	*Helotiales sp.* SM12-2 (EF093150) by 90.1%.
		151	17	55	*Articulospora tetracladia* M-Lob-30 (JN569103) by 88.7%.
		151	16	43	*Articulospora tetracladia* UMB-320.07 (GQ411292) by 89.4%.
		151	16	43	*Articulospora tetracladia* UMB-333.07 (GQ411290) by 89.4%.
		151	15	55	*Articulospora tetracladia* UMB-343.07 (GQ411293) by 90.1%.
		151	16	43	*Articulospora tetracladia* (GQ152144) by 89.4%.
		151	17	43	*Articulospora tetracladia* (GQ152145) by 88.7%.
		134	32	99	*Articulospora atra* CCM F-00684 (FJ000402) by 76.1%.
		134	32	99	*Articulospora atra* CCM F-01384 (FJ000396) by 76.1%.
		146	26	30	Uncultured fungus clone 162 (HM044618) by 82.2%.
		146	44	63	*Articulospora proliferata* (FJ000395) by 69.9%.
		151	0	99	***Articulospora* sp. Cry-FB2 (AB703292)** by 100%.

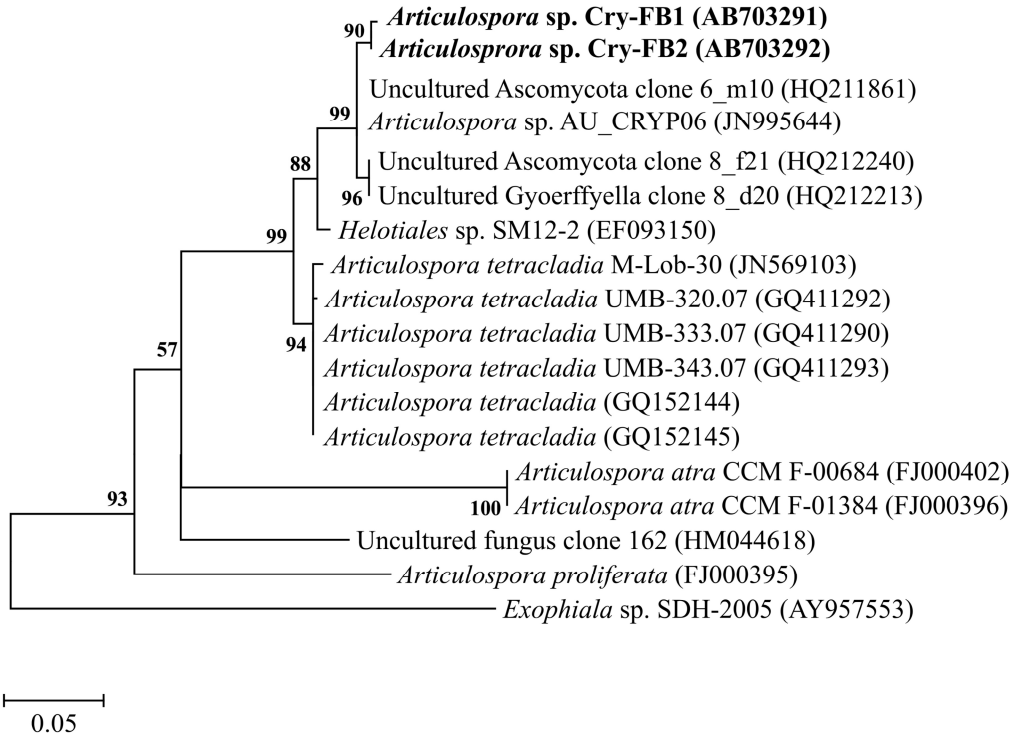


Fig. 26 Phylogenetic tree of putative novel species *Articulospora* sp. (using ITS region). The accession numbers of isolates are shown in parentheses. Tree was constructed with neighbor-joining method. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated substitutions per nucleotide position.

Sequence analyses of isolates *Thelebolus* sp. CRY-YB-240 (KT223585) and *Thelebolus* sp.CRY-YB-241 (KT223586) resembled *Thelebolus microsporus* CBS109799 (AY957552). *Rhodotorula* sp. Cry-FB3 showed their closest relationship to the species of *Rhodotorula svalbardensis* Cry-YB-1 (AB734690). The phylogenetic tree of *Thelebolus* sp. is shown in Fig. 26. After alignment the total sequence lengths, sequence similarities (%), and number of positions with base changes of *Thelebolus* sp. CRY-YB-240, *Thelebolus* sp.CRY-YB-241 and their closely related strains are shown in Table 22.

Table 22. ITS region gene sequences similarity (%) of *Thelebolus* species.

Sample detail	Sequence deposition no.	Total sequence length	No. of base changes	Bootstrap support %	ITS region gene sequences similarity (%)
Thelebolus sp. CRY-YB-240	KT223585	538	6	21	*Thelebolus microsporus* CBS109799 (AY957552) by 98.9%.
		504	6	63	*Thelebolus globosu* ANT03-221 (JX171196) by 98.8%.
		538	6	63	*Thelebolus microsporus* BI15-1-1 (GU004196) by 98.9%.
		482	12	39	*Thelebolus stercoreus* CBS709.69 (AY957549) by 97.5%.
		548	12	88	*Thelebolus ellipsoideus* ANT03-417 (JX171195) by 97.8%.
		480	12	88	*Thelebolus ellipsoideus* CBS113937 (AY957550) by 97.5%.
		1109	7	89	***Thelebolus* sp. CRY-YB-241 (KT223586)** by 99.4%.

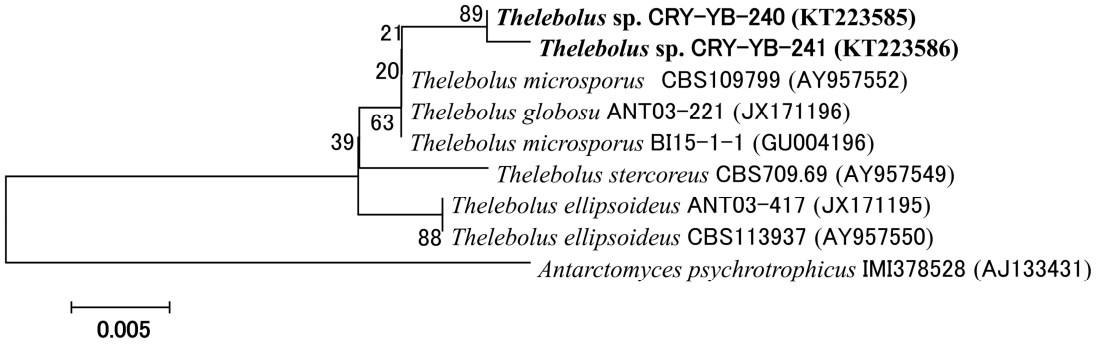


Fig. 27 Phylogenetic tree of *Thelebolus* sp. The accession numbers of isolates are shown in parentheses. Tree was constructed with neighbor-joining method. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated substitutions per nucleotide position.

3b.3.3 *Rhodotorula svalbardensis* sp. nov., a novel yeast species

3b.3.3.1 Phenotypic properties

The colony colour of MLB-I was creamy in appearance turning pale pink with time (Fig.28b); vegetative cells were ellipsoidal. The size of the yeast cells were measured to be 5.8-8.3 x 4.2-7.5 µm (mean 7 x 5.9 µm) and occurred singly or in groups (Fig. 28d-f). Budding was unilateral, occasionally multilateral. Short thin, septate hyphae [1.15- 1.84 µm wide (mean 1.45 µm)] were formed after about 4 weeks of incubation at 4^{o}C on PDA. The length of the hyphae was between 10 and 45 µm. Presence of septate hyphae, clamp connections and urease and amylase activity are characteristic features of strains MLB-I and CRY-YB1. These phenotypic features differentiate these strains from the closely related species of *R. himalayensis* (Shivaji *et al*. 2008). The strains were similar to *Bensingtonia* in possessing septate hyphae and urease activity. *Bensingtonia* however, produced ballistoconidia unlike the MLB-I and CRY-YB1 strains.

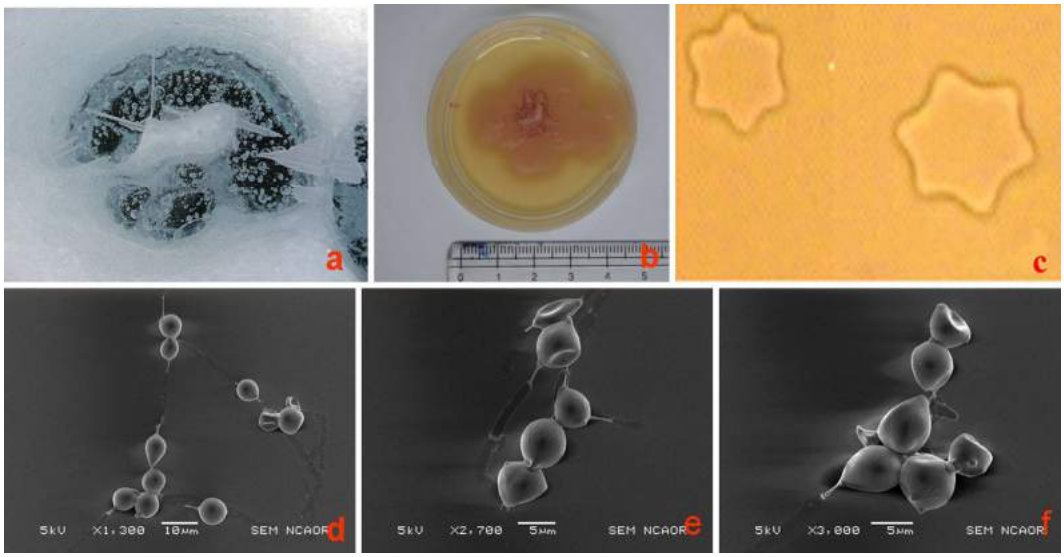


Fig. 28 a) Cryoconite hole, b) pure culture plate, c) AFPs Activity of yeast grown at -1^{o} C for 56 days, d-e) SEM photograph of MLB-I showing cell shape, f) SEM photograph of MLB-I showing cell shape and clamp connection

The strain MLB-I grew between 1 and 20^{o}C but not above 20^{o}C. The optimum temperature for growth (dry biomass wt.2.3 g/l) was 15^{o}C after 30 days of incubation in YMP broth, with luxuriant growth between 10 to 15^{o}C. On YM medium, cells grew between pH 5.0 and 8.0, and optimum pH for growth is between 6.0 to 7.0. Four-week grown culture of the isolates MLB-I and CRY-YB1 showed positive Diazonium Blue B reaction. All the six different media tested (MYP agar, YPD agar, MEA, PDA, PCA, SDA) showed good growth, but luxuriant growth was observed on PCA medium. The absence of sexual reproduction in MLB-I and CRY-YB1 strains indicate that these isolates are similar to other species of *Rhodotorula*. They are however, phenotypically different from *Rhodotorula psychrophila* CBS10440T, *R. glacialis* CBS10473T and *R. psychrophenolica* CBS10483T isolated from alpine soils (Margesin *et al*. 2007) (Table 23). Thus, it appears that MLB-I and CRY-YB1 strains, which are phylogenetically and phenotypically related to *Rhodotorula,* are novel strains of the genus. Earlier studies have reported various species of *Rhodotorula* from diverse cold habitats such as in Antarctica and Arctic (Vishniac 1999; 2006, Bergauer *et al*. 2005, Butinar *et al*. 2007, Shivaji *et al*. 2008). Thus, the occurrence of *Rhodotorula* strain MLB-I and CRY-YB1 in the frozen cryoconite holes in Arctic is acceptable.

Strains MLB-I and CRY-YB1 showed similar phenotypic characteristics but differed from the four nearest phylogenetic neighbours: *R. psychrophenolica* CBS10438T (8 tests), *R. himalayensis* 3AT (14 tests), *R. glacialis* CBS10437T (7 tests) and *R. psychrophila* CBS10440T (6 tests). Details of differences in phenotypic tests among closely related strains are shown in Table 23. The strain has the ability to split arbutin.

Table 23. Comparison of phenotypic characteristics of *Rhodotorula svalbardensis* sp.nov. with *R. himalayensis, R. glacialis, R. psychrophila* and *R. psychrophenolica.*

Phenotypic properties	***Rhodotorula* sp. MLB-I (CBS 12863)**	***R. glacialis*[2] (CBS10437T)**	***R. psychrophila*[2] (CBS10440T)**	***R. psychrophenolica*[2] (CBS10438T)**	***R. himalayensis*[1] 3AT, 4A, 4B and Rup 4B**
Growth temperature (°C)	1-20	1–20	1–15	1–20	5–22
Assimilation of carbon source					
D-glucose	+	+	+	+	+
Sucrose	+	+	+	+	+
Raffinose	+	+	+	+	+
Mannitol	+	+	+	+	+
D-gluconate	-	+	+	+	+
D-glucosamine	-	-	-	-	-
D-ribose	-	-	-	-	-
D-arabinose	+	-	-	-	-
Lactose	+	-	-	-	-
Glycerol	-	-	-	-	-
DL-lactate	+	-	-	-	-
Myo-Inositol	-	-	-	-	-
Methanol	-	-	-	-	-
D-galactose	-	-	-	-	+
L-sorbose	-	-	-	-	D
D-xylose	-	NA	-	NA	W
L-arabinose	-	NA	-	-	+
Maltose	+	-	-	-	+
Cellobiose	+	-	-	-	D
Melibiose	-	NA	-	-	+
Melezitose	+	NA	+	-	+
Erythritol	-	-	NA	-	+
Citrate	-	-	-	-	+
L-rhamnose	-	NA	-	+	-
D-glucuronate	-	+	+	+	-
Assimilation of nitrogen source and other tests					
Nitrate, ethylamine and D-tryptophan	+,-ND	+	+	+	+
Nitrite, lysine	+,+	-	-	-	-
Creatinine, creatine,cadaverine	+,W,+	+	+	+	-
Growth in 1% acetic acid or 50% D-glucose	+	-	-	-	-
Growth in 0.01% cycloheximide	-	-	NA	NA	-
Diazonium Blue B reaction	+	+	+	+	+
Urea hydrolysis	+	+	+	+	D

+ positive; - negative; W weak positive; D delayed; NA data not available, ND not detected,

The relative percentage of major fatty acids in *Rhodotorula svalbardensis* sp.nov. was oleic acid (C18:1n9c) followed by linoleic acid (C18:2n6c), linolenic acid (C18:3n3), palmitic acid (C16:0), stearic acid (C18:0), myristic acid (C14:0) and pentadecanoic acid (C15:0) (Table 24). Linoleic acid (C18:2n6c) and linolenic acid (C18:3n3) have contributed to the prevalence of PUFA (polyunsaturated fatty acids) in the organism. Oleic acid (C18:1n9c) was the most abundant MUFA (monounsaturated fatty acids) in all the samples, while palmitic acid (C16:0) was the major SFA (saturated fatty acids). PUFAs at colder temperatures modulate the membrane fluidity of organisms thereby enhancing the ability of the fungus to survive at low temperatures. Evidence suggests that membrane composition is critical for the ability of microorganisms to grow over specific temperature ranges (Robinson 2001). In the present study, the cultures at low temperatures accumulated high concentration of unsaturated fatty acids. Similar observations of higher concentrations of unsaturated fatty acids were also recorded from psychrophilic yeasts, viz. *Candida*, *Leucosporidium* and *Torulopsis* (Kerekes and Nagy 1980) and fungus *Microdochium nivale* (Istokovics *et al*. 1998). Pathan *et al.* (2010) made similar observations on different species of yeasts. Production of high concentration of oleic acid is an indication that the organism experienced environmental stress in cryoconite holes.

Table 24 The comparison of fatty acids composition of *Rhodotorula svalbardensis* sp. nov. grown at 1°C, 4°C and 15°C

	C14:0	**C15:0**	**C16:0**	**C18:0**	**C18:1n9c**	**C18:2n6c**	**C18:3n3**
15°C	0.62	0.72	7.29	4.69	44.54	21.94	9.28
4°C	5.26	1.70	9.83	4.01	39.00	19.62	18.84
1°C	0.96	0.89	10.43	3.27	50.04	13.86	12.06

Abbreviation: Myristic acid (C14:0), Pentadecanoic acid (C15:0), Palmitic acid (C16:0), Stearic acid (C18:0), Oleic acid (C18:1n9c), Linoleic acid (C18:2n6c), Linolenic acid (C18:3n3).

Phylogenetic placement

EMBOSS Matcher - Pairwise Sequence Alignment tool (http://www.ebi.ac.uk /Tools/ psa/ emboss _matcher /nucleotide.html) was used for sequence alignment. The sequence of D1/D2 domain of large-subunit rRNA gene (26S rRNA) of strain *Rhodotorula* sp. MLB-1 showed their closest relationship to species of *Rhodotorula psychrophenolica* CBS10483^T by 95.7%, with *R. himalayensis* 3A^T by 96.4%, with *R. glacialis* CBS10436^T by 96.0% and with *R. psychrophila* CBS10440^T by 95.8% (Fig. 28). This affiliation between MLB-I and the above mentioned four species of *Rhodotorula* is further confirmed by the NJ phylogenetic tree (Fig. 29). Phylogenetically the strains belong to a clade of psychrophilic yeasts. D1/D2 domain sequence of MLB-1 quite differed from the so far published, phylogenetically related species of *Rhodotorula* and had low identity (<95%) with *Bensingtonia yamatoana* CBS7243T (AF189896). Fell *et al*. (2000) noted *Rhodotorula* to be a polyphyletic genus. Similar observation has been made based on the D1/D2 domain phylogenetic tree in the present study (Fig. 29).

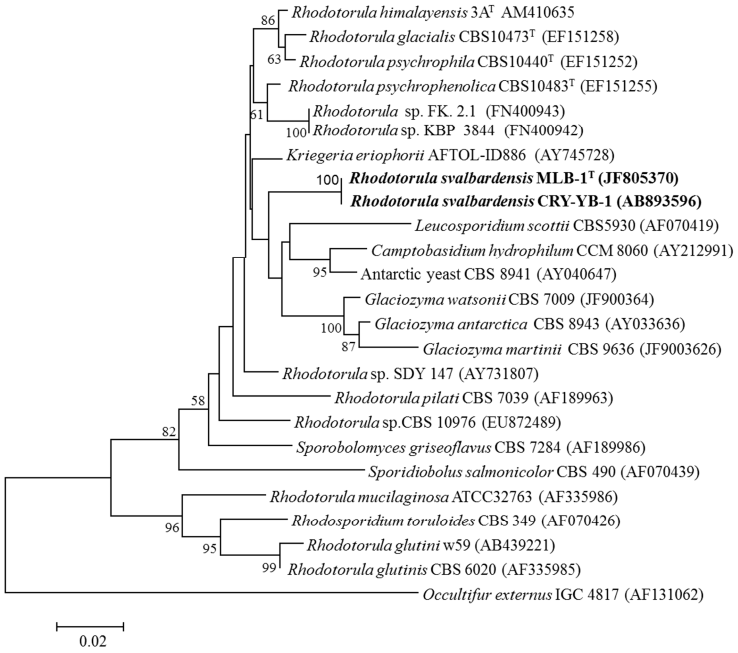


Fig. 29 Phylogenetic tree of *Rhodotorula svalbardensis* sp.nov based on D1/D2 domain. Neighbor-joining method was used for construction of phylogenetic tree.

The strain MLB-1 under the present investigation differed by more than two nucleotides in the D1/D2 domain. It differed from *Rhodotorula psychrophenolica* by 24nt, *R. himalayensis* by 22nt, *R. glacialis* by 24nt and *R. psychrophila* by 23nt. The total sequence length after alignment, percent sequence similarities and numbers of positions with base changes are given in Table 25. Thus, from these results, phylogenetic analysis of D1/D2 domain of MLB-I was strongly conjectured to have different characteristics from the D1/D2 sequence of known *Rhodotorula* spp. and related genera.

Sequence analysis of the ITS region showed their closest relationship with *Rhodotorula glacialis* cluster. MLB-I has only 89.3% sequence similarity with *R. glacialis* (EF151249), 88.2% with *R. glacialis* (EF151249), 89.7% with *R. psychrophila* (EF151244), 89.7 % with *R. psychrophila* (EF151245), 89.3% with *R. psychrophila* (EF151243), 88.3% with *R. psychrophenolica* (EF151246) and 89.5% with *R. himalayensis* (AM410635). Thus, these wild-type strains appear to be different from the so far published phylogenetically related species of *Rhodotorula* and related genera. The sequence of ITS1-5.8S rRNA gene-ITS2 region of the strains MLB-I and CRY-YB1, shows 99.6% similarity with each other and exhibit similar phenotypic properties. In the neighbour-joining phylogenetic tree, strains MLB-I and CRY-YB1, clustered with *R. glacialis* (EF151249), *R. himalayensis* (AM410635), *R. psychrophila* (EF151243) and *R. psychrophenolica* (EF151246) (Fig. 30).

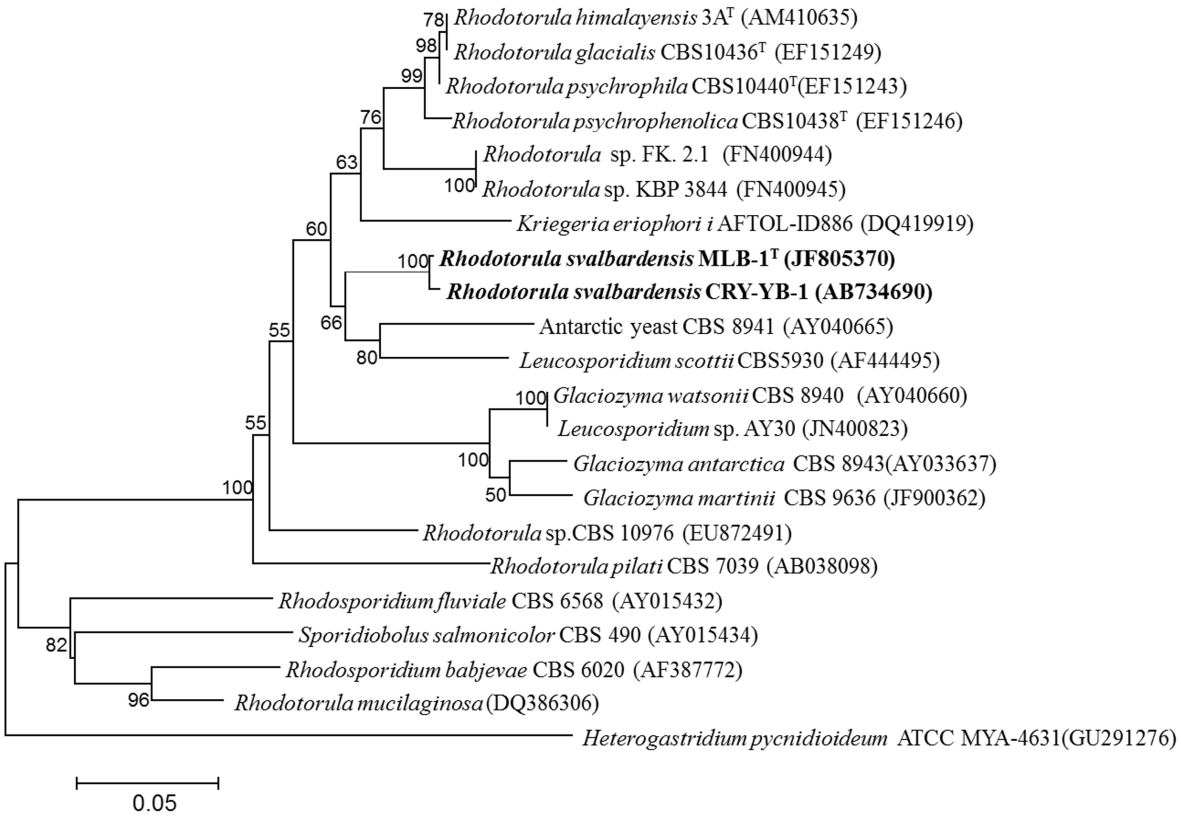


Fig. 30 Phylogenetic tree based on ITS region of sequences of *Rhodotorula svalbardensis* sp.nov.

Dearth of recombination and strict maternal inheritance render mitochondrial cytochrome-b gene a useful marker for inferring phylogeny of closely related species (Manceau *et al*. 1999, Biswas *et al*. 2001). Since no sequence data is available on mitochondrial cytochrome-b region for *Rhodotorula psychrophenolica*, *R. himalayensis*, *R. glacialis*, and *R. psychrophila*, no phylogenetic comparision was made. Nevertheless, for *Rhodotorula svalbardensis* the cytochrome b gene was sequenced and data deposited at the gene bank Accession no. AB704195) and phylogenetic tree was constructed with other *Rhodotorula sp.* available gene sequence data in NCBI. The length of the cytochrome-b gene sequence was 396 nt (Table 25, Fig. 31).

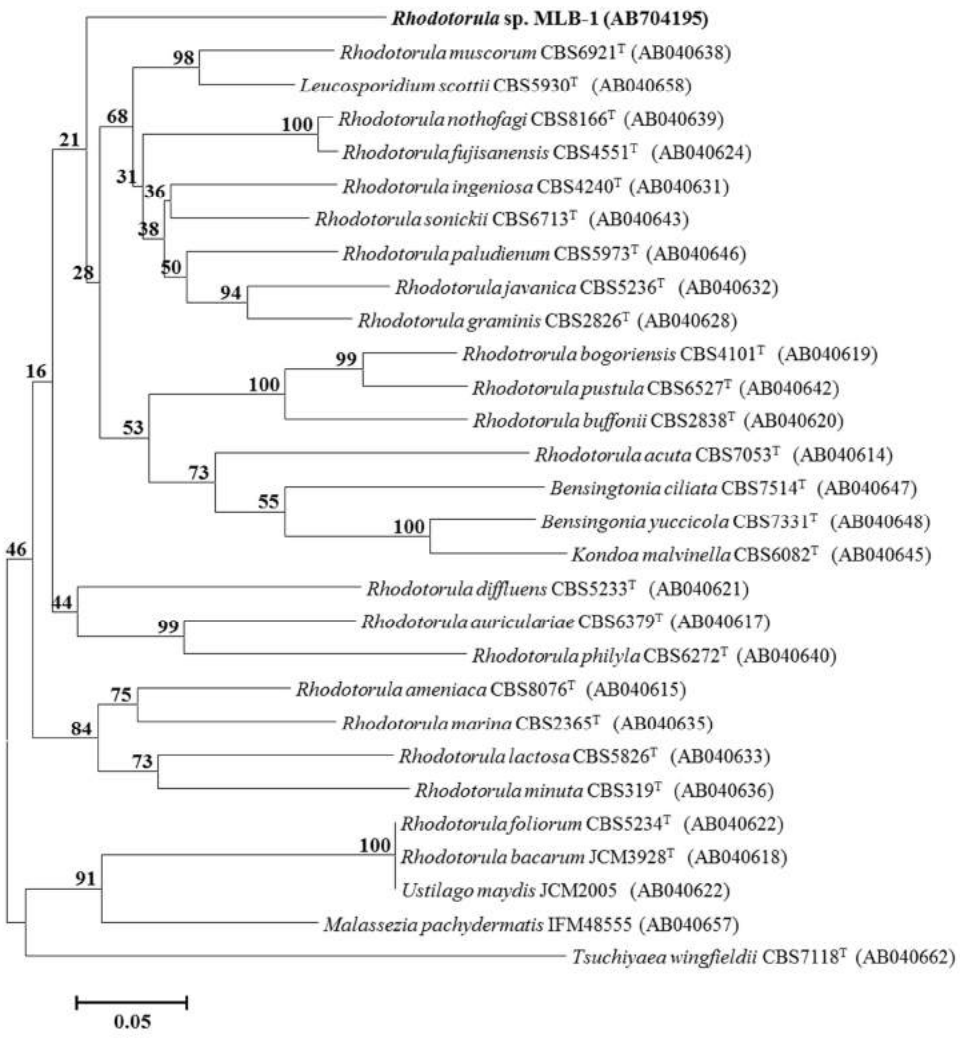


Fig. 31 Phylogenetic tree based on cytochrome-b region of sequences of *Rhodotorula svalbardensis* sp.nov.

Morphological and physiological properties, as well as sequences of 26S rRNA, D1/D2 domain, ITS region and mitochondrial cytochrome *b* gene, indicates that MLB-I belong to hitherto unknown species, for which the name *Rhodotorula svalbardensis* sp. nov. is proposed with strain MLB-I as the type strain.

Table 25. Details of D1/D2, ITS and Cyt-b gene sequences of *Rhodotorula svalbardensis* sp. nov. (CBS 12863, MTCC 10952) with sequences retrieved from Genbank of authentic strains related to species of the genus.

SPECIES	NO OF POSITIONS WITH BASE CHANGES (nt)			TOTAL SEQUENCE LENGTH AFTER ALIGNMENT(nt)			SIMILARITY* %)		
	D1/D2	Cyt b	ITS	D1/D2	Cyt b	ITS	D1/D2	Cyt b	ITS
Rhodotorula svalbardensis sp. nov	-	-	-	610	396	595	-	-	-
Rhodotorula psychrophenolica	24	-	71	553	-	607	95.7	-	88.3
Rhodotorula himalayensis	22	-	62	610	-	590	96.4	-	89.5
Rhodotorula glacialis	24	-	64	601	-	600	96.0	-	89.3
Rhodotorula psychrophila	23	-	62	547	-	579	95.8	-	89.3
Kriegeria eriophorii	23	-	79	564	-	537	95.9	-	85.3
Rhodotorula sp. KBP 3844	26	-	67	564	-	587	95.4	-	88.6
Rhodotorula sp. CBS 10976	49	-	86	552	-	546	91.1	-	84.2
Leucospridium scottii	34	-	61	564	-	599	94.0	-	89.8
Camptobasidium hydrophilum	35	-	-	564	-	-	93.8	-	-
Glaciozyma watsonii	21	-	102	518	-	622	95.9	-	83.6
Glaciozyma antarctica	34	-	111	568	-	628	94.0	-	82.3

3b.3.3.3 Description of *Rhodotorula svalbardensis* sp. nov.

(Etymology: sval.bar.den′sis. N.L. fem. adj. svalbardensis pertaining to Svalbard).

Rhodotorula svalbardensis sp. nov. was isolated from sediment of cryoconite holes, Midre Lovénbreen glacier, Ny-Ålesund, Spitsbergen, Svalbard archipelago, the Arctic. In liquid media (MYP) after 15 days at 10^{o}C, while on solid yeast-extract agar after 3-4 weeks at 10-15^{o}C, the colony colour is reddish to orange

with regular margin. Vegetative cells are ellipsoidal, the size measured to be 5.8-8.3 x 4.2-7.5 µm (mean 7x 5.9 µm), and occur singly or in groups. Budding was found unilateral, occasionally multilateral. Short thin hyphae [1.15- 1.84 µm wide (mean 1.45 µm)] were formed after 4 weeks of incubation at 4^{o}C. No sexual state was observed and spores did not form. Strains grew between 1 and 20^{o}C but not above 20^{o}C.

The strain assimilated D-glucose, Sucrose, Raffinose, D-arabinose, lactose, Maltose, Cellobiose, Melezitose, DL-lactate; but does not assimilate D-gluconate, D-glucoronate, methanol, D-glucosamine, D-ribose, glycerol, myo-inositol, D-galactose, L-sorbose, D-xylose, L-arabinose, Melibiose, Erythritol, Citrate, L-rhamnose as sole carbon sources. The strain assimilated nitrate, nitrite, L-lysine, creatinine, creatine (weak) and cadaverine; but did not assimilate ethylamine as sole nitrogen source. Salt tolerance was upto 1.5 M. Diazonium Blue B positive and hydrolyzed urea. Whole-cell fatty acids include oleic acid, linoleic acid, linolenic acid, myristic acid, pentadecanoic acid, palmitic acid and stearic acid. Optimum temperature for growth is 10–15^{o}C; produces extracellular amylase, cellulase, lipase, protease, urease, catalase and utilizes arbutin. AFP activity was observed at -1^{o}C.

Strains MLB-I (CCP-II), and CRY-YB-I are the strains of *Rhodotorula svalbardensis* sp. nov. Strain MLB-I is designated as type strain and deposited at and Centraalbureau voor Schimmelcultures (CBS), Netherlands (=CBS12863) and Microbial Type culture collection and Gene Bank, India (=MTCC 10952). The strains were isolated from sediment samples of cryoconite holes of Midre Lovénbreen glacier (Ny-Ålesund) Spitsbergen, Svalbard archipelago, the Arctic.

3b.3.4 Ice cores yeasts

The viable counts of yeast cells in ice core melted water ranged between 3 x 10^3 and 1 x 10^4 CFUs/ml. The mean viable counts in vertical profile of the ice core varied; in top (8.5 x 10^3 CFUs/ ml), middle (4.5 x 10^3 CFUs/ml) and bottom (3.5 x 10^3 CFUs/ml) layers. Colonies of the yeast isolates showed different colours, shape, size and physiological features (Fig 32).

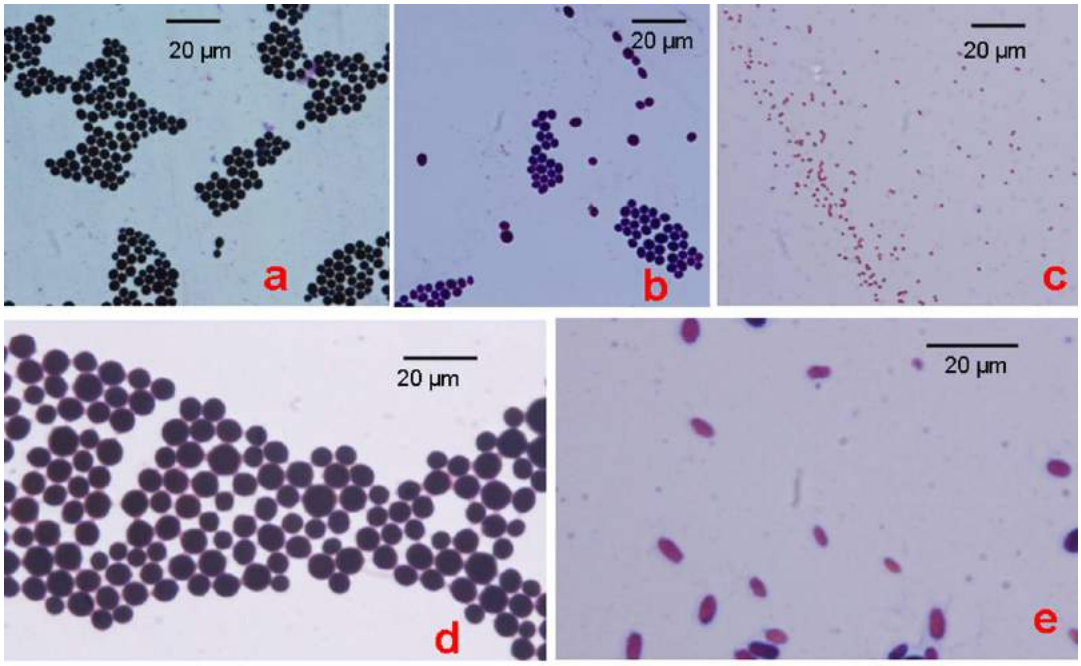


Fig. 32 Light Microscopic view of yeast species. a) MLB-25 JX192662 *Cryptococcus albidosimilis*, b) MLB-23 JX192660 *C. saitoi*, c) MLB-27 JX192664 *Rhodotorula mucilaginosa*, d) MLB-18 JX192655 *C. adeliensis*, e) MLB-20 JX192657 *Rhodosporidium* sp.

Based on morphological features, 10 isolates were selected for further characterization. Of these, five were from ice core sampling location G-4, two each from G-1 and G-3, and one from G-2 location. All the 10 isolates showed growth between temperature ranges of 1–20 ^oC, salt tolerance (NaCl) from 1.0 to 7.5 M, and positive activity for cellobiase and β-glucosidase.

3b.3.4.1 Phenotypic properties

The cryophilic yeast strains grew between 1 and 20 ^{o}C, good growth between 10 and 15 ^{o}C and poor growth above 20 ^{o}C. Cells grow between pH 6.0 and 8.0 with optimum pH between 6.5 and 7.0 on PDB medium (Table 26). No sexual state could be observed. Phenotypic characterization of the isolates suggest the utilization of sucrose, mannose, fructose, dextrose and xylose by all the isolates and none of the isolates could assimilate lactose, melibiose, sodium gluconate, dulcitol, inositol, sorbitol, mannitol, α-methyl-D-glucoside, a-methyl-D-mannoside, Ortho-Nitrophenyl-β-D-galactopyranoside (ONPG), D-arabinose or sorbose. Only a few isolates were able to weakly utilize raffinose, inulin, glycerol, adonitol and arabitol. Rest of the carbon sources were variably utililized by the isolates tested. Isolate *Cryptococcus* sp. MLB-26 utilized maximum carbon sources (13 positive and three weakly positive) followed by *Cryptococcus* sp. MLB-25 (13 positive) and MLB-19 (12 positive and three weakly positive). *Rhodotorula* sp. MLB-27 assimilated least number of carbon sources amongst tested (Table 27). Phenotypic properties, as well as 26S D1/D2 sequence indicate that ice core comprised of five species belonging to three genera (*Cryptococcus*, *Rhodosporidium*, and *Rhodotorula*).

Table 26 Distribution, morpho taxonomic features and physiological characteristics of yeast isolates.

Isolate Code	Ice Sampling Location				Cultural/Taxonomic characters				Physiological characters				
	G-1	G-2	G-3	G-4	Colony colour	Colony margin	Cell shape	Cell Size (µm)	Growth Temp. Range 1- 20oC	NaCl conc. (M) 1-7.5	Cellobiase	β-Glucosidase	AFPs
MLB-21	+				Cream	Regular	Sub-globose	3.6-4.2	+	+	+	+	-
MLB-22		+			Whitish cream	Regular	Sub-globose	3.3-3.7	+	+	+	+	-
MLB-23			+		White	Regular	Globose to Sub-globose	3.3-4.3	+	+	+	+	-
MLB-26			+		White	Regular	Globose to Sub-globose	2.4-3.1	+	+	+	+	-
MLB-27				+	Orange	Regular	globose	1.3-1.5	+	+	+	+	-
MLB-18				+	Peach colour	Regular	globose	4.5-8.7	+	+	+	+	-
MLB-19				+	white	Regular	globose	5.6-6.0	+	+	+	+	-
MLB-20				+	Pink	Regular	Ellipsoidal	L 4.0-7.0 W 2.2-5.6	+	+	+	+	-
MLB-24				+	Cream	Regular	globose	4.9-5.2	+	+	+	+	-
MLB-25	+				Cream	Regular	globose	2.3-4.5	+	+	+	+	-

Table 27. Phenotypic characters of the Ice core yeast isolates (+ positive; - negative; W weak positive).

Test	MLB-21	MLB-22	MLB-23	MLB-26	MLB-27	MLB-18	MLB-19	MLB-20	MLB-25
Lactose	-	-	-	-	-	-	-	-	-
Xylose	+	+	+	+	+	+	+	+	+
Maltose	-	+	+	+	-	-	+	-	+
Fructose	+	+	+	+	+	+	+	+	+
Dextrose	+	+	+	+	+	+	+	+	+
Galactose	-	-	-	-	w	-	-	+	+
Raffinose	-	-	-	-	w	w	-	w	-
Trehalose	-	-	-	-	-	+	+	+	+
Melibiose	-	-	-	-	-	-	-	-	-
Sucrose	+	+	+	+	+	+	+	+	+
L-Arabinose	-	w	-	w	w	w	w	-	+
Mannose	+	+	+	+	+	+	+	+	+
Inulin	-	-	-	w	-	-	-	-	-
Sodium gluconate	-	-	-	-	-	-	-	-	-
Glycerol	-	-	-	-	-	w	-	-	-
Salicin	+	-	+	+	-	+	-	-	+
Dulcitol	-	-	-	-	-	-	-	-	-
Inositol	-	-	-	-	-	-	-	-	-
Sorbitol	-	-	-	-	-	-	-	-	-
Mannitol	-	-	-	-	-	-	-	-	-
Adonitol	-	-	-	-	-	-	-	w	-
Arabitol	-	-	-	-	-	-	-	w	-
Erythritol	-	-	-	-	-	-	+	-	-
α-Methyl-D -glucoside	-	-	-	-	-	-	-	-	-
Rhamnose	-	-	+	w	-	-	-	-	+
Melezitose	-	+	w	+	-	-	w	-	-
α-Methyl-D -mannoside	-	-	-	-	-	-	-	-	-
Xylitol	+	+	+	+	-	+	w	+	+
ONPG	-	-	-	-	-	-	-	-	-
Esculin hydrolysis	+	+	+	+	-	+	+	-	+
D -Arabinose	-	-	-	-	-	-	-	-	-
Citrate utilization	-	+	+	+	-	-	+	-	-
Malonate utilization	-	+	+	+	-	-	+	-	-
Sorbose	-	-	-	-	-	-	-	-	-

3b.3.4.2 Phylogenetic analyses

The total sequence lengths after alignment, % sequence similarities, number of positions with base changes are given in Table 28. The sequences of all isolates (MLB-18 JX192655, MLB-19 JX192656, MLB-20 JX192657, MLB-21 JX192658, MLB-22 JX192659, MLB-23 JX192660, MLB-24 JX192661, MLB-25 JX192662, MLB-26 JX192663, and MLB-27 JX192664) were deposited in the DNA data bank (NCBI) and were subjected to a NCBI BLAST search. In the pairwise alignment performed using EMBOSS Matcher – Pairwise Sequence Alignment tool (http://www.ebi.ac.uk/Tools/ psa/emboss_ matcher/ nucleotide .html), the sequence analysis of the D1/D2 domain of large-subunit rRNA gene (26S rRNA) of isolates MLB-18 and MLB-21 indicated their closest relationship to species of the *Cryptococcus adeliensis* cluster. *Cryptococcus* sp. MLB-18 (JX192655) and *Cryptococcus* sp. MLB-21 (JX192658) resembled *C. adeliensis* CBS8351 (AF137603) by 99.6% and 100% sequence similarity in the D1/D2 region, respectively.

Sequence analysis of isolates MLB-19, MLB-24 and MLB-25 indicated their closest relationship to the species of *Cryptococcus albidosimilis* cluster. *Cryptococcus* sp. MLB-19 (JX192656), *Cryptococcus* sp. MLB-24 (JX192661), and *Cryptococcus* sp. MLB-25 (JX192662), resembled *C. albidosimilis* CBS7711 (AF137601) by 100%, 98.3% and 99.8% sequence similarity respectively. MLB-24 and MLB-25 differed by 10 and 1 nucleotide(s) in the D1/D2 region, respectively. Sequence analysis of isolates MLB-22, MLB-23 and MLB-26 indicated their closest relationship to the species of *Cryptococcus saitoi* cluster. *Cryptococcus* sp. MLB-22 (JX192659), *Cryptococcus* sp. MLB-23 (JX192660), *Cryptococcus* sp. MLB-26 (JX192663), resembles *C. saitoi* CBS1975 (AF181540) by 98.6–100% sequence similarity. MLB-26 differed by 8 nucleotides in the D1/D2 region.

Sequence analysis of isolate MLB-20 indicated its closest relationship with the species of *Rhodosporidium lusitaniae* cluster. *Cryptococcus* sp. MLB-20 (JX192657) resembles *R. lusitaniae* PYCC 4642 (JN246539) and *Rhodotorula*

colostri DBVPG 5006 (GQ911527) by 98.3–98.5% sequence similarity while it differed, in the D1/D2 region, from the two strains by 8 and 9 nucleotides, respectively. Sequence analysis of isolate MLB-27 indicated their closest relationship to the species of *Rhodotorula mucilaginosa* cluster. *Rhodotorula* MLB-27 (JX192664) resembles *R. mucilaginosa* KCTC7829 (AF257267) by 99.8% sequence similarity and differed by 1 nucleotide in the D1/D2 region.

A neighbor-joining phylogenetic tree was constructed using *Pseudozyma antarctica* as an outgroup (Fig. 33). The tree showed 66% bootstrap support to strains MLB-18-19, 21–26 and 83% bootstrap support to MLB-20 and MLB-27. Thus, these strains appear to be phylogenetically different from the published species of *C. antarcticus* CRUB1385 and *Rhodotorula nothofagi* CBS 8166, the nearest phylogenetic neighbors.

Table 28. Identification of isolates by D1/D2 domain of 28S rDNA sequences similarity (%).

Isolate Code	NCBI sequence deposition no.	Total sequence length after alignment	No. of base changes	18S rDNA gene sequences similarity (%)
MLB-21	JX192658	594	0	*Cryptococcus adeliensis* CBS8351 (AF137603) by 100%
MLB-22	JX192659	573	0	*Cryptococcus saitoi* CBS1975 (AF181540) by 100%
MLB-23	JX192660	593	0	*Cryptococcus saitoi* CBS1975 (AF181540) by 100%
MLB-26	JX192663	589	8	*Cryptococcus saitoi* CBS1975 (AF181540) by 98.6%
MLB-27	JX192664	546	1	*Rhodotorula mucilaginosa* KCTC7829 (AF257267) by 99.8%
MLB-18	JX192655	560	2	*Cryptococcus adeliensis* CBS8351 (AF137603) by 99.6%
MLB-19	JX192656	577	0	*Cryptococcus albidosimilis* CBS7711 (AF137601) by 100%
MLB-20	JX192657	533	8	*Rhodosporidium lusitaniae* PYCC 4642 (JN246539) by 98.5%
MLB-24	JX192661	582	10	*Cryptococcus albidosimilis* CBS7711 (AF137601) by 98.3%
MLB-25	JX192662	580	1	*Cryptococcus albidosimilis* CBS7711 (AF137601) by 99.8%

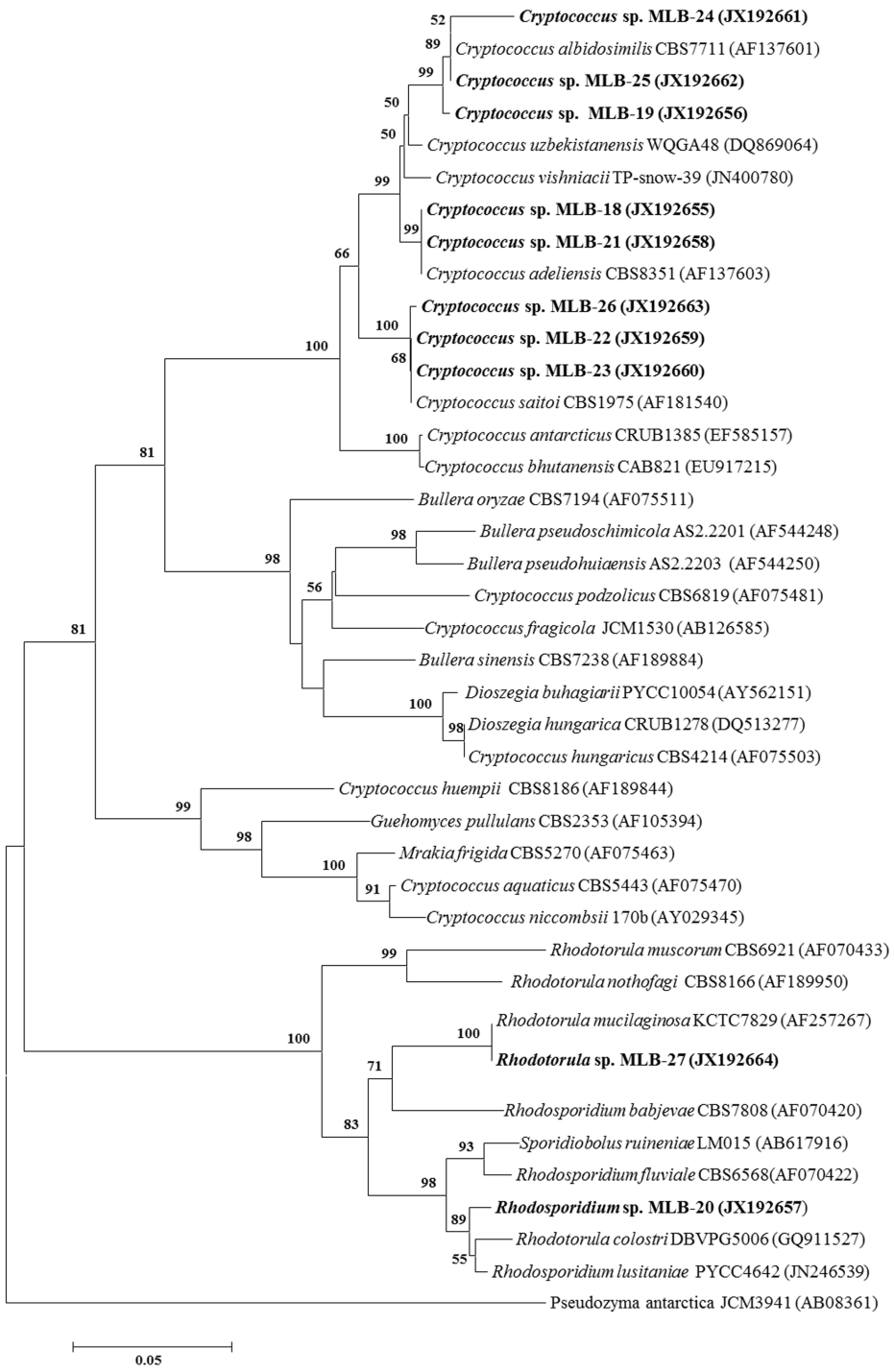


Fig. 33. Phylogenetic tree show the present yeasts strains with closely related species based on D1/D2 domain of sequences analysis of yeast species. The accession numbers of strains are shown in parentheses. Tree was constructed with neighbor-joining method by using the Jukes–Cantor distance estimation. The significance of each branch is indicated by a bootstrap value. The scale bar is estimated substitutions per nucleotide position.

3b.3.4.3 FAME analysis of ice-core yeast

FAME analysis data of culture grown at four different temperatures (1, 4, 15, and 20 ^{o}C) showed different composition of fatty acids (Table 29). The total unsaturated fatty acids in ice-core yeast species such as *C. adeliensis* was 81.13% at 1 ^{o}C, 75.40% at 4 ^{o}C, 77.82% at 15 ^{o}C and 74.38% at 20 ^{o}C; *C. saitoi* was 75.95% at 1 ^{o}C, 74.13% at 4 ^{o}C, 81.28% at 15 ^{o}C and 71.28% at 20 ^{o}C; *C. albidosimilis* was 87.15% at 4 ^{o}C, 76.23% at 15 ^{o}C and 88.20% at 20 ^{o}C; *Rhodosporidium lusitaniae* was 76.74% at 1 ^{o}C, 77.11% at 4 ^{o}C, 76.14% at 15 ^{o}C and 74.77% at 20 ^{o}C; and, *R. mucilaginosa* was 74.44% at 1 ^{o}C, 89.38% at 4 ^{o}C, 70.88% at 15 ^{o}C and 69.90% at 20 ^{o}C respectively. Overall, in all the five cultures, the relative percentage of unsaturated fatty acids was much higher as compared to the saturated fatty acids at all the temperatures tested.

The relative percentage of major fatty acids in the studied cultures (whole cell extract) at different temperatures taken on average shows that in case of *C. adeliensis*, *Rhodosporidium lusitaniae* and *R. mucilaginosa*, oleic acid (C18:1n9c) was the most abundant followed by linoleic acid (C18:2n6c), palmitic acid (C16:0) and linolenic acid (C18:3n3). In *C. saitoi*, linoleic acid (C18:2n6c) was the most abundant followed by oleic acid (C18:1n9c), palmitic acid (C16:0) and linolenic acid (C18:3n3) while in *C. albidosimilis* oleic acid (C18:1n9c) was the most abundant followed by linoleic acid (C18:2n6c), linolenic acid (C18:3n3) and palmitic acid (C16:0). In most cases, increase in temperature caused increase in concentration of oleic acid and decrease in the concentration of linolenic acid. In other words, unsaturation of fatty acids increased with decrease in temperature.

Table 29 Comparison of Fatty acids composition (relative %) of ice core yeasts grown at 1, 4, 15, and 20°C

Strain	Palmitic acid (C16:0)				Palmitoleic acid (C16:1)				Stearic acid (C18:0)				Oleic acid (C18:1n9c)				Linoleic acid (C18:2n6c)				Linolenic acid (C18:3n3)			
	1°C	4°C	15°C	20°C	1°C	4°C	15°C	20°C	1°C	4°C	15°C	20°C	1 °C	4 °C	15°C	20°C	1°C	4°C	15°C	20°C	1°C	4°C	15°C	20°C
Cryptococcus sp. MLB-21	16	19	-	0.7	-	-	14.5	19.1	-	1.7	2.5	2.2	25.7	28.7	36	39.1	31	29	29.6	28.7	22	16	8.8	6.6
Cryptococcus sp. MLB-23	22	23	-	-	-	-	19.4	25.3	-	-	-	-	16.8	17.9	29	36.8	46	45	41.2	34.5	13	12	11.7	-
Cryptococcus sp. MLB-24	ND	7.6	-	-	ND	-	8	10.8	ND	1.9	-	-	ND	28.3	32.6	45.6	ND	44	19.8	31.5	ND	15	6.9	11.1
Rhodotorula sp. MLB-20	17	18	3.1	5.7	4.7	6	17.5	18	2	-	1.1	1.6	23	34.7	39.8	41.2	23	21	22.9	19.3	25	16	8.8	8.2
Rhodotorula sp. MLB-27	14	25	10.4	20.8	10	7.1	19.9	18.8	-	-	2.3	-	16.5	19.5	33.8	32.8	23	23	20.3	21.9	24	26	6.5	7.8

3b.4 Discussion

Although microbial populations of the supra and subglacial habitats of Midre Love´nbreen glacier have been studied in the past (Säwström *et al.* 2002; 2007, Kastovska *et al.* 2005, Hodson *et al.* 2008), mycological investigation into the cryoconite holes have not been carried out so far. The viable cell counts obtained in the present investigation showed that the cryoconite holes at higher latitude (G1) support thriving of more yeast species than filamentous fungi. The counts plummeted along the slope of the glacier. At the lowermost point (G4), filamentous fungi were more abundant than the yeast. This could be likely because the temperature at higher altitudes is lower than the downstream segment of the glacier, and yeast rather than filamentous fungi survive at lower temperatures. This is in agreement with Gostinčar *et al.* (2006) and Butinar *et al.* (2007) who described the evolution of yeast and yeast-like fungal populations in the basal Arctic ice.

Taxonomic studies based on morphology and sequence data showed the presence of *Cryptococcus gilvescens*, *Mrakia* sp., *Rhodotorula* sp., *Phialophora alba* and *Articulospora tetracladia* from the cryoconite sediments. Species namely *C. gilvescens*, *Mrakia* sp. and *Rhodotorula* sp. were reported previously from glacier sediments of Alpine region (Turchetti *et al.* 2008). Taxonomic identification based on sequence data was followed according to Fell *et al.* (2000), who suggested that strains that differed from the closest related type strain by two or fewer nucleotides in the D1/D2 region are the same species. Simpson's and Shannon's diversity index (HO) indicated that the diversity at higher altitudes of the glacier was less and increased downstream as observed at G-4.

Chemical analysis of cryoconites revealed the presence of organic carbon, nitrogen and phosphorus in substantial amounts that might support the growth and activity of microbes in cryoconite holes. Similar data on nutrient availability was observed in the subglacial sediments by Foght *et al.* (2004) and Turchetti *et al.* (2008). From the soil data and the observations made related to the fungal diversity, it is evident that fungi prefer to grow and proliferate in organic soils as

compared to ahumic soils. Similar observations were made by Stonehouse (1989) in this contest.

Presence of heterotrophic organisms like filamentous fungi and yeasts in the cryoconite holes find the paramount importance as it drives the process of degradation of organic macromolecules through the secretion of extracellular hydrolytic cold-adapted enzymes and thus assists in nutrient cycling in subglacial environments. Evidence of cold-adaptation in the cryoconite isolates is evident from the fact that these isolates do not survive above 20^{o}C. Exhibition of cold-adapted extracellular enzymatic activity further confirms this observation.

Information on yeast diversity, distribution and biotechnological potential from the Arctic ice-cores is scanty. Butinar *et al.* (2007) studied the yeast from the ice cores of Austre Lovénbreen and Austre Brøgerbreen while Pathan *et al.* (2010) studied these from the puddles near the Midre Lovénbreen glacier. No information has been available so far on the diversity of yeast distributed in the ice of the Midre Love´nbreen glacier, their adaptation strategies and their biotechnological potential.

In the present study, the number of viable yeast cells decreased with the increase in the depth of ice core. This indicates that the yeast cells are reasonably equipped to thrive in the porous surface layer of ice compared to the deeper layers. Butinar *et al.* (2007) however, observed that the abundance of yeast is less in the surface layer as compared to the basal layer. Zhang and Zeng (2007) in case of bacterial deposition pattern explains that there may not always be a relationship between the cell counts and increasing depth since the microorganisms are deposited in the glacier episodically. D1/D2 domain sequence using phylogenetic analysis is a useful tool for basidiomycetous yeast and yeast like fungi (Fell *et al.* 2000). The sequence of D1/D2 domain of MLB-18 to MLB-27 isolates showed 98.3–100% similarity with validly published species in the genera *Cryptococcus*, *Rhodosporidium* and *Rhodotorula*. These isolates showed closest relationship with *C. adeliensis* CBS8351 (AF137603), *C. albidosimilis* CBS7711 (AF137601), *C. saitoi* CBS1975 (AF181540), *R. lusitaniae* PYCC 4642 (JN246539) and *R. mucilaginosa* KCTC7829 (AF257267).

Polyunsaturated fatty acids (PUFAs) at colder temperatures regulate the membrane fluidity of organisms thereby improving the ability of the organisms to survive at low temperatures (Robinson 2001). Enzymes such as desaturase present in yeasts, through repetitive desaturation, are responsible for the synthesis of PUFAs, like linoleic acid or linolenic acid (Russell 2008). At low temperatures, the membranes of psychrophilic yeasts accumulate high concentration of unsaturated fatty acids (McMurrough and Rose 1973, Arthur and Watson 1976, Watson 1984, Chintalapati *et al.* 2004, Pathan *et al.* 2010). Previous studies of genera *Mrakia*, *Leucosporidium* and *Rhodotorula* indicated that at low and sub-zero temperatures, linolenic and linoleic acids predominated in fatty acid composition. An increase in temperature led to the increase oleic acid percentage (Watson *et al.* 1976, Moat *et al.* 2002, Rossi *et al.* 2009). Increased concentrations of unsaturated fatty acids are also reported from psychrophilic yeasts, viz. *Candida, Leucosporidium* and *Torulopsis* (Kerekes and Nagy 1980) and fungus *Microdochium nivale* (Istokovics *et al.* 1998).

Rossi *et al.* (2009) observed that the psychrophilic strains comparatively produced more PUFA than the mesophiles. In the present study, when the mean percent concentrations of fatty acids in psychrophiles grown at different temperatures were compared, it was observed that while the PUFA concentration increased with decrease in temperature and the concentration of monounsaturated fatty acids (MUFA) was observed to decrease. The results agree with the observations made by Rossi *et al.* (2009). While studying the fatty acid composition of psychrophilic yeasts from the puddles in vicinity of Midre Lovénbreen glacier, Pathan *et al.* (2010) did not measure the percent concentration of linolenic acid (C18:3); the concentration of linoleic acid (C18:2) alone was higher than the MUFA or saturated fatty acid values obtained during the study. The present study reveals that linoleic acid (C18:2) values alone are higher than MUFA or saturated fatty acids (SFA) at low temperature.

The analysis on the fatty acid profile further reveals that decrease in temperature increases the concentration of total unsaturated fatty acids. The major fatty acids found were linoleic acid (C18:2n6c) and linolenic acid (C18:3n3),

contributing to the prevalence of PUFA. Oleic acid (C18:1n9c) was the most abundant MUFA in all the samples, while palmitic acid (C16:0) was the major SFA. These fatty acids possibly help the microbial strains to survive in glacial cold environment.

Biotechnologically PUFA production by microbes finds potential application as 'cell factories' in the production of these metabolites on a larger scale. Presence of oleic acid in abundance in the cell is related to tolerance for high concentration of ethanol was record by You *et al.* (2003). The yeasts strains found in this study fermented typical sugars as well as high concentration of oleic acid indicating the potential for bio-ethanol production at low temperatures. According to Nicholas (2002), PUFA producing organisms provide an opportunity for the study of genes and enzymes responsible for the fatty acid production.

Conclusion

Mycological investigation from cryoconites of AB, ML, VB glaciers, and yeasts from Ice cores of ML glacier were done for the first time. A novel species *Rhodotorula svalbardensis* sp.nov has been delineated.

4. OBJECTIVE 2: *SCREENING AND CHARACTERIZATION OF A FEW SELECTED ORGANISMS FOR THEIR BIOTECHNOLOGICAL POTENTIALS.*

4.1 Enzyme potentials of Bacteria, yeasts and filamentous fungi

4.1.1 Introduction

No concrete and systematic studies on the cold-active enzymes from the polar regions have been conducted so far (Medigue *et al*. 2005, Männistö and Häggblom 2006, Bej and Mojib 2010) despite their promising applications in the fields of health, agriculture and industry (Feller and Gerday 2003; Gawas-Sakhalkar and Singh 2011, Singh *et al*. 2011, Gawas-Sakhalkar *et al*. 2012, Singh *et al*. 2012a, Singh *et al*. 2013). Cold-active amylase production from the cold-adapted microbial isolates was reported from an actinomycete, *Nocardiopsis* sp. and fungus *Thelebolus microsporus* (Zhang and Zeng 2007, Singh *et al*. 2012b). These amylase enzymes can be used as detergent additive, in textile processing and in food industry (Männistö and Häggblom 2006, Margesin *et al*. 2007, Zhang and Zeng 2007, Bej and Mojib 2010). Degradation of cellulose and urea by these polar isolates at low temperatures demonstrates their immense biotechnological importance. Several reports on extracellular enzyme activities produced by Arctic bacteria were already chronicled by several investigators (Reddy *et al*. 2009, Srinivas *et al*. 2009 and Yu *et al*. 2009). While Srinivas *et al*. (2009) evaluated the effect of varying temperatures on the amylase and lipase activities, the effect of the same environmental parameter was studied for protease by Reddy *et al*. (2009). Yu *et al*. (2009) reported an extensive screening of bacterial isolates for production of protease, lipase, amylase, β-galactosidase and chitinase. These studies however were focused on sediment and sea ice bacteria.

In recent years, studies on psychrophilic yeasts from glacier habitats have been a gap area of research for the opportunities for biotechnology in Polar Regions (Cavicchioli *et al*. 2002, Buzzini *et al*. 2012). Glacier yeasts has been reported for degradation of phenol (Margesin *et al*. 2007) while Buzzini *et al*. (2005) found them to produce organic macromolecules at low temperatures. Strains of the genus *Rhodotorula* known to produce enzymes with

biotechnological potential (Cavicchioli *et al.* 2002, Shivaji *et al.* 2008) and also have the ability to degrade phenolic compounds (Sampaio 1999; Fell *et al.* 2000, Bergauer *et al.* 2005). Psychrophilic yeasts are reported to exhibit extracellular enzymatic activities (Brizzio *et al.* 2007, De García *et al.* 2007, Turchetti *et al.* 2008).

The present investigation has been the first ever enzymatic study on microbes of Arctic cryoconites, ice cores, and permafrost and is therefore has significance in terms of finding a potential for the future.

4.1.2 Materials and methods

4.1.2.1 Screening for enzymatic activities

Bacterial amylase activity was determined on agar plates containing (per litre) 5 g soluble starch, 1 g peptone and 1 g yeast extract. After 2 weeks of incubation starch hydrolysis was determined through iodine staining (Smibert and Krieg 1994). Cellulase activity was examined on media containing (per litre) 5 g carboxymethyl cellulose, 1 g peptone and 1 g yeast extract. After 2 weeks of incubation, the plates were treated with Congo red and NaOH as described by Teather and Wood (1982). Protease activity was determined on plates containing (per litre) 24 g of casein, 12 g of peptone, 12 g of yeast extract and 9 g agar-agar powder. Zone of clearance around the colonies indicated protease activity. Lipase was determined on media containing (per litre) 10ml Tween 80, 1 g peptone, 1 g yeast extract and 1 g $CaCl_2$. Formation of opaque halo around the colonies indicated lipase activity (Smibert and Krieg 1994). Urease activity was tested on urea agar base (M112, Himedia) plate and YNBG broth (6.7 g l^{-1} Yeast Nitrogen Base, 20 g l^{-1} glucose), containing 1g l^{-1} urea (pH 5.5) and change in colour of the media from orange to pink indicated positive activity (Pathan *et al.* 2010).

Fungal enzymatic activity for lipase (Tween-80), protease (Casein), amylase (starch), catalase and cellulase were also performed according to established procedures (Hankin and Anagnostakis 1975; Buzzini and Martini 2002, Buzzini *et al.* 2005). One loopful of yeast (~100 cells/ml) and filamentous fungi (106

spores/ml) culture grown for 10-15 days were used to inoculate the surface of enzyme-media-plates. Enzymatic activity was tested after 10 days of incubation at 4, 10 and 20 ^{o}C. Activity zone around the culture indicated positive activity while no zone suggested negative activity for the enzymes tested. Urease activity was tested on YNBG (6.7 g/l Yeast Nitrogen Base, 20 g/l glucose), containing 1 g/l urea solution (pH 5.5). Change in color from orange to pink was considered positive (Kurtzman *et al.* 2011).

Lipase activity

Cryptococcus sp. MLB-24 (10 µl) was inoculated in the lipase production medium (0.2% KH_2PO_4, 0.29% Na_2PO4, 0.02% NH_4Cl, 0.04% $CaCl_2$, 0.001% $FeCl_3$, 0.5% yeast extract, 1% Tween 80). MLB-24 was cultivated for 2 weeks at 10 ^{o}C at 120 rpm. After 2 weeks, cultures were centrifuged at 15,000 rpm for 10 min at 4^{o}C and tested for lipase activity.

Using the colorimetric method, lipase activity was measured keeping the p-nitrophenyl-palmitate as a substrate. 40 ml of 50 mM sodium phosphate buffer (pH 7.0) that contained 50 mg gum arabic and 0.2 g TritonX-100 was mixed with 3 ml 2-propanol containing 1 mM p-nitrophenyl-palmitate. 800 µl of prepared substrate was added to 200 µl of crude solution. The enzyme reaction was allowed to occur at 30^{o}C for 30 min. The release of p-nitrophenol was measured at A_{410}. One unit lipase activity was defined as the activity required for releasing 1 µmol of free fatty acids per minute at 30^{o}C. Optimum pH was measured using different 50 mM buffers: sodium citrate (pH 3.0–5.0), sodium phosphate (pH 6.0–8.0), glycine-NaOH (pH 9.0) and sodium carbonate (pH 10.0) at 30 ^{o}C for 30 min. Optimum temperature of enzyme activity was measured in 50 mM sodium phosphate buffer pH 7.0 for 30 min.

4.1.3 Results

4.1.3.1 Enzymes of Cryoconites bacteria

Extensive screening of the cultures for enzyme production indicated that all the 21 cultures studied were catalase positive, whereas 16 of the isolates were positive for amylase, 13 for cellulase, 12 for lipase, 11 for urease and 6 for protease activities (Fig. 34, Table 30). The study revealed that temperature influenced enzyme production. Enzyme activity of amylase and urease increased with increase in temperature from 4 to 20°C whereas in case of cellulase, enzyme production declined with increase in temperature. However, for enzymes (lipase and protease), the activity varied. MLB-32 was found to be the most promising culture as it produced all the enzymes tested while 6 other cultures (MLB 45, MLB 41, NA-b, MB-g, MLB 33, MLB 46) were able to produce 5 of the different enzymes tested. Five cultures (MLB 42, MLB 40, MLB 47, MLB 29 and MLB 33) were more specific enzyme producers and each produced only two of the enzymes tested.

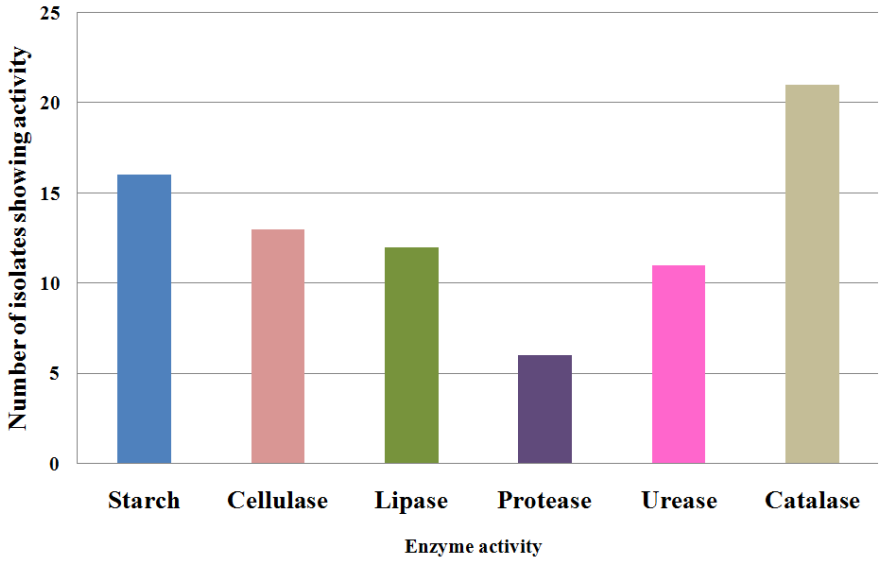


Fig 34. Cryoconites isolates showing enzyme activity

Table 30 Screening of enzyme activity of cryoconite bacteria

Cryoconite Bacteria	Amylase			Cellulase			Lipase			Protease			Urease			Catalase
	4^0C	10^0C	20^0C	4^0C	10^0C	20^0C	4^0C	10^0C	20^0C	4^0C	10^0C	20^0C	4^0C	10^0C	20^0C	4^0C
MLB-42	w	+	+	-	-	-	-	-	-	-	-	-	-	-	-	++
MLB-40	-	-	-	-	-	w	-	-	-	-	-	-	-	-	-	++
MLB-30	-	-	-	w	-	-	-	-	-	-	-	-	-	+	+	+
MLB-47	-	-	-	w	w	-	-	-	-	-	-	-	-	-	-	++
NA-9	-	+	w	w	-	-	w	w	+	-	-	-	-	-	-	+
MLB-34	-	w	-	w	w	-	-	-	-	-	-	-	-	-	-	++
MLB-44	-	-	-	-	-	w	-	w	++	-	-	-	-	-	+	w
MLB-45	-	-	+	-	-	-	+	++	+	-	w	w	-	-	+	+
MLB-36	+	++	-	-	-	-	-	-	-	-	-	-	w	-	w	++
MLB-41	w	++	++	w	w	-	-	-	-	-	-	++	-	w	+	++
MLB-35	-	-	w	-	-	-	+	+	+	-	-	-	-	-	+	+
MLB-38	-	-	+	-	-	w	+	+	-	-	-	-	-	-	-	+
MLB-39	++	+	-	-	-	w	+	+	-	-	-	-	-	-	-	+
NA-b	-	w	+	w	w	w	-	-	w	-	-	-	w	-	+	++
MLB-29	-	-	w	-	-	-	-	-	-	-	-	-	-	-	-	+
MLB-43	w	-	-	-	-	-	-	-	+	-	-	-	++	++	++	+
MB-g	-	-	w	-	w	-	+	+	+	-	+		-	-	-	++
MLB-32	++	++	++	+	+	-	+	++	-	++	++	-	-	w	w	+
MLB-33	w	+	-	w	-	-	-	++	-	-	-	-	-	+	w	w
MLB-33	-	-	-	-	-	-	-	-	-	+	+	+	-	-	-	+
MLB-46	-	-	+	-	-	-	-	++	+	w	-	-	-	w	-	+

++, Strong positive; +, Positive; W, Weak; -, Negative.
[Halo zone diameter (1-1.5cm) = + , (1.6-2.0 cm and above) = ++]

4.1.3.2 Enzymes of Ice core bacteria

Further the Ice core cultures were tested for enzyme activities such as amylase, cellulase, lipase, protease, urease and catalase at various temperatures (Table 31). While all the 14 cultures were catalase and protease positive, 13 of the isolates were positive for urease, 12 for lipase, 11 for cellulase and 10 for amylase (Fig. 35). In case of urease, enzyme production increased with increase in temperature from 4 to 20°C. For the rest of the enzymes the activity varied with variation in temperature. Isolates MLB-17, MLB-8, MLB-4, MLB-5, MLB-12, MLB-15 and MLB-16 were capable of producing all the six enzymes tested while isolate MLB-7 was more specific and produced only 2 (protease and catalase) of the enzymes tested. Rest of the cultures could each produce 5 of the different enzymes tested.

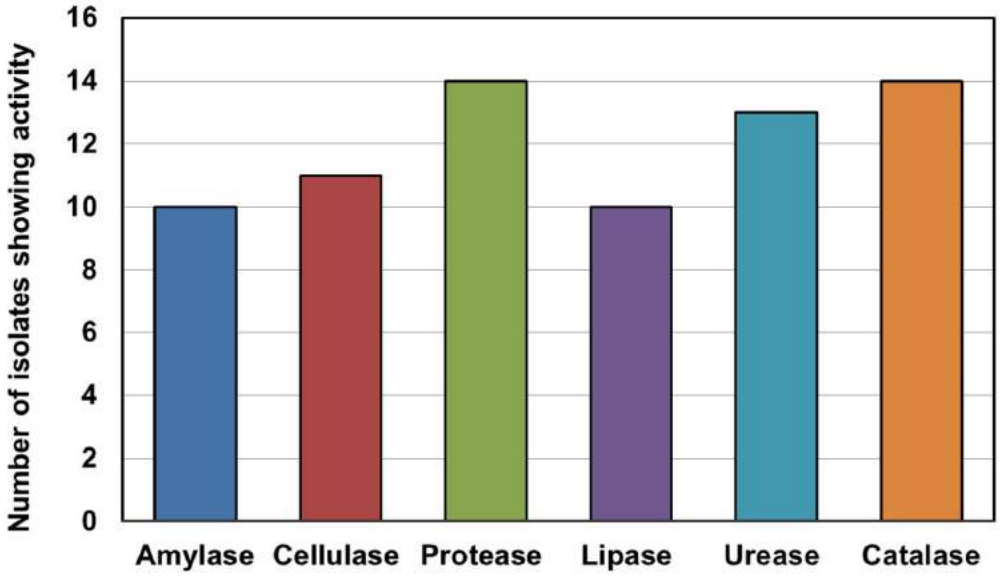


Fig. 35 Number of ice core isolates showing enzyme ability.

Table 31. Screening of extracellular enzyme activity of Ice-core bacterial isolates.

Ice core bacteria	Amylase			Cellulase			Protease			Lipase			Urease			Catalase		
	4^0C	10^0C	20^0C	4^0C	10^0C	20^0C	4^0C	10^0C	20^0C	4^0C	10^0C	20^0C	4^0C	10^0C	20^0C	4^0C	10^0C	20^0C
MLB-2	-	-	-	-	+	+	++	W	+	+	++	+	-	+	++	+	+	++
MLB-3	-	-	-	-	+	+	++	+	+	+	++	++	-	+	++	+	+	+
MLB-4	+	W	W	-	++	-	++	+	++	+	++	++	-	+	++	+	++	++
MLB-5	+	-	+	-	++	-	++	+	++	+	++	+	-	W	+	++	++	++
MLB-6	-	-	+	-	-	-	++	+	++	+	+	+	W	++	++	+	++	++
MLB-7	-	-	-	-	-	-	+	+	+	-	-	-	-	-	-	W	+	+
MLB-8	-	-	W	-	+	-	++	+	+	+	+	+	-	+	++	+	+	+
MLB-9	W	-	++	-	++	+	W	W	-	-	-	-	-	-	+	+	+	++
MLB-11	-	-	-	-	+	+	++	+	+	+	++	++	-	W	++	++	++	+
MLB-12	-	-	+	-	++	+	++	+	+	+	++	+	-	W	++	+	+	+
MLB-14	+	+	-	-	-	-	+	++	++	+	++	++	+	++	++	+	+	+
MLB-15	+	-	+	-	+	-	++	+	+	+	++	+	W	W	++	++	W	W
MLB-16	-	-	+	-	++	-	++	+	+	+	++	+	W	-	++	+	+	W
MLB-17	-	-	W	-	+	+	++	+	+	++	+	+	W	+	++	++	+	+

++, Strong positive; +, Positive; W, Weak; -, Negative.

[Halo zone diameter (1-1.5cm) = + , (1.6-2.0 cm and above) = ++]

4.1.3.3 Enzymes of Permafrost bacteria

The permafrost bacterial cultures were tested for enzyme activities such as amylase, cellulase, lipase, protease and urease at 15°C (Table 32). While most cultures (32) showed urease activity, 17 were protease positive, 14 were lipase positive and 6 were cellulase positive. Amylase activity was observed in only 3 of the isolates (PF1T1, PF2B4, PF3B5) Fig 36.

Pseudomonas mandelii strain PF3M2 and *Pseudomonas* sp. strain PF2M7 showed similar enzyme activity profile for lipase, urease and protease. Most isolates of *Pseudomonas* sp. strain PF3T5, PF3T4, PF3T2, PF5T1 and PF1B3 exhibited only urease activity. *Bacillus* sp. strain PF2B4 produced most of the enzymes tested (lipase, urease, amylase and protease).

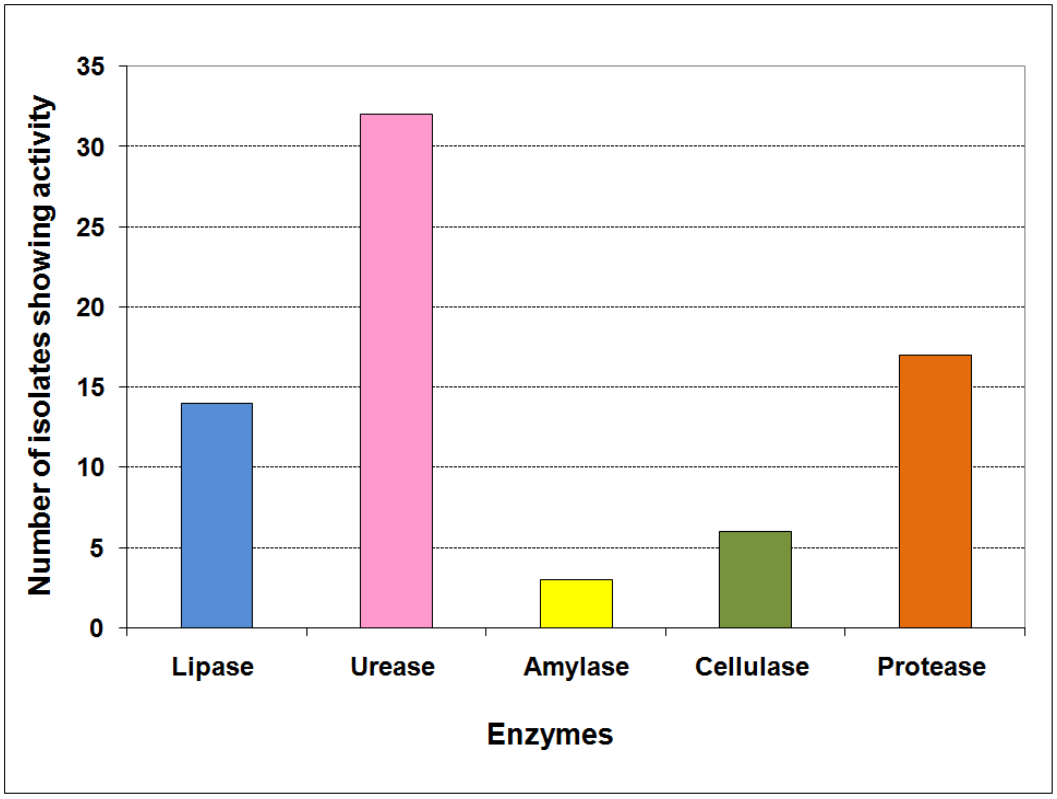


Figure 36. Number of permafrost isolates showing enzyme ability.

Table 32. Enzyme production by the isolated strains.

Identification	Strain no.	Amylase	Cellulase	Lipase	Protease	Urease
Acinetobacter johnsonii	PF1B1	-	-	+	-	+
Arthrobacter bergerei	PF1T1	+	-	-	+	+
Arthrobacter sp.	PF3B3	-	-	+	+	++
Arthrobacter sp.	PF3B2	-	-	-	-	++
Arthrobacter sp.	PF2M1	-	+	+	-	++
Arthrobacter sp.	PF3B4	-	-	-	+	-
Arthrobacter sp.	PF4B2	-	-	+	+	++
Arthrobacter sp.	PF2M3	-	-	-	-	+
Arthrobacter sp.	PF4M4	-	+	-	+	++
Arthrobacter sp.	PF1T4	-	+	-	-	++
Arthrobacter sp.	PF4T1	-	-	+	+	++
Arthrobacter sp.	PF4T2	-	-	-	+	+
Arthrobacter sp.	PF5B1	-	-	-	+	++
Arthrobacter sp.	PF5M1	-	+	-	-	+
Arthrobacter sulphonivorans	PF1T3	-	-	-	-	++
Arthrobacter sp.	PF3T1	-	+	+	-	++
Arthrobacter sp.	PF4M3	-	-	-	-	++
Bacillus sp.	PF2B4	+	-	+	+	++
Nesterenkonia sp.	PF2B19	+	-	-	-	+
Pseudomonas jessenii	PF2M8	-	-	+	+	++
Pseudomonas mandelii	PF2M12	-	-	-	+	+
Pseudomonas sp.	PF2M7	-	-	+	+	++
Pseudomonas mandelii	PF3M2	-	-	+	+	++
Pseudomonas sp.	PF1B3	-	-	-	-	++
Pseudomonas sp.	PF3B5	+	-	-	+	+
Pseudomonas sp.	PF2M9	-	-	+	+	++
Pseudomonas sp.	PF3T4	-	-	-	-	+
Pseudomonas sp.	PF3T5	-	-	-	-	+
Pseudomonas sp.	PF3T2	-	-	-	-	++
Pseudomonas sp.	PF5T1	-	-	-	-	+
Psychrobacter sp.	PF5B2	-	-	+	-	+
Sphingobacterium sp.	PF3T6	-	-	+	+	++
Stenotrophomonas sp.	PF1M2	-	+	+	+	++
Virgibacillus pantothenticus	PF1B6	-	-	-	-	++

4.1.3.4 Enzymatic activities from Yeast and filamentous fungi

i) Cryoconites Yeasts and filamentous fungi of Midre Love´nbreen glacier

The extracellular enzymatic activity of the strains is listed in Table 33. Almost all tested strains showed at least one extracellular enzyme activity. Lipase activity (hydrolysis of tributyrin/Tween-80) was the most widely expressed extracellular enzyme among the cultures tested. Almost all the strains showed the ability to hydrolyse at least one of the two lipophilic substrates, regardless of the temperature at which it was tested. *Mrakia* sp. (CCP-IIIWY) was the only isolate that exhibited phosphate solubilizing ability at 4 and 15^{o}C. The same strain expressed pectinase activity while cellulase activity was recorded only for *Phialophora alba* (CCP-I), at 15^{o}C.

Results obtained from the preliminary screening experiments of *Rhodotorula svalbardensis* sp.nov (MLB-1) for production of extracellular enzymes such as amylase, cellulase, lipase, protease, catalase and urease have been presented in Table 33. *Rhodotorula* sp. (MLB-1) expressed good amylase activity at 4 and 15^{o}C. All yeast strains expressed catalase activity at 10^{o}C. The isolate MLB-I exhibited a range of enzyme activities. It showed good amylase activity at all temperatures. With the increase of temperature, decrease in cellulase activity and increase in lipase activity was observed. Protease produced by the culture exhibited activity at 15°C. The influence of further rise in temperature on its activity was not studied. No activity was observed at low temperature (4°C). The strain exhibited good lipase activity at 20°C. The pychrophilic yeast, which grew at a temperature between 1 and 20°C too have good lipase activity at 20°C which is unusual. However, considering the adaptation and sustenance point of view, it is important that the isolate retains its enzyme activity at slightly higher temperatures.

Table 33 Enzyme activity of Cryoconite fungal isolates from Midre Love´nbreen glacier

Culture Code	Amylase			Cellulase			Lipase			Protease			Phosphate			Urease			Catalase		
	4°C	15°C	20°C	4°C	15°C	20°C	4°C	15°C	20°C	4°C	15°C	20°C	4°C	15°C	20°C	4°C	15°C	20°C	4°C	15°C	20°C
CCP-I	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+
CCP-V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	+	+
CCP-IWY	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	+	-	+	+	+
CCP-IOY	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	+	+	+	+	+
MLB-1	++	++	+	++	+	+	+	+	++	-	++	ND	-	-	-	-	+	-	+	+	+

Table 34 Enzyme activity of Cryoconite fungal isolates from Austre & Vestre Broggerbreen glaciers.

Culture Code	Identification of strains	Protease				Lipase				Cellulase				Amylase				Urease			
		1°C	4°C	10°C	20°C	1°C	4°C	10°C	20°C	1°C	4°C	10°C	20°C	1°C	4°C	10°C	20°C	1°C	4°C	10°C	20°C
Cry-FB1	*Articulospora* sp.	+	+	++	++	-	+	w	+	+	+	++	-	+	+	+	++	-	+	+	-
Cry-FB2	*Articulospora* sp.	+	+	++	++	+	++	+	+	+	+	++	+	+	+	-	++	-	+	+	-
Cry-FB3	*Rhodotorula* sp.	+	+	+	w	+	+	++	+	+	+	++*	-	+	+	+	++	-	w	+	-
Cry-YB 240	*Thelebolus* sp.	+	-	++	w	-	+	-	+	-	-	-	-	+	+	-	-	-	+	-	+
Cry- YB 241	*Thelebolus* sp.	-	-	-	w	-	-	-	-	-	-	+	-	+	+	-	+	-	++*	++	-

++, Strong positive; +, Positive; W, weak; -, Negative. [Halo zone size (1mm-15 mm) = + , (16-20 mm & above) = ++]

ii) Enzyme actvity of isolates from Austre and Vestre Broggerbreen glaciers

Most of the fungi isolated from the Arctic Broggerbreen glacier cryoconites were capable of growing between 1 and 25 ˚C, indicating that cryoconite fungi are cryophilic in nature. Isolates showed salt tolerance ranging from 1-7% NaCl. It was worth investigating the potential of *Articulospora* sp, *Rhodotorula* sp. and *Thelobolus* sp. in terms of enzyme production (Fig. 37a-e). The filamentous fungus like *Articulospora* sp., expressed high amylase, cellulase, protease activities whereas yeast *Rhodotorula* sp. showed high amylase, cellulase and lipase activity. *Thelebolus* sp. showed protease and urease activities as shown in Table 34.

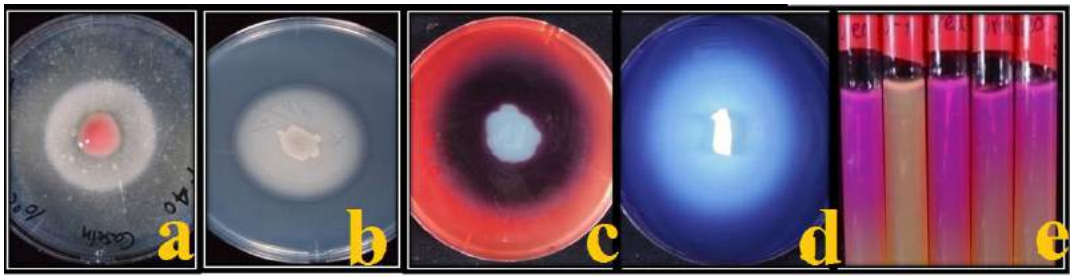


Fig. 37(a-e) Plate assay of various enzymes a) protease activity b) lipase activity c) cellulase activity d) amylase activity e) urease activity.

iii) **Screening of enzymes from Ice cores Yeasts:**

Results of the preliminary screening experiments for production of various extracellular enzymes indicate that out of six enzymes (amylase, cellulase, lipase, protease, catalase, and urease) studied the isolates exhibited significant activity for cellulase, protease and urease (Table 35). All the 10 isolates screened exhibited cellulase activity at all the temperatures tested. Protease activity was not observed at 4 ^{o}C while all the cultures showed positive activity at 10 and 20 ^{o}C. All 10 isolates produced urease at 4 and 10 ^{o}C, however at 20 ^{o}C only 6 isolates were tested urease positive. Catalase activity was also observed in all isolates while lipase activity was observed in nine of the cultures tested. Amylase was

produced in eight of the cultures at 4 ^{o}C while with increase in temperature the number of isolates possessing amylase activity decreased.

Table 35. Screening of extracellular enzyme activity of ice-core yeast isolates.

Ice Core Yeast	Amylase			Cellulase			Protease			Lipase			Urease			Catalase		
	4°C	10°C	20°C	4°C	10°C	20°C	4°C	10°C	20°C	4°C	10°C	20°C	4°C	10°C	20°C	4°C	10°C	20°C
MLB-21	+	ND	-	++	++	++	-	++	++	w	-	-	+	++	-	+	+	+
MLB-22	+	w	-	+	+	++	-	++	+	w	w	++	+	+	++	+	+	++
MLB-23	+	ND	-	++	+	+	-	+	++	w	+	++	+	+	+	++	++	+
MLB-26	+	+	-	++	+	++	-	++	++	+	+	++	++	++	-	+	+	++
MLB-27	-	ND	-	++	w	++	-	++	++	+	w	++	++	++	++	++	+	+
MLB-18	+	ND	-	+	++	++	-	++	++	w	w	-	+	+	++	+	+	+
MLB-19	+	+	-	++	++	++	-	++	++	w	w	++	+	+	++	+	+	+
MLB-20	-	-	-	w	++	++	-	++	++	-	+	-	++	++	++	++	+	++
MLB-24	w	ND	w	w	++	++	-	++	++	-	++	ND	+	+	ND	-	ND	ND
MLB-25	+	-	-	+	++	++	-	++	++	-	+	++	++	+	-	+	+	+

++, strong positive; +, positive; w, weak; - negative, ND not detected

[Halo zone diameter (1-15 mm) = + , (16-20 mm & above) = ++]

iv) Lipase activity

Lipase was extracted from *Cryptococcus* sp. MLB-24 by growing it on lipase production medium. The enzyme was found to show optimal activity at 40 ^{o}C. Further rise in temperature caused a decrease in activity. At 60 ^{o}C the enzyme possessed no activity. Similarly, the enzyme showed 80% activity at pH 6.0 and maximum (100%) activity at pH 7.0 beyond which the activity plummeted (Fig. 38).

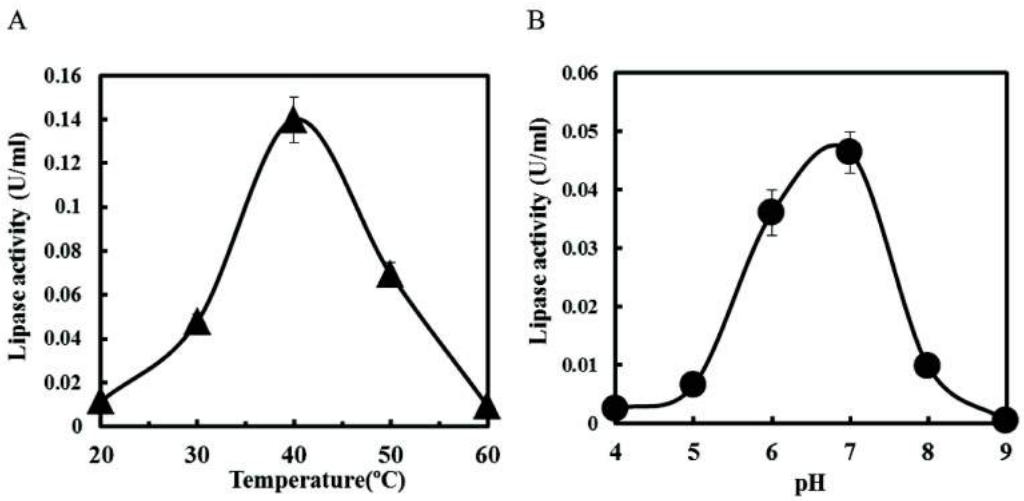


Fig. 38 Lipase activity of MLB-24 determined by using p-nitrophenyl-palmitate as substrate. (A) Effect of temperature on activity, and (B) effect of pH on activity.

4.1.4 Discussion

The ability to produce cold active enzymes renders the cryophilic microorganisms potential candidates for use in biotechnology, agriculture and medicine (Feller and Gerday 2003). All the 21 representative strains pertaining to the Cryoconites bacteria tested showed activities of one or more enzymes (amylase, cellulase, lipase, urease, protease, or catalase) either at 4 °C, 10 °C and (or) 20 °C (Table 30). The ability of the cryoconite bacterial strains, to produce different enzymes indicates their ability to utilize the available organic matter, within the micro-environment, as source of carbon and nitrogen for their subsistence and growth. Earlier reports have also stated the utilization carbohydrates and polyols by bacteria in Arctic ocean (Sala *et al.* 2008, 2010) and Antarctic environments (Sala *et al.* 2005). Cellulose and urea degradation potential by these isolates at low temperatures were also reported to indicate their biotechnological potential. Plethora of reports published by Yu *et al.* (2009), Reddy *et al.* (2009) and Srinivas *et al.* (2009) on extracellular enzyme activities by Arctic bacteria is available;

Srinivas *et al.* (2009) evaluated the amylase and lipase activities at various temperatures; Reddy *et al.* (2009) also demonstrate protease activity from Arctic bacterial strain. Yu *et al.* (2009) reported extensive screening of the bacterial isolates for production of protease, lipase, amylase, β- galactosidase and chitinase. The present study of screening cellulase, catalase and urease from cryoconite bacterial isolates has not been reported earlier.

All the 14 representative strains of Ice core bacteria tested also showed activities of two or more enzymes (amylase, cellulase, lipase, urease, protease, or catalase) either at 4 °C, 10 °C and 20 °C.

All the 33 representative strains of Permafrost bacteria tested showed enzyme activities. The ability of the bacterial strains, to produce different enzymes signifies their potential to consume the available organic matter, within this permafrost environment. Urea and protein degradation by these isolates at low temperatures demonstrate their biotechnological potential. Enzyme activities by bacteria from sea, fjords and glacier stream habitats were screened for amylase, lipase, protease, β-galactosidase and chitinase (Reddy *et al.* 2009, Srinivas *et al.* 2009, Yu *et al.* 2009, Singh *et al.* 2013a) in earlier studies, however there was no such study on permafrost bacteria of Svalbard. Microbial metabolic process in the frozen grounds can bring forth biochemical transformation that can tremendously impact on the geochemistry of the cryolithozone (Panikov 2009) and thus bears significance. Psychrophilic enzymes play an important role in the molecular adaptation to cold condition (Gerday *et al.* 1999, Lonhienne *et al.* 2000, Feller 2003, Feller and Gerday 2003). The ability to thrive under cold conditions and to maintain catabolic and anabolic processes microorganism depends on enzymes that are cold-active. The exoenzymes reported from these environments show low temperature optima close to environmental conditions in Antarctic soils (Pietr *et al.* 1983, Feller *et al.* 1994). There are also various interactions at the sub-cellular level, where certain enzymes act as inducers for chains of further processes, which are related to precursors of following chain reactions (Orange 1994, Booth 1999). Most of these physiological reactions are energy consuming and possibly

cold active enzymes/exoenzymes produced by these bacteria help them in cold adaptation in permafrost environment. Thus cold-active enzymes play an important role in survival of bacteria in oligotrophic permafrost environment.

Though cold-active enzymes such as amylases, catalases, cellulases, invertase, lactase, lipases, pectinases and proteases produced by Arctic fungal strains find potential applications in food, medicine and detergent industries, as of now only catalase (Fiedurek *et al.* 2003) and invertase (Skowronek *et al.* 2003) have been recorded from Spitsbergen fungi. Extreme habitats as cryoconites holes are, therefore rather conspicuous for further exploration to understand the molecular adaptations of these microbes and their biotechnological potentials.

Psychrophilic yeasts could grow at low and subzero temperatures as reported ~~by~~ previously by Panikov and Sizova (2007). These environmental conditions are generally considered to simulate oligotrophic conditions. From the present Ice core study it has been observed that the cryophilic yeasts possess the ability to produce extracellular enzymes by dint of which they are able to utilize traces of available nutrients which enable their survival and coexistence of other microbial communities persisting in the ice. These organisms therefore play a role of paramount importance in nutrient cycling in the ice and glacial habitats (Welander 2005). Yeast isolates exhibited a wide range of enzyme activities, *viz*, amylase, cellulase, lipase, protease, catalase and urease activities. It was observed that several of the enzymes showed good activity at low temperatures. This is one of the most well-understood adaptation strategies amongst psychrophiles (Gerday *et al.* 1997). In case of protease and lipase it was observed that the activity of the enzymes is higher at 20 ^{o}C as compared to 4 ^{o}C. This might be a certain case~~s~~ of adaptation and sustenance strategy wherein the isolates prefer to retain its enzyme activity at slightly higher temperatures.

Lipase secretion was studied in many microbes using the substrate Tween 80 (Li *et al.* 2001, Taoka *et al.* 2011). Lipase from psychrophilic yeasts was studied previously (Pathan *et al.* 2010, Brandão *et al.* 2011), however their studies were limited to qualitative screening on solid medium. Only a strain of psychrophilic yeast *Candida antarctica* (Rotticci-Mulder *et al.* 2001) was screened in liquid

media. In the present study, the isolate MLB-24 was subjected to solid medium and liquid medium lipase activity screening and characterization. The isolate showed an optimum activity at pH 7.0 and temperature 40 ^{o}C. The neutral pH activity of the enzyme renders it a potential source to be used in detergent industries and in the waste treatment of edible oil industries. Cold adapted organisms in general are thought to operate at low temperatures, however quiet a number of studies report high temperature enzyme activities by these microbes. An isolate of *Rhodotorula glutinis* was reported previously to have an optimum pH of 7.5 and temperature 35 ^{o}C (Papaparaskevas *et al.* 1992). In another study, an Alaskan psychrotolerant bacterium species of *Pseudomonas* showed a pH optima of eight and a temperature optima of 45 ^{o}C (Choo *et al.* 1998). Ohgiya *et al.* (1999) described several cold-adapted enzymes that got activated at high temperatures. An Antarctic isolate *Bacillus* sp. from Antarctica showed β-galactosidase activity at 40 ^{o}C while *Stenotrophomonas maltophila* and *Pseudoalteromonas* sp. exhibited protease activity up to 60 ^{o}C (Dhaked *et al.* 2005, Vàzquez *et al.* 2008, Vàzquez *et al.* 2005). Proffer *et al.* (2006) reported a thermotolerant lipase A from *C. antarctica* showing an optimum activity at 50 ^{o}C and at pH 7.0. In the present study similar results were observed with strain MLB-24 that produced lipase which showed the optimum activity at 40 ^{o}C. Recently optimum phosphatase activity at high temperature (60 ^{o}C) was reported from cold-tolerant Arctic strain of *Penicillium citrinum* (Gawas-Sakhalkar *et al.* 2012).

4.1.5 Conclusion

The present study is the first enzymatic study on microbes of Arctic cryoconites, ice cores, and permafrost and is therefore of significance. The ability of producing cold-active enzymes by cryoconite bacteria makes these microorganisms potential candidates for use in biotechnology, and medicine. All the representative bacterial strains tested showed activities of one or many enzymes between 4 and 20 °C.

Cellulose and urea degradation by these isolates at low temperatures demonstrates their biotechnological potential.

All the representative strains of ice core bacteria tested showed activities of two or more enzymes (amylase, cellulase, lipase, urease, protease, or catalase) either at 4 °C, 10 °C and (or) 20 °C. In case of urease, enzyme production increased with increase in temperature from 4 to 20°C to signify that temperature played an important role in enzyme production. From the present study it can be observed that the cryophilic yeasts possess the ability to secrete extracellular enzymes by virtue of which they are able to utilize traces of available nutrients which helps in their survival and in the survival of other microbial communities persisting in the ice.

All the representative permafrost strains tested showed enzyme activities. The ability of the bacterial strains, to produce different enzymes signifies their inherent ability to consume the available organic matter, within this permafrost environment. Urea and protein degradation by these isolates at low temperatures demonstrate their biotechnological potential. Metabolic activity of bacteria in the frozen grounds can bring about biochemical transformation that has tremendous impact on the geochemistry of the cryolithozone. Psychrophilic enzymes play an important role in the molecular adaptation to cold. The ability to live under cold conditions and to maintain catabolic and anabolic processes depends on enzymes that are cold-active. There are also possibly various interactions at the sub-cellular level, where certain enzymes act as inducers for chains of further processes, which are related to precursors of other reaction chain. Most of these physiological reactions are energy consuming and cold active enzymes or exoenzymes produced by these bacteria possibly help them in cold adaptation process and survival in oliogotrophic permafrost environment.

4.2 Antimicrobial activity of Arctic Bacteria, yeast and filamentous fungi

4.2.1 Introduction

Extremophilic environment has been predicted as the goldmine for the antimicrobial producing organisms (Soll 2000, Lindermuth *et al*. 2001). The antimicrobial compounds such as plectasin and novozymes tested against a range of bacteria, including *Bacillus subtilis, Enterococcus faecalis, Micrococcus luteus, Streptococcus pneumoniae, Staphylococcus epidermidis* and *Staphylococcus aureus* (Hans-Henrik Kristensen and Debbie Y 2004). *Pseudomonas* species was demonstrated to synthesize an iron-binding compound-pyochelin that showed activity against several species of *Candida* sp. and *Aspergillus fumigatus* (Phoebe *et al*. 2001). Another novel cyclic hexadepsipsepeptide named 'pipalamycin" (Uchita *et al.* 2002) was isolated from a *Streptomyces* sp. ML 297-90F8 as an apoptosis- inducing agent. It showed antibacterial activity on gram-positive bacteria such *S. aureus* and *Micrococcus luteus.*

Increasing number of bacteria are developing resistance to commercial antibiotics (Paul Strames 2001); therefore, it essential to search more strong antimicrobial molecules which has good activity against multidrug-resistant human pathogens with least adverse-effects.

4.2.2 Materials and methods

4.2.2.1 Screening for Antimicrobial activity

Individual isolates were grown in modified trypticase Soy broth (pH 7.2± 0.2)for 24-48 hrs at 15°C. Cell free supernatant were obtained after 48 hrs by centrifuging at 12000 rpm at 30 minutes at 4°C, followed by 0.2 µm disposable syringe filtration (PALL, Acrodisc Syringe filters). The cell free supernatant (CFS) was stored at -80°C until use. The cell free supernatant was further concentrated by lyophilization using either Martin-Christ lyophilizer or stirred

cell. The concentrate was used for the cut well agar assay. 30 to 35 ml of molten BHI agar was added to a sterile petri plate. Wells were made on the dried plates using a sterile stainless steel well borer of diameter 8 mm. 100 µl cell free supernatant and concentrated cell free supernatants were added into different wells. The plates were kept for complete diffusion, and then 100 µl freshly grown (12-16 hours) culture e.g. *S. aureus, Pseudomonas aeruginosa, Staphyloccus pneumonieae, Staphyloccus pyogenes* and *Klebsiella pneumoniae* were spread uniformly across each plate. All the plates were incubated at 37^{o}C for 24 hours and were inspected for the zone of inhibition.

4.2.3 Result

Of the 25 isolates of Cryoconites, 14 of Ice cores, and 106 of permafrost, most of them failed to produce any antimicrobial substance that could inhibit the 11 strain/indicator test (*Escherichia coli* MTCC 723, *E. coli* MTCC 729, *E. coli* DH5α, *Staphyloccus aureus* MTCC 5021, *S. aureus* MTCC 737, *S .aureus* MTCC 5021, *Staphyloccus pyogenes, Pseudomonas aeruginosa* MTCC 428, *Pseudomonas aeruginosa* MTCC 2582, *Enterococcus faecalis* VRE and *Micrococcus luteus*). However, the *Pseudomonas* sp. MLB-16 isolate showed an 8 mm inhibition zone for *Staphyloccus aureus* MTCC 5021 and a 4 mm inhibition zone for *Escherichia coli* MTCC 723.

4.2.4 Discussion

Though there is a significant diversity among bacteria of cryoconites, ice-cores and permafrost, and they do not share all of the same biochemical and physiological properties. However, most of them showed negative activity against tested multidrug resistant pathogens. This indicates that Arctic is still a pristine environment and there is negligible anthropogenic influences.

4.2.5 Conclusion

In the present study it is evident that no isolates have significant antimicrobial activity.

4.3 Antifreeze proteins (AFPs) of Arctic Bacteria, yeast and filamentous fungi

4.3.1 Introduction

Antifreeze proteins (AFPs) are considered to be multifarious class of ice-binding proteins having wide structural diversity with the capability of inhibiting the growth of ice either by depressing the freezing point of the solution [thermal hysteresis (TH) activity] or by inhibiting the recrystallization (RI activity) of ice crystals (Knight *et al*. 1988, Duman and Olsen 1993, Jia and Davies 2002). By virtue of AFP formation, the bio-membranes of many cold-adapted bacteria remain protected from injuries caused by freezing, thereby increasing the survival ability of microbial entities trapped in glacier ice (Garnham *et al*. 2008, Raymond *et al*. 2008). Recrystallization inhibition activity might also indicate the conservation of boundaries between ice grains in glacier ice. Such boundaries constitute a network of liquid veins that has tendency to accumulate ions and nutrients and may act as a microbial habitat in deep Antarctic ice (Price 2000). Microorganisms that do not produce an AFP in such an environment could derive survival advantage from commensal relationships with bacterial species that produces AFPs in glacier ice (Walker *et al*. 2006).

Evolution of AFPs in microorganisms takes place through regular phases of low temperature and freezing transition. Indeed they furnish evidence of long-standing existence of microorganisms in the frozen glacier conditions. Ice-active proteins were reported to be produced by the organisms inhabiting the frozen land. The true production of such proteins by a microorganism would suggest supporting evidence that the AFPs are not contaminating particles or macromolecules from ancient ice (Inman 2007, Raymond *et al.* 2008).

AFPs for the first time were isolated from Antarctic teleost fishes (DeVries *et al*. 1970). Thereafter, a number of AFPs have been discovered. Umpteen number of AFPs were reported from a diverse group of permafrost-dwelling organisms including bacteria (Duman and Olsen 1993, Sun *et al.* 1995, Xu *et al.* 1998, Mills

1999, Kawahara *et al*. 2001, 2004, Raymond and Fritsen 2001,Yamashita *et al*. 2002, Gilbert *et al*. 2004, Walker *et al*. 2006, Wilson *et al*. 2006), diatoms (Janech *et al*. 2006, Bayer-Giraldi *et al*. 2010; 2011), fungi (Duman and Olsen 1993, Hoshino *et al*. 2003a, b, Lee *et al*. 2010, Xiao *et al*. 2010), plants (Urrutia *et al*. 1992, Worrall *et al*. 1998, Meyer *et al*. 1999, Smallwood *et al*. 1999, Sidebottom *et al*. 2000, Griffith and Yaish 2004, Griffith *et al*. 2005), insects (Duman *et al*. 1998, Duman 2001), and fish (Fletcher *et al*. 2001, Ewart and Hew 2002). The effects of AFPs are influenced by the biological source of isolation. For example, insect AFPs typically show considerable TH activity, whereby they reduce the freezing point of their body fluids to prevent the tissue from damage occurring as a result of freezing (Feeney *et al*. 1986, Cheng and DeVries 1991, Graham *et al*. 1997, Ewart *et al*. 1999, Duman 2001, Fletcher *et al*. 2001, Marshall *et al*. 2004). AFPs deriving from bacteria and plants are characterized by a plummeted TH activity. They deploy AFPs to thwart the ice recrystallization, lessening injuries produced by ice crystals (Duman and Olsen 1993, Sun *et al*. 1995, Xu *et al*. 1998, Yamashita *et al*. 2002, Griffith and Yaish 2004, Kawahara *et al*. 2004, Raymond *et al*. 2008).

AFPs potentially may find applications in the medical sector for cryopreservation of blood and organs, in the food sectors, or as hydrate inhibitors to avoid the clogging in oil and gas pipelines (Griffith and Ewart 1995, Chao *et al*. 1996, Amir *et al*. 2004, Gordienko *et al*. 2010).

These prior information pursuaded us to generate for more valuable information of Arctic bacterial AFPs; therefore the study was undertaken to determine the presence of AFPs in bacteria, and yeasts isolated from cryoconites and ice cores on Arctic glaciers and to characterize their activity.

4.3.2 Materials and methods

4.3.2.1 Screening for Antifreeze protein (AFPs) activity

One loopful of pure culture was inoculated into 50 ml autoclaved broth medium (Nutrient Broth for bacteria and potato dextrose broth (PDB) for yeast and filamentous fungi), and incubated at -1 ^{o}C for 1– 2 months (56 days) in static condition. 50µl of top layer of broth culture was centrifuged and 5µl of this broth was placed on a rounded thin glass and covered with similar identical small size cover slip. Subsequently the sandwiched culture broth between two glass coverslips was kept on the groove on the stage of the microscope and observed under 50x magnifying lens. Leica DMLB 100 photomicroscope (Leica Microsystems AG, Wetzlar, Germany) equipped with a Linkam LK600 temperature controller (Linkam, Surrey, UK) was used to examine the extracellular and intracellular antifreeze activity. The culture medium of the isolate was frozen (at about -25 ^{o}C) for a brief while and warmed to 0 ^{o}C on sample stage of the photomicroscope to create several ice crystal seeds in solution. This solution was cooled to approximately -1 to -5 ^{o}C and the growth of ice crystal seeds was monitored. By observing the shape of the ice crystals in terms of AFP, the positive and negative activity of the strains were determined. Hexagonal crystals indicated that ice crystal was AFP-positive while rounded type indicated negative AFP activity.

4.3.3 Result

i) AFPs activity of Cryoconites bacteria and yeast

Fourteen bacterial strains were used for primary screening of AFP activity (Table 36). Eight bacterial strains (Cry-l, Cry-c, Cry-21 and Cry-k belonging to genus *Cryobacterium*, Cry-g, Cry-n and Cry-2 to *Pseudomonas*, and Cry-8 to genus *Subtercola*) showed positive AFPs activity, while six bacterial strains (Cry-9, Cry-5 belonging to genus *Cryobacterium*, Cry-6, Cry-b to *Leifsonia*, and Cry-15,

Cry-16 to *Polaromonas*) showed negative AFP activity. The most dominant species showing AFP activity belonged to genus *Cryobacterium* (*C. psychrotolerans, C. psychrophilum*) followed by *Pseudomonas* (*P. ficuserectae*) and *Subtercola* (*S. frigoramans*) were recorded in glacier cryoconite holes of high Arctic. Similarly, the culture media of a yeast strain MLB-I (*Rhodotorula svalbardensis* sp.nov.) showed unique hexagonal ice crystal structures (Fig.28c) when incubated undisturbed for 2 months.

Table 36 Strains exhibiting antifreeze activity in culture medium are indicated by '+' and those that did not exhibit ice modification are indicated by '–'.

Isolate Code	**Sequence deposition no.**	**Species Identification/ 16S rRNA gene sequences closest similarity (%)**	**AFPs Activity**
Cry-g (MB-g)	JF790967	*Pseudomonas ficuserectae* (AB021378) by 96.9%	+
Cry- n (MLB-46)	JX205212	*P. ficuserectae* (AB021378) by 97.3%	+
Cry-2 (MLB42)	JX205209	*P. ficuserectae* (AB021378) by 96.9%	+
Cry-l (MLB-33)	JX205200	*Cryobacterium psychrophilum* (AM410676)by 95.7%	+
Cry-c (MLB-29)	JX205196	*Cryobacterium psychrotolerans* (JN637331) 98.2%	+
Cry-21 (MLB-37)	JX205204	*Cryobacterium psychrotolerans* 99%	+
Cry-k	AB872307	*Cryobacterium* sp. AsdMX-L1 (JX123060) by 99.0%	+
Cry-5 (MB-5)	JF790972	*Cryobacterium* sp.DR9 (FJ464984) by 98.3%	-
Cry-9 (NA-9)	JF790966	*Cryobacterium* sp. (FJ464984) by 98.0%	-
Cry-6 (MLB-40)	JX205207	*Leifsonia antarctica* (AM931710) by 98.4%	-
Cry-b (NA-b)	JF790973	*Leifsonia* sp. (GU213322) by 99.3%	-
Cry-15 (MLB-44)	JX205211	*Polaromonas naphthalenivorans* (AY166684) by 99.0%	-
Cry-16 (MLB-45)	JX205214	*Polaromonas naphthalenivorans* (AY166684) by 97.8%	-
Cry-8 (MLB-47)	JX205212	*Subtercola frigoramans* (AM410673) by 97.8%	+

ii) Ice cores bacteria and yeast

All the isolates of ice core bacteria and yeasts showed negative result for extracellular and intracellular antifreeze proteins (AFPs).

4.3.4 Discussion

As a result of investigation, fourteen bacterial strains were isolated from the glacier cryoconite hole sediments. Only eight strains (four to genus *Cryobacterium*, three to *Pseudomonas*, and one to genus *Subtercola*) showed positive AFP activity. Raymond *et al.* (2008) screened one dozen of ice core bacteria (*Subtercola* sp., *Sphingomonas* sp., a Flavobacteriaceae, and nine unidentified bacteria), but a solitary isolate demonstrated antifreeze activity, while others exhibited no activity. The present investigation revealed more isolates with AFP activity in Arctic than in Antarctic (Raymond *et al.* 2008) because of the dichotomization in ecological and biogeographical characteristics. The microorganisms that fail to produce AFPs could derive benefits from commensal relationships with species capable of producing AFPs in glacial ice (Raymond *et al.* 2008) and cold-adapted soil bacteria (Walker *et al.* 2006). Glacial cryoconite holes include ice and sediment which might suggest commensalism amongst bacteria in the existing microecosystem. Some of the bacteria surviving in the cryoconite environment therefore produce AFPs. AFPs, when released get adsorbed to the ice crystals and potentially influence their growth pattern, causing an alteration in ice microstructure, thereby a structural change increasing the porosity (Raymond *et al.* 2008). Once ice porosity is increased it enables unhindered passage of solutes and nutrients, thus nourishing the bacterial cells. Inhibition of the growth of ice crystals also leads to depression of freezing point of water, thereby protecting the cells from injuries caused by freezing (Raymond *et al.* 2008). These AFPs probably play also an important role in the survival of other non-AFP-producing organisms coexisting in the cryoconite ecosystem.

Rhodotorula svalbardensis, a cryoconite yeast AFP was reported to provide protection of the cells in the freezing climate. Similar observations about AFPs

were also recorded in several cold-adapted basidiomycetous fungi (Duman and Olsen 1993, Hoshino *et al*. 2003, Xiao *et al*. 2010, Lee *et al*. 2010), AFPs were recorded to be produced by several cold-adapted fungi (Hoshino *et al.* 2003, Lee *et al.* 2010).

Quite interestingly, the study on the antifreeze protein from the ice-core yeasts revealed that the yeast isolates did not produce any AFP. Adaptation strategies in ice microbes vary from enzyme secretion, PUFA accumulation to antifreeze protein secretion. Amongst the enzymes reported, catalase is one of the important enzymes to enable the producer strain to overcome cold stress (Fiedurek *et al.* 2003). From the results obtained, it can be inferred that the ice core isolates acclimatize to the ultralow temperatures by enzyme and PUFA secretion rather than AFP production. The strains investigated so far may be considered a potential source of cold active metabolites of significant use. Streamlined investigations on the unsaturated fatty acids and enzymes elaborated by these strains can potentially contribute to important biotechnological findings.

4.2.5 Conclusion

This is the first study on Antifreeze protein (AFPs) in Arctic glaciers Bacteria and its characterasation. Further research on cloning and expression of this protein will help in biotechnological applications.

4.4 Chemical constituents and antioxidant activity of *Lycoperdon molle*

4.4.1 Introduction

The Arctic area covered approximately 2.3% fungal microbiota. In 1933, Sommerefelt for the first time launched the investigation on the macromycete flora of Svalbard and much later a comprehensive account on Svalbard Basidiomycota fungi was published (Gulden and Torkelsen 1996, Aarnæs 2002). Subsequently, the macromycete *Lycoperdon molle* Pers. (Lycoperdaceae, Agaricales, Agaricomycetidae, Agaricomycetes, Basidiomycota, Fungi) an edible puffball was found out in that region.

Studies of polar mosses (Rozema *et al.* 2001, Huttunen *et al.* 2005) showed that ultraviolet-B radiation and low temperatures influence the production of certain specific metabolites that protect these organisms from environmental stresses. Biochemical adaptations enable macromycete species to overcome extreme environmental hazards including low temperatures, intermittent freezing and relatively high exposure to ultraviolet rays (Jang *et al.* 2000, Feller and Gerday 2003, Hoshino *et al.* 2003). These acclimatizations might influence the production of cold tolerant enzymes and antimicrobial compounds (Fiedurek *et al.* 2003, Frisvad 2008), with biotechnological potential (Leary 2008). Therefore urgency was felt to screen Arctic mushrooms for biotechnological prospecting.

In recent years, mushrooms drew attention on account of their pharmacological characteristics and their high-protein/low-fat nutritional value (Barros *et al.* 2007). Wild-type mushrooms are endowed to produce fatty acid of compositions that may be beneficial to improve blood lipid profiles. Substitution of saturated fatty acids with monounsaturated fatty acids reportedly enhanced increased high-density lipoprotein cholesterol and decreased low-density lipoprotein cholesterol, triacylglycerol, lipid oxidation and low density lipoprotein susceptibility to oxidation (Kanu *et al.* 2007). Reports are relatively rare on

antioxidant activities in polar organisms, e.g., Antarctic fish (Ansaldo *et al.* 2000), lichens (Paudel *et al.* 2007) and moss (Bhattarai *et al.* 2008). The current study presents antioxidant activities from Arctic *L. molle*.

Human metabolism generates reactive oxygen species (ROS) in the form of free radicals such as superoxide, hydroxyl radicals and other different nonfree radicals (Halliwel and Gutteridge 1999, Yildirim *et al.* 2000, Gulcin *et al.* 2002). ROS reacts with various biomacromolecules namely proteins, lipids and DNA and create oxidative stress. These agents directly or indirectly create a large number of pathophysiological disorders such as arthritis, diabetes, inflammation, cancer and genotoxicity (Kourounakis *et al.* 1999, Gulcin *et al.* 2002). To overcome the physiological load of free-radical production in the body, needed is the supply of exogenous antioxidative compounds. Antioxidants scavenge free radicals and help in maintaining the physiological system (Halliwel and Gutteridge 1999). They are often used as food additives to provide protection from oxidative degradation of food and edible oils (Senevirathne *et al.* 2006). There are certain synthetic antioxidants like propylgallate, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tert-butylhydroquinone that are regularly used by many. These are however, suspected of causing hepatic damage and carcinogenesis (Grice 1986, Wichi 1988, Hettiarachchy *et al.* 1996).

Antioxidants from natural sources provide protection against ROS without producing any side effects by thwarting the progress of many age-related disorders (Pryor 1991, Kinsella et al. 1993, Lai *et al.* 2001, Gulcin *et al.* 2003). In this regard, mushrooms can play a positive roles as they produce a variety of compounds (Almendros *et al.* 1987, Sun and Xie 2004, Barros *et al.* 2008) believed to possess antioxidant property (Mau *et al.* 2002, Barros *et al.* 2007) and antimicrobial properties (Puttaraju *et al.* 2006, Barros *et al.* 2007, Coolak *et al.* 2009; Ramesh and Pattar 2010). In this study, the investigation on chemical composition, antioxidant potential and antimicrobial activity of the Arctic wild mushroom *L. molle* was carried with the aim of filling a knowledge gap regarding the properties of Arctic fungi beneficial to the commonalty.

4.4.2 Materials and methods

4.4.2.1 Collection and preparation of the specimens

The natural fruit bodies of *Lycoperdon molle* were collected at different localities in Ny-Ålesund (78°54'.605"N - 12°05'.365"E), on the west coast of Spitsbergen Island in the month of August from Arctic archipelago of Svalbard. The samples were placed in sterile Whirl-Pak bags (Nasco, Fort Atkinson, WI, USA) and a sample collector (HiMedia) and stored at -20^{o}C until studied. Specimens were identified using the standard literature (Moser 1983, Courtecuisse and Duhem 1995) and representative specimens were deposited at the National Fungal Culture Collection of India (NFCCIWDCM 932), Pune.

The fruit bodies (3 g) were extracted in 20 ml each of methanol, ethanol, acetone and dimethyl sulfoxide (DMSO) using a soxhlet extractor. The extract was dried overnight at 37^{o}C and weighed. The dried extracts were redissolved in the different solvents and used for the determination of antioxidant activities.

4.4.2.2 High-performance liquid chromatography of the extract

The fruit bodies were extracted in methanol using a soxhlet extractor. High-performance liquid chromatography (HPLC) analysis was carried out on a e2695 HPLC system (Waters, Milford, MA, USA) using a C18 column (Waters) and a 2998 photodiode detector (Waters), with a solvent system of methanol-water-phosphoric acid (80:20:0.9, v/v/v). The detection wavelength 254 nm was used and the injection volume was 10 µl, with a flow rate of 1 ml/min. Retention times at 3.003, 3.758, 5.294 and 6.002 min were recorded.

4.4.2.3 Mass spectrometric and nuclear magnetic resonance analyses of the compounds

Electrospray ionization mass spectrometry (ESI-MS) analysis was performed on a Qstar XL quadrapole time-of-flight mass spectrometer (Applied Biosystems, Rotkreuz Zug, Switzerland) equipped with analyst software. The compounds isolated from the *L. molle* samples were dissolved in methanol and directly

analysed by ESI-MS. The samples were introduced at a constant flow rate into the electrospray source using an integrated syringe pump. The mass/charge (m/z) range was from 100-2000 in positive and negative mode. The declustering potential and the collision energy were optimized for the tandem mass spectra (MS/MS) experiments so as to cause fragmentation of the selected molecular ion species, as evident by the appearance of fragment ions and decrease in the intensity of the molecular ion. 1H, 13C nuclear

magnetic resonance (NMR) spectra were recorded, in CD_3OD, on an Avance-300 spectrometer (Bruker, Switzerland) with tetramethylsilane as the internal standard. Silica gel, 60-120 mesh (Merck, Mumbai, India) was used for column chromatography, while pre-coated Kieselgel 60 F254 plates (Merck) were used for thin layer chromatography.

4.4.2.4 Free-radical scavenging activity

The free-radical scavenging (FRS) activity of the extracts was measured following DPPH using the method of Blois (1958), with minor modifications. Briefly, 0.1 mM solution of DPPH was prepared in ethanol. Two ml of this solution was added to one hundred ml of fruit bodies extract and was allowed to incubate at 25^{o}C for 2 h, and the absorbance was recorded at 517 nm against blank samples using a Specord 205 UV-VIS spectrophotometer (Analytik Jena, Jena, Germany). The percentage of inhibition was calculated against a negative control (only DPPH solution). Lower absorbance of the reaction mixture indicated higher FRS activity, as shown in this equation:

$$\% \text{ inhibition} = [(A_{C(0)} - A_{A(t)}) / A_{C(0)}] \times 100$$

where $A_{C(0)}$ is the absorbance of the control at t=0 h and $A_{A(t)}$ is the absorbance of mushroom extract at 2 h.

4.4.2.5 Inhibition lipid peroxidation assay

The inhibition of lipid peroxidation (ILP) activity of the extracts was determined following the method of Liegeois *et al.* (2000). 30µl of 16 mM linoleic acid dispersion was added to a ~~UV~~ cuvette containing 2.81 ml of 0.05M phosphate

buffer with a pH of 7.4 which had been brought to 40^{o}C. The oxidation reaction was allowed to take place at 37^{o}C in a water bath where in 150 µl of 40 mM AAPH solution was added. Oxidation was carried out in aliquots (20 µl) of extract (0.3 mg/ml). The peroxidation reaction rate at 37^{o}C was monitored by recording the increase in absorbance at 234 nm, caused by conjugated diene hydroperoxides. ILP in per cent was calculated using the following equation:

$$\% \text{ inhibition} = [(A_0-A_1)/A_0] \times 100,$$

where A_0 is the absorbance of the blank sample without extract and A_1 is the absorbance in the presence of the extract.

4.4.2.6 Trolox equivalent antioxidant capacity assay

The Trolox equivalent antioxidant capacity (TEAC) of the *Lycoperdon molle* extracts was measured using the assay described by Miller et al. (1995), with minor modifications. $ABTS^{\cdot+}$ radicals were generated by the interaction of $ABTS^{\cdot+}$ (100 µM), H_2O_2 (50 µM) and peroxidase (4.4 unit/ml) in the assay system. To measure the antioxidant capacity of the mushroom fruit bodies, 200 µl of mushroom extract (0.3 mg/ ml) was mixed with an equal volume of $ABTS^{\cdot+}$, H_2O_2, peroxidase and deionized water. Absorbance was measured at 734 nm for 10 min. The decrease in absorption at 734 nm after the addition of reactant was used to calculate the TEAC value. TEAC value is expressed as mM of Trolox solution having the antioxidant equivalent to a 0.1% (w/v) extract solution. Higher TEAC values suggest stronger antioxidant ability.

4.4.2.7 Total polyphenolic assay

To determine the correlation between the antioxidant activities and the phytochemicals present in the fruit bodies extracts, polysaccharide, protein and total polyphenolic content present in the fruit bodies extracts were estimated. Total soluble phenolics in the fruit bodies extracts were determined with Folin-Ciocalteu reagent, according to the method of Slinkard and Singleton (1977), using pyrocatechol as the standard. Briefly, 0.1 ml of extract solution and 1 ml of

Folin-Ciocalteu reagent were added and shaken well. After 5 min, 3 ml of Na_2CO_3 (2%) was added, and the mixture was incubated for 1 h with intermittent shaking. The absorbance was measured at 760 nm. Total polyphenol was determined as mg pyrocatechol equivalent using the equation given below (obtained from standard pyrocatechol curve):

$$\text{Absorbance} = 0{:}001 \text{ x Pyrocatechol } (\mu g) + 0.0033$$

4.4.2.8 Polysaccharide assay

The polysaccharide content in the fruit bodies extracts was determined using the method described by Dubois *et al.* (1956). One ml of extract solution was added to 25 µl of 80% phenol and one ml concentrated sulfuric acid (H_2SO_4). The mixture was shaken and allowed to stand at
30 ^{o}C for 30 min. The absorbance was measured using a UV-VIS spectrophotometer at 490 nm. Polysaccharide content was estimated by a standard curve, using known amounts of standard polysaccharide solution.

4.4.2.9 Protein assay

The protein content of the fruit bodies extracts was determined using the method described by Bradford (1976). The concentration of protein was estimated using a standard curve for a known amount of bovine serum albumin.

4.4.3 Results

4.4.3.1 Free-radical scavenging activity

The extracts showed DPPH FRS activity ranging from 44 to 89.60% (Fig. 38) while the methanol extract showed the highest activity at 89.60%. The FRS activity of the methanol extract was found to be higher than shown by the BHA extract (56%) but less than that of the BHT extract (69%). The extracts with ethanol, acetone and DMSO showed moderate FRS activity (87.27, 57.86 and 44%, respectively) as shown in Fig. 39). In general, the antioxidant activity of

fruit bodies is solvent specific, and the extraction preference for fruit bodies is probably more in methanol and ethanol than in other solvents, which would account for the variation in FRS activity.

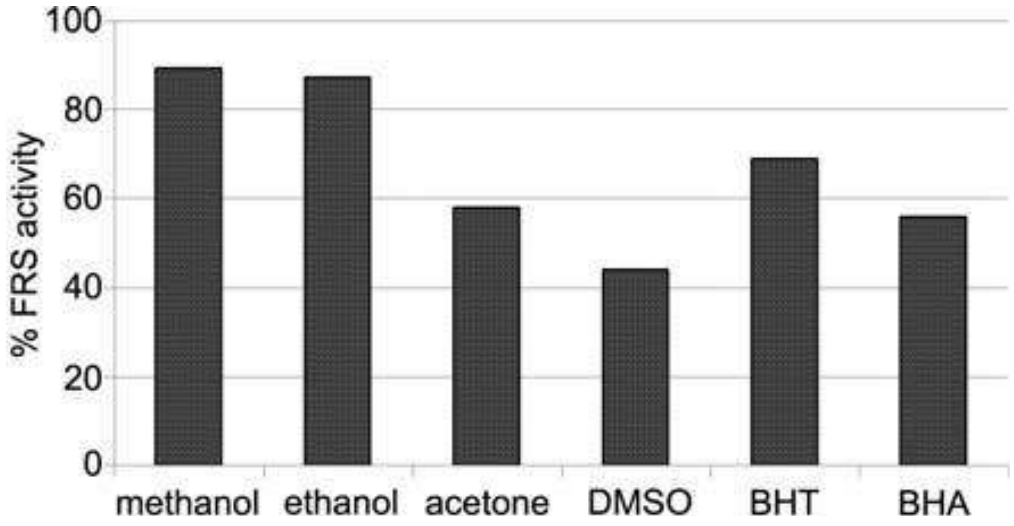


Fig. 39 Free-radical scavenging (FRS) activity of the Arctic mushroom Lycoperdon molle in various solvents: methanol, ethanol, acetone, DMSO, BHT and BHA.

4.4.3.2 Inhibition of lipid peroxidation

ILP values of the different solvent extracts are presented in Fig. 40. Almost all the solvent extracts showed greater percentages of ILP than the standard antioxidants, BHA (38%) and BHT (47%). The methanol extract showed the highest activity at 54.41%, followed by ethanol at 45.00%, acetone at 39.00% and DMSO at 32.00%, in that order.

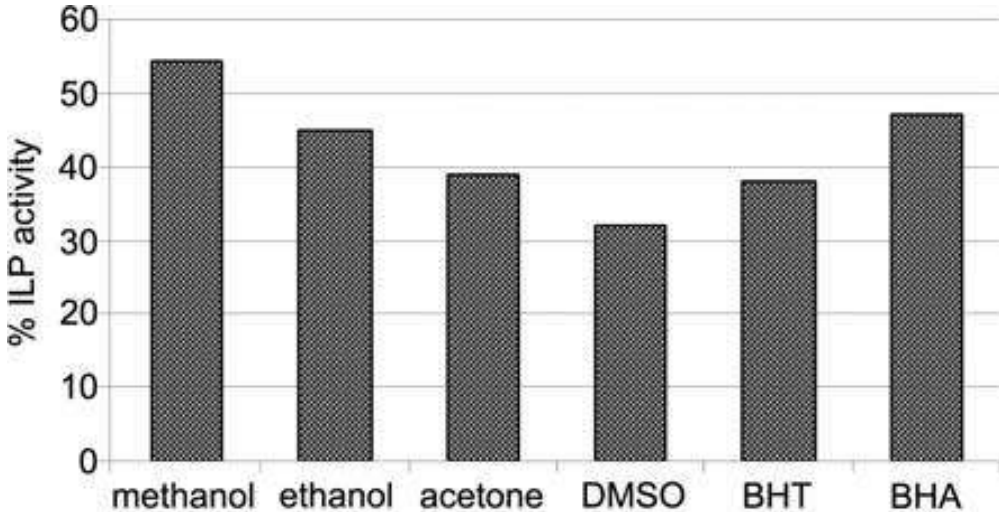


Fig. 40 Inhibition of lipid peroxidation (ILP) activities of *L. molle* in various solvents: methanol, ethanol, acetone, DMSO, BHT and BHA.

Trolox equivalent antioxidant capacity

The results of the TEAC assays of the various solvent extracts are presented in Table 37. All the extracts showed good activity. The acetone and ethanol extracts showed higher antioxidant capacity (7.4 mM - 7.0 mM, respectively) compared to other solvents. TEAC values of our mushroom extracts were found to be higher than the activity shown with standard Trolox (3.9 mM).

4.4.3.3 Protein, polysaccharide and total phenol content of the fruit bodies

The polysaccharide, protein and total phenol contents present in the mushroom extracts were estimated and are presented in Table 37. Regardless of the solvent used, the protein content obtained in different solvents ranged from 8.00 µg to 24.00 µg/g dry wt. The polyphenol content ranged from 0.031 mg to 0.325 mg/g dry wt. High polysaccharide dry content was found in all the solvent extracts, ranging from 4.00 mg to 14.92 mg/g wt. The acetone extract showed the highest polysaccharide content: 14.92 mg/g dry wt.

4.4.3.4 Antioxidant activities in relation to phytochemical content

Fruit bodies of *L. molle* produce primary and secondary compounds such as proteins, polysaccharides and polyphenols reported earlier were essential for various biological activities (Barros *et al.* 2008). Protein, polysaccharide and polyphenol contents were determined in the *L. molle* extract (Table 37) and correlated these with the antioxidant activities. A significant correlation (R^2= 0.968, $p < 0.01$) was found between polysaccharides content and antioxidant activities. From this study, it was deduced that the very high content of polysaccharide in *L. molle* is mainly responsible for the antioxidative activity that is essential for their survival in the Arctic. The results presented are also in agreement with those of Liu *et al.* (1997), who reported the ability of polysaccharide for scavenging free radicals.

Table 37. Antioxidant activity, polysaccharide, protein and total polyphenol content of *L. molle* extracts in various solvents. Data presented are based on the experiments performed in triplicate.

		Antioxidant activities			Polysaccharide content (mg/ml)	Protein content (µg/ml)	Total polyphenol Content (mg/ml)
Solvent extracts	**Extract dry wt. g**	**FRS[1] (%)**	**ILP[2] (%)**	**TEAC[3] (mM)**			
Acetone	0.06	57.87	39	7.4	14.92	24	0.031
Ethanol	0.08	87.27	45	7	14.85	8.04	0.09
Methanol	0.64	89.6	54.41	-	14.68	8.63	0.304
DMSO[4]	0.11	44	32	2.4	4	8	0.325

[1]Free-radical scavenging activity, [2]Inhibition of lipid peroxidation, [3]Trolox equivalent antioxidant capacity, [4]Dimethyl sulfoxide.

4.4.3.5 Purification and ESI-MS analysis of purified fractions A and B

The crude methanolic extract of *L. molle* exhibited six distinguishable spots on thin layer chromatography (TLC) in the solvent system butanol:acetic acid:water (60:15:25, BAW) and then detected with methanolic ninhydrin. These spots were separated using the preparative TLC technique to give six fractions. TLC of the concentrated fractions was carried out and only fraction 2, labelled fraction A, which showed a single spot (R_f = 0.7) weighing 5 mg. NMR and ESI-MS were carried out on fraction A. NMR was done in CD_3OD. Fraction 3 showed two spots but the amount was very small. Preparative TLC of fraction 3 gave one fraction, labeled fraction B, which showed only one spot, which weighed (10.8 mg, R_f =0.512). NMR and ESI-MS analyses were carried out on fraction B. The identification of the compounds from fractions A and B using ESI-MS analysis is explained in detail below. The remaining four fractions weighed less than 1 mg so a detailed spectral analysis could not be undertaken on them.

ESI-MS (negative ion mode) of fraction A

In the negative ion mode, the ESI-MS profile (Fig. 41) of one of the chromatographed fractions-fraction A- showed peaks in the mass range of m/z 200-350 that were consistent with assignment as carboxylate ions.

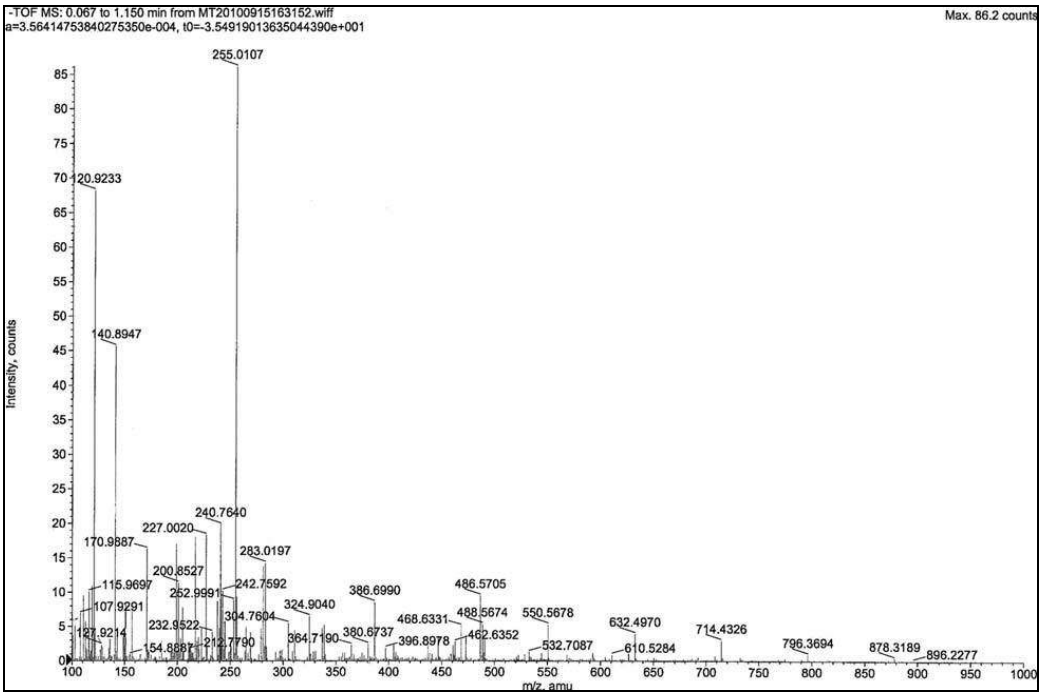
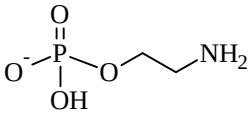
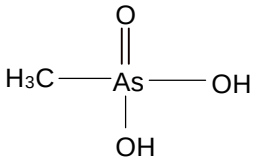


Fig. 41 Electrospray ionization mass spectrometry profile of fraction A in the negative mode.

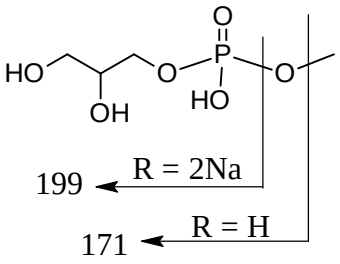
These peaks were m/z 227, 253, 255, 283, 305, 325 and 339 corresponding to C14:0 (myristic), C16:1 (methyl palmitoleate), C 16:0 (palmitic), C18:0 (stearic), C20:3 (methyl eicosatrienoate), C21:0 (heneicosanoic) and C22:0 (hehenic) carboxylate ions, respectively. These include ions formed from phospholipids as well as free fatty acids produced by the organism. Palmitic acid, at m/z 255, was the dominating ion, indicating it to be one of the major components. A peak at m/z 140.8 was assigned to the presence of phosphoethanolamine. It could also have been due to the presence of monomethyl arsenic acid, since it is known that arsenic molecular species are recorded from *L. molle*. An additional signal at m/z 135.9 could be due to trimethyl arsine oxides. The signals at m/z 171 and 199 corresponded to a phosphatidylglycerol moiety and the peak at 184.99 was due to phosphoserine unit. The signal at m/z 240 corresponded to phosphoionositol after the elimination of a water molecule.



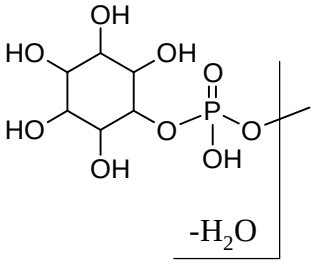
Phosphoethanolamine (PE)



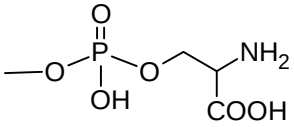
Monomethyl arsenic acid (MMA)



Phosphatidyl glycerol



Phosphoionositol



Phosphoserine

The mass range at m/z 500-1000 included peaks at 610.5 and 632.4 due to phosphatidic acids. It also included signals at 714.4 and 796.6, representing phosphatidylglycerols. Less intense signals at m/z 878 and 896 were consistent with the expected presence of digalactosyldiacylglycerol, but in the present sample a sugar moiety did not seem to be present as corresponding signals were not observed in the δ3.0-4.5 region of ^{1}HNMR.

ESI-MS (positive ion mode) of fractions A and B

The ESI-MS profile (Fig. 42) in the positive ion mode of fraction A showed signals in the range of m/z 200-500, which probably represented fragments of phospholipids.

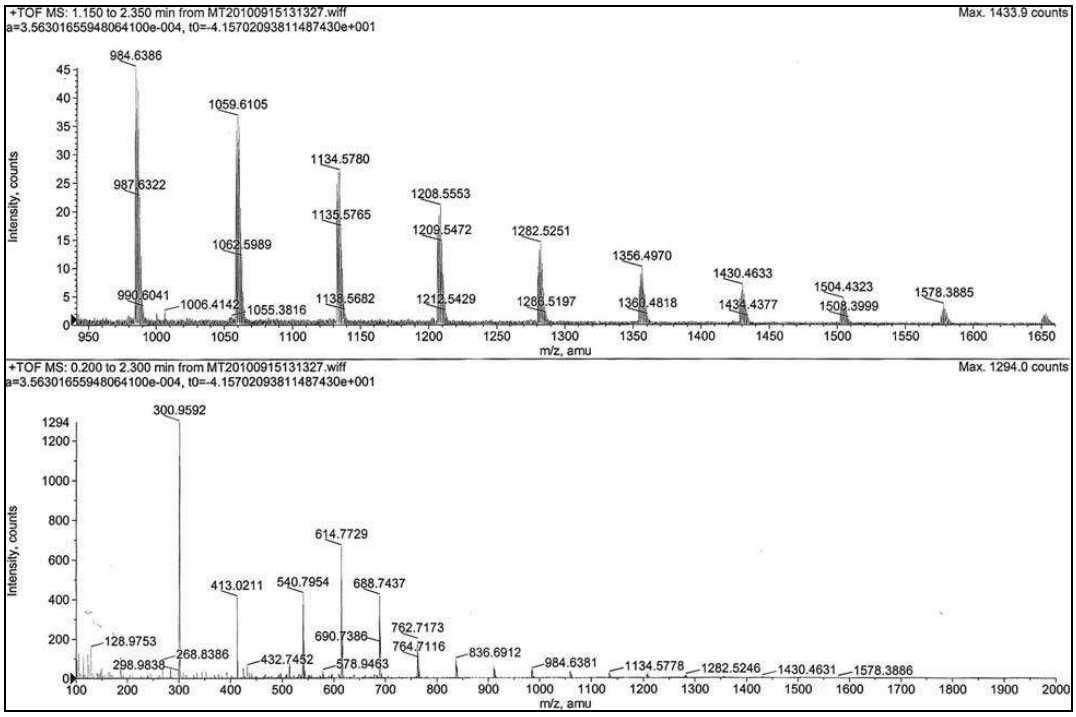
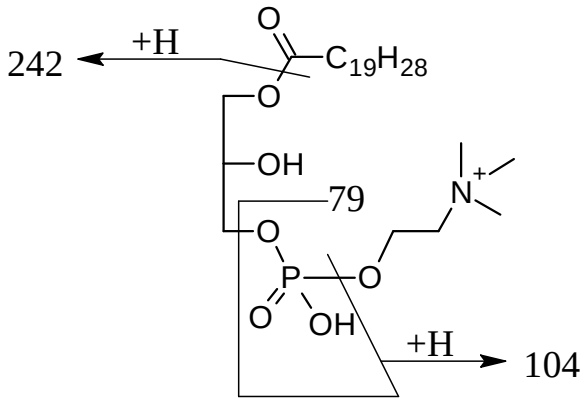


Fig. 42 Electrospray ionization mass spectrometry profile of fraction A in the positive mode.

A molecular species with m/z 540.7 was identified as lysophosphatidylcholine on the basis of fragmentation observed in its electrospray ionization-tandem mass spectrum (ESI-MS/MS; Fig. 43) and as shown in the diagram of its molecular structure.



LysoPhosphatidyl choline

An interesting observation here is that beyond molecular species with [M+H]+ at 540.7 the signals at m/z 614.77, 688.74, 762.71, 836.69, 910, 984, 1058, 1132, 1206, 1280, 1354, 1428, 1502, 1576 are observed.

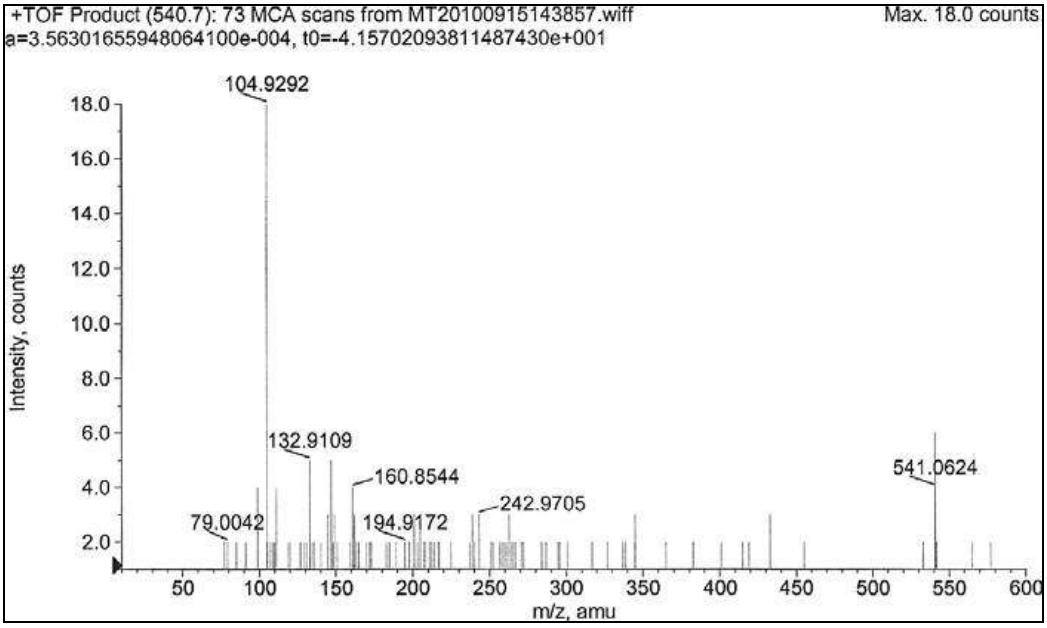
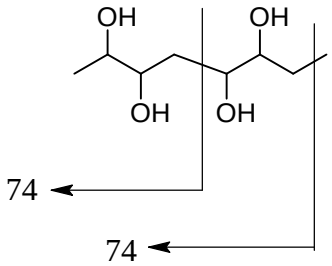


Fig. 43 Electrospray ionization mass spectrometry/tandem mass spectra of m/z 540.7.

A close observation at these values indicates that all these molecular species differ by 74 amu, which probably resulted from the oxidation of the double bonds to the corresponding vicinal diols, as shown below:



This indicates that addition of these units to the lysophosphatidylcholine (m/z 541) will lead to the formation of the structure of the remaining molecular species observed in fraction A. The presence of these groups is reinforced by the presence of NMR signals in the region of δ3.5-3.8 for protons and 73 ppm for carbons of

hydroxymethines. In addition, methylene groups were observed at δ1.2 and 30 ppm in ^{1}H and ^{13}C NMR spectra.

The ESI-MS analysis of fraction B showed similar pattern as A in its mass profile.

4.4.4 Discussion

Antioxidants play important roles in scavenging obnoxious free radicals produced during any physiological stress. Presumed antioxidant activities have been attributed to various mechanisms involving inhibition of chain initiation, transition metal ion catalysts binding, degradation of peroxides, prevention of continuous hydrogen abstraction and radical scavenging (Diplock 1997, Yilidirim *et al.* 2001). It was observed in the FRS assay that the DPPH free radicals generated by the action of alcohol were removed by the *Lycoperdon molle* extract while in the ILP test, *L. molle* extract prevents oxidation of lenoleic acid by the oxidizing agent 2,2'-azobis (2-aminopropane) dihydrochloride.

L. molle were reported to produce a number of secondary metabolites, several of which were phenolics and polysaccharides (Barros 1978 Ferreira *et al.* 2007). The correlations found between the polysaccharide content and the FRS, ILP and TEAC activity levels in the *L. molle* samples were rather significant; therefore it is supposed that the antioxidant activity showed by the extracts was probably due to high polysaccharide content (Table 37). These results are in partial agreement with the observations of Liu *et al.* (1997), who reported about the ability of polysaccharides that could scavenge free radicals. ESI-MS analysis of the *L. molle* extract also showed the presence of compounds namely phosphatidylglycerol, phosphoethanolamine, phosphoserine, phosphoionositol and lysophosphatidylcholine. The chemical reactions that generally take place in the Arctic, *viz*, ROS-induced peroxidation and de-esterification of glycerolipids, caused by dehydration and rehydration cycles, are responsible for cell membrane disruptions. Compounds such as polyols, might act as osmoprotectants, and quench the ROS, thereby protecting the cellular structures of the microbes against

the potentially deleterious effects of dehydration (Jennings *et al.* 1998). It can be inferred that the identified chemical constituents in the *L. molle* extracts contribute to the survival of this mushroom species in the Arctic's harsh environmental conditions.

4.4.5 Conclusion

The present study revealed strong antioxidant ability of *Lycoperdon molle*. The report on the antioxidant activity in any Arctic mushroom is very rare. Based on our findings, it can be concluded that the mushrooms available in the Arctic region can be a potential source of natural antioxidants applicable to the pharmaceuticals and therapeutics. Therefore Arctic mushroom species must essentially be explored systematically and cultivated in the laboratory conditions for mass production.

5. OBJECTIVE 3: *PURIFICATION AND BIOCHEMICAL CHARACTERIZATION OF THE MOST PROMISING SUBSTANCE OF BIOTECHNOLOGICAL INTEREST FROM THE SELECTED ORGANISM.*

Purification and Biochemical characterization of Antifreeze proteins

5. 1 Introduction

Antifreeze protein (AFP) producing organisms are abundant in areas such as the lakes (Gilbert *et al.* 2004), sea ice (Raymond *et al.* 2007, Bayer-Giraldi *et al.* 2011), ice cores (Raymond *et al.* 2008), and terrestrial environments (Raymond and Fritsen 2001) in the polar regions. Until recently documentation of AFP from the Arctic microorganisms were inadequate. An AFP from a yeast strain *Leucosporidium* sp., isolated from an ice core of an Arctic pond in Svalbard was reported by Lee *et al.* (2010). But much before that Sun *et al.* (1995), Xu *et al.* (1998), and Kawahara *et al.* (2001) reported about AFPs from *Pseudomonas putida*, isolated from plants in the high Canadian Arctic. A separate study in the Vestfold Hills, East Antarctica, contributed isolation of 11 bacterial strains with ice recrystallization activity (Gilbert *et al.* 2004). Another bacterial strain *Marinomonas protea* (presently known as *Marinomonas primoryensis*) was one of the most prolific producers of AFP was identified; the AFP activity of this bacterium was later observed to be Ca^{2+} dependent. Other bacterial strains exhibited AFP activity including *Micrococcus protea* was isolated from the ice–water interface of Ace Lake in the Vestfold Hills of Antarctica (Mills 1999) whereas species of *Moraxella* isolated from the McMurdo Dry Valleys region of Antarctica (Yamashita *et al.* 2002); besides this AFP was also isolated from an unidentified bacterial strain belonging to the Flavobacteriaceae isolated from the ice core of the subglacial Lake Vostok, Antarctica (Raymond *et al.* 2008).

Bacterial AFPs-1 belonging to ice binding protein (IBP-1) category were also purified from other than Arctic cryospheres (Xu *et al.* 1998, Yamashita *et al.* 2002, Gilbert *et al.* 2004, 2005, Kawahara *et al.* 2004; 2007, Muryoi *et al.* 2004, Gurian-Sherman and Lindow 1993, Kawahara 2002). These protein were reported from the bacterium *Colwellia* sp. (Raymond *et al.* 2007), the fungi *Coprinus*

psychromorbidus and *Typhula ishikarieneis* (Hoshino *et al.* 2003a, b), sea ice diatom *Fragilariopsis* sp. (Bayer-Giraldi *et al.* 2010), crustacean *Stephos longipes* (Kiko 2010), and algae (Raymond and Morgan-Kiss 2013).

In the present study, AFPs of six bacterial strains were subjected to purification and biochemical characterization.

5.2 Materials and methods

5.2.1 Purification of AFPs

Bacterial strains indicating AFP activity were selected for purification. They were cultured in nutrient broth (Eiken Chemical, Japan) at -1 °C under static conditions. After thirty days, the culture media were centrifuged and the cell free supernatants were dialyzed against 25 mM glycine–HCl buffer (pH 3.0). The dialysates were separately applied to an Econo-pac High-S column (Bio-Rad) and eluted with the same buffer containing 200 mM NaCl; however, dialyzate of one strain failed to bind to Q-resin at pH 6, and the fractions showing AFP activity were dialyzed against 25 mM MES–NaOH buffer (pH 6.0) and then loaded onto an Econo-pac High-Q column (Bio-Rad) and eluted with 25 mM MES–NaOH buffer (pH 6.0) containing 150 mM NaCl. Flow-through fractions showing AFP activities were subjected to dialysis against 25 mM sodium acetate buffer (pH 4.0) followed by reloading onto an Econo-pac High-Q column (Bio-Rad). The active fraction was eluted with the same buffer containing 150 mM NaCl.

5.2.2 Biochemical and molecular analyses of AFPs

The purity and molecular weights of AFPs were ascertained and measured by SDS-PAGE followed by staining by silver nitrate. The protein concentration for each AFP sample was estimated from the weight of lyophilized powders of the purified proteins. The N-terminal amino acid sequences of AFPs were determined by Edman degradation method, using 491cLC protein sequencer (Applied Biosystems).

5.2.3 Measurement of AFP activity

Thermal hysteresis activity of each purified AFP isoform groups (AFP solution) was measured with an in-house photo system consisting of a microscope, with a temperature controller, following the procedure of Takamichi *et al.* (2007), but using a cooling rate of 0.1 °C min^{-1}. Digital images of ice crystals were recorded using a CCD camera. To study the effect of AFP concentration on TH activity, serial dilutions of purified AFP solutions were made in 25 mM MES–NaOH buffer, pH 6.0. The measurements were repeated three times and averaged.

5.2.4 Ice recrystallization inhibition assay

The AFP solutions were dissolved in 100 mM ammonium bicarbonate containing 30% (w/w) sucrose, following the procedure of Xiao *et al.* (2010). One microlitre of sample solutions was frozen completely at -20 °C on the microscope stage, then warmed up to -6 °C and left for 1 h. During this incubation, images were taken every 5 min.

5.2.5 Characterization of the Antifreeze protein (AFP)

The Cry-c AFP from the strain *Cryobacterium psychrotolerans* (MLB-29) was purified by dialyzing the cell free supernatant followed by column chromatography using the Econo-pac High-Q column. The Cry-c AFP was ultrafiltered using Amicon (Millipore) with the membrane cut-off of 3kDa, to remove salts and placed in 25 mM ammonium acetate as final solvent to obtain final protein concentration around 40 µM. The molecular mass determination of the sample subsequently was carried out by Electropspray ionization quadrupole time-of-flight mass spectrometry (ESI-Q-TOF-MS) at a protein concentration of 10 µM in 30% acetonitrile, 0.5% formic acid (final) in 25 mM ammonium acetate.

5.2.6 Molecular mass determination

The ESI-Q-TOF-MS analysis was done on positive ion mode on SYNAPT G2 mass spectrometer (Waters). Calibration was performed using clusters of phosphoric acid in *m/z* range 50 to 3500, corresponding to raw spectra *m/z* acquisition range. Raw spectra were deconvoluted using the maximum entropy deconvolution technique (Max ent1 process in MassLynx software, (Waters).

5.2.7 Analysis of Cry c-AFP by nanoLiquid chromatography electrospray ionization mass spectrometry (nanoLC-ESI-MS/MS)

Sample was first digested using the MELD® protocol (the GIGA Proteomic facility, Université de Liège, Belgium). Further the proteins/peptides identification was accomplished by LC-ESI-MS/MS. The analyses were performed on an Acquity M-class ultra performance liquid chromatography (UPLC) system (Waters Corp.) hyphenated to a Q Exactive (Thermo Scientific, GIGA Proteomics), in nano-electrospray positive ion mode. The samples were loaded at 20 µL/min on the trap column (C18, 5 µm,180 µMx20 µM) in 100% solvent A (0.1% formic acid in water) during 3 minutes and subsequently separated on the analytical column (HSS T3 C18, 1.8 µm, 75x250 µm) with the flow rate of 600 nL/min, using the solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) as the mobile phase maintaining the linear gradient of 0 min, 98% A; 3 min, 93% A; 55 min, 70% A; 70 min, 60% A. The total run time was 100 min.

For the MS analysis, the parameters for MS spectrum acquisition were as follows: mass scanning range of m/z 400-1750, resolution of 70000 and maximum injection time of 200 ms; for the MS/MS study, the isolation window was set at 2.0 m/z with the collision induced dissociation energy set to 25% keeping the resolution of 17500 and maximum injection time of 50 ms.

5.2.8 *de novo* sequencing

Spectra were treated using Peaks 7.0 (Peaks Studio). *De novo* sequencing of all precursors having the same mass (tolerance 3 ppm) co-eluted (tolerance 0.3 min) were merged for further processes. *De novo* search was performed using PEAKS software with the following parameters: Parent Mass Error Tolerance: 3.0 ppm, Fragment Mass Error Tolerance: 0.015 Da, Variable Modifications: Carbamidomethylation: 57.02, Oxidation (M): 15.99 Deamidation (NQ): 0.98 and Max variable post translational modification per peptide: 3.

5.3 Results

5.3.1 SDS-PAGE analysis of bacterial AFPs

AFPs of six bacterial strains were subjected to biochemical characterization. SDS-PAGE analysis followed by silver staining showed that the AFPs were successfully purified and revealed that their apparent molecular weights were about 22 kDa (Fig. 44).

Probably, the AFPs include more than one isoform as can be seen in lanes (d), (e), and (g) of Fig. 44. Table 38 shows the results of N-terminal amino acid sequence analysis of the AFPs. These sequences revealed that the isolated bacterial AFPs belong to the family IBP-1 (Raymond and Morgan-Kiss 2013), which play a very crucial physiological role in the cold temperatures. AFPs of *Typhula ishikariensis* (molecular weight of 22 kDa) were used as standard (Fig. 44), and its N-terminal amino acid sequences have been used as a representative of IBP-1 family. These AFPs showed homology with the cryoconite bacteria (Table 38).

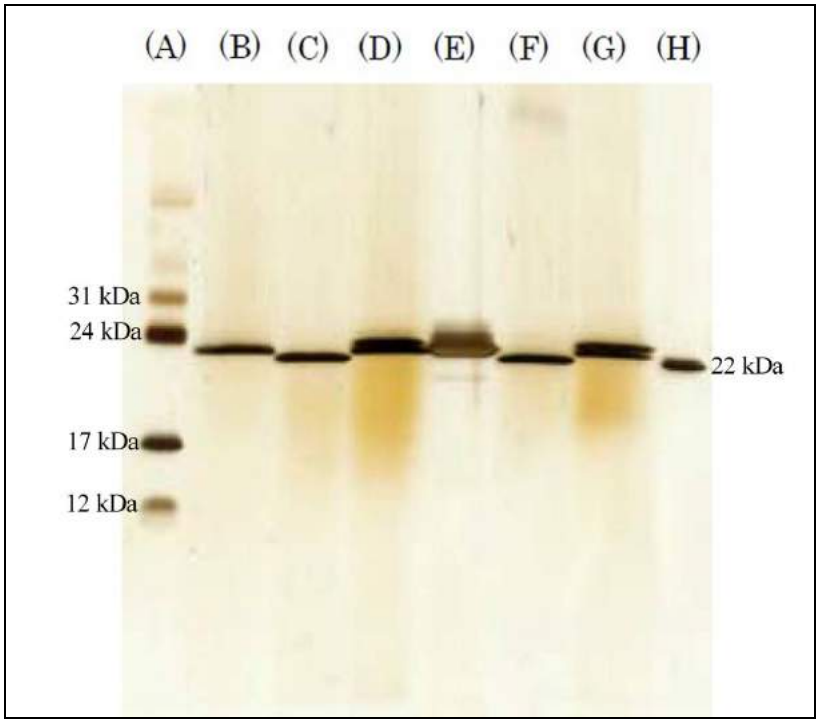


Fig.44 SDS-PAGE analysis of bacterial AFP purification. Six of bacterial AFPs were purified on SDS-PAGE followed by silver staining. (A) Polypeptide marker (Bio-rad), (B) cry-c, (C) cry-g, (D) cry-k, (E) cry-l, (F) cry-n, (G) cry-21, (H) *Typhula ishikariensis* AFP (TisAFP) with a molecular weight of 22 kDa (Hoshino *et al.* 2003) as a standard.

Table 38. Amino-terminal amino acid sequences of cryoconite AFPs studied. "Tis" denotes first 20 residues of an AFP from *Typhula ishikariensis.*

Isolate Code	Culture accession number	N-terminal amino acid sequences
Cry-g (MB-g)	JCM 19509	----NSNPSPVXLGSAXTFAILSQ-
Cry- n (MLB-46)		----NSNPSPVYLGSAXTFAILSQ-
Cry-l (MLB-33)		---DVMPQAPVNLGSTEXFSILS--
Cry-c (MLB-29)	JCM 19503	AVPXGSVXAXVXXGAATTFX-----
Cry-k	JCM 19507	AXPGGXRXXXVXXGAATXFI-----
Cry-21 (MLB-37)	JCM 19506	AVPVGSVRAPVALGLAXXFA-----
Tis		-----AGPSAVPLGTAGNYVILAST
		* * : :

X= unidentified amino acid, *****= conserved residues, **:** = partially conserved residues

ESI-Q TOF analysis of the Cry-c and Cry-g proteins revealed its exact molecular mass to be 22.14 kDa (Figure 45a, b) corroborating its above observation on SDS-PAGE analysis that was 22 kDa (approximately).

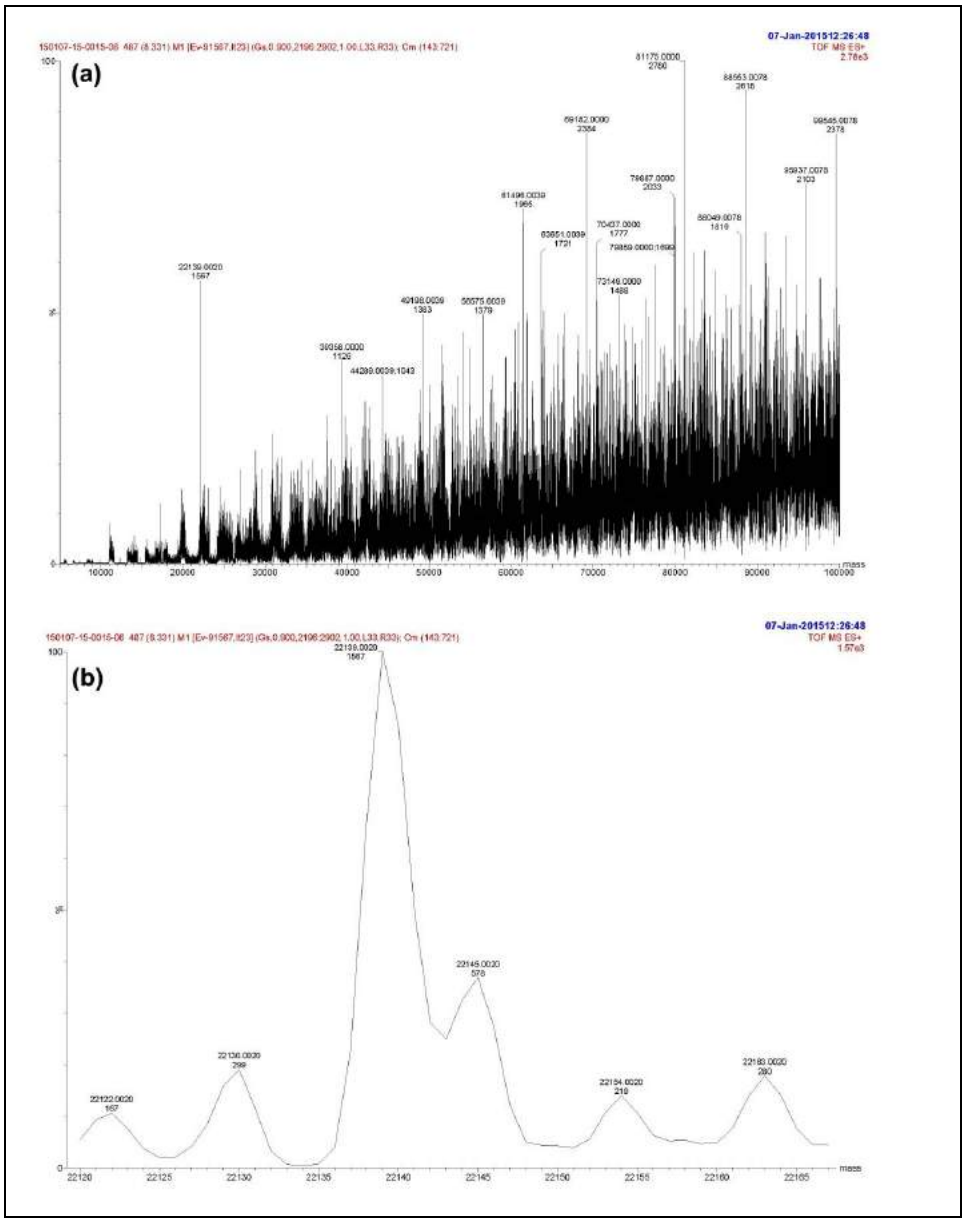


Fig. 45 a) Mass Determination by ESI-Q-TOF-MS. Peaks appearing at the mass of the protein divided or multiplied by an integer higher than 1 are usually artefacts arising from Max Entropy deconvolution data treatment. b) The peak showing the mass after zooming. The third peak showing the mass as 22.139 kDa.

5.3.2 AFP activity and ice crystal growth pattern

The six bacterial AFP solutions (extracellular proteins/AFP isoform groups) were tested for TH activity as a function of their concentration in 25 mM MES–NaOH buffer pH 6.0 (Fig. 46). High TH values of around 2 °C were obtained in the cry-g and cry-n strains. The AFP solutions of cry-k and cry-21 exhibited almost identical activity at the same concentration (TH of 0.78 °C at c. 1.8 mg mL^{-1}). TH activity of cry-c is about 1 °C at concentration 4 mg mL^{-1}, and Cry-l has TH value of 1.4 °C at 2.5 mg mL^{-1}.

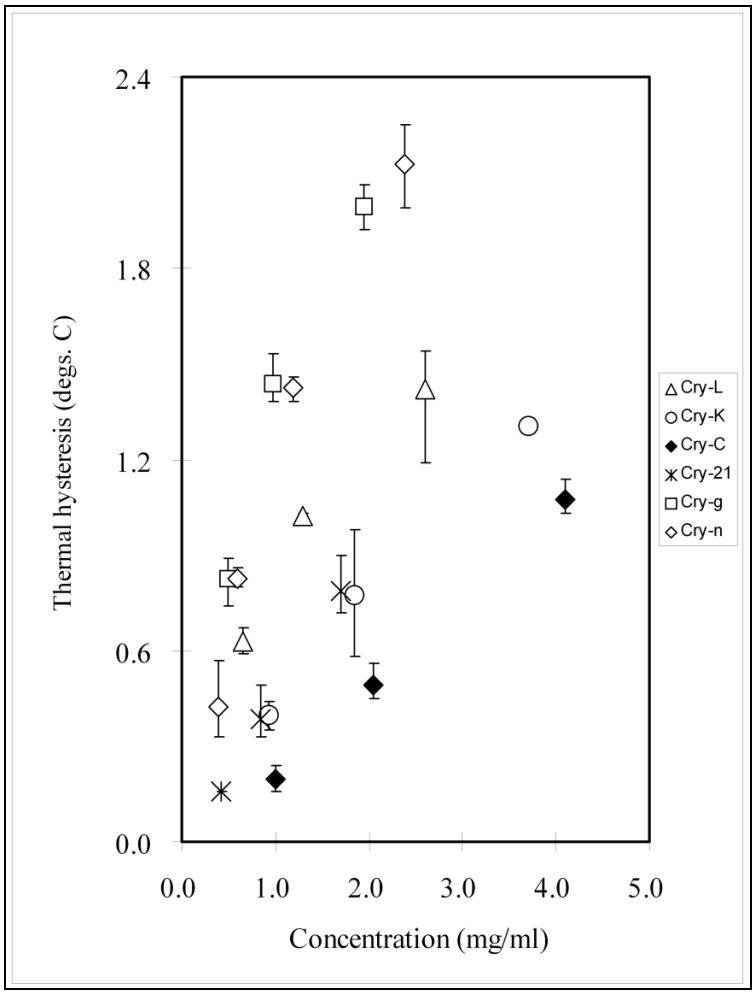


Fig. 46 TH activity of bacterial AFPs. TH activities were plotted as a function of protein concentration. Three replicates of each concentration were measured and averaged. Vertical bars represent the standard deviation.

The six bacterial AFP solutions were observed to produce round ice crystals that did not alter their size and morphology within the TH window. As shown in Fig. 47a, when the solution cooled below the freezing point, a rapid and sudden ice crystal growth ('burst') pattern in which ice crystal grows into six directions with hexagonal symmetry was observed. This pattern closely resembles the burst visualised in hyperactive AFP solutions rather than that of moderately active fish AFPs (Scotter *et al.* 2006).

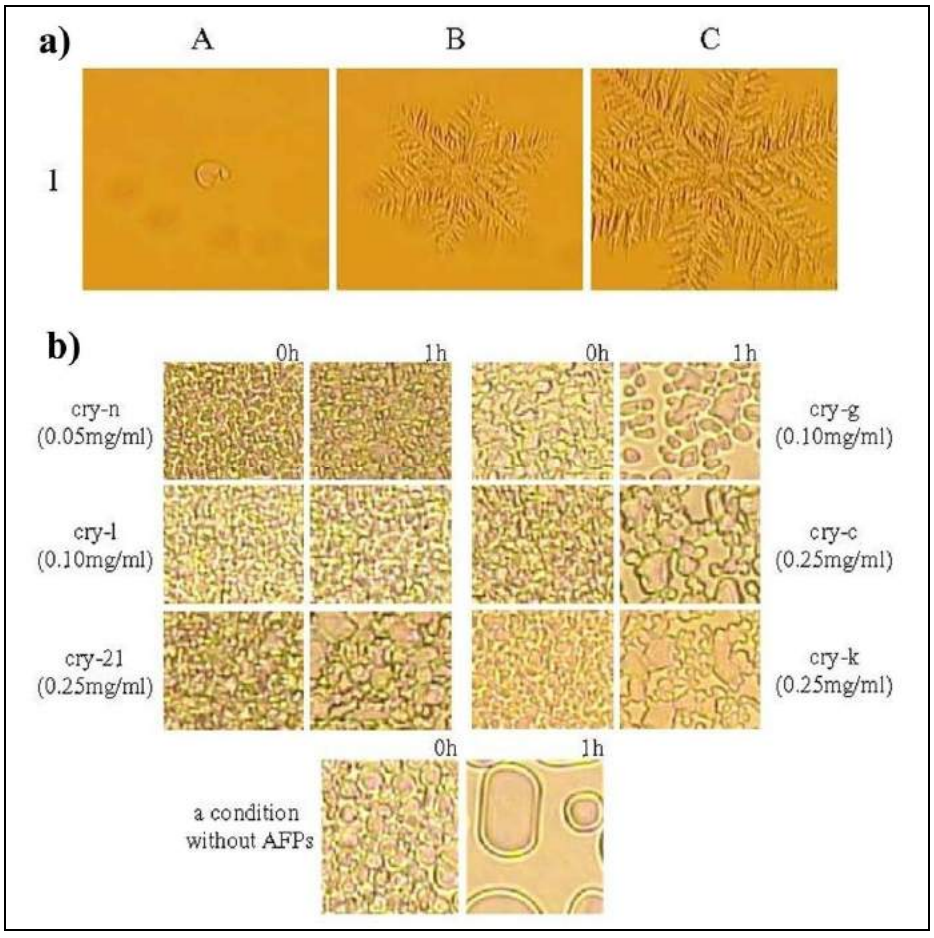


Fig. 46 a) Ice growth patterns in the presence of bacterial AFPs. These snapshots were taken during ice growth at temperatures above freezing point (frame A), around freezing point (frame B), below freezing point (frame C). b) Images of ice recrystallization observed in bacterial AFP solutions. Left panels show start points (i.e., 0 hour incubation) and right panels show end points (i.e., after 1 hour incubation). The lower figures indicate the measurements performed without AFPs as a negative control.

5.3.3 Ice recrystallization inhibition activity

The bacterial AFPs tested all had a different degree of ice recrystallization inhibition (IRI) activity (Fig. 47b). As mentioned above, the highest TH activity was obtained with both cry-g and cry-n AFPs. The IRI assay showed that cry-g AFP failed to inhibit ice recrystallization even at a concentration of 0.1 mg mL^{-1}, while cry-n AFP inhibited ice recrystallization at a concentration of 0.05 mg mL^{-1}. Therefore, it appears that the strength of IRI activity of AFPs does not relate to the strength of TH activity.

5.3.4 MS/MS spectra of identified peptides of antifreeze proteins from *Cryobacterium psychrotolerans* Cry-c and *Pseudomonas ficuseractae* Cry-g

Using PEAKS 7 software, de novo sequencing was performed on MS/MS spectra obtained by nanoLC-MS/MS. 21 peptides for Cry-c and 55 peptides for Cry-g were sequenced with a satisfying confidence. All the identified peptides (based on all data, i.e. not “ALC restricted” to 90%) are provided in table (39 and 40). It was not possible to distinguish leucine from isoleucine due to their common mass (only leucine “L” is reported).

In table, the ALC (Average Local Confidence) column indicates the probability of having a good assignment. The columns m/z, z, RT (retention time), Mass are related to the MS detection of the peptides and ppm represents the difference between the measured mass and the expected mass based on the found sequence. PTM column indicates if a PTM was found in the sequenced peptide. The column "local confidence" refers to the probability of having a true assignment for the given residue. Finally, proteins in which identified peptides probably belong are indicated.

MS/MS spectra obtained for the peptides has been presented (Fig 48 and 49). Ions that were assigned are indicated in blue or red, depending on their type. Vertical lines indicates the position of the fragmentation and horizontal line indicates of it is a y ion (if horizontal line is on the right side of the vertical line) or a b ion (if horizontal line is on the left side of the vertical line). For example, in

the given sequence L|A|A|D|D|F|R , the ions y1, y2, y3, y4, y5, y6 and the ions b2 and b3 were observed. The more fragments are observed, the more confident is the identification of the tag. The most important thing is to have every amino acid covered by a fragment, in other words having a vertical line between every amino acid.

Table 39. Peptides sequenced using PEAKS software. Cry-c shows 21 peptides consisting of 228 amino acid residues that combines to make 22.14 kDa.

Peptide (Length)	ALC (%)	m/z	z	RT	Mass	PTM	Alternate sequence
HKQ(+.98)MPFPK (8)	99	506.266	2	25.6	1012.516	Deamidation (NQ)	
KKLEK (5)	99	323.218	2	2.26	644.4221		
TGATVDGR (8)	98	388.696	2	9.8	775.3824		
YPLEAH (6)	98	365.182	2	21.3	728.3493		
LFQLAGNLTQASATR (15)	98	795.934	2	67.5	1589.853		
PVGSVR (6)	98	307.684	2	23.4	613.3547		
QPHGPPGKDGR (11)	98	382.532	3	12.9	1144.574		AGPHGPPGKDGR
QGPAGPNER (9)	97	463.228	2	11.6	924.4413		QGPAGPGGER
DMALNESKE (9)	96	502.74	2	20.4	1003.464		
VTLTGQLAK (9)	96	465.785	2	37.7	929.554		
AVGLLQNNSGAAKHATLR (18)	95	456.254	4	37.7	1820.986	Deamidation (NQ)	VAGLLENNSGAAKHATLR
TGQLAK (6)	95	309.184	2	7.27	616.3544		
NMALNPSKE (9)	94	502.248	2	26.6	1002.48		
YKVPQLQ (7)	94	438.745	2	45.8	875.4752	Deamidation (NQ)	YKVPQLE
VPDLKKPHYLYH (12)	93	378.211	4	37.6	1508.814		
GPSGEAGKMFVHQGPTGL (18)	92	590.627	3	26.9	1768.857		
GPAGPHGPPGKDGR (14)	92	433.89	3	12.9	1298.648		
LANPSKE (7)	92	379.706	2	12.7	757.397		
MHQPHQPLPPTHDFPPQ (17)	92	668.656	3	74.7	2002.947		
ASALTGNVGASPLTQALHL (19)	91	910.997	2	88.2	1819.979		
QAAGSVAVGTTAHFE (15)	90	723.355	2	45	1444.695		

Table 40. Peptides sequenced using PEAKS software. Table below shows 55 peptides of Cry-g.

Peptide	Tag length	ALC (%)	m/z	z	RT	Mass	ppm	PTM
VATVSLPR	8	99	421.758	2	23.4	841.5021	-0.2	
VATVSLPR	8	99	421.758	2	23.6	841.5021	-0.5	
YKVPQLE	7	99	438.744	2	26.3	875.4752	-2.1	
MTLDDFR	7	99	449.211	2	29.9	896.4062	1.5	
LPTVSLPR	8	99	441.774	2	30.5	881.5334	0	
LALSQSGLTDVYR	13	99	711.882	2	31.7	1421.752	-1.3	
LEMPFPK	7	99	431.231	2	32	860.4466	-0.2	
GPFPLEMPFPK	11	99	630.329	2	49.3	1258.642	0.6	
DMALNPSKE	9	98	502.74	2	19.6	1003.464	0	
DMALNPSKE	9	98	502.739	2	20.2	1003.464	-0.5	
EPVLGPVR	8	98	433.758	2	22.8	865.5021	-0.5	
GPFPLMAPK	9	98	479.264	2	32.1	956.5153	-1.1	
VYPFPGPL	8	98	445.245	2	47.9	888.4745	0.1	
VLPGGSAEQ(+.98)R	10	97	507.765	2	13.6	1013.514	2.1	Deamidatior
TLLDLDNTR	9	97	530.785	2	29	1059.556	0.3	
VYPFPGPL	8	97	445.245	2	48.1	888.4745	0	
SPGAGPGGERGPWPD	15	96	718.833	2	19.1	1435.648	1.9	
LTYLKGE	7	96	412.232	2	19.5	822.4487	0.3	
YEMPFPK	7	96	456.22	2	30.7	910.4258	-0.9	
KVPQLQ(+.98)L	7	96	413.755	2	31.6	825.496	-1	Deamidatior
KAFPSALNQDNYPNGGFTAEQL	24	96	871.096	3	31.8	2610.271	-1.6	
VYPFPGPLK	9	96	509.292	2	32.6	1016.57	0	
VYPFPGPL	8	96	445.245	2	47.7	888.4745	0	

VLPN(+.98)SAQ(+.98)ER	9	95	508.255	2	13.9	1014.498	-2.7	Deamidatior
DM(+15.99)ALNPSKE	9	95	510.738	2	14.3	1019.459	1.4	Oxidation (M)
LLQQVDTSTR	10	95	580.818	2	19.1	1159.62	1.1	
ESAPSFSDLPNPLHK	15	95	546.942	3	32.9	1637.805	-0.4	
GPSGPAAVR	9	94	406.225	2	15.8	810.4348	0	
YKVPQLE	7	94	438.745	2	26.7	875.4752	0.5	
VLPNQ(+.98)MPFPK	10	94	586.312	2	31.6	1170.611	-0.6	Deamidatior
LFPPQVVPE	9	94	513.287	2	42.7	1024.559	-0.5	
YQEPVLGPVRGLMPL	15	94	834.963	2	50.5	1667.907	2.5	
QGPAGASGAPGPR	13	93	561.788	2	13.3	1121.558	2.8	
LYQQ(+.98)PVLGVPR	11	93	635.861	2	30.8	1269.708	-0.4	Deamidatior
FPPLQVVPE	9	93	513.288	2	42.4	1024.559	1.9	
QLFPPVVPE	9	93	513.287	2	42.6	1024.559	0	
YALN(+.98)AQR	7	92	418.717	2	16.9	835.4188	0.5	Deamidatior
NMALNPSKE	9	92	502.247	2	17.6	1002.48	-0.4	
HLLPLPK	7	92	409.268	2	26	816.5222	-0.5	
YQEPVLGPVR	10	92	579.32	2	27.6	1156.624	0.4	
KGGVPAGTC(+57.02)TSLK	13	91	425.896	3	13.5	1274.665	0	Carbamidom
DMALNPSKE	9	91	502.74	2	18.8	1003.464	0.3	
LSSLSVAR	8	91	416.748	2	23.4	831.4814	-0.4	
TPPFTLPQHRGM	12	91	461.24	3	25.7	1380.697	1	
TSAVGDMKF	9	91	478.232	2	28.8	954.4481	0.7	
GPFPLSMKE	9	91	503.258	2	31.8	1004.5	1.3	
EPVLGPVRGPFPL	13	91	689.398	2	48.3	1376.782	-0.5	

GPSGEAGVAAGN(+.98)KQTGP	20	90	590.627	3	17.3	1768.859	-0.4	Deamidatior
HPDQGPSGPVGPAGPR	16	90	509.255	3	17.3	1524.743	0.7	
SPGGGGGGLGSAKDR	15	90	636.817	2	17.6	1271.622	-1.3	
FAYLDAQR	8	90	492.251	2	26.4	982.4872	-0.8	
GPFPLLNK	8	90	443.263	2	31	884.512	-0.6	
TPPVMFKDPLPQHPQSY	17	90	661.331	3	33.5	1980.977	-2.9	
ESLLLASGVVVGK	13	90	636.382	2	38.8	1270.75	-0.4	
TPVVVPPFLQPE	12	90	661.872	2	52.3	1321.728	1.6	

ALC denotes average local confidence; RT denotes retention time; PTM indicates if a post translational modification was found in the sequenced peptide.

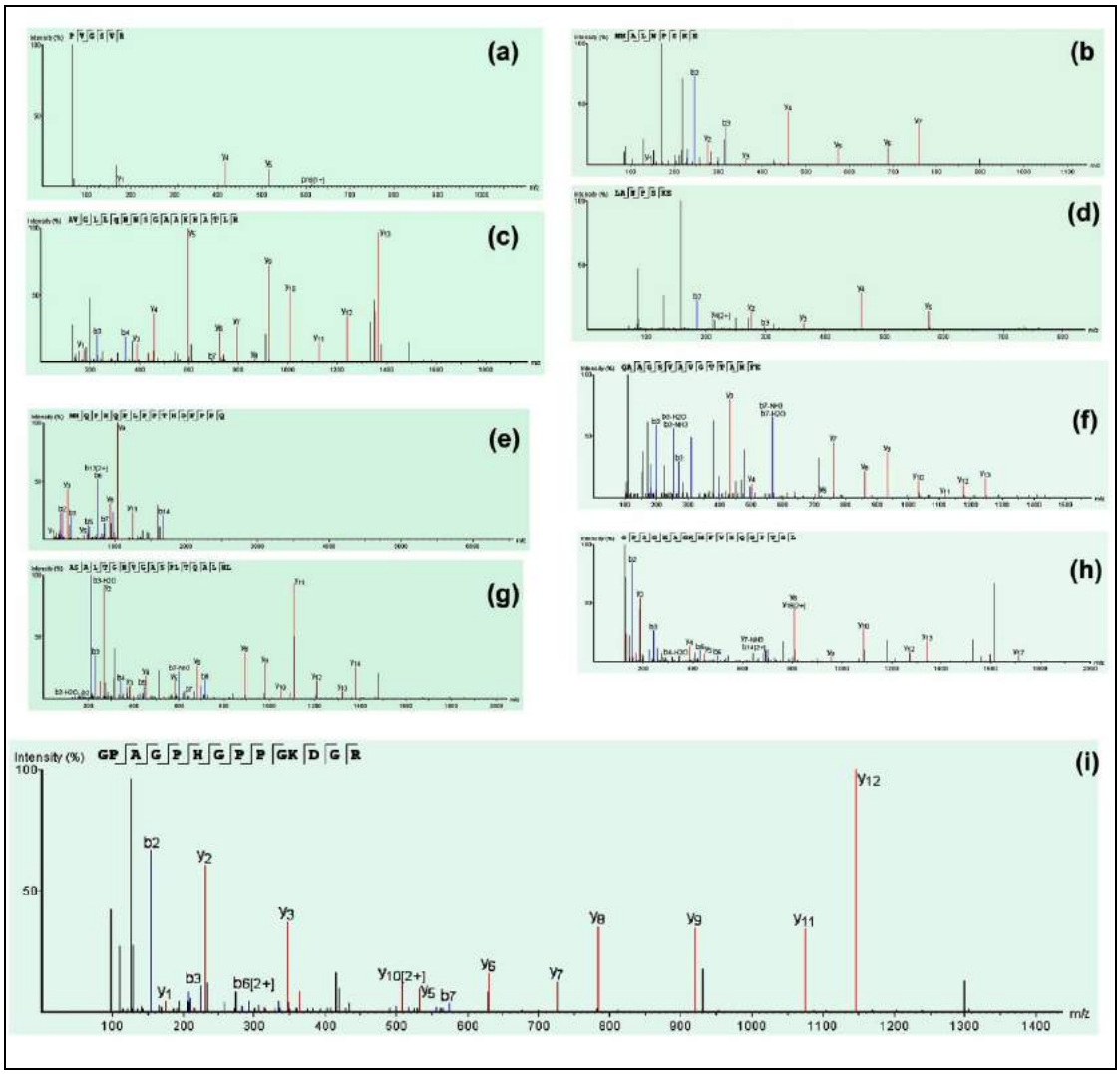


Fig. 48 a-i). *De novo* spectra of eight peptides of the Cry-c AFP. Vertical lines indicates the position of the fragmentation and horizontal line indicates of it is a y ion (if horizontal line is on the right side of the vertical line) or a b ion (if horizontal line is on the left side of the vertical line).

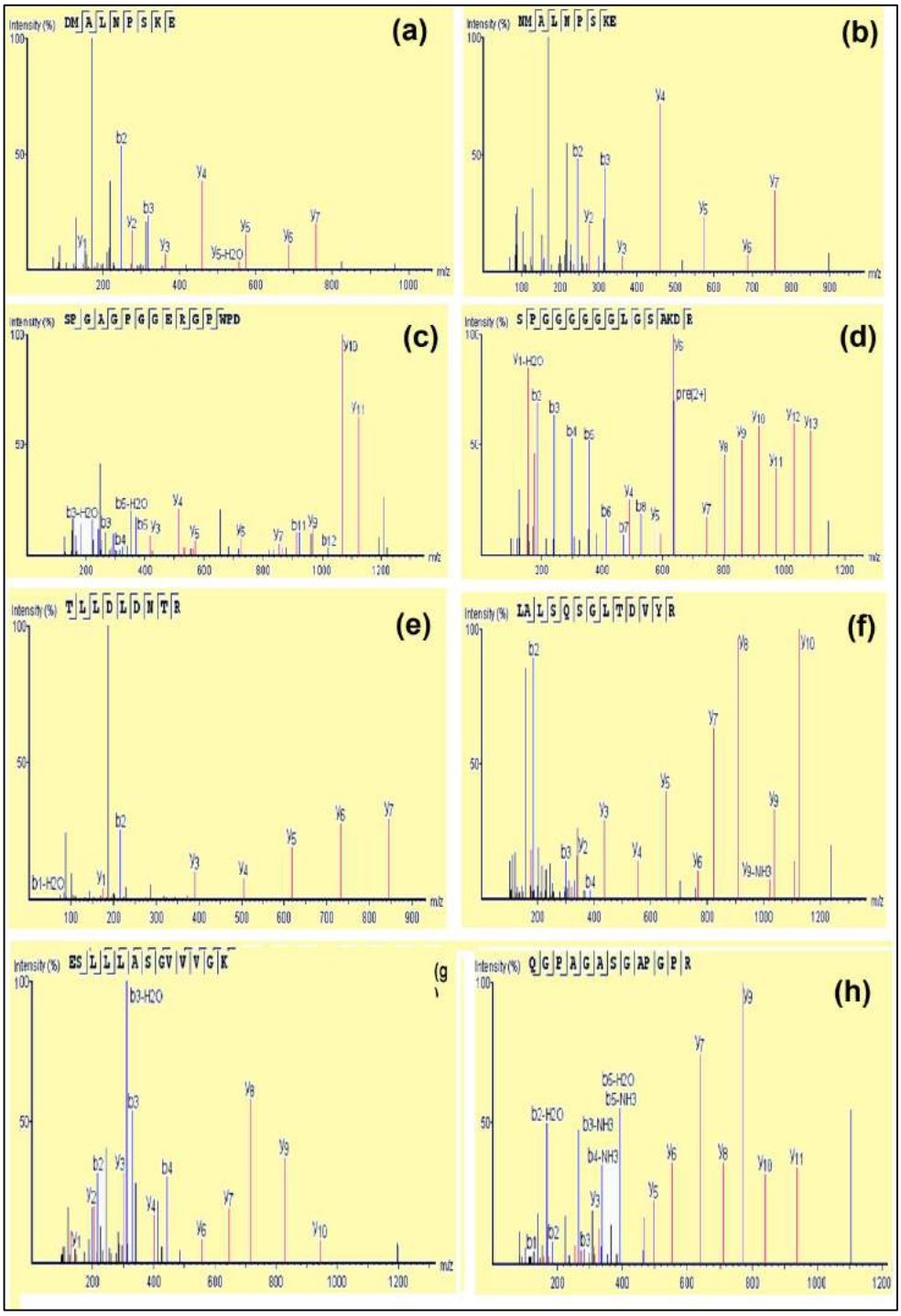


Fig. 49 a-g) *De novo* (MS/MS) spectra of seven identified peptides of the Cry-g AFP. (a,b) showing NPS-Motif, (c,d) showing Glycine rich poly G-motif (e-g) showing Leucine rich Motif (LRM), (h) showing metal binding sites GAS, GAP-repeats.

The AFPs produced by the Svalbard strains *Cryobacterium psychrotolerans* (Cry-c) and *Pseudomonas ficuseractae* (Cry-g) were preliminarily characterized and analyzed to comprehend the nature of the peptide/protein using the computational approach. ESI-Q-TOF analysis of the Cry-c and Cry-g AFPs generated a weak signal in the range of the molecular mass of 22.14 kDa (Fig. 45a, b) that corroborates its observation on sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis which was ~22 kDa (Singh *et al.* 2014) .

As a result of MELD and nanoLC-ESI-MS/MS analysis, 21 peptides of Cry-c (details summarized in Table 39) and 55 peptides of Cry-g (Table 40) were sequenced with a high-level confidence. Twenty one *de novo* sequenced peptides of Cry-c and fifty five peptides of Cry-g were subjected to the NCBI database search. However, no significant hits were obtained after the NCBI BLAST analysis. Out of the 21 peptides of the Cry-c antifreeze protein, an N-terminal sequence was identified as P**V**GSV**R** with an m/z value of 307.68 (Fig. 48a). This six amino acid peptide sequence is found to partially match with the previously determined Cry-c AFP N-terminal amino acid sequence AVPXGSVXAXVXXGAATTFX (Singh *et al.* 2014). In the previous study, though the first 20 amino acids of N-terminal sequence were revealed, the present analysis reveals the X- amino acids (4^{th} and 8^{th}) as V and R respectively. Thus the entire 20 N-terminal amino acid sequence is reconstructed as AVPVGSVRAXVXXGAATTFX. However, this sequence did not find any significant homology in the NCBI with any known antifreeze or ice binding protein.

The physico-chemical parameters, molecular mass, total number of positive and negative residues, aliphatic index and grand average hydrophathy (GRAVY) were computed using the Expasy's ProtParam (http:// us.expasy .org/ tools /protparam.html). The very high aliphatic index of all AFPs indicates that AFPs may be stable for a wide range of temperature; the calculated aliphatic index of this Cry-c

AFP is 64.30. The Grand Average Hydropathy (GRAVY) index of AFPs range from –0·1 to 0·9. The very low (─0.677) GRAVY index of AFP indicates that the AFP could result in a better interaction with water.

5.4 Discussion

In the present study, eight bacterial strains belonging to four species were reported to produce AFPs, and of these, six bacterial AFPs were characterized partially. AFP produced by cryoconite bacterial strains *Cryobacterium psychrotolerans* (Cry-c) and *Pseudomonas ficuseractae* (Cry-g) could be characterized to a greater extent.

On purification and SDS-PAGE analysis, the AFP was observed to ~22 kDa in size. However, ESI-Q TOF analysis of the Cry-c and Cry-g protein revealed its exact molecular mass to be 22.14 kDa. This is much smaller than the bacterial AFPs, which have a typical size of 52–164 kDa (Xu *et al.* 1998; Yamashita *et al.* 2002, Gilbert *et al.* 2004; 2005, Kawahara *et al.* 2004; 2007, Muryoi *et al.* 2004) and even smaller than the bacterial ice-nucleating proteins, which size between 120 and 180 kDa (Gurian-Sherman and Lindow 1993, Kawahara 2002). The proteins reported in the current study matched in molecular weight with proteins from the broad AFP family, IBP-1, isolated from the bacterium *Colwellia* sp. (Raymond *et al.* 2007), the fungi *Coprinus psychromorbidus* and *Typhula ishikarieneis* (Hoshino *et al.* 2003a, b), sea ice diatoms *Fragilariopsis* sp. (Bayer-Giraldi *et al.* 2010), crustacean *Stephos longipes* (Kiko 2010), and algae (Raymond and Morgan-Kiss 2013). It was cogently argued that widespread distribution of these AFPs in eukaryotic and prokaryotic polar organisms and their resemblance with one another point toward their possible spread through horizontal gene transfer (Sorhannus 2011). It was also suggested by Raymond *et al.* (2007) that the restricted gene occurrence theory as proposed by Doolittle (2002) supports the probable event of horizontal gene transfer.

It is assumed that TH activity of AFPs is low in microorganisms, fish, and plants as compared to that of insects existing in the cold. However, in the present study, it was recorded that the cry-g and cry-n bacterial isolates had a TH value as high as c. 2 °C at 2.4 mg mL^{-1}. Within microbial AFPs, relatively high TH values (c. 2 °C) were observed in *M. primoryensis* (Garnham *et al.* 2008) and *T. ishikarieneis* (Xiao *et al.* 2010). IRI activity was observed in AFPs produced by most cultures analyzed here. Only *P. ficuserectae* did not inhibit ice recrystallization, indicating that the IRI activity of the AFPs did not relate to the strength of TH activity. This is in agreement with Yu *et al.* (2010) and Olijve *et al.* (2016), who stated that there is hardly any correlation between TH and IRI activity. It is viewed that AFPs bind to the surface of ice grains and inhibit their aggregation. Two isolates may have comparable TH activity because they have similar ice-binding surfaces but they differ in non-ice-binding face because of which they may exhibit different levels of IRI activity. The significance of non-ice-binding face of AFPs was also observed by Nutt and Smith (2008). 'AFP isoforms groups' are present in bacterial cell free supernatant. TH activities in AFP isoforms mixture with unknown ratio were measured in the current study. Also, in this study, concentrations of AFPs were estimated by weighing the purified AFP powders (freeze-dried) which had isoforms. The different isoforms can display different activities, and that a mixture of isoforms can have different effect on ice than the isoforms individually. All the AFPs sequences reported so far belong to the same family, IBP-1, which is widespread throughout the polar organisms.

Out of 21 peptides detected in the *Cryobacterium psychrotolerans* Cry-c, twelve peptides are found to have glycine, and four peptides have shown Gly (G)-Pro (P) motif (Table 39); in these four peptides, the GP motif is found to be present more than once. AFP in our present study is relatively rich in glycine (11.8%) which imparts flexibility to the protein molecule, whereas hydrophobic residues proline and alanine constitute 11.8% and 11% respectively. The present finding is in partial agreement with snow-flea AFP rich in glycine reported earlier (Graham and Davies 2005, Mok *et al.* 2010). Table 39 indicates the presence of

several peptide fragments showing the presence of prolines, for example, a 17-amino acid peptide revealed the presence of six proline residues. As was reported by Sönnichsen *et al*., (1996), the ice-binding site is the most hydrophobic face of the protein and in several cases is very hydrophobic, like the alanine-rich ice-binding surface of winter flounder and shorthorn sculpin type I AFPs (Baardsnes *et al*., 2001).

Proline (11.8%) in Cry-c AFP has a unique characteristic of displaying kinks in polypeptide chains and disrupting ordered secondary structure. Previous studies on pshychrophiles reported the presence of compatible solutes such as proline, and glutamine (Russell 1990). In the ASALTGNVGASPLTQALHL peptide, proline might act as a helix breaker whereas small side chains presented by VGAS might create a metal binding site as apparently revealed by model structure (Fig. 50) which is yet to be established by an experimental validation. Studies of selected permafrost isolates indicated the dominance of glutamine and proline (as compatible solutes) when exposed to low temperatures. Proline has also been shown to depress the freezing point of water in some plants (Aspinall and Paleg 1981) and thus high proline content enables freezing tolerance. Rudolph *et al.*, (1986) argued that intercalating prolines between membrane phospholipids, helps membrane stabilization by maintaining hydrogen bonds.

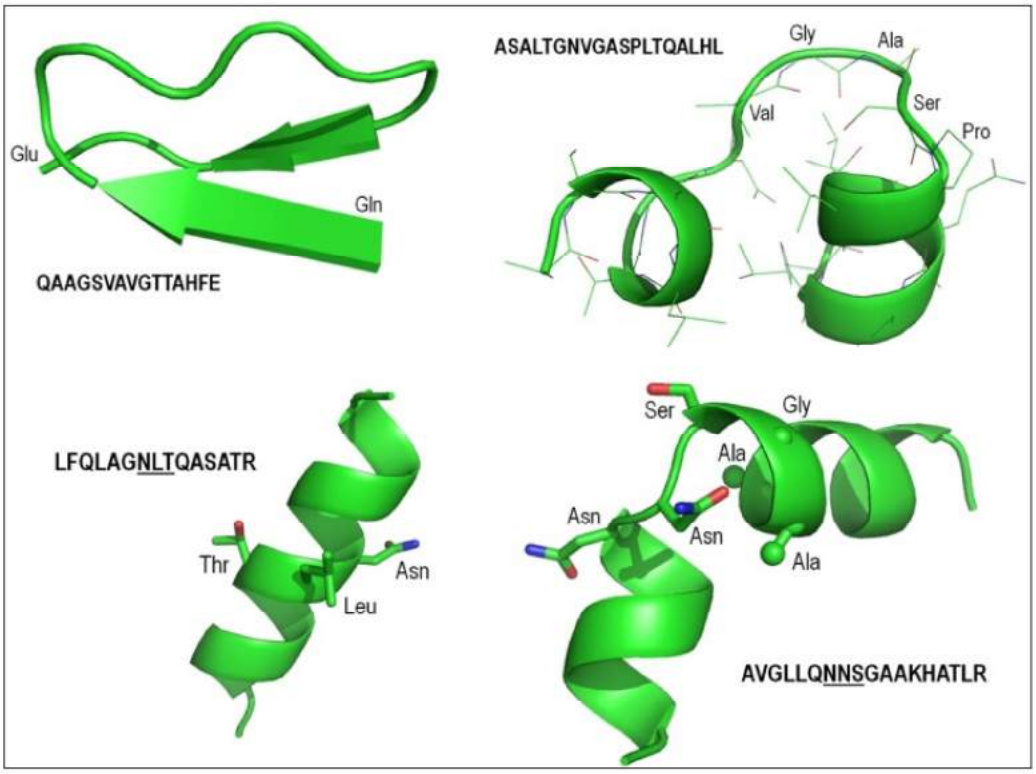


Fig. 50 Ribbon presentation of the selected alanine and glycine rich peptide fragment 3-D structure model generated by the PEP-FOLD algorithm. Sequence of amino acid residues in peptide fragments written in single letter codes, highlighted residues in 3-letter codes, motifs like NXS highlighted as underlined. The images are generated by using Pymol.

The detection of relatively glycine-rich sequences such as GPSGEAGKMFVHQGPTGL, GPAGPHGPPGKDGR, asparagines and alanine-rich sequences DMAL**NES**KE, NMAL**NPS**KE, LA**NPS**KE, AVGLLQ**NNS**GAAKHATLR, ASALTGNV**GAS**PLTQALHL and QAAGSVA VGTTAHFE (Table 39 and Fig. 48a-2i) signify the essential roles of those amino acids in the antifreeze activity (Lin *et al*. 2007). Meyer *et al*. (1999) reported the presence of NXS/T domain in the carrot AFP in the form of NPT, NFS and NVS. Our results are in partial agreement with their findings as two peptides are found to possess **NPS** motifs, one with NES which have not been reported so far in any AFP from the cryoconite bacterial isolates. The peptide fragment AVGLLQ**NNS**GAAKHATLR (Fig. 48b) modeled *in silico* (Fig. 50**)** using the

PEP-FOLD software has shown NXS motif present between two helices that might participate in interaction with aqueous environment; this motif is followed by GAA that might possibly assist in packing and enable the interaction of NXS/T motif with exterior aqueous environment. Interestingly the amino acid residues such as GAA, GAS and GPGG found in the large isoform of snow-flea AFP (Mok *et al.* 2010) are also recorded in the Cry-c AFP peptides (Table 39) that are in partial agreement with the observations made by Slaugther *et al.*, (1981) who referred to the sea raven AFP where alanine was the most common amino acid residue. The peptide LFQLAGNLTQASATR contains NXS motif that might be involved in helix packing (Fig.3) too.

The presence of amino acid residues threonine (T) and aspartic acid (D) in significantly high concentration in all of these AFPs and the interactions between these three amino acid (T, A and D) residues with water or ice might play a key role in producing thermal hysteresis and provide a unifying mechanistic thread for the action of all of the fish AFP (Slaugther *et al*. 1981). In the Cry-c AFP, threonine (6.1%), alanine (11%) and aspartic (2.6%) amino acid residues might play a similar role. α-helical AFP's having abundance of exposed hydrophilic amino acid residues such as threonine, glutamine, serine and asparagines on the surface are spaced out in the helix structures. It was proposed that these hydrophilic residues, and especially threonine, constitute hydrogen bonds to the surface of the ice crystal. When an AFP constitutes hydrogen bonds to the ice crystal, the hydrophobic side of the AFP gets oriented at the liquid water face. This hydrophobic interaction between one side of the AFP and water and the hydrophilic interaction between the other side of the AFP and the ice crystal is thought to be the mechanism by which AFP's inhibit ice growth (Chen *et al*. 1997). Though in our present study, we have not observed any ice-binding leucine rich repeat (LRR) as reported by Meyer *et al*. (1999) (in carrots and some of the Arctic fish), 5 peptides with leucine rich domains were observed in our study. Taken together, the Cry-c AFP is endowed with several unique and novel characteristics which tempt us to carry forward the work to look in to the molecular characteristics of this antifreeze protein.

In the Cry-g AFP, purified from the cryoconite isolate *Pseudomonas ficuserectae* JCM19509, the N-terminal domain of the N-terminally sequenced peptide NSNPSPVXLGSAXTFAILSQ showed the presence of NPS motif (Table 40). Besides this, the peptides DMALNPSKE and NMALNPSKE found in the same Cry-g peptide contain the NPS motifs (Fig.49a, b). These NPS motifs were found too in the purified AFP from *Cryobacterium* sp. JCM 19503. Meyer *et al*. (1999) reported the presence of NXS/T domain in the carrot AFP in the forms of NPT, NFS and NVS.

Another antifreeze protein AfpA also reportedly contained five calcium-binding motifs (G-X-G-X-D) that are less conserved than the G-G-X-G-X-D motif found in the cell surface proteins of other Gram-negative bacteria (Chabeaud et al. 2001). Though similar motif was not found in the AFPs of *Pseudomonas ficuserectae* (Cry-g) JCM19509, G-X-G-N-N- and non-conserved G-X-G-X-X-D (Lorv et al. 2014) motifs were found in AFP of other *Pseudomonas* sp.; in Cry-g AFP, for example, the peptide SPGAGPGGERGPWPD (Fig. 49c) with this kind of motif has been detected. The present finding is in partial agreement with snow-flea AFP rich in glycine reported earlier (Graham and Davies 2005; Mok *et al.* 2010).

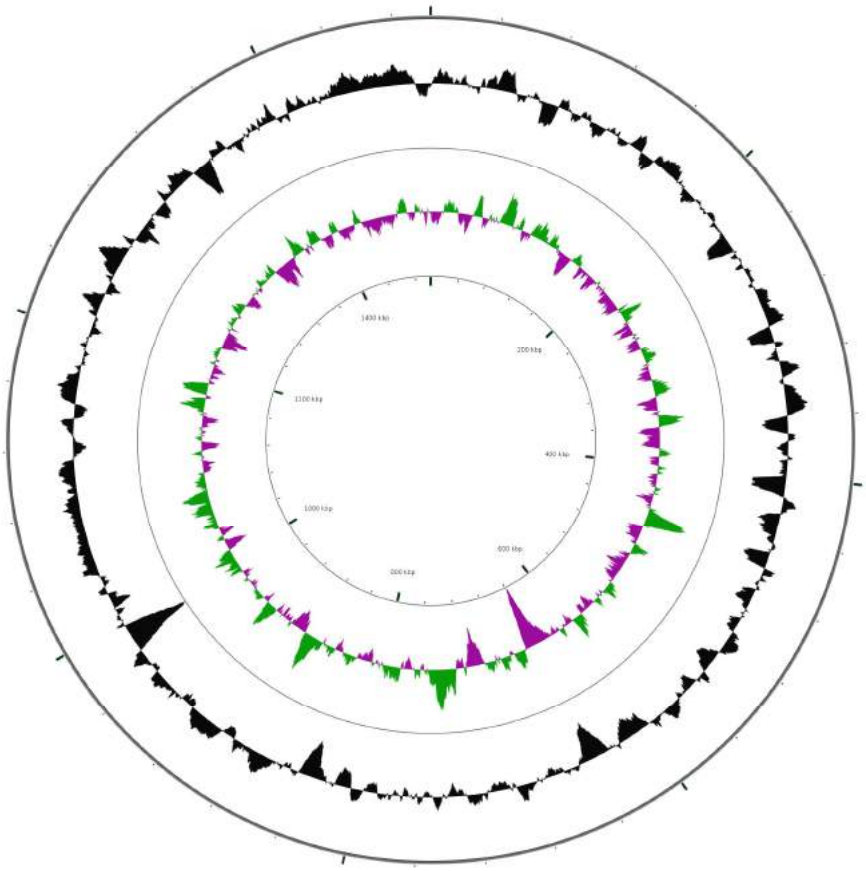
Likewise in several antifreeze peptides derived from the same cryoconite species were found the peptides with GPX (where X = S,T,P,W) motif (Table 40). Whether these sequences are calcium-binding motif can not be understood with certainty. In another case, the glycine-rich antifreeze protein found to be produced by snow fleas exhibited strong freezing point depression activity without adequately changing the melting point of its solution (TH). In present study, the presence of polyglycine motif $SP(G)_6LGSAKDR$ (Fig. 49d) in the AFP produced by *P. ficuserectae* (Cry-g) was not reported earlier anywhere else. Thus in several G-rich sequences, glycines may provide backbone carbonyl-amide hydrogen bonding to neighboring helices (Lin *et al.* 2007). The absence of side chains (in the G-rich motifs) probably allows the main chains to be packed into a hexagonal array with each chain hydrogen-bonded to six neighbors through back-

bone amide groups (C=O and N-H) that project normal to the helical axis (Lin *et al.* 2007). Additionally, five leucine rich peptides (Table 40) were detected in the Cry-g antifreeze peptide (Fig. 49 e-g). Leucine rich repeats (LRR) were previously reported in carrots and some of the arctic fish (Meyer *et al*. (1999). In carrots, an LT-up-regulated AFP showed a significant similarity (50-65%) to the polygalacturonase inhibitor family of plant leucine-rich repeat (LRR) proteins (Meyer *et al.* 1999).

5.5 Conclusion

The polar organisms probably, under the pressure of survival in cold condition, gained the novel property of AFP production through horizontal gene transfer. Besides this, AFP production is a conspicuous mechanism for the survival of mostly immovable microorganisms in ultralow temperatures that also involves minimization of frost damage to cells or tissues. The noticeable diversity existing amongst various microorganisms indicate that due to probable convergent evolution the AFPs produced by a particular living system might be unique to that microbe or milieu. For antifreeze proteins, merely handful bacterial sources have been studied in any detail and less number of AFPs have been isolated and characterized at the protein structure level. Determination of one of the molecular and physiological basis of adaptation strategies of microbial life in environments like cryoconite holes, yielded information that might unravel the process of evolution and ecological behaviour of microorganisms. The present study shed lights on compositional characteristics of the AFPs (Cry-c and Cry-g) and some of its unique structural features. Similar studies on AFPs from several microorganisms isolated from other cold habitats are essential in order to understand and compare their biochemical charateristics and biotechnological potentials.

6. Genome Sequencing of Selected Arctic Bacteria of Ecological and Biotechnological Significance



6a. Whole Genome sequence of *Cryobacterium* sp. MLB-32

6.1 Introduction

MLB-32 was isolated from cryoconite sample harvested from Vestre Brøggerbreen (VB) glacier, Svalbard, Arctic. MLB-32, a Gram-positive rod shaped, aerobic bacterium with an optimum growth at 4 °C, was identified as a member of the genus *Cryobacterium* based on 16S rRNA gene sequencing. MLB-32 exhibited highest 16S rRNA gene sequence (1379 nucleotides) homology of 95.4% with *Cryobacterium roopkundense* RuGI7(T). Digital DNA–DNA hybridization, performed as described by Auch *et al.* (2010), revealed only 37.90% +/−3.43 homology between MLB-32 and *C. roopkundense* RuGI7(T) indicating a distinct delineation between these two species and also depicting the novelty of strain MLB-32. These data suggested MLB-32 as a putative novel species of the genus *Cryobacterium*. In order to comprehend the genome organization of *Cryobacterium* sp. and decipher the cold adaptive strategies utilized by this microbe to survive at such low temperatures, whole genome sequencing was performed. To the best of our knowledge, draft genome sequence of this putative novel species of the genus *Cryobacterium* is reported for the first time. The strain MLB-32 has been deposited in Japan Collection of Microorganisms; under the Accession No. JCM 19504.

6.2 Materials and Methods

In the present study genomic DNA of *Cryobacterium* sp. MLB-32 was isolated using GenElute™ Bacterial Genomic DNA Isolation kit (Sigma, USA). The genome sequence was established with a whole-genome shotgun strategy using 316™ chip and 200-bp chemistry on the Ion Torrent PGM platform (Life Technologies, USA).

6.3 Results

Sequencing of the library generated 2,900,616 reads with an average read length of 208 bp. A total of 605 Mb of data was generated, with 523 Mb of data with a quality value above Q20. *De novo* assembly was performed using MIRA assembler version 4.0.5 (Chevreux *et al*. 1999) and produced 133 large contigs (>8000 bp) and 667 smaller contigs (>8000 bp) featuring G+ C content of 64.9%, an N_{50} contig size of 20,128 and N_{90} contig size of 2149 and the largest contig size of 130,596 (Table40).

Table 40 Cryobacterium sp. MLB-32 genome statistics.

Attribute	Value
Genome size	43,67,160
G + C content (%)	64.9
Total no. of subsystems	382
Total genes	3481
rRNAs	11 (5S, 16S, 23S)
tRNA genes	61
Protein coding genes (CDS)	3214

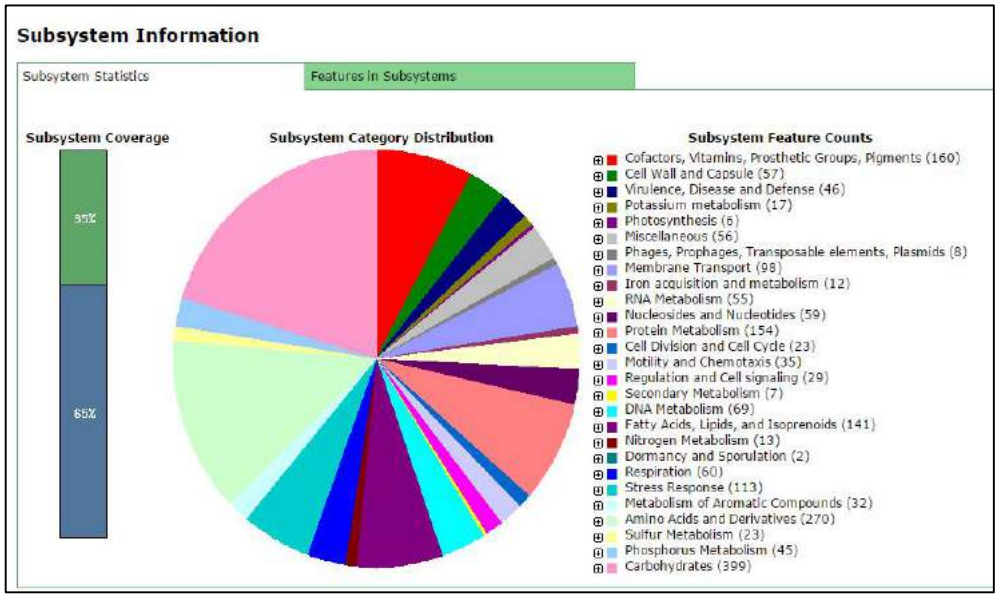


Fig. 51 Rapid Annotation using Subsystem Technology (RAST) Annotation

Gene prediction and functional characterization was performed with the help of Rapid Annotation using Subsystem Technology (Aziz *et al.* 2008) and the National Centre for Biotechnology Information Prokaryotic Genome Automatic Annotation Pipeline (PGAAP) version 2.6. RAST tool identified *Clavibacter michiganensis* sub sp. *michiganensis* NCPPB382 as the closest phylogenetic neighbor of MLB-32 (Score 507, Genome Id: 443906.13). The annotation predicted 3481 genes, including 3214 coding sequences (CDS), and 72 total RNAs (11 rRNAs and 61 tRNA genes).

A total of 339 unique genes associated with a subsystem (set of functional roles that make up a metabolic pathway, a complex, or a class of proteins) in *Cryobacterium* sp. MLB-32 were found when comparative analysis was performed with NCPPB382. These include genes associated with carbohydrate metabolism; synthesis of cell wall and capsule, cofactor, vitamin, prosthetic group, pigment, DNA, RNA and Protein metabolism; cell signaling; membrane transport; respiration; Sulfur, Nitrogen, Phosphorus and Potassium metabolism; stress response; virulence, disease and defense, motility and chemotaxis; metabolism of Aromatic compounds, fatty acids and lipids. These genes were identified as distinctive genomic elements that delineated MLB-32 from its phylogenetic relatives. Genome analyses revealed that the MLB-32 genome codes for a number of enzymes involved in resistance to fluoroquinolone, vancomycin and the β-lactam group of antibiotics. MLB-32 also harbored genes encoding resistance to toxic metal ions such as Arsenic and Mercury. The presence of stress-related proteins (three copies of genes encoding cold-shock proteins and an antifreeze protein of type I) were also detected. Cold-shock proteins (CSPs) are induced in response to temperature downshifts, thus illustrating the survival strategies in cold environments. Bacteria produce antifreeze proteins for adaptation to low temperature i.e. freeze-tolerance (Chattopadhyay 2006). Cold shock proteins are known to aid transcription and translation at low temperature in bacteria. CspA, acts as a transcriptional enhancer or as an mRNA chaperone (Bárria *et al*. 2013).

Cold active enzymes from psychrophiles are promising candidates with diverse applications in biotechnology, agriculture and medicine (Feller and Gerday 2003). The MLB-32 genome analysis revealed the presence of genes encoding industrially important enzymes, such as alcohol dehydrogenases, alpha-amylase, pullulanase, esterase, protease, hydantoinase and chitinase. These enzymes have wide applications in the food, feed, detergent, pharmaceutical and textile industries and have proven to be advantageous not only for their high specific activity, but also for their easy inactivation, reduced peril of microbial contamination, minimized energy consumption (Trincone 2011). Also, several genes associated with biosynthesis of plant growth promoting hormones like auxin, cytokinin, abscisic acid, ethylene, salicylic acid and indole acetic acid were detected indicating its immense potential to be used as a Plant Growth Promoting *Rhizobacterium* (PGPR) in cold environments.

Genes that can be associated to the psychrophilic and sea ice-specific lifestyle of MLB-32 for extreme environmental adaptations are given below.

1. Exopolysaccharide (EPS) : Glycosyl transferase - group 2 family protein (3 homologues), Undecaprenyl-phosphate galactosephosphotransferase (EC 2.7.8.6) (3 homologues), Exopolysaccharide production protein (2 homologues)

2. Polyunsaturated fatty acid (PUFA) biosynthesis: 3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100) (7 homologues) , 3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100) (3 homologues), Holo-[acyl-carrier protein] synthase (EC 2.7.8.7), Biotin carboxylase of acetyl-CoA carboxylase (EC 6.3.4.14) / Biotin carboxyl carrier protein of acetyl-CoA carboxylase (2). These are important to maintain membrane fluidity at low temperature and production of antifreeze lipoproteins.

3. Na+/H+ antiporters were detected. It is hypothesised that this pump may be specifically involved in both cold and salt tolerance in this microorganism (Karpinets *et al.* 2010).

2 Nucleotide sequence accession number

The draft genome sequence of *Cryobacterium* sp. MLB-32 was deposited in the DDBJ/EMBL/GenBank database under the Accession number JPRS00000000. The strain *Cryobacterium* sp. MLB-32 is available with the Japan Collection of Microorganisms under the accession number JCM 19504.

BASys annotation revealing protein profile of MLB-32

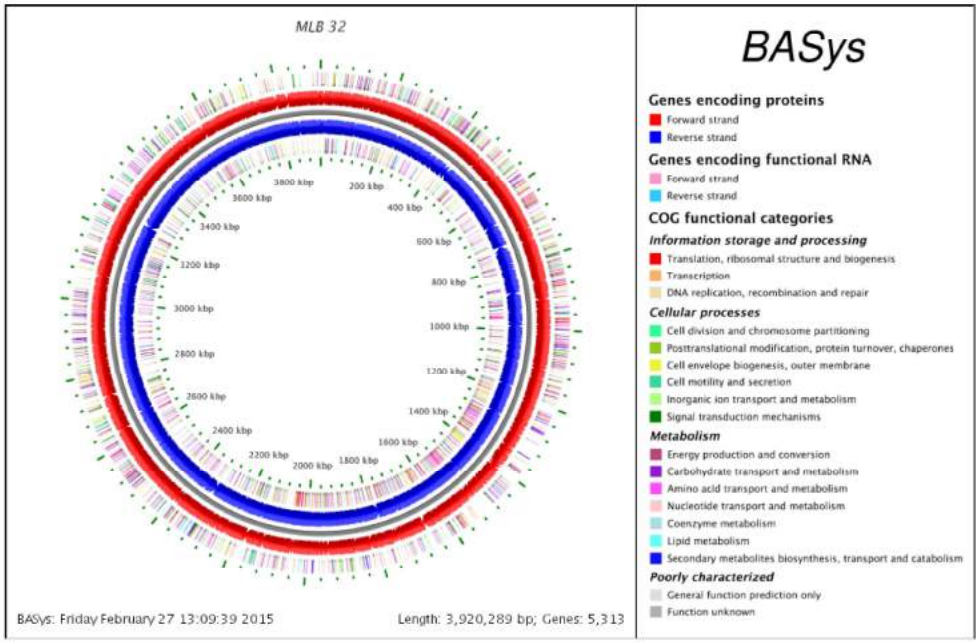


Fig. 52 COG analysis: The chromosome map from the outside inward: the first and second circles indicate predicted protein-coding regions on the plus and minus strands, respectively (colors were assigned according to the color code of the COG functional classes.

6.4 Conclusion

The first whole genome analysis of this putative novel species of the genus *Cryobacterium* revealed mechanistic insights into the metabolic adaptation of bacteria to Arctic ecosystems as well as the characterization of the biotechnological and agricultural potential of *Cryobacterium* sp. MLB-32.

7. Whole Genome sequence of *Nesterenkonia* sp. (PF2B19)

6.1 Introduction

PF2B19, a Gram-positive cocci, strictly aerobic bacterium, was identified as a member of the psychrophilic genus *Nesterenkonia* based on 16S rRNA gene sequencing. PF2B19 exhibited highest 16S rRNA gene sequence (1308 nucleotides) homology of 99.92% with *Nesterenkonia aethiopica* DSM 17733(T) and *Nesterenkonia suensis Sua*-BAC020(T). PF2B19 shared 97.09% homology with *Nesterenkonia alba* DSM 19423(T). However, the whole genomes of *Nesterenkonia aethiopica* DSM 17733(T) and *Nesterenkonia suensis* Sua-BAC020(T) are not available in the database, so *Nesterenkonia* JCM 19054, *Nesterenkonia alba* DSM 19423(T) and *Nesterenkonia* sp. AN1 were selected for Digital DNA–DNA hybridization. Digital DNA-DNA hybridization, performed as described by Auch et al. (2010), revealed only 30.20%, 26.50% and 27.40% homology between PF2B19 and *Nesterenkonia* JCM 19054, *Nesterenkonia alba* DSM 19423(T) and *Nesterenkonia* sp. AN1 respectively, indicating a distinct delineation between the species and also depicting the novelty of strain PF2B19. These data suggested PF2B19 as a putative novel species of the Genus *Nesterenkonia*. In order to elucidate the molecular mechanisms underlying the cold adaptation of this bacterium and identify potential novel biotechnologically relevant cold-adapted enzymes, the whole genome sequencing was performed.

6.2 Materials and Methods

In the present study, genomic DNA of *Nesterenkonia* sp. PF2B6 (PF2B19) was isolated using GenElute™ Bacterial Genomic DNA Isolation kit (Sigma, USA). The genome sequence was established with a whole-genome shotgun strategy

using 316™ chip and 200-bp chemistry on the Ion Torrent PGM platform (Life Technologies, USA).

6.3 Results

Genome attributes of Arctic *Nesterenkonia* sp. PF2B19

The genome of *Nesterenkonia* sp. PF2B19 was established with a whole-genome shotgun strategy using 316TM chip and 200-bp chemistry on the Ion Torrent PGM platform (Life Technologies, USA). Sequencing of the library generated 2,662,620 bp reads. The reads were de novo assembled using MIRA assembler version 4.0.5 (Chevreux *et al.* 1999). The draft genome comprises 2924 contigs, yielding a genome of ~2.6 megabases in size, with a mean GC content of 67.6%. These outcomes are similar to the sizes (2.59 to 2.81 Mb) and GC contents (62.2 to 71.5%) detected in the draft genomes of three temperate strains of *Nesterenkonia* which are available publically. The genome was annotated using the Rapid Annotations Subsystems Technology (RAST) server (Aziz *et al.* 2008). The genome codes for a predicted 3482 proteins, including 3434 coding sequences and 47 total RNAs (9 rRNA's as well as 38 tRNAs) (Table 42).

Table 42 *Nesterenkonia* sp. PF2B19 genome statistics.

Attribute	Value
Genome size	2,662,620 bp
G + C content (%)	67.6
Total no. of subsystems	382
Total no. of genes	3482
Number of RNAs	47
Protein coding genes (CDS)	3434

Comparative genomics identifies unique genes proteins in *Nesterenkonia* sp. PF2B19

RAST tool identified *Kocuria rhizophila* DC2201 as the closest phylogenetic neighbor of PF2B19. A total of 197 unique genes associated with a subsystem (set of functional roles that make up a metabolic pathway, a complex, or a class of proteins) in PF2B19 were found when comparative analysis was performed with DC2201 (Fig 53). These included genes associated with carbohydrate metabolism; synthesis of cell wall and capsule, cofactor; vitamin, prosthetic group, pigment; DNA, RNA and Protein metabolism; cell signaling; membrane transport; respiration; Sulfur, Nitrogen, Phosphorus and Potassium metabolism; stress response; virulence, disease and defense; motility and chemotaxis; metabolism of Aromatic compounds, fatty acids and lipids. These genes were identified as distinctive genomic elements that delineated PF2B-19 from its phylogenetic relatives.

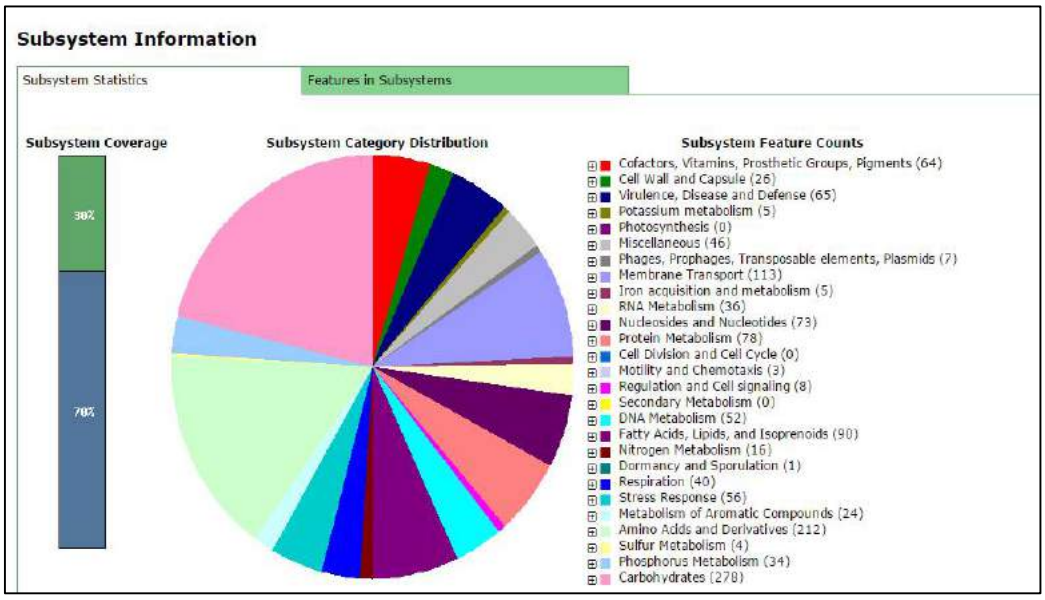


Fig. 53 Subsystem category distribution of *Nesterenkonia sp*. PF2B19

General genome comparisons of PF2B19 with its closest phylogenetic affiliates

To further prove novelty of PF2B19 and visualize differences between the available *Nesterenkonia* genomes, we generated the nucleotide alignments of the genomes shown in figure- 54, by BLASTn using the PF2B19 as the reference and the program BRIG (Alikhan *et al.* 2011). Diagram represents BLASTn results of each genome (*Nesterenkonia* JCM 19054, *Nesterenkonia alba* DSM 19423(T) and *Nesterenkonia* sp. AN1) against PF2B19 with results rendered using the BRIG program. Each genome is color coded as indicated by the legend. Relative shading density (from darker to lighter) within each circle represents relative levels of nucleotide homology. White regions indicate regions with no identity to the reference. Features of interest are annotated.

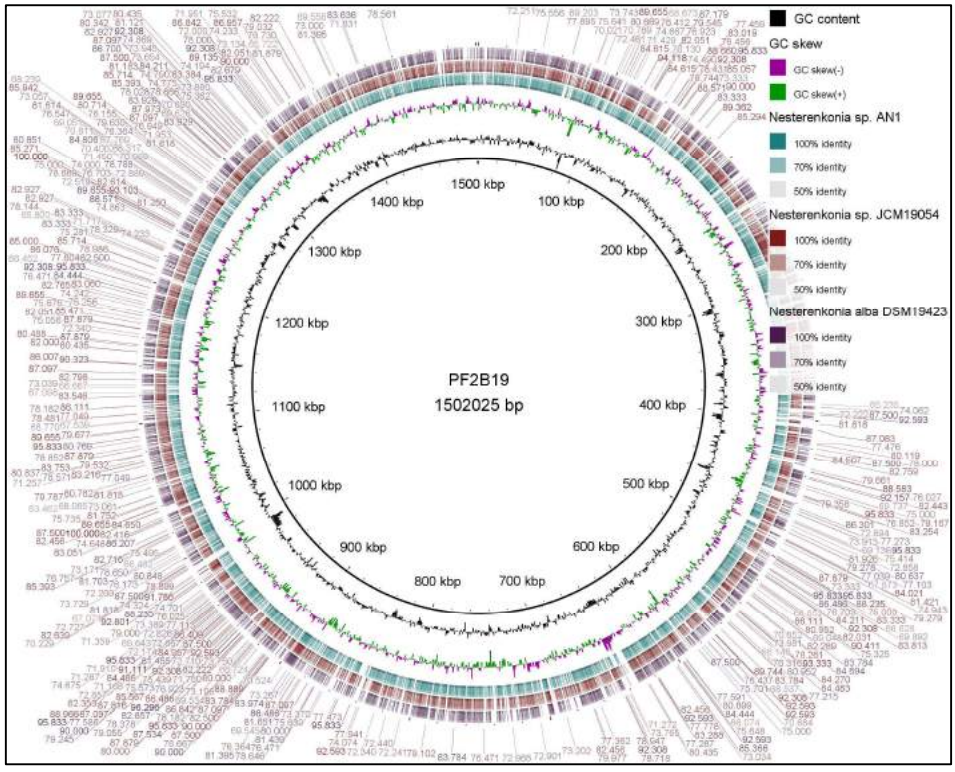


Fig. 54 Showing genome comparisons of PF2B19 with its closest phylogenetic affiliates.

Genomic traits linked to environmental stress-related adaptation:

Cold stress in Arctic is customarily allied with the effects of desiccation and sometimes with extremes of pH, high UV radiation, nutrient deficiency and salinity (Tehei and Zaccai 2005, De Maayer *et al*. 2014). A number of adaptive genes have been linked to the different environmental stresses associated with cold environments (Medigue et al. 2005, Methe et al. 2005, Gao *et al.* 2006, Riley *et al.* 2008, Overbeek *et al.* 2014). By means of genomic analysis, the genetic determinants of the adaptive strategies employed by *Nesterenkonia* sp. for survival in the cold desert soils of Arctic were determined. Analysis of the draft genome of *Nesterenkonia* sp. PF2B19 revealed a total of 78 putative stress response genes. These include 9 genes linked to cold stress response, 24 genes for oxidative stress response and 32 genes for osmotic stress response (Table 43)

Table 43. Genome features of *Nesterenkonia* sp. PF2B19 involved in adaptation to different stresses.

Sr. no.	Locus tag	Gene name	Gene products
	Cold stress		
1	FIG00001928	*cspC*	Cold shock protein CspC
2	FIG00004100	*cspA*	Cold shock protein CspA
3	FIG00000143	*infB*	Translation initiation factor 1
4	FIG00000391	*pnp*	Polyribonucleotide nucleotidyltransferase (EC 2.7.7.8)
5	FIG00000102	*infB*	Translation initiation factor 2
6	FIG00000325	*nusA*	Transcription termination protein NusA
7	FIG00023369	*dnaK*	Chaperone protein DnaK
8	----	*hrpA*	ATP-dependent helicase HrpA
9	---	*ysgA*	Uncharacterized tRNA/rRNA methyltransferase YsgA
	DNA repair		
10	FIG00000317	*recN*	DNA repair protein RecN
11	FIG00000190	*recR*	Recombination protein RecR
12	FIG00000065	*xthA*	Exodeoxyribonuclease III (EC 3.1.11.2)
13	FIG00035634	*mutM*	Formamidopyrimidine-DNA glycosylase (EC 3.2.2.23)

14	FIG00000234	*recA*	RecA protein
15	FIG00004099	*recX*	Regulatory protein RecX
16	FIG00000314	*ruvA*	Holliday junction DNA helicase RuvA
	Membrane fluidity		
17	FIG00621114	*fabG*	3-oxoacyl-[acyl-carrier protein] reductase (EC 1.1.1.100)
18	FIG00000928	*crtB*	Phytoene synthase (EC 2.5.1.32)
19	----	*idi*	Isopentenyl-diphosphate delta-isomerase (EC 5.3.3.2)
20	FIG00440067	*fabG*	short-chain dehydrogenase/reductase SDR
21	FIG00005243	*aas*	1-acyl-sn-glycerol-3-phosphate acyltransferase (EC 2.3.1.51)
	Oxidative stress		
22	FIG00001109	-	4-hydroxyphenylpyruvate dioxygenase (EC 1.13.11.27)
23	FIG00004487	*trxC*	Thiosulfate sulfurtransferase, rhodanese (EC 2.8.1.1)
24	FIG00007339	*ntcA*	transcriptional regulator, Crp/Fnr family
25	FIG00000013	*yrkH*	Hydroxyacylglutathione hydrolase (EC 3.1.2.6)
26	FIG00001181	*sodC*	Superoxide dismutase [Cu-Zn] precursor (EC 1.15.1.1)
27	FIG00008490	*cobB*	NAD-dependent protein deacetylase of SIR2 family
28	FIG01304149	---	Glutathione S-transferase domain protein
29	-----	*hcaC*	Ferredoxin, 2Fe-2S
30	FIG00050592	*sodA*	Superoxide dismutase [Mn] (EC 1.15.1.1)
31	FIG00000313	*katA*	Catalase (EC 1.11.1.6)
32	FIG00003291	*nrdH*	Glutaredoxin-like protein NrdH, required for reduction of Ribonucleotide reductase class Ib
33	FIG01318274	*trxA*	Thioredoxin
34	FIG01955919	*trxB*	Thioredoxin reductase
35	------	*linE*	Glyoxalase family protein
36	FIG01258694	*ahpC*	Alkyl hydroperoxide reductase subunit C-like protein
37	FIG00132548	*bcp*	Thiol peroxidase, Bcp-type (EC 1.11.1.15)
38	FIG01955919	*trxB*	Thioredoxin reductase (EC 1.8.1.9)
39	FIG00002441	*fur*	Transcriptional regulator, FUR family
40	----	*hcaC*	3-phenylpropionate dioxygenase ferredoxin subunit
41	FIG00011423	*bphG*	Ferredoxin reductase
42	FIG00022569	*pcaR*	Transcriptional regulator, IclR family
43	FIG00008490	*cobB1*	NAD-dependent protein deacetylase of SIR2 family
44	FIG00007339	*ntcA*	transcriptional regulator, Crp/Fnr family
45	FIG00005527	*bphC*	Catechol 2,3-dioxygenase (EC 1.13.11.2)
	Osmo-protection		
46	FIG00057416	*betA*	Choline dehydrogenase (EC 1.1.99.1)
47	FIG00001690	*betP*	High-affinity choline uptake protein BetT
48	FIG00000539	*gltB*	Glutamate synthase [NADPH] large chain (EC 1.4.1.13)

49	FIG00001198	*betC*	Choline-sulfatase (EC 3.1.6.6)
50	FIG00012746	*opuD*	Glycine betaine transporter OpuD
51	FIG00008556	*opuCA*	L-proline glycine betaine ABC transport system permease protein ProV (TC 3.A.1.12.1)
52	FIG00001349	*otsB*	Trehalose-6-phosphate phosphatase (EC 3.1.3.12)
53	FIG00001100	*otsA*	Alpha,alpha-trehalose-phosphate synthase [UDP-forming] (EC 2.4.1.15)
54	---	-	Na(+) H(+) antiporter subunit G
55	---	-	Na(+) H(+) antiporter subunit F
56	----	*mrpD*	Na(+) H(+) antiporter subunit D
57	---	*mrpE*	Na(+) H(+) antiporter subunit E
58	---	*mnhC1*	Na(+) H(+) antiporter subunit C
59	----	*mrpA*	Na(+) H(+) antiporter subunit A; Na(+) H(+) antiporter subunit B
60	FIG00008556	*opuCB*	Glycine betaine ABC transport system permease protein
61	-----	*mrpG*	Na(+) H(+) antiporter subunit G
62	----	*mrpC*	Na(+) H(+) antiporter subunit C
63	----	-	FIG152265: Sodium:solute symporter associated protein
64	----	-	Na(+) H(+) antiporter subunit F
65	-----	-	Na(+) H(+) antiporter subunit E
66	-----	*mrpD*	Na(+) H(+) antiporter subunit D
67	FIG00001690	*betT*	High-affinity choline uptake protein BetT
68	FIG00007957	*gltB*	Glutamate synthase [NADPH] small chain (EC 1.4.1.13)
69	FIG00002647	*ectA*	L-2,4-diaminobutyric acid acetyltransferase (EC 2.3.1.-)
70	FIG01269316	*gbsA*	Betaine aldehyde dehydrogenase (EC 1.2.1.8)
71	FIG00057416	*betA*	Choline dehydrogenase (EC 1.1.99.1)
72	FIG00000846	*baeS*	Osmosensitive K+ channel histidine kinase KdpD (EC 2.7.3.-)
73	FIG00000539	-	Glutamate synthase [NADPH] large chain (EC 1.4.1.13)
74	FIG00008556	*opuBB*	Glycine betaine ABC transport system permease protein
75	FIG00001101	*putA*	Proline dehydrogenase (EC 1.5.99.8) (Proline oxidase)
	General stress		
76	FIG00017615	-	Universal stress protein
77	FIG00017431	-	Serine phosphatase RsbU, regulator of sigma subunit
78	FIG01967571	*glbO*	Hemoglobin-like protein HbO

A. Cold stress response

A vital element in the cold stress adaptive tactics of bacteria is the rapid production of cold-inducible proteins (Phadtare *et al.* 1999). These are classified into two groups: cold shock proteins (CSPs) which are rapidly and

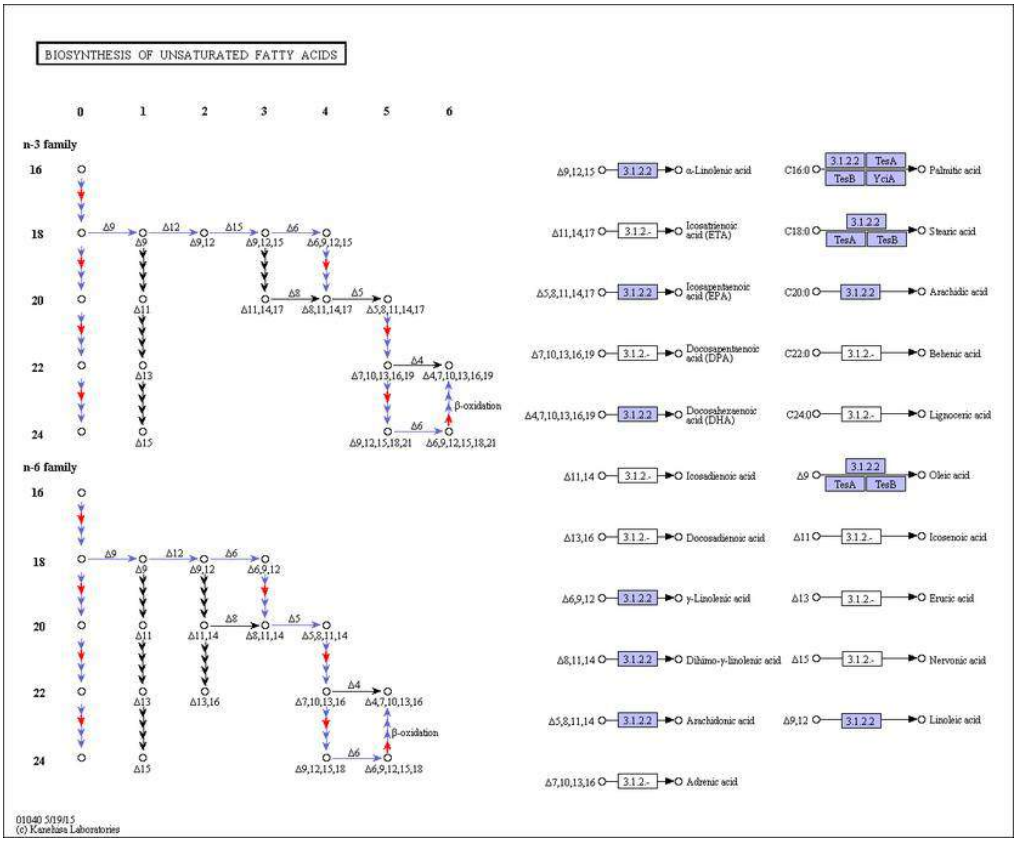
transiently induced after a downshift in temperature, and cold acclimation proteins (CAPs) which are continuously expressed throughout the period of low temperature exposure (Phadtare *et al.* 1999, Phadtare 2004). The genome of *Nesterenkonia* sp. PF2B19 encodes orthologs of the cold shock proteins CspA and CspC. CspA and other cold-inducible CspA-like proteins have been shown to act as RNA chaperones which destabilize mRNA secondary structures, thereby enhancing translation efficiency at low temperatures (Rabus *et al.* 2004, Chaikam and Karlson 2010). The genome also contains genes which encode the secondary CSPs polyribonucleotide nucleotidyltransferase (PNPase) and translation initiation factor (Inf2) which are typically induced via transcription antitermination (Bae *et al.* 2000). CspA mediates transcription antitermination and prevents the formation of hairpin loops in nascent transcripts and enhances the production of intact transcripts (Bae *et al.* 2000, Phadtare *et al.* 2007, Chaikam and Karlson 2010).

Modulation of membrane fluidity is an essential adaptation strategy in cold environments. It involves increased production of unsaturated fatty acids, modification of fatty acid branched chains and shortening of fatty acyl chains (Feller 2003, Feller and Gerday 2003, D'Amico *et al.* 2006). The *Nesterenkonia* sp. PF2B19 genome encodes 5 proteins involved in fatty acid biosynthetic pathways (Table 43). These include FabG which catalyse the onset of the reduction reaction in fatty acid biosynthesis, the condensation of fatty acids and the synthesis of branched fatty acids (Hoang et al. 2002, Methe *et al.* 2005). The genome also codes for an ortholog of 1-acyl-sn-glycerol-3-phosphate acyltransferase (PlsC), which catalyses conversion of intermediates in phospholipid synthesis, and 3-ketoacyl-(acyl-carrier-protein) reductases, which enhances the production of polyunsaturated lipids (Hoang et al. 2002, Methe *et al.* 2005). Also, the pathway for unsaturated fatty acid synthesis was detected in PF2B19 using KEGG pathway tool.

Pigments play a major role as modulators of membrane fluidity at low temperatures (Chintalapati *et al.* 2004, Morgan-Kiss *et al.* 2006, Dieser *et al.*

2010). The genome of *Nesterenkonia* sp. PF2B19 contains crtB (phytoene synthase) gene with putative roles in carotenoid biosynthesis (Table 43).

Sigma factors are dissociable units of bacterial RNA polymerase that control the conditional expression of a specific set of genes in response to a particular stress or stimulus. Copies of genes of the σ70 factor, RpoD, more commonly referred to as the house-keeping or general stress response sigma factor, were detected in PF2B19 genome. The presence of multiple copies of genes for RpoD is a common feature in psychrophilic bacteria such as *Planococcus halocryophilus* and *Psychromonas ingrahamii*.



B. Oxidative stress response

Various metabolic processes result in endogenous build-up of toxic hydrogen peroxide and other forms of ROS (Bayir 2005). The increased solubility of gasses at low temperatures makes microorganisms inhabiting cold

environments more prone to the toxic effects of ROS (Chattopadhyay 2006, D'Amico et al. 2006).

The second largest group of putative stress adaptation genes in the *Nesterenkonia* sp. PF2B19 genome can be linked to oxidative stress response (Table 43). Putative antioxidants encoded by *Nesterenkonia* sp. PF2B19 include catalase (kat), two superoxide dismutases (SodA; SodC), a thiol peroxidase (Bcp) as well as thioredoxin and thioredoxin reductase (TrxA and TrxB). Superoxide dismutase catalyzes the conversion of superoxide anion to hydrogen peroxide which subsequently undergoes disproportionation through a reaction that is catalyzed by catalases and peroxidases (Ebara and Shigemori 2008, Moustafa *et al.* 2010). TrxA and TrxB, while maintaining the intracellular thiol-disulfide balance, also scavenge ROS (Ballal and Manna 2010). Two putative dioxygenases were also identified in the genome of *Nesterenkonia* sp. PF2b19.

C. Osmo-protection

Microbial response to osmotic stress involves two major mechanisms: the salt-in strategy, involving the uptake of inorganic ions to balance the salinity gradient created by the high salt concentration in the external environment, and the accumulation of compatible solutes which are either synthesized or taken up from the environment (Mesbah and Wiegel 2008, Oren 2008). Compatible solutes play a dual role in stress response as both osmolytes and cryo-protectants (Welsh 2000). The genome of *Nesterenkonia* sp. PF2B19 encodes a repertoire of proteins involved in both strategies (Table 43). Similarly, the genome encodes transporters for glycine/betaine which are known osmo-protectants (Le Rudulier *et al.* 1984). In addition, several genes involved in the endogenous synthesis of compatible solutes are present. These include the trehalose biosynthesis genes otsA and otsB, known to be cold-inducible and essential for low temperature survival.

D. General stress response

Beside specific factors for cold, osmotic and oxidative stress response and tolerance, the *Nesterenkonia* sp. PF2B19 genome encodes a large number of other stress-related proteins, which can be considered as elements of a general stress response system (Table 43). At least 10 genes putatively involved in SOS response and DNA repair systems were identified. The genome also encodes two paralogs of the universal stress protein, UspA, which has been linked to cold acclimation (Garnier *et al.* 2010).

Biotechnological potential of PF2B19

Analysis of annotated genome sequence of *Nesterenkonia* sp. revealed the presence of genes involved in production of α-amylases, including maltose and maltodextrin and xylanase. The association of some *Nesterenkonia* strains with extreme niches highlights their potential as sources of biologically active molecules such as α-amylases and xylanases (Kui *et al.* 2010, Shafiei *et al.* 2012) and as models for understanding microbial adaptation strategies in harsh environments.

In addition, the genes encoding proteins involved in resistance to heavy metals and toxic compounds, including copper homeostasis, cobalt-zinc-cadmium resistance, arsenic resistance, and β-lactamase, were identified. These results show the genetic potential of the *Nesterenkonia* sp. to adapt to extreme lifestyles.

Genome-wide overview of carbohydrate active enzymes for *Nesterenkonia* sp. PF2B19

The draft genome analysis of *Nesterenkonia* sp. PF2B19 showed that this strain is a rich source of carbohydrate active enzymes. Genome annotation using RAST tools revealed that 20.8% of the subsystem categorized genes were classified as being involved in carbohydrate metabolism (Fig. 53). The genes

encoding carbohydrate active enzymes were grouped based on enzyme families (Table 44) and their substrate specificity (Table 45) compared with the only draft genome reported for *Nesterenkonia* spp., i.e., *Nesterenkonia alba* DSM 19423. This comparative genome study illustrated the superior ability of *Nesterenkonia* sp. PF2B19 compared with *Nesterenkonia alba* DSM 19423 to catabolize oligosaccharides and polysaccharides, owing to a higher variety and quantity of sugar hydrolysis encoding genes.

Table 44. Comparative analysis and classification of the genes encoding carbohydrate active enzymes based on the enzymes families between the *Nesterenkonia* sp. PF2B19 and *Nesterenkonia alba* DSM 19423 genomes.

Enzyme classes	EC number	Enzyme name	Abundance overview	
			N. alba DSM 19423	*Nesterenkonia* sp. PF2B19
Glycoside Hydrolase family	EC:3.2.-	Hydrolases. Glycosylases	1	1
	EC:3.2.1.1	α-amylase	0	1
	EC:3.2.1.20	α -glucosidase	0	1
	EC:3.2.1.21	β-glucosidase	1	3
	EC:3.2.1.22	α -galactosidase	1	0
	EC:3.2.1.23	β -galactosidase	2	0
	EC:3.2.1.24	α –mannosidase	1	0
	EC:3.2.1.52	β -N-acetyl hexosaminidase	1	0
	EC:3.2.1.55	Non-reducing end alpha-L-arabinofuranosidase	2	2
	EC:3.2.1.8	Endo-1,4-beta-xylanase	0	1
	EC:3.2.1.99	Arabinan endo-1,5-alpha-L-arabinosidase	0	2
Glycoside Transferase family	EC:2.4.1.-	Transferases. Glycosyl transferases. Hexosyl transferases	1	0
	EC:2.4.1.129	Peptidoglycan glycosyltransferase	1	10

	EC:2.4.1.15	α,-trehalose-phosphate synthase (UDP-forming)	1	1
	EC:2.4.1.187	N-acetylglucosaminyl diphosphoundecaprenol N-acetyl- β -D- mannosaminyl transferase	1	0
	EC:2.4.1.227	Undecaprenyl diphospho-muramoylpentapeptide β -N- acetyl glucosaminyl transferase	1	1
	EC:2.4.1.250	D-inositol-3-phosphate glycosyl transferase	1	10
	EC:2.4.1.251	GlcA- β -(1−>2)-D-Man- α-(1−>3)-D-Glc-β-(1−>4)- D-Glc-α-1- diphospho-ditrans,octacis-undecaprenol 4-β -mannosyl transferase	1	0
	EC:2.4.1.83	Dolichyl-phosphate β -D-mannosyl transferase	1	1
	EC:2.4.2.-	Transferases. Glycosyl transferases. Pentosyl transferases.	1	0
	EC:2.4.2.1	Purine-nucleoside phosphorylase	1	0
	EC:2.4.2.10	Orotatephosphoribosyl transferase	1	1
	EC:2.4.2.14	Amidophosphoribosyl transferase	1	1
	EC:2.4.2.17	ATP phosphoribosyltransferase	1	1
	EC:2.4.2.18	Anthranilate phosphoribosyltransferase	1	0
	EC:2.4.2.19	Nicotinate-nucleotide diphosphorylase (carboxylating)	1	0
	EC:2.4.2.28	S-methyl-5′-thioadenosine phosphorylase	0	0
	EC:2.4.2.4	Thymidine phosphorylase	1	1
	EC:2.4.2.7	Adenine phosphoribosyltransferase	0	0

	EC:2.4.2.8	Hypoxanthine phosphoribosyltransferase	1	1
	EC:2.4.2.9	Uracil phosphoribosyltransferase	2	1
Polysaccharide Lyase family	EC:4.2.2.2	Pectate lyase	0	1
Carbohydrate Esterase family	EC:3.1.1.1	Carboxylesterase	1	1
	EC:3.1.1.11	Pectinesterase	0	1
	EC:3.1.1.17	Gluconolactonase	0	0
	EC:3.1.1.24	3-oxoadipate enol-lactonase	0	0
	EC:3.1.1.29	Aminoacyl-tRNA hydrolase	1	0
	EC:3.1.1.31	6-phosphogluconolactonase	1	0
	EC:3.1.1.45	Carboxymethylenebutenolidase	1	0
Auxiliary Activities family	EC:1.10.3.-	Oxidoreductases. Acting on diphenols and related substances as donors. With oxygen as acceptor	2	43
	EC:1.11.1.15	Peroxiredoxin	1	0
	EC:1.11.1.6	Catalase	1	1

The genes encoding carbohydrate active enzymes were grouped based on enzyme families (Table 43) and their substrate specificity (Table 44) compared with the only draft genome reported for *Nesterenkonia* spp. PF2B19, i.e., *Nesterenkonia alba* DSM 19423. This comparative genome study illustrated the superior ability of *Nesterenkonia* sp. PF2B-19 compared with *Nesterenkoniaalba* DSM 19423 to catabolize oligosaccharides and polysaccharides, owing to a higher variety and quantity of sugar hydrolysis encoding genes.

Table 45. Comparative analysis and classification of the genes encoding carbohydrate active enzymes based on substrate specificity between the *Nesterenkonia* sp. PF2B19 and *Nesterenkonia alba* DSM 19423 genomes.

Carbohydrates class	EC number	Carbohydrate or derivates name	Carbohydrate active enzyme	Abundance overview	
				Nesterenkonia sp. strain PF2B-19	*N. alba* DSM 19423
Monosaccharide	EC:1.2.1.12	D-Glyceraldehyde	Glyceraldehyde-3-phosphate dehydrogenase (phosphorylating)	0	2
	EC:2.7.6.1	D-Ribose	Ribose-phosphate diphosphokinase	0	1
	EC:3.6.1.13		ADP-ribose diphosphatase	0	1
	EC:4.1.2.4		Deoxyribose-phosphate aldolase	1	1
	EC:5.3.1.6		Ribose-5-phosphate isomerase	0	2
	EC:5.3.1.4	L-Arabinose	L-arabinose isomerase	1	1
	EC:5.3.1.5	Xylose	Xylose isomerase	1	1
	EC:1.1.1.22	D-Glucose	UDP-glucose 6-dehydrogenase	3	1
	EC:1.1.1.49		Glucose-6-phosphate dehydrogenase (NADP(+))	0	1
	EC:2.3.1.157		Glucosamine-1-phosphate N-acety ltransferase	0	1
	EC:2.4.1.15		Alpha,alpha-trehalose-phosphate synthase (UDP-forming)	1	1
	EC:2.4.1.187		N-acetyl glucosaminyl diphospho undecaprenol N-acetyl-beta-D-mannosaminyl transferase	0	1
	EC:2.4.1.227		Undecaprenyl diphospho-muramoyl pentapeptide beta-N- acetyl glucosaminyl transferase	0	1
	EC:2.5.1.7		UDP-N-acetylglucosamine 1-carboxyvinyl transferase	1	1
	EC:2.7.1.63		Polyphosphate–glucose phosphotransferase	0	1
	EC:2.7.7.12		UDP-glucose–hexose-1-phosphate uridylyl transferase	1	1
	EC:2.7.7.23		UDP-N-acetyl glucosamine diphosphorylase	0	1

	EC:2.7.7.9		UTP–glucose-1-phosphate uridylyl transferase	1	1
	EC:2.7.8.33		UDP-N-acetyl glucosamine–undecaprenyl-phosphate N-acetyl glucosaminephosphotransferase	0	1
	EC:3.5.1.25		N-acetylglucosamine-6-phosphate deacetylase	2	0
	EC:3.5.99.6		Glucosamine-6-phosphate deaminase.	1	0
	EC:5.1.3.14		UDP-N-acetyl glucosamine 2-epimerase (non-hydrolyzing)	1	0
	EC:5.1.3.2		UDP-glucose 4-epimerase	3	1
	EC:5.3.1.9		Glucose-6-phosphate isomerase	0	1
	EC:5.4.2.10		Phosphoglucosamine mutase	3	1
	EC:1.1.1.336	D-Mannose	UDP-N-acetyl-D-mannosamine dehydrogenase	0	0
	EC:2.4.1.187		N-acetyl glucosaminyl diphospho undecaprenol N-acetyl-beta-D-mannosaminyl transferase	0	1
	EC:2.4.1.251		GlcA-beta-(1−>2)-D-Man-alpha-(1−>3)-D-Glc-beta-(1−>4)-D-Glc-alpha-1-diphospho-ditrans, octacis-undecaprenol 4-beta-mannosyl transferase.	0	1
	EC:2.4.1.83		Dolichyl-phosphate beta-D-mannosyl transferase	1	1
	EC:2.7.7.13		Mannose-1-phosphate guanylyl transferase	1	1
	EC:5.3.1.8		Mannose-6-phosphate isomerase	1	1
	EC:2.7.1.29	Glycerone	Glycerone kinase	0	1
	EC:5.1.3.1	D-Ribulose	Ribulose-phosphate 3-epimerase	3	1
	EC:5.1.3.4		L-ribulose-5-phosphate 4-epimerase	0	1
	EC:1.1.1.267	D-Xylulose	1-deoxy-D-xylulose-5-phosphate reductoisomerase	2	1
	EC:2.2.1.7		1-deoxy-D-xylulose-5-phosphate synthase	0	1

	EC:2.6.1.16	D-Fructose	Glutamine–fructose-6-phosphate transaminase (isomerizing)	0	1
	EC:3.1.3.11		Fructose-bisphosphatase	1	1
	EC:4.1.2.13		Fructose-bisphosphate aldolase	0	1
Oligosaccharides and Polysaccharides	EC:3.2.1.1	starch, glycogen	α –amylase	1	0
	EC:3.2.1.20	Oligosaccharides	α -glucosidase (Maltase)	0	0
	EC:3.2.1.21	Oligosaccharides	β-glucosidase (Cellobiase)	3	1
	EC:3.2.1.22	galactose oligosaccharides, galactomannans and galactolipids	α-galactosidase	0	1
	EC:3.2.1.23	beta-D-galactosides	β -galactosidase (lactozym)	0	2
	EC:3.2.1.24	alpha-D-mannosides	α -mannosidase	0	1

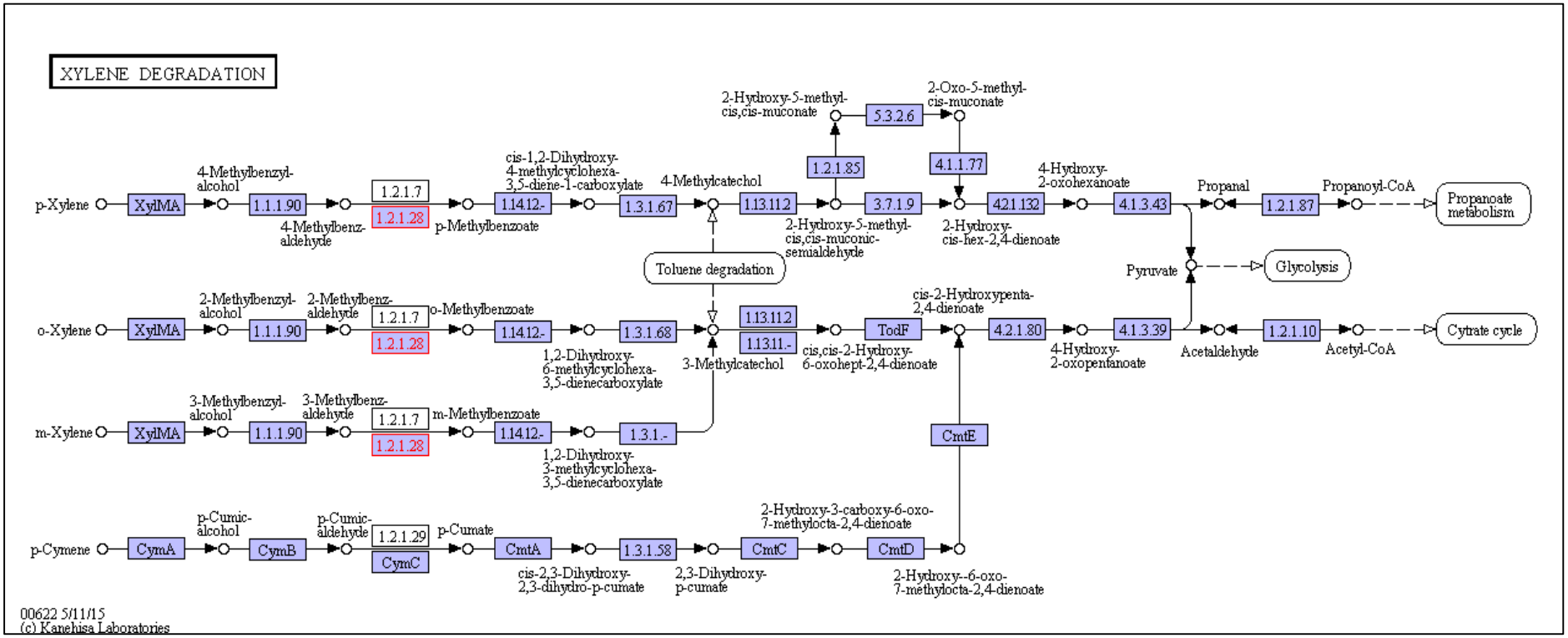
Oil bioremediation potential

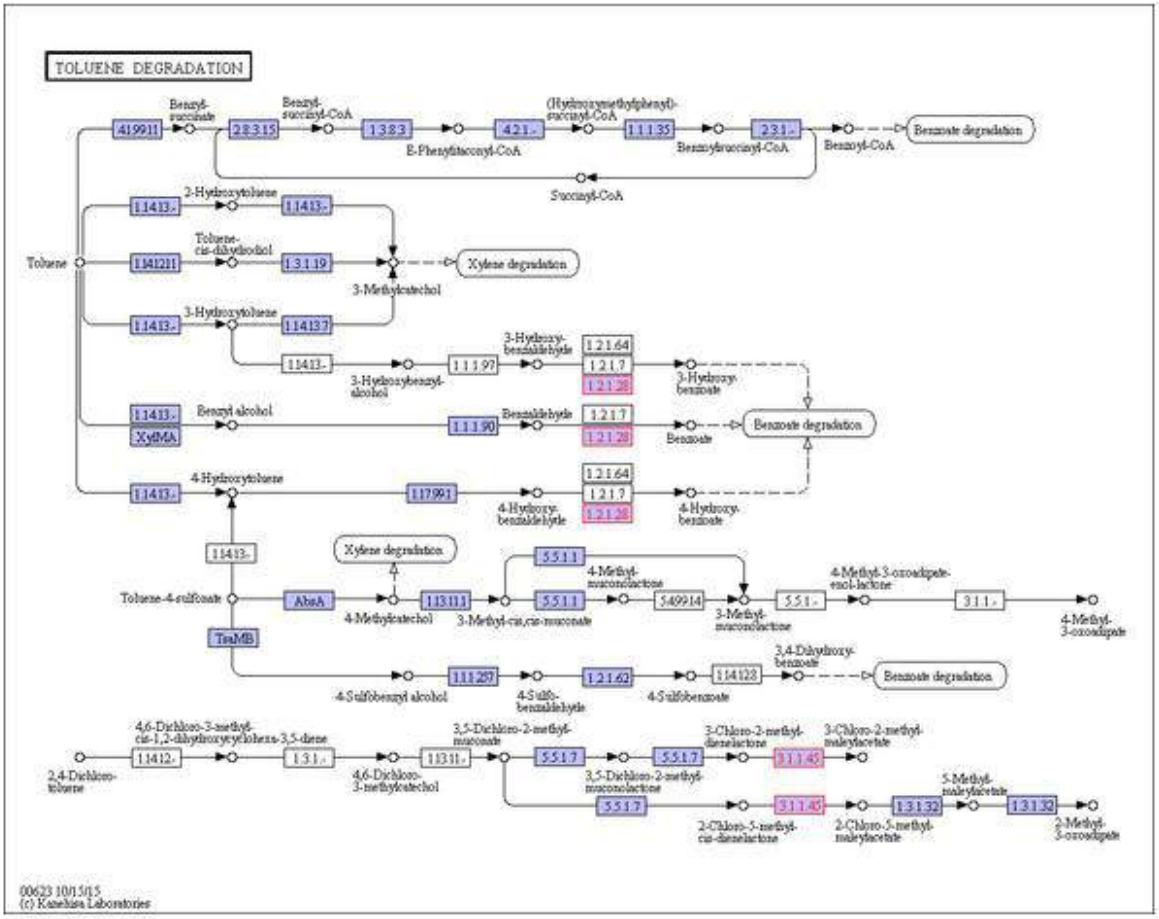
Genome analysis revealed the genes possibly responsible for hydrocarbon degradation. Genes encoding benzoate, catechol and alkanol monooxygenase were found in the genome.

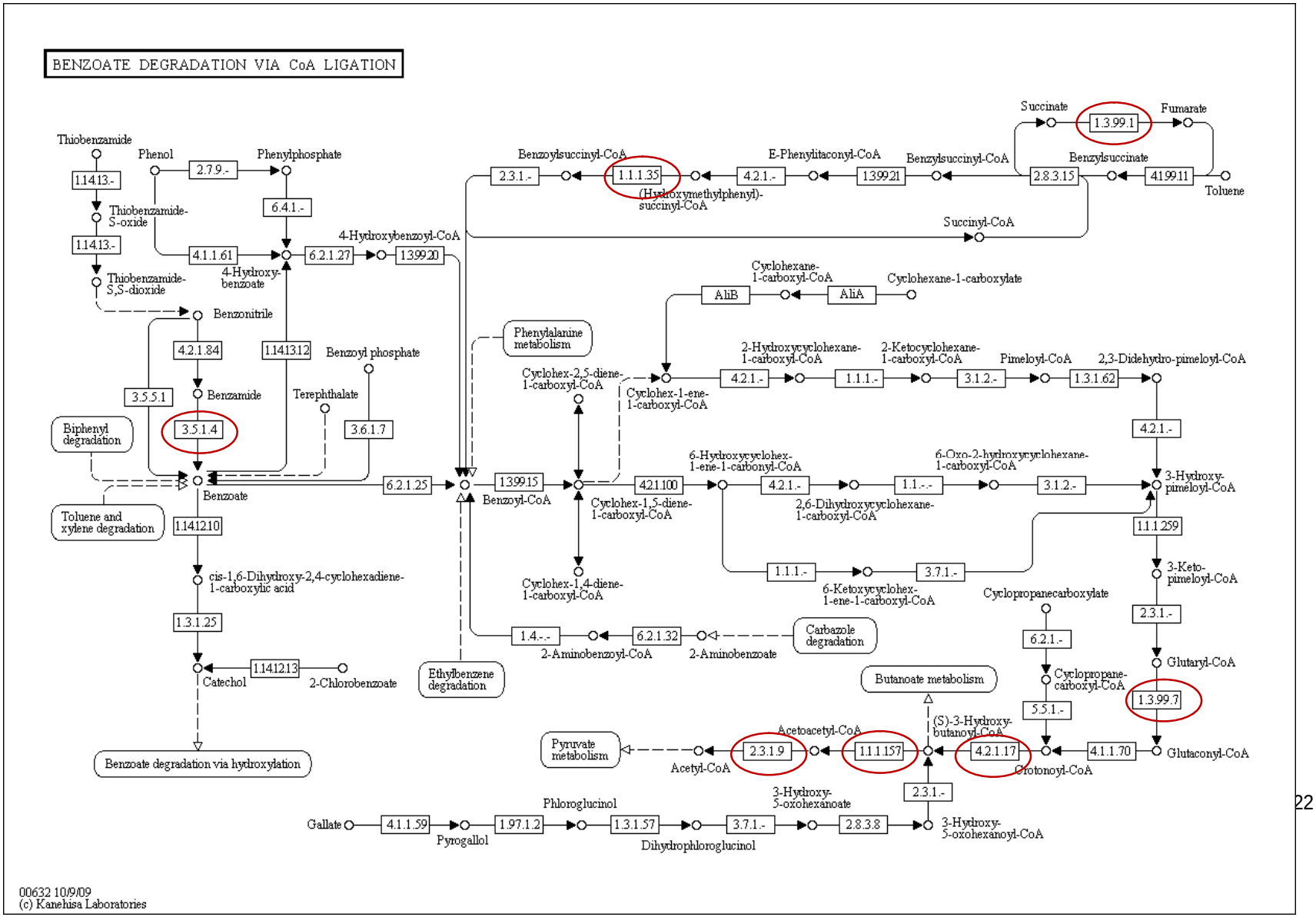
KEGG map Metabolic pathways for *Nesterenkonia* sp. PF2B19 (6666666. 176862)

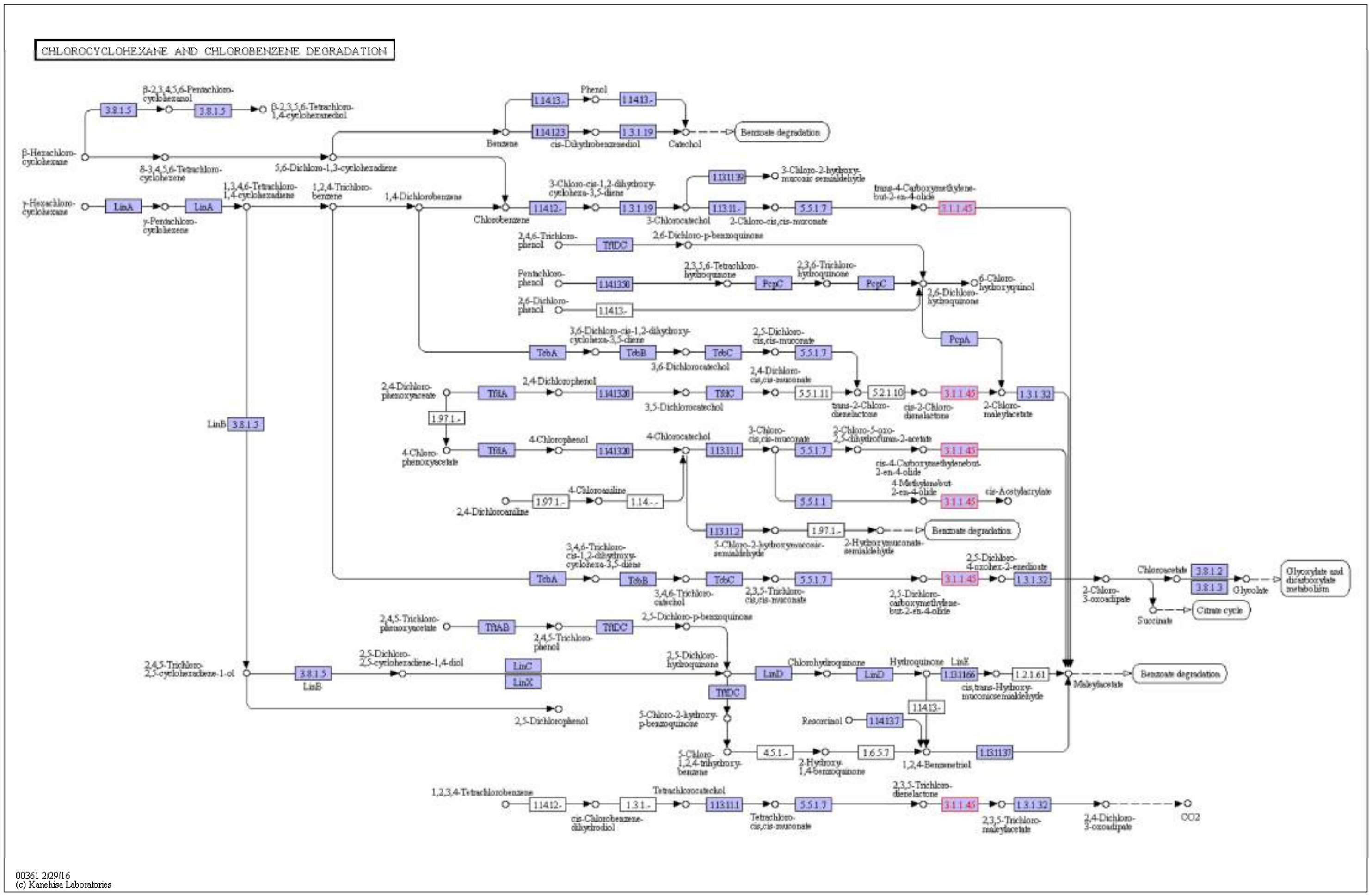
KEGG map	Distinct ECs	*Nesterenkonia* sp. PF2B19	*Kocuria rhizophila* DC2201
2,4-Dichlorobenzoate degradation	29	2 (6.9 %)	1 (3.4 %)
Benzoate degradation via CoA ligation	44	12 (27.3 %)	10 (22.7 %)
Benzoate degradation via hydroxylation	50	7 (14.0 %)	6 (12.0 %)
Glycerolipid metabolism	36	6 (16.7 %)	6 (16.7 %)
Glycolysis / Gluconeogenesis	41	12 (29.3 %)	20 (48.8 %)
Pyruvate metabolism	64	13 (20.3 %)	19 (29.7 %)
Toluene and xylene degradation	23	2 (8.7 %)	0
Biphenyl degradation	13	1 (7.7 %)	0
Butanoate metabolism	52	18 (34.6 %)	19 (36.5 %)
Carbazole degradation	11	1 (9.1 %)	0
Ethylbenzene degradation	11	3 (27.3 %)	2 (18.2 %)
Phenylalanine metabolism	51	9 (17.6 %)	5 (9.8 %)
Pyruvate metabolism	64	13 (20.3 %)	19 (29.7 %)
Toluene and xylene degradation	23	2 (8.7 %)	0
Benzoate degradation via CoA ligation	44	12 (27.3 %)	10 (22.7 %)
Ethylbenzene degradation	11	3 (27.3 %)	2 (18.2 %)
Glycolysis / Gluconeogenesis	41	12 (29.3 %)	20 (48.8 %)
Propanoate metabolism	47	11 (23.4 %)	16 (34.0 %)
Pyruvate metabolism	64	13 (20.3 %)	19 (29.7 %)
Styrene degradation	21	4 (19.0 %)	1 (4.8 %)

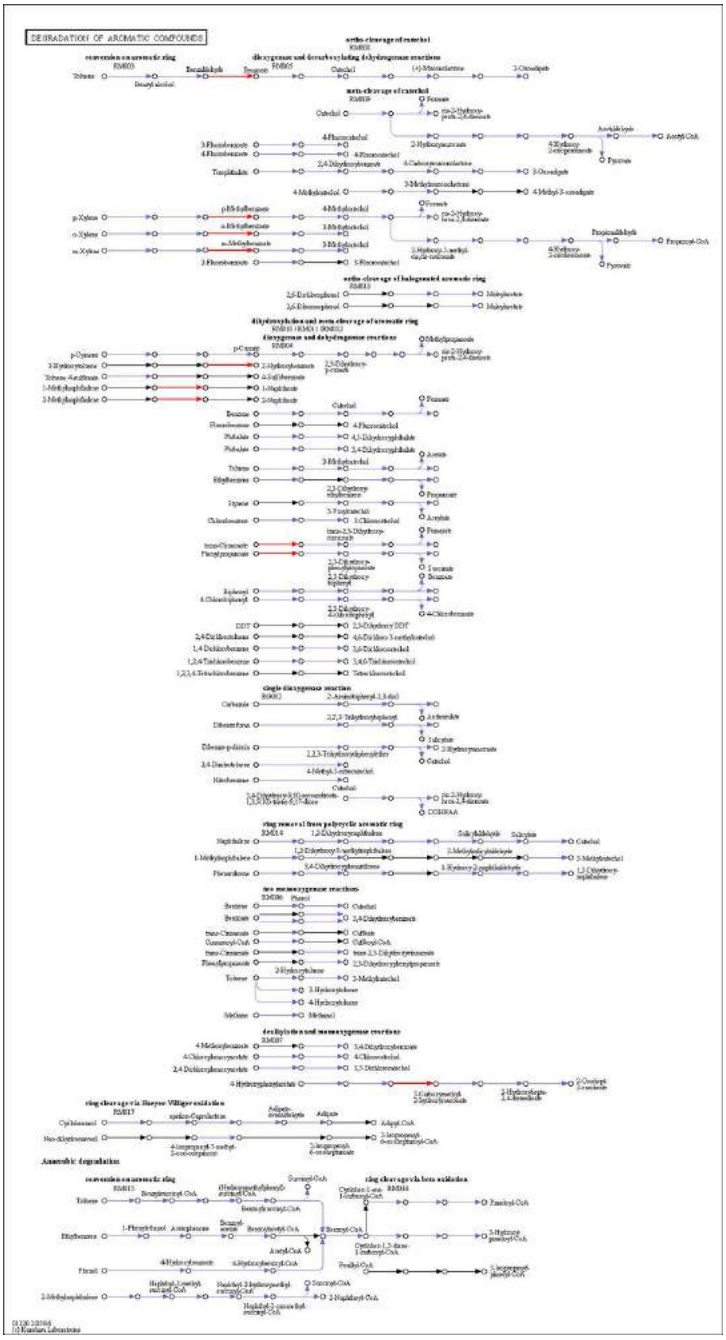
KEGG pathway for Xylene and Toluene degradation




TOLUENE DEGRADATION
Benzyl-succinate
Benzyl-succinyl-CoA
(Hydroxymethylphenyl)-succinyl-CoA
Benzoate degradation
E-Phenylitaconyl-CoA
Benzoylsuccinyl-CoA
Benzoyl-CoA
Succinyl-CoA
2-Hydroxytoluene
Toluene-cis-dihydrodiol
Toluene
Xylene degradation
3-Methylcatechol
3-Hydroxytoluene
3-Hydroxybenzyl-alcohol
3-Hydroxy-benzaldehyde
3-Hydroxy-benzoate
Benzyl alcohol
Benzaldehyde
Benzoate
4-Hydroxytoluene
4-Hydroxy-benzaldehyde
4-Hydroxy-benzoate
Toluene-4-sulfonate
4-Methylcatechol
3-Methyl-cis,cis-muconate
4-Methyl-muconolactone
3-Methyl-muconolactone
4-Methyl-3-oxoadipate-enol-lactone
4-Methyl-3-oxoadipate
3,4-Dihydroxy-benzoate
4-Sulfobenzyl alcohol
4-Sulfo-benzaldehyde
4-Sulfobenzoate
2,4-Dichloro-toluene
4,6-Dichloro-3-methyl-cis-1,2-dihydroxycyclohexa-3,5-diene
4,6-Dichloro-3-methylcatechol
3,5-Dichloro-2-methyl-muconate
3,5-Dichloro-2-methyl-muconolactone
3-Chloro-2-methyl-dienelactone
3-Chloro-2-methyl-maleylacetate
2-Chloro-5-methyl-cis-dienelactone
2-Chloro-5-methyl-maleylacetate
5-Methyl-maleylacetate
2-Methyl-3-oxoadipate
00623 10/15/15
(c) Kanehisa Laboratories


BENZOATE DEGRADATION VIA CoA LIGATION
Thiobenzamide
Phenol
Phenylphosphate
Succinate
Fumarate
1.3.99.1
1.14.13.-
2.7.9.-
Benzoylsuccinyl-CoA
E-Phenylitaconyl-CoA
Benzylsuccinyl-CoA
Benzylsuccinate
2.3.1.-
1.1.1.35
4.2.1.-
1.3.99.21
2.8.3.15
4.1.99.11
Toluene
(Hydroxymethylphenyl)-succinyl-CoA
Thiobenzamide-S-oxide
6.4.1.-
Succinyl-CoA
1.14.13.-
4-Hydroxybenzoyl-CoA
4.1.1.61
6.2.1.27
1.3.99.20
4-Hydroxy-benzoate
Thiobenzamide-S,S-dioxide
Cyclohexane-1-carboxyl-CoA
Cyclohexane-1-carboxylate
AliB
AliA
Benzonitrile
Phenylalanine metabolism
4.2.1.84
1.14.13.12
Benzoyl phosphate
2-Hydroxycyclohexane-1-carboxyl-CoA
2-Ketocyclohexane-1-carboxyl-CoA
Pimeloyl-CoA
2,3-Didehydro-pimeloyl-CoA
Cyclohex-2,5-diene-1-carboxyl-CoA
4.2.1.-
1.1.1.-
3.1.2.-
1.3.1.62
3.5.5.1
Benzamide
Terephthalate
Cyclohex-1-ene-1-carboxyl-CoA
4.2.1.-
Biphenyl degradation
3.5.1.4
3.6.1.7
6-Hydroxycyclohex-1-ene-1-carbonyl-CoA
6-Oxo-2-hydroxycyclohexane-1-carboxyl-CoA
3-Hydroxy-pimeloyl-CoA
6.2.1.25
1.3.99.15
4.2.1.100
4.2.1.-
1.1.-.-
3.1.2.-
Benzoyl-CoA
Cyclohex-1,5-diene-1-carboxyl-CoA
2,6-Dihydroxycyclohexane-1-carboxyl-CoA
Benzoate
1.1.1.259
Toluene and xylene degradation
1.14.12.10
1.1.1.-
3.7.1.-
3-Keto-pimeloyl-CoA
6-Ketoxycyclohex-1-ene-1-carboxyl-CoA
Cyclohex-1,4-diene-1-carboxyl-CoA
Cyclopropanecarboxylate
cis-1,6-Dihydroxy-2,4-cyclohexadiene-1-carboxylic acid
2.3.1.-
1.4.-.-
6.2.1.32
Carbazole degradation
6.2.1.-
1.3.1.25
2-Aminobenzoyl-CoA
2-Aminobenzoate
Glutaryl-CoA
Cyclopropane-carboxyl-CoA
1.14.12.13
Catechol
2-Chlorobenzoate
Ethylbenzene degradation
Butanoate metabolism
1.3.99.7
5.5.1.-
(S)-3-Hydroxy-butanoyl-CoA
Acetoacetyl-CoA
Pyruvate metabolism
2.3.1.9
1.1.1.157
4.2.1.17
4.1.1.70
Glutaconyl-CoA
Acetyl-CoA
Crotonoyl-CoA
Benzoate degradation via hydroxylation
2.3.1.-
3-Hydroxy-5-oxohexanoate
Phloroglucinol
Gallate
4.1.1.59
1.97.1.2
1.3.1.57
3.7.1.-
2.8.3.8
3-Hydroxy-5-oxohexanoyl-CoA
Pyrogallol
Dihydrophloroglucinol
00632 10/9/09
(c) Kanehisa Laboratories


CHLOROCYCLOHEXANE AND CHLOROBENZENE DEGRADATION
β-Hexachloro-cyclohexane
γ-Hexachloro-cyclohexane
LinA
LinB
Benzene
Phenol
Catechol
Benzoate degradation
Chlorobenzene
1,4-Dichlorobenzene
3-Chlorocatechol
2-Chloro-cis,cis-muconate
TfdC
PcpC
PcpA
TcbA
TcbB
TcbC
3,6-Dichlorocatechol
3,5-Dichlorocatechol
4-Chlorophenol
4-Chlorocatechol
4-Chloroaniline
2,4-Dichloroaniline
cis-Acetylacrylate
Chloroacetate
Glycolate
Glyoxylate and dicarboxylate metabolism
Citrate cycle
Succinate
LinC
LinX
LinD
LinE
Chlorohydroquinone
Hydroquinone
Maleylacetate
Resorcinol
1,2,4-Benzenetriol
2,5-Dichlorophenol
1,2,3,4-Tetrachlorobenzene
Tetrachlorocatechol
CO2
00361 2/29/16
(c) Kanehisa Laboratories



6.4 Conclusion

The first whole genome analysis of this putative novel species of the genus *Nesterenkonia* revealed mechanistic insights into the metabolic adaptation of bacteria to Arctic permafrost ecosystems as well as the characterization of the ecological and biotechnological potentials of *Nesterenkonia* sp. PF2B19.

7a. Summary and General Conclusions

Information was scarcely available on the diversity, adaptation strategies and biotechnological potential of cryophilic bacteria and fungi of cryoconites, ice cores and permafrost from Svalbard, Arctic. The isolates of cryoconites, ice cores and permafrost showed varied response to multiple antibiotics despite the limited bacterial diversity in these micro-ecohabitats. This adaptation strategy enables the bacterial strains to cope with the antagonistic stresses and survive or thrive in all possible stressed environmental conditions. Carbohydrate utilization tests on the other hand, indicated that the microbial isolates from cryoconite holes and ice cores are fond of easily utilizable simpler forms of carbohydrates, namely dextrose, mannose, rhamnose, dextrose and xylose, as carbon sources, while permafrost isolates indicate that the bacterial isolates prefer varied forms of carbon sources.

Studies on the occurrence of filamentous fungi in supraglacial cryoconite holes and their characterization for extracellular enzymes remained undescribed so far. In this study, the results on the isolation, identification and characterization of culturable yeasts and filamentous fungi from supraglacial Svalbard cryoconite holes are presented. Based on morphology and sequence data, these were identified as *Cryptococcus gilvescens*, *Mrakia* sp., *Rhodotorula* sp., *Thelebolus* sp., *Phialophora alba* and *Articulospora tetracladia*. Amongst these, *Phialophora alba*, *Cryptococcus gilvescens* and *Mrakia* sp. zhenx-1 are reported for the first time from Svalbard Arctic. Filamentous fungi and yeasts in the cryoconite holes drive the process of organic macromolecule degradation through cold-adapted enzyme secretion, thereby assisting in nutrient cycling in these subglacial environments. Further, these cold-adapted enzymes may also provide an opportunity for the prospect of biotechnology in Arctic.

Rhodotorula svalbardensis sp. nov. strains MLB-I and CRY-YB1 showed similar phenotypic characteristics but differed from the four nearest phylogenetic neighbours: *R. psychrophenolica* CBS10438T (8 tests), *R. himalayensis* 3AT (14

tests), *R. glacialis* CBS10437T (7 tests) and *R. psychrophila* CBS10440T (6 tests). D1/D2 domain of large-subunit rRNA gene (26S rRNA) indicated their closest relationship to species of *Rhodotorula psychrophenolica* CBS10483^T by 95.7%, with *R. himalayensis* 3A^T by 96.4%, with *R. glacialis* CBS10436^T by 96.0% and with *R. psychrophila* CBS10440^T by 95.8%. However, the sequence analysis of the ITS region showed 89.3% sequence similarity with *R. glacialis* (EF151249), 88.2% with *R. glacialis* (EF151249), 89.7% with *R. psychrophila* (EF151244), 89.7 % with *R. psychrophila* (EF151245), 89.3% with *R. psychrophila* (EF151243), 88.3% with *R. psychrophenolica* (EF151246) and 89.5% with *R. himalayensis* (AM410635). Thus, these strains appear to be different from the so far published phylogenetically related species of *Rhodotorula* and related genera. Morphological and physiological properties, as well as sequences of 26S D1/D2 domain, ITS region and mitochondrial cytochrome *b* gene, indicates that MLB-I and CRY-YB1 strains belong to hitherto unknown species, and named *Rhodotorula svalbardensis* sp. nov.

The results derived on the isolation, identification and characterization of culturable yeasts from glacier ice cores are also presented. The decrease in number of viable yeast cells was observed with the increase in the depth of ice core. This particular observation indicates that the yeast cells are capable of surviving in the porous surface layer of ice as compared to the deeper strata. The sequence of D1/D2 domain of isolates showed 98.3–100% similarity with validly published species in the genera *Cryptococcus, Rhodosporidium* and *Rhodotorula*. These isolates showed closest relationship with *C. adeliensis* CBS8351 (AF137603), *C. albidosimilis* CBS7711 (AF137601), *C. saitoi* CBS1975 (AF181540), *R. lusitaniae* PYCC 4642 (JN246539) and *R. mucilaginosa* KCTC7829 (AF257267).

The fatty acid profile from the present study reveals that decrease in temperature elevates the concentration of total unsaturated fatty acids. The relative percentage of major fatty acids in the whole cell extract was oleic acid (C18:1n9c) followed by linoleic acid (C18:2n6c), linolenic acid (C18:3n3), palmitic acid (C16:0), stearic acid (C18:0), myristic acid (C14:0) and

pentadecanoic acid (C15:0). Linoleic acid (C18:2n6c) and linolenic acid (C18:3n3) have majorly contributed to the dominating polyunsaturated fatty acids (PUFA) in the organism. Oleic acid (C18:1n9c) as the most abundant monounsaturated fatty acids (MUFA) and palmitic acid (C16:0) as the major saturated fatty acids (SFA) were recorded. PUFAs at colder temperatures modulate the membrane fluidity of organisms, thereby enhancing the ability to survive at low temperatures. Biotechnologically PUFA production by microbes finds potential use as 'cell factories' in the production of these metabolites on a larger scale.

The present study is the first enzymatic study on microbes of Svalbard cryoconites, ice cores, and permafrost and is therefore of immense significance. The ability to produce cold active enzymes by cryoconite bacteria makes these microorganisms potential candidates for use in biotechnology, agriculture and medicine. All the representative bacterial strains tested showed activities of one or more enzymes (amylase, cellulase, lipase, urease, protease, or catalase) either at 4, 10 and 20 °C. The ability of the bacterial strains, isolated from cryoconite holes, to produce different enzymes indicates their ability to utilize the available organic matter, within the restricted environment as source of carbon and nitrogen for their survival and growth. Cellulose and urea degradation by these isolates at low temperatures demonstrates their biotechnological potential.

All the representative strains of ice core bacteria tested showed activities of two or more enzymes (amylase, cellulase, lipase, urease, protease, or catalase) either at 4 °C, 10 °C and 20 °C. In case of urease, enzyme production increased with increase in temperature from 4 to 20°C. From the present study it is observed that the cryophilic yeasts possess the ability to secrete extracellular enzymes by virtue of which they are able to utilize traces of available nutrients which helps in their survival and in the survival of other microbial communities persisting in the ice.

All the representative permafrost strains tested showed enzyme activities. Urea and protein degradation by these isolates at low temperatures demonstrate their biotechnological potential. Metabolic activity of bacteria in the frozen grounds can bring about biochemical transformation that has tremendous impact on the geochemistry of the cryolithozone. Psychrophilic enzymes play an important role in the molecular adaptation to cold. Most of these physiological reactions are energy consuming and possibly cold active enzymes/exoenzymes produced by these bacteria help them possibly in cold adaptation in permafrost. Thus cold-active enzymes play an important role in survival of bacteria in oligotrophic permafrost environment.

Almost all strains of cryoconites yeasts and filamentous fungi showed the ability to hydrolyse at least one of the two lipophilic substrates. Lipase activity (hydrolysis of tributyrin/Tween-80) was the most widely expressed extracellular enzyme among the cryoconites fungal cultures tested. *Mrakia* sp. (CCP-IIIWY) was the only isolate that exhibited phosphate solubilizing ability.

Screening experiments for production of various extracellular enzymes by ice core yeasts indicate that out of six enzymes (amylase, cellulase, lipase, protease, catalase, and urease) studied, the isolates exhibited significant activity for cellulase, protease and urease. All the isolates screened exhibited cellulase activity at all the temperatures tested. Protease activity was not observed at 4 ^{o}C while all the cultures showed positive activity at 10 and 20 ^{o}C. All isolates produced urease at 4 and 10 ^{o}C, however at 20 ^{o}C only few isolates were tested urease positive. The isolate was characterized to have an optimum activity at pH 7.0 and temperature 40^{o}C. The neutral pH activity of the lipase makes it a potential enzyme to be used in detergent industries and in the waste treatment of edible oil industries.

In the present study, 14 bacterial strains iisolated from the glacier cryoconite holes were screened for AFPs activity. Only eight strains (four to genus *Cryobacterium*, three to *Pseudomonas*, and one to genus *Subtercola*) showed positive AFP activity. The six bacterial AFPs solutions (extracellular

proteins/AFP isoform groups) were tested for TH activity as a function of their concentration in 25 mM MES–NaOH buffer pH 6.0. High TH values of around 2°C were obtained in the cry-g and cry-n strains. The highest TH activity was obtained with cry-g and cry-n AFPs. AFPs of six bacterial strains were subjected to purification and biochemical characterization. The purified AFPs revealed their apparent molecular weights about 22 kDa. However, ESI-Q TOF analysis of the Cry-c and Cry-g protein revealed its exact molecular mass to be 22.14 kDa. N-terminal amino acid sequence analysis confirmed that the bacterial AFPs of cryoconites belong to the family IBP-1. In total 21 peptides from *Cryobacterium psychrotolerans* (Cry-c) and 55 peptides from *Pseudomonas ficuserectae* Cry-g were sequenced with a high-level confidence after multi-enzymatic digestion (MELD) and nanoLC-ESI-MS/MS analysis. Out of the 21 peptides of the Cry-c antifreeze protein, an N-terminal sequence was identified as PVGSVR with an m/z value of 307.68. AFP in our present study is relatively rich in glycine (11.8%) which imparts flexibility to the protein molecule, hydrophobic proline (11.8%) and alanine (11%). The N-terminal sequence of the Cry-C AFP was detected AVPVGS VRAX VXXGAA TTFX .

In whole genome analyses performed on one of the dominant taxa (*Cryobacterium*) of cryoconites, a total of 339 unique genes in *Cryobacterium* sp. MLB-32 were found when comparative analysis was performed with NCPPB382. These included genes associated with carbohydrate metabolism; synthesis of cell wall and capsule, pigment; DNA, RNA and Protein metabolism; cell signaling; membrane transport; respiration; Sulfur, Nitrogen, Phosphorus and Potassium metabolism; stress response; disease and defense; motility and chemotaxis; metabolism of aromatic compounds, fatty acids and lipids. These genes were identified as distinctive genomic elements that delineated MLB-32 from its phylogenetic relatives. Genome analyses revealed that the MLB-32 genome codes for a number of enzymes involved in resistance to fluoroquinolone, vancomycin and the β-lactam group of antibiotics. MLB-32 also harbored genes encoding resistance to toxic metal ions such as arsenic and mercury. The presence of genes encoding stress-related proteins (three copies of genes encoding cold-shock

proteins and an antifreeze protein of type I) were also detected. CSPs (cold-shock proteins) are induced in response to temperature downshifts, thus illustrating the survival strategies in cold environments. The first draft genome analysis of this putative novel species of the genus *Cryobacterium* revealed mechanistic insights into the metabolic adaptation of bacteria to Arctic ecosystems as well as the characterization of the biotechnological and agricultural potential of *Cryobacterium* sp. MLB-32.

Whole genome sequencing of *Nesterenkonia* sp. PF2B19 from 44800 yrs old permafrost soil was also completed. Analysis of annotated genome sequence of *Nesterenkonia* sp. PF2B19 revealed the presence of genes involved in production of α-amylases, maltase and mylanase. Five proteins involved in fatty acid biosynthetic pathways were detected in the genome of *Nesterenkonia* sp. PF2B19. Additionally, the genes encoding proteins involved in resistance to heavy metals and toxic compounds, including copper homeostasis, cobalt-zinc-cadmium resistance, arsenic resistance, and β-lactamase were identified. The genome of *Nesterenkonia* sp. contains gene with putative roles in carotenoid biosynthesis. These results show the genetic potential of the *Nesterenkonia* sp. to adapt to extreme lifestyles in permafrost environment.

7b. Specific Conclusions

- Contributed the culturable microbial diversity of Cryoconite holes, Ice-Cores and permafrost habitats for the first time from Svalbard, Arctic.

- Delineated a novel species *Rhodotorula svalbardensis* sp. nov.

- Investigated the biotechnological potentials (enzymes, PUFA and antioxidant) of Arctic organisms.

- First report on AFP from Arctic glacier bacteria and its characterizations.

- Whole genome sequencing of *Cryobacterium* sp. MLB-32 and *Nesterenkonia* sp. PF2B19 and the analyses.

8. Future Scope of work

The isolates (*Pseudomonas* sp. MLB-2, *Sphingomonas* sp. MLB-5, and *Sphingomonas* MLB-9, *Cryobacterium* sp. MLB-38, *Articulospora* sp. Cry-FB1 and Cry-FB2) exhibited unique genetic sequences need to be described as novel species in future.

All the representative strains of cryoconite, ice core and permafrost showed activities of one or more enzymes (amylase, cellulase, lipase, urease, protease, or catalase) either at 4 °C, 10 °C, 15°C and (or) 20 °C. Complete purification and characterization of these enzymes will make these microorganisms potential candidates for use in agriculture, health and industry.

The branched fatty acid and cold-adapted enzyme production are adaptation strategies to low temperature. These ecological adaptive strategies are of great interest and future studies needs to be focused on understanding their functioning at molecular level.

The organisms have probably, under the pressure of survival in cold environment, gained the property of AFP production through horizontal gene transfer. Determining the molecular and physiological basis of adaptation strategies of microbial life in cryoconite holes environments, would yield the vital information for understanding the process of evolution and ecological behaviour of microorganisms. Similar studies on AFPs from several microorganisms isolated from other cold habitats (Himalaya) need to be carried out in order to understand, and compare their ecological characteristics and biotechnological potentials. The whole genome of the AFP producing strain MLB-29 need to sequenced and the primers are to be designed to obtain the relevant amplicons and an attempt will be made to find the correlation between the AFP peptide sequences and genes.

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Appendices I

List of Publications and Presentations

PUBLICATIONS			
S.No	**Names of all authors**	**Title**	**Name of the Journal and Volume, Year and Page Number**
1.	**Singh P.**, Tsuji M., **Roy U**,	Characterization of yeast and filamentous fungi from Brøggerbreen glaciers of Svalbard, Arctic	***Polar Record,*** (2016) DOI: 10.1017/S0032247416000085 Cambridge University Press
2.	**Singh P**., Singh SM, **Roy U.**	Taxonomic characterization and the bio-potential of bacteria isolated from glacier ice cores in the High Arctic	***Journal of Basic Microbiology,*** 55 (2015): 1–11 **[IF: 1.823** WILEY-VCH Verlag GmbH, ISSN1521-4028]
3.	**Singh P**., Tsuji M., Singh SM, **Roy U**., Hoshino T	Taxonomic characterization, adaptation strategies and biotechnological potential of cryophilic yeasts from ice cores of Midre Lovénbreen glacier, Svalbard, Arctic.	***Cryobiology*** 66, (2013): 167–175. **[IF: 1.643,** ELSEVIER, ISSN: 0011-2240**]**
4.	**Singh P**., Singh A., D`Souza L.M., **Roy U**., Singh S.M. (2012).	Chemical Constituents and Antioxidant activity of Arctic Mushroom *Lycoperdon molle* Pers.	***Polar Research*** 31, 17329, DOI: 10.3402/polar.v31i0.17329. **[IF: 1.686**, Norwegian Polar Institute, Norway**]**.
Presentations: in International Symposium and Conference			
1.	**Singh P**., Singh SM, Srivastava A, **Roy U.**	Bacterial diversity and bio-potentials of culturable bacteria associated with different age sediments of Svalbard, Arctic	The 5th Symposium on Polar Science 2014 at National Institute of Polar Research, Tokyo, Japan

2.	**Singh P.**, **Roy U**., Hanada Y, Tsuda S	Antifreeze protein activity in glacier cryoconites.	6th International Conference on Polar and Alpine Microbiology (PAM 2015) University of South Bohemia, České Budějovice, Czech Republic.
3	**Singh P, Utpal Roy**	Biotechnological Potentials of Yeasts & Filamentous Fungi from Austre and Vestre Broggerbreen Glaciers of Svalbard, Arctic.[Poster presentation].	54th Annual Conference of Association of Microbiologists of India (AMI-2013) & International Symposium on "Frontier Discoveries & Innovations in Microbiology and its Interdisciplinary Relevance (FDMIR-2013), P.49, Nov. 17-20, at MDU, Rohtak, India.

Appendices II

Brief Biography of the Candidate

Mrs. Purnima Singh, completed her graduation from TDPG College Jaunpur, and post graduation in Biotechnology from VBS Purvanchal University, Jaunpur in 2006. She has qualified CSIR -NET Examination in 2009. She joined as a full time research scholar at BITS Pilani-K.K Birla Goa Campus in 2010. She is a recipient of DST-WOS-A-Woman Scientist fellowship from December 2009 to 2012, and subsequently from 2014 to till date. She has presented papers in two international conferences and published several research papers in journals of international repute.

Appendices III

Brief Biography of the Supervisor

Dr. Utpal Roy, Associate Professor, former Head, and presently DRC convener, department of Biological Sciences, BITS-Pilani KK Birla Goa campus obtained his Ph.D. degree in Microbiology from National Dairy Research Institute, Karnal. Dr. Utpal Roy has 17 years teaching and research experience in areas of Microbiology, Molecular biology and Biotechnology. He has undertaken systematic studies on proteomics and genomics. As PI he has handled multiple research projects of CSIR, DST, and UGC. He has published more than thirty research papers in international and national journals of repute apart from contributing papers in various national and international symposia. He has also published a book entitled “A Handbook of Genetic Engineering”. He has supervised several Ph. D. students, handled multiple research projects, and collaborations in India and abroad.