Microbiological Analysis of two Glacier samples from North Sikkim

A Thesis Submitted

To

Sikkim University

(***A Central University established by an Act of Parliament of India, 2007***)

In Partial Fulfilment of the Requirement for the Degree of Doctor of Philosophy

By

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CERTIFICATE

This is to certify that the PhD thesis entitled **"Microbiological Analysis of Two Glaciers of North Sikkim"** submitted to the **SIKKIM UNIVERSITY** in partial fulfilment for the requirement of the Doctor of Philosophy in Microbiology, embodies the work carried out by **Mr. Mingma Thundu Sherpa** for the award of PhD. Degree in Microbiology, Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok, Sikkim. The results are original and have not been submitted anywhere else for any other degree or diploma.

It is recommended this PhD thesis to be placed before the Examiners for evaluation.

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DECLARATION

I declare that the present PhD thesis entitled **"Microbiological Analysis of Two Glaciers of North Sikkim"** submitted by me for the award of the degree of **Doctor of Philosophy in Microbiology** of Sikkim University under the supervision of **Dr. Nagendra Thakur,** Assistant Professor, Department of Microbiology, School of Life Sciences, Sikkim University, is my original research work solely carried out by me in the Department of Microbiology, School of Life Sciences, Sikkim University, Gangtok. No part thereof has been submitted for any degree or diploma in any University/ Institution.

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ABBREVATION

Sq.	Square
Km.	Kilometres
CHGH	Committee on Himalayan glacier and hydrology
WMO	World Metrological Organization
IPCC	International Panel on Climate Change
CO_2	Carbon dioxide
CH_2	Methane
APHA	American public health association
°C	Degree Celsius
RNA	Ribonucleic acid
Mt.	Mount
DGGE	Denaturing gradient gel electrophoresis
PLFA	Phospholipids fatty acids analysis
PAST	Paleontological Statistics software
ARGs	Antibiotic resistance genes
UNESCO	United nation education, scientific and cultural organization
ICIMOD	International centre for integrated mountain development
GOLFs	Glacier lake outburst
KEGG	Kato encyclopaedia gene and genome
ELA	Equilibrium line altitude
UV	Ultraviolet
%	Percentage
Cfu	Colony forming unit

mL	Milliliter
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
SCFA	Short-chain fatty acids
BCFA	Branch chain fatty acids
AFPs	Antifreeze proteins
CAPs	Cold-acclimation proteins
pH	power of hydronium ions
Sp.	Species
GMEIA	Global market for enzymes
MTCC	Microbial Type Culture Collection Centre
USA	United State of America
GPS	Global positioning System
ICP-MS	Inductively couple plasma mass spectroscopy
IR Gun	Infrared Thermometers
TDS	Total dissolved solid
BOD	Biological oxygen demand
COD	Chemical oxygen demand
NaCl	Sodium chloride
HCL	Hydrochloric acid
O.D	Optical density
DNS	3, 5-dinitrosalicylic acid
TCA	Trichloroacetic acid
FAME	Fatty acid methyl ester
GC	Gas chromatography

SI	Similarity index
ICSB	International Committee on Systematic Bacteriology
SEM	Scanning electron microscope
MIDI	Microbial identification system
SRA	Sequence read archive
BLAST	Basic local alignment search tool
ARGs	Antibiotic resistance genes
ARBD	Antibiotic Resistance Gene Database
MRGs	Metal resistance genes
BACMET	Antibacterial Biocides and Metal Resistance
MHA	Muller Hinton agar
µg	Micro-gram
MIC	Minimum inhibitory concentration
CLSI	Clinical and laboratory standard institute
$NiCl_2$	Nickel chloride
$CuCl_2$	Copper chloride
$HgCl_2$	Mercury chloride
$ZnCl_2$	Zinc chloride
$CoCl_2$	Cobalt chloride
$FeCl_2$	Ferrous chloride
NTU	Nephelometric turbidity unit
CKG	Changme Khang glacier
CK	Changme Khangpu glacier
KGG	Kanchengayao glacier
KCTC	Korean Collection for Type Cultures

MUFA	Monounsaturated fatty acids
PUFA	Polyunsaturated fatty acids
PCA	Principal Component Analysis
COG	Cluster of orthologous group
NGS	Next generation sequencing

1. INTRODUCTION

Water is the nostrum of the earth, without it life on this planet is not possible. It has direct influences on animal and human life since ancient times. The Himalayas are vast and hot spots of the world, starting from Hindu Kush Mountain, stretching to Tibetan plateau covering surface area of over 4.3 million sq. km. across Afghanistan, Pakistan, India, Nepal, Bhutan, and China. Himalayan cryosphere contains the largest deposits of snowfall and houses huge number of glaciers apart from the Polar Regions. The glaciers of the Himalayas covers more than 33,000 sq. km **(Bajracharya and Shrestha, 2011)** and hence the region is regarded as "Third pole of the world". It is the driving force of life for trillions as in whole of Asia. It covers the young peaks of Himalayas. The Indian sub-continental climatic condition of monsoons directly influences and feeds the annual snow deposit in the Himalayas and supply fresh water in entire Asia. Ten main rivers originating from the Himalayas create a crucial ecological buffer and these are Yellow and Yangtze rivers, originating from the Qinghai Plateau, China; Mekong, Salween, and Irrawaddy rivers, which run south-ward from Tibet into South-East Asia, feed water to South-East Asia. The Ganges, Brahmaputra, and Meghna rivers, which accounts for lifeline to India, Pakistan and Bangladesh ensure around the year water supply, and these rivers also provide electricity to about 1.5 billion of people in Himalayas **(National Research Council Washington, 2012)**. In 2012, Committee on Himalayan Glaciers and Hydrology **(CHGH, 2012)**, they declared in ''Himalayan Glaciers: Climate Change, Water Resources, and Water Security'' agenda, emphasized the possible impacts of

climate change on Himalayan glaciers leading to water scarcity and which in turn may possibly play an escalating role in geopolitical tautness. Thus, the Himalayan region is the most critical region in the world in which melting glaciers will have a negative effect on water supplies in the next few decades**(Jianchu et al. 2007)**. The component that controls and regulates the Himalayan glacier dynamics is unknown **(Byers, 2012)**. In 2012, World Meteorological Organization (**WMO**) declared that Polar Regions were experiencing rapid decline in snow cover and the soil accounted for methane release from these frigid zones **(Jarraud, 2012)**.

The impact of climate change on glaciers is an extensively debated topic **(Førland et al. 2009; Meehl et al. 2007)**. The retreat of glaciers in the Himalayas directly affects the various atmospheric, climate, and ecological phenomena **(Bhutiyani et al. 2008)**. When the glaciers get the retreat, the volume of ice or snow decreases and the inner depth ice core gets exposed, the surface area of fore field increases and new top layer of soil develops ecological succession **(Garcia-Lopez and Cristina, 2017; Hagen et al. 2003)**. Due to global climate change the cryospheric conditions get altered and might influence the growth of extreme tolerant microbes of mesophilic nature which causes paradigm shift in transient flora over habitat flora resulting in new change of biodiversity **(Nowak and Hodson, 2014; Hell et al. 2013).**

Glacial ecosystem harboring extreme psychrophilic microbes might possibly potential bio-indicators of climate change **(Cavicchioli et al. 2002; Ramana et al. 2002)**. The soil beneath the glaciers carries out high diversity of ecological succession as the pores or

capillaries of glacier melt water provides the basal nutrition to the glacier covered soils and plays a crucial role in many bio-geochemical cycles **(Panikov, 1999)**. The main focus on dealing climate change and measuring its impact has always been on macro system dynamics whereas from microbiological point of view these microbes beneath the glaciers and within them can also help us to solve the global problem. International Panel on Climate Change (**IPCC, 2007)** report states that global warming is on rise at alarming rate. Cryosphere and water might help in growth of extreme tolerating mesophiles which might also significantly cause an increase in the production of greenhouse gases - CO_2, CH_4, N_2O, NOand also human activities such as waste disposal and agriculture have stimulated the production of greenhouse gases by microbes. Understanding the role of these microbes have as both contributors to and reactive components of climate change can help us determine whether they can be used to curb emissions or if they will push us even faster towards climatic disaster **(Davidson and Janssens, 2006)**. Microbial processes depends both on biotic and abiotic parameters which might influence the local climatic conditions **(IPCC, 2007)**. These subtle changes might accumulate and play various significant roles in nutrient cycling through biogeochemical cycles.

The study of the Himalayan glaciers is of prime significance as it feeds huge populations and a whole civilization depends on its source of water **(Edwards, 2015)**. The microbial ecology concerning the Himalayas are different than the other polar counterparts and hence the unexplored gene pool is of great interest. Being a young fold mountain, the Himalayas are always in great risk as they do not have stability and frequent earthquakes in this Himalayan belt makes it more vulnerable **(Griffiths, 2012)**. Recent changes in the

weather conditions where there is an increase of 2°C temperature all around the world also leading to retreating of some of the Himalayan glaciers which might be doom for all the organisms living in its proximity **(Rogers et al. 2004a)**.

During snowfall or rain, the atmospheric aerosols gets deposited and accumulated in the glaciers which is a replica of its ecological air flora **(Nijampurkar and Rao, 1992; Boutron and Delmas, 1980)**. In Himalayan glaciers, many researchers (Nijampurkar and Rao, 1992; Mayewski et al. 1986, 1984, 1983, 1981; Lyons et al. 1981) detected various dissolved minerals such as sodium, potassium, manganese, calcium, chlorine, silica, carbonate, and nitrate. One of the aspects of study the chemical constituents of glaciers is that the dissolved minerals in glaciers gets released into the stream and ultimately consumed by humans and animals, even applied in agriculture land, but at the same time when some elements present in high concentrations above their threshold levels can be harmful, for instance chloride, fluoride, sulphate, nitrates and phenolic compounds might cause illness when present at higher concentration according to WHO and APHA standards **(Tuladhar et al. 2015; Sherpa et al. 2013)**. Thus, the studies of chemicals in glaciers are important as peoples used glacial melt-water for various purposes such as drinking, sanitation, and bathing etc. Also under different concentrations of heavy metals, the microbes may achieve tolerance to these metals **(Santina et al. 2014)**. This tolerance of metals by various microbes can be exploited for bioremediation in cold regions and therefore, it is not surprising that the use of microbial biomass in heavy metal remediation has gained attention during the recent years **(Margesin and Feller, 2010)**.

The added essential characteristics feature of glaciers which has acquired the interest of researcher in recent years is the microbial diversity-owned by the glaciers. In the current state, glaciers are the hotspots of research in the arena of microbial ecology **(Miteva, 2008)**. The microorganisms present in such glaciers can flourish under extremely cold temperature. The microbes which show, minimum, optimum and maximum growth temperatures at or below or below 0°C, 15°C, and 20°C are called psychrophiles **(Morita, 1975)**. Psychrophilic bacteria isolated different habitats belongs to phyla *Proteobacteria* were (*Colwellia psychrerythraea, Colwellia demingiae, Colwellia hornerae, Colwellia rossensis, Glaciecola punicea, Psychromonas boydii, Psychromonas ingrahamii, Shewanella gelidimaria, Shewanella frigidimaria, Polaromonas vacuolatus, Octadecabacter arcticus, Octadecabacter antarcticus*) **(Auman et al. 2006; Huston et al. 2000; Gosink et al. 1997; Bowman et al. 1997; Irgens et al. 1996; Bowman et al. 1998a; D'Aoust and Kushner, 1972)**, similarly phylum *Bacteroidetes* were (*Flavobacterium gillisiae, Gelidibacter algens, Polaribacter irgensii, Polaribacter franzmannii, Polaribacter filamentus, Psychroflexus torques*) **(McCammon and Bowman, 2000; Bowman et al. 1997; Gosink et al. 1998; Bowman et al. 1998a)**and phylum *Firimcute* were (*Planomicrobium mcmeekinii*) **(Yoon et al. 2001;Junge and Rouge, 2011)**.The microbeswith growth maxima above 25°C but capacity to grow at lower temperature are called psychrotolerant **(Morita, 1975)**. Psychrotolerant bacteria isolated from different cold-habitats were (*Trichococcus patagoniensis, Proteocatella sphenisci, Pedobacter himalayensis, Exiguobacterium mindicum, Dyadobacter hamtensis, Leifsonia pindariensis, Bacillus cecembensis, Cryobacterium roopkundense, Cryobacterium pindariensis, Paenibacillus glacialis*) **(Kishore et al. 2010; Reddy et al.**

2010, 2009, 2008; Pikuta et al. 2009, 2006; Chaturvedi and Shivaji, 2006; Shivaji et al. 2005; Chaturvedi et al. 2005).

A psychrophilic prokaryote is characterized by those organisms which grow optimally below <15°C temperature, maximum growth at 20°C, with no growth above 20°C temperature **(Canganella and Wiegel, 2011)**. Another term is "psychrotolerant" which optimally grows above 20°C and can tolerate less than 5°C **(Canganella and Wiegel, 2011)**. True psychrophiles are called "stenopsychrophiles" **(Dalmaso et al. 2015)**. Psychrophiles are found in cold environments **(Dalmaso et al. 2015**; **Margesin and Miteva, 2011)**. Cold environments include permafrost, arctic and Antarctic ice, rocks in very cold regions, permanent cold sea water of polar regions, permanently cold marine (-2°C), fresh water, deep rock aquifers and all cold blooded organisms **(Canganella and Wiegel, 2011**; **Miteva and Brenchley, 2005**; **Miteva et al. 2004)**.

The psychrophilic microbes were detected in glacial ecosystem from different parts of the world long ago **(Punkari and Forsstrom, 1995)**. The research on Lake Vostok, Antarctica during the late 1980s created the curiosity to hunt cryosphere microbes grew immensely. They worked on ice core samples of 3,000m depth from the surface using microscopy and cultivation and they found viable bacterial cells at low concentrations (10^2-10^4 cells mL^{-1})**(Abyzov et al. 1998a, 1998b; 1993, 1982)**.Later on studies of ice core samples aging 20,000 years old **(Christner et al. 2002, 2000)** also suggested that diverse bacterial population can be recovered from those ice core samples. Till now many bacteria have been isolated from glaciers, among them *Actinobacteria, Firmicutes,*

and*Proteobacteria* were the dominant phyla of psychrophilic bacteria **(Christner et al. 2005b, 2003b; Miteva et al. 2004; Xiang et al. 2005)** and the predominant genera were *Acinetobacter, Arthrobacter, Chryseobacterium, Exiguobacterium, Frigoribacterium, Janthinobacter, Methylobacterium, Rhodococcus, Sphingomonas* and *Pseudomonas***(Mannisto and Haggblom, 2006; Belova et al. 2006; Gilbert et al. 2004)**.Recent studies and researches have reported many interesting and ecological rich biodiversity in cold ecosystem of Antarctica, Arctic and adjoin Polar Regions**(Bowman et al. 2000)**. However, the microbial diversity of Himalayan glaciers has been less investigated compared to other cold habitats around the world. Only a few culture-dependent and culture-independent bacterial diversity studies have been completed on Himalayan glaciers. From Mt. Qomolangma glacier (Everest) some groups have found *Actinobacteria*, *Firmicutes*, *Proteobacteria,* and *Deinococcus* **(Zhang, 2010**; **Hong et al. 2010; Liu et al. 2009).** From East Rongbuk Glacier, Mt. Everest and Muztag Ata Glacier, China ice core, bacterial candidates belonging to *Firmicutes*, *Proteobacteria*and *Actinobacteria* phyla were reported **(Shen et al. 2012; Liu et al. 2009; Xiang et al. 2005, 2004).**

Psychrophilic bacteria permanently adapts to these frigid conditions which mesophiles cannot due to their cellular physiology and biochemistry **(Bottos et al. 2008; Perreault et al. 2007)**. Adaptation of the organism at these low temperatures is due to cold shock proteins and RNA chaperons **(Dalmaso et al. 2015)**. These cold shock or cold adaptive proteins first binds with RNA molecule to maintain its single stranded (ss) conformation and these cold shock domain (Cold shock protein + RNA) then facilitate cold

adaptation**(Phadtare and Severinov, 2010)**. Other factors involved are secondary cold active metabolites, enzymes, antifreeze protein and more important membrane fluidity **(Casanueva et al. 2010)**. The higher content of alpha-helix in protein leads to maintain flexibility at low temperature **(Madigan et al. 2009)**. The high content of unsaturated fatty acid in lipid helps to maintain semi fluid state in membrane to adapt in these extreme temperatures **(Deming, 2009)**. Less side chain interaction among proteins also allows enzymes to be functional at minimal kinetic energy **(Satyanarayana et. al. 2005)**.

To survive under extremely cold conditions, psychrophilic and psychrotolerant bacteria requires many adaption strategies for its optimal functioning. In general, it has been known that the phospholipids composition of bacteria gets changes with the growth temperature **(Tribelli and Lopez, 2018)**. Thus they possess different absorption mechanisms as compared to mesophilic species. The psychrophilic bacteria are being recognized by their metabolic stability in cryo-habitats which is maintained by their cryoprotectant protein **(Feller, 2017)**. The cryostability of the psychrophilic enzymes has been established as valuable biocatalysts for various biotechnological and industrial applications. From the biotechnological point of view, the psychrophiles are the most attractive organisms due to their ability to produce enzymes that catalyze industrial appropriate process at low temperature **(Margesin and Schinner, 1994)**. Extracellular-polymer-degrading enzymes have several applications in biotechnology-based industries **(Cavicchioli et al. 2011)**.

The various researchers to determine the microbial diversity of the glaciers and other cold habitats have followed various culture dependent and culture independent methods. In view of some limitations of traditional culture-dependent techniques various new methodologies have been taken into consideration in nowadays such as PLFA, DGGE, and metagenomics to study the microbial diversity throughout the world **(Kayani et al. 2018; Fakruddin and Mannan, 2013; Rincon-Florez et al.2013)**. Aiming the geomicrobiological features from cold habitats, bacterial diversity from different glaciers, ice sheets and polar regions were studied such as Greenland ice sheets **(Stibal et al. 2015)**, Himalayan glaciers **(Sherpa et al. 2018; Pradhan et al. 2010; Liu et al. 2009; Zhang et al. 2008; Xiang et al. 2004)**,Arctic glacier **(Lutz et al. 2016; Cameron et al. 2012)**, Taylor glacier, Antarctica **(Doyle et al. 2013; Mikucki and Priscu, 2007),** GPSP2 Greenland ice core **(Miteva et al. 2009)**, Icelandic glaciers and ice caps, Iceland **(Lutz et al. 2015)**.

Since ancient periods nature and its products have the direct influence on human life. One of the most commercial exploited natural products is antibiotic. Antibiotic and their compound that kills or impede the development of causal microorganisms have extensive used in combating the bacterial diseases **(Penesyan et al. 2015)**. However, due to the overexploitation of ready to use drugs in combating the diseases contributed considerably resistance of microbes to antibiotic and arethe nowadays-growing problem around the world. One of the major facts is the transfer of antibiotic-resistant genes from affected population to various environments directly or indirectly associated with them. Over the

period of time, there may be the aggregation of multiple resistant traits among bacteria which makes them resistance to several classes of antibiotics **(Chang et al. 2015)**.

A new perspective of human health have aroused from antibiotic resistant bacterium harboring in glacial ecology. Many ARGs for antibiotics like aminoglycosides, lactams etc. were reported from glacier ecosystem of snow, ice, permafrost regions etc. **(Edwards, 2015; Segawa et al. 2013; Ushida et al. 2010)**. Functional analogs and structural homologues of various beta-lactamases were detected from ancient Alaskan soils by COG and KEGG analysis **(Allen et al. 2009)** andthe most probabilistic hypothesis put forward is due to the air borne bacteria carried as aerosols or in dust particles might help in the dispersal of these ARGs whereas some hypothesize them to be from migratory bird droppings **(Segawa et al. 2013)**. **Dancer et al**. **(1997)** also reported many ampicillin resistant coliforms in the 2000 years old glacial ice core samples from Canadian Arctic archipelago. The presence of ARGs in environment samples represents the anthropogenic closeness of human population which also might lead to transfer of genes through glacial melt water in future. Also, MRGs can also get transferred owing to inorganic pollutant contamination **(Edwards, 2015; Hodson, 2014; Tieber et al. 2009)**.

It is imperative to study the psychrophiles from glaciers as their chemical constituents and various metabolites are stable at various low temperatures. Sikkim, which harbors numerous glaciers and thus provides an opportunity to study psychrophilic/psychro-tolerant bacterial diversity, along with its antibiotic and heavy metal resistance and screening of some psychrophilic enzyme producing isolates.

In Sikkim, there are around 84 glaciers both in small and large in size covering over an area of 440 sq.km. of Sikkim **(Bahuguna et al. 2001)**. All these glaciers are located at Mt. Kanchenjunga range of North and West Sikkim, which is considered as the biological hotspot in Eastern Himalaya and also identified as World Heritage Site by UNESCO in 2016. The overall permanent snowfields in Sikkim was estimated 251 sq. km. area and in addition to glaciers, the whole snow-covered area attain around 691 sq. km **(Luitel and Shrestha, 2012)**. It is also reported that glacial and snow cover areas store roughly around 145 cu. km of water in Sikkim **(Pradhan et al. 2004)**.

The glaciers in Sikkim are the major perennial source of fresh water, the discharge water from these glaciers feed into the two main river system of Sikkim, the Teesta and the Rangit river. The main feeders' glaciers for Teesta river are Teesta Khangsa glacier, Tent peak glacier, Changme Khangpu glacier, Changme Khang glacier, South Lhonak glacier, North Lhonak glacier, Langbu glacier, Zemu glacier, Hidden glacier, Chungsang glacier, Talung glacier, Nepal gap glacier, Zumthal Phuk glacier. Similarly, for Rangit river the main feeder glacier is Rathong glacier **(Pradhan et al. 2004)**. Since limited microbiological studies have been conducted on Sikkim Himalayas glaciers, therefore our present study focuses on four glacial accumulation zones of North Sikkim (ChangmeKhangpu, ChangmeKhang, Chumbu and Kanchengayao glaciers) with special attention on the physiochemical analysis andmicrobial diversity.

2. RATIONALE AND SCOPE OF STUDY

The Himalayas are natural ecological and biological hubs for numerous vegetations, flora, fauna (both macro and micro) and animals and plants (**ICIMOD, 2009**). Sikkim Himalayas comes under the seismic zone and hence the probabilistic of extreme changes in local weather and climate might cause to glacier retreats in future (**Bajracharya et al. 2007**). Rapid changes in the abiotic parameters might decline the snowline and snow cover, irregular melting of glaciers with respect to local climatic changes, GLOFs etc. can be detrimental to huge populations present in the neighboring and adjoining states of Indian territory and countries near Sikkim in future.

Glaciological studies have been highlighted and are now in prime research arena owing to climate change analysis done globally. Thus, the microflora and fauna in cold cryosphere are now in focus and they are niches to psychrophilic or psychro-tolereant microbes (**Cavicchioli et al. 2002**). Cryosphere zones like glaciers hordes various kinds of microbial communities, which are, still obscure and evoke excitement and incite huge possibilities for the discovery of many new novel microbes posted at the gelid conditions. The Himalayas around Sikkim and adjoin areas are regarded as the hot spot zones of biodiversity in earth. This region has several kinds of habitat like the glacier, permafrost, cold desert, alpine lakes, and alpine soils **(Gurung and Bajracharya, 2012)**. There have been researches and reports of various committees and boards associated with climate change on Himalayas but they have been only from known sampling sites. Many regions are yet to be explored in Sikkim Himalayas.In Eastern Himalayas limited studies on microbiology of glaciers have been conducted with the aim of identifying few novel

species **(Kumar et al. 2016, 2015a, 2015b; Swarnkar et al. 2016)**whereas studies related to microbial diversity along with physic-chemical analysis of glaciers are lacking and therefore these kind of studies requires immediate attentions.The glacial with structural differences might harbor psychrophilic bacteria **(Bottos et al. 2008**;**Perreault et al. 2007; Bowman et al. 2000)**.

Glaciers are the important niche, habitat and analytical cosmos of the microbial consortium present in the 70% of the hydrosphere and which constitutes bio-indicating organisms**(Miteva, 2008)**. Global climate change and pertaining to it the effects of greenhouse gases, a lot is said about the glaciers to melt and change in the local weather. Microbiological analysis of the glaciers will be providing an immediate correlation between the climatic change and the differential data generated will help in the analysis of their current melt. The research focuses on studying the cold microbial dwellers of glaciers in Sikkim termed as "True Psychrophiles" and their presence along with the biomarker enzymes will help to connote the term psychrophily**(Miteva, 2008)**. Change in their geochemistry, seasonal kinetics, and characteristics along with their biodiversity variation will help to determine their nature of habitat and its trophic level.

Psychrophilic and psychrotolerant microorganism are crucial economic generator because of cryozymes production **(Margesin et al. 2007)** which have several industrial applications, such as cryo-enzyme based detergents, hydrolysis of lactose from milk, industrial dehairing of skins, stone washing, bio-polishing of textile products, food and meat processing, softening of wool or cleaning of contact lenses, bioremediation of waste

water and solids, and production of biofuels at frigid conditions **(Ueda et al. 2010)**. The benefit of these comes from its flexible structure, whichreimburses at cryo conditions **(Siddiqui and Cavicchioli, 2006)**. The change in the thermodynamics of the cellular physiology suits the biochemistry of the cellular functioning at cryo conditions **(Cavicchioli et al. 2011;Gerday et al. 2000)**.The psychrophiles are found in diverse cold habitats throughout the World and one of the major studied cold-habitats of psychrophiles are glaciers. In context to Sikkim, India, the present study of bacterial diversity in glaciers of Sikkim is unique as the microbiological diversity study among glaciers of Sikkim was not examined till date by using both culture-dependent and culture-independent techniques. The glaciers are regarded as God and Goddess in Sikkim and melt-water from these glaciers have been in use for religious and household purposes since ages.

Through metabolomics and ionomics, a detailed KEGG data can be obtained regarding each of the sampling sites. Then their genetic structures responsible for aiding the microbes to sustain the harsh cold climate will be correlated to the other microbes isolated from the various glaciers of the world. Metagenomics analysis provides the inevitable proof of the whole microbial community structure in extreme niches. During cultivation of microbes through media or nutrients, deciphering the complete microbiome community is very exclusive for many others those do not prefer *in vivo* conditions. It is biased whereas through metagenomics it removes the biasness of cultivation technique and rather relies on the genetic makeup, physiology and biochemistry of the microbe.

Omics have also enabled the researchers to study the metabolic pathways, energy or nutrient pathways and transcriptomic studies.

The next essential aspect is the study of resistance to antibiotics. The antimicrobial resistance is a main worry and threat to the current scenario throughout the world which is causing deadly infections, mortality and escalating healthcare costs **(Harbarth et al. 2015)**. The antibiotic resistance study was extensively targeted both pathogenic and non-pathogenic bacteria belonging to mesophiles by many researchers. But, very less research was carried out in the arena of antibiotic resistance pertaining to psychrophiles. As the antibiotic resistance is increasing at an alarming rate, the psychrophiles must be explored in order to get new brainwaves in the field so that the ways to manage the antibiotic resistance could be boomed.

There are numerous aspects to study psychrophiles isolated from glaciers of Sikkim in terms of antibacterial resistance. The first significance to study the antibiotic resistance of these psychrophiles is that they are considered to be causative agents of food poisoning along with seafood and spoilage of refrigerated food **(Karaft, 1992)**.As the glacier melt-water of Sikkim are used bythe huge number of people for various purposes such as drinking and agriculture purposes. Therefore, to study the antibiotic resistance bacteria dwelling in these glaciers is very crucial to take an account on the potential future effects and the consequence of these resistance genes. Further, it has been reported that there is a close nexus between antibiotic resistance phenomenon and heavy metal resistance phenomenon. Thus, the third and last important aspect of this study as psychrophiles are

less understood in terms of antibiotic and heavy metal resistances and very less information is present on the resistance profile.

In an order to address the above mentioned issues, four glaciers from North Sikkim were selected for the present study. Three glaciers, i.e., Chumbu, Changme Khangpu, and Changme Khang glaciersare closely located, whereas Kanchengayao glacier is distantly located. The culture-dependent microbiological studies including 16S rRNA sequencing were conducted for all the four glaciers, whereas culture independent studies were conducted on Changme Khangpu and Changme Khang glaciers.

Thus, the main aim of the thesis was to document the geographical coordinates of the glacier, conduct microbiological experiments to isolate psychrophilic or psychrotolerant bacteria from the glacier ice core samples identify them and screen useful enzyme producing bacteria from these frigid conditions.

The major objectives for the present studies are:

1) To document two glaciers of North Sikkim.
2) To perform the physicochemical analysis of the glacier samples.
3) To isolate psychrophilic or psychrotolerant bacteria from the glacier samples.
4) To perform the biochemical characterization of the bacterial isolates.
5) To check the enzymatic activity (amylase, protease and lipase) of the selected bacterial isolates.

6) To perform the bacterial identification of the isolates by the 16S rRNA gene sequencing.
7) To perform the fatty Acid Methyl Esters Analysis (FAME analysis) of the selected bacterial isolates.

3. REVIEW OF LITERATURE

3.1 General description of glaciers

Glaciers are usually described as a persistent body of dense ice that is constantly moving down under its own weight. Glaciers gradually deform and flow due to stresses induced by their weight, creating crevasses, moulin, and other unique features. They also erode rock and debris from their substrate to shape landforms such as cirques and moraines **(Mishra, 2007; Nath and Vaughan, 2003; Peterson 1999)**. A glacier consists of ice crystals, water and rocks debris. Out of these components, ice is a vital part of a glacier. Based on morphological characteristics glaciers are classified into two major groups, ice sheets and ice caps **(Thibaut, 2012; Sugden and John, 1976)**. Ice sheets are glacier that covers surrounding terrain area and is greater than 50,000 km^2 and may actually cover an entire continent such as Antarctic **(Kargel et al. 2011)**. They could be further being divided into terrestrial and marine based ice caps. For terrestrial based ice caps most of the base lies above sea level but for the marine-based ones, most of the base lies below sea level. Marine-based sheets are also called ice shelves. Ice caps are smaller than ice sheets. Depending on velocity glaciers might be classified as ice domes, ice shelves, and streams. Ice domes are huge areas of comparatively slow-moving ice **(Ronald and Pelt, 2010)**. Outlet glaciers flow faster and may be confined by topography, such as mountains, or by ice rises, slow-moving ice surrounding the outlet glacier. Glaciers, constrained by the adjacent landscape, whose flow is influenced by the shape of the valley, are classified as valley glacier, ice fields, and cirque glacier. Mountain glaciers as in the Himalayan regions, Alps, and Adens are chiefly constrained by topography and were mainly of the

valley type glaciers **(Basnett et al. 2011)** and were again categories into two types debris-free and debris-covered glaciers **(Shroder et al. 2000; Nakawo and Yabuki, 1999).**

In debris-free glacier, ablation zone is not covered by the deposition of rocks and boulders from the surrounding mountains and glaciers remains crystal clear. On the other hand, debris-covered glaciers are mainly responsible for sediment transport in glacial environments **(Kirkbride, 1995)**. Ablation zone of the debris-covered glacier is normally created by rockfall from the neighboring mountain, avalanches, soil erosion from high altitude moraines and debris fall through glaciated zones**(Benn et al. 2012;Hewitt, 2009; Hambrey et al. 1999)**. Among both the glaciers, two distinct main zones are formed i.e. ablation and accumulation zone. An upper portion of the glacier is called as accumulation zone where snow and ice accumulate by snowfall, hail, drifting snow, avalanche and frozen rainfall. Accumulation zone can be easily identified on a glacier as a snow or ice surface without any supra-glacier debris cover. Whereas, the lower portion of the glacier is called as ablation zone, where loss of ice takes place through the process of melting, evaporation, calving, and deflation **(Mishra, 2007)** (**Fig.1**). It is generally debris-covered and often glacier surface is marked by supraglacial lakes. The zone that separate accumulation and ablation zone on glacier surfaces is regarded as equilibrium line altitude (ELA) **(Kaser et al. 2003)**.

Microbiological studies of glacier ice from Himalayan areas are difficult for numerous reasons. The first reason is sample collection is pretty dangerous and highly expensive

from the Himalayan belt. Glacier ice core samples from the debris-covered glacier is even more difficult in comparison with debris-free glacier because of difficult topography and ice core drilling requires glacial expertise and requires costly instruments **(Christner et al. 2005a; Rogers et al.2005, 2004a; Willerslev et al. 2004b)**.

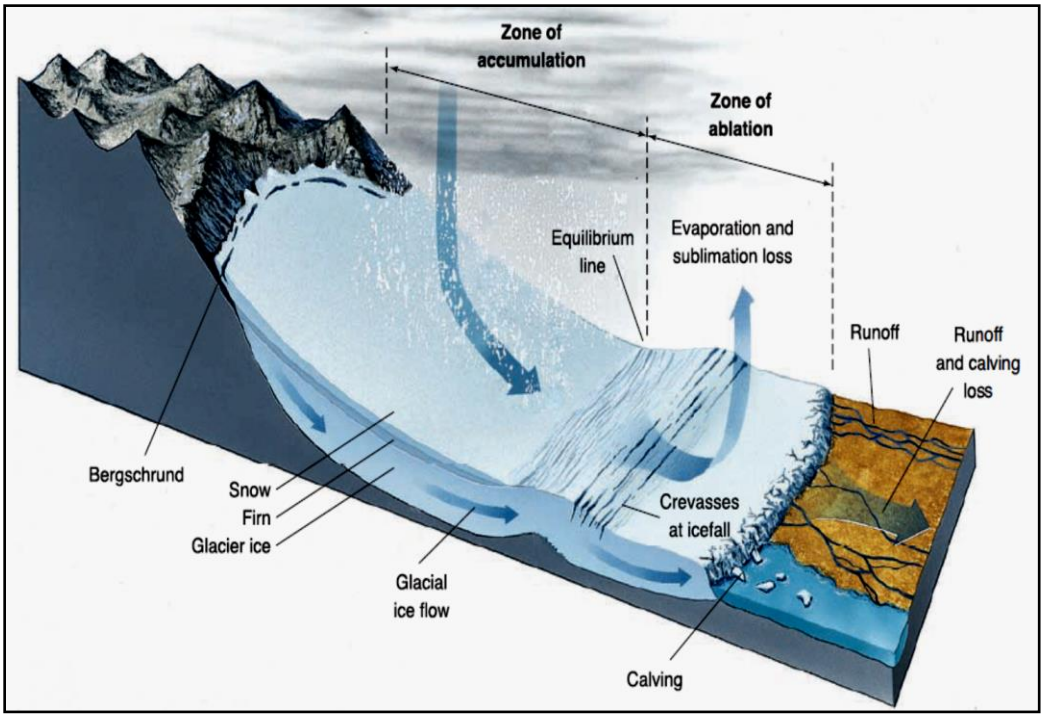


Fig.1. Morphology and Hydrological system of the Himalayan glacier(Adapted from **Bell, 2013)**.

3.2. Formation of mountain glaciers

Snowfall gets accumulated in the polar region and high altitudes globally. At high elevations, the snow fall above the glacier gets gradually compressed and pits on for ages. The translation of firm snowflakes into glacial ice is a complex process that depends on the rates and depths dependent on the amount of snowfall accumulation, air temperature,

the moisture content of the snow and whether the glacial surface experiences annual cycles of freezing and melting or not **(Christner, 2002; Peterson, 1994)**. As overlying firm snowflake applies great pressure and forms a solid crystal, these solid crystals slide and bond to other crystal planes which further squeezes the intervening space and creates ice trapped bubbles. Gases are not able to disperse through solid ice. Consequently, an air bubble within glacial ice does not originate from precisely the same time of point as the adjacent ice and variations range from hundreds to several thousand years. The glacier ice depth is often only a few meters in the mountain, but in a polar ice sheet, it can be a hundred meters depth **(Peterson, 1994)**. When a glacier accumulates to more than 20 meters in heights in accumulation zone the ice runs much like a viscous liquid. Gravitational force exerts a vertical force on the surface, causing the ice to be pressed out laterally and forms ablation zone of a glacier in Himalayas **(Hughes, 1998)**.

3.3. Microbial life in the glacier

The major portion of the ice of the glaciers on earth is accounted by Antarctica, Greenland ice-caps sheet, polar andnon-polar glaciers which covers roughly 10-12% of earth's land and generates approximately 70-75% of perennial fresh water on earth**(Junge and Rouge, 2011)**. Temperate and high altitude glaciers are found all over the word which represents a major source of freshwater for millions of peoples on the earth **(Junge and Rouge, 2011)**.

Microorganisms like (bacteria, archae, viruses, fungi and alage) are carried and deposited on the surface of snow by wind from different places. Glacier ice entraps and preserved aerosolized dust particles containing biological materials. The eroded materials in wind

varies in content and quality depending on the topology, local and global environmental conditions create different local snow ecological system and thus represents a dynamic nutrient and microbial reservoirs in glaciers**(Jones, 1999)**. Both in polar and non-polar glaciers viable bacteria and fungi have been detectedincluding some ancient glaciers also**(Miteva and Brenchley, 2005; Christner et al. 2003b; Christner et al. 2000;Abyzov et al. 1998b)**. **Priscu, 2004**reviewed the bacterial diversity in different glacial and sub-glacial environments.Generally, microorganism dwelling on glacier or ice habitats gets highest exposure to UV irradiation, high light intensity and experiences huge variations intemperature throughout different seasons.

In addition to the severe conditions associated with atmospheric transport, microorganisms deposited in glacial ice are exposed to stress conditions and extremely low temperature. Several studies in the field of microbial ecology and evolution reported the ability to adapt and survive the extreme cold climatic conditions prevalent in glaciers where availability of nutrient and liquid water is very low **(Karen et al. 2011).** The cold adapted higher macro-organisms or microbes produces intra cellular solutes like glycine and betaine which acts as cryo-protectant and also absorbs the cellular osmotic stress conditions **(Marthi and Lighthart, 1990)**. Along with solutes many bacteria produce thermal stress antifreeze proteins and cryo-protectant which prevent damage initiated by ice crystallization**(Gilbert et al. 2005, 2004)**. Cellular exopolysaccharides plays a crucial role as cryo-protectant in psychrophilic bacteria **(Kim and Yim, 2008; Krembs et al. 2002)**.

Another functional adaptation of psychrophilic bacteria is synthesis of dihydrouridine which maintain conformational flexibility of RNA and it helps the dwelling organisms to adapt at cryo conditions **(Moyer and Morita, 2007)**.

3.4. Microorganisms immured in glacier ice

The aerosol and dust particles from all over the earth carry the air flora and in the form of snow get deposited at the Polar Regions **(Jones, 1999)**. The Himalayas is the home to the largest concentrations of snow and glacier outside the polar region. These environments are niches for cryo-bacteria or psychrotolerant microbes. The term "psychrophiles" is defined by **(Moyer and Morita, 2007)** as bacteria or archaea having minimum, optimum and maximum growth temperatures at or below 0°C, 15°C and 20°C, respectively or psychrotolerant to describe those microbes which can grow both at low and high temperature range up to 20°C. According to Cavicchioli, microbes that prefer frigid conditions but can also survive in wide range of varied temperature as that of mesophiles are called Eurypsychrophiles**(Cavicchioli, 2006)**.

Bacteria found in the upper layers of glaciers can be considered as a recent deposition events and it showed community diversity profile **(Hell et al. 2013; Lopatina et al. 2013)** and relation to local environments **(Harding et al. 2011; Liu et al. 2009)**. Microbes from polar and non-polar environments was dominated by phylum *Proteobacteria, Cyanobacteria, Actinobacteria*, *Bacteroidetes* and *Firmicutes*, whereas *Beta-proteobacteria* are the dominant class detected by many researchers in the glaciers**(Moller et al. 2013; Hell et al. 2013; Lopatina et al. 2013; Larose et al. 2010; Liu et al. 2006)**. Many bacteria were isolated from various polar and non-polar glaciers.

Among them the dominant genus are *Arthrobacter, Clavibacter,* and*Mycobacterium* from phylum *Actinobacteria*;*Methylobacterium, Sphingomonas, Acinetobacter* and *Pseudomonas,* from phylum *Proteobacteria*; *Chryseobacterium,* and*Flavobacterium* from phylum *Bacteroidetes*and*Bacillus, Exiguobacterium, Paenibacillus,* and*Planococcus* from *Firmicutes***(Loveland-Curtze et al. 2010; Miteva et al. 2009, 2004; Raymond et al. 2008; Chaturvedi and Shivaji, 2006; Miteva and Brenchley, 2005; Xiang et al. 2005; Foght et al. 2004; Zhu et al. 2003; Christner et al. 2001, 2000)**.

Studies on glacier and ice sheet indicate that the concentration and diversity of microorganisms depends upon the geography, environment, niche and abiotic factors like wind **(Marshall and Chalmers, 1997; Fuzzi et al. 1997; Giorgio et al. 1996; Lighthart et al. 1995)**.**Abyzov et al**. **(1998a)** advocated that the concentration of microbes in debris free glacier ice are normally less and they have also found that the microbial cell number changes with depth and altitude, with a key role in these variations, might be assigned to the dust particles which vehicles away the microbes in glaciated zones **(Abyzov et al. 1998a)**. **Abyzov (1993)** studies in Antarctica demonstrated that these subtle differences are directly proportional to the volume of dust load experienced in annual snow precipitation. Similar reports have been found Greenland and Tibetan glaciers by different authors **(Zhang et al. 2006c; Yao et al. 2006; Xiang et al. 2005)**. The basal ice of alpine glaciers and from Antarctica and Greenland has been reported to contain very high percentage of microbes **(Price, 2007)**. In Greenland (GISP2), **Sheridan et al. 2003** detected 10^7 cfu mL^{-1} cell in the basal ice from eolian deposition matter. The high

percentage of minerals in ice core samples collected from 3,040 meters below the surface in GISP2 depicts the nutrient availability for microbial metabolism present in that niche **(Tung et al. 2006)**.

Cryoconite holes ("cryo" means"ice" and "conite" means"dust"),are another defined microbial habitat whichare present on glaciers **(Gajda, 1958)**. Cryoconite holes are water filled cylindrical melt-holes and are widely distributed in glaciers. All these glacier features harbors huge biodiversity of microbes and their discovery has caused a great thrill in research. These studies also reflect on the nature of origin and evolution of the limits of life on our planet.

3.5. Methodologies to study bacterial diversity

The first person to investigate the presence of microbial life was confirmed by Leeuwenhoek by employing his magnification lens, back in the late 1600s **(Kennedy et al. 1994)**. However, the velocity of research in microbial diversity has elevated due to the presence of industrially and ecologically significant bacteria from different environments **(Vitorino and Bessa, 2017)**. The reason behind the less understanding of the microbial world lies in the fact that about 99% of microbes are not cultivated by routine cultivation techniques in the laboratory **(Kellenberger, 2001)** and also these microbes are tiny and invisible to the naked eye **(Pace, 1997)**. On the basis of morphological and nutritional criteria it is very difficult to designate the taxonomical description of bacteria. The conventional culture-dependent methods were the principal methods to designate the bacterial diversity of the various ecosystems such as glaciers before the PCR era.

Development of rapid molecular techniques as polymerase chain reaction (PCR) based technologies boosted our information regarding the microbial diversity of environments **(Besseris, 2014)**. The comprehensiveness of 16s rRNA qualities makes them an ideal tool to study the phylogenetic and taxonomic grouping and thus it has aided a great understanding of microbial diversity **(Woese et al. 1975)**. The inability to cultivate the gigantic mass of microbes through culture based techniques paved the way to develop new tools to understand the biodiversity**(Hugenholtz et al. 1998; Amann et al. 1995; Staley and Konopka, 1985)**. To conquer this limitation, different molecular strategies were developed like DGGE, PLFA, and metagenomics. The advent of metagenomic techniques has deciphered many interests in microbial ecological researches. Another well-known technique to give an idea about the total biomass and the microbial diversity present in extreme environments is based on the presence of various signature fatty acids present in bacteria, which is generally known as Phospholipid Fatty Acid Analysis. This culture-independent approach is fascinatingand is used to evaluate the microbe community structure **(Willers et al. 2015)**. Earlier around late 70s, PLFA was only used to determine the marine and estuarine sediment biomass**(White et al. 1979)**. Phospholipids are unstable and it get rapidly degraded upon cell-death and thus PLFA analysis can measure the viable biomass as well as taxonomic evaluation of a microbial community **(Jenkinson and Ladd, 1981; White et al. 1983)**. The main disadvantage of PLFA is that it is unable decipher species composition or phylogenetic resolution **(Willers et al. 2015)**.

The limitations of culture dependent studies of microbial ecology and evolutions can be better represented by whole genome sequencing, microbiome analysis, PCR, DNA microarray etc. Phospholipids fatty acid analysis, and most recently metagenomic approaches. To recognizing the ecology of an environment metagenomic approach can explore the whole environmental microbiome. About 99% of bacteria from environmental samples remain 'unculturable' in the laboratory and hence remain ambiguous for their ecological functions and unexploited for biotechnological applications **(Kellenberger, 2001)**. The metagenomic techniques have resulted in discoveries that remain hidden from the traditional culturing techniques. It has opened the innovative facets in the advancement of biotechnology based on the utilization of uncultivated microbial species **(Bashir et al. 2014)**. The metagenomic technique also disclosed the information such as microbial diversity, uncharacterized metabolism, biogeochemical pathways and it also ensures to offer new enzymes and molecules with a variety of industrial applications **(Gilbert and Dupont, 2011)**.

3.6. Bacterial diversity in glacial ice

Christner, 2002 and his team constructed gene library from 500,000 year old ice core sample from Guliya, China glacier by targeting 16S rRNA gene primers. This study suggested the bacteria belonging to phylum *Proteobacteria*are predominantly present in those samples. Similar types of the dominance of were also reported by **Xiang et al. (2004)** from Tibetan plateau and Malan ice core samples **(Xiang et al. 2004)**.The identified bacteria from anaerobic liquid culture were dominated by phylum

Proteobacteria with *Eubacterium, Clostridium* and *Bacteroides* genuses from GISP2 (Greenland ice core) by **Sheridan et al. 2003**.

Even though the glacial were highly challenging, isolation of bacteria from glacial samples have been quite fruitful with many reports from Polar Regions. Different researchers or research groups have acquired bacteria from ice core retrieved from both polar and non-polar glaciers samples with different cultivation methods, however the most predominant bacterial flora to be reported were that of Gram-positive bacteria like *Marinobacter, Flectobacillus* and *Flavobacterium* which was found from Malan and Guliya ice core from the Tibetan plateau and and *Flavobacterium* predominantly from Polar ice caps **(Christner 2005b, 2001; Xiang et al. 2005, 2004; Miteva et al. 2004)**.

The uniformity of acquiring similar bacteria isolates suggested that almost all the microbes have similar cellular features to adapt these frigid cryo-conditions **(Christner et al. 2000)**.**Abyzov et al.(1998a)** identified spore-forming bacteria with the help of microscope, however they did not detect the presence of spore in these samples and hence reported that the vegetative cells might be more durable at cryo temperature for longer time period **(Yao et al. 2003)**.

Cryoconite hole as defined earlier, is water filled depression located in ablation zones on the glacier surface in both polar and non-polar glaciers **(Gribbon, 1979)**. It is considered as nutrient-rich habitats in the glacial habitats and also believed as one of the most studied glacial ecosystems in the globe **(Margesin et al. 2002)**. The first important

difference between glacier ice and Cryoconite hole is that the microbial communities like bacteria, algae, diatoms, and metazoan present in a Cryoconite hole are more abundant and more diverse than glacier ice. Among microbial communities the photosynthetic cyanobacteria are the most dominant in cryoconite holes**(Stibal et al. 2006; Sawstrom et al. 2002)**.

Christner et al.(2003a) for the first time reported the complete microbial community molecular characterization of cryoconite hole through universal bacterial and eukaryotic specific 16S/18S rRNA primers of total DNA extracted from an Antarctica Cryoconite hole. They found more bacterial and cyanobacterial sequences than algae in the library. Another interesting study found novel species *Pedobacter cryoconitis* from alpine glacial cryoconite**(Margesin et al. 2005)**.

3.7. Adaption of psychrophiles to low temperature

Psychrophiles endure under extreme temperatures pursuing many adaptations strategies than their mesophilic counterpart. These microbes acquire various physical (modification of membrane lipids), genomic and proteomic adoptions strategies in order to overcome their cell damages under low temperatures. Some of the adaptations are discussed below:

a. *Regulation of membrane fluidity*: In freezing environments, there are many changes at fatty acid level of intracellular membrane of bacteria to preserve the membrane fluidity. To overcome the deleterious effects of the harsh conditions bacterial cell membrane is the first line of barrier that can sense the environmental changes. According to **Shivaji and Prakash, 2010** bacterial membranes turn into more rigid at

cold temperature, which stimulates a membrane-associated sensor resulting in the genetical functionality responsible for membrane dynamics for substitute of metabolites from and to the cell. As a result, changes in the membrane lipid composition facilitate this process. This is achieved by modifications in the lipids' fatty acyl chains that serve to maintain optimum membrane fluidity.Generally, lower growth temperatures induce the activation of cold-shock-activated enzymes 'desaturases' which in turn promoted the synthesis of short-chain fatty acids (SCFA), branch chain fatty acids(BCFA), anteiso fatty acids to long chain fatty acids, straight chain fatty acids and iso-fatty acid **(Suutari and Laakso, 1994)**.

b. *Carotenoid pigments:*Synthesis of carotenoid pigments by numerous bacteria isolated from polar and non-polar regions ice has been described which helps in the maintenance of cell membrane structure, fluidity, and protection from UV radiation. A study carried out by **Fong et al. (2001)** in the psychrotrophic bacteria *Arthrobacter agilis* detected the abundance of carotenoid C-50, and Bacterioruberin and they hypothesis that accumulation of these pigments are responsible for membrane fluidity at low temperatures.Similarly, several carotenoid pigments are synthesized by Antarctic bacteria**,** i.e.,*Micrococcus roseus* and *Sphingobacterium antarcticus* **(Chattopadhyay, 2006).**

c. *Antifreeze proteins:* Antifreeze proteins (AFPs) are the ice-binding proteins which can reduce the freezing point of water and show extracellular ice re-crystallization inhibition action during latter stages of the warming cycle **(Christner, 2010; Gilbert et al. 2004)**. Duman and Olsen (1993) first reported the AFPs in bacteria through studying *Moraxella* sp. strains. **(Yamashita et al. 2002;Duman and Olsen,**

1993).Some psychrophilic bacterial AFPs have been detected from cell extracts of *Micrococcus cryophilus, Pseudomonas putida* and *Rhodococcus erythropolis***(Muryoi et al. 2004)**.

d. *Cryoprotectants;* Cryoprotectants are chemical substances (e.g. sugars, alcohols, and amino acids) which are generally produced in high amounts by many psychrophilic microorganisms and it was suggested that these cryoprotectants prohibits cryo-influenced protein aggregation and buffers the membrane dynamics at stress conditions **(Mancuso et al. 2005; Krembs et al. 2002). Ko et al. (1994)** proved that glycine betaine cryo-protectant enhanced the growth ability of *Listeria monocytogenes* at frigid temperature.Furthermore, trehalose demonstrated the evidence in preventing protein denaturation and aggregation in bacteria at low temperature **(Phadtare, 2004)**. Similarly in fungi trehalose is a stress protectant and stabilizer of the membrane at cryo level **(Tibbett et al. 1988)**.

e. *Cold Shock Proteins:* Theseare considered as a major response of cells to cold shock as they withstand the detrimental influences temperature downshift and they play a critical role in cold-adaption **(Hebraud and Potier, 1999)**. These stress proteins and are present both in mesophiles and psychrophiles whereas special group of proteins called as cold-acclimation proteins (CAPs) have been detected only in psychrophilic bacteria **(Chattopadhyay, 2006)**. These CAPs and CSPs get hyper synthesized when stress at cryo conditions is met with during the entire growth period **(Phadtare, 2004)**. Further, another cold accumulation protein (Hsc 25) was discovered which could refold the denatured cryozymes and it was synthesized by *Pantoea ananas*

KUIN-3, an ice nucleating bacterium following sudden temperature change **(Kawahara et al. 2000)**.

3.8 The biotechnological significance of Psychrophiles

The microbial biotechnology has undoubtedly altered our live style in many fascinating ways. Many of the feedbacks concerned in industrial or biotechnological procedures to formulate production need to take place on extremes of temperature, pH, pressure, and salinity **(Boehmwald et al. 2016)**. The mesophilic macromolecules can be exploited in these conditions, but being temperature susceptible, their macromolecules must be chemically or genetically modified in order to obtain suitable products. However, such modifications lead to lengthy processes and are not cost-effective **(Siddiqui et al. 2009)**. On the contrary, nature has excitedly offered us alternates in the form of cold-adaptive enzymes which are present in cold-loving microbes that can flourish in cold environments, pressure, salinity, and pH **(Sarmiento et al. 2015)**. These naturally cold-active macromolecules are being already used in diverse industrial processes.

The various applications which have made prototype alters in the field of microbial biotechnology are the detection of commercial cold active neutral protease is mainly obtained from *Bacillus subtitis* and marketed under the name of Neutrase**(Banerjee et al. 2016)**. The enzymes produced from *Bacillus sp*., *Clostridium sp., Staphylococcus sp*. are a very good source of enzymes for textile industry. Amylases have been used for desizing textile industry since middle of the last century**(Araujo et al. 2009)**.

Other applications of psychrophiles include bioremediation at low-temperatures using different cold-adaptive enzymes, **(Sarmiento et al. 2015)** psychrophiles were also used in textile industry, detergent, bioremediation, and Food **(Lee et al. 2017;Kirti et al. 2014).**

3.9. Application of Psychrophiles

Psychrophilic enzymes are cold-active biocatalysts acquired from cold-loving microbes. Such psychrophilic enzymes have a broad range of applications such as in dairy, food, bioremediation, and detergent industry. The cold-active milk coagulating, or curding enzymes have the benefit of controlledcasein coagulation for keeping the quality of whey in cheese industryfor manufacturing of whey cheese **(Samarzija et al. 2012;Ramana et al. 2002; Ren et al. 1988)**. A commercial microbial rennet accessible in the market in developed countries with the product name Marzyme® (Dupont, United States), Rennilase 50TL, and Moelilase are products of cold-active microbes **(Banerjee et al. 2016; Ramana et al. 2000)**. By using psychrophilic beta-galactosidase, around 70-80 percent product yields can be obtained, which is much elevated in contrast to the processes acquired using enzyme from mesophilic microbes **(Banerjee et al. 2016; Coker et al. 2003)**. The commercial cryo-protease is mainly obtained from *Bacillus subtilis* and is marketed under the name Neutrase **(Azarnia et al. 2006)**. The enzyme is known to increase the flavor intensity with a reduction in the ripening time of cheese.

3.10. Bioremediation

Human activities in cryosphere are leading to the accumulation of a pile of garbage, which poses a serious threat to the environment **(Reddy et al. 2016)**.With an urgent need of improving biodegradation, bioremediation and removal of toxic compounds and xenobiotic compounds from cold-regions, the psychrophilic enzymes have been receiving increased attention now a days **(Wang et al. 2010a; Joseph et al. 2008; Collins et al. 2005; Margesin et al. 2003)**.Numerous of studies from cold-regions revealed that cold-dwelling microbes and their enzymes such as catalase and oxidaseactivity areprerequisites at cold environments for bioremediation and biodegradation**(Violot et al. 2005)**. The biodegradation with cold-active enzymes presents various benefits over previous strategies. It was observed that management of contaminated soil with the application of cryozymes is cheap and sustainable **(Ramana and Singh, 2000)**. Till date, several studies have been carryout from Antarctic marine water to isolate the bacteria which can be utilized for bioremediation. **Sainai et al. (2006)** revealed that the recombinant expression of Toluene-o-Xylene Monooxygenase (ToMO) from *Pseudomonas stutzeri* OX1 in *Pseudoalteromonas haloplanktis* TAC125 (marine bacterium isolated from Antarctica) converted different types of aromatic compounds into their respective catechols at various temperatures**(Papa et al. 2009; Siani et al. 2006)**. These bacteria were utilized for bioremediation of marine contaminated habitats in Antarctica and other cold regions**(Brun et al. 1997)**.

3.11. Detergent industry

Detergent industries are the largest industry sector throughout the world and it requires a massive amount of enzymes. According to GMEIA (Global Market for Enzymes in Industrial Applications) detergent industry total growth will be around 25-30% in the year 2020. Microbial enzymes have added extensively to the growth and development of industrial detergents. Detergents are used in varied applications such as laundering, dishwashing, domestic, and industrial cleaning **(Schafer et al. 2002)**. These enzymes facilitate the elimination of proteinaceous stains and offer a great edge over conventional detergent technologies. The usage of cold active-enzymes in detergent is preferred due to the fabric restoration and an increased performance in cold temperature. The cold-active enzymes holds an important role in detergents due to their activity at varying range of temperatures, but the cost of production limits its usage **(Marx et al. 2007)**. Few cold-adapted enzymes are commercialized: this includes protease from Novozymes (trade name Savinase).

3.12. Food Industry

Cryozymes have great potential in food and beverage industries like dairy, meat, baking, meat etc. To remove lactose and to improve digestion and sweetness of milk, cold-active β-galactosidases from *Arthrobacter psychrolactophilus* were widely used **(Nam et al. 2011)**. Owing to functional activity at cryo-temperature it makes the transport and storage of milk convenient. Cryo-pectinases are used in juice extraction process in the juice industry for improving the extraction yield, clarification and taste of fruit juice **(Truong et al. 2001)**. Similarly,cryo-proteases have been extensively for the treatment of beverages such as beer and accelerated maturing of cheese. In the

meat processing industry, cryo-proteases have been used for enhancing freezed meat and tenderization of meat products. Apart from this, enzyme xylanase, extracted from *Pseudomonas haloplanktis* TAH3A a bacteria isolated from Antarctica is widely used in the baking industry. This enzyme is usually used to increasing the bread volume and improving the crumb quality **(Collins et al. 2005)**.

Table.1. Some relevant cryozymes of psychrophilic/psychrotolerant microbes and their application in food industry

Enzymes	Source	Application	References
ß-galactosidase	*Arthrobacter psychrolactophilus*	Conversion of cheese by-product to glucose and galactose; removal of lactose from milk	**(Nam et al. 2011)**
Xylanase	*Pseudomonas haloplanktis* TAH3A	Enhancing the quality of bread by xylan hydrolysis	**(Collins et al. 2005)**
Pectinase	*Bacillus*sp. SC-H	Pectin degradation in food processing	**(Cabeza et al. 2011)**
Proteases	*Colwellia psychrerythraea* strain 34H, *Bacillus amyloliquefaciens* S94	Meat tenderization, beer processing, bakery and cheese industries	**(Huston et al. 2004; Son and Kim, 2003)**

3.13. Textile Industry

The enzymes produced from *Bacillus*sp., *Clostridium* sp. and *Staphylococcus* sp. were reported todesignate a very good source of enzymes for the textile industries. Since the middle of the last centuryamylases has been in use as a desizing agent in the textile industry. The application of alpha-amylase enzyme for denim jeans and laccases for bleaching of textile effluents and textile bleaching is the most current commercial

advances in the textile industry **(Kuddus et al. 2013; Gerday et al. 2000)**. The psychrophilic enzyme also allows the improvement of environment-friendly strategies in fibre processing to enhance the final product quality **(Araujo et al. 2009)**.

3.14.Cryozymes/Psychrophilic enzymes

Psychrophiles turn into a key asset for bio-prospecting because of their unique ability to cold adoptions, which assist them to successfully thrive in such frigid conditions. The main imperative of these cold adaptations, which has huge potentials are yet to be explored like PUFA and cryozymes. The proteins and membranes in these organisms possess unique characteristics of property of improved structural flexibility which enhances the catalytic function. When temperature falls, psychrophiles make antifreeze proteins or CSP that boosts the enzyme activityby increasing stable microtubules to improve enzyme kinetics. Cryozymes have enormous advantage at cryo conditions with enhanced specific activity. Psychrophilic enzymes produced by such microbes' exhibits high catalytic activity at frigid temperaturewhich recommends considerable uses in detergent, textile, food, pharmaceutical, leather, brewing and wine etc. **(Kumar et al. 2011)**.The perspective of cryozymes and their uses have been extensively studied by many researchers **(Georlette et al. 2004; Cavicchioli et al. 2002; Deming, 2002; Margesin et al. 2002; Dumont et al. 2000)**.

3.15. Cold-adapted lipase

Next to carbohydrases and proteases, Lipase (triacylglycerols-acylhydrolases, EC 3.1.13) constitutes the third most important category of enzymes. They are ubiquitous enzymes, which are found in plants, animals, fungi, and bacteria and are of extensive potential in

industrial applications for a variety of purposes**(Aravindan et al. 2006)**. Cold-adapted lipases display high specific activitybetween 0°C-30°C**(Lee et al. 2017)** and have the ability to catalyze a variety of reactions related to inter-esterification of lipids **(Colla et al. 2010)**. Lipid degrading microbes at cryo-temperature is vital for waste water management **(Joshi and Satyanarayana, 2013)**.These interesting attributes create lipase the most versatile biocatalysts in industry.

Various kind of microbial lipases have been found from cryosphere **(Kanmani et al. 2015)**. The stability of lipase at a diverse range of temperature is an important prerequisite feature for industrial applications **(Nigam, 2013)**. The main disadvantages of enzymes were their sensitivity towards heat during food processing industries **(Margesin and Schinner, 2001)**. Therefore to prevent products from being denatured, cryozymes are useful. These microbes usually grow slowly even under appropriate conditions **(Ingraham and Strokes, 1959)**. However, modern advances in genetic or chemical engineering offer many tools which can alter the enzyme kinetics**(Cavicchioli et al. 2011)**. Genetic engineering has facilitated the vector based cloning of many cryozyme genes in *E. coli*host strain**(Siddiqui et al. 2009; Tutino et al. 2001)**.

Hence, recombinant clones of cryo-tolerant microbes which have high catalytic activities can bio-transform many compounds of industrial significance **(Margesin and Schinner, 1994)**.

Table.2. Cold-adapted lipase producing bacteria from isolated different habitats

Microorganism Name	Sources	References
Aeromonas hydrophila	Marine habitat	**Pemberton et al. 1997**
Bacillus sphaericus MTCC 7526	Gangotri glacier, India	**Joseph, 2008**
Photobacterium lipolyticum sp. nov.	Marine habitat	**Ryu et al. 2006**
Psychrobacter okhotskensis sp. nov.	Seacoast	**Yumoto et al. 2003**
Serratia marcescens	Raw milk	**Abdou, 2003**
Staphylococcus epidermidis	Frozen fish samples	**Joseph et al. 2008**

3.16. Cold-adapted protease.

Protease catalyzes the hydrolysis of peptide bonds linking amino acids together into its monomers. Cryo-proteases are mostly useful in detergent industries **(Joshi and Satyanarayana, 2013)**. Cryo-proteases are obtained from many psychrophilic environments mostly like cryospheric zones of our planet. Some of the microbial cryo-protease producers are *Bacillus licheniformis* from glacier soil **(Baghel et al. 2005)**, *Xanthomonas maltophilia* from alpine environments **(Kuddus and Ramteke, 2008)**, *Curtobacterium luteum* from glacier soil **(Kasana and Yadav, 2007)**, *Exiguobacterium sp*. from cold-desert **(Margesin et al. 2005)**, *Pedobacter cryoconitis* from glacier ice **(Margesin and Schimer, 1991)**. The first bacteria protease was launched in the market in 1959, named as Bio40, a detergent containing bacterial proteases developed by Swiss chemist Jaag a Swiss chemist **(Mesbah and Sarmiento, 2016)**. NovozymesCompany has established many novel cryo-proteases for various detergent applications. In 1998,

Novozymes developed the first cryo-protease for laundry which was functional at 10°C-20°C in commercial scale in the name of Kannase and Liquanase. Polarzyme is a serine protease which is functionally active at wide temperature range from 5°C-60°C developed by Novozymes **(Sarmiento et al. 2015)**. Genencor (Palo Alto, CA, USA), hasdeveloped two cold-active proteases (Purafect and Properase) for laundry detergents, with optimal temperatures between 20°C-40°C. Current research has focused on new cryo-protease isolated from psychrophilic or psychrotolearnt microorganism of cold cryosphere regions **(Chen et al. 2013)**.

3.17. Cold-active amylases

Amylases are starch degrading enzymes **(Smith et al. 2005)**which are abundantly found in almost every microbiota and macro flora. Cryospheric dwelling microbes produce enzyme amylase, which functions well at low temperatures and shows a high rate of catalytic specifictyon comparatively against mesophilic/thermophilic homologs **(Joshi and Satyanarayana, 2013)**. These cold-adapted amylases have a series cellular feature which provides them the required plasticity and specificity (**Struvay and Feller, 2012)**.Amylases have the widest applications as it one of the most important enzymes in different industries, such as waste-water treatment, bio-pulping, bioremediations, bioremediations, molecular biology, additives in processed food and detergent industry. The range of amylase application has broadened in many other fields, such as medical, clinical, analytical, starch saccharification, food, fermentation, papers, brewing, and distilling industry **(Pandey et al. 2000)**.

Cryo-amylase producers are found from various sources. Among bacteria, *Bacillus cereus* CA6was discovered at Gangotri glacier **(Kuddus et al. 2012)**,*Microbacterium foliorum* CA2 from Gangotri glacier **(Roohi et al. 2013)**, *Wangia* sp. C52 from southern Okinawa **(Liuet al. 2011)**, *Micrococcus antarcticus* from Antarctica soil **(Fan et al. 2009)***Arthrobacter psychrolactophilus* from Pennsylvania soil **(Munaganti et al. 2015)**.

4. MATERIALS

4.1 Materials used for measuring and mapping of the geographical features of the sampling sites

1.Global Positioning System-GPSMAP78S, Garmin 2010, Olathe Kansas, United States of America (USA).

2.Google Earth Software-Google, USA.

3. Arc GIS Software-Google, USA.

4.2 Materials used for sample collection

Cello chiller ice box, Capacity 20-liter Cello House, Corporate Avenue, Mumbai, India

4.3 Instrument used for the determination of a physical parameter of the glacial samples

The U-50 Series multi-parameter water quality meter, Horiba, Japan.

4.4 Instrument used for the determination of the Chemical Analysis of the glacial samples

Inductively Coupled Plasma Mass Spectroscopy (ICP-MS), Perkin Elmer, Massachusetts, USA.

4.5 Materials used for the Isolation and Cultivations of the microorganisms

4.5.1. Microbiological Media

Luria Bertani Agar	Composition stated in Appendix I
Antarctic Bacterial Medium	Composition stated in Appendix I
R2A Agar	Composition stated in Appendix I
Nutrient Agar	Composition stated in Appendix I

Gelatin Agar	Composition stated in Appendix I
Skimmed Milk Agar	Composition stated in Appendix I
Starch Agar	Composition stated in Appendix I
Tributyrin Agar Medium	Composition stated in Appendix I
Agar Agar Type-1	HiMedia, Mumbai, India
Peptone Bacteriological	HiMedia, Mumbai, India
Yeast Extract	HiMedia, Mumbai, India
Beef Extract	HiMedia, Mumbai, India
Starch	HiMedia, Mumbai, India
Mueller Hinton Agar	HiMedia, Mumbai, India
Gelatin	HiMedia, Mumbai, India
Antarctic Bacterial Medium	HiMedia, Mumbai, India
R2A Agar	HiMedia, Mumbai, India
Nutrient Agar	HiMedia, Mumbai, India
Gelatin Agar	HiMedia, Mumbai, India

4.5.2. Chemicals

Sodium Chloride	Merck, Mumbai, India
Mercury Chloride	Merck, Mumbai, India
Copper Sulfate	Merck, Mumbai, India
Nickel Chloride	Merck, Mumbai, India
Sodium Chloride	Merck, Mumbai, India
Mercury Chloride	Merck, Mumbai, India

Cobalt Chloride	Merck, Mumbai, India
Zinc Chloride	Merck, Mumbai, India
Ferric Chloride	Merck, Mumbai, India
Ethyl Alcohol	Merck, Mumbai, India
Phenol Red	Merck, Mumbai, India
Hydrogen peroxide	Merck, Mumbai, India
Trichloro acetic acid	Merck, Mumbai, India
Folin- Ciocalteu's	Merck, Mumbai, India

4.5.3. Materials for the staining of the microorganisms

Crystal violet	HiMedia, Mumbai, India
Gram's Iodine Solution	Merck, Mumbai, India
Safranine	HiMedia, Mumbai, India

4.5.4. Materials for the biochemical Analysis of the microorganisms

Carbohydrate Fermentation Test

D (-) Arabinose	HiMedia, Mumbai, India
D (+) Dextrose	HiMedia, Mumbai, India
D (-) Fructose	HiMedia, Mumbai, India
D (+) Galactose	HiMedia, Mumbai, India
D (+) Lactose	HiMedia, Mumbai, India
D (+) Mannose	HiMedia, Mumbai, India
D (+) Maltose	HiMedia, Mumbai, India
D (+) Rhamnose	HiMedia, Mumbai, India

D (-) Mannitol	HiMedia, Mumbai, India
D (+) Sucrose	HiMedia, Mumbai, India
D (+) Melezitose	HiMedia, Mumbai, India
D (+) Melibiose	HiMedia, Mumbai, India
D (+) Xylose	HiMedia, Mumbai, India
D (+) Cellubiose	HiMedia, Mumbai, India

4.5.5. Antibiotic used for Antibiotic Assay

Ampicillin 10µg	HiMedia, Mumbai, India
Chloramphenicol 30µg	HiMedia, Mumbai, India
Streptomycin 100µg	HiMedia, Mumbai, India
Erythromycin 15µg	HiMedia, Mumbai, India
Vancomycin 30µg	HiMedia, Mumbai, India
Methicillin 10µg	HiMedia, Mumbai, India
Tetracycline 30µg	HiMedia, Mumbai, India

4.6. Laboratory Equipment and Instruments

Cooling Incubator	Instrumentation Ltd, Kolkata, India
Spectrophotometers (UV-vis 1600-00BD)	Perkin Elmer, Massachusetts, USA
ICP Mass Spectrometry (ICP-MS)	Perkin Elmer, Massachusetts, USA
Laminar Air Flow System	Thermo Fisher Scientific, Bangalore, India
Digital Analytical Balance Weighing	CONTECH Instruments Ltd, Mumbai,

	India
ABS/Genetic 3500	Applied Bio System, HITACHI, Japan
Thermal Cycler	BIO-RAB, Singapore
Autoclave	Instrumentation India, Kolkata, India
Incubator cum Shaker	REMI, Kolkata, India
Freezer -20°C	Voltas, TATA Product, India
Freezer -80°C	Thermo Fisher Scientific, Bangalore, India
Freezer 4°C	LG/GL-205KAGES/2014, Noida, India.
Compound Microscope	Olympus CKX41, Japan
Water Purification System	Millipore (India) Pvt Ltd, Bangalore, India
Hot Air Oven	Instrumentation India/SS304, Kolkata, India
Trans Illuminator	Genei/107161 REMI, India
Electrophoresis Chamber	GeNei, Banglore, India
IR Gun (Infrared Thermometers)	Shenzhen P and C Electronic Technology/GM320, China

4.7. Glassware

Test Tubes	Borosil, India
Petri plate	Borosil, India
Spreader	Borosil, India

Pipettes	Borosil, India
Glass slides	Borosil, India
Conical Flask	Borosil, India
Reagent Bottle	Borosil, India
Glass Beaker	Borosil, India

4.8. Materials used for the Molecular Characterization

QIAamp DNA Mini kit	QIAGEN/51304, Germany
HiPurA Agarose Gel DNA Purification Kit	HiMedia Laboratories Pvt. Ltd, Mumbai/ MB511-50PR, India
Universal primer 27F	Imperial Life Sciences (P) Limited
Universal primer 1492	Imperial Life Sciences (P) Limited
Green Mix	Promega, Banglore, India

4.9. Others Materials

Inoculum loop	HiMedia, Mumbai, India
Spirit Lamp	Borosil, India
Test Tube Rack	Tarson, India
Cryo Vials	HiMedia, Mumbai, India
Micro-tips	Tarson, India
Absorbent Cotton	Bengal Surgical
Non-Absorbent Cotton	Bengal Surgical
Micropipette	Gilson, France

Cryo Vial Stand	Tarson, India
Paraffin Tape	Bengal Surgical
Blotting Paper	Bengal Surgical

4.10. Software used for data analyses

CLUSTAL W	Kyoto University Bioinformatics Center, Japan
Codon Code Aligner	Codon Code Corporation, Centerville USA
MEGA6	Pennsylvania State University, Pennsylvania

5. METHODS

5.1. Mapping of the sampling sites

With the help of Global Positioning System (GPS78SMAP), the geographical coordinates were measured **(Thakur et al. 2013)**. To describe any geographical location one has to know the longitude and latitude of the area. Thus, on the basis of coordinates, the exact area gets mapped. Subsequently, the elevation of the land from the sea level is calculated to spot the area of the land. An ascent of the land represents the height of the site at which it is present. With the help of a compass, the exact direction of the site can be easily quantified. After measuring all the coordinates, elevation, latitudes, longitudes, the elevation of the site, the ascent of the site and the atmospheric temperature of the site was measured and they were mapped in Arc GIS Software and Google Earth.

5.2. Glacier ice sampling

Samples were taken from the accumulation zone of Changme Khangpu, Changme Khang, Chumbu, and Kanchengayao glaciers, North Sikkim, India in October 2016 when the glacier was not covered with snow. Glacier ice was collected by employing clean sterilized digging bar and pick axe. The overlying 0.3m of glaciers ice was removed and discarded. About 2m depth ice pit was burrowed and the core was collected from each glacier accumulation zone into a sterilized polypropylene bags (HiMedia, Mumbai) and were stored in sterile Cello chiller ice box (Cello Mumbai, India). These Cello boxes were sealed tightly and were immediately brought to the laboratory.For microbiological analysis, aseptic measures were taken and the ice samples were cut with a sterilized saw-tooth knife and around 5mm annulus was discarded. The remaining inner core was rinsed

with cold ethanol (95%), and finally with cold autoclaved water. The sterile ice samples were placed in the sterile containers and melted at 4°C incubator. These handling procedures were undertaken at temperatures below 20°C aseptically using positive pressure laminar flow hood as described by**(Xiang et al. 2004; Zhang, 2003)**.

5.3. Physicochemical Analysis of the glacial samples

5.3.1 Methods used for the analysis and determination of the physical characteristics of the glacial samples.

With the help of U-50 Series multi-parameter water quality meter, Horiba, Japan, the physical parameters- pH, conductivity, Total Dissolved Solid (TDS) and color and electrical conductivity of the onsite glacial were measured **(Lutz et al. 2015)**.

5.3.2.Methods for the determination of the chemical characteristics of the glacial samples

A total of 25 different chemicals were analyzed from Changme Khang and Changme Khangpu glaciers ice samples with the help of using Inductive Couple Mass Spectroscopy (ICP-MS), Perkin Elmer, Nex-ION 300X ICPMS, USA. Along with the elemental analysis, various other parameters such as pH,total hardness, turbidity,total alkalinity, phenolic compounds, color, total dissolved solids (TDS), and electrical conductivity) were measured by the help of ICPMS and Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) as per APHA standards **(Ewa and Bosnak, 2012)** was done.

5.3.3. Statistical analysis of chemicals

Piper plotting was done to verify the chemical compositions of glaciers ice samples on the basis of relative concentration of its constituents **(Piper, 1944)**. A piper design is a method for study the science of water, soil and rocks. The piper diagram was plotted by using AqQA software (Version v1.x. Rockware). The Heat-map analysis was shaped to compare the elemental concentration of Changme Khang, Change Khangpu glaciers and other five Himalayan glaciers data using Heat-Mapper software **(Babicki et al. 2016)**.

5.4. Microbial Analysis of the glacial samples

5.4.1 Enumeration of the microbial load by total plate count.

The viable bacteria or microbes able of utilizing nutrients from the agar-based plate, when develops a colony, that is enumerated and counted and depending on the dilution factor, the approximate microbial load of the sample is determined**(Toubes-Rodrigo et al. 2016; Hameed et al. 2015)**. Thus a faint idea about the microbial load in the sample can be understood. Serial dilution of the sample was done and pour plated on PCA (Plate Count Agar) plates. First, the sample was diluted from 10^2 to10^7dilutions in a test tube containing 0.85% NaCl. Next, from each dilution tubes, 0.5mL of the sample was spread in Plate Count Agar plate. The plates after incubation demonstrated colonies of the viable bacteria present in the sample. Plates having >300 colonies were rejected as Too Numerous To Count (TNTC) and plates having <20 colonies were also rejected as Too Few To Count (TFTC). Plates having colonies ≥20 colonies and ≤300 colonies were accepted. After adequate incubation, the number of colonies developed was calculated by using the formula

Total Plate count or viable count of the sample =

$$\frac{\text{Number of colonies (average of three replicates)}}{\text{Amount Plated X Dilution}}$$

5.4.2 Isolation and culturing of bacteria

Thawed glacial melt water 200µL from each inner ice core sample was directly spread using Luria Bertani Agar and Antarctic bacterial medium(HiMedia, Mumbai) at 15°C for three weeks in cooling incubator and also 200µL thawed water sample was enriched in 200mL beaker containing 50mL Luria Bertani broth and was then incubated at 15°C for 14 days in incubator cum shaker at 120rpm. Then after enrichment, the culture was spread plate using Luria Bertani Agar and Antarctic Bacterial Medium incubated for 3 weeks at 15°C until the colonies became visible **(Reddy et al. 2010)**. Morphologically different colonies were selected and were subculture by standard streak plate method **(Antony et al. 2012)**. Cryopreservation of the pure bacterial isolates was done at -80°C in 50% (v/v) glycerol**(Rafiqet al. 2017)**.

5.4.3 Characterization of the bacterial isolates from glacier ice samples

5.4.3.1. Cultural characteristics and morphology

a) General colony morphologies of the isolates

General colony morphology of the bacterial isolates were described on the basis of their shape, arrangement, colony color, forms, margins, elevation, and density etc. (**Rafiq et al.2017**).

b) Gram Staining

Gram-staining was carried out with the help of Gram stain-kit K001L (Himedia, Mumbai, India), for each of the bacterial isolates as per the protocol given by the manufacturer. The primary Gram stain (Crystal Violet) was flooded to the heat fixed smear and was kept for 30s. Then the smear was washed with distilled water and Gram's Iodine solution was flooded to the stained slide and kept for 60s after that iodine was washed off with 95% ethanol followed by distilled water. Slides were then counter stained with 0.5% safranin for 30s and were washed off by distilled water. After air drying was observed under a light microscope x100 and the observations were noted down**(Dussault, 1955)**.

c) Endospore Staining

Bacterial endospore was checked for each of the isolates using Schaeffer and Fulton's spore stain kit (S028). An overnight growth culture smears were made on cleaned grease free slides. It was air dried and then heat fixed. Slides were then placed over hot water bath for 10mins. After that the slide was flooded with spore stain A (Schaeffer and Fulton stain) and again steamed for 3-6mins. Slides were then rinsed under tap water and counter stained with spore stain B (Schaeffer and Fulton stain) for 30s. The slides were then washed with tap water. After air drying, slides were observed under Olympus 100x light microscope per **Keast et al. (1884)** method.

5.4.4. Growth profile at various physical parameters

5.4.4.1. Tolerance to different degrees of Temperature

Aim of the experiment was to check the optimum growth temperature required by the bacterial isolates. Sterile Luria Bertani broths at pH.7.0 was added into autoclaved test tubes and then each test tube was inoculated with different bacterial isolates and

incubated at different temperatures i.e., 5°C, 10°C, 15°C, 20°C, 30°C and 40°C in the cooling incubator for 72hrs incubations**(Rafiq et al. 2017)**. Post incubation, the Optical Density (O.D.) of the broth was evaluated at 600nm and 660nm in uv/vis Spectrophotometer.

5.4.5. Tolerance to different pH

Aim of the experiment was to establish the optimal pH range of the bacterial isolates. The bacterial isolates were inoculated in a test tube containing various concentrations of pH ranges in Luria Bertani broth. The pH was adjusted by adding 0.1N HCl for making the condition acidic and 0.1N NaOH to make the conditions alkaline. The isolates were inoculated at the pH of 2.0, 0.4, 0.6, 0.8 and 10, and were incubated at 15°C in the cooling incubator as maximum growth at 15°C**(Rafiq et al. 2017)**. They were kept for 72hrs incubations. Post incubation, the O.D. of the broth cultures was measured at 600nm and 660nm in uv/vis Spectrophotometer. All the observations were noted down and the pH tolerance capability of the bacteria was interpreted with evaluating the respective O.D. values of broth cultures grown at pH8 was the Control. Blank was prepared by taking the uninoculated Luria Bertani broth.

5.4.6. Tolerance to different concentrations of NaCl

Tolerance potential or growth at NaCl concentrations was verified using Luria Bertani media supplemented 1%, 2%, 5%, 8% and 10% (w/v) with NaCl **(Rafiq et al. 2017)**. Separate test tubes containing varied NaCl concentrations were inoculated with different bacterial isolates and incubated at 15°C cooling incubator for 72hrs. After incubation, the O.D. of the cultures was measured at 600nm and 660nm in uv/vis Spectrophotometer **(Bowman et al. 1997)**.

5.5. Biochemical characterization of the bacterial isolates

5.5.1. Carbohydrate Fermentation Test

Fermentative degradation of various carbohydrates by microbes was carried out in a fermentative tube**(Madigan et al. 2009; Smibert and Krieg, 1994)**.

5.5.2. Catalase Test

To all the individual bacterial isolates, 3% H_2O_2 was added**(Cappuccino and Sherman, 2002)**. The observation was noted down. The release of free oxygen gas in the form of bubbles is a positive sign for the test.

5.5.3. Protease Test

0.1mL of fresh bacterial broth was added on Skimmed Milk Agar plate (HiMedia)**(Cappuccino and Sherman, 2002)**. It was then incubated at 15°C for 72hrs in cooling incubator. Formation of a clear zone around the bacterial colony suggests that the microbes have protease activity.

5.5.4. Starch Hydrolysis Test

All the individual bacterial isolates were spread onto a plate containing Starch Agar Media **(Cappuccino and Sherman, 2002)**and were kept at 15°C for 72hrs. After incubations, the 2% iodine solution was flooded to the starch Agar plate and was observed for any color change or halo zone formation.

5.5.5. Lipase Test

0.1mL of fresh bacterial culture broth was added on Tributyrin Agar plate (HiMedia) **(Cappuccino and Sherman, 2002)**. It was then incubated at 15°C for 72hrs in cooling

incubator and clear zone observations were noted down.Formation of a clear halo zone surrounds the bacterial colony will show that the bacteria have lipolytic activity.

5.5.6. Gelatin Hydrolysis Test

All the individual bacterial isolates were spread plated into Gelatin Agar Medium Plate **(Cappuccino and Sherman, 2002)**and were kept at 15°C for 72hrs. After incubation, the 1% Mercuric solutions were flooded to the Gelatin Agar plates and were observed for any color change.

5.6. Determination of the proteolytic and amylase activity

5.6.1. Screening of protease and amylase producing bacterial strains

All the isolates were streaked on Skim Milk Agar (protease) and Starch Agar (amylase) plate **(Cappuccino and Sherman, 2002)**for screening bacterial strains for proteolytic and amylolytic activity. The isolates were then incubated at 20°C for 24hrs. The bacterial colonies with a large zone of clearance around their colonies were selected and considered for future study.

5.6.2. Enzyme production

The protease positive isolates wereinoculated in M1 and M2 media **(Souissi et al. 2008)**similarly amylase positive isolates were also inoculated in starch media at 20°C in a rotary cum shaker operated at 120rpm for 24hrs incubation. Then the bacterial cells were separated by centrifugation at 15000rpm for 10mins and the clear supernatants were then used as the crude enzyme.

5.6.3. Estimation of enzymatic activity

The proteolytic activity was estimated according to the method of **Carrie (2008)** by using casein as a substrate and amylase activities were checked according to **Divakaranet al. (2011)** methodusing starch as substrate. In brief, 0.6% casein and starch solution was prepared separately in a 50mM phosphate buffer, pH 7.5. Then respective crude enzyme was added to the test sample and incubated at 20°C for 10mins. Trichloroacetic acid (TCA) for protease and 3, 5-dinitrosalicylic acid (DNS) for amylase was added to the assay mixture for stopping the enzyme reaction and incubates at 20°C for 20mins. For protease assay 500mM sodium carbonate and 0.5M Follin Ciocalteu's phenol reagent was added to the assay mixture and incubated at 20°C for 30mins. Finally, the observance was recorded at 660nm by using a spectrophotometer. Tyrosine curve for protease and maltose curve for amylase was used as a standard for estimation of respective enzyme assay. One unit of enzyme activity was defined as the amounts of enzymes that liberate 1µmole of maltose (amylase) and tyrosine (protease) per minutes under the assay condition was calculated **(Horikoshi et al. 1984)**.

$$\text{Enzyme activity (U/mL)} = \frac{\text{µmol tyrosine/maltose equivalent releases} \times \text{Total volume of assay}}{\text{The volume of enzyme taken} \times \text{incubation time}}$$

5.7. Identification of bacterial isolates by Culture-dependent Techniques

5.7.1 Genomic DNA extraction

The genomic DNA was extracted using the bacterial genomic DNA (prep) Kit (QIAGEN, Germany) as per the manufacturers' instructions**(QIAGEN, 2015)**which are as follows:

a) 1mL of overnight growth fresh bacterial culture was added into a 1.5mL microcentrifuge tube, and centrifuged for 5min at 7500 rpm.

b) Then ATL buffer supplied by Qiagen (QIAamp DNA Mini Kit) was added to a total volume of 180µL. For Gram-positive bacteria, bacterial pellet was suspended in 180 µL of enzyme solution (20mgmL^{-1} lysozyme; 20mM Tris·HCl, pH 8.0; 2mM EDTA; 1.2% Triton).

c) After that it was incubated for 30min at 37°C and 20µLof Proteinase K and 200µL Buffer ATL was added and mixed by vortex.

d) Then it was incubated at 56°C for 30min at 95°C.

e) After that 200µL ethanol (96–100%) to the sample was added, and mixed by vortexing for 15s. After mixing, briefly, the 1.5mL microcentrifuge tube was centrifuged.

f) Then carefully the mixture from step 5 (including the precipitate) was transferred to the QIAamp spin column (in a 2mL collection tube) without wetting the rimand centrifuged at 6000g (8000 rpm) for 1min and spin column was placed in a clean 2mL collection tube, and the tube containing the filtrate was discarded.

g) Then 500µL Buffer AW1 was added and centrifuged at 6000g (8000 rpm) for 1min and column was placed in a clean 2mL collection tube, and the collection tube containing the filtrate was discarded.

h) After that carefully 500µL Buffer AW2 was added without wetting the rimand centrifuged at full speed (20,000g or 14,000 rpm) for 3min.

i) Then the column was placed in a new 2mL collection tube and the collection tube containing the filtrate was discarded. It was centrifuged at full speed for 1min.

j) At last the column was placed in a clean 1.5mL micro-centrifuge tube, and the collection tube containing the filtrate was discarded. And carefully 30µL Buffer AE or d.w. was added. Incubation at room temperature for 1min was done and then it was centrifuged at 6000g (8000 rpm) for 1min and step 10 was repeated again.

k) Presence of DNA was verified on 1% agarose and visualized under ultraviolet by staining with ethidium bromide.

5.7.2. Polymerase Chain Reaction (PCR) Amplification

Total DNA from each of the bacterial isolate was used as a template for amplification of the 16S rRNA genes using two universal primer-27F(5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGTTACCTTGTTACGACTT-3') **(Shen et al., 2012)**. The polymerase chain reaction was performed in 25µL volume using 13µL GoTaq Green Master Mix 2X (Promega), 1µL (20pM) each forward and reverse primers, 2µL template DNA and 9µL Milli-Q water. Amplification was performed using BIO-RAD, Thermal cycler PCR machine. Reactions mixtures were subjected to the following temperature cycling profiles repeated for 35 cycles: Initial 94°C for 5min for denaturation, followed by 30 cycle of 94°C for 30s, 55°C for 1min, 72°C for 1min, and the final extension at 72°C for 10mins.The PCR products were analyzed by electrophoresis at 100V for 1hr in a 0.8% Agarose gel (Sigma Aldrich, USA) in 1×TAE buffer and visualized under ultraviolet by staining with ethidium bromide **(Sambrook et al. 1989)**.

5.7.3. Purification of PCR products

The PCR product was purified using the QIAquick PCR purification Kit protocol (QIAGEN, Germany) the manufacturer's instructions **(QIAGEN, 2008)** which are as follows:

a) QIAquick column was placed in a collection tube provided and then sample was added to the column and centrifuged for 30-60s at 14,000rpm.

b) After that washing was done by adding 750µL buffer PE to the QIAquick column and then centrifuged for 30-60s at 14,000rpm. The flow through was discarded and the column was placed in the same tube. Then the column was again centrifuged for 1min at 14,000rpm to remove residual wash buffer.

c) Before eluting QIAquick column was placed in clean 1.5mL micro-centrifuge tube. DNA was then eluted by adding 30µL elution buffer/Milli-Q water and then centrifuged the column for 1min at 14,000rpm.

5.7.4. DNA sequencing

The purified 16S rDNA was then sequenced using a BigDye^{TM}Terminator v3.1 cycle sequencing kit (Applied Biosystems, USA) as manufacturer's instructions using Automated DNA Sequencer (ABS/Genetic 3500 Analyzer). After 16S rRNA PCR amplifications, the sequencing of high-quality DNA products were completed. Prior to sequencing the next round of sequencing PCR amplifications was performed using only single primer at a time with the reaction mixture. That means two reaction mixture PCR tubes one including 27F primer and another PCR tube containing 1492R primer. The total reaction mixture of each PCR tube contained 10µL (1µL Master Mix RR-100, 1.5µL Terminate Buffer, 2µL primer, 1µL PCR purified DNA and 4.5µL Milli-Q). The

purification of the PCR product was done by manufacturer guidelines which are as follows:

a) 10µL of MilliQ water was added to the PCR tube containing the product and then mixed well and transferred to 1.5mL eppendorf tube.

b) After that 2µL (125mM) EDTA, 2µL (3M) Sodium acetate, and 50µL (100%) ethanol was added and incubated for 15mins at room temperature. After incubation the eppendorf tubes were kept at 4 ºC for 30mins.

c) Then centrifugation was done for 15mins at 14000rpm and the flow through was decanted.

d) After that 70% ethanol was added and centrifuged for 10mins at 14,000rpm.

e) After that the supernatant was removed and dried and 14µL HiDi Formamide was added.

f) The PCR tubes were then heated at 55 ºC for 5min and immediately transferred to ice for 5min.

g) After that 12-14µL of that reaction mixture was added to sequencer 96 well plate and the sequencing was performed in Automated DNA Sequencer at Department of Microbiology, Sikkim University, Sikkim.

5.7.5. Statistical analysis

Assembled sequences were compared with sequences in the public databases using Basic Local Alignment Search Tool (BLAST) in the National Center for Biotechnology Information website (http://blast.ncbi.nlm.nih.gov/BLAST). Alignment was done using CLUSTAL W 1.6 software. The 16S rRNA gene sequences with high similarity to those determined in the study were retrieved and added to the sequences for the study.

Sequencing alignment was done using Mega 7.0 **(Tamura et al. 2007)**.A phylogenetic tree was constructed to identify the evolutionary lineage of the isolates. The tree was constructed using the neighbor-joining method **(Saitou and Nei, 1987)** with Jukes-Cantor evolutionary distance measurement **(Erickson, 2010)**in MEGA 7.0 software.

5.8. Fatty acid methyl-ester Analysis

Fatty acid Methyl Ester (FAME) analysis was done as per the standard protocol **(Buyer and Sasser, 2012)** and were assessed using Sherlock-MIDI identification system. The calibrated standards were used by the microbial identification system (Sherlock-MIDI, KID biotech, New Delhi, India) for annotation of generated phospholipid peaks. The cultured bacteria are the prerequisite for Sherlock System. The fatty acid methyl ester involves various steps such as the extraction of fatty-acids by saponification in dilute sodium hydroxide and distilled water solution followed by methylation with dilute hydrochloric acid and methyl alcohol solution to give the respective methyl esters (FAMEs). The extraction of fatty acid methyl esters was done with the help of hexene and methyl tert-butyl ether, and base wash was completed by the use of sodium hydroxide and distilled water. The fatty acid methyl esters are then separated from the water phase by the utilization of an organic solvent and the subsequent concentrate was analyzed by Gas chromatography (GC). Fatty acid methyl esters are more appropriate to GC analysis because they are more volatile than their respective fatty acids **(Ichihara and Yumeto, 2010)**. The Sherlock programming at that point utilizes a particular strategy recognition algorithm and consequently coordinates the obscure FAME profile to the put away sections for Identification. The results in Sherlock system is represented by a numerical value called as Similarity Index (SI) **(Kunitsky et al. 2006)**. The similarity

index is actually a comparison of fatty acid esters of an unknown microorganism with the mean fatty acid composition of other closely related strains used to create a library entry listed as its match **(Heyrman et al. 1999)**.

5.9. Identification of Putative Novel Psychrotolerant bacteria

A putative novel bacterium was isolated and identified. The polyphasic categorization was doneas per ICSB rules and regulations of nomenclature (The International Committee on Systematic Bacteriology) **(Moorea et al. 2010)**, different phenotypic and genotypic tests has been made. Such tests are discussed below:

5.9.1. Morphological classification

A morphological characteristic such as colony color, form, margins elevation, density, motility, and staining was done**(Reddy et al. 2010)**.

5.9.2. SEM (Scanning Electron Microscope)

The SEM analysis was performed to measure the check size and shape of the novel isolate as per **(Golding et al. 2016; Kalab et al. 2008)** using Scanning Electron Microscope- JCM-5700 (JEOL USA, Peabody, MA, USA).

5.9.3. Growth Profile at various physical parameters

The growth at diverse physical parameters were checked such as temperature from (5°C-40°C), pH (4-10), and NaCl (1-10%) was verified as per the given procedures**(Reddy et al. 2010)**.

5.9.4. Biochemical characterization (by manual methods)

Various biochemical parameters were tested such as carbohydrate fermentation, enzymatic tests such as amylase, protease, Lipase, catalase, oxidase, nitrate reductase, methyl red test were performed as per the given procedures **(Reddy et al. 2008).**

5.9.5. Biochemical characterization (BIOLOG)

All of the physical and biochemical tests were also performed by BIOLOG system as per the **(Ray et al. 2015)** methods.

5.9.6. Fatty Acid Methyl Ester (FAME) analysis

The fatty acid analysis of the putative novel strain was performed at 20°C. Fatty acids were extracted and analyzed by following the instructions laid by MIDI (Microbial Identification System) **(Kunitsky et al. 2006)**. The RTSBA6 system was used and the results were investigated by Sherlock Version 6.2.

5.10. Identification of bacterial diversity by Culture-independent techniques

5.10.1. Phospholipids fatty acid (PLFA) analysis

Lipid extraction and PLFA analysis were performed according to **Margesin et al. (2009)** method. PLFA concentrations ($nmolesgm^{-1}$) were calculated on a dry mass basis in replicates **(Morgan and Winstanley, 1997)**.

5.10.2. Next Generation Sequencing

5.10.2.1. Metagenomic DNA extraction

Glacier ice community genomic DNA was extracted by using DNeasy Power water kits (MoBio Laboratories, Carlsbad, CA, USA) as per the manufacturer instructions. Quality

of the DNA was checked on 0.8% Agarose gel and the DNA was quantified using Qubit fluorimeter (Thermofisher, USA), with the detection limit of 10-100ng/µL.

5.10.2.2. Metagenomic Library preparation and Sequencing

Shotgun sequencing libraries were prepared using NEB Next UltraII DNA library preparation kit (Illumina) followed by the standard manufacturer's protocol**(Kayani et al. 2018)**. Bioanalyzer and High Sensitivity DNA Kit (Aligent) were used to check the fragment size distribution of each library. Each library was then quantified using qPCR and sequenced using 100bp paired-end, HiSeq 2000, Illumina platform.

5.10.2.3. Data availability and accession number

The raw metagenomics reads were submitted to Sequence Read Archive (SRA), NCBI**(Das et al. 2017).**

5.10.2.4. Bioinformatics analysis

The 18.72 million reads were optimally assembled using the metaSPAdes **(Nurk et al. 2017)**, and the optimal assemblies were performed with K-mer length of 20 in increments of 20 and also scaffolded. Out of the 5 assemblies (i.e. 41,61,81,101 kmers and scaffold) it was concluded based on N50, the number of contigs assembled and contig length distribution that scaffolded assembly performed the best and was taken for further analysis. The assembly statistics were calculated using QUAST software and metaSPAdes after scaffolding assembled into contig length greater than 500bp **(Gurevich et al. 2013)**. The reads from the sample were mapped onto the contigs using BOWTIE2 in order to check for the assembly quality. More than 60% of sequence reads mapped back to the contigs successfully **(Langmead et al. 2012)**. The microbial abundance was

estimated by using METAPHLAN-2 **(Truong et al. 2015)**, a tool that profiles and classifies the sequencing data from shotgun metagenome samples, with species-level resolution a set of one million clade-specific marker genes from more than 17,000 different microbes (~13,500 bacteria and archaea, ~3,500 virus, and ~110 eukaryotes).

5.10.2.5. Functional analysis of the Metagenome data

The assembled contigs were annotated using BLASTn against the NCBI-nucleotide custom metagenome database that included all nucleotide entries for organisms that belong to the archaea, bacteria, fungi and viruses; with minimum sequence similarity of 60% and e-value lesser than 1e-07 **(Altschul et al. 1990)**. The contigs were annotated with~80% contigs with similarity >90%. The gene prediction and annotation of the assembled contigs were carried out using PROKKA **(Seemann et al. 2014)**. The predicted genes were annotated further for KEGG orthology using the KEGG Automatic Annotation Server (KAAS) **(Moriya et al. 2007)**,and Cluster of Orthologous Groups (COG) by BLASTx against the COG database.

5.10.2.6. Antibiotic Resistance Genes (ARGs) analysis

For the Antibiotic Resistance Genes (ARGs) annotation, the metagenomic tags of each sample were blasted against the structured nun-redundant clear antibiotic resistance gene database (ARBD, htpp://ardb.cbcb.umd.edu)**(Liu et al. 2009)**. The sequence is considered as ARG like sequences according to its best BLAST hit in ARDB with a threshold of amino acid sequences identity of no <90% to the references sequences and had a query coverage of no <25 amino acid.

5.10.2.7. Metal Resistance Genes (MRGs) analysis

For annotation of Metal Resistance Genes (MRGs), metagenomic data of each MRG genes were BLAST against the structured nun-redundant clear metal resistance genes by searching on online BacMet database (htpp://bacmet.biomedicine.gu.se) **(Pal et al. 2017)**. The sequences were considered to be MRGs following the same cutoff; similarity no <90% to the references sequences and had query coverage of no <25 amino acid.

5.11. Antibiotic Sensitivity of Isolates

The bacterial isolates were tested for their response to various antibiotics by Disc Diffusion Method. Hence, a series of Antibiotic Sensitivity was carried out for the isolates**(Tomova et al. 2015)**. Kirby Bauer Antibiotic Sensitivity method was followed. The bacterial culture 0.1mL was streaked by sterile cotton swabs in different Muller Hinton Agar (MHA) plate respectively. Then after 15mins of standing, one sterile antibiotic disc was placed at the center of the MHA plate. For a single isolate, its Antibiotic Sensitivity was measured against seven various antibiotics. Thus all the isolates were tested against the following antibiotics; Ampicillin (Amp10µg), Chloramphenicol (C30µg), Streptomycin (S100µg), Vancomycin (Van30µg), Methicillin (Met10µg), Tetracycline (Tet30µg), and Erythromycin (Er15µg).The sterile discs dipped in sterile water were the control. The plates were incubated at 15°C for 72hrs. After incubation, the plates were observed for their zone of inhibition and their diameter was determined and recorded. By evaluating the diameter size of the zone of inhibition, with the standard chart provided for by the disk manufacturer, the isolates were interpreted as Resistant (R) or Intermediate (I) or Susceptible (S) to the respective antibiotics.

5.11.1. The minimum inhibitory concentration of antibiotics (MICs)

The minimum inhibitory concentration (MICs) assays were performed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines NCCLS, 2004 **(Lyamlouli et al. 2011)**. Tube dilution method was performed to check the MIC. Tubes containing 10mL of Luria Bertani broth and various antibiotics at different concentrations (between 0.5-8 $mgmL^{-1}$ using dilution factors of ½) were inoculated with 200µL of 72hrs old culture (O.D. at 600nm=>1) of each isolated bacterial strain. A negative control consisted of Luria Bertani broth (as it is antibiotic-deficient medium) inoculated with the isolate and negative control consisted of antibiotic supplemented medium without adding the isolates. Tubes were examined in *uv/vis* spectrophotometer at 600nm after incubation at 15°C for 72 hrs.

5.12. Heavy metal tolerance to isolates

5.12.1. Screening and assessment of metal toxicity

Based on two distinct locations, Changme Khang glacier (debris-free) and Changme Khangpu glacier (debris-covered) were selected. The isolates were screened and assessed for metal toxicity**(Lyamlouli et al. 2011; Jennifer, 2001)**. The heavy metal solutions were arranged from their chloride and sulfate salts as $CuSO_4$ [Copper (II) Sulfate], $MnSO_4$ [Manganese (II) Sulfate], $ZnCl_2$ [Zinc Chloride], $HgCl_2$ [Mercury (II) Chloride] and $CoCl_2$ [Cobalt (II) Chloride]. Heavy metals stock solutions were prepared in distilled waterand were autoclaved.

6.12.2. Tube dilution method

Tube dilution technique was executed to verify the MIC (minimal inhibitory concentrations) **(Lyamlouli et al. 2011; Jennifer, 2001)**. Both culture medium and metal solution were autoclaved. 0.5mL of appropriate metals concentrations were dissolved in LB broth with a final volume of 10mL. Three tubes were prepared for each metallic concentration, and then inoculated with 200µL of an18hr old culture of the studied bacterial isolates. A positive control consisted of LB broth without metal were inoculated with the isolate and the negative control consisted of a metal-containing medium without addition of isolate. To compose the metal containing medium, 1mL of respective metal solution was added to 9mL of LB broth. The glasswares used for the experiments was leached in 2N HNO_3 and then was rinsed three times with deionized double distilled water (Milli-Q) so as to rule out any chances of heavy metal contamination. Test tubes were read in *uv/vis* spectrophotometer (Perkin Elmer LAMBDA 40) at 600nm after incubation at 20°C for 24hrs **(Kim et al. 2007)**.

6. RESULTS

6.1. Description of sampling sites

Sikkim is the smallest state of India and it hosts many glaciers. The majority of these glaciers are valley-type debris-covered glaciers. Owing to a remotelocation, glaciers of Sikkim are less explored and not still properly documented. Therefore, we first checked the localization of Changme Khang, Changme Khangpu, Chumbu and Kanchengayao glacier of North Sikkim with the help of GPSMAP 78S. The coordinate of the sites of Changme Khang glacier and its sampling source was located and it lies between 27°56'38.80"N longitude and 88° 39'56.91" latitude and the elevation range of the location was 5221.2 m, similarly Changme Khangpu glacier were located at 27°58'04.16"N longitude and 88° 40'56.68" latitude and altitude range of the site was 5319m. These glaciers originated from south slope of Mt. Gurudongmar peak whereas Chumbu glacier originated from south slope of Chumbu peak and it was located between 27°55'06.52"N longitude and 88°40'10.89" latitude and altitude range of 5093.8m (**Fig. 2a**). Meltwater of these glaciers feeds into Sebu basin which is ultimately merged into Lachung river of North Sikkim. Kanchengayao glacier, on the other handis located at Lachen basin, in the upper catchment of one of its major tributaries, Lachen River, in the Thangu valley of North Sikkim, India.Kanchengayao glacier is adebris-free transverse valley glacier. Kanchengayao glacier originated from south slopeMt. Kanchengayao peak, trending north-south face having latitude 27°59'57.872'N and longitude 88°37'8.785'E at an altitude of 1393m (**Fig. 2b**). Melt water of this glacier feeds into Thangu River a tributary of LachenRiver, at Lachen North Sikkim.

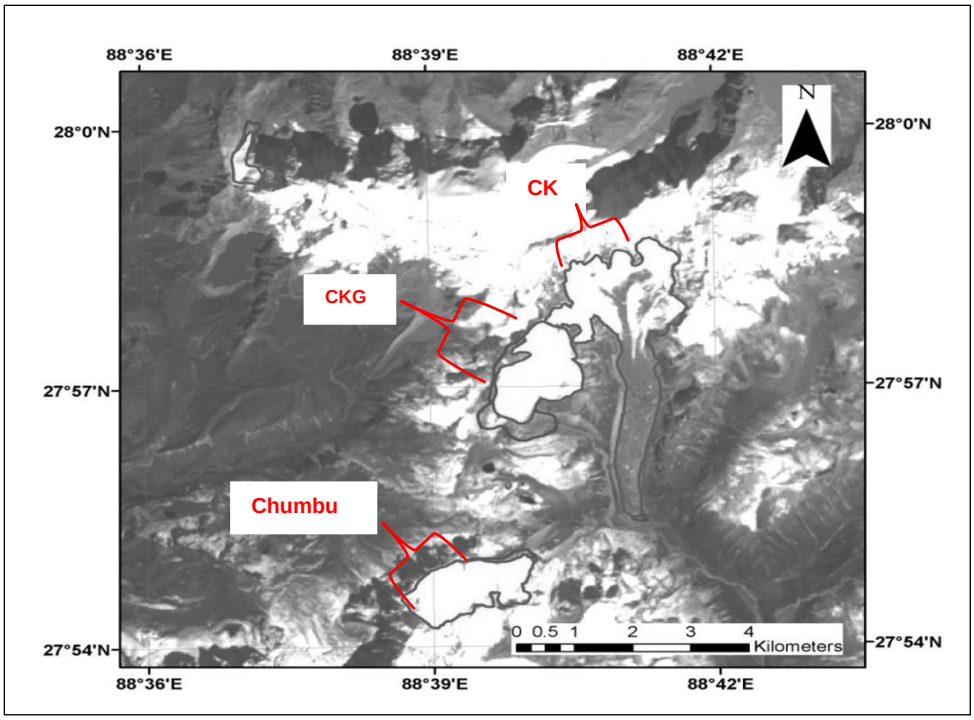


Fig: 2a. GoogleEarth map of Changme Khang (CKG), Changme Khangpu (CK) and Chumbu glaciers of North Sikkim.

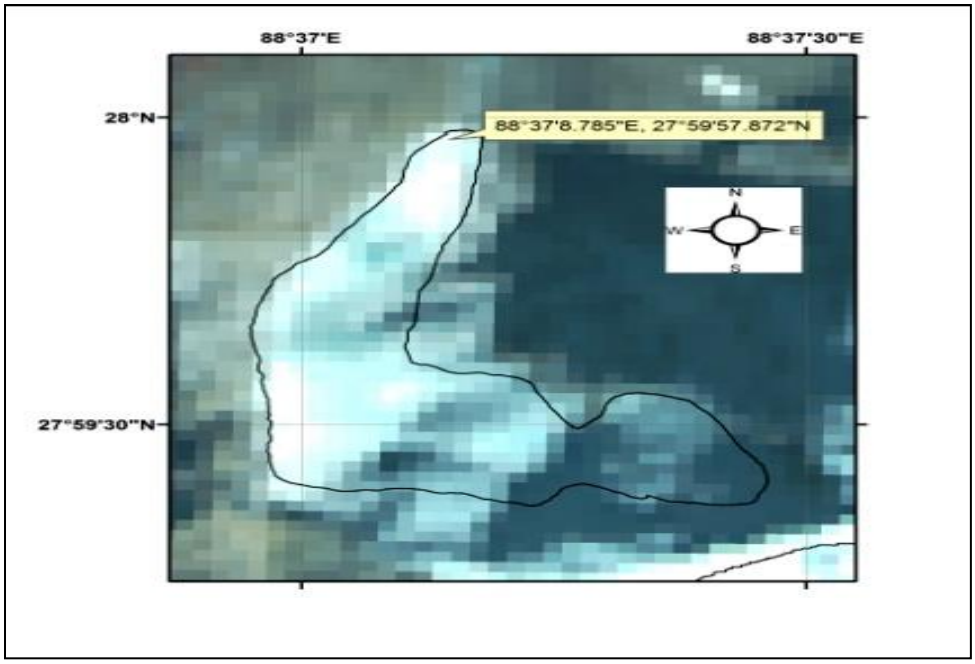


Fig: 2b. Google Earth map of Kanchengayao glaciers of North Sikkim.

6.2. Sampling and physiochemical analysis of Samples.

6.2.1. Sample collection and Pretreatment

The samples were collected by drilling in the accumulation zone of Changme Khang, Changme Khangpu, Chumbu, and Kanchengayao glaciers, North Sikkim, India by employing sterilized digging bar and pick axe. The 30cm glacier accumulation zone surface ice was removed and discarded. Around 2m depth ice pit was burrowed and the ice core was collected from each glacier accumulation zone into a sterilized polypropylene bags (HiMedia, Mumbai)and were stored in sterile Cello chiller ice box (Cello Mumbai, India).These Cello boxes were sealed tightly and were immediately brought to the laboratory. The ice core sample was processed and cut into small pieces about 6 inches squares. The aseptic measures were taken and the glacier ice core samples were cut with a sterilized saw-tooth knife and around 5mm annulus was discarded. The remaining inner core was rinsed with cold ethanol (95%), and finally with cold (4ºC) autoclaved water. Then the ice core samples were placed in the sterile containers and melted at 4ºC incubators. These handling procedures were undertaken at the temperature below 20ºC aseptically using positive pressure laminar flow wood as described by **Zhang, 2003; Xiang et al. 2004**.

6.2.2. Physical analysis of samples

The parameters which were used to describe the physical properties of water were temperature, turbidity, color, pH, and dissolved oxygen. The glacier accumulation zone temperature was monitored at approximately six-month interval for three years in the glacier ice pit at depths of 2m using IR Gun (China). The mean temperature showed by Changme Khangpu glacier was¯41°C followed by Changme Khang glacier sample ¯33°C.

Other two glaciers i.e. Chumbu glacier and Kanchengayao glacier showed ¯32°C and ¯ 30°C respectively. Other physical parameters were measured on the sampling site with the help of Horiba water analyzer. The nephelometric turbidity (8NTU) and total dissolved solids (1.03gL^{-1}) showed by Changme Khangpu glacier sample was highest as compared to other three glaciers (**Table 3a)**. pH of these glaciers were more or less similar. i.e., it was between 7-7.43. Thus, suggesting that pH of water sample are neural. Our result from Changme Khangpu glacier (Debris-cover glacier) accumulation zone sample showed positive correlated with**Takeuchi et al. (2012)** work, which also confirmed that the highest turbidity was confirmed in the debris cover glacier.

Table 3a. Physical Analysis of glaciers samples

Glaciers	**pH**	**Electrical Conductivity µScm**	**Turbidity NTU**	**D.O. mgL^{-1}**	**TDS gL^{-1}**	**Color Hazen**	**Temperature ºC**
Changme Khang	7.43	145	2.12	10.77	0.69	<1	-33
Changme Khangpu	7.42	144	8	10.23	1.03	<1	-41
Chumbu	7	133	4.12	9.5	0.75	<1	-32
Kanchengayao	7.2	105	3.22	12.4	0.65	<1	-30

The data was measured by Horiba water analyzer and IR Gun. The data present here is the average of the triplicates.

6.2.3. Chemical Analysis of the glaciers samples

The Inductive Couple Mass spectroscopy (ICP-MS) was used to determine the chemical properties of the water. It was observed that nitrate, magnesium and bicarbonate

concentration are maximum as compare to other chemical constituents. The Changme Khang glacier was found to be rich in calcium, magnesium, and total alkalinity as compare to Changme Khangpu glacier. Similarly from Changme Khangpu glacier chemicals such as nitrate, sulfate, colloidal sulfur as well as COD and BOD were showed higher than Changme Khang glacier (**Table 3b**).

Table 3b. Elemental analysis of Changme Khang (CKG) and Changme Khangpu (CK) glaciers.

SNO.	**1**	**2**	**3**	**4**	**5**	**6**	**7**
Test parameter	Color	Aluminum	Ammonium	Boron	Calcium	Chloride	Copper
Unit	Hazen	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
CK	<1	<0.03	<0.5	<0.5	18	17	<0.05
CKG	<1	<0.03	<0.05	<0.5	23	17	<0.05
SNO.	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Test parameter	Residual free chlorine	Iron	Magnesium	Manganese	Nitrate	Phenolic compounds	Sulfate
Unit	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
CK	0.2	0.06	5	<0.01	17	0.19	10
CKG	0.2	0.07	8	<0.1	11	0.13	9
SNO.	**15**	**16**	**17**	**18**	**19**	**20**	**21**
Test parameter	Total alkalinity	Zinc	Cadmium	Cyanide	Lead	Mercury	Nickel
Unit	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
CK	47	<0.05	<0.003	<0.05	<0.01	<0.001	<0.02
CKG	52	<0.05	<0.003	<0.05	<0.01	<0.001	<0.02
SNO.	**22**	**23**	**24**	**25**	**26**	**27**	**28**
Test parameter	Total arsenic	Total chromium	COD	BOD	Colloidal sulfur	Total phosphate	Bicarbonate
Unit	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}	mgL^{-1}
CK	<0.01	<0.05	60	26	11	<0.05	59
CKG	<0.01	<0.05	40	17	6	<0.05	63

Chemical Analysis of water from Changme Khang and Changme Khangpu glacier:The data was measured by Inductively Coupled Mass Spectroscopy (ICP-MS).

We also compared five different Himalayan glaciers chemical compositions by Heat-map analysis, and it was found that Changme Khang and Changme Khangpu glacier were different as both these glaciers have higher chloride concentration than other glaciers (**Fig. 3**). Similarly, concentration of calcium in Changme Khang and Changme Khangpu glacier was higher than Gangotri, Bagani, and Dokriani glacier but lesser than Kafni and Dudu glacier. Likewise, Kafni and Dokriani glacier showed higher bicarbonate than other glaciers (**Fig. 3**).

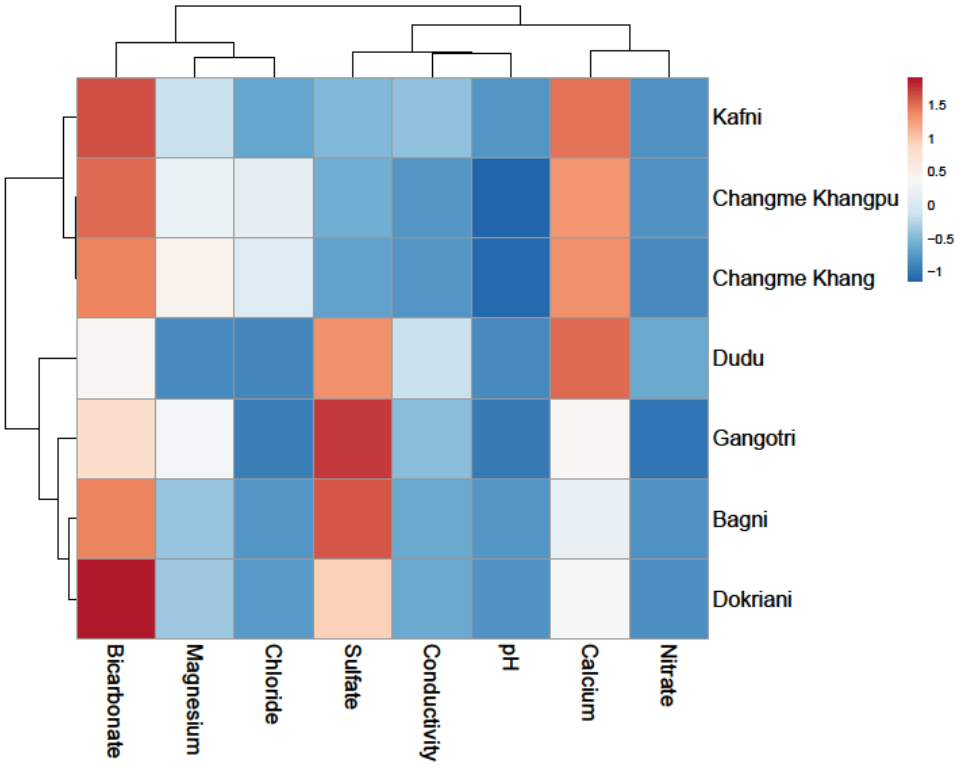


Fig 3. Heat-map analysis of Changme Khang and Changme Khangpu glacier and five other Himalayan glaciers chemical parameters

6.2.4. Statistical and comparative analysis of glacier samples

Ionic concentrations of elements in the glacier were plotted as piper diagram **(Fig. 4)** for classification on the basis of chemical composition (**Piper, 1944**). Piper diagram is a combination of triangle plots representing anionic and cationic element on a common baseline. The apexes of the cations plot were magnesium,calcium, sodium, and potassium cations, while the apexes of the anion plot were chloride,sulfate,carbonate, and hydrogen carbonate anions. The two ternary plots are then anticipated onto a diamond which can be used to describe different water types. Piper diagram divides water into four basic types conferring to their location near the four corners of the diamond. Water that plots at the top of the diamond is high in Ca^{2+} + Mg^{2+} and Cl^{-} + $SO4^{2-}$, which results in an area of permanent hardness. The water that plots near the left corner is rich in Ca^{2+} + Mg^{2+} and HCO^{3-} and is in an area of temporary hardness. Water plotted at the lower corner of the diamond is mainly composed of alkali carbonates (Na^{+} + K^{+} and HCO^{3-} + $CO3^{2-}$). Water present near the right-hand side of the diamond may be reflected as saline (Na^{+} + K^{+} and Cl^{-} + $SO4^{2-}$).

Both the glacier ice (CKG and CK glacier)are alkaline in nature. Cationic abundance order is Ca^{2+} > Mg^{2+}>, while anionic abundance order is HCO^{-}_{3} >Cl^{-}> SO^{2-}_{4}> NO^{-3}. The calcium and magnesium are the dominant cations, while bicarbonate is dominant anion followed by sulfate. The piper diagram showed that Changme Khang and Changme Khangpu glacier water fell under $Ca^{2+}HCO^{-}_{3}$ type which indicates the dominance of calcium and bicarbonate weathering as the major source of dissolved ions in the glacier ice.

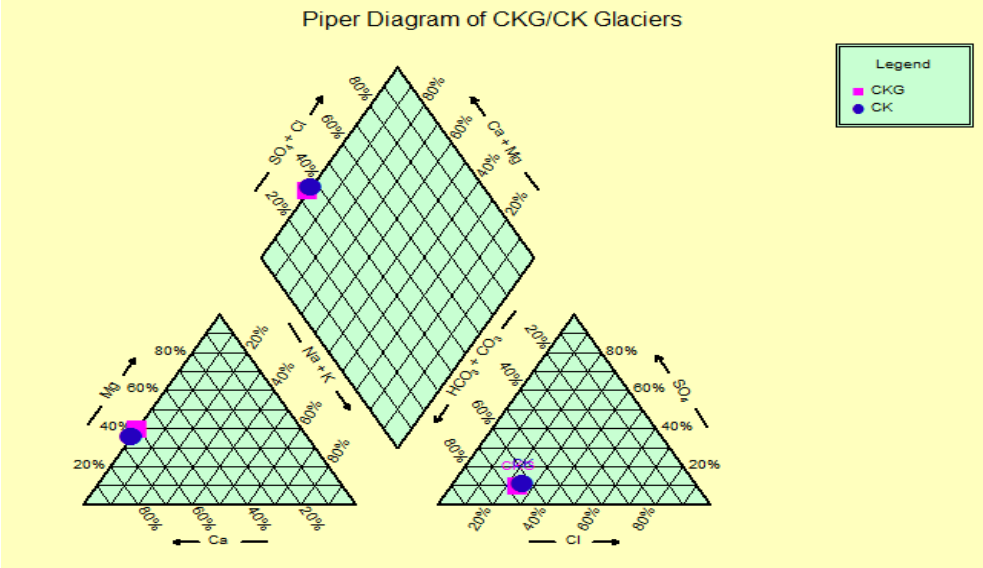


Fig 4. Piper diagram of Changme Khang (CKG) and Changme Khangpu (CK) glaciers physiochemical analysis.

6.3. Culture-dependent Analysis from glaciers samples.

6.3.1. Isolation of bacterial strains from glaciers.

A total of 136 psychrotolerant bacterial isolates were isolated from four glaciers of Sikkim and on the basis of morphological and biochemical characterization, 47 isolates were selected for further analysis. From Changme Khangpu glacier, 15 isolates were selected and from Changme Khang glacier 6 isolates were chosen for further characterization. Similarly, from Kanchengayao 22 isolates and Chumbu glacier, 4 isolates were preferred for further analysis.

6.3.2. General Morphology of the bacterial isolates

The 21 isolates were selected from Changme Khangpu (15 isolates) and Changme Khang (6 isolates) glacier on the basis of their pigmentation and shape of the colony formed on

the Luria Bertani plate (**Table 4a and 4b).** The isolates CK21 and CKG7 form pink colored colonies, whereas isolates CK10, CK1, CK15, CK20, and CKG6 formed yellow colonies, while isolates CK11, CK13, CK16, and CKG8 showed orange colored colonies and rest of the isolates showed white colored colonies.Majority of the isolates were with circular formation having entire margins and flat elevation. Similarly, from Kanchengayao glacier 22 isolates and Chumbu glacier 4 isolates were selected on the basis of their pigmentation and shape of the colony formed on Luria Bertani agar plate (**Table 4c and 4d**). Majority of the isolates from these two glaciers showed white colored colonies followed by yellow and orange colonies. All the isolates showed circular in a formation having flat elevations.

Table 4a. General Colony Morphology of Changme Khangpu (CK) glacier isolates.

	Growth on agar plates			
Isolates	**Colony color**	**Margin**	**Elevation**	**Form**
CK1	Yellow	Entire	Flat	Circular
CK3	White	Entire	Flat	Irregular
CK5	White	Entire	Flat	Circular
CK6	White	Entire	Raised	Irregular
CK9	White	Entire	Flat	Irregular
CK10	Yellow	Entire	Flat	Circular
CK11	Orange	Entire	Undulated	Irregular
CK13	Orange	Entire	Raised	Circular
CK15	Yellow	Entire	Raised	Circular
CK16	Orange	Entire	Flat	Circular
CK17	White	Entire	Flat	Circular
CK19	White	Entire	Flat	Circular
CK20	Yellow	Entire	Flat	Circular
CK21	Pink	Entire	Flat	Circular
CK22	White	Entire	Convex	Circular

Table 4b. General Colony Morphology of Changme Khang(CKG) glacier isolates.

Growth on agar plates				
Isolates	**Colony color**	**Margin**	**Elevation**	**Form**
CKG1	White	Entire	Flat	Circular
CKG2	White	Entire	Raised	Irregular
CKG4	White	Entire	Flat	Circular
CKG5	White	Entire	Raised	Circular
CKG6	Yellow	Entire	Flat	Circular
CKG7	Pink	Entire	Flat	Circular
CKG8	Orange	Entire	Flat	Circular

Table 4c. General Colony Morphology of Kanchengayao (KGG) glacier isolates.

Growth on agar plates				
Isolates	**Colony color**	**Margin**	**Elevation**	**Form**
KGG2	Yellow	Entire	Flat	Irregular
KGG6	White	Entire	Flat	Circular
KGG13	White	Entire	Flat	Circular
KGG14	White	Entire	Flat	Circular
KGG15	White	Entire	Flat	Circular
KGG16	White	Entire	Flat	Circular
KGG17	White	Entire	Flat	Circular
KGG20	Yellow	Entire	Flat	Irregular
KGG22	White	Entire	Flat	Circular
KGG25	White	Entire	Flat	Circular
KGG28	White	Entire	Flat	Circular
KGG29	White	Entire	Flat	Circular
KGG35	Brown	Entire	Flat	Circular
KGG38	White	Entire	Flat	Circular
KGG44	White	Entire	Flat	Circular
KGG50	White	Entire	Flat	Circular
KGG45	White	Entire	Flat	Circular
KGG51	White	Entire	Flat	Circular
KGG53	White	Entire	Flat	Circular
KGG59	White	Entire	Flat	Circular
KGG61	White	Entire	Flat	Circular
KGG62	White	Entire	Flat	Circular

Table 4d. General Colony Morphology of Chumbu (C) glacier isolates.

	Growth on agar plates			
Isolates	**Colony color**	**Margin**	**Elevation**	**Form**
C1	White	Entire	Flat	Circular
C2	White	Entire	Flat	Circular
C3	White	Entire	Flat	Circular
C4	White	Entire	Flat	Circular

6.3.3. Staining

Simple staining, Gram staining, and spore staining were performed as per the protocols given in material and methods section. On the basis of simple staining, it was observed that the majority of the isolates from Changme Khang and Changme Khangpu glaciers showed rod-shaped followed by cocci in shape (CK10, CK22, and CKG8) whereas long rods of Bacilli shapedwere showed by isolates CK9 and CK17 (**Table 4a and 4b**). In the same way, Kanchengayao and Chumbu isolates showed rod shape in majority followed by cocci in shape (**Table 4c and 4d**). The Gram Staining was performed to understand the physical morphology of the bacteria isolated from these glaciers. It was observed that from Changme Khang and Changme Khangpu glacier, all the isolates were Gram-positive except isolates CK 21, CKG5 and CKG8 which were Gram-negative in nature. Likewise, all the isolates from Chumbu glacier were Gram-positive in nature. However, all the isolates obtained from Kanchengayao glacier were Gram-negative. Similarly, spore staining was performed to check the ability of the isolates to form spores. Majority of the isolates from Changme Khang and Changme Khangpu glacier werespore formers and only a few isolates were spore negative such as CK10, CK13, CK,21, CK22, CKG5, CKG7 and CKG8 (**Table 4a and 4b**). From Chumbu glacier only one isolate was spore

positive (C1) where as none of the isolates were spore positive from Kanchengayao glacier (**Table 4gand 4h**).

Table 4e. Morphology characterization of Changme Khangpu (CK) glacier isolates.

General colony morphology on the basis of Staining			
Isolates	**Simple Staining**	**Grams Staining**	**Spore Staining**
CK1	Short Rods	Positive	Present
CK3	Short Rods	Positive	Present
CK5	Short Rods	Positive	Present
CK6	Short Rods	Positive	Present
CK9	Bacilli	Positive	Present
CK10	Cocci	Positive	Absent
CK11	Short Rods	Positive	Present
CK13	Short Rods	Positive	Absent
CK15	Short Rods	Positive	Present
CK16	Short Rods	Positive	Present
CK17	Bacilli	Positive	Present
CK19	Short Rods	Positive	Present
CK20	Short Rods	Positive	Present
CK21	Short Rods	Negative	Absent
CK22	Cocci	Positive	Absent

Table 4f. Morphology characterization of Changme Khang(CKG) glacier isolates.

General colony morphology on the basis of Staining			
Isolates	**Simple Staining**	**Grams Staining**	**Spore Staining**
CKG1	Short Rods	Positive	Present
CKG2	Short Rods	Positive	Present
CKG4	Short Rods	Positive	Present
CKG5	Cocci	Negative	Absent
CKG6	Short Rods	Positive	Present
CKG7	Short Rods	Positive	Absent
CKG8	Short Rods	Negative	Absent

Table 4g. Morphology characterization of Chumbu(C) glacier isolates.

General colony morphology on the basis of Staining			
Isolates	**Simple Staining**	**Grams Staining**	**Spore Staining**
C1	Short Rods	Positive	Present
C2	Short Rods	Positive	Absent
C3	Short Rods	Positive	Absent
C4	Cocci	Positive	Absent

Table 4h. Morphology characterization of Kanchengayao (KGG) glacier isolates.

General colony morphologyon the basis of Staining			
Isolates	**Simple Staining**	**Grams Staining**	**Spore Staining**
KGG2	Short Rods	Negative	Absent
KGG6	Short Rods	Negative	Absent
KGG13	Curve-rods	Negative	Absent
KGG14	Short Rods	Negative	Absent
KGG15	Short Rods	Negative	Absent
KGG16	Short Rods	Negative	Absent
KGG17	Short Rods	Negative	Absent
KGG20	Short Rods	Negative	Absent
KGG22	Short Rods	Negative	Absent
KGG25	Short Rods	Negative	Absent
KGG28	Short Rods	Negative	Absent
KGG29	Short Rods	Negative	Absent
KGG35	Short Rods	Negative	Absent
KGG38	Short Rods	Negative	Absent
KGG44	Short Rods	Negative	Absent
KGG50	Short Rods	Negative	Absent
KGG45	Short Rods	Negative	Absent
KGG51	Short Rods	Negative	Absent
KGG53	Short Rods	Negative	Absent
KGG59	Short Rods	Negative	Absent
KGG61	Short Rods	Negative	Absent
KGG62	Short Rods	Negative	Absent

6.3.4. Growth profiles at different physical parameters

Growth profiles of all the isolates were checked at the different temperature, pH and NaCl concentrations. The growth of isolates at various temperatures, i.e. 5°C, 10°C, 15°C, 20°C, 30°C and 40°C were carried out. The cultures were incubated for 72 hours in a shaker incubator. After incubation, Optical Density (O.D.) of the broth cultures was measured at 660nm. The results have shown that the isolates were able to grow at a wide range of temperature from 5°C-40°C. However, the optimum temperature for most of the isolates was 20°C. The isolates showing maximum growth at 20°C were CK1, CK3, CK5, CK6, CK9, CK10, CK11, CK13, CK15, CK16, CK20, CK22, CKG1, CKG2, CKG3, CKG4, CKG5, CKG7, KGG61, C1, C3 and C4. The isolates showing maximum growth at 30°C were KGG2,KGG6, KGG13, KGG14, KGG15, KGG16, KGG17, KGG22, KGG25, KGG28,KGG29, KGG35, KGG44, KGG45, KGG50, KGG53, KGG62 and C2. The highest growth at 10°C were showed by the isolates such as CKG6, CKG8, KGG20, KGG38 and KGG59, whereas at 15°C highest growth was shown by the isolates CK14, CK17, CK19, CK21 and KGG51 (**Fig 5a**). Thus these might be the optimum growth temperature for the above isolates. Overall, the isolates from Changme Khangpu showed better growth at 10, 15 and 20°C than the isolates obtained from other three glaciers. It also suggest that the majority of isolates can be categorized as psychrotolerant as their optimum growth condition is 30°C or 20°C and they grow in wide range of temperature.

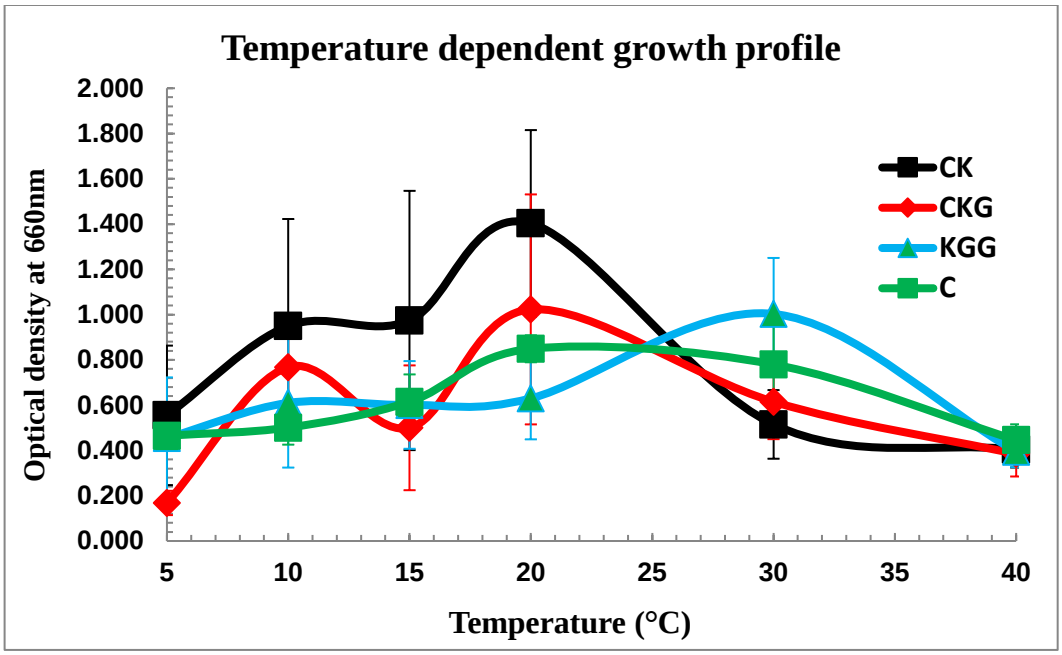


Fig 5a. Tolerance to diverse temperature ranges of Changme Khang (CKG), Changme Khangpu (CK), Kanchengayao (KGG), and Chumbu (C) glacier bacterial isolates.

The pH conditions play a significant role in deciphering the physiology of the bacteria. Depending on the bacterial optimum growth pH, they are categories as Acidophiles or Alkaliphiles. Acidophilic bacteria require an acidic condition for their growth and Alkaliphiles requires basic pH condition for their growth. Thus, the growth profiles all the isolates have been checked at various pH ranges i.e., from pH2 to pH10. The cultures were incubated at 15°C in a shaker incubator for 72 hours. The results have shown that the isolates were able to grow at wide ranges of pH, i.e., from pH4 to pH10. However, most of the isolates showed optimum pH between 6 and 8 (**Fig 5b)**.Most of our isolates required neutral pH for their growth, however few of the isolates KGG25, KGG35, KGG44, KGG51, and KGG53 had optimum pH of 4 (**Fig 5b)**.

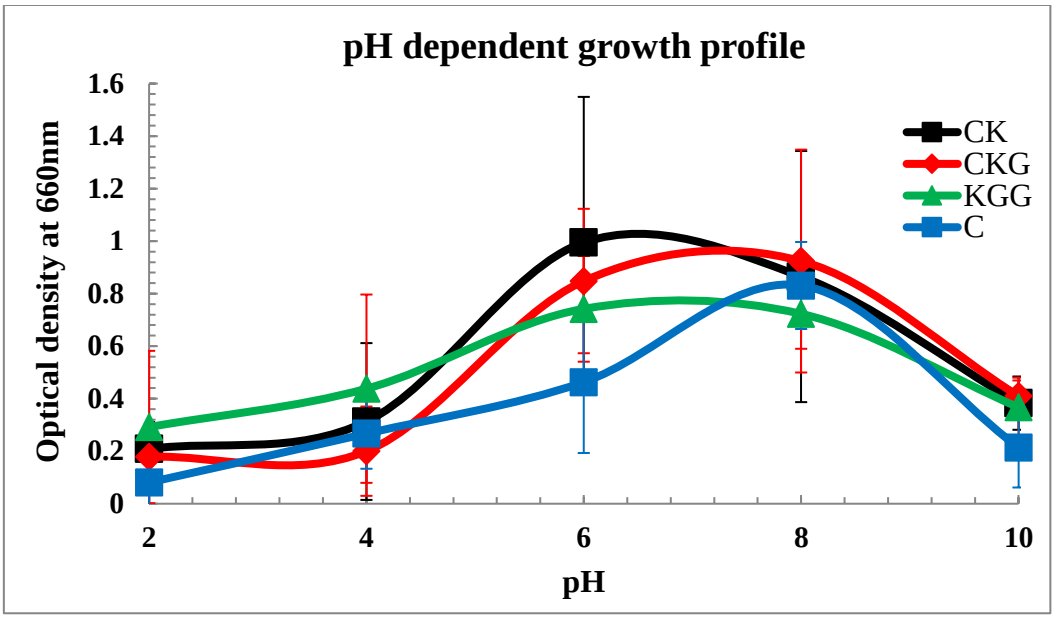


Fig.5b. Tolerance to various pH ranges of Changme Khang (CKG), Changme Khangpu (CK), Kanchengayao (KGG), and Chumbu (C) glacier bacterial isolates.

Saline conditions are one of the important characteristics in deciphering the physiology of the bacteria. The growth at various NaCl concentrations was carried out at a broad range from 1-10% at 15°C in a shaker incubator for 72 hours at pH7. The results have shown that the most of our isolates were able to grow up to 2% NaCl concentration but optimum growth showed by most of the isolates were at 1% NaCl concentration. The majority of the isolates showed optimum growth at 1% NaCl, CK5 and CK15 showed highest growth at 8% NaCl concentration **(Fig.5c)**. These isolates which showed optimum NaCl concentration of 5and 8% can be considered as halotolerantconcentration except CK10, CK11, CK13, CK16, CK21, CK22, KGG22, KGG59 and C1. Few isolates like CK6, CKG7 showed optimum growth at 5% NaCl concentration and CK3. The detection of halophiles in glacier ice samples is surprising as the glacier ice samples are not rich in NaCl. All the Gram negative bacteria from Kanchengayao glacier required normal saline

conditions whereas few Gram-positive bacteria from Changme Khang and Changme Khangpu glaciers were halotolerant.

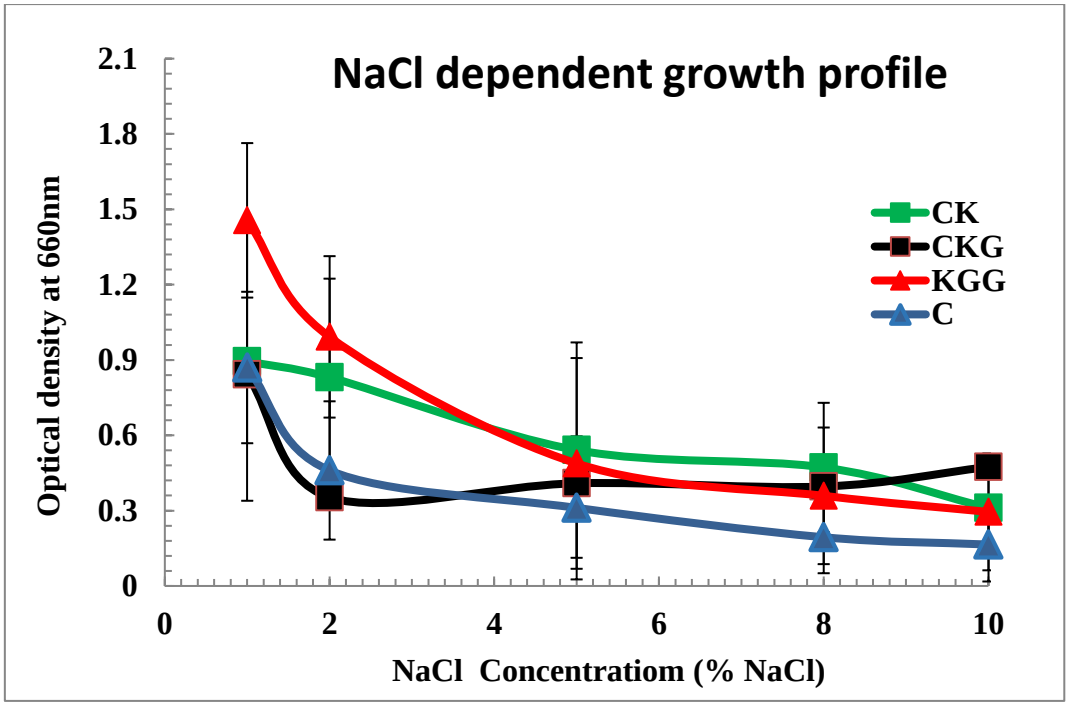


Fig.5c. Tolerance to different NaCl ranges of Changme Khang (CKG), Changme Khangpu (CK), Kanchengayao (KGG), and Chumbu (C) glacier bacterial isolates.

6.4. Biochemical characterization of the isolates

6.4.1. Carbohydrate Fermentation assessment

According to Bergey's manual of classification of bacteria, carbohydrate utilization is an essential characteristic feature of a bacterium through which differentiation and group classification can be completed. Thus, carbohydrate fermentation test was carried out with four different glaciers(47 isolates) with 6 diverse carbohydrates such as dextrose, ribose, fructose, xylose, mannose, and raffinose each of them were substituted and

checked as a carbohydrate substrate for the different isolates. The results have shown that the majority of the isolates prefer simple sugars such as dextrose and fructose. Other sugar like ribose was utilized by 25 isolates and xylose and mannose were also utilized by 15 each isolates similarly raffinose were utilized by 10 isolates (**Fig.6**). In addition to above test sugars seven other sugars such as cellubiose, arabinose, glucose, galactose, maltose, melezitose and rhammanose were checked against Changme Khang, Changme Khangpu and Chumbu glacier (25 isolates) isolates. The results showed that cellubiose were utilize by 13 isolates, glucose by 24 isolates, galactose by 22 isolates, maltose by 19 isolates, melezitose15 by isolates and rhammanose by 9 isolates respectively (**Table 5a, 5b & 5c**).

Similarly from Kanchengayao glacier (22 isolates) addition four different sugars were tested and the results shown that sucrose, dulcitol and mannose were utilize by 22, 5, and 3 isolates whereas none of the isolates utilize arabinose and lactose sugar (**Table 5d**).

Table 5a. Carbohydrate fermentation test of Chumbu glacier bacterial isolates.

	CARBOHYDRATE FERMENTATION														
Isolates	**Dulcitol**	**Cellubiose**	**Dextrose**	**Fructose**	**Glucose**	**Galactose**	**Mannose**	**Maltose**	**Mannitol**	**Melibiose**	**Melezitose**	**Ribose**	**Rhammanose**	**Raffinose**	**Xylose**
C1	-	+	+	+	+	+	-	+	-	+	+	+	-	-	-
C2	-	+	+	+	+	+	-	+	-	+	-	+	-	-	-
C3	-	+	+	+	+	+	+	+	+	-	-	-	-	+	-
C4	-	+	+	+	+	+	+	+	+	-	-	+	-	+	-

Table 5b. Carbohydrate fermentation test of Changme Khangpu glacier bacterial isolates.

Isolates	CARBOHYDRATE FERMENTATION														
	Arabinose	Cellubiose	Dextrose	Fructose	Glucose	Galactose	Mannose	Maltose	Mannitol	Melibiose	Melezitose	Ribose	Rhammanose	Raffinose	Xylose
CK1	-	-	+	+	+	+	-	-	-	-	-	+	-	-	-
CK3	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
CK5	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+
CK6	-	-	+	+	+	+	-	+	+	-	+	+	+	+	+
CK9	+	-	+	+	+	+	+	+	+	+	+	+	+	-	+
CK10	+	-	+	+	+	+	-	-	-	-	-	-	-	-	-
CK11	-	-	+	+	+	+	+	+	+	+	+	+	+	-	+
CK13	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-
CK15	-	-	+	+	+	+	+	-	+	+	+	+	-	+	+
CK16	-	-	+	+	+	+	+	+	-	-	-	-	-	-	-
CK17	-	+	+	+	+	+	+	-	-	-	-	-	-	-	+
CK19	+	-	+	+	+	+	+	-	-	-	+	+	-	-	-
CK20	-	+	+	+	+	+	+	+	-	+	+	+	+	+	+
CK21	+	-	+	+	+	+	+	+	+	+	+	+	-	-	+
CK22	+	-	+	+	+	+	+	+	+	+	-	+	-	-	-

Table 5c. Carbohydrate fermentation test of Changme Khang glacier bacterial isolates.

Isolates	CARBOHYDRATE FERMENTATION														
	Arabinose	Cellubiose	Dextrose	Fructose	Glucose	Galactose	Mannose	Maltose	Mannitol	Melibiose	Melezitose	Ribose	Rhammanose	Raffinose	Xylose
CKG1	+	+	-	+	+	+	-	+	+	+	+	+	-	-	+
CKG2	+	+	-	+	+	-	-	+	-	-	-	+	-	-	+
CKG4	+	+	-	-	+	-	-	+	+	+	-	+	+	-	+
CKG5	+	+	-	-	+	+	-	+	-	+	+	+	+	-	+
CKG6	+	+	-	-	+	-	-	+	+	+	+	+	+	-	+
CKG8	-	-	-	-	-	+	-	+	-	+	-	+	-	-	-

Table.5d. Carbohydrate fermentation test of Kanchengayao glacier bacterial isolates.

Isolates	CARBOHYDRATE FERMENTATION									
	Arabinose	Dextrose	Dulcitol	Fructose	Lactose	Mannose	Raffuinose	Ribose	Sucrose	Xylose
KGG2	-	+	-	+	-	+	-	-	+	-
KGG6	-	+	-	+	-	-	-	-	+	-
KGG13	-	+	-	+	-	-	-	-	+	-
KGG14	-	+	-	+	-	-	-	-	+	-
KGG15	-	+	-	+	-	-	-	-	+	-
KGG16	-	+	-	+	-	-	-	-	+	-
KGG17	-	+	-	+	-	-	-	-	+	-
KGG20	-	+	-	+	-	+	-	-	+	-
KGG22	-	+	-	+	-	+	-	-	+	-
KGG25	-	+	-	+	-	-	-	-	+	-
KGG28	-	+	-	+	-	-	-	-	+	-
KGG29	-	+	-	+	-	-	-	-	+	-
KGG35	-	+	-	+	-	-	-	-	+	-
KGG38	-	+	-	+	-	-	-	-	+	-
KGG44	-	+	-	+	-	-	-	+	+	-
KGG45	-	+	-	+	-	-	-	-	+	-
KGG50	-	+	+	+	-	-	+	+	+	-
KGG51	-	+	+	+	-	-	+	+	+	-
KGG53	-	+	+	+	-	-	+	+	+	-
KGG59	-	+	+	+	-	-	-	-	+	-
KGG61	-	+	-	+	-	-	-	-	+	-
KGG62	-	+	+	+	-	-	-	+	+	+

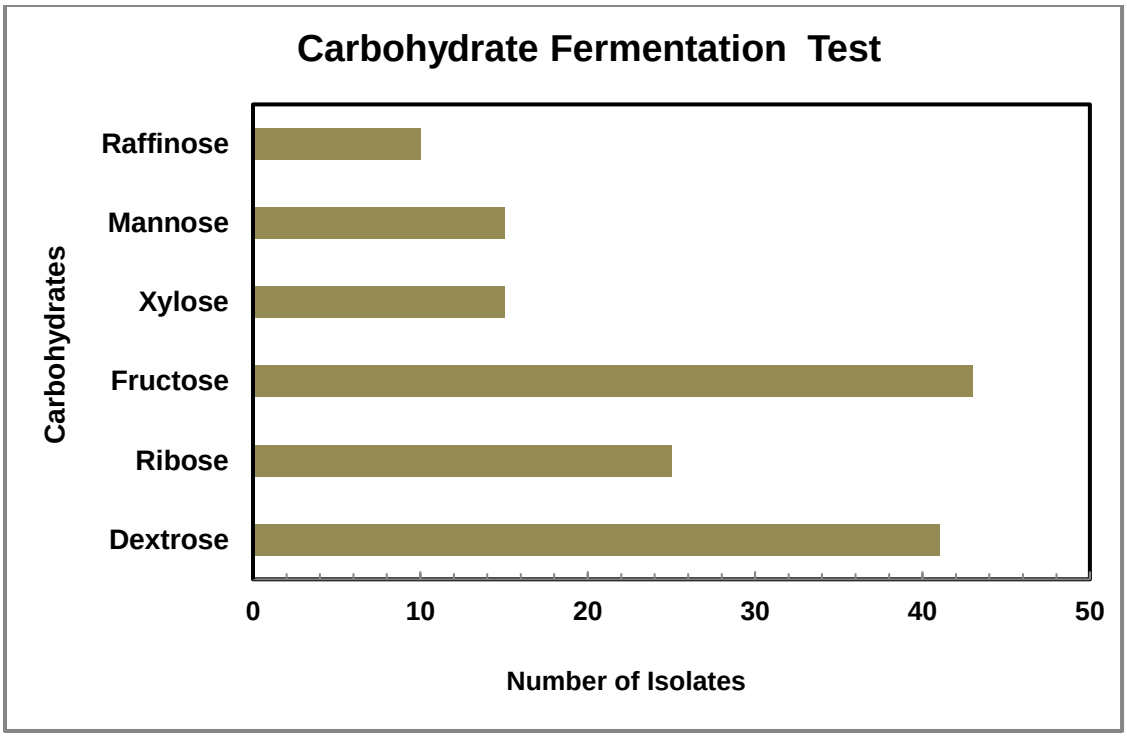


Fig.6. Carbohydrate fermentation of bacterial isolates from glaciers.

6.4.2. Enzymatic investigation of the glacier isolates

The additional important criterion to identify bacteria and differentiate it from others is the detection of various enzymatic activities of the isolate. Depending on the enzymatic activity, they can be classified into their relevant groups as per the Bergey's Manual of Systemic Classification. Thus, enzymatic activity was carried out with four different glacier isolates with 7 different enzymes. The catalase enzyme activities of the isolates were carried out and it was found that 33 isolates were positive as they produce bubbles when treated with 3% hydrogen peroxide. Similarly, protease activities of isolates were detected by growing these isolates on skimmed milk agar plates at 20°C for 72-hour incubation in a cooling incubator. The positive result in protease test infers that the microorganisms are able to degrade protein present in milk with the enzyme protease, and

can utilize it for its growth. It was found that 20 isolates (CK3, CK6, CK15, CK20, CKG1, CKG2, CKG4, CKG5, KGG2, KGG17, KGG20, KGG25, KGG28, KGG35, KGG38, KGG38, KGG44, KGG53, KGG59, C1, and C2) were having protease activity**(Table.6a,b,c, d)**. Amylase activity was also checked by growing the isolates on the starch agar plate at 20°C for 72-hour incubation in a cooling incubator and later flooded with iodine solution. The results showed that the 15 isolates were having amylase activity. Gelatin hydrolysis test and lipase activity were performed and it was found that none of the isolates were having gelatin and Lipase activity. The enzyme such as nitrate reductase and oxidase were also checked and it was found that 23 isolates having oxidase positive and 14 isolates were showing nitrate reductase activities (**Table 6a, b, c, d**).

Table 6a. Enzymatic analysis of Changme Khangpu glacier isolates

ISOLATES	ENZYMATIC ACTIVITY						
	Amylase	Catalase	Protease	Gelatinase	Oxidase	Nitrate	Lipase
CK1	-	+	-	-	+	-	-
CK3	+	+	+	-	+	-	-
CK5	-	+	-	-	+	+	-
CK6	-	+	+	-	+	+	-
CK9	-	+	-	-	+	+	-
CK10	-	+	-	-	+	-	-
CK11	-	+	-	-	+	-	-
CK13	-	+	-	-	+	-	-
CK15	-	-	+	-	-	-	-
CK16	-	+	-	-	-	-	-
CK17	+	-	-	-	-	-	-
CK19	-	+	-	-	+	-	-
CK20	-	-	+	-	-	+	-
CK21	-	+	-	-	+	-	-
CK22	-	+	-	-	+	+	-

Table.6b. Enzymatic analysis of Kanchengayao glacier isolates

ISOLATES	ENZYMATIC ACTIVITY						
	Amylase	Catalase	Protease	Gelatinase	Oxidase	Nitrate	Lipase
KGG2	-	+	+	-	+	+	-
KGG6	-	+	-	-	-	-	-
KGG13	-	+	-	-	+	+	-
KGG14	-	+	-	-	-	-	-
KGG15	+	+	-	-	+	-	-
KGG16	+	+		-	-	+	-
KGG17	-	+	+	-	-	-	-
KGG20	+	+	+	-	-	-	-
KGG22	+	+	-	-	-	+	-
KGG25	-	+	+	-	+	-	-
KGG28	-	+	+	-	-	-	-
KGG29	-	+	-	-	-	-	-
KGG35	+	+	+	-	+	+	-
KGG38	+	+	+	-	-	-	-
KGG44	+	+	+	-	+	-	-
KGG45	-	+	-	-	-	+	-
KGG50	-	+	-	-	+	-	-
KGG51	-	+	-	-	-	-	-
KGG53	+	+	+	-	+	-	-
KGG59	-	+	+	-	-	-	-
KGG61	+	+	-	-	-	-	-
KGG62	+	+	-	-	-	-	-

Table.6c. Enzymatic analysis of Chumbu glacier isolates

ISOLATES	ENZYMATIC ACTIVITY						
	Amylase	Catalase	Protease	Gelatinase	Oxidase	Nitrate	Lipase
C1	-	+	+	-	-	+	-
C2	+	+	+	-	-	-	-
C3	-	+	-	-	-	-	-
C4	-	-	-	-	-	-	-

Table.6d. Enzymatic analysis of Changme Khang glacier isolates

ISOLATES	ENZYMATIC ACTIVITY						
	Amylase	Catalase	Protease	Gelatinase	Oxidase	Nitrate	Lipase
CKG1	+	+	+	-	-	-	-
CKG2	-	+	+	-	+	+	-
CKG4	-	+	+	-	-	-	-
CKG5	+	+	+	-	-	+	-
CKG6	-	+	-	-	+	+	-
CKG7	-	+	-	-	+	-	-
CKG8	-	+	-	-	+	-	-

6.4.3. Screening of produced proteolytic and amylolytic bacteria

The isolates were screened for protease and amylase production by plate assay using protease specific medium, i.e., skimmed milk agar and starch agar for amylase. The clear zone diameters were measured after 24 hours of incubation at 20°C cooling incubator. After screening test 8 protease, and 4 amylase producer strains were selected for further studies.

6.4.4. Quantities estimation of amylase and protease enzyme

The purpose of the present investigation was to select the bacterial strains with a high level of protease and amylase producing ability in liquid medium. As expected, 8 protease and 4 amylase producer strains also produced protease and amylase in liquid medium. These strains were further tested for protease and amylase production at different temperature, pH and NaCl requirements. The protease and amylase activity was assayed at different temperature such as 5 ºC, 10 ºC, 15 ºC, 20 ºC, 30 ºC and 40ºC; pH 4, 6, 7, 8 and 10 and similarly for NaCl such as 1%, 2%, 3%, 4%, 6%, 8% and 10%

concentrations by adding equal amount of substrate along with the buffer as described earlier in methodology portion.

Maximum protease activity was observed at pH7, NaCl 1%, and at 15ºC temperature by all 8 isolates (CK3, CK6, CK15, CKG1, CKG2, CKG4, and CKG5) (**Fig.7**). Among these isolates it was found that the crude cell extract of isolate CKG2 showed (2.16 U/mL/min) highest protease activity. On the other hand, 4 isolates were tested for amylase activity such as CK3, CK17, CKG1, and CKG5. Similarly, efficient amylase activity was found at 15ºC temperature, pH8, and NaCl 1% (**Fig.8**). Among these isolates the crude cell extract of CK3 showed the best amylase activity (1.07 U/mL/min) as compared to other tested isolates (**Fig.8**).

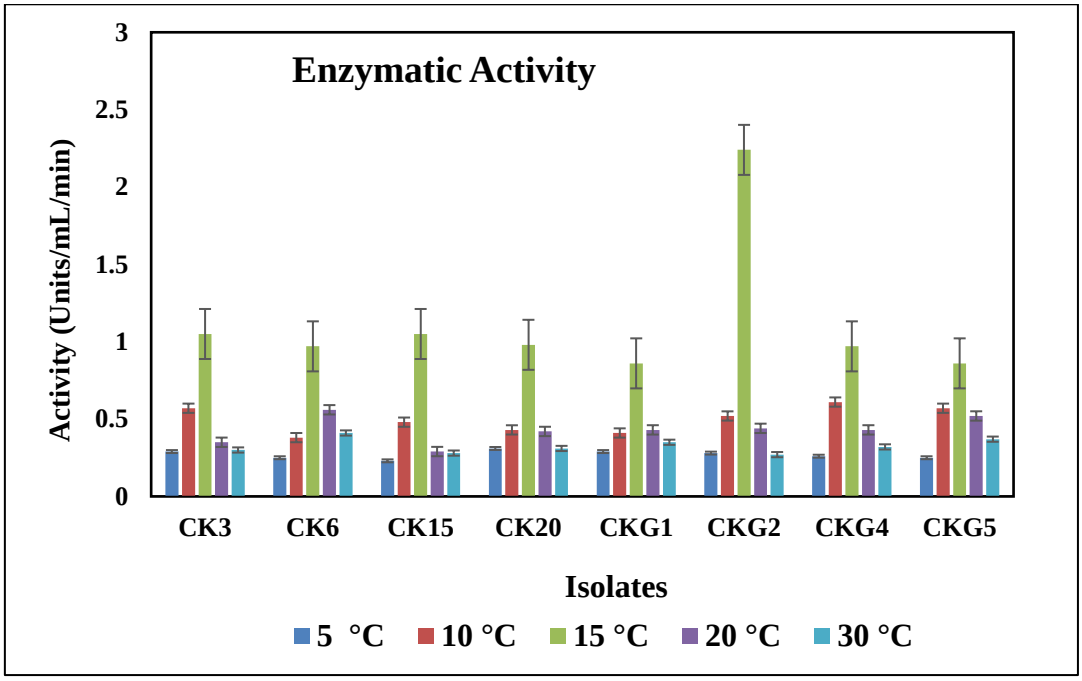


Fig.7. Protease activity of thebacteria isolated from Changme Khang and Changme Khangpu glacier.

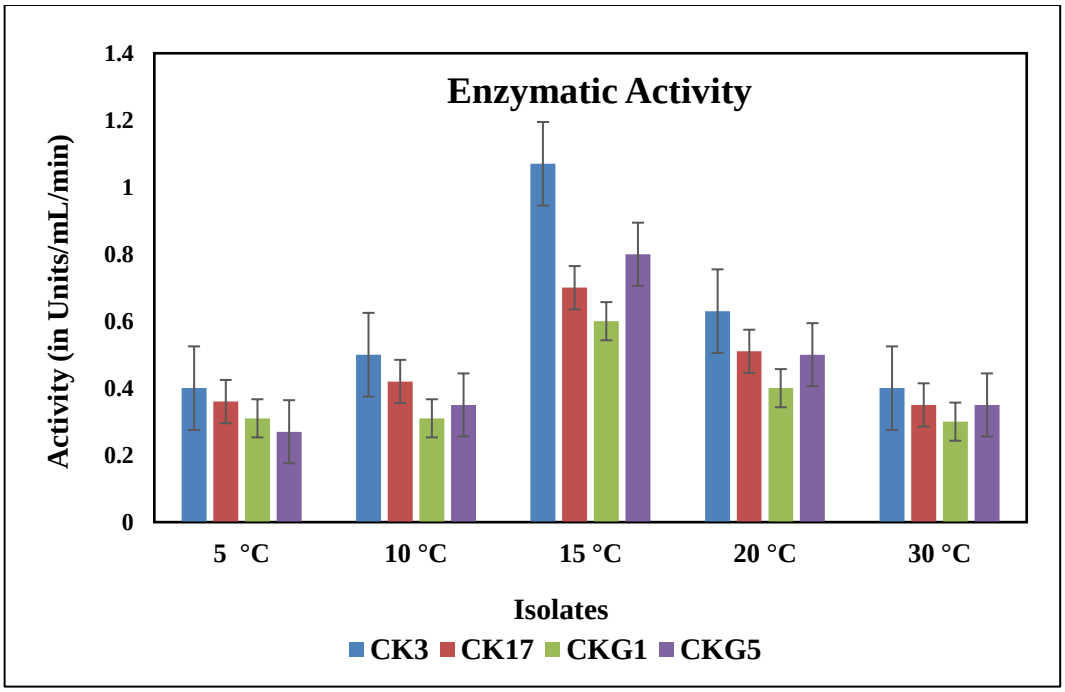


Fig.8. Amylase activity of the bacteria isolated from Changme Khang and Changme Khangpu glacier.

6.5. Identification of isolates by Culture-dependent techniques.

6.5.1. 16S rRNA sequencing

One of the most recognized molecular tools of identification is 16S rRNA sequencing. The16S rRNA sequencing was completed with the help ofBigDye terminationmethod by using Automated DNA sequencer (ABS/Genetic analyzer-3500). The data obtained from Genetic analyzer were assembled with Codon Code aligner software (version 5.2) and the data were compared with NCBI nr/nt database using BLAST sequence homology search for taxonomic identification. On the basis of molecular identification, the dominance of phylum *Proteobacteria* followed by *Firmicutes* and *Actinobacteria*was found. Major genus found in the study is *Pseudomonas* followed by *Bacillus*. Interestingly, it was found that the three glaciers located in close proximity, i.e, Changme Khangpu, Changme

Khang and Chumbu glacier were dominated by *Bacillus* species except CK21, CKG5 and CKG 8 which are Gram-negative bacteria. These results are in accordance with our Gram-staining results. On the other hand Kanchengayao glacier is mainly dominated by Gram negative bacteria belonging to *Psuedomonas* species. Identified isolates of Pseudomonas were *Pseudomonas fluorescens*KGG35, *Pseudomonas reactants* KGG59, *Pseudomonas hibisciola*KGG2, *Pseudomonas synxantha*KGG14, *Pseudomonas azotoformans*KGG6, *Pseudomonas poae*KGG51, and *Stenotrophomonas maltophilia*KGG25**(Table.7b)**. The representative isolates of genus *Bacillus* were *Bacillus cereus* CKG1, *Bacillus thuringinensis*CKG2, *Bacillus safensis* CKG4, *Bacillus oceanisediminis*CKG6, *Lysinibacillus mangiferahumi*CK1, *Bacillus nealsonii*CK3, *Bervibacillus brevis*CK6, *Bacillus aryabhattai*CK9, *Bacillus pumilus*CK16, *Lysinibacillus sphaericus*CK19,*Bacillus wiedmannii*C1, *Bacillus velezensis*C2, *Bacillus odorifer*C3 and *Bacillus fusiformis*C4 **(Table.7a,c,d)**. Other identified bacteria were *Staphylococcus haemolyticus*CK22, *Neomicrococcus lactis*CK10, *Pseudoclavibacter terrae* CK11, *Bervibacterium linens* CK13, *Paracoccus marcusii*CKG8and *Enterobacter cloacae* CKG5 **(Table.7a& 7c)**. The alignment and similarity search of 16S sRNA sequencing with NCBI database nr/nt have shown that majority of the isolates have <99% identity. A phylogenetic tree was constructed using the neighbor-joining method with jukes-cantor evolutionary distance measurement using the MEGA10 software (**Fig 9a,b,c,d**).

Table 7a. Identified bacterial isolates from Changme Khangpu glacier with the percentage of identity and accession numbers.

Isolates	Identification based on 16S sRNA sequencing	% Identity	Accession number
CK1	*Lysinibacillus mangiferahumi*	99%	MG163137
CK3	*Bacillus safensis*	98%	MF163138
CK5	*Bacillus nealsonii*	98%	MF163141
CK6	*Bacillus sp. 210-11*	99%	MF163139
CK9	*Bervibacillus brevis*	98%	MF191718
CK10	*Neomicrococcus lactis*	99%	MF163142
CK11	*Pseudoclavibacter terrae*	99%	MF163143
CK13	*Bervibacterium linens*	99%	MF163144
CK15	*Bacillus aryabhattai*	99%	MF163145
CK16	*Bacillus pumilus*	98%	MF191719
CK17	*Paenibacillus populi*	98%	MF191720
CK19	*Lysinibacillus sphaericus*	98%	MF163146
CK20	*Bacillus sp. gx13*	99%	MF191721
CK21	*Sphingomonas sp. PDD-69b-4*	98%	MF163147
CK22	*Staphylococcus haemolyticus*	99%	MF163148

Table 7b. Identified bacterial isolates from Kanchengayao glacier with the percentage of identity and accession numbers.

Isolates	Identification based on 16S sRNA sequencing	% Identity	Accession number
KGG2	*Stenotrophomonas hibiscicola*	98%	KY129838
KGG6	*Pseudomonas azotoformans*	98%	MH157226
KGG13	*Pseudomonas maltophilia*	99%	KY129834
KGG14	*Pseudomonas synxantha*	99%	MH079449
KGG15	*Pseudomonas azotoformans*	98%	MH079450
KGG16	*Pseudomonas azotoformans*	99%	MH157236
KGG17	*Pseudomonas azotoformans*	99%	MH157237
KGG20	*Stenotrophomonas maltophilia*	99%	MH157227
KGG22	*Pseudomonas azotoformans*	98%	MH157238
KGG25	*Stenotrophomonas maltophilia*	99%	MH157228
KGG28	*Pseudomonas azotoformans*	99%	MH157229
KGG29	*Pseudomonas azotoformans*	99%	MH157239
KGG35	*Pseudomonas fluorescens*	99%	KY129832
KGG38	*Pseudomonas azotoformans*	99%	MH157230
KGG44	*Pseudomonas azotoformans*	99%	MH157231
KGG45	*Pseudomonas azotoformans*	99%	MH157232
KGG50	*Pseudomonas azotoformans*	99%	MH157234
KGG51	*Pseudomonas poae*	98%	MH079451

KGG53	*Pseudomonas poae*	99%	MH157233
KGG59	*Pseudomonas reactants*	99%	KY129833
KGG61	*Pseudomonas azotoformans*	99%	MH157235
KGG62	*Pseudomonas azotoformans*	98%	MH079452

Table 7c. Identified bacterial isolates from Changme Khang glacier with the percentage of identity and accession numbers.

Isolates	Identification based on 16S sRNA sequencing	% Identity	Accession number
CKG1	*Bacillus cereus*	98%	KY982961
CKG2	*Bacillus thuringinensis*	98%	KY982962
CKG4	*Bacillus safensis*	99%	MG736309
CKG5	*Enterobacter cloacae*	99%	KY982963
CKG6	*Bacillus oceanisediminis*	99%	MF163139
CKG8	*Paracoccus marcusii*	98%	MF163140

Table 7d. Identified bacterial isolates from Chumbu glacier with the percentage of identity and accession numbers.

Isolates	Identification based on 16S sRNA sequencing	% Identity	Accession number
C1	*Bacillus wiedmannii*	99%	MH157240
C2	*Bacillus velezensis*	99%	MH157241
C3	*Bacillus odorifer*	99%	MH157242
C4	*Bacillus fusiformis*	99%	MH157243

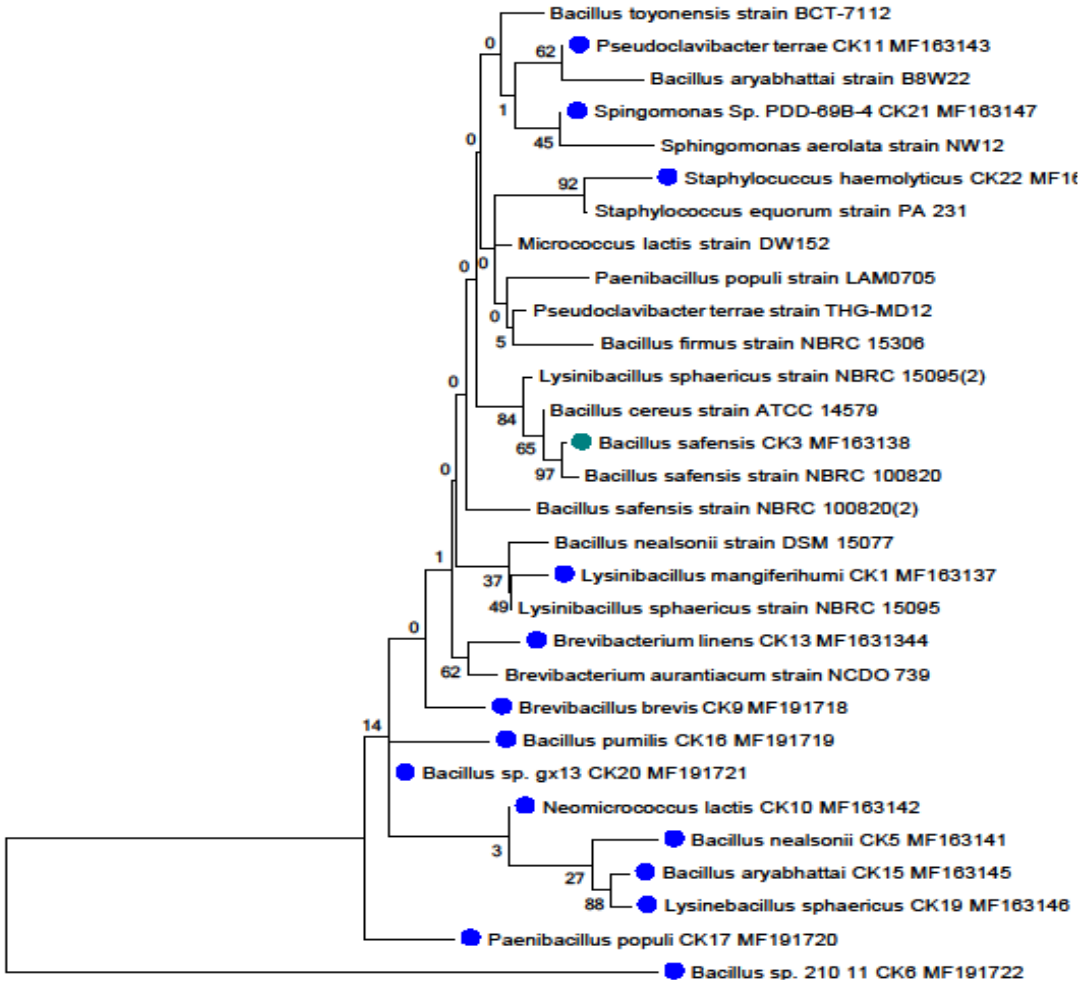


Fig 9a. The phylogenetic tree was made by the Neighbor-Joining method using the Jukes-cantor models showing the phylogenetic relationships of bacterial 16S rRNA gene sequences ofChangme Khangpu glacier ice core to closely related sequences from the GenBank database.

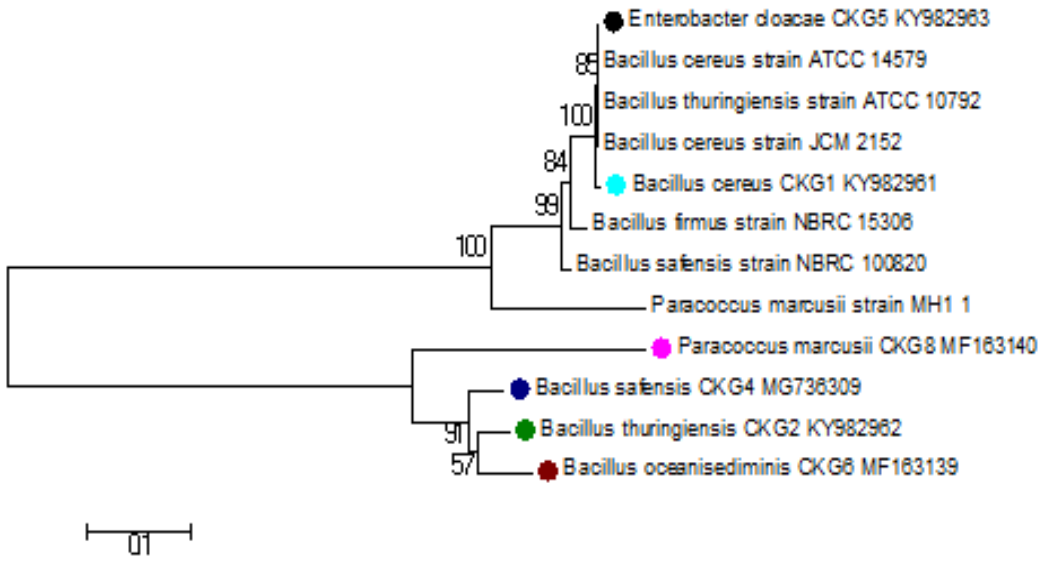


Fig 9b. The phylogenetic tree was made by the Neighbor-Joining method using the Jukes-cantor models showing the phylogenetic relationships of bacterial 16S rRNA gene sequences ofChangme Khang glacier ice core to closely related sequences from the GenBank database.

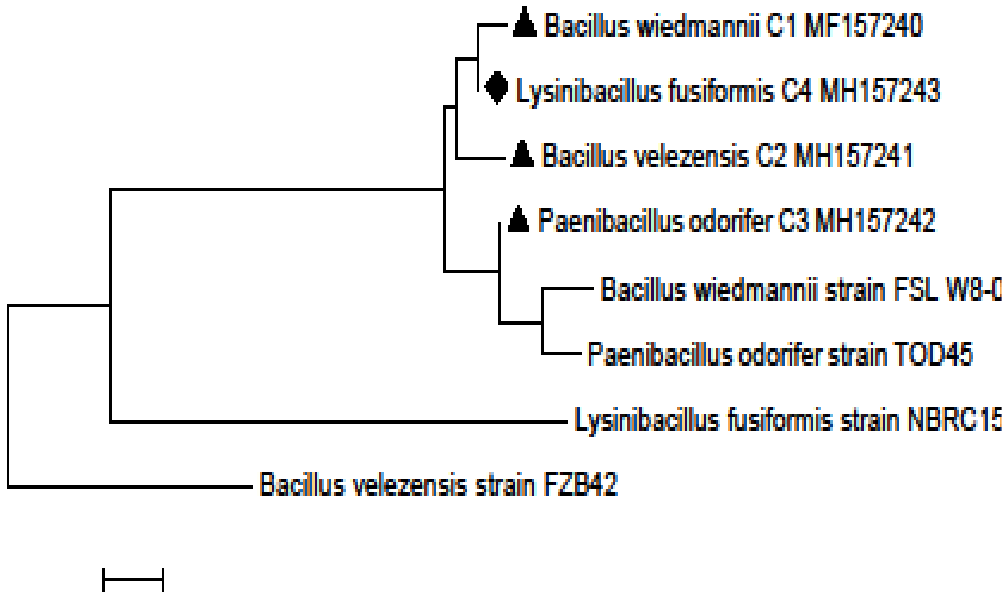


Fig 9c. The phylogenetic tree was made by the Neighbor-Joining method using the Jukes-cantor models showing the phylogenetic relationships of bacterial 16S rRNA gene sequences ofChumbu glacier ice core to closely related sequences from the GenBank database.

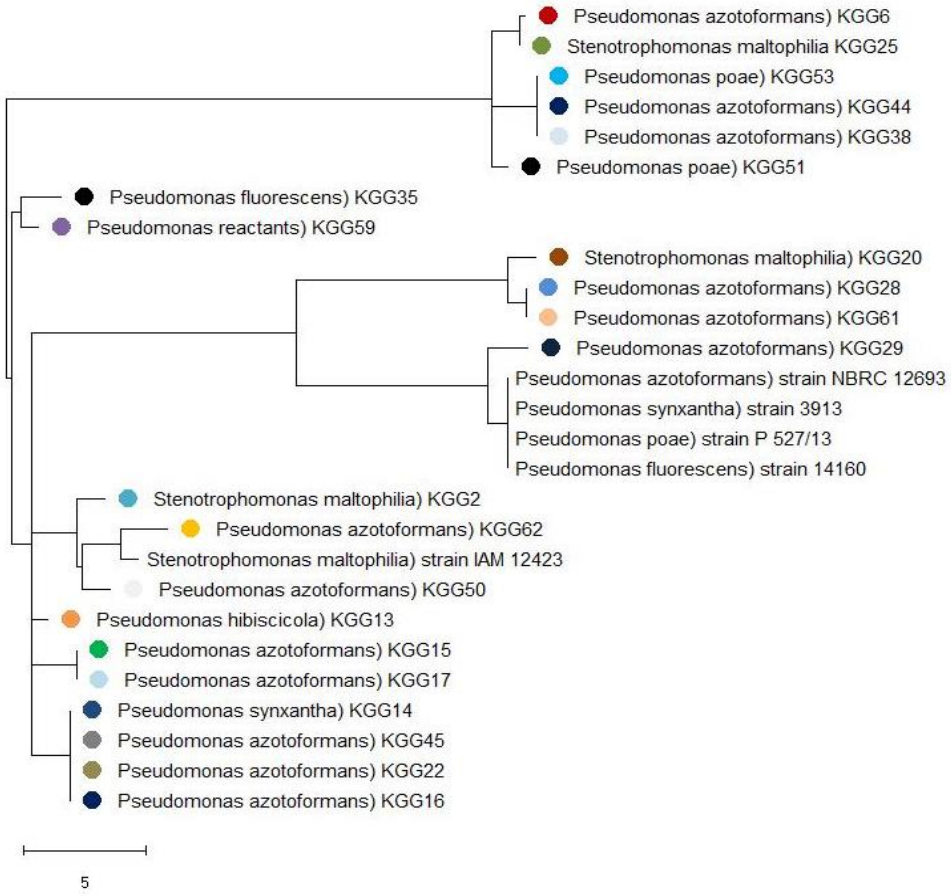


Fig 9d. The phylogenetic tree was made by the Neighbor-Joining method using the Jukes-cantor models showing the phylogenetic relationships of bacterial 16S rRNA gene sequences ofKanchengayao glacier ice core to closely related sequences from the GenBank database.

6.6. Identification of putative novelpsychrotolerant bacteria

16s rRNA sequencing result suggested that KGG13 isolate showed close similarity with *Stenotrophomonas maltophilia* KCTC 1773^{T}. However some of the biochemical tests and temperature dependent growth profile of our isolate KGG13 was not matching with *Stenotrophomonas maltophilia*. Therefore, we hypothesized that isolate KGG13 might be a novel species of *Stenotrophomonas*. In order to confirm it, we have carried polyphasic

characterization of KGG13 as per the guideline is given by “The International Committee on Systematic Bacteriology (ICSB)”. The bacterial strain KGG13 was isolated from Kanchengayao glacier on Luria Bertani agar medium incubated at 15°C for 72 hours.

6.6.1. Tentative identification by microscopy and biochemical test.

The colony color of strain KGG13 is white, elevation is flat and circular form. The cells are aerobic, curve-rod and motile. The isolate KGG13 is a Gram-negative and unable to form spores. The SEM analysis showed that the size of bacteria was 1μm diameter **(Fig.10a)**. The growth at various physical parameters such as temperature (range from 5-40°C), pH (range from 2-10) and NaCl concentration (range from 1-10%) was checked as per the protocols in material and methods. The result showed that the strain KGG13 can grow between 5-40°C with the optimal temperature at 20°C **(Fig. 10b)** and in the pH ranges from 6-10 with optimum growth at pH 6 **(Fig. 10c)**. The NaCl concentration for the growth of KGG13 was found to be in the range between 1-2%, with the optimum NaCl concentration of 1% at optimum pH and temperature and incubated at 72hrs incubation in a shaker cum cooling Incubator **(Fig. 10d)**. Diverse biochemical parameters were checked such as carbohydrate fermentation, enzymatic tests such as amylase, lipase, catalase, oxidase, nitrate reductase, and Gelatinase test as per the procedure is given in above material and method section. All of the biochemical tests were performed by BIOLOG system as per the guidelines is given by the manufacturer. The results have shown that the cells are catalase, oxidase, and protease was positive whereas lipase, nitrate, and amylase were shown negative by the isolate. Isolate utilize sugars such as glucose, sucrose, and salicin, whereas, other sugar like cellobiose, gentiobiose, lactose, maltose, melibiose, and turanose were weekly positive as shown in **Table 8a.**

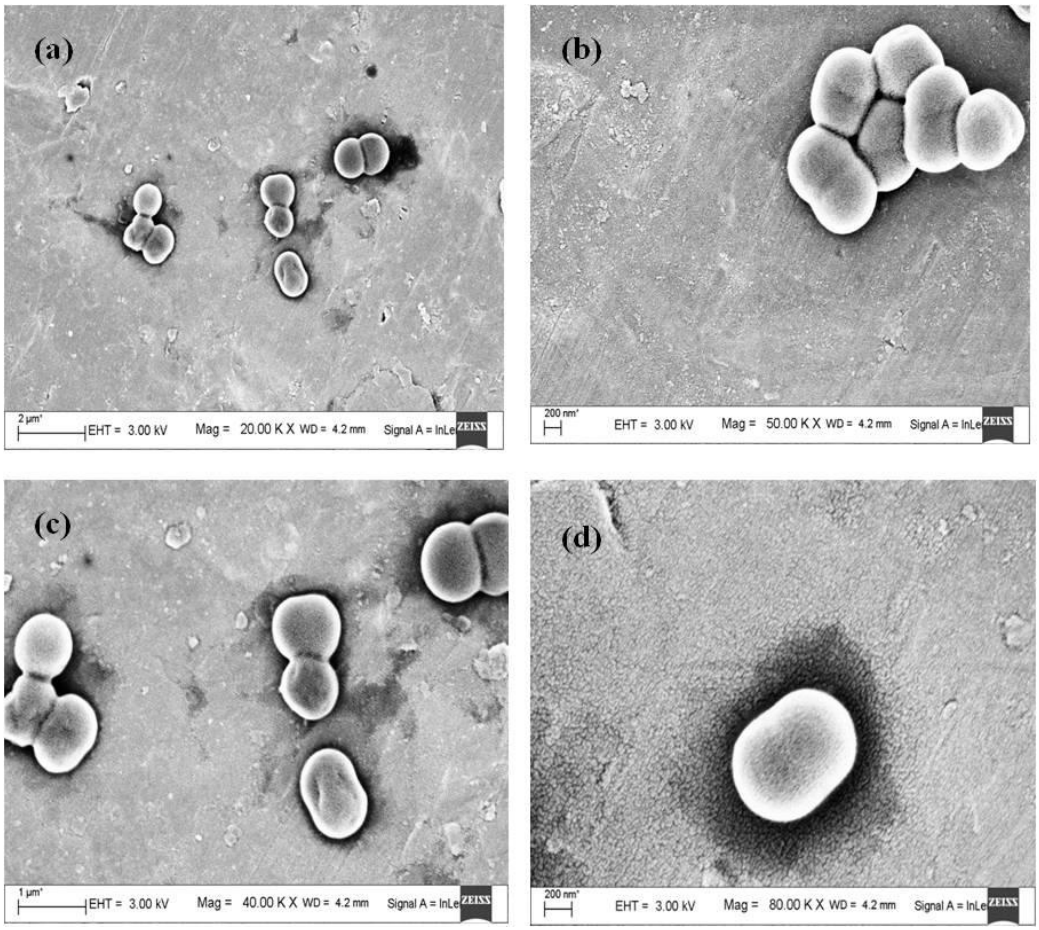


Fig 10a. Scanning Electron Microscopy (SEM) analysis (a, b, c, d) of KGG13 strain.

Table 8a. Differential phenotypic characteristics of KGG13 and Pseudomonas species: 1.*Putative Stenotrophomonas maltophilia* var. *kanchengayaoensis*KGG13; 2.*Stenotrophomonas maltophilia* KCTC^{T}data from **Palleroni(1984)**, **Palleroni & Bradbury(1993)**, **Finkmann et al.(2000);** 3. *Stenotrophomonas nitritireducens*KACC 10891^{T}data from **Finkmann et al**. **(2000)**, **Assih et al.** (2002);4.*Stenotrophomonas acidaminiphila* KACC 11356^{T} data from **Assih et al.(2002)**, 5.*S. rhizophila* DSM 14405^{T} data from **Wolf et al. (2002)**.

Characteristics	**1**	**2**	**3**	**4**	**5**
Oxidase	+	+	-	+	+
Growth at					
4°C	+	-	-	-	+
41°C	+	-	-	+	-
Growth at 5% (w/v) NaCl	+	+	-	-	-
Hydrolysis of:					
Aesculin	-	+	-	+	+
Gelatin	+	+	-	-	+
Tween 80	+	+	v	+	+
Assimilation of:					
D-Arabinose	-	-	-	-	-
D-Mannose	-	+	-	+	+
D-Xylose	-	-	-	-	+
Cellobiose	w	-	-	-	+
Gentiobiose	w	-	-	-	+
D-Glucose	+	+	-	+	+
Lactose	w	-	-	-	+
Maltose	w	+	-	+	+
Melibiose	w	-	-	-	+
Sucrose	+	+	-	-	+
Turanose	w	-	-	-	+
Salicin	+	w	-	-	+

Symbols: +, positive reaction; -, negative reaction; v, variable reaction; w, weakly positive.

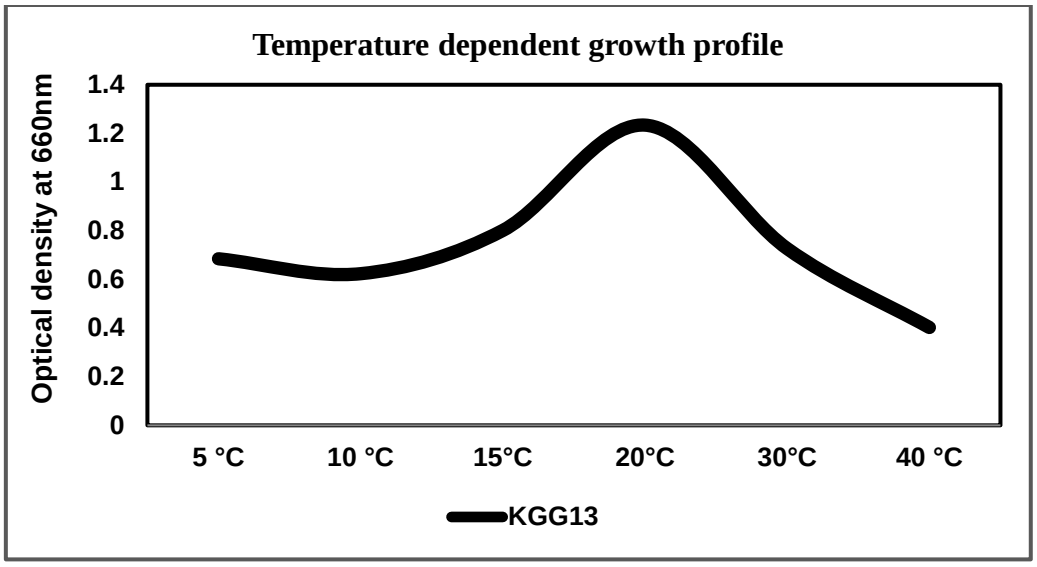


Fig 10b. Growth profile of strain KGG13 at different temperature was checked and it was shown that optimal temperature of strain KGG13 was found to be 20°C.

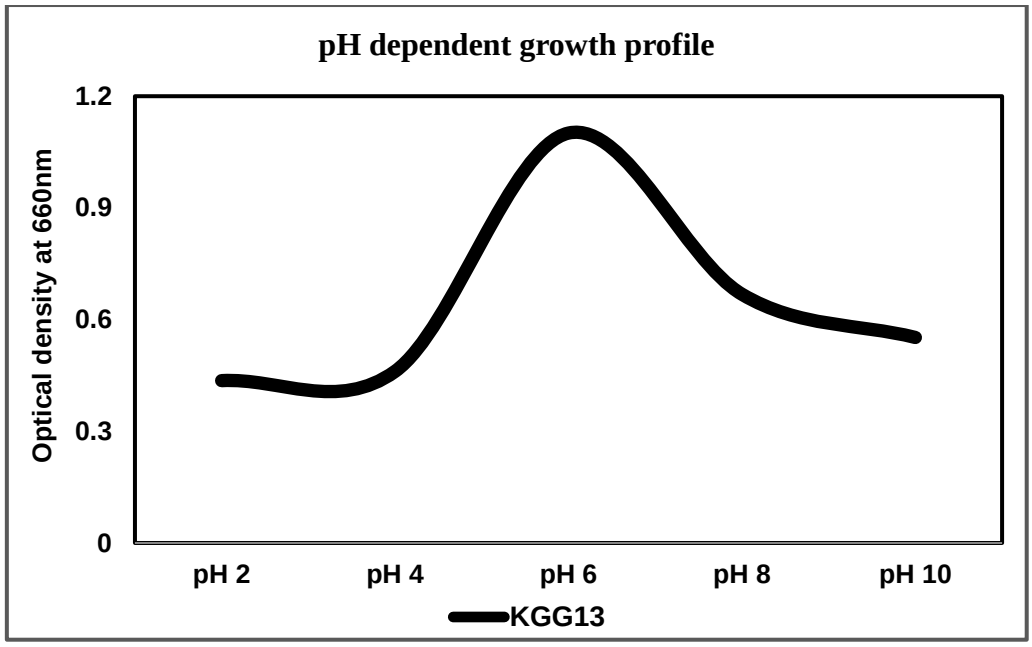


Fig 10c. Growth profile of strain KGG13 at various pH range from 2-10 was checked and it was shown that optimal pH of strain KGG13 was found to be pH 6.

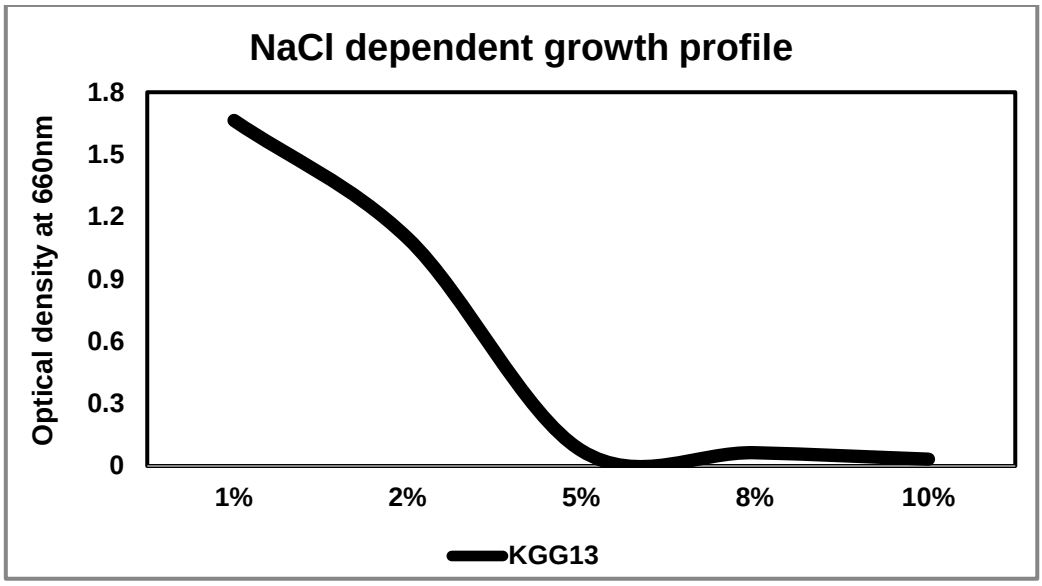


Fig 10d. Growth profile of strain KGG13 at various salt concentrations was checked and it was shown that optimal NaCl concentration is shown to be 1%.

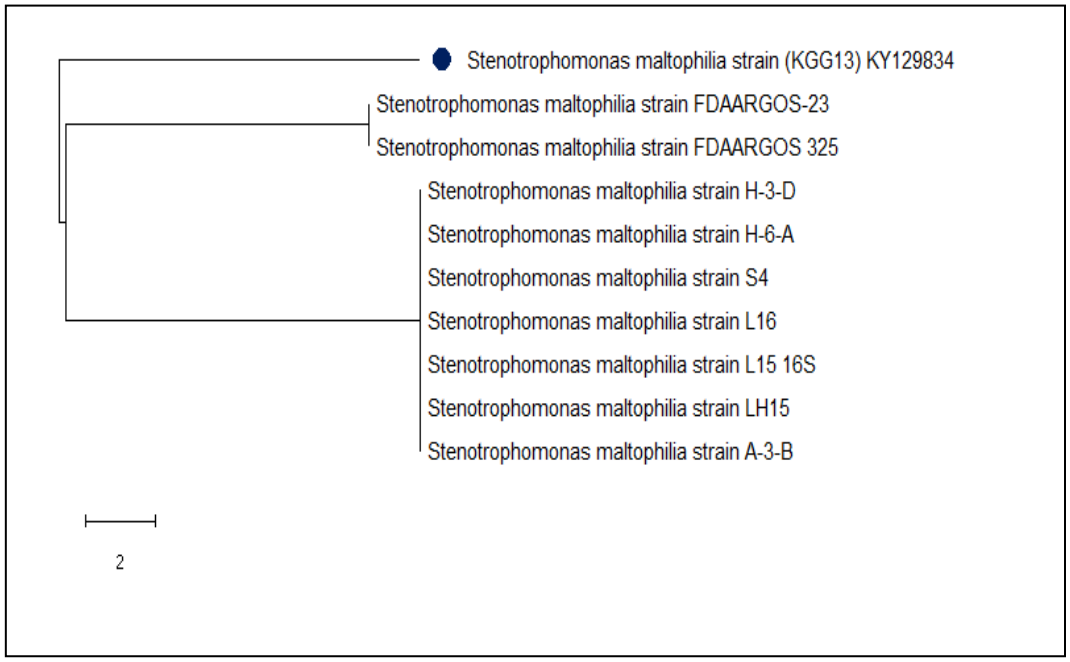


Fig 10e. Phylogenetic tree showing the position of *Putative Stenotrophomonas maltophilia* var. *kanchengayaoensis* KGG13among its related taxa. The evolutionary history was assumed by means of the Neighbor-Joining method. The proportion of repeat trees in which the allied taxa clustered collectively in the bootstrap test (1000 replicates) is shown next to the branches. The evolutionary distances were calculated using the Maximum Composite Likelihood technique. Evolutionary analyses were conducted in MEGA10.

6.6.2. Fatty Acid Methyl Ester (FAME) analysis

The fatty acid analysis of the putative novel strain was performed at 20°C. Fatty acid was extracted and analysis following the instructions of the Microbial Identification System (MIDI). The results have shown the predominance fatty acid were C16:0 (7.72%), *iso*-C11:0 (0.72%), *iso*-C15:0 (2.40%), *anteiso*-C15:0 (30.26%), and C12:03-OH (2.32%). By analyzing the results using RTSBA6 Sherlock libraries, it was found that the Similarity Index (SI-Index) of strain KGG13 was SI-Index 0.00.If the similarity index is <0.03 then the species might be novel with corresponding RTSBA6 libraries (Sherlock, MIDI). The comparison of various fatty acids of strain KGG13 and its closest relatives are shown in **Table 8b and Fig 10f.**

Table 8b. Cellular fatty acid profile of *Putative Stenotrophomonas maltophilia* var. *kanchengayaoensis* KGG13 and its closest phylogenetic neighbors. Strains: 1, KGG13 strain;2, *Stenotrophomonas maltophilia* KCTC 1773^T; 3, *Stenotrophomonas acidaminiphila* KACC 11356^T; 4, *Stenotrophomonas humi* DSM 18929^T; 5, *Stenotrophomonas nitritireducens* KACC 10891^T; 6,. *Stenotrophomonas daejeonensis* MJ03T

Fatty acid	1	2	3	4	5	6
Saturated						
C10:0	0.50	2.7	-	1.1	-	1.6
C14:0	0.63	1.6	-	1.1	2.3	1.6
C15:0	-	6.1	3.1	4.0	5.1	2.5
C16:0	**7.72**	-	1.9	1.8	-	1.9
iso-C10:0	-	7.2	11.5	7.0	12.0	10.1
iso-C11:0	**0.12**	0.6	5.0	16.3	6.6	9.6
iso-C14:0	0.29	39.9	26.8	14.6	27.7	33.7
iso-C15:0	2.40	2.7	3.4	16.4	4.0	2.7
iso-C16:0	8.43	10.1	8.6	5.4	8.4	4.2
anteiso-C15:0	**30.26**	-	-	-	-	-
Unsaturated						
iso-C 15:1 F	0.06	2.1	4.1	1.8	5.1	4.6
iso-C 17:1 w 9c	-	4.9	7.9	6.3	9.6	7.3
Hydroxy						
C12:0 3-OH	2.32	-	1.9	-	-	-
C13:0 2-OH	-	4.3	7.6	2.2	5.9	6.4
iso-C11:0 3-OH	**0.13**	3.1	5.1	8.5	5.1	4.1
iso-C12:0 3-OH	-	6.2	5.6	1.7	5.3	6.1
iso-C13:0 3-OH	-	3.0	3.0	7.3	-	1.5

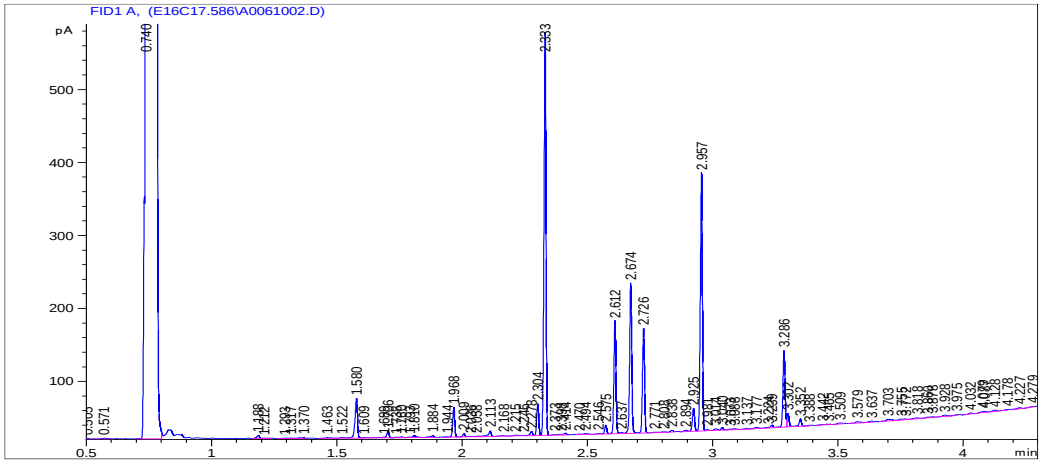


Fig 10f. RTSBA6 Sherlock libraries (MIDI) reference peaks.

On comparing the overall data obtained by various techniques the results have shown that the strain KGG13 grows well between 4°C to 40°C on the other hand its closest neighbour *Stenotrophomonas maltophilia* KCTC 1773^{T}were not able to grow between 4°C to 40°C temperature. Similarly KGG13 strain assimilates salicin and weakly assimilates other sugar such as cellobiose, gentibiose, lactose, maltose, and turanose whereas its neighbor strain KCTC 1773^{T} were not able to assimilate above mentioned sugars. Thus these results indicate that our isolate might be a novel species. The major fatty acid present in KGG13 were C16:0 (7.72), iso-C16 (8.43), anteiso C15:0 (30.26), and C12:03-OH (2.32), however these fatty acids were absent in the closest strain KCTC 1773^{T}. Thus on the basis of fatty acids comparison between KGG13 and its closest neighbour KCTC 1773^{T}, the strain KGG13 may be considered as novel species.

6.6.3. Description of Putative *Stenotrophomonas maltophilia* var. *kanchengayaoensis*

Putative *Stenotrophomonas maltophilia* var. *kanchengayaoensis* (kan.chen. gayao. en'sis N.L.fem.adj.*kanchengayaoensis*Kanchengayao referring to Kanchengayao glacier from where the type of strain was isolated).

Cells are Gram-negative, aerobic, slightly curve-rod, and 1µm in diameter. They didn't form endospore and the growth is observed between 4 and 40°C and the optimum growth temperature was 20°C. The pH for growth ranges from 4-8 with the optimum pH of 6 at the optimum growth temperature. The NaCl concentration for the growth is in the range between 1-5%, with the optimum growth at 1% NaCl at optimum pH and temperature. Cells showed positive results with catalase, oxidase, protease, and gelatinase positive,

whereas,lipase, nitrate, and amylase were negative. Isolate KGG13 was able to utilize aesculin, mannose, and salicin, whereas, other sugars such as gentibiose, lactose, maltose, melibiose, and cellobiose were weekly positive. The major fatty acids detected in KGG13 were C16:0, C12:03-OH, and antiso-C15:0. The isolate was isolated from Kanchengayao glacier accumulation zone ice, North Sikkim, India. The type of strain is KGG13, NCBI nr/nt accession no KY129834.

6.7. Analysis of bacterial diversity by Culture-independent techniques.

6.7.1. Phospholipid Fatty Acid Analysis (PLFA).

Phospholipids are fundamental components of microbial membranes and it has been described that they diverge between different species among prokaryotes which makes it a significant chemotaxonomic marker(**Powl and Anthony,2007; Joergensen and Wichem, 2008**). Phospholipid fatty acid analysis (PFLA) of two glaciers suggests that the major fatty acids significantly varied amongst the Changme Khang and Changme Khangpu glaciers. It was found that the straight and Branch chain fatty acids were abundant in case of Changme Khangpu glacier (51.34%) as compare to Changme Khang glacier (15.15%), whereas branch-chain fatty acids were higher in Changme Khang glacier (45.92%) than Changme Khangpu glacier (12.26). The polyunsaturated fatty acids (PUFA) were demonstrated high in Changme Khang glacier than Changme Khangpu glacier (CKG-34.25%; CK-22.60%). Similarly, monounsaturated fatty acids (MUFA) were slightly higher in Changme Khangpu glacier than Changme Khang glacier i.e., CK-7.59; CKG-3.33, whereas cyclopropane fatty acid was present only in Changme Khangpu

glacier **(Table.9b)**. The abundance of various fatty acids was represented by Plot matrix analysis as shown in **(Fig.11b).**

Table 9a. The abundance of various Fatty Acids in two glaciers.

Fatty acids	Changme Khang	Changme Khangpu
Straight	15.15	51.34
Branched	45.92	12.26
PUFA	34.25	22.60
MUFA	3.33	7.59
Cyclo	-	0.44
DMA	0.39	1.17
18:1w9c	0.40	2.84
18:1w6c,9c	0.10	0.57
10-methyl	0.46	1.20

Glaciers	Gram-Positive	GramNegative	Anaerobe	Actinomycetes	AMFungi	Fungi	Methanobacter	Eukaryote	Biomass content in (nmoles/gm)
Changme Khangpu	**24.84**	**22.65**	**2.43**	**1.77**	**--**	**1.19**	**--**	**47.12**	**801.22**
Changme Khang	**54.04**	**4.41**	**0.46**	**0.55**	**--**	**0.11**	**--**	**40.43**	**813.54**

Table 9b. Community structure analysis of glaciers based on PLFA studies.

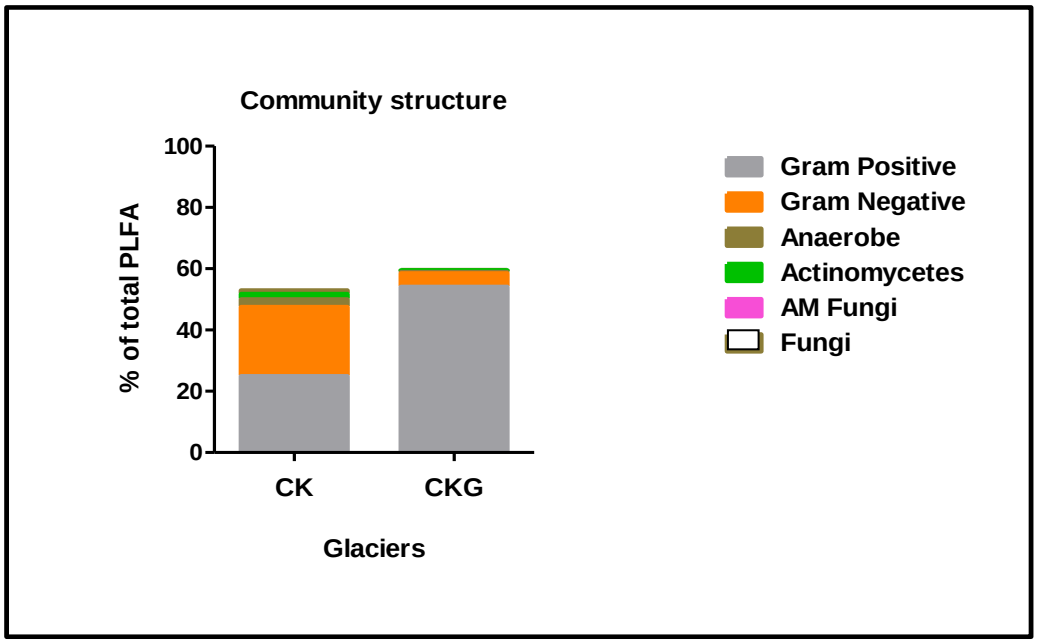


Fig.11a. Community structure of CK and CKG glacier based on PLFA studies.

The PLFA results showed that the two glaciers, i.e., Changme Khangpu and Changme Khang glacier were considerably different with respect to their biomass content. The biomass content of Changme Khang glacier was higher (365.05nmoles/g) than that Changme Khangpu glacier (60.31nmoles/g) **(Table.9a)**. Fatty acid marker analysis with Sherlock PLFA tool defined the community structure of two glaciers with the abundance of Gram-positive bacteria, Gram-negative bacteria, anaerobic bacteria, actinomycetes, fungi, and eukaryotes. The results showed that the Gram-positive bacteria were higher in Changme Khang glacier (54.04%) than Changme Khangpu glacier (24.84%), while Gram-negative bacteria were higher in Changme Khangpu glacier (22.65%) than Changme Khang glacier (4.41%) **(Table.9a & Fig.11a)**. The proportion of signature fatty acids related to fungi, anaerobe and actinomycetes were higher in Changme Khangpu glacier compare to Changme Khang glacier **(Fig.11a)**. The abundance of various fatty

types was also investigated and it was found that there was no such distinction between the fatty acids present in both the glaciers. The fatty acids found in glaciers were 11:0 *iso*, 12:0 *anteiso*, 12:0, and 23:3w3c. However, the fatty acids 13:0 iso, 15:0 *iso*, 17:0 *iso*, 19:3w3c, and 21:3ω9c was abundant in Changme Khang glacier which was not recorded in Changme Khangpu glacier **(Fig.11b)**.

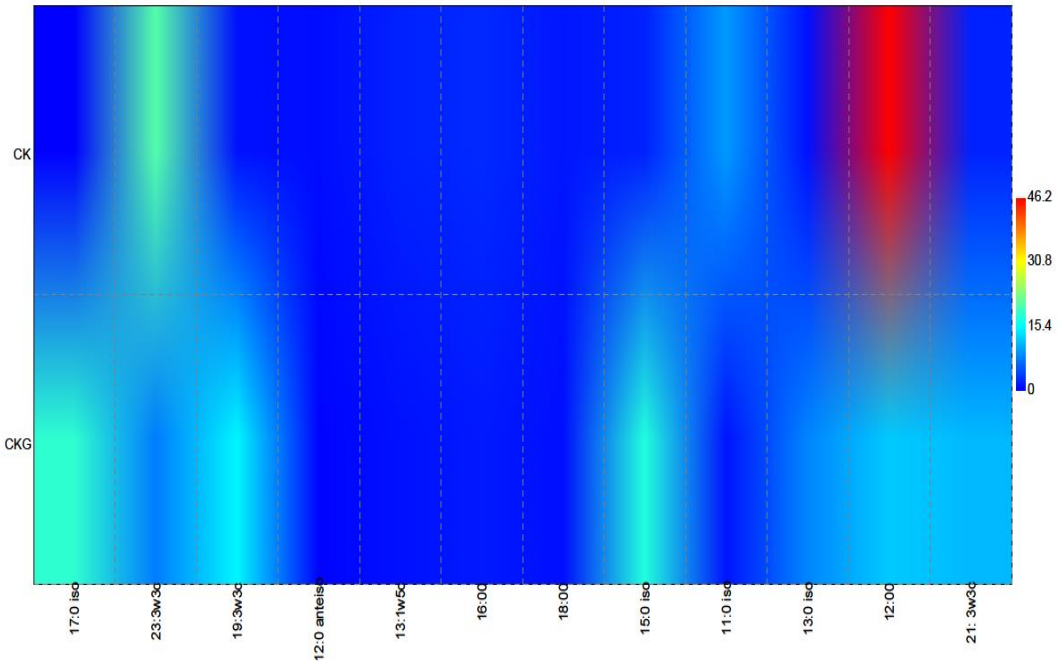


Fig.11b. Matrix plot showing the abundance of various fatty acids in two glaciers

6.7.2. Statistical Analysis

The correlation among fatty acids with respect to two different studied glacier were carried out with the help of Principal component analysis (PCA). The F1 component of the principal component analysis (PCA) of the fatty acids showed the relatively significant picture of the correlation between various fatty acids with respect to two glaciers. The F1-component acquiring (98.62%) variability with an eigen value of 1.97 as

shown in **Table.9c**. There was significant Pearson (n) correlation in two glaciers such as Changme Khang and Changme Khangpu glaciers with a significant p-value <0.005. The results have shown the positive correlation among 12:0 and 23:3w3c of Changme Khang and Changme Khangpu glacier **(Fig.11c)**.

Similarly, correlations of major fatty among two glaciers were carried out by principal component analysis. The results have shown that the F1-represents the maximum variability of (97.94%) as shown in (**Table.9d)**. There was significant Pearson (n) correlation in both the two glaciers such as Changme Khang and Changme Khangpu glacier with the significant p-value of <0.05. It has been shown that the Changme Khang and Changme Khangpu glacier are positively correlated with each other and with respect to parameters such as branch chain fatty acids. However, other fatty acids such as PUFA, Straight chain fatty acids, DMA, 18:1W6c, 9c and 18:1w9c were negatively correlated **(Fig.11d)**.

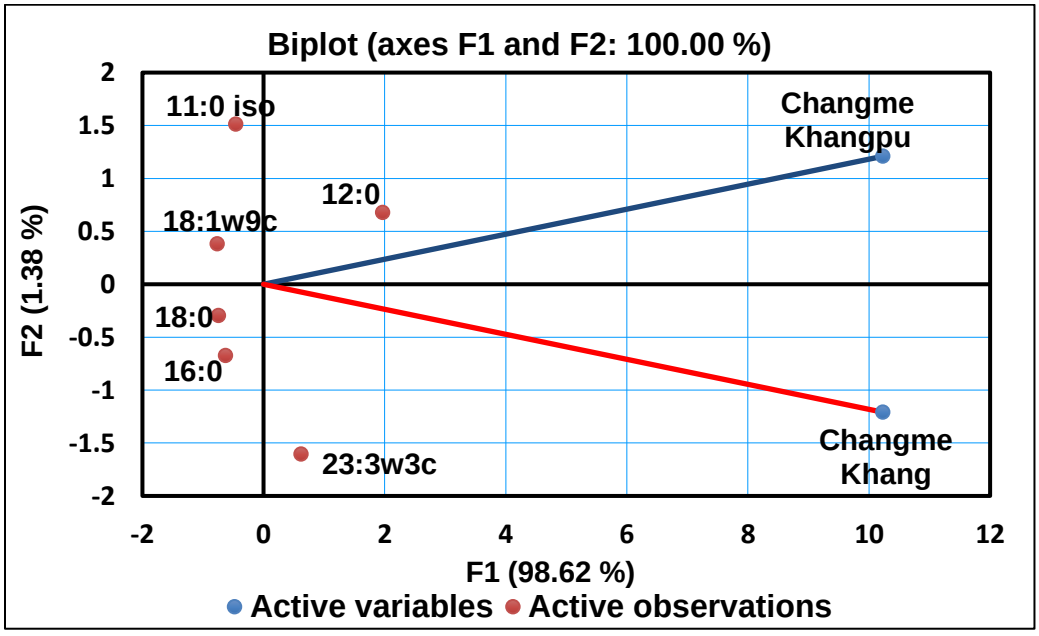


Fig.11c. Principal Component Analysis showing the correlation between fatty acids with respect to two different studied glaciers

Table.9c. Principal Component Analysis CKG and CK glacier fatty acids (Eigenvalues)

	F1	F2
Eigenvalue	1.972	0.028
Variability (%)	98.622	1.378
Cumulative %	98.622	100.000

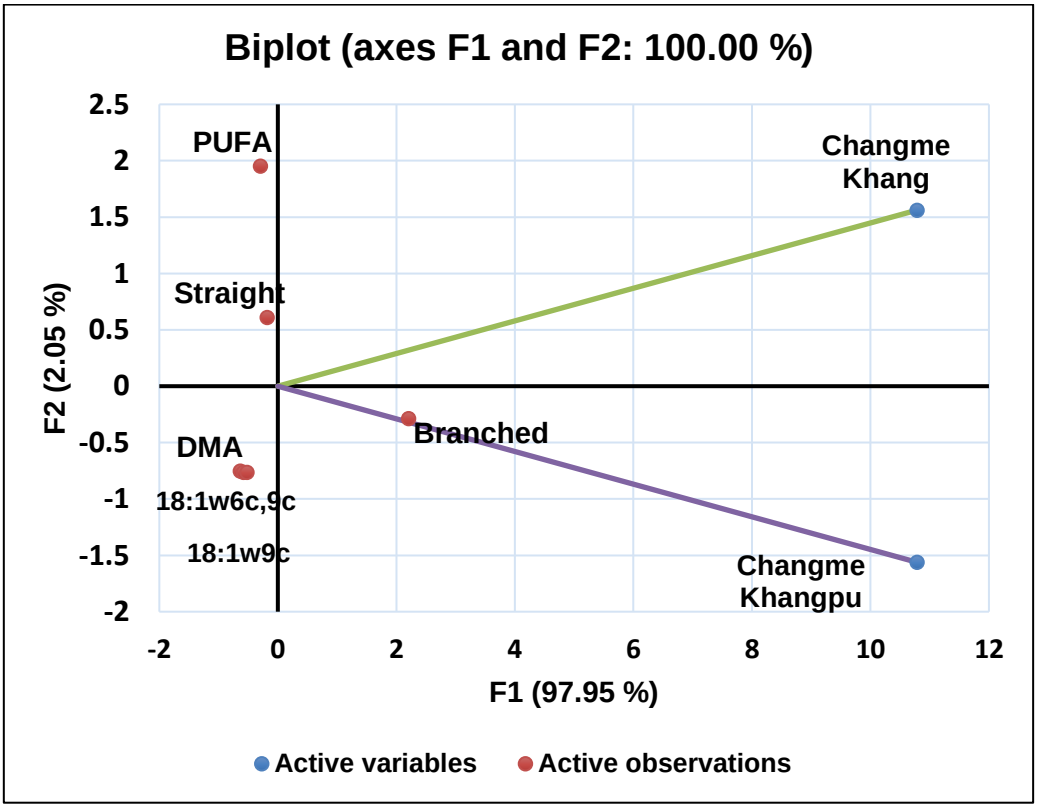


Fig.11d. Principal Component analysis of various fatty acids of Changme Khang and Changme Khangpu glacier.

Table.9d. Principal Component Analysis (Eigenvalues)

	F1	F2
Eigenvalue	1.959	0.041
Variability (%)	97.946	2.054
Cumulative %	97.946	100.000

6.7.3. Metagenomic Analysis

Shotgun metagenomic sequencing of Changme Khang glacier revealed a total of 18, 72,786 reads and 18,324 contigs with an average sequence length of 500bp**(Table.11)**. The G+C content was around 52%; while, 21, 12, 210 reads were obtained from Changme Khangpu glacier and 10, 211 contigs with an average sequence length of 500bp**(Table.11)**. The G+C content was estimated to be around 51%. The reads from both the samples were mapped onto the respective contigs using the bowtie2 software in order to check for the assembly quality. More than 60% of the sequence reads mapped back to the contigs successfully. The microbial abundance was estimated using Metaphlan2 software, a tool that profiles and classifies the sequencing data from shotgun metagenome samples, with species-level resolution using a set of 1 million clade-specific marker genes from more than 17,000 different microbes.

6.7.4. Diversity index and rarefaction curve

The diversity indices such as Shannon H, Fisher Alpha, and Chao1 were estimated using two software packages such as PAST and Estimate S-software. The results have shown that the Changme Khang glacier is more diverse than Changme Khangpu glacier. The Shannon index was 1.29 and 1.15 for Changme Khang and Changme Khangpu respectively **(Table.10)**. The Fisher alpha and Chao1 was also higher in the case of Changme Khang glacier **(Table.10)**. Rarefaction curve allows the calculation of species richness in a sample. The curve is a plot of a total number of species annotated as a function of the number of sequences sampled. The verticalangle at the beginning on the left side signifies the most common species which have been identified and the plateau at

the right side signifies further escalated sampling might lead to the identification of few rarest species **(Fig.12a)**(**Das et al., 2017**).

Table.10. Diversity indices of Changme Khang and Changme Khangpu glaciers microbial communities.

Glacier	Total number of reads	G+C content	Shannon H-indices	Fisher alpha	Chao 1
CK	1872786	52	1.15	3.56	12
CKG	2112210	51	1.29	4.43	14

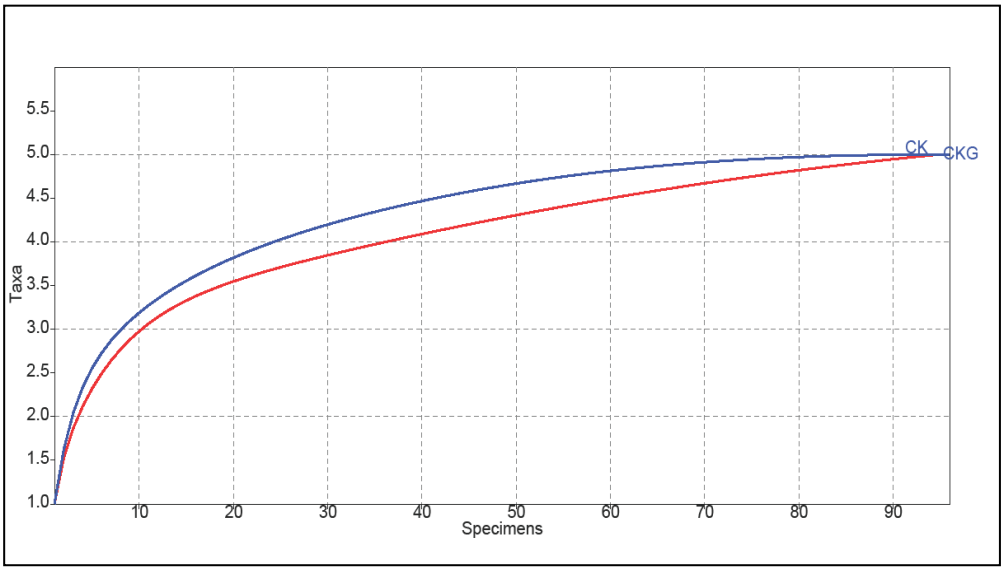


Fig.12a. Rarefaction curve, red curve shows species richness of Changme Khangpu glacier whereas blue line represents Changme Khang glacier. The x-axis represents the number of sequence reads while the y-axis represents the species counts.

6.7.5. Diversity Analysis of two glacial at Phylum, Genus and Species level

The bacterial community showed little variation between the glaciers. The phylum wise diversity showed that the dominance of *Proteobacteria*(75.5%), *Firmicutes* (5.1%), *Unidentified virus*(0.7%), *Actinobacteria* (7.2%), and *Ascomycota* (0.9%) in Changme Khangpu glacier **(Fig.12c)** whereas Changme Khang glacier was dominated by phylum

Proteobacteria (81.2%), followed by *Firmicutes* (7.1%) and *Actinobacteria* (3.1%) **(Fig.12b)**.

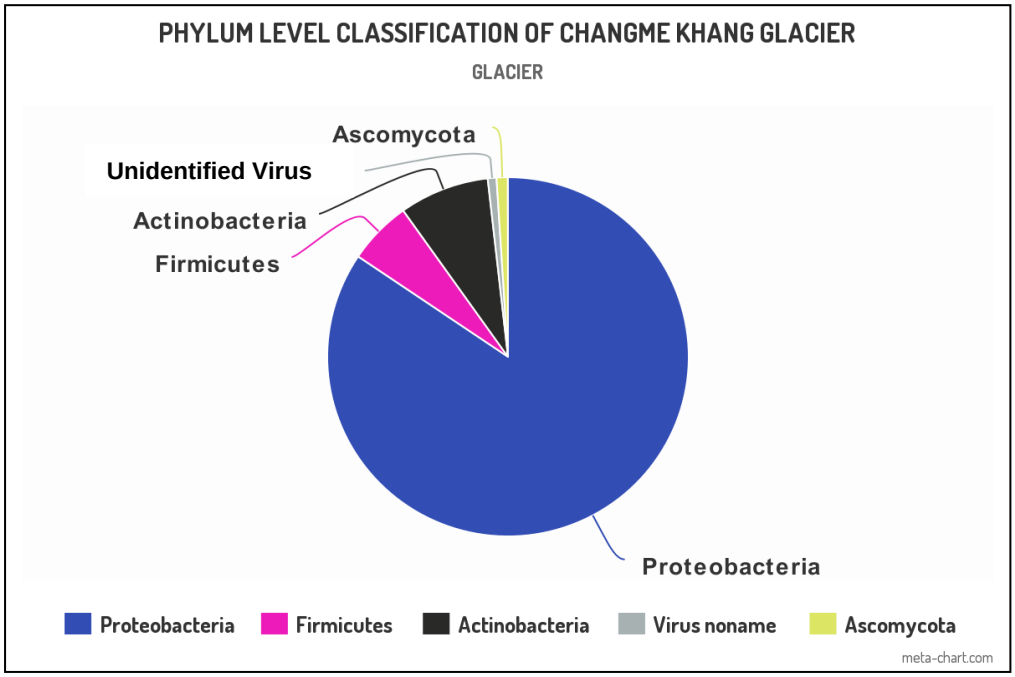


Fig.12b. Phylum level classification of Changme Khang glacier

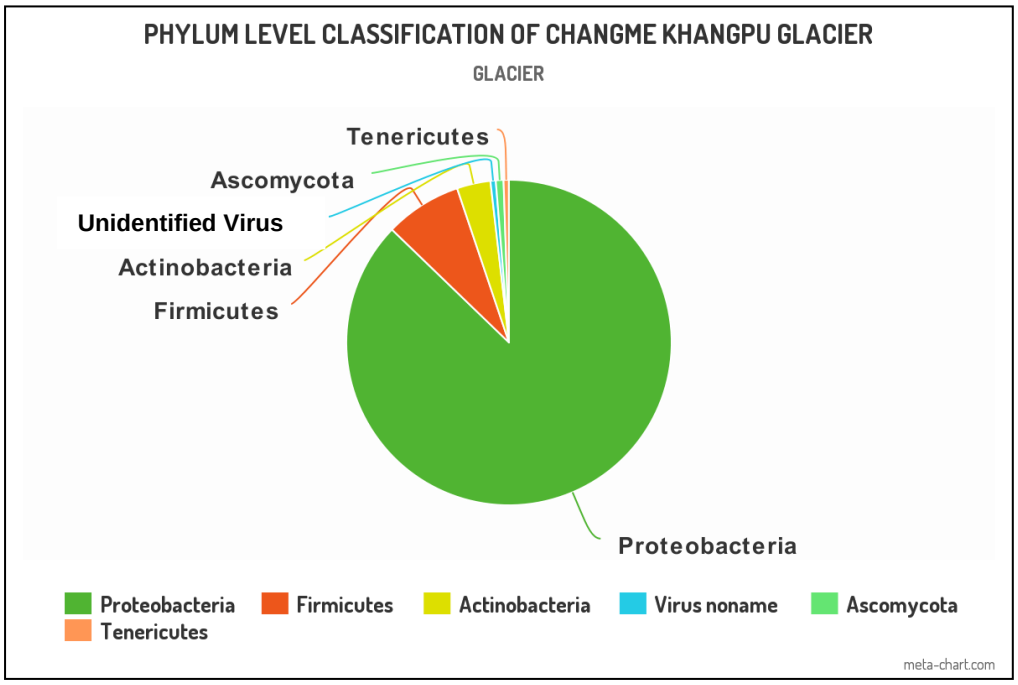


Fig.12c. Phylum level classification of Changme Khangpu glacier

The class hierarchy inphylum *Proteobacteria*, showed that the presence of *Beta-proteobacteria* in Changme Khang and Changme Khangpu glacier were (66.22% and 52.32%)followed by*Gamma-proteobacteria* in Changme Khangpu were 35.97% and 18.42% in Changme Khang glacier, similarly *Alpha-proteobacteria* which account for 11.27% in Changme Khang glacier and 14.61% in case of Changme Khangpu glacier respectively. The other class such as *Delta-proteobacteria*, *Epsilon-proteobacteria*, and *Zeta-proteobacteria* were not recorded in both the glacier.

At genus level classification there was not much variation in two glaciers. The major dominated genus demonstrated in Changme Khang glacier were *Delftia* (49.35%), *Serratia* (31.19%), *Brevundimonas*(11.23%), *Stenotrophomonas* (3.27%), *Massilia* (2.26%), *Commomonas* (0.66%), and *Pseudomonas* (0.51%) **(Fig.12e)**. Similarly, major genera demonstrated in Changme Khangpu glacier were *Delftia* (62.39%), *Serratia* (16.58%),*Brevundimonas* (14.52%), *Massilia* (2.95%), *Stenotrophomonas* (1.02%), and *Commomonas* (0.84%) as shown in **(Fig.12d)**.

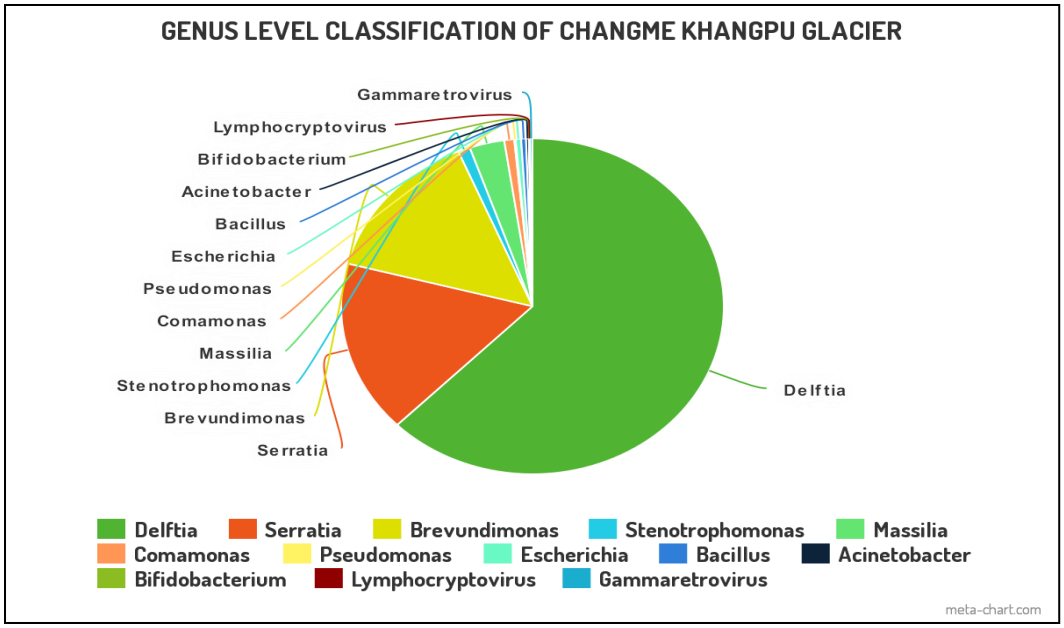


Fig.12d. Genus level classification of Changme Khangpu glacier

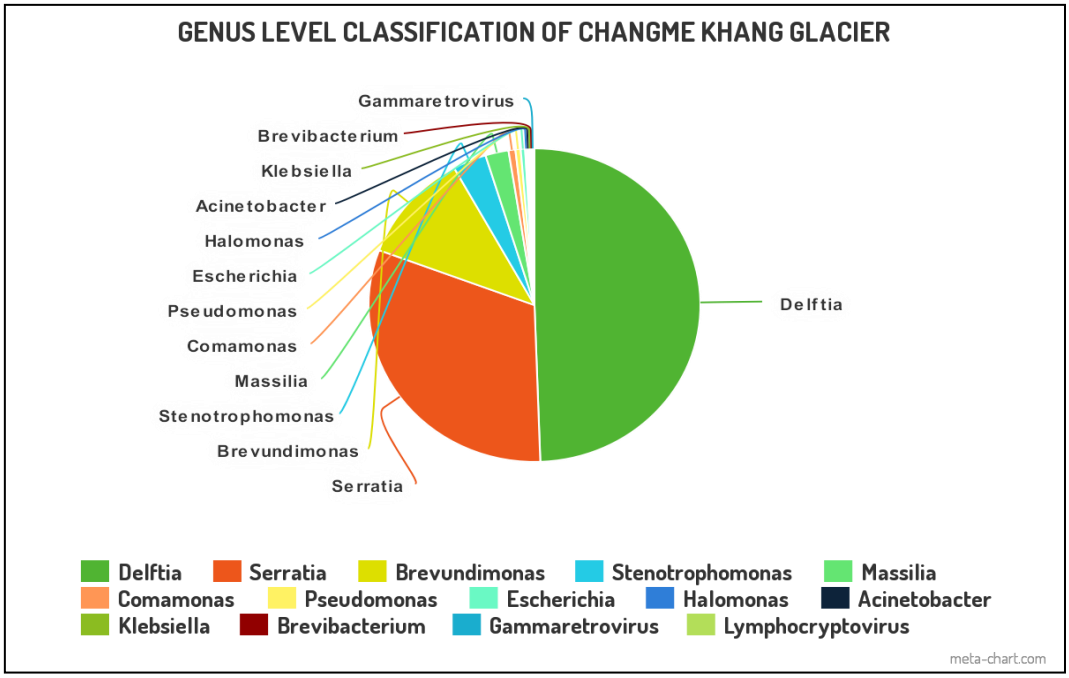


Fig.12e. Genus level classification of Changme Khang glacier

The diversity at species level varies significantly among two glaciers. In both the glacier the major bacterial flora was uncultured with 40.68% in Changme Khangpu glacier and 35.01% in Changme Khang glacier. Likewisein both the glaciers Gram-negative and Gram-positive bacteria were detected however the majority of them were Gram-negative psychrotolerant bacteria. In case of Changme Khang glacier, major species detected were *Serratia marcescens* (31.19%) followed by *Delftia acidovorans* (19.79%), *Stenotrophomonas maltophilia* (2.26%), *Pseudomonas putida* (0.51%) and *Escherichia coli* (0.44%) **(Fig.12g)**. In case of Changme Khangpu glacier major leading bacterial species were *Delftia acidovorans* (37.59%), subsequently by *Serratia marcescens* (24.80%) *Stenotrophomonas maltophilia* (0.86%), *Bacillus cereus* and *Bacillusthuringinensis*together account for about 0.41% of the total sequences, and *Pseudomonas putida* accounted for 0.11% of the total sequences, respectively**(Fig.12f)**.

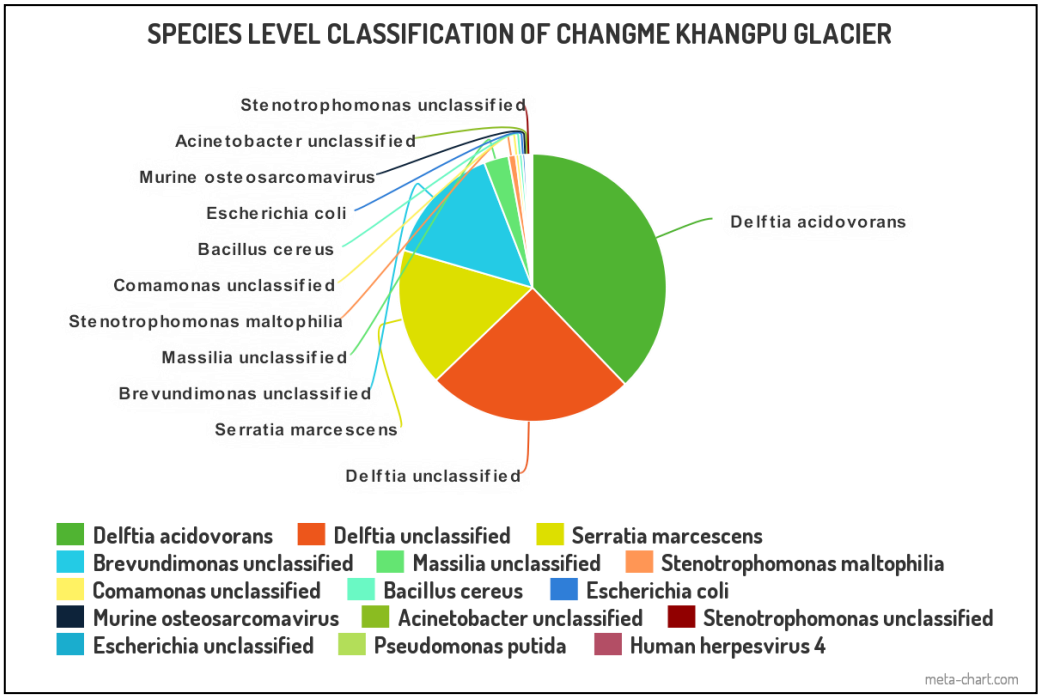


Fig.12f. Species-level classification of Changme Khangpu glacier

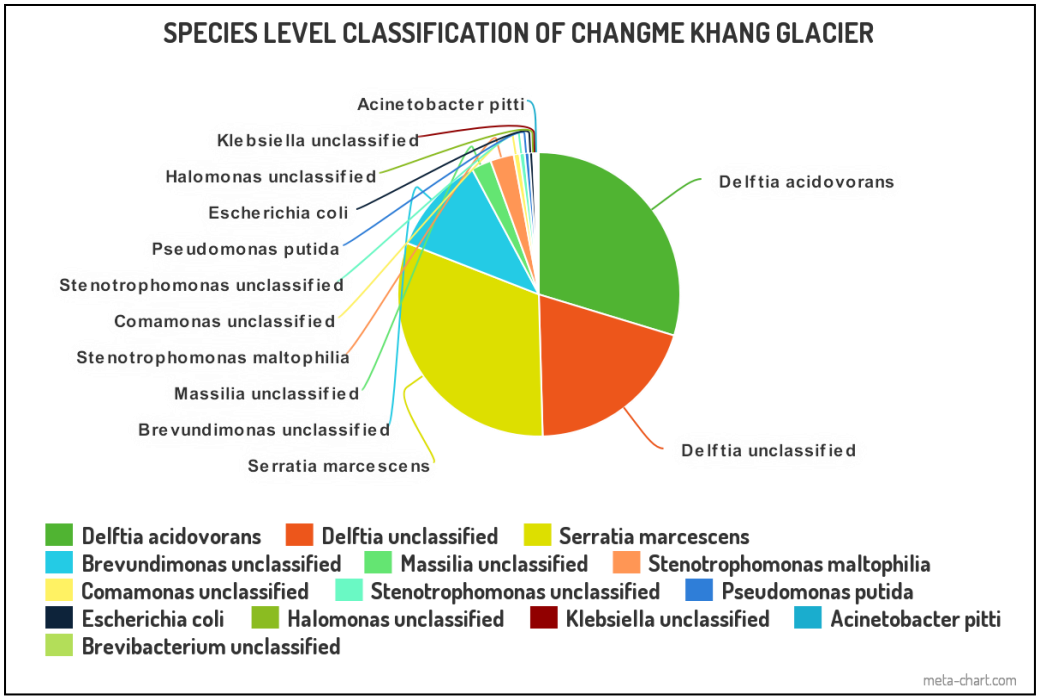


Fig.12g. Species-level classification of Changme Khang glacier

6.8. Antibiotic Sensitivity of Changme Khang and Changme Khangpu glacier Isolates.

Antibiotic sensitivity profiles of Changme Khang and Changme Khangpu glacier bacterial isolates were performed on Muller Hinton Agar plate by disk diffusion method. Six different antibiotics such as vancomycin, erythromycin, ampicillin, streptomycin, chloramphenicol, and methicillin were checked. These results were compared with the zone size of Gram-negative and Gram-positive bacteria such as *Enterobacter* and *Staphylococcus* as per CLSI guidelines. It suggests that the majority of the isolates showed resistance to ampicillin (sensitive >17mm, intermediate 14-16mm, resistant <13mm and methicillin (sensitive >15mm, intermediate 10-13mm, resistant <9mm) antibiotics as shown in (**Table.11a and 11b).**The results have shown that the resistance patterns of antibiotics were highest against ampicillin followed by methicillin, streptomycin, chloramphenicol, erythromycin, vancomycin respectively.

Table. 11a. Antibiotic Susceptibility of Changme Khangpuglacier isolates

Antibiotic disc with their Concentrationin mcg Zone of inhibition (in nm)						
Isolates	**Van30**	**Er15**	**Amp10**	**S100**	**C30**	**Met10**
CK1	30;S	40;S	19; S	0;R	40;S	0;R
CK3	36;S	40;S	14; S	37;S	33;S	40;S
CK5	40;S	40;S	0;R	39;S	40;S	0;R
CK6	28;S	36;S	0;R	40;S	40;S	12;I
CK9	30;S	35;S	10;R	32;S	40;S	0;R
CK10	32;S	32;S	14;S	38;S	0;R	25;S
CK11	36;S	18;I	10;R	16;R	16;R	25;S
CK13	29;S	0;R	10;R	40;S	40;S	0;R
CK15	28;S	30;S	10;R	39;S	25;S	26;S
CK16	29;S	40;S	22;S	40;S	40;S	0;R
CK17	12;R	20;S	21;S	40;S	40;S	0;R
CK19	29;S	40;S	21;S	0;R	40;S	0;R
CK20	25;S	35;S	0;R	35;S	36;S	0;R
CK21	20;S	40;S	0;R	0;R	40;S	0;R
CK22	15;S	24;S	10;R	30;S	30;S	12;I

S; Sensitive, I; Intermediate, R; Resistance, Van;Vancomycin, Er; Erythromycin, Amp; Ampicillin, S; Streptomycin, C; Chloramphenicol and Met; Methicillin.

Table.11b. Antibiotic Susceptibility of Changme Khangglacier isolates

Antibiotic disc with their Concentrationin mcg Zone of inhibition (in nm)						
Isolates	**Van30**	**Er15**	**Amp10**	**S100**	**C30**	**Met10**
CKG1	30;S	40;S	0;R	22;S	40;S	17;R
CKG2	29;S	40;S	0;R	31;S	40;S	0;R
CKG4	38;S	40;S	11;R	40;S	40;S	35;S
CKG5	29;S	40;S	0;R	40;S	40;S	0;R
CKG6	40;S	40;S	0;R	40;S	40;S	28;S
CKG7	0;R	0;R	0;R	40;S	0;R	0;R
CKG8	30;S	40;S	0;R	40;S	40;S	0;R

S; Sensitive, I; Intermediate, R; Resistance; Van;Vancomycin, Er; Erythromycin, Amp; Ampicillin, S; Streptomycin, C; Chloramphenicol and Met; Methicillin.

6.8.1. Minimum Inhibitory Concentration (MIC) of Changme Khang and Changme Khangpu glacier isolates.

The four different groups of antibiotics were tested against Changme Khang and Changme Khangpu glacier isolatesfor their Minimum Inhibitory Concentration (MIC) values. The test antibiotics were Beta-lactam (ampicillin), phenicol (chloramphenicol),aminoglycoside (streptomycin), and macrolide (erythromycin). Various dilutions from stock solution were made as 0.25, 0.5, 1, 2, 4, 8, and 16mgL^{-1}. The results have revealed that the minimum inhibitory concentration values forthree antibiotics (ampicillin, chloramphenicol, and erythromycin) were high. In the case of ampicillin antibiotic MIC values shown by the majority of the isolates were 4µgL^{-1} respectively. Against chloramphenicol antibiotic, the highest MIC value (8µgL^{-}1) was demonstrated by few of the isolates. Similarly, for erythromycin antibiotics, the majority of the isolates confirmed the MIC value of 4µgL^{-1} followed by MIC 8µgL^{-1}(**Fig.13**). The till-date there is not a single globally accepted guideline for susceptibility testing or breakpoint testing for psychrophilic/psychrotolerant bacteria was available. However, psychrotolerant bacteria such as *Pseudomonas* and *Enterobacter* species and their breakpoint as perCLSI, 27-edition data as well as two *Bacillus species* were taken as baselines to investigatethe resistance pattern of our isolates **(Rosenquist et al., 2005)**. In brief, the breakpoint values of the MIC for resistance were as follows: for aminoglycoside class of antibiotic such as Streptomycin breakpoint were ≥32 µgL^{-1}, similarly for Macrolides class of antibiotic such as Erythromycin breakpoint were ≥8 µgL^{-1}, Beta-lactam class of antibiotic for example Ampicillin was ≥4 µg$L^{-1}$and Phenicols group of antibiotic for instance Chloramphenicol ≥8 µgL^{-1}breakpoint value (**CLSI guidelines 2017; Rosenquist et al., 2005).**

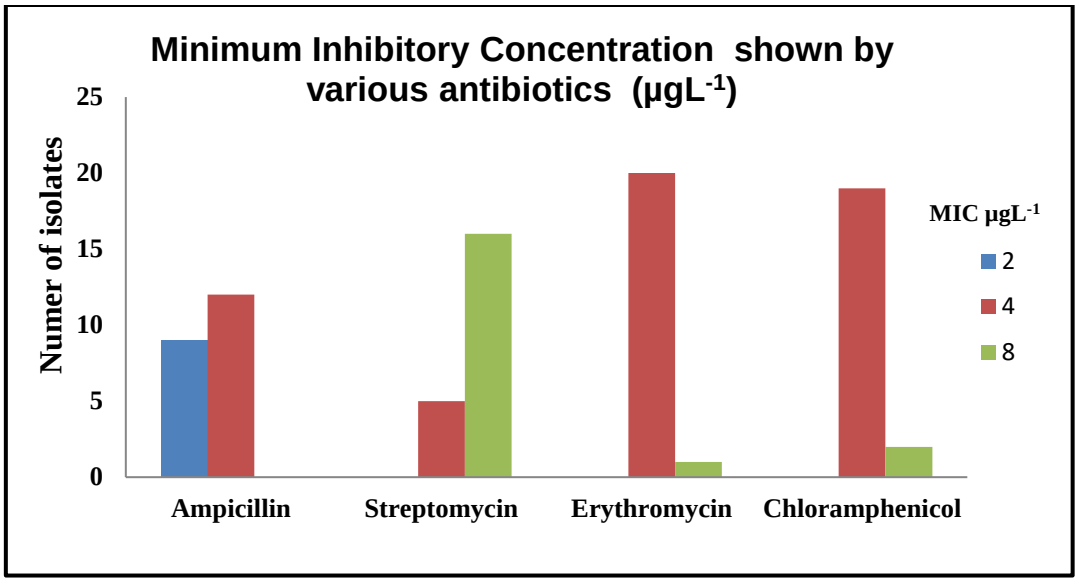


Fig.13. Minimum inhibitory concentration is shown by various antibiotics in µgL^{-1}

6.8.2. Metagenomic studies of antibiotic resistance genes (ARGs)

Metagenomic sequence data was generated using Illumina HiSeq and study of antibiotic resistance genes (ARGs) were carried out using arbAnno V.1.0. For functional metagenomic analysis of two glaciers, i.e., Changme Khang (Debris-free) and Changme Khangpu (Debris-covered) glacier were selected. The results have shown the different resistance genes in both the glaciers. Both the glaciers havethe resistance genes belonged to class aminoglycoside, tetracycline, ß-lactam, bacitracin, quinolone, and for multidrug resistance. Overall higher numbers of resistance genes were present in Changme Khangpu glacier than Changme Khang glacier. In both the glacier, the most dominant resistance genes were against bacitracin (*bacA*). *mexD*genes for multidrug resistance were present in Changme Khang glacier, whereas in Changme Khampu it was absent. On the other hand *mdtg* gene sequences were absent in Changme Khang glacier but present in Changme Khangpu glacier. The tetracycline resistance gene tet41 sequences reads

werehigherin Changme Khangpu glacier metagenomic data. Prevalence of different aminoglycoside resistance genes and their numbers were similar in both the glaciers. The aminoglycoside gene was showing closest identity (99%) with *Serratia marcescens* (*aac6ic*) gene, *Acinetobacter baumanii* (*aph3ia*) gene, and *Escherichia coli* (*KsgA*) gene. Similarly, bacitracin gene was showing 99% identity with *Bacillus cereus* (*bacA*) gene, Quinolone resistance gene showing 99% identity with *Serratia proteamaculans*(*qnrB*) gene. Tetracycline resistance gene illustrating 99% identity with *Serratia marcescens* (*tet41*) and *Bifidobacterium bifidum* (*tetC*) gene. Multidrug resistance genes demonstrate 99% identity with *Serratia proteamaculans*(qnrb)and (*rosb*) gene, *Pseudomonas putida* (*mexd*) gene and *Escherichia coli* (*mdtg*) gene **(Fig.14a and 14b)**.

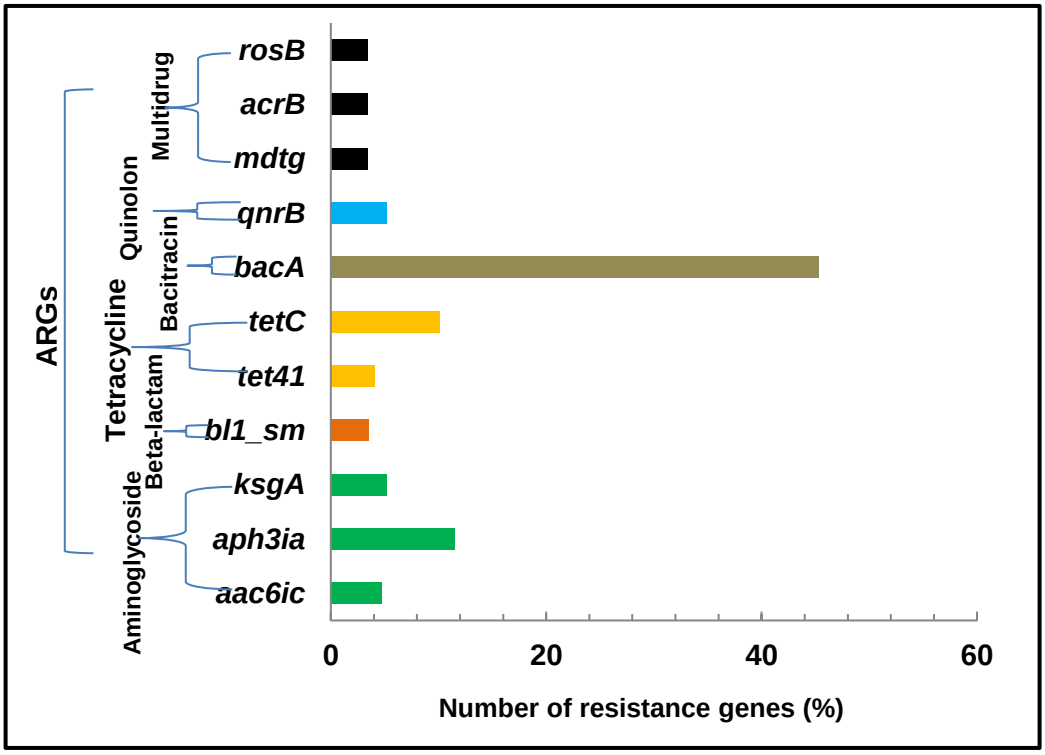


Fig.14a. Antibiotic Resistance genes (ARGs) distribution of Changme Khangpu glacier

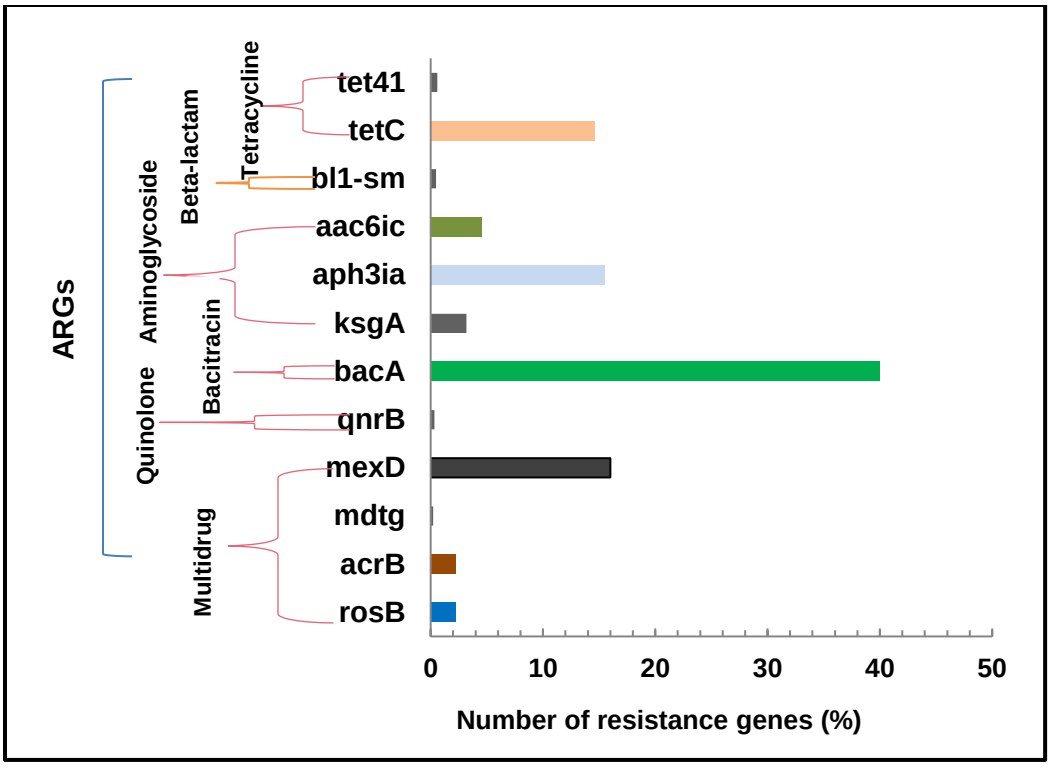


Fig.14b. Antibiotic Resistance genes (ARGs) distribution of Changme Khang glacier

6.9. Heavy metal tolerance to Changme Khang (CKG) and Changme Khangpu (CK) glacier isolates.

6.9.1. Screening and assessment of metal toxicity

Earlier to the introduction of antibiotics for the treatment of diseases, heavy metals were used for disease treatment for centuries and the close link between antibiotic resistant and heavy metal resistant phenomena has been reported by many researchers. Therefore, heavy metal tolerance was checked for our bacterial isolates. Five different metals such as $FeCl_2$, $ZnCl_2$, $CoCl_2$, $NiCl_2$, $CuCl_2$, and $HgCl_2$ were screened and assessed for metal toxicity. Tube dilution method was used to check the minimum inhibitory concentrations. An experiment in liquid culture was conducted to determine the precise concentrations of metals at which the isolates could grow. All the results were expressed in MIC (**Table.12a)**.

Mercury toxicity

The mercury was highly toxic to almost all the strains as only less concentration <0.1mM of HgCl2 was tolerable. In terms of minimum inhibitory concentrations against mercury, the majority of the isolates (CK3, CK5, CK6, CK10, CK11, CK13, CK15, CK16, CK17, CK19, CK20, CK21, CK22, CKG1, CKG2, CKG4, and CKG5) showed the MIC value of 1mM, whereas few isolates like CK1, CK9, CKG6, and CKG8 showed MIC value of 0.5 mM only **(Table.12a)**.

Zinc toxicity

Majority of the isolates showed MIC of Zinc was 3 and few isolates confirmed Zinc tolerant at a concentration of 1mM (MIC=2mM) such as strain CK10, CK15, CK20, and CKG1. Similarly isolate showing MIC=5mM were CK3, CK6, CK11, and CK21. All other remaining isolates showed tolerate at 2mM zinc (MIC=3mM) **(Table.12a)**.

Copper toxicity

Most of the studied isolates tolerated more than 0.5mM concentration of $CuSO_4.5H_2O$. It was observed that 9 isolates were having MIC=1mM. However, the most tolerant strains were CK1, CK3, CK5, CK6, CK9, CK10, CK11, CK13, CK19, and CKG6 (MIC=3mM) **(Table.12a)**.

Nickel toxicity

Majority of the isolates showed tolerant against nickel was 1mM (MIC=3mM). However, few isolates showed were tolerant 0.5mM (MIC=1mM) such as CK13, CK20, CK21, CKG1, CKG2 and CKG8 **(Table.12a)**.

Cobalt toxicity

Majority of the isolates were tolerating to cobalt at a concentration of 0.5mM however, 3 strains were tolerant at a concentration of 1mM (MIC=3mM) such as strain CK3, CK5, and CK13 **(Table.12a)**.

The numbers of isolates showing maximum tolerance to various heavy metals with minimum inhibitory concentration values are shown in **Fig.15a** isolates were showing higher MIC values of $ZnCl_2$,$CuCl_2$, $NiCl_2$, $HgCl_2$, and$CoCl_2$respectively. Metal tolerance was also checked in order to get some attractive psychrotolerant isolates tolerant to different metals that can be employed in bioremediation and also verify the co-occurrence of heavy metal resistance and antibiotic resistance. The result showed that metals such as zinc chloride isolates showing highest MIC value was 5mM, followed by cupper sulfate, nickel chloride, and cobalt chloride were 5mM each and mercury chloride was 1mM. As compare with heavy metals check point ($ZnCl_2$,$CuCl_2$,$NiCl_2$,$CoCl_2$(1mM each) and$HgCl_2$(0.5mM)descried by (**Nies, 1999**) our isolates showed higher values **(Fig.15a and 15b)**.

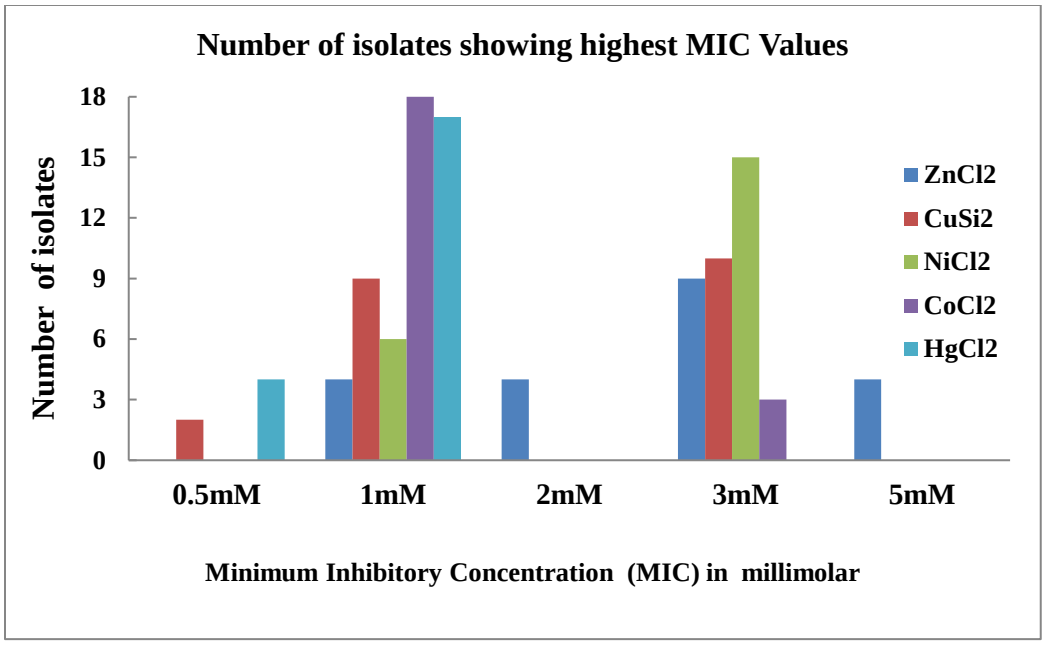


Fig.15a. The number of isolates showing the highest MIC values with each heavy metal.

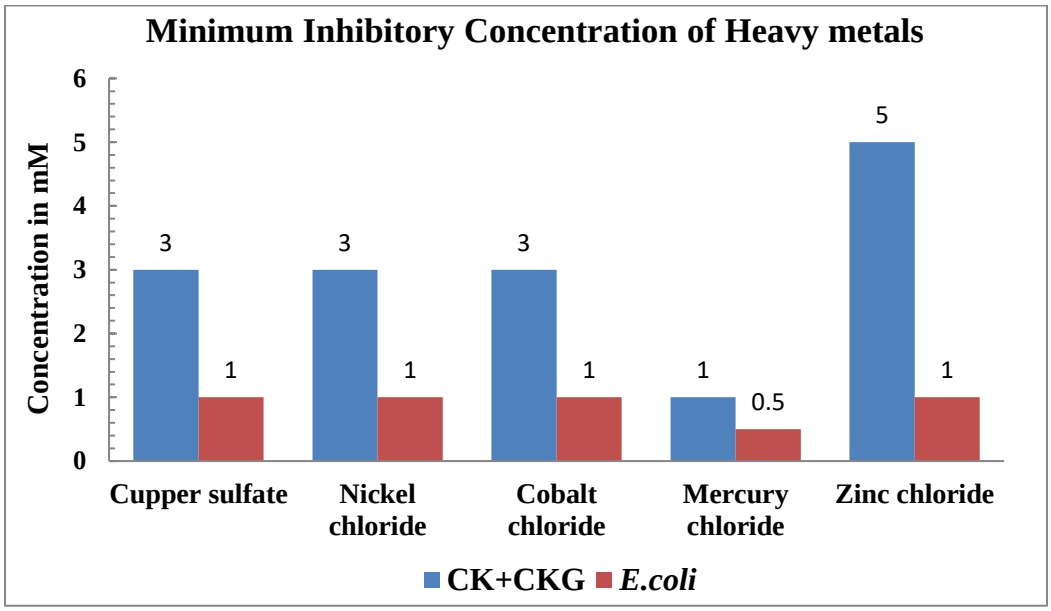


Fig.15b. Comparison of minimum inhibitory concentrations among CK+CKG isolates and *E. coli* (Nies, 1999) data.

Table.12a. Minimum Inhibitory Concentration of heavy metals (in liquid media)

Minimum Inhibitory Concentration (in liquid media)					
Isolates	**HgCl2**	**CuSO4**	**NiCl2**	**CoCl2**	**ZnCl2**
CK1	**0.5**	**3**	**3**	**1**	**3**
CK3	**1**	**3**	**3**	**3**	**5**
CK5	**1**	**3**	**3**	**3**	**1**
CK9	**0.5**	**3**	**3**	**1**	**3**
CK6	**1**	**3**	**3**	**1**	**5**
CK10	**1**	**3**	**3**	**1**	**2**
CK11	**1**	**3**	**3**	**1**	**5**
CK13	**1**	**3**	**1**	**3**	**3**
CKG6	**0.5**	**3**	**3**	**1**	**3**
CK15	**1**	**1**	**3**	**1**	**2**
CK16	**1**	**1**	**3**	**1**	**3**
CK17	**1**	**1**	**3**	**1**	**1**
CK19	**1**	**3**	**3**	**1**	**3**
CK20	**1**	**1**	**1**	**1**	**2**
CK21	**1**	**1**	**1**	**1**	**5**
CK22	**1**	**1**	**3**	**1**	**1**
CKG1	**1**	**0.5**	**1**	**1**	**2**
CKG2	**1**	**1**	**1**	**1**	**1**
CKG8	**0.5**	**0.5**	**1**	**1**	**3**
CKG4	**1**	**1**	**3**	**1**	**3**
CKG5	**1**	**1**	**3**	**1**	**3**

6.9.2. Statistical significance of heavy metal tolerance through principal component analysis (PCA)

Principal component analysis of heavy metals was calculated and interconnected with the studied isolates. The first two Eigen values among the five principal components were having >1values such as 1.876, and 1.210, correspondingly as shown in (**Table.12b)**. The overall variance of the principal component was 37.5%. The Bartlett's sphericity test was also completed which signify the test for null-hypothesis that correlation matrix has an identity matrix. Taking this into account, these tests offer the minimum standard to proceed to Factor Analysis. The results illustrated by these test with the p-value of 0.0092

(threshold value of <0.05) thus represent the factor analysis is valid and considerable as shown in**(Table.12c)**. PCA results were comparable to results achieved from minimum inhibitory concentration analysis ie, PCA confirms positive connection among individual heavy metals to those isolates which were having elevated MIC values with respect to that particular heavy metals. There was a positive correlation between $CuSo_4$, $ZnCl_2$, and $NiCl_2$. However, metals such as $CoCl_2$ and $HgCl_2$ were negatively correlated (**Fig.13c)**. The PCA result showed that the highly tolerant strains were present in the vicinity of the concerned heavy metal. The studied isolate such as CK19, CK17, CK10, CK13, CK11, CKG6, and CK16 are present in the vicinity of Cu, Zn, and Ni, which inferred that these isolates were positively correlated with Cu, Zn, and Ni (**Fig.13c)**. From Changme Khang and Changme Khangpu studied isolates, it can be inferred that the isolates showed the highest tolerance to Zn>Ni>Cu>Co>Hg on the basis of MIC results in liquid media.

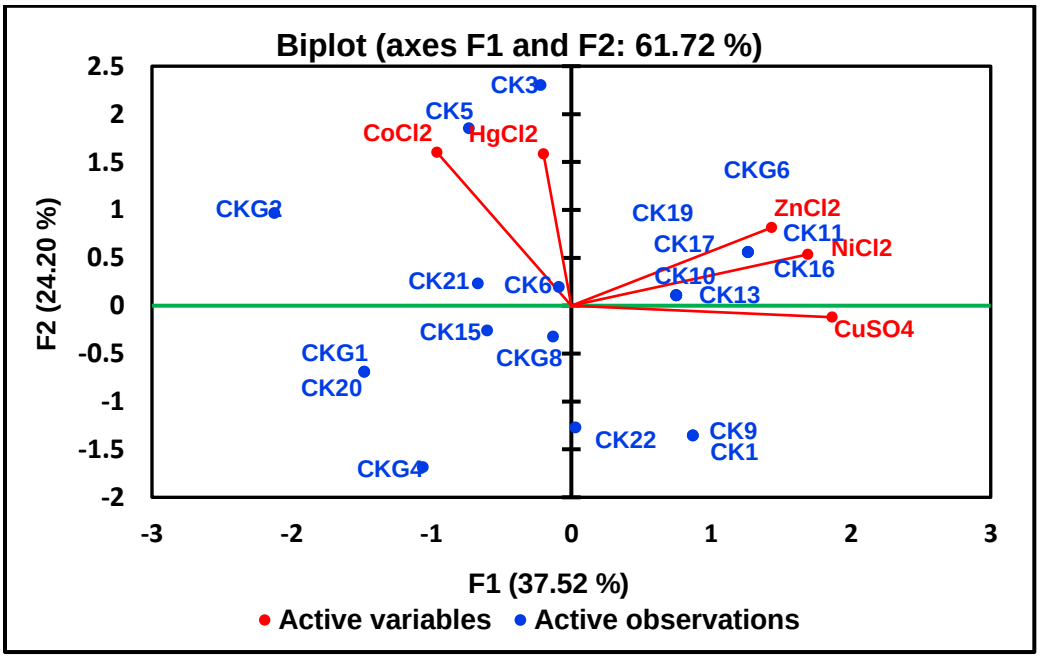


Fig.15c. Principal Component Analysis showing the correlation between heavy metals and various studied isolates

Table.12b. Principal Component Analysis the correlation between heavy metals and studied isolates (Eigenvalues)

	F1	F2	F3	F4
Eigenvalue	**1.876**	**1.210**	**0.893**	**0.655**
Variability (%)	**37.515**	**24.203**	**17.856**	**13.099**
Cumulative %	**37.515**	**61.718**	**79.574**	**92.673**

Table.12c.Bartlett's sphericity test:

Chi-square (Observed value)	11.896
Chi-square (Critical value)	**18.307**
DF	**10**
p-value (Two-tailed)	**0.0092**

6.9.3.Metagenomic studies of metal resistance genes (MRGs)

Metagenomic study of the metal resistance genes (MRGs) were carried out using BacMet Scan V.1.0. The results have shown the different resistance genes in both the glaciers. However, it was shown that the Changme Khangpu possess the higher diversity of resistance genes than the Changme Khang glacier. The resistance genes detected were belonging to various metals such as mercury, copper, chromium, and arsenic. The mercury, copper and chromium resistance gene were found in both the glacier but only arsenic resistance genes were detected in Changme Khangpu glacier. The copper resistance genes (*copC*and *cutF*) showed 96% identity *Pseudomonas fluorescens*and *Escherichia coli* K12. The mercury resistance genes (*merA*) are showing 98% identity with*Thiobacillus ferrooxidans*, *Staphylococcus aureus*, *Pseudomonas stutzeri*, and *Pseudomonas aeruginosa*.The chromium resistance gene (*chrA*) showing 96% identity with *Pseudomonasaeruginosa*. However, arsenic resistance gene (*arsH*) was showing 91%

identity with *Yersinia pestis Java* 9 which was detected only in Changme Khang glacier. Based on the cluster of orthologous groups (COG) classification, various metals resistance genes were envisaged subsequent to each metal resistance classes. However, metal resistances were diverse in Changme Khangpu glacier than of Changme Khangpu glacier. Two different metal resistance was detected in Changme Khang glacier were Copper with COG predicted genes such as *cutA, cutE, cutC, cutF, cueR, copC,*and*copB,* and Chromium with COG predicted genes were *yelf, ruvB, nfsA, chrR* and *chrA*(**Fig.16a**). Similarly, from Changme Khangpu glacier Cobalt with COG predicted genes for instance *mgtA, dmef, cord, corC, corB* and *cnrA* Iron with COG predicted genes such as *yefD, yefC, yefB,* and *yefA*(**Fig.16b**).

On the basis of metagenomic analysis biocide resistance genes were also detected and from Changme Khangpu glacier genes related to benzalkonium chloride (BAC) and acriflavin were detected in higher abundance than other biocides (**Fig.17a**) similarly acriflavin and hydrogen peroxide related genes were higher abundance than other biocides (**Fig.17b**).

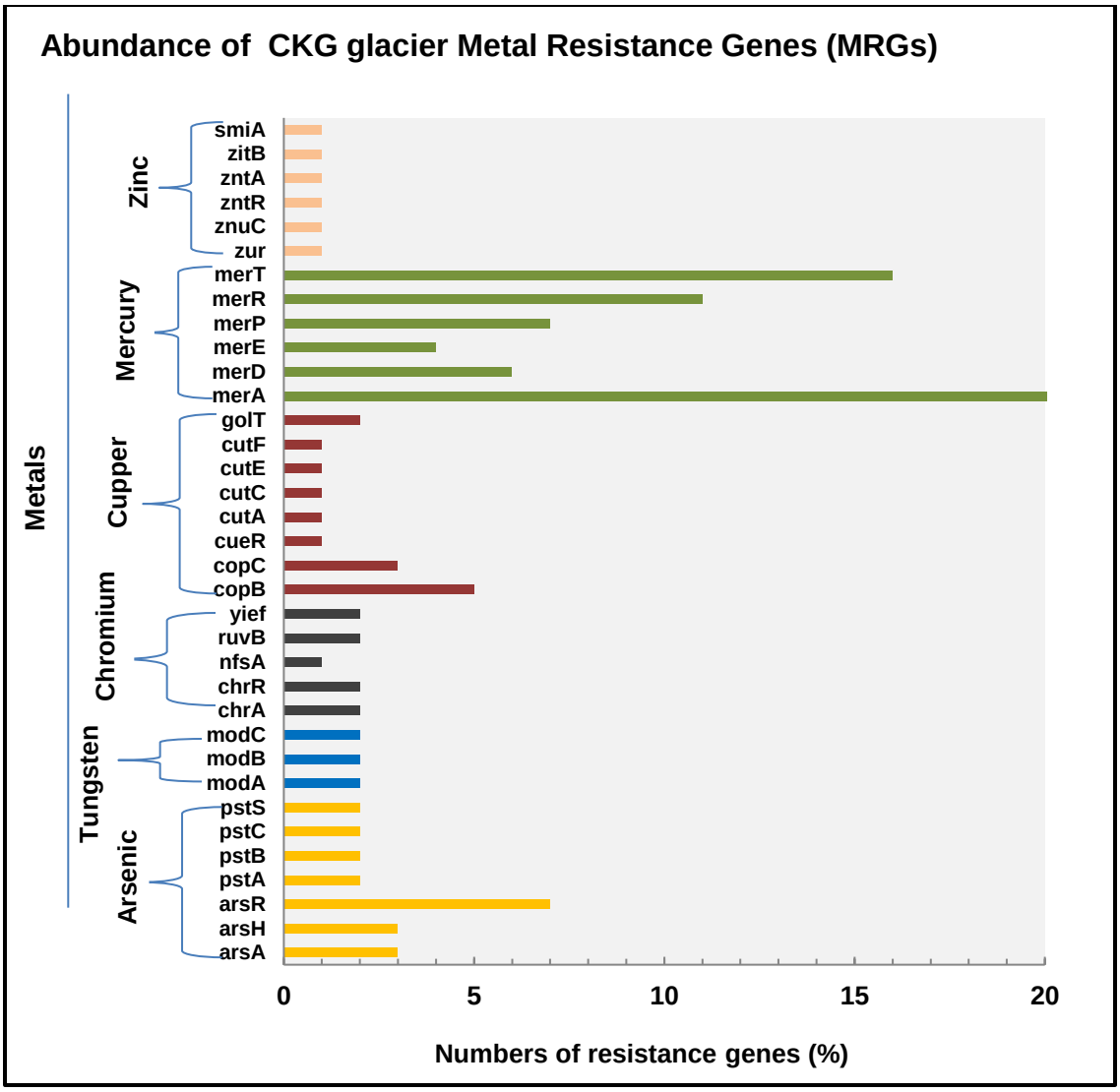


Fig.16a. The abundance of Changme Khang glacier Metal ResistanceGenes (MRGs)

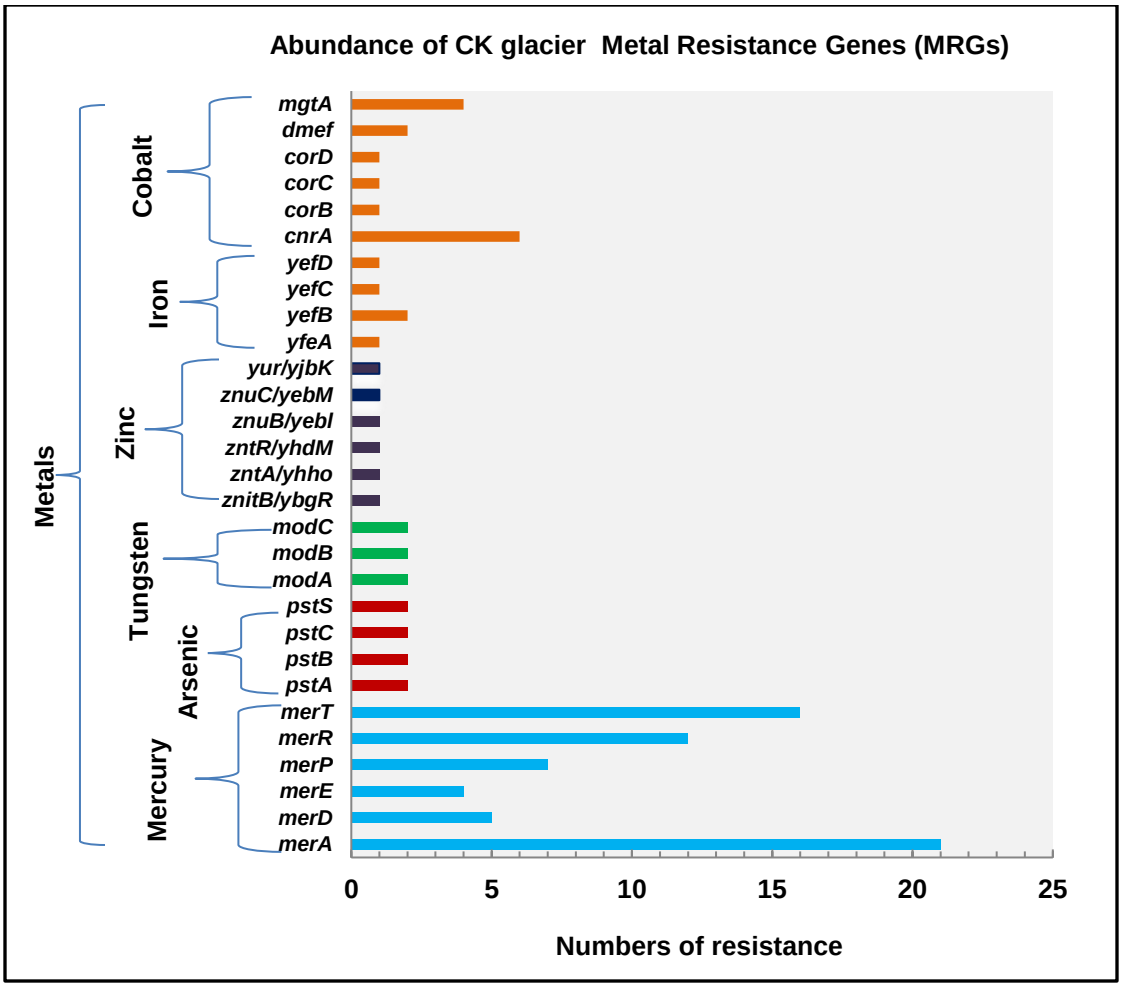
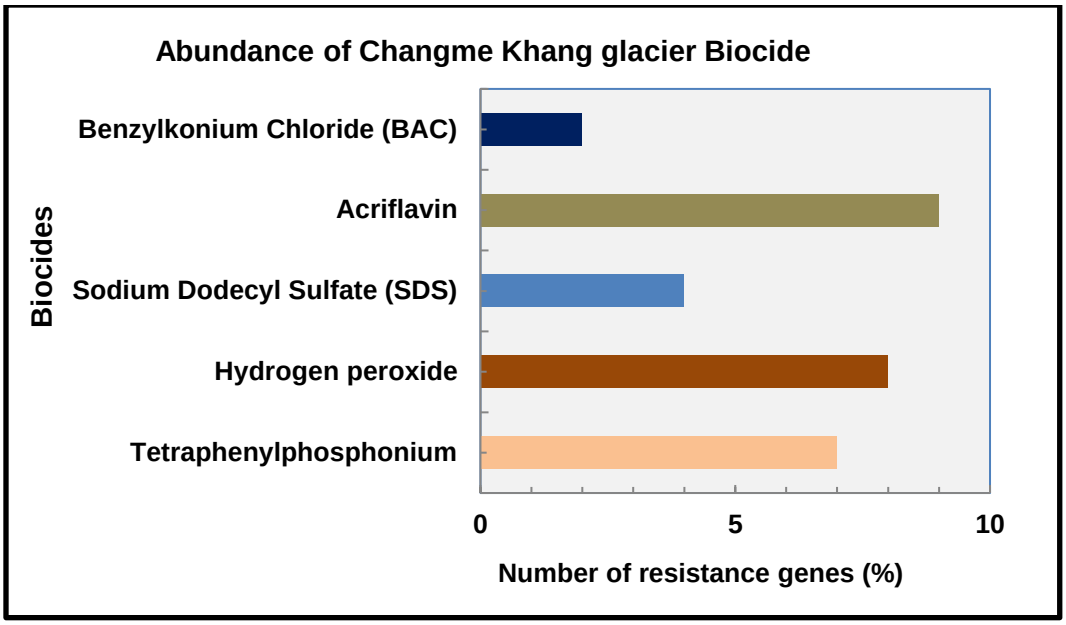
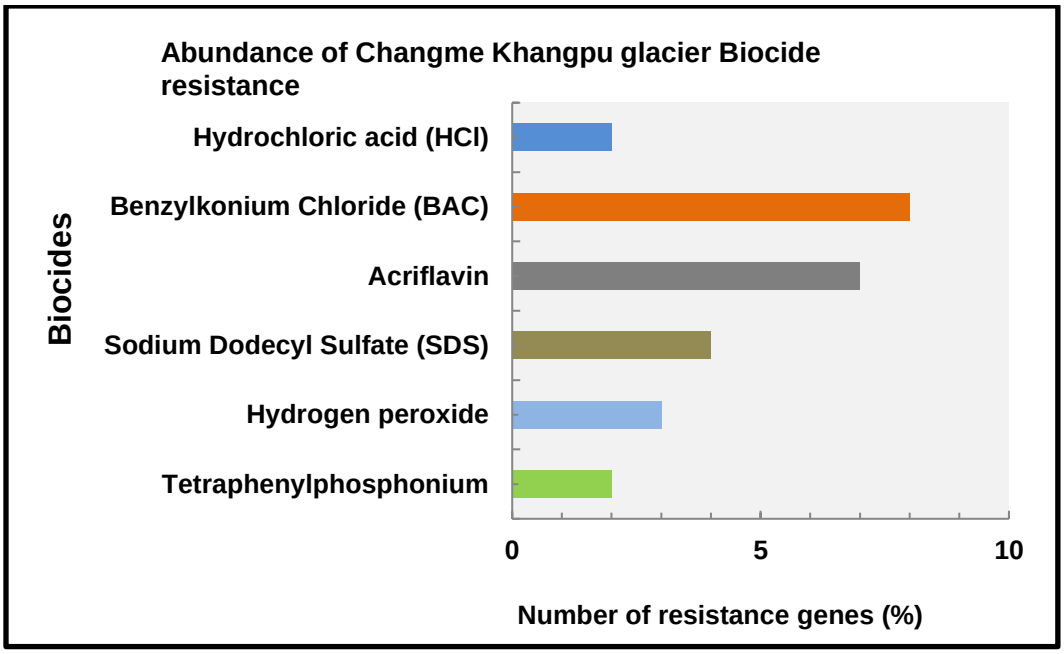


Fig.16b. The abundance of Changme Khangpu glacier Metal Resistance Genes (MRGs)



(a)



(b)

Fig.17. The abundance of (a) Changme Khang and (b) Changme Khangpu glacier Biocide resistance

6.10. Analysis of the metabolic potential encoded by Changme Khang and Changme Khangpu glacier.

6.10.1. Assessment of the functional diversity

To establish the functional diversity present in the Changme Khang and Changme Khangpu glacier, the shotgun metagenome-derived datasets were compared to the COG and KEGG databases. The gene prediction and annotation of the assembled contigs were carried out using PROKKA software and the complete functional annotation, gene function and sequences were carried out. The Gene or protein functions of all contigs from BLASTX output were parsed using practical Extraction and Report Languages (PERL) script. The predicted 4,975 genes of Changme Khang and 5,451 genes for Changme Khangpu glacier were annotated further for KEGG (Kyoto encyclopedia of genes and genomes) analysis based on taxons. Functional annotation of all the contigs is carried out by SEED Classification. MEGAN software was used to assign the function of each contig (**Huson et al. 2007**). The protein function of each contigs having the highest alignment score from BLASTX results was considered for functional assignment and relevant functions. Pathway annotation was done using KEGG pathways analysis performed for each contig sequence by assigning KEGG Orthology (KO) numbers obtained from known reference hits.

The functional subcategory metabolism in COG databases was represented by 24% in Changme Khangpu glacier and 25% in Changme Khang glacier, whereas incase of KEGG databases metabolism was represented by 64% in Changme Khangpu glacier and 76% in Changme Khang glacier sequences, respectively (**Fig.18b, c)**. Genetic Information and processing (KEGG) in Changme Khang showed 3.8% while, Changme Khangpu glacier showed 6.9%. In Changme Khangpu and Changme Khang glacier (KEGG) showed

comparatively similar percentage of cellular processes 9% and 7.2%, respectively. In Changme Khang and Changme Khangpu glacier subcategory such as environmental information processing (KEGG) yielded 6.5% and 12.9% of all classified sequences, respectively (**Fig.18a, b).**

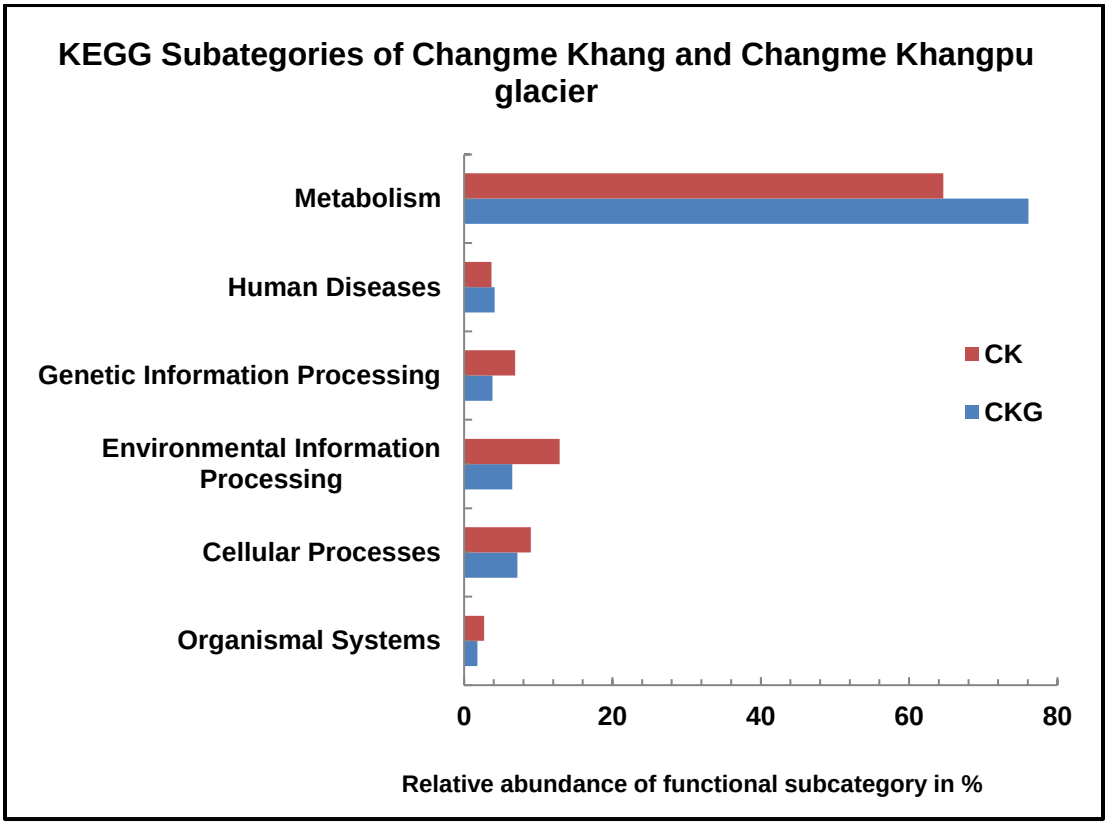


Fig.18a. Comparative graph showing six KEGG Orthologous (KO) categories between Changme Khang and Changme Khangpu glacier. Categories metabolism showed the highest percentage of abundance in both the glacier followed by environmental information processing. Abundance of these genes was determined by KAAS software (KEGG automatic annotation server).

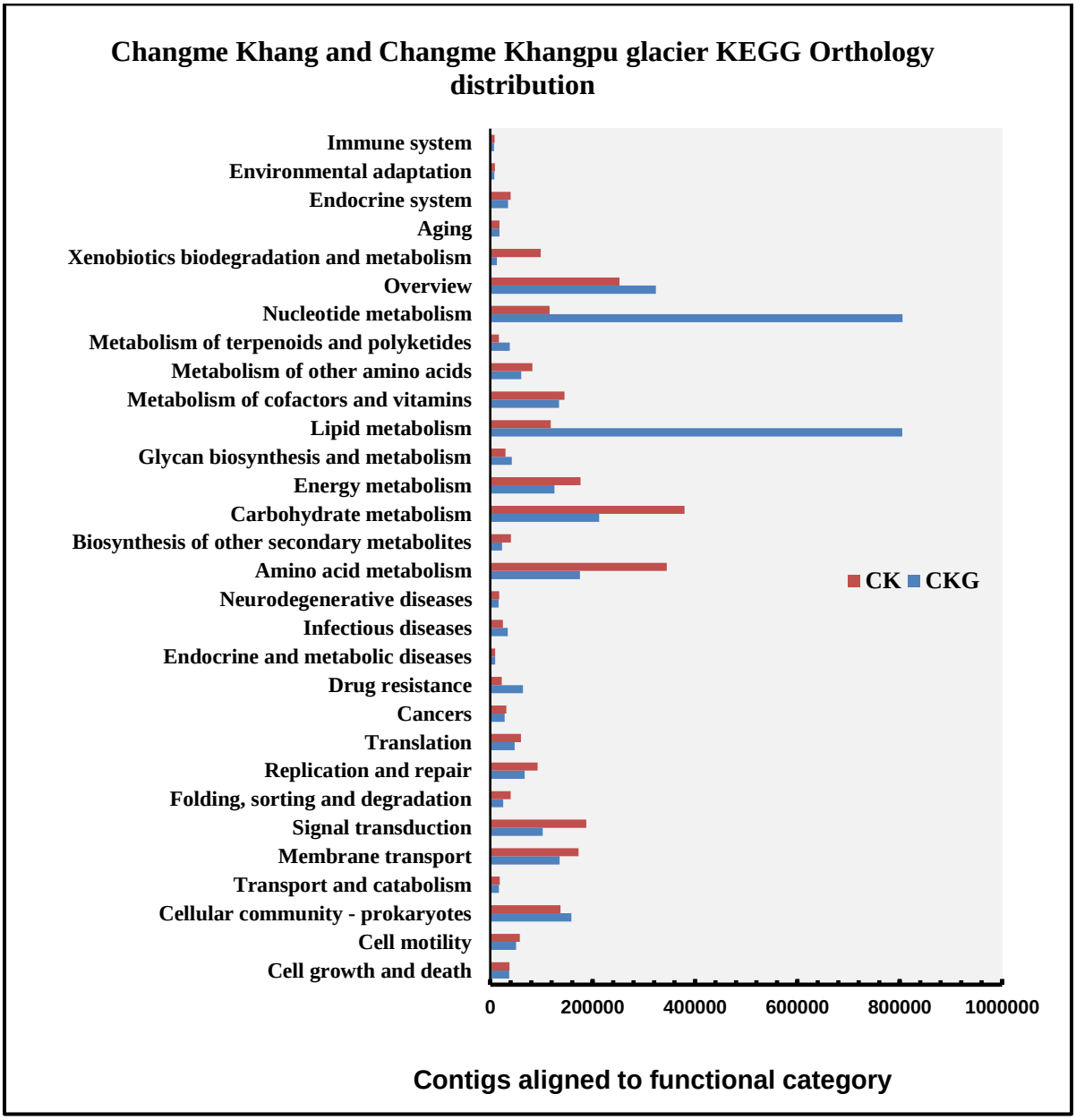


Fig.18b. Functional protein annotation of metagenomic profiles (a) KEGG annotation. Only 30 KEGG families with the most significant difference between Changme Khang and Changme Khangpu glaciers. Differential abundance of functions was determined by KAAS software (KEGG automatic annotation server).

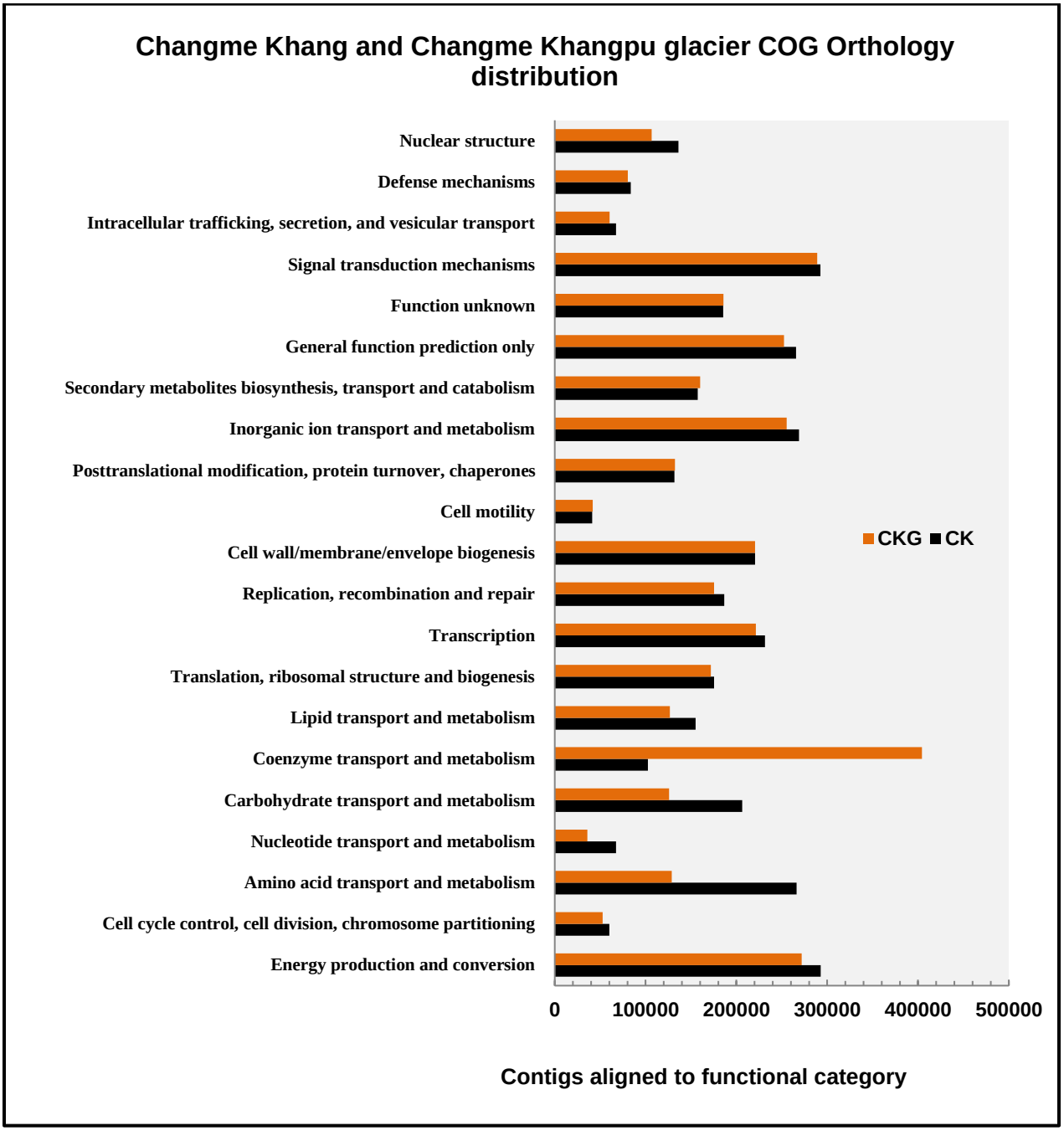


Fig.18c.Functional protein annotation of metagenomic profiles. **(b)**COG annotation. Only 21 COG families with the most considerable different between Changme Khang and Changme Khangpu glaciers. The degree of difference in abundance of functions was determined by KAAS software.

With the KEGG categories, matches were separated into different subcategories. Most of the glacial sequences in the carbohydrate metabolism subcategory shared homologies to known genes involved in glyoxylate (CKG glacier 4,061 sequences and CK glacier 3,562 sequences), butanoate (CKG glacier 1,899 sequences and CK glacier 2,033 sequences) and propanoate (CKG glacier 2,588 sequences and CKG glacier 2,594 sequences). In addition, sequences were homologous to genes responsible for glycolysis/gluconeogenesis (CKG 253 sequences and CK 193 sequences), citrate cycle (CKG glacier were 49,245sequences and CK glacier 3,478 sequences), and pentose-phosphate pathways (CKG glacier 4,675sequences and CK glacier 4,231 sequences), respectively **(Table.13a)**. The predominant of the glacial sequences in the subcategory energy metabolism were associated with genes that participate in oxidative phosphorylation (CKG 2,192 sequences and CK 1,739 sequences). The majority of the sequences in the category energy metabolism were associated to genes that contribute in methane metabolism (CKG 0.3% and CK 0.2%), nitrogen metabolism (CKG 3.1% and CK 3.7%) and sulfur metabolism were (CKG 3.8% and CK 4.4 %), respectively (**Table.13a**). The genes related to photosynthesis were *PsaB*gene(248 sequences in CKG glacier and 292 sequencesin CK glacier) and *PsaA* gene (11 sequences in CKG glacier and 24 sequences in CK glacier). Therefore a low degree of photosynthesis was indicated in both the glacier by shotgun metagenomic sequences. The lower number of genes related to photosynthesis and low abundance of photosynthetic organisms might be correlated to the fact that the ice core is covered by snow most of the year. Numerous genes related to the xenobiotics degradation were detected which indicate the degradative ability of microorganisms present in the glacial surface.

Table.13a. The number of sequences showing homologies to genes associated with KEGG pathways in the categories "carbohydrate metabolism", "energy metabolism" and Xenobiotics biodegradation.

KEGG class ID [ko number]	KEGG category	No. of matches (% relative abundance)	
	Carbohydrate Metabolism	**CKG 382640 reads**	**CK 379401 reads**
10	Glycolysis/Gluconeogenesis [PATHko00010]	253 **(0.06%)**	193 **(0.05%)**
20	Citrate cycle (TCA cycle) [PATHko00020]	14245 **(3.7%)**	3478 **(0.91%)**
30	Pentose phosphate pathways [PATHk00030]	4675 **(1.2%)**	4231 **(1.1%)**
31	Inositol metabolism [PATHko00031]	6922 **(1.8%)**	6403 **(1.6%)**
40	Pentose and glucuronate Interconversions [PATHko00040]	948 **(0.24%)**	1102 **(0.29%)**
52	Galactose metabolism [PATHko00052]	2833 **(0.74%)**	2403 **(0.63%)**
620	Pyruvate metabolism [PATHko00620]	16315 **(4.2%)**	16168 **(4.2%)**
630	Glyoxylate and decrboxylate metabolism [PATHko00630]	4001 **(1%)**	3562 **(0.93%)**
640	Propanoate metabolism [PATHko00640]	2588 **(0.67%)**	2594 **(0.68%)**
	Energy Metabolism	**175576 reads**	**176437 reads**
95	Methane metabolism [PATHko0095]	551 **(0.31%)**	413 **(0.23%)**
710	Nitrogen metabolism [PATHko00710]	5564 **(3.1%)**	6617 **(3.7%)**
920	Sulfur metabolism [PATHko00920]	6723 **(3.8%)**	7825 **(4.4%)**
	Xenobiotics biodegradation and metabolism	**92755 reads**	**98838 reads**
362	Benzoate degradation [PATHko00362]	1566 **(1.6%)**	1712 **(1.7%)**
622	Xylene degradation [PATHko00622]	3541 **(3.8%)**	4104 **(4.1%)**
365	Furfural degradation [PATHko00365]	3451 **(3.7%)**	1712 **(1.7%)**
984	Steroid degradation [PATHko00984]	1311 **(1.4)**	1234 **(1.2%)**

The values were determined by comparison of sequences derived from shotgun metagenome of the CK and CKG glacial DNA to the KEGG database

6.10.2. Functions linked to a psychrophilic lifestyle and to survive at low organic carbon

Glacial ice is considered a low nutrient environment. Most of the organic carbon present in glacial ice originated from airborne material and atmospheric deposition on the glacial

surface (**Stibal et. al., 2008**) Additional source of nutrients in the glacial surface, which is generated by glacial avalanches and erosion (**Stibal et al.,2008**). Abundant genes related to xenobiotic degradation and other carbon sources signified a high degradative capacity of the microorganisms present in glacial ice. This degradative activity of microorganisms in such habitat might be the result of survival in a habitat with low concentrations of different organic carbon sources. The significant number of carbon fixation genes involved in the Calvin cycle was detected in both the glacial ice which indicates that the autotrophic lifestyle in glacial habitat **(Table.13a)**.

To maintain the membrane fluidity the incorporation of unsaturated fatty acid into the membrane is vital characteristics of the bacteria which survive at low temperature. A large number of desaturases genes that are essential for the conversion of saturated to unsaturated fatty acids were detected in both the glacial ice **(Table.13b)**. The maintenance of protein folding in glacial habitat was demonstrated by the presence of genes encoding peptidyl-prolyl *cis*-trans isomerases.

At low-temperature solubility of gasses increases rapidly including oxygen. The ability to respond to reactive oxygen species is a crucial function for organisms thriving at low temperatures. It has been reported that the exposure to low temperature is connected with the enhancement of oxidative stress. Therefore, it was not surprising that the genes related to antioxidative enzymes such as dioxygenase and superoxidase dismutase were present in both the glacier samples which might be helping these microorganisms to prevent oxidative stress **(Table.13b)**. The averting of cell damage by the formation of ice crystals is essential for living at low temperatures. Similarly, many genes involved in the

synthesis of osmoprotectants and cryo-protectant genes were detected such as glycine, betaine, choline, and glutamate respectively **(Table.13b)**.

Table. 13b. Enzymes, amino acids, and compounds associated with a psychrophilic lifestyle and their functions detected in Changme Khang and Changme Khangpu glacier.

Keyword			**Number of match sequence reads**
	CK	**CKG**	**Selected functions and their reference**
Choline	**1025**	**1245**	The precursor of glycine betaine (**Methe et al., 2005**)
Sarcosine	**227**	**367**	Intermediate in the metabolism of choline to glycine (**Methe et al., 2005**)
Desaturase	**508**	**216**	Catalyzes the synthesis of unsaturated fatty acids (**Bolter, 2004**)
Peptidyl-prolyl*cis-trans* isomerase	**2137**	**1542**	Maintains protein-folding rates at low temperatures (**D'Amico et al., 2006**)
Glycine	**1253**	**1456**	Osmoprotectant (**Ludwig et al., 2004**)
Betaine	**2024**	**1213**	Osmoprotectant and cryoprotectant (**Methe et al., 2005**)
Glutamate	**2534**	**1827**	Osmoprotectant (**Medique et al., 2005**)
Dioxygenase	**2181**	**1598**	Antioxidative enzyme (**Medique et al., 2005**)
Superoxide dismutase	**1460**	**1163**	Antioxidative enzyme (**Methe et al., 2005**)

7. DISCUSSION

The cryosphere is the place in Earth‘s where the water is in the solid formincluding sea ice, alpine lake, glaciers, ice caps, and frozen ground. These environments contain the major reservoir of fresh-water throughout the world.According to the intergovernmental panel on climate change **(Barnett et al. 2005)** report, more than one-sixth of the world’s population directly or indirectly depends on glacier meltwater or snowmelt water. In Asia, the Hindu-Kush Himalaya region (HKH) and Tibetan Plateau are generally considered as the third pole of the Earth. These regions are the vital pool of global freshwater resources and provide fuel to around 150 million populations and also used for their agriculture, industry, and drinking purposes **(Singh and Kumar, 1997; Singh and Jain, 2002; Singh and Bengtsson, 2004).** The five main rivers which originated from the HKH region are the Indus, the Ganga, the Brahmaputra, the Mekong and the Yangtze. Despite having such major water resources, scarcity of water during dry seasons is a major dilemma for the rapidly growing population in these mountains region. Cryospheres present on Earth are very sensitive to global warming, especially those which are present in the polar as well as non-polar regions. According to **IPCC (2007)**, if temperature persistently raises then it is expected that some polar and non-polar glaciers will be depleted within a few decades and some within this century. The depletion of these glaciers and increase in temperature can result in threatening the livelihood of many organisms which play important ecological roles in the food chains of these cold environments. However microorganism that also performs critical ecological roles may respond to these climate changes differently and therefore requires attention **(Kirchman et al. 2009)**.

Psychrophiles and their enzymes have illustrated much interest because of their broad range of biotechnological applications and also to understand their biochemical mechanisms of adaptation to extremes low-temperature, pH and salinity. These psychrophiles and their enzymes are being employed in various bio-processes which are carried out at low-temperature. In addition, they are also utilized for natural decomposition of organic matter and nutrient recycling at low-temperature habitats **(Ramana et al. 2002)**. Most of the planets and its satellites of our solar system come under cold to very cold conditions and therefore from an astrobiological point of views studying the freezing environments or cryospheres present on Earth can offer important clues about the life that may present elsewhere in our solar system, such as on Mars and Europa (Jupiter's moon).

The main importance of these cryospheres including glaciers is the microbiota present in them. The microorganisms thriving in such glaciers can withstand extremely low temperatures. Depending upon their optimum growth temperature cold dwelling microbes were defined as psychrophiles or psychrotolerant. Glaciers are considered as large repositories of microbial life. Around 25% of the land surface on the earth is classified as cold environments **(Choudhari et al. 2014)**, and biological activity in these low-temperature habitats is generally thought to be restricted. Glaciers are simple and fairly closed-ecosystem which are inhabited by primary producer such as photosynthetic algae and bacteria **(Choudhari, 2015)**. However, due to the uncultivated status of the major taxa in the glaciers, culture-dependent technique led to recognized only a few distinct genera from the glaciers. The biodiversity of several cold habitats such Cryoconite hole

from Rotomoosferner glacier Austria **(Edward et al. 2013)**, Arctic soil **(Seok et al. 2016)**, Untersee lake, Antarctic and Matanuska Glacier, Alaska **(Koo et al. 2018)**, Kuytun 51 glacier, Kyrgyzstan **(Xiang et al. 2009)** were studied using shotgun metagenomic sequencing. These new molecular techniques have enhanced the investigation of diverse environments in great detail. The microbial diversity of Himalayan glaciers has been less investigated compared to other cold habitats around the world.The Himalayan glaciers might harbor microbial communities markedly different from those colonizing glaciers of Polar Regions, and thus studying their Microbiology is of extreme importance for several reasons; first, glacial ecosystems are considered as massive repositories of a virtually unexplored genomic diversity **(Edwards, 2015),** second, this largely unexplored biological diversity faces a real risk of extinction owing to the loss of its harboring ecosystem **(Griffiths, 2012),** third, rapid meltdown of these glaciers might contribute to the reactivation and release of human, animal, and plant pathogens that have remained contained in glacial ice for centuries and even thousands of years **(Rogers et al. 2004)**. Thus the aim of the present study was to explore the microbial diversity mainly bacteria present in the two glaciers of North Sikkim (Changme Khang and Changme Khangpu) to correlate these with the physiochemical parameters and to check enzymatic activity present in the microbes of such glacial environments.

There are 84 glacier of Sikkim the most important of which are Zemu glacier, Rathong glacier, Zumthul Phuk glacier, Onglathang glacier, Tasha Khang glacier, Lhonak glacier, Hidden glacier, Talung glacier, Tista Khangse glacier, North Lhonak glacier, South Lhonak glacier, Changme Khang glacier and Changme Khangpu glacier **Bahuguna et al.**

(2001). The water from these glaciers discharges into two major rivers of Sikkim, the Tista and the Rangit which are considered as the lifeline of Sikkim, Bengal and even Bangladesh also. The river Tista originates from Tista Khangse glacier in the northeast part of North Sikkim and similarly, the river Rangit origin from Rathong glacier in West Sikkim, both the river system take its course towards southern direction. The major tributaries of river Tista consist of Lachung Chu, Bakcha chu, Zemu Chu, Rangpu chu, Rangyoung chu, Dikchu chu, and many other small tributaries also contribute to river Tista. Similarly, major tributaries of river Rangit are Rathong Chu, Raammang Khola, and Kalej Khola.

The abiotic and biotic matters determine the suitability of glacier water for human use. Therefore several physical and chemical parameters of water from the glacier were checked. The physicochemical analysis of two glaciers Changme Khang (Debris-free glacier) and Changme Khangpu (Debris-covered glacier) glacier were checked. The physicochemical analysis of two glaciers suggested that besides being two different types of glaciers, i.e., debris covered and debris free glacier and closely located, these glaciers possess similar elemental concentrations. However, Changme Khang glacier had few physical and chemical parameters vary such as nitrate, total dissolved solids (TDS), biological oxygen demand (BOD) and chemical oxygen demand (COD) whereas in case of Changme Khangpu glacier magnesium were slightly higher than Changme Khang glacier. As per the American Public Health Association (APHA), the elements present in these glaciers are under permissible limits. The piper analysis shows the nature of glaciers and it has been predicted that both the glaciers are calcium bicarbonate thus it may be concluded that the calcium carbonate weathering regions. The total dissolved

solids (TDS) were highest in Changme Khangpu glacier as compare to other three glaciers and the results are not surprising as in debris-covered glacier fine sediments are mostly present in accumulation zone where the glacier get accumulated **(Lewis et al., 2003)**. These might be the reasons for elevated TDS in case of Changme Khangpu glacier.

Isolation of total 136 isolates was carried out and among them, 47 were selected for further analysis on the basis of morphology and biochemical characterization. On the basis of morphology, it was suggested that most of the isolates were Gram-positive, aerobic rod-shaped non-spore forming bacteria. The carbohydrate fermentation result demonstrates that the majority of the isolates were able to ferment simple sugars such as galactose, glucose, fructose, and dextrose but some of the isolates can also ferment complex sugar such as ribose, maltose, arabinose, and xylose etc. These results suggested that these isolates might belong to *Bacillus* and *Pseudomonas* groups. The additional biochemical attributes such as catalase, oxidase, nitrate reductase, amylase, lipase, and protease also add to the tentative identification of group *Bacillus* and *Pseudomonas.*

The growth profile of the bacterial isolates at various ranges of temperature, pH, and NaCl was also checked. The optimum growth temperature of the majority of the isolates was 20ºC, which indicate the psychrotolerant nature of these isolates. The optimal pH range of the isolates was found to be 6-8, thus proposed the neutrophilic or facultative alkalophilic character of the bacterial isolates. However few isolates like KGG25, KGG35, KGG44, KGG51 and KGG53 showed optimum growth at pH4, which suggest that these isolates are acidophilic in nature. The optimum NaCl concentration of <2%

suggested that the majority of isolates requires normal saline conditions. The pH of the glaciers was also in the range of 7-8 thus proposed the neutral to mild alkaline nature of glaciers. Alkaline nature of ice was also reported from Antarctic regions **(Sharma and Kumar, 2017; Ernest et al. 1964)**.

The biochemical tests and 16S rRNA sequencing results suggested that the three closely located glaciers, i.e., Changme Khangpu, Changme Khang and Chumbu glacier are dominated by *Bacillus* species, whereas as Kanchengayao glacier is mainly dominated by the genus *Pseudomonas*. The genus *Pseudomonas* was also isolated from Pico Bolivar's glacier, Venezuela **(Rondon et al. 2016)**. Similarly, genus *Paenibacillus, Staphylococcus, Bacillus, Stenotrophomonas, Paracoccus,* and *Brevundimonas* were isolated from East Rongbuk ice core Mt. Everest by **Shen et al. 2012** and from Dronning Maud, Antarctic ice core by **Antony et al. 2012**. **Shivaji et al. 1989** while working on Schumacher oasis, Antarctic, have shown that their isolated mainly dominated by *Pseudomonas* sp. which can grow between 4-30ºC, optimum at 20ºC andin pH range of (6-9) and NaCl tolerate up to 5.8%. Similarly, **Yao et al. 2006** studies on Malan ice core, Tibetan plateau suggest that their isolates are dominated by *Pseudomonas* sp. and *Bacillus* sp., which can grow between 2-40ºC. In accordance with above mentioned studies, our isolates also grow in broad range of temperature (5-40ºC), pH range of 4-9 and NaCl between 1-5%. In 2017 Rafi and his coworkers reported that bacteria both Gram-positive and Gram negative bacteria isolated from Siachen glacier, Pakistan were showed higher concentration of NaCl tolerance. Interestingly, from our study Gram negative bacteria from one of the studied glacier (Kanchengayao glacier) showed low

level of NaCl concentration, whereas from other two glaciers (Changme Khang and Changme Kangpu) Gram-positive bacteria showed variable preference for NaCl concentrated. The higher level of NaCl tolerance in glacial bacteria was correlated in the fact that in glacier water nuclei are formed mass and solutes from the surroundings diffuse towards these water nuclei making it hypertonic solution and make them higher level of NaCl tolerance was earlier reported by **(Clarke et. al. 2013)**.

Beside their optimal NaCl, temperature and pH, the isolates were capable to endure in a broad range of these environmental factors. The possible reasons might be assumed that this varied range of NaCl, pH, and temperature tolerance shown by these isolates may be due to melting, re-freezing, evaporation, and sublimation of ice in non-polar and mid-latitude glacier **(Nijampurkar et al. 1993)**. The physicochemical parameters may have the trend of getting change due to variations in dust and aerosol deposition on the glacier surface. The microbes present on the surface of the glacier may interact with fluctuation surrounding environments with sifting physiochemical parameters at different ice core depth. Thus these fluctuation situations concerning particular physiochemical parameters aid these microbes with becoming tolerant and flexible in the evolving state.It has been found that Changme Khang and Changme Khangpu glacier which is closely located glacier possess similar kinds of microbial diversity. The major determinedphylum was *Firmicutes* and few isolates showed *Proteobacteria* and *Actinobacteria*. Among phylum *Firmicutes*, many isolates belong to *Bacillus* species and few were *Staphylococcus*. Similarly, from phylum *Proteobacteria* dominant genus was*Paracoccus,Sphingomonas,* and *Enterobacter*. Likewise, from phylum *Actinobacteria*, three dominant phyla were

identified such as *Neomicrococcus, Pseudoclavibacter,* and *Brevibacterium* etc. The above results confirmed the dominance of genus *Bacillus* in Changme Khang and Changme Khangpu glacier. We compared the bacteria diversity from one closely located glacier and another distantly located glacier to check whether these glaciers also harbor similar kinds of diversity on the basis of culture-dependent methods or not. Thus we selected one closely located Chumbu glacier and another distantly located Kanchengayao glacier. Interestingly the result from these two glaciers shows that from Chumbu glacier *Bacillus* species were dominated whereas from Kanchengayao glacier predominance was *Pseudomonas s*pecies. Number studies from diverse parts of the World also suggested that the Gram-negative bacteria with predominance of γ-proteobacteria, α-proteobacteria and β-proteobacteria were more prevalent in glacial surface than Gram-positive bacteria **(Tamura et al. 2013; Mannisto and Haggblom, 2006; Musilova et al. 2015; Rafiq et al. 2017)**.

The present result concluded that in closely situated glaciers (Changme Khang, Changme Khangpu and Chumbu glacier) bacterial diversity was comparatively similar compared to that of the distantly located glacier (Kanchengayao glacier). These three glaciers are located in the adjoining area (Changme Khang, Changme Khangpu and Chumbu glacier), only a few miles apart and almost similar altitude with comparable chemical constituents. Consequently having related geological and geographical attributes; this might be the reasons for having similar bacterial diversity in the three glaciers.Another reason might be due to divergence in Indian monsoon air masses as well as due to regional or local mineral or aerosol deposition patterns in glaciers **(Zhang et al. 2007)**. Also globally it

has seen that the cultural bacterial diversity phylum *Proteobacteria*, *Firmicutes* and *Actinobacteria* were most versatile in the glacier ecosystem such as East Rongbuk glacier Mt. Everest **(Shen et al. 2012)**, East Rongbuk glacier, Mt. Qomolangma **(Zhang et al. 2010)**, Pico Bolvar's glacial and subglacial environments, Venezuela tropical Andes **(Rondon et al. 2016)**, Pindari glacier, India **(Shivaji et al. 2011)**, Mizuho, East Antarctic ice core **(Segawa et al. 2017)**. Other reasons for their dominance in glaciers might be due to the production of antifreeze protein and spores in Gram-positive bacteria **(Munoz et al. 2017; Singh et al. 2013; Zhang et al. 2016)**. But, to the best of our knowledge, this is the first report of bacterial diversity from glacier accumulation zone ice from Eastern Himalaya. Except for accumulation zone there are several other parts of glacier was studied by many researcher through the world such as glacier foreland **(Schuette et al. 2010; Wu et al. 2012)** and ablation zone of glacier **(Grzesiak et al. 2015, Zhang et al. 2015)**, cryoconite **(Franzetti et al. 2017; Cameron et al. 2012; Zdanowski et al. 2016; Edwards et al. 2013)** and Moraine Lake **(Yao et al. 2006; Liu et al. 2011)**. Possible reasons regarding less research in glacier accumulation zone may be due to difficult topography in Himalayan glaciers or might be due to high expenditure or less mountaineering expertise among researchers.

Survival of microbes in the cold habitats needs optimization of certain basic cellular processes which comprise enzymes modulation at structural and biochemical level, nutrient transport and membrane utility **(Rivkina et al. 2000)**. The diversity of both culturable and non-culturable microbes living under the extreme cold-habitats was enormous and these organisms can shape important gene pools for biotechnological

applications **(Sahay et al. 2013)**. In the current study, we got several psychrotolerant bacteria which are able to produce cold-active protease and amylase enzyme. The highest protease enzyme producing bacteria was CKG2 ($2.16UmL^{-1}min^{-1}$) which was isolated from Changme Khang glacier and identified as *Bacillus thuringensis* CKG2.Similarly, from Changme Khangpu glacier bacteria identified as*Bacillus safensis* CK3 showed the highest amylase activity ($1.07UmL^{-1}min^{-1}$). Cold-active proteases have also been reported from diverse cold habitats belonging to various genera such as *Bacillus, Pseudomonas, Serratia, Stenotrophomonas, Vibrio, Alcaligenes* and *Azospirillum***(Kaur et al. 2001; Kobayashi et al. 2007; Patil et al. 2011; Tariq et al. 2011)**. However, the majority of cold-active proteases were reported from *Pseudomonas* and *Bacillus*genera.

Although the culture-dependent method showed the dominance of *Pseudomonas* and *Bacillus* groups, but among them, plenty of species diversity was detected from these glaciers and also few isolates have found to be <98% at 16S rRNA sequence identity which suggests the novelty of these isolated bacteria. However, to get the complete bacterial diversity of an environment, culture-dependent approach is not sufficient. Therefore, it was important to check the left out phyla in these glaciers, using culture-independent methods such as Phospholipids Fatty acids Analysis (PLFAs) and metagenomic. The conventional culture-dependent approach fails to give a comprehensive microbial diversity profile of an environment due to the large number uncultivable status of the microbial world. The development of a broad array of culture-independent techniques has widened the scientific understanding of previously unknown microorganisms and their contributions in different biological processes. The culture-

independent methods such as phospholipids fatty acids analysis (PLFAs) was first used to estimate the microbial biomass from marine sediments by **White et al. 1979**. This methodis essential to establish viable microbial biomass, microbial community composition and metabolic activity in an environment **(Green and Scow, 2013)**. The phospholipid fatty acids are an essential structural component of microbial cell membrane and each group of bacteria acquires some signature fatty acids, which makes it the important chemotaxonomic marker. Till date, PLFA analysis is widely used to estimate total viable biomass and to monitor the changes in community composition of the microorganisms in soils and aqueous environments **(White et al. 1983)**. Interestingly from phospholipids fatty acids analysis (PLFAs) of Changme Khang and Changme Khangpu glacier Gram-positive bacterial signature fatty acids were in abundance. Our PLFAs results are correlated with culture-dependent methods as these two glaciers showed the dominance by Gram-positive bacteria. PLFA analysis are based on live microbiota present in the sample of any ecosystem, as the phospholipids are unstable and decompose rapidly after cell death **(Lanekoff and Karlsson, 2010)**. Therefore it is not surprising that our culture-dependent and PLFAs results are correlated with each other.

Astonishingly, the metagenomic analysis of Changme Khang and Changme Khangpu glacier suggested the dominance of Gram-negative bacteria followed by high-G+C Gram-positive bacteria and low-G+C Gram-positive bacteria. These glaciers were closely located with each other; the bacterial community shows little variation between the two glaciers. The phylum wise diversity showed the dominance of *Proteobacteria* (81.2%) followed by *Firmicutes* (7.1%), *Unidentified virus* (0.5%), *Actinobacteria* (3.1%), and

Ascomycota (0.7%) in Changme Khangpu glacier, similarly Changme Khang glacier also dominated by phylum *Proteobacteria* (75.5%), *Actinobacteria* (7.2%), *Unidentified virus* (0.7%), and *Firmicutes* (5.1%). However, the result showed that at phylum *Ascomycota, Tenericutes,* and *Unidentified virus*were detected in very small percentage (<1%) of all classified sequences in both the glacial samples. The dominant phylogenetic group established by metagenomic approach was the phylum *Proteobacteria.* Most proteobacterial sequences were assigned to the class *Betaproteobacteria* (Changme Khangpu glacier 66.22% and Changme Khang glacier 52.32% of all the classified sequences). This corresponded to other studies of glacier ice **(Foght et al., 2004**), subglacial habitats (**Cheng et al., 2007**), and mountain snow (**Segawa et al., 2005**). Phylum *Actinobacteria* appear to be second abundant phyla but their representation was also less than 1% of all classified sequences. It has been shown that *Actinobacteria* have a higher ability to survive in a cold environment than low-G+C Gram-positive bacteria and Gram-negative bacteria **(Willerslev et al. 2004b)**. This is probably caused by the ability of *Actinobacteria* to developed resting forms with low metabolic activity. Thus *Proteobacteria, Actinobacteria*, and *Firmicutes* represented the predominant phyla in accumulation zone glacier ice from Changme Khang and Changme Khangpu. These groups were also predominant in ice derived from several other glaciers **(Miteva et al. 2004, Willerslev et al. 2004b; Turchetti et al. 2008)** and other permanently cold habitats, such as sub-glacial melt-water **(Cheng and Foght, 2007; Foght et al. 2004)**, snow **(Segawa et al. 2005)**, Antarctic lakes **(Mosier et al.2006)**and Cryoconite hole **(Edwards et al. 2013)**. These results support the insight that interrelated phenotypes

persist in geography diverse cold habitats because of analogous strategies for survival and enduring activity at low temperature **(Priscu and Christner, 2004)**.

The functional analysis of the shotgun metagenome analysis provides the functional and metabolic repositories of the microbial community members dwelling the glacial ice of Changme Khang and Changme Khangpu glacier. The sequences were compared to KEGG and COG orthology, and a broad metabolic diversity was estimated. The high flexibility of carbohydrate metabolism was recognized, including a large degradative capacity and the ability to assimilate inorganic and organic compounds. In our results, we also found a lot of genes involved in the carbon and nitrogen cycle, methane generation and oxidation and organic matter degradation that might have an imperative role in climate change or glacier degradation.

Until now not many studies have been conducted about the metabolic functions of microbial communities in alpine glacial environments. Previously, it was proposed that most of the organic material derives from production sites somewhere else from atmospheric deposition on the glacier surface and maybe allochthonous **(Stibal et al. (2008)**. The existences of inorganic and organic forms of sulfur, nitrogen, and phosphorus have been reported by many researchers in the past from cold environments **(Bottrell and Tranter, 2002; Hodson et al. 2014)**. The functional metagenomic results obtained from Changme Khang and Changme Khangpu glacier indicates that the genes for dissimilatory nitrate reduction namely nitrate to ammonia, alanine, aspartate, and glutamate metabolism were prevalent in both the glacier along with genes responsible for glycolysis/gluconeogenesis, citrate cycle and pentose-phosphate pathways. In the energy

metabolism category, many reads encoding genes for nitrogen, methane, and sulfur metabolism were detected which suggested that the microbes present in these glaciers ice can also exhibit anaerobic respiration. These bacteria use methane, nitrogen, and sulfur as electron acceptors and such type of metabolism are referred to as lithotrophic metabolism. Based on the above metabolic pathways it can be concluded that the microbial communities present in the glacial ice are aerobic/facultative aerobes along with few anaerobes. The above metagenomic results were also supported by culture-dependent microbial diversity studies as most of the bacteria detected were aerobic (*Lysinibacillus sphaericus, Paracoccus marcusii, Bacillus thuringiensis, Lysinibacillus mangiferahumi,* and*Neomicrococcus lactis*) **(Prakash et al. 2015; Sanchis et al. 2007; Harker et al. 1998)** and facultative aerobes (*Sphingomonas sp.* PDD, *Paenibacillus populi* and *Enterobacter cloacae*) **(Hardoim et al. 2013; Han et al. 2005)**.

In addition to the above metabolism, several sequences read showing high similarities with an enzyme involved in the conversion of fatty acids to unsaturated fatty acids were also detected. These fatty acids are required by the microorganisms at the low temperature to maintain their membrane fluidity **(Bolter, 2004)**. Several metabolic pathways were detected by functional metagenomic analyses which were responsible for the formation of cryoprotectants. These cryoprotectants are essential to maintain a psychrophilic condition in cold-induced accretion of proteins and also to maintain membrane flexibility at low temperature **(Methe et al. 2005).** Such low-temperature adaption strategies of microbes awaken interest among scientific fraternity that can not only divulge the life at sub-zero condition but also extraterrestrial environments. Such

studies advocate the knowledge which is expected to be useful in the future for controlling pathogenic microbes, which endure and flourish in cold-stored food and feeds materials **(Chattopadhyay, 2006)**. The significance of psychrophilic lifestyle involves the modification of enzyme functionality, stability and their cell membrane fluidity **(Bowman, 2008)**. Our present metagenomic data also divulge essential information of genes encoding processes for low-temperature adaption of microbes and information of their functionality.

One of the most important aspects of a functional metagenomic approach is the clarification of antibiotic and metal resistance-related genes and its functions. An antibiotic-resistant bacterium which is threatening the human race has become a great concern and a large number of researches have been carried out to curb from this menace throughout the world**(Roca et al.2015).** Metagenomics analysis of resistance gene has played a potent role and has become a promising culture-independent method of resolving ARGs and MRGs diversity and abundance from various environments. From the several environments, ARGs and MRGs gene were detected by many researchers such as Estuary and deep-sea sediment **(Chen et al. 2013)**, drinking water **(Fernando et al. 2016)**, glacier environments **(Segawa et al. 2013)**.

In the present study, we also aim to check the antibiotic and metal resistance, ARG and MRG genes pattern in two closely located glaciers of Sikkim. The study was carried out using culture-dependent and independent methods. We checked the antibiotic resistance profile of six antibiotics by disc diffusion method such as ampicillin (30mcg),

erythromycin (15mcg), chloramphenicol (30mcg), and streptomycin (100mcg), methicillin (10mcg), vancomycin (30mcg) and interestingly found that some of the isolates were resistant against major tested antibiotics. Further, the minimum inhibitory concentrations of these antibiotics against our isolates from Changme Khang and Changme Khangpu glaciers were also checked. In the case of chloramphenicol and erythromycin, the MIC values were $8\mu gL^{-1}$ respectively. The least MIC of $4\mu gL^{-1}$was showed by ampicillin and streptomycin. No globally accepted guidelines are available for psychrophilic/psychrotolerant bacteriato test the susceptibility testing or for breakpoints for susceptible or resistant isolates.However, psychrotolerant bacteria such as *Pseudomonas* and *Enterobacter* species and their breakpoint as perCLSI, 27thedition data as well as two *Bacillus species*were investigated and their breakpoints have been established**(Rosenquist et al. 2005)**.On the basis of these guidelines, we found that the majority of our isolates were resistant against various tested antibiotics and also their MIC values were high. **Rafiq et al. 2017** studied on Siachen glacier were shown that their isolates were resistant to many antibiotics, e.g., 64% isolates were resistant to vancomycin and 58% against methicillin. From our studied glacier we have detected higher number of isolates was showed resistances against ampicillin (73%) followed by methicillin (68%) antibiotics but less number of isolates were detected resistance against antibiotics such as vancomycin (9%), erythromycin (13.6%), streptomycin (18%), and chloramphenicol (1.6%) respectively. Our result such as high number of isolates against methicillin antibiotic was correlate with **Rafiq et al. (2017)** studied on Siachen glacier; however other classes of antibiotics are showing relatively less resistant. The majority of the studies from pristine environments like ancient Siberian permafrost, alpine glacier

cryoconite, and alpine soil suggested that the bacterial isolates from these cold environments were generally sensitive to antibiotics **(Gupta et al. 2015; Zhanget al. 2010; Berge and Gilles, 2005).** According to Segawa et al. (2013) the distribution of antibiotic genes resistance in geography remote locality might be due to transmission through airborne bacteria and migratory birds **(Segawa et al., 2013)**.

Metagenomic study discloses the presence of various antibiotic resistance genes belonging to quinolone, bacitracin, tetracycline, beta-lactam, aminoglycoside. These genes also showed the maximum identity with Gram-negative bacteria and psychrotolerant bacteria. For example, the gene related to beta-lactam was showing identity with *Serratia marcescens* strain SR50 and tetracycline was showing identity with *Bifidobacterium bifidum* strain NCIMB 4117. Similarly, all the other predicted genes showed in functional metagenomic results were having closest similarity with psychrotolerant Gram-negative bacteria and many similar types of bacteria were also detected through metagenomic analysis **Segawa et al. 2013** studied glacier samples from different part of the glaciers Word and detected diverse antibiotic resistance genes such as quinoline resistance genes were detected from Yala glacier, Nepal, Qiyi glacier, China, and Gulkund glacier, Alaska. Similarly, beta-lactam resistance genes were recovered from Yala glacier, Nepal, Qiyi glacier, China, Takeyama glacier, Japan and genes related to tetracycline resistance were also reported from Yala glacier, Nepal, Qiyi glacier, China, and Takeyama glacier, Japan.

Thus it can be concluded that the resistance gene detected through metagenomics may be the biologically transmitted from microorganism to microorganism in particular micro-environments where dense microbial communities are often exposed to an intensive use of antibiotics **(Segawa et al. 2013)**. Many researchers reported that heavy metal resistance genes (MRGs) as a close link to antibiotic resistance phenomenon and heavy metal resistant phenomenon **(Pal, 2017)**. Metals such as copper and zinc are ample in ecology and their resources in environmental samples are innumerable. As early days these heavy metals have been directly correlated and connected to the increase of heavy metal tolerance in environmental microflora **(Seiler and Berendonk, 2012; Wales and Davies, 2015)**. In ecological samples, copper and zinc have always been found along with the related presence of antibiotic-resistant microbial communities **(Holman and Chenier, 2015)**. According to **Hu et al. (2016)** the chances of transmitting mobile genetic elements (MGEs) increases with increase in the concentration of copper.

There are several reports stated that metals and biocide promote antibiotic resistance via co-resistance such as resistance gene to copper (*tcrB*) linked to resistance genes to erythromycin, vancomycin, and tetracycline was reported by **Amachawadi et al. 2011; Hasman and Aarestrup, 2002**.The genes *oqx*AB encode an efflux pump, conferring resistance to triclosan, acriflavine, chlorhexidine, and fluoroquinolones, were shown to be co-located with beta-lactamases (*bla*CTX-M), the copper (*pco)* and silver (*sil)* operons on the same plasmid, therefore showed a potential for co-selection **(Fang et al. 2016)**. The MRGs (Metal Resistance Genes) were closely correlated between the antibiotic-resistant

phenomena and heavy metal resistant phenomena by many researchers. Thus this led us to hunt for heavy metal resistance genes (MRGs) in a glacial environment.

From the ancient period, heavy metals have been directly correlated to the increase of heavy metal tolerance in environmental microbiotas **(Seiler and Berendonk, 2012)**. The Copper and Zinc have always been detected with the parallel presence of antibiotic-resistant microbial communities in the environmental samples **(Peltier et al. 2010; Becerra-Castro et al. 2015)**. The chances of transfer of mobile genetic elements increase with an increase in the concentration of copper,acquiring the ability of copper-dependent antibiotic resistant genes (ARGs) as readily accessible **(Dupont et al. 2011)**. Antibiotic also form strong metallo-ligand coordination complexes with metal ions and their ionic interaction can illustrate broad results **(Niebergall et al. 1966).** The inactivation of penicillin **(Cressman et al. 1966)** by zinc was first reported by Eisner by promoting hydrolysis of β-lactam **(Eisner and Porzecanski, 1946)**. Thus, we primarily screened the metal tolerance if present among our bacterial isolates from the glaciers of study. Heavy metal tolerant psychrophilic/ psychrotolerant bacteria species was also obtained in an order to get some resistant bacteria which can be exploited in bioremediation in cold-regions and also to check co-occurrence of heavy metal resistance to antibiotic resistance. Fascinatingly, it was found that many of our bacterial isolates were tolerant to heavy metals, demonstrating higher MICs than *E.coli* **(Nies, 1999)**.

The functional metagenomic analysis also shows the presence of heavy metal resistance genes in glacial samples. However, similar to antibiotic resistance gene detection through

metagenomics, the metal resistance genes also showed maximum identity, i.e., more than 97% with psychrotolerant bacteria. For example, the gene related to copper resistance (copper resistance protein C and F) showed (96%) identity with *Pseudomonas fluorescens*and *Escherichia coli* K12. Similarly, mercury resistance genes (*merA*) showing 98% identity with*Thiobacillus ferrooxidans*, *Staphylococcus aureus*, *Pseudomonas stutzeri*, and *Pseudomonas aeruginosa*. The chromium resistance gene (*chrA*) showed (96%)identity with *Pseudomonas aeruginosa*. Gene related to arsenic resistance (*arsH*) was showed(98%) identity with *Bacillus subtilis* strain 168.

Therefore it may be considered that the metal tolerances detected in culturable bacteria arepossibly due to the diverse elemental presence in the glaciers. The various metal resistant genes detected by functional metagenomics were related to genus *Pseudomonas* and *Bacillus.* These bacteria were also detected by metagenomics and culture dependent study of Changme Khang, Changme Khangpu glacier and Kanchengayo glacier.

Thus our results supportedthe theory that the antibiotic and heavy metal resistance genes detected through metagenomic may be co-occurrence of heavy metals resistance to antibiotic resistance. Therefore it might be suggested that the there might be co-occurrence or co-selection of these genes in such environments. Hence, this is a broad hypothesis which needs to get counter to verify with other cold-habitats.

8. SUMMARY

More than 85% of the Earth's biosphere permanently experiences temperatures below 5ºC. The major parts of these cold habitats are successfully colonized by a broad diversity of cold-dwelling microbes, including bacteria, archaea, yeasts, filamentous fungi, and algaeinsects, and fish that are capable to dwell and even maintain metabolic activity to sub-zero temperature. The colonizing places for these psychrophilic microbes are scattered all over the globe from the Arctic to the Antarctic and from high-mountain regions to the deep oceans. The major fraction of these low-temperature environments is represented by the deep seas, followed by snow, permafrost, sea ice and finally glaciers. Other cold environments are alpine lakes, cold soils, cold deserts, and caves. The prime advantage is the psychrophilic nature of these microbes and their enzymes thus this characteristics made them the choice of various industrial and biotechnological applications. Among all the microbes thrive in such extreme conditions; psychrophiles (cold-loving microbes) got widespread devotion from various researchers in recent decades. There are almost 80 glaciers in Sikkim and most of them are debris-covered valley type glacier. Majority of these glaciers are located under Kanchenjunga National Park which is recognized as UNESCO heritage site in 2016, one of the biodiversity hotspot states of India. No work has been carried out till date in the context of bacterial diversity in the glaciers of Sikkim. Therefore, our works are first of its kind from glaciers of Sikkim.

Therefore there is an immediate need to explore the microbial diversity of these glaciers, which may lead to the understanding and accomplishing novel microbes and their

enzymes for industrial and biotechnological significance. Thus present study focused to investigate the microbial diversity of these glaciers by culture-dependent growth on agar-based media and 16S rRNA sequencing and culture-independent techniques (NGS and PLFA approach).

The first aim of our study was to document the two glaciers from North Sikkim, India. Therefore, we first check the localization of Changme Khang, Changme Khangpu, Chumbu and Kanchengayao glacier of North Sikkim with the help of GPSMAP 78S (Garmin, India) instrument. The coordinate of the sites of Changme Khang glacier and its sampling source lies between 27°56'38.80"N longitude and 88° 39'56.91" latitude and the elevation range of the location was 5221.2m, similarly Changme Khangpu glacier is located at 27°58'04.16"N longitude and 88°40'56.68" latitude and altitude range of the site is 5319m. These glaciers originates from south slope of Mt. Gurudongmar peak whereas Chumbu glacier originates from south-slope of Mt. Chumbu peak and located between 27°55'06.52"N longitude and 88°40'10.89" latitude and elevation range of 5093.8m. Kanchengayao glacier, on the other hand, is originates from south slope Mt. Kanchengayao peak, trending north-south face having latitude 27°59'57.872'N and longitude 88°37'8.785'E at an altitude of 1393m. All these glaciers melt-water merge into Teesta River in Chungthang, North Sikkim.

The second aim of our study was to check the physiochemical properties from two glaciers of Sikkim such as Changme Khangpu (debris-cover glacier) and Changme Khang (debris-free glacier) glacier. It was found that despite being different types of

glaciers and on the other hand closely located, these glaciers possess a similarelemental composition. The concentrations of various elements were also similar between the two glaciers; however, Changme Khangpu glacier had higher concentration of sulfate, total alkalinity, sulfur, and nitrite. The bicarbonate and magnesium in Change Khang glacier little higher in concentration as compare to Changme Khangpu glacier. The pH analysis shows that both the glaciers are neutral in nature. Piper analysis suggested that the Changme Khang and Changme Khangpu glacier water fell under Ca^{2+} and HCO^{-3}types which confirm that the calcium bicarbonate weathering is the major source of dissolved ion in the region.

The third and fourth aims were related to isolation and characterization psychrophilic/psychrotolerant bacteria from two glaciers of North Sikkim, India and their enzymatic analysis (protease, amylase, and lipase). Total 136 isolates were isolated from four glaciers of North Sikkim and on the basis of morphological and biochemical characterization, 47 isolates were selected for further analysis. Majority of these isolates were Gram-negative, aerobic, rod-shaped and most of them were non-endospore former. Carbohydrates fermentation test suggested that the majority of the isolates were able to utilize simple sugars such as dextrose, fructose, galactose, and glucose and few of the isolates can metabolize complex sugars like melezitose, cellubiose, mannitol, rhamnose, and raffinose. Based on morphological, biochemical characterization and carbohydrate fermentation test it can be assumed that majority of the isolates might belong to group *Bacillus* and *Pseudomonas.* Our results showed that 8 isolates were detected as protease positive, 4 amylase positive isolates whereas none of the isolates were positive for lipase.

Based on the enzyme activity test it was found that one isolate which is identified as *Bacillus thuringensis* (CKG2), its crude cell extract showed ($2.16UmL^{-1}min^{-1}$) highest protease activity than other tested isolates. Similarly, isolate strain CK3 which is identified as *Bacillus safensis*, its crude extract showed the best amylase enzyme activity ($1.07UmL^{-1}min^{-1}$).

The fifth and last objective was to identify the cultivable bacteria on the basis of 16S rRNA sequencing and Fatty Acid Methyl Ester Analysis (FAME). The overall from four glacier dominance of phylum *Proteobacteria* followed *by Firmicutes* and *Actinobacteria*wereobserved. The main genus detected was *Pseudomonas* and *Bacillus.* On the basis of alignment and similarity search of16S rRNA sequencing with nr/nt NCBI database most of the isolates has similarity index of 99% but isolate KGG13 isolated from Kanchengayao glacier has shown 98% identity with *Stenotrophomonas*which suggested that it might be a novel species. The polyphasic characterization such as biochemical identification, Biolog analysis, FAME, SEM analysis was carried out. These results suggested that there is a significant difference between our isolate KGG13 and closely related *Stenotrophomonas* sp. thus can be confirmed as a novel species. The putative strain was named as *Stenotrophomonas maltophilia var. kanchengayaoensis* KGG13 based on the glacier from which the strain was isolated. This isolate was Gram-negative, non-spore former, aerobic cocci with 1μm diameter. The cells are catalase, oxidase, protease positive and lipase, oxidase, nitrate, amylase, and gelatinase negative. Assimilation of sugars like glucose, sucrose, salicin and other sugar such as cellobiose,

gentiobiose, lactose, maltose, melibiose, and turanose were weekly positive. The strain grows a wide range of pH (2-10), NaCl (1-10%), temperature (5-40°C).

It is generally accepted that less than 1% of the microorganisms present in the nature are culturable and the rest of them are viable but in non-culturable state **(Bogosian and Bourneuf, 2001)**. The bacteria diversity detected by culturable-technique in our study was also low which led us to expand the work and to verify the bacterial diversity by using culture-independent techniques such as NGS and PLFA analysis.

The Phospholipid fatty acids analysis of Changme Khang and Changme Khangpu glacier showed that both the glaciers were dominated by Gram-positive bacteria followed by Gram-negative bacteria, which was also comparable with our culture-dependent analysis. The results showed that the Gram-positive bacteria were higher in Changme Khang glacier (54.04%) than Changme Khangpu glacier (24.84%), while Gram-negative bacteria were higher in Changme Khangpu glacier (22.65%) than Changme Khang glacier (4.41%). The major fatty acids such as straight, cyclo, MUFA, DMA, 18:1w9c and 10-methyl were higher in Changme Khangpu glacier;however, PUFA, and Branched were abundant fatty acids in Changme Khangpu glacier. The total biomass was found to be highest in Changme Khang glacier than Changme Khangpu glacier. The metagenomic analysis shows the dominance of *Proteobacteria* followed by *Firmicutes* and *Actinobacteria.* Most proteobacterial sequences were assigned to the class *Betaproteobacteria.* Similar kind of bacterial diversity was also observed from other Polar and non Polar glaciers.

We also checked the antibiotic resistance profile of 5 antibiotics such as ampicillin, methicillin, streptomycin, chloramphenicol, erythromycin, and vancomycin, and excitingly MIC values were generally around 2µgL^{-1} followed by 4µgL^{-1}, and few of the bacterial isolates showed MIC at 1µgL^{-1}. The minimum inhibitory concentrations (MIC) are the concentration at which an antibacterial agent occurrence completes inhibition of organism growth **(Vipra et al. 2013)**. We have detected higher resistance against ampicillin and methicllin but lower resistance against other antibiotics such as vancomycin, erythromycin, streptomycin and chloramphenicol.

The Antibiotic resistance genes (ARGs) were also investigated by metagenomic analysis. It was found that the resistance gene detected by metagenomic analysis was >95% similarity with psychrotolerant bacterial genes, thus suggesting the existence of resistance genes in psychrotolerant bacteria. Heavy metal tolerance was also checked against five heavy metals and it suggested that our isolates were more resistant than *E. coli*. The metagenomic analysis also showed the presence of heavy metal resistance genes and the percentage of identity was also higher with psychrotolerant bacterial species. Hence the results supported the hypothesis that the heavy metal and antibiotic resistance detected through metagenomic may be due to the co-occurrence of metals and antibiotics. However, this is an open hypothesis which needs further research is needed in such habitats.

Till now not much study have been carried out in relation to the metabolic functions of microbial communities in alpine glacial environments. This our first report of functional

metagenomic analysis from glaciers of Sikkim by Shotgun metagenomic analysis. The data were compared with KEGG and COG orthology database. Based on these results, the genes responsible for dissimilatory nitrate reduction namely nitrate to ammonia, alanine, aspartate, and glutamate metabolism were detected. The category energy metabolism, many reads encoding genes for nitrogen, methane, and sulfur metabolism were detected which suggested that the microbes present in these glacier ices can exhibit anaerobic respiration.Furthermore, several reads showing high similarities with enzyme engage in the conversion of fatty acids to unsaturated fatty acids were also detected. Similarly, many genes involved in the synthesis of osmoprotectants and cryo-protectant genes were detected such as glycine, betaine, choline, and glutamate respectively. The adaption strategies of microbes in sub-zero conditions also attracted the attention among scientific fraternity to understand the extraterrestrial environments. Such studies also promote the knowledge which is assumed to be useful in the future for controlling pathogenic microbes, which endure and flourish in cold-stored food and feed materials.

9. CONCLUSION

The Himalayas which is also called as “Third pole of the world” houses large deposits of snowfall and many glaciers. It is the largest source of fresh water from billions of people. However, the cryosphere located in Himalayas are sensitive to climate change and in recent years these Polar Regionsare experiencing rapid decline in snow cover and thus it will have a negative effect on water supplies in the next few decades. Despite being playing important roles on livelihood of billions of people and different organisms, limited microbiological studies have been carried out on Himalayan glaciers. Few studies from western Himalayas have been carried out, however very limited literature is available on Eastern Himalayas glaciers. It prompted us to carry out microbiological studies of two glaciers, i.e., Changme Khangpu and Changme Khang glacier of North Sikkim, India by both culture dependent and culture independent studies, such as PLFA and metagenomics studies which were supported by physic-chemical analysis of these glaciers water samples. These two glaciers are closely located, however Changme Khangpu glacier is debris covered glacier whereas Changme Khang is debris free glacier. Also, for microbiological studies two more glaciers were selected, i.e., Chumbu glacier and Kanchengayao glacier. Chumbu glacier is again closely located to Changme Khang and Changme Khangpu glacier, whereas Kanchengayao glacier is distantly located.

The culture-dependent analysis revealed the abundance of *Pseudomonas* and *Bacillus* in these glaciers; however three closely located glaciers have predominance of *Bacillus* species, whereas *Pseudomonas* species were dominant in distantly located Kanchengayao glacier. The detection of *Pseudomonas* and *Bacillus* species in glacier samples are not

surprising, as similar kind of bacteria have been detected in many other glaciers located in Polar and Non-Polar Regions. The isolates obtained from four these four Himalayan glaciers are capable of growing at wide range of temperature, pH and NaCl concentrations. Also, some of isolates can produce amylase and protease enzyme which can exploited for biotechnological and industrial applications.

The phospholipid fatty acid analysis and culture-dependent analysis of Changme Khang and Changme Khangpu glacier were correlated with each other as both the methods revealed the abundance of Gram-positive psychrophilic bacteria as compared to Gram-negative bacteria. PLFA suggested the abundance of Gram-positive bacteria in both the glaciers, however, the Changme Khang glacier possess the higher percentage of Gram-positive bacteria than Changme Khangpu glacier. Similarly, the percentage of fungi, anaerobe, actinomycetesand Eukaryotes were higher in Changme Khangpu glacier. The present study has limitations as the condensational PLFA and culture-dependent method fail to give a compressive diversity of an environment due to a large number of an uncultivable status of the microbes. Therefore there was further scope to study these environments by using culture-independent technique such as metagenomics.

On the basis of the metagenomic analysis, a wide and bacterial diversity in both the glacier were detected. These glaciers harbor many novel and unknown microbes which are indicated by the abundance of 40% and 35% unclassified bacterial sequences showed by metagenomic in Changme Khangpu and Changme Khang glacier respectively. However, the most abundant phyla from both the glacier were *Proteobacteria,*

Firmicutes, and *Actinobacteria.* To the best of our knowledge, this is the first study which revealed community composition and diversity from the accumulation zone of the Alpine glacier.

Interestingly we found that majority of the isolates were resistance against the most of antibiotic used.These results were supported by metagenomic analysis. Metagenomic study disclosed the presence of various antibiotic resistance genes belonging to quinolone, bacitracin, tetracycline, beta-lactam, aminoglycoside. These genes also showed the maximum identity with Gram-negative bacteria and psychrotolerant bacteria. Also, our isolates have shown substantial tolerance towards five heavy metals as compare to *E.coli.* Also, the different types of metal resistant genes were detected by metagenomic analysis. Thus our isolates which are resistant to some antibiotics and also tolerant to heavy metals support the hypothesis there might be co-occurrence or co-selection of antibiotics resistant genes and metal resistance genes in such environments, which was further supported by detection different kinds of metal resistance genes by metagenomic analysis. However, this is an open assumption which needs further research in such habitats.

The functional metagenomic analysis with KEGG and COG orthology database showed the presence of genes involved in the carbon and nitrogen cycle, methane generation and oxidation and organic matter degradation that might have an imperative role in climate change or glacier bio-degradation. The enzyme related to converting fatty acids into unsaturated fatty acids was also found and these fatty acids are required by microbes at an extreme temperature to keep their cell membrane flexibility. The bacterial

cryoprotectant such as betaine and glycine was also detected from metagenomic analysis such pathways is essential to maintain the psychrophilic microbes to maintain their cell membrane fluidity at sub-zero conditions. The adaption strategies of such microbes also attracted the interest among scientific fraternity to comprehend with extraterrestrial environments. Such a study may lead to promoting in future for controlling pathogenic microbes, which create the dilemma in cryo-environments.

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Appendix-I

Luria Bertani Agar	**gL^{-1}**	**Tributyrin Agar Base**	**gL^{-1}**
Casein enzymatic hydrolysate	10	Peptic digest of animal tissue	5
Yeast extract	5	Yeast extract	3
Sodium chloride	10	Agar	20
Agar	20		
		Gelatin Agar	**gL^{-1}**
Antarctic Bacterial Medium	**gL^{-1}**	Gelatin	30
Peptone/Tryptone	5	Casein enzymic hydrolysate	10
Yeast Extract	2	Sodium chloride	10
Agar	20	Agar	20
R2A Agar	**gL^{-1}**	**Skimmed Milk Agar**	**gL^{-1}**

Casein acid hydrolysate	0.5	SM powder	28
Yeast extract	0.5	Tryptone	5
Proteose peptone	0.5	Yeast extract	2.5
Dextrose	0.5	Dextrose (Glucose)	1
Starch, soluble	0.5	Agar	20
Dipotassium phosphate	0.3		
Magnesium sulphate	0.024	**Plate Count Agar**	**gL^{-1}**
Sodium pyruvate	0.3	Casein Enzyme Hydrolysate	5
Agar	20	Yeast extract	2.5
		Dextrose	1
Nutrient Agar	**gL^{-1}**	Agar	20
Peptone	5		
Sodium chloride	5	**Mueller Hinton Agar**	**gL^{-1}**
Yeast extract	1.5	Meat infusion	300
Beef Extract	1.5	Casein Enzyme Hydrolysate	17.5
Agar	20	Starch	1.5
		Agar	20
Actinomycete Isolation Agar	**gL^{-1}**		
Sodium caseinate	2	**50X TAE buffer**	**gL^{-1}**
L-Asparagine	0.1	Tris-base	242
Sodium propionate	4	Glacial acetic acid	57.1 ml
Dipotassium phosphate	0.5	EDTA (0.5M)	100 ml
Magnesium sulphate	0.1		
Ferrous sulphate	0.001		
Agar	20		

List of Media used for the isolation of Psychrophilic/Psychrotolerant Bacteria

Starch Agar	**gL^{-1}**	**Urea Agar**	**gL^{-1}**
Peptone	5	Urea	20
Yeast Extract	1.5	Peptone	1
Beef Extract	1.5	Dextrose	1
Starch Soluble	2	Monopotassium phosphate	2
NaCl	5	Phenol Red	0.01
Agar	20	Agar	20
Carbohydrate Fermentation Broth	**gL^{-1}**	**1X TE Buffer (pH 8)**	100ml
Sugar	5	Tris (1M)	1ml
Peptone	10	EDTA (0.5M)	0.2
NaCl	5	Distilled water	98.8ml
Phenol Red	20		

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ORIGINAL RESEARCH ARTICLE

Bacterial Diversity in an Alpine Debris-Free and Debris-Cover Accumulation Zone Glacier Ice, North Sikkim, India

Mingma Thundu Sherpa[1] · Ishfaq Nabi Najar[1] · Sayak Das[1] · Nagendra Thakur[1]

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Abstract The Himalayas are water tower for billions of people; however in recent years due to climate change several glaciers of Himalaya are receding or getting extinct which can lead to water scarcity and political tensions. Thus, it requires immediate attention and necessary evaluation of all the environmental parameters which can lead to conservation of Himalayan glaciers. This study is the first attempt to investigate the bacterial diversity from debris-free Changme Khang (CKG) and debris-cover Changme Khangpu (CK) glacier, North Sikkim, India. The abundance of culturable bacteria in CKG glaciers was 1.5×10^4 cells/mL and CK glacier 1.5×10^5 cells/mL. A total of 50 isolates were isolated from both the glacier under aerobic growth condition. The majority of the isolates from both the glaciers were psychrotolerant according to their growth temperature. Optimum growth temperatures of the isolates were between 15 and 20 °C, pH 6–8 and NaCl 0–2%. The phylogenetic studies of 16S RNA gene sequence suggest that, these 21 isolates can be assigned within four phyla/class, i.e., Firmicutes, Beta-proteobacteria, Gamma-proteobacteria and Actinobacteria. The dominant phyla were Firmicutes 71.42% followed by Actinobacteria 14.28%, Alpha-proteobacteria 9.52% and Beta-proteobacteria 4.76%. The isolate *Bacillus thuringiensis* strain CKG2 showed the highest protease activity (2.24 unit/mL/min). Considering the fast rate at which Himalayan glaciers are melting and availability of limited number of research, there is urgent need to study the microbial communities confined in such environments.

Keywords Psychrophiles · Psychrotolerant · Changme Khang · Changme Khangpu · Glacier

Introduction

The Himalayas, termed now as the Third Pole, is not only the water tower for billions of people, but also the climate driving force for the entire Asia. The Himalaya still covers around 10% of ice (glaciers and ice niches), and cryospheric area which could be as much as ~ 20% more than solid glacier cover. Committee on Himalayan Glaciers, Hydrology, Climate Change, 2012 in its report "Himalayan Glaciers: Climate Change, Water Resources, and Water Security", highlighted the potential impacts of climate change on Himalayan glaciers leading to water scarcity and which in turn could play an increasing role in political tensions. The factors that control and affect the Himalayan glacier dynamics is still poorly understood including the other peri-glacial/permafrost affected areas [1]. The World Meteorological Organization, 2012 has already reported that the polar areas have already undergone rapid decrease in snow and ice; thus releasing methane from permafrost regions causing rise in sea-level [2]. The role of cryosphere

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ORIGINAL ARTICLE

Culture independent bacterial diversity of Changme Khang and Changme Khangpu glaciers of North Sikkim, India

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Abstract
Microbial communities at cryosphere are the cosmopolitan buffers of important biogeochemical processes stationed at extreme archaic and frigid conditions. In the present study microbial diversity analysis from accumulation zone of two glaciers of North Sikkim, India has been carried by two culture independent methods. The phospholipid fatty acids analysis of *Changme Khang* and *Changme Khangpu* glacier showed that both of these were dominated by Gram-positive bacteria followed by Gram-negative bacteria. Among the two glaciers, *Changme Khang* (54.04%) had higher percentage of Gram-positive bacteria than *Changme Khangpu* (24.84%), while Gram-negative bacteria were higher in *Changme Khangpu* (22.65%) than *Changme Khang* (4.41%). The metagenomic analysis shows the dominance of *Proteobacteria* followed by *Firmicutes* and *Actinobacteria*. *Betaproteobacteria* were the dominant class among *Proteobacteria*. Similar kind of bacterial diversity was also observed from other polar and non-polar glaciers.

Keywords Psychrophiles · *Changme Khang* · *Changme Khangpu* · Glacier · Phospho lipid fatty acid analysis (PLFA) · Metagenomics

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RESEARCH ARTICLE

The Diversity of *Pseudomonas* species from the Accumulation Zone of Kanchengayao Glacier, North Sikkim, India

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Abstract

Glaciers are the cryospheric niches which support concealed microbial life. They inhabit broad-spectrum culturable and non-culturable bacterial diversity. There is virtually very little information on the psychrophilic/psychrotolerant bacterial diversity found in the glaciers in India. Indian Himalayas are regarded as the world heritage of flora and fauna. As it houses many largest glaciers in its lap, a new venture into glaciers has been started. Microbiological investigation of the glaciers in North-East India will help us to have an insight into the hidden treasure of microflora. We are providing the first report on the *Psuedomonas* sp. diversity from Kanchengayao glacier, North Sikkim, India. It is one of the most dominant genera isolated from glacier ice samples. This genus is one of the most medically and ecologically important groups of Gamma-proteobacteria present in environment. In the present study, the diversity of *Pseudomonas* species isolated from ice core sample was carried out based on the phenotypic and genotypic analysis. It was found that the glacier was abundant in *Pseudomonas azotoformans; Pseudomonas poae; Stenotrophomonas maltophilia; Pseudomonas fluorescens; Pseudomonas reactants; Pseudomonas hibiscicola* and *Pseudomonas synxantha*. Interestingly, the antibiotic susceptibility test showed that all the isolates were resistant to Ampicillin (10mcg) but all were sensitive to Streptomycin (10mcg), 19 isolates were resistant to Vancomycin (30mcg) and six were resistant against Tetracycline (30mcg) whereas majority of the isolates showed intermediate response. The antibiotic resistance found in this unexplored area is an important study and first of its kind reported from this glacier.

Keywords: *Pseudomonas*, Kanchengayao glacier, Sikkim, 16S rRNA, antibiotic resistance.

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DIVERSITY OF *BACILLUS* SPECIES FROM CHUMBU GLACIER

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