Studies on Microbial Communities from Satopanth Glacier Western Himalaya and Evaluation of their Biotechnological Applications

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Submitted by

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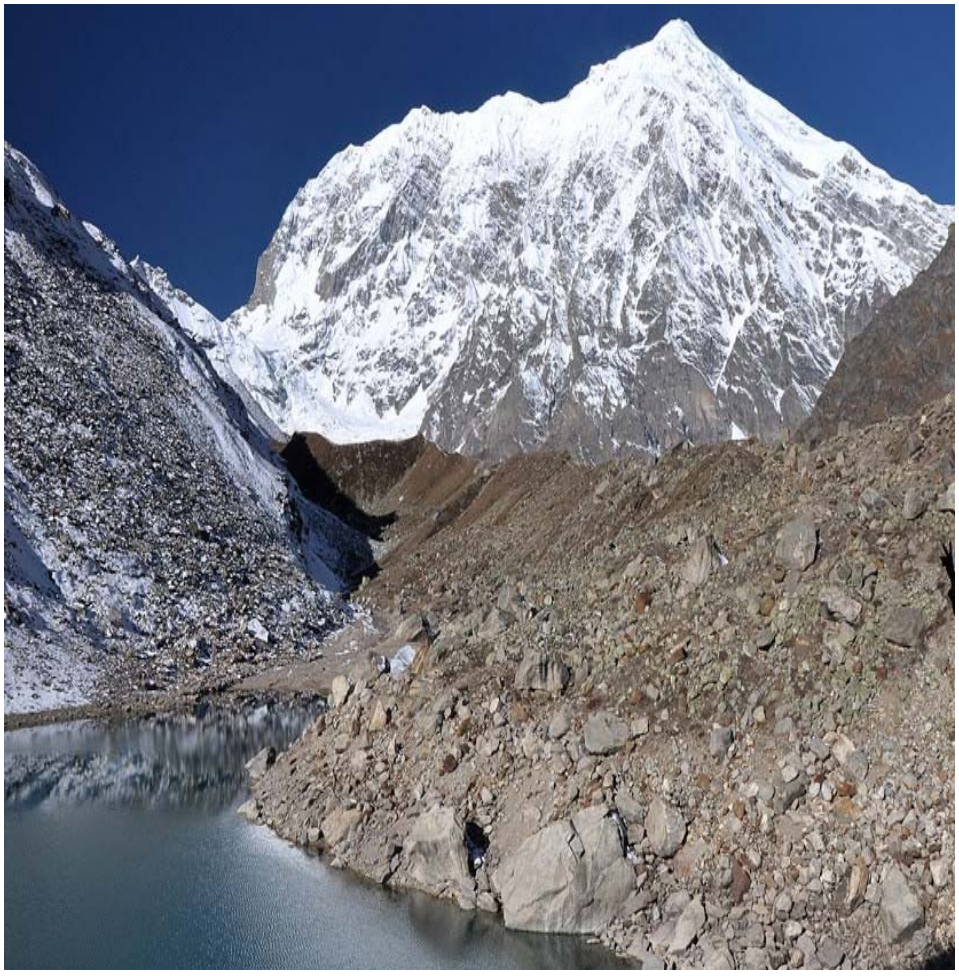

Dedicated to my beloved

Parents

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This is to certify that the thesis titled **"Studies on Microbial Communities from Satopanth Glacier Western Himalaya and Evaluation of their Biotechnological Applications"** submitted by **Ms. PRAGATI KATIYAR** is an original research work and has not been previously submitted in part or full for the award of any other degree or diploma to this or any other university.

The thesis submitted to Babasaheb Bhimrao Ambedkar University, Lucknow satisfies all the requirements as stipulated in the *Doctor of Philosophy (Ph.D.) Regulations-1999 as amended in 2008/2010/2013* and it is fit for submission and evaluation for the award of the degree of Doctor of Philosophy of the University.

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STUDENT DECLARATION

This is to certify that the material embodies in the present Ph.D. work entitled **"Studies on Microbial Communities from Satopanth Glacier Western Himalaya and Evaluation of their Biotechnological Applications"** is my original research work done by me. It has not been submitted in part or full for any other diploma or degree in any other University. In this thesis, matter written, data presented plagiarism if any is the sole responsibility of the student Ms. Pragati Katiyar. If any allegations/query/question arises regarding the thesis myself Ms. Pragati Katiyar will be solely responsible and answerable.

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Pragati 21/09/17

(PRAGATI KATIYAR)

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ABBREVIATIONS

–	Negative
%	Percentage
+	Positive
°C	Degree Celsius
µg	Microgram
A	Absorbance
CAB	Cold adapted bacteria
CFU	Colony Forming Unit
Co	Cobalt
Conc.	Concentrated
Cr	Chromium
Cu	Copper
DNA	Deoxyribose nucleic acid
EDTA	Ethylene diamine tetra acetate
et al.	et alia (and others)
gm	Gram
hr	Hour
Kb	Kilobase
kDa	Kilo Dalton
km	Kilo meter
masl	Meter above sea level
mg	Milligram
min.	Minute
Ml	Microlitre
ml	Millilitre
mm	Millimeter
NaOH	Sodium hydroxide

ND	Not determined
Ni	Nickel
Nm	Nanometer
OD	Optical Density
PCR	Polymerase Chain Reaction
ppm	Parts per million
PUFA	Poly Unsaturated Fatty Acid
RNA	Ribose nucleic acid
RPM	Rotation per minute
SDS	Sodium Dodecyl Sulphate
U	Units
UV	Ultra violet
W	Watt
w/v	Weight/Volume
Zn	Zinc

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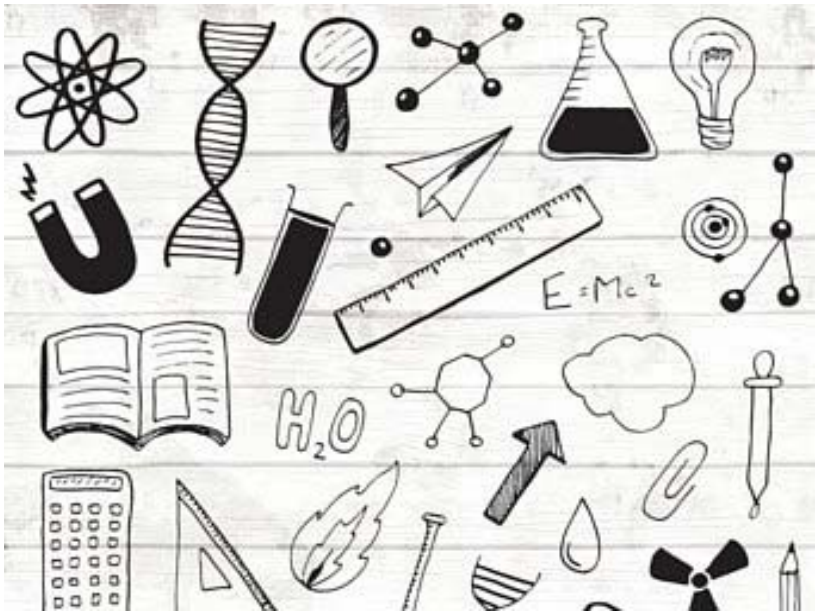


CHAPTER 1

Introduction

1. INTRODUCTION

Satopanth Glacier, a major peak of Garhwal division is situated in Western Himalaya, Uttarakhand between 30º42'55"-30º50'32" N latitude and 79º13'55"-79º29'40" E longitude and altitude is 4,600 masl. Satopanth glacier is situated at the head of Alaknanda River and draining in north-westerly direction. It is approximately 15 km long and with an average width of 750 m and covering an area of 21.17 sq. km. (Figure 1.1) (Nainwal et al., 2008). The first expeditions to the Himalayan glaciers go back to the East India Company, when the Schlagintweit brothers under the aegis of Alexander von Humboldt, explored many glaciers in the Himalayas and Karakoram during the years 1854-1857 (Kick, 1960). The Himalayan mountain ranges extend between latitudes 26°20' and 35°40' N and between longitudes 74°50' and 95°40' E (Ives and Messerli, 1989) are habitat to some of the largest mountain glaciers outside the polar and sub-polar regions. Our study interest, Alaknanda river, which rise about 30 miles north from Satopanth glacier merge to Bhagirathi at Dev Prayag to form Ganga which flows through the northern Indian planes, providing drainage and water for around 400 million people. Upper Alaknanda watershed covers an area of 234.35 sq. km. out of which 70.70 and 107.22 sq. km. is covered by Satopanth and Bhagirathi Kharak sub water shed respectively. While east facing Satopanth and Bhagirath Kharak glacier are separated by a linear Balakun ridge and together give rise to Alaknanda River (Tangari et al., 2014).

Glaciers cover roughly 10% of earth's land surface. As the earth warms, losses in glacial mass will lead to an export of freshwater to marine ecosystems and ultimately a rise in sea level. Due to the unreceptive climatic conditions prevailing in the Glacier of Western Himalaya and makes it an interesting habitat to study phylogenetic and functional diversity.

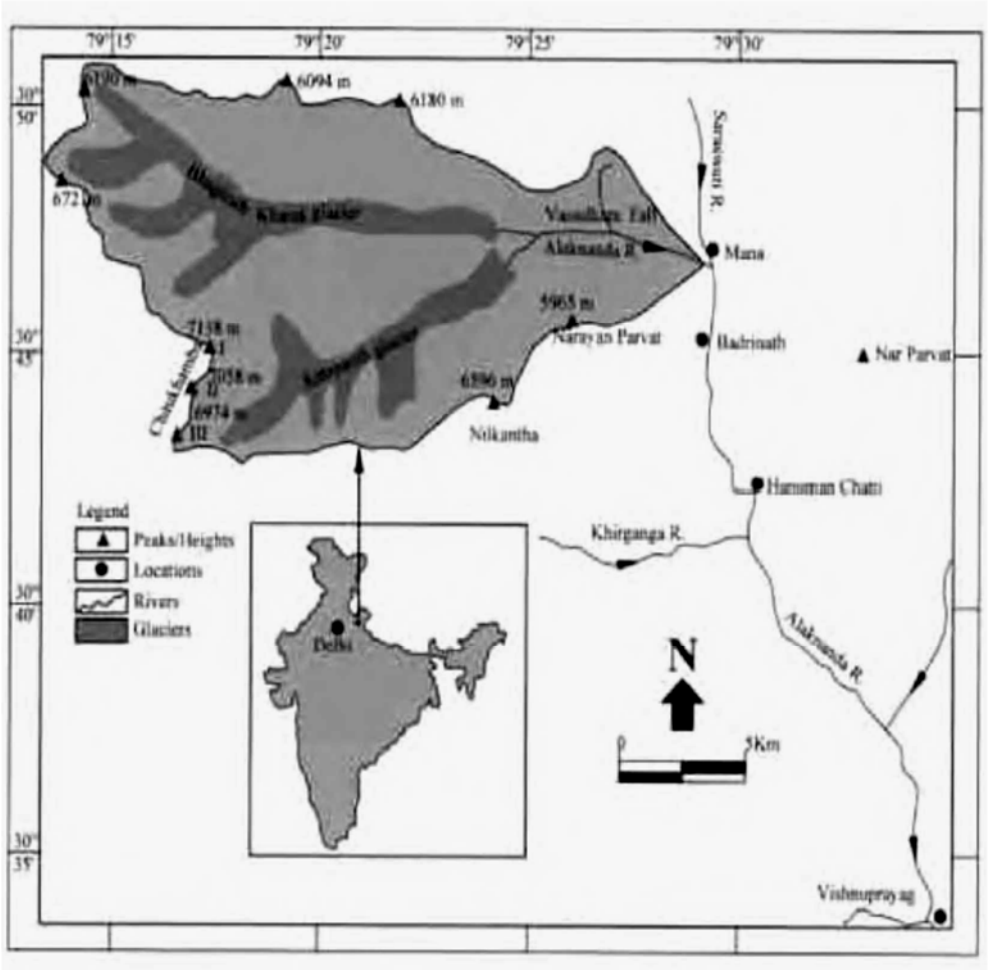


Fig. 1.1: Location map of Satopanth Glacier (Nainwal et al., 2008)

Therefore this study improved our understanding for the bacterial community in glacier ice and also helps to provide a better foundation for more understanding about ecophysiology and ecology of glacier microflora in general and cold adapted bacteria in particular.

The aim of study is twofold. Firstly, to monitor bacterial contamination and incidence of antibiotic resistance pattern among coliforms inhabiting runoff of Satopanth glacier. As well as the high-speed deprivation of glacier is expected because of anthropogenic activities which may lead to ecological imbalance as also threat to water balance. Due to the fact in past research assess the quality of glacier water runoff and rivers through bacteriological monitoring. Bacteriological indicators such as coliforms, faecal coliform, and faecal streptococci are reliable parameters, which indicated water suitability for human utilization (Okawasili and Akujobi, 1996; McLellan et al., 2001; Pathak and Gopal, 2001; Harwood et al., 2004; Baghel et al., 2005). A wide range of pathogenic microorganisms can be transmitted to humans via contaminated water with faecal material. These include enteropathogenic agents such as coliform, faecal coliform, *E. coli*, *Streptococcus faecalis*, *Stayphylococcus aureus*, *Pseudomonas aeruginosa, Clostridium walchi, Vibrio cholerae, Vibrio parahaemolyticus, Salmonella* and *Shiegella,* and *Klebsiella* (Hodegkiss, 1988). Enterococci are found in the faeces of warm blooded animals, so its presence in a given environment strongly indicates (99% certainty) the presence of animals, like mammals and birds, or humans in the area (Stevens and Ashbolt, 2003). Because of their ubiquity in human and warm-blooded animal faeces and their long-term survival in the environment, enterococci have been adopted as indicators of faecal contamination in aquatic bodies (Nevers and Whitman, 2011). However indicator organisms presently used for the monitoring of drinking water, although the reliance on indicator organisms as the main source of information about the

safety of drinking water is under review in many jurisdictions (WHO, 2004). In best of our knowledge, inadequate data is available on the bacteriological monitoring and assessment from glacier runoff of Himalayan region. Therefore monitoring and biological assessment of the Himalayan glaciers is important to assess the overall reservoir health.

Antibiotic resistance is a problem of global concern and is frequently associated with human activity. The extent of antibiotic resistance in the bacterial communities of a given environment has been frequently linked to anthropogenic activities. Therefore, in areas in which human activity is limited, bacteria are expected to contain few antibiotic-resistance genes. An example of this is the glacier; bacteria have negligible or no antibiotic resistance, although the anthropogenic impact has increased in recent years with tourist and scientific activities (Skurnik et al., 2006; Bonnedahl et al., 2008; Cowan et al., 2011). Whereas studying antibiotic resistance in bacteria isolated from pristine environments, extends our perceptive for these fragile ecosystems and can assess to its maintenance. Bacterial antibiotic resistance is the result of water contamination with faecal debris from homeotherms by coincidental or co-selection of resistance (Kaspar et al., 1990). The continuous exposure of microbial population such as *Escherichia coli*, the predominant faecal indicator bacteria (FIB), to antibiotics and heavy metals opts for resistant strains (Maloo et al., 2017). Resistant bacteria are seen as useful tools to study ecology and microbiology in polluted environments. Several studies have reported the presence of coliforms and antibiotic resistance pattern of isolated bacteria from cold habitats suggesting that tourist and pilgrimage and research workers are an extrinsic source of non-native microorganisms in the cold region (Baghel et al., 2005; Segawa et al., 2012; Rabbia et al., 2016). However, in best of our knowledge not as much as study available for bacteriological contamination from runoff of Satopanth and Alaknanda

river system, which have great relevance to geological exploration and tourism, in addition to environment and public health safety. Since my observation may be valuable in monitoring of water quality and conservation of glaciers and the associated riverine system.

Secondly, was to study bacterial diversity of glacier and to isolate bacteria in order to identify and characterize cold adapted bacteria for estimation of their biotechnological potential. Microbial diversity is the functional backbone of any ecosystem and they perform numerous functions which are essential for the life and biosphere. Glaciers are important constituents in the Earth's hydrological and carbon cycles, with predicted warming leading to increases in glacial melt (Smith et al., 2016). As we know not only is the sheer volume of freshwater of concern, but also the release of geochemical constituents in glacial ice from microorganism are also equivalent important. Due to the importance of cold environments such as arctic, Antarctic and glaciers, several studies focused on investigating microbial diversity and evaluation of their extracellular enzyme (Reddy et al., 2009; Srinivas et al., 2009; Singh et al., 2014). On the other hand, the estimation of species richness is important for comparing communities in conservation and management of biodiversity, for assessing the effects of human disturbance on biodiversity, and for making environmental policy decisions. Therefore researchers around the globe are putting a lot of thrust towards harnessing and understanding the biodiversity of cold environment and production of novel secondary metabolites and extracellular enzymes from them.

Earth's biosphere is largely adapted to extreme condition below the freezing temperature in arctic to above the boiling temperature in hydrothermal valves (Deming, 2002). The cold habitats span from the Arctic to the Antarctic and include the high-mountains such as Himalayas, the alpine glaciers, the deep ocean, snow, permafrost, sea

ice, lakes, cold soils (especially sub-soils), cold deserts, and caves. The glacier is one of the extreme environments among them and represents several distinct habitats such as, glacial ice, permafrost, tundra wetlands, subglacial soil, periglacial soil, tundra soil etc. Microbial flora exists in these extreme locations all around the globe are categorized in to thermophiles, mesophiles and psychrophiles according to their optimum temperature requirements. Currently, only ~2% of the microorganisms on the earth have been commercially exploited and amongst these there are only a few examples of extremophiles (Gomes and Steiner, 2004). These cold habitats constitute more than three-quarters of our planet and all these permanently cold habitats have been successfully colonized by a class of microorganisms known as psychrophiles. In 1975 Morita defined them as psychrophiles or "cold loving" that could grow at a temperature between -20 and 10^{o}C, and unable to grow at temperatures >15^{o}C. Unlike psychrophiles, psychrotrophs or "cold adapted" that could grow at temp near the freezing point of water but fastest growing over 20^{o}C (Cavicchioli et al., 2002). The term "Psychrophilic" was first used in 1902 by Schmidt-Nielsen to describe such cold-adapted organisms. Psychrophiles include a large range of representatives from all three domains: *Bacteria*, *Archaea* and *Eukarya*. More than 100 species of psychrophiles have been identified and reported which comprises of both Gram-negative and Gram-positive bacteria from various habitats. Various species within the genera *Alcaligenes*, *Alteromonas, Aquaspirillum, Arthrobacter, Bacillus, Bacteroides, Brevibacterium, Gelidibacter, Methanococcoides, Methanogenium, Methanosarcina, Microbacterium, Micrococcus, Moritella, Octandecabacter, Phormidium, Photobacterium, Polaribacter, Polaromonas, Psychroserpens, Shewanella* and *Vibrio* have been reported to be psychrophilic (Morita and Moyer, 2001). Throughout the world the majority of these microorganisms especially in cold habitats still remain hidden and need to be explored. Therefore,

microbial expeditions are encouraged globally to create novel and better techniques for bio prospecting of these novel microorganisms and utilization for humankind. Indeed microbes colonizing the high-mountains have adapted, superbly, through millions of years, to the prevailing, harsh climatic conditions. As a result of the adaptation to the cold environment microorganisms have evolved unique properties, such as colony pigmentation, ability to grow at low temperature with low nutrient concentration, specific membrane structure, and synthesis of cryoprotectants, exopolymers, cold active enzymes and ice binding proteins (Margesin et al., 2007; Bej and Mojib, 2010). However, a key determinant of adaptation to life is the synthesis of such cold active enzymes is the main physiological adaptation at the enzyme level. Therefore, researchers are now trying to exploit psychrophiles as valuable source of novel enzymes (Chakraborty, 2008). Recently there has been growing interest in psychrophilic enzymes as models in basic studies both to investigate the thermal stability of proteins and to understand the relationship between their stability, flexibility, catalytic efficiency, and as potential candidates for industrial and biotechnological applications (Gerday et al., 2000; Siguroardottir et al., 2009). In addition to look into what kind of microbes is present in these cold habitats, cold active enzymes as well as proteases, lipases and α-amylases, are another attraction to study (Pulicherla et al., 2011; Zheng et al., 2011; Joshi and Satyanarayana, 2013; Rojas-Contreras et al., 2015). The majority of the bacterial strains were able to secrete a broad range of cold-active hydrolytic enzymes into the medium at a cultivation temperature of 4ºC. Therefore the practical utilization of enzymes from cold-adapted organisms would constitute a considerable progress towards the saving of energy at large scale. Cold adapted enzymes or psychrozymes, which have high specific activities at low temperature often up to an order of magnitude higher than those observed for their mesophilic counterparts and comparatively low thermostability

therefore reducing the free energy barrier of the transition state (Feller and Gerday, 2003). For example, detergent replacements (Lipases and Proteases) to allow colder wash cycles and low-temperature digestion of industrial wastes and also cold adapted enzymes involved in the food industry, would allow product modification at low temperatures where other microbial growth is minimized.

The cold adapted microorganisms are source of commercially and industrially important enzymes like protease, lipase and amylase (Margesin and Schinner, 1994; Kasana et al., 2008). The application of cold-active enzymes enables lowering of temperature without loss of efficiency which results in saving of energy consumption and have great potential for various biotechnological processes (Ramteke and Bhatt, 2007; Kuddus and Ramteke, 2008a). Among cold-active enzyme α-amylase (Aghajari et al., 1996; Feller et al., 1998), lipase (Suzuki et al., 2001; Jeon et al., 2009), aspartate transcarbamylase (Feller et al., 1992) $Ca^{+}Zn^{+2}$ protease, metalloprotease (Villeret et al., 1997), malate dehydrogenase (Narinx, 1997) xylanase (Reddy et al., 2003b), proteases constitute an important class of hydrolytic enzymes that are found in all life forms as they are essential in physiological, metabolic and regulatory functions. Proteases (also called proteinases and proteolytic enzymes) are ubiquitous proteins that catalyse the cleavage of peptide bonds of other proteins (in some cases having auto proteolytic activity) and are essential for cell growth and differentiation (Rao et al., 1998; Gupta et al., 2002). They are enzyme of class hydrolases and subclass peptide hydrolases or peptidase. Proteases are classified into six broad group viz., serine, threonine, cysteine, aspartate, glutamic acid and metalloprotease, according to the character, mode of action and catalytic active site (Dubey et al., 2007).

Microbial alkaline proteases dominate the worldwide enzyme market, accounting for two-third of the share of the detergent industry. The global market for industrial

enzymes is estimated at $3.3 billion in 2010 and account for nearly 65% of the world enzyme market and this market is expected to reach $4.4 billion by 2015, a compound annual growth rate (CAGR) of 6% (PR Newswire, 2011). The two most important properties of cold active proteases that make them suitable candidate for biotechnological applications are their high catalytic activity at low temperature and low thermo stability at elevated temperature. Cold-adapted proteases may be applied in many fields e.g. in production of detergents for washing at low temperatures, in tannery, in the food industry (haze removal from beer, bakery, cheese-making, production of fermented foods, meat tenderisation) or high protein waste degradation (Margesin and Schinner, 1994; Brenchley, 1996). Proteases are being used in the food and detergent industries since long time, but their application in leather industry and for organic waste treatment is a relatively new development with added biotechnological importance. Proteases are currently used in basic and applied arenas of research as well as in a wide range of product design and manufacturing processes. Due to the versatile requirement for proteases, attempts are made continually to isolate newer sources of protease producing microorganisms with a potential for industrial application.

In past studies meanwhile protease isolated from cold adapted bacteria occupy a pivotal position in cold adapted enzymes but in recent development lipases also have emerged as an essential tool industrially due to their potential for application in low temperature processes. The survival at extreme environmental conditions involves the optimisation of enzymatic tools allowing metabolic rates that are able to exist at low or high temperatures, of lipid rich habitats. Therefore several psychrophilic and psychrotrophic bacteria have been exploited for the production of a variety of cold-active lipases across different cold habitats. Numerous lipases were isolated and characterized

from animals, plants, and microorganisms. Among them, microbial lipases are the most diverse enzymes in their enzymatic properties (Jaeger and Eggert, 2002).

Lipases (Triacylglycerol acylhydrolase, E.C. 3.1.1.3) are hydrolytic enzymes, which act on the carboxylester bonds present in acylglycerol to liberate fatty acids and glycerol. Lipases were first discovered in 1856 by Claude Bernard when he studied the role of the pancreas in fat digestion (Peterson and Drabløs, 1994). Lipase constitute an important class of hydrolytic enzymes that are found in all life forms as they are essential in physiological, metabolic and regulatory functions and an important group which have high catalytic efficiencies at lower temperatures. The commercial use of lipases of cold origin is a billion dollar business. It possesses many industrial applications specifically, they are employed in waste water treatment (degreasing of lipid clogged drains), pharmaceutical (resolution of racemic mixtures), dairy (hydrolysis of milk, fat), leather (removal of lipids from hides and skin), detergent (removal of oil/fat stains) and medical (diagnostic tool in blood triglyceride assay) biosurfactants and bioremediation etc. (Kumar et al., 2005; Paniker et al., 2006; Pogori et al., 2007; Sharma and Shukla, 2011). A well renowned example due to of economic importance is the polar yeast *Candida antarctica*. It is producer of two types of lipases, A and B. among them, lipase B is involved in wide range of applications related to pharmaceuticals, cosmetics food and animal feed processing (Struvay and Feller, 2012; Lohan et al., 2014). In view of the present limited understanding and availability of cold-active lipase with diverse characteristics, it is essential to explore earth's surface more in search of an ideal cold-active lipase.

Amylase (EC3.2.1.1, 1, 4-D-glucan glucanohydrolase, and endoamylase) hydrolyzes starch, glycogen, and related polysaccharides by randomly cleaving internal 1,4-glucosidic linkages to produce different sizes of oligosaccharides. Cold active

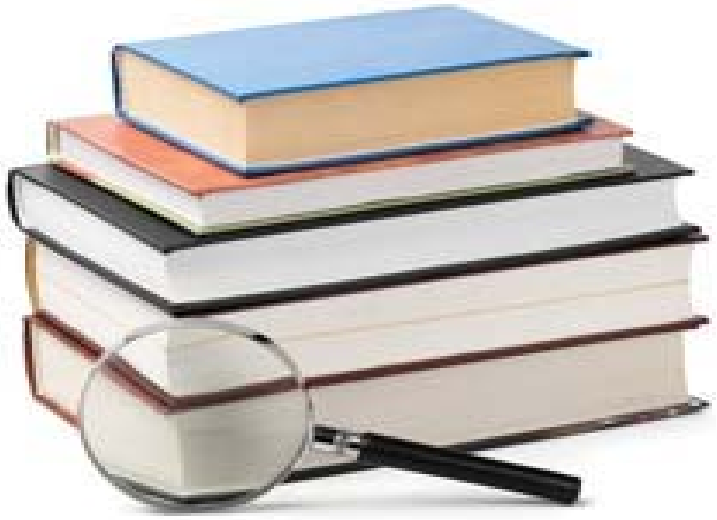
amylase is one of the important cold active enzymes, which is widely distributed in microorganisms existing at low temperature with high rates of catalysis in comparison to the amylases from mesophiles or thermophiles which shows little or no activity at low temperature. These amylases have evolved a range of structural features that confer a high level of flexibility, particularly around the active site are translated into low activation enthalpy, low-substrate affinity and high specific activity at low temperatures. Among amylases α-amylase is in maximum demand due to its wide range of applications in the industrial front and this group of enzymes represents one of the three largest groups of industrial enzymes and accounts for approximately 25–33% of the world enzyme market, in second place after proteases (Nguyen et al., 2002). Cold-active α-amylases isolated from living and metabolically active psychrotolerant microorganisms confers low activation energies and high activities at low temperature which are encouraging properties for the production of relatively insubstantial compounds that catalyzed at low temperature (Kuddus and Roohi, 2010). Alpha-amylases are ubiquitous in nature produced by plants, animals and microbes, where they play a dominant role in carbohydrate metabolism while microbial amylases are among the most important hydrolytic enzymes and have been studied extensively (Sheoran et al., 2014). They have numerous applications in the industrial processing of different items, namely, starch liquefaction process, improve flour in the baking industry and produce modified starch for paper industry and in laundry detergent formulations (Kim et al., 1995; Pandey et al., 2000). Thus, Cold-active amylases are becoming promising enzymes to replace successfully the conventional enzyme for biotechnological industries running at low temperature and serve as a world-wide choice for biotechnologists, microbiologists, biochemists, pharmacists and process engineers (Kuddus and Roohi, 2011).

More in general, the aim of current study was an attempt to determine bacterial abundance and viable bacterial diversity, first to investigate whether glacier microbial communities exhibited variability within glacier and bacteriological assessment in runoff of glacier. Secondly, to characterize the physiological features of dominant bacterial strain and screen them for their biotechnological applications. In the biotechnological perspective, they can be looked at as potential source of cold adapted enzyme and their industrial implications.

Objectives

Hence the present study entitled **"Studies on Microbial Communities from Satopanth Glacier Western Himalaya and Evaluation of their Biotechnological Applications"** was conducted **with the following objectives**:

- Quantitative and qualitative estimation of bacteria from Satopanth Glacier, Western Himalayan, India.
- Microbiological assessment and evaluation of water pollution using various indicator organisms and to screen for antibiotic resistance pattern among them.
- Isolation, characterization and screening of protease, lipase and amylase producing microorganisms and partial purification of extracellular enzymes, secreted by cold adapted bacteria isolated from soil sample of Satopanth Glacier.



CHAPTER 2

Review of Literature

2. REVIEW OF LITERATURE

2.1. Genesis of the study

In our milky wave galaxy only Earth fulfilled all the criteria for living organism although recent investigation proved that some microorganism may exist on other planet like on Moon as well as Mars. For the origin of life as well as to sustain life water and air made first priority. The Earth's biosphere is largely adapted to extreme conditions below the zero temperatures in the polar ice area, to boiling temperature in the hydrothermal valves (Blöchl et al., 1997; Deming, 2002). Among extreme environments, low temperature is very common in both natural and man-made environments. About 80% of the biosphere and more than 90% of marine environments have temperature lower than 5°C (Margesin and Schinner, 1994; Brenchley, 1996). In spite of this fact, comparatively less attention has been paid to microorganisms which can grow at low temperature, perhaps because of the slow growth rate and difficulty in handling these bacteria (Morita, 1975). These low temperature environments are inhabited by cold adapted microorganisms, which include psychrotrophs and psychrophiles. During the past couple of decades it has been recognized that cold adapted microorganisms provide a wide biotechnological potential, offering numerous economic and ecological advantages over the use of organisms and their enzymes which operate at higher temperatures. The cold adapted microorganisms are source of commercially and industrially important enzymes like protease, lipase and amylase (Margesin and Schinner, 1994; Kasana et al., 2008).

2.2. Cold environment and microbial diversity

Earth is unique in the solar system as being the only planet which is able to support life in all its forms, from basic living microorganism to highly sophisticated and intellectual human beings. Breathable atmosphere, suitable climate, presence of water, sunlight, Sun, Ozone layer, Earth's magnetic field are some factors that made earth suitable for life. Earth biosphere is divided in to hydrosphere, lithosphere and atmosphere. Hydrosphere constitutes about 70% of Earth surface of which 70% is in the form of glaciers. Earth's $3/4^{th}$ part is water and $1/4^{th}$ part is land. Water is present in form of oceans, seas, rivers; lakes and glacier among them glaciers are frozen water bodies which are like slow moving mountain. According to the National Snow and Ice Data Centre, glacier store around 75% of the world's fresh water. Glacier is primitive water bodies with some as the Arctic ice glacier sheet being more than 40 million years old. Hodson et al. (2008) differentiate glacier in to superglacial (cryconite holes) and sub glacial (deep ice) part having different thermal regimes, physical, hydrological and geothermal characteristics. Glacier are characterize by sub freezing temperature (-56^{o}C to -10^{o}C) high hydrostatic pressure, low nutrient and water availability. Despite of this glaciers are source of enormous diversity of microbial life. Some of them are uniquely preserved in chronological layers for thousands of years. These are considered as dynamic ecosystem microbial abundance varies between glacier with depth, altitude and ranges from $<10^2$ to 10^6-10^7 cells per ml (Priscu and Christner, 2004; Priscu et al., 2007). Therefore cold habitats that are widely spread on the Earth include glaciers, sea ice, glacial ice, tundra wetlands, oceanic water, sub glacial soil, per glacial soil etc. and contribute as a potential region for identifying novel psychrophilic bacteria with biotechnological prospects.

Glacier microbial diversity is mostly occupied by major phylogenetic group Actinobacteria, Firmicutes, Proteobacteria, CFB and included microbial eukaryotes

(fungi and yeast), some plant and bacterial viruses and few Archaea. Microbial diversity and ecology of cold habitat provide considerable impetus for the isolation of novel microorganism for possible biotechnological applications. Furthermore, the glacier has become a model system for global warming and the glaciers are regarded as key sites for Himalayan biodiversity monitoring. Earlier studies of cold environment have mainly focused on the abundance and biomass of bacteria in Arctic and Antarctic glaciers instead of Himalaya Glacier. According to Xiang et al. (2006), Miteva et al. (2008) reported that the biomass of glacier microorganism is low and Gram +ve bacteria are dominant group so it is difficult to isolate sufficient quantity of DNA, further DNA extraction of environmental microorganism involves sample pre-treatment, cell lysis, extraction, preparation and purification of DNA that further impose restriction on recoverable quantity of DNA (Peiying et al., 2012). In the past some researchers isolates some novel psychrophilic species from ancient ice samples of Greenland, Antartic and Tibet and Himalayan glaciers (Abyzov, 1993; Christner et al., 2000, 2001, 2003b; Miteva and Brenchley, 2005; Miteva et al., 2008; Turchetti et al., 2008; Zhang et al., 2008, 2009b; Liu et al., 2009a; Xiang et al., 2009a). Liu et al. (2009) studied bacterial community diversity of Tibetan Plateau and documented significant differences between glaciers with only 15 out of 82 bacterial genera common for all glaciers. In earlier Pradhan et al. (2010) explore diversity of two soil samples collected from the periphery of the Roopkund glacial lake and soil sample from the surface of the Roopkund glacier in the Himalayan ranges was determined by constructing three 16S rRNA gene clone libraries. Kohshima et al. (2002), Nakazawa et al. (2004), Segawa et al. (2006) retrieved snow algae from glacial ice cores collected at lower altitudes from Himalaya, Petagonia and Altai mountains have been used as environmental markers for studies of past environmental conditions. These environmentally important issues should be discussed

based on microbial ecology in glaciers however studies of bacterial communities on the surface of glacier cap is lacking with more studies focusing on bacteria present in deep ice cores (Christner et al., 2003; Miteva and Brenchley, 2005; Willerslew et al., 2007).

Segawa et al. (2010) analysed the bacterial communities on Gulkana Glacier using 16srRNA gene clone libraries and divided bacterial communities in to three types corresponding the snow covered, snow and ice covered and bare ice areas. Gulkana glacier which is located in the Alaskan range was dominated by Betaproteobacteria and Gammaproteobacteria and bacteriodetes and proteobacteria were abundant in the glacier (Yoshimura et al., 2010). Microbial communities on Gulkana glacier showed altitudinal variations and the pattern of distribution differed among the taxa.

2.3. Indicator bacteria

Microbial contamination in such an ecologically sensitive stream, with faecal waste from man and animals, is a great risk to public health (Scott et al., 2003). Therefore coliforms, faecal coliforms and faecal streptococci are established ecological indicators of faecal contamination in water, and are used for determination of water suitability for human use (Okawasili and Akujobi, 1996). Among these, *Escherichia coli*, a faecal coliform, occurs most commonly in faecal waste of homeotherms, including man, and has been found to be the best biological indicator of faecal contamination in drinking water (Leclerc et al., 2000). Enterotoxigenic *E. coli* (ETEC), is a chief source of traveller's diarrhoea to travellers in developing countries. Accordingly, there is an emerging trend toward a risk-management, multiple barrier approach to providing safe drinking water. At present, *E. coli* appears to provide the best bacterial indication of faecal contamination in drinking water. This based on following characteristics:

(1) The prevalence of thermo tolerant (faecal) coliform in temperate environments as compared to the rare incidence of *E. coli*, and

(2) The prevalence of *E. coli* in human animal faeces as compared to other thermo tolerant coliform, and

(3) The availability of affordable, fast, sensitive, specific and easier to perform detection methods for *E. coli*.

2.4. Microbiological assessment

Glaciers and icecaps constitute about 1.76% of global water resources (WQMD and ILEC, 1995) and contribute to most of the riverine system. Because glaciers are enormous bodies of freshwater and often serves as drinking water source of many populations, these should be kept preserved and free from contamination. As after melting of the ice of glaciers it gets converted to water and gives birth to river. River passes through populated lands hence the presence of coliforms in river is quite common and obvious because waste water of the population is mingled into river. Because of the presence of faecal materials it often serves as residence of coliform bacteria. Every year, thousands of pilgrims, tourists, students, and explorers visit glaciers. Microbial contamination in such an ecologically sensitive stream, with faecal waste from man and animals, is a great risk to public health (Scott et al., 2003). According to Pipes (1981) presence of bacteria in water can be seen as an indication of faecal contamination of water and it can be taken as an indication of the potential health risk that faecal contamination poses. According to WHO recommended standards for safety water, pollution indicator bacteria (PIB) that is coliform used as sanitary parameter for evaluation for potability of water.

Himalaya is the largest valley-type glacier of the Indian continent. Recently, it has been realized that in addition to various anthropogenic activities, a changing global climate is also exerting a significant impact on stream flow and water availability, as evidenced by regular regression of the glacier snout along with shrinking and thinning of the glacier area (Milly et al., 2005). Poindexter (2017) described influence of temperature indirectly through water and directly through organic molecules of living cells on the survivalists of the organism. Subba and Subba (1995) advocates the improper management of water bodies may arise serious problem in availability and quality of water. Sharma et al. (2010) and Sadat et al. (2011) revealed that coliform count has direct relation with anthropogenic activities and the places with greater anthropogenic pressure experience a comparatively higher bacterial load. Fang et al. (2007) also described that *E. coli* is faecal bacterium which is resident in the intestine of the warm-blooded animals including man and is regularly discharged with faeces.

Antibiotic resistance has been recognized as a global threat to humans and veterinary medicine both in developed and developing countries (Chandran et al., 2008b), but nowhere is it as stark as in India (Ganguly et al., 2011). In 2010, India was the world's largest consumer of antibiotics for human health (Laxminarayan and Chaudhury, 2016). Over the last years, human antibiotic use has grown substantially (increasing 36% between 2000 and 2010), mainly in developing countries (Boeckel et al., 2014). However the emergence of multidrug resistant enterococcal infections has generated considerable attention in the recent past. The present study was conducted to analyse the species distributions of *enterococcus*, the factors facilitating their persistence like the resistance properties in the isolates from glacial water runoff. Antimicrobial agents used in human therapy as well as their residues reach the sewage systems via urine and faeces. Their concentrations in wastewater, although significantly lower than

therapeutic dosages, are suspected to affect the susceptible bacteria and select resistant strains (Al-Ahmad et al., 1999; Backhaus and Grimme, 1999; Kim et al., 2007). More often antibiotic resistance among bacterial population is a plasmid-mediated phenomenon and is transferable in nature, which results into its spread among the sensitive aquatic bacterial species (Chen et al., 2005). Pathak and Gopal in (2007) evaluate bacterial contamination and antibiotic resistance in faecal coliforms from Gangotri glacial water runoff.

Boeckel (2014) aimed to assess variations in consumption to assist monitoring of the rise of resistance and development of rational-use policies and to provide a baseline for future assessment. Antibiotic drug consumption is a major driver of antibiotic resistance i.e. variations in antibiotic resistance across countries are attributable, in part, to different volumes and patterns for antibiotic consumption. Baghel et al. (2005) investigate the incidence of these indicator organisms, coliforms, faecal coliforms and faecal streptococci in runoff of the Gangotri glacier, Western Himalaya, India. Hernandez et al. (2012) reported that strains with resistance patterns were isolated from the Fildes Peninsula, so the results of this study demonstrate the persistence of antibiotic resistance in the seawater around the Antarctica.

2.5. Cold adapted bacteria and cold adapted enzymes

Community composition also varies seasonally, geographically and with changes of the local physico-chemical parameters (Edwards et al., 2010). Cold biosphere chiefly colonized by psychrophilic and psychrotolerant microorganism. Psychrophiles are true extremophiles as they are not only surviving at low temperature but frequently also to further environmental constraints. Psychrophiles include a large range of representatives from all three domains: Bacteria, Archaea, and Eukarya. Psychrophiles are mainly

represented by microorganisms such as bacteria (Morita, 1975; Gounot, 1991; Russel, 1998), Archaea (Cavicchioli, 2006), algae (Morgan et al., 2006), or yeast (Buzzini et al., 2012) but also by plants and animals (Margesin et al., 2007; Doucet and Win, 2009). Notably cold adapted microorganism colonized extreme environment shows reduced enzymatic reaction rates and exposed to limited bioavailability of nutrients, extreme in pH and salinity. The amount of water also poses constraint to succeed above mentioned harsh condition. In spite of such constraints, psychrophilic representatives of Gram-negative bacteria (for example, *Pseudoalteromonas*, *Moraxella*, *Psychrobacter*, *Polaromonas*, *Psychroflexus*, *Polaribacter*, *Moritella*, *Vibrio* and *Pseudomonas* species), Gram-positive bacteria (for example, *Arthrobacter*, *Bacillus* and *Micrococcus* species), Archaea (such as *Methanogenium*, *Methanococcoides* and *Halorubrum* species), yeast (*Candida* and *Cryptococcus* species), fungi (such as *Penicillium* and *Cladosporium*) and microalgae (*Chloromonas*) can be found in these environments and display striking cold-adaptive characteristics (Moyer and Morita, 1989; Russell, 1990; Gounot, 1991; Allen et al., 2001; Deming, 2002; Margesin, 2003). The psychrophilic and barophilic bacteria belong to α-Proteobacteria which includes *Shewanella, Photobacterium, Colwellia, Moritella* and *Alteromonas haloplanktis*. For the first time, *Leifsonia aurea, Sporosarcina macmurdoensis* and *Kocuriaolaris* (Reddy et al., 2003a; 2003b) have been reported from Antarctica. Bacterial diversity studies of Antarctic sediments both from fresh water sediments (Bratina Island, Sjöling and Cowan, 2003; Ardley Island, Li et al., 2006a,b), marine sediments (Vestfold Hills, Bowman et al., 2000; Ross ice shelf, Carr et al., 2013) and glacial sediments (Wright Glacier, Stibal et al., 2012) indicated that *Alphaproteobacteria, Betaproteobacteria, Gammaproteobacteria, Bacteroidetes, Actinobacteria, Acidobacteria Gemmatimonadetes, Firmicutes* and *Cyanobacteria*, are common to soils and sediments. Cold adapted bacteria belong to extremely diverse

genera and, in addition to mechanisms developed during the course of evolution, adaptation to identical thermal constraints may imply common molecular mechanisms that allow the maintenance of vital cellular functions at low temperatures. Psychrophiles do not merely survive or endure such extremely inhospitable conditions but are irreversibly adapted to these environments, as most of them are unable to grow at mild (or mesophilic) temperatures. Psychrophiles and their biomolecules have already found various applications. The high diversity among microbial psychrophilic enzymes, high yield, immense stability, high catalytic activity and economic feasibility highlighted its biotechnological potential and industrial applications (Gurung et al., 2013) in addition knowledge about the role of these microbes in their habitat.

In past various cold environments like Antarctic (Tytgat et al., 2014), Arctic (Frank-Fahle et al., 2014), Siberian tundra (Schnecker et al., 2014) have been explored for diversity of such cold adapted microbes. A wide variety of native microbial life forms with the ability to produce biologically active compounds has been discovered and studied. Gesheva (2012) suggesting their potential use in diverse biotechnological applications. Therefore, psychrophilic microorganisms mainly bacteria and their enzymes-have assumed considerable importance in the production of fine chemical and bioremediation processes in the detergent and food industries. According to Zhang et al. (2008) dust concentration played more important role in determining bacterial community diversity than bacterial concentration in Himalayan glacier. Reddy et al. (2009) studied bacterial diversity of Midtre Loven Breen Glacier, an Arctic glacier of Ny-Alesund. They used culturable bacterial technique and based on 16SrRNA gene sequence analysis report that mostly Gram positive bacteria with high G+C content belongs to four major phyla *Actinobacteria, Bacilli*, *Flavobacteria* and *Proteobacteria*. Their optimal growth conditions are 4-37^{o}C temperature range and pH range 2-3,

tolerance to Salt NaCl (0.1-1M). Some strains show extracellular enzymatic activities such as amylase, lipase and protease. Study revealed that Psychrophiles bacterial membrane have greater proportion of short chain unsaturated, branched, cyclic, cis fatty acids making them suitable to survival at low temperature and these fats could be used as dietary supplement. Margesin et al. (1997) and Master (1998), documented cold-adaptive enzymes for degradation of xenobiotic compounds including diesel oil and poly chlorinated biphenyls from northern hemisphere cold habitat. Amoroso et al. (2010) studied role of snow associated microbes in nitrogen cycling. Cavanagh (1998) reported ability of endogeneous Antarctic coastal bacteria to degrade n-alkane and aromatics (phenanthrene) hydrocarbons in a field-full spill experiment conducted in Easteren Antartica. Cold-active enzymes can be a better option to treat polluted water and soil at low temperature in competitive bioremediation of waste by the endogenous microflora which is reduced due to low temperatures.

It is generally thought that cold-adapted enzymes have evolved toward a high conformational flexibility, which would be responsible for their increased catalytic efficiency at low temperature and their low thermal stability (Feller et al., 1997). Crystal structures (Aghajari et al., 1998; Alvarez et al., 1998) and three-dimensional models (Narinx et al., 1997; Wallon et al., 1997) of cold-adapted enzymes have shown that these enzymes contain a reduced number of protein stabilization factors, such as salt bridges, hydrogen bonds, and aromatic-aromatic contacts, and reduced proline and arginine contents compared with their mesophilic counterparts. However transcription and translation are temperature-sensitive steps and psychrophiles have obviously adapted the process of protein synthesis to low temperature. It is expected that the enzymatic activities that are involved in protein synthesis have the general traits of cold-adapted enzymes - high activity associated with low stability - but this aspect has not been

consistently analysed so far. However, low temperature strengthen the interactions between DNA strands in the double helix and in the super coiled state, therefore impairing unwinding and access to RNA polymerase (Fellar and Gerday, 2003).

It was realized that some microorganisms in particular of inhabiting extreme environment produce enzymes similar to that of plants and animals in terms of actions and microorganisms represent an excellent source of enzymes due to their broad biochemical diversity and their susceptibility to genetic manipulation. Hence, they evolved a number of adaptation strategies (Margesin et al., 2005, 2007; Bej and Mojib, 2010):

I. Increase of membrane fluidity at low temperatures (by changing the composition of fatty acids)

II. Synthesis of protecting cold shock proteins as a response to thermal stresses

III. Synthesis of cryoprotectant macromolecules for reducing the presence of cytoplasm ice crystals

IV. Sub cellular, molecular and metabolic changes reduction of growth rates

V. Synthesis of cold-active enzymes.

Even though one important adaptation skill is to survive low temperature is to synthesize cold-adapted enzymes which catalyses biochemical reactions at low temperature and stability in enzyme activity (D'Amico et al., 2006; Puibgo et al., 2008). Low temperatures strongly inhibit the rates of chemical reactions, and the main challenge that is faced by psychrophiles is therefore to maintain an appropriate rate for the enzyme-catalysed reactions that are involved in essential cellular processes (Somreo, 1995). This is achieved by synthesizing cold-active, but heat-labile, enzymes with an activity that is up to 10 times higher at low temperatures than that of their mesophilic homologues. Such high activity at low temperatures seems to be achieved by destabilization of the active

site or the whole protein, allowing the catalytic centre to be more mobile or flexible at temperatures that tend to freeze molecular motions. Although other molecular adjustments can lead to cold activity, the adaptive strategy that is observed in natural cold environments seems to be a result of a lack of selective pressure for stable proteins, in conjunction with a strong selection for highly active enzymes. Thus high activity of cold active enzymes at low and moderate temperatures offers potential economic benefits (Russell, 1998; Allen, 1999; Margesin, 1999; Gerday, 2000; Cavicchioli, 2002) for example, through substantial energy savings in large-scale processes that would not require the expensive heating of reactors.

And all these specific traits are responsible for compatibility of cold active enzymes to industrial process.

I. They exhibit optimum activity at lower temperature than mesophilic and thermophilic counterpart.

II. Cold activity, they remain capable at tap water or ambient temperature, therefore avoiding over-heating at domestic or industrial levels.

III. High activity, a lower concentration of the enzyme catalyst is required, therefore reducing the cost of enzyme preparation in process.

IV. Heat liability, they can be efficiently and sometime selectively inactivated after a process by moderate heat input, efficient and selective inactivation in complex mixtures

V. Prevent undesired chemical transformations

VI. Running processes at low temperatures reduces the risk of contamination

VII. Cold-adaptive enzymes avoid degradation of reaction components and it also save metallic reactors from corrosion.

According to Chattopadhyay (2014) cold active extracellular hydrolytic enzymes produced by different groups of bacteria are a valuable source for biotechnological applications and for gathering knowledge about the eco-physiological role of these microbes in their habitat. In order to understand the geo-microbiological features of this unexplored habitat, a total of fifty one heterotrophic bacterial isolates were isolated from Ny-Ålesund soil sample. More than 74% (38 isolates) of the isolates showed significant extracellular hydrolytic enzyme activity, such as amylase, protease, lipase, DNase, phosphatase etc. Most of the work conducted on cold active bacteria has indicated highly cold-active enzymes such as amylase, protease, lipase and cellulase (Zeng et al., 2006; Kuddus, 2008; Gerday, 2013; Pandey, 2013).

Yadav et al. (2015) investigate psychrotrophic *Bacilli* for their cold active enzymatic activity isolated from three sub-glacial lakes of NW Indian Himalayas and conclude in his finding that the representative isolates from each cluster were screened for cold-active enzyme activities such as amylase, pectinase, and protease activities at 4°C were detected in more than 80% of the strains while approximately 40, 23, 14, 11% of strains possessed cellulase, xylanase, b-galactosidase, laccase, chitinase, and lipase activity, respectively. Recent investigations have provided numerous evolved structural and biochemical data on cold-active enzymes and significantly improved our knowledge regarding their functional and structural characteristics. More surprising is to discover a thermophilic enzyme with high activity at low temperatures. This was the case of a b-galactosidase isolated from *Pyrococcus furiosus* with optimal activity at 90ºC (130U/mg) and was still active at 0ºC, retaining 8% of its activity (Dong et al., 2014).

In conclusive remarks scientist explored various cold active enzymes from cold adapted bacteria are illustrated in Table 2.1.

Table 2.1: List of reported cold active enzymes from cold environment

Protease	Zhang et al., 2011
α-amylase	Aghajari et al.,1996; Feller et al., 1998
Lipase	Suzuki et al.,2005; Jeon et al., 2009
Aspartate transcarbamylase	Feller et al., 1992
$Ca^{+}Zn^{+2}$ protease	Villeret et al., 1997
Citrate synthetase α-lactamase	Feller et al., 1992
Malate dehydrogenase	Hwang et al., 1999
Triose-phosphate isomerase	Alvarez et al., 1998
DNA ligase	Georlette et al., 2004
Xylanase	Collins et al., 2002
Citrate synthase	Russell et al., 1998
Metalloprotease	Villeret et al., 1997
Polygalacturonase	Birgisson et al., 2003
Cellulases and xylanase	Akila and Chandra, 2003
Chitinase	Mavromatis et al., 2003
Endo-arabinanase	Sakamoto et al., 2003
Pectinase	Nakagawa et al., 2004

Due to these characteristic, cold-active enzymes are useful in biotechnology in order to shorten process times, save energy costs, decrease the enzyme concentration required, prevent undesired chemical transformations, and the loss of volatile compounds. To fulfill the demand of industries, enzyme technology needs extension of biotechnological approach in terms of both quality and quantity. Various molecular approaches such as protein engineering, r-DNA technology and metagenomic approach

could be established to achieve qualitative and quantitative improvements and develop radically novel cold-active enzymes (Kuddus, 2015).

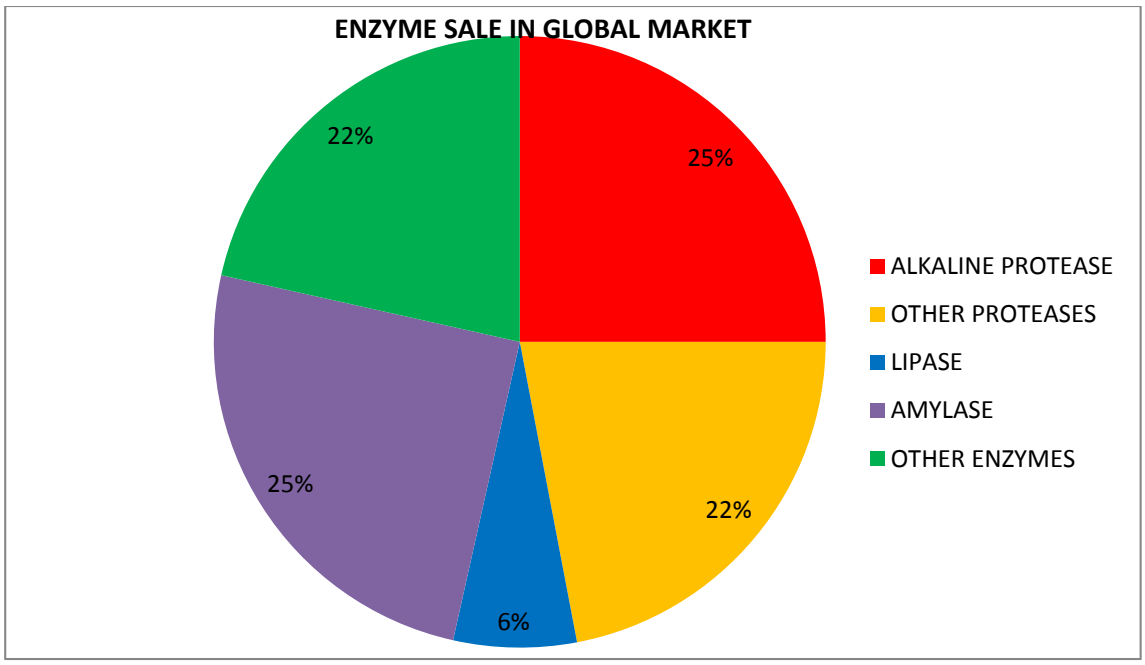


Fig. 2.1: Figure depicting sale percentage of cold active enzymes in global market

2.6. Protease

Glaciers are habitats for and provide a unique opportunity for studying the microbial diversity, ecology, and adaptation strategy of cold-adapted microbes (Segawa et al., 2005; Singh et al., 2014). In the early in 2000s Indian scientists started to explore microbial diversity and began to isolate cold adapted microorganism and their biotechnological implications (Trivedi et al., 2012). Their ability to survive in the cold environments is based on the capacity to synthesize cold-adapted enzymes, such as amylases, protease, lipase, amylase, and pectinases, cellulases etc. along with other specific characteristics (Margesin and Schinner, 1994; Kasana et al., 2008). Enzymes are non-toxic, biodegradable, and efficient/selective biocatalysts with outstanding catalytic properties, offering high levels of safety, low energy consumption, and an overall environmentally friendly production procedure (Saha and Demirjian, 2001; Dunn, 2012; Wang et al., 2012,

2016). Proteases from microorganisms represent one of the three largest groups of industrial enzymes. Nowadays, approximately 60% of the total enzyme market is shared by proteases in various industries, and according to a recent report from Business Communications Company (BCC, 2008), the global market for industrial enzymes had been estimated to reach US $ 4.9 billion by 2013 (Godfrey, 1985; Gaur et al., 2010) (Figure 2.1). According to the Enzyme Commission [EC] classification, proteases are members of the group 3 [Hydrolases], and sub-group 4 [hydrolyzing peptide bonds].

Currently, proteases are classified on the basis of four major criteria (Figure 2.2).

(I) On the basis of pH

(I) Type of reaction catalyzed,

(II) Chemical nature of catalytic site, and

(III) Evolutionary relationship with reference to structure

Proteases have been divided into two broad groups on the basis of their ability to hydrolyze N- or C- terminal peptide bonds (exopeptidases) or internal peptide bonds (endopeptidases). Although exopeptidases are used in some commercial applications, endopeptidases are industrially more important than the former. Exopeptidases are subdivided as aminopeptidases that cleave the N-terminal peptide linkage and carboxypeptidases that cleave the C-terminal peptide bond. Several other features have also been used in classifying proteases into different groups such as occurrence of charged moieties at sites relative to susceptible bond.

In 1974, the first report appeared on isolation and purification of protease from a psychrophilic *Escherichia freundii* of soil origin (Nakajima et al., 1974). After this protease producing psychrotrophic bacteria belonging to different genera ranging from *Azospirillum* to *Xanthomonas* have been isolated and characterized.

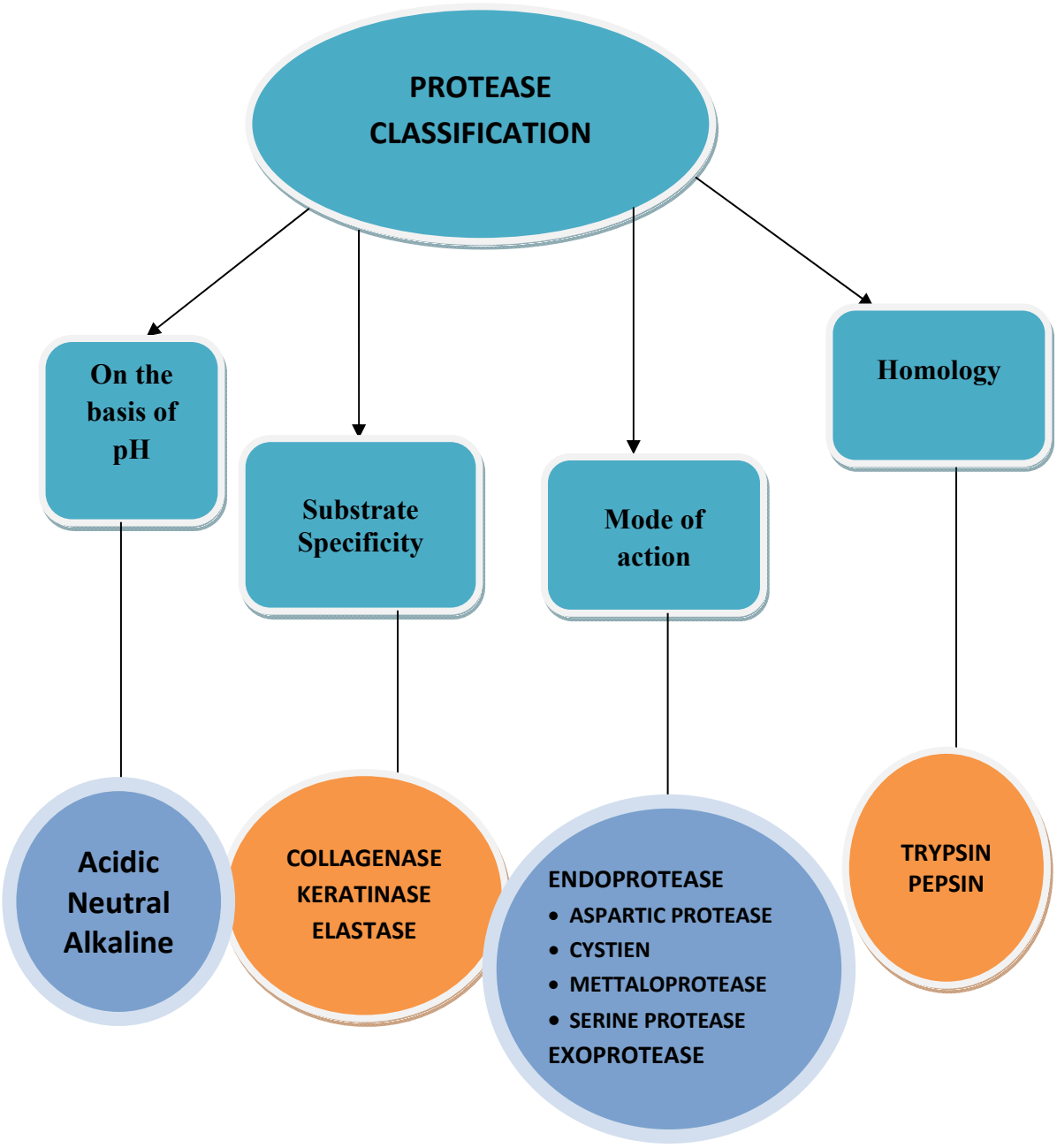


Fig. 2.2: Categorization of protease enzyme

Protease producing psychrotrophic bacteria and yeasts has been isolated from various environments such as *Azospirillum* sp. from mountain soil (Oh et al., 1999), *Bacillus licheniformis* from glacier soil (Baghel et al., 2005)*, Pedobacter cryoconitis* from glacier ice (Margesin and Schinner, 2005), *Clostridium* sp. from Antarctic region (Alam et al., 2005), *Colwellia* sp. from sea ice (Wang et al., 2008), *Curtobacterium luteum* from glacier soil (Kuddus et al., 2008), *Pseudoalteromonas* sp. P96-47 strain from Antarctic (Vázquez et al., 2008) and *Exiguobacterium* sp. from cold desert soil (Kasana, and Yadav, 2010), Gamma-proteobacterium (Sahay et al., 2013). Yu et al. (2011) screened organisms from the sandy sediment of Nella Fjord, Eastern Antarctica for the cold-active hydrolytic enzymes. Saba et al. (2012) isolated a psychrotolerant alkaline protease producing bacterium IIIM-ST045 was isolated from a soil sample collected from the Thajiwas glacier of Kashmir, India and identified as *Stenotrophomonas* sp. on the basis of its biochemical properties and 16S ribosomal gene sequencing. Cold active protease reported from *Chryseobacterium gleum* (Chaudhari et al., 2013). Borchert et al. (2017) isolate *Pseudoalteromonas* spp. from deep sea sponges to evaluate their biotechnological potential.

According to Gupta et al. (2002) proteases represent one of the three large groups of industrial enzymes and find application in detergents, leather, food, pharmaceutical industries and bioremediation processes silver recovery from x-ray films, waste management and others. Proteases play significant role in biotechnology and many industrial technologies, and are convenient tool whenever protein removal is needed (Rao et al., 1998). Cold-active proteases have been reported from various microorganisms, but detailed investigations on their adaptation to cold environments and structure and bioenergetics are scarce. Thermo and alkalostability (stability under alkaline conditions) of protease is considered to be another important character, which is

a useful criterion for application as a detergent additive due to the requirement of retention of activity during storage. Their application potential has not yet been exploited fully for the benefit of mankind. Microbes with high potential are still waiting in the cold and harsh niches. Cold active protease can be used in peeling process of leather for energy saving. For example instead of heating and bringing the temperature during the industrial peeling process of leather by conventional protease from mesophilic or thermophilic microbes, the process can be performed at the temperature of tap water by using cold-active proteases.

The highest quantities of various enzymes (protease, lipase, phosphatase, amylase, cellulase, chitinase, pectinase, etc.) are obtained when the strains are cultivated at temperatures that correspond to that of their natural environment. Most microorganisms with an optimal growth temperature around 20-25°C produce the highest enzyme yields when cultivated at 4-10°C (Margesin, 2011). The proteases from different psychrotrophic microorganisms differ in their thermo and alkalostability as reviewed by various authors (Nakajima et al., 1974; Matta and Punj, 1998; Huston et al., 2004; Tondo et al., 2004; Vazquez et al., 2004; Margesin et al., 2005; Vazquez et al., 2005). Protease from *Colwellia* sp. retained about 50% of its maximum activity for 50 min at 40°C (Wang et al., 2008) whereas; protease from *Exiguobacterium* sp. was stable at 50°C for 1 h (Kasana and Yadav, 2007). The enzyme from *Shewanella* sp. was stable at room temperature (20°C) for a week and for at least 3 h at 37°C (Irwin et al., 2001). Protease from *Clostridium* sp. retained 80% of its activity when exposed to 40 and 50°C for 30 min (Alam et al., 2005).

2.7. Lipase

Lipases (EC 3.1.1.3: Triacylglycerol acyl hydrolases) constitute the third most important category of enzymes, next to carbohydrases and proteases. They are unique in hydrolysing and synthesizing fatty acid esters in aqueous and non-aqueous media. Cold active lipases (CLPs) demonstrate high specific activity in the temperature range of 0−30°C (Feller et al., 1996). In addition to hydrolyze and or modify the carboxyl ester bonds of lipids, lipases also catalyze esterification, interesterification, acidolysis, alcoholysis and aminolysis activity on triglycerides. Structural organization of lipases consists of the alpha/beta hydrolases fold (Ollis et al., 1992; Nardini et al., 1999).

Microbial lipase was first found from *Penicillium oxalicum* in 1935 by Kirsh. Microbial lipases now a day have become backbone to industries because of their versatility with regard to various practical processes. According to BCC research report (2014), the global market size of lipases in particular is projected to reach $590.5 million by 2020, at a CAGR of 6.5% between 2015 and 2020 (Figure 2.1) (Research and Markets 2015, in report-lipase market by source, application and geography- Global forecasts to 2020 for the $590.5 million industry). Notably structure of lipase, a consequence of difference in protein sequences are main factor behind this. Hormone sensitive lipase (HSL) is a super family of lipase. Lipase has emerged as one of the leading biocatalysts with proven potential to contribute billions of dollars to be used in the biotechnology industry and is used as in the metabolism of lipids in situ and ex-situ multiple industrial applications (Benjamin and Pandey, 1998; Pandey et al., 1999). In microorganism, lipase activity identified by the formation of clear halos around colonies grown on tributyrin containing agar plates and colorimetric method used as a reference method for determination of the specificity and kinetic parameters of lipases. Soil infused with vegetable oils is a reservoir of a large and diverse microbial population.

The presence of lipases has been noticed as early as in 1901 for *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* which are now called *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively (Jaeger et al., 1999; Hasan et al., 2006). A number of cold active lipases from different sources have been studied in recent years from *Candida antarctica* lipase B, *Bacillus subtilis* lipase (Ahmad et al., 2012), *Staphylococcus hyicus* (Tiesinga et al., 2007), *Pseudomonas fluorescens* (Bofill et al., 2010). The properties of these enzyme high activity in the range of 0-20°C and high catalytic efficiency in fatty acid hydrolysis at 20°C indicate that this enzyme which can be potentially applied industrially to hydrolyze fatty acid. Another potential source of cold-active bacterial lipases is deep-see sediments. Few bacterial genera have been isolated and characterized from deep sea sediments where temperature is below 3°C. They include *Aeromonas* sp. (Lee et al., 2003), *Pseudoalteromonas* sp. and *Psychrobacter* sp. (Ohmae et al., 2012), *P. lipolyticum* (Ryu et al., 2006). Bacterial genera including *P. fragi* (Trodler et al., 2008), *Pseudomonas fluorescens* (Kim et al., 1997) and *S. marcescens* (Abdou, 2003) which produces cold-active lipases were isolated from refrigerated milk and food items. Cold adapted lipases have been screened from mountain soil (Chow et al., 2012; Ko et al., 2012), high altitude soil Taishan, China (Wei et al., 2009), deep-sea sediments (Jeon et al., 2009), marine sediment (Chu et al., 2008) and tidal flat sediment (Wu et al., 2009) Recently, *Janibacter* sp. HTCC2649 isolated from marine environment was reported to produce cold active lipase.

Lipases as biocatalysts have many favourable properties that make them suitable for specific applications compared with chemical catalysts. A number of relatively straightforward reasons for applications of cold active enzymes in biotechnology have been mentioned by various authors (Rusell, 1998; Margesin and Schinner, 1999; Gerday et al., 2000; Cavicchioli et al., 2002). Due to the specificity of lipases, the production of

unwanted products in the waste stream are decreased or eliminated. Moreover, the use of enzymes decreases the side reactions and makes post-reaction separation problems simpler (Pandey et al., 1999; Hasan et al., 2006). Thus, it can be said that lipases are environmental friendly. Additionally, lipases can carry out reactions under mild conditions of pH and temperature and this reduces energy needs to direct reactions at unusual temperatures and pressures. As a result, unstable reactants and products are protected from destruction.

Lipase production by psychrotrophic bacteria varies with species, as does the optimum temperature, optimum pH and enzyme specificity (Thomas, 1973). Choo et al. (1998) isolated a psychrotrophic bacterium producing cold adaptive lipase from Alaskan soil and identified as a *Pseudomonas* strain. They cloned and sequenced lipP gene in *E. coli*, which is correspond to a 924 bp (308 AAresidues) and was stable between pH 6.0-9.0 with optimal activity at pH 8.0.

Lipases have found broad applications in the modern food industry instead of traditional chemical processes. Nowadays, lipases are commonly used in the production of a variety of products like fruit juices, baked foods, fermented vegetables, cheese, butter, dressings, soups and sauces Tripathi et al. (2014) identified as *Microbacterium* sp. can be a good source for the production of lipase enzyme to be used in transesterification reaction for biodiesel production. Recent advances in biotechnology such as in protein engineering, recombinant methods and metabolic engineering have been employed but are yet to impact lipase engineering for cost-effective production of biodiesel (Hwang et al., 2014).

Lipases are largely employed as additives to detergents, which are used commonly in household and industrial laundry and in household dishwashers. Approximately 1000 tons of lipases are sold every year in this area. Lipases are generally

added to the detergents primarily in association with proteases and cellulases (Pandey et al., 1999). In 1994, the first commercial lipase was introduced by Novo Nordisk while Genencor International produced two bacterial lipases, which are Lumafast™ from *Pseudomonas mendocina* and Lipomax™ from *Pseudomonas alcaligenes* (Jaeger and Reetz, 1998) and also emerged as an important biocatalyst in environmental bioremediations (Digesters, composting, oil or xenobiotic biology applications), biotransformation and molecular biology applications, heterologous gene expression in psychrophilic hosts to prevent formation of inclusion bodies (Feller et al., 1996; Kuddus et al., 2008). They also help in the removal of lipid stains during paper recycling and to avoid the formation of sticky materials (Guncheva and Zhiryakova, 2011). Joseph, (2006) stated that cold active lipase has a remarkable capacity to retain its activity in presence of commercially available detergents and exhibited high efficiency for the removal of lipid stains (kitchen oil stains and used engine oil stain) from fabrics. Based on the findings of Qamsari (2011), lipase of *P. aeruginosa* KM110 is a potential alkaline lipase and a good candidate for industrial applications such as detergent, leather and fine chemical industries.

2.8. Amylase

Starch converting enzymes such as amylases are of great significance for biotechnology and represent a class of industrial enzymes having approximately 25% of the world enzyme market (Reddy et al., 2003; Rajagopalan and Krishnan, 2008) (Figure 2.1). Alpha amylase (endo-1, 4-α-D-glucan glucohydrolase, EC 3.2.1.1) belongs to the enzyme class of hydrolases which cleaves internal glycosidic linkages in starch molecules to hydrolyze them and yield dextrins and oligosaccharides. The α-amylase family is the largest family of glycoside hydrolases, transferases and isomerases

comprising nearly 30 different enzyme specificities (Henrissat, 1991). A large variety of enzymes are able to act on starch. These enzymes can be divided basically into four groups: endoamylases, exoamylases, debranching enzymes and transferases (Maarel et al., 2002).

a) Endoamylases: cleave internal α-1, 4 bonds resulting in α-anomeric products,

b) Exoamylases: cleave α-1, 4 or α-1, 6 bonds of the external glucose residues resulting in α- or β anomeric products,

c) Debranching enzymes: hydrolyze α-1, 6 bonds exclusively leaving long linear polysaccharides, and

d) Transferases: cleave α-1, 4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor forming a new glycosidic bond.

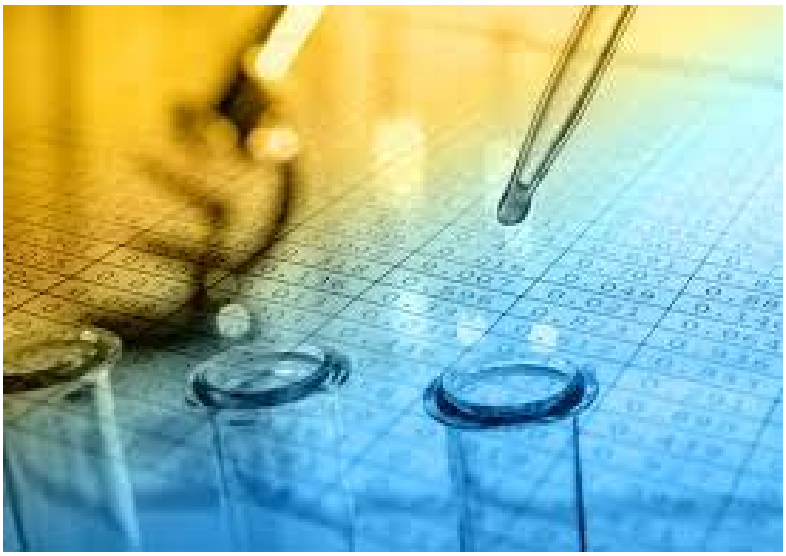
The potential of psychrophiles and enzymes produced by them have been reviewed from time to time cold-active amylase are mainly sourced from microorganisms from cold habitats such as arctic regions, polar regions, deep sea and glacier soils, glacier ice, permafrost, cold desert soil, and other cold regions on Earth. This is the most important hydrolytic enzymes and has been studied extensively. This group of enzymes represents one of the three largest groups of industrial enzymes and accounts for approximately 30% of the world enzyme market, in second place after proteases. Cold-adapted amylolytic microorganisms produce amylases, which function effectively at cold temperatures with high rates of catalysis in comparison to the amylases from mesophiles or thermopiles, which shows little or no activity at low temperature. Moreover, the maximum level of activity of these amylases is shifted towards lower temperatures with a concomitant decrease in thermal stability (Kuddus et al., 2011). The knowledge of cold active amylolytic enzymes is increasing at a rapid and exciting rate. Various cold-active or cold-adapted amylases have been reported from

microbial origins (Feller et al., 1999; Groudieva et al., 2004; Amico et al., 2006; Siddiqui et al., 2006). In order various cold adapted amylase producing bacteria have been reported by researcher worldwide such as strain A201 from Snow-covered soil of Ishikawa, Japan (Morita et al., 1997), *Arthrobacter psychrolactophilus* ATCC 700733 from Pennsylvania soil (Michael et al., 2005), *Streptomyces* 4 Alga from Antarctic vegetation (East Antarctica) (Mihaela et al., 2009).

According to Hussain et al. (2013) amylases; especially cold-active alkaline amylases can be used in detergents since washing clothes at low temperatures protect the colours of fabrics and reduce energy consumption. In food industry cold active α-amylase can be used for the reduction of haze formation in juices and retardation of staling in baking industry. Cold active α-amylase is also very useful for paper industry as it reduces the viscosity of starch for appropriate coating of paper. In pharmaceutical industry they can be used as a digestive aid. Among bacteria, *Bacillus* species is widely used for α-amylase production to meet industrial needs. Zhao et al. (2011) isolated a total of 126 bacterial strains from soil samples. Among them, 11 isolates were found positive for amylase production. Strain was identified as *Bacillus* sp. based on morphological and physiochemical characterization.

The investigation indicated that enzyme produced by the organism was stable in temperature range of 30°C to 40°C for period of 30 minutes, with maximum stability at 35°C and least at 50°C. Irfan et al. (2011) reported that α-amylase produced by *Bacillus* sp. showed optimum activity at 30°C for 20 minutes. Singh et al. (2014) partially purified and characterized *α*-amylase from *Streptomyces* sp. MSC702 in her study. Akcan et al. (2011) reported the bacterial strain *Bacillus subtilis* RSKK96 to produce extracellular α-amylase. The isolate (potential strain) was identified as *Bacillus marini* by microscopic, biochemical and molecular experiments. Optimization and

characterization can greatly affect the production cost and can lead either to profit or loss in an industry based on production of bioactive compounds by microorganisms (Swati and Narayana, 2012). Some scientist reported optimum activity in higher range such as Syed et al. (2009) reported optimal activity at 45ºC for *α*-amylase from *S. gulbargensis*. Hussain et al. (2013) reviewed the literature on the microorganism associated by the production of α-amylase on using different substrate, thermostability profile and its industrial application. In conclusion Himalayan mountain region in the subcontinent of India extends from West to East and is one of the richest bio resource of unique microflora particularly bacteria which have immense bio potential (Trivedi et al., 2012). However, in best of our knowledge, not sufficient data is available on the bacteriological analysis and enumeration of indicator bacteria from Satopanth glacier runoff for the quality assessment. Therefore monitoring and biological assessment of the glaciers is important to assess the overall reservoir health. Cold active microbial enzymes have biotechnological potential because these are active at extreme environmental conditions. Therefore current review of our study suggested that scientist should accomplish more expedition in this field.



CHAPTER 3

Materials & Methods

3. MATERIALS AND METHODS

3.1. Collection of sample

Seven soil samples were collected from seven different sites of the Satopanth glacier, for evaluation of their biotechnological applications and twelve water samples were collected from covering the stretch of Satopanth top to Badrinath ghat for the bacteriological monitoring. Satopanth glacier is situated at the head of Alaknanda valley in Western Himalaya, India. It is located between latitude 30º42'- 30º50' N and longitude 79º13'-79º29'E, and altitude is 4,600 M. It lies in the North West side of Nilkantha a major peak of the Garhwal division of the Himalayan. It sits below 2500 m (8200 ft) face of the peak originating at a height of 7000m from the peak of the Chaukhambha (7068) and the Badrinath (6974) Mountains. The glacier spreads over a long stretch of 13 km with an average width of 750 m. covering an area of 21.17 km. and the glacier melts in water at a position of 3810 m (Nainwal et al., 2008). Samples were collected with sterile spatula in sterile poly bags. The samples were brought to the laboratory under cold condition. Extreme care was taken at all times during the whole sampling processes to ensure the minimal contamination. The samples were transported to the laboratory in ice and stored at -20ºC.

3.2. Total viable counts/viable plate count method

For enumeration of the bacterial count in soil sample, one gram of respective sample were homogenized in 9 ml of cold sterilized distilled water and the suspensions was serially diluted upto 10^{-6}. The diluted bacterial suspension (0.1 ml) of various dilutions were inoculated in triplicate for each dilution of nutrient agar media and incubated for 48

hours at 20ºC. The numbers of colonies were counted and bacterial load was recorded as CFU per gram of soil by applying the formula (Devi et al., 2012a).

$$\text{No. of cell/gm soil} = \frac{\text{Mean plate count x Dilution factor}}{\text{Weight of soil}}$$

3.3. Biochemical identification and characterization of isolate

Twenty isolates were identified by studying morphological and biochemical characteristic according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). Cell shape and motility were examined on freshly prepared wet mounts by light microscopy of exponentially growing liquid culture. Gram's staining was performed as described (Madigan et al., 2004) and shape and Gram's reaction was observed under the microscope. A drop of 3% hydrogen peroxide was placed on the smear of microbial strain and observed for the formation of bubbles of oxygen as an indicative of Catalase enzyme. Reduction of nitrate to nitrite was checked by adding 0.5% α-naphthylamine and 0.8% sulphanilic acid, both prepared in 5N acetic acid. Oxidase was detected by appearance of pink or violet colour within 30sec after streaking a colony on oxidase disk. Starch and hydrolysis was checked as described by Gonzalez et al. (1978). Gelatine hydrolysis was tested in the solid medium containing 1% gelatine and a zone of clearance around the colonies was observed after flooding the colonies with 1% amido black which stains the unhydrolyzed gelatin. Acid production from dextrose, mannitol, fructose, xylose and maltose was tested in unbuffered growth medium supplemented with 1% of the above mentioned carbohydrates and sugar alcohols.

3.4. Assessment of bacterial contamination and antibiotic resistance among coliforms isolated from glacial water runoff and Alaknanda river system

3.4.1. Collection of sample

Sampling stations were selected on the basis of pollution level and population along the study stretch. Water samples were collected from twelve different sampling sites from the runoff of Satopanth glacier, which is source of Alaknanda River, in sterile glass bottles, transported on ice to the laboratory and processed within 24-h of collection. Samples were collected during the winter seasons. Study area constitute twelve sampling sites viz; Satopanth Top, Satopanth Tal, Sahshatra dhara, Chakratirath, Alkapuri, Laxmiban, Basudhara falls, Saraswati river, Narayan Parvat, Mana village, Badrinath Dham, Hanuman Chatti (SR1-SR12). Sampling sites are illustrated in Figure 4.2.

3.4.2. Multiple tube fermentation test or most probable number (MPN)

Coliform, faecal coliform and faecal streptococci count was determined by the standard most probable number (MPN) method (APHA, 1998 & 2005). The water quality was determined by the standard most probable number (MPN) method. Coliforms were detected by inoculation of samples into tubes of MacConkey broth and incubation at 37^{o}C for 48 h. The positive tubes were subcultured into brilliant green bile broth (BGBB) and were incubated at 37^{o}C for 48 h. Gas production in BGBB at 37^{o}C was used for the detection of faecal coliform after 48 h incubation. Faecal streptococci were detected by inoculation of water samples into Azide Dextrose broth and incubation at 37.5^{o}C for 24–48 h.

a. Procedure

Presumptive test: Differential medium for the isolation of coliforms was MacConkey broth Purple. Three broth tube series, the first series containing 3 double strength broth tubes and the remaining two series comprising 6 single strength broth tubes were inoculated with 10 mL, 1 mL and 0.1 mL of water (ratio 3:3:3) respectively. Tubes were incubated at 37°C and observed at 24 and 48 hours. Presumptive test is positive for coliforms if acid and gas are produced in Durham tubes.

Confirmed test: To eliminate false positives from noncoliform organisms, eosin methylene blue (EMB) agar plates were inoculated with a loopful from each positive presumptive broth tube by streaking across the agar surface. Plates were incubated for 24 h at 37°C.

Completed test: Finally, nutrient agar slants and MacConkey broth tubes were inoculated with distinct colonies picked from cultured isolates on EMB agar plates. After incubation for 24 h at 37°C, broth cultures were observed for acid and gas production and cultured isolates on agar slants was gram stained using standard technique (Chattopadhya and Basu, 1986; Niemi et al., 2003).

b. Biochemical characterization

Besides IMViC which stands for indole, methyl red, Voges-Proskauer and citrate tests, four other biochemical tests, i.e. catalase, gelatine liquefaction, starch hydrolysis and sugar fermentation were performed to confirm the identity of test isolate according to standard methods (Aneja, 2003; Cheesbrough, 2006).

3.4.3. Antibiotic susceptibility

a. Disc diffusion method

The coliform isolates identified as *E. coli* were subjected to antibiotic susceptibility by disc diffusion method (Bauer et al., 1996). Discs impregnated separately with appropriate concentrations (μcg/disc) of seven antibiotics, viz., Streptomycin (S10), Chloramphenicol (C30), Nalidixic Acid (NA30), Kanamycin (K30), Penicillin-G (P10), Tetracycline (T30) and Gentamycin (GEN10) were used in this study. Coliform isolates were subjected to antibiotic susceptibility by disc diffusion method. Antibiotic susceptibility test was performed by following method **(**Bauer et al., 1966). A loopful of fresh culture of bacterial isolates was inoculated in nutrient broth and seeded onto Muller Hinton Agar plates. The antibiotic impregnated discs (Himedia) were placed on freshly prepared lawns of each strain on agar plates, incubated at 20-37^{o}C for 24 h and examined for the inhibition zones diameters were measured and classified using reference values.

b. Procedure

Smeared inoculums from nutrient broth culture of isolated organism were spread evenly on the agar surface with a sterile swab stick. Using sterile forceps, antibiotic multidisc were placed at the centre of inoculated media. Plates were inverted and incubated at 25°C for 24 h. Thereafter, zones of inhibition around the discs were observed, their diameters measured and classified as resistant (R), susceptible (S) or intermediate (I) according to interpretive criteria defined by the Clinical and Laboratory Standards Institute (CLSI, 2007).

3.5. Screening and isolation of protease producing bacteria

Soil and water samples were collected from different regions of Satopanth glacier; seven soil samples were aseptically collected from topsoil surface. One gram of soil and one ml of water sample of each site was suspended in distilled water and mix thoroughly. Serial dilutions were prepared using sterile distilled water and 100>l aliquots plated on skimmed agar plates (pH 10.0). Plates were incubated at 4°C and 20°C temperature for 72 h in order to obtain colonial growth. Zone of casein solubilization and colony diameter were measured at every 24 h interval for 5 days for selection of alkaline protease producing organisms on solid media. Depending upon the size of the zone of solubilisation and the ratio of solubilisation zone front to colony size on media, potential strains were screened and subjected for further analysis. Total twenty one bacterial colonies were isolated on nutrient agar media from seven soil sediment of Satopanth Glacier. Out of twenty one, on the basis of zone formation, eleven isolates were screened for protease production on PSC solid agar medium containing skimmed milk at temperature 4°C and 20°C. After incubation, five isolates were found to be capable of producing protease at pH 10.0 and at two different temperatures 4^{o}C and 20^{o}C. Among them on the basis of diameter of hydrolysis zone one potent protease producing bacterial isolate SGPR10 has been taken for further purification and characterization of enzyme.

3.6. Screening and isolation of lipase producing bacteria

The bacterial isolates were screened for lipolytic activity on tributyrin agar plates. A loopful of isolate was streaked into the tributyrin medium and incubated at 20°C for 24 hours. After incubation the isolates were observed for lipolysis i.e. zone of hydrolysis around the colony. Lipase production is indicated by the formation of clear halos around

the colonies grown on tributyrin-containing agar plates (Patricia et al., 1997; Jaeger et al., 1998; Ertuğrul et al., 2007).

The bacterial isolates were screened for lipolytic activity on tributyrin agar plates. After that, all Twenty-one isolates were screened for extracellular lipase production. Out of twenty-one, on the basis of hydrolysis zone, seven isolates were screened for lipase production at low temperature i.e. 20°C. After that these colonies were incubated at two different temperature 4°C and 20°C at pH 8.0. After incubation, four isolates were found to be capable of producing lipase at both temperatures. Among them on the basis of large zone diameter one potent lipase producing isolate, SGPR4 selected for further studies.

3.7. Screening and isolation of amylase producing bacteria

The isolated colonies were streaked onto starch agar medium containing soluble starch and casein enzyme hydrolysate as main ingredients. The zone of hydrolysis was visualized by flooding the plates with Gram's iodine solution. The colonies with clear zones were evaluated as amylase producers (Chessa et al., 1999).

Out of twenty one, on the basis of zone formation, five isolates were screened for production of cold-adapted extracellular amylase on starch agar media at low temperature (20°C). Among these, three isolates were found to be capable of producing amylase at alkaline pH and at different temperature 4ºC and 20ºC. On the basis of larger clear zone formation at low temperature, i.e. on the basis of diameter of hydrolysis zone among them one potent amylase producing bacterial isolate, SGPR6 has been taken for further purification and characterization of enzyme.

3.8. Identification of potential isolates

3.8.1. Morphological and biochemical characterization

Three isolates (one protease producing, one lipase producing and one amylase) were identified by studying morphological and biochemical characteristic according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994).Cell shape and motility were examined on freshly prepared wet mounts by light microscopy of exponentially growing liquid culture. Gram's staining was performed as described (Madigan et al., 2004) and shape and Gram's reaction was observed under the microscope. A drop of 3% hydrogen peroxide was placed on the smear of microbial strain and observed for the formation of bubbles of oxygen as an indicative of Catalase enzyme. Reduction of nitrate to nitrite was checked by adding 0.5% α-naphthylamine and 0.8% sulphanilic acid, both prepared in 5N acetic acid. Oxidase was detected by appearance of pink or violet color within 30sec after streaking a colony on oxidase disk. Starch and hydrolysis was checked as described by Gonzalez et al. (1978). Gelatine hydrolysis was tested in the solid medium containing 1% gelatine and a zone of clearance around the colonies was observed after flooding the colonies with 1% amido black which stains the unhydrolyzed gelatin. Acid production from dextrose, mannitol, fructose, xylose and maltose was tested in unbuffered growth medium supplemented with 1% of the above mentioned carbohydrates and sugar alcohols.

3.8.2. 16S rRNA identification

The identification of the bacteria was confirmed by 16S rRNA gene sequencing studies. Genomic DNA was isolated by inoculating with a single bacterial colony in 50 ml LB broth to an absorbance 600 nm of 0.5–1.0 and cells were collected by centrifugation at

5000 rpm, at 4^0C, for 10 min. The genomic DNA was isolated from the given organism. Amplification of the 16s rRNA gene was performed using the universal primers.

Forward primer: 5'-AGAGTTTGATCCTGGCTCAG-3'

Reverse Primer: 5'-ACGGCTACCTTGTTACGACTT-3'

16S rDNA region amplified by PCR were sequenced by automated DNA sequencer. Sequences were compared to the non-redundant NCBI database by using BLAST, with the default settings used to find the most similar sequence. A representative sequence of most similar neighbours was aligned using CLUSTAL W2 for multiple alignments with the default settings (Sarikaya, 2000; Khan and Priya, 2011; Mahajan, 2011). The multiple alignment file was then used to create phylogram using MEGA 6 & 7 software.

3.8.3. Estimates of evolutionary divergence between sequences

The number of base substitutions per site from between sequences is shown. Analyses were conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). The analysis involved 8 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 207 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). The evolutionary history was inferred using the Neighbour-Joining method (Saitou and Nei, 1987). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches (Felsenstien, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum

composite likelihood method (Tamura et al., 2004, 2013) and are in the units of the number of base substitutions per site.

3.9. Determination of protease activity

Protease activity was measured by the modified method of Anson (1938) using casein as substrate. 0.5 ml of suitably diluted enzyme was added to 2 ml of 1% casein prepared in glycine-NaOH buffer and incubated at 37^{o}C for 10 minutes. The reaction was stopped by the addition of 3 ml of 5% tricholoroacetic acid (TCA). The mixture was allowed to stand for 30 minutes at 10^{o}C and then mixture was filtered through Whatman filter paper No. 1.

One millilitre of filtrate was placed in a 25 ml test tube with 5 ml of 0.44M sodium carbonate and incubated at 37^{o}C for 10 minutes. After that the six fold diluted Folin & Ciocalteu's Phenol reagent (one ml) was added and allowed to stand for 30 min. at 37^{0}C. The absorbance of the supernatant was measured at 660 nm by spectrophotometer. Control consisted of reaction mixture to which the enzyme was added after the reaction was stopped by addition of TCA. A standard curve was generated using tyrosine as standard (50-250 µg/ml). One unit (U) of protease activity is defined as the amount of enzyme required to liberate 1µg tyrosine per millilitre per minute under the standard assay conditions. The protein content of the cell free culture broth, crude enzyme and the partially purified fraction were determined from standard curve using BSA as standard protein.

3.9.1. Protein estimation

For total protein estimation, broth was harvested at 10,000 rpm at 4°C for 10 minutes. Appropriate aliquots from these supernatant were taken and total protein was estimated by Lowry method using bovine serum albumin (BSA) as control (Lowry et al., 1951).

3.9.2. Enzyme recovery and purification procedure

a. Ammonium sulphate precipitation

A trial was run to determine the optimal concentration required for the enzyme precipitation with various concentrations of ammonium sulphate. For this purpose, the supernatant obtained after centrifugation was subjected to ammonium sulphate fractionation. Ammonium sulphate was added at different concentrations ranging from 40 to 80% saturation. The precipitates so obtained were suspended in cold saline solution (2 ml) and tested for protease activity and total protein content. The salting out concentration of the crude enzyme was established to be 60% on the basis of enzyme activity. To obtain complete precipitation of the crude enzyme, the remaining harvest fluid was subjected to ammonium sulphate precipitation at 60% saturation. For this purpose, solid ammonium sulphate (195g) was added gradually with mechanical stirring to harvest fluid (2x600ml) at 4ºC to a saturation of 60%. The precipitate so formed was separated by centrifugation 10000 rpm for 15min., resuspended in cold saline solution (100ml) and dialyzed in cold against 1L of 0.05M Tris-HCl-0.1M NaCl (pH 10.0) for 20 hrs. After dialysis, the solution was centrifuged and supernatant obtained was designated as fraction.

3.9.3. Characterization of alkaline protease

a. Effect of temperature

The alkaline protease activity of the crude enzyme was determined after incubating the crude extract at temperatures (4ºC, 10ºC, 20ºC, 30ºC, 40ºC, 50ºC) for 1 hour and then the remaining activity was determined under the standard assay conditions. In order to determine thermal stability, the partially purified enzyme was first preincubated at different temperature and pH 10.0 for 1 h and then the enzyme assay was performed.

b. Effect of pH

The effect of pH on the activity of crude enzyme was studied by incubating the enzyme in the buffers (200 mmol) of different pH (sodium glycine, pH 11.0–12.0; diethanolamine, pH 9.0–10.0; Tris-HCl, pH 8.0; phosphate buffer, pH 6.0-7.0; and Acetate buffer, pH 4.0-5.0) for 1 hour. The remaining activity of alkaline protease was determined under standard assay conditions. For the pH stability, the partially purified enzyme was mixed/diluted in various pH buffer and incubated for 1 h at 10ºC and then the enzyme assay was performed (1%, v/v).

c. Compatibility with detergent

Compatibility with detergents/commercial detergents was performed with partially purified enzyme sample by incubating with different ionic and non-ionic detergent viz. SDS, Tween 80 and different commercial detergents like Wheel, Tide (1%, w/v or v/v) for 1 h at 20ºC and then enzyme assay was performed.

3.10. Lipase production and partial purification

The microorganism was grown at 20^{o}C in twelve, 500 ml Erlenmeyer flasks containing tributyrin broths (pH 8.0) for 24 h at incubator shaker of 150 rpm. The culture was centrifuged at 10,000 rpm for 10 min at 4^{o}C to obtain cell free supernatant. Supernatant was subjected to ammonium sulphate fractionation of 0-40%, 40-60% and 60-80%. Ammonium sulphate fraction (60-80%) having maximum activity was dialyzed (Dialysis membrane Himedia) against Tris HCL buffer (pH 8.0) for 24 h at 4^{o}C. Lipase activity was determined spectrophotometrically by measuring the amount p-nitro phenol palmitate as a substrate by following methodology of Beisson et al. (2000) with some modification.

a. Desalting (dialysis)

The dialysis tubing was boiled in distilled water (4 L) containing sodium bicarbonate (2% w/v) for 3 hours followed by boiling for 10 minutes in 1 mmol EDTA (pH 8.0). The prepared tubing was stored in ethyl alcohol at 4°C and extensively washed by distilled water before use. Ammonium sulfate precipitates were taken in a prepared dialyzing bag and placed in 2 L of diethanolamine (20 mmol, pH 9.0) containing 0.1% w/v ($ZnCl_2$, $CaCl_2$ and $MgCl_2$) at 4°C with stirring for 24 hours against 6 changes of buffer.

b. Ion exchange chromatography (DEAE-cellulose)

The ionization of proteins is pH dependent because they are amphoteric in nature. At a pH identical to the protein's isoelectric point (pI), the net charge of a protein is zero. At a pH lower than pI, the protein is positively charged, and at a pH higher than pI, the protein is negatively charged. Thus, in anion exchange chromatography the fixed charges

(stationary phase) are positive, and the displaceable charges (proteins) in the mobile phase are negative (Lee et al., 2003).

Principle

Proteins of similar charge (either positive or negative) interact with opposite charges in the stationary phase, leaving other proteins of a charge identical to the charges of stationary phase. The bound proteins can then be eluted or displaced from the stationary phase by a new counter ion or exchanger (usually NaCl), with a greater affinity for the fixed charges of stationary phase than the protein.

Procedure

In first treatment, 15 g (w/v) of the anion exchanger (DEAE-Cellulose) was suspended in 500 mmol hydrochloric acid and was allowed to stand for 30 minutes. The supernatant was decanted and the exchanger was washed until the effluent was at pH 4.0 (intermediate pH).

3.10.1. Lipase assay

Lipase activity was measured by spectrophotometer using *p* nitrophenyl palmitate as substrate. In this assay, amount of released *p*-nitrophenyl was measured at 405 nm. The reaction mixture contained 980 µl 100 mM sodium phosphate buffer including 150 mM sodium chloride and % 0,5 triton X-100, pH 7,2, 10 µl 50 mM *p*-nitrophenyl laurate and finally 10 µl enzyme solution. Blank contained the same components except enzyme solution.

3.10.2. Characterization of lipase

a. Effect of temperature

To study the effect of temperature on the activity of purified lipase enzyme, assay mixture was incubated at different temperatures ranging from 10 to 60ºC for 15 min and activity was determined. To study the enzyme stability at different temperature, purified enzyme was dissolved in 50 mM Tris-HCl buffer (pH 8.0), pre-incubated at different temperatures ranging from 10 to 60ºC for 1 h, rapidly residual activities were measured by the standard assay procedure.

b. Effect of pH

Following buffers were used for study of activity of the purified lipase at different pH citrate buffer (pH 5.0-6.5), as Tris-chloride buffer (pH 7.0-9.0), glycine-NaOH buffer (pH 11.0). Activity of the purified lipase at different pH was measured by adjusting pH of the reaction mixture using (0.1 M). The enzyme activity was assayed by method described before. To study the stability at different pH, purified lipase was dissolved in above-mentioned buffers. These enzyme solutions were pre-incubated at 20ºC for 1 h and residual activity was measured at pH 5.0-11.0.

3.11. Amylase activity

Amylase activity was determined by detecting the amount of reducing sugars liberated. The reaction mixture (1 ml) contained 0.25 ml of 2% soluble starch, 0.25 ml of 0.4MTris-HCl buffer (pH 8.0), and 0.5 ml of enzyme. The reaction was terminated by addition of 2 ml of 3, 5-dinitrosalicylic acid reagent after incubation at 35ºC for 30 min (Sengupta et al., 2000). One unit of enzyme activity was defined as the amount of enzyme that released 1 mmol of reducing sugar as glucose per minute under the assay conditions. The protein

concentration was measured with bovine serum albumin as a standard (Lowry et al., 1951). All measurements in this experiment were made in triplicate.

3.11.1. Amylase assay

The alpha amylase assay was carried out according to the method of Okolo et al. (1995). The reaction mixture consisted of 1.25 ml of 1% soluble starch, 0.5 ml of 0.2 M phosphate buffer (pH 7.0), and 0.25 ml of crude enzyme extract. After 10 min of incubation at 20°C, 3 ml of 3, 5-dinitrosalicylic acid (DNS) reagent was added and boiled for 5 minutes to stop the reaction. The liberated reducing sugars (glucose equivalents) were estimated by glucose standard curve using the 3, 5- dinitrosalicylic acid (DNS) (Miller, 1959). The colour developed was read at 510 nm using a UV-spectrophotometer. The blank contained 0.75 ml of 0.2 M phosphate buffer (pH=7) and 1.25 ml of 1 % starch solution. One unit (IU) of α- amylase was defined as the amount of enzyme releasing one μmol of glucose equivalents per minute under the assay conditions. The enzyme activities used for representations were the average values of three independent experiments. One unit of activity is defined as the amount of enzyme that produces 1 mmol of 4- nitrophenol per minute. For measurement of pH activity dependence, amylolytic activity was determined by the dinitrosalicylic method (Bernfeld 1955) using1% soluble starch (Sigma) as substrate in the above-mentioned buffer.

Total protein content was estimated by the method of Bradford (1976). An aliquot of 0.1ml of supernatant in test tube was taken and the volume made to 1ml with phosphate buffer (0.1M, pH 7.5). Then 5ml of Bradford reagent was added in test tube and mixed thoroughly. A blank containing 0.1ml of distilled water with 5ml of Bradford reagent was run parallel. The absorbance was recorded at 595 nm on a UV/VIS spectrophotometer after 5 minutes of reaction. A standard curve was plotted between

micrograms of protein (using Bovine serum albumin) as a standard and corresponding O.D. Protein contents were determined from the standard curve of BSA.

3.11.2. Characterization of amylase

a. Effect of temperature

To study the effect of temperature on the activity of purified amylase enzyme, assay mixture was incubated at different temperatures ranging from 10^{o}C to 60^{o}C for 15 min and activity was determined. To study the enzyme stability at different temperature, purified enzyme was dissolved in 50 mM diethanolamine buffer (pH 9.0), pre-incubated at different temperatures ranging from 10^{o}C to 60^{o}C for 1 h, rapidly residual activities were measured by the standard assay procedure.

b. Effect of pH

Following buffers were used for study of activity of the purified amylase at different pH citrate buffer (pH 5.0-6.5), as Tris-chloride buffer (pH 7.0-9.0), glycine–NaOH buffer (pH 11.0). Activity of the purified lipase at different pH was measured by adjusting pH of the reaction mixture using (0.1 M). The enzyme activity was assayed by method described before. To study the stability at different pH, purified amylase was dissolved in above-mentioned buffers. These enzyme solutions were pre-incubated at 20^{o}C for 1 h and residual activity was measured at pH 5.0-11.0.

Protein estimation

The method of Lowry et al. (1951) was used for the estimation of protein taking BSA (bovine serum albumin) as standard. Four solutions were prepared as following.

Procedure

From 10 mmol stock solution of BSA, different dilutions ranging from 10 to 100 µmol were prepared in 1ml of distilled water. Freshly prepared solution C (1 ml) was added in each test tube and kept at room temperature for 10 minutes. About 0.1ml of solution D was and incubated at 37°C for 30 minutes. The O.D recorded at 650 nm was plotted to calculate the slope for standard curve. The same procedure was performed for protein estimation of samples.

3.12. Calculations

Standard curve was prepared using BSA as standard and concentration of total protein in the sample was determined by the following formula:

$$\text{Protein (mg/ml)} = \frac{\text{Optical density of sample}}{\text{Optical density of standard}} \times \frac{\text{Concentration of standard}}{\text{ml of sample used}}$$

a. Specific activity

Specific activity of the sample was calculated by dividing the enzyme units (U) with the protein content.

$$\text{Specific activity} = \frac{\text{Total enzyme units (U)}}{\text{Total protein (mg/ml)}}$$

The activity of the sample is expressed in units (U) and wherever necessary as specific activity (U/mg).

b. Relative activity

It is the percentage enzyme activity of the sample with respect to the sample for which maximum activity is obtained.

$$\text{Relative activity} = \frac{\text{Activity of sample (U)} \times 100}{\text{Maximum enzyme activity (U)}}$$

c. Residual activity

It is the percentage enzyme activity of the sample with respect to activity of the control (untreated sample).

$$\text{Residual activity} = \frac{\text{Activity of sample (U)} \times 100}{\text{Activity of control (U)}}$$

3.13. Culture media / reagent

List of media and their composition

Nutrient agar (pH 7.0)

Ingredients	**g/l**
Peptone	5.0
Beef extract	3.0
Sodium chloride	5.0
Agar	18

Nutrient broth

Ingredients	**g/l**
Peptone	5.0
Beef extract	3.0
Sodium chloride	5.0

MacConkey broth

Peptone	20g
Lactose	10g
Sodium chloride	5g
Bile salt	5g
Neutral red solution	10 ml
Distilled water	1000 ml

MacConkey agar

Peptone	0g
Lactose	10g
Sodium chloride	5g
Bile salt	5g
Neutral red solution	10 ml
Distilled water	1000 ml
Crystal violet	0.001g
Agar	13.5g

Mueller-Hinton agar (pH 7.4)

Beef infusion	300
Acid hydrolysate of casein	17.5
Starch	1.5
Agar	17.0

Antibiotics Used

- Kanamycin (K 30)
- Streptomycin (S 10)
- Penicillin (P 2)
- Nalidixic acid (NA 30)
- Chloramphenicol (C 30)
- Tetracycline (T30)
- Gentamicin (HLG 120)

Luria bertani broth (LB) (pH 7.5)

Ingredients	**g/l**
Bacto-tryptone	10
Bacto-yeast extracts	5
Nacl	10

Skimmed Milk Agar (7.2)

Ingredients	**g/l**
Skim milk powder	100
Peptone	5.0
Agar	18.0

Agar was dissolved in 700ml distilled water by boiling. Poured into one litre flask and sterilized at 121ºC for 15 min. Milk powder was dissolved in 300ml water by heating at 50ºC, Sterilized at 113-115ºC (8 lbs) pressure for 20 min. The two solutions were cooled at 55ºC and mixed in one flask aseptically.

PSC Medium

Ingredients	**g/l**
Potato starch	10
Yeast extract	10
K_2HP0_4	1.0
Peptone	10
Agar	30
Trace element	1ml/l
Skim milk powder	10 (sterilized separately at 5 lb/10 min)

Tributyrin medium (pH 8.0)

Ingredients	**g/l**
Tryptone	1
Yeast extract	0.5
NaCl	1
Tributyrin	1
Gumacacia	1
Agar	1.5

Composition of trace element

Ingredients	**g/l**
$ZnSO_4$	3.5
$CaCl_2$	3.0
$MnSO_4$	1.2
$MgSO_4$	3.2

Tween 80 lipolytic agar (pH 7.2) (w/v)

Ingredients	%
Peptone	1.5%
Sodium chloride	0.5%
Calcium chloride	0.1%
Tween 80	1.0%
Agar	1.5%

Starch Agar

Ingredients	%
Meat-extract	3%
Peptic digest of animal tissue	5%
Starch, soluble	2%
Agar	15% Final
pH (at 25°C)	7.2±0.1

Directions Suspend 25 grams in 1000 ml distilled water and heat to boiling to dissolve the medium completely. Sterilize by autoclaving at 15 lbs pressure (121°C) for 10 minutes. Mix well and pour in sterile Petri plates.

Peptone water (7.2)

Ingredients	**g/l**
Peptone	10.0
Sodium chloride	5.0

Modified Luria Bertani (LB) agar, per liter

Tryptone	10 g
Yeast extracts	5 g
NaCl	5 g
Gellan gum	15 g
dH_2O up to	1 L

Rhodamine B solution (0.1% w/v)

0.1 g rhodamine B in 100 ml dH_2O.

X-GAL Stock Solution (50 mg/ml)

0.1 g x-gal in 2 ml N-N di-methyl formimide.

IPTG Stock Solution (0.1 M)

1.2 g IPTG was dissolved in 50 ml of deionized water

5X minimal salt solution

$Na2HPO_4.7H_2O$	64g
KH_2PO_4, 2,5g	15g
NaCl and NH_4Cl	5g
Distilled water	1 liter

Preparation of Reagents and Buffers

DNS reagent

Sodium sulphite	0.05 g
Potassium sodium tartarate	40.0 g
3, 5 Dinitrosalicylic acid	1.0 g
Sodium hydroxide	1.0 g
Phenol	0.2 ml
Distilled water	100 ml

Mixed the entire above ingredient in distilled water except. Reagent was stored in dark glass stopper bottle in refrigerator.

Reagent for protein estimation

Copper reagents

(a) 1% cupric sulphate (hydrated)

(b) 2% sodium potassium tartarate

(c) 3% alkaline sodium carbonate

All three a, b and c was taken in 1:1:100, respectively.

Foline and Cicalteu's Phenol reagent

Commercially available 2N, finally used 1N for protein estimation.

Bovine serum albumin (BSA)

20 gm of BSA was dissolved in 100ml of water to get final concentration of 200/ml.

Bradford Reagent

Brilliant blue dye	100 mg
Ethanol (95%)	50 ml
Phosphoric acid (85%)	100 ml

Mixed all above ingredient and dilute to 1 litre with distilled water and filtered through whatmann filter paper and kept in glass stopper bottle (Amber bottle) in refrigeration.

Phosphate buffer (pH 7.0) 50 mM

KH_2PO_4	13.609 GM/500 ml
K_2HPO_4	17.418 GM/500 ml

94.7 ml of B was taken and added 5.3 ml of (A) to get the desired pH 7.0 and finally made up to volume 200 ml with H_2O for the preparation of 200 Mm buffer.

Sodium phosphate buffer (100 mM), pH 7.2

0,3g $NaH_2PO_4.H2O$ and 1,09g Na_2HPO_4 (anhydrous) dissolved in 100 ml water and then 150 mM NaCl and % 0.5 Triton-X was added.

Sodium acetate buffer (50 mM), pH 4 to 5

0.4 g Sodium acetate dissolved in 100 ml water and then its pH was adjusted with acetic acid.

Potassium phosphate buffer (50 mM), pH 6 to7

0.68 g potassium dihydrogen phosphate dissolved in 100 ml water and then pH was adjusted with phosphoric acid.

Tris-HCL biffer (50 mM), pH 8

0.6 g tris base dissolved in 100 ml water and then pH was adjusted with HCL.

Glycine-NaOH buffer (50 mM), pH 9 to 12

0.36 g gylcine dissolved in 100 ml water and then pH was adjusted with NaOH.

***p*-nitrophenyl laurate solution (50 mM)**

1, 6 g *p*-nitrophenyl laurate dissolved in 100 ml acetonitrile.

Citrate phosphate Buffer

0.2M Di sodium hydrogen phosphate	3.56 g
Distilled water	100 ml
0.1M Citric acid solution	1.92 g
Distilled water	100 ml

30% Acrylamide & N, N-methylene bis acrylamide

29% (w/v) acrylamide

1% (w/v) N, N-methylene bisacrylamide

Should be prepared in deionised warm water pH=7 or less. Store the solution in dark bottle at RT (fresh solution should be prepared every few months)

Acrylamide and Bisacrylamide are potent neurotoxins.

10 % SDS

10% (w/v) should be prepared in deionised water and store at RT.

TEMED

TEMED accelerates the polymerization of acrylamide & bisacrylamide by catalyzing the formation of the free radical from ammonium per sulphate. Polymerisation inhibited at low pH.

Ammonium per sulphate

It provides free radicals. 10% (w/v) prepared in deionized water stored at 4°C.

Tris Glycine electrophoresis buffer (Tank Buffer)

25 mM Tris base

250 mM Glycine

0.1% SDS

For 5X buffer

Tris base 15.1 gm + 94 gm glycine dissolve in 900 ml deionized water. Then add 50 ml of a 10% (w/v) stock solution of SDS and adjust to 1000ml with water.

Staining solution

0.25 gm of Coomassie Brilliant Blue R250 Dissolve in 90 ml of methanol + water (1:1) + 10 ml glacial acetic acid

Destaining solution

Distilled water	530 ml
Acetic acid	70 ml
Methanol	10 ml

Methanol:	Water	90 ml
	Acetic acid	10 ml

Gel Loading Buffer

2 X SDS-gel Loading Buffer	
100 mM Tris. Cl (pH 6.8)	1 ml
SDS	400gm
Bromophenol blue	2g
Glycerol	200 ml
β-mercaptoethanol	1.5 ml
D.W	1000 ml
Store at RT	

Separating buffer (1.5 M Tris HCl, pH 8.8)

The 1.5 M Tris HCl buffer was prepared by dissolving 181.5 g Tris base in 900 ml of distilled water with constant stirring to adjust the pH 8.8 by adding concentrated HCl (32 % v/v) drop wise. After pH adjustment raised the final volume up to 1000 ml with distilled water.

Stacking buffer (1 M Tris HCl, pH 6.8)

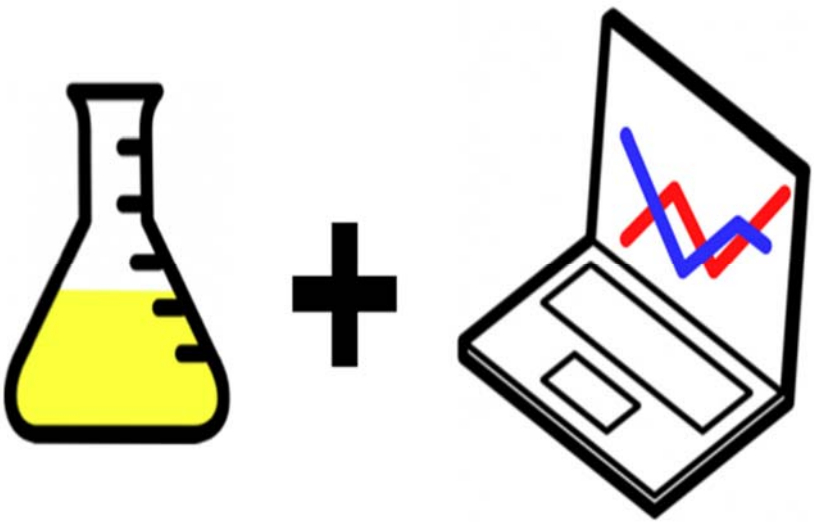
The 1 M Tris HCl, buffer was prepared by dissolving 157.6 g Tris base in 900 ml of Distilled water with constant stirring to adjust the pH at 6.8 by adding concentrated HCl (32 % v/v) drop wise. After pH adjustment, the final volume was raised up to 1000 ml with distilled water.

0.1 M Potassium phosphate buffer (pH 7):

Dissolve 34.0 gm of KH_2PO_4 in 250 ml of water and 45.6 gm of K_2HPO_4 in 200 ml of water. Add the 2^{nd} solution to the 1^{st} one to get 0.1 M of potassium phosphate buffer of pH 7.

0.1 M Glycine NaOH buffer (pH 10):

Add 75 gm of glycine and 10gm of NaOH to 1L of water and pH is adjusted to 10 to obtain 0.1M glycine-NaOH buffer of pH 10.



CHAPTER 4

Results & Discussion

4. RESULTS AND DISCUSSION

4.1. Quantitative and qualitative estimation of study area

The Himalayas possesses one of the larger resources of snow and ice and there by act as a large reservoir of diverse microbial community. Due to the unreceptive climatic conditions prevailing in Himalaya makes it an interesting habitat to study phylogenetic and functional diversity. In 1992, the International Centre for Integrated Mountain Development (ICIMOD) highlighted the necessity of considering mountain biodiversity as a repository of elements for future prospection and insisted on the urgent need of protecting this biodiversity (Jodha et al., 1992).

The cold habitats span from the Arctic to the Antarctic include the high-mountains such as Himalayas, the alpine glaciers, the deep ocean, snow, permafrost, sea ice, lakes, cold soils (especially sub-soils), cold deserts, and caves. Among them glacier region has several distinct habitats such as glacial ice, and soil sediment, rivers, and lake, etc. These habitat colonized by microorganism that have been adapted to survive at low temperature. Microorganisms living on these cold places are mainly bacteria, yeasts, fungi and algae, and this biodiversity has been extensively reviewed (Cowan et al., 2013; Yumoto, 2003). Although the widely held of biological studies done on cold environment have focused on mainly bacteria. Appropriate management of microbial diversity include mainly soil inhabiting bacteria, has an important role in towards large-scale industrial and commercial applications and in addition helpful for scientist who monitoring glacial retreat and recession. Recently researchers around the world are putting a lot of plunge towards understanding the pathways involved in production of novel secondary metabolites from cold adapted microbes and to understand their functional and phylogenetic diversity and response under changing abiotic and biotic

factors as they are answerable for functioning of the ecosystem (Bulat et al., 2004; Fell et al., 2006). Although over the world the majority of the cold adapted microorganism, still remain hidden and need to be explored and can be utilized for humankind. Therefore there is huge scope to explore the unexplored habitats such as study area, to isolate, characterize and report the microbial diversity. Furthermore, Monitoring and biological study of the glaciers is important to assess the overall reservoir health.

The availability of soil and ice from the Satopanth glacier provided a special opportunity to examine the diversity and possible activity of bacteria present in ice and soil sediment. Plate counts were performed in triplicate at seven sampling site and the results are presented as the means of the plates. Total viable count of seven soil samples from Satopanth glacier are summarized in Figure 4.1. Results indicate that total viable count in soil from Satopanth glacier varied from 2.7×10^4 to 12.7×10^4 cfu g^{-1}soil at 20±2ºC although in glacier runoff and Alaknanda River system, total viable count increase dramatically because of human activity. The lowest bacterial count was found at (SG-4) while highest bacterial count was found at prior to Satopanth Tal (SG-7). In similar study, soil samples collected from Gangotri glacier contains elevated bacterial count varying from 10×10^6 to 15×10^6 cfu/gm of soil the total viable count of aerobic heterotrophs ranged from 3.4×10^3 to 8.0×10^3 cfu/ml of water (Joseph et al., 2012). Comparatively to present study Baghel et al. (2005), Sood et al. (2008) have also observed high TVC values in the Gangotri glacier, Western Himalaya region. The results suggested that the soil of Satopanth glacier could be an affluent source of technologically significant microbial pool. One of the objectives of the present study is to evaluate the bacterial diversity of the soil samples from the different regions of Satopanth Glacier, Western Himalaya. With this in view, seven soil sediment samples were collected from uppers stretch of Satopanth and twelve water samples from glacier runoff for further study of biotechnological implication of cold adapted bacteria

and for assessment of pollution load respectively. The viable bacteria in samples determined on the basis of the colony forming units ranged from 10^4 to 10^7 cells per gram of soil sediment sample. Of the numerous CFUs appearing on the culture media plates, fifty seven distinct representative isolates were picked both based on unique morphotypes and also randomly from each plate for further studies. Due to their biotechnological importance twenty one isolates from upper stretch of glacier were selected and studied in detail to establish their taxonomic identity and their phylogenetic position.

Random selected twenty one isolates from nutrient agar plates and their morphological and biochemical identification are summarized in Table 4.1 and Table 4.2 respectively. The culture colonies were of different colours as white, cream, yellow, orange and grey (Annexure I). The colonies were characterized by entire margin; opaque and smooth texture; and convex, pulvinate to raised elevation. The shapes of bacterial cells were cocci and short to long rods. Most of isolates showed growth temperature at 4°C and 20°C, except three isolate at 30°C. Optimum temperature for growth was 15°C. The representative strains were, therefore, obligately and psychrotrophic in nature. All the isolates were analyzed by polyphasic taxonomy (Cowan and Steel, 1993) where in the phenotypic, chemotaxonomic characteristics were studied in an attempt to identify all the isolates up to the genus level. The results indicated that the twenty isolates belong to five different genera.

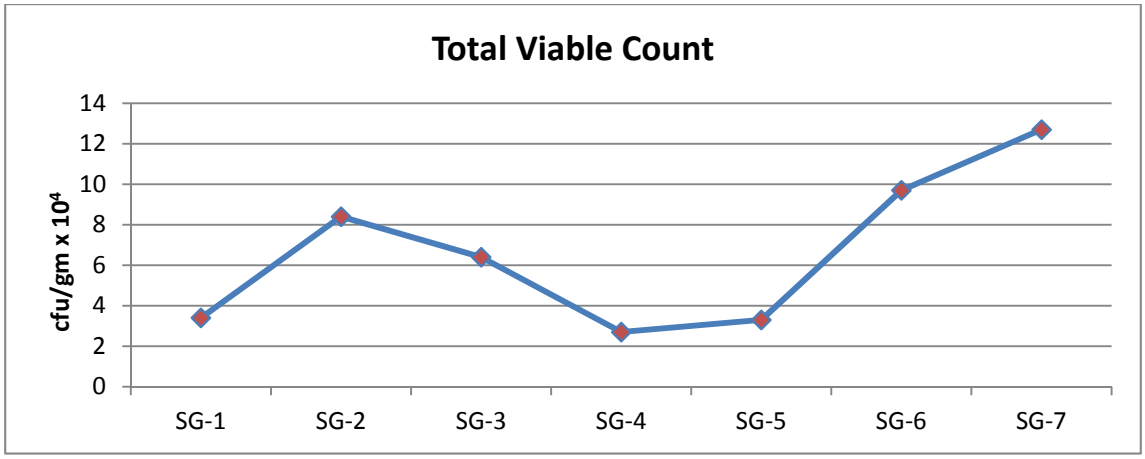


Fig. 4.1: Total viable count from soil samples of Satopanth glacier

Table 4.1: Random selection of isolates from seven different soil samples from Satopanth Glacier

Sl.No	Sampling Site		Isolate Code
1.	Satopanth Glacier	SG-1	SGPR1, SGPR2
2.		SG-2	SGPR3, SGPR4, SGPR5
3.		SG-3	SGPR6, SGPR7, SGPR8, SGPR9
4.		SG-4	SGPR10
5.		SG-5	SGPR11, SGPR12
6.		SG-6	SGPR13, SGPR14, SGPR15, SGPR16
7.		SG-7	SGPR17, SGPR18, SGPR19, SGPR20, SGPR21

Table 4.2: Morphological and biochemical characteristics of isolates

Isolate code	SGPR1	SGPR2	SGPR3	SGPR4	SGPR5	SGPR6	SGPR7	SGPR8	SGPR9	SGPR10	SGPR11	SGPR12	SGPR13	SGPR14	SGPR15	SGPR16	SGPR17	SGPR18	SGPR19	SGPR20	SGPR21
Colony Morphology																					
Colony Colour	Cream	Cream	Yellow	Greenish	Cream	Orange	Greenish	Creamy White	Red	Peach	Yellow	Yellow	Orange	Creamy White	Yellow	Reddish	Greenish	Greenish	Creamy	Yellow	Creamy White
Colony Shape	Round	Round	Round	Rod	Round	Round	Round	Rod	Round	Rod	Round	Round	Rod	Cocci	Round	Rod	Cocci	Round	Cocci	Cocci	Round
Colony Texture	Rough	Rough	Rough	Smooth	Rough	Smooth	Rough	Smooth	Rough	Smooth	Rough	Rough	Smooth	Smooth	Smooth	Rough	Rough	Rough	Smooth	Rough	Smooth
Colony Margin	Undulate	Undulate	Undulate	Undulate	Rays	Entire	Undulate	Rays	Undulate	Entire	Entire	Undulate	Undulate	Entire	Entire	Flat	Flat	Entire	Undulate	Undulate	Entire
Colony Elevation	Raised	Raised	Convex	Raised	Convex	Convex	Raised	Convex	Raised	Raised	Convex	Raised	Raised	Convex	Raised	Convex	Convex	Convex	Raised	Raised	Raised
Colony Opacity	Opaque	Opaque	Translucent	Opaque	Translucent	Opaque	Translucent	Translucent	Opaque	Translucent	Translucent	Opaque	Opaque	Translucent	Opaque	Translucent	Opaque	Translucent	Opaque	Opaque	Opaque
Cell Size	Long	Long	Long	Long	Short	Short	Short	Short	Long	Short	Short	Long	Long	Long	Long	Long	Long	Long	Long	Long	Long
Gram reaction	+	+	_	_	+	+	+	_	+	+	_	_	+	+	_	+	+	+	+	+	+
Motility	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Spore	+	+	-	-	ND	_	_	-/+	_	_	_	_	–	+	_	+	–	+	+	+	+
Biochemical characterization																					
Catalase	+	+	+	+	+	+	_	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	_	+	+	-	+	+	+	+/-	+	+/-	+	+/-	+	+	+
Methyl Red	_	_	_	_	_	_	+	-	–	+	-	-	–	ND	_	ND	–	ND	_	_	_
Indole	_	_	+	+	+	_	_	+	+	_	+	+	+/-	_	+	_	+	_	_	_	_
Voges Pras-okauer Test	+	+	_	_	_	+	_	_	–	_	_	_	–	ND	_	ND	+	ND	+	+	+

Citrate Utilization	+/-	+/-	+	+	_	ND	_	+	ND	+	+	+	ND	_	+	_	_	_	+/-	+/-	+/-
Nitrate Reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND	+	ND	+	+	+
Nitrite Reduction	+	+	+	+	+	+	+	+	+	+	+	+	+	ND	+	ND	+	ND	+	+	+
Starch Hydrolysis	+	+	+	+	ND	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+
Urea Hydrolysis	_	_	_	_	ND	_	+	_	+	+	_	_	+	_	_	-	+	_	_	_	_
Gas Production from Glucose	_	_	_	_	_	_	–	_	+	_	_	_	–	ND	_	ND	–	ND	_	_	_
H2S Production	_	_	_	_	ND	_	+	_	+	_	_	_	+	ND	_	ND	+	ND	_	_	_
Gelatine Liquefaction	+	+	+	+	_	ND	–	+	-/+	ND	+	+	–	ND	+	ND	–	ND	+	+	+
Acid Production from Carbohydrates																					
Dextrose	+	+	_	_	_	+/-	+	_	+	-	_	_	_	_	_	_	+	+	+	+	+
Mannitol	+	+	+	+	ND	+	+	+	+	+	+	+	ND	+	+	+	ND	+	+	+	+
Sucrose	+	+	_	_	_	+	–	_	–	+	_	_	+	+	-	+	+	+	_	+	+
Xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	_	+	+	ND	_	+	+	_
Galactose	+	+	+	+	+	+	–	+	–	+	+	+	–	_	+	_	_	_	+	+	+

(+ Positive, - Negative, ND =Not determined, +/- Variable)

Tables 4.2 summarizes the phenotypic and chemotaxonomic characteristics of representative isolates, which were identified as the genera *Bacillus, Pseudomonas, Micrococcus*, *Stenotrophomonas*, *Arthrobacter*. Among twenty one isolates SGPR (1, 2, 6, 10, 19, 20 & 21) showing characteristics similarity with other *Bacillus*. Some of them such as SGPR4, SGPR6, SGPR10, have been identified at the species level for the duration of further study. In past more than 100 species of psychrophiles have been identified and reported which comprises of both Gram-negative and Gram-positive bacteria from various habitats ranging from soil, sandstone, fresh water and marine lakes, sea ice and oceans. Various species within the genera *Alcaligenes, Alteromonas, Aquaspirillum, Arthrobacter, Bacillus, Bacteroides, Brevibacterium, Gelidibacter, Methanococcoides, Methanogenium, Methanosarcina, Microbacterium, Micrococcus, Photobacterium, Polaribacter, Polaromonas, Psychroserpens, Shewanella* and *Vibrio* have been reported to be psychrophilic (Margesin and Miteva, 2011). Moreover, culture dependent and culture independent based microbial diversity from soils, snow and water of three major glaciers of Western Himalayas, Pindari (Shivaji et al., 2011b), Kafini (Srinivas et al., 2011) and Roopkund (Pradhan et al., 2010) were reported recently. These microbial collections from cold environment provide ready availability for carrying out specific investigations and developing the novel products.

4.2. Microbiological monitoring and assessment

According to Water Quality Management District (WQMD) Glaciers and icecaps constitute about 1.76% of global water resource and contribute to the majority of the riverine system (WQMD and ILEC, 1995). Glaciers are formed solely on land and are distinct from the much thinner sea ice and lake ice that form on the surface of water bodies and its store of enormous water resources but with increasing anthropogenic

activities around the glacier region, deterioration of glacier runoff is expected. Although glacier water considered as pristine or contamination free while in past coliforms have been reported, from the Scott expedition in Antarctica over 50 years previously (Sneath, 1962). Water pollution through various pathways and agents can cause in clinical disorders of intestinal infections both in animals and humans. The detection of pathogenic bacteria would, of course, be the most direct evidence of risky contamination; But these pathogens are present, are so poor that the technical difficulty of isolating them, and they are very sensitive, difficult to isolate. Therefore, Pipes (1981) suggested that the higher the level of indicator bacteria, the higher the level of faecal contamination and the greater the risk of water-borne diseases. The use of bacteria as water quality indicators can be viewed in two ways, first, the presence of such bacteria can be taken as an indication of faecal contamination of the water, taken as a signal to determine why such contamination exists, how serious it and what steps can be taken to eradicate it; second, their presence can be taken as an indication of the potential danger of health risks (Papini et al., 2005; McQuaig et al., 2006). The riverine area of Indian Himalayan region consists of a number of microorganisms including *Enterobacter*, *Proteus* and *Staphylococcus* etc. (Shah et al., 2006). Enteric bacterial pathogens have been reported to thrive for long periods in water in spite of a large number of antagonistic populations i.e. there is a high possibility of resistance being spread by ARB (antibiotic resistant bacteria) from the environment to related human pathogenic microorganisms through numerous routes thereby suppressing the effectiveness of antibiotics (Threedeach et al., 2012). The incidence of antibiotic-resistant bacteria in aquatic environments have increased worldwide as a consequence of the widespread use of antibiotic (Borgen et al., 2000; Iversen et al., 2002; Baghel et al., 2003; Martinez et al., 2009a; 2009b; Davies and Davies, 2010). Antibiotic resistance has become a major health concern; thus, there is a growing interest in exploring the

occurrence of antibiotic resistance as well as the factors that contribute to their emergence (Rodriguez et al., 2015; Tripathi and Tripathi, 2017). Commensal bacteria from the intestinal microbes, such as coliforms, can play a decisive role in the spread of resistance within a community (Purohit et al., 2017). Satopanth, in Western Himalaya, is the largest valley-type glacier after Gangotri, Dokiryani glaciers of the Indian continent. Every year, thousands of pilgrims, tourists, students, and explorers visit Satopanth tal, Badrinath dham and Alaknanda rivers. Since our observations may be useful in monitoring of water quality and conservation of glaciers and the associated riverine system.

Water samples have been collected from twelve different sampling sites from glacier runoff for microbiological monitoring and assessment (Figure 4.2). The TVC from glacial runoff and Alaknanda river system value showed a regular trend (Figure 4.3). The highest TVC was noted in Badrinath ghat of Alaknanda River and Mana village, where the values were as high as 86.3 $\times 10^4$ and 68.5 $\times 10^4$, respectively. The lowest value 8.4 $\times 10^4$ and 12.3 $\times 10^4$ recorded in Satopanth top and Narayan Parvat, respectively. Log count of total coliform, faecal coliform faecal streptococci count has been represent in Figure 4.4 while, plates and tubes showing positive growth represented in Annexure V (A & B). The total coliform count was moderate in all water samples, values ranged from 8/100 to 1100/100 ml MPN. The highest MPN (1100/100 ml) was recorded at Badrinath temple ghat of Alaknanda, the least count MPN (8/100 ml) was obtained from Satopanth top. Pathak and Gopal (2007) attempt to assess the bacteriological contamination in glacial water runoff from the Gangotri glacier and Gangetic river system (Gaumukh to Rishikesh) by enumerating aerobic heterotrophs count and the total viable count of aerobic heterotrophs ranged from $3.4x10^3$ – $8.0\ x10^3$ cfu/ml. In the present study, moderate coliform load has been registered during the entire

course of study. The higher values of MPN for coliforms were recorded at SR-10 and SR-11, mainly attributed to the domestic sewage and municipal wastes.

Results for FC and FS counts have also shown a similar trend to TVC and TC. The MPN of faecal coliform organisms fluctuated from 2 to 75 MPN/100 ml i.e., Highest FC count was observed in Alaknanda at Badrinath (SR-11) 75 MPN/100 ml and lowest count was at Satopanth top (SR-2) 2 MPN/100 ml. Similar trend was also observed in FS, the higher count in Alaknanda was at Hanuman Chatti 25 MPN/100 ml, lowest at Chakratirath (SR-4) 4 MPN/100 ml, while in Satopanth top (SR-2) and Sahshatra dhara (SR-3) no faecal streptococci has been reported. Due to strict guidelines of government there is less disturbance in natural environment of Gangotri glacier national park, however some sites showing lack of anthropogenic activity and its reflects low FC/FS count.

Sati et al. (2011) also study was undertaken to investigate the water quality of Alaknanda and Bhagirathi rivers (tributaries of River Ganges) in Garhwal Himalayan region during the periods of monsoon, summer and winter seasons. They reported the highest MPN (310/100 ml) was recorded during monsoon at Basudhara falls of Alaknanda, the least count MPN (24/100 ml) was obtained in summer and winter season from Bharionghat of Bhagirathi.

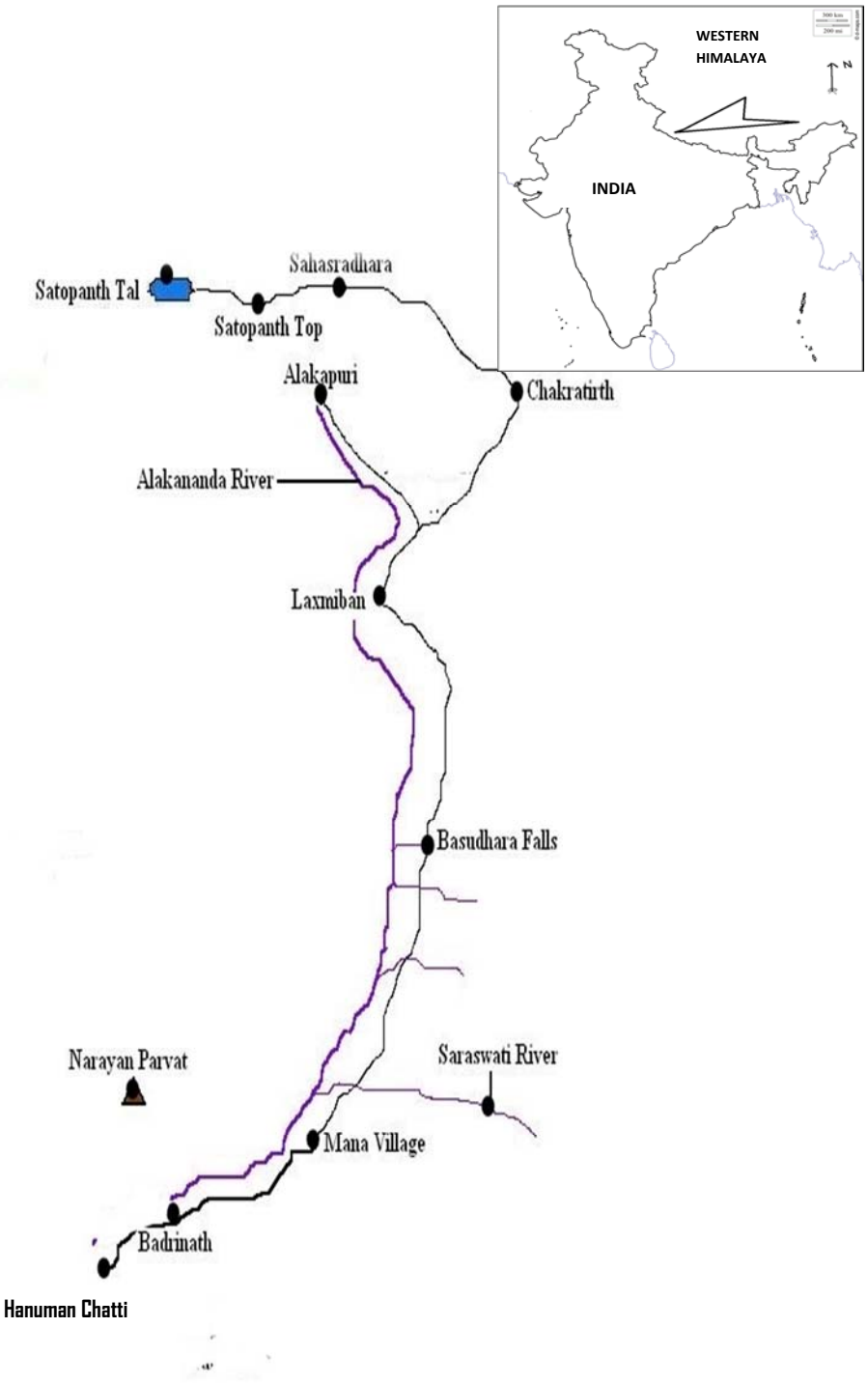


Fig. 4.2: Sampling sites in the study stretch of the Satopanth glacier and the Alaknanda river system in the Himalayan region of India

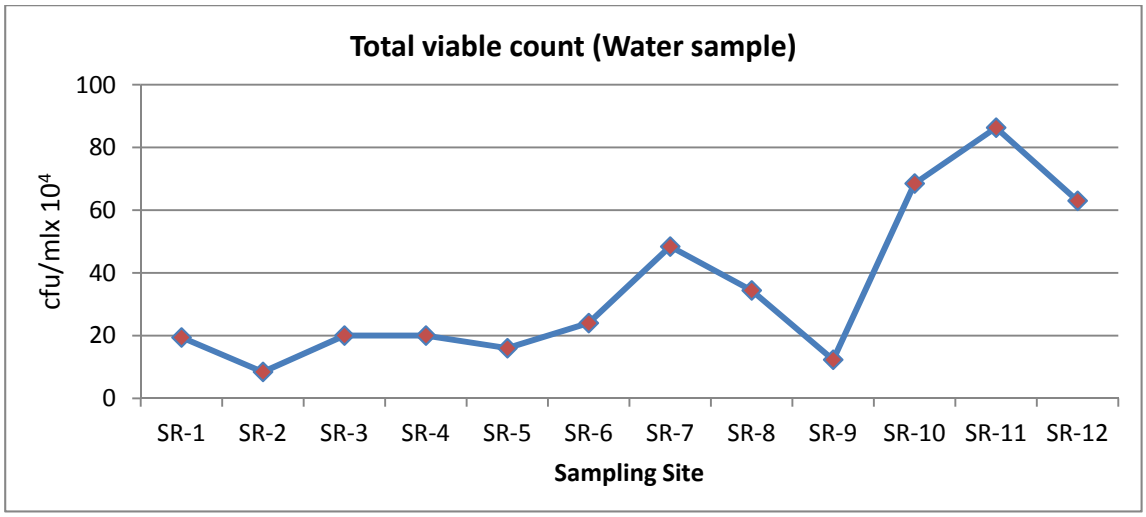


Fig. 4.3: Total viable count of runoff of Satopanth glacier (Water Sample)

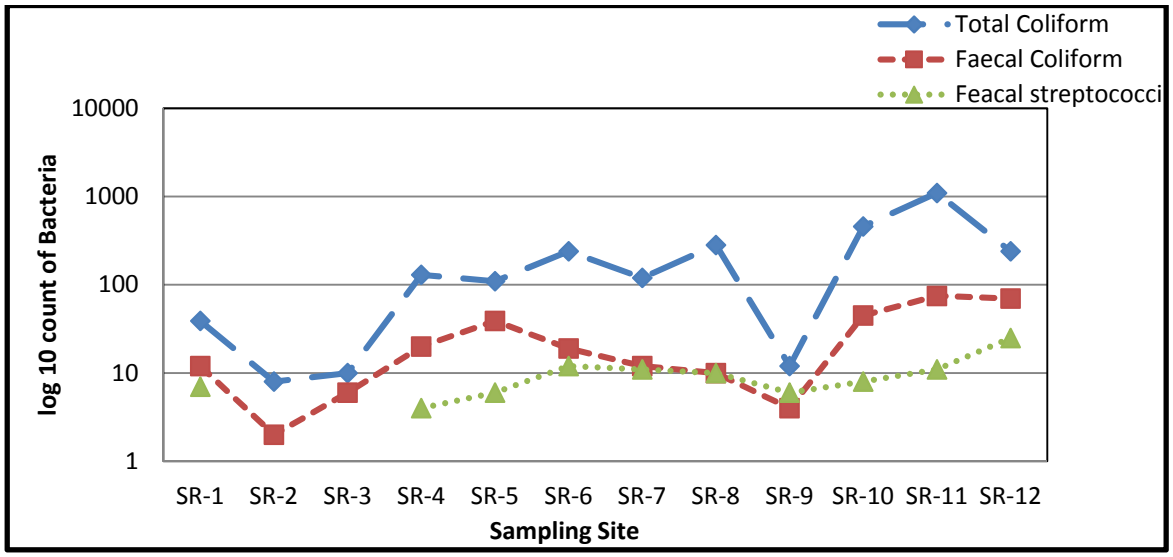


Fig. 4.4: Population density of pollution indicator bacteria (___♦__Total coliform), (-----■----- Faecal coliform) and (.....▲..... Faecal streptococci) in samples of glacial water runoff of Satopanth glacier and Alaknanda river system

In the present study the highest TVC was noted in Badrinath ghat of Alaknanda River and Gangotri of Bhagirathi River, where the values were as high as 22.2×10^3 and 19.8×10^3, respectively and total coliform count was values ranged from 24/100 ml to 310/100 ml. Moreover, Baghel et al. (2005) find the range of MPN 100 ml^{-1} for total coliform was found 34-1600, 300-1600 and 40-1600, for faecal coliform, it was 2-500, 2-900, 2-900 and for faecal streptococci, it was 33-900, 40-900 and 40-1600 in winter, summer and monsoon, respectively from Gangotri glacier. The bacteriological analysis revealed that all the samples collected from 12 different sites of glacier runoff and Alaknanda river system were contaminated with coliforms, faecal coliform while faecal streptococci is not abundant. This may be because large number of pilgrims and trackers visiting the Satopanth tal and Badrinath Dham. It is clear from the result that faecal streptococci were found minimum in glacial runoff and maximum in Alaknanda river system.

Water quality of an Alaknanda river shows significant deterioration in view of global standards. Almost all of the water samples were contaminated with coliform bacteria, resulting mainly due to anthropogenic activities, predominantly discharging of domestic and agricultural wastes directly into the river as some recent studies revealed that coliform count has positive relation with anthropogenic activities (Sadat et al., 2011). Occurrence of coliforms in the glacier regions is also confirmed by study conducted by Lateef et al. (2005) reporting high coliform counts in the spring water of Kashmir valley. Our study revealed that in upper stretch, large number of wild animals can put some load of faecal streptococci, which may be due to runoff of water having excreta of these animals from upper stretch to lower stretch of river. Probably, faecal coliform (FC) / faecal streptococci (FS) ratio more than one may have indicated contamination by human faecal matter (Araujo et al., 1989).

Although, resistance against common antibiotics among the bacterial population in glacier and glacial runoff regions with low anthropogenic activities is very unusual, some of the *E. coli* isolates from Alaknanda river system exhibited multiple antibiotic resistance (MAR). There have been twelve coliform isolated from twelve different sampling site (Table 4.3) and their resistant patterns have been illustrated in Table 4.4. Maximum resistance (for four out of seven antibiotics) was observed for Nalidixic followed by Kanamycin, Tetracycline, and Gentamycin in the isolates from Badrinath Dham (Table 4.4; Annexure VI). While minimum resistance (for one antibiotic) was found in isolates from three stations namely, Chakratirath, Alkapuri and Narayan parvat. While isolates from three stations viz; Satopanth tal, Satopanth top and Shahsradhara showing susceptibility for all seven antibiotics. From past research, Pathak and Gopal (2007) reported resistance against common antibiotics among the bacterial population in glacial and glacio-fluvial regions with low anthropogenic activities is very unusual, most of the *E. coli,* isolates from pre-Gangotri samples exhibited multiple antibiotic resistance (MAR). Rabbia et al. (2016) also reported strains isolated from Antarctic bird faeces, concluded that naturally occurring antibiotic resistance in *E. coli* strains isolated from Antarctic bird faeces and Fildes Peninsula treatment plants.

In general, in present study the isolates were remarkably susceptible to antibiotics, which would be expected as regards environmental factor. Significant increase in bacterial contamination in glacier region shown by pollution indicator bacteria can pose a risk to public health, particularly due to the emergence of MAR among them. Therefore this study may be relevant and useful for conservation of glacial as well as river system for the safety of aquatic life environment and human health.

Table 4.3: Selection of isolates (*E. coli*) for study of antibiotic resistance pattern

Sampling site		**Isolated strains**
Satopanth Glacial runoff and Alaknanda river system	SR-1	SGPRTC-1(b)
	SR-2	SGPRTC-2(a)
	SR-3	SGPRTC-3(d)
	SR-4	SGPRTC-4(a)
	SR-5	SGPRTC-5(a)
	SR-6	SGPRTC-6(b)
	SR-7	SGPRTC-7(c)
	SR-8	SGPRTC-8(d)
	SR-9	SGPRTC-9(c)
	SR-10	SGPRTC-10(a)
	SR-11	SGPRTC-11(c)
	SR-12	SGPRTC-12(d)

Table 4.4: Resistance to selected concentration (µg) of antibiotics among coliforms in water samples from different stations of Satopanth glacial water runoff and Alaknanda river system

Isolates Code	ANTIBIOTIC DISC						
	Streptomycin S10	Chloram-phenicol C30	Nalidixic Acid NA30	Kanamycin K30	Penicillin-G P10	Tetracycline T30	Gentamycin GEN10
SGPRTC-1(b)	S	S	S	S	S	S	S
SGPRTC-2(a)	S	S	S	S	S	S	S
SGPRTC-3(d)	S	S	S	S	S	S	S
SGPRTC-4(a)	S	S	S	S	R	S	S
SGPRTC-5(a)	S	S	S	S	R	S	S
SGPRTC-6(b)	S	S	S	S	R	R	S
SGPRTC-7(c)	S	S	S	S	R	S	R
SGPRTC-8(d)	S	S	S	R	S	R	S
SGPRTC-9(c)	S	S	S	S	S	S	R
SGPRTC-10(a)	R	S	S	R	S	R	S
SGPRTC-11(c)	S	S	R	R	S	R	R
SGPRTC-12(d)	S	S	S	S	R	R	R

(S=Sensitive, R= Resistant, I=Intermediate)

Consequently, the main purpose of analyzing the microbiological parameters is to develop measures that can serve as indicators of water pollution and protection of these systems can be regulated by regular monitoring of microbial load.

4.3. Protease

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields (Rao et al., 1998). Although proteolytic enzymes can be obtained from animals and plants but microorganisms are the preferred source for industrial applications in view of scientific and economical advantage (Kasana, 2010). Among various groups of microbes, psychrotrophs are ideal candidates for enzymes production keeping in mind that enzyme active at low temperature and stable under alkaline condition, these enzymes have applications in different industries like detergent, food, pharmaceutical, silk, leather and also for environmental bioremediation (Anisworth, 1994; Gupta et al., 2004).

4.3.1. Isolation, Characterization, Identification and Phylogenetic Analysis of Protease Producing Bacteria

The potential use of microorganism as biotechnological source of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Alva et al., 2007). The cold adapted enzymes have been evolved in psychrophilic bacteria as a strategy for low temperature adaptation (Singh et al., 2012; Gerday, 2013). The cold adapted enzymes are being characterized on the basis of their catalytic activity with respect to temperature and these enzymes exhibit optimum activity at <200°C (William et al., 1985; Theresa et al., 1998). Economic benefits can be achieved by using cold-active proteases as they allow working at low temperatures even

in an industrial scale. Moreover, research on cold adapted microorganisms is far behind that of thermophile for several years, psychrophiles have been employed to develop cold-active detergents, cosmetic treatments, and processed foods.

Cold adapted bacteria are a goldmine of proteases and represent the source option of enzymes in view of rapid growth at low temperature, less space required for cultivation and access to genetic manipulation (Margesin, 2011). Glaciers are supposed to be highly diverse in bacterial population surviving at low temperature therefore various proteolytic bacteria such as *Pseudomonas aeuroginosa*, *Bacillus subtilis* and *Bacillus licheniformis* were also isolated from the soil sample of Gangotri glacier (Baghel et al., 2005, Kuddus et al., 2014). Among cold adapted bacteria, *Bacillus* sp. is specific producers of extracellular alkaline proteases. The high density of proteolytic bacteria in glacier may be due to increasing human activities in the form of pilgrims and scientific expedition that causes environmental pollution and ecological disturbances. Moreover, the study of these bacterial isolates and their extra cellular enzymes will be valuable to build up desired microbial consortium for industrial applications.

Twenty one isolates from seven soil samples of glacier were tested for protease production on PSC agar medium containing skimmed milk at low temperature 20ºC. Eleven isolates screened for protease production, among these, five isolates were found to be capable of producing protease at alkaline pH and at two different temperature 20ºC and 4ºC and alkaline pH 10.0 (Table 4.5 and 4.6). Whereas strain SGPR10, yielded highest proteolytic activity with a ratio of solubilisation zone front to colony diameter followed by strain SGPR21, SGPR9, SGPR1 and SGPR3 at both temperature 4°C and 20°C. Solubilisation zone diameters of these five isolates are depicted in the Figure 4.5. Therefore one potent protease producing bacterial isolate, designated as SGPR10 has been taken for further purification and characterization of enzyme.

Table 4.5: Isolates showing protease activity at temperature 20°C and pH 10.0

Sl. no.	Isolates	Temperature (°C)	pH	Zone
1.	SGPR1	20	10	++
2.	SGPR3	20	10	+
3	SGPR5	20	10	+
4	SGPR8	20	10	+
5	SGPR9	20	10	++
6	SGPR10	20	10	+++
7	SGPR14	20	10	+
8	SGPR16	20	10	+
9	SGPR18	20	10	+
10	SGPR19	20	10	+
11	SGPR21	20	10	++

(+Positive, ++Moderate Positive, +++Strong Positive, -Negative)

Table 4.6: Isolates showing protease activity at temperature 4°C and pH 10.0

Sl. no.	Isolates	Temperature (°C)	pH	Zone
1	SGPR1	4	10	++
2	SGPR3	4	10	++
3	SGPR5	4	10	+
4	SGPR8	4	10	+
5	SGPR9	4	10	+
6	SGPR10	4	10	+++
7	SGPR14	4	10	+
8	SGPR16	4	10	+
9	SGPR18	4	10	+
10	SGPR19	4	10	+
11	SGPR21	4	10	+++

(+Positive, ++Moderate Positive, +++Strong Positive, -Negative)

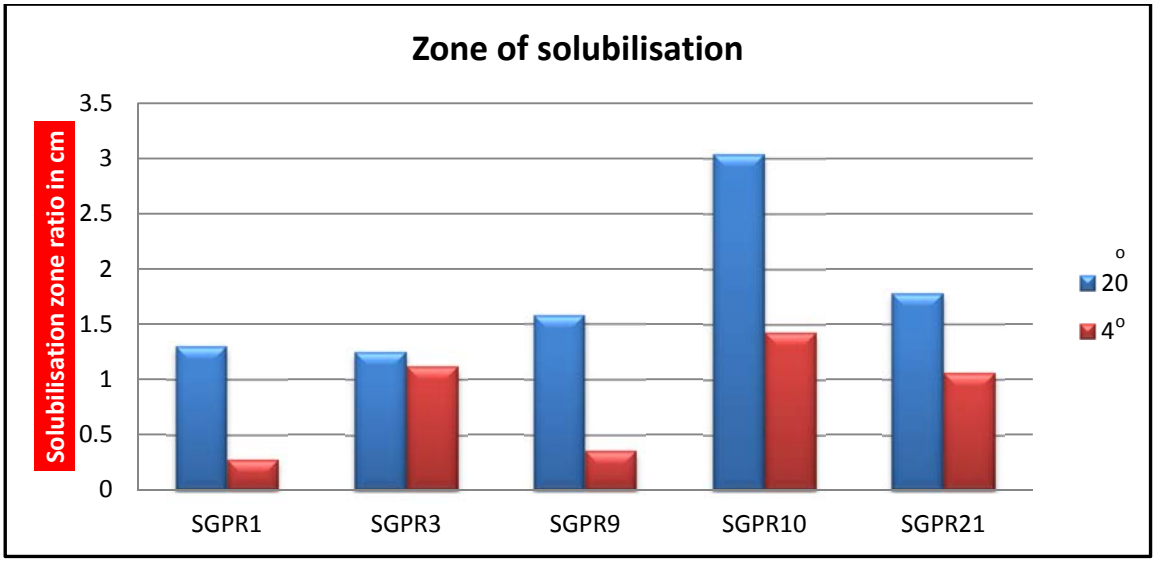


Fig. 4.5: Ratio of solubilisation zone front to colony diameter

4.3.2 Identification of potential protease producing strain, SGPR10

The isolate were identified by studying morphological and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1994). Detailed morphological, physiological and biochemical tests of the isolate are given in Table 4.7. SGPR10 was a Gram positive non-motile rod. The strain was found to be positive for catalase, Vogues-Proskauer and starch hydrolysis. The strain utilized citrate and was found to be negative for indole production. However acid production from sugar such as glucose and maltose got variable results. The data obtained with regard to the biochemical properties taken together confirmed that the strain belong to the *Bacillus* genera. The isolate SGPR10, was found to grow well between 4°C to 30°C showing an optimal growth at 20°C and variable result for 40°C and grow well between pH between 6.0-10.0. The isolated strain SGPR10 thus can be classified as a psychrotroph according to the definition of Morita et al. (1997).

Phylogenetic analysis

The evolutionary distances were computed using the Maximum composite likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 12 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 1450 positions in the final dataset (Annexure VII). The optimal tree with the sum of branch length 0.07813360 is shown. Taxonomic affiliation of the 16S rRNA sequences of the isolate, SGPR10 was retrieved from classifier programme of Ribosomal Database Project II version 9.0 (http://rdp8.cme.msu.edu/html). The 16S rRNA sequence of the isolate was blasted using megablast tool of GenBank (http:// www.ncbi.nlm.nih.gov). Phylogenetic and molecular evolutionary analysis was conducted using MEGA version 7 (Tamura et al., 2013). 5µl of DNA samples were resolved on 1% Agarose gel at 80V for 60 min and gel was visualized under UV light and the image was capture (Figure 4.6). For the tree construction four different out groups were used which were shown in the Figure 4.7 The forward and reverse sequences SGPR10 which we got after sequencing were aligned with the maximum homology sequence of *Bacillus aryabhattai* B8W22 and *Bacillus megaterium* ATCC 14581, respectively (Table 4.8). A phylogenetic tree was constructed based on bacterial 16S rRNA sequences, showed a close relationship between the strain, SGPR10 and the genus *Bacillus*. Based on the morphological, physiological, biochemical characteristics and phylogenetic analysis the isolate, SGPR10 was identified as *Bacillus aryabhattai.*

Table 4.7: Morphological, physiological and biochemical characteristics of the isolate

Morphological Tests	**SGPR10**
Colony morphology	
Configuration	Irregular
Shape	Round
Margin	Entire
Elevation	Flat
Surface	Smooth
Pigment	Initial brown later peach
Opacity	Raised
Size	Short
Cell shape	Short Rod
Gram's reaction	Positive
Spore(s)	+
Motility	Non Motile
Physiological tests	
Growth at temp.	
4ºC	+
10ºC	++
20ºC	++
30ºC	+
40ºC	+/-
Growth at pH	
pH 5.0	+/-
pH 6.0	+
pH 7.0	++
pH 9.0	+++
pH 10.0	++
pH 11.0	+
Growth under anaerobic condition	-
Biochemical Tests	
Indole	-
Voges proskauer Test	+
Citrate utilization	+
Starch hydrolysis	+
Urea hydrolysis	+
H_2O_2 production	+
Catalase test	+
Acid production from carbohydrates:	
Arabinose	+
Sucrose	+
Galactose	+
Glucose	+/-
Dextrose	+
Maltose	+/-

(+Positive, ++Moderate Positive, +++Strong Positive, -Negative, +/Variable)

Table 4.8: Alignment of nucleotide sequence of sample SGPR10 with respect to 11 closely related homologous sequence

Sl. No.	Description	Max score	Total score	Query cover	E value	Ident	Accession
1	*Bacillus aryabhattai* strain B8W22 16S	2727	2727	100%	0	99%	NR_115953.1
2	*Bacillus megaterium* strain ATCC 14581	2711	2711	100%	0	99%	NR_117473.1
3	*Bacillus megaterium* strain NBRC 15308	2707	2707	99%	0	99%	NR_112636.1
4	*Bacillus megaterium* strain IAM 13418 1	2684	2684	99%	0	99%	NR_043401.1
5	*Bacillus flexus* strain IFO15715	2615	2615	100%	0	99%	NR_024691.1
6	*Bacillus flexus* strain NBRC 15715	2607	2607	99%	0	99%	NR_113800.1
7	*Bacillus megaterium* strain ATCC 14581	2600	2600	95%	0	99%	NR_116873.1
8	*Bacillus flexus* strain SBMP3	2580	2580	97%	0	99%	NR_118382.1
9	*Bacillus qingshengii* strain G19	2559	2559	97%	0	99%	NR_133978.1
10	*Bacillus simplex* strain DSM 1321	2532	2532	97%	0	99%	NR_115603.1
11	Bacillus *taiwanensis* strain FJAT-14571	2236	2236	94%	0	99%	NR_136461.1

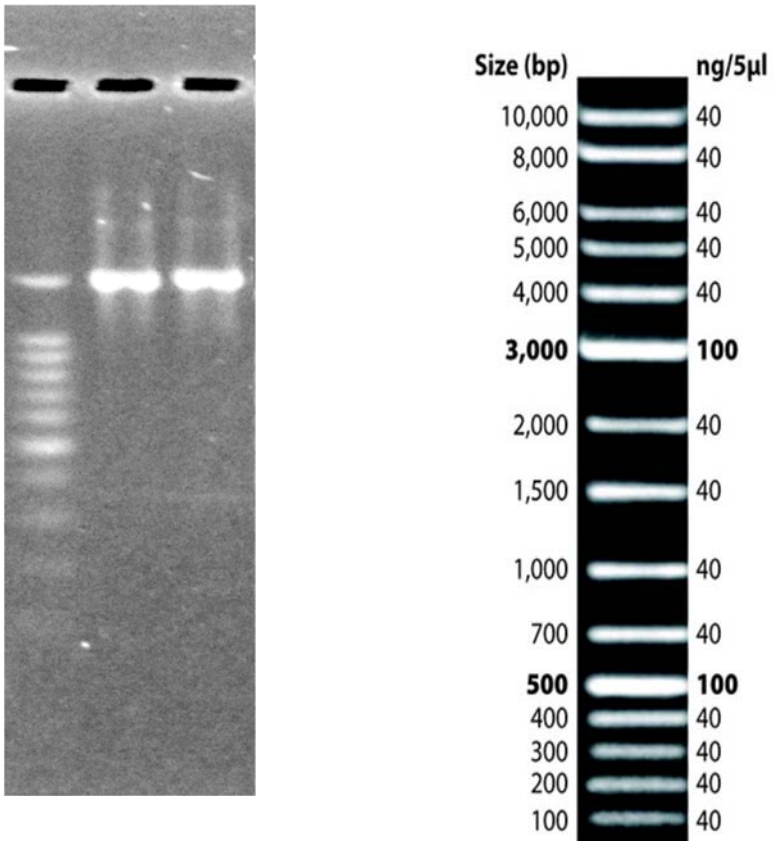


Fig. 4.6: Electrophoretic Analysis of Amplified Product

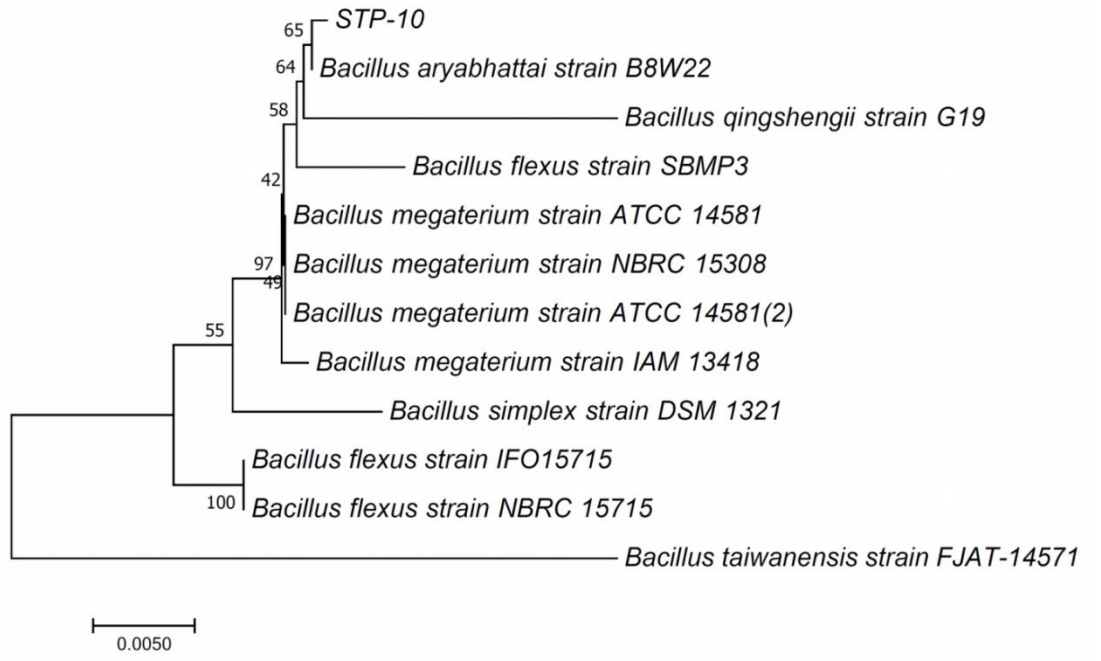


Fig. 4.7: Phylogenetic tree showing relationship of a novel protease producing *Bacillus aryabhattai,* SGPR10 (STP10) with other *Bacillus* species

4.3.3. Partial Purification of Alkaline Protease from Isolated Strain *Bacillus aryabhattai*, SGPR10

Proteases have been screened and partially purified from different sources. Different strategies have been employed for purifying cold-active proteases from diverse sources. The objective of purification was to get rid of unwanted protein, while retaining the enzyme activity. Five isolates were found to be capable of producing protease at alkaline pH and at different temperature ranging from 4ºC and 20ºC. Among them one potent protease producer SGPR10 had been selected for partial purification.

Extracellular protease from *Bacillus aryabhattai*, SGPR10 culture was subjected to partial purification and the summary of the partial purification procedure is illustrated in Table 4.9. Enzyme was partially purified to 3.1 purification fold with the increase of specific activity 1328.32 U/mg and yield of 31.5% by ammonium sulphate fractionation and dialysis.

Table 4.9: Partial purification summary of protease from *Bacillus aryabhattai,* SGPR10

Steps	Total activity (Unit)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	76320	178.2	428.28	1	100
Ammonium Sulphate precipitation (40-60%)	24056	18.11	1328.32	3.1	31.5

In an enzyme catalyzed process, enzyme cost is a single obstacle limiting expansion of industrial enzyme technology (Ward and Young, 1988). Psychrotolerant proteolytic bacterium *S. maltophilia* MTCC 7528 was isolated from soil of Gangotri glacier, Western

Himalaya, India that produced maximum protease (56.2 U/ml) at 20°C and pH 9.0 by Kuddus and Ramteke (2011). Baghel et al. (2005) also reported *Bacillus subtilis,* from soil of Gangotri glacier, produced protease (48.76 U/mg) at 20ºC and pH 10.0.

4.3.4. Characterization of Partially Purified Protease Enzyme

Effect of Temperature on Activity and Stability

Proteases produced by microorganisms are predominantly extracellular in nature and are greatly affected by nutritional and physicochemical factors. Numerous studies have shown that cold active proteases are generally characterized by higher turnover numbers (Kcat) and catalytic efficiencies (Kcat/Km) at lower temperature compared to their mesophilic counterparts (Kasana, 2010). The optimum temperature for enzyme production was depending on the type of organism, the medium conditions and the type of enzyme (Bamidele, 2014).

The optimum temperature for enzyme activity of *Bacillus aryabhattai,* SGPR10, was measured by determining its hydrolytic activity at different temperatures 4ºC to 50°C for 30 min at pH 10.0. The relative activities at different temperatures were plotted with respect to the maximum activity at particular temperature considered as 100%. The enzyme in present study displayed significant activity within a temperature range of 4ºC-50ºC with optimum activity at 10ºC.

In order to examine the temperature stability of the protease, the protease solution in Glycine-NaOH buffer (pH 10.0) was determined by assaying the caseinolytic activity at different incubation temperatures 4, 10, 20, 30, 40, and 50ºC. The enzyme retained more than 90% of its maximum activity after 1 h exposure to temperatures of 10–30ºC and 1520% after 1 h exposure at a temperature of 40–50ºC. Results is also similar to the

report of Saba et al. (2012) in which she reported that *Stenotrophomonas* sp. isolated from Kashmir, India shows optimal activity at low temp i.e. 15^{o}C.

The effect of temperature on the activity and stability of protease enzyme have shown in Figure. 4.8 and 4.9, respectively. Enzyme from *Bacillus aryabhattai*, SGPR10 shows optimum activity at temperature 10^{o}C and the enzyme was found stable between 10-30^{o}C.

Cold active protease from *Bacillus* reported by Furhan and Sharma (2014) showed maximum activity at pH 12.0 and at temperature 10^{o}C, proving that the isolate is cold active and can be very useful tool in biotechnology industries. Moreover, Antarctic isolates produced active extracellular proteases when growing at 4^{o}C and 18^{o}C but not at 30^{o}C (Rosales and Sowinski, 2011).

At temperatures above and below these points, there was reduction in protease activity. It was also earlier reported that a *Bacillus subtilis* ITRCGG3 isolated from Gangotri glacier showing optimum activity at 20^{o}C and stability between 10-30^{o}C (Baghel et al., 2005). Moreover, in my study alkaline protease was also found stable at temperature between 10-30^{o}C. Similar to the result reported by Kuddus and Ramteke (2008) and Turkiewicz et al. (2003), cold active protease from psychrotrophic bacteria belonging to various genera display maximal activity at 20–30°C. The poor thermal stability of psychrophilic enzymes, which facilitates their rapid inactivation by a moderate rise in temperature, is also advantageous in some technologies.

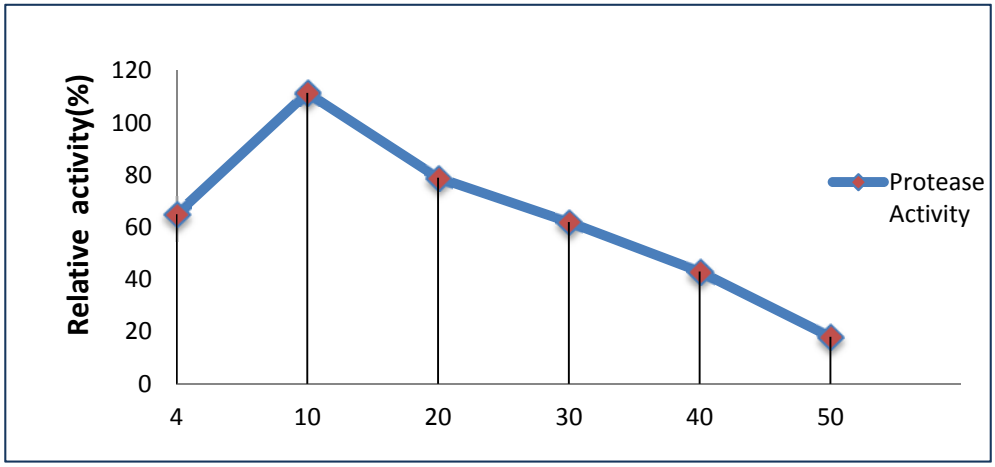


Fig. 4.8: Effect of temperature on activity of protease enzyme

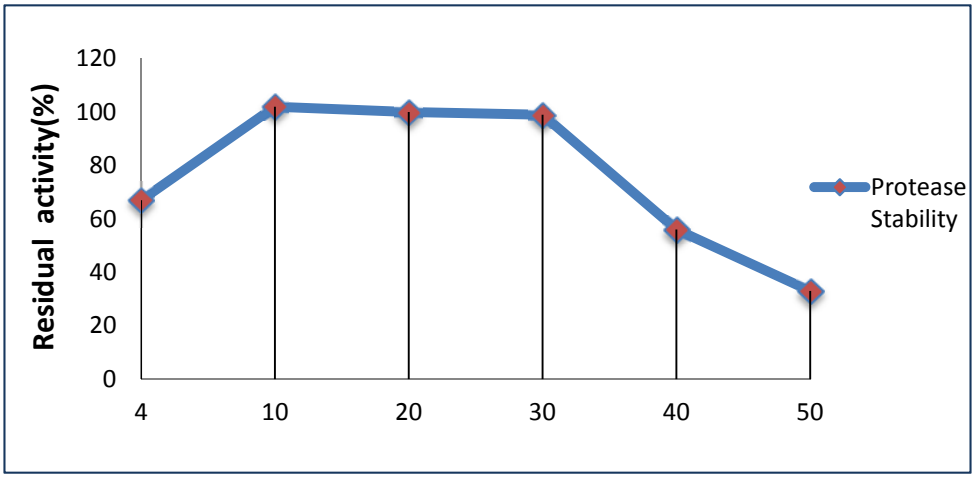


Fig. 4.9: Effect of temperature on stability of protease enzyme

Effect of pH on activity and stability

The optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (pH 5.0-11.0) at temperature 10^{o}C. Substrates (1% casein) were prepared in different buffers, 50 mM: Sodium acetate buffer pH 5.0; Tris-acetate buffer pH 6.0; Tris-acetate buffer pH 7.0; Tris-HCl buffer pH 8.0 and 9.0; Glycine-NaOH buffer pH 10.0 and 11.0. The values were expressed in terms of relative activity at different pH with respect to the maximum activity at particular pH considered as 100%.

The enzyme exhibited highest activity in the pH range 9.0-10.0 with maximum activity at pH 10.0. Activities at pH 6.0, 9.0 and 11.0 were 25.6%, 98.3% and 33% respectively, of the optimum activity at pH 10.0. No protease activity was obtained below pH 5.0 and at pH above 11.0.

The effect of pH on stability of the enzyme was studied by using casein as a substrate under the standard assay condition. The pH-stability profile of the protease as determined by the residual activity measurement showed 90% of its original activity was retained between pH 8.0–10.0.

The effect of pH on the activity and stability of protease enzyme have shown in Figure 4.10 and 4.11, respectively. Enzyme from *Bacillus aryabhattai*, SGPR10 shows optimum pH 10.0 and the enzyme was found stable between 8.0-10.0.

These results indicated that the extracellular protease from *Bacillus aryabhattai,* SGPR10 is a neutral to alkaline in nature. Optimal proteolytic activity occurred at neutral or moderate alkaline pH for the protease and this value is in accordance to those reported by Doddapaneni et al. (2007) and Tariq et al. (2011). The optimum pH range of *Bacillus* alkaline proteases is generally between pH 9.0 and 11.0 with a few exceptions of higher pH optima of 11.5 (Fujiwara and Yamamoto, 1987), 11-12 (Kumar and Takagi, 1999),

12-13 (Takami et al., 1989). Almost similar results were observed by Margesin et al. (1992) alkaline protease of the genus *Bacillu*s show an optimal activity at stability at alkaline pH values. In broad-spectrum, all currently used detergent compatible proteases are alkaline in nature therefore they are suitable for laundry detergents, which is generally in the range of 8.0 to 12.0 (Rao, 1998; Gupta et al., 2002).

4.3.5. Compatibility with commercially available detergents

Partially purified enzyme from *Bacillus aryabhattai*, SGPR10 was incubated with different detergent viz. SDS, Tween 80 and different commercial detergents like Wheel, Tide(1%, w/v or v/v) for 1 h at 20ºC and then enzyme assay was performed. The enzyme exhibited unusual stability in presence of 1% SDS with 60.61% residual activity after 1 h at 20ºC. The enzyme also exhibited enhanced activity in detergent and commercial detergents Tween 80, Wheel and Tide respectively (Figure 4.12; Table 4.10).

The economic importance of alkaline proteases came to light when bacterial alkaline proteases from *Bacillus* were introduced in 1960s to the detergent industry, accounting for about 35% of the total microbial enzyme sales (Kalisz, 1988; Outtrup, 1990). On the other hand nowadays pollution is great concern towards mankind in this way many researchers are seeking optional treatment for waste at low temperature therefore it can be better option to treat waste at low temperature.

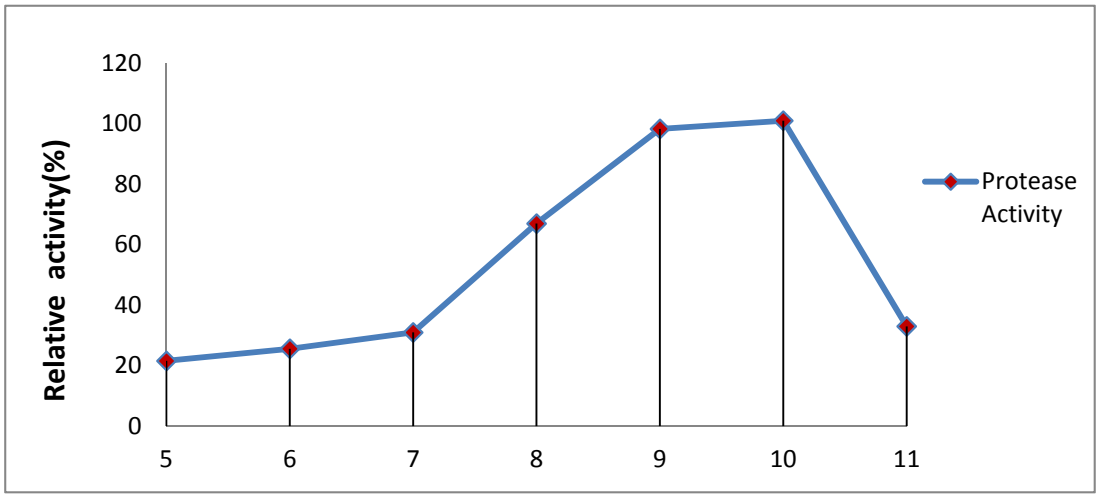


Fig. 4.10: Effect of pH on activity of protease enzyme

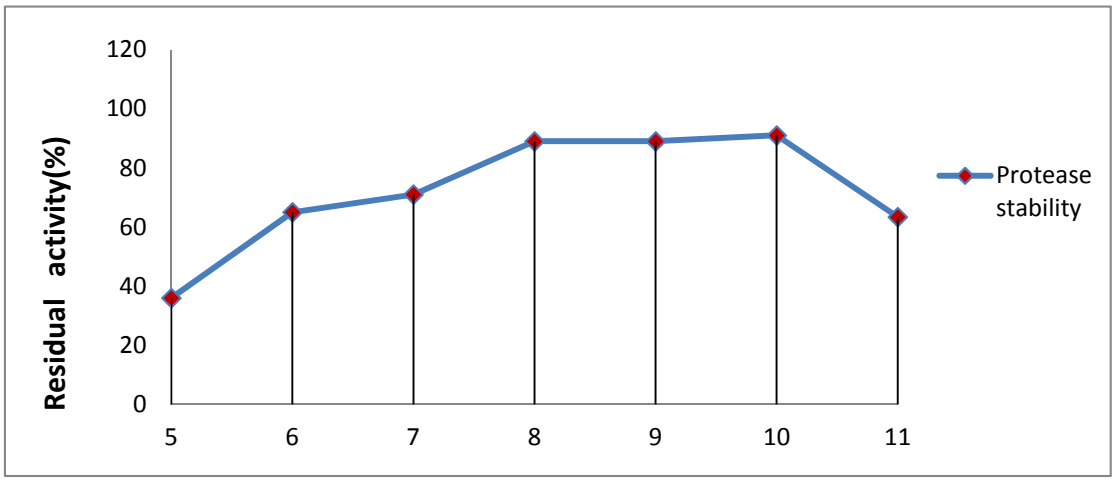


Fig. 4.11: Effect of pH on stability of protease enzyme

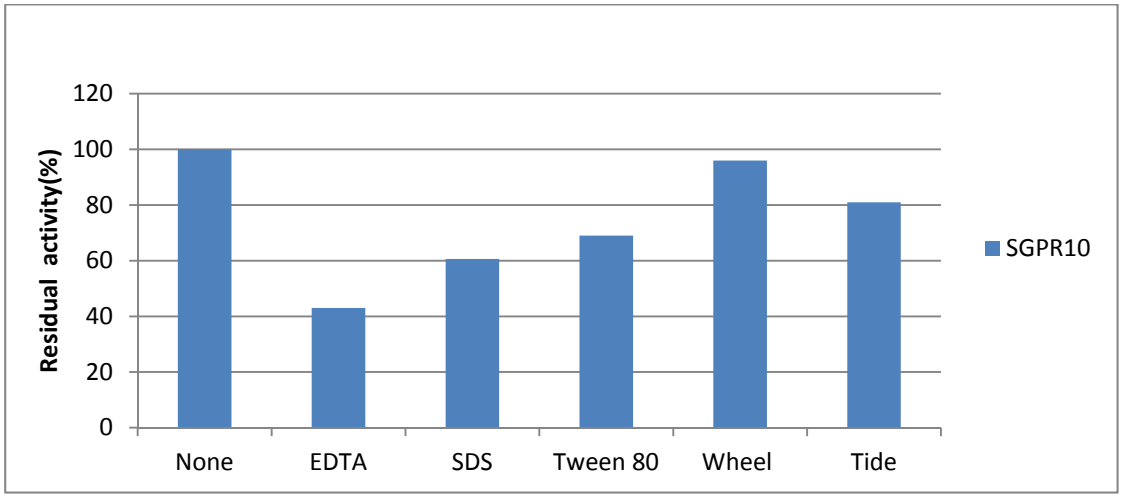


Fig. 4.12: Compatibility of detergent / commercial detergent on protease enzyme activity

Table 4.10: Effect of inhibitors and detergent on the activity of protease enzyme

Inhibitors (1 mM)	Residual activity (%)
None	100
EDTA	43
SDS	60.61
Tween 80	69
Wheel	96
Tide	81

The cost of enzyme production is the major obstacle in the successful application of proteases in industries. Moreover, the constrain for cost-cutting lies in the heating or cooling steps of industrial processes and increase in the upturn of the products of enzymatic reaction strength can give a much attention to the use of protease isolated from cold loving microorganisms (D'Amico et al., 2006). It can be concluded that psychrophiles have much more to contribute to the field of biotechnology (Gerday et al., 2000). That is why; the recent increasing interests on psychrophilic microorganisms not only focus on the genomic and proteomic study to establish the relationships but also on production of industrial important substitutions from psychrophiles. Although the psychrophilic enzymes have high specific activity but small half life makes a major drawback in the utilization of these enzymes at commercial aspect.

4.4. Lipase

Lipase (Triacylglycerol hydrolases EC 3.1.1.3) is an enzyme able of hydrolyzing lipids into fatty acids and glycerol (Yasuo et al., 2002). Several reports have shown that lipase have emerged as an significant enzyme in the fast-growing biotechnologies for numerous properties that may find use in a wide range of industrial applications, such as food

technology, pharmaceuticals, detergents, agrochemicals, biosurfactants and bioremediation etc. (Pandey et al., 1999; Pogori et al., 2007; Sharma and Shukla, 2011).

4.4.1. Isolation, characterization, identification and phylogenetic analysis of lipase producing bacteria

In the context of global needs for sustainability and clean manufacturing technologies, biocatalysts are an attractive alternative for the achievement of chemical transformations (Wohlgemuth, 2010; Bornscheuer et al., 2012). The survival at extreme environmental conditions involves the optimization of enzymatic tools allowing metabolic rates that are able to exist at low or high temperatures, of lipid-rich habitats. Thus, biocatalysis using psychrophiles as well as psychrozymes is rapidly being transformed from an academic science to an industrially viable technology. Cold-adapted bacteria produce lipases which work effectively at low temperatures, with high rates of the catalyst compared to mesophilic or thermophilic lipases show little or no activity at low temperatures. In addition, the maximum level of activity of the lipase is shifted to lower temperatures and reduced thermal stability. The presence of lipases has been noticed as earlier in *Pseudomonas aeruginosa* and *Pseudomonas fluorescens*, respectively (Jaeger et al., 1999; Hasan et al., 2006).

Lipase production is indicated by the formation of clear halos around the colonies grown on tributyrin-containing agar plates (Jaeger et al., 1997; Ertuğrul et al., 2007). Lipolytic bacteria are widely distributed in nature, with around 20% of several thousand microbes isolated from soil are found to be lipase producers as tested on solid media for lipase production (Jaeger and Eggert, 2002). Out of twenty-one isolate, on the basis of hydrolysis zone, seven isolates, were screened for lipase production on tributyrin agar at low temperature 20±2^{o}C. Then a loopful of isolate was streaked into the tributyrin

medium and incubated at two different temperature 4°C and 20°C. After incubation, four isolates (SGPR2, SGPR4, SGPR9 and SGPR15) were found to be capable of producing lipase at both temperature 20ºC and 4ºC (Table 4.11 and 4.12). Solubilisation zone diameters of these four isolates are depicted in the Figure 4.13. The ability to degrade lipid is used as a criterion for the determination of lipase production by a microbe depending upon the zone of hydrolysis produced. In view of the above, On the basis of zone diameter, one potent lipase producing isolate, SGPR4 was selected for further identification, partial purification and characterization.

Table 4.11: Isolates showing lipase activity at temperature 20ºC and pH 8.0

Sl. no.	Isolates	Temperature (ºC)	pH	Zone
1.	SGPR2	20	8	++
2.	SGPR4	20	8	+++
3.	SGPR5	20	8	+
4.	SGPR6	20	8	+
5.	SGPR9	20	8	+
6.	SGPR15	20	8	++
7.	SGPR20	20	8	+

(+Positive, ++Moderate Positive, +++Strong Positive**, -**Negative)

Table 4.12: Isolates showing lipase activity at temperature 4º C and pH 8.0

Sl. no.	Isolates	Temperature (ºC)	pH	Zone
1.	SGPR2	4	8	++
2.	SGPR4	4	8	+++
3.	SGPR5	4	8	-
4.	SGPR6	4	8	+
5.	SGPR9	4	8	++
6.	SGPR15	4	8	+++
7.	SGPR20	4	8	-

(+Positive, ++Moderate Positive, +++Strong Positive, -Negative)

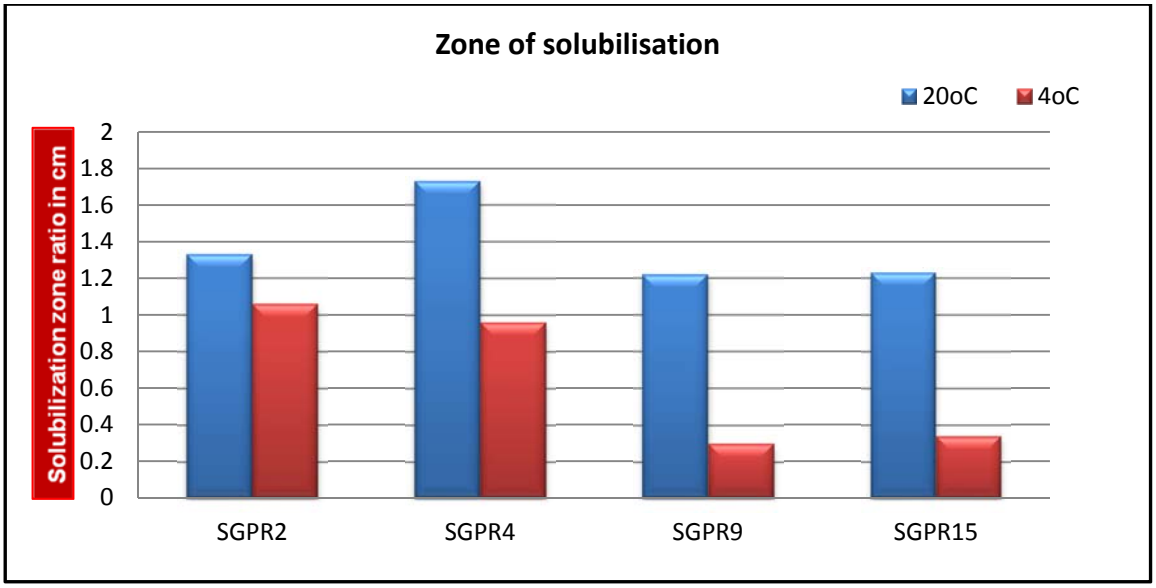


Fig. 4.13: Ratio of solubilisation zone front to colony diameter

4.4.2. Identification of potential lipase producing strain, SGPR4

On the basis of a clear zone around the colony on tributyrin agar a potential isolate, designated as SGPR4 was selected for morphological and biochemical characterization (Holt et al., 1994). Detailed morphological and biochemical tests of the isolate are given in Table 4.13. Colony morphology was circular, pulvinate, opaque and smooth in appearance while pigment is greenish in colour. The isolate studied was Gram negative, aerobic, rod shaped, motile bacteria and was able to grow at 4-30ºC and at a wide range of pH 6.0– 10.0. Wherever, not showing much growth beyond 30ºC and alkaline pH 5.0 It was positive for catalase, starch hydrolysis, citrate utilization, casein hydrolysis. Positive carbon sources were galactose, dextrose while showing variable activities for glucose. The bacterial isolate was negative for methyl red, V-P test and hydrogen sulfide production and assimilation for arabinose, sucrose.

Phylogentic analysis

Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013). To identify the experimental strain exactly according to 16S rRNA sequence analysis as well as taxonomical studies, genomic DNA of the strain was used as template to amplify partial 16S rRNA using universal bacterial primers. All positions containing gaps and missing data were eliminated and analysis clearly demonstrated that strain SGPR4 was a member of the genus *Pseudomonas* and exhibited maximum similarity with the 16S rRNA sequence of *Pseudomonas aeruginosa* LMG 1242T (Z76651) (98.94% sequence similarity). Finally, the obtained partial 16S rRNA sequence 1478 bp of this strain was analyzed with BLAST (Annexure VIII). It was found to have 95-99% identity with different strains of *Pseudomonas.* Among them, it showed high similarity (98.98%) with *Pseudomonas fluorescens* strain CIP 105469. A phylogenetic tree was also constructed based on the homology of known 16S rRNA sequences (Figure 4.14). Based on cultural characteristics, colony morphology, microscopic observations, biochemical, and physiological properties the isolate SGPR4, was identified as *Pseudomonas fluorescens.*

A large number of cold active lipolytic bacteria have been reported by different researcher such as *Pseudomonas vancouverensis, Pseudomonas ficuserectae, Cryobacterium psychrophilum*, *Pseudoalteromonas* sp., from Antarctic marine, glaciers, arctic and polar region (Singh et al., 2013; Gupta and Prakash, 2014). A cold active lipase producing potential psychrophilic bacteria (B11-1) was isolated and identified by 16S rRNA molecular studies as *Pseudomonas* (Choo et al., 1998). Strains of the genus *Pseudomonas* are prevalent and can be isolated from different sources especially from soil samples.

Table 4.13: Morphological, physiological and biochemical characteristics of the isolate

Morphological Tests	**SGPR4**
Colony morphology	
Configuration	Round
Surface	Smooth
Margin	Undulate
Elevation	Pulvinate
Surface	Smooth
Pigment	Greenish
Opacity	Opaque
Density	Translucent
Size	Short
Gram's reaction	Negative
Cell shape	Rod
Spore(s)	-
Motility	Motile
Physiological tests	
Growth at temp.	
4ºC	+
10ºC	++
20ºC	+++
30ºC	++
40ºC	+/-
50ºC	-
Growth at pH	
pH 5.0	-
pH 6.0	+
pH 7.0	++
pH 8.0	++
pH 9.0	+
pH 10.0	+
Growth under anaerobic condition	-
Biochemical Tests	
Methyl red test	-
Voges-Praoskauer Test	-
Citrate utilization	+
Starch hydrolysis	+
Urea hydrolysis	+/-
H_2S production	-
Catalase test	+
Casein hydrolysis	+
Acid production from carbohydrates:	
Arabinose	-
Sucrose	-
Galactose	+
Glucose	+/-
Dextrose	+
Maltose	-

(+Positive, ++Moderate Positive, +++Strong Positive, -Negative, +/Variable)

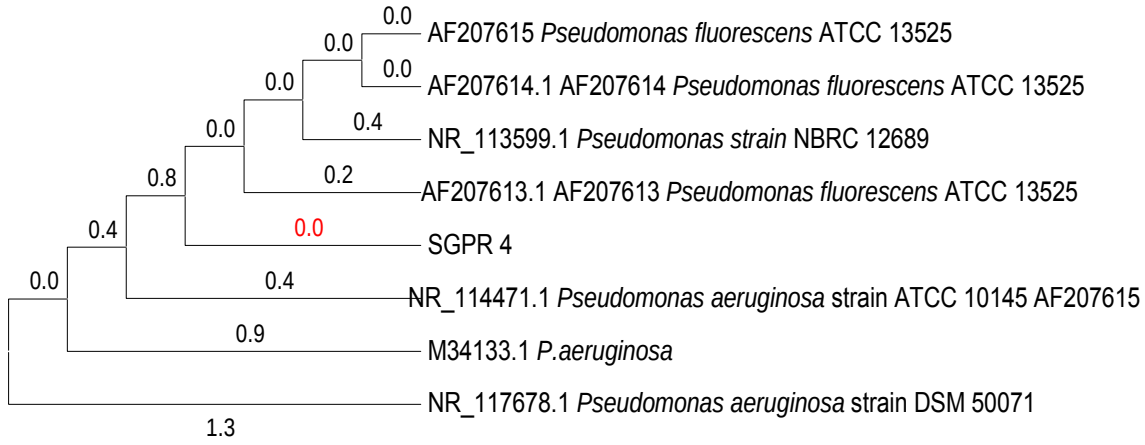


Fig. 4.14: Phylogenetic tree showing relationship of a lipase producing *Pseudomonas* sp. SGPR4 with other *Pseudomonas* species

4.4.3. Partial purification of lipase from isolated strain *Pseudomonas* sp. SGPR4

The objective of purification was to get rid of unwanted protein, while retaining the enzyme activity. Most purification schemes for lipases are based on multi step strategies. Cold active lipase was partially purified by precipitating with ammonium sulfate (60%) and using a single step ion-exchange chromatography on a DEAE-cellulose. Initially after ammonium sulphate fractionation specific activity was 51.48 U/mg with purification fold, 2.75. Finally, the enzyme was subjected to DEAE cellulose column to obtain a homogeneous amylase with specific activity of 120.47 U/mg and 6.44-fold of purification with 9.9 % yield. Partial purification results are summarized in Table 4.14. The purity of the enzyme after DEAE cellulose chromatography was more than 6.44-fold when compared to the crude extract of enzyme supernatant.

In similar study of Sharon et al. (1998) reported an extracellular lipase from *Pseudomonas aeruginosa* KKA-5 was purified using ammonium sulphate precipitation and successive hydroxyl apptite chromatography and the enzyme was purified 518-fold.

Table 4.14: Partial purification summary of lipase from *Pseudomonas* sp. SGPR4

Purification	Total protein (mg)	Total enzyme activity(U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	135.5	2532	18.68	1	100
$(NH_4)_2SO_4$ ppt.	33.6	1730	51.48	2.75	68.3
dialysis					
DEAE Cellulose	2.1	253	120.47	6.44	9.9

Cold-active lipase was partially purified with 17.74-fold purification and specific activity of 3,244.44 U mg^{-1} (Joseph and Ramteke, 2012). A new strain of *Pseudomonas aeruginosa* (KM110), yield 3-fold enhanced lipase production (0.76 U mL^{-1}) (Qamsar et al., 2011).

4.4.4 Characterization of partially purified lipase enzyme

Effect of temperature on activity and stability

There is a significant effect of temperature on cold active lipase production. Beyond the optimum temperature a sharp fall in the lipase production was observed. The activity of cold active lipase was determined at a wide range of temperature 10-60ºC. Enzyme was showing 48% and 71% at temp 10ºC and 30ºC respectively. Enzyme at temperature 20ºC showing 93% activity while at 60ºC remained only 24% of maximum activity. This marked liability of lipase together with its high catalytic efficiency near 20ºC clearly denotes that it is a cold active enzyme. Cold-adapted enzymes from microorganism have been found to exhibit both high activity and high stability (Saito and Nakayama, 2004; Kato et al., 2008) a remarkable property that goes against the trend of trade-off between activity and stability that has been observed for many enzymes

(Siddiqui and Cavicchioli, 2006). Low activation energy thus indicates its high catalytic efficiency (Choo et al., 1998). In our study the enzyme activity was almost constant within 20-30°C and gradually declined at a temperature beyond 30°C. Similarly, cold active lipase from *Psychrobacter okhotskensis* completely lost its activity above 36°C (Yumoto et al., 2003).

The effect of temperature on the activity and stability of lipase enzyme have shown in Figure 4.15 and 4.16 respectively. Enzyme from *Pseudomonas* sp. SGPR4 shows optimum activity at temperature 20°C and the enzyme was found stable between 20-30°C.

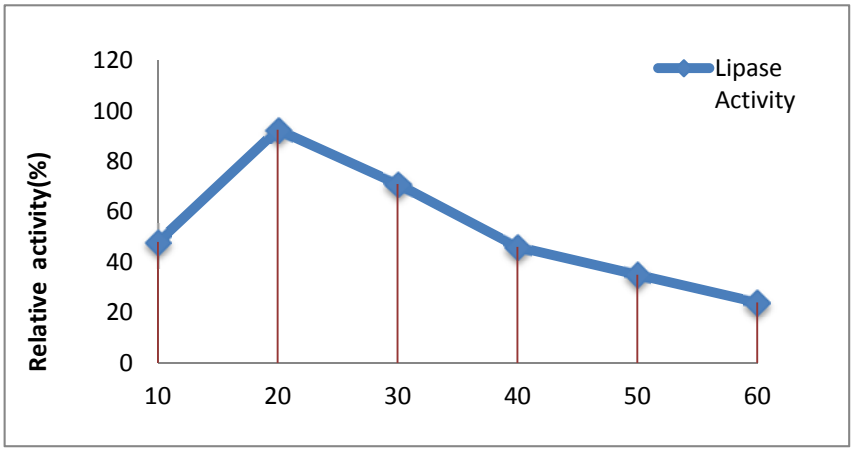


Fig. 4.15: Effect of temperature on activity of lipase enzyme

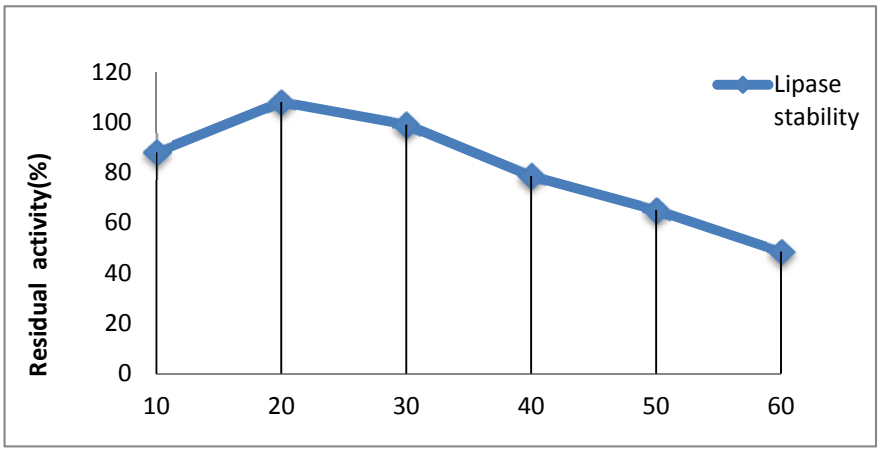


Fig. 4.16: Effect of temperature on stability of lipase enzyme

Assessment of the thermostability of lipase was performed by measuring the residual activity at various times, at different temperature incubation ranging from 10ºC to 60ºC. The enzyme retained 88% and 99% of its maximum activity at 10ºC and 30ºC, respectively.

The enzyme was stable up to 20ºC for 1 h and decreased at higher temperature (Figure 4.16). As for the stability of the enzyme, 99% activity remained after 3h of storage at 30°C and 78% at 40°C. While the stability of cold active lipase from *Aeromonas* sp. LPB4 was up to 50ºC and a dramatic decrease thereafter (Lee et al., 2003). *Moraxella* sp., isolated from Antarctican habitat grows well at 25°C and produced a cold active lipolytic enzyme (Feller et al., 1990). In present study the stability of the lipase decreased sharply after 1 h of incubation at high temperatures. It indicates that *Pseudomonas fluorescens,* lipase is a psychrotrophic enzyme. Moreover, *Pseudomonas* lipases, such as those from *P. fragi* (Mencher et al., 1967) and *P. mendoncina* were found to be optimally active at 35–45°C.

Effect of pH on activity and stability

The pH of medium strongly affects many enzymatic processes and transport of compounds across the cell membrane (Kuddus and Ramteke, 2008). For determination of activity at different pH, buffers were used. The effect of pH on enzyme activity was examined at different pH values (pH 5.0 to 11.0) at 20°C. The lipase exhibited activity in the pH range of 5.0-11.0, and the optimum activity was observed at pH 8.0. In the acidic range, significant reduction in enzyme activity was observed. For instance, at pH 5.0 the lipase retained only 3.5% of its maximum activity and also retained 23% at pH 11.0. The highest lipase activity (113%) was found to be at pH 8.0 using phosphate buffer (Figure 4.17). An increase in the enzyme activity in the alkaline range suggests that the enzyme

is slightly alkaline in nature. The data obtained indicated that there was strong influence of pH on enzyme production.

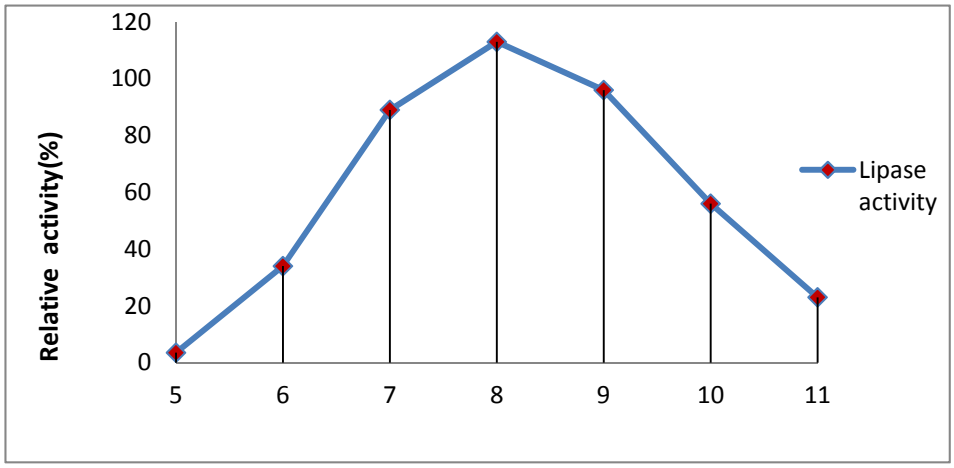


Fig. 4.17: Effect of pH on activity of lipase enzyme

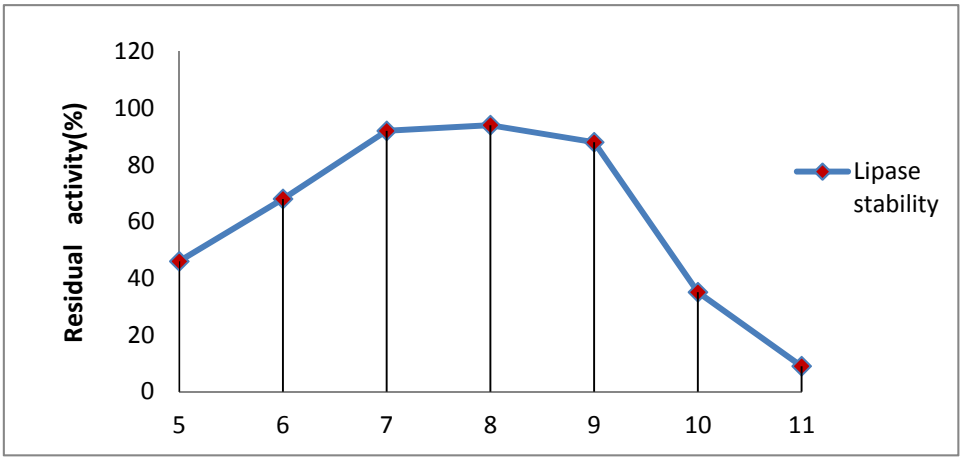


Fig. 4.18: Effect of pH on stability of lipase enzyme

The stability of alkaline lipase was determined by preincubating the partially purified cold active enzyme in various buffers of different pH for 1 h. The optimum pH for the activity of the enzyme is 94% at 8.0 pH. Lipases performing high stability and activity over a wide range of pH and activity under non-traditional conditions are of great interest.

The effects of pH on the lipase activity and stability have shown in Figure 4.17 and 4.18 respectively. Lipase from *Pseudomonas fluorescens*, SGPR4 shows optimum pH at 8.0 and the enzyme was found stable between pH 7.0-9.0.

A comprehensive review of all bacterial lipases done by Gupta et al. (2004) states that maximum activity of lipases at pH values higher than 7.0 has been observed in many cases. Interestingly, other *Pseudomonas* lipases from *P. pseudomalei* 12 (Kanwar and Goswami, 2002) and *P. aeruginosa* YS-7 (Shabtai and Daya, 1992) both isolated from *Pseudomonas* sp. growing in different water-restricted environments are stable within the pH ranges of 7.0-10.5 and 6.5-7.5, respectively. In the previous study of Joseph et al. (2012) a bacterial strain *Microbacterium luteolum*, produced maximum lipase at 15^{o}C, at a pH of 8.0. Lipase obtained in present study was stable from pH 7.0 to 9.0. However, it was not stable at acidic pH range. These properties can be extremely useful in various applications and are both innovative and invaluable. An interesting avenue for the application of cold-adapted lipase is their potential use in situ bioremediation or bio augmentation of fat contaminated cold environments (Suzuki et al., 2001). The potential application due to relatively high activity at low temperature and at alkaline pH could be used in detergent additives or for processing of volatile substances thereby making it possible to reduce temperature and thus bring down the energy costs.

4.5. Amylase

Amylase, (EC 3.2.1.1, 1, 4-α-D-glucan glucanohydrolase, and endoamylase) hydrolyzes starch, glycogen, and related polysaccharides by randomly cleaving internal α-1, 4-glucosidic linkages to produce divergent sizes of oligosaccharides (Najafi et al., 2005). Amylases from microbial sources have greater stability as compared to plant and animal amylases (Tanyildizi et al., 2005) and are among the most important cold active enzyme

account for approximately 30% of sales in the world market for enzymes, after protease that sharing 60% of it (Nguyen et al., 2002).

4.5.1. Isolation, characterization, identification and phylogenetic analysis of amylase producing bacteria

Cold-adapted amylolytic microorganisms produce amylases, which function effectively at cold temperatures with high rates of catalysis in comparison to the amylases from mesophiles or thermopiles, which shows little or no activity at low temperature (Kuddus and Roohi, 2010). Cold adapted bacteria are able to produce enzymes like amylase, cellulase, xylanase, pectinases, chitinase, protease and lipase for adaptation to extreme environment (Margesin et al., 2002; Burg, 2003; Fellar and Gerday, 2003) in the midst of all researcher develop interest toward cold-adapted amylolytic microorganisms, because these function efficiency. Therefore knowledge of cold active amylolytic enzymes is increasing at a rapid and exciting rate. A wide variety of microorganisms in nature, mainly fungi and bacteria have complex amylolytic enzymatic system and responsible for hydrolysis of starch into simple sugars (Singh et al., 2014). Recently, several members of group actinomyces provided a remarkable alternative to these traditional groups but for commercial applications α-amylase is mainly derived from the genus *Bacillus* (Pandey et al., 2000). *Bacillus* strains are distributed ubiquitously in the environment and frequently isolated from habitats with moderate to low temperature. The one objective of this study was to isolate amylase producing bacteria from soil samples of Satopanth glacier and to characterize them genetically and biochemically, in order to establish their phylogeny and their ability to grow at low temperature, and to generate cold adapted amylase. Out of twenty one, on the basis of zone formation, five isolates (SGPR1, SGPR6, SGPR11, SGPR20 and SGPR21) were screened for production

of cold-adapted extracellular amylase. Among these, three isolates were found to be adequate capable of producing amylase at both temperature 20ºC and 4ºC and at pH 8.0 (Table 4.15 and 4.16). On the basis of diameter of hydrolysis zone among three, one potent amylase producing bacterial isolate, SGPR6 has been taken for further purification and characterization of enzyme (Figure 4.19).

Table 4.15: Isolates showing amylase activity at temperature 20ºC and pH 8.0

Sl. no.	Isolates	Temperature (ºC)	pH	Zone
1.	SGPR1	20	9	+
2.	SGPR6	20	9	+++
3	SGPR11	20	9	+
4	SGPR20	20	9	++
5	SGPR21	20	9	++

(+Positive, ++Moderate Positive, +++Strong Positive, -Negative)

Table 4.16: Isolates showing amylase activity at temperature 4ºC and pH 8.0

Sl. no.	Isolates	Temperature (ºC)	pH	Zone
1	SGPR1	4	9	_
2	SGPR6	4	9	++
3	SGPR11	4	9	+
4	SGPR20	4	9	+
5	SGPR21	4	9	+

(+Positive, ++Moderate Positive, +++Strong Positive, -Negative)

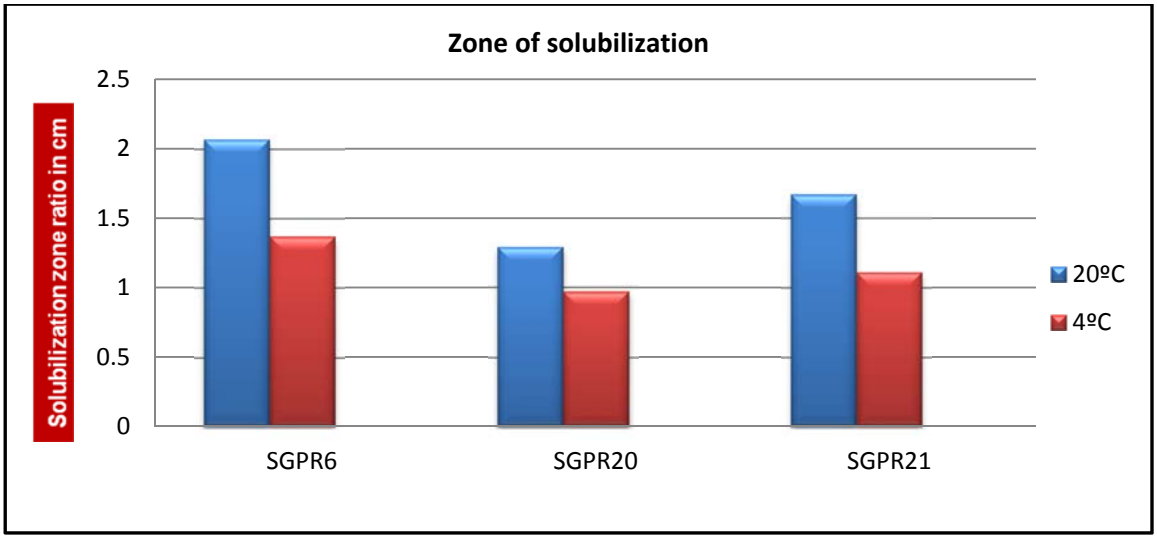


Fig. 4.19: Ratio of solubilisation zone front to colony diameter

4.5.2. Identification of potential amylase producing strain, SGPR6

The isolate were identified by studying morphological and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology (Holt et al., 1989). Detailed morphological, physiological and biochemical tests of the isolate are given in Table 4.17. On solid media colonies were entire, circular, undulate, and dull and dry in appearance creamy white. The isolate studied was gram positive, facultative aerobe, rod shaped and non motile bacteria. It was positive for Voges-Proskauer, catalase, citrate utilization, casein hydrolysis. Positive carbon sources were sucrose, galactose, glucose dextrose and maltose.

The strain was found to be methyl red and urea hydrolysis negative and assimilation for L-arabinose is negative. Considering the biochemical and physiological tests performed the strain SGPR6 was identified as *Bacillus.* The upper temperature growth limit of the strain SGPR6 was close to 20°C and at alkaline pH 8.0 not able to grow at 50°C. Considering the physiological features the strain SGPR4 was identified as psychrotrophic bacteria (Morita et al., 1975).

Table 4.17: Morphological, physiological and biochemical characteristics of the isolate SGPR6

Morphological Tests	**SGPR6**
Colony morphology	
Configuration	Irregular
Margin	Undulate
Elevation	Raised
Surface	Dull and dry
Density	Translucent
Pigment	Creamy white
Size	Long
Gram's reaction	Positive
Cell shape	Rod
Spore(s)	Positive
Motility	Non motile
Physiological tests	
Growth at temp.	
4ºC	-/+
10ºC	+
20ºC	+++
30ºC	++
40ºC	+/-
50ºC	-
Growth at pH	
pH 5.0	+/-
pH 6.0	+
pH 7.0	+
pH 8.0	++
pH 9.0	+
pH 10.0	+/-
Growth under anaerobic condition	-
Biochemical tests	
Methyl red test	-
Voges-Proskauer Test	+
Citrate utilization	+
Starch hydrolysis	+/-
Casein hydrolysis	+
Urea hydrolysis	-
H_2S production	-
Catalase test	+
Acid production from carbohydrates:	
Arabinose	-
Sucrose	+
Galactose	+
Glucose	+
Dextrose	+
Maltose	+

(+Positive, ++Moderate Positive, +++Strong Positive, -Negative, +/Variable)

In our best knowledge, we first report the purification and characterization of a cold adaptive amylase secreted by a *Bacillus* sp. SGPR6 strain that was isolated from soil samples of Satopanth glacier, Western Himalayan.

Phylogenetic analysis

Taxonomic affiliation of the 16S rRNA sequences of the isolate SGPR6 was retrieved from classifier programme of Ribosomal Database Project II version 9.0 (http://rdp8.cme.msu.edu/html). Phylogenetic and molecular evolutionary analysis was conducted using MEGA version 6 (Tamura et al., 2007) and aligned sequences are shown in Annexure IX. For the tree construction six different out groups were used which were shown in the Figure 4.20**.**

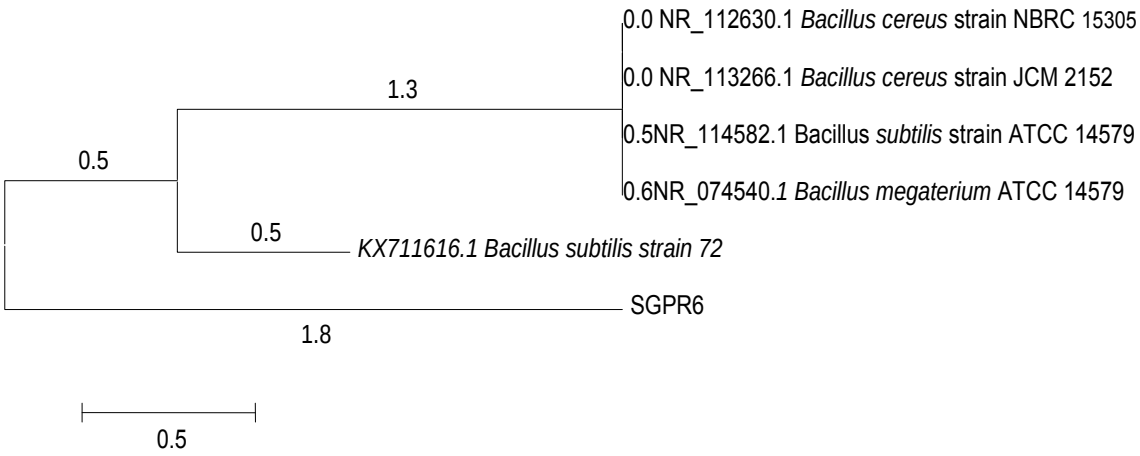


Fig. 4.20: Phylogenetic tree showing relationship of amylase producing *Bacillus cereus,* SGPR6 with other *Bacillus* species

A phylogenetic tree was constructed based on bacterial 16S rRNA sequences, showed a close relationship between the strains, SGPR6 with the genus *Bacillus*. The forward and reverse sequences SGPR6 which we got after sequencing were aligned with the maximum homology sequence of *Bacillus subtilis* and *Bacillus cereus*, respectively. Based on the biochemical characteristics and phylogenetic analysis, the isolate SGPR6 was identified as *Bacillus cereus*. A psychrophilic amylase producing bacteria on the

basis of 16S rRNA, TAC 240B identified as *Alteromonas*, had been reported by Chessa et al. (1999).

4.5.3. Partial purification of amylase from isolated strain *Bacillus cereus*, SGPR6

Amylase from *Bacillus cereus,* SGPR6 was subjected to partial purification. Most purification schemes for amylase are based on multi step strategies. Cold active amylase was partially purified by precipitating with ammonium sulfate (40-60%) and using a single step ion-exchange chromatography on a DEAE-cellulose. The purification procedure of the *Bacillus cereus,* SGPR6 extracellular amylase is summarized in Table 4.18. The amylase exhibited a specific activity of 116.23 U/mg, corresponding to a purification factor of 1.62- fold and a total yield of 12.29%.

Table 4.18: Partial purification summary of amylase from *Bacillus cereus,* SGPR6

Purification	Total protein (mg)	Total enzyme activity (U)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude enzyme	134.50	9672	71.91	1.0	100
Ammonium sulphate precipitation (60%)	46.23	4052	87.64	1.22	41.89
DEAE-Cellulose	10.23	1189	116.23	1.62	12.29

The culture supernatant (134.50 mg protein) was used as a starting material for the purification of amylase from *B. cereus*, SGPR6. The recovery of dialysed enzyme was 41.89% followed by 12.29% in ion-exchange chromatography. The overall purification strategy attained 1.6-fold purification of amylase and 12.29% yield with specific activity of 116.23 U/mg. In earlier study of Swain et al. (2007), alpha amylase was partially purified using ammonium sulphate fractionation. Roohi et al. (2011) isolated psychrotrophic strain

Microbacterium foliorum, GA from Gangotri glacier and reported maximum amylase production 3572(U).

4.5.4. Characterization of partially purified amylase enzyme

Effect of temperature on activity and stability

Recently cold active microorganisms have received rising attention, due to their relevance for both basic and applied research. The aim of the investigation was to isolate potential amylolytic bacteria with novel properties. Optimum temperature for amylase produced by *B. cereus*, SGPR6 was tested by incubating the enzyme at various temperatures such as 10°C, 20°C, 30°C, 40°C, 50°C and 60°C. The temperature for the amylase activity from *B. cereus*, SGPR6 was in a broad range of 10-60ºC (retained >56% relative activity at the temperature up to 40ºC) with optimum activity (89%) at 20ºC. Though, at temperature 10ºC and 60ºC, the retained relative activity of α-amylase was 32.45% and 10.65%, respectively. Thermal stability of amylase produced by *B. cereus,* SGPR6 was tested by incubating the enzyme at various temperatures such as 10ºC to 60°C for 30 minutes and then assay of enzyme was performed. The amylase retained more than 87% of the highest activity at 20ºC and 84% at 40ºC while residual activity decreased to 34% at 60°C.

The effect of temperature on activity and stability of amylase have shown in Figure 4.21 and 4.22 respectively. Amylase from *B. cereus,* SGPR6 shows optimum activity at temperature 20ºC and the enzyme was found stable at a temperature between 20-40ºC.

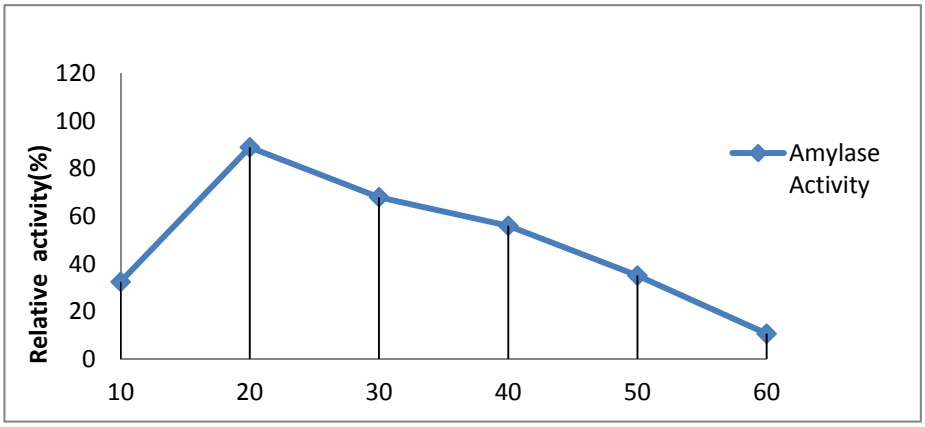


Fig. 4.21: Effect of temperature on activity of amylase enzyme

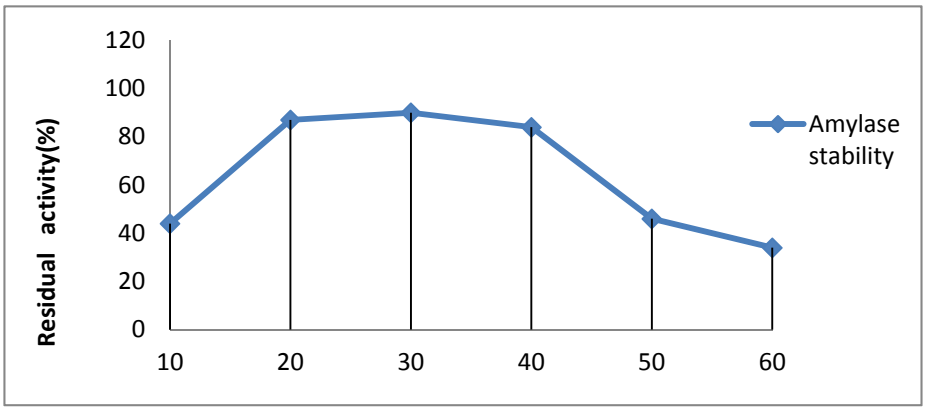


Fig. 4.22: Effect of temperature on stability of amylase enzyme

In comparable study the optimum temperature for enzyme activity of α-amylase from *Bacillus* sp. YX-1 was found to be 40-50°C (Liu and Xu, 2008), that is quite high comparatively of our study. Zhang et al. (2007) studied optimal activity of α-amylase from novel *Nocardiopsis* sp., 7326 at 35ºC. Similar findings were also reported for cold-adapted *Pseudoalteromonas arctica* GS230 producing maximum amylase at 20ºC (Lu et al., 2010). However, *Flavobacterium balustinum* A201 (Morita et al., 1997) and *Bacillus* sp. A-001 (Lealem and Gashe, 1994) showed its highest enzyme production at 30ºC and 35ºC, respectively.

Amylase from *B. cereus*, SGPR6 exhibited thermostable properties as indicated by retention of 84% of residual activity at 40ºC and 46% of residual activity at 50ºC. The investigation indicated that enzyme produced by the organism was stable in temperature

range of 20°C to 40°C for period of 30 minutes, with maximum stability at 20°C and least at 60°C. Irfan et al. (2011) reported that α-amylase produced by *Bacillus* sp., showed optimum activity at 30°C for 20 minutes. Therefore research suggests considerable different approach of *B. cereus,* SGPR6 towards temperature among all other previous studied psychrotrophic amylolytic bacteria. Results from present study provide lines of evidence that α-amylase from *Bacillus cereus,* SGPR6 could be a good candidate for the industrial application at low temperature without consumption of higher amount of energy.

Effect of pH on activity and stability:

The effect of pH on enzyme activity was examined at different pH values (pH 5.0 to 11.0) at 20°C for determination of relative activity at pH, different buffers were used. In the present study the alpha amylase activity of *B. cereus,* SGPR6 was found to be active in pH range from 5.0-11.0 with optimum activity (81%) at pH 9.0. (Figure 4.23). Although a decline in enzyme activity was observed between pH 10.0 and pH 11.0, retaining its 50.43 % and 8.3 % activity respectively. On the other hand, numerous α-amylases from *Bacillus* sp. had been studied Kuddus et al. (2011) up to now, which showed the highest activity at 20°C-70°C and pH 5.0-11.0. Rai and Solanki (2014) also reported pH activity profile of α-amylase produced from *Bacillus acidocaldarius* with an optimum activity at pH 7.0.

To determine the pH stability, the purified α-amylase from *B. cereus,* SGPR6 was dissolved in different buffers systems and the purified α-amylase was incubated in buffers of different pH for 30 minutes at 20°C. The pH-stability profile of the amylase as determined by the residual activity measurement showed 90% of its original activity was retained between pH 8.0–10.0.

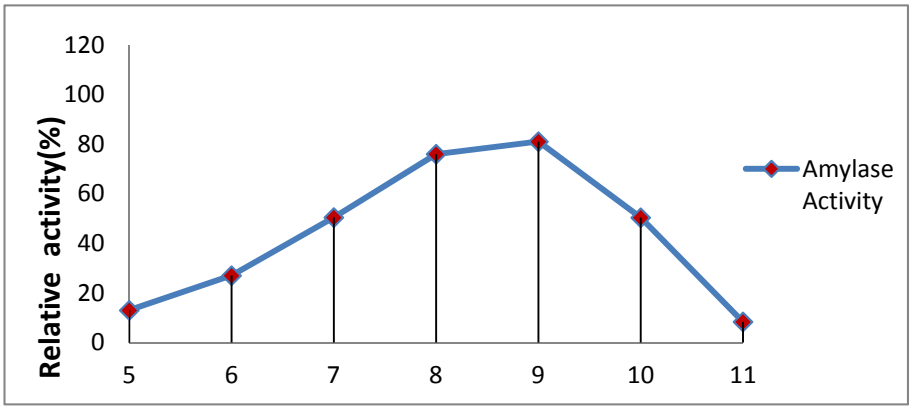


Fig. 4.23: Effect of pH on activity of amylase enzyme

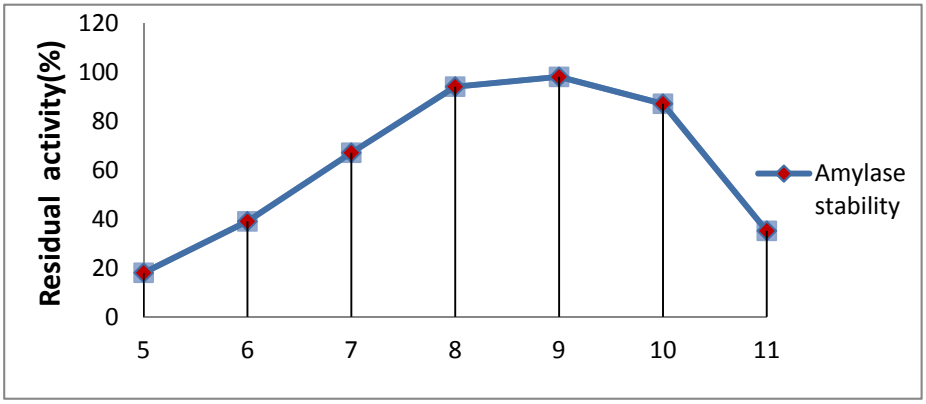


Fig. 4.24: Effect of pH on stability of amylase enzyme

The effect of pH on the activity and stability of amylase have shown in Figure 4.23 and 4.24 respectively. Amylase from *Bacillus cereus,* SGPR6 shows optimum activity at pH 9.0 and the enzyme was found stable between pH 8.0-10.0.

In similar findings on pH optimum analyses, *B. Cereus,* SGPR6 produced a peak in alkaline side i.e. at pH 9.0. Comparable to the results, Roohi et al. (2013) also reported maximum activity of α-amylase from *Bacillus cereus* GA6 at low temperature (4-37°C) in alkaline medium (pH 7-11). In similar findings on pH optimum analyses, *B. Cereus,*

SGPR6 produced a peak in alkaline side i.e. at pH 9.0. Comparable to the result of Poornima et al. (2008) optimum pH was 9.0, 8.0 and 7.5 for Actinomycete strain AE-19

In this study, the most desirable properties of amylase from *Bacillus cereus*, SGPR6 was its high activity at low temperature and stability at slightly alkaline pH, which also permitted its biotechnological application in various industries. For example, it could be applied as a detergent additive, as a desizing agent in textile processing and leather processing and as starch liquefaction agent in the food industry and a best alternative of decomposition of household waste at low temperature. The use of amylases that are active and stable around the saccharification pH is attractive because it could avoid or reduce the use of acid to lower the pH from liquefying to saccharifying range and also simplify the procedures during biotechnological processing.

CHAPTER 5

Summary & Conclusion

5. SUMMARY AND CONCLUSION

Satopanth Glacier, a major peak of Garhwal division is situated in Western Himalaya, Uttarakhand between 30°42'55"- 30°50'32" N latitude and 79°13'55"- 79°29'40" E longitude and altitude is 4,600 masl. Satopanth glacier is situated at the head of Alaknanda River and draining in north-westerly direction. It is approximately 15 km long and with an average width of 750 m and covering an area of 21.17 sq. km. (Nainwal et al., 2008). Glacier is one of the richest bio resource of unique microflora particularly bacteria, fungi and actinomycetes which have immense bio potential (Trivedi et al., 2012). Therefore monitoring and biological assessment of the Satopanth glaciers is important to assess the overall reservoir health. Cold adapted microorganisms are abundant and play an important role in the cold environment. Cold adapted microorganisms have distinct features due to their unique physiological and metabolic characteristics; henceforth, they are easily adapted to the extreme environments such as low temperature, acidic or alkali environment, and low nutritional content. These unique features have attracted many scientists to explore in depth. Cold adapted microorganisms were already proven as a potential agent for the production of many metabolites including industrial enzymes. These organisms are active not only in permanently cold areas but also in habitats which experience seasonal variation in temperature during late fall and spring. Therefore, it would be of much interest to study the nature of extracellular enzymes. The one main objective of the present study is to evaluate the biotechnological importance of cold adapted bacteria isolated from the soil samples of Satopanth Glacier, Western Himalaya. Present research is an also attempt to assess the bacteriological contamination in glacial water runoff from Satopanth and Alaknanda river system. With this in view, seven soil samples were collected from uppers stretch of

Satopanth and twelve water samples from glacier runoff for further study of biotechnological implication of cold adapted bacteria and for assessment of pollution load respectively.

The present studies revealed the presence of bacteria in samples of Satopanth glacier. Result indicate that total viable count in soil from Satopanth glacier varied from 2.7×10^4 to 12.7×10^4 cfu g^{-1} soil at 20±2°C. The lowest bacterial counts were found at SG-4 while highest bacterial count was found at SG-7. Of the numerous CFUs appearing on the culture media plates, fifty seven distinct representative isolates were picked both based on unique morphotypes and also randomly from each plate for further studies. Due to their biotechnological importance twenty one isolates from upper stretch of glacier were selected and studied in detail to establish their taxonomic identity and their phylogenetic position. The results indicated that the twenty isolates may belong to five different genera on the basis of morphological and biochemical characteristics. These were primarily identified as the genera, *Bacillus, Pseudomonas, Micrococcus*, *Stenotrophomonas*, *Arthrobacter*. These glaciers are supposed to be highly diverse in bacterial population surviving at low temperature. The results suggested that the soil of Satopanth glacier could be an affluent source of technologically significant microbial pool.

To assessment of bacteriological contamination of study area further research has been done. The Total viable count from glacial runoff and Alaknanda river system value showed a regular trend. The highest TVC was noted in Badrinath ghat of Alaknanda River and Mana village, where the values were as high as 86.3 $\times10^4$ and 68.5 $\times10^4$, respectively. The lowest Value 8.4 $\times10^4$ and 12.3 $\times10^4$ recorded in Satopanth top and Narayan Parvat, respectively. Results for FC and FS counts have also shown a similar trend to TVC and TC. The MPN of faecal coliform organisms fluctuated from 2 to 75

MPN/100 ml. i.e., highest FC count was observed in Alaknanda at Badrinath (SR-11) 75 MPN/100 ml and lowest count was at Satopanth top (SR-2) 2 MPN/100 ml.

The present study revealed resistance against common antibiotics among the bacterial population in glacier and glacial runoff regions with low anthropogenic activities is very unusual, most of the *E. coli* isolates from Alaknanda river system exhibited multiple antibiotic resistance (MAR). Maximum resistance (for four out of seven antibiotics) was observed for Nalidixic followed by Kanamycin, Tetracycline, and Gentamycin in the isolates from Badrinath Dham. While minimum resistance (for one antibiotic) was found in isolates from three stations namely Chakratirath, Alkapuri and Narayan parvat. While isolates from three stations viz; Satopanth tal, Satopanth top and Shahsradhara showing susceptibility for all seven antibiotics.

In order to establish their phylogeny and their ability to grow at low temperature, and to generate cold adapted enzymes, we screened protease, lipase and amylase producing bacteria and further purification and characterization of all these three enzymes. In best of our knowledge till the date this part of Himalayas, remained unexplored for microbial diversity and their biotechnological potential. Therefore keeping in mind the potential applications of cold adapted microbes and their extracellular enzymes, we screened bacteria resembling protease, lipase and amylase activity from study area.

Twenty one isolates from seven soil samples of glacier were examined for protease production on PSC agar medium containing skimmed milk at temperature 20±2^{o}C. Eleven isolates screened for protease production, among the isolates, five isolates were found to be capable of producing protease at alkaline pH and at two different temperature 4^{o}C and 20^{o}C. Among them strain SGPR10 yielded highest proteolytic activity with a ratio of solubilisation zone front to colony diameter i.e. one potent protease

producing bacterial isolates, SGPR10 has been taken for further purification and characterization of enzyme.

This isolate SGPR10 was subjected to the morphological and biochemical characterisation according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994) and phylogenetic analysis (Tamura et al., 2013). Based on the morphological, physiological, and biochemical characteristics and phylogenetic analysis, the isolate SGPR10 was identified as *Bacillus aryabhattai.*

Extracellular protease from *Bacillus aryabhattai*, SGPR10 culture was subjected to partial purification. Enzyme was partially purified to 3.1-purification fold with the increase of specific activity 1328.32 U/mg and yield of 31.5% by ammonium sulphate fraction and dialysis.

The optimum temperature for purified enzyme activity was determined by assaying the caseinolytic activity at different incubation temperatures 4, 10, 20, 30, 40, and 50^{o}C. Enzyme from *Bacillus aryabhattai*, SGPR10 shows optimum activity at temperature 10^{o}C and found stable between 10-30^{o}C. The proteases showed to be thermolabile, compared with the commercial enzymes, when they were incubated for 1 h at various temperatures.

The optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (5.0-11.0). Enzyme from *Bacillus aryabhattai*, SGPR10 shows optimum pH 10.0 and the enzyme was found stable between 8.0-10.0. These results indicated that the extracellular protease from *Bacillus aryabhattai*, SGPR10 is an alkaline protease. In broad-spectrum, all currently used detergent compatible proteases are alkaline in nature therefore they are suitable for laundry detergents, which is generally in the range of 8.0 to 12.0 (Rao et al., 1998).

Partially purified enzyme from SGPR4 was incubated with different detergent viz. SDS, Tween 80 and different commercial detergents like Wheel Tide (1%, w/v or v/v) for 1 h at 20ºC and then enzyme assay were performed. The enzyme exhibited unusual stability in presence of 1% SDS with 60.61% residual activity after 1 h at 20ºC. The enzyme also exhibited enhanced activity in detergent Tween 80, commercial detergent Wheel and Tide. In conclusion, results obtained in our study suggest that among the twenty one strains, SGPR10 proved to be potential alkaline protease producer; hence it was subjected to further characterization & identified as *Bacillus aryabhattai* strain SGPR10 by 16S rDNA identification and used for further study. The protease produced by strain SGPR10, was an alkaline protease and was active in alkaline conditions as well as stable at low temperature, indicating its potential use in detergent formulations & other industrial applications.

Isolation of lipase producing strains was carried out using tributyrin agar medium. Lipolytic bacteria are widely distributed in nature, with around 20% of several thousand microbes isolated from soil are found to be lipase producers as tested on solid media for lipase production (Jaeger and Eggert, 2002). Total twenty-one bacterial colonies were isolated on nutrient agar media from seven soil samples from Satopanth Glacier, Western Himalaya. Out of twenty-one, on the basis of zone formation, seven isolates were showing positive results while among them, four isolates were found to be capable of producing lipase at two different temperature 4ºC and 20ºC both. Among them, on the basis of zone diameter, one potent lipase producing isolate SGPR4, has been selected for further identification, partial purification and characterization.

On the basis of a clear zone around the colony on tributyrin agar a potential isolate, designated as SGPR4 had been selected for morphological and biochemical characterization (Holt et al., 1994). On the basis of colony morphology, microscopic

observations, biochemical and phylogenetic analysis the isolate SGPR4, was identified as *Pseudomonas* sp. maximum similarity with *Pseudomonas fluorescens.*

Most purification schemes for cold active lipases are based on multi step strategies. Cold active lipase was partially purified by precipitating with ammonium sulfate (60%) and using a single step ion-exchange chromatography on a DEAE-cellulose. Partially purified lipase was eluted out as fractions (with 0.1–1M NaCl gradient) from DEAE-cellulose column with 6.44-fold purification and specific activity of 120.47 U/mg.

There is a significant effect of temperature on cold active lipase production. Beyond the optimum temperature a sharp fall in the lipase production was observed. The activity of cold active lipase was determined at a wide range of temperature 10ºC-60ºC. Enzyme from *Pseudomonas* sp., SGPR4 shows optimum activity at temperature 20ºC and the enzyme was found stable between 20ºC-30ºC.

The pH of medium strongly affects many enzymatic processes and transport of compounds across the cell membrane (Kuddus and Ramteke, 2008). For determination of activity at different pH, buffers were used. In the present study cold active lipase from *Pseudomonas fluorescens,* SGPR4 shows optimum activity at pH, 8.0 and the enzyme was found stable between pH 7.0- 9.0. However, it was not stable at acidic pH. Lipases performing high stability and activity over a wide range of pH and activity under non-traditional conditions are of great interest. The relatively high thermolability of cold-adapted enzymes may therefore be advantageous as in order to avoid changes to food ingredients caused by undesirable side-reaction that would otherwise occur at higher temperatures.

Twenty-one isolates were examined for extracellular amylase production on starch agar media at 20±2ºC. Out of twenty-one, on the basis of hydrolysis zone, five

isolates (SGPR1, SGPR6, SGPR11, SGPR20 and SGPR21) were screened for production of cold-adapted extracellular amylase. Among these, three isolates were found to be capable of producing amylase at pH 8.0 and at temperature 4ºC and 20ºC. On the basis of solubilisation zone formation, i.e. on the basis of diameter of hydrolysis zone among them one potent amylase producing bacterial isolate, SGPR6 has been taken for further purification and characterization of enzyme.

On the basis of a clear zone around the colony on starch agar a potential isolate, designated as SGPR6 was selected for morphological and biochemical characterization (Holt et al., 1994). Based on the morphological, physiological, and biochemical characteristics and phylogenetic analysis, the isolate SGPR6 was identified as *Bacillus cereus.*

Amylase from *Bacillus cereus*, SGPR6 was subjected to partial purification. Amylase from *Bacillus cereus*, SGPR6 was subjected to partial purification. Most purification schemes for amylase are based on multi step strategies. Cold active amylase was partially purified by precipitating with ammonium sulfate (40-60%) and using a single step ion-exchange chromatography on a DEAE-cellulose. The amylase exhibited a specific activity of 116.23 U/mg, corresponding to a purification factor of 1.62-fold and a total yield of 12.29%.

Optimum temperature for alpha amylase produced by *Bacillus cereus,* SGPR6 was tested by incubating the enzyme at various temperatures such as 10°C, 20°C, 30°C, 40°C, 50°C and 60°C. *α*-amylase from *Bacillus cereus,* SGPR6 shows optimum temperature at 20ºC and the enzyme was found stable at a temperature between 20-40ºC. Therefore research suggests considerable different approach of *Bacillus cereus,* SGPR6 towards temperature among all other previous studied psychrotrophic amylolytic bacteria. Results from the present study provide baselines of evidence that *Bacillus*

cereus, SGPR6 could be a good candidate for the efficient for biotechnological application.

The effect of pH on enzyme activity was examined at different pH values (pH 5.0 to 11.0) at 20°C for determination of relative activity at pH, different buffers were used. The optimum pH for α-amylase activity from *Bacillus cereus,* SGPR6 ranged from pH 5.0 to 11.0 with an optimum activity at pH 9.0. and the enzyme was found stable between pH 8.0-10.0. In the present work, the most desirable properties of amylase from *Bacillus cereus*, SGPR6 was its high activity at low temperature and stability at slightly alkaline pH, which also permitted its biotechnological application in various industries. For example, it could be applied as a detergent additive, as a desizing agent in textile processing and leather processing and in the food industry.

Researchers around the world are putting a lot of plunge towards understanding the pathways involved in production of novel secondary metabolites and to understand their species richness, functional and phylogenetic diversity and response under changing abiotic and biotic factors as they are answerable for functioning of the ecosystem. This study improved our understanding for the bacterial community in glacier ice and this study will also help to provide a better foundation for more understanding about ecophysiology and ecology of glacier microflora in general and cold adapted bacteria in particular. However, in our best knowledge not as much as study available for bacteriological contamination from runoff of Satopanth and Alaknanda river system, which have great relevance to geological exploration and tourism, in addition to environment and public health safety. It is also necessary to understand the pathogenic bacteria genera in the Himalayas river system and to develop measures that can serve as indicators of water pollution. Therefore this study may be relevant and useful for conservation of glacial as well as river system for the safety of aquatic life environment

and human health. This is a one attempt also for search of novel cold active enzymes from glacier colonizing cold adapted bacteria. Research findings may give clear picture and base line data about cold active bacteria present in Indian glaciers and search of their biotechnological and industrial implications. In view of the implications of cold adapted bacteria and prospects of their enzymes it is high time for scientist to give their attentions for more focused study. Conclusive statement is that the cold active enzymes might also have tremendous applications in industrial processes, which require low temperatures. Therefore it could be cost effective and environment friendly. More organized study is required to explore biotechnological implications of cold adapted bacteria and their enzymes isolated from Indian glaciers and in this regard attention is needed from government, regularity authorities, research institutes and other agencies concern.

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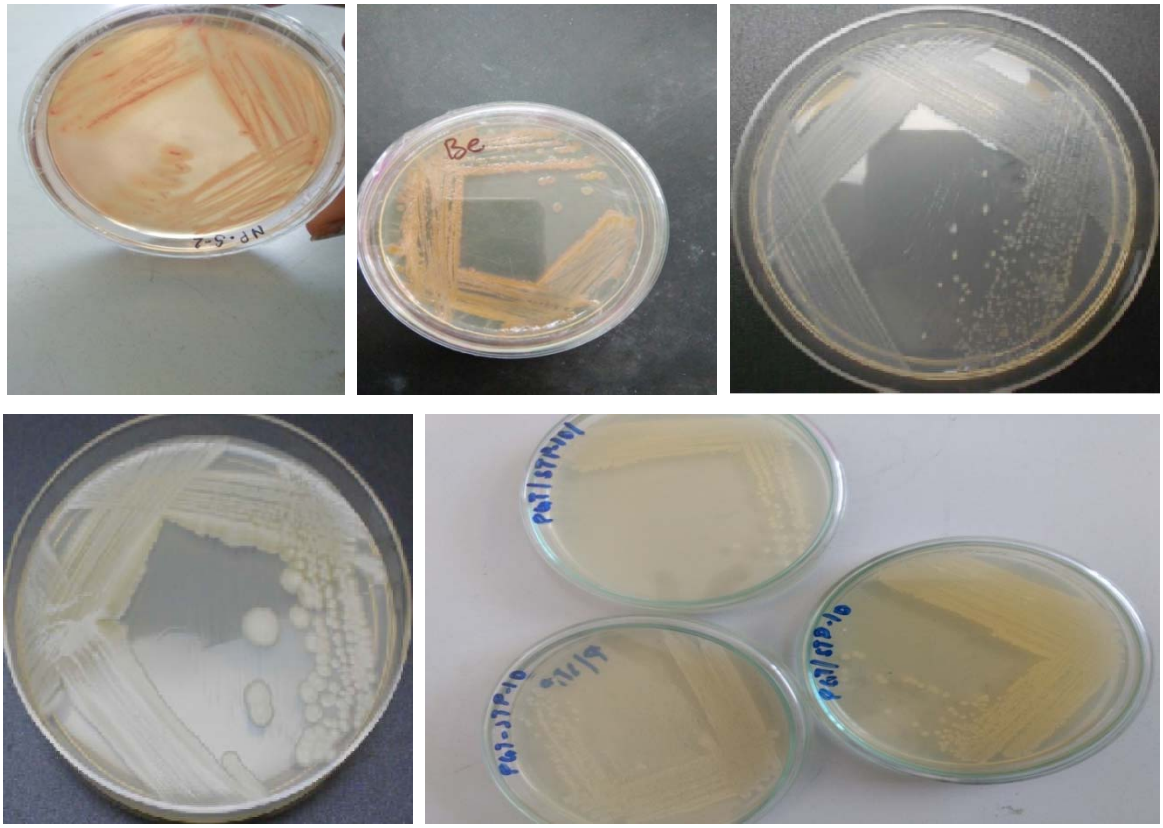
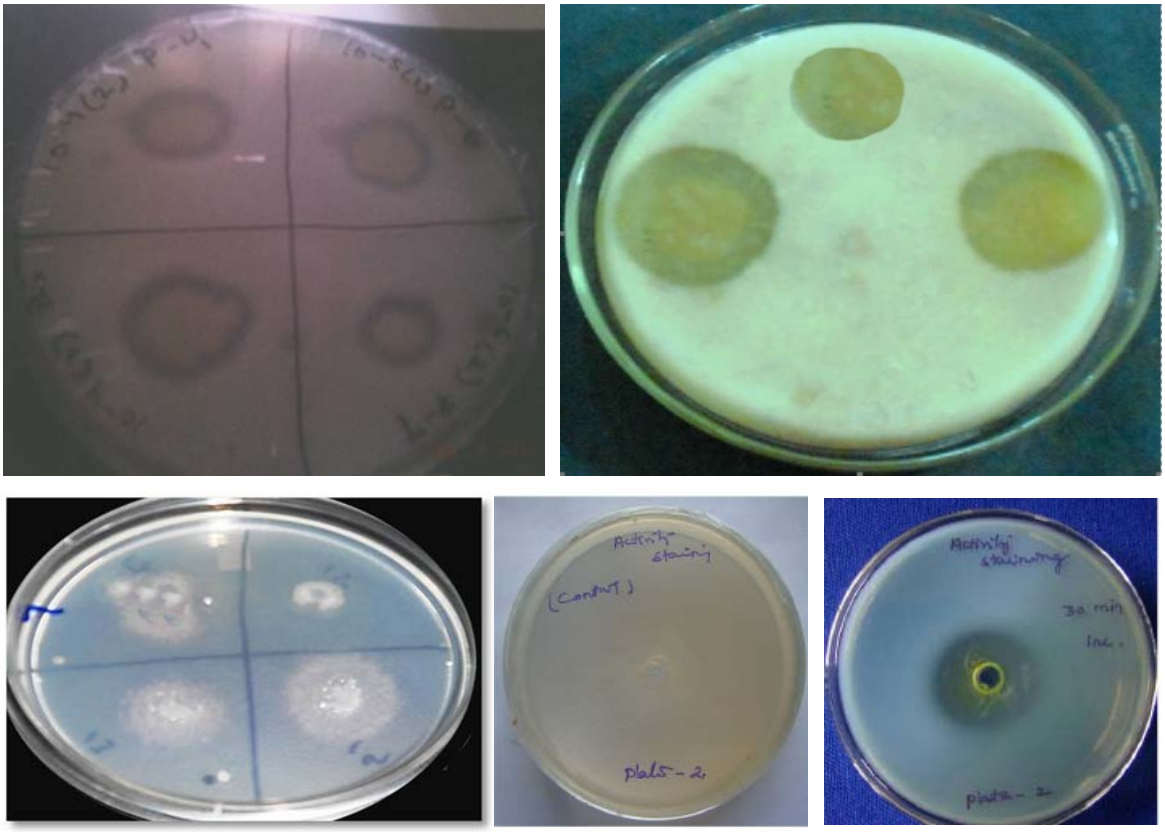
ANNEXURES

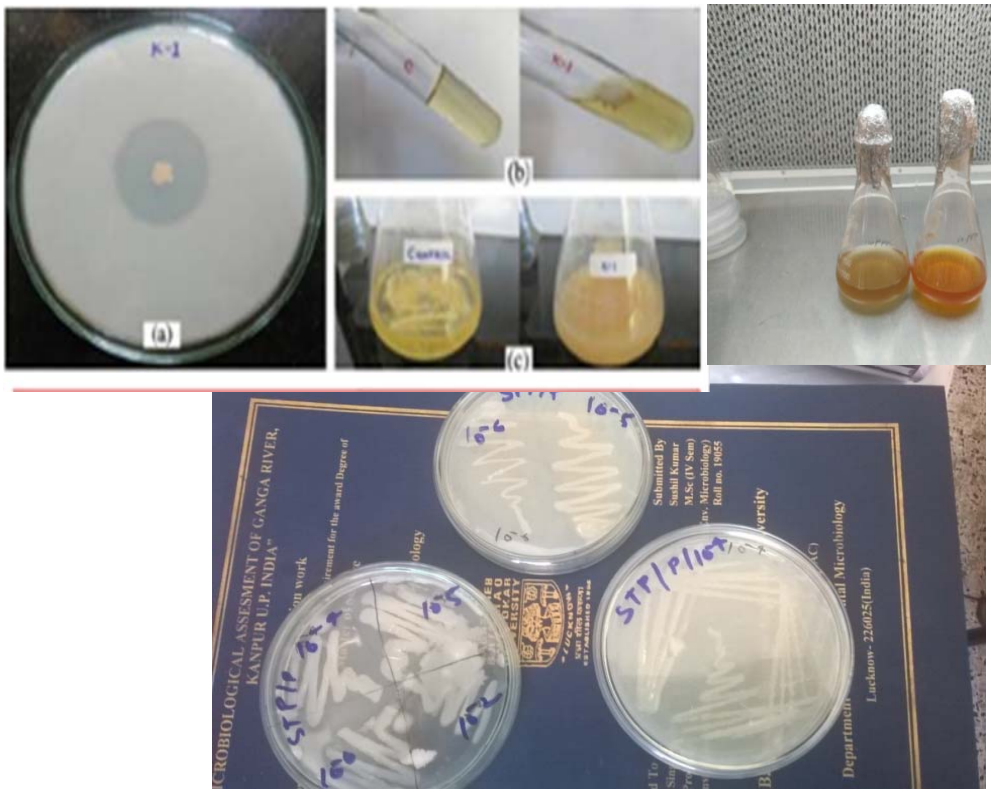
ANNEXURES



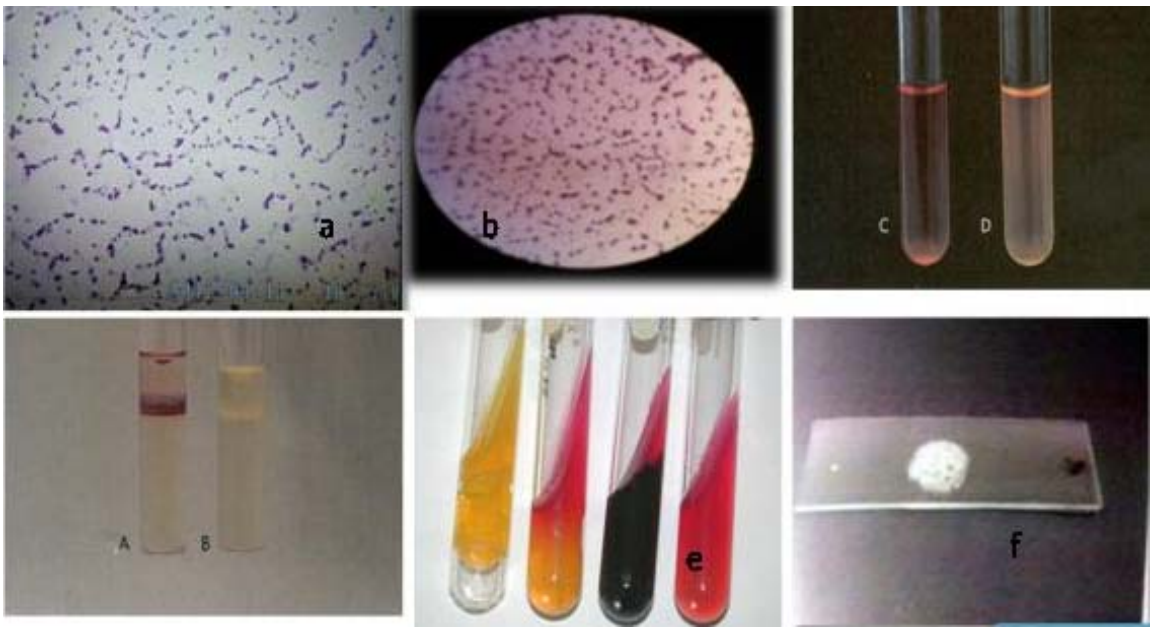
Annexure I: Plates showing bacterial growth on nutrient agar



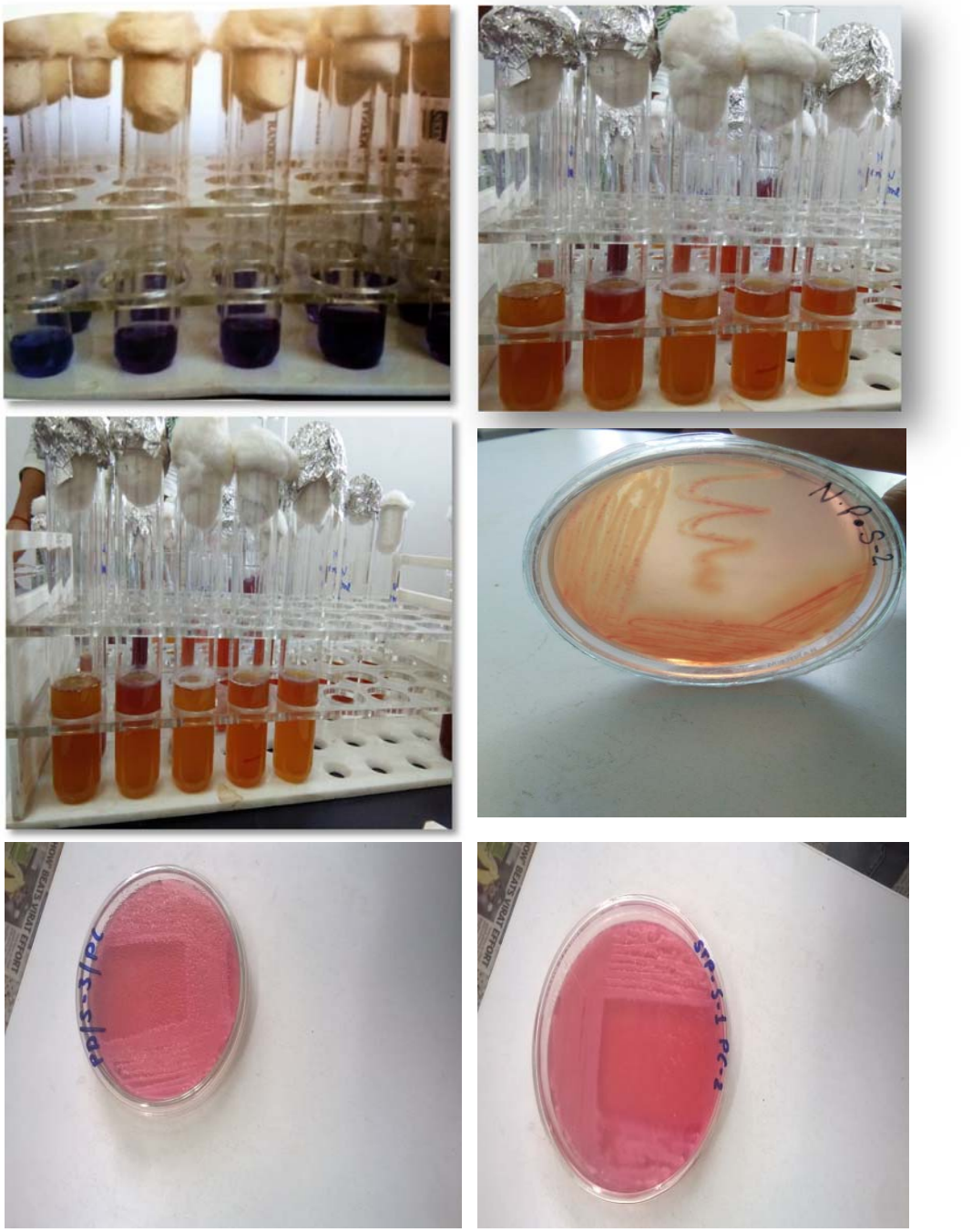
Annexure II: Plates showing protease and lipase activity on skimmed milk agar and tributyrin agar respectively



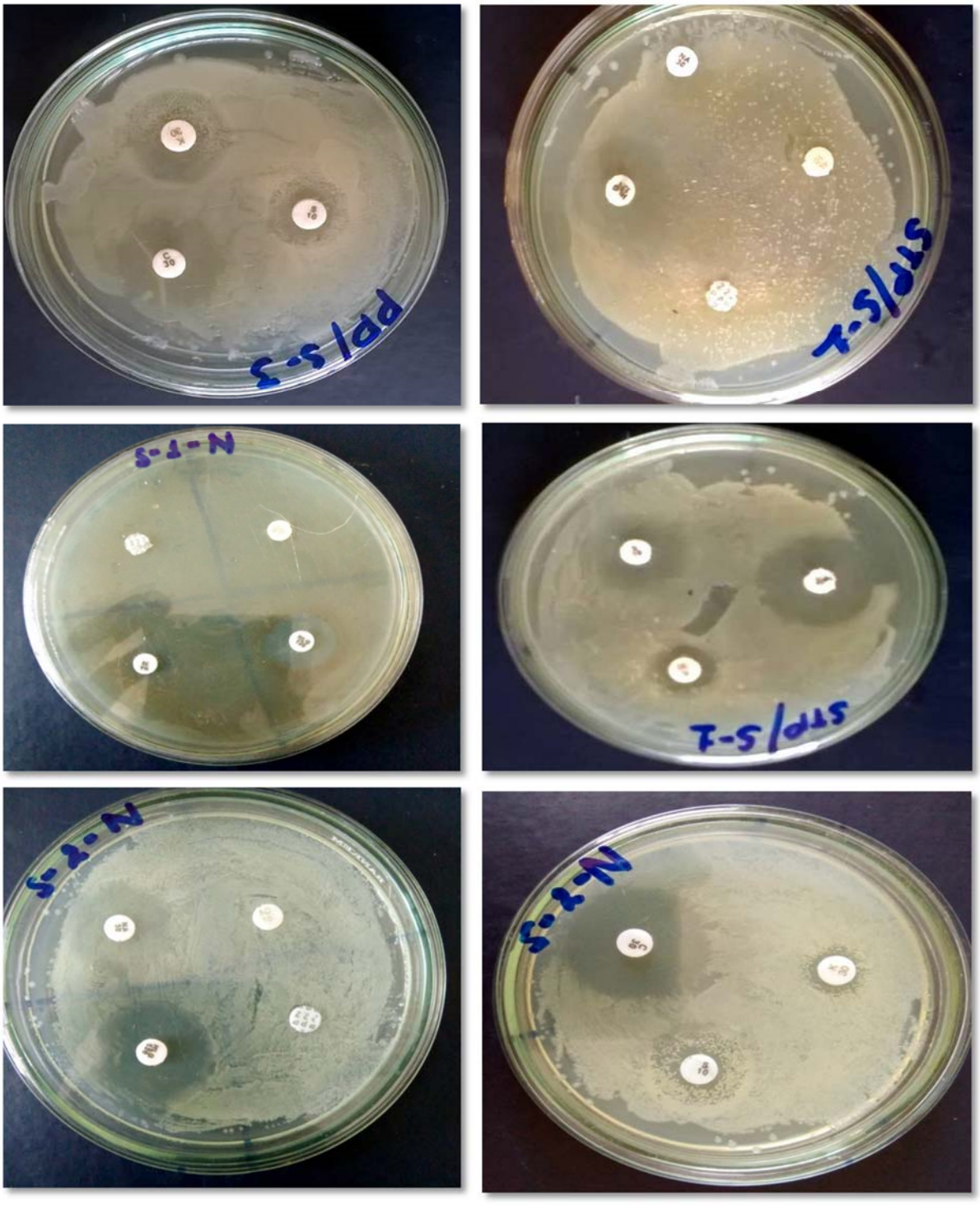
Annexure III: Plates and flask showing positive isolates for amylase activity on starch agar and broth



Annexure IV: Morphological and biochemical identification of isolates



Annexure V (A): Tubes of MacCkonkey broth showing results before inoculation and after inoculation (B) MacCkonkey agar plates showing coliform isolates



Annexure VI: Plates showing antibiotic sensitivity and resistance pattern among coliforms

Annexure VII: Aligned Sequence of Sample SGPR10

Sample PGT/SGPR10

AGGCGGCTAGCTCCTTACGGTTACTCCACCGACTTCGGGTGTTACAAACTCTC
GGGTGTGACGGGCGGTGTGTACAAGGCCCGGGAACGTATTCACCGCGGCAT
GCTGTCCGCGATTACTAGCGATTCCAGCTTCATGTAGGCGAGTTGCAGCCTA
CAATCCGACTGAGAATGGTTTTATGGGATTGGCTTGACCTCGCGGTCTTGCA
GCCCTTTGTACATCCATTGTAGCACGTGTGTAGCCCAGGTCATAAGGGGCAT
GATGATTTGACGTATCCCCACCTTCCTCCGGTTTGTCACCGGCAGTCACCTTA
GAGTGCCCAACTAAACTGGCAACTAAGATCAAGGGTTGCGCTCGTTGCGGGA
CTTAACCCAACATCTCACACACGAGCTGACGACAACCATGCACCACCTGTCA
CTCTGTCCCCCGAAGGGGAAGCTCTATCTCTAGAGTTGTCAGAGGATGTCAA
GACCTGGTAAGGTTCTTCGCGTTCTTCGAATTAAACCACATGCTCCACCGCTT
GTGCGGGCCCCCGTCAATTCCTTTGAGTTTCAGTCTTGCGACCGTACTCCCCA
GGCGGAGTGCTTAATGCGTTAGCTGCAACTAAAGGGCGGAAACCCTCTAACA
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ATGCACCGCGGGCCCATCTGTAAGTGATAGCCGAAACCATCTTCAATCATCT
CCCATGAAGGAGAAGATCCTATCCCGTATTAGCTTCGGTTTCCCGAGTTATCC
CAGTCTTACAGGCAGGTTGCCCACGTGTTACTCACCCGTCCGCCGCTACGTC
ATAGAAGCAAGCTTCTAATCAGTTCGCTCGACTTGCATGTATAGCACGCCCA
GCGTTCATCCTGA

Annexure VIII: Aligned Sequence of Sample SGPR4

Sample PGT/SGPR4

GAACTGAAGAGTTTGATCATGGCTCAGATTGAACGCTGGCAGCAGGGGCCTT
CAACACATGCAAGTCGAGCTTATGAAGGGAGCTTGCCTTGGATTCAGCGGCG
GACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGATAACGTCCG
GAAACGGCCGCTAATACCGCATACGTCCTGAGGGAGAAAGTCGGGGATCTT
CGGACCTCACGCTATCAGATGAGCCTAGGTCGGATTAGCTAGTTGGTGGGGT
AAAGGCCTACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTC
ACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGA
ATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAA
GGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTAAT
ACCTTGCTGTTTGACGTTACCAACAGAATAAGCACCGGCTAACTTCGTGCCA
GCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGAATTACTGGGCGT
AAAGCGCGCGTAAGTGTTCAGCAAGCTTGATGTGAAATCCCCGGGCTCAACC
TGGGAACTGCATCCAAAAGCTACTGAGCTAGAGTCGGTAGAGGTGGTAGAA
TTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGA
AGGCGACCACCTGGACTGTACTGACACTGAGGTGCGAAAGCGTGGGGAGCA
AACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCG
TTATCCTTGAGATCTTAGTGGCGCACGTAACGCGATAAGTCGACCGCCTGGG
GAGTACGGCCGCAAGGTTACTCAAATGAATTGACGGGGGCCCGCACAAGCG
GTGGAGCATGTGGTTTAATCGAAGCAACGCGAAGAACCTTACCTGGCCTTGA
CATGCTGAGAACTTTCCAGAGATGGATTGGTGCCTTGGGAACAGAGACACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGT
AACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAA
GGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATC
ATGGCCCTTACGGCCAGGGCTACACACGTGCTACAATGGTCGGTACAAAGGG
TTGCCAAGCCGCGAGTGGGAGCTAATCCCATAAAACCGATCGTAGTCCGGAT
CGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATC
AGAATGTCACGGTGAATACGTCCCCGGGCCTTGTACACACCGCCCGTCACAC
CATGGGAGTGGGTGCTCCAGAAGTAGCTAGTCTAACCGCAAGGGGGACGGT
TACCACGGAGTGATTCATGACTGGGGTGAAGTCGTAACAAGGTAGCCGTAG
GGGAACCTGCGGCTGGATCACCTCCTTA

Annexure IX: Aligned Sequence of Sample SGPR6

Sample PGT/SGPR6

CGTCTCCATGCGGTCTGTTCACTGCGTTAGCTGCACTACTAAGGGGCGGAGC
CCCCTAACCTTATGACTCATCTTTTAGGGTGTGGACTACCGGGGTATCAAATC
GTGTGCTCTCCCCACTCTTTCGCGCCTCAGCGTCTGTTACCGACCGAAGAGGC
CCCCTCCCCCCTGGTGTTCCTCCACATCTCTACACTTTTCAGCGCTACACGTG
AAATTCCCCTCTCCTCATCTGCACTCAAGTTCCCCAGTTTCCTGTGACCCTCC
CCGGTTGATCCGGGAGCTTTCATATCTCATTTAAAAAACCGCCCGCGCGCTCT
TTACCCCCAATATTTCAGGACAACGCGCGCCCCATACATAATACCGCGGGTG
CGGGCACATATTTCCCCGGGGCTTTCTGGTTATGTACCGTCTTTGTCCCGCCA
TATTCCAACGGTATTTGTTCTTCGCTAACAATGCATTTTTACCATCCGAAAAC
CTTCATCACTCCGGCGGGGTTGCTCCATCGGACTTTCCTCTGTTGCGGAATAT
TCCCTACTGCTGCCTCCAGGAGGATTCTGGGCCGTGTCTCACTCCTGTTGTGG
CCCATCATCCTCTCACGTCTGCTACTCATCGTCGTGTTGATGAGCTATTACCT
CAACAACTAGATCATGCGCCGCGGGTTCTTCTGTAGGTGGTAGCTGAGATCC
CCTGTTTTTGTATTGCACGGGGTATGCCGTACTAGCTACCGGTTATCTACGCC
CCTGATTTCCCCGCTATTTATCGCACGTTCTCAGATAGCATTGATTCCCCACG
TTCTTACTCTCGCCATCCAGCACGCGTAAGATCTCATGCTAGCCAATCCTCAC
ATTGCTATGCCGCTCGACATCCCACGTAGCGTGCAATCCTGACCCACGATTCT
ATCACTCGTGCCGAGATACAAAAAACCATGGGGGCGGCGGAGTGTTTTTCCC
CTA

List of Publications

LIST OF PUBLICATIONS

1. **Pragati Katiyar**, Pratibha, Vishwas Hare, V.S. Baghel (2017**) "Isolation, Partial Purification and Characterization of a Cold Active Lipase from *Pseudomonas* Sp., Isolated from Satopanth Glacier of Western Himalaya, India."** International Journal of Science Research and Management, 5(7) 6106-6112.
2. **Pragati Katiyar**, V.S. Baghel (2017**) "Monitoring and Assessment of Bacterial Contamination in runoff of Satopanth Glacier, India."** Bulletin of Environment, Pharmacology and Life Sciences, 6(6): 89-92.
3. Pratibha, **Pragati Katiyar**, V.S. Baghel (2016) "**Isolation and Characterization of Psychrotrophic Protease Producing Bacteria from Gangotri Glacier, India."** International Advanced Research Journal in Science, Engineering and Technology 3(11): 79-82.
4. Pratibha, **Pragati Katiyar**, V.S. Baghel (2016) "**Isolation and Characterization of Lipase producing bacteria from Gangotri glacier, Western Himalaya, India."**International Journal of Advance Research Science Engineering and technology, 3(12): 1-9.
5. Vishwas Hare, **Pragati Katiyar**, V.S. Baghel (2017) "**Isolation, Biochemical and molecular identification of arsenic resistant bacteria from arsenic affected area from contaminated site of Uttar Pradesh, India."** International Journal of applied and advanced scientific research, 2(2):1-7.