BIOCHEMICAL AND BIOTECHNOLOGICAL IMPLICATIONS OF COLD ADAPTED BACTERIA ISOLATED FROM GANGOTRI GLACIER, WESTERN HIMALAYA, INDIA

THESIS

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Submitted by

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Enrolment No.: 389/11 **Year 2016**

Dedicated to my

Parents

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CERTIFICATE

This is to certify that the thesis titled **"Biochemical and Biotechnological Implications of Cold Adapted Bacteria Isolated from Gangotri Glacier, Western Himalaya, India"** submitted by **Ms. PRATIBHA** 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.

(V.S. Baghel)
Date: **Supervisor**

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DECLARATION

This is to certify that the material embodies in the present work entitled **"Biochemical and Biotechnological Implications of Cold Adapted Bacteria Isolated from Gangotri Glacier, Western Himalaya, India"** 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 University.

Pratibha

(Candidate)

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(Pratibha)

Lucknow

ABBREVIATIONS

%	Percentage
+	Positive
–	Negative
°C	Degree Celsius
A	Absorbance
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
mg	Milligram
min.	Minute
ml	Millilitre
mm	Millimeter
Mg	Microgram
Ml	Microlitre
NaOH	Sodium hydroxide
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

INTRODUCTION

Gangotri glacier is located in Uttarkashi district of Uttaranchal state, India between 30°44´-30°56´N latitude and 79°04´-79°15´E longitude. It originates from the Chaukhamba group of peaks at an elevation of 7100 masl. It is about 30 km long flowing in a NW direction and its width varies from 0.5 to 2.5 km. Numerous small sized glaciers feed and contribute into the main glacier to form the Gangotri group of glaciers. Snow and glacier ice of these mountains serve as the major water source for Indian rivers such as the Indus, the Ganges and the Brahmaputra. Bhagirathi river emerging from central Himalayas, flows through the north Indian planes provide water and drainage for over 350 million people.

Glaciers represent possible analogs of extraterrestrial cold habitats. They are also important as long term, chronological repositories of microorganisms. Despite their relevance, studies of diversity, viability, and physiology of the organisms in glacial ice are just beginning. Bacteria that are especially adapted to low-temperature habitats have been described as either psychrotrophic or psychrophilic. Temperature is one of the most important environmental factors for life, as it influences most biochemical reactions. In permanently cold habitats, low temperatures have constrained psychrophiles to develop enzymatic tools allowing metabolic rates compatible to life that are close to those of temperate organisms. Cold biosphere which is highly rich in microbial diversity would be most appropriate to screen microbes for the production of

enzymes. The improved understanding of the bacterial community in glacier ice studied in 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.

Only about 2% of the world's microorganisms have been tested as enzymes sources. The enzymes of psychrophilic microorganisms adapted to permanently low temperatures have attracted much less attention than the enzymes of thermophiles. There are many cold active enzymes such as proteases, lipases, and amylases isolated from cold active bacteria. Cold-adapted enzymes with high levels of catalytic activity at low temperatures are believed to have acquired, through evolution, increased flexibility in their protein structures in order to increase their catalytic abilities.

Proteases are enzymes that catalyze the hydrolysis of peptides and proteins. They represent a very large and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima and stability profile. The nature of the amino acid and other functional groups (aromatic or aliphatic or sulphur-containing) close to the bond being hydrolysed determine the specificity of proteolytic. These biocatalysts hydrolyze peptide bonds in proteins and hence are classified as hydrolases and categorized in the subclasses peptide hydrolases or peptidases.

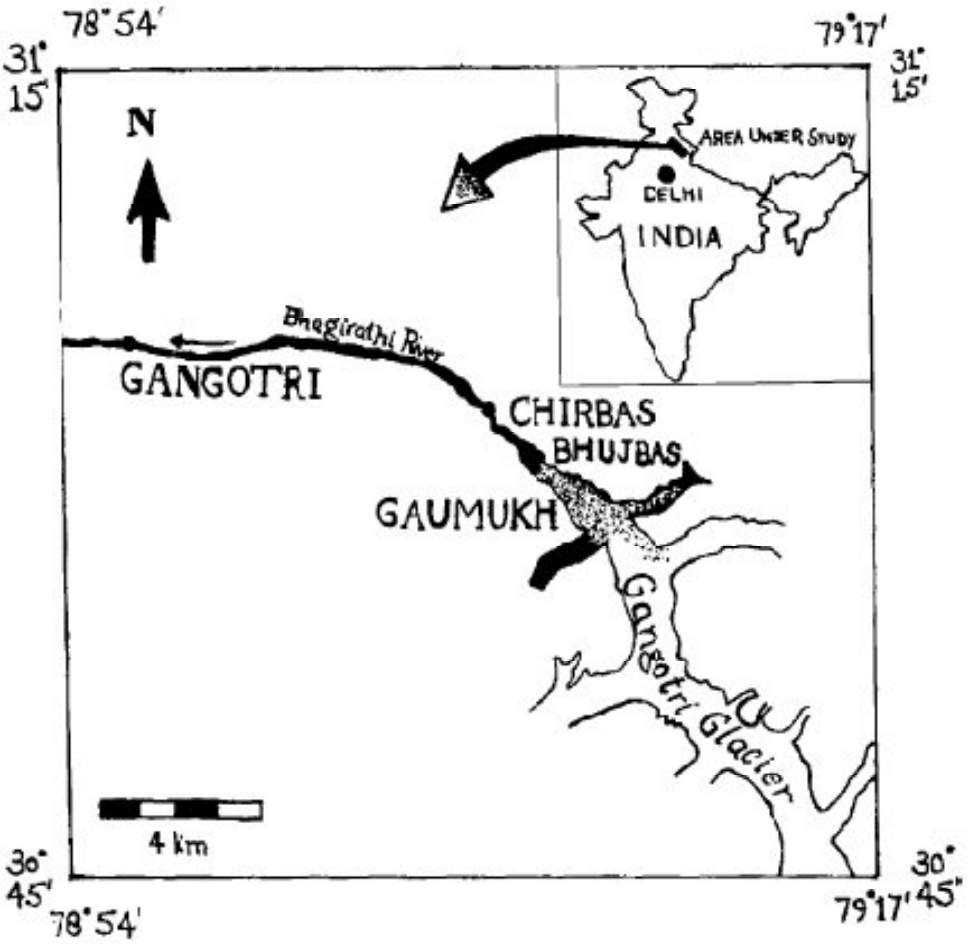

Fig. 1.1: Location map of Gangotri glacier

Lipases catalyze the hydrolysis of acylglycerides and other fatty acid esters. Lipases have been recognized as very useful biocatalysts because of their wide-ranging versatility in industrial applications. The temperature stability of lipases has regarded as the most important characteristic for use in industry.

Cold active enzymes are characterized by high catalytic efficiency at low and moderate temperatures at which homologous mesophilic enzymes are not active. This property is useful in biotechnology in order to-

- Shorten process times for processes operated at low temperatures
- Save energy costs
- Decrease the enzymes concentrations required
- Obtained high yields from reactions involving thermosensitive components
- Prevent undesired chemical transformations
- Prevent the loss of volatile compounds
- Perform on-line monitoring under environmental temperature conditions

Proteases are important industrial enzymes accounting for 60% of total global enzyme sales. Protease production is an inherent capacity of microorganisms. Among them bacterial proteases show more promise than animal and fungal proteases, accounting for 20% of the world market. Proteases and their producing organisms attracted attention of scientific community to understand the protein chemistry and protein engineering to enhance their utilization niche. However, novel proteases with high activity

profile at versatile environments have major application potential in pharma, diagnostic, detergent, tannery, amino acid production and biodegradation.

Psychrophilic lipases have lately attracted attention because of their increasing use in the organic synthesis of chiral intermediates due to their low optimum temperature and high activity at very low temperatures, which are favorable properties for the production of relatively frail compounds. Cold-adapted microorganisms are potential source of cold-active lipases and they have been isolated from cold regions and studied. Cold active lipases offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperature and low thermostability and unusual specificities. Indeed, the cold enzymes, along with the host microorganisms cover a broad spectrum of biotechnological applications. They include additives in detergents (cold washing), additives in food industries (fermentation, cheese).

From the past decade it has been found that cold adapted microorganism and their enzymes provided a large biotechnological potential, offering numerous economical and ecological advantages over the use of organisms and their enzymes which operates higher temperatures. Typically cold enzymes show higher specific activity than that on their mesophilic enzymes that there is a huge biotechnolgical potential of enzymes from psychrophiles. So there is a urgent need to exploit them for stimulating further advances in this fields. Further we will focus on psychrophilic enzymes and their cold adaptations and at structural and molecular level activity parameters and their applications in

various industries. This study will give base line data about biodiversity of cold adapted bacteria in Indian glaciers and more importantly the outcome of the study may give clear picture of biotechnological implications of cold adapted bacteria. The work may be useful for national research laboratories and industries. Industrially important specific proteases and lipase may also isolate and characterize which may have great role in any Indian enzyme industry.

Keeping above in mind following objectives has been taken for study.

- Estimation of micro-orgasnisms.
- Glacier microflora: Genotypic identification and phylogenetic analysis.
- Isolation and characterization of protease producing microorganisms.
- Partial purification, characterization and useful application of proteases.
- Isolation and characterization of lipase producing microorganisms.
- Partial purification, characterization and useful application of lipase.

CHAPTER 2

Review of Literature

REVIEW OF LITERATURE

2.1 Cold adapted bacteria

Life exists in a diverse range of habitats including extremes of temperature, pH, salt, pressure etc. The extremophilic organisms adopted several strategies to survive at such conditions involving physiological modifications of the intracellular environment and the synthesis of biomolecules displaying adequate properties. The most widespread extreme condition for life is represented by low-temperature environments. Organisms adapted to cold environment (e.g. Antarctic or Arctic cold) produce antifreeze proteins (AFP) that prevents the organisms from freezing and allows them to survive below 0^0C (Vries, 1971). "Coping with our cold planet" (Rodrigues and Tiedje, 2008), the title of review unambiguously stresses a frequently overlooked aspect: the Earth's biosphere is predominantly cold and permanently exposed to temperatures below 5^0C. Cold environments, such as polar regions, as well as oceans, which, below a depth of 1000 m, maintain an average temperature of 1–3^0C, make up about 70% of the earth's surface. Cold environments represent a large proportion of Earth's area, including the Arctic, the Antarctic, oceans, and mountain areas (Cowan et al., 2007). Despites of great challenges at low temperature various microorganisms survive at these harsh conditions. Microorganisms which are able to grow at low temperature have been known for long time (Morita, 1966; Farrell and Rose, 1967). Microorganisms have successfully colonized all permanently cold environments from the deep sea to mountain and polar region. Cold adapted microorganisms are of particular importance in global ecology since the majority of terrestrial and aquatic

ecosystems of our planet is permanently or seasonally submitted to cold temperatures: the world's oceans occupy 71% of the earth surface and 90% of their volume is below 5^0C, the polar regions represent 14% of the earth surface and if one includes alpine soils and lakes, snow and ice fields, fresh waters and caves, more than 80% of the earth biosphere is below 5^0C. Microorganisms capable of coping with low temperatures are widespread in these natural environments where they often represent the dominant flora and they should therefore be regarded as the most successful colonizers of our planet (Russell, 1990). Extremophiles successfully colonized on these eternally cold environments which we can call as psychrophiles (which literally means cold-loving). Diverse range of psychrophilic microorganisms, belonging to bacteria, achaea, yeast and fungi have been isolated from these cold environments (Cavicchioli et al., 2002; Deming, 2002; Margesin et al., 2002; Feller and Gerday, 2003; Georlette et al., 2004; Yadav et al., 2014; Yoshida-Mishima, 2016). Recent isolation and characterization of a strain of *Pedobacter himalayensis* from a water sample of the snout of Hamta glacier in the Himalayan mountain range of India (Shivaji et al., 2005a), culturable bacterial diversity under subzero temperature conditions from Leh Ladakh region (Yadav et al., 2015) and a number of novel Antarctic species belonging to the genus *Psychrobacter* (Shivaji et al., 2004, 2005b), *Pseudonocardia* (Prabahar et al., 2004), *Halomonas* (Reddy et al., 2003a), *Sporosarcina* (Reddy et al., 2003b), *Leifsonia* (Reddy et al., 2003c), *Planococcus* (Alam et al., 2003) and *Pseudomonas* (Reddy et al., 2004) have the scope of research in the area of bacterial cold adaptation. Investigations on various aspects

involving these organisms are likely to generate more information on the mechanism of cold adaptation, in near future (Chattopadhyay, 2006).

2.2 Biotechnological and industrial significance of cold adapted bacteria

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. Cold-adapted bacteria, which include psychrophilic and psychrotolerant bacteria are widespread in the natural environment. This ability to cope with low temperature shifts must be accompanied by adaptive changes in response to alterations of numerous physical and biochemical parameters, including solubility, reaction kinetics, membrane fluidity, protein conformation and stability and changes in gene expression. In this respect, they are interesting since, while being able to grow at temperatures close to or below freezing, they kept their ability to survive at mild temperatures. In spite of their importance in natural environments and in refrigerated food, cold-adapted bacteria have been largely ignored and their biotechnological exploitation has remained untapped. This situation is changing and recent interest has focused on the biotechnological implications of cold adapted bacteria and their cold-active enzymes. A better knowledge of the physiology and genetics of these bacteria is necessary to control their degradative abilities in natural cold environments, to optimize the production of their enzymes for biotechnological purposes. Several studies on molecular adaptation to cold conditions have increased interest in cold-adapted bacteria and exhibited the immense biotechnological potentials, for

example, the production of polyunsaturated fatty acids (Russell and Nichols, 1999) and the utilization of cold-active enzymes in specific biotransformations and environmental bioremediations (Margesin and Schinner, 1994; Feller et al., 1996; Russell, 1998).

There is considerable interest in the use of cold adapted bacteria for food biopreservation and in the understanding of cold adaptation mechanisms. The psychrotrophic biopreservative *Lactococcus piscium* strain CNCM I-4031 was studied for its growth behavior and proteomic responses after cold shock and during cold acclimation. Cold acclimation thus constitutes a real advantage in bacterial competition against spoiling and pathogenic psychrotrophic bacteria in terms of food preservation (Garnier et al., 2010). From biotechnological point of view, the cold temperatures adapted features of different microorganisms would be useful for adopting strategies to control refrigerated food-spoiling as well as food contaminating pathogenic bacteria.

Micro-organisms growing at low temperatures and possessing low proteolytic activities might be suitable tools for the production of commercially and medically useful molecules which are very susceptible to proteolysis at higher temperatures in other organisms. The considerable potential of cold enzymes for biotechnological exploitations at low temperature has been recognized. The potential for the biotechnological application of psychrophilic bacteria is receiving increasing attention (Aguilar, 1996). Psychrophilic bacteria are also potential sources of novel pigments (as food additives) and cold-adapted enzymes for industrial application (Feller et al., 1996).

Bioremediation (Timmin and Pieper, 1999) is a recently-established technology that utilizes microorganisms for the restoration of contaminated environments. Many cold-adapted isolates that were obtained are of potential interest for bioremediation and biotechnological applications (Ferror et al., 2003; Gentile et al., 2003). Due to their unique specificity at low and moderate temperatures and catalytic efficient, cold adapted microorganism are ideal for bioremediation purposes and would enable low energy treatment (Margesin, 1998, 1999; Timmis, 1999). Cold-adapted microorganisms show a great potential for the low-temperature biodegradation of hydrocarbons (Margesin et al., 2003). Cold-adapted indigenous microorganisms play a significant role in the *in situ* biodegradation of hydrocarbons in cold environments, where ambient summer temperatures often coincide with their growth temperature range. The potential of hydrocarbon-degrading microorganisms has led to the development of bioremediation techniques for contaminated soil and water (Dua et al., 2002). In some cases, bioremediation can be enhanced by adding microorganisms with specific metabolic functions, a procedure that is referred to as bioaugumentation (Tanaka, 1998). Bioremediation strategies in Arctic and temperate areas that use cold-adapted PCB-degrading bacteria may be more efficient than strategies using mesophilic PCB degrading bacteria, since heating requirements for growth and PCB degradation activity may be reduced. Biodegradation of petroleum hydrocarbons in cold environments, including Alpine soils, is a result of indigenous cold-adapted microorganisms able to degrade these contaminants (Margesin et al., 2003). A large number of degrading bacteria from contaminated cold soils have been identified, including representatives of gram negative and

gram-positive genera (Whyte, 1996; Whyte et al., 1998; Aislabie et al., 2000; Bej et al., 2000; Juck et al., 2000; Baraniecki et al., 2002). The psychrophilic microorganisms as well as their enzymes have been proposed as alternative to physicochemical methods for bioremediation of solids and waste waters polluted by hydrocarbons, oils and lipids (Margesin and Miteva, 2011). Belousova and Shkidchenko (2004) isolated 30 strains including *Pseudomonas* sp. and *Rhodococcus* sp. capable of oil degradation at 4-6^{0}C and maximum degradation of ethanol benzene resins were observed in *Pseudomonas* sp. and maximum degradation of petroleum oils and benzene resins were observed in *Rhodococcus* sp. Further, they stated that the introduction of psychrophilic microbial degraders of oil products into the environment is most important in the contest of environmental problems in temperate regions. In conclusion, cold-adapted microorganisms show a great potential for the low-temperature biodegradation of hydrocarbons. The treatment of waste water, contaminated as a result of human activities would probably be the easiest way to start check the potential application of cold adapted microorganism in biotransformation of chemical (Vazquez et al., 1995) and lowering the amount of toxic compound likes nitrate, hydrocarbons, aromatic compounds, heavy metals and biopolymers, aromatic compounds, heavy metals and biopolymers such as cellulose, chitin, lignin, Proteins and tryglycerol. The psychrotrophic harmless bacteria might be useful for expression of temperature labile pharmaceutical products, low temperature fermentation process to avoid contamination, and degradation of environmental pollutants in oceanic or other low temperature ecosystems (Ray, 1994).

2.3 Cold active enzymes and their implications

Cold active enzymes from cold adapted bacteria have attracted much less attention than the enzymes of thermophiles. Kobori et al. (1984) reported the existence of cold- active enzymes from cold adapted bacteria. Cold adapted bacteria have developed adaptive mechanisms to perform their metabolic functions at low temperatures by incorporating unique features in their proteins and membranes (Cavicchioli et al., 2002; Deming, 2002; Margesin et al., 2002). Compared to proteins from mesophiles, psychrophilic proteins show decreased ionic interactions and hydrogen bonds, possess less hydrophobic groups and more charged groups on their surface and longer surface loops (Cavicchioli et al., 2002; Deming, 2002; Margesin et al., 2002). Due to these modifications, at low temperatures psychrophilic proteins lose their rigidity and gain increased structural flexibility for enhanced catalytic function. Enzymes from psychrophilic organisms have high catalytic activity at low temperatures and low thermal stability (Margesin and Feller, 2010; Margesin and Miteva, 2011). Ohgiya et al. (1999) have described three groups of enzymes according to their thermolability and catalytic properties. Group I comprises those enzymes that have similar activity and more heat-sensitivity than the equivalent mesophilic enzymes, whereas group II enzymes have higher activity at low temperatures and more heat sensitivity. In comparison, enzymes with higher activity at low temperatures but similar thermostability (group III) are rare. 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., 1996). Crystal structures (Aittaleb et al., 1997;

Aghajari et al., 1998; Alvarez et al., 1998) and three-dimensional models (Aittaleb et al., 1997; Feller et al., 1997; 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 (Xie et al., 2009), and aromatic-aromatic contacts, and reduced proline and arginine contents compared with their mesophilic counterparts. The commonly accepted hypothesis for this cold adaptation is the activity-stability-flexibility relationship, which suggests that psychrophilic enzymes increase the flexibility of their structure to compensate for the 'freezing effect' of cold habitats (Johns and Somero, 2004). Recently there has been growing interest in psychrophilic/psychrotolerant enzymes as models in basic studies to investigate the thermal stability of proteins and to understand the relationship between their stability, flexibility, catalytic efficiency, and as potential candidates for R&D and biotechnological applications (Gerday et al., 2000; Siguroardottir et al., 2009). The growing interest on these enzymes is due to unique kinetic and molecular properties, which are attractive catalysts for enthalpy deficient conditions and to develop potentially useful products. The properties of cold-active enzymes provide numerous avenues for industrial application; however, specific properties may be improved through enzyme engineering. This may include enhancing inherent properties, such as increased thermolability and/or catalytic activity at low temperatures, or the modification of pH profiles or other biochemical properties (Cavicchioli, 2002).

The practical utilization of such cold adapted enzymes enables lowering of the temperature and shortening of processing times without a loss of efficiency, which constitutes considerable progress in energy saving. At higher temperature

undesirable chemical reactions occur resulting rapidly inactivation of enzyme. The cold active enzyme can be used in such a unwanted reaction where enzyme required to work at low temperature due to heat sensitivity of substrate (Jeon et al., 2009a). These properties are of particular useful to the food and feed industry where it is important to avoid spoilage, and change in nutritional value and flavour of the original heat-sensitive substrates and products (Russell, 1998; Gerday et al., 2000; Cavicchioli et al., 2002; Tutino et al., 2009). The use of cold active enzymes has a great potential in terms of lower energy costs, use in therapeutics, lowers microbial contamination in industrial processes (Marshall, 1997; Alquati et al., 2002). Various cold adapted bacterial enzyme can be used in food, dairy industry improving cream and whey, cheese ripening (Takasawa et al., 1997), fruit juice and wine industry, animal feed (Iyo and Forsberg, 1999), food storage (Yokoigawa, 2001), biosensors, biotransformation (Alvarez et al., 1997; Feller et al., 1997; Oikawa et al., 2001), health products (Lonhienne et al., 2001), textiles. low temperature acrylamide synthesis (Watanabe et al., 1987), detergent, cosmetics (Mayordomo et al., 2000; Irwin et al., 2001), chemical synthesis and molecular biology (Kawalec et al., 1997; Lanes et al., 2000). Molecular biosciences involve various sequential processes because the actions of an enzyme need to be terminated before the next process. Heat-labile enzymes enable heat inactivation to be performed at temperatures that do not cause double stranded DNA to melt, and the use of heat-labile enzymes obviates the need to use chemical extraction processes (Cavicchioli, 2011).

2.4 Cold active enzyme protease

Enzymes that cleave peptide bonds in proteins are also known as proteases, proteinases, peptidases, or proteolytic enzymes (Kuddus and Ramteke, 2008). 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 (Rao et al., 1998).

According to the Enzyme Commission (EC) classification, proteases belong to group III (hydrolases), and sub-group IV (which hydrolyse peptide bonds). Proteases can be separated into two major groups based on their ability to cleave N- or C- terminal peptide bonds (exopeptidases) or internal peptide bonds (endopeptidases). Exopeptidases are subdivided as aminopeptidases that cleave the N-terminal peptide linkage and carboxypeptidases that cleave the C-terminal peptide bond. Though exopeptidases find commercial applications (such as leucine aminopeptidase, in the debittering of protein hydrolysates), endopeptidases are industrially more important than exopeptidases. 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 (Ward et al., 1985), their pH optima (as acidic, neutral or alkaline), substrate specificity (collagenase, keratinase, elastase), or their homology to previously characterized proteases such as trypsin, pepsin and others (trypsin-like, pepsin-like) (Joshi and Satyanarayana, 2013). Hartley (1960) classified endoproteases into four groups on the basis of their active site and sensitivity to various inhibitors as Aspartic or Carboxyl proteases, Cysteine or thiol proteases, Metallo- proteases and serine proteases.

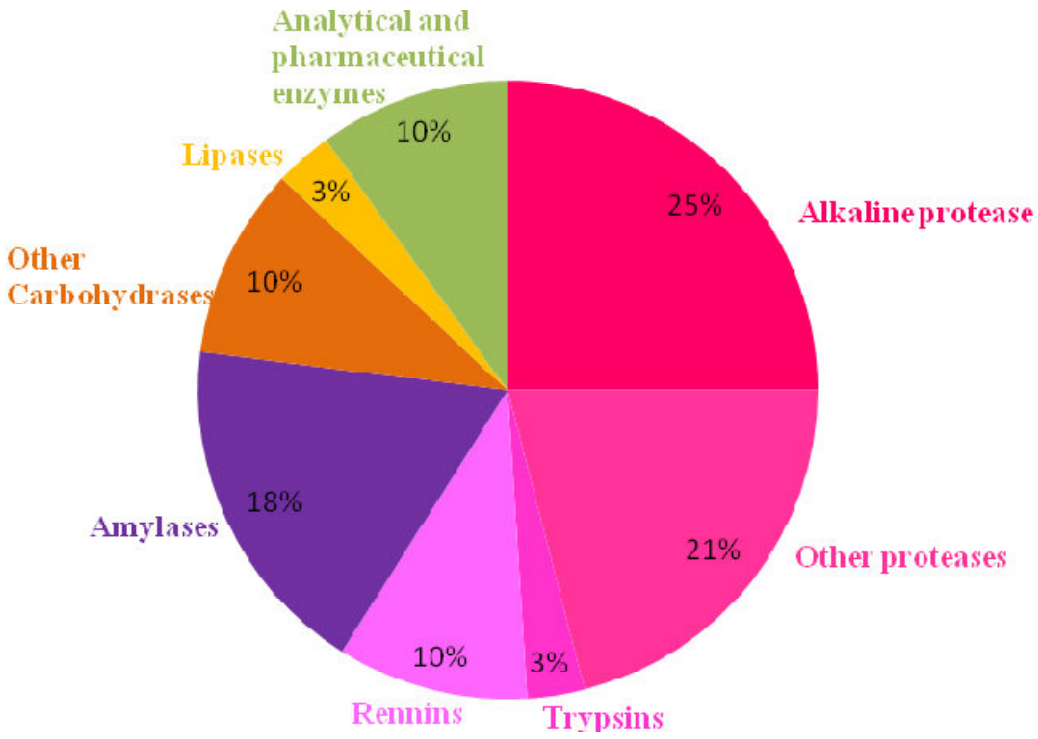

Fig. 2.1: Scenario of enzyme sales in world

Psychrotrophs are also credited to produce heat-stable extracellular proteases (Kasana and Yadav, 2007). Therefore, there is also an increasing number of studies which report on proteases active at low temperature from psychrophilic and psychrotrophic bacteria (Kamata et al., 1992; Hamamoto et al., 1994; Hoshino et al., 1997; Kumeta et al., 1997; Villeret et al., 1997; Liao and Mccallus, 1998; Kristjansson et al., 1999; Kulakova et al., 1999; Irwin et al., 2000; Wang et al., 2005; Pascale et al., 2008). Cold-active proteases 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, sub-Antarctic sediments, sub-glacial water, alpine regions and other cold regions on earth. Vazquez et al. (2004) isolated extracellular proteases from eight psychrotolerant antarctic strains which are with reduced thermal stability but active at a quite broad range of temperature and pH. Martınez-Rosales and Castro-Sowinski (2011) also isolated Antarctic bacteria that produces cold-active extracellular proteases at low temperature but are active and stable at high temperature. Cold-active protease producing microorganisms have been isolated from different geographical regions such as *Bacillus licheniformis* from glacier soil (Baghel et al., 2005), *Exiguobacterium* sp. from cold desert soil (Kasana and Yadav, 2007), *Clostridium* sp. from Antarctic region (Alam et al., 2005), *Colwellia* sp. from sea ice (Wang et al., 2008) and sub-Antarctic sediments (Olivera et al., 2008) *Curtobacterium luteum* from glacier soil (Kuddus and Ramteke, 2008), *Pedobacter cryoconitis* from glacier ice (Margesin et al., 2005), *Penicillium chrysogenum* from cold marine environment (Zhu et al., 2009), *Pseudomonas* sp. from deep sea (Zeng et al., 2003), *Psychrobacter proteolyticus* from Antarctic krill *Euphasia superba*

Dana (Denner et al., 2001), *Serratia* sp. from coastal water (Larsen et al., 2006), *Vibrio* sp. from marine water (Kristjansson et al., 1999). Yu et al. (2011) isolated two genus *Arsukibacterium ikkense* from Greenland and a related strain, *Arsukibacterium* sp. MJ3, from Antarctica, were further characterized with respect to protease production (Lylloff et al., 2016). Saba et al. (2012) Purified and characterized the cold active alkaline protease from *Stenotrophomonas* sp., isolated from Kashmir, India while Kuddus and Ramteke (2009) also isolated the bacterial protease from *Stenotrophomonas* sp. from Gangotri, India. The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Poldermans, 1990; Fox, 1991).

2.5 Biotectnological application of cold active protease

Proteases are important industrial enzymes accounting for 60% of total global enzyme sales (Maugh, 1984; Ward, 1985; Outturp and Boyce 1999; Chu, 2000; Nunes and Martins, 2001; Singh et al., 2001; Beg et al. 2003; Chun et al., 2007).

Commercially Proteases play an important role in various cellular and metabolic processes by taking part in e.g. protein digestion and turnover, enzyme modification and regulation of gene expression (Rao et al., 1998). Apart from their biological importance proteases have gained high relevance in technical enzyme applications (Anwar and Saleemuddin, 1998; Gupta et al., 2002). They are used in the manufacture of detergents, food, cheese making, baking, brewing, pharmaceuticals and leather, in the production of protein hydrolysate, in the wine

industry, in meat tenderization, peptide synthesis, medical diagnosis, and certain medical treatments. The application of proteases in detergent, leather, silk, bakery, soy processing, meat tendering and brewery industries is well documented (Rao et al., 1998; Gupta et al., 2002). On the other, the operation of proteases in organic media is an interesting developing area of biotechnology and biochemistry (Ogino and Ishikawa, 2001). It has also wide application in Bioremediation process (Godfrey and Reichelt, 1985; Anwar and Saleemudin, 1998; Gupta et al., 2002). Because of the functional property, they are widely used in laundry detergents, leather processing, protein recovery or solubilization, meat tenderization, paper and pulp, silk industries, the biscuit and cracker industries (Johnvesly and Naik, 2001; Abidi et al., 2008). In addition, cold-active proteases with high catalytic efficiencies at low-temperatures can be used as of taste enzyme in food industry. For example, cold active protease can improve taste of refrigerated meat treated (He et al., 2004). Wang et al. (2008) reported the cold-active protease from the psychrophilic or psychrotrophic bacterium *Pseudoalteromonas* sp. NJ276 protease had broad substrate specificities and potential application in low-temperature food processing. They treated milk protein by this protease released more free amino acids than those treated by mesophilic papain at 4^0C which would be a good candidate not only for industrial applications, but as additives in baking flour and food processing and preservation.

Tenderness is one of the most important sensory qualities of meat (Huff-Lonergan et al., 2010). Mesophilic or thermophilic proteases enzymes have optimal temperatures of 50–80^0C and only retain less than 10% of its highest activity at 20^0C. This large decrease in activity is a negative attribute for these

enzymes when used as meat tenderizers because meat tenderization is often carried out at room temperature before cooking (Tappel et al., 1956; El-Gharbawi and Whitaker, 1963; Allen Foegeding and Larick, 1986). In addition, the mesophilic or thermophilic proteases are usually stable, even during cooking, and therefore, over-tenderization can occur (Cronlund and Woychik, 1986). An ideal meat tenderizer should be a specific enzyme with high activity at room temperature and be easily inactivated during cooking. Cold adapted proteases may have potential use as meat tenderizers. MCP-01, the most abundant extracellular protease secreted by the psychrophilic bacterium *Pseudoalteromonas* sp. SM9913, is a novel serine protease of the subtilase family (S8), and also a cold-adapted enzyme (Chen et al., 2007). MCP-01 could significantly reduce the beef meat shear force and kept the fresh colour and moisture of the meat when applied at 4^0C (Zhao et al., 2012). Another important use of the protease of *P. aeruginosa* MCM B-327 was found for dehairing hides (Zambare et al., 2011).

In view of its possible applications, alkaline protease from extreme organisms should be produced commercially in high yield at a low-cost method (Patel et al., 2005). For example, commercial applications include a protease from Novozyme (trade name Savinase) sold as an encapsulated detergent. Researchers in Japan at the Hokkaido National Industrial Research Institute have isolated organisms from cold soil and water that have yielded a cold-active protease from *Pseudomonas* PL-4. Japan Advanced Institute of Science has two patents for cold-active proteases CP-58 and CP-70 (Quamrul et al., 1998). Another Japanese company, Kao Corporation, has filed a number of patents for cold-active proteases for use in detergents (Hiromi et al., 2006).

More importantly, cold-active proteases could offer economic benefits through energy savings: they negate the requirement for expensive heating steps, function in cold environments during the winter season, provide increased reaction yields, accommodate a high level of stereo specificity, minimize undesirable chemical reactions that can occur at higher temperatures and exhibit thermal liability for rapidly and easily inactivating the enzyme when required (Cavicchioli et al., 2006). For example, the ability to heat-inactivate cold-active proteases has particular relevance to the food industry where it is important to prevent any modification of the original heat-sensitive substrates and product.

Several studies have indeed shown that cold-active proteases are often characterized by higher turnover numbers (kcat) and catalytic efficiencies (kcat/Km) at low temperatures compared to their mesophilic counterparts (Turkiewicz et al., 1999). Their properties make them potentially useful for industrial applications on processes carried out at neutral pH and ambient temperature in temperate-climate zones, as these enzymes have their optimal activity at temperatures where the mesophilic enzymes show a considerable reduction of their maximal activity. An attempt has been made to isolate microorganisms capable of producing cold active alkaline proteases (Saba et al., 2011). They describe the production and characterization of a cold active alkaline protease from *Stenotrophomonas* sp. isolated from the high altitudes of Thaji glacier. They isolated enzyme that can be used as an ingredient for the preparation of detergents for cold washing. The Protease from *Stenotrophomonas* sp. IIIM-ST045 was significantly more stable in commercial detergents for wool finishing treatments aimed at increased comfort (reduced prickle, greater softness) as well

as improved surface appearance and also gave a good finish to silken cloth by performing the washing at lower temperature. The cold active protease of *Serrratia marcescens* strain TS1found to more active to remove the dirts and stains from the clothes at low temperature in a short time without damaging the nature of the cloth (Tariq et al., 2011). So the economic values and enhancer actions will be boom to the detergent industry as detergent additives. Cold-active detergents are commonly advertised because washing is now frequently carried out at environmental temperatures. Patents involving enzymes such as proteases from cold-adapted bacteria have already been filed as Baeck and Quamrul, ZA9610820; Mikio and Katsuhisa, WO9743406; Quamrul and Eiichi, US6200793; Quamrul and Eiichi, WO9730172; and Eiichi, ZA9601237 (Marx et al., 2006).

Cold active protease can be used in peeling process of leather for energy saving. So economic benefits can be achieved by using cold-active proteases as they allow working at low temperatures even in an industrial scale. 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.

In textile industry, proteases may also be used to remove the stiff and dull gum layer of sericine from the raw silk fiber to achieve improved luster and softness. Protease treatments can modify the surface of wool and silk fibers to provide new and unique finishes. The increasing use of synthetic fibers which cannot tolerate temperatures above 50–60^{0}C has changed the washing habits

during the past years toward the use of lower washing temperatures (Nielson et al., 1981).

In addition to these applications, they have also shown promise as therapeutic modalities for cosmeceutical applications (by reducing glabellar lines) and a number of disease conditions, including bacterial infections (by disrupting biofilms to prevent bacterial infection), topical wound management (when used as a debridement agent to remove necrotic tissue and fibrin clots), oral/dental health management (by removing plaque and preventing periodontal disease), and in viral infections (by reducing the infectivity of viruses, such as human rhinovirus 16 and herpes simplex virus (Fornbacke and Clarsund, 2013). Furthermore, data from post market studies suggest that the use of ColdZyme (Enzymatica AB, Lund, Sweden) as mouth spray, an oral solution containing glycerol and a cold-adapted cod trypsin, can reduce the incidence of the common cold. Furthermore, the cold adapted trypsin used in ColdZyme mouth spray has shown high efficiency in reducing the infectivity of human rhinovirus 16 (ColdZyme (product information). Lund, Sweden: Enzymatica AB; 2011) and herpes simplex virus 1 *in vitro* (Hilmarsson et al., 2010).

2.6 Cold active lipase

Lipase belongs to the enzyme class of hydrolases (E.C.3). It acts on ester bonds (E.C.3.1) of carboxylic esters (E.C.3.1.1). They hydrolyze triacylglycerols to fatty acids, diacylglycerol, monoacylglycerol, and glycerol (Carriere et al., 1994) and known as triacylglycerol acyl hydrolases (E.C.3.1.1.3). They resemble esterases, but differ markedly from them in their ability to act on water-insoluble

esters (Brockerhoff and Jensen, 1974). Lipolytic enzymes are involved in the breakdown and thus in the mobilization of lipids within the cells of individual organisms as well as in the transfer of lipids from one organism to another (Beisson et al., 200).

Cold adapted lipases are largely distributed in microorganisms existing at low temperatures nearly 5^0C. Although a number of lipase producing sources are available, only a few bacteria were exploited for the production of cold adapted lipases (Joseph, 2006). The organism *Curtobacterium* sp. Isolated from the soil samples of Gangotri glaciers was able to produce cold active lipase at low temperature (15^0C) and alkaline pH (8.0) (Jaiswal and Joseph, 2011). Cold adapted bacterial strains were isolated mostly from Antarctic and Polar regions, which represents a permanently cold (0 ± 2^0C) and constant temperature habitat. Another potential source of cold active lipases is deep sea bacteria. A marine bacterium *Aeromonas hydrophila* growing at a temperature range between 4 and 37^0C was found to produce lipolytic enzyme (Pemberton et al., 1997). *Aeromonas* sp. (Lee et al. 2003), *Pseudoalteromonas* sp. and *Psychrobacter* sp. (Zeng et al., 2004), *Photobacterium lipolyticum* (Ryu et al., 2006). Purification and Characterization of cold active lipase from psychrotrophic *Aeromonas* sp. LPB 4 was done by Lee et al. (2003). Recently, the genes of cold-adapted lipases from psychrotrophic bacteria *Moraxella* TA144 (Feller et al., 1991) and *Psychrobacter immobilis* B10 (Arpigny et al., 1993) isolated from antarctica were cloned and sequenced. The enzymes showed high activities at temperatures as low as 3^0C. A psychrotrophic bacterium producing a cold-adapted lipase upon growth at low temperatures was isolated from Alaskan soil and identified as a Pseudomonas

strain (Choo et al., 1998). A number of cold active lipases from different sources have been studied in recent years from *Bacillus subtilis* lipase (Ahmad et al., 2012), *Pseudomonas fluorescens* (Bofill et al., 2010) and *Pseudomonas vancouverensis* (Gupta and Prakash, 2014). The properties of these enzyme high activity in the range of 0-20^{0}C and high catalytic efficiency in fatty acid hydrolysis at 20^{0}C indicate that this enzyme which can be potentially applied industrially to hydrolyze fatty acid. The bacterial strains which were isolated from cold habitat such as Antarctic and Polar regions which represents a permanently cold and constant temperature habitat. 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^{0}C. They include *Aeromonas* sp. (Lee et al., 2003), *Pseudoalteromonas* sp., *Psychrobacter* sp. (Ohmae et al., 2012) and *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. Permanently cold regions such as mountain and glaciers are another habitat for cold active enzymes producing microorganisms.

2.7 Cold active lipase and their biotechnological implications

Cold active lipases offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperature and low thermostability and unusual specificities. Recently, much attention has been made to the application of psychrophiles, psychrotrophs, and their cold-active enzymes

in biotechnology (Choo et al., 1998, Kulakova et al., 1999, Gerday et al., 2000). New applications of the microbial lipases such as hydrolysis of fats, transesterfication, stereospecific hydrolysis of racemic esters, organic synthesis, and the use of lipases in detergents have been developed (Mckay et al., 1993; Gerday et al., 1997; Marshall et al., 1997). A number of reports mentioned straightforward reasons why cold-active enzymes have application in biotechnology (Russell et al., 1998; Margesin and Schinner, 1999; Ohgiya et al., 1999; Gerday et al., 2000; Cavicchioli et al., 2002). Lipases are very commonly used in pharmaceutical industry because of its regioselective property. Lipases have found broader 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 (Griebeler et al., 2011).

The use of cold-active lipase in the formulation of detergents would be of great advantage for cold washing that would reduce the energy consumption and wear and tear of textile fibers (Hasan et al., 2009). Enzymes can reduce the environmental load of detergent products since they save energy by enabling a lower wash temperature to be used. Addition of cold-active lipsase in detergent become biodegradable, leaving no harmful residues, have no negative impact on sewage treatment processes and do not present a risk to aquatic life. The low temperature active lipase can be added to detergents to hydrolyze oily stains at the temperature of tap water to reduce energy consumption and protect the color of fabrics (Siddiqui and Cavicchioli, 2006).

Surfaces that are at ambient temperatures, such as buildings, carpets and benches, cannot easily be heated or immersed in cleaning solutions and tend to be cleaned using sprays or wipes, providing good avenues for the use of cold-adapted enzymes. lipase have been used in a cleaning solution in a building conservation project to improve the removal of mould from stone and reduce the damage normally associated with the use of standard cleaning agents (Valentini et al., 2010). The industrial dehairing of hides and skin at low temperature using psychrophilic lipase would not only save energy but also reduce the impacts of toxic chemicals used in dehairing.

Lipases from some of the industrially important chemicals manufactured from fats and oils by chemical processes could be produced with greater rapidity and better specificity under mild conditions. The use of industrial enzymes allows the technologists to develop processes that more closely approach the gentle, efficient process in nature. Microbial lipases are be utilized for food processing, a few, especially psychrotrophic bacteria of *Pseudomonas sp*. Cold-active lipase from *Pseudomonas* strain P38 is widely used in non-aqueous biotransformation for the synthesis of n-heptane of the flavoring compound butyl caprylate (Kazlauskas and Bornscheuer, 1998; Joseph et al., 2008).

Biodiesel is a non-toxic, biodegradable, environmental friendly and non-fossil fuel (renewable) that produces significantly lower emissions than petroleum-based diesel when it is burned, whether used in its pure form or blended with petroleum diesel. Lipases have an important application in the field of bioenergy, especially in biodiesel production, which is an expanding sector, as a result of the worldwide rising demand on the use of renewable energy (Colla et al., 2010). The

lipase is methanol-stable and is capable of production of biodiesel from olive oil and waste cooking oil (Yang et al., 2009). Lipase MA1-3 performed transesterification reactions using waste oils, palm oil, and methanol, which suggests that this enzyme represents a potential catalyst for the production of biodiesel (Kim et al., 2012). Only recently a psychrophilic *Pseudomonas fluorescens* lipase for a transesterification reaction in the production of biodiesel under low-temperature conditions was described (Luo et al., 2006). *Photobacterium lipolyticum* M37 was isolated from an intertidal flat in Korea. This bacterium produces a cold-adapted lipase (Ryu et al., 2006). The application of such psychrophilic enzymes to catalyse reactions at low temperatures offers enormous industrial potential; however, the requirement for continued research in this area is reflected in the current industrial status of such cold-active lipases within the biodiesel production process (Joseph et al., 2007).

Ramani et al. (2010) purified lipase from the *P. gessardii* had the highest hydrolytic activity towards olive oil (100%), palm oil (96%), fried cooking oil (96%) and animal tallow such as beef (146%), goat (112%) and chicken tallow (97%) although it could hydrolyze all the oils and fats tested in study, suggesting that the purified lipase has high potential application in digestion of lipids. A large amount of lipid wastes are discharged from households, slaughterhouses and different industries, each day and causes heavy pollution in fresh water and seawater. Therefore, the lipase with high hydrolytic activity towards animal fats and vegetable oil may have high potential applications in degradation of these lipid wastes (Ramani et al., 2010).

In the pharmaceutical industry, lipases can be used for racemic mixtures resolution or as biocatalysts for the synthesis of chiral synthesis intermediates (Gotor-Fernández et al., 2006; Ghanem, 2007). Besides this enzymes can be used as efficient catalysts in organic synthesis (Reetz, 2000; Bertau, 2002; Secundo and Carrea, 2003). Many examples of using lipases in organic synthesis have been reported in the literature. Lipases are versatile biocatalysts that not only serve to achieve hydrolysis but are also capable of catalysing the reverse synthesis reaction to produce esters under certain conditions. Martinelle and Hult (1995) reported kinetics of acyl transfer reaction in organic media catalysed by lipase from Antarctica. Applications of lipase isolated from Antarctica in organic synthesis has been reported (Anderson et al., 1998). Lee et al. (2003) isolated cold active lipase producing psychrotrophic bacterium, *Aeromonas* sp. LPB4. It was interesting that purified lipase from *Aeromonas* sp. LPB 4 maintained the lipolytic activity at low temperature.

The overwhelming interest for screening lipases for use in the cosmetic and perfume industry has mainly been due to its activity in surfactant and aroma production, which are the main ingredients in cosmetics and perfumes (Kao, 1995). Generally in cosmetic industry Lipases are widely used, especially cold-adapted lipases (Pandey, 1999; Joseph et al., 2008). Cold active lipase has high activity so it can be used in catalysis of various reaction for cosmetic preparation.

Cold-adapted lipases have great potential in the field of wastewater treatment, bioremediation in fat contaminated cold environment and active compounds synthesis in cold condition (Buchon et al., 2000). Suzuki et al. (2001) identified a psychrotrophic strain of the genus Acinetobacter strain no. 6 as it

produced extracellular lipolytic enzyme that efficiently hydrolyzed triglycerides such as soybean oil during bacterial growth even at 4^0C, the bacterium is potentially applicable to *in situ* bioremediation or bioaugumentation of fat contaminated cold environments. The wide application of psychrophilic enzyme in industrial scale are the recently exploiting, and a lot to find the future aspect in various applications.

It can be concluded that cold adapted bacteria have much more to contribute to the field of biotechnology (Gerday et al., 2000). Thus it is necessary to identify further key feature of these psychrophilic enzymes to evaluate and upgrade their biotechnological potential (Pulicherla et al., 2011). Wide and constant screening of cold adapted bacteria for their cold active enzymes at low temperature will open novel and simpler routes for the industrial processes. Consequently, this may pave new ways for biotechnological applications.

CHAPTER 3

Materials & Methods

MATERIALS AND METHODS

3.1 Collection of sample

Soil and water sample were collected from eight different sampling sites from the run off of glaciers, covering the stretch from Gaumukh to Haridwar for the isolation of cold adapted protease and lipase producing bacteria. The glacier is situated in between $30^0$04'-$79^0$15'E and around 30 Km in length covering an area of 143 km^2 following North West. 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 -20^0C (Foght et al., 2004).

3.2. Culture media / Reagent

Luria bertani broth (LB) (pH 7.5)

Ingredients	**g/l**
Bacto-tryptone	10
Bacto- yeast extract	5
NaCl	10

Mueller-Hinton Agar (pH 7.4)

Ingredients	**g/l**
Beef infusion	300
Acid hydrolysate of casein	17.5
Starch	1.5
Agar	17.0

Skim 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 121^0C for 15 min. Milk powder was dissolved in 300ml water by heating at 50^0C, sterilized at 113-115^0C (8 lbs) pressure for 20 min. The two solutions were cooled at 55^0C and mixed in one flask aseptically.

Tributyrin medium (pH 7.0)

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

Nutrient Agar (pH 7.0)

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

PSC Medium

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

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%

Peptone water (7.2)

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

Nutrient broth

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

Phosphate buffer (pH 7.0) 50 mM

A. KH_2PO_4 13.609 GM/500 ml

B. 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 200ml with H_2O for the preparation of 200 Mm buffer.

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) were 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.

Buffer for enzyme characterization

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.

3.3. 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 37^0C. For colony counting plates, appropriate dilutions which contain colonies ranging from 30 to 250 was selected. The numbers of colonies were counted and bacterial load was recorded as CFU per gram of soil by applying the formula (Devi et al., 2012).

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

3.4 Screening and isolation of cold active protease producing bacteria

One gram of the soil sample was suspended in 10 ml of phosphate buffer saline and after appropriate dilution sample was spread on PSC Agar media containing 10% skimmed milk. The proteolytic activity was assayed using skimmed milk agar and expressed as diameter of clear zone.

One hundred twenty bacterial colonies were isolated on PSC solid agar medium from eight samples of Gangotri glaciers. These one hundred twenty isolates were found to be capable of showing protease activity by clear zone formation. On the basis of diameter of zone hydrolysis on PSC solid agar media at temperature 20 ± 2^0C. Ten potential isolates, designated as AAPRT-3, BBPRT-7, BBPRT-13, CCPRT-12, DDPRT-6, EEPRT-5 FFPRT-10, GGPRT-6, GGPRT-8, and HHPRT-8 were selected as potent protease producing strains. These isolates were subjected for further taxonomical characterization. Among these, three isolates were found to be capable of producing protease at alkaline pH at different temperature ranging from 10 to 37^0C.

3.5 Screening and isolation of cold active lipase producing bacteria

For the preliminary screening of lipase producing bacteria trybutyrin agar media was used. Lipase production is indicated by the formation of clear halos around the colonies grown on tributyrin containing agar plates (Jaeger, 1994; Ertuğrul, 2007). These one hundred isolates were found to be capable of showing lipase by clear zone formation at 20 ± 2^0C. On the basis of clear zone around the

colony on trybutyrin agar, eight potential isolate was selected as potent lipase producing strain. These lipase producing lipase producing strains are designated as AAPRTL-10, BBPRTL-5, CCPRTL-8, DDPRTL-14, EEPRTL-5, FFPRTL-9, GGPRTL-6, GGPRTL-5. The isolates were subjected for further taxonomical characterization. Among these, one isolates was found to be capable of forming larger zone indicates production of lipase at alkaline pH at different temperature ranging from 10 to 20^0C.

3.6 Identification of potential isolates

Morphologicl and biochemical characteristics

All the isolates (ten protease producing and eight lipase producing) were identified by studying morphological and biochemical characteristic according to Bergey's Manual of Systematic Bacterialogy (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% amidoblack 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.

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'

PCR was performed as follows in a total volume of 50 µl in a 0.2 ml thin walled PCR tube. The related sequences were preliminarily aligned with CLUSTALW (Thompson et al., 1994). 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 MEGA5 software.

3.7 Assay 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 37^0C for 10 min. 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 10^0C 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 37^0C for 10 min. After that the six fold diluted Folinecioelteae reagent (one ml) was added and allowed to stand for 30 min. at 37^0C. 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.

Protein content was measured by the method of Hartree-Lowry et al. (1951) with Bovine Serum Albumin (BSA) as the standard.

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).

Relative activity

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

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

Residual activity

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

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

3.8 Protease production

The bacteria was grown at 20^0C in twelve, 500 ml Ehlernmeyer flask containing 100 ml PSC medium (pH 10) with 10% skimmed milk for 24 h at incubator shaker at 200 rpm. Then culture was centrifuged at 10000 rpm for 20 min. at 4^0C.

3.9 Partial purification of protease enzyme

The protease was purified as per the standard protein purification procedures which involved various steps such as centrifugation, ammonium sulphate precipitation, diafiltration.

Ammonium sulphate precipitation

Different types of salts such as ammonium sulfate and sodium sulfate are known to precipitate protein effectively. But ammonium sulfate is most widely used for precipitation of proteins as it is highly soluble (solubility is 3.9 M at 0^0C and 4.1 M at 25^0C), inexpensive, available in highest purity (as enzyme grades), does not alter the protein solution to extremes of pH and in most cases does not denature proteins. Moreover, as the dissolution process is endothermic, the enzyme solution gets cooled and stabilizes the enzymes. Furthermore, it does not absorb significantly at 280 nm. During ammonium sulfate precipitation, the salt has to be added in small portions under constant stirring to prevent increase of high salt local concentrations. At 90% saturation, ammonium sulfate precipitates almost all the proteins. When large amount of salt is added to an aqueous solution of proteins, the salt requires large number of water for its own dissolution and hence competes for the water molecules on the proteins. As the completely ionized salt has more affinity for water molecules than the proteins, more and more addition of salt takes up the water molecules from the proteins. Therefore, the ionic interactions between water molecules and proteins are reduced. As a consequence the hydrophobic interactions dominate. The 'hydrophobic amino acid patches' present indifferent proteins attract each other and aggregates. Thus, the

protein precipitates are nothing but the aggregates of the protein molecules large enough to be visible.

In salt precipitation, the anions appear to be more important. Temperature, pH and most importantly the protein concentration affect ammonium sulfate precipitation of protein to large extent. Higher ammonium sulfate concentration is required for precipitating highly soluble proteins. Precipitating proteins with ammonium sulfate is also known as "salted out".

Procedure

Cell free supernatant was subjected to protein precipitation by means of ammonium sulfate. Fractionation of protein was made by addition of solid ammonium sulfate by slow continuous stirring in a cold room. The amount of ammonium sulfate required for percentage saturation was calculated by the method of Deutscher (1990). The saturated solution was left over night at 4^0C and centrifuged at 10,000 rpm for 20 minutes at the same temperature. The supernatant was used as the starting material for the next fractionation. Fractions were precipitated at 0- 40%, 40-60%, 60-80% saturation. The precipitate was dissolved in small volume of 0.1M phosphate buffer, (pH 6.0) and dialyzed. All subsequent steps were carried out at 4^0C. The fraction giving the precipitate was checked for the enzyme activity as well as protein content.

Dialysis

The partially purified enzyme was diafiltered using dialysis membrane against glycine NaOH buffer (pH 10.0 for 24 hr at 4^0C). This was done to remove

ammonium sulphate. The dilysed partially purified enzyme served as a enzyme source for characterization and its possible application.

3.10 Characterization of protease

Effect of pH

Activity of the protease was measured at different pH using the following buffers [10M: Phosphate, (pH 7-8)], Glycine NaOH buffer pH 9-11.5 at 20^0C. To study the pH stability, the enzyme was dissolved in the above mentioned buffer and incubated for 1 hr at 20^0C then enzyme assay was performed without substrate and then the residual activity was estimated as per standard assay procedure.

Effect of temperature

The proteolytic activity of protease was performed at different temperature for the study of optimum temperature (10-50^0C). To study the enzyme stability, enzyme was first preincubated (pH 10.0) at designated temperature for 1 hr. After the incubation the proteolytic activity was determined by the protease assay, and optical density was measured at 280 nm.

Effect of detergent / commercial detergent on protease activity

The effect of detergent (1%w/v) such as SDS, Tween 80, Surf, Wheel and tide on enzyme stability were studied by preincubating the enzyme with respective detergent for 1 h at 20^0C. The residual activity was measured by employing standard assay procedure. The activity of the enzyme without any addition was considered as 100% activity.

Effect of metal ion

Metal and inhibiting agent present in surroundings play important role in the growth of bacteria. The protease was preincubated for 1 hr with different metal ion including, Cu^{+2} ($CuSO_4$), Zn^{+2} ($ZnCl_2$), Hg^{2+} ($HgCl_2$) at a concentration of 5.0 mM and with inhibitor like cystein, EDTA remaining activity was determined after the incubation period.

3.11 Assay of lipase

The lipase activity in the culture supernatant was determined by p-nitrophenylpalmitate (p-NPP) substrate as described by Winkler and Stuckmann (1979) with some modifications. In brief, the reaction mixture consisted of 135 ml of 0.4% (w/v) Triton-X, 0.1% (w/v) gum Arabic in 50 mM Tris-Cl buffer, pH 7.0) and 15 ml of substrate (16.6 mM r-nitrophenyl substrate in 2-propanol). The mixture was pre-reacted at 10^0C for 10 min and 50 ml of enzyme solution was subsequently added. The color change was measured at 405 nm using multi-well plate reader after 30 min of incubation at 10^0C. Protein concentration was measured by the method of Bradford (Bradford, 1976) with bovine serum albumin as a standard.

One unit of lipase activity is defined as the amount of enzyme releasing 1 µ mol p-NPP min^{-1} under assay condition.

3.12 Production of lipase enzyme

The stock culture was maintained in nutrient broth and glycerol (50:50, v/v) at -20^0C. Working cultures were prepared by two successive transfers of

stock culture to trybutyrin agar plates and incubated for 48 h at 15^0C. The extracellular lipase production was carried out in a medium composed of 3% w/v yeast extract, 1% w/v KH_2PO_4, 0.1 w/v $MgSO_4.7H_2O$, 0.5% w/v maltose and 0.2 v/v olive oil at pH 7.2. Medium was sterilized and incubated with 1% inoculum prepared in nutrient broth followed by incubation at 15^0C for 48 h at 160 rpm in a shaker incubator. The cells were centrifuged at 10000 rpm for 15 min and the supernatant was used directly as crude preparation of lipase for further studies.

3.13 Partial purification of lipase enzyme

The cells were centrifuged at 10000 rpm for 15 min and the supernatant was subjected to ammonium sulphate fractionation of 0-40%, 40-60% and 60-80%. Fraction was dialyzed against glycine NaOH buffer. Cold-active lipase was purified to homogeneity by precipitating with ammonium sulphate fractions and using a single step ion exchange chromatography on a DEAE-cellulose. Enzyme was eluted from the column as unbound fractions with 0.7M NaCl gradient. The purified enzyme served as a enzyme source for further characterization.

3.14 Characterization of lipase

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 50^0C for 15 min and activity was determined. To study the enzyme stability at different temperature, purified enzyme was dissolved in 50 mM phosphate buffer (pH 8.0),

pre-incubated at different temperatures ranging from 10 to 50^{0}C for 1 h, rapidly residual activities were measured by the standard assay procedure.

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 10). 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 15^{0}C for 1 h and residual activity was measured at pH 9-11.

Effect of inhibitors and metal ion

The effect of organic compound and inhibitor on the stability of purified lipase was investigated by the modified procedure of Ogino et al. (2000). The enzyme was incubated with various inhibitors (1 mM) compounds such as EDTA, SDS, and dithiothreitol (DTT) and Tween 80 that may inhibit the enzyme, metal ions Zn^{2+}, Na^{+}, Mg^{2+}, Ca^{2+}, Fe^{2+},$CO^{+2,}$ Cu^{+2}, Hg^{2+} in the form of (5 mM): $ZnSO_4$, $NaSO_4$, $MgSO_4$, $CaCl_2$, $FeSO_4$, $COCl_2$, $CuSO_4$ and $HgCl_2$ at 20^{0}C for 30 min and the residual activity was measured with *p*-nitrophenyl palmitate as the substrate at 25^{0}C. Residual activities were measured and compared with a control (without metal ions/inhibitors/detergents).

CHAPTER 4

Results & Discussion

RESULTS AND DISCUSSION

4.1 Quantitative and qualitative study of microorganism

In 1992, the International Center 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 to harness its potential for developing technological options for sustainable agriculture (Jodha et al., 1992). Following these recommendations, in the early 2000s Indian scientists started to bioprospect diverse Himalayan biotopes to explore their microbial wealth and diversity and began to isolate microorganisms with potential applications in various fields (Trivedi et al., 2012). Recent research in the field of Cryobiology in India has gained momentum with workers such as Srinivas et al. (2009), Reddy et al. (2009), Shivaji et al. (2009) on bacterial diversity of the cold active enzymes in the Ny Alesund glaciers. Psychrophilic microorganisms have successfully colonized all permanently cold environments from deep sea to mountains. Bacterial abundance in permafrost varies depending on the environment. According to direct microscopic counts, Siberian and Antarctic permafrost yield 10^7-10^8 and 10^5-10^6 cells per gram of dry mass, respectively (Gilichinsky et al., 2008). A large proportion of cold adapted bacteria existing indicate the ability of continued growth and metabolism at low temperature. They are commonly found in ice dust (Hodson et al. 2008; Mac Donell and Fitzsimons, 2008), glaciers, in all Antarctic zones and in artificial cold systems like refrigerators and freezers either at domestic or industrial level (Baross and Morita, 1978; Willerslev et al., 2004).

Microorganisms have been documented in ancient glacier ice (Christner et al., 2003; Miteva and Brenchley, 2005), sub glacier water (Mikucki et al., 2009), basal ice (Sheridan et al., 2003; Miteva et al., 2008), sub glacier lakes and accreted ice (Karl et al., 1999; Priscu et al., 1999; Gaidos et al., 2004).

The composition and characteristics of the bacterial isolates might be related to the variation of climatic and environmental conditions. Glacial ice is an intriguing habitat for studying of bacteria, because it represents an extreme environment with low nutrient concentrations and subzero temperatures. The related researches will expand our knowledge of microbial diversity and provide insights into microbial survival for extended times. The availability of a soil and ice from the Gangotri glacier provided a special opportunity to examine the diversity and possible activity of bacteria present in ice. Microbiological studies revealed the presence of bacteria in samples of Gangotri glacier. Plate counts were performed in duplicate at eight sampling sites and the results are presented as the means of the plates. Total viable bacterial soil samples of different places of Gangotri glacier are summarized in Fig. 4.1. Total bacterial count varied from $10x10^6$ to $16x10^6$ cfu g^{-1} of soil at 37^0C. The lowest bacterial counts were found at Gaumukh, Gangotri and Bhatwari while highest bacterial counts were found at Haridwar. Soil samples collected from Gangotri glacier contains high bacterial count varying from $10x10^6$ to $15x\ 10^6$ cfu g^{-1} of soil (Joseph et al., 2012). The lowest bacterial count was found at Gaumukh, Gangotri, Bhatwari while highest bacterial count were found at Haridwar. 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.

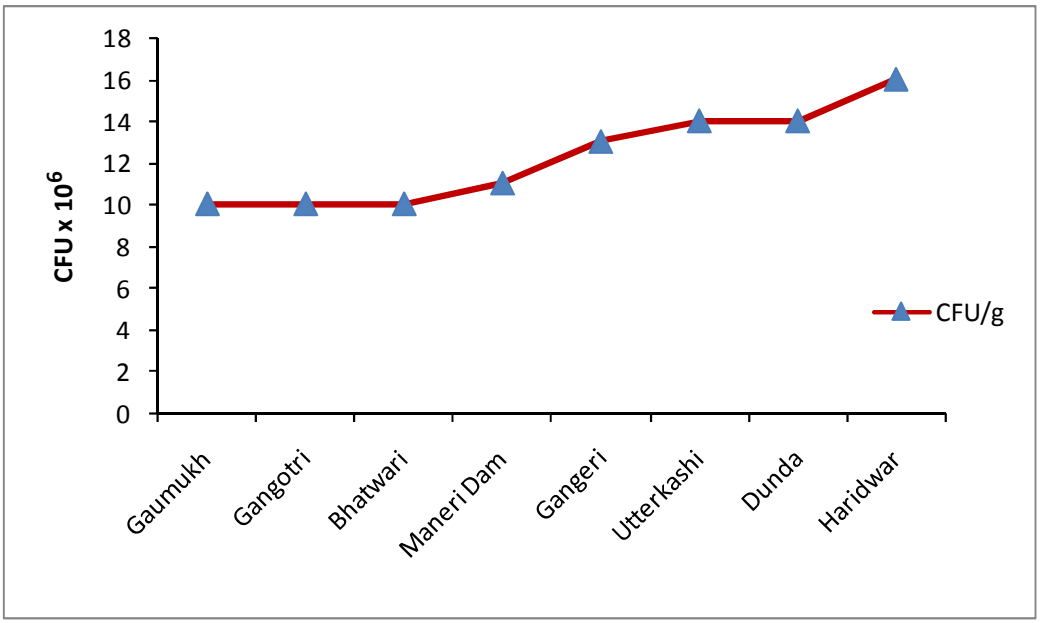

Fig. 4.1: Microbial load of study samples

The total viable count of aerobic heterotrophs ranged from $3.4x10^2$ – 8.0 $x10^3$ c.f.u./ml. 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. 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 $\times 10^3$, respectively. Total viable count exceeded the maximum permissible limits in all the samples irrespective to different seasons. Baghel et al. (2005), Sood et al. (2008) have also observed high TVC values in the entire stretch of river Ganga in Uttarakhand region. The present work is intended to reveal culturable bacterial communities on Gangotri glacier.

4.2 Isolation, characterization, identification and phylogenetic analysis of protease producing bacteria

Cold biosphere which is highly rich in microbial diversity would be most appropriate to screen microbes for the production of enzymes. Moreover, research on psychrophilic microorganisms is far behind that of thermophiles. Keeping in mind the potential applications of psychrophilic microbes and their biomolecules, we isolated bacteria from sample of Gangotri glaciers, India. This region is a part of Himalayas, which remained unexplored for microbial diversity. Cold-adapted microorganisms are a potentially exploitable source for cold-active enzymes (Gerday et al. 2000, Margesin 2002). Thus, knowledge about the type and characteristics of the extracellular proteases produced by the microorganisms is essential to understand the dynamics of the environment where such

microorganisms live. Therefore, an attempt has been made to isolate microorganisms capable of producing cold active alkaline proteases. Here we describe the production and characterization of a cold active protease from cold adapted bacteria isolated from Gangotri glaciers. The current study was initiated to isolate the psychrotrophic bacteria from the sample and screen them for the protease enzyme, which finds applications in various industries.

One hundred twenty bacterial isolates were screened from the eight sample of Gangotri sample on PSC solid agar medium containing skimmed milk. The proteolytic activity was assayed using skimmed milk agar and expressed as diameter of clear zone. Out of 120 positive isolates ten isolates showed good zone of hydrolysis revealing to be capable of producing protease at temperature 20 ± 2^0C (Table 4.1). On the basis of diameter of hydrolysis zone on PSC solid agar media at temperature from 20 ± 2^0C ten isolates, designated as AAPRT-3, BBPRT-7, BBPRT-13, CCPRT-12, DDPRT-6, EEPRT-5 FFPRT-10, GGPRT-6, GGPRT-8, and HHPRT-8 were selected as potent protease producing strains. These isolates were subjected to the taxonomical characterization.

The isolates were identified by studying morphological biochemical characteristics and. according to Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). Detailed morphological and biochemical tests of the isolates are given in Table 4.2.

Table 4.1: Isolates showing protease activity at temperature 20±2^{0}C

Sl. No.	**Isolates showing protease activity**
1	AAPRT-3
2	BBPRT-7, BBPRT-13
3	CCPRT-12
4	DDPRT-6
5	EEPRT-5
6	FFPRT-10
7	GGPRT-6, GGPRT-8
8	HHPRT-8

Table 4.2: Morphological and biochemical identification of protease producing bacteria

	Results									
	AAPRT-3	**BBPRT-7**	**BBPRT-13**	**CCPRT-12**	**DDPRT-6**	**EEPRT-5**	**FFPRT-10**	**GGPRT-6**	**GGPRT-8**	**HHPRT-8**
Colony morphology										
Configuration	Round	Round	Round	Round	Round	Round	Round	Round	Round	Round
Margin	Undulate	Undulate	Undulate	Undulate	Undulate	Undulate	Undulate	Undulate	Undulate	Undulate
Elevations	Raised	Raised	Opaque	Raised	Raised	Opaque	Opaque	Raised	Opaque	Raised
Surfaces	Rough	Rough	Smooth	Rough	Smooth	Smooth	Smooth	Rough	Smooth	Rough
Pigment	Cream	Cream	greenish	Reddish	Cream	Cream	Greenish	Orange yellow	Greenish	Cream
Gram's reaction	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive	Positive
Shape	Rods	Rods	Rods	Rods	Rods	Cocci	Rods	Rods	Rods	Rods
Size	Long	Short	Long	Long	Long	–	Short	Long	Short	Long
Motality	+	+	+	+	+	–	+	+	+	+
Spore										
Endospore	+	+	–	+	+	–	–	–	–	+
Position	Central	Central	ND	Central	Central	ND	ND	ND	ND	Central
Biochemical tests										
Catalase	+	+	+	+	+	+	+	+	+	+
Oxidase	+	+	+	+	+	+	+	+	+	+
Methyle Red	–	–	–	–	–	–	–	–	–	–
V-P Test	+	+	–	+	+	–	–	–	–	–
Indole	–	–	–	–	–	–	–	–	–	+
Citrate utilization	±	+	±	+	+	+	+	+	+	+
Nitrate reduction	+	+	+	+	+	–	+	+	+	+
Nitrite reduction	+	+	+	+	+	–	+	+	+	+
Starch hydrolysis	+	+	±	+	+	–	–	+	–	+
Urea hydrolysis	–	–	±	+	–	–	±	–	±	–
Gas production from glucose	-	–	–	–	–	–	–	–	–	–
H_2S production	–	–	–	+	–	–	–	–	–	–
Gelatin liquification	+	+	+	+	+	–	+	+	+	+
Acid production from carbohydrate										
Dextrose	+	+	+	+	+	+	+	–	+	–
Mannitol	+	+	+	+	+	+	+	+	+	+
Sucrose	+	+	–	+	+	+	–	–	–	–
Xylose	+	+	+	+	+	+	+	+	+	+

+ = Positive, – = Negative, ND = Not detected

4.2.1 Phylogenetic diversity

Phylogenetic analysis using 16S rRNA indicated that strain AAPRT-3 and DDPRT-6, shared high similarities with *Bacillus subtilis* strain NRRL B-4219 (NR116183.1) (99.2%) and with *Bacillus subtilis* strain TRC-4118(|AF047177) (100%). BBPRT-7 shared high similarity with *Bacillus licheniformis* strain DSM 13(118996)(100%) and with *B. licheniformis* strain NCDO 1772 (118959) (96.9%), BBPRT-13 with *Pseudomonas mandelii* (99.2%), GGPRT-8 With *Pseudomonas fluorescens* strain ATCC13525(114476.1) and *Pseudomonas fluorescens* strain NBRC 14160(NR_113647.1 (99.1%). FFPRT-10 with *Pseudomonas fluorescens* strain ATCC13525(114476.1)(98.5%) and *Pseudomonas fluorescens* strain NBRC 14160(NR_113647.1 (98.5%). HHPRT-8 and CCPRT-12 with *Pseudoalteromonas flavipulchra* (AF297958) (100%), EEPRT-5 with *Staphylococcus aureus* strain (100%), GGPRT-6 with *Exiguobacterium alkaliphilum* (94.5%) (Fig. 4.2, Table 4.3).

Among these ten potent protease producing isolates three isolates were selected as potent protease producer strain designated as DDPRT-6 (*Bacillus subtilis*), BBPRT-7 (*Bacillus licheniformis*) and FFPRT-10 (*Pseudomonas fluorescens*) were taken for further studies on the basis of their larger clearance zone at alkaline pH and different temperature ranging from 4-37^{0}C (Tables 4.4, 4.5, 4.6). The isolated strain thus can be classified as a psychrotroph according to the definition of Morita et al. (1997) which describes them as psychrotrophs (now psychrotolerants), as they are able to grow at 0^{0}C but have optimum growth temperatures 15–25^{0}C.

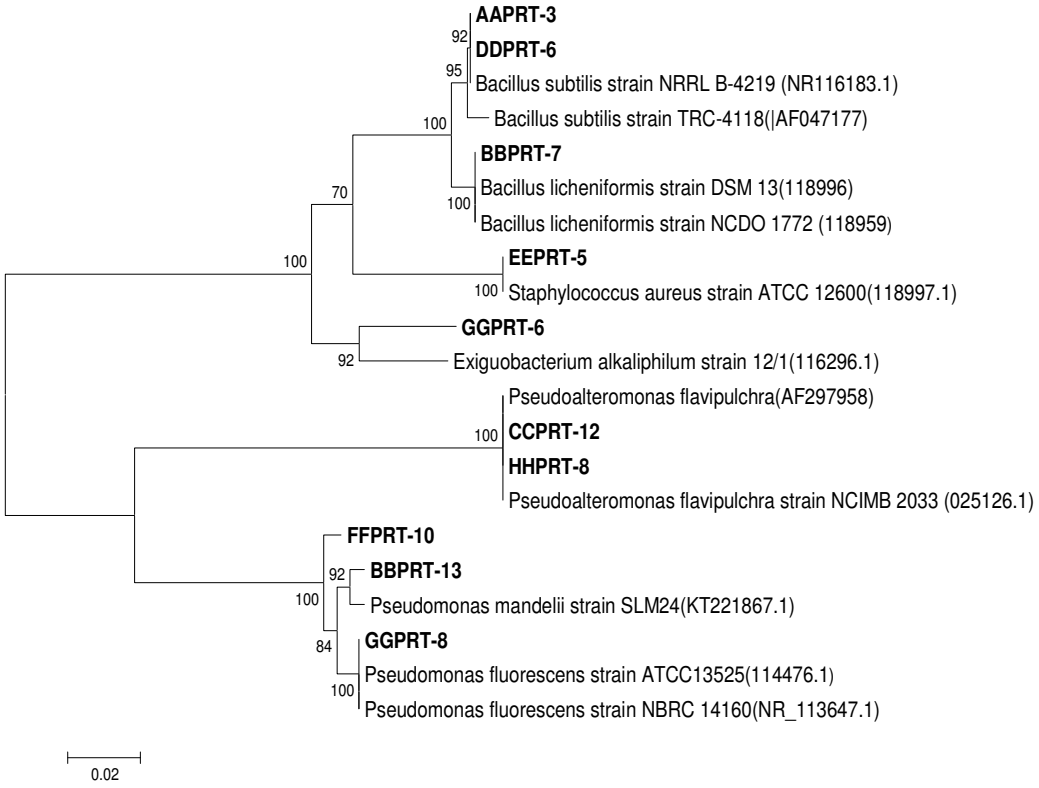

Fig. 4.2: Neighbor-joining tree of 16S rRNA sequence of isolates showing protease activity

Table 4.3: Similarity of 16S rRNA gene sequences in the GenBank database (Sequence Identity Matrix) of protease producing isolates

Seq->	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**
AAPRT-3	ID	1.000	0.992	1.000	0.986	0.986	0.957	0.799	0.800	0.769	0.926	0.926	0.769	0.769	0.797	0.803	0.803	0.913	0.919	0.769	0.805
DDPRT-6	1.000	ID	0.992	1.000	0.986	0.986	0.957	0.799	0.800	0.769	0.926	0.926	0.769	0.769	0.797	0.803	0.803	0.913	0.919	0.769	0.805
B. subtilis TRC-4118	0.992	0.992	ID	0.992	0.981	0.981	0.952	0.794	0.795	0.766	0.926	0.926	0.766	0.766	0.792	0.798	0.798	0.908	0.917	0.766	0.799
B. subtilis NRRL B-4219	1.000	1.000	0.992	ID	0.986	0.986	0.957	0.799	0.800	0.769	0.926	0.926	0.769	0.769	0.797	0.803	0.803	0.913	0.919	0.769	0.805
BBPRT-7	0.986	0.986	0.981	0.986	ID	1.000	0.969	0.796	0.797	0.766	0.926	0.926	0.766	0.766	0.794	0.800	0.800	0.915	0.922	0.766	0.801
B. licheniformis DSM 13	0.986	0.986	0.981	0.986	1.000	ID	0.969	0.796	0.797	0.766	0.926	0.926	0.766	0.766	0.794	0.800	0.800	0.915	0.922	0.766	0.801
B. licheniformis NCDO1772	0.957	0.957	0.952	0.957	0.969	0.969	ID	0.773	0.774	0.746	0.899	0.899	0.746	0.746	0.771	0.777	0.777	0.889	0.897	0.746	0.779
BBPRT-13	0.799	0.799	0.794	0.799	0.796	0.796	0.773	ID	0.992	0.856	0.800	0.800	0.856	0.856	0.977	0.986	0.986	0.805	0.792	0.856	0.981
P. mandelii SLM24	0.800	0.800	0.795	0.800	0.797	0.797	0.774	0.992	ID	0.855	0.797	0.797	0.855	0.855	0.977	0.986	0.986	0.803	0.791	0.855	0.981
P. flavipulchra NCIMB2033	0.769	0.769	0.766	0.769	0.766	0.766	0.746	0.856	0.855	ID	0.772	0.772	1.000	1.000	0.846	0.853	0.853	0.777	0.773	1.000	0.849
EEPRT-5	0.926	0.926	0.926	0.926	0.926	0.926	0.899	0.800	0.797	0.772	ID	1.000	0.772	0.772	0.793	0.800	0.800	0.903	0.891	0.772	0.804
S. aureus ATCC 12600	0.926	0.926	0.926	0.926	0.926	0.926	0.899	0.800	0.797	0.772	1.000	ID	0.772	0.772	0.793	0.800	0.800	0.903	0.891	0.772	0.804
HHPRT-8	0.769	0.769	0.766	0.769	0.766	0.766	0.746	0.856	0.855	1.000	0.772	0.772	ID	1.000	0.846	0.853	0.853	0.777	0.773	1.000	0.849
P. flavipulchra(AF297958)	0.769	0.769	0.766	0.769	0.766	0.766	0.746	0.856	0.855	1.000	0.772	0.772	1.000	ID	0.846	0.853	0.853	0.777	0.773	1.000	0.849
GGPRT-8	0.797	0.797	0.792	0.797	0.794	0.794	0.771	0.977	0.977	0.846	0.793	0.793	0.846	0.846	ID	0.991	0.991	0.797	0.783	0.846	0.976
P. fluorescens ATCC13525	0.803	0.803	0.798	0.803	0.800	0.800	0.777	0.986	0.986	0.853	0.800	0.800	0.853	0.853	0.991	ID	1.000	0.803	0.789	0.853	0.985
P. fluorescens NBRC14160	0.803	0.803	0.798	0.803	0.800	0.800	0.777	0.986	0.986	0.853	0.800	0.800	0.853	0.853	0.991	1.000	ID	0.803	0.789	0.853	0.985
GGPRT-6	0.913	0.913	0.908	0.913	0.915	0.915	0.889	0.805	0.803	0.777	0.903	0.903	0.777	0.777	0.797	0.803	0.803	ID	0.945	0.777	0.806
E. alkaliphilum 12/1	0.919	0.919	0.917	0.919	0.922	0.922	0.897	0.792	0.791	0.773	0.891	0.891	0.773	0.773	0.783	0.789	0.789	0.945	ID	0.773	0.790
CCPRT-12	0.769	0.769	0.766	0.769	0.766	0.766	0.746	0.856	0.855	1.000	0.772	0.772	1.000	1.000	0.846	0.853	0.853	0.777	0.773	ID	0.849
FFPRT-10	0.805	0.805	0.799	0.805	0.801	0.801	0.779	0.981	0.981	0.849	0.804	0.804	0.849	0.849	0.976	0.985	0.985	0.806	0.790	0.849	ID

Table 4.4: Isolates showing protease activity at temperature 37^0C and pH 10

Sl. no.	Isolates	Temperature (^{0}C)	pH	Zone
1.	AAPRT-3	37	10	+
2.	BBPRT-7	37	10	+
3	BBPRT-13	37	10	+
4	CCPRT-12	37	10	+
5	DDPRT-6	37	10	+++
6	EEPRT-5	37	10	+
7	FFPRT-10	37	10	+
8	GGPRT-6	37	10	+
9	GGPRT-8	37	10	+
10	HHPRT-8	37	10	+

Table 4.5: Isolates showing protease activity at temperature 10^0C and pH 10

Sl. no.	Isolates	Temperature (^{0}C)	pH	Zone
1.	AAPRT-3	10	10	+
2.	BBPRT-7	10	10	+++
3	BBPRT-13	10	10	+
4	CCPRT-12	10	10	+
5	DDPRT-6	10	10	+
6	EEPRT-5	10	10	+
7	FFPRT-10	10	10	+
8	GGPRT-6	10	10	+
9	GGPRT-8	10	10	+
10	HHPRT-8	10	10	+

Table 4.6: Isolates showing protease activity at temperature 4^0C and pH 10

Sl. no.	Isolates	Temperature (^{0}C)	pH	Zone
1	AAPRT-3	4	10	+
2	BBPRT-7	4	10	+
3	BBPRT-13	4	10	+
4	CCPRT-12	4	10	+
5	DDPRT-6	4	10	+
6	EEPRT-5	4	10	+
7	FFPRT-10	4	10	+++
8	GGPRT-6	4	10	+
9	GGPRT-8	4	10	+
10	HHPRT-8	4	10	+

Similar results were reported by Helmke and Weyland (1991), who found that extracellular proteases from permanently cold environments had optimum growth temperatures of about 10–20^{0}C lower in comparison with proteases from mesophilic isolates. The genera Exiguobacterium (Gram-positive and facultatively anaerobic) and Psychrobacter (Gram-negative) have been repeatedly isolated from ancient Siberian permafrost (Rodrigues et al., 2009; Vishnivetskaya et al., 2009). Martınez-Rosales and Castro-Sowinski (2011) reported the isolation and identification of bacteria *Pseudomonas* (growth between 4 and 30^{0}C) and *Flavobacterium* (growth between 4 and 18^{0}C) that produce extracellular cold-active proteases from water samples collected at Fildes Peninsula King George Island, South Shetlands. Pradhan et al. (2010) reported the presence of *Bacillus* from a Roopkund glacier, though an isolate was also reported from the Pindari glacier of Himalayas earlier (Reddy et al., 2008).

Despite the variable metabolic capabilities and other differences in environmental conditions, the key feature of all cold-adapted microorganisms is the successful surviving of the negative effects of low temperatures by evolving a range of structural and functional adaptations (Bej et al., 2010; Margesin and Miteva, 2011). Baghel et al. (2005) isolated bacteria *Bacillus subtilis* at the growing temperature from 10-37^{0}C. The protease released by arctic bacteria showed optimal activity at low temperatures (Huston et al., 2000). Extracellular cold-active serine-proteases have been identified in psychrophilic and psychrotrophic bacteria such as *Pseudoalteromonas* (Wang et al. 2008). Various bacteria like *Pseudomonas, Bacillus, Arthrobacter, Exiguobacterium, Mycetecola, Pantoea, Acinetobacter* and *Serratia* have been identified as hydrolytic enzyme

producer in previous study on Himalayas (Salwan et al., 2010; Venkatachalam et al., 2015).

Exploitation of glacier may result in the discovery of cold adapted that produce enzymes with promising properties**.** The biotechnological potential of secreted proteases produced by bacteria adapted to both cold and alkaline conditions, prompted us to perform a screen for protease producing isolates from cold regions.

4.3 Purification and characterization of protease

Owing to the characteristics of the cold adapted enzymes, much attention is being focused on isolation and characterisation of extracellular enzymes produced by psychrophiles and psychrotolerant microorganisms (Brenchley, 1996).

Extracellular protease from DDPRT-6 (*Bacillus subtilis*), BBPRT-7 (*Bacillus licheniformis*) and FFPRT-10 (*Pseudomonas fluorescens*) was subjected to partial purification. The summary of partial purification procedure is illustrated in table respectively.

Enzyme from DDPRT-6 (*Bacillus subtilis*) was partially purified to 2.49 purification fold with the increase of specific activity 561.98 U/mg and yield of 82.4 % by ammonium sulphate fraction and dialysis (Table 4.7).

Enzyme from BBPRT-7 (*Bacillus licheniformis*) was partially purified to 5.39 purification fold with the increase of specific activity 379 U/mg and yield of 66.6 % by ammonium sulphate fraction and dialysis (Table 4.8).

Table 4.7: Summary of partial purification of protease enzyme from DDPRT-6

Purification step	**Total activity (units)**	**Total protein (mg)**	**Sp. activity (U mg^{-1})**	**Purification fold**	**Yield (%)**
Crude enzyme	26450	117.4	225.29	1	**100**
(Ammonium sulfate fractionation (40-60%)	21805	38.8	561.98	2.49	82.4

Table 4.8: Summary of partial purification of protease enzyme from BBPRT-7

Purification step	**Total activity (units)**	**Total protein (mg)**	**Sp. activity (U mg^{-1})**	**Purification fold**	**Yield (%)**
Crude enzyme	29133	415	70.2	1	**100**
(Ammonium sulfate fractionation (40-60%)	19404	51.1	379	5.39	66.6

Enzyme from FFPRT-10 (*Pseudomonas fluorescens*) was partially purified to 3.3 purification fold with the increase of specific activity 1430.55 U/mg and yield of 45.04 % by ammonium sulphate fraction and dialysis (Table 4.9).

Saba et al. (2012) studied the Protease from stenotrophomonas being extracellular was partially purified by ammonium sulphate precipitation followed by a series of chromatography steps in order to give an overall 18.45 folds purification with a specific activity of 41.2 U/mg of protein. Psychro-tolerant 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). Vázquez et al. (2008) isolated Antarctic marine *Pseudoalteromonas* sp. P96-47 strain have the highest proteolytic activity in culture supernatant (63.2 EU/ml) and the maximum protease production yield (33.3 EU/g) at 15^0C.

Effect of pH on enzyme activity and stability was examined in the pH range 7-11.5 (Figs. 4.3, 4.4). The optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (pH 7-11.5).

Enzyme from DDPRT-6 (*Bacillus subtilis*) shows maximum activity at pH 10 and enzyme was more than 80% stable at pH range 9-10.

The BBPRT-7 (*Bacillus licheniformis*) shows maximum activity at pH 10 and had the widest pH range for activity, with more than 100% stable at pH range 9-10.

Enzyme from FFPRT-10 (*Pseudomonas fluorescens*) shows maximum activity at pH 9 and more than 100% stable at pH range 9-10.

Table 4.9: Summary of partial purification of protease enzyme from FFPRT-10

Purification step	**Total activity (units)**	**Total protein (mg)**	**Sp. activity (U mg^{-1})**	**Purification fold**	**Yield (%)**
Crude enzyme	80030	190.4	420.32	1	**100**
(Ammonium sulfate fractionation (40-60%)	36050	25.2	1430.55	3.3	45.04

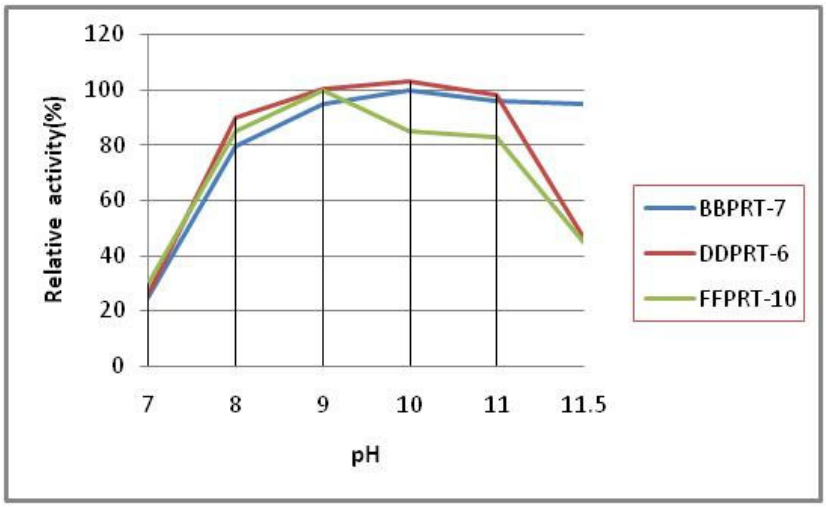

Fig. 4.3: Effect of pH on protease enzyme activity

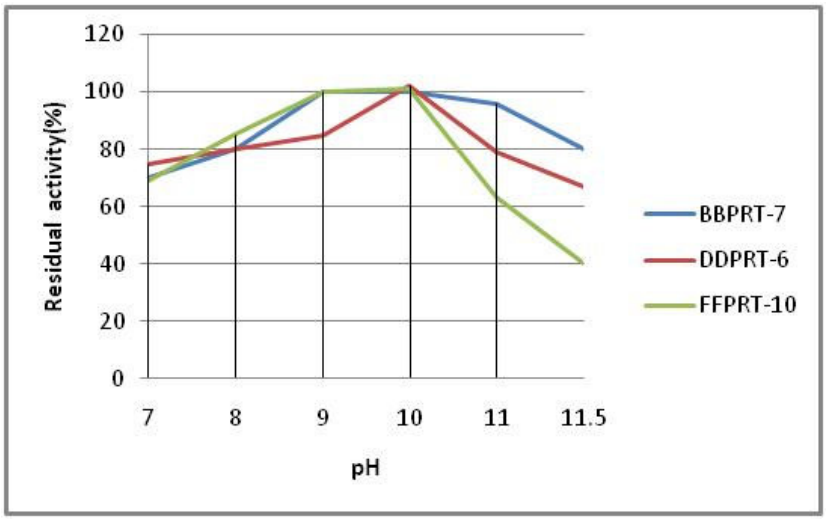

Fig. 4.4: Effect of pH on protease enzyme stability

The protease was active over a broad pH range. Optimal proteolytic activity occurred at neutral or moderate alkaline pH for the three protease enzymes. None of the enzymatic activities had a sharp dependence on pH. All of them were inactivated at pH 11.5. Enzymes from BBPRT-7, DDPRT-6 and FFPRT-10 had the widest pH range for activity, with more than 80% of their maximal activity occurring at pH 9.0–11.5. All proteases had maximal activity at neutral pH and showed high residual activity over a broad range of pH. Many cold-active proteases have previously been isolated from cold-adapted bacteria living at near-neutral pH (Joshi and Satyanarayana, 2013). Vázquez et al. (2008) reported a strain of *Pseudoalteromonas* sp. P96-47, the optimal proteolytic activity was registered at neutral and slightly alkaline pH. The pH-dependence profile showed maximal activity between pH 7.0 and pH 9.0, with relative activity above 50% between pH 6.0 and 10.0, and more than 80% between pH 7.0 and 9.0. This pH dependence for activity makes these enzymes interesting for industrial applications, in contrast with other cold proteases with a narrow pH profile (Fernandez et al., 1996; Irwin et al., 2001; Secades et al., 2001; Vazquez et al., 2004).

When incubations at different temperatures with azocasein as substrate were done, the effect of temperature on the purified proteases was observed at temperature range 10-50^{0}C. The effect of temperature on the protease activity and stability shown in Fig. 4.5 and 4.6, respectively.

Enzyme from DDPRT-6 (*Bacillus subtilis*) shows optimum temperature at 20^{0}C and the enzyme was found stable at a temperature between 10-30^{0}C.

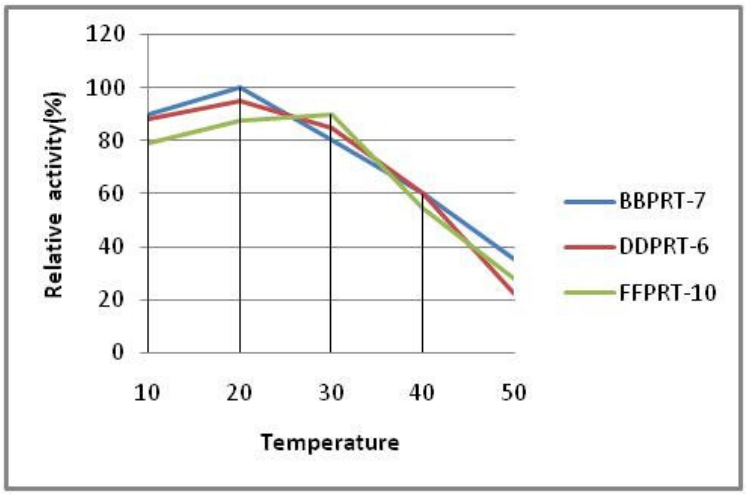

Fig. 4.5: Effect of temperature on protease enzyme activity

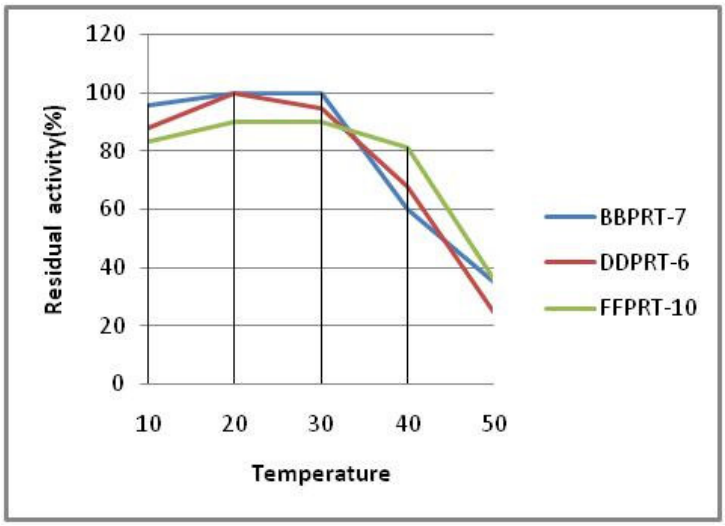

Fig. 4.6: Effect of temperature on protease enzyme stability

The BBPRT-7 (*Bacillus licheniformis*) shows optimum temperature at 20^0C and the enzyme was found stable at a temperature between 10-30^0C.

Enzyme from FFPRT-10 (*Pseudomonas fluorescens*) optimum temperature at 30^0C and the enzyme was found stable at a temperature between 10-35^0C.

BBPRT-7, DDPRT-6 was the most active at 20^OC and FFPRT-10 at 30^0C. The three proteases showed to be thermolabile, compared with the commercial enzymes, when they were incubated for 1 h at various temperatures. The all three psychrotolerant proteases reach thermal inactivation at temperatures 10^0C lower than the mesophilic metalloprotease. The enzyme was found stable at temperature betwen 10-30^0C. Antarctic isolates produced active extracellular proteases when growing at 4 and 18^0C but not at 30^0C, even though *Pseudomonas* isolates were able to grow at this high temperature (Martı´nez-Rosales and Castro-Sowinski, 2011). Psychrophilic and psychrotolerant bacteria never have to cope with temperatures higher than 30^0C in their natural habitats, so their enzymes can shift the cardinal temperatures for activity towards lower values (Feller et al., 1996). For them, to have good performance for the hydrolysis of substrates at low temperature is a requirement for survival, and this strategy is vital for their adaptation to live in cold environments.

In addition to pH, a good detergent protease is expected to be stable in the presence of commercial detergents at low temperature for cold washing. Partially purified enzyme from DDPRT-6 (*Bacillus subtilis*), BBPRT-7 (*Bacillus licheniformis*) and FFPRT-10 (*Pseudomonas fluorescens*) was incubated with different detergent viz. SDS, Tween 80 and different commercial detergent like surf, wheel and Tide for 1 hr at 20^0C and then enzyme assay was performed.

Purified protease from DDPRT-6 (*Bacillus subtilis*) exposed tremendous stability and compatibility with a wide range of locally available commercial detergents at 20°C (Fig. 4.7). Similar results are also obtained by other workers from different strains of *Bacillus* sp. but at 60^0C (Adinarayana et al., 2003; Beg and Gupta, 2003). It was most stable with Wheel detergent after 1.0 h incubation. The enzyme also shows enhance activity surf while inhibited with tide and Tween 80. The enzyme was sensitive to anionic SDS addition, indicating that hydrogen bonds may play a pivotal role in maintaining enzyme activity (Wang et al., 2005).

Purified protease from BBPRT-7(*Bacillus licheniformis*) shows enhanced activity with tween80, wheel, surf and enzyme was sensitive to tide and SDS.

The purified enzyme from FFPRT-10 (*Pseudomonas fluorescens*) was stable in presence of Tween 80, Surf, SDS and Wheel while inhibited by Tide.

The effect of various metal ions on protease production was evaluated (Table 4.10). The purified proteases of DDPRT-6 and BBPRT-7 were inhibited by EDTA while protease activity of FFPRT-10 was increased. Proteolytic activities of all three enzymes were affected in the presence of 5mM Zn^{2+}, Hg^{2+} and Cu^{2+}. DDPRT-6 was the most sensitive among these protease producing strains. However, Zn^{2+} and Cu^{2+} strongly inhibited enzyme production (Saba et al., 2012). The DDPRT-6 protease seems to belong to the metalloprotease rather than to the serine protease family, because the activity of this enzyme was strongly inhibited by a chelating agent, EDTA and $HgCl_2$ (Hutadilok-Towatana et al., 1999; Tariq et al., 2011).

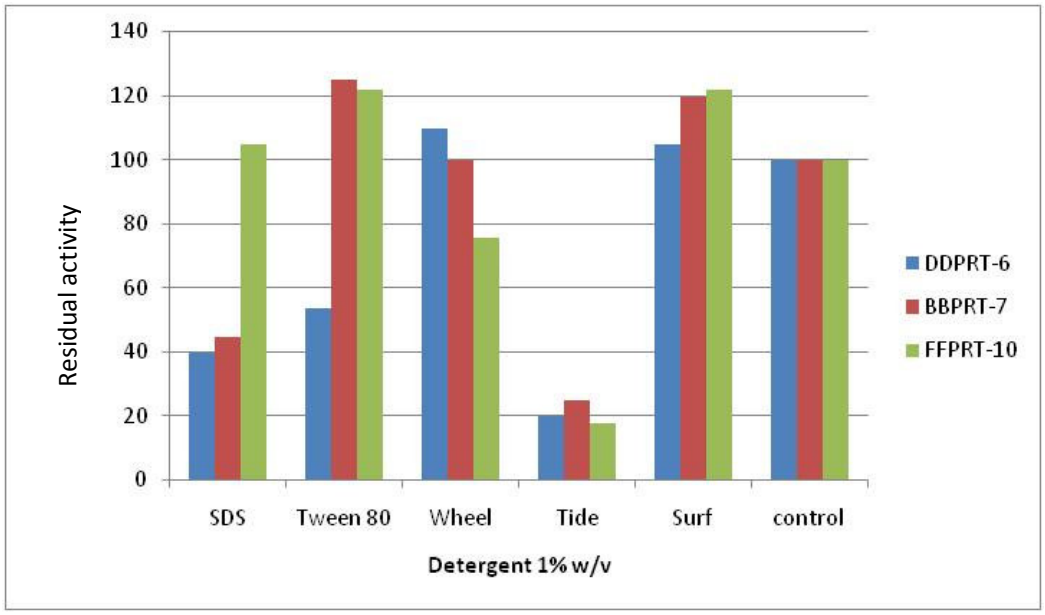

Fig. 4.7: Compatibility of detergent / commercial detergent on protease enzyme activity

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

Inhibitor/Salt	**Residual activity**		
	DDPRT-6	**BBPRT-7**	**FFPRT-10**
None	100	100	100
EDTA	2.1	32	79
Cystein	3.4	55	45
$ZnCl_2$	8	19	45
$HgCl_2$	5	12	16
$CuSO_4$	12	30	93

The major use of detergent compatible proteases is in the laundry detergent formulation (Anstrup and Anderson, 1974). The economic importance of alkaline proteases came to light when bacterial alkaline proteases from Bacillus sp. were introduced in 1960s to the detergent industry, accounting for about 35% of the total microbial enzyme sales (Kalisz, 1988; Outtrup, 1990). The cost of enzyme production is the major obstacle in the successful application of proteases in industries. Over the past 30 years, the proteases in detergents have changed from being minor additives to being the key ingredients. There is always a need for newer enzymes with novel properties that can further enhance the wash performance of currently use enzyme-based detergents. Conventionally, detergents have been used at elevated washing temperatures, but at present, there is considerable interest in the identification of alkaline proteases which are effective over a wide temperature range (Oberoi et al., 2001). Banic and Prakash (2004) isolated extracellular protease from *B. cereus* was stable over a wide range of alkaline pH and temperature. It also showed excellent compatibility with various laundry detergents tested and the stability of the enzyme in detergents was much better for longer time period than the endogenous enzymes of laundry detergents. In addition, the current consumer demands and increased use of synthetic fibers, which cannot tolerate high temperatures, have changed washing habits towards the use of low washing temperatures (Nielsen et al., 1981; Kitayama, 1992; Hasan and Tamiya, 1997). This has pushed enzyme manufacturers to look for novel enzyme that can act under low temperatures. Other potential fields of application of cold-active proteases are food processing and bioremediation in cold climates (Ohgiya et al., 1999).

4.4 Isolation, characterization, identification and phylogenetic analysis of lipase producing microorganism

Lipases catalyze the hydrolysis of acylglycerides and other fatty acid esters. A variety of microbial lipases with different enzymological properties have been found. Cold-active lipases have true enzyme potentialities for industrial applications (Giudice et al., 2004) in fields of pharmaceutical preparations, cosmetics, food production, waste management, biosensors (Joshi et al., 2006), organic synthesis of unstable compounds, fine chemicals, additives in laundry detergents for cold washing, bioremediation (Timmin and Pieper, 1999). Cold active lipases are largely distributed in microorganisms surviving at low temperatures near 5^0C. Although a number of lipase producing bacterial sources are available, only a few cold adapted bacteria were exploited for the production of cold active lipases. Attempts have been made from time to time to isolate cold active lipases from these bacteria having high activity at low temperatures. Choo et al. (1998) screened various psychrotrophic bacteria from tundra soils of Alaska and Siberia. They isolated total 88 strains for bacterial strains showing high lipase activity at cold temperatures.

One hundred bacterial colony were isolated on trybutyrin agar plates from eight samples of Gangotri glaciers. Out of 100 positive isolates eight isolates showed good zone of hydrolysis revealing to be capable of producing lipase. Eight lipase producing strains are designated as AAPRTL-10, BBPRTL-5, CCPRTL-8, DDPRTL-14, EEPRTL-5, FFPRTL-9, GGPRTL-06, GGPRTL-5 (Table 4.11). These isolates were subjected to the taxonomical characterization. Detailed morphological and biochemical tests of the isolates are given in Table 4.12.

Table 4.11: Isolates showing lipase activity at temperature 20±2^{0}C

Sl. no.	**Isolates showing lipase activity**
1	AAPRTL-10
2	BBPRTL-5
3	CCPRTL-8
4	DDPRTL-14
5	EEPRTL-5
6	FFPRTL-9
7	GGPRTL-5
8	GGPRTL-6

Table 4.12: Morphological and biochemical identification of lipase producing bacteria

	Results							
	AAPRTL-10	**BBPRTL-5**	**CCPRTL-8**	**DDPRTL-14**	**EEPRTL-5**	**FFPRTL-9**	**GGPRTL-5**	**GGPRTL-6**
Colony morphology								
configuration	Round	Round	Round	Round	Round	Round	Round	Round
Margin	Undulate	Entire	Undulate	Undulate	Undulate	Undulate	Undulate	Wrinkled
Elevations	Raised	Flat	Raised	Flat	Raised	Raised	Raised	Raised
Surfaces	Rough	Smooth	Rough	Smooth	Rough	Rough	Rough	Rough
Pigment	Cream	Cream	reddish	Lemon yellow	Cream	Cream	Cream	Cream
Gram's reaction	Negative	Positive	Positive	Positive	Positive	Positive	Positive	Positive
shape	Rods	Rods	Rods	Cocci	Rods	Rods	Rods	Rods
size	Long	Long	Long	Long	Long	Long	Long	Long
Motality	+	+	+	+	+	+	+	+
Spore								
Endospore	–	+	–	–	–	–	–	+
Position	ND	Central	ND	ND	ND	ND	ND	Central
Biochemical test								
Oxidase	+	+	_	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+
V-P Test	+	_	+	_	_	_	_	_
Indole	_	+	+	+	+	+	+	+
Citrate	+	–	+	–	–	–	–	–
Nitrate reduction	+	+	+	+	–	+	+	+
Starch hydrolysis	+	+	+	+	–	+	+	+
Nitrate reduction	+	±	+	+	–	–	+	_
Nitrite reduction	+	+	+	+	+	–	+	+
H_2S production	–	+	–	–	–	–	–	+
Acid production from carbohydrates								
Lactose	+	+	+	_	+	+	+	_
Dextrose	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Sucrose	+	+	–	+	+	+	–	+
Xylose		+	+	+	+	+	+	+

+ = Positive, – = Negative, ND = Not detected

4.4.1 Phylogentic analysis

Phylogenetic analysis using 16S rRNA indicated that strain AAPRTL-10 shared high similarity with *Pseudoalteromonas lipolytica* (96.5%), FFPRTL-9 with *Bacillus sonorensis* (99.3%), BBPRTL-5 with Bacillus sphaericus (99.4%), DDPRTL-14 with *Arthrobacter psychrolactophilus* strain D5 (134182.1) (99.4%) with *Arthrobacter psychrolactophilus* strain D2 (134181.1) (99.8), GGPRTL-5 with *Stenotrophomonas maltophilia* strain strain KB2 (98.6%) with *Stenotrophomonas* sp. (066098) (98%), EEPRTL-5 with *Pseudomonas vancouverensis* (100%), CCPRTL-8 with *Aeromonas veronii* (90.8%), GGPRTL-6 with *Bacillus licheniformis* strain NBRC12200 (113588.1) (99.7%) (Fig. 4.8, Table 4.13).

A large number of cold active lipase producing bacterial have been reported by different researchers as *Bacillus sphaericus* MTCC 7526 from Gangotri glacier, Western Himalayas (Joseph and Ramteke, 2012), *Staphylococcus aureus* (Alford and Pierce, 1961), P*seudomonas* (Choo et al., 1998; Tan et al., 1996; Rashid et al., 2001; Gupta and Prakash, 2014), and *Aeromonas* sp. (Lee et al., 2003). A cold active lipase producing potential psychrophilic bacteria (GN) was isolated and identified by 16S rRNA molecular studies as *Pseudomonas vancouverensis* (Gupta and Prakash, 2014).

Among these isolates GGPRTL-5 is potent lipase producing isolates at temperature 20^0C (Table 4.14). The strain GGPRTL-5 was selected as the best producer of lipase and was identified as *Stenotrophomonas multophilia* on the basis of its taxonomical characterization. An extracellular cold-active lipase producing bacterium was isolated from oil-contaminated soil samples, and identified taxonomically as *Stenotrophomonas maltophilia* (Li et al., 2013).

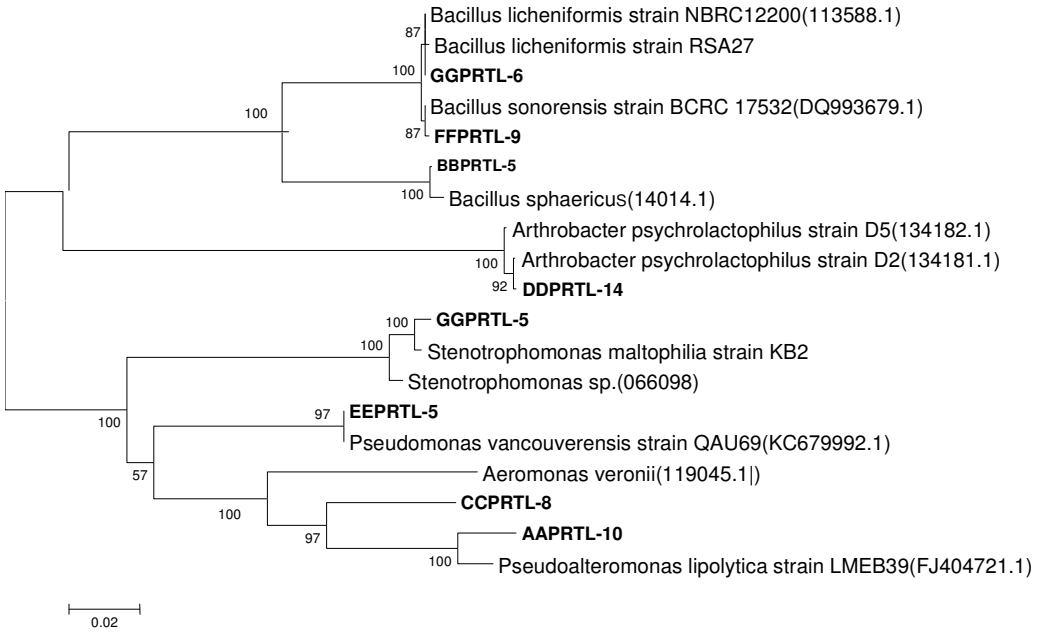

Fig. 4.8: Neighbor-joining tree of 16S rRNA sequence of isolates showing lipase activity

Table 4.13: Similarity of 16S rRNA gene sequences in the GenBank database (Sequence Identity Matrix) of lipase producing isolates

Seq->	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**
AAPRTL-10	ID	0.965	0.749	0.747	0.904	0.764	0.764	0.765	0.877	0.759	0.759	0.759	0.835	0.835	0.844	0.759	0.756	0.861	0.861
P. lipolytica LMEB39	0.965	ID	0.756	0.755	0.908	0.764	0.766	0.765	0.873	0.765	0.765	0.765	0.835	0.835	0.844	0.766	0.764	0.860	0.860
BBPRTL-5	0.749	0.756	ID	0.994	0.758	0.779	0.779	0.778	0.757	0.905	0.904	0.905	0.779	0.776	0.784	0.907	0.904	0.794	0.794
B. sphaericus (14014.1)	0.747	0.755	0.994	ID	0.756	0.777	0.777	0.776	0.756	0.901	0.900	0.901	0.777	0.774	0.782	0.902	0.900	0.792	0.792
CCPRTL-8	0.904	0.908	0.758	0.756	ID	0.768	0.770	0.769	0.884	0.771	0.771	0.771	0.827	0.827	0.835	0.772	0.771	0.860	0.860
A. psychrolactophilus D2	0.764	0.764	0.779	0.777	0.768	ID	0.995	0.998	0.772	0.786	0.786	0.786	0.769	0.768	0.772	0.788	0.786	0.772	0.772
A. psychrolactophilus D5	0.764	0.766	0.779	0.777	0.770	0.995	ID	0.994	0.772	0.786	0.786	0.786	0.771	0.770	0.774	0.788	0.786	0.772	0.772
DDPRTL-14	0.765	0.765	0.778	0.776	0.769	0.998	0.994	ID	0.773	0.785	0.785	0.785	0.768	0.767	0.771	0.787	0.785	0.773	0.773
A. veronii (119045.1)	0.877	0.873	0.757	0.756	0.884	0.772	0.772	0.773	ID	0.770	0.770	0.770	0.831	0.832	0.842	0.772	0.770	0.857	0.857
GGPRTL-6	0.759	0.765	0.905	0.901	0.771	0.786	0.786	0.785	0.770	ID	0.997	0.997	0.783	0.783	0.791	0.994	0.990	0.802	0.802
B. licheniformis NBRC122	0.759	0.765	0.904	0.900	0.771	0.786	0.786	0.785	0.770	0.997	ID	0.996	0.782	0.782	0.790	0.993	0.989	0.802	0.802
B. licheniformis RSA27	0.759	0.765	0.905	0.901	0.771	0.786	0.786	0.785	0.770	0.997	0.996	ID	0.783	0.783	0.791	0.994	0.990	0.802	0.802
GGPRTL-5	0.835	0.835	0.779	0.777	0.827	0.769	0.771	0.768	0.831	0.783	0.782	0.783	ID	0.986	0.980	0.784	0.781	0.849	0.849
S. maltophilia KB2	0.835	0.835	0.776	0.774	0.827	0.768	0.770	0.767	0.832	0.783	0.782	0.783	0.986	ID	0.981	0.784	0.781	0.848	0.848
Stenotrophomonas sp.	0.844	0.844	0.784	0.782	0.835	0.772	0.774	0.771	0.842	0.791	0.790	0.791	0.980	0.981	ID	0.792	0.789	0.860	0.860
B. sonorensis BCRC 17532	0.759	0.766	0.907	0.902	0.772	0.788	0.788	0.787	0.772	0.994	0.993	0.994	0.784	0.784	0.792	ID	0.993	0.801	0.801
FFPRTL-9	0.756	0.764	0.904	0.900	0.771	0.786	0.786	0.785	0.770	0.990	0.989	0.990	0.781	0.781	0.789	0.993	ID	0.800	0.800
EEPRTL-5	0.861	0.860	0.794	0.792	0.860	0.772	0.772	0.773	0.857	0.802	0.802	0.802	0.849	0.848	0.860	0.801	0.800	ID	1.000
P. vancouverensis QAU69	0.861	0.860	0.794	0.792	0.860	0.772	0.772	0.773	0.857	0.802	0.802	0.802	0.849	0.848	0.860	0.801	0.800	1.000	ID

Table 4.14: Isolates showing lipase activity at temperature 20^0C and pH 10

Sl. No.	Isolates	Temperature (^{0}C)	pH	Zone
1	AAPRTL-10	20	10	+
2	BBPRTL-5	20	10	+
3	CCPRTL-8	20	10	+
4	DDPRTL-14	20	10	+
5	EEPRTL-5	20	10	+
6	FFPRTL-9	20	10	+
7	GGPRTL-6	20	10	+
8	GGPRTL-5	20	10	+++

Cold-adapted microorganisms tend to have good growth rate at low temperature. The production of cold active lipase is considered temperature dependent and thermolabile (Rashid et al., 2001). *Moraxella* sp. isolated from Antarctican habitat grows well at 25^0C and produced cold active lipolytic enzyme (Feller et al., 1990). Lee et al. (2003) isolated cold adapted lipase from *Aeromonas* sps. These isolates producing lipase were selected because they can be valuable in food processing industry, in detergent industry, in desizing and for bioremediation in cold regions. Lipolytic enzymes have been also proposed as interesting alternative to the commercially available microbial agents of mesophilic origin for the removal of fats from aqueous systems (Suzuki et al., 2001).

The isolation and characterization of lipolytic-enzyme producers that are able to efficiently remove lipids at low temperatures will provide insight into the possibility to use different cold-adapted bacteria as a source of extremophilic enzymes (Lo Giudice et al., 2006).

4.5 Partial purification and characterization of lipase

Enzymes from psychrotrophic and psychrophilic microorganisms have recently received increasing attention, due to their relevance for both basic and applied research. This effort has been stimulated by the recognition that cold-adapted enzymes might offer novel opportunities for biotechnological exploitation based on their high catalytic activity at low temperatures, low thermostability and unusual specificities. 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).

One hundred lipase producing bacterial isolates were isolated from samples. However, eight isolates were identified as potent lipase producing species. The lipase producing isolates GGPRTL-5 was selected as best one on the basis of zone formation on TBA plates was selected for further studies. Lipase from GGPRTL-05 (*Stenotrophomonas maltophilia*) was subjected to partial purification. 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 (40-60%) and using a single step ion-exchange chromatography on a DEAE-cellulose. The summary of partial purification procedure is illustrated in Table 4.15.

Partially purified lipase was eluted out as fractions (with 0.1–1M NaCl gradient) from DEAE-cellulose column with 28.80 fold purification and specific activity of 82.10 Umg^{-1}. The aim of the investigation was to isolate potential lipolytic bacteria with novel properties. Lipase production by psychrotrophs varies with species, as does the optimum temperature, optimum pH and enzyme specificity (Thomas and Thomas, 1973). 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).

Specific physiological conditions may support the production of lipolytic enzymes. Most lipases can act in a wide range of pH and temperature, although alkaline bacterial lipases are the most common (Gupta et al., 2004). For determination of relative activity at pH, different buffers were used. The maximum activity of lipase of GGPRTL-5 at Ph 8 at 20^0C and the enzyme was stable between pH 8 and 9 at the indicated pH range when incubated at 20^0C for 24 h, but its activity decreased at pH at 10 (Fig. 4.9).

Table 4.15: Summary of partial purification of lipase from GGPRTL-5

Purification step	**Total activity (units)**	**Total protein (mg)**	**Sp. activity (U mg^{-1})**	**Purification fold**	**Yield (%)**
Crude enzyme	3708	1 300	2.85	1	100
$(NH_4)_2SO_4$ precipitation (dialyzed)	2300	136.57	16.84	6.2	5.90
DEAE-cellulose	156	1.9	82.10	28.80	4.2

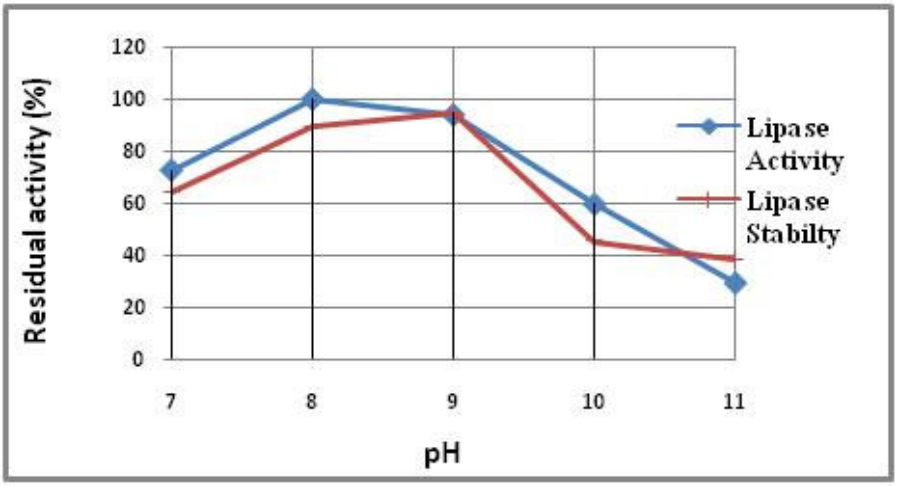

Fig. 4.9: Effect of pH on enzyme activity and stability

pH of 8.0 was optimum for cold active lipase activity from *Corynebacterium* sp. (Suxcena et al., 2003). Lipases showing high stability and activity overa wide range of pH and activity under non-conventional conditionsare of great interest. The major commercial applicationfor alkaline stable lipases is the use in laundry and householddetergents. These properties can be extremely useful in various applications.

The activity of cold active lipase was determined at a wide range of temperature 10-50^{0}C (Fig. 4.10). The optimum temperature for lipolytic activity of GGPRTL-5 was determined to be 20^{0}C. The enzyme activity was almost constant within 25–30^{0}C and gradually declined at temperature beyond 35^{0}C. Similarly, cold active lipase from *Psychrobacter okhotskensis* completely lost its activity above 36^{0}C (Yumoto et al., 2003). M37 was a typical cold-adapted lipase also displayed a maximum activity at 25^{0}C and maintained its activity at a low temperature range 5–25^{0}C (Ryu et al., 2006).

Thermal stability of cold active lipase was tested at different temperature ranging from 10 to50^{0}C. The enzyme was stable up to 30^{0}C for 1 h and decreased at higher temperature. While stability of cold active lipase from *Aeromonas* sp. LPB4 was up to 50^{0}C and a dramatic decrease thereafter (Lee et al., 2003). This marked liability of lipase together with its high catalytic efficiency near 20^{0}C clearly denotes that it is cold active enzyme. The increased catalytic activity at low temperatures and decreased thermostability of psychrophilic enzymes suggest that there is a relationship between stability and activity to maintain the activity at low temperature.

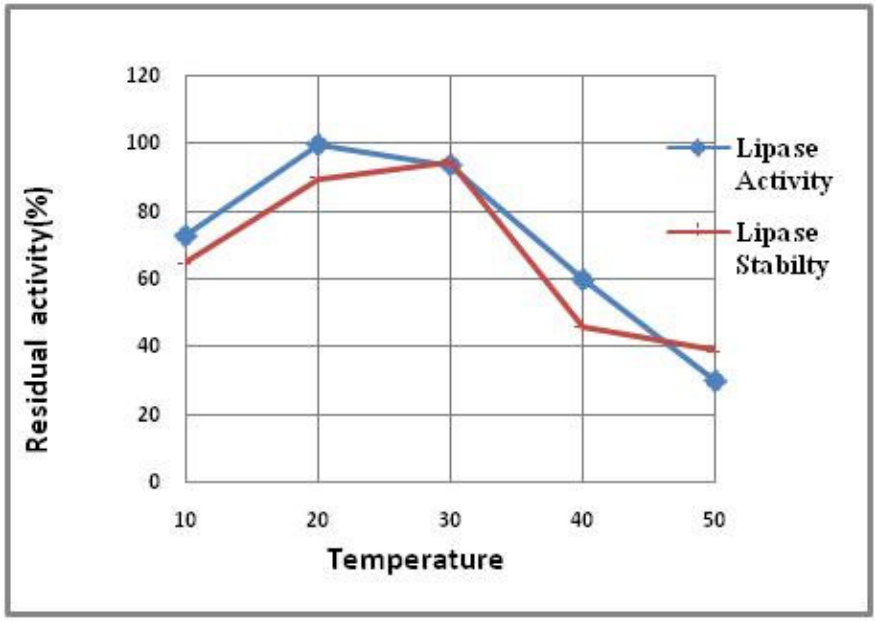

Fig. 4.10: Effect of temperature on lipase enzyme activity and stability

The poor thermal stability of psychrophilic enzymes, which facilitates their rapid inactivation by a moderate rise in temperature is also advantageous in some technologies. The lipase thus exhibiting stability at ambient temperature can be employed owing to its high catalytic efficiency and unique specificity at low and moderate temperatures for biotechnological or industrial processes.

The enzyme was incubated with various compounds that may inhibit the enzyme, and the remaining activity was measured with *p*-nitrophenyl butyrate as the substrate at 25^0C (Table 4.16). The metal ion like Ca^{2+}, Mg^{2+}, Na^{+}, Fe^{2+} and Zn^{2+} at a concentration of 5.0 mM were able to enhance the activity of purified lipase while other ion like Hg^{2+}, Cu^{2+}, CO^{2+} had shown negative effect on the enzyme activity. Among the different inhibitors EDTA inhibit the cold active lipase. In contrast, lipase is stable in presence of DTT, SDS and Tween 80. Nearly one third of all known enzymes require metal ion for its catalytic activity (Voet et al., 1999). The metal ion like Ca^{2+}, Mg^{2+}, Na^{+}, Fe^{2+} and Zn^{2+} were able to enhance the activity of purified lipase. Enhanced activity of enzyme was due to ion shown that the lipase was metal dependent enzyme. Enzyme inhibition studies primarily give an insight in to the nature of the enzyme, its cofactor requirements and the nature of the active centre (Sigma and Mooser, 1975). The influence of divalent metal ions on the activity of LipEH166 was tested by adding 1, 5, or 10 mM $CaCl_2$, $CuSO_4$, $MgSO_4$, $FeSO_4$ and $ZnSO_4$. The activity was enhanced by 30% in 5 mM $CaCl_2$ and was slightly increased by $MgSO_4$. The presence of $CuSO_4$ or $ZnSO_4$ strongly inhibited lipolytic activity (Kim et al., 2008).

Table 4.16: Effect of metal ions and inhibitors on lipase from GGPRTL-5

	Residual activity (%)
Inhibitors (1 mM)	
None	100
EDTA	32
SDS	92
Dithiothreitol	83
Tween 80	89
Metal ion (5 mM)	
$FeSO_4$	91
$CoCl_2$	32
$NaSO_4$	87
$CuSO_4$	25
$HgCl_2$	19
$CaCl_2$	58
$MgCl_2$	76
$ZnSO_4$	58

It was found that the enzymes showed maximum activity at pH 9.0 and temperature 20^0C and thus we can say that the enzymes produced from isolate GGPRTL-5 is cold active and may have various applications in industry. Now a days it in on demand in detergent industry as additives or for processing of volatile substances thereby making it possible to reduce temperature and thus bring down energy costs.

CHAPTER 5

Summary & Conclusion

SUMMARY AND CONCLUSION

Gangotri glacier is located in Uttarkashi district of Uttaranchal state, India between $30^{o}44´$-$30^{o}56´$ N latitude and $79^{o}04´$-$79^{o}15´$E longitude. It is about 30 km long flowing in a NW direction and its width varies from 0.5 to 2.5 km. Bacteria that are especially adapted to low-temperature habitats have been described as either psychrotrophic or psychrophilic. Gangotri glacier is highly rich in microbial diversity would be most appropriate to screen microbes for the production of cold active enzymes. The present work was focused on isolation and characterization of cold adapted bacteria capable of producing cold active enzyme protease and lipase.

The present studies revealed the presence of bacteria in samples of Gangotri glacier. Total bacterial count varied from $10x10^{6}$ to $16x10^{6}$ cfu g^{-1} of soil at 37°C. The lowest bacterial counts were found at Gaumukh, Gangotri and Bhatwari while highest bacterial count were found at Haridwar.

One hundred twenty bacterial isolates were screened from the eight sample of Gangotri sample on PSC solid agar medium containing skimmed milk at temperature from 20 ± 2^{0}C. Ten isolates, designated as AAPRT-3, BBPRT-7, BBPRT-13, CCPRT-12, DDPRT-6, EEPRT-5 FFPRT-10, GGPRT-6, GGPRT-8, and HHPRT-8 were selected as potent protease producing strains. These isolates were subjected to the taxonomical characterization. Phylogenetic analysis using 16S rRNA indicated that strain AAPRT-3 and DDPRT-6, shared high similarities with *Bacillus subtilis*. BBPRT-7 shared high similarity with *Bacillus licheniformis*, BBPRT-13 with *Pseudomonas mandelii*, GGPRT-8 With

Pseudomonas fluorescens, FFPRT-10 with *Pseudomonas fluorescens*, HHPRT-8 and CCPRT-12 with *Pseudoalteromonas flavipulchra*, EEPRT-5 with *Staphylococcus aureus* and GGPRT-6 with *Exiguobacterium alkaliphilum*.

Among these ten potent protease producing isolates three isolates were selected as potent protease producer strain designated as DDPRT-6 (*Bacillus subtilis*), BBPRT-7 (*Bacillus licheniformis*) and FFPRT-10 (*Pseudomonas fluorescens*) at alkaline pH different temperature (4-37^{0}C) were taken for further studies.

Extracellular protease from DDPRT-6 (*Bacillus subtilis*), BBPRT-7 (*Bacillus licheniformis*) and FFPRT-10 (*Pseudomonas fluorescens*) was subjected to partial purification. Enzyme from DDPRT-6 (*Bacillus subtilis*) was partially purified to 2.49 purification fold with the increase of specific activity 561.98 U/mg and yield of 82.4 % by ammonium sulphate fraction and dialysis. Enzyme from BBPRT-7 (*Bacillus licheniformis*) was partially purified to 5.39 purification fold with the increase of specific activity 379 U/mg and yield of 66.6 % by ammonium sulphate fraction and dialysis. Enzyme from FFPRT-10 (*Pseudomonas fluorescens*) was partially purified to 3.3 purification fold with the increase of specific activity 1430.55 U/mg and yield of 45.04 % by ammonium sulphate fraction and dialysis.

Effect of pH on enzyme activity was examined in the pH range 7-11.5.The optimum pH for maximum alkaline protease activity was determined by performing protease activity assay in buffers with different pH (pH 7-11.5). Enzyme from DDPRT-6 (*Bacillus subtilis*) shows maximum activity at pH 10 and enzyme was more than 80% stable at pH range 9-10. The BBPRT-7 (*Bacillus*

licheniformis) shows maximum activity at pH 10 and had the widest pH range for activity, with more than 100% stable at pH range 9-10. Enzyme from FFPRT-10 (*Pseudomonas fluorescens*) shows maximum activity at pH 9 and more than 100% stable at pH range 9-10. The protease was active over a broad pH range. Optimal proteolytic activity occurred at neutral or moderate alkaline pH for the three protease. None of the enzymatic activities had a sharp dependence on pH. All of them was inactivated at pH 11.5. Enzymes from BBPRT-7, DDPRT-6 and FFPRT-10 had the widest pH range for activity, with more than 80% of their maximal activity occurring at pH 9.0-11.5. All proteases had maximal activity at neutral pH and showed high residual activity over a broad range of pH.

When incubations at different temperatures with azocasein as substrate were done, the effect of temperature on the purified proteases was observed at Temperature range 10-50^{o}C. Enzyme from DDPRT-6 (*Bacillus subtilis*) shows optimum temperature at 20^{0}C and the enzyme was found stable at a temperature between 10-30^{0}C. The BBPRT-7 (*Bacillus licheniformis*) shows optimum temperature at 20^{0}C and the enzyme was found stable at a temperature between 10-30^{0}C. Enzyme from FFPRT-10 (*Pseudomonas fluorescens*) optimum temperature at 30^{0}C and the enzyme was found stable at a temperature between 10-35^{0}C. BBPRT-7, DDPRT-6 was the most active at 20^{0}C and FFPRT-10 at 30^{0}C. The three proteases showed to be thermolabile, compared with the commercial enzymes, when they were incubated for 1 h at various temperatures. The all tree psychrotolerant proteases reach thermal inactivation at temperatures 10^{0}C lower than the mesophilic metalloprotease. The enzyme was found stable at temperature between 10-30^{0}C.

Purified protease from DDPRT-6 (*Bacillus subtilis*) exposed tremendous stability and compatibility with a wide range of locally available commercial detergents at 20^0C. Purified protease from BBPRT-7 (*Bacillus licheniformis*) shows enhanced activity with Tween 80, wheel, surf and enzyme was sensitive to tide and SDS. The purified enzyme from FFPRT-10 (*Pseudomonas fluorescens*) was stable in presence of Tween 80, surf, SDS and wheel while inhibited by tide. The effect of various metal ions on protease production was evaluated. The purified proteases of DDPRT-6 and BBPRT-7 were inhibited by EDTA while protease activity of FFPRT-10 was increased. Proteolytic activities of all three enzymes were affected in the presence of 5 mM $Zn^{2+,}$ Hg^{2+} and Cu^{2+}. DDPRT-6 was the most sensitive among these protease producing strains.

One hundred bacterial colony were isolated on trybutyrin agar plates from eight samples of Gangotri glaciers. Out of 100 positive isolates eight isolates showed good zone of hydrolysis revealing to be capable of producing lipase. Eight lipase producing strains are designated as AAPRTL-10, BBPRTL-5, CCPRTL-8, DDPRTL-14, EEPRTL-5, FFPRTL-9, GGPRTL-06, GGPRTL-5. These isolates were subjected to the taxonomical characterization. Phylogenetic analysis using 16S rRNA indicated that starin AAPRTL-10 shared high similarity with *Pseudoalteromonas lipolytica*, FFPRTL-9 with *Bacillus sonorensis*, BBPRTL-5 with *Bacillus sphaericus*, DDPRTL-14 with *Arthrobacter psychrolactophilus*, GGPRTL-5 with *Stenotrophomonas maltophilia*, EEPRTL-5 with *Pseudomonas vancouverensis*, CPRTL-8 with *Aeromonas veronii*, GGPRTL-6 with *Bacillus licheniformis*.

Among these isolates GGPRTL-5 is potent lipase producing isolates at temperature 20^0c. The strain GGPRTL-5 was selected as the best producer of lipase and was identified as *Stenotrophomonas multophilia* on the basis of its taxonomical characterization.

Most purification schemes for lipases are based on multi step strategies. Cold active lipase was partially purified by precipitating with ammonium sulfate (40-60%) and using a single step ion-exchange chromatography on a DEAE-cellulose. Partially purified lipase was eluted out as fractions (with 0.1-1 M NaCl gradient) from DEAE-cellulose column with 28.80 fold purification and specific activity of 82.10 U mg^{-1}.

The maximum activity of lipase of GGPRTL-5 at pH 8 at 20^0C and the enzyme was stable between pH 8 and 9 at the indicated pH range when incubated at 20^0C for 24 h, but its activity decreased at pH at 10.

The activity of cold active lipase was determined at a wide range of temperature 10-50^0C. The optimum temperature for lipolytic activity of GGPRTL-5 was determined to be 20^0C. The enzyme activity was almost constant within 25-30^0C and gradually declined at temperature beyond 35^0C. The enzyme was stable up to 30^0C for 1 h and decreased at higher temperature.

The metal ion like Ca^{2+}, Mg^{2+}, Na^{+}, Fe^{2+} and Zn^{2+} at a concentration of 5.0 mM were able to enhance the activity of purified lipase while other ion like Hg^{2+},Cu^{2+},Co^{2+} had shown negative effect on the enzyme activity. Among the different inhibitors EDTA inhibit the cold active lipase. In contrast, lipase is stable in presence of DTT, SDS and Tween 80. The metal ion like Ca^{2+}, Mg^{2+}, Na^{+}, Fe^{2+} and Zn^{2+} were able to enhance the activity of purified lipase. Enhanced activity of

enzyme was due to ion shown that the lipase was metal dependent enzyme. Cold active enzymes produced from isolate GGPRTL-5 (*Stenotrophomonas maltophilia*) showed maximum activity at pH 9.0 and temperature 20°C and thus we can say that the enzyme may have various applications in industry.

The conclusions resulting from these investigations would provide a baseline data about cold adopted bacteria and their industrial significance. The improved understanding of the nature of the bacterial community in glacier ice developed in this study will also help provide a better foundation for more detailed studies of the ecophysiology and ecology of glacier ice bacteria and for the biotechnological applications of cold adapted bacteria in general. We have isolated three cold active protease producing strain DDPRT-6 (*Bacillus subtilis*), BBPRT-7 (*Bacillus licheniformis*), FFPRT-10 (*Pseudomonas fluorescens*). BBPRT-7, DDPRT-6 was the most active at 20^0C and FFPRT-10 at 30^0C. Enzymes from BBPRT-7, DDPRT-6 and FFPRT-10 had the widest pH range for activity, with more than 80% of their maximal activity occurring at pH 9.0-11.5. This pH dependence for activity makes these enzymes interesting for industrial applications, in contrast with other cold proteases with a narrow pH profile. Purified protease from DDPRT-6 (*Bacillus subtilis*) exposed tremendous stability and compatibility with a wide range of locally available commercial detergents at 20^0C.The current consumer demands and increased use of synthetic fibers, which cannot tolerate high temperatures, have changed washing habits towards the use of low washing temperatures. It was found that the lipase enzymes produced by cold adapted bacteria GGPRTL-5 showed maximum activity at pH 9.0 and temperature 20°C may have application in detergent industries.

This study is a one step effort for search of a novel cold active enzyme. Research outcome 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 cols adapted bacteria and prospects of their enzymes it is high time for scientist to give their attentions for more focused study. More organized study is needed to explore 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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List of Publications

LIST OF PUBLICATIONS

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, ISSN (Online): 2393-8021 and ISSN (Print) 2394-1588.

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