CHAPTER 1
INTRODUCTION

1.1 Extremophiles

Earth’s biosphere is diverse with the extremities of climatic conditions, for example extremes of temperature, pressure, salinity and pH. From past few decades, it was found that few microbial communities are able to survive in these forbidden regions and has been classified as ‘extremophiles’ (Burg, 2003). Extremophilic microorganisms have been classified according to their habitats, a) thermophiles are the organisms that are able to grow at temperature between 60-80°C and hyperthermophiles grow at 80–122°C, b) Psychrophiles/cryophiles grows below 15°C, c) acidophiles grow at pH 3 or below; d) alkaliphiles grow at pH 9 or above; e) endoliths organisms live in microscopic spaces; f) metallotolerants are capable of tolerating high levels of heavy metals, such as copper, cadmium, arsenic, and zinc, g) oligotrophs show growth in limited nutrition; h) osmophiles grow in high sugar concentration i) piezophiles grow at high hydrostatic pressure and common in deep terrestrial subsurface as well as in oceanic trenches j) radioresistants are resistance to high level of ionizing radiation such as ultraviolet radiation, k) thermo-acidophiles are the combination of thermophiles and acidophiles that prefer temperature between 70–80°C and pH at 2-3, l) xerophiles grow in extremely dry, desiccating conditions, such as Atacama Desert, South America.

1.2 Industrial application of Extremophilic enzymes

The Extremophilic microorganisms are surviving these extreme conditions due to their cellular structure and active biocatalysts. The extremophilic biocatalysts have been studied for their properties and applications of these biocatalysts in various industries have led new cutting edge technology. Various enzymes have been commercialized due to usefulness of their properties like isolation of Taq polymerase, a thermostable enzyme from Thermus aquaticus, α-amylase, β-amylase, glucoamylases, pullulanases etc. (Kelly & Fogarty, 1983), which have been purified from Bacillus amyloliquefaciens, Bacillus subtilis, Bacillus stearothermophilus (Bessler et al., 2003) and Thermus sp. (Shaw et al., 1995). For biochemicals many micro-organisms such as Cellovibrio, Cytophaga, Clostridium thermocellum, Thermoanaerobium acetigenum, Penicillium funiculorum, Cellulomonas sp., Aspergillus sp., Talaromyces emersonii,
Sporotrichum pulverulentum, Trichoderma ressii, Melanocarpus albozymes, Thermomyces lanuginosus and actinomycetes, Thermoactinomycetes sp., Thermonospora cutvata (Sukumaran et al., 2005) have been exploited. Proteases have been isolated from Psuedomonas sp. (Griffith et al., 1981) and Bacillus licheniformis (Aunstrup 1993). Lipases have been isolated from Bacillus sp. (Sigurgisladottir et al., 1993), Bacillus alcalophilus (Ghanem, 2004). For biosorption of heavy metal ions and transition metals like Bacillus sp., Pseudomonas sp. and Staphylococcus sp. (Zolgharnein, 2010) have been utilized. For lactose treatment in milk industries, β-galactosidase enzyme from psychrophilic bacteria Arthrobacter sp. and Psuedoalteromonas haloplanktis (Hildebrandt et al., 2009 and Hoyoux et al., 2001) have been exploited. Other examples have been shown in table 1.

Table 1.1 : Classification of extremophiles and application of biocatalysts in extremozymes

<table>
<thead>
<tr>
<th>S. No.</th>
<th>Type</th>
<th>Growth characteristics</th>
<th>Enzymes</th>
<th>Application</th>
<th>References</th>
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</table>
| 1.     | Thermophilic | 1. Thermophile Temp. > 60-80° C
         | 2. Hyperthermophile Temp. > 80° C       | 1. Proteases
         | 2. Glycosyl hydrolysis e.g. amylases, pullulanases, glycoamylases, glucosidases, cellulases, xylanases
         | 3. Chitinases
         | 4. Lipases
         | 5. Estrases
         | 6. Xylanases
| 2.     | Pyschrophiles| Temp. < 15°C.                           | 1. Oxidases
         | 2. Proteases
         | 3. Amylases            | Oxidation reactions Detergents Food | Kim et al., 1999. Cavicchioli |
3. **Halophiles**

   - High salt (2-5 M NaCl)
   - 1. Proteases
   - 2. Amylases
   - Peptide synthesis
   - Biocatalysis in organic media
   - Applications and dairy products
   - Detergents
   - Feed and cosmetics

4. **Alkaliphiles**

   - pH > 9
   - 1. Protease
   - 2. Cellulases
   - Detergent
   - Food and feed

5. **Acidophiles**

   - pH < 2-3
   - 1. Amylases
   - 2. Glucoamylases
   - 3. Proteases
   - 4. Cellulases
   - 5. Oxidases
   - Starch processing
   - Detergents
   - Food components
   - Desulfurization of coal

### 1.3 Molecular adaptation of extremophiles

Increasing demand of biocatalysts to cope up industrial conditions and organic synthesis for better chemical precision leading more efficient production of single stereo isomers, fewer side reactions and lower environmental burden has attracted scientist to study active biocatalysts from extremophiles (Rozzell, 1999). More than 3000 different enzymes have been identified and many of them have been exploited in biotechnological and industrial processes, most of the enzymes do not meet the demands of industrial reaction conditions and therefore, study of extremophiles and their active biocatalysts working in extreme conditions have received great attention.

### A. Thermophiles

At the molecular level, thermophiles are adapted to high temperature due to increased number of hydrogen bonds, salt bridges, improved core packing, shorter and tighter surface loops, enhanced secondary structure propensities or oligomerization (Matthews, 1993, Vogt et al., 1997, Vogt et al., 1997, Szilagyi et al., 2000).
Thermophilic enzymes have been exploited for the production of proteases, lipases and polymer-degrading enzymes, such as cellulases, chitinases and amylases. They were found more stable in elevated temperatures and have improved polymeric substrate reactions. They were found more useful to reduce the risk of contamination as reaction to take place in higher temperature.

B. Halophiles

Halophiles survive in hyper saline habitats by maintaining osmotic balance. The active enzymes adapted to such environment are due to acquiring a relatively large number of negatively charged amino acid residues on their surface to prevent precipitation. This property has been applied in various processes like in aqueous/organic and non-aqueous media. Protease from *Halobacterium halobium* has been exploited for efficient peptide synthesis in water /N'-N’ dimethylformamide (Kim *et al*., 1997, Klibanov, 2001).

C. Alkaliphiles/acidophiles

Microorganisms inhabiting high pH (8-11) generally maintain pH 8 inside the cells. A majority of alkaliphiles require Na⁺ ions for their growth. There is a mechanism known as Na⁺/H⁺ system studied in *Bacillus firmus* and *Exiguobacterium auranticum* which is replaces respiration-coupled extrusion of Na⁺ by at least 2 antiporter proteins for the uptake of protons (Ivey *et al*., 1998).

Bacteria generally work on primary transport system that is maintained by proton-motive force, which is total sum of the electrical membrane potential and pH gradient. At high pH level, alkaliphilic organisms reverse their pH gradient in order to maintain their external pH near to neutral and produce high electrical membrane potential by increasing negative component in proton-motive force, yielding more ATPs (Ni *et al*., 1998). Very less is known about the alkaliphiles, but cell wall analysis showed that alkaliphiles exhibit correlation between density of high charge on cell wall and degree of pH regulation.

Various enzymes exploited are protease in gelatin removal on X-ray films, removing clogs in drain pipes, cellulase in wastewater treatment, xylanse in biological bleaching processes, Rayon modification. Pectinase in paper manufacturing, waste water treatment, cyclodextrins as
food additives, antibiotic stabilizers as well as hormones or vitamins are other examples of alkaliphilic enzymes.

Acidophiles have high reverse membrane potential (ΔΨ) formed by influx of K⁺ ions inhibiting proton entry into the cell. Another factor is Donnan potential equilibrium, which is formed when diffusions of ions in one direction equals the electromigrational flux of ions in opposite direction, resulting net zero mass and charged transport. Acidophilic constitutes secondary transproters which are membrane proteins that use transmembrane electrochemical gradients of protons or sodium ions to drive transport (Baker-Austin et al., 2007).

D. Psychrophiles

In psychrophiles, transcription and translation are controlled by cold active nucleic-acid-binding proteins, relieving adverse effects of low temperature (Michel et al., 1997, Cavicchioli et al., 2000, Tendeng et al., 2003,). The other factors responsible for cold adaptations are cold-acclimation proteins (CAP). It has been observed that a set of ~20 proteins are permanently synthesized during growth at low temperature (Hebraud et al., 1994, Berger et al., 1996, Hebraud et al., 2000).

Another reason of their survivability is by synthesizing anti-freeze proteins (AFP), widely studied in polar fish. They are composed of glycopeptides and peptides generally decreasing freezing point of cellular water by binding to ice crystals (Jia et al., 1996). Antifreeze protein have been used in cryosurgery, preservation of tissues for transplant and transfusion, for lengthening the shelf life of frozen food and in agriculture increasing freeze tolerance of crops, extending harvest season in cool climate (Feller et al., 2003).

Application of psychrophilic enzymes could be promised in domestic processes like application of subtilisin, lipase and glycosidases in laundry. Removal of lactose present in the milk by psychrophilic β-galactosidase. Other cold-active enzymes have been utililized for example, pectinases to reduce viscosity and clarify fruit juices at low temperatures and use of a heat-labile alkaline phosphatase in molecular biology (which does not interfere with end labeling by polynucleotide kinase after heat treatment).
1.4 Cold adaptive β-galactosidase enzyme

The cold active β-galactosidase enzyme is of high demand in dairy industries because milk and dairy products are generally stored and transported at 4-8°C. β-galactosidase enzyme catalysis the hydrolysis of lactose into galactose and glucose. In dairy industries, usually membrane technology is applied, which is an expensive procedures. The other technique is application of β-galactosidase enzyme, but till date only thermophilic or mesophilic β-galactosidase has been employed. Surprisingly, the treatment of milk at higher temperature suffers from off flavours, reduced organoleptic properties and high energy cost. Unfortunately till date membrane technology and β-galactosidase enzyme treatment are the only options available to the industries which has risen the the price about 20-30% but is still a choice of manufacturing lactose free milk with genuine taste.

Till date many efforts have been made to isolate the β-galactosidase enzyme from psychrophiles like; activity e.g. *Arthrobacter* sp. (Gutshall *et al.*, 1988, Coker *et al.*, 2003), *Pseudoalteromonas* sp. 22b (Cieslinski *et al.*, 2005), *Arthrobacter psychrolactophilus* (Nakagawa *et al.*, 2006, Nakagawa *et al.*, 2007), *Carnobacterium piscicola* (Coombs *et al.*, 1999) *Planococcus* sp. (Sheridan *et al.*, 2000, Hu *et al.*, 2007), *Pseudoalteromonas haloplanktis* (Houyox *et al.*, 2001) and *Pseudoalteromonas* sp. (Turkiewicz *et al.*, 2003), but all of them expressed the β-galactosidase intracellularly with low activity. Therefore, more efforts are required to screen more microorganisms secreting extracellular β-galactosidase enzyme. Extracellular enzymes offer benefits like, loss in the enzyme activity during cell breakage. The only β-galactosidase enzyme will be considered which have high activity between 4-8°C and should not be inhibited by ions such as Ca²⁺ and Na⁺ and sugars like lactose, galactose and glucose which are present in milk at higher concentration. Thus, present study was undertaken to isolate the psychrophilic microbes producing extracellular β-galactosidase.

In present studies, we characterized bacterial isolates from the glaciers’ soil samples of Kafnu and Sangla valley of Himachal Pradesh, India, which constitute permafrost regions of large and old glaciers covered by snow throughout the year. Our prime consideration was on the bacterial isolates, who exhibited the extracellular β-galactosidase enzyme. For that purpose following objectives were proposed for the isolation of psychrophilic bacteria secreting β- galactosidase,
1.5 Objectives

1. Isolation of psychrophilic bacteria and screening for the production of extracellular, constitutive β-galactosidase enzyme

3. Characterization of β-galactosidase enzyme secreting bacterial isolates for morphological, biochemical and genotypic properties

4. Biochemical characterization of the extracellular β-galactosidase enzyme by physical parameters (temperature, pH, metal ions and co-factors) and kinetic parameters/ constants ($K_m$, $V_{max}$, $K_{cat}$) of the β-galactosidase mediated catalysis