PRODUCTION AND CHARACTERIZATION OF ALKALINE PHOSPHATASE FROM PSYCHROPHILIC BACTERIA

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Production and Characterization of Alkaline Phosphatase from Psychrophilic Bacteria

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With the name of ALLAH, Beneficent, Merciful

Declaration

The material contained in this thesis is my original work. I have not previously presented any part of this work elsewhere for any other degree.

Bashir Ahmad

CERTIFICATE

This thesis, submitted by **Bashir Ahmad** is accepted in its present form by the Department of Microbiology, **Quaid-i-Azam University, Islamabad** as fulfilling the thesis requirement for the degree of Doctor of Philosophy in Microbiology.

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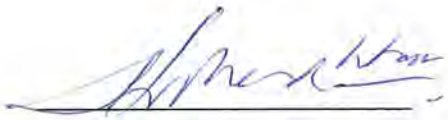

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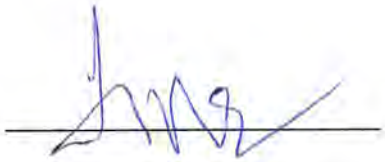

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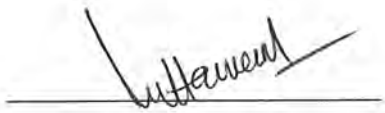

(Prof. Dr. Abdul Hameed)

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LIST OF ABBREVIATIONS

Abbreviation	Definition
Ala	Alanine
ALP	Alkaline phosphatase
$(NH_2)SO_4$	Ammonium Sulphate
ASP	Asparagine
β-ME	β-mercaptoethanol
BSA	Bovine Serum Albumin
CFU	Colony forming units
°C	Degree Celsius
DNA	Deoxyribose nucleic acid
DEAE	Diethyl amino ethyl
DMSO	Dimethyl sulfoxide
K_2HPO_4	Dipotassium hydrogen phosphate
Na_2HPO_4	Disodium hydrogen phosphate
DO	Dissolved oxygen
EDTA	Ethylenediamidetetracetic acid
Gln	Glutamine
Gly	Glycine
g/L	Grams/litre
H_2O_2	Hydrogen per oxide
OH-Group	Hydroxyl group
kDa	Kilo Dalton
LB Medium	Luria Bertani medium
V_{max}	Maximum velocity
K_m	Michaelis constant
mL	Milli litre
mM	Milli moles per litre
M	Moles per litre
mmol	Milli mole
mol	Mole

nm	Nano meter
Nt	Nucleotides
O.D	Optical density
*p*NPP	Para-Nitrophenyl phosphate
*p*NP	Para- Nitrophenol
1-NPP	1-Naphthyl phosphate
1-NP	1-Naphthol
Rpm	Revolution per minute
rRNA	Ribosomal Ribose nucleic acid
SDS-PAGE	Sodium dodecyl sulphate- Polyacrylamide gel electrophoresis
Na K Tartarate	Sodium potassium tartarate
Sp.	Species

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ABSTRACT

The normal flora entombed in ice of glaciers and freezers may have adapted the severe physiological conditions and scarce source of macronutrients for their survival. Aim of this study was to isolate, identify and characterize psychrophilic bacteria from glacial and non glacial samples, and to purify and characterize alkaline phosphatase from a selected strain. Three cold active bacteria, morpho-physiologically, identified as *Bacillus subtilis* MRLBA7, *Bacillus licheniformis* MRLBA8 and *Bacillus megaterium* MRLBA9 were isolated from -20°C freezer of Microbiology Research Laboratory (MRL), Quaid-i-Azam University, Islamabad, Pakistan. These strains were able to grow aerobically at 6°C but not at 40°C except MRLBA8 that could grow at 48°C. None of the isolates showed inhibition of growth in presence of glycerol. Isolate MRLBA7, bearing central spore, grew in the presence of 30% glycerol at 0°C after 48 hrs of incubation and showed maximum growth without glycerol at 25°C after 24 hrs. Isolate MRLBA8 showed growth in the presence of 50% glycerol at 4°C after 72 hrs of incubation and maximum growth was observed at 20°C in the absence of glycerol. Isolate MRLBA9 showed growth at 6°C in the presence of 40% glycerol after 48 hrs of incubation and maximum growth was observed at 25°C in the absence of glycerol. Isolates were susceptible to antibiotics except *Bacillus subtilis* MRLBA7 that exhibited antibiotic resistance against penicillin and fosphomycin, *Bacillus licheniformis* MRLBA8 against aztreonam and fosphomycin, and *Bacillus megaterium* MRLBA9 against vancomycin and penicillin. The growth profile and biochemical characteristics of all three isolates were rather similar to that of mesophilic counterparts except adaptation to low temperature.

On the basis of morphology, biochemical tests and 16S rRNA analyses, six cold active bacteria identified as *Pseudomonas* sp. MRLBA1, *Pseudomonas* sp. MRLBA2, *Pseudomonas* sp. MRLBA3 *Pseudomonas* sp. MRLBA4, *Arthrobacter* sp. MRLBA5 and *Stenotrophomonas* sp. MRLBA6 were isolated from ice, water and soil samples obtained from Batura, Hopper and Passu glacier, Northern Areas of Pakistan. All of the glacial isolates were aerobic, asporous, non-motile and Gram-negative rods except MRLBA5 that was Gram variable, motile and exhibited rod-coccus growth cycle. *Pseudomonas* sp. MRLBA1 was capable of

growing at 2-30°C, and pH 4-11; *Pseudomonas* sp. MRLBA2 at 4-30°C, and pH 4-10; *Pseudomonas* sp. MRLBA3 at 4-35°C, and pH 5-10; *Pseudomonas* sp. MRLBA4 at 4-37°C, and pH 5-10; *Arthrobacter* sp. MRLBA5 at 4-37°C, and pH 4-9; and *Stenotrophomonas* sp. MRLBA6 at 4-30°C, and pH 4-11. The glacial Isolates were susceptible to antibiotics except *Pseudomonas* sp. MRLBA1 that exhibited antibiotic resistance against vancomycin and penicillin, *Pseudomonas* sp. MRLBA2 against aztreonam and fosphomycin, *Pseudomonas* sp. MRLBA3 against vancomycin and penicillin, *Pseudomonas* sp. MRLBA4 against fosphomycin to vancomycin and penicillin, *Arthrobacter* sp. MRLBA5 against aztreonam and fosphomycin, and *Stenotrophomonas* sp. MRLBA6 against aztreonam and vancomycin

Pseudomonas sp. MRLBA1 selected for the production of alkaline phosphatase showed highest extracellular alkaline phosphatase activity at pH 8.0 and 18°C when inoculated with 24 hrs old inoculum (5%), after 48hrs of incubation in shake flask experiments. After precipitation with 60% ammonium sulfate, the enzyme was purified with gel permeation (134.81 U/mg) and ion exchange chromatography (225 U/mg) with 9.75 and 16.27 fold of purification, respectively. A single active peak of 54-58 KDa was estimated by gel permeation column and a single band of ~54-56 KDa was determined from SDS-polyacrylamide gel electrophoresis. The purified alkaline phosphatase was stable between pH 4-13 and 0-55°C but maximally active at pH 9.0 and 37°C. The enzyme was concluded as the thermo-labile in nature. The chloride salts of calcium zinc, magnesium, mercury and copper increased the specific activity of alkaline phosphatase but iron and potassium decreased it to some extent. The enzyme was stable when assayed along with 45% of glycerol but showed decrease in activity from 50-90% glycerol, sharply. The kinetic constants, Km and Vmax, were calculated as 122 µmol and 28 µmol.min^{-1} from Eadie-Hofstee plot. The potassium ferricyanide did not have any inhibitory or stimulatory effect on alkaline phosphatase whereas potassium ferrocyanide showed uncompetitive inhibition.

INTRODUCTION

Extreme is a relative term, which is quoted in comparison to what is normal for human beings. Extreme environments include upper and lower limits of temperature, pH, pressure, salt concentration, nutrients and water availability, radiations, harmful heavy metals and toxic compounds (organic solvents). Combinations of a number of extreme physical parameters are common. To live and survive under extreme conditions requires structural and/or physiological adaptations of the organism. Simple and early life like microbes has adapted the extreme environments mostly during evolutionary history (Satyanarayana *et al.*, 2005).

Extraterrestrial ice on planets like Mars and Moon like Europa, meteorites, satellites with delayed stay and clouds could be used as source of isolation of psychrophilic archaea (Christner, 2007). Aeroplankton are viable microbes from atmosphere (Sattler *et al.*, 2006). Bichteler (2000) revealed a highly active microbial assemblage which can be characterized by their different origins (terrestrial, airborne or meltwater). Ice from polar and mountain glaciers are potential source for isolation of bacteria. A glacier is a permanent (on a human time scale, because nothing on the Earth is really permanent) body of ice, consisting largely of recrystallized snow, that shows evidence of down slope or outward movement due to the pull of gravity (Nelson, 2003). Generally, six types of glacier (Mountain, Valley, Cirque, Niche, Ice caps, and Ice aprons) are observed in [Hindu Kush-Karakoram-Himalayan (HKH)] region of Pakistan.

Extremophiles are microorganisms that harbor extreme physical conditions to thrive. The important factors for growth of extremophilic microorganisms are; high or lower limits of temperature, pH, and high salinity. The resulting environments, based on the elevated or low conditions, are then qualified as thermophilic, psychrophilic, alkalophilic, acidophilic and halophilic, respectively. Much of the Earth's surface experiences low temperatures. The oceans which cover 71% of the surface have an average yearly temperature of 5°C and the ocean depths have a constant temperature between 1 and 4°C throughout the year due to the combined hydrostatic pressure and water density. Polar regions, representing 14% of the surface of the Earth, are permanently frozen or above freezing temperatures only for a short period of time. These environments, which are

dominant on Earth, are favorable to psychrophiles able to grow at any cold temperature at which water is still liquid (Madigan *et al.*, 1997). Strictness of microbes to their optima defines their types. Many extremophiles face more than one extreme parameter and they manage to live under those combinations. Extremophiles are subdivided according to their physico-chemical conditions of the atmosphere they live in. The most important extreme environments found in nature and typical microbial groups or species that have been observed in them are listed in Table 1.1.

Extremophiles have evolved several structural and biochemical adaptations, which allow them to survive and grow in extreme environments (Satyanarayana *et al;* 2005). They are a source of enzymes called *Extremozymes* with extreme stability and activity at harsh conditions of the system (Hough and Danson, 1999; Satyanarayana *et al;* 2005). The applicability of extremozymes is often hampered by their limited availability from natural sources (Rossi, 2003). Used in combination with aqueous media, extremozymes show great potential as shown by their unique properties. Low temperature processes using psychrophilic (cold-active) enzymes may enhance yields of heat-sensitive products and reduce energy consumption (Sellek and Chaudhuri, 1999).

The low temperature environments dominate most of the Earth's surface which is made up of extremely cold parts such as Arctic, Antarctic, Greenland, moderately cold parts like mountains and glaciers and oceans that occupy 70% of the planet (Baross and Morita, 1978). These ecosystems are cold and exert a big selective pressure to life on other environments. Though they are extreme but different organisms colonized the habitat and developed strategies to adapt the ecosystem largely, to survive or occupy the niche successfully (Gerday *et al.*, 1997). Thermodynamically, life is possible up to -12°C at minimum which is consistent with the known physical state of aqueous solutions at sub-zero temperatures.

Table 1.1 Characteristics of extreme environments and their habitants

Stress and attribute		Ecosystem	Environ. conditions	Microorganisms observed	References
Temperature	*Psychrophiles*	Glaciers	0-20°C	*Pseudomonas* sp.	Moyer and Morita (2007)
		Polar regions	-2.5 - 0°C	*Flavobacterium*	Shi *et al.*, 1997
		Deep marine trenches	0 - 4°C	*Pseudomonas*	Ravenschlag, 2001
	Thermophiles	Terrestrial hot springs	65 to 95°C	*Thermus* spp.	Kristjansson and Hreggvidsson, 1995
	Hyperthermophiles	Submarine vents	>100 to 121°C	*Thermococcus barophilus*	Marteinsson *et al.*, 1999
Oxygen	*Anaerobe*	Petroleum depths	(no O_2)	*Thermotoga* spp.	Pysz *et al.*, 2004
	Aerobe	Fresh water	20% O_2	*Bacillus* sp.	Vossenberg *et al.*, 1999
Salt	*Halophiles*	Salt brines, salterns	>6%	*Halobacterium* sp.	Sleator and Hill, 2002
pH	*Acidophiles*	Sulfide-rich zones	pH 3 or lower	*Thiobacillus*	Kristjansson and Hreggvidsson, 1995
	Alkaliphiles	Soda lakes	pH 10 or above	*Bacillus* sp.	Rees *et al.*,2004
Pressure	*Barophiles*	Deep marine trenches	500 to 1034 atm	*Moritella yayanosii*	Nogi and Kato, 1999
Radiation	*Radiophiles*	Nuclear power plants	3 - 5 Mrad	*Deinococcus radiodurans*	Rainey *et al.*, 1997

Psychrophiles ('psychro' Greek 'cold') are microorganisms that can not thrive at temperatures above 20°C. Baross and Morita, (1978) defined some associated terms like cryophile, psychrorobe, rhigophile, Glaciale Bakterien, thermophobic bacteria, facultative psychrophile, psychrocartericus, psychrotrophic and psychrotolerant to designate a psychrophile. Depending the strictness to obey

their optima, there are two groups of psychrophiles: obligate and facultative. *Obligate psychrophiles* are those organisms having a growth temperature optimum of 15°C or lower and cannot grow in a climate beyond a maximum temperature of 20°C. They are largely found in icy places (such as in Antarctica) or at the freezing bottom of the ocean floor. This separation is becoming more difficult as more organisms with a fairly large growth temperature range are discovered (Morita and Moyer, 2004). *Facultative psychrophiles* also termed as psychrotrophs can grow at 0°C up to 40°C, and exist in much larger numbers than obligate psychrophiles. They are generally not able to grow much below 0°C, though they may maintain basic functioning. They have evolved to tolerate cold, but they are not as physiologically specialized as obligate psychrophiles and are usually not found in the very cold environments (Morita and Moyer, 2004).

Psychrophilic microorganisms are ubiquitous and could be a bacterium or an archaeal candidate. They have a lot in common. Psychrophilic bacteria are distributed widely in cold environments. The permafrost is considered as the extreme environment for microorganisms because they must experience exposure to subzero temperatures for long time in scarce water and nutrients and limited metabolite transfer (Steven, 2006). Psychrophiles may be present in clouds in metabolically active form. Hence they may be involved in atmospheric chemistry (Amato *et al.*, 2006; Sattler *et al.*, 2006). Psychrophiles study provokes molecular adaptations, biodiversity and microbial dynamics in the cold ecosystems and its relatedness to other frozen environments elsewhere in the solar system.

For survival and proliferation, psychrophiles have to cope with the key barriers inherent to permanently cold environments. These challenges include: decreased membrane fluidity and permeability, inadequate uptake of nutrients, insufficient energy-generating systems, reduced enzyme activity, inadequate biodegradative enzymes, altered transport waste products, challenging protein synthesis machinery, inappropriate protein folding, intracellular ice formation and cold-denaturation of proteins (Russell, 2000; D'Amico *et al.*, 2006). The cellular levels of ATP and ADP increase at low temperature. Psychrophilic bacteria have evolved mechanisms like, increased membrane lipid unsaturation, increased

enzyme concentration and cold-shock protein induction, to combat these problems. Alternatively, accumulation of ATP and ADP at sub-zero temperatures might provide an energy reserve for repair and maintenance pathways or enable faster resumption of normal metabolism when favorable growth conditions are reintroduced (Christner, 2008). Psychrophilic microorganisms should have elevated levels and highly efficient phosphatases due to elevated levels of ATP, ADP and psychrophilic life style, respectively.

To thrive at their habitats, extremophiles have evolved their metabolism accordingly. The proteins from extremophiles especially enzymes (extremozymes) exhibit unique properties as compared to mesophilic ones. Due to their extended optima, extremozymes have interesting uses in common life. Also, commercially they have attracted a big interest and focus due to their spectrum of applications (Table 1.2).

Table 1.2: Some extremozymes and their applications (Burg, 2003)

Microorganism	Enzymes	Applications
Psychrophiles	Proteases	Detergents, food applications (e.g. dairy products)
	Amylases	Detergents and bakery
	Cellulases	Detergents, feed and textiles
	Dehydrogenases	Biosensors
	Lipases	Detergents, food and cosmetics
	Alkaline phosphatase	Molecular biology, biosensors
Thermophiles	Proteases	Detergents, hydrolysis in food and feed, brewing
	Glycosyl hydrolases	Starch, cellulose, chitin, pectin processing, textiles
	Chitinases	Chitin modification for food and health products
	Xylanases	Paper bleaching
	Lipases, esterases	Detergents, stereo-specific reactions
	DNA polymerases	Molecular biology (e.g. PCR)
	Dehydrogenases.	Oxidation reactions.
Halophiles	Proteases	Peptide synthesis
	Dehydrogenases	Biocatalysis in organic media
Alkaliphiles	Proteases, cellulases	Detergents, food and feed
Acidophiles	Amylases,	Starch processing
	glucoamylases	Feed component
	Proteases, cellulases	Desulfurization of coal
Piezophiles	To be defined	Food processing and antibiotic production

To compete with homologues of moderate temperatures and survive, Psychrophiles need an efficient adaptation of metabolic flux ultimately demanding adequate rates of chemical reactions. Since enzymes from psychrophiles are also cold adapted, their molecular structure complement the statement i.e. they catalyze the biochemical reactions efficiently at low temperature. Understanding the adaptations of psychrophiles to thrive in, and their enzymes to catalyze at low temperatures is an area to be explored. The key determinant for adaptation of psychrophiles to cold environment is appropriate *membrane fluidity* for exchange of metabolites from and to the cell, achieved by shortening of acyl chains of lipids in membranes and unsaturation of fatty acids (Garedy *et al.*, 1997).

Others include changes in *isomers distribution* and altered *methyl branching* (Russell, 1995) and finally the expression of *cold acclimatization proteins* (the proteins overexpressed after all cold shocks) which are expressed by psychrophiles in response to cold shocks. Such proteins have been reported in psychrotrophic *Bacillus cereus* and *Arthrobacter globiformis* (Mayr *et al.*, 1996; Berger *et al.*, 1996) whose essential role is still unsettled. The specific cold shock proteins (csp) in psychrophiles enable them to go in permanently cold environments efficiently. They are regulatory and xenobiotic factors for genes exposed to low temperatures and antibiotics respectively (Geradey *et al.*, 1997). Many csp genes (*cspA*, *cspB* and *cspG*) have been described in *E. coli* (Jones and Inouye, 1994; Nakashima *et al.*, 1996) and in other bacteria. Some psychrophilic antifreeze proteins (protect the cells from physical damages caused by ice crystals) have been purified from extracts and cells of microorganisms like *Micrococcus cryophilus*, *Pseudomonas putida* and *Rhodococcus erythropolis* (Geradey *et al.*, 1997).

Psychrophilic microorganisms produce enzymes that exhibit a high catalytic efficiency at low temperatures and rapid inactivation at temperatures as low as 30°C. Due to their high specific activity and thermo sensitivity, these enzymes and their producing microorganisms, offer a great potential in biotechnology (Feller *et al.*, 1996).

An Interesting property of psychrophilic or psychrotrophic enzymes is high specific activity and stability at moderate or low temperature as compared to mesophilic counterpart. This is attributed to conformational changes in the structure of cold enzymes that contribute for its flexibility to function at low temperature that render it instable for denaturing agents. The specific activity of cold enzymes is estimated over a temperature range of 0-30°C and by a relative instability (Geradey *et al.*, 1997). The high specific activity of cold enzymes can be harnessed by a very low activation free energy (Johnston and Walesby, 1977). The activation state of a cryozyme (cold enzyme) catalyzed reaction is energetically less costly and enthalpy (*energy difference*) driven interactions are broken quite early during activation of Enzyme-Substrate complex. Hence entropy (*order difference*) of the cold enzyme depends upon 3D status of the protein and movement of water molecules (Johnston and Walesby, 1977). The productive binding of the substrate, which may involve a conformational change, is largely rate limiting for turnover of the enzyme at low substrate concentrations (Wang and Guo, 2007).

The enzyme catalyses by binding substrates to its active site and lowering the energy needed in order for a reaction to occur (Mikkelsen, 2004) hence they show affinity towards substrates that are physically and geometrically complementary to their binding site (Donald and Judith, 1995). Alkaline phosphatase (ALP) catalyzes the phosphate-derived molecules to produce inorganic phosphate and a hydrolyzed molecule (Fig 1.1).

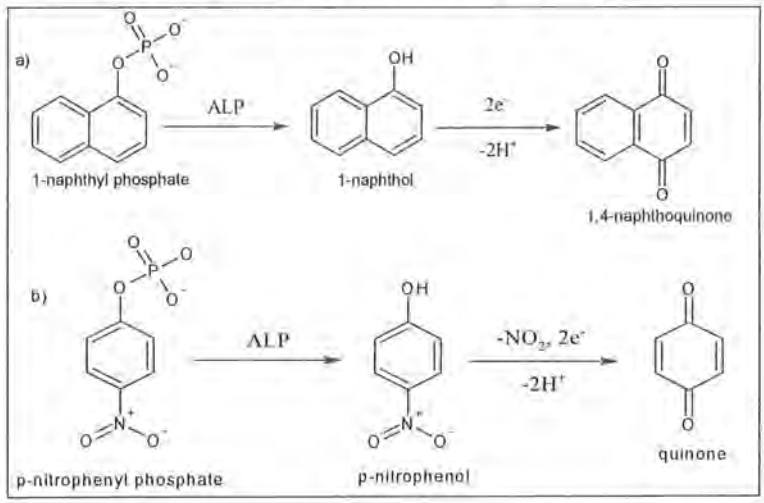


Fig 1.1: Alkaline Phosphatase catalyzed reactions

Substrates: a) 1-naphthyl phosphate, b) *p*-nitrophenyl phosphate.

Alkaline phosphatase (ALP) (E.C 3.1.3.1) is a hydrolase enzyme responsible for removing phosphate groups (dephosphorylation) from many types of molecules, including nucleotides, proteins, and alkaloids. As the name suggests, alkaline phosphatases are most effective in an alkaline environment. In bacteria, alkaline phosphatase is usually located in the periplasmic space to generate free phosphate groups for uptake and use. This hypothesis is in accordance with the fact that alkaline phosphatase is usually secreted by bacteria during the phosphate starvation only (Garen and Levinthal, 1960). The commonly used alkaline phosphatases used in research are; Bacterial alkaline phosphatase (BAP), Shrimp alkaline phosphatase (SAP), and Calf intestinal alkaline phosphatase (CIAP) (Table 1.3). Bacterial alkaline phosphatase however, shows higher stability and the rate of activity.

Bacterial alkaline phosphatase is an active catalyst for the hydrolysis of N-phosphorylated pyridines. Previously, alkaline phosphatase had been found to contain only one zinc (Ahlers, 1975) but Labow *et al*; (1980) found monomers of ALP containing two zinc ions as cofactors that were directly involved in the catalytic reaction. The magnesium ion is another activating cofactor that causes ALP to undergo a conformational change that allows the substrate bind to the active site (Ahlers, 1975).

Table 1.3: Sources of isolation of alkaline phosphatase

	Sources	Localization	References
Bacteria	*E.coli*	Extracellular	Boulanger and Kantrowitz, 2003
	Bacillus sp. P9	Extracellular	Dhaked *et al.*, 2005
	Bacillus subtilis	Extracellular	Hulett *et al.*, 1991
	Arthrobacter D10	Extracelluar	Prada *et al.*, 1996
	Vibrio sp.	Periplasmic	Hauksson *et al.*, 2000
	E. coli	Extracelluar	Boulanger and Kantrowitz, 2003
	Schewanella sp.	Extracelluar	Ishida *et al.*, (1998)
	Pyrococcus abyssi	Extracelluar	Zappa *et al.*, 2001
	P. aeruginosa PA01	Periplasmic	Tan and Worobec, 1993
	HK47 Antarctic bacterium	Extracellular	Kobori *et al.*, 1984
Algae	*Ulva pertusa Kjellm*	homogenate	Yang *et al.*, 2003
Plants	*Pisum sativum*	Thylakoid	Kieleczawa *et al.*, 1992
	Chenopodium rubrum	Extracellular	Chaidee *et al.*, 2007
Fish	*Cyprinus carpio*	Gut/membrane bound	Villanueva *et al.*, 1998
Shrimp	*Pandalus borealis*	Extracellular	Nilsen *et al.*, 2001
Mammals	Brain	Homogenate	Goldstein and Harris, 1981
	Placenta	Homogenate	Skynner *et al.*, 1999
	Liver/Bone/Kidney	Homogenate	Goldstein *et al.*, 1980
	Bile duct	Secretion	Kaplan *et al.*, 2007
	Intestinal mucosa	Homogenate	Fourniere *et al.*, 1995

World market for enzymes in 2009 is $ 5.1 billion and the demand will grow 6.3 percent annually through 2013 (World Enzymes to 2013, 2009). For hydrolases market for hydrolases in 1996 was 2 billion dollar (Godfrey and West, 1996). The diagnostic enzymes world market was reported as 100 million dollar, increasing continuously. Alkaline phosphatase has the biggest market volume (Enzymes for clinical purpose, 2004) share of 20 M dollar. The global market for biosensors and other bioelectronics is projected to grow from 6.1 billion dollar in 2004 to 8.2

billion dollar in 2009, at an AAGR (average annual growth rate) of 6.3% (Biosensor/Bioelectronics Market, 2005). Low temperature efficient alkaline phosphatase can interestingly be used in enzyme biosensors, efficient in cold environments (Mikkelsen, 2004).

Entombed within archives of Karakorum glacial ice cores ranging in age from less than 50 to above 750,000 years, are abundant microbial flora, important with respect to biotechnology. Exploring such a unique niche for novel extremophiles will provide with new experimental models i.e. Bacteria and their enzymes exhibiting activity at stressed conditions. A thermo labile alkaline phosphatase should have higher specific activity and can be used further in electrochemical biosensors (e.g. detection of hazardous chemicals in water bodies at low temperature). Hence, study of a model protein, alkaline phosphatase catalyzing a variety of substrates to colored products, extracted from microbes dwelling at such a stressed environment with scarce amount of energy was proposed.

India (Indo-Pak subcontinent) was tectonically part of Antarctica and Africa 200 million years ago (Redfern, 2001). The chronological biodiversity particularly those which are entombed in ice since millions of years should have conserved DNA bases of ancestors. The exploration of novel psychrophilic bacteria from ice and soil samples expedited from such biogeographically imperative location i.e. ancient glaciers of Pakistan and China border for phylogenetic clues to relate with Continental Drift and Plate Tectonic Theory is first study of its kind. In addition to its high commercial importance, the thermo labile alkaline phosphatase extracted from such microorganisms is uniquely important for its use in enzyme inhibition based electrochemical biosensors for detection of metabolites. The production and characterization of alkaline phosphatase purified from novel isolates from such a niche is a new topic of research and comply high commercial output, and merit for research.

AIM AND OBJECTIVES

The aim of this study was to explore the Karakorum glaciers of Pakistan for isolation of psychrophilic bacteria in view of their potential for biotechnological applications. Following were the objectives of the present study.

1. To obtain samples of ice, water and soil from glaciers of Pakistan.
2. Isolation of psychrophilic and/or psychrotrophic bacteria from these samples.
3. Effect of pH and temperature on the growth of bacteria.
4. Identification of bacteria by macroscopic, microscopic, biochemical (API kits) and 16S rRNA sequencing.
5. Screening of isolates for their potential to produce alkaline phosphatase.
6. To study the effect of various factors (incubation period, pH, temperature, size of inoculum and age of inoculum) on the production of alkaline phosphatase.
7. Purification of alkaline phosphatase through precipitation, dialysis, gel permeation and anion exchange chromatography.
8. To study the effect of various factors (pH, temperature, metals and glycerol) on the activity of purified enzyme.
9. Molecular wt. determination by gel permeation chromatography and SDS-PAGE analysis.
10. To calculate the kinetic constants, K_m and V_{max} using Eadie-Hofstee plot.
11. To check the inhibitory effects of potassium ferricyanide and potassium ferrocyanide on the activity of alkaline phosphatase.

REVIEW *of* LITERATURE

Extremophile is an organism that thrives in extreme condition for life in contrast to a neutrophile or mesophiles that live at neutral pH or moderate temperature, respectively. Extreme environment provide extraordinarily cold, hot, dry, acidic, basic or under immense pressure niche for life. Mostly, extremophiles are microbes present in diverse but numerous genetic lineages of archaea and bacteria. Based upon their habitats, extremophiles receive attributes like; temperature (psychrophiles, thermophiles), pH (neutrophiles, acidophiles, alkaliphiles), salt (halophiles), extremely dry conditions (xerophiles), rocks (endoliths), high sugar contents (osmophiles) and higher atmospheric pressure (piezophiles). Other than their applications as biompass, model organisms to explore novel genes and biochemical pathways, extremophiles are potent sources of metabolites (enzymes, antibiotics, heat and cold shock proteins, antifreeze proteins etc) due to their unique characteristics attained during adaptation to harsh conditions of environment as demonstrated by Burg (2003).

Based upon lower, optimum and upper limit of temperature for growth, Morita, (2001) classified bacteria according to the habitats they thrive in. Psychrophiles usually grow at or below zero (0°C) and have an optimum growth temperature ≤15°C and an upper limit of ≤20°C. In contrast, psychrotrophs, can also grow close to zero, have optima and upper limits above 30°C; hence they could be considered as being cold-tolerant mesophiles (Russell, 2006). Moyer and Morita (2007) defined the psychrophiles as "cold loving extremophilic bacteria or archaea having an optimal temperature (15°C or lower), a maximal temperature (20°C) and a minimal temperature (0°C or lower) for growth whereas psychrotrophs are cold-tolerant bacteria or archaea that have optimal and maximal growth temperatures above 15 and 20°C, respectively. Thermolability and higher specific activity at low cost of energy are important adaptations of psychrophiles.

The millions of years old glaciers have entombed ancient simple life that may be representative of primitive life (like bacteria) on earth, transferred across the other planets (if it existed there). The biomolecules isolated from these bacteria are very important, biotechnologically. Isolates from glacial samples, capable to grow at lower temperature are selected for the present study. A periplasmic protein

"alkaline phosphatase" exclusive to bacteria for restoration of energy was suggested to produce from these isolates. Low temperature active alkaline phosphatase has vast commercial applications.

PSYCHROPHILIC BACTERIA: Sources, Isolation and Characterization

There are three main methods for the detection of microorganisms in different ecosystems: inoculation into nutrient media, direct microscopy, and registration of metabolic activity (Abyzov *et al.*, 2004). Depending on the habitats and sources of isolation, bacteria have been attempted to retrieve and characterize accordingly. Cold active microorganisms can be isolated from cold storage, ice cabins, ice cream freezers, deep freezers, frozen oceans and glaciers.

Ice cabins and freezers

Ice cream and milk containers are potent sources of cold active microorganisms. Shehata and Collins (1971) isolated and characterized four psychrotrophic bacilli from milk samples while (Olofsson *et al.*, 2007) isolated various bacteria including *Pseudomonas* spp. from refrigerated beef. The growth of bacteria is halted in freezer, but not killed (Gidus, 2005). The spoilage agents (psychrotrophs) and food poisoning agents (psychrophiles) are active at 5°C in fridge (Palumbo, 2009).

Unfrozen soils

The sampling methodology for unfrozen and temperate soils is same except the texture of the soil e.g. the Antarctic soil is gravelly and heterogeneous and larger samples are difficult to obtain due to environmental protection limitations (Russell, 2006).

Clouds

Distilled droplets up in air contain bacteria as typhoons, winds, birds and aircrafts can contaminate the upper atmosphere up to acceptable limit for microbial dispersion (Hamilton and Lenton, 1998). Amato *et al.*, (2007) isolated 71 bacterial, 42 fungal and 15 yeast strains from water phase of tropospheric clouds. Bacteria, identified on the basis of their 16S rRNA gene sequence, were found belonging to *Actinobacteria*, *Firmicutes*, *Proteobacteria* (*Alpha*, *Beta* and *Gamma* subclasses) and *Bacteroidetes* phyla, and mainly to the genera *Pseudomonas*,

Sphingomonas, *Staphylococcus*, *Streptomyces*, and *Arthrobacter*. The isolates can grow at low temperatures (5°C): most of these are Gram-negative bacteria, and a few were psychrophiles.

Hamilton and Lenton (1998) suggested the 'phase change catalysis' as means of dispersal for nuclei between their aquatic, terrestrial or epiphytic growth sites. *Pseudomonas syringae*, release heat energy of phase change, thus contributing to local air movements that can be used both for lofting and for lateral dispersal of their producers. Small phytoplankton and bacteria take off from water through bubble-burst processes especially in 'white-caps', these often themselves caused by convective winds. Sattler *et al.*, (2001) isolated bacteria actively growing and reproducing at temperatures at or below 0°C, from supercooled cloud droplets collected from high altitudes.

Water and snow

The methodology for sampling from temperate and cold waters was described by Darwish and Bloomfield (1995). Water samples are filtered through membrane filters of 0.45 µm for vegetative cells or 0.22 µm for spores. Freshly-fallen snow can be collected without extraneous contamination in sterile bags and melted slowly at <5°C prior to filtration as for water samples.

Romanenko (2004) isolated Gram-negative, aerobic, psychrotolerant, non-motile, non-pigmented *Psychrobacter maritimus* sp. nov. and *Psychrobacter arenosus* sp. nov., from coastal sea ice and sediments of the Sea of Japan. Li *et al.*, (2009) analyzed 463 clones using 16S rDNA sequencing technique and identified 13 distinct major lineages of bacteria (α, β, γ, δ and ε-Proteobacteria, Acidobacteria, Bacteroidetes, Chloroflexi, Actinobacteria, Firmicutes, Planctomycetes, Spirochetes, and Verrucomicrobia). The community composition was different among sampling sites, which potentially was related to geochemical differences.

YongQin *et al.*, (2007) found that bacteria were abundant in snow as compared to Antarctic and increased with altitude but had no correlation to chemical parameters. Bacteria in the cryosphere on Mt. Everest were closely related to those isolated from soil, aquatic environments, plants, animals, humans and other frozen environments. The *Cytophaga- Flavobacterium-Bacteroides* (CFB)

group absolutely dominated in glacial meltwater, while β-*Proteobacteria* and the CFB group dominated in serac ice, and β-*Proteobacteria* and *Actinobacteria* dominated in surface snow.

Permafrost soils and ice

A number of protocols have been developed for collecting and handling permafrost soils and ice cores, and for their decontamination (Rogers *et al.*, 2004; Christner *et al.*, 2005). Christner *et al.*, (2006) discussed the limnological conditions and possible sampling measures from subglacial Lake Vostok of Antarctica, located ~4 km beneath the surface of the East Antarctic Ice Sheet and have been isolated from the atmosphere since 1.5 million years. A low temperature active, halophilic *Psychrobacter* sp. was isolated from ancient Siberian permafrost (Zheng *et al.*, 2006).

Sinclair and Stokes (1964) Isolated obligately anaerobic psychrophilic bacteria from soil, mud, and sewage. The organisms grew well at 0°C in liquid and on solid media, and grow only in the complete absence of oxygen. On the basis of shape, sporulation, flagellation, and strictly anaerobic growth, all of the organisms were classified as Clostridia.

Bai *et al.*, (2006) reported the culturable bacteria from alpine permafrost in the Tianshan Mountains, northwestern China. Aerobic $2.5–6.0\times10^5$ CFU/gdw (CFU per 1 gram of dry weight) on modified PYGV medium were recovered from alpine permafrost samples at 4°C; with different morphotypes such as morphology, colony pigmentation, Gram staining, endospore formation and temperature range of growth. The most abundant and diverse isolates were members of Gram-positive bacteria, particularly the *Arthrobacter* as a dominant group in alpine permafrost culturable populations.

Extraterrestrial sources

Samples obtained from other cold planets, belongings of astronauts and space shuttles in contact with extraneous environment, and meteorites may be explored for isolation of psychrophiles. In filterate of meltwater from Europa's ice, microbial life could exist up to detectable limit (Chyba, 2000).

Glaciers

A glacier is a large mass of moving ice that flows slowly over the land. Glaciers are usually permanent (on a human time scale, because nothing on the Earth is really permanent) body of ice, consisting largely of re-crystallized snow, that shows evidence of down slope or outward movement due to the pull of gravity. The bacteria in dormant and vegetative forms are present under ice of glacier and adapt the ecosystem with any one or combination of different mechanisms of adaptation (Miteva, 2008). During the process of glaciation (formation of glaciers), small droplets nuclei, airborne, dust, mud and snow; all contribute to add the spores, microbes or their vegetative structures which are entombed for years. Some of the compromised candidates adapt this ecosystem to survive at such stress of the environment.

Cryosphere; the part of Earth that remains below the freezing point of water, is majorly formed by glaciers like Arctic, Antarctic and mountain glaciers (McNamara and Kane, 2009).

Polar glaciers

Christner and Priscu (2008) believe that lakes under Antarctic sheets may contain some of the most unusual, extreme microbial ecosystems on Earth. Junge *et al.*, (2004) studied the bacterial abundance, activity and diversity of *Cytophaga-Flavobacteria-Bacteroides* (CFB), and *Archaea,* at -2 to -20C in winter ice samples of Arctic sea. Tindall (2004) studied the diversity in the ice and water samples of Iceberg, Antarctica; using direct cultivation and molecular techniques.

Sogin *et al.*, (2006) studied the microbial diversity of North Atlantic and diffuse flow hydrothermal vents, a very ancient"rare biosphere" that may represent a nearly inexhaustible source of genomic innovation. Shivaji (2005) characterized the *Psychrobacter vallis* sp. nov. and *Psychrobacter aquaticus* sp. nov., isolated from Antarctica. Zhang *et al.*, (2008) obtained ice samples from Skavrvsnes, Antarctic Lakes, isolated bacteria at 4°C and 20°C, and characterized them, using 16S rDNA sequence analysis. Phylogenetically, the isolates were members of *Flavobacteria*, *Bacillus*, *Actinobacteria*, *α-proteobacteria*, and *γ-proteobacteria*.

Psychrophilic *Leifsonia rubra* sp. nov. and *Leifsonia aurea* sp. nov. were isolated by Reddy (2003) from a pond in Antarctica. Bowman (1997) studied the diversity and association of pscychrophilic bacteria in Antarctic Ice using 16S rRNA analysis. Overall, four phylogenetic groups, the alpha and gamma subdivisions of the *Proteobacteria*, the Gram-positive branch, and the *Flexibacter-Bacteroides-Cytophaga* phylum were found. The 16S rDNA sequence analysis revealed that psychrophilic strains belonged to the genera *Colwellia*, *Shewanella*, *Marinobacter*, *Planococcus*, and novel phylogenetic lineages adjacent to *Colwellia* and *Alteromonas* and within the *Flexibacter-Bacteroides- Cytophaga* phylum. Psychrotrophic strains were found to be members of the genera *Pseudoalteromonas*, *Psychrobacter*, *Halomonas*, *Pseudomonas*, *Hyphomonas*, *Sphingomonas*, *Arthrobacter*, *Planococcus*, and *Halobacillus*.

Minna (2006) characterized the psychrotolerant heterotrophic bacteria from Finnish Lapland including forest soil, arctic alpine-tundra soil, stream water, lake and mire sediments, lichens and snow algae. Whole cell fatty acid, 16S rRNA gene sequence analysis and microscopy indicated that most of the isolates were members of α-, β-, γ-Proteobacteria, Gram-positives with low G+C content, Actinobacteria and the Cytophaga/Flexibacter/Bacteroides group. The frequently isolated Gram negative bacteria include *Pseudomonas* sp., *Burkholderia* sp., *Collimonas* sp., *Pedobacter* sp., *Janthinobacter* sp., *Duganella* sp., *Dyella* sp. and *Sphingomonas* sp.

Bozal (2003) characterized two novel psychrobacter strains (*Psychrobacter luti* sp. nov. and *Psychrobacter fozii* sp. nov.) isolated from Antarctic environments. The isolates were oxidase-positive, halotolerant, Gram-negative, non-motile coccobacilli with a strictly oxidative metabolism. The DNA G+C content ranged from 44 to 47 mol%.

Major microbial communities from Antarctic sea ice were isolated and studied by (Garrison *et al.*, 1986) however the mechanisms for cold adaptations were explored by (Nichols *et al.*, 2004) who attributed the cold adaptation of *Methanococcoides burtonii* to unsaturation of its lipid contents in membrane. The

structural adaptation is important for cold-activity of enzymes (Sheridan *et al.*, 2000).

Bacteria were retrieved from Guliya ice cap (750000 years) and Lake Vostok accretion ice (420,000 years) old samples. Based on 16S rDNA Sequences, the isolates were found as members of the α- and β-proteobacteria, actinobacteria and low-G+C Gram-positive bacteria; and *Brachybacteria*, *Methylobacterium*, *Paenibacillus* and *Sphingomonas*, respectively. The direct amplification of 16S rDNA from Guliya and Lake Vostoc ice melt water evidenced the presence of *Pseudomonas* and *Acinetobacter* γ- proteobacterial; and α- and β-proteobacteria, low- and high-GC Gram-positive bacteria and a member of the *Cytophaga/Flavobacterium/Bacteroides*, respectively (Christner *et al.*, (2002; 2003; 2008).

Borriss *et al.*, (2003) isolated and characterized marine psychrophilic phage-host systems from Arctic sea ice. On the basis of 16S rDNA sequences, the three bacterial phage hosts exhibited the greatest similarity to the species *Shewanella frigidimarina* (96.0%), *Flavobacterium hibernum* (94.0%), and *Colwellia psychrerythraea* (98.4%), respectively. Sheridan *et al.*, (2000) studied the structural basis of low temperature enzyme activity. They determined low temperature activity by comparing the enzymes having different optima, purified from phylogenetically related organisms.

Siegert *et al.*, (2001) explained the physical, chemical and biological processes in Lake Vostok and other Antarctic subglacial lakes (over 70 identified so far) beneath the Antarctic ice sheet. The hypothetical lake water chemistry has revealed small quantities of microbes. These findings suggest that Lake Vostok is an extreme, yet viable, environment for life. All subglacial lakes are subject to high pressure (~350 atmospheres), low temperatures (about -3°C) and permanent darkness. Microbes found in Lake Vostok's accreted ice are relatively modern, but the probability of ancient lake-floor sediments leads to a possibility of a very old biota at the base of subglacial lakes.

Bacterial density is two to sevenfold higher in accretion ice than the overlying glacial ice, implying that Lake Vostok is a source of bacterial carbon beneath the

ice sheet. Accretion ice has phylotypes from β-, γ-, and δ-Proteobacteria. Although the exact nature of the biology within Lake Vostok awaits direct sampling of the lake water, the data from the accretion ice support the working hypothesis that "a sustained microbial ecosystem is present in this subglacial lake environment, despite high pressure, constant cold, low nutrient input, potentially high oxygen concentrations, and an absence of sunlight" (Christner *et al.*, 2006).

Denner *et al.*, (2001) isolated a strictly aerobic, Gram-negative, oxidase-positive, psychrotrophic, halotolerant, non-motile and coccobacillus, *Psychrobacter proteolyticus* sp. nov., from the Antarctic Krill *Euphausia superba* Dana, excreting a cold-adapted metalloprotease. The G+C content of the DNA was 43.6 mol%. The isolate differentiated from *P. glacincola* based upon tolerance to 5% bile salts, nitrate reduction, citrate utilization, acid production from carbohydrates, alkaline phosphatase, acid phosphatase, C4 esterase, C14 lipase and valine arylamidase. Bowman (1998) isolated a psychrotrophic, halotolerant bacterium, *Pseudoalteromonas prydzensis* sp. nov., from Antarctic sea ice and possess a DNA G+C content of 38-39 mol%. A few isolates were recognized by phenotypic, DNA-DNA hybridization and 16s rRNA-based phylogenetic studies to represent a distinct genospecies clustering at the periphery of the non-pigmented *Pseudoalteromonas* species clade.

Irina *et al.*, (2007) studied the bacterial diversity of a deep ice bore hole (3650m) at east of Antarctica above vostok and defined the contamination free sampling method. The two dominant out of six phylotypes were members of *Sphingomonas* genus. Chattopadhyay and Jagannadham, (2001) found that enhanced biosynthesis of some fatty acids that increase membrane fluidity, and also of polar carotenoids has been evidenced in one Gram-positive and one Gram-negative psychrotrophic bacterium, isolated from Antarctic soil.

Non Polar Glaciers

During study of glacial history of Batura glaciers, Meiners (2005), found that the great Karakorum main ridge retreated about 8 kilometers at Kukuar and Baltar glaciers (Pakistan) since 1915 till to date. The geomorphological and glacial perspective of the Batura morain history, confirm a late to high glacial connection of the Bar glacier to a Hunza glacier, as postulated by Kuhle (2005).

Kim *et al.*, (2008) isolated an aerobic, motile, Gram-negative, ice-active substance-producing, rod-shaped psychrophilic bacterium *Moritella dasanensis* sp. nov., from a glacier in Kongsfjorden, Svalbard Archipelago, Norway. Isolate grew optimally at 9 °C, with a maximum temperature for growth of 18 °C. The genomic DNA G+C content was 46.9 mol%. Isolate was validated based on DNA–DNA relatedness, physiological and biochemical tests and ice-controlling activity, where it was found different, genetically and phenotypically.

Segawa (2005) studied the bacterial diversity in snow of Tateyama Mountains, Japan, using ARDRA and 16S rRNA Gene Sequence Analysis. The samples of snow contained psychrophilic bacterium, *Cryobacterium psychrophilum*, and two psychrotrophic bacteria, *Variovorax paradoxus* and *Janthinobacterium lividum*. The growth of the isolates was increased along with melting season and nutrients availablity. All three of these are also reported to be present in a glacier in Antarctica and a Greenland ice core, they seem to be specialized members of the snow biota that are distributed in snow and ice environments in various parts of the world.

Bacterial diversity in the snow over Tibetan Plateau Glaciers (Guoqu, Zadang, East Rongbuk and Palong No. 4) was investigated by Liu *et al.*, (2009) through culture-independent molecular analysis of 16S rRNA gene clone library and flow cytometry approaches. Bacterial diversity found, included 15 common genera distributed widely among the glaciers. Campen *et al.*, (2003) studied the microbial consortia metabolizing within South American mountain glacier. They studied the gravitational fractionation, thermal fractionation, gases trapped in refrozen meltwater, atmospheric heterogeneity, abiotic chemical reactions, and biologically mediated chemical reactions and its relation to metabolism of a consortium of microorganisms within the ice.

The cultivation-independent and -dependent characterization of bacteria from John Evans Glacier (Canada) was studied by Foght *et al.*, (2004). The 16S rRNA gene RFLPs of 341 clones were consisted of β-proteobacteria (25% of clones, particularly Comamonadaceae) Bacteroidetes (23%, particularly *Flavobacterium* spp.) and Actinobacteria (4%). A second water sample had 51% β-

proteobacteria, 5% Bacteroidetes and no Actinobacteria, and a sediment sample was dominated by β-proteobacteria (15%) and Bacteroidetes (38%).

Zhu *et al.*, (2003) isolated novel psychrophilic *Flavobacterium xinjiangense* sp. nov. and *Flavobacterium omnivorum* sp. nov., from the China No. 1 glacier. Strains were Gram-negative and both had an optimal growth temperature of 11°C. Strain *F. xinjiangense* was able to grow at 0–20 °C, the G+C content of its genomic DNA is 34.4 mol% and the major fatty acids of *F. xinjiangense* were C16 : 1ω7c (17.7%) and C15 : 1ω6c (12.7 %). Strain *F. omnivorum* showed a strong ability to degrade organic macromolecules such as starch, CM-cellulose, pectin and chitin. Its DNA G+C content is 35.1 mol%, and the major fatty acids are C16: 1ω7C (18.2%) and C15: 0 (9.9 %).

The four facultatively psychrophilic, Gram negative, aerobic straight amphitrichous rods were isolated by Yumoto *et al.*, (2009) from Rumoi, Hokkaido, Japan. The isolates were catalase-, oxidase-, nitrate, gelatinase positive but amylase negative, and able to grow at 4°C, but not at 40°C. The 16S rRNA sequencing indicated that the bacteria were belonging to the genus *Pseudomonas*. The type strains were named according to the origin of samples.

Christner *et al.*, (2001) isolated and identified bacteria from ancient and modern ice core that range from 5 to 20,000 years in age and that originating from both polar and non-polar regions. Direct plating of melt-water from a 200-year old sample of ice from the Guliya ice cap on the Tibetan Plateau (China) generated ~180 bacterial colonies per ml whereas melt water from late Holocene ice from Taylor Dome in Antarctica contained only 10 cfu/ml, and <10 cfu/ml were present ice of the same age from the Antarctic Peninsula and from Greenland. Mostly, the isolates were *Bacillus* and *Actinomycete* genera when identified using 16S rDNA analysis.

The immured bacteria from Guliya ice cap on the Tibetan Plateau (China) (>180 cfu ml^{-1}) and Sajama (Bolivia) (>20 cfu ml^{-1}) were recovered which is higher due to the closer proximity of major biological ecosystems. The 16S rDNAs analyses revealed that most of isolates belonged to spore-forming *Bacillus* and *Actinomycetes* species, or to non-sporulating Gram positive bacteria (Christner,

2000). The diversity of life in glassy margins of ocean floor basalt was studied by Thorseth (2001). The 16S rRNA analysis shows that bacteria belong to γ- and ε-Proteobacteria and the Cytophaga/Flexibacter/Bacteroides subdivision of the Bacteria, while the Archaea all belong to the Crenarchaeota kingdom. Their closest relatives belong to Arctic and Antarctic, deep-sea sediments and hydrothermal environments.

The diversity and distribution of alkaliphilic psychrotolerant bacteria in the permafrost region of Qinghai–Tibet Plateau were examined by Zhang *et al.*, (2007). The study revealed that viable bacteria on modified PYGV agar were varied between 102 and 105 CFU/g of dry soil. Isolates fell into three categories: high G + C gram positive bacteria (82.3%), low G + C Gram positive bacteria (7.2%), and Gram negative proteobacteria (10.5%). The strains could grow at pH (6.5 to 10.5) and below 37°C with optimum pH (9–9.5) and temperatures (10 to 15°C).

Baghela (2005) studied the psychrotrophic proteolytic bacteria from Gangotri Glacier, Western Himalaya, India. Liu *et al.*, (2001) studied diversity of culturable bacteria from East Rongbuk Glacier melt water and found that the major micro biota included members of α, β, and γ -Proteobacteria, Actinobacteria, and Firmicutes. Based upon Biolog bioassay and growth test patterns, the surviving culturable bacteria could be divided into three categories: Group I (sensitive to temperature change but versatile in utilization of carbon substrates; Group II (tolerant to variable temperature and less capable of carbon utilization); and Group III (slow in growth and weak in carbon utilization).

Isolates from Malan Ice Cap (Tibet) indicated that micro flora consisted of α, β, and γ -Proteobacteria, and the LGC, HGC, and CFB group by means of the results of 16S rRNA sequence analysis and physiological characteristics. Microbial population varied along the depth of glacier core, morphologically, that was correlated to climatic and environmental changes directly (Yao *et al.*, 2006).

The 16S rDNA analyses of the bacteria isolated from refrigerated beef indicated *Pseudomonas* spp. as the dominating group (99% of the population-sequences). *Pseudomonas panacis/Pseudomons brennerii* was the dominating taxon (99%

similarity to type strain), but sequences with highest similarity to *Pseudomonas lundensis* (99%), *Pseudomonas beteli* (99%) and *Pseudomonas koreensis* (100%) were also found (Olofsson, 2007).

COPING WITH COLD: Metabolism and adaptations for psychrophily

Psychrophiles owe the ability to cope with the challenges like, reduced enzyme activity; decreased membrane fluidity; altered transport of nutrients and waste products; decreased rates of transcription, translation and cell division; protein cold-denaturation; inappropriate protein folding; and intracellular ice formation, to survive and proliferate in such conditions. Cold-adapted organisms have successfully evolved features, genotypic and/or phenotypic, to surmount the negative effects of low temperatures and to enable growth in these extreme environments (D'Amico *et al.*, 2006). Psychrophiles use the freezing ability as their energy source for metabolism at low temperatures. The biochemical and molecular properties that allow psychrophiles and psychrotrophs, to survive and grow in extremely cold conditions are increasingly well characterized (Jermy, 2008).

The role of the cell membrane in survival of bacteria and archaea living in extreme environments were examined by Konings (2002) and concluded that proton and sodium permeabilities of all biological membranes increase with the temperature and vice versa. Psychrophilic and mesophilic bacteria, and mesophilic, hyper-/thermophilic and halophilic archaea are capable of adjusting the lipid composition of their membranes in such a way that the proton permeability at the respective growth temperature remains low and constant (homeo-proton permeability). Transport of solutes across the bacterial and archaeal membrane is mainly catalyzed by primary ATP driven transport systems or by proton or sodium motive force driven secondary transport systems.

The mechanism of molecular adaptation in psychrophiles and their biotechnological applications was discussed by Russell (1998). To survive in cold environments, psychrophilic bacteria have modified; membranes, energy-generating systems, protein synthesis machinery, biodegradative enzymes and the components responsible for nutrient uptake. The applications of metabolites of pcychrophiles include; use of cold-active enzymes in the detergent and food

industries, in specific biotransformation and environmental bioremediations, to specialized uses in contact lens cleaning fluids and reducing the lactose content of milk; ice-nucleating proteins have potential uses in the manufacture of ice cream or artificial snow; for lipids, the uses include dietary supplements in the form of polyunsaturated fatty acids from some Antarctic marine psychrophiles. Deming (2002) commented on molecular adaptation, biodiversity and microbial dynamics in the cold, along with the concept of eutectophiles (phase of change of water to ice).

The starvation conditions may be a significant factor in providing thermal tolerance as a survival mechanism in a psychrophilic marine bacterium, *Vibrio* Ant-300 (Janet, 1993). The genome sequence analysis of Antarctic *Pseudoalteromonas haloplanktis* reveals that increased solubility of oxygen at low temperature is due to dioxygen scavenging while deleting whole pathways producing reactive oxygen species (Medigue *et al.*, 2005).

Knight and Duman (1986) proposed that that AFP (antifreeze proteins) inhibits ice recrystallization in the extracellular regions during the latter stages of the warming cycle. Carpenter and Hansen (1992) demonstrated that antifreeze proteins modulate cell survival during cryopreservation. Relatively high concentrations of AFP (1.54 mg/ml) are much more effective at inhibiting extracellular recrystallization. The proteins specific to growth at 4°C versus optimum temperature (23°C) indicated that growth at 'optimal' temperatures was stressful for the cold-adapted Antarctic *Methanococcoides burtonii* (Goodchild *et al.*, 2004). *Colwellia psychrerythraea* thrives in the colder regions of the seas (Holden *et al.*, 2005).

The mechanism of cold-adaptation has been obtained from investigations on some psychrotrophic bacterial strains, isolated from the Schirmacher oasis of Antarctica (Chattopadhyay 1995). Other mechanisms of maintaining an optimum fluidity involve reduction in the acyl chain length and synthesis of branched-chain fatty acids (Suutari and Laakso 1994; Klein *et al.* 1999). A marked deficiency of the branched chain fatty acid was also observed in cold sensitive mutants (Annous *et al.* 1997).

The *Photobacterium profundum* and *M. roseus* exhibited enhanced synthesis of membrane fluidizing fatty acids (branched-chain and unsaturated fatty acids) (Allen *et al.* 1999). Synthesis of unsaturated fatty acids was also enhanced when the two (Gram positive and negative) organisms were grown at lower temperature (Jagannadham 1998; Jagannadham *et al.* 2000). The homeoviscous adaptation of membrane fluidity (Sinensky 1974) in Antarctic bacteria at low temperature is ensured by enhanced biosynthesis of some fatty acids that increase membrane fluidity.

Chintalapati (2004) studied the fluidity of the membrane by special fatty acids, in cold adaptation of psychrophiles. Other strategies include; by altering the lipid head group, the protein content of the membrane, the type of carotenoids synthesized, the fatty acid chain length and the proportion of *cis* to *trans* fatty acids and a two-component signal transduction pathway. Bakermans (2003) studied the isolation and metabolism of spore forming, Gram positive and Gram negative (-10°C) psychrophiles from Siberian permafrost. The cells capable of growing at -10°C exhibited morphological changes at the ultra-structural level.

Sabri *et al.*, (2007) studied the effect of temperature on growth of psychrophilic and psychrotrophic members of *Rhodotorula aurantiaca*. The thermodependence of growth kinetic parameters was investigated for the Antarctic psychrophilic strain *Rhodotorula aurantiaca* and a psychrotrophic strain of the same species isolated in Belgium (Ardennes area).

Awano (2007) studied the *csdA* mutant, cold sensitive *E. coli*, and its correlation to induction of cold shock proteins by downshift of temperature from 37°C to 15°C. Another gene RhlE, an RNA helicase can act as a counter part to *csdA* protein activity. Two other cold shock-inducible proteins, namely, CspA, an RNA chaperone, and RNase R, an exonuclease, can also complement the cold shock function of *csdA*.

ENZYMES FROM PSYCHROPHILES

The major driving force for isolation of psychrophiles is low temperature biotechnology (Margesin and Schinner, 1999), either as living organisms (environmental biosensors for example) or their isolated enzymes (washing

powders for example). Psychrophiles have a broad scope and applications in industries, including those concerned with food production, waste processing, mining, environmental bioremediations, specialty chemicals, agriculture, and medicine and molecular diagnostics (Russell, 2006). Key enzymes of metabolic pathways of psychrophiles have interesting properties (Gerday *et al.*, 1997).

The cold-active or cold adapted enzymes may be produced by organisms existing in permanently cold habitats located in polar zones, at high altitudes or in the deep sea (Nichols and McMeekin, 2001). These enzymes provide opportunities to study the adaptation of life to low temperature and the potential for biotechnological exploitation (Aguilar 1996; Morita *et al.*, 1997). Applications exist in a range of industries for cold-active enzyme applications, e.g. cleaning agents, leather processing, degradation of xenobiotic compounds in cold climates, food processing (fermentation, cheese manufacture, bakery, confectionery, meat tenderization) and molecular biology (heterologous gene expression) (Margesin and Schinner, 1999). Cold-active enzymes typically have maximal catalytic activity at temperatures below 40°C and usually display some degree of thermolability.

A number of psychrophilic microorganisms have been studied for the production and characterization of different enzymes, previously (Table 2.1).

Table 2.1. Enzymes From Psychrophilic Microorganisms

Enzymes	Microorganisms	References
adenylate kinase	*Bacillus globisporus*	Bae and Phillips, (2004).
alanine dehydrogenase	PA-43	Irwin *et al.*, (2001; 2003)
alanine dehydrogenase	*Shewanella* sp. strain Ac10	Galkin *et al.*, (1999)
alanine dehydrogenase	*Carnobacterium* sp. strain St2	Galkin *et al.*, (1999)
alanine racemase	*Bacillus psychrosaccharolyticus*	Okubo *et al.*, (1999; 2000)
alcohol dehydrogenase	*Moraxella* sp. TAE123	Tsigos *et al.*, (1998), Papanikolau *et al.*, (2005)
alcohol dehydrogenase	*Flavobacterium frigidimaris* KUC-1	Kazuoka *et al.*, (2007)
aldehyde dehydrogenase	*Cytophaga sp. KUC-1*	Yamanaka *et al.*, (2002)
alkaline phosphatase	*Vibrio* sp.	Hauksson *et al.*, (2000), Asgeirsson *et al.*, (2001)
alkaline phosphatase	*Arthrobacter* sp. Strain D10	De Prada and Brenchley (1997)

alkaline phosphatase	*Shewanella* sp.	Ishida *et al.*, (1998), Murakawa *et al.*, (2002)
alkaline phosphatase	Strain TAB5	Rina *et al.*, (2000), Wang *et al.*, (2007)
alkaline phosphatase	*Vibrio* sp.	Hauksson *et al.*, (2000), Asgeirsson *et al.*, (2001)
alkaline phosphatase	Strain HK47	Kobori *et al.*, (1984)
alpha-amylase	*Pseudoalteromonas haloplanktis*	Aghajari *et al.*, (1998), D'Amico *et al.*, (2001; 2002; 2003; 2005; 2006 and 2007)
aspartase	*Cytophaga* sp.KUC-1	Kazuoka *et al.*, (2003)
aspartate aminotransferase	*Pseudoalteromonas haloplanktis* TAC125	Birolo *et al.*, (2000)
aspartate carbamoyltransferase	*Moritella profunda*	De Vos *et al.*, (2005; 2007)
ATPase (membrane-bound)	*Vibrio sp.* strain ABE-1 (*Colwellia maris* strain ABE-1)	Harashima *et al.*, (1996)
β -galactosidase	*Arthrobacter psychrolactophilus* F2 (soil Japan)	Nakagawa *et al.*, (2006)
β -galactosidase	*Arthrobacter* sp. C2-2	Karasova-Lipovova *et al.*, (2003) Skalova *et al.*, (2005)
β-galactosidase	*Pseudoalteromonas* sp. TAE 79b	Fernandes *et al.*, (2002)
β -galactosidase	*Pseudoalteromonas haloplanktis* TAE79	Hoyoux *et al.*, (2001)
β -galactosidase	*Pseudoalteromonas* sp. 22b	Turkiewicz *et al.*, (2003; 2003), Cieslinski *et al.*, (2005), Makowski *et al.*, (2006)
β -galactosidase	*Planococcus* sp.	Sheridan *et al.*,(2000)
β -galactosidase	*Carnobacterium piscicola* BA	Coombs and Brenchley, (1999)
β -galactosidase	*Arthrobacter* sp. SB	Coker *et al.*, (2003), Coker and Brenchley (2006)
β -glucosidase	*Paenibacillus* sp. strain C7	Shipkowski and Brenchley (2005)
β -lactamase	*Shewanella frigidimarina*	Poirel *et al.*, (2005)
β -lactamase	*Shewanella livingstonensis*	Poirel *et al.*, (2005)
β -lactamase (class C)	*Psychrobacter immobilis* A8	Feller *et al.*, (1995)
β -lactamase (class C)	*Psychrobacter immobilis* A5	Feller *et al.*, (1997)
Catalase	*Vibrio salmonicida*	Lorentzen *et al.*, (2006) Riise *et al.*, (2006; 2007)
Catalase	*Vibrio rumoiensis* S-1	Ichise *et al.*, (2000), Yumoto *et al.*, (2000)
cellulase	*Pseudoalteromonas haloplanktis*	Violot *et al.*, (2005), Garsoux *et al.*, (2004)

cellulase	*Pseudoalteromonas* sp. DY3	Zeng *et al.*, (2006)
chitinase A	*Arthrobacter* sp. TAD20	Lonhienne *et al.*, (2001), Mavromatis *et al.*, (2003)
chitinase B	*Arthrobacter* sp. TAD20	Lonhienne *et al.*, (2001), Mavromatis *et al.*, (2003)
chitobiase	*Arthrobacter* sp. TAD20	Lonhienne *et al.*, (2001)
citrate synthase	*Arthrobacter* sp. strain DS2-3R	Gerike *et al.*, (1997; 1998; 2001), Russell *et al.*, (1998), Kumar (2004), Spiwok *et al.*, (2007)
dihydrofolate reductase	*Moritella profunda*	Xu *et al.*, (2003)
DNA ligase	*Pseudoalteromonas haloplanktis*	Georlette *et al.*, (2000), Georlette *et al.*, (2003)
DNA-polymerase	*Cenarchaeum symbiosum*	Schleper *et al.*, (1997)
endonuclease	*Vibrio salmonicida*	Altermark *et al.*, (2007)
esterase	*Psychrobacter* sp. ANT300	Kulakova *et al.*, (2004)
esterase	*Pseudomonas* sp. strain B11-1	Suzuki *et al.*, (2003)
esterase	*Acinetobacter* sp. strain No. 6	Suzuki *et al.*, (2002)
esterase	*Acinetobacter* sp. strain No. 6	Suzuki *et al.*, (2002)
fructose-1,6-bisphosphate aldolase	*Vibrio marinus*	Jones *et al.*, (1979)
glutamate dehydrogenase (NAD+)	*Psychrobacter* sp. TAD1	Di Fraia *et al.*, (2000), Camardella *et al.*, (2002)
glutamate dehydrogenase (NADP+)	*Psychrobacter sp. TAD1*	Camardella *et al.*, (2002)
histidinol dehydrogenase	*Bacillus psychrophilus* A.T.C.C. 23304	Lindsay *et al.*, (1977)
iron superoxide dismutase	*Marinomonas* sp. NJ522	Zheng *et al.*, (2006)
isocitrate dehydrogenases	*Colwellia psychrerythraea*	Maki f *et al.*, (2006)
isocitrate dehydrogenases	*Colwellia psychrerythraea*	Maki f *et al.*, (2006)
isocitrate dehydrogenases	*Colwellia maris* strain ABE-1 (*Vibrio* sp.strain ABE-1)	Ishii *et al.*, (1987; 1993)
isocitrate dehydrogenases	*Colwellia maris* strain ABE-1 (*Vibrio* sp.strain ABE-1)	Ishii *et al.*, (1987; 1993)
isocitrate lyase	*Colwellia maris*	Watanabe *et al.*, (2001; 2002; 2004)
isocitrate lyase	*Colwellia psychrerythraea*	Watanabe *et al.*, (2002)
Lipase	*Pseudomonas* sp. strain KB700A	Rashid *et al.*, (2001)
Lipase	*Pseudomonas* sp. strain B11-1	Choo *et al.*, (1998)
L-threonine dehydrogenase	*Cytophaga* sp. KUC-1	Kazuoka *et al.*, (2003)

malate dehydrogenase	*Aquaspirillium articum*	Kim *et al.*, (1999), Spiwok *et al.*, (2007)
malate dehydrogenase	PA-43	Irwin *et al.*, (2001)
malate dehydrogenase	*Moritella* sp (Vibrio sp) 5710	Ohkuma *et al.*, (1996), Saito *et al.*, (2004)
malate dehydrogenase	*Flavobacterium frigidimaris* KUC-1	Oikawa *et al.*, (2005)
malate dehydrogenase	*Moritella sp.* strain 2D2	Saito *et al.*, (2004; 2006)
malate synthase	*Colwellia maris*	Watanabe *et al.*, (2001)
ornithine carbamoyltransferase	*Moritella abyssi*	Xu *et al.*, (2003)
pectate lyase	*Pseudoalteromonas haloplanktis* strain ANT/505	Truong *et al.*, (2001)
pectate lyase	*Pseudoalteromonas haloplanktis* strain ANT/505	Truong *et al.*, (2001)
phosphatase (protein-tyrosine phosphatase)	*Shewanella* sp.	Tsuruta and Aizono (1999; 2000; 2003), Tsuruta *et al.*, (1998; 2005)
phosphatidylcholine-hydrolysing phospholipase C	*Pseudomonas fluorescens*	Preuss *et al.*, (2001)
phosphoglycerate kinase	*Pseudomonas* sp. TACII18	Bentahir *et al.*, (2000), Mandelman *et al.*, (2001), Zecchinon *et al.*, (2005)
protease (alkaline metalloprotease)	*Pseudomonas* sp. strain TAC II 18	Villeret *et al.*, (1997), Chessa *et al.*, (2000), Aghajari *et al.*, (2003), Ravaud *et al.*, (2003) Spiwok *et al.*, (2007)
protease (aminopeptidase)	*Colwellia psychrerythraea* 34H	Huston *et al.*, (2004)
protease	*Yersinia ruckeri*	Secades and Guijarro (1999)
protease	*Xanthomonas maltophilia*	Margesin and Schinner (1991)
protease MPC-01	*Pseudoaltermonas* sp. SM9913	Chen *et al.*, (2003)
protease MPC-02	*Pseudoaltermonas* sp. SM9913	Chen *et al.*, (2003)
protease	*Pseudomonas fluorescens* 164/03	Margesin and Schinner (1992)
protease	*Pseudomonas fluorescens* 177/30	Margesin and Schinner (1992)
protease	*Pseudomonas fluorescens* strain 165/14	Margesin and Schinner (1992)
protease	*Pseudoalteromonas issachenkonii* UST041101-043	Hairong *et al.*, 2007
protease Fpp1 (metalloprotease)	*Flavobacterium psychrophilum*	Secades *et al.*, (2001)
protease Fpp2 (metalloprotease)	*Flavobacterium psychrophilum*	Secades *et al.*, (2003)
protease (metalloprotease)	*Pseudomonas fluorescens* 114	Hamamoto *et al.*, (1994), Kumeta *et al.*, (1999)
protease (metalloprotease)	*Pseudomonas fluorescens*	Margesin and Schinner (1992)
protease (proteinase k-like)	*Serratia* sp.	Helland *et al.*, (2006), Larsen *et al.*, (2006)
protease (serine alkaline protease)	*Pseudomonas* sp. strain DY-A	Zeng *et al.*, (2003)
protease (serine peptidase)	PA-43	Irwin *et al.*, (2001)
protease (serine protease)	*Colwellia* sp. NJ341	Wang *et al.*, (2005)
protease (subtilisin)	*Bacillus sp.* strain TA41	Davail *et al.*,(1994), Miyazaki *et al.*, (2000)

protease (subtilisin)	*Bacillus sp.* strain TA39	Narinx *et al.*, (1992;1997), Tindbaek *et al.*, (2004)
protease (subtilisin-like protease)	*Pseudoalteromonas* sp. AS-11	Dong *et al.*, (2005)
protease (subtilisin-like serine proteinase)	*Vibrio* sp. PA-44	Arnorsdottir *et al.*, (1999; 2002; 2005)
RNA polymerase	*Shewanella violacea* DSS12	Kawano *et al.*, (2005)
RNA polymerase	*Pseudomonas syringae* Lz4W	Uma *et al* (1999)
superoxide dismutase	*Pseudoalteromonas haloplanktis* TAC125	Castellano *et al.*, (2006)
triose phosphate isomerase	*Vibrio marinus*	Alvarez *et al.*, (1998)
triosephosphate isomerase	*Moraxella* sp. TA137	Rentier-Delrue *et al.*, (1993)
triosephosphate isomerase	*Clostridium sp.* strain 69	Shing (1972; 975)
uracil-DNA glycosylase	Strain BMTU 3346	Sobek *et al.*, (1996), Jaeger *et al.*, (2000)
valine dehydrogenase	*Cytophaga* sp. KUC-1	Oikawa *et al.*, (2001)
xylanase	*Pseudoalteromonas* sp. strain TAH3a	Collins *et al.*, (2002; 2003; 2005; 2005; 2006), Petegem *et al.*, (2002; 2003), Dutron *et al.*, (2004), De Vos *et al.*, (2006), Spiwok *et al.*, (2007)

Recent research has focused on determining the structural characteristics which confer cold adaptation in enzymes. The tertiary and quaternary structures of cold-active enzymes have more open and flexible arrangements, thus providing better access of substrates to the active site at lower temperatures. Rigid secondary structures and disulfide bridges are practically absent, thus accounting for increased thermolability (Feller *et al.*, 1997; Feller and Gerday, 1997). Individual enzyme types possess different structural strategies to gain overall increased flexibility. Certain structural features thought to be indicative of cold adaptation have also been found in similar non-cold adapted enzymes (Schroder *et al.*, 1998). The same enzyme from different organisms, containing an identical amino acid sequence, has been found to possess different thermal properties (Love *et al.*, 2004). This suggests that protein folding has a critical role in conferring activity at low temperature.

Table 2.2: Optimal temperature and relative activity of cold-active enzymes from a variety of Antarctic sea ice bacteria. Adapted from Buia, 1997; and Nichols *et al.*, 1999.

Bacterial strains	Enzymes	Optimal Temp.(°C)	Residual activity (%)	
			10°C	40°C
Colwellia demingae	Protease (azocasein)	28	75	25
	Protease (azoalbumin)	30	39	30
	Trypsin	14	90	29
	Phosphatase	23	90	85
Cytophaga-like strain	Protease (azocasein)	20	68	65
	Protease (azoalbumin)	27	70	55
	Trypsin	30	72	60
	β-galactosidase	15	100	46
	α-amylase	25	65	60
	Phosphatase	19	85	85
Colwellia-like strain	Trypsin	12	100	53
	Phosphatase	17	85	85
	β-galactosidase	26	75	70
Pseudoalteromonas sp.	Protease	29	55	37
	Trypsin	22	90	23
Cytophaga-like strain	Phosphatase	19	85	85
Shewanella gelidimarina	β-galactosidase	24	65	<20

Preliminary data has been obtained for a variety of psychrophilic and psychrotolerant (cold-tolerant) enzymes from Antarctic bacterial isolates (Buia 1997). The isolates were obtained from sea ice and lake habitats in the Vestfold Hills. Whole cell and cell-free assays indicated the presence of protease, β-galactosidase, phosphatase, and amylase exhibiting strong cold adaptation in several strains (Table 2.1). The Cytophaga-like strain IC166 showed particular promise as it elaborates several cold adapted enzymes. From this research, it is apparent that Antarctic bacteria, especially those isolated from ice, are good sources of cold-active enzymes (Buia, 1997).

ALKALINE PHOSPHATASE

A hydrolase enzyme that can optimally remove phosphate group from molecules like alkaloids, nucleotides and proteins in alkaline range of pH, is called alkaline phosphatase (ALP) (EC 3.1.3.1). Bacterial alkaline phosphatase is usually located in periplasmic space and fairly stable to resist inactivation, denaturation and degradation, and also has a higher rate of activity. Alkaline phosphatase is usually produced during phosphate starvation and sporulation (Sebastian and Ammerman, 2009). The wide distribution of alkaline phosphatase in nature, ranging from bacteria to man, indicates that APs are ubiquitous and involved in fundamental biochemical processes (Millan, 1983).

Types of alkaline phosphatase

Nozawa *et al.*, (1984) reported that ALP is now subdivided by biochemical methods into three major groups e.g. tissue-unspecific, intestinal and term placental isoenzymes. Furthermore, term placental ALP in cancer is classified into L-leucine insensitive-Regan and L-leucine sensitive-Nagao isoenzymes. Alkaline phosphatase activity from 21 and 11 respective cases of normal and endometrial cancer measured by Fishman's method at 65 degrees C and amino acid inhibition tests showed the biochemical characteristics of tissue-unspecific ALP isoenzyme. The results suggested that the ALP isoezyme underwent "enzyme deviation" occurred during the course of endometrial carcinogenesis.

Biochemical localization and solubilization of the alkaline phosphatase from *Bacillus licheniformis* MC14 was achieved by fractionation and use of nonionic detergents, ionic detergents, bile salts, and various concentrations of magnesium and sodium, repectively. Hexadecyl pyridinium chloride (0.03 mol) and magnesium and sodium salts (above 0.2 mol) were found effective solubilizing agents (Glynn *et al.*, 1977).

Spencer *et al.*, (1982) extracted 80% of the membrance *B. lichenidomis* MC14 alkaline phosphatase activity usining 1 mol magnesium and remaining was solubilized with hexadecyl pyridinium chloride. The 60 kDa protein was localized 100% on the outer surface of the cytoplasmic membrane. Doonan and Jensen (1977) found that alkaline phosphatase is located in periplasmic space of *Plectonema boryanum* when grown on phosphate-free medium for 5 days.

Mechanism of hydrolysis by alkaline phosphatase

Yang and Metcalf (2004) concluded that phosphite (Pt) is oxidized to phosphate either by 14-gene phn operon, which encodes the enzyme C-P lyase and/or by phoA locus, which encodes bacterial alkaline phosphatase (BAP) in *E. coli*. Highly purified BAP (also acting as a hydrogenase) catalyzed Pt oxidation with specific activities of 62-242 milliunits/mg and phosphate ester hydrolysis with specific activities of 41-61 units/mg. The cell bound and cell free forms of alkaline phosphatase of *Plectonema boryanum* were extracted using lysozyme or polymyxin B treatment (Doonan and Jensen, 1980).

Induction and expression

The alkaline phosphatase in cell-free extract of *Proteus mirabilis* alkaline phosphatase was induced under conditions of inorganic phosphate deficiency in the medium (Salikhova *et al.*, 2003). A ubiquitous gene for alkaline phosphatae (PhoX) from *Silicibacter pomeroyi* exhibited 90% of phosphatase activity upon P-starvation only in comparison to PhoA that is induced by both, starvation and sporulation. It can act as a marker of P(i)-stress during P cycle (Sebastian and Ammerman, 2009).

A mutant Apase produced by *Pseudomonas aeruginosa* in PO_4 deficient medium, hydrolysed β-glycerol phosphate (betaGP) and p-nitrophenyl phosphate with specific activities of 45:1 in favour of betaGP versus pNPP (Marceau-Day *et al.*, 1978). Chesnut *et al.*, (1991) found that expression of phoAIII gene of *B. subtilis* causes expression of APase both during phosphate-starvation and sporulation. The set of three genes (spoIIA, spoIIG and spoIIE) regulated by PS promoter (-119 bp) and (phoP, phoR and phoS) regulated by PV promoter (-37 bp) affected the expression of phoAIII gene by sporulation and phosphate starvation induction, respectively.

Extraction of alkaline phosphatase

Ihlenfeldt and Gibson (1975) optimized the production of alkaline phosphatase from *Anacystis nidulans* in a medium of low phosphate (0.1 mmol), with doubling time of 5 hrs at 30°C. An increase in enzyme production also increased the specific activity (10-15 fold) when PO_4 level was decreased up to 4 mμmol in medium.

Hofmann *et al.*, (1989) detected four isozymes of alkaline phosphatase (ALP) from germ cell tumours, cell lines and precancerous cells. Two transgenic rabbit lines created by pronuclear microinjection with the whey acidic protein promoter-humanTNAP minigene (WAP-hTNAP); produced biologically active human (tissue non specific alkaline phosphatase) TNAP, not available normally. The phosphatase activity was two orders of magnitude higher compared to normal human serum levels. The demonstration that this TNAP is physiologically active would provide the clue to use transgenic animals as bioreactor for bulk production of the TNAP in milk, valuable option to attenuate the LPS mediated inflammatory responses (Bodrogi *et al.*, 2006).

Sharipova *et al.*, (2000) established that in the presence of mononucleotides, the content of extracellular alkaline phosphatase in both strains i.e. streptomycin-resistant strains *Bacillus intermedius* S3-19 and S7 was increased. The maximal effect was caused by 5'-AMP at a concentration of 20 µg/ml. *Neurospora crassa* produced a constitutive 74A ALP while growing at 30°C on medium containing acetate and inorganic phosphate (Pi) after 72 hours (Morales *et al.*, 2000).

Bacillus licheniformis MC14 produced 35 times higher yield of alkaline phosphatase in defined minimal salt medium supplemented with cobalt than cobalt less medium. The enzyme activity was increased when 0.075 mmol phosphate was added to medium during late-logarithmic and early-stationary-phase of culture growth. Presence of 3.65 +/- 0.45 g-atoms were found sufficient for each mole of purified active alkaline phosphatase. MC14 ALP was present in both, soluble (periplasmic) and membrane bound forms (Spencer *et al.*, 1981). *Thermotoga neapolitana* produced a hyperthermophilic alkaline phosphatase at 100°C that was purified by gel filtration chromatography to 2,880 fold with 44% yield (Dong and Zeikus, 1997).

Purification of Microbial Alkaline Phosphatase

Neurospora crassa os-1 alkaline phosphatase was purified by Phenyl-Sepharose CL-4B chromatography and Sephadex G-200 gel filtration (Bogo *et al.*, 2006). Schaffel and Hulett (1978) purified a membrane-associated alkaline phosphatase in buffer containing 0.2 mol Mg^{2+} from thermophilic *Bacillus licheniformis* MC14. A termostable alkaline phosphatase from *A. caespitosus* was purified 42-fold with

32% recovery by DEAE-cellulose and concanavalin A-Sepharose chromatography (Guimaraes *et al.*, 2007). The 38.5 kDa recombinant protein purified through glutathione-Sepharose 4B column showed a specific activity of 49.4 units and exhibited high catalytic activity at low temperature (Tsuruta and Aizono, 2000).

An orthophosphate-repressible extracellular alkaline phosphatase isolated from *Micrococcus sodonensis* was purified by sieve chromatography, disc gel electrophoresis, and ultracentrifugation (Glew and Heaths, 1971). The thermostable alkaline phosphatase from *Thermotoga neapolitana* was purified by gel filtration chromatography to 2,880 fold with 44% yield (Dong and Zeikus, 1997).

Micrococcus sodonensis produced an extracellular alkaline phosphatase purified by molecular sieve chromatography, disc gel electrophoresis, and ultracentrifugation (Glew and Heath, 1971). *Aspergillus caespitosus* alkaline phosphatase was purified using DEAE-cellulose and concanavalin A-sepharose in Sepharose CL-6B (Guimaraes *et al.*, 2004).

Ishibashi *et al.*, (2005) purified a holphilic alkaline phosphatase up to 510-fold (21% yeild) from *Halomonas* sp. 593. Yeh and Trela (1976) isolated a repressible alkaline phosphatase from *Thermus aquaticus* that could depress up to 1,000.fold by starving the cells for phosphate. Yamane and Maruo (1978) purified the APases with APDase from the membrane fraction of *B. subtilis* 6160-BC6 and from the culture fluid of *B. subtilis* RAN 1.Two kinds of phosphodiesterase (PDase) without phosphatase activity were detected in supernatants of RAN1 and *B. subtilis* SP25.

Bhatti *et al.*, (2000) purified constitutive (CAPase) and inducible (IAPase) alkaline phosphatases from *Serratia marcescens* through $(NH_4)_2SO_4$) precipitation, DEAE-cellulose and elution with linear gradient of sodium chloride. Rodrigues *et al.*, (2006) purified an acid and an alkaline phosphatase monomer from venom of social spider *Parawixia bistriata*, using Concanavalin A-Sepharose column chromatography. Yamane and Maruo (1978) purified a membrane-bound insoluble 6160-BC6 APase (46+/-1 kDa) and an extracellular soluble RAN1

APase (45+/-1 kDa) from a *B. subtilis* 6160-BC6, mutated to produce APase constitutively (Yamane and Maruo, 1978).

Effect of pH and temperature on the activity of microbial alkaline phosphatase

The activity of enzymes is dependent upon temperature and concentration of hydrogen or hydroxyl ions. The extracellular alkaline phosphatase from *Micrococcus sodonensis* hydrolysed mono-, di-, and triphosphates, and inorganic phosphate at pH 9- 9.5 (Glew and Heath, 1971). The *Aspergillus caespitosus* alkaline phosphatase was optimally active at 75°C, pH 8.5, stable at 50°C (Guimaraes *et al.*, 2003). The thermostable ALP from *Aspergillus caespitosus* showed maximum activity at pH 9.0 and 80°C (Guimaraes *et al.*, 2007).

Prada *et al.*, (1996) characterized a heat labile alkaline phospahatase with 45 and 55°C temperature as optimal and maximal temperature for activity, from a psychrophilic *Arthrobacter* sp. D10. The alkaline phosphatase from *P. chrysogenum* hydrolyzed the *p*-nitrophenyl phosphate at pH 9.0 and 45 °C and showed PI of 5.5. Both, phosphatase and phosphodiesterase from *B. subtilis* 6160-BC6 were optimum to function at pH 9.5 (Yamane and Maruo, 1978). Maximum hydrolysis of *p*-nitrophenylphosphate was observed at pH 10.5 for the alkaline phosphatase that irreversibly lost activity (>90%) at 55°C, 3 mol urea or 10 mmol β- mercaptoethanol for 30, 30 and 10 min incubations, respectively (Ikehara *et al.*, 1978).

The optimal temperature for the enzymatic activity was 25°C that completely lost activity at 55°C in 10 minutes. A novel enzyme for DNA dephosphorylation, HK phosphatase, is completely and irreversibly inactivated at 65°C (Kobori *et al.*, 1984). The thermophilic alkaline phosphatase from *Thermotoga neapolitana* showed maximum activity at pH 9.9 and 85°C (Dong and Zeikus, 1997). The repressible alkaline phosphatase from *T. aquaticus* showed maximum activity at pH 9.2 in Tris buffer and 75-80°C (Yeh and Trela, 1976).

The T59R monomeric AP showed a melting temperature (*Tm*) of 43°C, whereas the wild-type AP dimer exhibited its Tm as 97 °C. The catalytic activity of the T59R enzyme was reduced by 104-fold, whereas the T59A enzyme exhibited an activity similar to that of the wild-type enzyme (Boulanger and Kantrowitz, 2003).

The alkaline phosphomonoesterase from *Micrococcus sodonensis* was optimally active at pH 9 to 9.5 (Glew and Heaths, 1971). The CAPase and IAPase from *Serratia marcescens* showed maximum enzyme activity at pH 9.5 and pH 8.5-10.5 respectively. CAPase was more thermolabile than IAPase at 95°C (Bhatti *et al.*, 2000).

The phosphatases from *B. intermedius* (phosphomonoesterase and phosphodiesterase) exhibited an optimum temperature of 50 °C, optimum pH of 9.5 and were stable until 60 °C at pH 8-10 (Sharipova *et al.*, 1996). The other recombinat SIB1 APase from psychrotrophic *Shewanella* sp. shows maximum activity at 50 °C and 3.1 fold higher specific activity than that of *E. coli* APase at 80°C. SIB1 and *E. coli* APases lost their activitis with a half-life of 3.9 and >6 min at 70 and 80°C, respectively (Suzuki *et al.*, 2005). The two isozymes of *Mycobacterium bovis* BCG showed maximal activity at pH 10.0 and pH 12.0 (Braibant and Content, 2001). The streptomycin-resistant *Bacillus intermedius* S3-19 alkaline phosphatase exhibited maximum activity at pH 9.5 and 55 °C and was stable until 60 °C at pH 8.0-10.0 (Sharipova *et al.*,1998).

Molecular characterization of alkaline phosphatase

Amino acid residues comprising active site especially and 3-D structure generally are important to describe the nature and activity of the enzyme. The cold-active protein-tyrosine phosphatase (CAPTPase) from *Shewanella* sp., has three amino acid sequences, Asp-Xaa-His, Gly-Asp-Xaa-Xaa-Asp-Arg and Gly-Asn-His-Glu, that are observed in many protein-serine/threonine phosphatases (PS/TPases) (Tsuruta *et al.*, 2005). The DNA sequence analysis of regulatory region of ALP gene of *Bacillus licheniformis* MC14 expressed an open reading frame of 129 amino acids containing the amino-terminal sequence of the mature APase protein. The protein sequence was preceded by a putative signal sequence of 32 amino acid residues. Heterologous expression of promoter fusing with lacZ gene indicated that it functions as a very strong inducible promoter in *B. subtilis* that is tightly regulated by phosphate concentration (Lee *et al.*, 1991).

EPR spectroscopy of alkaline phosphatase revealed that serine-to-cysteine mutations caused a considerable loss of activity but improvement in thermal stability. Mutations at W274 (K328 in *E. coli* AP), caused a reduction in mobility of

C67 suggesting that it was interconnected to S65 for catalytic events (Heidarsson *et al.*, 2009). Akiyama and Ito (1993) found that PhoA (Alkaline phospahtase, lacking the signal sequence) undergoes a series of folding-assembly steps, some of which are of measurable speeds *in vivo* and mimicable *in vitro*.

Effect of substrates on catalysis by alkaline phosphatase

Out of two isomers, the D10A hydrolyzed both X-phos (5-bromo-4-chloro-3-indolyl phosphate) and *para*-nitrophenyl phosphate for pH range of 7 to 11. The other calcium ion dependant D10B, lacked activity against X-phos and had a narrow pH range of about 8 to 9 (Prada *et al.*, 1996).

Effect of metal ions, inhibitors and activators on the activity of alkaline phosphatase

Salts of different metals stimulate or inhibit some enzymes particularly ones requiring buffering mechanisms provided by metallic ions. Alkaline phosphatase activity was enhanced by Mg^{+2} and Mn^{+2} ions (Politino *et al.*, 1996). The purified alkaline phosphatase was stimulated by Mg^{2+} and Zn^{2+} in Tris-HCl buffer, and inhibited by Be^{2+}, histidine and EDTA. Also, 0.3 mol Tris-HCl buffer protected the purified enzyme against heat inactivation at 70 °C (half-life of 19.0 min, k = 0.036 min^{-1}) as compared to 0.3 mol CHES (half-life of 2.3 min, k = 0.392 min^{-1}) in the same experiment (Morales *et al.*, 2000).

The alkaline phosphomonoesterase from *Micrococcus sodonensis* was competitively inhibited by orthophosphate, arsenite, and arsenate; and activated by Ca^{2+} (90%); Mn^{2+} (22%); Co^{2+} (15%); and ST^{2+} (13%). Calcium was found a potent central metal atom that confers structural stability to enzyme (Glew and Heaths, 1971). The mutated ALP (H412Q) of *E. coli* with Gln at His-412 exhibited same K_{cat}, 50 fold increased K_m, lower zinc contents and inhibited by Tris; as compared to wild type enzyme. All characters were restored by addition of zinc (Ma and Kantrowitz, 1996).

Trotman and Greenwood (1971) found that the stability of the enzyme in 6 mol-urea was followed as a function of its zinc content and was found to be dependent on the first two of the four zinc atoms bound by apo-(alkaline phosphatase). The phosphatase activity was mostly dependent on a second pair

of zinc atoms. Mn^{2+}, Co^{2+}, Cu^{2+} or Cd^{2+} also restored structural stability. The alkaline phosphatase from *Halobacterium cutirubrum* showed no increase or decrease in activity in presence or absence of Calcium and magnesium ions. Protein could hydrolyze p-nitrophenyl phosphate, 5'-dATP, 5'-dTMP and 5'-dTTP (Peter *et al.*, 1976). Alkaline phosphatase was activated by Mg^{2+} (20%) and inhibited by Zn^{2+} (95%) at 0.5 mmol, L-histidine and imidazole (Ikehara *et al.*, 1978).

Coleman (1998) found that protein-bound Zn^{2+} functions catalytically and form protein-Zn^{2+}-substrate bonds that position the substrate or polarize its electron distribution to facilitate the catalysis. Christianson (1991) emphasized that zinc plays important role in catalytic and regulatory functions of alkaline phosphatase. The *Micrococcus sodonensis* ALP was activated by Ca^{2+}, Mn^{2+}, Co^{2+} and ST^{2+} with 90, 22, 15, and 13%, respectively. The orthophosphate, arsenite, and arsenate inhibited the enzyme competitively (Glew and Heath, 1971).

Yamane and Maruo, 1978 found that the *B. subtilis* 6160-BC6 APase and RAN1 APase were competitively inhibited by phosphate, arsenate and activated by Ca^{2+} but not by Zn ions. The *p*NPPase activity was inhibited by 0.1 mmol vanadate (46%), 0.1 mmol ZnCl2 (68%), 1 mmol levamisole (66%), 1 mmol arsenate (44%), 10 mmol phosphate (21%) and 1 mmol theophylline (72%) (Simao *et al.*, 2007). The alkaline phosphatase from *P. chrysogenum* was inhibited by EDTA (Politino *et al.*, 1996) while alkaline phosphatase from *Plectonema boryanum* was inhibited by mercuric chloride (Doonan and Jensen, 1980).

The phosphatasel from *Shewanella* sp. was inhibited with 1, 0.88 and 1 mol of diethylpyrocarbonate (DEPC), monoiodoacetic acid (MIAA), and monoiodoacetamide (MIAM) respectively (Tsuruta and Aizono, 1999). The inhibitory effects induced by binding of inorganic phosphate to active site of alkaline phosphatase were evidenced by weak infrared changes around 1631 and at 1639 cm^{-1}, during its study using infrared spectroscopy (Zhang *et al.*, 2004).

Effect of mutation

Enzyme engineering is an area of interest to modify the catalytic sites and improve the turn over and efficiency of enzymes. The products of mutagenesis of TAB5 ALP (substitution of Ala at Gly262 and Gly261), resulted in an inactive form and low stability of enzyme, respectively. The double mutant G261A/Y269A however, restored the energy of activation like native enzyme meaning that Gly cluster was significant for cold adaptation (Mavromatis *et al.*, 2002). The site-directed mutagenesis of *Shewanella* sp. ALP revealed that His148 acts role for acid catalyses, Asp115 assists the His148 for protonation, and, Asp76 and Asp112 were involved in binding to magnesium ions (Tsuruta *et al.*, 2004).

An insertion mutation in phoAIII gene of *B. subtilis* 168 reduced the starvation and sporulation APase specific activity by 40 and 45%, respectively. The native APase III corresponding to APase III gene has been mapped to approximately 50 degrees on the *B. subtilis* chromosome (Bookstein *et al.*, 1990). The X-ray crystallography of the mutated ALP (H412Q) of *E. coli* protein showed that the only α-carbon was displaced over 1°A, near the mutation site. The carbonyl oxygen of Gln-412 rotated by 3.2°A that was too away to coordinate to Zn1 (Ma and Kantrowitz, 1996).

Substitution of Ser102 by glycine, alanine and cysteine in *E. coli* alkaline phosphatase yielded isozymes like S102G (6 x 10(5)-fold), S102A (10(5)-fold) and S102C (10(4)-fold) lower than the wild-type enzyme. The crystal structure of isozyems reveals that only S102C has displaced the phosphate group by 2.5°A (Stec *et al.*, 1998). The site directed substitution of Leu to Arg in the hydrophobic domain of PI-glacan (membrane anchoring protein of ALP) converts it to a protein that is secreted into the medium. With this finding, (Lowe 1992) suggested that an essential signal for the correct sorting of PI-glycan anchored proteins versus secreted proteins resided in the hydrophobic domain.

The center-to-center distances of two zinc and one magnesium ions in alkaline phosphatase are 3.9, 4.9 and 7.1 °A for (Zn1-Zn2), (Zn2-Mg3) and (Zn1-Mg3), respectively. Zn1 coordinates to ester oxygen of Ser102 and water molecule while Zn2 coordinates to oxygen of seryl phosphate during catalysis (Coleman, 1992).

Cloning and Sequence analyses

Hulett (1984) cloned an 8.5 kb DNA fragment for alkaline phosphatase from *Bacillus licheniformis* MC14 into the Pst1 site of pMK2004. A restriction map of subcloning of a 4.2 kb DNA fragment revealed that the coding region of the gene was localized to a 1.3 kb region. The cloned alkaline phosphatase from *Thermus caldophilus* GK24 (Tca) consisted of 501 amino acid residues (including signal peptide) corresponding to 760 Da. The Tca showed 20% identity with *E. coli* ALP and 22% with *B. subtilis* (Bsu). Heat-stable Tca alkaline phosphatase activity was detected in *E. coli* YK537, harboring pJRAP ALP (Park *et al.*, 1999).

The gene sequence of a monomeric, psychrophilic G15-21 AP from *Vibrio* sp. reveals that the catalytic and metal ligating residues of the enzyme are conserved. Other than difference of Mg binding site, Asp-153 and Lys-328 of *E. coli* AP are His-153 and Trp-328 in *Vibrio* AP (Asgeirsson and Andresson, 2001). The deduced amino acid sequence analysis of Inverted PCR of previously cloned gene for cold active alkaline phosphatase from *Shewanella* sp. (Ishida *et al.*, 1998) showed a high content of hydrophobic amino acid residues and the lack of single α-helix as compared to *E. coli* alkaline phosphatase (Murakawa *et al.*, 2002).

Cloning, sequencing, and comparisons of deduced amino acid sequence with *Escherichia coli* alkaline phosphatase and three-dimensional structure of *Bacillus subtilis* alkaline phosphatases III and IV showed 64 and 63% identity at DNA and deduced primary amino acid sequence level. The deduced primary amino acid sequence of the mature protein showed 25-30% identity with other sequenced alkaline phosphatases from *Escherichia coli*, yeast, and humans (Hulett *et al.*, 1991).

Two recombinant alkaline phosphatases phoAIII and phoAIV were cloned from *Bacillus subtilis* and expressed with 64 and 63% identity at DNA and deduced amino acid sequence level. The mature protein showed 25-30% identity when compared with other sequenced alkaline phosphatases from Escherichia coli, yeast, and human (Hulett *et al.*, 1991). A previously cloned psychrophilic phosphatase I gene from *Shewanella* sp. was found to be a novel protein-tyrosine-phosphatase (PTPase) with a histidine as its catalytic residue (Tsuruta

and Aizono, 1999). The deduced amino acid sequence of an inverted PCR contained a conserved region of protein-serine/threonine-phosphatase (PPase) (Tsuruta and Aizono, 2000).

Eder *et al.*, (1996) purified an extracelluar phosphodiesterase/alkaline phosphatase, APaseD, from a culture of *Bacillus subtilis* JH646MS. The phoD gene was found mutated by deletion of single base pair and produced 49 kDa APaseD. The expression of the cloned phoD-lacZ promoter showed that the phoD gene was phosphate-starvation-induced and dependent on PhoP and PhoR for expression.

The *Thermus thermophilus* alkaline phosphatase (Tap) cloned in *E. coli* translocated through twin-arginine pathway (like its origin) instead of secretory system in host; meaning that two sequence and functional related enzymes are exported by distinct protein transport systems, which may play an integral role in the bacterial adaptation to their environment during the evolution (Angelini *et al.*, 2001). *Escherichia coli* ALP (AP) was fused to a C-terminal region (containing a signal sequence) of *Pseudomonas* sp. MIS38 lipase (PML) and examined for secretion using the *E.coli* cells carrying the heterologous Type I sectretion system (TISS). The fusion protein efficiently secreted to the extracellular medium existed as a homodimer and indistinguishable from AP (Angkawidjaja *et al.*, 2006).

Kinetics

Kinetic parameters particularly V_{max} and K_m have been determined for enzymes of different origin. A half-life of 238 min, Km 183 µmol and Vmax of 1,352 U mg^{-1} were observed at 90°C for a thermostable alkaline phosaphtase from *Thermotoga neapolitana* (Dong and Zeikus, 1997). The alkaline phosphatase from *T. aquaticus* exhibited Michaelis constant (Km) as 8.0 x l0 mol (Yeh and Trela, 1976).

The directed evolution of TAB5 AP gene yielded three thermostable and six thermolabile variants. Mutations located close to the active site only, affected the cold-adapted properties of the enzyme. The destabilized variants H135E and H135E/G149D had 2 and 3 fold higher k_{cat}, respectively, than the wild-type enzyme. Comparison of the wild-type and mutated TAB5 APs demonstrated that

it is possible to improve the activity and thermostability simultaneously (Koutsioulis *et al.*, 2008). The G15-21 AP from *Vibrio* G15-21 has highest K_{cat} value as compared to *E. coli*, human placenta, shrimp and TAB5 (Helland *et al.*, 2009).

To calculate the K_m and V_{max}, Eadie-Hofstee plot with "linear regression" is somewhat more precise and accurate than Hanes plot, and much better than Lineweaver–Burk method (Mikkelsen, 2004).

Molecular wt. determination of microbial alkaline phosphatase

There is considerable variation in the molecular weight of alkaline phosphatase from various organisms. The dimer of 140 kDa denatured by β- mercaptoethanol to two 72 kDa subunits had PI of 4.7 (Ikehara *et al.*, 1978). The purified monomers of 74A ALP from *Neurospora crassa* were determined as 58 kDa and 56 kDa using size exclusion chromatography and SDS-PAGE, respectively (Morales *et al.*, 2000). The 58 kDa active monomer of ALP from *Penicillium chrysogenum* was purified by DEAE and size exclusion chromatography (Politino *et al.*, 1996). Say *et al.*, (1996) purified a 145 and 110 kDa conidial alkaline phosphatases from Neurospora crass, using gel filteration chromatography, in presence and absence of magnesium ions, respectively. *Neurospora crassa* os-1 produced alkaline phosphatase exhibiting a native protein of 137 kDa but two protein bands (36 and 62 kDa) when denatured with SDS (Bogo *et al.*, 2006).

A single band of 41 kDa recombinant alkaline phosphatase from *Shewanella* sp. showed a specific activity of 1500 units/mg (Murakawa *et al.*, 2002). The phoAIII and phoAIV genes in *B. subtilis* code for predicted proteins of 47.1 kDa and 45.9 kDa, respectively (Hulett *et al.*, 1991). The phosphodiesterase from cpdP gene of *Vibri fischerit* MJ-1 was predicted as a subunit 33 kDa for the mature CpdP protein (36 kDa less 2 kDa for the leader peptide) which was found consistent with the molecular weight of 34 kDa estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (Dunlap and Callahan, 1993).

The *Mycobacterium bovis* BCG yielded two peaks suggesting the presence of two isozymes (Braibant and Content, 2001). The streptomycin-resistant *Bacillus intermedius* S3-19 produced a 46-47 kDa alkaline phosphatase (APase)

(Sharipova *et al.*, 1998). An ALP from *A. caespitosus* showed native and denaturing bands of 138 and 71 kDa respectively, indicating a homodimer (Guimaraes *et al.*, 2007). A thermostable alkaline phosaphtase from *Thermotoga neapolitana* showed an active band of 87 kDa but inactive band of 45 kDa at SDS-PAGE presuming it as a dimer (Dong and Zeikus, 1997). The *Thermus aquaticus* alkaline phosphatase produced a native protein of 143 kDa that exhibited 51 kDa for each of the 3 subunits on SDS- PAGE (Yeh and Trela, 1976).

A native alkaline phosphatase of 134.8 kDa was purified from *Aspergillus caespitosus* that showed a single denatured protein (SDS-PAGE) of 57 kDa suggesting that the native enzyme was a homodimer (Guimaraes *et al.*, 2003). An APase of 80 kDa was isolated from isolate 6160-BC6 (Yamane and Maruo, 1978). Kim *et al.*, (1998) found that *B. licheniformis* and eucaryotic alkaline phosphatases are 60 kDa while other bacterial alkaline phosphatases (APases) are usually of 45 kDa. The DNA sequence of (APasel) gene of *B. licheniformis* MC14 coded for a 60-kDa alkaline phosphatase. The extracellular alkaline phosphatase from *Bacillus intermedius* showed a singe band of 47 kDa on SDS-PAGE (Sharipova *et al.*, 1996). *Micrococcus sodonensis* produced an extracellular alkaline phosphatase of 80 kDa (Glew and Heath, 1971). An antarctic bacterium HK47 produced a 68 KDa heat labile alkaline phosphatase (Kobori *et al.*, (1984).

Applications of alkaline phosphatase in a biosensor

Biosensor technology is expected to play a significant analytical role in medicine, agriculture, food safety, homeland security, environmental and industrial monitoring. However, the commercialization of biosensor technology has significantly lagged behind the research output as reflected by a plethora of publications and patenting activities. Biosensors pose a great challenge of detection of single or a few target analytes. Successful biosensors must be versatile to support interchangeable bio-recognition elements, miniaturization, automation and ease of operation at a competitive cost. A significant upfront investment in research and development is a prerequisite in the commercialization of biosensors (John *et al.*, 2008).

A microbial biosensor consists of a transducer along with immobilized viable or non-viable microbial cells. Non viable cell containing periplasmic enzymes have been used as an economical substitute for enzymes while Viable cells make use of the respiratory and metabolic functions of the cell, the analyte to be monitored being either a substrate or an inhibitor of these processes. Microbial biosensors have wide application in environmental monitoring, use in food, fermentation and allied fields (D'Souza, 2001).

Cyclic voltammetry of the corresponding hydrolysis products, 4-aminophenol and 1-naphthol, of 4-Aminophenyl phosphate (4-APP) and 1-naphthyl phosphate (1-NP) at the surface of screen-printed carbon base transducers, uncoated or coated with anti-progesterone monoclonal antibody (mAb) showed well-defined anodic responses for both species. 1-NP possesses the advantages over 4-NPP of being inexpensive, easy to obtain and soluble (1-naphthol cf. 4-aminophenol) at high pH. Hence the preferred substrate for use is proposed milk progesterone biosensor (Pemberton *et al.,* 1999).

Serra *et al.*, (2005) designed an amperometric graphite-Teflon composite tyrosinase biosensor for the rapid monitoring of alkaline phosphatase (ALP), with no need of an incubation step and using phenyl phosphate as the substrate. The reaction was optimized to occur at pH 8.5, current -0.10 V for 5 min after the addition of ALP. A linear calibration plot was obtained for ALP between 2.0×10^{-13} and 2.5×10^{-11}, with a detection limit of 6.7×10^{-14} mol. Adsorption is an accumulation of species bound to the electrode surface. The species interacts with the electrode surface to form a partial or complete layer. The layer formed on the electrode can be an electro inactive species that can inhibit an electrode's interaction with the reactive solution (Wang, 2000). A layer could also accelerate reactions occurring at the electrode due to a double-layer effect which acts similar to that of a capacitor. Certain embodiments like singe walled carbon nanotubes, disposed in interconnected networks are used as electrodes. The device, methods and kits like this have applications for detection and measurement of biomolecular species including polynucleotides, proteins, polysaccharides and the others (WIPO/045799/Nanomix, Inc. 2008).

MATERIALS AND METHODS

Sampling

Samples of ice, water and soil were collected from 4 glaciers (Batura, Passu, Hopper and Shishkit) in Northern Areas of Pakistan (Fig 3.1; Appendix V). Microbiological prospects like sterility of instruments, personnels and handling of samples were performed according to standard microbiological techniques. A total of 45 samples were collected from 18 different sites (Table 3.1).

Handling of samples

Samples of ice, water and soil were collected carefully with intense care and transferred to respective portable ice boxes.

Materials

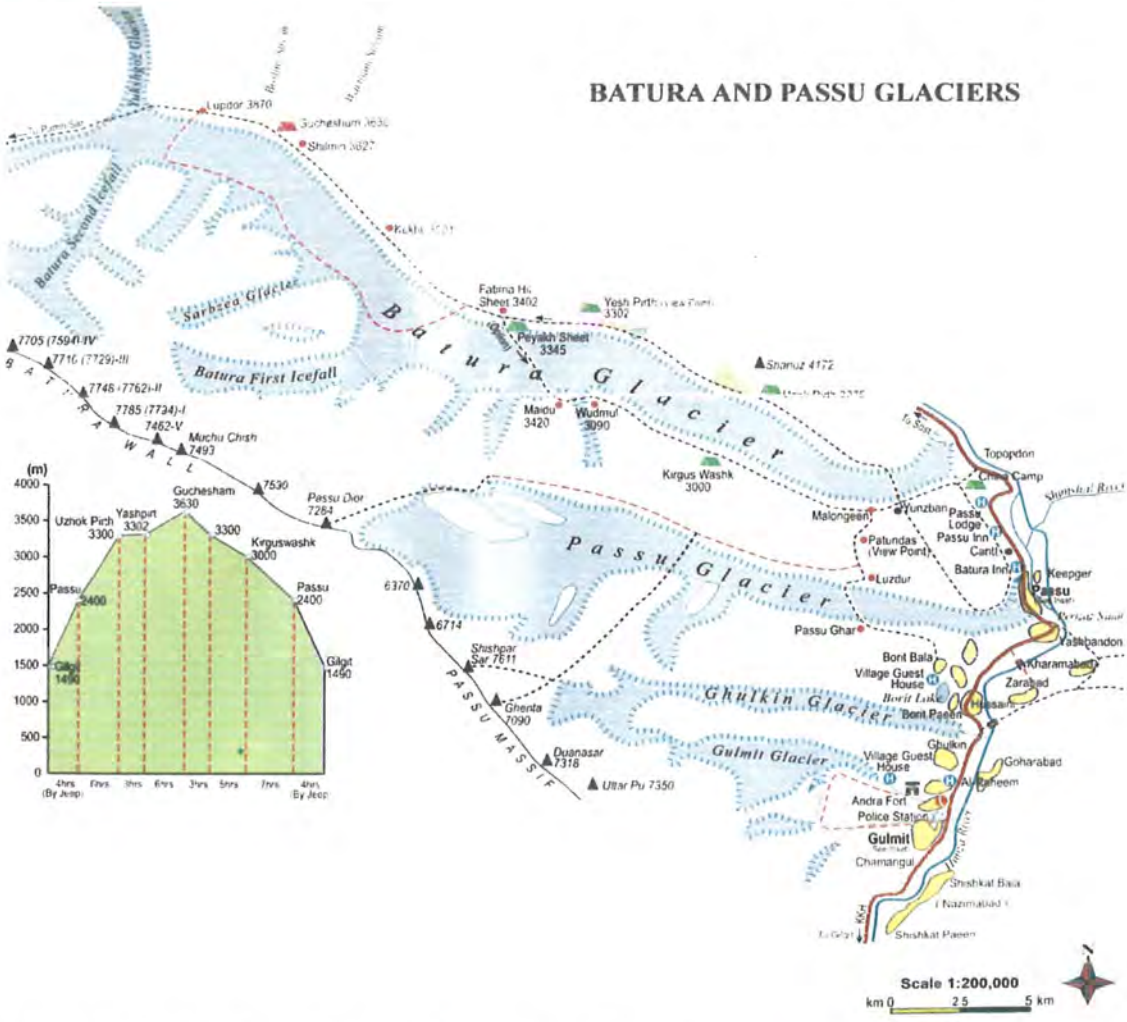
The materials included a manual auger, sampler, sterile gloves and sample bags, pH strips, thermometer, GPS, DO meter, Ice cabins (Portable), ethanol, methylated spirit, spray bottles, tissues and cotton, prepared Petri plates with nutrient agar medium.

Method for collection of samples

Geographic coordinates, height and atmospheric pressure were recorded using a GPS (Garmin 60). Dissolved oxygen was measured by using Portable Dissolved Oxygen Meter (DO21 Kalestead USA). The pH was recorded using pH indicator strips (Mini Science Inc. USA). The ice was cut into pieces and collected in sterile sample bags. The water samples were obtained in sterile bottles by opening their lids inside the water. The soil samples were collected in sterile bags.

Transportation and Preservation

All samples were transported to the laboratory in intact physical conditions. The soil and water samples were preserved at 4°C while ice was preserved at -70°C.



Adapted from: (http://www.silkroutelodge.com/Maps/batura&passuglaciers_map.jpg)

Fig 3.1. Geographical Location of the sampling site: (Indus Basin Report, 2005).

Fig 3.2a. Hopper Glacier Fig 3.2b. Passu Glacier

Fig 3.2a and 3.2b: Site of sampling at Hopper and Passu glaciers: Adapted from inventory of glaciers, glacial lakes and identification of potential glacial lake outburst floods (GLOFS), In: Indus Basin-Pakistan (2005).

Media, Culture conditions and Isolation of bacteria

Samples of ice, water and soil (Table 3.2) were used for isolation of bacteria. Isolation from soil samples was done by making dilutions of 1 g of soil to 9 ml of distilled water. This stock was serially diluted. About 100 µl of each dilution and lake or ice melt water were spread on nutrient agar medium (Oxoid, Basingstoke, U.K). Duplicate spread plates were incubated aerobically at 4° or 10° and 20°C for 7 days.

The microorganisms isolated in this study were *Pseudomonas* spp. (MRLBA1, MRLBA2, MRLBA3, and MRLBA4), *Arthrobacter* sp. MRLBA5, *Stenotrophomonas* sp. MRLBA6, *Bacillus subtilis* MRLBA7, *Bacillus licheniformis* MRL MRLBA8 and *Bacillus megaterium* MRLBA9 in Microbiology Research Laboratory (MRL), Department of Microbiology, Quaid-i-Azam University Islamabad, Pakistan.

Table 3.1: Isolation of psychrophilic bacteria from various samples collected from glaciers and Microbiology Research Laboratory

Source		Sample Code	Isolates
Glacial samples and isolates	Passu glacier: Lake Ice	1.6	MRLBA1
	Batura glacier: Pond water	2.1	MRLBA2
	Hopper glacier: Deep ice	4.4	MRLBA3
	Passu glacier: Pond water	1.2	MRLBA4
	Hopper glacier: Surface soil	4.5	MRLBA5
	Passu glacier: Deep ice	1.3	MRLBA6
Non glacial Isolates	-20°C freezer: Laboratory	4.9	MRLBA7 MRLBA8 MRLBA9

Maintenance of cultures:

The cultures were routinely maintained at 2-5 °C (glacial and non glacial isolates, respectively) on nutrient agar slants with following composition:

Contents	Amount
Agar	20.0 g
Beef extract	3.0 g
Peptone	5.0 g
Distilled water	1000 ml
pH	8

IDENTIFICATION OF BACTERIA

Isolates were identified on the basis of morphology, biochemical characteristics and 16S rRNA sequencing. Results were interpreted according to Bergey's Manual of Determinative Bacteriology, 8th edition (Buchanan and Gibbons, 1974) and NCBI data base.

Morphological, biochemical, and physiological characterization

Morphological characteristics were determined on the basis of colony morphology (color, shape, elevation, margins, and odor) and Gram's staining behavior. Physiologically, the isolates were studied for optimum temperature, pH, respiration using Gas Generating Kit, (Oxoid, U.K), growth rate and carbohydrate assimilation using Analytical Profile Index (API® 50CHB, BioMérieux® France).

Sequencing of 16s rRNA and phylogenetic analysis

The molecular signatures of the isolate were studied by partial sequencing of 16S rRNA gene and comparing it with known sequences. Bacterial genomic DNA was extracted according to a protocol modified from Janarthanan and Vincent (2007). The set of universal primers; 27F 5'AGAGTTTGATCCTGGCTCAG3'and 1492R 5'TACGGTTACCTTGTTACGACTT3' (Lane, 1991; Reysenbach and Pace, 1995) was used to amplify the desired sequence (Fig 3.3).

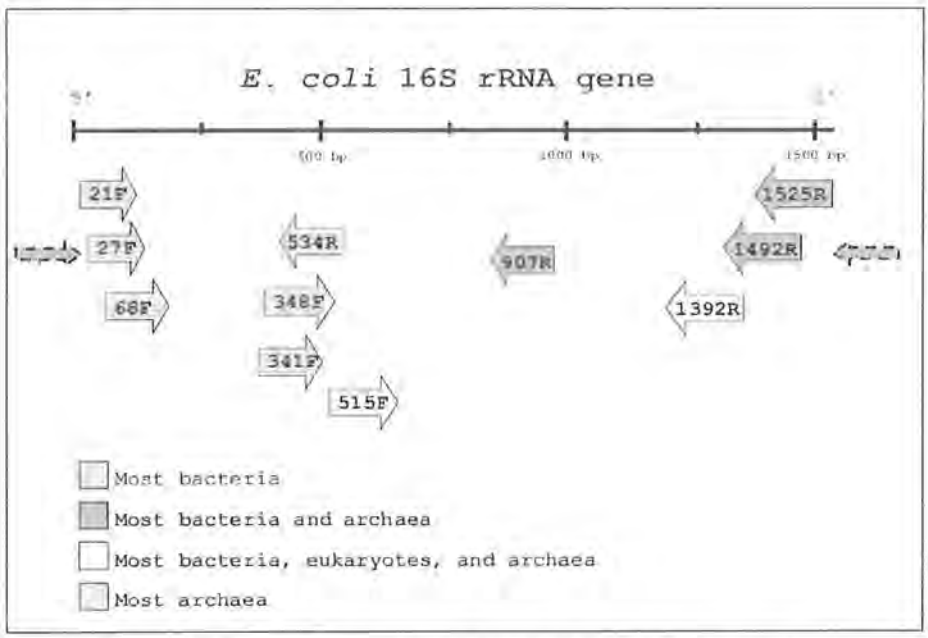


Fig 3.3. *E. coli* 16S rRNA gene: Adapted from (Christner, 2002)

PCR Reaction

The following reaction mixture was used to amplify 16S rRNA of isolates (Table 3.2). The total reaction volume was 50 µL for each reaction.

Table 3.2: Optimized PCR Conditions

No.	Ingredients	Concentration	Volume µL
1	Template (0.2 ng/ µL)	1ng	5
2	primers (10 µmol) each	1µL	1
3	dNTPs Mix (10 mmol)	1 µL	1
4	PCR Standard Buffer	5 µL	5
5	*Taq* DNA Polymerase	1 Unit	0.25
6	ddH_2O	35.75 µL	35.75
7	$MgCl_2$(50 nmol)	1 µL	1

Amplification

The following conditions were optimized for temperature, time and number of cycles for amplification of 16s rRNA using above mentioned set of primers:

No.	Step	Temperature (°C)	Time (Min)	Cycles
1	Denaturation	94	3	30
		94	1	30
2	Annealing	56	1	30
3	Extension	72	2	30
	Final Extension	72	10	1
	Hold	4	∞	1

The PCR products were analyzed on 0.9% agarose gel (Lane, 1991; Reysenbach and Pace, 1995) and subsequently stained with ethidium bromide (1 µg/ml in TAE buffer) (Williams, 2001). A 2 kb DNA marker (Trackit Invitrogen, United Kingdom) was used to compare the size of amplicons. Purification of amplicons was done by using QIAquick® PCR Purification Kit (Qiagen Ltd., Crawley, United Kingdom). After removal of primers and other reagents, the PCR product was visualized by running the samples on 0.9% (w/v) agarose gel for 35 minutes at 80 V and 400 mA. The bands were compared with Hyperladder I and IV (Bioline Ltd., London, United Kingdom). The DNA was quantified on Nano-Drop spectrophotometer (Nanodrop™

1000) by finding concentration at 230, 260 and 280 nm and checking contamination by 260:280 nm ratio and analyzed on 0.9% agarose gel.

Sequencing

The nucleotide sequence of the PCR purified fragments was determined by using the ABI PRISM® BigDye™ Terminator cycle sequencing kit (Applied Biosystems Inc., Warrington, United Kingdom). The same set of primers as those for amplification was used for sequencing, with an additional nucleotide (A) at its end to make it a nested primer. The reaction conditions for 25 cycles include:

No.	Step	Temperature °C	Time
1	Denaturation	96	10s
2	Annealing	50	5s
3	Extension	60	4 min
	Final Extension	60	4 min

Phylogenetic analyses

The nucleotide homology search was performed against the partial 16s rRNA sequences of 1050 base pairs using the nucleotide blast program "Blastn" (Altschul *et al.*, 1997) in non-redundant (nr) data base. The sequences having maximum score and percentage for sequence homology was retrieved from the NCBI GenBank and aligned by Clustal W program (Thompson *et al.*, 1994) in the Molecular Evolutionary Genetics Analysis Program (MEGA) version 4.0.1 (Tamura *et al.*, 2007). The regions in the sequences corresponding to the MRLBA1, MRLBA2, MRLBA3, MRLBA4, MRLBA5 and MRLBA6 sequence were retained and all non aligned sequence parts were trimmed. This alignment was used to neighbor joining (NJ) tree and finally construct the maximum parsimony (MP) tree with bootstrapping using 1000 replicates.

Sequence submission to Genbank and Accession Numbers

After interpretation, respective sequences were submitted to NCBI Genbank using *Sequin*, online software by NCBI and accession numbers were assigned to the submitted partial sequences.

Antibiotic Sensitivity

Antibiotic sensitivity of the isolates was performed was tested by Kirby Bauer Method (NCCLS, 1999; Jørgensen, 1999), using prepared disks (Oxoid, U.K) of different antibiotics as given below and incubating at 25 °C for 48 hours:

Groups of antibiotics	Antibiotics
β-Lactam	Penicillin
synthetic monocyclic β-lactam	Aztreonam
Glycopeptides	Vancomycin
	Tecoplanin
Aminoglycoside	Neomycin
	Streptomycin
Phosphonomycin	Fosphomycin

The zones of inhibition were measured (mm) using a manual scale.

Preservation of strains

Purified and characterized strains were preserved in glycerol at -20 and -70°C , microbe bank containing beads submerged in cryo-preservative solution (Microbank™ Richmond Hill, Ontario, Canada) at 0°C and lyophilized form (Labconco: Freezone 2.5 lyophilizer).

Growth curve

The time taken by the cells to become double in number or the generation time was determined from microbial growth curve. The log of the population number was plotted against time as:

Growth rate = specific growth rate x number of cells)

or $dx/dt=\mu.x$ (Widdel, 2007)

where x= biomass (g/L) or O.D

μ= specific growth rate [h^{-1}]

To calculate μ;

$$\ln Nt - \ln N_o = \mu(t - t_o) \quad \text{or} \quad \log_{10} N_t - \log_{10} N_o = (\mu/2.303)(t - t_o)$$

So $$\mu = ((\log_{10} N - \log_{10} N_0)\ 2.303) / (t - t_0)$$

Nutrient broth (300 ml) was prepared in 1000 ml Erlenmeyer flask. The pH of the medium was adjusted at 8 using 0.1 N NaOH and 0.1 N HCl. Then 10 ml of this stock was taken out in another 50 ml flask to use it as blank. Both the vessels were autoclaved at 121°C, under 15 lbs pressure for 20 min. A 24 hour old inoculum of 10 ml was added to 290 ml of nutrient broth and incubated at 25°C and 150 rpm for 7 hours. Samples, each of 5 ml, were collected after every 1 hour and their O.D and determined their O.D. and enzyme activity.

Screening of isolates with potential to produce alkaline phosphatase

The production of enzyme was carried out in basal medium (0.5% peptone, 0.2% glucose, 0.08 mol NaCl, 0.2 mmol $CaCl_2$, 0.02 mol NH_4Cl, 0.02 mol KCl, 1 mmol $MgSO_4$ and 0.004 mmol $ZnCl_2$). Sodium phosphate (0–200 µmol) and calcium (0–50 mmol) were added in the basal medium to study the regulation of phosphatase production as given in the text below. The effect of pH on growth and enzyme production was studied by growing cells in basal medium. The pH of the media was adjusted to 5 –13 using appropriate buffers (10 mmol) (Dhaked *et al.*, 2005). Growth was measured spectrophotometerically at 600 nm (Agilent 8354).

The *Pseudomonas* sp. MRLBA1 was selected for the production of alkaline phosphatase (Fig 4.13).

Alkaline phosphatase Assay

Activity of alkaline phosphatase was measured by absorbance (OD_{405}) to monitor the release of *p*-nitrophenol from *p*-nitrophenyl phosphate (*p*NPP) as mentioned by Dhaked *et al.*, (2005). A typical reaction mixture contained 300 µl of enzyme diluted in 1 mol diethanolamine buffer (pH 9.8), 0.5 mmol $MgCl_2$, 0.5 mmol $CaCl_2$, and 150 mmol *p*-nitrophenyl phosphate (*p*NPP), in a final volume of 3 ml.

Table 3.3: Alkaline phosphatase assay

Contents	Test	Blank
Enzyme	500 µl	-
Substrate (150 mmol)	1.5 ml	1.5 ml
$MgCl_2$ (0.5 mmol)	50 µl	50 µl
$CaCl_2$ (0.5 mmol)	50 µl	50 µl
Diethnolamine (1mol)	900 µl	1.4 ml
Total volume	3.0 ml	3.0 ml

The reaction performed at 37°C for 30 min was stopped by addition of 50 µl of 4 mol sodium hydroxide. One unit is defined as the amount of alkaline phosphatase which hydrolyses 1 µmol of *p*-nitrophenyle phosphate to *p*-nitrophenol in 1 min at pH 9.8 and 37°C. The calculations related to enzyme activity were calculated as: Phosphatase activity (U/ml), Protein concentration (mg/ml), Specific activity (U.ml/mg.ml), K_{cat} (V_{max}/K_m), Kinetic efficiency (K_{cat}/K_m).The quantification of enzyme activity was done by standard curve of *p*-nitrophenol (0–500 µmol) at 405 nm.

***p*-Nitrophenol**

A 20 mmol solution of *p*-nitrophenol (Sigma, U.K) was prepared in 1 mol diethanol amine buffer in 50 ml volumetric flask in the dark room and shifted to amber bottle to avoid photoreactivity. Dilutions of 10 to100 µmol were prepared in 1 mol diethanolamine buffer and O.D was recorded spectrophotometrically at 405 nm. The mean of triplicate sets of data was used to plot a standard curve. The amount of *p*-nitrophenol produced during reactions according to Beer–Lambert law was calculated.

Selection of the production medium

Effect of composition of medium on the production of alkaline phosphatase was studied by using the following media. All experiments were performed in triplicate. The pH of the media was adjusted to 7 –10 using appropriate buffers (10 mmol).

Medium 1. Dhaked *et al.*, (2005)

Contents	Amount
Peptone	0.5 %
Glucose	0.2 %
$(NH_4)_2SO_4$	3.0 g/L
$CaCl_2$	0.2 mmol
NaCl	0.08 mol
KCl	0.02 mol
NH_4Cl	0.02 mol
$MgSO_4$	1 mmol
$ZnCl_2$	0.004 mmol
Na_3PO_4	200 µmol
$Ca(NO)_3$	50 mmol

Medium 2: Prada *et al.*, (1996)

Contents	Amount
Peptone	0.5 %
Glucose	0.2 %
$CaCl_2.2H_2O$	0.2 mmol
NaCl	0.08 mol
$MgSO_4$	1 mmol
KCl	0.02 mol
$ZnCl_2$	0.004 mmol
Na_3PO4	200 µmol

The medium showing best results regarding production of enzyme was selected for further studies.

Production of crude enzyme

The production of crude enzyme was done in two steps

1. Inoculum preparation
2. Fermentation for alkaline phosphatase production

Preparation of Inoculum

The Inoculum was prepared by inoculating the 100 ml medium (Table 3.5) with 3 full loops of 24 hours growth on nutrient agar and allowed to grow at 25°C and 150 rpm.

Table 3.4: Composition of medium for preparation of Inoculum

Contents	Amount (g/L)
$(NH_4)_2SO_4$	3.0
$MgSO_4$	0.70
NaCl	0.50
$Ca(NO)_3$	0.40
KH_2PO_4	1.0
K_2HPO_4	0.1
Glucose	5.0
Na_3PO_4	0.002
$Ca_3(PO_4)_2$	0.05

Fermentation for production of alkaline phosphatase

Batch culturing was done in shake flask and various parameters were optimized for the production of alkaline phosphatase.

Batch culturing in shake flask

The production medium (100 ml) prepared in 250 ml Erlenmeyer flask was autoclaved, inoculated with 24 hours old inoculum (5%) and incubated in shaker incubator at 150 rpm at 18°C (except for temperature optimization experiment) for 144 hours. At regular intervals of 24 hours, the samples were collected and cells were separated by centrifugation at 13,000× g for 20 minutes (Dhaked *et al.*, 2005) at 4°C. The cells and supernatant were collected separately and stored at -20°C till the assay for alkaline phosphatase activity was performed. Alkaline phosphatase

activity was determined by method described before and total protein was estimated by the method of Lowry *et al.*, (1951).

Optimization of various parameters for the production of alkaline phosphatase in shake flask

Effect of pH on the production of alkaline phosphatase

Effect of pH on the growth of *Pseudomonas* sp. MRLBA1 and enzyme production was studied at pH 5, 6, 7, 8 and 9 at 18°C and 150 rpm. Alkaline phosphatase activity, change in pH and total protein concentration was measured in each sample.

Effect of temperature on the production of alkaline phosphatase

The production of alkaline phosphatase was carried out at 5, 10, 15, 20, 30 and 35°C at 150 rpm and pH 8.0. Alkaline phosphatase activity, change in pH and total protein concentration was measured.

Effect of incubation period on the production of alkaline phosphatase

Growth of *Pseudomonas* sp. MRLBA1 and the production of alkaline phosphatase were carried out for 144 hours at 18°C, pH 8 at 150 rpm. The samples were collected after every 24 hours to check the production of alkaline phosphatase. Alkaline phosphatase activity, change in pH and total protein concentration was determined.

Effect of size of inoculum on the production of alkaline phosphatase

The inocula of different size (1, 2, 3, 4 and 5%) were used to study their effect on the production of alkaline phosphatase at at 18 °C, pH 8.0 and 150 rpm. Alkaline phosphatase activity, change in pH and total protein concentration was measured.

Effects of age of inoculum on the production of alkaline phosphatase

The inocula with of 24, 48, 72, and 96 hours were used to study the effect of their age on the production of alkaline phosphatase in medium adjusted at pH 8.0, incubated at 18 °C with 150 revolutions per minute. Alkaline phosphatase activity, change in pH and total protein concentration was measured.

PROTEIN ESTIMATION

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

Solution A

Na_2CO_3	1.0 g
NaOH (0.1N)	0.4 g
NaK tartarate	1.0 g
Distilled water	100 ml

Solution B

$CuSO_4.5H_2O$	0.5 g
Distilled H_2O	100 ml

Solution C

Solution A	25 ml
Solution B (fresh)	0.5 ml

Solution D

Folin phenol and distilled water	1:1

Procedure

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

Calculations

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

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

Determination of specific activity of crude extract

The specific activity of crude extract (mg/ml/min/mg protein) was determined by dividing the enzyme activity of crude extract by its protein content (U/mg).

Total viable count

Total viable counts of bacteria from inoculum and crude enzyme extracts of 24, 48 and 72 hrs were done by using the method of Sharpley (1960). One ml of inoculum was taken after desired incubation and serially diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) in sterilized normal saline (0.9 ml). Each dilution (100 µL) was spread on nutrient agar plates and incubated at 37°C for 24 hrs. The plates with 30-300 colonies were selected for calculations.

Calculations

No. of colonies in 1 ml of sample	= No. of colonies in 0.1 ml of sample x 10
Total viable count	= No. of colonies in 1ml x dilution factor of bacteria
Colony forming units (CFU)	= Cells per ml

PURIFICATION OF ALKALINE PHOSPHATASE

In present study, the *Pseudomonas* sp. MRLBA1 was cultured in shake flask for 48 hrs at optimized conditions. The following steps were carried out for purification of alkaline phosphatase.

Separation of bacteria from fermentation medium (crude extract)

After 48 hours of incubation, the culture was centrifuged (Kokusan Model H-251; Kokusan Ensinki Co., Japan) at 12,000 rpm for 30 minutes at 4°C. The supernatant was filtered through 0.4 micron Millipore filter and used as crude enzyme and stored at -20°C till further use. Alkaline phosphatase activity was determined in supernatant by the method given above.

Extraction of enzyme from periplasmic space

Freezing is another technique used for lysis of cells (Jang *et al.*, 2007). After filtration, the harvested cells were exposed to -70°C for 10 minutes to lyse the cells. The lysed cells were resuspended in lysis solution (1N NaOH, 10% SDS and

distilled H_2O; 200 mmol, pH 8.0) and incubated at 4 °C with stirring for 2 hours to release the enzyme freely present in the periplasmic space. After 3 washes of cells with alkaline lysis solution, the pooled extract was centrifuged at 12,000 rpm for 30 min at 4°C. The alkaline phosphatase activity of cell free lysate and resuspended lysed cells was determined by the method described earlier.

Ammonium sulfate precipitation

For precipitation of alkaline phosphatase, 100 ml of crude enzyme solution was saturated with 40% ammonium sulfate [$(NH_4)_2SO_4$]. After sufficient shaking, solution was placed in the cold room (4°C) for one hour and then precipitates were collected by centrifugation at 14000 rpm for 20 min at 4°C. Phosphatase activity was determined in the supernatant and in the precipitates. The salt (50, 60 and 70%) was added continually till no activity was observed in supernatant. The precipitates thus obtained were suspended in 20 mmol diethanolamine (pH 8.5).

Desalting (Dialysis)

The dialysis tubing was boiled in distilled water (4 L) containing sodium bicarbonate (2% w/v) for 3 hours followed by boiling for 10 minutes in 1 mmol EDTA (pH 8.0). The prepared tubing was stored in ethyl alcohol at 4°C and extensively washed by distilled water before use.

Ammonium sulfate precipitates were taken in a prepared dialyzing bag (12 kDa cut off) and placed in 2 L of diethanolamine (20 mmol, pH 9.0) containing 0.1% w/v ($ZnCl_2$, $CaCl_2$ and $MgCl_2$) at 4°C with stirring for 24 hours against 6 changes of buffer.

Gel Permeation Chromatography (Sephadex G-75)

In gel filtration, liquid inside the stationary phase (gel particles) is in equilibrium with a mobile phase (solution of proteins and butter). Smaller solute molecules (proteins) enter the pores of the gel particles while larger ones who cannot enter, travel very fast through "the interstitial spaces of the gel particles" or void volume (V_o). Smaller molecules that enter the gel pores move slowly through the column, and their elution

volumes are designated as Ve. The total volume (Vt) represents the sum of the external and internal volume within the beads (Ahmed, 2004).

Principle

Solute molecules are eluted in order of decreasing molecular weight i.e. larger molecules elute first while smaller remain entrapped in gel beads and come later.

Reagents

Sepahdex G-75

20 mmol diethanolamine buffer (pH 9.0)

Dextran blue solution (0.5% w/v)

12.5 mg of Dextran blue dissolved in 2.5 ml of distilled water

Procedure

About 10.0 g of Sephadex G-75 was soaked in 500 ml of 20 mmol diethnolamine buffer (pH 9.0) containing 1% sodium azide as bacteriostatic agent and was kept at room temperature for 72 hrs. After deaeration for 42 minutes, the gel was packed in a 0.9 × 60 cm column followed by a continuous washing with same buffer. The void volume was determined by using dextran blue (0.05% w/v). About 5 ml of the precipitated enzyme was applied to column and eluted with automatic fraction collector (Advantec SF-100). The fractions of 3 ml each were eluted at the rate of 1 ml/5 min and total protein (absorbance at 280 nm), enzyme assay and protein estimation by Lowry's method, was determined in each fraction. Active fractions were pooled and used in further study.

Ion exchange chromatography (DEAE-Cellulose)

The ionization of proteins is pH dependent because they are amphoteric in nature. At a pH identical to the protein's isoelectric point (pI), the net charge of a protein is zero. At a pH lower than pI, the protein is positively charged, and at a pH higher than pI, the protein is negatively charged. Thus, in anion exchange chromatography the fixed charges (stationary phase) are positive, and the displaceable charges (proteins) in the mobile phase are negative (Ahmed, 2004).

Principle

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

Reagents

Diethyl amino ethyl cellulose (DEAE-Cellulose)

Sodium chloride (100, 200, 300, 400 and 500 mmol)

Hydrochloric acid (0.5 mol)

Procedure

In first treatment, 15 g (w/v) of the anion exchanger (DEAE-Cellulose) was suspended in 500 mmol hydrochloric acid and was allowed to stand for 30 minutes. The supernatant was decanted and the exchanger was washed until the effluent was at pH 4.0 (intermediate pH). In second treatment, the exchanger was stirred into 15 volumes of 0.5 mol sodium hydroxide and allowed to stand for an additional 30 minutes. The second treatment was repeated using distilled water till the pH of effluent was neutral. Pretreated exchanger was placed in buffer (pH less than 4.5) and sonicated for 40 minutes. The exchanger was then titrated with the basic component of the same buffer (pH 8.0), filtered and suspended in fresh buffer to complete the pretreatment. After decanting the fines above the settled exchanger, fresh buffer was added to the exchanger so that the final volume of the slurry was 150% of the settled wet volume of the exchanger.

The slurry of exchanger was packed in column 1.5 x 30 cm (Pharmacia K 15/30, Sweden) and it was equilibrated overnight with 0.02 mol diethanolamine buffer (pH 8.6). The column was loaded with the enzyme which was then eluted with 50 ml of 0.02 mol diethanolamine buffer (pH 8.6) followed by step wise flush of NaCl gradients (0.1-0.5 mol). Using an automatic collector (Advantec SF-100), each fraction of 3 ml was eluted at the rate of 1 ml/5 min and subjected to absorbance at

280 nm, enzyme assay and protein estimation. The fractions with alkaline phosphatase activity, were pooled, lyophilized and used in further study.

Lyophilization

Lyophilization is a process based on the principle of sublimation. The desalted protein solution and fractions from column chromatography were concentrated by lyophilizer (Freezone 2.5, Labconco Corp. USA).

CHARACTERIZATION OF CRUDE ALKALINE PHOSPHATASE

Effect of temperature on the activity of crude alkaline phosphatase

The alkaline phosphatase activity of the crude enzyme was determined after incubating the crude extract at temperatures (0, 4, 10, 15, 20, 25, 30, 37, 40, 45, 50, 55 and 60°C) for 1 hour and then the remaining activity was determined under the standard assay conditions.

Effect of pH on the activity of crude alkaline phosphatase

The effect of pH on the activity of crude enzyme was studied by incubating the enzyme in the buffers (200 mmol) of different pH (sodium glycine, pH 11.0–12.0; diethanolamine, pH 9.0–10.0; Tris-HCl, pH 8.0; phosphate buffer, pH 6.0-7.0; and Acetate buffer, pH 4.0-5.0) for 1 hour. The remaining activity of alkaline phosphatase was determined under standard assay conditions.

CHARACTERIZATION OF PURIFIED ALKALINE PHOSPHATASE

The purified alkaline phosphatase was then characterized for activity and stability of enzyme at different temperatures, pH, metal ions, inhibitors and solvent (glycerol).

Effect of temperature on the activity of purified alkaline phosphatase

Activity of the purified alkaline phosphatase was determined by incubating the enzyme at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55 °C for 1 hour and then the remaining activity was measured under the standard assay conditions. The percentage stability was also calculated.

Effect of pH on the activity of purified alkaline phosphatase

The effect of pH on the activity of alkaline phosphatase was observed by incubating the purified enzyme in the buffers (200 mmol) of different pH (sodium glycine, pH 11.0–12.0; diethanolamine, pH 9.0–10.0; Tris-HCl, pH 8.0; phosphate buffer, pH 6.0-7.0; and Acetate buffer, pH 4.0-5.0) for 1 hour at 37°C. The remaining activity of alkaline phosphatase was determined under standard assay conditions.

Effect of metal ions on the activity of purified alkaline phosphatase

The effect of metal ions on the activity was examined by assaying the remaining activity after incubating the enzyme with 1 mmol metal salt solutions ($CaCl_2$, $ZnCl_2$, $MgCl_2$, $HgCl_2$, $CuCl_2$, $FeCl_3$ and KCl) for 1hour at 37°C.

Effect of organic solvent on the activity of purified alkaline phosphatase

The residual alkaline phosphatase activity was determined after incubating the purified enzyme in the presence of different concentrations (0, 10, 20, 30, 40, 50, 60, 70, 80 and 90%) of glycerol for 1 hr, under the standard assay conditions.

Effect of inhibitors on the activity of purified alkaline phosphatase

Effect of inhibitors such as potassium ferricyanide and potassium ferrocyanide was determined. The remaining alkaline phosphatase activity was determined after incubating the reaction mixture [45 µL of enzyme, 1.48 ml of substrate (*p*NPP), and 1.48 mL of inhibitor (0.5 mmol)], for 1 hour at 37°C.

Determination of the kinetic constants and inhibition: K_m and V_{max}

The concentration of substrate at which the enzyme has half of its maximum velocity (V_{max}) during hydrolysis, is called Michaelis constant (K_m). The K_m and V_{max} were calculated from the Eadie (1942) and Hofstee (1952), plot.

Procedure

A fixed quantity of the purified alkaline phosphatase was incubated for 15 minutes with varying amounts of the substrate solution (ranging from 0 to 20 mmol) at optimum temperature and pH of the enzyme. The reciprocal of enzyme activity (V) was plotted against the reciprocal of substrate (S) concentration. K_m was calculated from the graph.

Calculations

For Eadie-Hofstee plot, the calculations were made using the equation:

$$V_{max}\text{-}K_m(v/[S])$$

For inhibition studies; the 1/V was plotted against 1/[S], and a straight line with its intercept on the ordinate ($1/V_{max}$) and abscissa ($K_m/[S]$) was obtained by Lineweaver and Burk (1934) plot.

The K_m was determined by the following equation:

$K_m=Y/X \times V$

Where $Y=1/V$

$X= 1/[S]$ and

$V= V_{max}$ (maximum velocity)

MOLECULAR WEIGHT DETERMINATION

The molecular weight of the purified protein was determined using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli *et al.*, (1970).

Principle

The SDS being an anionic detergent can bind to the protein molecule giving it a large negative charge. During polyacrylamide gel electrophoresis, the complexes from each of different proteins are separated on the basis of their molecular size. The molecular weight of an unknown protein can thus be determined by using marker proteins of known molecular weight.

Reagents

Acrylamide (30%)/ Bis-acrylamide (0.8%)

The acrylamide (30 g) and bis-acrylamide (0.8 g) were dissolved in distilled water and the volume was made upto 100 ml. The solution was filtered and stored at 4°C.

Resolving Gel Buffer (1.5 mol Tris-HCl) pH 8.8

The trizma base (36.3 g) was dissolved in distilled water. The pH was adjusted to 8.8 with HCl and the volume was made upto 200 ml.

Stacking Gel Buffer (0.5 mol Tris-HCl) pH 6.8

The trizma base (3.0 g) was dissolved in distilled water. The pH was adjusted to 6.8 with HCl and the volume was made up to 50 ml.

Sample Buffer [0.125 mol Tris-HCl (pH 6.8), SDS (4%), glycerol (20%), β-mercaptoethanol (10%)]

The Tris (12 g), glycine (57.6 g) and 10% SDS (40 ml) were dissolved and the final volume (1 liter) was made using distilled water. The buffer was diluted with distilled water (1:4) before use.

Sodium dodecyl sulfate (SDS 10%)

The SDS (10 g) was dissolved in distilled water and the volume was made up to 100 ml

Ammonium persulfate (APS) (20 mg/ml) (Freshly prepared)

Ammonium persulfate (0.06 g) was dissolved in 3.0 ml of distilled water.

N, N, N', N', tetra-methyl-ethylethylene-diamine (TEMED)

(Used as purchased)

Fixative Solution

Glacial acetic acid	70 ml
Methanol	400 ml
Distilled water	530 ml

Staining Reagent

Coomassie brilliant blue	2.5 g
Fixative solution	1000ml

Destaining Reagent

Methanol	50 ml
Glacial acetic acid	75 ml
Distilled water	875 ml

Procedure

Preparing and Loading the Gels

The plates were cleaned and dried at room temperature. The two glass plates were used to make a sandwich by using spacers (0.75 or 1.5 mm) between them and assembled to the gel caster.

For 50 ml of 10% resolving gel, the reagents were mixed as:

Resolving gel (10%)	Volume (ml)
30% acrylamide	16.7
1.5 mol Tris (pH 8.8)	12.5
10% SDS	0.5
Distilled water	19.8l
APS (10%)	0.5
TEMED	0.02

The reagents were mixed as given above in a 250 ml reagent bottle and deaerated for 5 minutes. The APS and TEMED were added and mixed by swirling. The gel was poured immediately between the two glass plates using a 10 ml micropipette by touching with the surface of the glass plate to avoid bubble formation. Keeping about 1.5 cm empty at the top for stacking gel, a 500 µl of isopropanol was layered to keep the gel layer from contact with air/oxygen and facilitate polymerization. The gel was allowed to polymerize at room temperature.

For 10 ml of 10% stacking gel, the reagents were mixed as:

Stacking gel (10%)	Volume (ml)
30% acrylamide	3.4
1.0 mol Tris (pH 6.8)	2.5
10% SDS	0.2
Distilled water	6.8
APS (10%)	0.2
TEMED	0.02

The mixture was deaerated for 5 minutes. The APS and TEMED were added and mixed by swirling.

After polymerization of resolving gel, the isopropanol was flicked off and the gel was washed with separating gel buffer. Finally the stacking gel was loaded on top of the separating gel. A clean and dry comb was inserted into the gel sandwich and allowed to polymerize.

Preparing and loading sample

About 20 µL of enzyme was added to 5 µl of sample buffer, placed in boiling water (100 °C) for 2 minutes, spun on microfuge to remove the debris and loaded onto the gel. A high range ColorBurst™ protein marker (30-220 kDa) (Sigma, USA) was used for comparison.

Running Gels

After adjusting the gel sandwich in electrophoresis tank, it was submerged with electrophoresis buffer and comb was removed gently and protein samples were loaded into the wells. The lid of electrophoresis tank was closed; the chamber electrodes attached to the power supply and run at a constant voltage of 100 V for 3 hours at room temperature.

Staining and destaining

After electrophoresis, the gel was placed in fixative solution for 10 minutes and then stained with Coomassie Brilliant Blue R-250 for 1 hour. The gel was destained and observed in gel documentation system.

Data analyses

The results from triplicate experiments for production and characterization of enzymes were analyzed for their statistical significance using ANOVA: Two Factor with Replication in MS Excel 2003.

RESULTS

Glaciers in Karakorum Range of mountains (Pakistan) are archives of psychrophilic bacteria. The laboratory freezers adjusted at subzero temperatures since decades represent the conditions like glaciers except atmospheric pressure. Bacterial alkaline phosphatase is usually produced during starvation and sporulation other than stress of pH and temperature etc. Three facultatively psychrophilic bacteria were isolated from freezer (-20°C) and six from glacial samples. A thermolabile alkaline phosphatase was purified and characterized from one of the glacial isolate identified as *Pseudomonas* sp. MRLBA1.

Sampling

Ice samples were obtained from -20°C freezer of Microbiology Research Laboratory; and soil, water and ice samples were obtained from glaciers (Passu, Batura, Shashkit and Hopper) in Northern Areas of Pakistan (Table 4.1). On average, glaciers were found at 36´27 and 74´52 at globe with average physical parameters as: height, 3000 m; Atmospheric pressure, ~790mb; Temperature range, -3 to 18°C; and pH 5.5 to 7.0 in the month of June, 2006.

Isolation of bacteria from samples obtained from glaciers and -20°C freezer

Out of 45 samples, a total of nine isolates were found capable of growing at low temperature (2-37°C). The glacial isolates include MRLBA1, MRLBA2, MRLBA3, MRLBA4, MRLBA5 and MRLBA6 isolated from different samples of ice, water and soil (sample code 1.6, 2.1, 4.4, 1.2, 4.5 and 1.3 (Table 4.1), respectively. The isolates from ice obtained from -20°C freezer of the laboratory were MRLBA7, MRLBA8 and MRLBA9 (sample code 4.9) (Table 4.1).

Table. 4.1 Sampling Data Sheet

I. Glacial Isolates								
A. PASSU GLACIER (23/05/2006)								
			GPS Coordinates					
Sr.#	Location	Transect name	N°	E°	Temp (°C)	Pressure (mb)	pH	Height (m)
1.1	Lake head	Stagnant lake water	36°27'24.4	074° 52'33.3	6	796mb	6	2815
1.2	Pond	Stagnant water(lpf2)	36°27'23.6	074° 52'34.0	15	793	6.5	2840
1.3	Glacier	Passu glacier ice	36°27'23.6	074° 52'33.8	-1	790	6	2870
1.4	Water fall	Water from glacier ice	36°27'21.8	074° 52'34.2	0	792	6	2840
1.5	Water	Lake water	36°27'21.0	074° 52'33	6	794	~6.0	2830
1.6	Ice	Lake ice	36°27'21.9	074° 52'32.8	0	794	~6.0	2830
B. BATURA GLACIER (24/05/2006)								
2.1	Pond	Batura Moraine pond water	36°21'34.1	074° 51'19.2	15	790	6	2800
2.2	Soil	Batura moraine dry soil	36°21'34.5	074° 51'20.2	18	790	6	2800
C. SHUSHKIT GLACIER (25/05/2006)								
3.1	Shshkit glacier	Glacier ice	36°21'38.6	074° 51'20.8	-2	790	6	2700
3.2	-do-	Glacier ice	36°21'38.5	074° 51'20.3	-3	792	6	2680
D. HOPPER GLACIER (26/05/2006)								
4.1	Pond water	Glaciel top pond	36°12'52.8	074°46'17.2	4	785	6	2900
4.2	Pond ice	Pond edge ice	36°12'52.0	074° 46'17.0	-1	784	6	2900
4.3	Soil	Pond soil	36°12'52.6	074° 46'17.2	4	784		2900
D. HOPPER GLACIER B								
4.4	Ice	Deep ice	36°12'52.8	074° 46'17.2	-2	779	5.5	2910
4.5	Soil	Surface soil	36°12'52.0	074° 46'17.0	1	778	6	2912
4.6	Soil	Deep soil	36°12'52.0	074° 46'17.0	-1	779	6	2910
D. HOPPER GLACIER C								
4.7	Soil	Lichens from rock surface	36°12'52.0	074° 46'16	9	770	6	2950
4.8	Soil	Soil	36°12'52.0	074° 46'19	9	772	6	2955
II. Non Glacial Isolates: MIROBIOLOGY RESEARCH LABORATORY (09/04/2006)								
4.9	Ice	- 20°C freezer	33° 44.8202'	073°8.5402'	-20			

GROWTH CHARACTERISTICS OF NON GLACIAL ISOLATES

Morphological, Physiological and Biochemical Characteristics

The morpho-physiological characteristics of non glacial isolates are summarized in Table 4.2. Colonies of all of the three isolates were white and the cells were Gram positive rods with spores. The isolate MRLBA7 was non-motile, chains present in late growth phase; grew at pH 4-9 and 0-37°C. The, isolate MRLBA8 was motile, grew at pH 4-10 and 4-40°C; and the isolate MRLBA9 was also motile, able to grow at pH 4-10 and 0-37°C.

Viable cell count and enumeration of bacteria in ice samples

A total of 37, 49 and 33 colonies of the strains MRLBA7, MRLBA8 and MRLBA9 respectively, were observed, when ice melt water (0.1 ml) was plated on nutrient agar medium and incubated at room temperature (20°C).

Biochemical characteristics

The three bacterial isolates showed activity of different enzymes in the cell free supernatant (Table 4.2). Amylase and catalase tests were positive and urease and Triple Sugar Iron tests were negative in case of all the three isolates. The Simmons citrate test was positive only in case of MRLBA8. The gelatinase, methyl red and nitrate reductase tests were positive for isolate MRLBA7 only. However, Voges-Proskauer test was positive both, for isolate MRLBA7 and MRLBA8.

Table 4.2: Morpho-physiological and biochemical characterization for the identification of non-glacial Isolates

Characteristics	MRLBA7	MRLBA8	MRLBA9
Color	Off White	White	Creamy White
Margin and elevation	Irregular, flat elevation	Irregular form, lobate margin	Smooth entire margin, convex
Gram's staining	+	+	+
Shape	Cocco-bacilli	Rods	Long rods
Spore	+	+	+
Motility	-	+	+
pH range			
Lower limit	4	4	4
Upper limit	9	11	10
Temperature limits			
Lower limit (°C)	0	4	0
Upper limit (°C)	37	40	37
Biochemical tests			
Amylase	+	+	+
Catalase	+	+	+
Gelatinase	+	-	-
Simmon Citrate	-	+	-
Triple sugar Iron	-	-	-
Urease	-	-	-
Methyl Red	+	-	-
Voges-Proskauer	+	+	-
Nitrate Reductase	+	-	-
Identified microorganisms	*Bacillus subtilis*	*Bacillus licheniformis*	*Bacillus megaterium*

Optimization of pH and temperature for non-glacial isolates

Bacillus subtilis MRLBA7 was able to grow in pH range 4-9, with optimum growth (OD_{600} 2.5) at pH 7 at 25°C. For temperature optimization, *Bacillus subtilis* MRLBA7 started to grow after 48 and 36 hours when incubated at 0 and 4°C respectively, but started to grow readily after 12 hours when incubated at higher temperatures 10, 25, 30 and 37°C. The optimum growth (OD_{600} 2.9) was observed at 25°C; where its growth was observed after 6 hours of incubation and showed exponential growth after 12 hours up to 60 hours (Fig 4.1).

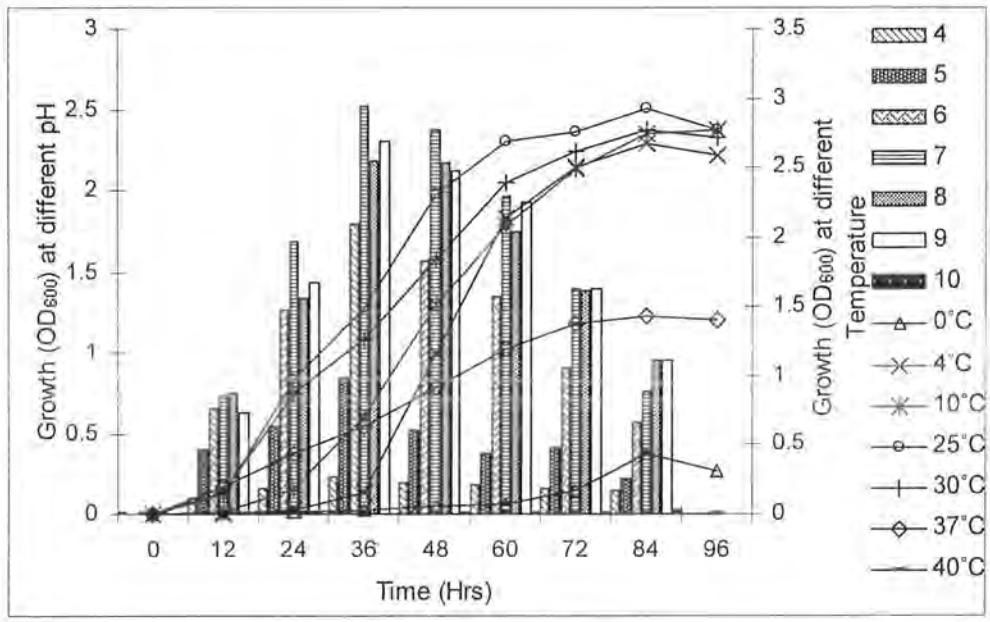


Fig 4.1: Optimization of pH and Temperature: *Bacillus subtilis* MRLBA7

Bacillus licheniformis MRLBA8 was able to grow at pH 4-11, with optimum growth (O.D. 2.9), at pH 9. For temperature, *Bacillus licheniformis* MRLBA8 started to grow after 96 and 36 hours when incubated at 0 and 4°C, respectively. However, the strain exhibited its growth after 12 hours when incubated at 10 and 25°C and 6 hours when incubated at 30, 37, 40 and 48°C. Maximum growth (O.D. 4.3) was observed at 37°C; where it started its growth after 6 hours of incubation and predominantly showed its start of lag phase after 10 hours that lasted till 72 hours. The stationary phase continued till recorded time of 96 hours (Fig 4.2).

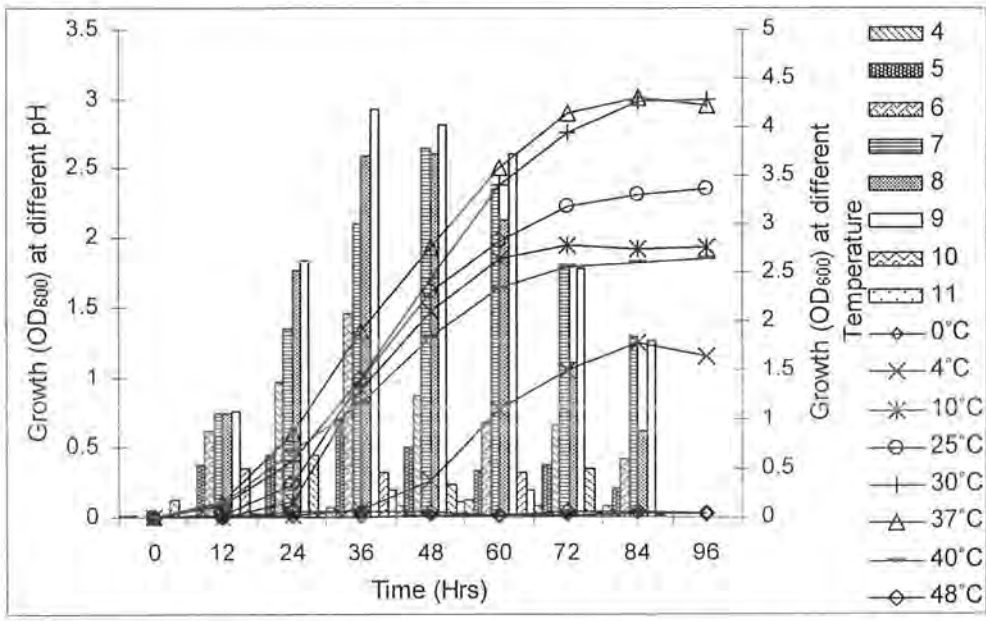


Fig 4.2: Optimization of pH and Temperature: *Bacillus licheniformis* MRLBA8

Bacillus megaterium MRLBA9 was able to grow at pH 4-10, with optimum growth (O.D 2.4) at pH 8. For temperature, *Bacillus megaterium* MRLBA9 started to grow after 48, 24, 18, 12, 4, 6 hours when incubated at 0, 4, 10, 25, 30, 37 and 40°C respectively. The maximum growth (O.D. 3.6) was observed at 30°C; where it started its growth after 4 hours and predominantly showed its start of log phase after 12 hours that lasted up to 72 hours where stationary phase was reached that continued till 96 hours (Fig 4.3).

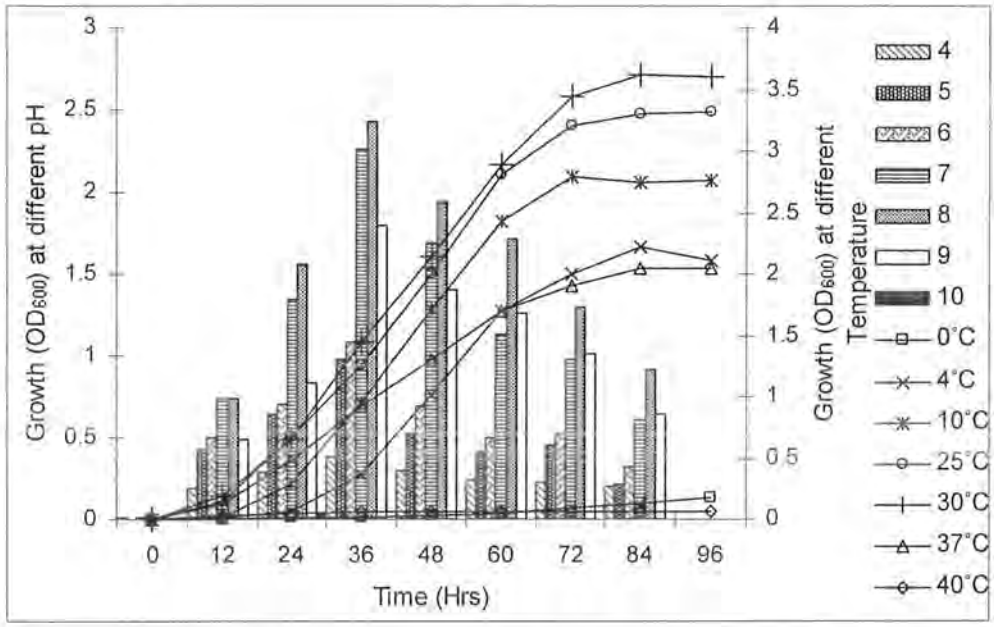


Fig 4.3: Optimization of pH and Temperature: *Bacillus megaterium* MRLBA9

Antibiotic Sensitivity of non-glacial bacteria

Seven β-lactam antibiotics (cell wall synthesis inhibitors and protein synthesis inhibitors) were tested (in duplicate) against spore forming Gram positive psychrotrophs (Table 4.3). *Bacillus subtilis* MRLBA7 revealed sensitivity to neomycin (26 mm), streptomycin (23 mm); vancomycin (9 mm), aztreonam (11 mm) and tecoplanin (12 mm) but was resistant to penicillin and fosphomycin. *Bacillus licheniformis* MRLBA8 was sensitive to neomycin (29 mm), streptomycin (25 mm), penicillin (21 mm), tecoplanin (11 mm) but resistant to aztreonam and fosphomycin. *Bacillus megaterium* (MRLBA9) was sensitive to neomycin (27 mm), streptomycin

(28 mm), tecoplanin (13 mm), aztreonam (9 mm) and fosphomycin (7 mm) but resistant to vancomycin and penicillin.

Effect of glycerol on growth and preservation of non-glacial isolates

There was no inhibitory effect of glycerol on viability of any of the isolates during preservation at freezing temperature for storage. Maximum growth was observed in the presence of 30, 50 and 40% of glycerol by *Bacillus subtilis* MRLBA7, *Bacillus licheniformis* MRLBA8 and *Bacillus megaterium* MRLBA9, respectively, when incubated for 72 hours at their optimum temperatures required for growth (Fig. 4.4).The isolates were inoculated in optimized, glycerol containing media and preserved at -20°C (Fig 4.5).

Table 4.3: Antibiotic sensitivity of non-glacial bacteria (MRLBA7-MRLBA9)

Class	Antibiotics (groups of antibiotics)	Zone of Inhibition (mm)		
		MRLBA7	MRLBA8	MRLBA9
Protein synthesis inhibitors	**Streptomycin** (aminoglycoside)	S (23)	S (25)	S (28)
	Neomycin (aminoglycoside)	S (26)	S (29)	S (27)
Cell wall synthesis inhibitors	**Vancomycin** (glycopeptide)	S (9)	S (20)	R
	Tetracycline (glycopeptide)	S (12)	S (11)	S (13)
	Penicillin (β-lactam)	R	S (21)	R (NA)
	Aztreonam (synthetic monocyclic β-lactam)	S (11)	R	S (9)
	Fosphomycin (phosphonomycin)	R	R	S (7)

R= Resistant, S= Susceptible

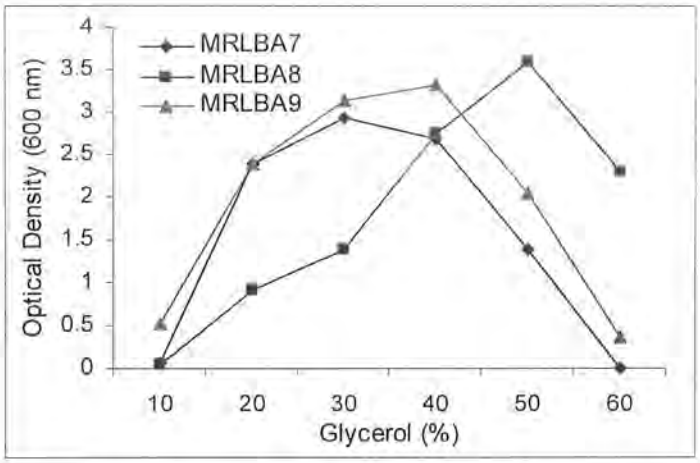


Fig 4.4: Growth pattern of *Bacillus subtilis* MRLBA7, *B. licheniformis* MRLBA8 and *B. megaterium* MRLBA9 in different concentrations of glycerol

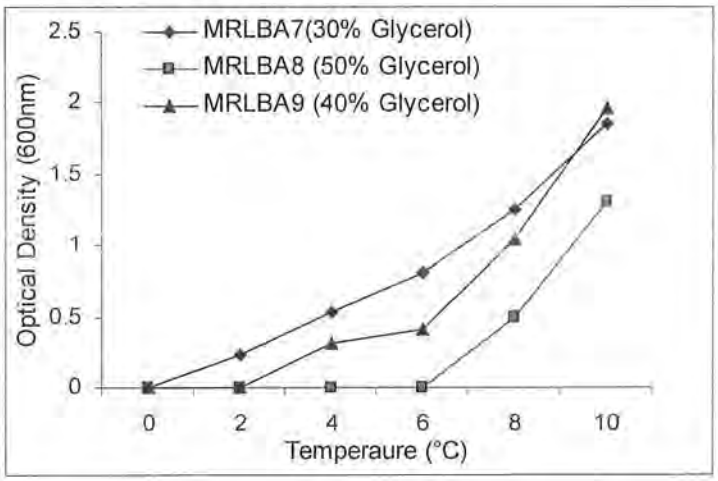


Fig 4.5: Viability of *Bacillus subtilis* MRLBA7, *B. licheniformis* MRLBA8 and *B. megaterium* MRLBA9 at low temperature (0-10°C) in presence of glycerol

GLACIAL BACTERIA

Morphological, Physiological and Biochemical Characteristics

All of the glacial isolates were Gram negative rods except MRLBA5 that was Gram variable short rod exhibiting colonies of yellow color and coccus shape in the late stationary phase of growth. None of the glacial isolate exhibited sporulation and motility. Isolates MRLBA1, MRLBA2 and MRLBA6 were strictly aerobic while MRLAB3, MRLBA4 and MRLBA5 could grow in absence of oxygen as well. The morpho-physiological characteristics of glacial isolates are summarized in Table 4.4.

Biochemical tests for glacial isolates

The six glacial isolates showed activity of different enzymes in the cell free supernatant (Table 1). Isolate MRLBA1 was positive for amylase, catalase, urease and Voges Proskauer tests, isolate MRLBA2 was positive for catalase, Simmon citrate, urease and Voges Proskauer tests, isolate MRLBA3 was positive for amylase, catalase and Voges Proskauer tests, MRLBA4 positive for catalase, urease and Voges Proskauer tests, MRLBA5 positive for amylase, catalase and Voges Proskauer tests, and MRLBA6 positive for amylase, triple sugar iron, urease and nitrate reductase tests (Table 4.4).

Effect of pH and temperature on growth of bacteria isolated from glaciers

The lower and upper limits of the pH required for growth of 6 selected isolates was observed as; 4-11 (MRLBA1); 4-10 (MRLBA2); 5-10 (MRLBA3); 5-10 (MRLBA4); 4-9 (MRLBA5); and 4-11 (MRLBA6). The lower and upper limits of temperature for growth of isolates was observed as; 2-20°C (MRLBA1); 4-30°C (MRLBA2); 4-35°C (MRLBA3); 4-37°C (MRLBA4); 4-37°C (MRLBA5) and 4-30°C (MRLBA6) (Table 4.4).

Oxygen requirements for glacial bacteria

Isolate MRLBA1, MRLBA2 and MRLBA6 were obligate anaerobes while MRLBA3, MRLBA4 and MRLBA5 were capable of growing in anaerobic conditions as well (Table 4.4).

Identification by Analytical Profile Index (API)

The isolates were tested for their carbohydrate fermentation pattern using API kit and revealed the pattern accordingly (Table 4.5). All the glacial isolates i.e. MRLBA1, MRLBA2, MRLBA3, MRLBA4, MRLBA5 and MRLBA6 assimilated the carbohydrates including glycerol, D-arabinose, L-arabinose, D-ribose, D-xylose, D-glactose, D-glucose, D-fructose, D-mannose, inositol, D-mannitol, D-cellobiose, D-maltose, D-lactose, D-mallobiose, D-sucrose, D-trehalose, D-raffinose, gentiobiose, D-fucose, L-fucose, D-arabitol, L-arabitol, potassium gluconate, potassium 2-ketoglutarate and potassium 5- ketogluconate but none of them could assimilate the

carbohydrates like erythritol, L-xylose, D-adonitol, methyl β-D-xylopyranoside, L-sorbose, L-ramnose, dulcitol, D-sorbitol, methyl α-D mannopyrunoside, methyl α-D glucopyranoside, N-acetyl glucosamine, amygdaline, arabutin, asculin, salicin, inuline, D-melezitose, amidon, glycogen, xylitol, D-turanose, D-lyrose, D-tagatose.

Table 4.4: Morpho-physiological and biochemical properties of glacial Isolates

Characteristics	MRLBA1	MRLBA2	MRLBA3	MRLBA4	MRLBA5	MRLBA6
Color	white	white	white	white	yellow	white
Margin	smooth	smooth	smooth	smooth	smooth	smooth
Gram's staining	-	-	-	-	variable (- to +)	-
Shape	rod	rod	rod	rod	variable rod-coccus	rod
Spore	-	-	-	-	-	-
Motility	-	-	-	-	-	-
Oxygen utilization	aerobic	aerobic	facultative	facultative	facultative	aerobic
pH range						
Lower limit	4	4	5	5	4	4
Upper limit	11	10	10	10	9	11
Temperature limits						
Lower limit (°C)	2	4	4	4	4	4
Upper limit (°C)	30	30	35	37	37	30
Biochemical tests						
Alk. phosphatase	+	+	-	-	-	+
Amylase	+	-	+	-	+	+
Catalase	+	+	+	+	+	-
Gelatinase	-	-	-	-	-	-
Methyl Red	-	-	-	-	-	-
Nitrate Reductase	-	-	-	-	-	+
Simmon citrate	-	+	-	-	-	-
Triple sugar Iron	-	-	-	-	-	+
Urease	+	+	+	+	-	+
Voges-Proskauer	+	+	+	+	+	-

Table 4.5: Analytical Profile Index (API® 50CH)

Tests	MRLBA1	MRLBA2	MRLBA3	MRLBA4	MRLBA5	MRLBA6
Glycerol	+	+	+	+	+	+
Erythritol	-	-	-	-	-	-
D-Arabinose	+	+	+	+	+	+
L-Arabinose	+	+	+	+	+	+
D-Ribose	+	+	+	+	+	+
D-Xylose	+	+	+	+	+	+
L-Xylose	-	-	-	-	-	-
D-Adonitol	-	-	-	-	-	-
Methyl β-D-Xylopyranoside	-	-	-	-	-	-
D-Galactose	+	+	+	+	+	+
D-Glucose	+	+	+	+	+	+
D-Fructose	+	+	+	+	+	+
D-Mannose	+	+	+	+	+	+
Methyl α-D Glucopyranoside	-	-	-	-	-	-
L-Sorbose	-	-	-	-	-	-
L-Ramnose	-	-	-	-	-	-
Dulcitol	-	-	-	-	-	-
Inositol	+	+	+	+	+	+
D-Mannitol	+	+	+	+	+	+
D-Sorbitol	-	-	-	-	-	-
Methyl α-D Mannopyranoside	-	-	-	-	-	-
Amygdaline	-	-	-	-	-	-
Arbutin	-	-	-	-	-	-
N-Acetyl Glucosamine	-	-	-	-	-	-
Esculin	-	-	-	-	-	-
Salicin	-	-	-	-	-	-
D-Cellobiose	+	+	+	+	+	+

D-Maltose	+	+	+	+	+	+
D-Lactose	+	+	+	+	+	+
D-Melibiose	+	+	+	+	+	+
D-Sucrose	+	+	+	+	+	+
D-Trehalose	+	+	+	+	+	+
Inuline	-	-	-	-	-	-
D-Melezitose	-	-	-	-	-	-
D-Raffinose	+	+	+	+	+	+
Starch	-	-	-	-	-	-
Glycogen	-	-	-	-	-	-
Xylitol	-	-	-	-	-	-
Gentiobiose	+	+	+	+	+	+
D-Turanose	-	-	-	-	-	-
D-Lyxose	-	-	-	-	-	-
D-Tagatose	-	-	-	-	-	-
D-Fucose	+	+	+	+	+	+
L-Fucose	+	+	+	+	+	+
D-Arabitol	+	+	+	+	+	+
L-Arabitol	+	+	+	+	+	+
Potassium Gluconate	+	+	+	+	+	+
2-Ketogluconate	+	+	+	+	+	+
5-Ketogluconate	+	+	+	+	+	+

Antibiotic sensitivity of glacial isolates

Seven β-lactam antibiotics (cell wall synthesis inhibitors and protein synthesis inhibitors) were tested (in duplicate) against low temperature active bacteria isolated from glaciers (Table 4.6). Isolate MRLBA1 was found sensitive to neomycin (18 mm), streptomycin (22 mm); vancomycin (11 mm), aztreonam (24 mm) and tetracycline (25 mm) but was resistant to penicillin and fosphomycin. Isolate MRLBA2 was sensitive to neomycin (17 mm), streptomycin (24 mm), penicillin (32 mm), and tetracycline (16 mm) but resistant to aztreonam and fosphomycin. Isolate

MRLBA3 was sensitive to neomycin (23 mm), streptomycin (30 mm), tetracycline (24 mm), aztreonam (23 mm) and fosphomycin (49 mm) but resistant to vancomycin and penicillin. Isolate MRLBA4 was sensitive to neomycin (20 mm), streptomycin (28 mm), tetracycline (22 mm) and aztreonam (20 mm) but resistant to fosphomycin, vancomycin and penicillin. Isolate MRLBA5 was sensitive to neomycin (16 mm), streptomycin (22 mm), tetracycline (10 mm), vancomycin (24 mm) and penicillin (32 mm) but resistant to aztreonam and fosphomycin. Isolate MRLBA6 was sensitive to neomycin (18 mm), streptomycin (23 mm), tetracycline (19 mm) and penicillin (13 mm) and fosphomycin (22 mm) but resistant to aztreonam and vancomycin.

Table 4.6: Antibiotic sensitivity of glacial bacteria

Class	Antibiotics (groups)	Zone of Inhibition (mm)					
		MRLBA1	MRLBA2	MRLBA3	MRLBA4	MRLBA5	MRLBA6
Proteins synthesis inhibitors	**Streptomycin** (aminoglycoside)	S (22)	S (24)	S (30)	S (28)	S (22)	S (23)
	Neomycin (aminoglycoside)	S (18)	S (17)	S (23)	S (20)	S (16)	S (18)
Cell wall synthesis inhibitors	**Vancomycin** (glycopeptide)	S (11)	S (24)	R	R	S (24)	R
	Tetracycline (glycopeptide)	S (25)	S (16)	S (24)	S (22)	S (10)	S (19)
	Penicillin (β-lactam)	R	S (32)	R	R	S (32)	S (13)
	Aztreonam (monocyclicβ-lactam)	S (24)	R	S (23)	S (20)	R	R
	Fosphomycin (phosphonomycin)	R	R	S (49)	R	R	S (22)

R= Resistant, S= Susceptible

16s rRNA sequencing of glacial Isolates

The universal primers (27F′ and 1492R′) for the amplification of 16S rRNA were able to amplify the region giving ~1.1kb size fragment in all of the isolated strains like MRLBA1, MRLBA2, MRLBA3, MRLBA4, MRLBA5 and MRLBA6 (Fig 4.6). Amplicons visualized on 0.9% agarose gel with 1 X Tris acetate EDTA buffer at constant voltage of 80V (Fig 4.1). The absorbance ratios (A 260/280) of the preparations were in range of 1.80-1.90 (Nanodrop™ 1000) which was subsequently

sequenced for ~1.1kb fragment. The resulted nucleotide sequences were blasted using BLAST tool at National Centre for Biotechnology Information (NCBI) website to identify these strains on molecular level.

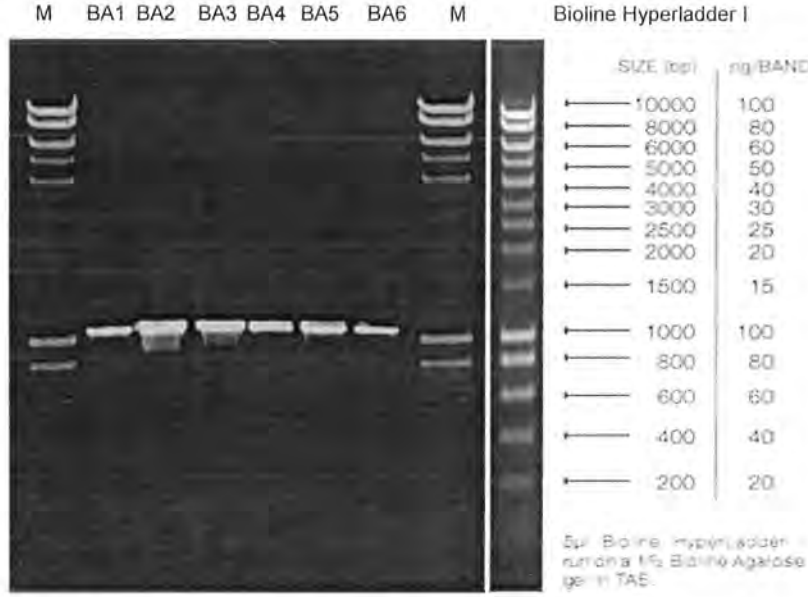


Fig 4.6: PCR amplification for 16S rRNA of glacial isolates on 0.9% agarose gel

Genotypic analyses confirmed strains MRLBA1, MRLBA2, MRLBA3 and MRLBA4 as *Pseudomonas* spp., MRLBA5 as *Arthrobacter* sp. and MRLBA6 as *Stenotrophomonas* sp. The 16S rRNA sequences of all these isolates were submitted to NCBI GenBank that assigned accession numbers as given in Table 4.7.

Table 4.7. Identity and accession numbers of selected strains

Isolate	Identified microorganisms	Accession Numbers
MRLBA1	*Pseudomonas* sp.	FJ415981
MRLBA2	*Pseudomonas* sp.	FJ415982
MRLBA3	*Pseudomonas* sp.	FJ415983
MRLBA4	*Pseudomonas* sp.	FJ415984
MRLBA5	*Arthrobacter* sp.	FJ415985
MRLBA6	*Stenotrophomonas* sp.	GU581318

Phylogenetic analyses of glacial isolates

Pseudomonas sp. MRLBA1

The Blastn search showed that *Pseudomonas* sp. MRLBA1 (FJ415981) has highest sequence similarity with the Antarctic soil isolate *Pseudomonas* sp. Nj-70 (AM409370) and Mingyong Glacier ice isolate *Pseudomonas* sp. MY1420 (EF062807) with 98% sequence identities and 99% sequence coverage with (e-value 0.0) (Table 4.32). The maximum parsimony analysis was performed with 1000 bootstrap replicates, with the values shown at the nodes. The scale bar represents 2 evolutionary steps (Fig 4.7).

Pseudomonas sp. MRLBA2

The Blastn search showed that *Pseudomonas* sp. MRLBA2 (FJ415982) has highest sequence similarity with the Antarctic soil isolate *Pseudomonas* sp. Nj-70 (AM409370), Mingyong Glacier ice isolate *Pseudomonas* sp. MY1420 (EF062807) and Pakistani glacier ice isolate *Pseudomonas* sp. MRLBA1 (FJ415981) with 98% sequence identities and 99% sequence coverage with (e-value 0.0) (Table 4.33). The maximum parsimony analysis was performed with 1000 bootstrap replicates, with the values shown at the nodes. The scale bar represents 2 evolutionary steps (Fig 4.8).

Pseudomonas sp. MRLBA3

The Blastn search showed that *Pseudomonas* sp. MRLBA3 (FJ415983) has highest sequence similarity with the Antarctic surface soil isolate *Pseudomonas sp.* NJ-22 (AM421975) and Mingyong Glacier ice isolate *Pseudomonas* sp. MY1404 (E082805) with 98% sequence identities and 99% sequence coverage with (e-value 0.0) (Table 4.34). The maximum parsimony analysis performed with 1000 bootstrap replicates, with the values shown at the nodes. The scale bar represents 0.5 evolutionary steps (Fig 4.9).

Pseudomonas sp. MRLBA4

The Blastn search showed that *Pseudomonas* sp. MRLBA4 (FJ415984) has highest sequence similarity with *Pseudomonas* sp. MRLBA2 (FJ415982) isolated from water

sample of Passu glacier, *Pseudomonas* sp. MRLBA1 (FJ415981) isolated from ice sample of Hopper glacier, *Pseudomonas sp.* NJ-70 (AM409370) isolated from soil sample of Antarctica and *Pseudomonas* sp. MY1420 (EF062807) isolated from ice sample of Mingyong Glacier, with 98% sequence identities and 99% sequence coverage with (e-value 0.0) (Table 4.35). The maximum parsimony analysis performed with 1000 bootstrap replicates, with the values shown at the nodes. The scale bar represents 2 evolutionary steps (Fig 4.10).

***Arthrobacter* sp. MRLBA5 (DSM 23633)**

The Blastn search showed that *Arthrobacter* sp. MRLBA5 (FJ415985) has highest sequence similarity with *Arthrobacter citreus* MVLI (FM955881) isolated from water of the Midre Lovenbreen Glacier, and *Arthrobacter citreus* (X80737) 96% sequence identities and 97% sequence coverage with (e-value 0.0) (Table 4.36). The maximum parsimony analysis performed with 1000 bootstrap replicates, with the values shown at the nodes. The scale bar represents 5 evolutionary steps (Fig 4.11).

***Stenotrophomonas* sp. MRLBA6**

On the basis Blastn search, *Pseudomonas* sp. MRLBA1 was found closely related to the *Stenotrophomonas maltophilia* NPQ01 (EU622536.1) and *Stenotrophomonas maltophilia* ISSDS-585 (EF620455.1) with 69% sequence identities and 92% sequence coverage with (e-value $1x10^{-32}$) (Table 4.37). The maximum parsimony analysis performed with 1000 bootstrap replicates, with the values shown at the nodes. The scale bar represents 0.5 evolutionary steps (Fig 4.12).

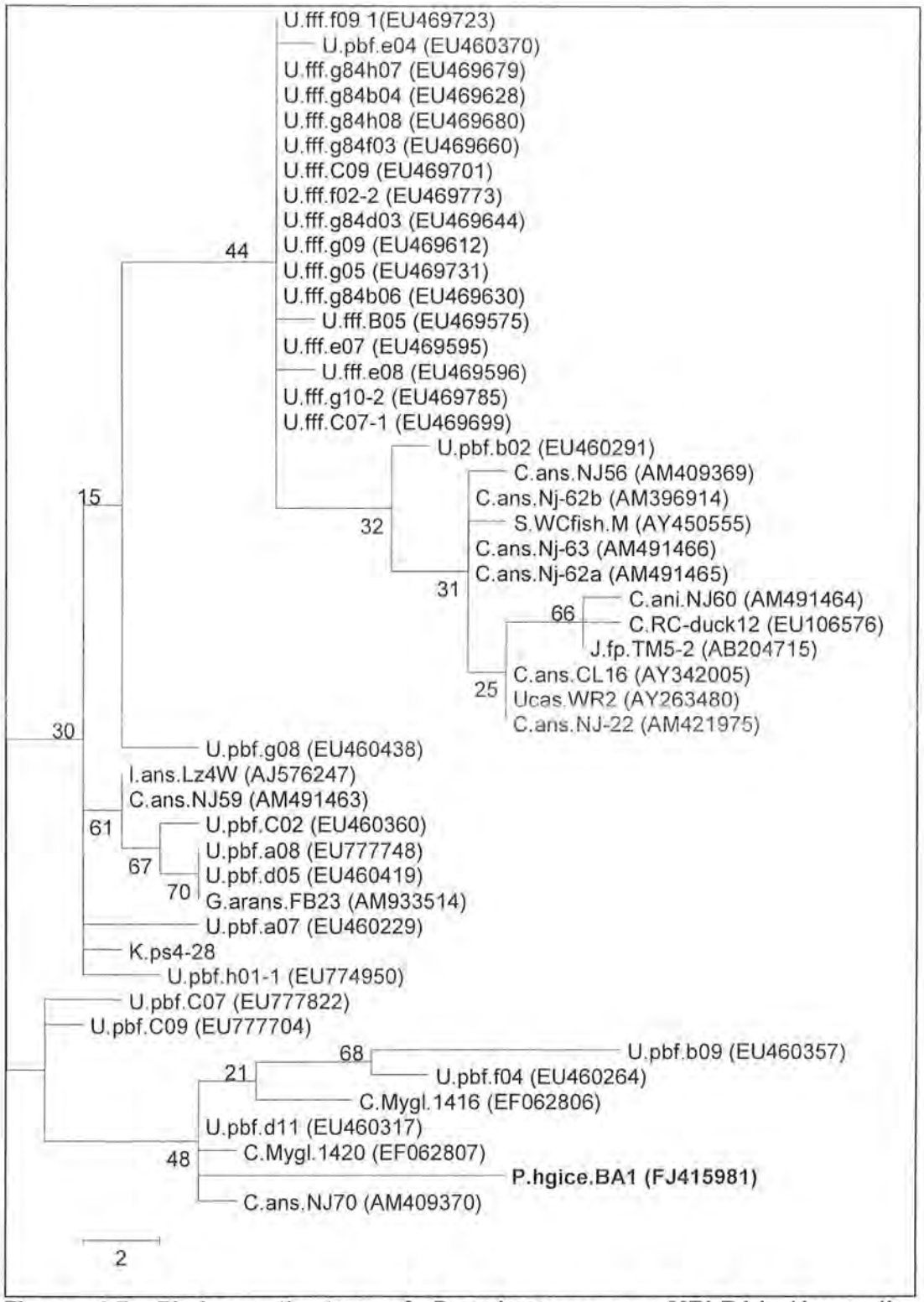


Figure 4.7: Phylogenetic tree of *Pseudomonas* sp. MRLBA1 (Appendix IV-1).

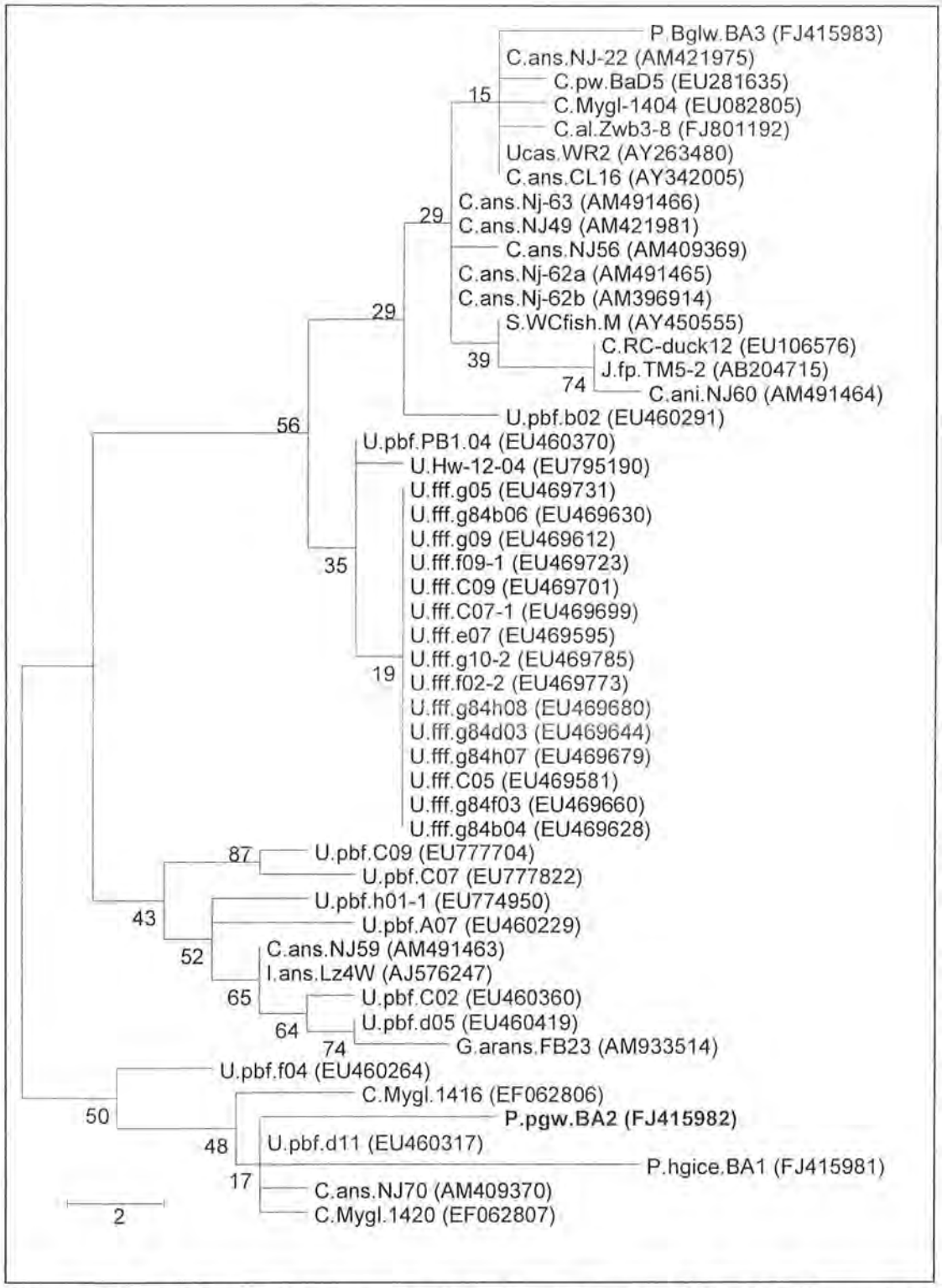


Figure 4.8: Phylogenetic tree of *Pseudomonas* sp. MRLBA2 (Appendix IV-2)

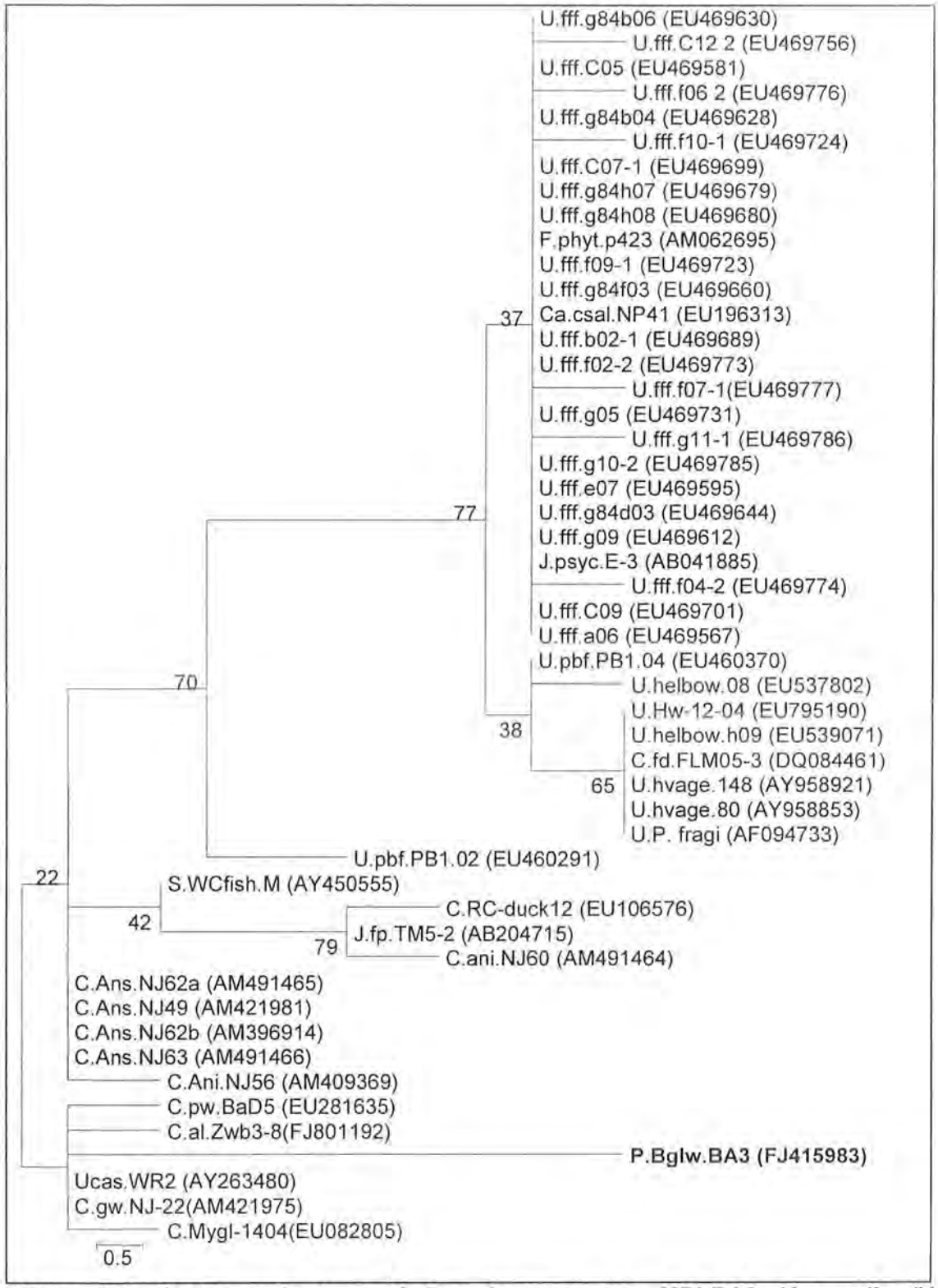


Figure 4.9: Phylogenetic tree of Pseudomonas sp. MRLBA3 (Appendix IV-3)

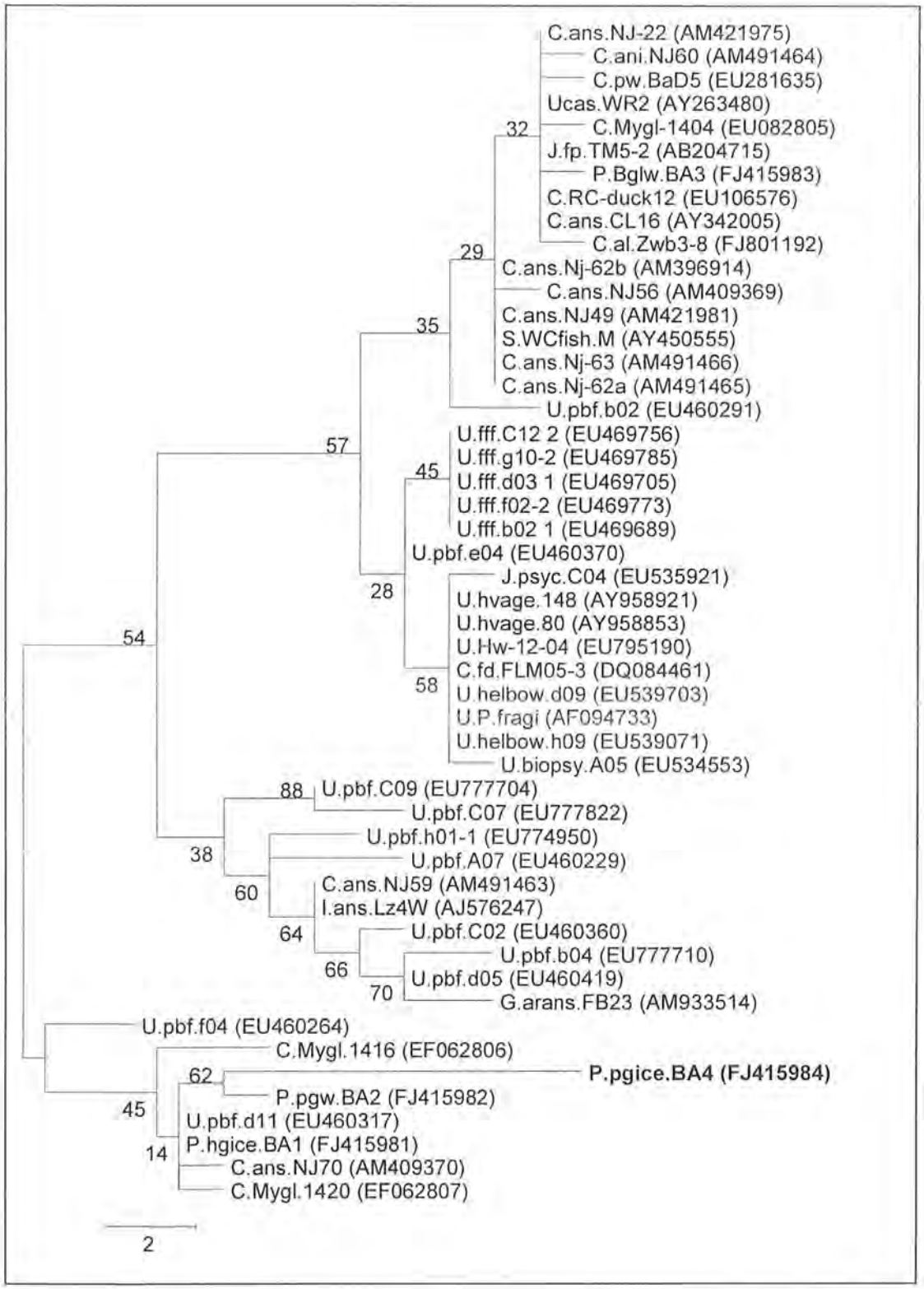


Figure 4.10: Phylogenetic tree of *Pseudomonas* sp. MRLBA4 (Appendix IV-4)

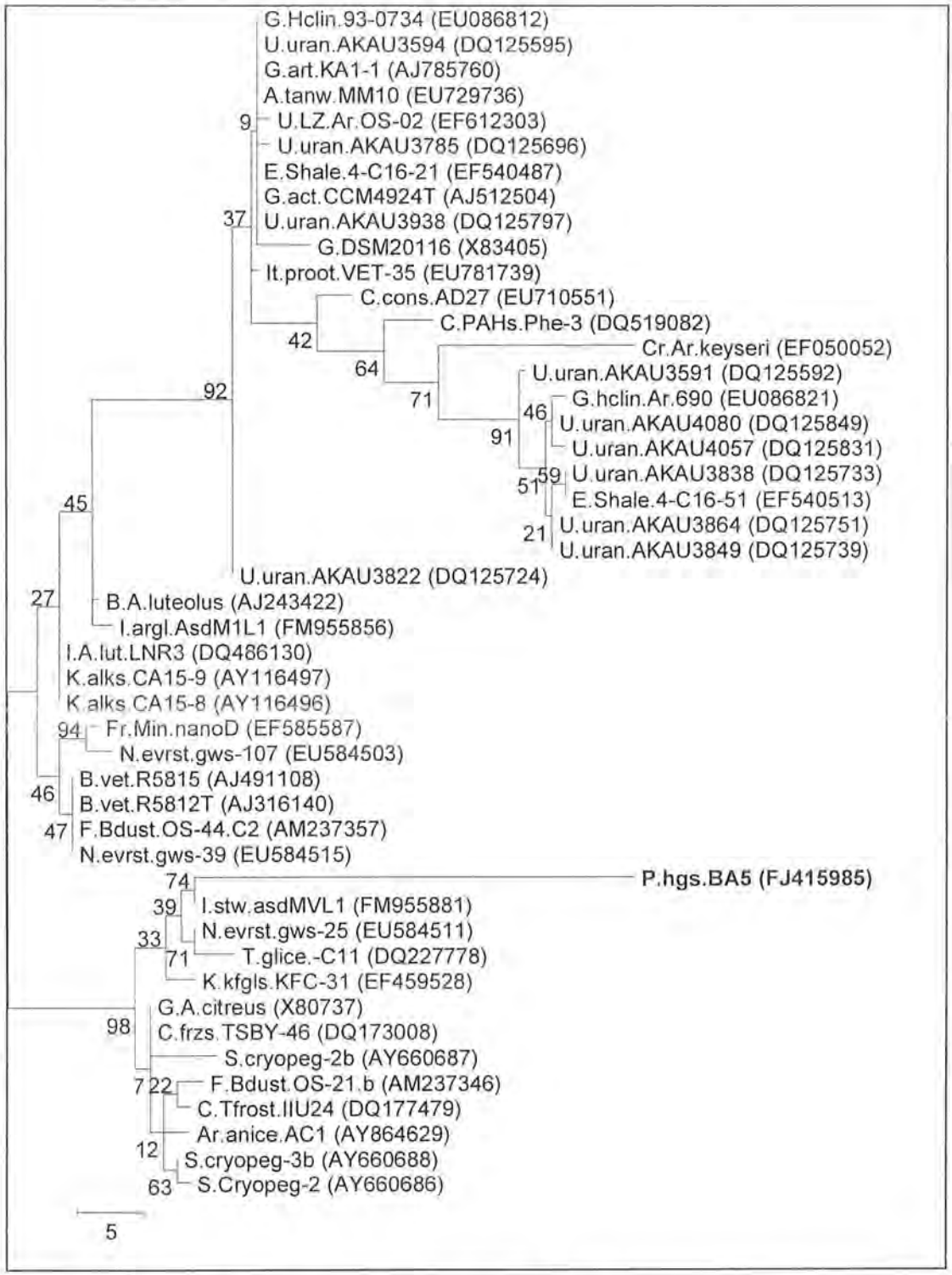


Figure 4.11: Phylogenetic tree of *Arthrobacter* sp. MRLBA5 (Appendix IV-5)

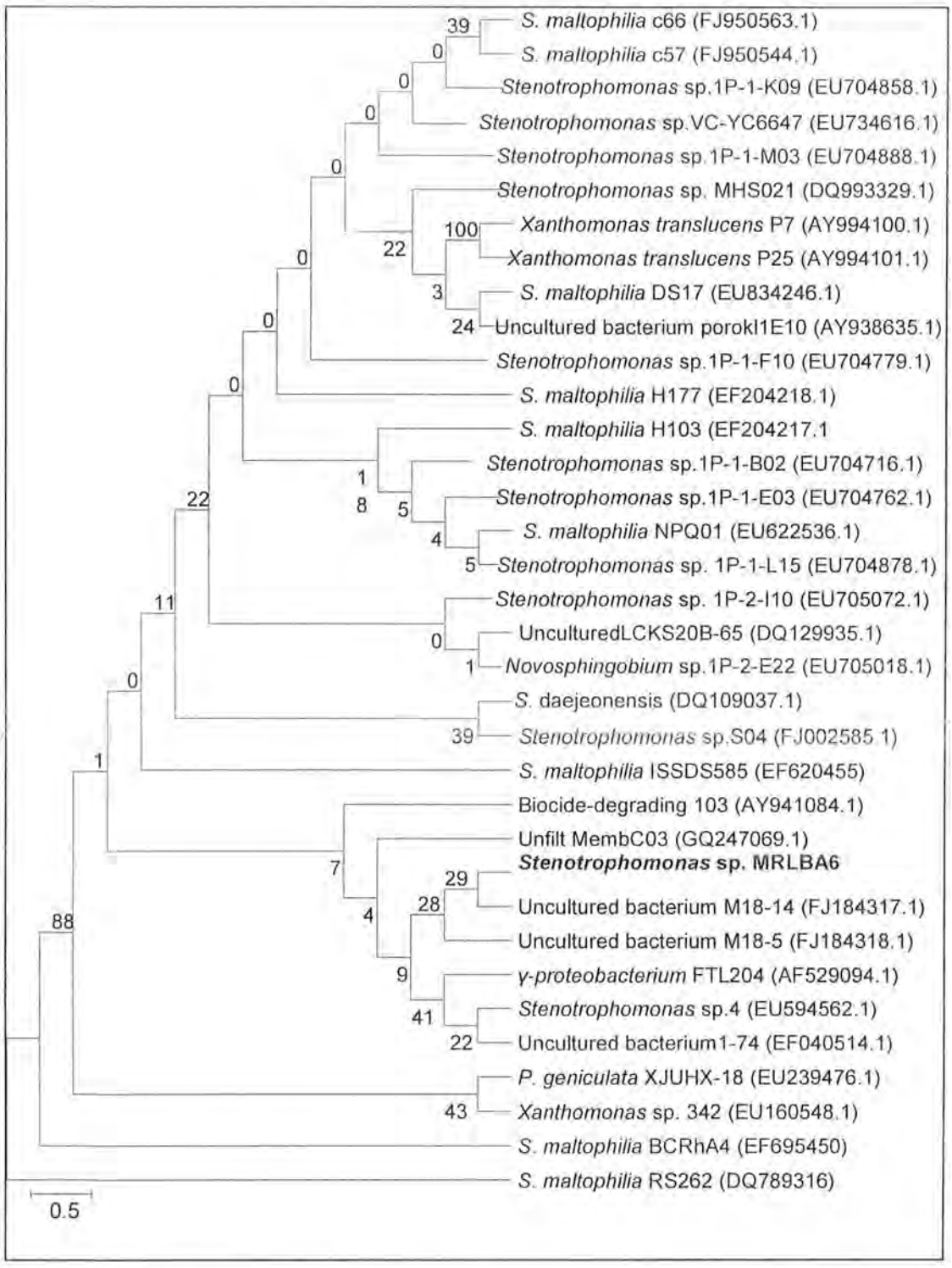


Figure 4.12: Phylogenetic tree of *Stenotrophomonas* sp. MRLBA6 (Appendix IV-6)

PRODUCTION OF ALKALINE PHOSPHATASE

All of the six glacial isolates were screened for the production of alkaline phosphatase using standard assay conditions. *Pseudomonas* sp. MRLBA1, *Pseudomonas* sp. MRLBA2 and *Stenotrophomonas* sp. MRLBA6 exhibited alkaline phosphatase activity in the medium however *Pseudomonas* sp. MRLBA1 showed significantly higher phosphatase activity ($p<0.017614$) both in supernatant (3.62 U/ml) and pellet (0.396 U/ml) (Fig 4.13); therefore it was selected for further study.

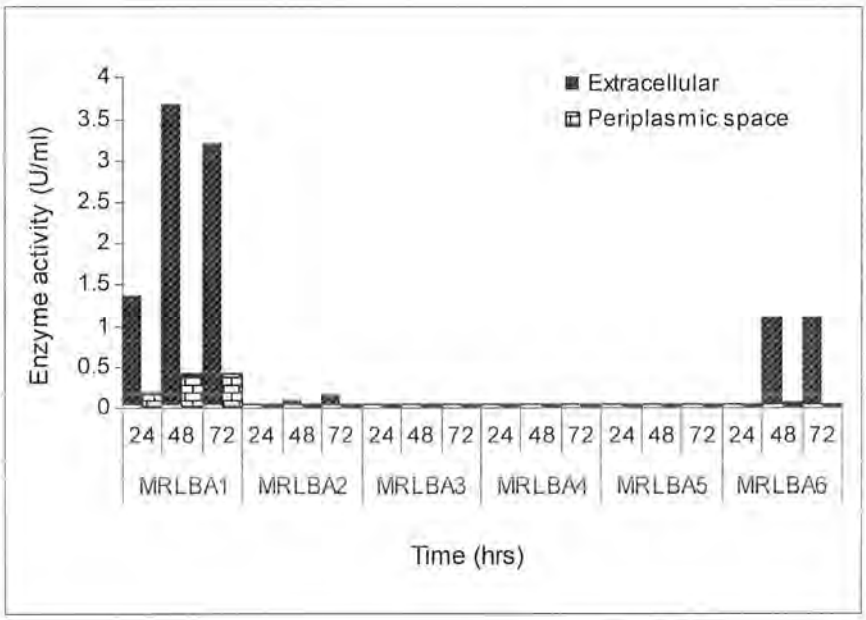


Fig 4.13: Screening for alkaline phosphatase activity in supernatant and periplasmic space of bacterial cells

Growth characteristics of *Pseudomonas* sp. MRLBA1

Optimization of pH

The *Pseudomonas* sp. MRLBA1 showed maximum growth (OD_{600}) 0.75, 1.38, 2.08, 1.91, 1.35, 0.85 and 0.60 after 12, 24, 36, 48, 60, 72 and 84 hours of incubation, respectively, at pH 8.0 and 25°C. Minimum growth was observed at pH 4 and pH 10 (Fig 4.14).

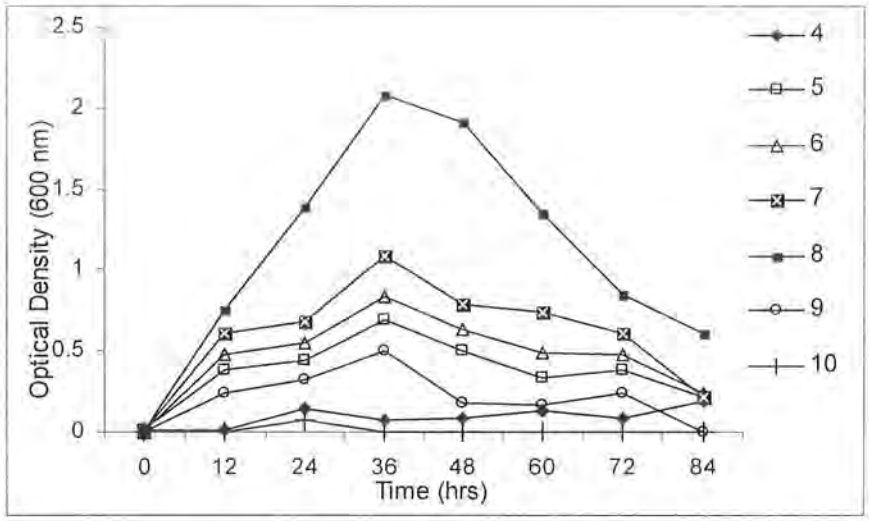


Fig 4.14: Optimization of pH for the growth of *Pseudomonas* sp. MRLBA1

Optimization of temperature

The *Pseudomonas* sp. MRLBA1 showed maximum growth (OD_{600}) 0.19, 0.97, 1.59, 2.21, 2.81, 3.08 and 2.93 after 12, 24, 36, 48, 60, 72 and 84 hours of incubation in nutrient broth, respectively, at 25°C pH 8.0. Minimum growth was observed after 0 and 30°C (Fig 4.15).

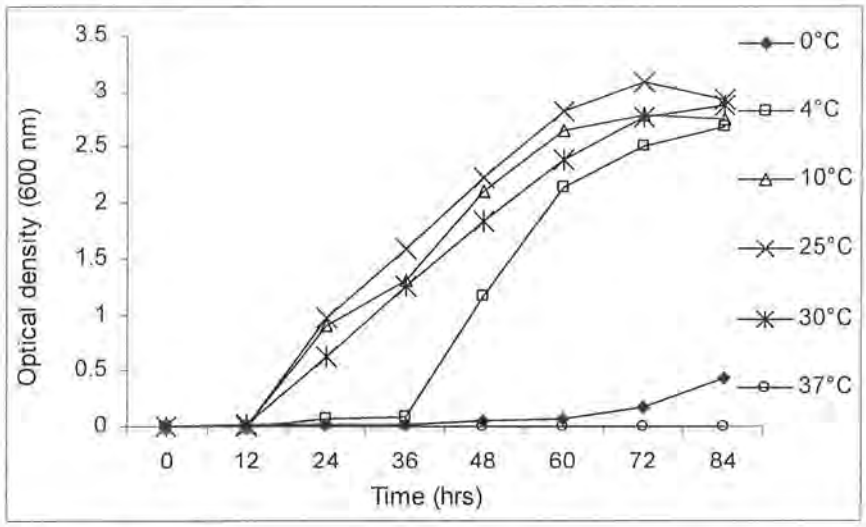


Fig 4.15: Optimization of temperature for the growth of MRLBA1

Growth rate of Pseudomonas sp. MRLBA1

For growth between 24 to 32 hours (exponential phase), the number of cells were found as;

$\mu = (6.73 - 3.57) \times 2.303) / (32-24) = \sim 1 \text{cell hour}^{-1}$

Growth rate= μ x biomass (g/L)

=1x6.4

$=6.4 \text{ g } L^{-1}h^{-1}$

Growth curve of Pseudomonas sp. MRLBA1

The growth of *Pseudomonas* sp. MRLBA1 was monitored under optimized conditions of pH (8.0) and temperature (25°C) spectrophotometerically at 600 nm. A plot of growth (O.D) versus time (hours), yielded a typical growth curve with a prolonged lag phase of about 10 hours. The exponential phase appeared as a steep curve after 24 hours and lasted up to 36 hours followed by a long stationary phase that lasted up to120 hours (Fig 4.16).

Growth of Pseudomonas sp. MRLBA1 *and the production of alkaline phosphatase*

The production of alkaline phosphatase was observed along with the growth curve of bacterium. Most of the phosphatase activity was secreted into the medium with very little activity bound to cell (Fig 4.16).

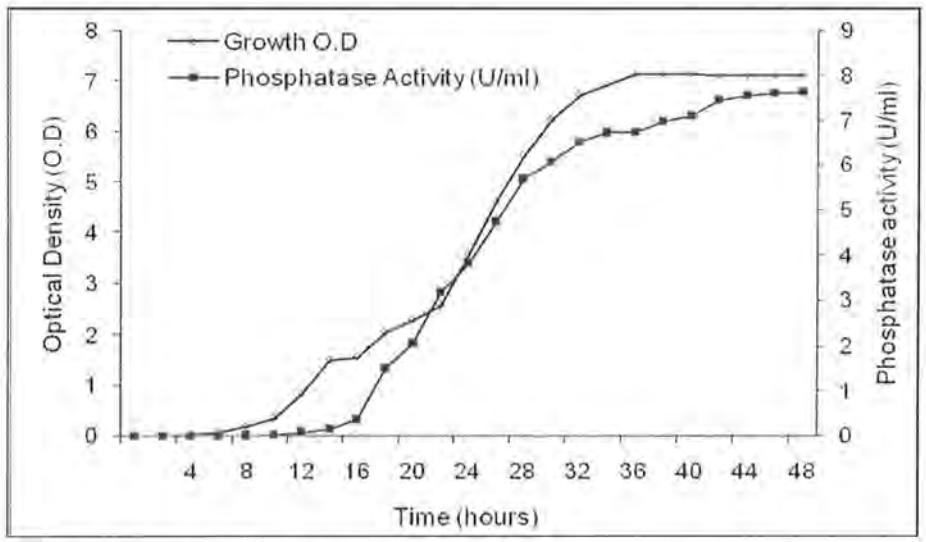


Figure 4.16: Production of alkaline phosphatase at different stages of bacterial growth

BATCH CULTURING IN SHAKE FLASK

Effect of incubation period

The inoculated production medium revealed specific activities of 4.42 U/mg (24hrs), 54.01 U/mg (48 hrs) and 59.43 U/mg after 72 hours when incubated at pH 8, 18°C for 72 hours and 150 rpm (p0.07) (4.17).

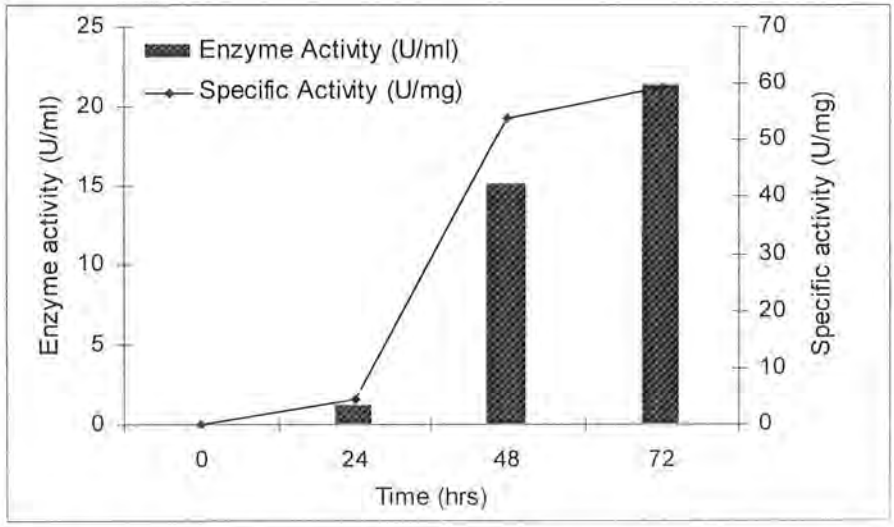


Fig 4.17: Effect of Incubation period on the production of alkaline phosphatase

Effect of temperature on the production of alkaline phosphatase

The maximum alkaline phosphatase was produced (22.41 U/ml) with specific activity (53.72 U/mg) at 18°C after 48 hrs of incubation ($P<0.0001$). At lower temperatures i.e. 4, 10 and 15°C, the production of enzyme (6.73, 11.13 and 14.57 U/ml) with specific activities of 28.56, 41.17 and 45.81 U/mg, respectively (Fig.4.10). The yield of enzyme was enhanced when harvested cells were exposed to -70°C for 10 minutes and harvested again after resuspension in 20 mmol Tris HCl (pH 8.0) (Fig 4.18).

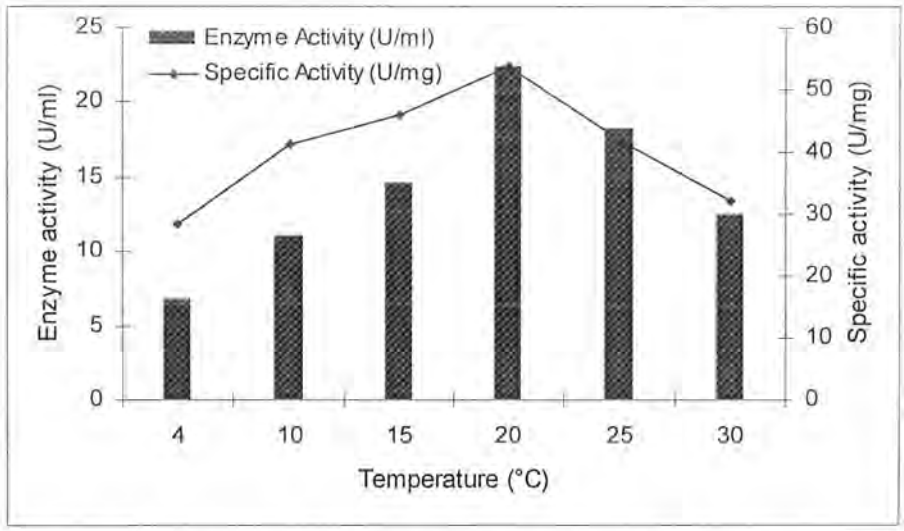


Figure 4.18: Effect of temperature on production of alkaline phosphatase

Effect of pH on the production of alkaline phosphatase

The *Pseudomonas* sp. MRLBA1 produced alkaline phosphatase maximally at pH 8.0 after 72 hrs of incubation at 18°C and 150 rpm with specific activity of 57.56 U/mg protein ($P<0.0001$). The production of enzyme (specific activity) was examined as 33.05, 44.32, 47.14, 56.28 and 35.39 U/mg at pH 5.0, 6.0, 7.0, 9.0 and 10.0 respectively (Fig 4.19).

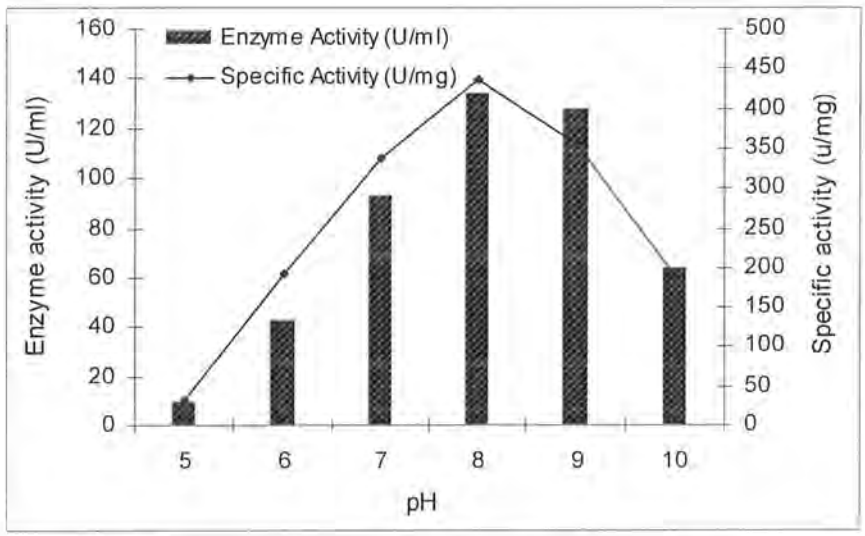


Fig 4.19: Effect of pH on production of alkaline phosphatase

Effect of size of inoculum on the production of alkaline phosphatase

The maximum alkaline phosphatase (specific activity; 77.02 U/mg) was produced in case of 5% inoculum whereas in case of 1, 10, 15 and 20% inoculum sizes, the specific activity was 38.71, 32.37, 18.85 and 12.7 U/mg respectively, with significant value ($p>0.001$) (Figure 4.20). The pH of the medium increased up to 8.3 starting from initial pH 8.0.

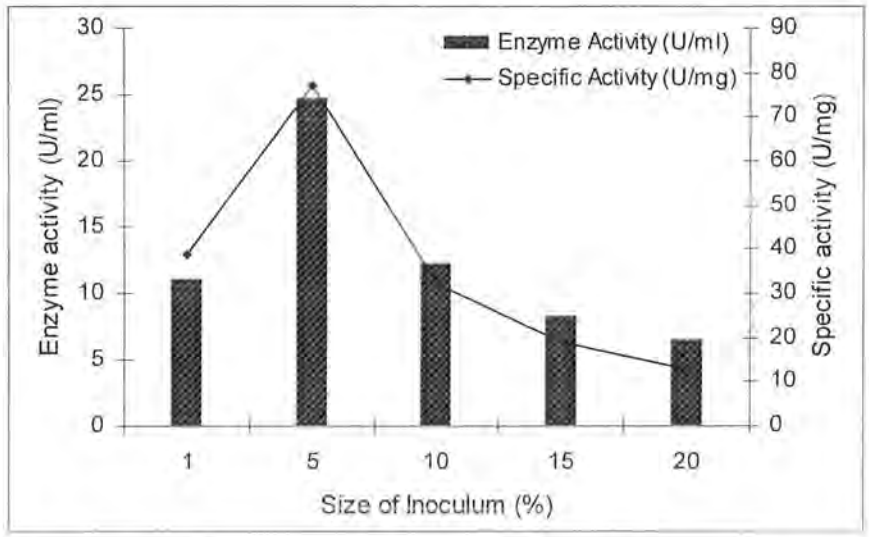


Fig 4.20: Effect of size of inoculum on production of alkaline phosphatase

Effect of age of inoculum on the production of alkaline phosphatase

The maximum production of alkaline phosphatase (56.42 U/mg) was observed in production medium inoculated with a 24 hour old inoculum after 48 hours of incubation at pH 8.0, 18°C, 5% inoculum and 150 rpm ($p<.001$). When 12, 48 and 72 hour old inocula were used, specific activities of phosphatase were recorded as 34.19, 39.53.12 and 16.8 U/mg respectively (Fig. 4.21).

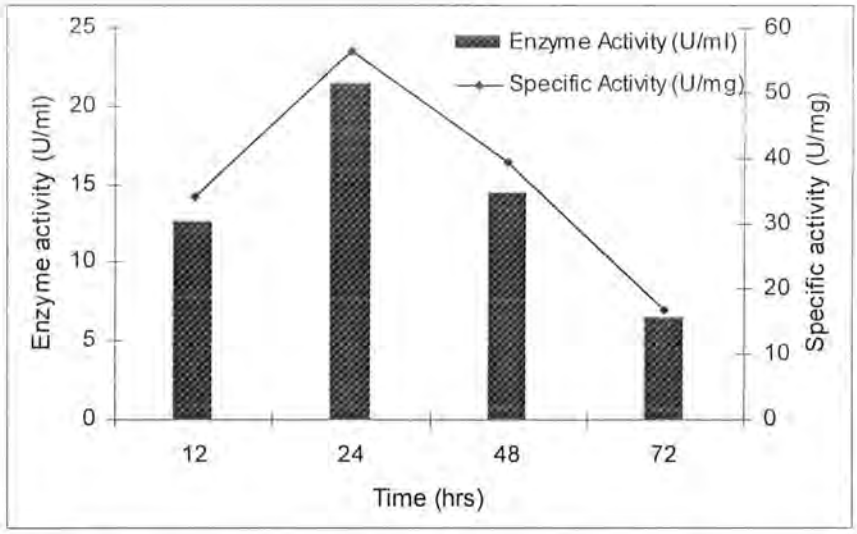


Fig 4.21: Effect of age of inoculum on the production of alkaline phosphatase

PURIFICATION OF ALKALINE PHOSPHATASE

Ammonium Sulfate precipitation

After saturation of the culture filtrate with 40% ammonium sulfate, the maximum pellet was obtained at 60% ammonium sulfate with minimal activity of alkaline phosphatase (0.01 U/ml) in supernatant ($p<0.28$). The activities of 34, 20, 16, 12, and 1.6 U/ml were calculated for 10, 20, 30, 40 and 50 % of ammonium sulfate suspensions, respectively (Fig. 4.22). The specific activity of the enzyme increased from 13.83 to 26.57 U/mg with 1.92 fold of purification (Table 4.8).

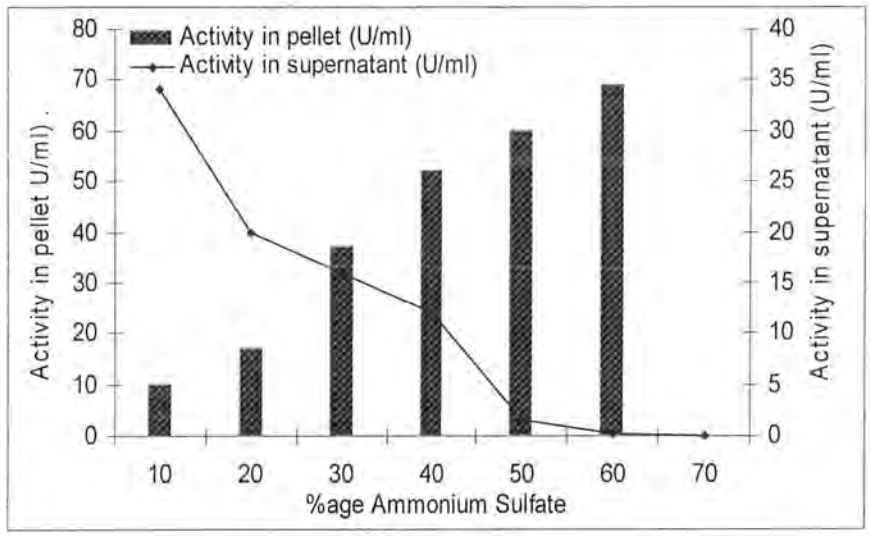


Fig 4.22: Ammonium sulfate precipitation

Dialysis

The desalting process effectively removed the salts and specific activity increased from 26.57 U/mg to 33.03 U/mg and purification reached up to 3.16 fold (Table 4.8).

Gel permeation chromatography (Sephadex G-75)

A sample of 5 ml eluted at the rate of 12 ml per hour from G-75 sephadex column exhibited peaks with phosphatase activity in fractions showing absorbance 280 nm spectrophotometerically. Fractions 6, 7, 8, 9, 10, 11 and 12 showed phosphatase activity (0.029, 0.40, 0.28, 0.21, 0.005, 0.0043, and 0.0015 U/ml), respectively. The specific activity and purification increased up to 134.81 U/mg and 7.24 fold, respectively (Fig. 4.23).

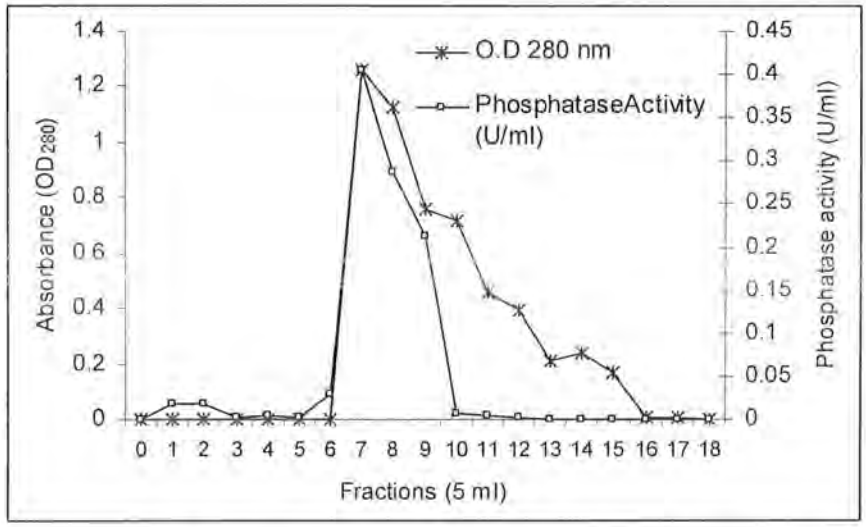


Fig 4.23: Purification of alkaline phosphatase by gel permeation chromatography

V_o= 10 ml; V_t= 40 ml; Flow rate= 12 ml/hr; Fraction volume= 5 ml

Eluent= 0.02 mol diethanolamine buffer (pH 8.6)

Ion exchange chromatography

The pooled fractions from gel chromatography were eluted from DEAE cellulose column by linear gradient of NaCl (0.1-0.5 mol). The phosphatase activity in fractions (4, 5, 6, 7, 8, 9, 10, 11, 12, 13 and 14) showing absorbance at 280 nm, was recorded as (1.21, 1.694, 3.63, 2.25, 1.97, 1.40, 1.08, 0.36, 0.37, 0.22 and 0.10

U/ml), respectively (Fig 4.24). The pooled fractions revealed a specific activity of 225 U/mg with 8.9 folds of purification (Table 4.8).

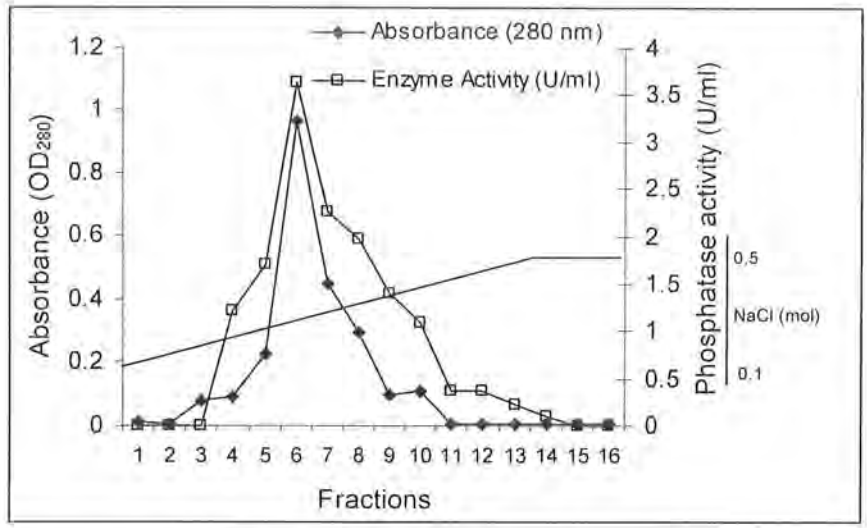


Figure 4.24: Purification of alkaline phosphatase by Ion Exchange Chromatography

V_o= 10 ml; V_t= 40 ml; Flow rate= 12 ml/hr; Fraction volume= 5 ml

Eluent= NaCl (0.1- 0.5 mol)

Lyophilization

About 30 ml purified pooled fractions from ion exchange chromatography were concentrated up to 10 ml with specific activity of 300 U/mg with 10.23 folds of purification.

Table 4.8: Purification of alkaline phosphatase from MRLBA1

Purification Step	Volume (ml)	Total Protein (mg)	Enz. Activity (U)	Spec. Activity (U/mg)	Yield (%)	Fold Purification
Culture supernatant	160	389.6	5390	13.83	100	1
Amm. Sulfate Precipitation (10-60%)	96	172	4571	26.57	85	1.92
Dialysis	90	123	4063	33.032	75	3.16
Gel permeation	50	16	2157	134.81	40	7.24
Ion Exchange	30	8	1800	225	33	8.9
Lyophilization	10	5	1500	300	27	10.23

Estimation of molecular weight of purified alkaline phosphatase by column chromatography

Proteins of known molecular weight (mammalian alkaline phosphatase, BSA and lysozyme) along with purified alkaline phosphatase of *Pseudomonas* sp. MRLBA1 were pooled and passed through Sephadex G-75 column. Fractions showing absorbance at 280 nm (9, 10, 11, 12 and 13) revealed phosphatase activity (0.11, 4.39, 5.87, 3.41 U/ml), respectively. The known proteins eluted in order of decrease in their molecular weight (140>66>44 kDa). The fractions showing phosphatase activity exhibited a single peak after 66 but before 44 kDa proteins meaning that the *Pseudomonas* sp. MRLBA1 alkaline phosphatase (ALP) has molecular weight in range of ~ 50-60 kDa (Figure 4.25).

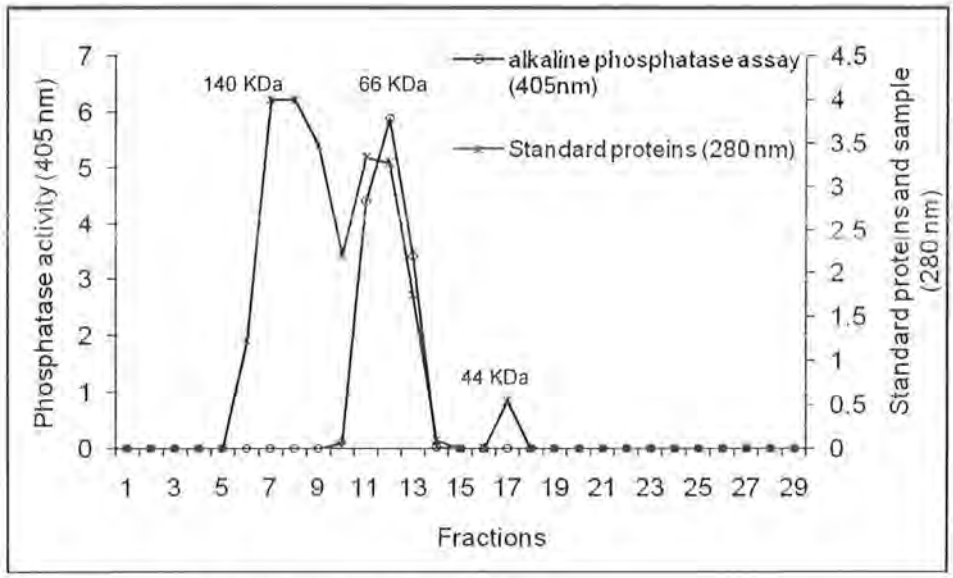


Figure 4.25: Molecular weight estimation

Molecular weight determination by Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

The SDS-PAGE of purified samples from gel filtration and anion exchange chromatography showed a single band of approximately 54 KDa (Fig 4.23). The appearance of a single band on SDS-PAGE from fractions with phosphatase activity was consistent with the estimated molecular size of 56 KDa and confirms that it was a monomer (Fig 4.26).

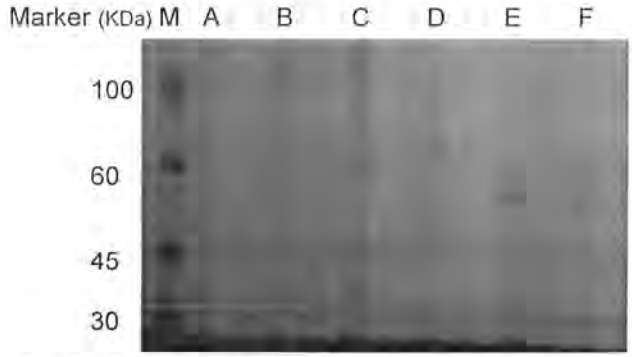


Figure 4.26: SDS-PAGE of purified alkaline phosphatase

A= supernatant; B= supernatant from cell debris; C= wash 1; D= dialyzed; E= lyophilized fraction with alkaline phosphatase activity eluted from gel filtration; F= lyophilized fraction with alkaline phosphatase activity eluted from anion exchange column; M= standard protein marker; 220, 100, 60, 45 and 30 KDa. A single band of ~54-56 KDa protein was observed.

CHARACTERIZATION OF CRUDE ALKALINE PHOSPHATASE

Effect of pH on the activity of crude alkaline phosphatase

The crude alkaline phosphatase exhibited maximum activity (52.21 U/mg) when incubated at 37°C at pH 9.0 for 30 min ($p<0.001$). The incubations at pH 4, 5, 6, 7, 8, 10, 11 and 12 exhibited residual activities of 23.2, 28.5, 45, 68, 79, 93, 98, 87 and 56 % respectively (Fig 4.27).

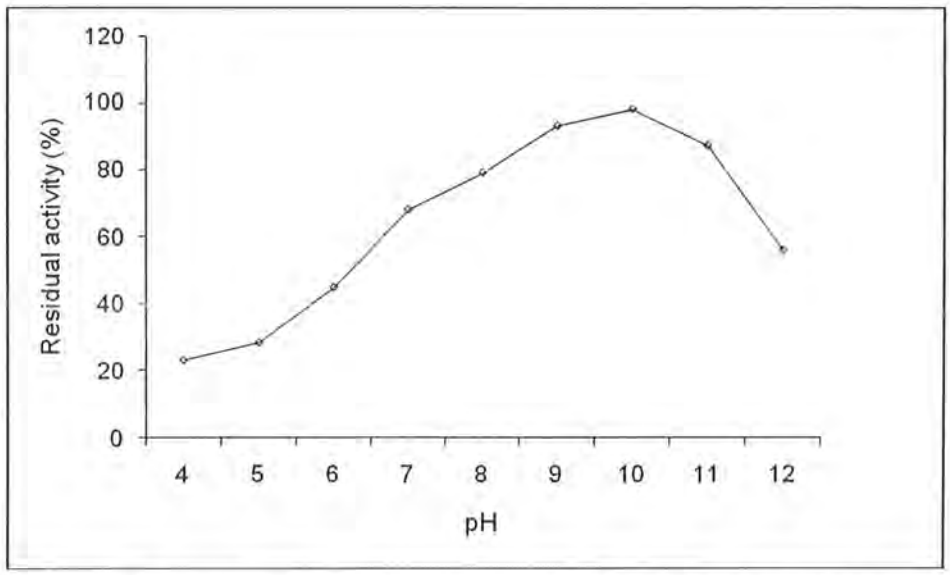


Fig 4.27: Effect of pH on the activity of crude alkaline phosphatase

Effect of temperature on the activity of crude alkaline phosphatase

The crude alkaline phosphatase showed maximum residual activity (100%) when incubated at 4, 10, 15, 20, 25, 30 and 37°C for 30 minutes ($p<0.01$). However, it decreased slightly through 40, 45, 50, 55 and 60°C as 96, 82, 63, 42 and 5 %, respectively (Fig 4.28).

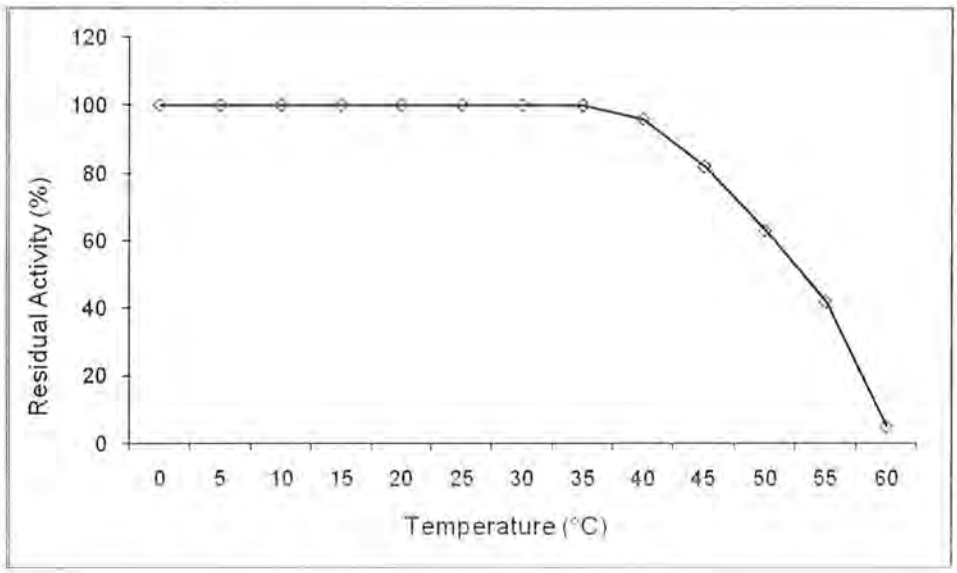


Fig 4.28: Effect of temperature on the activity of crude alkaline phosphatase

CHARACTERIZATION OF PURIFIED ALKALINE PHOSPHATASE

Effect of pH on the activity of purified alkaline phosphatase

The incubation of purified alkaline phosphatase at pH 4, 5, 6, 7, 8, 9, 10, 11 and 12 for 1 hour showed residual activities of 10, 21, 36, 60, 90, 100, 91, 70, 48 and 40%, respectively ($p<0.01$) (Fig 4.29).

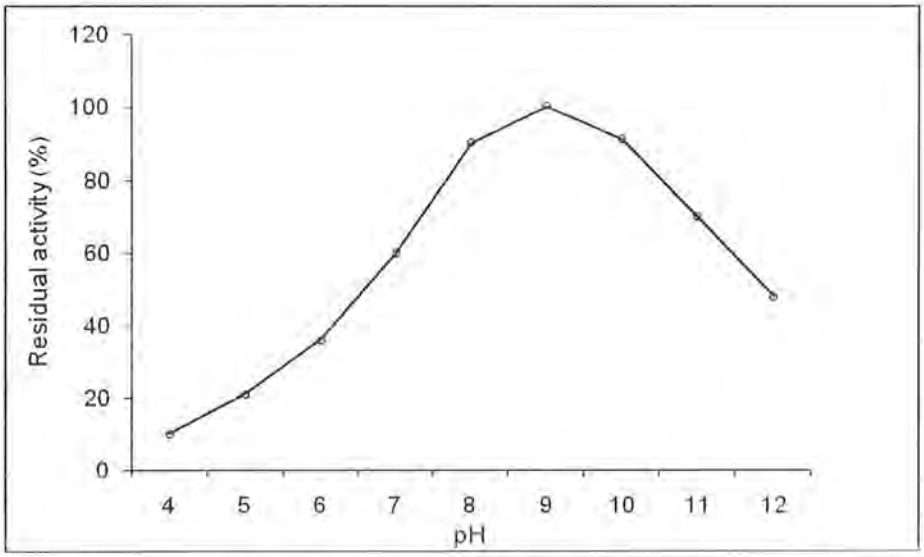


Figure 4.29: Effect of pH on the activity of alkaline phosphatase from *Pseudomonas* sp. MRLBA1

Effect of temperature on the activity of purified alkaline phosphatase

The incubation of purified alkaline phosphatase at 5, 10, 15, 20, 25, 30, 35, 40, 45, 50 and 55°C for 1 hour followed by enzyme assay under standard assay conditions showed residual activities of 100, 100, 100, 100,100, 100,100, 83, 51, 20 and 0 %, respectively. The enzyme was active from 5 to 50°C with maximum activity at 37°C ($p<0.0001$) (Fig 4.30).

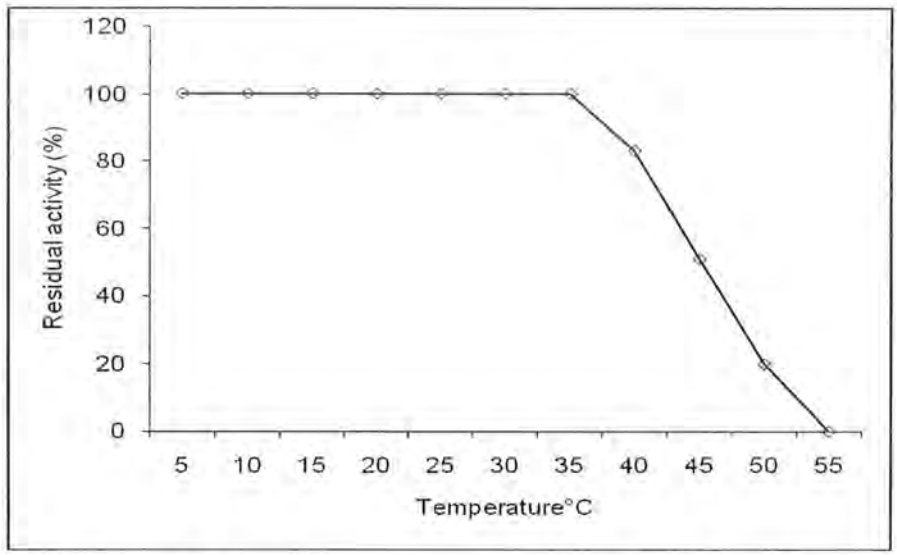


Figure 4.30: Effect of temperature on the activity and stability of alkaline phosphatase from *Pseudomonas* sp. MRLBA1

Effect of metal ions on the activity of purified alkaline phosphatase

The chloride salts of calcium, zinc, magnesium, mercury and copper stimulated the enzyme and showed residual activities (stimulation of activity) of 134.6, 140, 139.25, 111.47 and 126%, respectively. The iron and potassium showed inhibitory effect and revealed 12.5 and 10% residual activities of phosphatase, respectively ($p<0.0001$) (Fig 4.31).

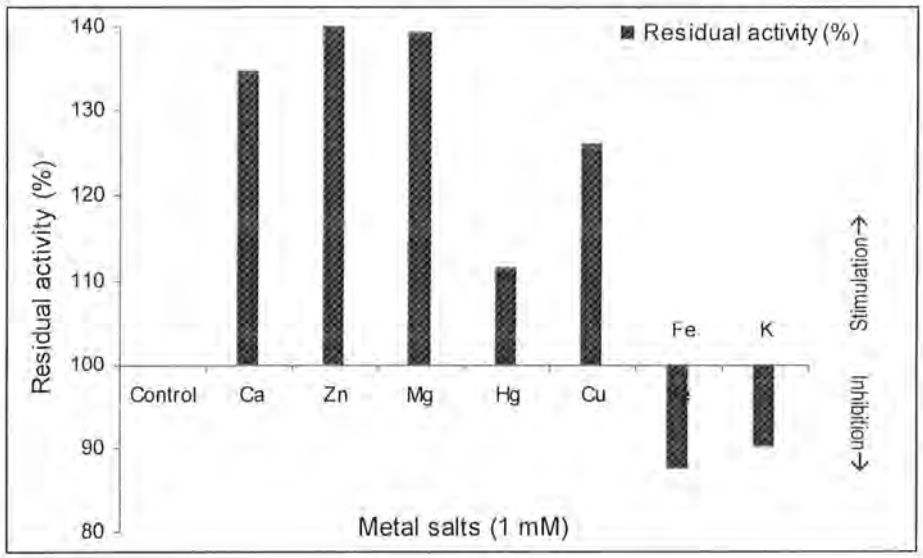


Figure 4.31: Effect of Metal ions on the activity of alkaline phosphatase

Effect of glycerol (%) on the activity of purified alkaline phosphatase

The residual phosphatase activity observed in the presence of 10, 20, 30, 40, 50, 60, 70, 80 and 90 % of glycerol was observed as 99, 99, 98.5, 98, 86, 76, 54, 27 and 16 %, respectively. The activity of control (without glycerol) was considered as 100 % (Fig 4.32).

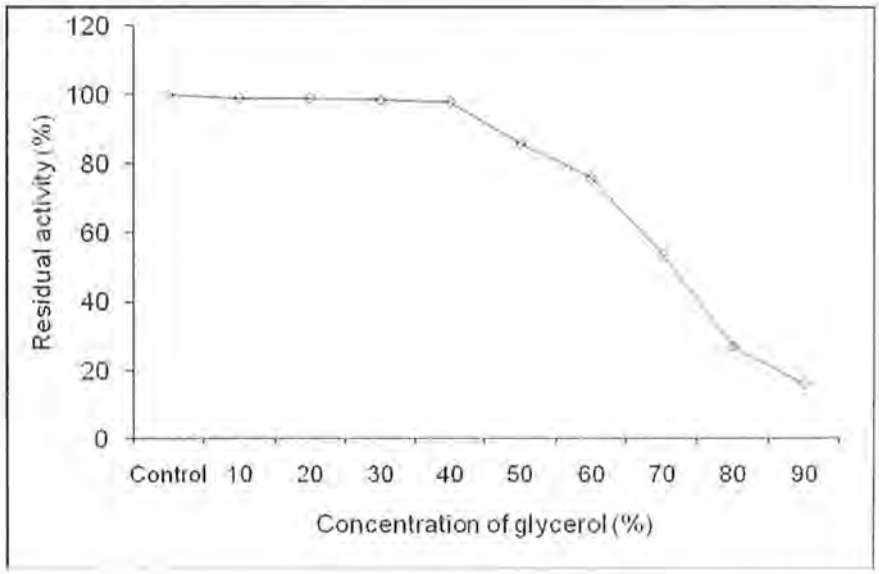


Figure 4.32: Effect of glycerol on the activity of alkaline phosphatase

Inhibition of alkaline phosphatase by potassium ferricyanide

Potassium ferricyanide (0.5 mmol) did not show any inhibition of alkaline phosphatase and residual activity of the enzyme remained intact (100%) after incubations for 5, 10, 15, 20, 25 and 30 minutes at 37°C.

Inhibition of alkaline phosphatase by potassium ferrocyanide

A decrease in residual activity of alkaline phosphatase was observed when incubated with 1.5 mmol potassium ferrocyanide at 37 °C for 5, 10, 15, 20, 25 and 30 minutes (Fig 4.33).

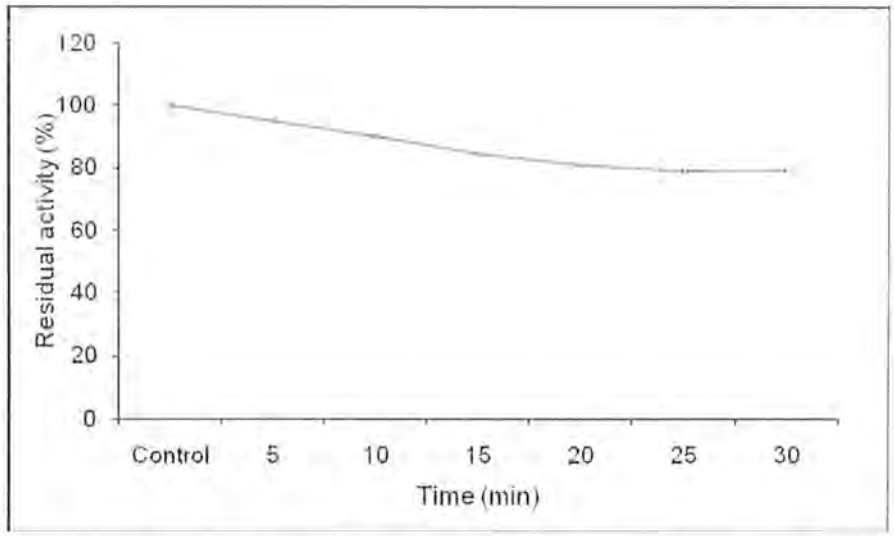


Fig 4.33: Inhibition of alkaline phosphatase by potassium ferrocyanide (Qualitative)

Kinetic studies

Alkaline phosphatase in presence of 0-1.5 mmol potassium ferrocyanide was allowed to undergo hydrolysis of *p*NPP at 37°C for 2 minutes. A graph was plotted for reciprocals of [V] and [S]. The apparent V_{max}, $1/V_{max;app} = \{1+[I]/K_i\}/V_{max}$, and the apparent Km, $1/K_{m;app} = -\{1+[I]/K_i\}/K_m$. The trend lines thus obtained from data series exhibited typical plot of uncompetitive inhibition (Fig 4.34).

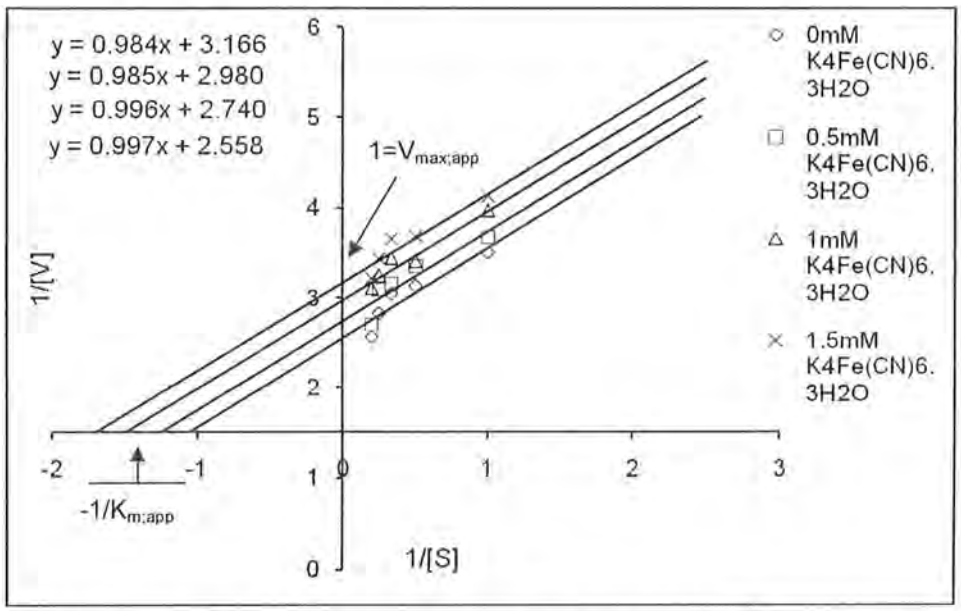


Fig 4.34: Inhibition of alkaline phosphatase by potassium ferrocyanide

Eadie-Hofstee Plot

The kinetic parameters were solved by Eadie-Hofstee Plot using the equation:

$$v = V_{max} - K_m(v/[S])$$ (Eadie, 1942; Hofstee, 1952)

A plot of n (as y) against n/[S] (as x) yielded, after linear regression, a y intercept of V_{max} and a slope of $-K_m$. The enzyme assay was performed at 405 nm for *p*-nitrophenol. The kinetic constants, K_m and V_{max}, were calculated as 122 µmol and 28 µmol.min^{-1} from Eadie-Hofstee plot and were chosen as the most reliable values for the analysis of ALP. The turnover number, k_{cat} and kinetic efficiency were calculated as 0.229 min^{-1} and 1.8x10^{-3}, respectively (Figure 4.35).

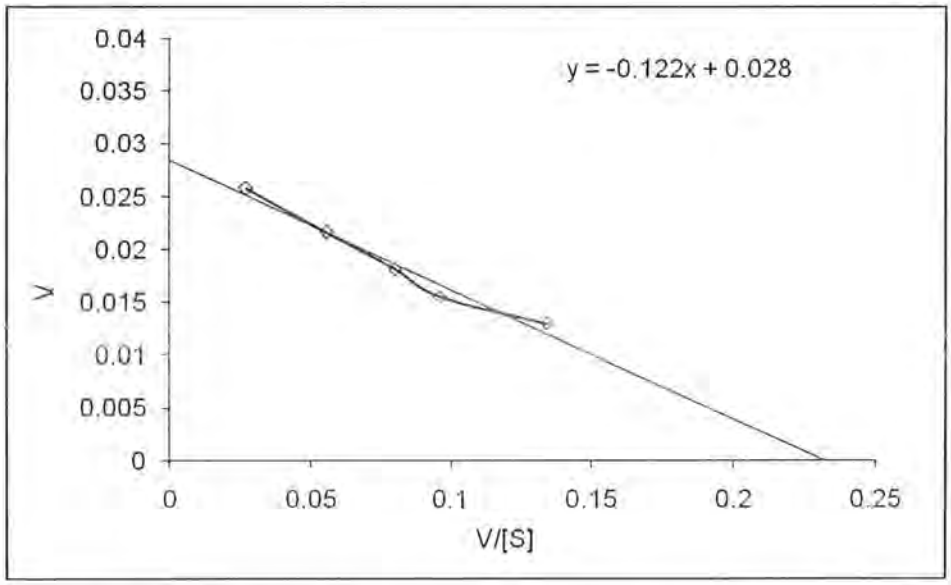


Fig 4.35: Eadie-Hofstee plot of reaction between *p*-nitrophenyl phosphate and ALP (K_m=0.122 mmol or 122 µmol and V_{max}=0.0284 mmol/min or 28 µmol.min^{-1}).

DISCUSSION

Psychrophiles are biotechnologically important candidates especially due to their enzymes active at lower than usual temperature. The enzymes from psychrophiles have adaptive features in their structure, like; Fewer salt links, Reduced aromatic residues, Reduced hydrophobicity, and Increased number of interactions between the enzyme and the Solvent (Moncheva *et al.*, 2001).

The cold ecosystems are natural laboratories for exploring and monitoring *in situ* interactions between the environments and dynamics of microbial biodiversity, and to study influence of geography and climate change on the composition of entrapped species, examine survival mechanisms and their preservation (Hoffman *et al.*, 1997; Kirshvink 1992). The isolates from glacial ice cores of Karakorum Range of mountains (0.75 million years old) exhibited their physiology like obligate or facultative psychrophiles. Secretion of secondary metabolites (antibiotics) as an adaptation mechanism for survival, secreted by some of these survivors may have been habituated by competitors due to their continuous exposure. The proteins like alkaline phosphatase produced by these stressed bacteria function optimally at same conditions with higher specific activity. The studies on survival and physiological adaptation of isolates from freezers show similar results as exhibited by those isolated from glaciers.

Site of sampling and its importance

The Karakorum, Himalayas and Hindu Kush are three mightiest mountain ranges of the world that diverge from a "junction point" (36´27N and 74´52 E) near Gilgit, Pakistan. The Gojal and Hunza valleys are located at the base of Karakorum Range along Karakorum Highway (KKH) and Indus River beneath, with world's famous glaciers like Batura (36° 32′ 0″ N, 74° 39′ 0″ E), Passu (36°29′16″N, 74°35′16″E) and Hopper (36°21′38.6″N, 74°51′20.8″E) near Khunjerab Pass on Sost border of Pakistan and China.

We expedited and collected samples from the above mentioned three glaciers. The parameters we considered during sampling e.g. geographic location, physical state and condition of samples, pH, temperature, dissolved oxygen, pressure and height; correlate with Christner (2002) who adapted the strategies of sampling from frozen terrestrial environments analogs for Mars and Europa.

The laboratory freezers set at -20°C since decades seem analogues of glaciers except few concerns like atmospheric pressure, height and time. For the comparative study, some non glacial bacterial strains having the ability to survive at low temperature were also isolated from the -20°C freezer in the laboratory. The spore forming bacteria have better chances of survival and adaptability in the freezers because they have sufficient time to opt for physiological changes. Survival in low temperature environment requires physiological and metabolic adaptations (Thomas and Diekmann, 2002). Low temperature dwellers possess high specific activity (K_{cat}) at low temperature, weak thermostability and incomplete adaptation to temperature optima (D'Amico *et al.*, 2002).

The *Bacillus subtilis* MRLBA7, *Bacillus licheniformis* MRLBA8 and *Bacillus megaterium* MRLBA9; isolated from ice obtained from -20 °C freezer showed survival even at sever physiological conditions and scarce amount of available nutrients. Bacteria have been isolated from glacier ice, sub glacial ice cores and ice cream freezers previously (Abyzov, 1993; Hamilton and Lenton, 1998). Christner *et al.*, (2000) documented the recovery of viable, *Bacills subtilis and Bacillus licheniformis* and phage (tomato mosaic genomic segments) from the *Bacillus subtilis,* Castello and Rogers (2005) from 100,000 years old ice core.

Plate count method as we used here in our study, is fast, inexpensive and can directly provide information on the active, heterotrophic component of the population (Bing-Ru *et al.*, 2006).

The range of temperature required for the growth of three strains was observed as; 0-37°C for *Bacillus subtilis* MRLBA7, 4-40°C for *Bacillus licheniformis* MRLBA8 and 0-37°C in case of *Bacillus megaterium* MRLBA9, with maximum growth at 25, 37 and 30°C and pH 7, 9 and 7, respectively. Psychrotrophs can grow near zero but have optimum temperature for growth above 20°C (Russell, 1990; Hebraud and Potier, 1999) and continue to adapt further (Gerday, 2000). Survival at temperature like -20°C and scarce inorganic nutrients, stresses the marine protozoans to develop a robust resistant cyst (Stoecker *et al.*, 1998). Alteration of physiology like solubility, reaction kinetics, membrane fluidity and protein conformation are adapted to cope with concurrent changes in physical and biochemical parameters (Hebraud and Potier, 1999). The long term viability

of only spore former, Gram positive bacteria seem to be adaptive of the environment and acquired the physiological attributes like psychrotrophy.

Presence of an appreciable number of viable cells of spore forming bacteria (dormant in ice or in transition of growth) adapted any combination of mechanisms for adaptation in addition to role of spores. The membrane fluidity and cold shock response are key factors for adaptation to such environments. Membrane fluidity at low temperature can be achieved by increasing the ratio of unsaturated fatty-acyl residues and/or cis double bonds, chain shortening and sometimes by methyl branching (Russell, 1990; Hebraud and Potier, 1999). Other adaptive mechanisms of psychrotrophs include cold shock proteins (csps) (Schroder *et al.*, 1993; Berger *et al.*, 1996; Michel *et al.*, 1997) and cold acclimation proteins (Caps) (Roberts and Inniss, 1992; Hebraud and Potier, 1999; Berger *et al.*, 1996). The cold shock response is a complex process, connected with heat shock and general stress response associated with cold induced proteins (CIPs) in *B. subtilis* and bears a broad spectrum of functions. A sudden decrease of temperature (10-15°C) in *Bacillus subtilis* creates a stress situation to which cells respond by specific adaptive mechanisms that allow them subsequent growth at the lower temperature.

To examine the levels of antibiotic sensitivity and resistance of microbes entombed away from natural exposure of communities and in the absence of current use of antibiotics, we studied two major groups of antibiotics i.e. protein and cell wall synthesis inhibitors. All the three isolates were found susceptible to neomycin and streptomycin. Tecoplanin and Fosphomycin, bearing a different β-lactam structural property among cell wall inhibitor group showed weak potency against these isolates. Antibiotic resistance was observed against three antibiotics i.e. vancomycin, penicillin and aztreonam but less than that of protein synthesis inhibitors. Cell wall synthesis inhibitors were not effective class of antibiotics against low temperature dwelling *Bacillus* spp. studied here. Levy (2002) described that antibiotics are pivotal in the selection of bacterial resistance and spread of the resistant genes. Many psychrotrophs exhibiting susceptibility/resistance also showed multiresistance to β-lactams (Munsch-Alatossava and Alatossava, 2007; Miller *et al.*, 2009).

To study the proliferation and preservation of strains near freezing temperature, we optimized growth of isolates in presence of glycerol in media. The *Bacillus subtilis* MRLBA7, *B. licheniformis* MRLBA8 and *B. megaterium* MRLBA9 grew to maximum number in 30, 50 and 40% of glycerol at 25, 37 and 30°C respectively. Also, they were able to grow at 2, 4 and 8°C respectively when incubated in media containing optimized concentration of glycerol. Previously, (Rashid *et al.*, 1999) characterized KB700A that showed severe inhibition of growth in presence of 10% glycerol but Howard (1956) preserved different bacteria in 15% glycerol at -70°C for 2 months and -10°C without losing viability. Nanninga (1971) preserved *B. subtilis* in 20% glycerol concentration during physical and chemical fixation protocols.

Glacial Bacteria

The *Pseudomonas* sp. MRLBA1, *Pseudomonas* sp. MRLBA2, *Pseudomonas* sp. MRLBA3, *Pseudomonas* sp. MRLBA4, *Arthrobacter* sp. MRLBA5 and *Stenotrophomonas* sp. MRLBA6 were isolated from different glaciers in Pakistan (Table 4.1). Among recent reports on isolation of bacteria from glacial ice cores (Abyzov, 1993; Hamilton and Lenton, 1998), *Bacills subtilis*, *Arthrobacter agilis*, and *Stenotrophomonas africae* have been documented from 5 to 20,000 years old ice cores from Greenland, China, Bolivia and Antarctica (Christner *et al.*, 2000). The frequent isolation of related genera from geographically different sampling sites (Fig. 1.1, 1.2a and 1.2b; Table 4.1) suggests that these bacteria are adapted to low temperature or freezing conditions.

The genus *pseudomonas* comprised about 100 species but characterization and validation by modern tools provided a long list of members by reclassifying into different genera (Kersters *et al.*, 1996; Anzai *et al.*, 2000). Among recent reports on Pseudomonas spp., *Pseudomonas peli* sp. nov. and *Pseudomonas borbori* sp.nov. (Vanparys *et al.*, 2006); *Pseudomonas antarctica* sp. nov. and *Pseudomonas proteolytica* sp. nov. (Reddy *et al.*, 2004); *Pseudomonas guineae* sp. nov. (Bozal *et al.*, 2007); *Pseudomonas alcaliphila* sp. nov. (Yumoto *et al.*, 2001); *Pseudomonas stutzeri* and *Pseudomonas balearica* sp. nov. (Bennasar *et al.*, 1996); *Pseudomonas putida* P8 (Diefenbach *et al.*, 1992); *Pseudomonas putida* S12 (Hartmans *et al.* 1990); *Pseudomonas flavescens* sp. nov.

(Hildebrand *et al.*, 1994); *Pseudomonas psychrophila* sp. nov. (Yumoto *et al* 2001) are well characterized.

The *Pseudomonas* spp. from our study are facultatively psychrophilic and grew in the range of 2-37 °C and pH 7 on average while *Pseudomonas guineae* sp. nov., (Bozal *et al.*, 2007) and *Pseudomonas alcaliphila* sp. nov. (Yumoto *et al.*, 2001) exhibited their grow range of -4 to 30 °C and 4 to 37 °C, respectively. The biochemical profile like catalase, nitrate reductase and most of the carbon sources utilized by *P. guinea* and *P. alcaliphila* are in accordance with our results however *P. alcaliphila* being alkaliphilic did not show tolerance to broad range of pH like ours (pH ~4 -11). In a comparative analysis of enzyme production *P. guinea* M8^T (Bozal *et al.*, 2007) and *P. peli* LMG 23201^T produced alkaline phosphatase in supernatant but not nitrate reducatase. However *P. anguilliseptica* LMG 21629^T (Vanparys *et al.*, 2006) produced both of them like some of our *Pseudomonas* spp.

Bacteria with coryneform morphology such as low temperature dwelling Arthrobacters, have been isolated from samples obtained from soil, ponds and lakes of Antarctica (Johnson *et al.*, 1981; Johnson and Bellinoff, 1981; Madden *et al.*, 1979; Siebert & Hirsch, 1988; Shivaji *et al.*, 1989; Loveland-Curtze *et al.*, 1999; Reddy *et al.*, 2000, 2002; Stibor *et al.*, 2003), Arctic soils (Juck *et al.*, 2000) and glacier silts (Moiroud & Gounot, 1969). Other than yellow-pigmented *A. nicotianae*, *A. aurescens*, *A. ilicis*, *A. citreus*, *A. protophormiae*, *A. uratoxydans*, *A. sulfurous* and *A. mysorens*, there are many others that are non-pigmented like *A. globiformis*, *A. crystallopoietes*, *A. pascens* and *A. histidinolovorans*, *A. crystallopoietes* (Gram variable), *A. nitroguajacolicus*, *A. sanguinis*, *A. bergerei*, *A. flavus*, *A. scleromae* and *A. globiformis* (Valdes-Stauber *et al.*, 1997; Bockelmann *et al.*, 1997; Kotouc kova *et al.*, 2004; Huang *et al.*, 2005; Irlinger *et al.*, 2005; Margesin *et al.*, 2004; Ward & Claus, 1973; Mages *et al.*, 2008; Reddy *et al.*, 2000; Keddie *et al.*, 1986; Schleifer, 1986, and Koch *et al.*, 1995). Among the cold active arthrobacters *A. psychrophenolicus* and *A. ardleyensis* (Loveland *et al.*, 1999, Chen *et al.*, 2005) are common while those typically isolated from glacial samples include; *Arthrobacter* sp. cryopeg_2b, *Arthrobacter* sp. TSBY-46, *A.citreus*, *Arthrobacter* sp. cryopeg_3b, *Arthrobacter* sp. cryopeg_2, *Arthrobacter*

citreus OS-21.b, *Arthrobacter* sp. Tibet-IIU24, *Arthrobacter* sp. PIC-C11, *Arthrobacter* sp. Everest-gws-25) (Table 4.35).

Previously, *Arthrobacter scleromae* (Huang *et al.*, 2005) was reported to grow at temperatures between 15-37 °C and pH 6-9, but not at 42 °C and pH 5. Irlinger *et al.*, (2005) reported *Arthrobacter bergerei*, a bacterium with yellow colonies (2mm), Gram-positive, catalase positive, non-spore-forming, with rod–coccus growth cycle (10-30°C); and *Arthrobacter arilaitensis*, with same characteristics except size of colonies that is 2-3 mm in diameter. Kotouc kov *et al.*, (2004) reported an *Arthrobacter nitroguajacolicus*; yellow colonies, rod-coccus life cycle, Gram positive, asporous that grows at 4-37 °C (25-30 °C optimum) and pH (6-8). Reddy *et al.*, (2000) reported *A. flavus*: aerobic, Gram-positive, non-spore-forming, non-motile, exhibited a rod–coccus growth cycle and produced a yellow pigment that was insoluble in water and grows at (5-30 °C) and pH (6-9). The *A. globiformis*, *A. polychromogenes* and *A. psychrolactophilus* grow at (10-37 °C), (10-37 °C) and (0-30 °C) repectively (Loveland *et al.*, 1999). Growth of *A. psychrophenolicus*, *A. sulfureus* and *A. nicotianae* (Margesin *et al.*, 2004) was optimum at (25 °C, pH 8-10), (25 °C, pH 7–9) and (20-30 °C, pH 7–8), respectively.

Funke *et al.*, (1996) studied the antibiotic susceptibility of a wide range of *Arthrobacters*. Many psychrotrophs exhibiting susceptibility/resistance also showed multi-resistance against β-lactams (Munsch-Alatossava and Alatossava, 2007). De Souza *et al.*, (2006), Miller *et al.*, (2009) and Foti *et al.*, (2009) reported the antibiotic resistance pattern of Antarctic and marine isolates. Belliveau *et al.*, (1991) described antibiotic resistance profile of marine bacilli. Boon and Cattanach, (1999) analyzed the antibiotic resistance pattern of the microbes from terrestrial deep subsurface.

The all *Pseudomonas* spp. i.e. MRLBA1, MRLBA2, MRLBA3 and MRLBA4 from geographically different glaciers and samples of ice and water (Table 3.2), indicate that species coming from glacial samples are most closely related to species reported from freshwater ecosystems (Fig 4.7 to Fig 4.12).

Microorganisms sharing a 16S rRNA gene sequence similarity lower than 97% are usually regarded as belonging to different bacterial species (Stackebrandt

and Goebel, 1994). In the genus *Pseudomonas*, several recently reported *Pseudomonas* species have shown 16S rRNA gene sequence similarities of more than 99% to other established species (Achouak *et al.*, 2000; Andersen *et al.*, 1991; Hauser *et al.*, 2004; Kwon *et al.*, 2003; Sikorski *et al.*, 2001). This situation also seems to be relevant for the subgroup of pseudomonads analysed in case of isolate MRLBA6. Thus from biochemical, physiological and 16S sequenc analyses, it is evident that *Pseudomonas* spp. MRLBA1, MRLBA2, MRLBA3, MRLBA4, *Arthrobacter* sp. MRLBA5 and *Stenotrophomonas* sp. MRLBA6 represent distinct, previously undescribed species within the genus *Pseudomonas*, *Arthrobacter* and *Stenotrophomonas*, respectively. In conclusion, phylogenetically and physiologically these strains are unique and represent novel species within the genera *Pseudomonas*, *Arthrobacter* and *Stenotrophomonas* and named as (*Pseudomonas passus*, *Pseudomonas baturus*, *Pseudomonas hopperus*, *Pseudomonas fruitius*; *Arthrobacter stikyeus*; and *Stenotrophomonas amylus*), respectivlely.

We can relate the climatic changes and geographic variations to local microbial population once we identify and enumerate the bacteria from glacial ice. Similarly by characterizing individual isolates, we can obtain information that contributes to discussions of the possibility that microorganisms might survive frozen in extraterrestrial environments and prevalence of antibiotic resistance before the advent of antibiotic therapies (Hoffman *et al.*, 1998). A total of 6 Gram negative and facultatively psychrophilic bacteria were isolated from various samples obtained from mountain glaciers. At geographic location of these glaciers (36° N and 74° E), temperature varies along with four equal seasons in the region. Hence isolation of none of the obligate psychrophile is an expected result. It is in agreement with Kobori *et al.*, (1984) who considered it paradoxical that out of 155 Antarctic isolates, only 23 % were found obligately psychrophilic and all of the 16 examined, were Gram negative.

BIOGEOGRAPHIC ORIGIN OF ISOLATES

India (Indo-Pak subcontinent) was tectonically part of Antarctica and Africa 200 million years ago (Redfern, 2001). The chronological biodiversity of this region should have conserved DNA bases of ancestors.

Pangeae (Greek: *Pan-entire; geae-earth*), is a term that refers to a sole continent of land (all continents joined together) on Earth about 200 million years ago, floating on water; thereby leaving a single ocean Panthalassa. Earth's land surfaces were indeed joined in a single super continent (Redfern, 2001). Plate tectonics gave a comprehensive description of lithosphere breakdown into eight continents or plates (Yi and Oldroyd, 1989). Under influence of its *Rotation* (movement of Earth about its axis) and *Revolution* (movement of Earth in an orbit around the Sun), about 135 years ago, Pangeae had split along and east-west fault just above its equator according to bridges; in convergent, divergent and transform boundaries (directions). The major two components that arose first were Laurasia (north) and Gondwanaland (south). After that, Laurasia splitted into North America and Eurasia (Europe and Asia later on); and Gondwanaland broke into Africa, Antarctica, Australia, South America, and India (Redfern, 2001; Briggs, 2003). India made most spectacular journey of all plates that travelled 5500 miles in 180 million years to join Asia after rifting from Africa and Antarctica (Redfern, 2001). The marine sediments between these two continents were deformed like molding clay such that world's highest mountains like Himalaya and Karakorum tops appear that were ocean floors, once (Redfern, 2001).

Microorganisms recovered from glacial ice likely to have freezing (a period of frozen dormancy) and thawing exposures and could be pigmented (Christner *et al.*, 2003). Interestingly, the isolated bacteria closely resemble to others isolated from glaciers separated by great distances like Greenland's, Chinese and Antarctic glaciers, suggesting that a possible novel specie have evolved that attained such features to survive at variable conditions of freezing and thawing. Frozen environments like ice crevices (Thomas 2002), permafrost (Mary Ann Liebert Inc. 2007), glacial ice (Skidmore *et al.*, 2000) and surface snow (Carpenter *et al.*, 2000) have microbial activity. Super cooled clouds are habitat and conveyor of condensation nuclei (Sattler *et al.*, 2001). Aeolian dust particles in glacial ice are a means of microbial transport (Griffin *et al.*, 2003). Thin films of

liquid water may exist between ice crystals, even within apparently solid ice (Price, 2000), and studies of permafrost (Rivkina *et al.*, 2004), basal glacial ice (Skidmore *et al.*, 2000), and surface snow (Carpenter *et al.*, 2000), have all demonstrated microbial activity under freezing conditions. Ice cores from high-altitude glaciers generally contain more recoverable bacteria than polar ice cores, presumably because the Andes and Himalayas are closer to major sources of airborne biological materials. Similarly, polar ice from regions adjacent to the exposed soils and rock surfaces in the Taylor Valley (Antarctica) contains more recoverable bacteria than polar ice from remote regions. We have established that bacteria remain viable when frozen in glacial ice for >500,000 years and, based on other studies of *Bacillus* spore longevity (Cano and Borucki, 1995; Vreeland *et al.*, 2000), this is almost certainly an underestimate. Therefore, it seems plausible that desiccation-resistant microorganisms, possibly cyptoendolithic microbial communities, could similarly be entombed and preserved in a frozen but viable state in ice on Mars. It is also possible that some microorganisms might even maintain some metabolic activity while apparently frozen within ice.

Though solely this data is not sufficient to prove a theory but in view of above discussion about distribution of horizontal microbial gene pool across the continents and its conserved clue from isolates and gene sequences indicate the possibility of truth about tectonics and continental drift theory. Simply based upon this data we cannot say that India originated from Antarctica and Africa and adapted its current biogeographic environmental conditions at south east China but is a least attempt to relate microbial signatures of life to evolutionary, geographic and paleontologic claims of origin and adaptation of life especially in part of India (Indo-Pak today).

ALKALINE PHOSPHATASE

Freezing and thawing of an enzyme causes loss of catalytic efficiency. Enzymes from psychrophilic or psychrotrophic isolates are usually thermo-labile but possess sufficiently high specific activity. Since the psychrophiles have to adapt as low as freezing and subzero range of temperature, scarce sources of energy, high radiation if residing at altitudes and low atmospheric pressure; the expression of alkaline phosphatase is induced as a stress protein, a secondary

metabolite or tool to release energy from high energy bonds (phosphoanhydrides) (Seufferheld *et al.*, 2008, Antelmann *et al.*, 2000, Kornberg *et al.*, 1999).

Production of alkaline phosphatase

Alkaline phosphatase (ALP) have been produced from several bacterial species like *Escherichia coli* (Sayer, 1968), *Halobacterium cutirubrum* (Fitt and Baddoo, 1979), *Lysobacter enzymogenes* (Tigerstrom and Stelmaschuk, 1989), *Enterococcus faecalis* (Lee *et al.*, 1999), thermophiles; *Pyrococcus abyssi* (Zappa *et al.*, 2001), or psychrophiles; *Arthrobacter* spp. (Kobori *et al.*, 1984; De Prada *et al.*, 1996), Antarctic TAB5 (Rina *et al.*,2000), *Bacillus sphaericus* P9 (Dhaked *et al.*, 2005). The production of alkaline phosphatase from Gram negative bacteria (Cheng and Costerton, 1977) especially *Pseudomonas aeruginosa* (Cheng *et al.*, 1970), *Pseudomonas fluorescens* E2 and *Pseudomonas* sp. 8E3 (Pratt-Lowe *et al.*, 1988) has also been reported, previously.

The growth and production of ALP from facultatively psychrophilic *Pseudomonas* sp. MRLBA1 is similar to growth and production characteristics of psychrotolerant *Bacillus* sp. P9 who excreted thermo-labile alkaline phosphatase in the supernatant (Dhaked *et al.*, 2005) i.e. it grows and produces alkaline phosphatase optimally at 25°C but produces maximum cell mass at 10–15°C. Also this is in accordance with the notation that growth rates at different temperatures do not reflect the physiological state of bacterium and cell mass at early stationary phase can differentiate between a psychrophile and psychrotroph (Feller *et al.*, 1994).

Qualitatively, the production of ALP was recorded in the late stationary and death phase of the growth of bacterium when grown in production medium at optimized conditions for growth. The phosphatase activity per unit number of cells (OD_{405}/OD_{600}) did not show a major difference at pH above or below the optimized one (pH 8.0). A very little activity of alkaline phosphatase (0.396 U/ml) was found associated to periplasmic space during growth curve.

To complement with the periplasmic fraction of the phosphatase activity, the cells were ruptured by freezing at -70°C and thawing subsequently as practiced earlier by (Heppel, 1967; Bhatti *et al.*, 1976; Cheng *et al.*, 1970) to release the periplasmic alkaline phosphatase from *Pseudomonas aeruginosa*. There are reports on generating osmotic shock through treatment with magnesium ions (Ingram *et al.*, 1973) or sonication (Chattopadhyay *et al.*, 1995) only. The Isolate MRLBA1 yielded 16 grams (10 mg/ml) of wet cells after 72 hours from 1.6 Liter biomass at optimized conditions.

Purification and Molecular Weight Determination

The extracellular alkaline phosphatase from *Pseudomonas* sp. MRLBA1 was precipitated by 60% ammonium sulfate and resulted in 1.92 fold of purification, with a rise in specific activity from 13.83 to 26.57 U/mg as discussed in previous reports by Ishida *et al.*, (1998) and Boulanger and Kantrowitz, (2003) from *Schewanella* sp. and *E. coli*, respectively. In the present study, ALP remained stable and purified to 2.38 fold with rise in specific activity from 26.57 to 33.03 U/mg during dialysis with 0.02 mol diethanolamine at 4°C like psychrophilic phosphatase from *Shewanella* sp. that was desalted in 0.2 mol sorbitol at 4°C for 72 hours by Ishida *et al.*, (1998).

Elution of 60 fractions using 1mmol diethanolamine at a rate of 0.2 ml/min from Sephadex G-75 improved the specific activity up to 134.81 U/mg with 9.75 fold of purification. Previously, Hussin et al., (2006) eluted 50 fractions of alkaline phosphatase from Bacillus megaterium at a rate of 0.5 ml/min and showed specific activity of 36.6 U/mg for the monomer. Boulanger and Kantrowitz (2003) eluted a recombinant alkaline phosphatase using a 40 mmol phosphate buffer at a rate of 0.5ml/min. Peter and Peterkin (1976) used Sephadex G-100 to elute a halophilic alkaline phosphatase from analytical gel filtration columns using standard proteins. Ishida et al., (1998) eluted a psychrophilic phosphatase at a rate of 0.5ml/min from Sephacryl S-100 HR16/60 gel column. Anion exchange chromatography using DEAE cellulose with subsequent 0.1-0.5mol sodium chloride like Boulanger and Kantrowitz, (2003) that used 0 to 0.1 mol NaCl in TMZP buffer but contrary to Kobori et al., (1984) who used 0.19 mol NaCl to elute a single peak of pooled fractions DEAE-Sephacel column.

The purification with anion exchange column increased the specific activity up to 225 U/mg with 16.27 fold of purification. However, the protein was further concentrated from 30 to 10 ml and increased the specific activity up to 300 U/mg with 21.69 fold of purification. This is in accordance with Kobori *et al.*, (1984) who purified a phosphatase up to 160 fold with 800-1600 units of APase activity per mg of protein. Demir *et al.*, 2005 purified four ancient elephant phosphatase isozymes for 47, 34, 50 and 40 fold with 14.10, 5.12, 3.00 and 43.36 U/mg and TEAE-cellulose 82, 76, 69, 83 fold with 23.57, 11.40, 4.16 and 86.50 U/mg of specific activity for outer peripheral, cytosolic inner peripheral and integral phosphatases, respectively.

A single peak showing alkaline phosphatase activity was eluted after 66 kDa and 44 kDa known protein. Hence a 50-60 kDa protein was approximated with G-75 (0.9 x 60cm). Peter and Peterkin (1976) used Sephadex G-100 to elute a halophilic alkaline phosphatase from analytical gel filtration G-150 (3x70cm) columns using standard proteins. Kobori *et al.*, (1984) approximated a 65-70 KDa monomer of alkaline phosphatase using P-700 gel filteration.Demir *et al.*, 2005, reported four phosphatase isozymes from bone of an extinct elephant eluting at rate of 0.33 ml/min.

A single band of 56 KDa from SDS-PAGE is in agreement with column chromatography and confirms that MRLBA1 ALP is a monomer. HK47 Apase (Kobori *et al.*, 1984) and *P. aeruginosa* ALP (Day *et al.*, 1973) appeared as 68 kDa while T59R ALP (Boulanger and Kantrowitz, 2003) appeared as a single band of 44 kDa against staining with coomassie brilliant blue after running at SDS-PAGE. Zappa *et al.*, (2001) purified a thermophilic 54KDa phosphatase monomer from *Pyrococcus abyssi.* Hulett *et al.*, (1991), calculated the molecular weight of *Pho AIII* amino acids as 45.9 KDa and *Pho AIV* amino acids as 47KDa from *Bacillus subtilis.* Ishida *et al.*, (1998) purified a 41.8 KDa phosphatase from *Schewnella* sp.

Characterization of Alkaline Phosphatase

Purified MRLBA1 ALP showed maximum activity at pH 9 which is almost similar as HK47 Apase that showed maximum activity and stability at pH 9.5, and pH 7.0-9.5, respectively (Kobori *et al.*, 1984). The D10B alkaline phosphatase from

psychrophilic *Arthrobacter* sp. D10 exhibited best activity at pH 8 to 9 (Prada *et al.*, 1996). The thermo-labile P9 ALP exhibited maximum activity at pH 9.0 (Dhaked *et al.*, 2005). Hence our protein is stable and active at more alkaline conditions than HK47, D10B and P9 ALPs.

The maximum enzyme activity of MRLBA1 ALP was recorded at 37°C which was lost completely at 55°C irreversibly. However, the crude alkaline phosphatase was a bit stable up to 60°C. In crude enzyme, the other metabolites produced during process of its production, might be stabilizing the enzyme and it showed activity at prolonged range of temperature than purified one. The HK47 ALP (Kobori *et al.*, 1984) showed maximum activity at 25°C and lost it completely at 50°C that did not resume till incubations up to 72 hours while trout intestinal ALP exhibited a half-life of 10 min at 40°C. The *Sphingobacterium* ALP showed maximum activity at 37°C but lost it completely at 62°C (Chattopadhyay *et al.*, 1995). The *Ulva pertusa kjellm* ALP (Yang *et al.*, 2003) showed maximum activity at pH 9.8 and 37°C. A psychrophilic phosphatase studied by Ishida *et al.*, (1998) showed best activity at 40°C, and stability below 10°C.

The stability of proteins depends upon hydrogen bonds, salt bridges, Vander-Waals and hydrophobic interactions between polypeptide backbones and side chains of amino acids. Psychrophilic proteins should have weaker hydrophobic interior and stronger hydrophilic exterior as compared to meso and thermophilic counterparts. There is no trigger of heat shock proteins by fall in temperature suggesting that there could be some unique mechanism regarding low temperature stress (Russell *et al.*, 1990). Enzymes of cold adapted species have higher catalytic efficiencies as compared to higher temperature adapted (Hochachka and Somero, 1984). Usually, the psychrophilic enzymes have optimum temperature for activity and stability above the upper growth temperature limit of microorganisms (Russell *et al.*, 1990).

The activity of ALP in case of *Pseudomonas* sp. MRLBA1 was increased in presence of calcium (134.6%), zinc (140%) and magnesium (139.25), mercury (111.47%) and copper (126%) while iron (87.5%) and potassium (90%) had slight inhibitory effect on activity of purified enzyme. Coleman (1992) reviewed the ALP as a first enzyme with Zn and Mg in its structure. A significant rise (Mg 123%)

and (Zn 104%) in catalytic efficiency of the enzyme is signature of strong dependency on divalent cations and confirm the metalloenzymatic nature of *P. abyssi* ALP (Zappa *et al.*, 2001). The HK47 ALP exhibited 6 fold elevated activity in the presence of calcium ions (Kobori *et al.*, 1984) while P9 ALP showed 25 and 22 % rise in production and enzyme activity, respectively, by addition of calcium ions (Dhaked *et al.*, 2005). Contrary to these reports on steep increase in enzyme activities, T59R AP (Boulanger and Kantrowitz, 2003) showed a very little increase in activity by addition of magnesium and zinc in the reaction mixture. In view of above arguments, *Pseudomonas* sp. MRLBA1 ALP seems to be a metalloenzyme with magnesium and calcium or magnesium and zinc divalent centre.

The MRLBA1 ALP showed stable activity in the presence of 10 to 50% glycerol but started to decrease afterwards till almost complete loss of activity at 90% glycerol emulsion. This is in agreement with Zappa *et al.*, (2001) who optimized 50% glycerol to preserve *P. abyssi* ALP at -20°C.

The enzyme kinetic constants from the Eadie-Hofstee plot were chosen as the most reliable values for the analysis of alkaline phosphatase. From the Eadie-Hofstee plot, the K_m was 122 µmol and V_{max} was 28 µmol.min^{-1} (Fig 4.35). The turnover number, kcat, had value of 0.229 min^{-1}. The kinetic efficiency was equal to 1.8x10^{-3}. Also the enzyme showed uncompetitive inhibition pattern when resolved the equation; $v=V_{max}/\{(K_m/[S])+ ([I]/K_i)+1\}$ using Lineweaver–Burk plot (Fig 4.34). The *P. abyssi* ALP exhibited the V_{max1} of 4.07 µmol.min^{-1} and K_{m1} 166.33 µmol; and V_{max2} 8.31 µmol.min^{-1} and K_{m2} 1204.98 µmol (Zappa *et al.*, 2001).

The *Pseudomonas* sp. MRLBA1 ALP is a thermolabile metalloenzyme of 54 kDa with sufficiently higher specific activity than some of previously reported alkaline phosphatases and should be used in commercial applications.

CONCLUSIONS

* Spore forming Gram positive strains of *Bacillus subtilis* MRLBA7, *Bacillus licheniformis* MRLBA8 and *Bacillus megaterium* MRLBA9 were isolated from ice obtained from -20 °C freezer of the Microbiology Research Laboratory and identified by morpho-physiological and biochemical methods.
* Asporous Gram negative strains like *Pseudomonas* sp. MRLBA1, *Pseudomonas* sp. MRLBA2, *Pseudomonas* sp. MRLBA3, *Pseudomonas* sp. MRLBA4, *Arthrobacter* sp. MRLBA5 and *Stenotrophomonas* sp. MRLBA6 were isolated from ice, water and soil samples were obtained from different glaciers in Pakistan and identified by morphological, biochemical and molecular methods.
* The *Pseudomonas* sp. MRLBA1 showed antibiotic resistance to fosphomycin; *Pseudomonas* sp. MRLBA2 to aztrenam and fosphomycin; *Pseudomonas* sp. MRLBA3 to vancomycin and penicillin; *Pseudomonas* sp. MRLBA4 to vancomycin, penicillin and fosphomycin; *Arthrobacter* sp. MRLBA5 to aztreonam and fosphomycin; *Stenotrophomonas* sp. MRLBA6 to vancomycin and aztrenam; *Bacillus subtilis* MRLBA7 to penicillin and fosphomycin, *Bacillus licheniformis* MRLBA8 to aztreonam and fosphomycin; and *Bacillus megaterium* MRLBA9 to vancomycin and penicillin.
* All of the nine isolates in study (non-glacial and glacial) were found to be facultatively psychrophilic.
* The *Pseudomonas* sp. MRLBA1 showed highest extracellular alkaline phosphatase activity.
* Optimization of growth parameters revealed that the maximum alkaline phosphatase was produced at pH 8.0 and 18°C when inoculated with 24 hours old inoculum (5%), after 48hrs of incubation.
* All of the protein in sample was precipitated with 60% ammonium sulfate.
* The purification steps showed an increase in specific activity of alkaline phosphatase; ammonium sulfate precipitation (26.57 U/mg), dialysis (33.03 U/mg), gel permeation chromatography (134.81 U/mg) and ion

exchange chromatography (225 U/mg), lyophilization (300 U/mg) with 1.92, 2.38, 9.75, 16.27 and 21.69 folds of purification respectively.

* A single active peak of 54-58 kDa was observed in samples eluted from gel permeation column and a single band of ~54-56 KDa was determined from SDS-polyacrylamide gel electrophoresis.
* The crude alkaline phosphatase showed maximum activity at 37°C and pH 9.0 (52.21 U/mg).
* The purified alkaline phosphatase was stable between 4-13 and 0-55 °C but maximally active at pH 9.0 and 37°C. The enzyme was concluded as the thermo-labile in nature.
* The chloride salts of calcium, zinc, magnesium, mercury and copper increased the specific activity of alkaline phosphatase but iron and potassium decreased it to some extent.
* The enzyme was stable when assayed along with 45% of glycerol but showed decrease in activity from 50-90% glycerol, sharply.
* The kinetic constants, K_m and V_{max}, were calculated as 122 µmol and 28 µmol.min^{-1} from Eadie-Hofstee plot.
* The potassium ferricyanide did not have any inhibitory or stimulatory effect on alkaline phosphatase whereas potassium ferrocyanide showed uncompetitive inhibition.

FUTURE PROSPECTS

* Exploration of novel obligate psychrophiles from glacial samples in the laboratory by cultured and uncultured techniques.
* Validation of isolates and submission to culture collection center.
* Study of biomass applications of facultatively psychrophilic strains for bioremediation of pollutants in contaminated water bodies of the country.
* Amino acid sequencing of the enzyme and study of residues involved in thermolability.
* Applications of thermolabile alkaline phosphatase in an amperometeric biosensor.

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APPENDICES

Appendix I. Protocol for isolation of bacterial genomic DNA

1. The cells were grown overnight in nutrient broth.
2. Transferred 1.5 ml of the culture to a micro centrifuge tube and centrifuged at 10,000 rpm for 2 minutes. Collected the pellet and repeated with another 1.5 ml of culture containing cells. The supernatant was decanted using a micropipette.
3. Re-suspended the pellet in 450 µL of TE buffer.
4. Added 45 µl of 10% SDS and 5 µl of 20 mg/ml proteinase K, mixed them by inverting tubes and incubated for 1 hour at 37°C.
5. Added 500 µl of phenol-chloroform and mixed well by inverting the tube till the phases were completely mixed.
6. Centrifuged the mixture at 10,000 rpm in a microfuge for 2 minutes.
7. Transferred the upper aqueous phase to a new tube and re-extracted by adding an equal volume (about 500 µl) of phenol-chloroform. Again centrifuged the mixture at 10,000 rpm in a microfuge for 5 min. Transferred the upper aqueous phase to a new tube.
8. Added 50 µl of the sodium acetate and mixed by dispensing with micropipette.
9. Added 300 µl of isopropanol and mixed gently to precipitate the DNA.
10. Spooled out the DNA with the help of an inoculation loop (or centrifuge at 10,000 rpm for 2-5 min).
11. Washed the DNA by adding 1 ml of 70% ethanol. Mixed by inverting several times and then drained the tube on blotting paper.
12. Re-suspended the DNA in 100-200 µl TE buffer.
13. Stored the DNA at 4°C for short-term and -20°C or -70°C for long term storage.

Appendix II: Reagents

Reagent	Composition
TE Buffer (100 ml)	
100mmol Tris-HCl pH 8.0	1ml from 1mol Tris-HCl pH 8.0
1mmol EDTA pH 8.0	0.5 ml from 0.5mol EDTA pH 8.0
Distilled Water	98.5 ml
10% SDS (100 ml)	Dissolve 10 g SDS in 100 ml of distilled water. Heat gently to get SDS into solution.
Proteinase K (20 mg/ml)	Dissolve 20 mg proteinase K in 1 ml of distilled water.
Phenol-Chloroform (1:1)	Mix 50 ml of phenol with 50 ml of chloroform
3mol Sodium Acetate (pH 5.2) (500 ml)	Dissolve 123 g in 450 ml of distilled water. Adjust the pH to 5.2 with glacial acetic acid. Make up to 500 ml.
DNA Marker	Direct load™ Wide Range DNA Marker 50bp-10,000bp (D7058)
Loading Dye	6x loading dye solution (Fermentas Cat. No. R0611)
Taq DNA Polymerase (Native without BSA)	(Fermentas Cat. No. EP0281/ Cat. No. EP0402)
Taq Buffer 10x	(with ammonium sulfate $(NH_4)_2SO_4$)-$MgCl_2$)
Taq Buffer 10x (with KCl-$MgCl_2$)	(Fermentas Cat. No. B38)
dNTP Mix, 10mmol each	(Fermentas Cat. No. R0192)
$MgCl_2$	(Fermentas Cat. No. R0971)
Water, Nuclease Free	(Fermentas Cat. No. R0582) used for dilution of primers

Appendix III: Results Data

Appendix III-1: Qualitative test for activity of alkaline phosphatase

Strain	Time (hrs)	Periplasmic space	Supernatant
MRLBA1	24	0.152	1.3289
	48	0.3962	3.6278
	72	0.3739	3.158
MRLBA2	24	0.001	0.00379
	48	0.004952	0.06253
	72	0.003201	0.138
MRLBA3	24	0	0
	48	0	0
	72	0	0
MRLBA4	24	0	0
	48	0	0
	72	0	0
MRLBA5	24	0	0
	48	0	0
	72	0	0
MRLBA6	24	0	0.002
	48	0.04261	1.07232
	72	0.01385	1.0679

Appendix III-2 : Maximum cell density (OD_{600}) of MRLBA1 at different temperatures

Temp (°C) / Time (hrs)	0	4.0	10	25	30	37
0	0.000686	0.000686	0.000683	0.000689	0.000689	0.000051196
12	0.008885	0.006096	0.002822	0.001935	0.00987	0.000756
24	0.01206	0.072866	0.906392	1.17747	0.87359	0.0009627
36	0.023734	0.08711	1.3101	1.2955	1.26079	0.0003458
48	0.053729	1.1673	2.10562	1.7166	1.8396	0.00051604
60	0.07523	2.13792	2.64386	2.58114	2.3833	0.00072269
72	0.1741	2.50412	2.78731	2.60829	2.7615	0.00083428
84	0.43325	2.669	2.74698	2.9301	2.867	0.00089968

Appendix III-3: Maximum cell density (OD_{600}) of MRLBA1 at different pH

pH / Time (hrs)	4	5	6	7	8	9	10
0	0.006859	0.006317	0.005566	0.002689	0.005892	0.00112	0.001593
12	0.007932	0.37873	0.4741	0.60829	0.75213	0.23428	0.001671
24	0.13792	0.44386	0.5523	0.68114	1.3833	0.32269	0.067068
36	0.0669	0.69746	0.83325	1.093	2.0867	0.49968	0.000213
48	0.081673	0.50562	0.633729	0.793166	1.091262	0.17604	0.001534
60	0.12866	0.33917	0.49595	0.747	0.83552	0.16867	0.00102
72	0.0832	0.37873	0.4741	0.60829	0.85213	0.23428	0.001671
84	0.18711	0.21101	0.23734	0.20955	0.60709	0.003946	0.000251

Appendix III-4: Production of alkaline phosphatase along with growth curve of MRLBA1

Time (hrs)	Growth (OD_{600})	Enzyme assay (OD_{405})
0	0.00269	0.00013
2	0.005129	0.000156
4	0.025518	0.000572
6	0.062991	0.00521
8	0.188572	0.00863
10	0.3556	0.01489
12	0.8376	0.08727
14	1.50389	0.14793
16	1.5678	0.36281
18	2.0627	1.5
20	2.29305	2.0629
22	2.5735	3.1786
24	3.572168	3.828
26	4.593415	4.7372
28	5.51916	5.693
30	6.23254	6.07561
32	6.729	6.539
34	6.93885	6.732
36	7.131839	6.7518
38	7.142791	6.9731
40	7.142577	7.1139
42	7.11947	7.463
44	7.1178	7.563
46	7.119	7.6218
48	7.1169	7.6432

Appendix III-5: Effect of Incubation period on the production of alkaline phosphatase

Time (hrs)	Enzyme Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)
0	0	0.23571	0
24	1.1963	0.27032	4.42549571
48	3.1792	0.2782	11.4277498
72	2.2943	0.3583	6.40329333

Appendix III-6: Effect of temperature on the production of alkaline phosphatase

Temperature (°C)	Enzyme Activity (U/ml)	Total Protein (mg/ml)	Specific activity (U/mg)
4	6.73	0.23	28.56
10	11.12	0.27	41.16
15	14.57	0.31	45.81
20	22.40	0.33	53.72
25	18.17	0.53	41.72
30	12.55	0.39	32.04

Appendix III-7: Effect of various pH on production of alkaline phosphatase

pH	Enzyme Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)
5	0.8733	0.28571	3.056596
6	0.9522	0.22032	4.321895
7	1.9758	0.2766	7.143167
8	2.3317	0.3083	7.563088
9	2.2698	0.36137	6.281097
10	1.81	0.3359	5.388508

Appendix III-8: Effect of size of inoculum on the production of alkaline phosphatase

Inoculum Size	Enzyme Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)
1	11.06	0.28571	38.71058
5	24.6711	0.32032	77.02017
10	12.1938	0.3766	32.37865
15	8.2657	0.4383	18.85854
20	6.5245	0.5137	12.70099

Appendix III-9: Fig: Effect of age of inoculum on the production of alkaline phosphatase

Time (hrs)	Enzyme Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)
12	2.5794	0.3679	7.011144
24	11.4602	0.38032	30.13305
48	8.4531	0.3656	23.12117
72	6.5209	0.3883	16.79346

Appendix III-10: Standard curve of *p*-nitro phenol

Concentration (mmol)	Optical density (OD_{405})
1	0.1508
2	0.33101
3	0.48142
4	0.63144
5	0.79176
6	0.9553
7	1.1318
8	1.2892
9	1.5712

Appendix III-11: Standard curve of BSA

Concentration (µmol)	Optical density (OD_{650})
10	0.19947
20	0.26734
30	0.31773
40	0.39179
50	0.41841
60	0.47145
70	0.49297
80	0.59276
90	0.61742
100	0.66788

Appendix III-12: Precipitation of Alkaline Phosphatase using ammonium sulfate

Ammonium Sulfate (%)	Activity in pellet (U/ml)	Activity in supernatant (U/ml)
10	10	34
20	17	20
30	37	16
40	52	12
50	12	1.6
60	0.3	0.2
70	no pellet	0

Appendix III-13: Effect of temperature on the activity of crude ALP

Temperature (°C)	Residual Activity (%)
0	100
4	100
10	100
15	100
20	100
25	100
30	100
37	100
40	96
45	82
60	63

Appendix III-14: Effect of pH on the activity of crude alkaline phosphatase

pH	Phosphatase activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)
4	2.32	0.1	23.2
5	5.7	0.2	28.5
6	9.17	0.3	30.56
7	22.0	0.5	44.0
8	27.51	0.6	45.85
9	36.47	0.7	52.10
10	36.54	0.7	52.2
11	36.55	0.7	52.21
12	34.79	0.69	50.42

Appendix III-15: Effect of temperature on the activity of purified alkaline phosphatase

Temperature (°C)	Residual Activity (%)
5	100
10	100
15	100
20	100
25	100
30	100
35	100
40	83
45	51
50	20
55	0

Appendix III-16: Effect of pH on the activity of purified alkaline phosphatase

pH	Residual Activity (%)
4	0
5	1
6	10
7	40
8	90
9	100
10	87
11	60
12	48
13	32
14	3

Appendix III-17: Effect of Metal ions on the activity of alkaline phosphatase

Metal salts	Phosphatase Activity (U/ml)	Residual activity (%)
Control	29	100
Ca	57.19	134.6
Zn	63	140
Mg	61.9	139.25
Hg	38.7	111.47
Cu	46	126
Fe	21	87.5
K	27	90

Appendix III-18: Effect of glycerol on alkaline phosphatase activity

Glycerol (%)	Residual activity (%)
Control	100
10	99
20	99
30	98.5
40	98
50	86
60	76
70	54
80	27
90	16

Appendix III-19: Effect of potassium ferrocyanide on the activity of alkaline phosphatase

1/[S]	Slopes 1/[V]			
	0 mmol	0.5 mmol	1.0 mmol	1.5 mmol
1	3.500175	3.67242	3.969829	4.116921
0.5	3.131851	3.359086	3.402518	3.676471
0.33	3.045067	3.152585	3.436426	3.648304
0.25	2.825657	3.090235	3.243594	3.425831
0.2	2.56476	2.704164	3.098853	3.210273

Appendix III-20: Eadie-Hofstee plot of reaction between *p*-nitrophenyl phosphate and ALP

v/[S]	Vi
0.026943	0.025767
0.055687	0.0216
0.080084	0.01812
0.095906	0.0155
0.134062	0.013

Appendix IV: Phylogenetic analysis

Appendix IV-1: Origin and Accession Nos. of isolates similar in 16s rRNA sequence to *Pseudomonas* sp. MRLBA1

No.	Isolate	Geographical region		Accession Nos.
		Source material	Country	
1.	P.hgice.MRLBA1	Hopper glacier deep Ice	Pakistan	FJ415981
2.	U.fff.e07	Flying fox feces	USA	EU469595
3.	U.fff.g09	Flying fox feces	USA	EU469612
4.	U.fff.g84b04	Flying fox feces	USA	EU469628
5.	U.fff.g84b06	Flying fox feces	USA	EU469630
6.	U.fff.g84d03	Flying fox feces	USA	EU469644
7.	U.fff.g84f03	Flying fox feces	USA	EU469660
8.	U.fff.g84h07	Flying fox feces	USA	EU469679
9.	U.fff.g84h08	Flying fox feces	USA	EU469680
10.	U.fff.C07-1	Flying fox feces	USA	EU469699
11.	U.fff.C09	Flying fox feces	USA	EU469701
12.	U.fff.f09_1	Flying fox feces	USA	EU469723
13.	U.fff.g05	Flying fox feces	USA	EU469731
14.	U.fff.f02-2	Flying fox feces	USA	EU469773
15.	U.fff. g10-2	Flying fox feces	USA	EU469785
16.	U.Hw-12-04	Water of Hot site	ALOHA	EU795190
17.	K.ps4-28	Upland and Paddy soil	Korea	AY303300
18.	U.pbf. g08	Polar bear feces	Antarctica	EU460438

19.	U.pbf. a08	Polar bear feces	Antarctica	EU777748
20.	U.pbf. a07	Polar bear feces	Antarctica	EU460229
21.	U.pbf. b02	Polar bear feces	Antarctica	EU460291
22.	U.pbf. b09	Polar bear feces	Antarctica	EU460357
23.	U.pbf.e04	Polar bear feces	Antarctica	EU460370
24.	C.ani.NJ60	Antarctic ice	Antarctica	AM491464
25.	U.pbf.C02	Polar bear feces	Antarctica	EU460360
26.	U.pbf.d05	Polar bear feces	Antarctica	EU460419
27.	J.fp.TM5-2	Pseudomonas TM5	Japan	AB204715
28.	G.arans.FB23	Arctic and Antarctic soil	Greenland	AM933514
29.	S.WCfish.M	Fresh water crayfish	Spain	AY450555
30.	C.ans.NJ56	Antarctic ice	Antarctica	AM409369
31.	C.RC-duck12	Duck erythrocytes surface	China	EU106576
32.	U.pbf.h01-1	Polar bear feces	Antarctica	EU774950
33.	I.ans.Lz4W	Antarctic lake soil	Antarctica	AJ576247
34.	Ucas.WR#2	Cold alpine soil	Unknown	AY263480
35.	Cpm.YJQ-10	Pink microbial mat	China	AY569287
36.	C.ans.Nj-62b	Antarctic soil	Antarctica	AM396914
37.	C.ans.NJ59	Antarctic soil	Antarctica	AM491463
38.	C.ans.Nj-62a	Antarctic soil	Antarctica	AM491465
39.	C.ans.Nj-63	Antarctic soil	Antarctica	AM491466
40.	C.ans.NJ-22	Antarctic soil	Antarctica	AM421975
41.	C.ans.CL16	Antarctic soil	Antarctica	AY342005
42.	U.pbf.C07	Polar bear feces	Antarctica	EU777822
43.	U.pbf.f04	Polar bear feces	Antarctica	EU460264
44.	U.pbf. C09	Polar bear feces	Antarctica	EU777704
45.	C.Mygl.1416	Mingyong glacier ice	China	EF062806
46.	C.Mygl.1420	Mingyong glacier ice	China	EF062807
47.	C.ans.NJ70	Antarctic soil	Antarctica	AM409370
48.	U.pbf.d11	Polar bear feces	Antarctica	EU460317
49.	U.fff.B05	Flying fox feces	USA	EU469575
50.	U.fff.e08	Flying fox feces	USA	EU469596

Appendix IV-2: Origin and Accession Nos. of isolates similar in 16s rRNA sequence to *Pseudomonas* sp. MRLBA2

No.	Isolate	Geographical region		Accession Nos.
		Source material	Country	
1.	P.pgw.BA2	Passu glacier morain water	Pakistan	FJ415982
2.	U.pbf.d11	Polar bear feces	Antarctica	EU460317
3.	C.ans.NJ70	Antarctic soil	Antarctica	AM409370
4.	C.Mygl.1420	Mingyong glacier ice	China	EF062807
5.	P.hgice.BA1	Glacier ice	Pakistan	FJ415981
6.	U.pbf. C09	Polar bear feces	Antarctica	EU777704
7.	U.pbf.C07	Polar bear feces	Antarctica	EU777822
8.	U.pbf.f04	Polar bear feces	Antarctica	EU460264
9.	C.Mygl.1416	Mingyong glacier ice	China	EF062806
10.	C.ans.CL16	Antarctic soil	Antarctica	AY342005
11.	C.ans.NJ49	Surface soil	Antarctica	AM421981
12.	C.ans.Nj-63	Antarctic soil	Antarctica	AM491466
13.	C.ans.Nj-62a	Antarctic soil	Antarctica	AM491465
14.	C.ans.Nj-62b	Antarctic soil	Antarctica	AM396914
15.	Cpm.YJQ-10	Pink microbial mat	China	AY569287
16.	P.Bglw.BA3	Batura glacier morain water	Pakistan	FJ415983
17.	C.ans.NJ-22	Antarctic soil	Antarctica	AM421975
18.	C.ans.NJ59	Antarctic soil	Antarctica	AM491463
19.	C.ans.NJ56	Antarctic ice	Antarctica	AM409369
20.	S.WCfish.M	Fresh water crayfish	Spain	AY450555
21.	I.ans.Lz4W	Antarctic lake soil	Antarctica	AJ576247
22.	C.al.Zwb3-8	Zoige Alpine Wetland	China	FJ801192
23.	U.pbf.h01-1	Polar bear feces	Antarctica	EU774950
24.	C.pw.BaD5	Pesticide water water	China	EU281635
25.	C.Mygl-1404	Mingyong glacier	China	EU082805
26.	Ucas.WR#2	Cold alpine soil	Unknown	AY263480
27.	U.pbf.d05	Polar bear feces	Antarctica	EU460419
28.	U.pbf.C02	Polar bear feces	Antarctica	EU460360
29.	U.pbf. b02	Polar bear feces	Antarctica	EU460291
30.	J.fp.TM5-2	Pseudomonas TM5	Japan	AB204715
31.	G.arans.FB23	Arctic and Antarctic soil	Greenland	AM933514
32.	U.pbf.PB1.04	Polar bear feces	Antarctica	EU460370
33.	C.RC-duck12	Duck erythrocytes surface	China	EU106576
34.	C.ani.NJ60	Antarctic ice	Antarctica	AM491464
35.	U.pbf.A07	Polar bear feces	Antarctica	EU460229
36.	U.Hw-12-04	Water of Hot site	ALOHA	EU795190
37.	U.fff. g10-2	Flying fox feces	USA	EU469785
38.	U.fff.f02-2	Flying fox feces	USA	EU469773
39.	U.fff.g05	Flying fox feces	USA	EU469731

40.	U.fff.f09_1	Flying fox feces	USA	EU469723
41.	U.fff.C09	Flying fox feces	USA	EU469701
42.	U.fff.C07_1	Flying fox feces	USA	EU469699
43.	U.fff.g84h08	Flying fox feces	USA	EU469680
44.	U.fff.g84h07	Flying fox feces	USA	EU469679
45.	U.fff.g84f03	Flying fox feces	USA	EU469660
46.	U.fff.g84d03	Flying fox feces	USA	EU469644
47.	U.fff.g84b06	Flying fox feces	USA	EU469630
48.	U.fff.g84b04	Flying fox feces	USA	EU469628
49.	U.fff.g09	Flying fox feces	USA	EU469612
50.	U.fff.e07	Flying fox feces	USA	EU469595
51.	U.fff.C05	Flying fox feces	USA	EU469581

Appendix IV-3: Origin and Accession Nos. of isolates similar in 16s rRNA sequence to *Pseudomonas* sp. MRLBA3

Sr. No.	Isolate Code	Geographical region		Acession Nos.
		Source material	Country	
1.	P.pgice.BA4	Ice from Passu Glacier	Pakistan	FJ415984
2.	P.pgw.BA2	Passu glacier morain water	Pakistan	FJ415982
3.	U.pbf.d11	Polar bear feces	Antarctica	EU460317
4.	P.hgice.BA1	Hopper glacier deep Ice	Pakistan	FJ415981
5.	C.ans.NJ70	Antarctic soil	Antarctica	AM409370
6.	C.Mygl.1420	Mingyong glacier ice	China	EF062807
7.	C.Mygl.1416	Mingyong glacier ice	China	EF062806
8.	P.Bglw.BA3	Batura gl. morain water	Pakistan	FJ415983
9.	U.pbf. C09	Polar bear feces	Antarctica	EU777704
10.	U.pbf.C07	Polar bear feces	Antarctica	EU777822
11.	U.pbf.f04	Polar bear feces	Antarctica	EU460264
12.	C.ans.NJ49	Surface soil	Antarctica	AM421981
13.	C.ans.Nj-63	Antarctic soil	Antarctica	AM491466
14.	C.ans.Nj-62a	Antarctic soil	Antarctica	AM491465
15.	C.ans.Nj-62b	Antarctic soil	Antarctica	AM396914
16.	Cpm.YJQ-10	Pink microbial mat	China	AY569287
17.	C.ans.NJ-22	Antarctic soil	Antarctica	AM421975
18.	C.ans.NJ59	Antarctic soil	Antarctica	AM491463
19.	C.ans.NJ56	Antarctic ice	Antarctica	AM409369
20.	S.WCfish.M	Fresh water crayfish	Spain	AY450555
21.	I.ans.Lz4W	Antarctic lake soil	Antarctica	AJ576247
22.	C.ans.CL16	Antarctic soil	Antarctica	AY342005
23.	C.al.Zwb3-8	Zoige Alpine Wetland	China	FJ801192
24.	U.pbf.h01-1	Polar bear feces	Antarctica	EU774950

25.	C.pw.BaD5	Pesticide water water	China	EU281635
26.	C.Mygl-1404	Mingyong glacier	China	EU082805
27.	U.helbow.d09	Human inner elbow	USA	EU539703
28.	Ucas.WR#2	Cold alpine soil	Unknown	AY263480
29.	U.Hw-12-04	Water of Hot site	ALOHA	EU795190
30.	U.pbf.d05	Polar bear feces	Antarctica	EU460419
31.	U.pbf.C02	Polar bear feces	Antarctica	EU460360
32.	U.pbf. b02	Polar bear feces	Antarctica	EU460291
33.	C.RC-duck12	Duck erythrocytes	China	EU106576
34.	J.fp.TM5-2	Pseudomonas TM5	Japan	AB204715
35.	C.fd.FLM05-3	food	China	DQ084461
36.	U.hvage.148	Human vaginal epithelium	USA	AY958921
37.	U.hvage.80	Human vaginal epithelium	USA	AY958853
38.	U.P.fragi	ATCC4973	USA	AF094733
39.	U.fff.b02_1	Flying fox feces	USA	EU469689
40.	G.arans.FB23	Arctic and Antarctic soil	Greenland	AM933514
41.	U.fff.C12_2	Flying fox feces	USA	EU469756
42.	U.fff.d03_1	Flying fox feces	USA	EU469705
43.	U.pbf.e04	Polar bear feces	Antarctica	EU460370
44.	U.helbow.h09	Human inner elbow	USA	EU539071
45.	J.psyc.C04	Psychrophilic strain	Japan	EU535921
46.	U.biopsy.A05	Ear punch biopsy of female	USA	EU534553
47.	C.ani.NJ60	Antarctic ice	Antarctica	AM491464
48.	U.pbf.A07	Polar bear feces	Antarctica	EU460229
49.	U.pbf.b04	Polar bear feces	Antarctica	EU777710
50.	U.fff. g10-2	Flying fox feces	USA	EU469785
51.	U.fff.f02-2	Flying fox feces	USA	EU469773

Appendix IV-4: Origin and Accession Nos. of isolates similar in 16s rRNA sequence to *Pseudomonas* sp. MRLBA4

Sr. No.	Isolate Code	Geographical region		Acession Nos.
		Source material	Country	
1.	P.pgice.BA4	Ice from Passu Glacier	Pakistan	FJ415984
2.	P.pgw.BA2	Passu glacier morain water	Pakistan	FJ415982
3.	U.pbf.d11	Polar bear feces	Antarctica	EU460317
4.	P.hgice.BA1	Hopper glacier deep Ice	Pakistan	FJ415981
5.	C.ans.NJ70	Antarctic soil	Antarctica	AM409370
6.	C.Mygl.1420	Mingyong glacier ice	China	EF062807
7.	C.Mygl.1416	Mingyong glacier ice	China	EF062806
8.	P.Bglw.BA3	Batura gl. morain water	Pakistan	FJ415983
9.	U.pbf. C09	Polar bear feces	Antarctica	EU777704
10.	U.pbf.C07	Polar bear feces	Antarctica	EU777822
11.	U.pbf.f04	Polar bear feces	Antarctica	EU460264
12.	C.ans.NJ49	Surface soil	Antarctica	AM421981
13.	C.ans.Nj-63	Antarctic soil	Antarctica	AM491466
14.	C.ans.Nj-62a	Antarctic soil	Antarctica	AM491465
15.	C.ans.Nj-62b	Antarctic soil	Antarctica	AM396914
16.	Cpm.YJQ-10	Pink microbial mat	China	AY569287
17.	C.ans.NJ-22	Antarctic soil	Antarctica	AM421975
18.	C.ans.NJ59	Antarctic soil	Antarctica	AM491463
19.	C.ans.NJ56	Antarctic ice	Antarctica	AM409369
20.	S.WCfish.M	Fresh water crayfish	Spain	AY450555
21.	I.ans.Lz4W	Antarctic lake soil	Antarctica	AJ576247
22.	C.ans.CL16	Antarctic soil	Antarctica	AY342005
23.	C.al.Zwb3-8	Zoige Alpine Wetland	China	FJ801192
24.	U.pbf.h01-1	Polar bear feces	Antarctica	EU774950
25.	C.pw.BaD5	Pesticide water water	China	EU281635
26.	C.Mygl-1404	Mingyong glacier	China	EU082805
27.	U.helbow.d09	Human inner elbow	USA	EU539703
28.	Ucas.WR#2	Cold alpine soil	Unknown	AY263480
29.	U.Hw-12-04	Water of Hot site	ALOHA	EU795190
30.	U.pbf.d05	Polar bear feces	Antarctica	EU460419
31.	U.pbf.C02	Polar bear feces	Antarctica	EU460360
32.	U.pbf. b02	Polar bear feces	Antarctica	EU460291
33.	C.RC-duck12	Duck erythrocytes	China	EU106576
34.	J.fp.TM5-2	Pseudomonas TM5	Japan	AB204715
35.	C.fd.FLM05-3	food	China	DQ084461
36.	U.hvage.148	Human vaginal epithelium	USA	AY958921
37.	U.hvage.80	Human vaginal epithelium	USA	AY958853
38.	U.P.fragi	ATCC4973	USA	AF094733
39.	U.fff.b02_1	Flying fox feces	USA	EU469689

		samples		
27.	U.LZ.Ar.OS-02	Lead-zinc mine soil	Arizona	EF612303
28.	E.Shale. 4-C16-21	semi-coke	Estonia	EF540487
29.	F.Bdust.OS-44.C2	Barnyard dust	Finland	AM237357
30.	U.uran.AKAU3838	uranium contaminated soil	USA	DQ125733
31.	U.uran.AKAU3822	uranium contaminated soil	USA	DQ125724
32.	U.uran.AKAU3785	uranium contaminated soil	USA	DQ125696
33.	U.uran.AKAU3594	uranium contaminated soil	USA	DQ125595
34.	U.uran.AKAU3591	uranium contaminated soil	USA	DQ125592
35.	G.art.KA1-1	Unknown	Germany	AJ785760
36.	G.act.CCM 4924T	Actinobacterium	Germany	AJ512504
37.	B.vet.R5815	Veterinary origin strains	Belgium	AJ491108
38.	B.vet. R5812T	Veterinary origin strains	Belgium	AJ316140
39.	G. DSM20116	Human skin	Germany	X83405
40.	A.tanw.MM10	tannery waste contaminated site	Australia	EU729736
41.	C.cons.AD27	Contaminated soil	China	EU710551
42.	N.evrst.gws-39	Mount everest meltwater	Nepal	EU584515
43.	I.hims.K22-12	soil from cold desert	Himalayas India	EU333870
44.	E.Shale. 4-C16-51	semi-coke	Estonia	EF540513
45.	Cr.Ar.keyseri	Atrazine soil	Croatia	EF050052
46.	C.PAHs.Phe-3	Soil	China	DQ519082
47.	U.uran.AKAU4080	uranium contaminated soil	USA	DQ125849
48.	U.uran.AKAU4057	uranium contaminated soil	USA	DQ125831
49.	U.uran.AKAU3864	uranium contaminated soil	USA	DQ125751
50.	U.uran.AKAU3849	uranium contaminated soil	USA	DQ125739

Appendix IV-6: Origin and Accession Nos. of isolates similar in 16s rRNA sequence to *Stenotrophomonas* sp. MRLBA6

Sr. No.	Geographical Region		Isolate	Accession No
	Source material	Country		
1.	Ice block from Passu Glacier	Pakistan	*Stenotrophomonas* sp. MRLBA6	GU581318
2.	Oxytetracycline production waste water	China	*Stenotrophomonas maltophilia* c66	FJ950563.1
3.	Oxytetracycline production waste water	China	*Stenotrophomonas maltophilia* c57	FJ950544.1
4.	Kennedy Space Centre clean room floor	USA	*Stenotrophomonas* sp.1P-1-K09	EU704858.1
5.	Vermi compost	Korea	*Stenotrophomonas* sp. VC-YC6647	EU734616.1
6.	Kennedy Space Centre clean room floor	USA	*Stenotrophomonas* sp. 1P-1-M03	EU704888.1
7.	Unknown	China	*Stenotrophomonas* sp. MHS021	DQ993329.1
8.	Unknown	Australia	*Xanthomonas translucens* P7	AY994100.1
9.	Unknown	Australia	*Xanthomonas translucens* P25	AY994101.1
10.	Dokdo island soil	Korea	*Stenotrophomonas maltophilia*	EU834246.1

Appendix V: MRL Glacier Expedition 2006 (Hopper Glacier, Fig 3.2a)