Biological characterization of selected alkaline protease producing alkaliphilic bacteria from coastal ecosystems of Goa

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BY

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STATEMENT

I hereby state that this thesis for Ph.D. degree on "**Biological characterization of selected alkaline protease producing alkaliphilic bacteria from coastal ecosystems of Goa**" is my original contribution and that the thesis and any part of it has not been previously submitted for the award of any degree/ diploma of any University or Institute. To the best of my knowledge, the present study is the first comprehensive work of its kind from this area.

Date:

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Brenda D'Costa

Dedicated to my

parents…

For their endless love, care and encouragement

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CHAPTER I

INTRODUCTION

INTRODUCTION

1.1 Extremophilic microorganisms

Extremophiles are microbes having unique adaptive mechanisms that allow them to survive and thrive under extreme environmental conditions viz. pH, temperature, salinity and pressure. Thus these organisms are referred as alkalophiles, acidophiles, thermophiles, psychrophiles, halophiles and barophiles (Lederberg *et al*., 2000; Burg, 2003; Gomes and Steiner, 2004). Thermophiles grow readily at the temperature > 45 ^{0}C whereas some of them favour temperature > 80 ^{0}C and are referred as hyperthermophiles. Extremely hyperthermophilic microorganisms which survive above 100 ^{0}C include thermal vent bacteria viz. *Thermus aquaticus*, *Pyrococcus furiosus*, *Pyrolobus fumarii* and *Methanopyrus* sp. (Blochl *et al*., 1997; Gao *et al*., 2003). Psychrophiles are actually more common than thermophiles since the oceans maintain an average temperature of 1 to 3 ^{0}C and cover over half the earth's surface including Arctic and Antarctic Ocean. The psychrophiles include photosynthetic eukarya, notably algae and diatoms such as *Polaromonas vacuolata*. Acidophiles thrive in the rare habitats with pH < 5 and alkaliphiles favour habitats with pH 9 or > 9. Highly acidic environments have resulted naturally due to geochemical activities and metabolic activities of certain acidophiles. They are also found in the debris left over from coal mining. *Thiobacillus ferroxidans* and *Thiobacillus thiooxidans* are best studied acidophiles which prefer extremely low pH i.e. 2-3 (Yates and Holmes, 1987; Rawlings, 2005). Whereas alkaliphiles flourish in soil laden with carbonate and in soda lakes, such as those found in Egypt, the Rift valley of Africa and the Western U.S. Similarly barophiles are microorganisms which grow best under pressure > 1 atmosphere. They live deep under the surface of the earth or oceans. Halophiles or salt-loving microorganisms are found in the aquatic environment with high

concentrations of salts especially NaCl. Most common habitats for halophiles are hypersaline lakes such as the Great Salt Lake, the Dead Sea and solar salt evaporation ponds. Commonly known and well studied halophiles are *Halobacterium salinarum, Halothermothrix orenii* (Mijts and Patel, 2002).

1.2 Extremophilic microorganisms as a source of novel enzymes

Majority of extremophiles that have been identified belong to the domain archaea. However, extremophiles from the eubacterial and eukaryotic kingdoms have also been identified and characterized (Van den Burg, 2003). The extremophilic microorganisms are valuable sources of novel enzymes with unique properties and these biocatalysts are referred as extremozymes (Herbert, 1992; Madigan and Marrs, 1997). Extremozymes produced by extremophiles are proteins which function under extreme conditions and due to their stability under extreme conditions they offer new opportunities for biocatalysis and biotransformation of raw materials to the desired quality of the final product. Examples of extremozymes include cellulases, amylases, xylanases, proteases, pectinases, keratinases, lipases, esterases, catalases, peroxidases and phytases which have great potential in several biotechnological processes to produce value added products (Table 1.1). The extremozymes are used in various biotechnological industries due to specific requirement of the industrial processes such as high temperature, pressure and/or pH. At present only a very small percentage of microorganisms on the planet earth have been identified and tapped commercially, out of which only few are extremophiles (Gomes and Steiner, 2004). However commercial interest of biotechnologists to explore extremophiles with valuable extremozymes has got renewed due to advancement in the field of metagenomics and

recombinant DNA technology in order to enhance the production of novel extremozymes.

Table 1.1 Microbial Extremozymes and their applications (Van den 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

1.3 Extremophilic microorganisms as source of proteases

Proteolytic enzymes are ubiquitous, since they are found in all living organisms and essential for cell growth and differentiation. Proteases are intracellular and extracellular. The extracellular proteases are of commercial significance as they have multiple applications in various industrial sectors (Gupta *et al.*, 2002). Whereas intracellular proteases are important for various cellular and metabolic processes viz. sporulation, differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are also important for hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolyzed products (Kalisz, 1998). They are one of the largest group of enzymes (Fig. 1.1) that make up 60 % of total worldwide sale of enzymes (Joshi *et al.,* 2007). The extracellular proteases have also been commercially exploited to facilitate protein degradation in various industrial processes (Outtrup and Boyce, 1990; Kumar and Takagi, 1999).

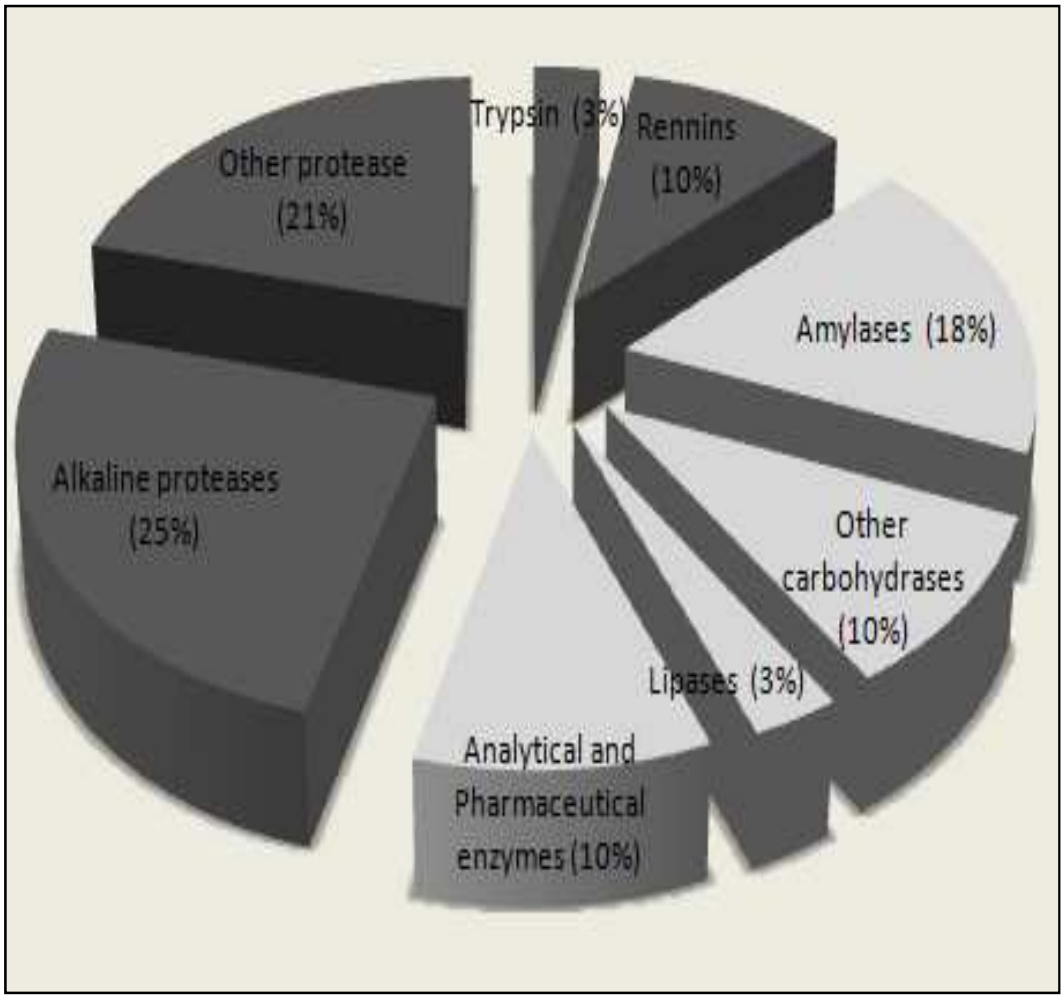


Fig 1.1 Worldwide distribution of enzyme sales. The contribution of different enzymes to the total sale of enzymes is indicated. The shaded portion indicates the total sale of proteases (Rao *et al.*, 1998)

1.4 Classification of proteases

Based on the source of proteases they have been categorized as plant proteases, animal proteases and microbial proteases:

➢ PLANT PROTEASES

Plants produce several proteases in various plant parts. Moreover, protease production from plants is a time-consuming process. Examples of plant proteases include papain, bromelain, keratinases and ficin which have been well characterized.

Papain

Papain is a traditional plant protease which is extracted from the latex of fruits of *Carica papaya,* which is usually grown in the sub-tropical areas of west and central Africa and India. The crude enzyme has a broader specificity due to presence of several proteinase and peptidase isozymes. The performance of this enzyme depends on the plant source, the climatic conditions for growth, and methods used for its extraction and purification. The enzyme is active between pH 5 and 9 and is stable upto 90 ^{0}C in the presence of substrates and extensively used in food industry for the preparation of highly soluble and flavored protein hydrolysates (Teixeira da Silva *et al*., 2007; Amri and Mamboya, 2012).

Bromelain

Bromelain is a cysteine protease enzyme active at pH 5 to 9 which is extracted from the stem and juice of pineapples (Rowan and Buttle, 1994; Tochi *et al*., 2008). It gets inactivated at 70 ^{0}C. The major supplier of the enzyme is Great Food Biochem, Bangkok, Thailand.

Keratinase

Some bacteria and fungi produce proteases capable of degrading hair e.g. *Bacillus licheniformis, Streptomyces* (Williams *et al*., 1990; Mohamedin, 1999). Degradation of hair is important for the production of essential amino acids such as lysine and for the prevention of clogging of wastewater drainage system (Rao *et al*., 1998).

➢ ANIMAL PROTEASES

The well-known proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennin (Boyer, 1971; Hoffman, 1974).

Trypsin

Trypsin (MW: 23,300 Da) is the main intestinal digestive enzyme responsible for hydrolysis of food proteins. It is a serine protease and hydrolyzes peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues. Based on the ability of protease inhibitors to inhibit the enzyme from the insect gut, this enzyme has been useful as a target for biocontrol of insect pests. Trypsin has limited applications in the food industry, since the protein hydrolysates generated by its action possess highly bitter taste. Trypsin is used commercially in preparation of bacterial growth media and in some specialized medical applications.

Chymotrypsin

Chymotrypsin (MW: 23,800 Da) is found in the pancreatic extract. Pure chymotrypsin is an expensive enzyme used only in diagnostic and analytical applications. It is specific for the hydrolysis of peptide bonds in which the carboxyl groups are provided by one of the three aromatic amino acids, i.e. phenylalanine, tyrosine, or tryptophan. It is used extensively in deallergenizing of milk protein hydrolysates. It is stored in the pancreas in the form of a precursor, i.e. chymotrypsinogen and is activated by trypsin in a multi-step reaction process.

Pepsin

Pepsin (MW: 34,500 Da) is an acidic protease found in the stomach of almost all vertebrates. The active enzyme is released from its zymogen, i.e., pepsinogen, by autocatalysis in the presence of hydrochloric acid. Pepsin is an aspartyl protease and resembles human immunodeficiency virus type 1(HIV-1) protease, responsible for the

maturation of HIV-1. It exhibits optimal activity between pH 1 and 2, while the optimal pH of the stomach is 2 to 4. Pepsin is inactivated above pH 6.0. This enzyme catalyzes the hydrolysis of peptide bonds between two hydrophobic amino acids.

Rennin

Rennin is a pepsin-like protease produced as an inactive precursor, pro-rennin in the stomach of all nursing mammals. It is converted to active rennin (MW: 30,700 Da) by the action of pepsin or through autocatalysis. It is used extensively in the dairy industry to produce stable curd with good flavour. The specific activity of this enzyme is attributed to its specificity in cleaving a single peptide bond in k-casein to generate insoluble para- k- casein and C-terminal glycopeptide.

➢ MICROBIAL PROTEASES

The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial protease production worldwide (Table 1.2). Microorganisms are excellent source of several industrially important extremozymes including proteases due to their broad biochemical diversity, stability and ease of genetic manipulation. It is important to note that microbial proteases account for approximately 40 % of the total worldwide enzyme sales (Godfrey and West, 1996). Proteases from microbial sources are preferred than plant and animal sources since they possess almost all the important characteristics desired for their biotechnological applications.

Table 1.2 Worldwide production of Microbial protease (Kumar *et al.*, 2008)

Supplier	Product trade name	Microbial source	Application
Novo Nordisk, Denmark	Alcalase	*Bacillus licheniformis*	Detergent, silk degumming
	Savinase	*Bacillus* sp.	Detergent, textile
	Esperase	*B. lentus*	Detergent, food, silk degumming
	Biofeed pro	*B. licheniformis*	Feed
	Durazym	*Bacillus* sp.	Detergent
	Novozyme 471MP	n.s.	Photographic gelatin hydrolysis
	Novozyme 243	*B. licheniformis*	Denture cleaners
	Nue	*Bacillus* sp.	Leather
Genencor International, USA	Purafact	*B. lentus*	Detergent
	Primatan	Bacterial source	Leather
Gist-Brocades, The Netherlands	Subtilisin	*B. alcalophilus*	Detergent
	Maxacal	*Bacillus* sp.	Detergent
	Maxatase	*Bacillus* sp.	Detergent
Solvay Enzymes, Germany	Opticlean	*B. alcalophilus*	Detergent
	Optimase	*B. licheniformis*	Detergent
	Maxapem	Protein engineered variant of *Bacillus* sp.	Detergent
	HT- proteolytic	*B. subtilis*	Alcohol, baking, brewing, feed, food, leather, photographic waste
	Protease	*B. licheniformis*	Food, waste
Amano Pharmaceuticals, Japan	Proleather	*Bacillus* sp.	Food
	Collagenase	*Clostridium* sp.	Technical
	Amano protease S	*Bacillus* sp.	Food
Enzyme Development, USA	Enzeco alkaline protease	*B. licheniformis*	Industrial
	Enzeco alkaline protease-L FG	*B. licheniformis*	Food
	Enzeco high alkaline protease	*Bacillus* sp.	Industrial
Nagase Biochemicals, Japan	Bioprase concentrate	*B. subtilis*	Cosmetics, pharmaceuticals
	Ps. protease	*Pseudomonas aeruginosa*	Research
	Ps. elastase	*Pseudomonas aeruginosa*	Research
	Cryst. protease	*B. subtilis* (K2)	Research
	Cryst. protease	*B. subtilis* (bioteus)	Research
	Bioprase	*B. subtilis*	Detergent, cleaning
	Bioprase SP-10	*B. subtilis*	Food
Godo Shusei, Japan	Godo-Bap	*B. licheniformis*	Detergent, Food
Rohm, Germany	Corolase 7089	*B. subtilis*	Food
Wuxi Synder Bioproducts, China	Wuxi	*Bacillus* sp.	Detergent
Advance Biochemicals, India	Protosol	*Bacillus* sp.	Detergent

1.5 Sources of microbial proteases

➢ Bacteria

Bacteria are major producers of proteases with genus *Bacillus*, as the prominent source (Patel *et al*., 2006). Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus.* Others include *Streptomyces cellulasae, Aeromonas hydrophila, Arthrobacter ramosus, Pseudomonas* sp. and *Nesterenkonia* sp. strain AL-20 (Morita *et al*., 1998; Van den Burg, 2003).

Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8). Due to their intermediate rate of reaction, neutral proteases cause less bitterness in hydrolyzed food proteins as compared to animal proteinases thus are valuable in the food industry. Neutrases (neutral proteases) are resistant to natural plant proteinase inhibitors therefore used in the brewing industry. Some neutral proteases belong to the metalloprotease group as they require divalent metal cations viz. Zn and Co for their activity. Others are serine proteinases not affected by chelating agents.

Bacterial alkaline proteases are characterized by their high activity at alkaline pH range of 9-11 and their broad substrate specificity. Their optimal temperature is around 40-60 ^{0}C. These characteristics of bacterial alkaline proteases render them more suitable for use in detergent industry as detergent additives. Most commercial serine proteases, mainly neutral and alkaline, are produced by microorganisms belonging to the genus *Bacillus.* Some of the gram negative bacteria producing alkaline proteases include *Vibrio cholerae* (Deane *et al*., 1987) and *Xathomonas maltophila* (Debette, 1991).

Halophilic bacteria are also known to produce alkaline proteases for example *Halobacterium* sp. (Ahan *et al.*, 1990). Similarly alkaline proteases are also produced by other bacteria viz. *Thermus caldophilus, Desulfurococcus mucosus, Streptomyces griseus* and *Escherichia coli.*

- **Fungi**

Fungi produce more diverse varieties of enzymes than do bacteria. For example, *Aspergillus oryzae* produces acid, neutral, and alkaline proteases. The fungal proteases are active over a wide range of pH (pH 4 to 11) and also exhibit broad substrate specificity. However, they have lower reaction rate and poor heat tolerance as compared to the bacterial enzymes. Fungal proteases can be conveniently produced in a solid-state fermentation process. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. They are especially useful in the cheese making industry due to their narrow pH and temperature specificities. Fungal neutral proteases are metalloproteases which are active at pH 7.0 and inhibited by chelating agents. Fungal alkaline proteases produced by fungal strains viz. *Aspergillus oryzae, Fusarium graminearum* and *Acremonium chrysogenum* are also commonly used in food protein modification through hydrolysis.

- **Viruses**

Viruses also produce different kind of proteases. Viral proteases have gained importance due to their functional involvement in processing of viral proteins which cause fatal diseases in humans and animals viz. AIDS, cancer, Hepatitis, Foot and mouth disease and Herpes labialis. Several viruses produce serine, aspartic and cysteine peptidases (Rawlings and Barrett, 1993) and all viral peptidases are endopeptidases. Extensive research has focused on the three-dimensional structure of viral proteases and their interaction with inhibitors in order to design potent inhibitors

to combat the relentlessly spreading epidemic of AIDS (Martin, 1992; Wlodawer and Gustchina, 2000).

Thus, although proteases are widespread in nature, microbes serve as a preferred source due to the ease with which they can be genetically manipulated to generate new enzymes with altered properties desirable for their various biotechnological applications.

1.6 Alkaliphilic microorganisms producing alkaline protease

Alkaliphilic microorganisms producing alkaline proteases include *Bacillus alcalophilus* (Kanekar *et al*., 2002), *B. amyloliquefaciens* (George *et al.,* 1995), *B. circulans* (Chislett and Kushner, 1961), *B. coagulans* (Gajju *et al.,* 1996), *B. firmus* (Moon and Parulekar, 1991), *B. intermedius* (Itskovich *et al*., 1995) and *B. lentus* (Bettel *et al.,* 1992).

Some of the Gram-negative bacteria producing alkaline proteases include *Pseudomonas aeruginosa* (Morihara, 1963), *Pseudomonas maltophila* (Kobayashi *et al.,* 1985), *Pseudomonas* sp. strain B45 (Chakraborty and Srinivasan, 1993), *Xanthomonas maltophila* (Debette, 1991), *Vibrio alginolyticus* (Deane *et al*., 1987) and *Vibrio metschnikovii* strain RH530 (Kwon *et al.,* 1994). Alkaline proteases are also produced by some rare microorganisms such as *Kurthia spiroforme*, a spiral shaped Gram-positive bacterium possessing a distant relationship to genus *Bacillus* (Steele *et al.,* 1992). A bacterial isolate symbiotic with a marine ship worm, *Psiloteredo healdi*, has also been reported to produce alkaline protease (Greene *et al.,* 1989).

Halophilic bacteria also produce alkaline proteases which include *Halobacterium* sp. (Ahan *et al*., 1990), *Halobacterium halobium* ATCC 43214 (Ryu *et al.,* 1994) and *Halomonas* sp. strain ES-10 (Kim *et al*., 1992).

Thermoalkaliphiles are also known to produce alkaline proteases viz. *Thermus aquaticus* YT-1, *Desulfurococcus, Bacillus thuringiensis, Anoxybacillus mongoliensis* and *Bacillus stearothermophilus* strain F1 (Salleh *et al*., 1977; Matsuzawa *et al*., 1988; Kunitate *et al*., 1989; Hanazawa *et al*., 1996; Namsaraev *et al*., 2010).

Fungi have also been reported to produce extracellular alkaline proteases (Matsubara and Feder, 1971). The alkaline protease of *Aspergillus* sp. has been studied in detail. Yeasts also produce alkaline proteases which include *Candida lipolytica* (Tobe *et al*., 1976), *Yarrowia lipolytica* (Ogrydziak, 1993) and *Aureobasidium pullulans* (Donaghy and McKay, 1993). Few alkaliphilic actinomycetes are also reported to produce alkaline proteases (Mikami *et al*., 1986).

1.7 Classification of proteases

Proteases are one of the hydrolases which cleave peptide bonds and are also referred as peptidases, proteinases or proteolytic enzymes (Fig. 1.2).

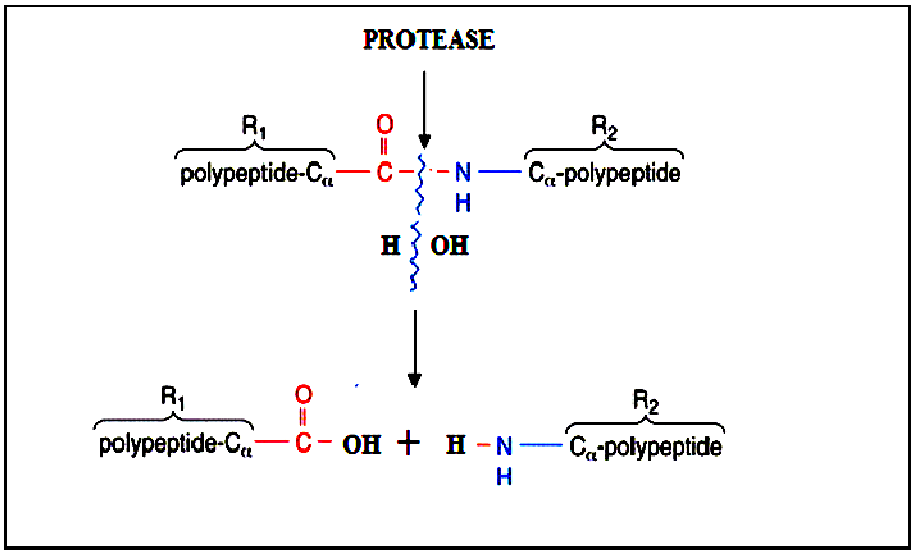


Fig 1.2 Mechanism of protease activity

Among the various proteases, bacterial proteases are the most important, since they can be cultured in large quantities in a relatively short time by various fermentation methods and produce an abundant supply of the desired product. In addition,

microbial proteases have a longer shelf life without significant loss of activity (Gupta *et al.*, 2002).

Proteases have also been classified based on 3 major criteria: i) type of reaction catalyzed; ii) chemical nature of the catalytic site and iii) evolutionary relationship as revealed by the structure. Proteases may be further grouped in two subclasses depending on the location of enzymatic action, i.e. exopeptidases and endopeptidases. Exopeptidase cleaves peptide bonds at the amino-terminus or carboxy-terminus of a peptide substrate while endopeptidase cleaves peptide bonds internally, away from either termini of the protein substrate.

EXOPEPTIDASES

Exopeptidases act only close to the ends of polypeptide chains. Based on their site of action i.e. N or C terminus, they are classified as **amino** and **carboxy peptidases** respectively.

➢ Aminopeptidases

Aminopeptidases act near the free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. They are also known to remove the N-terminal Met that may be present in heterologously expressed proteins but not in mature proteins. Aminopeptidases occur in a wide variety of microbial communities including bacteria and fungi (Watson, 1976). In general, aminopeptidases are intracellular enzymes, except a single report on an extracellular aminopeptidase produced by *A. oryzae* (Labbe *et al.*, 1974). Aminopeptidase I from *Escherichia coli* is a large protease (400,000 Da). It has broad pH optima in the range of 7.5 to 10.5 and requires Mg^{+2} or Mn^{+2} ions for its activity (De Marco and Dick, 1978). Whereas aminopeptidase II from *B. stearothermophilus* is a dimer with

molecular weight of 80,000 to 100,000 Da and is activated by Zn^{+2}, Mn^{+2} and Co^{+2} ions (Stoll *et al*., 1976).

- **Carboxypeptidases**

Carboxypeptidases act at C terminal of the polypeptide chain and liberate a single amino acid or a dipeptide. They can be divided into three major groups i.e. serine, metallo and cysteine carboxypeptidases based on the nature of the amino acid residues present at the active centre of the enzyme. The serine carboxypeptidases isolated from *Penicillium* sp., *Saccharomyces* sp. and *Aspergillus* sp. are similar in their substrate requirements but differ slightly in properties like optimum pH, stability, molecular weight and sensitivity to inhibitors. Metallocarboxypeptidases from *Saccharomyces* sp. and *Pseudomonas* sp. require Zn^{+2} or Co^{+2} for their activity. These enzymes also hydrolyze peptides in which the peptidyl group is replaced by a pteroyl moiety or by acyl groups.

Based on the functional group present at the active site, proteases are further classified into four main groups i.e. serine, aspartic, cysteine and metallo proteases (Hartley, 1960). Serine peptidases have serine involved in the active centre of the catalytic process (Kato *et al.*, 1992). The cysteine peptidases have a cysteine residue in the active centre, while the aspartic peptidases depend on two aspartic acid residues for their catalytic activity and the metallopeptidases use a metal ion (commonly Zn^{+2} or Co^{+2}) in the catalytic mechanism (Gupta *et al*., 2002).

Based on their amino acid sequences proteases are also classified into different families (Argos, 1987) and further subdivided into “clans” which have diverged from a common ancestor (Rawlings and Barrett, 1993). Each family of peptidases has been assigned a code letter denoting the type of catalysis, i.e. S, C, A, M, for serine, cysteine, aspartic or metalloprotease respectively.

ENDOPEPTIDASES

Endopeptidases act on the peptide bonds present in the inner regions of the polypeptide chain away from the N and C termini. The endopeptidases are divided into four subgroups based on their catalytic mechanism i.e. (i) serine (ii) aspartic (iii) cysteine and (iv) metalloproteases.

➢ Serine protease

Serine proteases are characterized by the presence of a serine amino acid in their active centre. They are more common and widespread among viruses, bacteria and eukaryotes, suggesting that they play crucial role in the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase and omega peptidase groups. Serine proteases are recognized by their irreversible inhibition by 3, 4-dichloroisocoumarin (3,4-DCI), L -3- carboxytrans 2, 3- epoxy propyl-leucyl amido (4- guanidine) butane (E.64), di-isopropylfluorophosphate (DFP), phenyl methyl sulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Few serine proteases are inhibited by thiol reagents such as *p*-chloro-mercuri benzoate (PCMB) due to the presence of a cysteine residue near the active site.

Serine proteases are usually active at neutral and alkaline pH with an optimum range (7-11). They exhibit broad substrate specificities including esterolytic and amidase activity. Their molecular masses range between 18 to 35 kDa. The isoelectric points of serine proteases are usually between pH 4 to 6. Serine alkaline proteases that are active at highly alkaline pH is among the largest subgroup of serine proteases (Saeki *et al*., 2002; Haddar *et al*., 2009; Jayakumar *et al*., 2012).

(i) Serine alkaline proteases

Serine alkaline proteases are produced by numerous bacteria, molds, yeasts and fungi. They are inhibited by DFP or a potato protease inhibitor. Their substrate requirement is similar to chymotrypsin but less stringent. They hydrolyze a peptide bond which has tyrosine, phenylalanine or leucine at the carboxyl side of the splitting bond. The optimum pH is 10 and their isoelectric point is pH 9. Their molecular weight ranges from 15 to 30 kDa. Although alkaline serine proteases are produced by several bacteria such as *Arthrobacter* sp., *Streptomyces* sp., and *Flavobacterium* sp. (Boguslawski *et al*., 1983), but subtilisins produced by *Bacillus* spp. are more common. Alkaline proteases are also produced by *S. cerevisiae* (Mizuno and Matsuo, 1984) and filamentous fungi such as *Conidiobolus* sp., *Aspergillus* sp. and *Neurospora* sp. (Lindberg *et al.,* 1981; Phadatare *et al*., 1993).

(ii) Subtilisins

Subtilisins of *Bacillus* origin belong to the second largest family of serine proteases. Two different types of alkaline proteases i.e. Subtilisin Carlsberg and Subtilisin Novo or bacterial protease Nagase (BPN9) have been reported (Wells *et al*., 1983; Hubner *et al*., 1993; Putten *et al*., 1996; Phadatare *et al*., 1997). Subtilisin Carlsberg produced by *Bacillus licheniformis* was discovered in 1947 by Linderstrom, Lang and Ottesen at the Carlsberg laboratory. Whereas Subtilisin Novo or BPN9 is produced by *Bacillus amyloliquefaciens*. Subtilisin BPN9 is commercially less important compared to Subtilisin Carlsberg. Subtilisin Carlsberg is widely used as additives in detergent industries. Both subtilisins have a molecular weight of 27.5 kDa but differ from each other by 58 amino acids. They have similar properties such as an optimal pH and temperature of 10 and 60 ^{0}C respectively. Both the enzymes exhibit an active-site triad made up of serine, histidine and aspartic acid amino acid residues. The Carlsberg

enzyme has broader substrate specificity and does not require Ca^{2+} ions for its stability. The active site conformation of subtilisins is similar to that of trypsin and chymotrypsin with difference in overall molecular arrangements (Pattabiraman and Lawson, 1972).

- **Aspartic proteases**

Aspartic acid proteases also referred as acidic proteases are endopeptidases which depend on aspartic acid residues for their catalytic activity. These aspartic acidic proteases have been grouped into three major families such as pepsin (A1), retropepsin (A2) and enzymes from para-retroviruses (A3) (Barrett, 1995). Most aspartic acid proteases show maximum enzyme activity at low pH i.e. 3 to 4 and possess isoelectric points in the range of pH 3 – 4.5. Their molecular weight ranges between 30 to 45 kDa. The active centre of aspartic acid residue is situated within the motif Asp-Xaa-Gly, in which Xaa can be Ser or Thr. The aspartic acid proteases are inhibited by pepstatin (Fitzgerald *et al.*, 1990) and also sensitive to diazoketone compounds such as diazoacetyl-DL-norleucine methyl ester (DAN) and 1, 2-epoxy-3-(*p* nitrophenox) propane (EPNP) in the presence of Cu^{+2} ions. Microbial acid proteases exhibit specificity against aromatic amino acid residues on both sides of the peptide bond, which is similar to pepsin. Microbial aspartic proteases can be broadly divided into two groups i.e. pepsin-like enzymes produced by *Aspergillus*, *Penicillium, Rhizopus* and *Neurospora* and rennin type enzymes produced by *Endothia* sp. and *Mucor* spp. (Da Silveira *et al.*, 2005; Kumar *et al.*, 2005; Wang, 2008).

- **Cysteine proteases**

Cysteine proteases occur in both prokaryotes and eukaryotes and approximately 20 families of cysteine proteases are known so far. The activity of all cysteine proteases

depends on a catalytic dyad consisting of cysteine and histidine. Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like (ii) trypsin-like with preference for cleavage at the arginine residue (iii) specific to glutamic acid and (iv) others. Among these Papain is the best known cysteine protease.

Cysteine proteases have neutral pH optima, although a few of them viz. lysosomal proteases are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents. Clostripain produced by the anaerobic bacterium, *Clostridium histolyticum* exhibits a stringent specificity for arginyl residues at the carboxyl side of the splitting bond and differs from papain in its obligate requirement for calcium. Clostripain has an isoelectric point of pH 4.9 and molecular mass of 50 kDa (Barrett and Rawlings, 2001; Manabe *et al*., 2010).

- **Metalloproteases**

Metalloproteases are the most diverse kind of proteases (Barrett, 1995). They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Shannon *et al.*, 1989). About 30 families of metalloproteases have been reported, of which 17 contain only endopeptidases, 12 contain only exopeptidases, and 1 contains both endo- and exopeptidases. Families of metalloproteases have been grouped into different clans based on the nature of the amino acid that completes the metal-binding site.

Based on the specificity of their action, metalloproteases can be divided into four

groups: (i) neutral, (ii) alkaline, (iii) *Myxobacter* I, and (iv) Myxobacter II. The neutral proteases show specificity for hydrophobic amino acids, while the alkaline proteases possess a very broad specificity. *Myxobacter* protease I is specific for small amino acid residues on either side of the cleavage site, whereas protease II is specific for lysine residue on the amino side of the peptide bond. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP (Murphy *et al*., 1981).

Thermolysin, a neutral protease, is the most thoroughly characterized member of clan MA. Thermolysin produced by *B. stearothermophilus* is a single peptide without disulfide bridges and has a molecular mass of 34 kDa. It contains an essential Zn atom embedded in a cleft formed between two folded lobes of the protein and four Ca atoms which impart thermostability to the protein. Thermolysin is a very stable protease, with a half-life of 1 hr at 80 ^{0}C (Takii *et al*., 1987; Fujio and Kume, 1991).

Collagenase, another important metalloprotease, was first discovered in the broth of the anaerobic bacterium *Clostridium hystolyticum* as a component of toxic products. Later, it was found to be produced by the aerobic bacterium *Achromobacter iophagus* and other microorganisms including fungi. The action of collagenase is very specific since it acts only on collagen and gelatin but not on any other usual protein substrates (Harrington, 1996).

Elastase produced by *Pseudomonas aeruginosa* is another important member of the neutral metalloprotease family. The alkaline metalloproteases produced by *Pseudomonas aeruginosa* and *Serratia* sp. are active in the pH range 7 - 9 and have molecular masses in the region of 48 to 60 kDa. *Myxobacter* protease I has a pH optimum of 9.0 and a molecular mass of 14 kDa and can lyse cell walls of *Arthrobacter crystellopoites,* whereas protease II cannot lyse the bacterial cells.

Matrix metalloproteases play a prominent role in the degradation of extracellular matrix during tissue morphogenesis, differentiation and wound healing and may be useful in the treatment of diseases such as cancer and arthritis (Browner *et al.*, 1995).

1.8 Various applications of proteases

Proteases are one of the most important industrial enzymes used in various industries viz. detergent, food, feed, leather, medical, pharmaceutical, silk (Fujiwara *et al*., 1991; Ainsworth, 1994; Outtrup *et al.,* 1995). In addition proteases are also used in recovery of silver from used X-ray films and clean up of household drains and industrial wastes. Proteases have found extensive applications in leather and waste treatment processes as environmental friendly alternative. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as skin ointments and creams for treatment of burn injuries. Crude preparations of the protease enzyme are used in large amounts in food and detergent industries, but those used in medicine are used in small quantities and require extensive purification before they can be used (Rao *et al*., 1998).

Proteases as Detergent Additives

Proteases are one of the key ingredient of different types of detergents used in household laundry, contact lens cleaning solution and denture cleaning reagent. The proteases in laundry detergents account for approximately 25 % of the total worldwide use. "**Burnus"** was the first protease based biodetergent prepared in 1913 which consisted of sodium carbonate and crude pancreatic extract (Crutzen and Douglas, 1999; Khoo and Ibrahim, 2009). BIO-40 was the first bacterial protease containing detergent which was introduced in 1956. For an ideal enzyme based detergent, the detergent formulation should fulfill few important criteria. The enzymes must be stable at high temperature, pH and possess stability in presence of other

detergent ingredients viz. bleaches, surfactants, chelating and oxidizing agents (Aehle *et al.*, 1993; Kumar *et al*., 1998; Gupta *et al*., 1999; Oberoi *et al*., 2001; Beg *et al*., 2002). Therefore, the enzymes showing extreme stability towards oxidizing agents are of significant commercial value for detergent industry and peroxides and perborates have been commonly used as ingredients of the bleach-based detergents (Kumar *et al*., 1998). Performance of a protease in a detergent depends on its pI value. It is interesting to note that a protease is most suitable for use in detergent if its pI value coincides with the pH of the detergent solution (Rao *et al*., 1998). Esperase and Savinase T (Novo Industry, Denmark), produced by alkaliphilic *Bacillus* spp. are good examples of commercial protease preparations with very high isoelectric point (i.e. pI-11) and withstand highly alkaline conditions. Removal of blood stain is another important parameter to examine the suitability of protease enzyme based detergent formulation.

Proteases from alkaliphilic bacteria and *Pseudomonas aeruginosa* PD100 are known to remove the blood stain from the cotton cloth in the absence of detergents (Kanekar *et al.*, 2002; Najafi *et al.*, 2005). Due to the present energy crisis and need for energy conservation, it is desirable to use proteases showing optimum activity at lower temperatures. A combination of other enzymes such as lipase, amylase and cellulase is expected to enhance the performance of protease in laundry detergents (Ito *et al*., 1998). The success of enzyme based detergents has led to the discovery of a number of detergent enzymes with specific applications. Alkazym (Novodan, Copenhagen, Denmark) is an important enzyme used for cleaning of membrane systems and Pronod 153 L, a protease enzyme based cleaner is used to clean up blood stains from surgical instruments (Gupta *et al*., 2002). Protease solution has also been used for cleaning the packed columns of stainless steel particles fouled with gelatin and β-

lactoglobulin (Sakiyama *et al*., 1998). In addition, alkaline proteases are also used in cleaning contact lenses (Nakagawa *et al*., 1994).

Use of Proteases in Leather industry

Alkaline protease is also used commonly in leather industry and processing of leather involves several steps viz. soaking, dehairing, bating, and tanning. The major components of skin and hair are proteins. The traditional methods of leather processing involve use of hazardous chemicals such as sodium sulfide and 80 % of other suspended solids which cause environmental pollution and problems in effluent disposal. The use of enzymes as alternatives to chemicals has proved useful in improving leather quality and in reducing environmental pollution (Andersen, 1998).The main purpose of soaking step is to allow the hide to swell. Traditionally, this step was performed with alkali, but now microbial alkaline proteases are used to ensure faster absorption of water and minimise the time required for soaking. The conventional method of dehairing and dewooling consists of development of an extremely alkaline condition followed by treatment with sulfide to solubilize the proteins of the hair root. Currently, alkaline proteases with hydrated lime and sodium chloride are used for dehairing, resulting in speeding up the process of dehairing, because the alkaline conditions enable the swelling of hair roots and subsequent attack of protease on the hair follicle protein promotes easy removal of hair and a significant reduction in the amount of wastewater generated.

Dehairing of leather has been reported by alkaline protease produced by a mutant strain of *B. pumilus* BA06 (Wang *et al*., 2007). Earlier methods of bating used animal feces as a source of protease. Since these methods were unpleasant and unreliable they were replaced by alternative methods involving use of pancreatic trypsin. At present trypsin is used in combination with other proteases produced by *Bacillus* and

Aspergillus for bating. The selection of the enzyme depends on its specificity for matrix proteins such as elastin and keratin, and the amount of enzyme needed depends on the type of leather (i.e. soft / hard) to be produced. Similarly alkaline protease produced by *B. subtilis* K2 has been used in bating and leather processing (Hameed *et al*., 1996). Increased usage of enzymes for dehairing and bating not only prevents pollution problems but also is effective in saving energy (Rao *et al*., 1998). Novo Nordisk (Denmark) manufactures three different types of proteases which are commercially known as Aquaderm, NUE and Pyrase and are used in different steps of leather processing.

Use of Proteases in Food industry

Microbial proteases are used in various ways in food industry viz. preparation of protein hydrolysates of high nutritional value (Muzaifa *et al*., 2012), cheese making (Ohmiya *et al.*, 1979), baking (Linko *et al*., 1997; Kara *et al*., 2005) and tenderization of meat (O'Meara and Munro, 1984). The protein hydrolysates play an important role in blood pressure regulation and are also used in infant food formulations, specific therapeutic dietary products and fortification of fruit juices and soft drinks (Ward, 1985; Neklyudov *et al*., 2000). The commercial protein hydrolysates were derived from casein, whey and soy protein and were known as Miprodan, Lacprodan and Proup respectively.

Fujimaki *et al.*, (1970) used alkaline protease for the production of soy protein hydrolysates, whereas Perea *et al*., (1993) used alkaline protease for the production of whey protein hydrolysate, using cheese whey in an industrial whey bioconversion process. It is interesting to note that the proteases produced by GRAS (genetically regarded as safe) microbes such as *Mucor michei*, *Bacillus subtilis* and *Endothia parasitica* are gradually replacing chymosin in cheese making. Production of fish

hydrolysates of high nutritional value has been reported using proteases of *B. subtilis* (Rebeca *et al*., 1991).

In the baking industry wheat flour is a major component involved and contains an insoluble protein, gluten which determines the properties of the bakery doughs. Endo and exoproteinases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. Enzymatic treatment of the dough facilitates its handling and machining and permits the production of a wider range of bakery products. The addition of proteases reduces the mixing time and results in increased loaf volumes. Bacterial proteases are used to improve the extensibility and strength of the dough. Up-gradation of lean meat waste to edible products following alkaline protease mediated hydrolysis of the meat waste has already been reported (O'Meara and Munro, 1984). Interestingly, keratinolytic activity of alkaline proteases B72 and PWD-1 from *B. subtilis* and *B. licheniformis* has proved valuable in the production of proteinaceous fodder from waste feathers and keratin containing materials (Dalev, 1994; Cheng *et al*., 1995).

Proteases have also been used in the enzymatic synthesis of aspartame which is a dipeptide composed of L-aspartic acid and the methyl ester of L-phenylalanine. The L-configuration of the two amino acids is responsible for the sweet taste of aspartame therefore used as a non-calorific artificial sweetener. Maintenance of the stereospecificity is crucial but it adds to the cost of production by chemical methods therefore enzymatic synthesis of aspartame is usually preferred. Immobilized preparation of thermolysin (protease) from *Bacillus thermoproteolyticus* is used for the enzymatic synthesis of aspartame. Toya Soda Company of Japan and DSM from Netherlands are the major industrial producers of aspartame.

Use of Proteases in Photographic industry

Alkaline proteases are also used in silver recovery from gelatin coated used X- ray films. The used X- ray films contain 1.5 – 2.0 % silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Conventionally, this silver is recovered by burning the films, which causes undesirable atmospheric pollution. Furthermore, base film made of polyester cannot be recovered using this method. Since the silver is bound to gelatin, it is possible to extract silver from the protein layer by proteolytic treatments. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled. Alkaline protease obtained from *B. subtilis* could decompose the gelatin layer within 30 mins at 50–60 ^{0}C and release the silver (Fujiwara *et al.*, 1989). The alkaline proteases of *B. coagulans* PB-77 are also efficient in decomposing the gelatinous coating on used X-ray films from which the silver could be recovered (Gajju *et al.*, 1996). Therefore hydrolysis of gelatin using alkaline protease is an environmentally friendly process (Gupta *et al.*, 2002).

Use of Proteases in Silk degumming

Proteases are also used to treat silk. Sericin, which is a proteinaceous substance comprising 25 % of the total weight of raw silk, thus providing the rough texture to the silk fibers. This sericin is conventionally removed from the inner core of fibroin by conducting shrink-proofing and twist-setting for the silk yarns, using starch (Kanehisa, 2000). The process is generally expensive and therefore an alternative method is the use of enzyme preparations, such as protease, for degumming the silk prior to dyeing. Silk degumming efficiency of an alkaline protease have been studied from *Bacillus* sp. RGR-14 and the treated silk fiber was observed by scanning electron microscopy (SEM). SEM of the fibers revealed that clusters of silk fibers had

fallen apart as compared with the smooth and compact structure of untreated fiber (Puri, 2001).

Protease mediated degradation of household and industrial wastes

Proteases can solubilize proteinaceous wastes and thus help lower the biological oxygen demand of aquatic ecosystems (Gupta *et al*., 2002). Thus proteases have been used in degradation of proteinaceous wastes from various food-processing industries and domestic activities. Alkaline protease from *B. subtilis* has been used for degradation of waste feathers from poultry slaughterhouses (Dalev, 1994). It is interesting to note that waste feathers make up approximately 5 % of the total body weight of poultry and are considered a high protein source of food and feed, provided their rigid keratin structure is completely broken. Keratinolytic protease activity to degrade food and feed industry waste is well known (Ichida *et al.*, 2001) and protease has also been used as a depilatory agent to remove hair from the household drains (Takami *et al*., 1992). A formulation containing proteolytic enzymes from *B. subtilis*, *B. amyloliquefaciens* and *Streptomyces* sp. and a disulfide reducing agent, thioglycolate which enhances hair degradation and helps in clearing pipes clogged with hair containing deposits, is currently available in market which is patented by Genex (Jacobson *et al.*, 1985).

Medical usage

Alkaline proteases are also used for developing products of medical importance. The elastolytic activity of *B. subtilis* 316M has been exploited for the preparation of elastoterase which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses (Kudrya and Simonenko, 1994). Oral administration of proteases from *Aspergillus oryzae* has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes (Rao *et al*., 1998). The use of

alkaline protease from *Bacillus* sp. strain CK 11- 4 as a thrombolytic agent having fibrinolytic activity has also been reported (Kim *et al*., 1996).

1.9 Main objectives of research

1) Screening and isolation of alkaliphilic bacteria from Mangrove, Coastal and Estuarine ecosystems (econiches) of Goa.
2) Further screening of potential isolates which produce high quantity of protease.
3) Identification of isolates by Morphological characterization, Biochemical characterization (as per Bergey's manual) and 16S rRNA sequencing followed by BLAST search (Krieg and Holt, 1984; Altschul *et al.*, 1990; Olivera *et al*., 2007).
4) Physiological characterization of selected potential bacterial isolates for growth and enzyme (protease) production with respect to following environmental conditions/factors:

   (a) pH (b) salinity (c) temperature (Denizci *et al.,* 2004) (d) Carbon sources (e) Nitrogen sources (f) Effect of metal ions (g) Effect of inhibitors (Thangam, 2002).

5) Biochemical characterization of selected potential bacterial isolates with reference to:

   (a) Protease activity assay (unit/mg of protein)

   (b) SDS- PAGE analysis with silver staining (Laemmli, 1970)

   (c) Native -PAGE & Zymography

CHAPTER II

Screening, isolation and identification of alkaliphilic protease producing bacteria from Mangrove, Estuarine and Coastal ecosystems (econiches) of Goa

Screening, isolation and identification of alkaliphilic protease producing bacteria from Mangrove, Estuarine and Coastal ecosystems (econiches) of Goa

MATERIALS AND METHODS

2.1 Details of sampling sites

I have selected seven sampling sites for isolation of alkaliphilic protease producing bacteria which include one mangrove, two estuaries and four beaches of Goa (Fig. 2.1). Among the mangroves of Goa, Ribandar mangrove was selected for sample collection to screen protease producing bacteria. Several sampling sites were considered in Mandovi and Zuari estuaries which were located near cortalim bridge in case of Zuari estuary and Ribandar ferry point and a point near Mandovi bridge for Mandovi estuary. Coastal sampling sites included Miramar beach, Cocco beach, Colva beach and Velsao beach (Fig. 2.2).

2.2 Collection of environmental samples and physicochemical analysis

Surface water as well as sediment samples were collected from various selected sampling sites in sterile polycarbonate bottles and polythene bags respectively. Physicochemical analysis of the collected environmental samples was done within 24 hours of collection. The bottles containing water samples were shaken prior to use and kept for 10 minutes on the working bench to allow the heavy suspended particles to settle down. Physicochemical analysis of water samples was done to determine pH using digital pH meter (Eutech pH meter-700, Spectronic Camspec Ltd. U.K.),

temperature using thermometer and salinity (Atago Refractometer, India Pvt. Ltd.) using salinometer as per the standard procedures.

2.3 Screening and isolation of alkaliphilic bacteria producing alkaline protease

Sediment and water samples from selected mangrove, estuarine and coastal sites were used to isolate alkaliphilic bacteria producing alkaline protease. Serial dilutions of the environmental samples (i.e. sediment and water samples) were done using sterile saline and 0.1 ml of serially diluted samples were spread plated on 5 % skim milk agar plates with pH 9 (Appendix A). These plates were incubated at room temperature for 24 hrs. Discrete colonies showing proteolytic activity as clear zone around the colony were picked up and further purified by repeated streaking on fresh 5 % skim milk agar plates (pH 9). Total count of alkaline protease producing bacteria was determined as colony forming units (cfu/ml) based on the number of colonies showing proteolytic activity.

Further screening of the potential protease positive bacterial isolates was carried out by repeated streaking on 5 % skim milk agar plates with pH 9 and incubated at room temperature for 24 hrs. The colony size and clear zone size were recorded in millimeter (mm). Based on the zone size to colony size ratio (Z : C) the prominent protease producing isolates were selected for identification and further characterization.

2.4 Identification of the selected alkaline protease producing bacterial isolates

Identification of the selected alkaline protease producing bacterial isolates was done based on morphological and biochemical characteristics following Bergey's manual of systematic bacteriology (Krieg and Holt, 1984) (Appendix B1 and C). Further confirmation was done by 16S rRNA sequencing followed by BLAST search (Altschul *et al*., 1990).

2.4.1 Identification based on morphological and biochemical characteristics

Morphological characterization of selected bacterial isolates was done with reference to colony morphology, pigmentation and motility in order to tentatively identify them. Gram staining was conducted to reveal cell wall composition and several biochemical tests were also performed. Oxidative-fermentative tests were conducted to determine whether organism is oxidative, fermentative or facultative anaerobe. Carbohydrate fermentation test was also done to confirm fermentative utilization of sugars with the production of acid and gas. Methyl Red test detects formation of large quantities of acid in the medium resulting from fermentation of glucose and Voges - Proskauer's test detects production of non-acidic or neutral end products such as butanediol and acetoin. Citrate utilization test determines that a bacterium can use citrate as the sole carbon source and indole production test determines ability of bacteria to convert tryptophan into indole. Activity of several microbial enzymes viz. urease, amylase, catalase, gelatinase, oxidase and nitrate reductase for the selected bacterial isolates was also determined (Krieg and Holt, 1984).

2.4.2 Identification of the isolates based on 16S rRNA sequencing and BLAST search analysis

16S rRNA of the selected bacterial isolates was PCR amplified as per the standard molecular biological procedure using genomic DNA of isolates as template and following eubacterial primers:

8F (5'-AGAGTTTGATCCTGGCTCAG-3')

1492R (5'-ACGGCTACCTTGTTACGACTT-3')

PCR amplification reaction was performed using PCR amplification kit (Bangalore Genei, India) and DNA sequencing was done by Xcelris laboratories, Ahmedabad, Gujarat, India. 16S rRNA sequence of selected bacterial isolates was compared with 16 S rRNA data of bacterial isolates available in the 16S ribosomal DNA data base of GenBank using NCBI- BLAST search (Sambrook *et al*., 1989; Altschul *et al.*, 1990). A phylogenetic tree was constructed with MEGA version 4.0 using the neighbor-joining method. The 16S rRNA sequence has already been deposited in the GenBank database and accession numbers were obtained.

2.5 Antibiotic susceptibility test

Antibiotic susceptibility test of protease producing bacterial isolates was performed following Kirby–Bauer disc diffusion method (Bauer *et al*., 1966) using Muller–Hinton agar (Appendix A3) and antibiotic discs (Himedia, India).Various antibiotics tested are as follows: cephalothin (Ch), clindamycin (Cd), cloxacillin (Cx), gentamycin (G), oxytetracycline (O), penicillin G (P), co-trimoxazole (Co), erythromycin (E), amikacin (Ak), carbenicillin (Cb), ciprofloxacin (Cf), co-trimazine (Cm), kanamycin (K), nitrofurantoin (Nf) , streptomycin (S) and tetracycline (T). Diameter of zone of inhibition of test isolates caused by individual antibiotics was

compared with standard chart (Himedia) and based on these results resistance or sensitivity of bacterial isolates to the tested antibiotics was determined.

2.6 Morphological characterization of protease positive *Bacillus altitudinis* strain BR1 at 50 ^{0}C

Scanning electron microscope (SEM-JEOL JSM-5800LV,USA) was employed to examine morphological changes at 30 ^{0}C and 50 ^{0}C respectively during their growth phase. Cells were grown in nutrient broth at two different temperatures and effect of temperature on cell morphology was analyzed by fixing bacterial cells in 3 % glutaraldehyde overnight with 50 mM Potassium phosphate buffer at 4 ^{0}C. Cells were washed thrice with phosphate buffer, pH 9 and dehydrated in gradually increasing concentration of ethanol i.e. 10, 20, 50, 70, 80, 90, 95 and 100 % for 15 min each, air dried and stored in vacuum chamber prior to SEM analysis (Neumann *et al.*, 2005).

Fig. 2.1 Map of Goa along with sampling sites

Sampling sites:

1. Ribandar mangroves
2. Mandovi estuary
3. Zuari estuary
4. Miramar beach
5. Cocco beach
6. Colva beach
7. Velsao beach

Fig. 2.2 Different sampling sites

RESULTS AND DISCUSSION

Goa is one of the most important international tourist destinations of India with a vast coastline of 104 kms and several important beaches in its northern and southern parts. Goa has got several rivers and fresh water bodies which ultimately merge with the Arabian sea naturally creating estuaries such as Mandovi and Zuari. The back water of Arabian sea naturally supports luxuriant growth of mangrove forest as the climatic conditions are most favorable for various types of mangroves. Estuaries are also considered one of the most productive ecosystems with diverse and rich microbial flora and microfauna since rivers and creeks drain down their inorganic and organic nutrients into them from uplands through run off from agriculture fields and other terrestrial econiches (Abreu *et al*., 2001; Kisand *et al*., 2002). Similarly mangroves and coastal sites are also enriched with diverse microorganisms along with several macroorganisms. Marine coastal and estuarine ecosystem along with mangrove possesses highly rich pool of proteins and polypeptides which is attributed to large number of micro and macro-invertebrates along with vertebrates such as fish and bivalves. It is interesting to note that large number of microbes including bacteria and fungi are involved in scavenging and degradation of detritus and proteinaceous waste of marine coastal, estuarine and mangrove econiches employing diverse varieties of enzymes viz. proteases, esterases and lipases with lot of biotechnological potential due to their catalytic metabolic activities (Gupta *et al*., 2002). Inspite of such important attributes, mangrove, estuarine and coastal econiches of Goa have been less explored to find out potential alkaliphiles producing industrially important proteases.

2.7 Physicochemical characteristics of the environmental samples

The sediment sample from Ribandar mangroves showed slightly alkaline condition with pH 7.8, whereas the water sample showed pH 7.6 at 30 ^{0}C. However there was significant variation in salinity of water and sediment samples which ranged from 25 to 28 psu (Table 2.1). Our results are very close to Ting Kok mangrove from Hong Kong as the salinity of water samples ranged between 27 - 30 psu with an exception that pH of the water samples was slightly lower as compared to Ribandar mangroves i.e. 6.4 - 7.2 (Tam *et al*., 1997). High level of variation in salinity of sediment and water samples of mangroves from Kachchh, Gujarat has also been reported which ranged between 34 to 44 psu (Saravanakumar *et al*., 2008). Similar variation in salinity has also been reported for water and sediment samples of mangroves along the east coast of India which ranged from 48.10 to 54.65 psu and pH 7.0 to 8.4 respectively (Thamizhmania and Senthilkumaran, 2012).

It was interesting to note that pH of estuarine water and sediment samples was alkaline (pH 7.7 -7.8) and temperature was 29 ^{0}C - 30 ^{0}C. Since these sites are close to the open sea they show a fluctuation in salt concentration due to constant mixing of sea water with fresh water (Abreu *et al*., 2001). However it was interesting to note that salinity of Zuari and Mandovi estuary water samples was significantly high as it was in the range of 21- 24 psu. Whereas the sediment samples of the Zuari and Mandovi estuary recorded comparatively low salinity i.e. 20 psu (Table 2.1). High salinity of estuarine water samples may be attributed to continuous mixing of mineral salts in the aquatic ecosystem (Lionard *et al*., 2005; MacDonald and Horner-Devine, 2008). Water normally contains dissolved gases, such as oxygen, and a variety of organic and inorganic matter but due to mixing of fresh water and sea

water in an estuary, it contributes to its unique physicochemical characteristics and supports a wide variety of mangrove vegetation, microorganisms and animals.

Physicochemical analysis of coastal (beach) sediment and water samples clearly demonstrated that pH ranged from 7.6 to 7.8, temperature from 28 ^{0}C to 30 ^{0}C and salinity from 26.24 psu to 28.22 psu respectively at various coastal sites (Table 2.1). Salinity of coastal marine samples was significantly higher as compared to mangrove and estuarine samples due to predominance and high levels of mineral salts (Klemas, 2011). Salinity and temperature are important abiotic factors that determine the density of sea water since density plays an important role in driving the currents in the oceans. Moderately alkaline and saline conditions of the selected econiches would selectively favour the growth of alkaliphilic and halotolerant microbes including bacteria producing various industrially important enzymes including proteases.

2.8 Screening of protease producing alkaliphilic bacteria

The total viable count of bacteria isolated from water samples of Ribandar mangrove was 11 x 106 cfu/ml, whereas only 2 isolates showed proteolytic activity on 5 % skim milk agar plate (pH 9). Whereas the viable count of bacteria from sediment samples was 25 x 107 cfu/ml but 5 isolates were protease positive (Table 2.2; Fig. 2.3). Mangroves are rich in organic and inorganic nutrients due to continuous process of mangrove leaf litter decomposition mediated by diverse kind of decomposer microorganisms including bacteria. Large pool of proteinaceous wastes and detritus in these unique econiches is degraded and hydrolysed by diverse kinds of protease producing bacteria which are responsible for maintenance and regulation of nitrogen cycle in the mangrove ecosystems. Our results are also in agreement

with other researchers involved in screening and characterization of protease producing bacteria (Venugopal and Saramma, 2006; Kathiresan and Manivannan, 2007).

Among the estuarine water samples collected, the total viable count of bacteria from Zuari estuary was 19 x 10^5 cfu/ml with 6 protease positive colonies and 17 x 10^5 cfu/ml for Mandovi estuary with 10 protease positive colonies. Whereas the sediment samples from Zuari estuary showed a viable count of 12 x 10^7 cfu/ml and 11 x 10^7 cfu/ml for Mandovi estuary. But the number of protease producing colonies from sediment samples of Zuari and Mandovi estuary were 2 and 5 respectively (Table 2.2; Fig. 2.3).

Sediment samples from Miramar and Cocco beach showed bacterial viable count of 20 x 10^9 cfu/ml and 18 x 10^9 cfu/ml. The number of protease positive colonies was 15 and 6 for Miramar and Cocco beach sediment samples respectively. Water samples of these beaches showed a viable count of 8 x 10^9 cfu/ml and 6 x 10^9 cfu/ml respectively but 2 protease positive colonies were detected from Miramar samples and only 1colony showed positive result from Cocco beach sample. Similarly water samples from Colva and Velsao beach showed total viable count of 10 x 10^9 cfu/ml and 5 x 10^9 cfu/ml respectively whereas only 2 protease positive colonies were detected from Colva water sample, but none from Velsao beach water sample. Viable count of bacteria from Colva and Velsao beach sediment samples was 22 x 10^9 and 15 x 10^9 respectively, whereas 4 protease positive colonies were detected for both the beach sediment samples (Table 2.2; Fig. 2.3).

It is evident from our studies that among the various sampling sites sediment samples from coastal sites showed the highest number of protease producing colonies. This may be attributed to extensive fishing activities along with disposal of

dead fish on the various coastal sites of Goa which is very common phenomenon. It results in accumulation of fish wastes and trash in the coastal sediments which allows proteolytic bacteria to flourish under these conditions (Kristensen, 1995; Cowie, 2005).

Further characterization of 64 protease producing isolates obtained from water and sediment samples from mangrove, estuarine and coastal ecosystems revealed presence of 24 morphologically different and distinct bacterial isolates. Out of these morphologically distinct isolates, 11 potential protease producers were finally selected for identification based on morphological and biochemical characteristics and 16S rRNA sequencing followed by BLAST search (Table 2.3).

Based on different sampling sites and type of the sample collected to the protease positive isolates were designated as follows:

MS - Isolate from mangrove sediment

MW - Isolate from mangrove water

EW - Isolate from estuarine water

ES - Isolate from estuarine sediment

CW - Isolate from coastal water

CS - Isolate from coastal sediment

2.9 Identification of the selected alkaline protease producing bacterial isolates

Alkaline protease producing bacterial isolates were identified as *Bacillus* sp. strain CS1, *Brevibacterium* sp. strain CS2, *Halomonas* sp. strain CS3, *Bacillus* sp. strain CS6, *Lactobacillus* sp. strain CW1, *Bacillus* sp. strain CW2, *Cellulomonas* sp. strain ES1, *Pseudomonas* sp. EW1, *Brochothrix* sp. EW2, *Bacillus* sp. EW3 and

Micrococcus sp. strain MS2 respectively based on morphological and biochemical characteristics (Table 2.4.1).

2.10 Identification of the isolates based on 16S rRNA sequencing and BLAST search analysis

Out of 11 tentatively identified potential protease positives 3 bacterial strains were selected for further identification based on their 16S rRNA sequence data and BLAST search analysis (Sambrook *et al.*, 1989; Altschul *et al.*, 1990). 16S rRNA sequence data of these 3 alkaline protease producing strains has already been submitted to Genbank and accession numbers have already been allotted (Table 2.4.2).

BLAST analysis of 16S rRNA sequence of these 3 protease positive strains revealed different percent homology with 3 different species of *Bacillus*. Strain BR1 showed 100 % homology with *Bacillus altitudinis* strain S-29, strain CS1 showed 99 % sequence homology with *Bacillus cereus* strain WA6-16 whereas strain CW2 showed 100 % homology with *Bacillus firmus* UST 981101-006 respectively. Based on these molecular studies strains BR1, CS1 and CW2 have been finally identified as *Bacillus altitudinis, Bacillus cereus* and *Bacillus firmus* respectively. Phylogenetic analysis of 16S rRNA sequence data of 3 protease positive bacterial strains viz. BR1, CS1 and CW2 clearly revealed that they are closely related to the genus *Bacillus* (Fig. 2.4).

2.11 Antibiotic susceptibility test

It is interesting to note that *Bacillus altitudinis* strain BR1 (earlier designated as EW3) is resistant to commonly used antibiotics viz. clindamycin, cloxacillin, gentamycin, oxytetracycline, penicillin G, amikacin, carbenicillin, nitrofurantoin and tetracycline, whereas sensitive to antibiotics cephalothin, co- trimoxazole, erythromycin, ciprofloxacin, co-trimazine, kanamycin and streptomycin. *Bacillus cereus* strain CS1 showed resistance against cephalothin, clindamycin, cloxacillin, gentamycin, oxytetracycline, co-trimoxazole, amikacin, carbenicillin, co-trimazine, kanamycin, nitrofurantoin, streptomycin and tetracycline and sensitivity to antibiotics penicillin G, erythromycin and ciprofloxacin. Similarly *Bacillus firmus* strain CW2 showed resistance to clindamycin, cloxacillin, gentamycin, erythromycin, amikacin, carbenicillin kanamycin, streptomycin and tetracycline and sensitivity to antibiotics cephalothin, oleondamycin, penicillin G, co-trimoxazole, ciprofloxacin, co-trimazine and nitrofurantoin (Table 2.5).

Natural bacterial isolates inherently possess different resistance mechanisms to withstand high concentrations of commonly used antibiotics. These mechanisms are governed by genes located on chromosomal genome, plasmids or transposons. Therefore it is not surprising that these proteolytic bacterial isolates may possess antibiotic resistance genes. It would be advantageous to use selected antibiotics in the growth medium during large scale production of protease using fermentors, since contamination can be avoided (Barbosa and Levy, 2000; Davies and Davies, 2010).

2.12 Morphological characterization of protease positive *Bacillus altitudinis* strain BR1 at 50 ^{0} C

It is interesting to mention that *Bacillus altitudinis* BR1 isolated from water sample of Mandovi estuary, Goa could tolerate and show growth even upto 50 ^{0}C. The cell morphology was normal when grown in nutrient broth at 37 ^{0}C, but significant change in cell morphology as reduction in cell size and formation of oval cells was observed after exposure to 50 ^{0}C which was clearly demonstrated by Scanning electron microscopic analysis of bacterial cells (Fig. 2.5 A, B).

Morphological alterations of cells is one of several strategies bacteria adapt to withstand unfavourable environmental conditions. Temperature is one of the most important selection factors affecting all organisms at all levels of biological organizations. Adaptive responses of *Pseudomonas pseudoalcaligenes* cultured in increasing temperature has already been reported (Bihong and Xuhua, 2003). It has also been observed that morphological alterations disturb the production of enzymes and secondary metabolites (Yun *et al*., 2009). In case of *Pseudomonas aeruginosa* strain 4EA reduction in cell size was also noticed when bacterial cells were exposed to 0.8 mM lead nitrate which was clearly revealed by SEM analysis (Naik and Dubey, 2011).

Table 2.1 Physicochemical characteristics of water and sediment samples from various sampling sites

S.N.	Sampling sites	pH	Temperature (^{0}C)	Salinity (psu)
1	Ribandar mangroves			
	i) water sample	7.6	30	25
	ii) sediment sample	7.8	30	28
2	Zuari estuary			
	i) water sample	7.8	29	24
	ii) sediment sample	7.7	30	20
3	Mandovi estuary			
	i) water sample	7.8	30	21
	ii) sediment sample	7.7	29	20
4	Miramar Beach			
	i) water sample	7.7	30	27.11
	ii) sediment sample	7.6	29	28. 12
5	Cocco beach			
	i) water sample	7.7	29	26.24
	ii) sediment sample	7.8	28	28.15
6	Colva beach			
	i) water sample	7.7	30	26.34
	ii) sediment sample	7.6	29	27.37
7	Velsao beach			
	i) water sample	7.6	30	27.10
	ii) sediment sample	7.7	30	28.22

Table 2.2 Screening of protease producing bacteria from various sites

Sampling sites	Total viable count (cfu/ml)	Protease producing bacteria (cfu/ml)
Ribandar mangroves		
water sample	11×10^6	2
sediment sample	25×10^7	5
Mandovi estuary		
water sample	17×10^5	10
sediment sample	11×10^7	5
Zuari estuary		
water sample	19×10^5	6
sediment sample	12×10^7	2
Miramar beach		
water sample	8×10^9	2
sediment sample	20×10^9	15
Cocco beach		
water sample	6×10^9	1
sediment sample	18×10^9	6
Colva beach		
water sample	10×10^9	2
sediment sample	22×10^9	4
Velsao beach		
water sample	5×10^9	none
sediment sample	15×10^9	4

Table 2.3 Screening of alkaline protease producing strains on 5 % Skim milk agar (pH 9)

Protease Positive Bacterial isolates	Colony diameter (C) (mm)	Zone of clearance(Z) (mm)	Zone of clearance : Colony diameter (Z:C)
MS2	5	16	3.2
ES1	3	6	2.0
EW1	3	12	4.0
EW2	3	7	2.3
EW3	5	20	4.0
CS1	3	6	2.0
CS2	4	7	1.75
CS3	3	6	2.0
CS6	5	9	1.8
CW1	3	11	3.6
CW2	2	14	7.0

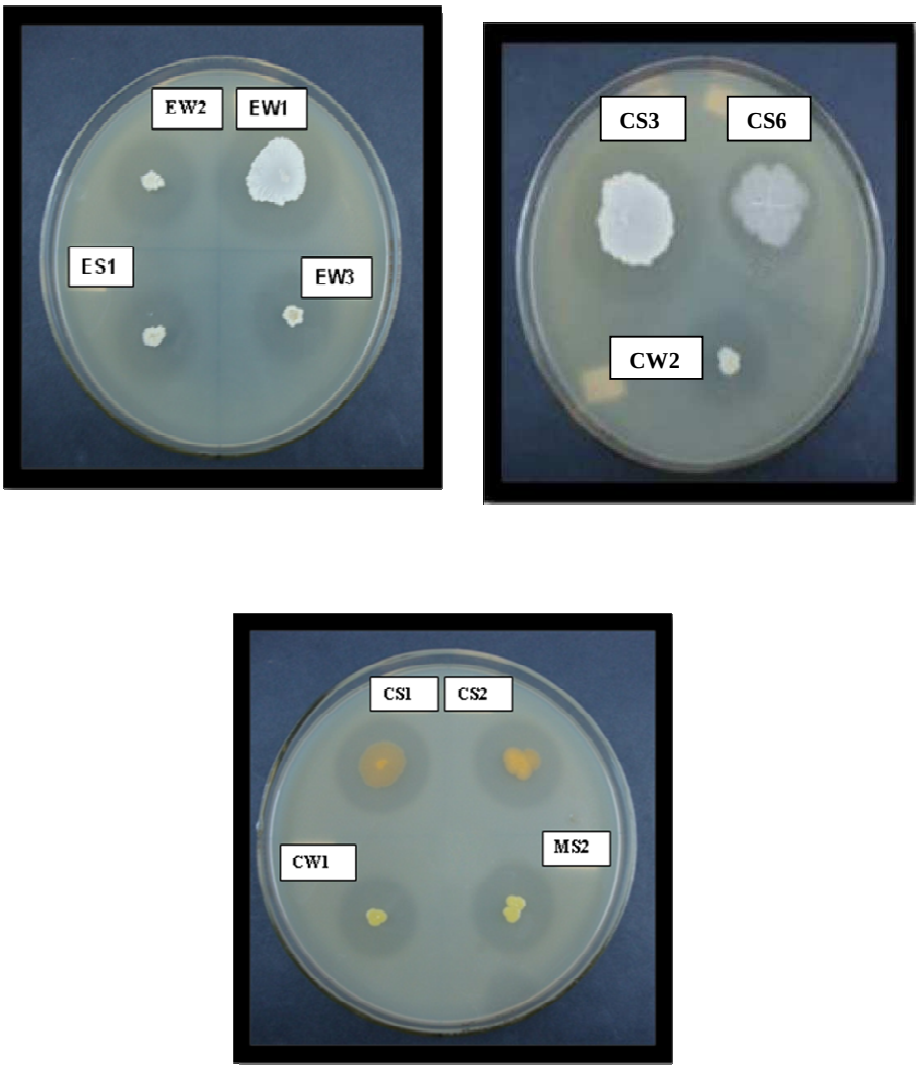


Fig 2.3 Screening of the potential protease producing isolates on 5 % skim milk agar (pH 9) from different ecosystems.

Table 2.4.1 Biochemical characteristics of alkaline protease producing alkaliphilic bacterial strains

TESTS	Strain CS1	Strain CS2	Strain CS3	Strain CS6
Gram staining	Gram positive short rods	Gram positive short rods	Gram negative rods	Gram positive short rods
Motility	Non- motile	Non- motile	Non- motile	Non- motile
HL- test	Oxidative+ fermentative	Oxidative+ fermentative	oxidative	oxidative
Indole	-	-	-	-
MR- test	-	-	-	+
VP- test	-	-	-	-
Citrate	-	-	-	-
Catalase	+	-	+	+
Oxidase	-	-	+	+
Urease	-	-	-	-
Nitrate reduction	-	-	-	-
H_2S	-	-	-	-
Phenylalanine deaminase	-	-	-	-
Starch hydrolysis	+	+	+	+
Gelatinase	+	+	+	+
ONPG	-	-	-	-
Lysine δc	-	-	-	-
Ornithine δc	-	-	-	-
Esculin hydrolysis	-	-	+	+
Malonate δc	-	-	-	-
Pigment	Yellow orange	Yellow orange	-	-
Sugar fermentation				
Glucose	+	+	+	+
Saccharose	+	+	+	+
Rhamnose	-	-	-	-
Arabinose	-	-	-	-
Xylose	-	-	-	-
Raffinose	-	-	-	-
Trehalose	-	-	-	-
Lactose	+	-	-	-
Adonitol	-	-	-	-
Melibiose	-	+	-	-
Cellobiose	-	-	-	-
Tentatively Identified as	***Bacillus* sp.**	***Brevibacterium* sp.**	***Halomonas* sp.**	***Bacillus* sp.**

Key: - = Negative; + = Positive

Table 2.4.1 continues

TESTS	Strain EW1	Strain EW2	Strain EW3	Strain MS2
Gram staining	Gram negative short rods	Gram negative short rods	Gram positive bacilli	Gram positive cocco bacilli
Motility	Non- motile	Non- motile	Non- motile	Non- motile
HL- test	Oxidative+ fermentative	Oxidative+ fermentative	Oxidative+ fermentative	Oxidative
Indole	-	-	-	-
MR- test	-	-	-	-
VP- test	-	-	-	-
Citrate	+	+	-	-
Catalase	-	-	-	+
Oxidase	-	-	-	-
Urease	-	-	-	-
Nitrate reduction	+	-	-	-
H_2S	-	-	-	-
Phenylalanine deaminase	-	-	-	-
Starch hydrolysis	-	+	+	-
Gelatinase	-	-	-	-
ONPG	-	-	+	-
Lysine δc	-	-	-	-
Ornithine δc	-	-	+	-
Esculin hydrolysis	-	+	-	-
Malonate δc	-	-	-	-
Pigment	-	-	-	-
Sugar fermentation				
Glucose	+	+	+	+
Saccharose	+	+	+	+
Rhamnose	-	-	-	-
Arabinose	-	-	-	-
Xylose	-	-	+	-
Raffinose	-	-	+	-
Trehalose	+	+	+	+
Lactose	-	-	-	-
Adonitol	-	-	-	-
Melibiose	-	-	+	-
Cellobiose	-	-	-	-
Tentatively Identified as	***Pseudomonas* sp.**	***Brochothrix* sp.**	***Bacillus* sp.**	***Micrococcus* sp.**

Key: - = Negative; + = Positive

Table 2.4.1 continues

TESTS	Strain CW1	Strain CW2	Strain ES1
Gram staining	Gram positive cocco bacilli	Gram positive cocci	Gram positive short rods
Motility	Non- motile	Non- motile	Non- motile
HL- test	Oxidative+ fermentative	Oxidative+ fermentative	Oxidative+ fermentative
Indole	-	-	-
MR- test	+	-	-
VP- test	-	-	-
Citrate		+	-
Catalase	-	-	+
Oxidase	-	-	-
Urease	+	-	-
Nitrate reduction	-	-	-
H_2S	-	-	
Phenylalanine deaminase	-	-	-
Starch hydrolysis	-	+	-
Gelatinase	-	+	-
ONPG	-	+	-
Lysine δc	-	-	-
Ornithine δc	+	-	-
Esculin hydrolysis	-	-	-
Malonate δc	-	-	-
Pigment	Light yellow	-	-
Sugar fermentation			
Glucose	+	+	+
Saccharose	+	+	+
Rhamnose	-	-	-
Arabinose	-	-	+
Xylose	-	-	-
Raffinose		-	-
Trehalose	+	+	-
Lactose	-	-	-
Adonitol	-	-	-
Melibiose	-	-	-
Cellobiose	-	-	+
Tentatively Identified as	***Lactobacillus* sp.**	***Bacillus* sp.**	***Cellulomonas* sp.**

Key: - = Negative; + = Positive

Table 2.4.2 Identified alkaline protease producing bacterial strains based on 16S rRNA sequence and BLAST search along with their Genbank accession numbers

Bacterial strain	Homology with	% Homology	Genbank accession no.
BR1	*Bacillus altitudinis* strain S-29	100 %	JN 712305
CS1	*Bacillus cereus* strain WA6-16	99 %	JQ 657725
CW2	*Bacillus firmus* strain HNS012	100 %	JX 901027

Table 2.5 Antibiotic susceptibility test

Antibiotics / Bacterial strains	Ch 30 µg	Cd 2 µg	Cx 5 µg	G 10 µg	O 30 µg	P 1 units	Co 25 units	E 15 units	Ak 10 µg	Cb 100 µg	Cf 10 µg	Cm 25 µg	K 30 µg	Nf 300 µg	S 10 µg	T 30 µg
BR1	-	+	+	+	+	+	-	-	+	+	-	-	-	+	-	+
CS1	+	+	+	+	+	-	+	-	+	+	-	+	+	+	+	+
CW2	-	+	+	+	-	-	-	+	+	+	-	-	+	-	+	+

Key: Resistant (+); Sensitive (-)

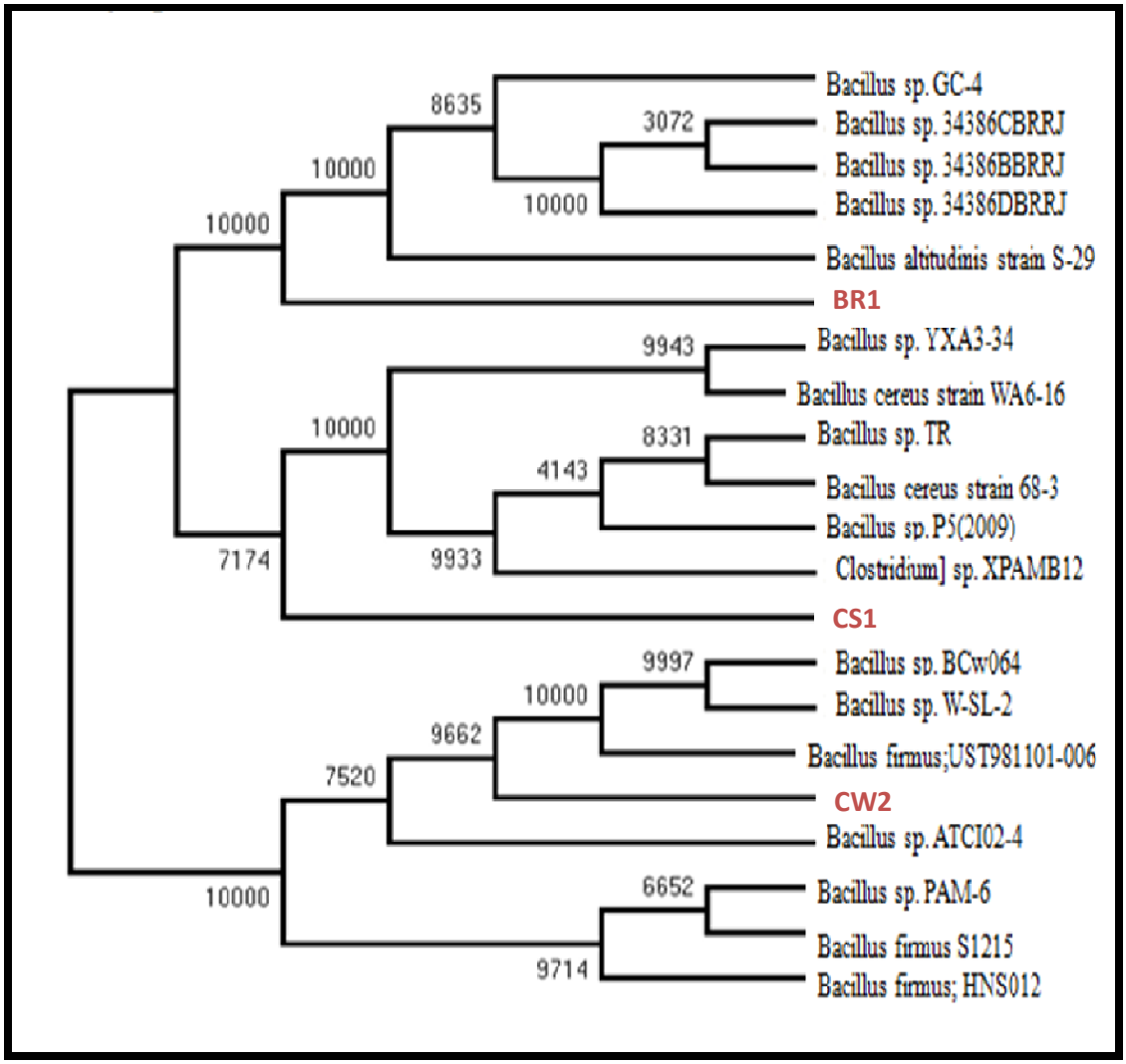


Fig. 2.4 Dendogram showing phylogenetic relationship between protease positive strains BR1, CS1 and CW2 with different *Bacillus* spp. based on their 16S rRNA sequence data

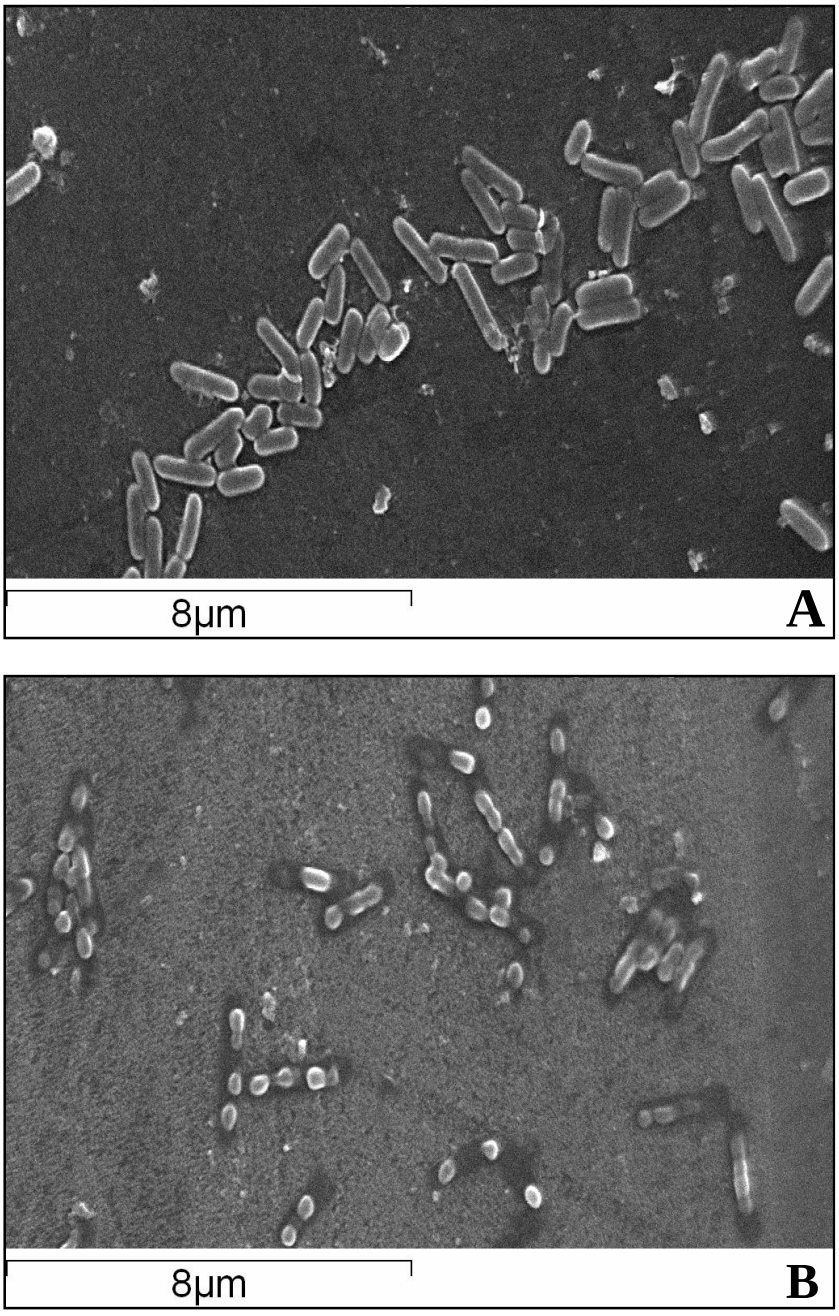


Fig 2.5 A, B Scanning electron microscopic analysis of *Bacillus altitudinis* strain BR1 grown in Nutrient broth (magnification, X 9000)

A- Control cells (at 37 ^{0}C); B- Cells exposed to 50 ^{0}C

CHAPTER III

Physiological characterization of selected potential bacterial isolates for growth and protease production with reference to environmental factors

Physiological characterization of selected potential bacterial isolates for growth and protease production with reference to environmental factors

MATERIALS AND METHODS

3.1 Determination of environmental optimas (pH, salinity, temperature, carbon and nitrogen sources) for growth and protease production

3.1.1 Determination of optimum pH for growth and protease production

Bacterial growth and protease activity at various pH values ranging from 7 to 12 was tested to investigate optimum pH required by the potential alkaline protease producing isolates in Horikoshi-I medium (Horikoshi, 1996). Overnight grown culture was inoculated in fresh growth medium to get zero hour absorbance of 0.05 at 600 nm. Bacterial cultures were grown at pH 7, 8, 9, 10, 11 and 12 at 30 ^{0}C and 150 rpm separately. Glucose (0.5 %) was used as the sole carbon source and growth was monitored every 3 hrs by measuring absorbance of culture suspension at 600 nm.

Protease enzyme activity of the bacterial cultures was also measured by modified Kunitz assay (Kunitz, 1947). Protease was assayed using casein as a substrate. 2 ml of culture suspension was centrifuged at 10, 000 rpm for 10 mins at 4 ^{0}C and the cell free supernatant was used to determine protease activity. 200 µl of cell free supernatant was taken in an eppendorf tube along with 800 µl of mixture was incubated for 20 mins at room temperature. The reaction was terminated by adding 1ml of 10 % Trichloroacetic acid (TCA) and sample was incubated for 10 mins for

complete precipitation of proteins. Mixture was centrifuged at 10,000 rpm for 15 mins at 4 ^{0}C. Absorbance of the supernatant was recorded at 280 nm against a blank (containing 0.5 % casein in Tris- HCl buffer (pH 9) and 10 % Trichloroacetic acid).

All spectrophotometric determinations were carried out using Double beam Spectrophotometer (UV- 2450 Shimadzu Corporation, Japan). Tyrosine standard solution, in the range of 0-100 μg/ml was prepared to obtain a standard curve (Appendix D). One unit of enzyme activity (U) is defined as the amount of enzyme required to liberate 1 μg of tyrosine/ml/min under standard assay conditions.

3.1.2 Determination of optimum salinity (% NaCl) for growth and protease production

Similarly bacterial growth and protease activity at various salinity (% NaCl) ranging from 0 - 6 % were determined to find out optimum salinity required by the potential alkaline protease producing isolates. Selected bacterial isolates were grown in Horikoshi-I medium (Horikoshi, 1996) supplemented with different NaCl concentrations (0 %, 2 %, 4 % and 6 %) at pH 9, 30 ^{0}C and 150 rpm. Glucose (0.5 %) was used as the carbon source and growth was monitored every 3 hrs by measuring absorbance at 600 nm. Protease activity was measured by modified Kunitz assay (Kunitz, 1947).

3.1.3 Determination of optimum temperature for growth and protease production

Similarly bacterial growth and protease activity at various temperatures ranging from 30 - 60 ^{0}C were determined to find out optimum temperature required by the potential alkaline protease producing isolates. Selected bacterial isolates were grown in Horikoshi-I medium (Horikoshi, 1996) and incubated at different temperature (30 ^{0}C, 37 ^{0}C, 45 ^{0}C, 50 ^{0}C and 60 ^{0}C) at pH 9 and 150 rpm. Glucose (0.5 %) was used

as the carbon source and growth was monitored every 3 hours by measuring absorbance at 600 nm and protease activity was measured by modified Kunitz assay (Kunitz, 1947).

3.1.4 Determination of best carbon source for growth and protease production

Similarly bacterial growth and protease activity in presence of various carbon sources viz. glucose, fructose, maltose, sucrose and lactose was determined to find out best carbon source required by the potential alkaline protease producing isolates. Selected bacterial isolates were grown in Horikoshi - I medium (Horikoshi, 1996) supplemented with 1 % carbon source at pH 9, 30 ^{0}C and 150 rpm. Growth was monitored every 3 hrs by measuring absorbance at 600 nm and protease activity was measured following modified Kunitz assay (Kunitz, 1947).

3.1.5 Determination of best nitrogen source for growth and protease production

Similarly bacterial growth and protease activity in presence of various organic and inorganic nitrogen sources viz. peptone, yeast extract, tryptone, casein, ammonium nitrate [$NH_4 (NO_3)_2$] and potassium nitrate (KNO_3) was determined to find out best nitrogen source required by the potential alkaline protease producing isolates. Selected bacterial isolates were grown in Horikoshi - I medium (Horikoshi, 1996) supplemented with 0.2 % nitrogen source at pH 9, 30 ^{0}C and 150 rpm. Growth of the bacterial isolates was monitored every 3 hrs by measuring absorbance at 600 nm and protease activity was measured by modified Kunitz assay (Kunitz, 1947).

3.2 Effect of metal ions and inhibitors on enzyme activity of protease producing bacteria

In order to study the effect of metal ions viz. Cu^{+2}, Ca^{+2}, Mg^{+2}, Pb^{+2} and Hg^{+2} on alkaline protease activity of bacterial isolates, 100 µl of the partially purified enzyme in Tris- HCl buffer (pH 9) was supplemented with different concentrations of metal ions viz. 1 mM, 2.5 mM and 5 mM in separate eppendorf tubes and the sample was incubated for 1 hour. Effect of Hg^{+2} ions on protease activity was assessed using 0.01 mM, 0.1 mM and 1mM of $HgCl_2$ in the enzyme assay. Alkaline protease activities was measured under standard assay conditions using the Kunitz assay (Kunitz, 1947).

Effect of various protease inhibitors viz. SDS (sodium dodecyl sulphate), DTT (dithiothreitol), PMSF (phenyl methyl sulphonyl fluoride), β-ME (β-mercaptoethanol) and Na_2-EDTA (disodium ethylenediamine tetraacetic acid) on alkaline protease activity was monitored using 100 µl of the partially purified enzyme supplemented with 1 mM of different inhibitors separately. The mixture was incubated for 1 hr at the optimum temperature of the respective bacterial isolates and protease activity was measured using standard assay procedure (Kunitz, 1947).

3.3 Proteolytic activity of *Bacillus firmus* strain CW2 on different substrates

Alkaline protease activity for partially purified enzyme of *Bacillus firmus* strain CW2 on various protein substrates such as bovine serum albumin (BSA) and gelatin was determined by incubating 200 µl of enzyme sample in eppendorf tube with 800 µl of different protein substrates (0.5 %) in Tris- HCl buffer (pH 10). The enzyme

sample with different substrates was incubated at 37 ^{0}C and the standard assay procedure was followed to determine the alkaline protease activity (Kunitz, 1947).

RESULTS AND DISCUSSION

3.4 Determination of environmental optimas (pH, salinity, temperature, carbon and nitrogen sources) for growth and protease production

Three potential alkaline protease producing bacterial isolates viz. *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 , *Bacillus firmus* strain CW2 were selected to determine environmental optimas for growth and protease activity (production).

3.4.1 Determination of optimum pH for growth and protease production

Enzymes are amphoteric molecules containing a large number of acidic and basic groups, usually located on their surface. The charges on these groups vary according to their acid dissociation constants with the pH of the environment. This affects the total net charge of the enzymes which will overall affect the enzyme activity, structural stability and solubility of the enzyme (Chaplin and Bucke, 1990). In case of estuarine bacterium *Bacillus altitudinis* strain BR1 although growth and enzyme activity was determined in pH range 7- 12, significant increase in enzyme activity was observed within pH range 8-11 with pH optimum of 9.0 for growth and protease activity (i.e. 92 U/ml). Whereas coastal sediment isolate *Bacillus cereus* strain CS1 showed growth and enzyme activity in the pH range of 8-11, but optimum pH was 9.0 for growth and enzyme activity (i.e. 125 U/ml). Similarly coastal water isolate *Bacillus firmus* strain CW2 showed that pH 10.0 was optimum for growth and enzyme activity (i.e. 142 U/ml). This clearly demonstrated that

bacterial strains viz. BR1, CS1 and CW2 are alkaliphiles showing optimum alkaline protease activity in pH range 9 to 10 (Figs. 3.1 a-c). Interestingly all three bacterial isolates showed a rapid decline in growth and protease activity beyond pH 11. It has been reported that the optimum pH for alkaline proteases of *Bacillus spp.* generally range from 9 to 11 with a few exceptions with pH optima of 11-12 (Kumar *et al*., 1999) and 12-13 (Takami *et al*., 1992; Fujiwara *et al*., 1991; Margesin *et al*., 1992; Kumar and Takagi, 1999). These studies have clearly demonstrated that a wide range of pH optima is required for different alkaliphilic bacteria producing commercially important proteases.

Our findings are in agreement with few earlier reports showing pH optima of 9.0 for protease activity in *Bacillus licheniformis* and *Bacillus firmus* 7728 (Manachini and Fortina, 1998 ; Rao and Narasu, 2007). But *Bacillus sp*. AR-009, *Bacillus sp.* SB5 and *Bacillus sp.* SSR1 showed pH optima of 10 (Gessesse, 1998; Gupta *et al*., 1999; Singh *et al*., 2001). In general all currently used detergent compatible proteases are alkaline in nature with a high pH optima and are suitable as laundry detergent additives mostly in the range of 8-12. It is interesting to mention that majority of commercially available subtilisin-type proteases are also active in the pH range 8 -12 (Gupta *et al*., 2002). Subtilisin Carlsberg and subtilsin Novo which show maximum protease activity at pH 10.5 are best examples of commercial proteases (Banerjee *et al.*, 1999).

The alkaliphilic protease producing bacteria isolated from coastal and estuarine environment of Goa are also valuable source of industrially important alkaline proteases as they have shown optimum enzyme activity within the pH range 8 to 10.

3.4.2 Determination of optimum salinity (% NaCl) for growth and protease production

Saline environment causes stress to non-halophilic microorganisms by decreasing water activity and increasing NaCl concentration. However some microorganisms including bacteria and fungi viz. *Halomonas haloplanktis, Halothermothrix orenii, Halobium sp., Hortaea werneckii* and *Aureobasidium pullulans* can withstand high salinity as they can grow in presence of NaCl even upto 10 % and are referred as halotolerant microorganisms (Setyorini *et al*., 2006).

Although all three potential protease producing isolates showed growth at NaCl levels in the range of 0-6 %, optimum NaCl level for high protease activity of estuarine isolate *Bacillus altitudinis* strain BR1 and coastal sediment isolate *Bacillus cereus* strain CS1 was 2 % with maximum activity of 86 U/ml and 91 U/ml respectively (Fig. 3.2 a, b). While for coastal water isolate *Bacillus firmus* strain CW2 optimum NaCl concentration was recorded 4 % which induced protease activity of 96 U/ml (Fig. 3.2 c). Therefore *Bacillus firmus* strain CW2 may be a good candidate for optimum activity as a detergent additive as NaCl is one of the core material components of enzyme based granulated detergents.

Growth and enzyme activity (production) was significantly repressed above 2 % NaCl in case of estuarine isolate *Bacillus altitudinis* strain BR1. All the three bacterial isolates viz. BR1, CS1 and CW2 are halotolerant as protease activity and growth was observed with and without NaCl in the growth medium. Halotolerance is an added advantage to the alkaline proteases as it enhances its efficacy in industrial processes where the concentration of salt varies (Mohaparta *et al*., 1998; Ventosa *et al*., 1998; Gupta *et al*., 2005). Salt tolerant protease producing

mesophilic and thermophilic *Bacillus sp.* has also been reported earlier (Joshi *et al*., 2007; Ningthoujam and Kshetri, 2010).

3.4.3 Determination of optimum temperature for growth and protease production

Temperature is one of the critical parameters for regulating synthesis and secretion of alkaline protease by bacteria. High content of hydrophobic amino acids in the enzyme molecule provides a compact structure, which is not easily denatured by change in the environment. In addition, disulfide bridges and other bonds provide high resistance both against heat inactivation and chemical denaturation.

When tested at different temperature range i.e. 30 ^{0}C - 60 ^{0}C it was clearly revealed that *Bacillus altitudinis* strain BR1 showed a sharp decline in cell biomass but enhanced protease activity (i.e. 98 U/ml) at 50 ^{0}C (Fig. 3.3 a). Thus the protease of this estuarine isolate appears to be thermostable and can be successfully used as a promising detergent additive at alkaline pH (pH 9) and high temperature (50 ^{0}C). Similar findings on optimum temperature range of thermostable microbial proteases have also been reported which showed a broad range of temperature optima from 27 ^{0}C- 50 ^{0}C (Kasana and Yadav, 2007; Bajaj and Jamwal, 2013).

Bacillus cereus strain CS1 and *Bacillus firmus* strain CW2 exhibited growth in temperature range of 30 ^{0}C - 45 ^{0}C but optimum temperature for growth and enzyme activity was 37 ^{0}C as maximum protease activity was 142 U/ml and 121 U/ml respectively (Figs. 3.3 c, d). Majority of the alkaliphilic *Bacillus* strains produce alkaline proteases with temperature optima ranging between 30 ^{0}C- 37 ^{0}C and are mostly mesophiles (Takami *et al*., 1989; Banerjee *et al*., 1999; Kanekar *et al*., 2002; Joo *et al*., 2003). Our studies have clearly demonstrated that protease produced by selected alkaliphilic bacterial isolates *Bacillus cereus* strain CS1 and

Bacillus firmus strain CW2 may successfully be used as valuable detergent additives and in pre-treatment of heat sensitive food items in the temperature range of 30 ^{0}C-37 ^{0}C.

3.4.4 Determination of best carbon source for growth and protease production

Among various carbon sources tested viz. lactose, glucose, fructose, maltose and sucrose the best carbon source for growth was found to be 1 % sucrose for *Bacillus altitudinis* strain BR1 and *Bacillus cereus* strain CS1.Whereas *Bacillus firmus* strain CW2 showed best growth in the Horikoshi-I medium supplemented with 1 % glucose. However it is interesting to note that the protease activity was highest (i.e. 66 U/ml) in presence of 1 % fructose for *Bacillus altitudinis* strain BR1. Similar finding has been reported in case of *Bacillus cereus* MTCC 6840 (Joshi *et al.*, 2007). *Bacillus coagulans* PSB-07 growth was maximum in presence of glucose as carbon source but maximum protease production (760.4 U/ml) was observed in growth medium supplemented with 0.5 % sucrose (Olajuyigbe and Ehiosun, 2013). Sucrose was also reported best substrate for production of extracellular protease by Madzak *et al.*, (2000). Some earlier reports also indicated that production of protease was enhanced in the presence of maltose and glucose (Mehrotra *et al.*, 1999; Gupta *et al.*, 2005). However repression of protease production in presence of glucose, maltose and sucrose have been previously reported (Sen and Satyanarayana, 1993).

Coastal isolates *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 exhibited better growth and protease activity (70 U/ml) and (92 U/ml) respectively in presence of 1 % glucose as compared to other carbon sources viz. lactose, fructose, maltose and sucrose (Fig. 3.4 a-c). Our results are in agreement with

earlier findings showing glucose as the preferred carbon source for protease production and growth shown by haloalkaliphilic *Bacillus sp.* and *Bacillus subtilis* (Patel *et al*., 2006; Muhammad *et al*., 2008; Verma *et al*., 2011; Prabhavathy *et al*., 2013). We have also observed that enzyme production in all the three selected bacterial strains is not growth dependent. Therefore selection of optimas for high level enzyme production is more relevant than the one which enhances only biomass.

3.4.5 Determination of best nitrogen source for growth and protease production

Nitrogen sources play a vital role in the synthesis of proteins, nucleic acids and secondary metabolites along with enzymes. Organic nitrogen sources tested viz. peptone, yeast extract, tryptone and casein at the level of 0.2 % showed a significant increase in growth and enzyme activity for all the selected bacterial strains (Fig. 3.5 a-c). Peptone was found preferred nitrogen source for growth and enzyme production (i.e. 101 U/ml) for *Bacillus altitudinis* strain BR1 which clearly indicates that peptone was utilized better as compared to other organic nitrogen sources. The addition of peptone to the growth media shortened the lag period and increased the exponential period that resulted in enhanced enzyme production. Similarly *Bacillus sp*. PCSIR EA-3 and *Bacillus subtilis* demonstrated maximum enzyme production in presence of (1 % & 0.5 %) peptone (Shah *et al*., 2009; Das and Prasad, 2010).

In case of *Bacillus cereus* strain CS1, casein was preferred nitrogen source for growth but tryptone induced high level of protease enzyme activity (i.e. 158 U/ml). However casein was observed to be the best nitrogen source for growth as well as protease activity (i.e. 115 U/ml) for *Bacillus firmus* strain CW2 (Fig. 3.5 a-c). *Bacillus subtilis* PE-11 and *Bacillus cereus* also showed high specificity for casein

as compared to other organic nitrogen sources (Adinarayana *et al.*, 2003; Kavitha and Thankamani, 2010; Kuberan *et al.*, 2010).

Besides organic nitrogen sources, inorganic nitrogen sources such as ammonium nitrate [NH_4 $(NO_3)_2$] and potassium nitrate (KNO_3) also regulate the production of microbial proteases (Do Nascimento and Martins, 2004; Sindhu *et al.*, 2009; Kuberan *et al.*, 2010). Growth medium supplemented with ammonium nitrate and potassium nitrate inhibited growth and protease enzyme activity of all three selected bacterial isolates. Growth as well as enzyme activity was significantly repressed (i.e. 10 U/ml) for *Bacillus cereus* strain CS1 in presence of ammonium nitrate. It seems that inorganic nitrogen adversely affects the biosynthesis of alkaline proteases (Zvidzai and Zvauya, 2001).

3.5 Effect of metal ions and inhibitors on protease activity of selected bacterial strains

Divalent cations viz. Ca^{+2}, Mn^{+2}, Mg^{+2}, Zn^{+2}, Co^{+2}, Cu^{+2} and Fe^{+2} regulate protease activity of microorganisms including bacteria in diverse manner (Joshi *et al.*, 2007; Venugopal and Saramma, 2007; Mala and Srividya, 2010; Kavitha and Thankamani, 2010). In general Ca^{+2}, Mn^{+2} and Mg^{+2} ions enhance the protease activity whereas Hg^{+2}, Pb^{+2} and Cd^{+2} repress the enzyme activity. It is interesting to note that Ca^{+2} ions enhance the thermal stability of alkaline protease in *Bacillus* spp. thereby protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation at high temperatures (Steele *et al.*, 1992; Kumar and Takagi, 1999).

Among various metal cations tested viz. Ca^{+2}, Mn^{+2}, Cu^{+2}, Pb^{+2} and Hg^{+2} for protease activity of *Bacillus altitudinis* strain BR1, Ca^{+2} and Cu^{+2} ions enhanced the

enzyme activity at 1 mM since residual enzyme activity was recorded as 6 % and 3 % respectively compared to the control. Whereas at 2.5 mM & 5 mM levels of Ca^{+2} and Cu^{+2} ions gradual decrease in activity was recorded (Fig. 3.6 a). Interestingly, protease residual activity of the isolate was 78 % at 5 mM Mn^{+2} and 22 % in presence of 5 mM Pb^{+2} ions which may be attributed to significant inhibition of enzyme activity due to lead toxicity (Fig. 3.6 a).

The coastal sediment isolate *Bacillus cereus* strain CS1 demonstrated significant increase in residual protease activity (i.e. 7 %) as compared to control with increase in concentration of Ca^{+2} ions upto 5 mM. Calcium ions are known to be stabilizers of many enzymes, protecting them from conformational changes. The Ca^{+2} binding sites examined for some bacterial proteases contain a number of co-coordinating aspartate and glutamate residues (Vordouw *et al.*, 1976). Whereas at 5 mM Mn^{+2} ions residual activity was 56 % as compared to control.

Similar findings have been reported which indicates that Ca^{+2} and Mn^{+2} significantly increase the protease activity of *Bacillus subtilis* PE-11 and *Bacillus sp*. SMIA-2 respectively (Adinarayana *et al*., 2003; Do Nascimento and Martins, 2004). Our coastal sediment isolate demonstrated significant repression of protease activity in presence of 1 mM Pb^{+2} and Cu^{+2} as activity was only 13 % and 9 % respectively (Fig. 3.6 b).

Coastal water isolate *Bacillus firmus* strain CW2 exhibited significant increase in enzyme activity (i.e. 57 %) as compared to control in presence of 5 mM Cu^{+2} ions. This study suggests that Cu^{+2} ions facilitate proper conformation and stabilization of protease structure (Ozturk *et al*., 2012). Alkaline protease produced by *B. licheniformis* was also stimulated in presence of Cu^{+2} ions (Sinha and Satyanarayana, 1991). Incidentally only a few alkaline proteases have shown

increased protease activity in the presence of Cu^{+2} ions e.g. *B. mojavensis* (Beg and Gupta, 2003), *Serratia rubidaea* (Doddapananeni *et al*., 2007), and *B. aquimaris* VITP4 (Shivanand and Jayaraman, 2011).

Whereas residual protease activity of *Bacillus firmus* strain CW2 in presence of 5 mM Mn^{+2} and Ca^{+2} ions was 47 % and 41 % respectively. Pb^{+2} ions induced sharp decrease in residual enzyme activity (i.e. 23 %) at 5 mM due to lead toxicity at high levels (Fig. 3.6 c).

All three bacterial isolates *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 showed drastic inhibition in protease activity at 1 mM Hg^{+2} ions since residual activity was only 18 %, 18 % and 7 % respectively (Fig. 3.7 a-c). Hg^{+2} ions are well known to inhibit enzyme activity significantly due to their binding with –SH groups of enzymes. Alkaline protease from *Bacillus brevis* and *Bacillus circulans* BM15 lost its activity completely in the presence of Hg^{+2} ions (Banerjee *et al*., 1999; Venugopal and Saramma, 2007). While activity of metalloprotease from the wild basidiomycete mushroom *Lepista nuda* was reported to be inhibited in presence of 5 mM Hg^{+2} ions (Wu *et al*., 2011).

Enzyme inactivation studies provide an insight into the nature of enzymes, their cofactor requirements and nature of the active sites. Therefore we have selected several protease inhibitors and reducing agents viz. PMSF, DTT, SDS, Na_2-EDTA and β-mercaptoethanol to find out exact nature of alkaline proteases produced by all the selected bacterial isolates. Phenyl methyl sulfonyl fluoride (PMSF) and di-isopropyl fluorophosphate (DFP) selectively inhibit serine proteases by sulfonation of the essential serine residues in the active site of the enzyme. Some alkaline proteases are metalloproteases since they need metal cations such as Co^{+2} and Zn^{+2} ions for protease activity. Therefore they are sensitive to metal chelating agents viz.

Na_2 –EDTA, EGTA and o-phenanthroline. Thiol inhibitors have little effect on alkaline proteases of *Bacillus* spp., although they do affect the alkaline enzymes produced by *Streptomyces* sp. (Rao *et al*., 1998; Kumar and Takagi, 1999).

Protease inhibitors viz. PMSF, DTT, SDS, β- ME and Na_2–EDTA at 1 mM level were tested on alkaline protease activity. It is interesting to note that protease activity of *Bacillus altitudinis* strain BR1 was significantly inhibited by PMSF as the percent residual activity was only 40 % as compared to control. This clearly demonstrated it to be a serine protease because PMSF represses enzyme activity through sulphonation of the serine residues in the active site. PMSF has also been reported to completely inhibit alkaline protease activity in *Bacillus* sp. KSM-K16, *Bacillus pumilus*, *Bacillus* sp., *Bacillus subtilis* PE-11, *Bacillus* sp. GUS1 and *Streptomyces* sp. AB1 (Kobayashi *et al*., 1995; Feng *et al*., 2001; Singh *et al*., 2001; Adinarayana *et al*., 2003; Seifzadeh *et al*., 2008; Jaouadi *et al*., 2010). Whereas residual protease activity was recorded 82 % in presence of chelating agent Na_2-EDTA which clearly indicates high enzyme stability in presence of this chelating agent and metal ion cofactors are not required. This characteristics of the protease is important for its use as detergent additives since detergents contain high amounts of chelating agents which remove divalent cations responsible for water hardness and also facilitate stain removal (Oberoi *et al*., 2001; Walsh, 2002; Beg and Gupta, 2003; Ghorbel *et al*., 2003). Significant residual protease activity (i.e. 60 %) was also observed in the presence of SDS which clearly suggested that it can be successfully used as a detergent additive. Similarly 89 % and 50 % residual protease enzyme activity of isolate was observed in presence of thiols such as β- ME and DTT which interestingly demonstrated significant stability in presence of reducing agents such as β- ME and DTT (Fig. 3.8 a). Psychrotrophic *Exiguobacterium* sp.

SKPB5 showed 20 % increase in alkaline protease activity in presence of β- ME and DTT (Kasana and Yadav, 2007). Whereas proteases of *Vibrio metschnikovii* and *Bacillus circulans* BM15 retained 65 % residual activity with β – ME whereas DTT induced significant increase in residual protease activity i.e. 114.9 % (Kwon *et al*., 1994; Venugopal and Saramma, 2007).

Coastal isolates *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 showed significant inhibition of protease activity in presence of Na_2-EDTA as the residual protease activity was recorded 21 % for both the isolates confirming it to be metalloprotease. Therefore it could be a metal dependent protease. Our findings are in agreement with earlier reports (Secades *et al*., 2001; Stensvag *et al*., 2006; Amoozegar *et al*., 2007; Sousa *et al*., 2007; Kuddus and Ramteke, 2008).

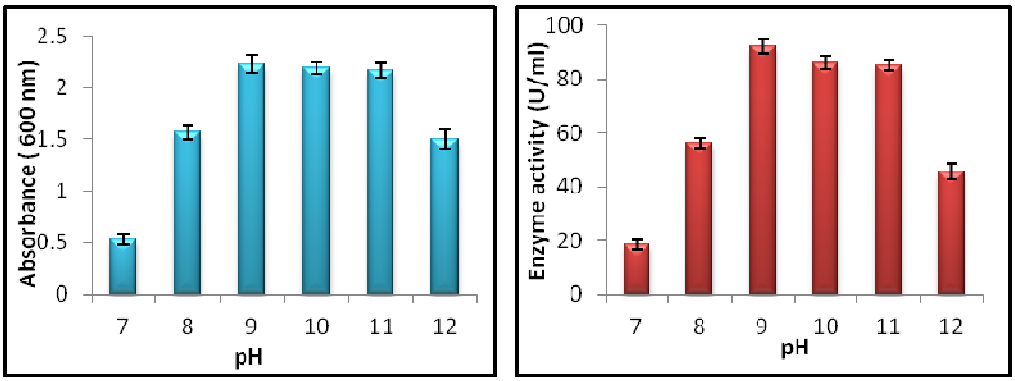
Residual protease activity of *Bacillus cereus* strain CS1 was recorded 52 %, 51 %, 50 % and 45 % respectively when partially purified protease was exposed to 1 mM of SDS, β- ME, DTT and PMSF separately (Fig. 3.8 b). Whereas partially purified enzyme of *Bacillus firmus* strain CW2 when incubated with β- mercaptoethanol, PMSF and DTT separately showed significantly high residual activity of 112 %, 104 % and 93 % respectively (Fig. 3.8 c). But a moderate repression of enzyme activity (i.e. 69 % residual activity) was observed in case of SDS. Similarly significant enhancement (i.e. 164.8 % and 127 %) in residual protease activity was also observed in the presence of 1 mM PMSF and 0.1 % β- mercaptoethanol for *Bacillus cereus* and *Bacillus* sp. JB99 respectively (Sousa *et al*., 2007; Kainoor and Naik, 2010). Whereas proteases from *Bacillus* sp. GUS1 retained 90 % of its activities in presence of 5 mM

SDS (Seifzadeh *et al*., 2008) and *Pseudomonas* sp. DR89 also showed residual protease activity (i.e. 80 %) in presence of 0.05 % SDS (Asoodeh and Musaabadi, 2012).

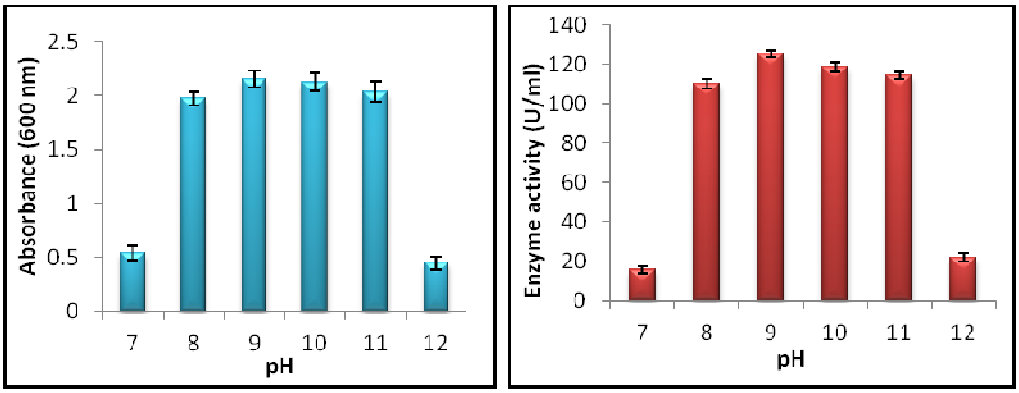
3.6 Hydrolysis of different protein substrates by crude protease enzyme

In order to assess the proteolytic activity of alkaline protease produced by coastal isolate *Bacillus firmus* strain CW2 different protein substrates viz. casein, gelatin and bovine serum albumin (BSA) were used. This isolate hydrolyzed gelatin more efficiently than other protein substrates (Fig. 3.9) as the enzyme activity was 158 U/ml as compared to control (i.e. 100 U/ml) whereas in presence of BSA enzyme activity was slightly repressed i.e. 83 U/ml (Fig. 3.9). These results confirm that gelatin is a preferred protein substrate for protease of *Bacillus firmus* strain CW2 and we can also refer this enzyme as gelatinase (Hamza *et al*., 2006; Sai-Ut *et al*., 2013).

The ability of an organism to hydrolyze a wide range of substrates may be advantageous for its use in detergents against a wide variety of stains. Also due to its ability to use gelatin as a substrate, the alkaline protease enzyme from *Bacillus firmus* strain CW2 can be advantageous in the recovery of silver from photographic films (Fujiwara *et al*., 1989, 1991; Ishikawa *et al*., 1993; Gajju *et al*., 1996 and Vijayalakshmi *et al*., 2011). Earlier studies have revealed that casein was preferred protein substrate for alkaline proteases as compared to other protein substrates including BSA (Freeman *et al.*, 1993; Rahman *et al*., 1994).



(a)



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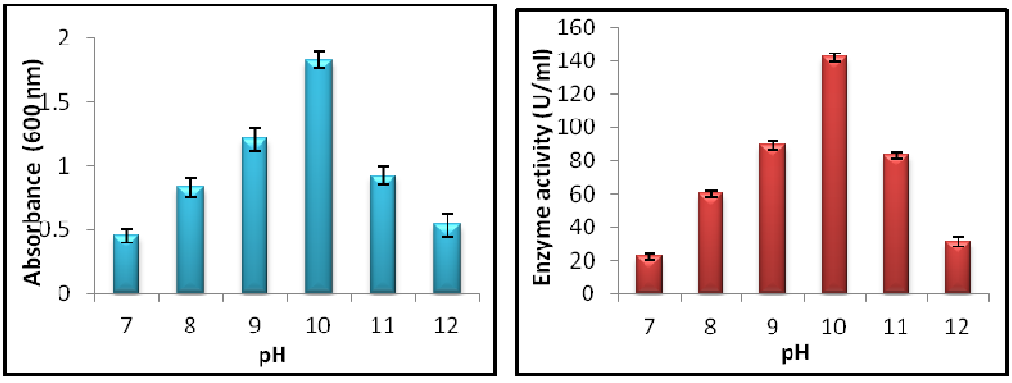
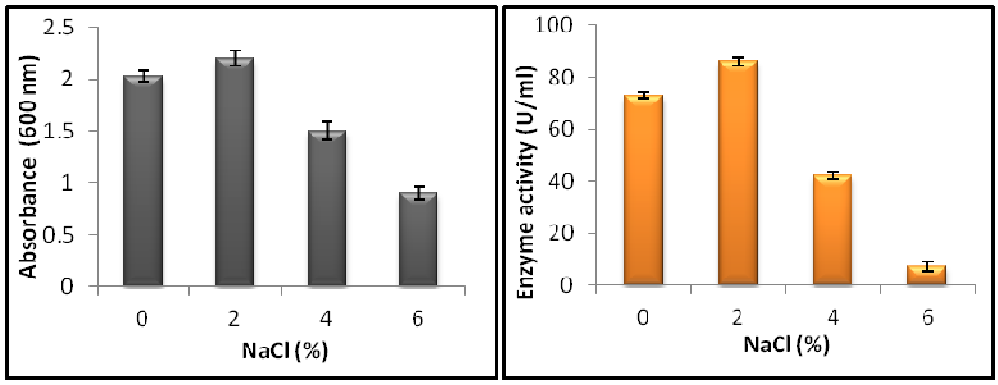


(c)

Fig. 3.1 **Effect of pH on growth and enzyme activity of *Bacillus altitudinis* strain BR1 (a), *Bacillus cereus* strain CS1 (b) and *Bacillus firmus* strain CW2 (c)**



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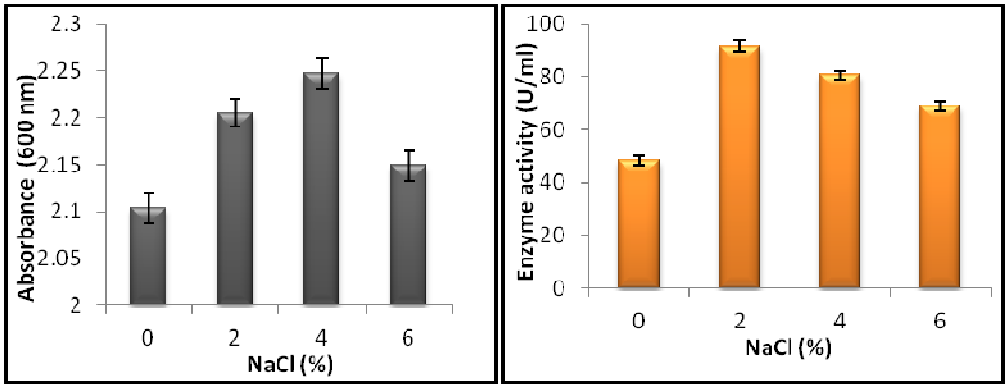

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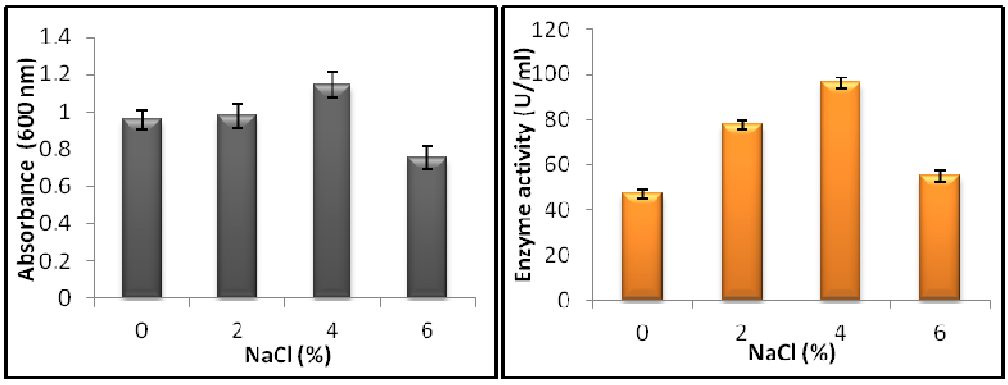
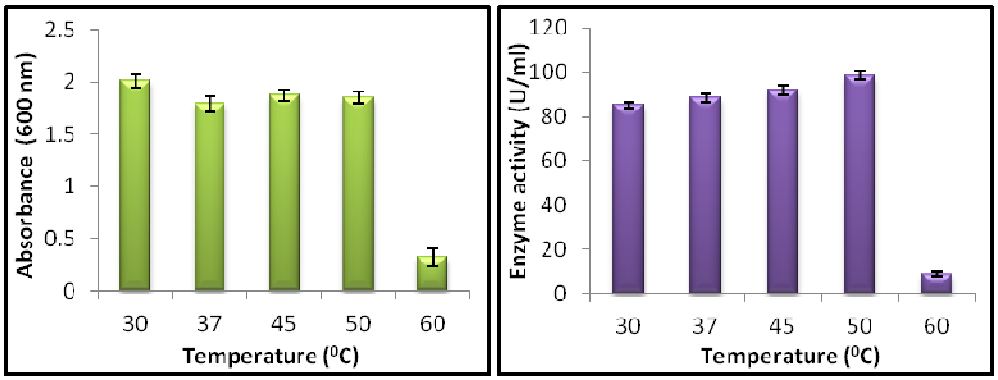


(c)

Fig. 3.2 **Effect of salinity (NaCl %) on growth and enzyme activity of *Bacillus altitudinis* BR1 (a), *Bacillus cereus* CS1 (b) and *Bacillus firmus* CW2 (c)**



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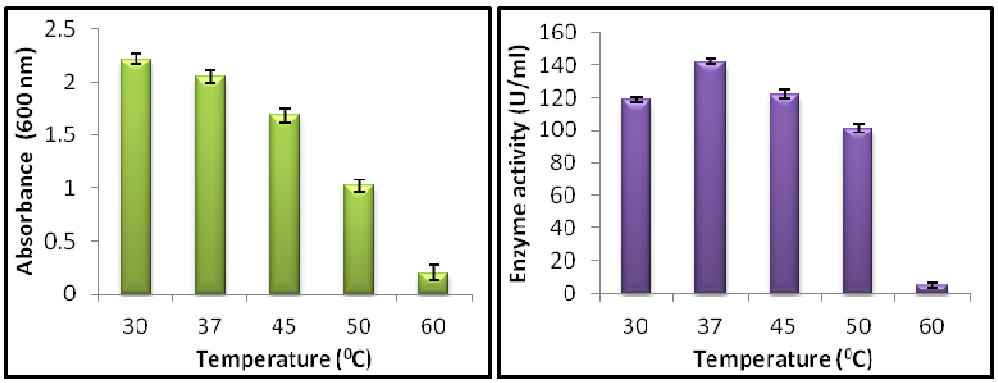

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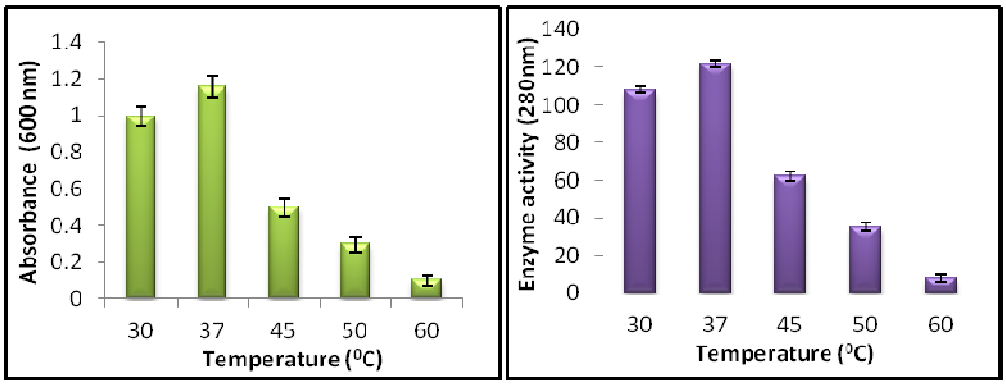
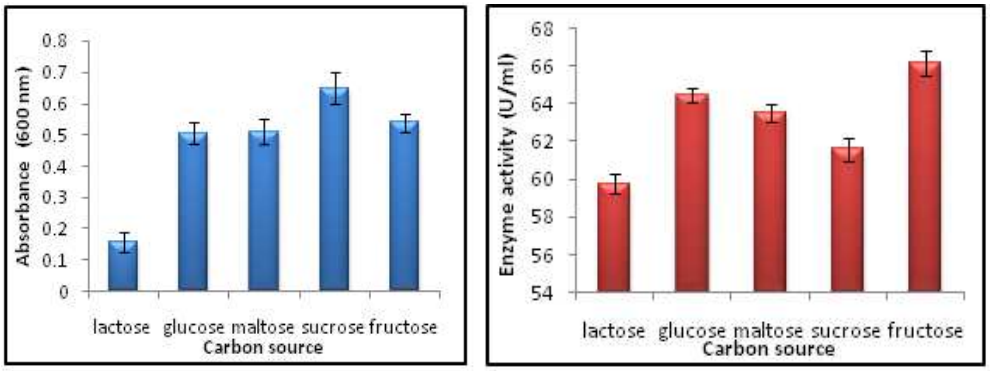
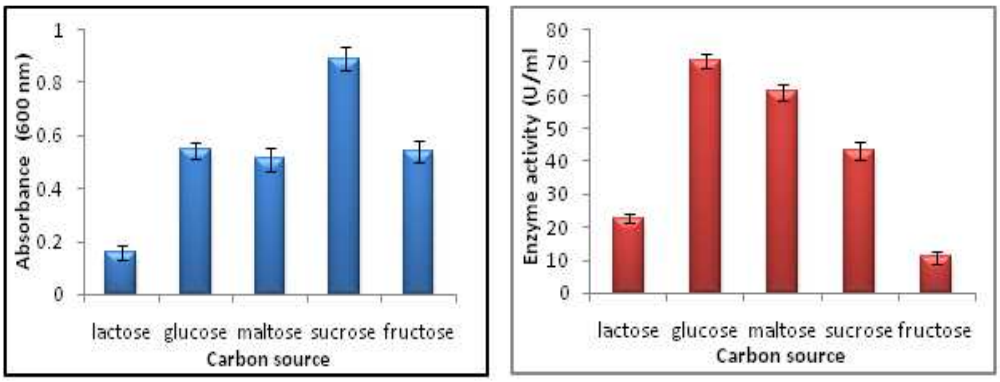


(c)

Fig. 3.3 Effect of temperature on growth and enzyme activity of *Bacillus altitudinis* strain BR1 (a), *Bacillus cereus* strain CS1 (b) and *Bacillus firmus* strain CW2 (c)



(a)



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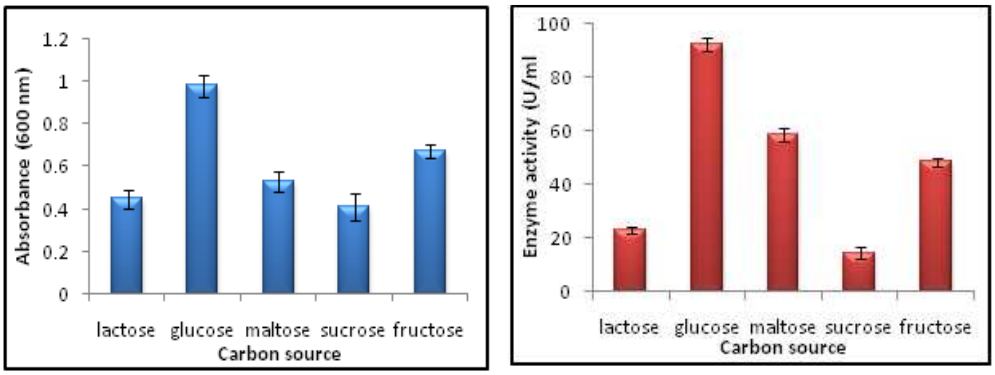
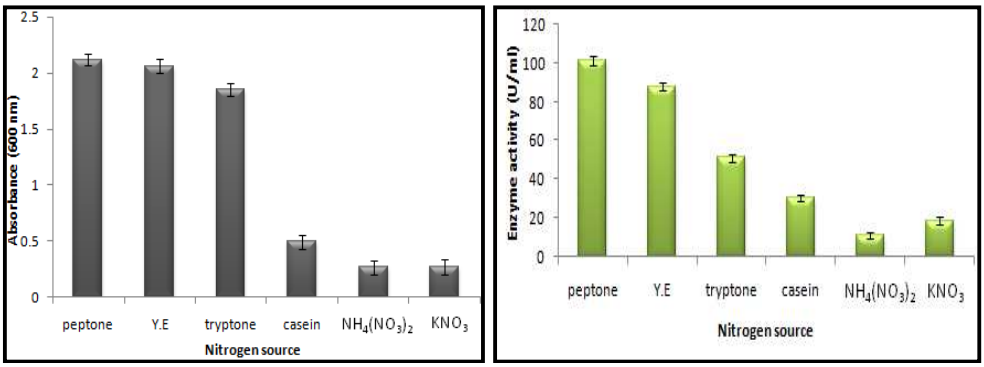
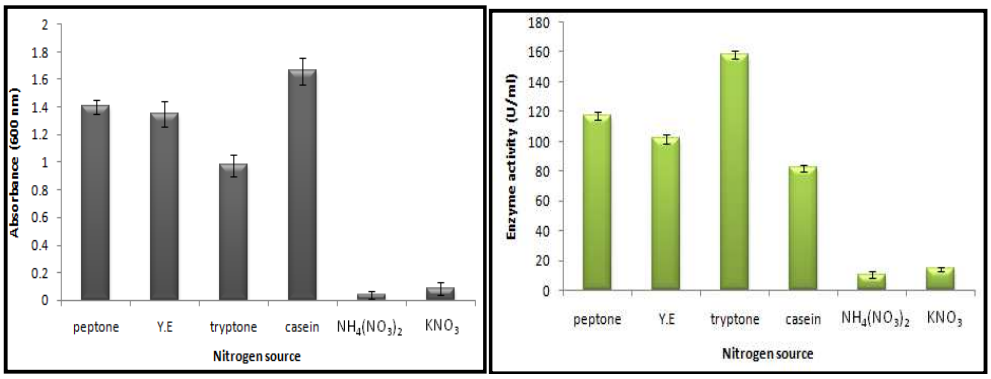


(c)

Fig. 3.4 Effect of carbon sources on growth and enzyme activity of *Bacillus altitudinis* strain BR1 (a), *Bacillus cereus* strain CS1 (b) and *Bacillus firmus* strain CW2 (c)



(a)



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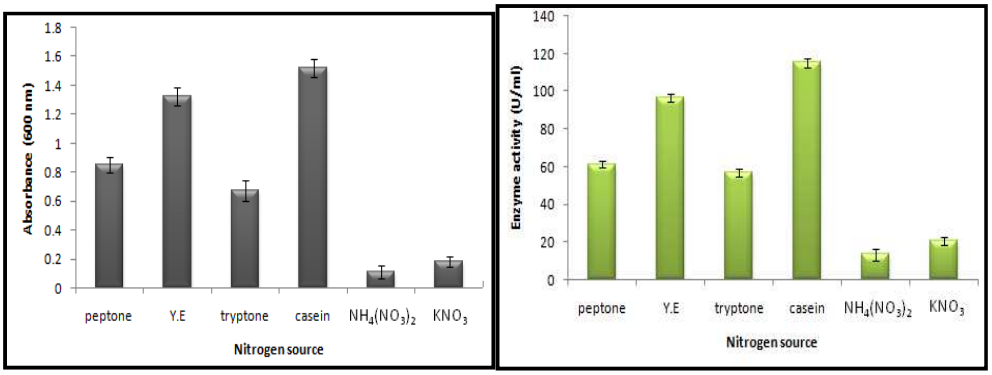
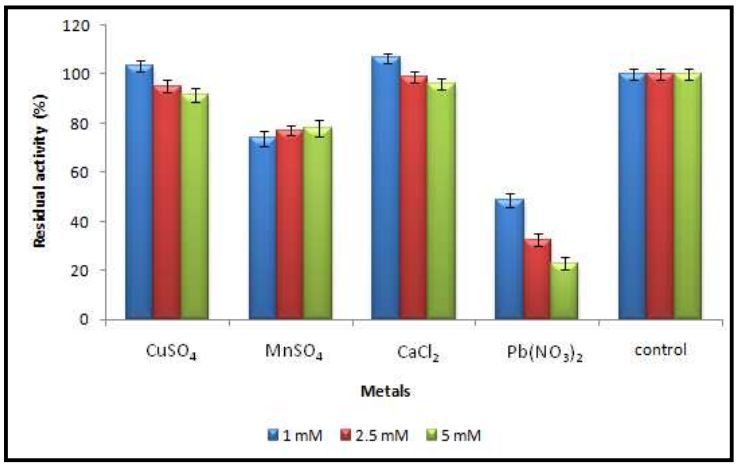
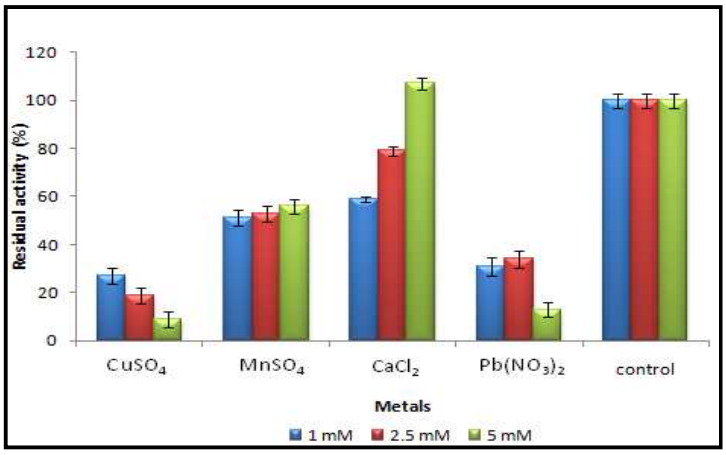


(c)

Fig. 3.5 Effect of nitrogen sources on growth and enzyme activity of *Bacillus altitudinis* strain BR1 (a), *Bacillus cereus* strain CS1(b) and *Bacillus firmus* strain CW2



(a)



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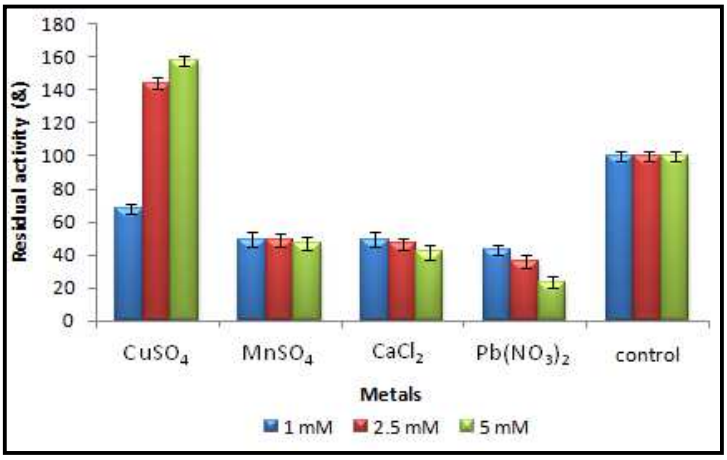
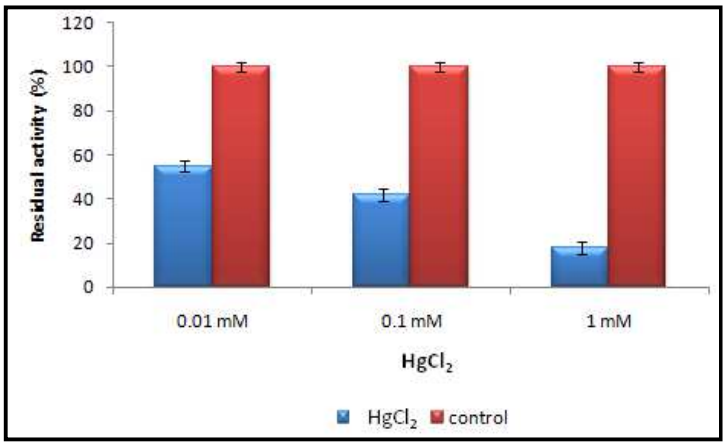


(c)

Fig. 3.6 Effect of metals on residual protease activity of *Bacillus altitudinis* strain BR1 (a), *Bacillus cereus* strain CS1(b) and *Bacillus firmus* strain CW2 (c)



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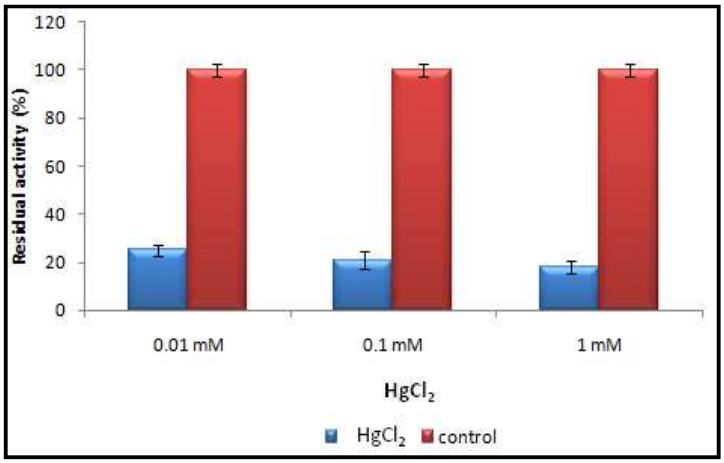

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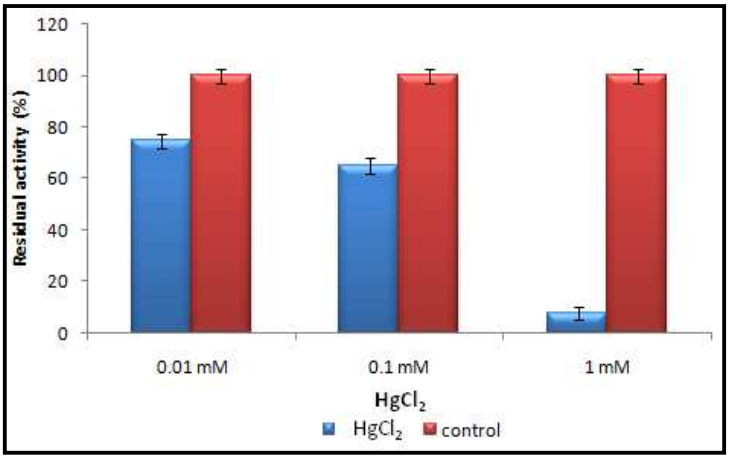
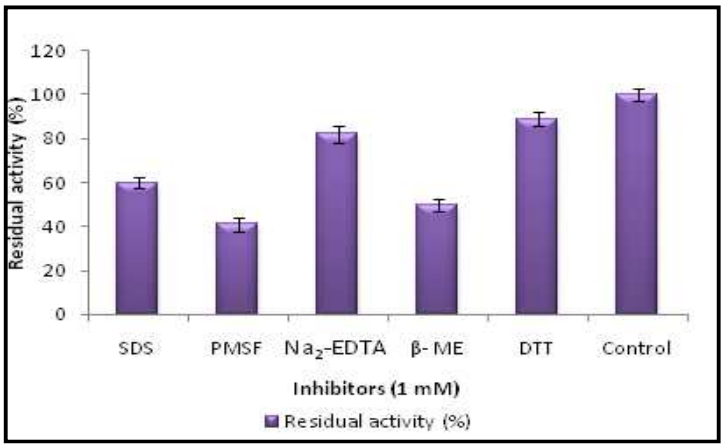
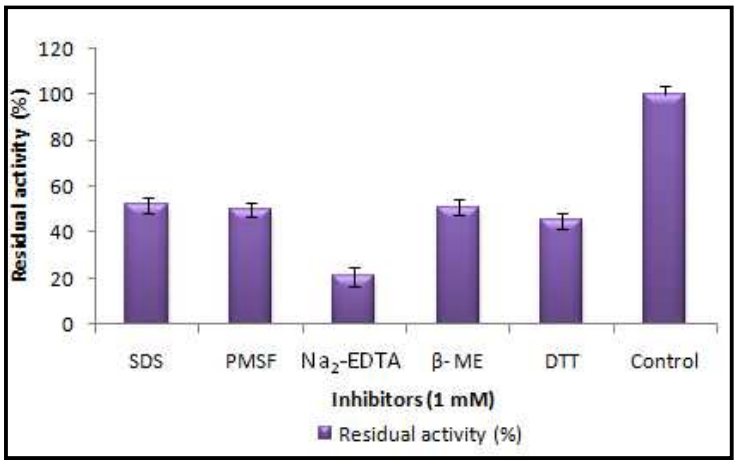


(c)

Fig. 3.7 Effect of $HgCl_2$ on residual protease activity of *Bacillus altitudinis* strain BR1 (a), *Bacillus cereus* strain CS1(b) and *Bacillus firmus* strain CW2 (c)



(a)



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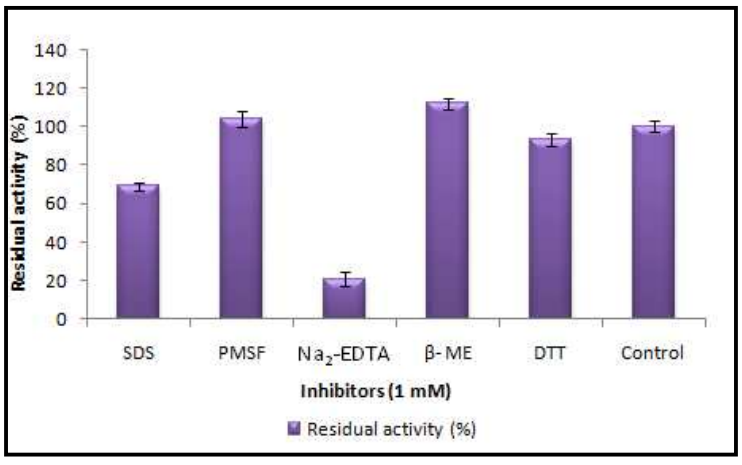


(c)

Fig. 3.8 Effect of inhibitors on residual protease activity of *Bacillus altitudinis* strain BR1 (a), *Bacillus cereus* strain CS1(b) and *Bacillus firmus* strain CW2 (c)

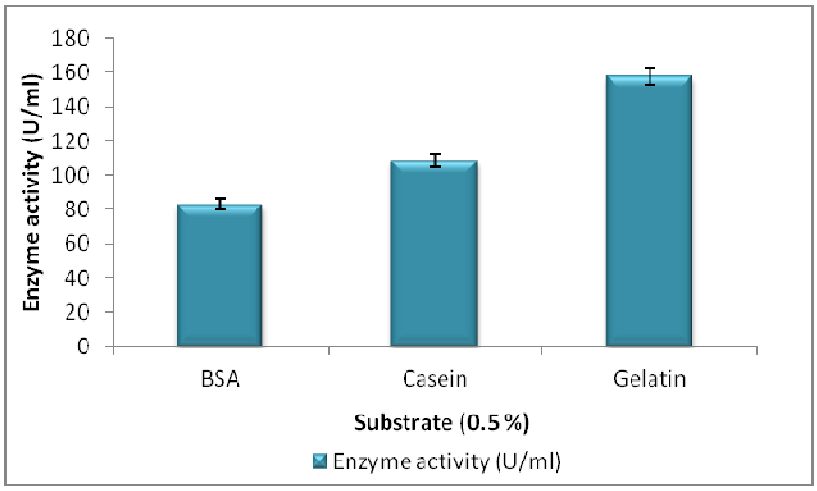


Fig. 3.9 Effect of different protein substrates on protease activity of *Bacillus firmus* strain CW2

CHAPTER IV

Biochemical characterization of potential protease producing bacterial isolates

Chapter IV

Biochemical characterization of potential protease producing bacterial isolates

MATERIALS AND METHODS

4.1 Partial purification of protease enzyme

4.1.1 Ammonium sulphate precipitation

The selected bacterial isolates *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 were inoculated (0.5 % inoculum) in three different flasks containing 50 ml of Horikoshi- I medium (Horikoshi, 1996). The pH of the growth medium was maintained at pH 9 for *Bacillus altitudinis* strain BR1 and *Bacillus cereus* strain CS1, whereas pH 10 was maintained for *Bacillus firmus* strain CW2 respectively. Flasks containing bacterial isolates were incubated on a rotary shaker at 150 rpm at 50 ^{0}C for *Bacillus altitudinis* strain BR1 whereas at 30 ^{0}C for *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 for 24 hrs. 5 % inoculum from each flask was transferred into a 1 litre flask containing 500 ml of the fresh growth medium and the culture flasks were incubated at same pH and temperature used earlier. The culture suspensions of individual bacterial isolates were harvested after 18 hrs of incubation at 10, 000 rpm and 4 ^{0}C for 20 mins. The cell free supernatant (CFS) was used for further purification of extracellular protease.

Different concentrations of ammonium sulphate (i.e. 10 - 80 %) were used to find out the percent saturation of ammonium sulphate which results in highest level of protein precipitation as well as protease activity [Appendix H]. According to

ammonium sulphate percent saturation table appropriate amount of ammonium sulphate was added to 500 ml of the cell free supernatant, sample was incubated at 4 ^{0}C for 1 hr while stirring and subsequently incubated at 4 ^{0}C overnight. The precipitate was resuspended in 0.1 M sodium carbonate (pH 10) and dialyzed out several times against 0.05 M sodium carbonate (pH 10) using dialysis bag (12kDa). The dialysed sample was centrifuged at 9000 rpm at 4 ^{0}C for 15 mins. This supernatant was further dialyzed against 1 M sucrose solution for 1 hr to concentrate the protease enzyme. This partially purified protease enzyme was stored at 4 ^{0}C and used for further characterization. The protease enzyme activity as well as total protein content of the sample before and after ammonium sulphate precipitation was estimated.

4.1.2 Estimation of protein concentration

Standard procedure was followed to estimate total protein content of partially purified enzyme samples (Lowry *et al.*, 1951). Protein sample (1 ml) was supplemented with 5 ml alkaline copper sulphate solution and incubated at room temperature for 10 mins in the dark. 0.5 ml Folin and Ciocalteau's Phenol reagent was also added to the sample and incubated for 20 mins in the dark [Appendix D]. Absorbance of the sample was measured at 660 nm against blank and concentration of protein in the samples was determined using Bovine serum albumin (BSA) as standard.

4.2 SDS-PAGE analysis and Zymography

4.2.1 One- dimensional gel electrophoresis (SDS-PAGE)

Sodium dodecyl sulphate – Polyacrylamide gel electrophoresis (SDS - PAGE) analysis of partially purified protease was done using 10 % resolving gel and 5 %

stacking gel as per the standard method of Laemmli, 1970 [Appendix E]. The partially purified enzyme sample (15 µl) with approximately 50 µg protein was mixed with equal volume of 2X sample solubilizing buffer (tracking dye) containing bromophenol blue [Appendix E.10] and mixture was placed in boiling water bath for 5 mins followed by centrifugation at 10,000 rpm at 4 ^{0}C for 2 mins. The clear supernatant was loaded on the 10 % SDS-PAGE gel [Appendix E] and electrophoresis was carried out in 1X Tris-Glycine electrophoresis buffer at 25 ^{0}C following standard procedure. Protein samples were initially electrophoresed at 35 mA until tracking dye entered the resolving gel, subsequently current was increased to 80 mA and electrophoresis was done till the tracking dye migrates slightly above the bottom of the resolving gel. Broad range protein molecular weight marker (MWM, Banglore Genei, India) was used to determine size of the proteins. The SDS-Polyacrylamide gel was visualized after silver staining [Appendix E.13]. The SDS-PAGE gel was recorded using gel documentation system (Alpha-Innotech, USA). The molecular weight of the proteases was determined by comparing with protein standard.

4.2.2 Silver staining

After completion of the electrophoresis, the protein gel was stained using silver stain. Firstly, the protein gel was fixed by incubating it for 4 hrs at room temperature with gentle shaking in at least 5 volumes of fixing solution [Appendix E.13]. The fixing solution was discarded and gel was incubated in 30 % ethanol for 30 mins at room temperature with gentle shaking. The above step was repeated one more time. 30 % ethanol was discarded and 10 gel volumes of deionised water was added. The gel was incubated for 10 mins at room temperature with gentle shaking. This step was repeated twice. After completion of the water washes, silver stain

[Appendix E.13] was added and the gel was incubated for 30 mins at room temperature with gentle shaking. The gel was then washed with distilled water and then incubated in freshly prepared silver developer solution [Appendix E.13]. Once stained bands of protein appeared, the reaction was quenched by adding 1 % acetic acid.

4.2.3 Native PAGE and Zymogram analysis of partially purified enzyme

Native PAGE and Zymogram analysis was carried out to detect the proteolytic activity of proteases directly in the Native-PAGE gel. Skim milk was co-polymerized with 10 % Polyacrylamide gel. The gel was prepared similar to SDS-PAGE but without SDS and with (0.5 %) skim milk as substrate in the separating gel. 25 µl of the protein sample was loaded onto the Native-PAGE gel and electrophoresis was carried out at 80 V for 3 hrs. After the electrophoresis run was over, the gel was rinsed in 2.5 % Triton-X 100 [Appendix E.11] for 1 hour at room temperature to remove SDS. This was followed by washing the gel in developing buffer (pH 10) [Appendix E.9]. Enzyme activity in the Native-PAGE gel is detected by incubating the gel in developing buffer at 37°C overnight followed by silver staining. The clear bands observed in the zymogram were compared with NATIVE-PAGE molecular weight marker (NMWM, Banglore Genei) to determine the molecular mass of the proteases showing activity in the gel. The gel was recorded using gel documentation system (Alpha-Innotech, USA).

4.3 Determination of specific protease activity

Specific protease activity (U/mg protein) of selected bacterial isolates *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 was measured under optimum environmental conditions viz. pH, temperature, salinity, carbon and nitrogen source.

RESULTS AND DISCUSSION

4.4 Partial purification of protease enzyme

4.4.1 Ammonium sulphate precipitation

Although various ammonium sulphate saturation levels (10-80 %) were checked for protein precipitation using cell free supernatant, 70 % saturation was responsible for highest level of precipitation and alkaline protease activity. Enzyme activity of partially purified protease from *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 was 152 U/ml, 212 U/ml and 179 U/ml respectively. Significant purification of protease enzyme was achieved using ammonium sulphate precipitation method since 3.61 fold and 3.16 fold purification was possible in case of *Bacillus altitudinis* strain BR1and *Bacillus firmus* strain CW2 , whereas 2.8 fold purification was achieved for *Bacillus cereus* strain CS1(Table 4.1). Similarly 2.76, 2.4, 1.2 fold purification was achieved using ammonium sulphate precipitation of alkaline protease from *Lactobacillus brevis*, *Bacillus* sp. B001 and *Bacillus subtilis* PE-11(Femi-Ola and Oladokun, 2012; Deng *et al.,* 2010; Adinarayana *et al*., 2003) whereas 4.1 and 4 fold purification was reported for protease obtained from *Aspergillus* sp. strain KH17 and *Bacillus subtilis* (Palanivel *et al*., 2013; Yang *et al*., 2000).

Partial purification also resulted in 1.55 %, 1.46 % and 1.47 % recovery of protease enzyme for *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus*

firmus strain CW2 respectively (Table 4.1). Further purification of protease using column chromatography will certainly result in highly purified enzyme.

4.4.2 SDS-PAGE and Zymography analysis

Resolution of protease enzyme by SDS-PAGE has a limitation because some of them do not renature and hence can't be detected following treatment with SDS. Therefore zymography is done which is a two-stage technique involving protein separation on Native-PAGE followed by detection of enzyme activity in the gel.

The partially purified enzyme was detected and characterized by SDS-PAGE, Native-PAGE and zymography. *Bacillus altitudinis* strain BR1 clearly revealed presence of five distinct bands in the gels confirming presence of five protease isozymes with molecular weight of 17, 22, 43, 64 and 88 kDa respectively when compared with Native-PAGE protein marker (Fig. 4.1a). Similarly three protease isozymes with molecular weight of 20, 35 and 45 kDa were reported earlier in *Bacillus subtilis* isolated from midgut of fresh water fish (Geetanjali and Subhash, 2011).

Native-PAGE and zymographic analysis of coastal sediment isolate *Bacillus cereus* strain CS1 revealed presence of two distinct bands in the gels confirming presence of two protease isozymes with molecular weight of 210 and 215 kDa respectively when compared with Native-PAGE marker protein marker (Fig. 4.1b).

Coastal isolate *Bacillus firmus* strain CW2 on Native-PAGE and zymographic analysis clearly revealed presence of three distinct bands showing proteolytic activity indicating three protease isozymes with molecular weight of 17, 67 and 212 kDa respectively when compared with Native-PAGE protein marker (Fig. 4.1c). Therefore it is interesting to note that the halotolerant alkaliphilic bacterial isolates

viz. *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 possess alkaline proteases with different molecular weight.

Alkaline protease isozymes are commonly present in microorganisms e.g. *Bacillus* sp. GUS1 possessed three alkaline protease isozymes with molecular mass ranging from 30 to 47 kDa (Seifzadeh *et al*., 2008), *Pseudoalteromonas* sp. strain P96-47 isolated from King George island, Antarctica showed five metalloprotease isozymes with molecular mass 30.5, 40, 41, 42.5 and 52 kDa (Vazquez *et al*., 2008) and two protease isozymes with molecular mass 18 and 66 kDa have been reported from *Bacillus* sp. Y (Mala and Srividya, 2010).These studies clearly demonstrated diversity in the size of protease isozymes which is also evident from our results (Fig. 4.1 a).

The molecular mass of alkaline proteases generally ranges between 15 - 36 kDa with a few exceptions of high molecular mass proteases e.g. 42 kDa in *Bacillus* sp. PS719 (Hutadilok- Towatana, 1999) and 90 kDa in *Bacillus subtilis* (Kato *et al*., 1992).

4.4.3 Specific activity of alkaline proteases of selected bacterial isolates

Partially purified protease from selected bacterial isolates i.e. *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* CW2 showed specific activity of 1.82, 3.16 and 1.43 U/mg protein respectively (Fig. 4.2). Among the three bacterial isolates highest specific activity was recorded for isolate *Bacillus cereus* strain CS1. Similar results have also been reported for *Bacillus subtilis* and *Bacillus circulans* BM15 where specific activity of partially purified protease was 3.88 and 5.57 U/mg protein respectively (Yang *et al*., 2000; Venugopal and Saramma, 2007). Whereas high specific activity of 12.1 and 10.02 was also reported

of partially purified alkaline protease from *Bacillus subtilis* PE-11 and *Lactobacillus brevis* (Adinarayana *et al*., 2003; Femi-Ola and Oladokun, 2012).

Therefore the coastal sediment bacterial isolate, *Bacillus cereus* strain CS1 was selected for further characterization to study enzyme stability, compatibility and efficacy as detergent additive.

Table 4.1 –Alkaline protease activity (production), specific activity, level of purification and percent recovery of enzyme activity for selected bacterial strains

Purification step	Bacterial isolate	Enzyme activity (U/ml)	Total activity (U)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Purification fold	% recovery of activity
Cell free supernatant (500 ml)	***Bacillus altitudinis* strain BR1**	98.02	49010	55.31	27655	1.772	-	-
Ammonium sulphate (70 % saturation) precipitation followed by dialysis (5 ml)		152	760	23.7	118.5	6.41	3.61	1.55
Cell free supernatant (500 ml)	***Bacillus cereus* strain CS1**	145	72500	35.63	17815	4.069	-	-
Ammonium sulphate (70 % saturation) precipitation followed by dialysis (5 ml)		212	1060	18.8	94	11.2	2.8	1.462
Cell free supernatant (500 ml)	***Bacillus firmus* strain CW2**	122	61000	80.41	40205	1.52	-	-
Ammonium sulphate (70 % saturation) precipitation followed by dialysis (5 ml)		179	895	37.34	186.7	4.8	3.16	1.47

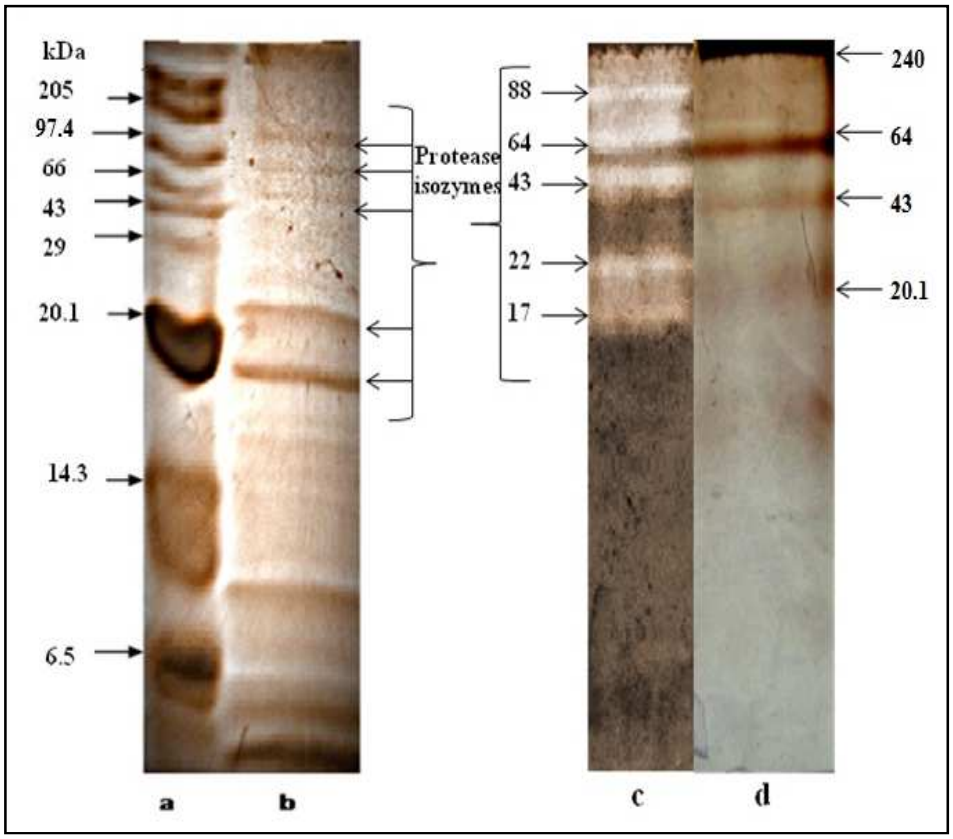


Fig. 4.1a SDS-PAGE and Zymogram analysis of *Bacillus altitudinis* strain BR1

a- SDS-PAGE marker

b- SDS-PAGE of partially purified enzyme sample

c- Zymogram of partially purified enzyme sample

d- Native-PAGE marker

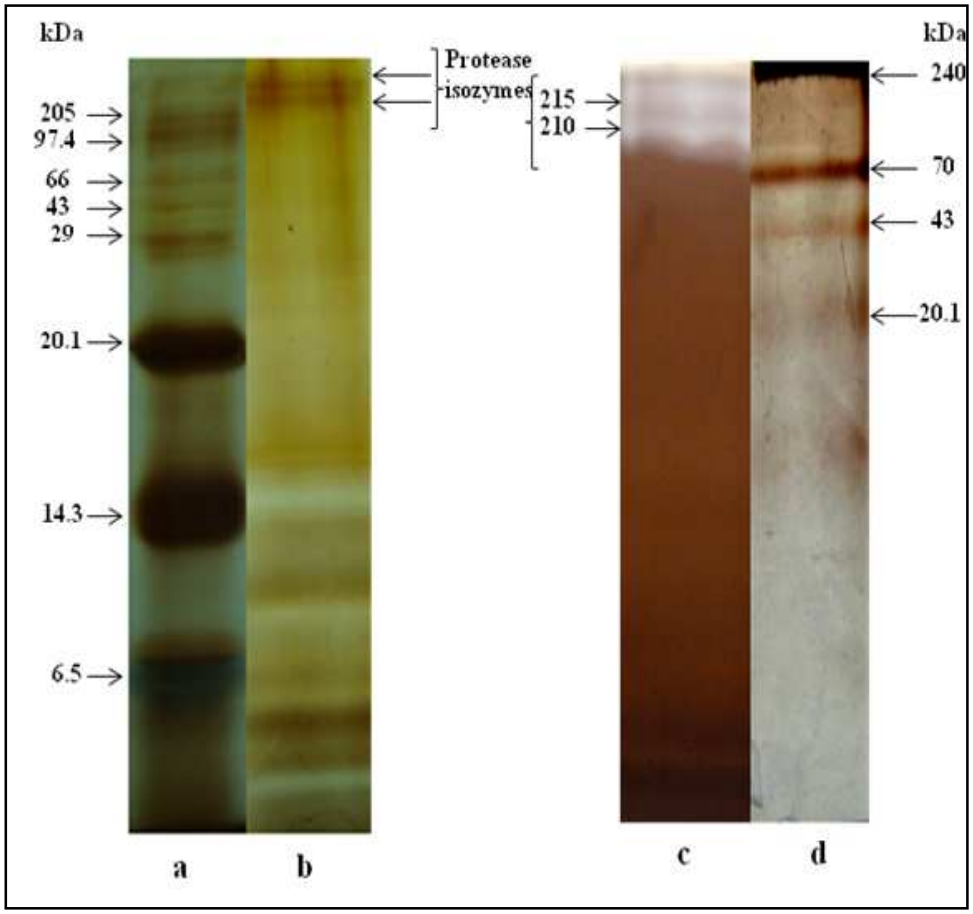


Fig. 4.1b SDS-PAGE and Zymogram analysis of *Bacillus cereus* strain CS1

a- SDS-PAGE marker

b- SDS-PAGE of partially purified enzyme sample

c- Zymogram of partially purified enzyme sample

d- Native-PAGE marker

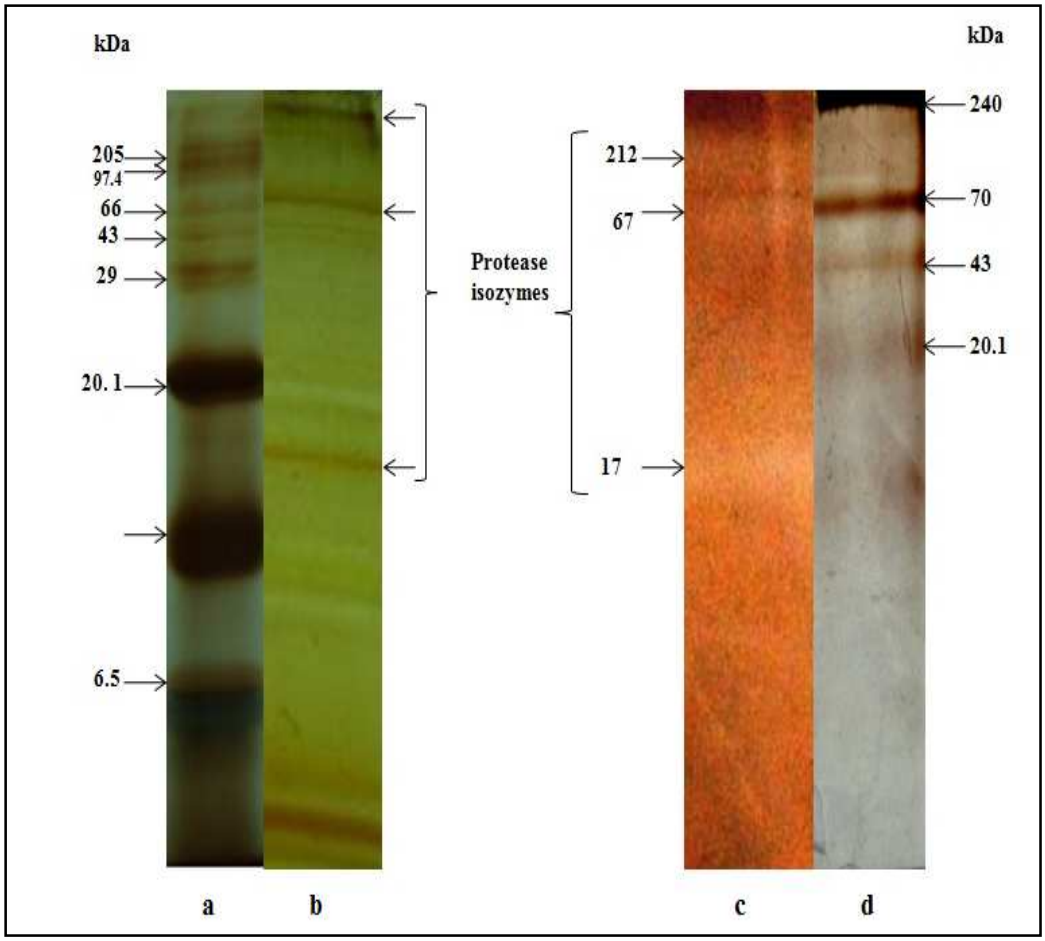


Fig. 4.1c SDS-PAGE and Zymogram analysis of *Bacillus firmus* strain CW2

a- SDS-PAGE marker

b- SDS-PAGE of partially purified enzyme sample

c- Zymogram of partially purified enzyme sample

d- Native-PAGE marker

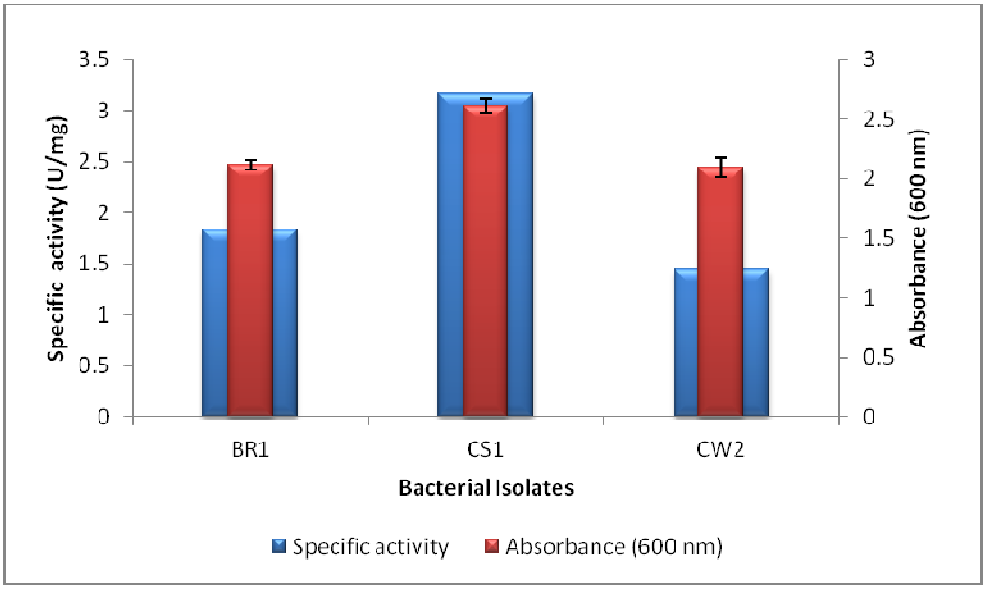


Fig. 4.2 Specific protease activity of selected bacterial isolates

CHAPTER V

Studies on protease stability, compatibility and application as detergent additive

Chapter V

Studies on protease stability, compatibility and application as detergent additive

Microbial alkaline proteases dominate commercial applications with a major share of market being occupied by subtilisins and/or alkaline proteases from *Bacillus* spp. for laundry detergent applications due to their high stability and activity over a broad range of temperature and pH (Ward, 1985). Other parameters involved in the selection of a good detergent protease includes compatibility and stability with detergent components e.g. surfactants, sequestering agents and bleaches (Bech *et al*., 1993; Kumar *et al*., 1998), good activity at washing pH and temperature (Aehle *et al*., 1993; Oberoi *et al*., 2001), compatibility with the ionic strength of the detergent solution, stain degradation and removal potential, stability and shelf life (Showell, 1999). Over the period of years, the proteases in detergents have changed from being minor additives to being the key ingredients. There is always a need to explore for newer enzymes with novel properties that can further enhance the wash performance of currently available enzyme-based detergents.

Since the coastal sediment isolate, *Bacillus cereus* strain CS1 demonstrated highest specific activity (i.e. 3.16 U/mg protein) as compared to other two isolates, it was selected for further characterization to study enzyme stability, compatibility and efficacy as detergent additive.

MATERIALS AND METHODS

5.1 Stability and compatibility of protease enzyme in presence of various detergent additives and detergents

5.1.1 Assessment of stability of partially purified protease enzyme in presence of various surfactants and oxidants

Different levels of various surfactants viz. SDS (0.05 %, 0.1 %, 0.2 % and 0.4 %), Tween- 20 (1 %, 2 % and 4 %), Triton X-100 (0.1 %, 0.4 %, 0.7 % and 1 %) and oxidizing agent i.e H_2O_2 (1 %, 2 %, 3 % and 4 %) were tested to check stability of partially purified protease enzyme of *Bacillus cereus* strain CS1 in order to evaluate its suitability and effectiveness as a detergent additive. 100 µl of these surfactants and oxidants were pre-incubated separately with 100 µl of partially purified protease enzyme sample followed by incubation for 30 mins at 37 ^{0}C. Control was also taken simultaneously in the absence of surfactants and oxidants. Protease activity of these samples was measured by standard Kunitz assay (Kunitz, 1947).

5.1.2 Determination of compatibility and stability of protease enzyme in presence of various laundry detergents

The compatibility and stability of the protease enzyme with few common laundry detergents viz. Ariel® (Procter and Gamble, India), Henko® (Henkel Spic India Ltd., India), Surf Excel® & Wheel® (Hindustan Lever Ltd, India) and Rin® (Hindustan Unilever Ltd, India) was assessed following standard procedure of Mukherjee *et al*., 2008 with slight modifications. Partially purified protease sample (100 µl) from *Bacillus cereus* strain CS1 was mixed with 0.7 mg/ml of various detergents (containing heat inactivated indigenous protease) and the final volume of 1.0 ml was adjusted using milli Q ultra pure water. The sample containing detergent

and partially purified enzyme was incubated at 37 ^{0}C for 1 hr and the enzyme activity was determined by standard assay of Kunitz (Kunitz, 1947). The results were compared with the control (partially purified protease enzyme diluted to 1.0 ml with 100mM Tris-HCl, pH 9). The relative protease enzyme activity was expressed as percent activity considering control as 100 % (Mukherjee *et al*., 2008). The experiment was carried out in triplicates and the values were expressed as mean± SD.

5.2 Assessment of efficacy of partially purified enzyme preparation as a detergent additive

In order to assess the efficacy of partially purified protease of *Bacillus cereus* strain CS1 as a detergent additive, procedure of Kanmani *et al*., 2011 was followed. White cotton pieces (5×5 cm) stained with blood and fish curry were taken in separate flasks and the following sets were prepared and studied:

1. Flask with distilled water (100ml) + blood stained cloth/ fish curry stained cloth
2. Flask with distilled water (100ml) + blood stained cloth/fish curry stained cloth + 1ml (7.0 mg/ml) detergent
3. Flask with distilled water (100ml) + blood stained cloth/fish curry stained cloth + 1ml (7.0 mg/ml) detergent + 1ml enzyme preparation

The above flasks were incubated at 37 ^{0}C for 15 mins. After incubation, cloth pieces were taken out, rinsed with water and dried. Untreated cloth pieces stained with blood and fish curry were taken as control.

RESULTS AND DISCUSSION

5.3 Stability and compatibility of partially purified protease enzyme in presence of various surfactants and oxidants

5.3.1 Stability of protease enzyme in presence of surfactants and oxidants

Laundry detergents contain several surfactants, bleaching agents, oxidants, builders, fabric softners, fillers, minor additives and several enzymes (i.e. amylase, protease, lipase, esterase etc). Surfactants are compounds which lower the surface tension of a liquid when used in low concentration in a detergent. They play an important role in cleansing, wetting, dispersing, emulsifying and anti-foaming activity. e.g. SDS, H_2O_2 and Triton- X100. Surfactants and oxidants make up 50 % of an enzyme-based detergent. The purpose of using surfactants is to soften the water so that it can wet the fibres and cloth surfaces, loosen and encapsulate the dirt and prevent redeposition of dirt on the cloth surface. Whereas bleaching agents such as H_2O_2, Sodium hypochlorite, Per-borates (Sodium perborate tetrahydrate) and Per-carbonates bleach the natural substances present in cotton fibres and cellulose fibres which make the fabric yellowish.

Proteases and other enzymes used in detergent formulations should be stable with various detergent components along with oxidizing and sequestering agents. Broad substrate specificity is a desirable character for enzyme based detergents. When checked for stability of protease enzyme in the presence of various detergent additives, it was found that the activity increased upto 96 % after incubation with 0.1 % Triton-X 100 for 30 mins at 37 ^{0}C. While retained 95 % and 83 % of the residual activity with 0.2 % SDS and 4 % H_2O_2 respectively (Fig. 5.1a). Protease enzyme

from *Bacillus cereus* strain CS1 is more effective and stable in presence of H_2O_2 (bleaching agent) as compared to protease enzyme reported by Manachini *et al*., 1988 which showed only 70 % residual enzyme activity in presence of 3 % H_2O_2 after 1 hour at 40 ^{0}C. Interestingly we have observed 83 % residual activity of protease in presence of H_2O_2 (Fig. 5.1a).

There are only few reports on H_2O_2 stable protease enzymes (Joo *et al*., 2003). Alkaline protease of *Bacillus* sp. RGR-14 which is a soil isolate retained 60 % residual activity in the presence of 1 % H_2O_2 (Oberoi *et al*., 2001) and protease of *Bacillus circulans* BM15 which is a mangrove isolate retained 70 % protease enzyme activity in presence of 0.05 % of H_2O_2 and 56 % in 0.4 % SDS respectively (Venugopal and Saramma, 2007). Proteases produced by *Oerskovia xanthineolytica* TK- 1 and *Streptomyces* sp. YSA-130 was also inhibited by 0.1 % SDS (Sheki *et al*., 1994; Yum *et al*., 1994).

In presence of 4 % Tween-20, protease of *Bacillus cereus* strain CS1 retained 50 % of its residual activity. Similar reports on protease stability have also been reported by alkaline protease secreted by *Bacillus* sp. RGR-14, *Bacillus megatarium* and *Bacillus* sp. S17110 retaining 35 %, 71 % and 73 % and 35 % of its residual activity in the presence of 1 %, 100 mM and 1 % Tween-20 (Oberoi *et al*., 2001; Sumathi *et al*., 2012 and Jung *et al*., 2007).

5.3.2 Stability of protease enzyme in presence of various commercially available detergents

Commercial available common laundry detergents contain anionic surfactants, bleaching agents and water softening agents, which may influence the stability of protease enzyme present. A pre-requisite for any substance to be used in the detergent formulation is its stability and compatibility in the presence of various

detergent components (Maurer, 2004; Stoner *et al*., 2004). Some of the components of the detergents e.g. ethoxylated surfactants, non-ionic co-polymeric builders and sucrose may have a stimulatory effect on protease activity (Anwar and Saleemuddin, 1998; Rai *et al*., 2010). Ideally, proteases or other hydrolytic enzymes to be used in detergent formulations should be effective at low levels ranging from 0.4 % to 0.8 % (Kumar and Takagi, 1999; Kumar *et al*., 2004).

The protease of *Bacillus cereus* strain CS1 when tested with common laundry detergents demonstrated promising results with reference to stability and compatibility. Interestingly the enzyme activity was retained 98 % and 87 % in presence of Rin® and Wheel® at 37 ^{0}C (Fig. 5.1b). Whereas 64 %, 62 % and 59 % residual enzyme activity was retained activity in presence of Surf Excel®, Ariel® and Henko®. Similar findings showing stability of protease isolated from thermophilic strain *Paenibacillus tezpurensis* sp. nov. AS-S24-II and *Bacillus* sp. Y showing 70-80 % residual activity at room temperature in presence of commercially available detergents have also been reported (Rai *et al*., 2010; Mala and Srividya, 2010).

Whereas alkaline protease from *Bacillus licheniformis* KBDL4 retained 97 %, 95 % and 92 % of its original activity in the presence of laundry detergents i.e. Surf Excel, Nirma and Tide respectively at 40 ^{0}C, pH 10 (Pathak and Deshmukh, 2012) and SDS-stable alkaline protease from *Bacillus* sp. RGR-14 retained more than 70 % relative activity for 1 hr at 25 ^{0}C in presence of various commercial detergents (Oberoi *et al*., 2001).

5.4 Efficacy of partially purified protease as detergent additive

Partially purified enzyme preparation of *Bacillus cereus* strain CS1 when used as detergent additive with Rin as well as Wheel significantly improved their cleansing efficiency since blood and fish curry stains on the cotton cloth fabric disappeared within 15 mins (Fig. 5.2).

Similar findings have been reported for protease from *Bacillus licheniformis* strain N-2 which indicated blood stain removing capability (Nadeem *et al*., 2008). Wash performance analysis of grass and blood stains on cotton fabric also showed an increase in reflectance (14 and 25 % with grass and blood stains, respectively) after enzyme treatment. However, enzyme in conjunction with detergent proved to be the best (Oberoi *et al*., 2001). Similarly protease from alkaliphilic bacteria and *Pseudomonas aeruginosa* strain PD100 also possessed capability to remove blood stain from the cotton cloth in the absence of detergents (Najafi *et al*., 2005; Kanekar *et al*., 2002; and Banerjee *et al*., 1999). Whereas protein stains (i.e. egg yolk) were removed within 10 mins from cotton fabric in 100 U/ml protease enzyme from *Bacillus* sp. APR-4 at pH 9.0 with 1 % detergent and it took 30 mins to remove blood stains (Kumar and Bhalla, 2003).

Thus protease produced by *Bacillus cereus* strain CS1 is a valuable and potential candidate in detergent industry as detergent additive where alkaline conditions, higher processing temperatures and certain additives containing metal ions are necessary to boost the effectiveness of detergents. The supplementation of the enzyme preparation in two detergents i.e. Rin and Wheel could significantly improve the cleansing performance towards the blood and fish curry stains (Fig. 5.2).

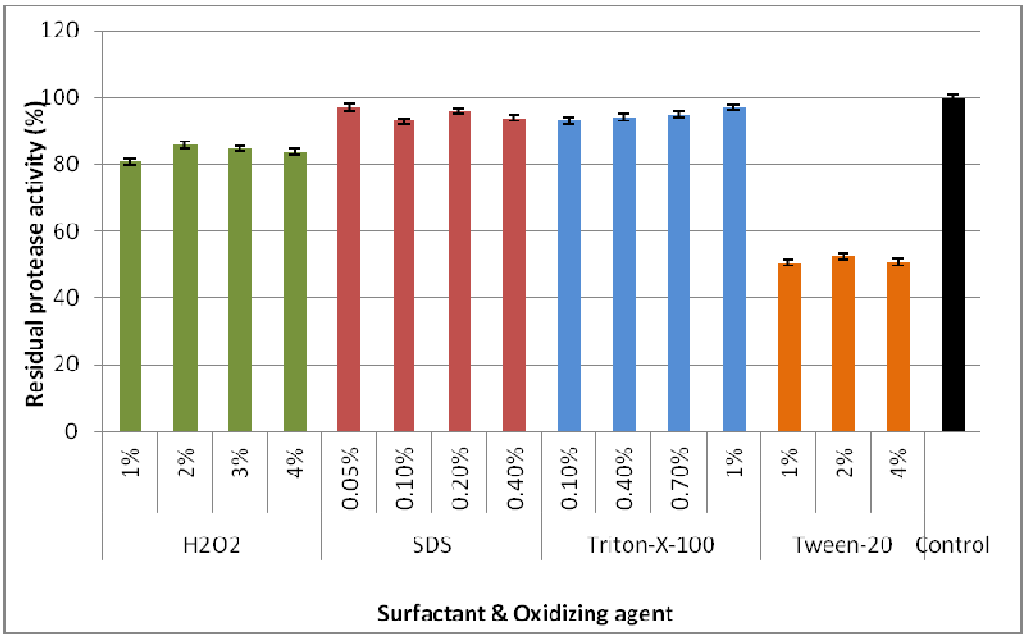


Fig. 5.1a Effect of surfactants and oxidizing agents on the residual protease activity (%) of *Bacillus cereus* strain CS1

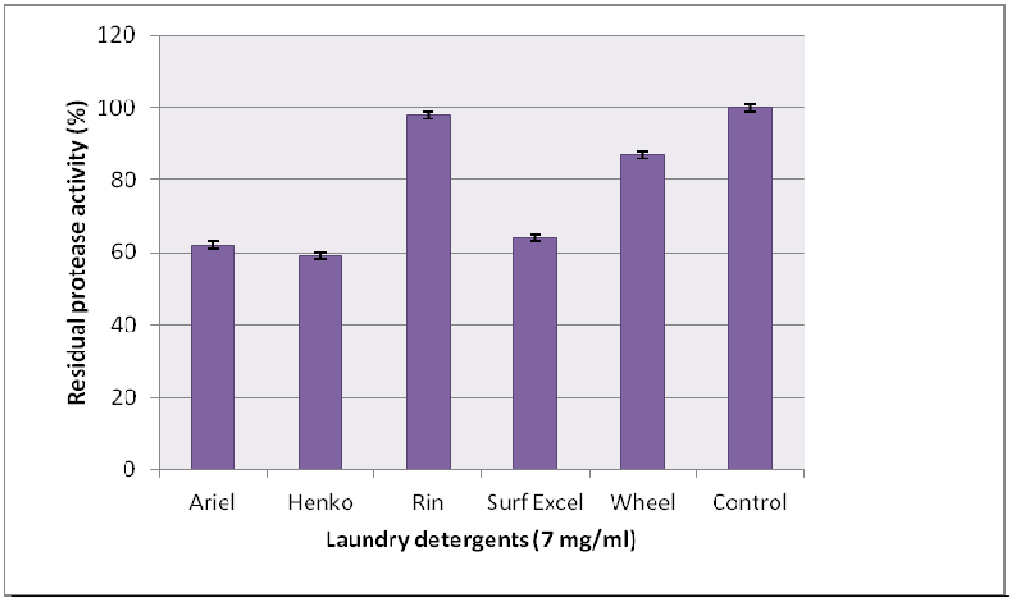


Fig. 5.1b Effect of laundry detergents on residual protease activity (%) of *Bacillus cereus* strain CS1

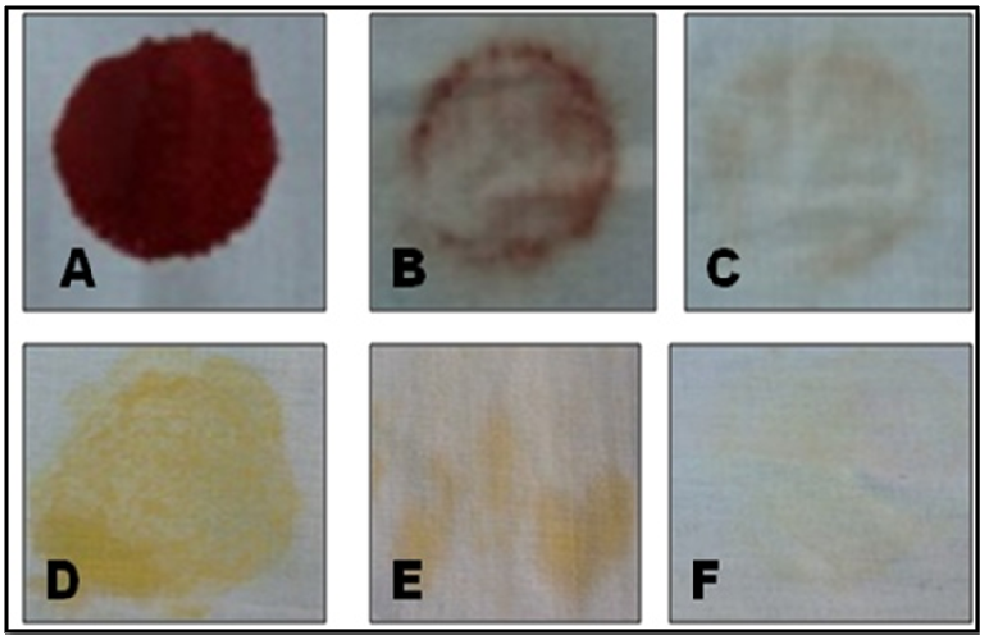


Fig. 5.2 Washing test to assess activity of partially purified alkaline protease preparation from *Bacillus cereus* strain CS1 as a detergent additive (A) Cloth stained with blood; (B) Blood-stained cloth washed with detergent (Rin) only; (C) Blood-stained cloth washed with detergent (Rin) supplemented with enzyme preparation; (D) Cloth stained with fish curry; (E) Fish curry stained cloth washed only with detergent (Wheel); (F) Fish curry stained cloth washed with detergent (Wheel) supplemented with enzyme preparation.

SUMMARY

SUMMARY

- Soil and water samples were collected from various mangroves, estuarine and coastal sites of Goa and their physicochemical characteristics viz. pH, temperature and salinity were determined. Alkaline protease producing colonies were selectively isolated and purified and their further characterization was carried out. Among various sampling sites coastal sediment samples gave rise to highest number of alkaline protease positive colonies indicating that these microbes use these enzymes to degrade the protein rich organic waste.

- Out of 64 protease positive colonies from different sampling sites, 24 morphologically different colonies were selected. Among theses 11 potential protease producing isolates were identified based on morphological and biochemical characteristics following Bergey's Manual of Systematic Bacteriology. Based on proteolytic efficiency of these 11 isolates best three isolates were selected for identification by 16S rRNA sequencing and BLAST search, and they were identified as *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2. 16S rRNA data was submitted to Genbank and accession numbers were received. Phylogenetic analysis of all these three potential protease producing isolates revealed that they were closely related to *Bacillus* sp.

- These three isolates were further characterized based on their growth and protease production with reference to various environmental optimas viz. pH, temperature, salinity, carbon and nitrogen sources. All three isolates i.e. *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 showed active growth and enzyme activity (U/ml) in the alkaline pH range of (9-11) confirming that they were **alkaliphiles.** *Bacillus altitudinis* strain BR1 and *Bacillus cereus* strain CS1 showed maximum protease activity (i.e. 92 U/ml and 125 U/ml) at pH 9.0 whereas *Bacillus firmus* CW2 showed maximum activity (i.e. 145 U/ml) at pH 10 respectively.

- Study related with optimum temperature for growth and protease activity revealed that all three isolates grew best at 37 ^{0}C. Optimum temperature for maximum protease activity was 37 ^{0}C for *Bacillus cereus* strain CS1 (i.e. 142 U/ml) and *Bacillus firmus* strain CW2 (i.e. 121 U/ml). Cultures incubated at 50 ^{0}C showed maximum enzyme activity for *Bacillus altitudinis* strain BR1 (i.e. 98 U/ml).

- When tested optimum salinity for growth and enzyme activity, *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 showed growth and enzyme activity in presence of NaCl in the range of 0 – 4 % indicating that they were **halotolerant.** Optimum NaCl was 2 % to induce maximum enzyme activity for *Bacillus altitudinis* strain BR1 (i.e. 86 U/ml) and *Bacillus cereus* strain CS1(i.e. 91 U/ml), whereas 4 % NaCl was optimum for *Bacillus firmus* strain CW2 (i.e. 96 U/ml) respectively.

The halotolerance of the enzymes may play an important role in survival of a halotolerant bacterial strain in moderately saline environment.

- It was observed that carbon sources did not influence the enzyme activity much, but nitrogen sources had a significant effect on the growth and enzyme activity. Among various carbon sources, fructose (1 %) was observed best carbon source for *Bacillus altitudinis* strain BR1 for maximum protease activity (i.e. 70 U/ml) whereas *Bacillus cereus* strain CS1 showed 92 U/ml protease activity and *Bacillus firmus* strain CW2 showed 66 U/ml enzyme activity in presence of glucose (1 %) which clearly indicated it to be the best carbon source.

- Among various nitrogen sources tested, inorganic nitrogen viz. $NH_4(NO_3)_2$ and KNO_3 adversely affected the growth and enzyme activity of all three isolates. Among various organic nitrogen sources, peptone was found best organic nitrogen source for growth and protease activity (i.e. 101 U/ml) of *Bacillus altitudinis* strain BR1, whereas tryptone was preferred nitrogen source for maximum enzyme activity in *Bacillus cereus* strain CS1(i.e. 158 U/ml). Similarly casein was best nitrogen source for *Bacillus firmus* strain CW2 for enzyme activity (i.e. 115 U/ml).

- When tested for effect of metal ions and protease inhibitors on enzyme activity of the above selected isolates, Ca^{+2} and Cu^{+2} ions (1 mM) enhanced the residual enzyme activity of *Bacillus altitudinis* strain BR1 by 6 % and 3 % respectively. Whereas 7 % increase in residual enzyme activity of *Bacillus*

cereus strain CS1 was observed in presence of 5 mM Ca^{+2} ions. Residual enzyme activity of *Bacillus firmus* strain CW2 was significantly enhanced by 57 % above control in presence of Cu^{+2} ions (5 mM). All three selected bacterial strains showed significant inhibition of enzyme activity in presence of Pb^{+2} ions as 13 % residual enzyme activity was observed for *Bacillus cereus* strain CS1 at 5 mM Pb^{+2}. Whereas (1 mM) Hg^{+2} caused significant repression of protease activity i.e. 7 % residual activity was recorded for *Bacillus firmus* strain CW2. Significant repression of protease may be attributed to high lead toxicity in case of Pb^{+2} ions and binding of Hg^{+2} ions with –SH groups of enzyme.

- Enzyme inactivation studies were conducted to provide an insight into the nature of the protease enzyme and their active sites. Effect of various protease inhibitors viz. DTT, PMSF, Na_2-EDTA, SDS and β-ME on activity of proteases from all three selected isolates revealed coastal isolates *Bacillus cereus* strain CS1and *Bacillus firmus* strain CW2 that showed significant repression of protease (i.e. 21 % residual protease activity) as compared to in presence of Na_2-EDTA. This clearly demonstrated that they are **metalloproteases**. While *Bacillus altitudinis* strain BR1 in presence of PMSF (1 mM) showed only 40 % residual protease activity which clearly confirmed it to be a **serine protease** since PMSF represses activity through sulphonation of the serine residues present in the active sites of protease. *Bacillus altitudinis* strain BR1 showed interestingly 82 % residual protease activity in presence of Na_2-EDTA indicating its stability in presence of this chelating agent. This characteristic of this serine protease makes it a valuable

detergent additive since detergents contain high amounts of chelating agents which act as softening agents and stain removers.

- Proteolytic activity of *Bacillus firmus* strain CW2 when assessed in presence of different protein substrates viz. BSA, Gelatin and Casein at 0.5 % level clearly revealed that gelatin was hydrolyzed more efficiently as enzyme activity was 158 U/ml compared to BSA (i.e. 83 U/ml). These results confirm that gelatin is a preferred protein substrate for protease of *Bacillus firmus* strain CW2 and due to its ability to use gelatin as a substrate, the enzyme from *Bacillus firmus* strain CW2 can be effectively used in recovery of silver from photographic films.

- Partial purification of protease enzyme of *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 was carried out by ammonium sulphate precipitation followed by dialysis. **70 % ammonium sulphate saturation** for highest level of precipitation and alkaline protease activity. Enzyme activity of partially purified protease from *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* strain CW2 was 152 U/ml, 212 U/ml and 179 U/ml respectively. Significant purification of protease enzyme was achieved using ammonium sulphate precipitation method since 3.61 fold and 3.16 fold purification was possible in case of *Bacillus altitudinis* strain BR1 and *Bacillus firmus* strain CW2 , whereas 2.8 fold purification was achieved for *Bacillus cereus* strain CS1. Partial purification also resulted in 1.55 %, 1.46 % and 1.47 % recovery of enzyme for *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and

Bacillus firmus strain CW2 respectively. Further purification of protease using column chromatography will certainly result in highly purified enzyme.

- Biochemical characterization of protease produced by three potential bacterial strains was carried out using SDS-PAGE & silver staining as well as Native-PAGE followed by **zymography**. Native-PAGE and zymographic analysis clearly revealed that *Bacillus altitudinis* strain BR1 possessed five distinct bands in the gel confirming presence of five protease isozymes with molecular weight of 17, 22, 43, 64 and 88 kDa respectively, whereas coastal sediment isolate *Bacillus cereus* strain CS1 clearly showed presence of two distinct bands in the gel confirming presence of two protease isozymes with molecular weight of 210 and 215 kDa. Whereas *Bacillus firmus* strain CW2 revealed presence of three distinct bands showing proteolytic activity indicating three protease isozymes with molecular weight of 17, 67 and 212 kDa when compared with Native-PAGE protein marker.

- **Specific activity** (U/mg of protein) of the partially purified protease of *Bacillus altitudinis* strain BR1, *Bacillus cereus* strain CS1 and *Bacillus firmus* CW2 was recorded as 1.82, 3.16 and 1.43 U/mg protein respectively. Among these three bacterial isolates highest specific activity was recorded for coastal sediment isolate *Bacillus cereus* strain CS1. Therefore this potential isolate was selected for further characterization with reference to enzyme stability, compatibility and efficacy as detergent additive.

- **Stability** and **compatibility** of protease enzyme of *Bacillus cereus* strain CS1 was also studied in presence of various surfactants, oxidants and laundry detergents. It was interesting to note that protease activity increased upto 96 %, 95 % and 83 % in presence of Triton-X 100 (0.1 %), SDS (0.2 %) and H_2O_2 (4 %) respectively for 30 mins at 37 ^{0}C. Therefore this protease was found more effective and stable in presence of H_2O_2 as a bleaching agent which is one of the ingredients used in commonly used detergents.

- Protease when tested with common laundry detergents demonstrated promising results with reference to stability and compatibility. Interestingly the enzyme activity was retained 98 % and 87 % in presence of Rin® and Wheel® at 37 ^{0}C. Whereas residual activity was 64 %, 62 % and 59 % respectively in presence of Surf Excel®, Ariel® and Henko®.

- **Efficacy** of partially purified enzyme of *Bacillus cereus* strain CS1 as a detergent additive was also assessed which clearly revealed that laundry detergents (i.e. Rin & Wheel) significantly improved their cleansing efficiency when mixed with partially purified protease of *Bacillus cereus* strain CS1. It was noticed that blood and fish curry stains on the cotton cloth fabric disappeared within 15 mins of application of mixture of detergents with protease. Therefore protease produced by *Bacillus cereus* strain CS1 is a valuable and **potential candidate in detergent industry** as detergent **additive.**

APPENDIX

APPENDIX

APPENDIX- A

A. Media Composition

A.1 5 % Skim Milk Agar for 1L:

Skim milk	50 ml
Nutrient broth	950 ml
Agar	15 gm

A.2 Nutrient Agar (Himedia) for 1L:

Peptone	10 g
Beef extract	3.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml
Agar	2 gm

(Final pH 7.2- 7.4)

Dissolve the components in distilled water and make volume upto 1000 ml with distilled water. Adjust pH to desired pH with 0.1 N NaOH. Sterilize by autoclaving at 121 ^{0}C at 15 p.s.i for 20 mins.

A.3 Muller Hinton Agar

Commercially available Muller Hinton agar (Himedia, India) was used.

A.4 Horikoshi- I medium (Horikoshi, 1996)

Glucose	10 gm
Yeast extract	5 gm
Peptone	5 gm
KH_2PO_4	1 gm
$MgSO_4$	0.2 gm
Na_2CO_3	10 gm
d/w	1000 ml

A.5 Mac Conkey's agar (Himedia)

Peptone	20 gm
Sodium taurocholate	5.0 gm
Lactose (10 %)	100 ml
2 % Neutral red	3.5 ml
Agar	25 gm
d/w	1000 ml
Final pH	7.5

APPENDIX- B

B. Composition of stains, buffers and reagents

B.1 Stains

i) Gram stain reagents

Crystal violet

Solution A- 2 gm of crystal violet in 20 ml ethanol

Solution B- 0.8 gm ammonium oxalate dissolved in 80 ml d/w

Mixed solution A and B and filter through Whatman filter paper No. 1.

Gram's Iodine

Dissolve 1gm iodine and 2 gm Potassium iodide in 300 ml d/w. Filtered through Whatman filter paper No. 1(diameter =12.5 cm).

Saffranine

2.5 gm Saffranine was dissolved in 10 ml ethanol and make up the volume to 100 ml with d/w and filter through Whatman filter No.1.

Procedure for gram staining

Prepare smear of the organism on a clean glass slide and heat fixed it. Flood the smear with crystal violet for 1.5 mins and gently wash with running tap water, then flood smear with Gram's iodine for a minute. Gently washed with tap water and decolourized with 90 % ethanol prepared in d/w till colour oozes out. Counter stained with saffranine for 1 min. Washed with tap water, air dry the slide and then put a drop of oil on smear to examine under oil immersion lens of microscope.

ii) Endospore stain reagents

B.2 Buffers

i) Sodium carbonate buffer (0.05 M), pH 10

Molecular weight of Na_2CO_3 - 105.99,

Dissolve 52.995 gms of Na_2CO_3 in d/w and make the volume to 1 L with d/w.

APPENDIX-C

C.1 Biochemical media used for identification.

i) Sugar fermentation medium

Peptone	5.0 gm
Sugar	1 gm
Sodium chloride	5.0 gm
Phenol red	0.01 gm
pH	7.0
d/w	make volume to 1 L

Media with Durham's tube was autoclaved at 15 psi for 20 mins. Tubes inoculated and incubated at room temperature for 24- 48 hrs. Change in colour from pink to yellow indicated sugar fermentation and presence or absence of gas was noted. Uninoculated tube served as control.

ii) Nitrate reduction test

Nitrate Broth

Peptone	5 gm
Beef extract	3 gm
KNO_3	1 gm
NaCl	5 gm
pH	7.0
d/w	make volume to 1 L

Nitrate broth was inoculated and incubated at room temperature for 24 hours. After incubation, 5 drops of sulphanilic acid and 5 drops of α- naphthylamine were added. Red coloration indicated a positive test while in a negative test, red coloration is observed after addition of 5 mg of zinc. Uninoculated tubes served as the control.

iii) Citrate utilization test

Simon's citrate agar

Ammonium dihydrogen phosphate	1 gm
Diammonium phosphate	1 gm
Sodium chloride	5 gm
Magnesium sulphate	0.2 gm
Sodium citrate	5 gm
Bromothymol blue	0.3 gm
d/w	make volume to 1 L
Agar	20 gm
pH	7.0

Inoculate Simon's citrate slant and incubate for 24- 48 hrs at room temperature. Citrate utilization is indicated by presence of growth and media becomes dark blue colour.

iii) Catalase test

3 or 4 drops of 3 % (v/v) hydrogen peroxide was mixed with a loop full of culture in a plate. Evolution of gas bubbles caused by liberation of free oxygen was indicative of catalase positive organisms.

iv) Oxidase test

A filter paper strip was soaked in tetramethyl-p-phenylenediamine dihydrochloride (TMPD) dye. A loop full of fresh bacterial culture was smeared on the moist filter paper. Production of deep purple colour in 5- 10 seconds indicated a positive oxidase test. This dye act as electron acceptor and get reduced.

v) Gelatin liquefaction

Nutrient gelatin

Peptone	5 gm
Beef extract	3 gm
Gelatin	20 gm
pH	7.0
d/w	make volume to 1L

Inoculate tubes and incubate at room temperature for 24 hrs, the tubes were refrigerated for 30 mins and the medium was observed. Liquid medium after refrigeration indicated positive test.

vi) Starch hydrolysis

Starch agar medium

Peptone	5 gm
Beef extract	3 gm
Soluble starch	2 gm
Agar	20 gm
pH	7.0
d/w	make volume to 1L

Inoculate starch agar plates by spot inoculation. Incubate the plates at room temperature for 24- 48 hrs then flood the plates with Gram's iodine for 1 min and pour off the excess stain. Clear zone around the colony indicated positive test and rest of the plate appear blue.

vii) Casein hydrolysis

Inoculate milk agar plates (milk added to nutrient agar) and incubate at room temperature for 24- 48 hrs then examine the plates for the presence of a clear area around the colony. A zone of clearance around the bacterial colony indicates positive proteolytic activity.

viii) Hugh Leifson's medium

Peptone	2 gm
Sodium chloride	5 gm
Di potassium hydrogen phosphate (K_2HPO_4)	0.3 gm
Glucose	10 gm
Bromothymol blue	0.01 gm
pH	7.0
d/w	make volume to 1 L

Adjust pH to 7.0 before adding Bromothymol blue. Autoclave at 121 ^{0}C for 15 mins. Media after sterilization is cooled immediately to avoid diffusion of air in medium. Inoculate in two tubes and overlay one tube with sterile paraffin oil and incubate. Facultative anaerobes showed change in colour in both tubes from green to yellow whereas aerobic (oxidative) organisms showed yellow colour only in tube which is not overlaid with paraffin anaerobes. Strict anaerobes show yellow colour in tube overlaid with paraffin oil.

ix) Methyl red- Voges Proskaur's test (MR- VP)

Glucose phosphate broth is recommended for the performance of the MR- VP tests in differentiation of the coli- aerogenes group.

Peptone	7 gm
Glucose	5 gm
Di potassium hydrogen phosphate (K_2HPO_4)	5 gm
pH	7.0
d/w	make volume to 1 L

Methyl red test is employed to detect the production of sufficient acid during the fermentation of glucose phosphate in the medium. In Voges- Proskauer, test organisms which produce acetyl methyl carbinol or its reduction product 2, 3 butanediol from glucose are detected. O'meara reagent is added and incubated at 37 ^{0}C for 2 hrs and appearance of pink colour indicates positive test.

x) Indole test

The medium used is tryptone water which is rich in amino acid tryptophan. Some organisms produce an enzyme tryptophanase which breakdown tryptophan into indole and pyruvic acid. Xylene is added in the medium to concentrate indole and then 2- 3 drop's of Kovac's reagent is added slowly to give pink colour in the xylene layer.

xi) Urease test

Christensen's medium

Peptone	1.0 gm
Sodium chloride	5.0 gm
Potassium dihydrogen phosphate	2.0 gm
Phenol red (0.2 %)	6.0 ml
Glucose (10 %, sterile)	10 ml
d/w	1000 ml of animal tissue
Urea (20 %, sterile)	100 ml

Sterilize the glucose and urea solutions by filtration. Prepare the basal medium without glucose and urea, adjust pH 6.8 to 6.9 and sterilize by autoclaving at 121 ^{0}C for 30 mins. Cool to about 50 ^{0}C, add glucose and urea and dispense into test tubes.

xii) Triple Sugar Iron (TSI) medium

Peptone	10 gm
Tryptone	10 gm

Yeast extract	3.0 gm
Beef extract	3.0 gm
Ferrous sulphate	0.2 gm
Sodium thiosulphate	0.3 gm
Phenol red	0.024 gm
d/w	1000 ml
Lactose (10 %)	
Sucrose (10 %)	
Glucose (1 %)	
Final pH	7.4

* Sterilize sugar solutions separately

xiii) SIM medium

Peptic digest of animal tissue	30 gm
Beef extract	3 gm
Peptonized Iron	0.2 gm
Sodium thiosulphate	0.0025 gm
Agar	3 gm
pH	7.3 ± 0.2
d/w	1000 ml

xiv) Motility agar

Nutrient broth	1000 ml
Agar	4 gm

C.2 Reagents for biochemical tests

i) Reagents for nitrate reduction

Solution A (Sulphanilic acid)

Sulphanilic acid	8 gm
Acetic acid (5 N)	one part of acetic acid to 2.5 parts of d/w, make final volume to 1 L with d/w

Solution B (α- naphthylamine)

α- naphthylamine	5 gm
Acetic acid (5 N)	1000 ml

Before use, mix equal volume of solution A and B to give test reagent.

ii) Oxidase reagent

Tetra methyl-p- phenylenediamine dihydrochloride	1.0 gm
d/w	100 ml

(Freshly prepared before use)

iii) O'Meara's reagent

Potassium hydroxide (KOH)	40 gm
Creatine	0.3 gm
d/w	200 ml

(used as purchased from Himedia R031)

iv) Kovac's reagent

p- dimethylamino benzaldehyde	5.0 gm
Isoamyl alcohol	75 ml
HCl (37 %)	25 ml

(used as purchased from Himedia R008)

APPENDIX- D

D. Chemical estimation and standard graphs

D.1 Folin Lowry's method for Protein estimation (Lowry *et al.*, 1951)

Reagent A: 2 % Na_2CO_3 in 0.1 N NaOH

Reagent B: 0.5 % $CuSO_4$ in 1 % Potassium sodium tartarate

Reagent C: Alkaline Copper solution- Mixed 50 ml A and 1 ml B prior to use.

Reagent D: Folin Ciocalteau's phenol reagent

Commercially available reagent diluted with equal volume of d/w on the day of use. This reagent is a solution of sodium tungstate and sodium molybdate in phosphoric and hydrochloric acids.

Standard bovine serum albumin solution: 0.1 mg of BSA dissolved in 1 ml d/w

Procedure: To 1 ml of the sample, 5 ml of $CuSO_4$ solution was added and kept at room temperature in the dark for 10 mins. 0.5 ml of Folin Ciocalteau's phenol reagent was then added and kept in dark for 20 mins. Absorbance was measured at

660 nm against blank and concentration of the samples determined from standard graphs using Bovine serum albumin as the standard (0- 100 µg/ ml).

D.2 Stock solution of Tyrosine

Tyrosine	0.006 mg
Tris Buffer (pH 9)	60 ml

APPENDIX- E

E. Composition of stock solutions, buffers used in SDS-PAGE, Native- PAGE and Zymography

E.1 Acrylamide–bis-acrylamide solution (Monomer solution): 29 gm acrylamide and 1 gm (w/v) N, N-methylene bis-acrylamide was dissolved in 70 ml d/w. pH was adjusted to 7.0 and volume was made up to 100 ml with d/w. Stored in amber coloured bottles at 4°C.

E.2 4X Resolving gel buffer (1.5 M Tris- HCl pH 8.8): Prepared by dissolving 18.615 gm Tris base, in 70 ml d/w. The pH of the solution was adjusted to 8.8 using 6N HCl and volume was made upto 100 ml with d/w. The solution was stored at 4°C.

E.3 4X Stacking gel buffer (1 M Tris- HCl pH 6.8): Prepared by dissolving 12.11 gm Tris base, in 70 ml d/w. The pH of the solution was adjusted to 6.8 using concentrated HCl and the volume was made up to 100 ml with d/w. The solution stored at 4°C.

E.4 10 % Sodium dodecyl sulphate (SDS)

SDS	10 gm
d/w	100 ml

E.5 Ammonium per sulphate (APS, 10 %): Prepared by dissolving 0.1 gm of APS in 1.0 ml d/w. The solution was prepared fresh each time.

E.6 Electrophoresis buffer:

Composition of 5 X buffer is as follows:

Tris- base	15.1 gm
Glycine	94 gm
SDS (10 %)	50 ml
pH	8.3

d/w to make total volume 950 ml

E.7 5 X Sample buffer:

1 M Tris- HCl	15.5 ml
1 % Bromophenol blue	2.5 ml
Glycerol	25 ml
d/w	7 ml

E.8 TEMED:

As purchased from Himedia (RM 1572)

E.9 Developing buffer:

Tris- HCl	50 mM
Sodium chloride	0.2 mM
Calcium chloride ($CaCl_2$)	5 mM

E.10 Sample solubilising buffer:

Composition of 4 X buffer is as follows:

Tris- HCl (1 M, pH 6.8)	0.04 ml
Glycine	0.04 gm
SDS	0.004 gm
β-mercaptoethanol	0.004 ml
d/w	to make total volume 10 ml

E.11 2.5 % Triton- X 100

Triton- X 100	2.5 ml
d/w	98.5 ml

E.12 Tracking dye:

50 % Sucrose	10 ml
Bromophenol blue	10 mg

E.13 Solutions for Silver staining

i) Gel fixing solution:

Methanol	40 ml
Acetic acid	10 ml
d/w	50 ml

ii) Gel washing solution:

Ethanol	10 ml
Acetic acid	5 ml
d/w	85 ml

iii) Silver stain:

Silver nitrate ($AgNO_3$)	0.1 gm
d/w	100 ml

iv) Silver Developer solution:

Sodium Carbonate (Na_2CO_3)	2.5 gm
Formaldehyde	0.02 ml
d/w	100 ml

v) Destaining solution:

Acetic acid	1 ml
d/w	100 ml

E.14 Preparation of gel monomer

Composition of Separating gel (10 %) and Stacking gel (4 %)

Solution	Resolving gel (10 %) 5 ml	Stacking gel (4 %) 5 ml
30 % acrylamide mix	1.7	0.83
1.5 M Tris (pH 8.8)	1.3	-
1.0 M Tris (pH 6.8)	-	0.63
10 % SDS	0.05	0.05
10 % APS	0.05	0.05
d/w	1.9	3.4
TEMED	0.002	0.005

APPENDIX- F

F.1 Preparation of stock solutions

F.1 Metal stock solutions

1. Copper sulphate ($CuSO_4$) (M.W- 249.69)

Stock solutions (1 M) - $CuSO_4$ (249.69 gm) was dissolved in 1 Litre de-ionized double distilled water. The solution was filter sterilized and stored at 4 ^{0}C in dark places. And required concentrations (1 mM, 2.5 mM and 5 mM) were prepared from the stock solution.

2. Calcium chloride ($CaCl_2$) (M.W- 147.02)

Stock solutions (1 M) - $CaCl_2$ (147.02 gm) was dissolved in 1 Litre de-ionized double distilled water. The solution was filter sterilized and stored at 4 ^{0}C in dark places. And required concentrations (1 mM, 2.5 mM and 5 mM) were prepared from the stock solution.

3. Magnesium sulphate ($MgSO_4$) (M.W- 246.48)

Stock solutions (1 M) – $MgSO_4$ (246.48 gm) was dissolved in 1 Litre de-ionized double distilled water. The solution was filter sterilized and stored at 4 ^{0}C in dark

places. And required concentrations (1 mM, 2.5 mM and 5 mM) were prepared from the stock solution.

4. Lead Nitrate (Pb $[NO_3]_2$) (M.W- 331.20)

Stock solutions (1 M) – Pb $[NO_3]_2$ (331.20 gm) was dissolved in 1 Litre de-ionized double distilled water. The solution was filter sterilized and stored at 4 ^{0}C in dark places. And required concentrations (1 mM, 2.5 mM and 5 mM) were prepared from the stock solution.

5. Mercuric chloride ($HgCl_2$) (M.W- 271.50)

Stock solutions (1 M) – Pb $[NO_3]_2$ (331.20 gm) was dissolved in 1 Litre de-ionized double distilled water. The solution was filter sterilized and stored at 4 ^{0}C in dark places. And required concentrations (0.01 mM, 0.1 mM and 1 mM) were prepared from the stock solution.

F.2 Inhibitors stock solutions

1. Phenyl methyl sulphonyl fluoride (PMSF) (M.W- 174.19)

Stock solutions (1 M) – PMSF (174.19 gm) was dissolved in 1 Litre isopropanol. The solution was at -20 ^{0}C. And required concentration (1 mM) was prepared from the stock solution.

2. Sodium dodecyl sulphate (SDS) (M.W- 288.38)

Stock solution (1 M) - SDS (288.38 gm) was dissolved in 1 Litre de-ionized double distilled water. The solution was filter sterilized and stored at 4 ^{0}C. And required concentration (1mM) was prepared from the stock solution.

3. Dithiothreitol (DTT) (M.W- 154.25)

Stock solution (1 M) – DTT (154.25 gm) was dissolved in 1 Litre de-ionized double distilled water. The solution was filter sterilized and stored at 4 ^{0}C. And required concentration (1mM) was prepared from the stock solution.

4. Disodium ethylenediamine tetraacetic acid (Na_2-EDTA) (M.W- 292.24)

Stock solution (1 M) - Na_2-EDTA (292.24 gm) was dissolved in 1 Litre de-ionized double distilled water. The solution was filter sterilized and stored at 4 ^{0}C. And required concentration (1 mM) was prepared from the stock solution.

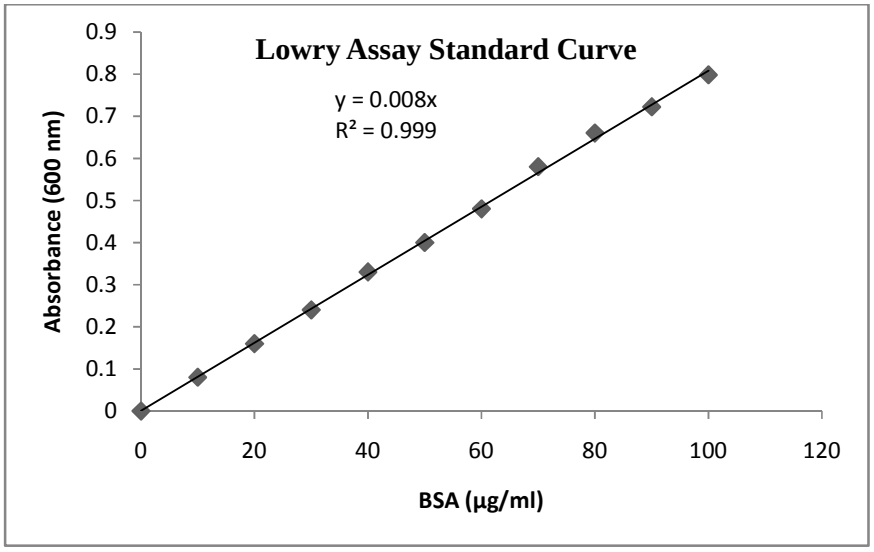
5. β- mercaptoethanol (Density- 1.170 g/ml) (M.W- 82.08)

Stock solution (1 M) - β- mercaptoethanol (70.17 ml) was dissolved in 1 litre de-ionized double distilled water. The solution was stored at 4 ^{0}C. And required concentration (1 M) was prepared from the stock solution.

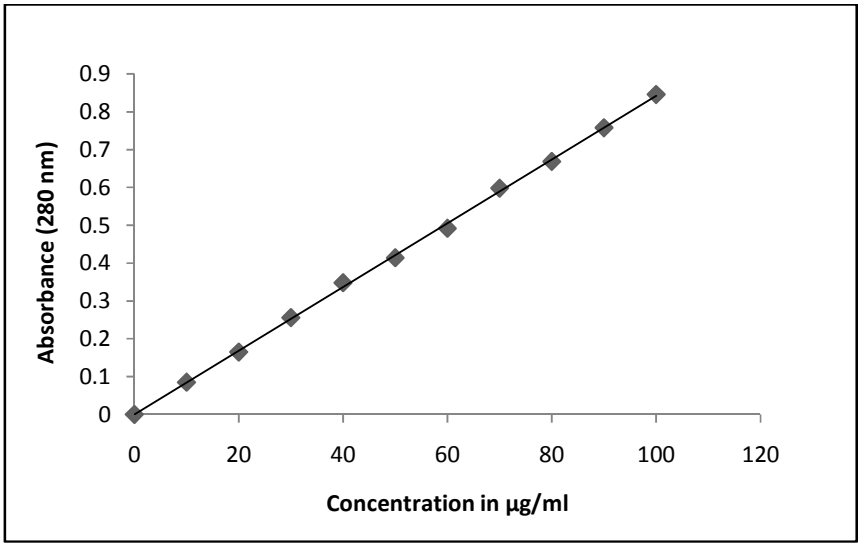
APPENDIX- G

G. Standard Graphs

a) Standard graph of protein (Bovine serum albumin)



b) Standard graph of tyrosine



APPENDIX- H

Quantities of Ammonium Sulphate required to reach given degrees of saturation at 20°C.

	Final percent saturation to be obtained																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
Starting percent saturation	Amount of ammonium sulphate to add (grams) per liter of solution at 20 °C																
0	113	144	176	208	242	277	314	351	390	430	472	516	561	608	657	708	761
5	85	115	146	179	212	246	282	319	358	397	439	481	526	572	621	671	723
10	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15	28	58	88	119	151	185	219	255	293	331	371	413	456	501	548	596	647
20	0	29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25		0	29	60	91	123	157	191	228	265	304	344	386	429	475	522	571
30			0	30	61	92	125	160	195	232	270	309	351	393	438	485	533
35				0	30	62	94	128	163	199	236	275	316	358	402	447	495
40					0	31	63	96	130	166	202	241	281	322	365	410	457
45						0	31	64	98	132	169	206	245	286	329	373	419
50							0	32	65	99	135	172	210	250	292	335	381
55								0	33	66	101	138	175	215	256	298	343
60									0	33	67	103	140	179	219	261	305
65										0	34	69	105	143	183	224	267
70											0	34	70	107	146	186	228
75												0	35	72	110	149	190
80													0	36	73	112	152
85														0	37	75	114
90															0	37	76
95																0	38

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RESEARCH PUBLICATIONS

- **Brenda D'Costa**, Kashif Shamim and Santosh K. Dubey (2013). Characterization of thermostable serine protease from *Bacillus altitudinis* BR1. Journal of Scientific and Industrial Research. 72: 166-171 **(Published)**.

- **Brenda D'Costa**, Dnyanada Khanolkar and Santosh K. Dubey (2013). Partial purification and characterization of metalloprotease of halotolerant alkaliphilic bacterium *Bacillus cereus* from coastal sediment of Goa, India. African Journal of Biotechnology. 12: 4905-4914 **(Published)**.

- **Brenda D'Costa** and Santosh K. Dubey (2014). Characterization of Cu-induced metalloprotease from *Bacillus firmus* CW2. Annals of Microbiology. **(Communicated)**.