PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE PROTEASE FROM ALKALIPHILIC AND THERMOPHILIC *Geobacillus sp.*

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ABSTRACT

PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THERMOSTABLE PROTEASE FROM ALKALIPHILIC AND THERMOPHILIC *Geobacillus sp.*

Proteases are the hydrolase enzymes that catalyze the hydrolysis of the peptide bonds in the primary structure of proteins and peptids. They are used to cleave the proteins specifically to produce useful peptides in the processes. Proteases are present in a wide variety of living organisms and they also show different physicological, physicochemical, biological, chemical functions on the earth. They are the most important enzymes in the industry, accounting for 60% of the total enzyme scales in the world.

The microorganisms that were previously isolated and characterized as a *Bacillus sp.* from Balçova Geotermal region in İzmir were used in the experiments. The aim of this study was to produce the protease enzyme from alkaliphilic and thermophilic *Bacillus sp.*, purify and determine the properties of the enzyme with the characterization steps. When the screening studies and growth conditions were investigated, it was understood that the alkaliphilic and thermophilic *Bacillus sp.* produced extracellular protease enzyme. This extracellular protease enzyme was purified by ammonium sulphate precipitation and ion exchange chromatography chromatograpy. The yield and purification fold after purification of the enzyme were 33% and 1.41, respectively.

In the characterization studies, the results indicated that the protease enzyme had highest activity at pH 8.0 and 55 ºC. The protease enzyme lost 20% of its activity at pH 4.0 and it lost 10% of its activity at pH 10.0. The protease enzyme at temperatures below 55 ºC lost 15% of its activity and also the protease enzyme at temperatures above 55 ºC lost 25% of its activity. The protease enzyme was stable at different pH values during 3 hours and at different temperature values during 6 hours. When compared the substrates, casein showed higher activity. The effect of organic solvents and surfactants on protease activity was investigated and the results indicated that the protease enzyme was stable in the presence of 10% of the organic solvents and 1% of the surfactants. PMSF and the protease inhibitor coctail decrease the activity of the protease.

ÖZET

ALKALİFİLİK VE TERMOFİLİK *Geobacillus sp.*'den TERMAL KARARLI PROTEAZ ENZİMİNİN ÜRETİLMESİ, SAFLAŞTIRILMASI VE KARAKTERİZASYONU

Proteazlar proteinler ve peptitlerdeki peptit bağlarının hidrolizini katalizleyen hidrolaz enzimlerdir. Proseslerde yararlı peptitleri üretmek için proteinleri spesifik bir şekilde kesmede kullanılırlar. Proteazlar büyük çeşitlilik gösteren canlı organizmalarda bulunurlar ve aynı zamanda yeryüzünde farklı fiziksel, fizikokimyasal, biyolojik, kimyasal fonksiyonlara sahiptirler. Dünya enzim skalasının %60'ını oluşturan, endüstrideki en önemli enzimlerdir.

İzmir Balçova Jeotermal bölgesinden daha önce izole edilen ve *Basillus sp.* olarak karakterize edilen mikroorganizmalar deneylerimizde kullanıldı. Bu çalışmanın amacı, alkalifilik ve termofilik *Basillus sp.*'den protease enzimini üretmek, saflaştırmak ve karakterizasyon basakları ile enzimin özelliklerini belirlemektir. Görüntüleme çalışmaları ve büyüme koşulları incelendiğinde, alkalifilik ve termofilik *Basillus sp.*'nin ekstraselüler proteaz enzimi ürettiği anlaşılmıştır. Bu ekstraselüler proteaz enzimi amonyum sülfat çökürmesi ve iyon değiştirici kromatografi ile saflaştırılmıştır. Saflaştırma sonrası verim ve saflık katsayısı sırasıyla %33 ve 1.41'dir.

Karakterizasyon çalışmalarında, sonuçlar enzimin pH 8.0 ve 55 ºC'de en yüksek aktiviteye sahip olduğunu göstermiştir. Enzim pH 4.0'te aktivitesinin %20'sini ve pH 10.0'da aktivitesinin %20'sini kaybetmiştir. 55 ºC'nin altında proteaz enzimi aktivitesinin %15'ini kaybetmiştir ve aynı zamanda 55 ºC'nin üzerinde proteaz enzimi aktivitesinin %25'ini kaybetmiştir. Proteaz enzimi farklı pH değerlerinde 3 saat ve farklı sıcaklık değerlerinde 6 saat boyunca stabildir. Substratlar kıyaslandığında, kazein daha yüksek aktivite göstermiştir. Organik çözücülerin ve yüzey aktif maddelerin proteaz aktivitesi üzerine etkisi incelenmiştir ve sonuçlar proteaz enziminin %10'luk organik çözücü ve %1'lik yüzey aktif madde varlığında stabil olduğunu göstermiştir. PMSF ve proteaz inhibitör kokteyli protease activitesini düşürmüştür.

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LIST OF ABBREVIATIONS

APS	Ammonium persulfate
BSA	Bovine serum albumin
CBB	Commasie brilliant blue
DMSO	Dimethyl sulfoxide
DIFP	Diisopropylmethylfluoro phosphate
DTT	Dithiothreitol
EDTA	Ethylenediamine tetra acetic acid
LB	Luria-Bertani broth
PAGE	Polyacrylamide gel electrophoresis
PMSF	Phenylmethylsulphonyl fluoride
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
sp.	Species
TCA	Trichloroacetic acid
TEMED	Tetramethylethylenediamine
TLCK	Tosyl-L-lysine chloromethyl ketone
UV	Ultraviolet
µl	Microliter
ml	Milliliter
mM	Millimolar

CHAPTER 1

INTRODUCTION

1.1. Proteases (EC 3.4)

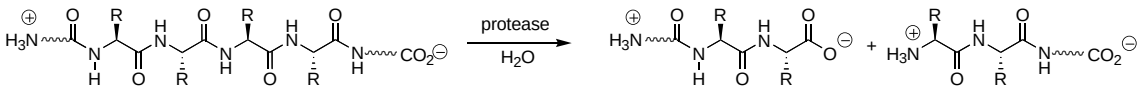


Figure 1.1. The mechanism of protease hydrolysis

Proteases are the hydrolase enzymes which have small size, compact molecules, spherical structures and they catalyze the hydrolysis of the peptide bonds in the primary structure of proteins and peptids (Dixon and Webb 1979; Polgar 1989). They are used to cleave the proteins specifically to produce useful peptides in the processes (Amara et al. 2009). Proteases are present in a wide variety of living organisms and they also show different physicological, physicochemical, biological, chemical functions on the earth (Hase and Finkelstein 1993). They are the most important enzymes in the industry, accounting for 60% of the total enzyme scales in the world (Outtrup et al. 1990; Banerjee et al. 1999). Because they are used in the regulation and transportation of metabolism, gene expression, enzyme modification, pathogenicity and the processes of the industry (Rao et al. 1998).

1.2. Classification of Proteases

The characterization of the proteases is very difficult due to the biochemical diversity in their structures. Firstly, they were categorized depending on the molecular size, charge or substrate specificity and then, they were started to be categorized based on the catalytic-active sites, mechanism of action, and the three-dimensional structure (Beynon et al. 1989; Barett 1994; Rao et al. 1998).

Proteases belong to the hydrolase enzymes in the Nomenclature Committee of

the International Union of Biochemistry and Molecular Biology (International Union of Biochemistry 1992). According to the committee, proteases have four classes with the six families. They are serine I, serine II, cysteine (plants), cysteine (animals), aspartic and metalloproteases (Kumar and Savitri 2008).

In the literature, you can review the studies about spesific capabilities in the hydrolytic mechanism, specificity for a particular site, maximum activity under particular conditions (temperature, salt concentration, endo/exopeptidase ratio, activity in organic solvents) (García-Carreńo 1992).

- Proteases are divided into three groups depends on their origin. They are animal-origin proteases, plant-origin proteases and microbial-origin proteases. Recently, the reserchers are interested in the microbial-origin proteases in the genus *Bacillus* because of the fact that they are of vital importance in the industry (Priest 1977).
    - **Plant proteases:** The places for cultivation and the climatic circumstances affect the growing of the plant and production of the enzyme. The disadvantage of the production of the enzyme is losing time. For example; papaya, pineapple, papain, bromelain, keratinases, ficin (Rao et al. 1998).
    - **Animal Proteases:** The political and agricultural strategies affect the obtaining the animals and production of the enzyme from them. For example; pancreatic trypsin, chymotrypsin, pepsin, and rennins (Rao et al. 1998).
    - **Microbial Proteases:** The use of microbial proteases are generally common (nearly 40% of the total enzyme sales) in comparison to plant, animal and fungal proteases, due to having improved biological, biochemical and molecular diversity in the nature (Godfrey and West 1996; Rao et al. 1998). For examples; Bacteria, fungi, viruses. Microbial proteases are of the vital importance because of the biochemical diversity, the rapid growth of the microorganisms and the limited space required for cell cultivation. Bacterial neutral proteases (pH 5-8, low thermotolerance, includes metallopreotease and serine protease) and bacterial alkaline proteases (pH 10, high thermotolerance, used in detergent technology) are the best known examples of the microbial proteases (Rao et al. 1998). The microbial preteases play a crucial role in not only cellular metabolic processes, but also industrial processes (Mahendran et al. 2010). In the literature, you can examine the microbial proteases (especially serine and metalloprotease) isolated by *Bacillus subtilis*, B. *amyloliquefaciens*, *Pseudomonas sp.*, *Lysobacter enzymogenes* and

Escherichia coli (Fujishige et al. 1992). It is important to identify and characterize the microbial proteases with the sensitive experimental designs in order to understand their role and improve their applications in the industry (Lantz and Ciborowski 1994).

- Proteases are divided into three groups depends on the secretion of the enzyme from the microorganism. They are intracellular-protease (in the cell), periplasmic (with the cell wall) and extracellular-protease (in the media) (Kohlmann et al. 1991; El-Safey et al. 2004; do Nascimento and Martins 2004). Intracellular proteases are of vital importance in the cellular, metabolic and regulatory processes. Extracellular proteases are of vital importance in the hydrolysis of proteins in cell-free environments, obtaining hydrolytic products in the commercial processes (Kalisz 1988; Kumar and Takagi 1999; Gupta and Beg 2002). Recently, not only the extracellular protease enzyme but also the intracellular protease enzyme are investigated due to the important roles in metabolic and regulatory processes in the industries (Zeigler 2001).
- Proteases are divided into four groups depends on the optimum pH. They are acidic protease, neutral protease, alkaline protease and high-alkaline protease (Guangrong et al. 2006). Acidic proteases are found in animal cells, moulds, yeasts and rarely bacteria. A few of them include aspartic acid residue. The aromatic and bulky side chains at the cleaving bonds reveal the specificity of the acidic proteases. Neutral proteases contain the cystein proteases (Papain, bromelain, ficin) isolated from botanical origin and the metalloproteases (Sumantha et al. 2006). The production of bacterial neutral proteases is commonly occured at neutral (pH 5-8) conditions. They have low thermo tolerance, so they control their activity and stability in the food hydrolysis (Rao et al. 1998; Siddalingeshwara et al. 2010). Alkaline proteases are produced at alkaline conditions and also they have wide application spectra because of having their catalytic nature (Asokan et al. 2010).
- Proteases are divided into different groups depends on the substrate specificity. They are collagenases, keratinases, elastases, etc (Sumantha et al. 2006).
- According to Enzyme Commission (EC) classification, proteases are divided into two groups depends on the site of action and the cleavage sites within the target molecule in the catalytic mechanism. They are exopeptidases or endopeptidases (García-Carreńo 1991; García-Carreńo 1993; Liao and McCallus 1998).

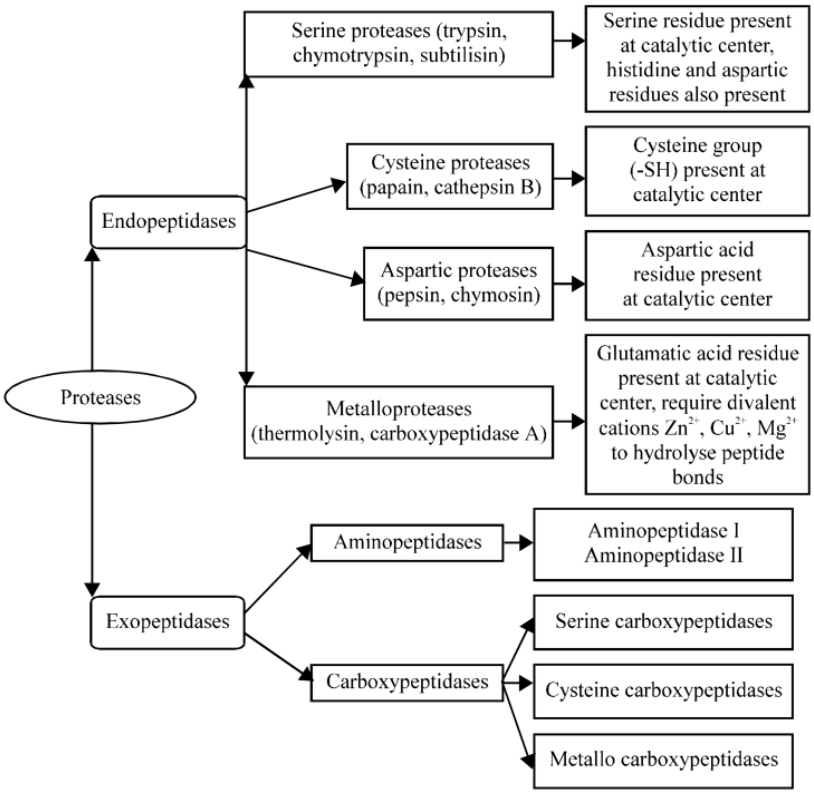


Figure 1.2. The classification of proteases
(Source: Kumar and Savitri 2008)

1.2.1. Endopeptidases (Proteinases) (E.C. 3.4.21-99)

Endopeptidases are the enzymes which cleave and hydrolyze the internal peptide bonds. They are divided into three groups based on the catalytic mechanism and the functional groups at the active site of the proteases (Hase and Finkelstein 1993; Rao et al. 1998).

If the enzyme cleaves the peptide bond proximal to the amino or carboxy terminus of the substrate, they are classified as **exopeptidases**. If the enzyme cleave peptide bonds distant from the termini of a substrate, they are classified as **endopeptidases.** Based on the functional group present at the active site and their catalytic mechanism, proteases are then categorized into four prosthetic groups; serine proteases, aspartic proteases, cysteine/thiol proteases, or metalloproteases. Four classes of endopeptidases have been identified in living organisms and three of the four classes of endopeptidases have been isolated and purified in bacteria; serine, cysteine, and metalloproteases (Liao and McCallus 1998).

Table 1.1. The characteristic features of endopeptidases
(Source: Sumantha et al. 2006)

Properties	EC No.	Molar mass range/kDa	pH optimum	Temperature optimum/°C	Metal ion requirement(s)	Active site amino acid(s)	Major inhibitor(s)	Major source(s)
Aspartic or carboxyl proteases	3.4.23	30–45	3–5	40–55	Ca^{2+}	Aspartate or cysteine	Pepstatin	*Aspergillus, Mucor, Endothia, Rhizopus, Penicillium, Neurospora,* animal tissue (stomach)
Cysteine or thiol proteases	3.4.22	34–35	2–3	40–55	–	Aspartate or cysteine	Indoacetamide, *p*-CMB	*Aspergillus,* stem of pineapple (*Ananas comorus*), latex of fig tree (*Ficus* sp.), papaya (*Carica papaya*), *Streptococcus, Clostridium*
Metallo proteases	3.4.24	19–37	5–7	65–85	Zn^{2+}, Ca^{2+}	Phenyl-alanine or leucine	Chelating agents such as EDTA, EGTA	*Bacillus, Aspergillus, Penicillium, Pseudomonas, Streptomyces*
Serine proteases	3.4.21	18–35	6–11	50–70	Ca^{2+}	Serine, histidine and aspartate	PMSF, DIFP, EDTA, soybean trypsin inhibitor, phosphate buffers, indole, phenol, triamino acetic acid	*Bacillus, Aspergillus,* animal tissue (gut), *Tritirachium album* (thermostable)

1.2.1.1. Serine Proteases (E.C. 3.4.21)

Serine proteases are commercially important, the most studied and best understood class in the protease groups. They have a essential serine residue in their active center (García-Carreńo 1993). The serine residue binds with substrates, activators or inhibitors in their catalytic mechanisms. Exopeptidases, endopeptidases, oligopeptidases, and omega peptidases includes serine proteases.

The serine proteases are divided into twenty families and six clans according to the similarities and differences in their three-dimensional structures, amino acid sequences and active site configurations (Barett 1994; Zeigler 2001). Subtilisin family (serin alkaline proteases and extracellular alkaline proteases from *Bacillus species*) and trypsin family (cymotrypsin, tripsin, mammalian elastases, several bacterial proteases) are the best known subgroups in the serin proteases. Subtilisin family includes –SH group, thermitase and proteinase K in their mechanism (Zeigler 2001).

In the literature, the microorganisms producing thermostable alkaline proteases are *Bacillus species* (*Bacillus licheniformis*, *Bacillus thermoruber*, *Bacillus stearothermophilus*, *Bacillus alcalophilus*, *Bacillus amyloliquefaciens*, *Bacillus circulans*,

Bacillus firmus, Bacillus intermedius, Bacillus lentus, Bacillus proteolyticus, Bacillus pumilus, Bacillus sphaericus, Bacillus subtilis, Bacillus thuringiensis) (Kumar and Takagi 1999).

The stability of the alkaline proteases is provided via the immobilization methods in a good way. The methods are based on the ground of the binding between enzymes and carriers by carrier binding (physical adsorpsion, ionic bonding, covalent bonding), cross-linking with bifunctional reagents, entrapping into gels / beads / fibres (lattice-matrix type, microencapsule type). Thanks to the immobilization methods, the enzymes are re-used and stabilized easily for a long time in the applications in the industry (Anwar and Saleemuddin 1998).

The production of serine proteases is commonly occured at neutral (pH 7) and alkaline (pH 11) conditions. They have broad substrate specificities. The molecular mass of serin proteases generally changes from 18 kDa to 35 kDa and the isoelectric point of them changes from pH 4 to pH 6. *Bacillus species* are the best known producer of serine protease in comparison with the other microorganisms (Rao et al.1998). Also, they are inhibited by PMSF (phenylmethylsulfonyl fluoride), DIFP (diisopropylfluoro phosphate), 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), tosyl-L-lysine chloromethyl ketone (TLCK) and they are active at alkaline pH (9 – 11) (Priest 1989; Kamal 1993; Rao et al. 1998).

In some of the serin proteases, the peptide bonds are attacked by the hydroxyl group of a serine residue which carries a proton donor/general based on the serin protease enzyme. On the other hand, it is called nucleophilic attact in the catalytic mechanism. The proton donor should be a histidine residue which includes imidazolium ring. But, an aspartate or an histidine residue is required because of the catalytic triad.

In some of the serin proteases, the peptide bonds are attacked by the catalytic dyad of a serine residue which carries a proton donor/general base on the serin protease enzyme. The proton donor should be a lysine residue and there is no need another catalytic residue in the catalytic mechanism (Supuran et al. 2002).

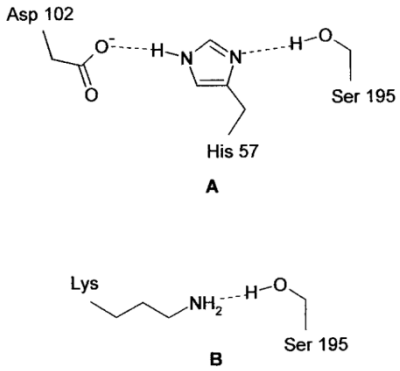


Figure 1.3. The schematic representation of the serine protease amino acid residues involved in the proteolytic scission. (Source: Supuran et al. 2002)
A: Catalytic triad (chymotrypsin numbering)
B: Catalytic dyad (a lysine residue activates the hydroxyl group of serine residue essential for catalysis)

Table 1.2. Optimized production conditions for alkaline proteases (Source: Gupta and Beg 2002)

Microorganism	pH	Temperature (°C)
Bacteria		
Alcaligenes faecalis	8	30
Bacillus sp. IS-3	10.5	37
Bacillus sp. JB99	10	55
Bacillus sp. K2	7	37
Bacillus sp. P-2	9.5	30
Bacillus sp. RGR-14	7	37
Bacillus sp. SSR-1	10	40
B. brevis MTCC B0016	10.5	37
B. licheniformis ATCC 21415	7	30
B. mojavensis	7	50
B. pumilis MK6–5	9.6	35
B. sphaericus	n.s.[b]	30
B. subtilis 168	n.s.	36
Flavobacterium balustinum P104	7.4	10
Serratia marcescens ATCC 25419	n.s.	30; 36

1.2.1.2. Cysteine/Thiol Proteases (E.C. 3.4.22)

Cysteine proteases are produced by prokaryotes and eukaryotes. The cystein proteases have cysteine (SH-) and histidin groups in their active sites (Garcia-Carreno 1993). They are divided into twenty families depends on the order differences between cysteine and histidin residues (Barett 1994). On the other hand, they are categorized into four groups. They are papain-like, trypsin-like, glutamic acid-like and others. The production of cystein proteases is commonly occured at neutral conditions. The molecular mass of serin proteases generally changes from 32 kDa to 50 kDa and the isoelectric point of them changes from pH 4.9 to pH 8.4 (Rao et al. 1998). Also, they are inhibited by thiol reagents (heavy metals, alkylating-oxidizing agents), sulphydryl reagents, (*p*-chloromercuribenzoate and iodoacetamide) (Kamal 1993; Zeigler 2001).

The peptide bonds are attacked by the –SH group of a cysteine residue which carries a nucleophile and a proton donor/general base on the serin protease enzyme. On the other hand, it is called nucleophilic attact in the catalytic mechanism. The proton donor should be a His residue which includes imidazolium ring (Supuran et al. 2002).

1.2.1.3. Aspartic Proteases (Acidic Proteases) (E.C. 3.4.23)

Aspartic proteases have an aspartic acid residue in the active sites (Garcia-Carreno 1993). The aspartic proteases are divided into three families. They are pepsin, retropepsin and enzymes from pararetroviruses. The production of aspartic proteases is commonly occured at acidic conditions owing to the fact that the aspartic proteases show maximum activity at acidic pH. The molecular mass of serin proteases generally changes from 30 kDa to 45 kDa and the isoelectric point of them changes from pH 3 to pH 4.5. Also, they are inhibited by pepstatin, diazoacetyl norleucine methyl ester (DAN), and 1,2-epoxy*p*-nitrophenoxy propane (EPNP) (Rao et al. 1998; Zeigler 2001).

1.2.1.4. Metalloproteases (E.C. 3.4.24)

Metalloproteases are the enzymes which cleave the peptide bonds thanks to the nucleophilic attact of a water molecule which is coordinated to a divalent metal ion

(catalytic zinc, manganese, cobalt, nickel or copper) or bridged to a dimetallic center. The metal ion is complexed by three conserved amino acid residues that can be glutamic acid (Glu), aspartic acid (Asp), histidin (His) or lysin (Lys) in their active sites (Hase and Finkelstein 1993; Supuran et al. 2002; Mansfeld 2007).

The properties of catalytic and structural metal-binding sites are defined by the x-ray crystallographic analyses of the metalloproteases. The most studied metalloprotease, zinc-containing metalloproteases have a catalytic zinc atom (coordinated to three amino acid residues) and an active water molecule in their cristal structures. Also, the residues (His, Glu, Asp, Cys) coordinates the catalytic zinc atom, the tridentate active zinc site occures and the coordination sphere is completed by the activated water (Vallee and Auld 1990).

The metalloproteases are divided into thirty families and the families are categorized into fourteen different clans according to the amino acid sequences and the relation between the aminoacids and the metal binding sites. Clan MA, MB, MD, ME, MJ, MK, MM, MO and MP include only one divalent cation and clan MF, MG, MH, MN and MQ include two divalent cations acted co-catalytically on the substrate (Mansfeld 2007). The clan MA has the HEXXH-E sequence (for exp: thermolysin) and the clan MB has the HEXXH-H sequence.

On the other hand, the metalloproteases are divided into four groups according to the catalitic action definitely. They are neutral, alkaline, Myxobacter I and Myxobacter II. The production of metalloproteases is commonly occured at neutral conditions (Rao et al. 1998). The pH optima of metalloproteases is commonly in the range of 5-9 (Zeigler 2001). Also, the metalloproteases are inhibited by chelating agents (for example: EDTA, 1,10-phenantroline) (Matsubara and Feder 1971; Kamal 1993).

1.2.2. Exopeptidases (Peptidases) (E.C. 3.4.11-19)

Exopeptidases are the enzymes which cleave and hydrolize the external peptide bonds in the terminal amino end or carboxylic end of the substrate (Garcia-Carreno 1993). They are divided into two subgroups based on the cleavage sites.

1.2.2.1. Aminopeptidases

Aminopeptidases are the enzymes that hydrolize and split off the N-terminal residues (Kamal 1993). They are generally intracellular enzymes.

1.2.2.2. Carboxypeptidases

Carboxypeptidases are the enzymes that hydrolize and split off the C-terminal residues (Kamal 1993). Carboxypeptidases are divided into three groups due to having different amino acid residues at the active site of the proteases. They are serine carboxypeptidases, metallocarboxypeptidases, and cysteine carboxypeptidases.

1.3. Industrial Applications of Proteases

75% of the commercial enzymes are hydrolase enzymes. The protease enzymes from the hydrolatic enzymes are the most important groups in the industrial enzymes, accounting for nearly 60% of the total enzymes in the world (Figure 1.5) (Rao et al. 1998)

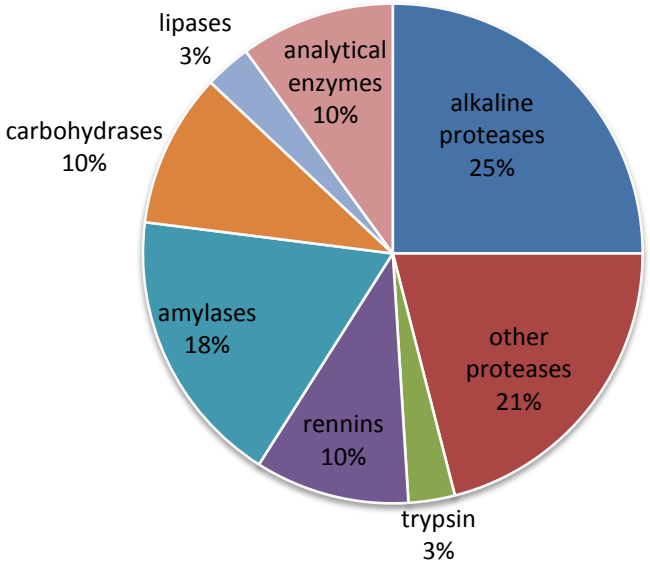


Figure 1.4. The distribution of enzyme sales
(Source: Rao et al. 1998)

Proteases are the commercial enzymes, because of this reason, they are of vital importance in the biochemical and biotechnological applications in the industry and also they are commonly isolated from the genus *Bacillus* (including *Bacillus spp., Bacillus subtilis, Bacillus firmus, Bacillus stearothermophilus, Bacillus kaustophilus, Bacillus cereus, Bacillus brevis*).

Proteases are used in the modification of proteins, digestive supplements, protein processing, peptide synthesis, determining the structure of proteins and polypeptides (Kalisz 1988; Bhosale, Rao et al. 1995; Anwar and Saleemuddin 1998; Rao et al. 1998; USP Enzyme Workshop 2009).

Table 1.3. Different applications of some industrally important proteases (Source: Anwar and Saleemuddin 1998)

Species	Source	pH Optimum/ stability	Industrial application(s)
Streptococcus sp.	Bacterial	8·0	Dairy/cheese production
Bacillus stearothermophilus	Bacterial	9·5	Detergents and heavy duty laundry powders
Tritirachium album (proteinase T)	Fungal	9·0–12·0	Laundry detergents formulations
Tritirachium album (proteinase R)	Fungal	7·0–10·0	Laundry detergent formulations
Conidiobolus coronatus (alkaline proteinase B)	Fungal	9·7	Resolution of racemic mixtures of D,L-phenyl alanine and glycine
Bacillus sp. Y. (BYA)	Bacterial	10·0–12·5	Detergent formulations
Bacillus lichenformis (Alcalase)	Bacterial	8·2	Catalyst for *N*-protected amino acids
Bacillus sp. (AH-101)	Bacterial	12·0–13·0	Dehairing/leather industry
Rhizopus oryzae (RO, IIT, KGP)	Fungal	3·0–11·0	?
Conidiobolus coronatus (NCl 86.8.20)	Fungal	8·5	Commercial detergents
Bacillus firmus	Bacterial	8·0	Detergent industry
Bacillus sp. (P-001A)	Bacterial	9·5	Production of biomass from natural waste
Bacillus sp. (B 18)	Bacterial	12·0	?
Bacillus sp.	Bacterial	12·0	?
Bacillus sp.	Bacterial	8·5	Dehairing/leather industry
Thermus Rt 41A	Bacterial	11·0	?
Bacillus sp. (Savinase/ Durazym)	Bacterial	9·0–11·0	Detergent formulations
Bacillus licheniformis (Alcalase)	Bacterial	8·2	Synthesis of biologically active peptides
Bacillus subtilis	Bacterial	8·5	Bating agent in leather industry
Bacillus sp.	Bacterial	8·5	?
Bacillus subtilis	Bacterial	–	Contact lens cleansing agent

Table 1.4. Application of proteases in industry
(Source: Kumar and Savitri 2008)

Industry	Protease	Application
Baking	Neutral protease	Dough conditioner
Beverage	Papain	Chill proofing, removal of haze in beverages
Dairy	Fungal proteases, chymosin, other proteases	Replacement of calf rennet, whey protein processing, production of enzyme modified cheese (EMC)
Detergent	Alkaline protease, subtilisin	Laundry detergents for protein stain removal
Food processing	Several proteases	Modification of protein rich material i.e., soy protein or wheat gluten
Leather	Trypsin, other proteases	Bating of leather, dehairing of skins
Meat and fish	Papain, other proteases	Meat tenderization, recovery of protein from bones and fish waste
Medicine	Trypsin	Dead tissue removal, blood clot dissolution
Photography	Several proteases	Recovery of silver from used X-ray and photographic films
Sweetner	Thermolysin	Reverse hydrolysis in aspartame synthesis

- ***Detergent Industry***

The serine proteases (subtilisins) from *Bacillus* subtilis and *Bacillus* lichneformis are generally used in the laundry detergent technology and also they are used nearly 25% of the total worldwide sales of enzymes in the detergent industry (Fogarty et al. 1974). In the detergent industry, the alkaline proteases should be active and stable at alkaline pH and high temperatures in the presence of chelating and oxidizing agents. The cleaning mechanism is based on forming complex structure with detergents and the hydrolysis of the proteins on the proteinaceous stains due to the food (milk, egg, meat, fish), keratin, blood, body secretions in the laundry detergents (Anwar and Saleemuddin 1998; Beg and Gupta 2003). For this purpose, the protease enzymes are the most suitable enzymes not only in the household laundering and industrial-institutional cleaning (cleaning ultrafiltration membranes, laundry detergent, dishwashing detergents, cleaning hard surface), but also in the reagents (cleaning contact lens, presoak formulations) because of exhibiting high performance when the pI of the enzyme is nearly similar the pH of the detergent (Rao et al. 1998).

Table 1.5. Serin proteases (Subtilisin) used in detergents
(Source: Maurer 2004)

Trade mark	Producer	Origin	WT/PE[c]	Production strain
Alcalase®	Novozymes	*B. licheniformis*	WT	*B. licheniformis*
FNA[a]	Genencor	*B. amyloliquefaciens*	PE	*B. subtilis*
Savinase®	Novozymes	*B. clausii*	WT	*B. clausii*
Purafect™	Genencor	*B. lentus*	WT	*B. subtilis*
KAP[b]	Kao	*B. alkalophilus*	WT	*B. alkalophilus*
Everlase™	Novozymes	*B. clausii*	PE	*B. clausii*
Purafect OxP™	Genencor	*B. lentus*	PE	*B. subtilis*
FN4[a]	Genencor	*B. lentus*	PE	*B. subtilis*
BLAP S[b]	Henkel	*B. lentus*	PE	*B. licheniformis*
BLAP X[b]	Henkel	*B. lentus*	PE	*B. licheniformis*
Esperase®	Novozymes	*B. halodurans*	WT	*B. halodurans*
Kannase™	Novozymes	*B. clausii*	PE	*B. clausii*
Properase™	Genencor	*B. alkalophilus PB92*	PE	*B. alkaliphilus*

Table 1.6. Commercial bacterial alkaline proteases, sources, applications and their industrial suppliers. (Source: Gupta and Beg 2002)

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 Pharmaceticals, 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*	Cosmetic, 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

The enzyme should be stable and active in the presence of detergent ingredients (surfactants, builders, bleaching agents, bleach activators, fillers, fabric softeners,

detergent composition, formulation aids) and the different conditions (pH of detergent, ionic strength, wash temperature, mechanical handling) (Anwar and Saleemuddin 2000; Beg and Gupta 2003).

The thermostable proteases show high activity and stability in the high temperatures, so using them in the detergents is of vital importance because of having high yield and low cost. They also used in the dishwashing and the laundry detergents in the hospitals, due to the fact that the sterilization at high temperatures is too important to overcome the microbial contaminations (Zeigler 2001).

- ***Food Industry***

Proteases are very significant in the food technology because of the fact that they are used as a catalyst and they try to convert the untreated materials to the developed food materials. The importance of substrate specificity, kinetic parameters, catalitic activity should be taken into the consideration while doing the experiments under the different conditions (García-Carreńo 1991).

The researchers' aim is to save time, enegy, money and to reach high yield, high catalytic activiy, high substrate specificty, large amount of product. Because of this reason, they tried to find new approaches (fermantation processes, protein engineering, synthetic enzymes, three dimensional structures) in food technology (Wasserman 1990). For example; dairy industry, baking, cheese production, gluten development, whipping, preparation of soya hydrolysates, aspartame synthesis, meat tenderization, chill-haze prevention, brewing, dietetic, health products (Rao et al. 1998).

Further, proteases play a prominent role in meat tenderization, especially of beef. Thermophilic alkaline proteases are used in the meat tenderization by hydrolyzing the tissue proteins. Firstly, the enzyme was given into the meat by injecting. Nowadays, the enzyme is given into the circulatory system of the animal (Kumar and Takagi 1999).

Hydrolysis of the proteins is required in the nutritions and also it is used in different situations such as food formulations, therapeutic dietary foods and the addition to the fruit juices and soft drinks (Neklyudov et al. 2000; Gupta and Beg 2002).

Proteases are useful addition matters in the food industry because of having improved digestibility, solubility, flavor & palatability, processing (viscosity reduction, improved drying), modified functional properties (emulsification, fat-binding, water-binding, foaming properties, gel strength, whipping properties) (USP Enzyme Workshop 2009).

- **Dairy Industry:** The improvements are freezing storage of raw-pasteurized milk for a long time, staying at higher temperatures in the pasteurization and obtaining high quality of milk in the dairy industry (Meer et al. 1991; Meer, Wodburn et al. 1993; Stadhouders 1992; Baker and Griffiths, 1993). The proteases are used as milk-clotting agents for cheese production in the dairy industry (Sumantha et al. 2006).
- **Baking industry:** The neutral proteases from *Bacillus* spesies are generally used in baking industry. The soft dough in the biscuit and crackers are taken into consideration not to be confronted with a problem about bending and wrinkling in the oven (Bryce 1966). Thanks to using the protease enzyme, the mixing time is reduced and the loaf volumes are increased (Rao et al. 1998).
- **Brewing industry:** Proteases from the *Bacillus* species are generally used in brewing industry in order to cleave the peptide bonds in the proteins. If you don't want to be confronted with a problem about turbidity in the beverages, you should add the protease into the cold English and German beer. (Fogarty et al. 1974)
- **Animal protein processing** includes animal feed additives, improving digestibility, reducing allergenicity, improving flavor and meat tenderization in the food industry. In the meat tenderization, if the protease enzyme should be active and have thermal stability during the cooking and storage processes, good tenderization and developed taste of the meat will be obtained in these applications (Wilson et al. 1992; Zeigler 2001). In the fish industry, proteases from the *Bacillus* species are generally used in the fish industry. The aim is to obtain fishmeal and enhanced oil recovery from the uneatable fish (Lovern 1955).

- ***Leather Industry***

Initial soaking (neutral proteases), de-hairing of animal hair, hides and skin (alkaline proteases), bating (acidic proteases), tanning and removing the waste materials (animal glue) are the stages of leather preparation. The animal hair, hides and skin include the materials with protein. The proteases are used instead of hazardous chemicals (sodium sulfide) to prevent the pollution problems, obtain high quality, easy control, speed up dehairing and reduce the waste materials in the leather industry. The most improtant advantage is being eco-friendly (Grimm 1958; Rao et al. 1998; Gupta and Beg 2002) The

unwanted proteinaceous pigments are removed by using protease enzyme and the clean skin-hide are obtained. The alkaline conditions should be choosen because it is easy to remove the hair for the alkali protease enzyme (Varela et al. 1997; Gupta and Beg 2002).

- ***Silver Recovery***

The photographic films and the X-ray films which are used before include silver (1.5-2.0%) in their gelatin layers. Because of this reason, alkaline proteases are used in order to hydrolise the gelatin layers on the films and release of the silver materials in the industrial applications (Kumar and Takagi 1999). There are lots of advantages in recovering silver from photographic films by digestion of gelatin emulsion coating. The pollution of silver is prevented and the silver is used in another purposes after recovering in the environment. Thus, recovering silver is very important in the silver industry (Gupta and Beg 2002).

- ***Medical and Pharmaceutical Industry***

Proteases are used in the medical and pharmaceutical industry because of having broad diversity and specificity. The usage of this enzyme is common in developing therapeutic agents, preparation of medicines, medical diagnosis, biopharmaceutical products (contact-lens enzyme cleaners, enzymatic debriders) and cosmetics (skin care ointments) (Anwar and Saleemuddin 2000). In the treatment of burns, wounds, carbuncles, furuncles and abscesses, the subtilisin (or clostridial collagenase) is chosen to be used with the advanced antibiotics (Rao et al. 1998; Gupta and Beg 2002).

- ***Waste Treatment (Industrial and household)***

Alkaline proteases are used to solubilize and hydrolyze the proteins in the wastes. The wastes include proteinaceous materials which come from not only the industries but also the house (Kumar and Takagi 1999). The aim is to obtain bioconversion of the proteinaceous waste into the useful biomass and to decrease the biological oxygen demand of aquatic systems in the biotechnological hydrolising and bioremediation prosesses (deproteinization of shrimp and crabshell waste) (Yang et al. 2000; Gupta et al. 2002). Conversion the waste materials which include fibrous proteins (horn, feather, wool, nail, hair) into the useful biomass is of vital importance in the waste treatment industry (Venugopal et al. 1989).

1.4. Thermophiles

Microorganisms are categorized into three groups depends on the optimal growth temperatures. They are **psychrophiles** (below 20°C), **mesophiles** (moderate temperatures) and **thermophiles** (high temperatures, above 55°C) (Brock 1986; Turner et al. 2007).

Thermophiles are adapted to live at high temperatures. The enzymes isolated from thermophiles are used in most of the commercial applications due to their thermostability and thermoactivity. Thus, the isolation, identification and characterization of the thermophiles from natural sources are of vital significance in terms of discovering new commercial enzymes (Yavuz et al. 2004).

All of the microorganisms have adaptation mechanism to the environment which they live. Thus, thermophiles are used to live at high temperatures and need this condition to survive. They are isolated from terrestrial, subterranean and submarines. Thermophiles include lots of proteins and produce lots of enzymes, so they have resistance to denaturation and proteolysis (Kumar and Nussinov 2001).

Thermophiles are divided into three groups depends on their minimal and maximal growth temperatures. They are **moderate thermophiles** (35 - 70 ºC), **extreme thermophiles** (55 - 85 ºC) and **hyperthermophiles** (75 - 113 ºC) (Baker et al. 2001).

Thermophiles are also classified into three different groups based on the growth and not growth temperatures. They are **obligate thermophiles** (optimum growth temperatures between 65-75 ºC, do not grow below 40 ºC), **facultative thermophiles** (optimum growth temperatures between 50-60 ºC and around 37 ºC), **thermotolerant thermophiles** (optimum growth temperatures between 45-50 ºC and 30 ºC) (Hughes and Williams 1977).

1.4.1. Thermophilic and Alkaliphilic Microorganisms

Thermophilic and alkaliphilic *bacillus* produce thermophilic protease enzyme which has resistance to high temperature, pH, organic solvents, detergents, oxidizing and denaturing agents (Johnvesly and Naik 2001; Hawumba 2002).

1.4.1.1. Thermophilic Bacillus

Bacteria of the genus *Bacillus* is aerobic, facultatively anaerobic, endospore-forming, Gram-positive and rod-shaped. The representatives of this genus are widely distributed in soil and water (deep layers in the ocean, arctic regions, etc.). The ability of certain strains to tolerate high or low temperatures and extreme pHs and to secrete high levels of proteins has made bacilli important as sources of commercial enzymes (Norris et al. 1981). Strains of *Bacillus* are used for the production of enzymes, antibiotics, fine biochemicals and insecticides (Harwood 1989). *Bacillus species* produce several extracellular proteases. For example; *Bacillus cereus, Bacillus sterothermophilus, Bacillus pumilus, Bacillus licheniformis, Bacillus mojavensis, Bacillus megaterium* and *Bacillus subtilis* (Mahmoud et al.; Ammar et al. 1991; Sookkheo et al. 2000; Beg and Gupta 2003; Banik and Prakash 2004; Gerze et al. 2005; Soares et al. 2005).

The genus *Bacillus* is divided into different groups based on the different conditions in the environments. They are temperature (thermophilic and psychrophilic bacteria), pH (acidophilic and alkalophilic bacteria) and carbon sources (freshwater and halophilic bacteria) (Nazina et al. 2001).

The genus *Bacillus species* are categorized into seven phylogenetic groups that are *Alicyclobacillus, Paenibacillus, Brevibacillus, Aneurinibacillus, Virgibacillus Salibacillus* and *Gracilibacillus* (Wisotzkey et al. 1992; Ash et al. 1993; Heyndrickx et al. 1997; Wainø et al. 1999; Sung et al. 2002).

The thermophilic *Bacillus species* are divided into different groups depends on the temperature range (45-70°C) in their growing conditions. They are genera *Bacillus, Alicyclobacillus, Brevibacillus, Aneurinibacillus, Sulfobacillus, Thermoactinomyces* and *Thermobacillus* (Sneath 1986; Wisotzkey et al. 1992; Dufresne et al. 1996; Heyndrickx et al. 1997; Touzel et al. 2000, Nazina et al. 2001).

The groups in the termophilic *Bacillus species* are transferred to the new genus *Geobacillus*. The *Geobacillus* are isolated from the geothermal areas, oilfield, shallow marine vents-hot springs, deepsea hydrothermal vents, artificial hot environments (hot water pipelines, heat exchangers, waste treatment plants, burning coal refuse piles and bioremediation biopiles (McMullan et al. 2004).

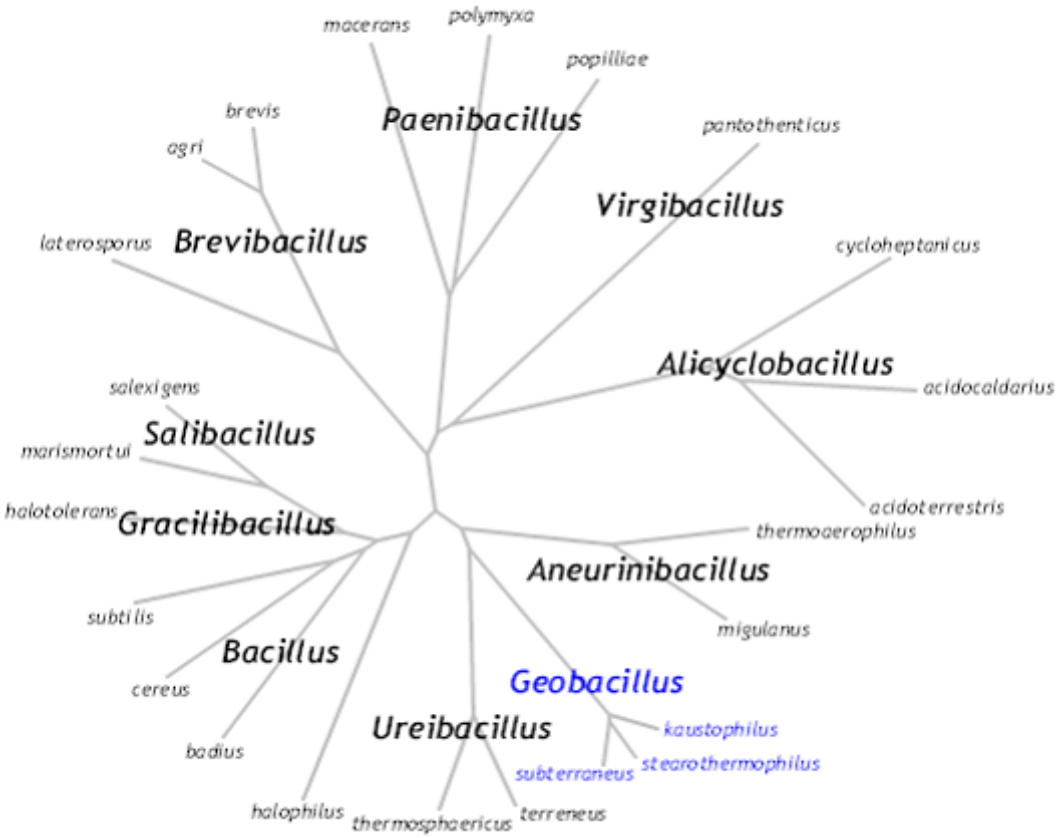


Figure 1.5. Phylogenetic tree based on 16S rRNA gene alignments
(Source: Zeigler et al. 2001)

The genus *Geobacillus* is categorized into five groups. They are *Geobacillus subterraneus*, *Geobacillus uzenensis*, *Geobacillus caldoxylosilyticus*, *Geobacillus toebii*. The *Geobacillus species* are phenotypically and phylogenetically grups of thermophilic *bacillus* due to the similarities of 16S rRNA sequence (98.5-99.2%) *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* are transferred to *Geobacillus stearothermophilus* comb. nov., *Geobacillus thermoleovorans* comb. nov., *Geobacillus thermocatenulatus* comb. nov., *Geobacillus kaustophilus* comb. nov., *Geobacillus thermoglucosidasius* comb. nov. and *Geobacillus thermodenitrificans* comb. nov. in the new genus *Geobacillus*. Most of the thermophilic species grow at temperatures above 55 °C. On the other hand, *Geobacillus species* are the important source of thermophilic enzymes in the biotechnological processes (Nazina et al. 2001; Sung 2002; McMullan et al. 2004).

Table 1.7. Description of the *Geobacillus* genus
(Source: Zeigler 2001; Nazina et al. 2001).

Morphology	
Vegetative cell	Rod-shaped cells, occurring either singly or in short chains and motile by means of peritrichous flagella. The cell wall structure is Gram-positive, but the Gram-stain reaction may vary between positive and negative.
Spore	One ellipsoidal or cylindrical endospore per cell, located terminally or subterminally in slightly swollen or non-swollen sporangia
Colony	Variable shape and size; pigments may be produced on certain media.
Metabolism	
Energy	Chemo-organotrophic
Oxygen	Aerobic or facultatively anaerobic. O_2 is the electron acceptor, replaceable in some species by nitrate.
Temperature	Obligately thermophilic. The growth-temperature range is 37-75°C, with an optimum at 55-65°C.
pH	Growth occurs in a pH range of 6.0 to 8.5, with an optimum at pH 6.2-7.5.
Requirements	Growth factors, vitamins, NaCl and KCl are not required by most species.
Identification tests	
Carbohydrates	Acid but no gas is produced from glucose, fructose, maltose, mannose and sucrose. Most species do not produce acid from lactose.
Enzymes	Most species form catalase. Phenylalanine is not deaminated, tyrosine is not degraded, indole is not produced, the Voges-Proskauer reaction is negative. Oxidase-positive or negative.
Biochemicals	The major cellular fatty acids are iso-15:0, iso-16:0 and iso-17:0, which make up more than 60% of the total. The main menaquinone type is MK-7.
DNA characterization	
G-C content	48.2-58 mol% (thermal denaturation method)
16S rRNA	Sequence identities higher than 96.5% among the members of this genus
Ecology	
Prevalence	Most species are widely distributed in nature.

1.4.1.2. Alkaliphilic Bacillus

Alkaliphilic microorganisms show higher activity in the alkaline environments (for examples: soda soils, desert soils, forest soils, soda lakes, deserts).

The alkaliphilic microorganisms are divided into two groups depends on the range of the optimum pH. They are **alkalophiles** and **alkalotolerants**. The alkalotolerants show activity above pH 10, maximum activity nearly pH 7 (Krulwich 1986). The alkalophiles show activity above pH 10, maximum activity pH 9 and minimum activity belove pH 7. The extreme alkalophiles are subdivided into two groups. They are **facultative alkalophiles** and **obligate alkalophiles**. The facultative alkalophiles grow in not only alkali conditions (pH 10 or above), but also neutral conditions (nearly pH 7). The obligate alkalophiles grow in the neutral conditions (Krulwich 1989).

Alkaline proteases are divided into three goups. They are bacterial-origin fungal-origin and insect-origin. The alkaline protease by bacterial-origin organism is isolated from alkalophiles, which grow at high pH (Anwar and Saleemuddin 1997).

When the enzimatic and physicochemical properties are compared in genus *Bacillus* strains, it is understood that they are distinct from eachother. The genus *Bacillus* and the alkaliphilic *Bacillus spp.* are prolific producers of extracellular proteolytic enzymes and they are the best known source of alkaline proteases and the secretion of these enzymes are used in vaarious industrial applications (Horlkoshi 1971; Kobayashi et al. 1996; Kumar and Takagi 1999).

Alkaline proteases are of vital significance due to being active and stable at higher pH and temperature values (Ward 1993). They also withstand to harsh conditions. That's why, they are chosen to be used in the industrial applications.

With a view to develop an economically feasible technology, research efforts are mainly focused on the improvement in the yields of alkaline proteases and the optimization of the fermentation medium and production conditions (Kumar and Takagi 1999).

Table 1.8. Properties of some alkaline proteases from different microbial sources (Source: Gupta and Beg 2002)

Microorganism	pH optima	Temperature optima (°C)	Substrate specificity	MW (kDa)
Bacteria				
Alcaligenes faecalis	9	55	Casein, BSA, gelatin, azocoll, azocasein	67
Arthrobacter nicotianae 9458	9; 9.5	55–60; 37	α_{S1}- and β-casein	55; 70–72
Bacillus sp. JB99	11	70	Casein	29
Bacillus sp. NG-27	9.2	40	Casein	n.s.
Bacillus sp. KSM-KP43	11	70	Casein	n.s.
Bacillus sp. NCDC-180	11; 12	50; 55	Casein, synthetic *p*-nitroanilides	28; 29
Bacillus sp. PS179	9	75	Azocasein	42
Bacillus sp. SSR1	10	40	Azocasein	29
B. brevis MTCC B0016	10.5	37	Azocasein	n.s.
B. mojavensis	10.5	60	Casein	30
B. pumilis MK6–5	11.5	50–55	*p*-Nitroanilides	28
Oligotropha carboxydovorans DSM 1227	9	60; 50	Casein, azocasein, azocoll, carbon monooxide dehydrogenase	23
Pimelobacter sp. Z-483	9	50	Casein	23
Pseudomonas aeruginosa PST-01	8.5	55	Casein	38
Serratia marcescens ATCC 25419	9.5	48	Azocasein	66.5

1.4.2. Thermophilic Enzymes

Thermophiles produce the thermophilic enzyme based on the fermentation of the microorganisms or the cloning of the genes by using the recombinant DNA technology. Thermophilic and thermostable enzymes are used in the industry because of thermo-activiy and thermo-stability at higher temperatures, chemical and pH stability, reducing the risk of microbial contamination, viscosity. These enzymes are required in the different industrial applications such as detergents, food, paper industry and wastes etc (Haki and Rakshit 2003).

All of the thermophiles grow above 55 ºC and also they are divided into three subgroups depends on the optimal growth temperatures. They are **moderate thermophiles** (above 65 ºC), **extreme thermophiles** (above 75 ºC) and **hyperthermophiles** (above 90 ºC) (Bouzas et al. 2006).

Lipases, proteases, esterases, xylanases and amylases are the most studied thermophilic enzymes.

Table 1.9. Bioconversion reactions and applications of thermostable enzymes (Source: Haki and Rakshit 2003)

Enzyme	Temperature range (°C)	Bioconversions	Applications
α-Amylase (bacterial)	90–100	Starch → dextrose syrups	Starch hydrolysis, brewing, baking, detergents
α-Amylase (fungal)	50–60	Starch → dextrose syrups	Production of maltose
Pullulanase	50–60	Starch → dextrose syrups	Production of glucose syrups
Xylanase	45–65, 105[a]	Craft pulp → xylan + lignin	Pulp and paper industry
Chitinase	65–75[b]	Chitin → chitobiose	Food, cosmetics, pharmaceuticals, agrochemicals
		Chitin → *N*-acetyl glucosamine (chitibiase)	
		N-acetyl glucosamine → glucosamine (deacetylation)	
		Chitin → chitosan (deacetylase)	
Cellulase	45–55, 95[c]	Cellulose → glucose	Cellulose hydrolysis, polymer degradation in detergents
Protease	65–85	Protein → amino acids and peptides	Baking, brewing, detergents, leather industry
Lipase	30–70	Fat removal, hydrolysis, interesterification, alcholysis, aminolysis	Dairy, oleo chemical, detergent, pulp, pharmaceuticals, cosmetics and leather industry

The thermostability of the enzyme was affected by the structures of the protein, but not affected by the purification and crystallization assays. There were two assumptions of the structures in thermostability of the enzyme.

- The enzyme has coiled molecules, so it doesn't lose activity by heating.

- The enzyme has a rigid structure, so it doesn't lose activity by temperature, organic solvents or etc. (Cambell 1955).

The studies of the thermophilic enzymes in the literature, the concentration methods (alcohol-acetone-ammonium sulphate precipitations, ultrafiltration, lyophilization), the purification methods (ion-exchange, gel-filtration, size-exclusion, affinity, hydrophobic interaction, dye ligand chromatography) and the characterization (temperature, pH, molecular, weight, isoelectric point, effects of inhibitors, various agents, metal ions, kinetic parameters, Km-Vmax) are investigated.

1.4.2.1. Thermophilic and Thermostable Proteases

Thermophilic and thermostable proteases are used in most applications due to the fact that they show high activity and stability at high temperatures (Zeigler 2001). Reaching higher temperatures, faster reaction rates, increasing products in the solubility, and reducing risks of microbial contamination are the advantages of the thermostable proteases (do Nascimento 2004). That's why, the thermophilic proteases have increasingly usages in a wide range of commercial applications of the industry (Wasserman 1984; Cowan et al. 1985; Gusek et al. 1988; Sookkheo et al. 2000).

In the biological diversity of the *Bacillus species*. There is no exact evidence that the thermophilic and thermostable proteases are produced not only the the thermophilic microorganisms but also the other species (Rahman 1994; Kaur et al. 2001). The best known sources of thermostable proteases are the thermophilic bacteria as *Bacillus thermoproteolyticus, Thermus aquaticus spp., Bacillus stearothermophilus* in thermal environments.

Table 1.10. Source microorganisms and properties of thermostable proteolytic enzymes (Source: Haki and Rakshit 2003)

Organism	Enzyme properties	
	Optimal temperature (°C)	Optimal pH
Bacillus brevis	60	10.5
Bacillus licheniformis	70	9.0
Bacillus stearothermophilus	60	–
Bacillus stearothermophilus	85	–
Bacillus sp. JB-99	80	6–12
Bacillus stearothermophilus TP26	75	–
Bacillus sp. no. AH-101	80	12.0–13.0
Bacillus thermoruber	45	9
Pyrococcus sp. KODI	100	7
Staphylothermus marinus	–	9
Thermoacidophiles (archeal and bacterial origin)	60–70	7.0–8.5
Thermococcus aggreganes	90	7.0
Thermococcus celer	95	7.5
Thermococcus litoralis	85	8.5
Thermotoga maritema	95	9.5

1.4.3. The Applications of Thermophiles in Biotechnology and Industry

The aim of the termophiles is to catalyze the chemical, biological and biochemical reactions at higher temperatures. Thanks to the thermophiles, high thermostability, thermoactivity and high resistance are obtained against to the chemical denaturants (organic solvents, surfactants, oxidizing agents). Higher reaction rates and reducing the risk of contamination are the advantages in the prosesses of the industry (Kristjansson 1989).

Table 1.11. Main advantages of high temperature and thermostable enzymes (Source: Kristjansson 1989; Niehaus et al.1999; Yavuz et al. 2004)

Property	Advantage in process
-Thermostability - High optimum temperature	Tolerate high temperatures Little activity at low temperatures, long shelf life
-Resistance to denaturing agents	Tolerate organic solvents, high and low pH
-Solubility	High concentrations of poorly soluble compounds
-Viscosity	Decreases, mixing and pumping can be accelerated, mass transfer rate increases
-Microbial contamination	Growth of pathogens and undesired contaminants are prevented
-Reaction rates	Diffusion and chemical reaction rates are accelerated

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

Casein, Folin & Ciocalteu's Phenol reagent, L-tyrosine and Coomassie Brilliant Blue G-250 were purchased from Merck. TCA was purchased from Sigma Aldrich. Cellulose Ion Exchanger, DEAE-Cellulose resin was purchased from Servacel and the other chemicals for electrophoresis and characterization studies were purchased from Sigma Chem. Co.

All of the analyses were carried out triplicate and mean values were reported. The preparation of the buffers, reagents, solutions, the procedure of the assays and the standart curves are given in Appendix A, Appendix B and Appendix C.

2.2. Bacterial Strain and Growth Conditions

In this study, the bacterial strain (alkaliphilic and thermophilic *Geobacillus sp.*) was isolated from an uncontrolled thermal leak of Balçova Geothermal Region in İzmir (Yavuz et al. 2004). In biochemistry research laboratory, the optimum growth conditions of these thermophilic bacterial strains and their bacterial growth curves were determined. The bacterial strain number-53 which has been stored at -86 °C for nearly 6 years was used in this study.

Two different media was used in order to compare the secretion of the proteins in the bacterial strain.

- Alkaliphilic and thermophilic *Geobacillus* was cultivated overnight in 250 ml Erlenmayer flask containing 200 ml LB media, at 55 ºC and 200 rpm. Luria-Bertani broth medium contains 10.0 g/L tryptone, 5.0 g/L yeast extract and 5.0 g/L NaCl per liter with a final pH of 7.0. The medium was sterilised by autoclaving at 121°C for 15 minutes under high pressure.

- Alkaliphilic and thermophilic *Geobacillus* was cultivated overnight in 250 ml Erlenmayer flask containing 200 ml complex media, at 55 ºC and 200 rpm. Complex medium contains 10.0 g/L glucose, 5.0 g/L yeast, 2.5 g/L peptone, 2.5 g/L casein, 0.3 g/L $MgSO_4$, 0.02 g/L $FeSO_4$, 0.2 g/L $ZnSO_4$, 1.0 g/L $CaSO_4$, 1.0 g/L KH_2PO_4, 1.0 g/L K_2HPO_4 per liter with a final pH of 7.0. The medium was autoclaved for sterilization for 15 minutes at 121 °C under high pressure.

2.3. Screening of Protease Activity in Thermophilic *Bacillus sp.*

There are two methods to exhibite the protease activity in the bacterial strains.

- Firstly, the media which consists of 8 g/L nutrient broth, 10 g/L skim milk and 15 g/L agar agar, was prepared. The skim milk was dissolved in deionized water by putting a magnet into the bottle to prevent the precipitation and autoclaved at 110 °C for 5 minutes (two times) and added to the medium. Nutrient broth and agar agar were dissolved in deionized water by putting a magnet into the bottle to prevent the precipitation and they sterilised by autoclaving at 121 °C for 15 minutes. When the bottles' temperatures became nearly 50-55 °C, the skim milk was poured into the nutrient-agar agar. The mixture mixed without heating. The prapared medium was poured into the plates quickly and the plates were put into the refrigerator at +4 °C (Priest et al. 1989).

  The bacterial culture number-53 stored at -86 °C was inoculated onto the plate and incubated for 24 hours - 3 days at 55 ºC. The clear zones around colonies indicated the protease activity.
- Secondly, the bacterial culture was cultivated overnight in LB media or complex media, at 55 ºC and 200 rpm. Bacterial culture was centrifuged at 5000 rpm, 4 ºC for 20 minutes. The activity of the obtain culture supernatant was determined by recording the change in absorbance at 660 nm with the assay mixture at 55 ºC.

2.4. Determination of Enzyme Activity

Protease activity of the samples were determined by the protease activity method using L-Tyrosine (0-1000 mg/L) as the standart. L-Tyrosine standarts were prepared in different concentrations and the standard calibration curve was drawn with response to

their absorbance values. Total protein activity was calculated from standard calibration curve equation. The spectrophotometric assays were performed using Shimadzu UV-VIS spectrophotometer (UV-2450) with a constant temperature water circulator. While determining the protease activity of the samples, the steps should be taken into consideration. 100 µl 0.5% (w/v) casein in 50 mM Tris-HCl pH 7.2 is added to 100 µl enzyme solution and the assay mixture was incubated for 10 minutes at 55 ºC in the water bath. 100 µl 15% TCA in deionized water is added to enzyme-substrate solution to terminate the reaction. The mixture was put in ice bath for 10 minutes and centrifuged at 13500 rpm, room temperature for 10 minutes. The pellet was removed. 1 ml NaOH, 200 µl supernatant and 200 µl Folin & Ciocalteu's Phenol reagent were put into the cuvette, respectively. The reference cuvettes had the same composition except for the enzyme. The changing of the activity was recorded spectrophotometrically in absorbance at 660 nm after an hour. The enzyme activities were given as unit (U) and one unit will hydrolyze casein to produce color equivalent to 1.0 µmole (181.0 µg) of tyrosine per minute under the defined assay conditions.

Calculations: (Folin et al. 1929; Anson 1938)

$$\text{Units/ml enzyme} = \frac{(\mu\text{mole Tyrosine equivalents released})\ (a)}{(b)\ (c)\ (d)} \qquad (2.1)$$

a = Total volume (in milliliters) of assay
b= Time of assay (in minutes) as per the Unit Definition
c = Volume of enzyme (in milliliter) of enzyme used
d = Volume (in milliliters) used in Colorimetric Determination

$$\text{Units/mg solid} = \frac{\text{units/ml enzyme}}{\text{mg solid/ml enzyme}} \qquad (2.2)$$

$$\text{Units/mg protein} = \frac{\text{units/ml enzyme}}{\text{mg protein/ml enzyme}} \qquad (2.3)$$

2.5. Determination of Protein Concentration

Total protein concentration of a sample was determined by Bradford method by using bovine serum albumin (BSA, 0.2 mg/ml) as the standart (Bradford 1976). BSA standards were prepared in different concentrations and the standard calibration curve was drawn with response to their absorbance values. Total protein content was calculated from standard calibration curve equation.

In the Bradford method, the Coomassie Brilliant Blue G-250 dye binds to the proteins which have arginine, lysine and histidine residues and changes the colour. The changing of the absorbance was recorded spectrophotometrically at 595 nm in UV-visible spectrophotometer.

The blank includes 800 µl water and 200 µl bradford reagent. The sample included 2 µl protease enzyme, 798 µl water and 200 µl bradford reagent. The solutions were kept at room temperature for 5 minutes followed by the addition of bradford reagent. The changing of the protein concentration of the sample was recorded spectrophotometrically in absorbance at 595 nm.

According to the results of the absorbance, the total protein concentration was calculated from the standart curve and formula. The spectrophotometric assays were performed using UV-VIS spectrophotometer with a constant temperature water circulator.

2.6. Preparation of Crude Enzyme Extract

Bacterial growth was carried out in erlenmeyer flask in two ways. One of the erlenmeyer flask contained 200 ml of LB media and the other one contained complex media. Bacterial culture was centrifuged at 5000 rpm, 4 ºC for 20 minutes to obtain culture supernatant. The culture supernatant is the crude extracellular enzyme extract. The pellet including cell debris was removed.

2.7. Enzyme Purification Procedure

The crude extracellular enzyme extract was subjected to total protein precipitation with 80% saturation (w/v) ammonium sulphate. The ammonium sulphate (104.6 g) should be slowly added into the enzyme extract by stirring at 4 ºC. After addition, the resulting precipitate was collected by centrifugation at 9500 rpm, 4 ºC for 15 minutes and the pellet was resuspended in 10 ml 50 mM Tris-HCl buffer, pH 7.2.

The enzyme solution was loaded on to the weak anion exchange (DEAE-Cellulose) column (2.5 cm x 10 cm) which was equilibrated with 50 mM Tris HCl buffer (pH 7.2) in loading step. The column was washed with 50 mM Tris HCl buffer (pH 7.2) in washing step. Then, the fractions of 38 ml were collected by gradient eluting with 50 mM Tris HCl buffer (pH 7.2) and 2 M NaCl+50 mM Tris HCl buffer (pH 7.2) in the elution step. The chromatographic assays were performed using FOXY 2000 low pressure liquid chromatography system. The collected fractions were assayed for their protein concentrations at 280 nm with Thermo Scientific nanodrop, 595 nm with Bradford method and protease activity at 660 nm with UV-VIS spectrophotometer. The active fractions were pooled and stored at -20 ºC until using for the electrophoretic and characterization experiments.

2.8. Electrophoretic Studies (SDS- PAGE)

In the electrophoretic study, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used in order to find out the approximate molecular weight of protease enzyme following the Laemmli method (Laemmli, 1970). The electrophoretic assays were performed using Biorad Mini-Protean Tetra System and Biorad Protean II Xicell gel electrophoresis device.

SDS-PAGE is used to seperate the proteins according to their molecular sizes in an electrical and estimate the molecular weight of a protein. Firstly, the seperating gel (acrylamide concentrations of 12%) and stacking gel (acrylamide concentrations of 4%) were prepared. The samples which were in all of the purification steps were diluted by a volume ratio of 1:1 with sample buffer, kept in boiling water at 100 ºC for 10 minutes to denature proteins. 4 µl of molecular weight marker and 20 µl of sample-sample buffer mixture were loaded onto gel. The electrophoretic run was applied at 100 V for 2 hours.

After electrophoretic run, SDS-polyacrylamide gel was stained in two ways.

- **Coomassie Staining:** SDS-polyacrylamide gel was fixed with 20% TCA and incubated for 30 minutes. It was washed with ultrapure water for 3 times to remove the TCA. Then, it was stained by using coomassie staining solution and incubated for 30 minutes. Lastly, the gel was destained with the destaining solution and incubated for 15 minutes for 3 times.
- **Silver Staining:** SDS-polyacrylamide gel was fixed with fixation solution and incubated for 1 hour. It was washed with 50% ethanol for 3 times and incubated for 20 minutes. Then, it was pre-treated with pre-treatment solution and incubated for 1 minute. In order to remove the materials from the gel, it was rinsed with ultrapure water for 3 times and incubated for 20 seconds. In the impregnate step, the gel was impregnated with silver nitrate solution and incubated for 20 minutes. In order to remove the materials from the gel, it was rinsed with ultrapure water for 3 times and incubated for 20 seconds. Later, it was developed with developing solution. When the color development was observed, ultrapure water was added to stop the reaction. Lastly, the gel was kept in the stop solution and incubated for 10 minutes

At the end of these steps, the image of the gels were taken with a special camera under white light by using Biorad Versadoc Gel Visualizing System. The procedure for preparation of gels and reagents that were used in SDS-PAGE are given in Appendix C.

2.9. Characterization Studies

2.9.1. Effect of pH on Enzyme Activity and Stability

The effect of pH on protease activity was investigated by using 50 mM Tris-HCl buffer at different pH values at 55 ºC. The optimum pH of the protease enzyme was determined under the standart assay conditions by measuring activity in the presence of buffers at different pH values (pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0).

While determining the pH stability of the enzyme, 100 µl enzyme solution was mixed with 100 µl 0.5% (w/v) casein in 50 mM Tris-HCl pH 4-7-10. After incubation time (1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} hour), the activity assay was applied. The changing of the absorbance was recorded spectrophotometrically at 660 nm and the residual activity was obtained.

2.9.2. Effect of Temperature on Enzyme Activity and Stability

The effect of temperature on protease activity was investigated by using 50 mM Tris-HCl buffer (pH 7.2) at different temperatures. The optimum temperature of the protease enzyme was determined under the standart assay conditions by measuring the activity at different temperatures (25, 35, 45, 55, 65, 75 ºC). The protease enzyme and the specific substrate casein were heated to relevant temperature before the assay and during the assay.

While determining the temperature stability of the enzyme, 100 µl enzyme solution was mixed with 100 µl 0.5% (w/v) casein in 50 mM Tris-HCl (pH 7.2) at 55-85 ºC. After incubation time (1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} hour), the activity assay was applied. The changing of the absorbance was recorded spectrophotometrically at 660 nm and the residual activity was obtained.

2.9.3. Substrate Specificity of Enzyme

The substrate specificity of the enzyme was determined by measuring activity towards different substrates (casein, azocasein, hammers casein, skimmilk, BSA). The activity of the enzyme was measured by using these different substrates prepared in 50 mM Tris-HCl buffer (pH 7.2) at concentrations of 0.5% (w/v) under the standart assay conditions. The changing of the absorbance was recorded spectrophotometrically at 660 nm and the residual activity was obtained.

2.9.4. Effect of Metal Ions on Enzyme Activity

The effect of the metal ions on protease activity was measured in the presence of different metal ions ($CaCl_2$, $CuSO_4$, $MgSO_4$, NaCl and $ZnCl_2$). 100 µl protease enzyme solution and 100 µl 10 mM metal ion were incubated 55 ºC for 10 minutes. After the incubation period, the substrate was added and the reaction was initiated. Then, the activity of the enzyme was measured under the standart assay conditions. The changing of the absorbance was recorded spectrophotometrically at 660 nm and the residual activity was obtained.

2.9.5. Effect of Various Agents on Enzyme Activity

2.9.5.1. Effect of Organic Solvents on Enzyme Activity

The effect of some organic solvents (ethanol, methanol, hexane, benzene, dimethyl sulfoxide (DMSO)) on enzyme activity were investigated.

100 µl protease enzyme solution and 100 µl 10% (v/v) of the agents were incubated 55 ºC for 10 minutes. After the incubation period, the substrate was added and the reaction was initiated. Then, the activity of the enzyme was measured under the standart assay conditions. The changing of the absorbance was recorded spectrophotometrically at 660 nm and the residual activity was obtained.

2.9.5.2. Effect of Surfactants on Enzyme Activity

The effect of some surfactants (sodium dodecyl sulfate (SDS), Triton X-100, Tween-20, Tween-80, H_2O_2) on enzyme activity were investigated. SDS is an anionic agent and H_2O_2 is an oxidizing agent. Triton X-100, Tween-20 and Tween-80 are nonionic agents.

100 µl protease enzyme solution and 100 µl 1% (v/v) of the agents were incubated 55 ºC for 10 minutes. After the incubation period, the substrate was added and the reaction was initiated. Then, the activity of the enzyme was measured under the standart assay conditions. The changing of the absorbance was recorded spectrophotometrically at 660 nm and the residual activity was obtained.

2.9.6. Effect of Inhibitors on Enzyme Activity

The effect of some inhibitors (phenylmethylsulphonyl fluoride (PMSF), dithiothreitol (DTT), ethylenediaminetetraacetic acid (EDTA), iodoacetamide, protease inhibitor cocktail) on enzyme activity were investigated. PMSF is a serine protease inhibitor and EDTA is a metalloprotease inhibitor. DTT and iodoacetamide are the cysteine protease inhibitors. Protease inhibitor cocktail contains AEBSF (inhibition of serine proteases), EDTA (inhibition of metalloproteases), bestatin (inhibition of

aminopeptidases), pepstatin A (inhibition of acid proteases), E-64 (inhibition of cystein proteases).

100 µl protease enzyme solution and 100 µl agents were incubated 55 ºC for 10 minutes. The concentrations of the agents: 1% (v/v) β-mercaptoethanol and 1 mM of other agents in the assay mixture. After the incubation period, the substrate was added and the reaction was initiated. Then, the activity of the enzyme was measured under the standart assay conditions. The changing of the absorbance was recorded spectrophotometrically at 660 nm and the residual activity was obtained.

Table 2.1. Inhibitors of the endopeptidases
(Source: Garcia-Carreno 1993)

Class	Inhibitor; abbreviation, recommended concentration and suggested enzyme control
Serine	Phenylmethylsulfonyl fluoride (PMSF), 100 mM,* in DMSO or 2-propanol, 2mM†
	Soybean trypsin inhibitor (SBTI), 12 mM,* 240 µM†
	Tosyl-lysine chloromethyl ketone (TLCK), 10 mM,* in 1 mM HC1, pH 3, 100 µM.† Trypsin-inhibitor
	Tosyl-phenylalanine chloromethyl ketone (TPCK), 5 mM,* in MeOH or EtOH, 100 µM.† Chymotrypsin-inhibitor
	Control: trypsin and chymotrypsin (bovine or porcine)
Cysteine	Iodoacetamide (IA), 100 mM,* 2 mM†
	p-Hydroxy-mercuribenzoic acid (PHMB), 50 mM,* 1 mM†
	N-Ethyl-maleimide (NEM), 100 mM,* 2 mM†
	trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (e64), 500 mM,* 10 mM†
	Control: papain
Aspartic	Pepstatin A (assay at acid pH), 50 µg/ml,* in DMSO or MeOH, 1µg/ml†
	Control: cathepsin D, pepsin
Metallo	EDTA 2–20 mM†
	EGTA 2–20 mM†
	1,10-phenanthroline, 100–200 mM,* in DMSO or MeOH, 2-4 mM†
	Control: thermolysin, carboxypeptidase A

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Screening of Protease Activity in Thermophilic *Bacillus sp.*

There are two methods to exhibite the protease activity in the bacterial strain, alkaliphilic and thermophilic *Bacillus sp.*

- Firstly, the bacterial culture number 33, 41, 53 which were positive controls and the bacterial culture number 17, 50, 77, 85 which were negative controls were inoculated on to the plates. After incubation time (24 hours - 3 days), the clear zones around colonies indicated the protease activity in the positive control-plates but there was no clear zones around the colonies in the negative control-plates. Bacterial culture number-53 exhibited highly hydrolysis of the substrate and specific clear zones around the colonies (Figure 3.1).

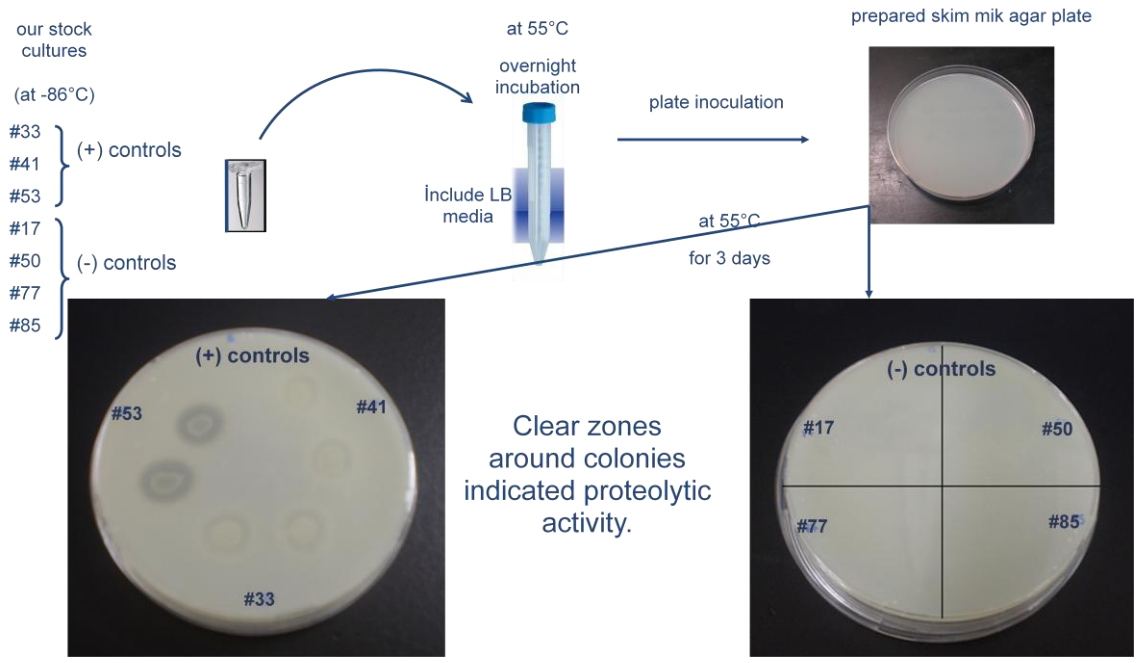


Figure 3.1. Screening of the protease activity

- Secondly, the bacterial culture number 33, 41, 53 were cultivated overnight in LB media. Following the bacterial growth, the activities of the crude enzymes were determined by recording the change in absorbance at 660 nm with the assay mixture at 55 ºC. When compared the results, there was an increase in absorbance in the activity test of bacterial culture number-53.

According to these results, the bacterial culture number-53 produced protease enzyme more than the other bacterial cultures and it was used in the experiments. The bacterial culter number was chosen and also the specific growth media should be chosen to obtain high activity and high yield during the experiments. That's why, a complex medium including different ions and Luria-Bertani broth medium were prepared. Following the bacterial growth, the activities of the crude enzymes were determined by recording the change in absorbance at 660 nm with the assay mixture at 55 ºC. In comparison to the LB media, there was an increase in absorbance by using the complex medium. According to these results, the complex media was chosen as a specific growth media.

3.2. Purification of Protease

In this study, the protease enzyme from alkaliphilic and thermophilic *Bacillus sp.* was purified by using the ammonium sulphate precipitation (80%) and ion exchange chromatography.

Firstly, it should be decided that the protease enzyme was an extracellular or intracellular enzyme. Thus, the crude extracellular enzyme extract and the crude intacellular enzyme extract were produced. Following the bacterial growth, the activities of them were determined by recording the change in absorbance at 660 nm with the assay mixture at 55 ºC. When compared with the results, the extracellular enzyme showed high activity. These results suggested that the protease enzyme from alkaliphilic and thermophilic *Bacillus sp.* was an extracellular enzyme. That's why, the crude extracellular enzyme was used as a starting material in the experiments.

Later, the crude extracellular enzyme was subjected to one of the precipitation methods to precipitate, purify and concentrate total protein. Ammonium sulphate precipitation with different saturations from 20% to 90%, ethanol precipitation and aseton precipitation were used. Following precipitations, the activities of the partial

purified enzymes were determined by recording the change in absorbance at 660 nm with the assay mixture at 55 ºC. In comparison to other techniques and different saturations, there was an increase in absorbance by using the ammonium sulphate precipitation with 80% saturation. The partial purified enzyme with ammonium sulphate precipitation (80% saturation) was assayed for the protein concentrations at 280 nm with Thermo Scientific nanodrop, 595 nm with Bradford method and protease activity at 660 nm with UV-VIS spectrophotometer. The yield and purification fold after this step was 80% and 1.17, respectively (Table 3.1).

Finally, the precipitate was dissolved in 50 mM Tris HCl buffer (pH 7.2) and loaded on to the weak anion exchange (DEAE-Cellulose) column which separates the proteins according to their ionic strength. After the washing step, the fractions were collected by gradient eluting from the column. The protein concentrations at 280 nm with Thermo Scientific nanodrop and the protease activity at 660 nm with UV-VIS spectrophotometer were determined. The active fractions were collected and pooled to be used in the experiments of characterization (Figure 3.2). The resulting enzyme solution which had a specific activity of 135.66 U/mg, was purified 1.41 fold and contained 33% of the activity (Table 3.1).

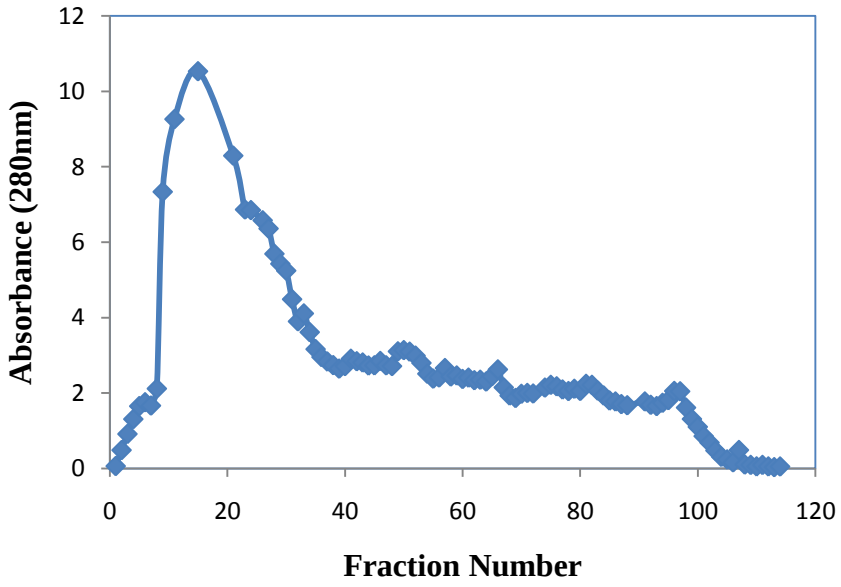


Figure 3.2. The concentration of the protease enzyme fractions after ion exchange chromatography

Table 3.1. Purification of protease from alkaliphilic and thermophilic *Bacillus sp.*

Purification Step	Total Activity (U/ml)	Total Protein (mg/ml)	Specific Activity (U/mg)	Yield (%)	Purification (Fold)
Crude Extract	894.75	9.33	95.85	100	1
Ammonium Sulphate Precipitation	723	6.43	112.27	80	1.17
Ion Exchange Chromatography	296	2.182	135.66	33	1.41

When the purification fold and yield values of proteases were compared in the literature, similar and different results were observed. *Bacillus stearotherrnophilus* RM-67 protease which had 39.5 fold purification and an overall recovery of 8.21% was purified by ammonium sulfate fractionation (40 to 70% saturation), gel filtration through Sephadex G-100, and diethylaminoethyl-Sephadex A-50 ion-exchange chromatography (Chopra and Mathur 1983). *Bacillus polymyxa* B-17 protease which had 40.38 fold purification and an overall recovery of 78.35% was purified by precipitation with ammonium sulfate and gel filtration through Sephadex G-100 (Matta and Punj 1998). *Bacillus cereus* protease which had 14.5 fold purification and an overall recovery of 7.2% was purified by ammonium sulfate fractionation, acetone precipitation, Bio-Gel filtration and column chromatography on DEAE-cellulose (DE-52 cellulose) (Sierecka 1998). *Bacillus cereus* protease which had 77 fold purification and an overall recovery of 38% was purified by ion exchange chromatography (DEAE-cellulase and CM-Toyopearl 650) and gel filtration through Sephadex G-100 (Takii et al. 1998). Alkalophilic *Bacillus* spp. protease which had 27.7 fold purification and an overall recovery of 6% was purified by acetone precipitation, DEAE- and CM-Sepharose CL-6B ion exchange and Sephacryl S-200 gel filtration chromatographic techniques (Kumar 1998). Alkaliphilic and thermophilic *Bacillus sp.* PS7 19 protease which had 18.5 fold purification and an overall recovery of 39% was purified by ammonium sulfate precipitation, DEAE-cellulose and a-casein agarose column

chromatographies (Hutadilok-Towatana et al. 1999). Thermostable proteases from *Bacillus stearothermophilus* TLS33 which had 2.6 fold purification and an overall recovery of 7% was purified by lysine affinity chromatography, strong anion exchange Q HyperD chromatography, and Ultrogel AcA44 gel filtration (Sookkheo et al. 2000). Heat-stable alkaline protease from *Bacillus stearothermophilus* F1 which had 4.0 fold purification and an overall recovery of 99% was purified by heat treatment (Fu et al. 2003). Heat-stable alkaline protease from *Bacillus subtilis* which had 7.87 fold purification was purified by ammonium sulfate precipitation and Sephadex G200 filtration (El-Safey et al. 2004). An alkaline protease from haloalkaliphilic *Bacillus sp.* which had 10.0 fold purification and an overall recovery of 82% after purification was purified by a single step method on Phenyl Sepharose 6 Fast Flow column (Gupta and Roy et al. 2005). Thermophilic neutral protease from *Thermophilic bacillus* strain HS08 which had 4.25 fold purification and an overall recovery of 5.1% was purified by ammonium sulfate precipitation, with columns of DEAE-Sepharose anion exchange chromatography and Sephacryl S-100HR on AKTA purifier 100 protein liquid chromatography (Guangrong 2006). An extracellular alkaline protease from a novel haloalkaliphilic bacterium (Ve1) which had 112.0 fold purification and an overall recovery of 6% was purified by ammonium sulphate precipitation, DEAE cellulose and Sephadex-200 (Patel et al. 2006). Thermophilic extracellular proteases from *Lactobacillus helveticus* which had 10.31 fold purification and an overall recovery of 15.2% was purified by aceton precipitation, Sephadex G-25 and QAE Sephadex A-50 column (Valasaki et al. 2008).

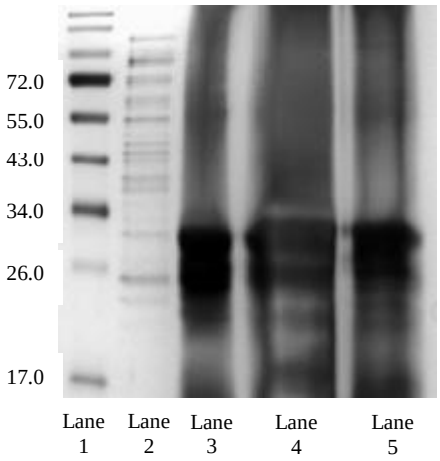
The purification fold and overall recovery of protease enzyme from alkaliphilic and thermophilic *Bacillus* was lower than the values from the literature because the protease enzyme in here was partially purified. On the other hand, the impurities in the partially purified enzyme content may mask the protease activity.

3.3. Electrophoretic Studies (SDS-PAGE)

The samples which were from all of the purification steps were applied to SDS-PAGE. After electrophoretic run, SDS-polyacrylamide gel was stained by coomassie and silver in two ways. The image of the gels were taken with a special camera under white light after silver staining is given in Figure 3.3. The image of the gels were taken

with scanner after silver staining is given in Figure 3.4 and after colloidal coomassie staining is given in Figure 3.5.

The sample from extracellular crude extract prepared by using LB media was very complex so lots of protein bands were observed on the gel. The sample from extracellular crude extract prepared by using complex media was nearly specific. Only two bands were observed on the gel because complex media contains specific substrate in growth conditions. The molecular weight of the enzymes could be determined by these two bands on the gel and they were 25.37 kDa and 29.09 kDa.



- ***Lane-1:*** Protein marker,
- ***Lane-2:*** Extracellular crude extract prepared by using LB media,
- ***Lane-3:*** Extracellular crude extract prepared by using complex media,
- ***Lane-4:*** Sample prepared by using complex media after ammonium sulphate precipitation,
- ***Lane-5:*** Sample prepared by using complex media after ion exchange chromatograpy.

Figure 3.3. SDS-PAGE image with silver staining under UV light.

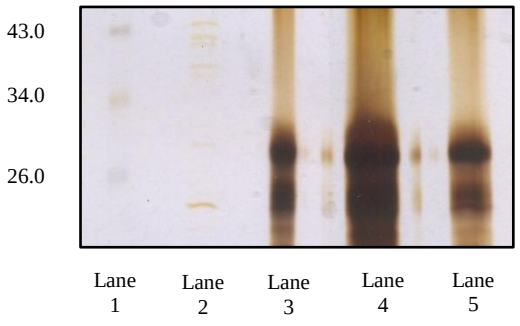


Figure 3.4. SDS-PAGE image with silver staining.

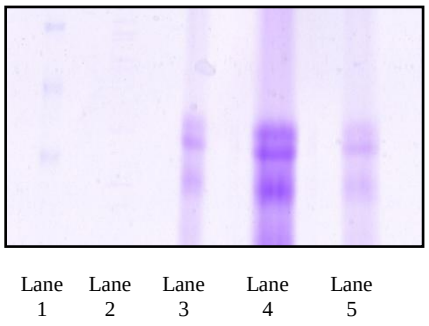


Figure 3.5. SDS-PAGE image with coomassie staining.

When the molecular weights of proteases were compared in the literature, similar and different results were observed.

The molecular weight of protease from thermophilic *Bacillus* strain HS08 is 30.9 kDa (Guangrong et al. 2006), molecular weight of thermostable protease from *Geobacillus sp.* YMTC 1049 is 59.2 kDa (Zhua et al. 2007), molecular weight of thermostable protease from *Hyperthermophilic Bacillus* strain HUTBS71 is 49 kDa (Akel et al. 2009), molecular weight of an alkaline protease from haloalkaliphilic *Bacillus sp.* is 29 kDa (Gupta, Roy et al. 2005), molecular weight of an alkaline protease from haloalkaliphilic *Bacillus sp.* is 30 kDa (Patel et al. 2005), molecular weight of extracellular protease isolated from alkaliphilic and thermophilic *Bacillus sp.* PS719 is 42 kDa (Hutadilok-Towatana et al. 1999),

The molecular weights of proteases from *Bacillus stearothermophilus* RM-67 are 67.610 and 19.950 kDa (Chopra and Mathur 1985), molecular weights of thermostable proteases from *Bacillus stearothermophilus* TLS33 are *36* kDa, 53 kDa and 71 kDa (Sookkheo et al. 2000).

The molecular weight of thermophilic alkaline proteases resistant to SDS and EDTA from *Bacillus sp.* GUS1 is 30-47 kDa (Seifzadeh et al. 2008), molecular weight of protease from *Bacillus* strain SAL1 is 27 kDa (Almas et al. 2009), molecular weight of a surfactant-stable high-alkaline protease from *Bacillus sp.* B001 is 28 kDa (Deng et al. 2010).

The molecular weight of an extracellular protease from *Bacillus subtilis* EAG-2 strain is 27 kDa (Ghafoor and Hasnain 2010), molecular weight of an extracellular alkaline protease produced by *Bacillus subtilis* (MTTC N0-10110) is 20.5 kDa (Ramakrishna et al. 2010).

The molecular weight of neutral protease from *Bacillus cereus is 29 kDa* (Sierecka 1998), molecular weights of alkaline proteases produced by an alkalophilic *Bacillus* are *28* kDa and 29 kDa (Kumar et al. 1999), molecular weights of proteases from *Bacillus cereus* MCM B-326 are 36 kDa and 45 kDa (Nilegaonkar et al. 2006), molecular weight of a solvent and detergent-stable protease from *Bacillus cereus* is 28 kDa (Doddapaneni et al. 2009), molecular weight of a calciumdependent protease by *Bacillus cereus* BG1 is 34 kDa (Ghorbel-Frikha et al. 2005), molecular weight of a solvent, detergent and oxidizing agent tolerant protease from *Bacillus cereus* is 38 kDa (Shah et al. 2010).

The molecular weight of an alkaline protease from *Bacillus circulans* BM15 is 30 kDa (Venugopal and Saramma 2007), molecular weight of thermo- and detergent stable serine protease from isolated *Bacillus circulans* is 39.5 kDa (Rao et al. 2009).

The molecular weight of a surfactant-stable alkaline serine-protease from *Bacillus mojavensis* A21 is 20 kDa (Haddar et al.2009), molecular weight of an extracellular, thiol-dependent and oxidation-stable alkaline serine protease from *Bacillus mojavensis* is 30 kDa (Beg and Gupta 2003).

The molecular weight of thermostable extracellular protease from *Bacillus polymyxa* B-17 is 30 kDa (Matta and Punj 1998), molecular weight of an extracellular serine alkaline protease of *Bacillus clausii* GMBAE 42 is 26.5 kDa (Kazan et al. 2005), molecular weight of protease from *Bacillus Firmus* Tap5 is 34 kDa (Joshi 2010), molecular weight of an alkaline-protease from *Bacillus licheniformis* MP1 is 30 kDa (Jellouli et al. 2011), molecular weights of proteases from Pseudomonas fluorescens are between 47 kDa and 51 kDa (Margesin and Schinner 1992).

3.4. Characterization Studies

3.4.1. Effect of pH on Enzyme Activity and Stability

The effect of pH on protease activity was examined by measuring enzyme activity in the presence of buffers at different pH values (pH 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0). In the Figure 3.6, the effect of pH showed a bell shaped curve with the highest activity at pH 8.0. On the other hand, the optimum pH of the protease enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* was pH 8.0. The protease enzyme at acidic pH values exhibited a little lower activity than the protease enzyme at alkaline pH values. The enzyme lost 20% of its activity at pH 4.0 and it lost 10% of its activity at pH 10.0. When the activities at different pH values were compared, it was understood that this enzyme was nearly stable in these conditions because of having above the 80% of its activity at all of the pH values.

When the pH values were compared in the literature, similar and different results were observed in acidic, neutral or alkaline conditions.

The proteases which were secreted in the acidic conditions were isolated from *Geobacillus* at pH 6.5 (Hawumba et al. 2002).

The proteases which were produced in the neutral conditions were isolated from *Bacillus thermoproteolyticus* (Ohta 1966), *Bacillus circulans* (Venugopal and Saramma 2007) and *Lactobacillus helveticus* (Valasaki 2008) at pH 7.0. *Bacillus polymyxa* (Matta and Punj 1998), *Bacillus* strain HS08 (Guangrong et al. 2006), *Geobacillus sp*. YMTC 1049 (Zhu et al. 2007) produced protease enzyme at pH 7.5. They were also isolated from *Bacillus stearothermophilus* TLS33 at pH 7.0-7.5 (Sookkheo et al. 2000), *Bacillus brevis* MIB001 at pH 7.6 (Takii et al. 1998), hyperthermophilic *Bacillus* strain HUTBS71 at pH 7.8 (Akel et al. 2009).

The proteases which were secreted in the alkaline conditions were isolated from *Bacillus stearotherrnophilus* RM-67(Chopra and Mathur 1983), thermophilic *Bacillus* (do Nascimento and Martins 2004) and *Bacillus cereus* BG1 (Ghorbel-Frikha et al. 2005) at pH 8.0. They were also isolated from thermophilic *Bacillus sp*. at pH 8.5 (Silva et al. 2007), alkaliphilic and thermophilic *Bacillus sp*. PS719 at pH 9.0 (Hutadilok-Towatana et al. 1999), *Bacillus species* at pH 9.0 (Naidu and Devi 2005), *Bacillus sp.* P-2 at pH 9.6 (Kaur et al. 2001), *Bacillus mojavensis* at pH 10.5 (Beg and Gupta 2003), thermophilic and alkaliphilic *Bacillus sp*. JB-99 at pH 11 (Johnvesly and Naik 2001), haloalkalophilic *Bacillus clausii* at pH 11.5 (Kumar et al. 2004), alkalophilic *Bacillus spp.* at pH 11-12 50-55 ºC (Kumar et al. 1999).

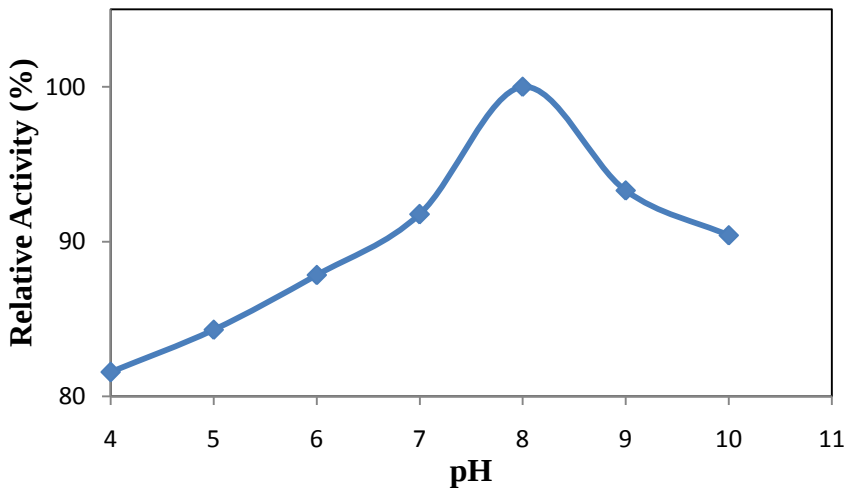


Figure 3.6. Effect of pH on protease activity (at 55 ºC, for 10 min.)

The pH stability of the protease enzyme was investigated by incubating the enzyme in the buffers at different pH (pH 4-7-10) during 6 hours. After the incubation

time (1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} hour), the residual activities were measured spectrophotometrically under standart assay conditions. They were calculated and plotted against pH values as in Figure 3.7. While preparing the control, the pH treatment was not applied to the enzyme and the activity of the protease enzyme was regarded as hundred percent.

Three pH values (pH 4-7-10) were examined to compare the differences of the stabilities. It was understood from the figure that,

- The enzyme at pH 4 retained 85% of its activity after an hour and lost 50% of its activity after six hours incubation time.
- The enzyme at pH 7 retained more than 90% of its activity after an hour and lost 40% of its activity after six hours incubation time.
- The enzyme at pH 10 retained more than 95% of its activity after an hour and lost 45% of its activity after six hours incubation time.

The results showed that the protease enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* was stable at different pH values (pH 4-7-10) during 3 hours but after 3 hours, there was a significant loss in the stability.

When the pH stabilities were compared in the literature, similar and different results were observed. For examples; the purified protease I was stable in the pH range of 8.8-10.5 and protease II was stable at pH 11.5 when incubated at 37 °C for 30, 60, and 90 min (Chopra and Mathur 1983). In the acidic conditions, the activity of the protease enzyme was stable in the range of 4.0-6.0. The protease showed high stability at pH 9.0. When the pH values were lower or higher than 9.0, the enzyme activity decreased nearly 70% at pH 7.0 and 50% at pH 11.0 (Hutadilok-Towatana et al. 1999). The thermostable protease S showed nearly 60% of proteolytic activity in the pH range of 6-10. Proteases N and B retained little activity above pH 9.0 (Sookkheo 2000). The protease was stable at pH 9.6 and retained more than 80% of its activity in the pH range of 7-10 (Kaur et al. 2001). The purified protease enzyme was stable at 70 °C for 24 hours in the pH range from 8.0 to 10.0 (Fu et al. 2003). There was a decrease of nearly 51% of the activity at pH 5.5, 18% of the activity at pH 8.0 and 66% of the activity at pH 9.0 for 24 hours (do Nascimento and Martins 2004). A decrease of about 15% of the activity at pH 8.5 and of the activity at pH 10.0 were observed (Silva et al. 2007).

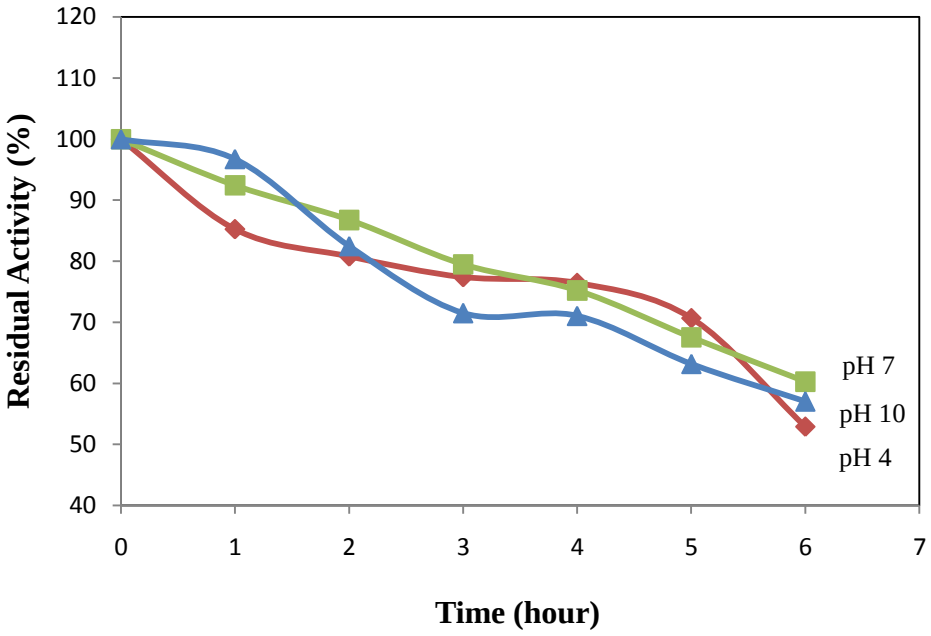


Figure 3.7. pH stability of protease (at 55 ºC, for 6 hours)

3.4.2. Effect of Temperature on Enzyme Activity and Stability

The effect of temperature on protease activity was examined by measuring enzyme activity at different temperatures ranging from 25 ºC to 75 ºC. In the Figure 3.8, the effect of temperature showed a bell shaped curve with the highest activity at 55 ºC. On the other hand, the optimum temperature of the protease enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* was 55 ºC. The protease enzyme at temperatures below 55 ºC lost 15% of its activity and also the protease enzyme at temperatures above 55 ºC lost 25% of its activity. When the activities at different temperatures were compared, it was understood that this enzyme was nearly stable in these conditions because of having above the 75% of its activity at all of the temperatures.

When the optimum temperature values were compared in the literature, similar and different results were observed. The protease was highly produced by *Bacillus circulans* at 40 ºC (Venugopal and Saramma 2007), *Bacillus polymyxa* at 50 ºC (Matta and Punj 1998), alkalophilic *Bacillus spp.* at 50-55 ºC (Kumar et al. 1999), *Bacillus species* 55 ºC (Naidu and Devi 2005), *Bacillus mojavensis* at 60 ºC (Beg and Gupta 2003), thermophilic *Bacillus* at 60 ºC (do Nascimento and Martins 2004), *Bacillus*

cereus BG1 at 60 ºC (Ghorbel-Frikha et al. 2005), *Lactobacillus helveticus* at 60 ºC (Valasaki 2008), *Bacillus* strain HS08 at 65 ºC (Guangrong et al. 2006), hyperthermophilic *Bacillus* strain HUTBS71 at 65 ºC (Akel et al. 2009), *Bacillus stearotherrnophilus* RM-67 at 70 ºC (Chopra and Mathur 1983), thermophilic and alkaliphilic *Bacillus sp.* JB-99 at 70 ºC (Johnvesly and Naik 2001), *Geobacillus* at 70 ºC (Hawumba et al. 2002), thermophilic *Bacillus sp.* at 70 ºC (Silva et al. 2007), *Bacillus stearothermophilus* TLS33 at 72-78 ºC (Sookkheo et al. 2000), *Bacillus brevis* MIB001 75 ºC (Takii et al. 1998), alkaliphilic and thermophilic *Bacillus sp.* PS719 at 75 ºC (Hutadilok-Towatana et al. 1999), *Bacillus thermoproteolyticus* at 80 ºC (Ohta 1966), *Geobacillus sp.* YMTC 1049 at 85 ºC (Zhu et al. 2007), *Bacillus sp.* P-2 at 90 ºC (Kaur et al. 2001), haloalkalophilic *Bacillus clausii* at 80 ºC (Kumar et al. 2004).

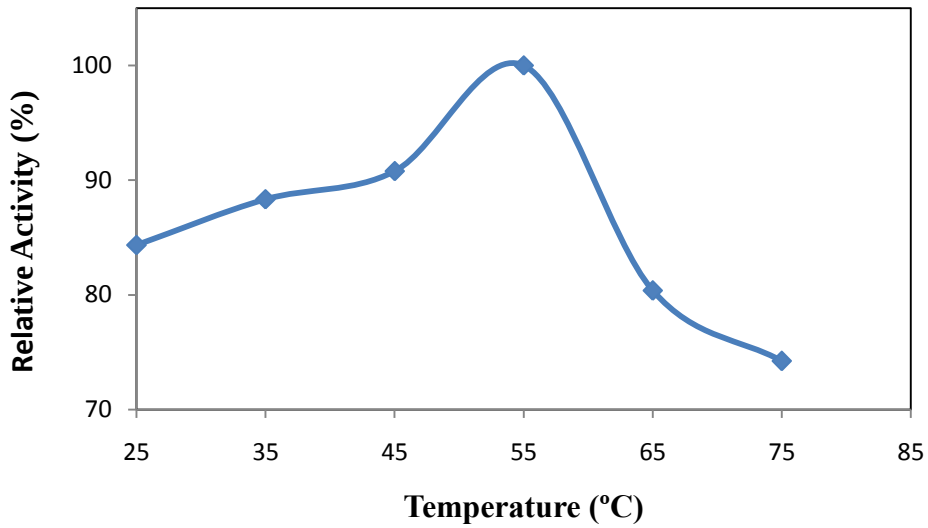


Figure 3.8. Effect of temperature on protease activity (at pH 7.2, for 10 min.)

The temperature stability of the protease enzyme was investigated by incubating the enzyme at different temperatures (55-85 ºC) during 6 hours. After the incubation time (1^{st}, 2^{nd}, 3^{rd}, 4^{th}, 5^{th} and 6^{th} hour), the residual activities were measured spectrophotometrically under standart assay conditions. They were calculated and plotted against temperature values as in Figure 3.9. While preparing the control, the temperature treatment was not applied to the enzyme and the activity of the protease enzyme was regarded as hundred percent.

Two temperature values (55-85 ºC) were examined to compare the differences of the stabilities. It was understood from the figure that,

- The enzyme at 55 ºC retained more than 95% of its activity after an hour and lost about 3% of its activity after six hours incubation time.
- The enzyme at 85 ºC retained more than 95% of its activity after an hour and lost about 15% of its activity after six hours incubation time.

The results showed that the protease enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* was stable at different temperature values (55-85 ºC) during 6 hours because there was not a significant loss in the stability.

When the temperature stabilities were compared in the literature, similar and different results were observed. For examples; protease I and II from *Bacillus stearotherrnophilus* RM-67 retained 100% activity at 60 °C for 30 min. The purified protease enzymes were stable at 55 °C for 30 min. Protease I retained 63% of its activity and protease II retained 20% of its activity after 30 min at 65 °C (Chopra and Mathur 1983). Thermostable neutral protease was stable up to 75 °C and it lost 60% of its activity at 85 °C (Takii et al. 1998). The protease enzyme retained 35% activity at 70 °C for 10 min. There was no activity at 85 °C after 10 min (Matta and Punj 1998). The purified enzyme was stable at temperatures below 40 °C for 15 min. This protease enzyme was retained 60% of its activity at higher temperatures and lost the activity at 90°C 15 min later (Hutadilok-Towatana et al. 1999). Highly thermostable proteases S (at 60 °C), N (at 65 °C), and B (at 70 °C) retained more than 90% of the activity. They lost 50% of the activity at 72, 78, and 90 °C, respectively. After the incubation at 100 °C for 30 min, the protease B, S and N had 31%, 5%, 10% of their initial activities, respectively (Sookkheo 2000). The enzyme was stable at 90 °C for more than 1 hour. It also retained 95% of its activity at 99 °C and 37% of its activity at 121 °C after 1 hour. The half life of protease at 121 °C was 47 minutes (Kaur et al. 2001). The enzyme was stable for 2 hours at 30 ºC. 14% of the activity was lost at 40 ºC and 84% of the activity was lost at 80 ºC (do Nascimento and Martins 2004). The protease enzyme retained 80% of the activity at 60 ºC 2 hours later. 70% of the activity was retained at 70 ºC, 15 min later (Silva et al. 2007). The present enzyme was stable at 50 ºC and 60 ºC for 2 hours and it retained 84% of its activity (Akel et al. 2009).

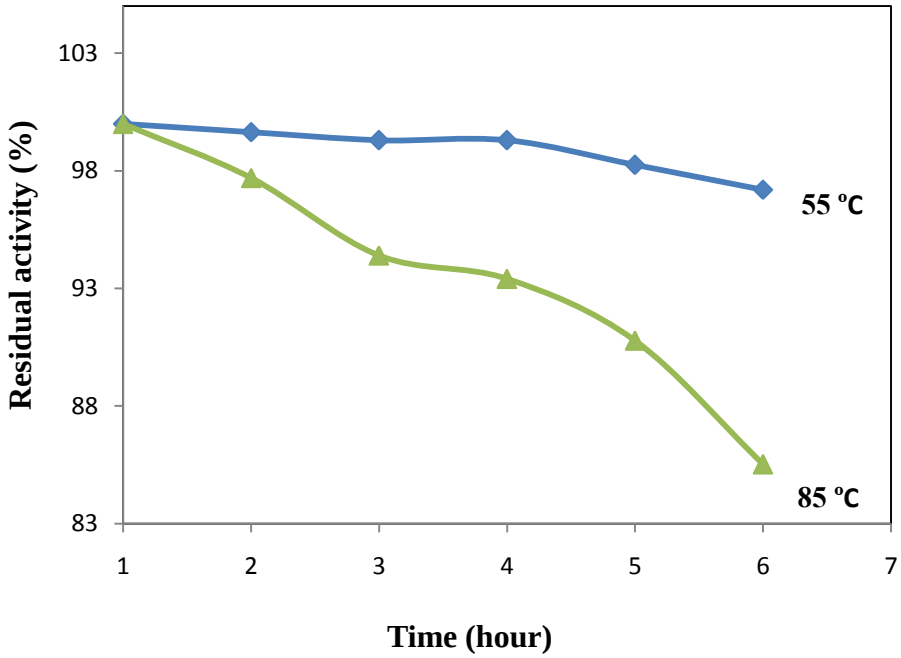


Figure 3.9. Thermal stability of protease (at pH 7.2, for 6 hours)

3.4.3. Substrate Specificity of Enzyme

The substrate specificity of the protease enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* was determined by measuring activity towards casein (5%), azocasein (5%), hammersten casein (5%), skimmilk (5%), BSA (5%) under the assay conditions. In the Table 3.2, the results showed that the casein was the best substrate among them and the azocasein decrease the activity of the protease.

Table 3.2. Substrate specificity of protease (at 55 ºC, pH 7.2, for 10 min.)

Substrate	Wavelength (nm)	Relative Activity (%)
Casein	660 nm	100
Skim milk	660 nm	98.57
Hammersten casein	660 nm	97.5
BSA	660 nm	87.14
Azocasein	440 nm	6.68

When the substrate specificities were compared in the literature, similar and different results were observed. The substrates which were used in the experiments were haemoglobin, albumin and casein (Sierecka 1998), gelatin (El-Safey and Abdul-Raouf 2004), starch (do Nascimento and Martins 2004), hammarsten casein (Kazan et al. 2005). In the previous studies, alkaline proteases exhibited highest activity towards casein or modified proteins including haemoglobin, ovalbumin, BSA, fibrin, collagen, elastin, keratin, azocasein and azocoll. The purified proteases hydrolyzed the protein substrates such as casein, elastin, keratin, albumin and the synthetic chromogenic peptide substrates Glu-Gly-Ala-Phe-pNA and Glu-Ala-Ala-Ala-pNA and the results exhibited thar casein was the best substrate among them (Kumar et al. 1999). The protease hydrolyzed the substrates, such as gelatin, elastin, albumin, haemoglobin, and skim milk (Beg and Gupta 2003). Substrates specificity results showed that azocasein was the best substrate among the azocasein, casein and BSA (Guangrong et al. 2006).

3.4.4. Effect of Metal Ions on Enzyme Activity

The effect of the metal ions on protease activity was measured in the presence of different metal ions (Ca^{+2}, Cu^{+2}, Mg^{+2}, Na^{+1} and Zn^{+2}). The protease enzyme solution and 10 mM metal ion were incubated for 10 minutes at 55 ºC. After the incubation period, the substrate was added and the reaction was initiated. Then, the activity of the enzyme was measured under the standart assay conditions. While preparing the control, the metal ion was not added into the enzyme solution and its activity was regarded as hundred percent. In the Figure 3.10, the results showed that Ca^{+2} and Cu^{+2} increase the activity of the protease, but Mg^{+2}, Na^{+1} and Zn^{+2} did not affect the activity of the protease so much. According to these results, these metal ions protected the enzyme against thermal denaturation. It was also known that Ca^{+2} is the most effective ion on the activity of the proteases.

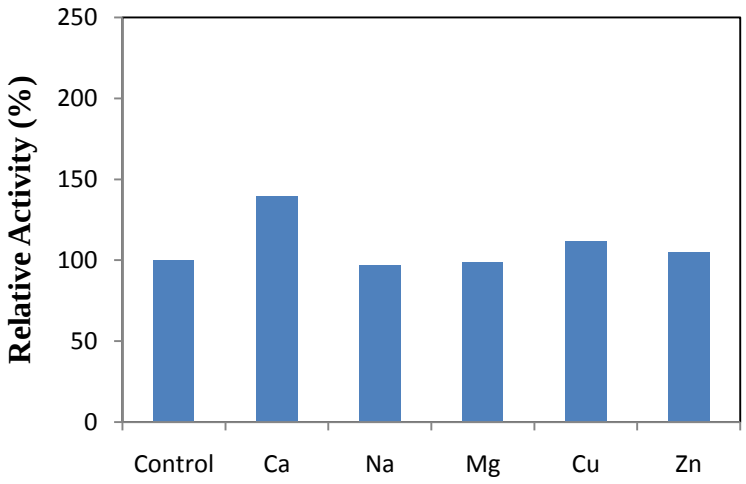


Figure 3.10. Effect of metal ions on protease activity (at 55 ºC, pH 7.2, for 10 min.)

When the effects of metal ions on protease activity were compared in the literature, similar and different results were observed. In the presence of manganese, the protease I enzyme from *Bacillus stearotherrnophilus* RM-67 exhibited 100% stability at 65°C for 30 min (Chopra and Mathur 1983). There was an inhibition on the enzyme activity in the presence of Na, K, Mg and Co. However, Ca and Mn increased the activity (Matta and Punj 1998). Ca^{2+}, Mg^{2+} and Mn^{2+} increased the enzyme activity of the enzymes. Hg^{+2} ions (1 mM) exhibited highly inhibition of the enzymes, by 74% (Kumar et al. 1999). Ca^{2+} was activated the enzyme causing 85% stimulation. However, Mn^{2+} and Mg^{2+} showed fewer positive effects. Zn^{2+}, Fe^{2+}, Co^{2+} and Cu^{2+} inhibited the enzyme activity, bringing about 15%, 33%, 57% and 65% decreases, respectively. 2 mM Ca^{2+} concentration on the enzyme activity was determined (Hutadilok-Towatana et al. 1999). Addition of Ca^{2+} (10 mM) and glycine (1 M) increased the half life of protease. The enzyme retained more than 50% activity after 4 days at 60°C in the presence of them (Banerjee et al. 1999). The proteolytic activity was increased in the presence of $ZnCl_2$. The addition of Na^{+}, K^{+}, Li^{+}, Ca^{2+}, and Mn^{2+} did not affect the activities of the apoenzymes. The addition of Zn^{2+} increased the activities of proteases changing from 54% to 72% of the enzyme activity (Sookkheo et al. 2000). The addition of 10 mM Ca^{2+} retained 78% activity after 1 hour at 80 °C. The enzyme activity was increased in the presence of 10 mM metal ions namely Mn^{2+}, Mg^{2+}, Cu^{2+} and Co^{2+}. The activity also was inhibited in the presence of 10 mM Fe^{3+}, Hg^{2+} and Zn^{2+} (Johnvesly and Naik 2001).

1mM K^{+}, 1mM Hg^{2+} and 1mM Cu^{2+} were strongly inhibited the enzyme activity. Mn^{2+} and Ca^{2+} were stimulated the activity and they also had a functional role in the molecular structure of the enzyme (do Nascimento and Martins 2004). There was no effect in the presence of Mn^{2+}, Zn^{2+} and Mg^{2+}. The enzyme was activated with Ca^{2+} (1 mM) and Cu^{2+} (5 mM) (Gupta et al. 2005). The enzyme activity was increased by $Ca^{2+}$and Mg^{2+} but inhibited by Ba^{2+}, Zn^{2+}, Pb^{2+}, Co^{2+}, Mn^{2+} and Cu^{2+} (Zhu et al. 2007). The activity of the enzyme was increased in the presence of 5 mM Mn^{2+}, $Ca^{2+}$and Na^{2+}; not affected or slightly inhibited by Fe^{2+}, Co^{2+}, and Zn^{2+}, but inhibited remarkably by EDTA, $Cu^{2+}$and Cd^{2+} (Akel et al. 2009).

3.4.5. Effect of Various Agents on Enzyme Activity

The effect of the organic solvents, surfactans and detergents on the enzyme activity were investigated to determine whether they acted as inhibitor or activator. The protease enzyme solution and 10% of the organic solvent or 1% surfactant were incubated for 10 minutes at 55 ºC. After the incubation period, the substrate was added and the reaction was initiated. Then, the activity of the enzyme was measured under the standart assay conditions. While preparing the control, the organic solvents or the surfactants were not added into the enzyme solution and its activity was regarded as hundred percent.

3.4.5.1. Effect of Organic Solvents on Enzyme Activity

Benzene did not cause much effect on the activity and the protease enzyme showed nearly 96% of its activity in the presence of benzene. On the other hand, ethanol caused much effect on the activity among the other organic solvents and the protease enzyme showed nearly 84% of its activity in the presence of ethanol. In the Table 3.3, the results indicated that alkaliphilic and thermophilic protease enzyme was stable in the presence of 10% of these organic solvents because there was a little loss on the protease activiy in the experiments.

Table 3.3. Effect of organic solvents on protease activity (at 55 ºC, pH 7.2, for 10 min.)

Organic Solvents	Concentrations	Relative Activity (%)
Control	-	100
Ethanol	10%	84.77
Methanol	10%	87.24
Hexane	10%	92.6
DMSO	10%	92.18
Benzene	10%	96.7

When the effects of the organic solvents on protease activity were compared in the literature, similar and different results were observed. All the organic solvents such as methanol, ethanol and isopropanol induced the enzyme activity of both the proteases (Kumar et al. 1999). The protease activity was increased by the water immiscible solvents except butanol as compared to water miscible solvents. The protease activity was higher in the presence of decane, hexadecane, hexane, cyclooctane and toluene, benzene. On the other hand, the protease activity was decreased in the presence of water miscible solvents like ethanol (70% activity) and DMSO (80% activity) (Shah 2010).

3.4.5.2. Effect of Surfactants on Enzyme Activity

Triton X-100 did not cause much effect on the activity and the protease enzyme showed nearly 97% of its activity in the presence of Triton X-100. On the other hand, H_2O_2 caused much effect on the activity among the other surfactants and the protease enzyme showed nearly 81% of its activity in the presence of H_2O_2. In the Table 3.4, the results indicated that alkaliphilic and thermophilic protease enzyme was stable in the presence of 1% of these surfactants because there was a little loss on the protease activiy in the experiments. Later, higher concentrations (5%-10% mM) of these surfactants were used in the experiments, but the enzyme activity could not be determined because of the precipitation and turbidity in the activity tests.

Table 3.4. Effect of surfactants on protease activity (at 55 ºC, pH 7.2, for 10 min.)

Surfactants	Concentrations	Relative Activity (%)
Control	-	100
SDS	1%	83.2
Triton X-100	1%	97.99
Tween-20	1%	94.6
Tween-80	1%	90.6
H_2O_2	1%	81

When the effects of the surfactants on protease activity were compared in the literature, similar and different results were observed. There was no increasing or decrasing of the enzyme activity when the non-ionic surfactants such as Brij-58, Triton X-100 and Tergitol were added. The anionic surfactant, 0.1% sodium dodecyl sulphate (SDS) caused an inhibition of 23% and 0.5% sodium dodecyl sulphate increased the activity (Kumar et al. 1999). The enzyme was stable in the presence of 5% H_2O_2 (Johnvesly and Naik 2001). The alkaline protease was stable towards SDS and H_2O_2. 5% SDS retained its activity above 96% and 5% H_2O_2 retained its activity 75% for 72 hours (Kumar et al. 2004). The activity was enhanced with 0.1% SDS and 0.1% Triton X-100 but it was not affected by 0.1% Tween 80 (Gupta et al. 2005). The enzyme was stable with SDS and Triton X-100 (Patel 2006). The protease enzyme was resistant to denaturation by SDS, dithiothreitol, urea and guanidine hydrochloride (Zhu 2007). The alkaline protease showed highly stability towards non-ionic surfactants (5% Tween-20 and 5% Triton X-100) and anionic surfactants (0.5% SDS) for 60 min at 40 °C (Sellami-Kamoun et al. 2008)

3.4.6. Effect of Inhibitors on Enzyme Activity

Firstly, 1 mM of the inhibitors were used. According to the results, PMSF and the protease inhibitor coctail decreased the activity of the protease. In the presence of 1 mM PMSF, the protease enzyme showed nearly 83% of its activity. The protease inhibitor coctail, which is regarded as a typical inhibitor for all kinds of proteases, did not exhibit a strong inhibitory action on the protease enzyme. In the Figure 3.11, the

results indicated that alkaliphilic and thermophilic protease enzyme was stable in the presence of 1 mM of these inhibitors because there was a little loss on the protease activiy in the experiments. Thus, higher concentrations of this agent may be required for the inhibiton of the enzyme. Later, higher concentraitons (5-10 mM) of these agents were used in the experiments, but the strongest inhibitor could not be observed because of the precipitation and turbidity in the activity tests. Thus, the group of enzyme could not be classified.

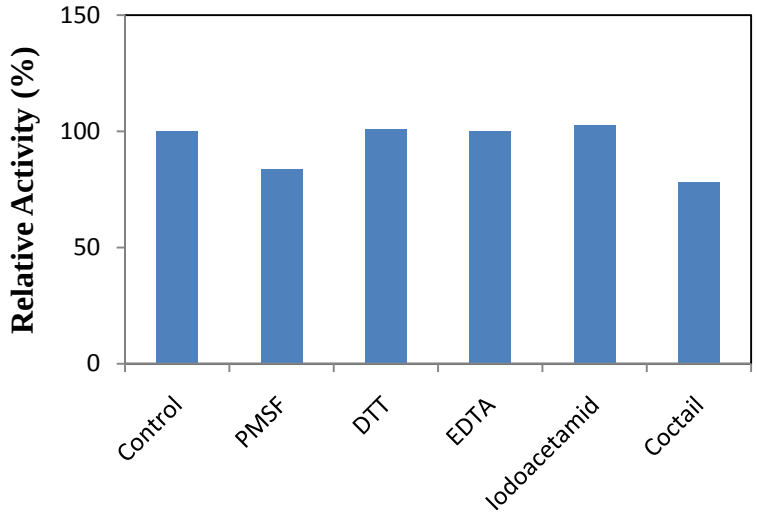


Figure 3.11. Effect of inhibitors on protease activity (at 55 ºC, pH 7.2, for 10 min.)

When the effects of the inhibitors on protease activity were compared in the literature, different results were observed. The *B. stearotbermopbilus* RM-67 protease I was inhibited by metal chelating agents 8-hydroxyquinoline-5-sulfonic acid (100%), ethylenediaminetetraacetate (EDTA) (45%), and 1,10- phenanthroline (29%) but not by dinitrofluorophosphate (DFP) the inhibitor of alkaline proteases. Thus, it may be classified as a neutral protease. The *B. stearotbermopbilus* RM-67 protease II was inhibited by DFP Thus, the protease should be classified as a serine protease (Chopra and Mathur 1983). The protease enzyme was inhibited strongly by metal chelating agent EDTA (Matta and Punj 1998). 1 mM PMSF inhibited the proteases completely (Kumar et al. 1999). The metal chelators EDTA and 1,10-phenanthroline exhibited strong inhibition towards the enzyme activity. The protease enzymes were 90% inhibited in the presence of 10 mM EDTA and 10 mM 1,10-phenanthroline. Thus, they can thus be classified as metalloproteases (Sookkheo 2000). The proteolytic activity was inhibited

by 1 mM PMSF and TPCK. There was no inhibition in the presence of EDTA and 1,10-phenanthroline (Johnvesly and Naik 2001). The proteolytic activity was inhibited by PMSF and it was suggested that the enzyme was serine type protease (Gupta et al. 2005). The protease enzymes were inhibited by phenylmethylsulfonyl fluoride (PMSF). In the presence of 2-mercaptoethanol and iodoacetate, 80-90% enzyme activities were retained (Seifzadeh et al. 2008).

CHAPTER 4

CONCLUSION

The microorganisms that were previously isolated and characterized as a *Bacillus sp.* from Balçova Geotermal region in İzmir by Elif Yavuz were used in the experiments. The aim of this study was to produce the protease enzyme from alkaliphilic and thermophilic *Bacillus sp.* by purifing and determine the properties of the enzyme with the characterization.

Firstly, the bacterial culture number-53 was decided to be used in the experiments. When the stock cultures were inoculated to the plate including media used for protease screening, the clear zones around the colonies were observed. This result indicated the proteolytic activity. After screening the protease activity, complex medium to grow the bacteria in a specific way was decided to be used. In order to maintain the purification steps, the enzyme was examined whether it was extracellular or intracellular enzyme. When compared with the results, it was observed that the extracellular enzyme showed higher activity than the intracellular enzyme. This extracellular protease enzyme was purified by ammonium sulphate precipitation (80% saturation) and ion exchange chromatograpy by using a weak anion exchange (DEAE-Cellulose) column. The yield and purification fold after purification of the enzyme were 33% and 1.41, respectively.

Later, the characterization studies were examined. The results indicated that the protease enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* had highest activity at pH 8.0 and 55 ºC. The protease enzyme at acidic pH values exhibited a little lower activity than the protease enzyme at alkaline pH values. The enzyme lost 20% of its activity at pH 4.0 and it lost 10% of its activity at pH 10.0. It was understood that this enzyme is nearly stable in these conditions because of having above the 80% of its activity at all of the pH values. The results showed that the protease enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* was stable at different pH values (pH 4-7-10) during 3 hours but after 3 hours, there was a significant loss in the stability. The protease enzyme at temperatures below 55 ºC lost 15% of its activity and also the protease enzyme at temperatures above 55 ºC lost 25% of its activity. It was understood

that the protease enzyme is also nearly stable at different temperatures because of having above the 75% of its activity at all of the temperatures. The results showed that the protease enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* was stable at different pH values (55-85 ºC) during 6 hours because there was not a significant loss in the stability. When compared the substrates, casein showed higher activity and azocasein showed lower activity in the experiments. In the effect of metal ions, the results shows that Ca^{+2} and Cu^{+2} increase the activity of the protease. When the effect of organic solvents and surfactants on protease activity was investigated, the results indicated that alkaliphilic and thermophilic protease enzyme was stable in the presence of 10% of these organic solvents and 1% of these surfactants because there was a little loss on the protease activiy in the experiments. In order to determine the type of the protease, the effects of inhibitors on enzyme activity were examined. PMSF and the protease inhibitor coctail decrease the activity of the protease. In the presence of 1 mM PMSF, the protease enzyme showed nearly 83% of its activity. The protease inhibitor coctail, which is regarded as a typical inhibitor for all kinds of proteases, did not exhibit a strong inhibitory action on protease. The results indicated that alkaliphilic and thermophilic protease enzyme was stable in the presence of 1 mM of these inhibitors because there was a little loss on the protease activiy in the experiments. Thus, higher concentrations of this agent may be required for the inhibiton of the enzyme.

In conclusion, the protease enzyme isolated from alkaliphilic and thermophilic *Bacillus sp.* was purified and characterized during the experiments. In the further studies, it can be immobilized on to organic or inorganic supports to be used in industrial processes and it is also produced by using recombinant DNA technology to improve its application in biotechnology. On the other hand, X-ray crystallographic analyses of the proteases can be defined the features of the catalytic and structural metal-binding sites.

REFERENCES

Akel H., Al-Quadan F., Yousef T.H. 2009. Characterization of a Purified Thermostable Protease from Hyperthermophilic *Bacillus* Strain HUTBS71. *European Journal of Scientific Researc.* 31(2): 280-288.

Almas S., Hameed A., Shelly D., Mohan P. 2009. Purification and characterization of a novel protease from *Bacillus* strain SAL1. *African Journal of Biotechnology* 8(15): 3603-3609.

Anson M.L. 1938. *J. Gen. Physiol.* 22: 79-89.

Amara A.A., Salem S.R. and Shabeb M.S.A. 2009. The Possibility to Use Bacterial Protease and Lipase as Biodetergent. *Global Journal of Biotechnology & Biochemistry* 4(2): 104-114.

Ammar M.S., El-Louboudy S.S. and Abdulraouf U.M. 1991. Protease(s) from *Bacillus anthracis* S-44 and *B. cereus* var. mycoids, S-98 isolated from a temple and slaughter house in Aswan city. *Az. J. Microbiol*. (13): 12-29.

Anwar A. and Saleemuddin M. 1998. Alkaline proteases: A review. *Bioresource Technology* 64(3): 175-183.

Ash C., Priest, F.G. & Collins M.D. 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus*. *Antonie van Leeuwenhoek* 64: 253-260.

Asokan, Jayanthı S., C. 2010. Alkalıne protease production by *Bacillus licheniformis* and *Bacillus coagulans*. *Journal of Cell and Tissue Research* 10(1): 2119-2123.

Baker J.M., Griffiths M.W. 1993. Predictive modelling of psychrotrophic *Bacillus cereus*. *J. Food Protect.* 56: 684–688.

Baker G., Gaffar S., Cowan D.A., Suharto A.R. 2001. Bacterial Community Analysis of Indonesian Hotsprings. *FEMS Microbiology Letters* 200: 103-109.

Banerjee U.C., Sani R.K., Azmi W., Soni R. 1999. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additive. *Process Biochemistry* 35(1-2): 213-219.

Banik R.M. and Prakash M. 2004. Laundery detergent compatibility of the alkaline protease from *Bacillus cereus*. *Microbiol. Res*. 159(2): 135-140.

Barett A.J. 1994. Proteolytic enzymes: serine and cysteine peptidases. *Methods Enzymol*. 244: 1–15.

Beg Q.K., Gupta R. 2003. Purification and characterization of an oxidation-stable, thiol-dependent serine alkaline protease from *Bacillus mojavensis*. *Enzyme and Microbial Technology* 32: 294–304.

Beynon J. and Bond. J.S. 1989. Proteolytic enzymes, a practical approach. IRL *Press, New York, NY*.

Bhosale S.H., Rao M.B., Deshpande V.V., Srinivasan M.C. 1995. Thermostability of high activity alkaline protease from Conidiobolus coronatus (NCL 86.8.20). *Enzyme Microbiol Technol.* 17: 136-139.

Bouzas T.D., Barros-Velazquez J., Villa T.G. 2006. Industrial applications of hyperthermophilic enzymes: A review. *Protein Peptide Lett.* 13(7): 645-651.

Bradford M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 72: 248–251.

Brock T.D. 1986. Introduction, an overview of the thermophiles. In *Thermophiles: General, Molecular and Applied Microbiology* Edited by: Brock TD. New York: John Wiley & Sons 1-16.

Bryce W.W. 1966. Manufacturing uses of enzymes. *Food Manual* 41(1): 18-20.

Campbell, L.L. 1955. *Arch. Biochem. Biophys*. 64: 154.

Casella M. and Whitaker J. 1990. Enzymatically and chemically modified zein for improvement of functional properties. *J. Fd Biochem.* 14: 453-475.

Chopra A. K. and Mathur D. K. 1985. Purification and Characterization of Heat-Stable Proteases from *Bacillus stearothermophilus* RM-67. *J Dairy Sci.* 68: 3202-3211

Cowan D., Danial R., and Morgan H. 1985. Thermophilic protease: Properties and potential applications. *Trends Biotechnol.* 3: 68–72.

da Silva C. R., Delatorre A. B., Martins M. L. L. 2007. Effect of the culture conditions on the production of an extracellular protease by thermophilic *Bacillus sp.* and some properties of the enzymatic activity. *Brazilian Journal of Microbiology* 38: 253-258.

Deng A., Wu J., Zhang Y., Zhang G., Wen T. 2010. Purification and characterization of a surfactant-stable high-alkaline protease from *Bacillus sp.* B001. *Bioresource Technology* 101: 7100–7106.

do Nascimento, W.C.A. and M.L.L. Martins. 2004. Production and properties of an extracellular protease from thermophilic *Bacillus sp. Brazilian Journal of Microbiology* 35(1-2): 91-96.

Doddapaneni K. K., Tatineni R., Vellanki R. N., Rachcha S., Anabrolu N., Narakuti V., Mangamoori L. N. 2007. Purification and characterization of a solvent and

detergent-stable novel protease from *Bacillus cereus*. *Microbiological Research* 164: 383-390.

Dufresne S., Bousquet J., Boissinot M. & Guay R. 1996. *Sulfobacillus disulfidooxidans sp.* nov., a new acidophilic, disulfide-oxidizing, gram-positive, spore-forming bacterium. *Int. J. Syst. Bacteriol.* 46: 1056-1064.

El-Safey E. M., Abdul-Raouf U. M. 2004. Production, purification and characterization of protease enzyme from *Bacillus subtilis*. Botany and Microbiology Department, Faculty of Science, Al-Azhar University.

Folin O., and Ciocalteu V. 1929. *J. Biol. Chem.* 73: 627.

Fogarty W.M., Grilffin P. H. & Joyce A.M. 1974. Enzymes of *Bacillus species*- Part 2. *Process Biochemistry* 9(7): 27-35.

Fu Z, Hamid S. B. A., Razak C. N. A., Basri M., Salleh A. B. and Rahmanb R. N. Z. A. 2003. Secretory expression in Escherichia coli and single-step purification of a heat-stable alkaline protease. *Protein Expression and Purification* 28: 63–68.

Fujishige A., Smith K.R., Silen J.L., Agard D.A. 1992. Correct folding of a-lytic protease is required for its extracellular secretion from Escherichia coli. *J Cell Biol.* 118: 33-42.

Garcica-Carreno F. L. 1991. Proteases in food technology. *Biotechnology Education* 2(4): 150-153.

Garcica-Carreno Fernando Luis. 1992. Protease inhibition in theory an practice. *Biotechnology Education* 3(4): 145-150.

Genencor. 2009. Proteases in the Food Industry. *USP Enzyme Workshop Rockville, MD.*

Gerze A.; Omay D. and Guvenilir Y. 2005. Partial purification and characterization of protease enzyme from *Bacillus subtilis* and *Bacillus megatherium. Appl Biochem Biotechnol*. 121(124): 335-45.

Ghafoor A., Hasnain S. 2010. Purification and characterization of an extracellular protease from *Bacillus subtilis* EAG-2 strain isolated from ornamental plant nursery. *Polish Journal of Microbiology* 59(2): 107-112.

Ghorbel-Frikha B., Sellami-Kamoun A., Fakhfakh N., Haddar A., Manni L., Nasri M. 2005. Production and purification of a calcium-dependent protease from *Bacillus cereus* BG1. *J Ind Microbiol Biotechnol.* 32: 186–194.

Godfrey T., and West S. 1996. Industrial enzymology, 2nd ed., p. 3. *Macmillan Publishers Inc., New York, N.Y.*

Grimm O. 1958. Advances in the bating field. *Leder* 9: 308-311.

Guangrong H., Tiejing Y. 2006. Purification and characterization of a protease from Thermophilic *Bacillus* strain HS08. *African Journal of Biotechnology* 5(24): 2433-2438.

Gupta A., Roy I., Patel R.K., Singh S.P., Khare S.K., Gupta M.N. 2005. One-step purification and characterization of an alkaline protease from haloalkaliphilic *Bacillus sp. Journal of Chromatography A.* 1075: 103–108.

Gupta R., Beg Q. K. 2002. Bacterial alkaline proteases: molecular approaches and industrial applications. *Applied Microbiology and Biotechnology* 59(1): 15-32.

Gupta R., Beg Q. K., Khan S., Chauhan B. 2002. An overview on fermentation, downstream processing and properties of microbial alkaline proteases. *Appl Microbiol Biotechnol.* 60: 381–395.

Gusek T., and Kinsella E. 1988. Properties and potential applications of a unique heat-stable protease. *Food Technol.* 49: 102–107.

Haddar A., Bougatef A., Agrebi R., Sellami-Kamoun A., Nasri M. 2009. A novel surfactant-stable alkaline serine-protease from a newly isolated *Bacillus mojavensis* A21. Purification and characterization. *Process Biochemistry* 44: 29–35.

Haki G.D.; Rakshit S.K. 2003. Developments in industrially important thermostable enzymes: a review. *Bioresource Technol*. 89(1): 17-34.

Harwood C.R. 1989. Introduction to the biotechnology of *Bacillus*, in: Biotechnology handbooks 2: *Bacillus* (Harwood, C.R. ed.) *Plenum Press, New York*, pp 1-4.

Hase C.C. and Finkelstein R.A. 1993. Bacterial extracellular zinc-containing metalloproteases. *Microbiological Reviews* 57(4): 823-837.

Hawumba J.F., Theron J., Brözel V. S. 2002. Thermophilic Protease-Producing *Geobacillus* from Buranga Hot Springs in Western Uganda. *Current Microbiology* 45: 144–150.

Heyndrickx M., Lebbe L., Vancanneyt M. & 7 other authors 1997. A polyphasic reassessment of the genus *Aneurinibacillus*, reclassification of *Bacillus thermoaerophilus* as *Aneurinibacillus thermoaerophilus* comb. nov., and emended descriptions of *A. aneurinilyticus* corrig., *A. migulans*, and *A. thermoaerophilus*. *Int. J. Syst. Bacteriol.* 47: 808-817.

Horlkoshi K. 1971. Production of alkaline enzymes by alkalophilic microorganisms. I. Alkaline protease produced by *Bacillus* no. 221. *Agric. Biol. Chem.* 35: 1407-1414.

Huges R.A.P and Williams R.A.D. 1977. Yelow-pigmented Strains of Thermus ssp. from Icelandic Hot Springs. *Journal of General Microbiology* 102: 375-383.

Hutadilok-Towatana N., Painupong A. and Suntinanalert P. 1999. Purification and Characterization of an Extracellular Protease from Alkaliphilic and Thermophilic *Bacillus sp.* PS719. *Journal Of Bioscience And Bioengineering* 87(5): 581-587.

International Union of Biochemistry. 1992. Enzyme nomenclature. *Academic Press, Inc.*, Orlando, Fla.

Jellouli K., Ghorbel-Bellaaj O., Ayed H.B., Mani L, Agrebi R., Nasri M. 2011. Alkaline-protease from *Bacillus licheniformis* MP1: Purification,characterization and potential application as a detergent additive and for shrimp waste deproteinization. *Process Biochemistry* 9.

Johnvesly B., Naik G.R. 2001. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic *Bacillus sp.* JB-99 in a chemically defined medium. *Process Biochemistry* 37: 139–144.

Joshi B.H. 2010. Purification and Characterization of a Novel Protease from *Bacillus Firmus* Tap5 Isolated from Tannery Waste. *Journal of Applied Sciences Research.* 6(8): 1068-1076.

Kalisz H.M. 1988 Microbial proteinases. *Adv. Biochem. Eng. Biotechnol.* 36: 1–65.

Kristjansson J.K. 1989. Thermophilic Organisms as Sources of Thermostable Enzymes. *TIBTECH* 7: 349-353.

Matta H., Punj V. 1998. Isolation and partial characterization of a thermostable extracellular protease of *Bacillus polymyxa* B-17. *International Journal of Food Microbiology* 42: 139-145.

Mustafa Kamal. 1993. Purification and characterization of protease(s) from *Bacillus species*. Depertmant of microbiology universitey of Karachi.

Kaur S., Vohra R.M., Kapoor M., Beg Q. K. and Hoondal G.S. 2001. Enhanced production and characterization of a highly thermostable alkaline protease from *Bacillus sp.* P-2. *World Journal of Microbiology & Biotechnology* 17: 125-129.

Kazan D., Denizci A.A., Kerimak Öner M.N., Erarslan A. 2005. Purification and characterization of a serine alkaline protease from *Bacillus clausii* GMBAE 42. *J. Ind. Microbiol. Biotechnol*. 32: 335–344.

Kobayashi T., Hakamada Y., Hitomi J., Koike K., and Ito S. 1996. Purification of alkaline proteases from a *Bacillus* strain and their possible interrelationship. *Appl. Microbial. Biotechnol.* 45: 63-71.

Kohlmann K.L., Nielsen S.S., Steenson L.R. and Ladisch M.R. 1991. Production of proteases by psychrotrophic microorganisms. *Journal of Dairy Science* 74(10): 3275-3283.

Kristjansson J.K. 1989. Thermophilic organisms as sources of thermostable enzymes. *Trends in Biotechnology 7*(12): 349-353.

Krulwich T.A. 1986. Bioenergetics of alkalophilic bacteria. *J. Membrane Biol.* 89: 113–25.

Krulwich T.A, Guffanti AA. 1989. Alkalophilic bacteria. *Ann. Rev. Microbiol.* 43: 435–63.

Kumar C. G. and Takagi H. 1999. Microbial alkaline proteases: From a bioindustrial viewpoint. *Biotechnology Advances* 17(7): 561-594.

Kumar C. G., Joo H.-S., Koo Y.-M., Paik S. R., Chang C.-S. 2004. Thermostable alkaline protease from a novel marine haloalkalophilic *Bacillus clausii* isolate. *World Journal of Microbiology & Biotechnology* 20: 351–357.

Kumar D., Savitri, Thakur N., Verma R. and Bhalla T.C. 2008. Microbial proteases and application as Laundry Detergent additive. *Research Journal of micribiology*. 3(12): 661-672.

Kumar S., Nussinov R.. 2001. How do thermophilic proteins deal with heat? *CMLS, Cell. Mol. Life Sci.* 58: 1216–1233.

Laemmli U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227(5259): 680-5.

Lantz M.S., Ciborowski P. 1994. Zymographic techniques for detection and characterization of microbial proteases. *Methods Enzymol.* 235: 563-594.

Liao C.H. and McCallus D.E. 1998. Biochemical and genetic characterization of an extracellular protease from Pseudomonas fluorescens CY091. *Applied and Environmental Microbiology* 64(3): 914-921.

Lovern J.A. 1955. Recent development in the fish by products industry. *J. Sci. Food Agric.* 6: 233-239.

Mahendran S., Sankaralingam S., Shankar T. and Vifayabaskar P. 2010. Alkalophilic protease enzyme production from estuarine *Bacillus aquimaris*. *World Journal of Fish and Marine Sciences* 2(5): 436-443.

Mahmoud M.A., Al-Agamy M.H.M., Ashour M.S. and El-Loboudy S.S. Purification and characterization of neutral protease from soil strain of *Bacillus subtilis*. *Microbiology and Immunology department, Faculty of Pharmacy, Botany and Microbiology department, Faculty of Science, Al-Azhar university, Nasr City, Cairo, Egypt*.

Mansfeld J. Metalloproteases. 2007. Department of Biochemistry, Institute of Biotechnology, Martin-Luther University. *Industrial Enzymes* 221–242.

Margesin R. and Schinner F. 1992. A comparison of extracellular proteases from three psychrotrophic strains of pseudomonas fluorescens. *J Gen. Appl. Microbiol*. 38: 209-225.

Matsubara H. & Feder J. 1971. In: The Enzymes (Boyer, P., ed.) *Academic Press, New York* 3: 721-792.

Maurer K.-H. 2004. Detergent proteases. *Current Opinion in Biotechnology* 15: 330–334.

McMullan G., Christie J.M., Rahman T.J., Banat I.M., Ternan N.G., Marchant R. 2004. Habitat, applications and genomics of the aerobic, thermophilic genus *Geobacillus*. *Biochemical Society Transactions* 32(2): 214-217.

Meer R.R., Baker J., Bodyfelt F.W., Griffiths M.W. 1991. Psychrotrophic *Bacillus* species in fluid milk products a review. *J. Food Protect.* 54: 969–979.

Meer R.R., Wodburn M.J., Bodyfelt F.W. 1993. Identification and characterization of heat resistant psychrotrophic bacteria in Oregon grade A raw milk. *Dairy Food Environ. Sanitation* 13: 631–637.

Naidu K. S. B., Devi K. L. 2005. Optimization of thermostable alkaline protease production from species of *Bacillus* using rice bran. *African Journal of Biotechnology* 4(7): 724-726.

Nazina T.N., Tourova T.P., Poltaraus A.B., Novikova E.V., Grigoryan A.A., Ivanova A.E., Lysenko A.M., Petrunyaka V.V., Osipov G.A., Belyaev S.S., Ivanov M.V. 2001. Taxonomic study of aerobic thermophilic *Bacilli*: descriptions of *Geobacillus subterraneus* gen. nov., *sp.* nov. and *Geobacillus uzenensis sp.* nov. from petroleum reservoirs and transfer of *Bacillus stearothermophilus*, *Bacillus thermocatenulatus*, *Bacillus thermoleovorans*, *Bacillus kaustophilus*, *Bacillus thermoglucosidasius* and *Bacillus thermodenitrificans* to *Geobacillus* as the new combinations *G. stearothermophilus*, *G. thermocatenulatus*, *G. thermoleovorans*, *G. kaustophilus*, *G. thermoglucosidasius* and *G. thermodenitrificans*. *International Journal of Systematic and Evolutionary Microbiology* 51: 433–446.

Neklyudov A.D., Ivankin A.N., Berdutina A.V. 2000. Properties and uses of protein hydrolysates (review). *Appl. Biochem. Microbiol.* 36: 452–459.

Niehaus F., Bertoldo C., Kahler M., Antranikian G. 1999. Exteromophiles as a Source of Novel Enzymes For Industrial Application. *Applied Microbial Biotechnology* 51: 711-729.

Nilegaonkar S.S., Zambare V.P., Kanekar P.P., Dhakephalkar P.K., Sarnaik S.S. 2007. Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326. *Bioresource Technology* 98: 1238–1245.

Norris J.R., Berkeley R.C., Logan N.A. & O'Donnel A.G. 1993. The genera *Bacillus* and *Sporolactobacillus* in: The prokaryotes: a handbook on habitants, isolation and identification. (Starr, M., Stolp, H., Truper, H.G., Balows, A. and Schlegel, H. eds.) *Springer Verlag, Berlin* pp. 1711-1742.

Ohta Y. 1966. Thermostable Protease from Thermophilic Bacteria. *The Journal Of Biological Chemistry* 242(3): 509-515.

Patel R., Dodia M., Singh S. P. 2005. Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus sp.*: Production and optimization. *Process Biochemistry* 40: 3569–3575.

Patel R. K., Dodia M. S., Joshi R. H., Singh S. P. 2006. Purification and characterization of alkaline protease from a newly isolated haloalkaliphilic *Bacillus sp. Process Biochemistry* 41: 2002–2009.

Priest F.G. 1977. Extracellular enzyme-synthesis in genus *Bacillus*. *Bacteriological Reviews* 41(3): 711-753.

Priest F.G. 1989. Products and applications. In: *Bacillus*.(Harwood, C.R., ed.), *Biotechnology Handbooks*. Vol.2, (Atkinson,T. and Sherwood, R.F., series eds.), Plenum Press, New York, London, pp. 293-320.

Rahman R.N.Z.A., Razak C.N., Ampon K., Basri M., Yunus W.M.Z.W. & Salleh A.B. 1994. Purification and characterization of heat-stable alkaline protease from *Bacillus stearothermophilus* F1. *Applied Microbiology and Biotechnology* 40: 822-827.

Ramakrishna D.P.N., Gopi Reddy N., Rajagopal S.V. 2010. Purification and properties of an extracellular alkaline protease produced by *Bacillus subtilis*. *International Journal of Biotechnology and Biochemistry* 6(4): 493–504.

Rao Ch Subba, Sathish T., Ravichandra P., Prakasham R.S. 2009. Characterization of thermo- and detergent stable serine protease from isolated *Bacillus circulans* and evaluation of eco-friendly applications. *Process Biochemistry* 44: 262–268.

Rao M. B., Tanksale A. M., Ghatge M.S., and Deshpande V.V. 1998. "Molecular and biotechnological aspects of microbial proteases. *Microbiology and Molecular Biology Reviews* 62(3): 597-635.

Seifzadeh S., Sajedi R.H., Sariri R. 2008. Isolation and characterization of thermophilic alkaline proteases resistant to sodium dodecyl sulfate and ethylene diamine tetraacetic acid from *Bacillus sp.* GUS1. *Iranian Journal of Biotechnology* (6)4: 214-221.

Sellami-Kamoun A., Haddar A., Ali N. E.-H., Ghorbel-Frikha B., Kanoun S., Nasri M. 2008. Stability of thermostable alkaline protease from *Bacillus licheniformis* RP1 in commercial solid laundry detergent formulations. *Microbiological Research* 163: 299-306.

Shah K., Mody K., Keshri J., Jha B. 2010. Purification and characterization of a solvent, detergent and oxidizing agent tolerant protease from *Bacillus cereus* isolated from the Gulf of Khambhat. *Journal of Molecular Catalysis B: Enzymatic* 67: 85–91.

Siddalingeshwara K.G, Uday J., Huchesh C.H., Puttaraju H.P., Karthic J., Sudipta K.M., Pramod T. and Vishwanatha T. 2010. Screening And Characterization Of Protease From Bacıllus Sp. *International Journal of Applied Biology and Pharmaceutical Technology* 1(2): 575-581.

Sierecka J. K. 1998. Purification and partial characterization of a neutral protease from a virulent strain of *Bacillus cereus*. *The International Journal of Biochemistry & Cell Biology* 30: 579-595.

Sneath P. H. A. 1986. Endospore-forming Gram-positive rods and cocci. In *Bergey's Manual of Systematic Bacteriology*, vol. 2, pp. 1104-1207. Edited by P.H.A. Sneath, N.S. Mair, M.E. Sharpe & J.G. Holt. Baltimore: William & Wilkins.

Soares, V.F.; Castilho, L.R.; Bon, E.P. and Freire, D.M. 2005. High-yield *Bacillus subtilis* protease production by solid-state fermentation. *Appl. Biochem. Biotechnol*. 121-124: 311-9.

Sookkheo B., Sinchaikul S., Phutrakul S. and Chen S.T. 2000. Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS33. *Prot. Exp. Pur.* 20: 142-151.

Stadhouders J. 1992. *Bacillus cereus* in milk and milk products. *Int. Dairy Fed. Bull*. 275, 3.

Sumantha A., Larroche C. 2006. Microbiology and industrial biotechnology of food-grade proteases: A perspective. *Food Technology and Biotechnology* 44(2): 211-220.

Sung M.-H., Kim H., Bae J.-W., Rhee S.-K., Jeon C. O., Kim K., Kim J.-J., Hong S.-P., Lee S.-G., Yoon J.-H., Park Y.-H. and Baek D.-H. 2002. *Geobacillus toebii sp.* nov., a novel thermophilic bacterium isolated from hay compost. *International Journal of Systematic and Evolutionary Microbiology* 52: 2251–2255

Supuran C. T., A. Scozzafava, et al. 2002. Bacterial protease inhibitors. *Medicinal Research Reviews* 22(4): 329-372.

Takii Y., Urata Y., Ueno N. 1998. Thermostable neutral protease resembling thermolysin derived from *Bacillus brevis* MIB001. *Biosci. Biotechnol. Biochem.* 62(5): 1028-1030.

Touzel J.P., O'Donohue M., Debeire P., Samain E. & Breton C. 2000. *Thermobacillus xylanilyticus* gen. nov., *sp.* nov., a new aerobic thermophilic xylan-degrading bacterium isolated from farm soil. *Int. J. Syst. Evol. Microbiol.* 50: 315-320.

Turner P., Mamo G. and Karlsson E.N. 2007. Potential and utilization of thermophiles and thermostable enzymes in biorefining. *Microbial Cell Factories* 6(9):1-23.

Valasaki K., Staikou A., Theodorou L.G., Charamopoulou V., Zacharaki P., Papamichael E.M. 2006. Purification and kinetics of two novel thermophilic extracellular proteases from *Lactobacillus helveticus*, from kefir with possible biotechnological interest. *Bioresource Technology* 99: 5804–5813.

Vallee B.L. and Auld D.S. 1990. Zinc coordination, function, and structure of zinc enzymes and other proteins. *Biochemistry* 29(24): 5647-5659.

Varela H., Ferrari M.D., Belobradjic L., Vazquez A., Loperena M.L. 1997. Skin unhairing proteases of *Bacillus subtilis*: production and partial characterization. *Biotechnol Lett.* 19: 755–758.

Venugopal M., Sarama A.V. 2007. An alkaline protease from *Bacillus circulans* BM15, newly isolated from a mangrove station: characterization and application in laundry detergent formulations. *Indian J. Microbiol.* 47: 298–303.

Venugopal V., Alur M.D. & Nerkar D.P. 1989. Solublization of fish proteins using immobilized microbial cells. *Biotechnol. Bioeng.* 33: 1098-1103.

Wainø M., Tindall B.J., Schumann P. & Ingvorsen K. 1999. *Gracilibacillus* gen. nov., with description of *Gracilibacillus halotolerans* gen. nov., *sp.* nov.; transfer of *Bacillus dipsosauri* to *Gracilibacillus dipsosauri* comb. nov., and *Bacillus salexigens* to the genus *Salibacillus* gen. nov., as *Salibacillus salexigens* comb. nov. *Int. J. Syst. Bacteriol.* 49: 821-831.

Ward 0. P. Proteinases, 1983. In Forgarty, W.M. (ed.), Microbial enzymes and biotechnology. *Applied Science Publishers, London* p. 251-317.

Wasserman, B. P. 1984. Thermostable enzyme production. *Food. Technol.* 2: 78–89.

Wasserman B. 1990. Evolution of enzyme technology: progress and prospects. *Food Technol.* 118-122.

Wilson S.-A., Young O., Coolbear, T. and Daniel, R.M. 1992. The Use of Proteases from Extreme Thermophiles for Meat Tenderising. *Meat Science* 32: 92-103.

Wisotzkey J.D., Jurtshuk P., Fox G.E., Deinhard G. & Poralla K. 1992. Comparative sequence analyses on the 16S rRNA (rDNA) of *Bacillus acidocaldarius*, *Bacillus acidoterrestris*, and *Bacillus cycloheptanicus* and proposal for creation of a new genus, *Alicyclobacillus* gen. nov. *Int. J. Syst. Bacteriol.* 42: 263-269.

Yang J.-K., Shih I.-L., Tzeng Y.-M., Wang S.-L. 2000. Production and purification of protease from a *Bacillus subtilis* that can deproteinize crustacean wastes. *Enzyme and Microbial Technology* 26: 406–413.

Yavuz E., Gunes H., Harsa S. and Yenidunya A.F. 2004. Identification of extracellular enzyme producing thermophilic bacilli from Balcova (Agamemnon) geothermal site by ITS rDNA RFLP. *Journal of Applied Microbiology* 97(4): 810-817.

Zeigler D.R. 2001. The Genus *Geobacillus*. *Bacillus Genetic Stock Center Catalog of Strains.* 3(7): 1-25.

Zhua W., Chab D., Chenga G., Peng Q., Shen P. 2007. Purification and characterization of a thermostable protease from a newly isolated *Geobacillus sp.* YMTC 1049. *Enzyme and Microbial Technology* 40: 1592–1597.

APPENDIX A

PREPARATION OF BRADFORD REAGENT, PROTEIN STANDARTS AND STANDART CURVE FOR BRADFORD ASSAY

A.1. Preparation of Bradford Reagent:

10.0 mg Coomassie Brilliant Blue G-250 (CBB G-250), 5 ml 95% ethanol and 10 ml 85% phosphoric acid were used. Firstly, 10.0 mg CBB G-250 is dissolved in 5 ml 95% ethanol and 10 ml phosphoric acid is add. Later, the mixture is diluted to 100 ml with ultra pure water. The solution is filtered through Whatman No. 1 paper and store at 4 ºC.

A.2. Preparation of Protein Standarts:

Bovine serum albumin (BSA) was used as protein standart. For the stock solution with a concentration of 0.2 mg/ml; 0.02 g BSA was dissolved in 1 ml dH_2O. 10 µl stock solution and 990 µl dH_2O were mixed and the final concentration became 0.2 mg/ml. While preparing the standarts, necessary amounts of water, BSA and bradford reagent were put into cuvettes respectively (Table A.1) and incubated at room temperature for 5 minutes. The absorbance was measured at 595 nm using a spectrophotometer.

Table A.1. Preparation of BSA standarts

	BSA (µl)	**dH_2O (µl)**	**Bradford Reagent (µl)**
Blank	0	800	200
Standart 1: (1 µg/ml)	5	795	200
Standart 2: (2 µg/ml)	10	790	200
Standart 3: (4 µg/ml)	20	780	200
Standart 4: (6 µg/ml)	30	770	200
Standart 5: (8 µg/ml)	40	760	200

Table A.2. Absorbance values of BSA standarts

Concentration (µg/ml)	**Absorbance (595 nm)**
1	0.0495
2	0.1007
4	0.2052
6	0.2825
8	0.3462

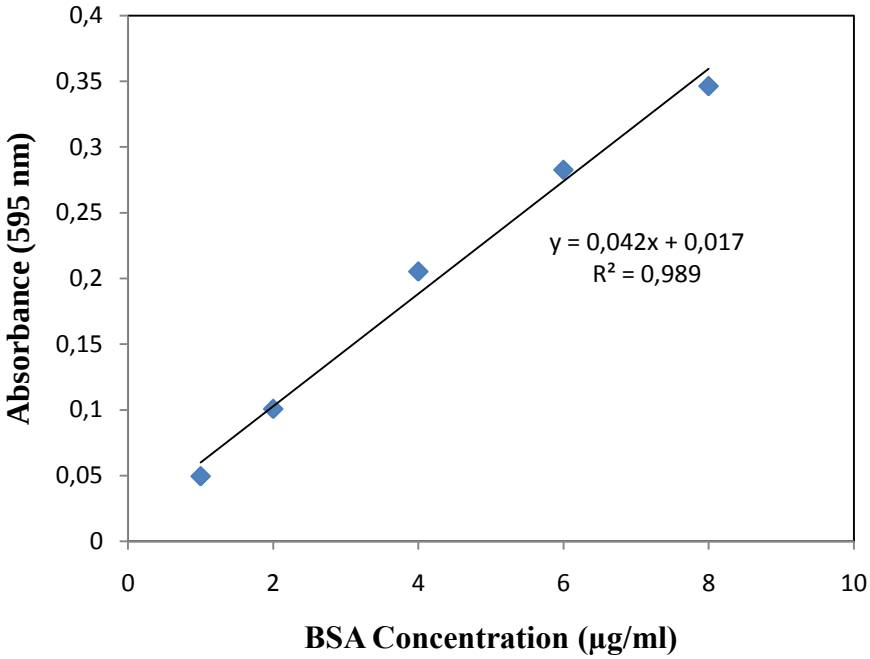


Figure A.1. BSA standart curve for Bradford assay

APPENDIX B

PROTEOLYTIC ACTIVITY DETERMINATION STANDARTS AND STANDART CALIBRATION CURVE FOR L-TYROSINE ASSAY

Table B.1. Preparation of L-Tyrosine standarts

	L-Tyrosine (µl)	**dH_2O (µl)**
Blank	0	1000
Standart 1: (0.2 mg/ml)	200	800
Standart 2: (0.4 mg/ml)	400	600
Standart 3: (0.6 mg/ml)	600	400
Standart 4: (0.8 mg/ml)	800	200
Standart 5: (1.0 mg/ml)	1000	0

Table B.2. Absorbance values of L-Tyrosine standarts

Concentration (mg/ml)	**Concentration (µmole)**	**Absorbance (595 nm)**
0.2	0.00625	0.054
0.4	0.0125	0.128
0.6	0.01875	0.184
0.8	0.025	0.238
1	0.03125	0.313

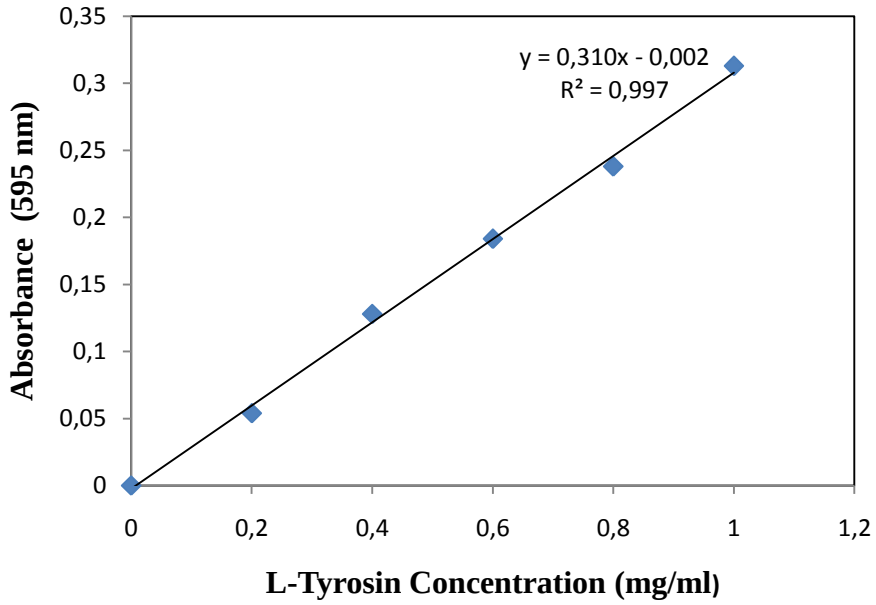


Figure B.1. Standart calibration curve for L-Tyrosine assay

APPENDIX C

MEDIA, BUFFERS, REAGENTS AND SOLUTIONS

C.1. Media and Buffers

- *Luria Bertani (LB) broth, per liter*

10.0 g tryptone, 5.0 g yeast extract, 5.0 g NaCl and dH_2O up to 1 liter. The mixture was autoclaved for sterilization for 15 minutes at 121 °C under high pressure.

- *Complex medium, per liter*

10.0 g glucose, 5.0 g yeast, 2.5 g peptone, 2.5 g casein, 0.3 g $MgSO_4$, 0.02 g $FeSO_4$, 0.2 g $ZnSO_4$, 1.0 g $CaSO_4$, 1.0 g KH_2PO_4, 1.0 g K_2HPO_4 and dH_2O up to 1 liter. The mixture was autoclaved for sterilization for 15 minutes at 121 °C under high pressure.

- *Media Used For Protease Screening, per liter*

8 g nutrient broth, 10 g skim milk, 15 g agar agar and dH_2O up to 1 liter.

Ingredients except skim milk were dissolved in deionized water. Medium was sterilised by autoclaving at 121 °C for 15 minutes. Skim-milk is autoclaved separately at 110 °C for 5 minutes (two times) and added to the medium.

- *50 mM Tris-HCl pH 7.2*

6.055 g tris base was dissolved in 800 ml of deionized water. pH was adjusted to 7.2 with concentrated HCl. Volume is brought to 1 liter with deionized water.

C.2. Reagents Preparation for SDS-PAGE

C.2.1. Stock Solutions in Coomassie Staining

- *30% Acrylamide Mixture*

(29.2 g acrylamide, 0.8 g N'N'-bis-methylene-acrylamide)

Make up to 100 ml with ultrapure water. Filter and store at 4 ºC in the dark for at least one month.

- *1.5M Tris-HCl, pH 8.8*

(18.15 g Tris Base, ~80 ml deionized water)

Dissolve Tris base in deionized water, adjust to pH 8.8 with HCl. Make up to 100 ml with deionized water and store at 4 ºC.

- *0.5M Tris-HCl, pH 6.8*

(6 g Tris Base, ~80 ml deionized water)

Dissolve Tris base in deionized water, adjust to pH 6.8 with HCl. Make up to 100 ml with deionized water and store at 4 ºC.

- *10% SDS*

(10 g SDS, ~90 ml deionized water)

Dissolve SDS in deionized water with gentle stirring and bring to 100 ml with ultrapure water.

- *Sample Buffer*

(3.0 ml ultrapure water, 1.0 ml 0.5M Tris-HCl (pH 6.8), 1.6 ml Glycerol, 1.6 ml 10% (w/v) SDS, 0.4 ml 2-mercaptoethanol, 0.4 ml 0.5% (w/v) bromophenol blue)

Dilute the sample at least 1:4 with sample buffer. Heat 100 C for 10 minutes.

- *5X Running (Electrode) Buffer*

(15 g Tris Base, 72 g Glycine, 5 g SDS)

Dissolve Tris base, glycine and SDS in ~800 ml deionized water and make up to 1L with water. Store at 4 ºC. For electrophoretic run, dilute 5X stock solution to 1X with deionized water.

- *10% Ammonium persulfate (APS)*

(0.1 g APS, 1 ml deionized water)

Dissolve APS in 1 ml deionized water. This solution should be prepared fresh daily.

- *Fixation Solution*

(20% (w/v) TCA solution)

- *Colloidal Coomassie Staining Solution*

Dissolve 40 g ammonium sulfate in ~300 ml water, add 8ml 85% o-phosphoric acid and add 0.5 g Coomassie Brilliant Blue G-250. Make up to 400 ml with water, add 100 ml methanol to 500 ml total volume. Store at 4 ºC.

- *Destaining Solution*

(25% (v/v) methanol solution, 12.5% acetic acid, 82.5% dH_2O)

C.2.2. Stock Solutions in Silver Staining

- *Fixer Solution*

(150.0 ml ultrapure water, 36.0 ml acetic acid, 150.0 µl 37% formaldehyde)

Complete to 300 ml with dH_20.

- *50% EtOH*

(600.0 ml ultrapure water, 600.0 ml pure EtOH)

- *Pretreatment Solution*

(0.08 g $Na_2S_2O_3.5H_2O$, 400.0 ml ultrapure water)

- *Silver Nitrate Solution*

(0.08 g silver nitrate, 400.0 ml ultrapure water, 300.0 µl 37% formaldehyde)

- *Developing Solution*

(400.0 ml ultrapure water, 9.0 g potassium carbonate, 8.0 ml pretreatment solution, 300.0 µl 37% formaldehyde)

- *Stop Solution*

(200.0 ml MeOH, 48.0 ml acetic acid)

C.2.3. Gel Preparation

- *Separating Gel*

Table C.1. Preparation of 12% SDS-PAGE separating gel (for 10 ml)

	Volume
Deionized water	2.35 ml
1.5 M Tris-HCl, pH 8.8	2.5ml
10% SDS	100.0 µl
Acrylamide/Bis (30% Stock)	5.0 ml
10% APS	50.0 µl
TEMED	5.0 µl

- *Stacking Gel*

Table C.2. Preparation of 4% SDS-PAGE stacking gel (for 5ml)

	Volume
Deionized water	3.05 ml
0.5 M Tris-HCl, pH 6.8	1.25ml
10% SDS	50.0 µl
Acrylamide/Bis (30% Stock)	650.0 µl
10% APS	25.0 µl
TEMED	5.0 µl