

**PARTIAL PURIFICATION AND CHARACTERIZATION OF  
ALKALINE PROTEASES FROM ISOLATED *BACILLUS*  
STRAINS**

**M.Sc. Thesis by**

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**İZOLE EDİLEN *BACILLUS* TÜRLERİNDEN  
ALKALİN PROTEAZ ENZİMİNİN KISMI  
SAFLAŞTIRILMASI VE KARAKTERİZASYONU**

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## ABBREVIATIONS

|                 |                                                              |
|-----------------|--------------------------------------------------------------|
| <b>APS</b>      | : Ammonium persulfate                                        |
| <b>BCA</b>      | : 2,2'-Bichinoyl-4,4'-dicarbonic acid, Na <sub>2</sub> -salt |
| <b>BSA</b>      | : Bovine serum albumin                                       |
| <b>BSP</b>      | : <i>Bacillus subtilis</i> protease                          |
| <b>BLP</b>      | : <i>Bacillus licheniformis</i> protease                     |
| <b>BCP</b>      | : <i>Bacillus cereus</i> protease                            |
| <b>BPP</b>      | : <i>Bacillus pumilus</i> protease                           |
| <b>EDTA</b>     | : ethylene-diaminetetraacetic acid                           |
| <b>FPLC</b>     | : Fast protein liquid chromatography                         |
| <b>PMSF</b>     | : Phenylmethylsulfonyl fluoride                              |
| <b>SDS-PAGE</b> | : Sodium dodecyl sulfate polyacrylamide gel electrophoresis  |
| <b>TEMED</b>    | : N,N,N', N' Tetramethylethylamin                            |
| <b>TCA</b>      | : trichloroaceticacid                                        |
| <b>U</b>        | : Unit                                                       |
| <b>KDa</b>      | : kilo Dalton                                                |
| <b>β-me</b>     | : β-mercaptoethanol                                          |

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## **PARTIAL PURIFICATION AND CHARACTERIZATION OF ALKALINE PROTEASES FROM ISOLATED *BACILLUS* STRAINS**

### **SUMMARY**

Proteases are enzymes that hydrolyze proteins to short peptides or free amino acids and catalyze peptide synthesis in organic solvents. Proteases have a large variety of applications in the detergent, food, leather, pharmaceutical and other biotechnology industries.

Alkaline proteases are produced by a wide range of microorganisms including bacteria, moulds, yeasts. Among bacteria, *Bacillus* species are specific producers of extracellular proteases.

In this study, different *Bacillus* species were isolated from the waste waters of tannery industry and they were screened for their ability to produce alkaline protease. Samples collected from different leather factories were screened for their content of microorganisms, specially for the genus. Four potential producers were examined and identified as *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus* and *Bacillus cereus*. Identification of the genus and detection of protease production were carried out by using detection kits.

Extracellular alkaline proteases produced by these *Bacillus* isolates were purified to homogeneity by ammonium sulfate precipitation. Concentrated solution of *Bacillus pumilus* protease precipitates was loaded onto UNO Q-6 ion exchange column in a fast protein liquid chromatography system. Active fractions were concentrated with Millipore centrifugal filter unit. Their molecular weights were estimated by SDS-PAGE analysis. Effect of pH and temperature on the stability and activity of the purified enzymes were further studied. Effect of metal ions and chemicals on the activity of purified enzymes were determined.

## İZOLE EDİLEN *BACILLUS* TÜRLERİNDEN ALKALİN PROTEAZ ENZİMİNİN KISMİ SAFLAŞTIRILMASI VE KARAKTERİZASYONU

### ÖZET

Proteazlar, proteinleri kısa peptitlere veya serbest aminoasitlere parçalayan ve organik çözücüler içinde peptit sentezini katalizleyen enzimlerdir. Proteazların deterjan, gıda, deri, ilaç ve diğer biyoteknoloji endüstrilerinde birçok uygulama alanları vardır.

Alkali proteazlar, bakteri, küf, maya gibi birçok çeşitli mikroorganizmalar tarafından üretilirler. Bakteriler arasında hücre dışı proteaz üretimi özellikle *Bacillus* türüne özgüdür.

Bu çalışmada, deri sanayinin atık sularından farklı *Bacillus* türleri izole edilmiş ve proteaz enzimi üretimleri araştırılmıştır. Çeşitli deri fabrikalarından alınan atık su numunelerinde mikroorganizmalar, özellikle de *Bacillus* türü aranmıştır.

Dört farklı proteaz üreticisi olabilecek tür gözlenmiş ve *Bacillus subtilis*, *Bacillus licheniformis*, *Bacillus pumilus*, *Bacillus cereus* olarak tanımlanmıştır. Türlerin tanımlanması ve proteaz üretiminin gözlenmesi standart test kitlerinin kullanımıyla gerçekleştirilmiştir.

*Bacillus* türlerince üretilen proteazlara, amonyum sülfat ile çöktürme yöntemi uygulanmıştır. Elde edilen *Bacillus pumilus* proteazının çökeltileri konsantre edilmiş çözeltiler halinde UNO Q-6 iyon değiştirme kolon kromatografisi sistemine yüklenmiştir. Kromatografi sonunda elde edilen aktif fraksiyonlar Millipore santrifüj üniteleri ile konsantre edilmiştir. Bu örneklerin molekül ağırlıkları SDS-PAGE yöntemi ile belirlenmiştir. Kısmi olarak saflaştırılan proteaz enzimlerinde sıcaklık ve pH'nın enzim aktivitesi ve stabilitesi üzerine olan etkileri incelenmiştir. Ayrıca metal iyonları ve çeşitli kimyasalların enzimlerin aktivitesi üzerinde olan etkileri de belirlenmiştir.

## **1. INTRODUCTION**

### **1.1. Proteases**

Proteases catalyse the hydrolysis of proteins to peptides and amino acids. These enzymes are important from an industrial perspective and serve the requirements of nearly 60% of the world enzyme market.

Proteases are classified according to three major criteria. These are: (i) the reaction catalysed, (ii) the chemical nature of the catalytic site, and (iii) the evolutionary relationship, as revealed by the structure (Gözükara, 1997).

Proteases can be further divided into families and clans after initial classification. By the reactions they catalyse, protease may be divided into sub-subclasses of peptide hydrolases (class 3.4) depending on the location of the enzymatic action, either exopeptidase or endopeptidase. Exopeptidases cleave peptide bonds at the amino terminus (aminopeptidase) or the carboxy terminus (carboxypeptidase) of a peptide substrate. Endopeptidases, on the other hand, cleave peptide bonds internally, away from either termini of the protein substrate.

Proteases may be then subdivided on the basis of their catalytic mechanism as serine, cysteine, aspartic and metalloproteases depending on which residue is essential for the active site.

#### **1.1.1. Serine proteases**

This class comprises two distinct families. The chymotrypsin family which includes the mammalian enzymes such as chymotrypsin, trypsin or elastase or kallikrein and the subtilisin family which include the bacterial enzymes such as subtilisin. The general 3D structure is different in the two families but they have the same active site geometry and the catalysis proceeds via the same mechanism. The serine proteases show different substrate specificities. These are related to amino acid substitutions in

the various enzyme subsites that interact with the substrate residues. Some enzymes have an extended interaction site with the substrate whereas others have a restricted specificity.

There are three residues which form the catalytic triad and they are essential in the catalytic process; His 57, Asp 102 and Ser 195 (chymotrypsinogen numbering). The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential Serine (Figure A.1). For the formation of this covalent intermediate, a negatively charged tetrahedral transition state intermediate is firstly formed and then the peptide bond is cleaved. During the second step or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme. The deacylation is the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of the Ser residue. The His residue provides a general base and accept the OH group of the reactive Ser.

#### **1.1.2. Cystein proteases**

This family includes the plant proteases such as papain, actinidin or bromelain, several mammalian lysosomal cathepsins, the cytosolic calpains (calcium-activated) as well as several parasitic proteases (e.g Trypanosoma, Schistosoma). Papain is the archetype and the best studied member of the family.

Like the serine proteases, catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue (Figure A.2). The essential Cys25 and His159 (papain numbering) play the same role as Ser195 and His57 respectively. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with imidazolium group of His159. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps. A water molecule is not required.

#### **1.1.3. Aspartic proteases**

Most of aspartic proteases belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin and chymosin and processing enzymes such as renin, and certain fungal proteases (penicillopepsin, rhizopuspepsin, endothiapepsin). A second family includes viral proteases such as the protease from the AIDS virus

(HIV) also called retropepsin. Crystallographic studies have showed that these enzymes are bilobed molecules; their active site is located between two homologous lobes. Each lobe contributes one aspartate residue of the catalytically active aspartates. These two aspartyl residues are in close geometric proximity in the active molecule. One aspartate is ionized whereas the second one is unionized at the optimum pH range of 2-3. Retropepsins, are monomeric; carry only one catalytic aspartate and then dimerization is required to form an active enzyme.

In contrast to serine and cysteine proteases, catalysis by aspartic proteases do not involve a covalent intermediate because a tetrahedral intermediate exists. The nucleophilic attack is achieved by two simultaneous proton transfer: one from a water molecule to the diad of the two carboxyl groups and a second one from the diad to the carbonyl oxygen of the substrate. There is then a CO-NH bond cleavage (Figure A.3). This general acid-base catalysis leads to the formation of a non covalent neutral tetrahedral intermediate.

#### **1.1.4. Metalloproteases**

The metallo proteases are one of the older classes of proteases and are found in bacteria, fungi as well as in higher organisms. They are widely different in their sequences and their structures. But the great majority of enzymes contain a zinc atom which is catalytically active. In some cases, zinc may be replaced by another metal such as cobalt or nickel without loss of the activity.

Bacterial thermolysin has been characterized and its crystallographic structure showed that zinc is bound by two histidines and one glutamic acid. Many enzymes contain appropriate sequence which provides two histidine ligands for the zinc; the third ligand is either a glutamic acid or a histidine. In other families, there is a distinct mode for the binding of the Zn atom.

The catalytic mechanism leads to the formation of a non covalent tetrahedral intermediate. This intermediate is formed after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is then decomposed by transfer of the glutamic acid proton to the leaving group.

X-ray crystallographic analyses of Zn containing proteins have given information about features of the catalytic and structural Zn-binding sites. In all Zn enzymes, a catalytic Zn atom is coordinated to three residues of the protein and an active water

molecule, and structural Zn atoms are coordinated to four Cys residues (Figure A.4). A combination of His, Glu, Asp or Cys residue creates a tridentate active Zn site and an activated water molecule fills and completes the coordination sphere.

## **1.2. *Bacillus* Species**

The genus *Bacillus* contains a number of industrially important species. Approximately half of the present commercial production of bulk enzymes derives from the strains of *Bacillus* sp. The *B. subtilis* group is the major source of commercial alkaline protease production world-wide. This group comprises four species: *B. amyloliquefaciens*, *B. licheniformis*, *B. pumilis*, and *B. subtilis* itself. Recent ecological studies, however, have identified some very close relatives of *B. subtilis*, such as, *B. mojavensis* and *B. vallismortis* (Beg and Gupta, 2003).

Members of the genus *Bacillus* are widely used as sources of industrially important products as hydrolytic enzymes, antibiotics, insecticides and fine biochemicals. Members of this genus have the capacity to secrete large quantities of particular proteins, mainly hydrolytic enzymes, into the growth medium. This has resulted in the development of species of *Bacillus* for the production and export of heterologous proteins (Harwood and Archibald, 1990).

Representatives of the genus *Bacillus* are aerobic, endospore-forming, rod-shaped Gram-positive bacteria. The vegetative cells are straight, round or square-ended rods ranging from 0.5 by 1.2 to 2.5 by 10  $\mu\text{m}$ . They occur singly or in chains, their length ranges from a few to many cells. The traditional methods used for the identification of *Bacillus* species rely mainly on spore morphology. Species have been classified into three groups depending on whether they have oval spores that do not distend the sporangium, oval spores that have a larger diameter than sporangium or spherical spores (Turnbull and Kramer, 1991).

*Bacillus* species have adopted a range of strategies to difficult conditions. These include carbon and nitrogen repression, stringency, chemotaxis and associated motility, and the production of antibiotics and various hydrolytic enzymes. Both the latter responses are associated with other soil organisms. They have probably evolved because organisms need to increase their fitness by limiting competition from other microbes and enlarging the range of carbon/nitrogen sources that can be

utilised. The most notable response is sporulation, it involves major morphological, physiological and chemical changes to form heat-, desiccation and radiation-resistant endospores (Harwood and Archibald, 1990).

Sporulation in *Bacillus* species is series of responses to the cessation of exponential growth. It is initiated after the end of cell growth. It consists of a number of stages which were originally classified by morphological features. Cells which are still growing are designated as stage 0. At stage I, two nucleoids of the vegetative cell condense to form a single axial chromatin filament. During stage 0-I, transition sporulating cells synthesize a number of mainly extracellular enzymes, including amylase, proteases, and nucleases, as well as a variety of extracellular antibiotics (Nicholson and Setlow, 1990).

*Bacillus* species are mostly saprophytes, they are widely distributed in nature, particularly in soil, spread in dust, in water and on materials of plant and animal origins. There is a broad range of physiologic characteristics within the genus. There are facultative variants of mesophilic species, facultative and obligate thermophiles that are capable of survival in spore form or even of growth at environmental extremes (Turnbull and Kramer, 1991).

### **1.2.1. Isolation and screening**

The primary stage in the development of an industrial fermentation process is to isolate strains capable of producing the target product in commercial yields. This requires intensive screening. A large number of strains is tested to identify high producers which have novel properties. The conventional method for extracellular microbial products is to grow a large number of organisms on agar plate media. Then production capability of each organism is determined related to the radius of the product's zone of diffusion around the colony.

To design a medium for screening proteases, the medium should contain inducers of the product and there should not be any constituents that may repress enzyme synthesis. Normally, organisms are isolated by surface plating on a medium and subsequent screening for the desired characteristics. The organisms are further grown on specific media for estimating proteolytic, amylolytic, lipolytic, or cellulolytic activities using appropriate substrates such as skim milk or casein, starch, tributyrin,

butter fat, or carboxymethylcellulose. The isolates which exhibit desired level of activity are chosen and maintained on slants for further use.

Of all the alkalophilic microorganisms that have been screened for use in various industrial applications, members of the genus *Bacillus* were found to be predominant and a profitable source of alkaline proteases (Kumar and Takagi, 1999).

### **1.3. Production of Alkaline Proteases**

Most alkalophilic microorganisms produce alkaline proteases, but only those that yield substantial amounts are interested in. It is essential that these organisms be provided with optimal growth conditions to increase enzyme production. The culture conditions that promote protease production are significantly different from the culture conditions that promote cell growth. In the industrial production of alkaline proteases, technical media that were usually employed contained very high concentrations (100–150 g/litre) of complex carbohydrates, proteins, and other media components (Moon et al, 1991). To develop an economically feasible technology, research efforts are mainly focused on: (i) improvement in the yields of alkaline proteases; and (ii) optimization of the fermentation medium and production conditions.

#### **1.3.1. Improvement of yield**

Strain improvement plays a key role in the commercial development of microbial fermentation processes. The wild type strains usually produce limited quantities of the desired enzyme for commercial application. However by adopting simple selection methods, such as spreading of the culture on specific media, it is possible to pick colonies that show a substantial increase in yield. The yield can be further improved by the use of mutagens or antibiotics and the adoption of special techniques or procedures for detecting useful mutants (Glazer et al., 1995).

The protein engineering and sophisticated molecular technologies have offered possibilities for screening variants of enzymes and tailor-made proteins from alkalophilic microorganisms. This can enhance production yields for specific commercial applications. New constructions have been made by the transfer of genes between organisms to produce high yielding variants (Takami et al., 1992).

Currently, two different strategies are available for generation of protein-engineered variants: random and site-directed mutagenesis. With random mutagenesis, a large number of variants are produced, but the success largely depends on efficient screening procedures to identify variants which have improved properties. In site-directed mutagenesis, structural or biochemical data are used to reduce the number of variants to be constructed. Every protein variant is purified and individually tested for improvements. For producing mutated enzymes, the two approaches are optimally used in combination with each other. Promising variants which are generated and identified by random mutagenesis can be further improved by site-directed introduction of known advantageous mutations.

### **1.3.2. Optimization of fermentation medium**

Alkaline proteases are generally produced by submerged fermentation. In addition, some solid state fermentation processes have been assayed for production of these enzymes (George et al., 1995). Research efforts have been directed mainly toward: (i) evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes; (ii) requirement of divalent metal ions in the fermentation medium; and (iii) optimization of environmental and fermentation parameters such as pH, temperature, aeration, and agitation. In addition, no defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme production.

Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium (Cruegar, 1984).

Studies have also indicated a reduction in protease production due to catabolite repression by glucose (Kole et al., 1998). In commercial practice, high carbohydrate concentrations repressed enzyme production.

Divalent metal ions such as calcium, cobalt, copper, boron, iron, magnesium, manganese, and molybdenum are required in the fermentation medium for optimum production of alkaline proteases (Kumar and Takagi, 1999). However, the requirement for specific metal ions depends on the source of enzyme.

The important characteristic of most alkalophilic microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production. For increased protease yields, the pH of the culture medium must be maintained above 7.5 throughout the fermentation period. The culture medium pH also strongly affects many enzymatic processes and transport of various components across the cell membrane. Because there is a close relationship between protease synthesis and the utilization of nitrogenous compounds, pH variations during fermentation may indicate kinetic information about the protease production, such as the start and end of the protease production period (Moon et al., 1991).

Temperature is another critical parameter that has to be controlled and varied from organism to organism. The mechanism of temperature control of enzyme production is not well understood. However, studies by Frankena et al. (1986) showed that a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake.

### **1.3.3. Correlation between growth and protease production**

The production of an enzyme shows a relationship with the growth phase of that organism. In general, the synthesis of protease in *Bacillus* species is constitutive or partially inducible. It is controlled by numerous complex mechanisms during the transition state between exponential growth and the stationary phase (Priest, 1977). The production of extracellular proteases during the stationary phase of growth is characteristic of many bacterial species. The sequence and the rate of enzyme production are, however, variable with the specific organism. At early stationary phase, two or more proteases are secreted. The ratio of the amount of the individual proteases produced also varied with the *Bacillus* strains (Priest, 1977). In several cases, the function of the enzyme is not very clear, but its synthesis is correlated with the onset of a high rate of protein turnover and often sporulation.

The growth environment can also influence enzyme synthesis, since protease production in *Bacillus* sp. is extracellular in nature. There is little or no enzyme production during the exponential growth phase. In other cases, the synthesis and secretion of the protease was initiated during the exponential growth phase. A substantial increase was seen near the end of the growth phase and there were maximum amounts of protease produced in the stationary growth phase.

## **1.4. Purification of Alkaline Proteases**

Crude preparations of alkaline proteases are generally employed for commercial use. Nevertheless, the purification of alkaline proteases is important for the better understanding of the enzyme function.

### **1.4.1. Recovery**

After fermentation, when the fermented medium leaves the controlled environment of the fermenter, it is exposed to a drastic change in environmental conditions. It is indispensable to rapidly lower the temperature of the fermented medium to below 5°C, to prevent microbial contamination and maintain enzyme activity and stability. The removal of the cells, solids, and colloids from the fermentation broth is the primary step in enzyme downstream processing, for which filters and centrifuges are commonly used.

### **1.4.2. Concentration and precipitation**

Because the amount of enzyme present in the cell-free filtrate is usually low, the removal of water is a primary objective. Recently, membrane separation processes have been widely used for downstream processing. Ultrafiltration (UF) is one such membrane process that has been largely used for the recovery of enzymes. This pressure-driven separation process is inexpensive; it results in little loss of enzyme activity. It offers both purification and concentration, also diafiltration, for salt removal or for changing the salt composition (Strahtman, 1990). However, a disadvantage of this process is the fouling or membrane clogging because of precipitates formed by the final product. This clogging can be overcome by treatment with detergents, proteases, or acids and alkalies.

Precipitation is the most commonly used method for the isolation and recovery of proteins from crude biological mixtures. It also performs both purification and concentration steps. It is generally affected by the addition of reagents such as salt or an organic solvent, which lowers the solubility of the desired proteins in an aqueous solution.

Molecules are separated according to their physical properties as size, shape, charge, hydrophobic interactions; chemical properties as covalent binding or biological properties as biospecific affinity. Enzymes are very complex protein molecules that

possess both ionizable and hydrophobic groups which interact with the solvent. Proteins can be agglomerated and precipitated by changing their environment. High salt concentrations act on the water molecules surrounding the protein and change the electrostatic forces responsible for solubility.

Ammonium sulfate is commonly used for precipitation; it is an effective agent for concentrating enzymes. Enzymes can also be fractioned by using different concentrations of ammonium sulfate. The optimal concentration of salt required for precipitation must be determined experimentally, and generally ranges from 20 to 80 % saturation (Wilson and Walker, 2000).

Although precipitation by ammonium sulphate has been used for many years, it is not the precipitating agent of choice for detergent enzymes. Ammonium sulphate found wide utility only in acidic and neutral pH values and developed ammonia under alkaline conditions. Despite better precipitating qualities of sodium sulphate over ammonium sulphate, its poor solubility at low temperatures restricted its use for this purpose (Shih et al.,1992). Precipitation of enzymes can also be achieved by the use of water-soluble, neutral polymers such as polyethylene glycol.

#### **1.4.3. Ion-exchange chromatography**

Ion-exchange chromatography is a separation technique based on the charge of protein molecules. Enzyme molecules possess positive and negative net charges. The net charge is influenced by pH, and this property is used to separate proteins by chromatography on anion exchangers (positively charged) or cation exchangers (negatively charged). The sample is applied in aqueous solution at low ionic strength, and elution is best carried out with a salt gradient of increasing concentration.

Ion-exchange is very useful because large volumes can be processed and the elution of dilute sample components in concentrated form is possible. The matrix used to produce ion-exchange resins should be sufficiently hydrophilic to prevent enzyme denaturation. They should also have a high capacity for large molecules at fast equilibration. Industrial applications require ion exchangers that give good resolution, allow high flow rates to be used, suffer small changes in volume with salt gradients or pH changes, and can be completely regenerated (Wilson and Walker, 2000).

Alkaline proteases are generally positively charged and are not bound to anion exchangers. However, cation exchangers can be a rational choice and the bound molecules are eluted from the column by an increasing salt or pH gradient.

#### **1.4.4. Stabilization**

The enzyme preparations used commercially are impure and are standardized to specified levels of activity by the addition of some diluents and carriers. The conditions for maximum stability of crude preparations may be quite different than for purified enzymes. Storage stability is of prime concern to enzyme manufacturers because there is a loss of activity during storage in the factory and shipment to clients. Protease solutions are subject to proteolytic and autolytic degradation that results in rapid inactivation of enzymatic activity.

Addition of stabilizers like calcium salts, sodium formate, borate, propylene glycol, glycerine or betaine, polyhydric alcohols, protein preparations, and related compounds has been successful to maintain the enzyme activity and provide stability (Weijers and Van't Riet, 1992). Also, to prevent contamination of the final commercial crude preparation during storage, addition of sodium chloride at 18-20% concentration has been suggested. In certain cases, the liquid enzyme preparations are brought to powder form because it is more convenient in handling and storage. However, handling of dry enzymes poses potential health hazards and also it is customary to maintain the enzyme preparations in stabilized liquid form.

The stabilization of alkaline proteases has also been made possible. Numerous examples have been cited in literature with the use of protein engineering. Site-directed mutagenesis has been also used. It is based on specific protein design strategies, including change of electrostatic potential, introduction of disulfide bridges, replacement of oxidation labile residues, modification of side chain interactions, improvement of internal packaging, strengthening of metal ion binding, reduction in unfolding entropy, residue substitution or deletion based on homology and modification of substrate specificity (Kumar and Takagi, 1999).

### 1.5. Properties of Alkaline Proteases

The enzymatic and physicochemical properties of alkaline proteases from several microorganisms have been studied extensively.

The optimum pH range of alkaline proteases is generally between pH 9 and 11, with a few exceptions of higher pH optima. They also have high isoelectric points and are generally stable between pH 6 and 12. The optimum temperatures of alkaline proteases range from 50 to 70°C.

Alkaline proteases require a divalent cation like  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  or a combination of these cations, for maximum activity. These cations were also found to enhance the thermal stability of a *Bacillus* alkaline protease. It is believed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures. In addition, specific  $\text{Ca}^{2+}$  binding sites that influence the protein activity and stability apart from the catalytic site were described for Proteinase K (Bajorath et al., 1988).

Inhibition studies give information about the nature of the enzyme, its cofactor requirements, and the nature of the active site. In some of the studies, catalytic activity was inhibited by  $\text{Hg}^{2+}$  ions. In this regard, the poisoning of enzymes by heavy metal ions has been documented in the literature.

Alkaline proteases are completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP). PMSF sulfonates the essential serine residue in the active site and results in the complete loss of activity. This inhibition profile classifies these proteases as serine hydrolases. In addition, some of the alkaline proteases were found to be metal ion dependent; they were sensitive to metal chelating agents, such as EDTA. Thiol inhibitors have little effect on alkaline proteases of *Bacillus* spp.

### 1.6. Industrial Application

Alkaline proteases are robust enzymes with industrial potential in detergents, leather processing, silver recovery, medical purposes, food processing, feeds, and chemical industries, as well as waste treatment. The different applications currently using alkaline proteases are:

### 1.6.1. Detergent additives

Microbial alkaline proteases dominate commercial applications with a significant share of the market captured alkaline proteases from *Bacillus* spp. for laundry detergent applications. Alkaline proteases added to laundry detergents enable the release of proteinaceous material from stains. The increased usage of these proteases as detergent additives is mainly due to the cleaning capabilities of these enzymes in environmentally acceptable, nonphosphate detergents. In addition to improved washing efficiency, the use of enzymes allows lower wash temperatures and shorter periods of agitation, often after a preliminary period of soaking.

Ideally, proteases and other enzymes used in detergent formulations should have high activity and stability over a broad range of pH and temperature. The enzymes used should be effective at low levels and should also be compatible with various detergent components such as oxidizing and sequestering agents. They must also have a long shelf life.

The usage of alkaline enzymes in automatic dishwashing detergents has also increased recently. The in-place cleaning of ultrafiltration (UF) and reverse osmosis (RO) membranes are two important aspects of modern dairy and food industries. The UF and RO membranes are put to a variety of uses, including concentration, fractionation, clarification and sterilization of liquid foods such as milk, whey, egg white, fruit juices, wines, and other beverages (Glover, 2002).

The enzyme detergent preparations presently marketed for cleaning of membrane systems are *Alkazym* (Novodan A/S, Copenhagen, Denmark), *Terg-a-zyme* (Alconox, Inc, New York, USA), *Ultrasil 53* (Henkel KGaA, Dusseldorf, Germany) and *P3-paradigm* (Henkel-Ecolab GmbH, Düsseldorf, Germany). These enzyme-based cleaners rely on the proteases to cleave and solubilize the protein foulant.

The use of a cocktail of proteases and lipases to degrade and solubilize protein and fat foulants have also been beneficial. In addition, contact lens cleaning solutions using an alkaline protease from a marine shipworm bacterium cleaned the contact lens at low temperatures. In India, one such enzyme-based optical cleaner in the form of tablets containing *Subtilopeptidase A* is presently marketed by M/s Bausch and Lomb (India) Ltd.

### **1.6.2. Tannery industry**

Alkaline proteases that possess elastolytic and keratinolytic activity provide an effective biotreatment of leather. It includes the dehairing and bating of skins and hides. Hair roots are swelled in alkaline conditions, then with subsequent attack of proteases on the hair follicle protein, the hair is easily removed. This process is safer than traditional methods using sodium sulfide treatment because it contributes to 100% of sulfide and over 80% of the suspended solids in tannery effluents.

The bating following the dehairing process involves the degradation of elastin and keratin, removal of hair residues, and the deswelling of collagen. At the end of the bating process a good, soft leather mainly used for making leather clothes and goods is produced (Taylor et al., 1987). Studies carried out by different workers have demonstrated the successful use of alkaline proteases in leather tanning *B. amyloliquefaciens*, and *B. subtilis*.

### **1.6.3. Silver recovery**

Alkaline proteases find potential application in the bioprocessing of used X-ray films for silver recovery. Used X-ray film contains approximately 1.5 to 2.0 % (by weight) silver in its gelatin layers. The conventional method of silver recovery is carried out by burning film. But this causes a major environmental pollution problem. The enzymatic hydrolysis of the gelatin layers on the X-ray film enables not only the silver, but also the polyester film base, to be recycled.

The alkaline proteases from *Bacillus* sp. B21-2, *Bacillus* sp. B189 and *B. coagulans* PB-77 decomposed the gelatinous coating on the used X-ray films from which the silver was recovered (Ishikawa et al., 1993).

### **1.6.4. Medical uses**

Collagenases with alkaline protease activity are used for therapeutic applications in the preparation of slow-release dosage forms. A new semi-alkaline hydrolyzed various collagen types without amino acid release and liberated low molecular weight peptides of potential therapeutic use (Barthomeuf et al., 1992).

Similarly, *Elastoterase*, a preparation with high elastolytic activity from *Bacillus subtilis* 316M, was immobilized on a bandage for therapeutic application in the treatment of burns and purulent wounds, carbuncles, furuncles, and deep abscesses (Kudrya and Simonenko, 1994) .

#### **1.6.5. Food industry**

Alkaline proteases can hydrolyze proteins from plants, fish, or animals to produce hydrolysates of well-defined peptide profile. The commercial alkaline protease, *Alcalase*, has a broad specificity for terminal hydrophobic amino acids. A less bitter hydrolysate and a debittered enzymatic whey protein hydrolysate were produced by using this enzyme.

The protein hydrolysates commonly generated from casein, whey protein and soy-protein find major application in hypoallergenic infant food formulations. They can also be used for the fortification of fruit juices or soft drinks and in manufacturing protein-rich therapeutic diets.

Proteases play an important role in meat tenderization, especially of beef. They possess the ability to hydrolyze connective tissue proteins as well as muscle fibre proteins (Takagi et al., 1992).

#### **1.6.6. Waste treatment**

Alkaline proteases provide potential application for the management of wastes from various food processing industries and household activities. These proteases can solubilize proteins in wastes to recover liquid concentrates or dry solids of nutritional value for fish or livestock.

Many keratinolytic alkaline proteases were used in feed technology for the production of amino acids or peptides, for degrading waste keratinous material in household refuse. They were also used as a depilatory agent to remove hair in bath tub drains, which caused bad odors in houses and in public places.

#### **1.6.7. Chemical industry**

Some studies have demonstrated the possibility of using alkaline proteases to catalyze peptide synthesis in organic solvents. Proteases immobilized on insoluble supports have been employed to synthesize peptides enzymatically (Gololobov et al., 1994).

*Proleather*, a commercial alkaline protease preparation from *Bacillus* sp. was used for a sucrose-polyester synthesis in anhydrous pyridine. The polyester is extremely water-soluble and also soluble in polar organic solvents. It finds its application as a biodegradable plastic. The *Proleather* also catalyzes the transesterification of D-glucose with various acyl donors in pyridine.

The enzyme *Alcalase* acted as catalyst for resolution of N-protected amino acid esters and alkaline proteases from *Conidiobolus coronatus* were found to replace subtilisin Carlsberg in resolving the racemic mixtures of DL-phenylalanine and DL-phenylglycine.

#### **1.6.8. Other uses**

Alkaline proteases from *Conidiobolus* sp. were also able to act as a substitute for trypsin used in the preparation of animal cell cultures. Kwon et al. (1994) reported the use of alkaline proteases from *Vibrio metschnikovii* RH530 as an alternative for Proteinase K in DNA isolation.

## **2. MATERIALS AND METHODS**

### **2.1. Materials**

#### **2.1.1. Bacterial culture media**

##### **2.1.1.1. Nutrient agar medium**

5 g peptone, 3 g meat extract, 15 g agar (Merck) were dissolved in distilled water, the pH was adjusted to 7.0-7.5. The solution was completed to a total volume of 1 l and sterilized for 15 minutes under 1.5 atm pressure at 121°C.

##### **2.1.1.2. Nutrient broth medium**

5 g peptone, 3 g meat extract, 10 mg  $\text{MnSO}_4 \cdot \text{H}_2\text{O}$  (Riedel-de Häen) were dissolved in distilled water, the pH was adjusted to 7.0-7.5. The solution was completed to a total volume of 1 l and sterilized for 15 minutes under 1.5 atm pressure at 121°C.

#### **2.1.2. Solutions and buffers**

##### **2.1.2.1 Britton-Robinson Buffer (B-R Buffer)**

**Solution A:** 2.71 ml of 85%  $\text{H}_3\text{PO}_4$  (Riedel-de Häen), 2.36 ml of acetic acid (Riedel-de Häen), 2.47 g of  $\text{H}_3\text{BO}_3$  (Riedel-de Häen) were mixed and distilled water was added to a final volume of 1 l.

**Solution B:** 8 g of NaOH (Riedel-de Häen) was dissolved in 1 l of distilled water.

**B-R Buffer; pH 7:** 100 ml of solution A was mixed with 52.5 ml of solution B.

**B-R Buffer; pH 8:** 100 ml of solution A was mixed with 60 ml of solution B.

**B-R Buffer; pH 9:** 100 ml of solution A was mixed with 67.5 ml of solution B.

**B-R Buffer; pH 10:** 100 ml of solution A was mixed with 80 ml of solution B.

##### **2.1.2.2. Enzyme assay solutions and buffers**

**Tris-HCl Buffer (0.5 M; pH 7.2):** 60.6 g Tris(hydroxymethyl)aminomethane (Merck) was dissolved in minimum amount of distilled water, its pH was adjusted to 7.2 with 10 M HCl (Merck), and distilled water was added to a final volume of 1 l.

**Sodium Acetate Solution (1M):** 20.5 g sodium acetate (J.T. Baker) was dissolved in 250 ml of distilled water.

**Acetic Acid Solution (1M):** 18.8 ml of %37 fuming acetic acid (Merck) was added to distilled water to a final volume of 330 ml

**TCA Solution (50 %):** 18 g trichloroacetic acid (Riedel-de Häen) was dissolved in 36 ml of distilled water.

**TCA Mix Solution:** 220 ml of 1 M sodium acetate solution, 330 ml of 1M acetic acid solution and 36 ml of 50 % TCA solution were mixed and distilled water was added to a final volume of 1l.

**Casein Solution (0.6 %):** 0.6 g casein (Fluka) was first dissolved in 10 ml of 0.1 M NaOH, then distilled water and 4 ml Tris-HCl were added to this solution. Its pH was then adjusted to 7.2 with 0.1 M HCl and distilled water was added up to 100 ml.

**NaOH (10M):** 40 g sodium hydroxide was dissolved in 100 ml of distilled water.

**HCl (10M):** 83 ml hydrochloric acid 37% was completed to 100 ml with distilled water.

#### **2.1.2.3. BCA assay solutions**

**Solution A:** 1 g BCA, 2 g sodium carbonate (Merck), 0.16 g sodium tartrate (Riedel-de Häen), 0.4 g sodium hydroxide and 0.95 g sodium bicarbonate (Riedel-de Häen) were dissolved in a minimum amount of distilled water, its pH was adjusted to 11.25 with sodium hydroxide, then distilled water was added to complete the volume to 100 ml.

**Solution B:** 4 g copper sulfate (Riedel-de Häen) was dissolved in 100 ml of distilled water.

**Solution C:** 100 ml of solution A was mixed with 2 ml of solution B.

#### **2.1.2.4. SDS-PAGE solutions and buffers**

**Monomer Solution (30% T/ 2.7% Cbis):** 58.4 g acrylamide (Sigma) and 1.6 g bis-acrylamide (Sigma) were dissolved in 200 ml of distilled water and stored at 4°C in the dark.

**4 x Separating Gel Buffer (1.5 M Tris-HCl, pH 8.8):** 36.3 g Tris was dissolved in a minimum amount of distilled water, pH of the solution was adjusted to 8.8 and the solution and distilled water was added to a final volume of 200 ml.

**4 x Stacking Gel Buffer (0.5 M Tris-HCl, pH 6.8):** 3 g Tris was dissolved in a minimum amount of distilled water, pH of the solution was adjusted to 6.8 and the solution reached a volume 50 ml. with distilled water.

**SDS Solution (10 %):** 10 g SDS (Sigma) was dissolved in 100 ml distilled water.

**Ammonium Persulfate (APS) solution (10 %):** 0.1 g APS (Sigma) was dissolved in 1 ml distilled water.

**TEMED (Gel Catalyst):** N,N,N', N' Tetramethylethylamin (Carlo Erba).

**2 x Sample Buffer :** 2.5 ml of 4 x Stacking Gel Buffer, 4 ml of 10 % SDS solution, 2 ml glycerol (Fluka), 1 ml  $\beta$ -Mercaptoethanol (Merck) and 0.05 % Bromophenol blue were dissolved in 10 ml of distilled water.

**Tank Buffer :** 3 g Tris, 14.4 g glycine (Merck), 10 ml SDS (0.1%) were mixed and distilled water was added to reach a final volume of 1l.

**Coomassie Blue Stain:** 0.1 g CBB R-250 (Biorad), 50 ml methanol (Riedel-de Häen), 10 ml acetic acid were mixed and total volume of the solution reached 100ml with distilled water.

**Gel Destain Solution:** 5 ml methanol, 10 ml acetic acid were mixed and distilled water was added to reach a volume of 100 ml.

#### 2.1.2.5. Metal ion solutions

**MnCl<sub>2</sub> (10 mM):** 0.0162 g MnCl<sub>2</sub>.2H<sub>2</sub>O (Merck) was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**MgCl<sub>2</sub> (10 mM):** 0.0203 g MgCl<sub>2</sub>.6H<sub>2</sub>O (Carlo Erba) was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**KCl (10 mM):** 0.0075 g KCl (Riedel-de Häen) was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**NiCl<sub>2</sub> (10 mM):** 0.0238 g NiCl<sub>2</sub>.6H<sub>2</sub>O (Merck) was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**NaCl (10 mM):** 0.0058 g NaCl (Riedel-de Häen) was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**ZnCl<sub>2</sub> (10 mM):** 0.0136 g ZnCl<sub>2</sub> (Riedel-de Häen) was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**CuSO<sub>4</sub> (10 mM):** 0.025 g CuSO<sub>4</sub>.5H<sub>2</sub>O (Riedel-de Häen) was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**CuCl<sub>2</sub> (10 mM):** 0.017 g CuCl<sub>2</sub>·2H<sub>2</sub>O was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**FeSO<sub>4</sub> (10 mM):** 0.0278 g FeSO<sub>4</sub>·7H<sub>2</sub>O (Riedel-de Häen) was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**CaCl<sub>2</sub> (10 mM):** 0.0147 g CaCl<sub>2</sub>·2H<sub>2</sub>O (Merck) was dissolved in 10 ml of 0.5 M Tris-HCl buffer pH 7.5.

**HgCl<sub>2</sub> (10 mM):** 0.0271g HgCl<sub>2</sub> (Merck) was dissolved in 10ml of 0.5 M Tris-HCl buffer pH 7.5.

#### **2.1.2.6. Chemical solutions**

**SDS (1mM):** 0.0029 g of SDS was dissolved in 10ml of 0.5 M Tris-HCl buffer pH 7.5.

**Sodium azide (1mM):** 0.0065 g of sodium azide (Merck) was dissolved in 100ml of 0.5 M Tris-HCl buffer pH 7.5.

**EDTA (1mM) :** 0.0037 g EDTA was dissolved in 10ml of 0.5 M Tris-HCl buffer pH 7.5.

**β-Mercaptoethanol (1mM):** 7 µl of β-Me was mixed to 10ml 0.5 M Tris-HCl buffer pH 7.5.

**PMSF (1mM):** 0.00174 PMSF (Merck) was dissolved in 10ml of 0.5 M Tris-HCl buffer pH 7.5.

#### **2.1.3. API Test Kit media and reagents**

##### **2.1.3.1. API 50 CH**

**API CHB/E Medium Composition:** 2 g ammonium sulphate, 0.5 g yeast extract, 1 g tryptone, 3.22 g disodium phosphate, 0.12 g monopotassium phosphate, 10 ml trace elements, 0.18 g phenol red were mixed in 1000 ml of demineralized water.

**API 50 CH Strip Composition:** Strips include different kinds of substrates to study the carbohydrate metabolism of microorganisms (Table 2.1).

**Table 2.1.** Compositon of API 50 CH Strip

| Tube | Active Ingredients        |    |                           |
|------|---------------------------|----|---------------------------|
| 0    | Control                   | 25 | Esculin ferric citrate    |
| 1    | Glycerol                  | 26 | Salicin                   |
| 2    | Erythritol                | 27 | D-Cellobiose              |
| 3    | D-Arabinose               | 28 | D-Maltose                 |
| 4    | L-Arabinose               | 29 | D-Lactose                 |
| 5    | D-Ribose                  | 30 | D-Melibiose               |
| 6    | D-Xylose                  | 31 | D-Sachharose              |
| 7    | L-Xylose                  | 32 | D-Trehalose               |
| 8    | D-Adonitol                | 33 | Inulin                    |
| 9    | Methyl-βD-Xylopyranoside  | 34 | D-Melezitose              |
| 10   | D-Galactose               | 35 | D-Raffinose               |
| 11   | D-Glucose                 | 36 | Amidon                    |
| 12   | D-Fructose                | 37 | Glycogen                  |
| 13   | D-Mannose                 | 38 | Xylitol                   |
| 14   | L-Sorbose                 | 39 | Gentiobiose               |
| 15   | L-Rhamnose                | 40 | D-Turanose                |
| 16   | Dulcitol                  | 41 | D-Lyxose                  |
| 17   | Inositol                  | 42 | D-Tagatose                |
| 18   | D-Mannitol                | 43 | D-Fucose                  |
| 19   | D-Sorbitol                | 44 | L-Fucose                  |
| 20   | Methyl-αD-Mannopyranoside | 45 | D-Arabitol                |
| 21   | Methyl-αD-Glucopyranoside | 46 | L-Arabitol                |
| 22   | N-AcetylGlucosamine       | 47 | Potassium gluconate       |
| 23   | Amygdalin                 | 48 | Potassium 2-ketogluconate |
| 24   | Arbutin                   | 49 | Potassium 5-ketogluconate |

**2.1.3.2. API ZYM**

**ZYM A reagent:** 25 g Tris-hydroxymethyl-aminomethane, 11 ml hydrochloric acid (37%), 10 g sodium lauryl sulfate are mixed in of 100 ml of distilled water.

**ZYM B reagent:** 0.35 g Fast Blue BB is dissolved in 100 ml of 2-methoxyethanol.

**NaCl (0.85 %):** 8.5 g sodium chloride is dissolved in 1000 ml of distilled water.

**API ZYM Strip Composition:** This strip includes 20 microwells including different substrates to test the enzymatic activity. The names of the substrates, the enzymes assayed and colors developed after the assay are listed in the reading table of the kit (Table B.1).

#### 2.1.4. Laboratory Equipments

**Autoclave:** 2540 ml benchtop autoclave, Tuttbauer GmbH Labor- Systemtechnik.

**Balances:** Precisa BJ 610C and Precisa 125A SCS, Precisa Instruments AG, CH-Dietikon.

**Centrifuges:** Avanti J-301 and Microfuge 18, Beckman Coulter.

**Centrifuge rotors:** JA-30.50 Ti and JLA-16.250, Beckman Coulter.

**Chromatography column:** UNO Q-6, Catalog No 720-0003, Bio-Rad.

**Electrophoresis equipment:** Mini- PROTEAN 3 cell and systems, Bio-Rad.

**FPLC:** BioLogic Duo-Flow, Bio-Rad.

**Fraction Collector:** Model 2128, Bio-rad

**Glassware:** Teknik Cam, ISOLAB.

**Ice Machine:** AF 10, Scotsman.

**Incubators:** EN400, Nüve; Memmert GmbH-Co.

**Laminar flow cabinet:** ÖZGE.

**Magnetic stirrer:** AGE 10.0164, VELP Scientifica srl.

**Microscope:** Olympus Corp.

**Microplate Reader:** Model 3550 UV Microplate, Bio-Rad.

**Pipettes:** 1000, 200, 20 µl ; Eppendorf.

**pH meter:** Inolab pH Level1, Wissenschaftlich Technische Werkstätten GmbH Co.

**Refrigerator and deep freezer:** 4240 T refrigerator, 2021 D deep freezer, Arçelik.

**Spectrophotometer:** Lambda 25 UV/Vis Spectrometer, Perkin Elmer Instruments.

**Vortex apparatus:** NM 110, Nüve.

## 2.2. Methods

### 2.2.1. Isolation, screening and identification of protease producing strains

Samples of tanning wastewater collected from various leather factories were analyzed for their content of microorganisms, specially for the genus *Bacillus*. 200µl of each sample was spread onto different nutrient agar plates and cultured at 37°C for 24 hours. Some colonies were detected. Each of these colonies was then transferred to new agar plates and was grown under same conditions. First the purity of the strain was checked. Then by gram staining test, they were examined under microscope if they have the shape of spore-forming rod and also if they are gram

positive. Those with matching characteristics were further grown on agar plates to have enough amounts of bacteria.

#### **2.2.1.1. Gram staining procedure**

Both Gram-positive (Gr+) and Gram-negative (Gr-) organisms form a complex of crystal violet and iodine within the bacterial cell during the Gram-staining procedure. Gr+ organisms are thought to resist decolorization by alcohol or acetone because the alcohol/acetone mixture causes dehydration of the multilayered peptidoglycan in the Gram-positive cell wall, decreasing the space between the molecules; it means that cell wall permeability is markedly decreased. Thus, the dye complex is entrapped within the cell and resist being washed out by the solvents, and Gr+ bacteria remain purple following this differential stain. In contrast, cell wall permeability of Gr- organisms is increased by ethyl alcohol washing because being a lipid solvent it removes the outer membrane from the Gram-negative cell wall. The thin layer of peptidoglycan is unable to retain the crystal violet-iodine complex. This allows the removal of the crystal violet-iodine complex from within the cell. The decolorized Gr- cell can then be rendered visible with a suitable counterstain, in this case Safranin O, which stains them pink. Pink which adheres to the Gr+ bacteria is masked by the purple of the crystal violet.

For the staining of isolated microorganisms, a small drop of liquid medium was placed on a clean slide. A small amount of bacteria was aseptically removed from the agar surface and mixed thoroughly with the liquid medium using the loop. This suspension was spread over the entire slide to form a thin film. This thin suspension was allowed to completely air dry. The slide (film-side up) was passed through the flame 3 or 4 times for heat-fixation. The slide was stained by crystal violet for two minutes. Then it was gently washed with water. The excess water was shaken off. The slide was then stained with lugol solution for two minutes and gently washed with water. The slide was decolorized by adding ethanol- acetone mixture drop by drop until the purple color stopped flowing. It was then immediately washed with water. It was then stained with safranin for 30 seconds and washed with water. The slide was left to dry on a blotting paper and observed using oil immersion microscopy.

#### **2.2.1.2. API 50 CH System**

Specific identification of bacterial strains was done with API 50 CH test kit (Biomérieux, France) which is a standardized system associating 50 biochemical tests for the study of the carbohydrate metabolism of microorganisms. The API 50 CH strip consists of 50 microtubes used to study fermentation of substrates belonging to the carbohydrate family and its derivatives (heterosides, polyalcohols, uronic acids). The fermentation tests were inoculated with a suspension of microorganisms prepared with the suitable API CHB/E medium. This medium served to rehydrate the substrates. During incubation, fermentation was revealed by a color change in the tube, caused by the anaerobic production of acid and detected by the pH indicator present in the medium.

#### **Preparation of the inoculum**

All the bacteria from the culture plates were picked up using a loop. Then, in a sterile solution of NaCl (% 0.85) all the bacteria was suspended to prepare a heavy suspension (S). To another tube of 5 ml NaCl (% 0.85) solution, drops were added from suspension (S) to have a turbidity equivalent to 2 McFarland. Number of drops was recorded as (n) number. The medium used to inoculate the strips (API 50 CHB/E medium 10 ml) was then inoculated adding twice the number of drops (2n) from the suspension (S).

#### **Inoculation of the strips and Incubation**

Each tube was inoculated with the bacteria-medium suspension and incubated at 37°C for 48 hours.

#### **Reading of the strip**

Strips were read after 24 and 48 hours of incubation. A positive test corresponded to an acidification revealed by the phenol red indicator contained in the medium changing to yellow. For the Esculin test (tube no.25), a change from red to black was observed. A positive test may become negative at the second reading . This was because of the production of ammonia from peptone which neutralizes the acids. Such tests were recorded as positive. The results recorded as + or – were entered in the identification software. Four different bacterial strains were identified as *Bacillus cereus*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis*.

### **2.2.1.3. API ZYM System**

To determine the best protease producers among these strains, API ZYM enzymatic activity research system was used. API ZYM is a semi-quantitative micromethod designed for the detection of enzymatic activities. It allows systematic and rapid study of 19 enzymatic reactions using very small sample quantities. It permits the detection of enzymatic activities in a complex sample which has not been purified. Using the same inoculation principle as API 50 CH, these strips containing 19 different substrates were inoculated by with a dense suspension of organisms to rehydrate the enzymatic substrates. The base of these strips were made of non-woven fibers containing synthetic substrates. This allowed enzymatic reaction to take place even if the substrates were insoluble. The metabolic end products produced during the incubation period were detected through colored reactions revealed by the addition of reagents. Next experiments were carried out by the most protease producers of these four different strains detected by this test.

#### **Preparation of the inoculum**

All the bacteria from the culture plate were picked up using a loop. Then, in a sterile solution of NaCl (% 0.85) all bacteria were suspended to prepare a heavy suspension with a turbidity equivalent to 5-6 McFarland.

#### **Inoculation of the strips and Incubation**

Each cupule of the strip was inoculated with 65 µl of specimen and incubated for 4 hours at 37°C.

#### **Reading of the strip**

After incubation, 1 drop of ZYM A reagent and 1 drop of ZYM B reagent were added to each cupule. By placing a surface-active agent (ZYM A reagent) in the cupule, solubilization of ZYM B reagent in the medium was facilitated. Color was developed after five minutes. The strip was put under a light source or daylight for a few minutes to eliminate any yellow color which might appear in the cupules due to any excess of Fast Blue BB which has not reacted.

A value ranging from 0-5 can be assigned corresponding to the colors developed: 0 corresponds to a negative reaction, 5 to a reaction of maximum intensity (Table B.1).

### **2.2.2. Production and partial purification of enzyme**

The cultivation of isolates was carried out in 1 litre Erlen Mayer flasks containing 200 ml nutrient broth as liquid media. The isolates were cultivated for 20-22h at 37°C with agitation of 200-250 rev/min.

After incubation, the culture broth was centrifuged at 4°C and 20,000 x *g* for 20 min. The supernatants were collected. The enzyme in the cell-free supernatant was precipitated between 40 and 80% saturation of ammonium sulfate. For 40% and 80% saturations, 22.6 g and 26.3 g ammonium sulfate were added respectively for 100 ml of supernatant. Saturated supernatant solutions were left over night after addition of salt and the resulting precipitates were collected by centrifugation at 25,000x *g* for 30 min. The precipitates were then dissolved in a minimal volume of 50 mM Tris-HCl-5 mM CaCl<sub>2</sub>, (pH 7.5). These solutions were dialyzed overnight against 500 ml of the same buffer at 4°C.

After dialysis, concentrated solutions were loaded onto a Q- Sepharose anion exchange column (2.5x13.5 cm) in a fast protein liquid chromatography system. The column was previously equilibrated with 50 mM Tris-HCl-5 mM CaCl<sub>2</sub>, (pH 7.5). The column was washed at a flow rate of 1 ml min<sup>-1</sup> with this buffer and the bound proteins were eluted with a linear gradient of 0 to 1 M NaCl in the same buffer. Active fractions were collected and concentrated using a centrifugal filter unit (NMWCO;10,000) at 4000 x *g* at 4°C. Centrifugation continued until the solution was concentrated to a volume of 500 µl. Samples from this concentrated solution was loaded on a SDS-PAGE system and bands were detected.

### **2.2.3. Characterization of protease**

#### **2.2.3.1. Effect of temperature on enzyme activity and stability**

The temperature stability was determined by measuring the residual activity. Enzyme solution (50 µl) was incubated in 950 µl of 0.05M Tris-HCl buffer (pH 7.5) for 30 minutes at different temperatures ranging from 30 to 80 °C. They were then put on ice and relative activities were assayed at standart assay conditions.

The activity of partially purified enzyme was assayed at the same temperature range for 10 min. % 0.6 casein solution was preincubated at respective temperatures for 5 min before the enzymatic assay.

#### **2.2.3.2. Effect of pH on enzyme activity and stability**

The optimum pH was determined at 30°C with casein solution prepared in Britton-Robinson (B-R) Buffer ranging from pH 6 to pH 12.

pH stability was also determined at the same pH range in the same B-R buffer systems. 50 µl enzyme solution was incubated with 150 µl B-R buffer for 20 h. at 30°C. Then it was diluted with 0.05 M Tris-HCl buffer (pH 7.5) to 1 ml. For the reference value, 50 µl enzyme was left at + 4°C for 20 h. in 150 µl of 0.05 M Tris-HCl buffer (pH 7.5) then diluted with the same buffer to 1 ml.

#### **2.2.3.3. Effect of metal ions on protease activity**

The effect of 10 mM cations on protease activity was examined. Partially purified proteases at the concentration of 200 µg/ml were preincubated for 30 min at 30°C with tested cations ( $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^{+}$ ,  $\text{Ni}^{2+}$ ,  $\text{Na}^{+}$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Hg}^{2+}$ ) in 0.05 M Tris-HCl buffer (pH 7.5). Residual activities were determined using the standart enzyme assay. Residual activities are the percentages of control with no additions.

#### **2.2.3.4. Effect of chemicals on protease activity**

The effects of protease inhibitors were studied using phenylmethylsulfonyl fluoride (PMSF), ethylene-diaminetetraacetic acid (EDTA), β-mercaptoethanol, sodium dodecylsulfate, sodium azide. Partially purified proteases at the concentration of 200 µg/ml were preincubated for 30 min at 30°C with these chemicals in 0.05 M Tris-HCl buffer (pH 7.5) except PMSF(1-propanol was used as a solvent). Residual activities were determined as a percentage of the activity in control samples without reagent.

### **2.2.4. Experimental Methods**

#### **2.2.4.1. Protease assay**

For measuring protease activity, a diluted enzyme solution (0.5 ml) was mixed with 2.5 ml of 0.6 % casein solution (in Tris-HCl buffer pH 7.2) and incubated for 10 min at 30°C. The reaction was terminated by adding 2.5 ml of trichloroacetic acid mixture solution. The reaction mixture was filtered through Whatman no.1 filter paper. The amount of tyrosine in the solution was measured by reading the

absorbance at 275nm. One unit of activity was defined as the amount of enzyme required to liberate 1 $\mu$ g of tyrosine in one minute at 30°C.

(1U/ml= 1 $\mu$ g tyrosine /min.ml)

A tyrosine standart was prepared by dissolving different amounts of tyrosine in TCA solution (Fig B.1).

Enzyme activity was calculated as:

1U/ml = (Abs.sample/slope )x (1/min)x (reaction volume/enzyme volume)x dilution coefficient

1U/ml = (Abs.sample/0.0072)x(1/10)x(5.5/0.5)x1

1U/ml= Abs.sample x 152,78x dilution coefficient

#### **2.2.4.2. Protein determination with BCA assay**

Protein concentration was determined with 2,2'-Bichinoly-4,4'-dicarbonic acid (BCA), Na<sub>2</sub>-salt at 570nm (Stoscheck, 1990) in a Bio-Rad Model 3550 UV Microplate Reader (Hercules CA,USA). Bovine serum albumin was used as a standart.

BCA assay measures the formation of Cu<sup>+1</sup> from Cu<sup>+2</sup> by the Biuret complex in alkaline solutions of protein. There are two distinct reactions that take place with copper ions unique to the BCA assay. The first reaction occurs at lower temperatures and is the result of the interaction of copper and BCA with the following residues: cysteine, cystine, tryptophan and tyrosine. At elevated temperatures it has been shown that the peptide bond itself is responsible for color development. This is why performing the assay at 37°C or 60°C increases the sensitivity and reduces the variation in the response of the assay to protein composition. The BCA reagent replaces the Folin-Ciocalteu reagent used in the Lowry assay with bicinchoninic acid. The BCA reagent forms a complex with Cu<sup>+1</sup>, which has a strong absorbance at 562 nm (Wilson, 2000).

BCA is advantageous in that it does not interact with as many substances as the Folin-Ciocalteu reagent, especially detergents and buffers. The BCA assay is limited in that it interacts with most reducing agents and copper chelators. In general, these are not critical components of buffers and can be easily eliminated prior to assay. The BCA assay has many advantages over other protein determination techniques:

the color complex is stable; there is less susceptibility to detergents, it is applicable over a broad range of protein concentrations.

To prepare working reagent, 50 parts of Reagent A was mixed with 1 part of Reagent B. 10 µl of each standart, blank or unknown sample were put into the appropriate microtitre plate wells. 200 µl of working reagent was added to each well. Samples were mixed for 30 seconds on a plate shaker. The microtitre plate was covered and incubated at 37°C for 30 minutes for color development. Absorbance values were read at 570 nm with microtitre plate reader. All readings were carried out in duplicate. Using BSA standart curve, protein concentration for each sample was determined (Fig B.2). As the protein concentration values were greater than 0.1 mg/ml, the use of the linearized graph didn't make any error for the calcuations.

#### **2.2.4.3. SDS-polyacrylamide gel electrophoresis**

Sodium dodecyl sulfate polyacrylamide gel electrophoresis was performed according to the protocol modified from Hoefer catalog. Separating gel (10%; pH 8.8) and stacking gel (3%; pH 6.8) were used and approximately 10 µg enzyme protein was loaded. Electrophoresis was run one hour with a voltage of 150 mV. Protein bands were stained with Coomassie brilliant blue R250. Molecular size standarts used were Protein Molecular Weight Marker (Fermentas) including β-galactosidase (116 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45 kDa), lactate dehydrogenase (35 kDa), endonuclease Bsp 981 (25 kDa), β-lactoglobulin (18.4 kDa) and lysosym (14.4 kDa).

### 3. RESULTS and DISCUSSION

#### 3.1. Identification of Protease Producing Strains

##### 3.1.1. API 50 CH System

Four different bacterial strains were identified as *Bacillus cereus*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus subtilis*. A positive test corresponded to an acidification revealed by the phenol red indicator contained in the medium changing to yellow (Fig 3.1). The results recorded as (+) or (–) were entered in the identification software (Table 3.1). Interpretations for the other three *Bacillus* strains are also presented (Table C.1-3.).

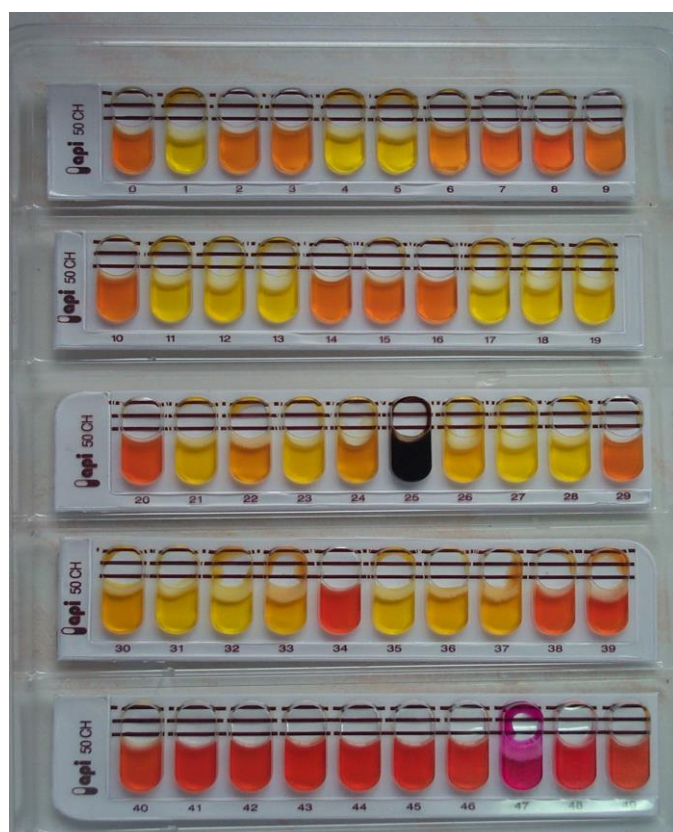


**Figure 3.1** API 50 CH strip view for *Bacillus subtilis*.

**Table 3.1.** Interpretation of API 50 CH strip for *Bacillus subtilis*.

|   |   |   |   |   |   |   |   |   |   |    |    |    |    |
|---|---|---|---|---|---|---|---|---|---|----|----|----|----|
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| - | + | - | - | + | + | - | - | - | - | -  | +  | +  | +  |

|    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| -  | -  | -  | +  | +  | +  | -  | +  | -  | +  | -  | +  | +  | +  |

|    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|
| 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 |
| +  | -  | V  | +  | +  | V  | -  | +  | V  | V  | -  |

|    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|
| 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |
| -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

### 3.1.2. API ZYM System

Four isolated strain were identified as potential producers of alkaline phosphatase, esterase (C 4), esterase lipase (C 8), leucine arylamidase, trypsin,  $\alpha$ -chymotrypsin, acid phosphatase,  $\alpha$ -glucosidase and  $\beta$ -glucosidase. Trypsin and  $\alpha$ -chymotrypsin are proteases in nature. *Bacillus pumilus* was also capable of producing lipase (C 14). API ZYM strip and colors developed after the assay of Bacillus are presented in the figure.



**Figure 3.2** API ZYM strip view after color development.

### 3.2. Partial Purification of the Enzyme

For BSP, BLP and BCP purifications were carried out by ammonium sulfate precipitation (80%). BPP also submitted the anion-exchange chromatography step. BSP was purified to about 3.6 fold with a specific activity of 180 U mg<sup>-1</sup> and final yield of 14% (Table 3.2).

**Table 3.2.** Partial purification of protease from *Bacillus subtilis*.

|                    | Volume (ml) | Activity (U/ml) | Protein conc (mg/ml) | Specific activity (U/mg) | Total protein (mg) | Total activity (U) | Yield (%) | Purification fold |
|--------------------|-------------|-----------------|----------------------|--------------------------|--------------------|--------------------|-----------|-------------------|
| Crude extract      | 178         | 66              | 1.32                 | 50                       | 235                | 11748              | 100       | 1                 |
| A.S.fraction (80%) | 10          | 166             | 0.92                 | 180                      | 9                  | 1660               | 14        | 3.6               |

BLP was purified to about 3.3 fold with a specific activity of 83 U mg<sup>-1</sup> and final yield of 11% (Table 3.3).

**Table 3.3.** Partial purification of protease from *Bacillus licheniformis*.

|                    | Volume (ml) | Activity (U/ml) | Protein conc (mg/ml) | Specific activity (U/mg) | Total protein (mg) | Total activity (U) | Yield (%) | Purification fold |
|--------------------|-------------|-----------------|----------------------|--------------------------|--------------------|--------------------|-----------|-------------------|
| Crude extract      | 166         | 38              | 1.54                 | 25                       | 256                | 6308               | 100       | 1                 |
| A.S.fraction (80%) | 10          | 72              | 0.86                 | 83                       | 8.6                | 715                | 11        | 3.3               |

BCP was purified to about 8.5 fold with a specific activity of 373 U mg<sup>-1</sup> and final yield of 23% (Table 3.4).

**Table 3.4.** Partial purification of protease from *Bacillus cereus*.

|                    | Volume (ml) | Activity (U/ml) | Protein conc (mg/ml) | Specific activity (U/mg) | Total protein (mg) | Total activity (U) | Yield (%) | Purification fold |
|--------------------|-------------|-----------------|----------------------|--------------------------|--------------------|--------------------|-----------|-------------------|
| Crude extract      | 170         | 68              | 1.57                 | 44                       | 267                | 11611              | 100       | 1                 |
| A.S.fraction (80%) | 10          | 272             | 0.73                 | 373                      | 7.3                | 2720               | 23        | 8.5               |

The BPP could not bind to UNO-Q column, suggesting it to be a basic protein. Unbound fractions were pooled together and concentrated. After this step, the enzyme was purified to about 21 fold with a specific activity of 1433 U mg<sup>-1</sup> and final yield of 8% (Table 3.5).

**Table 3.5.** Partial purification of protease from *Bacillus pumilus*.

|                    | Volume (ml) | Activity (U/ml) | Protein conc (mg/ml) | Specific activity (U/mg) | Total protein (mg) | Total activity (U) | Yield (%) | Purification fold |
|--------------------|-------------|-----------------|----------------------|--------------------------|--------------------|--------------------|-----------|-------------------|
| Crude extract      | 215         | 93              | 1.38                 | 67                       | 297                | 19995              | 100       | 1                 |
| A.S.fraction (80%) | 6           | 471             | 0.63                 | 748                      | 3.78               | 2826               | 14        | 11                |
| UNO-Q              | 4           | 387             | 0.27                 | 1433                     | 1.07               | 1543               | 8         | 21                |

As it can be seen from the tables, more the enzyme is purified to a higher degree more there is less amount of enzyme that can be used for any application. From the industrial point of view, it is important to decide whether an enzyme of high purity is needed or a higher amount of non pure enzyme with a appreciable activity is better for the usage.

### 3.3. Characterization of Protease

Optimum pH and T values of BSP (*Bacillus subtilis* protease), BPP (*Bacillus pumilus* protease), BLP (*Bacillus licheniformis* protease), BCP (*Bacillus cereus* protease) and their pH and thermal stabilities were determined. Effect of metal ions and chemicals on enzyme activities were also determined. SDS-PAGE was carried out to determine the molecular mass of BPP.

#### 3.3.1. Effect of temperature on enzyme activity and stability

The optimum temperature for BSP was found to be 70°C. The optimum temperature for BPP, BLP and BCP was found to be 60°C (Fig 3.3). These optimum T values are higher than other recorded proteases that have optimum temperature values at 40°C (Sierecka, 1998), 45°C (Singh et al. 2001) and 50°C (Matta and Punj, 1998).

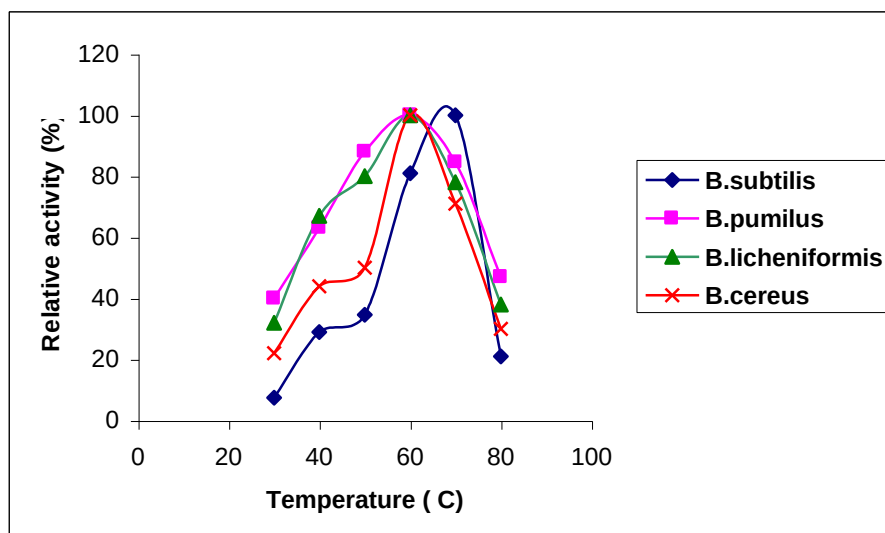


**Figure 3.3** Effect of temperature on the activity of partially purified protease from *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus cereus*.

This value is observed to be same as the *Bacillus pumilus* protease recorded by BCP retained 40% of its activity at 80°C for 30 min exposure time. BLP showed a higher thermal stability by retaining 70% of its original activity at 80°C (Fig 3.4).

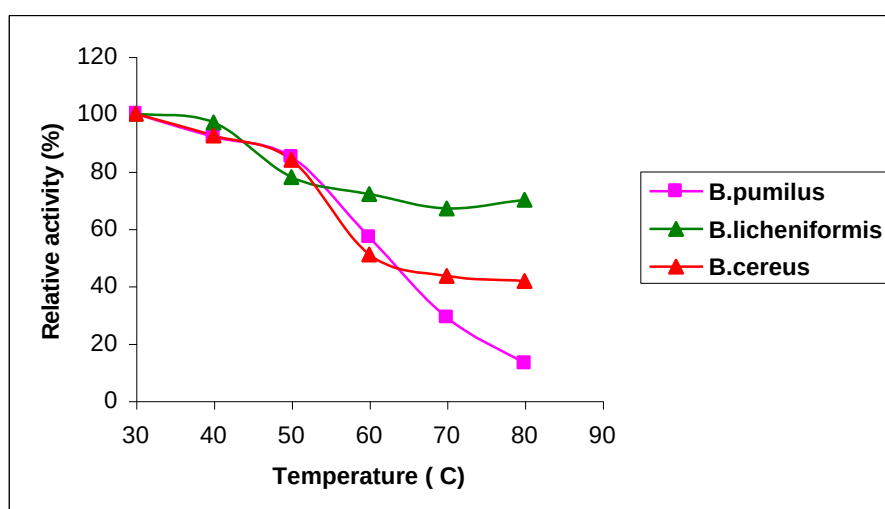


**Figure 3.4** Thermostability of partially purified protease from *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus cereus*.

### 3.3.2. Effect of pH on enzyme activity and stability

The pH optimum of BPP for hydrolysis of casein was around 10-12, with maximum activity at pH 11. The enzyme was almost stable over a pH range of 7-10, but unstable under more acidic conditions. Although enzyme activity was maintained even at pH 12, activity decreased under acidic conditions, to 60% of its maximal

value at pH 6. The pH optimum of BSP was 11. But it couldn't retain its activity at pH 12, on the other hand it was more stable on acidic conditions such as pH 6 when compared with BPP (Fig 3.5).

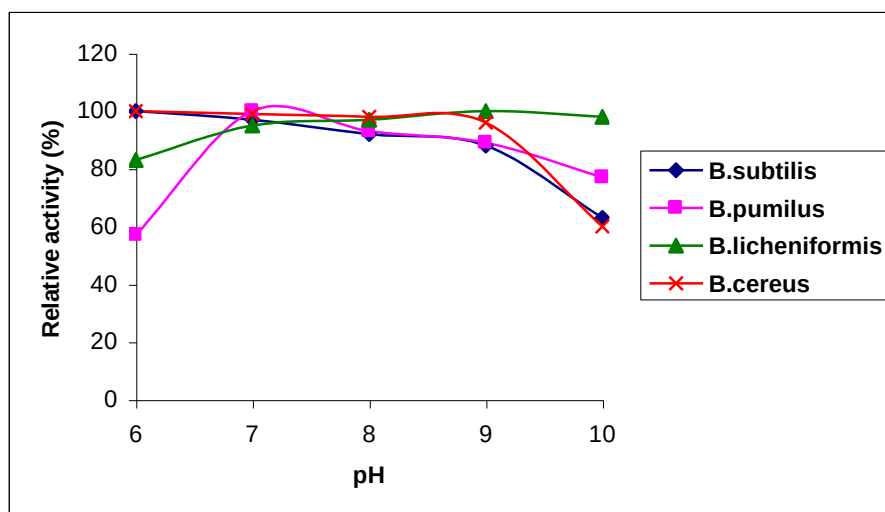


**Figure 3.5** pH stability of partially purified protease from *Bacillus subtilis*, *Bacillus pumilus*, *Bacillus licheniformis*, *Bacillus cereus*.

The optimum pH of 11 recorded for BPP and BSP is higher than the pH optima values, viz. pH 9 (Dhandapani and Vijayaragavan, 1994; Joo et al. 2002); pH 10.5 (Banerjee et al. 1999; Mehrotra et al. 1999) reported earlier for other alkalophilic *Bacillus* species. The pH optimum of BLP and BCP was 10. BLP is stable over a pH range of 6-10, but there is a decrease on the activity of BCP under more alkaline conditions such as pH 10 (Fig 3.5).

### 3.3.3. Effect of metal ions on protease activity

The effects of divalent ions of various metals were examined in order to find out if these ions might influence catalysis and which ions are stimulators and which are inhibitors of the catalytic process. It is generally known that metal ions may participate in the following enzyme mechanisms of increasing the reaction rate: (i) overall acid-base catalysis; (ii) covalent catalysis; (iii) approach of reagent and (iv) induction of structural changes in the enzyme or substrate (Sierecka, 1998). In addition to the activating effect on the catalysis mechanism, metal ions especially heavy metal ions also act as inhibitors.

Metalloproteases contain in their structure a metal cation which is indispensable to their catalytic activity and whose role consists in ensuring the proper spatial

orientation of the substrate through the coordination sphere of metal, polarizing its carbonyl group and increasing its susceptibility to the nucleophilic attack of a water molecule (Kumar et al. 1999b).

The binding site for bivalent metal cations in many enzymes is believed to be the imidazole ring of histidine and SH groups of cysteine.

BSP was activated with  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $K^+$ ,  $Na^+$ ,  $Zn^{2+}$ ,  $Ca^{2+}$  ions (Table 3.6).

**Table 3.6.** Effect of metal ions (10 mM in 0.05 M Tris-HCl buffer, pH 7.5) on the activity of purified extracellular protease of *Bacillus subtilis*.

| Metal ions             | Residual protease activity (%) |
|------------------------|--------------------------------|
| Control                | 100                            |
| $Mn^{2+}$ ( $MnCl_2$ ) | 124                            |
| $Mg^{2+}$ ( $MgCl_2$ ) | 112                            |
| $K^+$ (KCl)            | 122                            |
| $Ni^{2+}$ ( $NiCl_2$ ) | 100                            |
| $Na^+$ (NaCl)          | 117                            |
| $Zn^{2+}$ ( $ZnCl_2$ ) | 111                            |
| $Cu^{2+}$ ( $CuSO_4$ ) | 102                            |
| $Cu^{2+}$ ( $CuCl_2$ ) | 109                            |
| $Fe^{2+}$ ( $FeSO_4$ ) | 98                             |
| $Ca^{2+}$ ( $CaCl_2$ ) | 116                            |
| $Hg^{2+}$ ( $HgCl_2$ ) | 51                             |

BLP was activated with  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $K^+$ ,  $Ni^{2+}$ ,  $Na^+$ ,  $Zn^{2+}$ ,  $Cu^{2+}$ ,  $Ca^{2+}$  ions. In the presence of  $Mn^{2+}$  the protease activity was 41% higher than that observed in the control solution (Table 3.7).

BCP was activated with  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $K^+$ ,  $Ni^{2+}$ ,  $Na^+$ ,  $Zn^{2+}$ ,  $Fe^{2+}$ ,  $Ca^{2+}$  ions.  $Mn^{2+}$ ,  $Mg^{2+}$ ,  $K^+$ ,  $Na^+$ ,  $Ca^{2+}$  were strong activators for this protease increasing its activity about 50-60% compared to the original activity. BCP was inhibited with  $Cu^{2+}$  ions (Table 3.8).

**Table 3.7.** Effect of metal ions (10 mM in 0.05 M Tris-HCl buffer, pH 7.5) on the activity of purified extracellular protease of *Bacillus licheniformis*.

| Metal ions                            | Residual protease activity (%) |
|---------------------------------------|--------------------------------|
| Control                               | 100                            |
| Mn <sup>2+</sup> (MnCl <sub>2</sub> ) | 152                            |
| Mg <sup>2+</sup> (MgCl <sub>2</sub> ) | 143                            |
| K <sup>+</sup> (KCl)                  | 154                            |
| Ni <sup>2+</sup> (NiCl <sub>2</sub> ) | 130                            |
| Na <sup>+</sup> (NaCl)                | 166                            |
| Zn <sup>2+</sup> (ZnCl <sub>2</sub> ) | 118                            |
| Cu <sup>2+</sup> (CuSO <sub>4</sub> ) | 104                            |
| Cu <sup>2+</sup> (CuCl <sub>2</sub> ) | 82                             |
| Fe <sup>2+</sup> (FeSO <sub>4</sub> ) | 119                            |
| Ca <sup>2+</sup> (CaCl <sub>2</sub> ) | 157                            |
| Hg <sup>2+</sup> (HgCl <sub>2</sub> ) | 80                             |

**Table 3.8.** Effect of metal ions (10 mM in 0.05 M Tris-HCl buffer, pH 7.5) on the activity of purified extracellular protease of *Bacillus cereus*.

| Metal ions                            | Residual protease activity (%) |
|---------------------------------------|--------------------------------|
| Control                               | 100                            |
| Mn <sup>2+</sup> (MnCl <sub>2</sub> ) | 141                            |
| Mg <sup>2+</sup> (MgCl <sub>2</sub> ) | 124                            |
| K <sup>+</sup> (KCl)                  | 124                            |
| Ni <sup>2+</sup> (NiCl <sub>2</sub> ) | 131                            |
| Na <sup>+</sup> (NaCl)                | 124                            |
| Zn <sup>2+</sup> (ZnCl <sub>2</sub> ) | 122                            |
| Cu <sup>2+</sup> (CuSO <sub>4</sub> ) | 120                            |
| Cu <sup>2+</sup> (CuCl <sub>2</sub> ) | 113                            |
| Fe <sup>2+</sup> (FeSO <sub>4</sub> ) | 98                             |
| Ca <sup>2+</sup> (CaCl <sub>2</sub> ) | 126                            |
| Hg <sup>2+</sup> (HgCl <sub>2</sub> ) | 56                             |

BPP was activated with  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$ ,  $\text{K}^+$ ,  $\text{Ni}^{2+}$ ,  $\text{Na}^+$ ,  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ ,  $\text{Ca}^{2+}$  ions. In the presence of  $\text{Mn}^{2+}$  and  $\text{K}^+$  the protease activity was 40% higher than that observed in the control solution (Table 3.9).

**Table 3.9.** Effect of metal ions (10 mM in 0.05 M Tris-HCl buffer, pH 7.5) on the activity of purified extracellular protease of *Bacillus pumilus*.

| Metal ions                           | Residual protease activity (%) |
|--------------------------------------|--------------------------------|
| Control                              | 100                            |
| $\text{Mn}^{2+}$ ( $\text{MnCl}_2$ ) | 140                            |
| $\text{Mg}^{2+}$ ( $\text{MgCl}_2$ ) | 129                            |
| $\text{K}^+$ ( $\text{KCl}$ )        | 140                            |
| $\text{Ni}^{2+}$ ( $\text{NiCl}_2$ ) | 128                            |
| $\text{Na}^+$ ( $\text{NaCl}$ )      | 138                            |
| $\text{Zn}^{2+}$ ( $\text{ZnCl}_2$ ) | 135                            |
| $\text{Cu}^{2+}$ ( $\text{CuSO}_4$ ) | 124                            |
| $\text{Cu}^{2+}$ ( $\text{CuCl}_2$ ) | 104                            |
| $\text{Fe}^{2+}$ ( $\text{FeSO}_4$ ) | 98                             |
| $\text{Ca}^{2+}$ ( $\text{CaCl}_2$ ) | 139                            |
| $\text{Hg}^{2+}$ ( $\text{HgCl}_2$ ) | 44                             |

$\text{Hg}^{2+}$  ions showed maximum inhibition of BSP, BLP and BPP by 49, 44 and 56% respectively. BCP was only 20% inhibited. The inhibitory effect of heavy metal ions is well documented in the literature (Vallee and Ulmer, 1972). It is known that the ions of mercury, cadmium and lead react with the protein thiol groups (converting them to mercaptides) and as well as with histidine and tryptophan residues. Moreover, by action of silver and mercury, the disulphide bonds were found to be hydrolytically degraded.

Most of the metal ions tested had exhibited a slight or strong stimulatory effects. Some of the metal ions such as  $\text{Mn}^{2+}$ ,  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  increased and stabilized the protease activity and this is possibly because of the activation by metal ions. These cations have been reported to increase the thermal stability of other *Bacillus* alkaline proteases (Paliwal et al. 1994; Rahman et al. 1994). These results suggest that these metal ions apparently protected the enzyme against thermal denaturation and played

a vital role in maintaining the active conformation of the enzyme at high temperatures.

### 3.3.4. Effect of chemicals on protease activity

Inhibition studies primarily give an insight of the nature of the enzyme, its cofactor requirements and the nature of the active centre. The effects of different inhibitors on the enzyme activity of purified proteases were determined.

**Table 3.10.** Effect of chemicals (1mM in 0.05 M Tris-HCl buffer, pH 7.5) on the activity of purified extracellular protease of *Bacillus subtilis*.

| Chemicals             | Residual protease activity (%) |
|-----------------------|--------------------------------|
| None                  | 100                            |
| Sodium dodecylsulfate | 81                             |
| Sodium azide          | 94                             |
| EDTA                  | 56                             |
| $\beta$ -Me           | 98                             |
| PMSF                  | 90                             |

**Table 3.11.** Effect of chemicals (1mM in 0.05 M Tris-HCl buffer, pH 7.5) on the activity of purified extracellular protease of *Bacillus licheniformis*.

| Chemicals             | Residual protease activity (%) |
|-----------------------|--------------------------------|
| None                  | 100                            |
| Sodium dodecylsulfate | 83                             |
| Sodium azide          | 92                             |
| EDTA                  | 95                             |
| $\beta$ -Me           | 94                             |
| PMSF                  | 42                             |

Of the inhibitors tested, PMSF at 1 mM concentration inhibited both the BLP and BCP at values of 58 and 65% respectively. In this regard, PMSF sulphonates the essential serine residue in the active site of the protease resulting in the complete loss of enzyme activity (Gold and Fahrney, 1964). This inhibition profile suggest that BLP and BCP are alkaline serine proteases.

The anionic surfactant, SDS, caused a moderate inhibition of 19, 17 and 27% of BSP, BLP and BPP respectively. This inhibition may be the result of the combined effects of factors such as reduction in the hydrophobic interactions that play a crucial role in

holding together the protein tertiary structure and the direct interactions with protein molecule (Creighton, 1989).

No inhibition was detected when cystein type inhibitor mercaptoethanol was added. This shows that none of the proteases tested are cystein type.

Metal chelator EDTA showed 44% and 35% inhibition capability for BSP and BPP respectively. They can be classified as metalloproteases.

**Table 3.12.** Effect of chemicals (1mM in 0.05 M Tris-HCl buffer, pH 7.5) on the activity of purified extracellular protease of *Bacillus cereus*.

| Chemicals             | Residual protease activity (%) |
|-----------------------|--------------------------------|
| None                  | 100                            |
| Sodium dodecylsulfate | 91                             |
| Sodium azide          | 93                             |
| EDTA                  | 100                            |
| $\beta$ -Me           | 93                             |
| PMSF                  | 35                             |

**Table 3.13.** Effect of chemicals (1mM in 0.05 M Tris-HCl buffer, pH 7.5) on the activity of purified extracellular protease of *Bacillus pumilus*.

| Chemicals             | Residual protease activity (%) |
|-----------------------|--------------------------------|
| None                  | 100                            |
| Sodium dodecylsulfate | 73                             |
| Sodium azide          | 100                            |
| EDTA                  | 65                             |
| $\beta$ -Me           | 100                            |
| PMSF                  | 100                            |

### 3.3.5. Molecular mass determination with SDS-PAGE

The molecular masses of alkaline proteases ranges from 15 to 30 kDa, with few reports of higher molecular masses (Kumar and Tagaki, 1999). Concetrated sample of BPP after anion exchange chromatography was run on SDS-PAGE. To get a more dense band view, higher amount of protein should have been loaded onto the gel.

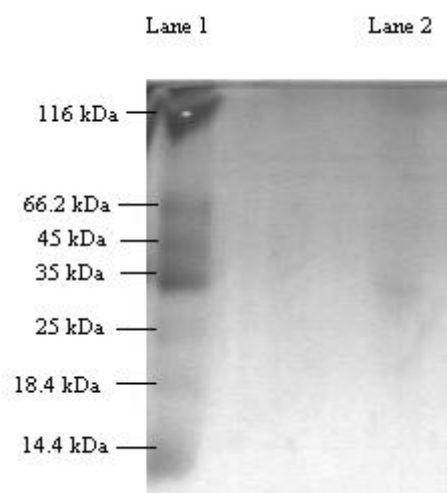


**Figure 3.6** SDS-PAGE of BPP. Lane 1, molecular mass markers; lane 2, concentrated BPP after ion exchange chromatography step.

Molecular mass of BPP was determined to be approximately 30 kDa (Fig 3.6).

#### 4. CONCLUSION

In this study, proteases produced by four different *Bacillus* strains showed maximum activity at high pH values as 10 and 11. The high optimum pH is a feature of alkaline proteases.

The optimum temperature for BSP was found to be 70°C. The optimum temperature for BPP, BLP and BCP was found to be 60°C. These optimum T values are higher than other recorded proteases.

BLP showed a higher thermal stability by retaining 70% of its original activity at 80°C.

After the ion-exchange chromatography step, the BPP enzyme was purified to about 21 fold with a specific activity of 1433 U mg<sup>-1</sup> and final yield of 8%.

Molecular mass of BPP was determined to be approximately 30 kDa.

Most of the metal ions tested had exhibited a slight or strong stimulatory effects. Some of the metal ions such as Mn<sup>2+</sup>, Mg<sup>2+</sup> and Ca<sup>2+</sup> increased and stabilized the protease activity and this is possibly because of the activation by metal ions.

Hg<sup>2+</sup> ions showed maximum inhibition of BSP, BLP and BPP by 49, 44 and 56% respectively. BCP was only 20% inhibited.

Metal chelator EDTA showed 44% and 35% inhibition capability for BSP and BPP respectively. They can be classified as metalloproteases.

PMSF at 1 mM concentration inhibited both the BLP and BCP at values of 58 and 65% respectively. This inhibition profile suggest that BLP and BCP are alkaline serine proteases.

These alkaline proteases can be suitable for inclusion in detergent compositions and can have potential usages in other biotechnological applications which would need higher working temperatures.

Additional work is to be done to enable the full characterization of the examined proteases.

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[www.cgat.ukm.my/protease](http://www.cgat.ukm.my/protease)

## APPENDIX

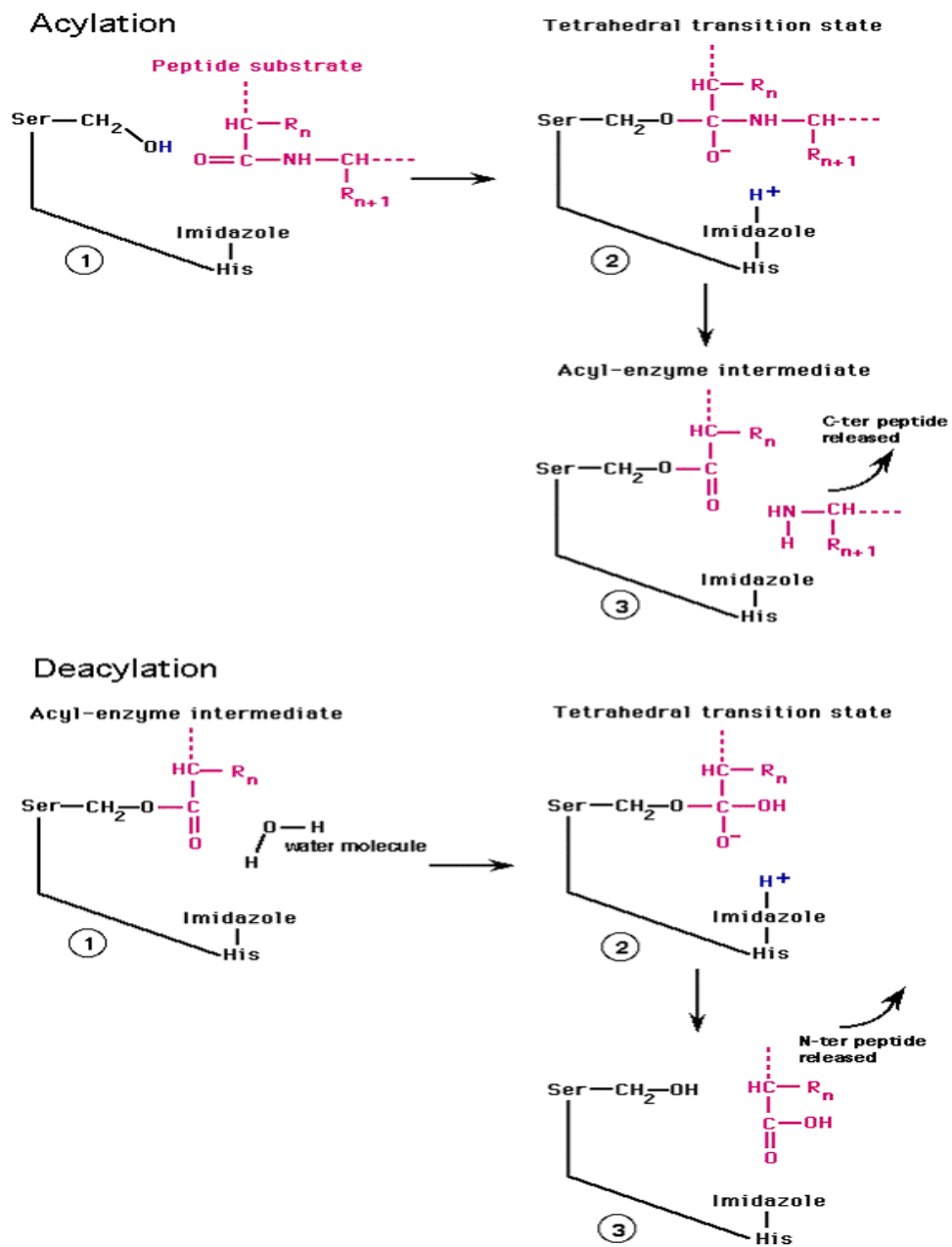


**Figure A.1** Catalytic mechanism of serine proteases.

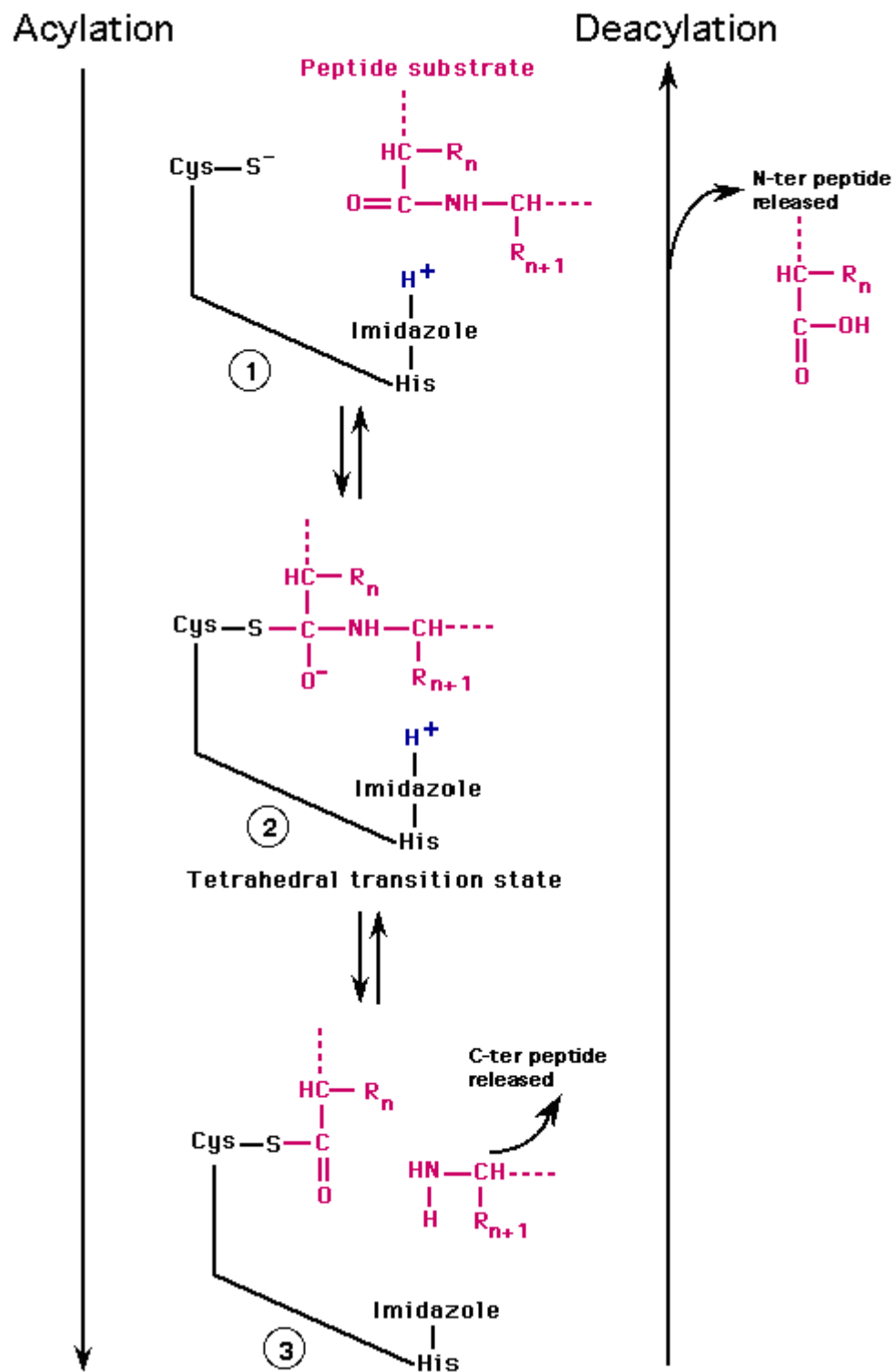


**Figure A.2** Catalytic mechanism of cysteine proteases.

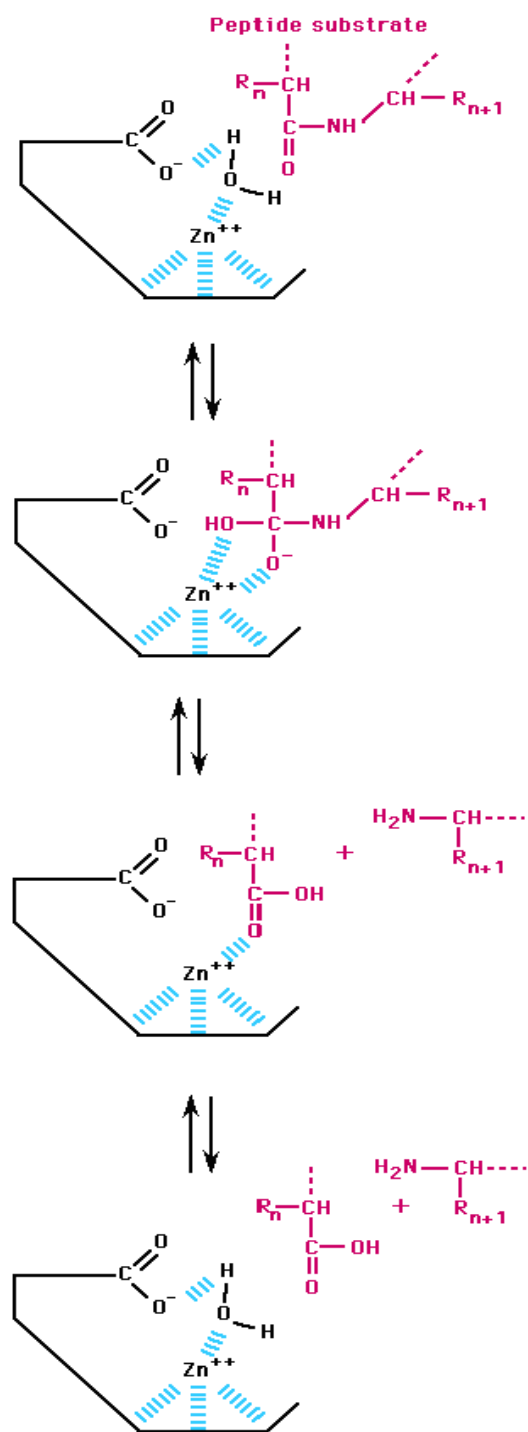


**Figure A.3** Catalytic mechanism of metalloproteases.

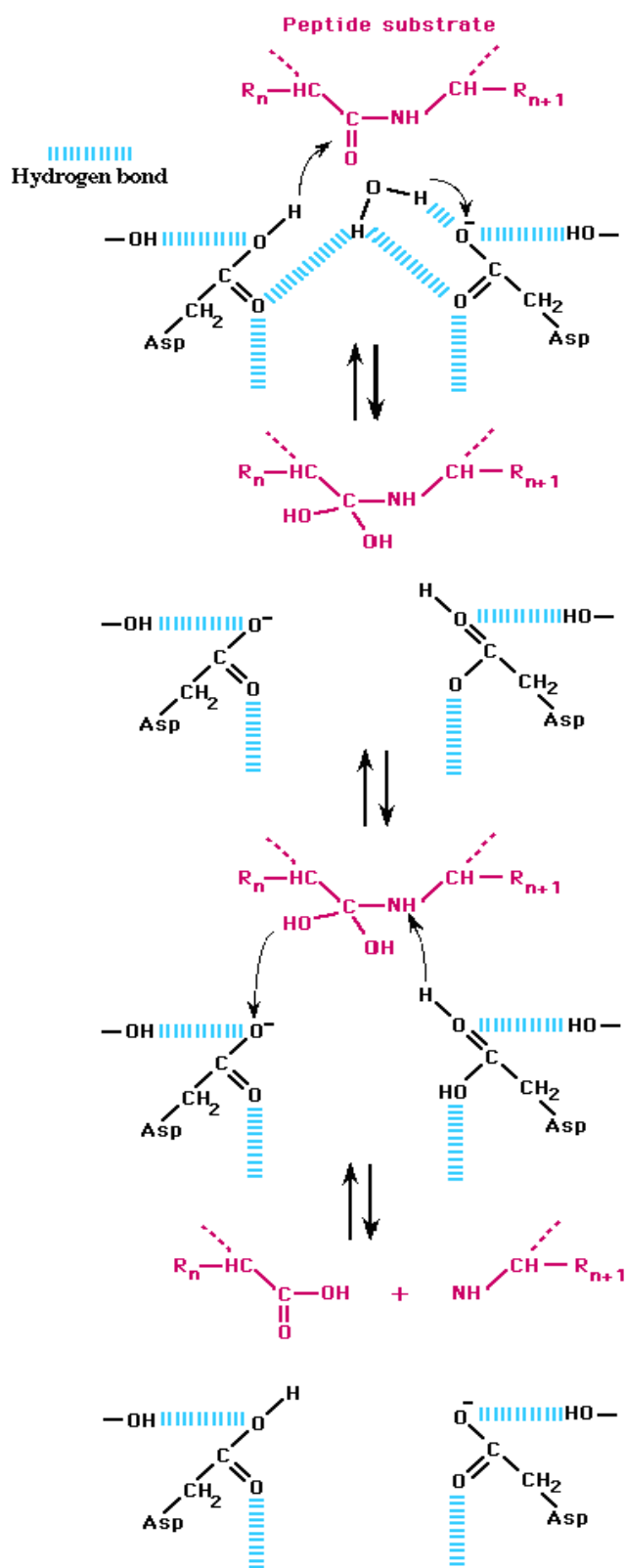


**Figure A.4** Catalytic mechanism of aspartic proteases.

**Table B.1.** API ZYM Reading Table

| No | Enzyme Assayed For                 | Substrate                                     | pH  | Result                                                           |                               |
|----|------------------------------------|-----------------------------------------------|-----|------------------------------------------------------------------|-------------------------------|
|    |                                    |                                               |     | Positive                                                         | Negative                      |
| 1  | Control                            |                                               |     | Colorless or color of the sample if it has an intense coloration |                               |
| 2  | Alkaline phosphatase               | 2-naphthyl phosphate                          | 8.5 | Violet                                                           | Colorless or very pale yellow |
| 3  | Esterase (C 4)                     | 2-naphthyl butyrate                           | 6.5 | Violet                                                           |                               |
| 4  | Esterase Lipase (C 8)              | 2-naphthyl caprylate                          | 7.5 | Violet                                                           |                               |
| 5  | Lipase (C 14)                      | 2-naphthyl myristate                          | "   | Violet                                                           |                               |
| 6  | Leucine arylamidase                | L-leucyl-2-naphthylamide                      | "   | Orange                                                           |                               |
| 7  | Valine arylamidase                 | L-valyl-2-naphthylamide                       | "   | Orange                                                           |                               |
| 8  | Cystine arylamidase                | L-cystyl-2-naphthylamide                      | "   | Orange                                                           |                               |
| 9  | Trypsin                            | N-benzoyl-DL-arginine-2-naphthylamide         | 8.5 | Orange                                                           |                               |
| 10 | $\alpha$ -chymotrypsin             | N-glutaryl-phenylalanine-2-naphthylamide      | 7.5 | Orange                                                           |                               |
| 11 | Acid phosphatase                   | 2-naphthyl phosphate                          | 5.4 | Violet                                                           |                               |
| 12 | Naphtol-AS-BI-phosphohydrolase     | Naphtol-AS-BI-phosphate                       | "   | Blue                                                             |                               |
| 13 | $\alpha$ -galactosidase            | 6-Br-2-naphthyl- $\alpha$ D-galactopyranoside | "   | Violet                                                           |                               |
| 14 | $\beta$ -galactosidase             | 2-naphthyl- $\beta$ D-galactopyranoside       | "   | Violet                                                           |                               |
| 15 | $\beta$ -glucuronidase             | Naphtol-AS-BI- $\beta$ D-glucuronide          | "   | Blue                                                             |                               |
| 16 | $\alpha$ -glucosidase              | 2-naphthyl- $\alpha$ D-glucopyranoside        | "   | Violet                                                           |                               |
| 17 | $\beta$ -glucosidase               | 6-Br-2-naphthyl- $\beta$ D-glucosaminide      | "   | Violet                                                           |                               |
| 18 | N-acetyl- $\beta$ -glucosaminidase | 1-naphthyl-N-acetyl- $\beta$ D-glucosaminide  | "   | Brown                                                            |                               |
| 19 | $\alpha$ -mannosidase              | 6-Br-2-naphthyl- $\alpha$ D-mannopyranoside   | "   | Violet                                                           |                               |
| 20 | $\alpha$ -fucosidase               | 2-naphthyl- $\alpha$ L-fucopyranoside         | "   | Violet                                                           |                               |

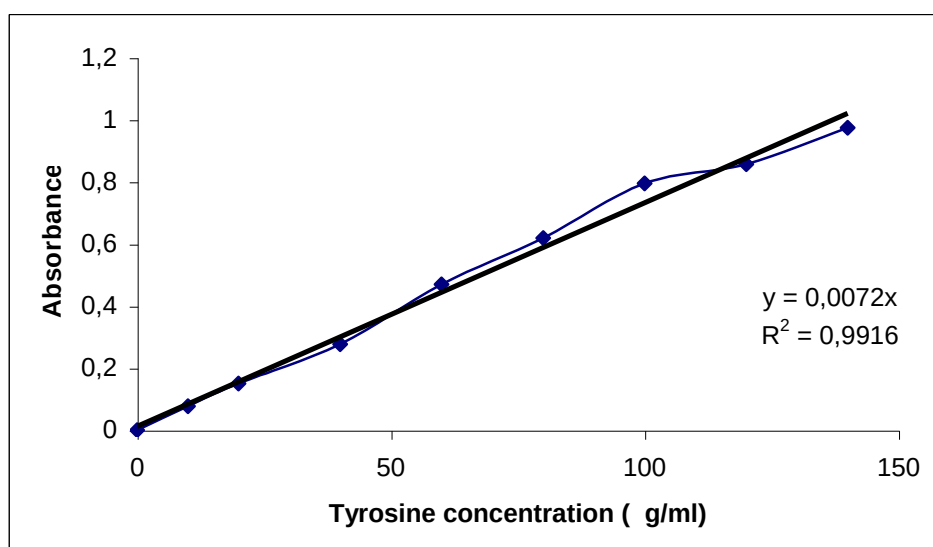


**Figure B.1** Tyrosine standart curve.

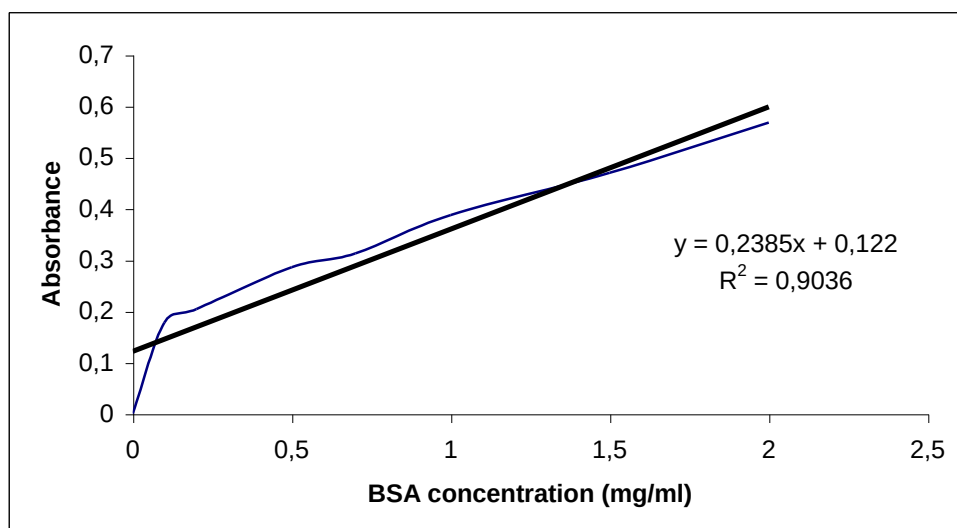


**Figure B.2** BSA standart curve.

**Table C.1.** Interpretation of API 50 CH strip for *Bacillus licheniformis*.

|   |   |   |   |   |   |   |   |   |   |    |    |    |    |
|---|---|---|---|---|---|---|---|---|---|----|----|----|----|
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| - | + | - | - | + | + | + | - | - | - | -  | +  | +  | +  |

|    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| -  | -  | -  | +  | +  | +  | -  | +  | -  | +  | +  | +  | +  | +  |

|    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|
| 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 |
| +  | -  | +  | +  | +  | +  | -  | +  | +  | +  | -  |

|    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|
| 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |
| -  | +  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

**Table C.2.** Interpretation of API 50 CH strip for *Bacillus cereus*.

|   |   |   |   |   |   |   |   |   |   |    |    |    |    |
|---|---|---|---|---|---|---|---|---|---|----|----|----|----|
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| - | - | - | - | - | + | - | - | - | - | -  | +  | +  | -  |

|    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| -  | -  | -  | -  | -  | -  | -  | -  | +  | -  | +  | +  | +  | +  |

|    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|
| 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 |
| +  | -  | -  | +  | +  | -  | -  | -  | +  | +  | -  |

|    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|
| 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |
| -  | -  | -  | -  | -  | -  | -  | -  | -  | -  | -  |

**Table C.3.** Interpretation of API 50 CH strip for *Bacillus pumilus*.

|   |   |   |   |   |   |   |   |   |   |    |    |    |    |
|---|---|---|---|---|---|---|---|---|---|----|----|----|----|
| 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 |
| - | + | - | - | + | + | + | - | - | - | -  | +  | +  | +  |

|    |    |    |    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 | 24 | 25 | 26 | 27 |
| -  | -  | -  | -  | +  | -  | -  | +  | +  | +  | -  | +  | +  | +  |

|    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|
| 28 | 29 | 30 | 31 | 32 | 33 | 34 | 35 | 36 | 37 | 38 |
| +  | -  | -  | +  | +  | -  | -  | -  | -  | -  | -  |

|    |    |    |    |    |    |    |    |    |    |    |
|----|----|----|----|----|----|----|----|----|----|----|
| 39 | 40 | 41 | 42 | 43 | 44 | 45 | 46 | 47 | 48 | 49 |
| +  | +  | -  | +  | -  | -  | -  | -  | -  | -  | -  |

## **CURRICULUM VITAE**

M.Hale Öztürk was born in Istanbul in 1980. She got her high school diploma from Saint Joseph in 1998. She entered the same year to the department of Chemical Engineering at Istanbul Technical University. She graduated in 2002 and the same year, she started the master programme at the department of Molecular Biology and Genetics in the same university. She is still pursuing her studies in the same department. She is also student at Anatolian University, at the fourth and last year of the Business Administration programme.