CHAPTER II

LITERATURE REVIEWS

Protease

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Protease catalyze the cleavage of peptide bonds in other proteins. Proteases are degradative enzymes which catalyze the total hydrolysis of proteins (Figure 1).

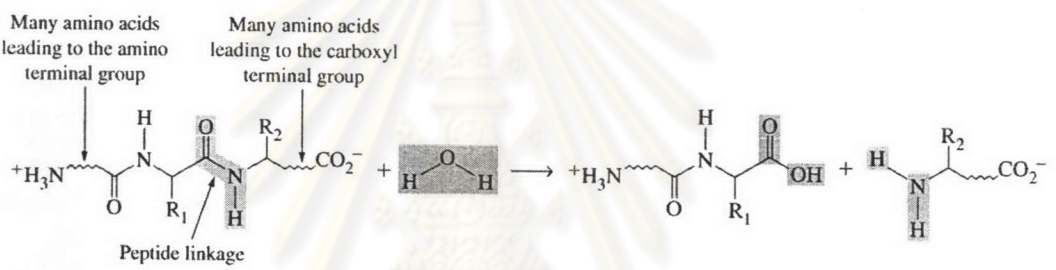


Figure 1. Catalytic reaction of protease

Proteolytic enzymes are involved in a great variety of physiological processes and their action can be divided into two different categories:

1) Limited proteolysis, in which a protease cleaves only one or a limited number of peptide bonds of a target protein leading to the activation or maturation of the formely inactive protein *e.g* conversion of prohormones to hormones.

2) Unlimited proteolysis, in which proteins are degraded into their amino acid constituents. The proteins to be degraded are usually first conjugated to multiple molecule of the polypeptide ubiquitin. This modification marks them for rapid hydrolysis by the proteasome in the presence of ATP. Another pathway consists in the compartmentation of proteases *e.g* in lysosomes. Proteins transferred into this compartment undergo a rapid degradation.

1. Classification of protease

According to the Nomenclature Committee of the Internatioal Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases). However, proteases do not comply easily with the general system of enzyme nomenclature due to their huge diversity of action and structure. Currently, proteases are classified on the basis of three major criteria (Barett, 1994).

1.1 Type of reaction catalyzed. Proteases are grossly subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on the location of the enzymatic action, either exopeptidase or endopeptidase. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate.

1.2 Chemical nature of the catalytic site. Based on the functional group present at the active site, proteases are further classified into four prominent groups. There are serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Guzzo et al., 1990)

1.2.1 Serine proteases: Serine proteases are characterized by the presence of a serine group in their active site. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. This class comprises two distinct families. The chymotrypsin family which includes the mammalian enzymes such as chymotrypsin, trypsin or elastase or kallikrein and the substilisin family which include the bacterial enzymes such as subtilisin.

Serine proteases are recognized by their irreversible inhibition by 3,4-dichloroisocoumarin (3,4-DCI), L-3-carboxytrans 2,3-epoxypropyl-leucylamido (4-guanidine) butane (E.64), diisopropylfluorophosphate (DFP), phenylmethylsulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Some of the serine proteases are inhibited by thiol reagents such as *p*-chloromercuribenzoate (PCMB) due to the presence of a cysteine residue near the active site. Serine proteases are generally

active at neutral and alkaline pH, with an optimum between pH 7 and 11. They have broad substrate specificities including esterolytic and amidase activity.

1.2.2 Aspartic proteases. Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin, retropepsin, and enzymes from pararetroviruses.

The aspartic proteases are inhibited by pepstatin (Fitzgerald et al., 1990). They are also sensitive to diazoketone compounds such as diazoacetyl-DL-norleucine methylester (DAN) and 1, 2-epoxy-3-(*p*-nitrophenoxy) propane (EPNP) in the presence of copper ions. Microbial acid proteases exhibit specificity against aromatic or bulky amino acid residues on both sides of the peptide bond, which is similar to pepsin, but their action is less stringent than that of pepsin. Microbial aspartic proteases can be broadly divided into two groups, (i) pepsin-like enzymes produced by *Aspergillus*, *Penicillium*, *Rhizopus*, and *Neurospora* and (ii) rennin-like enzymes produced by *Endothia* and *Mucor* spp.

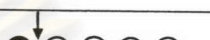
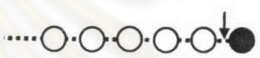
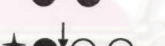
1.2.3 Cysteine/thiol proteases. Cysteine proteases occur in both prokaryotes and eukaryotes. The activity of all cysteine proteases depends on a catalytic dyad consisting of cysteine and histidine.

Generally, cysteine proteases are active only in the presence of reducing agents such as HCN or cysteine. Based on their side chain specificity, they are broadly divided into four groups: (i) papain-like, (ii) trypsin- like with preference for cleavage at the arginine residue, (iii) specific to glutamic acid, and (iv) others. Papain is the best-known cysteine protease. Cysteine proteases have neutral pH optima, although a few of them, e.g., lysosomal proteases, are maximally active at acidic pH. They are susceptible to sulfhydryl agents such as PCMB but are unaffected by DFP and metal-chelating agents.

1.2.4 Metalloproteases. Metalloproteases are the most diverse of the catalytic types of proteases. They are characterized by the requirement for a divalent metal ion for their activity. The metallo proteases may be one of the older classes of proteases and are found in bacteria, fungi as well as in higher organisms. They differ widely 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. Because of they require a metal ion for their activity, so inhibited by metal chelating agents such as EDTA but not by sulfhydryl agents or DFP. For example carboxypeptidase, thermolysin, collagenase.

Table1. Classification of protease

Protease	Mode of action[a]	EC no.
Exopeptidases		
Aminopeptidases		3.4.11
Dipeptidyl peptidase		3.4.14
Tripeptidyl peptidase		3.4.14
Carboxypeptidase		3.4.16-3.4.18
Serine type protease		3.4.16
Metalloprotease		3.4.17
Cysteine type protease		3.4.18
Peptidyl dipeptidase		3.4.15
Dipeptidase		3.4.13
Omega peptidases		3.4.19
		3.4.19
Endopeptidases		3.4.21-3.4.34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24
Endopeptidases of unknown Catalytic mechanism		3.4.99

[a] Open circles represent the amino acid residues in the polypeptide chain. Solid circles indicate the terminal amino acids, and stars signify the blocked termini. Arrows show the sites of action of the enzyme.

1.3 Evolutionary relationship with reference to structure: Based on their amino acid sequences, proteases are classified into different families (Argos, 1987) and further subdivided into "clans" to accommodate sets of peptidases that have diverged from a common ancestor (Rawling et al., 1993). Each family of peptidases has been assigned a code letter denoting the type of catalysis, i.e., S, C, A, M, or U for serine, cysteine, aspartic, metallo-, or unknown type, respectively.

2. Industrial application

Proteases have a large variety of applications, mainly in the detergent and food industries. In view of the recent trend of developing environmentally friendly technologies, proteases are envisaged to have extensive applications in leather treatment and in several bioremediation processes. The worldwide requirement for enzymes for individual applications varies considerably. Proteases are used extensively in the pharmaceutical industry for preparation of medicines such as ointments for debridement of wounds, etc. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used.

Table 2. Markets for Microbial proteinases. Estimates of Annual Sales Turnover and Market Share for the Major Applications of Microbial Proteinases. Data used with permission

	Sales (million US$)	Share of industrial proteinase Market (%)
Detergent proteinases	140	89-2
Microbial rennets	12	7-6
Baking proteinases	3	1-9
Leather	1	0-6
Miscllaneous	1	0-7
Totals	157	100-0

2.1 Detergents. Proteases are one of the standard ingredients of all kinds of detergents ranging from those used for household laundering to reagents used for cleaning contact lenses or dentures.The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes. The preparation of the first enzymatic detergent, "Burnus," dates back to 1913; it consisted of sodium carbonate and a crude pancreatic extract. The first detergent containing the bacterial enzyme was introduced in 1956 under the trade name BIO-40.

In Table 3 it can be seen that two microbial proteinases dominate detergent products today. While these appear as seven products from four suppliers, the microbial sources tend to be either *Bacillus licheniformis* or the alkalophilic Bacilli.

Table 3. Major detergent proteases.

Trade name	Producer	Source
Alcalase	Novo-Nordisk	*B. licheniformis*
Maxatase	Ibis	
Optimase	Miles-Kali Chemie	
Esperase	Novo-Nordisk	Alkalophilic *Bacilli*
Savinase	Novo-Nordisk	
Maxacal	Ibis	
Kazusase	Showa Denko	

All detergent proteases currently used in the market are serine proteases produced by *Bacillus* strains. Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe-free enzyme. An alkaline protease from *Conidiobolus coronatus* was found to be compatible with commercial detergents used in India (Phadatare, et al., 1993) and retained 43% of its activity at 50°C for 50 min in the presence of Ca^{2+} (25 mM) and glycine (1 M) (Bhosale, et al., 1995).

2.2 Food industry. The use of proteases in the food industry dates back to antiquity. They have been routinely used for various purposes such as cheesemaking, baking, preparation of soya hydrolysates, and meat tenderization.

2.2.1 Dairy industry. The major application of proteases in the dairy industry is in the manufacture of cheese. The milk-coagulating enzymes fall into three main categories, (i) animal rennets, (ii) microbial milk coagulants, and (iii) genetically engineered chymosin. Both animal and microbial milk-coagulating proteases belong to a class of acid aspartate proteases and have molecular weights between 30,000 to 40,000.

In cheesemaking, the primary function of proteases is to hydrolyze the specific peptide bond (the Phe105-Met106 bond) to generate *para*-k-casein and macropeptides. Chymosin is preferred due to its high specificity for casein, which is responsible for its excellent performance in cheesemaking.

Whey is a by-product of cheese manufacture. It contains lactose, proteins, minerals, and lactic acid. The insoluble heatdenatured whey protein is solubilized by treatment with immobilized trypsin.

2.2.2 Baking industry. Wheat flour is a major component of baking processes. It contains an insoluble protein called gluten, which determines the properties of the bakery doughs. Endoand exoproteinases from *Aspergillus oryzae* have been used to modify wheat gluten by limited proteolysis. Enzymatic treatment of the dough facilitates its handling and machining and permits the production of a wider range of products. The addition of proteases reduces the mixing time and results in increased loaf volumes. Bacterial proteases are used to improve the extensibility and strength of the dough.

2.2.3 Manufacture of soy products. Soybeans serve as a rich source of food, due to their high content of good-quality protein. Proteases have been used from ancient times to prepare soy sauce and other soy products. The alkaline and neutral proteases of fungal origin play an important role in the processing of soy sauce. Proteolytic modification of soy proteins helps to improve their functional properties. Treatment of soy proteins with alcalase at pH 8 results in soluble hydrolysates with high

solubility, good protein yield, and low bitterness. The hydrolysate is used in protein-fortified soft drinks and in the formulation of dietetic feeds.

2.2.3 Debittering of protein hydrolysates. Protein hydrolysates have several applications, e.g., as constituents of dietetic and health products, in infant formulae and clinical nutrition supplements, and as flavoring agents. The bitter taste of protein hydrolysates is a major barrier to their use in food and health care products. The intensity of the bitterness is proportional to the number of hydrophobic amino acids in the hydrolysate. The presence of a proline residue in the center of the peptide also contributes to the bitterness. The peptidases that can cleave hydrophobic amino acids and proline are valuable in debittering protein hydrolysates. Aminopeptidases from lactic acid bacteria are available under the trade name Debitrase. Carboxypeptidase A has a high specificity for hydrophobic amino acids and hence has a great potential for debittering. A careful combination of an endoprotease for the primary hydrolysis and an aminopeptidase for the secondary hydrolysis is required for the production of a functional hydrolysate with reduced bitterness.

2.2.4 Synthesis of aspartame. The use of aspartame as a noncalorific artificial sweetener has been approved by the Food and Drug Administration. Aspartame is a dipeptide composed of L-aspartic acid and the methyl ester of L-phenylalanine. The L configuration of the two amino acids is responsible for the sweet taste of aspartame. Maintenance of the stereospecificity is crucial, but it adds to the cost of production by chemical methods. Enzymatic synthesis of aspartame is therefore preferred. Although proteases are generally regarded as hydrolytic enzymes, they catalyze the reverse reaction under certain kinetically controlled conditions. An immobilized preparation of thermolysin from *Bacillus thermoprotyolyticus* is used for the enzymatic synthesis of aspartame. Toya Soda (Japan) and DSM (The Netherlands) are the major industrial producers of aspartame.

2.3 Pharmaceutical Industry. The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents. Oral administration of proteases from *Aspergillus oryzae* has been used as a digestive aid to correct certain lytic enzyme deficiency syndromes. Clostridial collagenase or subtilisin is

used in combination with broad-spectrum antibiotics in the treatment of burns and wounds. An asparginase isolated from *E. coli* is used to eliminate aspargine from the bloodstream in the various forms of lymphocytic leukemia. Alkaline protease from *Conidiobolus coronatus* was found to be able to replace trypsin in animal cell cultures.

2.4 Leather Processing. Two operations in comverting animal skins to leather use proteases, at least, to some extent: unhairing of hides and bating. Use of microbial enzymes in these steps can be characteized as, 'limited', for both, but for defferent reasons.

2.4.1 Unhairing. For unhairing ofhides microbial serine proteases are competing againxt inexpensive chemicals: lime and sodium sulfide. The alkaline swelling and dehairing is very effective and reasonably fast (Aunstrup, 1980). Microbial proteases from alkalophilic Bacilli technically can replace the chemical treatment, but at an increased cost. The emphasis on controlling pollution and worker safety (hydrogen sulfide fumes) has pressured the leather industry in some countries to find replacement processes or to relocate to countries with more relaxed environmental rules/enforcement. The first option has led to an opening for microbial proteases, however the second option has been the more popular elective.

2.4.2 Bating. Leather bating is the process which gives leather a degree of flexibility and suppleness. Erroneously, consumers often refer to this quality as leather 'softness'. For example, Leather for gloves has undergone extensive bating while leather for shoe-soles has had little or no treatment.

Bating has traditionally been an enzymatic process, but the main soure of protease has been pancreatic glands. Hence, trypsin or the cruder preparation, pancreatin, has been the main agent for bating leather.

Bacterial serine proteinases have attempted to displace animal trypsin as an economical substitute, but with little success. In the 1970s trials with these alkaline proteinases produced poor results due to the greater proteolytic activity of the serine proteinases. Only more recently have improved microbial proteinase products met with success. Thus today, proprietary mixtures

of chemicals and proteinases *from Aspergillus oryzae, B. amyloliquefaciens or B. licheniformis* can be found in the bating house supply room.

2.5 Other Applications. Besides their industrial and medicinal applications, proteases play an important role in basic research. Their selective peptide bond cleavage is used in the elucidation of structurefunction relationship, in the synthesis of peptides, and in the sequencing of proteins. In essence, the wide specificity of the hydrolytic action of proteases finds an extensive application in the food, detergent, leather, and pharmaceutical industries, as well as in the structural elucidation of proteins, whereas their synthetic capacities are used for the synthesis of proteins.

3. Sources of proteases

Since proteases are physiologically necessary for living organisms, they are ubiquitous, being found in a wide diversity of sources such as plants, animals, and microorganisms.

3.1 Plant Proteases. The use of plants as a source of proteases is governed by several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process. Papain, bromelain, keratinases, and ficin represent some the well-known proteases of plant origin.

3.1.1 Papain. Papain is a traditional plant protease and has a long history of use (Schechler and Bweger, 1967). It is extracted from the latex of *Carica papaya* fruits. The crude preparation of the enzyme has a broader specificity due to the presence of several proteinase and peptidase isozymes. The performance of the enzyme depends on the plant source, the climatic conditions for growth, and the methods used for its extraction and purification.It is extensively used in industry for the preparation of highly soluble and
flavored protein hydrolysates.

3.1.2 Bromelain: Bromelain is prepared from the stem and juice pineapples. The major supplier of the enzyme is Great Food Biochem., Bangkok,

Thailand. The enzyme is characterized as cysteine protease and is active from pH 5 to 9. Its inactivation temperature is 70°C, which is lower than that of papain.

3.1.3 Keratinases. Some of the botanical groups of plants produce proteases which degrade hair. Digestion of hair and wool is important for the production of essential amino acids such as lysine and for the prevention of clogging of wastewater systems.

3.2 Animal Proteases. The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennins . These are prepared in pure form in bulk quantities. However, their production depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies.

3.2.1 Trypsin. Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. It is a serine protease and hydrolyzes peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues. Based on the ability of protease inhibitors inhibit the enzyme from the insect gut, this enzyme has received attention as a target for biocontrol of insect pests. Trypsin has limited applications in the food industry, since the protein hydrolysates generated by its action have a highly bitter taste. Trypsin is used in the preparation of bacterial media and in some specialized medical applications.

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

3.2.3 Pepsin. Pepsin is an acidic protease that is found in the stomachs of almost all vertebrates. The active enzyme is released from its zymogen, i.e., pepsinogen, by autocatalysis in the presence of hydrochloric acid. Pepsin is an aspartyl protease and resembles human immunodeficiency virus type 1 (HIV-1) protease, responsible for the maturation of HIV-1. It exhibits optimal activity between pH 1 and 2,

while the optimal pH of the stomach is 2 to 4. Pepsin is inactivated above pH 6.0.The enzyme catalyzes the hydrolysis of peptide bonds between two hydrophobic amino acids.

3.2.4 Rennin. Rennet is a pepsin-like protease (rennin, chymosin; EC 3.4.23.4) that is produced as an inactive precursor, prorennin, in the stomachs of all nursing mammals. It is converted to active rennin by the action of pepsin or by its autocatalysis. It is used extensively in the dairy industry to produce a stable curd with good flavor. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in k-casein to generate insoluble *para*-k-casein and C-terminal glycopeptide.

3.3 Microbial Proteases. The inability of the plant and animal proteases to meet current world demands has led to an increased interest in microbial proteases. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Microbial proteases account for approximately 40% of the total worldwide enzyme sales (Godfrey and West, 1996). Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications.

3.3.1 Bacteria. Most commercial proteases, mainly neutral and alkaline protease, are produced by organisms belonging to the genus *Bacillus*. Bacterial neutral proteases are active in a narrow pH range (pH 5 to 8) and have relatively low thermotolerance. Due to their intermediate rate of reaction, neutral proteases generate less bitterness in hydrolyzed food proteins than do the animal proteinases and hence are valuable for use in the food industry. Neutrase, a neutral protease, is insensitive to the natural plant proteinase inhibitors and is therefore useful in the brewing industry. The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity during the production of food hydrolysates with a low degree of hydrolysis. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity, while others are serine proteinases, which are not affected by chelating

agents. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10, and their broad substrate specificity. Their optimal temperature is around 60°C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry.

3.3.2 Fungi. Fungi elaborate a wider variety of enzymes than do bacteria. For example, *Aspergillus oryzae* produces acid, neutral, and alkaline proteases. The fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity. However, they have a lower reaction rate and worse heat tolerance than do the bacterial enzymes. Fungal enzymes can be conveniently produced in a solid-state fermentation process. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. They are particularly useful in the cheesemaking industry due to their narrow pH and temperature specificities. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating agents. In view of the accompanying peptidase activity and their specific function in hydrolyzing hydrophobic amino acid bonds, fungal neutral proteases supplement the action of plant, animal, and bacterial proteases in reducing the bitterness of food protein hydrolysates. Fungal alkaline proteases are also used in food protein modification.

3.3.3 Viruses. Viral proteases have gained importance due to their functional involvement in the processing of proteins of viruses that cause certain fatal diseases such as AIDS and cancer. Serine, aspartic, and cysteine peptidases are found in various viruses (Rawling and Barrett, 1993). All of the virus-encoded peptidases are endopeptidases; there are no metallopeptidases. Retroviral aspartyl proteases that are required for viral assembly and replication are homodimers and are expressed as a part of the polyprotein precursor. The mature protease is released by autolysis of the precursor. An extensive literature is available on the expression, purification, and enzymatic analysis of retroviral aspartic protease and its. Extensive research has focused on the three-dimensional structure of viral proteases and their interaction with synthetic inhibitors with a view to designing potent inhibitors that can combat the relentlessly spreading and devastating epidemic of AIDS.

Thus, although proteases are widespread in nature, microbes serve as a preferred source of these enzymes because of their rapid growth, the limited space required for their cultivation, and the ease with which they can be genetically manipulated to generate new enzymes with altered properties that are desirable for their various applications.

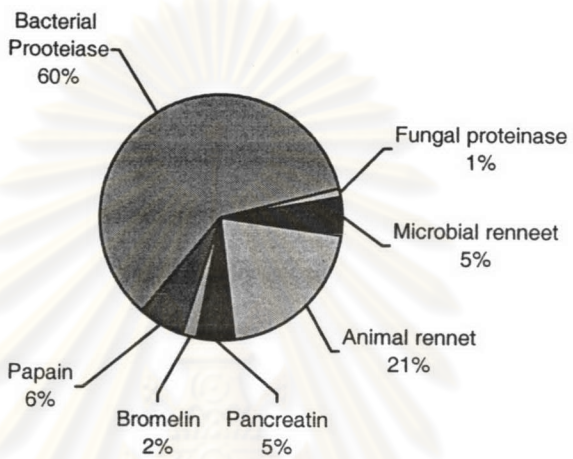


Figure 2. Piechart of the industrial proteinase market. Market shares for each proteinase are given as percent of a total estimated sales turnover of US$242 m. Microbial proteinases are depicted in pie slices with shading.

Although there are many microbial sources available for producing protease, only a few are recognized as commercial producers. For that reason, it would be of great importance to have available enzymes showing optimal activities at different values of salt concentrations and temperature. Halophiles are the most likely source of such enzymes, because not only are their enzymes salt-tolerant, but many are also thermotolerant. (Sanchez-Porro, 2003)

4. Halophilic bacteria

Halophiles are salt-loving organisms that inhabit hypersaline environments. They include mainly prokaryotic and eukaryotic microorganisms with the capacity to balance the osmotic pressure of the environment and resist the denaturing effects of salts. Among halophilic microorganisms are a variety of heterotrophic and

methanogenic archaea; photosynthetic, lithotrophic, and heterotrophic bacteria; and photosynthetic and heterotrophic eukaryotes.

Although salts are required for all life forms, halophiles are distinguished by their requirement of hypersaline conditions for growth. They may be classified according to their salt requirement (Kushner, 1985). (Figure 2)

4.1 Slight halophiles. The slight halophile grow optimally at 0.2–0.85 mol/L (2–5%) NaCl, such as *Pseudomonas, Moraxella, Flavobacterium, Acinetobacter* and *Vibrio*, they isolated from sea fish and shell.

4.2 Moderate halophiles. The moderate halophiles grow optimally at 0.85–3.4 mol/L (5 –20%)NaCl. Moderately halophilic bacteria constitute a large group of organisms encompassing a great diversity of bacteria. The moderately halophilic bacteria, from the taxonomic point of view, constitute a very heterogeneous group of microorganisms which include species from different genera. Archae bacteria; Methanohalophilus, Halomethanococus, Gram-negative; *Deleya*, *Arhodononas, Chromohalobacter*, *Dichotomicrobium*, *Flavobacterium*, *Haloanaerobium*, *Halobacteroides*, *Haloincola*, *Halomonas*, *Halovibrio*, *Pseudomonas*, *Spirochaeta*, *Sporohalobacter*, *Vibrio*, *Volcaniella*, Gram-positive; *Halobacillus*, *Bacillus*, *Clostridium*, *Marinococcus*, *Micrococcus*, *Salinicoccus*, *Salibacillus*, *Lentibacillus*, *Virgibacillus*, and *Sporosarcina*, and facultative anaerobe; *Flavobacterium salegens* and *Arhodomonas aquaeolei*; Actinomycetes; Genera *Actinopolyspora*. Characteristic of some bacteria in this group show in Table 4.

4.3.Extreme halophiles. The extreme halophiles grow optimally above 3.4–5.1 mol/L (20–30%) NaCl. The extremely halophilic archaeobacteria are well known because, they often were (and still are) simply called"red halophiles", such as Genera *Halobacterium*, *Haloferax*, and *Haloarcula*, *Halococcus*, *Natronomanas, Haloterrigena*.

In contrast, nonhalophiles grow optimally at less than 0.2 mol / L NaCl. Halotolerant organisms can grow both in high salinity and in the absence of a high concentration of salts. Many halophiles and halotolerant microorganisms can grow over

a wide range of salt concentrations with requirement or tolerance for salts sometimes depending on environmental and nutritional factors.

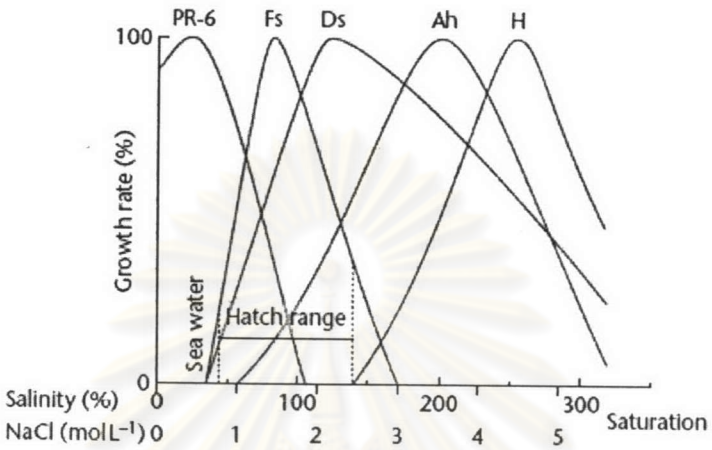


Figure 3. Salt-tolerance of halophilic organisms. Relative growth rate is plotted against both percentage salinity and NaCl concentration. The five microorganisms are *Agmenellum quadraplicatum* (PR-6), a slightly halotolerant cyanobacterium, *Fabrea salina* (Fs), a moderately halophilic protozoan, *Dunaliella salina* (Ds), a halophilic green alga, *Aphanothece halophytica* (Ah), an extremely halophilic cyanobacterium, and *Halobacteriumsp.* (H), an extremely halophilic archaeon. The salinity of sea water and the hatch range for brine shrimp are noted.

Table 4. Salient features of some genera of aerobic, endospore-forming bacteria and *Marinococcus*

Character	*Virgibacillus*	*Bacillus*	*Paenibacillus*	*Aneurinibacillus*	*Brevibacillus*	*Halobacillus*	*Sporosarcina*	*Marinococcus*
No. of species	1	>60	13	2	10	3	1	3
Murein	DAP	V*	DAP	DAP	DAP	Orn-D- Asp	L-Lys-Gly-D-Glu	DAP
Cell shape	Rods	Rods	Rods	Rods	Rods	Rods and cocci	Cocci	Cocci
Spore	+	+	+	+	+	+	+	-
Spore shape	E-S	E,C,S,B	E	E	E	E,S	S	NA
Sporangia swollen	+	V	+	+	+	+/na	NA	NA
Anaerobic growth	+	V	V	-	+/-	-	-	-
Optimum temperatur(°C)	37	15-55	30	37	30-48	35	30	30
Optimum pH	7	7-9.5	7	7	7	7.5	8	7.5
Growth in 10% NaCl	+	V	-	-	-	+	-	+
Major isoprenoid quinone	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7	MK-7(MK-8)
Major cellular fatty acids	Iso+anteiso-$C_{15:0}$	V	Anteiso-$C_{15:0}$	Iso-$C_{15:0}$- $C_{16:0}$ Iso-$C_{16:0}$	Anteiso-$C_{15:0}$+ iso-$C_{15:0}$ or just iso-$C_{15:0}$	ND	ND	ND
G+C (T_m)	36.9†-38.3‡	32-69	40-54	41.1-43.4	42.8-57.4	40-43	40-41.5	44.9-49.3

Abbreviations: DAP, direct-linked meso-diaminopimilic acid; E, ellipsoidal; S, spherical; E-S, ellipsoidal to spherical; C, cyclindrical; B, ellipsoidal or cylindrical spores bent into kidney or banana shape; V, character varies accocrding to species. NA, Not applicable; ND, No data available; * Predominant type is DAP; †, According to Fahmy et al (1985); ‡, Mean of two measurements (T_m Method).

5. Protease-producing halophillic bacteria

Although halophilic microorganisms have attracted much attention in recent years, most studies have been performed in halobacteria. However, moderately halophilic bacteria represent an excellent model of adaptation to frequent changes in extracellular osmolality and constiute an interesting group of microorganisms from a biotechnological point of view. Thus, many of them accumulate intracellular organuc osmolytes named "compatible solutes" which can be used as stabilizers of enzymes and whole cells (da Costa et. al., 1997: Ventosa et. al., 1998; Nieto et. al., 2000) and they produce halophilic exoenzymes that could br of commercial interest and could be used in biodegradation processes. They have the advantage that most species are able to grow in a wide range of salinities, in contrast to the more strict requirements of salt presented by halobacterium. For example *Gracilibacillus* and *Tetragenococcus* strains can growth in absence NaCl and cell reduce size in low concentration of salts(Thongsanit et al., 2002)

5.1 Protease-producing extremely halophilic bacteria. A few protease from extreme halophiles, member of the archael phylogenetic branch, have been characterized by Norberg and Hofsteen (1969) ; Stepnov et al.(1992); Studdert et al. (1997) ; Ryu et al. (1994) ; Gimenez et al.(2000). Some poperties of protease from extreme halophiles show in Table 5.

5.2 Protease-producing moderately halophilic bacteria. protease from moderately halophilic bacteria have not been extensively studied. Some of their works on protease from moderately halophilic bacteria are as follows : Duong Van Qua et al. (1981), Sanchez-Porro et al. (2003), and Namwong et al., (2005).

Previously study, only studies extracellular protease produced by unidentified moderately halophilic bacterium, designated *Psseudomonas* sp. strain A-14, was purified. The molecular weight of this enzyme was estimated to be 12,000 Da, The optimum pH for activity was 8.0, and the enzyme presented maximal at 18% NaCl concentration (Qua et. al., 1981).

In 2003, the protease CP1 produced by the moderately halophilic bacterium, *Pseudoalteromonas* sp. CP76 has been purified and characterized in detail

by Sanchez-Porro et. al. (2003). The enzyme is a homodimer with a subunit size 38 kDa. The enzyme is moderately thermophilic , presenting optimum activity at 55 °C, at pH 8.5. An interesting feature of this protease is salt tolerance over a wide range of NaCl concentration (0-20% NaCl). These characteristic make the protease CP1 interesting for its application in biotechnological process. The protease activity was inhibited by EDTA, PMSF and Pefabloc. No significant inhibition was detected with E-64, bestatin, chymostatin or leupeptin. According to this result and the sequencing of the amino terminal region of the purified enzyme, the protease CP1 has been classified as a serine metalloprotease.

In order to improve the production of the protease CP1 for industrial application, the growth condition of *Pseudoalteromonas* sp.CP76 for optimum protease activitty were studied. The production was optimal in saline medium containing 7.5% NaCl, supplemented with sucrose, fructose and glycerol. This study constitutes the first report on the purification and in-depth characterization of proteolytic enzyme from a moderately halophilic microorganism.

In a recent study, Hiraga, et al. (2005) studied the protease – producing bacteria were screened from fish sauce in Thailand. An isolated moderately halophilic bacterium, RF2-5 was identified and named as *Fillobacillus*. The molecular weight of the purified enzyme was estimated to be 49 kDa. The enzyme showed the highest activity at 60 °C and pH 10-11 under 10%NaCl and was highly stable in the presence of about 25% NaCl. The activity was strongly inhibited by PMSF, Chymostatin, and α-microbial alkaline proteinase inhibitor (MAPI). The N-terminal 15 amino acid sequence of the purified enzyme showed about 67% identity of the serine proteinase from *Bacillus subtilis* 168 and *Bacillus subtilis* (Natto). The proteinase from *Fillobacillus* sp RF2-5. might be useful for the degradation of fish protein during fermentation at high salt concentrations and might be useful in reduction of the fermentation period.

Table 5. Properties of protease from extreme halophlies

Extrem halophile	Native molecular mass(kDa)	Optimal condition			Inhibitor
		NaCl	pH	Temp (°C)	
Natrialba magadii	45	1.5M	8-10	60	DFP,PMSF chymostatin
Halophilic archaebacterium 172P1	42-46	-	10.7	75	-
Halobacterium mediterranei 1538	41	4.5M	8-8.5	55	-
Halobacteriu halobium ATCC 43214	66	4M	10	-	DMF
Natronocuccus occultus NCMIB 2129	45	1-2M	-	-	DFP,PMSF chymotrypsin
Halobacterium sp. strain TuTA	60	-	-	-	PMSF,DFP, leupeptin