FATTY ACID METHYL ESTER ANALYSIS OF BACTERIAL ISOLATES FROM SALT LAKE, TURKEY AND CHARACTERIZATION OF THEIR EXTRACELLULAR ENZYMES

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ABSTRACT

FATTY ACID METHYL ESTER ANALYSIS OF BACTERIAL ISOLATES FROM SALT LAKE, TURKEY AND CHARACTERIZATION OF THEİR EXTRACELLULAR ENZYMES

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In this study, 11 bacterial isolates from Salt Lake,Turkey were identified by using fatty acid methyl ester (FAME) analysis. They were screened for production of industrially important enzymes xylanase, cellulase, α-amylase and protease. These enzymes were characterized in terms of enzyme activity, stability, optimum temperature and optimum pH.

One of the isolates was identified as *Bacillus pumilus*, and two of them were identified as *Bacillus subtilis*. Other isolates were determined to be *Bacillus licheniformis*.

All the isolates were determined to produce xylanase. Optimum temperatures and optimum pH values of xylanases were 50-55 ℃ and pH 7.0-8.0. Xylanases were quite stable up to pH 8.0 and 70 ℃. Isolates were not significant cellulase

producers. Four of the isolates did not produce any cellulase enzyme and the rest produced negligible amounts of cellulase. Therefore, xylanases from the isolates were promising for pulp and paper industry, which requires cellulase free and stable xylanases.

All the isolates produced appreciable quantities of α-amylase. Optimum temperatures and optimum pH values of α-amylases 60-80 ℃ and pH 7.0-8.0. α-Amylases were quite stable up to pH 9.0 and 80 ℃. α-Amylases from the isolates were promising for starch processing industry, which requires α-amylases stable at high temperatures and for detergent industry, which requires α-amylases stable at alkaline pH values.

Considerable protease productions were achieved by all the isolates. TTG 2 was the best protease producer with 271 U/ml. Optimum temperatures and optimum pH values of proteases were 50-60 ℃ and pH 7.0-7.4. Proteases were quite stable up to pH 9.0 and 80 ℃. Proteases from the isolates were promising for detergent and leather industry, in which proteases must be stable at alkaline pH values.

Keywords: *Bacillus*, FAME identification, xylanase, cellulase, α-amylase, protease

ÖZ

TUZ GÖLÜ BAKTERİ İZOLATLARININ YAĞ ASİDİ METİL ESTER ANALİZİ VE HÜCRE DIŞI ENZİMLERİNİN KARAKTERİZASYONU

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Bu çalışmada, Tuz gölünden izole edilmiş 11 bakterinin yağ asidi metil ester profillerine göre kesin tanısı yapılmıştır. İzolatların endüstriyel öneme sahip ksilanaz, selülaz, alfa-amilaz ve proteaz enzimlerini üretip üretmedikleri belirlenmiştir. Bu enzimlerin enzim aktivitesi, stabilitesi, optimum sıcaklık ve optimum pH değerleri belirlenmiştir.

Bir izolat *Bacillus pumilus* ve iki izolat *Bacillus subtilis* olarak tanımlanmıştır. Diğer izolatların *Bacillus licheniformis* olduğu belirlenmiştir.

Bütün izolatların ksilanaz enzimi ürettiği belirlenmiştir. İzolatların ksilanaz enzimlerinin optimum sıcaklıkları ve optimum pH değerleri 50-55 °C ve 7.0-8.0 olarak belirlenmiştir. Ksilanazlar pH 8 ve 70 °C limitine kadar yüksek oranda stabilite göstermektedirle. İzolatların selülaz üretimi önemli miktarlarda değildir. 4 izolatın selülaz enzimi üretmediği, diğerlerinin ise önemsiz miktarlarda selülaz üretimine

sahip oldukları belirlenmiştir. Bu yönleriyle ksilanaz enzimleri selülaz içermeyen ve stabil ksilanaza ihtiyaç duyan kağıt endüstrisi icin umut vericidirler.

İzolatlar α-amilaz enzimini önemli miktarlarda üretmektedir. Enzimlerin optimum sıcaklıkları ve optimum pH değerlerinin 60-80 °C ve 7.0-8.0 olduğu saptanmıştır. α-Amilazlar pH 9 ve 80 °C limitine kadar önemli miktarda stabilite göstermektedir. Bu yönleriyle α-amilazlar yüksek sıcaklıklarda stabilite gerektiren nişasta işlenmesi endüstrisi ve yüksek pH değerlerinde stabilite gerektiren deterjan endüstrisi için umut vericidirler.

Bütün izolatlar önemli miktarlarda proteaz enzimi üretmektedir. İzolatların proteaz enzimlerinin optimum sıcaklıkları ve optimum pH değerleri 50-60 °C ve 7.0-7.4 olarak tesbit edilmiştir. Proteazlar pH 9 ve 80 °C limitine kadar önemli miktarda stabilite göstermektedir. Bu yönleriyle proteazlar yüksek pH değerlerinde stabilite gerektiren deterjan ve deri endüstrisi için umut verici enzimlerdir.

Anahtar kelimeler: *Bacillus*, FAME tanımlaması, ksilanaz, selülaz, α-amilaz, proteaz

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INTRODUCTION

1.1. Genus *Bacillus*

The genus *Bacillus* comprised a phylogenetically and phenotypically heterogeneous group of species. *Bacillus* species are rod-shaped, endospore-forming, aerobic or facultatively anaerobic, Gram-positive bacteria. The many species of the genus exhibit a wide range of physiologic abilities that allow them to live in every natural environment. Only one endospore is formed per cell. The spores are resistant to heat, cold, radiation, desiccation, and disinfectants. Endospores are so named because they are formed intracellularly, although they are eventually released from this mother cell or sporangium as free spores. Endospores have proven to be the most durable type of cell found in nature, and in their cryptobiotic state of dormancy, they can remain viable for extremely long periods of time, perhaps millions of years. The form of the endospore and shape of the spore-bearing mother cell, the sporangium, is a characteristic feature of *Bacillus* species (Wipat and Hardwood, 1999; Holt, 1994; Laskin and Lechevalier, 1974).

The genus Bacillus can be distinguished from other endospore-forming bacteria because it is rod shaped and either aerobic or facultatively anaerobic. Cells of *Bacillus* may occur singly or occur in chains which may be of considerable lenght. Most *Bacillus* species are motile by means of peritrichous flagella. Today, there are 65 validly described species in the genus (Wipat and Hardwood, 1999; Holt, 1994).

Due to the resistance of endospores and to their long term survival under adverse conditions, most aerobic spore formers are ubiquitous and can be isolated from a wide variety of sources. It is generally accepted that the primary habitat of the

majority of *Bacillus* species is the soil where they play an important role in the biological cycling of carbon and nitrogen. Other habitats like fresh water, polluted sea water, deep sea sediments, foods, milk, pharmaceuticals, etc., may have acquired these organisms from soil by runoff, from dust, from infected plant materials. Such habitats may provide conditions suitable for the growth of *Bacillus* species or may only harbor spores which may survive in any habitat for long periods (Wipat and Hardwood, 1999; Holt, 1994; Sonenshein *et al*, 1993).

Most *Bacillus* species are versatile chemoheterotrophs capable of respiration using a variety of simple organic compounds (sugars, amino acids, organic acids). In some cases, they also ferment carbohydrates in a mixed reaction that typically produces glycerol and butanediol. A few species, such as *Bacillus megaterium*, require no organic growth factors; others may require amino acids, B-vitamins, or both. The majority are mesophiles, with temperature optima between 30 and 45 degrees, but the genus also contains a number of thermophilic species with optima as high as 65 degrees. In the laboratory, under optimal conditions of growth, *Bacillus* species exhibit generation times of about 25 minutes (Wipat and Hardwood, 1999; Holt, 1994; Sonenshein *et al*, 1993; Laskin and Lechevalier, 1974).

Bacillus species are easily isolated and readily grown in the bacteriology laboratory. The simplest technique that enriches for aerobic spore formers is to pasteurize a diluted soil sample at 80 degrees for 15 minutes, then plate onto nutrient agar and incubate at 37 degrees for 24 hours up to several days. The plates are examined after 24 hours for typical *Bacillus* colonies identified as catalase-positive, Gram-positive, endospore-forming rods. Although many species contain sporangia and free spores within 24 hours, some cultures must be incubated 5-7 days before mature sporangia, and the size and shape of the endospore contained therein, can be observed (Wipat and Hardwood, 1999; Holt, 1994; Sonenshein *et al*, 1993).

It is possible to allocate many Bacillus species to one of six taxa that have distinguishable physiologies and, perhaps surprisingly, are generally consistent with the division of the genus based on spore morphologies. Figure 1.1. illustrates phylogenetic tree of some members of the genus *Bacillus* based on 16S rRNA sequence analysis. Members of the ***B. polymyxa*** **group**, recently renamed *Paenibacillus*, are auxotrophs, most commonly associated with rotting plant

materials, composts and the rhizosphere. They are true facultative aerobes. Some members of this group are able to fix nitrogen and may contribute significantly to the uptake of nitrogen by crops such as Canadian wheat. There is also evidence that members of this group produce plant hormones such as gibberellin. *B. macerans, B. circulans and B. lentimorbus* are other representatives of this group beside *B. polymyxa*. Members of the ***B. brevis* group**, renamed *Brevibacillus*, are found in both soil and water habitats. These bacteria are strict aerobes that do not produce acid from sugars. *B. brevis, B. badius, B. azotoformans* and *B. laterosporus* are the representatives of this group. The ***B. sphaericus* group** are most noted as insect pathogens and are found in the sediments of pools, lakes and drainage ditches where insect larvae thrive. These bacteria are strictly oxidative and in most cases will not use sugars as a source of carbon or energy, preferring acetate or aminoacids such as glutamate as carbon sources. *B. sphaericus, B. aminovorans* and *B. insolitus* are examples of this group. Two groups of **thermophiles**, which include members of the genus *Alicyclobacillus* and *B. stearothermophilus*, are found predominantly in soils from a variety of thermal and non-thermal sites (Wipat and Hardwood, 1999; Sonenshein *et al*, 1993).

The ***B. subtilis* group** includes the most intensively studied of the bacilli, including species such as *B. subtilis* itself, *B. licheniformis* and *B. amyloliquefaciens* which are of industrial importance. Species of this group are phylogenetically and phenetically consistent. These bacteria all produce acids from a range of sugars and they are an intermediate stage between the true facultative anaerobes of the *B. polymyxa* group and the stricts aerobes of *B. brevis* and *B. sphareicus* groups. The *Bacillus* species *subtilis, licheniformis,* and *pumilus* are closely related and there has been difficulty distinguishing among the three species (Wipat and Hardwood, 1999; Sonenshein *et al*, 1993).

1.1.1. *Bacillus subtilis*

B. subtilis is a ubiquitous soil microorganism that contributes to nutrient cycling when biologically active due to the various enzymes produced by members of the species. However, under most conditions the organism is not biologically active but exists in the spore form. It is thought that 60 to 100% of soil bacilli populations exist

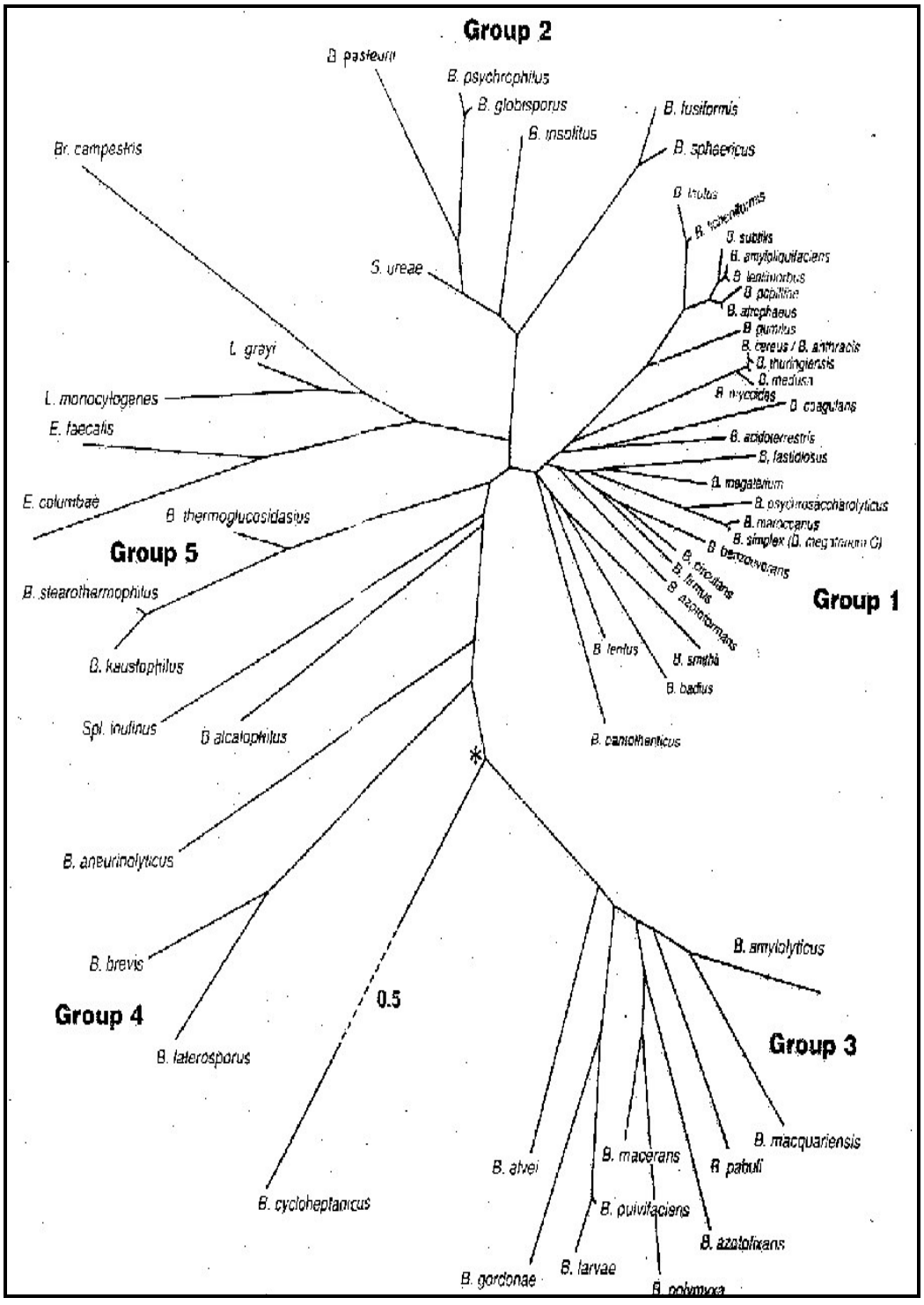


Figure 1.1: Phylogenetic tree of some members of the genus *Bacillus* based on 16S rRNA sequence analysis.

in the inactive spore state. *B. subtilis* is widely distributed throughout the environment, particularly in soil, air, and decomposing plant residue. It has shown a capacity to grow over a wide range of temperatures including that of the human body *B. subtilis* grows as a unicellular rod, seldom as chains. Like most members of the genus, *B. subtilis* is aerobic, except in the presence of glucose and nitrate, some anaerobic growth can occur. This bacterium is the "*E. coli*" of Gram-positive bacteria. Much of the information we have on the biology, biochemistry and genetics of the Gram-positive cell, indeed, of bacteria in general, has been derived from the study of *B. subtilis. B. subtilis* is considered a benign organism as it does not possess traits that cause disease. The potential risk associated with the use of this bacterium in fermentation facilities is low, therefore *B. subtilis* is one of the most widely used bacteria for the production of enzymes and specialty chemicals. Industrial applications include production of amylase, protease, inosine, ribosides, and amino acids (Holt, 1994; **www.epa.gov/opptintr/biotech/fra/fra009htm**).

1.1.2. *Bacillus licheniformis*

Bacillus licheniformis is a saprophytic bacterium that is widespread in nature and thought to contribute substantially to nutrient cycling due to the diversity of enzymes produced by members of the species. Typically, the cells are motile by peritrichous flagella and are facultatively anaerobic, allowing for growth in additional ecological niches. Certain members of the species are capable of denitrification; however, their importance in bacterial denitrification in the environment is considered to be small as the bacilli typically persist in soil as endospores. Strains of *B. licheniformis* are widely distributed in the environment: they are common in most soils and dominate in nutrient-poor soils such as moorland and deserts. *B. licheniformis* has been used in the fermentation industry for production of proteases, amylases, antibiotics, or specialty chemicals. The ATCC Catalogue of Bacteria and Phages lists strains which are capable of producing alkaline proteases, alpha-amylases, penicillinases, pentosanases, bacitracin, proticin, 5'-inosinic acid and inosine, citric acid, and substituted L-tryptophan. *B. licheniformis* is not a human pathogen nor it is toxigenic (De Boer *et al*, 1994; Holt, 1994; **www.epa.gov/opptintr/biotech/fra/fra005.htm**).

1.1.3. *Bacillus pumilus*

Bacillus pumilus is a naturally occurring bacterium that is especially common in soil, water, air and decomposing plant tissue. It is a gram positive, rod shaped and endospore-forming bacterium. Spores of *B. pumilus* are ubiquitous in nature and occur in soil more frequently than those of *B. subtilis*. It is not considered pathogenic or toxigenic to humans, animals, or plants.

1.2. Microbial Identification

Mainly, three commonly applied methods are used for the identification of unknown microorganisms. First method is based on biochemical, physiological and morphological criteria which suffer two major disadvantages: They rely on time-consuming and labor-intensive procedures which often yield ambiguous and unspecific results. In addition, classical tests, e.g., based on assimilation or fermentation of sugars, do not allow differentiation between strains. Second method is based on RNA/DNA profile of unknown microorganism, yielding the most accurate results. But this technique uses tremendously expensive equipment, chemicals, radiochemicals and enzymes, is very time consuming and labor intensive and therefore not appropriate for large numbers of samples and automated data analysis. Moreover, since the sequence of the universal primers is based on cultured organisms, the applicability of this technique for community analysis in environmental samples remains questionable. A promising approach to bacterial detection and identification is chemotaxonomy. In chemotaxonomy, chemical and physical techniques are employed to elucidate the chemical composition of whole bacterial cells and/or their individual cellular components in order to produce a chemical signature or profile of taxonomic significance. One successful and commercialized chemotaxonomic approach for obtaining bacterial fatty acid profiles is based on the analysis of the methyl esters of their fatty acids by gas chromatography (GC) (Basile *et al*, 1998; Fang *et al*,2000).

Fatty acid methyl esters (FAMEs) derived from lipids and free fatty acids have long been used for microbial diagnostics and taxonomy. Whole cellular fatty acid methyl esters (FAME) content is a stable bacterial profile, the analysis method is rapid, cheap, simple to perform and highly automated. The fatty acids between 9 and 20

carbons in length have been used to characterize genera and species of bacteria. Simple mutations or plasmid loss or gain do not alter the fatty acid composition of an organism. The fused silica capillary columns, now commonly used with gas chromatography, allow recovery of hydroxy acids and resolution of isomers which in turn make the identification of whole cell fatty acids methyl esters (FAME) over a wide range of microorganisms possible and practical (Giacomini *et al*, 2000; Xu *et al*, 2003).

Currently, there is an automated system, the MIDI Sherlock Microbial Identification System (MIS), which identifies microorganisms based on unique FAME patterns of known strains. The peaks are automatically named and quantitated by the system. Branched chain acids predominate in some Gram positive bacteria, while short chain hydroxy acids often characterize the lipopolysaccharides of the Gram negative bacteria. The structures of a few of these compounds are shown in Figure1.2. (Whittaker *et al*, 2003).

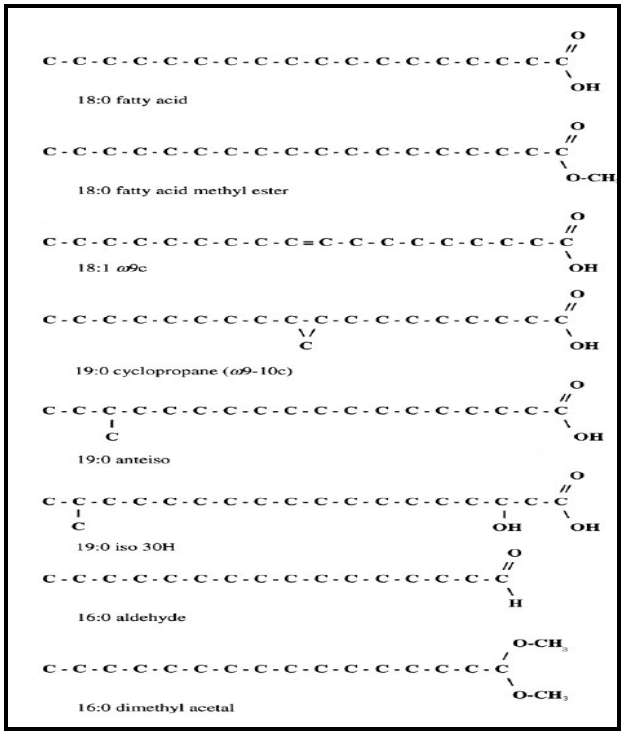


Figure 1.2: Structure of fatty acids

For using MIDI system, the microorganism is first purified and then grown on a standard medium, incubated at a certain temperature and time for that organism. The material is harvested and placed in a glass test tube for the extraction and methylization. This part of the analysis uses common reagents, heated water baths and mixers and is relatively easy to perform, the only critical factor being the timing of events. A dozen samples with the appropriate controls and blanks can be easily ready for the gas chromatograph in two to three hours. Patterns or FAME extract profiles are then compared to a standard database and possible / most likely hits are then presented to the user as shown in Figure 1.3. The databases currently have over 2000 entries, including aerobic bacteria, anaerobic bacteria and yeasts (Basile *et al*, 1998).

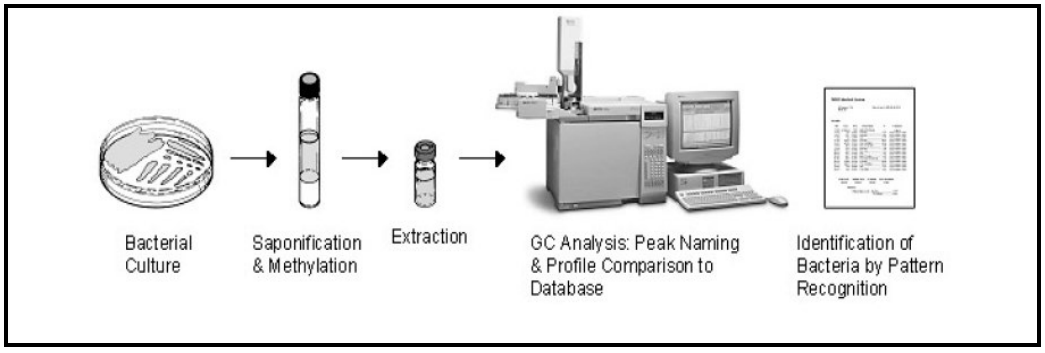


Figure 1.3: Diagram of the MIDI system.

This method has some advantages over biochemical tests. For example, fatty acid analysis is more objective and less prone to human error. And in contrast to biochemical tests, fatty acid composition is not influenced by plasmid loss or gain and is rarely influenced by organism mutations. Fatty acid analysis is based on species databases (accounts for normal species variability) versus a series of yes/no answers. Furthermore, biochemical-based identification can take up to several days, compared to just several hours for fatty-acid-based identification. It also has advantages over DNA-based identification. The cost/sample for fatty acid analysis is low compared to DNA techniques. Technical proficiency required for DNA techniques is high. Fatty acid analysis can identify to the strain level versus the

species level for most DNA-based methods. DNA-based identification is very labor intensive. Moreover, DNA-based identification can take up to several days, compared to just hours for fatty-acid-based identification.

1.3. Microbial Enzymes

Enzymes are a certain class of protein molecules that accelerate, or catalyse, the chemical reactions of living cells. Without enzymes, most biochemical reactions would be too slow to carry on life processes. These reactions are the basis of the metabolism of all living organisms, and provide tremendous opportunities for industry to carry out elegant, efficient and economical biocatalytic conversions.

Each organism produces a large variety of enzymes, most of which are made in only small amounts and are involved in cellular processes. However, certain enzymes are produced in much larger amounts by some organisms, and instead of being held within the cell, they are excreted into the medium. Extracellular enzymes are usually capable of digesting insoluble nutrient materials such as cellulose, protein, and starch, the products of digestion then being transported into the cell where they are used as nutrients for growth. Some of these extracellular enzymes are used in the food, dairy, pharmaceutical, and textile industries and are produced in large amounts by microbial synthesis. They are especially useful because they often act on single chemical functional groups, they easily distiguish between similar functional groups on a single molecule, and in many cases, they catalyze reactions in a stereospecific manner producing only one of two possible enantiomers (Madigan *et al*, 1997).

Enzymes found in nature have been used since ancient times in the production of food products, such as cheese, sourdough, beer, wine and vinegar, and in the manufacture of commodities such as leather, indigo and linen. All of these processes relied on either enzymes produced by spontaneously growing microorganisms or enzymes present in added preparations such as calves' rumen or papaya fruit. The enzymes were, accordingly, not used in any pure or well-characterized form. The development of fermentation processes during the later part of the last century, aimed specifically at the production of enzymes by use of

selected production strains, made it possible to manufacture enzymes as purified, well-characterized preparations even on a large scale. This development allowed the introduction of enzymes into true industrial products and processes, for example, within the detergent, textile and starch industries. The use of recombinant gene technology has further improved manufacturing processes and enabled the commercialization of enzymes that could previously not be produced. Furthermore, the latest developments within modern biotechnology, introducing protein engineering and directed evolution, have further revolutionized the development of industrial enzymes. These advances have made it possible to provide tailor-made enzymes displaying new activities and adapted to new process conditions, enabling a further expansion of their industrial use. Industrial enzymes are mainly produced from micro-organisms by a process of fermentation and extraction. Although some enzymes are produced by extraction of plant and animal tissues, fermentation is replacing these processes as it is more efficient and has a lower impact on the environment (Kirk *et al*, 2002; Beilen and Li, 2002). Microbial enzymes and their applications are illustrated in Table 1.1.

The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Proteases remain the dominant enzyme type, because of their extensive use in the detergent and dairy industries and constitute more than 65% of the world market. Various carbohydrases, primarily amylases and cellulases, used in industries such as the starch, textile, detergent and baking industries, represent the second largest group.

The starch hydrolytic enzymes comprise 30% of the world's enzyme consumption.The technical industries, dominated by the detergent, starch, textile and fuel alcohol industries, account for the major consumption of industrial enzymes as shown in Figure 1.4. Overall, the estimated value of the worldwide use of industrial enzymes has grown from $1 billion in 1995 to $1.5 billion in 2000 (Haki and Rakshit, 2003; Kirk *et al*, 2002).

The enzyme industry as we know it today is the result of a rapid development seen primarily over the past four decades thanks to the evolution of modern biotechnology. The food, feed, agriculture, paper, leather and textile industries are

Table1.1: Microbial enzymes and their applications

Enzyme	*Source*	*Application*	*Industry*
Amylase	Fungi	Bread	Baking
	Bacteria	Starch coatings	Paper
	Fungi	Syrup and glucose manufacture	Food
	Bacteria	Cold-swelling laundry starch	Starch
	Fungi	Digestive aid	Pharmaceutical
	Bacteria	Removal of coatings(desizing)	Textile
	Bacteria	Removal of stains; detergents	Laundry
Protease	Fungi	Bread	Baking
	Bacteria	Spot removal	Dry cleaning
	Bacteria	Meat tenderizing	Meat
	Bacteria	Wound cleansing	Medicine
	Bacteria	Desizing	Textile
	Bacteria	Household detergent	Laundry
Invertase	Yeast	Soft-center candies	Candy
Glucose oxidase	Fungi	Glucose removal, oxygen removal	Food
		Test paper for diabetes	Pharmaceutical
Glucose isomerase	Bacteria	High fructose corn syrup	Soft drink
Pectinase	Fungi	Pressing, clarification	Wine, fruit juice
Rennin	Fungi	Coagulation of milk	Cheese
Cellulase	Bacteria	Fabric softening, brightening; detergent	Laundry
Lipase	Fungi	Breaks down fat	Dairy, laundry
Lactase	Fungi	Breaks down lactose to glucose and galactose	Dairy, health foods
DNA polymerase	Bacteria Archaea	DNA replication in PCR	Biological research forensics

well suited for enzyme technology because products as well as raw materials consist of biomolecules, which can be produced, degraded or modified by enzymatic processes. Many enzymes are commercially available, and numerous industrial applications have been described . In the pulp and paper industries, enzymes are increasingly used for cleaner production processes, resulting in 1994 enzyme sales in the United States and Europe of about $25 million. The availability of cost-effective and clean enzymatic or biological alternatives to chemical procedures strongly promotes technological improvements. Hence, the resulting market value of biotechnology for clean production in the paper industry ($31–62 billion) is very significant indeed. Enzymes have a similar role in textiles production: the use of laccases for bleaching or catalase to remove hydrogen peroxide cause a significant reduction in the use of raw materials and production of waste. Enzymes are also used in a wide range of agrobiotechnological processes, such as enzyme-assisted silage fermentation, bioprocessing of crops and crop residues, fibre processing and

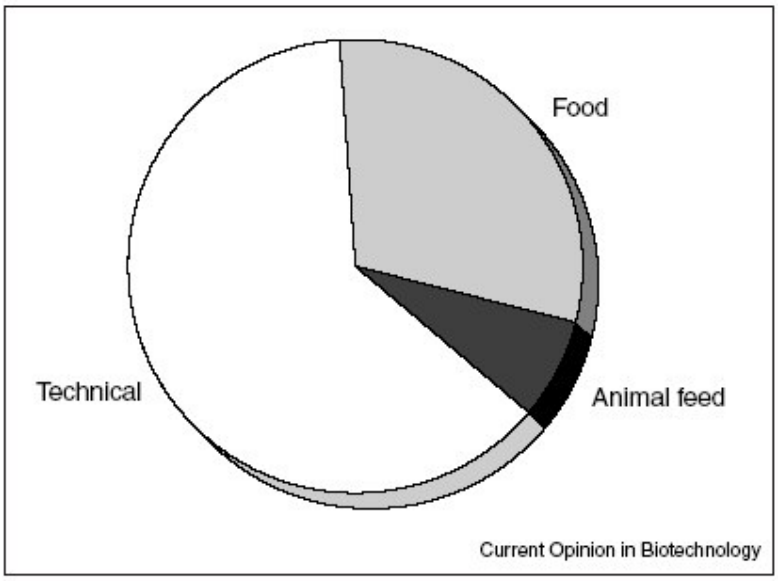


Figure 1.4: Segmentation of the industrial enzyme market. In the year 2000, the enzyme market totalled $1.5 billion. The technical industries segment comprises the detergent, starch, textile, fuel alcohol, leather, and pulp and paper industries.

production of feed supplements to improve feed efficiency. The feed enzyme market now amounts to $150 million. Several developments have started to tie in the agricultural sector with the chemical and pharmaceutical industries. Plants are being modified by genetic engineering for the production of polymers and pharmaceuticals such as antibodies or for improved nutritional value, for example, by increasing lysine or carotenoid content. Agricultural waste biomass may soon turn into a valuable resource for the production of chemicals and fuels. Biobased renewables have many advantages, such as reduced CO_2 production, flexibility, and self-reliance. This was also recognized by the chemical industry. For example, it is estimated that by the year 2050 renewable resources could supply 30% of the worldwide chemical and fuel needs, resulting in a biomass market of $150 billion (Beilen and Li, 2002; Kirk *et al*, 2002).

Despite the fact that to date more than 3000 different enzymes have been identified and many of these have found their way into biotechnological and industrial applications, the present enzyme toolbox is still not sufficient to meet all demands. A

major cause for this is the fact that many available enzymes do not withstand industrial reaction conditions. Mesophilic enzymes are often not well suited for the harsh reaction conditions required in industrial processes because of the lack of enzyme stability. For this reason, the use of biocatalysts in organic reactions represented only a small fraction of the potential industrial market in the past. The discovery of new extremophilic microorganisms and their enzymes has had a great impact on the field of biocatalysis. Extremophiles are organisms that have evolved to exist in a variety of extreme environments and fall into a number of different classes that include thermophiles, acidophiles, alkalophiles, psychrophiles, and barophiles (piezophiles) and others. They have adapted to thrive in ecological niches such as deep-sea hydrothermal vents, hot springs, and sulfataric fields . As a result, these microorganisms produce unique biocatalysts that function under conditions in which their mesophilic counterparts could not survive, permitting the development of additional industrial processes. Psychrophilic enzymes produced by cold-adapted microorganisms display a high catalytic efficiency that offers considerable potential to the biotechnology industry, for example, in the detergent and food industries, and for the production of fine chemicals . The industrial potential for halophilic enzymes is increasing as the approaches to study the genetic processes of halophiles and our understanding of haloadaptation becomes more sophisticated. Acidophiles typically share other extremophilic habitat properties such as thermophilicity, halophilicity or heavy-metal resistance, and they have found a niche in the bioprocessing of minerals. Alkaliphiles thrive in alkaline environments and have made a great impact in industrial applications, especially alkaline proteases and cellulases in biological detergents. Deep-sea organisms are the source of piezophiles (barophiles), and research has focused mostly on the identification of pressure-regulated operons showing the relationship between pressure and microbial growth. Only recently has attention been shifted to the potential biotechnological applications of piezophiles compared with those of other extremophiles. Radiophiles are a class of extremophiles typically ignored but receiving a lot of attention recently, because of their ability to survive under conditions of starvation, oxidative stress, and high amounts of DNA damage. Most of the extremophiles that have been identified to date belong to the domain of the Archaea. However, many extremophiles from the eubacterial and eukaryotic kingdoms have also been recently identified and characterized (van den Burg, 2003; Demirjian *et al*, 2001).

Enzymes are produced commercially from both fungi and bacteria. The microbial enzymes produced in the largest amounts on an industrial basis are the bacterial proteases, used as additives in laundry detergents. Many enzymes are isolated from species of *Bacillus*. Many bacteria belonging to the genus *Bacillus* excrete large amounts of enzymes into the culture medium. About two-thirds of the industrially important enzymes are produced by members of the genus *Bacillus*. *Bacillus subtilis* is well known for the production of the industrially important enzymes such as amylase and protease. The typical enzyme yield from a *Bacillus* fermentation process is estimated to be around 20 g/liter of secreted material in a relatively short time with very low-cost carbon and nitrogen sources. This demonstrates the abilities of *Bacillus* spp. to produce large quantities of enzymes at competitive cost (Madigan *et al*, 1997; Sonenshein *et al*, 1993).

Reasons of the predominance of *Bacillus* spp. in enzyme industry are several. First, they comprise a group of chemoorganotrophs that can be easily maintained and cultivated and yet are markedly heterogenous in character. Psychrophiles, mesophiles, and thermophiles, in addition to alkalophilic, neutrophilic, and acidophilic species are well represented. Furthermore, virtually all of the species of the genus secrete a variety of extracellular enzymes, which reflects the diversity of the parental habitats. Amylases that can liquefy starch under pressure at 110 ℃ and proteases that are stable and active at pH 12.0 are extreme examples of enzyme adaptation. Moreover, the fact that these bacteria have been used for decades to produce substances generally recognized as safe makes *Bacillus* spp. an excellent choice for the production of recombinant proteins (Priest, 1977; Sonenshein *et al*, 1993).

Historically, *Bacillus licheniformis* and *Bacillus amyloliquefaciens* have been the organisms of choice for fermentation production, largely because of the properties of their extracellular enzymes, which are more suitable for certain industrial applications. The major drawback with the industrial strains is that they are very recalcitrant to most genetic manipulation. Another drawback is that these bacteria are also the active producers of numerous proteases. Because the industrial strains are difficult to manipulate genetically, it is troublesome to isolate and characterize mutants deficient in production of proteases or having altered secretion

characteristics. These makes *Bacillus subtilis*, with its exquisitely developed genetics, the leading candidate to become the production host of choice (Sonenshein *et al*, 1993).

1.3.1. Xylanases

Plant cell walls can be considered to be the main renewable resource formed in the process of photosynthetic fixation of carbon dioxide. They are composed of three major polymeric constituents: cellulose (35-50 %), hemicellulose (20-30 %) and lignin (20-30 %). Hemicelluloses are noncellulosic polysaccharides that are found in plant tissues. Xylan is the second most abundant biopolymer after cellulose and the major hemicellulosic polysaccharide found in the plant cell wall. It is a complex molecule composed of β-1,4-linked xylose chains with branches. The xylan structure is extremely variable and depends on its source. The structure ranges from an almost linear unsubstituted chain, e.g., in some grasses, to highly branched heteropolysaccharides in cereal seeds. The prefix hetero indicates the presence of sugars other than D-xylose. The main chain is usually substituted to various degrees by residues of 4-O-methyl-D- glucuronic acid, D-glucuronic acid, or L-arabinofuranose, and in some cases is also esterified by acetyl groups. Xylanases are a group of enzymes that catalyse the hydrolysis of xylans (Damiano *et al*., 2003; Whitaker, 2002; Dhillon *et al*., 2000; Blanco *et al*., 1995; Whitaker, 1994).

Due to the heterogeneity of xylan, its hydrolysis requires the action of a complex enzyme system.This is usually composed of β-1,4-endoxylanase, β -xylosidase, α -L-arabinofuranosidase, α-glucuronidase, acetylxylan esterase, and phenolic acid esterases. All these enzymes act cooperatively to convert xylan to its constituent sugar as shown in Figure 1.5.

Endoxylanases and β-xylosidases have the most important activities among the xylanolytic enzymes involved in xylan hydrolysis. **β-1,4-endoxylanase** (1,4-β-D-xylan xylohydrolase; EC 3.2.1.8) cleaves the internal glycosidic linkages of the heteroxylan backbone and the main products formed are xylooligosaccharides. **β-D-Xylosidases** (β-D-Xyloside xylohydrolase; EC 3.2.1.37) are exoglycosidases that hydrolyze short xylooligosaccharides and xylobiose from the nonreducing end to liberate xylose.

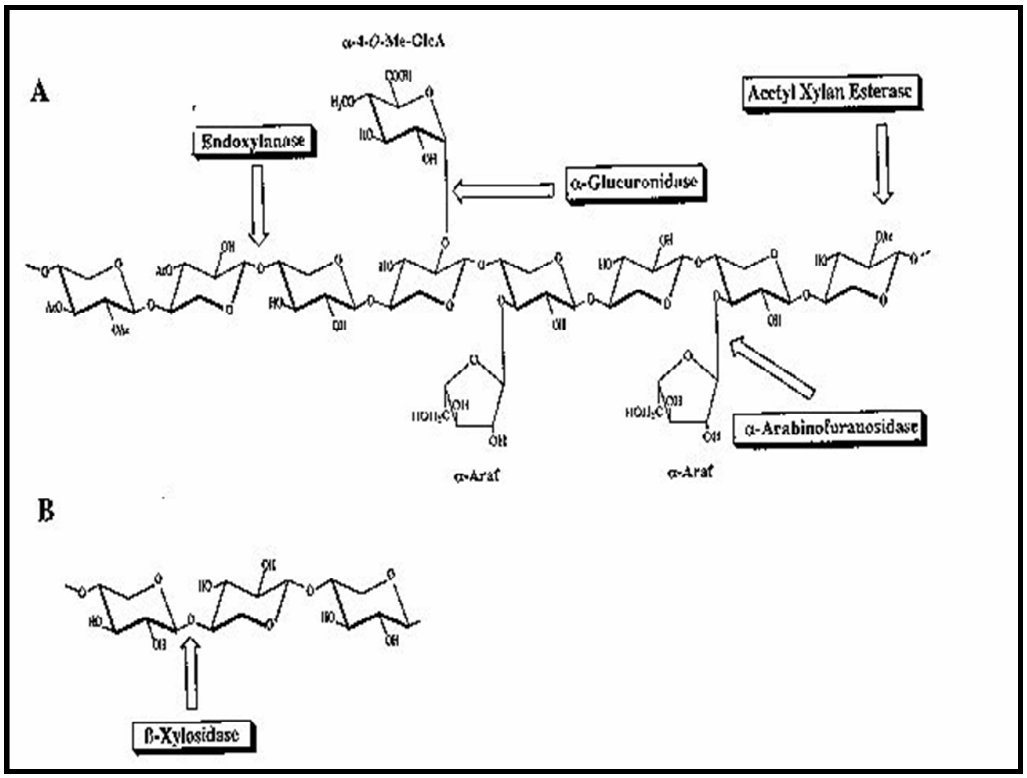


Figure 1.5: (A)The xylanolytic enzymes involved in the degradation of xylan.Ac: Acetyl group; α-Araf: α-arabinofuranose; α-4-O-Me-GlcA: α-4-O-methylglucoronic acid. (B) Hydrolysis of xylooligosaccharide by β-xylosidase.

Side-chain cleaving enzymes like α-arabinofuranosidase, α -glucuronidase and acetylxylan esterase play important roles in the removal of side substituents of heteroxylans. While **α–arabinofuranosidase** (E.C.3.2.1.55) hydrolyse the arabinose side chains, **α-glucuronidase** hydrolyzes the α -1,2 linkages between glucuronic acid and xylose residues in glucuronoxylan. **Acetylxylan esterases** (EC 3.1.1.6) catalyzes the removal of *O*-acetyl substituents at the C-2 and C-3 positions of xylose residues in acetylxylan. Among the xylanolytic enzymes, endoxylanases (1,4-β-D-xylan xylohydrolase; EC 3.2.1.8) are the most abundant components and the main enzymes required in most applications (Breccia *et al*., 1998; Sunna and Antranikian, 1997).

1.3.1.1. Panorama of Microbial Xylanases

Xylanases are mainly used in pulp and paper industry. However, the use of xylanases in the paper industry has been slow down by the lack of large scale availibility of cellulase free xylanase active at pH above 8 and temperatures around 60 ℃, which are conditions prevailing in many bleaching processes.The usable pH and temperature ranges of some of the commercial enzymes are pH 5-7 and 55℃ for Irgazyme10, pH 3-5 and 30-50℃ for Cartazyme HS-10, pH 5-6 and 50-55℃ for Ecopulp, pH 5-6 and 55℃ for Iogen and pH 6-8 and 50-55℃ for Pulpazyme HB. These enzymes require previous neutralization of pulp with acid to pH 6-7 for optimal activity, while Novozyme, pH 8 and 40℃ requires cooling of the pulp for optimal enzyme activity. Both neutralization and cooling of incoming pulp for optimal activity are additional steps which add to the operational costs. Therefore, it is of interest to study the production of xylanase which had both thermostability and alkalitolerance (Dhillon *et al*., 2000).

Xylanases are produced by a plethora of organisms like bacteria, algae, fungi, protozoa, gastropods, and arthropods. Most of the bacteria and fungi secrete extracellular xylanases which act on the hemicellulosic material to liberate xylose as a directly assimilable end product allowing the organisms to grow heterotrophically on xylan .

Filamentous fungi are particularly interesting producers of xylanases since they excrete the enzymes into the medium and their enzyme levels are much higher than those of yeast and bacteria. However, fungal xylanases are generally associated with cellulases and they are often acidic to neutral in nature. On the other hand, bacterial xylanases range from acidic to alkaline and from moderate to extremely thermostable depending on the type of organism (Kulkarni *et al* ., 1999). Table 1.2. illustrates the characteristics of the xylanases from different microbial sources.

1.3.1.2. Applications of Xylanases in Biotechnology

Xylan degrading enzymes have considerable potential in several biotechnological applications.

Table 1.2: Characteristics of the xylanases from different microbial sources.

Enzyme Source	Optimum pH	pH stability	Optimum temp.(℃)	Temp. stability	Reference
Aspergillus fumigatus	6.0-6.5	75% at 8.0	60	7,12 min halflife at70,60℃	Anthony *et al*, 2003
Aspergillus niger	4.0	80,75,45 min halflife at pH 3.5,4.0,4.5	60	135,55 min halflife 70,75 ℃, stable 4h at 55℃	Pandey and Pandey, 2002
Aspergillus giganteus	6.0	low stability <4.5->10.5	50	120, 1 min halflife at 40,60℃,	Coelho and Carmona, 2003
Acrophialophora nainiana	6.5	—	55	1 h halflife at 60℃	Cardoso and Filho, 2003
Rhizopus oryzae	4.5	—	55	—	Bakır *et al*, 2001
Fusarium proliferatum	5.0-5.5	94% stability at pH 6.0	55	22% stability at 60℃	Saha, 2002
Penicillum capsulatum	3.8	—	48	lost activity in 3 min at 70℃	Ryan *et al*, 2003
Sporotrichum thermophile	5.0	stable at 6.0-9.0 after 1h at 4℃	70	stable 40-50℃, 33% stability after 1h at 60℃	Topakos *et al*, 2003
Trichoderma longibrachiatum	5.0-6.0	lost activity≥ 9.0	45	50% stability after 4.5 hour at 50℃	Chen *et al*,1997
***Thermomonospora fusca* BD25**	8.0-8.5	—	70	stable 27h at 50℃, 30m halflife 80℃	Tuncer, 2000
Pleurotus ostreatus	6.0	stable 3.0-7.0	25-40	43% stability after 15min at 50℃	Quinnghe*et al*, 2003
***Streptomyces* sp. Ab106**	6.0	70% stability at 60 ℃ pH 9.0	60	70% stability after 144h at 60℃	Techapun *et al*, 2002
Streptomyces thermoviolaceus	7.0	—	70	—	Tsujibo *et al*, 1992
Streptomyces roseiscleroticus	6.7-7.0	75% stability at pH 8.0	60	—	Grabski and Jeffries, 1991

Table 1.2 (continued)

***Streptomyces* sp. QG-11-3**	8.6	stable 1h at pH 5.4-9.4	60	stable at 55-65℃, 10% stability after 2h at 80℃	Beg *et al*, 2000
***Talaromyces emersonii* CBS 814,70**	4.2	—	78	—	Subramaniyan and Prema, 2000
Thermomyces lanuginosus	6.5	—	50	—	Subramaniyan and Prema, 2000
Thermococcus zilligii	6.0	—	75	80% stability after 8h at 85℃ 4h,8min halflife at 95,100℃	Uhl and Daniel, 1999
Bacillus thermantarcticus	5.6	—	80	24h stable at 60 ℃, 50min halflife at 80℃	Lama *et al*, 2004
***Bacillus circulans* AB 16**	6.0-7.0	—	80	67% stability after 1h at 70℃,pH9.0	Dhillon *et al*,2000
B. amyloliquefacies	6.8-7.0	71-43% stability at pH 9.0-10.0	80	stable up to 55℃, lost activity at 15 min at 75℃	Breccia *et al*, 1998
***B. licheniformis* A99**	6.0	70% stability at pH 8.0	60	stable 4h at 50℃, 8h halflife at 60℃	Archana and Satyanarayana, 2003
***B. stearothermophilus* T-6**	9.0	—	65	—	Kulkarni *et al*,1999
***Bacillus* sp. Sam-3**	8.0	—	60	75% stability after 2h at 60℃, pH8.0	Shah *et al* ,1999
***Bacillus* sp. 41M-1**	9.0	—	50	stable up to 55℃	Nakamura *et al*, 1993
***Bacillus* sp. SPS-0**	6.0	stable at 5.0-9.0	75	stable 4h up to 70 ℃	Bataillon *et al*, 2000
***Bacillus* sp. BP-23**	5.5	72-35% stability at pH9.5-11.0	50	stable up to 55℃	Blanco *et al*, 1995

Xylan constitutes 20-30 % of the weight of wood and agricultural wastes.Thus xylan is a potential significant resource for renewable biomass which can be utilised as a substrate for the preparation of many products such as liquid fuels, solvents, single cell proteins, artificial low calorie sweeteners (xylitol) and pharmaceuticals. For most bioconversion processes, xylan must first be converted to xylose or xylooligosaccharides, which can be done by the use of xylanolytic enzymes (Chivero *et al*., 2001).

Recently, the use of xylanolytic enzymes in pulp bleaching has been considered as one of the most important new biotechnological applications of these enzymes. Most paper industries have used a bleaching sequence based on chlorine dioxide and extraction by alkaline solutions.The chlorine dioxide provides an excellent bleaching for the pulp, and although it is significantly less dangerous to the environment than chlorine gas, it is still toxic and accumulate in the biotic and abiotic components of the ecosystem. Faced with market, environmental and legislative pressures the pulp and paper industry is modifying its pulping and bleaching technologies to reduce the environmental impact of mill effluents. The use of xylanases in pulp bleaching as an alternative to the chlorine based methods, helps mainly in reducing the kappa number and increasing the brightness of the pulp. The enzymatic pulp prebleaching resulted in a 20 to 30 % reduction of chlorine requirements. The biobleaching of the paper pulp requires xylanases that remain active even above pH 9.0 and lacks all cellulase activity (Tseng *et al*., 2002; Sunna and Antranikian , 1997; Damiano *et al*., 2003; Dhillon *et al*., 2000).

Xylanases have significant importance in the food industry. In bakeries the xylanases act on the gluten fraction of the dough and help in the even redistribution of the water content of the bread, thereby significantly improving the desirable texture, loaf volume and shelf life of the bread. Xylanases also play a key role in liquefaction of coffee mucilage for making liquid coffee, extraction of flavors and pigments, plant oils and starch. Along with that of cellulases and pectinases, the use of xylanases has also been suggested in applications such as the clarification of juices, the preparation of dextran for use as food thickeners, and the production of fluids and juices from plant material (Wong *et al*., 1992; Kulkarni *et al* ., 1999).

Another application of xylanases is the use of these enzyme in animal diets. The dietary hemicelluloses have little nutritional significance for non-ruminant organisms as they lack the appropriate digestive enzymes. These undigested fibers increase the viscosity of the food in the gut, which interferes with penetration of digestive enzymes, absorption of digested food and may support pathogenic conditions, especially in broiler chicks. The use of xylanase together with other hemicellulases corrects the problems and also increases the nutritive value of the feed (Kulkarni *et al* ., 1999).

1.3.2. Cellulases

Cellulose is the most abundant organic macromolecule on earth. It has been estimated that total biomass (fosil fuels excepted) amounts to about $1{,}8x10^{12}$ tonnes, $1x10^{11}$ tonnes being replenished each year by photosynthesis. Since 40% of this biomass consist of cellulose, one may calculate that $7x10^{11}$ tonnes of this material exist, mainly in higher plants. The magnitude of these figures can be appreciated by noting that the rate of cellulose synthesis is equivalent to 70 kg per person per day, or to 50 000 barrels of oil per second in energy terms.

Cellulose is mainly produced in higher plants, e. g., woods as well as annual plants in which it forms the rigid skeleton of the plant. In addition, all algae produce highly crystalline cellulose. Some bacteria, especially *Acetobacter xylinum*, are also able to synthesize cellulose. It is a linear homopolymer of β-1,4-linked anhydro-D-glucose residues in the chair configuration as shown in Figure 1.6. The number of such residues, i.e. the degree of polymerization, is approximately 14 000 in native plant cellulose, about 3500 in that produced by bacteria while that of commercial celluloses ranges from 50 to 5000 Since adjacent glucose molecules alternate by 180° in orientation, the basic repeating unit is cellobiose. Hydrogen bonds support each glucosidic bond in maintaining the rigidity of cellulose molecule. Coupling of adjacent cellulose molecules by hydrogen bonds and van der Waal's forces result in their paralel alignment to produce a crystallite structure resulting in a resistance to hydrolysis (Whitaker, 2002; Fogarty and Kelly, 1990; Sharrock, 1988).

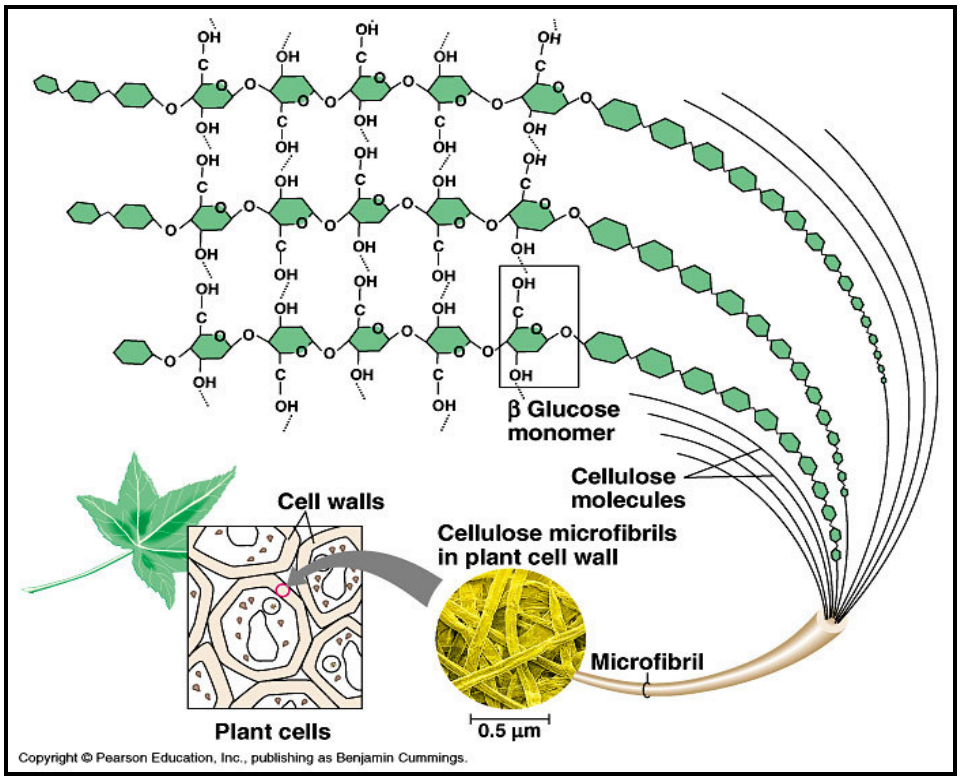


Figure 1.6: Cellulose structure

Cellulases are the group of hydrolytic enzymes able to hydrolyze insoluble cellulose to glucose. They are members of the glycoside hydrolase families of enzymes, which hydrolyse oligosaccharides and/or polysachharides. Celulases playing the key role in organic carbon turnover demostrate a striking diversity of forms resulting from the convergent evolution of genetically distant producing species (Rabinovich *et al.*, 2002; Klyosov, 1990 ; Bayer *et al.*, 1998).

The widely accepted mechanisms for the enzymatic degradation of cellulose is based on studies of the cellulase system of mesophilic fungal species. In these organisms, cellulase capable of degrading crystalline forms of cellulose is composed of essentially three enzyme species : endo-β-1,4-glucanases, exo- β-1,4-glucanases (or cellobiohydrolases), and β-glucosidases (or cellobiases).The three enzyme groups work synergistically to effect the hydrolysis of crystalline cellulose (Table 1.3).

All cellulases act on the chemically identical bond, the α-1,4 linkage between two anhydroglucose units. However, they differ in terms of their site of attack on the cellulose chain as illustrated in Table 1.3. **Endo-β-1,4-glucanases** (E.C.3.2.1.4) hydrolyze cellulose chains in a random fashion, thereby converting long chains to oligosaccharides. The net effect is a rapid decrease in polymer lenght coupled with a slow increase in reducing groups. In contrast, **exo- β-1,4-glucanases** (E.C.3.2.1.91) are cellulases which remove glucose or cellobiose units from the nonreducing end of the cellulose chain. The result of this type of hydrolysis is a rapid increase in reducing sugar with little change in overall chain lenght over the short term. β-glucosidases (E.C.3.2.1.21) hydrolyze cellobiose to two glucose units. Although the **β-glucosidases** are not cellulases, they generally significantly enhance cellulose degradation by relieving feedback inhibition of endo- and exo- β-1,4-glucanases by cellobiose. The classical action of cellulase is envisioned as an initial attack by endoglucanases, followed by the combined action of cellobiohydrolases and endoglucanases, with final hydroysis of the small oligosaccharides to glucose by cellobiase (Goyal *et al*., 1991; Robson and Chambliss, 1989).

Although cellulose is chemically homogenous, it is structurally diverse and comprises amorphous regions where the cellulose chains are not closely linked, and crystalline areas where inter- and intra-chain hydrogen bonding results in an ordered array of cellulose microfibrils. It is the latter form of the polysaccharide which is particularly recalcitrant to enzyme attack (Gilbert and Hazlewood, 1993). It was well known that cellulase systems from some biological sources are able to hydrolyze both amorphous and crystalline cellulose (“complete”, “full value”, or “true” cellulases), whereas other cellulase systems are active only toward amorphous cellulose (“low value”). The latter are practically inactive toward crystalline cellulose. Full value cellulase contain endo-β-1,4-glucanases, cellobiohydrolases, and β-glucosidases .

Table 1.3: Celluases and their mode of actions.

Cellulase type	E.C. no	Synonym	Mode of action
Endo-(1,4)-β-D-glucanase	3.2.1.4	Endoglucanase or endocellulase	--G—G—G—G-- ▲ ▲ cleaves linkages at random
Exo-(1,4)-β-D-glucanase	3.2.1.91	Cellobiohydrolase or exocellulase	G—G—G—G—G—G-- ▲ releases cellobiose either from reducing or nonreducing end
β -glucosidase	3.2.1.21	Cellobiase	G—G G—G—G—G ▲ ▲ releases glucose from cellobiose and short chain cellooligosaccharides

1.3.2.1. Panorama of Microbial Cellulases

Microorganisms that degrade cellulose are both abundant and ubiquitous in nature. They include fungi, bacteria and actinomycetes, aerobes and anaerobes, mesophiles and thermophiles.

Fungal cellulases are produced in large amounts, which include all components of a multi-enzyme system with different specificities and mode of action, i.e. endoglucanases, cellobiohydrolases and β-glucosidase, acting in synergism for complete hydrolysis of cellulose (Mawadza *et al.*,2000). Cellulases from *Trichoderma* reseii (Ogava *et al.*, 1991), *Aspergillus* sp. (Lusta *et al.*, 1999), *Schizophillum commune* (Willick and Seligy, 1985), *Fusarium lini*, *Penicillium funiculosum* (Fogarty and Kelly, 1990) are among the better characterized "complete" system fungal cellulases. Today a large number of cellulases are marketed by different enzyme companies from *Trichoderma* spp., *Aspergillus* spp., and *Humicola insolens* (Godfrey and West,1996)

Cellulolytic enzymes have been reported in several bacterial genera, namely *Clostridium* (*Clostridium thermocellum* is the best characterized bacterial cellulase

system) (Ng and Zeikus, 1981), *Cellulomonas* (Stoppok *et al*., 1982), *Ruminococcus*, *Acetovibrio*, *Thermomonospora*, *Erwinia* and *Bacillus* (Robson and Chambliss, 1989). Bacterial cellulases have in many cases been reported to have much higher specific activities than fungal cellulases. However, bacteria generally produce extracellular cellulases in much lower quantities than fungi and most of these organisms cannot significantly degrade crystalline cellulose. These bacteria may produce only endoglucanases rather than a cellulase complex. Moreover, several cellulolytic bacteria secrete large amount of cellulases as tightly associated clusters of multienzyme complexes called cellulosomes which are difficult to distrupt without loss of activity as well as of individual components.

Among bacteria, *Bacillus* species produce a number of extracellular polysaccharide hydrolysing enzymes. A variety of *Bacillus* species secrete cellulases, including strains of *B.subtilis* (Park *et al.,* 1991), *B.pumilus* (Christakopoulos *et al*., 1999), *B.licheniformis* (Dhillon *et al*., 1985), *B.polymyxa*, *B.cereus* (Robson and Chambliss,1989), *Bacillus* sp.KSM-330 (Ozaki and Ito, 1991) and KSM-635 (Ito *et al*., 1989). A fairly common observation has been that bacilli lack the complete cellulase system to degrade crystalline cellulose.

1.3.2.2. Applications of Cellulases in Biotechnology

Active research on cellulases began in the early 1950s owing to their enormous potential to convert cellulose, the most abundant and renewable source of energy on earth, to glucose and soluble sugars. Extensive basic and applied research during the 1970s and 1980s demonstrated that the enzyme-induced bio-conversion of cellulose to soluble sugar was rather difficult and uneconomical. Nevertheless, continued research on cellulases revealed their biotechnological potential in various industries, including food, brewery and wine, animal feed, textile and laundry, agriculture as well as in research and development.

One of the major industrial application of cellulases is in textile industry for bio-polishing of fabrics and producing stonewashed look of denims. During bio-polishing process, the cellulases act on small fibre ends that protrude from the fabric surface, where the mechanical action removes these fibres and polishes the fabric. Bio-

polishing process requires acid and endoglucanase rich cellulase. The indigo blue used in denim production is attached throuhout the cloth via binding to the free ends of cotton fibres. To reduce the intensity of the dye, the cutting of fibre ends will release the dyed portion. As an alternative to the stone- washing, cellulases act on the cotton fabric and break off the small fibre ends on the yarn surface, thereby loosining the indigo, which is easily removed by mechanical abrasion in the wash cycle. Using the cellulase in biostoning has the advantages of reduced cost and time, increased product quality and ease of operation. For this application, preferably neutral and endoglucanase rich cellulase is used (Bhat, 2000; Godfrey and West, 1996).

The detergent industry is the other major user of enzymes. The fibrils and fibres sticking out from the textile surface scatter light and form pills giving the fabric a greyish and dull appearance.The cellulase preperations capable of modifying the structure of cellulose fibrils are added to laundry detergents to improve the color brightness, hand feel and dirt removal from cotton and cotton blend garments (Bhat, 2000; Godfrey and West, 1996; Whitaker, 2002).

Cellulases have potential applications in food industry. A combination of cellulases, pectinases and hemicellulases (macerating enzymes) are used in the extraction and clarification of fruit and vegetable juices. The use of macerating enzymes in olive oil production process increase the oil yield, antioxidant and vitamin E content of the oil (Bhat, 2000). In brewery and wine industries, cellulases are used to hydrolyze β-1,3 and β-1,4 glucan which is present in low grade barley and help in the filtration of beer and to increase the aroma in wines. The recombinant yeasts have already been used in brewery industry (Bhat and Bhat, 1997).

Cellulases are also used in animal feed industry. In many cereals, part of their energy content is locked up in the form of nonstarch polysaccharides, undigestible for several animals resulting in poor food conversion rate and slow weight gain. Therefore, selected enzymes, such as cellulases and hemicellulases, can be added to break down the cell walls leading to increased metabolizable energy . Currently, there is a great deal of interest in using enzyme preparations containing high levels of cellulase and hemicellulase activities for improving the feed utilization, milk yield and body weight gain by ruminants (Bhat, 2000; Whitaker, 2002).

Moreover, cellulases can be used in research and development . As a research tool, cellulases with hemicellulases and pectinases are used in the production of plant or fungal protoplasts by solubilizing the cell wall materials. Since cellulases and related enzymes from certain fungi are capable of degrading the cell wall of plant pathogens, they have potential to be used in bio-control of plant pathogens and diseases. Cellulose-binding domains of fungal cellulases, which functions normally when fused to heterologous proteins, have been succesfully used either as an affinity tag for the purification of proteins or immobilization of fusion proteins (Mawadza *et al*.,2000; Bhat, 2000).

1.3.3. Amylases

Starch is a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. Starch-containing crops form an important constituent of the human diet and a large proportion of the food consumed by the world's population originates from them. Starch is contained in the roots, stems, tubers, fruit and seeds of a wide variety of plants throughout nature. Plants synthesize starch as a result of photosynthesis, the process during which energy from the sunlight is converted into chemical energy.

Chemically, starch is a polymer of glucose. The glucose units are linked together by α-1,4 and α-1,6 linkages. Structurally, starch is not a homogenous polymer as it is composed of two high molecular weight homopolysaccharides known as amylose and amylopectin, with the relative proportion varying from one grain source to another as shown in Figure 1.7. Amylose, a linear polymer consisting of up to 6000 glucose units with α-1,4 glycosidic bonds, generally comprises 17-30 % of the total starch composition. Amylopectin consists of short α-1,4 linked linear chains of 10–60 glucose units and α-1,6 linked side chains with 15–45 glucose units. The average number of branching points in amylopectin is 5%, but varies with the botanical origin. The complete amylopectin molecule contains on average about 2 million glucose units, thereby being one of the largest molecules in nature (Maarel *et al*, 2002; Godfrey and West, 1996).

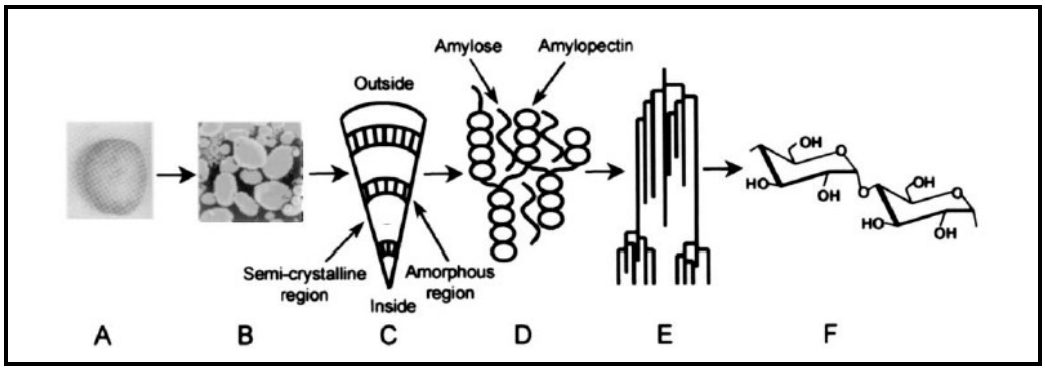


Figure 1.7: Zoom in of how a potato starch tuber is built-up. A, tuber; B, electron microscopic image of starch granules; C, slice of a starch granule showing the growth rings consisting of semi-crystalline and amorphous regions; D, detail of the semi-crystalline region; E, organization of the amylopectin molecule into the tree-like structure; F, two glucose molecules with an α,1-4 glycosidic bond.

Starch granules are organized into amorphous and crystalline regions. In tuber and root starches, the crystalline regions are solely composed of amylopectin, while the amylose is present in the amorphous regions. In cereal starches, the amylopectin is also the most important component of the crystalline regions. While amylopectin is soluble in water, amylose and the starch granule itself are insoluble in cold water. Hence, starch is often resistant to chemicals and enzymes (Hamilton *et al,*1999).

Utilization of starch requires degradation to maltooligodextrins and glucose which is done in nature by a palette of amylolytic enzymes of varying specificity (Sogaard *et al,* 1993). There are basically four groups of starch-converting enzymes: (i) endoamylases; (ii) exoamylases; (iii) debranching enzymes; and (iv) transferases (Figure 1.8).

Endoamylases are able to cleave α-1,4 glycosidic bonds present in the inner part (endo-) of the amylose or amylopectin chain. α-Amylase (E.C. 3.2.1.1) is a well-known endoamylase (Maarel *et al*, 2002). α-Amylase can be found in microorganisms, plants and higher organisms where they play a dominant role in carbohydrate metabolism (Kandra, 2003). The end products of α-amylase action are oligosaccharides with varying length with an α-configuration and α-limit dextrins, which constitute branched oligosaccharides.

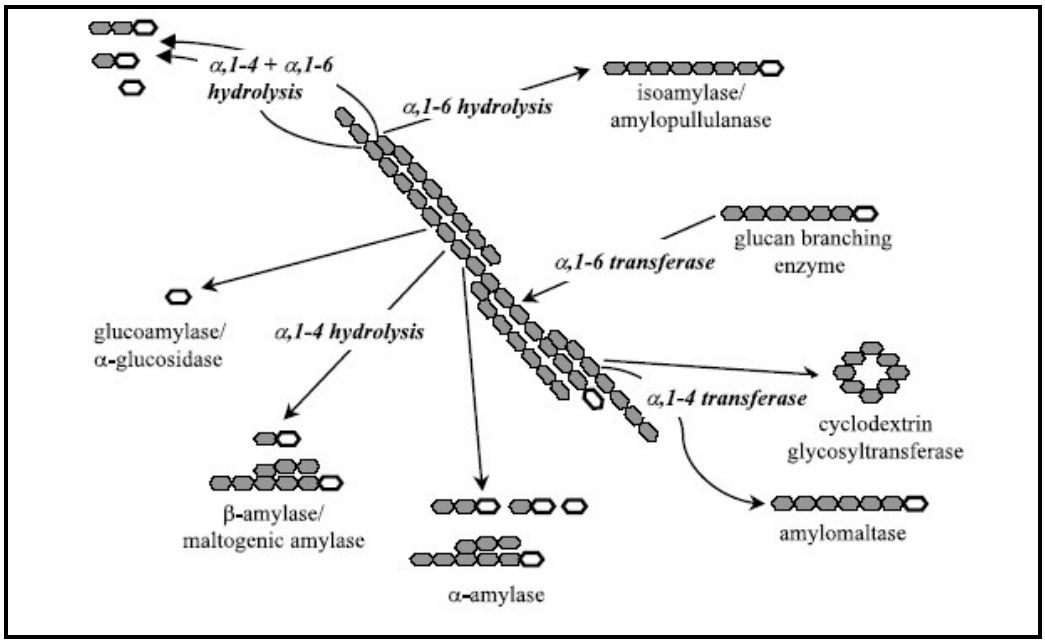


Figure 1.8: Different enzymes involved in the degradation of starch. The open ring structure symbolizes the reducing end of a polyglucose molecule.

Enzymes belonging to the second group, the **exoamylases**, either exclusively cleave α-1,4 glycosidic bonds such as β-amylase (E.C. 3.2.1.2) or cleave both α-1,4 and α-1,6 glycosidic bonds like amyloglucosidase or glucoamylase (E.C. 3.2.1.3) and α-glucosidase (E.C. 3.2.1.20). The third group of starch-converting enzymes are the **debranching enzymes** that exclusively hydrolyze α-1,6 glycosidic bonds: isoamylase (E.C.3.2.1.68) and pullanase type I (E.C. 3.2.1.41). The fourth group of starch-converting enzymes are **transferases** that cleave an α-1,4 glycosidic bond of the donor molecule and transfer part of the donor to a glycosidic acceptor with the formation of a new glycosidic bond. Enzymes such as amylomaltase (E.C. 2.4.1.25) and cyclodextrin glycosyltransferase (E.C.2.4.1.19) form a new α-1,4 glycosidic bond while branching enzyme (E.C. 2.4.1.18) forms a new α-1,6 glycosidic bond (Maarel *et al*, 2002).

1.3.3.1. Panorama of Microbial α-Amylases

Amylases are of ubiquitous occurrence, produced by plants, animals and microorganisms. However, microbial sources are the most preferred one for large

scale production. The major advantage of using microorganisms for the production of amylases is the economical bulk production capacity and microbes are easy to manipulate to obtain enzymes of desired characteristics. A wide variety of microorganisms produce extracellular amylases, having different specificities, properties and action patterns depending on their source. These amylases are used extensively in various industries and the suitability of any one amylase to a particular process will depend on its specific characteristics such as the selection of thermostable amylases for use in the liquefaction of starch (Hamilton *et al* , 1999).

α-Amylases from fungal origin have some advantages. Since the enzymes are excreted into the medium, downstream process cost is economical. Moreover, fungi produce enzymes in large quantities. However, fungal α-amylases are generally acidic to neutral in nature and less thermotolerant when compared to bacterial α-amylases. On the other hand, bacterial α-amylases range from acidic to alkaline and from moderate to extremely thermostable depending on the type of organism. The genus *Bacillus* produces a large variety of extracellular enzymes of which amylases and proteases are of significant industrial importance. The microbial α-amylases for industrial purposes are derived mainly from *Bacillus licheniformis*, *Bacillus amyloliquefaciens* and *Aspergillus oryzae* (Konsula *et al*, 2003). Table 1.4. illustrates characteristics of alpha amylases from different microbial sources in literature.

1.3.3.2. Applications of Amylases in Biotechnology

α-Amylases and related amylolytic enzymes which constitute a class of industrial enzymes having approximately 25-33 % of the enzyme market, in second place after proteases, are among the most important enzymes and of great significance in the present day biotechnology. Although they can be derived from several sources, such as plants, animals and microorganisms; enzymes from microbial sources generally meet industrial demands. Several different amylase preparations are available with various enzyme manufacturers for specific use in varied industries.

A large-scale starch processing industry has emerged in the last century. Three stages can be identified in starch modification. Firstly, amylases liberate maltodextrin by the liquefaction process. Specific maltooligosaccharides have a

Table 1.4: Characteristics of alpha amylases from different microbial sources.

Enzyme source	pH optimum	pH stability	Temp. Optimum (℃)	Temp. stability	Reference
Aspergillus oryzae	5.4	5.0-9.0	50	30min stable at 50℃	Gupta *et al*,2003
Aspergillus niger	4.0-5.0	2.2-7.0	60	15 min stable at 60℃	Gupta *et al*,2003
Aspergillus carbonarius	6.0-7.0	stable for 2h at 3.0-9.0	40	>85% stable at 30-80℃, 65% stable at 90℃	Okolo *et al*, 2000
Aspergillus tamarii	4.5 6.5	stable for 2h at 3.0-8.0	50-55	stable up to 65℃, 30min halflife at 70℃	Moreira *et al*, 2004
Trichoderma viride	5.0-5.5	stable at 4.0-7.0	—	10 min stable at 60℃	Gupta *et al*,2003
Thermomyces lanuginosus	4.6-6.6	stable at 4.0-7.0	70	—	Nguyen *et al*, 2002
Cryptococcus flavus	5.5	—	50	90% stable after 1h at 50℃	Wanderley *et al*, 2004
Thermococcus profundus	5.5	—	80	—	Kwak *et al*, 1998
Lactobacillus plantarum	5.5	stable at 3.0-8.0	65	—	Gupta *et al*,2003
Lactobacillus manihotivorans	5.5	—	55	stable at 40℃, 70% stable at 50℃	Aguilar *et al*, 2000
Rhodothermus marinus	6.5	stable at 5.0-9.0	85	35% stable at 95℃	Gomes *et al*, 2003
Streptococcus bovis	5.0-6.0	stable at 5.5-8.8	—	1 hr stable at 50℃	Gupta *et al*,2003
Bacillus licheniformis	9.5	—	91	1 hr stable at 91℃, 40-50% stability at 110℃	Medda and Chandra, 1980

Table 1.4 (continued)

B.licheniformis CUMC305	9.0	pH 7(18 hr) pH10(95%)	90	91% stability after 3 hr at 100℃	Krishnan and Chandra, 1983
B.licheniformis NCIB 6346	7.0	24 h stable at 7.0-9.0	90	15 min stable at 50℃, 60% stable at 90℃	Morgan and Priest, 1981
B.licheniformis	9.0	—	76	—	Saito, 1973
B.licheniformis NRLL B14368	5.0-7.0	—	76	—	Bose and Das, 1996
Bacillus licheniformis	6.0-6.5	—	90	—	Ivanova *et al*, 1993
B. stearothermophilus	6.9	24 h stable at 7.5-8.5	80	90% stable after 30 min at 50℃	Srivastava, 1987
B. amyloliquefaciens	6.8	—	36	—	Castro *et al,*1993
B. subtilis	6.5	stable ≤7.0	50	stable ≤50℃	Gupta *et al*,2003
B.acidocaldirus	3.5	—	75	—	Buonocore *et al*, 1976
B. globisporus	4.5	95% stable at 5.0-6.5 90% stable at 8.0	60	10 min stable at 55℃, 75% stable at 75℃	Bandyopadhyay *et al*, 1993
Bacillus sp. IMD434	6.0	4.0-9.0	65	1 h stable at 40℃	Hamilton *et al*, 1999
Bacillus sp. IMD435	6.0	—	65	—	Hamilton *et al*, 1999
Bacillus sp.WN11	5.5	80% stable at 5.0-8.0	75-80	4 h stable at 80℃	Mamo *et al*, 1999
Bacillus sp. ANT-6	10.5	83% stable at 9.0-13.0	80	85% stable at 60-100℃	Burhan *et al*, 2003

range of potential uses in the food, pharmaceutical and fine chemical industries because of their unique nature and special properties. They are all highly soluble and produce clear viscous solutions which are palatable and superior nutrient foods for infants and aged. Maltopentaose has been used as nutrient food for patients having renal failure and those in a condition of calorie deprivation. Maltotetraose is also being examined as a food additive to improve texture and moisture retention in food. Such maltodextrins are not very sweet as they contain dextrins and oligosaccharides.The dextrins and oligosaccharides are further hydrolysed by enzymes such as pullulanase and glucoamylase in a process known as saccharification. Complete saccharification converts all the limit dextrans to glucose, maltose and isomaltose. The resulting syrups are moderately sweet and are frequently modified further. Treatment of glucose/maltose syrups with glucose isomerase converts a large proportion of the glucose to fructose which is sweeter than glucose. This isomerisation process is usually performed with immobilised glucose isomerase and results in syrups with approximately 50 % fructose and 50 % glucose. Such products are known as high fructose syrups and are frequently used as sugar replacements in the manufacture of foods and beverages. This processes require the use of a highly thermostable α-amylase for starch liquefaction. It is desirable that a-amylases should be active at the high temperatures of gelatinization (100-110 ℃) and liquefaction (80- 90 ℃) to economize processes (Kandra, 2003; Burhan *et al*, 2003; Gupta *et al*, 2003; Maarel *et al*, 2002).

The baking industry has made use of these enzymes for hundreds of years to manufacture a wide variety of high quality products. For decades, enzymes such as malt and microbial α-amylases have been widely used in the baking industry. These enzymes were used in bread and rolls to give these products a higher volume, better colour, increased shelf-life and a softer crumb (Gupta *et al*, 2003; Maarel *et al*, 2002).

Modern production processes for textiles introduce a considerable strain on the warp during weaving. The yarn must, therefore, be prevented from breaking. For this purpose a removable protective layer is applied to the threads. The materials that are used for this size layer are quite different. Starch is a very attractive size, because it is cheap, easily available in most regions of the world, and it can be

removed quite easily. Good desizing of starch sized textiles is achieved by the application of α-amylases, which selectively remove the size and do not attack the fibres. It also randomly cleaves the starch into dextrins that are water soluble and can be removed by washing (Gupta *et al*, 2003).

A growing new area of application of α-amylases is in the fields of laundry and dish-washing detergents. A modern trend among consumers is to use colder temperatures for doing the laundry or dishwashing. At these lower temperatures, the removal of starch from cloth and porcelain becomes more problematic. Detergents with α-amylases optimally working at moderate temperatures and alkaline pH can help solve this problem. α-Amylases have been used in powder laundry detergents since 1975. Nowadays, 90% of all liquid detergents contain α -amylase and the demand for α -amylases for automatic dishwashing detergents is growing (Gupta *et al*, 2003; Maarel *et al*, 2002).

The spectrum of amylase application has widened in many other fields, such as clinical, medicinal and analytical chemistry. In humans, α-amylase is one of the major secretory products of the pancreas and salivary glands, playing a role in digestion of starch and glycogen. Human amylases of both salivary (HSA) and pancreatic origin (HPA) have been extensively studied from the viewpoint of clinical chemistry, because they are important as indicators of pancreatic and salivary glands disorders (e.g. acute pancreatitis, parotitis) (Kandra, 2003).

1.3.4. Proteases

Proteases are the single class of enzymes which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes catalyze the cleavage of peptide bonds in other proteins. Proteases are degradative enzymes which catalyze the total hydrolysis of proteins (Rao *et al*, 1998).

Proteases are essential constituents of all forms of life on earth, including prokaryotes, fungi, plants and animals. Microorganisms elaborate a large array of

proteases, which are intracellular and/or extracellular. Intracellular proteases are important for various cellular and metabolic processes, such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products. At the same time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes (Gupta *et al*, 2002).

Currently, proteases are classified on the basis of three major criteria: (i) type of rection catalyzed, (ii) chemical nature of catalytic site, and (iii) evolutionary relationship with reference to structure. Proteases are grossly subdivided into two major groups, exopeptidases and endopeptidases, depending on their site of action as shown in Table 1.5. 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. Based on the functional group present at the active site, proteases are further classified into four prominent groups: serine proteases, aspartic proteases, cysteine proteases and metalloproteases.

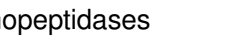
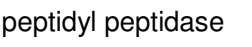
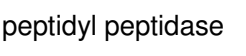
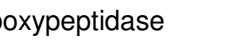
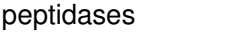
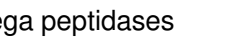
The **exopeptidases** act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively. Aminopeptidases act at a free N terminus of the plypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide. Carboxypeptidases act at a C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups serine carboxypeptidases, metallocarboxypeptidases and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of enzymes.

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N or C termini. The endopeptidases are divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases, and (iv) metalloproteases (Rao *et al*, 1998).

Serine proteases (E.C. 3.4.21) are characterized by the presence of a serine group in their active site. They are generally active at neutral and alkaline pH, with optima at pH 7–11. They are numerous and widespread among viruses, bacteria, and eukaryotes, suggesting that they are vital to the organisms. Alkaline serine proteases are used as detergent additives and, thus, represent the largest volume of microbial enzymes in the industrial sector. This class comprises two distint families. The chymotrypsin family which includes the mammalian enzymes such as chymotrypsin and trypsin and the subtilisin family which include the bacterial enzymes such as subtilisin. Chymotrypsin is found in animal pancreatic extract. It is specific for the hydrolysis of peptide bonds in which the carboxyl groups are by one of the three aromatic amino acids phenylalanine, tyrosine, and tryptophan. Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. It hydrolyzes the peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues. Subtilisins of *Bacillus* origin represents the second largest family of serine proteases. They are generally secreted extracellularly for the purpose of scavenging nutrients. Two different types of alkaline proteases, subtilisin Carlsberg (*Bacillus licheniformis*) and subtilisin Novo (*Bacillus amyloliquefaciens*) have been identified. Subtilisin Carlsberg is widely used in detergents. Its annual production amounts to about 500 tons of pure enzyme proteins (Rao *et al*, 1998; Gupta *et al*, 2002; Fogarty and Kelly, 1990).

Aspartic acid proteases (E.C.3.4.23), commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Most aspartic proteases show maximal activity at low pH (pH 3 to 4). Acidic proteases have been grouped into three families, namely pepsin, retropepsin, and enzymes from pararetroviruses. Most of aspartic proteinases belong to the pepsin family. The pepsin family includes digestive enzymes such as pepsin and chymosin as well as lysosomal cathepsins D and processing enzymes such as renin. Pepsin is an acidic protease that is found in the stomachs of almost all vertebrates. Rennin (chymosin) is a pepsin-like protease that is produced as an inactive precursor, prorenin, in the stomachs of all nursing mammals. It is converted to active renin by the action of pepsin. It is used extensively in the dairy industry to produce a stable curd with good flavor. Recombinant calf chymosin produced in genetically modified *E. coli , A. awamori, and Kluyveromyces lactis* are approved by FDA for use in cheese making (Rao *et al*, 1998; Gupta *et al*, 2002; Fogarty and Kelly, 1990).

Table 1.5: Proteases an their mode of action.

Protease	*Mode of action*	*E.C. no*
Exopeptidases		3.4.11
Aminopeptidases		3.4.14
Dipeptidyl peptidase		3.4.14
Tripeptidyl peptidase		3.4.16-18
Carboxypeptidase		3.4.16
Serine type protease		3.4.17
Metalloprotease		3.4.18
Cysteine type protease		3.4.15
Peptidyl dipeptidase		3.4.13
Dipeptidases		3.4.19
Omega peptidases		3.4.19
Endopeptidases		3.4.21-34
Serine protease		3.4.21
Cysteine protease		3.4.22
Aspartic protease		3.4.23
Metalloprotease		3.4.24

Cysteine proteases (E.C.3.4.22) occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. Cysteine proteases have neutral pH optima. This family includes the plant proteases such as papain from the latex of unripe fruit of the Carica papaya tree, ficin from the fig tree (*Ficus carica*), actinidin from kiwi fruit (*Actinidia chinensis*), and bromelain from the pineapple (*Ananus comosus*), several mammalian cathepsins, the cytosolic calpains as well as several parasitic proteases. Papain, the archetype and the best studied member of the family, is a traditional plant protease and has a long history of use. The enzyme is active between pH 5 and 9 and is stable up to 80 or 90 ℃ in the presence of substrates. It is extensively used in industry for the preparation of highly soluble and flavored protein hydrolysates. The lysosomal cathepsins are important because of

their physiological roles in turnover of proteins in the body and their possible role in diseases, such as muscular dystrophy, osteoporosis, inflammatory heart diseases, and tumor invasion (Rao *et al*, 1998; Gupta *et al*, 2002; Fogarty and Kelly, 1990).

Metalloproteases (E.C. 3.4.24) are most diverse of the catalytic types of proteases. They differ widely in their sequences and their structures but the great majority of the 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. Thermolysin is the best characterized member of metalloproteases. It is a neutral protease which is very stable, with a half-life of 1 h at 80°C. Collagenase and elastase are other important member of the neutral metalloprotease family (Rao *et al*, 1998; Gupta *et al*, 2002; Fogarty and Kelly, 1990).

1.3.4.1. Panorama of Microbial Proteases

Protease production is an inherent capacity of all organisms including bacteria, moulds, yeasts and also mammalian tissues. Microorganisms account for a two-third share of commercial protease production in the world (Gupta *et al*, 2002).

Fungi as protease producers have many advantages, considering that the produced enzymes are normally extracellular, making easier its recuperation from the fermentation broth. Besides, the use of fungi as enzyme producer is safer than the use of bacteria, since they are normally recognised as GRAS (generally regarded as safe) (Germano *et al*, 2003).However, among the various proteases, bacterial proteases are the most significant, compared with animal and fungal proteases (Banerjee *et al*, 1999). Among bacteria, *Bacillus sp*. are specific producers of extracellular proteases. Currently, a large proportion of commercially available alkaline proteases are derived from Bacillus strains. Table 1.6 illustrates the characteristics of proteases from different microbial sources in literature.

Table 1.6: Characteristics of proteases from different microbial sources.

Enzyme source	Optimum pH	pH stability	Optimum temp. (℃)	Temp. stability	Reference
Aspergillus parasiticus	8.0	45% stable at 12.0, 80% stable at 5.0	40	stable 1h at 40℃ lost activity>40℃	Tunga *et al*,2003
Aspergillus tamarii	6.0-10.0	stable 1 h at 5.0-9.5	45	75,20min halflife at 50,55℃, stable 10h at 40-45℃	Boer and Peralta, 2000
Aspergillus flavus	7.5 9.5	stable at 2.0-7.0 lost activity>7.0	32	—	Malathi and Chakrabort y, 1991
Penicillum sp.	6.5	stable at 6.0-8.0 for 24h	45	60% at 45℃, 90% at 35℃, lost activity>50℃	Germano *et al*, 2003
Kluyveromyces marxianus	8.5	stable at 6.0-9.0	45	15% stable at 70℃	Zavala *et al*, 2004
Nocardiopsis sp.	8.0	55% stable at 12.5, 62% stable at 5.0	50	60% stable at 50℃ for 2h, lost activiy in 1h ≥60℃	Moreira *et al*,2003
Thermoactinomyces vulgaris A60	9.0	most stable at 7.0-9.0	60-70	40,60% stability at 80,90℃	Desai and Dhala, 1969
Thermomonospora fusca YX	9.0	—	80	stable up to 75℃, 88,54,0% stability at 80,85,90℃	Gusek and Kinsella, 1987
Thermomyces lanuginosus	5.0 9.0	—	70	160,60min halflife at 60,70℃, lost activity in 5min at 80℃	Li *et al*, 1997
Streptomyces sp.	6.0	50% stable at 5.0-8.0	55-70	lost activity>70℃	Azeredo *et al*,2004
Streptomyces tandae	6.0	—	70	stable at 50℃ 70% stable at 60℃ for 30min	Seong *et al*, 2004
Pseudoaltermonas sp. SM9913(MCP-01-02)	6.5-7.0 8.0	—	30-35 50-55	stable 20min at 30℃, stable<50℃,	Chen*et al*, 2003
Bacillus sphaericus	10.5	>70% stability at 8.5-11.5	50-55	3,42 h halflife at 50℃, 27min halflife at 60℃	Singh *et al*, 1999
B. mojavensis	10.5	>70% stability at 7.5-11.5	60	86% stability for 1h at 60℃, 7m halflife at 70℃	Beg and Gupta, 2003

Table 1.6 (continued)

B. polymyxia B-17	7.5	—	50	35% stable for 10m at 70℃, lost activity in 10min at 85℃	Matta and Punj, 1998
Bacillus cereus	7.0	—	40	55% stable at 40℃, lost activity at 70℃	Sierecka, 1998
B. brevis	10.5	77% stable at 7.5, 92% stable at 11.0 for 1h	60	7h halflife at 60℃	Banerjee *et al*, 1999
B. stearothermophilus	7.5	—	75	—	Kim *et al*, 2002
B. pumilus	11.0	stable at 8.0-11.0	40	87-65% stable at 50-60℃ for 30 min	Feng *et al*, 2001
B. licheniformis	7.0	—	47	—	Manczinger *et al*, 2003
B. subtilis	7.0	stable at 6.0-8.0	50-55	97% stable at 30℃	Varela *et al*, 1997
Bacillus sp. SSR1	10.0	stable at 7.0-11.0, >75% stable for 30min, 11.0	40	6-1h halflife at 50-60℃	Singh *et al*, 2001
Alkalophilic Bacillus sp.	11.0 12.0	>50% stability at 10.0-13.0	50 55	—	Kumar *et al*, 1999
Bacillus sp. PS719	9.0	stable 3h at 9.0, 70-50% stable at 7.0-11.0	75	40% stable for 15min at 90℃	Towatana *et al*, 1999
Bacillus sp. P-2	9.6	—	90	95% stable for 1h at 90℃, 37% stable for 1h at 99℃	Kaur *et al*, 2001
Bacillus sp. JB-99	11,0	—	75	stable at 40-70℃	Johnvesly and Naik, 2001
Bacillus sp. GX6638	10.3	88% stable for 24h at 12.0	65	8min halflife at 50℃	Durham *et al*, 1987
Bacillus sp.	12-13	60% stable for1h at 10.0	85	50% stable at 80℃, lost activity at 90℃	Fujiwara and Masui, 1993

1.3.4.2. Applications of Proteases in Biotechnology

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Gupta *et al*, 2002).

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 history of detergent enzymes dates back to 1914, when two German scientists used pancreatic proteases and sodium carbonate in washing detergents. The product was named Burnus. The first detergent containing the bacterial enzyme was introduced into the market in 1956 under the trade name Bio-40. Over the past 30 years, the importance of proteases in detergents has changed from being the minor additives to being key ingredients. Today, detergent enzymes account for 89% of the total protease sales in the world; and a significant share of the market is captured by subtilisins and/or alkaline proteases from many *Bacillus* species. 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. An ideal detergent enzyme should be stable at high pH and temperatures up to 40°C, withstand oxidizing and chelating agents, and be effective at low enzyme levels in detergent solutions. Moreover, it should also have broad substrate specificity. Various commercial detergent proteases such as Subtilisin, Carlsberg, Subtilisin BPN', Alcalase, Esperase and Savinase are available in the market (Beg and Gupta, 2003; Anwar and Saleemuddin, 1998; Rao *et al*, 1998; Gupta *et al*, 2002; Kumar and Takagi, 1999).

Another industrial process which has received attention is the enzyme assisted de-hairing of animal hides and skin in the leather industry.The major building blocks of skin and hair are proteinaccous. The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental

pollution and safety hazards. Thus, for environmental reasons, the biotreament of leather using an enzymatic approach is preferable as it offers several advantages, like easy control, speed and waste reduction, thus being ecofriendly. Alkaline proteases with elastolytic and keratinolytic activity can be used in leather-processing industries. Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced. Bating is traditionally an enzymatic process involving pancreatic proteases. However, recently, the use of microbial alkaline proteases has become popular. Alkaline proteases speed up the process of dehairing, because the alkaline conditions enable the swelling of hair roots; and the subsequent attack of protease on the hair follicle protein allows easy removal of the hair (Anwar and Saleemuddin, 1998; Rao *et al*, 1998; Gupta *et al*, 2002; Kumar and Takagi, 1999).

The use of proteases in the food industry dates back to antiquity. They have been routinely used for various purposes such as dairy, baking, preparation of soya hydrolysates, and meat tenderization. The major application of proteases in the dairy industry is in the manufacture of cheese. In cheesemaking, the primary function of proteases is to hydrolyze the spesific peptide bond to generate *para*-κ-casein and macropeptides. Chymosin is preferred due to its high specificity for casein, which is responsible for its excellent performance in cheesemaking. The proteases produced by GRAS-cleared microbes such as *Mucor michei*, *Bacillus subtilis*, and *Endothia parasitica* are gradually replacing chymosin in cheesemaking. In 1988, chymosin produced through recombinant DNA technology was first introduced to dairy industry. In baking industry proteases are used for the degradation of protein gluten in wheat flour. The addition of proteases reduces the mixing time and results in increased loaf volumes. Bacterial proteases are used to improve the extensibility and strenght of the dough. Proteases have also 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. Moreover, alkaline proteases have been used in the preparation of protein hyrolysates of high nutritional value. The protein hydrolysates play an important role in blood pressure regulation and are used in infant food formulations, specific therapeutic dietary products and the fortification of fruit juices and soft drinks.

Further, proteases play a prominent role in meat tenderization, especially of beef. An alkaline elastase and thermophilic alkaline protease have proved to be successful and promising meat tenderizing enzymes, as they possess the ability to hydrolyze connective tissue proteins as well as muscle fibre proteins (Rao *et al*, 1998; Gupta *et al*, 2002; Kumar and Takagi, 1999).

The proteases have frequently been used for peptide synthesis. Enzymatic peptide synthesis offers several advantages over chemical methods, e.g. reactions can be performed stereospecifically and reactants do not require side-chain protection, increased solubility of non-polar substrates, or shifting thermodynamic equilibria to favor synthesis over hydrolysis. There is less need for expensive protecting-groups, organic solvents, or hazardous chemicals, resulting in production costs competitive with those of chemical methods (Gupta *et al*, 2002).

Alkaline proteases play a crucial role in the bioprocessing of used X-ray or photographic films for silver recovery. These waste films contain 1.5–2.0% silver by weight in their gelatin layer, which can be used as a good source of silver for a variety of purposes. Conventionally, this silver is recovered by burning the films, which causes undesirable environmental pollution. Furthermore, base film made of polyester cannot be recovered using this method. Since the silver is bound to gelatin, it is possible to extract silver from the protein layer by proteolytic treatments. Enzymatic hydrolysis of gelatin not only helps in extracting silver, but also the polyester film base can be recycled (Gupta *et al*, 2002).

Alkaline proteases are also used for developing products of medical importance. 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 *Conidioolus coronatus* was found to be able to replace trypsin in animal cell cultures (Rao *et al*, 1998).

1.4. Scope of the Study

Previously, 11 bacterial isolates have been isolated and characterized by Firdevs Yalçın (2000) during her thesis work. Their identifications were made by using classical microbial identification tests and API 50 CHB tests.

The aim of this study is the definite identification of the 11 Bacillus isolates from the Salt Lake,Turkey, and determination of the variety and amount of extracellular enzymes of industrial interest produced by these strains.

To achieve these goals, firstly, microoorganisms are identified according to their short chain fatty acid composition by using Sherlock Microbial Identification System, which is based on gas chromatographic analysis and comparisons of the peaks with the database. Since these isolates are from an extremophilic environment, Salt Lake, their industrially important extracellular enzymes xylanases, cellulases, α-amylases and proteases are characterized in terms of enzyme activities, optimum temperatures and pH as a second step. Finally, the stabilities of these enzymes at different temperatures and pH values are determined.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

The chemicals used and their suppliers are listed in Appendix A.

2.2. Media, Reagents and Buffers

Composition and preparation of the media, reagents and buffers used in this study are presented in Appendix B, C and D respectively

2.3. Microorganisms

A total of 11 bacterial strains were from our laboratory culture collection. Nine HTG strains have been previously isolated from Salt Lake, Turkey and 2 TTG were kindly supplied by Prof. Dr. Sedat Dönmez from Department of Food Engineering, University of Ankara. Microorganisms were activated and maintained on Nutrient Agar and stored in 20 % glycerol and in microbanks at -80 ℃.

2.4. Microbial Identification

Isolates were identified by using Sherlock Microbial Identification System (MIS), MIDI in Atatürk University, Erzurum. This system identifies microorganisms based on fatty acid methyl ester (FAME) patterns of microorganisms. FAME profiles are then compared to a standard database and most likely hits are then presented to the user. The database includes aerobic bacteria, anaerobic bacteria and yeasts.

2.5. Characterization of the Isolates in Terms of their Enzyme Production

2.5.1. Xylanase

2.5.1.1 Screening for Xylanase Production

Microorganisms were cultivated for 16 hours in Nutrient Broth at 37 ℃ and inoculated to Congo Red Xylan Agar plates (Williams, 1983) (Appendix B). Plates were incubated at 37 ℃ for 48 hours for the formation of clear zones around colonies.

2.5.1.2. Cultivation Conditions for Xylanase Production

Fifty ml of the xylan containing liquid media (Appendix B) in 100 ml flasks were used for cultivation and inoculated with 5 ml of inoculum culture grown in the same media for 16 hours and incubated at 37℃ . Cultivations were carried out at 45℃ and 150 rpm for 72 hours. Culture samples were collected at 6, 12, 24, 30, 36, 48, 54, 60 and 72nd hours. The samples were spun immediately after collection at 12000 rpm for 15 min and the culture supernatants were used for xylanase activity assays.

2.5.1.3. Xylanase Assay

Culture supernatants were assayed for reducing sugars by DNS stopping method (Miller, 1959; Bailey, 1992) at 50 ℃ with 1% (w/v) birchwood xylan as a substrate in 0.05 M sodium phosphate buffer, pH 7.0 . Crude enzyme samples were mixed with the substrate solution prewarmed to 50℃ in a 9:1 ratio and placed in a waterbath at 50℃. One ml samples were started to withdrawn at 15 second intervals during 2 minutes to measure initial reaction rates. After the reactions were stopped by the addition of 1.5 ml DNS reagent, tubes were incubated in a boiling waterbath for 10 minutes and cooled immediately under tap water. Absorbances were measured at 540 nm against a blank containing buffer instead of enzyme. Xylose was used as a standard (Appendix E). One international unit (IU) is defined as the amount of enzyme causing the release of 1µmole of xylose per minute at 50 ℃ and pH 7.0. All

experiments were made as triple and their mean values were used. The composition of the DNS reagent and the preparation of 1% xylan substrate are given in Appendix C.

2.5.1.4. Determination of Optimum Temperature for Xylanase

The same procedure of xylanase assay was used within a temperature range of 40-70 °C.

2.5.1.5. Determination of Optimum pH for Xylanase

The same procedure of xylanase assay was used in a pH range of 4.0-10.0 (Subramaniyan *et al*, 1997). Buffers used were 0.05 M sodium acetate buffer (pH 4.0 and 5.0), 0.05 M sodium phosphate buffer (pH 6.0, 7.0 and 8.0) and 0.05 M carbonate buffer (pH 9.0 and 10.0) prepared as shown in Appendix D.

2.5.1.6. Determination of Xylanase Stability

Stabilities were determined by diluting the crude enzyme with the same pH 7.0, 8.0, 9.0 and 10.0 buffer solutions (20% v/v) and then incubating at waterbath at 50, 60, 70, 80 and 90 °C for 1 hour. At the end of this period, the residual xylanase activities were measured .

2.5.2. Cellulase

2.5.2.1. Screening for Cellulase Production

Microorganisms were cultivated for 16 hours in Nutrient Broth at 37 °C and inoculated to carboxymethyl cellulose (CMC) and congo red containing plates (Appendix B). Plates were incubated at 37 °C for 48 hours for the formation of clear zones around colonies.

2.5.2.2. Cellulase Assay

Culture supernatants of the xylanase cultivation medium were also used for cellulase activity determination. Total cellulase activities were assayed by measuring the formation of reducing sugars by the Filter Paper assay method based on DNS method (Ghose, 1987). Whatman no.1 filter paper strips (1x6cm) were used as substrate and placed in test tubes. One ml 0.05 M sodium phosphate buffer (pH 7.0) and 0.5 ml crude enzyme were added to tubes and tubes were incubated in waterbath at 50 ℃ for exactly 1 hour. Reactions were stopped by the addition of 3.0 ml DNS reagent, tubes were incubated in boiling waterbath for 10 minutes and cooled under tap water. Tubes were diluted with the distilled water in a 1:5 ratio and absorbances of the resulting solutions were measured at 540 nm against a blank containing buffer instead of enzyme. Glucose was used as a standard (Appendix F). All experiments were made as triple and their mean values are used. One unit (U) is defined as the amount of enzyme causing the release of 1 mmole of glucose per minute at 50 ℃ and pH 7.0.

2.5.3. α-Amylase

2.5.3.1. Screening for α-Amylase Production

Microorganisms were cultivated for 16 hours in Nutrient Broth at 37 ℃ and inoculated to soluble starch containing plates (Saito, 1973)(Appendix B). Plates were incubated at 37 ℃ for 48 hours for the formation of clear zones around colonies, and stained with the gram's iodine to make clear zones visible.

2.5.3.2. Cultivation Conditions for α-Amylase Production

Five ml soluble starch containing liquid media (Haq *et al*, 2003) were inoculated and incubated at 37 ℃ for 16 hours. These cultures were used as the inoculum for 50 ml of the same media in 100 ml flask. Cultivations were carried out at 45℃ and 150 rpm for 72 hour. At 6, 12, 24, 30, 36, 48, 54, 60 and 72nd hours, samples were withdrawn from the flasks aseptically and α-amylase activities were determined from the culture supernatant, taken by 10 minutes centrifugation at 15000 rpm.

2.5.3.3. α- Amylase Assay

α-Amylase activity was determined by using the iodometric method of De Moraes (1995). Two hundred µl crude enzymes were mixed with 1 ml 0.5 % soluble starch substrate solution in 0.05 M sodium phosphate buffer, pH 7.0 and incubated in waterbath at 50℃ for 10 minutes. From this reaction mixture, 200 µl was added to 5 ml iodine solution to stop the reaction. Absorbances were measured at 620 nm against a blank containing buffer instead of substrate solution and enzyme. Measured values were also compared with the control containing buffer instead of enzyme. Starch was used as a standard (Appendix G). All experiments were made as triple and their mean values are used. One unit (U) is defined as the amount of enzyme causing the removal of 1 mg soluble starch per minute at 50 ℃ and pH 7.0. Composition and preparation of iodine solution and starch substrate solution are given in Appendix C.

2.5.3.4. Determination Optimum Temperature for α-Amylase

Optimum temperatures of the α-amylases were determined by the same procedure of α-amylase assay within a temperature range of 30-90℃.

2.5.3.5. Determination of Optimum pH for α-Amylase

Optimum pH values of the α-amylases were determined by using the same procedure of α-amylase assay in a pH range of 4.0-10.6. Buffers used were 0.05 M citrate phosphate buffer (pH 4.0 and 5.0), 0.05 M sodium phosphate buffer (pH 6.0, 7.0 and 8.0) and 0.05 M glycine-NaOH buffer (pH 9.0, 10.0 and 10.6) prepared as shown in Appendix D.

2.5.3.6. Determination of α- Amylase Stability

Crude enzymes were diluted with the same pH 7.0, 8.0, 9.0 and 10.0 buffer solutions (20% v/v) and then incubated at waterbath at 50, 60, 70, 80 and 90 ℃ for 1 hour. At the end of this period, the residual α-amylase activities were measured.

2.5.4. Protease

2.5.4.1. Screening for Protease Production

Microorganisms were cultivated for 16 hours in Nutrient Broth at 37 ℃ and inoculated to skim-milk agar plates (Nehete *et al*, 1986)(Appendix B). Plates were incubated at 20 ℃ for 96 hours for the formation of clear zones around colonies.

2.5.4.2. Cultivation Conditions for Protease Production

Five ml peptone containing liquid media (Fujiwara and Masui, 1993)(Appendix B) inoculated and incubated at 37 ℃ for 16 hours were used as the inoculum for 50 ml of the same media in 100 ml flasks. Cultivations were carried out at 45℃ and 150 rpm for 120 hour. At 12, 24, 36, 48, 60, 72, 84, 96, 108 and 120th hours, samples were taken from the flasks aseptically and protease activities were determined from the culture supernatant, result of15 minutes centrifugation at 15000 rpm.

2.5.4.3. Protease Assay

Protease activities were assayed by using the azocasein method (Kole *et al*, 1988). One ml of 0.5% azocasein substrate solution in 0.05 M Tris-HCl buffer, pH 7.4 and 1 ml crude enzyme were mixed and incubated in waterbath at 50 ℃ for exactly 1 hour. Reactions were stopped by adding 2 ml 10% trichloroacetic acid (TCA) solution and the mixtures were centrifuged at 3000 rpm for 10 minutes. From the supernatants, 1.2 ml was added to 1.4 ml 1.8 N NaOH solution. Absorbances were read at 380 nm against a blank in which enzyme was added after the addition of TCA. All experiments were made as triple and their mean values are used. One unit (U) is defined as the amount of enzyme which yielded an 0.01 increase in absorbance at 380 nm in 1 hour at 50 ℃ and pH 7.4.

2.5.4.4. Determination of Optimum Temperature for Protease

The same procedure of protease assay was used within the temperature range of 30-90℃ to determine the optimum temperatures of proteases.

2.5.4.5. Determination Optimum pH for Protease

The same procedure of protease assay was used in a pH range of 4.0-10.6 to determine the optimum pH values of proteases. Buffers used were 0.05 M citrate phosphate buffer (pH 4.0 and 5.0), 0.05 M sodium phosphate buffer (pH 6.0, 7.0 and 8.0), Tris-HCl buffer (pH 7.4) and 0.05 M glycine-NaOH buffer (pH 9.0, 10.0 and 10.6) prepared as shown in Appendix D.

2.5.4.6. Determination of Protease Stability

Stabilities were determined by diluting the crude enzyme with the same pH 7.0, 8.0, 9.0 and 10.0 buffer solutions (20% v/v) and then incubating at waterbath at 50, 60, 70, 80 and 90 ℃ for 1 hour. At the end of this period, the residual protease activities were measured.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Microbial Identification of Salt Lake Isolates

Bacillus isolates from the Salt Lake,Turkey have been previosly isolated, characterized and identified by Firdevs Yalçın (2000) during her thesis work. These 11 isolates were named as HTG 1, 2, 3, 4, 5, 6, 8, 9, 10, TTG 1 and 2. Identifications were made according to classical microbial identification tests like gram and endospore staining, motility test, catalase test, oxygen utilization test and cultural characteristics. API 50 CHB tests were also performed for more precise identification of isolates. API tests use series of biochemical and assimilation tests and results are compared with a computerized database of known bacteria. API tests were not conducted for HTG 9 and 10 since they appeared to have the same phenotypic characters as HTG 2. Isolates were identified as given in Table 3.1.

Table 3.1: Identification of *Bacillus* strains from Salt Lake by API 50 CHB tests; ND: not determined (Yalçın, 2000)

Strain	***Identification***	***% Identification***
HTG 1	*Bacillus circulans*	65.6
HTG 2	*Bacillus licheniformis*	99.0
HTG 3	*Bacillus licheniformis*	99.9
HTG 4	*Bacillus licheniformis*	99.9
HTG 5	*Bacillus licheniformis*	93.2
HTG 6	*Bacillus licheniformis*	99.9
HTG 8	*Bacillus licheniformis*	99.9
HTG 9	ND	ND
HTG 10	ND	ND
TTG 1	*Bacillus licheniformis*	89.9
TTG 2	*Bacillus licheniformis*	82,6

In this study, isolates were identified according to their fatty acid methyl esters (FAME) profile in order to support the results of API tests. As a result, FAME results were consistent with the API results about the identity of 8 isolates. On the other hand, different identification results were obtained for three isolates by FAME analysis and API tests. (Table 3.2). While HTG1 was identified as *Bacillus circulans* by API tests, it was identified as *B. subtilis* by FAME analysis. According to the API values differentiating *B. circulans* and *B. subtilis*, there are 6 basic difference in fermentation of 49 carbohydrate source. *B. subtilis* can ferment glycerol, however, *B. circulans* can not and while *B. circulans* can ferment adenitol, N-acetyl glucosamine, melezitose, D-L-xylose and glucanate, *B. subtilis* can not. API results in Firdevs Yalçın's thesis illustrated that HTG1 was able to ferment glycerol, whereas it was not able to ferment adonitol, melezitose and D-L-xylose like *B. subtilis*. On the contrary, HTG 1 can ferment N-acetyl glucosamine like *B. circulans*. After all, HTG 1 is more likely to be *B.subtilis* although it was identified as *B. circulans* with an identification percentage of 65.6 by API tests.

Table 3.2: Identification of *Bacillus* strains from Salt Lake according to FAME profile;

	First	***Choice***	***Second***	***Choice***
	S.I.	**Identification**	**S.I.**	**Identification**
HTG 1	0.867	*Bacillus subtilis*	—	—
HTG 2	0.555	*Bacillus pumilus*	0.343	*Bacillus marinus*
HTG 3	0.551	*Bacillus licheniformis*	0.370	*Bacillus subtilis*
HTG 4	0.670	*Bacillus licheniformis*	0.399	*Bacillus subtilis*
HTG 5	0.647	*Bacillus licheniformis*	0.422	*Bacillus pumilus*
HTG 6	0.545	*Bacillus licheniformis*	0.539	*Bacillus subtilis*
HTG 8	0.441	*Bacillus licheniformis*	0.294	*Bacillus subtilis*
HTG 9	0.558	*Bacillus subtilis*	0.529	*Bacillus licheniformis*
HTG 10	0.699	*Bacillus licheniformis*	0.617	*Bacillus subtilis*
TTG 1	0.740	*Bacillus licheniformis*	—	—
TTG 2	0.749	*Bacillus licheniformis*	0.477	*Bacillus subtilis*

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Similarly, API results contrasted with the FAME analysis in regard the identity of HTG 2. Although this strain was identified as *Bacillus licheniformis* by API tests, it was determined as *Bacillus pumilus* by FAME analysis. While *Bacillus licheniformis* can ferment galactose, inositol, sorbitol, α-methyl-D-glucoside, maltose, inulin, stach, glycogen and D-turanose, *Bacillus pumilus* cannot ferment these carbohydrates. According to the API results of previous work, HTG 2 can ferment all these carbohydrates like *B. licheniformis*, but it was identified as *B. pumilus* with FAME analysis. These differences may result from many reasons. First of all, *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* are very closely related species and confusion about the identity of these microorganisms are quite common consequences. In addition, the percent identity of *Bacillus circulans* in API tests was too low to be accepted as a good match. Moreover, API tests based on the colour change in fermentation media and use the numbers 0, 1 and 2 for negative and 3 and 4 for positive results, but the difference between 2 and 3, for example, is not always clear. Brown and Leef (1996), compared the API tests and the FAME analysis. According to their results, FAME analysis yielded 27.8 % excellent and 69.4 % good matches, and two different API tests yielded 27.5 and 6% excellent and very good matches. While these two different API tests were unable to identify 27.5 and 44.5 % of the isolates, all the microorganisms were identified succesfully with FAME analysis. Moreover, previously Santos *et al* (1993) found that the API system could not accurately identify some strains of bacteria when compared with biochemical tube and plate tests.

Microorganism identification using fatty acid methyl ester (FAME) analysis is based on Similarity Index. Similarity Index is a numerical value, which expresses how closely the fatty acid composition of an unknown sample compares with the mean fatty acid composition of the strains used to create the library entry listed as its match. The database search presents the best matches and associated similarity indices. An exact match of the fatty acid make-up of the unknown sample to the mean of a library entry results in a similarity index of 1.000. The similarity index will decrease as each fatty acid varies from the mean percentage. Strains with a similarity of 0.700 or higher and with a separation of 0.200 or more between first and second choices are considered an excellent match. If the similarity index is between 0.500 and 0.700 with a seperation of 0.100 between the first and second choices,

then the match is considered good. A Similarity Index between 0.300 and 0.500 could be an acceptable match, but would indicate an atypical strain. Values lower than 0.300 suggest that the species is not in the database, but those listed provide the most closely related species (Brown and Leef, 1996). According to these values, HTG 1, TTG 1 and TTG 2 were excellent matches, HTG 2, HTG 3, HTG 4 and HTG 5 were good matches. Similarity index of HTG 8 was 0.441, which makes it an acceptable match. On the other hand, although their similarity indexes of first and second choice were higher than 0.500, there was no 0.100 difference in similarity indexes of first and second choice of HTG 6, HTG 9 and HTG 10. So, their identifications were not absolute.

FAMEs are named by a standard nomenclature: the total number of carbon atoms, followed by a colon and the number of double bonds. The suffixes ‘‘c’’ and ‘‘t’’ refer to cis and trans conformations, respectively. Methyl branching at the iso and anteiso positions and at the 10th carbon atom from the carboxyl end is designated by the prefixes ‘‘i’’, ‘‘a’’ and ‘‘10 Me’’, respectively. The fatty acid composition of the bacteria, including *Bacillus*, are significantly different from those of higher organisms in having no polyunsaturated fatty acids. Fatty acid pattern of *Bacillus* are strikingly uniform in many respects. The predominance of terminally methyl-branched iso and anteiso fatty acids having 12 to 17 carbons is a characteristics observed in all species of *Bacillus*. The two branched series (iso and anteiso) of the fatty acids account for 55-95 % of the total acids. Unsaturated fatty acids are generally absent or present only in very small amounts. The fatty acid pattern of the various species of *Bacillus* can be divided into two groups: either the *anteiso*-15 or the *iso*-15 is most abundant among the fatty acids. The group, in which the *anteiso*-15 is most abundant, includes most *Bacillus* species (*B. subtilis* group), whereas the other group, in which *iso*-15 is most abundant, includes only three species (*B. cereus* group) which are taxonomically closely related. The percentages of the cellular fatty acids in *Bacillus* isolates are summarized in Table 3.3. FAME analysis results match with the theoretical *Bacillus* characteristics, such as chain lenght of the fatty acids were between 12 and 17. Unsaturated fatty acid amount did not exceed 4 % and iso and anteiso branched fatty acids constituted more than 90 % of all fatty acids as expected. So, isolates can be accepted as *Bacillus*. In all of the isolates, anteiso-15 was most abundant, therefore they can be accepted as a member of *Bacillus subtilis*

group. *B. subtilis, B. licheniformis* and *B. pumilus* share common characteristics, i.e. amount of *iso*-15 acid is almost equal to that of *anteiso*-15 acid and most of the fatty acids are *iso*-15 acid and *anteiso*-15 acid. Also, *iso*-17 acid and *anteiso*-17 acid are the second most abundant cellular fatty acid group in these microorganisms (Kaneda, 1968; Kaneda, 1977; Haack *et al*, 1994).

Table 3.3: Percentage of major cellular fatty acid components from the Bacillus isolates of Salt Lake. "i" before the carbon atoms denotes iso-branched chain fatty acids; "a" refers to anteiso-branched fatty acids; "al" stands for a kind of fatty alcohol.

Isolates	**i14:0**	**i15:0**	**a15:0**	**i16:0**	**16:1 w11c**	**16:0**	**al16:1 w7c**	**i17:0**	**a17:0**	**i17:1 w10c**
B. subtilis HTG1	0.74	26.23	40.17	2.52	0.75	2.33		11.87	11.95	
B. pumilus HTG2		35.56	42.71	3.69				6.59	11.45	
B.licheniformis HTG3		33.44	32.52	2.99		3.00		11.79	13.36	2.89
B.licheniformis HTG4		32.90	36.33	3.74		3.15		7.82	13.44	2.61
B.licheniformis HTG5		32.96	38.43	3.41		4.24		7.72	13.24	
B.licheniformis HTG6		30.87	38.95	3.96		3.38	1.69	5.97	10.70	2.37
B.licheniformis HTG8		34.45	37.70			5.22		8.78	13.86	
B. subtilis HTG9		29.76	38.46	3.91		3.27	1.31	6.24	11.19	2.54
B.licheniformis HTG10		32.12	37.95	3.25	1.61	2.83		6.42	11.23	2.27
B.licheniformis TTG1		37.10	30.34	2.76	1.16	2.14	1.23	9.33	9.74	3.28
B.licheniformis TTG2		31.23	34.95	4.04		4.37		9.69	13.26	2.47

3.2. Characterization of the Isolates in Terms of their Enzyme Production

3.2.1. Xylanase

3.2.1.1 Screening for Xylanase Production

Microorganisms were screened for the production of xylanase on Congo Red Xylan Agar. Congo Red is a dye making complex with the polysaccharides including xylan. Therefore, if microorganisms use that polysaccharide, its degradation by the microorganisms would create clear zones around colonies on a red background. Formation of the clear zones around the colonies showed the production of xylanolytic enzymes (Figure 3.1). *Micrococcus* spp. was used as a negative control.

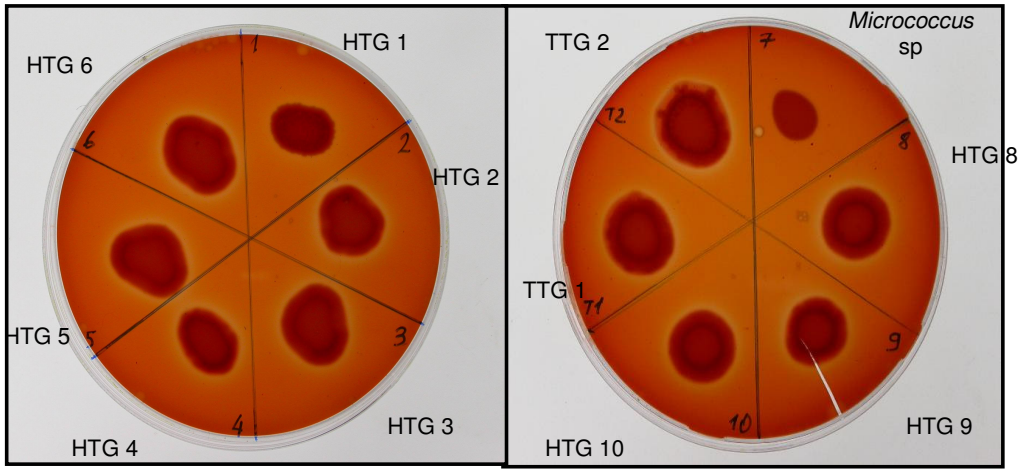

Figure 3.1: Screening of *Bacillus* isolates for xylanase production.

3.2.1.2. Quantitative Determination of Xylanase Activities

Isolates were cultivated for production of xylanases for 72 hours. Samples were withdrawn at regular time intervals and assayed for xylanase production. Maximum xylanase production was observed at 48th hour for all the isolates. Then xylanase activities started to decrease most probably because of protease action, which

started to increase after 48 hour (Figure 3.2). HTG 1 was the best xylanase producer having 54 U/ml. Except for TTG 1, xylanase activity of 6 U/ml, all the isolates produce considerable amount of xylanase. HTG 2, HTG 9 and TTG 2 were other important xylanase producers with 47, 40 and 41 U/ml. These results seems quite high among other Bacillus species. It can be concluded that HTG 1, 2, 9 and TTG 2 were promising isolates having higher xylanase activities among all isolates.

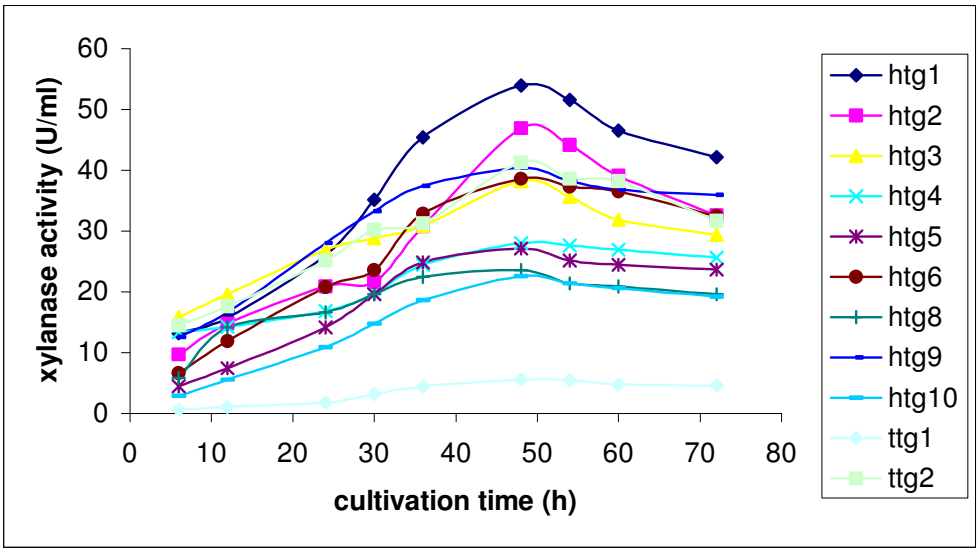

Figure 3.2: Xylanase production curves of *Bacillus* isolates at 50 ℃, pH 7

3.2.1.3. Determination of Optimum Temperature for Xylanase

Xylanase assays were conducted at various temperatures in the range of 40-70℃ at pH 7. While HTG 1, 4, 9 and TTG 1 showed maximum xylanase activity at 55 ℃, HTG 2, 3, 5, 6, 8,10 and TTG2 displayed best xylanase activity at 50 ℃ (Table 3.4). There exist many other microorganisms displaying their best xylanase activities at higher temperatures. Some of them are *Aspergillus niger*, 60 ℃ (Pandey and Pandey, 2002), *Thermomonospora fusca* BD25, 70 ℃ (Tuncer, 2000), *Streptomyces thermoviolaceus*, 70 ℃ (Tsujibo *et al*, 1992), *Talaromyces emersonii*

CBS 814-70, 78 ℃ (Subramaniyan and Prema, 2000), *Thermococcus zilligii*, 75 ℃ (Uhl and Daniel, 1999), *Bacillus thermantarcticus*, 80 ℃ (Lama *et al*, 2004), *Bacillus circulans* AB 16, 80 ℃ (Dhillon *et al*,2000), *B. amyloliquefaciens*, 80 ℃ (Breccia *et al*, 1998), *B. licheniformis* A99, 65 ℃ (Archana and Satyanarayana, 2003). Graphs showing optimum temperatures are given in Appendix H.

3.2.1.4. Determination of Optimum pH for Xylanase

Xylanase assays were performed in a pH range of 4.0-10.0 at 50 ℃. While HTG 2, 4 and TTG1 displayed best activities at pH 7.0, HTG 1, 3, 5, 6, 8, 9, 10 and TTG 2 showed maximum xylanase activity at pH 8.0. These results showed that the isolates produce generally more alkalophilic xylanases than fungi like *Aspergillus niger*, pH 4.0 (Pandey and Pandey, 2002), *Penicillum capsulatum*, pH 3.8 (Ryan *et al*, 2003), *Trichoderma longibrachiatum*, pH 5.0-6.0 (Chen *et al*,1997) and bacteria like *Streptomyces* sp. Ab106, pH 6.0 (Techapun *et al*, 2002), *Thermomyces lanuginosus*, pH 6.5 (Subramaniyan and Prema, 2000), *Thermococcus zilligii*, pH 6.0 (Uhl and Daniel, 1999), *Bacillus thermantarcticus*, pH 5.6 (Lama *et al*, 2004), *Bacillus circulans* AB 16, pH 6.0-7.0 (Dhillon *et al*,2000), *B. licheniformis* A99, pH 6.0 (Archana and Satyanarayana, 2003), *Bacillus* sp.BP-23, pH 5.5 (Blanco *et al*, 1995). However, xylanases from some *Bacillus* like *B. stearothermophilus* T-6, pH 9.0 (Kulkarni *et al*,1999) and *Bacillus* sp.41M-1, pH 9.0 (Nakamura *et al*, 1993) have higher pH optimum than the isolates. Results are summerized in Table 3.4 and graphs showing optimum pHs are given in Appendix I.

Paper pulp biobleaching process requires xylanases that remain active even above pH 8.0 and withstand temperatures around 60 ℃. HTG 2, 4 and TTG 1 seems to be promising isolates with respect to pH optimums, whereas according to optimum temperatures HTG 1, 4, 9 and TTG 1 are ideal for further studies.

3.2.1.5. Determination of Xylanase Stability

Stabilities of xylanases of pH 7.0, 8.0, 9.0 and 10.0 were measured after 1 hour incubation at 50, 60, 70, 80 and 90 ℃. All isolates retain 87-100 % of their xylanase activities at 50 ℃ and pH 7.0 and lost 78-97 % of their xylanase activities at 90 ℃ and pH 10.0 within 1 hour. HTG 3, 6 and TTG 1 retain 100 % of their xylanase

Table 3.4: Summary of the xylanase activities and optimum temperature and pH of xylanases from *Bacillus* isolates.

Isolates	***Max xylanase activity(U/ml)***	***Optimum temperature(˚C)***	***Optimum pH***
HTG1	54.0	55	7
HTG2	46.9	50	8
HTG3	38.2	50	7
HTG4	28.0	55	8
HTG5	27.1	50	7
HTG6	38.6	50	7
HTG8	23.6	50	7
HTG9	40.4	55	7
HTG10	22.6	50	7
TTG1	5.5	55	8
TTG2	41.3	50	7

activities at 50 ℃ and pH 7.0 for 1 hour. It seems that generally xylanases are quite stable up to pH 8.0 and 70 ℃. At 90 ℃ and pH 10.0, TTG 2, TTG 1 and HTG 9 remained 27%, 22 % and 21 %, repectively. Results are summerized in Table 3.5 and stabilities graphs are given in Appendix J.

In conclusion, HTG 1, 2, 9 and TTG 2 had considerable xylanase activities. Xylanases from HTG 1, 4, 9 and TTG 1 displayed their best activities at 55 ℃ and xylanases from HTG 2, 4 and TTG 2 showed their maximum activities at pH 8.0. Xylanases from HTG 3, 6 and TTG 1 remain 100% stable after 1 hour at 50℃, pH 7.0. TTG 2, TTG 1 and HTG 9 retained 27%, 22% and 21% of their xylanase activities after 1 hour at 90 ℃ and pH 10.0. Xylanases retain 82-100%, 65-86 %, 26-45% and 3-27% of their activities after 1 hour at 50 ℃ and pH 7.0, 60 ℃ and pH 8.0, 80 ℃ and pH 9.0, 90 ℃ and pH 10.0, respectively. Considering all the criteria, HTG 1 with the best xylanase activity and temperature optimum of 55 ℃, HTG 2 with considerable xylanase activity, stability and pH optimum 8.0, HTG 4 with considerable xylanase stability, 55 ℃ temperature optimum and pH optimum 8.0, HTG 9 with considerable xylanase activity, one of the best xylanase stability, 55 ℃ temperature optimum and pH optimum 8.0 and TTG 2 with considerable xylanase activity and one of the best xylanase stability are the promising isolates for industrial applications.

Table 3.5: Thermal and pH stability of xylanases after 1 hour incubation.

		Residual xylanase activities (%)				
Xylanase source	**pH**	**50 °C**	**60 °C**	**70 °C**	**80 °C**	**90 °C**
HTG 1	**7.0**	96	86	73	52	45
	8.0	85	78	66	47	38
	9.0	47	41	35	26	21
	10.0	21	17	14	12	3
HTG 2	**7.0**	96	88	81	69	58
	8.0	82	76	68	62	45
	9.0	63	51	43	34	23
	10.0	41	36	22	20	16
HTG 3	**7.0**	100	93	86	71	57
	8.0	89	81	71	57	48
	9.0	69	63	48	36	25
	10.0	44	37	29	19	8
HTG 4	**7.0**	98	95	81	69	54
	8.0	90	82	70	53	39
	9.0	72	65	49	36	23
	10.0	57	48	37	29	16
HTG 5	**7.0**	93	86	75	59	45
	8.0	87	81	73	50	36
	9.0	69	58	52	39	25
	10.0	41	36	27	19	10
HTG 6	**7.0**	100	92	83	65	54
	8.0	95	86	75	62	50
	9.0	79	68	59	43	37
	10.0	46	35	23	16	8
HTG 8	**7.0**	90	86	80	68	61
	8.0	87	79	70	58	49
	9.0	66	59	46	38	26
	10.0	43	38	31	27	18
HTG 9	**7.0**	94	90	78	71	64
	8.0	92	83	69	53	46
	9.0	83	65	53	43	36
	10.0	67	53	45	28	21
HTG 10	**7.0**	86	78	65	53	44
	8.0	77	65	56	48	35
	9.0	65	58	39	28	19
	10.0	36	28	21	13	5
TTG 1	**7.0**	100	87	76	69	62
	8.0	98	83	68	55	47
	9.0	72	61	54	40	29
	10.0	55	49	34	28	22
TTG2	**7.0**	97	90	82	71	58
	8.0	86	73	66	51	45
	9.0	78	67	59	45	33
	10.0	51	42	37	31	27

3.2.2. Cellulase

3.2.2.1. Screening for Cellulase Production

Microorganisms were screened for the production of xylanase on Congo Red CMC Agar. Congo Red is a dye making complex with the polysaccharides including cellulose. Therefore, if microorganisms use that polysaccharide, its degradation by the microorganisms would create clear zones around colonies on a red background. After 48 hours of incubation at 37 ℃, any clear zones could not be visualized. As a result, none of the isolates produces cellulase or the isolates produce insufficient amount of cellulase to be visualized by this method (Figure 3.3).

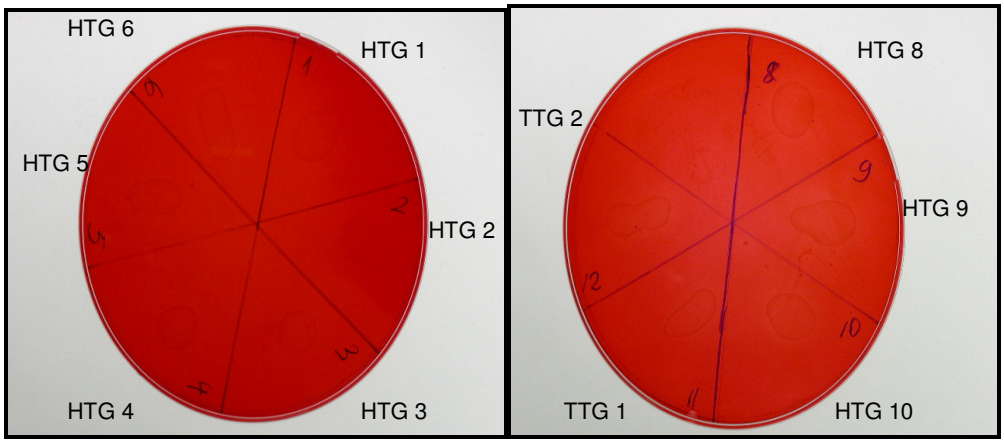

Figure 3.3: Screening of *Bacillus* isolates for cellulase production

3.2.2.2. Quantitative Determination of Total Cellulase Activities

All isolates were determined to produce insignificant amounts of cellulase. Even, no cellulase activities were observed at HTG 4, 5, 8 and 10. In other isolates maximum cellulase productions were observed at 48th hour. Then cellulase activities started to decrease most probably because of protease action, which started to increase after 48 hour (Figure 3.4). Cellulase producing isolates had cellulase activities between 0.000006 and 0,000015 (Table 3.6) and these values are quite negligible among other micrororganisms. In fact, xylanases used in paper industry have to be

cellulase free and remain active even above pH 8.0 and withstand temperatures around 60 ℃ (Dhillon *et al*, 2000). HTG 1, 2, 4, 9 and TTG 2, which are ideal for xylanase production, are good choices with their negligible cellulase activities.

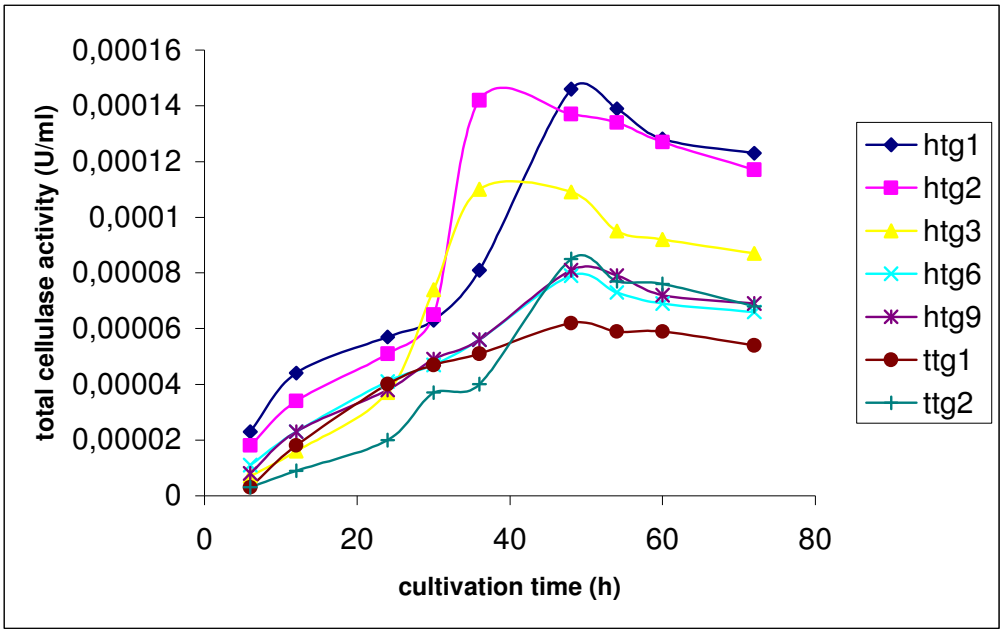

Figure 3.4: Cellulase production curves of *Bacillus* isolates at 50 ℃, pH 7

Table3.6: Summary of cellulase activities of *Bacillus* isolates

Isolates	***Total cellulase activity(U/ml)***
HTG1	0.00015
HTG2	0.00014
HTG3	0.00011
HTG6	0.00008
HTG9	0.00008
TTG1	0.00006
TTG2	0.00008

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3.2.3. α-Amylase

3.2.3.1. Screening for α-Amylase Production

Microorganisms were screened for the production of α-amylase on soluble starch containing plates. These plates were stained with the gram's iodine to make the clear zones visible after 48 hour growth of microorganisms at 37 ℃. Iodine forms blue coloured complex with starch and if the microorganism hydrolyses starch, transparent clear halo appear around the colonies on a dark blue background. According to screening results, all isolates produce considerable amount of α-amylase (Figure 3.5).

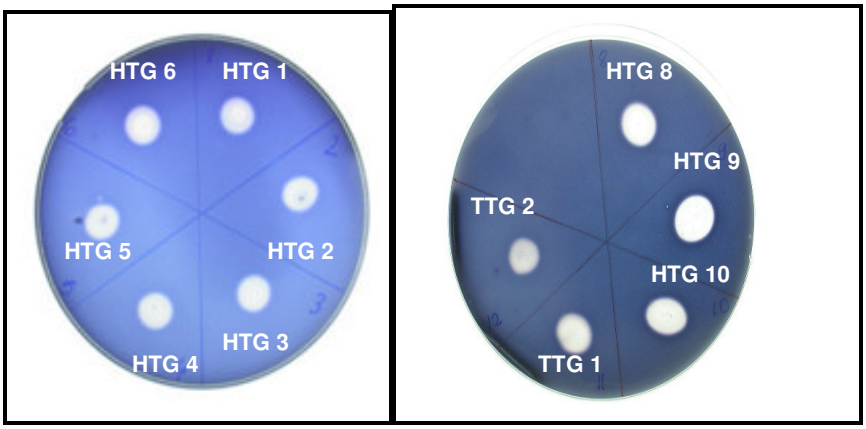

Figure 3.5: Screening of *Bacillus* isolates for α-amylase production.

3.2.3.2. Quantitative Determination of α-Amylase Activities

Isolates were cultivated for production of α-amylase for 72 hours. Samples were taken at regular time intervals and assayed for α-amylase production. Maximum α-amylase productions were observed at 48th hour for all the isolates. Then α-amylase activities started to decrease most probably because of protease action, which started to increase after 48 hour. HTG 1 was the best producer of α-amylase with 289 U/ml. HTG 2, 3, 4, 5 and 6 also produce considerable amounts of α-amylase among other isolates with 144, 153, 175, 133, and 158 U/ml respectively. Since there is no international unit used for α-amylase activities, results could not be

compared with other microorganisms. However, it is known that Bacillus species and especially *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* are good producers of α-amylase. α-Amylases used in industrial processes are mainly derived from *Aspergillus niger, Aspergillus oryzae, Bacillus amyloliquefaciens, Bacillus licheniformis, Bacillus stearothermophilus* and *Bacillus subtilis*, activities of which are given in specific activity units. Therefore, results could not be compared with industrial α-amylases too.

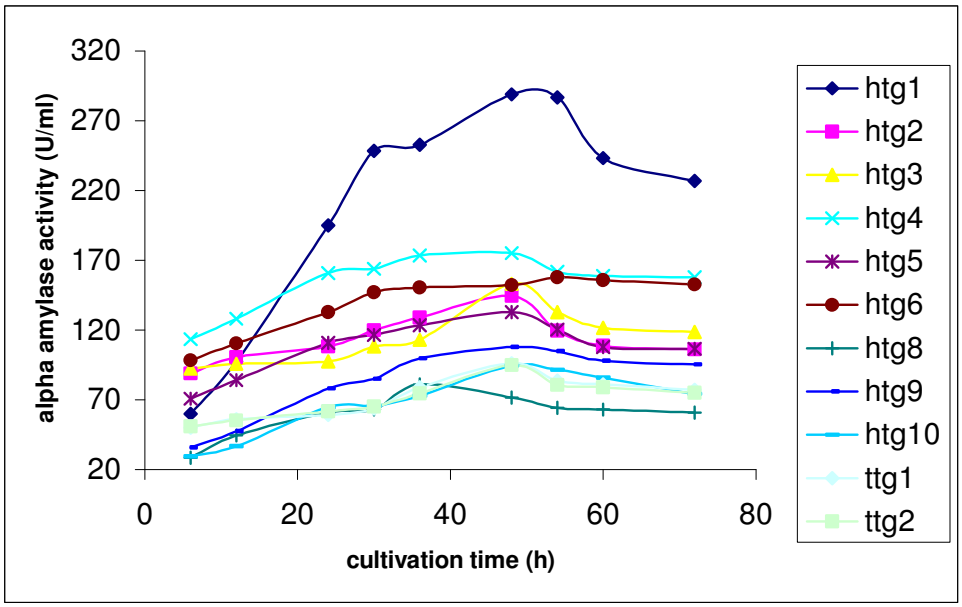

Figure 3.6: α-Amylase production curves of *Bacillus* isolates at 50 ℃, pH 7

3.2.3.3. Determination Optimum Temperature for α-Amylase

α-Amylase assays were carried out at various temperatures in the range of 30-90 ℃ at pH 7.0. HTG 2, 3, 4 and TTG 2 showed maximum α-amylase activities at 80 ℃, α-amylases from HTG 1 and TTG 1 had temperature optimum at 70 ℃ and HTG 5, 6, 8, 9 and 10 displayed maximum α-amylase activities at 60 ℃. Especially α-amylases from HTG 2, 3, 4 and TTG 2 have higher temperature optimum than many

microorganisms including *Aspergillus oryzae*, 50 ℃ and *Aspergillus niger*, 60 ℃ (Gupta *et al*, 2003), *Aspergillus carbonarius*, 40 ℃ (Okolo *et al*, 2000), *Cryptococcus flavus*, 50 ℃ (Wanderley *et al*, 2004), *Lactobacillus plantarum*, 65 ℃ (Gupta *et al*,2003), *B. amyloliquefaciens*, 36 ℃ (Castro *et al,*1993) and *B. subtilis*, 50 ℃ (Gupta *et al*,2003). However, α-amylases from most Bacillus species have similar temperature optimums, such as *B.licheniformis*, 76 ℃ (Saito, 1973), *B. stearothermophilus*, 80 ℃ (Srivastava, 1987), *B.acidocaldirus*, 75 ℃ (Buonocore *et al*, 1976) and *Bacillus sp.*WN11, 75-80 ℃ (Mamo *et al*, 1999). *B.licheniformis* CUMC305, 90 ℃ (Krishnan and Chandra, 1983) and *Bacillus licheniformis*, 91 ℃ (Medda and Chandra, 1980) have higher temperature optimum than α-amylases from the isolates. The major field in which α-amylases are used extensively is the processing of starch. It is desirable that α-amylases should be active at high temperatures of gelatinization (100-110 ℃) and liquefaction (80- 90 ℃) to economize these processes. HTG 2, 3, 4 and TTG 2 are promising isolates according to this criterium. Results are summarized in Table 3.7, and graphs showing optimum temperature are given in Appendix H.

3.2.3.4. Determination of Optimum pH for α-Amylase

α-Amylase assays were conducted in a pH range of 4.0-10.6 at 50 ℃. While α-amylases from HTG 1, 2, 3 and 5 had their pH optimum at 7.0, HTG 4, 6, 8, 9, 10, TTG 1 and 2 displayed best α-amylase activities at pH 8.0. These values are more alkaline with respect to many microorganisms like *Aspergillus oryzae*, pH 5.4 and *Aspergillus niger*, pH 4.0-5.0 (Gupta *et al*, 2003), *Aspergillus carbonarius*, pH 6.0-7.0 (Okolo *et al*, 2000), *Trichoderma viride*, pH 5.0-5.5 (Gupta *et al*,2003), *Thermomyces lanuginosus*, pH 4.6-6.6 (Nguyen *et al*, 2002), *Thermococcus profundus*, pH 5.5 (Kwak *et al*, 1998), *Rhodothermus marinus*, pH 6.5 (Gomes *et al*, 2003), *Streptococcus bovis*, pH 5.0-6.0 (Gupta *et al*,2003), *B.licheniformis* NRLL B14368, pH 5.0-7.0 (Bose and Das, 1996), *Bacillus licheniformis*, pH 6.0-6.5 (Ivanova *et al*, 1993), *B.acidocaldirus*, pH 3.5 (Buonocore *et al*, 1976) and *B. globisporus*, pH 4.5 (Bandyopadhyay *et al*, 1993). However, α-amylases from *Bacillus licheniformis*, pH 9.5 (Medda and Chandra, 1980), *B.licheniformis* CUMC305, pH 9.0 (Krishnan and Chandra, 1983; Saito, 1973) and *Bacillus sp.* ANT-6, pH 10.5 (Burhan *et al*, 2003) have more alkaline optimum pHs. A growing

new area of application of α-amylases is in the fields of detergent industry which requires α-amylases optimally working at alkaline pH. HTG 4, 6, 8, 9, 10, TTG 1 and 2 are the promising isolates according to this criterium. Results are summarized in Table 3.7 and graphs showing optimum pH values are given in Appendix I.

Table 3.7: Summary of the α-amylase activities and optimum temperature and pH of α-amylases from *Bacillus* isolates.

Isolates	***α-amylase activity(U/ml)***	***Optimum pH***	***Optimum temperature(℃)***
HTG1	288.9	7	70
HTG2	144.4	7	80
HTG3	153.4	7	80
HTG4	175.0	8	80
HTG5	133.0	7	60
HTG6	157.8	8	60
HTG8	81.1	8	60
HTG9	107.8	8	60
HTG10	94.4	8	60
TTG1	96.1	8	70
TTG2	95.0	8	80

3.2.3.5. Determination of α-Amylase Stability

Stabilities of α-amylases at pH 7.0, 8.0, 9.0 and 10.0 were measured after 1 hour incubation at 50, 60, 70, 80 and 90 ℃. All isolates retain 88-100 % of their α-amylase activities at 50 ℃ and pH 7.0 and lost 73-94 % of their α-amylase activities at 90 ℃ and pH 10.0 within 1 hour. HTG 5 retain 100 % of its α-amylase activity at 50 ℃ and pH 7.0 for 1 hour. It can be concluded that α-amylases from isolates are quite stable up to 80℃ and pH 9.0. α-Amylases from the isolates are more stable at alkali pHs and high temperatures than xylanase of the isolates. α-Amylase from HTG 9 remained 27% stable, HTG 2 retain 24 % of its α-amylase activity and α-amylases from HTG 4 and TTG2 remained 22% stable at 90 ℃ and pH 10.0. Results are summarized in Table 3.8 and stability graphs are given in Appendix J.

Table 3.8: Thermal and pH stability of α-amylases after 1 hour incubation.

		Residual α-amylase activity (%)				
α-Amylase source	**pH**	**50 °C**	**60 °C**	**70 °C**	**80 °C**	**90 °C**
HTG 1	**7.0**	95	93	86	73	61
	8.0	92	89	79	68	49
	9.0	79	75	61	56	41
	10.0	54	46	35	21	16
HTG 2	**7.0**	90	88	83	78	75
	8.0	82	79	77	71	67
	9.0	68	65	59	52	49
	10.0	46	42	38	31	24
HTG 3	**7.0**	88	85	83	76	73
	8.0	78	75	69	63	58
	9.0	51	49	43	38	32
	10.0	31	26	21	17	12
HTG 4	**7.0**	96	95	91	86	73
	8.0	91	88	79	68	61
	9.0	74	69	58	50	42
	10.0	56	49	41	29	22
HTG 5	**7.0**	100	98	89	83	69
	8.0	84	76	72	63	56
	9.0	66	59	49	36	27
	10.0	48	39	31	23	12
HTG 6	**7.0**	91	88	82	73	65
	8.0	89	79	75	67	51
	9.0	74	62	53	45	33
	10.0	39	30	26	18	16
HTG 8	**7.0**	98	92	85	78	69
	8.0	89	83	71	62	58
	9.0	67	59	54	48	29
	10.0	46	36	29	22	19
HTG 9	**7.0**	89	86	78	66	53
	8.0	89	76	70	58	49
	9.0	73	67	56	48	38
	10.0	52	44	39	36	27
HTG 10	**7.0**	97	89	81	75	66
	8.0	95	86	79	68	61
	9.0	68	64	59	51	46
	10.0	36	30	28	19	6
TTG 1	**7.0**	88	85	81	67	61
	8.0	83	76	71	63	56
	9.0	63	55	49	38	25
	10.0	42	39	27	22	15
TTG2	**7.0**	95	92	88	83	76
	8.0	92	87	81	75	66
	9.0	74	62	56	45	29
	10.0	61	50	38	31	22

As a conclusion, HTG 1, 2, 3, 4, 5 and 6 had appreciable quantities of α-amylase activities. α-Amylases from HTG 4, 6, 8, 9, 10, TTG 1 and 2 displayed their best activities at pH 8.0 and HTG 2, 3, 4 and TTG 2 showed maximum α-amylase activities at 80 ℃. α-Amylase from HTG 5 remain 100 % active after 1 hour at 50 ℃, pH 7.0. Most of the α-amylases remained more than 50 % stable after 1 hour up to pH 9.0 and 90 ℃. α-Amylases remained 78-100%, 75-89 %, 36-56% and 6-27 % stable at 50 ℃ and pH 7.0, 60 ℃ and pH 8.0, 80 ℃ and pH 9.0, 90 ℃ and pH 10.0, sequentially. Considering all the criteria, HTG 1 with its best α-amylase activity, HTG 2 with its considerable α-amylase activity, stability and 80 ℃ optimum temperature, HTG 3 with its considerable α-amylase activity and 80 ℃ optimum temperature, HTG 4 with its considerable α-amylase activity, stability, 80 ℃ optimum temperature and pH optimum 8.0, HTG 6 with its considerable α-amylase activity and pH optimum, HTG 8 and 9 with their considerable stability and pH optimum 8.0 and TTG 2 with its considerable stability, 80 ℃ optimum temperature and pH optimum 8.0 are the promising isolates for industrial applications.

3.2.4. Protease

3.2.4.1. Screening for Protease Production

Microorganisms were screened for the production of protease on Skim Milk Agar. After incubation of 96 hours at 20 ℃, protease producing colonies were surrounded with clear zones on white background. According to screening results, all isolates produce considerable amount of protease (Figure 3.7).

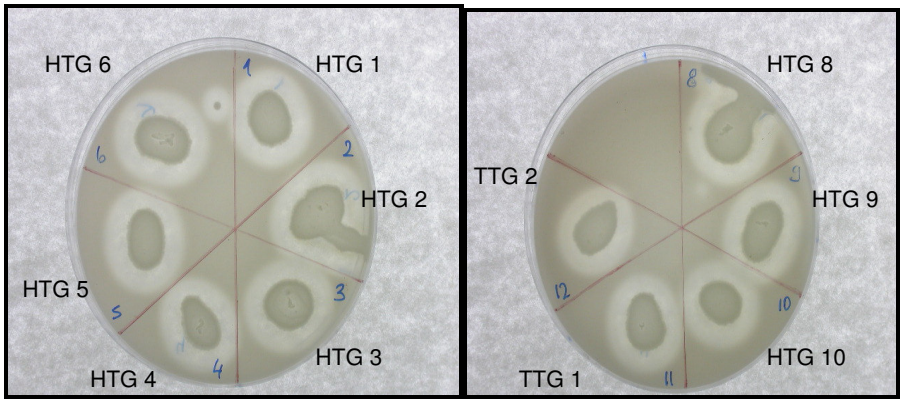

Figure 3.7: Screening of *Bacillus* isolates for protease production.

3.2.4.2. Quantitative Determination of Protease Activity

Isolates were cultivated for production of protease for 120 hours. Samples were withdrawn at regular time intervals and assayed for protease production. Assay results illustrated that, protease activities start to increase after 48th hour and all the isolates produce considerable amounts of proteases (Figure 3.8). Maximum protease productions were observed at 96th hous for all the isolates.TTG 2 is the best producer of protease with 271 U/ml, TTG 1, HTG 1 and HTG 9 are the next with 256, 227 and 204 U/ml respectively. All other isolates produce protease around 170-180 U/ml. Since there is no international unit used for protease activities, results could not be compared with other microorganisms. However, *Bacillus* species and especially *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* are known as good producers of protease. Bacterial proteases used in industrial processes are mainly derived from *Bacillus firmus, Bacillus licheniformis, Bacillus stearothermophilus*, *Bacillus subtilis* and *Bacillus lentus* activities of which are given in specific activity units. Therefore, results could not be compared with industrial proteases too.

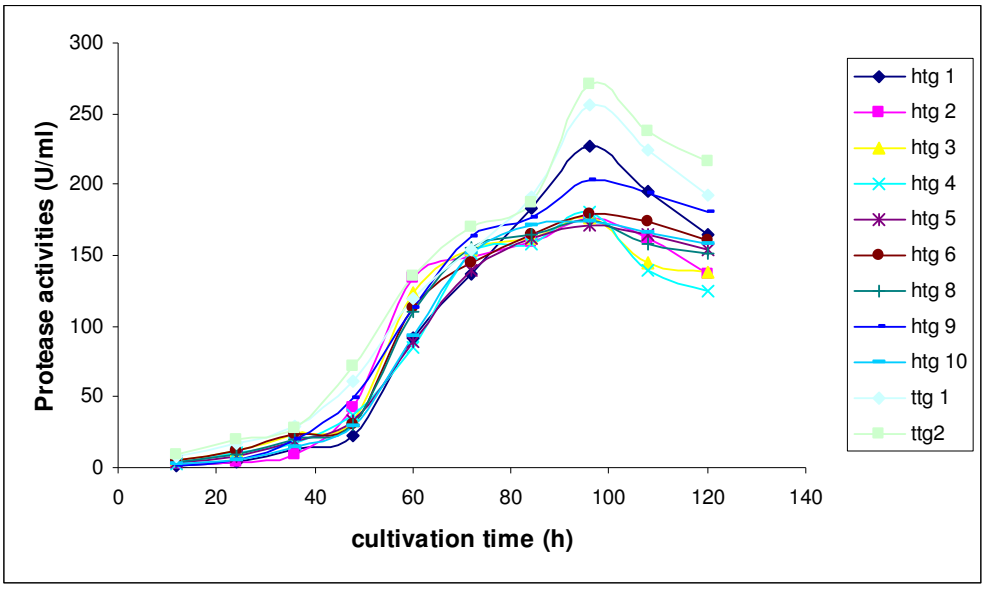

Figure 3.8: Protease production curves of *Bacillus* isolates at 50 ℃, pH 7.4

3.2.4.3. Determination of Optimum Temperature for Protease

Protease assays were carried out at temperatures in the range of 30-90 ℃ at pH 7.0. HTG 1 and TTG 1 displayed their best protease activities at 50 ℃, on the other hand proteases from all other isolates had temperature optimum at 60 ℃. Proteases from the isolates have higher temperature optimums than many microorganisms including *Aspergillus flavus*, 32℃ (Malathi and Chakraborty, 1991), *Aspergillus parasiticus*, 40 ℃ (Tunga *et al*,2003), *Penicillum* sp., 45 ℃ (Germano *et al*, 2003), *Kluyveromyces marxianus*, 45 ℃ (Zavala *et al*, 2004), *Bacillus cereus*, 40 ℃ (Sierecka, 1998), *B. licheniformis*, 47 ℃ (Manczinger *et al*, 2003) and *Bacillus* sp. SSR1, 40 ℃ (Singh *et al*, 2001). There exist many microorganisms having proteases with similar temperature optimum, such as *Bacillus sphaericus*, 50-55 ℃ (Singh *et al*, 1999), *B. mojavensis*, 60 ℃ (Beg and Gupta, 2003), *B. polymyxia* B-17, 50 ℃ (Matta and Punj, 1998), *B. brevis*, 60 ℃ (Banerjee *et al*, 1999) and *B. subtilis*, 50-55 ℃ (Varela *et al*, 1997). Nevertheless, there are some microorganisms having proteases with higher temperature optimum than the isolates like *Thermomyces lanuginosus*, 70 ℃ (Li *et al*, 1997), *Streptomyces tandae*, 70 ℃ (Seong *et al*, 2004), *B. stearothermophilus*, 75 ℃ (Kim *et al*, 2002), *Bacillus* sp. P-2, 90 ℃ (Kaur *et al*, 2001) and *Bacillus* sp., 85 ℃ (Fujiwara and Masui, 1993). Results are summarized in Table 3.9 and graphs showing optimum temperatures are given in Appendix H.

3.2.4.4. Determination Optimum pH for Protease

Protease assays were performed in a pH range of 4.0-10.6 at 50 ℃. While proteases from HTG 1, 6, TTG 1 and 2 had temperature optimum at pH 7.0, all other isolates displayed their best protease activities at pH 7.4. These values are more alkaline than some microorganisms like *Penicillum* sp, pH 6.5 (Germano *et al*, 2003), *Streptomyces tandae*, pH 6.0 (Seong *et al*, 2004) and *Streptomyces* sp., pH 6.0 (Azeredo *et al*,2004). On the other hand, proteases from *Aspergillus parasiticus*, pH 8.0 (Tunga *et al*,2003), *Thermoactinomyces vulgaris* A60, pH 9.0 (Desai and Dhala, 1969), *Thermomonospora fusca* YX, pH 9.0 (Gusek and Kinsella, 1987), *Bacillus sphaericus*, pH 10.5 (Singh *et al*, 1999), *B. mojavensis*, pH 10.5 (Beg and Gupta, 2003), *B. pumilus* , pH 11.0 (Feng *et al*, 2001), Alkalophilic *Bacillus* sp , pH 11.0-12.0 (Kumar *et al*, 1999), *Bacillus* sp., pH 12.0-13.0 (Fujiwara and Masui,

1993) are some of the microorganisms having more alkaline pH optimums than proteases of the isolates. Results are summarized in Table 3.9 and graphs showing optimum pH values are given in Appendix I.

Alkaline proteases are prerequisite for most of the industrial applications where proteases used. These applications include usage of proteases in detergent and leather industry, silver recovery from used X-ray films and medical applications. HTG 2, 3, 4, 5, 8,9 and 10 are promising isolates according to this criterium.

Table 3.9: Summary of the protease activities and optimum temperature and pH of proteases from *Bacillus* isolates.

Isolates	***Max. protease activity (U/ml)***	***Optimum Temp(°C)***	***Optimum pH***
HTG1	227.2	60	7.0
HTG2	176.4	50	7.4
HTG3	177.0	60	7.4
HTG4	180.4	60	7.4
HTG5	170.6	60	7.4
HTG6	179.2	60	7.0
HTG8	175.6	60	7.4
HTG9	203.6	60	7.4
HTG10	174.0	60	7.4
TTG1	255.6	50	7.0
TTG2	271.2	60	7.0

3.2.4.5. Determination of Protease Stability

Stabilities of the protease at pH 7.0, 8.0, 9.0 and 10.0 were measured after 1 hour incubation at 50, 60, 70, 80 and 90 °C. All the isolates retain 82-100 % of their protease activities at 50 °C and pH 7.0 and lost 91-98 % of their protease activities at 90 °C and pH 10.0 within 1 hour. HTG 3 and TTG 2 retain 100 % of their protease activities at 50 °C and pH 7.0 for 1 hour. It can be concluded that proteases were quite stable up to pH 9.0 and 80 °C. Proteases from TTG 1, HTG 6 and HTG 9 remained 19%, 17 % and 16% stable at 90 °C and pH 10.0, respectively. Results are summarized in Table 3.10 and stability graphs are given in Appendix J.

Table 3.10: Thermal and pH stability of proteases after 1 hour incubation.

		Residual protease activity (%)				
Protease source	**pH**	**50 ° C**	**60 ° C**	**70 ° C**	**80 ° C**	**90 ° C**
HTG 1	**7.0**	97	92	86	76	71
	8.0	90	85	78	70	62
	9.0	77	69	60	45	33
	10.0	32	23	18	15	7
HTG 2	**7.0**	89	86	77	73	65
	8.0	82	76	70	62	57
	9.0	65	58	49	41	28
	10.0	19	15	8	3	3
HTG 3	**7.0**	100	96	88	78	71
	8.0	91	85	80	73	65
	9.0	79	71	62	55	38
	10.0	35	28	19	14	8
HTG 4	**7.0**	93	88	80	73	64
	8.0	88	75	68	61	54
	9.0	66	58	51	48	40
	10.0	28	25	20	16	13
HTG 5	**7.0**	91	86	75	69	58
	8.0	79	66	58	49	43
	9.0	56	49	43	35	26
	10.0	23	19	16	9	4
HTG 6	**7.0**	98	88	81	74	66
	8.0	86	78	75	66	61
	9.0	62	55	43	38	24
	10.0	40	32	27	25	17
HTG 8	**7.0**	82	75	66	60	53
	8.0	73	65	50	42	36
	9.0	48	43	34	26	16
	10.0	20	16	13	7	2
HTG 9	**7.0**	95	90	78	75	66
	8.0	91	78	72	64	59
	9.0	80	71	63	58	48
	10.0	43	38	31	25	16
HTG 10	**7.0**	82	75	71	63	52
	8.0	74	69	62	55	49
	9.0	58	46	39	36	28
	10.0	26	24	20	17	13
TTG 1	**7.0**	94	90	83	77	69
	8.0	88	82	74	63	59
	9.0	70	66	64	48	43
	10.0	45	36	32	28	19
TTG2	**7.0**	10	96	87	78	70
	8.0	92	86	78	73	65
	9.0	74	68	59	43	27
	10.0	38	29	25	17	12

In summary, HTG 1, 9, TTG 1 and 2 had considerable protease activity. HTG 1, 3, 4, 5, 6, 8, 9, 10 and TTG 2 displayed best protease activities at 60 ℃ and HTG 2, 3, 4, 5, 8, 9 and 10 showed their maximum protease activities at pH 7.4. Protease from HTG 3 remain 100 % stable at 50 ℃, pH 7.0. TTG 1, HTG 6 and 9 retained 19%, 17% and 16% of their protease stabilities at 90 ℃, pH 10.0. Most of the proteases remain more than 50 % of their stabilities up to 90 ℃, pH 9. Proteases retained 82-100%, 65-86 %, 26-58% and 2-19 % of their stabilities at 50 ℃ and pH 7.0, 60 ℃ and pH 8.0, 80 ℃ and pH 9.0, 90 ℃ and pH 10.0, respectively. Considering all the criteria, HTG 1 with its considerable protease activity and 60 ℃ optimum temperature, HTG 6 with its considerable stability and 60 ℃ optimum temperature, HTG 9 with its considerable protease activity, stability, 60 ℃ optimum temperature and pH optimum at 7.4, TTG 1 with its considerable activity and stability, TTG2 with its best protease activity and 60 ℃ optimum temperature are promising isolates for industrial applications.

CHAPTER 4

CONCLUSION

Bacillus isolates from Salt Lake were identified using MIDI system based on the fatty acid methy ester (FAME) analysis by gas chromatography. HTG 2 was identified as *Bacillus pumilus*, HTG 1 and 9 were identified as *Bacillus subtilis* and other isolates were identified as *Bacillus licheniformis.*

All the isolates were determined to produce xylanase. HTG 1 was the best xylanase producer with 54 U/ml. Optimum temperatures of the xylanases from the *Bacillus* isolates were around 50-55 ℃ and optimum pH values were around pH 7.0-8.0. Xylanases retain 82-100%, 65-86 %, 26-45% and 3-27% of their activities after 1 hour at 50 ℃ and pH 7.0, 60 ℃ and pH 8.0, 80 ℃ and pH 9.0, 90 ℃ and pH 10.0, respectively. Three of the isolates were determined to retain 21-27 % xylanase activity after 1 hour at 90 ℃ and pH 10.0. Cellulase production of the *Bacillus* isolates were in negligible amounts. Even, HTG 4, 5, 8 and 10 did not produce cellulase enzyme. Paper and pulp industry requires cellulase free xylanases that remain active even above pH 8.0 and withstand temperatures around 60 ℃. HTG 1, 2, 4, 9 and TTG 2 are promising isolates due to their considerable xylanase activities, stabilities, optimum pH 7-8, optimum temperature 50-55 ℃ and negligible cellulase activities. Therefore, these Bacillus isolates could serve as a source of microorganisms that produce xylanases.

Considerable α-amylase productions were achieved by all isolates. HTG 1 was the best α-amylase producer with 288.9 U/ml. Optimum temperatures of the α-amylases from the *Bacillus* isolates were around 60-80 ℃ and optimum pH values were around pH 7.0-8.0. α-Amylases remained 78-100%, 75-89 %, 36-56% and 6-27 % stable at 50 ℃ and pH 7.0, 60 ℃ and pH 8.0, 80 ℃ and pH 9.0, 90 ℃ and pH 10.0,

sequentially. Four of the isolates were determined to maintain 22-27 % of their α-amylase activities for 1 hour at 90 ℃ and pH 10.0. Starch processing industry needs α-amylases that remain active at liquefaction temperature of 80-90 ℃ and gelatinization temperatures of 100-110 ℃. HTG 2, 3, 4 and TTG 2 are promising isolates according their higher optimum temperatures and stabilities. On the other hand, detergent industry requires alkaline stable α-amylases. HTG 4, 6, 8, 9, 10, TTG 1 and 2 are the promising isolates according to their alkaline pH optimums and stabilities.

All the isolates were determined to produce appreciable quantities of proteases. TTG 2 was the best protease producer with 271.2 U/ml. Optimum temperatures of the proteases from the *Bacillus* isolates were around 50-60 ℃ and optimum pH values were around pH 7.0-7.4. Proteases retained 82-100%, 65-86 %, 26-58% and 2-19 % of their stabilities at 50 ℃ and pH 7.0, 60 ℃ and pH 8.0, 80 ℃ and pH 9.0, 90 ℃ and pH 10.0, respectively. Alkaline proteases are used extensively in many industrial applications like detergent and leather industry. HTG 2, 3, 4, 5, 8, 9 and 10 are promising isolates according to their their alkaline pH optimums and stabilities.

Regarding these results, it can be concluded that some *Bacillus* isolates of Salt Lake produce considerable amount of xylanase, α-amylase and protease enzymes with high optimum temperatures and neutral to alkaline pH optimum. In addition, these are the results without any optimization. As next, optimization of cultivation parameters like carbon and nitrogen sources, temperature and pH and use of strain improvement techniques could be done for higher enzyme productions. Purification of these enzymes from the promising producers could be done for better characterization of those enzymes, most suitable ones could be choosen for industrial purposes and used in industrial applications comparing with the commonly used commercial enzymes. To our knowledge, there is no information about the nucleotide sequence of xylanase from *Bacillus licheniformis* in literature. Therefore, genetic studies could be done with the xylanase from the best xylanase producers of *Bacillus licheniformis*.

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APPENDIX A

CHEMICALS

Chemicals	***Supplier***
Birchwood Xylan	Sigma
Azocasein	Sigma
Congo Red	Sigma
Carboxymethy cellulose	Sigma
3,5- Dinitrosalysilic acid	Sigma
Xylose	Sigma
Lactose	Sigma
Glycine	Sigma
Soluble starch	Applichem
Trichloroacetic acid	Applichem
Sodium carbonate	Applichem
Sodium bicarbonate	Applichem
Acetic acid	Applichem
Sodium acetate	Applichem
Phenol	Applichem
Sodium hydroxide	Merck
Sodium chloride	Merck
Sodium sulphite	Merck

Iodine	Merck
Potassium iodide	Merck
Potassium chloride	Merck
Calsiyum chloride	Merck
NaH_2PO_4	Merck
Na_2HPO_4	Merck
KH_2PO_4	Merck
$MgSO_4$	Merck
HCl	Merck
Tris	Merck
Peptone	Merck
Citric acid	Merck
Nutrient Broth	Merck
Agar	Merck
Yeast Extract	Merck
Glycerol	Merck
Skim milk	Fluka
Tryptone	Oxoid
Glucose	Oxoid
Potassium Sodium Tartarate	Difco
Nutrient Agar	Difco

APPENDIX B

COMPOSITION OF THE MEDIA

1. Xylanase Cultivation Medium

Composition	**900 ml dH_2O**
Birchwood xylan	5 g
Yeast extract	5 g
NaCl	2.5 g
KH_2PO_4	1 g
$MgSO_4$	0.2 g

Composition	**100 ml dH_2O**
Na_2CO_3	1 g

Sterilized separately at 121 ℃ for 15 min. and mixed aseptically.

2. Congo Red Xylan Agar

Composition	**1 L dH_2O**
Agar	20 g
Birchwood xylan	5 g
Yeast extract	5 g
Tryptone	10 g
NaCl	5 g
Congo Red	125-150 mg

Sterilized separately at 121 ℃ for 15 min. and mixed aseptically.

3. Congo Red Carboxymethyl Cellulose Agar

Composition	**1 L dH_2O**
Agar	20 g
Carboxymethyl cellulose	10 g
Tryptone	10 g
Yeast extract	5 g
NaCl	5 g
Congo Red	125-150 mg

Sterilized separately at 121 ℃ for 15 min. and mixed aseptically.

4. α-Amylase Cultivation Medium

Composition	**1 L 0.05 M phosphate buffer (pH 7.0)**
Nutrient Broth	20 g
Soluble starch	10 g
Lactose	5 g
NaCl	5 g
$CaCl_2$	2 g

Sterilized at 121 ℃ for 15 min.

5. Starch Agar

Composition	**1 L dH_2O**
Agar	20 g
Soluble starch	5 g
Yeast extract	0.25 g
Sodium citrate	0.1 g
$MgSO_4$	0.5 g
$CaCl_2$	0.008 g
KCl	1.5 g

Sterilized at 121 ℃ for 15 min.

6. Protease Cultivation Medium

Composition	**900 ml dH_2O**
Glucose	20 g
Peptone	20 g
Yeast extract	0.5 g
KH_2PO_4	1 g
$MgSO_4$	0.2 g

Composition	**100 ml dH_2O**
Na_2CO_3	1 g

Sterilized separately at 121 ℃ for 15 min. and mixed aseptically.

7. Skim Milk Agar

Composition	**500 ml dH_2O**
Skim milk	50 g

Composition	**500 ml dH_2O**
Agar	10 g

Sterilized separately at 121 ℃ for 15 min. and mixed aseptically.

APPENDIX C

REAGENTS AND SOLUTIONS

1. DNS Reagent

Composition	Percentage (%)
Potassium sodium tartarate	27
3,5-dinitrosalicylic acid	1
NaOH	1
Phenol	0.2
Sodium sulphite	0.05

Stored in glass stoppered dark bottle at refrigiration temperature. Sodium sulphite was added just before use (Miller, 1959).

2. 1% Xylan substrate Solution

One gram of birchwood xylan was dissolved in 80 ml 0.05 M sodium phosphate buffer (pH 7.0), heated and boiled for 2-3 min. to solubilize the xylan. This solution was stirred overnight at room temperature, the volume was adjusted to 100 ml with the same buffer and centrifuged at 5000rpm for 20 min to get rid of insoluble xylan. Xylan substrate solution was stored at 4 ℃ for at most one week (Bailey *et al*, 1992).

3. Iodine Solution

Stock solution was prepared with 0.5% iodine and 5% potassium iodide. Iodine solution was prepared with mixing 1 ml of the stock solution, 5 ml of 5M HCl and 500 ml dH_2O (De Moraes *et al*, 1995).

4. 0.5% Starch Substrate Solution

A hundred ml 0.05 M sodium phosphate buffer, pH 7.0 was heated to boil and 0.5 g soluble starch was added with stirring. After 2-3 minutes boiling, volume was adjusted to 100 ml with the same buffer. Starch substrate solution was stored at 4 ℃ (De Moraes *et al*, 1995).

5. 0.5% Azocasein Substrate Solution

Azocasein substrate solution was prepared 0.5 g azocasein in 100 ml 0.05 M tris-HCl buffer, pH 7.4 and heated with stirring until azocasein was completely solubilized. Final volume was adjusted to 100 ml with the same buffer and this solution was stored at refrigiration temperature (Kole *et al*, 1988).

APPENDIX D

BUFFERS

1. Sodium Phosphate Buffer (0.05 M)

A: 0.1 M NaH_2PO_4 solution (13.9 g in 1 L)

B: 0.1 M Na_2HPO_4. $7H_2O$ solution (26.83 g in 1 L)

pH	A(ml)	B(ml)
6.0	87.7	12.3
7.0	39	61
8.0	5.3	94.7

Diluted to a total of 200 ml.

2. Sodium Acetate Buffer (0.05 M)

A: 0.1 M actic acid solution (5.78 ml in 1L)

B: 0.1 M sodium acetate solution (8.2 g of $C_2H_3O_2Na$ in 1 L)

pH	A(ml)	B(ml)
4.0	41	9
5.0	14.8	35.2

Diluted to a total of 100 ml.

3. Tris-HCl Buffer (0.05 M)(pH 7.4)

50 ml 0.1 M tris(12.1 g in 1 L) + 41.4 ml 0.1 M HCl, diluted to a total of 200 ml.

4. Carbonate Buffer (0.05 M)

A: 0.2 M sodium carbonate solution (21.2 g in 1 L)

B: 0.2 M sodium bicarbonate solution (16.8 g in 1 L)

pH	A(ml)	B(ml)
9.0	4	46
10.0	27.5	22.5

Diluted to a total of 200 ml.

5. Citrate Buffer (0.05 M)

A: 0.1 M citric acid solution (21.01 g in 1L)

B: 0.1 M sodium citrate solution (29.41 g in 1L)

pH	A(ml)	B(ml)
4.0	33	17
5.0	20.5	29.5

Diluted to a total of 100 ml.

6. Glycine- NaOH Buffer (0.05 M)

A: 0.1 M glycine solution (7.5 g in 1L)

B: 0.1 M NaOH

pH	A(ml)	B(ml)
9.0	50	8.8
10.0	50	32
10.6	50	45.5

Diluted to a total of 200 ml.

APPENDIX E

XYLOSE STANDARD CURVE

A stock solution of 1000µg/ml xylose was prepared in 0.05 M sodium phosphate buffer (pH 7.0) and dilutions were made according to following table.

Xylose(µg/ml)	0	25	50	100	150	200
Xylose stock(µl)	0	25	50	100	150	200
Buffer(µl)	1000	975	950	900	850	800

Procedure:

0.9 ml xylan substrates were incubated 5 min in waterbath at 50 ℃. 1.5 ml DNS reagent was added to each tubes and then 0.1 ml of each dilutions were added to tubes, boiled for 10 min. in boiling waterbath and cooled under tapwater. Absorbances were read at 540 nm against a blank containing 0.1 ml buffer instead of xylose dilutions. From the absorbances OD vs concentration graph was prepared and slope was calculated from the best line of graph.

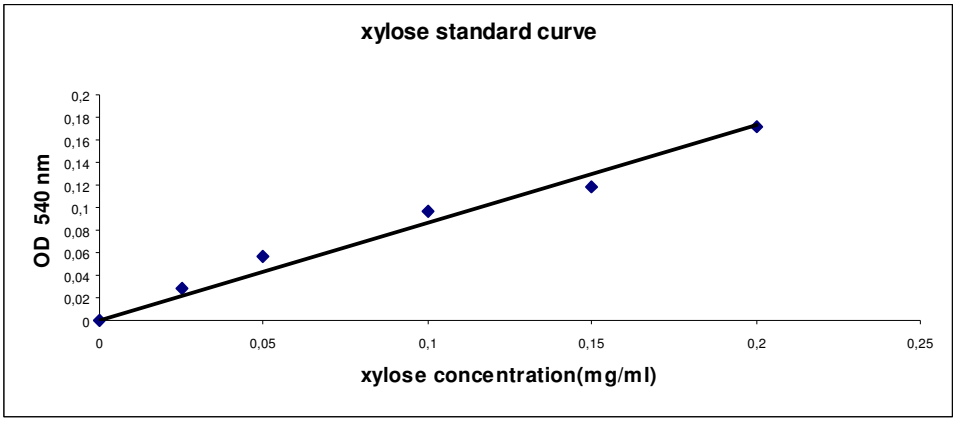


Figure E.1: Xylose standard curve

APPENDIX F

GLUCOSE STANDARD CURVE

A stock solution of 10 mg/ml glucose was prepared in 0.05 M sodium phosphate buffer (pH 7.0) and dilutions were made according to following table.

Glucose(mg/ml)	6.7	5	3.3	2	0
Glucose stock(ml)	1	1	1	1	0
Buffer(ml)	0.5	1	2	4	5

Procedure:

0.5 ml dilutions were added to 1 ml buffer containing tubes and incubated in waterbath at 50 ℃ for 1 hour. Reactions were stopped by adding 3 ml DNS reagent, tubes were boiled for 10 minutes in boiling waterbath and cooled under tapwater. Absorbances were read at 540 nm against a blank containing 0.5 ml buffer instead of glucose dilutions. From the absorbances OD vs concentration graph was prepared and slope was calculated from the best line of graph.

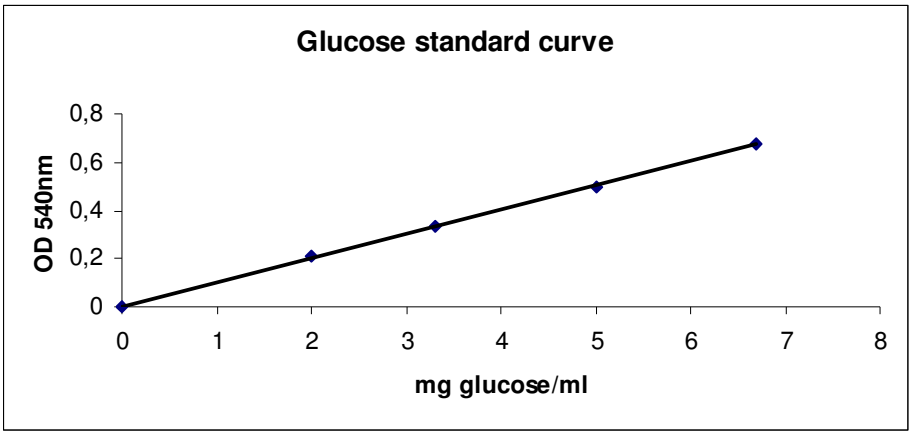


Figure F.1: Glucose standard curve

APPENDIX G

STARCH STANDARD CURVE

A stock solution of 0.5% (g/100ml) starch was prepared in 0.05 M sodium phosphate buffer (pH 7.0) and dilutions were made according to following table

Starch(%)	0.5	0.4	0.3	0.2	0.1	0
Starch stock(ml)	1	0.8	0.6	0.4	0.2	0
Buffer(ml)	0	0.2	0.4	0.6	0.8	1

Procedure:

1 ml of dilutions were mixed with 0.2 ml buffer and incubated in waterbath at 50 ℃ for 10 minutes. 5 ml iodine solution was added to 0.2 ml of reaction mixture. Absorbances were read at 620 nm against a blank containing buffer instead of dilutions. From the absorbances OD vs % starch graph was prepared and slope was calculated from the best line of graph.

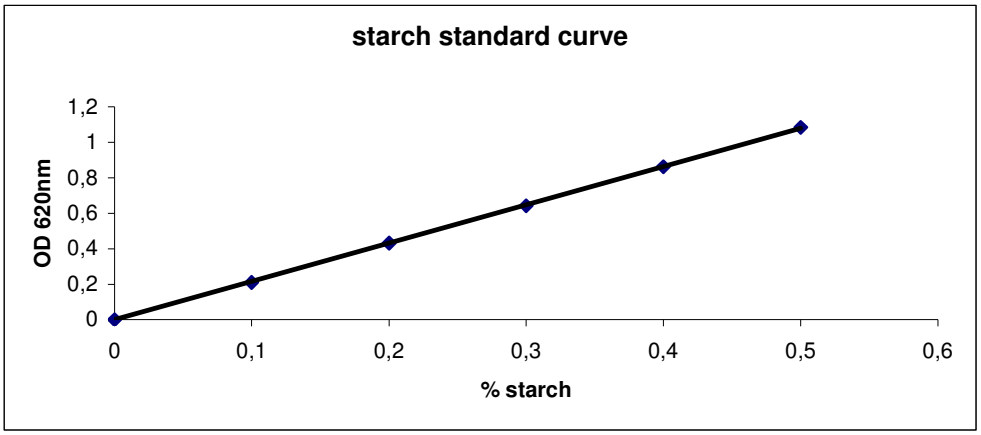


Figure G.1: Starch standard curve

APPENDIX H

ENZYME ACTIVITY- TEMPERATURE GRAPHS

1. Xylanase

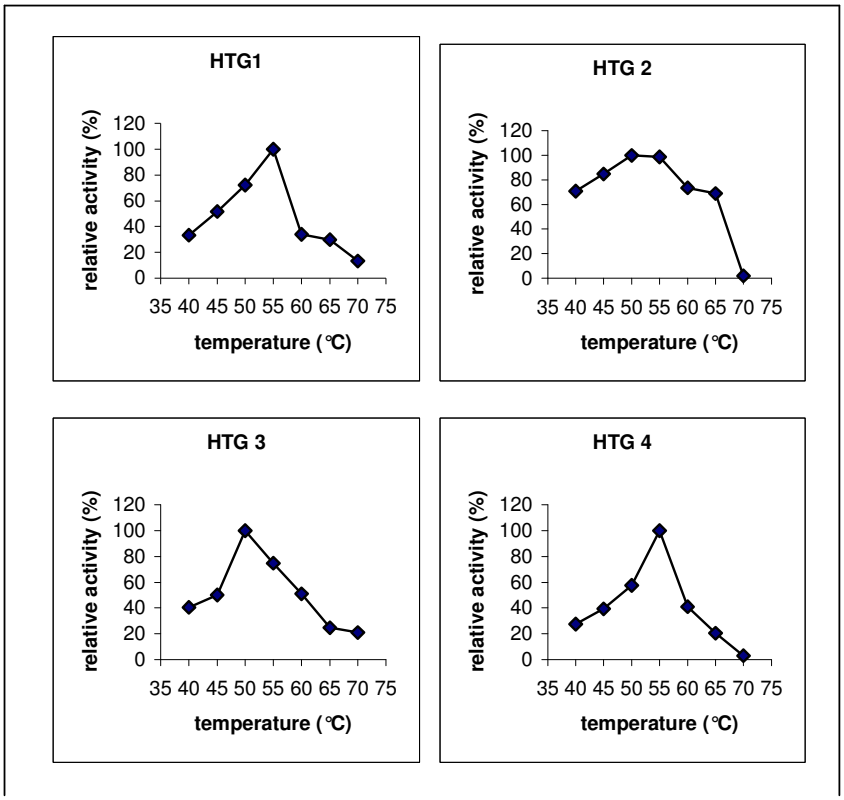


Figure H.1. (continued)

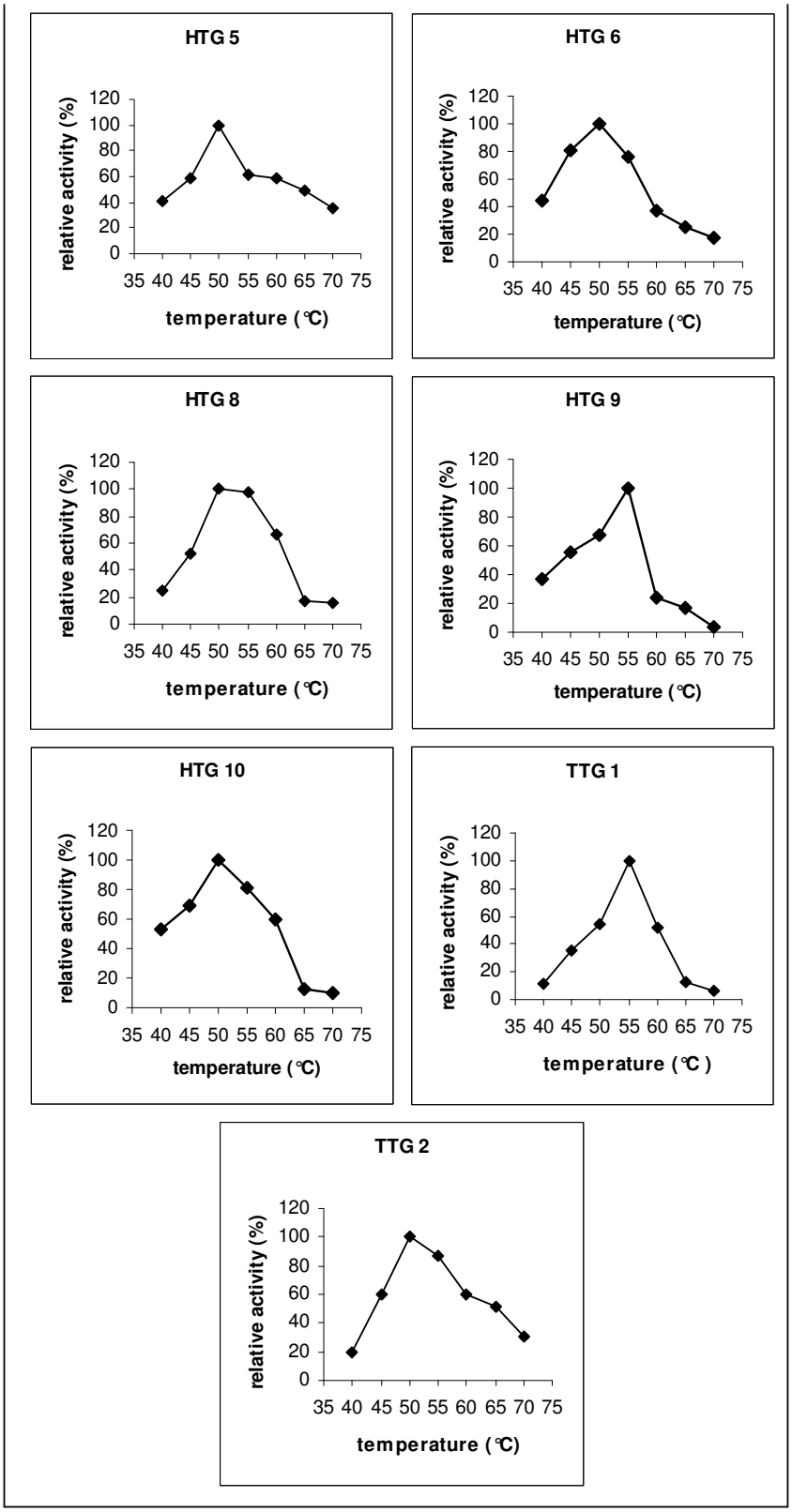


Figure H.1: Enzyme activity-temperature graphs of xylanases

2. α-Amylase

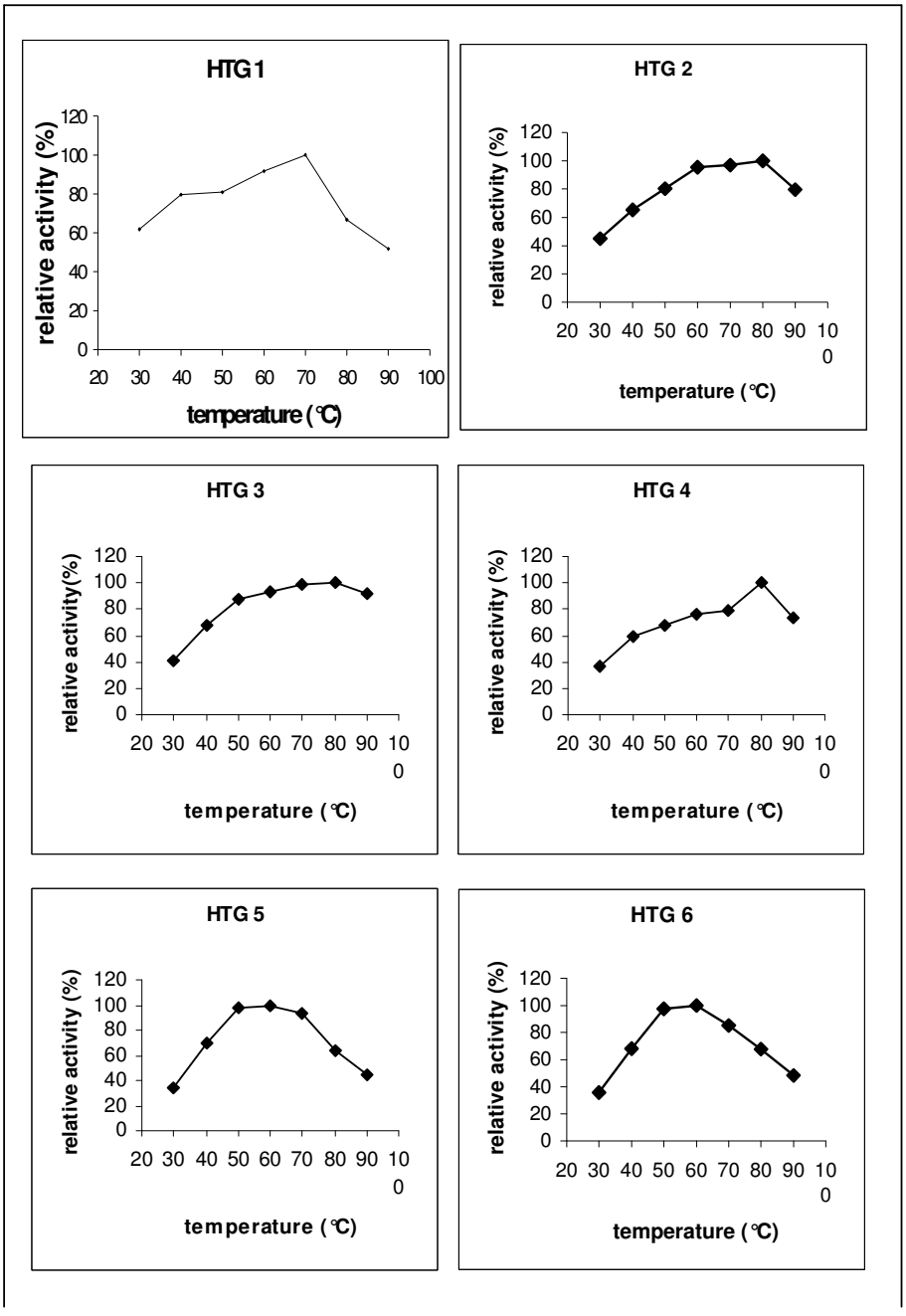


Figure H.2. (continued)

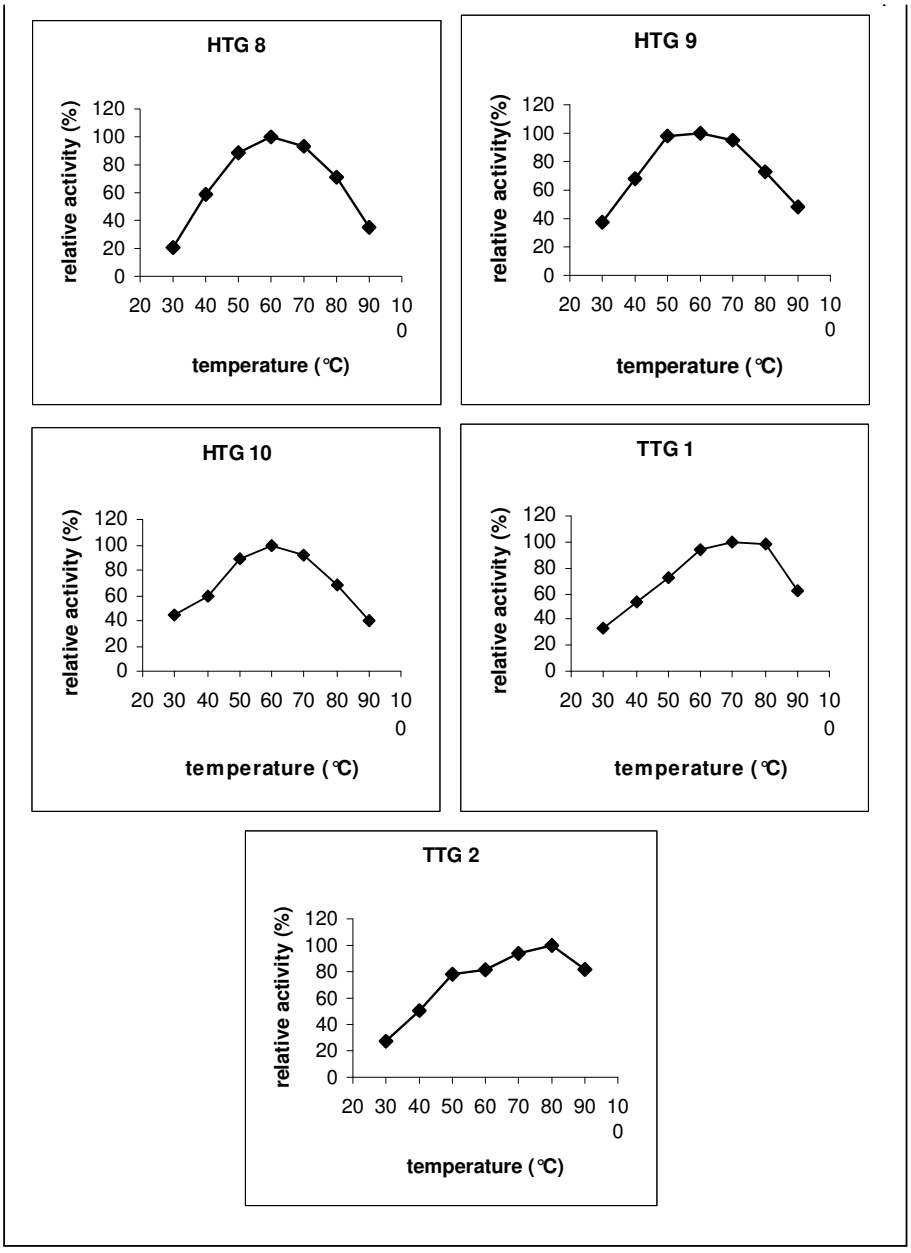


Figure H.2: Enzyme activity-temperature graphs of α-amylases

3. Protease

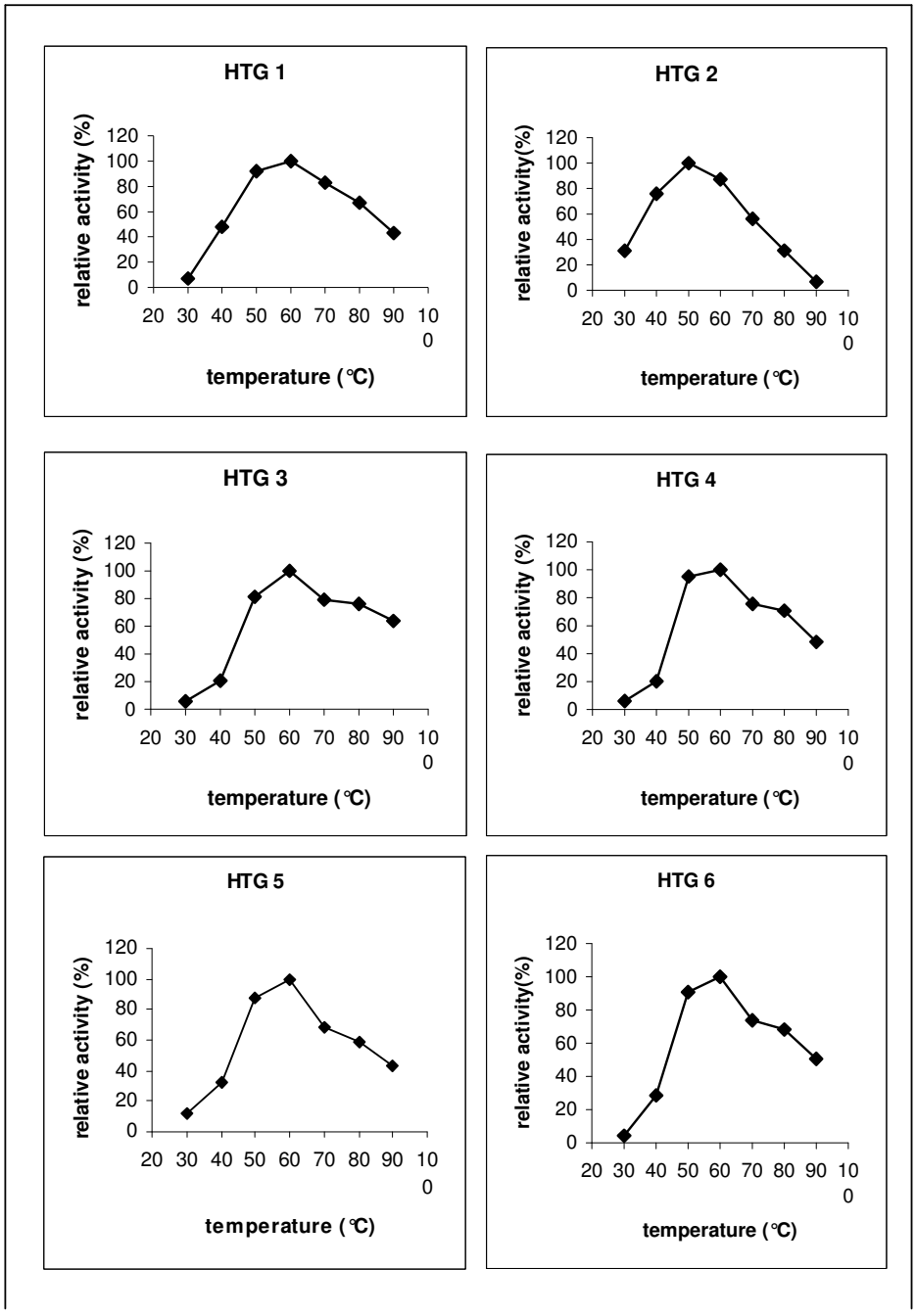


Figure H.3. (continued)

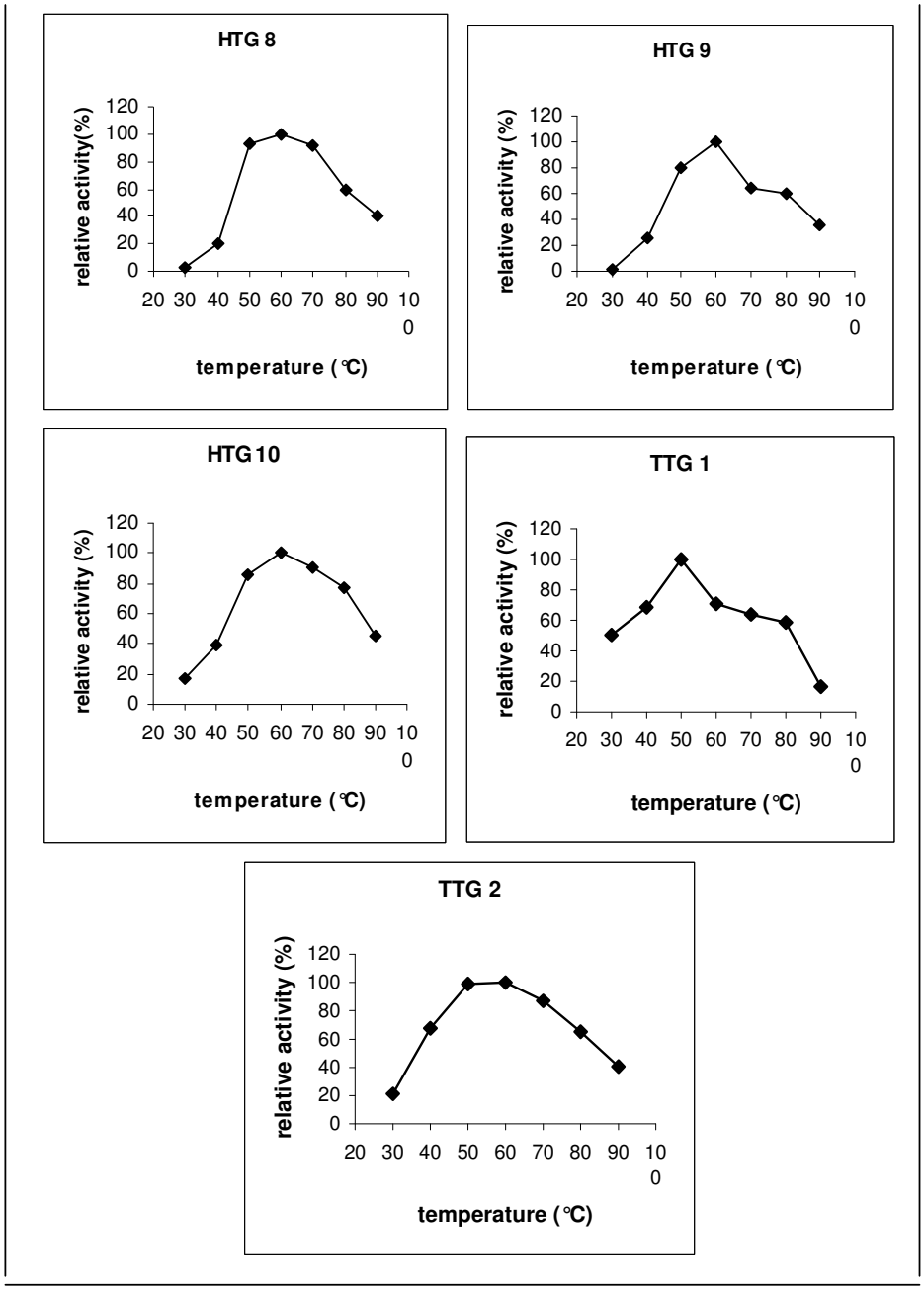


Figure H.3: Enzyme activity-temperature graphs of proteases

APPENDIX I

ENZYME ACTIVITY- pH GRAPHS

1. Xylanase

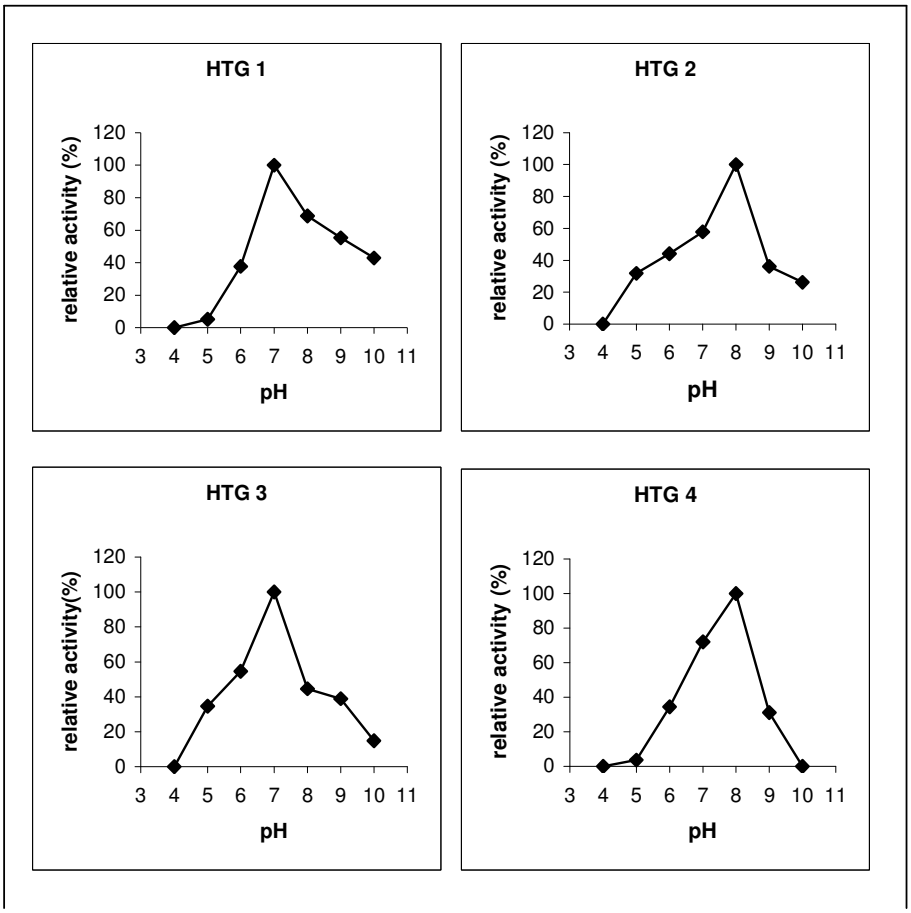


Figure I.1. (continued)

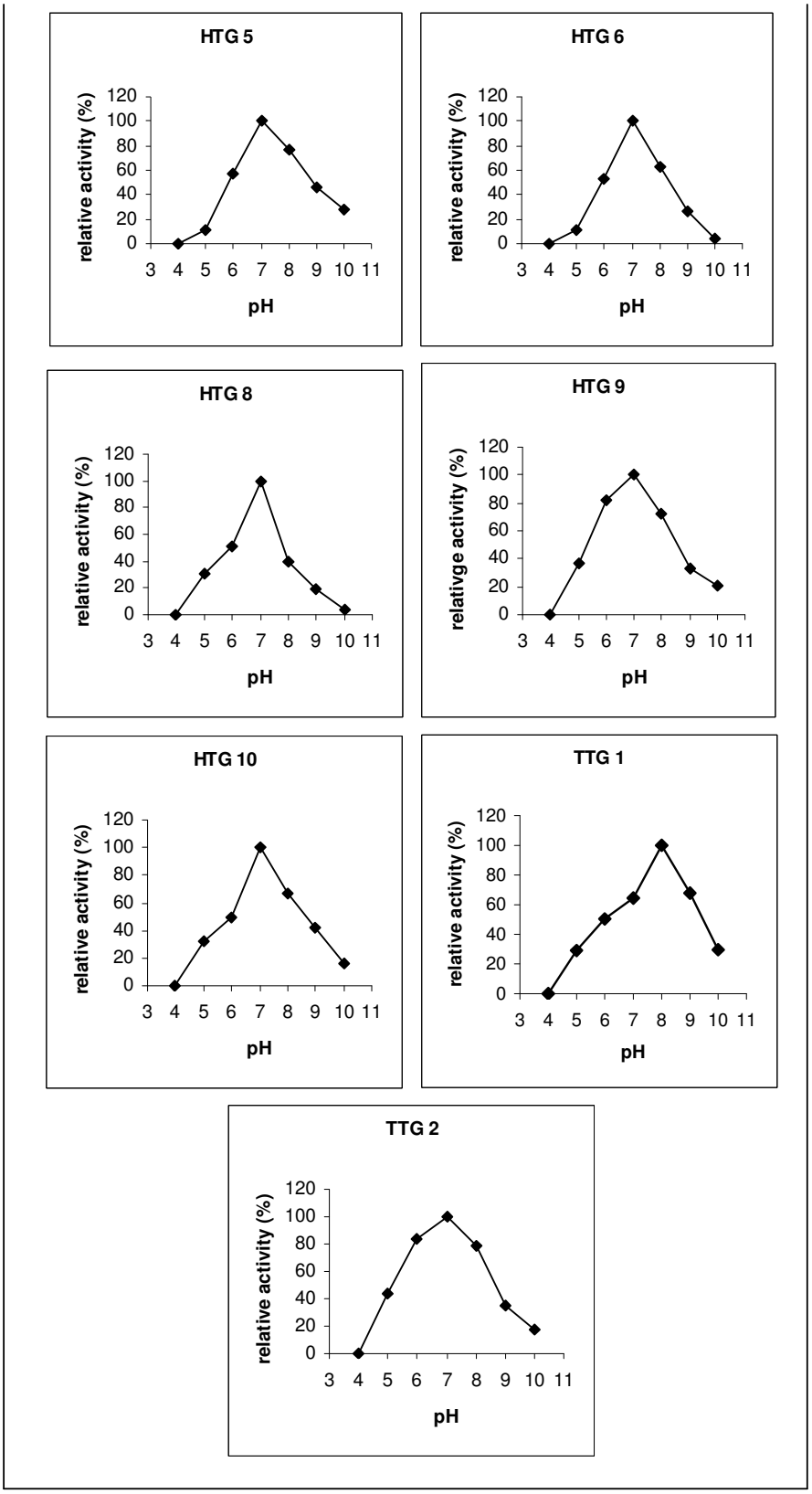


Figure I.1: Enzyme activity- pH graphs of xylanases

2. α-Amylase

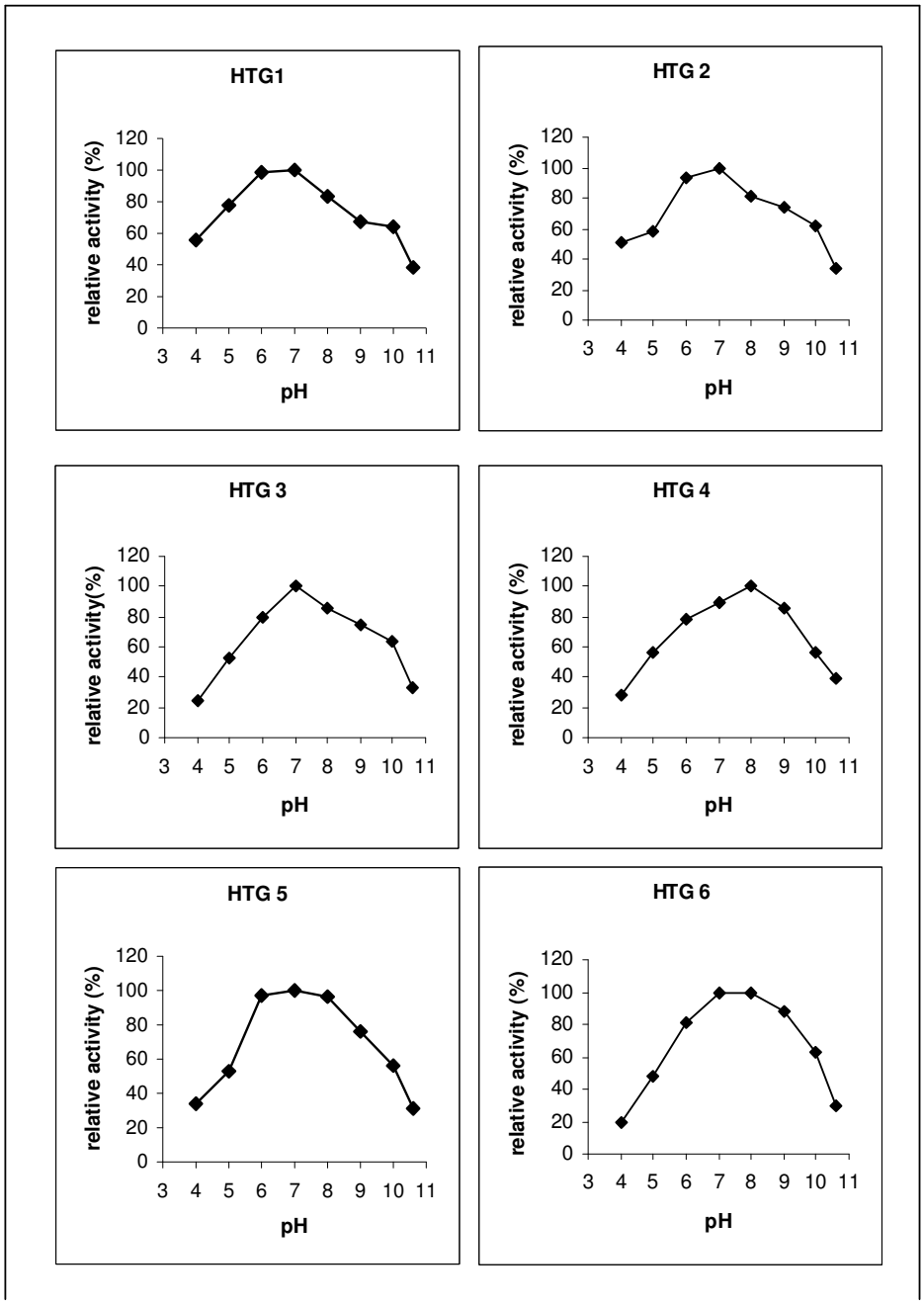


Figure I.2. (continued)

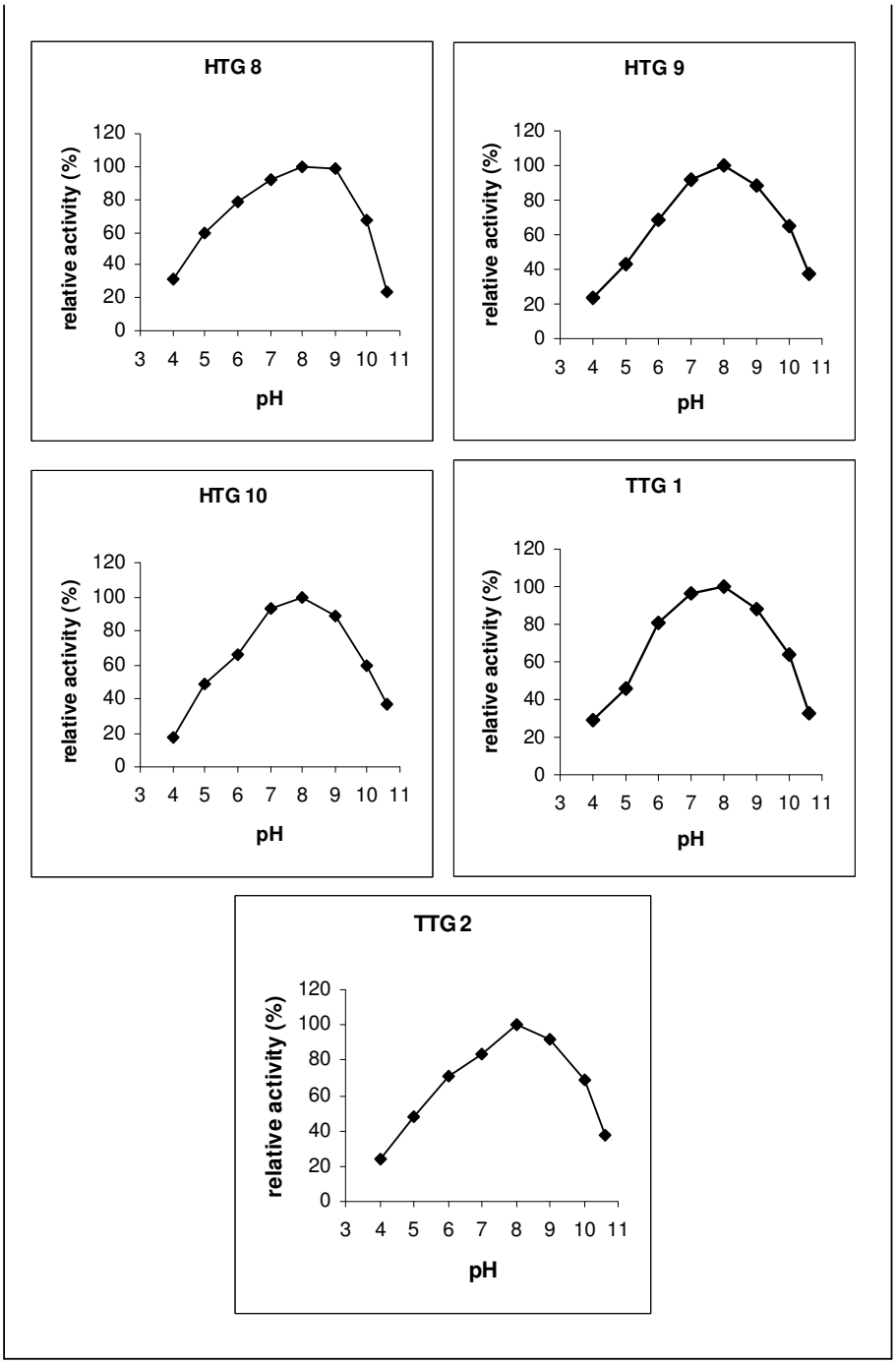


Figure I.2: Enzyme activity- pH graphs of α-amylases

3. Protease

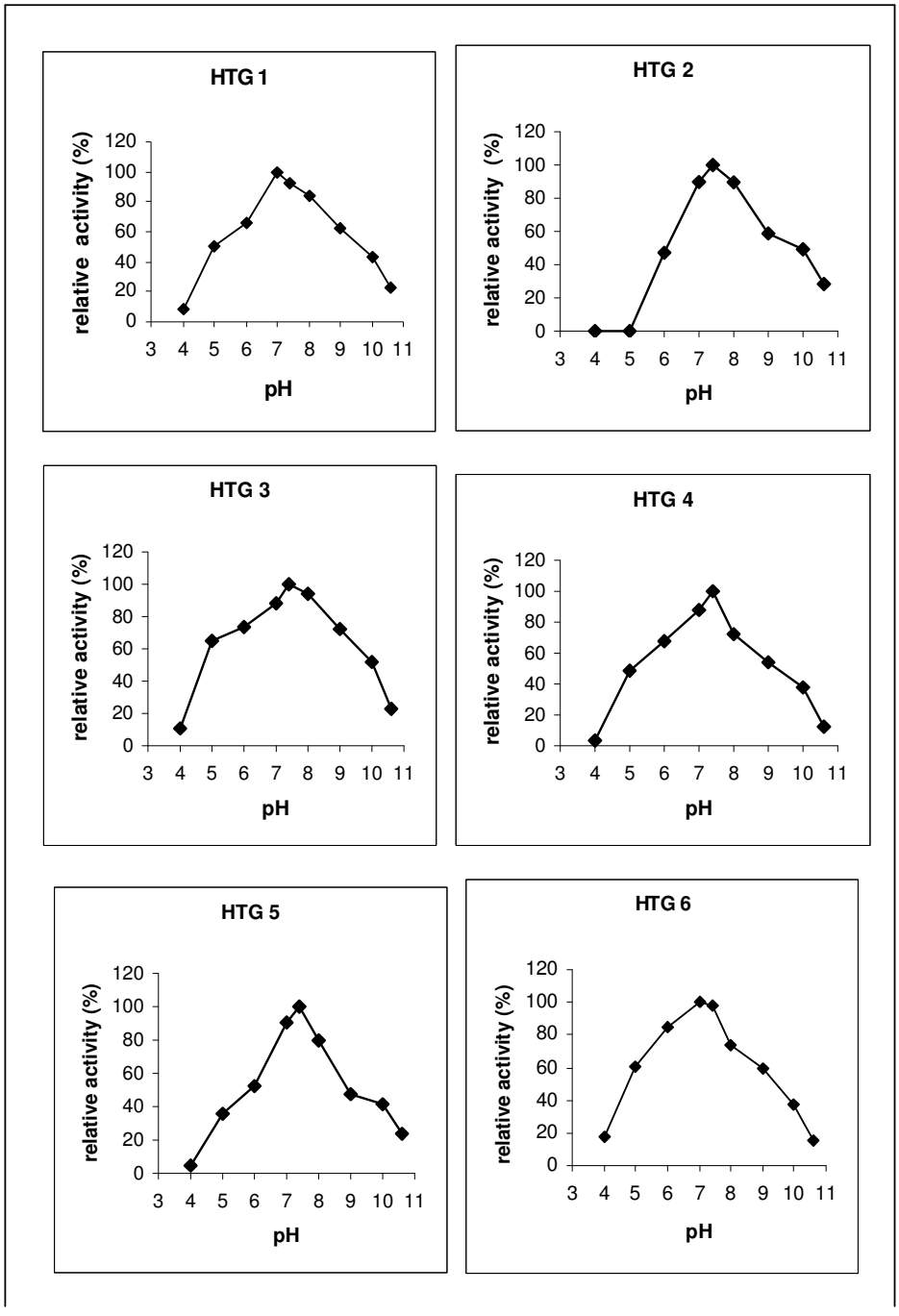


Figure I.3. (continued)

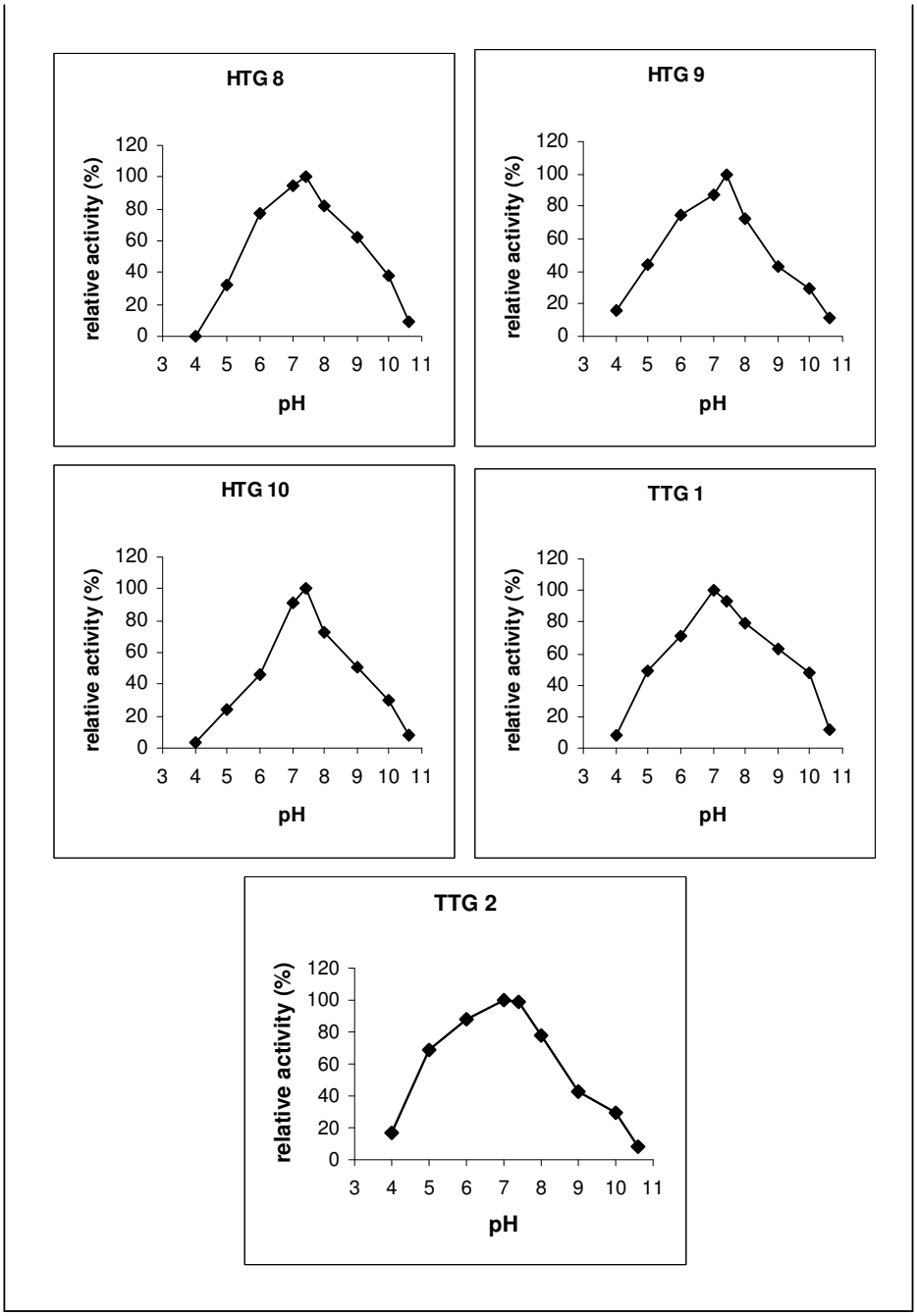


Figure I.3: Enzyme activity- pH graphs of proteases

APPENDIX J

STABILITY GRAPHS OF THE ENZYMES

1. Xylanase

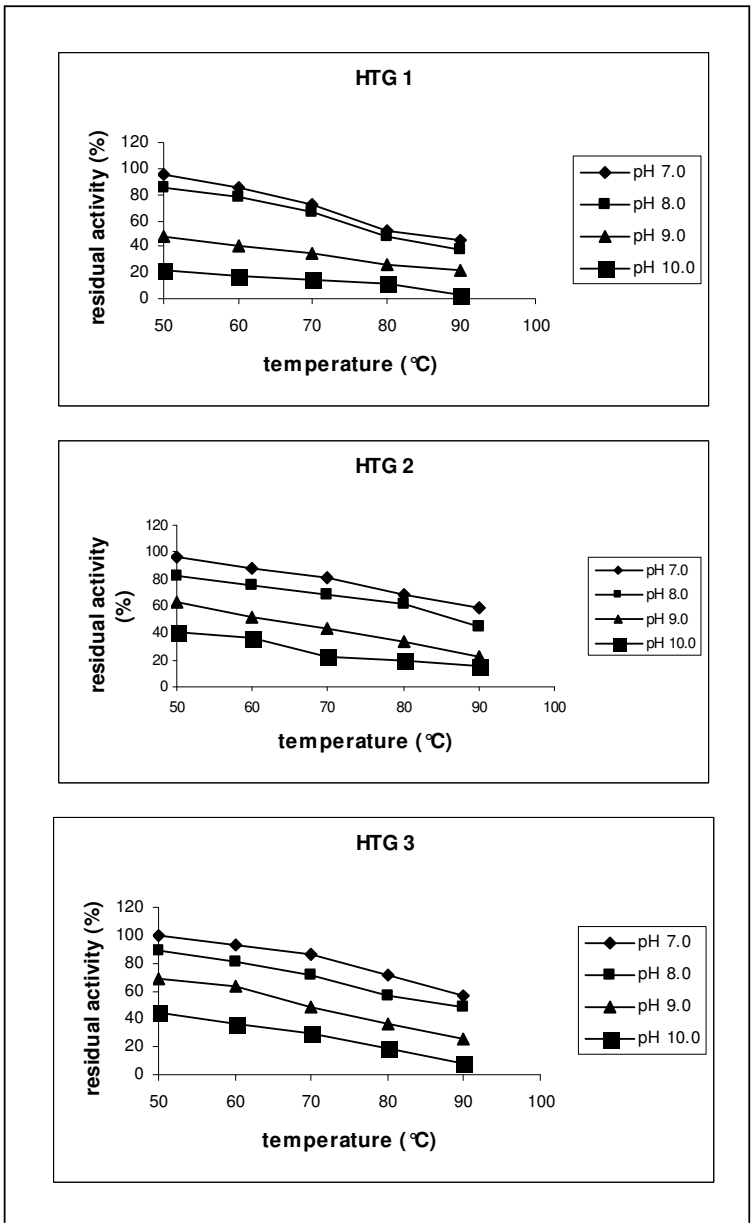


Figure J.1. (continued)

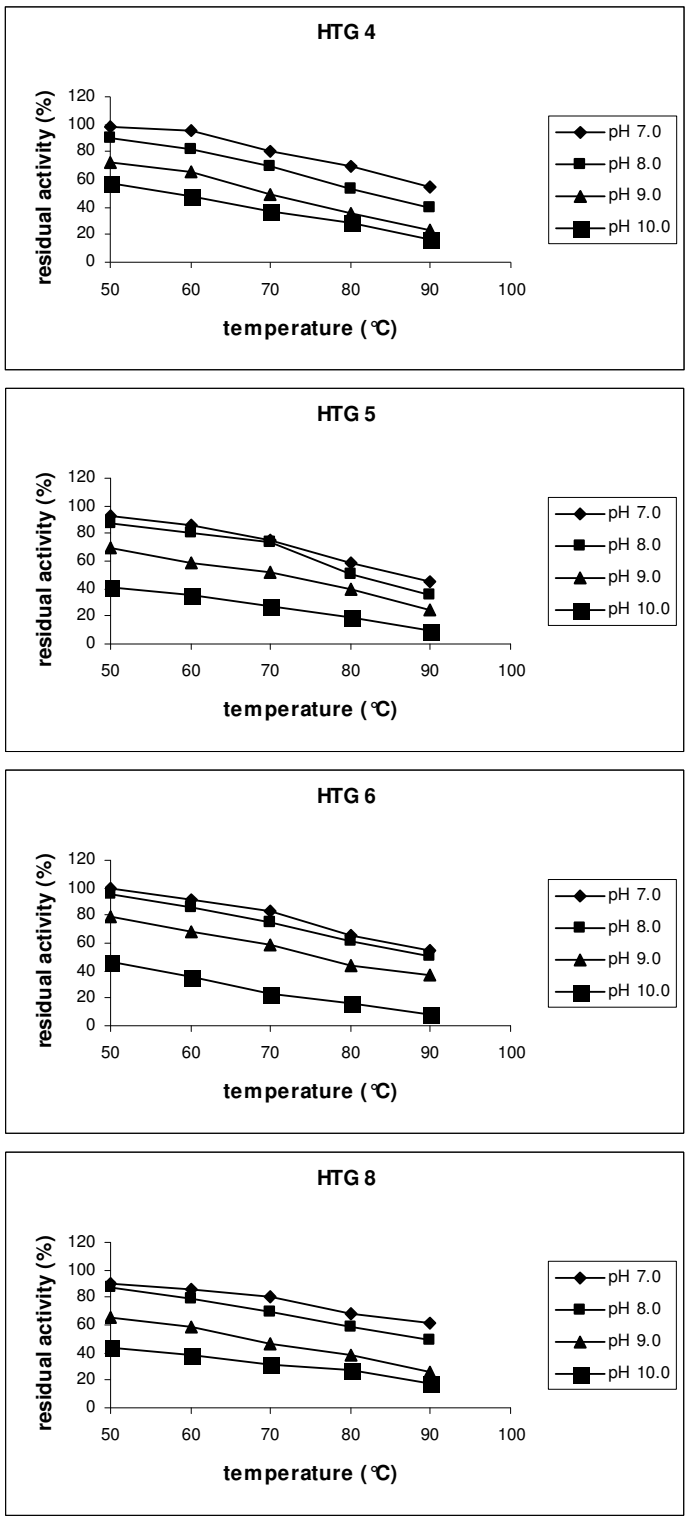


Figure J.1. (continued)

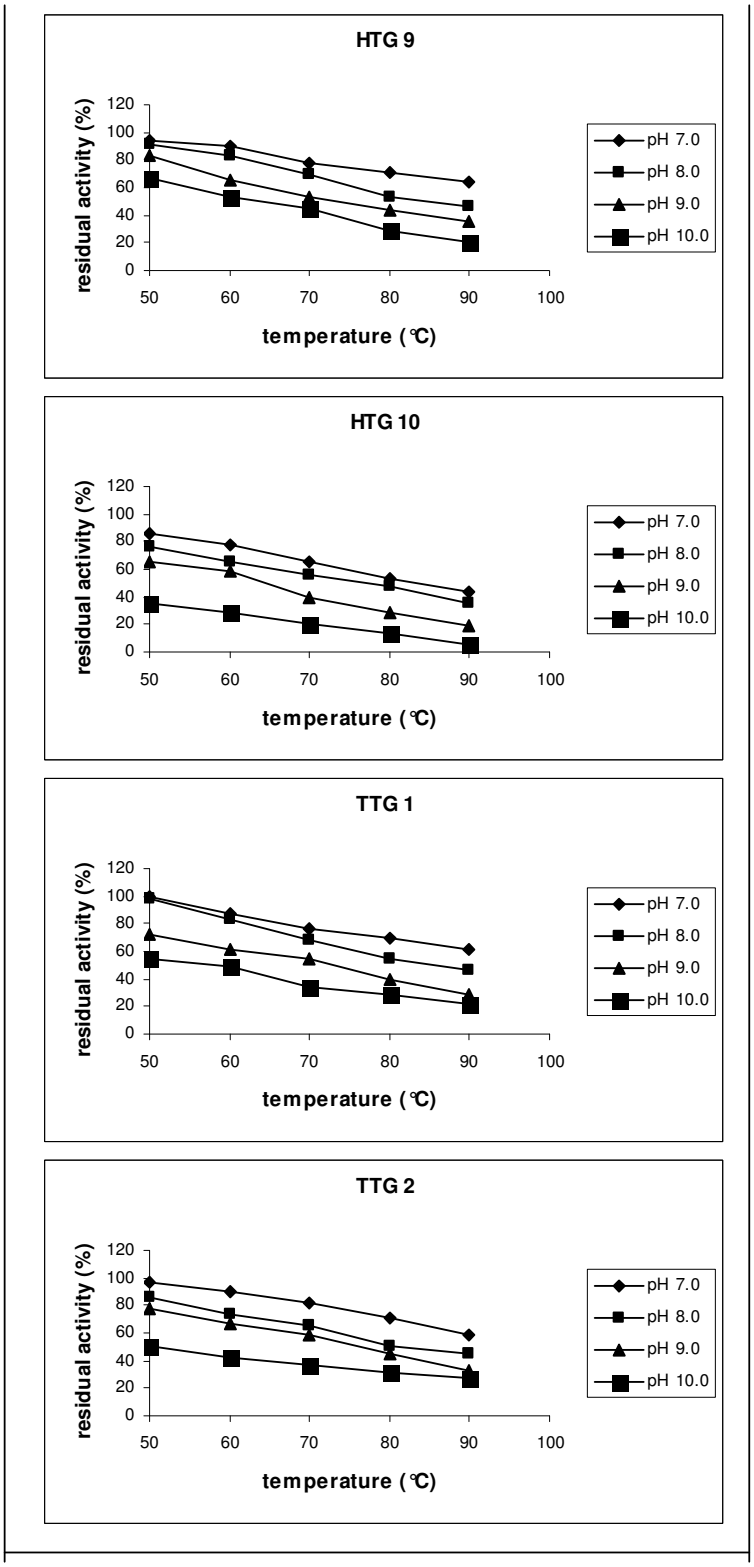


Figure J.1: Stability graphs of xylanases

2. α-Amylase

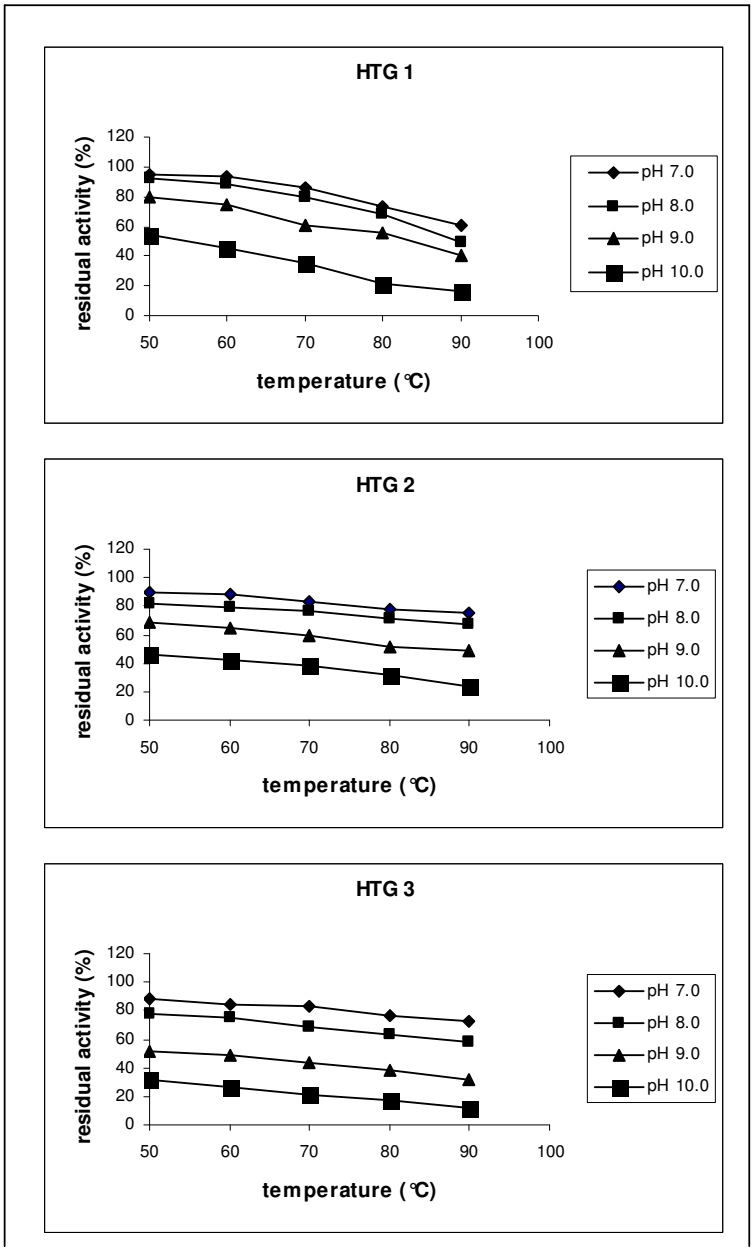
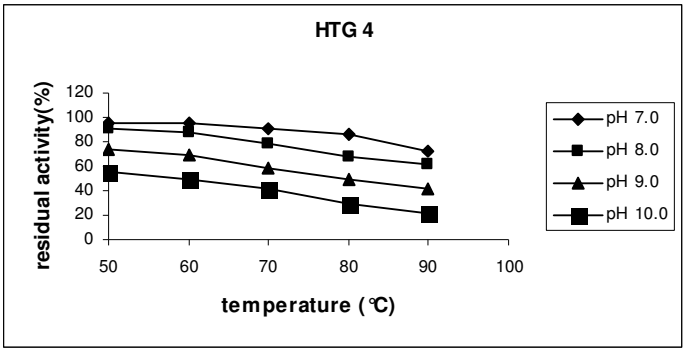
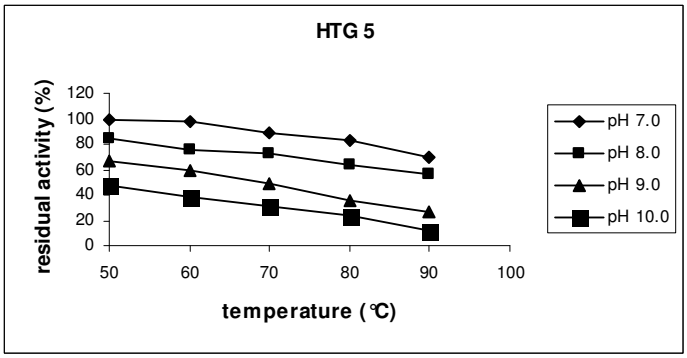
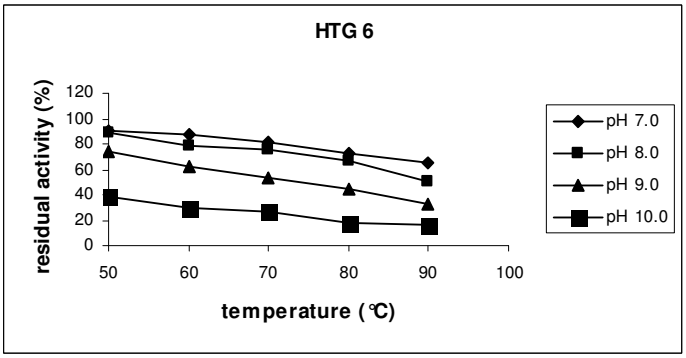


Figure J.2. (continued)







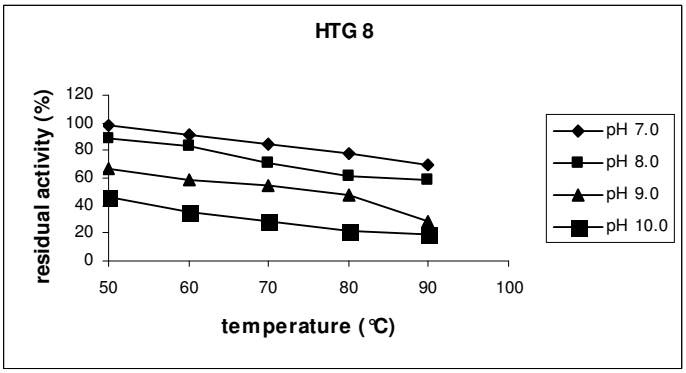


Figure J.2. (continued)

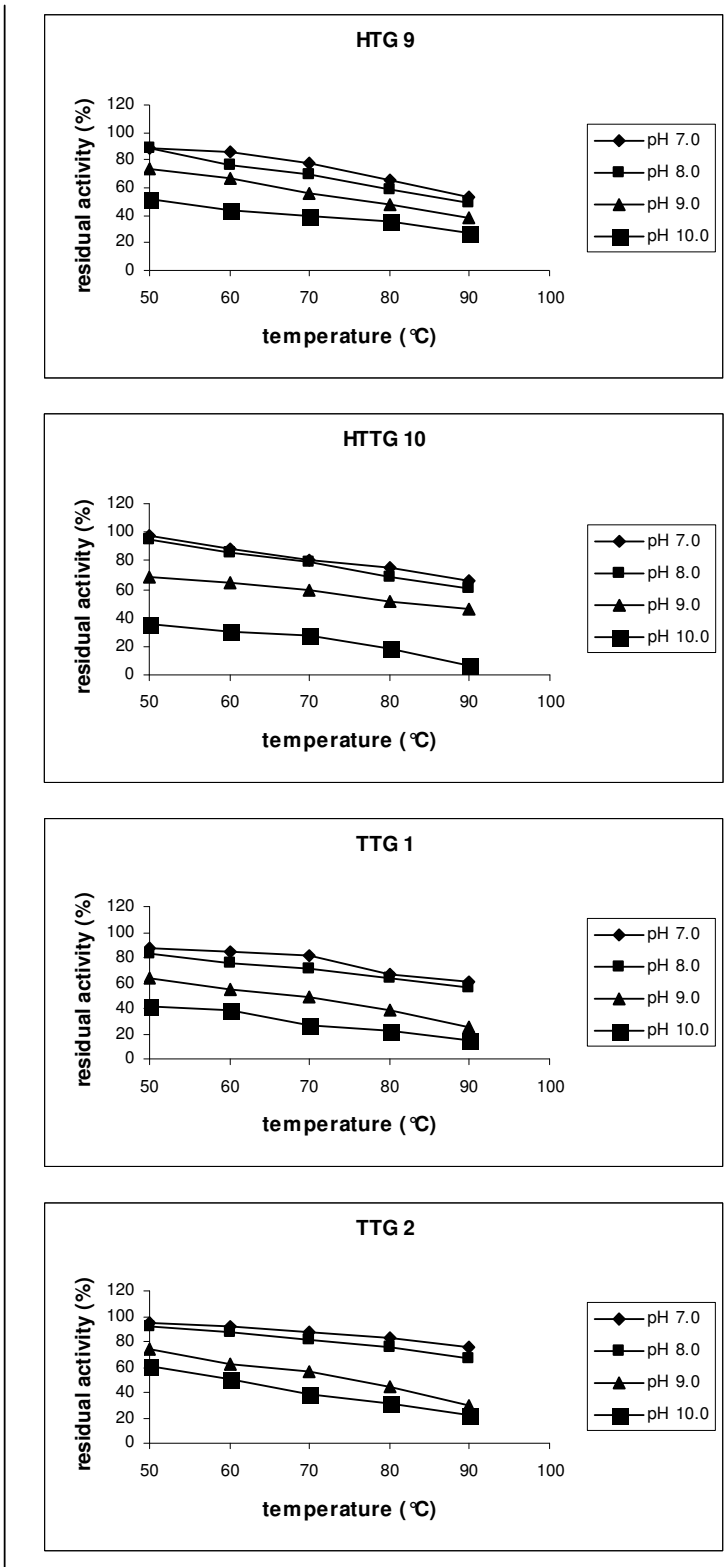


Figure J.2: Stability graphs of α-amylases

3. Protease

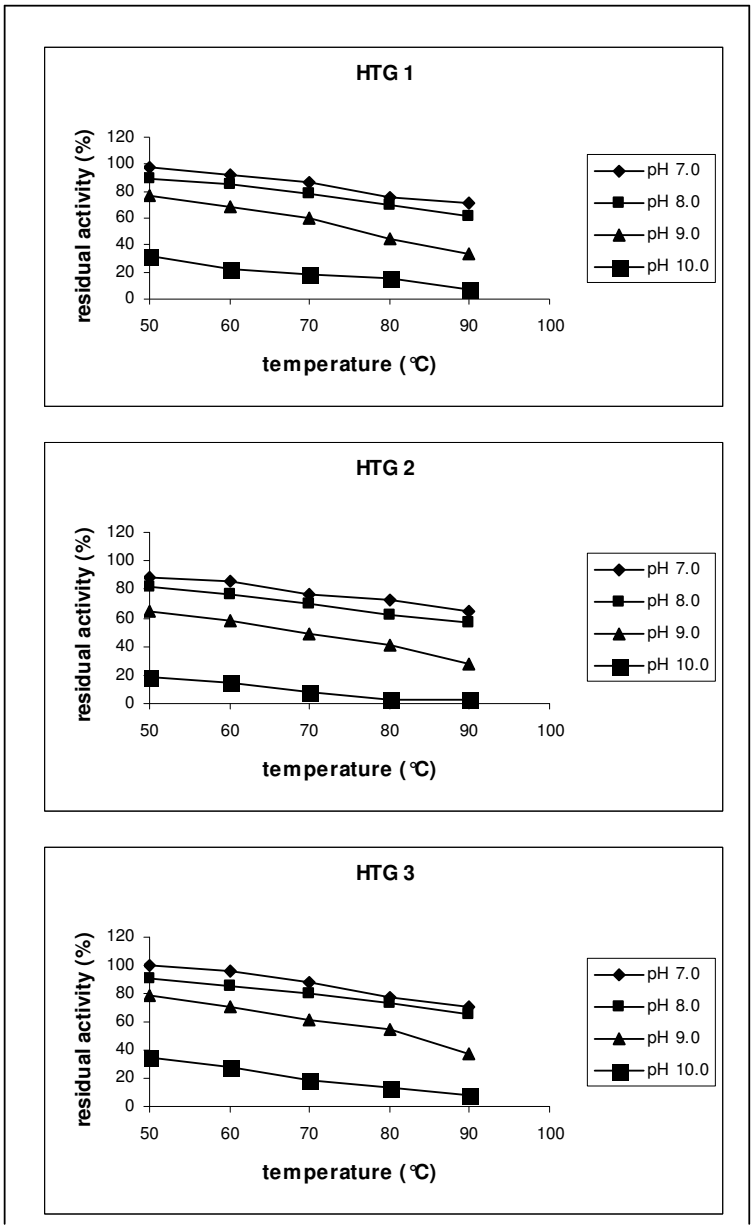
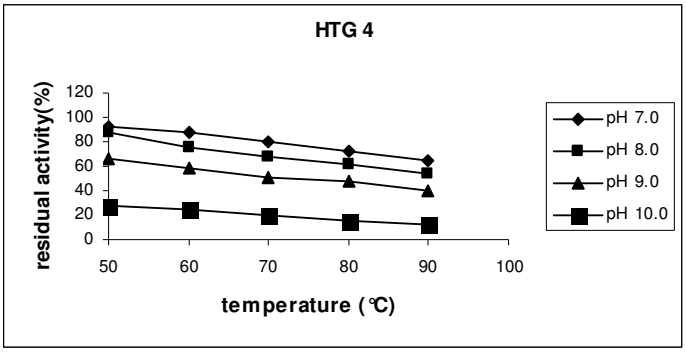
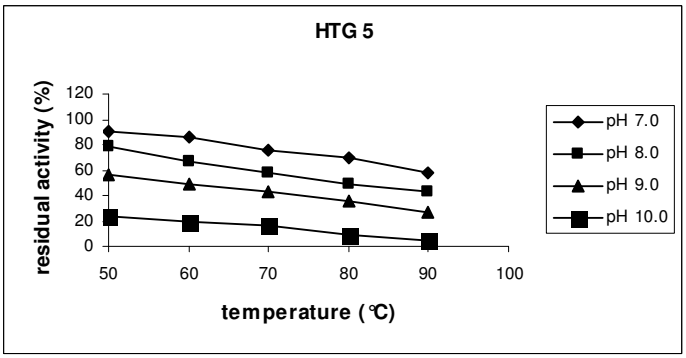
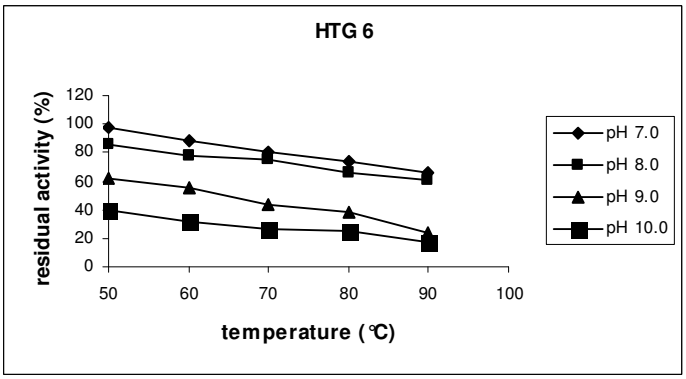


Figure J.3. (continued)







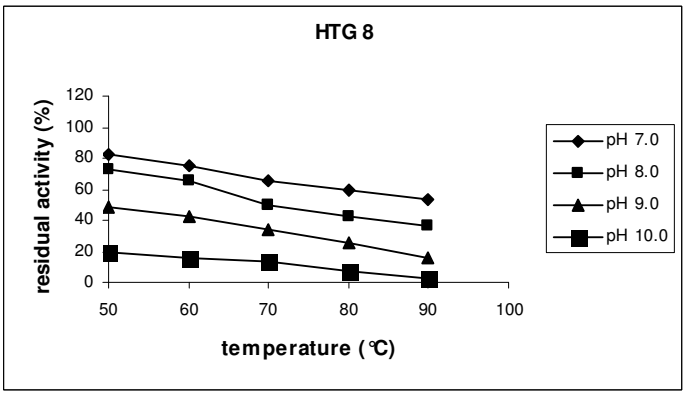


Figure J.3. (continued)

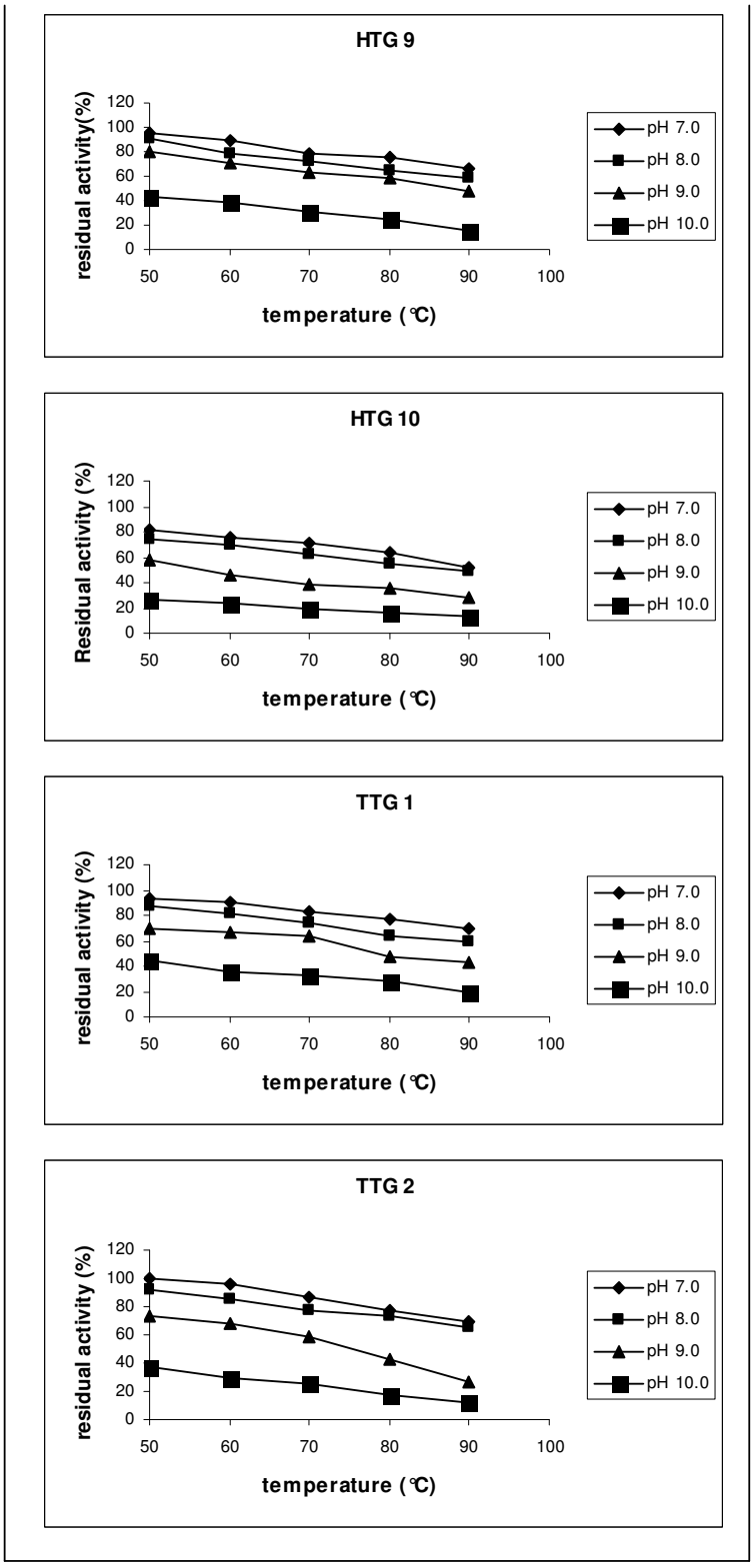


Figure J.3: Stability graphs of proteases