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Biotechnology Department

Genetic and biochemical study on protease produced from locally isolated *Bacillus stearothermophilus*

A Dissertation

Submitted to the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Biotechnology

By

Asmaa Ali Hussein

B.Sc. Biotechnology/ Al-Nahrain University/1999
M.Sc. Biotechnology/ Al-Nahrain University/ 2002

Supervised by

Dr. Hameed M. Jasim
Assistant Professor

Dr. Norrya A. Ali
Professor

December 2009 Thull-hijja 1430

Supervisors certification

We, certify that this dissertation entitled "Genetic and biochemical study on protease produced from locally isolated *Bacillus stearothermophilus*" under our supervision at the College of Science / Al-Nahrain University as a partial fulfillment of the requirements for the Degree of Doctorate of Philosophy in Biotechnology.

Signature:
Supervisor: Dr. Hameed M. Jasim
Scientific Degree: Assistant Professor
Date:

Signature:
Supervisor: Dr. Norrya A. Ali
Scientific Degree: Professor
Date:

In View of the available recommendations, I forward this dissertation for debate by the examining committee.

Signature:

Name: Dr. Kadhim M. Ibrahim

Scientific Degree: Professor

Title: Head of Biotechnology Department

Date:

Committee certification

We, the examining committee certify that we have read this dissertation entitled "Genetic and biochemical study on protease produced from locally isolated *Bacillus stearothermophilus*" and examined the student "Asmaa Ali Hussein" in its contents and that in our opinion, it is accepted for the Degree of Doctorate of Philosophy in Biotechnology.

(Chairman)

Signature:
Name: Dr. AbdulWahid B. Al-Shaibani
Scientific Degree: Professor
Date:

(Member)
Signature:
Name: Dr. Ghazi M. Aziz
Scientific Degree: Assist. Prof.
Date:

(Member)
Signature:
Name: Dr. Sanad B. Al-Arrji
Scientific Degree: Assist. Prof.
Date:

(Member)
Signature:
Name: Dr. Sawsan H. Othman
Scientific Degree: Assist. Prof.
Date:

(Member)
Signature:
Name: Dr. AbdulKareem Al- Kazaz
Scientific Degree: Assist. Prof.
Date:

(Member / Supervisor)
Signature:
Name: Dr. Hameed M. Jasim
Scientific Degree: Assist. Prof.
Date:

(Member / Supervisor)
Signature:
Name: Dr. Norrya A. Ali
Scientific Degree: Prof.
Date:

I, hereby certify upon the decision of the examining committee.

Signature:
Name: Dr. Laith Abdul Aziz Al-Ani
Scientific Degree: Assistant Professor
Title: Dean of the Science College
Date:

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Asmaa

Summary

Fifty three local bacterial isolates of a thermophilic *Bacillus* spp. were obtained from 93 soil samples taken from different locations over Basrah governorate (Iraq). All these isolates were subjected to identification by studying their morphological and microscopical and biochemical characteistics. Results showed that 6 of these isolates belong to *Bacillus stearothermophilus.* The local six *B. stearothermophilus* were screened according to their ability of protease production. Results showed that all these isolates were protease producers. Isolate *B. stearothermophilus* B17 was the most efficient in production when the specific activity of protease in its crude filtrate was 36.4U/mg protein.

Enhancement of protease production in the locally isolated B17 was achieved by two methods:

First. Cloning of protease gene in a cloning vector, and the recombinant molecules were used to transform suitable host. This was achieved first by amplification of protease gene from B17 using polymerase chain reaction (PCR) technique, then the amplified fragments coding for protease were cloned into pJET blunt cloning vector, and the recombinant molecule was used to transform *Escherichia coli* DH5α deficient in protease production (Pr^{-}). Results showed that many transformants of *E. coli* were able to make a halo of hydrolysis around each clone on skim milk agar medium after incubation at 37°C for 24hours. Specific activity in the crude filtrate of the most efficient clone (symboled DH5α5) in protease production was 42 U/mg protein.

In another attempt for cloning protease gene from *B. stearothermophilus*, genomic DNA of B17 was isolated, partially digested with *Sau*3A, and ligated to *Bam*HI digest of pUC18. The recombinant

molecules were used to transform *E. coli* DH5α (Pr^-), then from about 2000 ampicillin resist transformants, 6 clones (0.3%) were able to form a halo of hydrolysis on skim milk agar plates due to their ability in protease production. The specific activity of protease in crude filtrate of the most efficient clone (symboled DH5αP7) was 39.5 U/mg protein.

Second. Subjection to random mutagenesis using physical and chemical mutagens. This was achieved by subjection of the wild type B17 to physical mutagen using UV ray under different doses of irradiation (1, 2, 3, 4, 5 and 6 Jole/m^2). Results showed that 6 mutants out of 45 (13.3 %) were able to make a halo of hydrolysis on skim milk agar plates after incubation at 55°C for 24 hours. These mutants were screened for protease production, and it was found that all mutants were protease producers; the specific activity of protease in crude filtrate of the most efficient over producer mutant (B17U1) was 80.08 U/mg protein in comparison with 36.4 U/mg protein for the wild type B17.

Chemical mutagenesis was also used to enhance the ability of B17 in protease production by incubation with 1-methyl-3-nitro-1-nitroso-guanidine (MNNG) in a concentration of 200 µg/ml for different periods of time (20, 40, 60, 80, and 100 minutes). Results showed that 3 mutants out of 32 (9.3%) were able to make a halo of hydrolysis on skim milk agar plates after incubation at 55°C for 24h. After screening the mutants for protease production, all of these mutants were protease producers; the specific activity of protease in culture filtrate of the most efficient over producer mutant (B17M1) was 99 U/mg protein in comparison with 36.4 U/mg protein for the wild type B17.

Optimum conditions for protease production by the most efficient over producer mutant B17M1 was studied, and it was found that cultivation of this mutant in the production medium containing 1% of each of starch and yeast

extract, pH 7, for 18h. at 55°C gave higher productivity of protease when the specific activity of enzyme in crude filtrate that reached 99 U/mg protein.

Protease produced by the most effecient mutant under the optimum conditions was purified by several steps including precipitation by ammonium sulfate (80% saturation), dialysis, affinity chromatography using Bacitracin-silica column, and finally gel filtration using Superdex-75 column with 28.3 fold of purification and % 42.5 yield.

Result of characterization of the purified enzyme showed that the molecular weight of protease was 31,000 dalton; the enzyme was active at pH 7, and stable within a pH range 6-7, active at 60°C, stable till 70°C for 30 min. Results also showed that protease produced by *B. stearothermophilus* B17M1 was a metalloenzyme because its enzyme activity was completely inhibited after incubation with 2mM EDTA.

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List of Abbreviations

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
CFU	Colony forming unit
Da	Dalton
DEAE	Diethyl amino ethyl
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotide trisphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methyl sulphonate
FPLC	Fast protein liquid chromatography
IUBMB	International union of biochemistry and molecular biology
J/m^2	$Jole/meter^2$
MNNG	1-methyl-3-nitro-1-nitroso-guanidine
MR-VP	Methyl red- Voges Proskauer
O.D.	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PMSF	phenylmethansulfonyl floride
Pr^-	Protease deficient
Rm	Relative mobility
rpm	Rotation per minutes
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris acetate electrophoresis buffer
TCA	Trichloroacetic acid
TEMED	N,N,N,N-Tetramethylene diamine
TLPs	Thermolysin like protease
TNBSA	Trinitrobenzenesulfonic acid

List of Abbreviations

BCA	Bicinchoninic acid
BSA	Bovine serum albumin
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DEAE	Diethyl amino ethyl
DMSO	Dimethyl sulphoxide
dNTP	Deoxyribonucleotide trisphosphate
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethyl methyl sulphonate
FPLC	Fast protein liquid chromatography
IUBMB	International union of biochemistry and molecular biology
J/m^2	$Jole/meter^2$
MNNG	1-methyl-3-nitro-1-nitroso-guanidine
MR-VP	Methyl red- Voges Proskauer
O.D.	Optical density
ORF	Open reading frame
PCR	Polymerase chain reaction
PMSF	phenylmethansulfonyl floride
Pr^-	Protease deficient
Rm	Relative mobility
rpm	Rotation per minutes
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TAE	Tris acetate electrophoresis buffer
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TLPs	Thermolysin like protease
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Chapter One
Introduction
and
Literatures Review

1.1 Introduction

Microbial life does not seem to be limited to specific environments. During the past few decades it has become clear that microbial communities can be found in the most diverse conditions, including extremes of temperature, pressure, salinity, and pH. These microorganisms, called extremophiles, produce biocatalysts that are functional under extreme conditions and the unique properties of these biocatalysts have resulted in several novel applications of enzymes in industrial processes (Bertus, 2003). The recent advances in the field of biocatalysts have enabled the biological processes to compete successfully with conventional chemical processing. Combination of chemical and biocatalytic systems are being developed thereby utilizing the most attractive features of biocatalysts i.e. enzymes, namely high specificity with less side or waste products and higher yields, mild reaction conditions and usually low environmental impacts (Sorup *et al*., 1998). With better knowledge and purification of enzymes the number of applications has increased many folds, and with the availability of thermostable enzymes a number of new possibilities for industrial processes have emerged. Thermostable enzymes, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability (Demirijan *et al*., 2001; Haki and Rackshit, 2003; Richard and Jim, 2007).

Many species of the genus *Bacillus* produce a variety of extracellular and intracellular proteases, which are critical to the maintenance of cellular function (Ronald *et al*., 2002) They hydrolyze both external and internal nutrient sources and recognize and break down unneeded or abnormal polypeptides; the latter produced as a result of environmental stress, mutation or errors in biosynthetic processes (Tomoyasu *et al*., 2001). The protease enzyme constituted two third of the total enzyme used in various industries (Gupta *et al*., 2002a). It is applied in pharmaceutical, food, and detergent

industries, waste treatments and others (Marek and Sajja, 2004; Karel, 2006). Protease that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations, whereas those that are used in medicine are produced in small amounts but require extensive purification before they can be used (Sharmin and Rahman, 2007).

New industrial enzymes produced by *Bacillus* strains such as proteases have emerged as a result of mutations and cloning, with desired specificity, activity, and stability properties. By the same token, the cloning of a structural gene for an extracellular protease from the thermophilic *B. stearothermophilus* would be significant practically as well as academically (Marcus *et al*., 2004). Since the preferred method of obtaining enzyme with improved thermostability from naturally occurring thermophilic organisms, with yields may be low because of imprecise growth conditions, in addition, high- temperature fermentations may require specialized equipment is to apply recombinant gene technology to isolate, clone, and express thermophilic genes of interest in mesophilic organisms (Bryce *et al*., 1994). On the other hand, direct evolution tools have been increasingly used to improve enzymes and whole genomes for various bioprocessing applications and numerous molecular biology techniques have been developed to create genetic diversity through mutagenesis. Several attempts of mutagenesis aimed at increasing enzymes productivity using physical and chemical mutagenesis methods (Rivera *et al*., 2003). Also, the availability of cloned genes and the use of random mutagenesis have facilitated the production of enzymes with higher activities (Singh, 1999).

According to those mentioned above, this study was aimed to isolate a locally higher protease producer thermophilic *Bacillus* sp. and to improve its ability in protease production and this was achieved by:

1. Isolation of thermophilic *Bacillus* sp. from different soil samples.

2. Screening the ability of local isolates for ability in protease production and select the efficient one for enzyme production.
3. Cloning and expression of *Bacillus* protease gene in a suitable host of *E. coli* strain.
4. Improving the ability of the selected isolate in protease production by physical and chemical mutagenesis.
5. Optimization, purification and characterization of protease produced by the over-producer mutant.

1.2 Literatures review

1.2.1 The genus *Bacillus*

In 1872, Ferdinand Cohn, a student of Robert Koch, recognized a rod-like bacteria in the soil and named it *Bacillus subtilis*. This organism is part of large and diverse genus of bacteria, the genus Bacillus, and was placed in the family Bacillaceae. Members of the genus Bacillus are characterized as Gram-positive, rod-shaped, aerobic or facultative anaerobic, endospore-forming bacteria (Bergquist, 1987; Yang Yang, 2007). The genus includes thermophilic and psychrophilic, acidophilic and alkalophilic, fresh- water and halophilic bacteria that utilize a wide range of carbon sources for heterotrophic growth or grow autotrophically. *Bacillus* include both free living and pathogenic species .Under stressful environmental conditions, the cells oval endospore can stay dormant for extend periods (Turnbull, 1996). Born *et al*. (1999) mentioned that the nonpathogenity of most species and their ability to secrete proteins make these bacteria interesting for the use in the pharmaceutical, food, and cosmetics industry. Bacilli produce many different industrially important enzymes, including, proteases, amylases, glucanases, lipases, nucleases, and phosphatases. On the other hand Bacilli currently account for 60% of the commercially available proteins synthesized on an economical scale. Majority of these proteins are homologous proteins that are naturally secreted into the growth medium, such as proteases and amylases (Schallmey *et al*., 2004; Quax *et al*., 2004). As microbiologists in the 1990's explored earth's extreme environments, novel endospore-forming halophiles, acidophiles, alkaliphiles, and thermophiles turned up in large numbers. One might expect the list of approved *Bacillus*-like genera to expand rapidly during the coming decade (Figure 1.1) (Nazina *et al*., 2001).

1.2 Literatures review

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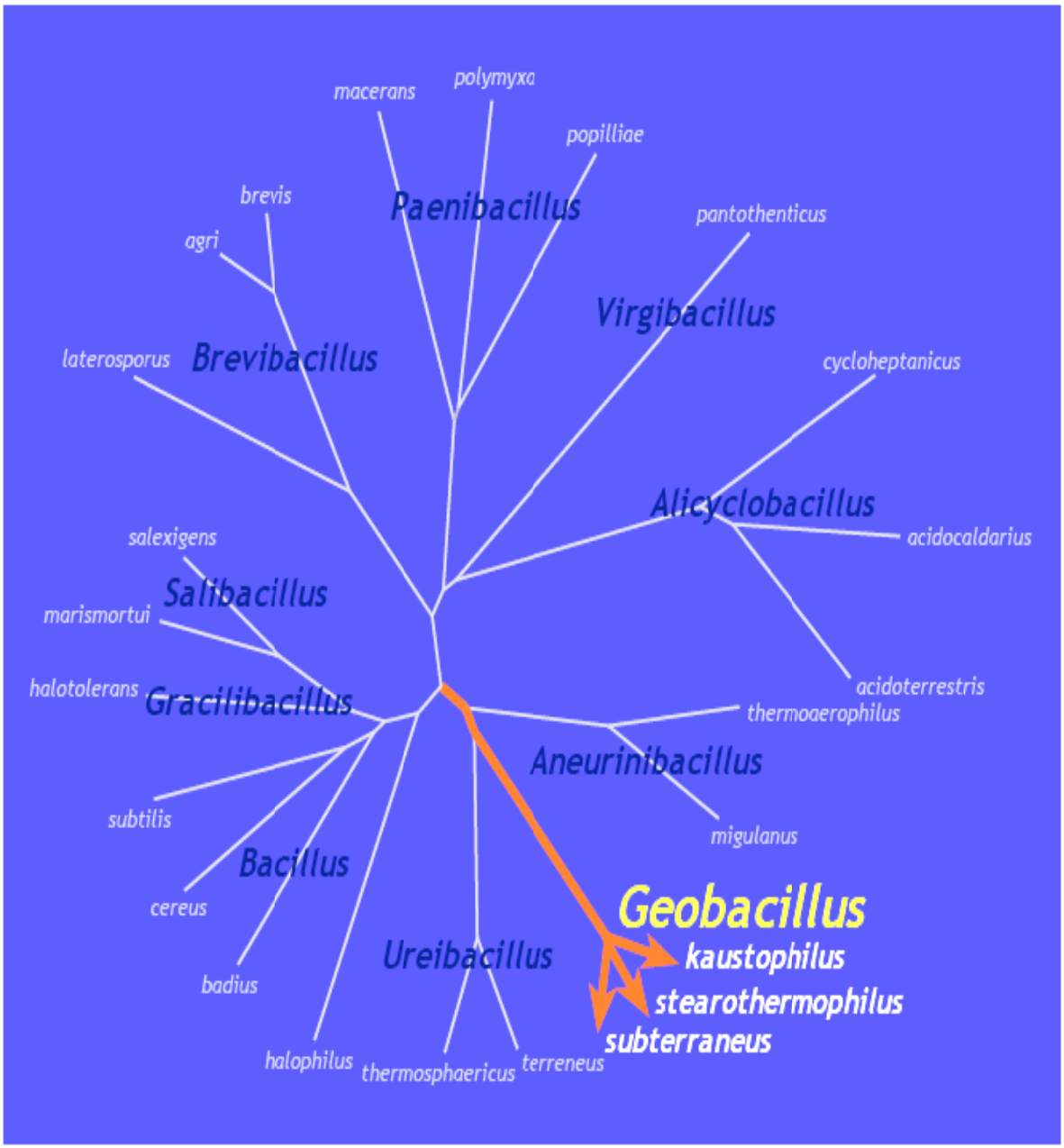

Figure (1.1): Phylogenetic tree based on 16S rRNA gene alignments

(Nazina *et al*., 2001)

1.2.1.1 *Bacillus stearothermophilus*

Bacillus stearothermophilus (or *Geobacillus stearothermophilus*) is a rod shaped, Gram-positive, strictly aerobic species of endospore-forming bacterium, and it's widely distributed in soil, hotspring, and ocean sediment. Among the characteristics which was identified the bacterium belonging to *Bacillus stearothermophilus* with "Bergy's manual of systematic bacteriology": It grows at 65°C, it is absolute aerobic, it forms spores, it is Gram positive, and it produces catalase (Imanaka *et al*., 1981).

Bacillus stearothermophilus is of special interest with respect to thermostability as well as chemostability of enzymes (Bergquist *et al*., 1987). The proteins and enzymes of thermophiles and hyperthermophiles are very heat stable, upon modifications of these proteins in a number of ways and through slight changes in their primary structures, accounts for their thermal stability (Claire and Gregory, 2001). On the other hand, the thermophilic *Bacillus* offer significant potential for biotechnological applications (α-arabinofuranosidase, acetate kinase, alpha-amylase, biological indicator for sterilization, restriction endonuclease, catalase, cellobiose fermentation, DNA polymerase, ethanol production, glucose-6-phosphate, dehydrogenase, liquefying starch, maleate dehydrogenase, neutral proteases, polynucleotide phosphorylase, prenyl diphosphate synthase, pyruvate kinase, riboflavin, glucoside, superoxide dismutase, xylanase) (Nazina *et al*., 2000).

1.2.2 Enzymes

Enzymes are biomolecules able to catalyze a variety of chemical reactions such as hydrolysis, polymerization (bond formation), functional group transfer, oxidation, reduction, isomerization and dehydration. Enzymes and chemical catalysts increase the rate of reaction and lowering the activation energy. The enhancements of enzyme-catalyzed reactions are typically several orders of magnitude over background, and take place under mild condition. Unlike most chemical catalysts, enzymes are able to catalyze specific and often different chemical transformation in aqueous solutions at room temperature and atmospheric pressure (Tao & Cornish, 2002). Furthermore, enzymes are generally environmentally friendly, economical and clean catalysts. Enzymes currently have commercial applications ranging from food or paper processing to fine chemical synthesis and diagnostic/research reagents. However, enzymes are often poor catalysts for non-natural substrates and they suffer from poor stability. Direct evolution methods have proven to be an effective strategy for improving enzyme properties (Wahler & Reymond, 2001).

1.2.2.1 Enzymes of extreme thermophilic environments

Driven by increasing industrial demands for biocatalysts that can cope with industrial process conditions, considerable efforts have been devoted to the search for such enzyme (Rozzell, 1999). Extremozymes have a great economic potential in many industrial processes, including agricultural, chemical and pharmaceutical applications. Thermophilic extremophile have attracted most attention, in particular extremophilic proteases, lipases, cellulases and, amylases have found their way into industrial applications. The reasons to exploit enzymes that are stable and active at elevated temperatures are obvious, since at these temperatures the solubility of many reaction components is significantly improved. Moreover, the risk of contamination, leading to undesired complications, is reduced at higher temperatures (Bertus, 2003), therefore, requirements for thermostable biocatalysts are far greater

than those of the mesophiles of which proteases contributes two thirds. Moreover, there are two strategies for the production of enzymes from extremophiles. First, production of the biocatalysts can be optimized by increasing the biomass production of the extremophile. Alternatively, the gene encoding the biocatalyst can be cloned and expressed in a suitable host (Blackman *et al*., 2002).

1.2.2.2 Proteases

Proteases are highly complex group of enzymes which occupy a central position with respect to their applications in both physiological and commercial fields. Proteolytic enzymes, which are produced intracellularly and extracellularly, play an important role in the metabolic and regulatory processes of animal and plant cells, as well as in those of prokaryotic and eukaryotic microorganisms. Extracellullar proteases are involved mainly in the hydrolysis of large polypeptide substrates, such as proteins into smaller molecular entities which can subsequently be absorbed by the cell. Different types of proteases include serine, cysteine, aspartic and metallo proteases (Barrett, 1999). Serine and cysteine proteases use these amino acid side chains as a nucleophile to attack a peptide bond carbonyl, while aspartic and metalloproteases use water molecules as the nucleophile. The protease enzymes constituted two thirds of the total enzyme used in various industries in 2002 (Gupta *et al*., 2002b). And this dominance in the industrial market has probably increased during the last three years. They have several applications, mainly in detergent and food industries. In view of the recent trend of developing environmentally friendly technologies; proteases have extensive applications in leather treatment and in several bioremediation processes. Proteases are used extensively in the pharmaceutical industry for preparation of medicines. Proteases that are used in the food and detergent industries are prepared in bulk quantities and used as crude preparations; whereas those that are used

in medicine are produced in small amounts but require extensive purification before they can be used (Garcia-Carreno and Del Toro, 1997).

❖ **Classification of proteases**

The classification system for microbial proteinases, recommended by the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (IUBMB), divides the proteolytic enzymes into two major groups on the basis of their nature of attack: endopeptidases and exopeptidases (Barret and McDonald, 1985).

Exopeptidases cleave the peptide bond proximal to the amino or carboxyl termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. The exopeptidase acting at a free amino-terminus may liberate a single amino acid residue (aminopeptidases), and those acting at a free carboxyterminus liberate a single residue (carboxypeptidase) (Rao *et al*., 1998). Both exopeptidases and endopeptidases exhibit "sequence specificity", i.e. they show varying preferences for particular amino acids near the peptide bonds to be cleaved. The active site of a protease is commonly located in a groove on the surface of the molecule, and substrate specificity is dictated by the properties of binding sites arranged along the groove on one or both sides of the catalytic site that is responsible for the hydrolysis of the peptide bond. Accordingly, the specificity of a peptidase is described by the use of a model in which each specificity subside is able to accommodate the side chain of a single amino acid residue the sides are numbered from the catalytic site (Figure 1.2). S1, S2 and so on towards the amino-terminus of the substrate, and S1', S2' and so on towards the carboxy-terminus. The substrate amino acids they accommodate are numbered P1, P2, etc., and P1', P2'. etc. respectively (Barret, 1994),

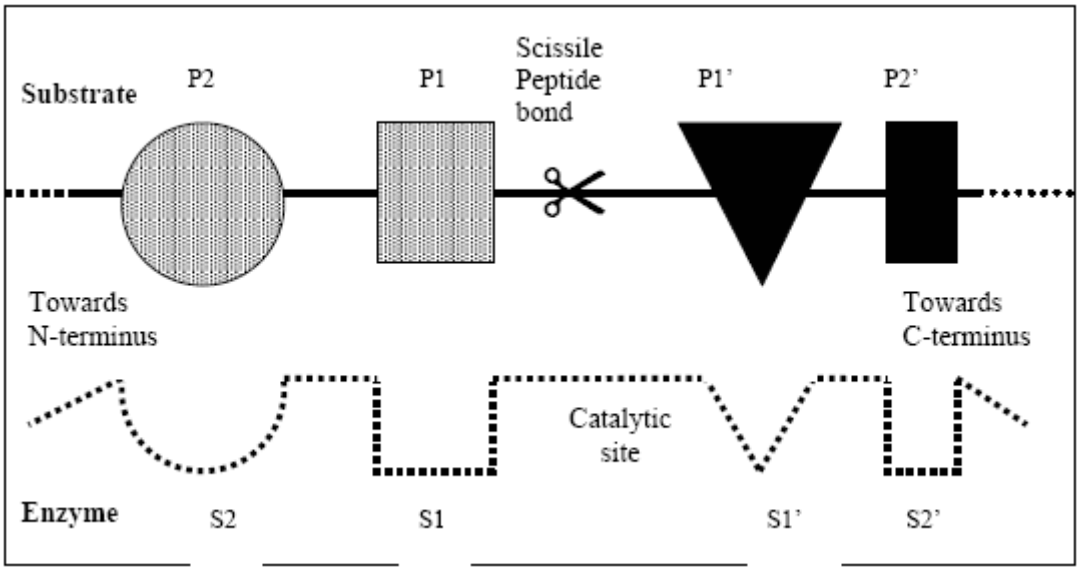

Figure (1.2): Scheme for the specificity subsites of the protease.

S1through S2 are the specificity subsite on the enzyme, while P1through P2 are the residues on the substrate accommodated by the subsite on the enzyme (Barret, 1994)

The endopeptidases are also called proteinases classified according to the chemical nature of the amino acid residues that are responsible for the catalytic activity of the enzyme (Table 1.1) (Barret *et al*., 2001).

Table (1.1): Five catalytic types of proteases (Barret *et al*., 2001).

Catalytic type of proteases	Catalytic group
Serine	Hydroxyl group of serine
Threonine	Hydroxyl group of therionine
Cysteine	Tiol group of cysteine
Aspartic	Carboxyl group of two aspartic acid residues
Metallo	Zinc atom (some times another metal)

Unlike the IUBMB system, the structure-based approach can be applied to a protease that is known only from its gene, and so can allow predictions, about properties and functions at a very early stage in the study of the enzyme, which is particularly important in the genomic area. The present form of the classification can be found in the MEROPS database on the World Wide Web (http://.merops.sanger.ac.uk). In the MEROPS system, individual proteases are first grouped into families on the basis of statistically significant similarities between the amino acid sequences of the parts that are most directly responsible for catalytic activity. The families are then grouped into clans when it is apparent from similar 3-D structures or amino acid sequence motifs that they have evolved ultimately from a single peptidase (Figure 1.3) (Barret *et al*., 2001).

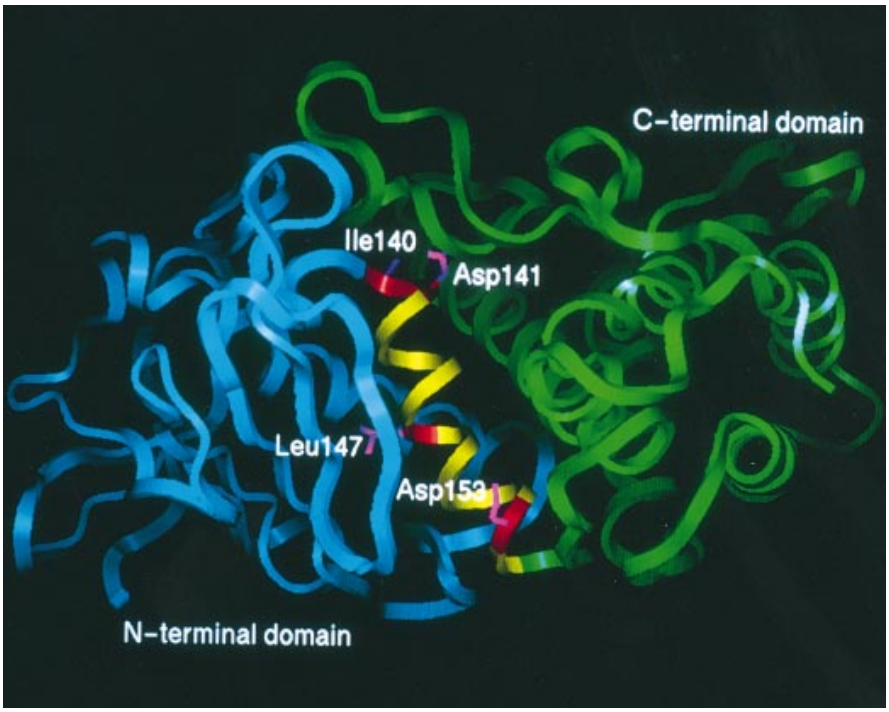

Figure (1.3): Molecular structure of neutral protease. The model structure was costructed based on the thermolysin molecule using a computer homology (Barret *et al*., 2001).

❖ Thermophilic Proteases

Most of proteases are stable in temperate (mesophilic) temperature; however, mesophilic enzymes are often not optimally adapted to conditions where they are to be applied. For this reason several strategies are being used to improve the characteristics of biocatalysts, such as stability, activity, specificity, and pH optimum. In this respect, isolation of enzymes from organisms that are able to survive under extreme conditions eventually in combination with directed evolution approaches has been shown to be an important source for new biocatalysts (Fujiwara *et al*., 1993). Because of that, in recent years much attention has been given to proteases from extremophilic microorganisms. During their evolution these microorganisms adapted themselves to grow over a wide range of temperature, pH and pressure. Much of interest has been especially focused on those living at high temperatures particularly on their enzymes (Niehaus *et al*., 1999). Thermostable proteases from thermophilic bacteria are given much attention due to their stability at higher temperatures. Because thermophilic enzymes are optimally active under more severely denaturing conditions than mesophilic enzymes, they need to be more rigid than mesophilic enzymes. Another advantage of applying thermostable enzymes for production purposes is lower viscosity of process fluids. At higher temperatures viscosity is usually reduced, which lowers shear and, consequently, the cost of pumping, filtration, and centrifugation. Moreover, at high temperature, more substrates will dissolve and this can shift the equilibrium to higher product yields. Thermophilic enzymes catalyze reactions at high temperatures with Km and Vmax values similar to those of their mesophilic counterparts at their respective optimal temperature (Bruins *et al*., 2001).

❖ Thermolysin Like Protease (TLPs)

Thermolysin (EC 3.4.24.27) is the first name given to an extracellular metalloendopeptidase secreted by the Gram positive thermophilic bacterium

Bacillus thermoproteolyticus (Bertus and Vincent, 2004). Several members of the bacterial genus *Bacillus* are known to produce extracellular neutral proteases that resemble thermolysin. These so-called thermolysin like proteases (TLPs) (EC 3.4.24.4) (Mansfeld *et al*., 2004). Published work on these enzymes such as TLPs from *B. subtilis, B. stearothermophilus , and Bacillus cereus* indicate that thermolysin and thermolysin like protease are generally quite similar in term of enzymological properties(De Kreij *et al*., 2000). TLPs that are produced by various species of *Bacillus* are characterized by having their optimum activity at neutral pH. The overall structure of TLPs consists of two roughly spherical domains with a deep cleft running across the middle of the molecule separating the two domains. The secondary structure of each domain is quite different, the N-terminal domain consists of mostly beta pleated sheet, while the C-terminal domain is mostly alpha helical in structure (Figure 1.4). These two domains are connected by a central alpha helix, they are also characterised by a zinc ion that is bound in the active site and that plays an essential role in catalysis, all TLPs bind two calcium ions in a double binding site, whereas the more stable TLPs bind two additional calcium ions in two single calcium binding sites (Matthews, 1988).

TLPs are enzymes of industrial importance. They are used in diverse applications such as the preparation of protein hydrolysates; the degradation of gluten from wheat (baking industry); the clarification of beer (brewing industry) and in dehairing or dewooling of skins (leather industry) (Eijsink and Venema, 1995). Thermolysin is also used in peptide synthesis, particularly in the production of artificial sweeteners like aspartame (Ager *et al.,* 1998).

TLPs are highly suitable for protein engineering experiments aimed at tailoring enzyme specificity and stability because they have been subject of many studies and because information can be extracted from studies of the differences between the many naturally occurring variants. For example,

bacilli vary widely in their optimum growth temperature and the stability of their enzymes, such as the TLPs, differs accordingly (Priest, 1993).

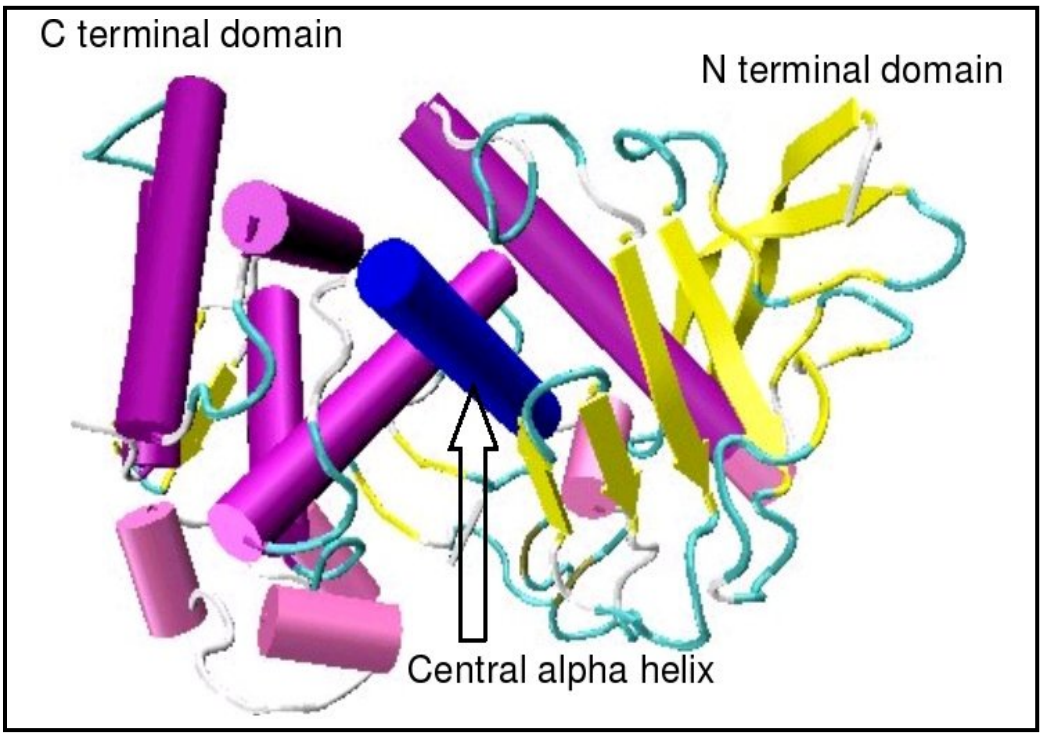

Figure (1.4): Tertiary structure of thermolysine like protease (Matthews, 1988).

1.2.3 Protease activity estimation

The ability to rapidly detect and inhibit proteases is important in assessing the quality of commercially available proteins. Therefore, the necessity to detect proteases has led to the development of numerous detection methods. Several techniques monitor the change in absorbance at 280nm following precipitation of a test protein with trichloroacetic acid (TCA). Degradation of the test protein releases small, acid-soluble peptides that absorb at 280nm. Other similar methods use dye-modified proteins, such as azocasein, where the acid soluble peptides released by proteolysis cause an increase in absorbance at a wavelength specific to the dye. More sensitive assays have been developed which use small peptides containing chromophores on their C-terminus. Cleavage after the last amino acid results in an absorbance change.

While sensitive, these methods are very specific for proteases that cleave after a distinct amino acid and may not detect other proteases (Sarath *et al*., 1989; Douglas *et al*., 1993).

Another method quantitates the generation of primary amine groups during proteolysis using a test protein and trinitrobenzenesulfonic acid (TNBSA). TNBSA reacts with primary amines and produces a compound detectable at 450 nm. This method, however, is not compatible with Tris- or amine-based buffers. Furthermore, crude extracts containing high protein concentrations or primary amines can cause high backgrounds (Twining, 1984).

1.2.4 Polymerase Chain Reaction (PCR)

Under natural circumstances, DNA is replicated by the cell machinery. When living cells divide, biological stimuli prompt the cell to generate an exact duplicate of its entire genomic sequence (Niel *et al*., 2007). Under specific *in vitro* conditions (thermal, chemical, etc.), similar duplication processes can be forced to occur in rapid succession. The most popular amplification process, called the polymerase chain reaction (PCR), was invented in 1985 (Li, 2006). PCRs are a very sensitive method of amplifying nucleic acid. Many specific copies of DNA are produced from each round of amplification. The original concept for PCR, like many good ideas, was an amalgamation of several components that were already in existence, the synthesis of short lengths of single-stranded DNA (oligonucleotides) and the use of these to direct the target-specific synthesis of new DNA copies using DNA polymerases were already standard tools in the repertoire of the molecular biologists of the time. The novelty in Mullis's concept was using the juxtaposition of two oligonucleotides, complementary to opposite strands of the DNA, to specifically amplify the region between them and to achieve this in a repetitive manner so that the product of one round of polymerase activity was added to the pool of

template for the next round, hence the chain reaction (Bartlett and Stirling, 2000).

Figure (1.5) shows two synthetic oligonucleotide primers that will hybridize to the DNA sequence of interest are mixed with the DNA sample (template), together with a heat-stable DNA polymerase and dNTPs substrates. When this mixture is heated to denature the template DNA and then cooled, the primers anneal to the template. The enzyme uses the primers to start synthesis of new DNA strands, complementary to each strand of the template, thus doubling the amount of the DNA fragment (Dale and Park, 2004).

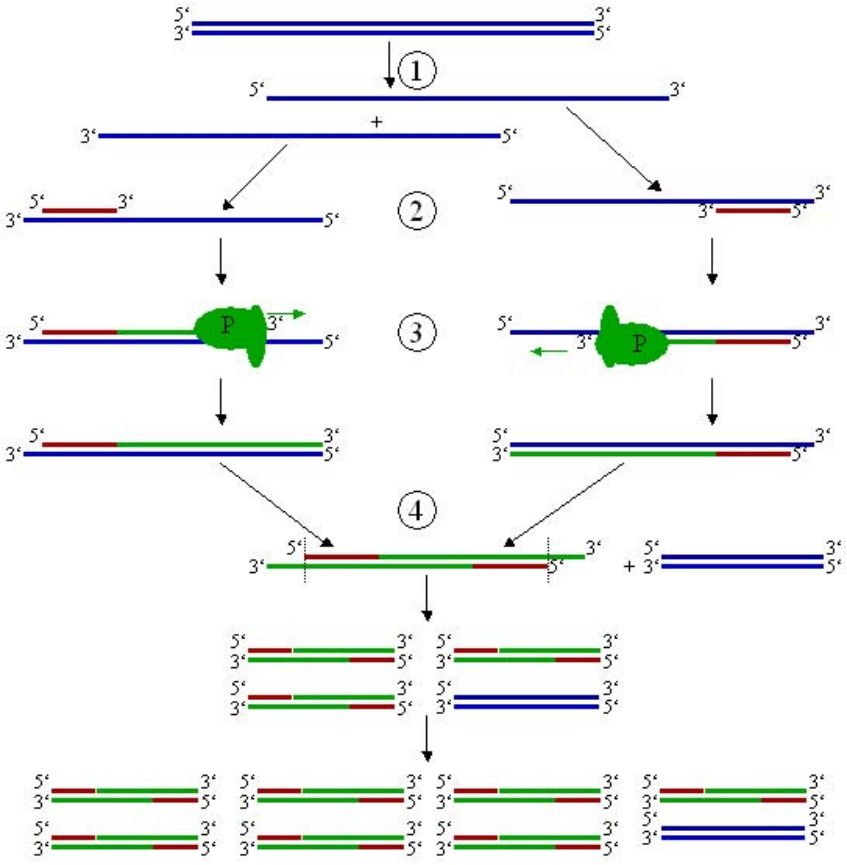

Figure (1.5): Schematic drawing of the PCR cycle. (1) Melting at 96°C. (2) Annealing at 68°C. (3) Elongation at 72°C (P=Polymerase). (4) The first cycle is complete. The two resulting DNA strands make up the template DNA for the next cycle, thus doubling the amount of DNA duplicated for each new cycle; each cycle again doubles the amount of the target DNA, so that after 20 cycles a single molecule of DNA will be amplified to about 1 million copies (Dale and Park, 2004).

1.2.5 Genetic engineering of microbial protease

Gene cloning is a rapidly progressing technology that has been instrumental in improving our understanding of the structure function relationship of genetic systems. It provides an excellent method for the manipulation and control of genes. More than 50% of the industrially important enzymes are now produced from genetically engineered microorganisms (Hodgson, 1994; Abo-Aba *et al*., 2006). Several reports have been published in the past decade on the isolation and manipulation of microbial protease genes with the aim of (i) enzyme overproduction by the gene dosage effect, (ii) studying the primary structure of the protein and its role in the pathogenicity of the secreting microorganism, and (iii) protein engineering to locate the active-site residues and/or to alter the enzyme properties to suit its commercial applications. Protease genes from bacteria, fungi, and viruses have been cloned and sequenced. The objective of cloning bacterial protease genes has been mainly the overproduction of enzymes for various commercial applications in the food, detergent and pharmaceutical industries. The virulence of several bacteria is related to the secretion of several extracellular proteases. Gene cloning in these microbes was studied to understand the basis of their pathogenicity and to develop therapeutics against them. Proteases play an important role in cell physiology, and protease gene cloning, especially in *E. coli* has been attempted to study the regulatory aspects of protease (Rao *et al*., 1998). The structural genes encoding the alkaline protease or subtiisin (Stahl and Ferrari, 1984), neutral protease A and B (Yang *et al*., 1984; Tran *et al*., 1991), extracellular protease (Sloma *et al*., 1988), bacillopeptidase F (Sloma *et al*., 1990), and metalloprotease have been cloned and characterized. Chromosomal DNA from *B. stearothermophilus* was partially digested with *Sau*3A and ligated with *Bam*H1 digest of pYB53 cloning vector. The ligation mix used to transform *B. subtilis* (neutral protease deficient mutant). The thermostable neutral protease gene nprT of *B. stearothermophilus* was se-

quenced (Figure 1.6). The DNA sequence revealed only one large open reading frame, composed of 1,644 bases and 548 amino acid residues. A Shine-Dalgarno sequence was found 9 bases upstream from the translation start site (ATG), and the deduced amino acid sequence contained a signal sequence in its amino-terminal region. The sequence of the first 14 amino acids of purified extracellular protease completely matched that deduced from the DNA sequence starting at GTC (Val), 687 bases (229 amino acids) downstream from ATG. This suggests that the protease is translated as a longer polypeptide. The amino acid sequence of the extracellular form of this protease (319 amino acids) was highly homologous to that of the thermostable neutral protease from *B. thermoproteolyticus* (Masahiro *et al*., 1985).

1.2.6 Cloning vectors

1.2.6.1 pJET1.2 cloning vector

pJET1.2/blunt is a linearized cloning vector, which accepts inserts from 6 bp to 10 kb. The 5'-ends of this vector was contained phosphoryl groups, therefore, phosphorylation of the PCR primers is not required.

Blunt-end PCR products generated by proofreading DNA polymerases can be directly ligated in just 5 min with the pJET1.2/blunt cloning vector. PCR products with 3’-dA overhangs generated using *Taq* DNA polymerase or other non-proofreading thermostable DNA polymerases are blunted in 5 min with a proprietary thermostable DNA blunting enzyme prior to ligation. All common laboratory *E. coli* strains can be directly transformed with the ligation product.

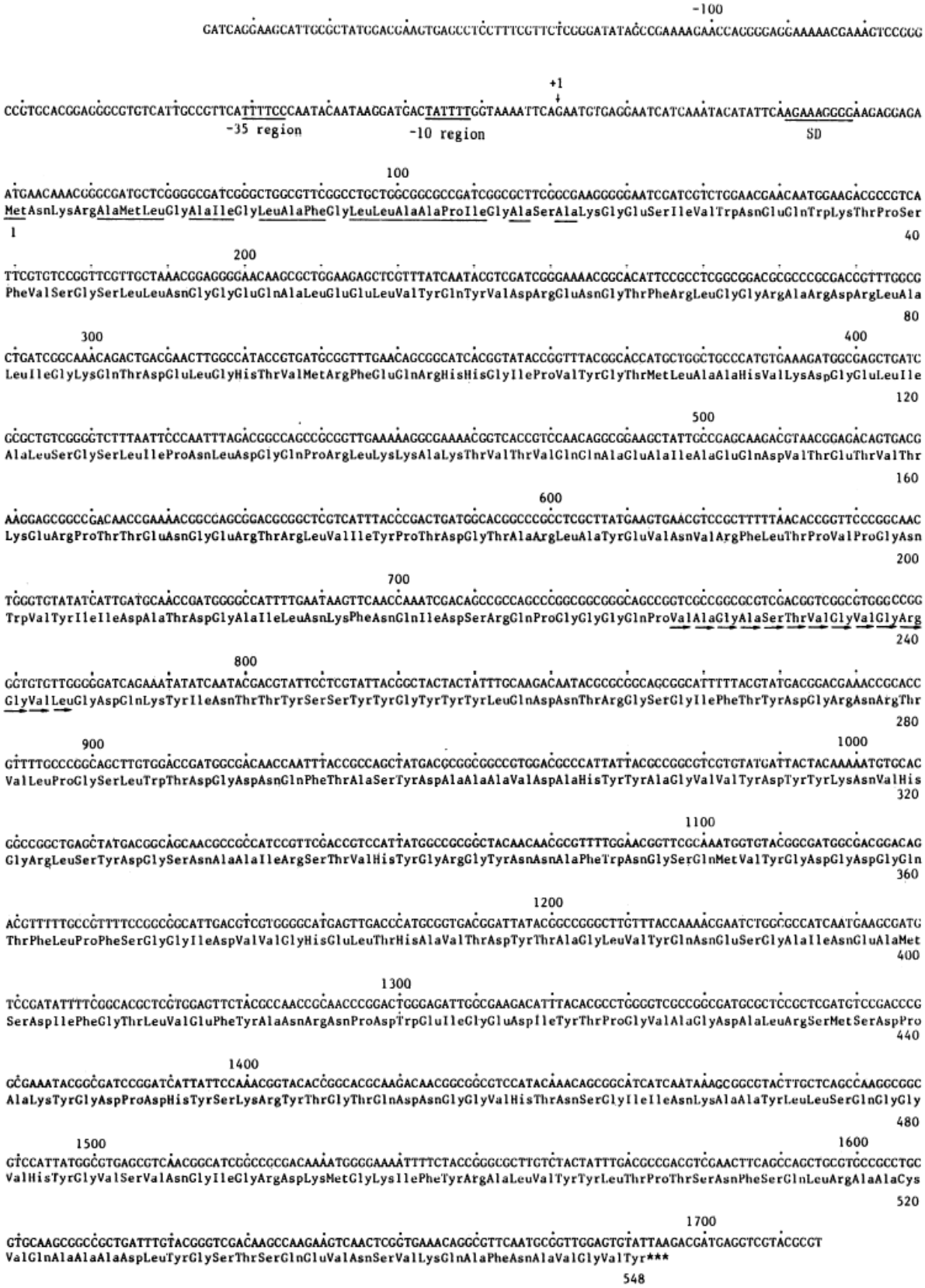

Figure (1.6): Nucleotide and amino acid sequences of neutral protease (Npr) gene in *Bacillus stearothermophilus* (Masahiro *et al*., 1985).

Recircularized pJET1.2/blunt vector expresses a lethal restriction enzyme after transformation and is not propagated. As a result, only recombinant clones containing the insert appear on culture plates. Therefore, blue/white screening is not required. The vector contains an expanded multiple cloning site, as well as a T7 promoter for *in vitro* transcription. Sequencing primers are included for convenient sequencing of the insert (Yang *et al*,. 2009).

pJET is 2974bp in length and contains:(Figure 1.7)

- Replicon (rep): from the pMBI plasmid responsible for the replication of pJET1.2 cloning vector at the position (1762-1148) bp.
- Replication start: for Initiation of replication at the position (1162 +1) bp.
- *bla* (Ap®): B-lactamase gene conferring resistance to ampicillin, used for selection and maintenance of recombinant *E. coli* cells (2782-1922) bp.
- *eco47IR*: Lethal gene *eco47IR* enables positive selection of recombinant plasmid (753-16) bp.
- P_{lacUV5}: Modified P_{lac} promoter for expression of the *eco47IR* gene at a level sufficient to allow for positive selection (892 -769) bp.
- T7 promoter: T7 RNA polymerase promoter for *in vitro* transcription of the cloned insert (305-324) bp.
- Multiple cloning site (MCS): Mapping, screening and excision of the cloned insert (422-328) bp (Yang *et al*,. 2009).

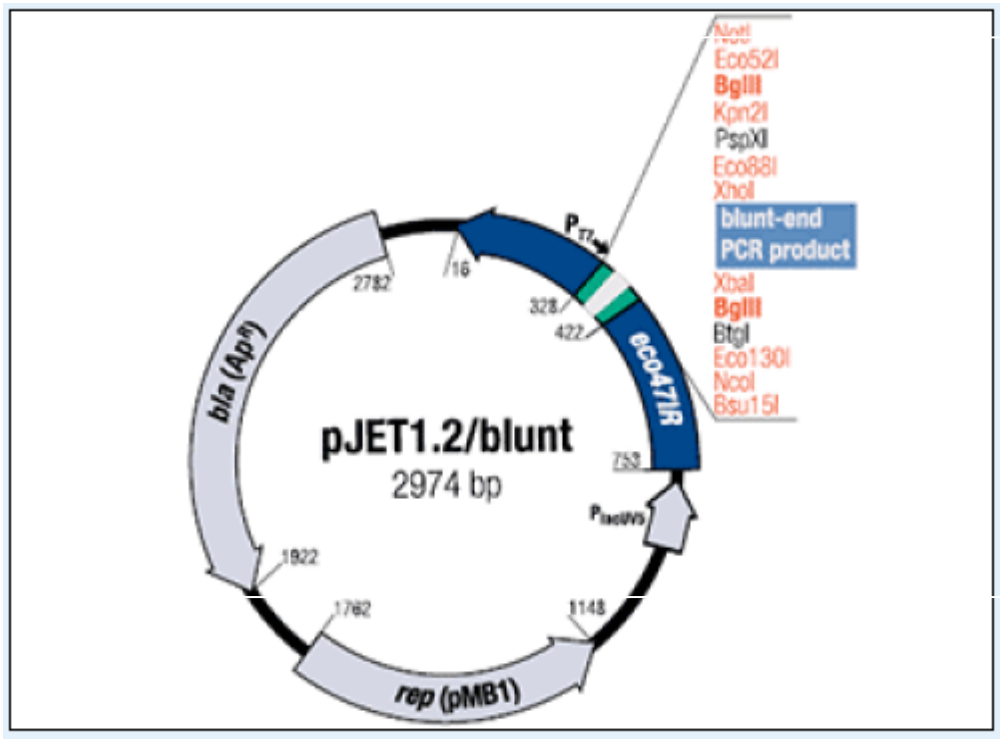

Figure (1.7): Clone JET 1.2/blunt vector map (Yang *et al.*, 2009).

1.2.6.2 pET expression vector

The pET expression vectors were originally constructed by Studier and Moffatt (1986). The newer pET vectors developed at Novagen offer enhanced features to permit easier cloning, detection, and purification of target proteins. The pET expression vectors (4600 bp), derived from the pBR322 plasmid, are engineered to take advantage of the features of the T7 bacteriophage gene *10* that promote high-level transcription and translation. The bacteriophage encoded RNA polymerase is highly specific for the T7 promoter sequences, which are rarely encountered in genomes other than T7 phage genome. First, this ensures that the T7 promoter will not be recognized by host cell RNA polymerase. Thus target genes are transcriptionally silent in the uninduced state--a feature that is very important if the gene to be expressed is toxic to the cell. Second, upon induction, the target gene is the only gene in the cell that will be transcribed by the highly active polymerase. The pET28b (+) vector (Figure 1.8) is designed to generate target protein with the features of: T7lac promo-

ter; Kanamycin resistance; Multiple cloning sites in all three reading frames (Studier *et al*., 1990).

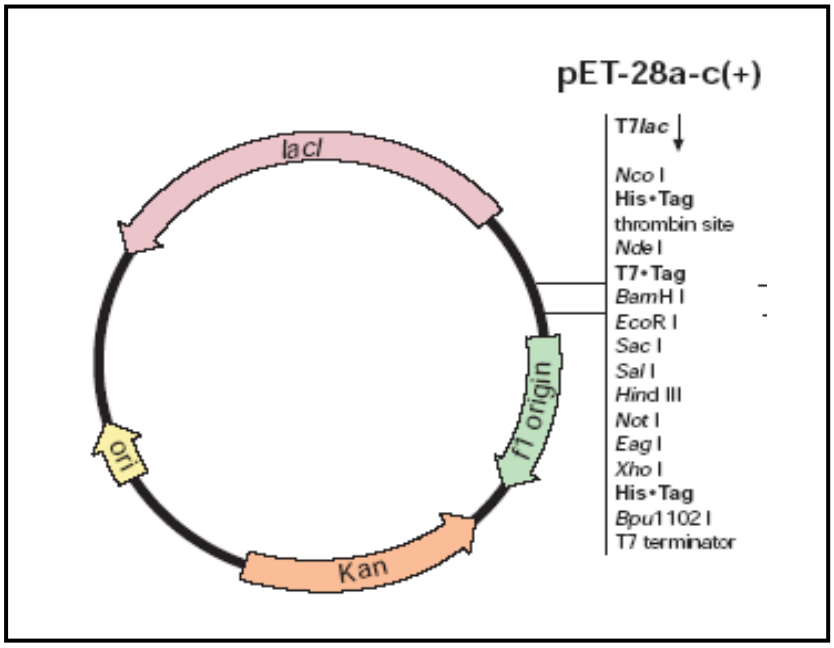

Figure (1.8): pET-28b(+) vector map (Studier *et al*., 1990).

1.2.6.3 pUC18 cloning vector

pUC18 vector is a small, high copy number, *E. coli* plasmid, and 2686 bp in length. pUC18 plasmid contain: (**1**) the pMB1 replicon *rep* responsible for the replication of plasmid (source - plasmid pBR322). The high copy number of pUC plasmids is a result of the lack of the *rop* gene and a single point mutation in *rep* of pMB1; (**2**) *bla* gene, coding for beta-lactamase that confers resistance to ampicillin (source - plasmid pBR322). It differs from that of pBR322 by two point mutations; (**3**) region of *E. coli lac* operon containing CAP protein binding site, promoter P_{lac}, *lac* repressor binding site and 5'-terminal part of the *lacZ* gene encoding the N-terminal fragment of beta-galactosidase. The exact position of the genetic elements is shown on the map (termination codon included) (Figure 1.9). The *bla* gene nucleotides 2486-2418 (complementary strand) code for a signal peptide. The LacZ polypeptide corresponding to beta-galactosidase and essential for blue/white

screening ends at position 236; another 30 codons in the same reading frame are derived from pBR322 (Yanisch-Perron *et al.,* 1985).

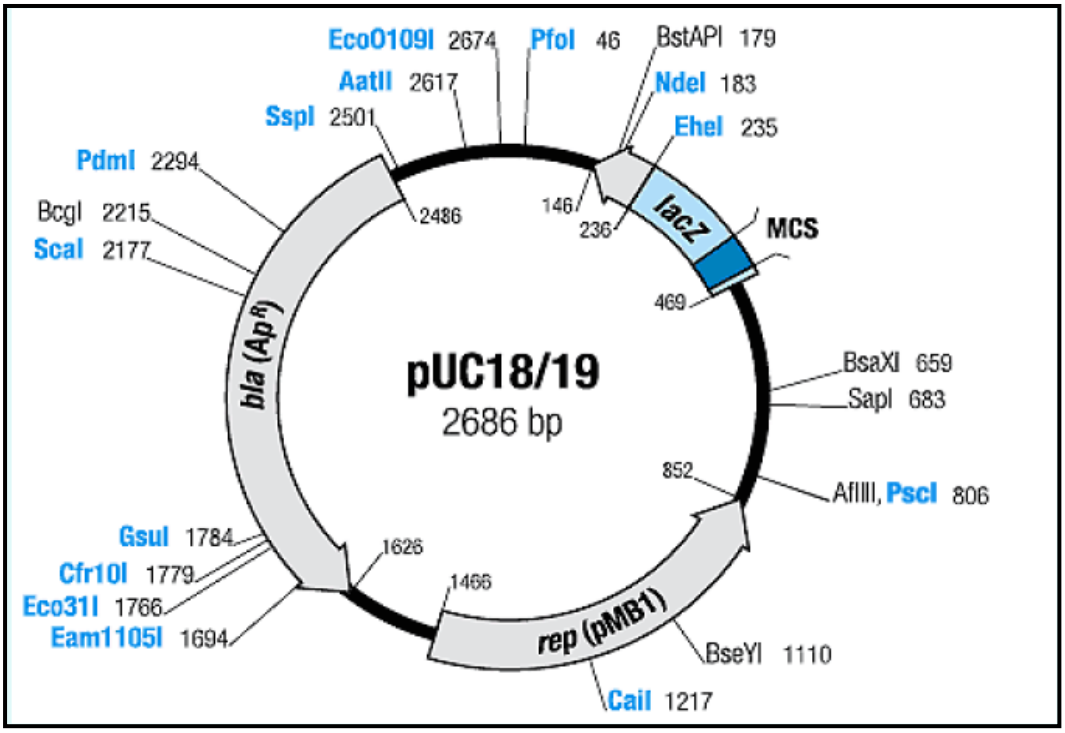

Figure (1.9): Description and restriction map of pUC18/19 (Yanisch-Perron, 1985).

1.2.7 Mutation and variation

The cornerstone of bacterial genetics was the isolation of specific mutants, i.e. strains in which the gene concerned is altered (usually in a deleterious fashion). This alteration shows up as a change in the corresponding characteristics of the organism. It is this change in the observable properties of the organism (the phenotype) that is used to follow the transmission of the gene. The genetic nature of the organism (the genotype) is inferred from the observable characteristics (Kurtzman *et al*., 2001)

1.2.7.1 Physical mutagen

Any agent that damages DNA can in principle lead either to the death of that organism or, amongst the survivors, to mutation. This is true of irradiation as well as of chemical agents. Many types of physical mutagen have been used to generate mutations. The higher energy rays such as X-rays and gamma rays however require expensive apparatus and safety equipment and are

not really suitable for routine use in a microbiology laboratory. In addition, they produce an excessive amount of chromosomal damage that is not easily repaired by the microorganism. Ultraviolet irradiation on the other hand is easily controlled (although eye and skin protection is necessary) and requires only comparatively inexpensive equipment (Mitra, 1996).

The principal effect of UV irradiation is the production of pyrimidine dimers (commonly referred to as thymine dimmers) (Figure 1.10). Where two pyrimidine residues are adjacent on the same DNA strand, the result of UV irradiation is the creation of covalent links between them. These pyrimidine dimers cannot be replicated and are therefore lethal to the cell unless it is able to repair the damage. It is the attempts to repair the damage caused by ultra-violet irradiation that can lead to mutagenic effects. Although most repair mechanisms are reasonably accurate (error-free repair), in the event of these mechanisms being unable to cope with the damage an additional defense comes into play (Jeremy and Simon, 2004).

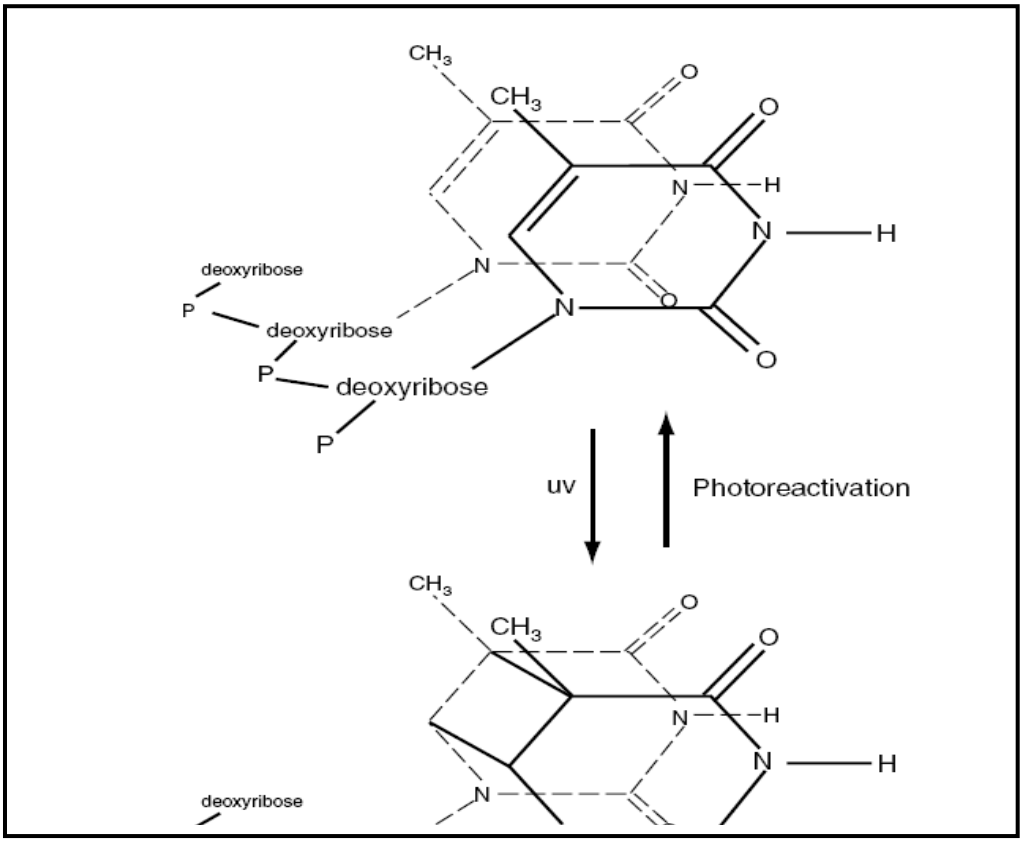

Figure (1.10): Structure of thymine dimmers (Jeremy and Simon, 2004).

1.2.7.2 Chemical mutagen

The natural rate of spontaneous mutation is much too low for convenient isolation of most types of mutants (apart from a handful of easily selected mutations such as antibiotic resistance). Ways must be found of enhancing that frequency. It is often possible to use *in vitro* mutagenesis or transposon mutagenesis, but there are still many situations where chemical or physical procedures are preferred or essential. Many different chemical agents interact with DNA or the replication machinery so as to produce alterations in the DNA sequence. Of these, the simplest to understand are those agents that act by chemically modifying a base on the DNA so that it resembles a different base. Alkylating agents such as ethyl methane sulphonate (EMS) and MNNG are extremely powerful mutagens and the latter in particular is extremely hazardous to use. They act by introducing alkyl groups onto the nucleotides at various positions, especially the O_6 position of guanine, and tend to cause multiple closely linked mutations in the vicinity of the replication fork (Gerhardt *et al*., 1981; Jeremy and Simon, 2004).

1.2.7.3 Enzyme evolution and mutagenesis

Organisms evolve to adjust to change in the environments, as explained by Darwin's Theory of Evolution. A changing environment challenges and forces them to evolve by modification, mainly through mutation. Darwinian evolution caused by an artificially controlled environmental selective pressure is a very attractive strategy for altering protein activity in the laboratory. This approach, referred to as laboratory directed evolution, is a promising approach to improve enzyme selectivity and evolve function (Zhao and Arnold, 1999). General steps of the laboratory directed enzyme evolution strategy consist of mutagenesis, expression of the resulting enzyme variants, and selection/screening for a desired new property as described by Farinas *et al.* (2001) and Tao and Cornish (2002). These steps can be repeated until the enzyme acquires the desired new activity level. Improved enzymes from di-

rect evolution have been applied to chemical synthesis, biological and medical research, commercial products in the food process and detergent industry, and even as practical pharmaceutical products. Direct evolution provides a powerful tool to understand the mechanisms of enzymatic reactions as well. Many successful examples of enzyme evolution have been reported using well-known mutagenesis methods (Powell *et al.*, 2001). Commercial proteases such as subtilisin (Ness *et al*., 1999) have been studied for 30 years to improve their commercial value. Random mutagenesis and screening resulted in a 470-fold improvement of activity compared to the wild-type (You and Arnold, 1996). Enzymes have also been evolved for improved activity at elevated and low temperatures (Taguchi *et al.*, 1999). In addition to stereospecificity, improvements in substrate specificity, thermostability, solvent tolerance and enzymatic activity over a broad pH range have been reported. Arnold *et al*. (2003) have produced mutants of bacterial cytochrome P450 BM-3 mutant enzyme that have 20-fold higher activity. One newly evolved P450 BM-3 mutant exhibited new substrate selectivity as well as higher turnover rates. Direct evolution of β-lactamase resulted in 32,000-fold higher enzymatic activity (Stemmer, 1994). There are many other successful directed evolution examples of enzymes, including atrazine chlorohydrolase, tRNA synthetase, alkyl transferase, amylase, cellulases (β-galactosidase or β-xalyanase), phytases, penicillin acylases, nitrile-hydrolyzing enzymes, and hydantoinases for improvements of activity, specificity and stability (Powell *et al.*, 2001).

1.2.7.4 Mutagenesis and protease production

Random mutagenesis can result from a few to many changes per gene. Artificial UV/chemical mutagenesis can be used to introduce changes (Paul and Leemor, 2007). This mutation method mimics the natural process of evolution, but the library sizes are relatively small because the number of changes is generally small. Based on X-ray can be a powerful way to produce proteins

with enhanced function. An advantage of the rational design approach is that only a few mutants need to be constructed. However, in practice, the function of a variant derived by rational design cannot generally be predicted, since enzymes are extremely complex. For example, guided by an X-ray structure, the conversion of trypsin to chymotrypsin was attempted by mutating Asp189 to Ser in the S1 site. Instead of transforming trypsin to chymotrypsin, this mutation resulted in a poor, nonspecific protease although the anionic residue Asp189 at the bottom of the S1 binding pocket of trypsin is thought to interact with positive charged residues Lys/Arg of substrate. In another attempt, the switching of 15 amino acid residues of the S1 pocket and two surface loop regions of chymotrypsin into trypsin gave similar specificity of chymotrypsin, but had considerably sacrificed enzymatic activity (Stoltze *et al*., 2000). Therefore, multiple, unpredictable changes are often needed in order to alter enzyme activity in a useful way. As an alternative to site directed mutagenesis, scientists have developed ways of mimicking the strategies of genetic variation found in natural biological evolution. In the laboratory, libraries of enzyme mutants can now be achieved by random mutagenesis (Manel and Lawrence, 2003).

1.2.8 Optimum conditions for protease production

1.2.8.1 Optimum culture conditions

Cheaper of both carbon and nitrogen sources are the attraction key for commercialization of the production process and thus, ability of the microbial agent to grow and produce enzymes, using these sources has a point of interest (Mehta, 2000; Kanekar *et al*., 2002). Most proteolytic bacteria showed variation in their requirement for both carbon and nitrogen sources as well as enzyme formation. Since nutritional factors are known to affect the production of microbial enzymes. Therefore, considerable changes in fermentation medium have to be made to increase the protease yield from *B. stearothermophilus* (Rahman *et al*., 2003). Earlier, Fujiwara and yamamoto

(1987), found that a combination of soy bean meal (3%) and yeast extract (1.5%) was an excellent nitrogen sources for protease production from their *Bacillus* sp. While, Takii *et al*. (1990) discovered that yeast extract (0.3%) and sodium nitrate (1%) induced the formation of protease by *Bacillus alkaliphilus*. The presence of carbon sources such as glucose was reported to be essential for enzyme production in some microorganisms (Macfarlane and Macfarlane, 1992; Homa *et al*., 1993) but was inhibitory to others (Joo *et al*., 2002; Johnvesly *et al*., 2002).

1.2.8.2 Optimum pH

The pH of the culture strongly affects many enzymatic processes and transport of compounds across the cell membrane. Majority of the thermophilic bacilli are found to grow at pH range of 5.8-8.0 (Krishna and Kodidhela, 2005). Safey and Abdul-Rauf (2004), showed that, when the production medium was adjusted at different pH values of different buffers, the best buffer was phosphate buffer at optimum pH (7.0) for production of protease with 177.83 units/ml-^{1}. A notable decline in the enzyme productivity occurred at both higher and lower pH values. Other studies on three *Bacillus* species produced protease over the entire range of pH investigated (pH 5-10) with maximum protease production at pH 8.0 (Folasade and Joshua, 2005).

1.2.8.3 Optimum temperature

Assuming that the temperature was the fastest-changing environmental parameter during the life of microorganisms, easy (and therefore rapid) evolutionary adaptation to these temperatures may have been important for survival. The studies with thermolysin like proteases showed that evolutionary processes resulting in molecular adaptation of these enzymes to many changes in the environments (Bertus *et al.*, 1998)

Studies on the production dynamics of extracellular protease from three *Bacillus* species showed that, protease production from these isolates were

occurred between 37 °C and 65°C with maximum growth and maximum enzyme production at 60°C (Folasade and Joshua, 2005). According to the research of Denizci *et al*. (2003), the temperature optimum for the production of commercial protease from *Bacillus clausii* used in detergent industry stay within (30-60) °C.

1.2.8.4 Optimum incubation period

It has been suggested that optimization of incubation period is needed to determine the highest amount of enzyme production (Razak *et al*., 1994). Gupta *et al.* (2002b) reported that protease production occurs in the stationary phase of the growth, while Safy and Abdul-Rauf (2004), founded that the optimum production of protease from *B. subtilis* was after 24h. incubation under the optimum conditions. While, thermolysin like protease has been produced from *B. stearothermophilus* I n the late stage of logarithmic growth (Nishiya and Imanaka, 1990).

1.2.9 Purification of protease

Protein isolation is endeavoring to purify a particular protein, from some biological (cellular) material, or from a bioproduct, since proteins are only synthesized by living systems. The objective is to separate the protein of interest from all non-protein material and all other proteins which occur in the same material. Removing the other proteins is the difficult part because all proteins are similar in their properties. In an ideal case, where one was able to remove the contaminating proteins, without any loss of the protein of interest, clearly the total amount of protein would decrease while the activity (which defines the particular protein of interest) would remain the same (Clive, 2002). The thermophilic proteases can be used as ideal models for studying thermal stability of protein, among these enzymes, the neutral proteases have been extensively purified and studied not only for industrial production, but also for the elucidation of mechanisms involved in thermostability of enzymes

(Helmann, 1995). Several reports on the purification of the extracellular protease. A thermophilic neutral protease from thermophilic *Bacillus* strainHS08 was purified with DEAE-Sepharose anion exchange chromatography and sephacryl S-100 HR liquid chromatography (Huang *et al*., 2006). Two proteases from newely isolated *Bacillus* sp. were purified to homogeneity using aceton precipitation, cation exchange chromatography CM-Sepharose CL-6B, followed by gel filtration Sephadex G-75 superfine (Mona, 2006). From the culture supernatant of *Bacillus amyloliquefaciens* an extracellular protease was purified to apparent homogeneity by successive purification steps using 80% ammonium sulfate precipitation, SP-Sephadex column chromatography, and Sephacryl S-100 column chromatography (Euo-Sun and Jong, 2003). An extracellular protease produced by *Bacillus megaterium* was purified by hydrophobic interaction combined with gel filtration techniques. After final purification step, the enzyme was purified 148% fold with an increase in specific activity from 0.09 to 13.33 U/mg protein (Siriporn *et al*., 2006).

1.2.10 Enzyme characterization

1.2.10.1 pH effects on protease activity and stability

Since enzymes are proteins, they are very sensitive to change in pH. Each enzyme has its own optimum range for pH where it will be most active. This is the result of the effect of pH on a combination of factors: (1) the binding of the enzyme to substrate, (2) the catalytic activity of the enzyme, (3) the ionization of the substrate, and (4) the variation of protein structure (Clive, 2002). The pH-activity profiles of thermolysin like protease follow a bell-shaped curve with maximum activity at or near pH 7.0 (Fujiwara and Yamamoto, 1987). The pH profile of purified thermolysin like protease from *Clostridium perfringens* was detectable within a range of pH 5.0 to 8.0, and the pH for optimum activity was 7.5(Jin *et al*., 1995). Other studies on the influence of pH on the protease purified from a thermophillic *Bacillus* strain HS08have also been carried out and the results showed that the activity of the

protease was very low at pH values between 4.0 and 5.5, and then increased sharply beyond pH 5.5. The protease remained active between pH 7 and 9 but had its maximum activity at pH 7.5 (Huang *et al*., 2006).

Biocatalyst stability, i.e. the capacity to retain activity through time, is undoubtedly the limiting factor in most bioprocesses, biocatalyst stabilization being then a central issue of biotechnology. In fact, biocatalyst operational stability will determine to a large extent the viability of the process. pH is also a factor in the stability of enzymes. As with activity, for each enzyme there is also a region of pH optimal stability (Andres, 1999).

1.2.10.2 Temperature effects on protease activity and stability

Functional studies of enzyme temperature linked properties have defined a so-called enzyme "temperature optimum" which is being derived from a complex mixture of both activity and thermal stability effects, and dependent on assay duration. Consequently, it is of limited value for measuring enzyme temperature adaptation (Lee *et al*., 2007). Studies on a thermopihilic *Bacillus* sp. protease revealed that, the optimum temperature of this enzyme was 60°C, the enzyme was stable for 2h. at 30°C, while at 40°C and 80°C, 14% and 84% of the original activities were lost. This result was similar to that described for other *Bacillus* proteases (Wellingta and Meire, 2004). The activity of the crude and purified thermostable *Bacillus* protease was determined at different temperature ranging from 30°C to 90°C and the optimum temperature recorded was at 60°C.the thermal stability was tested, the enzyme was almost 100% stable at 60°C after 350 minutes of incubations (Kunamneni *et al*, 2003).

1.2.10.3 Inhibitors effect on enzyme activity

Inhibition studies primarily give on insight of the nature of the enzyme and nature of the active center. Dynamic studies of the binding of inhibitors to extracellular metalloendopeptidase from thermophilic bacteria can be found in references from Giessener and Jacob (1989) to Murray *et al.* (1999) and Bohm and Klebe (2002). Thermolysin is reversibly inhibited by millimolar concentrations of zinc-chelating agents such as 1, 10-phenanthroline. While inhibition by EDTA is irreversible, as EDTA preferentially chelates the calcium ions (Fontana, 1988). Upon purification and characterization of caseinolytic extracellular protease from *B. amyloliqueficiens,* the result showed neither SDS nor Triton x-100 detergent strongly affected the enzyme activity. In the concetration (0.1%) of detergent, the enzyme maintained over 50% of its activity. Reducing agents such as dithiothreitol (DTT) and β-mercaptoethanol had partial or little effect on enzyme activity (Euo-Sun and Jong, 2003).

Chapter Two

Materials

and

Methods

2. Materials and Methods

2.1 Materials

2.1.1Culture media

2.1.1.1Ready made media

Medium	Manufactured company
Nutrient agar	Oxoid (England)
Nutrient broth	Oxoid
Simmon citrate medium	Difco (USA)
Tryptic soy agar	Biolife (Italy)
Urea agar base	Biolife

They were prepared as recommended by the manufacturing companies and sterilized by autoclaving.

2.1.1.2 Laboratory prepared media

- **Skim milk agar medium (Sneath *et al*., 1986)**

This medium was prepared by dissolving 5 g of skim milk in 50 ml distilled water (D.W.) and sterilized by autoclaving, then 2 g of agar were dissolved in 50 ml D.W. and sterilized by autocalving, cooled into 45°C, mixed together then distributed into sterilized plates.

- **Nitrate medium (Atlas *et al*., 1995)**

This medium was composed of 5g peptone supplemented with 0.2g KNO_3 in 1L of D.W: After pH was adjusted to 7.0, medium was distributed into tubes and sterilized by autoclaving.

- **Gelatin medium (Cruickshank *et al.*, 1975)**

This medium was prepared by dissolving 12g of gelatin and completed to 100 ml of nutrient broth medium, then distributed in tubes and sterilized by autoclaving.

- **Methyl Red and Voges-Proskauer (MR-VP) medium (Atlas *et al.*, 1995)**

This medium was prepared by dissolving peptone (5g) and K_2HPO_4 (5g) in 900 ml of D.W.; pH was adjusted to 7.6, the volume was completed to 950 ml with D.W. and sterilized by autoclaving, then 50 ml of 10% glucose solution was added (sterilized by filtration).

- **Peptone Water medium (Atlas *et al.*, 1995)**

This medium was prepared by dissolving peptone (20g) and Sodium chloride (5g) in 950 ml D.W.; pH was adjusted to 7.0, then the volume was completed up to 1L with D.W., mixed thoroughly, distributed into tubes and sterilized by autoclaving.

- **Urea agar medium (Collee *et al.*, 1996)**

It was prepared by adjusting pH of the urea agar base (Christensens media) to 7.0 and autoclaved, then cooled to 50°C, then 50 ml of 40% urea (sterilized by filtration) mixed together then distributed into sterilized tubes, kept as slant.

- **Semi-solid agar medium (Collee *et al.*, 1996)**

It was prepared by dissolving 0.4% of agar in nutrient broth or, in which the final medium should be quite clear and transparent. Then 10 ml was dispensed in test tubes, sterilized by autoclaving and left to stand in a vertical position.

- **Luria- Bertani (LB) medium (Nazina *et al*., 2001)**

This medium was prepared by dissolving tryptone (10g), yeast extract (5g), and NaCl (5g) in 950 ml D.W., pH was adjusted to 7.0, then the volume was completed to 1L with D.W., sterilized by autoclaving, cooled to 55°C then 1ml of 0.1M $MnCl.H_2O$ (Autoclaved separately) was added.

- **Minimal medium (Rahman *et al*., 2003)**

This medium was composed of the basal salts containing the followings:

Component	Concentration (g/L)
$CaCl_2.2H_2O$	0.5
K_2HPO_4	0.2
$MgSO_4.7H_2O$	0.5
KCl	0.2
NaCl	0.1

All components were dissolved in 950 ml D.W., pH was adjusted to 7.0, then the volume was completed to 1L with D.W. and sterilized by autoclaving.

2.1.2 Reagents and Dyes

2.1.2.1 Catalase reagent (Atlas *et al*., 1995)

This solution was consisted of 3% hydrogen peroxide.

2.1.2.2 Oxidase reagent (Atlas *et al*., 1995)

This solution was freshly prepared and consisted of 1% tetramethyl-*p*-phenylenediamine dihydrochloride in D.W.

2.1.2.3 Methyl red indicator (Collee *et al*., 1996)

This indicator was prepared by mixing the following components:

Methyl red	0.05 g
Ethanol (95% v/v)	150 ml
D. W.	100 ml

2.1.2.4 Barritt's reagent (Collee *et al*., 1996)

This reagent was consisted of two solutions:

Solution A: Potassium hydroxide (40%)

Solution B: It was prepared by dissolving 5 g of α-naphthol with absolute ethanol up to 100 ml.

Equal volumes of solution A and solution B were immediately mixed before use.

2.1.2.5 Nitrate test reagent (Atlas *et al*., 1995)

This reagent was consisting of two solutions:

Solution A: It was prepared by adding 0.8 g of sulfanilic acid to 5*N* acetic acid up to 100 ml.

Solution B: It was prepared by adding 0.5 g of Dimethyl-α-naphthylamine to 5*N* acetic acid up to 100 ml.

Equal volumes of solution A and B were immediately mixed before use.

C: Zinc Dust.

2.1.2.6 Kovac's reagent (Atlas *et al*., 1995)

Isoamyl alcohol	150 ml
ρ-Dimethyl-aminobenzaldehyde	10 g
Concentrated HCl	50 ml

ρ-Dimethyl-aminobenzaldehyde was dissolved in isoamyl alcohol with heating in a water bath at 50°C and acid was added slowly. The reagent was prepared in small quantities and stored in refrigerator.

2.1.2.7 Bromophenol blue (0.25%) (Blackshear, 1984)

It was prepared by dissolving 0.25g of bromophenol blue in D.W. up to 100 ml, mixed until dissolved completely.

2.1.2.8 Gram Stain (Atlas *et al.*, 1995)

2.1.2.9 Ethidium bromide solution 10 mg / ml (Maniatis *et al.*, 1982)

It was prepared by dissolving 0.2 g of ethidium bromide in 20 ml D.W. and stirred on magnetic stirrer for few hours till the ethidium bromide was dissolved completely then it was filtrated, and stored in a dark bottle at 4ºC.

2.1.3 Buffers and Solutions:

2.1.3.1 Potassium Phosphate buffer pH 7.0 (Atlas *et al.*, 1995)

K_2HPO_4	50 mM
KH_2PO_4	50 mM

2.1.3.2 Bicinchoninic acid (BCA) protein assay kit reagents

BCA reagent A, contains sodium carbonate, sodium bicarbonate, bicinchoninic acid and sodium tartarate in 0.1M sodium hydrooxide.

BCA reagent B, contain 4% cupric sulfate.

BCA working reagent (WR): it was prepared by mixing 50 parts BCA reagent A with 1 part of BCA reagent B (50:1, reagent A: B).

2.1.3.3 Protease assay

Tris-HCl buffer (0.05M) pH7.5, 5mM $CaCl_2$

Casein solution (1%): It was prepared by dissolving 1g of casein in 0.05M Tris-HCl buffer up to 100 ml.

Trichloroacetic acid (TCA) 10%.

2.1.3.4 Genomic DNA preparation (Qiagen kit):

Qiagen kit contain many buffer solutions. All solutions wete prepared to be consisting of the followings:

Buffer B1 (Bacterial Lysis buffer): 50mM Tris-Cl, pH 8.0; 50mM EDTA, pH 8.0; 0.5% Tween-20; 0.5% Triton x-100.

Buffer B2 (Bacterial Lysis buffer): 3M guanidine HCl; 20%Tween-20.

Buffer QBT (Equilibration Buffer): 750mM NaCl; 50mM MOPS; 15% isopropanol; 0.15% triton x-100, pH 7.0.

Buffer QC (Wash Buffer): 1.0M NaCl; 50mM MOPS; 15% isopropanol, pH 7.0.

Buffer QF (Elution Buffer): 1.25mM NaCl; 50mM tris-Cl; 15% isopropanol, pH8.5.

TE Buffer: 10mM Tris-Cl , pH8.0; 1mM EDTA, pH8.0.

RNase A solution (100mg/ml) in Buffer B1

Proteinase K (20mg/ml) in D.W.

Lysozyme solution (100mg/ml) in D.W.

2.1.3.5 Agarose gel electrophoresis

Tris-Acetate (50X), pH 8.0 (Maniatis *et al*., 1982)

2M Tris base	242 g
Glacial Acetic Acid	57.1 ml
0.5 M EDTA, pH 8	100 ml
D. W. to volume	1000 ml

Gel Loading Buffer 6X (Maniatis *et al*., 1982)

Bromophenol	0.25%
Sucrose	40 %(w/v)

2.1.3.6 DNA extraction from the gel

Binding buffer (1 M): (50 mM Tris 7.5, 1 M NaCl, 1% NP-40)

Washing buffer: (300 mM NaCl, 5 mM $MgCl_2$, 5 mM Dithiothretol (DTT), 0.5% NP-40)

Elution buffer: (10mM Tris-HCl, pH 8.5).

2.1.3.7 Plasmid DNA isolation (miniprep kit)

Buffer A2 (Bacterial Lysis buffer): 3M guanidine HCl; 20% Tween-20.

Buffer A1; A3 (Equilibration Buffer): 750mM NaCl; 50mM MOPS; 15% isopropanol; 0.15% triton x-100, pH 7.0.

Buffer A4 (Washing Buffer): 1.0M NaCl; 50mM MOPS; 15% isopropanol, pH 7.0.

Buffer AE (Elution Buffer): 1.25mM NaCl; 50mM tris-Cl; 15% isopropanol, pH 8.5.

2.1.3.8 Protease purification

A-Affinity chromatography

Wash buffer: 20 mM Na-acetate, 5 mM $CaCl_2$, 10% isopropanol, pH 5.3.

Equilibration buffer: Wash buffer + 2.5 M NaCl

Elution buffer: 20 mM Na-acetate, 5 mM $CaCl_2$, 2.5 M NaCl, 20% isopropanol, pH 5.3.

B-Gel filtration chromatography

Equilibration buffer: 20mM Tris-HCl, pH 7.5

2.1.3.9 Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) (Laemmli, 1970)

A. Stacking Gel Buffer (0.626 M Tris-HCl)

It was prepared by dissolving 7.56 g Tris base in 40 ml of D.W., the pH was adjusted to 6.8 by a slow addition of 1M HCl. The volume was completed to 100 ml with D.W.

B. Resolving Gel Buffer (1.5 M Tris-HCl)

It was prepared by dissolving 22.7g of Tris base in 80 ml of D.W., pH was adjusted to 8.8 by a slow addition of 1M HCl, and the volume was completed to 100 ml of D.W.

C. Sodium Dodecyl Sulphate (SDS) Solution 10% (W/V)

It was prepared by dissolving 10g of SDS in 90 ml of D.W. and the volume was completed to 100 ml with D.W.

D. Resovier Buffer (Tris-Glycine)

It was prepared by dissolving 3.0g Tris base, 14.4g Glycine and 10 ml SDS (10%) solution in D.W., the volume was completed to one liter with D.W.

E. Acrylamide-Bisacrylamide Solution

It was prepared by dissolving 30g of acrylamide and 0.8g bisacryamide in 60 ml of D.W., the volume was completed to 100 ml with D.W. and kept in a dark bottle in refrigerator**.**

F. Ammonium Persulfate Solution (10 %)

Ammonium persulfate (1g) was dissolved in 10 ml of D.W. (freshly prepared).

G. N,N,N,N-Tetramethylene Diamine (TEMED)

It was used as supplied by the manufacturer.

H. Fixing Solution

It was composed of 40% Methanol and 10% Tri-Chloroacetic Acid (TCA).

I. Destaining Solution

It was composed of 40% Methanol and 10% Acetic Acid.

J. Sample Buffer Solution (2X)

It was prepared by mixing 2 ml stacking gel buffer, 3.2 ml of 10% SDS, 1.6 ml glycerol, 0.8 ml of 2-mercaptoethanol and 0.4 ml of 0.25% bromophenol blue, mixed well and stored at -20°C.

2.1.3.10 Silver stain

Solution 1: 60 ml of 50% acetone in D.W.; 1.5 ml of 50% TCA; 25 µl of 37% HCHO

Solution 2: 100 µl of 10 % $Na_2S_2O_3H_2O$ in D.W.

Solution 3: 0.8 ml of 20% $AgNO_3$ in D.W.; 0.6 ml of 37 % HCHO; 60 ml D.W.

Solution 4: 1.2 g Na_2CO_3; 25 µl 37 % HCHO; 25 µl of 10 % $Na_2S_2O_3.5H_2O$ in D.W.

2.1.3.11 Protease characterization solutions

Sodium acetate 50mM (pH 5- 6)

Sodium phosphate 50 mM (pH 7- 8)

Tris-HCl 50 mM (pH 9- 10)

2.1.3.12 Inhibitor and metal ion solutions

Each one of EDTA, Iodoacetic acid, $CaCl_2$, $ZnSO_4$, and $MgCl_2$ was prepared singly by dissolving in D.W. as stock solution to give a final concentration of 2M, then 2mM was prepared from each stock with D.W., while PMSF was prepared by dissolving in isopropanol as stock solution to give a final concentration of 2M, then 2mM was prepared from this stock

solution with isopropanol.

2.1.3.13 Antibiotic stock solutions (Maniatis *et al*., 1982)

Ampicillin (50mg/ ml)

Ampicillin stock solution was prepared by dissolving 50 mg of ampicillin in 1ml of D.W.; it was sterilized by filtration and stored at -20°C.

Kanamycin (25 mg/ ml)

Kanamycin stock solution was prepared by dissolving 25mg in 1ml of D.W.; it was sterilized by filtration and stored at -20°C.

2.1.4 Bacterial standard strain

Competent *E.coli* DH5α (F' *lacZ M15 gyrA96 recA1 endA1 hsdR17*) was obtained from Dr. Mansfeld/ Department of Biochemistry and Biotechnology, Institute of Biotechnology, Martin Luther University Halle-Wittenberg/ Germany.

2.1.5 Enzymes and nucleic acids

Pfu DNA polymerase	Promega (USA)
Alkaline phosphatase	Promega
DNA ligase	Promega
Fast link ligase	Promega
BSA	Biolab (USA)
*Xho*I	Biolab
*Nco*I	Biolab
*Nde*I	Biolab
*Hind*III	Biolab
*Sau3*AI	Biolab
*Bam*HI	Biolab

2.1.6 Primers

Two PCR primers were used in this study that have the ability to introduce appropriate restriction sites supplied by MWG biotech (Germany):

Forward primer NTPro with *Nde*I restriction site:
(5'-CCCAAGCTTAAGGAGATACATATGAAGGGGGAATCG-3').

Reverse primer MatC-Term with *Hind*III restriction site:
(5'-GGGAAGCTTAATACACTCCAACCGCATTG-3').

1.2 Methods

2.2.1 Sterilization Methods

Three methods of sterilization were used:

(A) Moist Heat Sterilization (Autoclaving)

Media and solutions were sterilized by the autoclave at 121˚C (15 Ib/in^2) for 15 min.

(B) Dry Heat Sterilization

Electric oven was used to sterilize glasswares and others at 160-180 ˚C for 3-2 h.

(C) Membrane Sterilization (Filtration)

Millipore filtering was used to sterilize heat sensitive solutions by using millipore filters (0.22 μm) in diameter.

2.2.2 Isolation of protease producing thermophilic *Bacillus* spp. (Krishna and Kodidhela, 2005)

For isolation of protease producing thermophilic *Bacillus* species, 93 soil samples were collected from different locations in Basrah governorate. Aportion of 10 g of each sample were suspended in 90 ml sterile D.W., agitated vigorously in a shaker waterbath at 80°C for 40 min. Serial dilutions for each sample using sterilized phosphate buffer (pH 7.0) were

set up, then 0.1 ml aliquot of the appropriate dilution was taken, spread on skim milk agar plates, and incubated at 65°C for 24h. Replica plating was made for the growing colonies to examine the ability of different colonies in production of protease and make the colonies that showed a halo diameter zones ratio (Z/G); zone/ growth ratio.

2.2.3 Identification of the isolates

In order to identify the selected isolates, the following examination were performed:

2.2.3.1 Microscopic and morphological characteristics (Atlas *et al*., 1995)

The colonies morphology (size, shape, edge, color, and margin)of the bacterial isolates were studied. On the other hand, a loopfull of bacterial suspension was fixed on a slide, and stained by Gram stain to examine Gram reaction, shape grouping, and spore forming of the cells.

2.2.3.2 Biochemical tests

Catalase Test (Atlas *et al*., 1995)

A single colony of different isolates was placed onto a clean glass microscope slide with a sterile toothpick, then a drop of hydrogen peroxide (3%) was placed onto the colony. Production of gaseous bubbles indicates a positive result.

Oxidase Test (Atlas *et al*., 1995)

This test was done by using moistened filter paper with few drops of a freshly prepared solution of tetramethyl-ρ-phenylene diamine dihydro-chloride. Aseptically, a clump of cells was picked up from the slant growth with a sterile wooden stick and smeared on the moistened paper. Development of a violet or purple color within 10 seconds indicates a positive result.

Methyl Red Test (Collee *et al*., 1996)

MR-VP broth were inoculated with a single colony of bacterial isolate and incubated at 55°C for 48 hours, then five drops of methyl red reagent was added and mixed gently. Presence of bright red color indicates a positive result.

Voges-Proskauer Test (Collee *et al*., 1996)

MR-VP broth were inoculated with a single colony of bacterial isolates and incubated at 55°C for 24 hr. then 1 ml of Barritt's reagent (2.1.2.4A) and 3 ml of Barritt's reagent (2.1.2.4B) were added to 5 ml of cultured broth and shaked for 30 seconds. Formation of pink to red color indicates a positive result.

Nitrate Reduction Test (Atlas *et al*., 1995)

Single colony of each bacterial isolate was used to inoculate 5 ml of nitrate media, then test tubes were incubated at 55°C for 24 hr. After incubation, 0.1 ml of the test reagent (2.1.2.5) was added to each tube. The immediate formation of red color indicates a positive result.

Indole Test (Collee *et al.,* 1996)

Peptone water was inoculated with a single colony of bacterial isolates and incubated at 55˚C for 48 hours. Fifty microlitter of Kovac's reagent (2.1.2.6) was added and mixed gently. Appearance of a red ring on the surface of liquid media indicates a positive result.

Gelatin Liquefaction Test (Cruickshank *et al.,* 1975)

Tubes of gelatin media were inoculated with a single colony of bacterial isolates and incubated overnight at 55^{o}C, then transferred to refrigerator for an hour. Liquefaction of tubes indicated positive result.

Citrate Utilization Test (Atlas *et al*., 1995)

Simmon's citrate agar slants were inoculated with a single colony of bacterial isolates, and incubated for 24 hrs at 55ºC. Changing of color to royal blue indicates a positive result.

Urease Test (Atlas *et al*., 1995)

Urease activity was detected by streaking Christensen urea agar slants with the bacterial growth and incubated at 55°C for 24 hrs. Appearance of a red-violet color indicates a positive result.

Motility Test (Collee *et al*., 1996)

Semi-solid agar medium was inoculated with each bacterial isolates using a straight wire to make a single stab down the center of the tube to about half the depth of the medium. Motile bacteria typically give diffuse, hazy growth that spreads throughout the medium rendering it slightly opaque.

2.2.4 Maintenance of Bacterial Isolates

Maintenance of bacterial isolates was performed according to Maniatis *et al*. (1982) as follows:

2.2.4.1 Short-Term Storage

Bacterial isolates were maintained for few weeks on LB agar slants. They were tightly wrapped with parafilm, and then stored at 4°C.

2.2.4.2 Medium-Term Storage

Bacterial isolates were maintained as stab cultures for few months. Such cultures were prepared in small screw-capped bottles containing 2-3 ml of LB agar medium and stored at 4°C.

2.2.4.3 Long-Term Storage

Single colonies were cultured in LB broth and incubated for 24 h. and then 8.5 ml of bacterial culture mixed with 1.5 ml of glycerol, then stored for a long time.

2.2.5 Determination of protease activity (Manachini *et al*., 1989)

After the appropriate incubation period, bacterial culture was centrifuged at 15000 rpm for 20 min. at 4˚C. The clear supernatant was assayed for proteolytic activity by casein digestion method. Activity of protease was assayed in triplicate by measuring the release of TCA soluble peptides from 1% (w/v) casein solution. The assay mixture consisted of 0.8ml casein solution and 0.2ml of enzyme solution, incubated at 55˚C for 30min. The reaction was terminated by the addition of 1ml of TCA reagent, incubated in ice bath for 10-15min, and centrifuged at maximum speed for 20 min. the control was prepared using the same steps except the addition of 1ml of TCA reagent into casein solution before the addition of 0.2ml of enzyme solution. The absorbance was measured at 280 nm since one unit (U) of enzyme activity was defined as the amount of enzyme required to produce an increase in absorbance at 280 nm equal to 0.01in one minute under experimental conditions according to the following equation:

$$\text{Enzyme activity (U/ml)} = \frac{\text{Absorbance at 280 nm}}{0.01 \times 30 \times 0.2}$$

0.01: Constant

30 : Reaction time (min.)

0.2 : enzyme volume (ml)

$$\text{Specific activity (U/mg)} = \frac{\text{Activity (U/ml)}}{\text{Protein concentration (mg/ml)}}$$

2.2.6 Determination of protein concentration

Protein concentration was determined by the BCA protein assay kit (Pierce, Germany) using bovine serum albumin (BSA) as standard protein. The contents of one Albumin Standard (BSA) ampule (2mg/ml) were diluted using 0.05M phosphate buffer pH 7.0 to prepare a set of standards within the assay range of (0-700) µg/ml.

Assay protocol

A- Samples were added directly to the center of the well.

B- The plate was covered and mixed on a shaker at medium speed for one min. then incubated at 37°C for 15 minutes.

C- Two hundred µl of BCA working reagent (2.1.3.2) was added to each well. The plate was covered and mixed on a plate shaker for one minute; the plate was incubated at 37°C for 30 minutes.

D- Using the standard control as the blank, the absorbance of the standards and unknown samples were measured at 562 nm on a plate reader.

E- A standard curve was prepared by plotting the average blank-corrected 562 nm value for each BSA standard vs. its concentration (µg/ml). The standard curve was used to determine the protein concentration of each unknown sample (Figure 2.1).

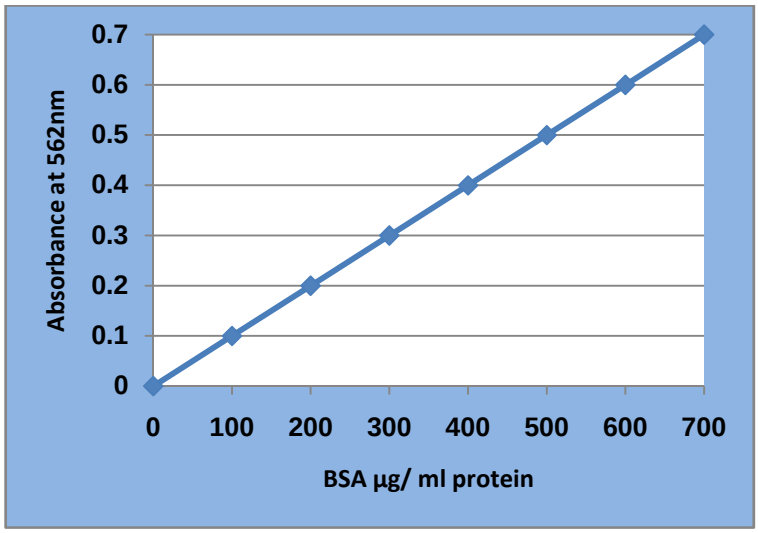

Figure (2.1): Standard curve to determine protein concentration using Bicinchoninic method.

2.2.7 Genetic analysis

2.2.7.1 Isolation of Genomic DNA with mid prep Qiagen kit (Genomic-tip100/G)

Part I: Sample preparation and lysis protocol for bacteria

A. *Bacillus stearothermophilus* was cultured in 50 ml LB broth with shaking (120rpm) for 24h. at 55°C until OD of the culture reach 0.6 at 600nm.

B. Bacterial cells were pelleted by at 6000 rpm for 10min, then the bacterial pellet was resuspended in 3.5ml of buffer B1(2.1.3.4.1)(with RNase A) by vortexing at maximum speed.

C. A portion of 80 µl of lysozyme stock solution (100mg/ml), and 100µl of proteinase K stock solution (20mg/ml) were added and incubated at 37°C for at least 30min.

D. A portion of 1.2 ml of Buffer B2 (2.1.3.4.2) was added and mixed by inverting for several times or by vortexing for a few seconds. This step is important for efficient deproteinization.

E. To get clear lysate, if necessary the particle matters were pelleted by centrifugation at 5000rpm for 10 min. at 4°C.

Part II: Genomic- tip prepatation

A- A Qiagen genomic 100/G was equilibrated with 4ml of buffer QBT (2.1.3.4.3) and allows the Qiagen genomic tip to empty by gravity flow.

B- The sample (from the last step of the lysis protocol) was vortexed for 10sec. at maximum speed and applied to the equilibrated Qiagen genomic-tip. Allowed it to enter the resin by gravity flow.

C- Qiagen Genomic-tip was washed twice with 7.5 ml of buffer QC (2.1.3.4.4) .

D- Genomic DNA was eluted with 2 × 1 ml of buffer QF (2.1.3.4.5).

E- DNA was precipitated by adding 3.5 ml isopropanol to the eluted DNA.

F- DNA was precipitated by inverting the tube 10 to 20 times, then DNA was spooled using a glass rod.

G- The spooled DNA was immedialtely transferred into a microcentrifuge tube containing 0.5ml of TE buffer, then DNA was dissolved at 37°C for 2h. and centrifuged at 5000 rpm for 10min at 4°C.

H- DNA pellet was then washed with 2ml of cold 70% ethanol, vortexed briefly and recentrifuged at 5000rpm for 10min at 4°C. The supernatant was carefully removed without disturbing the pellet layer; air dried for 10 min, and the DNA was resuspended in 0.5ml of TE buffer.

I- A portion of 8 µl of DNA sample was taken and added to 72 µl of sterilized distilled water in a quartz cuvette and the absorbancy at 260 nm and 280 nm was measured after calibration with distilled

water at 260 nm and 280 nm respectively, the final concentration of DNA was measured according to the following formula: Concentration of double strand DNA (µg/ml) = (A_{260} ×50 × 10). Pure DNA has an A_{260}/A_{280} ratio of 1.7-1.9.

2.2.7.2 Polymerase Chain Reaction (PCR)

- **PCR with thermal (T)gradient (52-70)°C:**

Amplification of mature sequence was carried out with the whatman Biometra Thermocycler gradient (Germany). PCR master mixture contains 2µl of genomic DNA, 1µl of 100 pmol of MatC and NTpro primers (the sequences of primers are given in 2.1.6, 2µl of 2U/µl *Pfu* DNA polymerase, 2µl of 10mM deoxynucleotide triphosphates (dNTPs), 6.8µl of 7.5% dimethylsulphoxide (DMSO), 9µl of 10X PCR reaction buffer, and 46.2 of sterile distilled water to complete the volume to 70µl. The reaction mixture was mixed well then divided into 7 PCR eppendorff tubes. PCR cycler was programmed as follows: One cycle of 95°C for 3min; 40 cycles of 95°C for 1min, gradient from (52-70) °C for 1min, 72°C for 2min; and final extension at 72°C for 8min.

- **PCR with T gradient (50, 51, and 53)ºC**

Amplification of mature sequence was repeated under the same conditions with lower gradient temperature (50, 51, and 53) °C.

- **Reamplification of PCR product**

PCR reaction was repeated with the same program and reaction mixture mentioned above (final volume of 50µl) with the annealing temperature of 52°C, the amplified PCR product was loaded on 0.8% agarose gel, desired band from PCR product was cut from the gel and eluted using peqGold Gel Extraction Kit.

2.2.7.3 Agarose Gel Electrophoresis

Electrophoresis was carried out on a horizontal submarine electrophoresis apparatus. 0.8% agarose gel was prepared in 1X TAE buffer, and ethidium bromide was added to give a 0.5 µg/ml final concentration in melted agarose gel. Electrophoresis was performed at 220 Volts for 45-60 min. The DNA bands were visualized on a shortwave UV transilluminator and photographed by using Vilber Lourmat Gel Imaging System. GeneRuler DNA marker (10kb) (Fermantas/Germany) was used to determine the molecular weights of DNA bands.

2.2.7.4 Purification of DNA fragment with peqGold Gel Extraction Kit (Biotechnonlogie Gmbh/ germany)

With pegGold gel extraction kit, a DNA fragment of interest was excised out of the gel with a sterile razor blade under a UV transilluminated, placed in 1.5ml sterile microfuge tube, then purified according to the protocol described by biotechnologie (Germany). The excised gel piece was estimated for the volume, an equal volume of binding buffer was added into microfuge tube. This microfuge tube was then incubated at 55ºC for 7min or until the gel melted completely. The HiBind spin column was placed in a 2 ml collection tube and 700µl of the DNA/ agarose solution was added, the spin column collection tube was centrifuged for 1 min at high speed, the flow- through was discarded and the collection tube was kept for further steps. 300µl of binding buffer was added, centrifuged at high speed for 1 min at room temperature, 750µl of washing buffer was added, centrifuged at high speed for 1 min. The column containing the DNA was placed into new microfuge tube and 30 µl elution buffer was added directly to the binding matrix and centrifuged for 1 min. at high speed to elute the DNA.

2.2.7.5 Subcloning in CloneJET PCR Cloning Kit (Fermantas/ germany)

For cloning blunt-end PCR product generated by *Pfu* DNA polymerase, this protocol was used:

- The ligation reaction

Component	Volume (µl)
Reaction buffer (2X)	5
PCR product (40ng/ µl)	3.5
pJET 1.2/ blunt cloning vector (50ng/µl)	0.5
T4 DNA ligase (5U/µl)	0.5
Adenosine Triphosphate ATP (20mM)	0.5
Total volume	10

- Vortexed briefly and centrifuged for 3-5sec.
- The ligation mixture was incubated at 25°C for 30 min. and heat inactivated at 65°C for 10min. then ligation mixture was used for bacterial transformation.

2.2.7.6 Isolation of plasmid DNA with NucleoSpin plasmid Miniprep kit (Machery- Angel/ germany)

A single colony of *E. coli*, which harbured engineered plasmids, was grown in 5ml of LB broth with Ampicillin (50µg/ml) overnight. The cell pellet from 3 to 5 ml of the culture was collected in a microfuge tube. The plasmid miniprep protocol for the kit was applied as followed: Pellet was resuspended in 250µl of buffer A1 resuspension solution (2.1.3.7.2), then 250µl of buffer A2 lysis solution (2.1.3.7.1), incubated at a room temperature for 5 min, and 300µl of neutralization (A3) buffer (2.1.3.7.2) was added respectively. Mixed solution was centrifuged at maximum speed

for 10 min. Supernatant was taken out and transferred into plasmid Nucleospin quick pure column which was placed in 2 ml collection tube. Centrifuged at high speed for 1 min, the flow through was discarded, then 600µl of washing (A4) buffer (2.1.3.7.3) was added, centrifuged and the flow through was discarded.

Plasmid DNA was purified by absorption by resin and eluted with 50µl of elution (AE) buffer (2.1.3.7.4) after centrifugation at high speed for 1min.

2.2.7.7 Digestion of pET-28b(+) cloning vector

- The digestion mixture was contained the following component volumes:

Component	Volume (µl)
Reaction buffer (10X)	8
pET-28b(+)cloning vector (50ng/µl)	15
*Nde*I (20U/µl)	2
*Hind*III (20U/µl)	2
Sterile D.W.	53
Total volume	80

- The mixture was incubated at 37°C for 4h.
- Aliquot of 0.2 µl of alkaline phosphatase (1U/µl) was added and incubated at 37°C for 30min and heat inactivated at 65°C for 15min.
- The mixture was analyzed on 0.8% agarose gel and the desired band was cut from the gel then extracted using Gene JET Gel Extraction Kit (Fermantas/ Germany).

2.2.7.8 Digestion of insert in Clone JET PCR Cloning vector

- The digestion mixture was contained the following volumes:

Component	Volume (µl)
Reaction buffer (10X)	8
Insert DNA (50ng/µl)	15
*Nde*I (20U/µl)	2
*Hind*III (20U/µl)	2
Sterile D.W.	53
Total Volume	80

- The mixture was incubated at 37°C for 4h, heat inactivated at 65°C

for 15min.

- The mixture was analyzed on 0.8% agarose gel, the desired band was cut from the gel then extracted using GeneJET Gel Extraction Kit (Fermantas/Germany).

2.2.7.9 Digestion of pUC18 cloning vector

- The digestion mixture was contained the following volumes:

Component	Volume (µl)
Reaction buffer (10X)	8
BSA (10mg/ml)	0.8
pUC18 (50ng/µl)	20
*Bam*HI (12U/µl)	3
Sterile D.W.	48.2
Total volume	80

- The mixture was incubated at 37°C for 4h.

- Aliquot of 0.2 µl alkaline phosphatase (1U/µl) was added and incubated at 37°C for 30min.
- The mixture was analyzed on 0.8% agarose gel and the desired band was cut from the gel then extracted using Gene JET Gel Extraction Kit (Fermantas/ Germany).

2.2.7.9 Partial digestion of genomic DNA

- Partial digestion mixtures was contained the following volumes

Component	Volume (µl)
Reaction buffer (10X)	5
BSA (10mg/ml)	0.5
Genomic DNA (8.6 µg/µl)	15
*Sau*3A (4U/µl)	0.5
Sterile D.W.	29
Total volume	50

- The reaction mixtures was incubated with *Sau*3A at 37°C for different times (60, 40, 20, 8, and 5) min.
- The reaction was stopped by the addition of 4µl of 0.5M EDTA.
- The mixture was analyzed on 0.8% agarose gel and the desired genomic size was cut from the gel then extracted using Gene JET Gel Extraction Kit (Fermantas/ Germany).

2.2.7.11 Purification of DNA fragment with GeneJET Gel Extracion Kit (Fermantas/ Germany)

The basic and steps are the same as with peqGold Gel Extraction Kit (2.2.6.4).

2.2.7.12 Ligation reactions

- Ligation of pET-28b(+) with the insert DNA and pUC18 with the genomic DNA were carried out using the following volumes:

Component	Volume (µl)
Fast link buffer (10X)	1
ATP (20mM)	0.5
pET-28(+) (50ng/µl); pUC18 (50ng/µl)	1
Insert DNA (50 ng/µl); Genomic DNA (8.6 µg/µl)	7
Fast link ligase (3U/µl)	0.5
Total volume	10

- The ligation mixture was incubated at a room temp. for 15 min and at 16°C for 5h.
- The reaction was stopped by heat inactivation at 70°C for 15 min, then used directly for transformation of the bacteria.

2.2.7.13 Transformation of competent *E. coli* DH5α (Maniatis *et al*., 1982; Inoue *et al*., 1990)

For transformation of competent *E. coli* DH5α, cells were kept on ice for 15 min. then 5µl aliquots of ligation products were added to the cells and mixed gently. The mixture was incubated on ice for 20 min. After a heat shock at 42°C for 45 sec, it was incubated on ice for 5 min., then 900µl of LB was added to the mixture and incubated at 37°C for 60 min by gentle agitation (100 rpm). Transformed cells were plated on selective medium for the expression of protease gene (skim milk agar media) containing appropriate antibiotic (50µg/ml ampicillin or 25 µg/ml kanamycin), incubated at 37°C for 24hrs. Positive colonies were surrounded by a clear zone on the selective medium, while negative control was *E. coli* DH5α before transformation. Positive transformant(s) were

assayed for the specific activity of protease encoded by the possible recombinant protease gene as mentioned in (2.2.4).

2.2.7.14 Mutagenesis of *B. stearothermophilus*

Physical mutagenesis by UV irradiation

Mutagenesis by UV irradiation was done according to David *et al*. (2005) by subjecting fresh culture of *B. stearothermophilus* to UV radiation in a dark place using the UV- transilluminator. The tray of the irradiation approximately 15X25 cm exposes sample in glass Petri dish and the distance between the UV source and irradiated suspension was 11 cm.

B. stearothermophilus was cultivated in LB broth at 55°C for 18h, followed by centrifuging 10 ml at 4000rpm for 10 min. The cell pellet was diluted in sterile phosphate buffer pH 7, and adjusted to a concentration of 10^6 CFU/ml, the cell suspensions were poured in sterilized Petri dishes and exposed to 1, 2, 3, 4, 5, and 6 J/m² UV irradiation under sterile conditions, then 0.1 ml of cell suspension was taken after each treatment diluted to appropriate dilution and plated on skim milk agar medium. Plates were then incubated over night at 55ºC to determine the viable count and survivals of *B. stearothermophilus*. Bacterial cells subjected to the dose at which 90% of the cells were killed (LD_{90}) were considered mutants and used for further study.

Chemical mutagenesis by MNNG

Mutagenesis by MNNG was done according to Cheng-gang *et al*. (2007) and as following:

B. stearothermphilus was cultivated in LB broth at 55˚C for 18 h, then bacterial cells were centrifuged at 4000rpm for 10 min. The cell pellet was diluted in sterile phosphate buffer (pH 7.0), and adjusted to a concentration of 10^6 CFU/ml, then 1ml of the cell suspension was incubated with 1 ml of 0.2 mg/ ml MNNG solution at 37°C for different periods of time (20, 40, 60, 80, and 100 min), 0.1 ml serially diluted aliquots were taken after each treatment and plated on skim milk agar plates and incubated at 55°C overnight to

determine the viable count and survivals of *B. stearothermophilus*. Bacterial cells subjected to the dose at which 90% of the cells were killed (LD_{90}) were considered mutants and used for further study.

2.2.8 Optimum conditions for protease production

The main optimal conditions for protease production were performed in order to achieve higher production and as following:

2.2.8.1 Optimum culture conditions for protease production

Various carbon (glucose, starch, sucrose, lactose, and maltose) and nitrogen sources (soybean meal, yeast extract, $(NH_4)_2SO_4$, meat extract and peptone) were supplemented individually to the minimal medium. All these sources were added in the medium at a final concentration of 1% (w/v) for carbon and nitrogen, respectively. The initial pH of the medium was set at 7.0. Protease activity was determined in the supernatants after inoculation of the medium with 1% of *B. stearothermophilus* log phase culture, and incubation at 55°C under shaking (200 rpm).

2.2.8.2 Optimum pH for protease production

Optimal pH for production of protease was determined by preparing the medium with different pH values (5, 6, 7, 8, 9, and 10). Protease activity was measured in the supernatant after inoculation of the production medium with 1% of *B. stearothermophilus* log phase culture, and incubation at 55°C under shaking (200 rpm).

2.2.8.3 Optimum temperature for protease production

B. stearothermophilus was grown in the production medium and incubated at different temperatures (40, 45, 50, 55, 60, 65, 70, 75) °C. The protease activity was determined in supernatants after centrifugation at 6000 rpm for 10min.

2.2.8.4 Optimum incubation period for protease production

The production medium was inoculated with fresh culture of *B. stearothermophilus* and incubated at 55°C for (6, 12, 18, 24, 30, and 36)

h. The protease activity was determined in supernatants after centrifugation at 6000 rpm for 10min.

2.2.9 Protease purification

The protease purification steps were carried out according to Bertus *et al*. (1989) and as follows:

2.2.9.1 Enzyme production and preparation of cell free filtrate

B. stearothermophilus was grown in the production medium under the optimum conditions. Crude filtrate (crude protease) was collected by centrifugation at 6000 rpm for 15 min. at 4ºC in order to obtain a cell free filtrate, then 200 ml of the cell free filtrates containing protease were collected and their proteolytic activities and protein concentration were determined.

2.2.9.2 Ammonium sulfate precipitation

Ammonium sulfate precipitation was achieved by adding ammonium sulfate to the crude enzyme gradually with continuous mixing on ice at saturation ratio of 80%, the mixture was centrifuged at 6000 rpm for 20 min at 4°C. The resulted pellet was dissolved in 40 ml of 0.05M Tris/ HCl buffer pH7.5, 5mM $CaCl_2$, The proteolytic activity and the protein concentration were determined.

2.2.9.3 Dialysis of crude enzyme

The obtained ammonium sulfate precipitate (in solution) was introduced into dialysis tube (special plastic bag) with 10000 MWcutoff against 0.05M Tris/ HCl buffer pH7.5, 5mM $CaCl_2$ for 3 h., followed by dialysis against the same buffer over night. The obtained protease enzyme preparation was kept at 4°C for further purification.

2.2.9.4 Application on column chromatographic technique

A. Affinity chromatography

The dialyzed protease (20 ml) with pH 5.3 was loaded onto a Bacitarcin-silica column (1x 11cm), equilibrated with 20 mM Na-acetate, 5

mM $CaCl_2$, 10% isopropanol, pH 5.3. After loading, the column was washed with 4 column volumes equilibration buffer and 4 volumes of the same buffer containing 2.5 M NaCl. The protease was eluted (2 ml/fraction) at flow rate of 30ml/hr with 20 mM Na-acetate, 5 mM $CaCl_2$, 2.5 M NaCl, 20% isopropanol, pH 5.3. Optical density was measured for each fraction at 280 nm. The enzymatic activity for each fraction was assayed as described in (2.2.4), the curve of enzymatic activity (unit/ml) was plotted against $O.D._{280}$ nm. The fractions that revealed significant peak of activity were pooled and kept for further steps of purification.

B. Gel filtration chromatography

Analytical gel filtration was performed with Fast Protein Liquid Chromatography (FPLC) system with a superdex 75 column (1x 30 cm, Pharmacia, Sweden). The fractions collected from Bacitracin-silica column chromatography were applied to a Sephadex 75 column previously equilibrated with 20mM Tris-HCl buffer, pH 7. Elution was performed with the same buffer, the fractions that revealed the protein and enzymatic activity in the same peak were mixed and transferred to a new sterile tube for further study.

2.2.10 Determination of protease purity and molecular weight estimation (Bollag *et al.*, 1996)

Polyacrylamide gel electrophoresis was performed in the presence of SDS (SDS-PAGE) according to Laemmli (1970) using 4.0 % stacking and 12.0 % polyacrylamide resolving gels (0.7 mm thickness). Gels were run using a Mini-Protein II Cell apparatus (Bio-Rad Laboratories, Hercules, CA). Low molecular weight proteins (phosphprylase b, Albumin, Ovalbumin, Carbonic anhydrase, Trypsin inhibitor, α Lactalbumin) were used as a standards (Amersham, Germany) to estimate the molecular weight of separated proteins.

For 4x gel preparations, the Separating (resolving) gel was prepared by adding 10 ml of (acrylamide/bisacrylamide) solution, 4.8 ml of resolving gel buffer pH 8.8, 240 µl of 10% SDS solution and 9 ml of distilled water, the solution was degassed for 10 min. using a vacuum pump, then 60 µl of 10 % ammonium persulphate and 10 µl of TEMED were mixed gently. Using pasteur pipet, the separating gel was transferred to the gel cassette, using another pipet, the top of the gel was covered slowly with isopropanol and allow the gel to polymerize about 1 hr at room temperature, then the layer of isopropanol poured off.

Stacking gel was prepared by adding 2.6 ml of (acrylamide/ bisacrylamide) solution, 3.2 ml of stacking gel buffer pH 6.8, 160µl of 10% SDS solution and 6.8 ml of distilled water, the solution was degassed for 10 min, using a vacuum pump, then 60 µl of 10 % ammonium persulphate and 10 µl of TEMED were added and mixed gently. Using pasteur pipette the stacking gel was transferred slowly over the separating gel at a level which allow the comb to be inserted with 5mm between the bottom and the well, the gel was allowed to polymerize about 2 h. at room temperature.

Samples for SDS-PAGE were prepared by adding an equal volume of sample buffer solution (1X) to the sample and mixed well. The samples were incubated in thermal block at 90ºC for 5 min. and cooled to 25ºC.

The PAGE casette were submerged in Resorvier buffer, and 20 µl of the prepared sample was loaded on the gel. The power supply was connected to the cell and run at 20 mA. The total run time for a gel was about 4hr. with cooling at 4ºC.

2.2.11 Detection of protein bands with silver stain (Shevchenko *et al.*, 1996; Soresen *et al.*, 2002)

Polyacryamide gel was removed from the PAGE casette and placed separately in a plastic container. Using buffer solutions prepared in (2.1.3.10) gel was covered with solution 1 for silver staining for 5min., fixing solution

was poured off, and the gel was washed with H_2O for 5 min., then pretreated with 60 ml of 50% aceton, the gel was pretreated with solution 2 for 1 min., after rinsing in water, the gel was stain with solution 3(silver stain) for 8min., then staining solution was poured off and the gel was immerged with solution 4 for 10- 20s. destaining was done by adding 1% glacial acetic acid. The destaining process continued until the bands were obtained. Gel was preserved in 7% acetic acid.

The relative mobility (Rm) of the markers were plotted against their known molecular weight on a logarithmic scale and the sample was calculated in accordance.

2.2.12 Detection of protein band with mass spectrometry (Shevchenko *et al*., 1996)

The protein band of interest was excised out of the PAGE gel with a sterile razor blade under sterile conditions, placed in 1.5ml sterile microfuge tube, and the tube containing protein band was sent to mass spectrometry unit.

2.2.13 Protease characterization

Some of the characteristics of partially purified protease were determined and as the following:

2.2.13.1 Determination of pH effects on protease activity

Using buffer solutions described in (2.1.3.11) which had been distributed evenly into aliquots, the pH was adjusted in each one according to the required value, 0.1 ml of partially purified protease was added to 0.9 ml from each one of different pH from (5 to 10).

The activity of protease was assayed and the remaining activity was plotted against the pH values to determine the optimal pH for protease activity.

2.2.13.2 Determination of pH effects on protease Stability

Equal volumes of partially purified enzyme and buffer solutions (2.1.3.11) with pH range (5 to 10) were incubated at a room temperature for 30 min. The enzymatic activity for each one was measured.

The remaining activity (%) for protease was plotted against the pH value of solutions to determine the optimal pH for protease stability.

2.2.13.3 Determination of optimal temperature for the activity and thermal stability for protease

The protease activity was measured at different temperatures (30, 40, 50, 60, 70, 80, 90, and 100) °C. The remaining activity was plotted against the temperature.

While for thermal stability, equal volumes of partially purified protease was incubated in water bath at (30, 40, 50, 60, 70, 80, 90, and 100) °C for 30min., and immediately transferred into an ice bath. Enzymatic activity was measured and the remaining activity (%) was plotted against the temperature.

2.2.13.4 Determination of metal ion and inhibitor effects on protease activity

The partially purified protease was pretreated for 30min at a room temperature with 2mM of the following reagents (1:1v/v) before assay: iodoacetic acid, EDTA (ethylenediaminetetraacetic acid, PMSF(phenylmethansulfonyl floride), $CaCl_2$, $ZnSO_4$, and $MgCl_2$. The enzymatic activity was measured under standard assay conditions.

Chapter Three
Results
and
Discussion

3. Results and Discussion

3.1 Isolation of *Bacillus* spp. producing protease

In order to isolate *Bacillus* spp. producing protease, 93 soil samples were collected from different places in Basrah (Iraq). One hundred eight bacterial isolates were obtained; of which 53 isolates (57%) were identified as thermophilic *Bacillus* spp. when subjected to morphological and microscopical tests in which growing colonies on the nutrient agar had a rough surface with an unacceptable odor. After cells were stained with Gram stain, results showed that they were Gram positive. This result agreed with that of Watanabe and Hayano (1993), they were identified 65 isolates of *Bacillus* spp. out of 100 soil samples.

Proteolytic activity was assayed using skim milk agar and expressed as diameter of clear zone in mm. Forty five of the 53 (83.39%) tested isolates were protease producing according to the clear zone around the growing colony on skim milk agar for 24h. at 65°C (Figure 3.1). A clear zone of skim hydrolysis gave an indication of protease producing organisims (Nunes and Martin, 2001).

Upon quantitative screening for the highest protease producing isolates on skim milk agar, 9 isolates were chosen for further biochemical tests and named (B3, B4, B9, B12, B17, B32, B35, B41, B44) to select an effecient *B. stearothermophilus* in protease production (Figure 3.2).

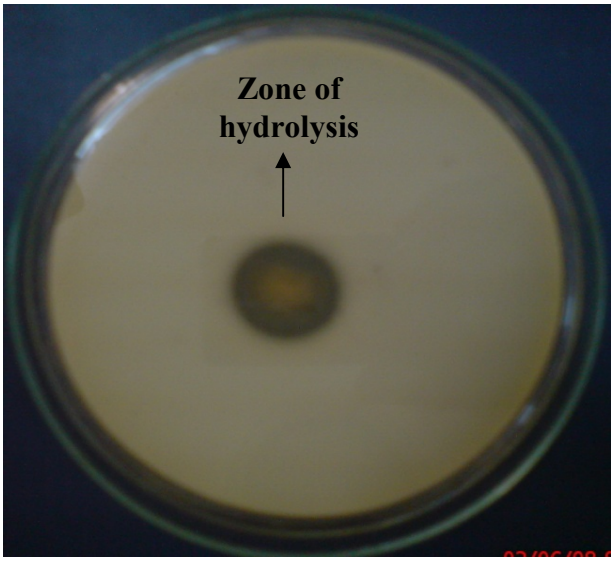

Figure (3.1): Proteolytic activity of a thermophilic *Bacillus* spp. on 10% skim milk agar at 65°C for 24h.

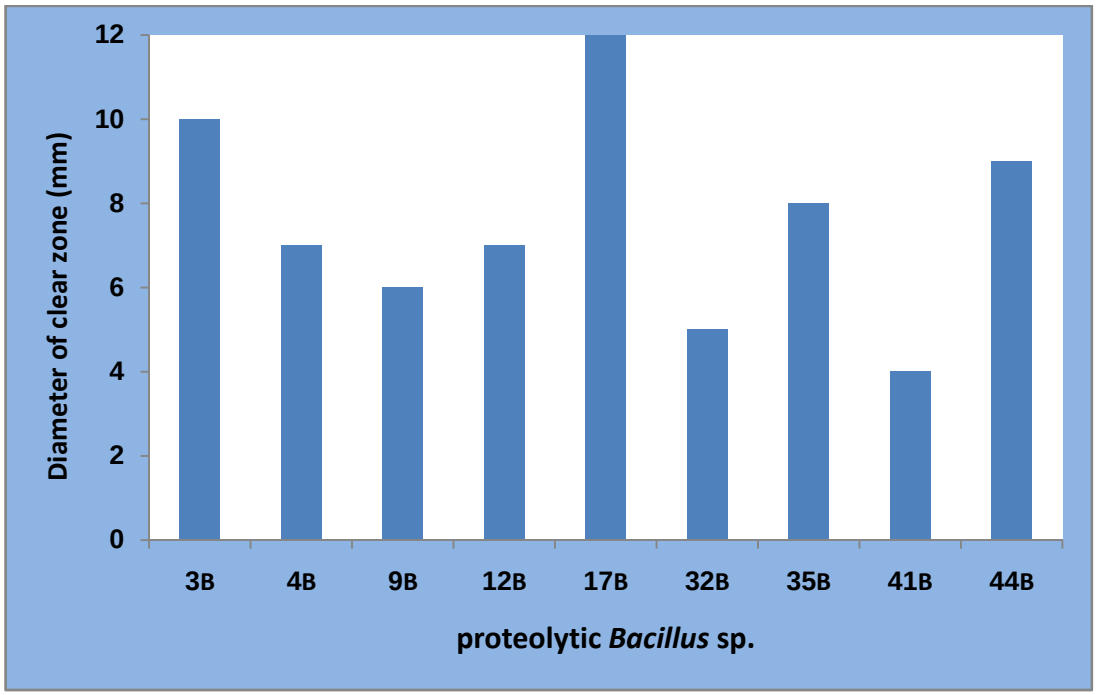

Figure (3.2): Clear zone of proteolytic *Bacillus* spp. on skim milk agar in mm.

3.2 Identification of the isolates

The nine selected *Bacillus* spp. isolates were subjected to the biochemical tests. The results (Table 3.1) indicate that only 6 isolates (B3, B4, B12, B17, B35, B44) from them were *B. stearothermophilus* according to the criteria of Bergey's manual of systematic bacteriology (Sneath *et al*., 1986).

Table (3.1): Biochemical and physiological characterization for identification of *Bacillus stearothermophilus* isolates

Isolate symbol / Test	*B. stearothermophilus* (standard)	B3	B4	B9	B12	B17	B32	B35	B41	B44
Catalase	+	+	+	-	+	+	-	+	-	+
Oxidase	ND	-	-	-	-	-	+	-	+	-
MR	+	+	+	+	+	+	-	+	-	+
VP	-	-	-	-	-	-	+	-	+	-
Nitrate reduction	+	+	+	-	+	+	-	+	-	+
Indole	-	-	-	+	-	-	+	-	+	-
Gelatin	+	+	+	-	+	+	-	+	-	+
Citrate utilization	+	+	+	+	+	+	+	+	-	+
Urease	ND	-	-	+	-	-	+	-	+	-
Motility	+	+	+	-	+	+	+	+	-	+
Growth at 40 °C	+	+	+	+	+	+	+	+	+	+
Growth at 50 °C	+	+	+	+	+	+	+	+	+	+
Growth at 60 °C	+	+	+	+	+	+	+	+	+	+
Growth at 65 °C	+	+	+	+	+	+	-	+	-	+

(+): Positive Result, (-): Negative Result, (ND): test is not performed

3.3 Screening for proteolytic activity

The six chosen *B. stearothermophilus* isolates were grown in LB broth at 55°C for 24hrs, then screened for their proteolytic activity by using casein digestion method. Results indicated in table (3.2) show that all the isolates were protease producers. Among them, *B. stearothermophilus* B17 was the most efficient one. The specific activity of protease in crude filtrate was 36.4 U/mg protein, while the specific activity for the other isolates were ranged between 23 and 27U/mg protein. According to these results, the isolate B17 was selected to be used for improving protease production. *Bacillus* strains continue to be dominant enzyme producing microorganisms in applied and industrial microbiology, these organisms are an important source of industrial extracellular enzymes, including proteases and amylases (Marcus *et al.,* 2004).

Table (3.2): Specific activities of protease produced by the six local isolates of *B. stearothermophilus* after 24h. incubation at 55°C in LB broth (pH 7.0).

Isolate	Specific activity (U/mg protein)
B3	27
B4	23.3
B12	23.0
B17	36.4
B35	24.2
B44	25

3.4 Gene manipulation of *B. stearothermophilus* B17 protease gene

3.4.1 Amplification of *B. stearothermophilus* B17 protease gene

Genomic DNA of the locally isolated *B. stearothermophilus* was extracted from this bacterium using mid prep Qiagen kit, the purity ratio ($O.D._{260}/O.D._{280}$) was 1.8 and can be used for the next cloning steps. The nitrogenous bases in nucleotides have an absorption maximum at about 260 nm. Also, the absorbance of a DNA sample at 280 nm gives an estimate of the protein contamination of the sample. The ratio of the absorbance at 260 nm/ absorbance at 280 nm is a measure of the purity of a DNA sample; it should be between 1.65 and 1.85 (Maniatis *et al*., 1982).

PCR of mature sequence using the genomic DNA as a template with an annealing temperature gradient between (52- 70) °C and NTPro and MatC-Term as forward and reverse primers respectively was done in order to amplify the protease gene of *B. stearothermophilus* B17. The expected 900 bp gene product belonging to the neutral protease *Npro* ORF (open reading frame) was obtained on 0.8% agarose gel (Figure 3.3 A). In order to check the desired annealing temperature for highest PCR product, amplification of mature protein sequence was repeated under the same conditions with lower gradient temperature (50, 51, and 53) °C (Figure 3.3B), the expected band was obtained with a lower sharpness. From these results we concluded that 52°C was the best annealing temperature to amplify protease gene with T gradient PCR. Therefore, reamplification of PCR product was done at 52°C (Figure 3.4C) and the amplified fragment (900bp) was purified from the gel with peqGold Gel Extraction Kit as described in Materials and Methods, then used for further study. The success in amplifying this region could be explained to the high degree of homology between all neutral proteases since, we have used PCR to amplify the region of mature sequence for protease gene and the primers used were originally designed to amplify a similar region for neutral protease gene from similar species, an organism with a high GC ratio.

All extracellular bacterial proteases are synthesized as inactive pre proenzymes. Sequence analysis of the neutral protease gene in *B. thermoproteolyticus* encoding thermolysin reveals that thermolysin is synthesized as a pre proenzyme consisting a signal peptide (28 amino acid residues), a pro-sequence (204 residues) and a mature sequence (316 residues) (Kiyoshi *et al.,* 2007). In a similar study the gene for a serine proteinase from a thermophilic *Bacillus* sp. was identified by PCR amplification, PCR of genomic DNA using an annealing temperature of 55°C and RM5 and RM6 yielded a 500 bp fragment. The 500 bp band was gel purified and used for further study (Bryce *et al*., 1994). Bach *et al*. (1999) worked on specific detection of the gene for the extracellular neutral protease of *Bacillus cereus* by PCR, the DNA region encoding the entire mature protein (951bp) was used as the template for PCR, with forward primer BcI and reverse primer BcII with the annealing temperature at 64°C, the amplified PCR products were analyzed on 0.8% agarose gel and the result confirmed the presence of neutral protease gene.

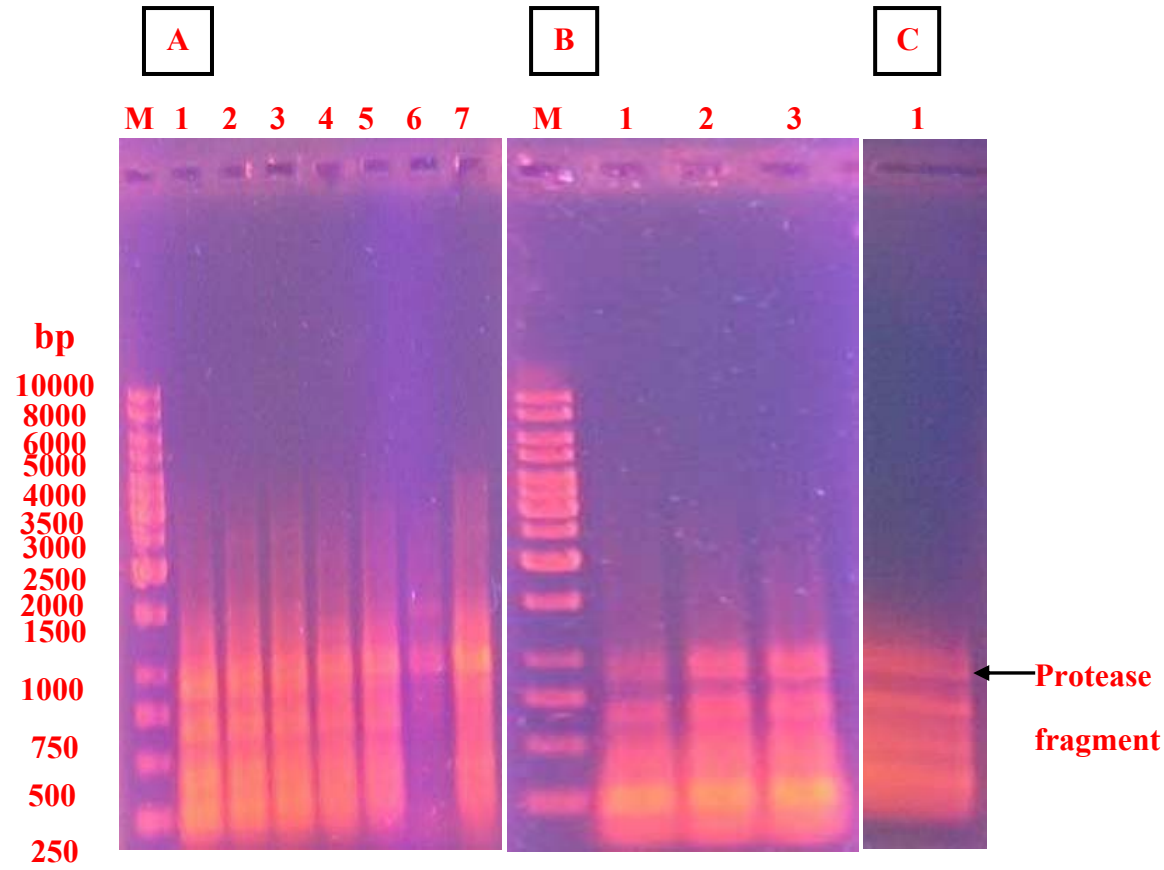

Figure (3.3): PCR Amplification of mature protease sequence with upstream primer (5'CCCAAGCTTAAGGAGATACATATGAAGGGGGAATCG-3') **and downstream primer** (5'-GGGAAGCTTAATACACTCCAACCGCATTG-3') **on 0.8% agarose gel where: {A}:(M)10kbDNA Ladder marker (1)52˚C, (2)55.6˚C, (3)57.8˚C (4):59.9˚C, (5)62.1˚C, (6)64.2˚C, (7)68.3˚C. {B}:(M)10kb-DNA-Ladder (1)50˚C, (2)51˚C, (3)53.6˚C. {C}:(1)52˚C, 5V/cm, 45 min.**

3.4.2 Molecular cloning and subcloning of *B. stearothermophilus* (B17) protease gene

The amplified PCR product (protease gene) was purified by peqGold Gel Extraction kit (Biotechnonlogie Gmbh/ germany) and then cloned into pJET 1.2 blunt cloning vector. Recombinant vector was transformed into *E. coli* DH5α for cloning purpose. Positive clones were picked and selected on the basis of their ampcillin resistance. Recombinant plasmid (from 10 colonies) was extracted and purified by NucleoSpin plasmid Miniprep kit (Machery- Angel/ germany), then analyzed on 0.8% agarose gel. The result (Figure 3.4) showed that the recombinant plasmid from clone no. 7 was in the

right size (about 2560 bp), by other mean the size of the pJET cloning vector was 2974bp and it was equal to 1960bp on the gel while the size of the insert was 600bp, so the size of the recombinant molecule (vector and the insert) must be about 2560 bp.

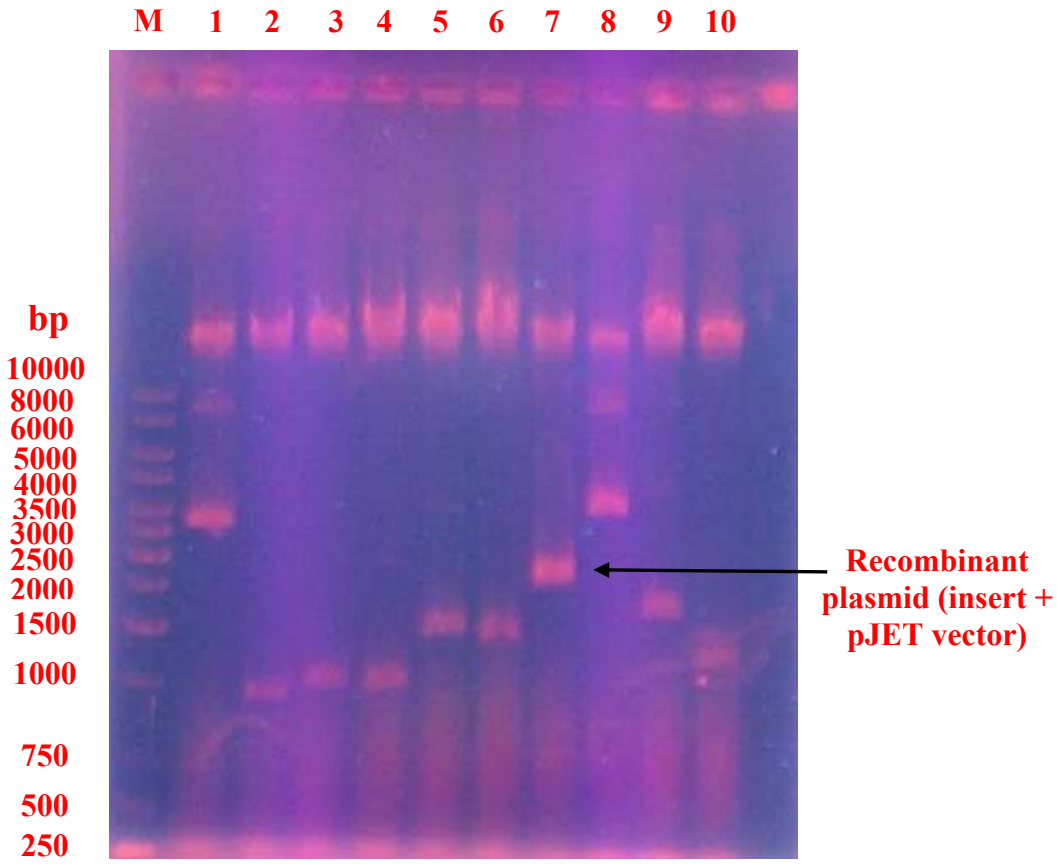

Figure (3.4): Screening of recombinant molecules (insert and pJET cloning vector) on 0.8% agarose gel where:
(M) 10kb-DNA-Ladder Marker
(1-10) positive clones of *B. stearothermophilus* (B17)
5V/cm, 45 min.

For sub-cloning, recombinant plasmid of clone no.7 was digested with *NdeI* , *Hin*dIII (Figure 3,5a) restriction enzymes and cloned into *NdeI*, *Hin*dIII digested pET-28b(+) cloning vector (Figure 3.5b). The recombinant plasmid was transformed into *E. coli* DH5α cells Pr^- (Protease deficient).
Many transformants were picked and selected on the basis of their Kanamycin resistance trait.

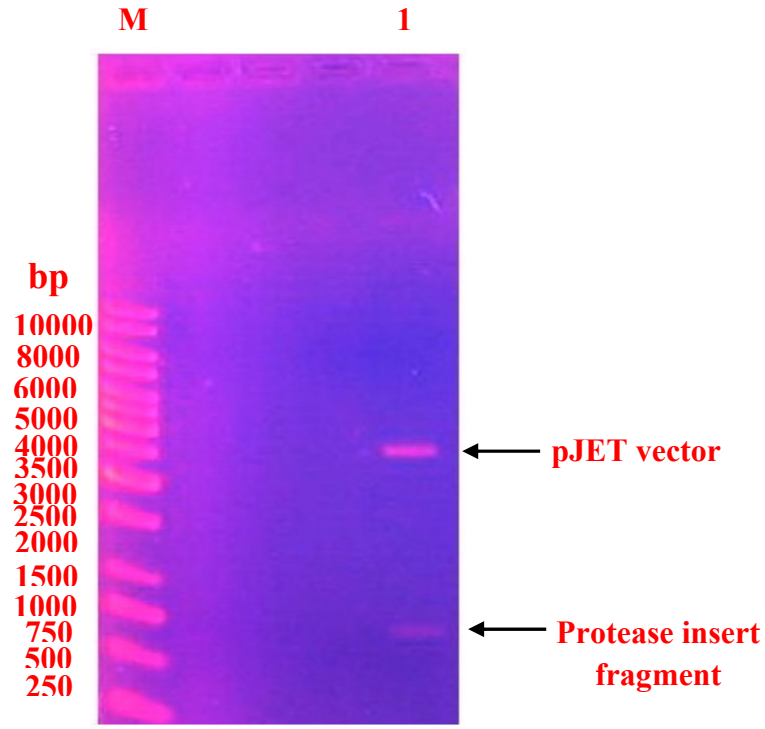

Figure (3.5a): Digestion analysis of recombinant plasmid (pJET + insert) on 0.8% agarose gel where:
(M) 10kb-Ladder marker, (1) Recombinant plasmid in clone no.7
5V/cm, 45 min.

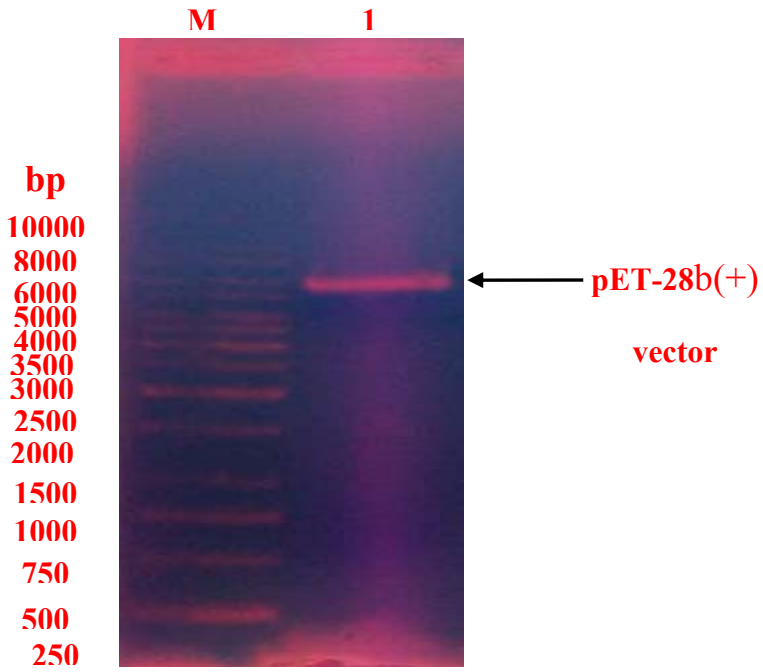

Figure (3.5b): Digestion analysis of pET-28b(+) cloning vector on 0.8% agarose gel where:
(M) 10kb-Ladder marker, (1) pET-(28)b(+) cloning vector
5V/cm, 45 min.

3.4.3 Expression of recombinant protease gene in *E. coli* DH5α harboring pET-28(+)

The transformation of *E. coli* DH5α with plasmid DNA was done as described previously, from about 605 transformants which were picked and selected on the basis of their Kanamycin resistance, 14 clones (2.3%) were able to produce protease (Pr^{+}) because these clones showed a halo of hydrolysis around the colonies on skim milk agar plates after incubation at 37°C for 24h. in comparison with the others non producer (Figure 3.6).

Figure (3.6): Expression of protease gene cloned into *E. coli* transformants on skim milk agar on the bases of zone of hydrolysis after incubation at 37°C for 24h.

The specific activity of protease was assayed in the crude extract of the 14 *E. coli* transformants (DH5α1, DH5α2, DH5α3, DH5α4, DH5α5, DH5α6, DH5α7, DH5α8, DH5α9, DH5α10, DH5α11, DH5α12, DH5α13, and DH5α14). Results indicated in table (3.3) showed that all these transformants were protease producers, DH5α5 was the highest protease producer among the other transformants. On the other hand results indicated in table (3.3) showed that all of these transformants have greater protease productivity than

the wild type of *B. stearothermophilus* B17 according to specific activity in their crude filtrates. It is obviously to conclude that all transformants (DH5α1-14) were carried the recombinant pET-28b (+) vector carrying protease gene, and DH5α5 was produced higher amount of protease than the others. It appeared that T7lac promoter in the vector did work efficiently in the competent *E. coli* DH5α5 clone and that the protease gene product could enhance transcription from this promoter. On the other hand the high level of expression may be due to the construction of recombinant pJET PCR cloning

Table (3.3): Specific activity of protease produced by *E. coli* DH5α transformed with recombinant pET-28b (+) carrying protease gene.

Transformant	Specific activity (U/mg protein)
DH5α1	36.7
DH5α2	40.9
DH5α3	36.7
DH5α4	37
DH5α5	42
DH5α6	37
DH5α7	40.1
DH5α8	40.5
DH5α9	40.3
DH5α10	40.3
DH5α11	41
DH5α12	40.9
DH5α13	39
DH5α14	38.7
B. stearothermophilus(W.T.)	36.4

vector after the ligation of the PCR amplified gene into this vector and subcloning of the gene into pET vector under the control of strong promoter (T7 promoter). Mansfeld *et al*. (2004) studied the expression of TLPase and its propeptide in *E. coli*. At first, the prosequence and the mature sequence

were cloned together into pET-28(+), the second strategy was based on a two-vector system. The prosequence was cloned into pALTER-Ex2 (*Nco*I/ *Hind*III). The mature sequence was inserted into the *Nco*I/ *Hind*III sites of pET-28(+), high amounts of the propeptide were produced with one (Mat Pro), and the two vector construct (Pro+Mat). A piece of chromosomal *B. stearothermophilus* DNA containing the neutral protease gene (npr) has been cloned by Fujji *et al*. (1983) into plasmid pTB90; the resulting plasmid was designated pNP22. The npr gene was subcloned from pNp22 into pTZ12 (Aoki *et al*., 1987), resulting in pGE501 (Eijsink *et al*., 1990). From plasmid pGE501 fragments containing parts of the neutral protease gene have been cloned into *E. coli* plasmids pMa and pMc (Stanssens *et al*., 1989; Eijsink *et al*., 1993).

3.4.4 Molecular cloning of *B. stearothermophilus* (B17) protease gene in pUC18 vector

Genomic DNA was isolated from this bacterium using mid prep Qiagen kit. It was partially digested with *Sau*3A, then the reactions were stopped at a time points ranging from (5-60) minutes by the addition of 0.5M EDTA. Aliquots from each time point were then analyzed by electrophoresis on 0.8% agarose gel. Results indicated in figure (3.7) showed that, reaction times between 60 and 5min. using 2U/µl *Sau*3A were not enough to give acceptable DNA fractions for protease (1000- 1500 bp). Therefore, 5min time point with diluted *Sau*3A (0.02U/µl) was chosen as the condition which gave the DNA in the size fractions between 1000-4000bp, the fractions between 1000-1500bp were cut from the gel then extracted using Gene JET Gel Extraction Kit and ligated to - *Bam*HI digested pUC18 (Figure 3.8) that had been treated with alkaline phosphatase.

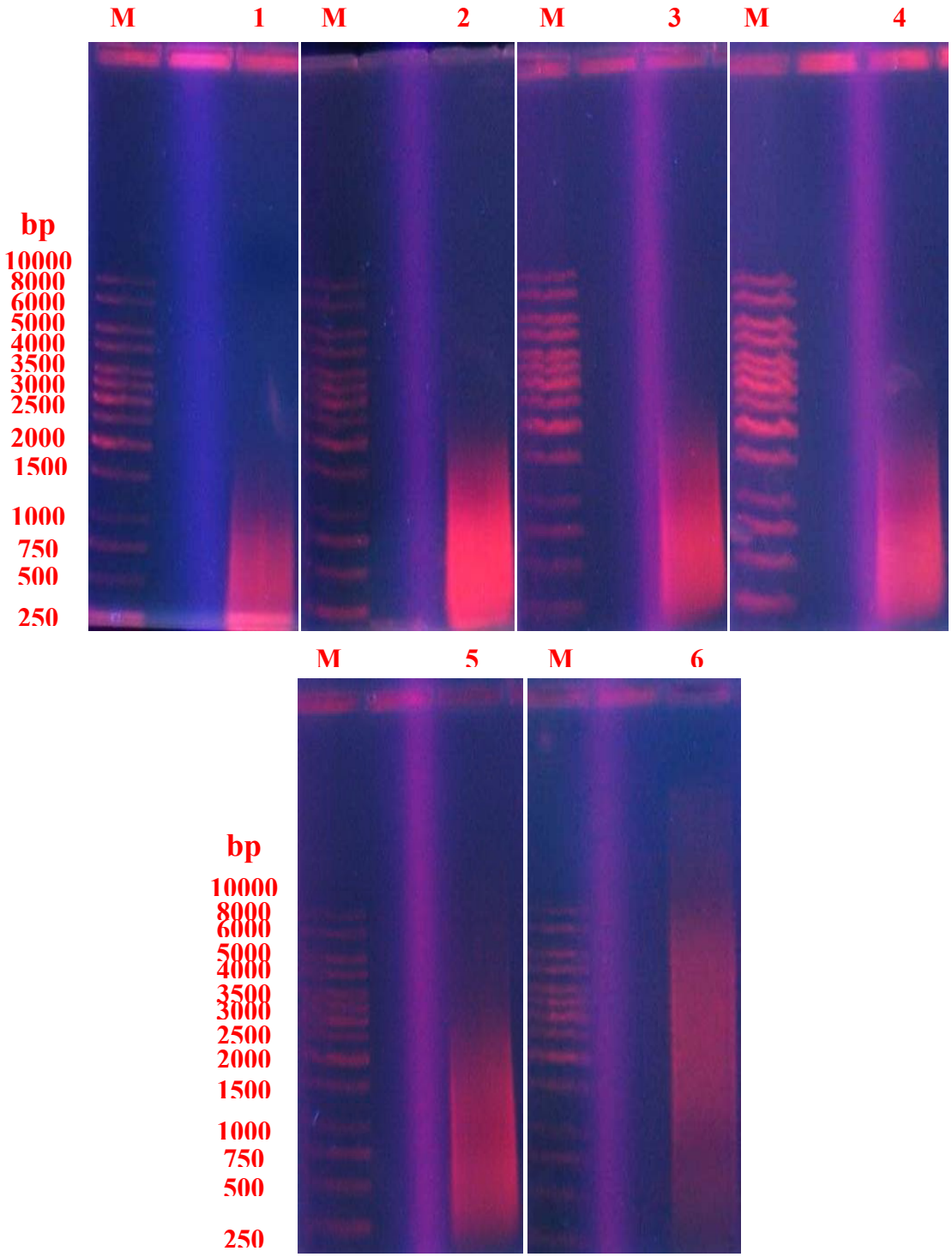

Figure (3.7): Partial digestion of *B. stearothermophilus* B17 genomic DNA at different time periods where:

(M) 10kb-ladder marker; (1) 60min. with 2U/µl *Sau*3A; (2) 40min. with 2U/µl *Sau*3A; (3) 20min. with 2U/µl *Sau*3A; (4) 8min. with 2U/µl *Sau*3A; (5) 5min. with 2U/µl *Sau*3A; (6) 5min. with 0.02U/µl *Sau*3A, 5V/cm

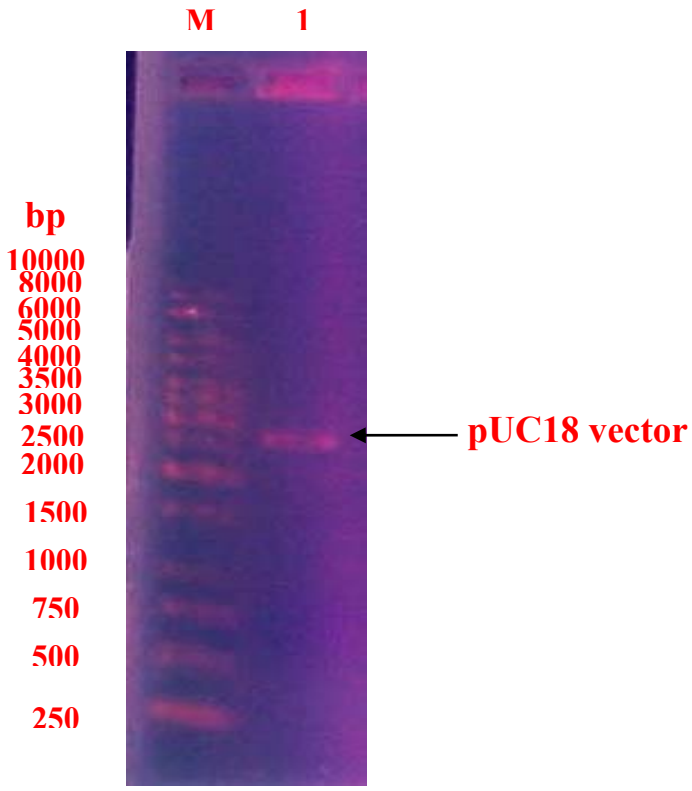

Figure (3.8): Digestion of pUC18 cloning vector using 12U/µl *Bam*HI

where:

(M) 10kb-ladder marker; (1) digested pUC18 cloning vector

The ligation was done at a 7:1ratio of insert to vector. The ligation mixture was then transformed in *E. coli* DH5α (Pr-). Many transfrmants (about 2000) were picked and selected on the basis of their ampicillin resistance trait (as described previously).

3.4.5 Expression of recombinant protease gene in *E. coli* DH5α harboring pUC18

From about 2000 ampicillin resistant transformants, a hallo of hydrolysis was occurred around 6 (0.3%) of these transformants on skim milk agar plate after incubation at 37°C for 24h., and as it was mentioned in figure (3.9).

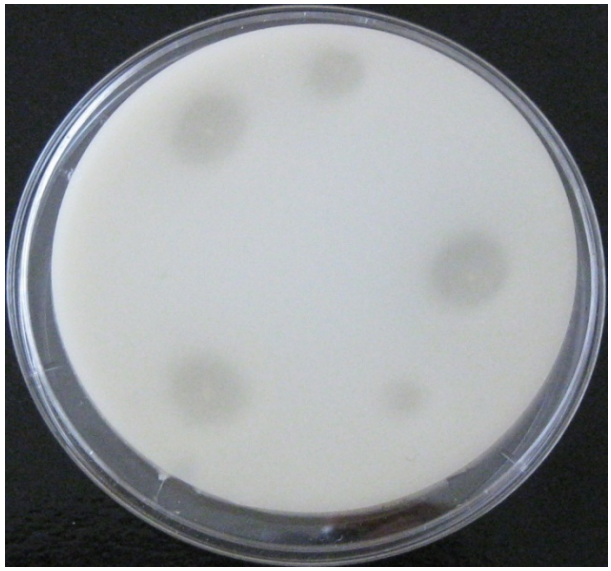

Figure (3.9): Expression of protease gene in *E. coli* DH5α transformed with the recombinant pUC18 on skim milk agar after incubation at 37°C for 24h.

The specific activity was assayed for the crude extract of the 9 transformants (DH5αP1, DH5αP2, DH5αP3, DH5αP4, DH5αP5, DH5αP6, DH5αP7, DH5αP8, andDH5αP9). Results indicated in table (3.4) showed that, DH5αP2, DH5αP3, DH5αP7 transformants were the highest protease producers among the other transformants and higher than that of the wild type *B. stearothermophilus* B17 in protease production according to the specific activity in their crude filtrates. The slight decrease in the activity for the other transformants could be attributed to the physiological differences between *Bacillus* sp. and *E. coli* DH5α transformants. Limited expression of the protease gene in *E. coli* appears to come from its own promoter system as evidenced by the results from recombinant plasmids pNZ1915 and pNZ1919. The formation of an-inframe fusion with α- peptide of the *Lac*Z gene in recombinant plasmid pNZ1917 or with lambda promoters p_L and P_R in plasmids pNZ1931 and pNZ1932 allowed much higher expression from the *E. coli Lac*Z promoter (Peek *et al*., 1993).

The primary purpose of this study is to isolate neutral protease gene from the genome of microorganism known to carry it, and to relocate the gene

in a gram negative host, such as *E. coli*, in which the gene is expressed regardless the amount of the produced protease. Still another object is to provide the neutral protease gene in a form in which it may be readily manipulated for further study or development, so locating the gene in a well characterized host, such as *E. coli*, will greatly facilitate the desired genetic manipulations (Deutch and David, 1990). Several attempts at cloning related protease enzyme in *E. coli* have yielded varying results. Yanagida (1986) discloses cloning of a serine protease DNA fragment from the microorganism *Serrattia marcescens* into *E. coli* in which there was specific secretion of the protease into the extracellular medium. By contrast, Nkahama (1986) founded no expression upon cloning *Serratia* sp. extracellular metalloprotease gene into *E. coli*, but reported excretion of the protease into the culture medium when the gene was cloned back into the *Serratia*. The gene encoding serine alkaline protease (SapSh) of the psychrotrophic bacterium *Shewanella* strain Ac10 was cloned in *E. coli,* The recombinant SapSh (rSapSh) was found to have a molecular weight of about 44,000 and to be highly active in the alkaline region (optimum pH, around 9.0) when azocasein and synthetic peptides were used as substrates. rSapSh was characterized by its high levels of activity at low temperatures (Ljudmila *et al*., 1999). The screening of a gene library of the milk-clotting protease from strain *Myxococcus xanthus* 422 constructed in *E. coli* allowed the description of eight positive clones. Only three of them (*cltA*, *cltB*, and *cltC*) encoded proteins that exhibited intracellular milk-clotting ability in *E. coli*, *Saccharomyces cerevisiae*, and *Pichia pastoris* expression systems (Poza *et al.*, 2004).

Table (3.4): Specific activity of protease produced by *E. coli* DH5α transformed with the recombinant pUC18 carrying protease gene.

Transformant	Specific activity (U/mg protein)
DH5αP1	35
DH5αP2	38.1
DH5αP3	38.8
DH5αP4	34.4
DH5αP5	34.3
DH5αP6	33.7
DH5αP7	39.5
DH5αP8	35.3
DH5αP9	35.8
B. stearothermophilus (W.T.)	36.4

3.5 Mutagenesis of *B. stearothermophilus* B17

3.5.1 Physical mutagenesis by UV irradiation

Since several publications have shown that there are variations in UV stimulation (Cockell *et al*., 2000), fresh culture of *B. stearothermophilus* (B17) were exposed to different doses (1, 2, 3, 4, 5, and 6 J/m²) of UV irradiation under sterile conditions. Results indicated in figure (3.10) show that, the (LD_{90}) for B17 was 4J/m^2, and the survival of B17 dramatically decreased with increasing dose of radiation. The killing effect was reached 100% at 6 J/m^2. UV mutagenesis at (LD_{90}) resulted in obtaining 6 mutants out of 45 (13.3%) from skim milk agar plate. The specific activity assay demonstrated that two of them B17U1 (80.08 U/mg protein), and B17 U2 (54.4 U/mg protein) were about 2.2, and 1.7 times more than that of the wild

type B17 (36.5U/mg protein) respectively. Moreover, the specific activity for the other mutants were less than that of the wild type (Table 3.5).

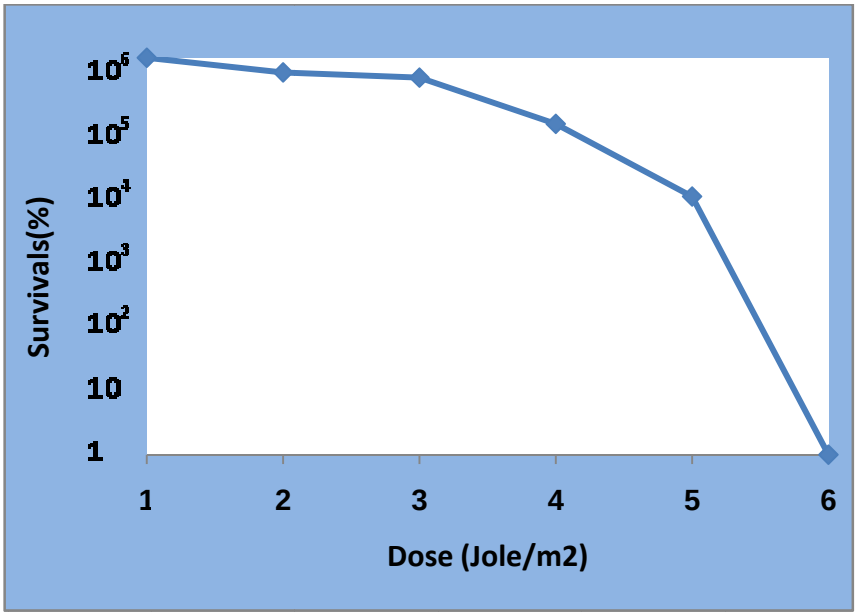

Figure (3.10): Survival curve for *B. stearothermophilus* B17 after exposure to different doses of UV irradiation

A system of random mutagenesis is specifically designed to facilitate the directed evolution of proteins (Camps *et al.,* 2003). *Pantonea dispersa* survivors of UV mutagenesis was assayed to determine the effect on their protease activity. Out of the 45 UV mutants examined for production of protease enzyme from *Pantonia dispersa* isolate was found to produce 9.52 U/ml of protease enzyme as compared to the wild type, which yielded 7.74 U/ml; this showing an increase in protease enzyme production by 1.23 fold (Gohel *et al*., 2004).

Table (3.5): Ability of *B. stearothermophilus* mutants in protease production after subjection to LD_{90} of UV irradiation

B17 mutant	Specific activity (U/mg protein)
B17U1	80.0
B17U2	54.4
B17U3	35.1
B17U4	30.3
B17U5	32.2
B17U6	29.9
B. stearothermophilus (W.T.)	36.5

David *et al*. (2005) studied the survival of spore forming microbes, 43 *Bacillus* spore forming isolates were screened, and 19 isolates showed resistance to UV light at 254nm, while the viable count for the others was reduced by 90%. In the same study, *B. pumilus*, and *B. subtilis* were exposed to UV_{254}, and results indicated that these strains exhibited inactivation rates up to a total dose of 50 J/m^2.

3.5.2 Chemical mutagenesis by 1-methyl-3-nitro-1-nitroso-guanidine (MNNG)

Several approach of traditional mutagenesis given the effectiveness for isolating mutant that produce improved yields of various microbial enzymes such as protease, lipase, and α-galactosidase (Tan *et al*., 2003; Wang *et al*., 2004). MNNG was used in this study as a mutagenic agent to generate mutants with higher protease activity. For this purpose, fresh culture of the

wild type *B. stearothermophilus* B17 was incubated with MNNG in a concentration of 200 µg/ ml MNNG solution at 37°C for different periods of time (20, 40, 60, 80, and 100 min). Results indicated in figure (3.11) show that the LD_{90} was reached after 60 min incubation with MNNG, and most of B17 cultivability was lost after 100 min. incubation. MNNG mutagenesis resulted in isolation of 3 out of 32 mutants (9.3%) from skim milk agar plate, designated as B17M1, B17M2, B17M3. The specific activity for protease assay demonstrated that one out of the three B17 M1 (99 U/mg protein) was about 2.7 times that of the wild type (36.3U/mg protein), while those for the other two mutants were less than the wild type (Table 3.6). Since B17M1mutant was the higher protease producer among the other mutants obtained from physical and chemical mutagen, it was chosen for further study.

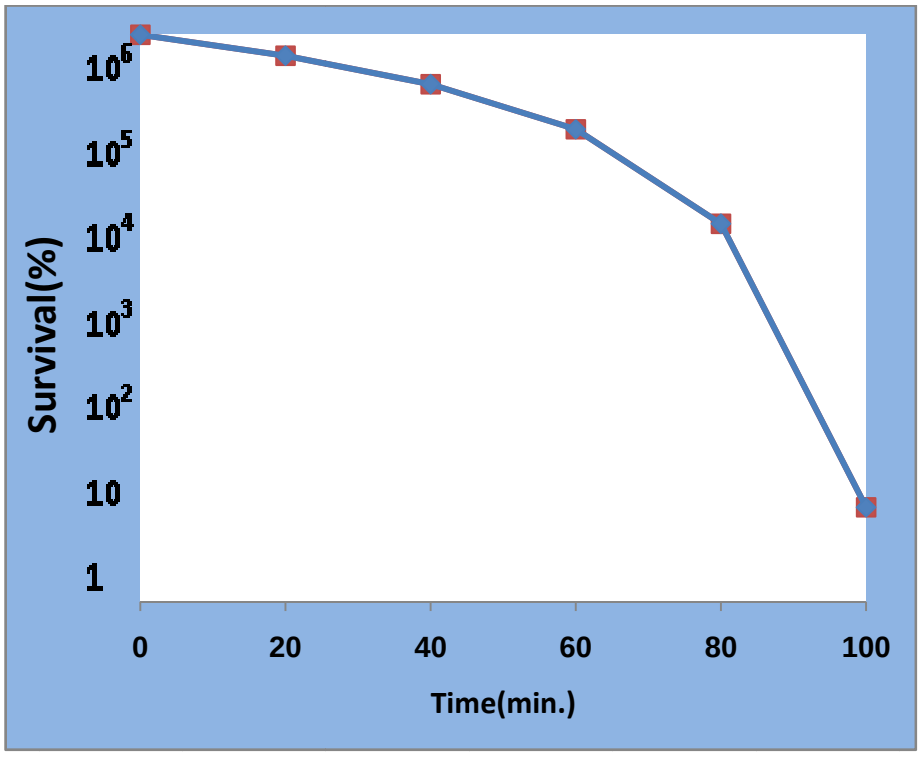

Figure (3.11): Survival curve of *B. stearothermophilus* B17 mutagenesis with 0.2mg /ml MNNG

Through random mutagenesis many advances are focuses for improvement of industrial enzyme (Tyler and Huimin, 2007). MNNG

mutagenesis of *B. subtilis* resulted in the isolation of two mutants with elevated protease activity from casein plate, designated as KD-N1 and KD-N2, respectively. Protease activity assay demonstrated that KD-N2 (60.9) U/ml was about 2.5 times that of the wild type strain (24.3) U/ml, while the activity for KD-N2 was less than those of the wild type strain (Cheng-gang *et al*., 2007).

Table (3.6): Ability of *B. stearothermophilus* B17 mutants in protease production at LD_{90} of MNNG mutagenesis

B17 mutant	Specific activity (U/mg protein)
B17M1	99
B17M2	34.2
B17M3	31.8
B. stearothermophilus (W.T.)	36.3

3.6 Optimum conditions for protease production from B17M1mutant

The overproducer mutant B17M1 of *B. stearothermophilus* characterized with its high ability in protease production was used to determine the optimum conditions for protease production.

3.6.1 Optimum culture conditions on protease production

Thermophilic *B. stearothermophilus* B17M1 mutant was cultivated in a minimal media containing 1% of various carbon and nitrogen sources. Results indicated in table (3.7) showed that, this mutant was capable of utilizing different carbon sources as a sole source for carbon and energy, while production of protease was varied according to the type of the carbon source. Results of same table show that starch and maltose were the best for protease production, while lactose and sucrose were the less effectives. Reasonable

amount of protease was produced in presence of glucose. Fereshteh *et al*. (2003) reported that higher concentration of glucose supported better growth and biomass but enzyme production was suppressed at glucose concentrations of 1% and above, this shows that protease synthesis is under catabolite repression. In a similar study, Camila *et al*. (2007) reported that thermophilic *Bacillus* sp. was capable of utilizing a wide range of carbon sources. Starch and maltose were the best carbon sources for protease production, and a good amount of protease was produced in presence of glucose. Huang *et al*. (2008) reported that the maximal protease activity of thermophilic *Bacillus* sp. was found bythe combinations of corn starch and soybean meal. Johnvesly and Naik (2001) showed that soluble starch was the best carbon source for protease production by *Bacillus* sp. JB-99. According to these authors, culturing this organism in 1% glucose repressed completely synthesis of protease.

Table (3.7): Effect of the carbon source on protease production by *B. stearothermophilus* mutant B17M1

Carbon source	Specific activity (U/mg protein)
Glucose	99.2
Starch	112.3
Sucrose	90.1
Lactose	90.4
Maltose	105.7

The type of nitrogen sources also affected enzyme production. Among the various nitrogen sources, maximum protease activity was obtained when peptone and yeast extract were used in production medium. The specific activity was 113.5 and 113.6 U/mg protein, respectively, as mentioned in

table (3.8). Moderate level of enzyme activity was obtained with soy bean meal and ammonium sulfate, and the enzyme activity was lower in presence of meat extract. Rahman *et al*. (1994) had reported that secretion of *B. stearothermophilus* protease was dependent on the composition of the culture medium. Similar results were obtained by Phadatare *et al.* (1993) on *Conidiobolus coronatus*; Banerjee *et al.* (1999) on *Bacillus brevis*; and Wellingta and Meire (2004) on *Bacillus* sp. They were reported enhancememnt of protease production by nitrogen sources like tryptone, peptone, and yeast extract. Takii *et al*. (1990) reported that maximum protease production from *Bacillus* sp. was obtained with yeast extract as a nitrogen source.

Table (3.8): Effect of the nitrogen source on protease production by *B. stearothermhilus* mutant B17M1

Nitrogen source	Specific activity (U/mg protein)
Yeast extract	113.6
peptone	113.5
Soybean meal	112
Ammonium sulfate	112.5
Meat extract	90.6

3.6.2 Optimum pH for protease production

A pH range between 5.0 and 10.0 was used to study the effect of medium pH on B17M1 protease production (Figure 3.12). The enzyme was produced at a pH range of 6- 8 with a maximum value at pH 7. Drastic decrease in bacterial growth and enzyme production was observed when the initial pH was in the range of 9-10. Effect of pH in enzyme production resulted from its role in the solubility of the nutritional substances of the

medium, its effect on the ionization of the substrate and its availability to the microorganism, in addition to its effect on the stability of the produced enzyme (Bull and Bushnell, 1976). Panuwan *et al*. (2003) reported that optimal pH of proteases from *B. subtilis* strain 38 was found to be at 6.5. In other study, effect of the initial pH of culture medium on the protease production from *Bacillus* sp.MIG was studied in pH range of 4-10, no growth was found at pH4, and the enzyme was produced at a pH range of 6-10 with a maximum value at pH7 (Mona, 2006).

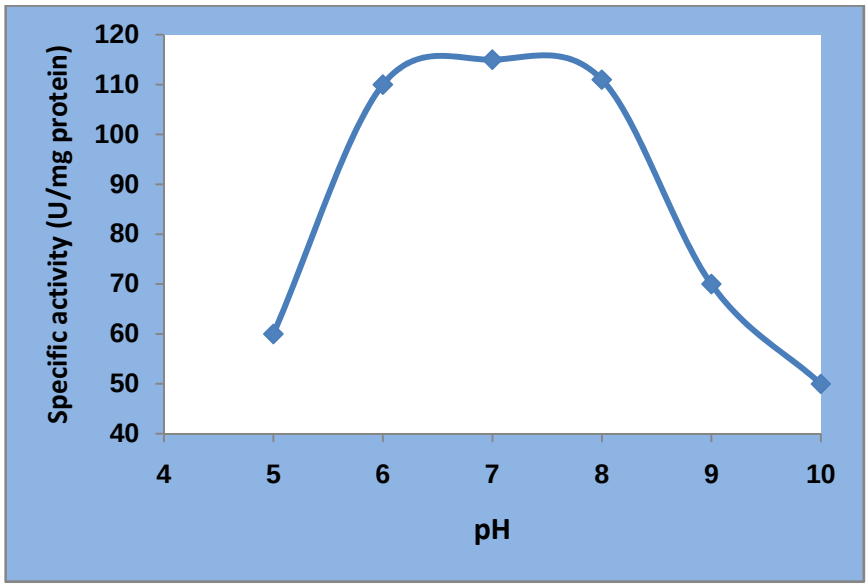

Figure (3.12): Effect of pH on protease production by *B. stearothermophilus* mutant B17M1

3.6.3 Optimum temperature for protease production

The effect of incubation temperature and pH of the production medium are critical and need to be optimized (Mona, 2006), therefore, protease activity was assayed at various temperatures (40, 45, 50, 55, 60, 65, 70, and 75) °C. The optimum temperature for protease production by the over producer *B. stearothermophilus* mutant B17M1 was found to be 55°C. However, the decrease or increase in the incubation temperature led to decrease enzyme production as it was illustrated in figure (3.13). Ray *et al*. (1992) reported that temperature could regulate the synthesis and secretion of

extracellular proteases by microorganisms. Protease activity of a thermophilic *Bacillus* sp. was assayed at different temperatures ranging from 40°C-100°C, and enzyme activity increased with temperature within the range of 40°C to 70°C; reduction in enzyme activity was observed at values above 70°C (Camila *et al*., 2007). A similar study on extracellular protease produced from thermophilic *Bacillus* sp. revealed that the optimum temperature for production of this enzyme was 60°C (Wellingta and Meire, 2004).

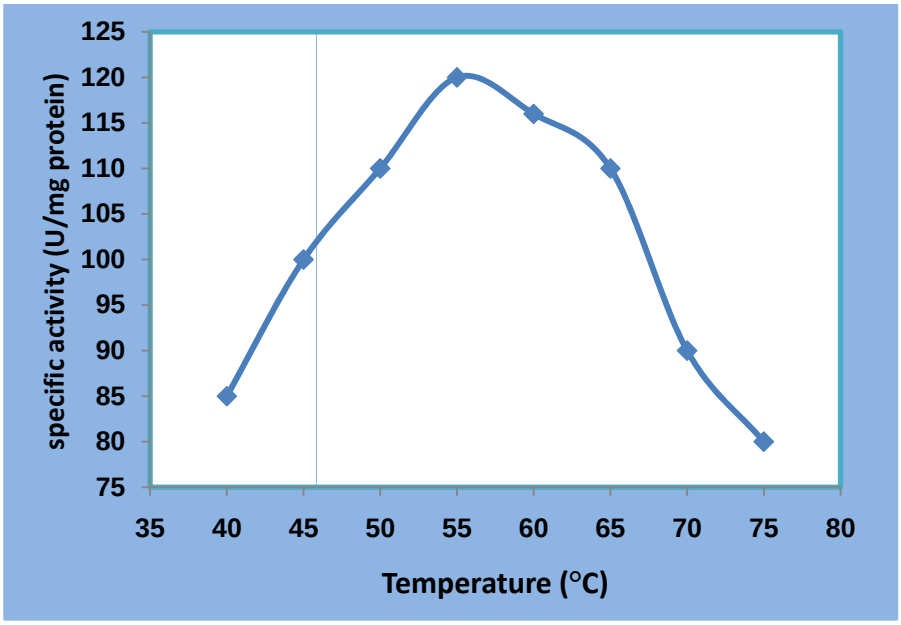

Figure (3.13): Effect of optimum temperature for protease production by *B. stearothermophilus* mutant B17M1

3.6.4 Optimum incubation period for protease production

Protease production by B17M1 was observed after (6, 12, 18, 24, 30, and 36) h. of incubation periods. Results (Figure 3.14) show that B17M1 isolate grew very fast and the formation of protease started from 6h. of the growth and reached its maximum in 18h. (123.4 U/mg protein) then began to decrease with increasing the incubation time. This suggests that protease production was directly linked to the culture being metabolically active. Different trends were reported for extracellular protease production and incubation periods, Camila *et al*. (2007) founded that protease production by thermophilic *Bacillus* sp. SIMA-2 reached its maximum at 14h., with a level

of 42 U/mg protein. Other researches for extracellular protease production by *Bacillus* sp. (Oberoi *et al*., 2001), *B. subtilis* PE-11 (Adinarayana *et al*., 2003), and by *Bacillus clausii* (Kumar *et al*., 2004) showed that cell density increased with time and protease activity reached its maximum after 24h. of incubation.

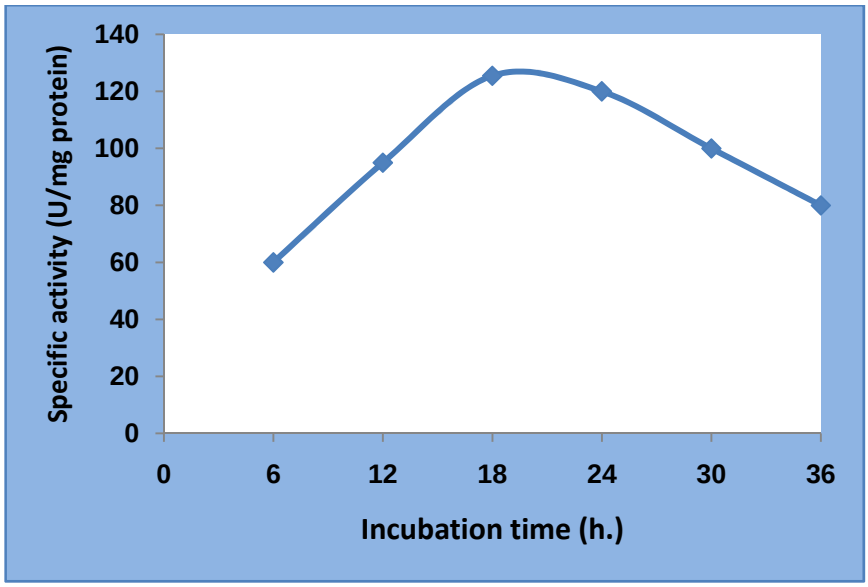

Figure (3.14): Effect of incubation periods on protease production by *B. stearothermophilus* mutant B17M1

3.7 Purification of B17M1 protease

Extracellular protease was purified from the cell free extracts of *B. stearothermophilus* B17M1 after production under the optimum conditions. Purification of protease was achieved at a sequence of steps including preparation of cell free filtrate (step 1), fractionation with ammonium sulfate (step 2), dialysis against buffer (step 3), affinity chromatography using Bacitracin-silica column (step4), and gel filtration chromatography using Superdex 75 column (step 5) and as follows:

3.7.1 Ammonium sulfate precipitation

Salting out using ammonium sulfate is one of the classical methods in protein biochemistry. Formarly it was widely used for the fractionation of proteins, it is rather used as an inexpensive way of concentrating a protein extract (Clive, 2002). Cell free extracts was subjected to ammonium sulfate

precipitation with 80% saturation ratio. It was found that this ratio gave specific activity 126.6 U/mg protein (Table 3.9). This result indicates that there was an increase in the specific activity compared to that of the crude extract (123.4 U/mg protein), on the other hand dialysis using 10000 cutoff dialysis bag against 0.05M Tris/ HCl buffer pH7.5, 5mM $CaCl_2$ results in an increase in the activity of protease (45 U/ml) together with specific activity (180 U/mg protein). Euo-Sun and Jong (2003) reported that fractions of 60-80% ammonium sulfate saturation were contained high protease activity and the specific activity was increased from (12 to 38) U/mg protein with 3.1 purifiaction fold as an initial step for the purification of caseinolytic extracellular protease from *B. amyloliquefaciens*. While Kunamneni *et al*. (2003) founded that *B. subtilis* supernatant was fractionated by precipitation with ammonium sulfate between 50% and 70% of saturation to give an increase in the specific activity from 10 to 12 U/g protein with 1.2 purifiaction fold.

3.7.2 Affinity chromatography

Using the elution procedure described in materials and methods, protease was efficiently bound to Bacitracin- silica after loading of dialyzed ammonium sulfate precipitates (80%) (Figure 3.15), while some contamination not showing proteolytic activity could be removed easily, by washing of the column with 2.5 M NaCl in starting buffer. The protease was efficiently eluted by the addition of 2.5 M NaCl and 20 % isopropanol to the starting buffer. Bacitracin-silica column chromatography purified the enzyme to about 10-fold purification with specific activity of 1266 U/mg (Table 3.9).

Affinity chromatography can certainly be considered as one of the most effective ways for protein separation and purification. In many cases this technique is fast, quite simple and very efficient. The method relies upon specific and reversible binding of a protein to a ligand, usually immobilized on an insoluble, porous and inert support (Antoni *et al*., 2003). Affinity

chromatography has been found to be convenient for separation of proteolytic enzymes and their inhibitors. An example of such an inhibitor is bacitracin, a branched cyclic peptide produced by *B. licheniformis*, which was used for purification of metalloproteinases (Eijsink *et al*., 1991), cysteine proteinase (Irvine *et al*., 1993) and serine proteinases (Rudenskaya *et al*., 1995). Several reported purification procedures for neutral proteases, including ion exchange and gel filtration chromatography, yielded unsatisfactory results in the purification of the neutral protease from *B. stearothermophilus*. Contamination by low molecular weight materials probably due to self digestion during chromatographic steps, good results were obtained when Bacitracin-silica was used (Bertus *et al*., 1989).

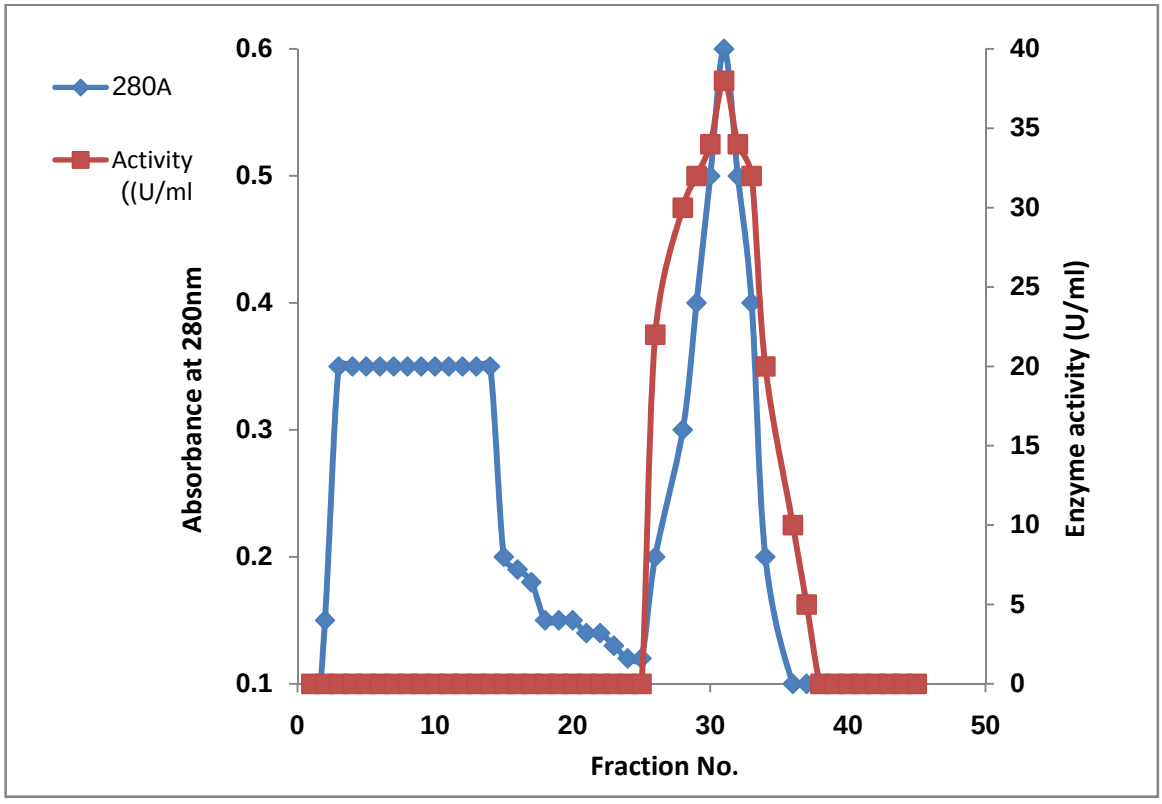

Figure (3.15): Purifiaction of protease from *B. stearothermophils* mutant B17M1 by affinity chromatography technique using Bacitracin-silica column (1x 11cm) equilibrated with 20 mM Na-acetate, 5 mM $CaCl_2$, 10% isopropanol, pH 5.3, 2ml/ fraction at flow rate 30ml/h.

3.7.3 Gel filtration chromatography

To obtain more purified protease, fractions (26-32) collected from the Bacitracin-silica column chromatography were applied to a Superdex 75 column (1×30 cm) previously equilibrated with 20mM Tris-HCl buffer, pH 7. Results illustrated in figure (3.16) showed that there is only one absorption peak which represents protease with maximum activity. The second column purification resulted in specific activity 3500 U/mg, 28 purification fold and 42.5 % yield (Table 3.9). Bertus *et al*. (1989) purified *Bacillus* neutral protease using Bacitracin-silica affinity column followed by gel filtration with an FPLC system with a Superose 12 column directly from the supernatant to give a specific activity of 5900 U/mg protein, while Poonyaras *et al*. (2000) purified the highly thermostable protease from *B. stearothermophilus* by lysine affinity chromatography, strong anion exchange Q hyperD chromatography, and Ultrogel gel filtration resulted in 2.7 purification fold and 27% yield.

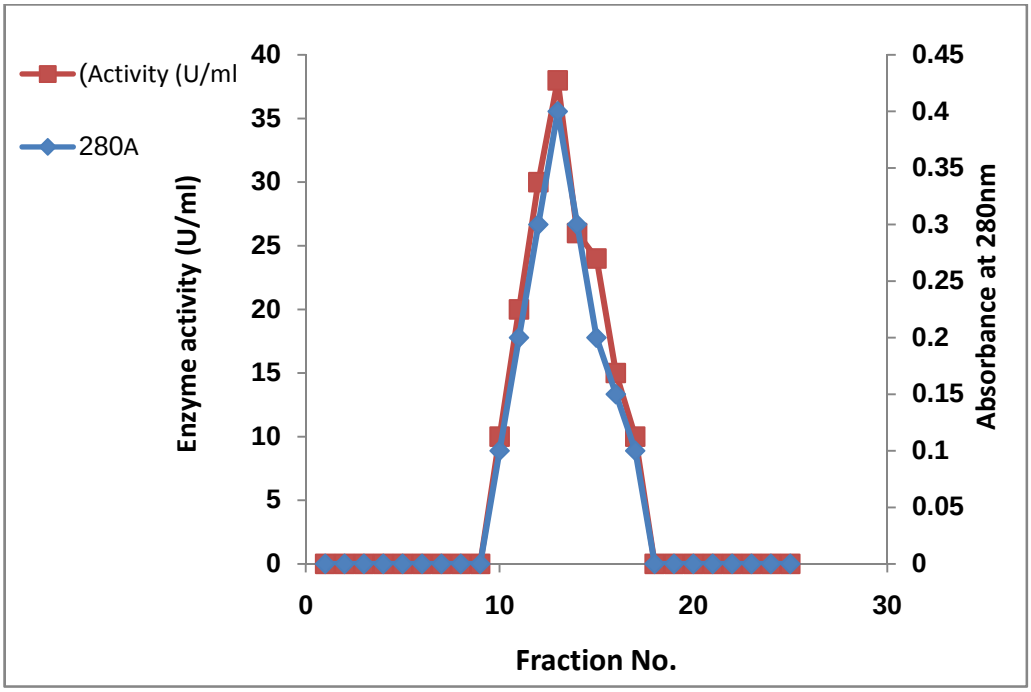

Figure (3.16): Gel filtration chromatography of *B. stearothermophilus* mutant B17M1 protease on a Superdex 75 column (1×30 cm) equilibrated with 20mM Tris-HCl buffer, pH 7, 3ml/ fraction at flow rate 30ml/h.

Table (3.9): Purification steps of neutral protease produced from *B. stearothermophilus* B17M1mutant

Steps Of Purification	Volume (ml)	Activity (U/ml)	Protein Conc. (mg/ml)	Specific Activity (U/mg)	Total Activity (U)	Folds of Purification	Yields (%)
Crude Extract	200	6.17	0.05	123.4	1234	1	100
80% Ammonium Sulfate Saturation	30	38	0.3	126.6	1140	1.02	92.3
Dialysis	20	45	0.25	180	900	1.45	72.9
Affinity Chromatography by Bacitracin–silica	14	38	0.03	1266	532	10.2	43.1
Gel Filtration Chromatography by Superdex 75	15	35	0.01	3500	525	28.3	42.5

3.8 Determination of enzyme purity and estimation of its molecular weight

As the intrinsic charge differences between proteins are masked by the SDS, separation of proteins is due to differences in size and hence the method can be used to determine molecular sizes. A linear relationship between mobility and log MW obtains over a molecular weight range dependent upon the gel pore size. The gel can thus be standardized with proteins of known molecular weight and subsequently used to estimate the molecular weights of unknowns**.**

The purified enzyme was electrophoresed under denaturing conditions using 12 % SDS-PAGE. Partial purified protease showed a single band upon

staining by silver stain (Figure 3.17); indicating that the enzyme was purified till homogeneity.

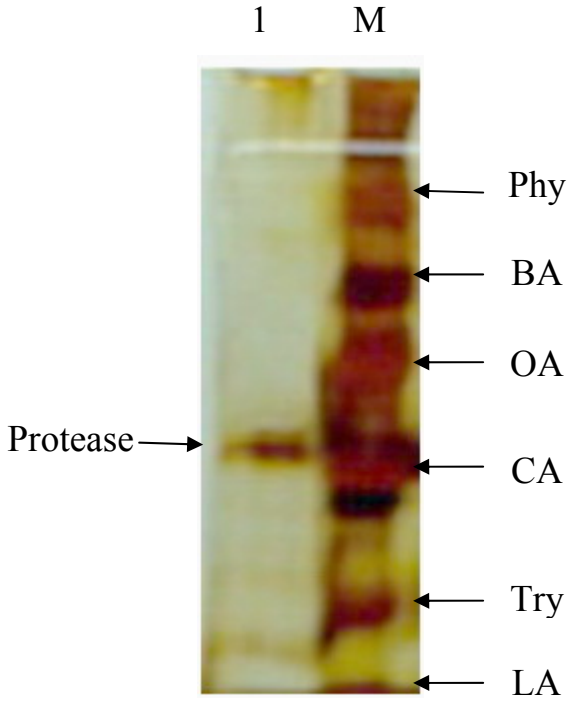

Figure (3.17): SDS-PAGE chromatograph (12%) of protease purified from *B. stearothermophilus* mutant B17M1 where:

(1) Purified enzyme visualized by silver staining.

(M) The molecular weight markers: Phosphorylase Phy (97,000), Bovine Albumin BA (66,000), Ova Albumin OA (45,000), Carbonic Anhydrase CA (30,000), Trypsin Inhibitor Try (20,000), and α-Lactalalbumin LA (14,000), 20mA, 4h.

The molecular weight of the protease was determined by comparison of the standard marker protein. The molecular weight was determined by interpolation from a linear molecular mass versus the R_m value (relative mobility). Depending on the relative mobility, the molecular weight of the protein band was calculated to be 31KDa, which coincided with the band of Carbonic anhydrase (Figure 3.18). The molecular masses of microbial proteases ranged between 15 and 36 KDa, with few exceptions of high molecular mass, such as 42 KDa from *Bacillus* sp. PS719 (Towatana *et al*.,

1999). Huang *et al*. (2006) reported that the molecular weight of the protease from thermophilic *Bacillus* strain HS08 was around 30.9 KDa by SDS-PAGE technique, While Siriporn *et al*. (2006) mentioned that the molecular weight of protease from *B. megaterium* was 27 KDa under denaturing condition.

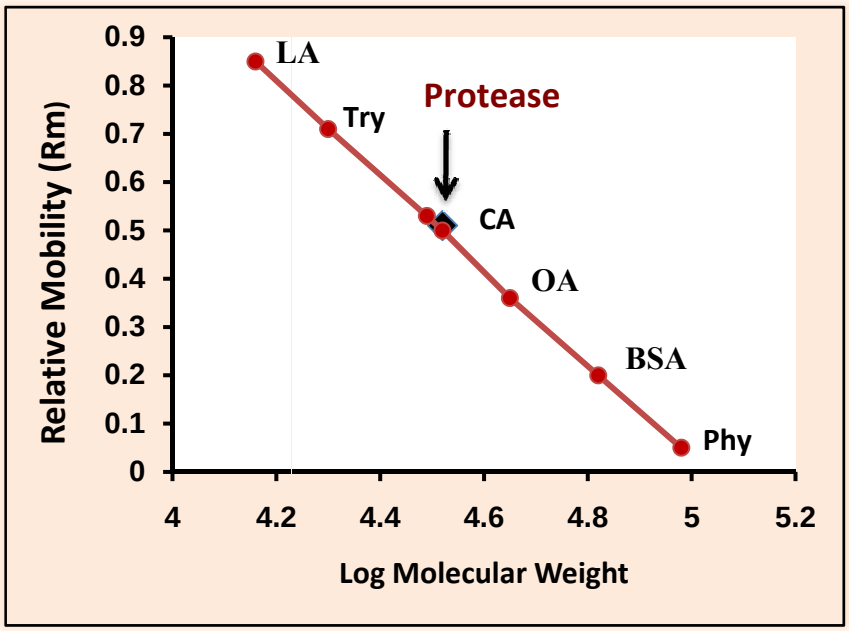

Figure (3.18): Log molecular weight of protease produced by *B. stearothermophilus* B17M1 after electrophoresis by 12% SDS-PAGE

3.9 Detection of protein band with mass spectrometry

Characterization and identification of proteins remains critically important in the biological sciences. In high throughput biology, separation of complex biological mixtures by gel electrophoresis, characterization of all bands by high sensitive tools including mass spectrometry, and assignment of biological function based on powerful database searches and protein profiling patterns. This trend will be accelerated as genomic sequences become available with increasing speed (Masahiro *et al*., 2001). Most scientists' expectation of mass spectrometry is that of determining the mass of an

unknown. In the postgenomic era, this has resulted in a fundamental shift in the ability to approach biological investigation (Andrew, 2000).

The protein band of interest was excised out of the PAGE gel and was analyzed by mass spectrometry unit. The mascot search result showed that the normal mass (Mr) of 31KDa, and the observed protein was associated with a functional category by comparing the amino acid sequences to a database of several protein sequences from a fully sequenced genomes of different microorganisms.

Matched peptide showed the following percentage similarity under these scores with *Bacillus* sp. Sequence:

K.ELADVVLVDIPQLENPTK.G (Ion score 36)

R.DDLVATNEK.I (Ions score 91)

R.VIGQSGILDTAR.F (Ion score 67)

R.VIGQSGILDTAR.F (Ion score 13)

R.TEVAQELNLSVK.D (Ion score 75)

K.DITGFVLGGHGDDMVPLVR.Y (Ion score 43)

R.YSYAGGIPLETLLPK.D (Ion score 45)

R.YSYAGGIPLETLLPK.D (Ion score 43)

K.DRIDAIVER.T (Ion score 59)

According to these results the purified protease sequence did not match any of the known genome sequence in the database of the matrixscience site, so we could conclude that this protein may be a new type of protein (Dr. Ulbrich-Hofmann, R. /Personal communication).

3.10 Enzyme characterization

3.10.1 pH effects on protease activity

A pH range of 5 to 10 was used to study the effect of pH on purified B17M1 protease activity (Figure 3.19). Optimum pH range was found to be between 7 and 6, with highest enzyme activity at pH 7, and with lower activity at pH 5 and pH 10, respectively. The pH can exert its effect on enzyme activity in different ways; on the ionization of groups in the enzyme's active site, on the ionization of groups in the substrate, or by affecting the conformation of either the enzyme or the substrate (Clive, 2002), which could explain the decrease in the activity at extreme value of acidity 5, and alkalinity 10. Sookkeheo *et al*. (2000) reported three proteases, S, N, and B from *B. stearothermophilus*, optimum pH values of 8.5, 7.5, and 7.0, respectively. The protease S was active over a broad pH range, and about 60% of proteolytic activity was still detectable at pH 6 and pH 10. In contrast, protease N and B retained little activity above pH 9.0. Li *et al.* (2008) showed that the optimal pH for the protease purified from *Streptomyces fradiae* was 7.8 and the enzyme showed stability over a pH range of 4.0-10.0.

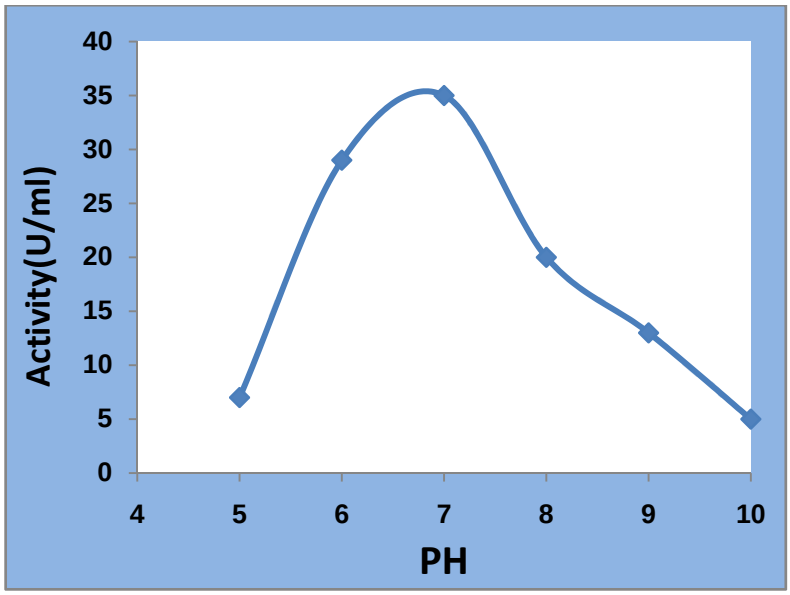

Figure (3.19): Effect of pH on purified *B. stearothermophilus* mutant B17M1 protease activity

3.10.2 pH effects on protease stability

After incubation of the purified enzyme solution at a room temperature (about 25°C) for 30 min. with the buffer in a pH range (5-10), decrease in the activity was observed of about 65% and 75% at pH 5 and pH 10, respectively (Figure 3.20). Thus protease of B17M1 seems to be active in a broad pH range (6-9), with highest activity at pH range (6-7).

Effect of pH on the enzyme stability could be explained in the formation of improper ionic form of enzyme or the active sites, irreversible inactivation. Stability of the enzyme depends on many factors such as temperature, ionic strength, chemical nature of buffer, concentration of various preservatives, concentration of metal ions, substrate and enzyme concentration (Segel, 1976). Wellingta and Meire (2004) observed decreases of 51%, 18%, and 66% of original *Bacillus* protease activity after incubation of the enzyme solution at a room temperature for 24h. at pH values of 5.5, 8.0, and 9.0, While Camila *et al*. (2007) showed that after incubation of enzyme solution from *Bacillus* sp. at a room temperature for 2h. at pH 6.0-10.0, a decrease of 15% of its original activity at pH 8.5 was observed and that at pH 10.0 was 24%.

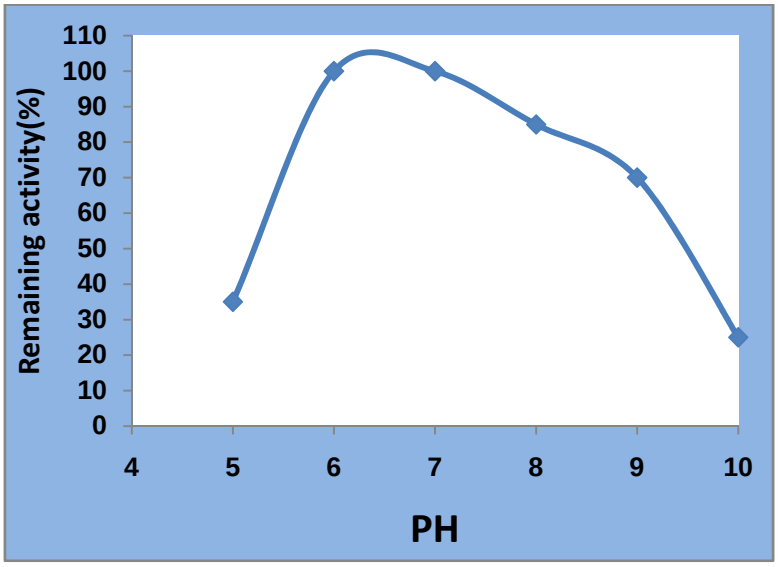

Figure (3.20): Effect of pH on purified *B. stearothermophilus* mutant B17M1 protease stability.

3.10.3 Temperature effects on protease activity

Protease activity was assayed at different temperatures ranging from 30°C-100°C (Figure 3.21). Enzyme activity increased with temperature within the range of 30°C to 70°C. A reduction in enzyme activity was observed at values above 70°C.

The increase in temperature resulted in imparting more kinetic energy to the reactant molecules, resulting in more productive collisions per unit time, but that should be within the intact and proper configuration of tertiary structure of an enzyme maintained primarily by a large number of weak non-covalent bonds, so if the molecule absorbs too much energy, the tertiary structure will disrupt and the enzyme will be denatured, that is, loss of catalytic activity (Segel, 1976).

The temperature profile of *Geobacillus caldoxylosilyticus* showed its maximum protease activity at 55-60 ºC, but at 70 ºC and 80 ºC the enzyme retained 82.07 % and 72.75 % of its activity (Mona *et al*., 2007). Similar studies on *Bacillus* proteases mentioned that the optimum temperature for purified protease activity was observed at 70°C (Horikoshi, 1990). Alkaline protease purified from *Bacillus majavensis* was optimally active at temperature of 60°C, with rapid loss of activity at temperature above 65°C (Beg and Gupta, 2003), while other proteases purified from *Nocardiopsis* sp. retained only 60% of activity at 50°C (Moreira *et al*., 2003). Li *et al*. (2008) reported that the optimal temperature for protease produced from *S. fradiae* was 60°C.

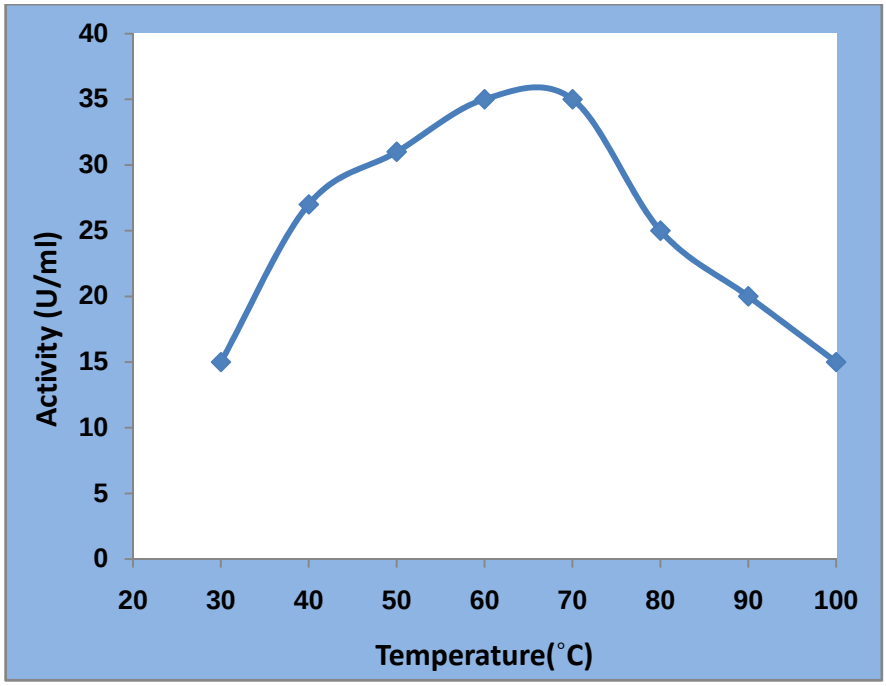

Figure (3.21): Effect of Temperature on purified *B. stearothermophilus* mutant B17M1 protease activity

3.10.4 Temperature effects on protease stability

The thermostability of the protease was examined after incubation of the purified enzyme at various temperatures 30 and 100°C for 30min. (Figure 3.22). Thermostability profile indicated that the enzyme was stable at a temperature range of 30-70°C for 30min. At 80°C and 90°C, 16% and 60% of the original activity were lost respectively, while most of activity (95%) was lost at 100°C.

Camila *et al*. (2007) founded that thermostability of the protease purified from a thermophilic *Bacillus* sp. was examined by measuring the remaining activities at 70°C, after incubation of the enzyme without substrate at various temperatures between 40 and 100°C for 2h., the enzyme retained 80% of the original activity at 60°C, and the activity decreased to 15% at 100°C (2007). Similar studies on protease produced from *Bacillus* sp. showed that the enzyme was stable after 30min. incubation at 80°C (Jasvir *et al*., 2007). Results of thermal stability test on other protease produced from *Bacillus* sp. showed a moderate temperature resistance when 45-50% of the

enzyme activity was lost at temperatures below 37°C and higher than 55°C (Najafi *et al*., 2003).

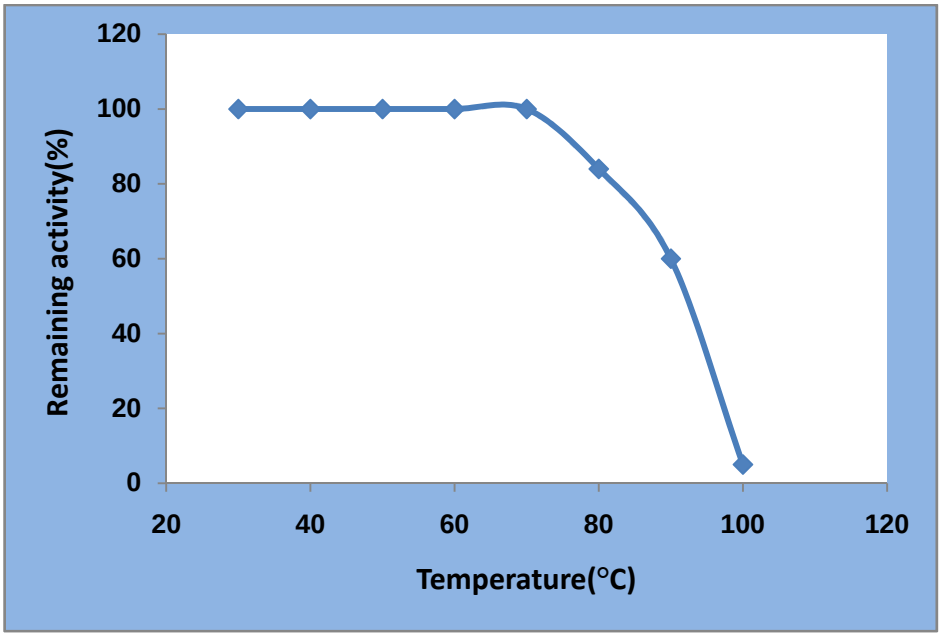

Figure (3.22): Effect of temperature on purified *B. stearothermophilus* B17M1 mutant protease stability

3.10.5 Metal ion and inhibitor effects on protease activity

The effect of some metal ions ($CaCl_2$, $ZnSO_4$, and $MgCl_2$) and inhibitors (Iodoacetic acid, EDTA, and PMSF) on the activity of the purified protease was tested. The results (Table 3.10) showed that, all the metal ions tested (Ca^{+2}, Zn^{+2}, and Mg^{+2}) led to increase the activity of the enzyme, confirming that these cations take parts in the stabilization of the protease structure and are required for protection against thermal denaturation (Paliwal *et al.,* 1994). They played a vital role in maintaining the active confirmation of the enzyme at high temperatures (Pan and Lin, 1991; Donaghy and Mckay, 1993).

When the inhibitors tested, only EDTA was able to inhibit the protease completely indicating presence of the metalloprotease. This finding was closed to those of Adinarayana (2003) and Siriporn *et al*. (2006) when 94% of of protease produced from *B. subtilis* and *B. megaterium*, respectively, was inhibited by the EDTA, Sharmin and Rahman (2007) also reported that

protease produced from *Bacillus* strain FS-1 was inhibited by the EDTA. The inhibitory activity increases with the increasing concentration of this reagent. Magda (2007) showed that the activity of protease purified from *Xenorhabdus nematophila* was totally abolished by 1 mM EDTA, but not affected by cysteine, serine and aspartyl protease inhibitors.

Table (3.10): Metal ions and inhibitors effect on protease activity produced by *B. stearothermophilus* B17M1 mutant.

Reagent	Concentration (mM)	Remaining activity (%)
Control		100
CaCl2	2	110
ZnSO4	2	105
MgCl2	2	108
Iodoacetic acid	2	100
EDTA	2	0
PMSF	2	100

EDTA (ethylenediaminetetraacetic acid), PMSF (phenylmethansulfonyl floride).

Chapter Four
Conclusions
and
Recommendations

4.1 Conclusions

1. Local isolate of *B. stearothermophilus* B17 was found to be one of the efficient protease producers among the local themophilc isolates, favored its use in the future for the industrial applications.
2. *E. coli* DH5α deficient in protease production was able to produce protease after cloning of *B. stearothermophilus* protease gene using different cloning vectors.
3. Ability of *B. stearothermophilus* in protease production was improved by random mutagenesis using physical and chemical mutagen.
4. Optimum conditions for protease production by *B. stearothermophilus* B17M1 was achieved when the production medium was supplemented with starch as a sole source for carbon and energy, and yeast extract as a nitrogen source, medium pH 7 and incubation for 18h. at 55°C.
5. Affinity charmatography using Bacitracin-silica and gel filtration chromatography using Superdex-75 are the proper techniques to purify protease after fractionation with ammonium sulfate and dialysis.
6. Protease produced by locally isolated *B. stearothermophilus* B17M1 is a novel enzyme among the other proteases according to mass spectrometry analysis.
7. Protease produced by *B. stearothermophilus* B17M1 is a metalloenzyme.

4.2 Recommendations

1. Biochemical and kinetic studies on the novel protease produced by the locally isolated *B. stearothermophilus*.
2. The use of a thermostable protease in different industrial applications.
3. Protein engineering for protease produced by locally isolated *B. stearothermophilus* by site directed mutagenesis to enhance the stability or the affinity of the enzyme to its substrate.
4. Studying the optimum conditions for protease produced by the genetically engineered *E. coli* DH5α containing *B. stearothermophilus* protease gene with an attempt to purify it.
5. Studying amino acid sequence of the novel protease produced by the locally isolated *B. stearothermophilus* and alignment with other proteases using protein database for molecular comparison.
6. Further study to enhance the production of the enzyme from any of the new hosts using immobilized cells technique.

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الخلاصة

عزلت ثلاث وخمسون عزلة محلية من بكتريا *Bacillus* spp. المحبة للحرارة من ثلاث وتسعون عينة تربة اخذت من مناطق مختلفة من محافظة البصرة (العراق), وقد درست الخصائص المظهرية والمجهرية لجميع هذه العزلات. اظهرت نتائج التشخيص والاختبارات الكيموحيوية ان 6 من هذه العزلات تعود الى جنس *Bacillus stearothermophilus* . تمت غربلة العزلات المحلية الستة لـ *B. stearothermophilus* تبعا لقابليتها في انتاج البروتييز, وقد اظهرت النتائج ان جميع هذه العزلات منتجة للبروتييز, وكانت العزلة B17 *B. stearothermophilus* هي الاحسن في انتاج البروتييز, اذ بلغت الفعالية النوعية للبروتييز في الراشح الخام لها 36.4 وحدة / ملغم بروتين.

تم تحسين انتاجية البروتييز في العزلة المحلية B17 باتباع طريقتين:

1. استنسال جين البروتييز في ناقل استنسال ثم استخدام الجزيئات الهجينة للتحول في مضيف مناسب, وقد تم هذا اولا بتضخيم جين البروتييز من بكتريا B17 باستخدام تقنية تفاعل سلسلة الدنا (PCR)ثم استنسلت الاجزاء المضخمة المشفرة للبروتييز في ناقل الاستنسال pJET, واستخدمت الجزيئات الهجينة لتحول بكتريا *Escherichia coli* DH5α غير القادرة على انتاج البروتييز (Pr^-) . اظهرت النتائج قابلية متحولات عديدة من بكتربا *E. coli* على تكوين هالة تحلل حول كل مستعمرة متحولة على وسط حليب الفرز المتصلب بالاكار بعد حضنها بدرجة 37م مدة 24 ساعة. بلغت الفعالية النوعية للراشح الخام في المتحولة الاكفأ (بالرمز DH5α5) في انتاج البروتييز 42 وحدة /ملغم بروتين.

في محاولة اخرى لاستنسال جين البروتييز من *B. stearothermophilus*. عزل مجين الدنا من B17, وهضم جزئيا بواسطة الانزيم القاطع *Sau*3A , ثم لحمت القطع الناتجة مع ناقل الاستنسال pUC18 المهضوم جزئيا بالانزيم القاطع *Bam*HI . استخدمت الجزيئات الهجينة لتحول بكتريا *E. coli* DH5α (Pr^-) , من حوالي 2000 متحولة مقاومة للامبيسيلين, كانت 6 متحولات منها(0.3%) لها القابلية على تكوين هالة التحلل على اطباق حليب الفرزالمتصلب بالاكار . اذ بلغت الفعالية النوعية للبروتييز في الراشح الخام للمتحولة الاكفأ (بالرمزDH5αP7) 39.5 وحدة / ملغم بروتين.

2. التعريض للتطفير العشوائي بواسطة المطفرات الفيزيائية والكيميائية. وقد تم انجازه بتعريض النوع البري للعزلة البكتيرية B17 الى المطفر الفيزيائي باستخدام الاشعة فوق البفسجية وبجرع اشعاعية

مختلفة (1, 2, 3, 4, 5, و 6 جول/م^2) . اظهرت النتائج ان 6 طافرات من 45 طافرة (13.3%) قابلة على تكوين هالة التحلل على اطباق حليب الفرزالمتصلب بالاكار بعد حضنها بدرجة حرارة 55م لمدة 24 ساعة. تم غربلة هذه الطافرات لمعرفة قابليتها في انتاج الروتييز, وكانت الفعالية النوعية في الراشح الخام للطافرة الاكثر انتاجا (B17U1) 80.08 وحدة / ملغم بروتين مقارنة مع 36.4 وحدة / ملغم بروتين للنوع البري.

كما استخدم التطفير الكيميائي لتحسين قابلية النوع البري لبكتريا B17 في انتاج البروتييز وذلك بحضنها مع مادة 1-methyl-3-nitro-1-nitroso-guanidine بتركيز 200 مايكروكرام/ مليلتر وبمدد زمنية مختلفة (20, 40, 60, 80, و 100 دقيقة). اظهرت النتائج ان 3 طافرات من 32 طافرة (9.3%) كانت لها القدرة على تكوين هالة التحلل على اطباق حليب الفرزالمتصلب بالاكار عند حضنها بدرجة حرارة 55م مدة 24 ساعة. بعد غربلة قابلية هذه الطافرات في انتاج البروتييز وجد ان كل هذه الطافرات منتجة للبروتييز, اذ بلغت الفعالية النوعية للبروتييز في الراشح الخام للطفرة الاعلى انتاجا (B17M1) 99 وحدة / ملغم بروتين مقارنة مع 36.4 وحدة /ملغم للنوع البري.

درست الظروف المثلى لانتاج البروتييز في الطفرة الاعلى انتاجا B17M1 , اذ وجد ان تنمية هذه الطافرة في وسط انتاجي يحتوي1 % لكل من النشا وخلاصة الخميرة وبرقم هيدروجيني مقداره 7 مدة 18 ساعة عند درجة حرارة 55 م اعطى اعلى انتاجا للبروتييز اعتمادا على الفعالية النوعية للانزيم في الراشح الخام التي بلغت 99 وحدة /ملغم بروتين.

نقي البروتييز المنتج من الطافرة الاعلى انتاجا تحت الظروف المثلى بعدة خطوات تضمنت الترسيب بكبريتات الامونيوم وبنسبة اشباع 80 % , الديلزه, كروماتوكرافيا الالفة بعمود Bacitracin- silica واخيرا كروماتوكرافيا الترشيح الهلامي بعمود Superdex-75, اذ بلغت عدد مرات التنقية 28.3 مع 42.5 % حصيلة انزيمية.

اظهرت نتائج توصيف الانزيم ان الوزن الجزيئي للبروتييز 31,000 دالتون , وقد كان الانزيم فعالا عند رقم هيدروجيني 7 واظهر الانزيم ثباتية ضمن المدى الهيدروجيني 6-7 . كان الانزيم فعالا عند درجة 60 م وثابتا الى درجة 70م عند حضنه لمدة 30 دقيقة. اظهرت النتائج ايضا ان البروتييز المنتج من *B. stearothermophilus* B17M1 هو من الانزيمات المعدنية نظرا لفقدان فعالية الانزيم بعد حضنه مع 2mM من مادة EDTA.

الاهداء

إلى . . .

عالم ومجتهد آل محمد (ص)

أهدي ثمرة جهدي المتواضع

أسماء

بِسْمِ اللَّهِ الرَّحْمَنِ الرَّحِيمِ

وَلَقَدْ كَتَبْنَا فِي الزَّبُورِ مِن بَعْدِ الذِّكْرِ
أَنَّ الْأَرْضَ يَرِثُهَا عِبَادِيَ الصَّالِحُونَ (١٠٥)

صدق الله العلي العظيم
سورة الانبياء

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وزارة التعليم العالي والبحث العلمي
جامعة النهرين
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قسم التقانة الاحيائية

دراسة وراثية وكيموحيوية على انزيم البروتييز المنتج من بكتريا *Bacillus stearothermophilus* المعزولة محليا

اطروحة

مقدمة الى مجلس كلية العلوم / جامعة النهرين وهي جزء من متطلبات نيل درجة دكتوراه فلسفه في التقانة الاحيائية

من قبل

اسماء علي حسين

بكالوريوس تقانة احيائية/ جامعة النهرين/ 1999
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باشراف

د. حميد مجيد جاسم
استاذ مساعد

د. نورية عبد الحسين علي
أستاذ

ذي الحجة 1430

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