Bioprocess Development for Production of Proteases by Bacillus Licheniformis

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Abstract

Bacterial proteases have enormous technical applications in food, pharmaceuticals and leather industries. Alkaline protease, however, has got important applications in eco-friendly leather processing. The bulk application of this hydrolyzing enzyme demands its cost effective production. Hence the present study was aimed at the development of a bioprocess for production of the alkaline protease by *Bacillus licheniformis* MZK05 strain which was previously isolated from tannery effluents and identified by 16S rRNA gene sequence analysis. Since the production of the enzyme by the wild *Bl*MZK05 strain was not adequate in a liquid medium namely Alkaline Protease Producing Broth (APPB), strain improvement through random mutagenesis followed by optimization of the enzyme fermentation by statistical methods were investigated. The mutation protocol involved the treatment of the *Bl*MZK05 cells of exponential phase with ultra violet (UV) irradiation and ethyl methanesulfonate (EMS) under varied combinations viz. combinatorial, single or sequential with different doses and exposure periods.

The potential mutants were selected from colonies of distinct characteristics, their clear zone ratio of casein hydrolysis on Skim Milk Agar (SMA), production capacity of the enzyme in liquid APPB medium and test for prolonged reproducible enzyme production ability. The distinct colonies were selected after mutagenic treatments that resulted in varied level of death rates. The highest death rates were revealed with EMS treatments for overnight. Of 182 colonies with distinct phenotypic characteristics, 100 mutants which exhibited casein hydrolysis in varying ratio yielded the enzyme activity between 46 – 270 U/ml in liquid APPB medium in shake flasks at 37°C, pH 7.5 and 150 rpm. The mutants with rather larger, raised and irregular colony appearance with respect to the parental organism produced greater protease activity than the other mutants. Most of the mutants tested for the reproducibility of their enzyme production capability for a prolonged period of about twelve months, were found inconsistent as “rise and fall” except two mutants namely *B. licheniformis* MZK05M9 (*Bl*M9) and *B. licheniformis* MZK05EO17 (*Bl*EO17). These mutants exhibited persistent enzyme activities of 135±3 U/ml and 235± 4 U/ml, respectively. The enzymes of two mutants *Bl*M9 and *Bl*EO17 were thus evaluated for their possible applications in leather processing based on their hydrolyzing capability of nonstructural

(albumin, globulin and elastin) and structural (collagen) leather proteins. Both the mutants were capable of hydrolyzing all but *Bl*M9 did not act on collagen indicating the suitability of *Bl*M9's enzyme in leather manufacturing processes. In this regard, optimization of the fermentation of alkaline protease by *Bl*M9 was performed using statistical methods which demonstrated effective medium components molasses as carbon source, soybean meal as nitrogen source and the salts NaCl, $MgSO_4.7H_2O$ and K_2HPO_4 by Plackett-Burman design. The concentrations of these medium components were optimized by Response Surface Methodology (RSM) based on Central Composite Design (CCD) in shake culture at pH 7.5, agitation 150 rpm and temperature 37°C. The optimum values for the tested variables for the maximum alkaline protease production were found as molasses (0.92%), soybean Meal (0.79%), NaCl (0.125%), $MgSO_4.7H_2O$ (0.125%) and K_2HPO_4 (0.59%) with the protease activity in the optimized medium 761 U/ml predicted by statistical software Minitab Version 17. The experimental value of the enzyme activity as obtained 765 U/ml was comparable and thus validated the predicted values of the software.

The granular sizes of the soybean meal affected the enzyme production revealing 4.7 mm mesh size supported the enzyme production 5 % higher than that of the mixed sizes between 6 to 4 mm. Fermentation in 7.0 L bioreactor cultivation with optimized medium at pH 7.5 and 37°C under cascade control of dissolved oxygen concentration, a high level of enzyme activity 1020 ± 10 U/ml was obtained after 28 hrs at stationery phase. This enzyme activity was 1.7 fold higher than that found in Molasses Soybean meal medium optimized by "one variable-at-a time" method.

The enzyme was stable up to 30 days at 4°C where as the enzyme lost its activity to 50% at 30°C after 40 days. However, it exhibited 100% stability up to 18 days at 30^oC in the presence of 0.5% polyethylene glycol. The partially purified protease had a pH optimum of 8.5 and temperature optimum of 55°C and the molecular mass of the enzyme was 27.2 kDa as judged by SDS–PAGE. According to the inhibition profiles obtained with the various protease inhibitors, it was confirmed that the partially purified protease belongs to the serine protease type. The activity of partially purified enzyme was enhanced by calcium, magnesium, barium, potassium and manganese ions and strongly inhibited by mercury ion. In addition, the protease showed remarkable stability in the presence of 1% SDS; 1, 3 and 5%

Triton X-100 and H_2O_2, which comprise the common bleach-based detergent formulation suggesting its possible usage in detergent as cleansing aids.

The application of the enzyme in dehairing of animal skin in leather processing demonstrated that treatment with enzymes removed 85% of hair from goat skin after 24 hrs under mild shaking condition at room temperature where as the treatment with enzymes and 5% lime together resulted in 100% dehairing under similar conditions. Sodium sulfide along with lime also removed 100% hair faster (with 20 hrs) than other two treatments. The grain surface of the enzyme treated skin was smoother and silkier than that of the chemical treated skin as revealed by Scanning Electron Microscopy.

The quality of the enzyme was found equally efficient to a commercial enzyme in bating of animal hide as proved by different physical tests such as tensile strength, percent of elongation, stitch tears strength, water vapor permeability, grain crack strength and tongue tear strength tests. In addition, the stability profile (pH, temperature and surfactants) also revealed its suitability for application in detergent industry. Thus these results will be a useful basis for commercial production of the alkaline protease by *Bl*M9 in Bangladesh.

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ABBREVIATION

Abbreviation	Meaning
%	Percentage
(v/v)	Volume/ volume e.g. (mL/mL)
(w/v)	Weight/ volume e.g. (g/mL)
°C	degrees Celsius
APPB	Alkaline Protease Producing Broth
BSA	Bovine serum albumine
CFU	Colony forming unit
cm	centimeter
DTT	1,4–dithio–D–threitol
EDTA	Ethylenediaminetetraacetic acid
EMS	Ethylmethane Sulphonate
et al	and others
EtBr	Ethidium Bromide
EtOH	Ethanol
FMB	Feather meal broth
GSMM	Glucose Soybean meal medium
Fig.	Figure
g	gram
hrs	Hours
Kg	kilo gram
L	litre
M	molar
mg	milligram
min	Minutes
mm	millimeter
mM	millimolar
MSMM	Molasses Soybean meal Medium
MW	Molecular weight
n	Number of replicates
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PEG	Polyethylene glycol
PMSF	Phenylmethylsulfonyl fluoride
rpm	Rotations per minute
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
SLPM	Standard liter per minute
SMA	Skim Milk Agar
SOM	Statistically optimized medium
TSA	Tryptic Soy Agar
TSB	Tryptic Soy Broth
µg	microgram
µl	microlitre
UV	Ultra violet
vvm	Gas volume flow per unit of liquid volume per minute

1.1. Introduction

Bioprocess is the use of biological materials (organisms, cells, organelles, enzymes) to carry out a process for industrial, medical or scientific reasons which has become widely used in several commercial areas. Bioprocesses offer numerous advantages as they usually require lower temperature, pressure, and pH; they can use renewable resources as raw materials; and greater quantities can be produced with less energy consumption. In most bioprocesses, enzymes are used to catalyze the biochemical reactions to produce valuable products. Unlike chemical catalysts, enzymes are biodegradable, having high specific catalytic activity under mild temperature and pressure conditions rendering them to be environmentally friendly. Proteases are one of the most important industrial enzymes which have applications in detergents, leather industry, food industry, pharmaceutical industry and bioremediation processes (Anwar and Saleemuddin, 1998; Gupta *et al*., 2002). Though these enzymes are widespread in nature, for industrial purposes, the preferred source of these enzymes are microbes because of their rapid growth, limited space required for their cultivation and their ready susceptibility to genetic manipulation to generate new enzymes with altered properties that are desirable for their various applications (Rao *et al*., 1998; Gupta *et al*., 2002). A wide range of bacteria, yeasts and molds produce the proteases (Walsh and Wilcox, 1970). Among them, bacteria are the most important source since bacterial proteases are mostly extracellular, easily produced in larger amounts, thermostable, and active at a wider pH range (Gupta *et al*., 2002). Bacteria belonging to the genus *Bacillus* are well known for protease production. The protease from *Bacillus* spp. has attracted attention of many researchers because of its tremendous technical applications (Bhunia *et al*., 2012). Since each microbial strain is unique in their molecular, biochemical, metabolic and enzyme production properties the newer strains of higher proteolytic activity with unique properties are always searched to meet the demand at commercial level (Prakasham *et al.*, 2006). In view of the above, Hoq *et al*. (2005) isolated eight *Bacillus* bacteria from the effluents of tannery and poultry farms and the bacteria were identified on the basis of physiological and carbohydrate assimilaton tests and 16S ribosomal RNA gene sequence analysis (Fig. 1.1). Among them, three isolates were revealed as strains of *Bacillus licheniformis*, two as *B. cereus*, and one of each as *B. subtilis*, *B. borstelensis* and *B. sphericus*. Among these *Bacillus* spp., *B. licheniformis* MZK03, *B.*

licheniformis MZK04, *B. licheniformis* MZK05 and *B. subtilis* MZK07 demonstrated chymotrypsin like (active towards Suc-Ala-Ala-Ala-pNA) and proteinase-K, elastase and subtilisin like (active towards Suc-Ala-Ala-Pro-Phe-pNa) protease activities. The protease inhibition studies with the enzymes from these strains exhibited the enzyme as serine protease (Hoq *et al*., 2005).

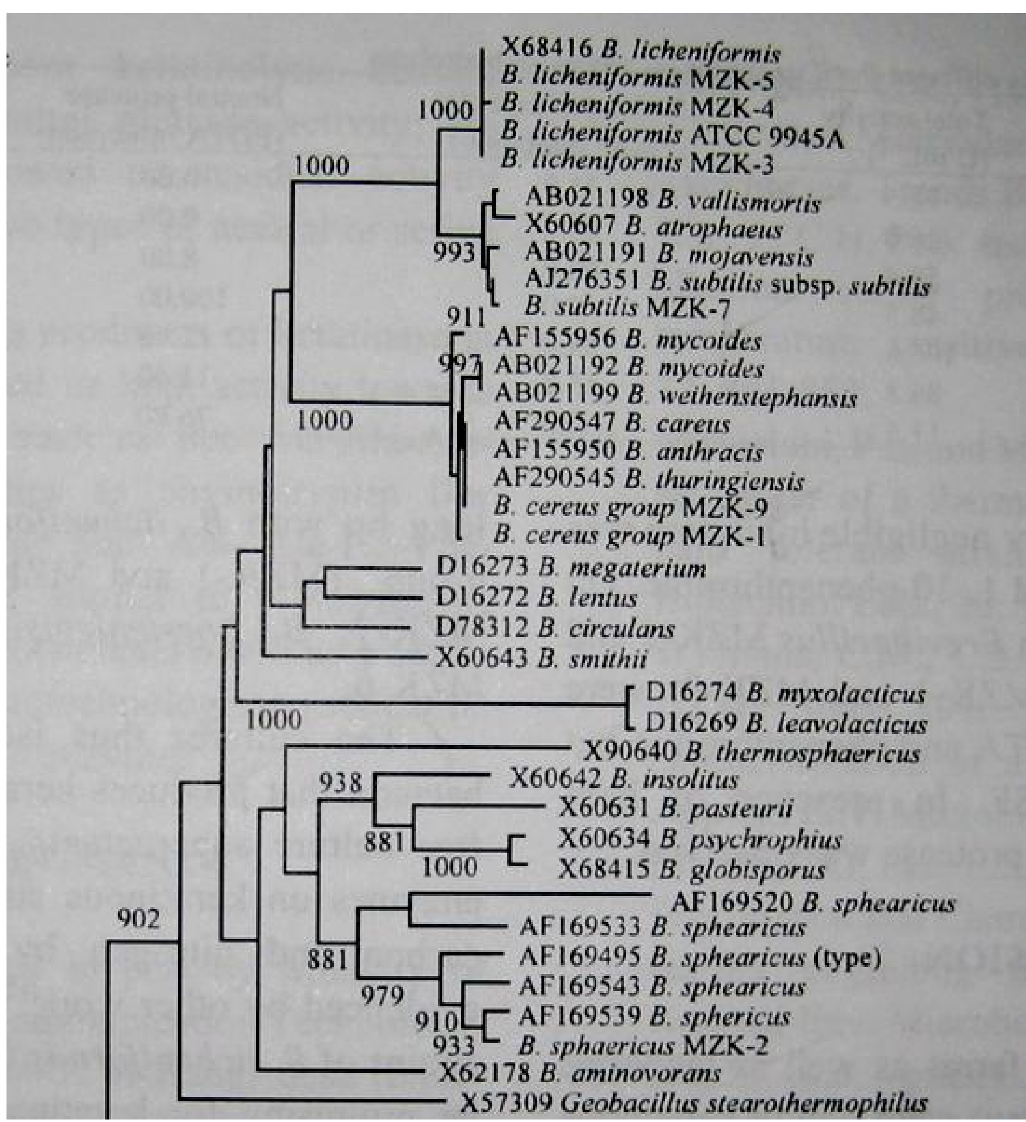

Fig. 1.1. Phylogenetic analysis based on the 16S rDNA sequence of *Bacillus* strains (*B. licheniformis* MZK05, *B. licheniformis* MZK04, *B. licheniformis* MZK03, *B. subtilis* MZK07, *B. cereus* MZK09, *B. cereus* MZK01 *B. sphericus* MZK02, *B. borstelensis* MZK06) and a type strain (*B. licheniformis* ATCC9945a).

B. licheniformis MZK03 and *B. licheniformis* MZK05 strains were tested for both alkaline protease and keratinase production in shake flask and bioreactor culture using Alkaline Protease Producing Broth (APPB) and Feather Meal Medium respectively (Hossain *et al.,* 2006, Hossain *et al.,* 2007). The enzyme preparations were tested for their technical applications in leather processing (Azad *et al*., 2002, Hossain *et al.,* 2008), feather solubilization (Hossain *et al.,* 2006) and as cleansing agent in detergents (Uddin *et al.,* 2006). It was found that the enzyme preparations were satisfactory in the technical applications. These applications of the enzyme preparations had encouraged to develop the process for large scale production. Since the production of the enzymes by natural strains of *Bacillus licheniformis* was not adequate, improvement of the wild strain through the genetic manipulation and development of a suitable medium was required for large-scale production of the proteases.

Bangladesh could be a major country for utilization of proteases in leather industry, and for this, there is a great need of production of cost effective proteases to meet the requirement for industrial sector to subside pollution hazards created by the leather industries. The leather industries require enzyme with unique properties that can degrade albumin, globulin, elastin specifically without causing any damage to the collagen, the main structure of the leather itself. Therefore, the study was designed to improve the natural strain *Bacillus licheniformis* MZK05 by mutation in order to obtain mutant strain with the unique properties suitable for the leather industries and develop a novel process that can increase the yield of proteases with respect to the industrial requirements coupled with lowering down the production cost. To achieve the goals, the research was conducted systematically to explore the means to reduce the protease production costs by genetic improvement through classical mutation followed by optimization of growth medium and conditions. Therefore, the objectives of the study were as follow.

1.2. Objectives of the study

1. Strain improvement of the *Bacillus licheniformis* MZK05 through classical mutation for enhanced production of protease.

2. Development of cost-effective medium based on agro-industrial residues using one variable-at-a-time and statistical (Placket-Burman and Response Surface Methodology based on Central Composite Design) methods.

3. Production of alkaline protease in shake-flask and bench-top bioreactor level.

4. Partial purification and characterization of the protease.

5. Application of the protease in bating of leather processing.

2. Literature review

2.1. Enzymes

Enzymes are the powerful and specific catalysts which are essential for all the living entities. Almost every biochemical reaction is catalyzed by an enzyme. With the exception of few catalytic RNAs, all known enzymes are proteins. Many of them require nonprotein coenzymes or cofactors for their catalytic function. Enzymes are classified according to the type of reaction they catalyze and there are six classes of enzymes (Table 2.1) (Nelson and Cox, 2005).

Table 2.1. International classification of Enzymes

No.	Class	Type of reaction catalyzed
1	Oxidoreductases	Transfer of electrons (hydride ions or H atoms)
2	Transferases	Group transfer reactions
3	Hydrolases	Hydrolysis reactions (transfer of functional groups to water)
4	Lyases	Addition of groups to double bonds, or formation of double bonds by removal of groups
5	Isomerases	Transfer of groups within molecules to yield isomeric forms
6	Ligases	Formation of C-C, C-S, C-O and C-N bonds by condensation reactions coupled to ATP cleavage

From an industrial standpoint, only a limited number of enzymes are commercially available and few of them have found applications in large quantities. Approximately, 75% of industrial enzymes are hydrolases (Rao *et al*., 1998). More than fifty commercial industrial enzymes are available and their number is being increased steadily. Today, enzymes are commonly used in numerous industrial applications and the demand for more stable, highly active and specific enzymes is growing rapidly. Presently the biotechnology industries are dominated by the leather, detergent, starch, textile and fuel alcohol industries accounting for the majority of the total enzyme market. The major hydrolytic enzymes used in industries are proteases, amylases, pectinases, cellulases, lipases, phytases, chitinases, lactase and xylanases. Among these industrial enzymes, proteases constitute around 60% of the total worldwide enzyme sales (Adinarayana *et al*., 2003; Merheb-Dini *et al*., 2009).

2.2. Proteases

Proteases, a group of enzymes hydrolyzing proteins into peptides and amino acids, are involved in every aspect of organism's function and constitute a very large and complex group of hydrolytic enzymes. Because of their broad substrate specificity, proteases have a wide range of applications such as in leather processing, detergent formulations, baking, brewing, meat tenderization, peptide synthesis, cheese manufacture, soysauce production, protein hydrolysate production, pharmaceutical industries, waste treatment, silk industries, organic synthesis, preparation of organic fertilizer, recovery of silver from waste photographic film, basic research as analytical tools and thus proteases have high commercial value (Godfrey and West, 1996; Rao *et al.*, 1998; Singh *et al.*, 1999; Chellappan *et al.*, 2006). These enzymes also have potential to contribute in the development of high value added products due to their characteristic nature of aided digestion. Therefore, there is a continued search for proteases having novel properties with known and newer applications.

2.2.1. General Classification of proteases

Proteases are subdivided into two major groups: exopeptidases cleaving the peptide bond proximal to the amino or carboxy terminal of the substrate, and endopeptidases cleaving peptide bonds distant from the termini. Based on the functional group present at the active site, proteases are further classified into four major groups: serine proteases, aspartyl proteases, cysteine proteases and metalloproteases (Kredics *et al*., 2005). According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified as like as Table 2.2 (Jisha *et al*., 2013).

Table 2.2. General classification of proteases with their enzyme commission (EC) code, coupled with specific mechanism of action of each subgroup

Protease	EC code	Mechanism
EXOPEPTIDASES		
Aminopeptidases	3, 4, 11	N-terminal residue released
Dipeptidases	3, 4, 13	Exopeptidases specific for dipeptides
Dipeptidyl peptidase	3, 4, 14	Release of an N-terminal dipeptide
Tripeptidyl peptidase	3, 4, 14	Release of an N-terminal tripeptide

Peptidyl dipeptidase	3, 4, 15	C-terminal dipeptide released
Carboxypeptidase (serine)	3, 4, 16	C-terminal residue released (serine at active site)
Carboxypeptidase (Metallo)	3, 4, 17	C-terminal residue released (metal requiring protease)
Carboxypeptidase (Cysteine)	3, 4, 18	C-terminal residue released (Cysteine at active site)
Peptidyldipeptidase	3, 4, 15	Release of free C-terminus liberate a dipeptide
Omega peptidases	3, 4, 19	Remove terminal residues that are linked by isopeptide bonds
ENDOPEPTIDASES		
Serine protease	3, 4, 21	Endopeptidases have an active centre serine involved in the catalytic process
Cysteine protease	3, 4, 22	Possesses a cysteine in the active centre
Aspartic protease	3, 4, 23	An aspartic acid residue for their catalytic activity
Metallo protease	3, 4, 24	Use a metal ion (often, but not always, Zn2+) in the catalytic mechanism
Endopeptidase with unkhown catalytic mechanism	3, 4, 99	Acting on peptide bonds

2.2.2. Sources of Proteases

Proteases are found in all forms of life: plants, animals and microorganism including viruses (Rao *et al*., 1998).

2.2.2.1. Plant Proteases

Plant proteases play pivotal roles against unfavorable conditions including water and environmental stress. They are also involved in various physiological processes, which include protein degradation, digestion, cell maintenance, signaling, differentiation, growth, development, apoptosis, ripening, regulatory mechanisms, wound healing, germination, senescence and necrosis. These proteases have also commercial usage in different industries including pharmaceutical and food industries for bioactive peptides production and meat tenderization (Zare *et al*., 2013). The use of plants as a source of proteases depends on

several factors such as the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process (Rao *et al*., 1998). The most common plant proteases are Bromelain, ficin, papain and zingibain (Lee *et al.*, 1986; Adulyatham and Owusu-Apenten, 2005; Sekizaki *et al.*, 2008).

2.2.2.1.1. Bromelain: Bromelain is a cysteine enzyme found in pineapple juice and in the pineapple stem. It has anti-inflammatory and immunomodulatory activities. The enzyme is active in pH 5 to 9 and its inactivation temperature is 70°C, which is lower than that of papain (Secor 2005). The name "bromelain" was originally used to describe any plant member of the Bromeliaceae family. It is, therefore, a collective term for proteolytic enzymes found in tissues, such as the stem, fruit and leaves, of the Bromeliaceae family, of which the pineapple, *Ananas comosus*, is the best known. Because of its anti-inflammatory and anti-cancer activities, as well as its ability to induce apoptotic cell death, bromelain has proved useful in several therapeutic areas. The market for this protease is growing, and several studies exploring various properties of this molecule have been reported. (De Lencastre Novaes *et al*., 2016).

2.2.2.1.2 Ficin: Ficin is a latex substance from the trunk of a tree called *Ficus insipida*. It is used as medicine, as well as in medical procedures and manufacturing. People take ficin for digestion problems and to get rid of intestinal worms. It is a sulfhydryl proteinase with cysteine at the active site extracted from latex of the ficus. It preferentially cleaves at tyrosine and phenylalanine residues. Ficin has proven to be a versatile low cost biocatalyst useful in peptide synthesis (Sekizaki *et al.*, 2008).

2.2.2.1.3. Papain: It is a cysteine protease extracted from latex of papaya (*Carica papaya*). In the active site of papain, Cys -25 and His -159 are thought to be catalytically active as a thiolate-imidazolium ion pair. Papain can be efficiently inhibited by peptidyl or non-peptidyl N-nitrosoanilines. The enzyme plays an important role in diverse biological processes in physiological and pathological states, drug designs and industrial uses such as meat tenderizers and pharmaceutical preparations (Amri and Mamboya, 2012).

2.2.2.1.4. Zingibain: Zingibain, zingipain, or ginger protease is a cysteine protease enzyme found in ginger (*Zingiber officinale)* rhizomes (Choi *et al*., 1999, Ohtsuki, *et al*.,1995, and Choi *et al*., 2000). Ginger proteases, all with a molecular weight around 31 kDa, are found to exist in 3 forms with isoelectric point values around 5.58, 5.40, and 5.22, respectively. These enzymes have very similar biochemical behavior, exhibiting optimal proteolytic activity from 40 to 60°C and maximum milk clotting activity at 70°C (Huang *et al*., 2011). Zingipain curdles milk, and has been suggested as vegetable rennet for cheese production (Huang *et al*., 2011). Like papain from papayas and bromelain from pineapples, it is used as a meat tenderizer (Ha *et al*., 2012).

2.2.2.2. Animal Proteases

The well-known proteases of animal origin are pepsin, trypsin, chymotrypsin and rennin. Production of these enzymes depends on available stock in slaughterhouse (Rao *et al*., 1998).

2.2.2.2.1. Trypsin: Trypsin is the main intestinal digestive enzyme responsible for the hydrolysis of food proteins. It is a serine protease and hydrolyzes peptide bonds in which the carboxyl groups are contributed by the lysine and arginine residues (Table 2). Based on the ability of protease inhibitors to inhibit the enzyme from the insect gut, this enzyme has received attention as a target for biocontrol of insect pests. Trypsin has limited applications in the food industry, since the protein hydrolysates generated by its action have a highly bitter taste. Trypsin is used in the preparation of bacterial media and in some specialized medical applications (Rao *et al*., 1998).

2.2.2.2.2. Chymotrypsin: Chymotrypsin is found in animal pancreatic extract. Pure chymotrypsin is an expensive enzyme and is used only for diagnostic and analytical applications. It is specific for the hydrolysis of peptide bonds in which the carboxyl groups are provided by one of the three aromatic amino acids, i.e., phenylalanine, tyrosine, or tryptophan. It is used extensively in the deallergenizing of milk protein hydrolysates. It is stored in the pancreas in the form of a precursor, chymotrypsinogen, and is activated by trypsin in a multistep process (Rao *et al*., 1998).

2.2.2.2.3. Pepsin: Pepsin is an acidic protease found in the stomachs of almost all vertebrates. The active enzyme is released from its zymogen, i.e., pepsinogen, by autocatalysis in the presence of hydrochloric acid. Pepsin is an aspartyl protease and resembles human immunodeficiency virus type 1 (HIV-1) protease, responsible for the maturation of HIV-1. It exhibits optimal activity between pH 1 and 2, while the optimal pH of the stomach is 2 to 4. Pepsin is inactivated above pH 6.0. The enzyme catalyzes the hydrolysis of peptide bonds between two hydrophobic amino acids (Rao *et al*., 1998).

2.2.2.2.4. Rennin: Rennet is a pepsin-like protease (rennin, chymosin) produced as an inactive precursor, prorennin, in the stomachs of all nursing mammals. It is converted to active rennin by the action of pepsin or by its autocatalysis. It is used extensively in the dairy industry to produce a stable curd with good flavor. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in k-casein to generate insoluble *para*-k-casein and C-terminal glycopeptides (Rao *et al*., 1998).

2.2.2.3. Microbial proteases

Microorganisms are attractive sources of proteases as they can be artificially cultured in large quantities in a relatively short time by established fermentation methods. Microbial alkaline proteases dominate the world enzyme market, accounting for a nearly two-thirds share of the detergent industry. Screening and characterization of these proteases from different sources serves many advantages from both environmental and industrial points of view (Mienda *et al.,* 2014).

Microbial proteases, involved in various cellular metabolic processes, represent one of the most important hydrolytic enzymes having various industrial applications. The industrial applications of proteases go back to 1914 as detergent additives (Khan, 2013).

Current world demand for proteases has led to an interest in microbial proteases because of their rapid growth, cost effectiveness and the ease with which they can be genetically modified to generate high yielding strains with more efficient enzymes with desirable properties required for their diverse applications.

2.2.2.3.1. Source of microbial proteases

The bacteria, virus and fungi are the main sources of microbial protease.

2.2.2.3.1.1. Bacteria: Most of the commercial proteases are of bacterial origin. Though proteases are produced by variety of bacteria such as *Pseudomonas aeruginosa*, *Flavobacterium*, *Clostridium*, *Staphylococcus aureus*, *Achromobacter*, *Thermoactinomyces* and species belonging to *Streptomyce, Bacillus* is the major source which secretes a variety of soluble extracellular enzymes (Prakasham *et al.*, 2006, Bhunia *et al*., 2012). Alkalophilic bacteria are also known to produce proteases. The first report of alkaline protease by an alkalophilic *Bacillus* sp. strain 221 was published in 1971 by Horikoshi (Horikoshi,1971). Alkaline proteases from bacterial source are widely used mainly in detergent formulations due to their activity and stability at high pH (9-11) and temperature (50-60°C). Neutral proteases of bacterial origin are active at pH 5-8 and between 35-40°C and generally used in food and brewing industries.

2.2.2.3.1.2. Virus: Viral proteases are enzymes encoded by the genetic material (DNA or RNA) of viral pathogens. The role of these enzymes is to catalyze the cleavage of specific peptide bonds in viral polyprotein precursors or in cellular proteins. In most cases these proteolytic events are essential for the completion of the viral infectious cycle. Viral proteases may use different catalytic mechanisms involving either serine, cysteine or aspartic acid residues to attack the scissile peptide bond (Steinkühler, 2008).Viral proteases are optimized to regulate and coordinate viral replication and assembly. Unlike digestive enzymes, they are highly selective catalysts performing limited proteolysis. The unique folds and structural complexities may lead to novel antiviral design which will selectively inhibit these enzymes while not affecting host functions (Babe and Craik, 1997).

2.2.2.3.1.3. Fungi: A great number of fungal strains have been used to produce protease belonging to genera *Aspergillus, Penicillium, Rhizopus,Mucor, Humicola, Thermoascus, Thermomyces,* among others (De Souza *et al*., 2015). The proteases of *Aspergillus* species, in particular, have been studied in detail since they are known for their capacity to secrete high levels of enzymes in their growth environment. Several of these secreted enzymes, produced

in a large-scale submerged fermentation, have been widely used in the food and beverage industry for decades. Example of species are *Aspergillus flavus* (Kranthi *et al.*, 2012; Macchione *et al*., 2008), *Aspergillus niger* (O'Donnell *et al*., 2001; Yang and lin, 1998) and *Aspergillus oryzae* (Ogawa *et al*., 1995; Vishwanatha *et al*., 2010; Vishwanatha *et al*., 2009). Various species of *Aspergillus* have been studied in detail for the production of proteases under various conditions. Alkaline proteases were reported to be produced by *A. flavus* and *A. oryzae* in solid state fermentation (SSF) system (De Souza *et al*., 2015). The fungus species, *Mucor pusillus* and *Mucor miehei*, secrete aspartate proteases, also known as mucor rennins, into the medium. The enzymes possess high milk-clotting activity and low proteolytic activity, enabling them to be used as substitutes for rennin in the cheese industry (Andrade *et al*., 2002). Thermophilic fungi produce hydrolases with important characteristics, such as higher thermostability, optimum activity at higher temperatures and high rates of hydrolysis. Thermostable proteases, that act in the temperature range 65–85 °C for the bioconversions of proteins into amino acids and peptides, have successful applications in baking, brewing, detergents and the leather industry (Haki and Rakshit, 2003; Merheb *et al*., 2007, De Souza *et al*., 2015).

2.2.2.3.2. Types of microbial protease:

More conventionally, microbial proteases can be classified into four important groups like serine, cysteine, aspartic and metallo proteases (Ellaiah *et al*., 2002).

2.2.2.3.2.1. Serine Protease: Among the microbial proteases, the serine proteases are the most important group of commercial enzymes (Kumar and Takagi, 1999). These are the most widely distributed in bacteria, fungi and viruses. The enzymes have a reactive serine residue in the active site and are generally inhibited by diisopropyl fluorophosphates (DFP) and phenyl methyl sulphonyl fluoride (PMSF). Three residues such as His (base), Asp (electrophile) and Ser (nucleophile) form the catalytic triad which is essential in the catalytic process (Rao *et al.*, 1998).The enzymes are generally active at neutral and alkaline pH with an optimum pH between 7-11. They have broad substrate specificities and are generally of low molecular weight (18-35 kDa) (Ellaiah *et al*., 2002).

2.2.2.3.2.2. Aspartic protease: The aspartic proteases (EC 3.4.23) are a group of proteolytic enzymes that can be found in different organisms. But best known sources of aspartic proteases are yeast and fungi (Szecsi, 1992). Most aspartic protease have molecular weights in the range of 30 – 45 kDa and their isoelectric points are usually in the range of pH 3.4 – 4.6. These enzymes are specific against aromatic or bulky amino acid residues on both sides of cleavage point and catalytic activities involve two aspartic acid residues. Aspartic proteases are characterized by maximum activity at low pH (3 – 4) and insensitivity to inhibitors of the other three groups of enzymes. Most aspartic protease are sensitive to epoxy and diazo-ketone compounds in the presence of copper cations. They are also inhibited by pepstatin or streptomyces pepsin inhibitor (Ellaiah *et al*., 2002).

2.2.2.3.2.3. Cysteine protease: Cysteine protease are sensitive to sulphydryl reagents such as *pCMB, Na*-tosyl-*L*-lysine chloromethyl ketone (TLCK), iodoacetic acid, iodoacetamide, heavy metals and are activated by reducing agents such as potassium cyanide or cysteine, dithiothreitol and ethylene diaminetetraacetic acid (EDTA). The occurrence of cysteine proteases has been reported in only a few fungi. Intracellular enzymes with properties similar to cysteine proteinases have been reported in *Trichoderma* species, *Oidiodendron kalrai* and *Nannizzia fulva*. Extracellular cysteine protease have been observed in *Microsporium* spp. *Aspergillus oryzae* and *Sporotrichum pulverulentum.* Most of these enzymes are active at pH 5 – 8. Some are stimulated by reducing agents (Kalisz, 1988, Ellaiah *et al*., 2002).

2.2.2.3.2.4. Metalloprotease: Metalloproteases are members of a group of proteases that contain a metal ion at their active site which acts as a catalyst in the hydrolysis of peptide bonds. (Hooper, 1994). All these enzymes have pH optima between pH 5-9 and are sensitive to metal-chelating reagents, such as EDTA, but are unaffected by serine protease inhibitors or sulphydryl agents. Most of the bacterial and fungal metalloproteases are zinc-containing enzymes, with one atom of zinc per moleculre of enzyme. The zinc atom is essential for enzyme activity. Calcium is required to stabilize the protein structure (Ellaiah *et al*., 2002).

2.2.2.3.3. Types of microbial protease based on substrate specificity:

2.2.2.3.3.1. Collagenase:. Collagenases are enzymes that break the peptide bonds in collagen, the structural component of the skin and hides. Collagenases are of two types, (i) low molecular weight serine collagenases (24-36 kDa) which are involved in the production of hormones and pharmacologically active peptides and (ii) high molecular weight metallocollagenases (30-150 kDa) containing zinc, which require calcium for stability and are involved in remodeling the extracellular matrix (Park *et al.*, 2002). Microbial collagenases are reported from bacteria, actinomycetes and fungi. The industrial applications of the collagenase enzymes are wide, including food industries, tannery industries, cosmetic industries and in the production of pharmaceutical compounds. In food industries the collagenases can be useful in several processes namely tenderization of meat. In tanneries the use of bacterial collagenases, after leather tanning, results in opening-up of the fibrous leather network enhancing the diffusion of dyes into the leather matrix. This results in an eco-friendly approach with an uptake of dye and in leathers with improved bulk properties like softness, fullness, grain smoothness, feel and general appearance (Duarte *et al*., 2014).

2.2.2.3.3.2. Elastase: Elastase is a protease that can solubilize elastin, extra cellular matrix proteins responsible for the resilience of skins and hides. Elastase production by microorganisms is relatively more promising due to its low cost, high production rate, and readily controlled conditions. Recently, it has attracted more and more interest because of the shortage of its sole source (pancreas) (Chen *et al*., 2007, EI-Aziz and Hassan, 2010). As elastase can degrade elastin that other proteases cannot; it has broad applications in medical therapy, food processing and daily use chemicals industry (Guo-qing, *et al*., 2004).

2.2.2.3.3.3. Keratinase: Keratinases are the proteolytic enzymes capable of hydrolyzing highly rigid, strongly cross-linked structural polypeptide, keratin which is recalcitrant to commonly known proteases such as trypsin, pepsin and papain. A vast variety of bacteria, actinomycetes and fungi are known to be keratin degraders. These enzymes are largely produced in the presence of keratinous substrates in the form of hair, feather, wool, nail, horn etc. during their degradation. Among bacteria, production of keratinase is mostly confined by

gram-positives, including *Bacillus, Lysobacter, Nesternokia, Kocurica and Microbacterium.* However, a few strains of gram-negative bacteria, viz. *Vibrio*, *Xanthomonas*, *Stenotrophomonas* and *Chryseobacterium* have also been reported as source of keratinase. In addition, a few thermophiles and extremophiles belonging to the genera *Fervidobacterium*, *Thermoanaerobacter*, *Bacillus* and *Nesternokia* have also been described as keratinase producers (Gupta and Ramnani, 2006). The most keratinolytic group among fungi belongs to fungi imperfectii including the following genera: *Chrysosporium*, *Aspergillus*, *Alternaria*, *Trichurus*, *Curvularia*, *Cladosporium*, *Fusarium*, *Geomyces*, *Gleomastis*, *Monodictys*, *Myrothecium*, *Paecilomyces*, *Stachybotrys*, *Urocladium*, *Scopulariopsis*, *Sepedonium*, *Penicillium*, *Doratomyces*. (Gradisar *et al*. 2000, Gupta and Ramnani, 2006)). Keratinases from *Bacillus* sp. particularly *B. licheniformis* and *B. subtilis* have been extensively studied due to their effectiveness in terms of feather degradation. *B. licheniformis* PWD1—a source of the Versazyme—the first commercial keratinase developed by Shih and coworkers at BRI (North Carolina), is used for feather meal generation and also promises to treat the dreaded mad cow's disease and turn feather protein into biodegradable plastic (Gupta and Ramnani, 2006).

Their application of the enzyme can also be extended to detergent and leather industries where they serve as specialty enzymes. Besides, they also find application in wool and silk cleaning; in the leather industry, better dehairing potential of these enzymes has led to the development of greener hair-saving dehairing technology and personal care products (Gupta and Ramnani, 2006).

2.2.2.3.3.4. Microbial Rennins: Rennin, an aspartic acid protease is an important enzyme in cheese manufacture. The enzymes possess high milk-clotting activity and low proteolytic activity, enabling them to be used as substitutes for calf chymosin in the cheese industry. Traditionally rennin is isolated from animal source (calf rennin) but increased demand of religious and ethnic regulations against animal derived enzyme has generated interest in microbial rennin. Among Mucorales, *M. miehe*i, *M. pusillus* (Arima *et al.*, 1967) and *M. bacilliformis* have been utilized in food industry and fermented beverage. The fungus, *Mucor miehei* are used for the production of rennin in large scale for industrial use (Seker *et al.*, 1999; Tubesha and Al-delaimy, 2003). Webb *et al.*, (1974) reported that the yield and quality

of cheese produced with the protease from *Mucor miehei* were the same as the calf rennet. Compared to calf rennin, microbial rennin ripened cheese more quickly and had no bitter flavours. The milk-clotting activity of the enzyme is due to its selective attack on the *k*-casein fraction, which stabilizes the casein micelle in milk. The split *k*-casein loses its stabilizing activity, and the micelles of casein coagulate in the presence of calcium.

2.2.2.3.4. Types of microbial protease based on pH optimal

Based on the pH optimal for their functioning, proteolytic enzymes can be characterized as alkaline, neutral or acidic proteases (Kredics *et al*., 2005, Mehta, 2010).

2.2.2.3.4.1. Acidic protease

These are mainly of fungal origin exhibiting maximum stability at pH 2.5-4.5 and having a molecular weight of around 35000 daltons. They are low in basic amino acid content and have low isoelectric points, but can hydrolyze a wide range of peptide bonds. These are not inhibited by di-isopropylflurophosphate, ethylene diaminetetraacetic acid or orthophenanthroline. These are much like animal enzymes and can be split into two subgroups, pepsin and rennin-like acid protease. Aspartic acid is present in their active centers (Mehta, 2010).

2.2.2.3.4.2. Neutral protease

They are widely spread both in bacteria and fungi having molecular weight in the range of 40,000-45,000 Da and several have been shown to be zinc containing metallo enzymes. They are inhibited by orthophenanthroline and EDTA but not by diisopropylflurophosphate. They exhibit some specificity towards bonds adjacent to leucine or phenyl alanine amino groups. They are used for food processing and have been shown to be active components in dental plague reaction, e.g., Bacillus subtilis and Bacillus thermoproteolyticus, which produce neutral protease montase and thermoase, respectively (Mehta, 2010).

2.2.2.3.4.3. Alkaline protease

These are frequent among bacteria and fungi. They resemble the animal enzymes, trypsin and chymotrypsin. The alkaline proteases have molecular weight in the range of 25000-30000

Daltons. The most studied are subtilisin carslberg and subtilisin novo, produced by various *Bacillus* spp. The alkaline protease have found extensive uses in detergents, leather tanning and food industries. The alkaline proteases are active in the pH range of 8 to 13 (Mehta, 2010).

2.2.3. Production of protease

2.2.3.1. Microorganism: Production is an inherent capacity of all microorganisms. A large number of bacterial species are known to produce proteases. But very few are recognized as commercial producers. In bioprocesses, selecting the proper microorganism is important in order to obtain the desired product. The microorganism that is to be used should give adequate yields, be able to secrete large amounts of proteases and should not produce toxins or any other undesired products. Potential hosts should be suitable for industrial fermentations and produce large cell mass per volume quickly on cheap media (Kirk & Othmer, 1994, Bhunia *et al*., 2012).

2.2.3.1.1. The Genus *Bacillus*: Bacillus is a genus of Gram-positive rod-shaped endospore forming bacteria and a member of the division Firmicutes. The endospores of the bacilli are more resistant than the vegetative cells to heat, drying, disinfectants, and other destructive agents and thus may remain viable for centuries. Cells stain Gram positive and are motile by peritrichous flagella. The genus *Bacillus* encompasses a great diversity of strains. Some species are strictly aerobic, others are facultative anaerobic. Although the majority is mesophilic, there are also psycrophilic and thermophilic species. Some are acidophiles while others are alkalophiles. Strains of some species grow well in a solution of glucose, ammonium phosphate and a few mineral salts, others need additional growth factors or amino acids, and still others have increasingly complex nutritional requirements (Laskin & Lechevalier, 1973). Bacilli are well known for their ability to excrete enzymes such as amylases and proteases and are, therefore, excellent candidates for large-scale production of proteases (Moon & Parulekar, 1991, Bhunia *et al*., 2012).

2.2.3.1.2. *Bacillus licheciformis:* *Bacillus licheniformis* is a Gram-positive, spore-forming saprophytic soil bacterium that is used in the biotechnology industries. This species is a close relative of *Bacillus subtilis*, an organism that is second only to *Escherichia coli* in the level of detail at which it has been studied. Unlike most other bacilli, which are predominantly aerobic, *B. licheniformis* is a facultative anaerobe, which may allow it to grow in additional ecological niches (Rey *et al*., 2004). There are numerous commercial and agricultural uses for *B. licheniformis* and its extracellular products. The species has been used for decades in the manufacture of industrial enzymes including several proteases, -amylase, penicillinase, pentosanase, cycloglucosyltransferase, -mannanase and several pectinolytic enzymes. The proteases from *B. licheniformis* are used in the detergent industry as well as for dehairing and bating of leather (Eveleigh 1981, Erickson, 1976). Specific *B. licheniformis* strains are also used to produce peptide antibiotics such as bacitracin and proticin in addition to a number of specialty chemicals such as citric acid, inosine, inosinic acid and poly- -glutamic acid (Gherna *et al*., 1989). Some *B. licheniformis* isolates can mitigate the effects of fungal pathogens on maize, grasses and vegetable crops (Neyra *et al*., 1996). As an endospore-forming bacterium, the ability of the organism to survive under unfavorable environmental conditions may enhance its potential as a natural biocontrol agent. *B. licheniformis* can be differentiated from other bacilli on the basis of metabolic and physiological tests (Logan and Berkeley, 1981; O'Donnell *et al*., 1980). Taxonomic studies indicate that *B. licheniformis* is closely related to *B. subtilis* and *Bacillus amyloliquefaciens* on the basis of comparisons of 16S rDNA and 16S-23S internal transcribed spacer (ITS) nucleotide sequences (Xu and Côté, 2003). Lapidus *et al.* (2002) constructed a physical map of the *B. licheniformis* chromosome using a PCR approach, and established a number of regions of colinearity where gene content and organization were conserved with the *B. subtilis* genome.

2.2.3.2. Media for production of microbial protease

Bacterium can be cultured for any purpose in the presence of appropriate biochemical and biophysical environment. The biochemical or nutritional environment is made available as a culture medium. Depending upon the special needs of particular bacteria a large variety and types of culture media have been developed with different purposes and uses (Todar, 2000). There are two major types of media depending on their composition or use. A chemically

defined or synthetic medium is one in which the exact chemical composition is known. A complex or undefined medium is one in which the exact chemical constitution of the medium is not known. Defined media are usually composed of pure biochemicals. A medium containing glucose, KH_2PO_4, $(NH_4)_2HPO_4$, and $MgCl_2$ is an example of a defined medium. Complex media usually contain complex materials of biological origin such as soybean, yeast extract, peptone, molasses or corn steep liquor, the exact chemical composition of which is obviously undetermined. In industry, complex media is preferred since the attainable enzyme activity and cell yields are much higher than that of the defined media due to the presence of necessary growth factors, vitamins, hormones, and trace elements (Bhunia *et al*., 2012).

The concentrations of media components are really important as they are tools for bioprocess medium design (Çalik *et al.*, 2001). Culture medium supplies the microorganism with all the essential elements for microbial growth. Certain microorganisms are capable of synthesizing all of their cellular constituents from carbon and nitrogen sources. However, most of the microorganisms require some source of micronutrients (i.e., amino acids, trace elements, vitamins, etc.). The culture conditions that promote production of proteases are significantly different from the culture conditions promoting cell growth (Moon & Parulekar 1991). Therefore optimization of media component is required for optimum cell growth and protease formation.

2.2.3.3. Cost-effective production of protease

For cost-effective large-scale production of protease a potential microorganism and economic optimized medium are required. Sometimes strain improvement through classical mutation or gene cloning are required to develop highly efficient strain for over production of protease.

2.2.3.3.1. Optimization of Media component

For commercial practice, the optimization of medium composition is done to maintain a balance between the various medium components in production media. Optimization helps minimizing the amount of unutilized components at the end of fermentation. Research efforts have been paying attention mainly toward: (i) Evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes; (ii) Requirement of

divalent metal ions in the fermentation medium; and (iii) Optimization of environmental and fermentation parameters such as pH, temperature, aeration, and agitation.

In addition, there are no defined medium established for the best production of alkaline serine proteases from different microbial sources. Each organism or strain has its own nutritional requirement for maximum enzyme production (Bhunia *et al*., 2012)

2.2.3.3.1.1. Effect of carbon source

Glucose is frequently used as carbon in bioprocesses for protease production. Studies have also indicated a reduction in protease production due to catabolic repression by glucose (Frankena *et al.*, 1986; Frankena *et al.*, 1985; Hanlon *et al.*, 1982; Kole *et al.*, 1988). Increased yields of alkaline proteases were also reported by several workers in the presence of different sugars such as lactose (Malathi & Chakraborty, 1991), maltose (Tsuchiya *et al.*, 1991), sucrose (Phadatare *et al.*, 1993) and fructose (Sen & Satyanarayana, 1993). However, a repression in protease synthesis was observed with these ingredients at high concentrations. In commercial practice, high carbohydrate concentrations repressed enzyme production. Therefore, carbohydrate was added either continuously or in aliquots throughout the fermentation to supplement the exhausted component and keep the volume limited and thereby reduce the power requirements (Aunstrup, 1980). In large-scale production of the protease complex organic substrates are used as carbon source (Bhunia *et al*., 2012).

2.2.3.3.1.2. Effect of Nitrogen source

Most microorganisms can utilize both inorganic and organic forms of nitrogen which are required to produce amino acids, nucleic acids, proteins and other cell wall components. The alkaline protease comprises 15.6% nitrogen (Kole *et al.*, 1988) and its production is dependent on the availability of both carbon and nitrogen sources in the medium (Kole *et al.*, 1988). The complex nitrogen sources are usually used for alkaline protease production.

The requirement for a specific nitrogen supplement differs from organism to organism. Low levels of alkaline protease production were reported with the use of inorganic nitrogen sources in the production medium (Chandrasekaran & Dhar, 1983; Chaphalkar & Dey, 1994; Sen & Satyanarayana, 1993). Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the

medium (Cruegar & Cruegar, 1984; Frankena *et al.*, 1986; Giesecke *et al.*, 1991). Several reports have demonstrated the use of organic nitrogen sources leading to higher enzyme production than the inorganic nitrogen sources. Soybean meal was also reported to be a suitable nitrogen source for protease production (Chandrasekaran & Dhar, 1983; Cheng *et al.*, 1995; Sen & Satyanarayana, 1993; Tsai *et al.*, 1988).

2.2.3.3.2. Criteria for the choice of raw materials as carbon and nitrogen source for industrial production of protease

In deciding the raw materials to be used in the large-scale production of protease, the following factors should be taken into account (Okafor, 2007).

2.2.3.3.2.1. Cost of the material: The cheaper the raw materials, the more competitive the selling price of the final product will be. No matter, therefore, how suitable a nutrient raw materials is, it will not usually be employed in an industrial process if its cost is so high that the selling price of the final product is not economic. Due to these economic considerations, the raw materials used in many industrial media are usually waste products from other processes. Corn steep liquor and molasses are, for example, waste products from the starch and sugar industries, respectively (Okafor, 2007).

2.2.3.3.2.2. Ready availability of the raw material: The raw material must be readily available in order not to halt production. If it is seasonal or imported, then it must be possible to store it for a reasonable period. Many industrial establishments keep large stocks of their raw materials for this purpose. There is also the important implication, which is not always easy to realize, that the material being used must be capable of long-term storage without concomitant deterioration in quality(Okafor, 2007).

2.2.3.3.2.3. Transportation costs: Proximity of the user-industry to the site of production of the raw materials is a factor of great importance, because the cost of the raw materials and of the finished material and hence its competitiveness on the market can all be affected by the transportation costs. The closer the source of the raw material to the point of use the more suitable it is for use, if all other conditions are satisfactory (Okafor, 2007).

2.2.3.3.2.4. Adequate chemical composition of medium and presence of relevant precursors: The demands of the microorganisms must also be met in terms of the compounds they can utilize. Some organisms grow better in one or the other substrate. The raw material must contain the precursors necessary for the synthesis of enzymes. Precursors often stimulate production of secondary metabolites either by increasing the amount of a limiting metabolite, by inducing a biosynthetic enzyme or both. These are usually amino acids but other small molecules also function as inducers. The medium must be complete and be able to provide the requirements for growth and enzyme production (Okafor, 2007).

2.2.3.3.3. Optimization of physical parameter

In fermentation some physical parameters such as oxygen transfer rate, pH and temperature show diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes (Çalik *et al.*, 2001).

2.2.3.3.3.1. Effect of temperature and pH: The incubation temperature and pH of the growth medium are important bioprocess parameters that is normally desired to keep both these variables constant and at their optimal values throughout the fermentation process. The influence of temperature and pH on a bioprocess can be very different, and since the growth process is the result of many enzymatic processes the influence of both culture parameters on the overall fermentation is quite complex (Çalik *et al.*, 2001).

The influence of temperature on the maximum specific growth rate of a microorganism is similar to that observed for the activity of an enzyme. Specific growth rate is gradually increased up to optimum temperature. But beyond that, decrease of specific growth rate can be observed (Nielsen & Villadsen, 1994). The mechanism of temperature control of enzyme production is not well understood. However, studies by Frankena *et al.*, (1986) showed that a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake.

Culture pH strongly affects many enzymatic processes and transport of several species across the cell membrane. Variation in pH alters acid-base equilibria and fluxes of various nutrients, inducers and growth factors between the abiotic and biotic phase (Moon & Parulekar, 1991).

The influence of pH on cellular activity is determined by the sensitivity of the individual enzymes to changes in pH. Enzymes are normally active only within a certain pH interval and the total enzyme activity of the cell is therefore a complex function of the environmental pH. Microbial cells have a remarkable ability to maintain the intracellular pH at a constant level even with large variations in the pH of the extracellular medium (Nielsen & Villadsen, 1994). The intracellular aqueous (cytoplasmic) pH of alkalophilic *Bacillus* species (e.g. *B. firmus*) is 8.2-8.5, whereas for neutrophilic *Bacillus* species (e.g. *B. subtilis*, *B. licheniformis*) this value is 7.5 (Çalik *et al.*, 2001).When ammonium ions were used, the medium turned acidic, while it turned alkaline when organic nitrogen, such as amino acids or peptides were consumed (Moon & Parulekar, 1993). The decline in the pH may also be due to production of acidic products (Moon & Parulekar, 1991). In view of a close relationship between protease synthesis and the utilization of nitrogenous compounds, pH variations during fermentation may indicate kinetic information about the protease production, such as the start and end of the protease production period.

2.2.3.3.3.2. Aeration and agitation

During fermentation, the aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth. Different dissolved oxygen profiles can be obtained by: (i) variations in the aeration rate; (ii) variations in the agitation speed of the bioreactor; or (iii) use of oxygen rich or oxygen deficient gas phase (appropriate air-oxygen or air-nitrogen mixtures) as the oxygen source (Michalik *et al.*, 1995; Moon & Parulekar, 1991). The variation in the agitation speed influences the extent of mixing in the shake flasks or the bioreactor and will also affect the nutrient availability. Optimum yields of alkaline protease are produced at 200 rpm for *B. subtilis* ATCC 14416 (Chu *et al.*, 1992) and *B. licheniformis* (Sen & Satyanarayana, 1993). However, lowering the aeration rate caused a drastic reduction in the protease yields (Moon & Parulekar, 1991). This indicates that a reduction in oxygen supply is an important limiting factor for growth as well as protease synthesis. Oxygen transfer shows diverse effects on product formation in aerobic fermentation processes by influencing metabolic pathways and changing metabolic fluxes. According to cell growth conditions and metabolic pathway analysis some bioprocesses require high oxygen transfer rate conditions while others require controlled oxygen transfer rate conditions (Calik *et al.*, 1999). It has

been extensively investigated in defined (Çalik *et al.*, 1998; Calik *et al.*, 1999 Calik *et al.*, 2000) and molasses based complex medium (Çalik *et al.*, 2003) for alkaline protease production and medium oxygen transfer conditions were found to be favorable for the enzyme production.

2.2.3.3.3.3. Effect of inoculum percentage and incubation time

Biomass and enzyme production was also influenced by the initial inoculum concentration. Optimum inoculum size was required for protease production. The increase in protease production using small inoculum sizes was suggested to be due to the higher surface area to volume ratio resulting in increased protease production. If the inoculum size is too small, insufficient number of bacteria would lead to reduced amount of secreted protease. This may be reasoned due to the limitation in nutrients in the fermentation medium components and reduced dissolved oxygen (Rahman *et al.*, 2005). The incubation also affects protease production. Normally protease is auto-degradable in nature. So protease yield can be increased by proper incubation time. This may also be reasoned due to the limitation of nutrients.

2.2.3.4. Strain improvement

Strain improvement plays a key role in the commercial development of microbial fermentation processes. As a rule, the wild strains usually produce limited quantities of the desired enzyme to be useful for commercial application. Conventionally, strain improvement has been achieved through mutation, selection, or genetic recombination. Overproduction of primary or secondary metabolites is a complex process, and successful development of improved strains requires knowledge of physiology, pathway regulation and control, including the design of creative screening procedures. The yield can be further improved by the use of mutagens or antibiotics and the adoption of special techniques or procedures for detecting useful mutants. However, in most cases, by adopting simple selection methods, such as spreading of the mutant culture on specific media, it is possible to pick colonies that show a substantial increase in yield. In addition, it requires mastery of the fermentation process for each new strain, as well as sound engineering know-how for media optimization

and the fine-tuning of process conditions for maximum production of protease (Parekh *et al*., 2000).

2.2.4. Uses of proteases

Alkaline proteases are one of the most important classes of proteases from an industrial point of view, occupying a major share of the total enzyme market. Because of the broad substrate specificity, proteases have a wide range of applications such as in leather processing, detergent formulations, baking, brewing, meat tenderization, peptide synthesis, cheese manufacture, soy sauce production, protein hydrolysate, pharmaceutical industry, waste treatment, silk industry, organic synthesis, recovery of silver from waste photographic film, as well as analytical tools in basic research and have high commercial value (Godfrey and West, 1996).

Use of alkaline proteases as active ingredients in detergents is the largest application of this enzyme. They are also widely used in leather industry, medical diagnostics, recovery of silver from X-ray films, silk degumming, food and feed industry etc. Due to their vast applications in the industrial processes, many companies started manufacturing them at commercial level. The table 2.3 gives the commercial manufacturers of alkaline proteases with their product trade name and different applications (Furhan and Sharma, 2014).

Table 2.3. Commercial manufacturers, trade name and applications of alkaline proteases

Manufacturer	Product trade name	Microbial source	Application
Novo Nordisk, Denmark	NovoCor SG	Not specified	Soaking
	Alcalase	*Bacillus licheniformis*	Detergent , Silk degumming
	Savinase	*Bacillus* sp.	Detergent, textile
	Esperase	*B. lentus*	Detergent , food, Silk degumming
	Biofeed pro	*Bacillus licheniformis*	Feed
	Durazym	*Bacillus* sp.	Detergent
	Novozyme 471 MP	Not specified	Photographic gelatin hydrolysis
	Novozyme 243	*B. licheniformis*	Denture cleaners
	Nue	*Bacillus* sp.	Leather processing
	RONOZYME Pro Act	Not specified	Maximize protein utilization and

			improving nutritional value of animal feed
	Novozyme 37020	Not specified	Meet and feed industry
	Ovozyme	Not specified	Remove egg soils
	Polarzyme	Not specified	Cold water hand wash
	Novobate 1547	Not specified	Leather, Bating
	Novolime	Not specified	Liming
Genecor International USA	Protex 6L	Not specified	Hydrolysis of milk fractions, baking, protein processing, pet food production, silver recovery
	Purafact	*B. lentus*	Detergent
	Primatan	Bacterial source	Leather
	Purafect Prime L	Not specified	High alkaline performance at lower dose rate
	Purafect OX L	Not specified	High alkaline protease with superior storage stability
	Purafect 4000L	Not specified	Soaking
Gist-Brocades, Netherlands	Subtilisin	*B. alcalophilus*	Detergent
	Maxacal	*Bacillus* sp.	Detergent
	Maxatase	*Bacillus* sp.	Detergent
Solvay Enzymes, Germany	Protease	*B. licheniformis*	Food, waste
	Optimase	*B. alcalophilus*	Detergent
	Opticlean	*B. licheniformis*	Detergent
	Maxapem	*Bacillus sp.*	Detergent
	HT-proteolytic	*B. subtilis*	Alcohol, baking, feed, food, leather, photographic waste
Amano pharmaceuticals, Japan	Amano protease S	*Bacillus sp.*	Food
	Collagenase	*Bacillus sp.*	Food
	Proleather	*Clostridium sp.*	Technical use
Enzyme development, USA	Enzeco alkaline protease	*B. licheniformis*	Industrial
	Enzeco alkaline protease- L FG	*B. licheniformis*	Food
	Enzeco high alkaline protease	*Bacillus sp.*	Industrial use
Nagase	Bioprase concentrate	*B. subtilis*	Cosmetic, pharmaceuticals

Biochemicals, Japan	Bioprase SP-10	*B. subtilis*	Food
	Bioprase	*B. subtilis*	Detergent, cleaning
	Cryst. protease	*B. subtilis*(*bioteus*)	Research
	Cryst. protease	*B. subtilis(K2)*	Research
	Ps. Elastase	*Pseudomonas aeruginosa*	Research
	Ps. protease	*Pseudomonas aeruginosa*	Research
Godo Shusei, Japan	Godo-Bap	*B. licheniformis*	Detergent, Food
Rohm, Germany	Corolase 7089	*B.Subtilis*	Food
	COROLASE H-PH	Not specified	Food and Feed Industry, Protein hydrolysis
	COROLASE LAP	Not specified	Bitterless Protein hydrolysis of animal or vegetable raw materials, e.g. whey, casein, soy.
Wuxi SynderBioproducts, China	Wuxi	*Bacillus Sp.*	Detergent
Advance Biochemicals, India	Protosol	*Bacillus Sp*	Detergent
	SEBsoak	Not specified	Leather industry, soaking
	SEBlime	Not specified	Dehairing, liming
	SEBateAlakli	Not specified	Cleaning and washing
Maps Enzyme Limited, India	Palkobate	Not specified	Leather industry, Bating
	Palkosoak	Not specified	Soaking
	Palkogent		Detergent industry Removal of protein stains
	Palkodehair	Not specified	Dehairing
Speciality enzymes and biotechnologies, USA	SEBpro A		Develop savory flavors and debitterment of protein hydrolyzate
	SEB Tender 70		Meat industry,Tenderization of meat
	SEBalase BP		Detergent industry, Removal of protein stains
	SEBDigest		Food and Feed industry, Protein hydrolysis
Rossari Biotech,	Lyserine ESD Powder	Not specified	Silk industry, Degumming of silk

India			
Bachauna Technology, India	Verma Batzyme	Not specified	Leather industry, Bating
Kao Corp., Tokyo, Japan	Proteinase K-16	Not specified	Detergebt Industry, Protein removal
Sunson, China	*PRAL800*	Not specified	Leather, silk and food industry, Leather depilation, silk degumming and protein hydrolysis

2.2.4.1. Detergent industry

Alkaline proteases have contributed greatly to the development and improvement of modern household and industrial detergents. They are effective at the moderate temperature and pH values that characterize modern laundering conditions in industrial & institutional cleaning. Various enzymes used in laundry industry are proteases, lipase, cellulases, amaylses etc. (Ito *et al.*, 1998). Of these, alkaline protease find a major application as detergent additives because of their ability to hydrolyze and remove proteinacious stains like blood, egg, gravy, milk etc in high pH conditions (Saeki *et al.*, 2007). Proteases and other enzymes used in detergent formulations should have high activity and stability over a broad range of pH and temperature. The enzymes used should be effective at low levels (0.4–0.8%). One of the most difficult design challenges that biotechnologists face is that the protease should be compatible with various commercially available detergents and its function is not hampered in the vicinity of typical detergent ingredients, such as surfactants, builders, bleaching agents, bleach activators, fillers, fabric softeners and various other formulation aids. Recently, alkaline proteases from *Bacillus cereus*, *Bacillus pumilus* strain CBS, *Streptomyces* sp. strain AB1, *Bacillus licheniformis*, *Aspergillus flavus*, *Aspergillus niger*, *Bacillus brevis*, *Bacillus subtilis* AG-1 have exhibited excellent deteregent compatibily in the presence of certain stabilizers such $CaCl_2$ and glycine (Abou-Elela *et al.*, 2011; Jaouadi *et al.*, 2011; Bezawada *et al.*, 2011). In order to survive the extremes of high alkalinity and chelator concentration in detergents, subtilisins have been improved with respect to their thermostability and resistance to chelators (Bryan, 2000). To prevent the loss of activity,

several oxidatively stable serine proteases (OSPs) suitable for use in detergents have been isolated from alkaliphilic *Bacillus* strains (Saeki *et al.*, 2000).

2.2.4.2. Leather industry

Although, leather industry is economically important, but the process of leather making has been clearly proved to be environmentally objectionable due to the discharge of effluent rich in BOD, COD and large quantities of toxic chemicals (Pepper and Wyatt, 1989). Proteases play a vital role in the treatment of the raw leather in tanneries. Soaking involves removal of blood, dung and dirt from hides and some structural changes. Most effective of leather treatment is the use of alkaline serine proteases. Alkaline proteases play an important role in solubilization of albumin and globulin, opening up of contracted fibrous proteins and washing dirt and excessive fat at soaking stage (Godfrey, 1996). The conventional method used for depilation involves the use of sodium sulfide and hydrated lime and is described as lime-sulfide process. The objective of this treatment is the removal of hair by hydrolysis of mucoids, swelling of collagen fibers and elastin digestion. This process is clearly objectionable worldwide now because of the release of dangerous chemical compounds in huge amount (Pepper and Wyatt, 1989). Proteases, which are mostly produced by bacteria are stable in alkaline range, have become more and more widespread for the depilation and opening up of the hide. These proteases may be used in conjunction with alkalies such as lime, sodium carbonate and with reducing agents such as sulfides or mercaptans at pH 10-14. Depending on the nature of finally prepared leather, the treatment can last for 6-24 hours. Enzymatically aided dehairing processes help to increase the surface area and facilitate the cleaning and the dying of the leather. Use of protease having high pH activity can enter in to the skin with more ease (Pepper and Wyatt, 1989). *Bacillus subtilis* produced proteases with keratinolytic activity that replace sodium sulfide in dehairing process of leather industry (Godfrey, 1996).Traditionally, bating is an enzymatic process mainly involving the use of pancreatic enzyme preparations containing trypsin. Today, microbial proteases have replaced the use of trypsin, as they are more economical. Quality of the finished leather mainly depends upon the bating efficiency (Haines and Barlow, 1975).

2.2.4.3. Chemical industry

It is established that enzymes in organic solvents can expand the application of biocatalysts in synthetic chemistry (Kumar and Takagi, 1999). A high stability in the presence of organic solvents is a feature which is highly desired in applications involving biocatalysis in non-aqueous medium for peptide synthesis. Alkaline proteases from *Aspergillus flavus*, *Bacillus pseudofirmus* SVB1, *Pseudomonas aeruginosa* PseA have shown promising results for potential of peptide synthesis due to their organic solvent stability (Prakasham *et al*., 2005). A sucrose-polyester synthesis was carried out in anhydrous pyridine using Proleather, a commercial alkaline protease preparation from *Bacillus* sp. (Patil *et al*., 1991). The polyester, which is extremely water-soluble and also soluble in polar organic solvents, finds its application as a biodegradable plastic. The Proleather also catalyzes the transesterification of D-glucose with various acyl donors in pyridine (Watanabe *et al*., 1995). Further, the enzyme Alcalase acted as catalyst for resolution of N-protected amino acid esters (Chen *et al*., 1991) and alkaline proteases from *Conidiobolus coronatus* were found to replace subtilisin Carlsberg in resolving the racemic mixtures of DL-phenylalanine and DL-phenylglycine (Sutar *et al*., 1992).

2.2.4.4. Medical uses

The use of immobilized alkaline protease from *Bacillus subtilis* possessing therapeutic properties has been studied for development of soft gel-based medicinal formulas, ointment compositions, gauze, non-woven tissues and new bandage materials (Davidenko *et al.*, 1999). Oral administration of proteases from *Aspergillus oryzae* has been used as a diagnostic aid to correct certain lytic enzyme deficiency syndromes (Rao *et al.*,1998). Alkaline-fibrinolytic protease have been reported to preferentially degrade fibrin suggesting its future application in thrombolytic therapy and anticancer drugs (Mukherjee *et al.*, 2011).

2.2.4.5. Feed and food industry

Alkaline proteases are widely used for production of protein hydrolysates for many years. Hydrolysates can be used as additives to food and mixed feed in order to improve their nutritional value. In medicine, they are administered to patients with digestive disorders and food allergies (Neklyudov *et al.*, 2000). Protein hydrosylates can be obtained from a variety of substrates such as whey, meat, soy and casein. Soluble meat hydrolysates can also be derived from lean meat wastes and from bone residues after mechanical deboning by solubilization with proteolytic enzymes. However, the hydrolysates are usually bitter when the degree of hydrolysis is above 10%, which is needed for sufficient solubilization. A patented method used a specific combination of neutral and alkaline proteases for hydrolyzing raw meat. The resulting meat hydrolysate exhibited excellent organoleptic properties and can be used as a meat-flavored additive to soup concentrates. Hydrolysis of over 20% did not show any bitterness when such combinations of enzymes were used. The reason for this may be that the preferential specificity was favorable when metalloproteinase and serine protease were used simultaneously (Pedersen *et al*., 1994, Kumar and Takagi, 1999). Further, proteases play a prominent role in meat tenderization, especially of beef. An alkaline elastase (Takagi *et al*.,1992) and thermophilic alkaline protease (Wilson *et al*., 1992) have proved to be successful and promising meat tenderizing enzymes, as they possess the ability to hydrolyze connective tissue proteins as well as muscle fibre proteins. The tenderization process can be achieved by sprinkling the powdered enzyme preparation or by immersion in an enzyme solution and/or by injecting the concentrated protease preparation into the blood stream or meat. A method has been developed in which the enzyme is introduced directly into the circulatory system of the animal shortly before slaughtering (Bernholdt, 1975) or after stunning the animal to cause brain death (Warren, 1992). An important application of proteases in the food industry is in the process of cheese-making. Lactic acid bacteria are used for the purpose of milk coagulation, an essential feature for yogurt production. These bacteria are dependent on their proteolytic enzymes which hydrolyze casein and fulfill their requirement of a nitrogen source for growth. Alkaline proteases from *Streptococcus cremoris*, *Amycolata* sp. and *Amycolatopsis* sp. have been used for cheese production industrially (Anwar and Saleemuddin, 1998).

2.2.4.6. Silver Recovery

Alkaline proteases find potential application in the bioprocessing of used X-ray films for silver recovery. Used X-ray film contains approximately 1.5 to 2.0 % (by weight) silver in its gelatin layers. The conventional practice of silver recovery by burning film causes a major environmental pollution problem. On the other hand, the enzymatic hydrolysis of the gelatin layers on the X-ray film enables not only the silver, but also the polyester film base, to be recycled (Kumar and Takagi 1999). The alkaline proteases from *Bacillus* sp. B21-2 (Fujiwara and Yamamoto, 1987), *Bacillus* sp. B189 (Fujiwara *et al*., 1991) and *B. coagulans* PB-77 (Gajju *et al*., 1996) decomposed the gelatinous coating on the used X-ray films from which the silver was recovered. Further, a continuous process for silver recovery was also reported (Fujiwara *et al*., 1989) on the basis of kinetic studies and mechanism of enzymatic hydrolysis of the gelatin layers on X-ray film and the resulting release of silver particles (Ishikawa *et al*., 1993).

2.2.4.7. Silk Degumming

Threads of raw silk must be degummed to remove sericin, a proteinaceous substance that covers the silk fiber. Traditionally, degumming is performed in soap or alkali. This is a harsh treatment because the fiber itself is attacked. It also have other disadvantages of high energy consumption, time consumption and also a loss in luster of silk due to the amounts of water used in this process (Gulrajani *et al.*, 2000). However, the use of selected protease enzymes is a better method because they remove the sericin without attacking the fiber. Bacterial enzymes are being widely used because of its significant action on the fibroins. The action of the enzyme can be controlled to avoid strength loss but at the same time uniformity of degummed silk can be obtained (Gulrajani and Gupta, 1996; Johnny and Chinnammal, 2012).

2.2.4.8. Waste treatment

Alkaline proteases provide potential application for the management of wastes from various food processing industries and household activities. These proteases can solubilize proteins in wastes through a multistep process to recover liquid concentrates or dry solids of nutritional value for fish or livestock (Kumar and Takagi, 1999). Keratinolytic proteases are

used in the processing of waste feathers from poultry slaughterhouses (Gupta and Ramnani, 2006). Similarly, the proteases are also used in feed technology for the production of amino acids or peptides, for degrading waste keratinous material in household refuse, and as a depilatory agent to remove hair in bath tub drains which causes bad odors in houses and in public places (Kumar and Takagi, 1999).

2.3. Scope of the present study

With the increasing demand for the enzymatic methods to replace chemical processes, researchers are now more and more focused on discovering microbial proteases with novel properties to meet the industrial requirements and also for the fulfillment of the demands of the global market.

In Bangladesh, leather is one of the exportable items of Bangladesh. There are about 220 tanneries and more than 75000 people are working as the direct employee. Hide processing into the final leather is a multi steps process including soaking, dehairing and bating of raw hide during which proteolytic action is necessary and important to produce clean and supple pelts, and the good quality leather. Most of the tanneries in Bangladesh exploit harsh chemicals for the processing of leather due to the high import costs of bating enzymes. Importing of enzymes also takes long time during which the activity of the enzymes is decreased causing further expenses and this has been discouraging the use of bating enzymes in leather processing (Hoq *et al*., 2013). On the other hand, local production of the protease enzyme will be cheaper not only due to its production on cheap raw materials but also enzyme preparation and uses in liquid state unlike dried enzyme being imported at high cost. With a view to develop an economically feasible technology for production of protease based on indigenous microbe and locally available cheap substrates as carbon and nitrogen source, the present study focused on strain improvement, optimization of medium as well as fermentation conditions, partial purification, characterization and application of the enzyme in leather processing.

Chapter 3. Improvement of *Bacillus licheniformis* MZK05 strain through classical mutation for increased production of protease

3.1. Introduction:

Bacterial proteases have important applications in various industries. Several *Bacillus* species are involved in protease production i.e., *B. cereus, B. stearothermophilus, B. mojavensis, B. megaterium, B. subtilis and B. licheniformis* (Sookkheo *et al*., 2000; Gupta *et al*., 2002; Beg and Gupta, 2003; Gerze *et al*., 2005; Hoq *et al*., 2005, Bhunia *et al*., 2012). Among them *Bacillus licheniformis* is well known for its production of several important extracellular enzymes that can be used for various industrial purposes. Protease is one of these enzymes that has found important applications in a number of industries like leather industry (George *et al*., 1995), detergents (Kobayashi *et al.,* 1996), food (Anwar and Saleemuddin, 1998); in cosmetics (Weiss and Maurer, 2003) and for the recovery of silver from used x-ray films (Ishikawa *et al*., 1993).

Several protease producer strains of *Bacillus licheniformis* are present in our laboratory but it is necessary to improve them further so that they can meet the industrial demands. There are several ways that can be adopted for improving these strains, such as gene cloning techniques, site specific mutagenesis, random mutation, optimization of growth conditions etc.

This study was aimed at improving the *Bacillus licheniformis* MZK05 for enhanced production of protease through random mutation and selection of the stable potential mutant in terms of production of enzyme titre with specific function. Four different mutational procedures have been employed in this experiment to improve protease activity of *Bacillus licheniformis* MZK05. This organism was treated with UV irradiation, Ethylmethane Sulphonate (EMS), EMS and UV (254nm) simultaneously (EMS + UV) and the EMS treated mutants that showed improved activity were also subsequently exposed to UV at 254nm (EMS →UV). Potential mutants were selected based on clear zone ratio on Skim Milk Agar plates as well as production of enzyme titre in Alkaline Protease Producing Broth (APPB). Stability of the mutants in terms of production of protease was studied over a twelve months period. These results are presented in this chapter.

3.2. Materials and Methods:

3.2.1. Chemicals, reagents and different protein substrates: Chemicals, reagents and different protein substrates used in this study were listed in Appendix A.

3.2.2. Media: Media were listed in Appendix B.

3.2.3. Buffers and Solutions: Buffers and solutions were presented in Appendix C.

3.2.4. Equipment: Appendix D.

3.2.5. Bacterial strain

The bacterial strain, *Bacillus licheniformis* MZK05 (*Bl*MZK05) was obtained from Enzyme and Fermentation Biotechnology Laboratory, Department of Microbiology, University of Dhaka. This bacterium was previously isolated from tannery effluent and was identified by 16S rRNA typing in the International Center for Biotechnology, Osaka University, Japan (Hoq *et al.,* 2005). It was preserved in 20% glycerol stock at -70°C. The organism from the stock culture was streaked carefully onto Tryptic Soy Agar (TSA) so that it did not confront any temperature shock. This strain was subjected to four different mutational treatments in order to improve the strain.

3.2.6. Mutagenesis

3.2.6.1. UV irradiation

The strain of *Bacillus licheniformis* MZK05 was grown overnight in 50 ml Tryptic Soy Broth (TSB) in a 250 ml Erlenmeyer flask at 37˚C and 150 rpm. Then one ml of appropriately diluted bacterial suspension was centrifuged at 6000 rpm for 10 minutes and pellet was resuspended in Phosphate buffer (1 ml, 0.1M, pH 7.4). 0.2 ml of this suspension was plated on Tryptic Soy Agar (TSA) media and treated under UV irradiation (Wave length: 254; lamp distance: 10 cm, Time: 15-90 minutes and overnight). Following the treatment the plates were incubated at 37°C for 24 hrs and then subsequent screening of the mutants was performed.

3.2.6.2. Ethyl methanesulfonate (EMS) treatment

The strain of *Bacillus licheniformis* MZK05 was grown overnight in 50 ml TSB in a 250 ml Erlenmeyer flask at 37˚C and 150 rpm. 5 ml of the bacterial culture was centrifuged at 6000 rpm for 10 min and then washed and resuspended in 5 ml normal saline. 99.99% EMS (Fluka, Lot no: 64292) was added to the bacterial cell suspension to make final EMS concentrations of 300 µl/ml, 250 µl/ml, 200 µl/ml, 150 µl/ml, 100 µl/ml and 50 µl/ml. One set of each concentration was incubated for 30 min and another set was incubated for overnight at 37°C. Following the treatments, the cells were washed with sterile normal saline three times and then resuspended and appropriately diluted in normal saline and spread plated on TSA. These plates were incubated at 37˚C for 24 hrs and then subsequent screening of the mutants was performed.

3.2.6.3. EMS followed by UV (EMS UV) treatment

In this case, some of the mutants with enhanced protease activity achieved from EMS treatments were subjected to UV irradiation at 254 nm. For this, the EMS mutants were grown overnight in 50 ml TSB in a 250 ml Erlenmeyer flask at 37˚C and 150 rpm. 15 ml of the bacterial culture was centrifuged at 6000 rpm for 10 min and then washed and resuspended in 15 ml normal saline in sterile Petri-dish. One set of the bacterial suspension of each was exposed under UV lamp at 254 nm with a distance of 10 cm from the lamp for 30 min and another set was exposed for overnight. Following mutagenic treatments the bacterial suspensions were appropriately diluted in normal saline and spread plated on TSA. These plates were incubated at 37˚C for 24 hrs and then subsequent screening of the mutants was performed.

3.2.6.4. EMS and UV simultaneous (EMS+UV) treatment

The strain of *Bacillus licheniformis* MZK05 was grown overnight in 50 ml TSB in a 250 ml Erlenmeyer flask at 37˚C and 150 rpm. 15 ml of the bacterial culture was centrifuged at 6000 rpm for 10 min and then washed and resuspended in 15ml normal saline in sterile Petri-dish. 99.99% EMS was added to the cell suspension to make final EMS concentrations of 50 µl/ml, 100 µl/ml, 150 µl/ml, 200 µl/ml, 250 µl/ml and 300 µl/ml. One set of each concentration was exposed under UV at 254 nm with a distance of 10 cm from the lamp for 30 min and another set was exposed for overnight. Following mutagenic treatments, the cells were washed in sterile normal saline three times and then

resuspended and appropriately diluted in normal saline and spread plated on TSA. These plates were incubated at 37˚C for 24 hrs and then subsequent screening of the mutants was performed.

3.2.7. Calculation of death rate

Using the cell counts before and after mutagenic treatments, the death rate was calculated using following equation:

$$\text{Death rate (\%)} = \frac{\text{Cells (CFU/ml) before treatment} - \text{Cells (CFU/ml) after treatment}}{\text{Cells (CFU/ml) before treatment}} \times 100$$

3.2.8. Screening of the mutants through clear zone ratio on Skim Milk Agar (SMA) plates

Colonies of the bacteria grown on TSA after mutagenic treatments were picked based on their morphological differences with respect to the parental organisms (For example, raised colonies or irregular or bigger colonies were selected) and transferred on Skim Milk Agar (SMA) plates by sterile needle. These plates were incubated at 37˚C for 48 hrs and the clear zone ration of the casein hydrolysis as a measure of initial proteolytic activity of the mutants were calculated following the equation:

$$\text{Clear zone ratio} = \frac{\text{Diameter of the clear zone (mm)}}{\text{Diameter of the colony (mm)}}$$

Mutants from these plates were chosen for further study based on their larger clear zone ratio compared with that of the wild organism and some were also chosen based on only the diameter of their clear zones and the appearance of the zone.

3.2.9. Selection of the mutants based on the enzyme titre in APPB medium in shake culture

3.2.9.1. Production of seed culture: The mutants selected from Skim Milk Agar plates, were streaked onto TSA plates and incubated overnight at 37°C. One loopful of the organisms from these plates was transferred to 250 ml Erlenmeyer flask containing TSB for overnight at 37˚C and 150 rpm. This was used as inoculum.

3.2.9.2. Fermentation: 5 ml of inoculum was transferred aseptically to 250 ml Erlenmeyer flasks containing 100 ml APPB medium, pH 7.5 and this was incubated in an orbital shaker at 37˚C for 48 hrs at 150 rpm.

3.2.9.3. Separation of culture filtrates from the culture: After 48 hrs of fermentation in the APPB medium, the extracellular protease was harvested by centrifugation of the fermented broth at 6000 rpm for 10 min. The supernatant was checked for alkaline protease activity using azo-casein as substrate according to the modified method described by Kreger and Lockwood, 1981.

3.2.9.4. Determination of enzyme activity: Enzyme activity was determined with azocasein (Sigma-Aldrich, St Louis, Mo) as substrate by a modified procedure described by Kreger and Lockwood (1981). 400 µl of 1% Azo-casein solution in 0.05M Tris -HCI buffer (pH 8.5) was added to 400 µl of culture supernatant and kept for 1 hour at 37°C in a water bath. The reaction was stopped by the addition of 135 µl of 35% trichloroacetic acid (TCA) and the mixture was kept at 4°C for 10 min. After centrifugation at 13,000 rpm for 10 min, 0.75 ml of supernatant was mixed with 0.75 ml of 1.0M NaOH and the absorbance was taken at 440 nm within one minute in a spectrophotometer. The control was prepared by adding TCA before mixing the culture supernatant with azo-casein solution. One unit of protease activity was determined as the amount of enzyme that produces an increase in absorbance of 0.01 under the above assay condition. The value obtained is expressed in U/ml.

3.2.10. Preservation of the mutants:

All the mutants obtained after treatment and screening were grown in TSB for overnight at 37°C and 150 rpm and preserved as 20% glycerol stock in sterile cryogenic tubes at -20˚C.

3.2.11. Selection of the mutants based on stability in producing enzyme activity and substrate hydrolyzing ability

3.2.11.1. Stability study: The proteolytic activities of the mutants in shake culture were checked every two months interval. For this purpose, the preserved mutants from the stock cultures were streaked carefully onto TSA so that they did not confront any temperature shock. The plates were incubated overnight at 37°C. The inoculum

preparation, fermentation, separation of the culture filtrate and enzyme assay were carried out as similar methods described in section 3.2.9.4. The mutants showing consistent activities for over 12 months were chosen for further study.

3.2.11.2. Hydrolysis of different protein substrates by the enzyme: The capability of the of the protease of the mutants to hydrolyze several substrates like elastin, collagen, albumin and globulin was tested according to the modified method described by Gradisar *et al*., 2000.

20 mg of the substrate was taken in 1 ml of 50 mM Tris-HCl (pH 7.5) and to this 250 µl of the crude enzyme was added. This mixture was incubated at 37˚C in a water bath for 30 min with constant agitation at 120 rpm after which the reaction was terminated by adding 500 µl of 10 % Trichloroacetic acid (TCA). This was then incubated at 4˚C for 30 min and then centrifuged for 10 min at 10,000g. Absorbance was taken at 280 nm against a control in a UV-visible spectrophotometer. The control was prepared in the same way except that TCA was added before mixing the enzyme with the substrate.

3.3. Result:

3.3.1. Screening of the UV treated mutants

Following treatments, mutants were initially screened through culturing on SMA. This process is quite laborious and therefore only mutants that had different morphological appearance in comparison to the parental strain were selected. Among the UV treated mutants 47 colonies with different morphological characteristics were selected to evaluate their ability of casein hydrolysis on SMA medium. From these, 24 mutants showing clear zone on SMA were cultivated in shake flask using APPB to check their capability of producing enzyme titre in liquid medium. Among them UVM9 showed highest activity 135 U/ml in APPB medium which initially showed clear zone ratio 1.33 on SMA (Table 3.1).

Table 3.1. Clear zone ratio and enzyme activities of UV treated mutants

UV exposure duration	Death rate (%)	Mutants	Clear zone ratio	Enzyme activity (U/ml)
15 min	98.09	UVM1	No clear zone	-
		UVM2	No clear zone	-
		UVM3	1.09	105
		UVM4	1.1	55
		UVM5	1.3	48
		UVM6	No clear zone	-
30 min	98.68	UVM7	No clear zone	-
		UVM8	No clear zone	-
		UVM9	1.33	135
		UVM10	1.9	85
		UVM11	1.76	65
		UVM12	1.7	54
		UVM13	No clear zone	-
		UVM14	No clear zone	-
		UVM15	1.15	58
		UVM16	1.45	49
		UVM17	1.8	74
45 min	98.98	UVM18	No clear zone	-
		UVM19	No clear zone	-
		UVM20	No clear zone	-
		UVM21	No clear zone	-
		UVM22	No clear zone	-
		UVM23	No clear zone	-
		UVM24	No clear zone	-
		UVM25	No clear zone	-
		UVM26	1.2	63
		UVM27	1.4	51
60 min	99.42	UVM28	1.25	47
		UVM29	1.1	55
		UVM30	No clear zone	-
		UVM31	1.2	112
		UVM32	1.0	46
		UVM33	1.13	59
		UVM34	No clear zone	-
		UVM35	1.04	60
90 min	99.83	UVM36	1.07	56
		UVM37	1.19	50
		UVM38	1.02	53
		UVM39	1.15	64
		UVM40	No clear zone	-
		UVM41	1.21	56
		UVM42	1.23	48
		UVM43	No clear zone	-
Overnight	99.98	UVM44	No clear zone	-
		UVM45	No clear zone	-
		UVM46	No clear zone	-
		UVM47	No clear zone	-

The mutants with relatively higher potentiality i.e., those gave enzyme activities greater than 100 U/ml were selected to study their stability. Three UV treated mutants were considered for stability study with respect to protease production in APPB medium. The protease activities of these mutants were checked 02 months interval for a twelve months period. Among these mutants, UVM31 showed stability up to 6 months where as UVM9 was found to be stable up to 12 months period (Table 3.2).

Table 3.2. Stability of the UV treated mutants in terms of proteolytic activity

Strains	Enzyme activity (U/ml)						
	Initial	2nd month	4th month	6th month	8th month	10th month	12th month
MZK05	40	40	38	40	42	39	40
UVM3	105	70	85	60	55	45	45
UVM9	135	135	133	134	137	133	135
UVM31	112	110	115	113	40	47	45

3.3.2. Screening of the EMS treated mutants

The wild strain MZK05 was treated with various concentrations of EMS (50-300 μl/ml) for 30 minutes and overnight. The highest death rates for 30 minutes and overnight treatments were 99.964% and 99.986% respectively.

Following treatments, mutants were initially screened through culturing on SMA. Among the EMS treated mutants 48 colonies with different morphological characteristics were selected to evaluate their ability of casein hydrolysis on SMA medium. From these, 24 mutants showing clear zone on SMA were cultivated in shake flask using APPB to check their capability of producing enzyme titre in liquid medium. Among them EO18 showed highest activity 270 U/ml in APPB medium which initially showed clear zone ratio 1.19 on SMA, while mutant EO17 showing highest clear zone ratio (1.85) exhibited the activity 235 U/ml (Table 3.3).

Table 3.3. Clear zone ratio and enzyme activities of EMS treated mutants

EMS concentration (µl/ml)	Duration of treatment	Death rate (%)	Mutants	Clear Zone ratio	Enzyme activity (U/ml)
50	30 min	90.357	E1	No clear zone	-
			E2	No clear zone	-
			E3	No clear zone	-
			E4	No clear zone	-
100	30 min	91.643	E5	No clear zone	-
			E6	No clear zone	-
			E7	No clear zone	-
			E8	No clear zone	-
150	30 min	95.714	E9	No clear zone	-
			E10	1.1	65
			E11	No clear zone	-
			E12	1.05	60
200	30 min	98.125	E13	1.7	148
			E14	1.33	150
			E15	1.15	191
			E16	1.13	217
			E17	1.43	181
			E18	1.19	93
250	30 min	99.937	E19	1.16	194
			E20	1.3	157
			E21	1.12	218
			E22	1.12	225
300	30 min	99.964	E23	No clear zone	-
			E24	No clear zone	-
			E25	No clear zone	-
			E26	1.4	138
50	Overnight	99.205	EO1	1.08	117
			EO2	1.8	157
			EO3	1.1	70
			EO4	1.45	160
100	Overnight	99.343	EO5	1.05	60
			EO6	No clear zone	-
			EO7	No clear zone	-
			EO8	No clear zone	-
150	Overnight	99.950	EO9	No clear zone	-
			EO10	1.12	62
			EO11	1.5	127
			EO12	No clear zone	-
200	Overnight	99.962	EO13	No clear zone	-
			EO14	1.07	61
			EO15	No clear zone	-
			EO16	1.8	55
250	Overnight	99.965	EO17	1.85	235
			EO18	1.19	270
300	Overnight	99.986	EO19	No clear zone	-
			EO20	No clear zone	-
			EO19	No clear zone	-
			EO20	No clear zone	-

The mutants with relatively higher potentiality i.e., those gave enzyme activities greater than 100 U/ml were selected to study their stability. Their stability with respect to protease production in APPB medium was checked 02 months interval. Among the EMS treated mutants three mutants E21, EO1 and EO18 showed stability up to 6 months where as EO17 showed consistent enzyme activity up to 12 months (Table 3.4).

Table 3.4. Stability of the EMS treated mutants in terms of proteolytic activity

	Enzyme activity (U/ml)						
Mutants	Initial	2nd month	4th month	6th month	8th month	10th month	12th month
E13	148	88	108	65	75	40	42
E14	150	65	54	45	40	47	44
E15	191	120	140	122	100	70	74
E16	217	70	40	35	38	50	48
E17	181	43	37	40	40	45	41
E19	194	130	130	110	70	52	50
E20	157	105	120	117	70	85	56
E21	218	210	220	220	105	58	42
E22	225	118	133	100	53	38	40
E26	138	77	63	67	60	35	37
EO1	117	115	118	115	70	49	45
EO2	157	65	90	29	48	55	50
EO4	160	105	98	90	87	75	62
EO11	127	120	112	80	75	60	60
EO17	235	230	233	230	234	235	236
EO18	270	272	265	260	210	140	95

3.3.3.Screening of the EMS followed by UV (EMS →UV) treated mutants

Among the EMS treated mutants, nine mutants (E13, E16, E17, E21, E22, EO2, EO4, EO17 and EO18) were further subjected to UV irradiation. The EMS mutants that were previously subjected EMS treatment for 30 min were exposed to UV for 30 min and those with overnight EMS treatments were exposed to UV overnight. When the EMS mutants were treated with UV (254nm) for 30 min, the highest death rate was observed in the mutant E17, which was exposed to 200 µl/ml EMS for 30 minutes in the previous phase of mutation, and its death rate was found 67.74%.

The EMS mutants that were exposed to overnight dose of UV radiation (254 nm) all showed approximately 99% death rate. However, the highest death rate was 99.97% for

the mutant EO2 that had been treated with 50 µl/ml of EMS overnight in the previous stage of mutagenesis.

Among the EMS followed by UV (EMS → UV) treated mutants, 39 with different morphological characteristics were selected to be screened on SMA medium. From these, 37 mutants showing clear zone were cultivated in APPB medium. Among them EFU10 showed highest activity 259 U/ml in APPB medium which initially showed clear zone ratio 1.75 while the mutant EFU8 showing the highest zone ratio 1.82 had the activity 227 U/ml. These results are represented in the Table 3.5.

Table 3.5. Clear zone ratio and enzyme activities of EMS followed by UV (EMS → UV) treated mutants

EMS mutants	EMS concentration in previous treatment (µl/ml)	Duration of the treatment	Death rate (%)	Mutants	Clear zone ratio	Enzyme activity (U/ml)
E13	200	30 min	33.33	EFU1	1.18	100
				EFU2	1.7	145
				EFU3	1.65	167
E16	200	30 min	0	EFU4	1.44	104
				EFU5	1.3	93
				EFU6	1.12	68
				EFU7	1.6	124
E17	200	30 min	67.74	EFU8	1.82	227
				EFU9	1.62	205
				EFU10	1.75	259
				EFU11	1.54	210
E21	250	30 min	26.71	EFU12	1.24	102
				EFU13	1.2	100
				EFU14	1.33	80
				EFU15	1.06	84
E22	250	30 min	16.79	EFU16	1.1	65
				EFU17	1.6	187
				EFU18	1.02	55
				EFU19	1.13	75
E02	50	Overnight	99.97	EFU20	1.47	116
				EFU21	1.26	98
				EFU22	1.25	172
				EFU23	1.17	153
E04	50	Overnight	99.79	EFU24	1.25	190
				EFU25	1.9	216
				EFU26	1.07	115
				EFU27	No zone observed	-

E017	250	Overnight	99.30	EFU28	1.1	104
				EFU29	1.7	172
				EFU30	1.13	63
				EFU31	1.17	84
				EFU32	1.6	150
				EFU33	1.12	77
E018	250	Overnight	99.30	EFU34	No zone observed	-
				EFU35	1.3	61
				EFU36	1.5	60
				EFU37	1.1	75
				EFU38	1.2	71
				EFU39	1.15	62

Among the EMS followed by UV (EMS → UV) treated mutants 21 showing activities more than 100 U/ml in APPB medium were selected to check their stability. No mutants showed consistent enzyme activity over 12 months period (Table. 3.6).

Table 3.6. Stability of the EMS treated mutants in terms of proteolytic activity

	Enzyme activity (U/ml)						
Strains	Initial	2nd month	4th month	6th month	8th month	10th month	12th month
EFU1	100	77	65	67	60	55	58
EFU2	145	100	41	55	43	40	43
EFU3	167	115	90	83	76	60	52
EFU4	104	80	75	54	67	50	43
EFU7	124	57	64	44	51	40	41
EFU8	227	200	180	95	55	40	39
EFU9	205	207	78	56	54	40	51
EFU10	259	170	70	64	47	44	41
EFU11	210	45	40	43	56	70	38
EFU12	102	72	59	50	58	50	44
EFU13	100	50	50	47	45	40	42
EFU17	187	114	60	65	40	45	40
EFU20	116	105	85	52	50	47	44
EFU22	172	140	73	66	60	40	40
EFU23	153	102	97	76	71	37	38
EFU24	190	90	61	40	45	40	43
EFU25	216	200	123	88	78	64	47
EFU26	115	46	40	45	47	45	40
EFU28	104	93	46	43	40	41	35
EFU29	172	47	55	50	46	40	43
EFU32	150	89	80	67	60	46	40

3.3.4. Screening of simultaneous EMS and UV (EMS + UV) treated mutants

B. licheniformis MZK05 cells were also treated simultaneously with UV (254 nm) and EMS (50-300 µl/ml) with varying treatment duration (30 min and over night). The highest death rate for 30 min and overnight treatments were 99.960% and 99.895% respectively, both of which were treated with 300 µl/ml EMS.

Following the treatments, 48 mutants with different morphological characteristics were selected to screen on SMA medium. Among them 33 mutants did not show clear zone. Other 15 mutants were cultivated in APPB medium to check their capacity for production of enzyme titre. The mutant SEU15 showed highest activity 190 U/ml in APPB medium (Table 3.7).

Table 3.7. Clear zone ratio and enzyme activities of simultaneous EMS and UV (EMS +UV) treated mutants

EMS concentration (µl/ml)	Duration of simultaneous UV exposure	Death rate (%)	Mutants	Clear zone ratio	Enzyme activity (U/ml)
50	30 min	99.799	SEU 1	No zone observed	-
			SEU 2	1.15	69
			SEU 3	No zone observed	-
			SEU 4	No zone observed	-
100	30 min	99.924	SEU 5	No zone observed	-
			SEU 6	No zone observed	-
			SEU 7	No zone observed	-
			SEU 8	No zone observed	-
150	30 min	99.941	SEU 9	1.4	148
			SEU 10	No zone observed	-
			SEU 11	1.65	172
			SEU 12	No zone observed	-
200	30 min	99.946	SEU 13	1.19	116
			SEU 14	No zone observed	-
			SEU 15	1.7	190
			SEU 16	No zone observed	-
250	30 min	99.958	SEU 17	1.06	77
			SEU 18	1.5	138
			SEU 19	1.66	174
			SEU 20	1.33	112
300	30 min	99.960	SEU 21	No zone observed	-
			SEU 22	No zone observed	-
			SEU 23	No zone observed	-
			SEU 24	1.2	163

50	Overnight	99.671	SEU 25	1.18	178
			SEU 26	1.38	180
			SEU 27	1.05	142
			SEU 28	No clear zone	-
100	Overnight	99.646	SEU 29	1.02	77
			SEU 30	No clear zone	-
			SEU 31	No clear zone	-
			SEU 32	No clear zone	-
150	Overnight	99.746	SEU 33	No clear zone	-
			SEU 34	No clear zone	-
			SEU 35	No clear zone	-
			SEU 36	No clear zone	-
200	Overnight	99.758	SEU 37	No clear zone	-
			SEU 38	No clear zone	-
			SEU 39	No clear zone	-
			SEU 40	No clear zone	-
250	Overnight	99.741	SEU 41	1.05	67
			SEU 42	No clear zone	-
			SEU 43	No clear zone	-
			SEU 44	No clear zone	-
300	Overnight	99.895	SEU 45	No clear zone	-
			SEU 46	No clear zone	-
			SEU 47	No clear zone	-
			SEU 48	No clear zone	-

Of the simultaneous EMS and UV (EMS+UV) treated mutants 11 were selected to study their stability, but no mutants showed stability in terms of enzyme production over 12 months period (Table. 3.8)

Table 3.8: Stability of the simultaneous EMS and UV (EMS +UV) treated mutants

	Enzyme activity (U/ml)						
Mutants	Initial	2nd month	4th month	6th month	8th month	10th month	12th month
SEU9	148	100	98	90	40	45	42
SEU11	172	86	64	38	42	40	25
SEU13	116	101	59	35	28	30	34
SEU15	190	90	110	64	48	55	60
SEU18	138	70	57	40	32	36	40
SEU19	174	75	66	55	50	47	40
SEU20	112	94	87	81	100	50	44
SEU24	163	88	92	90	60	45	40
SEU25	178	78	89	97	40	33	41
SEU26	180	170	170	110	60	42	40
SEU27	142	62	67	50	45	40	40

3.3.5. Protease activity of the mutants with different colony morphology:

Larger, wrinkled and raised colonies showed higher enzyme activities compared with smaller, regular and flat colonies. This pattern was observed every time when the mutants' proteolytic activity was checked. Nadeem *et al*. (2010) and Nehete *et al.* (1986) reported similar observations that rough and wrinkled *B. licheniformis* mutants produced higher yield of alkaline protease. Table 3.9 compares the proteolytic activities of some of the raised, larger, irregular colonies with those of the rather flat, smaller and less irregular colonies. The former mutant strains showed higher proteolytic activities than the latter mutants.

Table 3.9. Comparison of enzyme activity with colony morphology of the mutants

Mutants	Appearance	Colony characteristics	Proteolytic activity (U/ml)
EO16		Medium, round, flat and smooth	55
EO17		Large, raised, wrinkled, irregular	235
EFU24		Large, raised, irregular	190

3.3.6. Selection of the mutants based on stability in producing enzyme titre:

Upon mutagenic treatments, overall 182 mutants were selected based on different morphological characteristics for determining the enzyme activity based on zone of casein hydrolysis on SMA medium. Among them, total 100 mutants showed clear zone of casein hydrolysis on SMA which were cultivated in liquid APPB medium in shake flasks. After shake flask cultivation 51 hyperactive mutants were considered for stability study for 12 months period in terms of potentiality to produce enzyme (Table 3.10). All mutants showed instability in production of enzyme except two mutants UVM9 (30 min UV treated mutant) and EO17 (overnight 250 µl/ml EMS treated mutant), which showed

consistent enzyme production over the 12 months period. Therefore theses mutants were finally selected as stable potential mutant and named as *Bacillus licheniformis* MZK05M9 (*Bl*M9) and *Bacillus licheniformis* MZK05EO17 (*Bl*EO17).

Table 3.10. Overall effect of the mutagenic treatments on *B. licheniformis* MZK05

Treatments	Number of mutants selected based on different morphological characteristics	Number of mutants showing zone of casein hydrolysis	Highest enzyme activity (U/ml) achieved	Number of mutants selected for stability study	Number of Stable mutants
UV	47	24	135±3	3	1(*Bl*M9)
EMS	48	24	270±5	16	1(EO17)
EMS→UV	39	37	259±4	21	0
EMS +UV	48	15	190±3	11	0
Total	182	100	-------	51	2

3.3.7. Comparison of the mutants with wild MZK05 strain

The stable mutants *Bl*EO17 and *Bl*M9 were compared with the wild MZK05 in terms of their morphological characteristics and enzyme production. The fig.3.1 showed the colony morphology and table 3.11 summarize the phenotypic characteristics of the strains.

MZK05 *Bl*EO17 *Bl*M9

Fig. 3.1. Colony morphology of the stable mutants and wild strains

Table. 3.11. Comparison of colony morphology between wild and mutant strains

Characteristics	MZK 05	*Bl*EO17	*Bl*M9
Size	Small	Large	Large
Margin	Irregular	Irregular	Irregular
Texture	Smooth and dull	Rough and dull	Smooth and shiny
Elevation	Flat	Raised	Convex

Fig. 3.2(a) showed the clear zone of casein hydrolysis on SMA media where as fig. 3.2 (b) showed the enzyme activities of the mutant and wild strains.

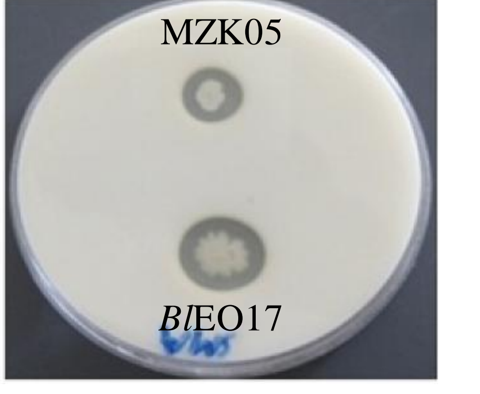

Fig.3.2 (a). Clear zone ratio of the stable mutants on SMA

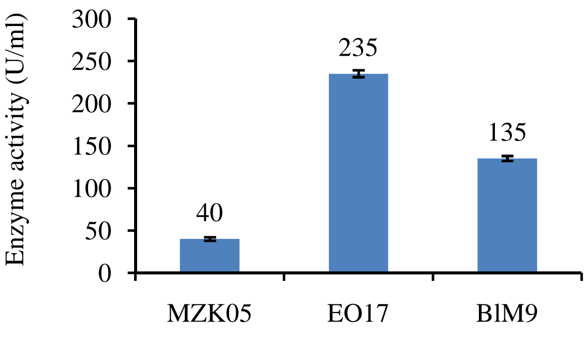

Fig.3.2 (b). Enzyme production by mutant and wild strains

3.3.8. Substrate specificity of the stable mutants

The hydrolytic ability of the crude protease of the mutant, EO17 and *Bl*M9 on several substrates like albumin, globulin, elastin and collagen were tested. The crude protease of EO17 showed 139 U/ml, 14 U/ml, 19 U/ml and 30 U/ml against albumin, globulin, Elastin and collagen respectively. The crude protease of *Bl*M9 also showed activities 85 U/ml, 73 U/ml and 54 U/ml against albumin, globulin and Elastin, respectively. However, *Bl*M9 did not show any activity against collagen (Fig. 3.3).

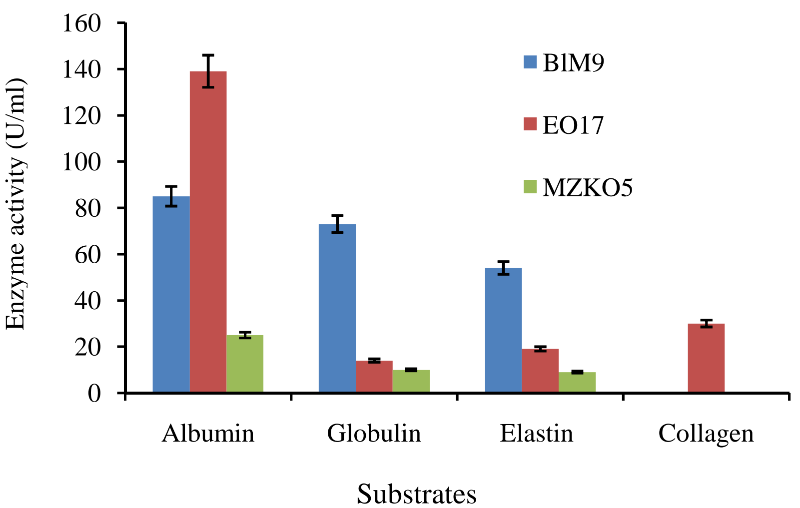

Fig. 3.3. Substrate specificity of the stable mutants

As the genetic improvement of the wild *Bacillus licheniformis* MZK05 was designed to develop a potential strain to overproduce the protease that could be used in leather industries. The leather industries require enzyme with unique properties that can degrade albumin, globulin, elastin specifically without causing any damage to the collagen, the main structure of the leather itself. From the substrate specificity study it was found that *Bl*EO17 showed activity against the albumin, globulin, elastin and also collagen where as the *Bl*M9 showed activity against all the substrates except collagen. Therefore the *Bl*M9 was finally selected as a unique strain in terms of having selective activities against non structural proteins of leather without affecting collagen, the main structural protein of leather. Therefore, the optimization of the fermentation conditions for production of protease was performed with the *Bl*M9.

Chapter 4.1. Optimization of fermentation conditions for protease production by *Bl*M9 in Alkaline Protease Producing Broth

4.1.1. Introduction

Among the different types of microbial proteases the most commercially important are those from the bacterial sources (Gupta *et al*., 2002). In this study, a suitable mutant *Bacillus licheniformis* MZK05 (*Bl*M9) strain has been selected for increased production of protease to be used in leather processing (described in chapter 3). Now, it is necessary to optimize the cultural conditions to maximize the enzyme production by the selected strain so as to reduce the cost of production.

Fermentation condition is an important parameter to consider that can influence the cost and rate of enzyme production (Beg *et al*., 2002). Extracellular protease production from *Bacillus* species is significantly influenced by medium composition and some physical factors, such as fermentation period, aeration, inoculum density, incubation temperature and pH of growth medium (Puri *et al*. 2002; Genckal and Tari 2006; Nadeem *et al*. 2006). The present study (described in this chapter 4.1) was carried out to optimize the factors like incubation temperature, pH, inoculum concentration, aeration and agitation for maximum yield of protease by *Bl*M9. All of these factors can significantly affected protease production. In this study, one variable-at-a-time strategies was applied to optimize the cultural parameters (incubation temperature, pH, size and age of inoculum, aeration and agitation rate) for maximum production of alkaline protease by *Bl*M9 in Alkaline Protease Producing Broth (APPB) medium.

4.1.2. Materials and Methods

4.1.2.1. Chemicals, reagents and different substrates: Chemicals, reagents and different substrates used in this study were listed in Appendix A.

4.1.2.2. Media: Media were listed in Appendix B.

4.1.2.3. Buffers and Solutions: Buffers and solutions were presented in Appendix C.

4.1.2.4. Equipments: Appendix D.

4.1.2.5. Bacterial strain

Bacillus licheniformis MZK05M9 (*Bl*M9), the mutant strain developed by UV irradiation was used in this study. It was preserved in 20% glycerol stock at -70˚C. The organism from the stock culture was streaked carefully onto Tryptic Soy Agar (TSA) so that it did not confront any temperature shock.

4.1.2.6. Production of inoculum

The mutant *Bl*M9 was streaked onto TSA plates and incubated overnight at 37°C. One loopful of the organism from this plate was transferred to 250 ml Erlenmeyer flask containing 50 ml TSB and incubated for 12 hrs at 37˚C and 150 rpm. This was used as inoculum.

4.1.2.7. Optimization of fermentation parameters for protease production by *Bl*M9 in shake flask

Optimization of the fermentation condition for protease production by *B. licheniformis* MZK05M9 (*Bl*M9) was studied. The experiments were carried out systematically in such a way that the parameter optimized in one experiment was maintained at its optimum level in the subsequent experiments. Various process parameters that enhance the yield of protease under submerged fermentation were investigated by one factor-at-a-time approach. All the experiments were conducted in triplicate and then the mean values were considered.

4.1.2.7.1. Effect of temperature on protease production by *Bl*M9: To study the effect of temperature on protease production by *Bl*M9, the fermentation experiments were carried out in APPB medium at various temperatures (30°C, 35°C, 37°C, 40°C, and 45°C) at 150 rpm, pH 7.5 for 48 hrs. After autoclaving, the medium was cooled and inoculated with 5% of 12 hrs old inoculum and incubated in orbital shaker at 150 rpm for 48 hrs.

4.1.2.7.2. Effect of pH on protease production by *Bl*M9: Effect of pH on the enzyme production by *Bl*M9 was carried out in 250 ml of Erlenmeyer flasks containing100 ml APPB medium with various initial pH ranging from 6.5 to 9.0 with an interval of 0.5 adjusted by using 0.1N NaOH and 0.1 N HCl. The volume of the medium was made up to desired level after adjusting the pH. After autoclaving, the medium was cooled and

inoculated with 5% of 12 hrs old inoculum and incubated in orbital shaker at 37°C at 150 rpm for 48 hrs.

4.1.2.7.3. Effect of size and age of inoculum on protease production by *Bl*M9: The effect of inoculums size (%) was studied for optimal protease activity. The sterile fermentation medium contained in flask was inoculated with 3 to 7% of 12 hrs old culture with 10^7CFU/ml viable cell concentration. The flasks were incubated at 37°C and 150 rpm for 48 hrs in the orbital shaker.

In order to understand the impact of inoculum age on protease production by *Bl*M9 in shake culture, the fermentation experiments were conducted using the cultures of different age (8 to 24 hrs old cultures as inoculum). The flasks with 100 ml APPB medium having pH 7.5 were inoculated with cultures of different age at 5% level. The flasks were incubated at 37°C at 150 rpm for 48 hrs in the orbital shaker.

4.1.2.8. Optimization of fermentation conditions in Bioreactor

4.1.2.8.1. Inoculum preparation for fermentation in bioreactor: For inoculum preparation the composition of the medium (pH 7.5) was as same as the medium (APPB) used in the bioreactor. After sterilization by autoclaving the inoculum medium was cooled and inoculated with the 16 hrs old culture (preparation of the culture has been described in section 4.1.2.6.) and incubated for 16 hrs at 37°C and 150 rpm in an orbital shaker.

4.1.2.8.2. Preparation of bioreactor: The bioreactor studies were carried out in a 7 L lab scale bioreactor (Bioflo 110, New Brunswick scientific) with 3.5 L working volume. The fermentor is equipped with digitally controlled pH electrode, temperature probe, dissolved oxygen (DO) probe (Metller-tolledo, Germany) and six-blade Rushton turbine impeller. The pH electrode was calibrated by using standard buffers (Fluka) at pH 4 and 7 prior to the sterilization of fermentor (121°C for 15 min). However, the calibration of DO electrode was conducted after sterilization by sparging the air until highest saturation achieved and DO cable was disconnected from the DO probe and the reading was taken as 0% saturation following by the reconnecting the DO cable to the probe and the reading was taken as 100 % saturation when DO level reached at highest saturation. The foam was controlled manually by adding few drops of silicone polymer based antifoam (Antifoam A, Sigma-Aldrich, St. Louis, Mo, USA) at the time of foaming.

4.1.2.8.3. Effect of different aeration and agitation rates on protease production by *Bl*M9: The bioreactor was operated with 3.5 L of APPB medium to optimize the aeration and agitation rates for the maximum yield of the protease. The pH of the growth medium was adjusted at 7.5 with 1 N HCl/NaOH before sterilization at 121°C for 15 min. Glucose was sterilized by filtration through 0.45μm filter separately and mixed with the growth medium at the time of inoculation. The fermentation medium was inoculated with 5% of 16 hrs old inoculum.

To evaluate the effect of aeration rate fermentation was carried out at different aeration rate ranging from 0.5 to 2.5 vvm while agitation speed was fixed at 300 rpm. To optimize the agitation rate the fermentation was also carried out with different agitation speed ranging from 200 to 400 rpm while the aeration rate was fixed at 1 vvm.

4.1.2.8.4. Time course for extracellular protein and protease production by *Bl*M9: To determine the time course for cell growth, extracellular protein and protease production by *Bl*M9, fermentation was carried out in the 7 L bench-top bioreactor with working volume of 3.5 L at agitation speed 300 rpm and aeration rate 1 vvm and 37°C. The fermenting medium APPB (initial pH 7.5) was inoculated with 5% of 16 hrs old inoculum. The dissolved oxygen level was recorded by Bio-command Plus Version 3.1 software. Sampling were performed 02 hrs intervals and the cell count, dry weight of the biomass, glucose concentration, enzyme activity and protein concentration were estimated according to the following methods.

4.1.2.9. Determination of viable cell concentration

The sample collected from the bioreactor was serially diluted in sterile normal saline and 100 μl of the diluted sample was spread plated on TSA plate. The plates were then incubated at 37°C for 24 hrs and then cell count was taken as colony forming unit (CFU). From this, the viable cell concentration was measured as CFU/ml.

4.1.2.10. Determination of bacterial cell mass

One ml of sample in microfuge tube was centrifuged at 8,000 rpm for 10 min and the cell pellet was washed with sterilized normal saline three times to remove the suspended particles. The washed cell pellet was freeze dried for 12 hrs in freeze dryer (Ilshin,

Korea). The bacterial cell mass was estimated by subtracting the weight of the microfuge tube from the total weight of the microfuge tube containing freeze dried cell.

4.1.2.11. Determination glucose concentration

Concentration of glucose was determines according to the method described by Miller (1969). For the construction of standard curve 1 gm of glucose (Sigma-Aldrich) was taken in 100 ml volumetric flask and the volume was adjusted to 100 ml by distilled water.

The solution was then diluted to 1.0, 0.8, 0.6, 0.5, 0.4, 0.2 and 0.1 mg/ml concentrations in different volumetric flasks. 1.0 ml of solution of the glucose of these dilutions was added in 7 different tubes. 3.0 ml of DNS reagent was added in each test tube and mixed well with the solution of the glucose. In the control 1.0 ml distilled water was added instead of solution of glucose. After boiling at 90°C for 5 min the absorbance was taken at 545 nm. A standard curve of absorbance versus glucose concentration was prepared by using the data of diluted standard glucose concentrations and their corresponding absorbance.

1.0 ml of appropriately diluted culture supernatant was mixed well with 3.0 ml of DNS reagent and after boiling at 90°C for 5 min the absorbance was taken at 545 nm. The concentration of glucose was determined from that standard curve using the following equation and expressed as mg/ml.

$$\mathbf{y = mx}$$

Where, y = absorbance at 545 nm

x = Glucose concentration (mg/ml)

m= slope of the standard curve

4.1.2.12. Determination of protein concentration

Soluble protein in the culture supernatant was estimated according to the Bradford method (Bradford, 1976). For the construction of standard curve 1 gm of Bovine Serum Albumin (BSA) was taken in 100 ml volumetric flask and the volume was adjusted to 100 ml by distilled water.

The solution was then diluted to 1.0, 0.8, 0.6, 0.5, 0.4, 0.2 and 0.1 mg/ml concentrations in different volumetric flask. 0.1 ml of solution of the BSA of these dilutions was added in 7 different tubes. 5 ml of Bradford reagent (Fluka) was added in each test tube and mixed well with the solution of the BSA. In the control 0.1 ml distilled water was added instead

of BSA solution. After 5 minutes the absorbance was taken at 595 nm. A standard curve of absorbance versus protein concentration was prepared by using the data of diluted standard protein concentrations and their corresponding absorbance.

0.1 ml of culture supernatant was mixed well with 5 ml of Bradford test solution and the absorbance was taken at 595 nm after 5 minutes. The concentration of soluble protein was determined from that standard curve using the following equation and expressed as mg/ml.

$$y = mx$$

Where, y = absorbance at 595 nm

x = protein concentration in mg/ml

m= slope of the standard curve

4.1.2.13. Determination of protease enzyme activity: Described in chapter 3, section 3.2.9.4.

4.1.3. Results

4.1.3.1. Effect of temperatures on enzyme production by *Bl*M9 in shake culture

The Alkaline Protease Producing Broth (APPB) medium was inoculated with 5% inoculum of *Bl*M9 at pH 7.5 and incubated at various temperatures ranging from 30-45°C at 150 rpm for 48 hrs. It is evident from the results that maximum protease production (133 ± 4 U/ml) was observed at 37°C after 48 hrs of incubation. The results clearly indicate that increase in the temperature from 30 to 37°C, production was increased and it was decreased with further increase in temperature. Therefore, 37°C was found optimum temperature for protease production by *Bl*M9 (Fig.4.1.1).

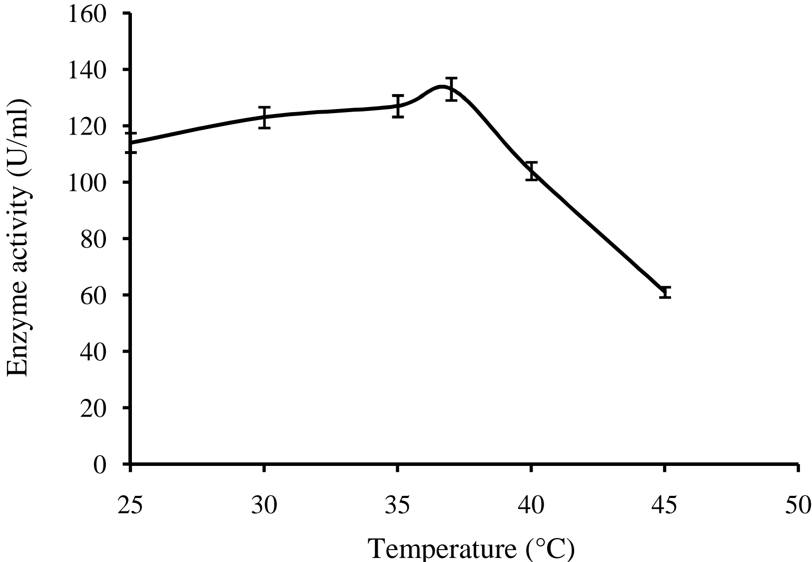

Fig. 4.1.1. Effect of temperatures on protease production by *Bl*M9 in shake flask

4.1.3.2. Effect of pH on protease production by *Bl*M9

The results described in fig. 4.1.2 showed that the optimum pH for production of protease by *Bl*M9 was 7.5 with highest enzyme activity 132 ± 4 U/ml at 37°C and 150 rpm. The decrease in enzyme production was observed clearly by decrease or increase in pH beyond the optimum level.

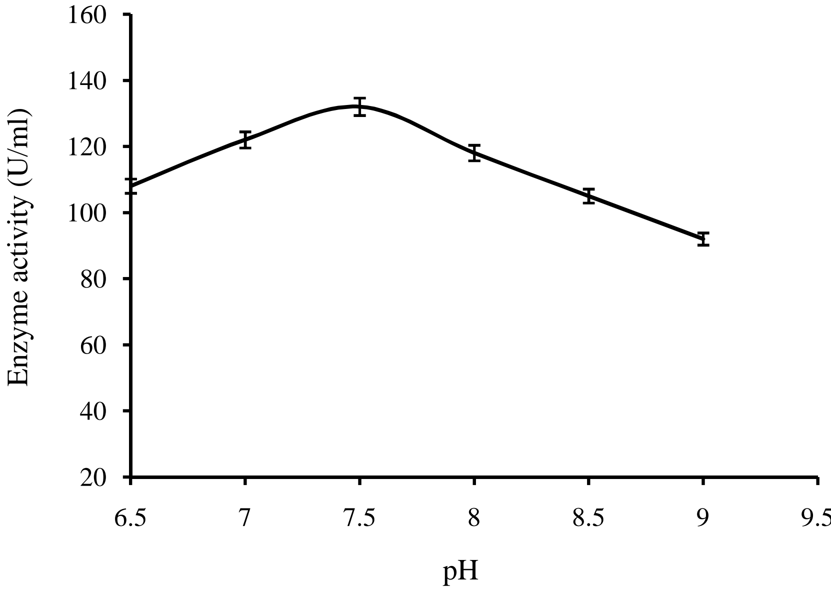

Fig. 4.1.2. Effect of pH on protease production by *Bl*M9 in shake flask

4.1.3.3. Effect of inoculum size and age on protease production by *Bl*M9

Size and age of inoculum are important factors affecting the cell growth and product formation in fermentation. To determine the optimal size of inoculum the fermentation experiments were carried out for 48 hrs at pH 7.5, 37°C and 150 rpm. The inoculum size of 5% (v/v) (10^7 CFU/ml viable cell concentration) was found to be optimal (Fig. 4.1.3 a) for protease production and a decrease in protease activity was observed due to lower or higher percentage of inoculum sizes.

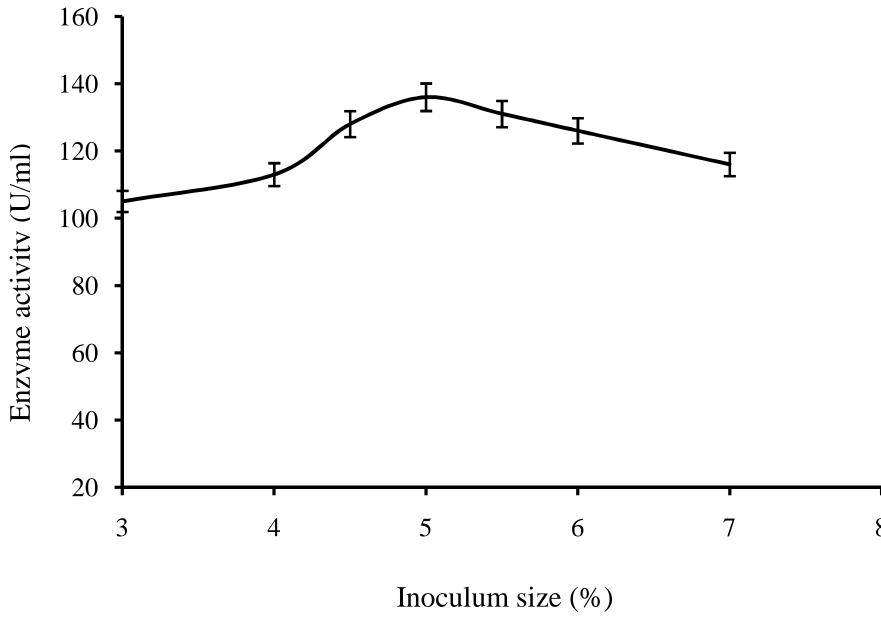

Fig. 4.1.3 (a). Effect of inoculum size on protease production by *Bl*M9 in shake flask

To determine the optimal age of inoculum fermentation experiments were carried out using 5% (v/v) inoculum of different ages (8 to 24 hrs). The results showed that the inoculum of 16 hrs had maximum protease production 135 ± 4 (Fig.4.1.3 b). Further increase in age of inoculum was found to have negative effect on protease production by *Bl*M9.

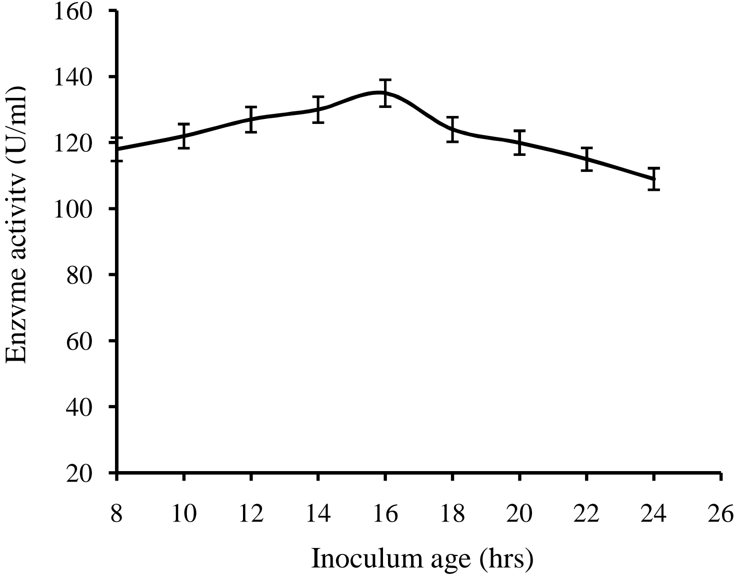

Fig. 4.1.3 (b). Effect of inoculum age on protease production by *Bl*M9 in shake flask

4.1.3.4. Optimization of fermentation conditions in Bioreactor

4.1.3.4.1. Effect of aeration rates on enzyme production

The effects of aeration rates from 0.5 vvm to 2.5 vvm on protease production during fermentation of *Bl*M9 in 7 L bioreactor with 3.5 L working volume at constant agitation speed of 300 rpm is shown in fig. 4.1.4. Maximum alkaline protease activity (208 U/ml) was observed at 1 vvm aeration rate after 34 hrs incubation where as 170, 180 and 150 U/ml enzyme activities were found at 0.5, 1.5 and 2 vvm aeration, respectively. Since aeration rate of 1 vvm produced maximum yield of alkaline protease by *Bl*M9, it was selected for the subsequent study.

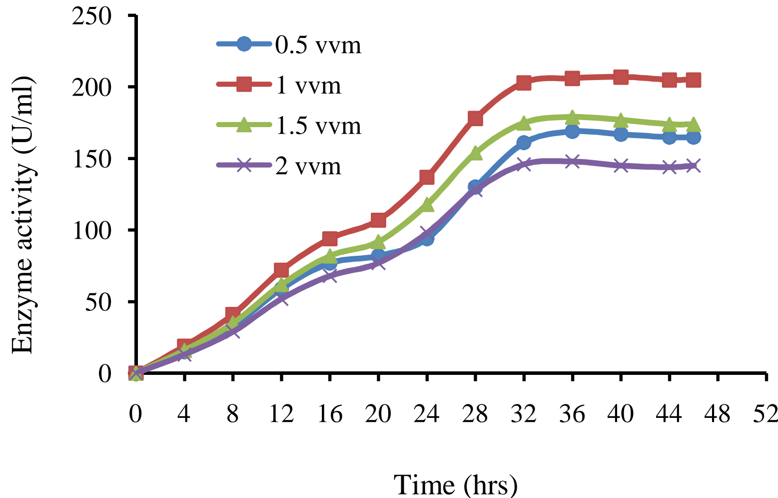

Fig. 4.1.4. Time course of protease production by *Bl*M9 in APPB medium at different aeration rate

4.1.3.4.2. Effect of agitation rates on enzyme production by *Bl*M9 in Bioreactor

Agitation rate is one of the indispensable parameter for proper oxygen transfer and homogeneous mixing of the nutrients in fermentation system. Therefore, the effects of five different agitation rates on enzyme yield are studied at constant aeration of 1 vvm (Fig. 4.1.5). However, the optimal production of protease yield (210) was obtained at agitation speed of 300 rpm. The higher agitations might reduce the protease production due to sheer stress and heterogeneous mixing effects. A decrease in enzyme production was also observed below 300 rpm resulting from incomplete mixing and/or oxygen transfer resistance at low agitation rates.

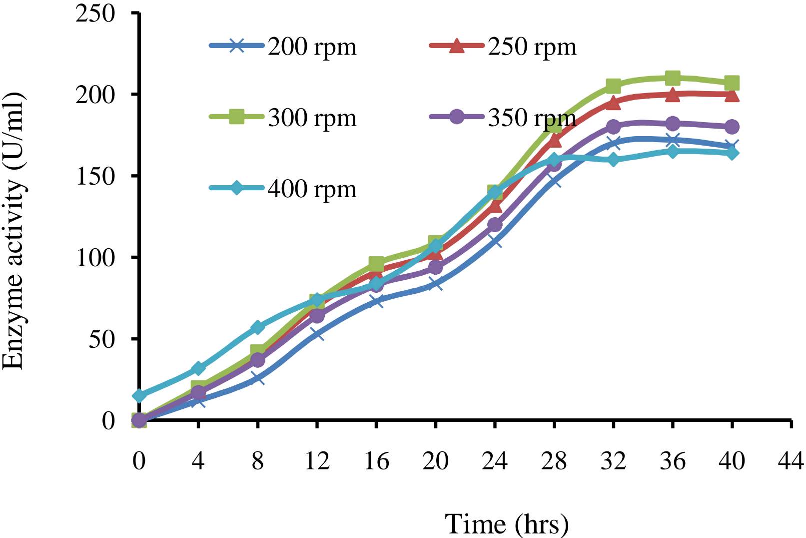

Fig. 4.1.5. Time course of protease production by *Bl*M9 in APPB medium at different agitation rate

4.1.3.4.3. Time course for extra cellular protein and protease production by *Bl*M9 in 7 L bioreactor at agitation 300 rpm and aeration 1.0 vvm

In bioreactor, the lag phase of the *Bl*M9 was found to be very short indicating that the organism has the potentiality to adapt with the condition of bioreactor cultivation which is very important to produce protease in large-scale. When cell concentration increased than dissolved oxygen level decreased. After 4 hrs the dissolved oxygen level decreased at 50% level when the cell concentration found 3.7 Log CFU/ml. After 6 hrs the dissolved oxygen level reached below 30 % level (Fig. 4.1.6.a)

The fig. 4.1.6.a also indicated that the consumption of the glucose was directly related to the growth of the *Bl*M9. From this figure the specific growth rate and substrate utilization rate was calculated (Described later).

From the bioreactor study it was found that highest extra-cellular protein concentration 0.73 mg/ml and highest protease activity 215 U/ml were achieved at stationary phase after 34 hrs (Fig. 4.1.6.b)

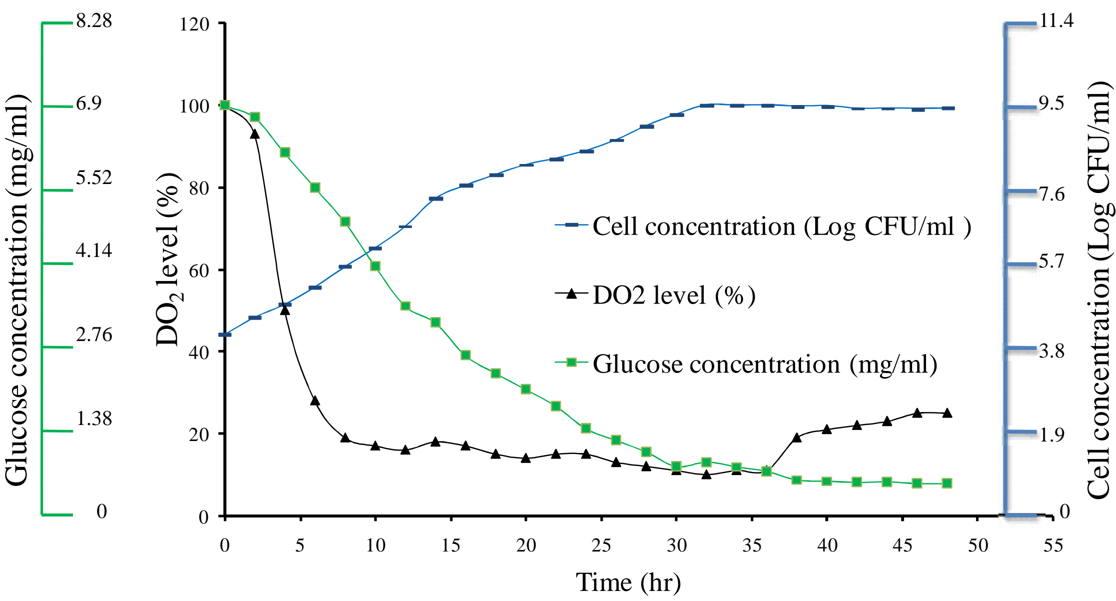

Fig. 4.1.6 (a). Time course of *Bl*M9 growth corresponding to DO_2 level and glucose concentration in APPB medium in 7 L bioreactor

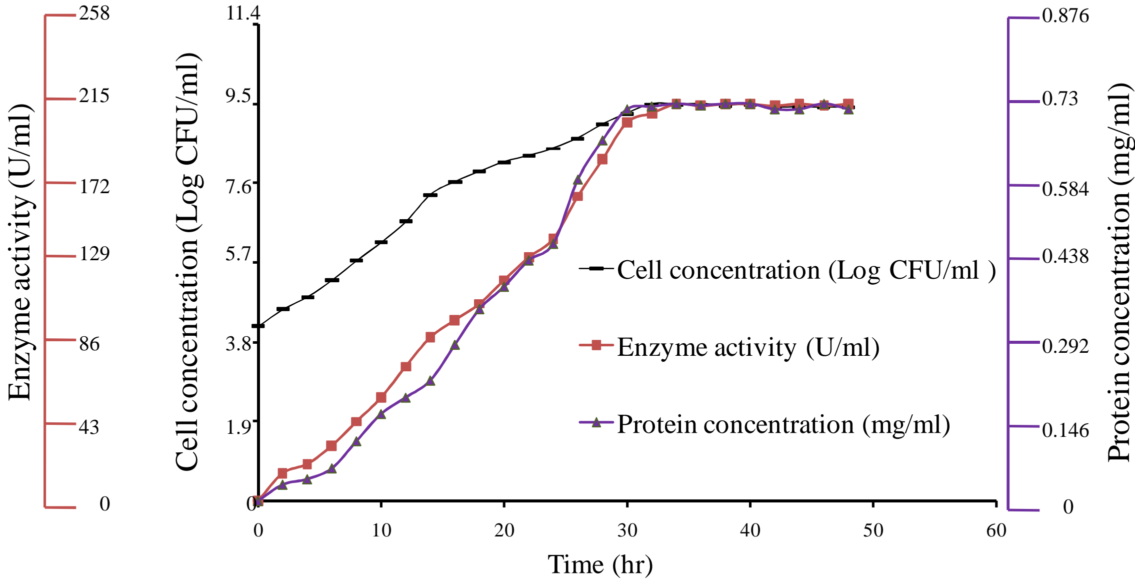

Fig. 4.1.6 (b). Time course of extracellular protein and protease production by *Bl*M9 in APPB medium in 7 L bioreactor

4.1.3.4.4. Determination of specific growth rate

Mathematically, the exponential growth of *Bl*M9 can be described by two methods; one is related to biomass (x) and the other to cell numbers (N). For cell biomass, growth can be considered as an autocatalytic reaction. Therefore, the rate of growth is dependent on the biomass concentration (Maier, 2008). This can be described as follows:

Rate of change of biomass is: $dx/dt = \mu x$ …………………………………………………. 1

Where, x = concentration of biomass (g/L), μ = specific growth rate (per hour) and t = time (h).

Equation 1 can also be rearranged to estimate the specific growth rate (μ):

$\mu = 1/x * dx/dt$ ……………………………………2

During any period of true exponential growth, equation 1 can be integrated to provide the following equation:

$x_t = x_o e^{\mu t}$ ……………………………………..3

Where, x_t = biomass concentration after time t, x_o = biomass concentration at the start of exponential growth, and e = base of the natural logarithm.

Taking natural logarithms, $\log_e$ (ln), gives, $\ln x_t = \ln x_o + \mu t$ ………………….4

This equation is of the form $y = c$ (intercept on y axis) + μx where, μ= gradient, which is the general equation for a straight-line graph. For cells in exponential phase, a plot of natural log of biomass concentration against time, a semilog plot, should yield a straight line with the slope (gradient) equal to μ

Or

$\mu = (\ln x_t - \ln x_o)/t$

$\mu = 2.303(\log x_t - \log x_o)/t$

According to the relationship between biomass and OD the equation can be expressed as following (Widdel, 2010):

$$\mu = \frac{\ln OD2 - \ln OD1}{(t2 - t1)}$$

The raw data of substrate utilization rates, growth rates and specific growth rates at different stage of fermentation by *Bl*M9 in APPB medium for production of protease has been shown in table 4.1.1.

Table 4.1.1: Substrate utilization rates, growth rates and specific growth rates (derived from cell concentration and OD of the culture)

T	Log OD	μ (from OD)	Log C	μ (from C)	lnx	μ (From x)	-ds/dt	dx/dt
0	-0.173		4.2		-0.679			
2	-0.133	0.0455	4.6	0.46	-0.587	0.046	0.1	0.0243
4	-0.106	0.0316	4.9	0.345	-0.5244	0.0313	0.3	0.0181
6	-0.072	0.0392	5.3	0.46	-0.4462	0.0391	0.29	0.024
8	-0.035	0.0424	5.766	0.536	-0.36	0.0431	0.29	0.0285
10	-0.003	0.0365	6.202	0.502	-0.289	0.0355	0.37	0.026
12	0.029	0.0383	6.697	0.57	-0.211	0.039	0.335	0.03
14	0.069	0.0459	7.343	0.74	-0.1199	0.0455	0.14	0.039
16	0.087	0.0204	7.742	0.459	-0.079	0.0204	0.275	0.0185
18	0.101	0.016	7.894	0.175	-0.047	0.016	0.15	0.015
20	0.113	0.014	8.122	0.262	-0.018	0.0145	0.135	0.014
22	0.121	0.009	8.174	0.0598	0	0.009	0.14	0.009
24	0.13	0.0104	8.436	0.0598	0.02	0.01	0.19	0.0105
26	0.143	0.0145	8.6	0.188	0.049	0.0145	0.0975	0.015
28	0.159	0.0183	9.021	0.484	0.086	0.0185	0.0975	0.0195
30	0.171	0.0143	9.284	0.302	0.1151	0.01455	0.122	0.016
32	0.181	0.0116	9.5	0.248	0.138	0.0114	0.037	0.0135
34	0.181		9.493		0.138		0.04	
36	0.181		9.5		0.138		0.0385	
38	0.181		9.473		0.138		0.0715	
40	0.18		9.48		0.138		0.01	
42	0.181		9.42		0.138		0.01	
44	0.181		9.424		0.138		0.005	
46	0.181		9.419		0.138		0.015	
48	0.181		9.424		0.138		0.01	

T-time (hr), OD-Absorbance at 600 nm of cell growth, C- cell concentration (CFU/ml), x-biomass (mg/ml), S-substrate concentration (mg/ml)

The graph is plotted for cell OD_{600} against time, the product is a curve with a constantly increasing slope (Fig. 4.1.7).

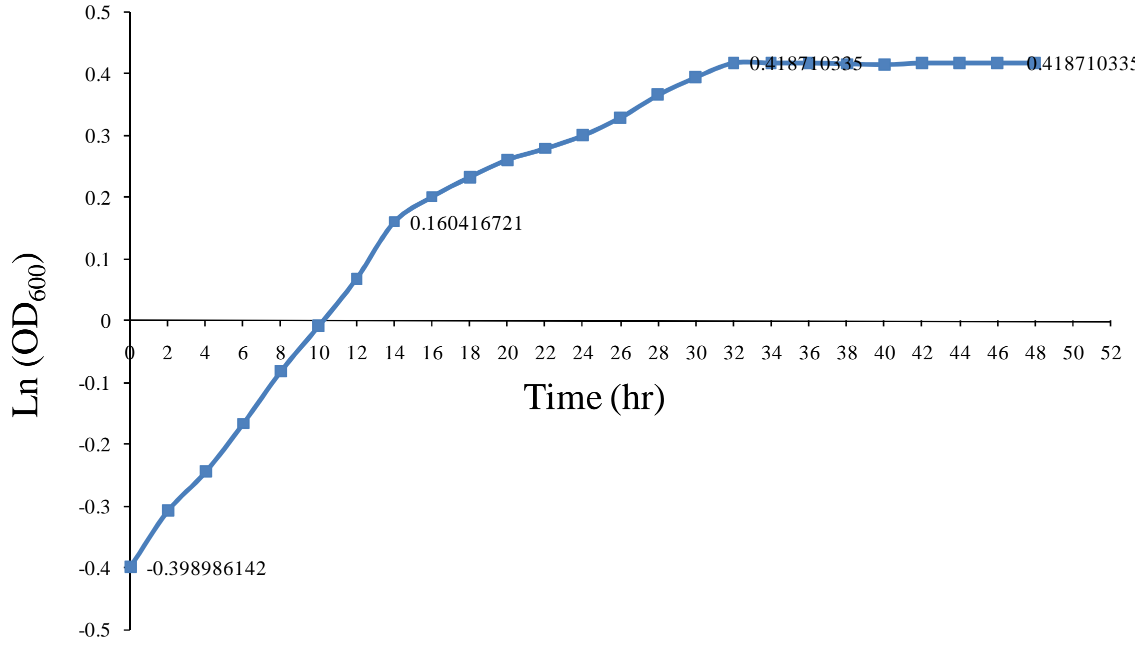

Fig. 4.1.7. Ln (OD_{600}) Vs time (hr) of the growth of *Bl*M9 in APPB medium

From the fig. 4.1.7, the specific growth rate can be calculated using following equation:

$$\mu = \frac{LnOD2 - lnOD1}{T2 - T1}$$

Table 4.1.2. Specific growth rate (μ) of *Bl*M9 in APPB medium

T1	T2	Ln (OD1)	Ln (OD2)	μ	Maximum μ
0	14	-0.398	0.1604	0.0398	
14	24	0.1604	0.3008	0.014	0.0398
24	32	0.3008	0.418	0.0146	

From the study it was found that the maximum specific growth rate (μ) of the *Bl*M9 was found 0.0398 in APBB medium. In addition, it was also found that the optimum temperature, pH, aeration and agitation for the maximum production of protease by *Bl*M9 in APPB medium were 37°C, 7.5, 1 vvm and 300 rpm with the 5% inoculum of 16 hrs old culture. These optimum levels of parameters were used in the subsequent study to develop a cost effective medium for production of protease by *Bl*M9.

Chapter 4.2. Development of a cost effective medium by one variable-at-a-time method

4.2.1. Introduction

About 30- 40% of the cost of industrial enzymes depends on the cost of the growth medium (Joo *et al*. 2003). Generally, proteases production from microorganisms is constitutive or partially inducible in nature. Under most culture conditions, *Bacillus* species produce extracellular proteases during post-exponential or stationary phases. Extracellular protease production in microorganisms is strongly influenced by media components, e.g. variation in C/N ratio, presence of some easily metabolizable sugars, such as glucose (Beg *et al.* 2002), and metal ions (Varela *et al*. 1996). Protease synthesis is also greatly affected by rapidly metabolizable nitrogen sources in the medium. Therefore it is necessary to optimize the concentration of the ingredients in the medium for production of protease. The 'one variable-at-a-time' approach is the most frequently used operation in biotechnology to obtain maximum cell density, high yields of the desired metabolic product, or enzyme levels in a microbial system. In this experiment the 'one variable-at-a-time' method was used to optimize the concentration of medium ingredients specially carbon and nitrogen sources.

4.2.2. Materials and methods

4.2.2.1. Production of inoculum for shake flask fermentation: Inoculum was produced according to the method described in section 4.1.2.6.

4.2.2.2. Optimization of the concentration of the carbon and nitrogen source for maximum protease production by *Bl*M9 in shake culture

Initially, *Bacillus licheniformis* MZK05M9 was cultivated in the Glucose Soybean meal medium containing Glucose 10.0 g/l, Soybean meal 10.0 g/l, K_2HPO_4 3.0 g/l, $MgSO_4$ 0.5 g/l, $CaCl_2$ 0.5 g/l and NaCl 0.5 g/l at initial pH 7.5, temperature 37°C and rpm 150 in an orbital shaker. To make the medium more cost-effective the glucose was aimed to replace

by molasses. Initially, the traditional one variable-at-a-time method was employed for selection of appropriate concentration of Molasses (carbon source) and Soybean meal (Nitrogen source) for maximum production of protease by *Bl*M9.

To optimize the concentration of molasses the media were prepared (100 ml in each 250 ml Erlenmeyer flasks) with various concentrations of molasses (5%, 4% 3%, 2%, 1%, 0.5% and 0.25%) instead of glucose. To optimize the concentration of soybean meal the media were prepared (100 ml in each 250 ml Erlenmeyer flasks) with various concentration of soybean meal (5%, 4% 3%, 2%, 1%, 0.5% and 0.25%). The pH of the media was adjusted to 7.5 with 0.1N NaOH and 0.1 N HCl. After sterilization by autoclaving the media were cooled and inoculated with the 16 hrs old culture and incubated at 37°C and 150 rpm for 48 hrs in an orbital shaker.

The experiments were carried out systematically in such a way that the concentration of one ingredient optimized in one experiment was maintained at its optimum level in the subsequent experiments.

4.2.2.3. Production of the protease enzyme by *Bl*M9 in 7 L bench-top Bioreactor

4.2.2.3.1. Inoculum preparation for fermentation in bioreactor

The composition of the inoculum medium for bioreactor cultivation was as same as the medium used in the bioreactor. After sterilization by autoclaving the inoculum medium (175 ml) was cooled and inoculated with 16 hrs old culture (preparation of the culture described in section 4.1.2.6) at 5% level. After inoculation, the inoculum medium for bioreactor was incubated for 16 hrs at 37°C and 150 rpm in an orbital shaker.

4.2.2.3.2. Preparation of bioreactor: The preparation of bioreactor described in the section 4.1.2.8.2**.**

4.2.2.3.3. Fermentation experiments in bioreactor

The fermentation experiments for the production of extracellular alkaline protease by *Bl*M9 were carried out in a laboratory scale 7.5 L batch bioreactor (Bioflo 110, New Brunswick Scientific, USA) with a working volume of 3.5 L. Controls were performed at different levels for different parameters. The vegetative bacterial inoculum was transferred to the sterilized optimized medium at a level of 5 % (v/v) based on total working volume of the bioreactor. The batch was carried out at an incubation temperature

of 37°C and initial pH 7.5. The dissolved oxygen level in the culture was controlled by cascading mode maintained by both agitation and aeration where high and low limit of agitation were 300 rpm and 150 rpm respectively and high and low limit of aeration were 3.5 SLPM and 1 SLPM, respectively. Sampling were performed 02 hrs intervals and the viable cell count, protein concentration and protease enzyme activity were estimated according to the methods describe previously in sections 4.1.2.9., 4.1.2.12. and 3.2.9.4, respectively.

4.2.3. Results

Initially, *Bacillus licheniformis* MZK05M9 was cultivated in the Glucose Soybean meal medium (GSMM) containing Glucose 10.0 g/l, Soybean meal 10.0 g/l, K_2HPO_4 3.0 g/l, $MgSO_4$ 0.5 g/l, $CaCl_2$ 0.5 g/l and NaCl 0.5 g/l at initial pH 7.5, temperature 37°C and rpm 150 in an orbital shaker. The *Bl*M9 produced 325±5 U/ml in this medium which is 2.5 fold higher than that in APPB medium (Fig. 4.2.1) in shake culture level.

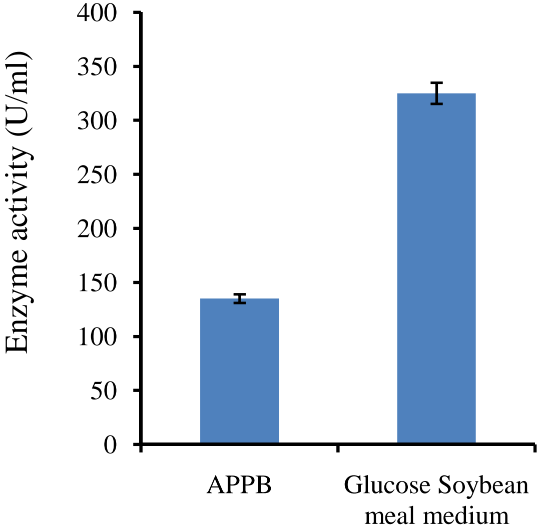

Fig. 4.2.1. Comparison of enzyme titre produced by *Bl*M9 in APPB and GSMM medium

To make the GSMM medium cost-effective the glucose was aimed to replace by molasses. Therefore, the concentration of the molasses instead of glucose was optimized. The traditional one variable-at-a-time method was employed for selection of appropriate concentration of molasses (carbon source) and soybean meal (Nitrogen source) for maximum production of protease by *Bl*M9. The experiments were carried out

systematically in such a way that the concentration of one ingredient optimized in one experiment was maintained at its optimum level in the subsequent experiments.

4.2.3.1. Effect of concentration of molasses in protease production by *Bl*M9

Among the various concentration of molasses (5%, 4% 3%, 2%, 1%, 0.5% and 0.25%) 0.5% demonstrated the highest enzyme activity 420 ± 5 U/ml (Fig. 4.2.2).

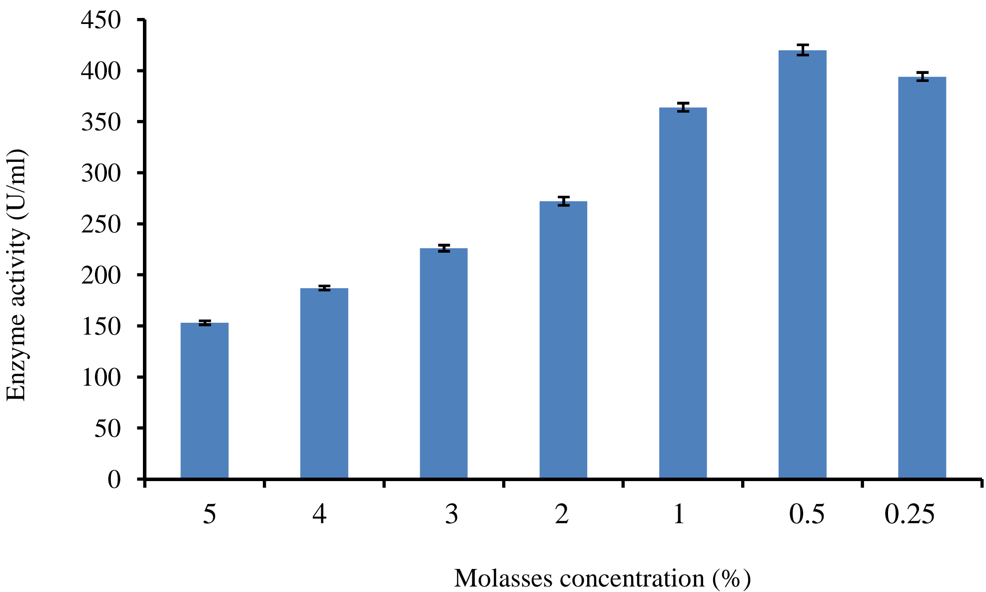

Fig. 4.2.2. Effect of molasses concentration in protease production by *Bl*M9 in shake flask

4.2.3.2. Effect of concentration of soybean meal in protease production by *Bl*M9

Among the various concentration of Soybean meal (5%, 4% 3%, 2%, 1% and 0.5%) 1% supported the highest enzyme activity 425 ± 5 U/ml (Fig. 4.2.3).

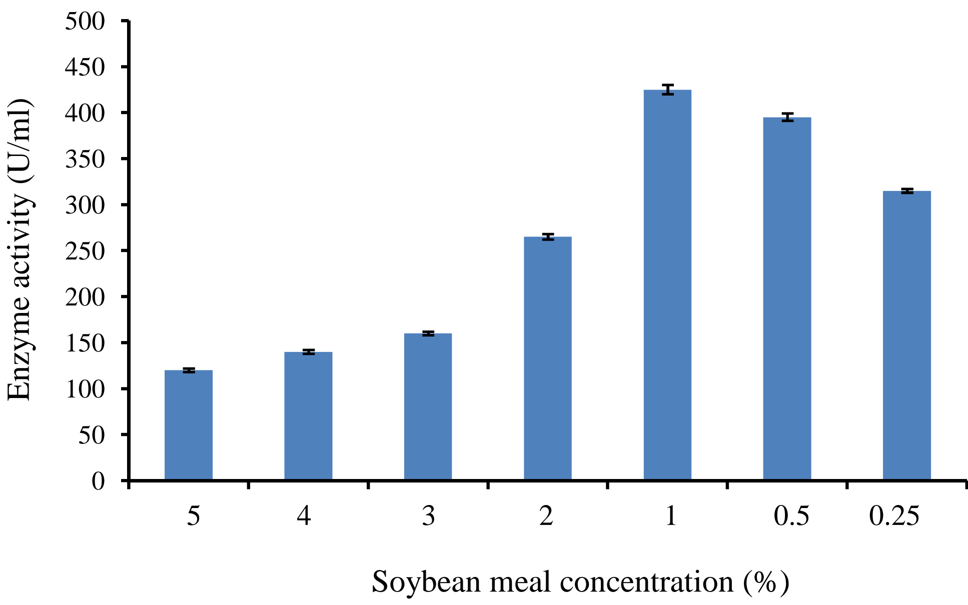

Fig. 4.2.3. Effect of Soybean meal concentration in protease production by *Bl*M9 in shake flask

Therefore finally the medium (Molasses Soybean meal medium- MSMM) containing molasses 5 g/l, soybean meal 10 g/l, K_2HPO_4 3.0 g/l, $MgSO_4$ 0.5 g/l, $CaCl_2$ 0.5 g/l and NaCl 0.5 g/l was selected for bioreactor study in 7.0 L bench-top fermentor.

4.2.3.3. Bioreactor study

The dissolved oxygen levels in the culture were controlled by cascading mode maintained by both agitation and aeration where high and low limit of agitation were 300 rpm and 150 rpm respectively and high and low limit of aeration were 3.5 SLPM and 1 SLPM respectively. In these experiments, the cascading mode which was aimed to control the dissolved oxygen level at 30% supported the enzyme productivity most (Table 4.2.1). To control dissolved oxygen level more than 30% required more vigorous agitation and aeration which might have negative effect on enzyme production. On the other hand, in lower dissolved oxygen level it was required slower aeration and agitation rate which might not mix the nutrient properly or did not supply the appropriate amount of oxygen. From the bioreactor study it was found that the highest protein concentration (1.0 mg/ml) and protease activity (608 U/ml) were found after 28 hrs at early stationery phase (Fig. 4.2.4). The highest cell concentration was found 9.61 log CFU/ml. This enzyme was

further partially purified, characterized (described later in Chapter 5) and applied for bating step in leather processing (described later in Chapter 6).

Table 4.2.1. Productivity of *Bl*M9 in different dissolved oxygen level in bioreactor

DO (%)	Highest cell count (Log CFU/ml)	Highest enzyme activity (U/ml)	Productivity (U/L/hr)
40	9.58	580 U/ml	18125
30	9.61	608U/ml	19000
20	9.54	575 U/ml	17968

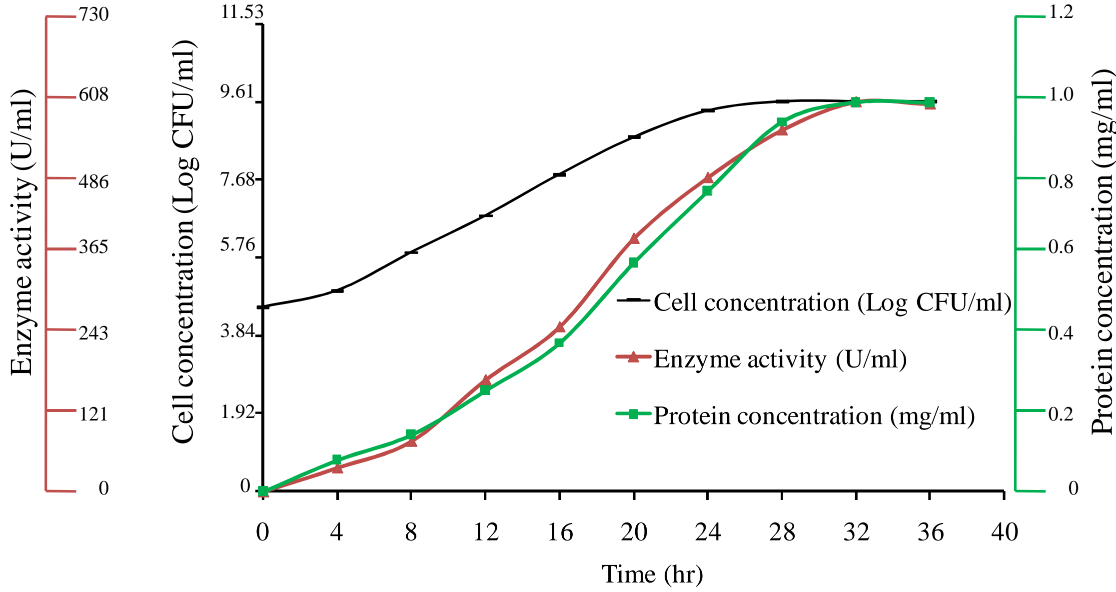

Fig. 4.2.4. Time course for cell concentration, protease activity and extra cellular protein production by *Bl*M9 in Molasses Soybean meal medium with cascading mode set to control dissolved oxygen level at 30%

Chapter 4.3. Statistical optimization of the medium composition for overproduction of the enzyme by *Bl*M9

4.3.1. Introduction

As a consequence of the research described earlier in chapter 4.1 and chapter 4.2 , the further optimization of the production medium was required for overproduction of the alkaline protease to make the production industrially as well as commercially viable. For optimization of the medium the statistical methodologies are generally preferred due to their recognized advantages of their use (Srinivas *et al*., 1994; Carvalho *et al*., 1997; Li *et al*., 2007; Xiao *et al.,* 2007). The Plackett–Burman factorial designs allow to screen of main factors from a large number of process variables, and these designs are thus quite useful in preliminary studies in which the principal objective is to select variables that can be fixed or eliminated in further optimization processes. In addition, response surface methodology (RSM) is an efficient strategic experimental tool by which the optimal conditions of a multivariable system may be determined. A number of reports have already been published regarding the role of a variety of carbon and nitrogen sources on protease production. The complex carbon and nitrogen source facilitate the large-scale protease production by providing some trace elements and vitamins. The principal objective of this study was to screen for the significant carbon and nitrogen sources and further to optimize the concentration level of the screened ingredients using statistical methodologies for alkaline protease production by employing the mutant *Bacillus licheniformis* MZK05M9 (*Bl*M9). These results are described in this chapter.

4.3.2. Materials and methods

4.3.2.1. Production of inoculum: Described in section 4.1.2.6.

4.3.2.2. Fermentation and separation of culture filtrates

5 ml of inoculum (10^7 CFU/ml) was transferred to 100 ml of fermentation medium containing (g/l): soybean meal, 10; molasses, 5.0; K_2HPO_4, 3.0; $MgSO_4.7H_2O$, 0.5; NaCl,

0.5 and $CaCl_2.2H_2O$, 0.5 in a 250 ml Erlenmeyer flask and incubated in an orbital shaker for 48 hours, at 37°C and 150 rpm. Samples were collected at certain intervals and centrifuged at 6000 rpm for 10 minutes. The cell free supernatant was used for enzyme assay.

4.3.2.3. Determination of enzyme activity: Described in section 3.2.9.4.

4.3.2.4. Selection of nutrients by statistical Plackett-Burman design (PBD)

In the present study, PBD was used for screening of major nutrients i.e. carbon source, nitrogen source and some salts those have effects on production of alkaline proteases. Since the complex carbon and nitrogen sources facilitate enzyme production by providing necessary growth factors, vitamins, and trace elements (Bhunia *et al.*, 2012), the locally available cost-effective complex carbon and nitrogen sources such as rice bran (A), wheat bran (B), mustard seed meal (Oil cake) (C), molasses (D) and Soybean meal (E) were studied. Salts such as $CaCl_2.2H_2O$ (F), $MgSO_4.7H_2O$ (G), K_2HPO_4 (H) and NaCl (J) were considered to find out their effects on protease production. As of Plackett-Burman factorial design, each factor was examined on two levels: -1 for a low level and +1 for a high level (Table 4.3.1).

Table 4.3.1. Low level and high level concentration of the variables for Plackett-Burman factorial design

	Ingredients	(1)High (%)	(-1)Low (%)
A	Rice bran	2	0.1
B	Wheat bran	2	0.1
C	Oil cake	2	0.1
D	Molasses	2	0.1
E	Soybean meal	2	0.1
F	$CaCl_2.2H_2O$	0.2	0.01
G	$MgSO_4.7H_2O$	0.2	0.01
H	K_2HPO_4	0.5	0.03
J	NaCl	0.2	0.01

The Plackett-Burman experimental design was based on a first-order model. The factors were screened linearly using the approach: Y= o + i xi (i=l-k). In this equation Y is the target function, o and i are the intercept and regression coefficient, respectively.

4.3.2.5. Optimization of the concentration of the selected ingredients by response surface methodology

The next step in the formulation of the medium was to determine the optimum concentration levels of the ingredients for protease production by *Bl*M9. For this purpose, the Response Surface Methodology (RSM) based on Central Composite Design (CCD) was adopted for the augmentation of alkaline protease production. In this study, the independent variables selected by PBD such as soybean meal as nitrogen source, molasses as carbon source, K_2HPO_4, $MgSO_4.7H_2O$ and NaCl were coded as A, B, C, D, and E respectively. Thus, the second-order polynomial equation can be represented as follows:

$$Y = \ o + {}_{1}A + {}_{2}B + {}_{3}C + {}_{4}D + {}_{5}E + {}_{11}A^2 + {}_{22}B^2 + {}_{33}C^2 + {}_{44}D^2 + {}_{55}E^2 + {}_{12}AB + {}_{13}AC + {}_{14}AD + {}_{15}AE + {}_{23}BC + {}_{24}BD + {}_{25}BE + {}_{34}CD + {}_{35}CE + {}_{45}DE.$$

4.3.2.6. Fermentation experiments in Bioreactor

The fermentation experiments for the production of extracellular alkaline protease by *Bl*M9 were carried out in a laboratory scale 7.5 L batch bioreactor (Bioflo 110, New Brunswick Scientific, USA) with a working volume of 3.5 L. The vegetative bacterial inoculum was transferred to the statistically optimized medium at a level of 5 % (v/v) based on total working volume. The batch was carried out at temperature of 37°C and initial pH 7.5. The dissolved oxygen levels in the culture were controlled by cascading mode maintained by both agitation and aeration where high and low limit of agitation were 300 rpm and 150 rpm respectively and high and low limit of aeration were 3.5 SLPM and 1 SLPM, respectively.

4.3.3. Results

4.3.3.1. Screening of significant nutrients by Plackett-Burman (PB) method

PB design was adopted to select the ingredients with most positive effects on the protease production by *Bl*M9. The *P* value is the probability which serves as a tool for checking the significance of each of the parameters. A low *P*-value indicates a "real" or significant effect. In our study, the *P* value for the model was 0.000 indicating that the model was significant. After 24 set of experiments (Table 4.3.2) designed by Minitab version 17 the

pareto chart (Fig.4.3.1) showed that the Soybean meal demonstrated the most positive effect on protease production. The rank order of the effect of the ingredients is: Soybean meal > Molasses > NaCl > Mustard seed meal > K_2HPO_4 > $MgSO_4.7H_2O$. On the other hand $CaCl_2.2H_2O$, Rice bran, Wheat bran had diminutive effect on the enzyme production. Therefore the soybean as nitrogen source, molasses as carbon source and other salts NaCl, K_2HPO_4 and $MgSO_4.7H_2O$ were selected for further optimization of the medium determining the concentration of the ingredients using Response Surface Methodology (RSM) based on Centre Composite Design (CCD).

Table 4.3.2. 24 set of fermentation experiments (in PB design) and their results (Y=enzyme activity U/ml)

RunOrder	A	B	C	D	E	F	G	H	J	Y
1	-1	-1	-1	-1	1	-1	1	-1	-1	53.8
2	1	1	-1	1	-1	1	1	1	1	647
3	-1	1	-1	1	1	1	1	1	-1	80.4
4	-1	-1	-1	1	-1	1	-1	-1	1	713.4
5	1	-1	-1	-1	-1	1	-1	1	-1	72.6
6	1	1	-1	-1	1	1	-1	1	-1	18.8
7	1	1	1	-1	-1	-1	-1	1	-1	58.2
8	-1	1	-1	-1	1	1	-1	-1	1	41.6
9	1	1	1	1	-1	-1	-1	-1	1	366.2
10	1	-1	-1	1	1	-1	1	-1	1	470.4
11	1	-1	1	-1	1	1	1	1	1	63.2
12	-1	-1	1	-1	1	-1	-1	1	1	84.2
13	-1	1	1	-1	-1	1	1	-1	1	293.2
14	1	1	1	1	1	-1	-1	-1	-1	15
15	1	1	-1	-1	-1	-1	1	-1	1	696
16	-1	-1	-1	-1	-1	-1	-1	-1	-1	397
17	1	-1	1	-1	-1	1	1	-1	-1	184.6
18	-1	1	1	-1	1	-1	1	1	1	95.8
19	-1	1	1	1	1	1	-1	-1	-1	300.4
20	-1	1	-1	1	-1	-1	1	1	-1	655.6
21	-1	-1	1	1	-1	1	-1	1	1	386.2
22	-1	-1	1	1	-1	-1	1	1	-1	218.8
23	1	-1	-1	1	1	-1	-1	1	1	273.4
24	1	-1	1	1	1	1	1	-1	-1	317.6

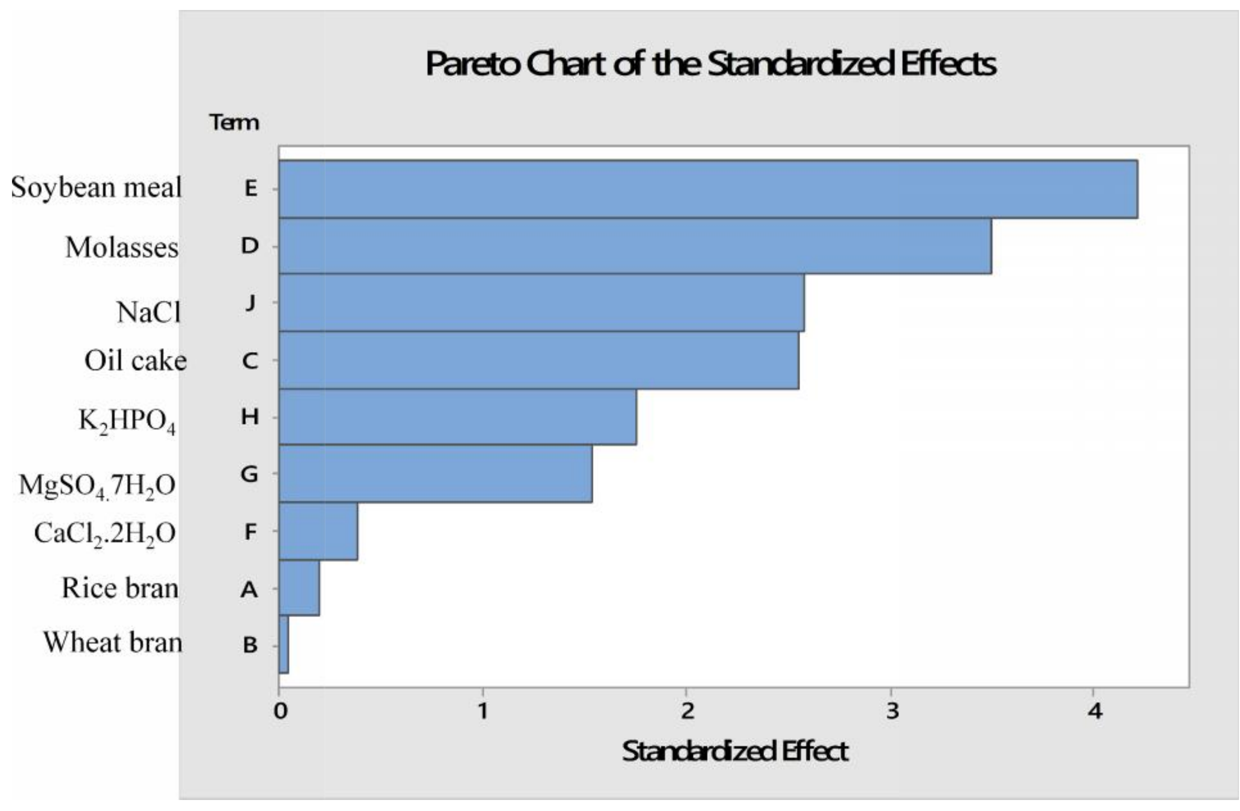

Fig. 4.3.1. Pareto chart showing the rank order of the effect of the ingredients on enzyme production by *Bl*M9

4.3.3.2. Optimization of the concentration of the selected ingredients by Response Surface methodology

4.3.3.2.1. Optimization of the Key Determinants: CCD was designed to study the effects of five selected independent variables, namely, soybean meal (*A*), Molasses (*B*), K_2HPO_4 (*C*), $MgSO_4.7H_2O$ (*D*), and NaCl (*E*). The designed CCD has been described in the Table 4.3.3.

Table 4.3.3. Experimental range and level of the independent variables

Independent variables		High (+1) (%)	Low(-1) (%)	Mean (0) (%)	(+) (%)	(-) (%)
Soybean meal	A	1.5	0.5	1	2	0
Molasses	B	1	0.5	0.75	1.25	0.25
K_2HPO_4	C	0.75	0.25	0.5	1	0
$MgSO_4.7H_2O$	D	0.1	0.05	0.075	0.125	0.025
NaCl	E	0.1	0.05	0.075	0.125	0.025

For five variables, = 2.

After 32 set of fermentation experimental run the enzyme yields were found which are given in Table 4.3.4.

Table 4.3.4. 32 set of fermentation experimental run and their enzyme yields

Std Order	Run Order	Variables					Protease activity (U/ml) Y
		A	B	C	D	E	
24	1	0	0	0	2	0	677
27	2	0	0	0	0	0	688
7	3	-1	1	1	-1	1	600
16	4	1	1	1	1	1	625
12	5	1	1	-1	1	-1	630
3	6	-1	1	-1	-1	-1	570
1	7	-1	-1	-1	-1	1	530
17	8	-2	0	0	0	0	570
21	9	0	0	-2	0	0	665
25	10	0	0	0	0	-2	655
10	11	1	-1	-1	1	1	600
14	12	1	-1	1	1	-1	580
8	13	1	1	1	-1	-1	590
6	14	1	-1	1	-1	1	580
23	15	0	0	0	-2	0	640
19	16	0	-2	0	0	0	505
29	17	0	0	0	0	0	690
31	18	0	0	0	0	0	691
13	19	-1	-1	1	1	1	565
15	20	-1	1	1	1	-1	620
9	21	-1	-1	-1	1	-1	510
11	22	-1	1	-1	1	1	650
26	23	0	0	0	0	2	692
20	24	0	2	0	0	0	510
2	25	1	-1	-1	-1	-1	655
22	26	0	0	2	0	0	645
18	27	2	0	0	0	0	575
28	28	0	0	0	0	0	691
30	29	0	0	0	0	0	692
5	30	-1	-1	1	-1	-1	540
32	31	0	0	0	0	0	685
4	32	1	1	-1	-1	1	560

Polynomial regression equation was found-

$$Y = 684.28 + 8.29 A + 10.21 B - 1.87 C + 7.46 D + 5.79 E - 41.78 A*A - 40.28 B*B - 17.78 C*C - 2.53 D*D + 1.22 E*E - 15.94 A*B - 5.31 A*C - 0.31 A*D - 15.31 A*E + 6.56 B*C + 19.06 B*D - 0.94 B*E + 3.44 C*D + 0.94 C*E + 8.44 D*E.$$

The Model *P*-value of 0.000 implies that the model is significant. Values of "prob > *F*" less than 0.0500 indicate model terms are significant. Seven model terms (B, A2, B2, C2, AB, AE, and BD) were found most significant. The correlation coefficient (R^2) of polynomial equation was found to be 0.9570 indicating that 95.70% of the variability in the response (protease production) could be explained by this model (Table 4.3.5).

Table 4.3.5. ANOVA for the experiments

DF	20
Adj SS	120205
Adj MS	6010.3
F-Value	12.24
P-Value	0.000
S	22.1573
R-sq	95.70%
R-sq(adj)	87.88%
R-sq(pred)	0.00%

The response surface plots of the RSM generated by the software Minitab version 17 showed the interactions of the ingredients. Fig. 4.3.2 shows the relative effects of soybean meal and molasses with strong degree of curvature of 3D surface where the optimum can be determined. The contour plot of the same equation also showed that maximum protease production was 685 U/ml when the levels of soybean meal and molasses were at their nearly central value of 1% and 0.75% respectively (Fig. 4.3.3).

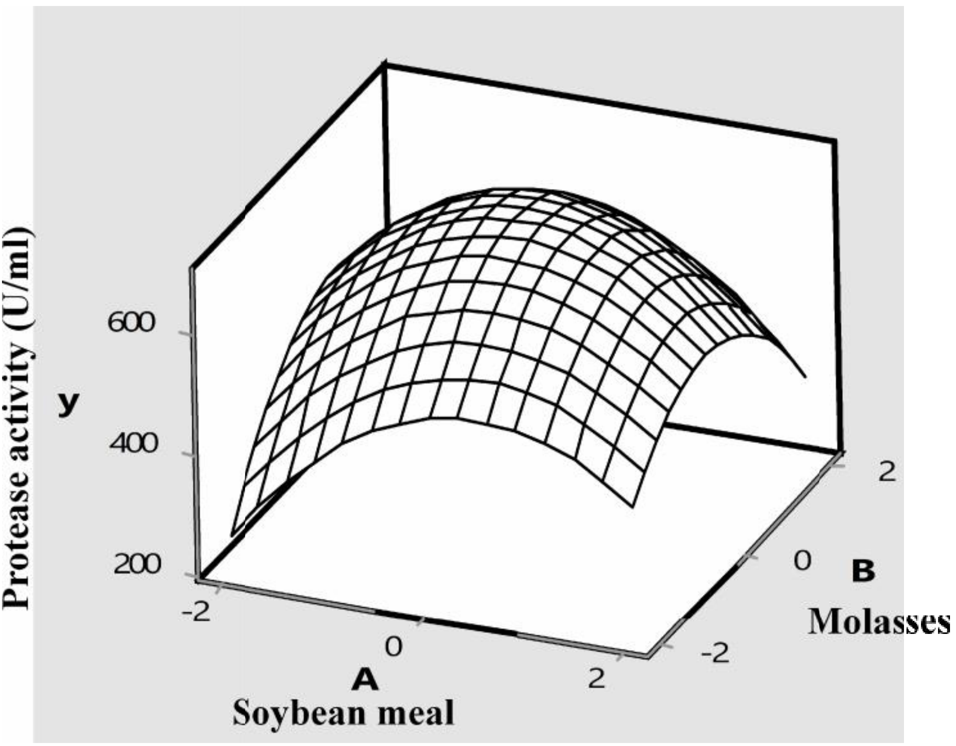

Fig. 4.3.2. Response surface plot of protease production by *Bl*M9 showing interaction between soybean meal and molasses

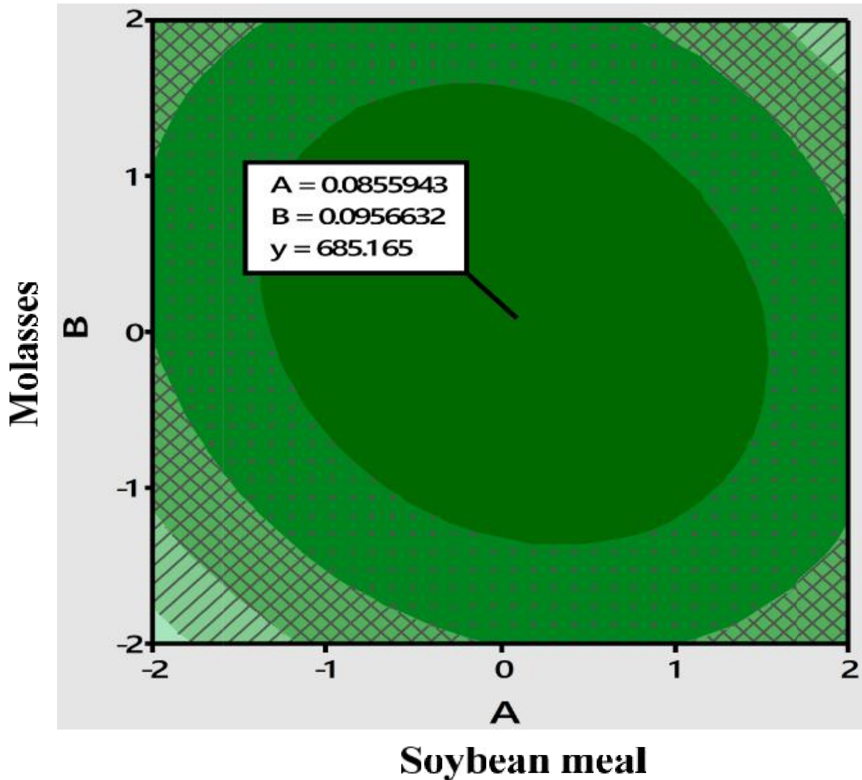

Fig. 4.3.3. Contour plot of protease production by *Bl*M9 showing interaction between soybean meal and molasses

4.3.3.3. Validation of the prediction of software at shake flask level (Validation of the model)

The software Minitab version 17 predicted that highest enzyme activity 761 U/ml would be found in the statistically optimized medium comprising of (g/L): Molasses (0.92%), Soybean Meal (0.79%), NaCl (0.125%), $MgSO_4.7H_2O$ (0.125%) and K_2HPO_4 (0.59%). However, the protease yield was found 765 U/ml in experimental fermentation which validated the prediction of the software.

4.3.3.4. Effect of mesh size of Soybean meal on enzyme production

The soybean meal was differentiated in different mesh size of 6.3 mm, 4.7 mm and 4 mm (Fig. 4.3.4) with a sieving machine. The mesh size 4.7 mm of soybean meal in the optimized medium supported the enzyme production most than those of other mesh sizes and original mixed sizes of soybean meal (Fig. 4.3.5).

Fig. 4.3.4. Different mesh size of Soybean meal. A= Mixed, B = 6.3 mm, C = 4.7 mm, D = 4.0 mm

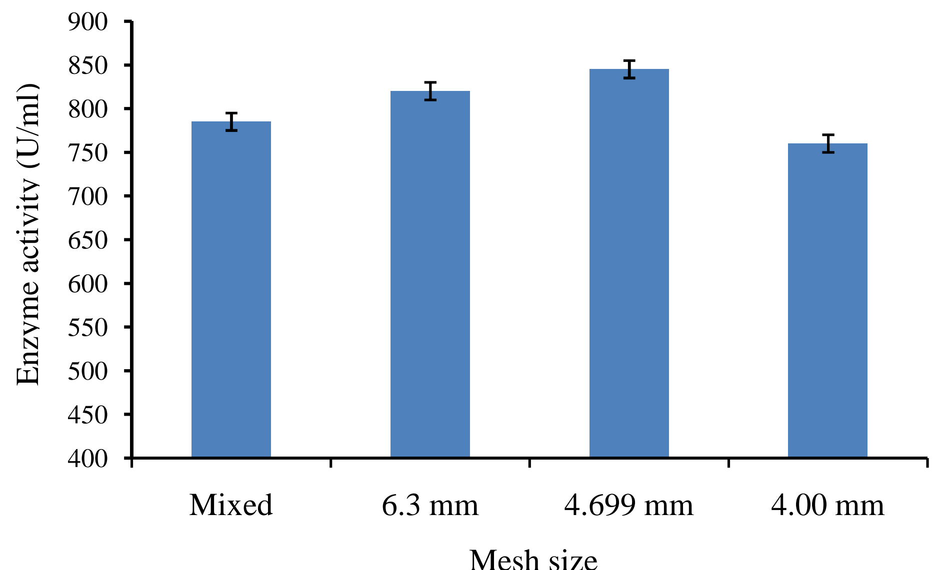

Fig. 4.3.5. Effect of mesh size of Soybean meal on protease production by *Bl*M9 in shake culture

4.3.3.5. Bioreactor cultivation of the *Bl*M9 for production of the protease in optimized medium

From the fermentation in 7 L bioreactor with 3.5 L working volume it was found that the highest protease activity 1020 ± 10 U/ml and extracellular protein concentration 0.87 mg/ml were achieved after 28 hrs at stationery phase (Fig. 4.3.7). A drastic fall of dissolved oxygen level was observed from the start to 12 hrs of the fermentation suggesting that the mutant *Bl*M9 has high growth rate and ability to reach exponential phase quickly under the fermentation conditions with the optimized medium.

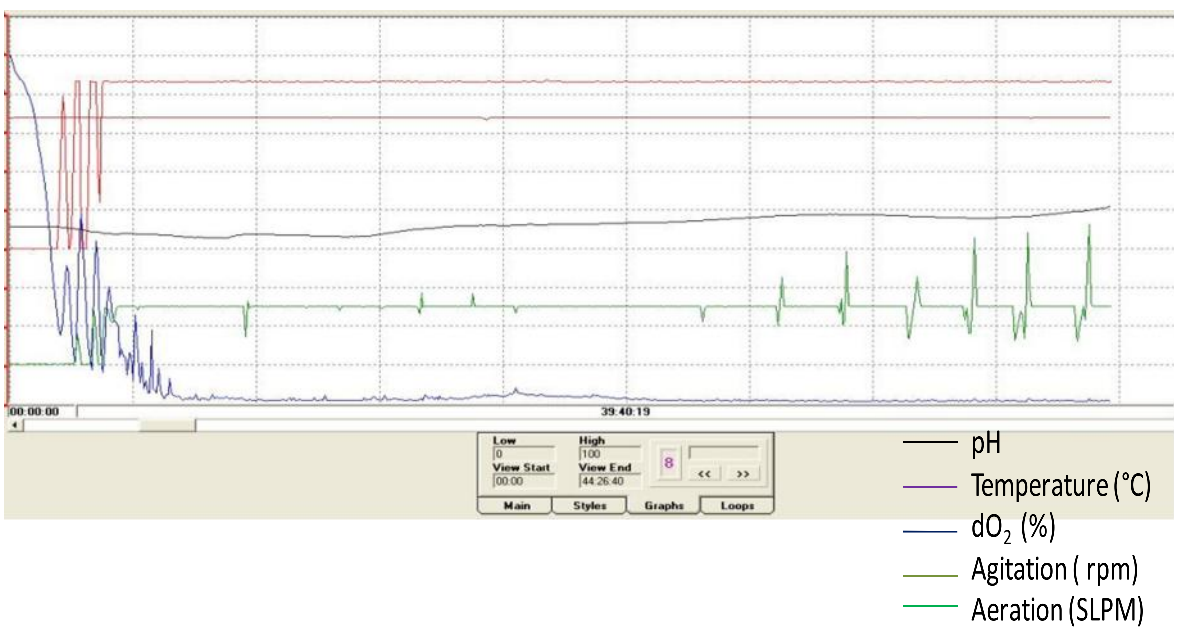

Fig. 4.3.6. Trend for different parameters collected automatically by Biocommand Plus software in the fermentation for production of protease by *Bl*M9 in statistically optimized medium

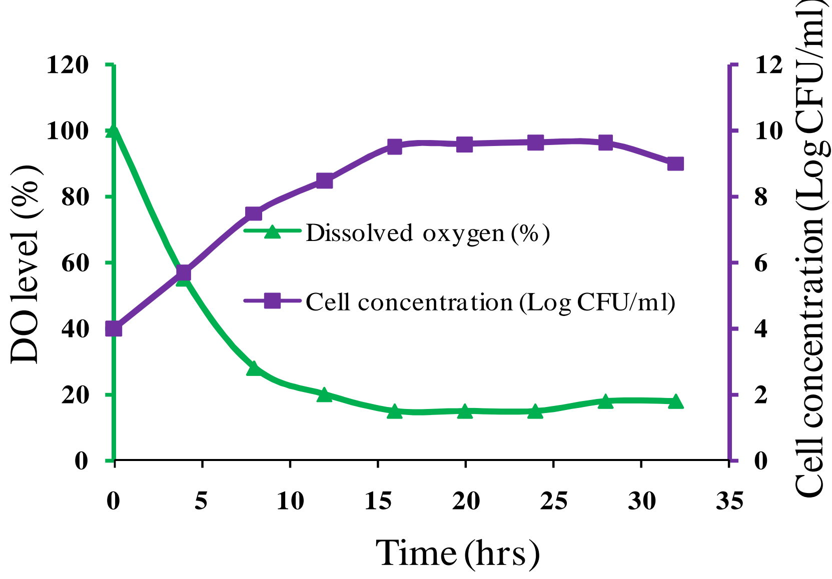

Fig. 4.3.7 (a). Time course for cell growth of *Bl*M9 in statistically optimized medium in 7 L bioreactor

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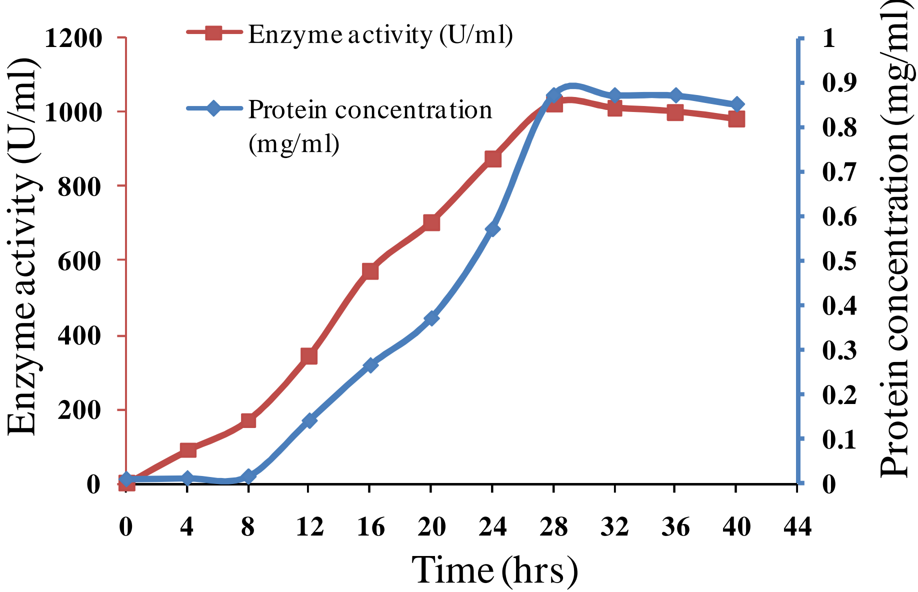
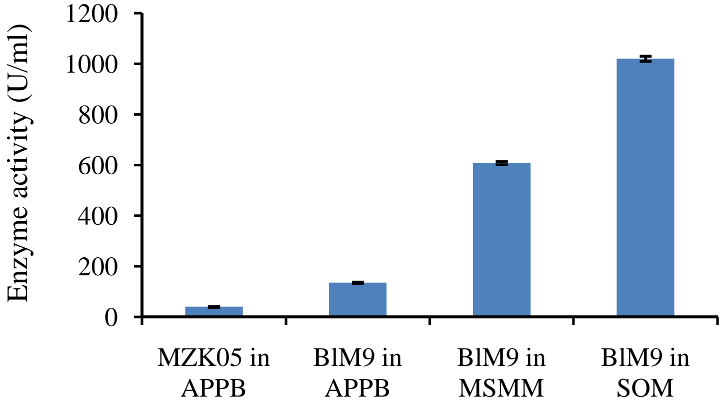


Fig. 4.3.7(b). Time course for protease production by *Bl*M9 in statistically optimized medium in 7 L bioreactor

From the study of development of suitable medium, it was found that the mutant *Bl*M9 finally showed proteolytic activity 1020 ± 10 U/ml in the statistically optimized medium (SOM). This mutant produced 135 U/ml in APPB medium where as the wild type produced 40 U/ml (Chapter 3). Therefore strain improvement and optimization of the medium together resulted in 25 fold augmentation in the enzyme titer than that produced by wild strain in APPB medium (Fig. 4.3.8)



APPB= Alkaline Protease Producing Broth, MSMM= Molasses Soybean meal medium, SOM=Statistically optimized medium

Fig.4.3.8. Development of protease production by strain improvement and medium optimization

Chapter 5. Partial purification and characterization of the *Bl*M9 enzyme

5.1. Introduction

Both crude as well as pure preparations of microbial protease are used in the industries. For example, crude proteases are used in bulk quantities in leather and detergent industries while pharmaceutical industries require small but pure protease preparations. In leather processing, crude proteases are being used increasingly to remove non-collagenous and globular proteins since they act under milder conditions and hair can be recovered as a valuable by-product (Rao *et al.*, 1998; Kumar and Takagi, 1999; Gupta *et al.*, 2002; Foroughi *et al.*,2006). However, care should be taken that the protease preparation is collagenase free and should not act on the skin protein, namely collagen.

Characterization and purification of an enzyme is highly important in its potential uses in industrial processes as the aptness of an enzyme depends on its unique characteristics such as optimum temperature, pH, mode of action, etc. (Zhang *et al*., 2011). Therefore different characteristics of the enzyme must be revealed before application in various processes. Thus in this study the protease of *Bl*M9 was partially purified and characterized for its better understanding which would allow for further improvements in the use and also enable us to identify other potential industrial applications of this enzyme.

5.2. Materials and methods

5.2.1. Protease enzyme

The production of the enzyme by *Bl*M9 was performed in a batch culture in 7 L bench-top bioreactor using the Molasses Soybean meal medium containing Molasses 5.0 g/l, Soybean meal 10.0 g/l, K_2HPO_4 3.0 g/l, $MgSO_4$ 0.5 g/l, $CaCl_2$ 0.5 g/l and NaCl 0.5 g/l (initial pH 7.5) which was previously optimized using one-variable-at a time approach. The fermentation was carried out in cascading mode at 37°C (described in chapter 4.2).

After the production of enzyme, the following steps were carried out for the purification of protease.

5.2.2. Separation of bacterial cells from fermentation medium

Separation of bacterial cells from fermentation medium was facilitated by using centrifugation technique. The fermentation medium was centrifuged at 8000 rpm for 10 min at 4ºC. The filtrate was separated and kept at 4ºC in refrigerator until the proteolytic activity and total protein estimation was performed.

5.2.3. Protease assay: Described in chapter 3, section 3.2.9.4.

5.2.4. Protein content determination: Described in chapter 4, section 4.1.2.12.

5.2.5. Determination of specific activity

To obtain the specific activity of the enzyme, the activity (U/ml) was divided by protein concentration (mg/ml);

$$\text{Specific activity (U/mg)} = \frac{\text{Protease activity (U/ml)}}{\text{Extracellular protein concentration (mg/ml)}}$$

5.2.6. Ammonium sulfate precipitation

Precipitation of protease was carried out by ammonium sulfate fractionation. Solid $(NH_4)_2SO_4$ was added to 100 ml of cell free enzyme solution for fractionation. Saturation between 30 to 90% was achieved by adding the ammonium sulfate. Ammonium sulfate was added slowly in the supernatant which was then stirred gently for 1 hr using a magnetic stirrer and left overnight at 4°C. Then the precipitates were collected by centrifugation at 10,000 g for 10 min at 4ºC. The proteolytic activity and protein concentration of each step were checked.

5.2.7. Dialysis

5.2.7.1. Preparation of dialysis tube: For dialysis of the precipitated enzyme the tube (D 2272, Sigma, St. Louis, Mo, USA) with molecular weight cutoff value 2 kD, useful for separating compounds having molecular weight greater than 2000 daltons was used. The tubing was prepared as follows:

i. Tube was washed in running water for 3-4 hours to remove glycerol

ii. Sulfur compounds were removed by treating with a 0.3% (w/v) sodium sulfide solution at 70°C for one minute, then washed with hot water (60°C) for two minutes, followed by acidification with 0.2% sulfuric acid, and rinsed with hot water to remove the acid.

5.2.7.2. Procedure for dialysis: The protein precipitates were suspended in small volume of 0.02M Tris-HCl buffer (pH 7.5), and taken into the dialyzing bag, and the bag was placed in 2 liters of 0.02M Tris-HCl buffer (pH 7.5) for overnight (12 hrs) at 4ºC with continuous mild stirring while 2 liters of the same buffer was changed three times. After dialysis, the sample was kept at 4ºC in refrigerator and used for gel filtration.

5.2.8. Gel permeation chromatography using Sephadex G-75

Sephadex G-75 was used for gel filtration. 10g of Sephadex G-75 was soaked in 500 ml of 20 mM Tris-HCl buffer (pH7.5) and was kept at room temperature for 48-72 hrs, for maximum swelling of beads in gel. The gel was de-aerated in sonicator for 20 minutes. After sonication, the gel was poured and packed in a 1.5×60 cm column. The packed column was washed with 25 mM Tris-HCl buffer (pH7.5) until fully packed. 2 ml of dialyzed enzyme was applied on the column and eluted with excess volume of 25 mM Tris-HCl buffer (pH7.5) and fractions of 1.5 ml were collected at a flow rate of 1 ml/min. Each fraction was then assayed for enzyme activity by azocasein method and the concentration of protein was determined by Bradford method. Fractions containing greater enzyme activity were pooled and used for further experiments.

5.2.9. Determination of molecular weight by polyacrylamide gel electrophoresis

SDS-PAGE was carried out using a 4% (w/v) stacking gel and 10% (w/v) separating gel as described by Laemmli (1970), under reducing conditions. The sample for SDS-PAGE was prepared by mixing the enzyme with sample buffer in a ratio of 1:1. The mixture was heated at boiling water for 5 minutes. After complete electrophoresis, the gel was transferred into a staining solution of Coomassie Brilliant Blue R-250 for over-night with gentle agitation at room temperature. After staining, the gel was destained using a destaining solution until the background had been satisfactorily removed and protein bands became clear.

5.2.10. Characterization of partially purified *Bl*M9 enzyme

5.2.10.1. Effect of temperature on enzyme activity and stability

The optimum temperature for partially purified protease activity was measured by incubating the enzyme with 1% (w/v) azocasein as a substrate at various temperatures ranging from 30°C to 75°C for 60 min. The thermostability of the protease was determined by pre-incubating the enzyme in absence and presence of 5mM of Ca^{2+} at temperature of 30, 40, 50, 60, and 70°C for 60 min followed by determination of relative activities under standard assay condition. The untreated enzyme was taken as control.

5.2.10.2. Effect of pH on proteolytic activity and stability of the protease

The optimum pH of the partially purified protease was determined with 1% (w/v) azocasein as substrate dissolved in different buffers (potassium phosphate, pH 5~7.5; Tris-HCl, pH 8.0~9.5; and Glycine-NaOH, pH 10~11). The enzyme was incubated with substrate of different pH and incubated at 37 ºC for 1 hr for the determination of enzyme activity.

The pH stability of partially purified protease was determined by pre-incubation the enzyme in different buffers of various pH (5~11) for 1 hr at room temperature. After incubation, the enzyme was assayed by incubating with 1% (w/v) azocasein as substrate at 37 ºC and the relative protease activity was determined. The untreated enzyme was taken as control.

5.2.10.3. Effect of various metal ions on protease activity

Effect of various metal ions (Ca^{2+}, Ba^{2+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, Hg^{2+}, Na^{+}, K^{+} and Zn^{2+}) on the protease activity was studied by pre-incubating the partially purified enzyme at room temperature in a specified ion (5mM final concentration) containing buffer solution for 60 min. After pre-incubation the enzyme assay was carried out under standard assay condition.

5.2.10.4. Effects of surfactants, oxidizing agents and detergents on protease activity

The effect of some surfactant and oxidizing agents (SDS, Tween-80, Triton X-100 and H_2O_2) at 1%, 3% and 5% concentration on partially purified enzyme was studied by determining the relative activity of the enzyme after pre-incubation for 60 min at 37°C. The enzyme activity of a control (without surfactants and oxidizing agents), incubated under the similar conditions, was taken as 100%.The compatibility of partially purified protease with local laundry detergents was studied using Surf Excel, Wheel, Chaka, Tibet, Jet and Fast Wash. The tap water was used to dilute the detergents to give a final concentration of 7 mg/ml to simulate washing conditions. The endogenous proteases contained in these detergents were inactivated by incubating the diluted detergents at 65°C for 60 min prior to the addition of enzyme. Protease was incubated in various detergents solutions for 60 min at room temperature and the relative activity was determined under standard assay condition. The enzyme activity of a control (without detergent), incubated under the similar conditions, was taken as 100%.

5.2.10.5. Effect of protease inhibitors on enzyme activity

The effect of various enzyme inhibitors on protease activity was studied using ethylenediaminetetracetic acid (EDTA) (inhibitor for metalloprotease), phenylmethylsulfonyl fluoride (PMSF) (inhibitor for serine alkaline protease), dithiothreitol (DTT) and β-mercaptoethanol (inhibitor for cysteine protease). The partially purified enzyme was pre-incubated with each inhibitor (5mM) for 60 min at room temperature and then the remaining enzyme activity was determined under standard assay condition. The activity of the enzyme without any inhibitor was taken as control.

5.3. Results

5.3.1. Separation of bacteria from fermentation broth

The fermentation medium was centrifuged at 8,000 g for 10 minutes at 4°C in a centrifuge. The supernatant was kept at 4°C in refrigerator until the proteolytic activity and total protein estimation was performed.

5.3.2. Ammonium sulfate precipitation

Ammonium sulfate precipitation was applied as initial step to purify the crude enzyme extract. Different concentrations of salt were applied to the cell free supernatant, which significantly affected the precipitation of the protease (Fig. 5.1). The results in figure indicated that the percentage of enzyme extraction was enhanced by increasing the concentration of the salt. It is clear from the figure that the maximum percentage of the alkaline protease can be precipitated by addition of 60% saturated $(NH_4)_2SO_4$ to the culture filtrate. Whereas minimum extraction was achieved at 20, 30, 40 and 50% saturation with 50, 53, 66 and 87% respectively. Hence, 60% saturation ammonium sulfate was found suitable for the extraction of the protease from culture filtrate with 1.23 fold purification.

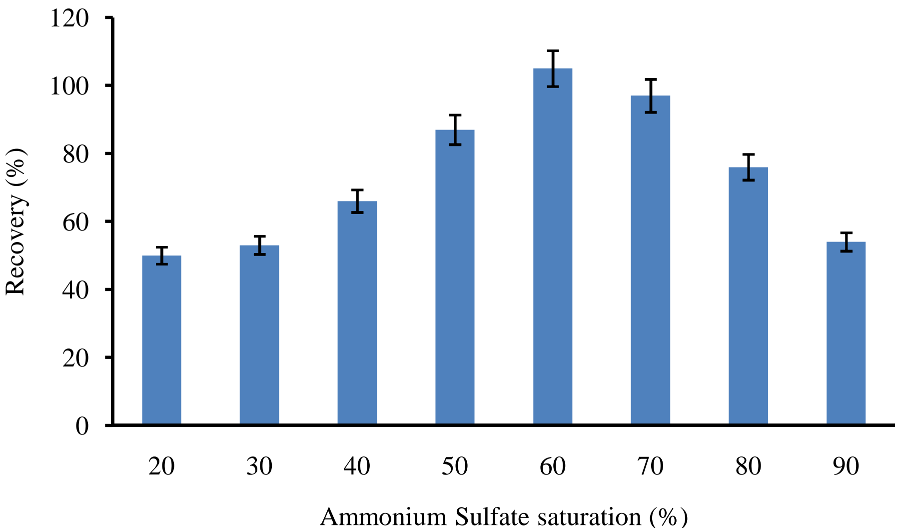


Fig. 5.1. Ammonium Sulfate precipitation of *Bl*M9 enzyme

5.3.3. Dialysis

After precipitation the dialysis was employed to remove the excess of salts from the precipitate and to elute out the molecules less than 2,000 D molecular weight. The precipitates collected after precipitation process were subjected to dialysation by using dialyzing bag against three changes of 0.02M Tris-HCl buffer (pH 7.5).

5.3.4. Gel permeation chromatography by using Sephadex G-75

After 2 ml loading of dialyzed enzyme on sephadex G-75 column for fractionation, the sample was eluted by Tris-HCl buffer and 30 fractions each of 1.5 ml were collected. Each fraction was assayed for proteolytic activity and its protein content was determined. From the specific activities only one prominent peak was observed (Fig. 5.2).

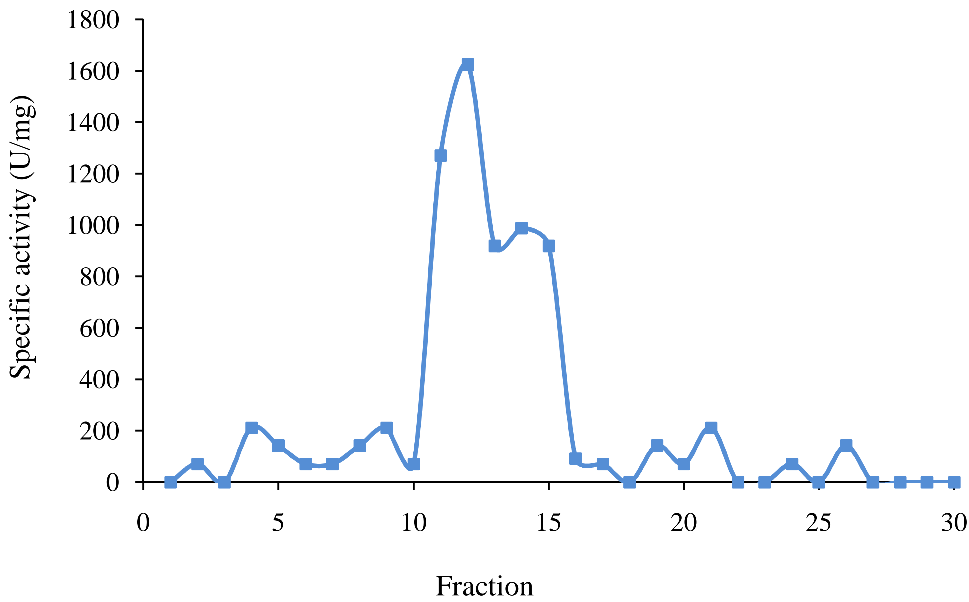


Fig.5.2. Purification of protease by Sephdex G-75 Chromatography technique

Gel filtration effectively removed the considerable amount of impurities present in crude enzyme extract with 2.7 fold purification (Table 5.1). The fractions obtained by gel filtration with proteolytic activity were pooled and subjected to further study.

Table 5.1. Purification of protease from *Bl*M9

Purification steps	Specific activity (U/mg)	Purification (fold)
Crude extract	601	1
Ammonium Sulfate (60%) precipitation	743	1.23
Sephadex G-75 column Chromatography	1624	2.7

5.3.5. Molecular weight of the *Bl*M9 enzyme

The molecular weight of *Bl*M9 alkaline protease was determined by SDS-PAGE. Comparison with the molecular weight of the marker revealed that *Bl*M9 alkaline protease has a molecular weight of about 27.2 kDa (Fig. 5.3).

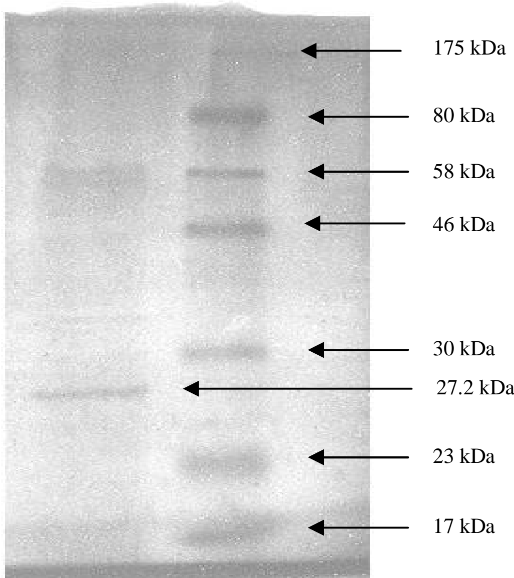


Fig. 5.3. SDS-PAGE analysis of partially purified *Bl*M9 protease. Lane (Left) = Partially purified enzyme, Lane (Right) = Marker.

5.3.6. Characterization of the *Bl*M9 protease

5.3.6.1. Effect of temperature on activity of the enzyme

The enzyme was active in a large temperature range, with an optimum at 55°C (Fig. 5.4). The relative activities at 50 and 60°C were about 83% and 75%, respectively, of that at 55°C.

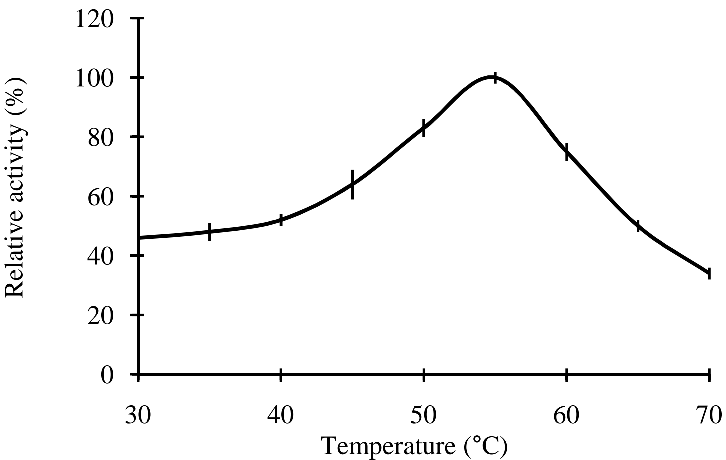


Fig. 5.4. Temperature activity of the protease from *Bl*M9

5.3.6.2. Thermostability of partially purified enzyme of *Bl*M9

The purified enzyme showed different temperature stability at various temperatures (30-70°C) (Fig. 5.5). Results indicate that the enzyme was stable at 40°C without loss of any activity. Whereas at 50°C the relative activity became half (50%) of the original activity and at 70°C it became zero (0%). However, addition of $CaCl_2$ stabilized the enzyme (at 100% level) up to 50°C.

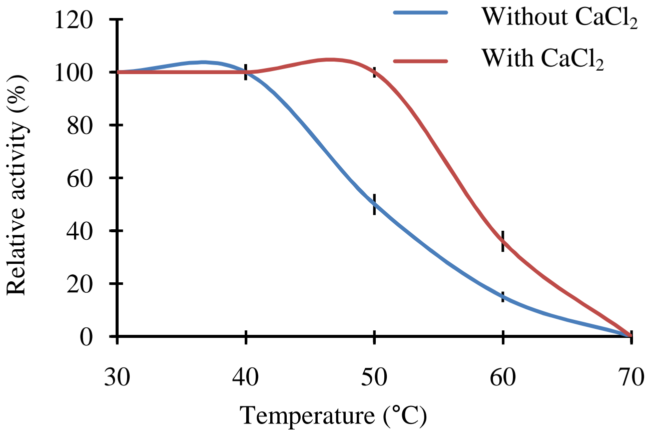


Fig. 5.5. Thermostability of the protease from *Bl*M9

5.3.6.3. Effect of pH on activity of the *Bl*M9 enzyme

The partially purified enzyme was found highly active between pH 7.0 and 9.5 with an optimum at pH 8.5, indicating its alkaline nature. The relative activities at pH 6.5 and 10.0 were about 74% and 69%, respectively, of that at optimal pH (Fig. 5.6).

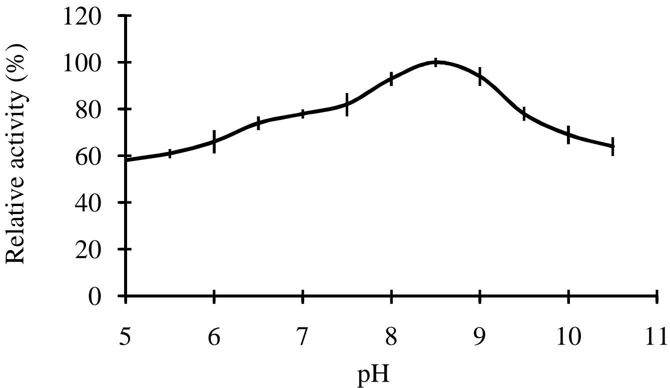


Fig. 5.6. pH Activity of the Protease from *Bl*M9

5.3.6.4. Effect of pH on stability of *Bl*M9 enzyme

The pH stability profile of alkaline protease showed that the partially purified enzyme was stable between 7.0 and 8.0 but in presence of $CaCl_2$, the enzyme showed stability in wide pH ranges 6.0 -9.0 (Fig. 5.7).

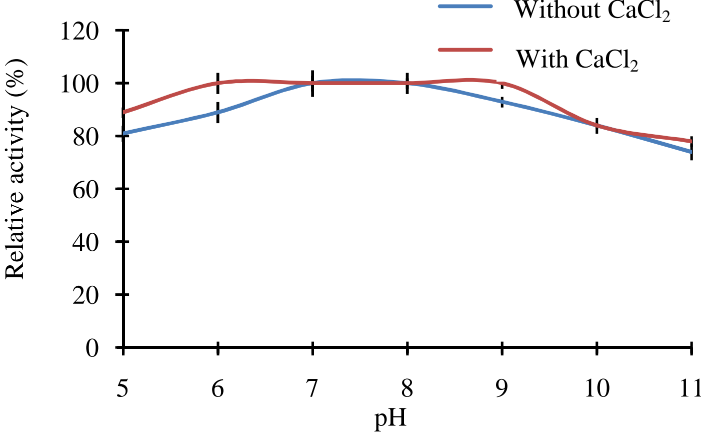


Fig.5.7. pH Stability of the protease from *Bl*M9

5.3.6.5. Effect of metal ions on *Bl*M9 enzyme

The results recorded (Fig. 5.8) indicating that in presence of Ba^{2+}, K^{+}, Ca^{2+}, Mg^{2+} and Mn^{2+} the proteolytic activity resulted in the discreet increase, while due to inhibitory effect it lost almost total activity in presence of Hg^{2+}. Metal like Na^{+} restored the enzyme activity as 97% of the original activity. Whereas other metals like Zn^{2+} and Cu^{2+} showed an appreciable inhibitory effect on enzyme with relative activity of 38 and 33%, respectively.

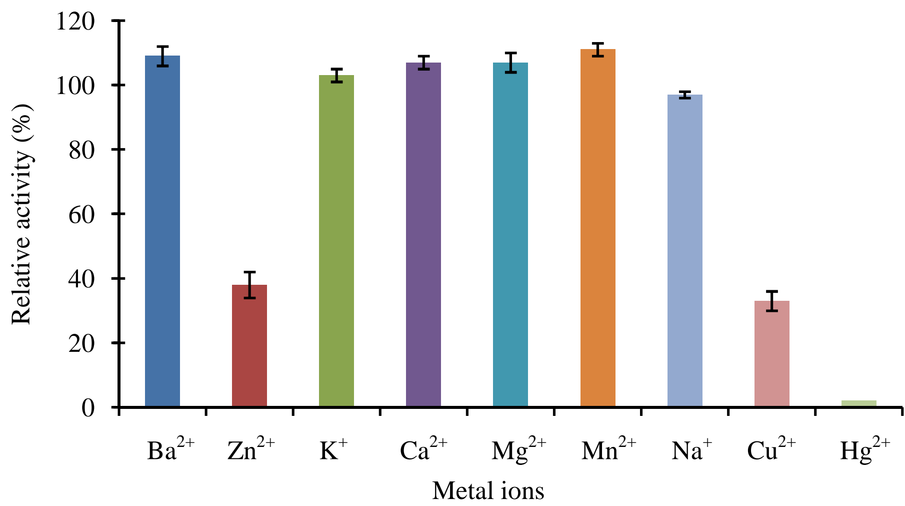


Fig. 5.8. Effect of metal ions on the *Bl*M9 protease

5.3.6.6. Effect of inhibitors on the protease activity

The specific influence of inhibitors on the proteolytic activity was determined by using inhibitors of a recommended concentrations (5 mM) and data obtained showed that enzyme was strongly inhibited (76% inhibition) by PMSF (Phenylmethylsulfonyl fluoride), a serine protease inhibitor, and EDTA, DDT and -mercaptoethanol showed only 29, 46, and 17% inhibition respectively indicating that this enzyme is a serine type protease (Fig. 5.9).

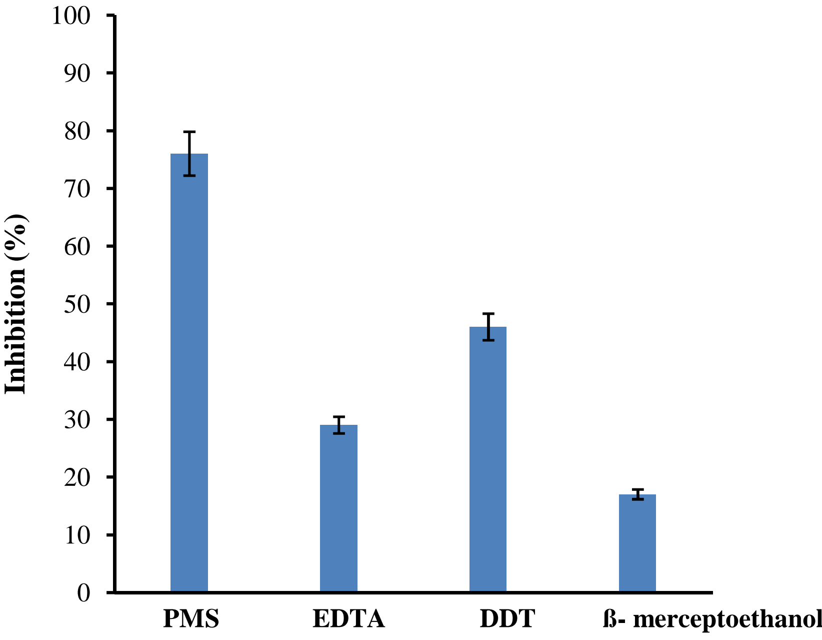


Fig. 5.9. Effect of inhibitors on the protease activity

5.3.6.7. Effect of surfactants, oxidant and detergent on the protease

The partially purified protease was stable at low concentration (1%) of SDS with relative activity of 62%. While it was found stable in Triton X-100 and H_2O_2, it was unstable in Tween-80 (Fig. 5.10).

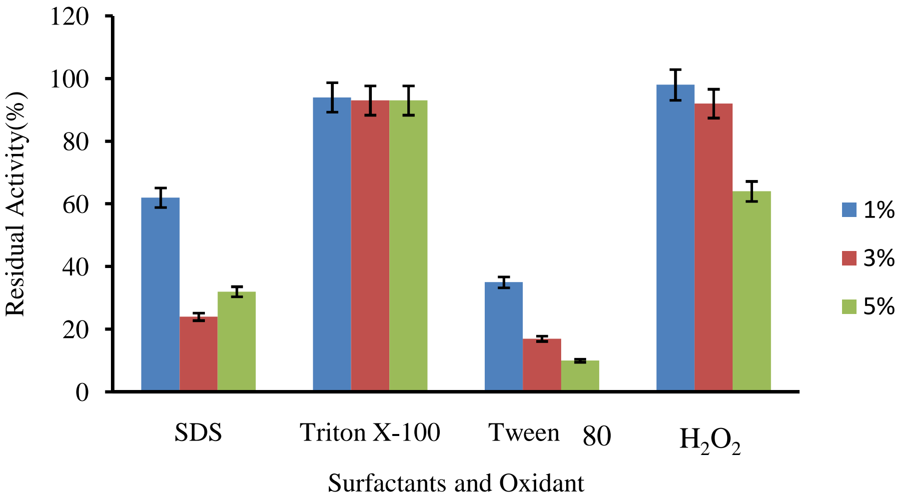


Fig.5.10. Effect of surfactants and oxidant on the protease

The enzyme retained activity at room temperature with Surf Excel more than 65%, in Wheel 86%, in Tibet 113%, in Keya 87%, in Jet 70% and in Fast Wash 98% after 60 min incubation (Fig. 5.11)

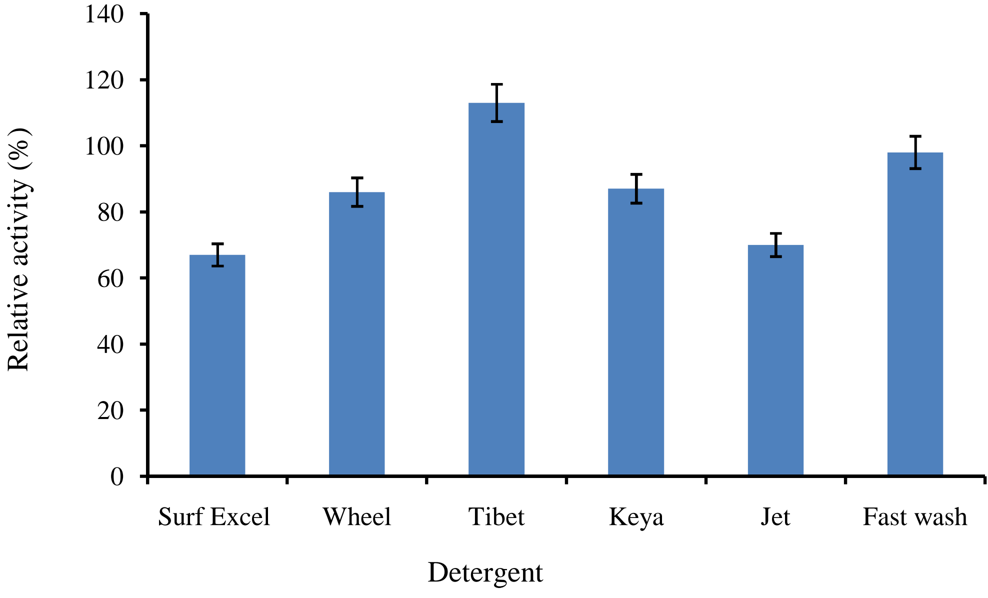


Fig. 5.11. Effect of detergents on *Bl*M9 Enzyme

Chapter 6.1. Stabilization of the *Bl*M9 enzyme

6.1.1.Introduction

The instability of enzymes is usually a limiting factor in most processes because this intrinsic instability leads to difficulty in production, processing and storage and subsequently leads to high costs of the process (Iyer and Ananthanarayan, 2008). This limited stability may be due to deleterious effects of aqueous environment and microbial contamination (Rainer, 2000; Rajini and Mattiasson, 1993). They may also be denatured by changes in temperature, pH, pressure and ionic strength (Michiaki *et al.,* 1997). In order to develop new proteases with better stability systemic efforts including cloning of protease genes into more suitable hosts or site directed mutagenesis of the microorganism can be carried out (Fagain, 2003). Generally, to stabilize the enzyme the most methods focus on effects of additives on enzyme stability rendering it the popular technique for enzyme stabilization. Various classes of additives being employed for enzyme stabilization are ligands, substrates, salts, polyols, sugars, DMSO, glycerol, polyethylene glycols, synthetic polymers and miscellaneous additives (Chaniotakis, 2004; Gouda *et al.,* 2003; Iyer and Ananthanarayan, 2008). Other methods in use are enzyme immobilization and crystallization (Grazu *et al.,* 2005). In this study various additives like salts, sugars, surfactants and polymers were used to check their effect on storage stability of *Bl*M9 enzyme. The results of storage stability of the *Bl*M9 enzyme are presented in this chapter.

6.1.2. Materials and methods

6.1.2.1.Excepients used

Various additives viz. Salts ($MnSO_4$ and NH_4SO_4), sugars (sucrose, fructose, and glucose), polymer (Polyethylene glycol, PEG 4000), Surfactants (Tween-80 and Triton X-100) were used to study their effect on enzyme storage stability.

6.1.2.2. Lyophilization of alkaline protease

After centrifugation the supernatant was taken in petri-dishes subjected to freezing in -20°C for an hour. The petri-dishes were then placed carefully to the lyophilization unit. The operation was carried out for 5 to 6 hrs with monitoring at regular intervals. Finally, the petri-dishes were removed from lyophilization unit and the powdered product was transferred to a duran bottle, the bottle was then tightly sealed. To assay the activity of the lyophilized product, it was re-suspended in distilled water and protease assay was performed. The product was stored at 30°C and the residual activity was checked after every 7 days. The recovery of lyophilization was calculated by the following formula.

$$\text{Recovery (\%)} = \frac{\text{Activity of the sample after lyophilization}}{\text{Activity of the sample before lyophilization}} \text{X100}$$

6.1.3. Result

6.1.3.1. Recovery of the enzyme activity upon lyophilization of the alkaline protease

After complete lyophilization solid powdered enzyme was kept in Duran bottle and tightly sealed.

Fig. 6.1.1. Lyophilized *Bl*M9 enzyme

By determining the enzyme activity before and after lyophilization it was found that the process of lyophilization recovered the enzyme at 98.5 % level (Table 6.1.1).

Table 6.1.1. Recovery of the lyophilized enzyme

Total loss of activities during lyophilization	1.5%
Recovery after lyophilization	98.5 %

6.1.3.2. Effect of temperature and lyophilization on enzyme storage stability

The enzyme retained 100% activity up to 30 days at 4°C whereas at 30°C the enzyme showed instability. The stability profile of *Bl*M9 protease in lyophilized form was investigated. The enzyme retained 98.5 % of its activity after lyophilization (Table 6.1.1). The lyophilized enzyme was then stored in a duran bottle (Fig. 6.1.1) at 30°C. Enzyme activity was taken 02 days intervals and was found that the lyophilized enzyme retained 100% of its initial activity even after 35 days (Fig. 6.1.2) and 90 % after 120 days which suggests that lyophilization stabilize the enzyme significantly. Lyophilization is an effective method of storage of enzyme as it dries up the product and protects the enzyme against denaturation due to chemical reactions that occur in aqueous environment. During the process of freezing and drying, changes occur in product that may denature the enzyme (Hanson & Raoun, 1992). But in case of *Bl*M9 enzyme, during lyophilization only 1.5% activity was denatured.

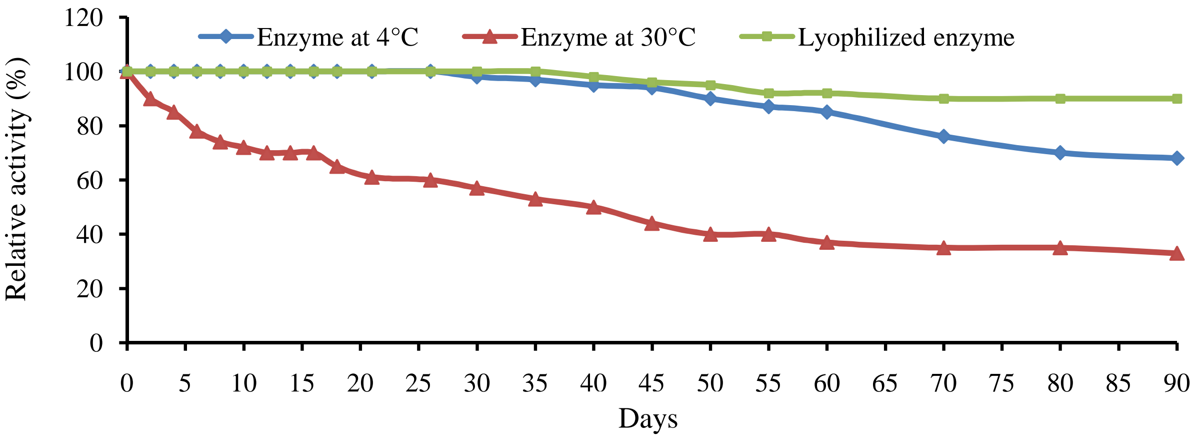


Fig. 6.1.2. Stability profile of *Bl*M9 enzyme

6.1.3.3. Effect of polymer (PEG-4000) and surfactants (Triton X-100 and Tween-80) on storage stability of the *Bl*M9 enzyme

Effect of PEG on the stability of alkaline protease was investigated by incubating the enzyme with various amount of PEG such as 0.25%, 0.5% and 1%. The stabilities of the enzyme were then compared by taking activities up to 90 days. At 30°C, the enzyme was significantly stabilized by 0.5% PEG with 100 % activity up to 18 days and 90% activity up to 30 days. PEG 0.25%, and PEG 1%, retained 100% activity up to 8 and 10 days, respectively (Fig. 6.1.3).

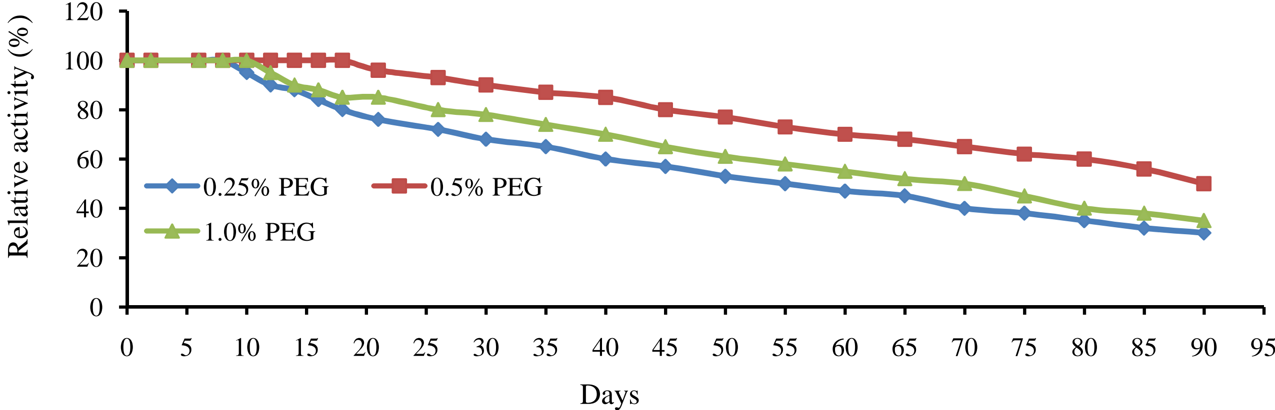


Fig. 6.1.3. Effect of polymer (PEG-4000) on storage stability of the *Bl*M9 enzyme

Triton X-100 stabilized the enzyme 100 % level up to 8 days with the half shelf life of 50 days where as the Tween -80 partially stabilized the enzyme with half shelf life of 40 days (Fig. 6.1.4).

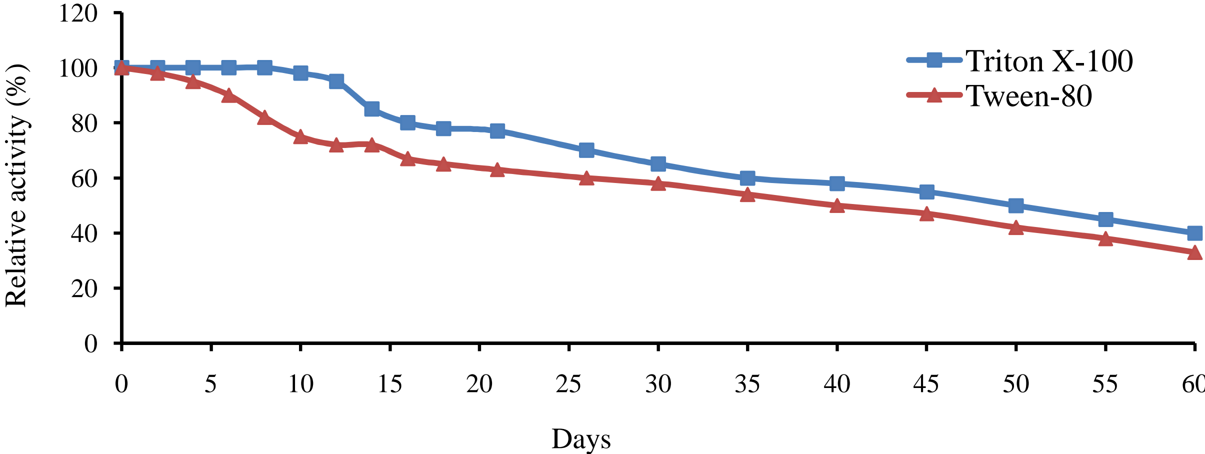


Fig. 6.1.4. Effect of surfactants (Triton X-100 and Tween-80) on storage stability of the *Bl*M9 enzyme

6.1.3.4. Effect of salts and sugars on storage stability of the *Bl*M9 enzyme

Effect of salts and sugars on the stability of alkaline protease was investigated by incubating the enzyme with sugars such as fructose, glucose and sucrose and salts such as $MnSO_4$ and NH_4SO_4 at 30°C. The stabilities of the samples were then compared by taking protease activity at 03 days intervals. Sugars such as fructose, glucose and sucrose did not show any noticeable stabilization effect on the alkaline protease rather enzyme incubated with glucose lost its activity more rapidly.

Salts stabilize the enzyme by salting out effect of hydrophobic residues from surface into the interior of enzyme molecule thereby compressing the enzyme leading to higher stability of the enzyme (Klibanov, 1983). However in this study, ions such as NH_4^+ and Mn^{2+}provided some protective effect (Fig.6.1.5).

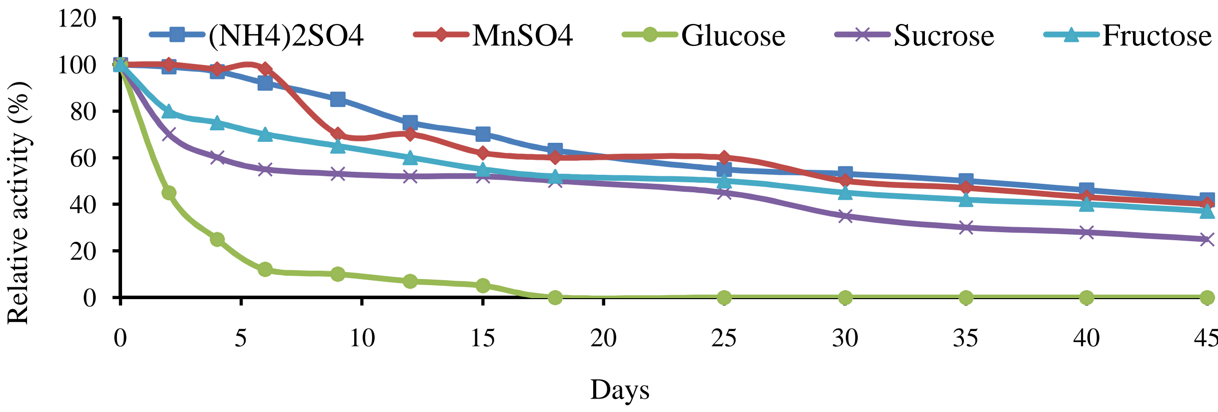


Fig. 6.1.5. Effect of salts and sugars on storage stability of the *Bl*M9 enzyme

6.1.3.5. Half shelf life of the *Bl*M9 enzyme

Half shelf life of an enzyme is very important to know about the stability of the enzyme. It gives information about how long the enzyme could be used. To determine the half shelf-life, the activity of the *Bl*M9 enzyme incubated with various additives at different temperature was assayed for certain time period. The period after which the half of the initial activity of the enzyme found due to degradation was considered as half shelf-life. Fig. 6.1.6 shows the half shelf-life of the *Bl*M9 enzyme. At 4°C enzyme showed the half shelf-life of 120 days where as at 30°C showed 40 days suggesting that if the enzyme is stored at 4°C it could be used long time for application. In case of additives, PEG-4000 rendered the half shelf-life of the enzyme up to 90 days.

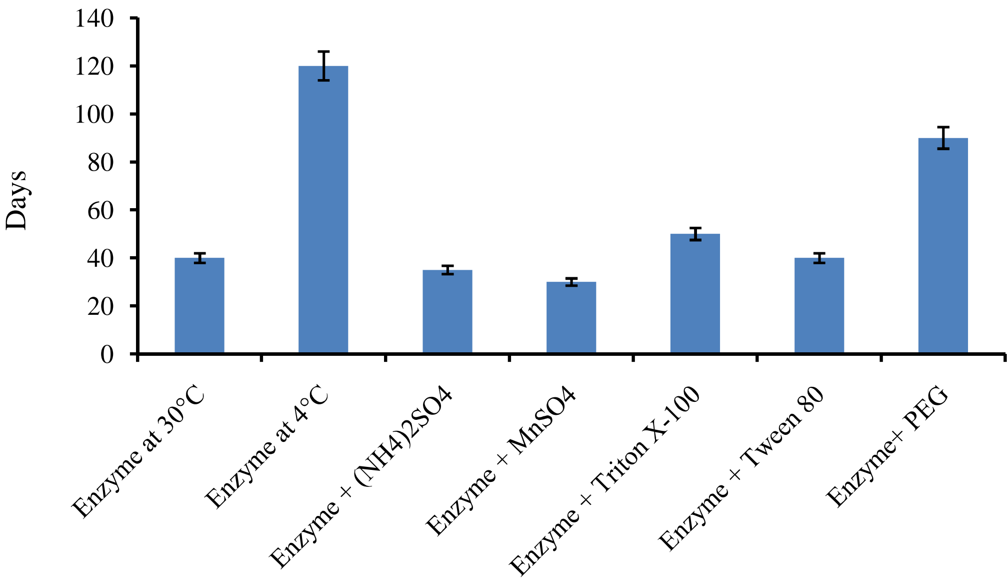


Fig. 6.1.6. Half shelf life of *Bl*M9 enzyme

Chapter 6.2. Application of the *Bl*M9 enzyme in dehairing of goat skin

6.2.1. Introduction

Leather industries (Tanneries) offer various advantages in terms of manufacturing capacity and export potential (Luthra, 2006). There are about more than 200 tanneries in Bangladesh producing and exporting quality leathers with good international reputation and have been ranked fifth in the country's export earning sectors (Paul *et al*, 2013). About 25 hectares of land area has been occupied by the tannery industries at Hazaribagh, the south-west part of the capital city Dhaka. The tanning industries of Hazaribagh are processing approximately 220 metric tons of hide daily with an associated release of 600 - 1000 Kg of solid waste resulting from the production of each ton processed hide (Hossain *et al.* 2009). Animal skins/hides, byproduct of meat industries, are composed of proteins (90-95% of solid, 35% by weight), lipids, carbohydrates, mineral salts and water. Among several classes of proteins (collagen, elastin, keratin, glycoproteins, albumins and globulins), collagen as structural protein present in largest amount and is responsible for the formation of leather by combining with tanning agent (Kanagaraj *et al.* 2006).The animal skins/hides have different processing steps like soaking, dehairing, deliming, bating, degreasing , pickling and tanning in beamhouse operations to prepare stabilized collagenous leather products (Zambare *et al.* 2013). For this purpose, all local tanneries still use traditional leather processing method utilizing mostly harsh chemicals. The conventional chemical leather processing generates huge amount of environmental pollutions (Kanagaraj *et al.* 2006 and Thanikaivelan *et al*., 2004). Dehairing process, based on the use of lime and sodium sulfide, excretes highly alkaline effluent which contributes to 50–60% of total aquatic pollution load in terms of biochemical oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS) and total suspended solids (TSS)(Marsal *et al*., 1999 and Taylor *et al*., 1987). The highly alkaline sodium sulfide having obnoxious odor is very toxic and can cause skin burn. Moreover, in oxidation-reduction reaction sodium sulfide produces hydrogen sulfide which is proven to be fatal even in concentrations as low as 200 ppm (Hannah and Roth 1991 and Roth *et al*., 1995). Hydrogen sulfide also reacts with water and produces sulfuric acid that causes health hazards to the tannery workers and damage buildings, bridge etc. (Puvanakrishnan and Dhar, 1988). The extensive use of hazardous sulfide not only leads to

unfavorable consequences on the health and environment but also undermines the efficiency of the effluent treatment plants (Davies, 1997).

The above mentioned situations necessitate the use of an alternative and eco-friendly process, which is most likely could be accomplished by a process involving enzymes, to reduce environmental pollution (Dettmer *et al*., 2011). So, this study was designed to develop an enzymatic unhairing process for total substitution of the toxic sodium sulfide, and to reduce the environmental impact of leather production. Goat skins were treated several times by enzyme assisted and enzyme mediated method to develop a suitable eco-friendly dehairing process.

6.2.2. Materials and methods

6.2.2.1. Enzyme production: Production of proteolytic enzyme by *Bl*M9 has been described in chapter 4.2.

For keratinase production, *Bl*M9 was cultivated in Feather meal broth (FMB) medium comprising of following constituents (g/l): NH_4Cl 0.5, NaCl 0.5, KH_2PO_4 0.3, K_2HPO_4 0.4, $MgSO_4$ 0.1, feather meal 10 and yeast extract 0.1 in bioreactor in the same condition as applied for alkaline protease production. After separation of solid liquid by centrifugation, the supernatant was used as enzyme and applied for deharing of the goat skins.

6.2.2.2. Proteolytic activity assay: Described in chapter 3, section 3.2.9.4.

6.2.2.3. Keratinolytic activity assay

The Keratinolytic activity of the enzyme preparations was determined using keratin powder (TCI Co. Ltd. Tokyo) as substrate according to the method described by Hossain *et al*. 2007. In brief, 1 ml of .05 M Tris-HCl, pH 7.5 containing 20 mg of keratin suspension was incubated with 250 µl of the enzyme solution for 30 min at 37°C with constant agitation at 120 rpm in a water bath. The reaction was stopped by adding 500 µl of 10% trichloroacetic acid (TCA) and then incubation at 4°C for 30 min. Then reaction mixture was centrifuged at 10,000 g for 10 min and the supernatant was used to read the absorbance at 280 nm against a control. The control was treated in the same way, except that the TCA was added before the

addition of enzyme solution. One unit of keratinolytic activity was determined as the amount of enzyme that produced an increase in absorbance of 0.01 under the assay conditions.

6.2.2.4. Dehairing methods

Freshly flayed goat skin was obtained from the local slaughter house and washed properly with water and detergent to eliminate blood, dirt and other unwanted particles. After removing the adipose tissue layer with a knife the skin was cut into pieces (6cm x 6cm) and weighted. These pieces were used for dehairing studies- conventional (control), enzyme mediated and enzyme assisted methods. The pieces of the goat skin placed into a conical flasks (1000 ml) containing water (120 ml) to dip the skin pieces. In conventional method, Na_2S 2% and CaO 5% (of the skin weight) and in enzyme mediated method keratinase 2.5% and alkaline protease solutions 2.5% (of the water volume) were used for dehairing of the goat skin. For enzyme assisted method the piece of goat skin was soaked in 5% CaO (w/w of the skin) solution for 6 hrs at 37°C. After CaO treatment, the skin piece was washed several times by tape water until pH of the skin dropped near 7.5. Then the piece of the goat skin dipped in 120 ml of water in a 1000 ml conical flask and 2.5% keratinase along with 2.5% alkaline protease solutions were added. All the flasks were transferred into orbital shaker rotating at 120 rpm for 26 hrs at 37°C.

The efficiency of the dehairing methods to remove the hair from skin was evaluated by determining the area of the dehaired portion and expressed as the percentage of the area of the treated skin. The smoothness of grain surface of dehaired skin was examined by Electron microscopy (JSM-6490LA, Jeol, Japan).

6.2.3. Results

6.2.3.1. Optimization of conditions for dehairing of goat skin by *Bl*M9 enzymes

Conventional dehairing process using CaO and Na_2S needed 20 hrs for complete dehairing of the goat skin. On the other hand, the enzyme mediated and enzyme assisted methods produced different percent of dehaired yield after different treatment durations. From the

results, it was found that the highest 85% and 100% dehaired area were resulted using enzyme mediated and enzyme assisted methods respectively (Fig. 6.2.1).

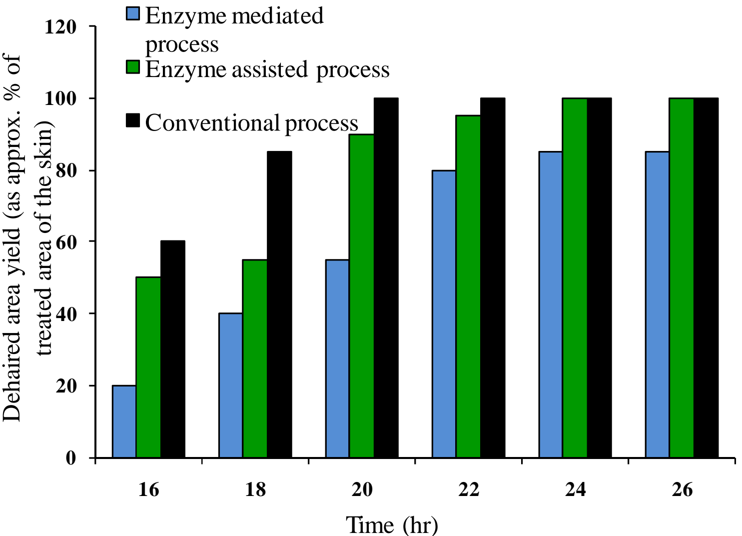
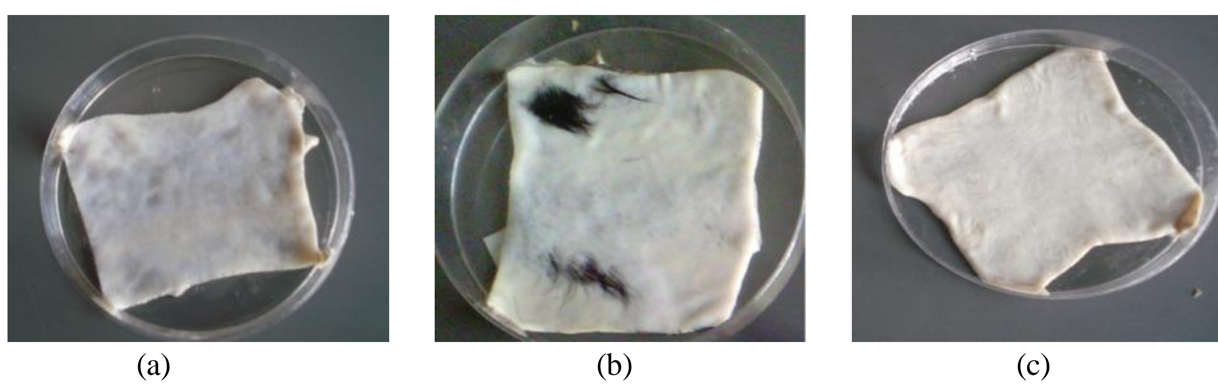


Fig. 6.2.1. Comparative dehairing efficiency of enzyme assisted (enzyme +lime) and enzyme mediated (only enzyme) methods

From the experiment it was revealed that the enzyme assisted method was comparable to conventional lime-sulfide method in complete deharing. Though the conventional lime-sulfide method required 20 hrs to dehair the goat skin completely as compared to 24 hrs for the enzyme assisted method (Table 6.2.1), the surface of the skin dehaired by later method was smoother than that of the conventional method as observed visually (Fig. 6.2.2).

Table 6.2.1. Effect of the *Bl*M9 enzymes and chemical treatments on the dehaired area yield of the skin

Type of method	Area of treated skin (cm^2)	Dehairing agents	Duration of treatment (hr)	Dehaired skin area obtained after treatment (cm^2)	Area yield (as % of treated area)
Conventional	36	2% Na_2S and 5% CaO	20	36	100
Enzyme mediated	36	2.5% keratinase + 2.5% alkaline protease	26	30	85
Enzyme assisted	36	5% CaO, 2.5% keratinase and 2.5% alkaline protease	24	36	100



(a) (b) (c)

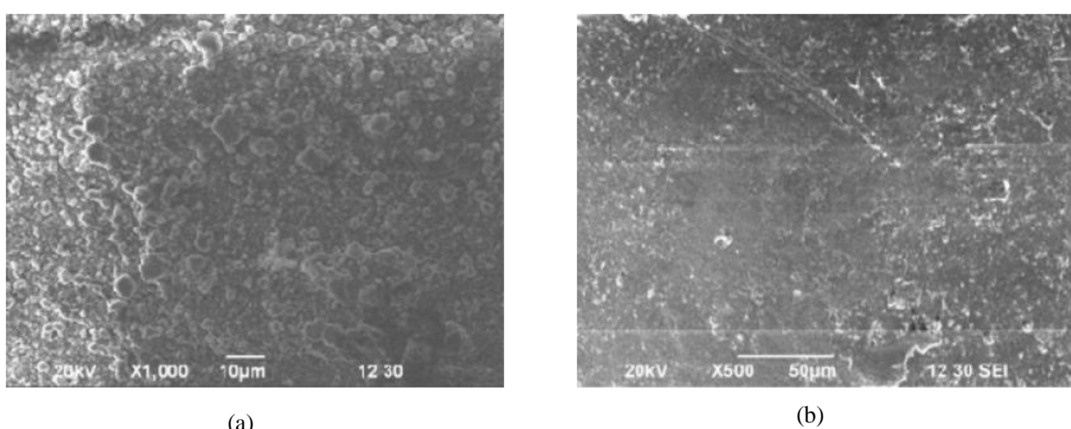
Fig. 6.2.2. Evaluation of different treatment methods for dehairing of goat skins (small scale): (a) Conventional lime-sulfide method (b) Enzyme mediated method and (c) Enzyme assisted method

Comparison between enzyme mediated and enzyme assisted method showed that complete dehairing was occurred by enzyme assisted method in small scale unhairing process. So, the only enzyme assisted method was applied to dehair a comparatively large (40 cm x 40 cm) piece of goat skin. As expected, it also provided complete unhairing of the goatskin in large scale unhairing process (Fig. 6.2.3).

Fig. 6.2.3. Efficiency of enzyme assisted method (5% CaO and 2.5% keratinase + 2.5% alkaline protease) for dehairing of large goat skin

6.2.3.2. Scanning electron microscopy (SEM) analysis of dehaired pelts

SEM analysis of the grain surface of dehaired pieces of goat skin demonstrated that there was no apparent damage to the collagen fibre in both process but the grain surface appeared to be smoother in enzyme assisted process than conventional one (**Fig. 6.2.4**).



(a) (b)

Fig.6.2.4. Scanning electron micrographs of the grain surface of goat skin dehaired by (a) Conventional lime-sulfide and (b) Enzyme assisted methods

Chapter 6.3. Application of the *Bl*M9 enzyme in Bating of cow hides

6.3.1. Introduction

Alkaline proteases offer an effective biotreatment of leather, especially the dehairing and bating of skins and hides (Taylor *et al*., 1987). The bating following the dehairing process involves the degradation of elastin and keratin, removal of hair residues, and the deswelling of collagen, which produces good, soft leather mainly used for making of leather clothes and goods. Previously the traditional method of bating used dung from cattle which was unpleasant and unhygienic (Puvanakrishnan and Dhar, 1988). The bating process as practiced today is by far more pleasant, safer and less offensive in odour as compared to the earlier process using the infusion of dung or manure. Modern bating procedure employed proteolytic enzymes of bacterial origin. Enzymes bate is one of the essential auxiliaries and its use is absolutely essential for the manufacture of leathers like glove, shrunken grain softie, nappa, garment and glace kid. Bating is the step in leather processing where enzymatic process cannot be substituted by chemical processes (Puvanakrishnan and Dhar, 1988).

In Bangladesh, leather industries import bating enzymes that adds much to the cost of leather processing. In this regard, local production of bating enzyme may reduce the cost of leather processing. However, care should be taken that the protease preparation should be collagenase free i.e. the enzyme should not act on the skin structural protein, namely collagen. The *Bl*M9 enzyme can degrade the non structural proteins of skin, albumin, globulin and elastin specifically but does not show any hydrolytic activity against collagen. Therefore the *Bl*M9 enzyme was applied in bating of pelts in leather processing.

6.3.2. Materials and methods

For determining the bating activity of *Bl*M9 protease, crude enzyme (2% of hide weight; 100 ml equivalent to 60,100 U =34,841 LVU for 5 kg hide) was applied to the cow hide in presence of water. Cow hide (after deliming) emerged in the enzyme preparations were rolled in a drum for about 60 minutes. In parallel, as a control, a commercial enzyme (Oropon K) as 0.5% (w/w) of leather weight was also used in bating experiment. Different stages of leather processing treatments before and after bating are illustrated in Fig. 6.3.1. After bating, the

qualitative tests such as the bubble, cross section and thumb tests as well as the physical tests such as tensile strength, percent of elongation, stitch tear strength, water vapor permeability, grain crack strength and tongue tear strength tests of crushed leather were performed to evaluate the potentiality of *Bl*M9 enzyme in bating of pelts.

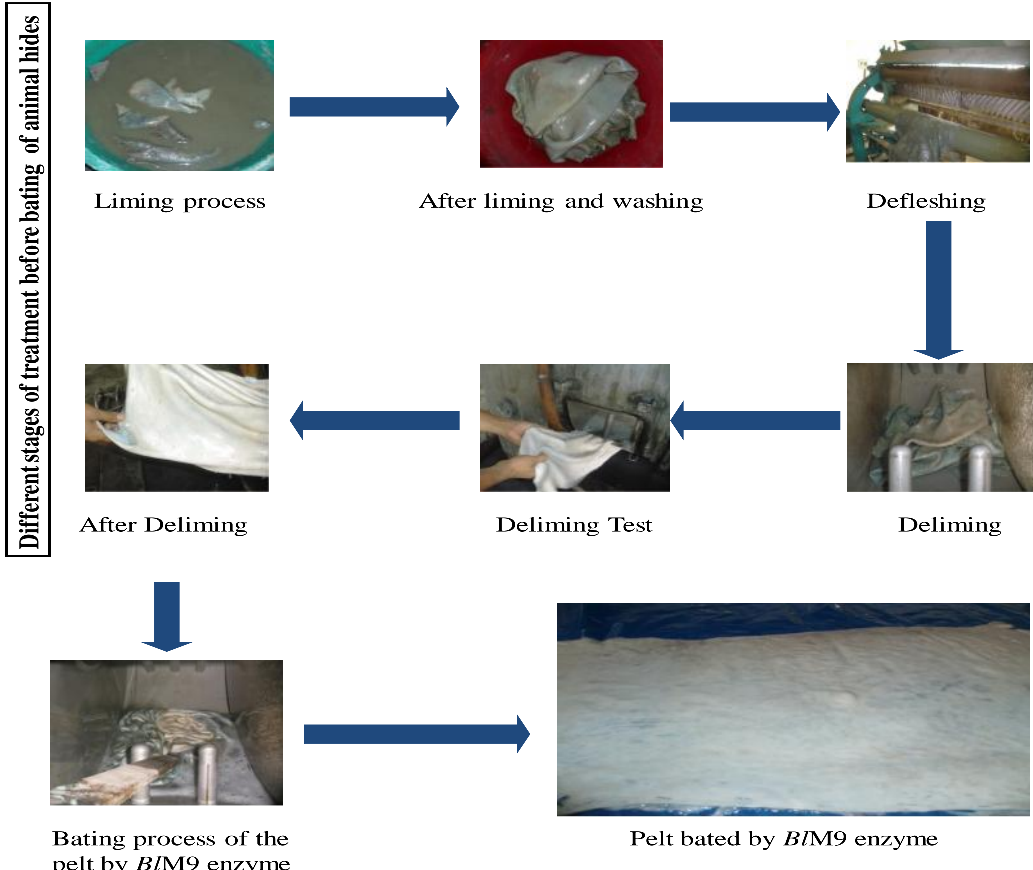


Fig. 6.3.1. Different stages of treatment before and during bating operation

6.3.2.1. Qualitative tests

Different qualitative tests of pelt bated by *Bl*M9 enzyme were performed to reveal the bating efficiency of the enzyme:

6.3.2.1.1. Thumb test: It is performed by pressing the pelt with thumb after bating. If the press left a print there that means bating has been performed properly by the enzyme.

6.3.2.1.2. Bubble test: The bated pelt is wrapped by hand and pressed. If bubble comes out then it is understood that the bating operation is done properly.

6.3.2.1.3. Cross section test: Phenolphthalein is dripped in the cross section of the pelt and if it turns into completely colorless, then it is understood that bating is finished.

6.3.2.2. Physical tests

Several other physical tests of crushed leather bated by *Bl*M9 enzyme were performed in ISO certified laboratory of Institute of Leather Engineering and Technology, Hazaribag, Dhaka.

6.3.2.2.1. Tensile strength: It is the force required to rupture a leather specimen of unit cross sectional area. It is determined with the tensile strength tester or tensometer.

$$\text{Tensile strength (Kg}/sq.cm) = \frac{Breaking\ load\ in\ Kg}{Cross\ sectional\ are\ in\ sq.cm}$$

Tensile strength is thus the combined breaking strength of all the fibres taking part to fight the applied load in a tensometer.

6.3.2.2.2. Tongue tear strength: It is the load required to tear the leather specimen of unit thickness between two tongues formed by splitting the leather perpendicular to its surface.

$$\text{Tongue tear strength (kg/cm thickness)} = \frac{Tearing\ load\ in\ Kg}{Leather\ thickness\ in\ cm}$$

The tongue tearing strength gives an idea about the fibre strength of the leather because small number of fibres are ruptured at a time in these experiments.

6.3.2.2.3. Stitch tear strength (Double hole): It is the load required to tear the leather between two holes (2 mm dia. Each and 6 mm apart) when the leather thickness is unity.

$$\text{Stitch tear strength (kg/cm thickness)} = \frac{Tearing\ load\ in\ Kg}{Leather\ thickness\ in\ cm}$$

6.3.2.2.4. Percentage of elongation at break: It is the increased length of a 100 cm long leather piece at the point of break. From this experiment an idea is obtained about the sliding effect between the fibres and also about the nature and degree of lubrication.

6.3.2.2.5. Grain crack strength: The pressure required to cause first sign of crackiness on the grain is the grain crack strength. It is generally determined with a lastometer in which a metallic rod exerts necessary pressure on the leather specimen.

The rod can also be replaced by compressed gas or a suitable fluid. With this instrument, percent of area extension, the percentage of radial extension the linear tension in kg/cm are also determined. From this experiment, the condition of grain layer can be known.

6.3.2.2.6. Water vapour permeability test: To perform this test the leather specimen was used as diaphragm with a region of high relative humidity on one side and one of low relative humidity on the other. The gain in weight per unit area on the low relative humidity side in unit time was expressed as the water vapor permeability of the leather specimen.

6.3.3. Results

The crude enzyme (2% of hide weight, 100 ml equivalent to 60,800 U for 5 kg hide) was used to check the bating activity of the *Bl*M9 protease. After deliming the hide was emerged in enzyme preparation and rolled in a drum about 60 minutes for bating. Comparison with the control (commercial bating enzyme Oropon K) clearly showed that *Bl*M9 protease was suitable for bating of the animal hides. Results of different qualitative tests have been shown in Fig.6.3.2. Cross section test resulted in whitish appearance as surface after bating and after chrome treatment pelt appeared light blue. Thumb test showed that bated pelt retain the spots created by the pressure of thumb for few minutes which was a indicative of properly bated pelt. Moreover, the pH of chrome treated pelts should be within 3.7-3.9. In this trial pH of skins bated by both commercial enzyme and *Bl*M9 enzyme were in the range of 3.7-3.9 after chrome treatment.

Name of the tests	Action of commercial enzyme on the pelt	Action of *Bl*M9 protease on the pelt
Bubble test		
Thumb test		
Cross section test (after bating)		
Pelt after chrome treatment		
Cross section test (after chrome treatment)		

Fig. 6.3.2. Results of different qualitative tests of pelt

The results of tensile strength, percent of elongation, stitch tear strength, water vapor permeability and grain crack strength tests (Table 6.3.1) of leather samples treated with *B. licheniformis* M9 bate indicated that this bate was equally efficient to the commercial bate.

Table 6.3.1. Physical test Report of crust leather bated by *Bl*M9 bate and commercial bate

Name of the tests	0.5% commercial bate	2% *Bl*M9 bate	Standard Value*
Tensile Strength test	202.49 Kg/cm^2	254.12 Kg/cm^2	200Kg/cm^2 (minimum)
% of Elongation Test	44.66%	55%	80% (maximum)
Stitch Tear Strength Test	121.20 Kg/cm	173.07 Kg/cm	80 Kg/cm (minimum)
Water Vapor Permeability Test	10.96 mg/cm^2	11.32 mg/cm^2	10 mg/cm^2 (minimum)
Grain Crack Strength (Lastometer)Test	28 Kg	20 Kg	20 Kg (minimum)
Tongue Tear Strength Test	63.06 Kg/cm	55.55 Kg/cm	45 Kg/cm (minimum)

7.1. Discussion

Bacillus spp. is one of the very suitable microorganisms known to secrete industrial enzymes. Since each species or strain has its own nutritional requirement there is no general medium has been established for production of any singly enzyme, also alkaline protease by different microbial strains (Pandey *et al*., 2000; Bhunia *et al*., 2012). Enzyme overproduction is essential to economize the production process and it can be achieved both by genetic manipulation and media engineering. Therefore the study was designed to develop and select an improved strain through classical mutation followed by optimization of fermentation conditions and establish a suitable cost-effective medium using both one variable-at-a-time and statistical approaches. In addition, the enzyme was partially purified, characterized and studied for its application in leather industries.

Strain improvement

Strain improvement has become an essential part for development of commercial fermentation process since the wild strain is usually unable to produce the bulk amount needed for industrial purposes (Glazer and Nikaido, 1995). Several approaches like random mutagenesis, site-directed mutagenesis and gene cloning are commonly used for mutant development. Though new constructions of high yielding variants have been made through gene cloning (Ikemura *et al.,* 1987, Takagi *et al.*,1988, Takami *et al.*, 1992, Aehle *et al.*, 1993, and Tsuchiya *et al.*, 1997), this process is difficult and takes longer time to obtain improved strain. For site directed mutagenesis, the access to the biochemical structure of the protein is a prerequisite to reduce the number of variants created (Damián-Almazo and Saab-Rincón, 2013) whereas random mutagenesis coupled with efficient screening, although laborious, is very effective and a faster process in many cases (Meraz *et al*. 2005, Nadeem *et al*. 2010). Keeping all these under consideration, the present work was designed to use random mutagenesis on previously studied strain, *Bacillus lichenifromis* MZK05 which has been isolated from tannery effluent and was identified by 16S rRNA typing in the International Center for Biotechnology, Osaka University, Japan (Hoq *et al.,* 2005) to enhance protease activity. Four different mutational strategies were used: UV irradiation, EMS treatment, EMS followed by UV (EMS UV) treatment, simultaneous EMS and UV treatment (EMS+UV). When the cells

of *Bacillus lichenifromis* MZK05 were treated with UV the death rate was found more than 98% in all duration of treatments with the highest death rate 99.98% for overnight treatment. In case of treatments with various EMS concentrations for 30 min, the death rate increased with increased dose up to a dose of 200 µl/ml, after which increase in death rate with increasing in EMS concentration was negligible. The death rate in these cases was greater than 90%; but when these EMS treated mutants were exposed to UV for 30 minutes after about 3 months, their death was quite variable with the lowest being 0% and the highest being 67.74%. In cases where MZK05 has undergone 30 min EMS+UV treatment, the death rate was always greater than 99%. For overnight treatments of MZK05 with EMS, EMS+UV and overnight treatment of EMS mutants with UV, the death rates were always found greater than 99%. Several different improved mutants from each treatment were observed for morphological differences. Mutants of EMS treatment and EMS+UV simultaneous treatment produced colonies that were rough and dull, whereas, mutants of EMS UV treatment had smooth and shiny texture.

Following treatments, the various mutants were initially screened through clear zone ratio in Skim Milk Agar (SMA) plates. Mutants with varied morphological appearance in comparison to the parental strain were screened. Larger, wrinkled and raised colonies showed higher enzyme activities compared with smaller, regular and flat colonies. Nadeem *et al*. (2010) and Nehete *et al.* (1986) reported similar observations that rough and wrinkled *B. licheniformis* mutants produced higher yield of alkaline protease. In the present study, it was found that, many mutants with relatively smaller clear zone ratio (diameter of the clear zone divided by diameter of the colony) but wide clear zone diameter with larger colony gave quite high enzyme activity in APPB medium.

Even though clear zone ratio was used for screening, many mutants with relatively smaller clear zone ratio gave quite high enzyme activity. Therefore mutants from these SMA plates were also selected based on the clear zone ratio as well as the diameter of clear zone. Screening through SMA was however a good indicator for simply detecting whether the new mutants had good enzyme activity. Upon mutagenic treatments, overall 182 mutants were selected based on different morphological characteristics for determining the enzyme activity based on zone of casein hydrolysis on SMA medium. Among them, total 100 mutants showed clear zone of casein hydrolysis on SMA which were cultivated in liquid APPB medium in shake flasks. Among them 51 hyperactive mutants showing proteolytic activity greater than 100 U/ml in APPB medium were

selected for further studies. These mutants were checked regularly for enzyme activity; that is, their stability in producing protease was monitored. For almost all mutants there was a fall in activity. This happened probably because mutagenic treatments have made the bacterial cell metabolism unstable or the mutated bacteria are constantly trying to revert back to their old form. Such patterns were observed in all mutants except for *Bl*EO17 generated through treatment with EMS at a concentration of 250 µl/m for overnight and *Bl*M9 generated through UV treatment for 30 min. These two stable mutants were selected for further analysis.

The morphological characteristics and different substrate hydrolytic capacity of the mutants were compared with the wild MZK05 strain. As the genetic improvement of the wild *Bacillus licheniformis* MZK05 was designed to develop a potential strain to overproduce the protease that could be used in leather industries. The leather industries require enzyme with unique properties that can degrade albumin, globulin, elastin specifically without causing any damage to the collagen, the main structure of the leather itself. From the substrate specificity study it was found that *Bl*EO17 hydrolyzed albumin, globulin, elastin and collagen where as the *Bl*M9 showed activity against all these substrates except collagen. Therefore the *Bl*M9 was selected as a unique strain in terms of having selective activities against non structural proteins of leather without affecting collagen, the main structural protein of leather. This seems to be suitable for bating operation of hides and skins. Hence, the subsequent studies for optimization of the fermentation conditions, development of the cost effective medium, partial purification and characterization of the enzymes and technical application of the enzyme were carried out using the mutant *Bl*M9.

Optimization of fermentation conditions for production of protease by *Bl*M9:

Effect of temperature: For protease production temperature and its regulation is one of the most critical parameters (Singh *et al.,* 2011). Cultivation temperature affects protein synthesis by influencing rate of biochemical reactions within the cell and consequently inducing or repressing enzyme production (Bakermans and Nealson, 2004). However, studies by Frankena *et al.*, (1986) showed that a link existed between enzyme synthesis and energy metabolism in Bacilli, which was controlled by temperature and oxygen uptake. In this study, *B. licheniformis* MZK05M9 (*Bl*M9) exhibited protease production at temperatures studied (30-45°C) but optimum temperature for protease production was

obtained at 37°C, increasing or decreasing the temperature beyond this led to the decrease in enzyme production. However, about 75% of the enzyme production was obtained at 40°C but decreased rapidly at its beyond, these results were in harmony with the findings of Asokan and Jayanthi (2010) and Mabrouk *et al.*, (1999). Rahman *et al.*, (2005) also observed optimum temperature for enzyme production in *Pseudomonas aeruginosa* strain K at 37°C and by increasing to higher temperature up to 40 and 45°C caused more than 24.0% and 53.0% loss of enzyme activity and no protease were observed at low 4°C or at high 50°C or higher temperature. Higher temperatures at (or) around 45°C have been reported to be optimum for the production of protease by bacteria such as *Bacillus* sp. P-001A (Atalo and Gashe, 1993), *Bacillus licheniformis* S40 (Sen and Satyanarayana,1993), *Bacillus sp.* (Sepahy and Jabalameli, 2011) and *Bacillus subtilis* SHS-04 (Olajuyigbe, 2013). However, Gouda, 2006 reported a lower optimum temperature of 30°C for protease production by *Bacillus sp*. MIG.

Effect of pH on protease production by *Bl*M9: The metabolic activities of the microorganisms are very sensitive for pH variation (Sher *et al*., 2012) and the optimum pH have important role in enzyme yield (Ali *et al.,* 1998). The effect of initial pH of the culture medium on the enzyme production by *Bl*M9 was studied in pH range 6.5-8.5. The initial pH was adjusted with NaOH or HCl. The enzyme activity of *Bl*M9 in the APPB medium was found to increase with increasing pH up to an optimum of 7.5, beyond which the activity was found to decline. The results clearly indicated that the enzyme was synthesized by *Bl*M9 in neutral as well as in alkaline pH but maximally produced at slight alkaline pH. Similar result was also observed by Seifzadeh *et al.*, 2008.

The optimal pH for other *Bacillus* bacteria reported in literature are pH 7.0 for *Bacillus* sp. K2 (Hameed *et al.*, 1999) and *B. licheniformis* ATCC 21415 (Mabrouk *et al*, 1999), pH 9– 9.5 for *Bacillus* sp. P-2 (Kaur *et al*., 2001) and *Bacillus subtilis* strain AKRS3 (Ravishankar *et al*., 2012).The important characteristic of most alkaliphilic microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production (Aunstrup, 1980). *Bacillus* sp. JB99 (Johnvesly and Naik, 2001), *Bacillus* sp. SSR-1 (Singh *et al*, 2001) and *Bacillus brevis* MTCC B0016 (Banerjee *et al.*, 1999) were reported to have optimum pH 10.0 – 10.5.

Effect of inoculum size on protease production by *Bl*M9: A very low inoculum size can extend lag phase leading to delay in production of extracellular enzymes. On the other hand, a very high inoculum density could also lead to growth inhibition due to presence of excessive concentrations of toxic, inhibitory metabolic substances (Ravishankar *et al*., 2012). A moderate inoculum size of 5 % was found to be sufficient for optimal enzyme production by the *Bl*M9. Optimum protease production was reported with inoculum size 2% for *Bacillus licheniformis* N-2 (Nadeem *et al*., 2008), *Bacillus alcalophilus* (Kanekar *et al*., 2002) and 4% for *Bacillus subtilis* strain AKRS3 (Ravishankar *et al*., 2012). However, higher inoculum size 10% was also found as optimum for protease production by *Bacillus subtilis* PCSIR-5 (Nadeem *et al*., 2006). All these findings indicate that inoculum size has an effect on protease production which depends on the characteristics of the strains

Effect of inoculum age on protease production by *Bl*M9: The age of the inoculum influences the pattern of growth and product formation by the microorganism (Ravishankar *et al*., 2012). An inoculum age of 16 hours was found to be optimum for alkaline protease production by the present bacteria *Bl*M9. Decreased or increased in age of inoculum were found to have negative effect on alkaline protease production by mutant *Bl*M9. Culture of 24h age had maximum protease productivity by *Bacillus licheniformis* BS2 (Rojarani and Ganesh, 2014), *Bacillus licheniformis* N-2 (Nadeem *et al*., 2008) and *Bacillus alcalophilus* (Kanekar *et al*., 2002).

Effect of aeration and agitation in protease production by *Bl*M9 in 7L Bioreactor using APPB medium:

To evaluate the effect of agitation speed and aeration rate on protease production by *Bl*M9 fermentation was carried out in 7 L bench-top bioreactor at different agitation speed and aeration rate. Results indicated that enzyme production was highest by *Bl*M9 when the agitation speed of 300 rpm and aeration rate of 1.0 vvm were selected. Any change beyond these selected values declined the enzyme production. Because agitation speed influence the mixing of medium ingredients as well as DO level in the growth medium (Nascimento *et al*., 2004). Pastor *et al*., 2001 observed highest yield of protease enzyme with the agitation speed of 750 rpm and aeration rate of 1vvm. Samarntarn *et al.*199 worked on alkaline protease production in 5 L fermentor using genetically engineered *Aspergillus oryzae* and observed best results with the aeration rate of 0.5vvm

and agitation speed of 500 rpm. Similar effects of higher agitation rates on cell growth had been documented by some other researchers (Demirtas 2003, Gibbs and Seviour, 1996, Hewitt *et al*., 2000).Some studies reported that agitation speed of 200 rpm results an enhanced protease production by *Halobacterium* sp. (Vijayanand *et al.,* 2010), *Teredinobacter turniae* (Elibola and Moreira, 2005) and *Bacillus halodurans* (Ibrahim and Al-Salamah, 2009). Agitation affects both air bubble dispersion and mixing of nutrients during fermentation process. In one study, *Bacillus* sp. B21-2 produced increased enzyme titre when agitation speed of 600 rpm and aeration rate of 0.5 vvm were selected (Fujiwara and Yamamoto 1987). Similarly, *Bacillus firmus* exhibited maximum enzyme yields at an aeration rate of 7.0 l /min and an agitation rate of 360 rpm (Moon and Parulekar, 1991). However, these results indicated that by lowering the aeration rate and agitation speed there was reduction in the protease yields.

The results of the present study showed that relationship of different process parameters are necessary for the maximum yield of alkaline protease production by *Bl*M9 during submerged fermentation.

Development of a cost effective medium:

Optimization by one variable-at-a-time approach: The demand of eco-friendly technology is increasing day by day to reduce the pollution at industrial level. Being an eco-friendly compound, enzymes got application at different industrial sectors. There is no general medium for protease production by different microbial strains (Pandey *et al.,* 2000). Every microorganism evidences its own nutritional requirements for growth and enzyme secretion (Reddy *et al*., 2008). In this study, enzyme production was carried out under shake flask culture at 37°C and 150 rpm for the production of alkaline protease by *B. licheniformis* MZK05M9 (*Bl*M9) in medium containing glucose 10.0 g/l, soybean meal 10.0 g/l, K_2HPO_4 3.0 g/l, $MgSO_4$ 0.5 g/l, $CaCl_2$ 0.5 g/l and NaCl 0.5 g/l at initial pH 7.5. After 48 hrs fermentation, enzyme activity of 325±5 U/ml was obtained. The extracellular protease production in microorganisms is greatly influenced by concentration of carbon and nitrogen sources (Bhunia *et al*., 2012). Hence, to increase the production of the enzyme, optimization of the concentration of carbon source and nitrogen source were studied. Initially, the traditional one variable-at-a-time method was employed for selection of appropriate concentration. The 'one variable-at-a-time' approach is the most

frequently used operation in biotechnology to obtain maximum cell density, high yields of the desired metabolic product, or enzyme levels in a microbial system (Puri *et al*., 2002). In the present study the glucose was used as carbon source. But, glucose is a readily metabolizable substrate (Lin *et al*., 1998) and due to cost of the glucose the overall cost of the medium might be increased. Therefore, pure glucose is rarely used for industrial scale fermentations (Waites *et al*., 2001). So, the attempt was taken to replace the glucose by molasses, a byproduct of cane and beet sugar production, a cheaper and more usual source of sucrose. This material is the residue remaining after most of the sucrose has been crystallized from the plant extract. It is dark coloured viscous syrup containing 50–60% (w/v) carbohydrates, primarily sucrose, with 2% (w/v) nitrogenous substances, along with some vitamins and minerals (Waites *et al*., 2001). In the study, 0.5% molasses supported the highest protease production (420 ± 5 U/ml) in the shake culture. Lakshmi *et al.* (2014) recorded maximum protease production from *Bacillus* sp. in the presence of molasses. In another research, Younis *et al.,* 2009 claimed that only molasses supported the production of considerable amount of protease by *Bacillus subtilis* KO. Another researcher used molasses 0.3% to enhance protease production by *Streptomyces* sp. (De Azeredo *et al*., 2004).

The concentration of soybean meal, the nitrogen source was also optimized to obtaim maximum level of enzyme titre. Soybean meal, the residues remaining after soya beans have been processed to extract the bulk of their oil are composed of 50% protein, 8% non-protein nitrogenous compounds, 30% carbohydrates and 1% oil (Waites *et al*., 2001). In this study, 1.0% Soybean meal was found optimum for maximum enzyme production by *Bl*M9. Soybean meal was also reported to be a suitable nitrogen source for protease production (Chandrasekaran and Dhar, 1983; Cheng *et al*., 1995; Sen and Satyanarayana, 1993; Tsai *et al*., 1988).

Optimization by statistical approach: Medium optimization by a conventional “one-at-a-time-approach” does leads to a substantial increase in enzyme yields, however, this approach is not only cumbersome and time consuming, but also has the limitations of ignoring the importance of interaction of various physiochemical parameters (Puri *et al*., 2002). The use of statistical models to optimize culture medium components has increased in present-day biotechnology, due to its ready applicability and aptness. The statistical approach using response surface methodology for medium optimization can find out the optimal concentration of the ingredients by establishing the relationship

between more than one variable and given response. In this present study selection and optimization of the concentration of ingredients for maximal alkaline protease production through *Bl*M9 fermentation were carried out. The mathematical analysis has been carried out by standard software Minitab version 17. The significant variables necessary for enhanced protease production, soybean meal, molasses, NaCl, K_2HPO_4 and $MgSO_4.7H_2O$ were selected using the Plackett–Burman design. Then RSM was used for optimization of the ingredients' concentration and to observe the interactions among various variables. When protease production was observed as a response to the interaction of soybean meal and molasses keeping other ingredients' concentration at central points, an enhancement in protease production was observed at the central concentration of Soybean meal and molasses. Thus, maximal enzyme production could be obtained at mid-value of Soybean meal and molasses. A high degree of similarity was observed between the predicted and experimental values that reflected the accuracy and applicability of RSM to optimize the process for *Bl*M9 enzyme production. Similar improved production was reported in other RSM experiments, most notably in the case of amylase from *Bacillus circulans* GRS313 and in the case of protease production using *Bacillus* sp. RGR-14 (Dey *et al*., 2001; Chauhan and Gupta, 2004). This present result confirms earlier reports regarding protease production, in which it has been observed that the complex carbon and nitrogen sources constitute better substrates for protease production (Reddy *et al*., 2008).

In addition, the granular sizes of the Soybean meal affected the enzyme production revealing 4.7 mm mesh size supported the enzyme production 5% more than that of the mixed sizes which ranged from 6.5 to less than 4 mm. The smaller granular size makes the medium condition more viscous which subsequently prevent the transfer of the oxygen to the medium. On the other hand, the larger size of the granules may interfere with the microbe in breaking down the granule and taking the nutrition. In bioreactor the optimized medium supported the enzyme production 1.3 fold higher than that in shake flask suggesting that the optimized medium is suitable for large-scale enzyme production by *Bl*M9.

To produce alkaline protease many researchers used inexpensive substrates coupled with expensive one as carbon and nitrogen source such as Glucose and Soybean meal (Nadeem *et al.,*2008; Saxena and Singh, 2010; Irfan *et al*., 2010), Soybean meal and Trypton (Zambare, 2013)**,** Wheat bran and Soybean meal (Saurabh *et al*., 2007), Starch and Soybean meal (Oberoi *et al*, 2001), Molasses and Potassium nitrate (Lakshmi

et al., 2014), Molasses and Urea (Wahyuntari and Hendrawati, 2012), Starch, Wheat bran and Soya flour (Zambare *et al*., 2007), Wheat bran and Beef extract (Naidu and Devi, 2005), Glucose and Ground nut meal (Olajuyigbe, 2013). However, there are fewer attempts reported to induce protease production using inexpensive both carbon and nitrogen sources (Reddy *et al*., 2008). In the present study, the established optimized medium containing locally available cheap agro-industrial residues molasses and soybean meal as carbon and nitrogen source respectively, will result in a significant reduction in the cost of medium constituents. Therefore, the present study will facilitate the effective economization of overproduction of the alkaline protease by *Bl*M9. In this regard, the developed medium for enhanced production of protease by mutant *Bl*M9 can be said unique.

Partial purification and characterization:

Purification of the *Bl*M9 enzyme:

After 28 hrs of cultivation, the culture liquid was centrifuged at 8,000 g for 10 minutes for solid liquid separation. After separation, the purification of the enzyme was followed. The first step used was precipitation of the enzyme protein by ammonium sulfate. The reaction between salt and crude enzyme solution resulted in precipitation of the protease, while the impurities remained in the supernatant. By gradual increase of the saturation of the ammonium sulfate salt 20% to 60% the enzyme was extracted from crude solution.

The precipitates so obtained were subjected to dialysis for further removal of excess of salts and impurities. The dialyzed extract was applied for gel filtration on sephadex G75 for further purification. The material under proteolytic peaks was obtained with a high purity. The partially purified protein showed a specific activity of 1624 U/mg protein and the purification factor was 2.70. SDS-PAGE analysis of purified enzyme showed a molecular mass of about 27.2 kDa. The protease emerged as a single symmetrical peak from a gel chromatography. Same kind of result was obtained by Kumar (2002) with serine alkaline protease having 28 kDa. Shimogaki ***et al.*** in 1991 purified by ammonium sulfate precipitation, ion exchange and gel filtration chromatography and obtained 8-fold purification and 52% recovery of the protease with 42 kDa.

Effect of temperature on enzyme catalysis and stability:

The enzyme was active in a wide temperature range from 30°C to 70°C, with an optimum at 55°C while catalyzed the azocasein for 60 min. The relative activities at 50 and 60°C

were about 83% and 75%, respectively. The optimum temperature of alkaline protease was similar to the values reported by Kumar *et al*., 2012.

The purified enzyme showed different temperature stability at various temperatures (30-70°C). Results indicate that the enzyme was stable at 40°C for 60 min without loss of any activity. Whereas at 50°C the relative activity become half (50%) of the original activity and at 70°C it becomes zero (0%). However, addition of $CaCl_2$ stabilizes the enzyme (100%) up to 50°C.

Effect of pH on enzyme activity and stability:

The partially purified enzyme showed higher activity between pH 7.0 and 9.5 with an optimum at pH 8.5, indicating its alkaline nature. The relative activities at pH 6.5 and 10.0 were about 74% and 69%, respectively, of that at optimal pH. In this study, the optimum pH 8.5 was recorded which is lower than the pH optima values, viz pH 11.5 (Kumar, 2002), pH 9.0 (Dhandapani and Vijayaragavan, 1994), pH 10.0 (Gupta *et al.*, 2008), pH 10.5 (Beg and Gupta, 2003) and pH 11.0 (Margesin *et al.*, 1992) reported earlier for other enzymes from alkaliphilic *Bacillus* spp.

The pH stability profile of alkaline protease showed that the partially purified enzyme was stable (100%) between 7.0 and 8.0 but in presence of $CaCl_2$, the enzyme showed 100% stability in wide pH ranges (pH 6.0-9.0).

However, the preset enzyme with the optimum pH and stability obtained will suitably comply its application in leather industries.

Effect of metal ions on the enzyme:

The results recorded indicating that in presence of Ba^{2+}, K^{+}, Ca^{2+}, Mg^{2+}, and Mn^{2+} the proteolytic activity resulted in discreet increase, while Hg^{2+} showed inhibitory effect. Metal like Na^{+} restored the enzyme activity as 97% of the original activity. Whereas other metals like Zn^{2+} and Cu^{2+} showed appreciable inhibitory effect on enzyme with residual activity of 38 and 33% respectively. Shimogaki *et al.*, (1991) also found stimulatory effect of Mn^{2+} on serine alkaline protease. Tsujibo *et al.*, (1990) and Aretz *et al.*, (1989) obtained best results with Ca^{2+} and Ba^{2+} with an increase in activity around two-fold. Many alkaline proteases were reported to be inhibited by mercury (Beg and Gupta, 2003) which is harmonious to this study.

Effect of inhibitors on the enzyme:

Results of sensitivity of partially purified *Bl*M9 protease to various protease inhibitors showed that strong inhibition (76% inhibition) was achieved by pre-incubation with PMSF indicating that the enzyme belongs to serine group proteases. However, EDTA, a metalloprotease inhibitor, and -mercaptoethanol, a cysteine protease inhibitors, were only slightly inhibited the protease with 29 and 17% inhibition respectively. Dhandapani and Vijayaragavan (1994) isolated *Bacillus stearothermophilus* strain AP-4 producing thermostable alkaline protease that was completely inactivated by PMSF, EDTA and -mercaptoethanol and proposed a metal ion-dependant alkaline serine protease.

Evaluation of the enzyme as detergent-additive:

Effect of bleach, surfactants and some detergents on enzyme activity: The feasibility of using the alkaline protease as a laundry detergent-additive was evaluated by determining its stability towards some surfactants, oxidant and some laundry detergents available locally. The partially purified protease was stable at low concentration (1%) of SDS with relative activity of 62%. While it was found stable in Triton X-100 and H_2O_2, it was unstable in Tween-80. Gupta *et al.*, (1999) reported that the protease from *Bacillus* sp. SB5 retained about 60 and 95% of its activity on treatment for 1 hr with 1% SDS and 5% H_2O_2, respectively, while the *Bacillus* sp. JB-99 protease retained 75 and >95% of its activity on treatment for 1 hr with 0.5% SDS and 5% H_2O_2, respectively (Johnvesly and Naik, 2001). Johnvesly and Naik (2001) also reported that in the presence of 3 and 5% Triton X-100, the enzyme retained 95 and 74% of original activity.

Compatibility of the *Bl*M9 enzyme in different detergents: A good detergent protease is expected to be stable in the presence of commercial detergents. The protease from the *Bl*M9 showed excellent stability and compatibility in the presence of locally available detergents (Surf Excel, Wheel, Tibet, Keya, Jet and Fast Wash) at room temperature. The enzyme retained activity at room temperature with Surf Excel more than 65%, in Wheel 86%, in Tibet 113%, in Keya 87%, in Jet 70% and in Fast Wash 98% after 60 min incubation. This suggests that the *Bl*M9 enzyme nearly compitable in the complex harsh components of the detergent formutalion and this might be suitable for its application as detergent additives. Uddin *et al.*, (2006) reported that protease from *Bacillus licheniformis* MZK05 retained 18, 24, 25, 54, 50, 32 and 27% of its original activity in

presence of Jet, Surf Excel, Wheel, Keya, Chaka, Tide and Rain detergents respectively. Singh *et al.*, (2001) reported that SSR1 protease retained 40-90% of its activity in presence local detergents. Hence, the present protease may be suitably used as cleansing aid in detergent formulation like Tibet.

Application of the enzyme:

Application of the enzyme in dehairing of goat skin:

Conventional dehairing of animal skins/hides in leather manufacturing, involves the generation of hydrogen sulfide with highly alkaline effluent due to the use of toxic chemicals like lime and sodium sulfide, can cause a serious health hazard to tannery workers and contributes to aquatic pollution. Thus the present work was performed to find out the possible eco-friendly dehairing process of animal skins using alkaline protease and keratinase preparation, obtained from the mutant *Bl*M9 strain. Investigation of the dehaired skin upon treatment with the enzymes was carried to measure the hair removal potential of the enzymes and as well as to determine visually the degree of its smoothness. The results exhibited that maximum area yield was found rapidly when skins were treated by conventional lime-sulfide method. However this treatment might have denatured and coagulated the skin proteins at high pH and subsequently the skin become wrinkled and lost its smoothness. On the other hand, skin treated by enzyme mediated (only *Bl*M9 enzymes treatment) method provided comparatively less area yield (85%). But in case of enzyme assisted method i.e. treatment with 5% CaO for 6 hrs followed by enzymatic action produced area yield comparable to the conventional method. This may be possible because of soaking the goat skin in 5% CaO solution, during which collagen fibers swelled osmotically by taking up water from the lime solution contributed by removing the electrical charge from the basic groups in collagen and changing the dimensions of its structure and this decreased the cohesive forces between the fibers by breaking H-bonds, causing the fiber structure to become looser (Herfeld and Schubert 1969, Menderes *et al.,* 2000) and opens up the collagen fiber bundles (Alexander *et al*., 1986).Thus the protease enzymes can enter more easily into the skin and open the fibrous structure by degrading the inter fibrillar substances (Sivasubramani *et al*., 2008). This is followed by loosening of hair with an attack by enzymes on the outmost sheath and subsequent swelling and breakdown of the inner root sheath (Alexarder, 1988).

One of the advantage of the present enzyme assisted method is the short duration of soaking in lime solution does not damage the collagen fiber structure rather it helps accelerating the penetration of alkaline protease and keratinase through collagen matrix to act upon anchoring proteins around the hair follicles eventually facilitating the removal of hair. The Scanning Electron Microscopy study revealed that the surface of the skin treated with enzyme assisted method was smoother and silkier than that treated with lime sulfide method. Because lime-sulphide removes the hair above the epidermis for which the leather doesn't become silky, but the proteases attack the hair below the dermis and improve the silkier quality of the final leather preparations (Malathi and Dhar, 1987).

Application of the enzyme in bating of hides:

Bating is the step in leather processing where enzymatic process cannot be substituted by chemical processes. The process of bating gives certain desired characteristics in the finished leather. The bating process as practiced today is by far more pleasant, safer and less offensive in odour as compared to the earlier process using the infusion of dog dung or manure. Modern bating procedure employs pancreatic enzyme or proteolytic enzymes of bacterial origin. Enzymes bate is one of the essential auxiliaries and its use is absolutely essential for the manufacture of leathers like glove, shrunken grain softie, nappa, garment and glace kid.

The main objective of bating is to remove some of the non-leather forming proteinous materials like albumins, globulin and mucoids from hides and skins and to allow the splitting up of collagen fibres so as to help in the penetration of tanning materials and other processing chemicals thereby giving the finished leather the desired feel, softness, pliability and other characteristics properties. After liming, the skins usually turn into swollen condition. The swollen pelt fibres must be reduced so that they will not be resilient when tanned. The main function of the bating is to remove all non-collagenous proteins and degraded collagen fibrils (Puvanakrishnan and Dhar 1988). Bating also brings about this reduction by removing the lime. Bating brings about the following other effects in the pelts:

I. Produces a silky grain
II. Bated pelts are slippery, non elastic and flaccid

III. The scud or dirt i.e. short hair, grease and lime soaps dark colored pigments and traces of epidermis are all loosened and are easily removable by scudding

IV. Remains of hypodermic tissue are loosened so that they can easily be removed by scrapping

V. Increases the degree of stretch

It is well known that one of the actions of bate is the removal of breakdown products such as grease, epidermal residue and glands, which is brought about by a combination of proteolytic enzymes present in the bate. Bating enzymes produce softening of the collagenous fibre structure (Pfannmueller, 1956) and this helps expel the degraded epidermal matter or scud from the interior of the skin. Further, mechanical agitation during bating loosens and also helps in the removal of scud and surface debris.

According to Ornes and Roddy (1960), 30-80% of elastin is removed by the use of pancreatic enzyme preparations.

For determining the efficiency in bating, *Bl*M9 alkaline protease was compared with a commercial enzyme Oropon K (one of the commercial bates imported in Bangladesh for bating purpose). The results of different qualitative tests such as the bubble, thumb and cross section tests and the physical tests of crushed leather such as tensile strength test, percent elongation test, stitch tear strength test, water vapor permeability test and grain crack strength (Lastometer) test indicated that *Bl*M9 enzyme was equally efficient to the commercial bate Oropon K. Such results are of great importance in the sense that if we can produce *Bl*M9 enzyme as bate locally in commercial scale it will save the currency by reducing the import of commercial bate and at the same time it will also save the environment by reducing the use of harsh chemicals in tannery industry.

7.2. Conclusion

The aim of this research was to develop a bioprocess for production of protease by *Bacillus licheniformis* strain. In this research the bioprocess for production of the protease has been established on local economic substrates by the mutant *Bl*M9.

- The mutant *Bl*M9 was selected for the production of protease with respect not only enzyme titre but also substrate specificity. The *Bl*M9 enzyme selectively can degrade the albumin, globulin, elastin but not the collagen, the main structure of the leather. Thus the *Bl*M9 is suitable to produce protease for application in bating of hides in leather processing.

- In the present study, it was found that strain improvement and optimization of the medium together resulted in 25 fold augmentation in the enzyme titer than that produced by wild strain in APPB medium. The large-scale enzyme production thus will be cost effective as the carbon and nitrogen source are based on cheap agro-industrial substrates.

- 0.5% polyethylene glycol (PEG 4000) found to be suitable for retaining 100 % and 90 % activity of the liquid enzyme for more than two weeks and 4 weeks, respectively in room temperature.

- The enzyme was partially purified, characterized and tested for its efficacy in some technical applications and the major use of the present protease was found in leather manufacturing industry where the results of different qualitative and quantitative tests suggested its potentiality in bating of skins and hides.

- The protease also demonstrated promising application in dehairing of animal skins and hides which can save the environment from toxic sodium sulfide.

- The protease demonstrated good stability towards surfactants and oxidants, which are usually used in the formulation of common bleach-based detergents.

Thus the application of the present enzyme will lead to develop eco-friendly hide processing of leathers reducing the disposal of hazardous chemicals into the environment.

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Publication and conference presentation

Publications:

1. **Mamun MAA**, Khan MM, Akand MNR, Khan SN, Hoq MM (2015) Characterization of an alkaline protease with high quality bating potential in leather processing from *Bacillus licheniformis* MZK05M9 mutant. International Journal of Biological Research. Vol. 3 issue 1, p. 36-41.

2. **Mamun MAA,** Hosain MA, Ahmed S, Zohra FT, Sultana R, Khan MM, Akhter MZ, Khan SN, Hoq MM (2015) Development of an alternative enzyme-assisted dehairing method of animal skins using proteases from *Bacillus licheniformis* MZK05M9. Bangladesh J Microbiol, vol. 32, issue (1&2), p.33-37

3. Hoq MM, **Mamun AA**, Shishir MA, Khan MM, Akand MNR and Khan SN (2013) Bioprocess development for eco-friendly microbial products and impacts on bio-industry establishment in Bangladesh. Proceedings of international conference on biotechnology, 25-26 May, 2013.

Conference presentations:

1. Hoq MM, **Mamun AA**, Nahar M, Mian MM, Khan TT, Karim MM and Khan SN, Key note presentation on, "Bioprocess development for eco-friendly industrial enzymes from *Bacillus licheniformis",* International conference on metabolic science, 20 – 23 October, 2016*,* Nanjiao hotel, Shanghai, China
2. **Mamun MAA,** Khan SN and Hoq, MM, Poster presentation on "Production of alkaline protease with bating activity by *Bacillus licheniformis* mutant in modified soy meal medium", 27th annual conference of Bangladesh Society of Microbiologist, 27 April 2014, Department of Microbiology, University of Dhaka, Dhaka-1000, Bangladesh
3. **Mamun MAA,** Poster presentation on **"**Enhanced production of protease by *Bacillus* MAS-6 Mutant and application in Bating of hides in leather", Sixth HOPE Meeting with Nobel Laureates, 11-15 March 2014, Tokyo, Japan
4. **Mamun MAA,** Amin MR, Hossain SR, Akand MNR, Ahmed S, Zohra FT, Khan SN and Hoq MM, Poster presentation on "Appropriate bating enzyme for use in Bangladesh tanneries", International conference on biotechnology organized by committee of action for research, extension and services (CARES), 25-26 May 2013, The Westin Dhaka, Dhaka, Bangladesh

APPENDIX – A

CHEMICALS AND REAGENTS

All chemicals were of analytical grade and were purchased from a variety of suppliers.

Name of chemicals/reagents/substrates	**Source**
Acrylamide	Carl Roth, Germany
Agar	Sigma, USA
Agarose	Promega, USA; Carl Roth, Germany
Albumin	Sigma
Aluminium sulfate	Merck, India
Ammonium persulphate	Wako, USA
Ammonium sulfate	Merck, India
Antifoam	Sigma, USA
Azo-casein	Sigma, USA
Bis-acrylamide	Carl Roth, Germany
Boric acid	Merck, India
Bromophenol Blue	Wako, USA
Buffer solution pH 4	Thermo scientific
Buffer solution pH 7	Thermo scientific
Buffer solution pH 10	Thermo scientific
Bovine serum albumin (BSA)	Sigma, USA
Calcium chloride (CaCl2)	Sigma, USA
Collagen	Sigma
Coomassie Brilliant Blue G250	Thermo Scientific, USA
Di potassium hydrogen phosphate	Merck, Germany
Di sodium hydrogen phosphate	Merck, Germany
Dithiothreitol (DTT)	American Bioanalytical, USA
Protein quantification kit	Fluka, Japan
EDTA	BDH, England
Elastin	Sigma
EMS	Fluka
Ethanol (EtOH)	Merck, Germany
Ethidium bromide (EtBr)	Sigma, USA
Ferric ammonium citrate	Sigma, USA
Glacial acetic acid	Merck, Germany
Globulin	Sigma, USA
Glucose	Sigma, USA
Glycerol	Sigma, USA
Glycine	Wako, USA
Hydrochloric acid (HCl)	Merck, Germany
Immersion oil	Merck, Germany
Iodine	Sigma, USA
Isoamyl alcohol	Merck, Germany
Isopropanol	Merck, Germany
Potassium chloride	Sigma, USA
Potassium dihydrogen orthophosphate	Merck, Germany
Lysozyme	Wako, USA

Methanol	Sigma, USA
Magnesium chloride	Sigma, USA
Magnesium sulfate	Sigma-Aldrich
Manganese chloride	Merck, Germany
Mercaptoethanol	Sigma-Aldrich
Molasses	Local market
Mustard seed meal	Local market
Na_2CO_3	Sigma, USA
Na_2HPO_4	Merck, Germany
Sephadex G-75	Sigma-Aldrich
Skim Milk Powder	Fluka
Sodium chloride	Sigma, USA
Sodium dihydrogen orthophosphate	Merck, Germany
Sodium sulfide	Sigma-Aldrich
Coomassie Brilliant Blue R-250	Sigma
Peptone	Oxoid, England
Phosphoric acid	Merck, Germany
Phenol red	Sigma, USA
Phenyl methyl sulphonyl fluoride	Sigma, USA
Polyethylene glycol-4000	BDH, England
Potassium hydrogen phosphate	Sigma, USA
Protein marker	Precision plus protein standards (All blue), Bio-Rad, USA; Pre-stained protein Marker, NEB, England
Rice bran	Local market
Sodium acetate	Merck, Germany
Sodium dodecyl sulphate (SDS)	Wako, USA
Sodium hydroxide (NaOH)	Sigma, USA
Soybean meal	Local market
Sucrose	Merck, Germany
Tri-chloro acetic acid	Sigma, USA
Tris base	Sigma, USA
Triton X-100	Daejung chemicals
Tryptone	BD, USA
Tryptose	BD, USA
Tween-80	Merck
Wheat bran	Local market
Yeast Extract	Sigma, USA
Zinc chloride	Sigma-Aldrich
Calcium chloride	Sigma
Barium chloride	Sigma-Aldrich
Copper sulphate	Sigma
Manganese chloride	Sigma-Aldrich
Mercuric chloride	Sigma-Aldrich
Potassium chloride	Sigma-Aldrich
Hydrogen peroxide	Sigma

APPENDIX – B

MEDIA COMPOSITION

Alkaline Protease Producing Broth

Glucose	10 g/L
Peptone	5.0 g/L
Yeast extract	5.0 g/L
NaCl	5.0 g/L
K_2HPO_4	5.0 g/L
$MgSO_4$	0.1 g/L

Tryptone Soya Broth (TSB)

Casein peptone (pancreatic)	17.0 g/L
Soya peptone (papain digest)	3.0 g/L
Glucose	2.5g/L
NaCl	5.0g/L
K_2HPO_4	2.5g/L

Tryptone Soya Agar (TSA)

Casein peptone (pancreatic)	17.0 g/L
Soya peptone (papain digest)	3.0 g/L
Glucose	2.5 g/L
NaCl	5.0 g/L
K_2HPO_4	2.5 g/L
Agar	16 g/L

Skim Milk Agar

Skim milk	100.0g/L
Agar	16.0g/L

APPENDIX - C
BUFFERS AND SOLUTIONS

Azo-casein solution (1.0%)
Azo-casein solution was prepared by dissolving 1.0 g of azo-casein in 100 ml 0.05 M Tris-HCL buffer (pH: 8.5). The solution was preserved at 4°C.

1× Laemmli buffer
Ingredients were mixed in a way so that the final concentration becomes 50 mM Tris-HCl (pH 6.8), 10% (v/v) glycerol, 2% (w/v) SDS, 100 mM DTT and 0.1% bromophenol blue.

NaOH (1 N)
40 g of NaOH pellet was dissolved in distilled water to final volume of 1000 mL. The solution was stored in an airtight bottle at RT.

Normal saline
Normal saline was prepared by dissolving 0.85g NaCl in 100ml of distilled water and sterilized by autoclaving.

Phenol Red Solution
0.1 g phenol red was dissolved in 28.2 ml 0.01M NaOH and 221.8 ml water to prepare a 250 ml solution of Phenol red.

Phosphate buffer
Na_2HPO_4 was dissolved in distilled water to make a 0.05 M solution and the pH was adjusted to appropriate value with 0.05 M NaH_2PO_4.

SDS (10%)
10g of SDS (Sodium dodecyl sulfate) (Siga) was added to 80 mL of distilled water and dissolved by stirring on a magnetic stirrer slowly to avoid foaming. The final volume was adjusted to 100 mL with distilled water and stored at RT.

Tris-HCl (1.0 M)
121.1 g tris-base was dissolved in 800 mL of distilled water. The pH was adjusted to the desired value by adding concentrated HCl and the final volume was made up to 1L with distilled water. The solution was sterilized by autoclaving and stored at room temperature (RT).

Reagents for SDS-PAGE

1. 30% acrylamide-bisacrylamide solution:

Acrylamide	: 29.0 g
Bisacrylamide	: 1.0 g
Distilled water	: 100 ml

2. 10% ammonium persulphate (APS)

APS	: 1.0 g
Distilled water	: 10 ml

Stored at 4°C

3. 0.1% BPB (Bromophenol blue solution) or tracking dye

Bromophenol blue	: 0.1 g
Distilled water	: 100 ml

4. Staining solution

Coomassie brilliant blue G-250	: 0.20 g
Phosphoric acid (85%)	: 20 ml
Aluminium sulfate	: 50 g
Absolute Ethanol	: 100 ml

Deionized water was added to adjust the volume up to 1000 ml.

5. Sample loading buffer

0.5 M tris-Cl (Upper gel buffer)	: 10 ml
10% SDS	: 10 ml
1.0M DTT	: 5 ml
Glycerol	: 10 ml
Distilled water	: 14 ml

6. Electrophoresis buffer (pH 8.3)

Tris-base	: 3.0 g
Glycine	: 14.4 g
10% SDS	: 10 ml
Distilled water	: 1000 ml

APPENDIX – D

EQUIPMENT

Equipment	Model/ Company/Origin
Autoclave	LTE Scientific/Touchclave, England
Balance	Shinko Denshi, Japan
Bioreactor,7.0 L	Bioflo 110, New Brunswick Scientific, USA
Biosafety cabinet	Telstar/Bioultra 4, Spain
Centrifugation	Hitachi/CT 15E, Japan
Centrifuge machine	Kokusan/H-9R, Japan
Electronic fine balance	KERN, ABS, Germany.
Balance balance	Vibra/AJ-320CE, Japan
Electrophoresis equipment	Bio-Rad, USA
Freezer (-30°C)	SIEMENS, Germany
Freeze dryer	Ilshin/FD 5512, Korea
Gel Documentation	Alphaimager mini, USA
Glassware sterilizer	Binder, USA
High speed refrigerated centrifuge	TOMY, MX-305, Japan
Incubator	Memmert/INE 500, Germany
Magnetic stirrer	IKA/C-MAG HS 7, USA
Micropipettes	Eppendorf research, USA
Orbital shaker	Excella E25 New Brunswick, USA
pH meter	Thermo Scientific/Orion 2 Star, Singapore
Microscope	BOECO/BM-180, Germany
Power supply	Powerpac Basic, Bio-Rad, USA
Refrigerator (4°C)	Walton, Bangladesh
Scanning Electon Microscopy	Jeol, Japan
Sonicator	Omni Ruptor-4000, USA
Spectrophotometer	Genesys 5, Thermo scientific, USA
Water bath	Selecta, Spain
Vortex mixer	Benchmark Scientific/ NJ 08818, USA