Optimization of alkaline protease production by *Bacillus licheniformis* MZK05M9 in batch culture using Response Surface Methodology

BRAC University

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Dedicated
To
My beloved parents

Declaration

I declare that, this thesis entitled "**Optimization of alkaline protease production by *Bacillus licheniformis* MZK05M9 in batch culture using Response Surface Methodology**" is the result of my own research under the joint supervision and guidance of Professor Dr. Naiyyum Choudhury, Coordinator, Biotechnology Program, BRAC university and Professor Dr. Md. Mozammel Hoq, Department of Microbiology, University of Dhaka in partial fulfillment of MS in Biotechnology, BRAC University, Dhaka. It is further declared that the research work presented here is original and has not been submitted to any other institution for any degree or diploma.

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Abstract

Bacterial alkaline protease has got its particular eco friendly technical applications in leather processing, detergent and feathers digestion to feed in Bangladesh. The present study is aimed at the optimization of major factors affecting submerged culture fermentation for production of alkaline protease by a *Bacillus licheniformis* MZK05M9 mutant strain using statistical Central Composite Design (CCD) and Response Surface Methodology (RSM). The molasses as carbon source, urea as nitrogen source and salt $CaCl_2.2H_2O$ were selected to design a cost-effective medium for production of protease by the *B. licheniformis* MZK05M9 mutant. The concentrations of the medium components such as molasses, urea and salt $CaCl_2.2H_2O$ were optimized at pH 7.5, agitation 150 rpm and temperature 37°C. The optimum values for the tested variables for the maximum alkaline protease production was found as molasses (0.63%, w/v), urea (0.16%, w/v) and $CaCl_2.2H_2O$ (0.11%, w/v) predicted by statistical software. The protease activity in unoptimized medium was 410 U/ml where as 463.1 U/ml was predicted in optimized medium by the software. But observed validated experimental value was 560U/ml in optimized medium. Therefore, the statistical optimization by RSM resulted in 1.3 fold increase in the production of the protease enzyme by the *B.licheniformis* MZK05M9 mutant. The productivity in shake culture was 11666.6 U/L/hr while 13888.8 U/L/hr in bioreactor using optimized medium. Partial purification of the protease was carried out by ammonium sulphate fractionation and ultrafiltration membrane resulting the enzyme purified to 16.59 folds with specific activity of 38,736.7 micromoles/min/ mg of protein. The molecular mass of the enzyme was about 28 kDa as judged by SDS-PAGE. The enzyme is very efficient in solubilisation of feathers and removal of blood stain data revealing its potential for application in detergent industries. Based on the productivity of the enzyme on locally available cheap ingredients, the present study will be a useful basis for developing a bioprocess for commercial production of alkaline protease in Bangladesh.

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Abbreviations

ANOVA	**Analysis of Variance**
APPB	**Alkaline Protease Producing Broth**
α	**Alpha**
BSA	**Bovine serum albumin**
CCD	**Central composite design**
$CaCl_2.2H_2O$	**Calcium chloride**
DF	**Degree of freedom**
EC	**Enzyme Commission**
et.al	**And others**
EDTA	**Ethylene diamine tetra acetic acid**
Fig	**Figure**
G	**Gram**
g/l	**Gram per litre**
hrs	**Hours**
MS	**Mean of square**
MSSM	**Modified Soya Mill Medium**
MW	**Molecular Weight**
ml	**Milliliter**
mg	**Milligram**
$MgSO_4.7H_20$	**Magnesium Sulfate**
pH	**Negative logarithm of hydrogen ion concentration**
RSM	**Response Surface Methodology**
rpm	**Rotation per minute**
SMM	**Soya Mill Medium**
SDS-PAGE	**Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE)**

°C	**Degree Centigrade**
Temp	**Temperature**
TCA	**Trichloroacetic acid**
TSB	**Tryptone Soya Broth**
U/ml	**Unit per milliliter**
U/L/hrs	**Unit per liter per hours**
V/v	**Volume Per volume**
W/v	**Weight per volume**
%	**Percentage**
/	**Per**

Chapter 1: Introduction and Literature Review

1.1 Introduction

Enzymes are organic catalysts. It plays a vital role in many aspects of life. These have been used since beginning of civilizations. Enzymes play a huge role in everyday life from simple fermentation to complicated gene expressions (Hindhumathi., et *al.,*2011).

Proteases are enzymes. It has the ability to degrade protein by the breaking of hydrogen bond that binds peptides into protein. It is also called proteolytic enzymes. Proteases occupy a pivotal position with respect to their physiological roles as well as their commercial applications. They perform synthetic functions. They are ubiquitous in nature and are produced by a wide range of microorganisms including bacteria, moulds and yeasts, actinomycetes etc. (Akcan and Uyar, 2011). These microorganisms are attractive sources of proteases. In bacteria, protease enzyme is produced mainly by *Bacillus licheniformis*, *B. horikoshii*, *B. sphaericus*, *B. furmis*, *B. alcalophilus*, *B.subtilis* (Adinarayana and Ellaiah, 2004).

Proteases constitute one of the most important groups of industrial enzymes, accounting for more than 65% of the total industrial enzyme market (Rao *et al.*, 1998; Banik and Prakash, 2004). According to a New Report by Global Industry Analysts Inc., the worldwide sales of industrial enzymes to exceeded $2.9 billion by the year 2012. Industrial enzyme sales in Asia-Pacific are estimated at US$327 million in 2008. Proteases are the largest product segment with an estimated share of 57.03% in 2008. (http://www.prweb.com/news/2008/1106/index.htm).

Bangladesh could be a major country for utilization of proteases in detergents and leather industries. In view of this reason, a research program on production and application of proteases (both keratinase and alkaline protease) has been undertaken at the Fermentation and biotechnology laboratory of Department of Microbiology, University of Dhaka. For this purpose, strains of *Bacillus licheniformis* MZK03 and MZK05 were isolated from tannery effluents and identified by 16S rRNA gene sequence analysis (Hoq *et al.,* 2005). These two strains were tested for both alkaline protease and keratinase production in shake flask and bioreactor culture using

Alkaline Protease Producing Broth (APPB) and feather mill medium respectively (Hossain *et al.,* 2006). The enzyme preparations were tested for their technical applications in hide processing (Azad *et al*., 2002), 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. Since the production of the enzymes by natural strains of *Bacillus licheniformis* is not adequate, genetic manipulation by mutation and gene cloning of both *kerA* and alkaline protease gene is being carried out in the laboratory. A mutant *Bacillus licheniformis* MZK05M9 has been obtained by random mutation which exhibited about three-fold higher enzyme activity than that of the natural strain.

Now we need a cost effective production of proteases to fulfil the requirement for industrial sector to subside pollution hazards created by detergent and leather industries. Alkaline protease should be produced commercially in high yields by a low-cost method by using agro-industrial residues.

Molasses as nutrient medium can be used as a relatively inexpensive and economic alternative to synthetic medium for the production and purification of many economic enzymes (Hezayen, *et.al*.,2009). Molasses is an interesting raw material, it is rich in nutrients and minerals, cheap in price as well as it present in plenty as a by product in Sugar industries.

Medium optimization is very important because we all know that medium components play a very important role in enhancing cell growth and increase the target product accumulation. In general, medium optimization by the traditional 'one factor at-a time' technique was used (Gokhade *et al*., 1991). This method is very laborious and time consuming. It also often leads to an incomplete understanding of the system behaviour and gave confusing results and it has a lack of predictive ability. To design an efficient medium is prerequisite for high product yields. Experimental factorial design and response surface methodology (RSM) can be employed to optimize the medium components.

RSM is a collection of statistical and mathematical techniques useful for the modeling and analysis of problems in which a response of interest is influenced by several variables, with the objective being to optimize this response (Montgomery, 2001). Response surface methodology has eliminated the drawbacks of classical methods and has proved to be powerful and useful for the optimization of the target metabolites production (Deepak *et al*. 2008; Liu and Wang 2007; Sayyad *et al*., 2007). Second-order models like Central Composite, Box-Behnken and Doehlert designs are widely used in RSM as they can take on a wide variety of functional forms, and this flexibility allows them to more closely approximate the true response surface (Srinivas *et al.,* 1994; Carvalho *et al.,* 1997; Adinarayan and Elliah 2002; Rahman and Gomes 2003; Li *et al*. 2007; Xiao *et al*. 2007).RSM has been recently used for the modeling and optimization of several bioprocesses, including fermentations (Sen 1997) enzymatic reactions (Ferreira *et al.* ,1998) product recovery (Annadurai *et al*., 1996) and enzyme immobilization techniques (Zhao *et al.,* 2007; Chang *et al.,* 2007). The application of experimental design and response surface methodology in fermentations process can result in improved product yields, reduced process variability and development time and overall costs (Rao *et al*., 2000).

In this study we have investigated the protease production levels in *Bacillus licheniformis* MZK05M9 mutant in commercial and various cheap source media. Alkaline Protease production from *Bacillus licheniformis* MZK05M9 mutant as a result of the interaction between three variables molasses, urea and $CaCl_2.2H_2O$ which had played a significant role in enhancing the production protease was optimized with response surface methodology.

Literature Review

1.2Enzymes

Enzymes are effective catalysts, responsible for thousands of coordinated chemical reactions involved in biotechnological process of living systems. Outstanding features of enzymes in comparison to chemical catalysts are their substrate specificity and specificity in promoting of only one biochemical reaction with their respective substrates ensuring synthesis of a specific bimolecular product without the concomitant production of by products (Chaplin *et al.,* 1990). With the understanding of the nature, enzyme has gradually been extended in a variety of fields, such as food production, brewing, pharmaceuticals, medicine textiles, leather and detergents (Rehm *et al.,* 1987). Of more than 3000 different enzymes described to date the majority have been isolated from mesophilic organisms. These enzymes mainly function in a narrow range of pH, temperature, and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes many of the currently known enzymes unparallel in some applications. Thus, the search for new microbial sources is a continual exercise, where one must respect biodiversity. The microorganisms from diverse and exotic environments called as extremophiles, are an important source of enzymes, whose unique properties are expected to result in novel process applications (Kumar and Takagi, 1999).

Enzymes can be classified into two categories: intracellular and extracellular. Intracellular enzymes occur inside living cells, where they catalyze and regulate reactions of biochemical pathways essential to the existence of the living system. Extracellular enzymes are originally defined as enzymes, which are external to the cell wall and in contact with the surrounding medium. Most of the industrial enzymes are extra-cellular and hydrolytic. And because of their amenability to simple separation and purification, fairly broad tolerance to p^H in activity and stability and fairly long-term storage stability, they have diversified uses.

1.3 Enzyme Nomenclature

Enzymes are identified by a common nomenclature system based on the description of what function it performs in the cell and ends with a common phrase- ase. The International Union of Biochemistry and Molecular Biology and the International Union of Pure and Applied Chemistry developed a nomenclature system where in each enzyme is given an Enzyme Commission Number called as the EC number.

Accordingly the top level classes based on the mechanism of operation of an enzyme are:

1. Oxidoreductases: catalyze oxidations/reactions.
2. Tranferases: transfer a functional group.
3. Hydrolases: Catalyse the hydrolysis of bonds with addition of water.
4. Lyases: removes group from their substrates.
5. Isomerases: Catalyze isomerisation changes within a single molecule.
6. Ligases: Join two molecules with two covalent bonds.

1.4 Proteases

Proteases are the single class of hydrolytic enzymes that attracted the attention of wide spectrum population due to their diversified properties properties. Proteases (EC 3.4.21-24 and 99; peptidyl-peptide hydrolases) are enzymes that hydrolyse proteins via the addition of water across peptide bonds and catalyses peptide synthesis in organic solvents and in solvents with low water content (Sookkheo *et al.,* 2000; Beg *et al.,* 2003). The hydrolysis of peptide bonds by proteases as shown in Figure 1.2 is termed as proteolysis; the products of proteolysis are protein and peptide fragments, and free amino acids.

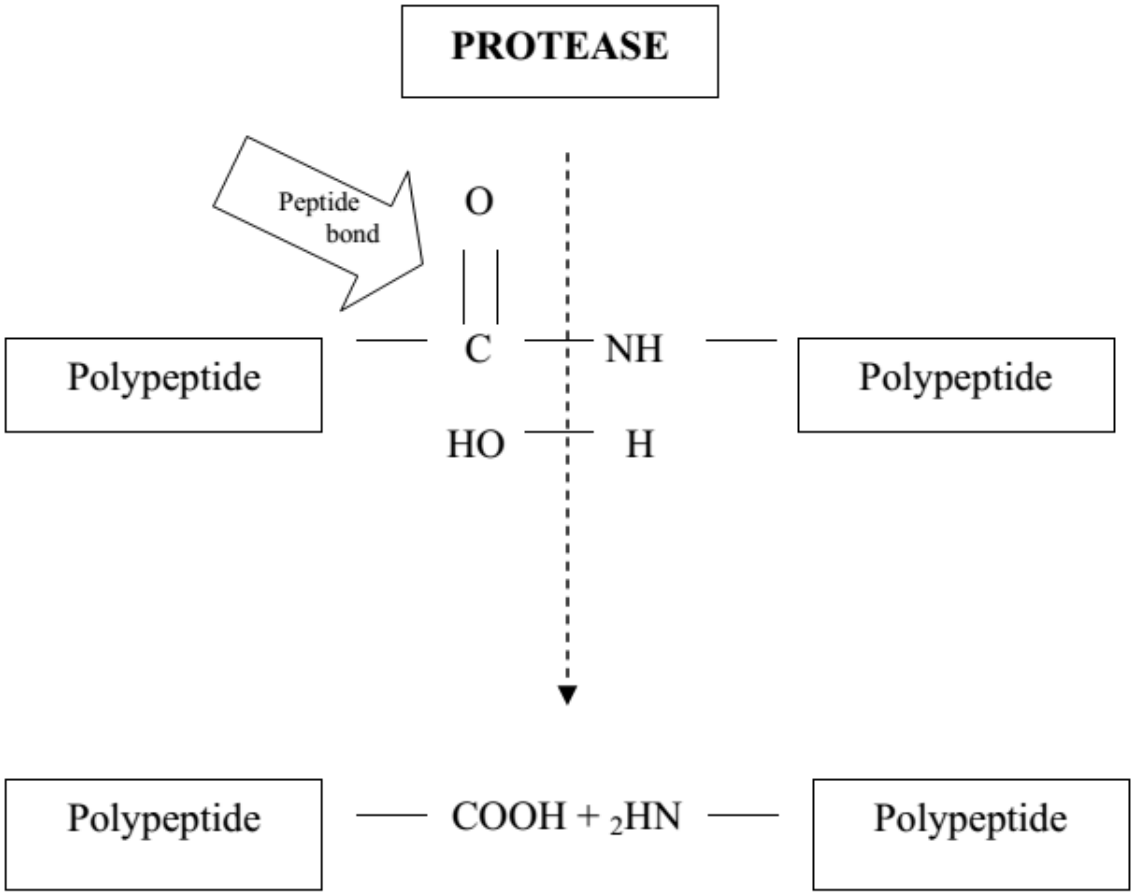


Fig 1.1: Catalysis of peptide bonds (Proteolysis) by proteases

Proteolytic enzymes are ubiquitous in occurrence, found in all living organisms, and are essential for cell growth and differentiation. There is renewed interest in the study of proteolytic enzymes, mainly due to the recognition that these enzymes not only play an important role in the cellular metabolic processes but have also gained considerable attention in the industrial community (Gupta *et al.,* 2002b). Proteases account for about 60% of the total worldwide sale of enzymes (Rao *et al.*1998). Detergent, tanning and fiber sizing industries are fully exploiting the power of these enzymes (Rao & Pande 1999).

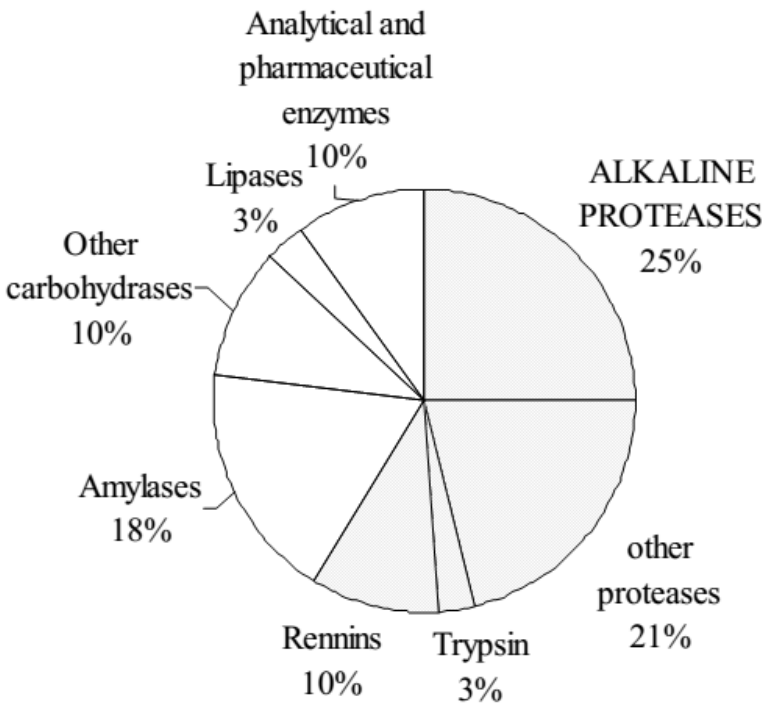


Fig 1.2: The contribution of different enzymes to the total sale of enzymes is.(Rao *et al.,*1998)

1.5 Source of Proteases

Proteases are physiologically necessary for metabolic activities of living organisms, they are ubiquitous in nature. They are found in plant, animal and microbial sources.

1.5.1 Plant proteases:

Most common plant proteases are bromelain, ficin, papain and zingibain (Lee *et al.*, 1986; Adulyatham and Owusu-Apenten, 2005).

Bromelain: Bromelain (EC 3.4.22.32) is a crude extract from the pineapple (*Ananas comosus*) plant. It is a mixture of sulfur-containing proteases. Bromelain is present in all parts of the pineapple plant but the stem is the most common commercial source. It is active between pH 5 to 9 and stable up to 70°C beyond which it is inactivated. It is used as meat tenderizer, anti-inflammatory agent and in debridement (Secor *et al.*, 2005). The major mechanism of action of bromelain appears to be proteolytic in nature, although evidence suggests an immune modulatory and hormone like activity acting via intracellular signalling pathways. Bromelain has also been

shown to reduce cell surface receptors such as hyaluronan receptor CD44, which is associated with leukocyte migration and induction of proinflammatory mediators (Tochi *et al.,* 2008).

Ficin: It is extracted from latex of ficus and is a sulfhydryl proteinase with cysteine at the active site (EC 3.4.22.3). 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).

Papain: It is a cysteine protease (EC 3.4.22.2) extracted from latex of papaya (*Carica papaya*). The crude enzyme has broad specificity due to mixture of several proteases. The enzyme is active between pH 5 to 9 and is stable up to 80-90°C in presence of substrates. It consists of 212 amino acids stabilized by 3 disulfide bridges. Its catalytic triad is made up of 3 amino acids - cysteine-25 (from which it gets its classification), histidine-159, and asparagine-158. It is extensively used in tenderization of the meat (to break down the tough meat fibers), preparation of highly soluble and flavoured protein hydrolysates, dissociate cells in the first step of cell culture preparations, to make single cell preparation and an ingredient in some toothpastes and mints as teeth-whitener (Kim *et al.,* 2004).

1.5.2 Animal proteases:

Pepsin, chymotrypsin, trypsin and rennin are widely used animal proteases. As the plant and animal proteases are unable to meet the current world demand of the enzyme an increased interest in microbial source has grown.

Chymotrypsin: It is found in the pancreatic extract of animals (EC 3.4.21.1). The enzyme cleaves peptides at the carboxyl side of tyrosine, tryptophan and phenylalanine although over time it also hydrolyzes other amide bonds, particularly those with leucine-donated carboxyls. It is present in zymogen form and is activated on cleavage by trypsin into two parts that are still connected via an S-S bond. Pure chymotrypsin has main applications in analytical and diagnostic field. It is extensively used in deallergenizing of milk protein hydrolysates.

Pepsin: It is a digestive protease (EC 3.4.23.1) released by the chief cell in the stomach of almost all vertebrates that function to degrade food proteins into peptides. Pepsin is produced in its

zymogenic form i.e pepsinogen, whose primary structure has additional 44 amino acids. This zymogen is activated by hydrochloric acid (HCl), which is released from parietal cell in the stomach lining. HCl creates an acidic environment which allows pepsinogen to unfold and cleave itself in an autocatalytic fashion, thereby generating pepsin. Pepsin functions best in acidic environments between pH 1 to 2 and is inactivated above pH 6. Pepsin cleaves preferentially after the N-terminal of aromatic amino acids such as phenyalanine and tyrosine. It is an aspartyl protease and has resemblance with HIV-1 protease. Pepsin is commonly used in the preparation of F(ab)2 fragments from antibodies.

Rennin: It is an aspartic acid protease (EC 3.4.23.4), produced as an inactive precursor, prorennin in stomachs of all nursing mammals but more specifically in the fourth stomach of calves. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in k-casein to generate insoluble para-k-casein and C-terminal glycopeptide. It cleaves the peptide bond between phenyalanine and methionine, the specific linkage between the hydrophobic (para-casien) and hydrophilic (acidic glycopeptide) group of casein in milk, since they are joined by phenylalanine and methionine. The hydrophobic group would unite together and would form a three dimensional network totrap the aqueous phase of the milk resulting in the formation of calcium phosphocaseinate. This specificity is used to bring about the extensive precipitation and curd formation in cheese making.

Trypsin: It is a serine protease (EC 3.4.21.4) found in the digestive system and is responsible for the breakdown of food proteins. Trypsin has an optimal operating pH and temperature of about 8 and 37°C respectively and predominantly cleaves proteins at the carboxyl side of the lysine and arginine. Trypsin is commonly used in proteomics, since it has a very well defined specificity. Trypsin is also used for the preparation of bacterial media, to dissolve blood clots, treat inflammation and to dissociate dissected cells. Trypsin has limited application in food because the protein hydrolysates generated have bitter taste. Based on the ability of protease inhibitors to inhibit the enzyme from the insect gut, trypsin is targeted for biocontrol of insect pests.

1.5.3. Microbial Protease:

Proteases are widely distributed in microbial population viz. bacteria, actinomycetes, viruses and fungi. Although proteases are widespread in nature, microbes serve as a preferred source of these enzymes and account for around two-thirds of commercial production worldwide. Alkaline serine proteases (EC 3.4.21) are the most important group of commercial enzymes (Kumar and Takagi, 1999). 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 producing more efficient enzymes with desirable properties required for their diverse applications.

Bacteria: Most of the commercial proteases are of bacterial origin (Prakasham *et al.,* 2006). 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. Alkaline proteases from bacterial source are widely used 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. Compared to alkaline proteases, neutral proteases have lower thermo-tolerance. 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, (1971).

Virus: Viral proteases are involved in processing of proteins that cause fatal diseases like AIDS and cancer. Mature enzymes encoded within the human immunodeficiency virus type 1 (HIV-1) genome protease (PR); reverse transcriptase (RT) and integrase (IN) are derived from proteolytic processingof a large polyprotein (Gag-Pol). The viral PR catalyzes Gag-Pol processing, which is active as a homodimer (Olivares *et al.,* 2007). Most of the viral proteases are endopeptidases rather than exopeptidases (Rawling & Barrell 1993).Due to the involvement of viral proteases in pathogenesis extensive studies on the three dimensional structure of viral proteases and their interaction with synthetic proteases inhibitors have been undertaken with a view to designing potent inhibitors that can combat the diseases like AIDS .

Fungi: Fungi synthesize wide variety of proteases than do bacteria. Filamentous fungi can effectively secrete various hydrolytic enzymes and one of the main groups of secreted enzymes in fungi is protease. They usually show better results when cultured in solid-state fermentation as compared to bacteria (Pandey *et al.,* 1999). Fungi are known to produce acid, neutral, alkaline and metallo proteases. A single organism can produce more than one type of protease (Lindberg *et al.,* 1982). Fungal proteases are active over a wide pH range (pH 4 to 11) and exhibit broad substrate specificity (Rao *et al.,* 1998). One of the first known representatives of proteases was proteinase K, an alkaline enzyme from Engyodontium album also known as Tritirachium album (Kotlova *et al.,* 2007).

1.5.4 Other Proteases:

Collagenase: Collagen is a fibrous protein and is a constituent of skin, bone, cartilage, tendon and other connective tissue. Its commercial importance in leather and in production of gelatin and glue has long been recognized. Collagen is converted into gelatin by boiling. Collagenases are enzymes capable of degrading collagen and 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).

Elastase: Elastin is a fibrous protein and together with collagen determines the mechanical properties of connective tissue. It imparts elasticity and allows the tissues to regain its original shape after stretching or contracting. Elastase is a protease, which breaks down elastin and has applications in food, pharmaceuticals and cosmetics industries (Chen *et al.,* 2007). Fungal elastases have been reported from Aspergillus (Markaryan *et al.,* 1994; Mellon and Cotty., 1995; Alp and Arikan, 2008) and entomopathogenic fungus Conidiobolus coronatus (Wieloch and Bogus, 2007). Mellon and Cotty (1996) purified and characterized elastase from Aspergillus flavus NRRL 18543.

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. Keratinases are widely distributed in nature and secreted by variety of organisms belonging to bacteria, actinomycetes and fungi. Traditionally keratinases have been in use for production of feather meal, fertilizers, glues etc.Their applications have been further extended to other areas such as detergent formulations, cosmetics, leather, medicine and animal feed. Keratinases are also finding applications in treatment of mad cow disease (degradation of prion) and biodegradable plastic (Gupta and Ramnani, 2006).

Microbial Rennins: Rennin, an aspartic acid protease is 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 and religious and ethnic regulations against animal derived enzyme has generated interest in microbial rennnis.

1.6. Classification of Proteases According To Enzyme Commission

According to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology, proteases are classified in subgroup 4 of group 3 (hydrolases) (Rao *et al.,* 1998).

Proteases can be classified according to 3 major criteria. Such as;

A). the reaction catalysed,

B). the chemical nature of the catalytic site,

C). the evolutionary relationship, as revealed by the structure (Rao *et al.,* 1998).

Proteases as shown in Table 2.1 are broadly classified as endo- or exoenzymes on the basis of their site of action on protein substrates (Rao *et al.,* 1998).

Table-1.1: Classification of proteases.

Proteases	Mode of action	EC NO.
EXOPEPTIDASES		
Aminopeptidases	●↓–O-O-O-O	3.4.11
Dipeptidyl peptidase	●-● ↓-O-O-O	3.4.14
Tripeptidyl peptidase	●-●-●↓-O-O	3.4.14
Carboxypeptidase		3.4.16-3.4.18
Serine type peptidase		3.4.16
Metalloproteases		3.4.17
Cystein type proteases		3.4.18
Peptidyl dipeptidase	---O-O-O-O↓-●-●	3.4.15
Dipeptidases	●↓-●	3.4.13
Omega peptidases	*-●↓-O-O---- ---O-O-O↓-●*	3.4.19 3.4.19
ENDOPEPTIDASES	--O-O-O↓-O-O-O--	3.4.21-3.4.34
Serine proteases		3.4.21
Cystein proteases		3.4.22
Asparic proteases		3.4.23
Metallo proteases		3.4.24
Endopeptidases of unknown catalytic mechanism		3.4.99

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

1.6.1 Exoproteases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively (Rao *et al.,* 1998).

1.6.1.1 Aminopeptidases

Amino peptidases act at a free N- terminus of the polypeptide chain liberating a single amino acid, a dipeptide or tripeptide. They are known to remove the N terminal Met that may be found in heterologously expressed proteins but not in many naturally occurring mature proteins. These enzymes occur in a wide variety of microbial species including, bacteria and fungi (Watson, M. 1976).Most of them require divalent ions like,Mg^{+2},Mn^{+2},Co^{+2}, or Zn^{+2} for their optimal activity(De-Marco & Dick 1978). In general aminopeptidases are intracellular enzymes, but there has been a single report on an extracellular peptidase produced by A. oryzae. (Rao *et al.,*1998).

1.6.1.2Carboxy peptidases

Carboxy peptidases digest the protein and liberate single amino acid or a dipeptide from carboxy end. Three are distinct groups of carboxypeptidases have been isolated. These are known as serine carboxypeptidases, metallo carboxypeptidass and cystein carboxy peptidases depending on the amino acid present at the active site of the enzymes. Serine carboxy peptidases isolated from different fungal sources have different pH optima, temperature sensitivity and molecular weight but similar substrate profiles. Metallo carboxy peptidases isolated *Pseudomonas* species & saccharomyces species require Zn^{+2} or Co^{+2} for their activity (Felix & Brouillet 1966).

1.6.2Endopeptidases

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

1.6.2.1 Serine Protease (EC.3.4.21)

Serine proteases are characterized by serine at the active site. They are wide spread and reported from bacteria, fungi and viruses. They are found in endopeptidases as well as exopeptidases. Three residues which forms the catalytic triad are essential in the catalytic process i.e His (base), Asp (electrophile) and Ser (nucleophile). The first step in the catalysis is the formation of an acyl enzyme intermediate between the substrate and the essential serine. Formation of this covalent intermediate proceeds negatively charged tetrahedral transition state intermediate and then the peptide bond are cleaved. During the second step or deacylation, the acyl-enzyme intermediate is hydrolyzed by a water molecule to release the peptide and to restore the Ser-hydroxyl of the enzyme (Figure 1.3). The deacylation, which also involves the formation of a tetrahedral transition state intermediate, proceeds through the reverse reaction pathway of acylation. A water molecule is the attacking nucleophile instead of Ser residue. The residues provide a general base and accept the OH group of the reactive Ser residue (Rao *et al.,* 1998).

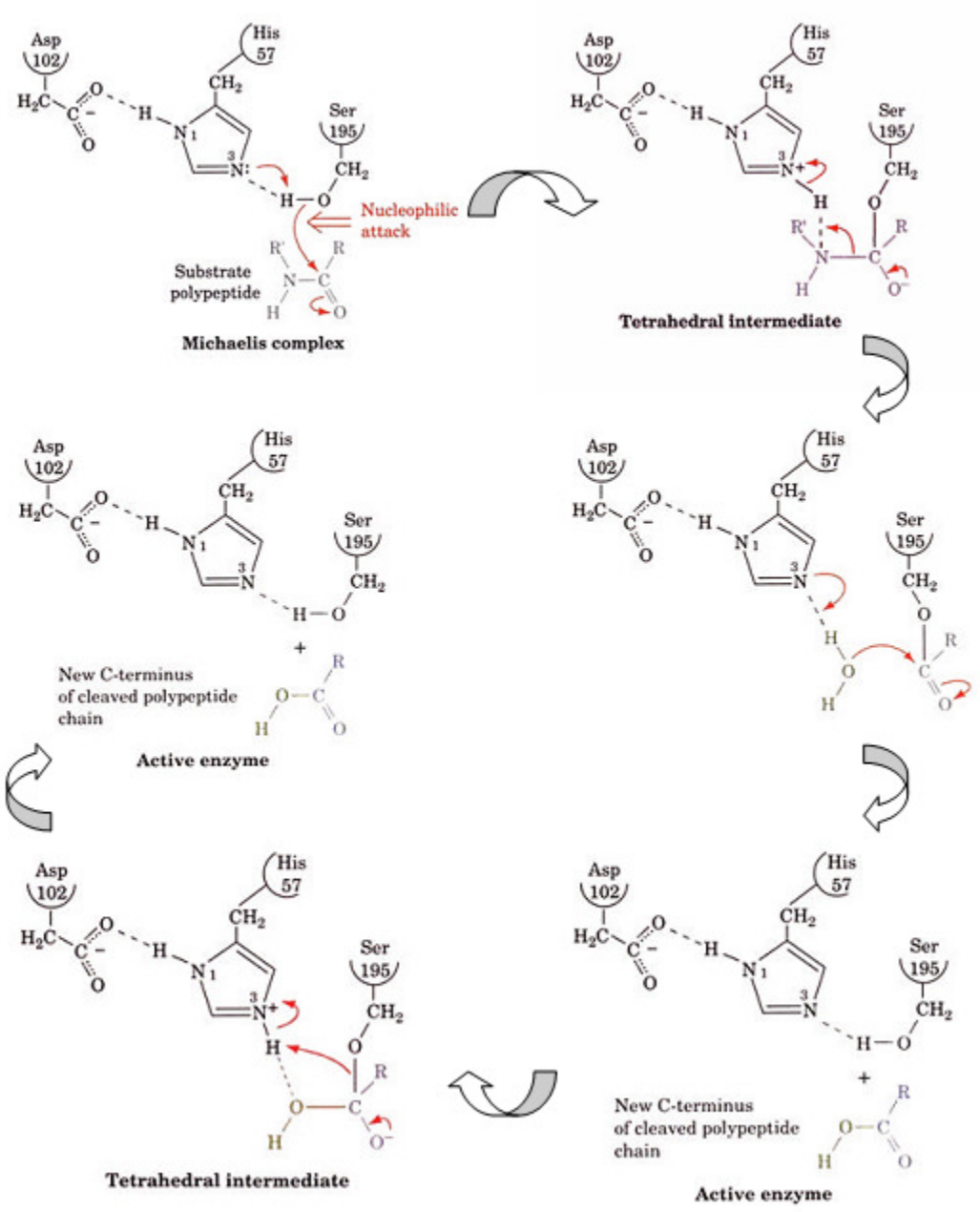


Fig1.3: Mechanism of action serine proteases
(http://info.bio.cmu.edu/courses/03231/Protease/SerPro.htm)

1.6.2.2 Cystein Protease (EC.3.4.22)

Catalysis proceeds through the formation of a covalent intermediate and involves a cysteine and a histidine residue. The essential Cys and His play the same role as Ser and His respectively as in serine proteases. The nucleophile is a thiolate ion rather than a hydroxyl group. The thiolate ion is stabilized through the formation of an ion pair with neighboring imidazolium group of His. The attacking nucleophile is the thiolate-imidazolium ion pair in both steps (Rao *et al.*, 1998).

1.6.2.3 Aspartic Protease (EC 3.4.23)

Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Acidic proteases have been grouped into three families, namely, pepsin, retropepsin and enzymes pararetroviruses. Most aspartic proteases show maximal activity at low pH and have isoelectric points in the range of pH 3 to 4.5.Their molecular weights are in the range of 30 to 45 kDa. The aspartic proteases are inhibited by pepstatin (Rao *et al.,* 1998).

1.6.2.4 Metalloprotease (EC 3.4.24)

The catalytic mechanism by metalloprotease involves the formation of a non-covalent tetrahedral intermediate after the attack of a zinc-bound water molecule on the carbonyl group of the scissile bond. This intermediate is further decomposed by transfer of the glutamic acid proton to the leaving group (Rao *et al.*, 1998).

1.7 Alkaline Proteases

Alkaline proteases exhibit optimal activity at a pH range between 8.0 and 11.0 are inactivated by serine active site inhibitors such as phenyl-methyl sulfonyl fluride (PMSF) and diisopropyl flurophosphate (DIFP). These enzymes are concentrated in laundry detergents and leather processing (Markland and smith, 1971; Chu *et al.*1992). Alkaline proteases secreted by both neutrophilic and alkalophilic bacilli are of interest because they represent a major source of commercially produced proteolytic enzymes (Horikoshi, 1971; Markland and Smith1971). They

are mostly produced extracellularly (Kalisz 1988) having a molecular weight ranging from 20,000 to 30,000 are stabilized by ca+2 And have characteristically high isoelectric point (Keay *et.al.*1970; Markland and Smith 1971).

Two type of amino alkaline proteases have been identified and characterized differing from each other by 58 amino acids (Aunstrup 1980; Kalisz, 1998). They are subtilisin Carlsberg produced by *Bacillus licheniformis* and subtilism Novo, or Bacterial proteases Nagase (BPN), Synthesized by *Bacillus amyloliquefaciens.* Recently Sutar *et.al* (1992) and Phadatare *et.al* (1953) isolated fungus *Conidiobulus coronatus* having high alkaline proteases activity and Steele *et.al.(*1992) isolated kurthia spiroforme from thermal spring which has alkaline protease activity. Tsuchiya *et,al* (1992) also reported the production of large amount of extracellular alkaline protease by alkalophilic *Thermoactinomyces* sp. 682.

1.8 Properties of enzyme

1.8.1 Optimum Temperature and Thermostability of Alkaline Proteases

The heat stability of enzymes is affected by at least two factors alone or in combination. The first one is the primary structure of the enzyme. A high content of hydrophobic amino acids in the enzyme molecule provides a compact structure, which is not denatured easily by a change in the external environment. In addition, disulfide bridges and other bonds provide a high resistance both to heat inactivation and chemical denaturation. Secondly, specific components such as polysaccharides and divalent cations, if any, can stabilize the molecule (Öztürk, 2001).

Even though there is no firm evidence to suggest that thermostable enzymes are necessarily derived from thermophilic organisms, nevertheless there is a greater chance of finding thermostable proteins from thermophilic bacteria (Rahman *et al.,* 1994). Therefore, a wide range of microbial proteases from thermophilic species has been extensively purified and characterized. These include *Thermus sp.,* Desulfurococcus strain Tok12S1and *Bacillus sp*. Among them alkaline proteases derived from alkaliphilic bacilli, are known to be active and stable in highly alkaline conditions (Rahman *et al.,* 1994). The earliest thermophilic and alkaliphilic *Bacillus sp.* was *B. stearothermophilus* strain F1 isolated by Salleh and friends in 1977, which was stable at 60ºC (Haki and Rakshit, 2003). Further studies on microbial alkaline

proteases have been done in view of their structural-function relationship and industrial applications, as they needed stable biocatalysts capable of withstanding harsh conditions of operation (Rahman *et al.,* 1994).

1.8.2 Optimum pH of Alkaline Proteases:

Enzymes are amphoteric molecules containing a large number of acid and basic groups, mainly located on their surface. The charges on these groups will vary, according to their acid dissociation constants, with the pH of their environment. This will affect the total net charge of the enzymes and the distribution of charges on their exterior surfaces, in addition to the reactivity of the catalytically active groups. These effects are especially important in the neighborhood of the active sites, which will overall affect the activity, structural stability and solubility of the enzyme (Chaplin and Bucke, 1990). Most of the commercially available subtilisin-type proteases are also active in the pH range of 8-12 (Gupta et al., 2002). Alkaline proteases of the genus *Bacillus* show an optimal activity and a good stability at high alkaline pH values (Margesin *et al.,* 1992).

1.8.3 Effect of inhibitors

The inhibition profiles of an enzyme give an insight of its nature. In fact the nature of the active site and co-factor requirement of an enzyme also can be assessed by these studies (Sigma and Moser, 1975). Alkaline proteases are completely inhibited by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphate (DFP). The loss of activity is attributed to the sulfonation of the essential serine residue at the active site by PMSF (Gold and Fahrney, 1964). This inhibition pattern shown by alkaline proteases categorizes them as serine hydrolases (Morihara, 1974).

1.8.4 Molecular Weight

Alkaline proteases are biocatalysts with molecular masses lying in the range of 15 to 30KDa (Kumar and Takagi, 1999). There are very few reports with alkaline proteases of higher molecular masses 32KDa (Huang *et al.*, 2003), 36.0KDa (Durham *et al.*, 1987) and 45 KDa (Kwon *et al.*, 1994). However an enzyme of extremely low molecular weight of 8KDa has been reported from *Kurthia spiroforme* (Steele *et al.*, 1992).

1.9 Regulation of Exoprotease synthesis

1.9.1 Induction of Extracellular proteases

Priest in 1977 pointed out that the extracellular enzyme synthesis is partly constitutive and partly inducible. Catabolic enzymes are normally induced by the enzymes substrate but the exoenzymes are an exception in that they are presumably secreted because the substrate is unable to enter the cell. The substrate therefore does not directly induce the enzyme. It is now established that a low basal level of constitutive exoenzymes degrades its exogenous substrate and the resultant low molecular weight products enter the cell and induce further enzyme synthesis. Therefore the mechanism of induction of exoproteases is more or less imperical (Priest 1977).

1.9.2 Feed back repression

The end products of exoenzyme may repress the synthesis of the enzyme in a manner resembling the end product repression of intracellular anabolic enzymes. Extracellular proteases synthesis is regulated in this way and is strongly repressed by the presence of amino acids or peptides in the environment (Doi R.H 1972). Individual amino acids do not repress effectively nor do the same amino acids cause effective repression in various *Bacillus* Mey and Elliot (1968) showed that of the 16 amino acids examined proline, isoleucin, glutamate and aspartate repress the exoproteases synthesis.

It has been observed that maximal extracellular proteases have been synthesized during late exoponential phase of growth (Schaeffer 1969). It has subsequently been shown that sporulation is related to serine proteases synthesis. Mutant isolation studies have shown that during sporulation two serine proteases is elaborated differing in their physico-chemical properties. The asporogenous mutant could synthesize extracellular serine proteases at non-permissive temperature. This suggests that sporulation may be separated from extracellular protein synthesis. But in absence of intracellular serine proteases sporulation and protein turnover do not take place. Batch-culture and continuous culture study reveal that proteases synthesis become maximum when sporulation begins. Dancer & Mandelstam 1975 showed that a neutral metalloexoproteases was synthesized during late log phase but has no connection with sporulation.

1.9.3 Transcriptional Control

Coleman (1976) has proposed a model for the transcriptional control of exoproteases synthesis in Bacillus. It is suggested that exoprotein mRNA synthesis is negligible during exoponential phase of growth because of the low affinity of RNA polymerase of initiation sites on exoprotein operons. At the end of exponential growth a nutritional limitation is increased. This inhibits rRNA synthesis with an accompanying reduction in cell protein mRNA formation. The net result is that more cores RNA polymerase is available to combine with exoprotein specific sigma factor, thus causing increased synthesis of exoproteases mRNA and its ultimate translation to protein. This model is not without substantial experimental evidence. Evidence in support of such switch mechanism in the transcriptional specificity of RNA polymerase during the growth cycle has been obtained from studies of the incorporation of labeled uracil into rRNA during growth ,sporulation and exoprotein synthesis. These studies point to the fact that modification of RNA polymerase takes place during exoenzyme synthesis.

1.10 Medium design

The concentrations of media components are really important as they are tools for bioprocess medium design (Çalık *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 enzymes like 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 product formation. To design a medium, it is important to know the composition of Bacterial cell.

1.10.1 Importance of Cell composition in medium design

Bacterial cells are composed of high molecular weight polymeric compounds such as proteins, nucleic acids, polysaccharides, lipids, and other storage materials. Formation of macromolecules which the major part of the cell mass requires production of the necessary building blocks followed by polymerization of the building blocks (Nielsen & Villadsen 1994). In addition to these biopolymers, cells contain other metabolites in the form of inorganic salts (e.g., NH_4^+, $PO4^{3-}$, K^+, Ca^{2+}, Na^+, SO_4^{2-}), metabolic intermediates (e.g., pyruvate, acetate), and vitamins. A typical bacterial cell is composed of 50% carbon, 20% oxygen, 14% nitrogen, 8% hydrogen, 3% phosphorus, and 1% sulfur, with small amounts of K^+, Ca^{2+}, Na^+, Mg^{2+}, Cl^-, and vitamins (Table 2&3) (Shuler & Kargi 2008).

Most of the enzymes formed by organisms are produced as a result of their response to media components, such as nutrients, growth hormones, and ions. The qualitative and quantitative nutritional requirements of cells need to be determined to optimize growth and product formation.

Nutrients required by the cells can be classified into two categories (Shuler & Kargi, 2008):

1. Macronutrients are needed in concentrations higher than 10^{-4} M, such as carbon, nitrogen, oxygen, hydrogen, sulfur, phosphorus, Mg^{2+}, and K^+. Table 4 lists the major macronutrients and their physiological functions.
2. Micronutrients are needed in concentrations less than 10^{-4}Trace elements such as Mo^{2+},Zn^{2+}, Cu^{2+}, Mn^{2+}, Ca^{2+}, Na^+, Vitamins, Growth hormones and metabolic precursors are known as micronutrients.

Table1.2: The major elements and their physiological functions.

Element	Physiological function	Required Concentration(mol/L)
Carbon	Constituents of organic Cellular material. Often the energy source.	$>10^{-2}$
Nitrogen	Constituents of proteins, nucleic acids, and coenzymes.	10^{-3}
Hydrogen	Organic cellular material and water.	-
Oxygen	Organic cellular material and water. Required for aerobic respiration.	-
Sulfur	Constituents of proteins and certain coenzymes.	10^{-4}
Phosphorus	Constituents of nucleic acids, phospholipids, nucleotides, and certain coenzymes	10^{-4}-10^{-3}
Potassium	Principle inorganic cation in the cell and cofactor for some enzyme	10^{-4}-10^{-3}
Magnesium	Cofactor for many enzymes and chlorophyls and present in cell walls and membranes.	10^{-4}-10^{-3}

1.11 Types of media

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_2PO4, $(NH4)_2HPO4$, 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 defined media due to the presence of necessary growth factors, vitamins, hormones, and trace elements.

1.12 Optimization Of Media Components

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.

1.12.1 Effect of Carbon source:

Glucose is frequently used 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). 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). Whey, a waste byproduct of the dairy industry containing mainly lactose and salts, has been demonstrated as a potential substrate for alkaline protease production (Donaghy & McKay 1993; McKay 1992). Similarly, maximum alkaline protease secretion was observed in the presence of pure cellulose

(Solka-floc) as the principal carbon source (Gusek *et al.* 1988). Molasses used as carbon source for protease production (Mabrouk *et al.,* 1998). Molasses contains sucrose, glucose, fructose and important minerals such as calcium, iron and magnesium.

1.12.2 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. 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). 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). Urea used as a nitrogen source for protease production (Mabrouk, *et al.,* 1998).

1.12.3 Effect of Metal Ion and salt:

Alkaline proteases require a divalent cation like Ca^2+, Mg^2+ and Mn^2+ or a combination of these cations, for maximum activity. These cations were also found to enhance the thermal stability of a Bacillus alkaline protease. It is believed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Kumar and Takagi, 1999). In particular metal ions like Ca^{2+}, Mg^{2+} and Mn^{2+} ions either individually or in combination have been found to give maximal activity to the enzyme. Especially the metal ion Ca^{2+} is reported to increase activity and thermal stability of alkaline protease at increased temperatures (Kumar, 2002). Other metal ions that are used for stabilizing proteases include Ba^{2+}, Co^{2+}, Fe^{3+} and Zn^{2+} (Johnvesly and Naik, 2001).

1.12.4 pH and temperature

The important characteristic of most alkalophilic microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production. For increased protease yields from

these alkalophiles, the pH of the medium must be maintained above 7.5 throughout the fermentation period (Aunstrup, 1980). The advantage in the use of carbonate in the medium for an alkaline protease has been well demonstrated (Horikoshi & Akiba, 1982).

Temperature is another critical parameter that has to be controlled and varied from organism to organism. The mechanism of temperature control of enzyme production is not well understood (Chaloupka, 1985). However, studies by Frankena *et al*. (1986) showed that a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake.

1.12.5 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 (Moon & Parulekar, 1991; Michalik1985). 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).

The production of an enzyme exhibits a characteristic relationship with regard to the growth phase of that organism. In general, the synthesis of protease in *Bacillus* species is constitutive or partially inducible and is controlled by numerous complex mechanisms operative during the transition state between exponential growth and the stationary phase (Priest, 1977; Strauch & Hoch, 1993). The production of extracellular proteases during the stationary phase of growth is characteristic of many bacterial species (Priest *et al*., 1977). The sequence as well as the rate of enzyme production is, however, variable with the specific organism. At early stationary phase, two or more proteases are secreted and the ratio of the amount of the individual proteases produced also varied with the *Bacillus* strains (Priest, 1977; Uehara *et al*., 1974). In several cases, the function of the enzyme is not very clear, but its synthesis is correlated with the onset of a high rate of protein turnover and often sporulation (Chu *et al*., 1992; Power & Adams, 1986).

1.13 Optimization techniques

1.13.1 Classical techniques

The traditional one-at-a-time optimization strategy is simple and useful for screening. Unfortunately, this simple method frequently fails to locate the region the optimum response because the join effects of factors on the response are not taken into account in such procedures. It was reported that the complexities and uncertainties associated with large-scale fermentation usually come from a lack of knowledge of the sophisticated interactions among various factors affecting fermentation (Liu *et al.,* 2005).

1.13.2 Statistical mathematical techniques

1.13.2.1 Response Surface Methodology (RSM)

Response surface methodology is a procedure for fitting a series of regression models to the output variable of a simulation model (by evaluating in at several input variable values) and optimizing the resulting regression function.

Statistically designed experiments are highly efficient in that they give a fixed amount of information with much less effort than the classical one-variable at-a time approach and many of them give additional information about interaction as a bonus. It will be shown that significant interaction are an important clue in the search for optimum conditions and substantial interactions mean that careful control will have to be exercised if a reproducible process is to ensure (Haines 2010). The broad aims of RSM are to investigate the nature of the response surface over a region of interest and to identify operating conditions associated with maximum or minimum response. RSM is generally conducted in three phases, as emphasized in Myers and Montgomery (2002).Phase 0 involves the screening the explanatory variables to identify those which have a significant effect on the response, Phase 1 is concerned with the location of optimum operating conditions by conducting a sequence of suitable experiments and phase 2 involves the fitting of an appropriate empirical model, usually a second order polynomial model, in order to examine the nature of the response surface in the vicinity of the optimum. RSM is a

design that has been described as the most powerful statistical techniques in technological research.

1.13.2.2 Response Surface Design

Sometimes simple linear and interaction models are not adequate. For example, suppose that the outputs are defects or yield, and the goal is to minimize defects and maximize yield. If these optimal points are in the interior of the region in which the experiment is to be conducted, you need a mathematical model that can represent curvature so that it has a local optimum. The simplest such model has the quadratic form

$$Y= \beta+\beta_1X_1+\beta_2X_2+\beta_{12}X_1X_2+\beta_{11}X_1^2+\beta_{22}X_2^2$$

Containing linear terms for all factors, squared terms for all factors, and products of all pairs of factors.

Designs for fitting these types of models are known as response surface designs. One such design is the full factorial design having three values for each input. Although the Statistics Toolbox is capable of generating this design, it is not really a satisfactory design in most cases because it has many more runs than are necessary to fit the model. The two most common designs generally used in response surface modeling are Central Composite Designs (CCD) and Box-Behnken designs. In these designs the inputs take on three or five distinct values (levels), but not all combinations of these values appear in the design.

The functions described here produce specific response surface designs:

- Central Composite Designs.
- Box-Behnken Designs.

1.13.2.3 Central Composite Design

The central composite design (CCD) is a design widely used for estimating second order response surfaces. Central composite designs are response surface designs that can fit a full quadratic model. Since introduced by Box and Wilson (1951), the CCD has been studied and used by many researchers. To picture a central composite design, imagine we have several factors that can vary between low and high values. For convenience, suppose each factor varies from -1 to +1.

One central composite design consists of cube points at the corners of a unit cube that is the product of the intervals [-1, 1], star points along the axes at or outside the cube, and center points at the origin. Central composite designs are of three types. Circumscribed (CCC) designs are as described above. Inscribed (CCI) designs are as described above, but scaled so the star points take the values -1 and +1, and the cube points lie in the interior of the cube. Faced (CCF) designs have the star points on the faces of the cube. Faced designs have three levels per factor, in contrast with the other types, which have five levels per factor. The following figure shows these three types of designs for three factors.

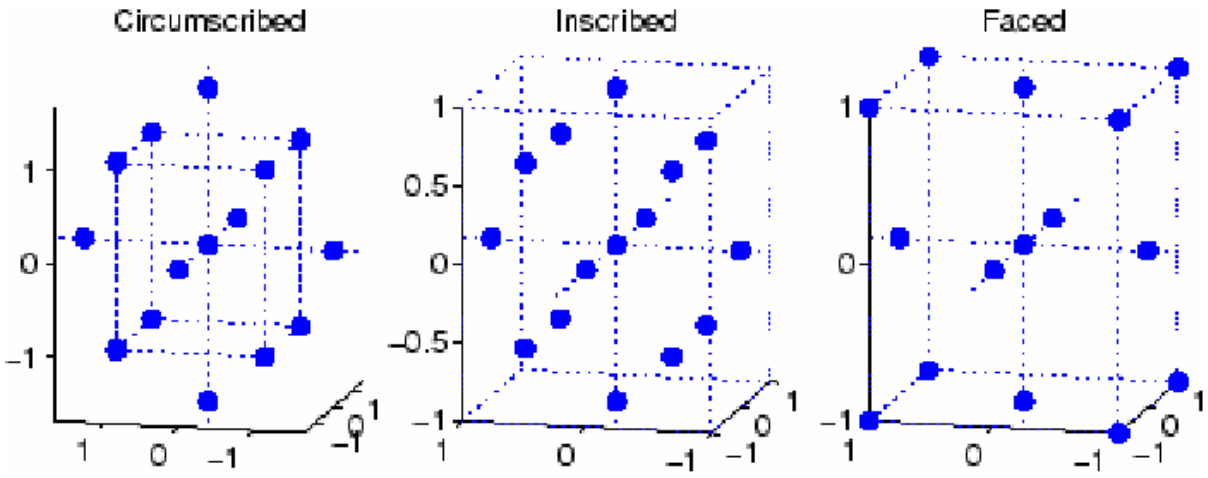


Figure1.4: Three types of Central Composite Design

1.13.2.4 Determining α in Central Composite Designs

The value of α is chosen to maintain rotatability. To maintain rotatability, the value of α depends on the number of experimental runs in the factorial portion of the central composite design:

$\alpha = [\text{Number of the factorial runs}]^{1/4}$

If the factorial is a full factorial then, $\alpha = [2^k]^{1/4}$

Table1.3: Illustrates some typical values of α as a function of the number of factors.

Number of factors	Factorial portion	Scaled value for α relative to ±1
2	2^2	$2^{2/4}$ =1.414
3	2^3	$2^{3/4}$=1.682
4	2^4	$2^{4/4}$=2.000
5	2^{5-1}	$2^{4/4}$=2.000
5	2^5	$2^{5/4}$=2.378
6	2^{6-1}	$2^{5/4}$=2.378
6	2^6	$2^{6/4}$=2.828

The value of α also depends on whether or not the design is orthogonally blocked. That is, the question is whether or not the design is divided into blocks such that the block effects do not affect the estimates of the coefficients in the 2nd order model.

1.13.2.5 Minitab Software

Minitab is a computer program designed to perform basic and advanced statistical functions. It combines the user-friendliness of Microsoft Excel with the ability to perform complex statistical analysis. In 1972, instructors at the Pennsylvania State University developed Minitab as a light version of OMNITAB, a statistical analysis program by NIST.

The software's success led its creators to form Minitab Inc. in 1983. The privately owned company is head quartered in State College, Pennsylvania, with subsidiaries in Coventry, England (Minitab Ltd.) and Paris, France (Minitab SARL). The company also maintains a worldwide network of resellers and representatives.

Today, Minitab is often used in conjunction with the implementation of Six Sigma and other statistics-based process improvement methods. Thousands of companies in more than 80 countries use it, including over half the companies in the Fortune 500.

1.13.2.6 Statistical methods

In recent years, there has been a great amount of research and development effort focusing on the use of statistical approach methods, using different statistical software packages (Table 1.4)

during process optimization studies, with the aim of obtaining high yields of alkaline protease in the fermentation medium (De Coninck *et. al.*2000; Puri *et. al.,* 2002; Varela *et. al.* 1996).

Table 1.4: Statistical methods used to improve protease production from microorganisms.(n.s. Not specified)

Microorganism	Design	Software	Yield improvement	Reference
Rhizopus oryzae	Levenberg-Marquardt technique	n.s	2.5 fold	Banerjee and Bhattacharyya(1992)
Bacillus subtilis **IIQDB32**	Plakett-Burman	n.s	3.0 fold	Varela *et. al., (*1996)
Tetrahymena thermophila **BIII**	Central composite design	Minitab	1.2 fold protease 1.4 fold biomass	De Coninck *et al.,* (2000)
Mucar miehei **ATCC 3420 (Acid protease)**	Box-Wilson	n.s	n.s	Ayhan *et al.,(*2001)
Streptomyces sp.**82(elastase)**	Box-Wilson	n.s	7.5 fold	Georeva and Vlahov (2001)
Bacillus sp.**RGR-14**	Face-centered central composite design	Design-Expert (Statease)	2.5 fold	Puri *et al.,(*2002)

1.14 Production of protease

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 these enzymes (Moon & Parulekar, 1991).

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 enzymes like 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 product formation.

Most of the commercial proteases are generally produced by submerged fermentation. There is no universal medium for protease production and media composition varies with organism to organism. The major factors influencing the production are nutrients like carbon and nitrogen sources, vitamins and metal ions, environmental factors such as pH and temperature, agitation/aeration etc. Response Surface Methodology (RSM) is a technique used in recent times to optimize the production and increase the yields of enzyme. RSM is a useful model for studying the effect of several factors influencing the response by varying them simultaneously and carrying out a limited number of experiments. Bioreactor operation conditions 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).

1.15 Bioreactor

A Bioreactor refers to the manufactured or engineered device that supports a biologically active environment for cultivation of biological cells for production of useful metabolites. Bioreactor is used in production of commercially useful chemical or fuel by a biological process such as microbial fermentation or degradation. It is a processing system which is operated to generate

information about the behavior of the system for use in design of larger facilities. Bioreactor (fermenter) is the heart of the fermentation process.

1.15.1 Component parts of a fermentation process

Regardless of the type of fermentation an established process may be divided into six basic component parts

- The formulation of media to be used in culturing the process organism during the development of the inoculums and in the production fermenter.
- The sterilization of the medium, fermenters and ancillary equipments.
- The production of an active, pure culture in sufficient quantity to inoculate the production vessel.
- The growth of the organism in the production fermenter under optimum conditions for product formation.
- The extraction of the product and its purification.
- The disposal of effluents produced by the process.

1.15.2 Modes of Bioreactor Operations:

Bioreactors can be operated in following three ways

- Batch Bioreactors
- Continuous Bioreactors and
- Fed –batch Bioreactors.

1.15.3 Use of Bioreactor

Microorganisms are exploited to produce a wide variety of products using fermentation. These include:

- Dairy products: cheese, yogurt
- Beverages: Beer, wine
- Single Cell Proteins (SCP)
- Antibiotics
- Fuels: Ethanol, Methanol, Methane
- Chemicals: Citric acid, amino acids, vitamin and enzymes

1.16 Purification of proteases

Purification is a crucial step in the study and application of a biocatalyst. Characterization, applicability and commercial potential of an enzyme depend on its ease of purification. As such there are no specific rules in this regard but in general purification strategy of an alkaline protease usually comprises of few steps like separation of culture from fermentation broth by centrifugation or filtration followed by concentration of the culture supernatant since most of the alkaline proteases are extracellular in nature. The fermentation broth is concentrated by any one of the methods like ultrafiltration (Kang *et al.*, 1999), salting out by solid ammonium sulfate (Kumar, 2002) or solvent extraction by using either acetone (Kumar *et al.*, 1999) or ethanol (El-Shanshoury *et al.*, 1995). Other methods used for concentration of alkaline proteases include lyophilization (Manonmani and Joseph, 1993) heat treatment of enzyme (Rahman *et al.*, 1994) or use of activated charcoal (Aikat *et al.*, 2001), or PEG-35,000 (Larcher *et al.*, 1996) or temperature-sensitive hydrogel (Han *et al.*, 1995).

1.17 Biological importance of proteases

Proteases play important role in the metabolic and regulatory process such as spore formation, spore germination, protein maturation in viral assembly, various stages of mammalian fertilization process, blood coagulation, fibrinolysis (Guyton 1991) complement activation, phagocytosis and blood pressure control (Bhagavan 1973). Besides all those biochemical roles of proteases, they have also a wide industrial applications.

1.18 Industrial application of proteases

Alkaline proteases are robust enzymes with considerable industrial potential in detergents, leather processing, silver recovery, medical purposes, food processing, feeds, and chemical industries, as well as waste treatment. These enzymes contribute to the development of high value-added applications or products by using enzyme-aided (partial) digestion. The different applications currently using alkaline proteases are:

1.18.1 Detergent Industry

Proteases are one of the standard ingredients those used for cleaning household goods or sophisticated materials like contact lenses. The use for proteases in laundry detergents accounts for approximately 25% of the worldwide sales of enzymes (Grant *et.al.,* 1990). The biggest market for detergents is laundry industry, accounting to a worldwide production of 13 billion tons per year. The ideal detergent proteases should posse's broad substrate specificity to facilitate the removal of large variety of stains due to food, blood and other body secretions.The key parameter for the best performance of a protease in a detergent.

The protease is most suitable for detergent application if its isoelectric pH (pI) coicides with the pH of the detergent solution. More recently a variety of bacterial proteases active and stable at alkaline pH also stable in oxidizing agents, bleach and SDS are found to be suitable for detergent applications (Kumar and Takagi, 1999; Gupta *et.al.,* 2002). Some of the fungal proteases are also reported to be suitable for detergent application (Phadatare *et.al.,* 1993; Tanksale *et.al.,* 2001;Hajji *et al.,* 2007). 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).

1.18.2 Food industry

Proteases have been used in processing various foods such as cheese making, baking, preparation of soya hydrolysate and meat tenderization.

Proteases used in cheese manufacturing industries belong to three categories. They are animal renets, microbial coagulants and genetically engineered chymosin. In cheese making the primary function of proteases is to hydrolyze the specific peptide bond(the phe 105-Met 106 bond) to generate para-k Casein and macro peptides. Chymosin is preffered because it has high specificity for casein which is responsible for its excellent performance in cheese making. Genetically engineered chymosin is now available for commercial production of cheese (Godfrey and West 1996).

Proteases are now a day used in the bakery industry in order to soften the glutelin present in the wheat. This makes the dough soft and facilities its handling and miching and permits the production of a wider range of products. Bacterial proteases are used in order to improve the extensibility and strength of the dough.

Soyabeans serve as a rich source of food proteins. Proteolytic modifications of soya proteins improve their functional properties (Yokotsuka 1985). Treatment of soya proteins as alcalase at pH 8 results in soluble hydrolysates with hogh solubility, good protein yield and low bitterness. The hydrolysate is used in protein fortified soft drinks and in the formulation of dietetic feeds.

Protein hydrolysates may be the constituents of dietetic and health products, infant formulae, clinical nutrition supplements and as flavoring agents. This bitterness of protein hydrolysate is a major barrier in their use as a food and health care product. The intensity of bitterness is due to the number of hydrophobic bonds present in the protein. The protease which can cleave hydrophobic amino acids are valuable in debittering protein hydrolysates (Rao *et. al.,* 1998).

1.18.3 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). Alkaline-fibrinolytic protease have been reported to preferentially degrade fibrin suggesting its future application in thrombolytic therapy and anticancer drugs (Mukherjee *et al.*, 2011).

1.18.4 Leather Industry

Alkaline proteases are used in three important steps of leather tanning. e.g

- Soaking
- Dehairing
- Bating

1.18.4.1 Soaking

In 1987, Taylor *et. al.* reported that Monsheimer and pfleider used alkaline proteases in soaking from Bacterial and fungal sources and they claimed that this reduced the need for the liming chemicals by 30 to 60%. Proteases such as alcalase 1.5 from novo industry and Milenzyme 8x from Miles laboratories are used in soaking as they are compatible with the surfactants and sodium chloride which are used to prevent microbial spoilage of the hide. The advantages of enzyme soaking include-

-Shortening of the wetting back time
-Loosening of the scud
-Initiating of opening of the fibre structure (Taylor *et al.,* 1987)

1.18.4.2 Dehairing

Dehairing removes hair from the skin. Alkaline proteases from the obligate alkalophilic *Bacillus spp* perform the dehairing of hides by attacking the various proteins of hide, especially the matrix proteins, elastin and keratin. Alkaline proteolytic keratinases from *Chrysosporium keratinophilum* can be important in leather tanning industries in preference to traditional methods involving sodium sulphide (Dozie *et.al.,* 1994). Proteases reduce the use of lime and sulphide upto 50% required in non enzymatic process (Kalisz1988). Alkaline proteases carry out the process at pH 8.0-10.0 and 35-40C for 6 hr. The hair is removed at the root rather than broken off the skin surface as in the case with the lime sulphide method.

1.18.4.3 Bating

Several species of bacteria including *Bacillus subtilis* produce neutral and alkaline protease which is suitable for bating (Pvanakrishnan Dhar, 1986).

1.18.5 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 (Shoemaker, 1986; Shih & Lee, 1993). Dalev, 1994 reported an enzymatic process using a *B. subtilis* alkaline protease in the processing of waste feathers from poultry slaughterhouses

1.18.6 Silver Recovery

Silver is one of the precious and noble metals used in large quantities in the photographic industry. Around 25% of the world's silver requirement is met through recycled silver of which 75% is obtained from photographic waste (Nakiboglu *et al.,* 2003). Alkaline proteases from *Bacillus sp* (Fujiwara and Yamamoto, 1987) and fungi (Ingale *et al.,* 2002; Shankar *et al.,* 2010) have been used successfully to recover silver from spent photographic films.

1.18.7 Silk Degumming

One of the least explored areas for the use of proteases is the silk industry and only a few patents have been filed describing the use of proteases for the degumming of silk. Sericin, which is about 25% of the total weight of raw silk, covers the periphery of the raw silk fibers, thus providing the rough texture of the silk fibers. This sericin is conventionally removed from the inner core of fibroin by conducting shrink-proofing and twist-setting for the silk yarns, using starch. The process is generally expensive and therefore an alternative method suggested is the use of enzyme preparations, such as protease, for degumming the silk prior to dyeing (Gupta *et al.,* 2002). Alkaline proteases remove serecin covering the silk fibroin and increase the luster without damaging the properties of the fiber (Gulrajani *et al.,* 2000).

1.18.8 Other Applications

Some of the other applications of proteases include their use in basic research, peptide synthesis, desizing of fabrics, optical resolution of amino acids, for dissociation of cells from monolayer animal cell culture etc. Alkaline protease from *Conidiobolus coronatus* was able to replace trypsin in animal cell cultures (Chiplonkar *et al.,* 1985). Alkaline protease from *Conidiobolus coronatus* was capable of resolving the racemic mixtures of DL-phenylalanine and DL-phenylglycine conventionally done by Subtilisin Carlsberg (Sutar *et al.,* 1992). Alcalase acted as catalyst for resolution of N-protected amino acid esters (Kumar and Takagi, 1999).

1.19 Prospect of Protease enzyme in the perspective of Bangladesh

Bangladesh and other south Asian countries produce and export leather as one of the major items, which are mostly being processed by chemical treatment, resulting in inferior quality of product as well as environmental pollution. Tanneries are constantly concerned about the obnoxious odor and pollution caused by the extremely toxic sodium sulfide used in the dehairing process. Other chemicals responsible for pollution in pre-tanning processes are lime, sodium sulphide and caustic soda. Many of the tanneries in Bangladesh however use commercial enzymes in bating step and import huge amount of bating powder. Huge amount of foreign exchange will be saved if proteases produced locally are employed in soaking, dehairing and bating process and it will also be safer to environment.

Objectives of the study

Microbial proteases, especially from *Bacillus* sp. have traditionally held the predominant share of the industrial enzyme market of the worldwide enzyme sales with major application in food and feed, leather, detergent, pharmaceutical, silk and recovery of silver from photographic films (Anisworth 1994; Outtrup *et al.,* 1995; Inhs *et al.,* 1999). At the Centre for Advanced Research in Sciences (CARS),University of Dhaka, a lot of work has been done with a view to develop bioprocess on alkaline serine protease by *Bacillus licheniformis* strains, (previously isolated and characterized (Hoq *et al,* Siddiquee *et al,* Hossain *et al.*) for its technical applications in leather processing, detergent, feather solubilisation to poultry feed component. However, protease production by microorganism is highly influenced by media components as carbon, nitrogen ratio, presence of some easily metabolizable sugars such as glucose (Gupta *et al*. 2002; Beg *et al.,* 2002, Ferrero *et al.* 1996) and metal ions. Beside this, the concentration of the components has the effect on protease production. So, optimization of the medium is very important in production of protease. Optimization is a topic of central importance in industrial production processes with particular regard to biotechnology(Reddy *et al.* 2008).Optimization of medium by classical method involves changing one independent variable while maintaining all others at a fixed level and is extremely time consuming and expensive. To overcome this difficulty, experimental factorial design and response surface methodology (RSM) can be employed to optimize the medium components.

Objective of the study:

The general objective of the study was to design a medium which is cheap and easy to prepare for production of protease by *Bacillus licheniformis* MZK05M9 mutant by optimization with Centre Composite Design (CCD) and Response Surface Methodology (RSM).

Specific Objectives of the Study:

The Specific objectives of this study were as follows:

- Production of extracellular protease in shake flask by submerged culture fermentation on various media by *B. Licheniformis* MZK05M9 mutant strain.
- Evaluation of some media for protease production by the mutant strain.

- Evaluation of the effect of $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ on protease production.
- Optimization of the fermentation medium for maximum alkaline protease production using Response Surface Methodology.
- Optimization of the parameters for production of protease enzyme in shake flasks like effect of temperature and pH.
- Production of protease enzyme in lab scale bioreactor.
- Assessment of the storage stability and some tests on applications of the protease.

Chapter 2: Materials and Methods

STUDY AREA

The study was performed at the Centre for Advanced Research in Sciences (CARS),University of Dhaka, Bangladesh from a study period of June 13,2013 to July 24,2014. The materials and procedures in the present study are described below:

2.1 Bacterial strain

The bacterial strain for this experiment was *Bacillus licheniformis* MZK05M9 a mutant obtained from the Enzyme and Fermentation Biotechnology Laboratory, Department of Microbiology, University of Dhaka.

2.2 Inoculum Preparation

The inoculum was prepared on Tryptone Soya Broth (TSB) medium having following compositions.

Casein peptone (pancreatic)	1.7 g
Soya peptone (papain digest)	0.3 g
Glucose	0.25g
NaCl	0.5g
K_2HPO_4	0.25g
Distilled H_2O	100 mL (pH 7.5)

In a test tube 5 ml of Tryptone Soya Broth TSB medium was prepared and the pH was adjusted at 7.5.The medium was then autoclaved at 121°C for 15 min at 15 lbs pressure in Touchclave. After autoclaving, 1 loopful of *Bacillus licheniformis* MZK05M9 mutant was inoculated aseptically in TSB medium and incubated overnight at 37^0C. The overnight culture was then used as an inoculum for shake flask cultivation.

2.3 Production of Alkaline Protease in Shake Flask

In 250mL Erlenmeyer flask, 95 ml of fermentation medium was poured and the pH was adjusted at 7.5 by using 0.1N NAOH and 0.1N HCl. The medium was then autoclaved at 121°C for 15

min at 15 lbs pressure. After autoclaving, 5 ml of overnight culture was added to the production medium contained in Erlenmeyer flask. The flask was then kept at shaking incubator at 37^0C at 150 rpm.

Various parameters such as pH and temperature were optimized for protease production in shake flasks. On the other hand various production media having different cheap substrates were used to check the maximum cost effective alkaline protease production by mutant *B. licheniformis* MZK05M9.

2.4 Selection of Media for Protease Production

Various media such as Alkaline Protease Producing Broth (APPB), Soya Meal Medium (SMM), Modified Soya Meal Medium (MSMM), Urea Molasses Medium (UMM) were used for alkaline protease production. The compositions of the media are given in the following.

Alkaline Protease Producing Broth (Abu Sayem *et al.*, 2006)

Glucose	10.0g/L
Peptone	5.0g/L
Yeast extract	5.0g/L
K_2HPO_4	5.0g/L
$MgSO_4.7H_2O$	0.1g/L

Soya Meal Medium (Nadeem *et al.*, 2008)

Soybean meal	10g/L
Glucose	10.0g/L
K_2HPO_4	3.0g/L
$MgSO_47H_2O$	0.5g/L
NaCl	0.5g/L
$CaCl_22H_2O$	0.5g/L

Modified Soya Meal Medium (Developed by Pilot Plant Research Laboratory, CARS, University of Dhaka):

Soybean meal	10g/L
Molasses	5.0g/L
K_2HPO_4	3.0g/L
$MgSO_47H_2O$	0.5g/L
NaCl	0.5g/L
$CaCl_22H_2O$	0.5g/L

Urea Molasses Medium (Wahyuntari .B and Hendrawati 2012)

Urea	2.0g/L
Molasses	5.0g/L

100 ml of production media (all the above 4 medium) was poured in four separate 250 ml Erlenmeyer flasks and autoclaved at 121°C for 15 min at 15 lbs pressure. After cooling, the flasks were inoculated with 5% of 18 hrs old inoculum. Flasks were placed in a shaking incubator (Excella E-25, New Brunswick) at 37°C at 150 rpm for 48 hrs. Samples were then collected at 24 hrs interval and centrifuged at 6,000 rpm for 10 min and the supernatant was then stored at 4°C till the time when assay for proteolytic activity and total protein estimation were performed.

2.5 Optimization of Various Parameters for Production of Protease Enzyme in Shake Flasks

2.5.1 Effect of Temperature on Protease Production

The production of protease was carried out at 30°C, 35°C, 37°C, and 40°C, and by keeping the agitation at 150 rpm, and pH at 7.5 for 48 hrs. The crude enzyme extract was separated by

centrifugation at 10,000 rpm for 30 min at 4°C. The supernatant was then used for assay of proteolytic activity and the total protein estimation was done by Bradford method.

2.5.2 Effect of pH on Protease Production

Effect of pH on the growth of *Bacillus licheniformis* MZK05M9 mutant and enzyme production was carried out at pH ranging from 6.5, 7.0, 7.5, 8.0, 8.5, 9, 9.5 and 10. For this, medium was prepared in 250 ml of Erlenmeyer flasks with 100 ml volume of medium and pH was adjusted in range 6.5, 7.0, 7.5, 8.0, 8.5,9,9.5 and 10 with 0.1N NaOH and 0.1 N HCl. The volume of the medium was made up to desired level after adjusting the pH. The all flasks were autoclaved at 121°C and 15 lbs pressure for 15 min. After autoclaving, the medium was cooled and inoculated with 5% of18 hrs old inoculum and kept at shaking incubator at 37°C at 150 rpm for 48 hrs. The proteolytic activity measurement was performed at 24 hrs of interval.

2.6 Protease assay

Enzyme activity was determined with azo-casein (Sigma Co. St. Louis. Mo.) as substrate by a modified procedure described by Krieger and Lockwood (1981). In this method 400µl of 1% Azo-casein solution in 0.05M Tris -HCI buffer (pH 8.5) was mixed with 400µl of culture supernatant and kept for 1 hour at 37°C. The reaction was stopped by the addition of 135µl of 35% trichloroacetic acid (TCA) and keeping the mixture at 4°C for 2 to 3 min. After centrifugation at 13,000 rpm for 10 min 0.75ml of supernatant was mixed with 0.75 ml of 1.0M NaOH and the absorbance was taken at 440 nm within one minute. The control was prepared by adding TCA before mixing the culture supernatant and 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.

2.7 Estimation of Extra-soluble protein Concentration

Soluble protein in the culture supernatant was estimated according to the Bradford method (Bradford MM, 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. 5 ml Bradford test solution was taken in 9 test tubes. 0.1 ml standard solution of different dilutions was added in 7 different tubes and was well mixed. In the control 0.1 ml distilled water was added instead of sample. After 5 minutes the absorbance was taken at 595 nm. 100 µl of culture supernatant was mixed well with 5 ml of Bradford test solution and the absorbance was taken at 595 nm after 5 minutes. A standard curve of absorbance versus protein

Concentration was prepared by using the data of diluted standard protein concentrations and their corresponding absorbance. The amount of soluble protein was determined from that standard curve using the following equation and expressed as mg per ml of test sample.

$$y = mx$$

Where,

y = absorbance at 595 nm

x = protein concentration in mg/ml

m= slope of the standard curve

2.8 Determination of Specific activity

The protease activity (U/ml) is divided by protein concentration (mg/ml) to obtain the specific activity;

$$\text{i.e., specific activity (U/mg)} = \frac{\text{Protease activity}}{\text{Extracellular protein concentration}}$$

2.9 Determination of Enzyme productivity

Using the following equation, we can calculate the productivity;

$$\text{i.e., Productivity (U/L/hrs)} = \frac{\text{Enzyme activity(U/ml) X 1000}}{\text{hrs}}$$

2.10 Optimization of media

The optimization of media for enzyme production was carried out using statistical design of experiments in two steps. In the first steps the selection of variables which was done by wet lab experiments. The second step involved the optimization significant variables by RSM employing the central composite design (CCD) implemented in Minitab (Version 17) statistical software.

2.10.1 Central composite design

The next step in the optimization was to determine the optimum levels of significant variables selection by wet lab experiments. For this purpose, RSM, using a central composite design (CCD) was adopted. The CCD is a statistical experimental design numeric factor is varied over 5 levels-alpha points (-1.682, +1.682), 1 factor (+1 -1) and one central point resulting in a total of 20 experiments. Three significant variables (Molasses, Urea, and $CaCl_2.2H_2O$) were chosen for the experiment.

Table 2.1 Level of independent variables established according to central composite design (CCD).

INDEPENDENT VARIABLES	HIGH(+1)	LOW(-1)	MEAN(0)	PLUS ALPHA(+α)	MINUS ALPHA(-α)
MOLASSES	1	0.5	0.75	1.17	0.33
UREA	0.2	0.1	0.15	0.234	0.066
$CaCl_2.2H_2O$	0.1	0.05	0.075	0.117	0.033

Table 2.2 Central Composite Design

Run	Molasses (%,w/v)	Urea (%,w/v)	$CaCl_2.2H_2O$ (%,w/v)
1	-1.00	-1.00	-1.00
2	1.00	-1.00	-1.00
3	-1.00	1.00	-1.00
4	1.00	1.00	-1.00
5	-1.00	-1.00	1.00
6	1.00	-1.00	1.00
7	-1.00	1.00	1.00
8	1.00	1.00	1.00
9	-1.68	0.00	0.00
10	1.68	0.00	0.00
11	0.00	-1.68	0.00
12	0.00	1.68	0.00
13	0.00	0.00	-1.68
14	0.00	0.00	1.68
15	0.00	0.00	0.00
16	0.00	0.00	0.00
17	0.00	0.00	0.00
18	0.00	0.00	0.00
19	0.00	0.00	0.00
20	0.00	0.00	0.00

2.10.2 Statistical analysis and Modelling

The statistical analysis of the data obtained from RSM for protease production was subjected to analysis of variance (ANOVA). A second order polynomial equation can be used to represent the function in the range of interest.

$$Y= \beta_0+\beta_1X_1+\beta_2X_2+\beta_3X_3+\beta_{11}X_1^2+\beta_{22}X_2^2+\beta_{33}X_3^2+\beta_{12}X_1X_2+\beta_{13}X_1X_3+\beta_{23}X_2X_3.$$

Where Y is the measured response, β_0 is the intercept term, β_1,β_2,β_3 are linear coefficient, β_{11},β_{22}, β_{33} are quadratic coefficient, $\beta_{12},\beta_{13},\beta_{23}$ are interaction coefficient and X_1,X_2,X_3 are coded independent variables.

2.11 Protease Enzyme production in Bioreactor

2.11.1 Inoculum Development

For production of 5% inoculum for fermentation in 4 liter of Optimized urea molasses medium, one loop-full of *B.licheniformis* MZK05M9 mutant was transferred from the stock culture to 10 ml of nutrient broth and was incubated for 18hours. This was then added to 200ml OUMM and incubated for 24hours and then finally added to the bioreactor containing 4000ml of Optimized urea molasses medium.

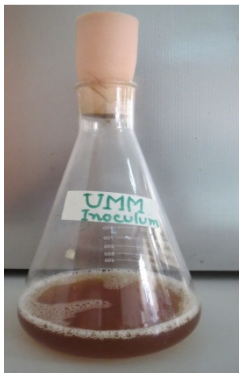


Figure 2.1: Inoculum

2.11.2 Growth conditions in Bioreactor

Optimized Urea Molasses medium was used for the cultivation of *B.licheniformis* MZK05M9 under controlled condition of bioreactor. Temperature and pH were controlled at 37˚C and 7.5 respectively. Aeration and agitation were controlled at cascading mode to maintain the dO_2 at 30%. Agitation high was set at 350rpm and agitation low at 150rpm, while the aeration high was set at 3SLPM and aeration low at 1SLPM.

2.11.3 Fermentation in Bioreactor

For this experiment bioreactor facilities in the Pilot Plant Lab at the Center for Advanced Research in Sciences in University of Dhaka were used. The vessel volume of that stirred tank bioreactor (model: BIO FLO 110 Fermenter / Bioreactor; company: NEW BRUNSWICK SCIENTIFIC) was 7.5 liter and the working volume was 4 liter.

Figure 2.2: BIO FLO 110 Bioreactor; NEW BRUNSWICK SCIENTIFIC

The bioreactor was equipped with instrumentation in order to measure and control the agitation, pH, temperature, foam, dissolve oxygen (dO_2) and exit gases. The medium was aerated by a pump (MTH) through a membrane filter. The agitator was equipped with six bladed impellers. These impellers homogenized air and the bio-controller displayed the dissolve oxygen concentration by sensing with an electrode. For temperature control, there was an outer jacket wrapping the vessel and a chiller was connected to supply cool water through a ring inside the vessel.

Before autoclaving, the vessel was washed carefully and then UMM. All the screw-able nuts were fastened tightly and the vessel was equipped with a pH probe. This probe was calibrated by dipping it in a pH 7 buffer and this value was set at zero. The probe was then rinsed with distilled water and dipped again in pH 4 buffer and set span. All the connection except air outlet was closed by tying with rubber.

Figure 2.3: Preparation of Bioreactor for autoclaving

In this experiment the pH and foam were not controlled but dO_2 was controlled by cascading mode. However, the soybean oil added acted as the anti-foam and pH probe detected the pH. The vessel was then placed into the autoclave machine (TOUCHCLAVE) very carefully and autoclaved at 121°C for 15 minutes at 15psi.

Figure2.4: **dO_2 probe was calibrated after autoclaving**

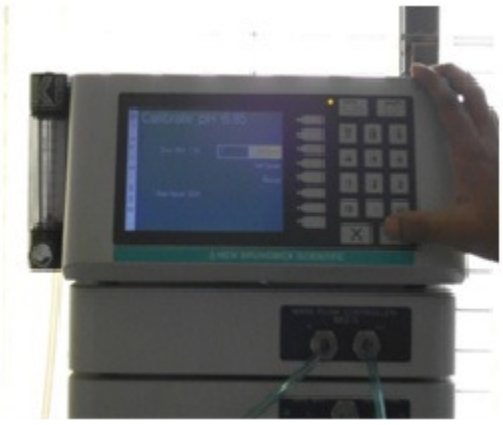


Figure 2.5: pH probe was calibrated before autoclaving

After autoclaving the vessel was placed near the bio-controller and all the connections such as pH electrode, dO_2 electrode, agitator, chiller inlet and outlet were connected carefully. When the temperature came down to 37°C, then aeration was continued to saturate the medium with dissolved oxygen. The dO_2 set point and set span were set at 30% and 100% respectively.

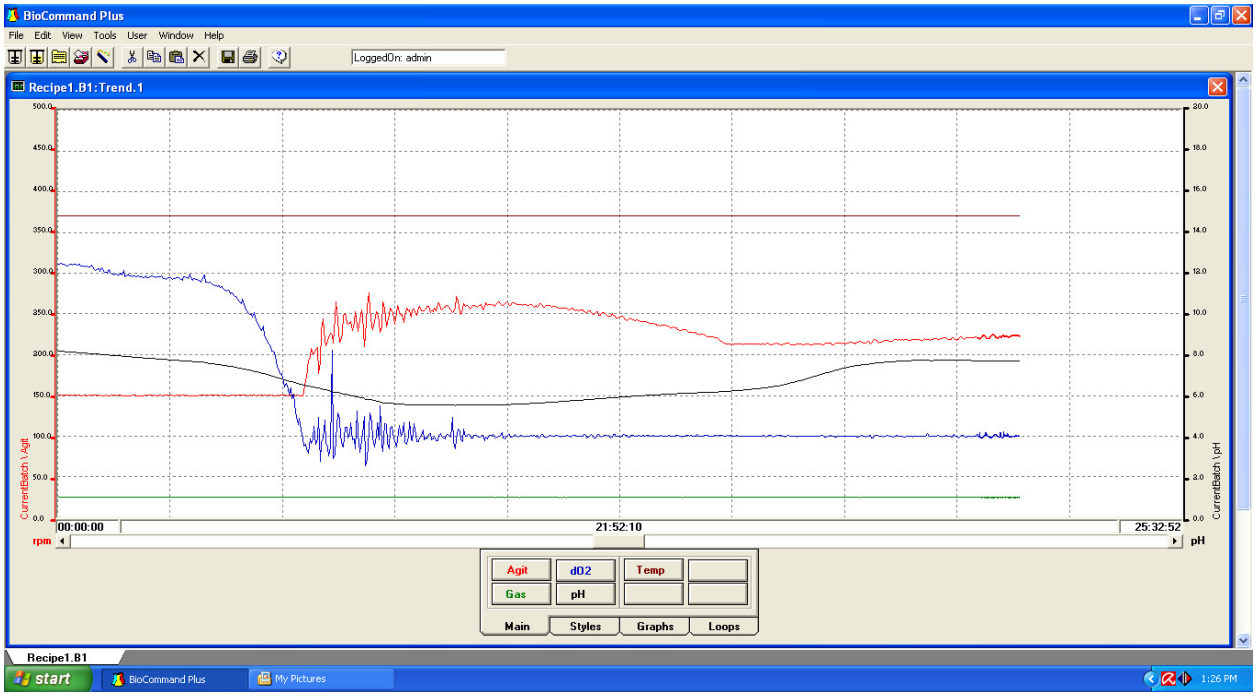


Figure 2.6: Control of different parameters during fermentation

Agitation and aeration were controlled at cascading mode to maintain dO_2 at 30%. Agitation and aeration were set between 250 to 120 rpm and 2.5 to 1 SLPM respectively. Inoculum was added. The controller was connected to a computer in order to obtain a graphical representation of change of various parameters during fermentation. Fermentation was carried out continuously for 42 hours.

Sampling was done at 4 hour interval, cell concentration estimated, and the enzyme activity was assayed by azo-casein digest method and protein concentration was measured by Bradford method after separating the cell mass from the fermented medium.

2.11.4 Separation of culture filtrates from culture media

The samples taken at 4 hour intervals were centrifuged at 6000×g for 20 minutes. The cell free supernatant was used for crude enzyme assay and protein estimation. The solid residue was discarded after autoclaving.

2.12 Determination of storage stability of protease

Storage stability of protease was determined at 4^0C, room temperature and room temperature (with chemical Sodium benzoate 0.60%). The residual enzyme activity of each line measured under standard assay condition at every 7 days of interval.

2.13 Purification of Protease Enzyme

A *Bacillus licheniformis* MZK05M9 was incubated with all determined optimized conditions in 7L bioreactor for production of bulk enzyme extract.

After the maximum production of enzyme, the following steps were carried out for the purification of protease. All the purification steps were carried out at 4ºC.

2.13.1 Ammonium Sulfate Fractionation

The required protease enzyme was fractionated by adding ammonium sulfate stepwise according to the method described below:

Culture supernatant was prepared according to the method described earlier. Every 150 ml of culture supernatant was taken in each of seven (500ml) conical flask, solid $(NH_4)_2SO_4$ was added into every conical flask gradually in order to make it 20,30,40,50,60,70,80 percent saturation. The amount of ammonium sulfate required for percentage saturation is given below table.

Table 2.3: The amount of ammonium sulfate required for percentage saturation.

Percentage of saturation	**gm of (NH4)2SO4/liter of solution**
20%	106
30%	162
40%	226
50%	291
60%	361
70%	436
80%	516
90%	603

2.13.2 Salt precipitation

Protease precipitation was carried out by ammonium sulfate. Solid $(NH_4)_2SO_4$ was added to 100mL of crude enzyme solution for partial purification. Saturation between 20 to 90% was achieved. 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 6,000 g for 10 min at 4ºC. The proteolytic activity and protein content was checked.

2.13.3 Ultrafiltration by Centricon-100

After salt precipitation the protein mixture was concentrated by centricon-100.The protein mixture was put on a centricon-100 tube and was subjected to centrifugation at 4^0C and 6000 rpm for 10 minutes.

The enzymatically active fraction was then subjected to ultrafiltration by centricon-100 Centrifugation was carried out at 4^0C and 6000 rpm for 10 minutes. The enzymatically active fraction was preserved at -20^0C.

2.13.4 Determination of Molecular Weight by Polyacrylamide Gel Electrophoresis

SDS–PAGE was carried out to determine the purity and molecular weight of the enzymes as described by Laemmli (1970) using a 4% (w/v) stacking and a 10% (w/v) separating gels. Samples were prepared by mixing enzymes with loading buffer containing 30% (w/v) glycerol, 2% SDS, 62.5 mM Tris, pH 6.8, 50 mM DTT, 5 mM EDTA, 0.02% NaN_3 and 0.01% bromophenol blue. Samples were heated at 100^0C for 5 min before electrophoresis.

After electrophoresis, gels were stained with 0.25% Coomassie Brilliant Blue R-250 in distilled water and destained with distilled water. The molecular weights of the proteolytic enzymes were estimated using a Bio-Rad Precision Plus Protein™ Unstained Standards (10-250 kDa).

2.14 Applications of Crude Protease

2.14.1 Washing Test with the Protease Preparations and Detergents

For the determination of applicability of protease enzyme as detergent additive clean cotton cloth pieces were soiled with blood and dried for 7 days at room temperature. The stained cloth pieces were taken in separate flasks. The following three sets were prepared and studied.

- Flask containing tape water (50mL)+cloth piece stained with blood
- Flask containing tape water (50mL)+cloth piece stained with blood+Jet(7mg/mL)
- Flask containing tape water (50mL)+cloth piece stained with blood+Jet(7mg/mL)+ 1.5mL of crude enzyme solution.

The above flasks were incubated at room temperature for 30 minutes. After incubation, the cloth pieces were taken out of flasks and rinsed with water and dried. Visual examination was performed and difference was checked. Untreated cloth piece stained with blood were taken as control.

2.14.2 Hydrolysis of Chicken feather

For the hydrolysis test of Chicken feather the following two steps are studied.

- Test-tube containing 10 ml tap water + one piece chicken feather.
- Text-tube containing 10 ml protease enzyme+ one piece chicken feather.

The above text tubes were incubated at 37°C for 14 days. After 7 days interval, two test tubes were checked. Water containing test-tube were taken as control.

2.15 Production cost of 1000 liter alkaline protease enzyme

To cost analysis, we have collected from different ingredient's price from different company.

Chapter 3: Results

3.1 Selection of Suitable Medium for Protease Production

Four media were evaluated to determine the maximum protease production in shake flask cultivation at 37^0C, 150 rpm for 48 hrs. Figure 3.1 shows that the maximum proteolytic activity (580U/ml) was observed with Modified Soya Meal medium (MSSM). The less proteolytic activity was found in Alkaline Protease Producing Broth (APPB) and Urea molasses media with proteolytic activity 220U/ml and 300U/ml respectively. Molasses is very cheap ingredients; it is rich in nutrients and minerals. Molasses is a carbon source and urea is a nitrogen source.

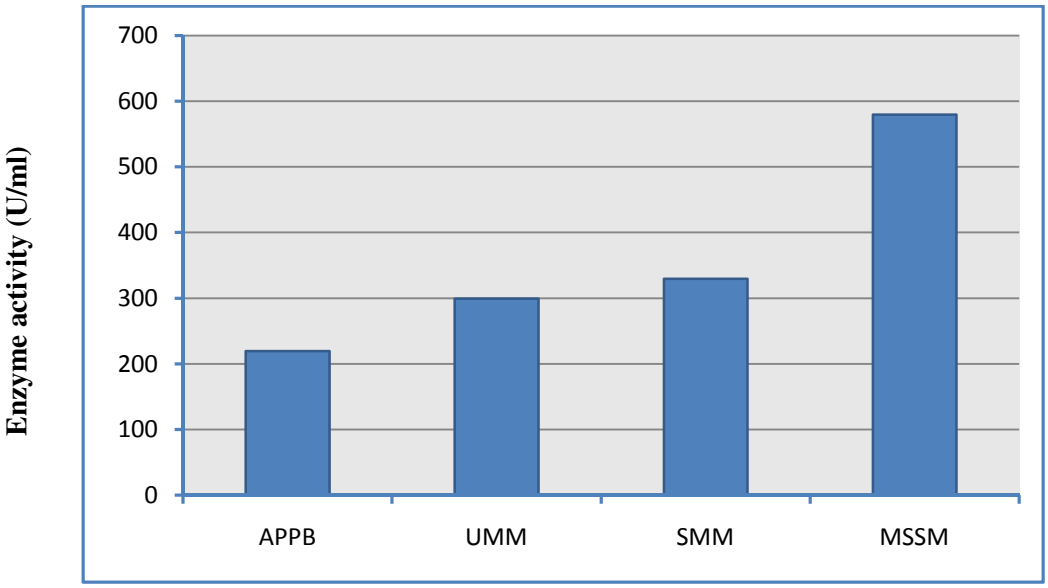


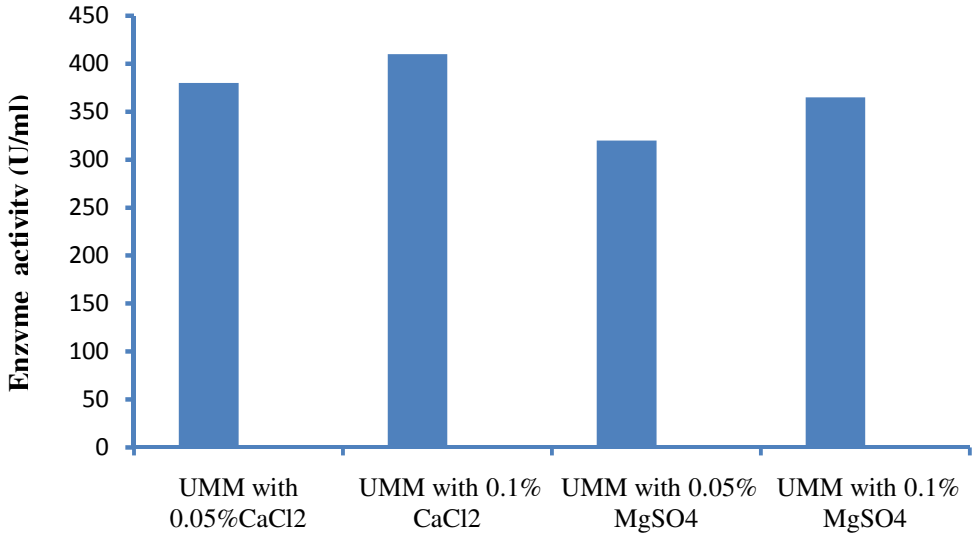
Figure 3.1Proteolytic activity of *B. licheniformis* MZK05M9 grown on MSMM=Modified Soya Meal Medium, SMM=Soya Meal Medium, APPB= Alkaline Protease Producing Broth, UMM= Urea Molasses Medium.

Urea molasses media contains only two ingredients. From literature it was found that the $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ have positive effect in protease enzyme production (Bhunia,B *et al.,* 2012). Therefore, to evaluate the effect of different salts on the

production of protease the salts were added in the Urea Molasses medium at 0.05% and 0.1% concentration.

3.2Effect of $CaCl_2.2H_2O$ and $MgSO_4.7H_2O$ on enzyme production in Urea-Molasses Media

From this study it was found that $CaCl_2$ showed better positive effect than $MgSO_4$ and 370 U/ml enzyme activity was found in case of 0.1% $CaCl_2$ in Urea Molasses medium.



\ Figure 3.2: Effect of different salts on enzyme production in Urea-Molasses medium.

So, finally, the urea, molasses and 0.1%$CaCl_2.2H_2O$ were taken into consideration to make the medium cheap and easy to prepare by optimization with Centre Composite Design (CCD) and Response Surface Methodology (RSM).

3.3. Optimization of Nutrients as well as medium components for Alkaline Protease Production by *Bacillus licheniformis* MZK05M9 with Response Surface Methodology

This study involves the optimization of the fermentation medium for maximum alkaline protease production by *Bacillus licheniformis* MZK05M9 as a result of the interaction between three variables (Molasses, Urea and $CaCl_2.2H_2O$) by employing three level designs for fitting response surfaces.

A quadratic model consisting of twenty trials experiments were designed (Table 3.1) The design matrix and the corresponding results of RSM experiments to determine the effects of three independent variables (Molasses, Urea and $CaCl_2.2H_2O$). In all, the 20 fermentation experiments were conducted in triplicate,(pH 7.5,temperature 37°C, agitation 150 rpm 48 hours of fermentation) and the average values of alkaline protease yields were tabulated, as given Table(3.1)

Table 3.1 Central composite design for the experimental design by the suggested model

Run	Molasses (%,w/v)	Urea (%,w/v)	$CaCl_2.2H_2O$ (%,w/v)	Experimental Enzyme activity (U/ml)	Predicted Enzyme activity (U/ml)	Residuals
1	0.5	0.1	0.05	320	333.035	-13.0352
2	1	0.1	0.05	397	398.268	-1.2678
3	0.5	0.2	0.05	450	432.516	17.4837
4	1	0.2	0.05	420	440.749	-20.7489
5	0.5	0.1	0.1	430	410.042	19.9580
6	1	0.1	0.1	402	420.275	-18.2746
7	0.5	0.2	0.1	455	454.523	0.4769
8	1	0.2	0.1	420	407.756	12.2443
9	0.33	0.15	0.075	360	375.177	-15.1771
10	1.17	0.15	0.075	407	390.704	16.2956
11	0.75	0.066	0.075	390	382.878	7.1224
12	0.75	0.234	0.075	450	456.004	-6.0039
13	0.75	0.15	0.033	432	421.935	10.0647
14	0.75	0.15	0.117	450	458.946	-8.9462
15	0.75	0.15	0.075	458	449.365	8.6347
16	0.75	0.15	0.075	454	449.365	4.6347
17	0.75	0.15	0.075	444	449.365	-5.3653
18	0.75	0.15	0.075	448	449.365	-1.3653
19	0.75	0.15	0.075	452	449.365	2.6347
20	0.75	0.15	0.075	440	449.365	-9.3653

The ANOVA analysis of the optimization study is given in table (3.2). The Model F-value of 8.80 implies the model is significant. There is only a 0.1% chance that a “Model F-Value” this large could occur due to noise. The model was found to be significant and sufficient to represent the actual relationship between the response and the significant variables as indicated by the

small model P-value (<0.001).The Lack of Fit F-value of 11.54 implies that it is less insignificant.

Table 3.2: Analysis of Variance for the three factorial design

SOURCE	DF	SEQ SS	CONTRIBUTION	ADJ.SS	ADJ MS	F-VALUE	P-VALUE
Model	9	21987.5	88.79%	21987.5	2443.05	8.80	0.001
Linear	3	8399.5	33.92%	8399.5	2799.83	10.09	0.002
A	1	291.0	1.18%	291.0	291.03	1.05	0.330
B	1	6454.9	26.07%	6454.93	6454.93	23.26	0.001
C	1	1653.5	6.68%	1653.5	1653.51	5.96	0.035
Square	3	8938.5	36.10%	8938.5	2979.49	10.74	0.002
A*A	1	7260.9	29.32%	7948.2	7948.23	28.64	0.000
B*B	1	1534.1	6.20%	1613.1	1613.13	5.81	0.037
C*C	1	143.5	0.58%	143.5	143.48	0.52	0.489
2 way interaction	3	4639.5	18.78%	4649.5	1549.83	5.58	0.016
A*B	1	1624.5	6.56%	1624.5	1624.50	5.85	0.036
A*C	1	1512.5	6.11%	1512.5	1512.50	5.45	0.042
B*C	1	1512.5	6.11%	1512.5	1512.50	5.45	0.042
Error	10	2775.5	11.21%	2775.5	277.55		
Lack of Fit	5	2554.2	10.31%	2554.2	510.83	11.54	0.009
Pure Error	5	221.3	0.89%	221.3	44.27		
Total	19	24763.0	100.0%				

R^2 =0.8879, ADJUSTED .R^2=0.7870

Notes: DF, Degree of freedom; SS, some of square; MS, mean of square; F: F ratio (F=MS/SS)

The regression equation coefficients (Table 3.3) were calculated and the data was fitted to a second-order polynomial equation. Thus the response (Y), (in terms of coded factors) protease production by the selected *Bacillus licheniformis* MZK05M9 mutant can be expressed in terms of the following regression equation.

Protease Yield = 449.37+4.62A+21.74B+11.00C-23.48A*A-10.58 B*B-3.16 C*C-14.25A*B-13.75A*C-13.75B*C

Where A is Molasses, B is Urea and C is $CaCl_2.2H_2O$

Table3.3: Coded Coefficients estimated by multiple linear regression.

Term	Coefficient	T -value	P-value
Constant	449.37	66.13	0.000
A	4.62	1.02	0.330
B	21.74	4.82	0.001
C	11.70	2.44	0.035
A*A	-23.48	-5.35	0.000
B*B	-10.48	-2.41	0.037
C*C	-3.16	-0.72	0.489
A*B	-14.25	-2.42	0.036
A*C	-13.75	-2.33	0.042
B*C	-13.75	-2.33	0.042

Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case B, C, A*A, B*B, A*B, A*C, B*C are significant model terms.

The regression equation obtained from the ANOVA (Table 3.2) showed that the R^2 (multiple correlation coefficient) was 0.8879. This is an estimate of the fraction of overall variation in the data accounted by the model, indicating that the model is capable of explaining 88.79% of the variation in response. [For a good statistical model, the R^2 value should be in the range of 0-1.0]

3.3.1: Residual plots of experimental vs. predicted values of protease production

3.3.1.1 Normal probability plot of residuals for Y (Enzyme Yield)

From the Figure 3.3 it was observed, the normal plot shows an approximately linear pattern that is consistent with a normal distribution.

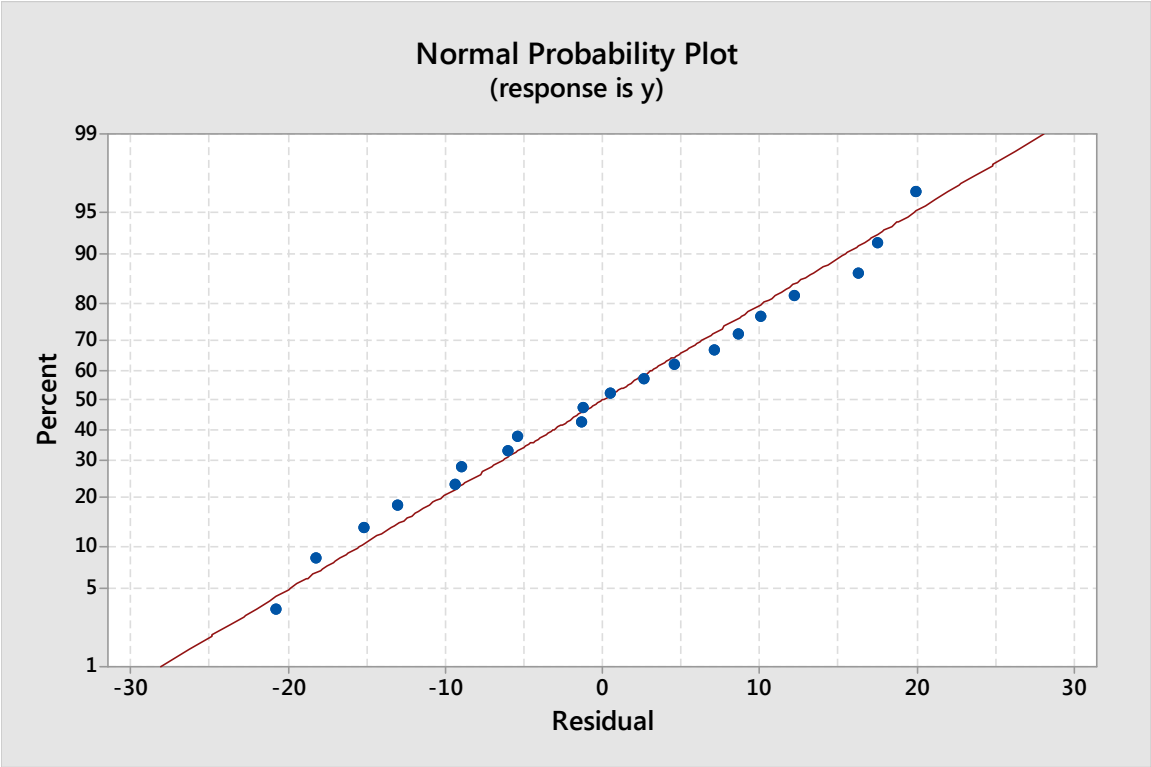


Figure 3.3: The normal probability plot of residuals

3.3.1.2 Residuals versus Fits for Y (Enzyme Yield)

From the Figure 3.4, it was found that, the plot of residuals versus fits shows that the fit tends to be better for higher predicted values

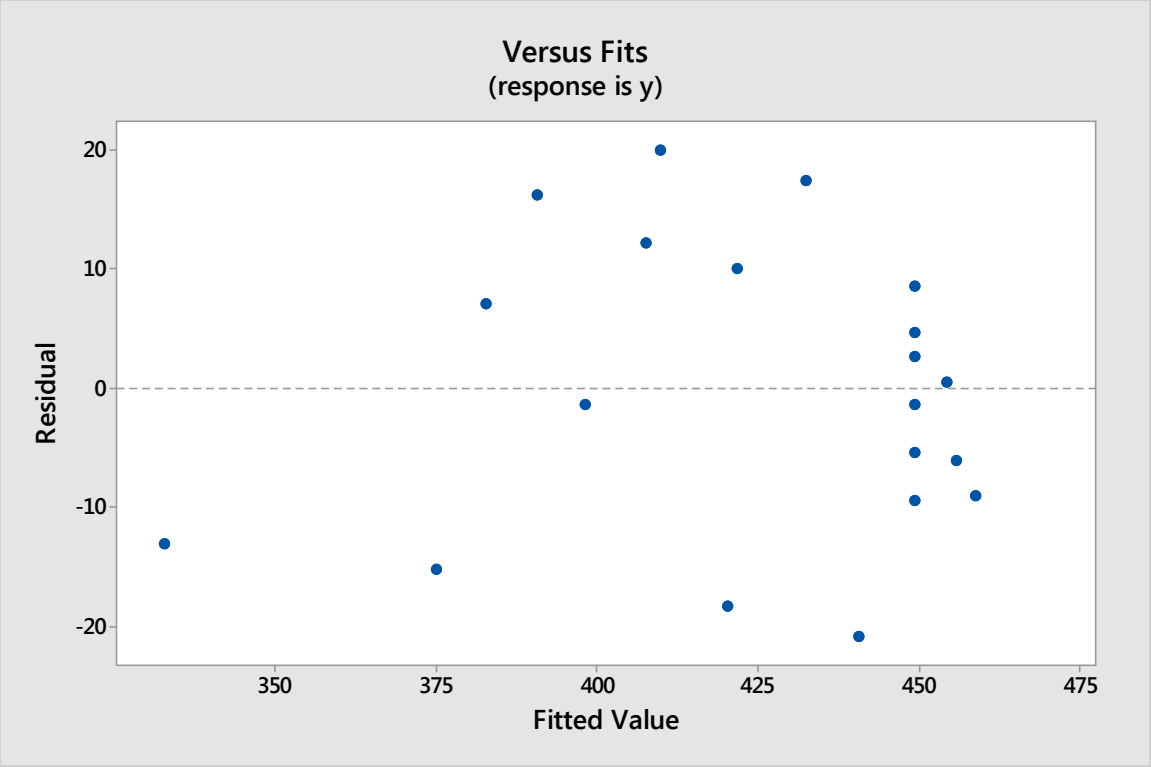


Figure 3.4: Distributions of residual vs. fitted values

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3.3.1.3 Residual Histogram for Y (Enzyme Yield)

From the Figure 3.5, it was found that, the histogram plot shows an approximately linear pattern that is consistent with a normal distribution.

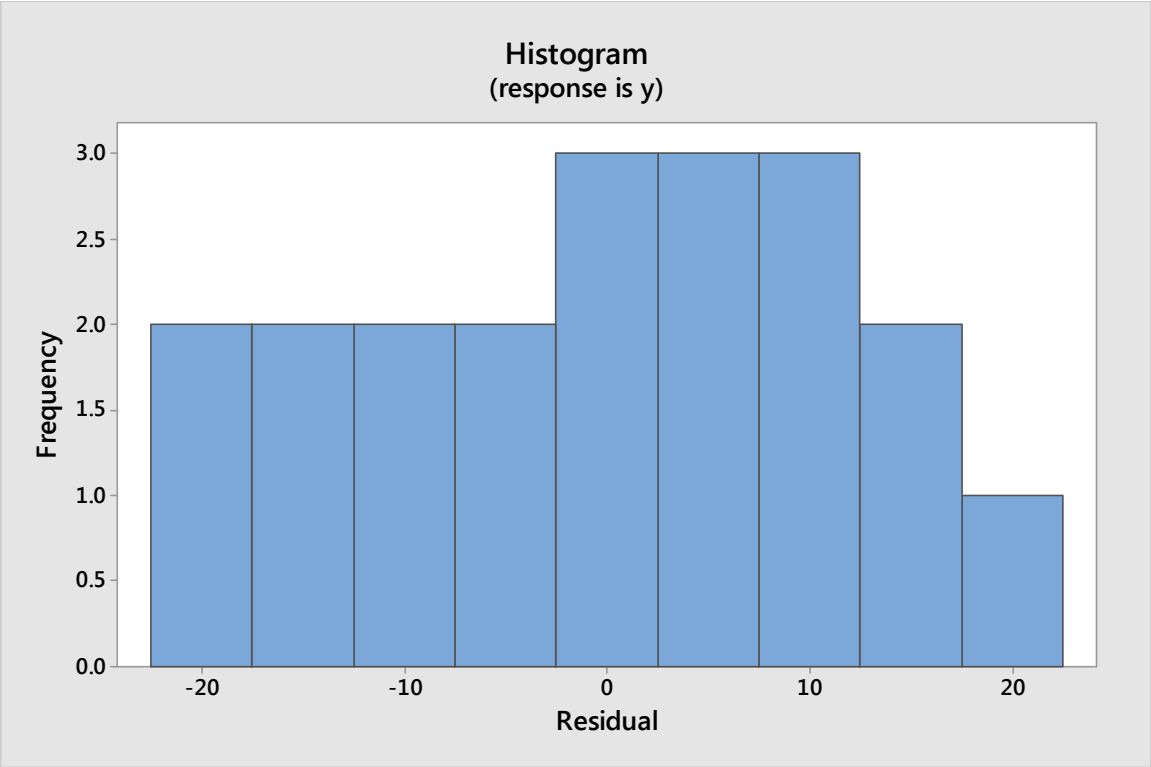


Figure 3.5: Residual Histogram for Y (Enzyme Yield)

3.3.1.4: Residuals Vs Order for Y (Enzyme Yield)

From the Figure 3.6 it was observed that, the control chart shows that all observations fall in the three sigma limits.

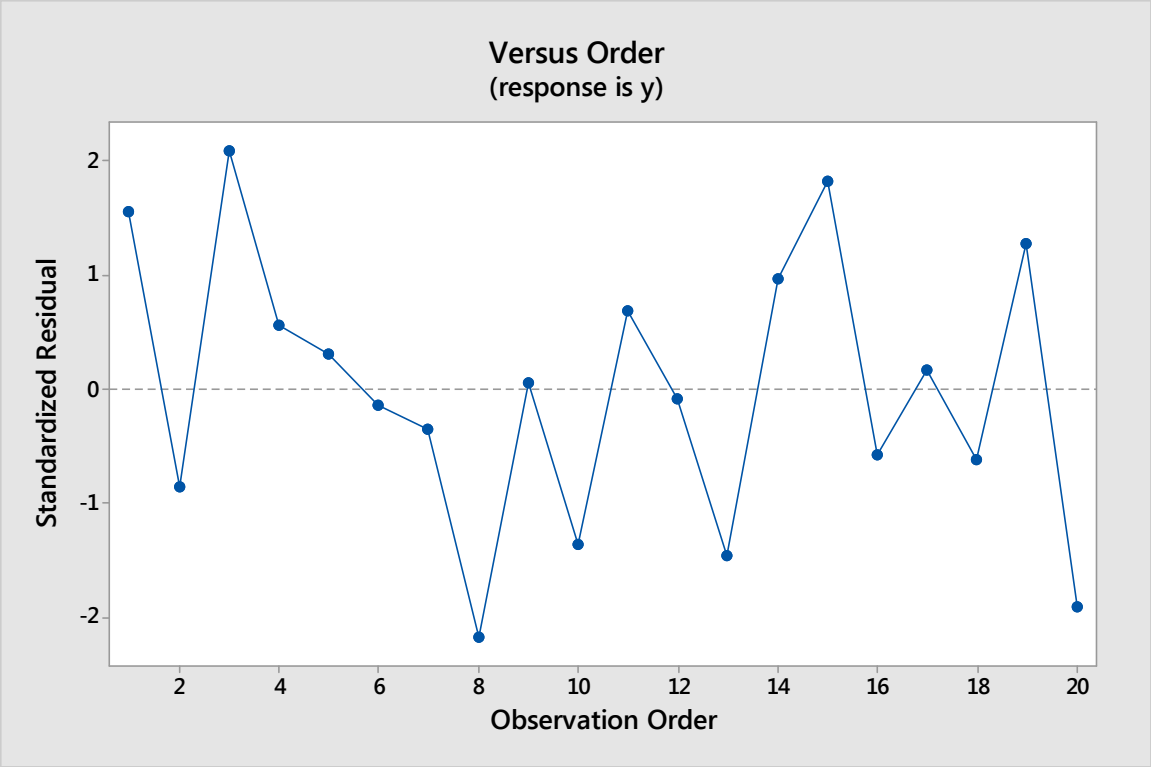


Figure 3.6: Residuals Vs Order for Y (Enzyme Yield)

3.3.2 Optimization plots for Protease production by *B.licheniformis* MZK05M9

3.3.2.1 Contour plots for Protease production by *B.licheniformis* MZK05M9

Figure 3.7A depicts the effect of molasses and urea on the microbial enzyme production while $CaCl_2.2H_2O$ was constant. The effect of molasses and urea is quadratic. The effect of urea is more than that of molasses on enzyme yield. (Y= Enzyme yield).

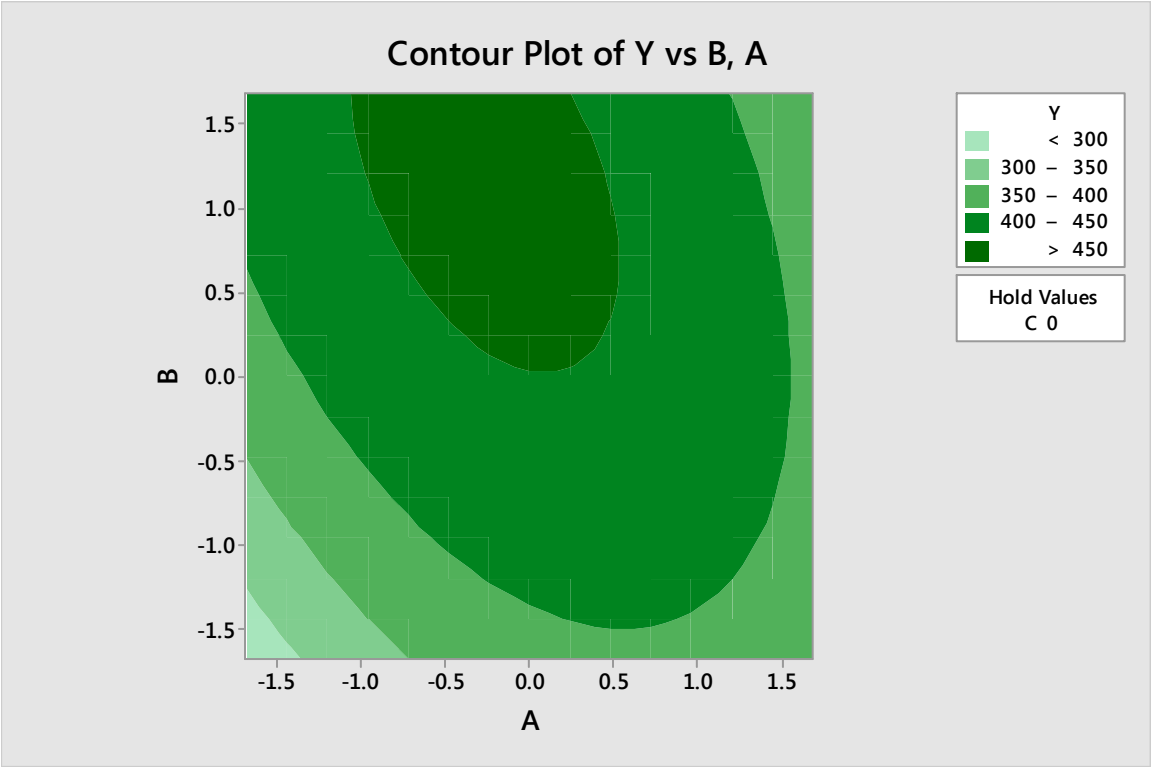


Figure 3.7A: Contour plot of the effects of molasses and urea on the protease enzyme production by *B.licheniformis* MZK05M9 mutant.(Where A=molasses, B=urea, C=$CaCl_2.2H_2O$ and Y= Enzyme yield).

Figure 3.7B depicts the effect of molasses and $CaCl_2.2H_2O$ on the microbial enzyme production while urea was constant. The effect of molasses and $CaCl_2.2H_2O$ is quadratic. The effect of $CaCl_2.2H_2O$ is more than that of molasses on enzyme yield. (Y= Enzyme yield).

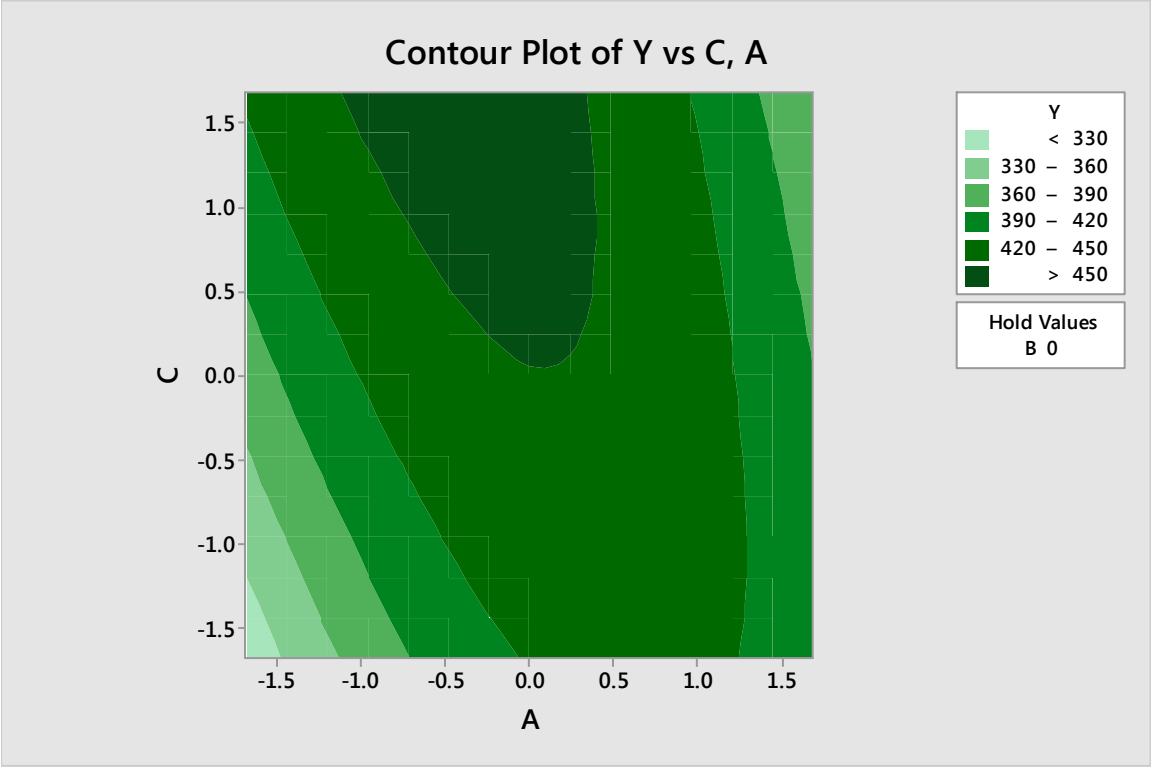


Figure3.7 B: Contour plot of the effects of molasses and $CaCl_2.2H_2O$ on the protease enzyme production by *B.licheniformis* MZK05M9 mutant. (Where A=molasses, B=urea, C=$CaCl_2.2H_2O$ and Y= Enzyme yield).

Figure 3.7C depicts the effect of urea and $CaCl_2.2H_2O$ on the microbial enzyme production while molasses was constant. The effect of urea and $CaCl_2.2H_2O$ is approximately linear. Both and Urea and $CaCl_2.2H_2O$ have similar effect on enzyme yield. (Y= Enzyme yield).

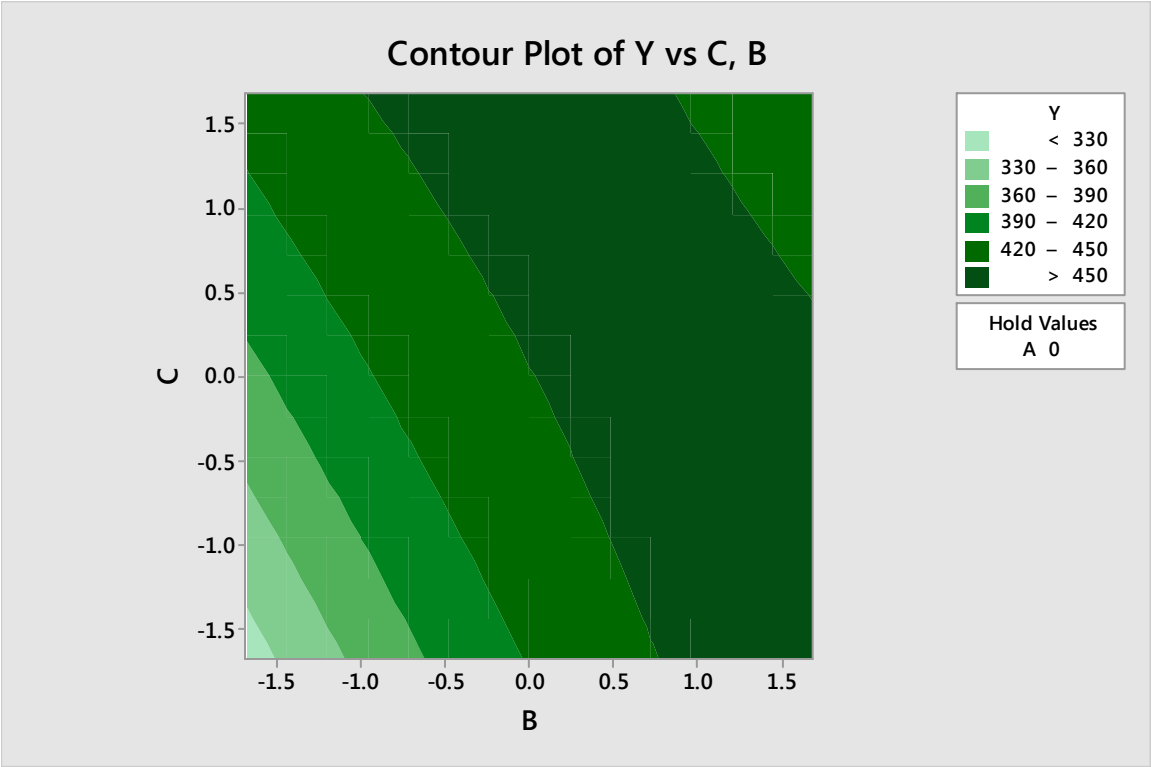


Figure 3.7 C: Contour plot of the effects of urea and $CaCl_2.2H_2O$ on the protease enzyme production by *B.licheniformis* MZK05M9 mutant. (Where A=molasses, B=urea, C=$CaCl_2.2H_2O$ and Y= Enzyme yield).

3.3.2.2 Response surface plots for protease enzyme production by *B.licheniformi* MZK05M9

Figure3.8A shows the effect of molasses and urea and their mutual effect on the production of alkaline protease by *B.licheniformis* MZK05M9. The surface plot shows that the highest yield is obtained when urea levels are high and molasses levels are low.

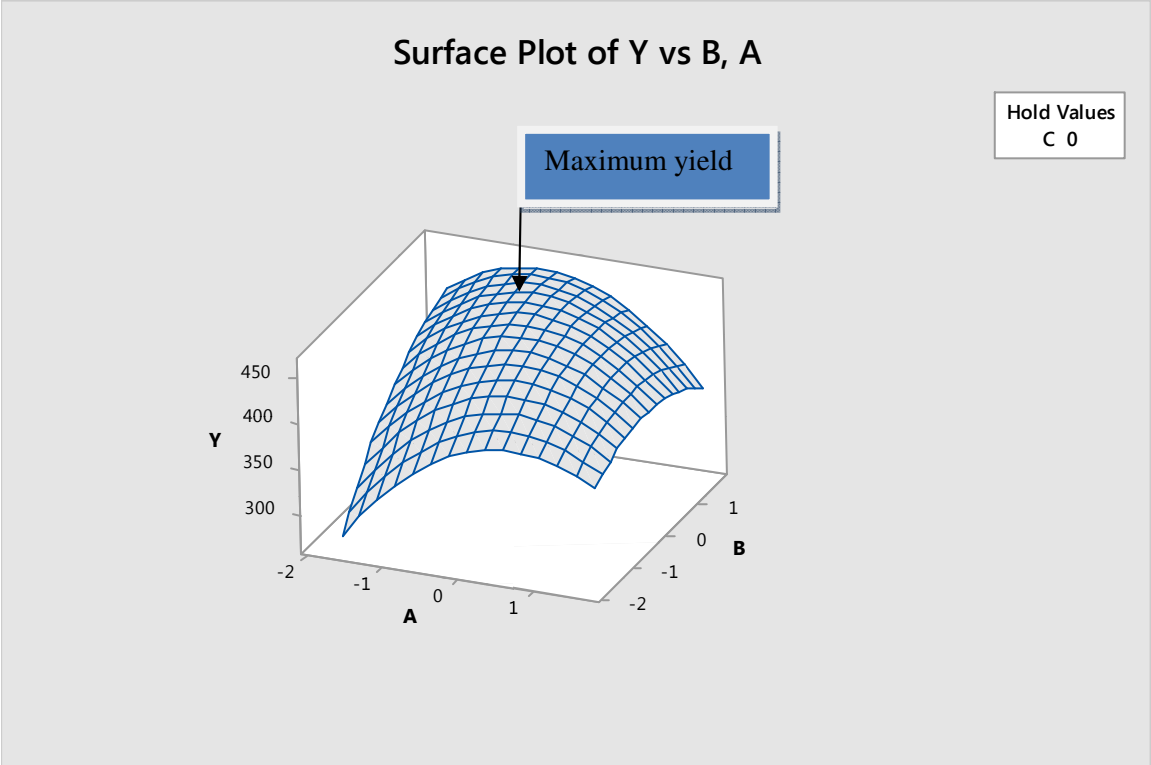


Figure3.8A: Three-Dimensional Response surface plot for the effects of molasses and urea on protease production by *B.licheniformis* MZK05M9 at a constant $CaCl_2.2H_2O$.(Where A=molasses, B=urea, C=$CaCl_2.2H_2O$ and Y= Enzyme yield).

Figure 3.8B shows the effect of molasses and $CaCl_2.2H_2O$ and their mutual effect on the production of alkaline protease by *B.licheniformis* MZK05M9. The surface plot also shows that the highest yield is obtained when $CaCl_2.2H_2O$ levels are high and molasses levels are low.

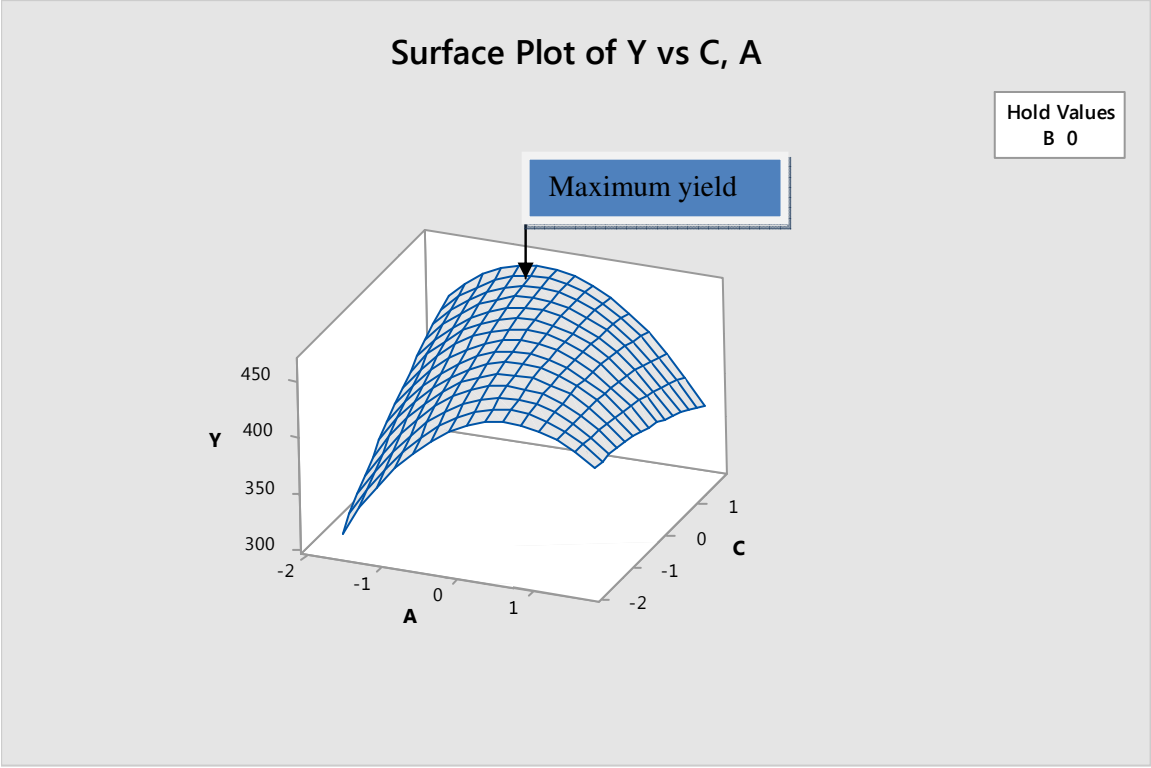


Figure3.8B:Three-Dimensional Response surface plot for the effects of molasses and $CaCl_2.2H_2O$ on protease production by *B.licheniformis* MZK05M9 mutant at a constant urea. (Where A=molasses, B=urea, C=$CaCl_2.2H_2O$ and Y= Enzyme yield).

Figure3.8C shows the effect of urea and $CaCl_2.2H_2O$ and their mutual effect on the production of alkaline protease by *B.licheniformis* MZK0509. The surface plot shows that the highest yield is obtained when both urea and $CaCl_2.2H_2O$ levels are high. But $CaCl_2.2H_2O$ has greater effect than that of urea.

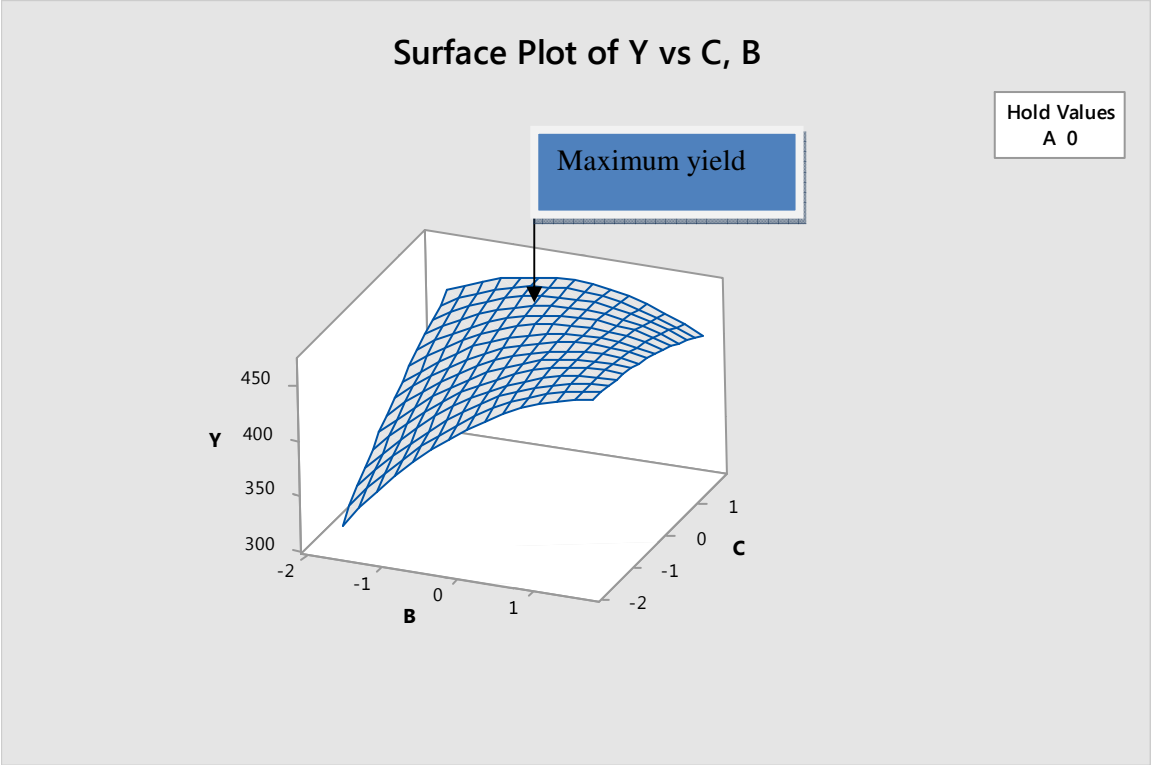


Figure3.8C: Three-Dimensional Response surface plot for the effects of urea and $CaCl_2.2H_2O$ on protease production by *B.licheniformis* MZK05M9 at a constant molasses.(Where A=molasses, B=urea, C=$CaCl_2.2H_2O$ and Y= Enzyme yield).

3.3.3 Prediction by the Software Minitab (Version 17)

A Prediction was found by the Software (Minitab Version 17) for stationary point of response surface for the alkaline Enzyme Production by *B.licheniformis* MZK05M9.

Table3.4: The Enzyme yield at Stationary point of response surface

Molasses (% w/v)	Urea (%w/v)	$CaCl_2.2H_20$(% w/v)	Enzyme Yield (U/ml)
0.6353	0.1627	0.1170	463.1

3.3.4 Validation of the model in the shake flask culture

The validation of the statistically optimized condition for the production of protease by the selected *Bacillus* strain was verified by carrying out shake flask fermentation in the laboratory using the composition suggested by the software (Minitab 17). The model was verified for the three variables within the design space and tested for the protease production. Under the final optimized conditions, the predicted response for protease production was 463.1 U/ml, and the observed result was 560 U/ml in statistically optimized medium (Figure3.9). These results confirmed the validity of the model, and the optimization of the media led to 1.3 fold increase in the enzyme activity than the initial activity (410U/ml) in unoptimized medium.

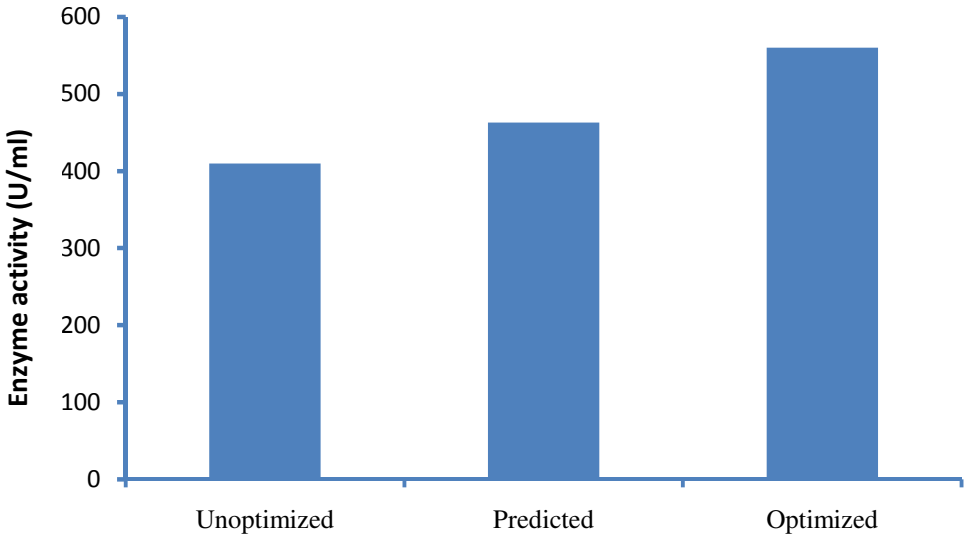


Figure 3.9: Validation of the model in the shake flask culture

3.4 Effect of temperature on Protease production in optimized Urea-Molasses medium

The effect of temperature on the production of alkaline protease by *Bacillus licheniformis* MZK05M9 mutant was determined by cultivating the microorganism at various temperature 30^0C, 35^0C, 37^0C and 40^0C at 150 rpm for 48 hrs. The maximum protease production (562.33± 10.78 U/ml) was observed at 37°C after 48 hours of incubation. The enzyme activity was found at 30°C, 35°C and 40°C as 502 ± 7.21 U/ml, 525.66±6.02 and 454.33±6.02 respectively after 48 hours of incubation.

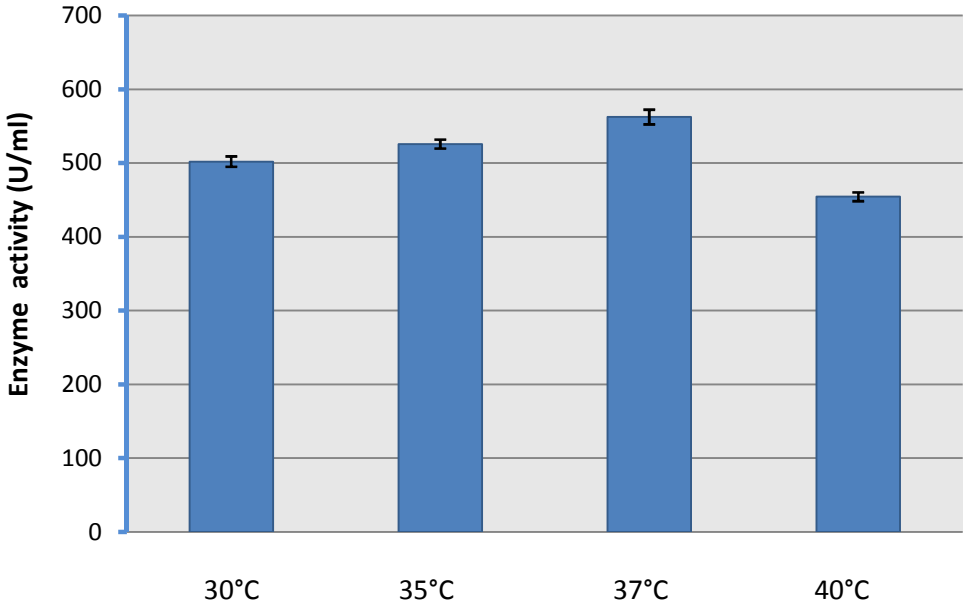


Figure 3.10: Effect of temperature on protease production by *B. licheniformis* MZK05M9

3.5 Effect of initial pH of Urea-Molasses medium on Protease production in shake flask

The effect of pH on the production of alkaline protease by *B. licheniformis* MZK05M9 mutant was determined by cultivating the microorganism at various pH 6.5, 7, 7.5,8, 8.5, 9, 9.5 and 10 at 150 rpm for 48 hrs. The optimum pH for *B. licheniformis* MZK05M9 was 7.5 after 48 hrs of incubation at 37°C and 150 rpm. The highest enzyme activity 551.66 ±10.40U/ml. was found at pH 7.5.On the other hand, The enzyme activity was found at pH 6.5, 7.0, 8.0, 8.5, 9.0, 9.5 and 10.0 as 411.66±7.63, 478.33±7.63, 500±10,498.33±10.40, 481.66±10.40, 463.33±2.88, 456.66±5.77U/ml respectively after 48 hrs of incubation(Figure 3.11) . From the experiment it was found that, the *B. licheniformis* MZK05M9 produced in a broad range of enzyme from 7.5 to 10.

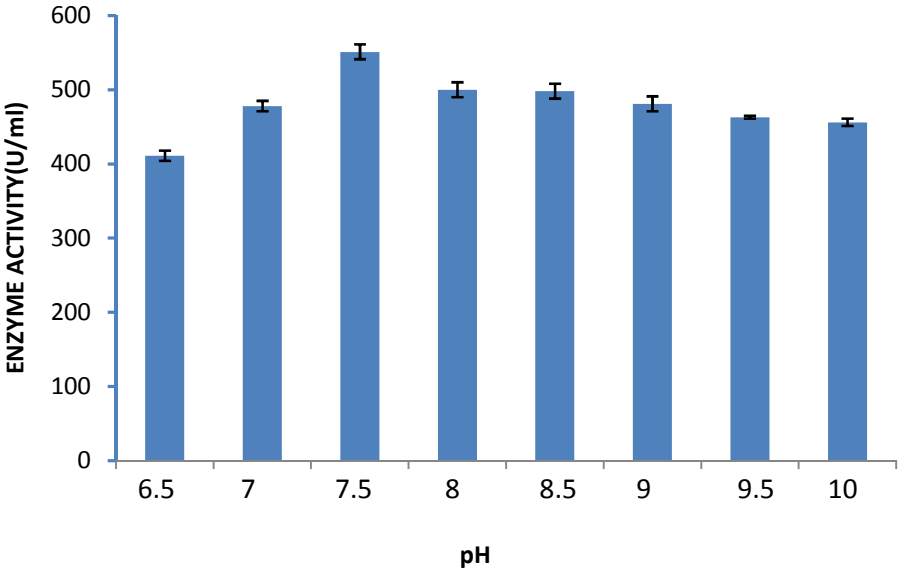


Figure 3.11: Effect of initial culture pH on protease production

3.6 Time course of enzyme production by *Bacillus licheniformis* MZK05M9 mutant in Shake flask

3.6.1 Time course of enzyme activity in shake flask

The enzyme activity was estimated at 4 hours interval. The maximum 560 ±5.56 U/ml enzyme activity was found after 48 hrs of fermentation. From the figure (3.12), it is found that the enzyme activity was increased exponentially after 20hrs.

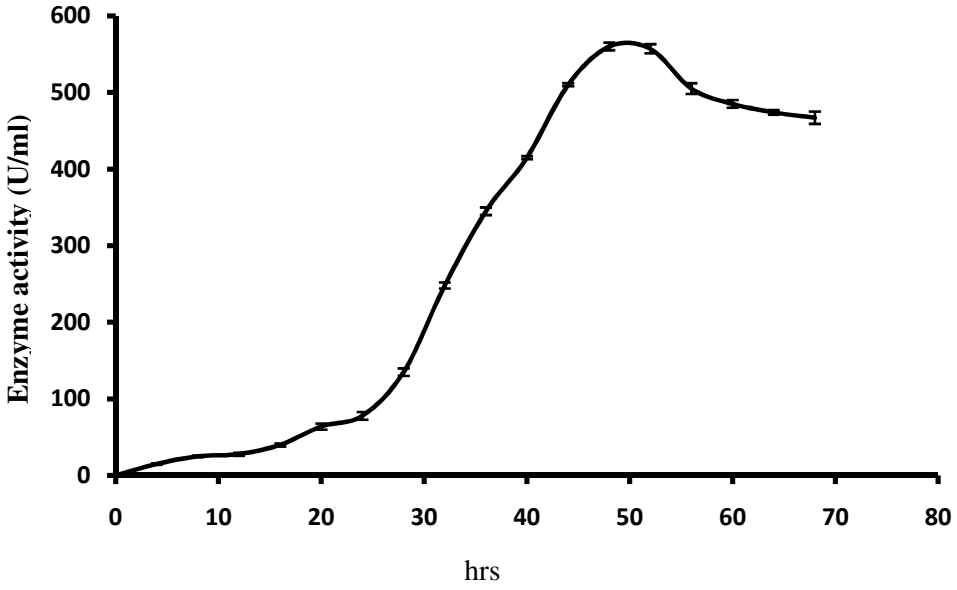


Figure 3.12: Time course of enzyme activity in shake flask

3.6.2 Time course of cell concentration in Shake flask

The Cell concentration was estimated after 4 hours interval. The maximum cell concentration was found after 48 hour 9.3 log cfu/ml. From the figure (3.13) it can be said that the culture was gone to death phage after 55 hrs.

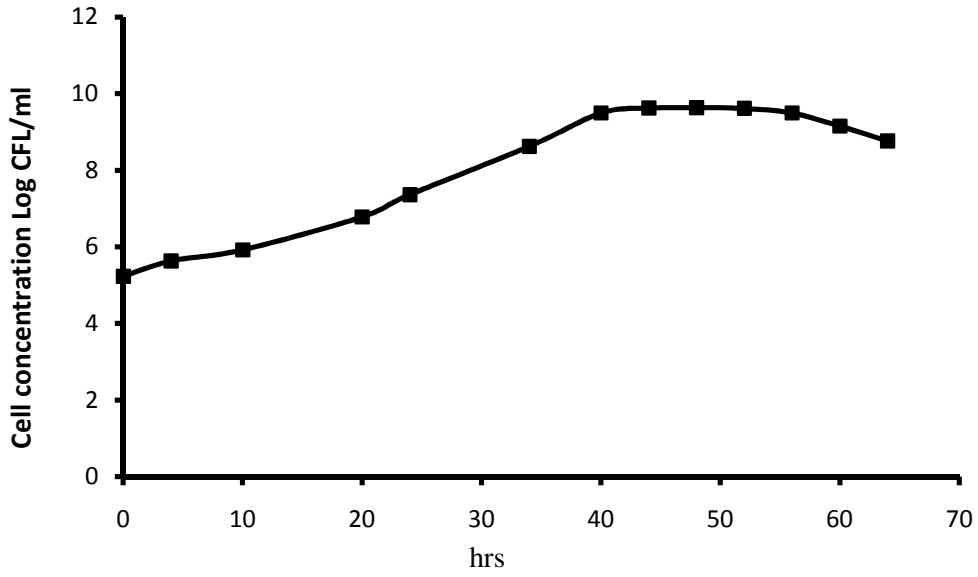


Figure 3.13: Time course of cell concentration in Shake flask

3.6.3Time course of protein concentration

The protein concentration was measured after 4 hours interval. This graph shows the production of extracellular protein by the *B. licheniformis* MZK05M9. The production of extracellular proteins begins at around the 4th hour and kept on increasing till 48 hrs. The maximum concentration was found at 48th hour of fermentation which was 0.726± 0.015 mg/ml.

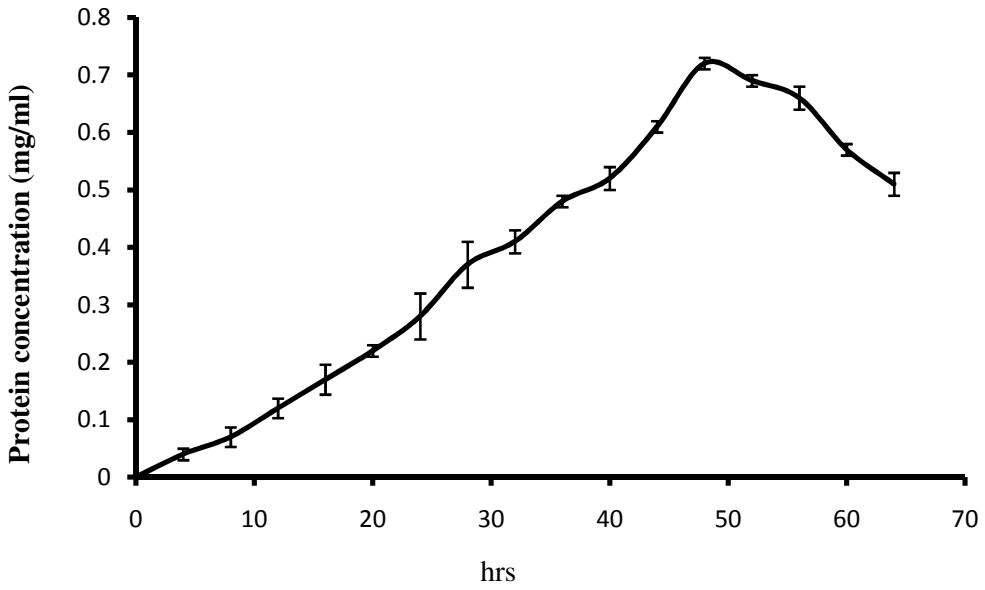


Figure 3.14: Time course of Protein concentration in shake flask

3.7 Enzyme production in Bioreactor in Optimized Urea-Molasses Medium

3.7.1 Time course of Enzyme activity in bioreactor

Protease production in 7 L bioreactor (New Brunswick Scientific, Bioflo 110) was performed using Optimized Urea-Molasses medium at 37^0C and pH 7.5 under 30% dissolved oxygen (dO_2) concentration which was controlled by cascading mode maintained with both agitation and aeration automatically (Figure 3.15). Under this condition, after 36 hrs of incubation proteolytic activity of the enzyme was measured as 500± 5.656 U/ml.

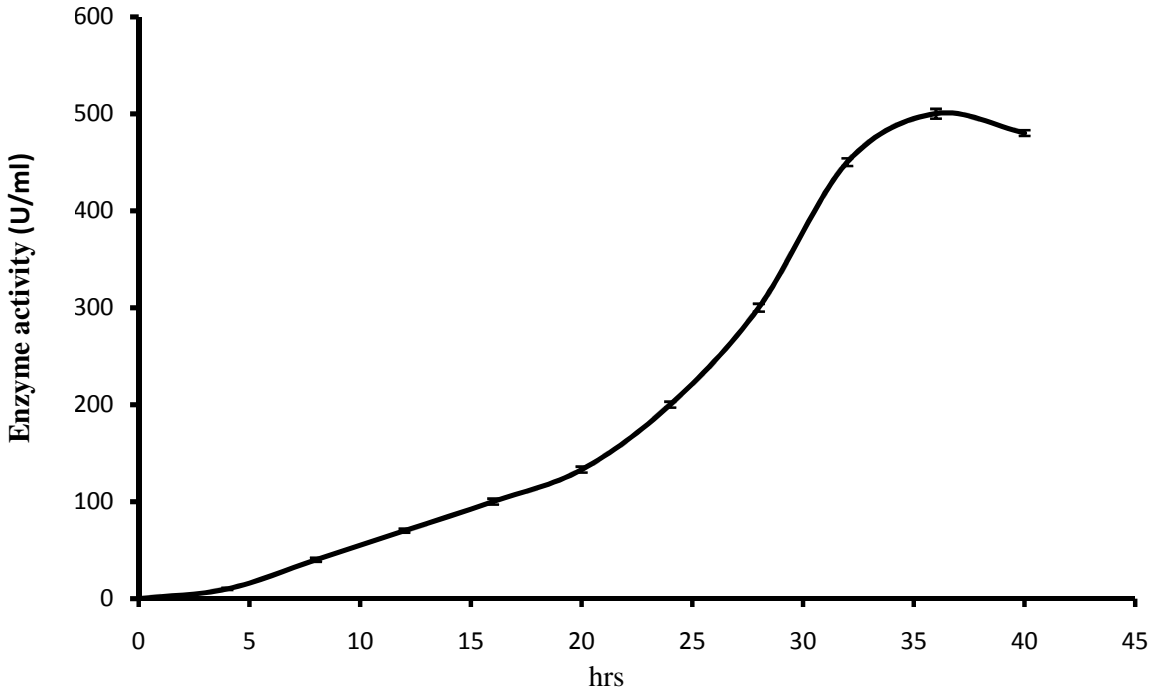


Figure 3.15: Time course of Enzyme activity in bioreactor

3.7.2 Time course of protein concentration in Bioreactor

The protein concentration was measured after 4 hours interval. This graph shows the production of extracellular protein by this *B. licheniformis* MZK05M9. The production of extracellular proteins begins at around the 4th hour and keeps on increasing till the end of fermentation. The maximum concentration was found at 36th hour of fermentation which was 0.69± 0.007071 mg/ml.

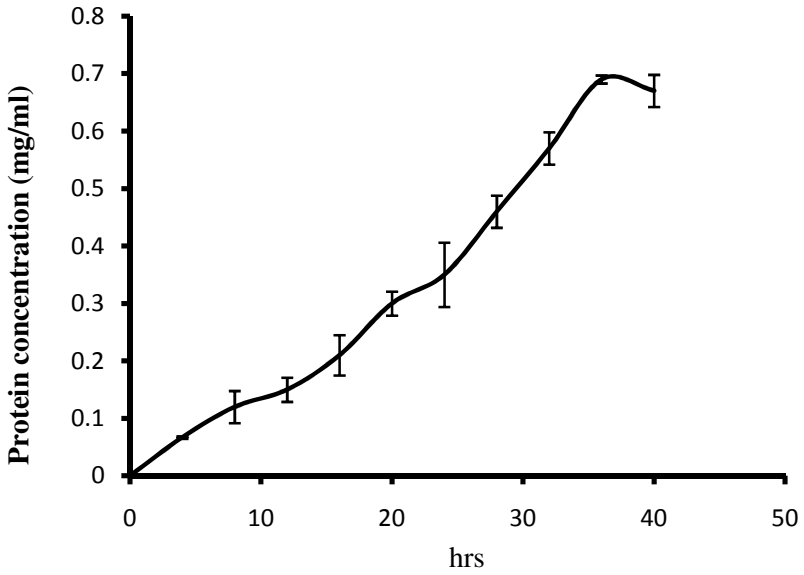


Figure 3.16: Time course of protein concentration in Bioreactor

3.8 Comparison of Productivity between Shake flask and Bioreactor

In shake flask, the highest enzyme activity was found 560 U/ml after 48 hours where as Bioreactor highest enzyme activity was found 500 U/ml after 36 hours. The comparison of the productivity between Shake flask and Bioreactor are given in the table (3.5)

Table 3.5: Comparison of Productivity between Shake flask and Bioreactor

Name	Time (hrs)	Productivity(U/L/hrs)
Shake flask	48	11,666.6
Bioreactor	36	13,888.8

3.9 Storage stability of Alkaline Protease from *B. licheniformis* MZK05M9

The crude extracellular protease enzyme was stored under three different condition namely 4°C,room temperature and room temperature with chemical (Sodium Benzoate 0.60%) for 4 weeks. The results are presented in figure (3.17). Enzyme activity was determined from each sample at seven days intervals and the process was continued for 4 weeks. It was found that the activity of the enzyme stored at room temperature declined slowly within seven days and thereafter enzyme activity declined moderately up to 4 weeks, and room temperature (with chemical sodium benzoate) declined slowly after 3rd weeks and four weeks. There was no significant decrease in enzyme activity when stored at 4°C within the above mentioned period of investigation. After fourth week the enzyme activity was found for 560U/ml (100%) at 4°C, room temperature 490U/ml (87.5%) and room temperature with chemical 540 U/ml (96.4%) respectively.

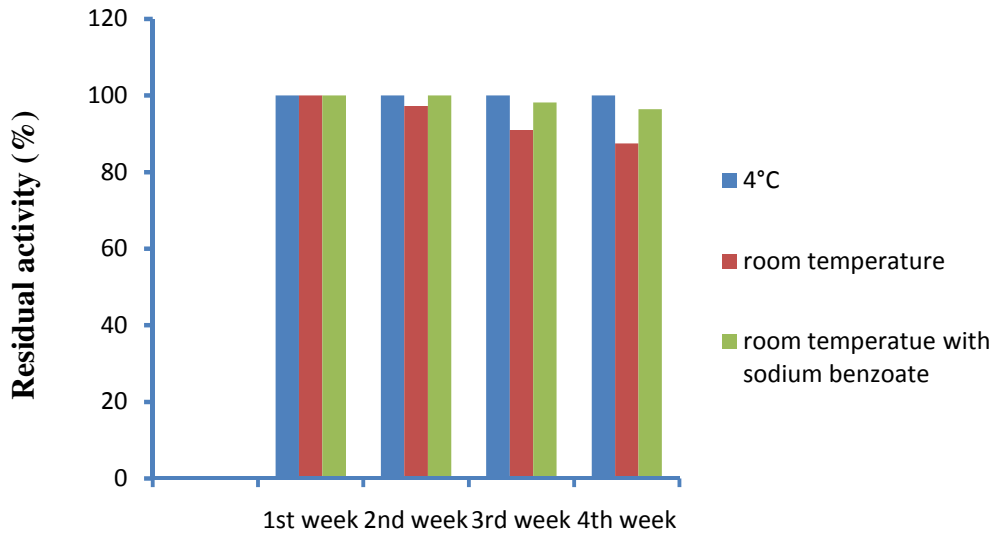


Figure 3.17 Storage stability of alkaline protease from *Bacillus licheniformis* MZK05M9

3.10. Purification of Protease

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 alkaline protease (Figure 3.18). 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% $(NH_4)_2SO_4$ to the culture filtrate. Whereas minimum extraction was achieved at 20, 30, 40 and 50% concentration with 50, 53, 66 and 87% respectively, Hence 60% ammonium sulfate was found suitable for the extraction of whole alkaline protease from culture filtrate.

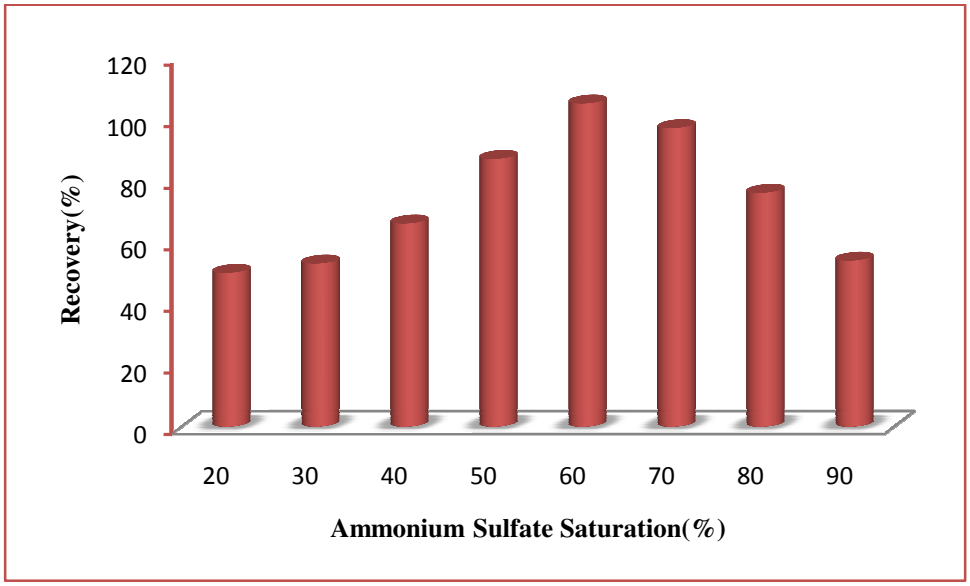


Figure 3.18: Ammonium Sulfate Precipitation of Protein

The following table gives an overall view of the purification process. The specific activity of the protease after ammonium sulfate fractionation (60%) was about 3.025 fold higher than that of culture supernatant. Ultrafiltration by centricon-100 effectively eliminated most of the impurities of the protease and raised the specific activity to about 16.59.

Table 3.6: Purification of extracellular protease from *B. licheniformis* MZK05M9

Name	Enzyme activity(U/ml)	Protein Concentration (mg/ml)	Specific activity(U/mg)	Purification Fold
Crude enzyme	560	0.726	771.34	1
Ammonium sulfate precipitation(60%)	10,758.2	4.6099	2,333.7	3.025
Centricon-100 Kda permeate	7,224.4	0.1865	38,736.7	16.59

3.11The molecular weight of protease *B. licheniformis* MZK05M9 *by* SDS-PAGE

The molecular weight of *Bacillus licheniformis* MZK05M9 alkaline protease (Centricon permeate partially purified) was determined by running the protein in SDS-PAGE. Comparison with the molecular weight marker revealed that *B. licheniformis alkaline* protease has a molecular weight of about 28 kDa.

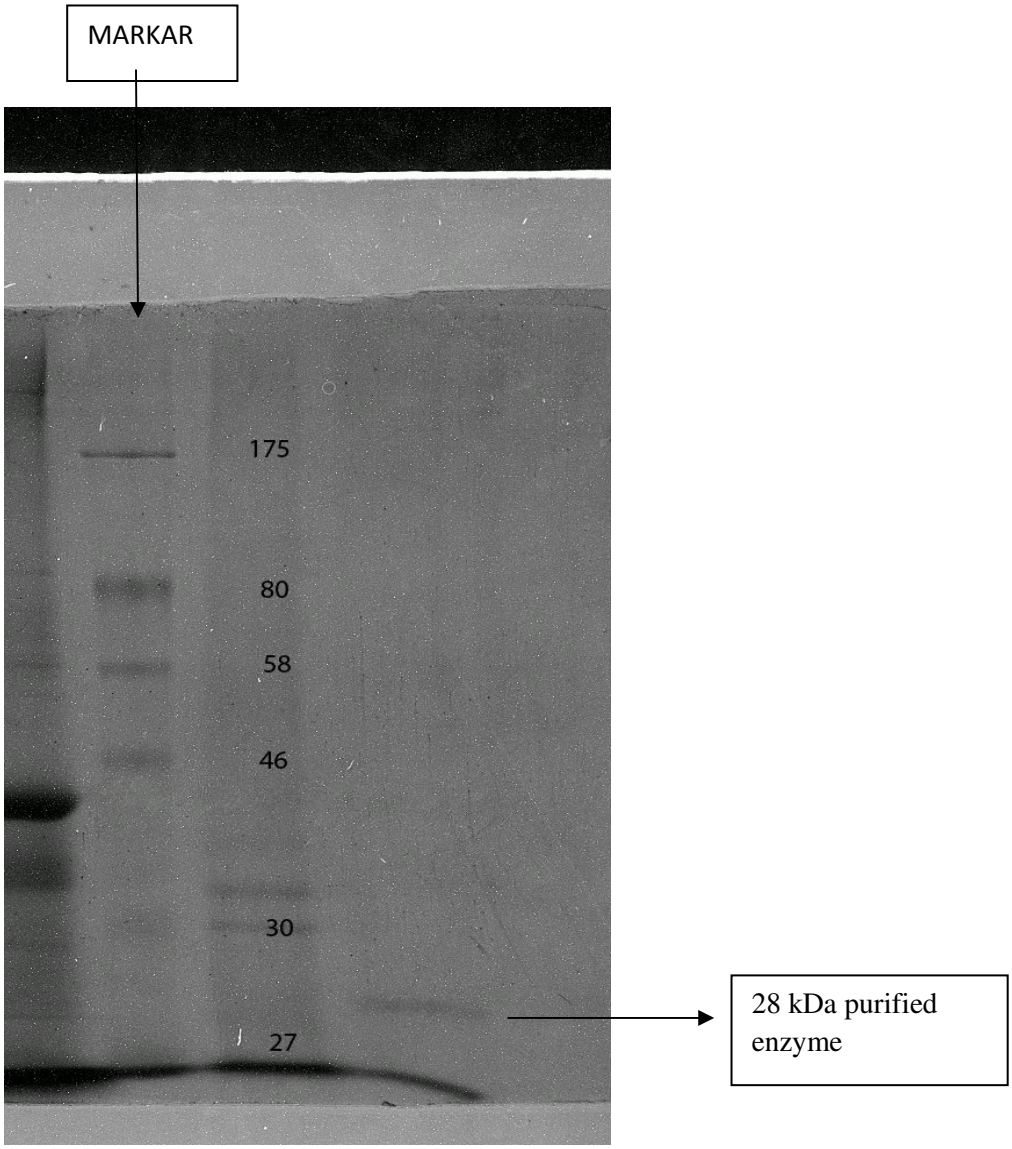


Figure 3.19 SDS-PAGE analysis of alkaline protease by *Bacillus licheniformis* MZK05M9

3.12 Application of crude enzyme

3.12.1 Washing test of Protease with Detergent

The washing performance analysis was carried out by enzyme with jet (detergent) for removal of blood stains on piece of cloth by incubating it at room temperature for 30 minutes. The observation (Figure 3.20) showed excellent stability and compatibility in presence of locally available jet (detergent).

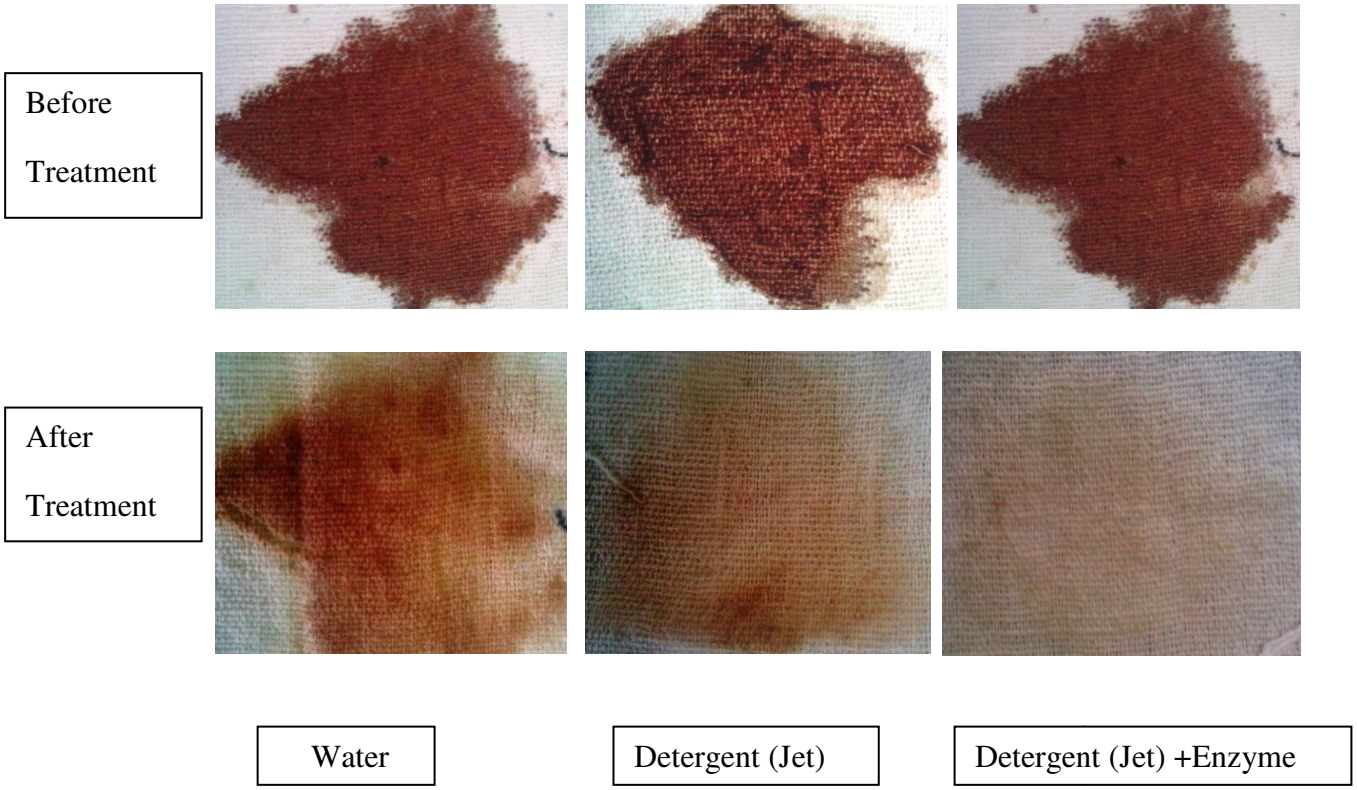
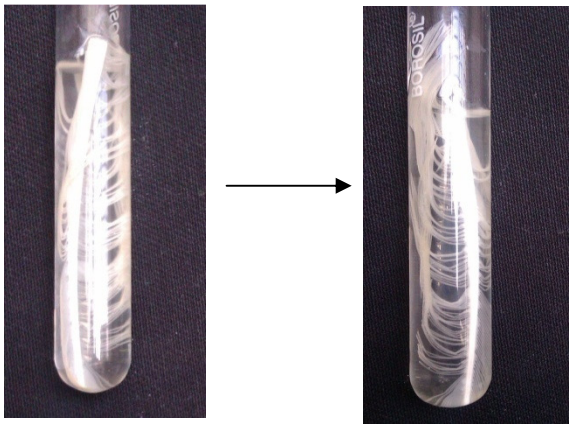


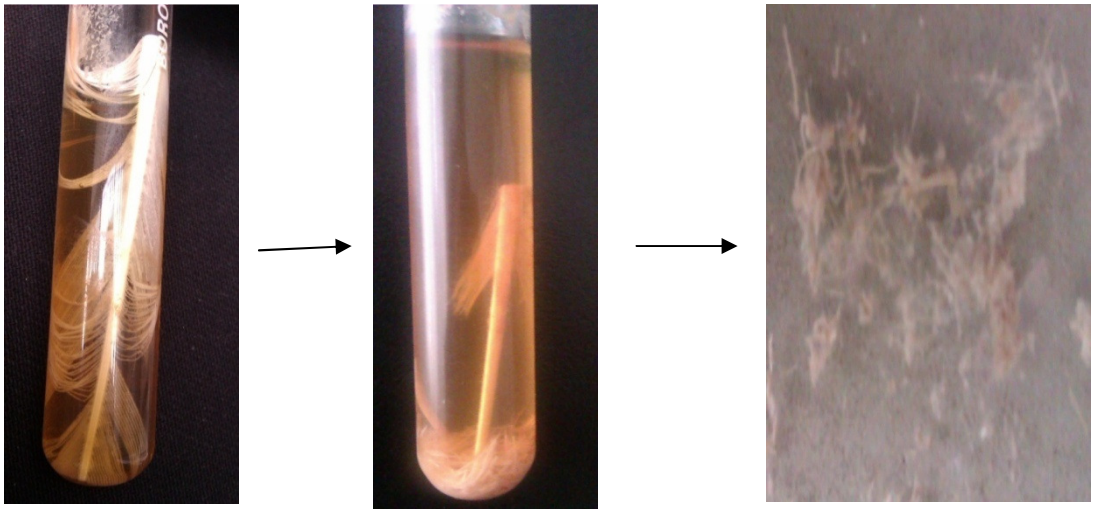
Figure 3.20: Washing performance of *Bacillus licheniformis* MZK05M9 alkaline protease.

3.12.2 Hydrolysis of chicken feathers

Degradation rate was examined during 14 days with day to day observation. From the figure (3.21) we found that, there was no degradation in control. On the other hand, after 7 days complete degradation was found in tested sample.



Control (1st day) 7th day (No change)



Tested sample (1st day) 7th day 14th day

Figure 3.21: Hydrolysis test of chicken feather

3.13. Production cost of 1000 liter Protease enzyme in four different media

From the Table 3.7 we found that, the cost of each medium was based on four different media such as Optimized Urea-Molasses Medium (OUMM), Modified Soya Meal Medium (MSSM), Soya Meal Medium (SMM), and Alkaline Protease Producing Broth (APPB). Total cost for 1000 L protease production in Urea-Molasses medium was only 6,739 TK where as 251,275 in APPB. So, it was revealed that the OUMM medium is 37 times cost effective than APPB medium.

Table3.7: Cost analysis of different media for 1000 liter protease enzyme production

Culture Media	Media composition (g/1000L)	Individual ingredient's cost for 1000L medium (TK)	Total cost of medium/ 1000L(TK)	Net cost difference between OUMM and other media used (Ratio)
OUMM	Molasses(6300), urea(1700) And CaCl2.2H2O(1100)	Molasses=189 Urea=2550 CaCl2.2H2O= 4000	6,739	1
MSSM	Soyabean mill(10,000), molasses(5000), K2HPO4 (3,000), MgSO4.7H2O (500), NaCl(500), CaCl2.2H2O(500)	(300+150+45+6000 +1125+2000)	9,620	1:1.4
SMM	Soyabean mill(10,000) Glucose(10,000),K2HO4 (3000),$MgSO_4.7H_2O$(500) NaCl(500),$CaCl_2.2H_2O$(500)	(300+70,000+45+ 6000+1125+2000)	79,470	1:11
APPB	Glucose(1000), peptone (5,000),yeast extract(5,000), K_2HPO_4(5,000) MgSO4.$7H_20$(100)	(70,000+55,000+125,000+75+1200)	251,275	1:37

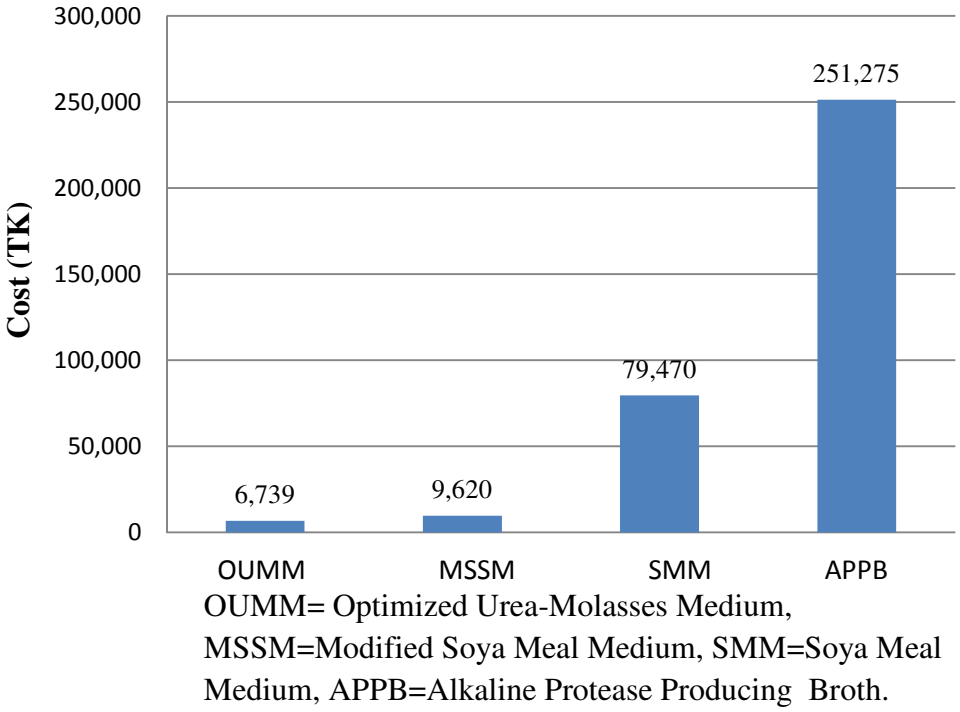


Figure 3.22: Production cost of 1000 Liter protease enzyme in four different media.

Chapter 4: Discussion

Microorganisms have been endowed with vast potentials of new biocatalysts (enzymes). They produce an array of enzymes, which have been exploited commercially over the years. In recent years, the potentials of using microorganisms as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzymatic activity in several microorganisms (Jayani *et al.,* 2005; Alva *et al.,* 2007).Among the bulk of industrial enzymes, proteases from plant, animal, and microbe constitute around 60 % of the total worldwide enzyme sales (Kunamnei *et al.*, 2003; Merheb-Dini *et al.*, 2009).

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively since the advent of enzymology (Gupta *et al.*, 2002). Microorganisms are large source of intracellular and/or extracellular proteases. Intracellular proteases are important for various cellular and metabolic processes such as sporulation and differentiation, protein turnover, maturation of enzymes and hormones and maintenance of the cellular protein pool. Extracellular proteases are important for the hydrolysis of proteins in cell-free environments and enable the cell to absorb and utilize hydrolytic products (Kalisz, 1988). At the same time, these extracellular proteases have also been commercially exploited to assist protein degradation in various industrial processes (Outtrup and Boyce, 1990; Kumar and Takagi, 1999).

For this purpose, a research program on production and application of proteases (both keratinase and alkaline protease) has been undertaken at the Fermentation and biotechnology laboratory of Department of Microbiology, University of Dhaka. A mutant *Bacillus licheniformis* MZK05M9 has been obtained by random mutation which exhibited about three-fold higher enzyme activity than that of the natural strain.

At present, the overall cost of enzyme production is very high (due to high cost of substrates and mediums used) and therefore, development of novel processes to increase the yield of proteases with respect to their industrial requirements coupled with lowering down the production cost is highly appreciable from the commercial point of view Furthermore, proteases produced by using commercial medium possess undesirable flavor, which are unsuitable for applications in food processing and pharmaceutical industries. Since the industrial use of proteases, particularly the alkaline proteases are expected to grow tremendously in the coming

decade; therefore microbial proteases producing industries are always in search of new cheaper methods to enhance the protease production as well as to decrease the market price of this enzyme (Mukherjee *et al.*, 2008). To achieve these goals, during the recent years, efforts have been directed to explore the means to reduce the protease production costs through improving the yield, and the use of either cost-free or low-cost feed stocks or agricultural by-products as substrate(s) for protease production (Sandhya *et al.,* 2005, Prakasham *et al.,* 2006).

Extracellular protease production by microorganisms is greatly influenced by media composition (Varela *et al.,* 1996).Extracellular protease production in microorganisms is also 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 affected by rapidly metabolizable nitrogen sources, such as amino acids in the medium.

Optimization of medium by classical method involves changing one independent variable while maintaining all others at a fixed level is extremely time consuming and expensive. To overcome this difficulty, experimental factorial design and response surface methodology (RSM) can be employed to optimize the medium components. Response surface methodology (RSM) is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching optimum conditions of factors for desirable responses (Myers *et al.,* 2002).This method has been successfully applied in many areas of biotechnology such as enzyme production (Bocchini *et al.,* 2002) with respect to protease production, it was utilized for *Bacillus* species (Puri *et al.,* 2002, Beg *et al.,* 2003).

In this study the agro-industrial residue molasses was used as carbon source and the urea was used for nitrogen source for protease production by mutant *Bacillus licheniformis* MZK05M9. Molasses is relatively inexpensive and economic alternative to synthetic medium for the production and purification of many economic enzymes (El-Enshasy, H.A.,*et al.,* 2008). Divalent metal ions such as calcium, cobalt, copper, boron, iron, magnesium, manganese, and molybdenum are required in the fermentation medium for optimum production of alkaline proteases.(Bhunia, *et al.,*2012).So, here we used $CaCl_2.2H_2O$.

The conventional method of optimization involves variation of one parameter One- at a time and keeping the others constant. This is extremely time consuming and expensive method when large numbers of variables are considered and also does not often bring up the effect of interaction of various parameters as compared to factorial design (Adinarayana and Ellaiah, 2002). These limitations of a single factor optimization process were eliminated by optimizing all the affecting parameters collectively by statistical experimental design using response surface methodology (RSM).(Response surface methodology consists of a group of empirical techniques devoted to the evaluation of relations existing between a cluster of controlled experimental factors and the measured responses, according to one or more selected criteria). In this regard, several experiment designs to study such model, and we selected the central composite design proposed by (Box *et al*., 1978). For this study, 2^3 factorial designs with six alpha points and six replicates at the centre points were employed to fit the second order polynomial model which indicated that 20 experiments were required for this procedure. The statistical software (Minitab version 17) was used for regression and graphical analysis of the data obtained.

The second order response surface model fitting in the form of analysis of variable (See Table3.2) the p value(>0.001) demonstrate a very high significance for the regression model(Akhnazarova *et al*., 1982; Khuri *et al*., 1987). The goodness of fit of the model was checked by the determination coefficient (R^2). In this case, the value of the determination coefficient (R^2=0.887) indicates that only 11.21% of the total variations are not explained by the model. The value of the adjusted determination coefficient (adj R^2=0.780) is also very high, which indicates a significance of the model (Akhnazarova *et al*., 1982; Khuri, . *et al*., 1987).

The significance of each coefficient was determined by student's t-test and P values, which are listed in Table 3.3. The larger the magnitude of the t-value and the smaller the p-value, the more significant is the corresponding coefficient (Akhnazarova *et al*., 1982; Khuri *et al*., 1987). From the Table 3.3 it was observed that, B, C, A*A, B*B, A*B, A*C,B*C are significant model terms in the present experiment, (Where A=Molasses, B=Urea and C=$CaCl_2.2H_2O$).

Response surface plots as a function of two factors at a time, maintaining all other factors at fixed levels. It is more helpful in understanding both the main and interaction effects of these two factors. These plots can be easily obtained by calculating (Minitab software version 17) from the model. The values taken by one factor where the second varies (from -2.0 to +2.0, step 0.5

for instance) with constraint of a given Y value. From the Figure 3.8A, when urea levels were high and molasses levels are low, we found highest enzyme yield, on the other hand (Figure 3.8B) highest enzyme yield was obtained when $CaCl_2.2H_2O$ levels were high and molasses levels were are low. And from the Figure 3.8C we found highest enzyme yield when both urea and $CaCl_2.2H_2O$ levels are high. But $CaCl_2.2H_2O$ showed a greater effect than that of urea. So, finally it was observed, that urea and $CaCl_2.2H_2O$ had a positive effect of highest enzyme production.

The Predicted values of the test variables are as follows; A=0.6353, B=0.1627, C=0.1170 with the corresponding Y= 463.1U/ml, and the observed validated experimental result was 560 U/ml. So, optimization of the media led to 1.3 fold increase in the enzyme activity than the initial activity (410U/ml) in unoptimized medium. Same kind of results was found by Saxena and Singh *(*2010) where they used *Bacillus sp*.

Besides nutritional composition of the fermentation media, several physical factors, such as aeration, inoculum density, pH, temperature and incubation, also affect the amount of protease produced (Hameed *et al.*, 1999; Puri *et al.*, 2002).

Temperature is one of the critical parameter that has to be controlled and varied from organism to organism and is most important factor affecting the enzyme production. 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 the Optimized Urea-Molasses Medium (OUMM) inoculated with 5% of 18 hours old seed culture at pH 7.5 and incubated at different temperatures 30°C, 35°C,37°C and 40°C at 150 rpm for 48 hours. At 37°C after 48 hours of incubation maximum enzyme production was observed 562±10.78 U/ml. So,the optimum temperature enzyme production was found to be 37°C. Mabrouk *et al.*,(1999) found optimum temperature as 37°C for *B. licheniformis*21415 after 5-days incubation and found that by increase in temperature the enzyme lost its activity more than 40%. Rahman *et al.*,(2005) also observed optimum temperature for enzyme production in *Pseudomna Aeruginosa* strain K as 37°C, and by increasing to higher temperature up to 40 & 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. Ray *et al.*,(1992) reported that temperature

could regulate the synthesis and secretion of extracellular proteases by microorganisms. Temperature strongly affects the synthesis of proteases either non-specifically, influencing the rates of biochemical reactions, or specifically, inducing or repressing their production (Rahman *et al.*, 2005).

Another important factor for alkaline protease production is the strong dependence on the extracellular pH for cell growth and enzyme production (Aunstrup, 1980). The pH of the culture medium plays a critical role for the optimal physiological performance of the cells and the transport of various nutrient components across the cell membrane aiming at maximizing the alkaline protease yields (Kumar and Bhalla, 2004).In the present experiment, the effect of initial pH of the culture medium on the enzyme production was studied in pH range 6.5-10. The initial pH of medium was adjusted with NaOH or HCl. In this experiment (figure 3.11) the maximum enzyme activity was found 551.66 ±10.40 at pH 7.5. Investigations on pH effect on production showed that the organism produced enzyme in the pH range of 6.5 to 10.0 with maximum activity at pH 7.5.

In shake flask, the highest enzyme productivity was obtained 11,666.6 U/L/hr as a compare to 13,888U/L/hr in the bioreactor cultivation. From this result (see table 3.5), it was found that the productivity is higher in bioreactor than the shake flask.

To determine the shelf life of the protease, the crude enzyme was stored at different temperatures such as 4°C, room temperature (without any preservative) and room temperature (with 0.60% sodium benzoate) for four weeks. It was observed (Figure 3.17) from the results that the protease remained stable at 4°C throughout this time period without any loss of the activity. Enzyme stored at room temperature (without any preservative) Showed a decrease in its activity was 87.5% at end of fourth week and room temperature (with 0.60% sodium benzoate)was stable for 4 weeks at the same temperature and its activity slightly decreased (96.4%) at the end of fourth weeks. Mukhtar and Ikram-Ul-Haq, 2012 studied that shelf life of alkaline protease at different temperatures (0^0C, 4^0C, 10^0C and 25^0C) decreased to zero (residual activity 0%) at the end of 4^{th} week.

The culture supernatant was separated by centrifugation at 10,000 g for 10minutes at 4°C. Partial purification of the enzyme was carried out by using ammonium sulphate fraction.60% saturation with ammonium sulphate; most of the enzymes present in the culture filtrate were precipitated at this stage of saturation. After separation, the purification was followed. The purpose of which was to concentrate the enzyme in a smaller volume of buffer. The reaction between salt and crude enzyme solution resulted in precipitation of alkaline protease, while the impurities remained in the supernatant.

The specific activity of the protease after ammonium sulphate fractionation was about 3.025 fold higher than that of culture supernatant. Ultrafiltration by centricon-100 effectively eliminated most of the impurities of the protease and raised the specific activity to about 16.59. As a result of ultrafiltration, the enzyme protein as detected was concentrated under single distinct peak with a molecular weight of 28 kDa. The protease emerged as a single symmetrical peak from a gel chromatography. Same results were obtained by Kumar (2002) with single serine alkaline protease having 28 kDa with 26.2% recovery, 36.6-fold purification by ammonium sulphate, ion-exchangeand gel filtration chromatography. Shimogaki *et al.* in 1991 purified by ammonium sulfate precipitation, ion exchange and gel filtration chromatography and obtained 8-fold and 52**%** recovery with 42 kDa.

As a cleansing aid of it was observed that the protease enabled the removal of blood stain very easily with detergent. Since the protease showed high capability for removing blood stain from cloth so it could be used in detergent powder or solution (Figure 3.20) resulting them as super one commercially. Anwar and Saleemuddin, 1997 reported usefulness of protease from *Spilosoma obliqua* for removal of blood stains from cotton cloth in the presence and absence of detergents but we believe that this protease is more effective.

For cost effectiveness, the four different media Optimized Urea-Molasses Medium (OUMM), Modified soya meal medium (MSSM), Soya meal medium (SMM) and Alkaline Protease Producing Broth (APPB) were evaluated. From the Figure 3.22 it was observed, total cost of 1000L protease production in OUMM medium was only Tk 6,739 compare to as Tk 251,275 in APPB So, it was revealed that the OUMM medium is 37 times cost effective than that of APPB medium.

Chapter 5: Concluding remarks

Statistical optimization method for fermentation process could overcome the limitations of classic empirical methods and has been proved to be a powerful tool for the optimization of protease enzyme production by *Bacillus licheniformis* MZK05M9. The use of Central Composite Design in Response Surface Methodology for determination of optimal medium composition for protease production is demonstrated in the present study. The optimization resulted in 1.3 fold increase in the production of the enzyme by the *B.licheniformis* MZKO5M9. The results show the effect of various factors on the enzyme production. The protease activity in unoptimized medium was 410 U/ml and optimized medium was 560 U/ml. The results of ANOVA and regression of the second-order model showed that the effects of Urea and $CaCl_2.2H_2O$ and the interactive effects of all variables are more significant for protease production. Other findings of this thesis work included:

- In shake flask highest enzyme productivity was found 11,666.6 U/L/hr and in Bioreactor it was 13,888 U/L/hr.
- For precipitation 60% ammonium sulfate was sufficient and by purification (after ultrafiltration by centricon 100) one single band was obtained in SDS- PAGE.
- The washing performance analysis of the protease with Jet (detergent) showed removal of blood stains.
- After cost analysis it was found that the medium was developed by RSM method was cost effective.

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Appendices

Appendix A

Media Composition

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

Alkaline Protease Producing Broth (APPB)

Glucose	10.0g/L
Peptone	5.0g/L
Yeast extract	5.0g/L
K_2HPO_4	5.0g/L
$MgSO_4.7H_2O$	0.1g/L

Soya Meal Medium

Soybean meal	10.0g/L
Glucose	10.0g/L
K_2HPO_4	3.0g/L
$MgSO_4 7H_2O$	0.5g/L
NaCl	0.5g/L
$CaCl_2 2H_2O$	0.5g/L

Modified Soya Meal Medium:

Soybean meal	10.0g/L
Molasses	5.0g/L
K_2HPO_4	3.0g/L
$MgSO_4 7H_2O$	0.5g/L
NaCl	0.5g/L
$CaCl_2 2H_2O$	0.5g/L

Urea Molasses Medium:

Urea	2.0g/L
Molasses	5.0g/L

Optimized Urea Molasses Medium:

Urea	1.6g/L
Molasses	6.3g/L
$CaCl_2.2H_2O$	1.1g/L

Appendix B

Solutions and Reagents

Preparation of Azocasein solution

Azocasein solution was prepared by dissolving 1.0 g of azocasein in 100 ml 0.05 M Tris-HCL buffer. The solution was preserved at refrigerator.

Bradford Reagent

A. Stock solution:

300 mg serva blue G (Comassie G-20) was dissolved in 300 ml methanol (BDH, England). 600.0 ml of 85% phosphoric acid (BDH, England) was then added and stirred well.

B. Test solution:

50.0 ml solution was dissolved with 850 ml distilled water and stirred well. The solution was then filtered with whatman filter paper.

Tris-HCL buffer (0.5 M)

Tris (Hydroxymethyl-aminomethane) was dissolved in distilled water to a 0.5-M solution and the pH was adjusted to the appropriate value with conc. HCl.

Glycine-NaOH buffer

15.01g glycine dissolved in 1L distilled water (0.2M) and pH is adjusted to the appropriate value with 0.2M NaOH.

30% acrylamide-bisacrylamide solution

Acrylamide	29.0 g
Bisacrylamide	1.0 g
Distilled water	100 ml

10% ammonium persulphate (APS)

APS	1.0 g
Distilled water	10 ml

0.1% BMB (Bromophenol blue solution) or tracking dye

Distilled water	100 ml
Stored at 4°C	
Bromophenol blue	0.1 g

Destaining solution

Glacial acetic acid	10 ml
Distilled water	90 ml

Staining solution

Coomassie brilliant blue G-20	0.20 g
10% acetic acid	100 ml

Sample loading buffer

0.5 M tris-Cl (Upper gel buffer)	10 ml
10% SDS	10 ml
2-mercaptoethanol	1 ml
Glycerol	10 ml
Distilled water	19 ml

Electrophoresis buffer (pH 8.3)

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

Upper gel buffer (pH 6.8)

Tris-base	18.17 g
SDS	0.4 g
pH adjusted to 8.8 by adding HCl	
Distilled water	Up to 11 ml

Lower gel buffer (pH 8.8)

Tris-base	18.17 g
SDS	0.4 g
pH adjusted to 8.8 by adding HCl	
Distilled water	Up to 11 ml

Appendix C

List of Apparatus

Apparatus	Model/ Company
Autoclave	Hirayama model HL-42, AE, Japan
Centrifugation	Biofuge Primo (Heraeus) and Hittch-Mikro-Rapid (Zrntrifugen D-72002, Japan)
Spectrophotometer, DR 4000U	HACH, USA.
Electronic balance	AJ-320CE, Shinko Denshi, Japan.
Glassware sterilizer	Heraeus model 0042, W. Germany
Incubator	Heraeus model D-6072, W. Germany
Laminar airflow	ESCO vertical Laminar flow cabinet
Magnetic stirrer	CIMAREC
Centricon tube	ROTH, Germany
Micropipettes	Eppendorf research and Nichiryo
Orbital shaker incubator scientific	Excella E-25, New Brunswick
pH meter	SNB43541, Thermo-scientific.
Refrigerator (4^{o}C)	Royal Frestech
Refrigeration (-20°C)	TOSHIBA,Japan.
Thermo stated shaking water bath	N-BIOTEK and MEMMERT
7 liter bioreactor	NEW BRUNSWICK SCIENTIFIC
Autoclave for bioreactor	TOUCHCLAVE

Appendix D

Chemicals Used

Name of chemicals	Source
Azocasein	Sigma, USA
Agar	Sigma, USA
TSB	Sigma, USA
BSA	Sigma, USA
Ammonium sulfate	BDH, England
SDS	Wako, Japan
Peptone	Oxoid, England
HCl	Merck, Germany
NaOH	Merck, Germany
K_2HPO4	Merck, Germany
Acrylamide	Merck, Germany
Molasses	Local Market
Na_2CO_3	Sigma, USA
$NaHCO_3$	Merck, Germany
NaCl	Merck, Germany
NaOH	Merck, Germany
Urea	Merck, Germany
Peptone	Oxoid, England
Phosphoric acid	Merck, Germany
Trizma base	Sigma, USA
Tri-chloro acetic acid	BDH, England

Appendix E

Preparation of standard curve by Bradford method

A standard curve of standard protein (BSA) concentration was prepared and the value of 'm' was taken from the graph prepared by following the standard Bradford method. (Fig. a)

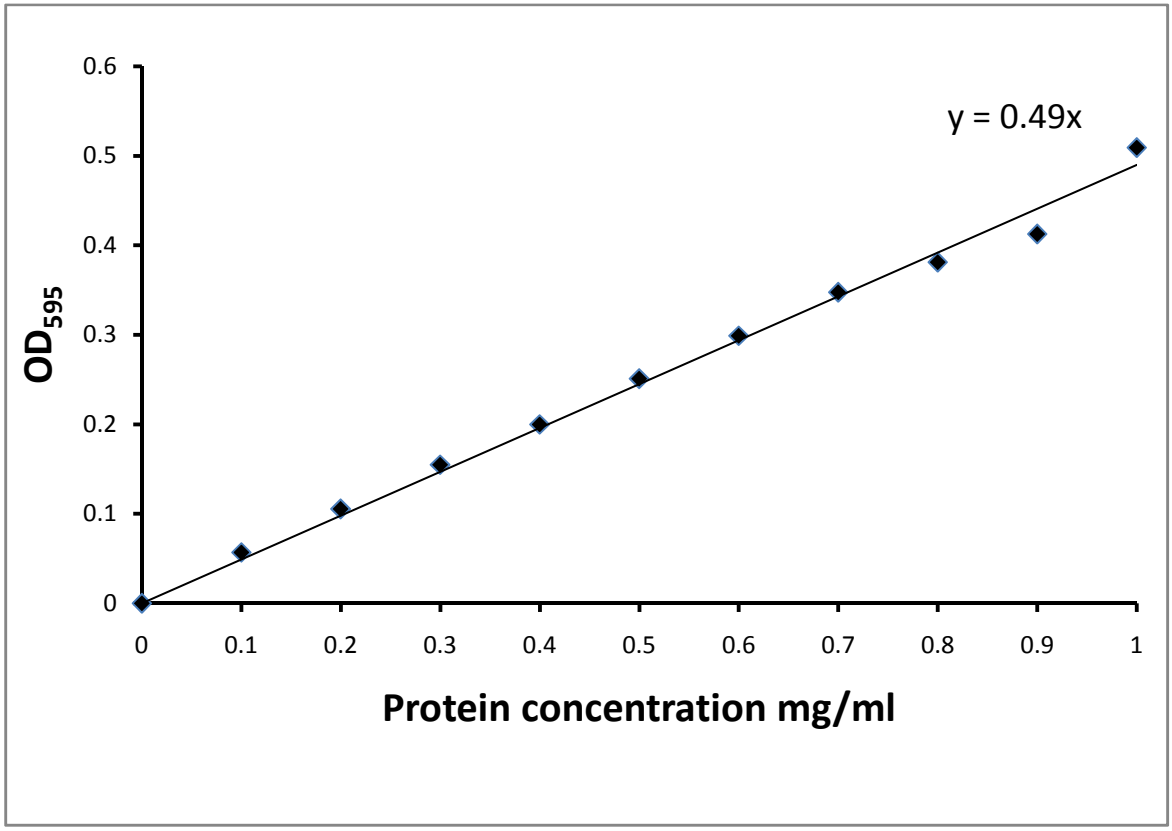

Figure (a): Standard curve for determination of protein concentration

Appendix F

Level of independent variables

INDEPENDENT VARIABLES	HIGH(+1)	LOW(-1)	MEAN(0)	PLUS ALPHA(+α)	MINUS ALPHA(-α)
MOLASSES	1	0.5	0.75	1.17	0.33
UREA	0.2	0.1	0.15	0.234	0.066
$CaCl_2.2H_2O$	0.1	0.05	0.075	0.117	0.033

α =1.68

Molasses +α (X-0.75)/0.25=1.68 X-0.75=0.42 X=0.42+0.75 X=1.17	Molasses -α (X-0.75)/0.25= -1.68 X-0.75= -0.42 X= -0.42+0.75 X=0.33
Urea +α (X-0.15)/0.05=1.68 X-0.15=0.084 X=0.084+0.15 X=0.234	Urea –α (X-0.15)/0.05= -1.68 X-0.15= -0.084 X= -0.084+0.15 X=0.066
$CaCl_2.2H_2O$ +α (X-0.075)/0.025=1.68 X-0.075=0.042 X=0.042+0.075 X=0.117	$CaCl_2.2H_2O$ –α (X-0.075)/0.025= -1.68 X-0.075= -0.042 X= -0.042+0.075 X=0.033