ADDIS ABABA UNIVERSITY

ADDIS ABABA INSTITUTE OF TECHNOLOGY

SCHOOL OF CHEMICAL AND BIO ENGINEERING

(BIOCHEMICAL ENGINEERING)

Eggshell and Membrane-Based Substrate for the Production, Optimization and Detergent Compatibility of Alkaline Protease Enzyme

A thesis submitted to Addis Ababa University Institute of Technology School of Chemical and Bio Engineering in partial fulfillment of the Degree of Master of Science in

Biochemical Engineering

By: Tsegazeab Kassa

Advisor: Solomon Kiros (PhD)

July 2019

Addis Ababa, Ethiopia

Addis Ababa University

Addis Ababa Institute of Technology

School of Chemical and Bio-Engineering

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Declaration

I hereby declare that the thesis is based on my original work except for assay protocols and citations which have been duly acknowledged. I also declare that it has not been previously or currently submitted for any other department at Addis Ababa University or other institute and that all resources of materials used in this thesis have been duly acknowledged.

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Abstract

In recent years the advancement of technology results not only development but also, environmental risks due to the use of chemicals, catalysts and environmentally hazardous materials moreover coasty. In spite of this, the use of biotechnological principles and products in the industrial sector is mandatory and expected to with stand the current problems. Though Microbial alkaline proteases are stimulating tremendous interests in the research and enzyme market. Hence, the aim of this thesis was the use of eggshell and membrane as a substrate for the production, optimization and detergent compatibility of alkaline protease using Bacillus subtilis mojavensis in submerged fermentation. Fermentation process for enzyme production was conducted using, four factors with three levels were used and 29 experiment had been studied. The optimum or maximum alkaline protease production was obtained at a temperature of 37 ^{0}C, eggshell concentration of 20 %, pH of 9.0 and incubation time of 48 h with a maximum activity of 214 U/ml. Optimum parameters for the production of alkaline protease was found using design expert optimizer at 9.08 pH, 39.74 ^{0}C, 19.78% eggshell and 48 h of incubation time were obtained. Alkaline protease enzyme was produced for further analysis such as, characterization and detergent compatibility of the enzyme. Enzyme activity was conducted in a pH range of 6-12, with maximum activity obtained at pH 10. It showed that more than 93.97% its original activity was retained using buffers pH ranging from 8-10, with the optimum activity at pH 10.0. Overall alkaline protease enzyme was stable between pH 8.0-10.0 attain 90%, 100% and 94% of residual activity. Its minimum residual activity was attained at pH value of 6.0 which is 39.6% of its original activity. Alkaline protease was found to be very active at all temperatures tested between 30 ^{0}C and 80 ^{0}C with maximum activity at 60 ^{0}C. Within temperature range of 40^{0}C -70^{0}C, alkaline protease was stable and retained more than 74% of its maximum activity. From 1M to 2.0M of NaCl concentration, exhibits > 97% of optimum activity was recorded at pH 8 and 10. Whereas; optimum stability was attained at these concentration range between 1.0 - 3.0 M NaCl. At pH 8.0 residual activity were raising as concentration increases and constant after 2.5 M. The detergent compatibility study was conducted. Results increasing the concentration of enzyme increases the degradation of blood stain. Therefore, alkaline protease enzyme has compatibility to industrial application including detergent as an alternative.

Key words: Alkaline protease, Eggshell, detergent, BSM, activity and stability

Acknowledgement

First and for most, I wish to express my utmost and profound thankfulness to almighty God and Saint Marry for giving strength to complete my thesis.

I would like to express my deepest respect and sincere gratitude to my advisor Dr Solomon Kiros (Ph.D.) for his constructive comments.

I wish to express my warm and genuine thanks to my family members especially mother and father' 'Emaye'', ''Gashe'' sisters and brothers Mitikuwa, Akale, Dr Tilahun, Wondwosen, Eng. Meron, Betelhem and to my cousin Mahlet and my uncle Debebe for their encouragement, guidance and comment on my thesis project.

My final gratitude goes to Addis Ababa University Bioengineering laboratory lab assistants for their support through the entire experimental work.

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Acronyms

AlkPE	Alkaline protease enzyme
ANOVA	One-way analysis of variance
BSM	Bacillus subtilis mojavensis
BBD	Box Behnken design
B. spp	Bacillus species
CSA	Central statistical Agency
DSP	Downstream process
ESM	Eggshell and membrane
FTIR	Fourier transformation infrared
SEM	Scanning Electron microscopy
SmF	Submerged fermentation system
SSF	Solid state fermentation system
TCA	Trichloro acetic acid

1 Introduction

1.1 Background

Many chemical transformation processes using inorganic catalysts in various industries have inherent drawbacks from an economic, energy and environmental point of view. However, all of these drawbacks can be virtually eliminated by using biocatalysts and or industrial enzymes. Biocatalysts offer green and clean solutions to chemical process and are emerging as a challenging and revered alternative to chemical technology (Sharma et al., 2014). As a result, enzymatic conversion has recently received attention and is becoming a favorable alternative to inorganic catalysts.

In the 21st century, extensive application of bioprocesses has created conducive environment for many scientists and engineers to expand knowledge and interest in biotechnology. One of the useful applications of biotechnology is the use of microorganisms to produce enzymes which are used in the laboratory, diagnostic and many industrial processes (Ravindran et al., 2016).

Proteases are essential constituents of all forms of life on earth. Microbial proteases are among the most important, extensively studied groups since the development of enzymology. Alkaline proteases are so far exploited as industrial catalysts in various industrial sectors. Neutralophilic and alkaliphilic microbial alkaline proteases possess a considerable industrial potential due to their biochemical diversity and stability at extreme pH environments, respectively. However, the demanding industrial conditions for technological applications and cost of alkaline proteases production resulted continuous exercise for search of new microbial resources (Gizachew et al., 2009).

Recycling of resources is becoming a valid and viable activity and is increasingly mentioned as a solution to some of the most pressing problems which will affect mankind's economic performance. Bioconversion of wastes is the natural way to return to the environment the resources previously extracted from it. This study address bioconversion of ESM wastes to produce alkaline protease enzyme. Recycling objective of the bioconversion the production of industrial products from wastes (Dunn et al., 2016).

For sustainable development, wastes should be recycled, reused, and channeled towards the production of value-added products. Efficient utilization of wastes helps to protect the environment

on one side and on the other side to obtain value added products while establishing a zero-waste standard. The utilization of the waste is a priority today in order to achieve sustainable development (Abdulrahman et al., 2014).

According to CSA Ethiopia is one of the few African countries with a significantly large population of chicken, estimated at 38.1 million. a local scavenging hen on average lays about 36–40 eggs/year. Ethiopia is rich in agricultural residues such as poultry waste like eggshell products. In Ethiopia, a local scavenging hen on average lays about 36–40 eggs/year according to (Moges & Dessie, 2010). The national poultry eggs consumption is difficult to estimate estimated, for example according to production manager of El'fora agro-industry (located in Addis Ababa) produces 50,000 eggs per day of which more than 5,000 eggs are goes to hatcheries. On an average to be 800,000 tons per annum. From this eggshell account 10- 11% of egg weight (King`ori et al., 2011; Nakano et al., 2003), 88,000 tons of ESM produced annually form home consumption, hatcheries, food companies and fast food cafeterias and hotels. Which can be employed as substrates for the production of AlkPE or other industrially important products, ESM waste causing environmental pollution like; and difficult to dispose and bad odor. Wide range of microorganisms including bacteria, molds and yeasts have been used for production of proteases.

Egg shell and membranes are non-edible wastes largely disposed but these are reserve of many bioactive compounds which can be extracted by efficient separation (Nakano et al., 2003). Disposal of egg shell and underlying membrane waste contributes to abrasiveness, odor and pollution (King`ori et al., 2011).

A study has suggested that the recycling of chicken eggshells is a way of improving the ecosphere; it reduces the need of management of waste and the eggshell can serve useful raw material for the production of AlkPE (Nagamalli et al., 2017).

Microbial proteases are among the most important hydrolytic enzymes and have been studied extensively. This group of enzymes represents one of the three largest groups of industrial enzymes and accounts for approximately 60% of the total enzyme sales in the world (Vasudeo Zambare, 2011). 90 % of world enzyme production is controlled by Europeans and North American companies (Amare et al., 2011).

Recently, industrial enzymes due to their environmentally friendly applications in industrial processes have become the focus of intense research by manufacturing biochemists and bioprocess Engineers. Proteases constitute 60–65% of the global industrial enzyme market most of which are alkaline proteases. Alkaline proteases account for 89% of the total protease sale (Jaouadi et al., 2009; Rai et al., 2010).

1.2 Statement of the Problem

Ethiopia now days shown promising development in industrial sectors. Apart from these developments, our country is not advantageous because of the wastes, raw materials cost as well as the chemicals (reagents), which are hardly locally produced or obtained. In spite of this, locally produced materials i.e. chemicals, raw materials, technologies must be encouraged to facilitate the advancement of the country industrially. Among the most imported materials apart from machineries enzymes are the leading ones in industries like textile, leather, detergent and food industries.

Though the newly constructed industrial parks attract many textile and leather processing industries. For example, in Ethiopia more than 150,000 kg of bathing enzyme is imported annually, so the thesis goal is to produce alkaline protease enzyme from waste egg shell & membrane (ESM) as using substrate for protease production. Reports shows that, above 8800 tons of ESM is generated intern this has environmental pollution issues. This proposal doesn't only give attention to low cost substrate it also has implication in reducing wastes by converting it to usable raw material using biotransformation.

Considering polluting chemical-based manufacturing/production technologies, there is immense emphasis on development of enzyme based environmentally-friendly/green technologies. However, enzymes being biological molecules are quite fragile for hostile industrial processes; therefore, there is quest for robust enzymes applications, the reason for not utilizing protease is that it's cost. In spite of this, there must be some mechanism to alleviate this problem. The proposed method is the use of eggshell and membrane as a substrate for the production of alkaline enzyme and optimization of the production parameters, this is the better choice because unlike agricultural wastes it doesn't have other uses rather it pollutes the environment.

Research trends in Ethiopia on enzyme production is at its developing stage and yet no enzyme producers are present. However, research is done and being conducted in the AAU and other research institution. This work is different from the rest is that, it is the first in our campus and also the first in Ethiopia to work on ESM based substrate for AlkPE production and optimization.

1.3 Objective of the Research

1.3.1 General Objective

- To produce, optimize and characterize crude alkaline protease enzyme using eggshell and membrane as a potential substrate at different concentration.

1.3.2 Specific Objectives

- Characterization of ESM
- Effect of concentration of ESM, effect of temperature and pH and fermentation time on AlkPE production
- Optimization of the process parameters in AlkPE production
- Blood stain removal efficiency as detergent compatibility

1.4 Significance of the Research

This thesis gives ways to optimize process parameters, decrease this cost and produce environmentally benign products using ESM as a potential substrate for the production of alkaline protease enzyme.

In general, the significance of this research can be seen from different perspectives.

1. Microbiological conversion of waste into valuable products enhances not only the sustainable economic development but also helps in management of waste.
2. Provide a means to exploit and manage local resources
3. Using waste materials to produce cheap protease and reducing environmental pollution
4. Finding environmentally friendly alternative substrate for the production of alkaline protease
5. Optimization of the process parameters for the production of alkaline protease enzyme using eggshell and membrane substrate.

6. Serve as a starting material for further research studies on the use of eggshell & membrane or agricultural wastes or byproducts as a substrate for the production of protease and other enzymes.
7. Serve as teaching aid for biochemical engineering students to integrate industrial microbiology and biochemical engineering principles.

1.5 Scope of the Study

This thesis project incorporates the production, characterization and optimization of alkaline protease enzyme by *Bacillus subtilis mojavensis using* eggshell & membrane as a substrate. Physicochemical characterization and detergent compatibility study of crude alkaline protease enzyme was conducted.

2 Literature Review

2.1 Alkaline Protease

Proteases are extremely useful enzymes with enormous application in the detergent, leather tanning, protein hydrolysis, chemical, and other industries. Currently alkaline proteases used for detergent application are known to account for 25% of the global industrial enzyme market. Proteases are also extremely useful in the leather industry where they are important for soaking, dehairing, and bating application. Today use of enzymes for leather bating is mandatory and all leather industries in the region import proteases as bating agents with the expenditure of foreign currency (Amare et al., 2011).

Alkaline proteases are those enzymes that are active at alkaline pH with optimum pH in between 9 to 11(Haddar, 2010; Olajuyigbe et al., 2013). The genus "*Bacillus*" is an important source of industrial alkaline proteases and are probably the only genera being commercialized for alkaline protease production. They are widely distributed in soil and water, and certain strains tolerate extreme environmental conditions including highly alkaline conditions. Isolation of alkaline protease producing *Bacillus spp* has been reported from a variety of sources including soils characterized by high pH and/or the presence of detergent contamination, sand soil, milk processing plant, slaughterhouses. The largest share of the enzyme market is occupied by detergent proteases, which are mostly alkaline serine protease and active at neutral to alkaline pH range (Sharma et al., 2011).

Amongst the industrially important enzymes (Wang et al., 2016), microbial alkaline proteases are stimulating tremendous interests in the enzyme market owing to the fact that there is a great demand for developing biotechnological alternatives for many industrial processes (Verma et al., 2016). (Kumar et al., 1999) have indicated that alkaline protease mediated process provides a viable alternative to the use of hazardous, expensive, and environmentally unsafe chemicals used in many industries. Today alkaline proteases are the largest groups of industrial enzymes and find application in detergent (Sridharan et al. 2012; Haddar et al. 2010; Marathe et al. 2018), leather tanning (Verma et al., 2016), textile (Chen & Du, 2007; Ozaki et al., 2007), and food and feed processing industries (Li & Kittikun, 2010). Other applications include silver recovery from used X-ray films and gelatin decomposition, cosmetics, medicine and pharmaceuticals (Getahun, 2016; Mukherjee et al., 2011), waste management (Amare et al., 2003).

At present different industries in the eastern African region import substantial quantities of enzymes for use in leather tanning, textile, and brewing industries. Other industries shy away from importing enzymes because of cost factor. For example, in Ethiopia more than 150,000 kg of bathing enzyme is imported annually. Tanzania is another country with high potential for leather tanning where the annual cost of bathing enzymes. Thus, the existing market for bathing enzymes in the East African region (Ethiopia, Tanzania, Kenya, Rwanda, Sudan, and Uganda) is estimated to have huge market (Amare et al., 2011).

To date no tannery in the region is using enzymes for soaking and dehairing applications, mainly because of cost and lack of availability of such enzymes with affordable price. If enzymes are used for these processes substantial quantity of chemicals imported could be replaced thus saving foreign currency expenditure (Amare et al., 2011).

Alkaline proteases are those enzymes that are active at alkaline pH with optimum pH in between 9 to 11. The genus "Bacillus" is an important source of industrial alkaline proteases and are probably the only genera being commercialized for alkaline protease production (Varela et al., 1997).

Furthermore, for successful industrial applications, it is necessary that bulk production of enzyme must be carried out in most cost-effective manner. One of the major cost-determining factors for bulk industrial enzyme production is substrate. Microbial growth medium for enzyme production at industrial scale takes about 30-40% production cost (Ravindran & Jaiswal, 2016). It is envisaged that relatively low-cost agricultural by products may have great potential as substrates for enzyme production.f

The main goal of my thesis is the use of low-cost substrate for the production of alkaline protease, since up to 30 % of total enzyme cost incurred to substrate. The substrate to be used in the in this research is waste egg shell and membrane. Efficient utilization of wastes reduces the environmental pollution problems caused by them. Egg shell and membranes are the poultry wastes generated during processing of egg, hatcheries, fast food centers and domestic sources produce large quantities of egg shell and membranes. Nearly 88,000 tons of egg shell waste is generated annually during processing. Outer cover of the egg, the shell, contributes 10–11% of total egg weight. Egg shell is composed of calcium carbonate (96%) and trace elements. Egg shell

membrane is composed of collagen type I, V and X, which has diverse applications in cosmetic, food, medical and pharmaceutical domains (King`ori et al., 2011).

2.2 substrate/ egg shell & membrane

Eggshells are waste materials from hatcheries, homes and fast food industries and can be readily collected in plenty. Earlier studies reported that CaCO3 induces the enzyme production significantly (Kanekar et al., 2011). Eggshell waste disposal contributes to environmental pollution. Challenges associated with disposal of eggshells include cost, availability of disposal sites, odor, flies and abrasiveness. However, they can be processed into saleable products like fertilizer, used in artwork, human and animal nutrition and building materials, substrate for some microbes specially those of bacteria, fungi etc and to produce to produce alkaline protease using *bacilli species* (Nagamalli et al., 2017).

2.2.1 Properties of eggshell and membrane

Egg Shell membranes consist of collagen as a component. The collagen is extracted and has diverse uses in medicine, biochemical, pharmaceutical, food and cosmetics industries. These uses

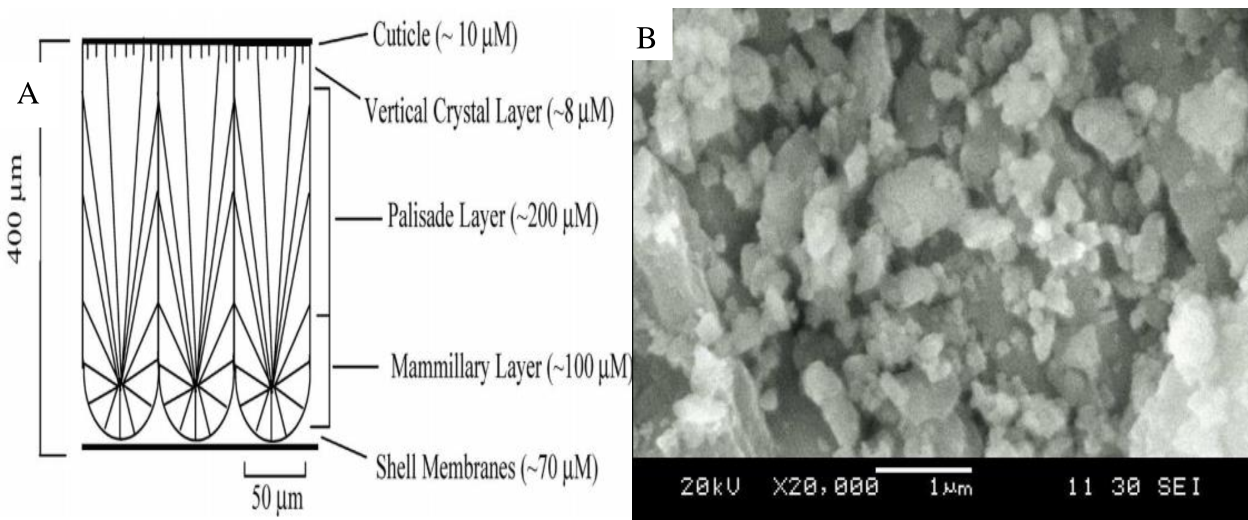


Figure 2-1. Schematic diagram of the structure and different layers within the eggshell [a] raw eggshell, [b] SEM images of ESM

Source: (A) (Hussain et al., 2009), (B) (Ahmad et al., 2015)

minimize their effect on environmental pollution. The eggshell and shell membranes make up 10.2% of the whole egg. The eggshell comprises of calcified shell and shell membranes

including inner and outer membranes. The organic matter of eggshell and shell membranes contains proteins.

2.2.2 Chemical composition of eggshell and membranes

The composition of the egg shell is approximately 98.2, 0.9, 0.9% Calcium carbonate, Magnesium and Phosphorous (phosphate) respectively. Shell membranes comprises of 69.2% protein, 2.7% fat, 1.5% moisture and 27.2% ash as major constituents with small amounts as carbohydrates and lipids (King`ori et al., 2011). Shell membranes protein comprises of approximately 10% collagen. Eggshell and shell membranes are non-edible by-products with little saleable value but they may contain biologically active compounds (Nakano et al., 2003).

2.3 Protease

Proteases are proteolytic enzymes that catalyze the breakdown of proteins by hydrolysis of peptide bonds. Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. Proteases are classified into acidic, neutral and alkaline proteases, of which alkaline protease plays very important role as most commonly used industrial enzyme in view of their activity and stability at alkaline pH such as from Bacillus subtilis (Swapna et al., 2014).

It is an important hydrolytic enzyme which is being studied extensively. These enzymes have a vital role not only in cellular metabolic processes but also in industrial aspects. More than 60% of global enzyme market is dominated by proteases (Devi et al., 2012; Varela et al., 1997).

Proteases can be produced from wide diverse sources such as plants, animals and microorganisms. All microorganisms that produce a substantial amount of extracellular protease have been exploited commercially.

2.3.1 Sources of proteases

Microbial proteases are the most significant source in the present scenario which has been derived from a wide variety of yeasts, molds, and bacteria. Among them bacterial protease has been used most as ease of isolation, purification and design at gene level (Rao et al., 2007).

Table 2-1. Different sources and uses of protease

Industry	Application	Source
Baking and milling	Improvement of dough texture, reduction of mixing time, increase in loaf volume	Fungal/bacterial
Brewing	Childproofing	Fungal/bacterial
Dairy	Manufacture of protein hydrolysates	Fungal /bacterial
	Stabilization of evaporated milk	Fungal
	Curdling milk	Fungal /bacterial
Laundry	Detergents	Bacterial
Leather	Dehairing, bating	Fungal /bacterial
Meat	Tenderization	Fungal
Pharmaceutical	Digestive aids	Fungal
Photography	Recovery of silver from spent film	Bacterial
Protein hydrolysates	Manufacture	Fungal /bacterial

All microorganisms that produce a substantial amount of extracellular protease have been exploited commercially. Microbial proteases are the most significant source in the present scenario which has been derived from a wide variety of yeasts, molds, and bacteria. Among them bacterial protease have been used most as ease of isolation, purification and design at gene level (Rao et al., 2007).

Bacterial Protease

Though the protease contributes 60% of industrial enzyme but contribution of bacterial protease is even more significant which is more than 70%. Among all the genera of bacteria Bacillus itself contributes proteases to a major extent for the industrial applications. Proteases find applications at various steps of leather processing, e.g., neutral proteases in soaking, alkaline proteases in dehairing and acid proteases in batting.

It was reported that protease are enzymes that have the ability to degrade protein by the breaking of the hydrogen bond and Bacillus is the major producer of protease (Verma et al., 2011).

Fungal Protease

Many of researchers have isolated and checked activity of various proteases for dehairing from fungal sources. The protease from Aspergillus flavus was earlier being used for dehairing, and

later it was reported that simultaneous dehairing and bating is possible with the protease of Aspergillus flavus.

Animal and Plant Sources

In the primitive time the gut of various animals itself used as the source of protease, many of enzyme like trypsin have been extracted and implemented for leather processing. Some plant like papaya is rich source of protease, the latex form papaya fruits has been used for the dehairing process. These proteases are not in industrial use in the current scenario as the isolation and purification complication. It is quite difficult to produce large scale enzyme in comparison to those from microbial sources. More ever in case of protease from animal source that is even more complicated and as many ethical issues regarding their isolation, these are not in practice.

Classification of protease

Proteases are classified based on chemical nature of the active site, the reaction they catalyze, and their structure and composition (Rao et al., 2007). The major classes are again classified in to sub classes based on pH, catalytic site on polypeptide, occurrence, and so on.

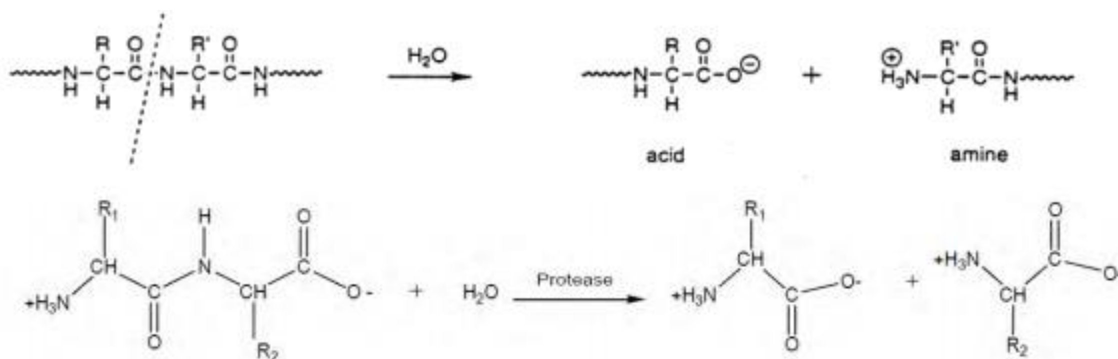


Figure 2-2. Schematic representation of peptide bond hydrolysis by proteases

Based on the catalytic site on the substrate, proteases are mainly classified in to endo-proteases and exo-proteases. Endo-proteases preferably act at the inner region of the polypeptide chain. By contrast, exo-proteases preferentially act at the end of the polypeptide chain. Exo-proteases are further classified in to amino peptidases (those proteases which act at the free N-terminus of the polypeptide substrate), and carboxypeptidases (those proteases which act at the free C-terminal of the polypeptide chain) (Kulkarni et al., 1999).

Similarly, endo-proteases are also classified based on the functional group present in active site and pH optimum. The different classes of proteases based on their catalytic active site include:

1) ***Serine proteases:*** Serine proteases are proteases having a serine group (-OH) in their active site.
2) ***Cysteine proteases:*** Cysteine proteases are proteases having a thiol (-SH) group in their active site.
3) ***Metalloproteases:*** Metalloproteases are proteases requiring divalent metal ion for their catalytic activity.
4) ***Aspartic protease:*** Aspartic proteases are proteases with aspartic residue at their catalytic active site.
5) Other rare proteases also contain other amino acid residues at their active site, such as threonine and glutamic acid.

Based on their optimal pH proteases are also classified as:

1) ***Acid proteases:*** Acid proteases are proteases which are active in the pH ranges of 2-6 (Tanksale et al., 2009)and are mainly of fungal in origin (Aguilar et al., 2008) Common examples in this subclass include aspartic proteases of the pepsin family. Some of the metalloprotease and cysteine proteases are also categorized in as acidic proteases.
2) ***Neutral proteases:*** Neutral proteases are proteases which are active at neutral, weakly alkaline or weakly acidic pH. Majority of the cysteine proteases, metalloproteases, and some of the serine proteases are classified under neutral proteases. They are mainly of plant in origin, except few fungal and bacterial neutral proteases (Aguilar et al., 2008).
3) ***Alkaline proteases:*** Alkaline proteases are optimally active in the alkaline range (pH 8-13), though they maintain some activity in the neutral pH range as well (Horikoshi et al., 2016). They are obtained mainly from neutralophilic and alkaliphilic microorganisms such as *Bacillus* and *Streptomyces* species. In most cases the active site consists of a serine residue, though some alkaline proteases may have other amino acid residue in their active site (Thanikaivelan et al., 2004)

Alkaline proteases are classified under serine proteases. They are produced by a wide variety of microbial species like *Bacillus subtilis*, *Aspergillus oryzae*, Streptomyces cellulase, and

Aeromonas hydrophilia species. Most commercial proteases, mainly neutral and alkaline, are produced by organisms belonging to the genus *Bacillus*. *Bacillus sp*. are attractive industrial tools for a variety of reasons, including their high growth rates leading to short fermentation cycle times, their capacity to secrete protease into the extracellular media and the GRAS (generally regarded as safe) status with the food and drug administration for species such as B. subtilis and Bacillus licheniformis. One of the most important and noteworthy features of many alkaliphiles is their ability to modulate their environment. They can convert any neutral or high alkaline medium in their favor to optimize external pH for their growth. Proteases can be produced from wide diverse sources such as plants, animals and microorganisms.

2.4 Characteristic features of *B. Subtilis* for being industrially important microorganism

The following attributes make *B. subtilis* industrially the important microorganism (Elander, 2003; Haddar et al., 2010; Nguyen et al., 2016).

2.4.1 General description

- Bacillus subtilis is a Gram-positive, rod-shaped and endospore-forming bacteria.
- It is regarded as an aerobe; however, it is also capable of growing under anaerobic conditions in the presence of nitrate, though growth is much slower.
- B. subtilis was one of the first bacteria studied by scientists.
- It was originally named Vibrio subtilis in 1835 by Christian Gottfried Ehrenberg and later renamed Bacillus subtilis by Ferdinand Cohn in 1872.
- Currently, B. subtilis is a significant microorganism in the scientific research field, as well as in biotechnology and industry.
- It is easily manipulated genetically and a good laboratory microorganism.
- Furthermore, B. subtilis produces a variety of enzymes used in biotechnology and industry, including antibiotic production.
- Its status as a “generally regarded as safe" (GRAS) organism makes it an excellent industrial microorganism.
- Bacillus subtilis is one of the best understood prokaryotes, in terms of molecular biology and cell biology.

- Research on Bacillus subtilis has been at the forefront of bacterial molecular biology and cytology, and the organism is a model for differentiation, gene/protein regulation, and cell cycle events in bacteria. Cell structure and metabolism.
- Bacillus subtilis is a prokaryotic cell, lacking membrane-bound organelles. It is enclosed by a cell wall, consisting of large amounts of peptidoglycan (Gram-positive).
- B. subtilis has the ability to produce acid from a variety of sugars.
- In response to nutrient limitation, it also produces several enzymes (including proteases, amylases, cellulases, and lipases) and antibiotics.
- These enzymes are believed to help search for and degrade macromolecules to be used as energy sources, while the antibiotics are used to limit bacterial competition for the newly discovered energy sources.

Ecology

Bacillus subtilis is an important soil microorganism. It produces a variety of enzymes, which allow it to degrade many natural substrates and thus cycle nutrients in the soil. This increases nutrient availability to plants, thereby stimulating plant growth. Moreover, *Bacillus subtilis* secretes antifungal antibiotics, which can control fungal diseases in plants and crops. *Bacillus subtilis* is capable of increasing the overall performance of crops and other plants.

Pathology

Bacillus subtilis is not pathogenic to humans, animals, or plants. Although it may contaminate certain foods, such as cocoa and spices, it rarely causes food poisoning. *Bacillus subtilis* does produce one extracellular toxin, the enzyme subtilisin. Subtilisin can result in allergic reactions in some individuals; however, the allergic reaction will only occur following prolonged and repeated exposure. Other species of *Bacillus* are pathogens, causing anthrax and food poisoning.

Microbial cell cultivation

Microbial cell cultivation is the process of cultivating microbial cells of different origin using growth medium under certain physical conditions.

Nutritional Requirements

To cultivate bacteria the nutritional medium should contain the organism's elemental composition which is listed in Table 2-2.

Table 2-2. Elemental compositions of bacteria

Component	Dry weight %
Carbon	50
Oxygen	20
Nitrogen	14
Hydrogen	8
Phosphorous	3
sulfur	1
Metals	4

All biological systems, from microorganisms to man, share a set of nutritional requirements:

1. Sources of energy:
   a. Prototroph: organisms which are capable of employing radiant energy.
   b. Chemotrophs: organisms which obtain the energy for their activities and self-synthesis from chemical reactions that can occur in the dark.
2. Source of carbon:

a. Autotrophs: organisms which can thrive on an entirely inorganic diet, using CO_2 or carbonates as a sole source of carbon.

b. Heterotrophs: organisms which cannot use CO2 as a sole source of carbon but require, in addition to minerals, one or more organic substances, such as glucose or amino acids, as sources of carbon.

3. Sources of nitrogen: atmospheric nitrogen, inorganic nitrogen compounds, or other derived nitrogen.
4. Sources of sulfur and phosphorus: elementary sulfur, inorganic sulfur, or organic sulfur.
5. Sources of metallic elements: sodium, potassium, calcium, magnesium, manganese, iron, zinc, copper and cobalt.
6. Sources of vitamins.

The other chemical elements in microorganisms is Ash composes approximately 5 percent of the dry weight of biomass with phosphorus and sulfur accounting, for respectively 60 and 20 percent. The remainder is usually made up of Mg, K, Na, Ca, Fe, Mn, Cu, Mo, Coy Zn and Cl (Bushell et al. 2003).

2.5 The industrial importance and applications of protease

2.5.1 Industrial applications of alkaline proteases

Alkaline proteases have several industrial applications. These include: as processing aid in leather tanning industries, as detergent additive, in protein hydrolysis, in pharmaceuticals production, and in chemical synthesis (Horikoshi et al., 1996).

Application of alkaline proteases in leather industry

Dehairing is an important operation in tanneries conventionally practiced using lime and sodium sulphide (Thanikaivelan et al., 2004). In this process, the skin/hide is painted with sulphide which helps to reduce the disulphide bond that is responsible for attachment of hair keratin in epidermis. This brings about complete removal of hair, but the hair root remained within skin (Ahmad et al., 2015). In this process, lime contributes to the dehairing process by opening up the collagen fiber structure. The use of alkaline protease has proven superior and efficient for selective removal of the non-collagen part of hide/skin.

The global environment is gradually deteriorating because of the socio-economic activities of humankind such as processing industries. Many industrial processes cause adverse changes in the immediate environmental change and therefore being challenged by society. Of these, leather industries and the increased amount of feathers generated by commercial poultry processing may represent a pollution problem and needs adequate management (Shh et al., 2012).

Leather processing involves a series of unit operations. At each stage, various chemicals are used and varieties of materials are expelled. Depilation or dehairing of hides and skins in leather industry is traditionally done with chemical methods using lime, sodium sulfide, etc. which contributes to 80-90% of the total pollution load in the leather industry and generates noxious gases as well as solid wastes, e.g. hydrogen sulfide and lime (Thanikaivelan et al., 2004). Therefore, leather industry is one of the industries looking up to enzymes to reduce the impact of tanning processes on the environment.

The advantages of enzymatic dehairing include hair-saving dehairing process, a reduction of sulfide content in the effluent, recovery of hair which is of good quality and elimination of the bate in the de-liming (Singh et al., 2016). Enzyme produced by *Bacillus subtilis* has a potential to complete elimination of the need for toxic sodium sulfide during dehairing process in leather industry (Macedo et al., 2008). Therefore, the ever-increasing attention to the environmental impact of leather industry has necessitated for the development of enzyme-based processes as potent alternatives to pollution causing chemicals.

Application of alkaline proteases as detergent additives

The idea of using proteases was first proposed by Rohm , who incorporated pancreatic enzymes into a detergent (Saeki et al., 2007). The economic importance of alkaline proteases became known when bacterial alkaline proteases from Bacillus sp. were introduced in 1960s to the detergent industry(Kazan et al., 2005; Saeki et al., 2007).

The use of enzymes in detergent formulations is now common in developed countries, with over half of all detergents presently available containing enzymes (Genckal, 2006; Khajuria et al., 2015). Proteases are one of the standard ingredients of all kinds of detergents ranging from household laundering to reagents used for cleaning contact lenses or dentures. The use of proteases in the laundry detergents accounts for approximately 25% (around 13 billion tons per year) of the total worldwide sales of enzymes (Jaouadi et al., 2009; Tanksale et al., 2001).

Removal of proteinaceous stains of blood, milk, egg, grass and sauces is very difficult using conventional surfactants. Removal of such a difficult- to- remove stain is now a day achieved by using alkaline proteases. Currently detergent protease account for more than 30% of the world enzyme market (Horikoshi, 2016; Maurer, 2004). To be used as detergent additives proteases need to be active and stable at an alkaline pH, be stable in presence of chelating agents, and must possess broad substrate specificity. In addition, stability and residual activity in the presence of detergent additives such as surfactants, builders, bleaching agents, bleach activating agents, fillers, and fabric softeners are also required (Maurer, 2004).

Application of alkaline proteases for protein hydrolysis

Protein hydrolysates of high nutritional value that can be used in blood pressure regulation, protein fortified soft drinks, and infant food formulations are produced by using alkaline proteases. In addition, alkaline proteases also contribute in improvement of fruit juice and in production of specific therapeutic dietary products from soya and other protein sources (Li et al., 2010).

Application of alkaline proteases in silver recovery from waste photographic and X-ray films

It is known that photographic and X-ray films are partly prepared using silver compounds. Black metallic silver spread in gelatin emulsion layer of waste X-ray films is about 1.5-2.0% silver by weight. Burning of X-ray films, and Striping methods are conventionally used to recover silver. But the conventional burning and chemical alkali methods are not pollution free and result in the generation of foul smell. Use of alkaline proteases that degrade the gelatin in few minutes for silver recovery has been reported. Using such enzyme treatment, about 99% pure silver recovery was achieved. In addition to silver, recovery of base film made of polyester is also possible using alkaline proteases (Sharma et al., 2014; Gethun et al., 2016).

Synthetic and pharmaceutical application of alkaline proteases

Protease by themselves or their degradation products such as peptides can be used as therapeutic agents. For instance, subtilisin is used in the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses to speed up healing process by producing anti-inflammatory response in patients reported. (Davidenko et al., 1999; Sharma et al., 2014) Peptides which can serve as chemotherapeutic agents, toxins, sweeteners, growth hormones, as inhibitors and antibiotics can be also synthesized using alkaline proteases (Saeki et al., 2007).

Application of alkaline proteases in management of waste

Alkaline proteases have interesting potential applications in the management of wastes from households and food processing industries. These include: cleaning of hair clogged pipe lines containing hairs, and in replacing alkali based feather processing from slaughterhouses to use as protein rich animal feed additive (Amare et al., 2003).

2.5.2 Miscellaneous applications

Silk Degumming and scale removal from wool fiber

Degumming of silk fabrics with neutral and alkaline proteases is employed in the lower enzymatic activities and indispensable pretreatment and subsequent finishing scouring which resulting in a long and complex process flow. In recent years, along with the increasing advances in biotechnology, some high-performance proteases are applied in silk degumming (Chen et al., 2007). Wool fiber exhibit fealty and shrinking feature because of the presence of scales that are found toward fiber tips. Conventionally, chlorine-Hircosity is applied to remove the scales by oxidation, or coating of fiber in polymers used. Wool treatment with hair and keratin degrading alkaline protease provide an option in the removal of scale tips from wool fiber (Macedo et al., 2008).

DNA isolation and in animal cell culture

Alkaline proteases of microbial origin also reported as a substitute of other proteases for molecular biotechnology applications. For instance, alkaline protease from Conidium bolus species is reported to substitute trypsin that used in the preparation of animal cell cultures i.e. in dissociation of cells for primary cell cultures, maintenance of cell lines and in production of G-band in Meta phase chromosome (Shin et al., 2016).

Applications in food and feed industry: Microbial proteases have been useful in the dairy industry in the process of cheese formation. Protease enzymes are added to milk during cheese production, to hydrolyze caseins (specifically kappa casein) which stabilize micelle formation preventing coagulation (Sharma et al., 2014; Li et al., 2010).

Applications in waste management: Chemical treatment of waste that can be very hazardous for the environment and its surroundings led researchers to find an alternate way or an eco-friendly way for the treatment of waste management. Waste feathers make up approximately 5% of the body weight of poultry and are considered to be a high protein source for food and feed, provided their rigid keratin structure is completely destroyed. The use of keratinolytic protease for food and feed industry waste, for degrading waste keratinous material from poultry refuse and as depilatory agent to remove hair from the drains has been reported (Sharma et al., 2014; Verma et al., 2016).

Applications in medical field: With the time, proteases have found their application in the medical field. Scientists have successfully used proteases for various purposes in the medical field. 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. Oral administration of proteases from *Aspergillus oryzae* has been used as a diagnostic aid to correct certain lytic enzyme deficiency syndromes (Davidenko et al., 1999).

2.6 Biological Process for Enzymatic Production

AlkPE are among the most important hydrolytic enzymes and have been studied extensively. Enzyme production has grown during the past century in volume and number of products in response to expanding markets and increasing demand for novel biocatalysts. Microorganisms constitute the major source of enzymes, but several enzymes are also obtained from renewable animal and plant sources. In biological transformation process there have three stages such as: upstream, fermentation and downstream process (Genckal et al., 2006; Hatti kaul; Singh et al., 2016).

Upstream process

Production of a new microbial enzyme starts with screening of microorganisms for desirable activity using appropriate selection procedures. The harsh environment to which several enzymes are subjected during process applications has given impetus to screening of extremophiles for enzymes having desirable features of activity and stability (Hatti-kaul; Falch & Nielsen, 1979). Bacterial species are the most widely used microorganism in enzyme production than other types of microbes such as; fungi and yeasts due to their resistance to harsh environment ad enzyme stability. Some of mostly utilized microbial species are listed in the following table.

Table 2-3. Microbial species used for the production of enzymes

Species	**Bacterial species**	**Bacterial species**
Bacteria	Amylases	Bacillus subtilis, Bacillus amyloliquefaciens, Bacillus licheniformis
	β- Glucanase	Bacillus subtilis
	Proteases	Bacillus subtilis, altitudinis
	Penicillinases	Bacillus subtilis
	Asperginase	Escherichia coli
	Pullulanase	Bacillus acidopullulyticus
	Maltase	Bacillus subtilis
	Mannanase	Bacillus leutus
	Xylanase	Bacillus subtilis
Yeast	Invertase	Saccharomyces cerevisiae
	Lactase	Saccharomyces fragilis
Fungal	Proteases	Aspergillus niger
	Pectinases	Aspergillus niger
	Glucose oxidase	Penicillium notatum
	Catalase	Aspergillus niger
	Lipase	Aspergillus niger
	Phytase	Aspergillus niger
	Xylanase	Aspergillus niger, Trichoderma longibrachiatum

2.6.1 Inoculum preparation

Seed inoculum was prepared by growing a loopful of slant culture of B. subtilis SHS-04 in 20 mL of seed medium containing 0.75% (w/v) peptone, 0.5% (w/v) glucose, 0.05% (w/v) NaCl and 0.01% (w/v) MgSO4.7H2O in a 200 mL conical flask with pH adjusted to 8.0. The inoculated seed medium was incubated at 37˚C for 24 h at 180 rpm in a shaking incubator (Olajuyigbe, 2013; Sarker et al., 2013).

2.6.2 Media Formulation and Preparation

Analysis of microbial cell composition shows that over 95% of cell dry weight is made up of a few major elements: carbon, oxygen, hydrogen, nitrogen, sulfur, phosphorus, potassium, calcium, magnesium, and iron. These are called macro elements or macronutrients because they are required by microorganisms in relatively large amounts. The first six (C, O, H, N, S, and P) are components of carbohydrates, lipids, proteins, and nucleic acids. The remaining four macro elements exist in the cell as cat-ions and play a variety of roles. For example, potassium (K^+) is required for activity

by a number of enzymes, including some of those involved in protein synthesis. Calcium (Ca^{2+}), among other functions, contributes to the heat resistance of bacterial endospores. Magnesium (Mg^{2+}) serves as a cofactor for many enzymes, complexes with ATP, and stabilizes ribosomes and cell membranes. Iron (Fe^{2+} and Fe^{3+}) is a part of cytochromes and a cofactor for enzymes and electron-carrying proteins (Elander, 2003; Warth et al., 1978).

Optimization of fermentation medium

Alkaline proteases are generally produced by submerged fermentation. In addition, solid state fermentation processes have been exploited to a lesser extent for production of these enzymes. In commercial practice, the optimization of medium composition is done to maintain a balance between the various medium components, thus minimizes the number of unutilized components at the end of fermentation. Research efforts have been directed 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, no defined medium has been established for the best production of alkaline proteases from different microbial sources. Each organism or strain has its own special conditions for maximum enzyme pro- duction (Kumar et al., 1999).

2.7 Fermentation Process

The term fermentation has different meaning to biochemist and microbiologist. In biochemical sense it means any energy-generation process in which organic compounds act as both electron donors and terminal electron acceptors. While for microbiologist it has the sense to mean any process for the production of products by the mass culture of microorganisms. The culture techniques in fermentation can be classified into batch, fed-batch, and continuous operation(James , 2009; McGraw Hill Companies, 2016). In batch processes, all the nutrients required for cell growth and product 20 formation are present in the medium prior to cultivation. Oxygen is supplied by aeration. The cessation of growth reflects the exhaustion of the limiting substrate in the medium (Prescott et al., 2002).

Figure 2-3. Typical fermentation process for enzymatic production

2.7.1 Types of fermentations

Based on the purpose they are intended for fermentations are categorized into five major industrially important groups (Ghosh, 2006; James, 2009; Prescott, 2002).

I. Those that produce microbial cells (or biomass) as the product.
II. Those that produce microbial enzymes
III. Those that produce microbial metabolites
IV. Those that produce recombinant products
V. Those that modify a compound which is added to the fermentation –the transformation process.

The transformations of substances into valuable products by microbial cells or enzymes in catalytic processes such as dehydrogenation, oxidation, hydroxylation, dehydrogenation, condensation, decarboxylation, amination, deamination and isomerization have greater advantages over chemical methods of transformation:

- Micro-organisms can behave as chiral catalysts with high positional and stereo specificity which enable us the addition, removal or modification of functional groups at specific sites on complex molecules to serve our purpose.
- Microbial processes can be operated at relatively low temperature and pressure without the requirement for heavy metal catalysts which are potentially pollutants.

All commercially important enzymes are produced by microorganisms. The enzymatic yield obtained from fermentation, cost of their production, and downstream processing cost determines the final cost of the enzyme(Ghosh, 2006). In speaking of substrate availability and cost should be given develop a viable industrial process, lowering production cost, and increasing enzyme productivity are very important. Selection of best fermentation technique and optimization of culture conditions contribute much in achieving enzyme productivity.

Solid State Fermentation

In microbial enzyme production surface method (solid state) and submerged fermentation process are used. Solid state fermentation is defined as the growth of microorganisms on moist solid substrates in the absence of free-flowing water. The microorganism obtains water, carbon, nitrogen, minerals, and other nutrients from the solid substrate. Microbial enzymes were produced by surface cultures, i.e., cultures of microorganisms in thin layers of liquid or moist, solid media. Control of infections and also uniform control of temperature, humidity, and aeration present difficulties (Falch & Nielsen, 1979).

Submerged Fermentation

Submerged culture methods today dominate in the production of enzymes because handling costs and the risk of infection are reduced, and because modern methods of control are more easily adapted to these processes. Yields are also generally higher. The production methods discussed below refer to submerged fermentation (Hatti-kaul; Chen et al., 2007).

The medium in the seed tank often resembles the production medium. Excessive heat sterilization of the medium retards the growth of the inoculum. The volume of the seed tank usually constitutes 3-10% of the volume of the production fermenter. The propagation time in the seed fermenter

varies from 10 to 80 hours, depending on the process. At present, more than 90 % of the commercial microbial enzymes, including protease, are produced using submerged fermentation.

The microorganisms used for enzyme production are grown in fermenters using an optimized growth medium. Both solid state- and submerged fermentation are applied commercially, however the latter is preferred in many cases because of a better handle on aseptic conditions and process control the comparison was listed in Appendix A of the document. The enzymes produced by the microorganism may be intracellular or secreted into the extracellular medium.

2.8 Down Stream Process

Downstream processing involves isolation and purification steps (fig 2-5.) and ends up in the formulation of the enzyme preparation (Roger, 2015).

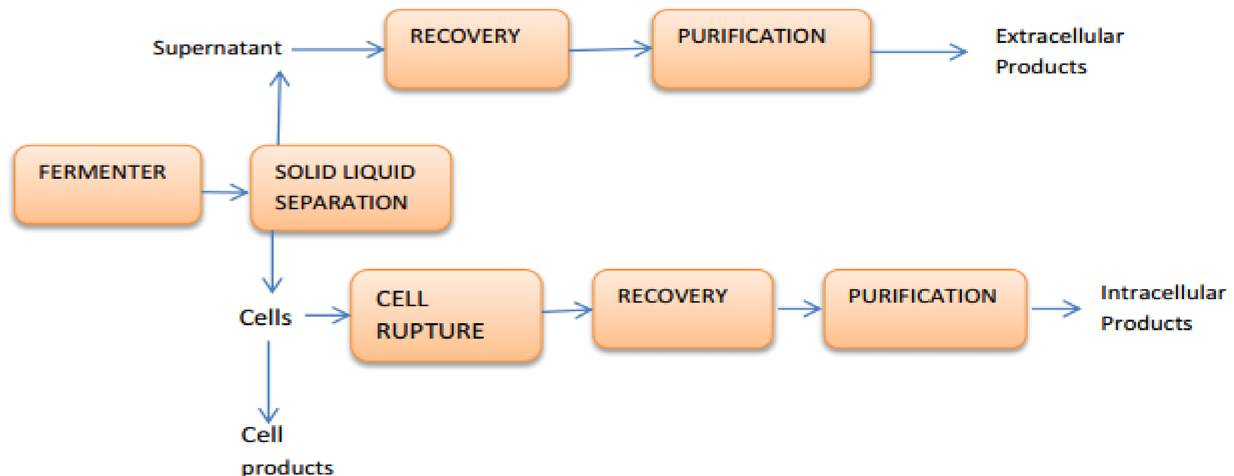


Figure 2-4. Major process steps in downstream processing

DSP has the following objectives

- The removal of unwanted impurities such as biomass, cellular debris, suspended solids and colloidal particles.
- Bulk volume reduction by concentration of the desired product.
- For protein products, it transfers the protein into an environment where it will be stable and active and ready for its intended application.
- To achieve required level of purification.

- To prepare the product for storage and /or distribution by preventing and /or avoiding contamination

2.8.1 Isolation and purification techniques

Downstream processing has been there over a very long period of time, especially in the area of chemical engineering where chemical engineers used to practice recovery of products using filtration, distillation, separations involving extractions and so on. Downstream processing of enzyme from the raw material constitutes the subsequent key stage in the production process. The desired level of purification depends on the ultimate application of the enzyme product. The industrial bulk enzymes are relatively crude formulations while especially enzymes undergo a thorough purification to yield a homogeneous product (Ghosh, 2006; Roger, 2015).

Primary unit operations of downstream processing

These are the ones which enable us to achieve partial purification of fermentation products that are intended for industrial applications like dehairing enzymes of this thesis concern. Hence, for this study these downstream processing is considered and performed and the efficacy of the products of each method is evaluated for removing the hair in reasonable period of time(Ghosh, 2006; Roger, 2015).

1. Cell recovery/removal

   Filtration and centrifugation are typical unit operations for cell removal.
2. Dewatering and partial purification. Usually it is done by:

***Microfiltration**:* it has an average pore size of 0.2 µm and prepares the broth for ultrafiltration by removing cells and other suspended particles to yield particle free clear solution.

Ultrafiltration (UF): The solution is forced under pressure through a membrane with average pore size ranging from 10 -200 kilo Daltons (depending on the molecular size of the target enzyme), which allows water, salts and small molecules to pass but rejects larger molecules i.e. the target enzyme.

***Precipitation**:* Salting out – addition of a high concentration of a soluble salt (typically ammonium sulphate) causes proteins to aggregate and precipitate.

Spray drying: Requires use of hot air to evaporate water from atomized sprays of the enzyme solution.

Secondary unit operations of downstream processing

a. Protein purification

- Adsorption chromatography
- Gel permeation chromatography

3. Protein processing
    - Immobilization
    - Beading

c. Protein packaging

- Sterilization
- Bottling etc.

Solid-liquid Separation (Removal of Insoluble)

There are different Gravity based separation such as: (sedimentation, precipitation, flocculation, electro-precipitation, and gravity settling), Centrifugation, Filtration

Centrifugation: Removal of solids/derbies is generally the first step in the downstream process for extracellular product and next to cell disruption for intercellular product processing. Biomass, dead biomass, cell debris and salts from the reactor are removed by one of solid-liquid separation techniques.

Product purification

There is a choice of different separation techniques for each stage. Chromatography is the major technique for high-resolution purification of enzymes.

Chromatography: used for separating biomolecules, proteins, carbohydrates, peptides and even small molecules metabolized small organic molecules. There are different types of chromatography, each one working on a certain principle. It has stationary phase the components to be separated is injected with a mobile phase, so that so the solute gets distributed based on

different principles physical principles. Hence, there is a separation of the various solutes so various solutes may have different separating or partitioning effects which may lead to a separation of components from a mixture (Bushell, 2003; Ghosh, 2006).

Enzyme activity Assay

The activity of protease was measured using casein as substrate according to (Amare, 1997) and (Amare et al., 2003) with a little modification first 2% of casein and 100mM of Glycine-NaOH buffer pH 10.0 were prepared and mixed together by vortex to give final concentration of 1% casein and 50mM Glycine-NaOH (pH 10.0) buffer. Then the reaction mixture containing 500 µl of the above prepared substrate was added to three clean labeled eppendorf tubes; two duplicates of enzyme and one enzyme blank.

Formulation of Enzyme

The enzyme is finally formulated as a liquid or solid product. In either case, stabilizing additives are added for rendering long shelf life to the product. Some enzymes are immobilized to solid supports or enzyme crystals are cross-linked to render them insoluble and stable for repeated or long-term use in a process application.

This thesis proposal covers upstream process like media preparation, fermentation process was done using submerged fermentation. Finally, extraction of enzymes or downstream process was taking place using centrifugation method for concentration of the crude enzyme. The last task was analytical methods for enzyme assay, characterization and detergent compatibility were conducted.

3 Materials and Methods

The materials and methods used to conduct this study described as follows:

3.1 Materials

3.1.1 Equipment and materials

I. Raw materials

ESM (egg shell and membrane)

II. Reagents and chemicals

- ✓ Trichloroacetic acid (TCA)
- ✓ Sodium Chloride
- ✓ Glycine
- ✓ Sodium hydroxide
- ✓ Distilled water
- ✓ NaOH
- ✓ Folin cio-calteou

Buffers:

- ✓ Potassium hydrogen di phosphate (KH_2PO_4)
- ✓ Dipotassium hydrogen phosphate (K_2HPO_4)
- ✓ Tris-HCl Glycine –NaOH
- ✓ Na_2PO4 and NaOH

Growth and production medium

- ✓ Source of carbon (glucose)
- ✓ Source nitrogen (yeast extract, peptone, soya bean)
- ✓ Source of inorganic salts (NaCl, $MgSO_4$, KH_2SO_4, ESM and $CaCl_2$)

III. Machines, Equipment and consumables

- ✓ Laminar air flow cabinet (Safety cabinet)
- ✓ Test tubes
- ✓ Shake flasks Shaker
- ✓ Petri dish and paraffin film
- ✓ Autoclave
- ✓ Graduated Measuring cylinders (10 ml, 25 ml. 50ml, 100 ml)
- ✓ Micropipette and tips
- ✓ Refrigerator
- ✓ Water bath
- ✓ Spectrophotometer

- ✓ Centrifuge
- ✓ Magnetic stirrer
- ✓ Dryer
- ✓ pH meter
- ✓ Incubator
- ✓ Closures such as cotton plugs, plastic foam, screw caps, metal caps and aluminum foil for closing end of culture vessels

IV. Culture for fermentation

- ✓ Bacillus subtilis bacterial species

V. Software

- ✓ Design expert 7.0

3.2 Methods

3.2.1 Collection and processing of ESM

Egg shells had been collected from the local fast food café and bakeries in Addis Ababa city with high consumption of egg. To remove impurity and the interference material such as; Insects and

Figure 3-1. Collection & processing of egg shell, (a) raw egg shell, (b) washing & dying, (c) grinding & sieving, (d) measuring

dirt (mud, dust, soil etc.), ESM was rinsed/ washed using distilled water and allow to dry under

sun light at 35–40 ^{0}C a day long. The eggshell was finely grounded using analytical mill and coffee grinder. The powder was sieved up to 100 µm geometrical size to use in ESM characterization and in the fermentation process.

3.2.2 Characterization of ESM

The samples of ESM were characterized by proximate analysis and Fourier transform infrared spectroscopy (FTIR).

Proximate Analysis

It consists of determination of moisture, ash, fixed carbon and volatile matter contents were done on Eggshell powder. The details of this analysis were described as follows:

Moisture content: The moisture content was determined using ASTM D2867-91 method. These is determined by loss on drying method. Then subsequent incubations weight was achieved at the interval of 30 min until constant reading. A crucible amount weighed, for of eggshell was taken and then the crucible was placed in an electric hot air oven maintained at 105^{0}C for 3 h. After 3 h, cool in dissector then measure the amount of the adsorbent, the evaporating dish amount are measured and returned to the oven at the same temperature as in the previous step. Finally, the moisture content is calculated using the equation 3.1.

Moisture content (%) = $\frac{(W1-W2)}{W1} * 100$ 3.1

Where: W_1 = weight of the sample before drying

W_2 = weight of the sample after drying

Ash content: Ash is defined as the quantity of mineral matter which, after application of the described working methods, remains as incombustible of testing substance. Ash content determination was done according to the ASTM D2866-94 method by 1g of the powdered ESM were accurately weighed and transferred into a crucible. The crucible and its contents were gently heated over Bunsen burner flame until it became free from moisture and completely charred. The heat was increased gradually until most of the carbon was vaporized and then heated strongly such that the inorganic ash could be seen (residue free from carbon) as almost white. Then after cooling the ash in desiccators, weighed and the weight was recorded. Then the heating continued until the weight became constant. Then the ash value was calculated using equation 3.2:

Ash content = $\frac{(W3-W1)}{W2} * 100$ 3.2

Where, W_1 = weight of the crucible, W_2 = weight of the crucible and sample before igniting,

W_3 = weight of crucible and sample after igniting

Volatile Matter content: 1g of air-dried, powdered sample was taken in crucible. The crucible was covered with silica lid. Then crucible was kept in a furnace for 7 min at the temperature of 800°C. The crucible was taken out of the furnace and allowed to cool in a dissector. The % volatile matter content in the sample was calculated by using the formula given below:

% Volatile matter (VM) = % loss in weight - % moisture content -----------------------------(3.3)

Fixed Carbon Content: Fixed carbon is a calculated value and it is the resultant of summation of percentage moisture content, ash content, and volatile matter content subtracted from 100 %.

Fixed carbon (%) = 100 – (moisture, % + ash, % + volatile matter, %) --------------------------3.4

3.2.3 Collection of bacteria and inoculum preparation

The bacteria *Bacillus subtilis m.* in broth *was* collected from EBI (Ethiopian Biodiversity Institute) and transferred to SCB laboratory and kept at 4^0C in refrigeration. Prior to seed inoculum preparation the bacteria in the broth was activated using nutrient agar and SMA medium in a plate and in slant for preservation. The plate and slant were incubated for 24 h to check the viability of *Bacillus subtilis m* prior to inoculate.

Seed inoculum was prepared by growing a loopful of plate/slant culture of *Bacillus subtilis m* in 30 ml of seed medium containing 0.75% (w/v) bacteriological peptone, 0.5% (w/v) glucose,

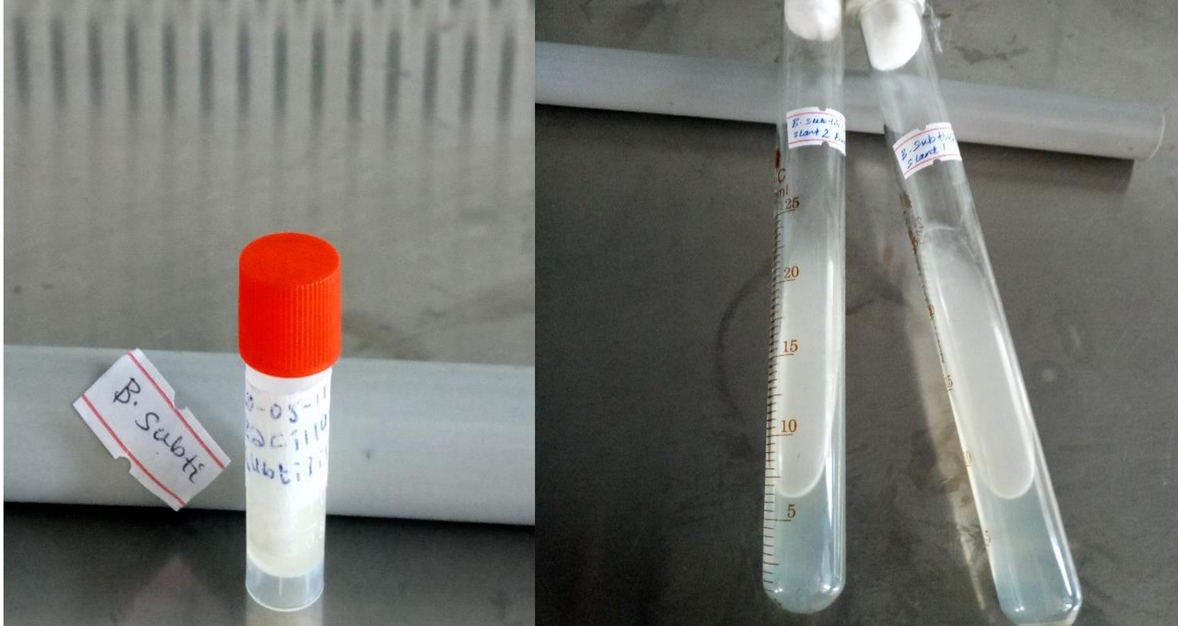


Figure 3-2. *BSM*. Broth from EBI & slant culture of *BSM*.

0.05% (w/v) NaCl and 0.01% (w/v) $MgSO_4.7H_2O$ in a 200 ml conical flask with pH adjusted to 9.0. The inoculated seed medium was incubated at 37˚C for 24 h at 180 rpm in a shaking incubator (Stuart, UK). The 24 h old seed culture had been used as seed culture and added to the working medium after cooling (Olajuyigbe et al., 2013).

3.2.4 Optimization of culture conditions

To select the optimum condition for maximum enzyme production, the level of parameters optimized, where the optimum value determined for one parameter was applied in the subsequent experiment.

In this part of the study, the relationship between controllable experimental factors (pH, temperature, ESM and incubation time) and the response (AlkPE production) was investigated by Response Surface Methodology (RSM) of Box-Behnken, which is the most accepted statistical technique for bioprocess optimization. It is a scientific approach in determining optimum conditions which combines special experimental designs with Taylor first and second order equations. The RSM process determines the surface of the Taylor expansion curve which describes the response (yield, impurity level, etc.). The response surface methodology approach has many advantages over other optimization procedures (European Commission, 2002).

Alkaline protease production using *BSM.* Cultivation of bacteria was done by inoculation using loop full of broth from EBI to prepare seed inoculum. 2 ml of the inoculum medium (corresponding to 10^8 cells per ml) of cultures of Bacillus subtilis inoculated to alkaline protease production media, which was made up of the components of Horikoshi-I alkaline medium (Marathe et al., 2018) (pH 9) i.e. D-glucose -10 g, Peptone -5 g, Yeast extract -5 g, KH_2PO_4-1 g, $MgSO_4$ $7H_2O$ -0.2 g, Na_2CO_3 -5 g and Distilled water-1000 ml (Horikoshi, 1971). Glucose and sodium carbonate were autoclaved separately and then added to rest of the autoclaved medium. The values used for investigation of the parameters were: initial pH (8.0, 9.0 and 10.0), temperature (30°C, 37°C , 45°C and 50°C) (Warth et al 1978), incubation time (36, 48, 72 h), ESM (15% , 20% and 25% w/v) (Nagamalli et al, 2017). After conducting 29 experiments maximum enzyme activity was obtained at 37°C, a pH of 9.0, ESM concentration of 20 % (w/v) and incubation time of 48 h.

3.2.5 Characterization of production media

Environmental or physical and chemical conditions allowing optimal growth and productivity of the culture were studied in terms of the following factors:

- pH
- Temperature
- Concentration of ESM and
- Time

pH, temperature, ESM and time effects were studied using lab grade standard media (Horikoshi alkaline media). The formulation of the synthetic or designed media used for the characterization of production media is shown in Table 3-1. Standard growth and production media formulation.

Table 3-1. Growth and production media

Ingredients for Inoculum preparation	% (w/v)	Ingredients for production media	% (w/v)
Bacteriological peptone	0.75		0.5
Yeast extract	----		0.5
Glucose	0.5		1
NaCl	0.05		----
KH_2PO4	---		0.01
$MgSO_4.7H_2O$	0.01		0.1

The effect of Temperature and pH on the production of Protease

he culture pH also strongly affects many enzymatic processes and transport of various components across the cell membrane. When ammonium ions were used, the medium turned acidic, while it turned alkaline when organic nitrogen, such as amino acids or peptides were consumed. The decline in the pH may also be due to production of acidic products. In view of a close relationship between protease synthesis and the utilization of nitrogenous compounds, pH variations during fermentation may indicate kinetic information about the protease production, such as the start and end of the protease production period.

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. However, studies showed that a link existed between enzyme synthesis and energy metabolism in bacilli, which was controlled by temperature and oxygen uptake. The optimum temperature values reported for maximum protease production are given in (Kumar et al., 1999).

The optimum conditions for maximum alkaline protease production under solid state fermentation were carried out according to the method described by (Warth et al., 1978) and (Nagamalli et al, 2017). The conditions are (30 ^{0}C, 37 ^{0}C, 45 ^{0}C), pH of (8,9, 10), ESM concentration of (15%, 20%, 25%) and incubation time of (36 h, 48 h, 72 h).

Effect of concentration of ESM and production of protease

According to (Nagamalli et al., 2017) ESM has effect on the production of Alkaline protease since calcium has the potential to induce the protease production. In spite of this, this thesis focusses on the use of ESM in different concentration and find the optimum concentration in which maximum production is attained and compare with protease enzyme produced without ESM.

Effect of temperature, pH, ESM and incubation period production

During optimization studies of fermentation process parameters in SmF, one factor at a time strategy was used using Box-Behnken analysis of design expert software. The software gives 29 combination of the factors to be used in the experiment. Each parameter was optimized independent of the others and at pH 7 and at 25 ^{0}C were done to have information on the lower level data points on the optimization process. Subsequently optimal conditions were employed in all experiments.

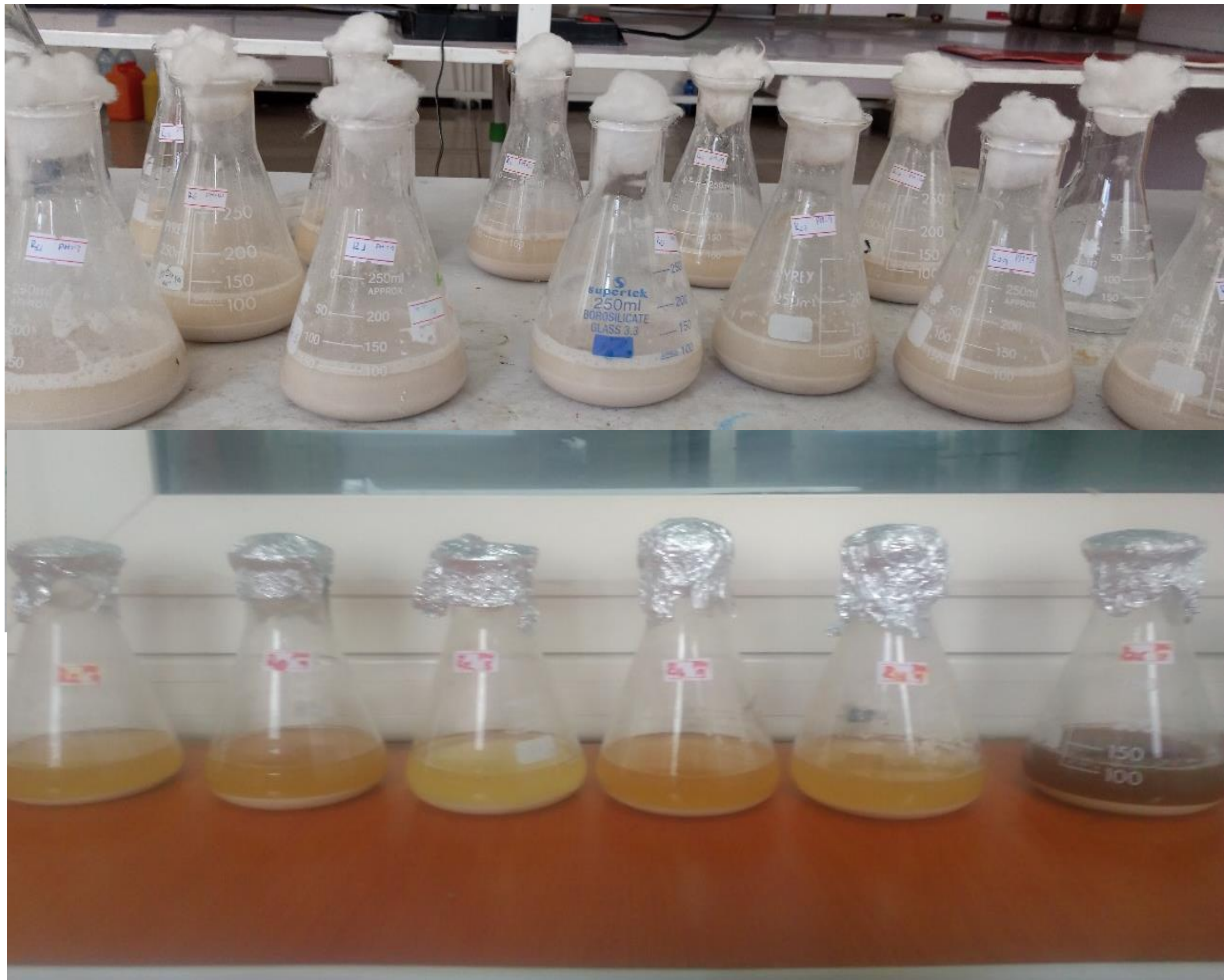


Figure 3-3. Production medium before and after sterilization

An experiment designed by Box-Behnken was used to investigate the effect of temperature, pH, ESM concentration and incubation time using standard medium.

Table 3-2. Lists the levels of the four independent variables studied

Variables (Factors)	Units	Level		
		Maximum (+1)	**Medium (0)**	**Minimum (-1)**
Temperature	^0C	45	37	30
pH	pH scale	10	9	8
ESM	Concentration % (w/v)	15	20	25
Incubation time	h	36	48	72

The levels of the variables were set based on the information obtained from the above characterization of these four factors.

Table 3-3. Bbd experimental factorial design matrix for the study of combined effect

Std	Run	Block	Factor1: pH	Factor 2: Temperature	Factor 3: ESM	Factor 4: Incubation time
28	1	Block 1	0	0	0	0
10	2	Block 1	1	0	0	-1
8	3	Block 1	0	0	1	1
11	4	Block 1	-1	0	0	1
20	5	Block 1	1	0	1	0
7	6	Block 1	0	0	-1	1
27	7	Block 1	0	0	0	0
2	8	Block 1	1	-1	0	0
9	9	Block 1	-1	0	0	-1
16	10	Block 1	0	1	1	0
21	11	Block 1	0	-1	0	-1
12	12	Block 1	1	0	0	1
18	13	Block 1	1	0	-1	0
13	14	Block 1	0	-1	-1	0
6	15	Block 1	0	0	1	-1
3	16	Block 1	-1	1	0	0
22	17	Block 1	0	1	0	-1
25	18	Block 1	0	0	0	0
23	19	Block 1	0	-1	0	1
19	20	Block 1	-1	0	1	0
24	21	Block 1	0	1	0	1
4	22	Block 1	1	1	0	0
1	23	Block 1	-1	-1	0	0
15	24	Block 1	0	-1	1	0
14	25	Block 1	0	1	-1	0
5	26	Block 1	0	0	-1	-1
26	27	Block 1	0	0	0	0
17	28	Block 1	-1	0	-1	0
29	29	Block 1	0	0	0	0

3.3 Physio-chemical characterization of the alkaline protease

3.3.1 Preparation of Tyrosine standard curve

In order to compute the activity of protease in unit basis, the standard curve of tyrosine was prepared using the standard proportions of tyrosine (Amare et al., 2003). The stock concentration of tyrosine used in the solution was 200 µg/ml.

Table 3-4. Standard concentrations of tyrosine for the determination of unit of alkaline protease

Run No.	Tyrosine(µl)	Buffer(µl)	Tyrosine] (µg/ml)
1	0	500	0
2	25	475	10
3	50	450	20
4	75	425	30
5	100	400	40
6	125	375	50
7	150	350	60

Then to generate the standard curve; 0.5M of Na2CO3, Glycine-NaOH buffer (50mM, pH 10.0), 1:10 diluted 2N Folin Cio-calteou phenol reagent and the tyrosine solution mixtures given in the table 3-4 were used. First 200 µg/ml of stock solution of tyrosine was prepared and appropriately diluted to attain a given concentrations of tyrosine as given in the above table.

1. 2.5 ml of 0.5M Na2CO3 was added in each test tube including blank.
2. Five hundred (500 µl) of the above mixture was added on it.
3. Following the mixtures vortexed and kept at room temperature for 10 min

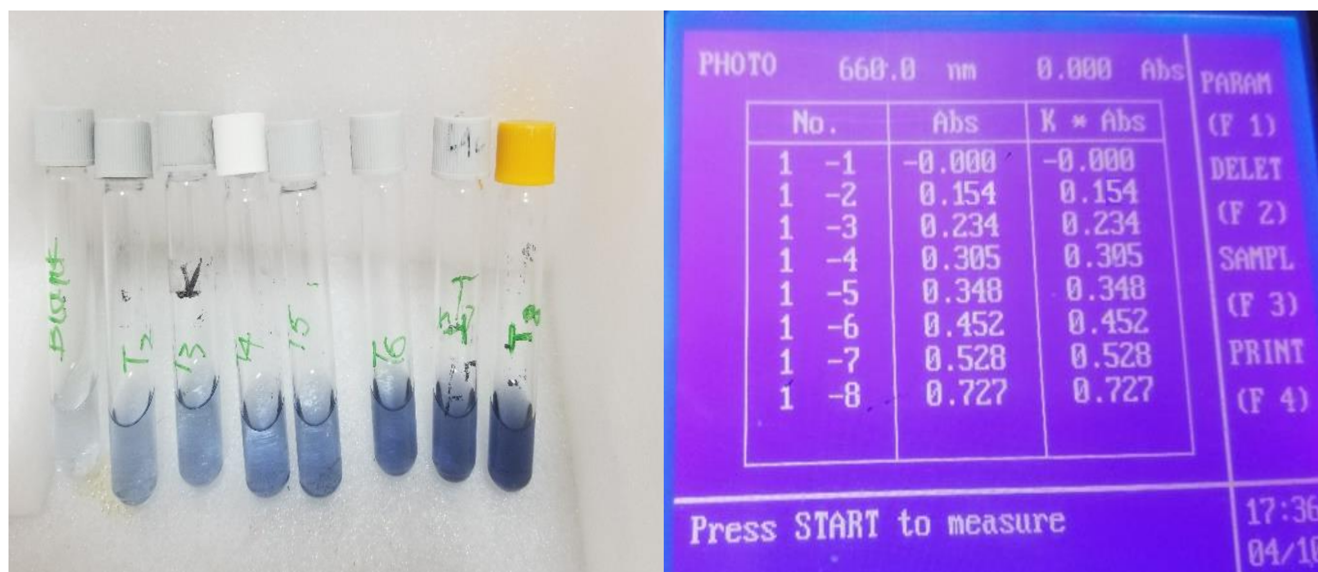


Figure 3-4. Tyrosine standard curve assay: a) assay mixtures after 30 min incubation, b) absorbance value @660nm

4. After this 500µl of 2N Folin Cio-calteou phenol reagent was added in each test tube.
5. Then solution was vortexed immediately and kept at room temperature for 30 min.
6. Finally, the absorbance (OD 660nm) was measured with spectrometer and standard curve was plotted by using Origin 8.0 software as shown in Figure 3-5.

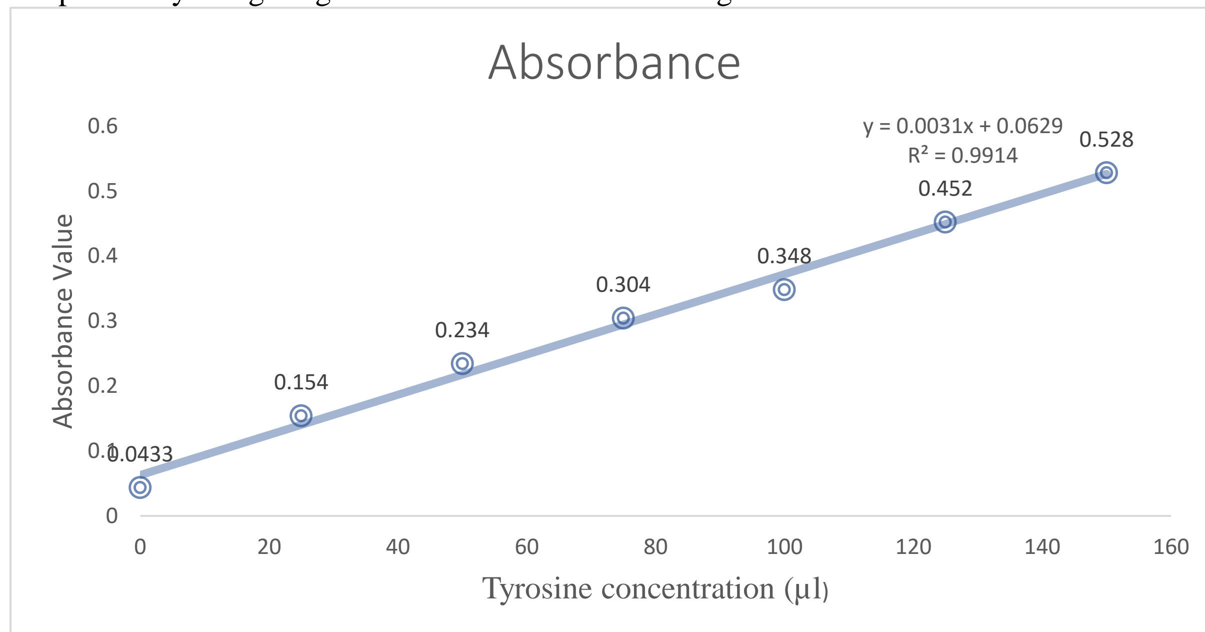


Figure 3-5. Tyrosine standard curve

The OD 660nm values from the above concentrations results in a regression equation of Y = 0.0031x + 0.056, with the regression coefficient (R^2 = 0.9954), which is strongly positively correlated. Then by rearranging, solving and substituting the absorbance values in to the above equation, we got;

$$\text{Enzyme Units (U/ml)} = \frac{(322.58)*OD@660nm*Dilution\ factor-(18)}{Incubation\ time}$$

3.3.2 Enzyme assay

The activity of protease was measured using casein as substrate according to (Amare. et al, 2003)with little modification. First 2% of casein and 100mM of Glycine-NaOH buffer pH 10.0 were prepared and mixed together by vortex to give final concentration of 1% casein and 50mM Glycine-NaOH (pH 10.0) buffer. Then the reaction mixture containing 500 µl of the above prepared substrate was added to three clean labeled eppendorf tubes; two duplicates of enzyme and one enzyme blank.

Similarly, one reagent blank was prepared except 1000 ml of TCA, which was used instead of appropriately diluted crude enzyme in others before incubation in the water bath. To the reaction mixture eppendorf tubes labeled as enzyme, 500 µl of crude enzyme was added and incubated at 40 ^{0}C for 30 min in the water bath. After 30 min 1000 µl of 10% TCA (Tri Chloro Acetic acid) was added to stop the reaction. Following this, 500 µl of crude enzyme was added to the enzyme blank. Then the reaction mixture left at room temperature for 10 min to allow undigested casein to precipitate. After this, the reaction mixtures were centrifuged at 10,000 rpm for 5 min. Then 1000 µl of clear supernatant from each reaction mixture was transferred to the respective labeled clean test tubes and 2.5 ml of 0.5 M sodium carbonate solution was added to it. Next 500 µl of 1:10 diluted folin-Ciocalteau phenol reagent was added to the solution and mixed thoroughly by vortex and incubated at dark room for 25 min. Finally, optical density (OD) was measured using double beam spectrophotometer (1800 UV/Vis) at 660 nm. Enzyme activity was measured against enzyme blank. One unit of alkaline protease is defined as the amount of enzyme that releases 1µg of amino acid equivalent to tyrosine per 1 min under standard assay conditions (Amare. et al, 2003).

$$\text{Enzyme Activity} = \frac{\left(\frac{1}{Slope}\right)*OD*DF-(Intercept/slope)}{Incubation\ time} \text{ -------------------3.1}$$

$$\text{Enzyme Units (U/ml)} = \frac{(322.58)*OD@660nm*Dilution\ factor-(18)}{Incubation\ time}$$

Relative activity: was calculated as the percentage enzyme activity of the sample with respect to the sample for which maximum activity is obtained.

$$\text{Relative Activity \%} = \frac{\left(\frac{1}{Slope}\right)*OD*DF-(Intercept/slope)}{Incubation\ time} \text{ ---------------------3.2}$$

Residual activity: was calculated as the percentage enzyme activity of the sample with respect to activity of control (untreated enzyme).

$$\text{Residual Activity(\%)} = \frac{\left(\frac{1}{Slope}\right)*OD*DF-(Intercept/slope)}{Incubation\ time} \text{ ---------------------3.3}$$

Where: Df = dilution factor, OD = absorbance value at 680 nm

3.3.3 Effect of enzyme concentration

The effect of dilution of alkaline protease on the proteolytic activity was determined. The original stock enzyme preparation was diluted by 100mM of Glycine - NaOH buffer (pH=10.0) to the ratio of 1:1,1:5, 1:10,1:15,1:20,1:25. Then casein hydrolysis was determined by standard protease assay procedure following the procedures for its activity.

Table 3-5. The effect of dilution ratio on activity of enzymes

Alkaline Protease (µl)	Glycine-NaOH buffer (µl)	Ratio
1000	1000	1:1
200	1000	1:5
100	1000	1:10
50	750	1:15
50	1000	1:20

3.3.4 The effect of pH on activity and stability of the enzyme

The effect of pH on activity of alkaline protease was examined by assaying the enzyme at different pH values. The buffers used are: KH_2PO_4 and K_2HPO4 (pH=6.0- 7.0), Tris-HCl (pH=7.5-8.5),

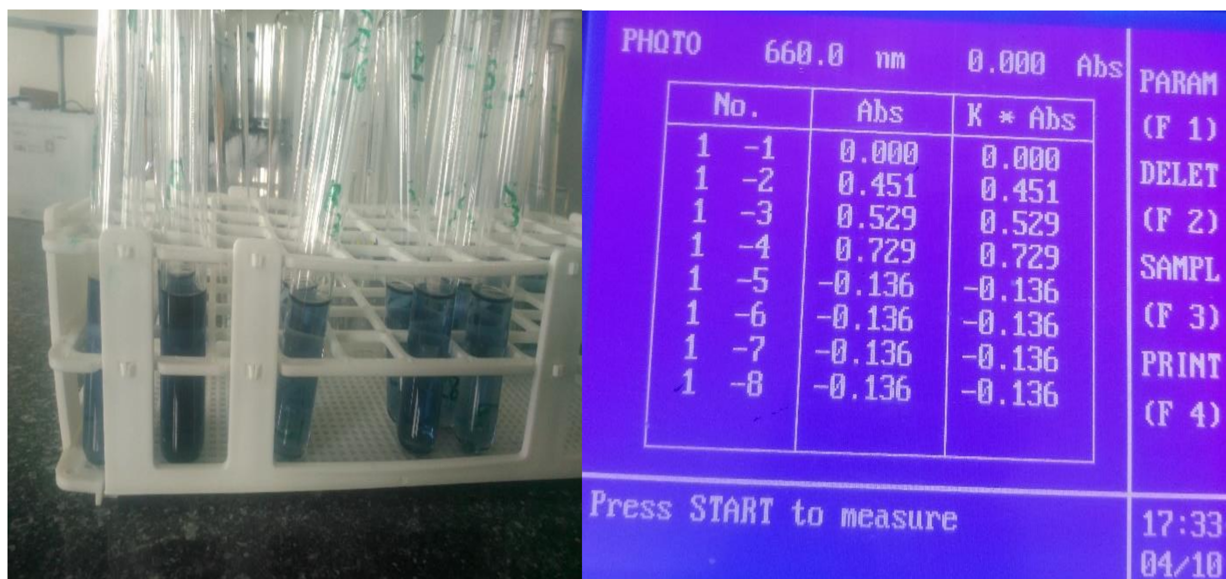


Figure 3-6. Enzyme assay mixtures & their respective absorbance value

Glycine –NaOH (pH=9.0-10) and Na_2PO4 and NaOH (pH=10.5-12.0). All the buffers were used at concentration of 50mM and within 0.5 differences at 50 using casein as substrate. The enzyme assay procedure was similar to the procedure described at section Enzyme assay and relative activity was calculated.

The pH stability of BSM alkaline protease was studied both at pH 8.0 and pH 10, between optimum pH for activity requirement of many industrial applications at which protease is tested and applied. The effect of pH on stability of the protease was conducted according to the method described by (Amare et al. 2003). The crude enzyme sample was mixed with different buffers and incubated at 40 ^{0}C for 1 h and residual activity was measured using casein in Glycine – NaOH (pH=10.0) and Tris - HCl (pH=8.0) as substrate using standard protease assay procedure. Buffers used and their concentrations were similar to the above mentioned in the enzyme activity part. Finally, residual activity was measured by taking untreated enzyme as 100%.

3.3.5 The effect of temperature on activity and stability of the enzyme

Temperature profile of the protease was determined by incubating the enzyme at different temperatures from 30-85 ^{0}C with in 5^{0}C interval in a water bath by using 1% casein in 50mM Tris-HCl buffer as substrate (pH =8.0) or using Glycine-NaOH buffer (pH=10.0). Then activity was determined following standard protease assay procedure. Relative active activity was calculated to determine the optimum temperature for proteolysis. Thermal stability of the enzyme was determined according to (Amare. et al., 2003). The enzyme appropriately diluted with Glycine-NaOH buffer (pH=10.0, 0.1M. For investigating temperature effect on stability of BSM alkaline protease, incubating the enzyme assay mixture for 1h prior to assaying at 40^{0}C, 45^{0}C, 50^{0}C, 55^{0}C and 60^{0}C in different Eppendorf or centrifuge tubes. The assay was done by taking aliquot sample at 10 minutes interval done for single temperature at a time. Following the enzyme samples withdrawn every 10 min and stored at 4 ^{0}C. Then the residual activity was determined by using casein as substrate following standard protease assay procedure. Untreated enzyme was taken as 100%.

3.3.6 The effect of Sodium Chloride on activity and stability of Protease

The ionic strength of the alkaline protease was determined by adding 0-3M (within 0.5 M interval) of NaCl to the reaction mixture and then by carrying out standard protease assay at pH 10.0. Finally, relative activity was calculated by taking the one with largest activity as 100%. The stability of alkaline protease in the different concentrations of sodium chloride was examined by preparing 0-3M NaCl (within 0.5 M intervals) according to modified method of (Devi et al., 2012). First a required concentration of NaCl prepared by dissolving it in100mM Glycine-NaOH buffer (pH=10.0). Then equal volumes of different [NaCl] in buffers and enzyme mixed together in clean

tubes and incubated at 60 ^{0}C for 1 h. After 1 h the mixture withdrawn and standard protease assay was carried out. The enzyme not treated with NaCl but incubated in the similar condition was taken as 100 %.

3.4 Potential applications of the alkaline Protease

3.4.1 Detergent applications and stain removal

Detergent compatibility study

The washing performance of purified protease with some commercial laundry detergents available in the local market such as Ariel, Largo (Repi detergent factory) used procedures described by (El-Hassayeb 2016; Rai et al. 2010) procedure. Appropriately diluted concentrations of protease (final concentration ranging from 25% to 100%) were mixed with a fixed concentration powder Ariel (7 mg/ml to stimulate the washing condition) prior to mix, the Ariel mixture was heated to destroy the indigenous protease activity, if any.

Removal of blood stain from cotton fabrics

Wash performance of crude protease was evaluated by subjecting the blood stain removal test from cotton fabrics as described by (Rai et al., 2010) liquid crude protease were used. Briefly, white cotton cloth was cut into 4x5 cm^2 pieces, and each piece was stained with 1.0 ml of fresh goat blood (obtained from slaughter house) and then dried at 40 °C for overnight. To test the wash performance, each piece of stained cloth was dipped in any one of the following flasks containing:

Using Ariel powder (diluted with)

- 25 ml of tap water (control)
- 20 ml of tap water and 5 ml of heated detergent (7 mg/ml),
- 20 ml of tap water and 5 ml of heated detergent (7 mg/ml) containing 25 % (v/v crude AlkPE).
- 20 ml of tap water and 5.0 ml of heated detergent 50 % (v/v crude AlkPE).
- 20 ml of tap water and 5.0 ml of heated detergent 75 % (v/v crude AlkPE).

Flasks were kept at 25 °C, 37 °C and 45 °C for 10 min followed by removal of the cloth pieces, and the leftover washes were used to determine the quantity of blood (hemoglobin) removed.

4 Data Analysis

Data generated from the optimization and application studies of alkaline protease were analyzed by using one-way analysis of variance (ANOVA) by BBD design. This Design analysis was done as stated in methodology stage the single and combined or interaction effect of the factors i.e. temperature, pH, concentration of ESM and incubation time were done. Analysis was stated in the form of graphical illustrations followed by description. Graphs are drown using origin pro8.0 and interaction effect was obtained from Design expert 7.0 software.

The results of the characterization studies of alkaline protease both on activity and stability were given by mean of two independent measurements and explained by graphs and columns using Origin pro 8.0 data analysis.

5 Results

5.1 Characterization of ESM

The moisture content, ash value, volatile matter was examined and their composition was 0.99%, 45.87%, 2.6 % and the fixed-carbon content of eggshell was determined by subtracting the percentages of moisture, volatile matter, and ash from a sample and it was obtained 50.54%. The moisture content has negative effect on the environment to cause odor and favorable for organisms to propagate, the ash content value and fixed carbon content, which contains Ca, Mg, K and trace minerals. The FT-IR result determines the presence of nutritional minerals of the *Bacillus subtilis Mojavensis*. Therefore, ESM can be used as substrate as well as inducer for production of AlkPE due to the presence of Ca and trace minerals.

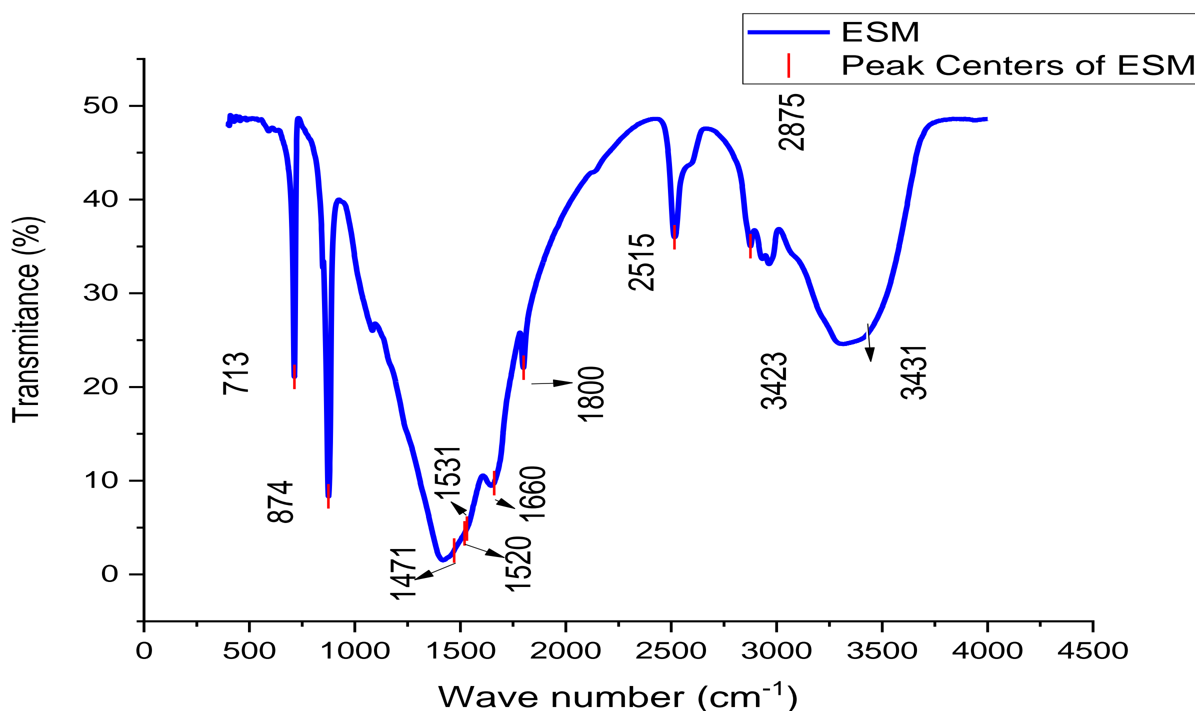


Figure 5-1. Fourier transform infrared (FT-IR) spectrum analyses of ESM

Form the FTIR analysis from the graph below it can be distinguished that in both spectra appears a prominent peak of carbonate CO_3^{2-} at 874 and 875 cm^{-1}respectivly. According to (Hariharan, et.al 2014) similar absorption peak for ESP is reported at 875 cm^{-1}. The presence of these bands justifies the carbonate content in ESP. Spectra of elements like Mg, Fe have a transmittance value between 1052-1520.

5.2 The effect of design factors on the production of alkaline protease

Effect of temperature and ESM concentration on production of AlkPE

As depicted on the graphs below single effect of the temperature a) and combined effect concentration of ESM b) on the production of alkaline proteas, as temperature increases maximum activity of alkaline protease was achieved 214.103 U/ml at a temperature 37 ^{0}C, pH 9 and ESM concentration of 20% and attain 157.066 U/ml and minimum activity of 130.803 U/ml was achieved at 30^{0}C and 45^{0}C.

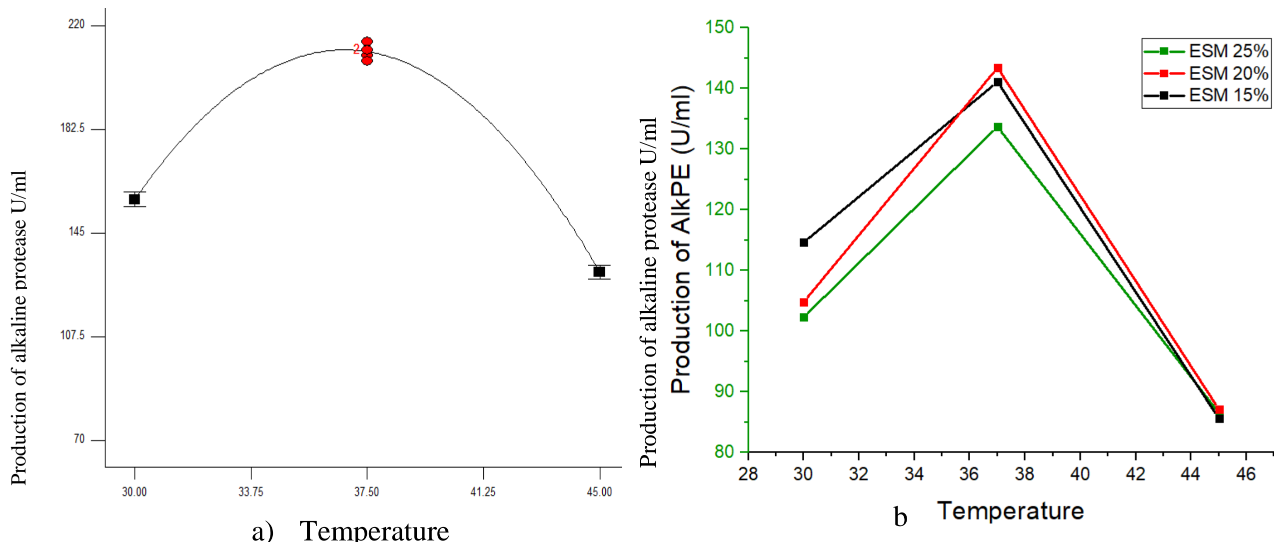


Figure 5-2. A) temperature effect b) temperature and ESM concentration on the production of AlkPE

Effect of ESM concentration on the production of alkaline protease

Effect of ESM concentration and temperature as shown in fig 12.b maximum alkaline protese activity was observed at 37 ^{0}C and at 20 % ESM concentration and at pH value of 9.0.

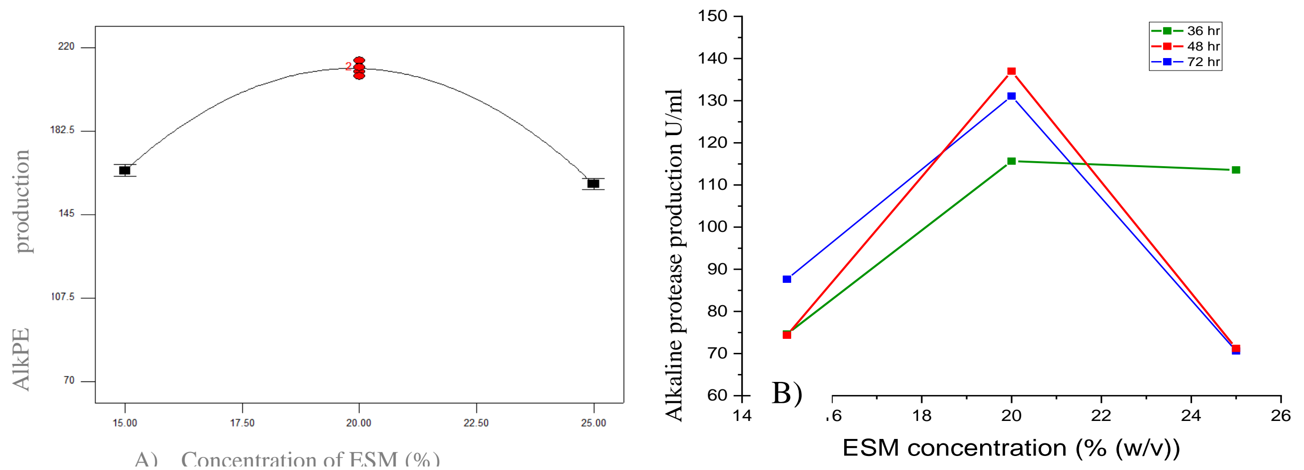


Figure 5-3. A) ESM concentration effect B) ESM & incubation time on the production of AlkPE

From the figure 5-3, we can conclude that production is increased as concentration of ESM and incubation time increases and reach maximum, thenafter it starts to decline. Maximum alkaline protease producton was achieved at 48 h incubation time and at 20% ESM concentration. Further more the experimental results showed that, at all concentration of ESM the maximum production was obtained at 48 h of incubation peiod.

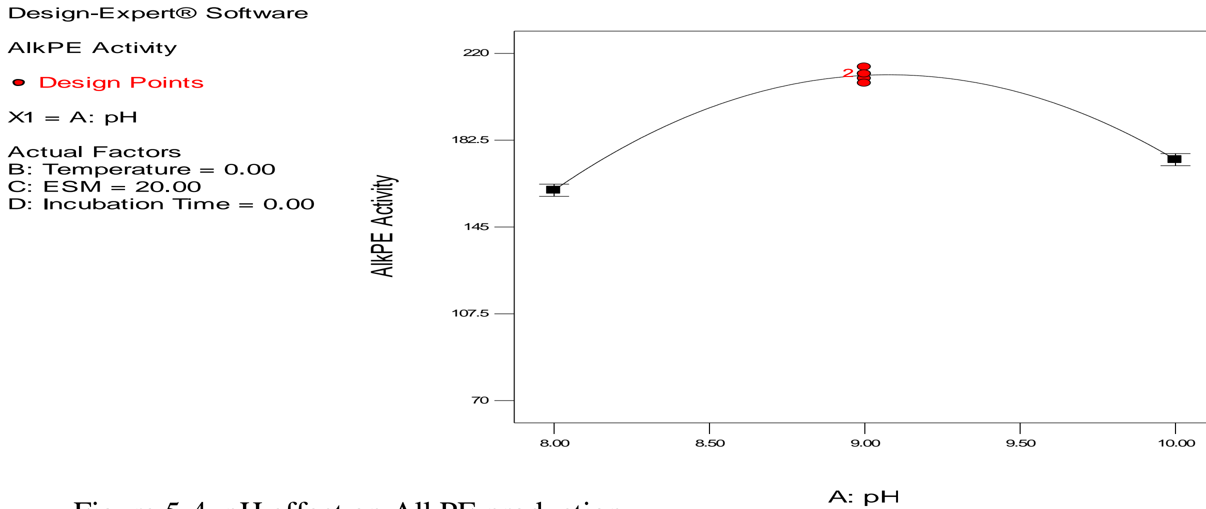


Figure 5-4. pH effect on AlkPE production

The graph indicates that, at middle level of other three factors as pH increases the productivity also increases. Moreover, over all interaction effect of all factors will be discussed after model evaluation.

5.3 ANOVA result analysis

5.3.1 Statistical Analysis of the Experimental Results

The resulting data obtained listed in Index-2, were analyzed using Design expert® 7.0 software to decide the effects of factors; pH, temperature, ESM concentration and incubation time. The dependent variable used as a response parameter was the production of alkaline protease enzyme. All experiments were carried out in a randomized order to minimize the effect of unexpected variability in the observed response due to extraneous factors. Design Summary for the production with four factors and three levels. The model is summarized as follows:

Table 5-1. Summary of factorial design

Study Type	Response Surface	**Runs**	29
Initial Design	Box-Behnken	**Blocks**	No Blocks
Design Model	Quadratic		

5.3.2 Development of Model Equation

The relationship between controllable experimental factors (pH, temperature, ESM, incubation time) was done using ANOVA and the response (AlkPE production production) and the interactions of the concerned variables was established and is given by the quadratic regression model equation in the form of coded factors.

Final equation in terms of coded factors:

AlkPE Activity = +210.50 + 6.61*A - 13.13*B - 3.03*C - 2.02*A*B - 7.01*A*C + 12.52*A*D + 5.40*B*C + 3.29*B*D + 32.40*C*D --- 5.1

Where A= PH, B= Temperature, C= concentration of ESM, D= Incubation time

ANOVA for response surface reduced quadratic model and analysis of variance listed in table 5-2.

Table 5-2. Summary of the ANOVA and parameter estimates for model coefficients of BBD

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F	Remark
Model	58237.48	12	4853.12	328.54	< 0.0001	significant
A-pH	523.73	1	523.73	35.46	< 0.0001	
B-Temperature	2069.29	1	2069.29	140.09	< 0.0001	
C-ESM	110.52	1	110.52	7.48	0.0147	
D-Incubation Time	31.58	1	31.58	2.14	0.1631	
AC	196.46	1	196.46	13.3	0.0022	
AD	627.03	1	627.03	42.45	< 0.0001	
BC	116.43	1	116.43	7.88	0.0126	
CD	4197.78	1	4197.78	284.18	< 0.0001	
A^2	12009.54	1	12009.54	813.01	< 0.0001	
B^2	28742.68	1	28742.68	1945.8	< 0.0001	
C^2	15465.5	1	15465.5	1046.97	< 0.0001	
D^2	20843.08	1	20843.08	1411.02	< 0.0001	
Residual	236.35	16	14.77			
Lack of Fit	209.15	12	17.43	2.56	0.1882	not significant
Pure Error	27.19	4	6.8			
Cor Total	58473.83	28				

Std. Dev.	3.84	R-Squared	0.9960
Mean	121.49	Adj R-Squared	0.9929
C.V. %	3.16	Pred R-Squared	0.9854
PRESS	852.78	Adeq Precision	55.403

Model adequacy check

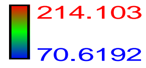
The fitness of the model was checked using values of p, F and regression coefficients. The Model F-value of 328.54 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

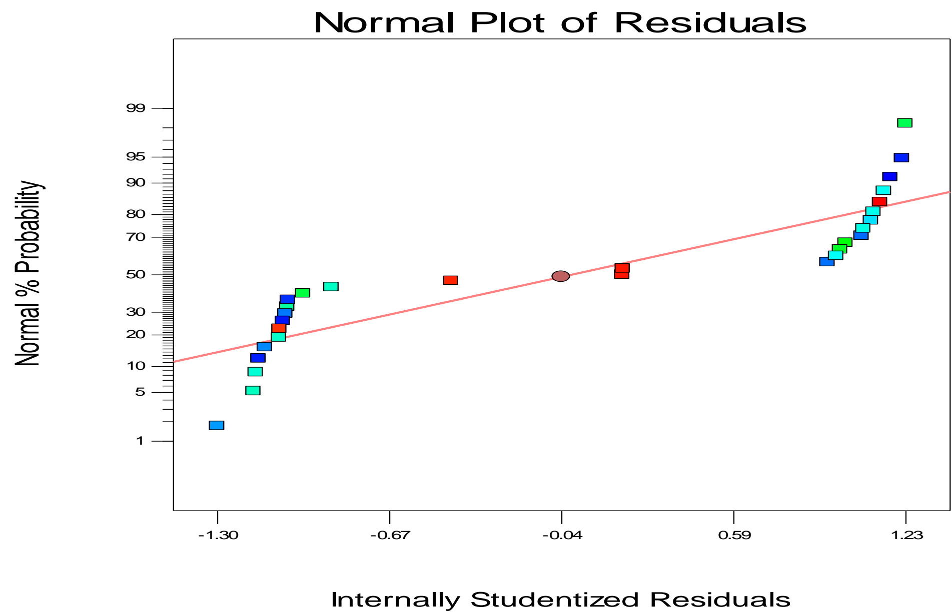
Values of "Prob > F" less than 0.0500 indicate model terms are significant. The contribution of each source terms for the model equation could be seen from table 5-2. Obviously, the model is significant as it has a lower p value of 0.0001 and a higher R^2 value of 0.9960. In general, the model is good enough as the R^2 value showed that 99.6% of the variations of the experiment could be accounted by the model. In this case A, B, C, AC, AD, BC, CD, A^2, B^2, C^2, D^2 are significant model terms. Values greater than 0.1 indicate the model terms are not significant model terms. There fore, not counting those insignificant model terms helps, in model reduction which inturn improove the model adequecy. The "Lack of Fit F-value" of 2.56 implies the Lack of Fit is not significant relative to the pure error. There is a 18.82% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good the model to fit, sothen, it is good for the qudratic model to be significant.

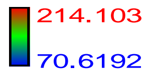
Table 5-3. Lack of fit test for each source of terms

Sum of		**Mean**	**F**	**p-value**	**Remark**	
Source	**Squares**	**df**	**Square**	**Value**	**Prob > F**	
Linear	55711.51	20	2785.58	409.76	< 0.0001	
2FI	50514.12	14	3608.15	530.76	< 0.0001	
Quadratic	149.46	10	14.95	2.20	0.2328	Suggested
Cubic	0.12	2	0.060	8.887E-003	0.9912	Aliased
Pure Error	27.19	4	6.80			

Lack of fit test: Want the selected model to have insignificant lack-of-fit. From the diagnosis graphs the actual versus predicted values using model in the equation 4 can be seenas follows.







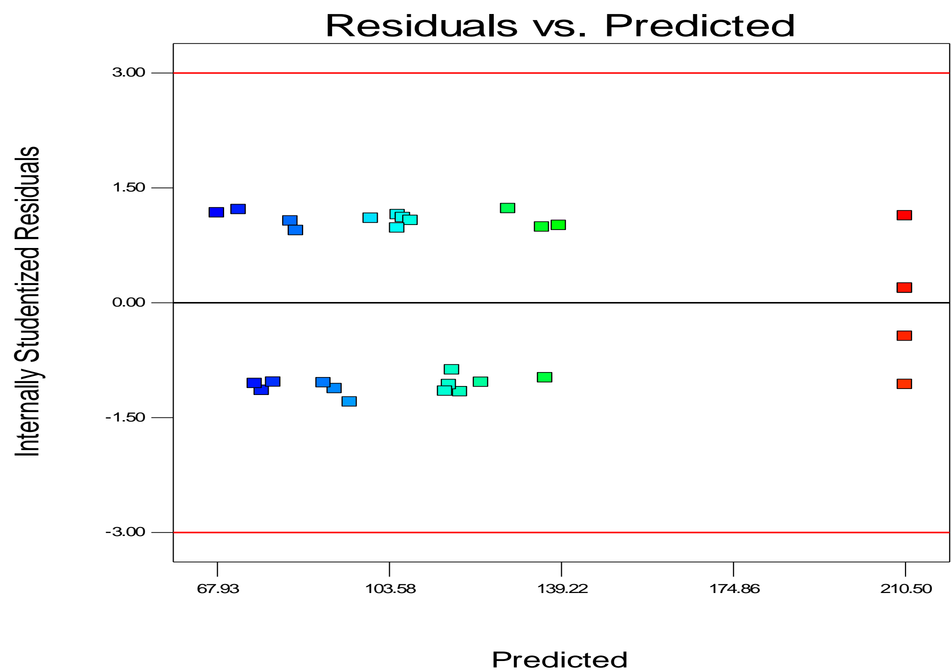
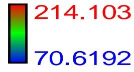
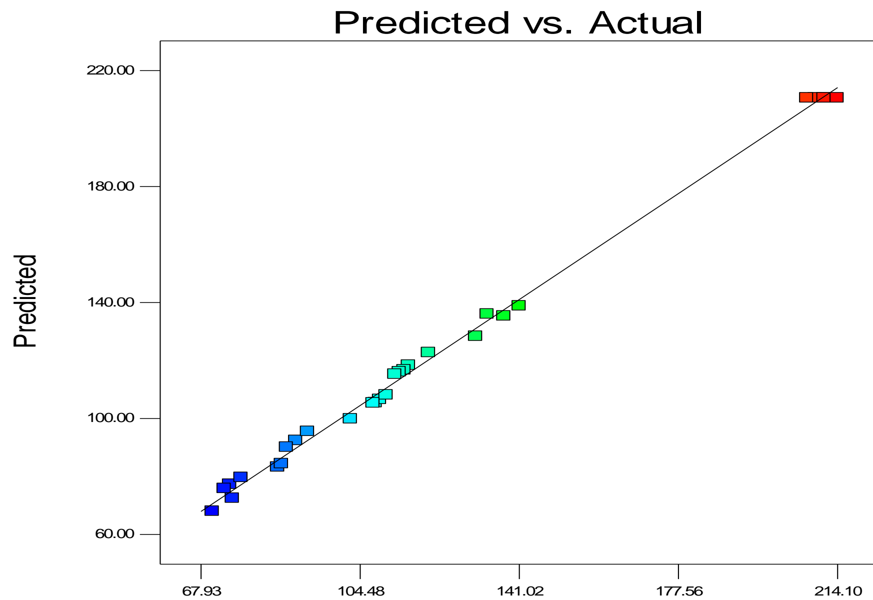
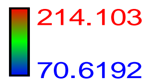


Figure 5-5. Normal and residual vs. Predicted

As shown abovein fig 5-5, the normal probability plot indicates the residuals following a normal distribution, in the case of this experiment the points in the plots shows fit to a straight line in the figure especially maximum values. this shows that the quadratic model satisfies the assumptions analysis of variance (ANOVA) i.e. the error distribution is approximately normal.







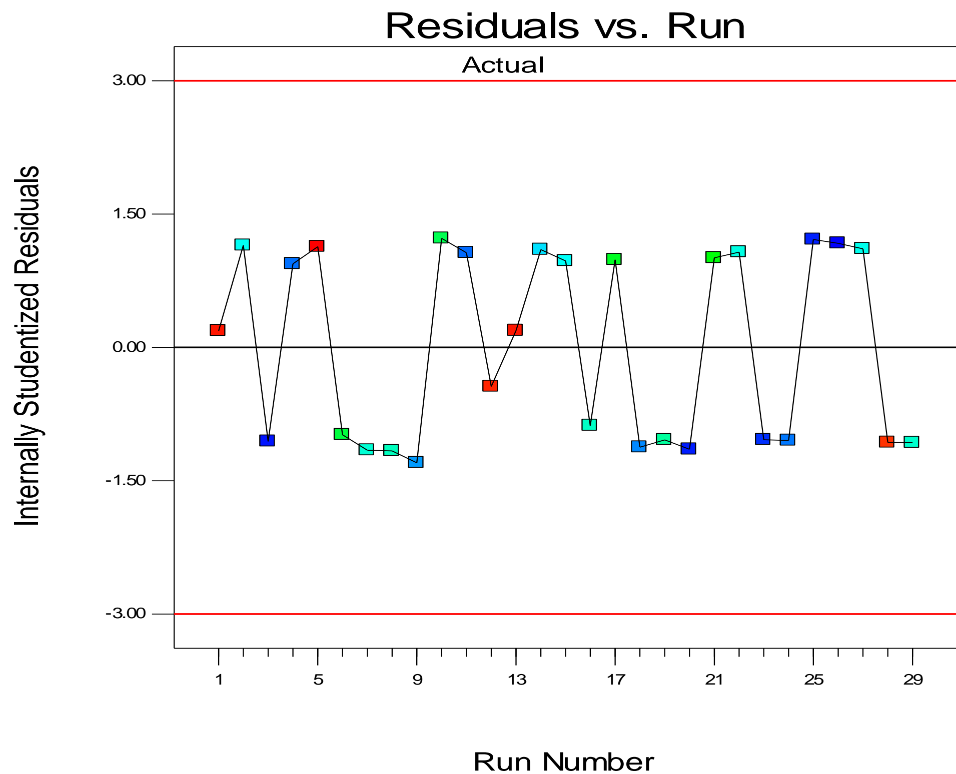


Figure 5-6. Predicted vs. Actual & residual vs. Run

A graph of the actual response values versus the predicted response values. It helps to detect a value, or group of values, that are not easily predicted by the model. The data points should be split evenly by the 45-degree line. Therefore, the model definitely can easily predict the data points.

If the model is correct and the assumptions are satisfied, the residuals should be structure less; in particular, they should be unrelated to any other variable including the predicted response. Simple check is to plot the residuals versus the fitted (predicted) values. A plot of the residuals versus the rising predicted response values tests the assumption of constant variance. The plot shows random scatter which justifying no need for an alteration to minimize personal error.

5.3.3 Interaction effect of the factors

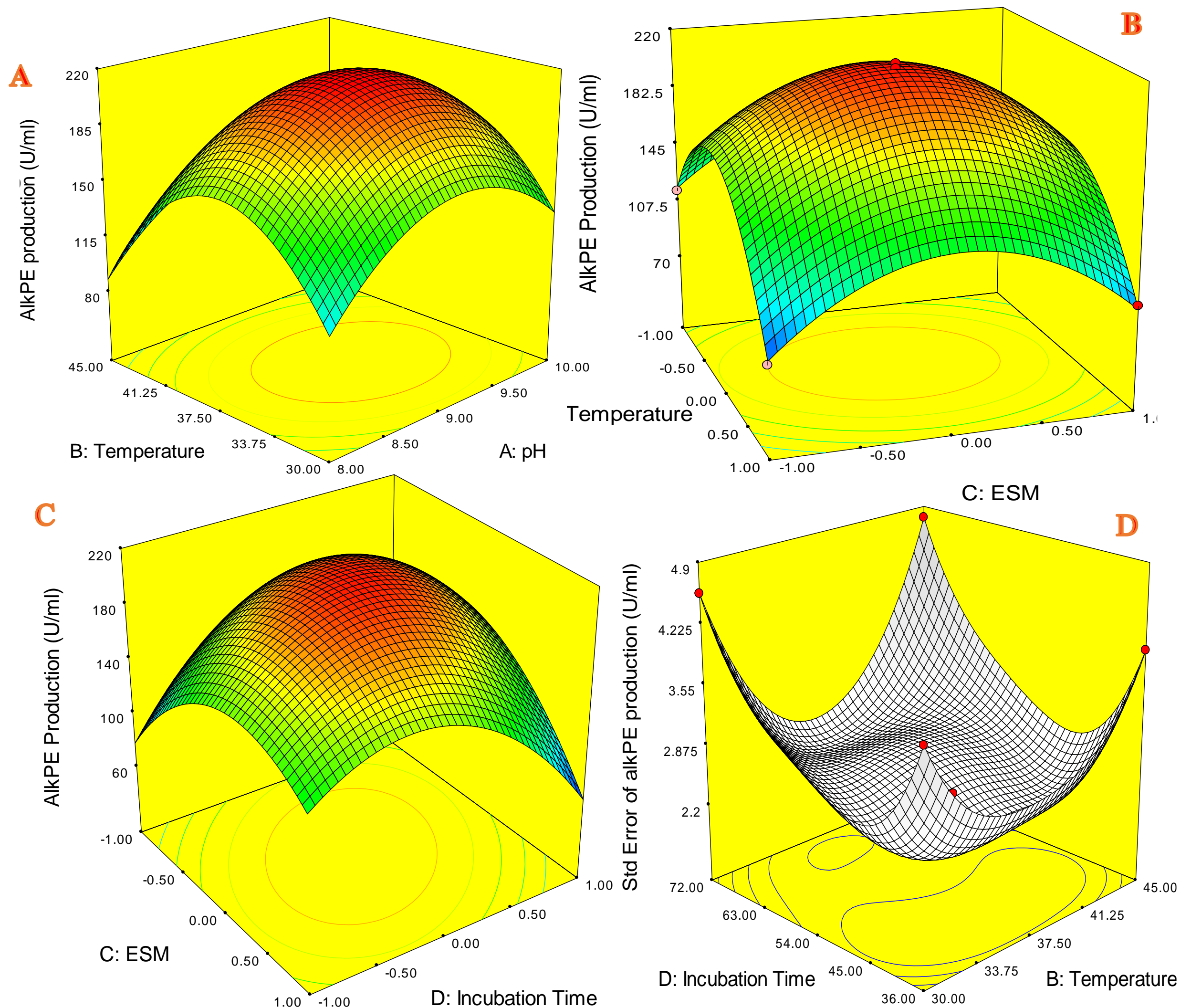


Figure 5-7. The interactive effect of temperature and pH on protease production a) temperature vs pH, b) temperature vs ESM concentration, c) ESM concentration vs incubation time and d) standard error between factors of incubation and temperature.

The codede numbers represented as follows:

Table 5-4. Coded and actual factors

Factor	Name	Units	Low Actual	Middle Actual	High Actual	Low Coded	Middle Coded	High Coded
A	pH	pH scale	8	9	10	-1	0	1
B	Temperature	^{0}C	30	37	45	-1	0	1
C	ESM	Conc %	15	20	25	-1	0	1
D	Incubation Time	h	36	48	72	-1	0	1

The 3D response surface plot for the interaction of the design factors summarized as follows:

A: The combined effect of pH and temperature on the production of alkaline protease at most of the design points of the two factors. Maximum alkaline protease was attained at 37^{0}C and pH of 9.0. Meanwhile, at a temperature of 30^{0}C and pH of 8 and 30^{0}C and pH 10 optimum production of AlkPE was observed. However, at 45^{0}C and pH of 10 production was minimum.

B) the maximum temperature and ESM interaction effect was observed at midpoint of the design factor levels, while at the extreme point of interaction was recorded as low level of production.

C) effect of interaction between the concentration of ESM and incubation time of the fermentation medium, shown. For both factors as we go from the lower level to the mid-point, increase in the production was observed. Optimum production was attained at 20% and 48h of ESM concentration and incubation time respectively. Again, when the level value increases from the average or med-point value production is becoming lower and lower.

5.4 Optimization of process parametrs

Optimization for pH, temperature, ESM concentration and incubation time was summarized as follows.

Table 5-5. Goals of optimization and its range

Parameters	Goal	Lower Limit	Upper Limit	Lower Weight	Upper Weight	Importance
pH	Is in range	8	10	1	1	3
Temperature	Is in range	30	45	1	1	3
ESM	Is in range	15	25	1	1	3
Incubation Time	Is in range	-1	1	1	1	3
AlkPE Activity	Maximize	70.6192	214.103	1	1	5

Using design expert 7.0.0 one solution was found for alkaline protease enzyme production.

Table 5-6. Optimum points with its desirability for the combined effects

Number	pH	Temperature	ESM	Incubation Time	AlkPE Activity	Desirability	
1	9.08	36.74	19.78	0	211.5076	0.981911	Selected

This section summarizes the criteria that was set for this optimization run.

To find solutions numerical optimization look through all the given solutions to see which ones best meet the needs to optimized. The best solution for your goal may not be the first one on the list, due to cost, efficiency, practicality, etc. Desirability range from zero to one for any given response, the program combines individual desirability into a single number and then searches for the greatest overall desirability. A value of one represents the ideal case. A zero indicates that one or more responses fall outside desirable limits.

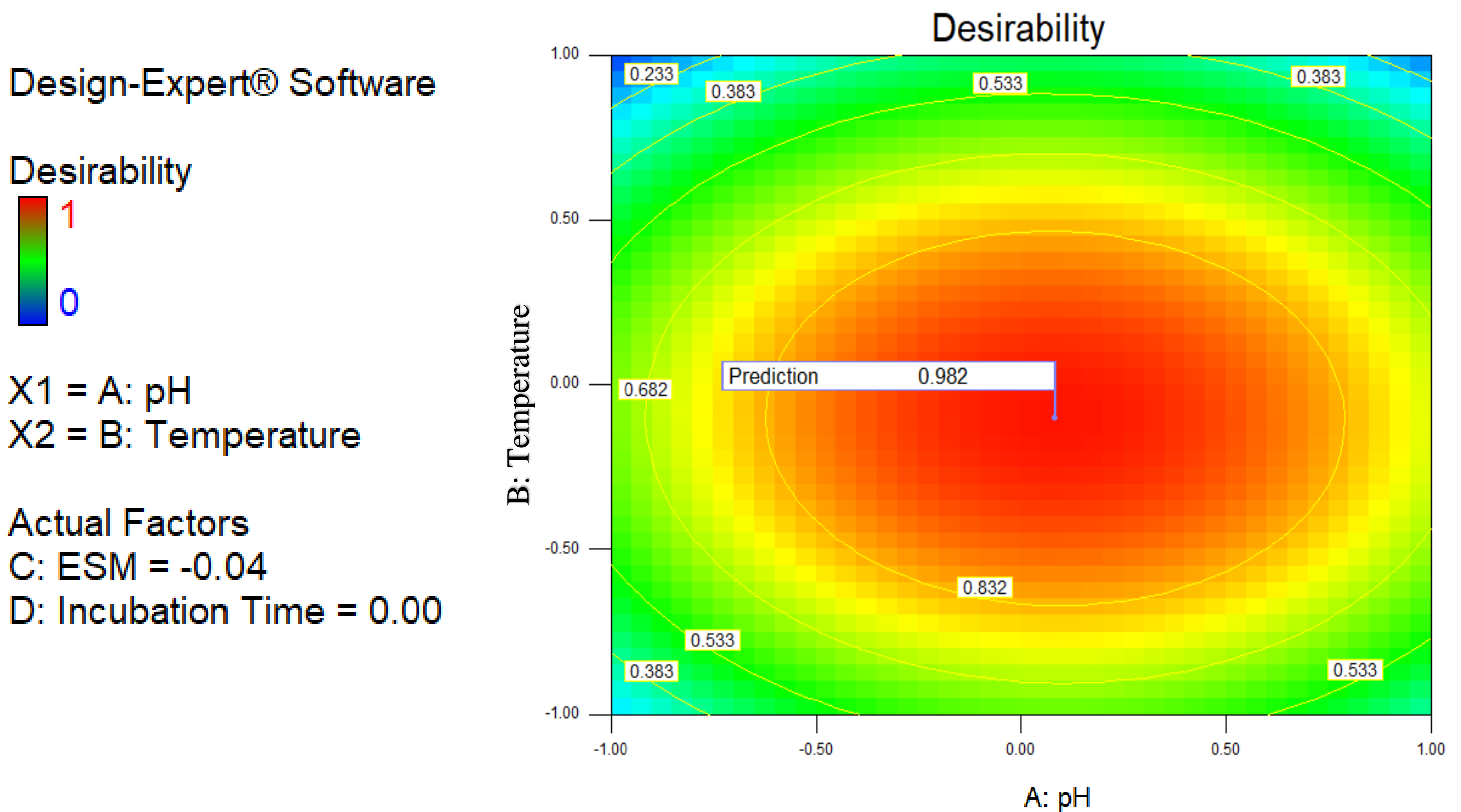


Figure 5-8. Desirability of the interaction effect based on the optimum solution

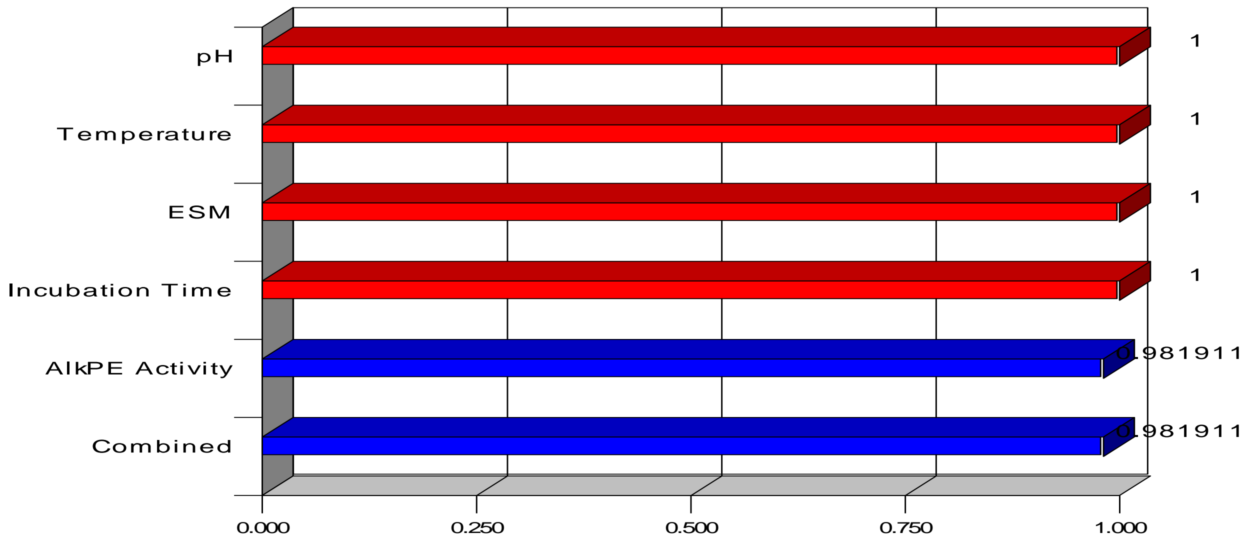


Figure 5-9. Desirability on response & combined based on the optimum solution

From the graph 5-8 & 5-9 the numerical optimization desirability is 0.9819 which is nearest to the ideal case & indicates the responses falls in the desirable limit. i.e. the optimum point was at a temperature of 36.74 ^{0}C, pH of 9.08, ESM 19.78 % and incubation time of 48 h. Hence, the maximum production or activity was attained at Std order 27 or Run order 5 (at the middle level of the factor levels), the optimized points are closer to the maximum production parameters but, with some optimized parameters.

5.4.1 Validation of the model

As we discussed in section 5.3.2 the model is fit and also considered to be accurate and reliable for predicting the activity of alkaline protease production using egg shell and membrane, at different oprearating parameters. Based on the second-order models, numerical optimization was carried out to maximize the AlkPE production, using the response optimizer in Design expert®7.0.0. The optimal values of the parameters were reported on table 5-6 pH of 9.08, a temperature of 36.74, concentartion of ESM 19.78% and an incubation time of 48 h. The analysis shows 211.51 U/ml of AlkPE was found with a diserability of 0.9819, which is strongly predictable.

5.5 Physico-chemical characterization of crude Alkaline protease

5.5.1 Effect of enzyme concentration on enzyme activity

The effect of dilution of alkaline protease on the proteolytic activity was determined. Then casein hydrolysis was determined by following standard protease assay procedure for its activity and obtained the following activity values.

Table 5-7. Activity of crude AlkPE at different dilution ratio

Alkaline Protease (µl)	Glycine-NaOH buffer (µl)	Ratio	Enzyme activity
1000	1000	1:1	43.23226
200	1000	1:5	162.8774
100	1000	1:10	229.4903
50	750	1:15	83.84516
50	1000	1:20	49.78539

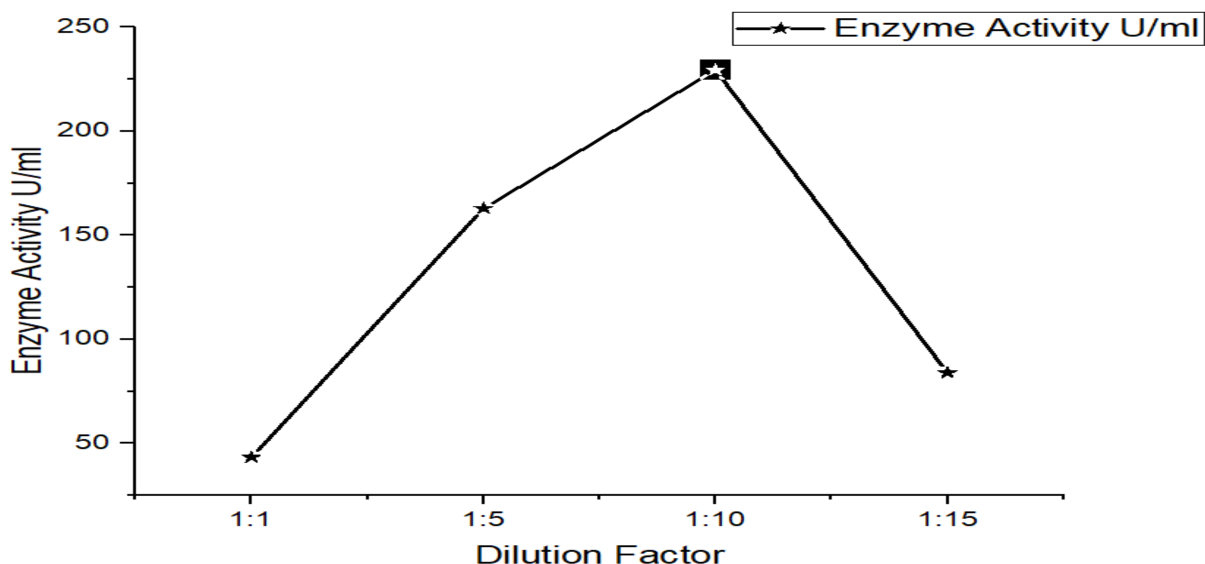


Figure 5-10. Dilution factor on enzyme activity

As shown in fig 5-9 as the dilution ratio increases activity also increases until dilution ration of 1:10, then it starts to decrease. For more conveniences all the assay protocols were done using a dilution factor of 1:5.

5.5.2 Effect of pH on activity and stability

AlkPE (alkaline protease enzyme) was active in a broad pH range of 6.0-12.0 with 87.495 % of its activity was retained. It showed that more than 93.97 % its original activity was retained using

buffers pH ranging from 8-10, with the optimum activity at pH 10.0 as indicated by the peak in figure 5-10.

The activity of AlkPE alkaline protease was found to increase progressively with the increase in pH range from 6.0 to 10.0, however beyond pH 10.0 it drops sharply in activity. AlkPE showed 89.24%, 99.16% and 87.49% of its maximal activity at pH 8.0, 9.0 and 11.0 respectively. Minimum relative activity was recorded at pH 6.0(76.57%) and pH 12.0(79.37% %).

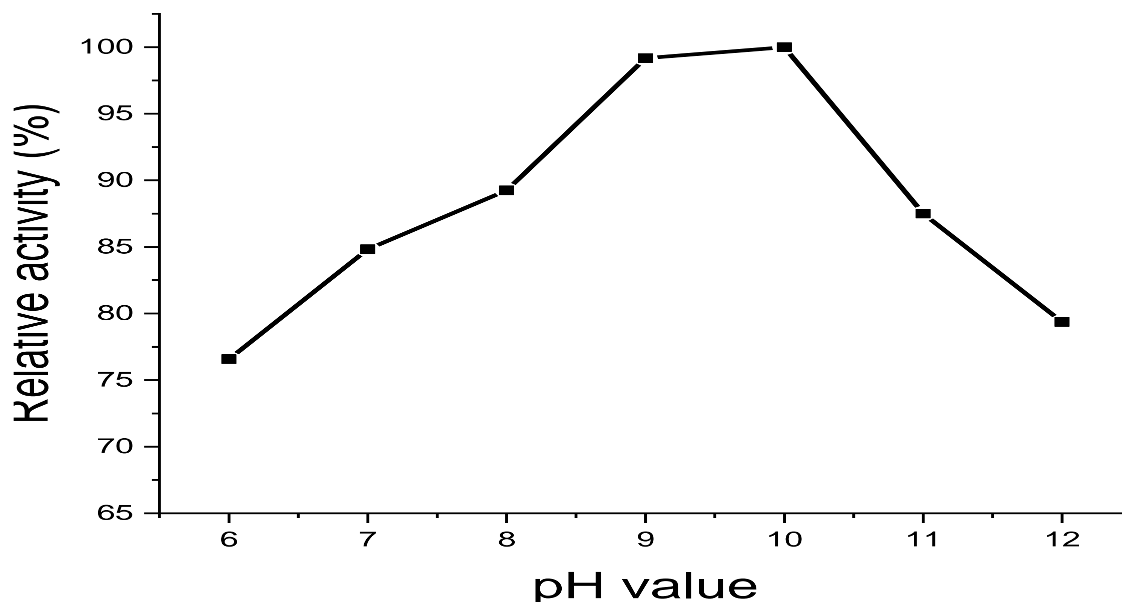


Figure 5-11. pH profile of alkaline protease enzyme

When assay reaction mixture was at pH 10.0, it shows increment from pH (6-8) then it is stable and maximum enzyme activity was obtained between pH values of (8-10). Within the stability zone with more than 94 % residual activity was retained. Beyond pH value of 10.0, it slightly decreases and at pH 12 it reaches 80%. Overall activity was more than 80% original activity was retained within the pH range of (8-10) were, 86%, 100% and 99 and 100% of its original activity was retained at pH 9.0.

When assay mixture was pH 8 pH stability profile shows increment from pH (6-8) and shows stability zone from pH value of 8 to 10 with more than 95% residual activity was retained. Beyond pH 10.0 it starts to decrease and reach 76% of residual activity. Overall alkaline protease enzyme was stable between pH 8.0-10.0 attain 90%, 100% and 94% of residual activity. Its minimum residual activity was attained at pH value of 6.0 which is 39.6% of its original activity.

As shown in stability profile alkaline protease enzyme at both assaying buffers shows a stability profile between pH values of 8-10. This means most of industrial alkaline process works at alkaline medium i.e. at pH 8 and 10 this AlkPE is best fits these requirements.

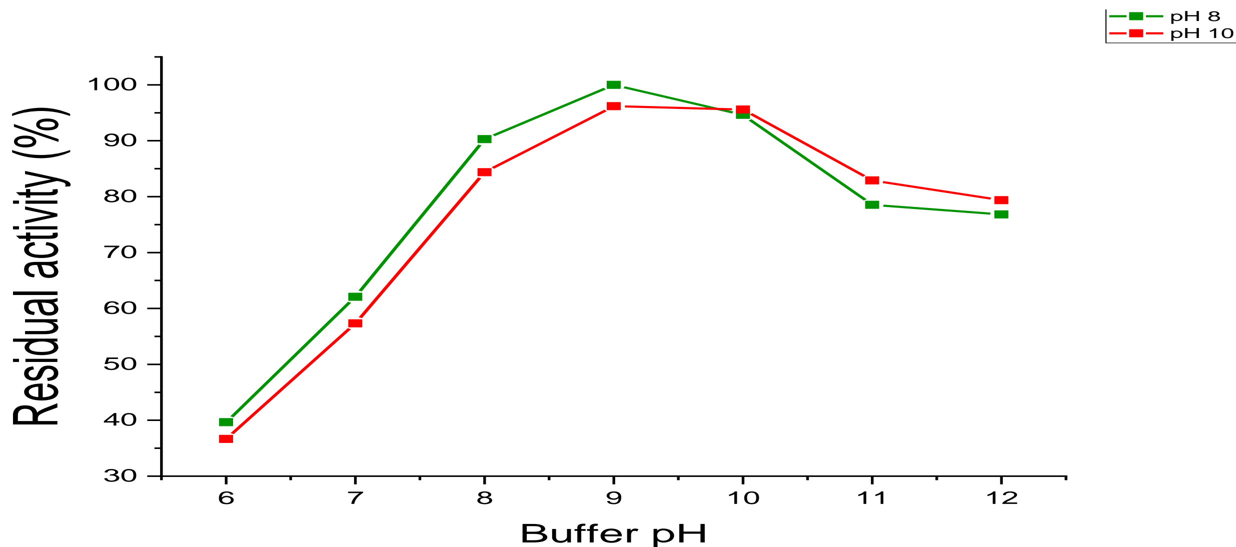


Figure 5-12. PH stability profile of BSM alkaline protease

5.5.3 Effect of temperature on activity and stability of the protease

Following the standard protease assay protocol for the temperature range of 30 ^{0}C– 70 ^{0}C measure the activity and calculate the relative activity (%) relative to the maximum value.

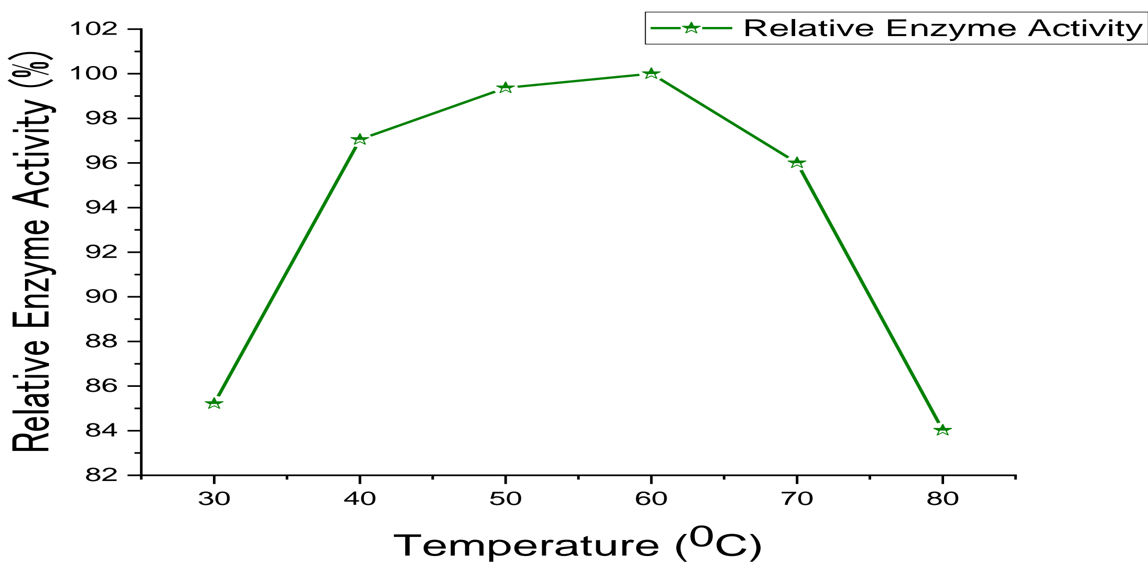


Figure 5-13. Temperature profile of BSM alkaline protease

The BSM alkaline protease was found to be very active at all temperatures tested between 30 ^{0}C and 80 ^{0}C with maximum activity at 60 ^{0}C as shown as peak in Figure 5-13. The activity of protease progressively increased as temperature increased from 30 ^{0}C to 60 ^{0}C and then gradually decline beyond its optimum temperature. Within temperature range of 40^{0}C -70^{0}C, BSM alkaline protease retained more than 74% of its maximum activity. The minimum relative alkaline protease was found at 80 ^{0}C and 30 ^{0}C, it attains 84% and 85% respectively of its maximum activity.

For investigating temperature effect on stability of BSM alkaline protease, incubating the enzyme assay mixture for 1h prior to assaying at 40^{0}C, 45^{0}C, 50^{0}C, 55^{0}C and 60^{0}C.

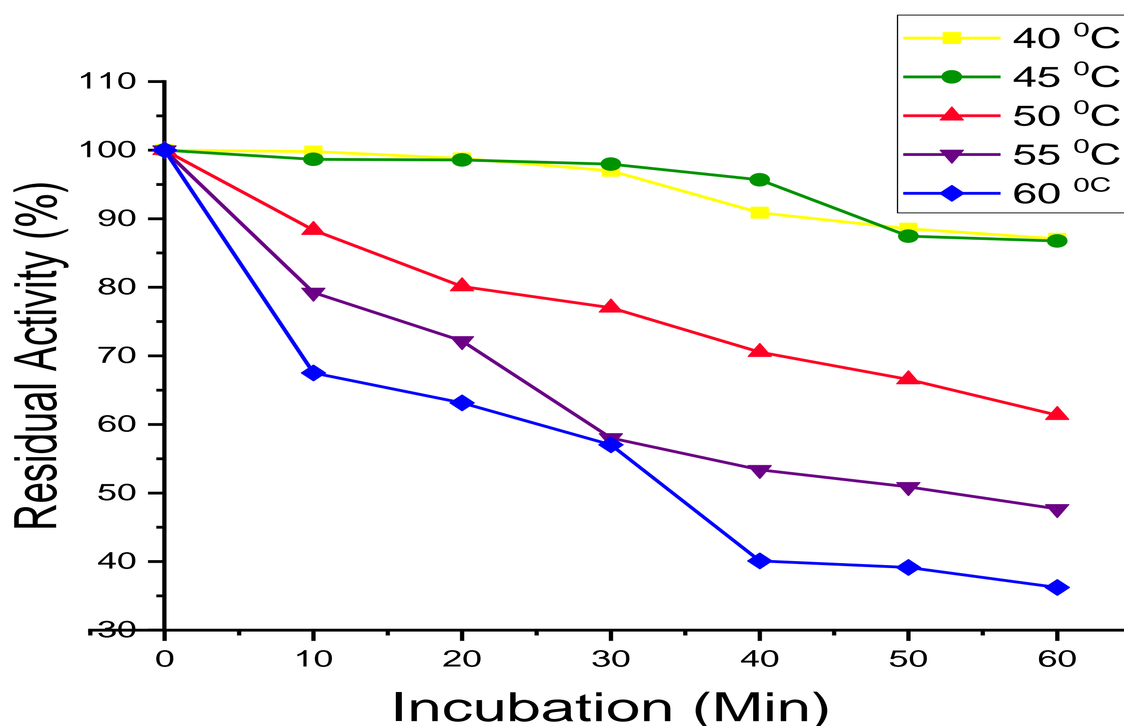


Figure 5-14. Thermal stability profile of BSM alkaline protease Ph

Thermal stability of BSM alkaline protease was tested at pH 10.0 as shown in Figure 5-14. The protease showed maximum thermal stability at 10-40 min 96% and 97% of residual activity for assaying at 40 ^{0}C and 45 ^{0}C respectively at 1h of incubation. After 40 min incubation for assaying temperatures of 40 ^{0}C, 45 ^{0}C, 50 ^{0}C, 55 ^{0}C and 60 ^{0}C, the residual thermal stability sharply drops 86%, 85%, 65%, 49% and 39% and beyond 50 ^{0}C becomes inactivated at 1h of incubation. Relatively higher thermal stability of BSM alkaline protease was obtained at temperatures 40 ^{0}C and 50 ^{0}C, 99% and 98% of residual activity respectively.

5.5.4 Effect of Sodium Chloride on activity and stability

Maximum alkaline protease activity was obtained at 1.5 M and 2M NaCl at pH 8.0 (98.87%) and 10.0 (99.25%). From 1M to 2.0M of NaCl, exhibits > 97% of optimum activity was recorded at pH 8 and 10. Even at highest concentration of NaCl (3M), BSM alkaline protease showed 89% and 90% of the relative optimum activity at pH 8 and 10. Yet at both tested pH range, further

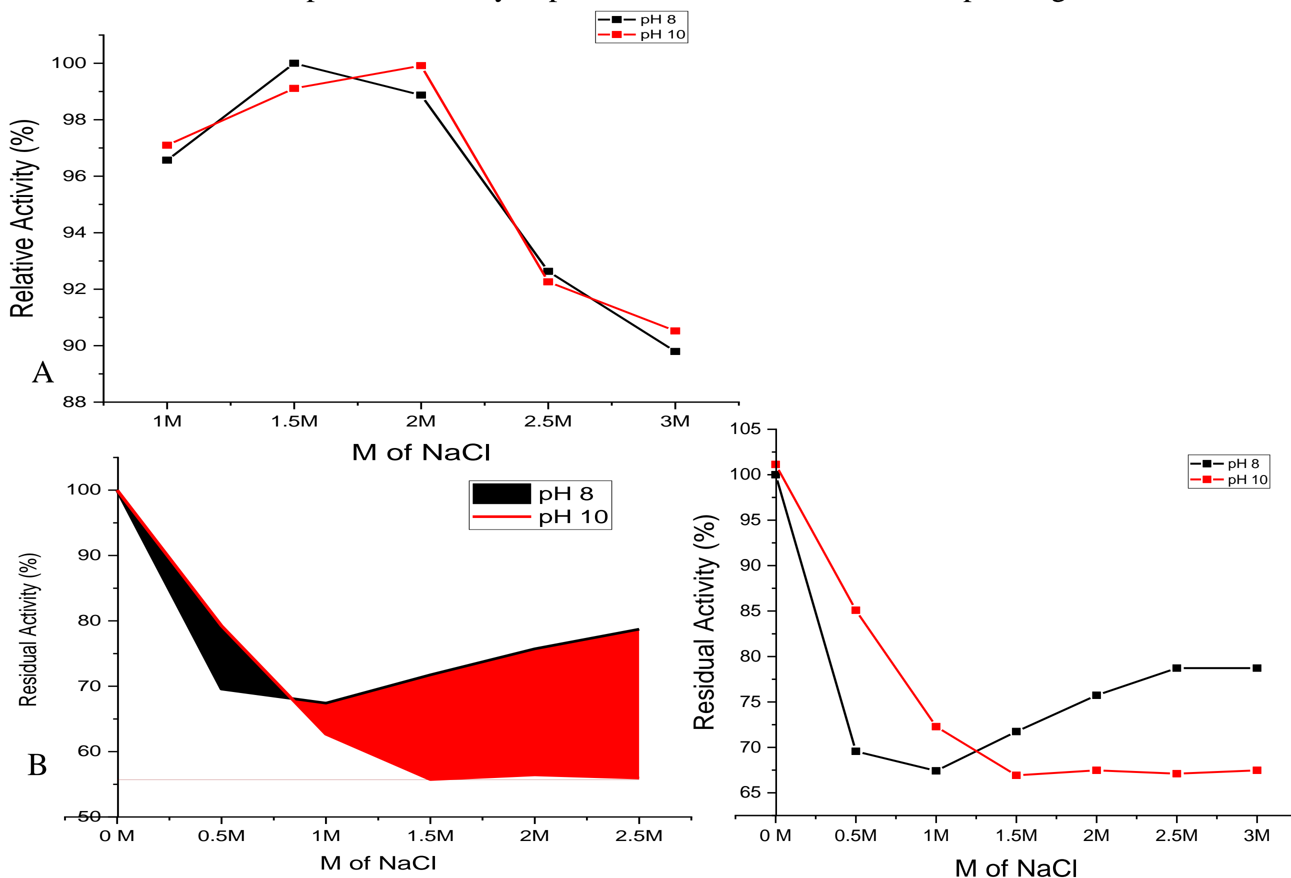


Figure 5-15. (a) the effect of [NaCl] on activity of BSM alkaline protease. (b) the effect of [NaCl] on stability of BSM alkaline protease

increasing in NaCl concentration above 1.5M for pH8 and 2M for pH 10 led to reduction in activity. In addition, at all studied concentrations of NaCl, comparatively higher relative activities. At each point of tested concentrations of NaCl, for pH 10 sharply decreases on the stability of BSM alkaline protease between 0.5-1.0 M with 70.9% residual activity was retained. However, for pH 8 it comparably stable between 0.5-1.0 M with residual activity of 68.5% was attained. As shown in fig (5-15 b) [NaCl] increases 1M – 3M After 1h of incubation at pH 10, residual activity was decreased and attain minimum value of 56% of residual activity was obtained. Whereas; optimum stability was attained at these concentration range between 1-3.0 M NaCl. At pH 8

residual activity were raising as concentration increases from1.0M, 1.5M, 2M, 2.5M and 3.0M from 67.4%, 71.7%, 75.7%, 78.7%, 78.7 % respectively of residual activity was recorded.

From the relative activity graph (fig 5-15. b) we can conclude that, with increasing concentration of NaCl, BSM alkaline protease is more stable in the range of 1.0M-3.0M at pH 10 and 55.7%, 56.4%, 55.9% and 56.4% residual activity was recorded. For pH 8 despite the rise in residual activity, between 1.0M-3.0M it is not stable at these range because of fluctuation. Relatively higher residual activity or halo-stability was obtained at pH 10.0 than 8.0.

5.5.5 Blood Stain Removal

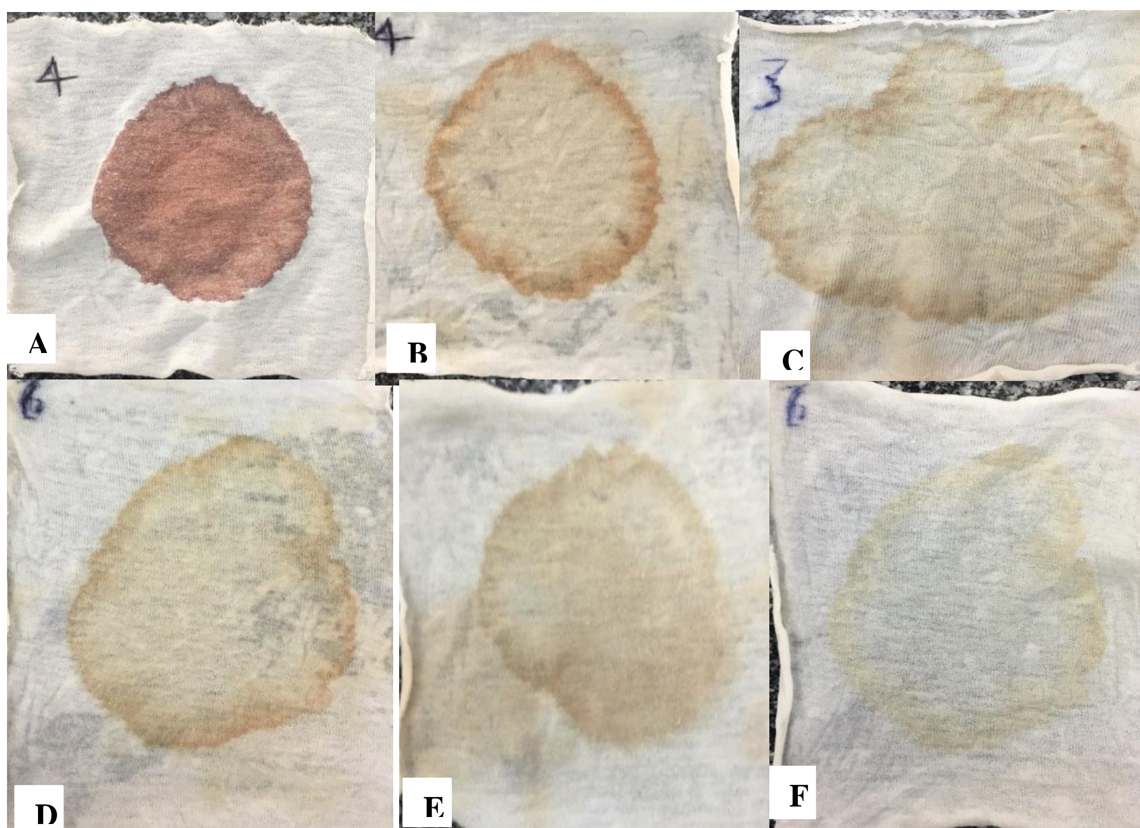


Figure 5-17. Blood stain removal: A) blood stain, B) treated with tap water C) treated with 100% detergent, D) treated with detergent (25% enzyme), E) treated with detergent (50% enzyme), F) treated with detergent (75% enzyme)

From figure 5-17 we can conclude that, the use of different concentration of enzyme were used to treat the blood stain. as we seen clearly there is a remarkable degradation of blood was occurred. The use of alkaline protease for blood stain removal reveals that the use of AlkPE for detergent applications.

6 Discussion

Now a day’s environmental pollution was the head ache of the world governments. In this regard the reuse of waste materials and waste free products and production process are developing. About 30 – 40% of enzyme production cost goes to substrate, the utilization of these resources must be used efficiently and effectively. *Bacillus subtilis mojavensis* was examined for the production of alkaline protease by (Haddar et al., 2010). Though, the use of ESM induces the production of alkaline protease by utilizing medium components effectively (Nagamalli et al., 2017). Egg shell and membrane is pollutant since it is not degradable, cause nuisance to the environment. The use of ESM for the production of alkaline protease was aimed at producing higher quantity of alkaline protease together with minimizing the waste egg shell as a substrate. This in fact, is two birds with one stone it doesn’t have uses like agricultural lignocellulosic materials and intern it used in the production of AlkPE.

In the selection of microorganism bacillus subtilis was found to be the most prominent microbe to produce different industrial enzymes (Elander, 2003; Wang et al., 2017), its sub species strain *Bacillus subtilis mojavensis* was studied in this thesis project. Incubation time for *Bacillus.s.m.* between 24 to 72 h, the pH and temperature range is in the range from 6-11 and 25-50^{0}C (Mukherjee & Rai, 2011). The investigation was conducted at pH of (8, 9 and 10) and temperature of (30, 37 and 45), ESM concentration (15%, 20% and 25%) and incubation time of (36h, 48h, and 732h) were investigated. Maximum alkaline production was obtained at, pH of 9.0, temperature of 37 ^{0}C, ESM concentration of 20% and 48 h of fermentation time.

Optimization of fermentation process parameters is done to establish favorable conditions to the growing microorganism and thereby minimize unutilized components at the end of fermentation. No defined medium has been established for the optimum production of alkaline proteases from different microbial sources including *Bacillus subtilis mojavensis*. Each organism or strain has its own special conditions and requirements for maximum enzyme production (Bushell, 2003; Elander et al., 2017). To find solutions numerical optimization look through all the given solutions to see which ones best meet the needs to optimized. The best solution for the production of AlkPE may not be the first one on the list, due to cost, efficiency, practicality, etc. Desirability range from zero to one for any given response, the program combines individual desirability into a single

number and then searches for the greatest overall desirability. A value of one represents the ideal case. A zero indicates that one or more responses fall outside desirable limits.

From the graph 5-8 & 5-9 the numerical optimization desirability is 0.9819 which is nearest to the ideal case & indicates the responses falls in the desirable limit. i.e. the optimum point was at a temperature of 36.74 ^{0}C, pH of 9.08, ESM 19.78 % and incubation time of 48 h. Hence, the maximum production or activity was attained at Std order 27 or Run order 5 (at the middle level of the factor levels), the optimized points are closer to the maximum production parameters but, with some optimized parameters.

The same experiment was done one without and with ESM (20% w/w) and finally revealed that, production of alkaline protease was higher using ESM than without ESM containing medium. According to (Nagamalli et al., 2017) using ESM as a substrate induces the production of AlkPE more than without employing ESM. The maximum alkaline protease was found without the use of ESM is 136.37 U/ml and employing ESM the activity is 214 U/ml. from this it is easily noticed that, using ESM as a substrate have made higher activity than without employing ESM as a substrate this has confirmed the effect of using ESM on the production of AlkPE.

Characterization of alkaline protease was done at different parameters as shown from section 3.3.3 to 3.3.6. Alkaline protease found to be active and stable in the following parameters:

Factor of dilution was studied between dilution ratio 1:1, 1:5, 1:10, 1:15 and 1:20 were assayed and maximum result obtained at 1:10 dilution factor. All assays were done with a dilution factor of 1:5.

The BSM alkaline protease was found to be very active at all temperatures tested between 30 ^{0}C and 80 ^{0}C with maximum activity at 60 ^{0}C the protease showed maximum thermal stability at 10 to 40 min 96% and 97% of residual activity for assaying at 40^{0}C and 45 ^{0}C respectively at 1h of incubation. Thermal stability of BSM alkaline protease was tested at pH 10.0 as shown in Figure 5-15. The protease showed maximum thermal stability at 10-40 min 96% and 97% of residual activity for assaying at 40 ^{0}C and 45 ^{0}C respectively at 1h of incubation. After 40 min incubation for assaying temperatures of 40 ^{0}C, 45 ^{0}C, 50 ^{0}C, 55 ^{0}C and 60 ^{0}C, the residual thermal stability sharply drops 86%, 85%, 65%, 49% and 39% and beyond 50 ^{0}C becomes inactivated after 1h of incubation. Similarly (Amare et al., 2003) reported that incubating at higher temperatures

for more than 1 h will denature the enzyme and thermal stability decreases and (Marathe et al., 2018) maximum activity was attained at pH 10 and 55 ^{0}C to 60 ^{0}C. Relatively higher thermal stability of BSM alkaline protease was obtained at temperatures 40 ^{0}C and 50 ^{0}C, 99% and 98% of residual activity respectively. This result was reported by (Kiranmayee & M, 2016)

The pH effect on activity and stability result has shown that, more than 93.97 % its original activity was retained using buffers pH ranging from 8-10, with the optimum activity at pH 10.0. AlkPE (alkaline protease enzyme) was active in a broad pH range of 6.0-12.0 with 87.495 % of its activity was retained. It showed that more than 93.97 % its original activity was retained using buffers pH ranging from 8-10, with the optimum activity at pH 10.0 as indicated by the peak in figure 5-10. This result converges to (Amare et al., 2003;Kumar et al., 1999). The activity of AlkPE alkaline protease was found to increase progressively with the increase in pH range from 6.0 to 10.0, however beyond pH 10.0 it drops sharply in activity. AlkPE showed 89.24%, 99.16% and 87.49% of its maximal activity at pH 8.0, 9.0 and 10.0 respectively. Minimum relative activity was recorded at pH 6.0(76.57%) and pH 12.0(79.37% %). This result shows in any industrial process most of alkaline media has a pH range between 8-10, therefore the result indicates the ability to be utilized in detergent as well as industrial process and not efficient to use under acidic environment. When assay reaction mixture was at pH 10.0, it shows increment from pH (6-8) then it is stable and maximum enzyme activity was obtained between pH values of (8-10). Within the stability zone with more than 94 % residual activity was retained. Beyond pH value of 10.0, it slightly decreases. This result is similar to (Kiranmayee & M, 2016). As shown in stability profile alkaline protease enzyme at both assaying buffers shows a stability profile between pH values of 8-10. The enzyme, used in this study, was found to have similar pH stability to commercially important detergent enzymes. (Kiranmayee & M, 2016; Swapna & Parcha, 2014). As shown in stability profile alkaline protease enzyme at both assaying buffers shows a stability profile between pH values of 8-10.

NaCl concentration on activity and stability was found such that, from 1M to 2.0M of NaCl, exhibits > 97% of optimum activity was recorded at pH 8 and 10. This result was concedes with (Rao & Narasu, 2007) they described that, between NaCl concentration range [0 - 3] at 2 M maximum activity was achieved. Whereas; optimum stability was attained at these concentration range between 1-3.0 M NaCl. At pH 8 residual activity were raising as concentration increases

from1.0M, 1.5M, 2M, 2.5M and 3.0M from 67.4%, 71.7%, 75.7%, 78.7%, 78.7 % respectively of residual activity was recorded. Thus, maximum activity was found at assay mixture 8 pH [1.5M] and pH 10 mixture assay [2M] of NaCl concentration. From this we can conclude that the stability of their proteolytic efficiency differs using assay mixture of pH 8 and 10.

Detergent compatibility study was studied as described in section 3.4.1 and as shown in fig 5-17, the compatibility as detergent removal was assessed using it efficiency in stain removal. Assessment were carried out at 25%, 50%, 75% and with water only and with enzyme without Ariel had been investigated and shows remarkable removal of stain. As we increase the concentration of crude enzyme the stain degradation is increases, liquid crude enzyme was used throughout the assay protocols.

Generally, the optimum production parameters and characterization of *BSM* alkaline protease enzyme was produced. Among the industrial uses its industrial application, detergent compatibility was tested and obtained capability of removing stain using different concentration of crude enzyme with water.

7 Conclusion and Recommendations

7.1 Conclusion

The media formulated using egg shell waste for alkaline protease production was low cost and applicable to produce alkaline protease enzyme. The enzyme is highly active at optimum temperature of 60 ^{0}C between 10-40 min incubation retaining more than 97 % of its original activity and pH 11. It is also active in broad pH and temperature ranges.

As shown in stability profile alkaline protease enzyme at both assaying buffers shows a stability profile between pH values of 8-10. This means most of industrial alkaline process works at alkaline medium i.e. at pH 8 and 10 this AlkPE is best fits these requirements.

The enzyme is highly efficient for stain removal and stable in the presence of detergents, bleaching agent and surfactants. All these properties infer that its suitable application as additive in detergent formulations.

The enzyme has a great potential for leather industry because of its dehairing potential and environmentally friendly approach.

7.2 Recommendations

Depending on the results obtained from this study, the following recommendations should be considered for future work:

The efficiency of crude alkaline protease showed its ability in stain removal as a result of this, detergent industries should use this finding. Instead of importing, locally produced enzymes have enormous impact on the socioeconomic aspect of the country

- ❖ Optimize and scale up enzyme production
- ❖ The enzyme should also be studied for its additional potential applications
- ❖ Further studies on the purification of the alkaline protease up to homogeneity level will give more information on the specific properties of the enzyme.
- ❖ Stabilize the enzyme using different stabilizers for commercial scale application
- ❖ Evaluate the potential applications of the enzyme for dehairing and detergent formulation at a pilot scale
- ❖ Study the feasibility of large-scale production for commercial purpose.

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Appendix A: Comparison of some important factors between submerged and solid-state fermentation

No	Factor	Submerged fermentation	Solid state fermentation
1	Substrate	Soluble substrates (usually sugars)	Insoluble polymeric substrates (starch, cellulose, pectin, lignin)
2	Aseptic condition	Heat sterilization and aseptic control	Vapor treatment, even non-sterile conditions can be used
3	Water	High volumes of water consumed and effluents discarded	Limited consumption of water; low amount or, no effluent produced and discarded
4	Metabolic heating	Easy control of temperature	Low heat transfer capacity, difficulty in control of temperature
5	Aeration	Limitation by soluble oxygen, high level of air required	Easy aeration and high surface exchange air/substrate
6	pH control	Easy pH control	Buffered solid substrates
7	Mechanical agitation	Good homogenization	Static conditions preferred
8	Scale up	Industrial equipment available	Need for engineering and new design equipment
9	Inoculation	Easy inoculation	Spore inoculation(fungi), batch
10	Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low rate growth fungi
11	Energetic considerations	High energy consuming	Low energy consuming
12	Volume of equipment	High volumes and high cost technology	Low volumes and low costs of Equipment
13	Effluent and pollution	High volumes of polluting effluents generated	No effluents, less pollution generated
14	Concentration of products	Low yield and diluted product	Highly concentrated product

Appendix B: Experimental runs from BBD and AlkPE production as response

Std	Run	Block	Factor 1 A: pH	Factor 2 B: Temperature	Factor 3 C: ESM	Factor 4 D: Incubation Time	Response AlkPE Activity
			pH scale	^{0}C	Conc %	Hour	U/ml
25	1	Block 1	9	37	20	48	211.097
1	2	Block 1	8	30	20	48	108.038
24	3	Block 1	9	45	20	72	73.3611
16	4	Block 1	9	45	25	48	86.4903
27	5	Block 1	9	37	20	48	214.103
6	6	Block 1	9	37	25	36	133.716
20	7	Block 1	10	37	25	48	112.555
9	8	Block 1	8	37	20	36	115.651
23	9	Block 1	9	30	20	72	92.4903
12	10	Block 1	10	37	20	72	131.103
3	11	Block 1	-8	45	20	48	85.635
26	12	Block 1	9	37	20	48	209.103
28	13	Block 1	9	37	20	48	211.103
15	14	Block 1	9	30	25	48	102.329
21	15	Block 1	9	30	20	36	107.522
13	16	Block 1	9	30	15	48	114.651
18	17	Block 1	10	37	15	48	137.555
4	18	Block 1	10	45	20	48	89.7805
2	19	Block 1	10	30	20	48	120.281
5	20	Block 1	9	37	15	36	74.6192
7	21	Block 1	9	37	15	72	141.103
17	22	Block 1	8	37	15	48	110.522
14	23	Block 1	9	45	15	48	77.2321
11	24	Block 1	8	37	20	72	87.635
22	25	Block 1	9	45	20	36	75.2321
8	26	Block 1	9	37	25	72	70.6192
10	27	Block 1	10	37	20	36	109.038
29	28	Block 1	9	37	20	48	207.103
19	29	Block 1	8	37	25	48	113.555