ADDIS ABABA UNIVERSTITY

SCHOOL OF GRADUATE STUDIES

CHEMICAL AND BIO - ENGINEERING DEPARTMENT

LEATHER TECHNOLOGY STREAM

DEHAIRING PROTEASE PRODUCTION AND EVALUATION OF THE EFFICACY OF PROTEASE PRODUCTS OF RELEVANT DOWNSTREAM PROCESSING

By

Birhanu Abebe

Advisor

Dr. Gowthaman M. Kuppuswami

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A thesis submitted to the School of Graduate Studies of Addis Ababa University in partial fulfillment of the Degree of Master of Science in Leather Technology

By

Birhanu Abebe

Approved by Board of Examiners

Dr. Ing. Berhanu Assefa (Head, chemical Engineering Department)	Signature	_____ Date
Dr.MK. Gowthaman (Advisor)	Signature	_____ Date
_____ (Internal Examiner)	Signature	_____ Date
_____ (External Examiner)	Signature	_____ Date

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ACRONYMS

BOD	Biological oxygen demand
COD	Chemical oxygen Demand
ANOVA	Analysis of Variance
ADLI	Agriculture Development Led Industrialization
SS	Suspended solids
RPM	Revolutions Per Minute
TDS	Total Dissolved solids
CCD	Central Composite Design
GRAS	Generally Regarded as Safe
ATP	Adenosine Triphosphate
DNA	Deoxyribonucleic Acid
NAD	Nicotinamide Adenine Dinucleotide
UV	Ultraviolet
TCA	Trichloroacetic Acid
LIDI	Leather Industry Development Institute
CLRI	Central Leather Research Institute
VVM	Volume of air per Volume of medium per Minute
UF	Ultrafiltration
RSM	Response Surface Methodology
OD	Optical Density

ABSTRACT

Globally as well as in Ethiopia the tanning industries are under a high pressure from strict legislation articulated for the purpose of protecting and preserving the environment. In the current situation of Ethiopia this industry is viewed from two perspectives; being an important economic activity and the most environment polluting industry. In order to operate within environmentally compatible limits and sustain the realization of Agriculture Development Led Industrialization (ADLI), the current development strategy of Ethiopia, with respect to the tanning industry, some sort of solutions should come out that mitigate the adverse effect of the conventional leather processing methods particularly that of lime-sulphide unhairing operation. This study deals with dehairing enzyme production as green technology alternative for the conventional unhairing practice.

The *B.subtilis* strain was obtained from Department of Biotechnology, CLRI, sub-cultured and characterized for its growth and dehairing protease production in terms of pH, temperature, incubation time and growth and production media composition. The combined effects of pH and temperature on protease production also investigated and they were found to have high interactive effect. Once the culture conditions for production were studied and known, the alkaline protease was produced in a pilot scale fermenter by submerged fermentation using soy bean flour media at optimal conditions of pH 6.5, temperature 31^0C and incubation period of 27 hours.

The final protease product was recovered, partially purified and stabilized by primary downstream processing such as crude enzyme formulation, ammonium sulphate precipitation, ultrafiltration and spray drying. The use of the protease products of each technique on sheep skins and cow hide resulted in a highly promising hair removal efficiency that can really compete with lime-sulphide chemical unhairing process.

1. INTRODUCTION

In Ethiopia the manufacturing of leather at industrial scale started in1920s by producing leather for horse seat back “koricha “, which was required as one of the elements in fighting against the invaders, at formerly named “asko koda” now called Addis Ababa tannery .Over this period the number of tanneries have increased and reached 31 and the industry has grown in terms of the type and quantity of leather it produces both for local and international market. Today the leather industry is generating about 110 million USD per year from exports made to different countries. However, this figure is believed to be very small and we need to do a lot to exhaustively exploit the sector.

In the five years Growth and Transformation Plan of Ethiopia the leather industry sector has been given high priority with the target of realizing 500 million USD at the end of the plan by transforming the industry through interventions in various areas. For this purpose some activities like benchmarking and twinning program are under taken and going on. However, it should be recognized that the work of creating conducive and competitive business environment for the sector should be more holistic in the sense that many of the inputs for manufacturing the leather need to be produced locally, local chemical producers and the leather industry should work jointly to represent Ethiopia in world leather market as one entity and due attention should be given to research to move the industry forward at fast pace. Although we are lucky in having comparative advantage of high livestock population, the past experiences show that the Ethiopia’s leather sector has not registered significant performance for a number of reasons. Poor management practice, lack of state of the art technology in certain production segments, lack of labor force with qualification that exactly fits to the industry , very poor practice of relevant research work in assisting the industry for various challenges they are facing such as environmental constraints among other major factors.

One of the serious and environmentally ill nature of the leather manufacturing industry is evident if we look at the use of lime-sulphide method for dehairing. The adverse effects of this dehairing method on the environment and human health is high notably due to large amount of COD and BOD from dissolved protein and process chemicals, high pH, SS,TDS and sulphide. Experiences show that poor environmental performance results in the loss of competitive advantage at global market, loss of public relation, image and credibility and also leads to legal liabilities for the adverse impact it creates to the human health, safety and the environment. For these reasons bioprocessing of leather is becoming an essential part in mitigating the environmental pollutions of conventional leather making processes. Bioprocess operations make use of microbial, animal and plant cells and components of cells such as enzymes to manufacture new products and destroy harmful wastes [6].Industrially useful enzymes such as dehairing protease are commercial products of bioprocessing. In terms of providing environmental benefits in processing industries, enzymes are probably the most important compounds produced by microorganisms. Enzymes have got applications in soaking, unhairing, degreasing, bating and post-tanning of leather processing operations. As a class, they are extraordinarily efficient. Minute quantities can accomplish at a low

temperature what, by ordinary means, would require a high temperature and greater amounts of strong chemical reagents [32].

In almost all tanneries in Ethiopia, the tendency to employ enzymatic dehairing method is very minimal because of the fact that high price of imported enzymes and enzymatic dehairing is disastrous if not carefully controlled as well as lack of access to ease of supply and technical services. In this research, as one means of encouraging the Ethiopian tanners in shifting from conventional to bioprocessing of leather processing, the production of dehairing protease from *Bacillus subtilis* by submerged fermentation and the hair removal efficiency of the protease products of relevant downstream processing are extensively studied. And also the potential benefits of producing the enzyme locally and its contribution to the leather industry and the country at large in environmental and some other dimensions is presented. The chosen fermentation technique is best suited for microorganisms such as bacteria that require high moisture content [33].

1. 1 Statement of the problem

The stringent environmental challenges faced by the tanning industry majorly arises from using sulphide for dehairing ,basic chromium sulphate for tanning, aldehyde and non-biodegradable as well as carcinogenic component containing auxiliary and main chemicals at various stages of leather making processes. Particularly the use of sulphide for dehairing is a big headache to the tanners in different parts of the world especially to the ones with very minimal practice of alternative dehairing operations like enzymatic hair removal methods. At present tanners in Ethiopian are fully dependent on lime- sulphide based dehairing practice which adversely affects the environment and attracts different penalties as a consequence.

It is apparent that poor environmental performance results in the loss of competitive advantage at global market, loss of public relation, image and credibility and also leads to legal liabilities for the adverse impact it creates to the human health, safety and the environment. As an example the China-Africa overseas and Hafde tanneries were closed for a couple of weeks in 2004 E.C for the environmental impact they have imposed (noxious smell and killing of cattle) on the surrounding community as a result of sulphide based unhairing practice. An ‘end-of-pipe’ treatment does provide a solution albeit with additional operation, time and investment. More meaningful solution would be an eco-friendly approach to replace this chemical based dehairing with a bioprocessing alternative. So as part of contribution to the development of the industry, this thesis aims at producing dehairing enzymes from *Bacillus subtilis* and evaluating the efficacy of the protease products of relevant downstream processes so as to spark the exploitation of bio-based cleaner technology alternatives in the current leather processing practice of Ethiopia.

1.2 Objectives

1.2.1 General Objectives

The main objective of this thesis work is producing dehairing protease enzyme from *Bacillus subtilis* by pilot scale submerged fermentation using soya bean flour medium of

production and evaluate the efficacy of the protease products of primary downstream processing in dehairing cow hides and sheep skins.

1.2.2 Specific objectives

- Studying and understanding microbiology protocols, preparations of various reagents, buffers, sub-culturing techniques, and enzyme activity assays.
- Studying and understanding the various media and their preparation for culture development and enzyme production
- Preparation of active and healthy pre-inoculum of preserved *Bacillus subtilis*
- Characterizing the process for maximum growth of the biomass in terms of pH, temperature, time and production medium at flask level.
- Producing the enzyme using pilot scale fermenter at optimum physical conditions
- Carrying out various downstream processing to recover, partially purify and concentrate the enzyme
- Apply the concentrated and partially purified enzyme obtained through various techniques as well as the crude enzyme on soaked raw cow hides and sheep skins to evaluate the efficacy of hair removal for each technique by comparing against the conventional unhairing method.

1.3 Significance of the research

Local production and distribution of dehairing protease from *B.subtilis* using cheaper production medium of agricultural products has multidimensional advantages:

- Getting the product relatively at attractive prices
- Possibly avoids the tying up of working capital in holding large stock as the Ethiopia's lead time for importing materials is 4 to 6 months and saving money by minimizing the degrading of the product quality(avoid or minimizes the product expiry)
- Promotes the linkage between researcher and the industry for mutual benefit and contributing to the technological and economic advancement of the country at large.
- It serves as one means of laying a foundation for the development of bioengineering in the country. In other words the principles of biotechnology used in the production of this enzyme can be used in other areas like producing other kinds of enzymes(for example food and pharmaceutical industries)
- It paves the way to better understanding of other courses of leather technology studies as raw hide and skins are biological materials and the leather making processes are bio-chemical processes.
- The reduction of the sulfide content in the effluent
- Recovery of the hair/wool which is of good quality
- An increased yield of leather area
- Easy handling of the pelts by workmen
- Simplification of the effluent pretreatment
- The elimination of the bate in the deliming stage and
- The production of a good quality pelts/leather

1.4 Scope of the paper

This thesis work covers the production of dehairing protease from *B.subtlis* by submerged fermentation using soy bean flour production media and evaluation of the efficacy of the protease products of relevant downstream processing in removing hairs of raw cow hides and sheep skins at the unhairing stage of leather making process.

2. LITERATURE REVIEW

2.1 Overview of beamhouse leather processing

In today's conventional leather processing, the term beamhouse encompasses all the processes conducted in a given tannery leading to the tanning step. Fig.2.1 shows the process steps, input and output in conventional beamhouse operations [15].

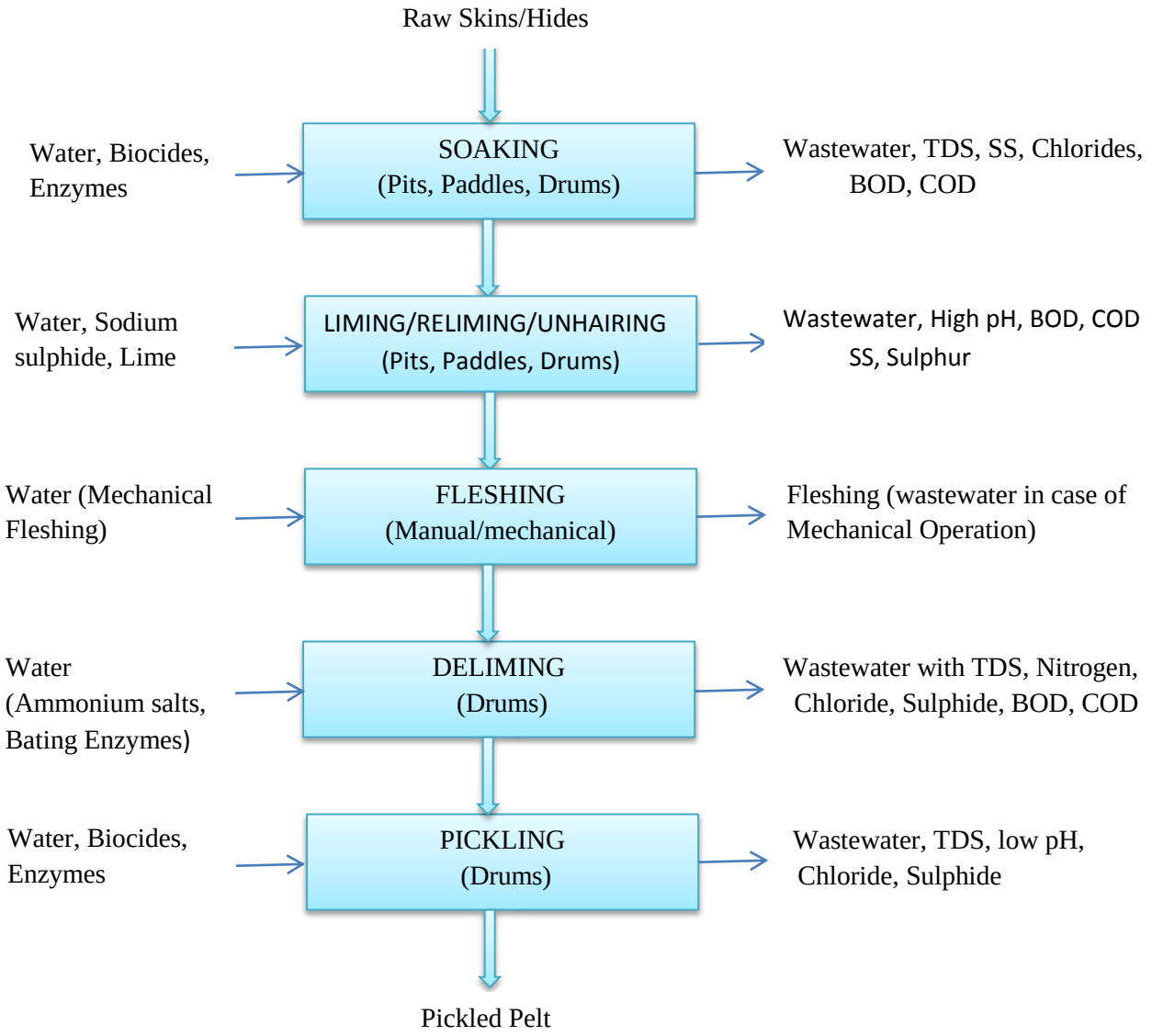

Fig. 2.1 Leather processing steps and input vs output in beahouse

The purpose of the beamhouse is to prepare the pelt for tanning. In other words the beamhouse is for purifying the pelt or 'opening up' the pelt structure. Opening up is a generic term that has two components: the removal of non-collagenous skin components and the splitting of the fiber structure at the level of the fibril bundles, to separate them [16]. The preparatory stages are when the hide/skin is prepared for tanning. Preliminary stages may include: preservation, soaking, liming, unhairing, fleshing, splitting, reliming, deliming, bating, degreasing, bleaching, pickling, and depickling. The raw hide has to undergo many of chemical treatments in a cascade manner before it turns into flattering leather, which is comprised of soaking, liming, dehairing, deliming, bating, degreasing, and pickling. For most of these steps the chemicals used are quite toxic and used in large amount in these pre-tanning operations which results in the leather processing industry becoming one of the worst offenders of the environment [21].

2.1.1 Soaking

Soaking is the first process applied to the raw stock. Hides and skins are received in a tannery in the following conditions according to the sources of supplies and soaking has three major objectives [17]:

i. As green or fresh just after flaying
ii. As wet-salted
iii. As dry-salted
iv. As dry

The hides and skins have to be soaked in water.

The objectives of soaking:

a) Cleaning the hides or skins (removing the dirt, blood and most of the salt used in curing).
b) Softening and swelling the fibers to bring the skins as far as possible back to the state of green hides, i.e., to rehydrate the fibers. Soaking is largely a reversal of the curing process.
c) Dispersal and removal of interfibrillar proteins which otherwise cement the fibers together and prevent their thorough rehydration.

The process of soaking can be classified into three stages [15].

- Dirt Soaking – In dirt soaking, 300-400 % of water is used to remove the unwanted materials
- Main Soaking – The purpose of main soaking is to re-hydrate the material. In this operation, water, non-ionic wetting agent (0.2 % concentrated soda ash and preservatives (0.0 5% concentration) are used.
- Final soaking – Only water is used for the washing purpose in this operation

Since the credit of good unhairing, opening up and scud loosening during subsequent liming is dependent on good soaking operation which must be carried out within a short period and under conditions which prevent injurious bacterial growth, tanners normally take recourse to some auxiliary soaking agents of which there are three categories [17]:

a) Chemical soaking agents: salt, sodium sulphide, sodium sulph-hydrate, sodium tetrasulphide, caustic soda, soda ash etc.

b) Surface active agents with wetting and emulsifying actions: non-ionic products, anionic products, and cationic products.
c) Proteolytic enzymes which are used in neutral or slightly alkaline pH range.
d) Biocides: acid amides and substances which may or may not contain chlorine.

2.1.2 Unhairing/ liming

Unhairing is defined as the removal of hair from hides. During chemical unhairing, the chemicals have a threefold purpose. They destroy the hair or attack the hair roots so that it will be detached from the hide. They loosen the epidermis, the hard outer layer covering the grain, and remove certain soluble skin proteins that lie within the hide substances [38].
Unhairing and liming are often linked because the traditional processes of hair dissolving and alkaline hydrolysis combine the process steps into one. However strictly they ought to be thought of as separate processes and, in modern processing, the steps are increasingly conducted separately.

As the term implies, unhairing is the process of removing the hair from the pelt.it is traditionally one of the dirtier aspects of leather processing, from the point of view of the odour created(typically from the sulfide employed and the decomposed protein) and the polluting load generated. The traditional method of unhairing is to dissolve it, called 'hair burning': this is an example of 'low tech.' processing, so called because it has the benefit of applying to closed drums and does not need any active process control as the reactions progress. It is technically more difficult to undertake the process of hair removal in alternative ways, 'hair saving', keeping the hair intact while removing it; each technology requires a different degree of process control [16].

Table 2.1 Comparison between hair burning and hair saving

	Hair burn	Hair save
Technology/process control	Simple technology Minimum process control Usually relies on undesirable chemicals	More complicated technology More process control. Reagents may be safer
Hair removal	Incomplete-staple remains within the follicle	Complete-allows better dyeing of cleaner grain
Environmental impact	High, due to BOD / COD from dissolved protein and process chemicals	Big reduction in BOD/COD
Environmental perception	Not clean	clean
Advantages	• Reliable • Capable of improvement, e.g. prior hair shaving • Combined with liming	• Cleaner • Better grain quality, e.g. for dyeing • Recovered hair has value
Disadvantages	• Dirty process	• Residual hair must be

	• Residual hair can reduce leather quality	dissolved by hair burning technique • Liming is a separate process step

The conventional unhairing /liming process employ sodium sulphide (Na_2S), sodium sulphydrate (NaHS) and lime.

2.1.3 Deliming

In short it has the purpose of removal of mechanically deposited and chemically bound lime and of capillary lime by conversion into readily soluble salts [28]. In conventional deliming process ammonium sulphate and chloride are the commonest deliming agents.

2.1.4 Bating

Bating is a generic term that refers to the use of enzymes in an early stage of leather making. Its purpose is to break down specific skin components: usually the non-structural proteins are the target. These proteins can be degraded by general proteases, because they do not have highly defined structure, even though they may be folded specifically. Their lack of structure means that scission of the peptide chain creates smaller molecules, making them more easily removable by rinsing. Intact collagen is resistant to such attack, because of its highly structured nature, and therefore requires specific collagenases to break it down. However, notably, alkali-damaged collagen is vulnerable to attack by general proteases, and so limed collagen can be significantly degraded during conventional bating. The usual effect of this type of damage is grain enamel loss and/or corium loosening [16].

2.1.5 Pickling

This operation, which is to be done after the bating and eventual degreasing operations, but prior to tanning, is done in the same drum. The purpose of pickling is to make the pelt's pH value for the tanning process, especially for carrying out the chrome tanning [17].

This is generally considered the most dangerous process because of the possibility of hydrogen sulphide coming out of the drum. This is a very toxic substance and can even cause death. For this reason, all the drums used for this process must be provided with an adequate extraction system for removing this toxic gas.

The pelts coming from bating and degreasing have a pH value of about 8. At this value, the amino acids of the collagen are disassociated with the predominance of free carboxylic ions, COO^-.In these conditions, they react very strongly with the cationic chrome used for tanning.

The reaction between the carboxylic and chrome groups cause the tanning substance to immediately attach itself to the surface of the hide, thus impeding uniform spreading or the chrome liquors inside the hide, thus producing a "non-tanning" action.

To prevent this reaction from taking place, the disassociation of the amino acids must be inverted to favour the reconstruction of the non-disassociated COOH groups and the

formation of NH_3^+ ions. This can be obtained by modifying the pH conditions of the pelt, bringing the acid field to a value of about 3.

The chemical products required to do conventional pickling are as follows [18]:

- 85% formic acid
- 96% sulphuric acid
- Sodium chloride

This is a preserving process in itself, and pelt can be kept in this state for extended periods of time without any deterioration.

2.2 Environmental impacts of conventional unhairing/liming method

The use of high concentration of lime and sodium sulphide creates and extremely alkaline environment resulting in the pulping of hair and its subsequent removal. While one cannot question the efficacy of this process one must recognize its inherent disadvantages. Significant amongst these are [1]:

i. It contributes in no small measure to the pollution load. Beamhouse processes generally account 70-80% of the total COD of effluent from all leather making processes. About 75% of the organic waste from a tannery comes from the beamhouse and 70% of this waste is from hair which is rich in nitrogen. These figures clearly illustrate the contribution made by the lime and sulphide method to the pollution problems of a tannery.
ii. Sulphide is highly toxic and has an obnoxious odour and if left untreated can cause major problems in the sewers.
iii. The severe alkaline condition is a health hazard for the workers.

Among the pretanning processes in leather manufacture, dehairing is an environmentally constrained operation wherein the sludge forming saturated lime liquor in conjunction with toxic sharpeners such as sodium sulfide are employed in high concentrations. Hydrogen sulfide, emanating from the dehairing process is proven to be fatal even in concentrations as low as 200ppm.The extensive use of hazardous sulfide not only leads to unfavorable consequences for the environment but also undermines the efficacy of the effluent treatment plants. The crisis has deepened and it has become a common occurrence that tanneries are forced to close down not only in developed countries but also in developing countries like China[37].

2.3 Bioprocessing of leather

Enzymatic processes, usually carried out at mild conditions are often replacing certain steps in traditional chemical processes which were carried out under harsh industrial environments (temperature, pressures, pH, and chemicals) so far. In this regard, in the leather industry, enzymes (predominately proteases) are used in several steps in the processing of skins and hides for the production of leather.

Animal's skins and hides go through a series of beamhouse operations prior to leather tanning. The discharges and refuges disposed from all these processing stages in the leather production, causes severe health hazards and environmental problems to the entire eco-system. The leather industry world over is therefore, coming under high pressure from environmental regulations to comply with the pollution and discharge legislation.

As a result, the leather industry is looking for cleaner option for the dehairing process. The current activity in this area indicates that the trend is shifting towards design and utilization of cleaner and safer technology like enzymatically enhanced process. A number of different enzymes (proteases, lipases, amylases) have been used in leather processing in these directions.

Interest in enzyme system is a result of a desire to utilize its vast catalytic potential, high specificity and high catalytic activity under mild environmental conditions of pH, temperature and pressure. Enzymes claim potential applications in agriculture, leather, food, and textile and in pharmaceutical industries. Enzymes also play a significant role in industrial effluent treatment.

Traditionally, enzymes found in dogs were used to treat leather to make it pliable by removing some protein components. The reason behind the use of protease lies in the fact that the protein is the major constituent of hair and skins. Hair is composed of α-keratin fibers, insoluble protein molecules containing a large fraction of cysteine residues and having an α-helix conformation. The α-keratin is arranged in piles of fibrils. Different skin layers are composed of collagens, α-keratin, and some elastin.

Dehairing is the single largest process in leather production, which requires huge amount of industrial enzymes like protease, amylases and lipases.

Enzymes have good potential to be exploited as an environmentally friendly option in dehairing as the trend is shifting towards cleaner technology. Therefore, the potential for the industrial use of enzymes in leather processing is very high because of their marked properties as highly efficient and selective catalysis. The resulting saving in process time increases efficiency and allows increased leather output as well. One of the main reasons for using enzymatic unhairing is the elimination of lime, and possibly sulfide, in order to alleviate the problems of effluent and stream pollution. This ultimately leads to a substantial reduction of effluent load and toxicity in addition to improvement in leather quality [13, 19, 37].

2.3.1 Enzyme applications in specific leather making processes

The various important processing methods involved in the leather manufacture are curing, soaking, liming, dehairing, bating, pickling, degreasing and tanning. All these successive steps in the leather production involve enzymatic action directly or indirectly for facilitating the procedures and enhancing the leather output of desired quality. The extent to which the enzymatic action has been involved at different stages of leather processing is shown in the Table 2.2.

Table 2.2 Enzymatic function and its involvement at different leather processing stages

Stages	Enzymes involved	Function of enzymes
Curing	Enzyme are directly not involved	To preserve hides and skins
Soaking	Alkaline and pancreatic proteases	To remove non fibrillar proteins
Dehairing	Alkaline and neutral proteases	To improve the waste water quality
Degreasing	Lipases and proteases	To remove fats
Bating	Trypsin and alkaline proteases	To make soft, supple and pliable
Tanning	Enzyme are directly not involved	To influence the quality of tanning
Waste processing	Trypsin and proteolytic enzymes	Chrome tanned waste processing

Acid protease has the ability to remove protein stains on the surface of chrome tanned blue stock and facilitate the lipases access to fats deposited in the hide. The use of enzymes in retanning is especially suited for the production of full grain aniline leathers including water-resistant. They could also be useful where surfactants are undesirable for water resistance [32].

2.3.2 Protease dehairing

The conventional methods in leather processing involve the use of hydrogen sulfide and other chemicals, creating environmental pollution and safety hazards. Thus, for environmental reasons, the biotreament of leather using an enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction, thus being ecofriendly. Alkaline proteases with elastolytic and keratinolytic activity can be used in leather-processing industries. Alkaline proteases speed up the process of dehairing, because the alkaline conditions enable the swelling of hair roots; and the subsequent attack of protease on the hair follicle protein allows easy removal of the hair [20].

The raw hide which is an ultimate source of leather comprises of epidermis and dermis layers. These layers contain many proteins and other biomolecules. The principal leather making protein, collagen, exists in hides and skins in association with various globular proteins, viz. albumin, globulin, mucoids; and fibrous proteins such as elastin, keratin, and reticulum. In the process of leather manufacture, the noncollagenous constituents of raw hide are removed partially or completely in the various pre-tanning operations; where leather dehairing is one main operation and the extent of removal of these constituents decides the characteristics of the final leather. Conventionally chemicals have been used for these pre-tanning operations. Since these chemicals are becoming problem for the environmental pollution, use of enzymatic treatments is also necessary to get optimum results without affecting the environment. A class of enzyme, proteases has been used successfully in last two decades. One such treatment, bating, is the only step in leather processing where

enzymatic process cannot be substituted by chemical processes. The dehairing by enzyme offers certain desired characteristics to the finished leather. Earlier, the process was carried out using dog dung or manure. The use of this was not only unhygienic but fermentation could also not be controlled.

In pre-tanning operations, the hides and skins are first subjected to a water soak. For loosening the hair, the oldest method is the 'sweating' process – a natural autolysis or breakdown process which is a mild putrefaction process induced at random. Since the type and quantity of the putrefying bacteria cannot be monitored, the process itself eludes control. Moreover, since the sensitivity to attack the epidermal proteins and the fibrous proteins of the corium by the proteolytic enzymes is more or less the same, the sweating may results in serious damage to the raw hide surface. So protease produced in today's biotechnology for dehairing hides and skins and also for bating is ecofriendly solution to conventional beamhouse pollution.

Enzyme based leather dehairing has been considered an environmentally friendly alternative to the conventional chemical process. The ability of enzymes to digests the basal cells of the hair bulb and the cells of the malphigian layer found more efficient without disturbing the native state of skin. As a result, by loosening of hair with an attack on the outermost sheath and subsequent swelling and breakdown of the inner root sheath and parts of the hair that are not keratinized hair removal is accomplished. Numerous significant advantages of enzymatic dehairing are [21]:

(i) Minimize or complete removal of use of sodium sulphide.

(ii) Enzymatic dehairing provides complete hair removal resulting in quality leather.

(iii) Creation of an ecologically conducive atmosphere for the workers.

(iv) Enzymatically dehaired leathers have shown better strength properties with surface area

(v) Simplification of pre-tanning processes by cutting down one step, viz. bating.

(vi) A significant nature of the enzymatic dehairing process is the time factor involved.

Additional advantages of enzymatic unhairing include recovery of higher quality hair, reduction in the chemical oxygen demand of the effluent, and easy handling by workers [38].

Proteolytic enzymes have great commercial importance and contributing to more than 60% of the world's commercially produced enzymes. Approximately 50% of the enzymes used as industrial process aids are proteolytic enzymes. Proteolytic enzymes are more efficient in enzymatic dehairing than amylolytic enzymes. The enzymatic hair loosening processes play a role wherever high-quality hair, wool or bristles are to be recovered.

Three methods of application are commonly used in the enzymatic dehairing process [21]:

(i) Paint method
(ii) Dip method

(iii) Spray method

In the case of paint method, the enzyme solution is mixed with an inert material like kaolin, made into a thin paste, adjusted to the required pH, applied on the flesh side of hides and skins, piled flesh to flesh, covered with polythene sheets and kept till dehairing takes place. In the dip method of enzymatic unhairing, the hides or skins are kept immersed in the enzyme solution at the required pH in a pit or tub. The disadvantage encountered in this method is the unavoidable dilution of the enzyme solution. Even though enzyme penetration is observed to be uniform, dehairing at backbone and neck is not up to the mark. A novel spraying technique has been adopted for the application of multienzyme concentrate in depilation.

The advantages of this method over the painting and dip methods are that:
(i) Even concentrated solutions can be sprayed,

(ii) When the enzyme solution is sprayed on the flesh side with force, entry becomes easier,

(iii) Backbone and neck can be sprayed with more amount of enzyme, thereby quick process

(iv) There is no effluent arising out of this method, and

(v) After depilation, hair will be almost free from all the adhering skin tissues.
Leather dehairing by drumming is being practiced, and industrially this should be feasible. Many of other methods are in the trials with the optimized operation for the improved dehairing.

2.4 The industrial importance and applications of protease

Protease enzyme has wider applications in detergent, pharmaceutical, photography, leather, food and agricultural industries. In exploiting its detergency, protease are applied to remove difficult stains and soil at low washing temperatures. It is also used in baking, brewing, meat tenderization, peptide synthesis, medical diagnosis, cheese making, certain medical treatment of inflammation and virulent wounds and in unhairing of skins [22]. The uses to which protease enzyme has been put are summarized in Table 2.3[2].Proteases are finding increasing application in leather processing. They are used in enzymatic unhairing to reduce the pollution load on the environment and in bating operations for improved yield and softer leather. The application of enzymes which replace chemicals in the unhairing process fundamentally eliminates the pollution in leather industry and may save the leather industry from a difficult situation [36].

Table 2.3 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	Chillproofing	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

2.4.1 Protease and its sources

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 [23].

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 [24].

Alkaline proteases are envisaged to have extensive applications in leather industry. In a tannery, a rawhide is subjected to a series of chemical treatments prior to tanning and finally converted to finished leather. Proteases may play a vital role in this treatment by replacing these hazardous chemicals especially involved in soaking, dehairing and bating. Increased usage of enzymes for dehairing and bating not only prevents pollution problems, but also is effective in saving energy. For unhairing skin and hides, the enzyme catalyzes the breakdown of the protein keratin in the hair and allows the hair to be easily removed [29].

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 hydrophila 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 proteins 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 alkalophiles is their ability to modulate their environment. They can convert any neutral or high alkaline medium in their favour to optimize external pH for their growth.

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 [25].

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.

2.4.1.1 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 [27].

2.4.1.2 Fungal Protease

Many of researchers have isolated and checked activity of various proteases for dehairing form fungal sources. The protease from Aspergilous flavus was earlier being used for dehairing, and later it was reported that simultaneous dehairing and bating is possible with the protease of Aspergilous flavus.

2.4.1.3 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's 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.

2.5 Characteristic features of *B.subtlis* for being industrially important microorganism

The following attributes make *B.subtilis* industrially the important microorganism [5, 36 41].

2.5.1 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 and sporulating under anaerobic conditions when necessary
- 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.
- B.subtilis has proved a valuable model for research

- Bacillus subtilis is one of the best understood prokaryotes, in terms of molecular biology and cell biology
- Its superb genetic amenability and relatively large size have provided the powerful tools required to investigate a bacterium from all possible aspects.
- Recent improvements in fluorescence microscopy techniques have provided novel and amazing insight into the dynamic structure of a single cell organism.
- 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).
- Cellular respiration in B. subtilis is mainly aerobic. However, it is able to grow under anaerobic conditions in the presence of nitrate, though growth is much slower.
- 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.
- B. subtilis often exists as an endospore.
- As an endospore, B. subtilis becomes resistant to heat, acid, salt, and other harsh environmental conditions.
- Endospore formation is usually triggered by a lack of carbon, nitrogen or phosphate in the environment.

2.5.2 Ecology

B. 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, B. subtilis secretes antifungal antibiotics, which can control fungal diseases in plants and crops. B. subtilis is capable of increasing the overall performance of crops and other plants.

2.5.3 Pathology

B. 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. B. 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.

2.5.4 Application to Biotechnology

B. subtilis is widely used in the field of biotechnology due to its ease at being manipulated and its generally low risk. B. subtilis produces a variety of enzymes useful to industry. The amylases it produces are used in the desizing of textiles and in starch modifications used to resize paper. The proteases it produces are used as additives in laundry detergents and products used for processing leather. B. subtilis also produces a wide array of antibiotics, including subtilin, surfactin, bacillomycin, bacilysin, and fengycin, which are used as antibacterial and antifungal medicines. The antifungal activity of B. subtilis strain QST713 is used as a biological control agent of fungal plant pathogens. B. subtilis is also used as a soil inoculant in horticulture and agriculture.

2.6 Microbial cell cultivation

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

2.6.1 Bacterial cell cultivation for the production of Dehairing protease

Despite the fact that enzymes used for unhairing skins and hides can be produced from animal and plant sources, the production of the enzyme using microbial sources has the advantages of high amount production of the enzyme in relatively shorter period of time using cheaper medium.

2.6.1.1Nutritional Requirements

To cultivate bacteria the nutritional medium should contain the organism's elemental composition which is listed in Table 2.4 [3].

Table2.4 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 CO_2 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 biological significance of the main chemical elements in microorganisms is given in Table 2.5. 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 [30].

Table 2.5. Physiological functions of the principal elements

Element	Physiological function
Hydrogen	Constituent of cellular water and organic cell materials
Carbon	Constituent of organic cell materials
Nitrogen	Constituent of proteins, nucleic acids and coenzymes
Oxygen	Constituent of cellular water and organic materials, as O_2, electron acceptor in respiration of aerobes
Sodium	Principal extracellular cation
Magnesium	Important divalent cellular cation, inorganic cofactor for many enzymatic reactions, incl. those involving ATP; functions in binding enzymes to substrates and present in chlorophylls
Phosphorus	Constituent of phospholipids, coenzymes and nucleic acids
Sulfur	Constituent of cysteine, cystine, methionine and proteins as well as some coenzymes as CoA and cocarboxylase
Chlorine	Principal intracellular and extracellular anion
Potassium	Principal intracellular cation, cofactor for some enzymes
Calcium	Important cellular cation, cofactor for enzymes as proteinases
Manganese	Inorganic cofactor cation, cofactor for enzymes as proteinases
Iron	Constituent of cytochromes

	and other heme or non-heme proteins, cofactor for a number of enzymes
Cobalt	Constituent of vitamin B_{12} and its coenzyme derivatives
Copper	Inorganic constituents of special enzymes
zinc	

The predominant atomic constituents of organisms, C, H, N, O, P, and S, go into making up the molecules of living matter. All living cells on earth contain water as their predominant constituent. The remainder of the cell consists largely of proteins, nucleic acids, lipids, and carbohydrates, along with a few common salts. A few smaller compounds are very ubiquitous and function universally in bioenergetics, e.g., ATP for energy capture and transfer, and NAD in biochemical dehydrogenation. Microorganisms share similar chemical compositions and universal pathways. They all have to accomplish energy transfer and conversion, as well as synthesis of specific and patterned chemical structures.

The microbial environment is largely determined by the composition of the growth medium. Using pure compounds in precisely defined proportions yields a defined or synthetic medium. This is usually preferred for researching specific requirements for growth and product formation by systematically adding or eliminating chemical species from the formulation. Defined media can be easily reproduced, have low foaming tendency, show translucency and allow easy product recovery and purification.

Complex or natural media such as molasses, corn steep liquor, meat extracts, etc., are not completely defined chemically, however, they are the media of choice in industrial fermentations.

In many cases the complex or natural media have to be supplemented with mainly inorganic nutrients to satisfy the requirements of the fermenting organism. The objective in media formulation is to blend ingredients rich in some nutrients and deficient in others with materials possessing other profiles to achieve the proper chemical balance at the lowest cost and still allow easy processing.

2.6.1.2 Physical conditions

It refers to the physical environment in which the organisms will grow best. The three major physical factors to be taken into consideration for the cultivation of bacteria are temperature, the gaseous environment and pH [3].

a. Temperature

Microbial growth is a function of temperature as microbial activity and growth are manifestations of enzymatic action which increase with temperature increment. Based on the temperature range in which it grows, bacteria are classified into the groups shown in table 2.6.

Table 2.6 Approximate Temperature Range for Growth of Various Bacteria

Types of Bacteria	**Approximate range for growth in ^{0}c**	**Optimum Temperature in ^{0}C**
Psychrophiles	-7 to 35	20 to 30
Mesophiles	7 to 45	30 to 40
Thermophiles	45 to 75	45 to 60

b. Gaseous environment

Based on oxygen requirement for growth bacteria is grouped into:

- Aerobic bacteria grow in the presence of free oxygen.
- Anaerobic bacteria grow in the absence of free oxygen.
- Facultatively anaerobic bacteria grow in either the absence or the presence of free oxygen.
- Microaerophilic bacteria grow in either the presence of minute quantities of free oxygen.

c. pH

For most bacteria the optimum pH for growth lies between 6.5 and 7.5.

2.7 Fermentation

Fermentation is defined and described in more general sense in a reference [8]. The term ‘fermentation’ was obtained from the Latin verb ‘fervere’, which describes the action of yeast or malt on sugar or fruit extracts and grain. The ‘boiling’ is due to the production of carbon dioxide bubbles from the aqueous phase under the anaerobic catabolism of carbohydrates in the fermentation media. The art of fermentation is defined as the chemical transformation of organic compounds with the aid of enzymes. The ability of yeast to make alcohol was known to the Babylonians and Sumerians before 6000 BC. The Egyptians discovered the generation of carbon dioxide by brewer’s yeast in the preparation of bread. In the process of ethanol production, carbohydrates are reduced to pyruvate with the aid of nicotinamide adenine dinucleotide (NADH); ethanol is the end product. Other fermentation processes include the cultivation of acetic acid bacteria for the production of vinegar. Lactic acid bacteria preserve milk; the products are yoghurt and cheese. Various bacteria and mold are involved in the production of cheese. Louis Pasteur, who is known as the father of the fermentation process, in early nineteenth century defined fermentation as life without air.

The term fermentation has different meaning to biochemist and microbiologist [2]. 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 micro-organisms.

The culture techniques in fermentation can be classified into batch, fed-batch, and continuous operation[10]. In batch processes, all the nutrients required for cell growth and product

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.

A fed-batch operation is that operation in which one or more nutrients are added continuously or intermittently to the initial medium after the start of cultivation or from the halfway point through the batch process. Fed-batch processes have been utilized to avoid substrate inhibition, glucose effect, and catabolite repression, as well as for auxotrophic mutants.

In a continuous operation, the feed medium containing all the nutrients is continuously fed at a constant rate (dilution rate) and the cultured broth is simultaneously removed from the fermenter at the same rate.

2.7.1 Types of fermentations

Based on the purpose they are intended for fermentations are catagorized into five major inductrially impotrant groups[2].

i. Those that produce microbial cells (or biomass) as the product.

The production of yeast and single –cell proteins(used as human or animal food) can be mentioned as examples for fermentation of this kind.

ii. Those that produce microbial enzymes

Microbes are preferred to plants and animals as sources of enzymes because[11,34]:

- They are generally cheaper to produce
- Their enzyme contents are more predictable and controllable
- Reliable supplies of raw material of constant composition are more easily arranged
- Plant and animal tissues contain more potentially harmful materials, including phenolic compounds (from plants), endogenous enzyme inhibitors and proteases.
- Can be produced in large quantities
- Comparatively productivity can easily be improved becauase enzyme production is closely controlled in micro-organisms like by induction by introducing inducers into the production medium or removing repression by mutation and recombination techniques.
- Enzymes of animal orgin can be synthesized by microorganisms through the application of recombinant DNA technology(the number of gene copies coding for the enzyme can be increased)
- Microorganisms also provide valuable services. They have proved to be particularly useful because of the ease of their mass cultivation, speed of growth, use of cheap substrates that in many cases are wastes, and the diversity of potential products.
- They have a high ratio of surface area to volume, which facilitates the rapid uptake of nutrients required to support high rates of metabolism and biosynthesis
- the facility to adapt to a large array of different environments, allowing a culture to be transplanted from nature to the laboratory flask or the factory fermenter, where it is capable of growing on inexpensive carbon and nitrogen sources and producing valuable compounds

- of simplicity of screening procedures, allowing thousands of cultures to be examined in a reasonably short time

The pH at which these enzymes are active ranges from acidic, in which pepsin and papain can be used, to neutral and alkaline for enzymes such as from *Bacillus subtilis.*

iii. Those that produce microbial metabolites

Bacterial growth in batch culture can be modeled with four different phases: **lag phase**, **log phase** or **exponential phase**, **stationary phase** and **death/decline phase** [45] (Fig.2.2).

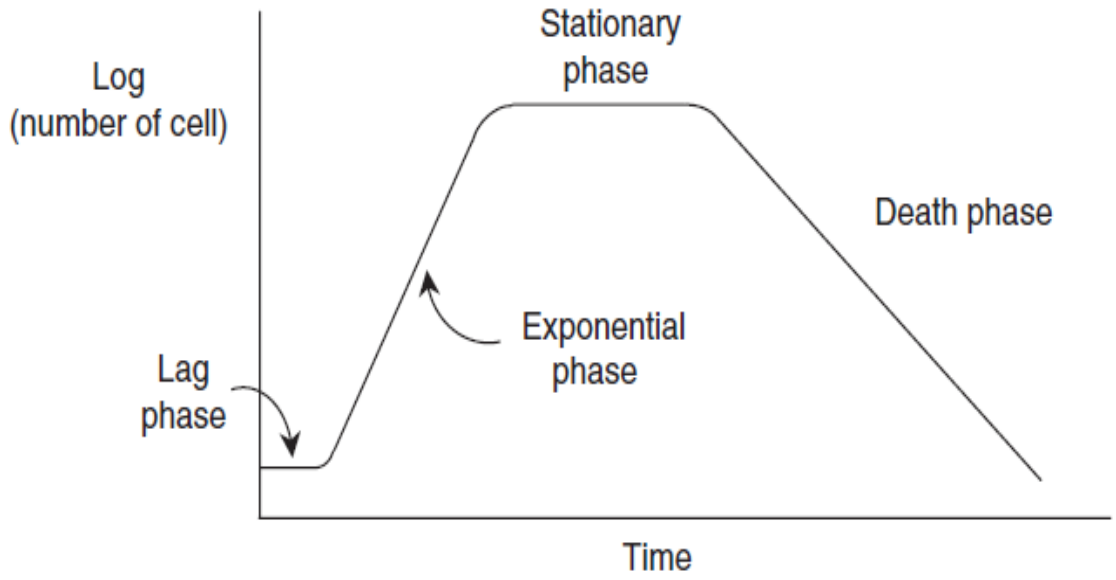

Fig.2. 2 Typical batch growth curve of a microbial culture

Lag phase

During **lag phase**, bacteria adapt themselves to growth conditions. It is the period where the individual bacteria are maturing and not yet able to divide. During the lag phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs [45].

This is the duration of time represented for adaptation of microorganisms to the new environment, without much cell replication and with no sign of growth. The length of the lag phase depends on the size of the inocula. It also results from the shock to the environment when there is no acclimation period. Even high concentrations of nutrients can cause a long lag phase. It has been observed that growth stimulants and trace metals can sharply reduce the lag phase [8].

Log or exponential phase

During this phase the growth rate of the cells gradually increases at a constant maximum rate.The metabolic products produced during this phase are refered to as primary products which have great economic importance in various areas as presented in Table 2.7 [2].

Table2.7 Some primary products of micrbial metabolism and their commercial significance

Lists of some of the primary metabolites	**Application**
Ethanol	'Active ingredient' in alcoholic beverages ;used as a motor-car fuel when blended with pertoleum
Citric acid	Used in the food industry
Glutamic acid	Flavour enhancer
Lysine	Feed supplement
Nucleotides	Flavour enhancers
Phenylalanine	Precursor of aspartame,sweetener
Polysaccharides	Applications in the food industry; enchanced oil recovery.
Vitamins	Feed supplements

Once there is an appreciable amount of cells and they are growing very rapidly, the cell number exponentially increases. The optical cell density of a culture can then be easily detected.The rate of cell synthesis sharply increases; the linear increase is shown in the semi-log graph with a constant slope representing a constant rate of cell population. At this stage carbon sources are utilised and products are formed.

Stationary phase

It is often due to a growth-limiting factor such as the depletion of an essential nutrient, and/or the formation of an inhibitory product such as an organic acid. Stationary phase results from a situation in which growth rate and death rate are equal. The number of new cells created is limited by the growth factor and as a result the rate of cell growth matches the rate of cell death. The result is a "smooth," horizontal linear part of the curve during the stationary phase.

Rapid utilization of substrate and accumulation of products may lead to stationary phase where the cell density remains constant. It is well known that the biocatalytic activities of the cell may gradually decrease as they age, and finally autolysis may take place. The dead cells and cell metabolites in the fermentation broth may create toxicity, deactivating the remaining cells [8].

Deceleration or death phase

At this stage, a death phase develops while the cell density drastically drops if toxic secondary metabolites are present. The death phase shows an exponential decrease in the number of living cells in the media while nutrients are depleted. In fact the changes are detected by monitoring the pH of the media.

During this phase the number of viable cells declines exponentially as a result of exhaustion of food. The products produced during the stationary and decelaration phases are referred to as secondary compounds of metabolism and usually exhibits certain characterstics :

- Many of them have antimicrobial activities and pharamaceutical properties
- Some are specific enzyme inhibitors
- Some are growth promoters

At this stage, a death phase develops while the cell density drastically drops if the toxic secondary metabolites are present. The death phase shows an exponential decrease in the number of living cells in the media while nutrients are depleted. In fact the changes are detected by monitoring the pH of the media[8].

iv. Those that produce recombinant products

In fermentations of this type genes from higher organisms are introduced to microbial cells such that the recipients are capapble of synthesizing 'foreign'or (hetrologous) proteins.*Escherichia coli,Saccharomyces cerevisiae* and filamentous fungi are the most commonly used microbial cells as hosts. Some of the products produced by such genetically engineered organisms are[2]:

- Insulin
- Human serum albumin
- Factors VIII and IX
- Calf chymosin and
- Bovine somatostatin

To effectively carry out the design of this job the factors that should be taken into consideration are:

- Secretion of the product
- Minimzation of the degradation of the product
- The control of the onset of synthesis during the fermentation and
- Maximizing the expression of the foreign gene.

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.

2.7.2 Unit operations of a fermentation process

A generalized process flow diagram of fermentation is shown in fig.2.3.

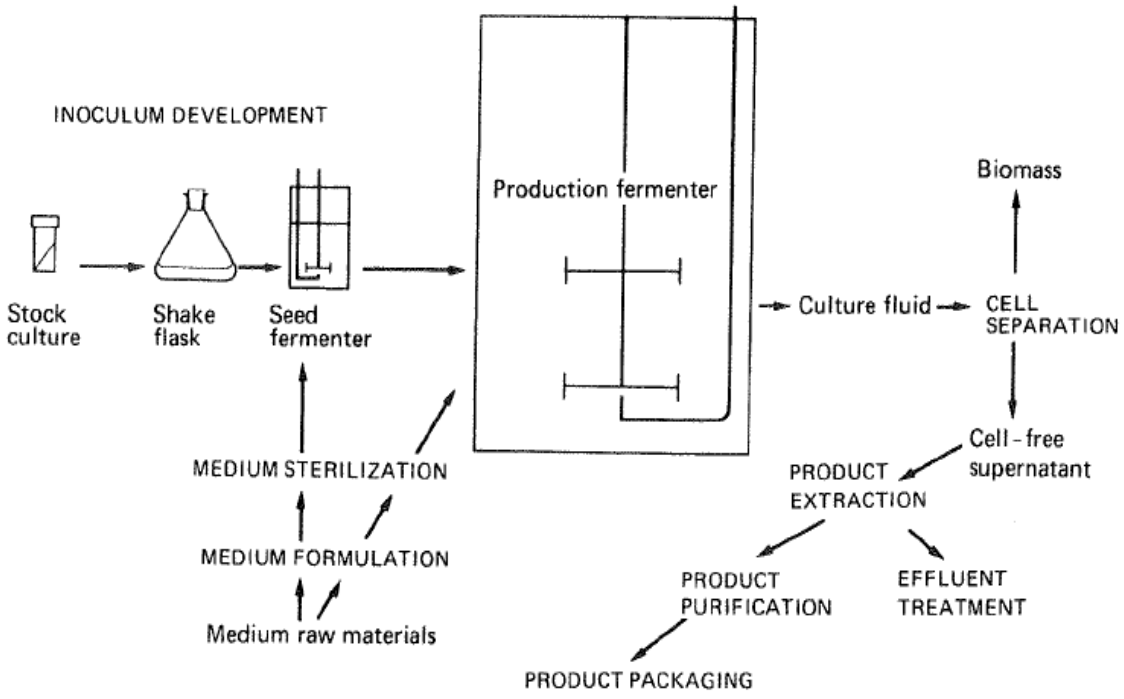

Fig. 2.3 Outline of a fermentation process

A well-established fermentation process involves the following operations [2]:

i. The formulation of media to be used in culturing the process organism during the development of the inoculum and in the production fermenter.

All micro-organisms require:

- Water
- Sources of energy
- Source of carbon
- Source of nitrogen
- Mineral elements and
- Possibly vitamins plus oxygen if aerobic

On a large scale one must normally use sources of nutrients to create a medium which will meet as many as possible of the following criteria:

- It will produce the maximum yield of product or biomass per gram of substrate used
- It will produce the maximum concentration of product or biomass.
- It will permit the maximum rate of product formation
- There will be the minimum yield of undesired products.
- It will be of a consistent quality and be readily available throughout the year
- It will cause minimal problems during media making and sterilization

- It will cause minimal problems in other aspects of the production process particularly aeration and agitation, extraction, purification and waste treatment.

ii. The sterilization of the medium, fermenters and ancillary equipment.

After a suitable culture medium is formulated for cultivating microorganism of interest, it is poured into culture vessels such as test tubes, shake flasks etc. and the openings of these vessels are covered with a suitable closure such that it allows the exchange of gases with the environment but prevent the entrance of foreign contaminants out of the medium. Cotton plugs, plastic foam, screw caps, metal caps and aluminum foil are commonly used materials as closures.

Then the medium, ancillary equipment and the culture vessel are sterilized to eliminate all microorganisms by suitable sterilization techniques such as moist heat (steam under pressure) in an autoclave at an approximate pressure of 15 psi and temperature of 121^0C for 15 to 20 minutes depending upon the type of vessel, the nature and volume of medium.

If the fermentation is invaded by a foreign micro-organism then the following consequences may occur:

a. The medium would have to support the growth of both the production organism and the contaminant, resulting in a loss of productivity.
b. If the fermentation is a continuous one then the contaminant may 'outgrow' the production organism and displace it from the fermentation
c. The foreign organism may contaminate the final product, e.g. single-cell protein where the cells, separated from the broth, constitute the product.
d. The contaminant may produce compounds which make subsequent extraction of the final product difficult.
e. The contaminant may degrade the desired product; this is common in bacterial contamination of antibiotic fermentations where the contaminant would have to be resistant to the normal inhibitory effects of the antibiotic and degradation of the antibiotic is a common resistance mechanism, e.g. the degradation of β -lactam antibiotics by β -lactamase-producing bacteria.
f. Contamination of a bacterial fermentation with phage could result in the lysis of the culture.

iii. The production of an active and pure culture in sufficient quantity to inoculate the production vessel.

The activity of inoculating the inoculum into a culture medium in a shake flask is done using a metal loop which is sterilized just before its use by heating it in a flame or using a sterilized pipette for liquid inoculum in laminar flow hood to minimize the risk of contamination. The inoculation of culture into fermenters is done by using sterilized rubber pipes and necessary ancillaries.

iv. The growth of the organism in the production fermenter under optimum conditions for product formation.

The process variables that need to be closely monitored and controlled to achieve the desired level of the growth of microorganisms at flask and/or fermenter level are pH, temperature, RPM, dissolved oxygen and nutrient concentration.

v. The extraction of the product and its purification.

In bioprocessing, any treatment of the culture broth after fermentation is known as *downstream processing***.** Downstream processing is a very important step in biotechnology because costs for collection, concentration and purification of the final product are substantial. High product concentrations in the supernatant or inside the cells and efficient purification are therefore important aspects in the overall economy of enzyme manufacture.

The degree of purity of commercial enzymes ranges from raw enzymes to highly purified forms and depends on the application. Raw materials for the isolation of enzymes are animal organs, plant material and microorganisms.

Often enzymes may be purified several hundred-fold but the yield of the enzyme may be very poor, frequently below 10% of the activity of the original material. In contrast, industrial enzymes will be purified as little as possible, only other enzymes and material likely to interfere with the process which the enzyme is to catalyze, will be removed. Unnecessary purification will be avoided as each additional stage is costly in terms of equipment, manpower and loss of enzyme activity. As a result, some commercial enzyme preparations consist essentially of concentrated fermentation broth, plus additives to stabilize the enzyme's activity. Downstream processing involves isolation and purification steps (Fig.2.4) and ends up in the formulation of the enzyme preparation [13].

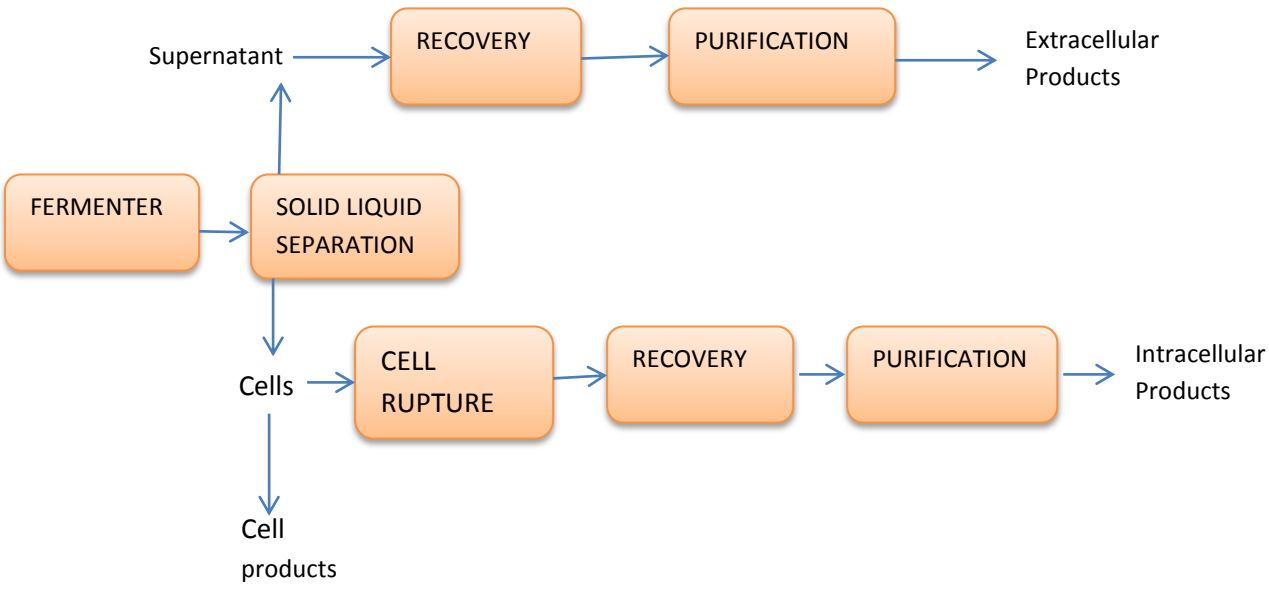

Fig.2.4 Major process steps in downstream processing

The purpose of downstream processing (DSP)

DSP has the following objectives [4]:

- 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

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.

a. Cell recovery/removal

Filtration and centrifugation are typical unit operations for cell removal.

b. 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

b. Protein processing

- Immobilization
- Beading

c. Protein packaging

- Sterilization
- Bottling etc.

vi. The treatment and disposal of effluents produced by the process.

Primary treatment

It includes physical and chemical methods. For example screening, sedimentation, coagulation etc.

Secondary treatment

It involves biological, methods like activated sludge, after primary treatment. Tertiary treatment: physical, chemical or biological methods to improve the quality of liquor from previous stages.

Sludge conditioning and disposal

Anaerobic digestion is often used to condition (make it more amenable to dewatering) the sludge produced in previous stages. Following dewatering the sludge can then be disposed of by incineration, landfilling, etc.

2.7.3 Applications of fermentation processes

Man has been using the fermentative abilities of microorganisms in various forms for many centuries. Yeasts were first used to make bread; later, use expanded to the fermentation of dairy products to make cheese and yoghurt. Nowadays more than 200 types of fermented food product are available in the market. There are several biological processes actively used in the industry, with high-quality products such as various antibiotics, organic acids, glutamic acid, citric acid, acetic acid, butyric and propionic acids. Synthesis of proteins and amino acids, lipids and fatty acids, simple sugar and polysaccharides such as xanthan gum, glycerol, many more fine chemicals and alcohols are produced by bioprocesses with suitable industrial applications. The knowledge of bioprocessing is an integration of biochemistry, microbiology and engineering science applied in industrial technology. Application of viable microorganisms and cultured tissue cells in an industrial process to produce specific products is known as bioprocessing. Thus fermentation products and the ability to cultivate large amounts of organisms are the focus of bioprocessing, and such achievements may be obtained by using vessels known as fermenters or bioreactors. The cultivation of large amounts of organisms in vessels such as fermenters and bioreactors with related fermentation products is the major focus of bioprocess.

A bioreactor is a vessel in which an organism is cultivated and grown in a controlled manner to form the by-product. In some cases specialized organisms are cultivated to produce very specific products such as antibiotics. The laboratory scale of a bioreactor is in the range 2–100 liters, but in commercial processes or in large-scale operation this may be up to 100 m^3. Initially the term ‘fermenter’ was used to describe these vessels, but in strict terms fermentation is an anaerobic process whereas the major proportion of fermenter uses aerobic

conditions. The term ‘bioreactor’ has been introduced to describe fermentation vessels for growing the microorganisms under aerobic or anaerobic conditions.

Bioprocess plants are an essential part of food, fine chemical and pharmaceutical industries. Use of microorganisms to transform biological materials for production of fermented foods, cheese and chemicals has its antiquity. Bioprocesses have been developed for an enormous range of commercial products. Most of the products originate from relatively cheap raw materials. Production of industrial alcohols and organic solvents is mostly originated from cheap feed stocks. The more expensive and special bioprocesses are in the production of antibiotics, monoclonal antibodies and vaccines. Industrial enzymes and living cells such as baker’s yeast and brewer’s yeast are also commercial products obtained from bioprocess plants [8].

3. MATERIALS AND METHODS

3.1 Materials

a. Raw materials

- wet salted sheep skins and
- wet salted cow hides

b. Reagents and chemicals

Trichloroacetic acid(TCA)
Agar
Ammonium sulphate
Buffer salts
- Sodium carbonate
- Sodium bicarbonate

Growth and production medium
- Source of carbon
- Source nitrogen (yeast extract, peptone, soya bean)
- Source of inorganic salts ($NaCl$,$MgSO_4$,KH_2SO_4 and $CaCl_2$)

Sodium benzoate
Bronopol
Tata salt
Distilled water
NaOH

c. Machines, Equipment and consumables

Laminar air flow cabinet
Test tubes
Shake flasks
Shaker
Petri dish and paraffin film
Graduated Measuring cylinders
Micropipette and tips
Refrigerator
Water bath
Auto clave

Spectrophotometer
Centrifuge
Magnetic stirrer
Fermenter
Micro filtration
Ultrafiltration
Spray dryer
Soaking liming pits/Drums
pH paper
tyrosine filter paper

Incubator	Closures such as Cotton plugs, plastic foam, screw caps, metal caps and aluminum foil for closing end of culture vessels

d. Culture for fermentation

Bacillus subtilis bacterial species

e. Software

Design expert 7.0 aprobado

3.2 Methods

To carry out this study the methodology followed involved studying and learning microbiology protocols, preparations of various reagents, preparation of pre-inoculum, characterization study of culture, production of dehairing protease using production media of soybean at pilot scale fermenter, carry out downstream processing and finally evaluating the efficacy of the protease products of each downstream processing employed.

3.2.1 Learning microbiology protocols and preparations of various reagents

Enzyme production and its application in the leather manufacturing industry is based on a profound understanding of fundamental microbiology concepts, protocols and know- how of various reagents involved in the area. So, in this section some of the basic topics relevant to the work of this research were addressed.

3.2.1.1Protease Assay

Samples were taken from the flasks periodically and centrifuged at 10000 rpm for 5 minutes. The collected supernatant was assayed for protease activity by the method of Kunitz (1947) using casein as substrate.

Principle

The absorbance of UV light by tyrosine and tryptophan residues is high at 280 nm. The peptides mainly tyrosine which are liberated in proteolytic digestion are measured in a filtrate of trichloroacetic acid (TCA), by means of their absorbance at 280 nm. The activity of protease is estimated in "Units". One unit is defined as the liberation of 1 mg of tyrosine in 10 min equivalent of substrate per ml of enzyme under standard assay conditions [42].

Preparation of reagents

1. 1% casein solution

1 g of Hammerstein casein in 100 ml of 0.05 M carbonate buffer, pH=9.5.

2. Carbonate buffer

A = 0.1 M Na_2CO_3 = 1.06 g / 100 ml distilled water

B = 0.1 M $NaHCO_3$ = 0.84 g / 100 ml distilled water

13 ml of A + 37 ml of B + 50 ml of distilled water

= 100 ml of buffer, 0.05 M, pH 9.5

3. 5% TCA

5 g of TCA in 100 ml of distilled water

Procedure

- 1.9 ml of casein solution was added to all test-tubes marked as test and control.
- 3 ml of TCA was added to the tubes marked as control.
- All the tubes were pre-incubated for 10 minutes at 40°C.
- 0.1 ml (100µl) of suitably diluted enzyme was added to all the tubes and incubated at 40°C for 10 minutes for the enzyme to react with the substrate.
- After incubation 3 ml TCA was added to all tubes marked as test to stop the reaction.
- The precipitate was filtered through Whatman filter paper (Grade A, 110 mm)
- The absorbance of the filtrate was read at 280 nm using spectrophotometer
- The amount of tyrosine released was calculated from the standard graph of tyrosine.

Tyrosine Standard

Tyrosine stock solution: 50 mg of tyrosine is dissolved in 1 ml of 0.5 N HCl and is made up to 100 ml with distilled water to give a concentration of 0.5 mg/ml.

Table 3.1 Procedure for tyrosine standard

Reagents	Blank	Standard
Vol. of stock (ml)	0	0.1 to 1.0
Buffer (ml)	2.0	Made up to 2.0
TCA (ml)	3.0	3.0
Read O.D. at 280 nm		

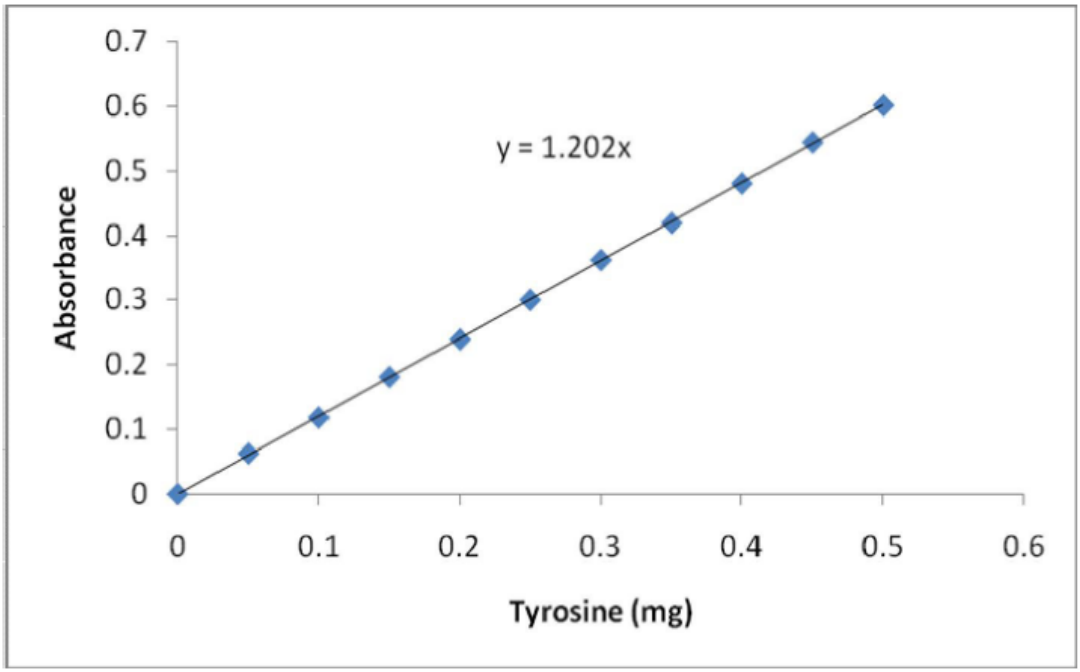

Fig.3.1 Standard graph for tyrosine

From the graph,

Slope (m) = 1.202 mg-1

$$\text{Protease Activity} = \frac{(\text{Test}-\text{Control})\text{x Dilution factor}}{\text{slope}(^{1.202}/_{mg})\text{xvolume of enzyme(0.1ml)}} \quad \text{----------3.1}$$

$$= (\text{Test} - \text{Control})\text{x } 8.3\text{mg/ml x Dilution factor}$$

3.2.1.2 Spectroscopy

A concise description of spectroscopy was extracted from a reference [31]. A spectrophotometer is an instrument for measuring the absorbance of a solution. Absorbance is a useful quantity. The Beer-Lambert law states that:

$$A = \varepsilon cl \text{ ----------3.2}$$

Where A is the absorbance of the sample at a particular wavelength,ε is the extinction coefficient for the compound at that wavelength in $(M \bullet cm)^{-1}$,c is the molar concentration of the absorbing species, and l is the path length of the solution in cm. Thus, if the extinction coefficient of an absorbing species is known, the absorbance of the solution can be used to calculate the concentration of the absorbing species in solution. (This assumes that the species of interest is the only material that absorbs at the wavelength being measured.)

The above is an explanation of why we measure absorbance: absorbance allows us to calculate the concentration of compounds in solution. However, it does not explain what absorbance is. Another definition of absorbance is:

$$A = \log \frac{(I_0)}{(I)} \quad \text{----------3.3}$$

Where I_0 is the amount of light entering the sample, and I is the amount of light leaving the sample. Absorbance is therefore a measure of the portion of the light leaving the lamp that actually makes it to the detector. A little thought will reveal that when absorbance = 1, only 10% of the light is reaching the detector; when absorbance = 2, only 1% of the light is reaching the detector.

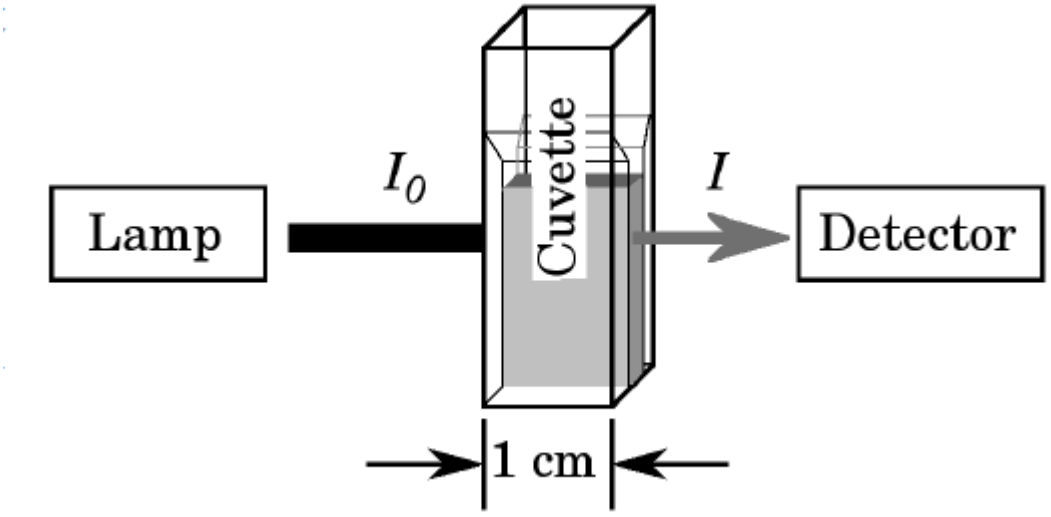

Fig.3.2 The typical internal arrangement of a Spectrophotometer

Absorbance values greater than 2 are unreliable, because too little light is reaching the detector to allow accurate measurements. When measuring absorbance, note the values; if the reading is greater than 2, dilute the sample and repeat the measurement.

Spectrophotometers measure the decrease in the amount of light reaching the detector. A spectrophotometer will interpret fingerprints on the optical face of the cuvette, or air bubbles, or objects floating in your solution as absorbance; you therefore need to look carefully at your cuvette before putting it into the spectrophotometer to make sure that your reading is not subject to these types of artifacts.

Spectrophotometers have the ability to specifically measure absorbance at specific wavelengths. The most commonly used method to allow this involves a “monochromator”, a device (either a prism, or more commonly, a diffraction grating) that splits the incident light into its component wavelengths, and allows only light of the desired wavelength to reach the sample. The ability to measure absorbance at different wavelengths is very useful, because the extinction coefficient of a compound varies with wavelength. In addition, the absorbance

spectrum of a compound can vary dramatically depending on the chemical composition of the compound, and depending on the environment (such as the solvent) around the compound.

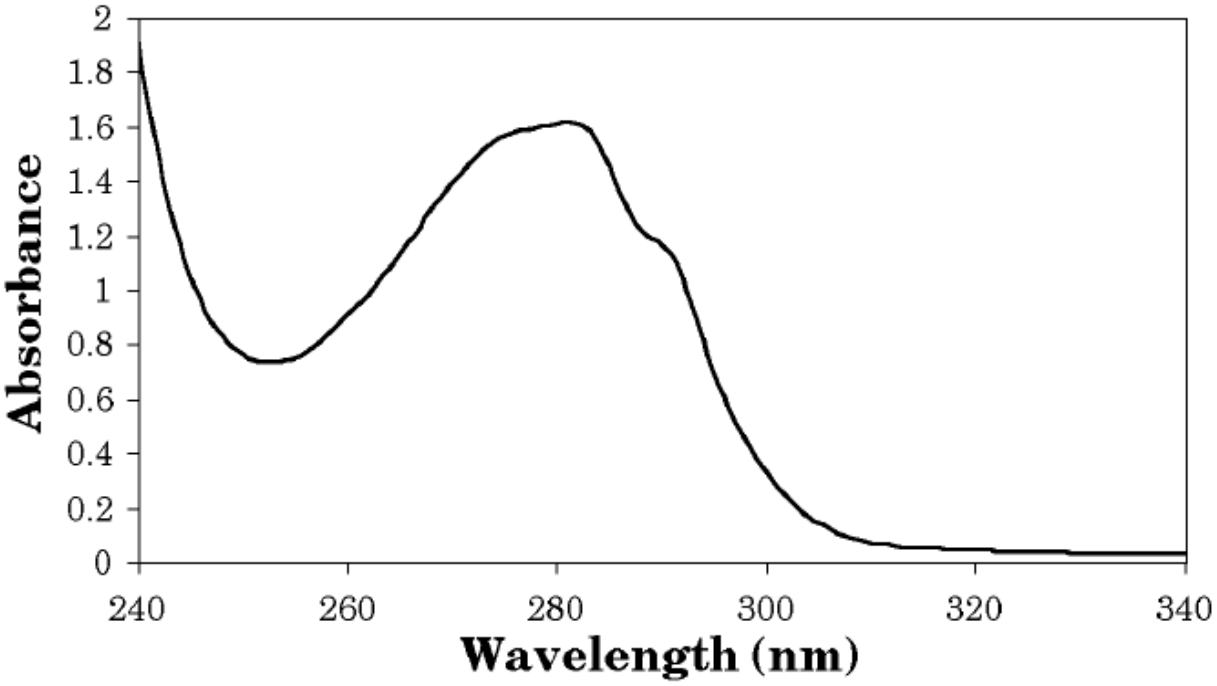

Fig.3.3 Absorbance of aromatic amino acids

The graph above shows the absorbance spectrum of a protein. The protein has a strong absorbance peak near 280 nm, but exhibits very little absorbance at longer wavelengths. For this protein, the only chromophores (chemical groups within a compound that absorb light) are the aromatic amino acids tryptophan and tyrosine.

For many proteins, these two residues are the only chromophores; because tryptophan and tyrosine only absorb in the ultraviolet portion of the spectrum, such proteins are colorless molecules. Colored proteins, such as hemoglobin, exhibit their color due to chromophores (heme, in the case of hemoglobin) that absorb in the visible portion of the spectrum.

The extinction coefficient of a molecule at a given wavelength can be calculated using the Beer-Lambert equation from absorbance measurements for solutions of known concentration.

3.2.1.3 Protein Precipitation

Many cytosolic proteins are water soluble and their solubility is a function of the ionic strength and pH of the solution. The commonly used salt for this purpose is ammonium sulphate; due to its high solubility even at lower temperatures, salting out effectiveness, pH versatility, low heat of solution, and low price. Proteins in aqueous solutions are heavily hydrated, and with the addition of salt, the water molecules become more attracted to the salt than to the protein due to the higher charge. This competition for hydration is usually more favorable towards the salt, which leads to interaction between the proteins, resulting in aggregation and finally precipitation. The precipitate can then be collected by centrifugation and the protein pellet is re-dissolved in a low salt buffer. Since different proteins have distinct

characteristics, it is often the case that they precipitate (or 'salt out') at a particular concentration of salt [34].

3.2.1.4 Common salt stock solution preparation

Preparing salt solution stock circumvents the wastage of time in taking the accurate and very small weight of each salt in every case of media preparation. Table 3.3 shows the percentage compositions of inorganic salts used in the preparation of media for cultivating the *B.subtilis* culture and producing the protease.

Procedure

1. Decide the amount to be prepared, for example 500ml.
2. Take 0.03 gram/ml of NaCl as a basis of calculation and calculate the amount each salt required for 500 ml of distilled water i.e.15, 7.5, 7.5 and 3.75 grams of NaCl, KH_2PO_4, $MgSO_4$ and $CaCl_2$ respectively.
3. For each case of media preparation, the amount of salt solution required for specific amount of media is determined and added, for example for preparing 200 ml media 8ml salt solution should be added.

3.2.2 Preparation of pre-inoculum

A preserved *B. subtilis* on agar slant obtained from a microbiologist was sub-cultured using LB(Luria-Bertani Broth medium) agar plate .Then the isolated individual colony with good microbial activity (i.e. large skim milk hydrolysis) was inoculated and cultivated in 100 ml standard shake flask using LB medium following standardized procedures. Periodically protease activity was measured by Kunitz method of enzyme assay.

Sub-culturing on LB Agar plates procedure

1. 4g of LB(ingredients shown in table 3.2) and 1g of agar was added to 75 ml distilled water in 250 ml shake flask and shaken well to ensure complete dissolve and uniform mixing
2. 1g of skim milk was dissolved in 25 ml distilled water in 100 ml shake flask
3. Autoclaved separately
4. Mixed them in laminar flow chamber which was already sterilized by UV for 10 minutes
5. It was poured to plates before it solidifies
6. The LB agar was allowed to solidify in laminar flow chamber
7. The plates were inoculated by streaking

8. The plates were sealed, labeled, dated, inverted and incubated at room temperature as shown in the following pictures.

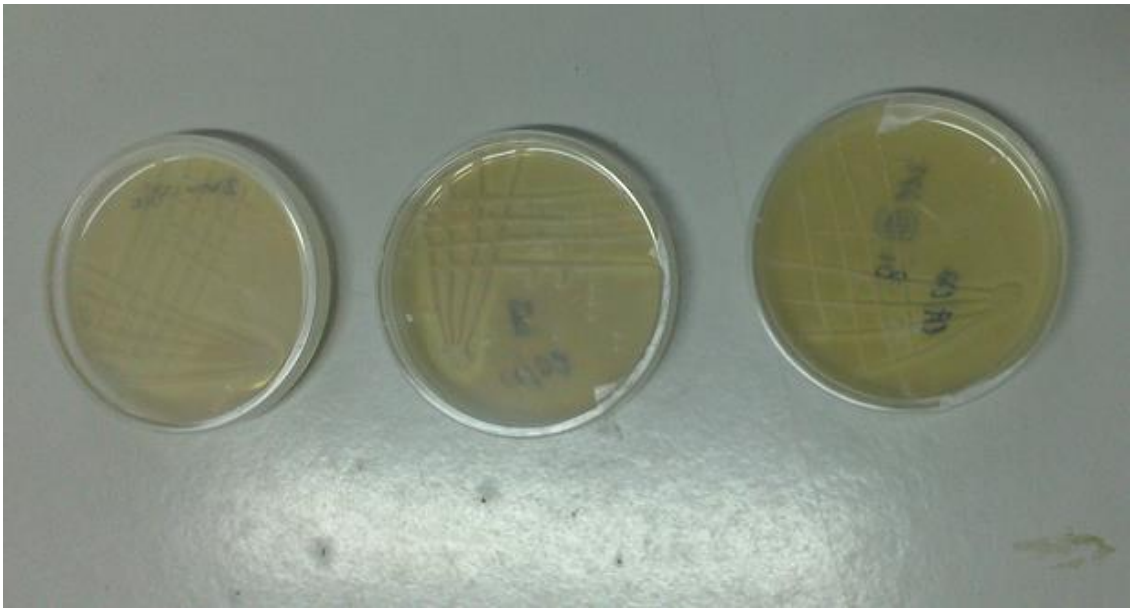

Fig.3.4a Quarter streaked LB agar plate

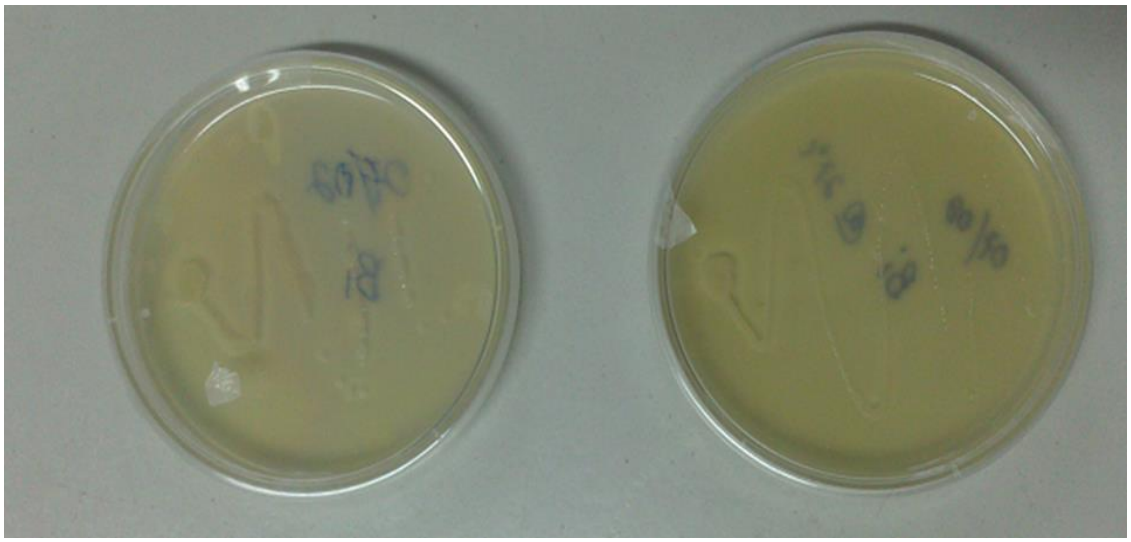

Fig.3.4b continuously streaked LB agar plate

9. The individual colony with good activity was also sub-cultured onto agar slant and preserved at 4^0C temperature for using it for further work.

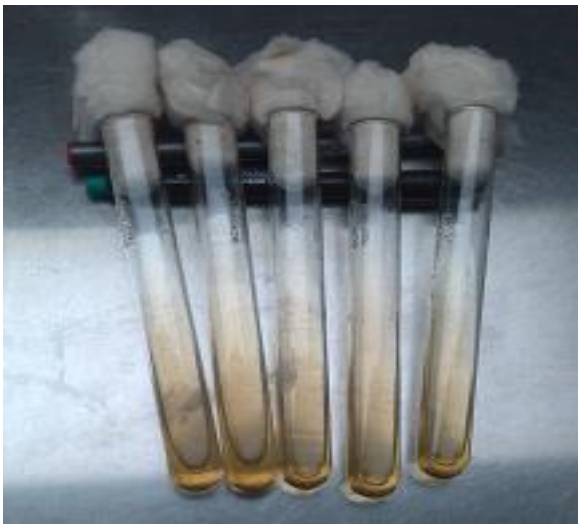

Fig.3.5 Sub-cultured *B.subtlis* on agar slants

Table 3.2 LB agar plate composition

Ingredients	Gram /liter
Casein enzymic hydrolysate	10.00
Yeast extract	5.00
Sodium chloride	10.00
Agar	15.00

Pre-inoculum preparation in shake flask procedure

1. In each of two 100 ml shake flasks 20 ml growth media was prepared
2. The media was autoclaved
3. Allowed to cool
4. A loopful of culture from agar plate that has shown good clear zone individual colony was inoculated into each flask
5. For each flask, enzyme activity was determined by Kunitz method.

3.2.3 Characterization study of culture

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

- pH
- temperature
- Time and
- Growth and production media: standard media vs soya flour.

pH, temperature and time effects were studied using lab grade standard media at flask level by applying 100 to 200_µl pre-inoculum. The formulation of the synthetic or designed LB media used for the characterization study is shown in table 3.3.

Standard growth and production media formulation

Table 3.3 LB growth and production media formulation for characterization study of *B.subtilis*

Ingredients	**% (w/v)**
Maltodextrine	0.5
Yeast extract	0.5
Skim milk	0.5
NaCl	0.12
KH_2PO_4	0.06
$MgSO_4$	0.06
$CaCl_2$	0.03

a. Effect of pH study

For the characterization study of pH a two replicates of experiments were conducted using 200 ml LB growth and production media in 250 ml shake flask.

Trial 1

In each of five 250 ml shake flasks 200 ml growth media was prepared and the pH of one of the flask was measured to be 5.5 and the other four flasks were adjusted to pH 6.0, 7.0, 8.0 and 9.0 by flakes of sodium hydroxide. Then each was sterilized, inoculated and incubated. Activity was checked by Kunitz method of enzyme assay.

Trial 2

In each of five 250 ml shake flasks 200 ml growth media is prepared and the pH of one of the flask was measured to be 6.5 and the other four were adjusted to pH 7.0, 8.0, 9.0 and 10.0 as illustrated in Fig 3.6. Then each was sterilized, inoculated and incubated. Activity was determined by Kunitz method of enzyme assay.

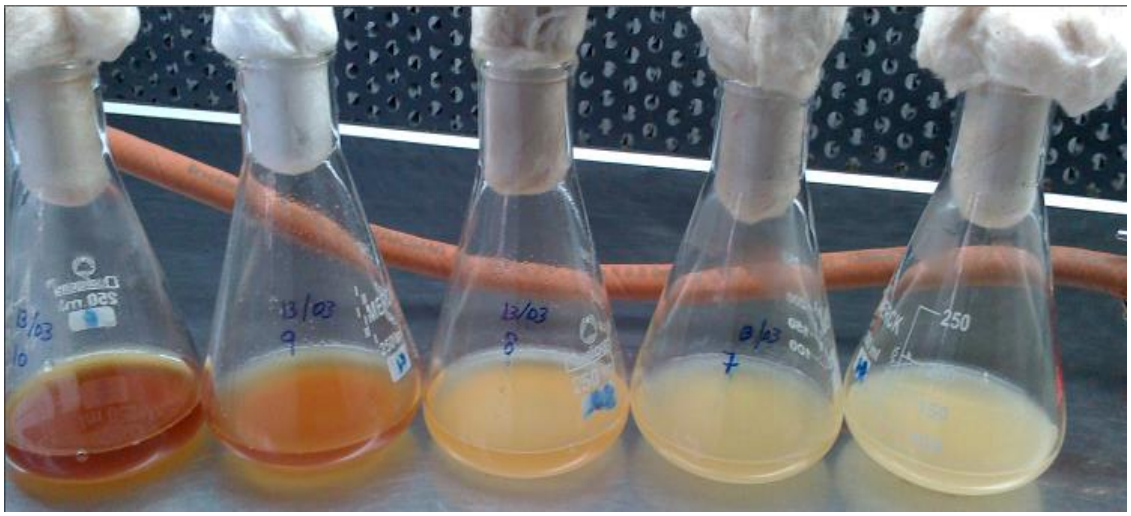

Fig.3.6 Growth media in shake flasks for characterization study of pH

b. Effect of temperature

For studying the effect of temperature on biomass growth two trials were carried out using LB standardized growth media. To do this, in each of three 250 ml shake flasks 200 ml growth media was prepared, sterilized, inoculated and incubated for 72 hours at 25, 30 and 33 degree Celsius. Activity was checked by Kunitz method of enzyme assay.

c. Effect of incubation period study

To get an idea of the effect of incubation period on the growth of culture and protease production, it was designed in such a way that it can be observed from the studies of pH, temperature and media.

d. Effect of growth and production media study

The effect of this culturing condition on the growth and protease activity was studied by comparing standard LB media against a media of soya flour. For this, 50 ml of standard LB media and 50 ml of soya flour media each in two 100 ml conical flask were taken.

All the flasks were autoclaved at 121^0C and 15 lb pressure for 20 minutes. After cooling the flasks were seeded with equal quantity of inoculum and incubated at 30^0C for 72 hours in shaker incubator. Protease activity was checked at time intervals of 24, 36, 48 and 72 hours by Kunitz method of enzyme assay.

e. The study of interaction effects of pH and temperature

An experiment designed by a Central Composite Design (CCD) was performed to investigate the effect of both the pH and temperature on protease production with time using standard medium. Table 3.4 lists the levels of the two independent variables studied. The levels of the variables were set based on the information obtained from the above characterization study of these two parameters.

Table 3.4 Process variables and levels used in the Full factorial Design

Variable (Factors)	Factor Coding	Unit	Levels	
			-1(low)	+1(high)
Temperature	A	^{o}C	25	35
pH	B	pH scale	5	9

The complete experimental design matrix of CCD for the factorial design of the study of the effect of pH and temperature on the production of protease at 48 hours of incubation time is given in Table 3.5.

Table 3.5 CCD experimental factorial design matrix for the study of combined effect of temperature and pH on protease production

Std	Run	Block	Factor 1 A:Temperature(^{0}C)	Factor 2 B:pH scale	Response 1 Protease Activity(U/ml)
6	1	Block 1	30.00	7.00	
2	2	Block 1	35.00	5.00	
7	3	Block 1	30.00	7.00	
5	4	Block 1	30.00	7.00	
1	5	Block 1	25.00	5.00	
3	6	Block 1	25.00	9.00	
4	7	Block 1	35.00	9.00	
12	8	Block 2	30.00	7.00	
13	9	Block 2	30.00	7.00	
9	10	Block 2	37.07	7.00	
8	11	Block 2	22.93	7.00	
14	12	Block 2	30.00	7.00	
10	13	Block 2	30.00	4.17	
11	14	Block 2	30.00	9.83	

The statistical analysis: analysis of variance (ANOVA), a regression analysis and the plotting of response surface was performed by Design expert 7.0 aprobado software.

3.2.4 Fermentation

a. Pre-inoculum preparation

In preparing the pre-inoculum, 200 ml of LB media in each of two 1000 ml shake flasks were taken. The flasks were autoclaved, cooled to room temperature, inoculated with one full slant of culture and incubated for 18 hours in a shake incubator. OD (optical density) was measured and protease activity measured by Kunitz method.

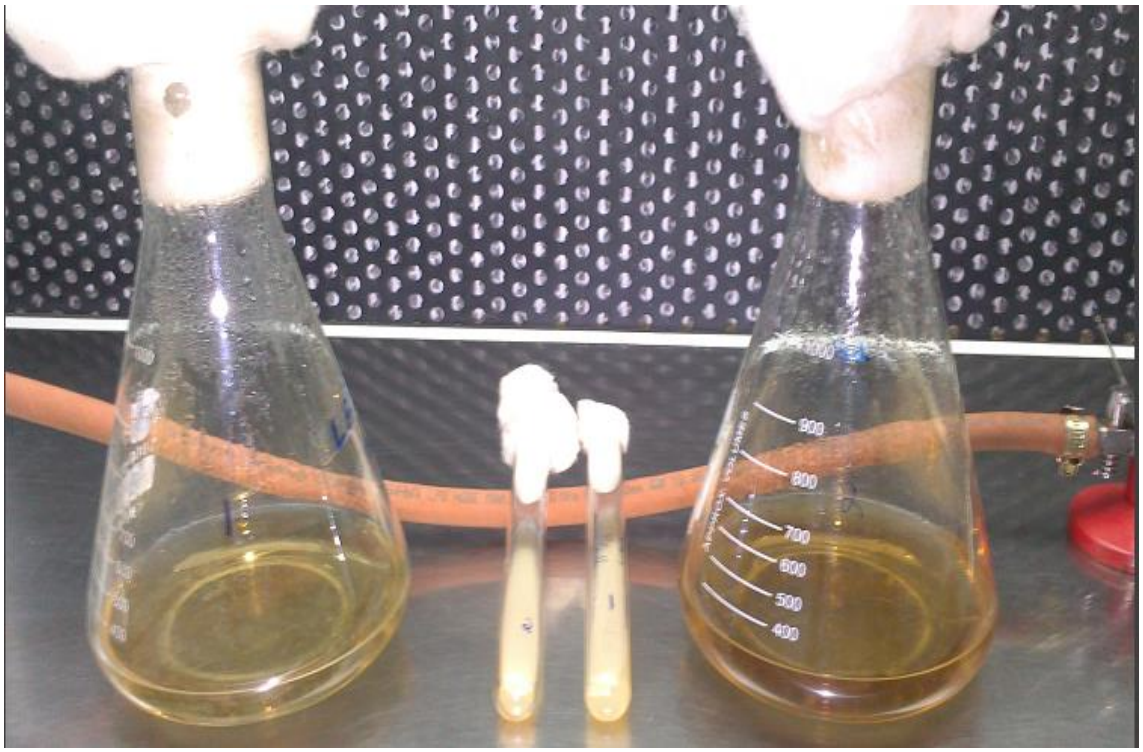

Fig.3.7 Preserved culture in agar slant and shake flasks containing LB media

b. Seed culture preparation

In going for pilot scale fermentation enough amount of inoculum was prepared. For this, in each of two 7 liters seed fermenters 4 liters of production media of soya flour (formulation shown in the table 3.6) were prepared, pH was measured to be 6.0 and adjusted to 6.5 by 1N NaOH, sterilized, cooled to process temperature of 30 ± 2 ^{0}c and each fermenter was inoculated with 200 ml pre-inoculum and fermentation was undertaken for 24 hours.

Fig.3.8 Pre-inoculum in a shake flask being transferred to seed fermenter

The OD was measured and the enzyme activity was determined at time intervals of 12, 18 and 24 hours. The one that has shown good activity was used for inoculating the main fermenter.

c. Pilot scale fermentation

In parallel to inoculum preparation, in pilot fermenter 40 liters of soya bean flour media was taken, sterilized and cooled to optimum operating temperature of 30 ± 2 ^{0}c. And also during this period, fermenter accessories like hoses that were used for transferring inoculum from flask to seed fermenter and from seed fermenter to pilot fermenter and needles, etc. were sterilized.

Table 3.6 Media formulation for the seed and pilot fermenters

Ingredients	**%(w/v)**
Soya flour	1.1
NaCl	0.12
KH_2PO_4	0.06
$MgSO_4$	0.06
$CaCl_2$	0.03

After making sure that all the necessary equipment and conditions were ready and seed culture was prepared, pilot scale fermentation was commenced and undertaken for 27 hours. The protease activity was measured periodically during the fermentation process until it started decreasing. Then the broth was collected and centrifuged for recovering the protease in the supernatant and stored at 4^0C for further downstream processing.

During the fermentation process, the factors affecting it were closely monitored and controlled:

- Temperature: 30 ± 2 ^{0}C
- PH:6.5-7.0
- Aeration:0.5-1vvm
- The RPM of agitator:100-150
- Foam formation: anti foam addition when required.
- Duration: until the growth reached stationary phase
- stationary phase

3.2.5 Downstream processing

After successful fermentation the enzyme was separated, purified and treated chemically to the required level of purity, stability and concentration to serve its ultimate purpose of effective and efficient dehairig activity. For this the primary downstream processing were employed:

a. Crude enzyme formulation

For this, 15 liters of fermentation broth was taken and 0.1% Bronopol, 0.2 % sodium Benzoate and 2% table salt were added to render it with enhanced stability. The formulated crude was kept in a refrigerator at a temperature of 4^0C until it was applied for dehairing.

Fig.3.9 Part of formulated crude protease ready for dehairing purpose

b. Ammonium sulphate precipitation

The ammonium sulphate precipitation of the protease was done based on well-established principles and basic procedures [43, 44]. In effecting this, 10 liters of crude enzyme was taken and the enzyme was precipitated from the crude extract by the gradual addition of solid ammonium sulphate with gentle and continuous overnight magnetic stirring to 80% saturation (following the guideline outlined in Annex 1) at a temperature of 4^0C. The protease pellet was collected by centrifugation at 10,000 rpm for 10 min and re-dissolved by bicarbonate buffer of pH 9.5 which should be 1-2 times the volume of the precipitate, since that will be enough to reduce the salt concentration to well below the precipitation point of the proteins present [46].The collected pellet was kept in a refrigerator at a temperature of 4^0C until it was applied for dehairing.

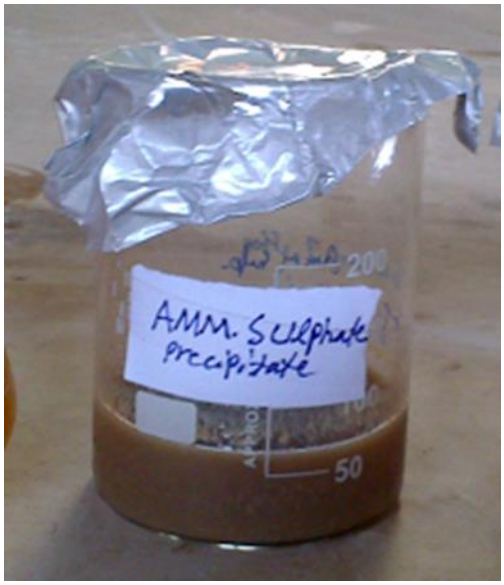

Fig.3.10 Sample of ammonium sulphate precipitated protease

c. Ultrafiltration (UF)

For this part of downstream processing, 8 liters of crude extract was taken and filtered through 0.2 µm pore size microfiltration membrane to remove the biomass that otherwise would clog the UF membrane. Then the extract was filtered and concentrated to about 20 times by UF having a membrane of 10 kDa pore size. The filtrate was kept in a refrigerator at a temperature of 4^0C until it was applied for dehairing.

Fig.3.11 Sample of protease prepared for dehairing skins and hides by U F

d. Spray drying

To get the protease in dry form, 5 liters of crude extract was taken and 20 % maltodextrin was added into it to serve as a carrier. This preparation was processed in a spray drier and the powder was made into a paste using bicarbonate buffer of pH 9.5. The paste was kept in a refrigerator at a temperature of 4^0C until it was applied for dehairing.

Fig.3.12 Paste of protease prepared by spray drying for dehairing skins and hides

3.2.6 Evaluations of the efficacy of the protease products of downstream processing

The enzyme prepared for use by ultrafiltration, spray drying and ammonium sulphate and crude enzyme were applied to pre-soaked raw cow hides and sheep skins to check hair removal efficiency by comparing against the conventional unhairing method.

a. Ammonium sulphate precipitate

As a rule of thumb, 3 to 4% offer of protease precipitate based on soaked hide or skin weight is accepted by most tanners for attaining virtually complete hair removal depending on the unit activity of the precipitate. So in exploring the efficacy of the precipitate in removing the hair, the upper limit i.e. 4% of the precipitate was taken. Then unit activity was measured and the total activity which would serve as a base in determining the required quantities of protease products of other downstream processing was determined. The protease precipitate was applied on the flesh side of soaked half piece of sheep skin and one side of cow hide. Then they were folded flesh to flesh and kept under cover for 5 hours. Hair loosening was rated every hour and finally hair removal efficiency was compared against the conventional lime-sulfide unhairing method.

b. Crude protease extract

For this, the unit activity of the crude extract was measured and the amount required was determined based on the weight of the material and the total activity of ammonium sulphate precipitate i.e. by dividing the total activity of protease precipitate by unit activity of the crude. Soaked half piece of sheep skin and one side of cow hide were soaked in protease crude for 5 hours. Hair loosening was rated every hour and finally hair removal efficiency was compared against the conventional lime-sulfide unhairing method.

a. Ultrafiltered concentrate

Similar to the case of the crude extract, first the unit activity of the protease filtrate was measured and the required amount was determined based on the total activity of ammonium sulphate precipitate. The filtrate was applied on the flesh side of soaked a half piece of sheep skin and one side of cow hide. Then they were folded flesh to flesh and kept under cover for 5 hours. Hair loosening was rated every hour and finally hair removal efficiency was compared against the conventional lime-sulfide unhairing method.

b. Spray dried formulation

The unit activity of the protease paste was measured and the required amount was determined in similar fashion to the other methods. The paste was applied on the flesh side of soaked a half piece of sheep skin and one side of cow hide. Then they were folded flesh to flesh and kept under cover for 5 hours. Hair loosening was rated every hour and finally hair removal efficiency was compared against the conventional lime-sulfide unhairing method.

4. RESULTS AND DISCUSSION

4.1 Preparation of pre-inoculum at shake flask level

In preparing pre-inoculum for the study, a preserved *B. subtlis* was obtained from Department of Biotechnology, CLRI and sub-cultured on LB agar plate. Subsequently the isolated individual colonies that have shown large clear zone (i.e. large skim milk hydrolysis) were inoculated into and cultivated in two 100 ml standard shake flasks by taking 20ml LB medium in each of them. The cultivation of the culture was done at a measured pH of 5.0

(flask A) and 5.5(flask B) and a temperature of 30^0C. Protease activity was determined for the samples taken from each flask within 12 to 72 hours by Kuntz method of enzyme assay. The result obtained was presented in Fig 4.1.

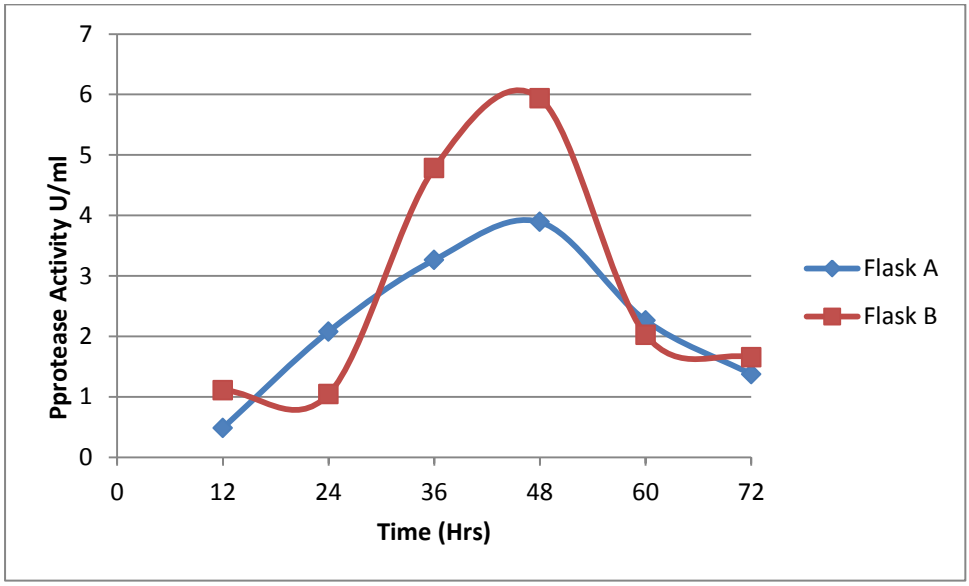

Fig.4.1 Protease activity of *B.subtilis* in LB culture growth and production media

As it was observed from the figure, the activity at this stage is promising since the environmental, physical and chemical conditions allowing optimal growth and productivity of the culture were further going to be studied and determined under the characterization aspect of the culture.

.4.2 Characterization of the culture

By performing a range of experiments under applicable different operating conditions i.e. by varying medium constituents, pH, temperature and incubation time, optimum growth/production conditions have been established.

a. Effect of pH study

It was reported that alkaline proteases belong to the group of proteases, which have either a serine center or metallo-type, exhibiting a wide pH range of 6-13 [6].Based on this knowledge, the influence of pH on the growth of biomass and protease production was studied by adjusting the production medium at various levels of pH. To accomplish this two trials or runs were carried out. For the case of the first trial, in each of five 250 ml shake flask 200 ml growth media was prepared and the pH of one of the flasks was measured to be 5.5 and the other four were adjusted to pH 6.0, 7.0, 8.0 and 9.0 by flakes of sodium hydroxide. In similar fashion the effect of pH for the second trial was studied; the media of one of the flask was measured to be 6.5 and the rest were adjusted to pH of 7.0, 8.0, 9.0 and 10.0. After measuring and adjusting the pH, each was sterilized, inoculated a full loop of inoculum and incubated in a shake incubator. Samples were taken from the flasks periodically and activity was determined by Kunitz method of enzyme assay. The results are presented in the Fig 4.2.

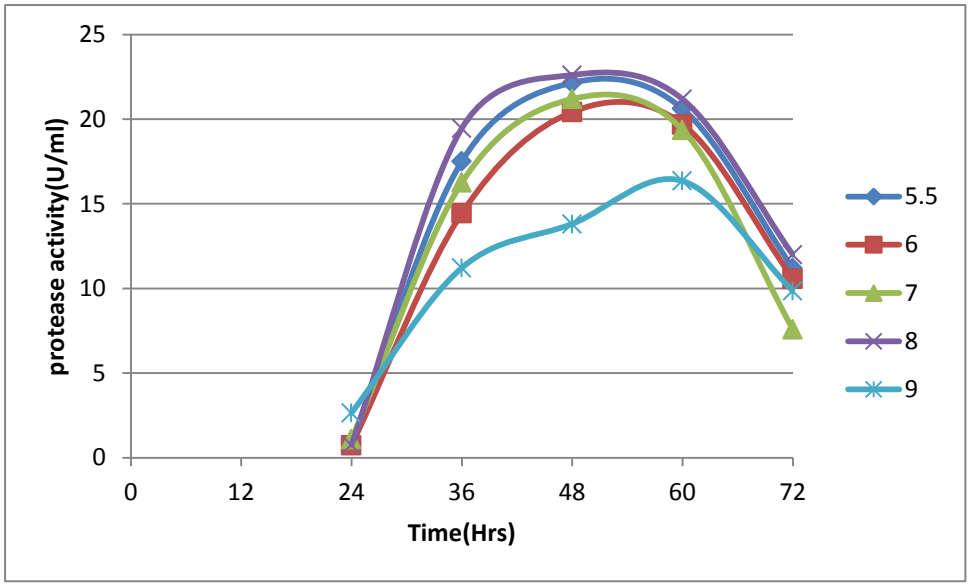

Fig.4.2a Effect of pH on protease production in flask 1

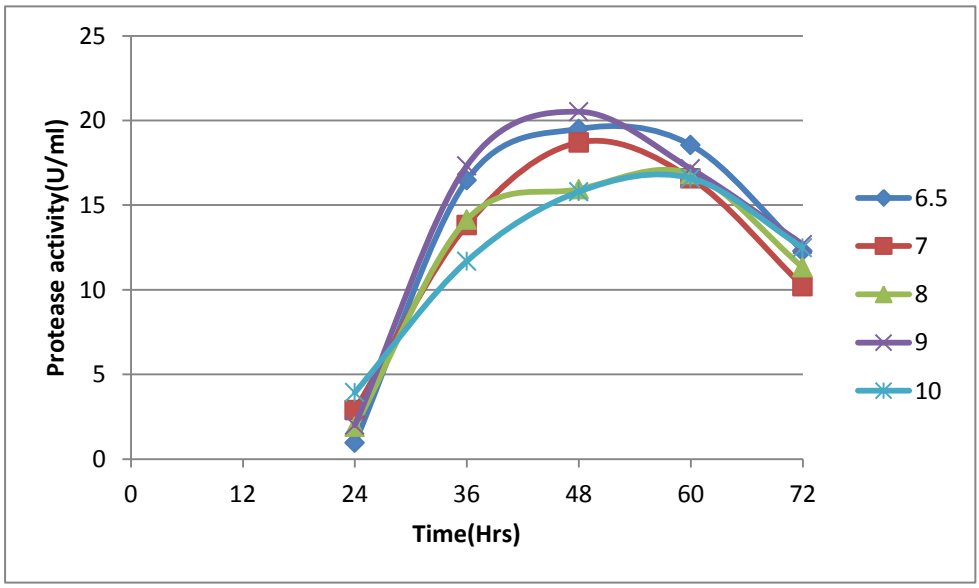

Fig.4.2b Effect of pH on protease production in flask 2

In investigating the effect of pH in the two above curves ,maximum protease production of about 13.8 to 22.6 U/ml at the end of 48th hour was observed for all ranges of pH considered (5.5 -10).The pH ranges of 6-8 can be deduced as optimum condition for the production of protease.

b. Temperature effect study

Descriptive studies have characterized B.subtilis as a mesophile, although a hardy one, growing at temperatures as low as 5 to 20^{0}C and as high as 45 to 55^{0}C[35].Taking this as an input, an attempt was made to observe the effect of temperature on the growth of culture and

protease production. In doing this, 200 ml of standard media was taken in each of three 250 ml shake flasks for two replicates. The flasks were then sterilized, inoculated with a full loop of inoculum and incubated at temperatures of 25, 30 and 35 degree Celsius. The response for this independent variable effect was protease activity which was determined by Kunitz method of enzyme assay. The result is illustrated in Fig.4.3.

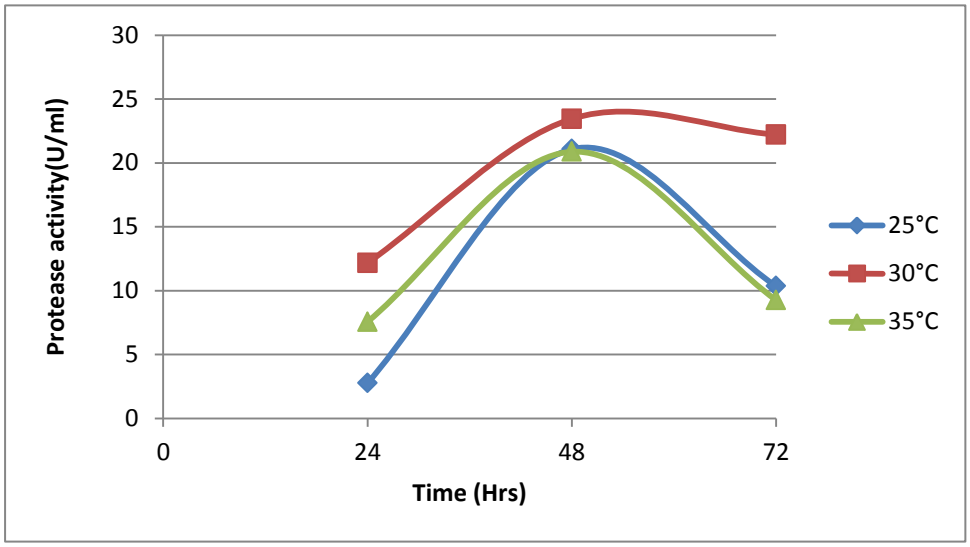

Fig.4.3a Effect of temperature on protease production in flask 1

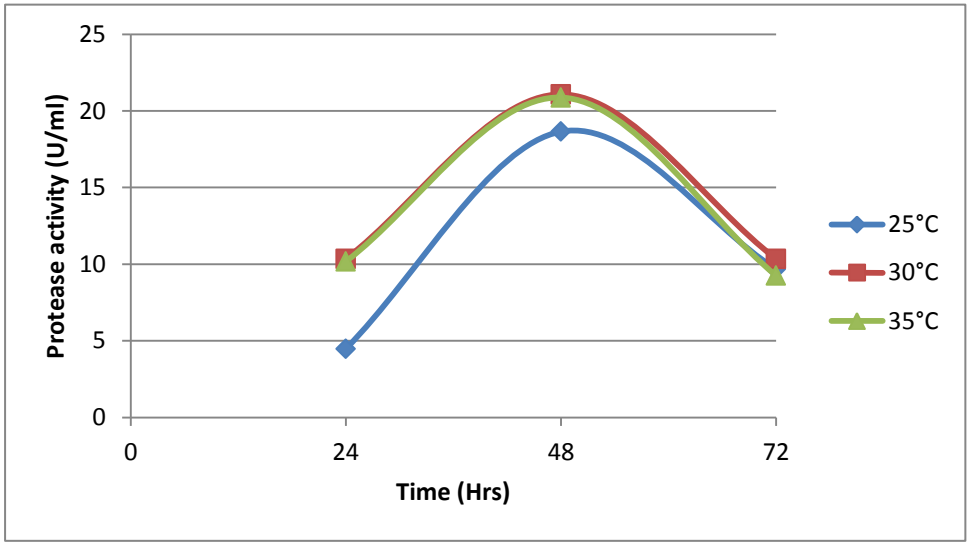

Fig.4.3b Effect of temperature on protease production in flask 2

From examining the graphs, we note that the temperature of 30^0C gives a maximum activity of 21 to 24 U/ml at an incubation time of 48 hours indicating optimum temperature for the growth of biomass of interest and protease production. A plus and/or a minus of 2 ^{0}C temperature range reasonably could be operating temperature for fermentation process.

c. Media

Growth and development of microorganisms are greatly affected by the chemical conditions of their environment. The rates of utilization of various nutrients differ in each substrate, and so does productivity [33]. For this reason, the impact of standard or designed LB and soy bean flour media on the growth and protease production by *B.subtilis* was investigated. The results were given in Fig.4.4

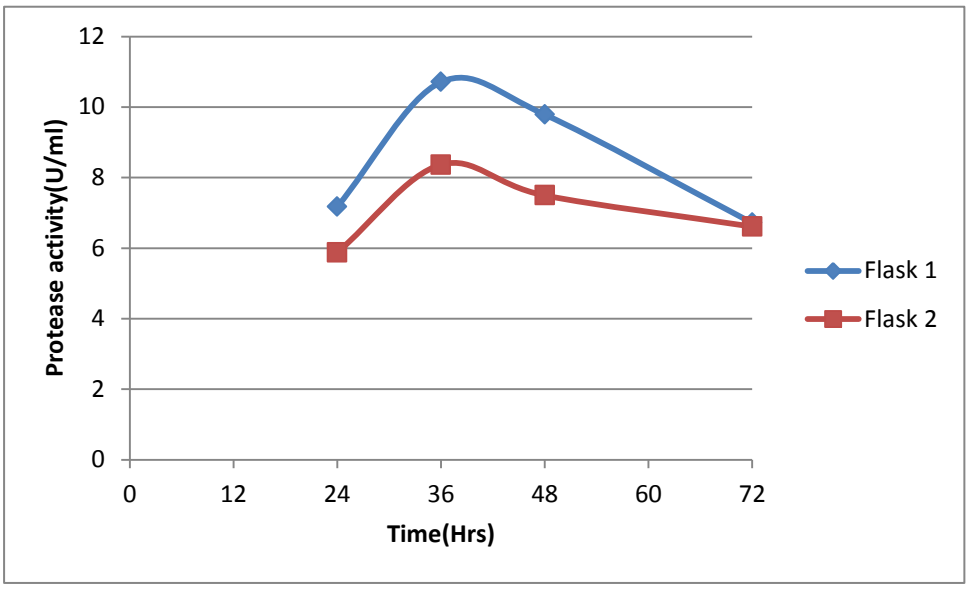

Fig.4.4a Effect of standard LB media on protease production

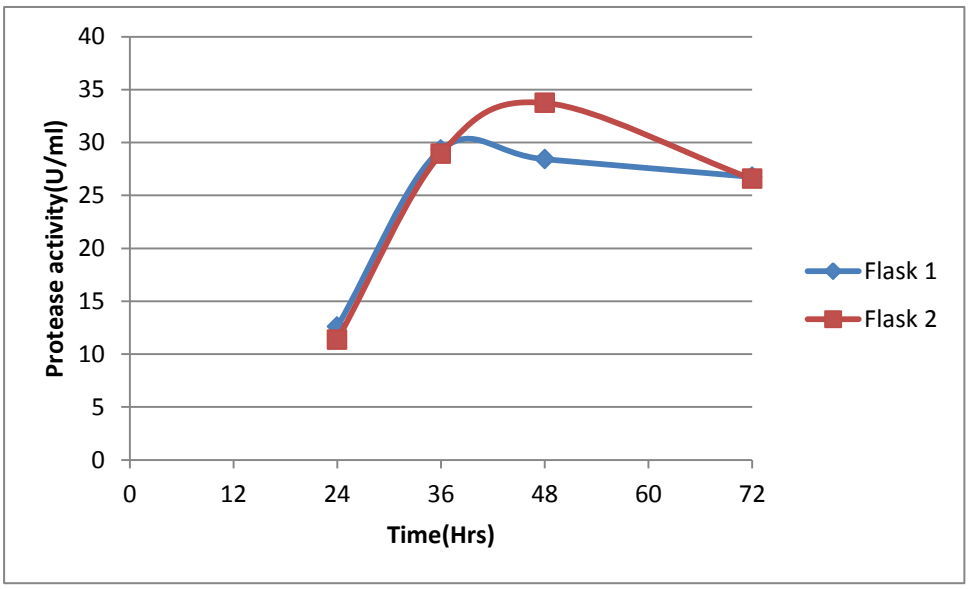

Fig.4.4b Effect of soy bean flour media on protease production

From examining the graphs, we note that the standard media gives a maximum activity of 8.2 to 10.5 U/ml at 36 hours incubation time while soy flour gives 30 to 34 U/ml at incubation time range of 36 to 38 hours.

d. Time

The effect of time can be observed from the studies of pH, temperature and media. Running the fermentation process up to the time range in which a higher and an increasing protease activity was observed (27 to 38 hours), could be logical and feasible.

e. The study of interaction effect of pH and temperature

In this part of the study, the relationship between controllable experimental factors (pH and temperature) and the response (protease production) was investigated by Response Surface Methodology (RSM), 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 [13].

Advantages of RSM

- Greatest amount of information from experiments.
- Forces you to plan.
- Know how long project will take.
- Gives information about the interaction between variables.
- Multiple responses at the same time.
- Gives information necessary for design and optimization of a process.

Disadvantages of RSM

- Tells what happens, not why.
- Notoriously poor for predicting outside the range of study.

To be economical in terms of all aspects of research resources including time, only these two major bioprocess influencing factors were given a particular attention and focus in studying their combination effect on protease production. The combination effect of the parameters was considered at incubation time of 48 hours as the characterization studies of both factors seem to give maximum protease production at this time. The experimental design performed by a CCD and the results for protease production was shown in Table 4.1. The range for pH was 5-9 and for temperature was 25-35 ^{0}C which were set based on the information available from characterization study experiments.

Table 4.1 experimental runs of the CCD and protease activity as a response

Run	Factor 1 A:Temperature(^{0}C)	Factor 2 B:pH scale	Response 1 Protease Activity(U/ml)
1	30.00	7.00	21.35
2	35.00	5.00	13.27
3	30.00	7.00	23.68
4	30.00	7.00	21.22
5	25.00	5.00	8.37
6	25.00	9.00	11.86
7	35.00	9.00	15.51
8	30.00	7.00	22.04
9	30.00	7.00	21.63
10	37.07	7.00	17.36
11	22.93	7.00	14.59
12	30.00	7.00	21.25
13	30.00	4.17	10.03
14	30.00	9.83	12.56

Development of Model Equation

On the basis of ANOVA, the relationship between controllable experimental factors (pH and temperature) and the response (protease production) and the interactions of the concerned variables was established and is given by the quadratic regression model equation….in terms of coded factors.

Protease Activity= 21.86 + 1.56*A +1.16*B -0.31*A*B-3.29A^2-5.63*B^2 ----------4.1

Where A= Temperature
B= pH

ANOVA for Response Surface Quadratic Model was shown table 4.2.

Table 4.2 Summary of the ANOVA and parameter estimates for model coefficients of CCD

Source	Sum of Squares	Degree of freedom	Mean Square	F Value	p-value Prob > F	Remark
Block	0.57	1	0.57			
Model	325.22	5	65.04	41.56	<0.0001	significant
A-Temperature	19.43	1	19.43	12.41	0.0097	
B-pH	10.83	1	10.83	6.92	0.0339	
AB	0.39	1	0.39	0.25	0.6327	
A^2	79.88	1	79.88	51.04	0.0002	
B^2	233.98	1	233.98	149.50	<0.0001	

Residual	10.96	7	1.57			
Lack of Fit	7.74	3	2.58	3.21	0.1447	Not significant
Pure Error	3.22	4	0.80			
Cor Total	336.75	13				
Std.Dev.	1.25		R-squared	0.9674		
Mean	16.77		Adj R-Squared	0.9441		
C.V.%	7.46		Pred R-Squared	0.8032		
PRESS	66.17		Adeq.Precision	14.586		

Model Adequacy Check

The fitness of the model was checked using values of p, F and regression coefficients. Each source of terms with the probability ("Prob > F") below 0.05 were eliminated for better fitness of model. The contribution of each source terms for the model equation could be seen from table 4.3. Obviously the model is significant as it has a lower p value of 0.0001 and a higher R^2 value of 0.9674.In general, the model is good enough as the R^2 value showed that 96.74% of the variations of the experiment could be accounted by the model.

Table 4.3 Lack of fit test for each source of terms

Source	**Sum of Squares**	**Degree of freedom**	**Mean Square**	**F Value**	**p-value Prob > F**	**Remark**
Linear	302.70	6	50.45	62.76	0.0007	
2FI	302.31	5	60.46	75.22	0.0005	
Quadratic	7.74	3	2.58	3.21	0.1447	Suggested
cubic	4.48	1	4.48	5.57	0.0776	Aliased
Pure Error	3.22	4	0.80			

The interactive effect of temperature and pH on protease production using standard or synthetic LB medium at 48 hours of incubation time is shown in fig.4.5.

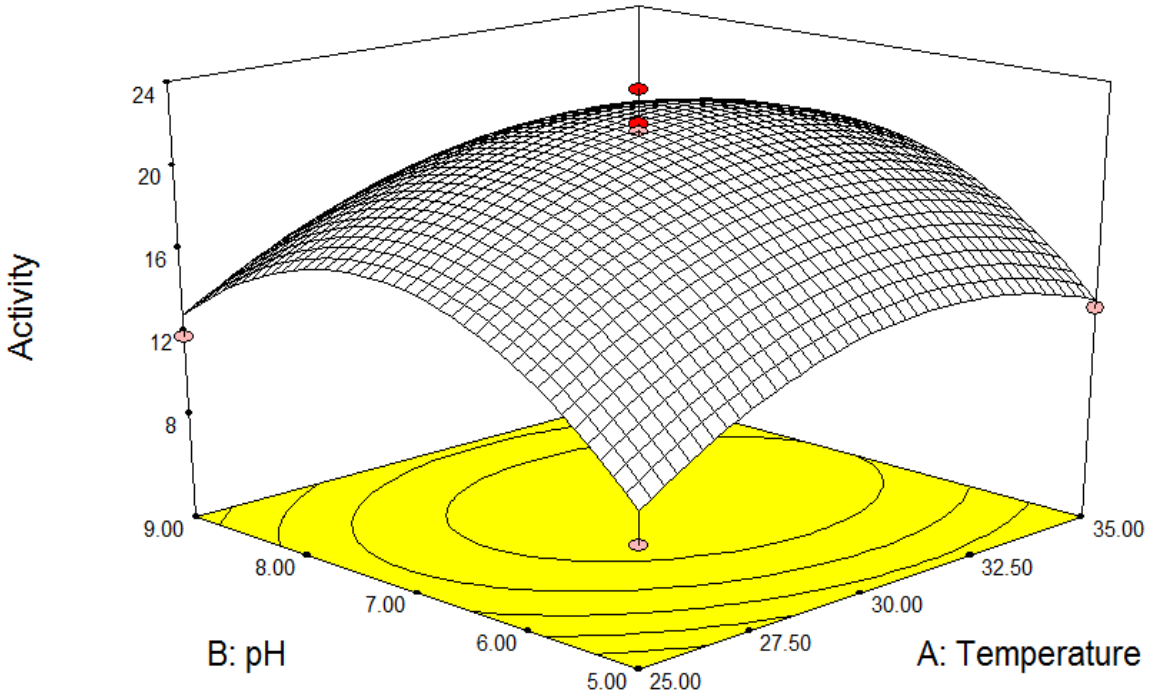

Fig.4.5 The interactive effect of temperature and pH on protease production

The 3D response surface plot for the interaction between pH and temperature shows the maximum protease activity was observed when the pH is 8 and temperature is 30.9 ^{0}C. A further increase or decrease of the two factors reduces the protease activity.

4.3 Fermentation

a. Seed culture preparation

In going for pilot scale fermentation enough amount of inoculum was prepared. For this, in each of two 7 liters seed fermenters 4liters of production media of soya flour were prepared, pH was measured to be 6.0 and adjusted to 6.5 by 1N NaOH, sterilized, cooled to process temperature of 30 to 31^{0}Cand each fermenter was inoculated with 200 ml pre-inoculum and fermentation was undertaken for 24 hours. Results are given in Fig 4.6.

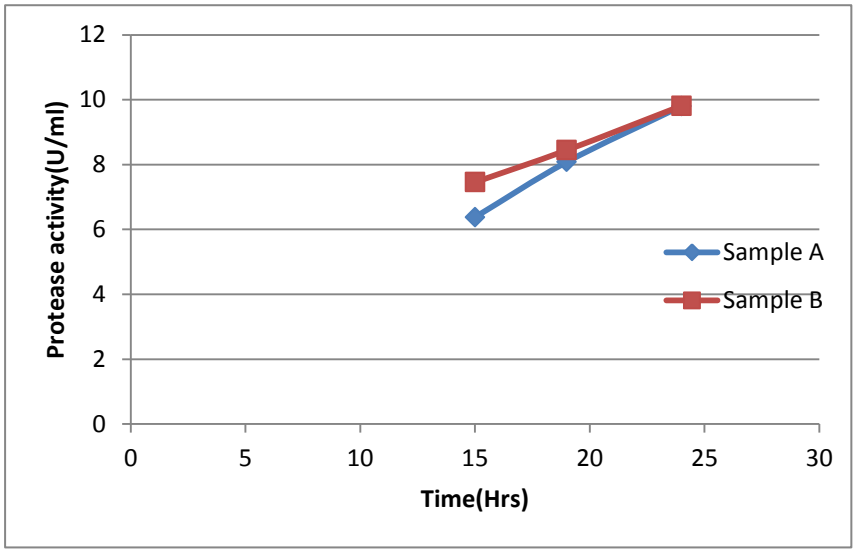

Fig.4.6a protease activity in soy bean flour media in Seed Fermenter 1

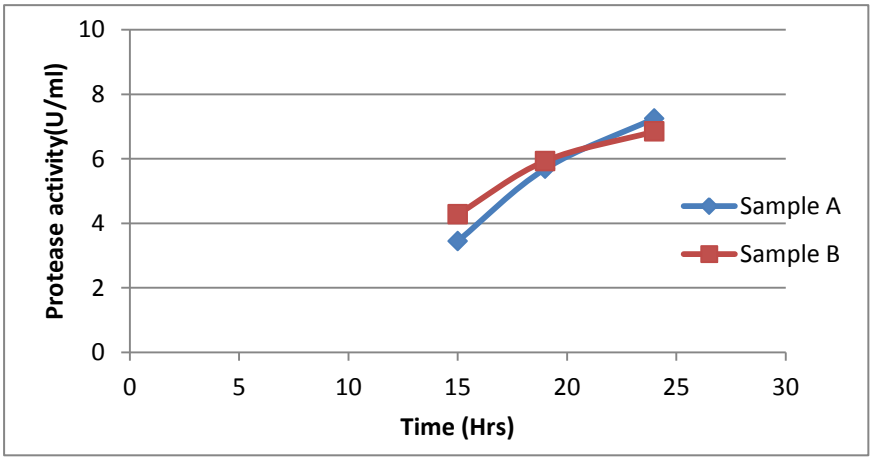

Fig.4.6b Protease activity in soy bean flour media in seed fermenter 2

From examining the graph, obviously seed culture prepared in fermenter 1 has to be used for inoculating the pilot scale fermenter.

b. The pilot scale Fermentation

At this scale of fermentation process, the biochemical engineering techniques involved in successful commercialization of very small (shake flask) lab –scale biological process into large-scale processes that are both technologically viable and economically efficient were studied. The pilot plant also provides the cultured broths needed for downstream processing and can generate information to determine the optimal cost structure in manufacturing and energy consumption as well as the testing of various raw materials in the medium [10].

During the fermentation process, the pH, DO (dissolved oxygen), temperature, agitation speed, culture volume and pressure can be measured with sensors located in the fermenter. The output of the individual sensors is accepted by the computer for the on-line, continuous and real-time data analysis. Information stored in the computer control system then regulates the gas flow valves and the motors to the feed pumps.
Automatic temperature control during the fermentation is accomplished by injecting either cold or hot water into the outer jacket and/or internal coils.

To prevent the risk of contamination, gases introduced into the fermenter should be passed through a sterile filter. A similar filter on the air exhaust system avoids environmental contamination. Sterile filtered air or oxygen normally enters the fermenter through a sparger system, and air flow rates for large fermenters rarely exceed 0.5–1.0 Volumes of air per Volume of medium per Minute (vvm).

Foam production in bioreactors is often a major problem, particularly in aerated fermentations. Formation of foam is due to the presence of surface-active agents, especially proteins, which produce stable foams. If not controlled this can lead to contamination and blockage of air filters. There are basically three methods used to control foam production: media modification, mechanical foam-breaking devices or the automatic addition of chemical antifoam agent.

During fermentation, methods are required for the routine determination of the microbial population, cell number and/or biomass, in order to monitor its progress. Numerous direct and indirect methods are available for this purpose. Direct procedures involve dry weight determination, cell counting by microscopy and plate counting methods. Indirect methods include turbidimetry, spectrophotometry, estimation of cell components (protein, DNA, RNA or ATP), and online monitoring of carbon dioxide production or oxygen utilization. For the case of this study microbial growth in the culture was monitored by Kunitz method of spectrophotometry technique. The result was given in Fig.4.7.

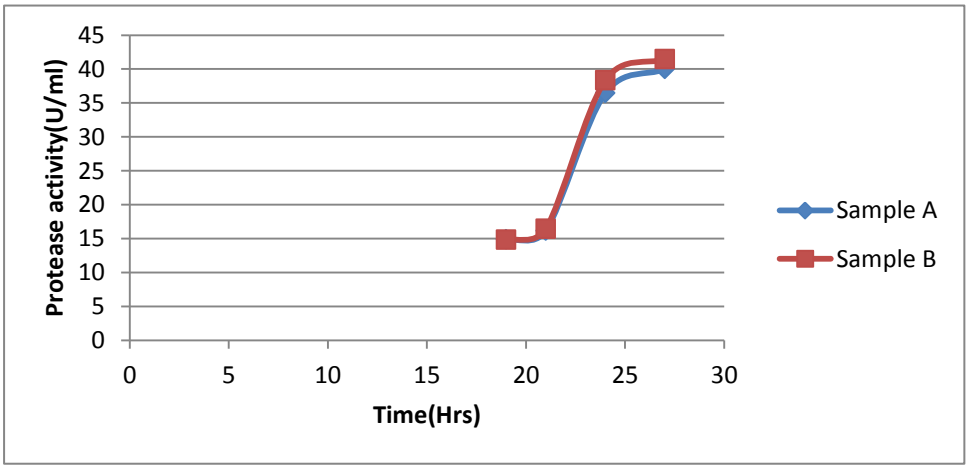

Fig.4.7 Protease activity during the fermentation process at pilot scale fermenter
As indicated by the curve, the fermentation was terminated just prior to the cessation of growth at protease activity of 40 U/ml and fermentation time of 27 hours. This cessation of

growth could be due to depletion of essential nutrients (carbon source, essential amino acids, etc.) or the build-up of toxic metabolites, such as ethanol and lactic acid, or a combination of nutrient depletion and toxin accumulation.

During batch fermentations certain environmental conditions continually change, particularly nutrient and product concentrations, as does the specific growth rate, because the cells must pass through the sequence of growth phases described above. Consequently, the system never achieves steady-state conditions. A further disadvantage is that several distinct practical stages are associated with the operation of batch fermentation [11]:

1. charging of the fermenter with fresh medium;

2 .sterilization of the fermenter and medium;

3 .inoculation of the fermenter;

4 production of microbial products;

5 .harvesting of biomass and spent fermentation broth; and finally

6 .cleaning of the vessel.

This has major economic implications for industrial processes. For a considerable period of time, the fermentation vessel is not producing microbial products, but is being cleaned, filled, sterilized, etc. The non-productive period is referred to as the down-time of the fermenter.

4.4 Evaluations of the efficacy of the products of downstream processing

DSP encompasses all processes following the fermentation. It has the primary aim of efficiently, reproducibly and safely recovering the target product to the required specifications (biological activity, purity, etc.), while maximizing recovery yield and minimizing costs. The target product may be recovered by processing the cells or the spent medium depending upon whether it is an intracellular or extracellular product. The level of purity that must be achieved is usually determined by the specific use of the product. Often, a product's purity will be defined by what is not present rather than what is. Purity of an enzyme, for example, is expressed as units of enzyme activity per unit of total protein. Not only is it important to reduce losses of product mass, but in many cases retention of the product's biological activity is vitally important.

It was reported [13] that DSP is very important step in biotechnology as it determines the overall economy of bio-product manufacturing, degrees of concentration, purity and stability of the final product. The degree of purity of commercial enzymes ranges from raw enzymes to highly purified forms and depends on the application. Often enzymes may be purified several hundred-fold but the yield of the enzyme may be very poor, frequently below 10% of the activity of the original material. In contrast, industrial enzymes will be purified as little as possible, only other enzymes and material likely to interfere with the process which the enzyme is to catalyze, will be removed. Unnecessary purification will be avoided as each additional stage is costly in terms of equipment, manpower and loss of enzyme activity. As a result, some commercial enzyme preparations consist essentially of concentrated

fermentation broth, plus additives to stabilize the enzyme's activity. Downstream processing involves isolation and purification steps and ends up in the formulation of the enzyme preparation. In this study, primary unit operations of downstream processing were employed to achieve partial purification of fermentation products. And then the efficacies of the products of each method were evaluated for removing the hair in reasonable period of time. The intention of doing this is to come up with the optimum formulation involving minimal downstream processing steps and hence, lower processing costs without compromising the efficacy. This in turn shows any possibilities of supplying the final protease at attractive prices so as to encourage the Ethiopian tanners for using the product for dehairing and save the environment. On top of this, giving the reader an insight into the techniques available for downstream purification of the enzyme of interest is another agenda of this section.

Bearing this in mind, the enzyme formulations were prepared for use by ultrafiltration, spray drying and ammonium sulphate and crude enzyme. Then protease product of each downstream processing technique was applied to pre-soaked raw cow hides and sheep skins to check hair removal efficiency by comparing against the conventional unhairing method. The results were presented in Fig. 4.8 to 4.16 and Tables 4.4 and 4.5. In this dehairing step, the use of protease enzyme instead of chemical treatment with lime and sodium sulphide only digests the cells of the root section of the hairs, leaving the keratinized (hornified) parts unaffected, thereby enabling the recovery of hair with good quality and strength. It was reported that [1,40] enzymatic method of unhairing has additional advantages; reduction in the COD and BOD of effluent, easy handling by workers and enzymatically dehaired skins show better strength properties and greater surface area. The enzymatic action during dehairing, to some degree, also digests non-leather forming proteins as albumins, globulin and mucoids, and split collagen fibres. Because of this, separate bating can be avoided.

a. Conventional method

In conventional unhairing processes of sheep skin and cow hide lime and sodium sulphide were employed. Annexes 3, 4 and 5 give the detail.

Fig.4.8**a** Sheep skin before unhairing

Fig.4.8**b** Sheep skin after unhairing

Fig.4.9**a** Cow hide before unhairing

Fig.4.9**b** Cow hide after unhairing

b. Ammonium sulphate precipitate

Reduction of protein solubility can be achieved by adding organic solvents, such as acetone, ethanol and isopropanol or by the addition of inorganic salts at high ionic strength like ammonium sulphate. Ammonium sulphate is popular because of its high solubility, low toxicity, salting out effectiveness, pH versatility, low heat of solution, and low cost [10, 34]. For evaluating the efficacy of the protease precipitate of this salt in removing the hair, unit activity was measured to be 360 units per ml. In enzymatic method of unhairing, experience revealed that 3-4 %w/w offer of precipitate on the basis of the weight of skin or hide would give higher degree of hair removal efficiency. For this case, 4% was taken and the total activity required to unhair one kg of skin/hide for this much offer of protease was calculated to be 14400 units per ml. For the respective weight of half piece of sheep skin and half side of cow hide, the required amount of protease precipitate was taken and applied on the flesh side .Then they were folded flesh to flesh and kept under cover for 5 hours. Hair loosening

was rated every hour and finally hair removal efficiency was compared against the conventional lime-sulfide unhairing method. The results are shown in fig.4.10 and 4.11.

Fig.4.10**a** Sheep skin before unhairing

Fig.4.10**b** Sheep skin after unhairing

Fig.4.11a Cow hide before unhairing

Fig.4.11b Cow hide after unhairing

c. Crude protease Extract

The unit activity of the crude extract was measured to be 20 units per gram and the amount required was determined based on the total activity of ammonium sulphate precipitate and the weight of the material. Soaked half piece of sheep skin and one side of cow hide were soaked in protease crude for 5 hours. Hair loosening was rated every hour and finally hair removal efficiency was compared against the conventional lime-sulfide unhairing method as presented in the fig.4.12 and 4.13.

Fig.4.12**a** Sheep skin before unhairing

Fig.4.12**b** Sheep skin after unhairing

Fig.4.13a Cow hide before unhairing

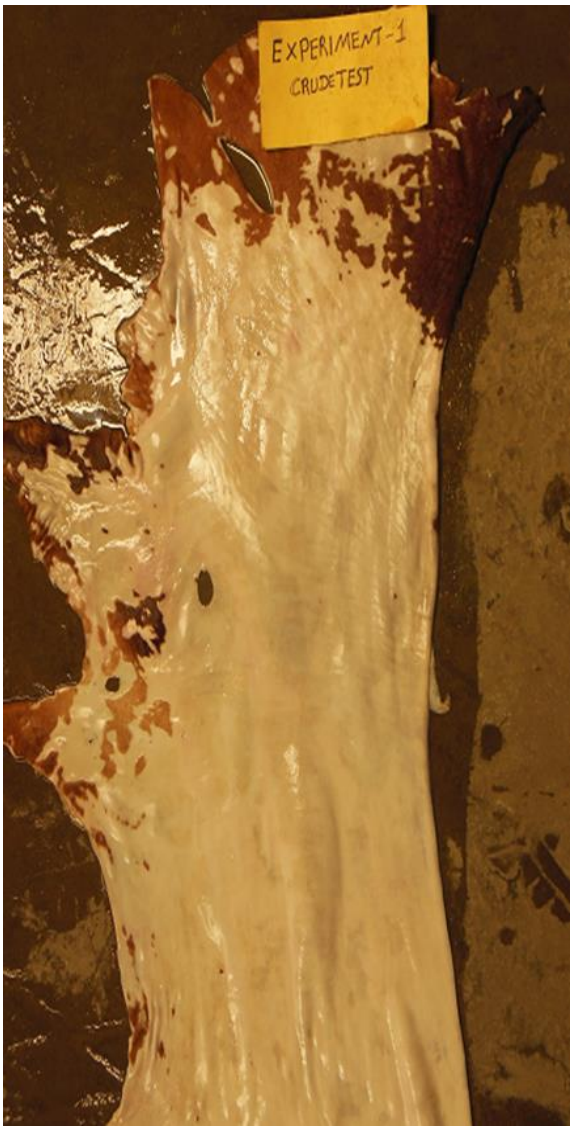

Fig.4.13a Cow hide before unhairing

d. Spray dried

It was reported [39] that many enzymes are subjected to spray drying process because the enzyme storage stability is better in that form rather than in a liquid formulation. So in transforming the prepared solution or suspension into a dry powder by lab scale spray dryer, the feed liquid flowing at a rate of 5.6 g/s was atomized into a fine spray which immediately contacts a flowing stream of hot air. The air was kept at inlet temperature of 120^0C and outlet temperature of 70^0C.The particles are dry in less than 5 s but remain in contact with the air for 20-30 s. The dried particles are separated from gases using cyclones. The presence of additives like polysaccharides in the enzyme preparations reduces enzyme inactivation by spray drying. After the enzyme was made into powder, the unit activity of the powder was measured to be 50 units per gram and it was made into paste using bicarbonate buffer of pH 9.5. The paste was kept in a refrigerator at a temperature of 4^0C until it was applied for dehairing. The amount of the paste required for efficient unhairing of 1kg of skin/hide was 288 grams which was calculated by dividing the required total activity of 14400 u/g by the

unit activity of the paste, 50 u/g. After the completion of soaking process of the skin and hide, the necessary amount of paste was taken and applied on the flesh side of half piece of sheep skin and half side of cow hide. Then they were folded flesh to flesh and kept under cover. The hair loosening was evaluated ever hour as shown in Tables 4.4 and 4.5. At the end, based on visual scale about 97% hair removal was achieved for sheep skin Fig4.14. This test was not done for cow hide as the prepared powder run out of stock.

Fig4.14**a**. Sheep skin before unhairing

Fig.4.14**b** Sheep skin after unhairing

e. Ultra filtration

Before ultrafiltration, the fermentation broth was treated by microfiltration with membrane of pore size 0.2 μm in order to separate particles including removal of microbial cells from the fermentation medium. Particles whose size is below the membrane 'cut-off' will pass through the membrane to become the **permeate** whereas the remainder is retained as the **retentate**. Then the permeate was purified and concentrated by ultrafiltration with membrane of pore size 10 kDa. In evaluating the efficacy of protease product of this unit

operation, first the unit activity of the protease filtrate was measured to be 90 units per gram and the amount required was determined based on the total activity of ammonium sulphate precipitate per 1kg of hide/skin. The filtrate was applied on the flesh side of soaked a half piece of sheep skin and one side of cow hide. Then they were folded flesh to flesh and kept under cover for 5 hours. Hair loosening was rated every hour and finally hair removal efficiency was compared against the conventional lime-sulfide unhairing method.

Fig.4.15**a** Sheep skin before unhairing

Fig.4.15**b** Sheep skeen after unhairing

Fig.4.16a Cow hide before unhairing

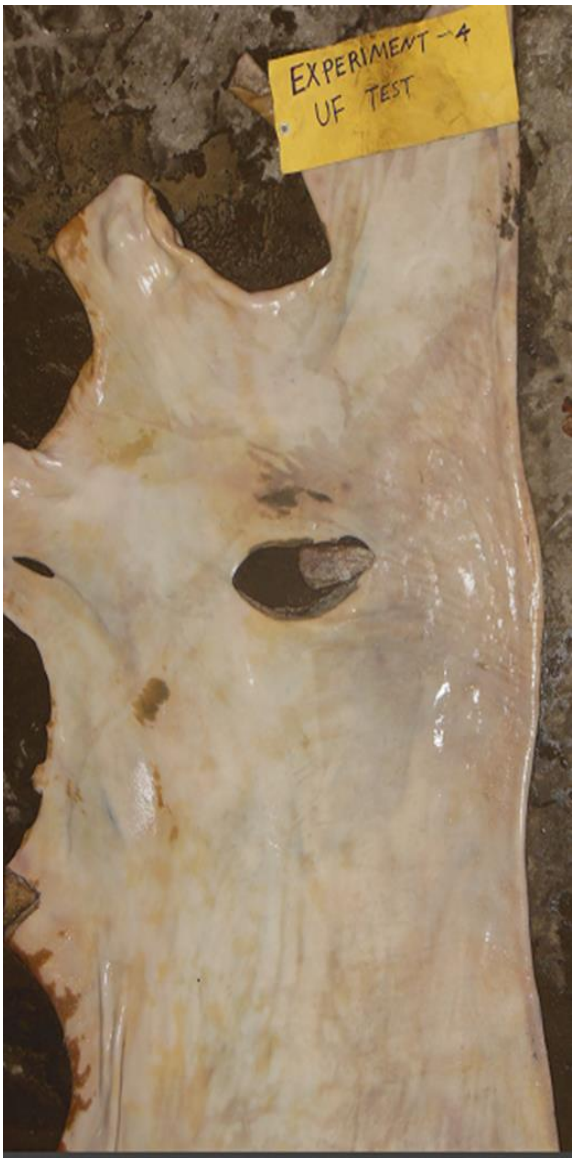

Fig.4.16b Cow hide after unhairing

Table 4.4 Periodic hair loosening rating of sheep skins for protease products of different downstream processing techniques against conventional unhairing method

Time in hour	Hair loosening rating (%)				
	Control	Crude	Amm.ppt	Spray dried	UF
1	40	30	60	70	50
2	60	40	70	80	70
3	80	60	80	90	80
4	90	80	90	95	90
5	100	95	96	97	96
6					

Table 4.5 Periodic hair loosening rating of cow hides for protease products of different downstream processing techniques against conventional unhairing method

Time in hour	Hair loosening rating (%)				
	Control	crude	Amm.ppt	Spray dried	UF
1	30	2	35	40	37
2	45	3	50	50	46
3	60	3	65	65	56
4	80	4	75	80	78
5	100	5	96	-	95
21(kept O/N)		96			

5. Conclusion and Recommendations

5.1 Conclusion

In an attempt mase in producing the dehairing protease as green technology option for the conventional lime-sulphide method of unhairing following the dictated methodology ,very encouraging results were obtained.The sub-culturing of the B.subtilis was done so effectively and it resulted in with good skim milk hydrolyses.The characterization studies of chemical and physical conditions in the cultvation of the culture revealed that an interesting optimization work was done as protease with promising activity was produced both at flask and pilot scale fermentations.The pH ranges of 6-8,temperature ranges of 30-32^{0}C,a media composition of soy bean flour and a fermentation period of 27-38 hours were found to be the optimal physical and chemical environments for the production of the enzyme. In addition, the existence of high degree interaction effects between pH and temperature on protease production was explored by RSM (Response Surface Methodology).

After successful production of protease at pilot scale fermentation,the partial purification of the broth was done by employing the primary downstream processing.In the downstream processings of crude enzyme formulation,ammonium sulphate precipetation,UF and spray drying,the difference in ease and simplicity techniques of processing and difference in the final protease product activity were observed.UF and spray drying are technicaly laborious and energy intensive and need higher capital investments than crude formulation and ammonium sulphate precipetation methods of downstream processings. The final protease product activities were 20 U/ml for crude extract, 360 U/ml for ammonium sulphate precipitate, 50 U/ml for Spray dried and 90 U/ml for UF.

Under the investigation of the efficacy of the protease products of the employed recovering and partial purification methods,use of the proease resulted in about 96% unhairing efficiency.Such a higher level of hair removal efficiency is a good indication for the protease products of each method in competing with the conventional chemical unhairing process with out taking other technical and economic factors into account.In this experiment the use of sodium sulphide was totally eliminated and the remaining hair was removed completely in the fibre opening up process of liming by 8 % offer of lime powder.

5.2 Recommendations

The experimental resulsts of this research indicated that about 96% hair removal efficiency was realized. This can be further improved if an intensive optimization work in terms of pH, temperature, media composition for growth and production and interaction effects of all main factors is performed. Formulation of the crude broth that undergoes minimum downstream processing should be maximized for activity while minimizing the cost. This improvement would essentially involve intensive research and would lead to an ideal enzymatic solution for depilation.

In this thesis work it was observed that the enzme production involved multidiciplinary work. It calls for the science of microbiology, biochemistry, genetic engineering, bioprocessing and chemical engineering. So to explore and exploit the different areas of biotechnology for the country's technological and economic advancement,science and engineering institutes of the area should work hand in hand at any time and place..

For the sake of issues of environment and economic factors of leather tanning industries of Ethiopia,socio-economic feasibility study was finilized for constructing CETP (common effluent treatment plant) in the area of Modjo town. So the wet end process sections (beamhouse, tanning and retanning) of each individual tannery in Addis Ababa and Modjo town are expected to be relocated to the CETP area. It will be a very good thing to do for the country's economic benefit if clusters or integrated industries of leather manufacturing, slaughter houses and necessary leather manufacturing input producers (chemicals,machineries,spare parts etc.) are created in this area. In this regard, the recommendation of establishing dehairing protease factory in the vicinity will provide certain advantages; the increament of production cost due to treatment to enhance stability of the product for very long time (usually 1-2 years), working capital tied up due to holding large stock and long distance transportation costs can be eliminated. Crude enzyme extract and ammonium sulphate precipitate can be used to effect unhairing and save the environment.

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ANNEXES

Annex 1: Table amount of Ammonium sulfate required for protein precipitation

initioal concentration of ammonium sulfate	Percentage saturation at 0^0																
	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
	solid ammonium sulfate (grams) to be added to 1 liter of solution																
0	106	134	164	194	226	258	291	326	361	398	436	476	516	559	603	650	697
5	79	108	137	166	197	229	262	296	331	368	405	444	484	526	570	615	662
10	53	81	109	139	169	200	233	266	301	337	374	412	452	493	536	581	627
15	26	54	82	111	141	172	204	237	271	306	343	381	420	460	503	547	592
20	0	27	55	83	113	143	175	207	241	276	312	349	387	427	469	512	557
25		0	27	56	84	115	146	179	211	245	280	317	355	395	436	478	522
30			0	28	56	86	117	148	181	214	249	285	323	362	402	445	488
35				0	28	57	87	118	151	184	218	254	291	329	369	410	453
40					0	29	58	89	120	153	187	222	258	296	335	376	418
45						0	29	59	90	123	156	190	226	263	302	342	383
50							0	30	60	92	125	159	194	230	268	308	348
55								0	30	61	93	127	161	197	235	273	313
60									0	31	62	95	129	164	201	239	279
65										0	31	63	97	132	168	205	244
70											0	32	65	99	134	171	209
75												0	32	66	101	137	174
80													0	33	67	103	139
85														0	34	68	105
90															0	34	70
95																0	35
100																	0

Annex 2: Table Nutritional categories of microorganisms

Physiological type	**Source of Energy**	**Electrons**	**Carbon**
Chemotroph	Chemical		
Phototroph	Light		
Organotroph		Organic compound	
Lithotroph		Inorganic molecule	
Autotroph			CO_2
Heterotroph			Organic compounds
Chemoorgano(hetero)troph (animals,fungi,ptotozoa,many bacteria)	Organic compound	Organic compound	Organic compound
Chemolitho(auto)troph (some bacteria)	Inorganic molecule	Inorganic molecule	CO_2

Photolitho(auto)troph (plants,most algae, some bacteria)	Light	Inorganic molecule	CO_2
Photoorgano(hetero)troph (algae, some bacteria)	Light	Organic compound	Organic compound

Annex: 3 LIDI model tannery wet salted sheep skin soaking process recipe

Operations & Chemicals	%	Temp.^{0}C	Kg/lit	Running time	Remark
Add wet salted sheep skin					
Add cold water up to the coverage of the skins				5 min.	Rest 20 to 30 min. then drain
Wash water	300	cold		15´x3	Drain 3x Check ^{0}Be <2.0
Wash water	100	Cold		5'	Rest 30'
Wetting agent	0.2				
Sodium silco fluoride	0.2				
Soda ash	0.2				
Soda ash	0.2			15´	Rest 30´
Soda ash	0.2			15´	Check pH 8.0-9.0
Soda ash for pH correction					
Run 3´ rest 27´ for 16 – 18 hours					
Next Day					
Run Drum				30'	Check pH & temp. then drain
Wash	300			15'x3	Drain 3x
Drain, unload and pile for 30' and make it ready for painting					

Annex: 4 LIDI model tannery recipes for Preparation of paint solution and unhairing/liming of soaked sheep skins

Operations & Chemicals	Remark
Add 50gm/lit sodium sulphydrate and 150 gm/lit Sodium sulphide	Stir well and check ^{0}Be 14-15 And leave O/N
Next day	
	check ^{0}Be 14-15
Add 270gm/lit lime	check ^{0}Be 27-30
Paint on the flesh side properly	Pile flesh to flesh for 2.5 -3 hours
Unhair and make it ready for liming	
Liming	
Add 200% cold water into liming drum	Run 5´

0.1% lime	
Put the unhaired skins into the drum	Run 15'
Add 0.5% sodium sulphide	Run20' and check sulphide content 6.5-7.5 gm/lit and then run 3' rest 27' for 16 -18 hours

Annex: 5 LIDI model tannery recipes for hide soaking and unhairing/liming processes

Operations & Chemicals	%	Temp.^{0}C	Kg/lit	Running time	Remark
Add wet salted cow hide					
Add cold water up to the coverage of the hides				5 min.	Rest 30 to 60 min. then drain
Wash water	300	cold		15'x3	Drain 3x Check ^{0}Be <2.0
Wash water	100	Cold		5'	Rest 60'
Wetting agent	0.2				
Sodium silco fluoride	0.2				
Soda ash	0.2				
Soda ash	0.2			60'	Rest 30'
Soda ash	0.2			60'	Check pH 8.0-9.0
Soda ash for pH correction					Check pH
Run 3' rest 27' for 16 – 18 hours					
Next Day					
Run Drum				30'	Check pH & temp. then drain
Wash	300			15'x3	Drain 3x
Water	50			60'	Rest 60'
Lime	3.0				
Sodium sulphydrate	1.0				
Sodium sulphide	1.0			90'	Check sulphide content 6.5-7.5 gm/lit
Run 3' rest 27' for 16 – 18 hours					