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PRODUCTION, PARTIAL PURIFICATION AND APPLICATION OF SERINE PROTEASE IN LEATHER MANUFACTURING

Submitted by

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ANNA UNIVERSITY: CHENNAI 600 025

BONAFIDE CERTIFICATE

Certified that this project report **"PRODUCTION, PARTIAL PURIFICATION AND APPLICATION OF SERINE PROTEASE IN LEATHER MANUFACTURING"** is the bonafide work of **"LAKSHMI BALRAM** and **SMRITI MARIAM RAJAN"** who carried out the project work under my supervision.

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Adyar, Chennai 600 020

Friday the 24th March 2006

BONAFIDE CERTIFICATE

This is to certify that the research work titled "**Production, Purification and Application of Serine Protease in Leather manufacturing**" is the bonafide work of **Miss. Lakshmi Balram, Miss. Smriti Mariam Rajan** and **Miss. Vinitha.G**, students of **B.Tech (Industrial Biotechnology)** of **Kumaraguru College of Technology**, Coimbatore, who carried out the project under my supervision in CLRI. It is further certified that the work reported herein does not form part of any work on the basis of which a degree has been awarded to these or any other candidate on an earlier occasion.

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The Report of the project work submitted by the above students in partial fulfillment for the award of Bachelor of Technology degree in Industrial Biotechnology of Anna University were evaluated and confirmed to be report of the work done by the above students. It was submitted for evaluation and viva-voce held on 28.4.06

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ABSTRACT

As natural biological substances, enzymes have much less impact on the environment, speed-up processes and are very specific in their activity than chemicals. The use of elastase proved to be successful for the process of unhairing in the leather industry. The use of porcine pancreatic elastase (Sigma) on the animal skins during soaking promotes better removal of salt, better rehydration and better removal of globular proteins. The enzyme increases the ease of unhairing and reduces the protease level by a significant amount resulting in a clean pelt. Moreover the methods do not interfere with the physical strength of the leather. However, to make it highly commercial, another approach is devised by producing elastase from the fungi *Aspergillus flavus*. The enzyme is extracted, partially purified by ammonium sulphate precipitation and subsequently characterized for its optimum pH and temperature.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

I.1 INTRODUCTION TO BIOTECHNOLOGY

Biotechnology is harnessing the power of the living organisms to improve biological and chemical processes thereby making them more effective, quicker, safer and more environmentally acceptable. Biotechnology is the manipulation of biological systems to make products that benefit human beings. It means any technological application that uses biological systems, living organisms or derivatives there of, to make or modify products or processes for specific use. It is the technology based on biology especially when used in diverse fields such as agriculture, food science and medicine where it contributes widely.

There are a number of sub-fields of biotechnology:

Red biotechnology is biotechnology applied to medical processes. Some examples are the designing of organisms to produce antibiotics and the engineering of genetic cures to cure diseases through genomic modification.

White biotechnology also known as **Gray biotechnology**, is biotechnology applied to industrial processes. An example is the designing of an organism to produce a useful chemical. It tends to consume less in resources than traditional processes when used to produce industrial goods.

Green biotechnology is biotechnology applied to agricultural processes. An example is the designing of an organism to grow under specific environmental conditions or in the presence (or absence) of certain agricultural chemicals. It is hoped that green biotechnology might produce more environmentally friendly solutions than traditional industrial processes. An example of this is the engineering of a plant to express a pesticide, thereby eliminating the need for external application of pesticides.

Blue biotechnology has also been used to describe the marine and aquatic applications of biotechnology.

Bioinformatics is an interdisciplinary field, which addresses biological problems using computational techniques. The field is also often referred to as computational biology. It plays a key role in various areas like functional genomics, structural genomics and proteomics that forms a key component in biotechnology and pharmaceutical sector.

The initial achievements in biotechnology were in food production, occurring about 5000 BC. Diverse strains of plants or animals were hybridized (crossed) to produce greater genetic variety. The offspring resulted from the hybridization were then selectively bred to produce the greatest number of desirable traits. Repeated cycles of selective breeding produced many present-day food staples. This method continues to be used in food-production programs.

Though biotechnology has existed since ancient times, some of its most significant advancements have taken place in more recent years. Modern achievements include the transferal of a specific gene from one organism to another (by means of a set genetic engineering of techniques known as transgenics); the maintenance and growth of genetically uniform plant and animal-cell cultures, called clones and the fusing of different types of cells to produce beneficial medical products such as monoclonal antibodies, which are designed to attack a specific type of foreign substance.

Biotechnology is often associated with the use of genetically altered microorganisms such as *E.coli* or yeast for the production of substances like insulin, antibiotics and organic products (examples include beer, milk

products, and skin). Genetically altered mammalian cells such as Chinese hamster Ovarian (CHO) cells are also widely used to manufacture pharmaceuticals. Another promising application of biotechnology among latest advancements is the development of plant made pharmaceuticals. Biotechnology also has great relevance with environmental protection through recycling, reuse, remediation etc.

Enzymology is one of the avenues of biotechnology. Biotechnology can provide an unlimited and pure source of enzymes as an alternative to the hazardous chemicals traditionally used in industries. Enzymes are found in naturally occurring microorganisms such as bacteria, fungi and yeast, all of which may or may not be genetically modified. Large quantities of enzymes are often needed for industrial use. Hence these microorganisms are multiplied through fermentation. When enzymes or the microorganisms are no longer needed, they are destroyed through exposure to heat or safe organic or inorganic materials.

Today Biotechnology is applied in various fields. In waste management for example, Biotechnology is used to create new biodegradable materials. One such material is made from the Lactic acid produced during the bacterial fermentation of discarded corn stalks. When individual Lactic acid molecules are joined chemically, they form a material that has the properties of plastics but is biodegradable. Widespread production of plastic from this material is expected to become more economically viable

The field of medicine employs Biotechnology for some of the most important applications. In 1986, factor VIII, a blood-clotting protein has been first produced in laboratory. This is boon for treating hemophilia. As a result of this condition, hemophiliacs are at risk of bleeding to death after

suffering minor cuts or bruises. Using Biotechnological procedure, the human gene that codes for the blood-clotting protein is transferred to hamster cells grown in tissue culture, which then produce factor VIII for use by hemophiliacs in the future.

There are also applications of Biotechnology that do not use living organisms. DNA micro arrays used in genetics and radioactive tracers used in medicine exemplify this. Biotechnology is also commonly associated with breakthroughs in new medical therapies and diagnostic devices.

I.2 INTRODUCTION TO INDUSTRIAL BIOTECHNOLOGY

Industrial biotechnology as mentioned before is the practice of using cells or components of cells like enzymes to generate industrially useful products. This growing field fosters sustainable, eco-friendly and cost-efficient production of chemicals, materials, consumer goods and alternative energy sources. It includes several developments in new tools and applications of Biotechnology for all industrial applications including biofuels, chemical and pharmaceutical biosynthesis, nano-biotechnology, food and feed processing, textiles, pulp and paper, bioplastics and biomaterials, and bio-defense.

Industrial biotechnology can provide an unlimited and pure source of enzymes as an alternative to the harmful industrial chemicals. Pulp and paper industry makes use of enzymes like cellulase that soften wood fibers, improve drainage and present alternatives to chemical bleaching. Enzymes are used to treat and modify fibers particularly during textile processing and in textile caring. For example, catalases are used to treat cotton fibers and

prepare them for the dyeing processes. Cellulases and xylanases are used to finish fabrics give Jeans a stonewashed effect.

Enzymes like protease, amylase and lipase are frequently used in laundry detergents to facilitate breaking down of dirt. They can also prevent the dulling of fabric colors. Very high temperatures and mechanical shaking are required for effective cleaning of clothes otherwise.

Synthetic fibers, which are made from renewable sources of Biomass are environmentally sustainable and are becoming increasingly economically sustainable. Biodegradable synthetic polymers include novel fibers such as polyglycolic acid and polylactic acid, which are made from natural starting materials like collagen, chitin and alginate. A prime example of a synthetic biomass fiber is Polylactic Acid (PLA), which is made by fermenting cornstarch or glucose into Lactic acid and then chemically transforming it into a polymer fiber. With properties similar to other synthetic fibers, PLA based materials are durable with a silky feel and can be blended with wool or cotton. PLA minimizes environmental waste, as it may be fully biodegraded by microorganisms. Unlike the non-renewable petroleum resources used to make traditional synthetic fibers, the supply of renewable corn biomass needed to make PLA is expected to surpass demand in the anticipated future. Some of the Biodegradable synthetic fibers and natural biological fibers can be used to make textiles for medical applications.

Biotechnology also has applications in the mining industry. In its natural state, Copper is found combined with other elements in the mineral chalcopyrite. The bacterium *Thiobacillus ferrooxidans* can utilize the molecules of Copper found in chalcopyrite to form the compound Copper

sulfate ($CuSO_4$), which in turn can be treated chemically to obtain pure Copper.

Enzymes can be used in the production of fuels from renewable sources of biomass. Such enzymes include cellulases, which convert cellulose fibers from feedstock's like corn into sugars. These sugars are subsequently fermented into Ethanol by microorganisms. A new process called "Simultaneous Saccharification and Fermentation" has improved Ethanol production. In this new process, cellulase enzymes and fermentation microorganisms are combined in a single reaction mixture to produce Ethanol in one step rather than producing sugars from cellulose and then fermenting them into Ethanol as a separate step.

I.3 INTRODUCTION TO ENZYMES

Enzymes (biocatalysts) are catalysts of biological origin and are characterized by their extraordinary specificity and reactivity. They are proteinaceous in nature and catalyze certain chemical reactions involving naturally occurring organic materials such as carbohydrates, proteins or fats. They can be used over and over again for performing the same specific catalytic cleavage or synthetic reactions.

However, there are quite a few limitations. Since enzymes are very much complicated and sensitive biomolecules, enzymatic activity is influenced by host, alkalinity, acidity, traces of metal ions and certain inhibitors.

The Committee on Enzyme Nomenclature of the International Union of Biochemistry has classified enzymes based on reaction types and reaction

mechanisms. The enzymes are divided into groups on the basis of the type of reaction catalyzed and this, together with the name of the substrate, provides a basis for naming individual enzymes. The systematic name of an enzyme is:

- In accordance with definite rules
- Indicative of its identity
- Informative about its reaction as precisely as possible
- Inclusive of the name of the substrate it reacts with

According to systematic nomenclature, any enzyme is indicated by a code number, unique for each enzyme. All enzymes are classified into six categories-

- Oxidoreductases catalyze the removal of H_2 (dehydrogenase) or addition of O_2 (oxygenase).
- Transferases catalyze the transfer of groups, which are not free during reaction from one substrate to another.
- Hydrolases catalyze the splitting of compounds by the addition of water across various bonds
- Lyases catalyze the non-hydrolytic addition or removal of groups which are free during reaction
- Isomerases catalyze internal transformations of optical, geometric and spatial isomers
- Ligases catalyze the condensation of two molecules coupled with the breakdown of pyrophosphate bond from a nucleotide triphosphate.

Carbohydrases, proteases, lipases and amidases are the sub-classes of hydrolases. Proteases are the major enzymes utilized in the pre-tanning

processes of leather manufacturing. They are obtained from three major sources viz. plants, animals and microorganisms. Microbial proteases, based on their properties viz. pH optimum, effect of inhibitor, metal involvement and specificity, etc., are further classified into-

- Acid proteases
- Thiol proteases
- Metal chelator-sensitive proteases
- Serine proteases

I.4 INTRODUCTION TO LEATHER MANUFACTURING

Leather industry has made a unique and independent position of its own due to its immense potential in employment generation, economic growth and export revenue. The art of leather processing is an ancient technique known to mankind.

Leather is obtained from animal hides and skins, by tanning. The raw animal skin and hide is vulnerable to microbial attack. Thus hides are restrained from the action of microorganisms by application of salt. This process of preserving skins is called as Curing. The salt applied reduces the moisture content to an extent that it prevents microbial growth and provides bacteriostatic action. Such cured hides and skins are then treated with water by a process called Soaking so as to make the skin or hide, soft and clean. Water rehydrates the dried inter-fibrillary protein and loosens the cementing substance of the fibers making the hide more flaccid and flexible. The hair, adipose layer of flesh and natural fats of the skin have to be removed and this is carried out by employing Lime and Sodium sulphide. The process is

called liming. The substrate on removal of hair, flesh and fat is called as pelt. The pelt containing Lime is now subjected to deliming, which employs Sulphate or Chloride of Ammonium for the removal of Lime. Bating is subsequently carried out using proteolytic enzyme to remove other proteinaceous materials from the pelt thereby the penetration of the tanning materials and other processing chemicals during further operations is enhanced. Excess fat in the grease skins and hides is removed by Degreasing using various organic solvents and enzymes. The pH is about 8.0 at this stage and has to be reduced depending on the subsequent process (2.8 for chrome tanning and 3.8 for vegetable tanning). The reduction in pH is achieved by treatment of the pelt with acid and salt. This process is called Pickling.

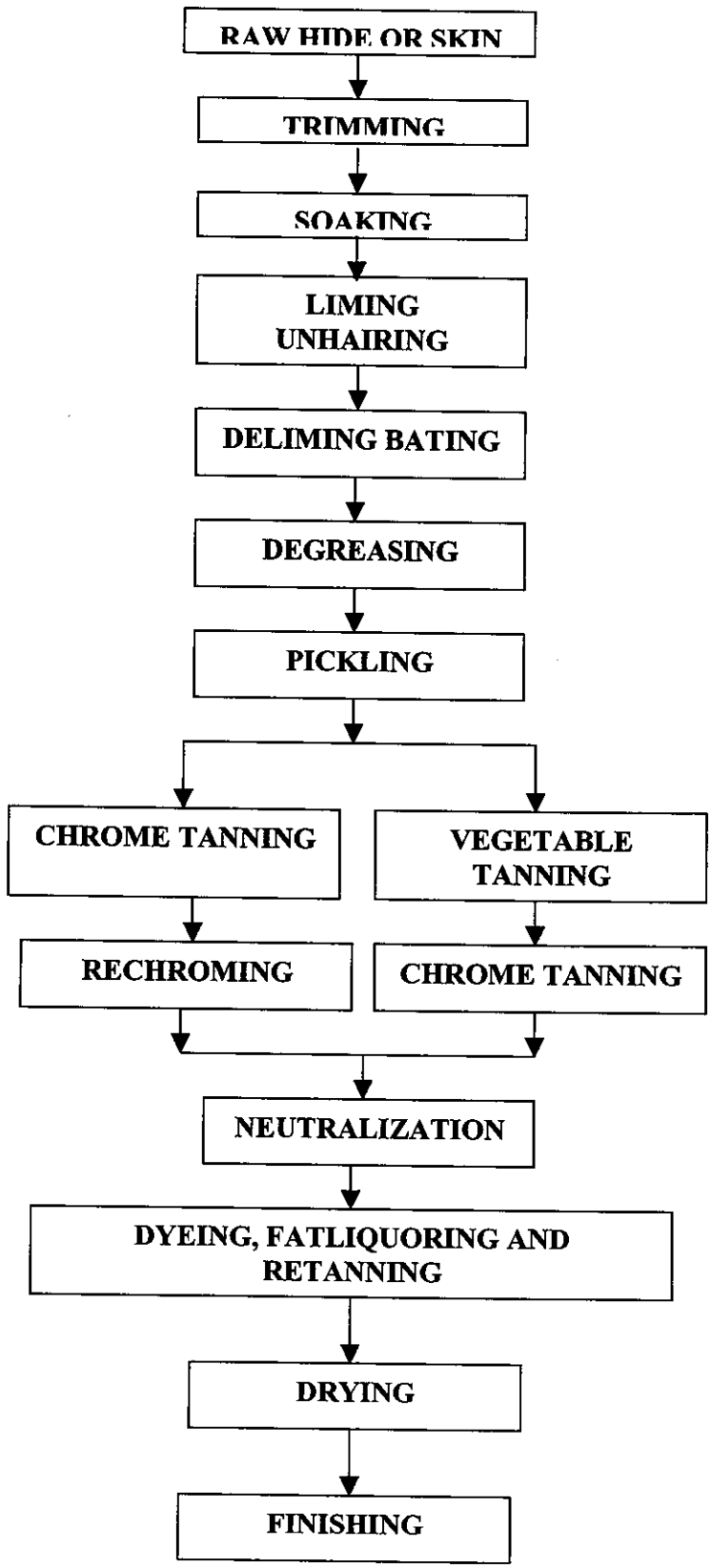
Action of the pelt with the tanning agent renders better stability to the substrate. On the basis of the nature of tanning agent, the process of tanning is classified as organic tanning and inorganic tanning. The former employs oils, aldehydes and plant materials viz Avaram, Babl, Myrabolan, Oak, and Quberacho while the latter utilizes mineral salts such as Chromium sulphate, Aluminium sulphate and Zirconium sulphate.

The post-tanning operations include retanning, dyeing and fat liquoring. Retanning supplements characteristics like tightness, strength and fullness to tanned materials. This is carried out with Syntans or synthetic tanning materials. Syntans could be phenol-formaldehyde resins of novolac type or polymers of acrylic. Dyeing the substrate with acidic dyes, direct dyes and basic dyes is generally carried out to impart desired color to the substrate. Fat liquors are auxiliaries that are oils in emulsifiable form. The process by

which substrate is treated with fat liquors is termed as fat liquoring. This procedure lubricates the fiber bundles and fibers making leather softer.

The substrate is now dried and finally the leather is coated with finish solution. The finish mixture consists of coloring agents (pigments or dye solutions, binder, plasticizer and medium (water). The operation of coating the leather with the finish mixture is called as finishing.

FIG 1.1: STEPS IN THE MANUFACTURE OF LEATHER



I.5 BIOTECHNOLOGICAL APPLICATIONS IN THE LEATHER INDUSTRY

Biotechnology, particularly the use of enzymes, offers an opportunity to add value by providing improved and cleaner processes for leather production. Enzymes have played an important role in the production of leather for centuries, but it is only in recent years that research and innovation have led to a greater understanding of how biotechnology can be harnessed by the industry. As natural biological substances, enzymes have much less impact on the environment, speed-up processes and are very specific in their activity than chemicals.

SOAKING

An efficient soaking stage is a prerequisite for the production of high quality leather. Enzymes during soaking accelerates the process by removing hyaluronic acid and improve quality through

- More effective rehydration of the skin
- Better opening up of the fiber structure
- Fat degradation and dispersion
- Better removal of carbohydrate or protein components

Both proteases and lipases aid soaking processes. They are particularly helpful when processing

- Fatty raw materials
- Very dry raw materials
- Fresh hides without salt where the removal of non structural proteins and carbohydrates is very difficult.

LIMING AND UNHAIRING

Enzymes especially proteolytic enzymes are very much useful for unhairing. The benefits include the following:

- Removal of hair or wool
- Removal of the epidermis
- Removal of residual, non-structural components
- Dispersal or removal of fatty components
- Reduction in effluent load and lower disposal charges

BATING

Efficient bating relies on the use of enzyme like proteases, amylases and lipases to

- Clean the hide or skin of degraded hair or epidermis
- Promote the further removal of non-structural protein
- Encourage the further removal of carbohydrates

Effective use of enzymes for bating will help in leaving the skins or hides clean, flat and soft.

DEGREASING

Degreasing is an essential process in the processing of fatty raw materials. Conventional methods of using organic solvents and surfactants give rise to environmental problems. Lipases play an important role by removing fats and grease from skins and hides during this process.

I.6 HAIR REMOVAL-PROBLEMS AND PROSPECTIVE SOLUTIONS

The hair, adipose layer of flesh and natural fats comprising the skin and hides should be removed and this is carried out by employing Lime and Sodium sulphide which denature hair and swell the flesh. Also the alkali saponifies the fats. This process is called Liming. Traditional chemical processes degrade the hair thus increasing the effluent load. Chemical-assisted enzyme unhairing systems have been used to remove the hair from the hide. The enzymes used for liming-unhairing operations belong to the class of proteolytic and amylolytic enzymes.

Proteases can be used for hair removal. Use of enzymes can eliminate the use of sulphides. Hence enzymatic process of hair removal is an environmentally benign alternate to sulphide-based system.

However, The use of enzymes is not widely accepted because of the exorbitant cost of the enzymes. Hence reduction in costs of enzyme is highly imperative to popularize this otherwise beneficial system. Fundamentally, there can be two broad strategies to reduce the cost of the enzymes. Firstly the cost of production of enzymes can be reduced by choosing better microorganisms or by designing a cost-effect production system. The second strategy can be the development of suitable pretreatment so as to reduce the requirement of enzyme for complete hair removal by many fields.

CHAPTER II

REVIEW OF LITERATURE

II.1 LITERATURE ON SKIN HISTOLOGY

Skin is the barrier between the organism and the environment. The function of the skin is decisive for its histological and physiological properties. Skin is one of the adaptation organs almost impermeable to water, aqueous solutions and pathogenic microorganisms. Nerve ends contained in it are receptors of pressure, heat, cold and pain stimuli. From physical view point, skin reveals a specific viscoelastic behavior permitting to avoid small injuries due to its suppleness in view of its stretching and shrinking. From chemical view point, the components of the skin reveal lower reactivity than the components of the other organs (Puvanakrishnan,1988).

II.1.1 Structure Of Animal Hide

An animal hide is stratified and has three layers as given below

- The epidermal layer
- The corium layer
- The adipose layer

The epidermal area is that portion of the animal skin or hide, which contains the hair, hair follicles, epidermis and its appendages such as the sebaceous glands and the sudoriferous glands. These are surrounded and supported by the collageneous fiber bundle structure. Also throughout this structure of collageneous fiber bundle are dispersed a network of elastic tissue fibers, erector pili muscles, blood vessels and nerves (Biekiewicz, 1983).

Developed epidermis covers about 1-5% of the total thickness of the entire skin. The epidermis is known as stratified epithelium because it is composed of four strata. Going from top to bottom of the strata are: stratum corneum, stratum lucidium, stratum granulosum, and stratum germinativum (Puvanakrishnan, 1988).

Hair is a typical epidermal structure and it is entirely a product of the epidermis. The cells of the epidermis dip down into the body of the dermis and form a hair pocket of follicle in which the hair grows.

The follicle is of complex structure being made of epithelial cells on the hair side and of connective tissue on the other. At the bottom the follicle is penetrated by a projection from the corium, which is called the hair papilla. The hair papilla contains both nerves and the blood vessels. Surrounding the papilla there are numerous epithelial cells which being nourished by the blood vessels of the papilla reproduce themselves. As new cells are formed the older ones are pushed upwards through the follicle, forming the hair. (Biekiewicz,1983).The rate of growth of the hair is determined by the rate at which the cell surrounding the papilla reproduces. The newly formed cells of the hair substance are soft like the cells of the Malpighian layer and slowly moving upward become elongated and hardened. In forming the hair they take the shape of the follicle. At the bottom of the hair follicle the hair root is expanded and has a bulb like shape. The expanded part is indented on its lower surface to support a capillary blood vessel and this arrangement gives rise to a pincer shape. From the bulb the hair extends upward through many vertical rows of cells arranged in a parallel manner. The portion of the hair

above the surface of the skin is called the shaft and lower portion the root (Puvanakrishnan,1988).

Erector pili muscle is attached to each hair follicle by elastic fibers. It passes just below the sebaceous gland (fat gland), and extends obliquely to the grain membrane. It consists of a bundle of non striated muscle fibers. This muscle is involuntary in type and contracts under the influence of temperature and excitement. When this muscle contracts the hair stands on its end produces the goose flesh (Biekiewicz, 1983).

The fat glands also called the sebaceous glands are connected to the hair follicle by means of capillary ducts through which the oily matter of the glands flows by the pressure of the erector pili muscles when they contract. This oily matter, on being pressed out of the gland flows on to the epidermis through the hair follicle making the surface of the skin, water and heat resistant and thereby protects the body against sudden change of temperature. They maintain the body temperature by covering the body with a film of oil and thus regulate the surface evaporation of water. The fat glands are made of cells having nuclei and are arranged like grapes in a bunch. The oil present can act as a barrier to water penetration during the soaking operation. Good deals of yellow pasty matter, which come out during liming operation, consist of the broken up fat glands (Puvanakrishnan,1988).

The sweat glands, also known as the sudoriferus glands, are made up of coiled tubes with spiral ducts having outer walls formed of a membrane of connective tissue and lined inside with a single layer of the large nucleated

cells, which secrete the perspiration through the ducts (Biekiewicz,1983).The sweat glands control the rise of temperature of the body by causing evaporation of the perspiration produced by heat.

This is the main layer of the hide or skin constituting about 98% of its thickness. In structure, corium is entirely different from epidermis. Corium is composed of fibers (mainly white fibers), which occur in bundles. The fibers of the corium consisting mostly of collagen to form a three-dimensional fiber weaves. The fibers of the bundle are held in sheaths of another kind of fiber known as reticulin fibers, which are fine delicate threads of a protein called reticulin. At various points along the length of the fiber bundles the reticulin threads surround the fibers of the bundles and hold them together. The fiber bundles are inter-woven at an angle to the surface of the hide or skin. In the middle of the corium, this angle varies between 45° and 75°. This is known as the angle of weave of the collagen fibers. At the lower part of the corium, the angle of weave is much lower and the fiber bundles are almost horizontal running parallel to the surface. The bundles are inter-woven in a wavy fashion. The fiber bundles diminish in size as they approach the grain layer and at the level of the hair roots they turn into smaller bundles running at random arrangements. The fibers are composed of extremely fine fibrils probably about.500 nm in diameter. The fibrils again are composed of micelles probably about 20 nm. These micelles are the ultimate units of fibrous structure. The fibrils are produced by certain white corpuscles of the blood called fibroblasts, which are unicellular. Fibroblasts leave the blood stream and migrate outwards to the skin and along their path of travel exude the protein collagen, which is transformed into collagen fibril. The fibrils unite with one another to form a fiber and a

number of fibers unite to form a fiber bundle. The fiber bundles are inter-woven producing corium or true skin (Puvanakrishnan, 1988).

The fibers are cemented together by a substance called inter-fibrillary material and the spaces between the fibers are filled with this substance.
The corium itself may be divided into two layers of different structures, viz

- The grain layer or papillary layer
- The corium proper or reticular layer

The grain layer is also called as 'the thermostat layer' because it keeps the body temperature constant through the action of sweat and fat glands present in it. The thermostat layer is also called corium minor. It constitutes the upper part of the corium and occupies about 10-25% of the entire thickness. The epidermal appendages like hair root, hair follicle, fat and sweat glands and also the erector pili muscles are in this layer. On the top of this layer is the grain membrane, which contains the negative imprint of the epidermis called papillae. These papillae contain nerve endings, which serve as the organ of the sense of touch. The papillae are well developed in some hides and skins. The arrangement of the hair follicles and pores and the development of nerve papillae give animal skins their distinct and characteristic grain pattern. The fibers in the grain membrane are extremely fine and generally run parallel to the surface. It is not definitely known whether the fiber of the grain surface is continuous of the white fibers of the corium, but they seem to possess somewhat different properties (Puvanakrishnan, 1988). When the unhaired skin is kept in boiling water, the fibers of the grain surface remain as a thin sheet, although some-what

changed, long after the larger collagen fiber below have passed into solution as gelatin.

It is of great importance that no damage is done to the grain surface in removing the epidermis because it determines the requirements of the finished leather. It is therefore fortunate for the tanner that the fibers in the surface are more resistant to the action of alkalis than the epidermis above it and more resistant to tryptic enzymes than the elastic fiber below it. The grain surface, however is readily attacked by proteolytic bacteria under certain conditions, resulting in what is known as pitted grain.

Some histologists are of the opinion that between the grain membrane and the lowest layer of the epidermis there is structureless thin transparent basal membrane called hyaline layer or glossy layer. This layer is not really seen and some histologists have doubted its existence. In this layer which gives the special optical properties of smoothness and polish to the grain surface.

Elastin fibers are present in the thermostat layer and also in the flesh layer underneath the reticular layer. Chemically, elastin fibers are different from collagen fibers. They are made of up protein called elastin whereas collagen fibers are made up of the protein, collagen. Physically also, the elastin fibers are different from collagen fibers. The elastin fibers do not combine with tannin like collagen fibers. They do not dissolve in boiling water either. Elastin fibers are hydrolyzed by proteolytic enzymes (pepsin. (Puvanakrishnan,1988).

The reticular layer is below the grain or thermostat layer constituting about 75-90% of the total thickness. It is called reticular layer on account of its

net-like woven structure. The fibers of this layer are ticker and longer than those of the thermostat layer. This layer is also called corium major or corium proper.

A thin layer appended to the corium is called flesh or adipose layer. Although this layer exists in all flayed hides and skins, it is not part of the hide or the skin. It is the loose connective tissue lying between the hide or the skin and the actual body of the animal (Biekiewicz,1983). The flesh consists of adipose tissue, elastic fibers, blood vessels, nerves and voluntary muscles.

II.1.2 Constituents Of The Skin

The constituents of the skin has been divided into two types:

- Non protein components
- Protein components

Non-Protein Constituents

Lipids

Lipids comprising of the phopsholipids, glycolipids, phosphatides and the derived lipids comprising of the fatty acids, alcohol and sterols. All the three types of lipids in the skin play an important role in the physiological functioning of the skin.

Fatty acids with glycerol and comprise an important means of food storage in animal life. They are widely distributed in the skin.Lipids in the skin are

concentrated mainly in the three regions: epidermal layer, the corium layer and the subcutaneous layer (Biekiewicz, 1983).

The epidermal layer represents the portion from the skin to the base of the deepest hair roots. This section includes the sebaceous and sudoriferous glands, the nucleated cells of the epithelial layers and the fibroblasts of the corium minor. The sebaceous glands contributes substantially to the lipid content of the skin for they are lined with cells that undergo degeneration, their lipid content is increased until its secreted into the hair follicle as sebum.

Lipid deposition in the corium can be demonstrated only in the fat cells, which are interspersed between the collagen fibers. This fat deposition is identical to the fatty tissue of the adipose or subcutaneous layer, results from the animals use of skin for the deposition of reserves of fat. Lecithin is the most important constituent of the phospholipid in the corium while very less amounts of cephalin was detected.

Subcutaneous tissue also referred to as the flesh or the adipose tissue consists of cellular deposits of fat in loose connective tissue fibers. These fats representing reserve deposits are mainly triglycerides.

Carbohydrates

The carbohydrates in the skin exist in the free form and in the polymeric form, the polysaccharides. Further, they also exist in intimate association with other substances such as proteins and lipids. The cells of the epidermal system, central mass of hair germ and developing follicle have large reserves

of glycogen which are essential as a source of energy that is supplied by aerobic glycolysis. Polysaccharides were detected in the blood vessel walls, eosinophilic leucocytes, mast cells, fibroblasts, hair follicles, connective tissue surrounding the sebaceous glands, smooth muscles but little was found in the horny layer.

Enzymes

Most of the enzymes common to the tissue occur in the skin, though its enzymatic ctivity is low owning to the low rate of metabolism. Arginase in the skin is confined to the epidermal layer especially in the malpighian layer. Other skin enzymes include amylase, diastase and alkaline phospahtase, the latter in the hair follicles and sweat glands. Enzymes are important in the degenerative processes, which are active during the post mortem period. Since enzymes are capable of digesting proteins, they become an important destructive factor in the loss of hide substance. The chief enzyme, which is active during this autolysis, is cathepsin, a proteinase that reduces complex proteins to their simpler components, proteoses and peptones (Puvanakrishnan, 1988).

Vitamins

It was found that ascorbic acid was concentrated chiefly in the non-cornified layer in the skin. Epidermis contains little free nicotinamide. Of the fat-soluble vitamins, the presence of 7-dehydrocholesterol, on ultraviolet irradiation yields vitamin D_3. Vitamin B_2 is believed to be present in all animal cells although its concentration is high mainly in the liver and kidney. The presence of biotin or vitamin H, in the skin has been detected. Inositol has also been detected in skin (Bienkiewicz, 1983).This has been

used to prevent the accumulation of fat and cholesterol in tissue and it exerts a great lipotropic action than choline.

Minerals

Most of the phosphorus is present in the epidermal layer and therefore results in high phospholipid content in this region. Iron is derived from the blood hemoglobin and from the nuclei and chromatin of cells as well as from the hair. Copper is related to the pigment found in skin and it functions either as a catalyst in mammalian pigmentation or a catalyst in keratinisation Silica has been found in the cell nuclei of the epidermal region. Arsenic is present in significant quantities and its believed that the epidermal system is the means by which this element is excreted from the body (Biekiewicz,1983). Calcium and magnesium are found in some concentration in the basal layers of the epidermal system.

Proteins

Approximately 80% of the dry matter of skin is made up of complex nitrogenous organic compounds known as proteins. All proteins belong to one of two large groups:fibrous proteins and globular (corpuscular) proteins. The corium or principle leather frming portion of the skin, consists of an inter-woven network of fiber bundles of collagen. Other fibrous proteins pesent in the skin are the keratins of the epidermal system and the elastin of the elastic fibers and blood vessels (Puvanakrishnan, 1988).

In contradiction to the structural functions of the fibrous proteins, the globular proteins are usually involved in the vital processes of the tissue.

Representatives of the globular proteins in the skin are albumins, globulins and other less defined proteins.

Keratin

Keratins, which are proteins of the protective coverings, may be divided into soft keratins and hard keratins. The soft keratins are the less highly structured condensed materials of which the outer layers of the epidermis are the chief examples. The hard keratins are the more highly structured condensed materials, generally showing a more highly differentiated morphological structure represented by hair, horn, nails, etc.

Collagen

The collagen, whose structure is found to be an alpha triple helix with three amino acid residues per turn with a rise of 0.1 nm and generally assuming a staggered form. From the standpoint of the tanner, this is the most important constituent of the skin, because it reacts with tanning agents to form the leather substance (Gross,1950). It must not be supposed that collagen is peculiar to the skin. It as bee estimated that approximately 30% of the total protein of the mammalian body is collagen. Glycine residues form about one-third of the molecule, and proline and hydroxyproline about a quarter. Moreover they show that there are approximately three polar side chains to nearly three and a half non-polar, and three and a half units without side chains, i.e. every ten resides carry three polar and three non-polar side chains. It is relatedly resistant to many proteolytic enzymes, including trypsin ad chymotrypsin and it is of interferes that certain anaerobic bacteria secrete a proteolytic enzyme which readily attacks native collagen, and

which is apparently quite specific for this protein. This enzyme has been called collagenase.

Reticulin

Reticulin fibers occur in most connective tissues as a network of fine fibrils which, in sharp distinction to the collagen fiber, exhibit marked branching and anastomosis. They are thought, in the skin, by many histologists to compose a network which surrounds collagen fiber bundles a helps to hold them together and are therefore formed by the union of collage with polysaccharides and other proteins like albumin, globulins and mucoids (Meyer,1945). This showed that reticulin is closely associated with carbohydrate material. The name "reticulin" referring to a specific protein, of which the reticular fibers have frequently been supposed to be composed, derives from their characteristic formation of a network, or reticulum (Biekiewicz,1983).Reticulin tissues are found in the grain surfaces of the hides and skins in comparison to the corium major. They are much finer, as they are strongly laterally bonded when compared to the collage fibers. The differences in chemical and physical properties of these two types of proteins naturally classify them into two distinct groups. Reticulin is a procollagen since there are many proofs, which support the view, that reticulin is n early stage of development of collagen. (Puvanakrishnan, 1988).

It would appear that the evidence arising from most of the recent investigations of reticulin strongly suggest that reticular fibers are essentially similar to collagen fibers, and that the observable differences in their behavior are due primarly to the association, in some unknown manner, of

the reticular material with the carbohydrate-rich material of the ground substance.

Elastin

Most connective tissues of the mammalian body contain yellow-colored elastic fibers (in their native condition) in addition to collagen fiber. The protein of which these fibers are composed is known as elastin. In some tissues, as in certain ligaments and the walls of blood vessels, elastin forms the predominating component and the collagen the minor one, while in other tissues the reverse is the case. Elastin occurs in very small quantities in the skin, the largest amounts being found in the grain layer (Biekiewicz,1983).

Elastin possesses rubber-like long-range elasticity, the property from which its name is derived. It is extremely resistant to water, in which it can be boiled with no apparent effect. It is much more resistant than collagen to the action of acids, alkalis and ordinary proteolytic enzymes. Elastin is apparently digested by trypsin obtained from crude pancreatins rather than crystalline trypsin. Studies have shown that pancreatic extracts contain an enzyme, which specifically attacks elastin, with a pH optimum at 8.7 to 9.3 and which does not attack collagen. Purifed trypsin and chymotrypsin did not attack elastin. The elastin-specific-enzyme is called the elastolytic enzyme or elastase. Appreciable amounts of sulphuric acid are liberated by the action of elastase on human elastic tissue but less from cattle ligament. Action of elastase on elastin involves the rupture of peptide bonds with the liberation of free α-amino groups. Evidence was shown in advance that elastase consists of two enzymes, which act on two components of an elastic tissue preparation, which is itself, dual in nature. Since elastin is extremely

resistant n nature, quite vigorous procedures have been used, where the danger of denaturation must always e considered. Conventional procedures for its purification involve the extraction of extraneous proteins with dilute salt solutions, the extraction of mucopolysaccharides with alkaline reagents such as dilute KOH solutions, removal of collagen by prolonged boiling in water, and defatting in orangic solvents (Puvanakrishnan,1988). It is mainly composed of non-polar side chains considerably high amounts of valine and proline.

II.2 LITERATURE ON ENZYMIC UNHAIRING

The hair or wool is usually collected in the tanneries after treating the skins with lime or lime-sulphide mixture, thereby damaging the hair. Moreover, the improper disposal of tannery effluents from the Beamhouse contains a lot of toxic and sludge forming chemicals and possibly some pathogenic organisms. Therefore, viewed from the environmental pollution, effluent disposal and optimal collection of hair or wool, sustained efforts are necessary to rationalize the dehairing processes using enzymes and enzymatic products in place of caustic chemicals.

The process of unhairing is achieved by one or the other of two general methods viz.

- By attacking the hair and reducing it to a pulp
- By destroying or modifying the epidermal tissue surrounding the hair bulb, so that the hair is loosened and can be removed mechanically (Merrill, 1956).

Hair destruction methods involve the rupture of the di-sulphide and other bonds which stabilize the hard keratins of the epidermis while hair loosening methods have been observed to involve only the partial or complete destruction or only a softening of the tissues that hold the hair in place.
Although there are five methods of unhairing viz.

- clipping process
- scalding process
- chemical process
- sweating process
- enzymatic dehairing process

Enzymes, being biological in nature, have less impact on the environment and are best considered when compared to the other processes. Contemporary chemical and biological technologies obtain enzyme preparation of high degree of specificity from plants, animals and microorganisms. They contain enzyme complexes, activators and buffers and are adjusted to proper pH. If the enzyme attack (most frequently an enzyme complex) is too vigorous, too much protein may be removed from the skin and collagen partly decompose making the resultant hides to be thin and stiff. If the enzyme action is too weak, insufficient amount of protein will be digested and additional operations are necessary such as alkaline swelling of hides before pickling. The enzymatically unhaired hides as a rule have to be tanned and dyed in different way than hides processed by other techniques. However, the enzymatically unhaired hides are considered to be spread, empty, and the economical aspect of this is still unsatisfactory, as a large amount of enzyme has to be used in order to obtain good results. Short liming with the help of NaOH, which is then stopped by the addition of

ammonium sulphate makes easier penetration of the enzyme into the hide. To increase the unhairing activity, the pelt has to be prepared by applying and inorganic chemical before the enzyme is used. The compounds giving optimal results are ammonium chloride, thiocyanate and sodium thiosulphate. Bacterial preparations and mould proteolytic enzymes produced leathers that were well-mellowed, soft and more resistant to stretch than the ones obtained in the common way. (Puvanakrishnan,1988).

Histological studies show that the Malpighian layer, basal cells of the hair bulb and hair medulla are less highly keratinized and contain fewer disulfide bonds, they are digested by the enzymes when compared to the cortex. Since so many different enzymes can loosen hair, it is likely that any different components of the Malpighian layer may be attacked. However, very little protein such as sulphur-containing proteins was solubilized in the enzyme bath of a multi-stage process for dehairing skins, much less than that in the preceding soak, alkaline swelling and deswelling baths thereby concluding that the function of the enzyme is merely to affect the partial hydrolysis of the proteins binding the hair and epidermis to the underlying connective tissue. It should be possible to unhair skins with mucolytic enzymes, i.e. enzymes that split off sugars from mucoids which are complex proteins containing one of the several different polysaccharides adjuncts (Schiller. S,1957).

It has been found that proteolytic and amylolytic enzymes derived from various sources have been applied individually or in combinations to produce effective unhairing of hides and skins (Biekiewicz,1983). However proteolytic enzymes are seen to be more efficient and find wider application

in enzymatic unhairing than amylolytic enzymes. Hair gets loosened by the action of autolytic or lysosomal enzymes present in the hides at a pH range of 7.0-8.5 after giving acetic acid treatment or by the autolytic action of proteolytic enzyme present in the skin or hide at optimum conditions of unhairing are formed to be incubated at 37°C in alkaline pH with a relative humidity of about 100%.

With the renewed emphasis on biotechnology, microbial enzymes have received increasing attention and processes that involve versatile utilization of microbial metabolic machinery for the production of enzymes are currently being studied with great interest. Microbial proteases are derived from a wide variety of yeasts, moulds and bacteria of which yeast proteases, being intracellular in nature, have not gained significant commercial interests (Puvanakrishnan,1988).

The protease from *Aspergillus flavus* has been observed to be suitable for unhairing (Rohm, 1935) and later on for simultaneous unhairing and bating (Rohm,1953). Enzyme preparations from cultures of *Aspergillus oryzae*, *Aspergillus parasiticus*, *Aspergillus fumigatus*, *Aspergillus effuses*, *Aspergillus ochraceus*, *Aspergillus wentii* and *Penicillum griseofulvum* (Gillespie, 1953).

A chemical-ezymatic method of liming and unhairing yields cattle hides with higher contraction temperature and degree of collagen crystallinity. When enzymatic treatment is preceded by alkaline pretreatment, the waste contains no toxic impurities and after removal of chromium salts, can be used as fertilizers. (Lisik,1978).

The process of enzymatic unhairing by means of proteolytic and amylolytic enzymes has been suggested to depend essentially on the hydrolysis and removal of the skin mucoids. (Bose et al, 1955). When the skin mucoids are removed, the cohesion of the cementing of the protein fibers is reduced and the entire structure gets loosened. The ground substance in the skin is a hyaluronic acid-protein complex, but no significant correlation could be observed between the apparent hyluronidase activity and depilatory action. The advantages of the above process are

- It is useful as a hair-saving method by providing both pelt and hair/wool in good condition, helps in easy handling of the pelts avoiding discomfort to tannery workers, reduces the tannery effluent disposal problem and simplifies the pre-tanning processes by cutting down bating (Hannan , 1982).
- The undisturbed nature of the original hide-structure
- It has been shown that the difficulty arising from the gelatinization of the sludge derived from the hair could be avoided by the use of enzymes
- Further upto 50% production in the BOD levels of the effluent, along with significant reduction in the chemical load of the effluent can also be achieved

II.3 LITERATURE ON ELASTASE

An enzyme is a protein that speeds up the rate of reaction of specific substance without being changed itself. These biological catalysts enable the specific organic substance to be degraded. As natural biological substances,

enzymes have much less impact on the environment than the chemicals used in various industries. The also speed up the process and are highly specific in their activity (Lansing , 1952).

Biotechnology especially the use of enzymes plays an important role in the leather industry. It has added value by providing improved, cleaner processes for leather production and enhanced environmental management in the industry. It has been found that bacterial enzymes can influence the properties of the final leather product by targeting the elastin fibers within the skin. Its also been proved that bacterial enzyme with specific elastase activity is much more effective in degrading elastin than the enzyme with general protease activity. Removing the elastin protein will make leather more softer and more flexible.

Elastase, like trypsin ad chymotrypsin, is an enzyme belonging to the class of proteases that break down proteins. It is a serine protease that also hydrolyses amides and esters. It is produced in the pancreas as an inactive zymogen, proelastase and activated in the deodenum by trypsin (Okumara, 2004). Elastase is distinctive in that it acts upon native elastin, an elastic fiber in the hide skin that together with collagen, determine the mechanical properties of connective tissues. Elastin is found in highest concentration in the elastic fibers of connective tissues, and is not attacked by trypsin, chymotrypsin or pepsin. Elastase is frequently used along with other proteolytic enzymes to dissociate tissues that contain extensive intercellular fiber networks.

Elastase, which is primarily produced in the pancreas, is also found in blood components. Elastolytic and elastase inhibitory activities were investigated for thirteen strains of *A. fumigatus*, three strains of *A.flavus* and three strains of *A niger*. While nine of the thirteen strains of *A.fumigatus* and all strains of *A.flavus* demonstrated elastase activity in more than one unit/ml, six of the thirteen strains of *A.fumigatus* and all strains of *A.flavus* expressed elastase inhibitory activity in more than 2 units/ml. However no elastase or elastase inhibitory activity were observed with *A.niger*. Moreover elastase from *Streptomyces griseus*, *Flavobacterium immotum* and mouse have also been reported (Okumara, 2004).

There exist two genes for elastase, namely, pancreatic (ELA-1) and neutrophil (ELA-2) elastase. Neutrophil elastase which is further seen in its two forms, termed II a and II b , is 218 amino acids long and is present in azurophil granules in the neutrophil cytoplasm. The gene for ELA-1 is located on chromosome 12q13 and that for ELA-2 is located on chromosome 12p13.3 and consists of fie exons.

On characterizing, porcine pancreatic elastase was found to have a molecular weight of 25.9 kDa. It is s ingle peptide chain of 240 residues and contains four disulphide bridges. Its optimum pH and isoelectric point were both found to be 8.5. Its extinction coefficient is 22. its active site is composed of several subsites and elastolytic activity is due only to its electrostatic adsorption onto elastin and the susceptibility of elastin to hydrolysis may be controlled by elastin ligands. It is inhibited by diisoproply-phosphofluoridate , alkylisocyanates and peptide chloromethyl ketone (Okumara, 2004). Derivatives of dipeptide and alanine, valine,

leucine and isoleucine are effective competitive inhibitors as well. However soybean trypsin inhibitor and kallikrein inhibitor suppress proteolytic but not elastolytic activity. Recent studies have shown that elastase is inhibited by the acute phase protein α_1 – antitrypsin (A1AT), which binds covalently 1:1 to it. α_1 – antitrypsin deficiency (A1AD) leads to uninhibited destruction of elastic fiber, resulting in pulmonary emphysema. Elastase is unstable at a pH lower than 3.5. When stored as a powder the enzyme is stable for 6-12 months t 2-8°C.It is stable at a pH between 4.0-10.4. One Unit of the enzyme is defined as the amount of enzyme that cleaves one micromole of the substrate, N-succinly-L-alanyl-L-alanyl-L-alanine-p-nitroanilide per minute at 25 °C, pH 8.0 (Okumara, 2004).

The apparent ability of *A.fumigatus* isolates to produce elastase in agar plates correlates with their ability to cause invasive pulmonary aspergillosis (Bezzera, 1999). In mice pretreated with cortisone, whose production governs the pathogenicity of particular isolates leading to the disruption of elastic layers within the blood vessel walls. *A.fumigatus*, when grown on he insoluble structural material obtained from murine and bovine lung, produces an extracellular 33-kDa elastolytic protease ultimately infecting the lung (Latge, 1999). Screening of hospital isolates of *A.flavus* showed great variation in the production of elastolytic activity much lower than that produced by *A.fumigatus* (Bezzera , 1999).

CHAPTER III

OBJECTIVES

OBJECTIVES

To develop practically and economically feasible biotechnological solutions to overcome the economical impedance of enzymatic hair removal (in Leather Manufacture).

- To verify and ensure the fundamental possibility of use of elastase in reducing protease requirement for unhairing.
- To identify suitable microorganisms for the production of elastase.
- To produce and partially purify the elastase.
- To characterize the elastase.

CHAPTER IV

MATERIALS AND METHODS

1V.1 MATERIALS

- Four fresh male goatskins.
- Elastase (EC 3.4.21.36)

Type I: From Porcine pancreas

IV.2 REAGENTS

IV.2.1 Media Used For Enzyme Production

Malt Extract Agar

Malt extract	-30.0gms
Dipotassiun Hydrogen phosphate	-1.0gm
Ammnium chloride	-1.0gm
Citric acid(1N)	-15.0mL
Agar	-12gms
Distilled water	-1000.0mL

The contents were mixed, autoclaved and prepared as slants.

Yeast Carbon Base

Succinic acid	-10.0gms
Sodium hydroxide	-6.0gms
Ammonium sulphate	-1.0gm
Distilled water	-900.0mL
Glucose(20%solution w/v)	-100.0Ml

Glucose is added from a sterile concentrated stock after autoclaving to prevent caramelization.

IV.2.2 Buffers Used For Enzyme Characterization

Tris/Hcl Buffer(50mM)

Stock solution A: 50mM solution of Tris (hydroxy methyl) aminomethane (6.05 gms in 1000 mL)

Stock solution B: 50mM HCl (1.825 mL in 1000 mL)

50 mL of A and 39.9 mL of B are diluted to a total of 200 mL to get the desired pH of 7.5

Sodium Acetate Buffer(20mM)

Stock solution A: 20mM solution of acetic acid (1.155 mL in 1000 mL)

Stock solution B: 20mM solution of sodium acetate(1.64 gm) or anhydrous sodium acetate(2.72 gms) in 1000 mL

41 mL of A and 9 mL of B are diluted to a total of 100 mL to get a desired pH of 4.0.

14.8 mL of A and 35.2 mL of B are diluted to a total of 100 mL to get a desired pH of 5.0

Sodium Phosphate Buffer(20mM)

Stock solution A: 20mM solution of monobasic sodium phosphate (2.78 gms in 1000 mL)

Stock solution B: 20mM solution of dibasic sodium phosphate (5.365 gms of $Na_2HPO_4.7H_2O$ or 7.17gms of $Na_2HPO_4.12H_2O$ in 1000mL)

X mL	Y mL	pH
87.7	12.3	6.0
39.0	61.0	7.0
16.0	84.0	7.5

X mL of A and Y mL of B are diluted to a total of 200 mL.

Sodium Borate Buffer (20mM)

Dissolve 1.236 gms of boric acid (H_3BO_3) and 0.16 gms of Sodium hydroxide in 1000 mL. By varying the volume of boric acid and sodium hydroxide the various desired pHs (7.5,8.0,8.5 and 9.0) are obtained.

Sodium Carbonate-Bicarbonate Buffer (20mM)

Stock solution A: 20mM solution of anhydrous sodium carbonate (2.12 gms in 1000 mL).

Stock solution B: 20mM solution of sodium bicarbonate (1.68 gm in 1000 mL).

13.0 mL of A and 37.0 mL of B are diluted to a total of 200 mL to get a desired pH of 9.5.

27.5 mL of A and 22.5 mL of B are diluted to a total of 200 mL to get a desired pH of 10.0.

IV.3 METHODOLOGY

IV.3.1 Experimental Design

- Four fresh male goatskins are marked as I, II, III and IV.
- The skins are trimmed.
- The skins are cut along the backbone, the left halves are marked as IL, IIL, IIIL and IVL and the right halves as IR, IIR, IIIR and IVR.
- Each of the eight samples is weighed
- All the left halves are kept as control and the right halves as experimental pieces.

IV.3.2 First Soaking

- All the skins are collectively soaked in 300% (of the total initial weight of the skin samples) distilled water for 30 minutes.
- The skins are taken out and the waster water is drained effectively.
- The wastewater is collected and measured.
- The skins are weighed individually.

IV.3.3 Second Soaking

- The control skins are collectively soaked in 300% (of the total initial weight of the control samples) distilled water overnight.
- The experimental skins are soaked individually in 300% (Of the initial weight of each sample) distilled water along with porcine pancreatic elastase enzyme (Sigma) in varying concentrations viz 2.59 X 10^{-5}%,

7.79 X 19^{-5}%, 2.34 X10^{-4}% and 7.0 X 10^{-4}% for experiments I to IV respectively.

- The skins are taken out and the wastewater is drained effectively for 20 minutes.
- The wastewater is collected and measured in each case.
- Sample of wastewater is taken for analysis.
- A small portion of the skin is cut from each sample for analysis of protein and chloride content.

1V.3.4 Liming And Unhairing

- All the skins are applied (on the flesh side) with the paste of 0.5% Biodart (SPIC) and 10% water.
- The skins are piled and unhairing efficacy is studied.
- After hair removal, the pelts are treated with 7% Lime and 300% water for 2 days.

4.1 UNHAIRING

4.2 REMOVED HAIR AND THE PELT

1V.3.5 Washing And Deliming

- The pelts (limed skins) are washed in drum with 250% (of the total pelt weight) water for 40 minutes.
- The water is drained out completely.
- 200% (of the total pelt weight) water and 1.5% (of the total pelt weight) Ammonium chloride (commercial reagent) are added to the drum and run for 40 minutes.
- The water is completely drained once again.
- The pelts are washed again with 250% water.

1V.3.6 Pickling

- 100% (of the total pelt weight) water and 10% (of the total pelt weight) Sodium chloride (commercial reagent) are added and run for 15 minutes.
- 1% (of the total pelt weight) Sulphuric acid is diluted in 10% (of the total pelt weight) water and administered in three feeds.
- After 1^{st} and 2^{nd} feed the drum is run for 15 minutes and after 3^{rd} feed it is run for 30 minutes.

1V.3.7 Chrome-Tanning

- Half of the pickling bath is drained out and to the remaining bath is added 8% (of the total pelt weight) Basic Chromium Sulphate (commercial reagent) and its drum is run for 45 minutes.
- 50%(of the total pelt weight) water is added to this and is run for 30 minutes.

- 1% (of the total pelt weight) Sodium formate (commercial reagent) is added and is again run for 20 minutes.
- 1%(of the total pelt weight) Sodium bicarbonate (commercial reagent) is diluted in 10%(of the total pelt weight) distilled water and is fed into the drum in three installments every 15 minutes and is allowed to run for 30 minutes after the final feed.
- The water is drained out completely and the pelts are piled after washing in water and are left overnight.
- The left over water is completely squeezed out without straining the samples and their individual weights are recorded.

1V.3.8 Re-Chroming

- 100%(of tanned leather weight) water and 0.5%(of tanned leather weight) Acetic acid (commercial reagent) is added with the tanned leather in the drum and is run for 15 minutes.
- 4%(of tanned leather weight) Basic Chromium Sulphate (commercial reagent) is added and is run again for 45 minutes.
- To this, is added 1%(of tanned leather weight) Sodium formate (commercial reagent) and is run for 20 minutes.
- 1%(of the tanned leather weight) Sodium bicarbonate (commercial reagent) is diluted in 10% (of tanned leather weight) distilled water and is fed into the drum in three installments every 15 minutes, and is allowed to run for 30 minutes after the final feed.

1V.3.8 Neutralization

- 200%(of tanned leather weight) water is added with the tanned leather in the drum and is run for 20 minutes.
- The water is drained out completely.
- 100%(of tanned leather weight) water and 1.0%(of tanned leather weight) Sodium formate (commercial reagent) is added and is run for 15 minutes.
- 1%(of the tanned leather weight) Sodium bicarbonate (commercial reagent) is diluted in 10% (of tanned leather weight) distilled water and is fed into the drum in three installments every 15 minutes, and is allowed to run for 30 minutes after the final feed.
- The water is drained out completely.
- 200%(of tanned leather weight) water is added and is run for 20 minutes and water is completely drained out.
- The pH of the leather is observed to be 5.0.

1V.3.9 Re-Tanning And Fat-Liquoring

- 100% (of tanned leather weight) water and 2.0%(of tanned leather weight) Relugan RE (commercial reagent) are added to the drum with the already tanned leather and is run for 30 minutes.
- 2% (of tanned leather weight) Balmol SXE is added run for 20 minutes.
- 1% (of the tanned leather weight) Formic acid (commercial reagent) is fed into the drum in three installments every 10 minutes, and is allowed to run for 20 minutes after the final feed.

- The water is drained out completely and leather is piled up and is left overnight.
- The left over water is completely squeezed out without straining the samples and henceforth is left for a few hours till they are dried.
- The leather is then tested for grain bursting and tensile strength

4.3 RE-TANNING AND FAT-LIQUORING USING A DRUM

IV.3.10 Determination Of Moisture Content

- A known amount of substance is taken in a previously weighed evaporating dish.
- The evaporating dish along with its contents is placed in a hot air oven at 100°C for 5 hours, cooled and weighed.

$$\text{Percentage moisture} = \frac{\text{Initial weight} - \text{Final weight}}{\text{Weight of the sample}} \times 100$$

Where, Initial weight = weight of the empty evaporating dish + weight of the skin

Final weight = weight of the evaporating dish with skin after keeping it in the oven.

IV.3.11 Determination Of Protein Content

The nitrogen content in the skin samples is estimated by kjeldahl's method.

- About 0.5 grams of sample is weighed accurately to a clean dry kjeldahl flask.
- Concentrated Sulphuric acid (volume 10ml) is added and contents are heated placing flask in an inclined position.
- About 1 spatula catalyst mix (Copper sulphate and Potassium) is added to the flask and heated briskly until solution turns colorless.
- A funnel is kept in the neck of the flask to prevent undue loss of acid during digestion.
- The contents of the flask are allowed to cool.

- After cooling, 200 mL of water is added to dilute contents.
- An excess of 40% sodium hydroxide is poured into the flask carefully through sides of the flask, which is then connected, to the distillation assembly.
- The contents are collected in 50mL of 4% boric acid solution containing few drops of mixed indicators (Methyl red and Methylene blue).
- When the evolution of Ammonia is completed the receiver is removed and distillate is titrated against 0.1N HCl.
- The end point is the color change from green to pink.

IV.3.12 Determination Of Chloride Content

- The pieces of skin along with distilled water are kept in a mechanical shaker.
- The supernatant solution is collected.
- A known volume of the supernatant (or waste water itself can be used as a sample) is added and is stirred for a minute.
- 2 drops of potassium chromate is also added.
- The solution is titrated against standardized silver nitrate till a reddish brown precipitate appeared.
- The same procedure is followed for blank and the difference in titre values are recorded.

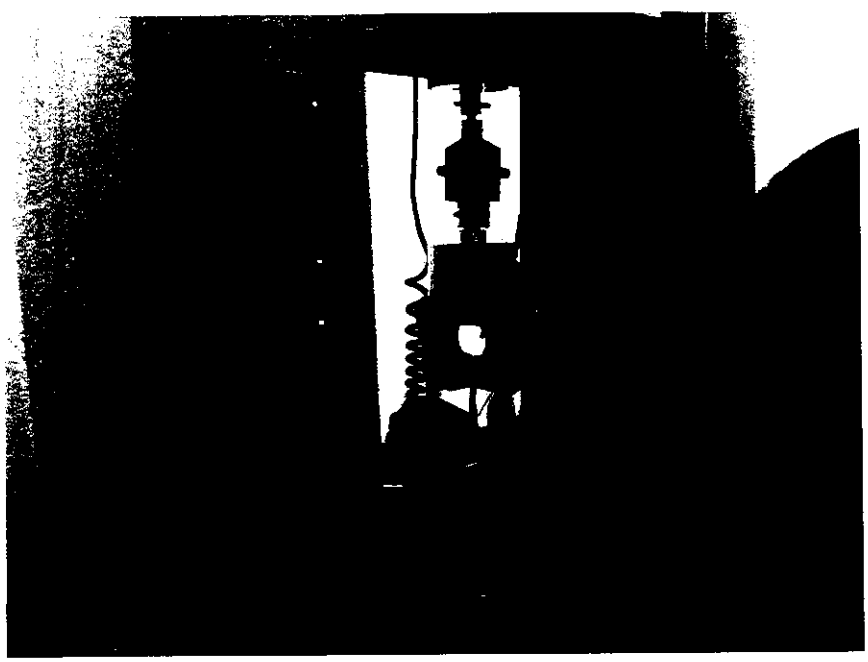
IV.3.13 Determination Of Tensile Strength

The tensile strength of the leather is tested using INSTRON.

- The leather samples are cut in the desired shape and thickness is measured using the thickness gauge.
- The distance between the upper and lower jaws is maintained at 5 cm in the INSTRON machine.
- The sample is clipped between the jaws and load is added.
- The load and the displacement for which the leather breaks is noted and the tensile strength is calculated.

4.4 MEASURING THE THICKNESS

4.5 TENSILE STRENGTH TESTED USING INSTRON



IV.3.14 Grain Bursting

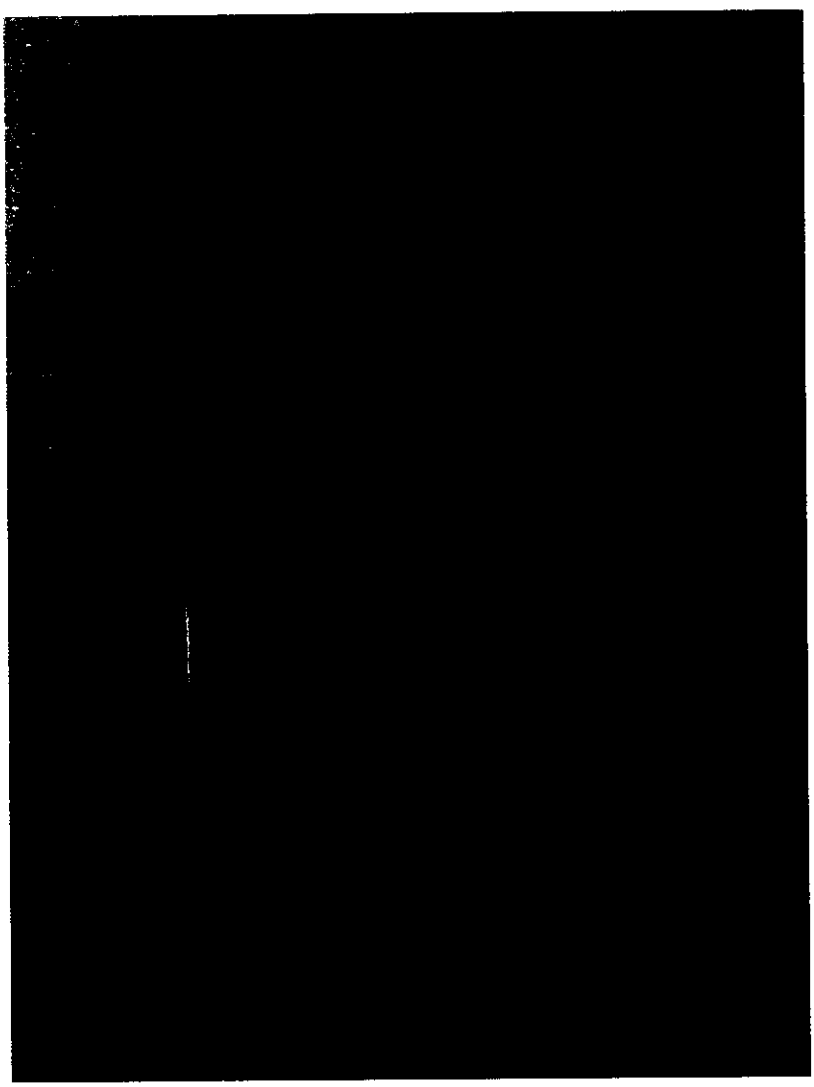
Grain bursting of the leather is tested using lastometer-LASRA

- The leather samples are cut in the desired shape and are fixed on the platform of the equipment.
- Care is taken too see that the load and the displacement reads zero before starting the experiment.
- The handle is rotated to add load, and as the sample moves down the leather breaks and the corresponding load is noted as the grain bursting load.

IV.3.15 Enzyme Production

- *Aspergillus flavus* is carried on slants of malt extract agar at 30°C.
- Stock cultures are stored in sterile distilled water and fresh cultures are prepared as needed.
- Flasks of yeast carbon base prepared in borate buffer containing elastin particles are inoculated to a starting concentration of $1 \times 10^5 - 2 \times 10^5$ conidia per ml.
- Flasks are incubated for 72 hrs at 37°C in a shaker.
- The culture medium is collected after being sequentially filtered through four-eight layers of cheesecloth and glass fiber filters to remove the gross solids obtaining the crude Aspergillus Elastase (AE)

4.6 SLANTS OF *ASPERGILLUS FLAVUS* IN MALT EXTRACT



IV.3.15.1 Determination Of Viability

- A drop of the conidial suspension is made from the liquid culture was placed on the engraved grid and the preparation is let to stand for 1-2 minutes to allow the conidia to settle at the bottom.
- The cover glass of the counting chamber is put over the grid carefully so that no air bubbles enter between the slide and the cover glass.
- The cover glass is slid backwards and forwards until coloured rings are visible as the two surfaces of the cover glass.
- Conidia of the fungus in the middle square is counted (since they are small in size), which consists of 25 groups of 16 small squares, each group 0.2mM square.

Spores/mL = Number of spores counted o the middle square of the grid×10000(each large square has a volume of $1/10cm \times 1/10cm \times 1/100cm^3 = 1/10^4 cm^3$)

IV.3.16 Ammonium Sulphate Precipitation

- The culture medium is centrifuged at 15000 rpm for 10 minutes at 4°C and the supernatant is taken.
- The supernatant is fractionated by adding 60% Ammonium sulphate at pH 7.0 for 2-3 hours at 4°C.
- The precipitate is removed by centrifugation at 12000 rpm for 20 minutes at 4°C.

IV.3.17 Enzyme Characterization

IV.3.17.1 ENZYME ASSAY (AE)

- 50mM Tris/HCl buffer and elastin Congo red were pipetted into a test tube according to the table.

Reagents	1	2	3	4	5
Tris/HCl buffer(mL)	1.49	1.45	1.40	1.35	1.30
Elastin Congo red (mG)	20	20	20	20	20
AE(mL)	0.01	0.05	0.10	0.15	0.20

- The appropriate amount of enzyme ias pipetted into the test tube as given in the table.
- The contents are mixed by inversion and pH is maintained at 7.5 under continuous shaking for 30 minutes at 37°C.
- The reaction is stopped by removing the substrate particles under mild centrifugation at 5000 rpm for 10 minutes.
- The orange supernatant obtained is placed in spectrophotometer and the absorbance was recorded at 495nm.
- Increase of 0.1 unit absorbance is equal to 1 unit enzyme activity.

IV.3.17.2 DETERMINATION OF OPTIMUM pH

The pH optimum is determined by assaying AE, against elastin Congo red particles suspended in the following buffers-

20mM sodium acetate buffer for pHs 4 and 5

20mM sodium phosphate buffer for pHs 6,7 and 7.5

20mM sodium borate buffer for pHs 7.5,8,8.5 and 9

20mM sodium carbonate-bicarbonate buffer for pHs 9 and 10

- A series of test tubes are set up each containing 20mM buffers of the following pH values: 4,5,6,7,7.5,8,8.5,9 and10.
- The experiment is started by adding 1.45mL of 20mM buffer (pH 4) containing 1.5 mL of elastin Congo red.
- The appropriate amount of enzyme is pipetted into the test tube as given in the table.
- The test tube contents are mixed by continuous shaking for 30 minutes at 37°C.
- The reaction is stopped by removing the substrate particles under mild centrifugation at 5000 rpm for 10 minutes.
- The orange supernatant obtained is placed in spectrophotometer and the absorbance was recorded at 495nm.
- The steps 2-6 are repeated for each of the indicated pH values.

IV.3.17.3 DETERMINATION OF OPTIMUM TEMPERATURE

The sensitivity of AE to temperature extremes is determined by incubating the enzyme at 0°C, 37°C, 45°C, 60°C, 80°C and 100°C.

- A series of test tubes are set up each containing 20mM sodium borate buffer of pH 8 and elastin Congo red.

- The enzyme is added into the cuvette and the contents were mixed thoroughly.
- The tubes are placed in boiling water baths and is adjusted to the following temperatures, 0°C, 37°C, 45°C, 60°C, 80°C and 100°C.
- The experiment is started with the tube kept at 0°C, mixed by continuous shaking for 30 minutes at 37°C.
- The reaction is stopped by removing the substrate particles under mild centrifugation at 5000 rpm for 10 minutes.
- The orange supernatant obtained is placed in spectrophotometer and the absorbance is recorded at 495nm.
- The steps 4,5 and 6 are repeated for each of the indicated temperature

CHAPTER V

RESULTS AND DISCUSSION

V.1 APPLICATIONS OF ELASTASE IN LEATHER MANUFACTURING

The weight of the skin samples prior to soaking is given in table 5.I.I:

TABLE 5.I-Weight of the skin prior to soaking

SAMPLE	WEIGHT (gms)
IL	430
IIL	400
IIIL	440
IVL	400
IR	420
IIR	400
IIIR	430
IVR	400

The chemical composition of the skin prior to soaking were calculated and is listed in table 5.I.2

TABLE 5.2- Characteristics of raw skin

MOISTURE CONTENT	CHLORIDE CONTENT	NITROGEN CONTENT
43%	13.4%	9.3%

V.1.1 Effect In Soaking

First Soaking

The skins were soaked in 300% water for half an hour. Degree of rehydration can be calculated by adding the chloride released from the skin to the weight gained after first soaking. Total amount of chloride released during first soaking is 107.07 gms (volume 2.9 L X 36.92 gms of salt). The weight gained after first soaking is 285.5 gms. Hence the total volume of water taken up by the skins after first soaking is 392.5 gms. Hence the degree of rehydration is 11.8%. The Chloride content and the Nitrogen content are tabulated and given in table 5.I.3.

TABLE 5.3-Chloride and Nitrogen content of the first soaked water.

CHLORIDE CONTENT	NITROGEN CONTENT
36919.05 mg/L	8.4%

Second Soaking

The control samples were collectively soaked in 300% distilled water and the experimental samples were individually soaked in 300% distilled water along with varying concentrations of Elastase. Chloride level in the waste water, degree of rehydration and nitrogen content were analyzed in table 5.1.4.

TABLE-5.4-Water loss and chloride content in the second soaked water

SAMPLE	WATER LOSS(%)	CHLORIDE CONTENT(mg/L)	NITROGEN (%w/w)
ALL CONTROLS (I L-V L)	7.3	12440.114	7.9
I R	8.1	11537.203	7.2
II R	8.5	15851.113	7.5
III R	8.5	16453.054	7.4
IV R	8.9	13343.026	7.2

V.1.2 Effect In Unhairing

The soaked skins were applied with protease enzyme for unhairing. The unhairing efficacy was assessed.

TABLE 5.5-Unhairing Index (1-10,10 being the best)

Expt.	Time				
	2 hr	**4 hr**	**6 hr**	**8 hr**	**10 hr**
ALL CONTROLS	1	1	3	5	7
I R	2	4	5	7	8
II R	2	5	7	8	9
III R	2	5	7	8	10
IV R	3	6	8	10	10

It was found that complete unhairing could be attained after eight hours and ten hours in experiment III and IV respectively.

V.1.3 Effect In Leather Quality

The quality of leather was tested for its physical characteristics by performing the Tensile strength and Grain bursting tests. The difference between the corresponding control and the experiment values were listed as in table 5.I.6 given below-

TABLE-5.6-Physical characteristics of leather

Sample no:	Difference in GBL (%)	Difference in TS (%)
I	3.4	5.1
II	4.9	4.3
III	2.5	3.1
IV	3.0	4.2

It could be understood the difference in the physical characteristics is insignificant. Hence it can be inferred that the Elastase treatment could not bring about any serious negative impact on the quality of leather

V.2 PRODUCTION OF ELASTASE

The use of Elastase proved to be successful for the process of unhairing in the leather industry bringing in with confident results, however, to make it highly commercial, another approach was devised by producing Elastase from the fungi *Aspergillus flavus.*

V.2.1 Innoculum

Aspergillus flavus was carried on slants of Malt extract agar at 30°C. Flasks of yeast carbon base (one liter) prepared in borate buffer containing elastin particles were inoculated to a starting concentration of 1 X 10^5 to 2 X 10^5 conidia per mL. Flasks were incubated for 72 hrs at 37°C in a shaker. The culture medium was collected after being sequentially filtered through four-eight layers of cheesecloth and glass fiber filters to remove the gross solids obtaining 827 mL of crude Aspergillus Elastase (AE).

V.3 PURIFICATION

The crude Aspergillus Elastase (AE) was partially purified by using 60% Ammonium sulphate. Ammonium sulphate acts as a precipitating agent due to its high solubility in water. The precipitation was carried out at 0 to 10°C to reduce denaturation. 220mL of the partially purified. Elastase enzyme was obtained. This was then used for further characterization.

V.4 CHARACTERIZATION

The partially purified Elastase enzyme was assayed and the specific activity was found to be 2.1 for 0.05 mL of the enzyme (optimum enzyme concentration) (fig 5.5.1). The optimum pH was found to be 8.0 (fig 5.5.2). Exposure of AE to pH extremes 2 and 11 resulted in the abolition of 97 and 100% of the enzyme activity respectively. Tris buffer of pH's 7.5 to 8.5 yielded specific activities equivalent to those obtained with borate buffer. The AE activity was very thermo-tolerant; optimal activity was obtained at 50°C (fig 5.5.3).

FIGURE 5.1

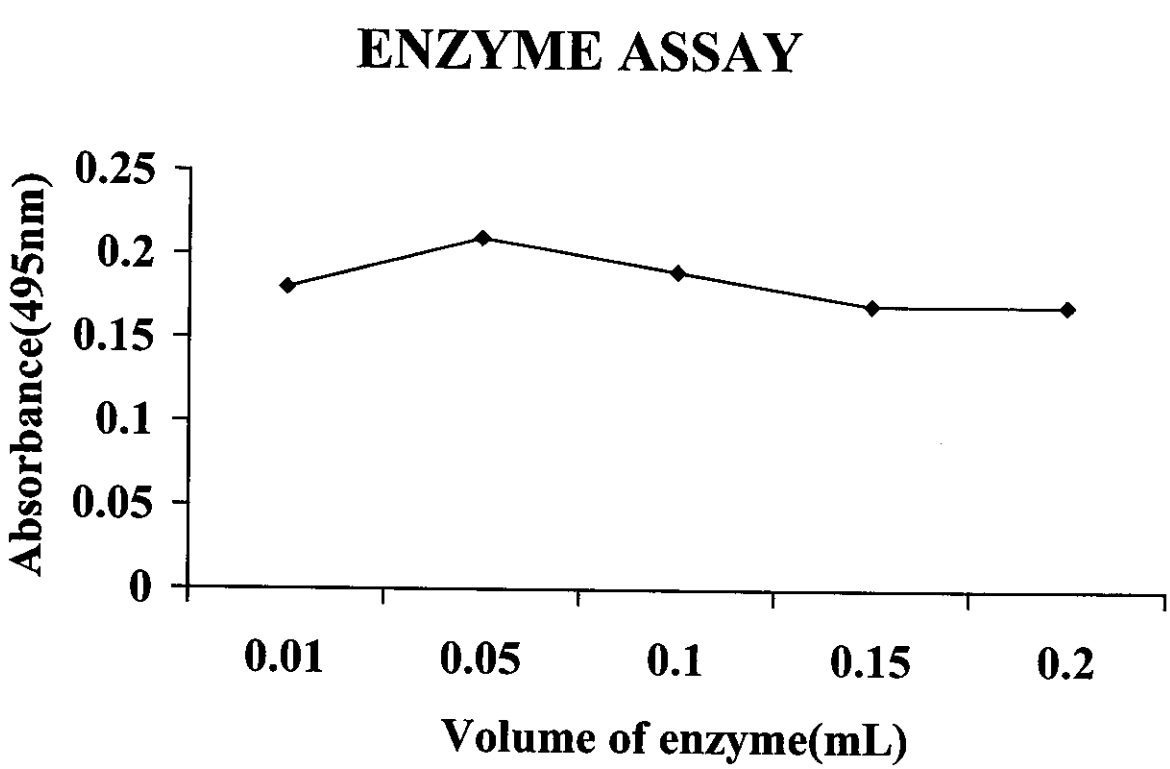

ENZYME ASSAY
Absorbance(495nm)
0.25
0.2
0.15
0.1
0.05
0
0.01
0.05
0.1
0.15
0.2
Volume of enzyme(mL)

FIGURE 5.2

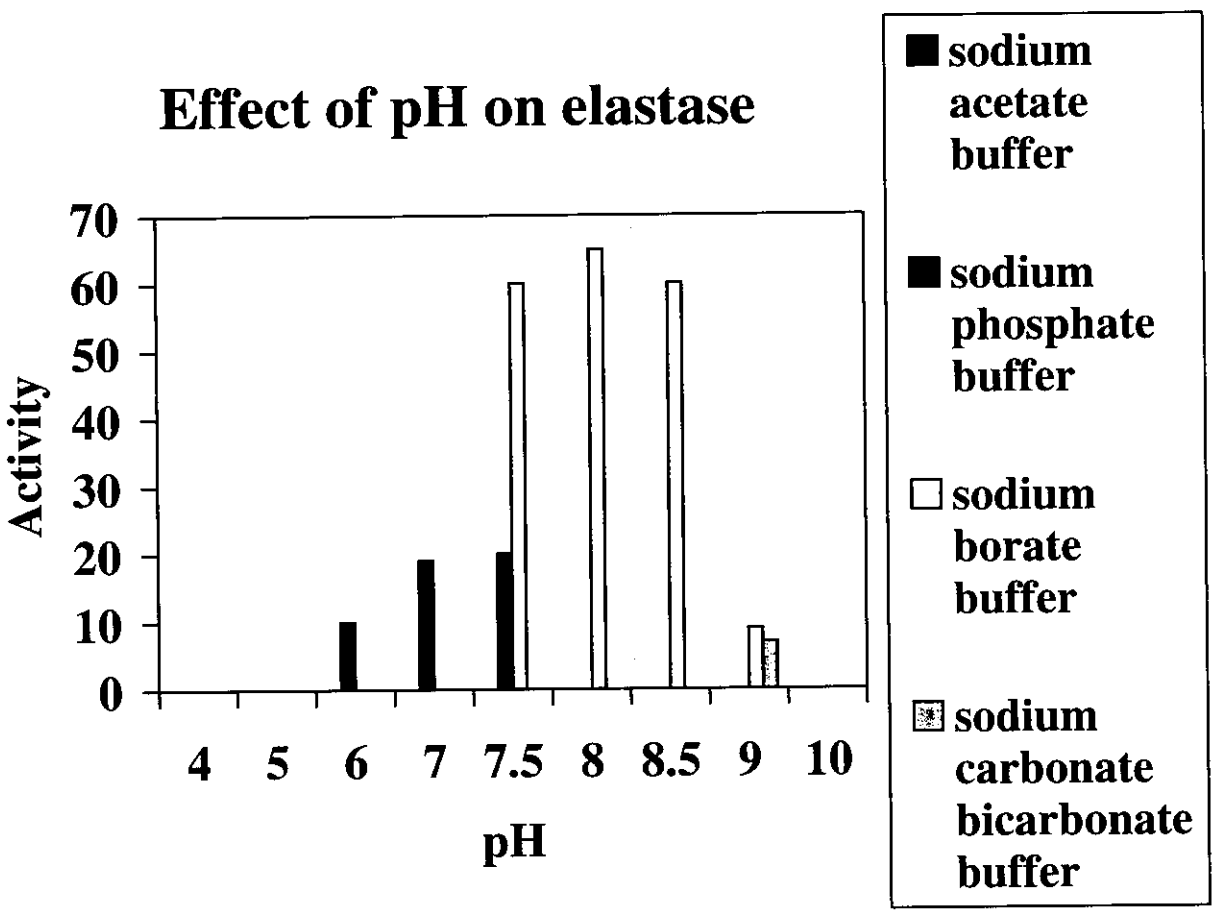
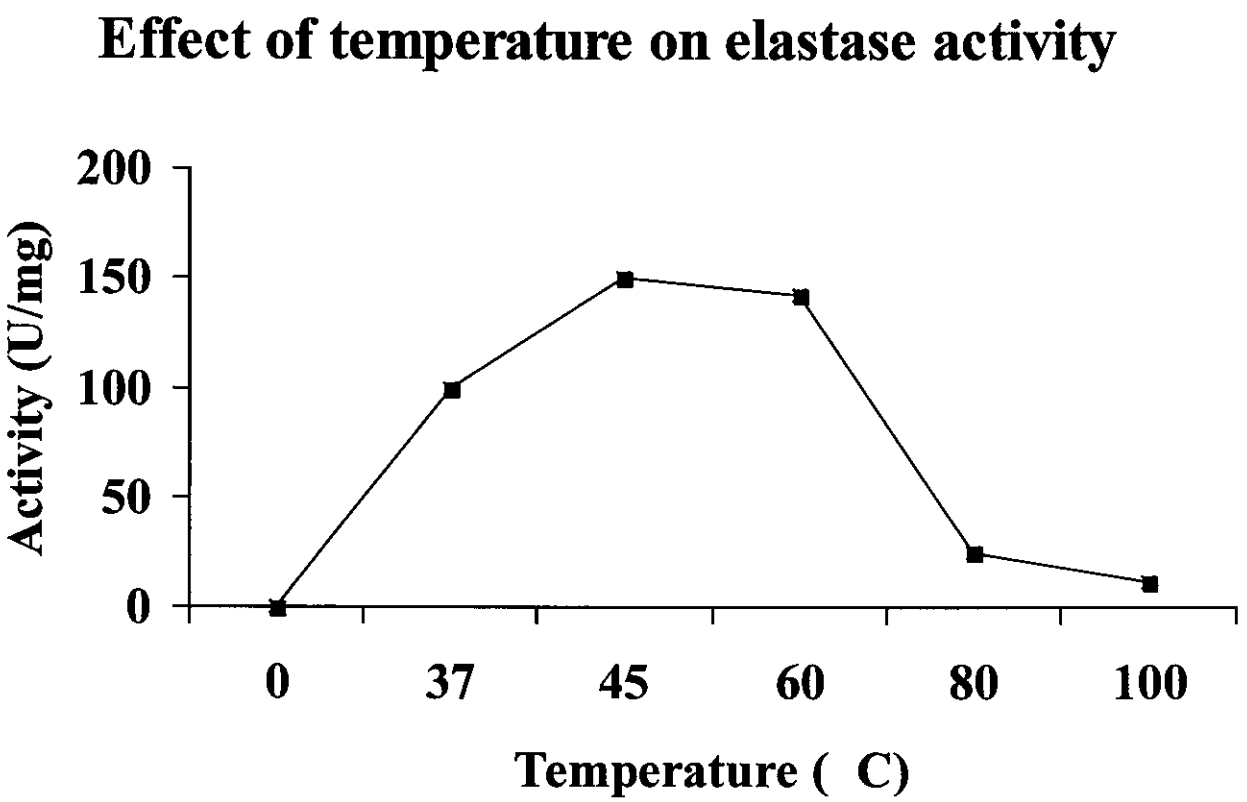

Effect of pH on elastase
Activity
70
60
50
40
30
20
10
0
4
5
6
7
7.5
8
8.5
9
10
pH
sodium acetate buffer
sodium phosphate buffer
sodium borate buffer
sodium carbonate bicarbonate buffer

FIGURE 5.3


Effect of temperature on elastase activity
Activity (U/mg)
200
150
100
50
0
0
37
45
60
80
100
Temperature (C)

CHAPTER VI

SUMMARY AND CONCLUSION

VI.1 SUMMARY

The present project has been pursued with the ultimate objective of development of a system for reducing the requirement of Protease for unhairing. It was aimed to use Elastase for reducing the Protease requirement for hair removal. Elastase (Sigma) in varying levels have been offered during second soaking prior to Pasting (with unhairing Protease). The degree of rehydration, salt removal and nitrogen content of skin after soaking were assessed. These parameters are indicative of the efficacy of Soaking as these are the performance indicators. After second Soaking, the skins are applied with a reduced level of protease (0.5%). Normally about 3.5% of the enzyme is employed for unhairing. Hence it was aimed for a seven-fold reduction. Hair removal efficacy was assessed. The effect of Elastase treatment on the quality of leather was also ascertained by testing the leathers for their physical characteristics.

Once it was understood clearly that the use of Elastase could bring down the Protease requirement, as the second phase of research Elastase was produced from *Aspergillus flavus.* The enzyme was characterized. It was found that this fungal species could be a potent and appropriate source for Elastase.

VI.2 CONCLUSION

Conclusively the use of Elastase has twin advantage. Use of this enzyme during second Soaking could bring about better Soaking evidently from degree of rehydration and salt removal. Use of this enzyme could bring down the requirement of unhairing Protease by a minimum of seven folds. Hence the cost of the process can be reduced by seven times. It can be understood from the physical testing data that the Elastase treatment doesn't render any negative impact to leather quality.

From the second part of the research, it can be concluded that *Aspergillus flavus* can be a potent and prospective source of Elastase.

VI.3 SCOPE FOR FUTURE USE

In our efforts to understand the role played by Aspergillus Elastase in effective unhairing in leather industry, further it has scope to study this enzyme, henceforth extending the pathway for further research in

- Studying the mechanism of the perturbance of the elastin fibers by the enzyme.
- Studying the change in microporous structure of the skin once treated with the enzyme.
- Decreasing the protease-level by a more significant fold

CHAPTER VII

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