STUDIES ON OPTIMIZATION OF PROTEASE PRODUCTION USING BACTERIAL ISOLATE (*CLRI STRAIN 5468)* AND ITS APPLICATION IN DEHAIRING AND HYDROLYSIS OF TANNERY FLESHINGS

(Solid Waste Management)

PROJECT REPORT

Submitted by

S. VIMALA DEVI

Register No: 1020203016

in partial fulfilment for the award of the degree

of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

(An autonomous institution affiliated to Anna University of Technology, Coimbatore-641 047)

COIMBATORE -641 049

APRIL 2012

STUDIES ON OPTIMIZATION OF PROTEASE PRODUCTION USING BACTERIAL ISOLATE (*CLRI STRAIN 5468)* AND ITS APPLICATION INDEHAIRINGAND HYDROLYSIS OF TANNERY FLESHINGS

(Solid Waste Management)

PROJECT REPORT

Submitted by

S. VIMALA DEVI

Register No: 1020203016

in partial fulfilment for the award of the degree

Of

MASTER OF TECHNOLOGY

IN

BIOTECHNOLOGY

KUMARAGURU COLLEGE OF TECHNOLOGY

(An autonomous institution affiliated to Anna University of Technology,Coimbatore-641 047)

COIMBATORE -641 049

APRIL 2012

KUMARAGURU COLLEGE OF TECHNOLOGY

COIMBATORE-641049

DEPARTMENT OF BIOTECHNOLOGY

PROJECT WORK

PHASE II

APRIL 2012

This is to certify that the project entitled

STUDIES ON OPTIMIZATION OF PROTEASE PRODUCTION USING BACTERIAL ISOLATE (*CLRI STRAIN 5468)*AND ITS APPLICATION INDEHAIRINGAND HYDROLYSIS OF TANNERY FLESHINGS (Solid Waste Management)

is thebonafide record of project work done by

S. VIMALA DEVI

Register No:1020203016

OfM.Tech (Biotechnology) during the year 2011-2012

…… ……..

Project Guide **Head of the Department**

Submitted for the project *Viva-Voce* examination held on.....

…….. ……

Internal Examiner External Examiner

DECLARATION

I affirm that the project work titled "**STUDIES ON OPTIMIZATION OF PROTEASE PRODUCTION USING BACTERIAL ISOLATE (*CLRI STRAIN 5468*) AND ITS APPLICATION INDEHAIRINGAND HYDROLYSIS OF TANNERY FLESHINGS (Solid Waste Management)**" being submitted in partial fulfilment for the award of M.Tech. (Biotechnology) is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other university.

S. VIMALA DEVI

(Reg.No. 1020203016)

I certify that the declaration made above by the candidate is true.

Signature of the Guide,

Mr.D.R.MANIMARAN

Assistant professor,

Department of Biotechnology,

Kumaraguru College of Technology,

Coimbatore-641 049.

ACKNOWLEDGEMENT

I wish to express my sincere thanks to **Dr.S.RAMACHANDRAN**, Principal, Kumaraguru College of Technology, Coimbatore, **Dr.A.MANICKAM** HOD, Biotechnology, Kumaraguru College of Technology, Coimbatore.

I wish to express my warm and sincere gratitude to my guide **Dr.C.ROSE**, Deputy Director and Head of the Department, Department of Biotechnology, Central leather Research Institute, Chennai, whose support from the initial to the final level enabled me to develop an understanding of the subject.

I wish to express my sincere thanks to my guide **Mr. D. R. MANIMARAN**, Assistant Professor, Department of Biotechnology, Kumaraguru College of Technology. Coimbatore,whose support from the initial to the final level of my project

I would like to extend my thanks to **Dr. P. RAMALINGAM**, Associate Professor, **Dr. A. SARASWATHY**, Associate Professor, Department of Biotechnology,Kumaraguru College of Technology. Coimbatore whoseexpertise, understanding, encouragement, guidance and patience, added considerably to my graduate experience and for the assistance they provided at all levels of the research project.

I am grateful to the teaching and non-teaching staff members of the Department of Biotechnology for their kind and patient help throughout the project.

All my family members have contributed significantly to bring this day in my life. My parents remained a constant source of strength throughout my educational career and to achieve my goals.

In the end I am thankful to the Almighty for his blessings without which nothing would have been possible.

S.VIMALA DEVI

CONTENTS

ABSTRACT

A potent Bacterial isolate which produces protease was originally isolated, characterized in Biotechnology laboratory at CLRI. Among the seven different media tested for protease production, 1% gelatin in Nutrient Broth was supported maximal enzyme elaborated. The crude enzyme preparation shows the optimum pH and Temperature at 8 and 30°C respectively. The age of inoculum was also studied. Among two inhibitors EDTA was shown to be maximum inhibition. Then the enzyme was observed for the unhairing of skin and the disadvantage in chemical treatment was studied. The confirmation of unhairing was studied using histology studies. The tannery waste (solid fleshings) as it cannot be directly disposed off to the environment. It was treated with the microbial proteases. The hydrolysis of waste was done using proteases. The solid waste was converted to protein, fat and the salt matter. Future work is to optimize the cheap media for the production of the enzyme for large scale applications in various industries.

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVATIONS

S.NO	ABBREVATION	EXPANSION
1	BOD	Biological Oxygen Demand
2	COD	Chemical Oxygen Demand
3	H_20_2	Hydrogen peroxide
4	ABTS	2, 2'-azinobis 3-ethylbenzothiazoline-6-sulphonic acid.
5	HBT	Hydroxybenzotriazole
6	VLA	Violuric acid
7	NHA	N-hydroxyacetanilide
8	SMF	Submerged Fermentation
9	EDTA	Ethylene Diamine tetra chloro acetic acid
10	v/v	Volume/volume
11	w/v	Weight/volume
12	M	Molar
13	OD	Optical Density
14	mM	Millimolar
15	nm	Nanometer
16	µg	Microgram
17	µl	Microlitre
18	h	Hour
19	l	Litre
20	min	Minute
21	TCA	Trichloroacetic acid
22	$CuSO_4$	Copper sulphate
23	NaOH	Sodium Hydroxide
24	%	Percentage
25	$Na_2 co_3$	Sodium bicarbonate
27	SDS PAGE	Sodium dodecyl sulphate- polyacrylamide gel electrophoresis.
28	UV	Ultra Violet
28	C	Centigrade
29	NaK	Sodium Pottassium Tartarate
30	SSF	Solid State Fermentation
31	KDa	Kilo Daltons
32	Conc	Concentration

1. INTRODUCTION

Leather is an intermediate industrial product with numerous applications in downstream sectors. It can be cut and assembled into shoes, clothing, leather goods, furniture and many other items of daily use. The raw material in the production of leather is a by-product of meat industry. Tanners recover the hides and skin from slaughterhouses and transform them into a stable material that can be used in the manufacture of a wide range of products. The Indian leather industry is one of the oldest industries that still occupy a place of prominence in view of its substantial export earnings, employment generation and growth.

The leather industry is under pressure from environmental authorities to comply with pollution and discharge legislations. Thus, the leather industry is being pressurized to look for cleaner options for processing raw hides and skins. Conventional methods of preparatory and tanning processes discharge enormous amounts of pollutants. Leather processing involves a number of unit operations. Generally, the conventional conversion process of hides to leather involves 'do- undo' operations like curing (dehydration)- soaking (rehydration), liming (swelling)- deliming (deswelling), pickling (acidification)- depickling (basification). Further, the skins are subjected to wide variations in pH 13.5- 2.8. Such changes in pH demand the use of acids and alkalis, which lead to the generation of salts. This results in a net increase in TDS (total dissolved solids) comprising chlorides, sulfates and other minerals in tannery wastewaters. Apart from this, solid wastes including sludge from tanneries and chrome sludge from effluent treatment plants are being generated.

India has about 3,000 tanneries with a total processing capacity of 700,000 tons of hides and skins per year. The leather processing industry is known to be associated with the generation of liquid, solid and gaseous wastes. The tanning process generates much greater quantities of byproducts and wastes than leather. One metric ton of wet salted hide yields only 200 kg of leather but over 600kg (about 250kg of tanned and 350kg of non-tanned) solid waste. In the international scenario, 1, 60,000 metric tons of chromium containing solid wastes i.e. chrome shavings are generated by the leather industry each year approximately. Solid wastes from the tanning industry are unavoidable. On the basis of the nature of the solid wastes generated from the leather processing, they can be categorized into chemical and protein- based solid wastes. The chemical wastes are generally dumped at the land sites or incinerated. Protein- based solid wastes acquire much attention due to their high value. Protein-based wastes can further be classified into untanned and tanned wastes. The untanned wastes such as fleshings and trimmings find use in glue/ gelatin manufacture and in several other applications. The tanned wastes primarily consist of chromium and protein. They include shavings, trimmings and splitting of chromium tanned leather. Chrome shavings contribute almost 10% of the solid wastes generated from processing raw hides/ skins accounting to 0.8 million ton globally.

A **protease** (also termed **peptidase** or **proteinase**) is any enzyme that conducts proteolysis that begins protein catabolism by hydrolysis of the peptide bonds that link amino acids together in the polypeptide chain forming the protein.

1.1. CLASSIFICATION OF PROTEASES

Proteases are currently classified into six broad groups:

- **Serine proteases**
- **Threonine proteases**
- **Cysteine proteases**
- **Aspartate proteases**
- **Metalloproteases**
- **Glutamic acid proteases**

The threonine and glutamic-acid proteases were not described until 1995 and 2004, respectively. The mechanism used to cleave a peptide bond involves making an amino acid residue that has the cysteine and threonine (proteases) or a water molecule (aspartic acid, metallo- and glutamic acid proteases) nucleophilic so that it can attack the peptide carboxyl group. One way to make a nucleophile is by a catalytic triad, where a histidine residue is used to activate serine, cysteine, or threonine as a nucleophile.

1.2. BY OPTIMAL PH

Alternatively, proteases may be classified by the optimal pH in which they are active:

- *Acid proteases*
- *Neutral proteases* involved in type 1 hypersensitivity. Here, it is released by mast cells and causes activation of complement and kinins. This group includes the calpains.
- *Basic proteases* (or *alkaline proteases*) Proteases occur naturally in all organisms. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood-clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase-activating cascade). Proteases can either break specific peptide bonds (*limited proteolysis*), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (*unlimited proteolysis*). The activity can be a destructive change, abolishing a protein's function or digesting it to its principal components; it can be an activation of a function, or it can be a signal in a signaling pathway.

1.3. BACTERIAL PROTEASES

Bacteria also secrete proteases to hydrolyse (digest) the peptide bonds in proteins and therefore break the proteins down into their constituent monomers. Bacterial and fungal proteases are particularly important to the global carbon and nitrogen cycles in the recycling of proteins, and such activity tends to be regulated in by nutritional signals in these organisms. The net impact of nutritional regulation of protease activity among the thousands of species present in soil can be observed at the overall microbial community level as proteins are broken down in response to carbon, nitrogen, or sulfur limitation.

A secreted bacterial protease may also act as an exotoxin, and be an example of a virulence factor in bacterial pathogenesis. Bacterial exotoxic proteases destroy extracellular structures. Proteases, also known as proteinases or proteolytic enzymes, are a large group of enzymes. Proteases belong to the class of enzymes known as hydrolases, which catalyse the reaction of hydrolysis of various bonds with the participation of a water molecule.

Proteases are involved in digesting long protein chains into short fragments, splitting the peptide bonds that link amino acid residues. Some of them can detach the terminal amino acids from the protein chain (**exopeptidases such as aminopeptidases, carboxypeptidase A**); the others attack internal peptide bonds of a protein (endopeptidases, such as trypsin, chymotrypsin, pepsin, papain, elastase).

Proteases are used throughout an organism for various metabolic processes. Acid proteases secreted into the stomach (such as pepsin) and serine proteases present in duodenum (trypsin and chymotrypsin) enable us to digest the protein in food; proteases present in blood serum (thrombin, plasmin, Hageman factor, etc.) play important role in blood-clotting, as well as lysis of the clots, and the correct action of the immune system. Other proteases are present in leukocytes (elastase, cathepsin G) and play several different roles in metabolic control. Proteases determine the lifetime of other proteins playing important physiological role like hormones, antibodies, or other enzymes—this is one of the fastest "switching on" and "switching off" regulatory mechanisms in the physiology of an organism. By complex cooperative action the proteases may proceed as cascade reactions, which result in rapid and efficient amplification of an organism's response to a physiological signal.

At this time there are huge amounts of wastes, which are leather fleshings, both in solid and liquid form from tanneries discarded or dumped without any attempt of recovery. During leather processing, tanneries in India produce annually 150,000 tonnes of offals as raw hide/skin trimmings, limed fleshings, hide splits and chrome shavings, which are wasted or under-utilized. This may be considered as unconventional or unexploited and this waste has a real potential for pollution and do pose a disposal problem. The question remains on what to do with the increasing amount of by products from leather industries. In a global context of tannery resources overexploitation, one of the main issues will be better use of landed biomass.

The solid wastes containing a major percentage dry matter are tissue proteins. Fleshing from animal hides/skins is one such waste that is high in protein content. Solid wastes, especially from pretanning operations, like fleshing contain mainly collagen (protein). Although notable progress has been made to increase production of vegetable waste proteins with high nutritive values by employing modern technology, the increase in production of meat and leather waste proteins still remains relatively expensive and ineffective. Generally, fleshing finds use in glue/gelatin manufacture. Fleshing hydrolysate prepared from fleshing after the liming operation in leather processing has been used as a chrome exhaust aid. However, glue/gelatin manufacture involves high initial investment, maintenance cost and is energy intensive. Also protein hydrolysates have been used for the nutritional management of individuals who cannot digest intact protein. The most prevalent use has been for feeding infants with food hypersensitivity

(Silvestre et al., 1997). Hence, the discovery of an appropriate use for fleshing will solve a significant portion of the solid waste disposal problem faced by tanners. For better management of tannery wastes it is therefore a challenge to utilize the valuable protein and lipid fractions from the by-products for use in aquaculture and animal feeds. Now a days works have been in progress for converting leather fleshings into potential resource instead of a waste.

Prior to analysis, the peptides and proteins have to be completely hydrolyzed to yield free amino acids. Hydrolysis is a critical step and an amino acid analysis can only be successful if the preceding hydrolysis has been performed properly. Many hydrolysis protocols exist and the application of the proper method depends on the aim of the individual analysis. Hydrolysis is usually performed by heating the sample to be analyzed in the presence of high concentrations of acids using thermal.

The effect of different acid-hydrolysis methods on the quantification of the amino acids and the degree of residue racemization in the hydrolysate, as well as the efficiency of the extraction methods .The results may be useful in the characterization and identification of proteins by amino acid compositional analysis for construction of protein maps.

1.4. ROLE OF ALKALINE PROTEASE IN LEATHER INDUSTRY

Enzymes are proteins that speed up biochemical reactions without being consumed or changed by the reaction. Enzymes like proteases, lipases and amylases have important role in soaking, dehairing, degreasing and bating operations of leather manufacturing. Proteases are the most commonly used enzymes in leather production. The criteria for selection of the best protease are that it should be non-collagenolytic and non-keratinolytic in nature. It has property to hydrolyze casein, elastin, albumin and globulin like proteins.

1.4.1 Alkaline protease

Proteases are also known as peptidyl-peptide hydrolases. It forms a large group of enzymes, ubiquitous in nature and found in a wide variety of microorganisms. They are molecules of relatively small size and are compact, spherical structures that catalyze the peptide bond cleavage in proteins. They are degradative enzymes which catalyze the total hydrolysis of protein.

Alkaline proteases are the main enzymes among the proteases which are known to 60-65% of the global industrial market. Most of them are used in the food industry in the meat tenderization process, peptide synthesis, for infant formula preparations, in baking and brewing. They are also used in the detergent industry as additives, in pharmaceuticals and medical diagnosis as well as in textile industry in the process of dehairing and leather processing.

Selection of specific enzymes are capable of totally or partially replacing chemical inputs in beam home processing of leather in aiding dehairing and enzymatic removal of other non-leather forming proteins such as reticulin, keratin, elastin, proteoglycan and other globular protein will be a significant breakthrough in beam home practices. This may markedly reduce considerable Total Dissolved Solids (TDS) generated into the environment and also an Eco-friendly recent approach than the existing conventional chemical processing. Hence, it is necessary to screen high potent microbial enzymes which lead to Eco-friendly approaches in leather manufacture.

Table 1.1: Enzymes used in Pre-tanning Operation

PROCESS	ENZYME	MICROORGANISMS
Soaking	Proteases	*Aspergillus flavus, A.oryzae and Bacillus subtilis, Rhizopus*
	Carbohydrates	*Aspergillus sp.*
Dehairing	Proteases	*Aspergillus flavus, Aspergillus sp. Bacillus subtilis, Lactobacillus sp, streptomyces sp*
Bating	Proteases	*A. parasiticus, and B. licheniformis, B. subtilis, Penicillium sp.*
Degreasing	Lipases	*Rhizopas nodosus, A.oryzae and A. flavus*

1.5. DEHAIRING

Dehairing is one of the main operations in the beam house. Five methods of dehairing are generally adopted, viz. (i) clipping process, (ii) scalding process, (iii) chemical process, (iv) sweating process, and (v) enzymatic process. Of these, the most commonly practiced method of dehairing of hides and skins is the chemical process using lime and sodium sulphide. However, the use of high concentrations of lime and sodium sulphide creates an extremely alkaline environment resulting in the pulping of hair and its subsequent removal. While one cannot question the efficacy of this process, its inherent disadvantages have to be taken note of. Significant amongst these are:

- It contributes in no small measure to the pollution load. Beam house processes generally account for 70–80% of the total COD (Chemical Oxygen Demand) of effluent from all leather making processes. About 75% of the organic waste from a tannery is from the beam house 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 process towards pollution.
- Sulphide is highly toxic with obnoxious odour. If left untreated, it can cause major problems in the sewers.
- The severe alkaline condition is a health hazard for the workers.

Enzymatic dehairing is suggested as an environmentally friendly alternative to the conventional chemical process. Alkaline proteases can be used as an alternative to sulfide. The alkaline condition enables the swelling of hair roots, and the subsequent attack of protease on the hair follicle protein (Gupta et al., 2002). This is followed 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.

The protease used to reducing environmental pollution by inputting the cleaner, safer technology in disposing the wastes by utilization of respective active enzymes into valuable products which lead to different applications in various industries such a leather, pharmaceutical, agriculture, animal feed, production of clay bricks, glue etc. In this paper, we describe the screening, identification, optimization and partial purification of a newly isolated alkaline protease producing bacteria. The alkaline protease produced by this strain has a prominent potential in the dehairing process.

1.6. TYPES OF PROTEIN HYDROLYSIS

The prerequisite of a successful compositional analysis is a complete hydrolysis of the peptides and proteins and a quantitative recovery of the residues in the hydrolysate. In order to increase the utilization of these waste products, chemical methods such as use of acid and alkali and biological (biochemical) method i.e. Uses of enzymes are most widely used for protein hydrolysis with chemical hydrolysis used more commonly in industrial practices.

1.6.1 Chemical methods:

Chemical methods of proteins are achieved by cleaving peptide bonds with either acid or base. Though several processes have been proposed for the acid or alkaline hydrolysis of fish, much work has not been done on leather fleshing hydrolysis. This has been the method of choice in the past for the industry primarily because it is relatively inexpensive and quite simple to conduct. But there are limitations of this method. Chemical hydrolysis tends to be a difficult process to control and almost invariably leads to products with variable chemical composition and functional properties. Use of strong chemicals, high pH and extreme temperatures cause the reduced nutritional, qualities, poor functionality and restricted use as flavour enhancers, of the products.

1.6.2. Biochemical methods of hydrolysis

Biochemical analysis to produce the protein hydrolysates is performed by utilizing enzymes to hydrolyze peptide bonds. This can done via proteolytic enzymes already present in the leather waste, or by adding enzymes from other sources i.e., autolytic hydrolysis.

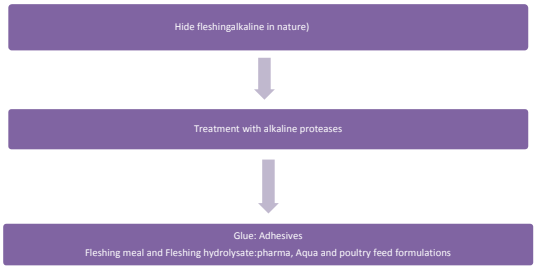
So the hydrolysis of the proteins of the leather fleshings had been considered to convert the wastes into certain more marketable and functional forms. Several enzyme, alkali and acid hydrolysis methods are examined to obtain protein hydrolysates and acid hydrolysis was found to be the best suited one. The investigation was done on the effect of different acid- hydrolysis methods on the compositional analysis of known proteins in solution and worked out the conditions for the processing of large numbers of samples. An accurate data was provided by conventional *propionic acid and formic acid* hydrolysis. Though production of *acid silage* is a common method to hydrolyse proteins

and lipids, lipids may get deteriorated by increasing the level of free fatty acids. Also there is a chance of destruction of tryptophan, which is an essential amino acid. Proteins are polymers of amino acids, which are linked to each other by polypeptide bonds. Comparatively weak forces responsible for maintaining secondary, tertiary and quaternary structures of proteins are readily disrupted by a variety of manipulations and disruption of original native structure of a protein is termed as denaturation. The denaturation of proteins may be brought about by heat, freezing, chaotropic agents (urea, guanidine), heavy metals, organic solvents, salt solutions and radiation. The use of organic solvents such as acids is in practice from the past several years.

Wet leather fleshings (LFs) from sheep and goatskins had high alkaline pH (12.1) and ash (18.1%) due to liming of fresh skins for leather production. Deliming of leather fleshings (LFs) with HCl produces toxic H_2S. Biological methods are being used to minimize industrial pollution resulting from tannery effluents. For deliming, 0.2N HCl was optimum although production of H_2S could not be avoided. H_2O_2 was employed to overcome H_2S problem during deliming of tannery fleshings. Collagen, a main protein in LFs, needs hydrolysis to be more useful as a nutrient in animal feeds. This study presents production of acid hydrolysate, which can be used in animal feeds. Response Surface Methodology is a useful tool applied towards the optimization of several operations (Diniz et al., 1996) and also for the investigation on complex processes. RSM defines the effects of the independent variables themselves, either alone or in combination, during the process. In addition, the method may generate a mathematical model that accurately describes the overall process.

1.7. SOLID WASTE MANAGEMENT IN TANNERY USING PROTEASES

- Experiments have shown that the substantial part of tanneries' solid waste can be used as valuable RAW MATERIALS for the production of protein feed (C.Rose *et al., 1998).*
- The biological treatment of tannery waste has been largely accepted field in recent years.
- These waste, in addition to collagen, contains Globular Protein which posses a more complete Amino acid composition particularly with respect to exogenous amino acids.



- Bating is the main process in the leather industry where the enzyme the bacterial proteases are used.
- Bating is the degradation process of non collagenous and globular proteins, which is done to soften the hide structure by removing the epidermis, hair, and fat residues, thereby preparing the hide for tannage.
- In recent years, the use of enzyme in the tannery solid waste management is becoming more and more important, namely in giving a better digestion of fleshing and shavings.

1.8. APPLICATION OF PROTEASES

The specific applications of proteases in different industries are reviewed in brief in the following sections:

(a) Food and feed industry

The food processing industry is highly dependent on enzymes and during processing of food, enzymes are added to modify or improve the characteristics. Food technologists are constantly improving enzyme technology by selecting those enzymes (protease) which can improve one particular unit operation of food production e.g. substituting fish protein hydrolysate for milk in place of calf milk (Diaz-Casteneda and Brisson, 2009), saving energy in production processes (Christensen, 2009), extracting juice (Kilara, 2002) and modifying the functional properties of proteins (Alder-Nissen et al., 2003).

Proteases are used in baking industry for hydrolysis of gluten for quick dough preparation. The proteases breakdown the gluten present in the raw flour whose high content is undesirable for the production of biscuits and cookies. Dairy is one of the important food industries where enzymes have been used since prehistoric time for processing of milk. In the beginning, the dairy industry used to employ rennet (chymosin) from the calf stomach for the manufacture of cheese (Chaplin and Bucke, 2000). Use of animal rennet has now been replaced by proteases from microbial sources (Gerhartz, 2000). In brewing industry, proteins are used to hydrolyse the cereal mash proteins so that the yeast can assimilate the peptides and amino acids during the fermentation.

(b) Leather industry

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 biotreatment of leather using an enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction, thus being ecofriendly (Anderson, 2008). Proteases find their use in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and thereby clean hide is produced. George et al. (2005) used B. amyloliquefaciens alkaline protease for unhairing hides and skins. Hameed et al. (2009) used B. subtilis K2 alkaline protease in bating and leather processing.

(c) Medical usage

Proteases are also used for developing products of medical importance. Kudrya and Simonenko, 2004 exploited the elastolytic activity of B. subtilis 316M for the treatment of burns, purulent wounds, carbuncles and deep abscesses. Kim et al. (2006) reported the use of alkaline protease from Bacillus sp. strain CK 11-4 as a thrombolytic agent having fribrinolytic activity. Oral administration of proteases from Aspergillus oryzae has been used as a diagnostic aid to correct certain lytic enzyme deficiency syndromes (Rao et al., 2008).

(d)Detergent Industry

Proteases find the largest single application in laundry detergents. Proteases are present in different brands of detergents for use in home and commercial establishments. Proteases have been added to laundry detergents for over 50 years to facilitate the release of proteinaceous materials in stains such as those of milk and blood. Detergents such as Era Plus (Procter and Gamble), Tide (Colgate Palmolive) and Dynamo (Procter and Gamble) contain proteolytic enzymes, the majority of which are produced by the members of the genus Bacillus (Samal et al., 2009).

2. OBJECTIVES

1. Revival of the bacterial culture for protease production,
2. Characterization and mass production of protease,
3. Dehairing of the goat skin using protease and
4. Hydrolysis of tannery fleshings using protease.

3. REVIEW OF LITERATURE

3.1. DISTRIBUTION OF PROTEASE IN BACTERIA

- The prokaryotic proteases from the rhizospheric bacterium *Azosprillium lipoferum (*Kamini *et al.,* 2009*),* where protease occurs as a multimeric enzyme capable of degrading the Fleshings obtained from tan yard.
- Protease has also been reported from a marine melanogenic bacterium M*arinosomonas mediterranea* producing two different alkaline proteases (Anwar *et al.*, 2008.)
- A protease like enzyme activity was also found in spores of B*acillus sphaericus* (Ellaiah *et al.*, 2003)

3.2. SCREENING OF BACTERIA SECRETING PROTEASE

- Microbes that produce protease have been screened on solid media containing Skim milk that enable the visualization of Protease production (Skirth *et al*; De jong *et al.*, 2009; Beg *et al.,* 2003).
- The traditional screening reagents such as tannic acid and Gallic acids (Haider *et al.,* 2009)
- **With casein a positive reaction** is indicated by the formation of a Zone of clearance. (Kamini *et al.,* 2009)

Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, and are essential for cell growth and differentiation. The extracellular proteases are commercial value and find multiple applications in various industrial sectors. Although there are many microbial sources available for producing proteases, only a few are recognized as commercial producers (Gupta, *et al*., 2002b). Of these, strainsB of *Bacillus sp*. dominate the industrial sector (Gupta *et al*., 2002a).

Kamini *et al.,* 2009 reported that the gram-positive, spore forming bacterium *Bacillus subtilis* produces and secretes proteases, esterases, and other kinds of exoenzymes at the end of the exponential phase of growth.

In addition to that, several workers investigated the production of protease and alkaline protease from *Bacillus subtilis (*Tsai *et al*., 2008; Despande *et al*., 2004). Some extracellular enzymes are used in the food, dairy, pharmaceutical, and textile industries and are produced in large amounts by microbial synthesis (Andrade *et al* 2002). Proteases are one of the most important groups of industrial enzymes and account for nearly 60% of the total enzyme sale (Berla *et al*., 2002). The major uses of free proteases occur in dry cleaning, detergents, meat processing, cheese making, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds (Oberoi, 2001).

3.3. CHARACTERISTICS OF FLESHINGS

Table 3.1 Characteristics of Fleshings

Ref. No.	Characteristic	Values	
		Values for limed bovine fleshings	Values for delimed bovine fleshings
1.	Appearance	Heterogeneous product made of gelatin and fat particles soaked with water.	Heterogeneous product made of gelatin and fat particles soaked with water.
2.	Colour	Grey	White
3.	Smell	Odourless in the first 48-60 hours at the room temperature, thereafter getting a characteristic smell of decaying protein.	Odourless in the first 48-60 hours at the room temperature, thereafter getting a characteristic smell of decaying protein.
4.	Moisture content, %	80 – 85%	23.00 – 72.00
5.	Ash, %	13.00 – 15.00*	2.50 – 15.00*
6.	Fatty matter, %	9.00 – 15.00*	4 – 11.50*
7.	Total nitrogen, %	12.00 – 14.00*	3.00 – 11.50*
8.	Hide matter, %	70.00 – 80.00*	40.00 – 62.00*
9.	Calcium oxide, %	6.00 – 10.00*	1.00 – 2.50*
10.	Sodium sulphide, %	2.00 – 4.00*	2.50 – 9.50*
11.	pH	10.5 – 12.5	7.5 – 8.0

* Values are based on the water-free weight

Equipment and technology for treating the limed fleshings resulted from Bovine hide processing (Kamini, *et al.,* 2009)

3.4. CHARACTERISTICS OF BY-PRODUCT SUET OBTAINED FROM BOVINE FLESHINGS

Table 3.2.Characteristics of By-Product Suet Obtained From Bovine Fleshings

Ref. No.	Characteristic	Values
1.	Appearance, colour, smell	Solid fatty product, of yellow-brownish colour, with a characteristic smell of bovine fat
2.	Solids, %	80.0, min.
3.	Fatty matter, %	90.0, min.*
4.	Iodine number, g/100g	45…55
5.	Saponification number, mg KOH/g	190…200
6.	Melting point, ^{0}C	40…45
7.	Specific gravity, g/cm^{3}	0.92-0.95
8.	Shelf life	6 months in the cold seasons 3 months in summer
* The value is based on moisture-free weight		

Equipment and technology for treating the limed fleshings resulted from Bovine hide processing (Kamini, *et al.,* 2009).

Animal fleshing, the major solid waste obtained during leather processing is proteinaceous in nature. It is used as a major substrate for the production of alkaline protease in the industries for the recycle of the solid waste from the tannery. (**Submerged fermentation** for production of alkaline protease by *Bacillus Cereus* 1173900 using proteinaceous tannery waste, (Kamini *et al., 2009)*

On an average, protein associated with the meat industry byproducts including leather fleshings constitute more than one-eighth of total protein in the lean meat (Webster *et al*., 1982). Protein hydrolysates were prepared and stabilized from H_2O_2 and HCl treated delimed leather fleshings (Wei-Hua *et al*., 2007). Also protein hydrolysate was prepared from pretreated sheep visceral mass (including stomach, large and small intestines) by enzymatic treatments at 40-42°C using fungal protease which readily solubilize proteins (Bhaskar., 2005). Protein hydrolysate was prepared from visceral waste proteins of Catla (*Catla catla*), an Indian fresh water major carp using a commercial protease and hydrolysis conditions like time, temperature, pH and enzyme to substrate level for preparing protein hydrolysates from the fish visceral waste proteins were optimized by Response Surface Methodology (RSM) using a factoral design (Wei-Hua *et al*., 2007).

Protein hydrolysates have been used for nutritional or technological purposes. Various methods used for the determination of the hydrolysis degree, the characterization

according to the peptide size, the evaluation of the molecular weight distribution and the estimation of the amino acid and peptide contents (Thanikaivelan *et al*., 2004). Studies showed improved solubility and enhanced or reduced emulsifying and most of other properties depending on the hydrolysis conditions and the starting protein materials (Sandhia *et al*., 2007). The combined effects of pH, temperature, and substrate/buffer ratio and enzyme concentration on protein recovery from Gold carp (*Carassius auratus*) processing waste with Flavourzyme were characterized. The effect of hydrolysis (DH) was described through Resource Surface Analysis (RSA) (Takami *et al*, 2009). The effect of the pH, ionic strength, temperature and such other parameters on the solubility of the protein was studied (Choudhary *et al*., 2003). There was major ongoing research on searching of bioactive compounds in marine organisms and development of new technology

For utilization of this so can assume that the future will bring more value out of what is told today as waste (Rohm *et al*., 1910). It is intended to provide a process for a highly safe seasoning by highly decomposing meat or bone remaining after taking meat with enzyme. It is also intended to utilize Nitrogen at an elevated ratio compared with the existing low Nitrogen utilization level (Nehete *et al.*, 2006). The work had been done to study how different raw material mixtures from Cod (*Gadus morhua*) by-products influenced the composition of the substrate for hydrolysis, yield and chemical composition of the different fractions after the enzymatic hydrolysis using different endo-peptidase (Flavourzyme) or exo- peptidase (Rajesh patel *et al.,2005*). There had been a brief survey of research and developments concerning enzymes and bioactive peptides from fish waste or by-catch applied for fish silage, feed or fish sauce production (Andrade, 2002).

3.5. ALKALINE PROTEASE

Alkaline proteases are defined as those proteases, which are active in a neutral to alkaline pH range. They either have a serine centre (serine protease) or are of metallo-type (metalloprotease); and they are the most important group of enzymes exploited commercially (Gupta *et al*., 2002). Alkaline proteases are most active at pH values of about pH 10 (Anwar *et al*., 2008).

Alkaline proteases of microbial origin, which dominate the worldwide enzyme market, possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Gupta *et al*., 2002).

Alkaline proteases are produced by a wide range of microorganisms including bacteria, molds, yeasts and also mammalian tissues (Singh *et al*., 2001). Various microbial sources of alkaline proteases identified, has been tabulated in Table 2. Despite this interest in other microbial sources, survey of the literature conclusively shows that bacteria are by far the most popular source of commercial alkaline proteases to date. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10 and their broad substrate specificity. Their optimal temperature is around 60^{o}C. These properties of bacterial alkaline proteases make them suitable for use in the different industries (Rao *et al*., 1998). From all the alkaliphilic bacteria that have been screened for use in various industrial application, members of the genus *Bacillus*, mainly strains *B. subtilis and B. licheniformis* were found to be predominant and a prolific source of alkaline proteases (Kumar and Takagi, 1999).

Most alkalophilic microorganisms produce alkaline proteases, though interest is limited only to those that yield substantial amounts. It is essential that these organisms be provided with optimal growth conditions to increase enzyme production. The culture conditions that promote protease production were found to be significantly different from the culture conditions promoting cell growth (Moon SH and Parulekar SJ, 1991). In the industrial production of alkaline proteases, technical media were usually employed that contained very high concentrations (100–150g dry weight/litre) of complex carbohydrates, proteins, and other media components (Frankena J *et al*., 2005).

3.5.1. Carbon source

Studies have also indicated a reduction in protease production due to catabolite repression by glucose (Frankena *et al*., 2005). On the other hand, (Zamost *et al.,* 2000) correlated the low yields of protease production with the lowering of pH brought about by the rapid growth of the organism. In commercial practice, high carbohydrate concentrations repressed enzyme production. Therefore, carbohydrate was added either continuously or in aliquots throughout the fermentation to supplement the exhausted component and keep the volume limited and thereby reduce the power requirements (Udani *et al*., 2008). Increased yields of alkaline proteases were reported by several workers who used different sugars such as lactose, maltose, sucrose and fructose (Kamini *et al.*, 2009). However, a repression in enzyme synthesis was observed with these ingredients at high concentrations. Whey, a waste byproduct of the dairy industry containing mainly lactose and salts, has been demonstrated as a potential substrate for alkaline protease production (Nehete *et al*, 2006). Similarly, maximum alkaline protease secretion was observed in *Thermomonospora fusca* YX, which used pure cellulose (Solka-floc) as the principal carbon source (Gupta *et al.*, 2002).

3.5.2. Nitrogen source

In most microorganisms, both inorganic and organic forms of nitrogen are metabolized to produce amino acids, nucleic acids, proteins, and cell wall components. The alkaline protease comprises 15.6% nitrogen and its production is dependent on the availability of both carbon and nitrogen sources in the medium. Although complex nitrogen sources are usually used for alkaline protease production, the requirement for a specific nitrogen supplement differs from organism to organism (Kole MM *et al.*, 2008).

Low levels of alkaline protease production were reported with the use of inorganic nitrogen sources in the production medium (Chaphalkar and Dey, 2004). Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium (Green GH *et al.*, 2001). However, one report indicated no repression in the protease activity with the use of ammonium salts (Nehete *et al*., 2006).

3.5.3. Optimum pH and temperature

The optimum pH range of alkaline proteases is generally between pH 9 and 11, with a few exceptions of higher pH optima of 11.5, pH 11–12, pH 12.3 and pH 12–13. They also have high isoelectric points and are generally stable between pH 6 and 12. The optimum temperatures of alkaline proteases range from 50 to 70ºC. In addition, the enzyme from an alkalophilic *Bacillus* sp. B189 showed an exceptionally high optimum temperature of 85ºC. Alkaline proteases from *Bacillus* sp., *Streptomyces* sp. and *Thermus* sp. are quite stable at high temperatures, and the addition of Ca^{2+} further enhanced enzyme thermostability (Folosade *et al.*, 2005).

3.6. CHARACTERIZATION OF PROTEASE

Zymographic technique is used for identification and characterization of microbial proteases, using SDS-PAGE and PAGE in non-dissociating gels. Techniques are described using copolymerized protein substrates, diffusible protein substrates, protein substrates incorporated into indicator gels, as well as synthetic esterase substrates. When a newly discovered protease is being characterized, it is advisable to try a variety of techniques, both to determine optimal conditions for enzyme detection and to characterize the protease. Zymography is a versatile two-stage technique involving protein separation by electrophoresis followed by detection of proteolytic activity. Each particular combination of protease separation and detection techniques had advantages and limitations. Protease separation by SDS-PAGE has a limitation, the fact that some proteases do not renature and hence cannot be detected following treatment with SDS. However, it has an advantage the fact that it allows estimation of the relative molecular weight of proteases. Protein separation using nondissociating PAGE is performed using much gentler protease inactivation conditions than those produced by treatment with SDS. Like SDS-PAGE, nondissociating PAGE permits detection of multiple forms of enzymes; however, a disadvantage is that it cannot be used to obtain molecular weight estimates of proteases. The main variable to control during development of zymograms is the length of time of incubations. Increasing incubation (development) time generally increases the sensitivity of protease detection; however, as the length of time of incubation increases so does the extent of diffusion of proteases and substrates. If incubations are prolonged, protease bands will diffuse, decreasing resolution. Additionally, zones of lysis produced by closely migrating proteolytically active species will merge, eliminating the possibility of detecting all proteolytic species in the sample. Zymographic techniques can be extremely useful in identification and characterization of microbial proteases. If a few properties of a protease are known, such as the pH range over which the enzyme is active, and whether it can renature after exposure to SDS, zymographic techniques can be specifically and readily adapted to optimize conditions for detection and assist in characterization of the enzyme (Lantz and Ciborowski 2004).

Analysis of the purified proteases by SDS–PAGE revealed that both proteases, AP-1 and AP-2 were homogenous with molecular weight estimates of 28 and 29 KDa, respectively. The optimum activity of AP-1 and AP-2 were at temperatures of 50 and 55°C and pH of 11 and 12, respectively. The enzymes were also stable in the pH range of 6.0-12.0 for a period of 4 h with

and without Ca^{2-} (5mM) and temperatures of up to 50°C. The half-lives of the enzymes recorded at 50°C were 50 and 40 min for proteases AP-1 and AP-2, respectively (Kumar *et al*., 2009).

3.7. ROLE OF PROTEASE IN DEHAIRING

The present investigation describes microbial production of an alkaline protease and its use in dehairing of buffalo hide. *Bacillus cereus* produced extracellular protease when grown on a medium containing starch, wheat bran and soya flour. The ammonium sulphate precipitated enzyme was applied for dehairing of buffalo hide. Microscopic observation of longitudinal section of buffalo hide revealed that the epidermis was completely removed and hair was uprooted leaving empty follicles in the hide. The ASP enzyme was stable for one month at ambient temperature between 25-35°C. Enzymatic dehairing may be a promising shift towards an environment-friendly leather processing method (Zambare *et al.,* 2007).

Enzymatic dehairing in tanneries has been envisaged as an alternative to lime-sulfide dehairing. Selection of specific proteolytic enzymes capable of totally replacing chemical inputs in dehairing process will be a significant breakthrough in leather processing due to substantial reduction in effluent load as well as decrease in toxicity besides improving leather quality (Cantera *et al* ., 2006; Kamini *et al*., 2009).

Bacterial strains secreting proteases were screened from biological wastes. Positive clones were further characterized by analysing their efficacy in dehairing and effects on collagen integrity. Among 171 colonies tested, a strain BA06, identified as *B. pumilus*, was picked owing to its efficient dehairing capabilities with minimal impact on collagen. By combined mutagenesis using UV, N-methyl-N'-nitro-N-nitrosdguanidine and Cobalt 60 c-rays, this strain was further improved with regard to its alkaline protease production. The alkaline protease activity of the mutant strain SCU11 was greatly improved up to 6000 U ml, in comparison with its parent strain BA06 of 1200 U ml (Wang *et al*., 2006).

Bacillus cereus MCM B-326, isolated from buffalo hide, produced an extracellular protease. Maximum protease production occurred (126.87 and 1.32U/ml) in starch soybean meal medium of pH 9.0, at 30°C, under shake culture condition, with 2.8 and 108 cells/ml as initial inoculum density, at 36 h. Ammonium sulphate precipitate of the enzyme was stable over a temperature range of 25-65°C and pH 6-12, with maximum activity at 55°C and pH 9.0. The enzyme required Ca^{2+} ions for its production but not for activity and/or stability. The partially purified enzyme exhibited multiple proteases of molecular weight 45 KDa and 36 KDa. The enzyme could be effectively used to remove hair from buffalo hide indicating its potential in leather processing industry (Nilegaonkar *et al.,* 2007).

Presently, several enzyme assisted dehairing methods by commercial enzymes use reduced quantity of lime or sulfide or both. Commercial enzyme preprations such as NUE 0.6 MPX from Novo-Nordisk, Riberzym MPX from Chromogenia, Biodart from SPIC and Microdep C from Textan chemicals are used in tanneries around the world. However, these enzymes do not completely eliminate the use of both lime and sulfide in deharing process (Frendrup., 2000). Enzyme assisted deharing process have been developed (Sivasubramanian *et al*., 2008a), which is better than conventional lime-sulfide process and enzyme assisted process using commercial dehairing enzyme with reduced quantities of lime-sulfide as it requires shorter duration of 6 hours and produces reduced effluent load in terms of biochemical oxygen demand, chemical oxygen demand, total dissolved solids and total suspended solids.

It is reported that a protease preparation from a fungal microbes, *Conidiobolus coronatus* (US Pat 6777219) is used along with a reduced quantity of sulfide in dehairing of skins and hides without using lime. Deharing enzymes which avoid the use of lime and sulfide are produced by microorganisms such as *Aspergillus flavus* (Malathy and Chakraborty, 2001), *Rhizopus oryzae* (Pal *et al.*, 2006) and *Bacillus sp.* strain (Raju *et al*., 2006). However, these enzymes are applied by painting method and use of these enzymes is limited to skins only and not for hides. It is reported that use of protease from *Bacillus subtilis* strain avoids the use of sulfide but employs lime for pH adjustment (Riffle *et al*., 2003). Schlosser *et al*., 2006) have reported a method of enzymatic dehairing in an acid medium containing *Lactobacillus* culture and this method suffers from the disadvantage of solubilization of collagen. Though reports are available for non-enzymatic dehairing methods, they lack commercial interest due to toxicity and inefficiency. (everett *et al*., 2006) have reported the use of oxidant, hydrogen peroxide in alkaline medium for dehairing; but, reduction in pollution load, especially COD is not significant. (Sehgal *et al*., 2006) have developed a non-enzymatic dehairing process by painting method; free of sulfide, using nickel compounds and Kaolin; but, the disposal of nickel compounds poses health hazards. A patent application (PCT/NO3/00074) has reported the use of animal and plant enzymes for total elimination of lime and sulfide in dehairing process. However, protease from microbes is more preferred over plant and animal sources since production from the latter sources are not readily inducible and scalable.

The use of soluble silicates as a lime substitute in dehairing process is reported recently (Munz and Sonnleitner, 2005; Saravanabhavan *et al*., 2005); yet, the reduction in pollution parameters is not significant. US Pat 6957554 describes an invention related to dehairing and fibre opening process using commercial protease formulations and silicates, wherein the use of lime and sulfide is eliminated in enzymatic dehairing step. However, use of silicate salt in dehairing contributes to a rise in total dissolved solids as well as chemical oxygen demand in effluent. Although many reports are available for enzymatic dehairing, either free of lime or sulfide and or both, none of these methods have found commercial application in tanneries.

3.8. PROTEASE IN TANNERY EFFLUENT TREATMENT

Anwar *et al.,* (2008) reported that the fleshing, the major solid waste generated at the pretanning operation of leather processing, were hydrolyzed using pancreatic enzymes with a view to evolve a simple method for solid waste management. The proteolytic activity of pancreatic homogenate with casein was found to be 80 units/ml fleshing treated with pancreatic enzyme preparation showed a six fold increase in proteolysis against the control at the end of 7 days. The total protein content, collagen and the free fatty acid in the hydrolysate supernatant were 80.0, 10.764 and 72.86 mg/ml respectively. The optimum pH for the enzyme preparation was 8.5. The hydrolysis was observed by almost total liquefaction of the fleshing. Asserted that up to 50% of total weights are lost as solid waste products in the course of tanning operations. They estimated that, on the world scale over five million tons of hides are processed annually some 15% of these in Italy alone. From this premise, they draw attention to the problem constituted by considerable volume of these waste products e.g.: fats and protein can be separated and recovered. A study was undertaken to obtain a measure of type and quality of such recoverable by products. The author arrives at an estimate of the total quantity of recoverable fats and proteins.

4. MATERIALS AND METHODS

Table 4.1: List of Instruments used during the whole experiment their manufacturers and function

Instruments	Manufacturer	Function
Analytical balance	Mettler Toledo AG 245	Weight measurement
Autoclave	Gambaks	Sterilisation
Incubator shaker	Orbitek	Proper shaking
Laminar air flow	Klenzaids	Aseptic environment
pH meter	Eu Tech instruments	Measurement of pH
Ultra low temperature freezer	Operon	Preservation of cultures
Lyophilizer	Virtis	Drying the samples
Ultra pure water system	Sartorius	Preparation of the stock solution, reagents
Spectrophotometer(UV/Vis)	Hitachi (Japan)	Estimation of biomass and Cr(VI) degradation
Ultra centrifuge	Remi	Collection of pellet and Cr(VI) estimation

4.1. REVIVAL OF THE CULTURE

The strain which produces protease was originally isolated and characterized in Biotechnology laboratory at CLRI and was maintained in a lyophilized vial. The vial was then revived and put in to use.

4.1.1. Procedure

- The glass vial containing the lyophilized culture was heated slightly in the flame.
- The vial was then cut opened using sterile lancet.
- A loop full of culture was transferred in to nutrient agar plate and the remaining culture was inoculated in to the liquid medium.
- They were kept at 37 °C incubator for 24-48 hrs and then were stored in cold room for future use.
- Sub culturing was done after every 24 hrs for three days.

4.2. PROTEOLYTIC ACTIVITY CONFORMATION

Qualitative assay rely on the formation of a clear zone of proteolysis on agar plates as a result of protease production. Both the parent and the UV exposed cultures were tested for the proteolytic activity on Gelatin-Agar plates.

4.2.1. Materials

- Medium:
  - Gelatin – 1%
  - Agar- Agar type I - 2%
  - Distilled water
- Sterile petri plates
- Sterile paper discs
- 10% tannic acid.

4.2.2.Procedure

- 5µl of the bacterial suspension was inoculated on to the hole made using gel puncture on the surface of the gelatin-agar plates.
- These plates were incubated for 48hrs at 30°C.
- After 48hrs, the plates were flooded with 10% tannic acid solution for 5 minutes.
- Protease secretion was detected by observing the zone of hydrolysis around the hole in agar gelatin plates.
- Both the parent and UV irradiated culture were streaked on skim milk agar plates and stored at 4°C.
- These plates can be used as master plates and sub cultured further.

4.3. ALKALINE PROTEASE PRODUCTION IN SUBMERGED FERMENTATION

4.3.1. Materials

- Medium
  - Nutrient broth
  - Gelatin
- 1000ml Erlenmeyer flasks
- Preinoculum

4.3.2. Procedure

- SMF is the technique employed to grow microorganisms on liquid media.
- The used medium is nutrient broth with 1% agar solution.
- About 6% (v/v) of the culture was used to inoculate production flasks.
- Incubate it in orbital shaker (200rpm) for 24hrs at 37°C.

4.3.3. Enzyme Extraction

- After 24hrs of incubation the enzyme from the fermented broth was extracted by simple centrifugation at 10,000 rpm for 10 mins at 4°C.
- The resultant supernatant was used as a source of enzyme.
- The proteolytic activity of the enzyme preparation was determined by Anson modified by Kunitz method (1938) using casein as substrate.
- By the enzyme activity determined, the production was continued for the further application.

4.4. ASSAY OF PROTEASE

The proteolytic activity of the enzyme preparation was determined by using casein as substrate.

4.4.1. Principle

The TCA soluble protein fraction released from casein by the hydrolytic action of enzyme alkaline protease was estimated by lowry's method to quantify the enzyme activity.

4.4.2. Reagents

- **Standard tyrosine solution:** 5mg of tyrosine was dissolved and made up to 50ml using distilled water.
- **5mM Copper Sulphate:** 12.5 mg of $CuSO_4$ was dissolved in 10ml of distilled water.
- **0.245N Sodium Hydroxide:** 980mg of NaOH was dissolved in distilled water and made up to 100ml.
- **4.8% Sodium Carbonate:** 2.45g of Sodium Carbonate was dissolved in distilled water and made up to 50ml.
- **0.25% Sodium Potassium Tartarate:** 25mg of Sodium Potassium Tartarate was dissolved in 10ml of distilled water.
- **Folin-Ciocalteau phenol reagent:** Diluted with distileed water in 1:2 ratio just before use.
- **1% Caesin solution:** 1 gm of casein was mixed with 100ml of phosphate buffer.
- **Carbonate Buffer (pH 9.2).**
- **10% Trichloroacetic Acid.**

4.4.3. Procedure

Reagents	**Blank(ml)**	**Test(ml)**	**Reagent blank(ml)**	Std**. tyrosine(ml)**
Sample(enzyme)	**0.1**	**0.1**	-	-
Buffer	**0.9**	**0.9**	-	-
TCA	**3**	-	-	-
Caesin	**1**	**1**	-	-
Incubated at 37°C for 10 mins				
TCA	-	**3**	-	-
Filter the entire sample				
Sample	**0.1**	**0.1**		**0.1,0.2**
Water	**0.9**	**0.9**	**1**	**0.9,0.8**
$cuso_4$	**0.2**	**0.2**	**0.2**	**0.2**
NaOH	**1**	**1**	**1**	**1**
$Na_2\ co_3$	**1**	**1**	**1**	**1**
NaK	**0.2**	**0.2**	**0.2**	**0.2**
Incubate at 37°C for 15 mins				
Folin:phenol(1:2)	**0.5**	**0.5**	**0.5**	**0.5**
Incubate it in dark for 20min OD was taken at 640nm				

4.4.4. Calculation: OD of unknown =OD of test - OD of blank

$$\frac{\text{OD of unknown}}{\text{OD of standard}} * \frac{\text{Conc of tyrosine}}{\text{Volume of unknown}} * \frac{5.0}{0.2} * \text{Dilution factor}$$

One unit of proteolytic activity (U) was defined as µg tyrosine liberated per ml per min of the enzyme extract.

4.5. DETERMINATION OF PROTEIN CONTENT

The protein from enzyme preparation samples were estimated by the method of *Lowry et al., 1951.*

4.5.1. Principle

Alkaline copper added in the form of copper sulphate reacts with the amino acids present in the sample. The copper present in copper sulphate forms a complex with the NH_3 groups of the amino acids. This complex reacts with Folin-Ciocalteau reagent containing phosphomolybdate and phosphotungstate. Phosphomolybdate is reduced by tyrosine and tryptophan present in the protein. This copper complex gives a blue color which is read at 640nm. Thus, the intensity of the color depends on the amount of aromatic amino acids present and will vary for different proteins.

4.5.2. Reagents

1. Standard BSA solution: 10mg of Bovine Serum Albumin (BSA) was dissolved and made up to 50ml using distilled water.
2. 5mM Copper Sulphate: 12.5mg of Copper Sulphate was dissolved in 10ml of distilled water.
3. 0.245N Sodium Hydroxide: 980mg of Sodium Hydroxide was dissolved in distilled water and made up to 100ml.
4. 4.8% Sodium Carbonate: 2.45g Sodium Carbonate was dissolved and made up to 50ml with distilled water.
5. 0.25% Sodium potassium tartarate: 25mg of sodium potassium tartarate was dissolved in 10ml of distilled water.
6. Folin-Ciocalteau reagent: Diluted with distilled water in 1:2 ratio just before use.
7. 1.0% Casein solution: 1.0gm of casein was mixed with 100ml of phosphate buffer.
8. 0.1M Carbonate buffer (pH 9.2).
9. 10% Trichloroacetic Acid (TCA)

4.5.3. Procedure

A 0.05ml of enzyme was made up to 1.0ml using distilled water. To this added 0.2ml of copper Sulphate and 1.0 ml of sodium hydroxide solution. After mixing, added 1.0ml sodium carbonate and 0.2 ml of sodium potassium tartarate and left at room temperature for 15 min, after shaking. To each test tube added 0.5 ml Folin's phenol reagent and incubated again at room temperature after vortexing. The blue color developed was read after 20 min, at 640nm in a spectrophotometer. A blank without protein was used.

The amount of protein present in the enzyme was calculated using BSA as standard

4.5.4. Calculation

$$\frac{\text{OD of unknown}}{\text{OD of standard}} * \frac{\text{Conc of tyrosine}}{\text{Volume of unknown}} * \frac{5.0}{0.2} * \text{Dilution factor}$$

One unit of proteolytic activity (U) was defined as µg tyrosine liberated per ml per min of the enzyme extract.

4.6. CHARACTERIZATION OF ENZYME

4.6.1 Molecular weight determination by SDS-PAGE (Sodium dodecyl sulphate-polyacrylamide gel electrophoresis)

4.6.1.1. Principle

Electrophoresis is the migration of charged molecules in solution in response to an electric field. Their rate of migration depends on the strength of the field; on the net charge, size and shape of the molecules and also on the ionic strength, viscosity and temperature of the medium in which the molecules are moving. As an analytical tool, electrophoresis is a simple, rapid and highly sensitive. It is used analytically to study the properties of a single charged species, and as a separation technique.

Agarose and polyacrylamide - provide a means of separating molecules by size, in that they are porous gels. A porous gel may act as a sieve by retarding, or in some cases completely obstructing, the movement of large macromolecules while allowing smaller molecules to migrate freely. Because dilute agarose gels are generally more rigid and easy to handle than polyacrylamide of the same concentration, agarose is used to separate larger macromolecules such as nucleic acids, large proteins and protein complexes. Polyacrylamide, which is easy to handle and to make at higher concentrations, is used to separate most proteins and small oligonucleotides that require a small gel pore size for retardation. Proteins are amphoteric compounds; their net charge therefore is determined by the pH of the medium in which they are suspended. In a solution with a pH above its isoelectric point, a protein has a net negative charge and migrates towards the anode in an electrical field. Below its isoelectric point, the protein is positively charged and migrates towards the cathode. The net charge carried by a protein is in addition independent of its size - i.e. the charge carried per unit mass (or length, given proteins and nucleic acids are linear macromolecules) of molecule differs from protein to protein. At a given pH therefore, and under non-denaturing conditions, the electrophoretic separation of proteins is determined by both size and charge of the molecules.

Sodium dodecyl sulphate (SDS) is an anionic detergent which denatures proteins by "wrapping around" the polypeptide backbone and SDS binds to proteins fairly specifically in a mass ratio of 1.4:1. In so doing, SDS confers a negative charge to the polypeptide in proportion to its length i.e.: the denatured polypeptides become "rods" of negative charge cloud with equal charge or charge densities per unit length. It is usually necessary to reduce disulphide bridges in proteins before they adopt the random-coil conFigureuration necessary for separation by size. This is done with 2-mercaptoethanol or dithiothreitol. In denaturing SDS-PAGE separations therefore, migration is determined not by intrinsic electrical charge of the polypeptide, but by molecular weight. This is done by SDS-PAGE of proteins or PAGE or agarose gel electrophoresis of nucleic acids of known molecular weight along with the protein or nucleic acid to be characterized. A linear relationship exists between the logarithm of the molecular weight of an SDS-denatured polypeptide, or native nucleic acid, and it's *Rf*. The *Rf* is calculated as the ratio of the distance migrated by the molecule to that migrated by a marker dye-front. A simple way of determining relative molecular weight by electrophoresis *(M*r) is to plot a standard curve of distance migrated vs. $\log_{10}$ MW for known samples, and read off the log*Mr* of the sample after measuring distance migrated on the same gel.

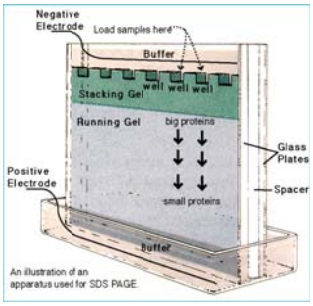


Figure 5.1. SDS-PAGE Apparatus

4.6.1.2.Reagents

1. **30% Acrylamide**: 29.2 g of acrylamide and 0.8 g of N, N methylene bis acrylamide was dissolved in 50ml of distilled water, and made up to 100ml with distilled water. Filtered and transferred to dark bottle.
2. **Separating gel buffer**: 18.17g of Tris buffer was dissolved in 70 ml of distilled water. The pH was adjusted to 8.8 with concentrated HCl and made up to 100ml with distilled water.
3. **Separating gel buffer:** 12.11g of Tris buffer was dissolved in 70 ml of distilled water. The pH was adjusted to 6.8 and made up to 100ml with distilled water.
4. **10% Sodium Dodecyl Sulphate**
5. **0.15% TEMED**
6. **Running buffer:** 3.03g of Tris and 14.41g of glycine were dissolved in a small quantity of distilled water. The pH was adjusted to 8.3 with concentrated HCl. To this 1g of SDS was added and made up to 1000 ml with distilled water.
7. **Sample buffer**

Stacking gel buffer (pH 6.8)	- 1.25ml
Glycerol	- 1.0ml
β -mercaptoethanol	- 0.50ml
SDS	-100mg
Distilled water	-7.25ml
Bromophenol blue	-pinch
10% ammonium persulphate	
Water saturated isobutanol	

8. **Staining solution**

Coomassie brilliant blue	-0.05%
Methanol	-50% (v/v)
Acetic acid	-10% (v/v)
Made up to 100ml with distilled water	

9. **Destaining solution**

Acetic acid	-7% (v/v)
Methanol	-5% (v/v)
Distilled water	-88%

10. **Sample**

To 50µl of sample 50µl of sample buffer was added.

11. **Separating gel (12%)**

Distilled water	- 3.3ml
30%Acrylamide	- 4.0ml
1.5M Tris buffer (pH8.8)	- 2.5ml
10%SDS	- 0.1ml
10%APS	- 0.1ml
TEMED	- 0.004ml

12. **Stacking gel (5%)**

Distilled water	- 3.4ml
30%Acrylamide	- 0.83ml
1.0M Tris buffer (pH6.8)	- 0.63ml
10%SDS	- 0.05ml
10%APS	- 0.05ml
TEMED	- 0.005ml

4.6.1.3. Procedure

Casting the gel

- The two casting plates were cleaned thoroughly with SDS powder to denature any contaminating proteins that may be present on the plates.
- The two gel plates were held at a distance apart by plastics spacers which were of uniform thickness both respect to each other and along their length to ensure good contact with the gel plates and formation of a gel with uniform thickness.
- Two spacers were used one for each vertical side. The plates were sealed using cellophane tape along the sides and the bottom and were then clamped together using clips to prevent leakage during gel casting and running.
- The clamp plate assembly was held vertically using a stand. The supporting gel was prepared, mixed well and poured without delay between the plates. This gel was used to seal the bottom of the plates.
- Once the supporting gel get polymerized, the separating gel was prepared, mixed gently and poured without delay between the plates to $3/4^{th}$ of its total volume and its surface was covered with distilled water to allow uniform polymerization and to prevent oxygen from interpretating with polymerization.
- After polymerization the water was blotted dry using blotting paper and the gel comb was placed between the plates for formation of wells. The stacking gel was prepared, mixed.
- Gently and poured without delay between the plates above the resolving gel and allowed to polymerize.
- After polymerization, the comb was carefully removed and the wells were shaped using syringe or needle.

Sample loading and electrophoresis

- The gel plates were removed from the casting stand, the cellophane tapes were removed and the gel was fixed in the electrophoresis tank unit.
- Tank or running buffer was poured into the lower upper reservoirs of the tank and any bubbles present were removed using a syringe to ensure uniform electrophoretic contact between the gel and the running buffer.

- Pre-dissolved samples (in sample loading buffer) were heated for 3-5 min and added to the wells in required aliquots (25µl) using a micropipette or syringe.
- The electrophoresis unit was connected to the power pack and was adjusted to deliver the necessary current (50V).
- The apparatus was switched on. The appearance of air bubbles at the cathode and indicate the passing of electric current and that the apparatus has been rightly set.
- The gel was allowed to run until the tracking dye has migrated to the appropriate distance, after which the power supply was cut off.

Staining and Destaining

The glass plates were separated with the help of a spatula, the gel was carefully removed and was transferred to a container with the staining solution and allowed to stand for overnight .The staining solution was then drained off and the gel was immersed in the destaining solution and left for 2-3 h. The bands developed can be viewed under illumination.

4.6.2 ZYMOGRAPHY

4.6.2.1. Principle

The term zymography was used by Granelli-Piperno and Reich to describe a technique in which protease separated by sodium dodecyl sulphate (SDS-PAGE) are allowed to diffuse from polyacrylamide gels into an underlying agarose indicator gel containing a protein substrate. In zymograms, zone of lysis, where proteolytically active bands have degraded the substrate in the indicator gel, are visualized as clear zones against an opaque background on dark-field illumination or as clear zones against a dark blue background after staining of the gels with coomassie brilliant blue.

4.6.2.2. Reagents

- **Separating gel (12%)**
  - Distilled water - 3.3ml
  - 30%Acrylamide - 4.0ml
  - 1.5M Tris buffer (pH8.8) - 2.5ml
  - 10%SDS - 0.1ml
  - 10%APS - 0.1ml
  - TEMED - 0.004ml
- **Stacking gel (5%)**
  - Distilled water - 3.4ml
  - 30%Acrylamide - 0.83ml
  - 1.0M Tris buffer (pH6.8) - 0.63ml
  - 10%SDS - 0.05ml
  - 10%APS - 0.05ml
  - TEMED - 0.005ml
- **Sample buffer**
  - Bromophenol blue - 10.0mg
  - 1.5M Tris-HCl (pH8.8) - 4.0ml
  - 60% (w/v) sucrose - 25.0ml
  - 20%SDS - 17.5ml
  - The volume was made up to 50 ml with distilled water.
  - Casein (10mg/ml)
- **Developing buffer**
  - Tris buffer (pH 7.4): 6.06 g of tris base was dissolved in distilled water, pH was adjusted to 7.4 with HCl and the volume was made up to 1000 ml with distilled water.
  - Triton X-100, 2.5% in distilled water (w/v): 2.5g of triton X - 100 was dissolved in 100ml of distilled water.
  - Triton X-100, 2.5%in tris buffer (w/v): 2.5g of triton X - 100 was dissolved in 100ml of tris buffer.
- **Staining solution**
  - Coomassie brilliant blue - 0.05%
  - Methanol - 50% (v/v)
  - Acetic acid - 10% (v/v)
  - Made up to 100ml with distilled water
- **Destaining solution**
  - Acetic acid - 7% (v/v)
  - Methanol - 5% (v/v)
  - Distilled water - 88%

4.6.2.3. Procedure

Casting gel

Casein (10mg/ml) was incorporated in 12% separating gel and 5% gel without casein was used as stacking gels. The separating gel solution was poured into the prepared casts and overlaid with n-butanol saturated with water. After polymerization n-butanol was removed, the stacking gel solution was added, and then well forming combs were placed.

Sample preparation

2mg of enzyme was dissolved in 100µl of 2X sample buffer and 100µl of deionized water. Pre-dissolved samples (in sample loading buffer) were allowed to stand at room temperature for 5-10 min and added to the wells in required aliquots (25µl and 35µl) using a micropipette or syringe.

Running gels

The gels were placed in the gel apparatus, and running buffer was placed in contact with the gels. Electrophoresis was performed with constant voltage (50 V), at 4^{o}C. Electrophoresis, which takes approximately 2 hours, was stopped when the tracing dye was within 2 mm of the bottom of the gel.

Development of Zymograms

Following electrophoresis, gels were placed in individual polystyrene Petri dishes and washed successively with 50ml portion of 2.5% (v/v) triton X-100 in distilled water (2 times, 10min each) 2.5% (v/v) triton X-100 in tris buffer (2 times, 10min each) to remove SDS. After washing was completed, tris buffer (50ml) was poured into dishes containing the gels, and the dishes were covered and incubated at 37^{o}C for 1.5h. Following incubations, gels were fixed for 10 min, stained with coomassie brilliant blue, and destained to reveal zones of substrate lysis.

4.7. BIOCHEMICAL CHARACTERIZATION OF THE ISOLATE

Identification of selected isolate was studied based on different morphological, physiological and biochemical characteristics. The purified bacterial culture was subjected to a range of biochemical tests for identification. The tests are:

1. Gram's staining
2. Indole Test
3. Methyl Red Test
4. Voges Proskauer Test
5. Citrate Utilization Test
6. Triple Sugar Iron Test
7. Urease Test
8. Catalase Test
9. Oxidase Test
10. Nitrate Reduction Test
11. Carbohydrate Utilization Test
12. Casein hydrolysis

4.8. APPLICATION STUDIES

4.8.1 Unhairing of Goat Skin

- The goat skin was taken and cleaned properly with water until the waste materials are washed off.
- Then it was cut in to 4 equal parts.
- The upper parts were used for the studies.
- As the hair root was longer in the backbone region.
- 300grams of skin was taken.
- The 15 %(v/w) enzyme was applied on the inner side of the skin and kept at room temperature, then observed for the hair removal after 18 hours.
- The other part of the skin was treated with the chemical treatment.
- At the end of the process, the skin pieces were gently scraped with fingers to remove loose hairs.
- This procedure was necessary because rubbing in this laboratory-scale process was not as vigorous as in industrial drums.
- The dehairing efficacy was assessed according to the depilated area of the skin at various time intervals and the quality of the dehaired skin was estimated according to the appearance observed by the naked eye and microscope after 18 hours of treatment.
- The dehaired skin with high quality showed clean hair pore, clear grain structure and no collagen damage.

4.8.1.1. Histological Study

The pelts were prepared for histopathalogical analysis by fixing in 10% (w/v) formaldehyde. The pelt was dehydrated with 80, 95 and 100% (v/v) of alcohol gradients followed by xylene treatment, and then embedded in paraffin. Longitudinal sections (L.S.) of hide embedded in paraffin wax were obtained using a microtome. The sections were fixed on slides using starch paste containing thymol, which acts as a preservative. The sections were stained with Harris's haematoxylin stain followed by 0.5% (v/v) HCl and dilute ammonia **(**John and Merriline 2002**)**.The slides were observed microscopically for (a) epidermis, (b) hair root follicles, and (c) corium (collagen layer).

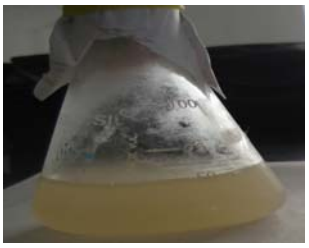
4.8.2. HYDROLYSIS OF TANNERY WASTE

- About 3 kg of waste flesh was collected from tannery and minced it properly using mincer.
- The initial pH was measured and adjusted the pH using H_2SO_4.
- Incubate the above mixture at 65-85°C for half an hour with proper mixing at regular intervals.
- Then cool the substrate to room temperature.
- Add enzyme to the mixture in the ratio of 10-15% V/W.
- Mix it thoroughly and incubate it at 37°C for 2-5hours rotating at 900rpm.
- Take the aliquots from the reaction mixture periodically at every one hour including an aliquot at 0^{th} hour after the addition of the enzyme.

5. RESULTS

5.1. REVIVAL OF THE CULTURE:

Solid Media **Liquid Media**



Nutrient agar Luria broth

Figure 5.1. Revival of the culture

5.2. CONFORMATION OF PROTEASE PRODUCTION

The proteolytic activity of the bacteria was confirmed in gelatin-agar plates were a circular zone of clearence is formed around the hole to which the inoculation was done.The zone appear only after the treatment with 10% tannic acid.

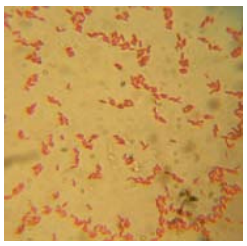
Zone of clearence in 1% GELATIN Nutrient agar medium confirms the presence of protease

Figure 5.2. Conformation of Protease Production

5.3. BIOCHEMICAL CHART FOR IDENTIFICATION OF BACTERIA.

Table.5.1. Biochemical Chart for Identification of Bacteria

S.NO	CHARACTERISATION	RESULT	INTERPRETATION
1	Shape	Rod	Rod shaped bacteria.
2	Gram staining	-ve	Gram negative bacteria.
3	Motility	+ve	Motile in nature.
4	Growth temperature	37^0C	Optimum growth.
5	Reduction of nitrate	+ve	The colour of the nitrate broth changed to cherry red on addition of the two reagents alpha-naphthalemine and sulphanilic acid. This indicated that the organism is blue to reduce nitrates to nitrites.
6	Production of indole	-ve	The organism does not produce the enzyme tryptophanase that converts the tryptophan present in the medium to indole pyruvic acid. No red colour formation.
7	Oxidase	+ve	Deep purple colour was formed on placing an isolated colony on the oxidase disc within 5 seconds. This indicates that the organism has the ability to produce the enzyme oxidase.
8	Catalase	+ve	Formation of bubbles was observed on addition of hydrogen peroxide to the slant surface. This indicates the organism produce the enzyme catalase which catalyses the conversion of hydrogen peroxide to oxygen gas and water.
9	Urease	+ve	The colour of Christensen's Urea Agar had changed to pink. This confirms the ability of the microorganism to produce Urease enzyme. This enzyme releases ammonia from urea and the resulting alkaline pH which leads to the colour change of phenol red indicator present in the medium from yellow to pink.
11	Voges Proskauer test	-ve	The organism does not produce non-acidic or neutral end products from glucose metabolism.
12	Citrate utilization test	+ve	Development of deep blue colour identifying that the organism able to utilize the citrate as a sole source of carbon. Citrate is broken down to oxaloacetic acid and acetic acid. These products are then enzymatically converted to pyruvic acid and carbon dioxide. Thus the color of bromothymol blue changes to blue colour in acidic conditions.
13	Triple sugar iron test	+ve	The organism exhibited alkaline slant and acid butt; this indicates glucose fermentation has occurred. The organism preferentially degrades glucose first. Since this substrate is present in minimum concentration, the small amount of acid produced on the slant surface is oxidized rapidly. The peptones in the medium are also used in the production of alkali. In the butt, the acid reaction is maintained because of reduced oxygen tension and slower growth of organisms. Absence of any black coloration in the butt or slant indicates that H_2S production has not occurred. No breaks or lifting up of slant indicates the absence of gas production.
14	Casein hydrolysis	+ve	Zone formed, indicates the presence of the enzyme casein.



GRAM- NEGATIVE RODS

Figure .5.3. Gram Staining

Biochemical Results for Identification of Bacteria

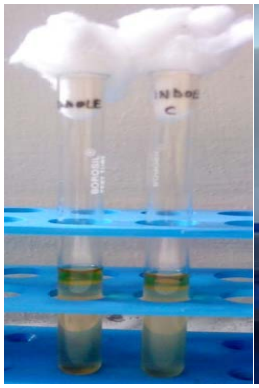


Figure .5.4. INDOLE TEST

Figure .5.5.UREASE TEST

Figure .5.6. MR TEST

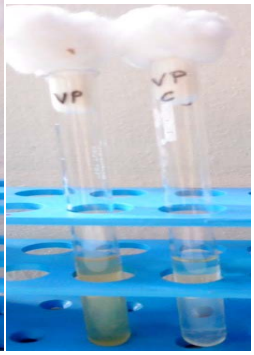


Figure .5.7.VP TEST

Figure .5.8. CITRATE TEST

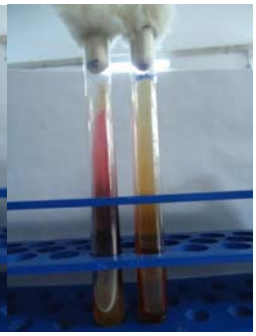


Figure .5.9.TSI TEST

5.4. OPTIMIZATION OF CULTURE CONDITIONS FOR ENZYME PRODUCTION

5.4.1. MEDIUM OPTIMIZATION

TABLE5.2. Medium Optimization (conditions; pH- 8.0, temperature-37°C).

MEDIA	TIME(HRS)			
	24	48	72	96
1	26.6	353.0	50.0	40.0
2	36.7	250.0	93.0	23.7
3	20.0	190.0	22.6	10
4	50.0	73.0	153.0	46..7
5	23.0	36.6	46.6	6.7
6	106.0.	263.0	46.0	56.6
7	143.3	533.0	820.0	296.0

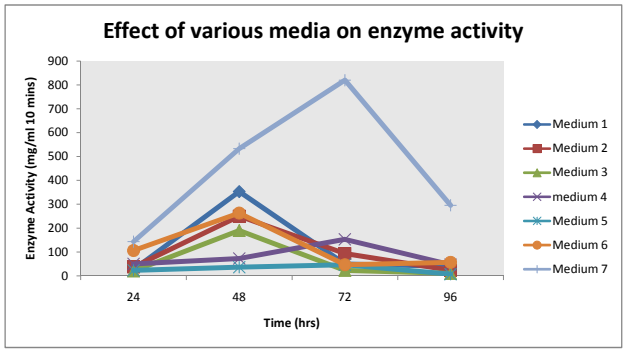


Figure 5.10. Effect of various medium on enzyme activity

Table 5.2.a. Medium Optimization (conditions; pH- 8.0, temperature-37°C).

Media	Time(hrs)			
	24	48	72	96
1	363	400	463	256.7
2	113	40	16.7	13.3
3	26.7	53.3	6.7	3.3

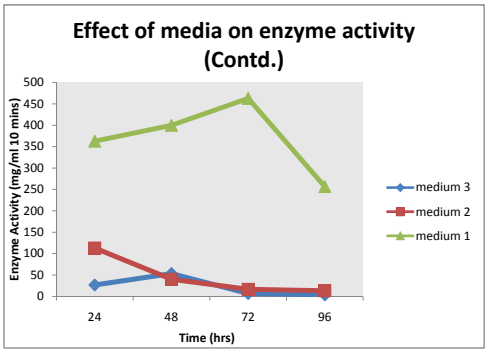


Figure 5.10.a Comparison of three medium

5.4.2. OPTIMIZATION OF TEMPERATURE

Temperature is one of the most important factors affecting the enzyme production. Protease activity was assayed at different temperatures viz. 25ºC, 30ºC and 37ºC at 96hrs. The results illustrated in Figure .5 referred to a positive relationship between protease production and incubation temperature up to 37ºC**.** A reduction in enzyme activity was observed at values above 37ºC. For using under the conditions present in a tannery, the proteases must be active at 37ºC.

Table.5.3. Effect of incubation time and temperature on protease production (conditions; pH- 8.0, temperature-37ºC).

Temp(ºC)	Time(Hrs)			
	24	**48**	**72**	**96**
25	30	100	170	76.7
30	36.7	503	983	246.7
37	50	593	990	296

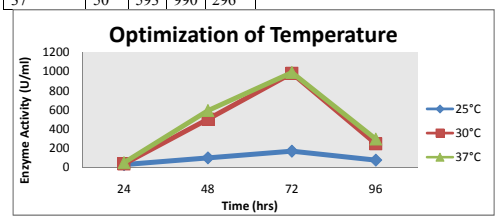


Figure 5.11. Temperature Optimization

5.4.3. OPTIMIZATION OF pH

The pH-activity profile of the enzyme is shown in **Table.4. And Figure: 5.6.** A pH range between6.0 and 10.0 was used to study the effect of pH on protease activity. Maximum enzyme activity was observed at pH 7 and decreased with pH beyond 7. At pH 10 the protease activity decreased. The protease activity was found to be stable over the pH range 7 – 9. The optimal pH for purified extracellular alkaline protease produced from the alkalophilic bacterium *Bacillus sps* was 7.0

Table.5.4.Effect of pH on protease production (conditions; pH- 8.0, temperature-37ºC).

pH	TIME(Hrs)			
	24	**48**	**72**	**96**
6	56.7	126.7	213.3	106.7
7	23.3	93.3	320.0	133.0
8	81.6	113.0	370.0	181.3
9	80.0	133.0	333.0	123.0
10	73.0	130.0	293.0	113.0

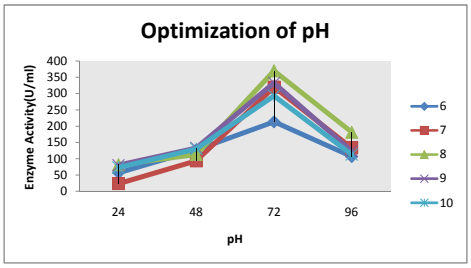


Figure 5.12 pH Optimization

5.4.4. DETERMINATION OF PROTEIN CONTENT IN THE ENZYME EXTRACT

Table.5.5. Protein content in the enzyme extract (conditions; pH- 8.0, temperature-37ºC).

Time(hrs)	OD	Enzyme activity (U/ml)
24	1.03	294.2
48	1.434	409.7
72	1.910	545
96	0.949	271

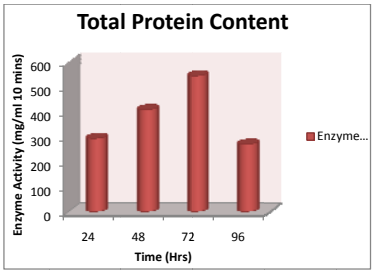


Figure 5.13 Total Protein Content

5.5. CHARACTERIZATION OF ENZYME.

5.5.1. EFFECT OF TEMPERATURE ON ENZYME STABILITY

Table .5.6.Thermal stability of enzymes (conditions; pH- 8.0, temperature-37ºC).

Temperature	Enzyme activity (U/ml)
25	3
30	33
37	36
40	60
50	60
60	90
70	183
80	26.6

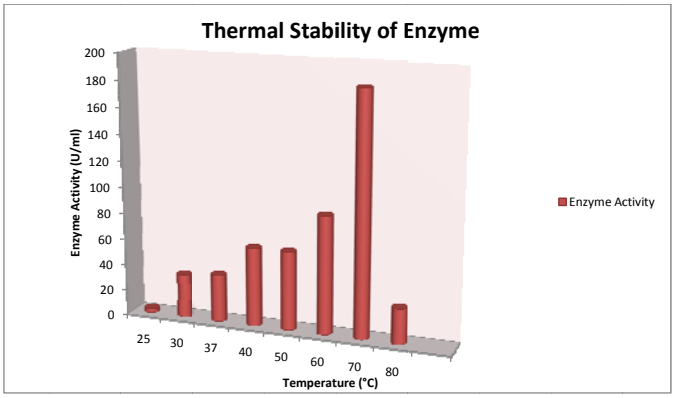


Figure 5.14. Effect of temperature on enzyme stability

5.5.2. EFFECT OF pH ON ENZYME STABILITY

Table.5.7. Effect of pH on enzyme stability (conditions; pH- 8.0, temperature-37ºC).

pH	Enzyme activity (U/ml)
4	5.8
5	19.2
6	21.6
7	36.6
8	438
9	516
10	391

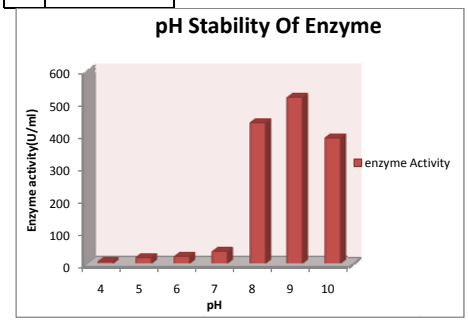


Figure 5.15.Effect of pH on enzyme stability

5.5.3. EFFECT OF METAL IONS ON ENZYME ACTIVITY

Table.5.8. Effect of Metal Ions on Protease Enzyme (conditions: pH- 8.0, temperature-37ºC)

Metal Ions	Relative enzyme activity (%)
$Mgso_4$	104
$Cocl_2$	14
$Cuso_4$	59
$Hgcl_2$	8
$ZnCOONH_2$	37
$Nicl_2$	6
$Cacl_2$	55
NH_4cl	14
Nacl	84
KOH	2

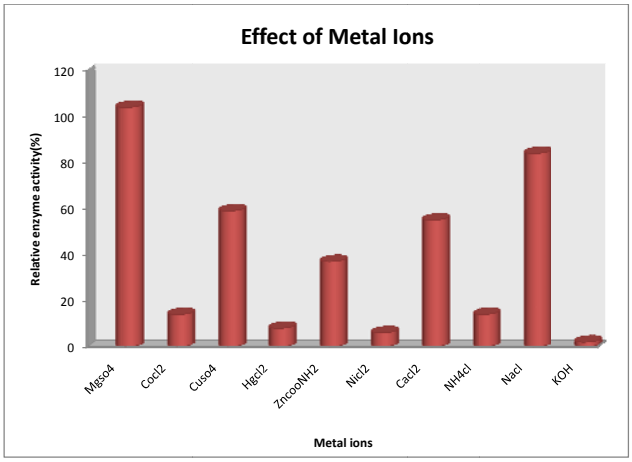


Figure 5.16. Effect of Metal Ions

5.5.4. EFFECT OF INHIBITOR, DETERGENT AND OXIDIZING AGENT, CARBON AND NITROGEN SOURCES ON STABILITY OF PROTEASE.

Table.5.9 Effect of Inhibitor, Detergent and oxidizing agent on protease enzyme (conditions; pH- 8.0, temperature-37°C).

Inhibitors, Detergent, Oxidizing agent		Relative enzyme activity (%)
Inhibitors	EDTA	65
	PMSF	45
Detergent	SDS	18
Oxidizing agent	H_2O_2	22

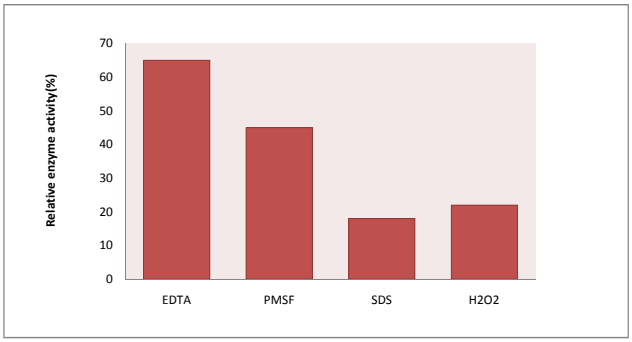


Figure 5.17. Effect of Inhibitor, detergent and oxidizing agent on enzyme

Table.5.10. Effect of Carbon sources on alkaline protease enzyme activity (conditions; pH- 8.0, temperature-37°C)

Percentage of carbon source	Glucose	Sucrose	Lactose	Fructose
0.5%	33.30	50.59	27.78	31.09
1.0%	42.86	63.10	33.48	39.55
1.5%	26.86	47.10	25.39	24.47

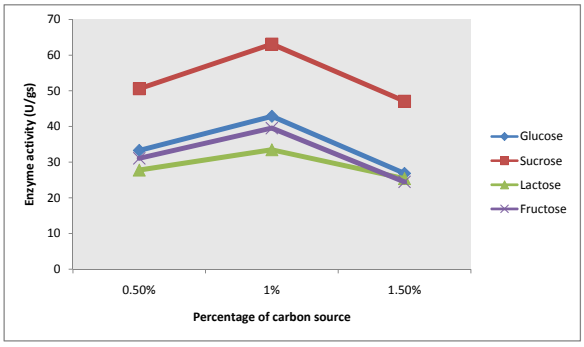


Figure 5.18. Effect of Carbon sources on alkaline protease enzyme activity

Table.5.11. Effect of Nitrogen sources on alkaline protease enzyme activity (conditions; pH- 8.0, temperature-37°C)

Percentage of Nitrogen source	Yeast extract	Peptone	Beef extract	Ammonium nitrate
0.5%	32.75	31.27	17.84	35.32
1.0%	38.82	34.95	22.07	29.25
1.5%	27.96	27.23	15.27	24.47

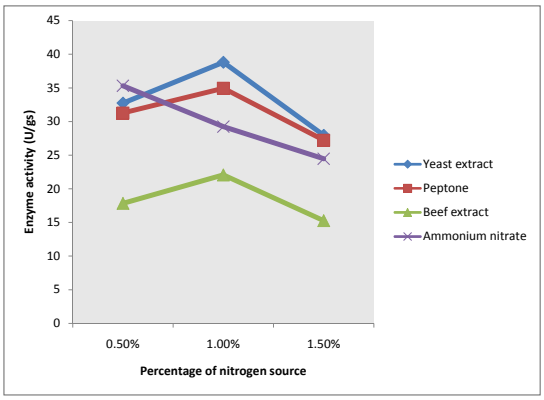


Figure 5.19. Effect of Nitrogen sources on alkaline protease enzyme activity

5.5.5. SDS- PAGE

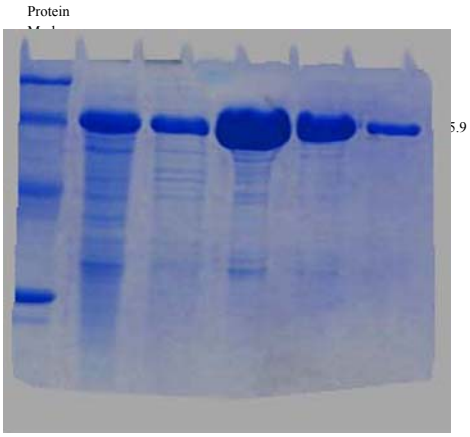


Figure 5.20. SDS- Page of protease enzyme

5.5.6. ZYMOGRAPHY

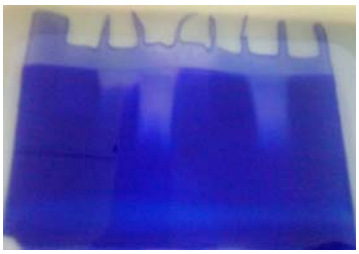


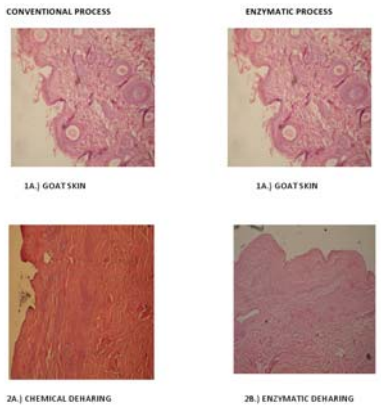
Figure 5.21. Zymography of protease enzyme

5.6. APPLICATION STUDIES

5.6.1. UNHAIRING OF GOAT SKIN

Figure 5.22. Unhairing of Goat Skin

5.6.1.1. HISTOLOGICAL CONFIRMATION OF UNHAIRING



Hair follicle in the skin

Absence of hair follicle in the skin without the damage of collagen

Figure 5.23. Histological confirmation of unhairing

5.6.2. HYDROLYSIS OF TANNERY FLESHINGS

Figure 5.24. Hydrolysis of Tannery Fleshings

5.6.3. HYDROLYSED PRODUCTS

Figure 5.25. Protein

Figure 5.26. Salt Content

Figure 5.27. Fat

5.6.4 Protein content in hydrolysed sample

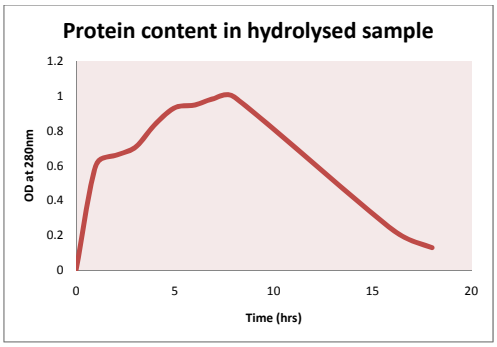


Figure 5.28. Protein content in hydrolysed sample

Table.5.12. PERCENTAGE RECOVERY OF HYDROLYSED PRODUCTS

HYDROLYSED PRODUCTS	Batch I (%)	Batch II (%)
Protein	3	6.6
Fat	3.5	5.06
SALT		
$CaSo_4$	8	11.6
$Ca(OH)_2$	4.25	6.348
Water	81.25	76.67

6. DISCUSSION

The present study aims to isolate *Bacillus sp* (MTCC 5468) from the tannery effluent and was identified for its potential to produce large amount of extracellular protease. The organism obtained was retrieved from lyophilized vial and used for enzymatic studies. The microorganism isolated by the Department of Biotechnology was streaked on nutrient agar plate and inoculated in Nutrient broth. It was incubated at 37ºC for 24 h. The microorganism which shows the high proteolytic activity after qualitative detection was maintained in the slant tube containing nutrient agar. After incubation the slant tubes were transferred to 4ºC. At this temperature the cells activity was arrested i.e. they are in inactive form. This type of culture is stable for 1-2 months after which they have to be sub cultured. The slant tubes stored has to be thawed to room temperature before they could be used.

Proteolytic activity is confirmed by Qualitative detection of protease. Qualitative assay rely on the formation of a clear zone of proteolysis on agar plates as a result of protease production. The proteolytic activity of the bacteria was confirmed in gelatin-agar plates were a circular zone of clearance is formed around the hole to which the inoculation was done

Identification of selected isolate was studied based on different morphological, physiological and biochemical characteristics. The purified bacterial culture was subjected to a range of biochemical tests for identification and the tests were studied according to Bergey's manual of systematic Bacteriology. The organism was found to be rod shaped gram negative motile bacteria that showed optimum temperature at 37ºC. This was observed fermentative in nature, able to hydrolyze gelatin substrate on gelatin agar medium and form a clear zone. From the above the results, it can be concluded that the isolated microorganism might belong to the genus of *Bacillus* (Holt et al., 1994).

Incubation time plays a substantial role in the maximum enzyme production. It has been reported that *Bacillus subtilis* PE-11 showed maximum protease production in 48hr (Pastor et al., 2001), where as *Bacillus subtilis* 3411 gave maximum production in 72 hr and Bacillus sp K-30 in 96hr (Naidu et al., 2005& Gibb et al., 1987).Results of the present study indicates that the production of protease depends upon the growth of the bacteria and the *Bacillus sp* (MTCC 5468) produced maximum enzyme activity at 72hr.

Culturing bacteria is the initial step in study its morphology and its identification. To culture bacteria, it is necessary to optimize the medium. All organisms require carbon and nitrogen source for their growth. It is necessary to check whether the medium is cheap and readily available. The cultural conditions (different sources of carbon and nitrogen, pH and temperature) were optimized for maximum enzyme production using various medium. Among these seven medium, Glucose, Potassium monophosphate, Dipotassium phosphate along with metal ions such as Sodium chloride, Calcium chloride, Dipotassium phosphate, Sodium carbonate, and Sodium nitrate is found to inhibit the growth of the medium. For example, In other protease producing organisms glucose and sucrose were shown to repress protease production (Glen.,1796); (Ingam et al.,1963); (Kaliz et al.,1988).In *Bacillus stearothermophilus* glucose repressed protease production each at concentration 1% (Razak et al.,1994).

Nitrogen sources like Peptone, Yeast extract and Beef extract enhances the growth of the culture and enzyme production. Conflicting reports regarding the effect of organic and inorganic source on alkaline protease production by *Bacillus* sp. are reported. Sinha and Sathyanarayana reported higher protease production with inorganic nitrogen sources, while many others have found inorganic sources better suited to *Bacillus sp.* for enzyme production.(Fujiwara&Yamamoto., 1987; Sen and Sathyanarayana., 1993; Gajju et al., 1996)

Apart from other sources Nutrient broth along with Gelatin which is a mixture of peptide and protein formed by partial hydrolysis of collagen is found to be an effective source for protease production with activity 820U/ml.

To check the effectiveness of the medium7 i.e., Nutrient broth and 1% Gelatin with carbon source is carried out further optimization in two different flasks containing NaCl and Glucose which shows lower activity 113U/ml, 53.3U/ml. This does not enhance the enzyme activity compared to medium7 (Nutrient broth and 1% Gelatin).

From the above results Nutrient broth with 1% gelatin were used for optimizing and characterizing parameters like temperature, pH.The activity of the enzyme at different temperatures 25°C, 30°Cand37°C were carried out. The maximum enzyme production is observed for 72hrs culture at 37^0C with the highest activity 990U/ml.Example, Han-Seung et al and Akcan et al showed maximum enzyme activity at 37^0C.

Similarly to determine the effect of pH on protease activity, the culture was incubated at different buffers with pH ranging from 6-10 for 96hrs.From this maximum enzyme

production was observed for 72hrs culture with enzyme activity 370U/ml at pH 8. Sandhiya et al showed the highest activity of protease at pH8.

The thermal stability of the crude protease was tested at different temperature range of 25°C-70°C for 30minutes.The enzyme was almost stable at 70°C.In earlier reports the enzyme was found to be active in the temperature at 55°C (Nilegaonkar et al., 2007), 60°C (Adinarayana et al., 2003) and the maximum stability of enzyme was reported as 80°C(Izmir et al.,2005)

The pH stability of protease enzyme was also determined by the pre-incubation of the enzyme in various buffers of different pH values. The enzyme was found to be stable over a range of pH8-10.The other report Adinarayana et al., 2003 shows that the enzyme was stable over pH8-11.

The metal ion Mg had a Strong stimulatory effect and other ions like Ca, Co, Cu, Hg, K, Na, NH_4 and Zn had inhibitory effect on enzyme.Mg increased and stabilized the protease activity of the enzyme, this is possible because of the activation by the metal ions which protected the enzyme against thermal denaturation and played a vital role in maintaining the active confirmation of the enzyme at high temperature (Adinarayana et al., 2003). Other metal ions such as Ca, Co, Cu, Zn, (Arlem et al., 2010), Hg, K, Na, NH_4 and Zn did not show any effect on enzyme activity. Similarly the activity of alkaline serine protease from *B. pumilus* is enhanced by Ca2+,Mg2+and Na+(Huang et al., 2003) In contrast to many reports Ca inhibited the activity of the enzyme produced by *Bacillus sp*(MTCC 5468).

Of the inhibitors tested at 5mM concentration, PMSF was able to inhibit the protease by 55%, while EDTA exhibited 35%inhibition.PMSF is found to sulphonate the essential serine residue in the active site of the protease and had result in the loss of enzyme activity (Adinarayana et al., 2003) This type of inhibition profile also was reported for proteases from *Pseudoperkinsus tapetis* (Ordás et al., 2001), *Vibrio fluvialis* (Venugopal and Saramma, 2006), *B. circulans* (Jaswal and Kocher, 2007)..These results suggested that the enzyme secreted by *Bacillus sp* could belong to the class of serine proteases (Izmir et al., 2005).

Oxidizing agent (H_2O_2) and Detergent (Sodium Dodecyl Sulphate [SDS]) tend to inhibit 18% and 22% and does not show any enhanced activity at 5mM concentration. Slight inhibition by H2O2 (Oberoi et al., 2001; Genckal and Tari, 2006; Hadj-Ali et al., 2007) has been observed for other bacterial proteases. At a concentration of 1%, SDS caused strong inhibitory effects (50%) on protease activity from *Pseudomonas aeruginosa* PD100 (Najafi et al., 2005). SDS (0.1%) reduced by 39% the activity of the protease enzyme produced by *P. aeruginosa* PseA (Gupta et al., 2005).But increase in SDS concentration may enhance the enzyme activity.

Separation of crude enzyme employed via using sodium Dodecyl sulphate, polyacrylamide gel electrophoresis (SDS-PAGE). The separation pattern of the crude enzyme, along with the protein molecular weight marker 3,000 to 205,000 Da can be seen. The electrophoresis patterns of the crude enzyme contain two enzymatically active proteins in the molecular weight range of 205 to 66 KDa. Therefore it is presumed that the molecular weight of the enzyme produced by the Bacterial species of this study could be between 66 to 205 KDa.

Fig.5.19 confirmed the proteolytic nature of the enzyme solution that hydrolyses the substrate casein that was incorporated in the gel system. A clear zone was observed in the 12% casein zymogram. The mobility of the enzyme protein on the gel containing casein is clearly seen by the clearance of opacity in the lane loaded with the enzyme. The Zone of clearance corresponds to a migration distance consistent with an approximate molecular weight of active proteins which ranges from 66,000 Da to 205,000 Da.

Crude enzyme was used for dehairing of 12 cm×12 cm piece of goat skin at pH 7.0 and temperature 28 ± 2°C for 24 hours. The enzyme activity was 1862.501 U/ml. The presence of lime or sulphide was not required for this process. The enzyme treated skin showed complete dehairing activity after 24 hrs of incubation. In the control sample, hair loosening was not observed, even by mechanical means such as plucking by forceps, whereas there was visible dehairing observed in the chemically treated sample after 24 hrs of incubation.

Microscopic examination of the pelt treated with chemical (Fig. 5.21) showed distorted collagen bundles with residual hair or hair shaft whereas enzyme- treated pelt showed complete removal of epidermis and presence of empty hair follicles (Fig. 5.19) suggesting removal of hair from hair root follicle. Collagen layer was not damaged or modified in enzyme-treated pelts. Similar results were observed by using multiple proteinase concentrate in goat skin (Chandrasekaran and Dhar 1985). In our studies, it was also observed that protease treated pelt swells moderately with adequate opening up of collagen fibre. Enzymatic dehairing did not damage the collagen layer. Likewise, a novel keratinase obtained from *B. subtilis* S_{14} exhibited remarkable dehairing capabilities without damage to bovine skin collagen, which was confirmed on SDS– PAGE using control collagen, and collagen treated with standard keratinase, collagenase and test enzymes (Alexandre *et al*., 2005). The enzyme treated hair was intact, with hair root, shaft, and sheath. In previous reports, the epidermis, hair shaft and bulbs were completely removed with better splitting of fibre bundles by the action of multiple protease concentrate (Chandrasekaran and Dhar 1985). Our study indicates that the hair loosening was due to enzyme action and not due to the swelling up or modification of the sheath.

7. SUMMARY AND CONCLUSIONS

The Bacteria- derived proteases were considered as major industrial workhorses because of their high production capacities and activities.

- The Organism isolated was found to be fermentative bacteria.
- Protease production by this bacterial species was shown to be affected by various environmental and nutritional conditions.
- The Organism produced an extracellular alkaline protease with optimum pH 8.0 and optimum Temperature around 30°C.
- Based on the optimization studies fructose and skim milk was the most effective Carbon and Nitrogen sources for protease production.
- The crude enzyme seemed to contain three enzymatically active proteins in the molecular weight range of 66 to 205 KDa. This was confirmed by Zymogram.
- The crude enzyme exhibited dehairing activity on goat skin without chemical assistance and without hydrolyzing fibrous proteins.
- Due to these properties, the enzyme could be potentially useful in leather industry for dehairing of goat skin without damaging collagen layer resulting in a better quality production and avoiding the pollution problem associated with the use of chemicals.
- The Enzyme was also studied for the hydrolysis of solid waste.

Future work is to optimize the cheap media for the production of the enzyme for large scale applications in various industries.

8. References

Adinarayana, K., P.Ellaiah and D.S Prasad. 2003. Purification and partial characterization of thermostable serine alkaline protease from a newly isolated Bacillus subtilis PE-II.AAPS. Pharm.Sci.Tech.4:1-9.

Ahmed, Muhammad Anjum Zia and Hafiz Muhammad Nasir Iqbal 2011. Purification and Kinetic Parameters Characterization of an Alkaline Protease Produced from *Bacillus subtilis* through Submerged Fermentation Technique. *World Applied Sciences Journal* 12 (6): 751-757, 2011.

Anwar, A., C.rose,. Saleemudin. 2008. Alkaline protease: A review. *Bioresource Technol.* 4: 175-183.

A.Annapurna Raju, N.K.Chandrababu, N.Samivelu, C.Rose and N.Muralidhara Rao, (2006). Eco – Friendly enzymatic dehairing using extracellular protease from a *Bacillus* species isolate, Bio-Products lab, CLRI, vol. 91:115-119.

Al-shehri, M.Abdulrahman and Mostafa, S. Yasser (2004). Production and some properties of protease produced by *Bacillus licheniformis* isolated from Tihamet Asser, Saudi Arabia, Pakistan journal of biological sciences 7 (9): 1631-1635.

Amare Gessesse and Berhanu A. Gashe (2007). Production of alkaline protease by an alkaliphilic bacterium isolated from an alkaline soda lake, Biotechnology Letters, Vol 19, No 5, pp. 479–481.

Andrade, V.S.; Sarubbo, L.A.; Fukushima, K.; Miyaji, M.; Nishimura, K. and de Campos - Takaki, G.M., (2002). Production of extracellular protease by *Mucor circinelloides* using D-Glucose as carbon sources/substrates. Brazilian Journal of Microbiology 33: 106-110

A.R.Shakoori, M.Makhdoom R.U. Haq (2000). Hexavalent chromium reduction by a dichromate –resistant gram- positive bacterium isolated from effluents of tanneries. Appl Microbiol Biotechnol (2000)53:348-351.

Banerjee R, Bhattacharyya BC (2002). Extracellular alkaline protease of a newly isolated *Rhizopus oryzae,* Biotechnol Lett; 14:301–4.

Babu, K.R., and H.Satyanarayana.2004. *J.Sci. Ind.* Res. 55:464-467.

Biswanath Bhunia, Debjani Dutta, Surabhi Chaudhuri Extracellular alkaline protease from Bacillus licheniformis NCIM-2042: Improving enzyme activity assay and characterization.

Beg Q.K., V. Sahai and R. Gupta (2003). Statistical media optimization and alkaline protease production from *Bacillus mojavensis* in bioreactor, Process Biochem. 39, 203-209.

Berla Thangam E and Suseela Rajkumar G (2002). Purification and Characterization of alkaline protease from *Alcaligenes faecalis* Biotechnol, Appl.Biochem. 35, 149–154.

Beynon, J. and J.S. Bond (2009). Proteolytic enzymes, a practical approach, Press, New York, NY.

Chaphalkar S, Dey S (2004). Some aspects of production of extracellular protease from *Streptomyces diastaticus,* J Microb Biotechnol; 9:85–100.

Choudhary, R.B., A.A.Jana and M.K.Jha. 2003. Enzyme technology applications in leather processing, *Ind. J. Chem. Technol.*11:659-671

Dayanandan, A., J.Kanagraj, L.Sounderraj, R. Govindaraju and G.s. Rajkumar.2003. Application of an alkaline protease in leather processing: an eco-friendly approach. *J. Clean. Product,* 11: 53-58.

Deshpande, V.V., Laxman, R.S., More, S.V., Rele, M.V., Rao, B.S.R., Jogdand, V.V., Rao, M.B., Naidu, R.B., Maniknandan, P., Kumar, D.A., Kanagaraj, J., Samayavaram, R., Samivelu, N., Rengarajulu, P., (2004). Process for preparation of an alkaline protease, 96, 723-745.

Ellaiah P, Adinarayana K, Rajyalaxmi P, Srinivasulu B (2003). Optimization of process parameters for alkaline protease production under solid state fermentation by alkalophilic *Bacillus* sp, Asian J Microbiol Biotechnol Environ Sci 5:49–54.

E. M. El-Safey and U.M.Abdul-Raouf., Production, purification and characterization of protease enzyme from bacillus subtilis.

Everett AL and Cordon TC (1956). Hydrolytic action of facultatively anaerobic microorganisms of raw skins. J.A.L.C.A. 51, 59.

Farhad Foroughi, Tajalli Keshavarz and Christine S Evans. 2006.Specificities of proteases for use in leather manufacture. Journal of chemical technology and Biotechnology 81:257-261 (2006).

Fereshteh Eftekhar, Jamsheed Fouladi and Mehrzad Faghihi (2005). Isolation and identification of an alkaline protease producing *Bacillus* from soil, Journal of Applied Microbiology, 99, 641–648.

Folosade M. Olajuyigbe and Joshua O. Ajele. 2005. Production dynamics of extracellular protease from *Bacillus* species. *African Journal of Biotechnology Vol. 4* (8), pp. 776-779,

Frankena, J.,H.W.Van and Versevild , A.H.Stouthamer.2005. A continuous culture study of the exocellular protease in *Bacillus Licheniformis, Appl Microbiol Biotechnol*. 22:169-76.

Gupta,R., Q.K Beg and P.Lorenze.2002. Bacterial alkaline protease: molecular approaches and Industrial applications. *Appl. Microbiol. Biotechnol.* 59:15-32.

Green GH (2001). Unhairing by means of enzymes. J.S.L.T.C. 36, 127-134.

Green GH(2003). A review of enzymes in the tannery, J.S.L.T.C. 45, 167-169.

Gupta R, Beg QK and Lorenz P (2000). Bacterial alkaline proteases: molecular approaches and industrial applications. Appl. Microbial. Biotechnol. 59, 15-32.

Haider, M., Hamzah, H.L.Anwar , G.Hamid and Hassan.2009. Physiological regulation of protease in pencillum sp using submerged and solid-state fermentation Techniques . *J . Engn. Sci,Techonol.* 4:81-89.

Han-Seung Joo,Chung-Soon Chang, 2009 Production of an oxidant and SDS-stable alkalineprotease from an alkaophilic Bacillus clausii I-52 by submergedfermentation: Feasibility as a laundry detergent additive.

Harrigan, W.F.,M.c.Cane,2006. Laboratory methory methods inb Microbiology *Academic press New York*.p:55-68.

Hermosinda Varela, Mario Daniel Ferrari, Leopoldo Belobrajdic, Adriana Vazquez and Milka Lyliam Loperensa (2007). Skin unhairing proteases of *Bacillus subtilis:* production and partial characterization. Biotechnology letters, Vol 19, pp. 755-758.

Holt, J.G., N.R krieg, P.H.A. J.T SneathStately and S.t Williams.2004. Bergey's Manual of Determinative Bacteriology, 9th ed, Baltimore, Williams and Wilkins, 787.

Ishtiaq Ahmed1,2, Muhammad Irfan1, Muhammad Nadeem1, Muhammad A. Zia2 , Bilal M. Ahmad3,and Hafiz M. N. Iqbal, 2006,Optimization of Media and Environmental Conditions for Alkaline Protease Production Using Bacillus Subtilis in Submerged Fermentation Process 43:195–267.

Joo HS, Kuamr G, Park GC, Kim KT, Paik R and Chang CS (2002) Optimization of the production of an extracellular alkaline protease from Bacillus Horikoshi. Process Biochem. 38, 155-159.

Kamini, N.R, Hemachandar, C., Mala, J.G.S and Puvanakrishnan, R (2009). Microbial enzyme technology as an alternative to conventional chemicals in leather industry. Current Science, 77: 80-86.

Kohlmann K.L, Nielsen S.S, Steenson L.R, and Ladisch M.R (2001). Production of proteases by psychrotrophic micro organisms, j. dairy Sci., 335:155-172.

Kole MM, Draper I, Gerson DF (2008). Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations, J Chem Technol Biotechnol; 41:197–206.

Kumar, C.G. and H. Takagi (2009). Research review paper: microbial alkaline proteases: from a bioindustrial viewpoint, Biotechnol. Adv., 17, 561-94.

Lantz MS and Ciborowski P (2004). Zymographic techniques for detection and characterization of microbial proteases, Methods Enzymol. 235:563-94.

Lowri O.H., Rosebrough N.J., Farr A.L., and Randall R.J (2001). Protein measurement with folin phenol reagent, Journal of biological chemistry, vol. 193, 265-273.

M. Vidyasagar S. Prakash S. K. Jayalakshmi. K. Sreeramulu (2007). Optimization of culture conditions for the production of halothermophilic protease from halophilic bacterium *Chromohalobacter sp.* TVSP101, World J Microbiol Biotechnol 23:655–662.

Mabrouk, S.S., A.M. Hashem, N.M.A. El-Shayeb,A.M.S. Ismail and A.F. Abdel-Fattah,(2009). Optimization of alkaline protease productivity by *Bacillus licheniformis* ATCC 21415, Bioresource Technol. 69, 155-159.

Manachini PL, Fortina MG, Parini C (2008). Alkaline protease produced by *Bacillus thermoruber* a new species of *Bacillus*, Appl Microbiol Biotechnol 28:409–413.

Manachini, P.L. and Fortina, M.G (2008). Production in sea water of thermostable alkaline protease by a halotolerant strain of *Bacillus licheniformis,* Biotechnol. Letters 20 565-568.

Mao W, Pan R, Freedman D (2002). High production of alkaline protease by *Bacillus licheniformis* in a fed-batch fermentation using a synthetic medium. J Ind Microbiol; 11:1–6.

M.Basu, S.Bhattacharya, A.K.Paul (2007). Isolation and Characterization of Chromium –Resistant Bacteria from Tannery Effluents. Environmental contamination and toxicology 58:(535-542).

Mehrotra S., Pandey P.K.,Gaur R.,Darmwal N.S (2009). The production of alkaline protease by a *Bacillus* species isolate.Biores.Technol. 67:201-203.

Nehete PN, Shah VD, Kothari RM (2006). Isolation of a high yielding alkaline protease variant of *Bacillus licheniformi,* Enzyme Microb Technol; 8:370–72.

Nilegaonkar, V.P. Zambare, P.P. Kanekar, P.K. Dhakephalkar, S.S. Sarnaik (2007). Production and partial characterization of dehairing protease from *Bacillus cereus* MCM B-326, Bioresource Technology 98; 1238–1245.

Norazizah Shafee, Sayangku Norariati Aris, Raja Noor Zaliha Abd Rahman, Mahiran Basri and Abu Bakar Salleh (2005). Optimization of Environmental and Nutritional Conditions for the Production of Alkaline Protease by a Newly Isolated Bacterium *Bacillus cereus* Strain 146, Journal of Applied Sciences Research 1(1): 1-8.

Oberoi R., Q.K.Beg, S.Puri, R.K.Sexena and R.Gupta. (2001). Characterization and wash performance analysis of an SDS –stable alkaline protease from *Bacillus* species, World .J. microbial.Biotechnol 17:493-497.

P.P. Kanekar, S.S. Nilegaonkar, S.S. Sarnaik, A.S. elkar (2002). Optimization of protease activity of alkaliphilic bacteria isolatedfrom an alkaline lake in India, Bioresource Technology 85; 87–93.

Rajesh Patel, Mital Dodia, Satya P. Singh (2005). Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: Production and optimization, Process Biochemistry 40: 3569–3575.

Riffle, A., Ortolan, S., Brandelli, A., (2003). Dehairing activity of extracellular proteases produced by keratinolytic bacteria, J. Chem. Technol. Biotechnol. 78, 855–859.

Rohm, O, (1910). Dehairing and cleaning of skins, German patent No. 268; 873.

Saurabh Saran, Jasmine Isar, Rajendra Kumar Saxena (2007). A modified method for the detection of microbial proteases on agar plates using tannic acid, J Biochem Biophys Methods.; 70(4):697-9.

Sandhia G. S. and Jijesh M 2007.,Studies on the production of proteinase by bacillus subtilis mtcc 1790 in different low cost Media.

Thanikaivelan, P., J.R Rao, B.V.Nair and T.Ramasami 2004. Progress and recent trends in biotechnological methods for leather processing. *Trends Biotechnol*, 22:181-188.

Udandi Boominadhan, Rajendran Rajakumar,Palanivel Karpaga Vinayaga Sivakumaar and Manoharan Melvin Joe 2008.,Optimization of Protease Enzyme Production Using Bacillus Sp. Isolated from Different Wastes.

Standard methods for the examination of water and waste water, 21st edition, 2005, Published by American Public Health Association (APHA), American Water Works Association (AWWA) and Water Environment Federation (WEF),.

Takami H; Akiba, T and Horikoshi, K (2009). Production of extremely thermostable alkaline protease from *Bacillus sp*. No. AH-101, Appl. Microbial. Biotechnol. 30(2): 120- 124.

Tsai YC, Juang RY, Lin SF, Chen SW, Yamasaki M, Tamura G (2008). Production and characterization of an alkaline elastase produced by alkalophilic *Bacillus* strain Ya-B, Appl Environ Microbiol; 54:3156–61.

V. P. Zambare S. S. Nilegaonkar P. P. Kanekar (2007). Production of an alkaline protease by *Bacillus cereus* MCM B-326 and its application as a dehairing agent, World J Microbiol Biotechnol 23:1569–1574.

Wei-Hua Chu, (2007). Optimization of extracellular alkaline protease production from species of *Bacillus,* J Ind Microbiol Biotechnol, 34: 241-245.

Zamost BL, Brantley QI, Elm DD, Beck CM (2000). Production and characterization of a thermostable protease produced by an asporogenous mutant of *Bacillus stearothermophilus,* J Ind Microbiol; 5

APPENDICES

CURRICULUM VITAE

NAME : S.VIMALA DEVI

REGISTRATION No. : 1020203016

E-MAIL : seenuvimmi@gmail.com

MOBILE No. : 9994089093

PERMANANT ADDRESS: 1/58, Periyavallikulam,
Virudhunagar,
626004.

UNDERGRADUATION : B.TECH

Course : Biotechnology

College : Bharathidasan Institute of Technology

Year : 2010

GATE: Nil

NET: Nil

PARTICIPATION IN

I) Conference/Seminar: NATIONAL SEMINAR ON BIOTECH INSTITUTE INDUSTRY INTERACTION 2012

ii) Workshop : Nil

PUBLICATIONS : Nil

SIGNATURE

STUDIES ON OPTIMIZATION OF PROTEASE PRODUCTION USING BACTERIAL ISOLATE (*CLRI STRAIN 5468)* AND ITS APPLICATION IN DEHAIRING AND HYDROLYSIS OF TANNERY FLESHINGS

(Solid Waste Management)

ABSTRACT

A potent Bacterial isolate which produces protease was originally isolated, characterized in Biotechnology laboratory at CLRI. Among the seven different media tested for protease production, 1% gelatin in Nutrient Broth was supported maximal enzyme elaborated. The crude enzyme preparation shows the optimum pH and Temperature at 8 and 30°C respectively. The age of inoculum was also studied. Among two inhibitors EDTA was shown to be maximum inhibition. Then the enzyme was observed for the unhairing of skin and the disadvantage in chemical treatment was studied. The confirmation of unhairing was studied using histology studies. The tannery waste (solid fleshings) as it cannot be directly disposed off to the environment. It was treated with the microbial proteases. The hydrolysis of waste was done using proteases. The solid waste was converted to protein, fat and the salt matter. Future work is to optimize the cheap media for the production of the enzyme for large scale applications in various industries.