Isolation, Partial Purification and Characterization of Protease Enzyme from Bacteria for Tannery use

A Thesis

SUBMITTED BY

Md. Sharifull Islam
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Approved As To the Style and Content By

Supervisor

Md. Emdadul Islam
Associate Professor
Biotechnology and Genetic
Engineering Discipline
Khulna University, Khulna-9208
Bangladesh

Co-supervisor

Md. GahangirAlam
Assistant Manager
Duncan Brothers Bangladesh Limited
Camellia House
22,KaziNazrul Islam Ave.Dhaka-1000
Bangladesh

11/10/2015

Dr. Sheikh Md. Enayetul Babar
Chairman
Examination committee
&
Professor
Biotechnology and Genetic Engineering Discipline
Khulna University, Khulna-9208
Khulna.

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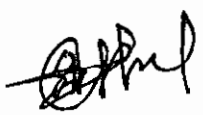
DECLARATION

This thesis report is the original research work of the author, except as otherwise stated. The material presented has not submitted either in whole or in part for a degree for this or any other university. The findings of the research has not been / will not be published anywhere without the concurrence of the concerned supervisor.



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ABBREVIATIONS

%	Percent
A.	*Aspergillus*
B.	*Bacillus*
°C	Degree in Celsius
et al.	And others
Fig.	Figure
Fuc	Fructose
G	Gram
Glc	Glucose
g/l	Gram per litre
hrs	Hours
L	Litre
Lac	Lactose
MW	Molecular weight
mM	Milimolar
min	Minutes
N	Normal
NB	Nutrient broth
nm	Nanometer
PEG	Polyethylene glycol
subsp.	Sub species
DNA	Deoxyribonucleic acid
EDTA	Ethaylene diamine tetraacetic acid
β-ME	β-Merceptoethanol
SDS	sodium dodosyle sulfate
$CaCl_2$	Calcium chloride
$ZnSO_4$	Zink sulfate
$Mg\ Cl_2$	Magnesium chloride
NaCl	Sodium chloride
KCl	Potassium chloride

TCA	Trichloro acetic acid
BSA	Bovin Serum Albumin
NaOH	Sodium hydroxide
HCl	Hydrochloric acid
Trise	Trisema base
EU	Enzyme unite
M	Molerity
O/N	Over night
kD	Kilodalton
ml	Mililiter
µl	Microliter
µg	Microgram
OD	Optical density
rpm	Revolution per minute
TSB	Trypticase Soya Broth
MR	Methyl Red
VP	Voges-proskaure
D/W	Distilled water
pH	Negative concentration of hydrogen ion
TSI	Triple Sugar –Iron
DMSO	Di methyl Sulfoxide
APS	Ammonium Per Sulphate
TEMED	N,N,N,N-Tetramethylenediamine Coomassie Brilliant Blu
BPB	Bromophenol blue
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
DEAE	Di ethyl amino ethyl

Introduction

Leather industry is one of the oldest industries in Bangladesh and plays a significant role in the national economy with a good reputation worldwide. In Bangladesh the leather industry is well established and ranked fourth in terms of earning foreign exchange. The leather products sector have huge opportunities in generating employment , entrepreneurship and investment by increasing export of higher value added products rather than finished leather and by utilizing locally made raw material (finished leather) to convert into more value added leather products (including footwear and other leather goods).[115]

In the process of leather manufacture, the noncollagenous constituents of raw hide are removed partially or completely in the various pre-tanning operations; where leather dehairing is one main operation and the extent of removal of these constituents decides the characteristics of the final leather. Conventionally chemicals(Lime, Sodium sulphide, Salt, etc) have been used for these pre-tanning operations. Since these chemicals are becoming problem for the environmental pollutions hence use of enzymatic treatments are also necessary to get optimum results without affecting environments. [114]

Most of the industries still use chemicals for dehairing which have environmental hazard.Use of protease enzyme for this purpose could be useful and no hazard for environment in this respect.Bacteria (psuedomonas sp. , bacillus,flavobacterium etc) is a good source of proteases.Most of the bacteria produce and secret protease as extracellular enzyme.Our study was to screen and identify a bacterial good source of protease focusing the activity of that protease enzyme in dehairing of leather.To perform this study we randomly collected soil sample where poultry waste (containig poultry feces) being dumped.We cultured and isolated different colonies, separated extracellular enzymes and confirmed presence of protease enzyme with azocasein test.

Bacterial colony which found good protease activity with azocasein test were selected and morphological and biochemical tests(catalase,oxidase,glucose fermentation etc) were carried out morphological and biochemical assay confirmed the colonies with best protease activity and it was pseudomonas sp. We therefore subcultured and enriched the population of pseudomonas sp.and conducted dehairing activityof the extracellular protease isolated from pseudomonas sp. We found significant dehairing activity of proteases isolated frompseudomonas sp. compairing with the activity of some other bacterial (Vibrio,

Bacillus,etc)proteases and conventional chemical dehairing method.We thereafter partially purified the protease from pseudomonas through amonium sulfate precipitation and DEAE cellulose column chromotagraphy and SDS PAGE which confirmed the presence of protease enzyme in compare with a molecular protien marker.This protease enzyme was characterized by investigating the effect of PH ,temperature,salt-ions and other effector mollecule.

The objective of this research work was

- Screening of high protease producing bacteria from soil sample.
- To study the de-hairing activity of protease enzyme isolated from the high protease producing bacteria

Review of Literature

2.1 Biochemical activity of microorganisms:

Extracellular Enzymes

a. Starch hydrolysis

b. Lipid hydrolysis

c. Caseinhydrolysis

d. Gelatinhydrolysis

Intracellular Enzymes

A. Carbohydrate fermentation

B .Litmus milk reaction

c. Hydrogen sulfide production

d. Nitrate reduction

e. Catalase reaction

f. Urease test

g. Oxidase test

h. IMViC test →

- Indole test
- Methyl red test
- VogesProskauer test
- Citrate test

When these tests are performed then the result can be incorporated in the table below to find out the genus of the bacteria.

This table of often-isolated chemoheterotrophic bacteria was put together in targeting likely names of genera to pin on the "nature isolates." An X marks the place where a certain pattern of characteristics matches up with a possible genus. Considering additional characteristics of the isolate, one can consult Bergey's Manual or The Prokaryotes for this genus and related genera (on nearby pages) for a more definitive identification. [112]

Table:1Probable identification chart of genus of unknown bacteria.[112]

Gram reaction (young culture)	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–	–
shape	coccus (clusters)	coccus (clusters)	coccus (chains)	coccus (tetrads)	rod	rod	irreg. rod	rod	rod	rod	rod	rod	rod	rod	rod	coccus (pairs)
aerobic growth	+	+	+	+	+	+	+	–	+	+	+	+	+	+	+	+
anaerobic growth	–	+	+	+	+	–	–	+	+	–	–	–	+	+	+	–
endospores	–	–	–	–	–	–	–	+	+	+	–	–	–	–	–	–
motility (Motility Medium)	–	–	–	–	–	+	–	+ or –	+ or –	+ or –	+ or –	+ or –	–	+	+	–
catalase reaction	+	+	–	–	–	+	+	–	+	+	+	+	+	+	+	+
benzidine reaction	+	+	–	–	–	+	+	–	+	+	+	+	+	+	+	+
oxidase reaction	+	–	–	–	–	–	–	–	+ or –	+ or –	+	+	–	–	+	+
glucose fermentation to acid or to acid+gas	–	+	+	+	+	–	–	+ (or –)	+	–	–	–	+	+	+	–
Glucose O/F Medium											–	O	F	F	F	O
Micrococcus	X															
Staphylococcus		X														
Streptococcus			X													

Lactococcus			X													
Enterococcus			X													
Leuconostoc			X													
Pediococcus			X	X												
Aerococcus				X												
Lactobacillus					X											
Kurthia						X										
Arthrobacter							X									
Clostridium								X								
Bacillus									X	X						
Alcaligenes											X					
Pseudomonas												X				
Klebsiella													X			
Shigella													X			
Salmonella														X		
Escherichia														X		
most other enteric genera														X		
Aeromonas															X	
Chromobacterium															X	
Neisseria																X

2.1.1 Bio-chemistry of hair with skin

The basic component of the skin is collagen, a fibrous protein. The latest research indicates That the basic collagen structure consists of twined triple units of peptide chains of differing lengths. The amino acid residues are joined together by peptide links. The peptides chains within the triple helices are held together by hydrogen bonding are as below

Peptide linkage
Amino Acid Glycine
Arginine side chain

There has been no conclusive evidence showing that the amino acid residues are arranged in any particular order within the peptide chain. Skin collagen is usually associated with keratin (the protein in hair, wool and nails). Most mammals have an outer coat of hair, wool or fur, which acts as an insulating layer and keeps the animal warm. Keratin is a fibrous protein and different from collagen in one very important aspect: the polypeptide chains are linked together by cystine linkages. The sulphur-sulphur linkage in cysteine is susceptible to the action of alkali, and breaks down quite readily in the presence of alkali and a reducing agent. The early part of leather production is the removal of hair from the skin, and the presence of thecystine linkage makes it possible for this to be acheived.[113]

The early part of leather production is the removal of hair from the skin, and the presence of thecystine linkage makes it possible for this to be acheived. [113]

2.2Preotease

Proteases (Proteinases, Peptidases or Proteolytic enzymes) as mentioned are enzymes that break peptide bonds between amino acids of proteins. The process is called proteolytic cleavage, a common mechanism of activation or inactivation of enzymes especially involved in blood coagulation or digestion. They use a molecule of water for this and are thus classified as hydrolases.[87, 88.90]

There are currently six classes of proteases:

1. Serine proteases
2. Threonine proteases
3. Cysteine proteases
4 .Aspartic acid proteases (e. g. plasmepsin)
5. Metalloproteases
6. Glutamic acid proteases

2.2.1 Occurrence

Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in multitude 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). Peptidases can break either 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 sign in a pathway. Protease split peptide bonds with water; they attack proteins via two modes, yielding different end products. Therefore, your choice of enzyme may be dictated by what you require as hydrolysis product. [87, 90]

On their modes of attack proteases are classified into two groups:

2.2.2 Exoprotease

These are proteases which can cleave off single amino acids from either end of the protein chain.Eventually under right conditions a protein can be reduced down to single amino acids[94].

2.2.3 Endoproteases

These are proteases which attack peptide bonds on the interior of the protein chain. The hydrolysis products are usually smaller polypeptides and peptides. Therefore most endoproteins will not produce a great deal of free amino acids as end products.[94]

2.2.4 Specificity

There are many different proteases, each with a different specificity toward protein substrates.[94]

2.2.5 Applications

Protease is a commercially important enzyme; it has a wide industrial application some of which are: 94

The greatest industrial use of protease is for laundry detergents where they help to remove protein based stains (such as blood and egg) from clothing.

The second largest use of protease is for cheese making. Enzymes from calf stomach and microbial sources are used to clot milk-one of the first steps in cheese making.

Proteases are also used for bating (softening) leather, modifying food ingredients (e.g. soy protein whipping agents), meat tenderizers, and flavor development

Proteases have also been studied for their role in blood clotting and inflammatory diseases.

2.2.6 Classification

Proteases have been classified on different criteria it can be on the bases of their sources, biochemical activity, and pH. On basis of pH Proteases are classified as: [(94)]

Acid Protease: These are proteases that show maximum activity at acidic PH.

Microbial Rennets: Highly specific endoproteases used to clot milk in cheese making.

Source: Mucormiehei.M. Pusillus, Endothiaparasitica

Renin: Highly specific endoprotease used to clot milk in cheese making

Source: Calf stomach

Pepsin: An endoprotease that will hydrolyze a broad range of synthetic peptides. Commercial products have exoprotease and esterase side activities.Pepsin prefers to cleave bonds near phenylalanine and leucine residues.

Source: Swine and bovine stomach

Fungal Acid Proteases: Hydrolyzes a wide range of peptide bonds. Preparations usually exhibit endo- and exo- protease activity.

Source: Molds- Aspergillus, Rhizopus

Neutral Protease: These are proteases that show maximum activity at Neutral pH.

Trypsin: Highly specific endoprotease which prefers to cleave bonds next to arginine and lysine residues.

Sources: Bovine and swine pancrease glands

Papain: Endoprotease that hydrolyzes a wide range of peptide bonds. It is a sulfhydryl protease requiring mild reducing agents.

Sources: Papaya latex

Bromelain/Ficin: Endoprotease that hydrolyze a wide range of peptide bonds and are similar to papain. Usually sold as a mixture of proteases.

Source: Pineapple and fig respectively

Bacterial Neutral Protease: An endoprotease that hydrolyses a wide range of peptide bonds.

Source: *Bacillus subtilis*

Alkaline Protease: These are proteases that show maximum activity at alkaline pH.

Subtilisin (and related protease): An endoprotease that hydrolyze a wide range of peptide bond preferences.

Source: *Pseudomonas sp* and others in this gene

2.3 Enzyme production

Some enzymes still extracted from animal and plant tissues. Enzymes such as papain, bromelain and ficin and other specialty enzymes like lipooxygenase are derived from plants and enzymes pepsin and rennin are derived from animal. Most of the enzymes are produced by microorganisms in submerged cultures in large reactors called fermentors. The enzyme production process can be divided into following phases:

1. Selection of an enzyme.
2. Selection of production strain.
3. Construction of an overproducing stain by genetic engineering.
4. Optimization of culture medium and production condition.
5. Optimization of recovery process.
6. Formulation of a stable enzyme product.

Criteria used in the selection of an industrial enzyme include specificity, reaction rate, pH and temperature optima and stability, effect of inhibitors and affinity to substrates. Enzymes used in the industrial applications must usually tolerant against various heavy metals and have no need for cofactors. [83, 98]

2.3.1 Microbial production strains

In choosing the production strain several aspects have to be considered. Ideally the enzyme is secreted from the cell. Secondly, the production host should have a GRAS-status. Thirdly, the organism should be able to produce high amount of the desired enzyme in a reasonable life time frame. Most of the industrially used microorganism have been genetically modified to overproduce the desired activity and not to produce undesired side activities.[83, 98]

2.3.2 Enzyme production by microbial fermentation

Once the biological production organism has been genetically engineered to overproduce the desired products, a production process has to be developed. The optimization of a fermentation process includes media composition, cultivation type and process conditions. The large volume industrial enzymes are produced in 50-500 m^3fermentors. The extracellular enzymes are often recovered after cell removal (by vacuum drum filtration, separators or microfiltration) by ultra-filtration.[83, 98]

2.3.3 Protein engineering

Often enzymes do not have the desired properties for an industrial application. One option is find a better enzyme from nature. Another option is to engineer a commercially available enzyme to be a better industrial catalyst. Another option is to engineer a commercially available enzyme to be a better industrial catalyst.

Two different methods are presently available: a random method called directed evaluation and a protein engineering method called rational design.[83, 98]

2.3.4 Enzyme technology

This field deals with how are the enzymes used and applied in practical processes. The simplest way is to use enzymes is to add them into a process stream where they catalyze the desired reaction and are gradually inactivated during the process. This happens in many bulk enzyme applications and the price of the enzymes must be low to take their use economical.

An alternative way to use enzymes is to immobilize them so that they can be reused. Enzyme can be immobilized by using ultra filtration membranes in the reactor system. The large enzyme molecule cannot pass through the membrane but the small molecular reaction products can. Many different laboratory methods for enzyme immobilization based on chemical reaction, entrapment, specific binding or absorption have been developed.[98]

2.4 Large scale Enzyme applications

2.4.1 Leather

Leather industry uses proteolytic and lipolytic enzymes in leather processing. Enzymes are used to remove unwanted parts. In dehairing and dewooling phases bacterial proteases enzymes are used to assist the alkaline chemical process. This results in a more environmentally friendly process and improves the quality of the leather. Bacterial and fungal enzymes are used to make the leather soft and easier to dye.[98]

2.4.2 Enzymes in DNA-technology

DNA-technology is an important tool in enzyme industry. Most traditional enzymes are produced by organisms, which have been genetically modified to overproduce the desired enzyme. The specific order of the organic bases in the chain of DNA constitutes the genetic language. Genetic engineering means reading and modifying this language. Enzymes are crucial tools in this process.[98]

2.4.3 Lipase based reactions

In addition to detergent applications lipases can be used in versatile chemical reactions since they are active in organic solvents. Lipases used in transesterification and also used for enantiomeric separation of alcohols and separate racemic amine mixtures. Lipases have also been used to form aromatic and aliphatic polymers and in leather industries.[98]

2.4.4 Other applications

The other applications of enzymes are in detergents, starch hydrolysis and fructose production, drinks, textiles, animal feed, baking, pulp and paper, analytics, fine chemical production, personal care, oligosaccharide synthesis etc.[98]

2.5 Leather industry: A major field for dehairing bacterial enzyme application

Leather industry contributes to one of the major industrial pollution problems facing the country, and the pollution causing chemicals, viz. lime, sodium sulphide, salt, solvents, etc. arise mainly from the pre-tanning process

es of leather processing. In order to overcome the hazards caused by the tannery effluents, use of enzymes as a viable alternative has been resorted to in pre-tanning operations such as soaking, dehairing, bating, degreasing and offal treatment. This review focuses on the use of microbial enzymes as an alternate technology to the conventional methods, and highlights the importance of these enzymes in minimizing the pollution load[99].

Environmental pollution has been a major irritant to industrial development. Chemical and chemical-based industries are the prime targets of the environmentalists for their crusade against pollution, and leather industry has also not been left out of the reckoning. The generation of pollution is significantly high in the pre-tanning operations compared to the post-tanning operations[1]. The chemicals mainly responsible for pollution in pre-tanning processes are lime, sodium sulphide, and caustic soda apart from common salt and degreasing chemicals. In fact, one third of the pollution caused by the leather industries results from the wastes generated during dehairing operations[2]. The wastes from the tanneries are let out into the drains which in turn empty into the main sewerage causing hazard to those who use this water. Many tanneries have been forced to close down because of their noncompliance with the standards laid down. In a short span of time, Indian leather industry has faced serious challenges such as German ban on pentachlorophenate, certain azo dyes, formaldehyde, etc. on one hand, and court order for compliance with environmental regula-tions on the other[3]. The attention of tanners is focused towards revamping the processing methods, recovery systems, and effluent treatment techniques to make leather processing eco-friendly. Intensive efforts are being directed towards using a viable alternative technology for pre-tanning processes using enzymes[4, 5].This could be one of the ways of solving the industrial pollution problems resulting from tannery effluents.

2.5.1 Conventional leather processing

The raw hide has to undergo a series of chemical treatments before it turns into flattering leather. This includes soaking, liming, dehairing, deliming, bating, degreasing, and pickling[1]. For all these steps, the chemicals used are quite toxic. Thus due to these pretanning operations, the leather processing industry is one of the worst offenders of the environment.

Besides chemical treatment, certain enzymatic treatments are also necessary to get optimum results. One such treatment, bating, is the only step in leather processing where enzymatic process cannot be substituted by chemical processes. The process of bating gives certain desired characteristics to the finished leather. Earlier, the process was carried out using dog dung or manure[6]. The use of this was not only unhygienic but fermentation could also not be controlled.

In pre-tanning operations, the hides and skins are first subjected to a water soak. For loosening the hair[7], the oldest method is the 'sweating' process – a natural autolysis or breakdown process. It is a mild putrefaction process induced at random. Since the type and quantity of the putrefying bacteria cannot be controlled, the process itself eludes control. Moreover, since the sensitivity to attack the epidermal proteins and the fibrous proteins of the corium by the proteolytic enzymes is more or less the same, the sweating may result in serious damage to the hide surface. Dehairing is used to be followed by opening up of fibre structure in 'liming'. The dehaired hide is transferred to an alkaline solution of lime milk where swelling occurs and the nonfibrillar proteins are dissolved. After mechanical removal of the subcutaneous tissue, deliming is performed in order to remove the adsorbed lime from the hide and to eliminate the lime swell.[99]

The fat present in the hide skins is removed either as soluble lime soap or hydrolysis products like fatty acids. Kerosene, chlorinated hydrocarbons, and white spirit are used in the degreasing system which adds to the toxicity of the environment and effluents[1].

The various steps of the pre-tanning processes of leather manufacture are shown in Figure1.

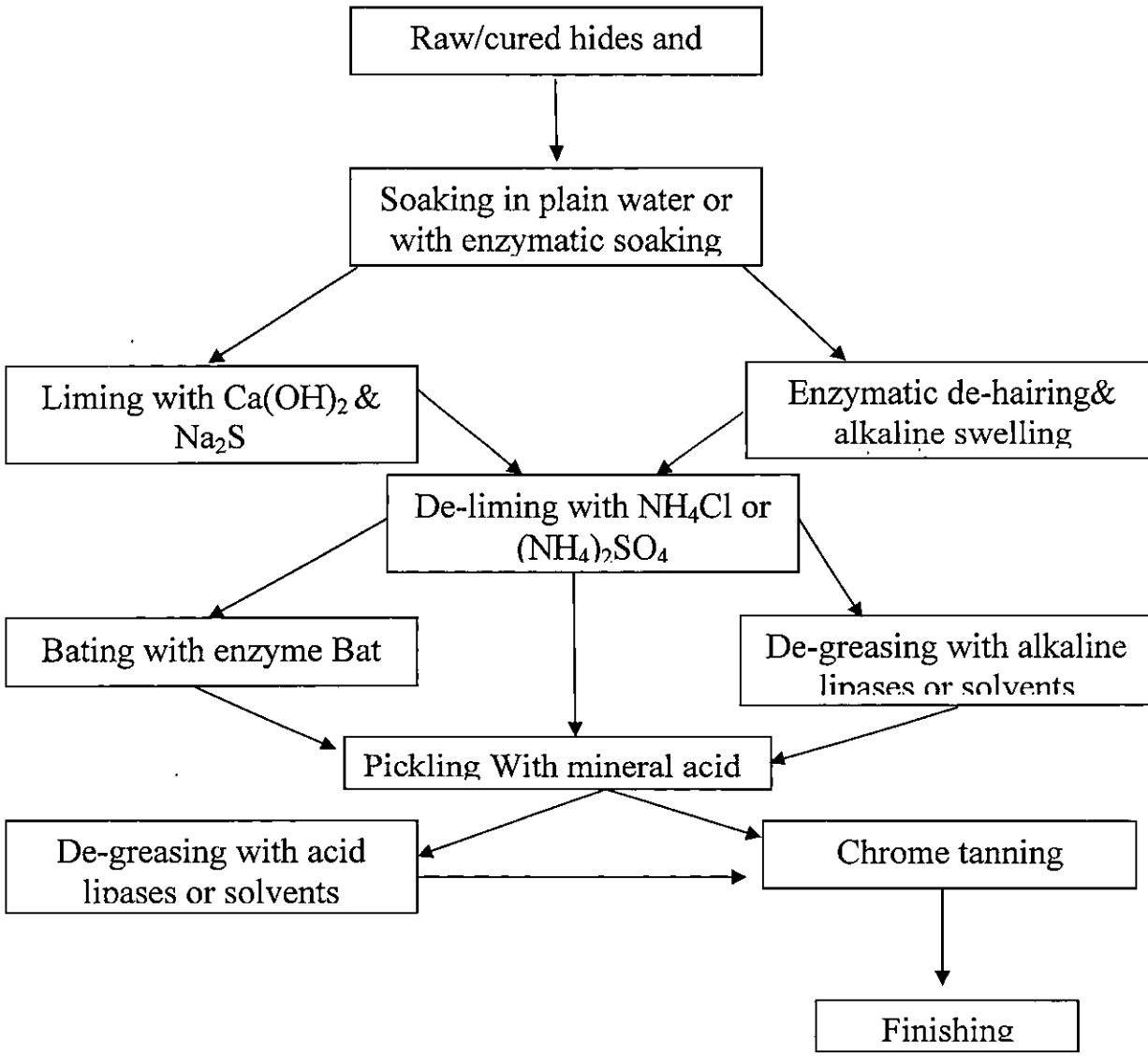

Figure 1: Flow chart of leather processing[99]

2.5.2 Enzymes in pre-tanning

An important enzyme used in pre-tanning processes belongs to the group of proteolytic enzymes, proteases. Obtained by microbial fermentation, the proteases are meant for use in the leather industry for dehairing, bating and soaking processes, and in the detergent industry for breaking down proteinaceous matter caused by body secretions, food stuffs, and blood[8]. The main advantages of the use of enzymes are specificity, stereospecificity, activity under mild conditions, and possibility of producing 'natural' products, nonpollutants, and biodegradability[9].Animal proteases and microbial proteases from bacteria and fungi are used in the pre-tanning processes of leather manufacture

.The animal proteases are mixtures of trypsin, chymotrypsin, and various peptidases which may contain amylase or lipase as secondary enzymes. Mainly for economic reasons, enzymes from microorganisms have come to play a significant role in recent years and enzyme products of microbial origin are already being produced on a wide scale[11].

Since microorganisms can be made to propagate rapidly and profusely, they are an ideal source for enzymes[12]. Mainly, neutral and alkaline proteases are obtained from bacteria, which differ in their pH activity range. Fungal proteases are also classified according to the pH activity range[13]: fungal acid proteases act between pH 2.5 and 6.0 and can be derived from *A. satoi*.

These are used for bating prior to pickling and serve to open up the fibre structure. Fungal alkaline proteases[14] belong to the same group of serine proteases as alkaline bacterial proteases. However, these are more heat sensitive and are quickly deactivated above 60° C. Fungal neutral proteases are mainly obtained from *Aspergillus* or *Penicillium* species

Table 2 shows the various enzymes produced by various microorganisms used in the leather industry.

Table 2: Enzymes used in pretanning operations[99]

Process	Enzyme	Microorganism
Soaking	protease	*Aspergillusparasiticus, A.flavus, A.oryzae, Bacillus subtilis*[19], *Rhizopus rhizopodiformis*[24]
	carbohydrase	*Aspergillus awamori*[30]
Dehairing	protease	*Pseudomonas, Aspergillus flavus*[33,35], *Aspergillus sp*[34]. *Bacillus subtilis*[41], *Lactobacillus sp*[42]. *Conidiobolus sp.*[34], *B. amiloliquefaciens*[64], *Streptomyces griseus, S. fradiae*[65], *S. moderatus*[6]
Bating	protease	*Pesudomonas, A. parasiticus*[67,68], *S. rimosus, B. licheniformis*[48], *Bacillus subtilis*[60], *Penicillium janthinellum*[70]
Degreasing	Lipase	*Rhizopus rodosus*[49], *A. oryzae and A. flavus*[71]

Apart from bacterial and fungal proteases, specific proteases like keratinases[15] are known. Keratinases which hydrolyse keratins are obtained from *Streptomyces fradiae* and can be used for dehairing[10]. Some of the important lipase-producing microorganisms used in degreasing are shown in Table 2. Lipases are used (i) in the oil and fat industry to modify fats for use in foods; (ii) in detergent compositions; (iii) for fatty acid production, lipid synthesis via reversal of hydrolysis and lipid modification by interesterification, and (iv) in degreasing of hides and skins[16].

2.5.3 Enzymes in soaking

Soaking is the first operation in the tannery wherein the hides and skins are cleaned and softened with water[17]. Wet-salted or freshly slaughtered hides and skins do not require any chemical agent for their proper soaking[4]. Soaking is necessary for solubilization and elimination of salts and globular proteins contained within the fibrous structure of hides and skins. It is carried out under alkaline conditions at low temperature between 10° C and 20° C in water treated with antiseptics such as sodium hypochlorite, sodium pentachlorophenate, formic acid, etc.[1]. It is accelerated by some of the nonionic detergents and additives such as sodium sulphide or sodium tetrasulphide.

The advantages of enzymatic soaking include loosening of the scud, initiation of the opening of the fibre structure, and production of leather with less wrinkled grain when used at an alkaline pH of less than 10.5 (ref. 6). Use of enzyme preparation in soaking of rabbit skins improves the softness and elasticity, and increases the area yield of the fur by 3.3% while reducing the processing time by 10–20 h (ref. 18).

Soaking is usually carried out using a combination of proteolytic enzymes that are optimally active in the neutral or alkaline pH range. For enzymatic soaking, the average soaking period for salted raw stock is about 4 h and for dried raw stock is about 8–10 h (ref. 26). A water soak without auxiliary agents takes 24 h for salted hides, and 36–48 h for dried hides.

2.5.4 Enzymes in dehairing

Dehairing is one of the main operations in the beamhouse. Five methods of dehairing are generally adopted, viz. (i) clipping process, (ii) scalding process, (iii) chemical process, (iv) sweating process, and (v) enzymatic process[1]. 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.

The major disadvantages of chemical dehairing areas follow:

1. It contributes in no small measure to the pollution load. Beamhouse processes generally account for 70–80% of the total COD of effluent from all leather making processes. About 75% of the organic waste from a tannery is from the beamhouse and 70% of this waste is from hair which is rich in nitrogen. These figures clearly illustrate the contribution made by the lime and sulphide process towards pollution[27].
2. Sulphide is highly toxic with obnoxious odor. If left untreated, it can cause major problems in the sewers.
3. 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[6]. The enzyme digests the basal cells of the hair bul band the cells of the malphigian layer. 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[28].

Advantages of enzymatic dehairing are:

i. Significant reduction or even complete elimination of the use of sodium sulphide.
ii. Recovery of hair of good quality and strength with a good saleable value.
iii. Creation of an ecologically conducive atmosphere for the workers.
iv. Enzymatically dehaired leathers have shown better strength properties and greater surface area.
v. Simplification of pre-tanning processes by cutting down one step, viz. bating.
vi. A significant nature of the enzymatic dehairing process is the time factor involved. The lime-sulphide process takes about 16 h, whereas the enzymatic dehairing would be also completed between 12 and 20 h (ref. 29).

Proteolytic enzymes are of great commercial importance, contributing to more than 40% of the world's commercially produced enzymes[30]. Approximately 50% of the enzymes used as industrial process aids are proteolytic enzymes[31]. Proteolytic enzymes are more efficient in enzymatic dehairing than amylolytic enzymes[1].

Microbial proteases are derived from a wide variety of yeasts, molds, and bacteria[32]. Yeast proteases are mainly intracellular in nature and therefore these enzymes have not gained significant commercial interest. The protease from *A. flavus* was earlier being used for dehairing, and later it was reported that simultaneous dehairing and bating is possible with the protease of *A. flavus*[33]. Gillespie[34] has observed that the enzyme preparation from cultures of *A. oryzae*, *A. parasiticus*, *A. fumigatus*, *A. ochraceus*, *A. wentii*, and *P. griseofulvum* exhibit marked depilatory activity on sheep skins.The use of this enzymatic depilation process completely eliminates the use of sulphide, a toxic pollutant.

The fungal culture, *Conidiobolus* sp., isolated at NCL, produces high yields of extracellular alkaline protease[36]. The enzyme is active at pH 10.0 and is being tried for many industrial applications.

Enzymes derived from bacteria have gained much commercial interest[37, 38] because of their easy production capabilities by submerged cultivation, high yield of enzyme, short duration for production, and easy recovery of the enzyme.

Proteolytic enzymes derived from a large number of *Pseudomonas* sp. And *Streptomyces* sp. Have been used in dehairing of hides and skins[39,40]. A lime and sulphide-free process of dehairing has been developed for the manufacture of suede from sheep skins using protease from *Pseudomonas sp.* Schlosser *et al.*[42] have reported a method of depilation in an acid medium containing *Lactobacillus* culture.

In dehairing, the hair loosening is effected at pH 10.0 using fungal or bacterial enzymes; the treatment period being approximately 12–16 h, followed by hair removal using mechanical means[10]. The treatment period can be substantially reduced if the enzyme solution is fed in from the flesh side under pressure[43]. Enzymatic hair loosening processes play a role wherever high-quality hair, wool or bristles are to be recovered.Three methods of application are commonly used in the enzymatic dehairing process: (i) paint method, (ii) dip method, and (iii) spray method.

2.5.5 Enzymes in bating

Bating is a very important process in which enzymes have been successfully employed for centuries. The concept of softening hides by treating them in a warm infusion of animal dung has been termed as 'bating' and the product used for such process is known as a bate. The main object of bating is to remove some of the nonleather-forming proteinous materials like albumins, globulin, and mucoids from hides and skins, and to allow splitting up of collagen fibres to facilitate the penetration of tanning materials and other processing chemicals, thereby giving the finished leather the desired characteristic properties like feel, softness, pliability, etc.[1].

Deliming and bating, the subsequent steps in the processing of the pelts after liming are really two separate operations although they are usually carried out in one step and often overlap each other. The principal materials which a bate contains are a proteolytic enzyme, a carrier for the enzyme like wood flour, and a suitable deliming agent like ammonium chloride or sulphate or both. The deliming agents are used for the removal of lime salts which are used during the dehairing process.

The comparatively richer source for the proteolytic enzyme is the pancreas from bovine and pig. The proteolytic enzymes in the pancreas are present in inactive forms; chymotrypsin as chymotrypsinogen, trypsin as trypsinogen, and carboxypeptidase as procarboxypeptidase. A process has been patented for the activation of pancreatic enzymes by the use of acid protease from *A. fumigatus*[45].A combination of both mold and pancreatic enzymes in suitable proportions will be an ideal bate for different types of leather.

In bating, pancreatic enzymes are used in combination with neutral and alkaline bacterial or fungal proteases. After loading the drum with the pelts, the float is fed in at 35–37° C and, then, the bating agent containing enzyme, ammonium salts and carrier material is added.

2.5.6 Enzymes in degreasing

Degreasing is an essential step in the production of glove and clothing leather. In this process there is removal of excess natural fats from greasy skins. The presence of natural grease in raw hides and skins, especially woolly sheep skins, results in various defects, viz. fatty spues, uneven dyeing and finishing, waxy patches in alum-tanned leathers, and pink stain on wet blues[1]. During the degreasing operation in the pretanning process, the fat or grease is removed from the interfibrillary spaces of the skins to facilitate the even penetration of tanning materials, fat liquors, and dyes, etc. Degreasing helps to obtain soft and pliable leather for garment manufacture.

Degreasing is carried out after pickling, using aqueous emulsification with detergents, or by solvent extraction. It is well known that organic solvents like kerosene, petrol, perchloroethylene and trichloroethylene are highly unsafe and hazardous to the workers and heavily pollute the environment. The detergents, though not hazardous while handling and storing, cause serious pollution problems. These detergents and solvents add to the BOD load of the pickling effluent, and the chlorinated hydrocarbons and solvents add to the toxicity of the effluent[49].

Enzymatic degreasing is suggested as a viable alternative to combat the pollution problems caused by the use of solvents and detergents. Lipases which are projected as alternatives for solvents and detergents, catalyze the breakdown of fats and can be obtained from animal, microbial and plant sources. The advantages of using enzymes for degreasing are the elimination of solvents, reduction in surfactants, and possible recovery of valuable by-products. The disadvantages are that the lipases do not remove all types of fats in the same way that solvents do, and they add cost to the process.

Enzymatic degreasing can be carried out with acidic or alkaline lipases of fungal or bacterial origin. For degreasing, pickled pelts are kept immersed in an enzyme bath containing microbial lipase and water pH of 3.6, and left in the same bath overnight at a temperature of 28–32° C. The degreased pelts are then removed from the bath and subjected to salt wash twice with water and common salt for 40 min. The washed pelts are repickled, chrome tanned and taken for further processing[50]. The use of an alkaline lipase at a pH of 9.0 to 9.3 in the degreasing of pig skin results in short degreasing time and high degreasing efficiency[58].

2.5.7 Enzymes for by-products utilization and effluent treatment

Enzymes could be used in the treatment of fleshings and effluent from tannery processes. A combination of hydrolytic enzymes, viz. proteases, carbohydrases, and lipases would be required. The advantages to be release include a protein by-product suitable for animal feed as well as energy conservation and fat recovery. Again, the major disadvantage would be the cost[6].

When raw hides are processed to leather, a number of by-products such as native hide material (claws, tails, necks, fleshings), pelt waste (trimmings, machine fleshings, gluestock, pelt cuts), and tanned material (shavings, leather cuts, buffing dust, chrome cuttings) are obtained[10].The problem of waste treatment can be approached (i) by getting rid of the pollution by proper effluent treatment, and (ii) by controlling pollution occurring at different stages of leather manufacture[2]. Biotechnology plays an important role in tannery effluent treatment. The secondary treatment of tannery effluents, which relies on living organisms, is normally by anaerobic lagoons and aerobic lagoons. Open waste-ponds or anaerobic lagoons are installed in few south Indian tanneries where the atmospheric temperature (20–40° C) is suitable for this operation. In these ponds, microorganisms which thrive in oxygen-less environments are allowed to digest the waste. Anaerobic lagoons can be used for cleaning wastes coming from both the vegetable tanning and chrome tanning procedures.

Closed type anaerobic systems are useful for tanneries situated in cold temperatures (5–10° C). Aerobic lagoon is a shallow water-tight pond of about 2–3 m depth. The wastes are kept for about a week. Fixed or floating type surface aerators blow oxygen or air into these for helping growth of organisms. This system requires less land and is economical for larger tanneries located in urban areas.

The necessity for chromium removal in tannery waste water is another area of waste management[2]. Microorganisms such as *A. fumigatus* and species of *Pseudomonas* when grown on chrome waste can 'leach' out chromium. Pentachlorophenol, a preservative used for raw as well as semi-processed skins, creates problems during handling and also during biological effluent treatment. *P. aeruginosa* could be used successfully to degrade pentachlorophenol[2]. Other potential techniques for reduction of pollution load are recycling of immobilized enzymes to hydrolyse the solid waste, and recycling of immobilized whole cells to absorb or detoxify toxic metals in the effluent.

Besides this the microscopic analysis of the pelts treated with enzymes showed that the epidermis is completely removed, appendages are completely absent, are absent sebaceous glands and there is no hair in the follicles. In the control, epidermis and hair are intact .Also there is indication of fibre structure being opened up moderately with the fibre bundles split to a medium extent .When hair remained in follicles it was observed that the outer root sheath was released from dermis or was destroyed. Collagen was not damaged its structure appears to be not modified in both control and treated pelts. Elastic tissue may become disorganized, but is still present and is not removed during depilation.

Table 3: Microscopic characteristics of the pelts de-haired by lime-sulfide and enzymatic methods

Enzymatic method	Lime-sulfide method[95,96]
Epidermis fully removed and hair roots and epidermal appendages absent	Epidermis completely removed and fine hair present
Grain layer fully merged into corium	Merging of the grain into the corium is fair
Skin structure partially opened up	Skin structure completely opened up
Fibre bundles were compactly woven horizontally and split a small amount	Fibre bundles were loosely woven and split to a medium extent without any separation

A protease is any enzyme that conducts proteolysis, that is, cuts through hydrolysis the peptide bonds that link amino acids together to form proteins.

Currently, proteases are classified on the basis of three major criteria, type of reaction catalyzed, chemical nature of the catalytic site and evolutionary relationship with reference structure. Alkaline proteases are referring to proteolytic enzymes which work optimally in alkaline pH (78, 79). The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications, e. g. food and feed industry, peptide synthesis, leather industry, management of industrial household waste, photographic industry, medical usage, silk gumming and detergents industry. Alkaliphiles are defined as organisms that grow optimally

at alkaline pH, with pH optima for growth being in excess of pH 8 (usually between 9 and 10), and some being capable of growing at pH > 11. Although they were once considered to be curiosities, awareness of alkaliphiles has blossomed in recent years due to an interest in their physiological adaptation to high pH and their potential uses in biotechnological applications. Soda lakes and soda deserts represent the major types of naturally occurring highly alkaline environments, in which the indigenous micro flora is subjected to number of extreme ecological pressures. They represent the most stable high pH environments on Earth, where large amounts of carbonate minerals can generate pH values >11.5.

2.6 Inhibitors of proteases

There are different kinds of biochemical and chemical substances that act as protease inhibitors. These inhibitors inhibits enzymatic reactions by different mode of action. These inhibitors may be competitive or noncompetitive in nature. The chemical inhibitors are PMSF, DMSO, SDS, EDTA, Methanol, Ethanol, β- Mercaptoethnol, Merhiolate etc.[93]

The function of proteases is also inhibited by protease inhibitor enzymes. Examples of protease inhibitors are the class of serpins (serine protease or peptidase inhibitors), incorporating alpha 1-antitrypsin. Other serpins are complement 1-inhibitor, antithrombin, alpha 1-antichymotrypsin, plasminogen activator inhibitor 1 (coagulation, fibrinolysis) and the recently discovered neuroserpin.[93]

Natural protease inhibitors include the family of lipocalin proteins, which play a role in cell regulation and differentiation. Lipophilic ligands, attached to lipocalin proteins, have been found to possess tumor protease inhibiting properties. The natural protease inhibitors are not to be confused with the protease inhibitors used in antiretroviral therapy. Some viruses, with HIV among them, depend on proteases in their reproductive cycle. Thus, protease inhibitors are developed as antiviral means.[93]

Materials and Methods

3.1 Apparatus

i. Balance

Weighing of all chemicals was carried out in an analytical electrical balance (Model: AS 60(S) OHAUS analytical standard, Ohaus Corporation, USA) and a top pan balance (Model: CT-200v, OHAUS portable advanced, Ohaus Corporation, USA).

ii. pH meter:

PH was measured using a digital pH meter (Model: 3305, JENWAY LTD, UK)

iii. Sterilization

All types of media, tips, petridish, flasks, and screw capped test tubes were sterilized at 121°C, 15lbs. Psi for 30 minutes in an autoclave (Model: Hirayana Manufacturing company, Japan). Some other glass wares (pipette, conical flask, measuring cylinder, screw capped tube, etc.) were sterilized in hot box oven (Gallenkamp, Hot box oven, England) at 200°C for 2 hours.

iv. Centrifuge

To collect the supernatant from cultured liquid media centrifugation is carried out in a sorvall RC 26 plus, refrigerated super speed centrifuge, USA.

v. Shaker incubator:

For incubation, psychrotherm incubator shaker, manufactured by New Bronswick Scientific Company, N.J was used.

vi. Incubator:

Bacterial cultures were incubated in an (Gallenkamp, Economy incubator size-1, England) incubator for overnight growth.

vii. Vortex mix:

For all types of mixing, a vortex mixer (SpinmixGallenkamp, U.K) was used.

3.2 Sample collection and streaking plates for isolation of single colonies

This was the toughest job that took long time and hard work. Soil sample from different areas of Savar were screened for bacteria efficient in dehairing hides & skins of cattle. Then sample was taken in a petridish and diluted in distilled water by a glass rod. After a few minutes when the soil settled down, the surface water (which contained the soil bacteria) was taken very carefully to liquid broth media by micropipette for overnight growth. Then the bacterial culture was diluted serially up to 10^{-6} times with distilled water and spread in an agar plate.

For many experiments involving bacteria, it is imperative to use a bacterial culture in which all cells are genetically identical. Because all cells in a colony develop from a single cell, colonies are excellent sources of genetically pure bacterial stocks. Single isolated colonies can be obtained by streaking small volumes of a liquid culture onto a.Petri plate containing nutrient media.

During the procedure, single bacterial cells are spatially isolated from each other on the surface of the media. These cells then developed into isolated colonies that can be picked off the plate using a wire loop or any sterile instrument. Streaking for isolation of single colonies was performed using a wire inoculating loop that is sterilized before and during the streaking procedure

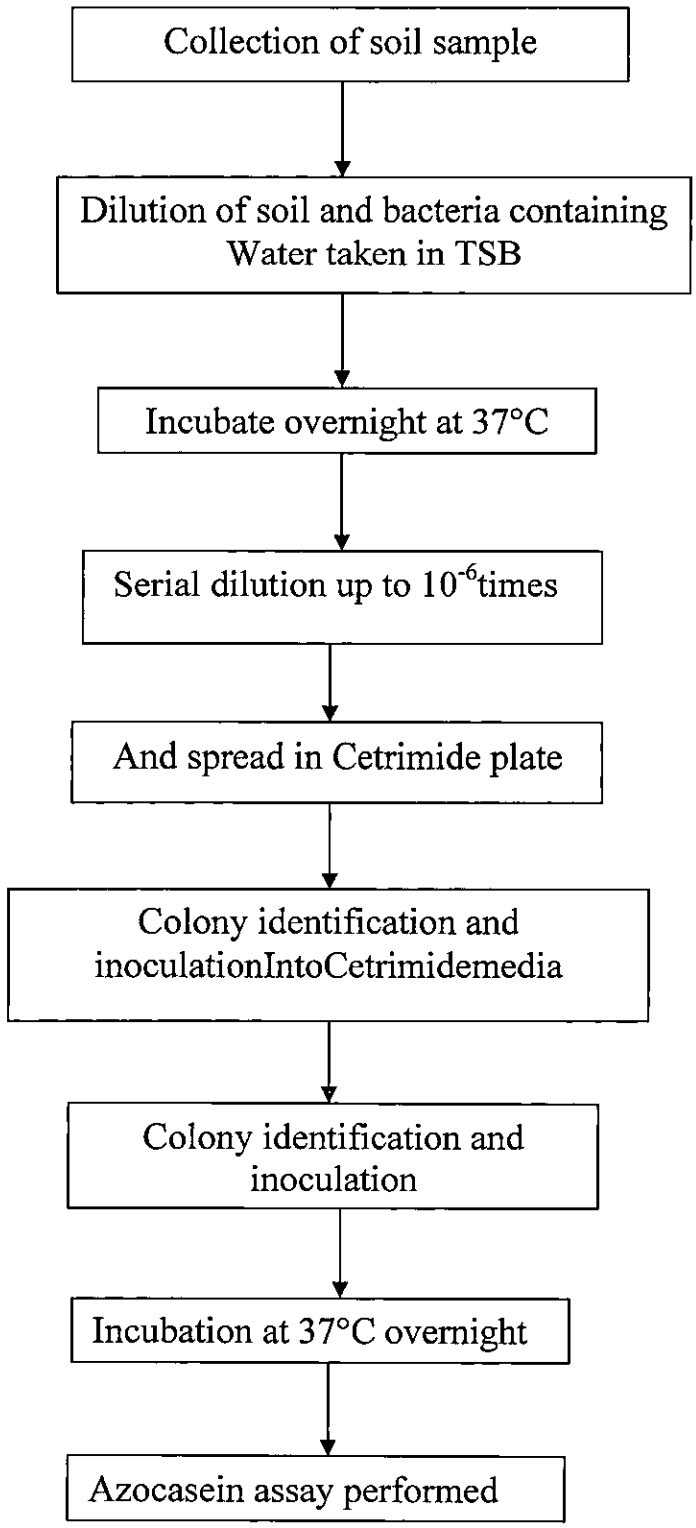

Figure 2: Flow chart of isolation of azocasein degrading bacteria from soil

3.3 Azocasein test

Azocasein test is a quantitative assay to measure the proteolytic activity described by Kreger and Lockwood (1981). Here azocasein is used as a substrate. For this test, the fresh bacterial culture is needed. 1.5ml culture was taken in an eppendorf tube and centrifuged at 4000 rpm for 5 minutes. Then 400 µl supernatant was taken and mixed with 400 µl 1% azocasein (pH 8.0) for the test sample. For control, 400 µl supernatant was mixed with 135 µl 35% TCA and incubated for 2-3 minutes. And then 400 µl 1% azocasein was mixed with the control solution. Then both test and control solution was incubated at 37°C for 1 hour.

After incubation, 135 µl 35% TCA was mixed with only test sample. Then both test and control solution was freezing at 4°C for 15 minutes. Then the solutions were centrifuged at 14000 rpm for 7 minutes. Then 750 µl

Supernatant was taken and mixed with 750 µl freshly prepared 1N NaOH. Then absorbance was measured at 440nm.

Enzyme unit:

One unit of proteolytic activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.01 at 440nm.

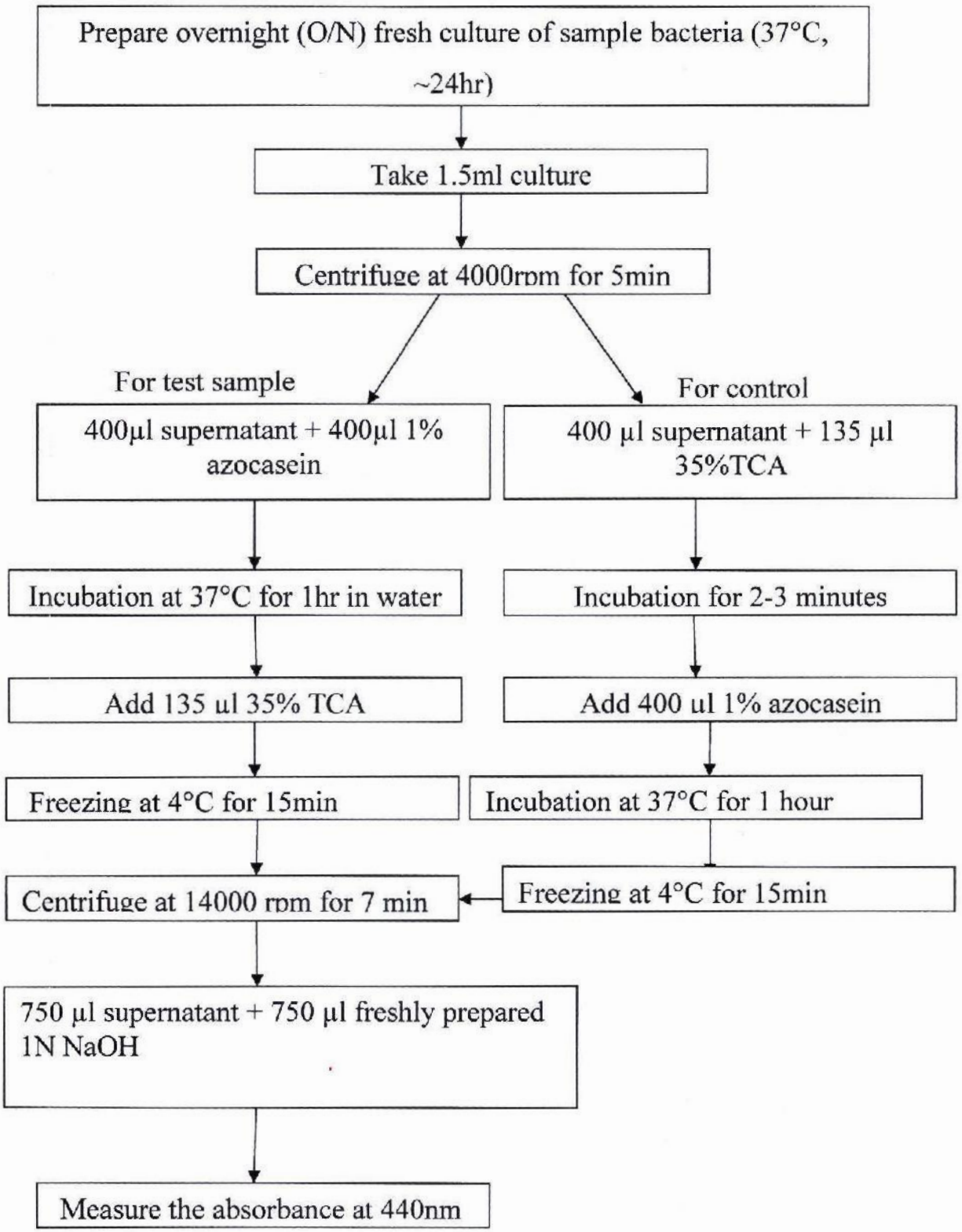

Figure 3: Flow chart of Azocasein test

3.4 Microbiological Examination

3.4. 1 Gram staining for the Bacteria

Staining materials (see appendix)

Procedure

A drop of sterilized distilled water was taken on the middle of the clear slide. Then a loopful bacterial suspension (young culture) was transferred to the sterilized drop of water and a very thin film was prepared on the slide by spreading uniformly. The film was fixed by passing it over the gentle flame for two or three times. The slide was flooded with crystal violet solution and allowed to stand for 30 sec and then washed thoroughly with gentle stream of tap water. The slide was then immersed in iodine solution for 1 minute and washed thoroughly with 95% alcohol for 10 sec. Alcohol was drained off and washed thoroughly with gentle stream of tap water. The slide was then covered with safranin for I minute. After washing with tap water and blotted dry it and examined under microscope.

3.5 Biochemical Test

To identify the biochemical properties of the organism different tests were performed. For correct interpretation of the results in every test Escherichia coli was taken as control.

3.5.1 Carbohydrate Test

The carbohydrate utilization tests are designed to detect the change in pH which would occur if fermentation of the given carbohydrate occurred. Acids lower the pH of the medium which will cause the pH indicator (phenol red) to turn yellow. If the bacteria do not ferment the carbohydrate then the media remains red. If gas is produced as a byproduct of fermentation, then the Durham tube will have a bubble in it.

The carbohydrate tests that were performed are the:

- Glucose
- Lactose
- Sucrose

Media

0.5% of different sugars were added with carbohydrate basal media (see Appendix) for different sugars test. Durham tube was added to each test tube. The medium was sterilized at 121°C for 15 minutes.

Procedure

All carbohydrate test media were inoculated with organism with the transfer loop from 24-48 hours culture grown in TSB media (see Appendix).

3.5.2 Catalase Test

Media

TSB media was used for organism growth.

Procedure

Catalase Test carried out of one drop of 30% hydrogen peroxide was placed on a slide. One loopful of the fresh bacterial culture was taken by a sterile needle and placed on the drop of hydrogen peroxide. Bubble production indicated positive result.

3.5.3 Hydrolysis of Starch

Media

Starch media (see Appendix) was used.

Procedure

A loopful of fresh bacterial culture was picked up by the sterile needle and stabbed on to the agar plate; After 24 hrs of incubation at 37° C, the plate was flooded with dilute iodine solution. Hydrolysis of starch was indicated by a clear zone around the growth and unchanged starch gave a blue color.

3.5.4 Methyl Red VogesProskauer Test

Media

MRVP media (see Appendix) was used.

Procedure

These tests are performed by inoculating a single tube of MRVP media with a transfer loop and then allowing the culture to grow for 3-5 days. After the culture is grown, about half of the culture is transferred to a clean tube. One tube of culture was used to conduct the MR test, the second tube served as the VP test.

MR (methyl red) test

Methyl red solution (see Appendix) was added to the MR tube. A red color indicates a positive result (glucose can be converted into acidic end products such as lactate, acetate, and formate. A yellow color indicates a negative result; glucose is converted into neutral end products.

VP (Vogues Proskauer) test: First alpha-napthol also called Barritt's reagent A (see Appendix) and then 40% potassium hydroxide also called Barritt's reagent B (see Appendix) were added to the VP tube. The culture allowed sitting for about 15 minutes for color development to occur. If acetoin was produced then the culture turns a red color (positive result); if acetoin was not produced then the culture appears yellowish to copper in color (a negative result).

3.5.5 Modified MRVP Test or MRVP Saline Test

Media

MRVP saline media (see Appendix) was used.

Procedure

Same as MRVP test It is therecommended medium for the performance of the Methyl Red and Voges-Proskauer Tests in the differentiation of the coli-aerogenes group.

3.5.6 Indole Production Test

Media

SIM media (see Appendix) was used.

Procedure

This test is done to determine if bacteria can breakdown the amino acid tryptophan into indole. SIM media is inoculated using a transfer needle. After incubating the bacteria for at least 48 hours, Kovac's reagent (see Appendix) was added to the media to detect if indole had been made by the bacteria. The development of a red/pink layer on top of the media is a positive result (the bacteria can breakdown tryptophan to form indole). Failure to see a red layer is a negative result (indole was not formed from tryptophan).

3.5.7 Nitrate Reduction Test

Media

Nitrate broth media (see Appendix) was used.

Procedure

Nitrate reduction test was carried out in nitrate broth. The freshly prepared cultures were inoculated in sterile nitrate broth containing tubes and incubated at 37° C for 24 hrs. At the end of incubation 0.1 ml of solutions A (see Appendix) was added followed by solution B (see Appendix) in equal volume. The appearance of pink deep color showed that bacterial isolates reduced nitrate to nitrite.

3.5.8 Gelatin test

Media

Nutrient gelatin media (see Appendix) was used.

Procedure

To inoculate this media, transfer needle was used to stab the gelatin. After incubating the inoculated media for at least 48 hrs, the tubes were transferred into a refrigerator. If the media is solid after refrigeration then the test is negative (the bacteria did not digest gelatin). If the

media is liquefied even after refrigeration, then the test result is positive if the bacterium is able to digest gelatin.

3.5.9 Citrate Utilization Test

Media

Simmon's Citrate media (see Appendix) was used.

Procedure

For the Citrate Utilization Test, slope culture with a 1 inch butt of Simmon's citrate agar was inoculated by streaking over surface with a wire needle and incubated at 37° C for up to 3 days. The color of the medium changed from green to bright blue due to the utilization of citrate and when citrate is not utilized, the color of the medium remain unchanged.

3.5.10 Motility and H2S Production Test

Media

For this purpose SIM media was used.

Procedure

Simply the media was stabbing in as straight a line as possible and the needle was withdrawn very carefully to avoid destroying the straight line. After incubating the sample for 24-48 hours observations can be made. If migration away from the line of inoculation is evident then the test organism is motile (positive test). Lack of migration away from the line of inoculation indicates a lack of motility (negative test result).

3.5.11 Oxidase Test

Media

TSB media was used for growth of the organism.

Procedure

Using a sterile swab, a heavy inoculum of the bacteria was transfered to a slide. The slide was placed on a white paper and the oxidase reagent (see Appendix) was added. A positive reaction appears pink, then maroon and finally black.

3.5.12 Urease Test

Media

Urea broth (see Appendix) was used.

Procedure

24-48 hours culture grown in TSB media was inoculated massively into the urea broth. Incubation period was up to 48 hours. Then color change was observed.

3.6 Preparation of Subculture

A 250 ml conical flask containing 50 ml of sterile nutrient broth was generously inoculated with *Pseudomonas sp.* It was grown overnight in a psychrotherm incubated shaker incubator for overnight at 31°C. This was the subculture used for the preparation of batch culture. A set of 10 conical flasks each of 500 ml capacity containing 100 ml sterile nutrient broth was aseptically inoculated with 5 ml subculture in a laminar flow hood. The culture was incubated in a psychrotherm incubated shaker at a temperature of 31°C for 15-20 hours until the absorbance of growing culture reached 1.5-1.8. The culture was then centrifuged at 10000 rpm in a sorvall RC-26 plus refrigerated super speed centrifuge for 10 minutes at 4°C. The supernatant was collected and used as crude enzyme sample.

3.7 Partial purification of the enzyme

3.7.1 Culture of the Bacteria.

The culture of the bacteria in TSB media was allowed to grow over night (24 h). Proper aeration and shaking was ensured in a water bath shaker. The culture was then centrifuge at 7000rpm to separate the cell from the crude enzyme.

3.7.2 Ammonium Salt Precipitation

The crude enzyme was then saturated with ammonium sulphate. The collected precipitate was dissolved in 0.1M Tris-HCl (pH 8) buffer and allowd for dialysis.

3.7.3 Dialysis :

Dialysis was carried out to remove the ammonium salts in a cellophane bag for 8-12 hours using Trise-HCl buffer.

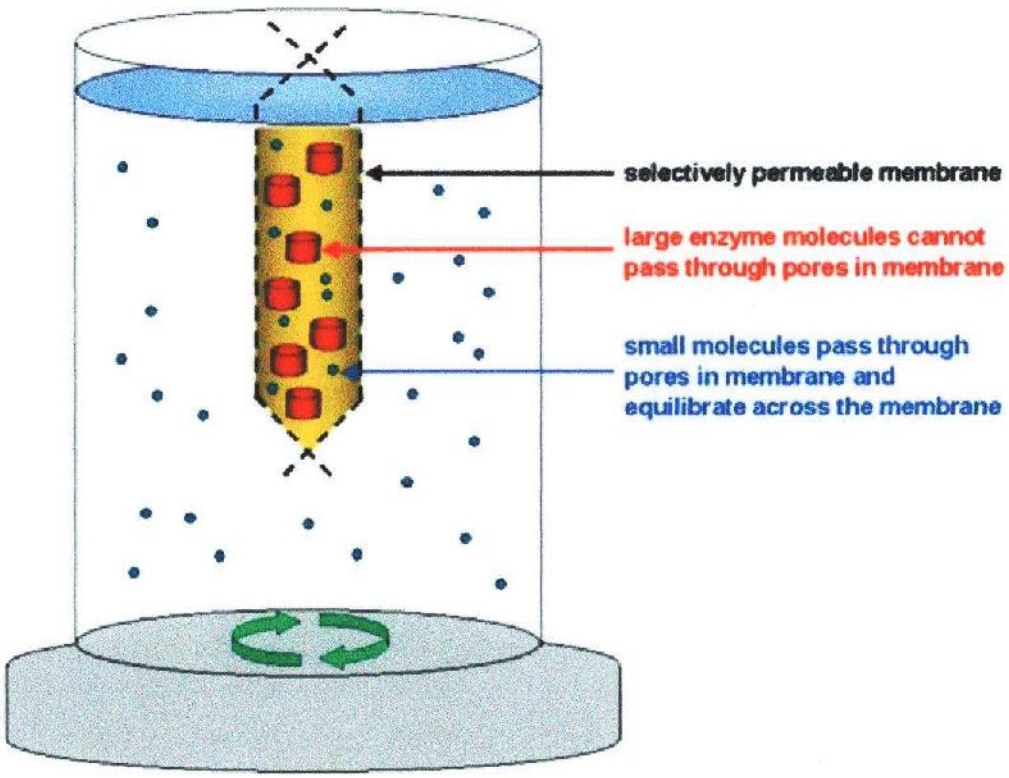

Fig 4:Removing the ammonium sulphate by dialysis

First, the concentrated protein solution is placed in dialysis bag with small holes which allow water and salt to pass out of the bag while protein is retained. Next the dialysis bag is placed in a large volume of buffer and stirred for many hours (8-12 hours), which allow the solution inside the bag to equilibrate with the solution outside the bag with respect to salt concentration: The dialyzed sample was loaded on to DEAE column at 4°C.The proteins were eluted from the column with the same buffer containing NaCl by liner and stepwise elution

3.7.4 DEAE-cellulose column Chromatography

3.7.4.1 Activation of DEAE-cellulose Powder

The DEAE-cellulose powder was suspended in 0.1M NaOH in a beaker and left it to swell for few hours. During swelling it was stirred gently at short intervals to prevent formation of lumps. Then it was transferred to a filtering funnel and washed with distilled water several times until its pH reached near to neutrality. The gel suspension was then transferred to another beaker containing 0.2 HCI and left for few hours. It was again washed with distilled water to neutralize its pH.

3.7.4.2 Packing of the column

The activate DEAE-cellulose suspension was taken in a filtering flask and de-aerated by vacuum pump. A column of desired length was packed with the activated column material. Precaution was taken to avoid trapping of air bubbles during packing.

3.7.4.3. Equilibration of the column

After packing, the column was equilibrated with 0.1M Tris-HCl buffer, pH 8.0

3.7.4.4. Preparation and application of sample

The dialyzed sample was loaded on to DEAE column at 4°C.The proteins were eluted from the column with the same buffer containing NaCl by liner and stepwise elution

3.7.5 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The protein patterns of the selected fractions were determined by 10%SDS-PAGE according to the method of Laemmli (1970) as modified by Smith (1984).

3.7.5.1 Reagents and solution

I. preparation of 50% TCA solution

5 g Tricloroacetic acid (TCA) was dissolved in about 30 ml distilled water in a 50 ml volumetric flask . The final volume was made up to the mark by adding distilled water.

II. Preparation of 30% acrylamide solution

14.4 g acrylamide and 0.5 g N, N'- methylene -bis acrylamide were dissolved in required amount of deionized water in a 50 ml volumetric flask and final value was made up to the mark with deionized water . The solution was filtered and stored in a dark battle at room temperature.

III. Preparation of 1.5 M Tris-HCI buffer (pH 8.8)

1.5M Tris-HCI was prepared by dissolving 18.17 g of Tris (MW121.14) in about 90 ml of deionized water. After adjusting the pH 8.8 with con^{c} .HCl, the final volume was made up to 100ml.

IV. Preparation of 0.5 M Tris-HCI buffer (pH 6.8)

0.5 M Tris-HCl was prepared by dissolving 6.057g of Tris in about 90ml of deionized water . After adjusting the pH to 6.8 with conc. HCl the final volume was made up to 100 ml.

V. Preparation of 0.75 M Tris-HCI buffer (pH 8.0)

0.7 m Tris- HCl was prepared by dissolving 9.085g of Tris in about 90 ml of deionized water . After adjusting the pH to 8.0 with conc. HCl the final volume was made up to 100ml.

VI. Preparation of 10% sodium dodecyl sulfate (SDS)

10% SDS was prepared by dissolving 5g of it in about 50ml deionized water . The final volume was made up to 50.

VII. Preparation of 10% ammonium persulfate (APS)

10% APS was prepared by dissolving 0.5g of it in about 5ml deionized water. The final volume was made up to 5ml. The solution was stored in Eppendorf tube (200ml in each tube) at -20°C

VIII. N, N, N`, N``_-Tetramethylenediamine (TEMED)

The commercially available preperation from Sigma Chemicals was used without modification.

IX. Preparation of sample buffer

The sample buffer prepared by mixing the components as follows and was stored 4°C

A.	0.5ml Tris- HCl buffer (pH 6.5)	0.5ml
B.	Glycerol	0.8ml
C.	SDS	0.184g
D.	2-mercaptoethanol	0.4ml
E.	Bromophenol blue (BPB)	1.0mg
F.	Deionized water	2.3ml
	Total	4.0ml

X .Preparation of Coomassie Brilliant Blue (CBB) staining solution

The CCB staining solution was prepared by mixing the component as follow:

A	CBBR-25	0.2g
B	Methanol	40ml
C	Glacial acetic acid	20ml
D	Deionized water	200ml
	Total	~260ml

XI .Preparation of Coomassie Brilliant Blue (CBB) destaining solution

The CCB destaining solution was prepared by mixing the component as follow:

A	Methanol	100ml
B	Glacial acetic acid	100ml
C	Deionized water	800ml
	Total	1000ml

XII. Preparation of the electrophoretic buffer

One liter of the electrophoretic buffer contained:

A	Tris base	6.0g
B	Glycine	28.0g
C	SDS	1.0g

De-ionized water was added to make the final volume 100ml

Sample preparation

The protein concentration in the fractions showing bacterial enzymes activity was too low to be used for SDS-PAGE. The protein was therefore precipitated using TCA.

Protocol for TCA method

STEPI: 1.0 ml of the eluent was taken in an Eppendorf tube and 300ml of 50%TCA solution was added to it.

STEP II: This was taken vortexed and kept at 4C for45-60 min

3.8 Characterization of the enzyme

3.8.1 Determination of the Effect of pH on Protease Activity

For determining the effect of pH on protease activity different buffer system with different pH were used. Azocasien was dissolved in different buffer solution and the enzyme assay was carried out within pH range (4.0 to 10.5) by azocasein assay method. All of them were used at 0.05M concentration.

Table 4: Different buffer used and their p^H ranges

Buffer	P^H range
Acetate buffer	4.0-5.6
Sodium phosphate buffer	5.6-8.0
TrisHCl buffer	7.5-8.9
Glycine-NaOH buffer	8.6-10.5

3.8.2 Determination of the Effect of Temperature on Protease Activity

For the determination of the effect of temperature, the reaction medium was incubated at varied temperature and the protease activity was determined. For this purpose the enzyme preparation was added to a mixture of 1 mg 1 % azocasein solution, 0.1 ml of 0.06 M CaCl2 and buffer (0.2 M Tris-HCl buffer, pH 8.0) and incubated at 37°, 40,°45°, 50°,°55,60°,65° ,70°C temperatures.

3.8.4 Determination of effect of other effectors on Protease Activity

The activity of the isolated protease was tested in the presence of various known protease effectors (all obtained from Sigma Chemical Co.): EDTA, 2-mercaptoethanol, DMSO, SDS, Methanol, and Ethanol. The azocasein assay was used with the addition of these effectors solution to achieve a final desired effectors concentration of 5mM. Control was taken where azocasein assay without these effectors were carried out.

3.9 Direct enzymatic de-hairing of bovine skin

For de-hairing studies, the organism was grown in TSB at 37°C for around 24 hours. Then it was centrifuged at 6000 rpm for 6 minutes. The cell free supernatant was added on detergent washed goat skin to observed enzymatic deharing capability of the organism. Sodium azide was used at 1% so that no organism can grow. TSB was used as control.

RESULTS

4.1 Isolation and characterization of the organism

The main object of this work was to isolate and characterize thermophilic enzyme which could specifically be used for deharing the hides and skins of cattle in the tannery industries. This is one of the important export oriented industries of Bangladesh.

In this connection two ways were planned. One was to isolate thermophilic organism from different natural sources. The other is to characterize the isolated organism. A thermophilic bacterium which produces extra cellular protease was isolated from soil of Dhamrai, Dhaka. The growth phenotype and some of the biochemical characteristics of the organism was determined. This organism was characterized and identified as a member of gram negative *Pseudomonas* family by several test but the species was not identified. So this bacteria is named here as a *Pseudomonas sp.* The results are presented here

Table 4: Identification and Morphological Characteristics of *Pseudomonassp*

Microbiological tests: Streak plate isolation		
Test performed	Observations	Results
Cetrimide agar plates at 37°C	greenish colonies	Positive
Gram stain	Small pink colonies singly or pairs	Gram Negative rods
Microbiological tests: Cultural characteristics		
Test performed	Observations	Results
Cetrimide agar plates	Growth on Cetrimide agar plates	small, pigmented, circular,raised,entire
TSB	growth on TSB	uniform fine turbidity
Biochemical Tests		
Catalase test	bubbles formed	Positive for catalase production
Oxidase test (young culture)	Black color formed	positive for oxidase production
Biochemical Tests : Acid & gas production		
Test performed	Observations	Results
Glucose	No bubbles formed	negative for acid and negative for gas
Lactose	Yellow color	Negative for acid and gas

Sucrose	No bubbles formed	Negative
Maltose	Yellow and no bubbles formed	Negative for acid and gas

Biochemical Tests : IMViC test		
Test performed	Observations	Results
Indole (SIM) test	No Red ring, growth away	negative for indole and motility
	from stab,no black color formed	negative for H2S production
Methyl red test	no red ring formed	Negative for mixed acid production
Voges-Proskauer test	no red ring formed	negative for acetoin production
Citrate test	change in color	positive for citrate utilization
Urease test	no bright pink color	negative for urea catabolism
Nitrate test	no color change after zinc dust addition	positive for nitrate reduction
Gelatin test	remain liquefied at 4°C	positive for gelatinase production
Starch test	No formed zone	negative for starch hydrolysis
Litmus Milk Reaction	Rapid peptonization	Positive for litmus milk reaction

4.4 Comparison of dehairing ability of *Pseudomonas*spp with other bacteria

Dehairing ability of the protease produced by our strain and other bacterial protease showed that our bacterial protease is very fast in dehairing compared to other three.

Table 5: Comparison of dehairing ability of *Pseudomonas*spp with other bacteria.

Organisms	Time of incubation for deharing	Change of color of leather
Pseudomonas	10h	no change
Vibriosp kr2	24h	no change
Flavobacteriumsp kr6	24h	no change
Bacillussp kr10	24h	no change

4.5 Effect of p^H on protease fromthe organism

The enzyme activity over a pH range between 4 and 11 was studied and the result is presented in the figure. The figure shows the optimum pH is 8.5 for the activity of protease enzyme in Tris-HCL buffers.

Table 6: Effect of pH on Protease Activity

pH	Activity of Enzyme(unit)
4.5	16.1
6.0	20.0
6.5	20.3
7.0	28.3
7.5	29.9
8.0	34.3
8.5	29.9
9.0	24.3
9.5	20.5

10	19.9
11	10.1

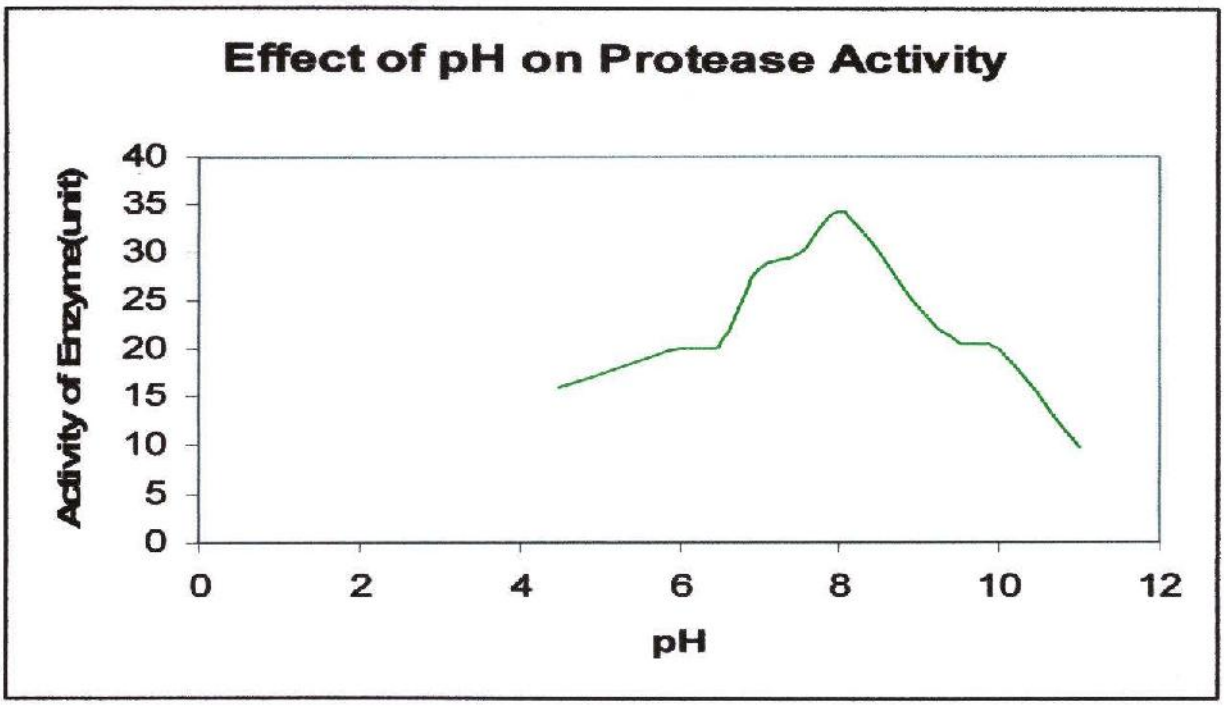

Figure 8: Graphical presentation of effect of pH on protease activity

4.6 Effect of temperature on enzyme activity

The activity of the enzyme was measured over a range of temperature (4°C, 20°C ,25°C, 30°C, 35°C,37°C, 40°C, 45°C, 50°C,55°C 60°C 65°C, 70°C) and the result is presented in Fig-3. There was a significant increase in enzyme activity between 35°C to 45°C .After 50°C the activity of the enzyme decrease gradually. The enzyme showed its maximum activity from 35°C to 45°C.

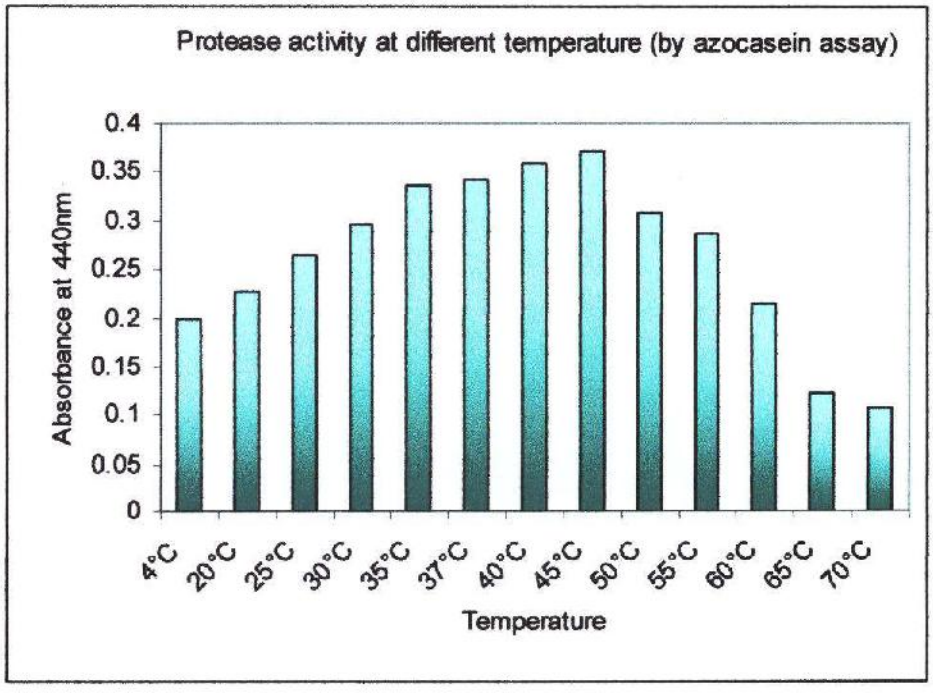

Figure 9: Graphical presentation of protease activities at different temperature

4.7 Effect of salts and other effectors on the protease activity

The effect of different salts ($MgCl_2$,$CaCl_2$, $AlCl_3$,KCl) and other effectors (EDTA, 2-mercaptoethanol,DMSO,SDS,Ethanol,Methanol,) at different concentration was measured. SDS increased the activity and2-mercaptoethanol decreased the activity of the enzyme. NaCl didn't change the activity. Others had little deactivating effect.

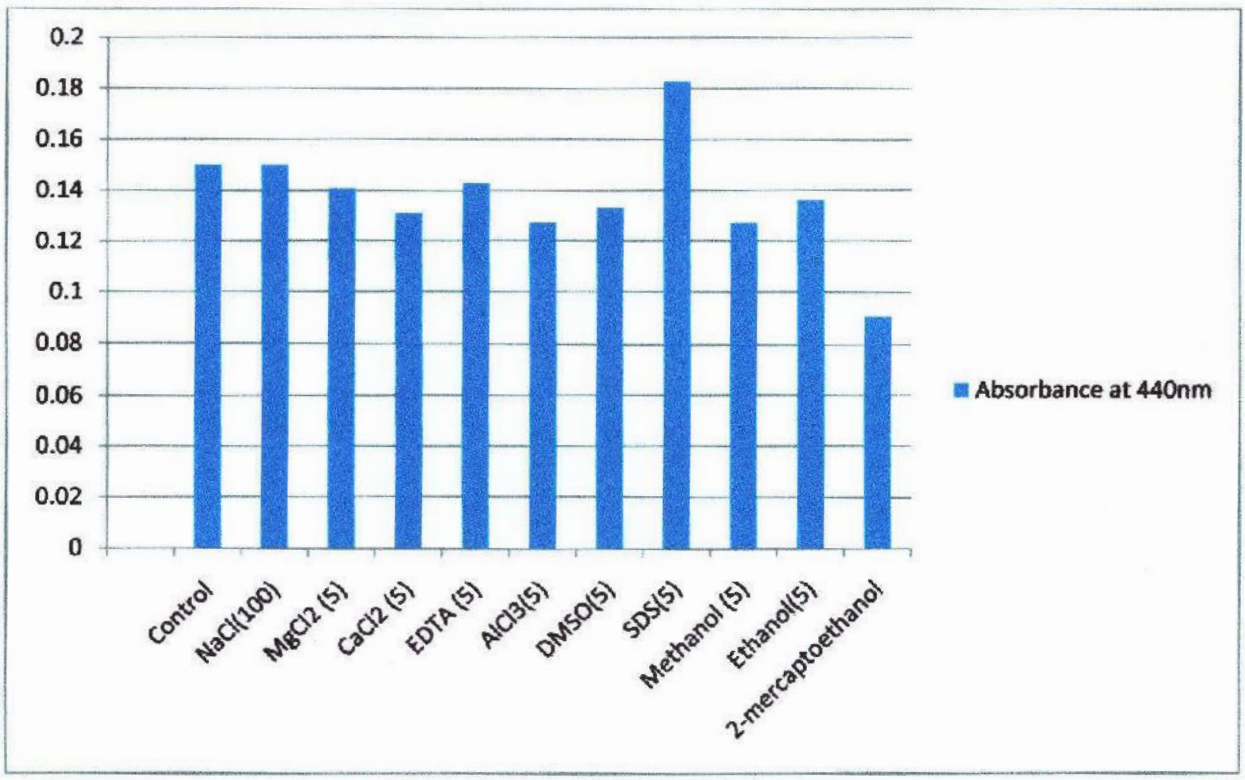

Figure 10: Graphical presentation of effects of salts and other chemicals on the activity on the protease activity

4.8 Ammonium sulphate precipitation:

Several concentration 20%,40% & 60 % were used to precipitate the enzyme but the enzyme were fully precipitate when 60% ammonium sulphate was used.

4.9 DEAE Column chromatography fraction

The fractions of column chromatography were collected by using fraction collector at different salt concentration. The fractions are given below:

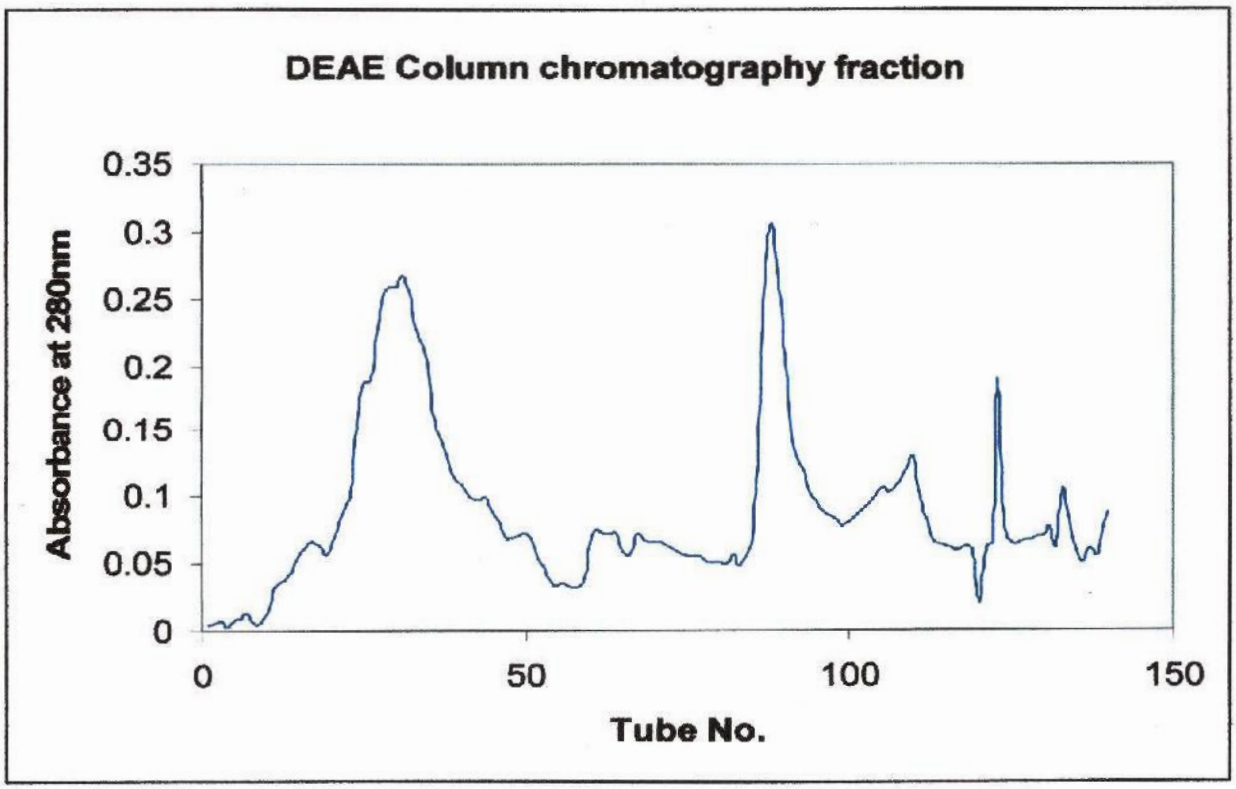

Figure 11: Graphical presentation of the result column chromatography

4.10 Result of SDS-PAGE

The single band in the figure indicate purification of the protease.Incomparism with the marker on the left side of the figure, it is clear that the molecular weight of the protein is approximately 66 kDa

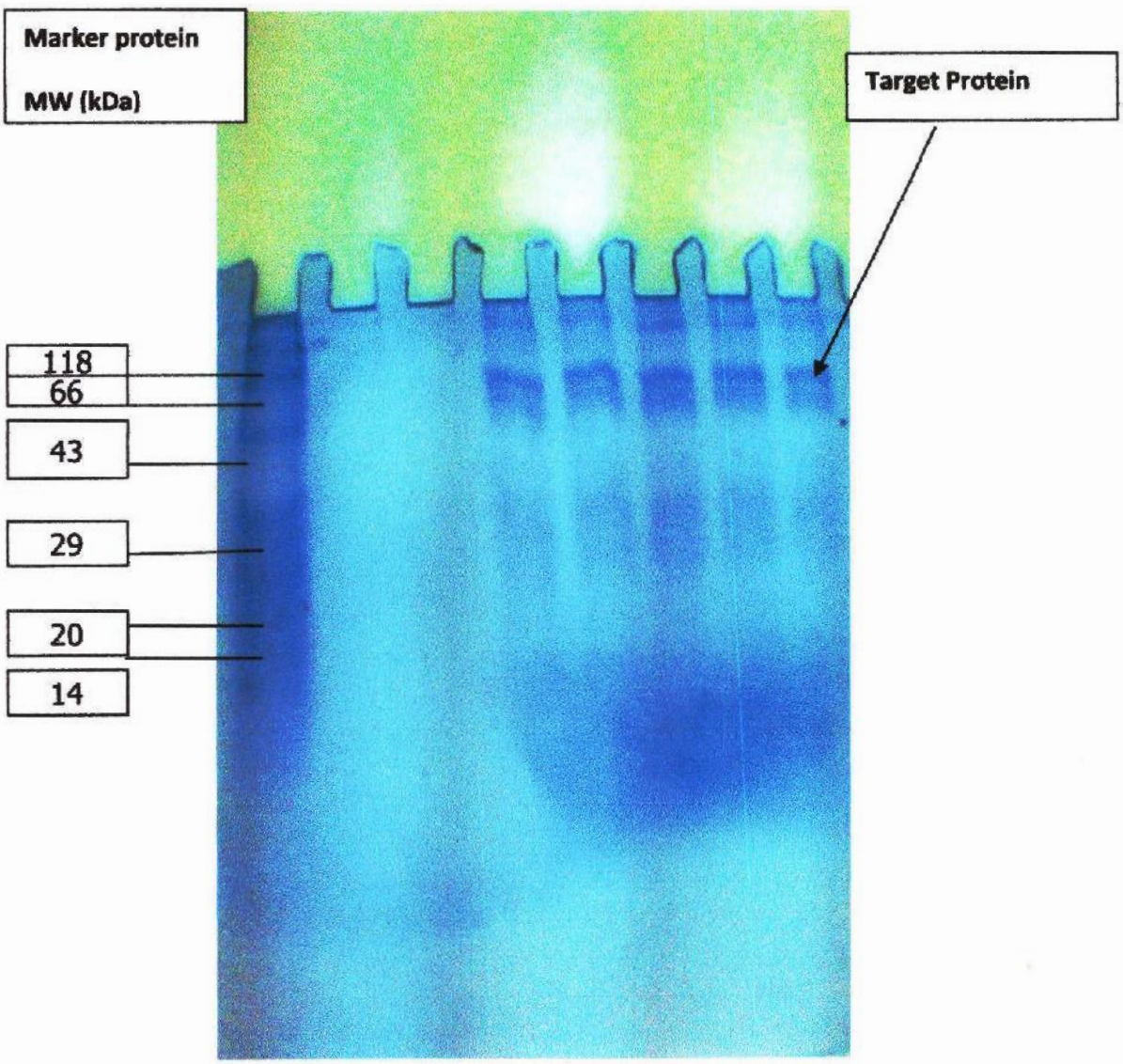

Figure 12: Band of SDS-PAGE

Discussions

Analysis on the bacteria showed that the bacteria had an optimum temperature at 37°C. The gram staining showed that the bacteria were Gram-Negative rod because the cells had a pink color under the microscope using a 100X (oil immersion) lens. The cellular arrangements of the bacteria were in chains.Gram Negative bacteria can be any one of the genus Pseudomonas, *E.coli, Klebsiella, Proteus,Citrobacter, Enterobacter, Acitinobacter, Salmonella,Shigella.* As our strain is Gram negative, it might be a member of any one of these genera. But as the organism is rod shaped so it doesn't belong to the Micrococcus,Staphylococcus, Streptococcus, Lactococcus, Enterococcus, Leuconostoc, Pediococcus, Aerococcus genera, as these are coccus. So the unknown bacterial strain can be a member of ,*E.coli, Klebsiella, Proteus,Citrobacter, Enterobacter, Acitinobacter, Salmonella,Shigella.*[112]

The unknown organism gave oxidase test. It also proved that the organism was capable of hydrolyzing casein.The Voges-Proskauer test confirmed that *Pseudomonas* VP negative, which meant that they don't produce non acidic products or neutral products (acetoin). [81] But our strain was VP negative. So it may be none of these families.Based on the result from the catalase test, the bacteria were catalase positive because of the formation of bubbles after addition of hydrogen peroxide. [73]In oxidase test, there was change in color after 10-15 seconds addition of the reagent which meant that the bacteria produced cytochrome oxidase thus resulting in positive result. The oxidase test narrowed down the possibility to *Enterobacteriaceae* or enteric bacteria because all enteric bacteria were oxidase negative [75]

The IMViC tests were performed to differentiate the enteric bacteria that are closely related to one another. In SIM test, the formation of a bright red ring after addition of Kovac's reagent was observed. This meant that the bacteria have no capability of producing indole thus giving negative result. [76] The SIM test also showed that the bacteria were none motile which can be seen, as there was growth away from the stab mark into the medium during inoculation. [73] However, hydrogen sulfide production was not seen on the medium.In Voges-Proskauer test, there was not a red ring formed on the MRVP medium that gave a negative toward this test. In

methyl red test, the negative result was not seen as the production of a deep red color after addition of reagents. [76]

Carbohydrate fermentation test was analyzed to narrow down the genera. The bacteria showed negative for glucose, sucrose and fermentation. The pH indicator showed that the broth changed color into yellow, which showed that fermentation had occurred. This result confirmed that this bacteria does not belong to *Enterobacter* species, which were not capable of fermenting lactose. [73]

The optimum temperature of the protease producing *Pseudomonas*sp was 35-45°C and the optimum pH was 8.0.The reducing agent β-Mercaptoethanol decreased the activity of the enzyme. β-Mercaptoethanol has been reported to stabilize cystein proteases by protecting the oxidation of sufhydral group in proteins.[84] But β-Mercaptoethanol will cause alteration of structure of enzyme by reducing disulfide bonds of the enzyme. Thus the protease could contain disulfide bonds.The result of the SDS-PAGE suggests the approximate molecular weight of the enzymes was about 66 KDa. It is the matter of further investigation that whether the protein was multi subunit or not.

Enzymatic de-hairing may be the ideal de-hairing process when the enzyme specificity is guided towards the components of the epidermis, its action on the structured proteins, especially the collagen, is minimized or avoided, and thus leather with desirable properties is developed.[86]these experimental results indicate that the bacterial depilatory systems tested have desirable characteristics for the de-hairing process. A significant feature of the enzymatic de-hairing process is complete hair removal by *Pseudomonas sp* within 10 hours where Vibrio sp kr2, Flavobacteriumsp kr6, Bacillus sp kr10 take 24 hours and minimal usage of sulfide and the decomposition products formed from the tannery wastewater, with great improvement in wastewater quality as a result.

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Appendix-1

Chemicals and Reagents

The chemicals and reagents which were used during this research work were obtained from different companies that are listed below. All other chemicals and reagents used were of analytical grade.

Composition of the media used

Nutrient Agar	**Grams/Liter**
Peptone	5.0
Bacto beef extract	3.0
NaCl	5.0
Agar	15.0
Distilled water	1000 ml
P^H	7.2

Sterilized at 121^0C under 15 1b/in^2 pressures for 15 minutes.

Nutrient Broth	**Grams/Liter**
Peptone	5.0
NaCl	5.0
Beef Extract	1.5
Yeast Extract	1.5
P^H	7.4
Distilled water	1000 ml

Sterilized at 121^0C under 151 b/in^2 pressure for 15 minutes.

Tryptic-soya broth (TSB)	**Grams/Liter**
Trypticase	15.0
Phytone	5.0
Sodium chloride	5.0
Glucose	5.0
pH	7.4
Distilled water	1000ml

Sterilized at 121^0C under 151b/in^2 pressure for 15 minutes

Sodium Chloride Broth	**Gram/Liter**
Beef extract	0.1
NaCl	0.375
Distilled water	1000 ml

Sterilized at 121^0C under 15 1b/in^2 pressures for 15 minutes.

Cetrimide agar	**Gram/Liter**
Gelatine	20.00
Agar	15.00
Potassium sulphate	10.00
Magnesium chloride	01.40
Cetrimide	00.30
Distilled water	1000ml

Sterilized at 121^0C under 15 1b/in^2 pressure for 15 minutes.

Normal Saline	**Gram/Liter**
NaCl	0.85
Distilled water	1000ml

Autoclaved at 121^0C for 15 minutes

APPENDIX-II

Composition of the media used in biochemical test

MR-VP broth	**Gram/Liter**
Peptone	7.0
Dextrose	5.0
Dipotassium phosphate	5.0
Distilled water	1000ml
P^H	6.9

Sterilized at 121^0C under 15 1b/in^2 pressure for 15 minutes.

Triple Sugar Iron (TSI) Agar	**Gram /Liter**
Peptone	10.0
Tryptone	10.0
Yeast Extract	3.0
Lactose	10.0
Saccharose	10.0
Dextrose	1.0
Ferrous Sulphate	0.2
Sodium Chloride	5.0
Sodium Thiosulphate	0.3
Phenol Red	0.024
Agar	12.0
P^H	7.4

Sterilized at 121^0C under 15 1b/in^2 pressure for 15 minutes.

Urea broth medium	**Gram/Liter**
Urea	20.0
Yeast extract	0.1
KH_2PO_4	9.0
K_2HPO_4	9.5
Phenol red	0.01
Distilled water	1000ml
P^H	6.8

Sterilized at 121^0C under 151 b/in^2 pressure for 15 minutes.

Indol tryptopon broth medium	**Gram/Liter**
Tryptone	10.0
Distilled water	1000ml

Sterilized at 121^0C under 15 1b/in^2 pressure for 15 minutes.

Nitrate broth	**Gram/Liter**
Peptone	5.0
Beef extract	3.0
NaCl	5.0
Potassium nitrate	1.0
Agar	1.0
Distilled water	1000 ml
P^H	7.2

Sterilized at 121^0C under 151 b/in^2 pressure for 15 minutes.

APPENDIX-III

Composition of chemicals and reagents

Crystal violet

Solution-A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20.0 ml

Solution-B

Ammonium oxalate	0.8
Distilled water	80.0 ml

Note-Mix the solution A and B

Gram's iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300.0 ml

Ethyl alcohol (95%)

Ethyl alcohol (100%)	95.5 ml
Distilled water	5.0 ml

Safranin

Safranin O	0.25 ml
Ethyl alcohol (95%)	10.0 ml
Distilled water	100.0 ml

Kovac's reagent (for detection of indole)

P-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Hydrochloric acid (concentrated)	25.0 ml

Concentrated P-Dimethylaminobenzaldehyde was dissolved in the amyl alcohol and HCl was added slowly.

Methyl red solution

Methyl red	0.04 g
Ethanol	40.0 g
Distilled water	100 .0 ml

Methyl red dissolved in ethanol and diluted water.

Barrit's reagent

Solution-A

α- naptho	15.0 g
Ethanol (Absolut)	95.0 g

α- naptho was dissolved in ethanol with constant stirring.

Solution-B

KOH	40.0 g
Creatine	0.3 g
Distilled water	100.0 ml

Hydrogen peroxide

3% aqueous solution of H_2O_2 was prepared from the H_2O_2 absolute solution. [9]

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Tryptone	Yeast extract
Peptone	Sodium chloride

BDH chemicals, LTD, Poole, England

Agar	Calcium chloride
Ethanol	Sodium carbonate
Iodine	

E. Merck (India)

Dipotassium hydrogen phosphate	Trichloroacetic acid (TCA)
Potassium dihydrogen phosphate	Zinc
Sodium hydroxide	

Sigma chemical company, St. Louis, USA.

Azocasein	Glucose
Bromophenol blue	Lactose
Ammonium per sulfate	Phenol red
Ferric	

Nutrient Gelatin:	**Grams/Liter**
Gelatine,	120
Meats extract	3
Peptone	5
Distilled water	1000 ml

Reagents for Quantitative Protein Assay

Total protein Estimation by Lowry's method

Trichloro acetic acid(TCA) (10%)	100mL
0.1M NaOH	200mL
Alkaline $NaHCO_3$	100mL
Na-K tertarate (10 gm/Lt)	200mL
CuSO4.5H2O :(5%)	100mL
Folin – Ciocalteau reagent solution (50%)	50mL
Bovin Serum Albumin (BSA) stock solution (1mg/ml).	

Reagents for Protein Purification

Solid ammonium sulfate	500gm
Sephadex G200.	50gm
Dye: Blue dextan	5mL
Buffer: 10mM Trise-HCl, pH-8	1000mL