PRODUCTION OF MICROBIAL PROTEASE AND ITS APPLICATION IN LEATHER SOLID WASTE BIOTREATMENT

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By

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DECLARATION

The material contained in this thesis is my original work and I have not presented any part of this thesis/work elsewhere for any other degree.

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Certificate

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AMI AND ABU

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LIST OF ABBREVIATIONS

Abbreviations	Full name
AIDS	Acquired immune deficiency syndrome
API	Analytical Profile Index
BPN	Bacterial proteases Nagase
BSA	Bovine serum albumin
CCLW	Chromium-containing leather waste
CZE	Capillary Electrophoresis
DCl	Dichloroisocoumarin
DEAE	Diethylaminoethyl cellulose
DFP	Diisopropylfluorophosphate
DI	Deionized water
DNA	Deoxyribonucleic acid
EC NO.	Enzyme Commission number
EDTA	Ethylenediaminetetraacetic acid
FALGPA	2-furanacryloyl –L-butylglycol-L-prolyl-L-alanine
HIV	Human immunodeficiency virus
PCR	Polymerase Chain Reaction
PMSF	Phenyl methyl sulfonyl fluoride
PVC	Polyvinyl chloride
SDS	Sodium dodecyl sulfate
PAGE	Polyacrylamide gel electrophoresis
TCA	Trichloroacetic acid

TKN	Total Kjeldahl Nitrogen
Tris	Tris (hydroxymethyl) aminomethane
TLCK	Tosyl-L-lysine chloromethyl ketone
MALDI-TOF	Matrix Assisted Laser Desorption Ionization- Time Of Flight

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ABSTRACT

The demand of leather items is increasing day by day with the growing population. Although, leather industry provides the necessities, such as leather shoes and garments while using the by-products of the meat industry, but the by-products generated during the process of leather making and the solid waste coming out of leather industry while making valuable leather items cannot be discarded. Only 25% of the wet salted hides are converted into commercial leather, while 70% becomes chromium containing leather waste, and the remainder of the hides (5%) is lost in waste water as fat, soluble protein, and solid suspended pollutants. Chrome shavings contribute 50% of the chromium containing solid wastes produced by leather industry which are disposed mostly in landfills and some of them are used for making of leather boards, artificial leather. An increasing environmental concern and decreasing number of disposal sites has triggered research to recover protein (collagen) and chromium from the chrome shavings.

Four strains of *Bacillus* sp. (SAL1, SAL2, SAL3, and SAL4) were isolated from soil and tannery wastes and screened for the protease producing ability. The SAL1 strain gave the maximum zone of hydrolysis when inoculated on agar plates containing 1% casein as substrate. The strain was identified as *Bacillus subtilis* on the basis of biochemical tests and molecular characterization. The *Bacillus* strain SAL1 was cultured in shake flask and various parameters were studied for the optimum production of protease.

Among the three different media, used for the production of protease, the medium containing casein, gelatin and glycerol gave the optimum production i.e. 285 proteolytic units/miligram (PU/mg). Among the various parameter tested for optimization of protease production, incubation period of 72hours,pH 8.5,temperature 37°C, 10% inoculum of 24 hours gave the maximum production of protease. Among the different carbon sources such as sucrose, fructose, manitol, dextrose, sorbitol, glycerol, glucose, maltose and lactose, maximum protease production was observed in case of glycerol and among all the nitrogen sources such as tryptone, peptone, beef extract, yeast extract and gelatin, maximum production was observed when yeast extract was used as nitrogen source. Addition of metal cations in the medium has a very profound effect on the production of protease. As a result of optimization studies, the yield of alkaline protease by the strain could be increased approximately up to 35 %.(387 PU/mg).

This protease from *Bacillus* strain SAL1 has been purified to homogeneity as judged by SDS-PAGE. The protease was purified to homogeneity by a combination of ammonium sulfate precipitation, DEAE Sephacryl ion exchange and Phenyl Sepharose hydrophobic interaction chromatography. The protease was purified up to 11.18 fold and had a specific activity of 4250 PU/mg. The enzyme was a monomeric protease with a relative molecular mass of 27 kDa as determined by SDS-PAGE. Proteolytic activity of the enzyme was detected by gelatin zymography, which gave a very clear protease activity zone on gel. Molecular mass of purified protease was also determined by matrix-assisted laser desorption ionization-time of flight-mass spectrometry (MALDI-TOF-MS) that corresponded to the mass determined by SDS-PAGE. The homogeneity of enzyme was also checked by capillary electrophoresis which gave one peak confirming the purity of the enzyme. The enzyme exhibited its optimal activity at 60 °C and at pH 9. The enzyme was stable in the pH range of 7.0–10.0 and was able to maintain its stability at 50 °C for 1 hour.

The proteases from *Bacillus* strain SAL1 were then tested for collagenolytic activity on leather dust. Maximum leather dust degradation was observed when leather dust was incubated with 5000 PU/mg of enzyme for 84 hours and the extent of degradation was measured by the release of hydroxyproline (9.0 μg/ml).This indicated that proteases produced from *Bacillus* strain SAL1 can be used successfully in the biotreatment of leather dust.

The protease from *Bacillus* strain SAL1 was also used for the biotreatment of chrome shavings. In this work, hydrolysis of chrome shaving was done by two ways. One is one step process in which hydrolysate protein is extracted by treatment with alkaline proteolytic enzyme at pH 8.5, temperature 60 °C, and agitation for 3 hours. Other is two-step process in which gelable proteins as well as hydrolysate proteins are extracted by treatment with alkaline proteolytic enzyme at pH 8.5, temperature 60 °C, and agitation for 11 hours. Hydrolysate proteins extracted from both processes contained high values of TKN (18.83%and 17.98% respectively) and showed a wide distribution of molecular weights (from16-97KDa). Because of the high nitrogen content, the isolated collagen hydrolysate has potential use as a fertilizer and as animal feed additives.

The effect of protease on the finished leather was also studied by applying it on the scraps of shoe upper leather. Firstly they were treated at high alkaline condition at 60 °C at pH 12, and

then the pH was decreased for the enzyme reaction. This process produced hydrolysate protein and chrome sludge. Because of the high nitrogen contents (15.87%), the isolated collagen hydrolysate has potential use as a fertilizer and as animal feed additives.

The most ideal way to minimize the waste generated from the leather industry is to convert the by-products into reusable items. For example, the hair pulp and shavings from the tannery solid waste, which are usually disposed, contain significant quantities of useful proteins like keratin and collagen, respectively. These proteins have demonstrated uses in pharmaceuticals, adhesives, cosmetics, films, encapsulations, etc. By employing the protease for the biodegradation of solid waste we can reduce them into gelatin and protein hydrolysate which can be used in organic nitrogenous fertilizer and animal feed and in preparation of films and adhesive. In this way, we can convert the harmful environmental pollutants into useful commercial items and find a way to make leather industry more profitable and less harmful to the environment.

INTRODUCTION

Most of enzymes are class of proteins that catalyze specific reactions in biological systems. They are present in all living cells, where they perform various vital functions ranging from cell signaling to metabolic processes. They speed up chemical processes without being consumed in the process and so, in principle, they could catalyze reactions indefinitely. Most of the organic catalysts have a limited stability and they break down over a period of time, exhibiting decreasing activity. The rate of activity along with enzyme efficacy characteristics such as optimal pH and temperature are found to be different for different enzyme catalysts (Uhlig, 1998).

Over the last four decades, the enzyme industry has grown significantly because of the evolution of modern biotechnology. Enzymes have been used for a long time. They have been used in the manufacture of different commodities like leather, indigo and linen. They have been employed in the production of so many food products such as cheese, beer, wine and vinegar. Most of these processes used unpurified and less known form of enzymes as the product of spontaneous growing microorganisms or the enzymes present in added preparations (calves, rumen, and papaya fruit). The fermentation processes focused specifically at the production of enzymes using selected strains. These processes have been used for the production of purified and well-characterized enzymes on a large scale. These developments allowed the use of enzymes into true industrial processes and products, such as detergent, textile and starch industries (Rao *et al.*, 1998); (Kirk *et al.*, 2002). Similarly, recombinant gene technology, protein engineering and directed evolution improved production processes and enabled the commercialization of the enzymes that could not be produced before (Neurath, 1999). These latest advancements within modern biotechnology have revolutionized the development of industrial enzymes. These developments made possible the expansion of their industrial application, by providing "tailor-made" enzymes exhibiting new activities and adapted to new production processes. The industry has made significant expansion because of these new findings and is still growing in size, diversity and complexity as shown in Table 1. Enzymes of industrial relevance are able to maintain their bio-catalytic activities in the absence of their respective productive cells, and this is the basis for their practical applications.

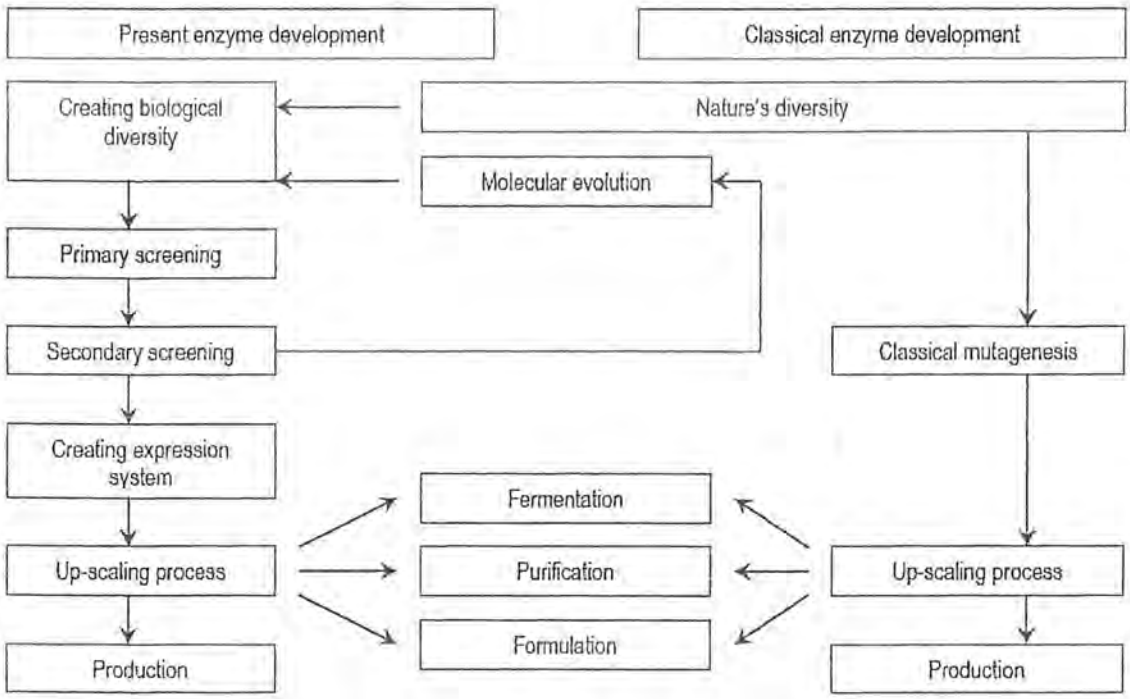


Fig. 1.1: Steps involved in classical values versus state-of-art of development of enzymes (Kirk *et al.*, 2002).

As they do not reproduce and are completely biodegradable, they represent an ecologically "clean" alternative to many chemical processes. A variety of applications is found in chemical industry, agri-food and house hold detergents, as they have never caused toxicological or environmental problems (Christner, 1995). A lot of credit should be given to the advancements in modern biotechnology which ultimately made possible the development of enzymes which are useful for a number of processes, no one would have considered a decade ago. Enzymes are currently used in several different industrial products and processes and new areas of application are constantly being added.

Table: 1.1 Enzymes used in various industrial segments and their applications. (Kirk *et al.*, 2002)

Industry	Enzyme class	Application
Detergent (laundry and dish wash)	Protease	Protein strain removal
	Amylase	Starch stain removal
	Lipase	Lipid stain removal
	Cellulase	Cleaning, color clarification, anti-redeposition (cotton)
	Mannanase	Mannanan stain removal (reappearing stains)
Starch and Fuel	Amylase	Starch liquefaction and saccharification
	Amyloglucosidase	Saccharification
	Pullulanase	Saccharification
	Glucose isomerase	Glucose to fructose conversion
	Cyclodextrin-glycosyltransferase	Cyclodextrin production
	Xylanase	Viscosity reduction (fuel and starch)
	Protease	Protease (yeast nutrition – fuel)
Food (including dairy)	Protease	Milk clotting, infant formulas (low allergenic), flavor
	Lipase	Cheese flavor
	Lactase	Lactose removal (milk)
	Pectin metyl esterase	Firming fruit-based products
	Pectinase	Fruit-based products
	Transglutaminase	Modify visco-elastic properties
Baking	Amylase	Bread softness and volume, flour adjustment
	Xylanase	Dough conditioning
	Lipase	Dough stability and conditioning (*in situ* emulsifier)
	Phospholipase	Dough stability and conditioning (*in situ* emulsifier)
	Glucose oxidase	Dough strengthening
	Lipoxygenase	Dough strengthening, bread whitening
	Protease	Biscuits, cookies
Animal Feed	Transglutaminase	Laminated dough strengths
	Phytase	Phytate digestibility – phosphorus release
	Xylanase	Digestibility
	B-Glucanase	Digestibility
Beverage	Pectinase	De-pectinization, mashing
	Amylase	Juice treatment, low calorie beer
	B-Glucanase	Mashing
	Acetolactate decarboxylase	Maturation (beer)
	Laccase	Clarification (juice), flavor (beer), cork stopper treatment
Textile	Cellulase	Denim finishing, cotton softening
	Amylase	De-sizing
	Pectate lyase	Scouring
	Catalase	Bleach termination
	Laccase	Bleaching
	Peroxidase	Excess dye removal
Pulp and Paper	Lipase	Pitch control, contaminant control
	Protease	Biofilm removal
	Amylase	Starch-coating, de-inking, drainage improvement
	Xylanase	Bleach boosting
	Cellulase	De-inking, drainage improvement, fiber modification
Fats and Oils	Lipase	Transesterification
	Phospholipase	De-gumming, lyso-lecithin production
Organic Synthesis	Lipase	Resolution of chiral alcohols and amides
	Acylase	Synthesis of semisynthetic penicillin
	Nitrilase	Synthesis of enantiopure carboxylic acids
Leather	Protease	Unhearing, baing
	Lipase	De-pickling
Personal Care	Amyloglucosidase	Antimicrobial (combined with glucose oxidase)
	Glucose oxidase	Bleaching, antimicrobial
	Peroxidase	Antimicrobial

In most of the applications, enzymes work as effective catalysts under mild conditions which save important resources such as energy and water for the benefit of both industry

and the environment. With a rapid growing population and approaching exhaustion of many natural resources, enzyme technology offers a great potential for many industries to meet the challenges they will face in years to come.

As illustrated in Fig. 2, the detergent, starch, textile, fuel, alcohol, leather, paper and pulp industries known as the technical industries, account for the major consumption of industrial enzymes.

Fig. 1.2: Segmentation of the Industrial Market. The technical industries segment comprises the detergent, starch, textile, fuel and alcohol, leather, pulp and paper industries (Kirk *et al.*, 2002).

The majority of currently used industrial enzymes are hydrolytic in action, being used for the degradation of various natural substances. Proteases remain the dominant enzyme type, because of their extensive application in detergent and dairy industries (Godfrey *et al.*, 1996).

1.1 PROTEASES

Proteases constitute a very large and complex group of enzymes; play an important nutritional and regulatory role in nature that accounts for 60% of the total world wide enzyme market which is estimated to be worth US$ 4 billion (Banerjee *et al.*, 1992, Chu *et al.*, 1992). These enzymes are important in a number of diverse and crucial biological processes; for example, they are involved in the regulation of metabolism and gene expression, enzyme modification, pathogenicity, and the hydrolysis of large proteins to smaller molecules for transport and metabolism (Rao *et al.*, 1998).

Analytical and pharmaceutical enzymes

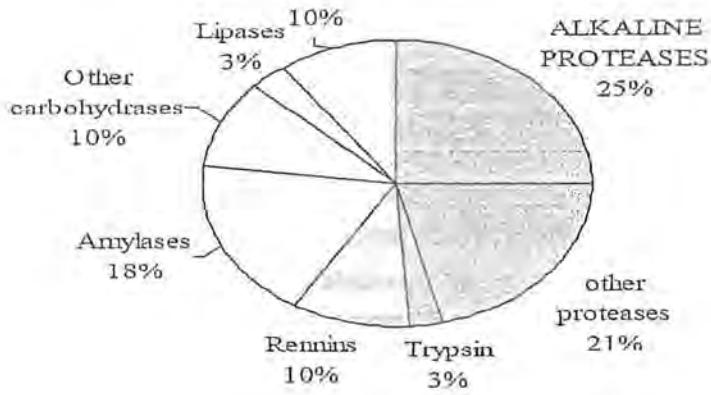


Fig. 1.3 The contribution of different enzymes to the total sale of enzymes is indicated. The shaded portion indicates the total sale of proteases (Rao *et al.*, 1998).

Proteases, the most important group of enzymes, are produced commercially and have been utilized in detergent, protein, brewing, meat, photographic, leather and dairy industries (Anwar *et al.*, 1998). Their application in the leather industry for dehairing and bating of hides to substitute currently used toxic chemicals, is a relatively new development and has conferred added biotechnological importance (Rao *et al.*, 1998). Since their introduction as an additive in detergent industry in 1914, these enzymes became a part of detergent industry as well (Gupta *et al.*, 2002a).

Proteases are difficult to characterize because of their diversity of action and structure. They were classified based on their molecular size, charge or substrate specificity. Proteases are broadly divided into exo- and endopeptidases depending on their site of action. If the enzyme cleaves the peptide bond proximal to the amino or carboxyl terminus of the substrate, they are classified as exopeptidases. If the enzyme cleaves peptide bonds distant from the termini of a substrate, they are classified as endopeptidases.

Proteases are further categorized into following four groups based on the functional group present at the active site and their catalytic mechanism;

A) serine proteases,
B) aspartic proteases,
C) cysteine/thiol proteases,
D) Metalloproteases.

Four classes of endoproteases have been identified in living organisms, whereas three of the four classes of endoproteases have been isolated and purified in bacteria which are serine, cysteine and metalloproteases (Liao *et al.*, 1998).

1.2 PROTEASES APPLICATIONS IN LEATHER INDUSTRY

Sodium sulfide and other toxic chemicals are generally employed in the leather processing industry which eventually creates environmental pollution and safety hazards. Hence, the bio-treatment of leather with biological methods such as using enzymatic approach is preferable as it offers several advantages, e.g. easy control, speed and waste reduction. Proteases are used in the soaking, dehairing and bating stages of preparing skins and hides. The enzymatic treatment destroys undesirable pigments, increases the skin area and therefore a clean hide is produced (Gupta *et al.*, 2002b).

The purpose of soaking is to swell the hide. Traditionally, this step was performed with alkali. Currently, microbial alkaline proteases are used to ensure faster absorption of water and to reduce the time required for soaking by 10 – 20 hours. The conventional method of dehairing and dewooling consists of development of an extremely alkaline condition followed by treatment with sulfide to solubilize the proteins of the hair root (the severe alkaline condition was a health hazard for the workers). At present, alkaline proteases with hydrated lime and sodium chloride are used for dehairing, resulting in a significant reduction in the amount of wastewater generated (Puvanakrishnan and Dhar, 1985). Leather processing industries can utilize alkaline proteases with elastolytic and keratinolytic activity. The hide is softened during bating by partial degradation of the interfibrillar matrix proteins (elastin & keratin). Therefore enzyme preparations with low levels of elastase and keratinase activity but no collagenase activity are particularly applicable for this process (Cowan, 1996). Bating is traditionally an enzymatic process

which utilizes pancreatic proteases. However, recently the use of microbial alkaline proteases has become popular. The substitution of chemical depilatory agents in the leather industry by proteolytic enzymes produced by *Bacillus* sp. can prove out to be economical and greener (Anwar *et al.*, 1998).

1.3 LEATHER MAKING

The leather processing industry is one of the oldest industries in the world. Leather is stabilized animal skins and hides. The skins and hides are the by-products of the meat industry and they consist of proteins, lipids, some carbohydrates, inorganic salts and water. Tanning is a process of converting readily putrescible raw animal skins and hides into leather, a stable product that can be conserved indefinitely, with adequate strength properties and resistance to various biological and physical agents. Three steps in the processing of leather are:

1. Removal of unwanted components such as hair and lipids, etc., leaving a network of fibers of skin protein;

2. Reacting this network with tanning materials to produce a stabilized fiber structure;

3. Building onto the tanned fibers characteristics of fullness, color, softness, and to finish the fiber surface to produce a useful product.

During tanning, additional cross-links are introduced into collagen (the principal protein of the skin) which binds to the active groups of tanning agents (Thorstensen, 1993).

With the passage of time, demand of leather products especially shoes, clothing, bags etc. is increasing. The manufacture of high quality leather results in an almost equal weight of solid waste materials (Maire *et al.*, 1980). To transform hides and skins from animals sacrificed for meat into leather, the tanner consumes water and chemicals; and produces waste water and solid waste with final leather (Alexander *et al.*, 1991). It is known that one ton of wet salted hide yields only 200Kg of leather but over 600kg of

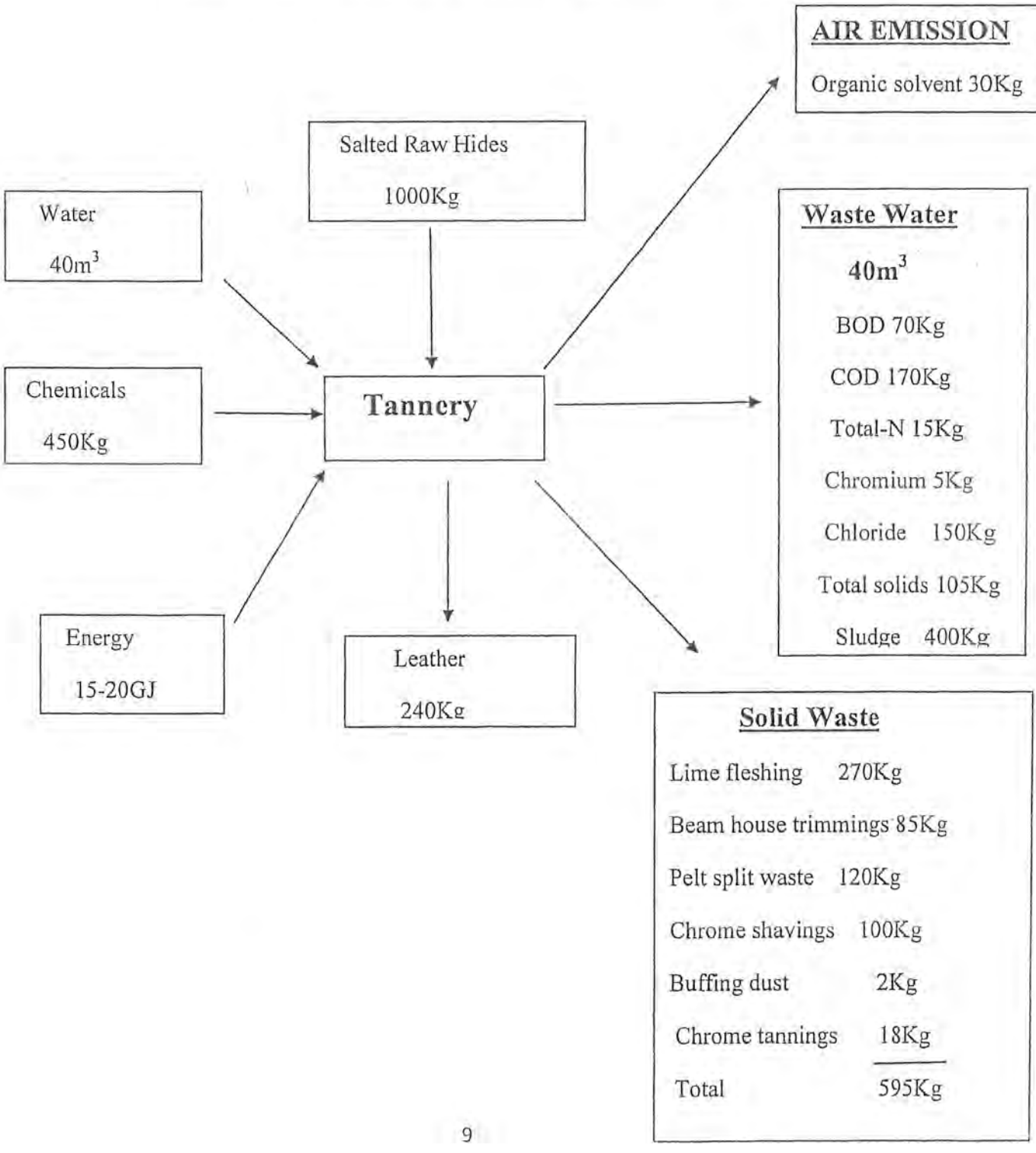
solid waste (Cot, 2004). Environmental pollution is a difficult problem for world leather industry (Cabeza *et al.*, 1998a).

1.4 CHROME SHAVINGS

Hides come to the tanner as a by-product of the meat industry. The tanning process, in turn, generates even greater quantities of by-products and wastes than of finished leather (Maire *et al.*, 1980). Of the solid wastes, chromium-containing shavings, trimmings and buffing are of most environmental concern (Cabeza *et al.*, 1998a). It has been stated by Berry *et al.*, 2002 that about 600,000 tonnes of solid waste is generated by leather industry each year worldwide and approximately 40–50% of the hides are lost to shavings and trimmings.

About 75% of the chromium-containing solid waste is produced when the tanned hide is shaved to a uniform thickness. These chrome shavings are small particles, in a variety of shapes, mainly consisting of collagen cross-linked with Cr (III) complexes which could be treated to give the potential resources of collagen protein and chromium (Heidemann, 1991). Historically shavings, trimmings and splits from the chrome tanning of hides and skins have been disposed of in the landfills. This is a rather expensive and environmentally inappropriate way of handling a waste material that has the potential for reutilization. Due to the restrictions on land disposal, tanning industry developed more useful and innovative methods to treat this waste product (Taylor *et al.*, 1997). Leather industries confront with disposal of solid wastes and they are responded with two strategies (I) minimizing the quantity of waste generated, and (II) maximizing the return on byproducts (Alexander *et al.*, 1991).

Typical Mass Balance for Leather Production using conventional technology (bovine hides)



1.5 LEATHER DUST AND LEATHER SCRAPS

When the treated animal skin (leather) is cut and shaped by the product manufacturing factories so that it can be used in making of shoes, garments, bags, and other leather items. During this process, a large quantity of minute leather dust falls off like saw dust. This leather dust is often disposed as such which causes severe environmental pollution as it contains chromium, sodium sulfide and other chemicals used during leather processing.

Disposal of leather scraps collected during the manufacturing of shoe uppers is also one of the major concerns for the footwear industry (Wilford, 1999). These solid leather wastes are often found to be unavoidable because of the nature of tanned leather and the pattern required by the shoe industry. Wilford in the year 1999 reported that approximately 0.4 million tons of waste lather is produced annually by the footwear industry. With small investment on further processing or transportation, some of these wastes may turn out to be marketable but the remainder still need to be disposed. Although, it is tedious and expensive but it has to be addressed because of their smell, noxious nature, or adverse effect on the surrounding land or water. It is vital for the shoe industry to devise a process that can treat and convert this waste into usable products. Extraction of gelatin and hydrolysable protein from wet blue shavings can serve as an option to reduce the disposal of leather scraps in landfills. It will be profitable for the shoe industry to convert this waste into value added products. This process may be extended to finished leather scraps as well

1.6 REUTILIZATION OF WASTE

Much of the waste generated from the leather industry has the potential to be reutilized. Some of the waste can be processed and recycled to produce useful by-products. For example, the hair pulp and shavings from the tannery solid waste, which are usually disposed, contain significant quantities of useful proteins like keratin and collagen, respectively. These proteins have demonstrated uses in pharmaceuticals, adhesives, cosmetics, films, encapsulations, etc (Taylor *et al.*, 1998a). The chromium from leather

shavings can be recycled to the tannery to produce good quality, sanitized hides for leather manufacture (Taylor *et al.*, 1998a). Reutilization of leather wastes will not only produce products of commercial value but will also result in the elimination of disposal costs, increasing the economic benefits of recycling.

For this purpose, the objective is three folds, (Brown *et al.*, 1996).

1) Development of methods for the recovery of valuable components from shavings and trimmings of chrome tanned leather.

2) Isolation of chrome free collagen fragments suitable for the use in leather finishing or for the manufacture of value-added products.

3) Separation of chromium from protein matrix.

The removal of chromium from solid wastes has been studied by methods such as air oxidation, (Okamura *et al.*, 1976) extraction, (Tancous *et al.*, 1981) incineration, (Veeger, 1994) and hydrolysis (Taylor *et al.*, 1996). The hydrolysis can be done by chemical and/or enzymatic methods (Shelly *et al.*, 1999).

Alkaline hydrolysis at elevated temperature and/or pressure has been used in many parts of the world for chrome recovery and isolation of protein fractions (Galatik *et al.*, 1988). During the last century, proteolytic enzymes have been used to solubilize collagen. Each proteolytic enzyme degrade molecule in a different way. Study by Cabeza *et al.*, 1997 shows that gelatin (collagen protein) is extracted without the use of enzyme but the hydrolysate (collagen protein) is isolated by enzymatic degradation of chrome shaving.

1.7 POTENTIAL USES OF PROTEIN HYDROLYSATES

The protein fractions isolated by the hydrolysis of chrome shavings have the following potential uses:

1. as organic nitrogenous fertilizer and animal feed (Brown *et al.*, 1996);

2. In preparation of films, (Brown *et al.*, 1996) inexpensive formaldehyde-free adhesives, cosmetics, paints, flocculating agents and fireproofing agents (Rose *et al.*, 1992).

3. as encapsulating agents for drugs, essential oils, perfumes etc. (Cabeza *et al.*, 1998);

4. As anti-skid agents in PVC and rubber compounds (Kolomaznik *et al.*, 2000);

5. As agents for increasing adhesion to textile backing in conveyor belt manufacture (Kolomaznik *et al.*, 2000);

6. As heat stabilizers in PVC paste (Kolomaznik *et al.*, 2000);

7. As an additive to concrete and plaster to influence setting time and rheological properties (Kolomaznik *et al.*, 2000);

8. In the manufacture of biodegradable polymers for agricultural applications (Kresalkova *et al.*, 2002);

9. In absorption of waste gases discharged from internal combustion engines (Okamura *et al.*, 1971);

10. As filler for leather to improve the uniformity (Chen *et al.*, 2001).

The end application for the protein hydrolysate depends on its composition, which in turn depends on the method of hydrolysis (Hafner *et al.*, 1987; Taylor *et al.*, 1989).

Aims and Objectives

Aims of the present study are,

- Isolation and identification of indigenous strain of *Bacillus subtilis* having potentials to produce extracellular protease.
- Production of extracellular protease in shake flask by submerged culture fermentation and optimization of different culture conditions for maximum enzyme production.
- Purification of crude enzyme filtrates using different biochemical techniques.
- Characterization of purified enzyme.
- To study the effect of this protease on leather dust degradation for biotreatment of leather industry solid waste.
- Biotreatment of chrome shaving with microbial protease for isolation of protein products.
- Development of method for recovery of gelatin from leather scraps from shoe upper leather and thereby, to reduce the amount of land filled solid waste produced by the footwear industries.

REVIEW OF LITERATURE

Enzymes catalyze chemical reactions without being consumed. Chemical reactions involving enzymes need lower amount of activation energy than reactions without enzymes for each molecule of substrate converted into product. Hence more number of molecules of substrates is consumed to yield the product. The reaction below depicts an enzyme-catalyzed chemical reaction in which an enzyme can be recovered and can be reused (Ritman and McCarty, 2001).

$$S + E \longrightarrow P + E$$

Where S represents the substrates, E represents the enzyme which is working as a catalyst, and P represents product of the reaction.

Enzymes are present in all living organisms. They are highly specific for the reaction they catalyze and one can not even think about the existence of life without enzymes. Enzymes can be produced from microorganisms, sometimes in remarkably large quantities by some strains of bacteria, yeast and fungi. Microorganisms provide an excellent source for enzymes as they can be cultured in large quantities in a short period of time. Microbial enzymes have an advantage over the enzymes obtained from plant or animal sources as microbial enzymes have longer stability under less than ideal conditions without significant loss of biological activity (Headon, 1994).

Industrial enzymes can be produced by employing the large scale fermentation process using the selected strains of harmless bacteria and yeast. Most of the enzymes obtained commercially from microbial fermentation procedures are hydrolases. They are either extracellular enzymes or intracellular enzymes. Extracellular enzymes are transported across the membrane, completely dissociated from cells and are found free into culture medium and can be separated from cells by filtration or centrifugation (Priest, 1984).

Enzymes play a major role in so many processes and their application is known for a long time. Precedence of using enzymes from microorganisms in baking, brewing alcohol, cheese making in ancient Greece are available. With better understandings and improvement in purification of enzymes, application of enzymes increases many folds. More and more enzymes have been engineered according to the demands of the processes

which could become possible only after an extensive research in the field of enzyme. Enzymes have widely been used in food, textile, and leather industries. Besides this, they are also used in meat and dairy industries, baking and flour processing, sugar production and wine brewing. Proteases and lipases are class of enzymes significantly used in leather industry for dehairing and degreasing the animal hides respectively. Enzymes also found application in textile industry as they are utilized in desizing, biopolishing, and biostoning of fabrics. Enzymes are also added in animal feed to degrade soluble fiber, reduce anti nutritional effects, and help supplement the animal's own digestive enzymes. Enzymes are also utilized in detergent industry as they bring down the washing temperatures and reduce cloth damage during washing (Godfrey and West, 1983).

2.1 PROTEOLYTIC ENZYMES

Proteases constitute one of the most important groups of industrial enzymes, accounting for at least 25% of global enzyme production (Herbert *et al.*, 1992). Proteolytic enzymes are found in all living organisms. They are essential for cell growth and differentiation. There is a renewed interest in the study of proteolytic enzymes. Recently, proteolytic enzymes had extensively been studied as they play a major role in the cellular metabolic processes besides being utilized in various industries (Gupta *et al.*, 2002a).

Proteolytic enzymes have received considerable attraction to elucidate the relationship between structure and function of enzymes (Noreau and Drapeau, 1979). Proteolytic enzymes such as EC 3.4.21-24 and 99; peptidyl-peptide hydrolases are employed to hydrolyze proteins by means of adding water across peptide bonds and catalyze peptide synthesis in organic solvents and in solvents with low water content (Sookkheo *et al.*, 2000; Beg *et al.*, 2003). Proteases of commercial importance are produced from microbial, animal and plant sources. They constitute a very large and complex group of enzymes with different properties of substrate specificity, active site and catalytic mechanism, pH and temperature activity and stability profiles. Industrial proteases have unique physical and catalytic properties which serves a major role in a range of industrial processes (Ward, 1991). Although, each protease is specific towards the reaction they take part in, but their physiological and biotechnological applications attracted researchers' worldwide (Rao *et al.*, 1998).

2.2 CLASSIFICATION OF PROTEASES

Proteases have been classified into 4 subgroups in group 3 (hydrolases) according to the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (Rao *et al.*, 1998). Proteases do not make an agreement with the general system of enzyme nomenclature as their mode of action is almost similar in all the reactions they participate. On the basis of the analysis of the vitro properties, proteases may be classified based on the following criteria:

i) type of reaction catalyzed,

ii) chemical nature of the catalytic site and

iii) evolutionary relationship with reference to amino acid sequence and protein structure (Rao *et al.*, 1998).

On the basis of their site of action on protein substrates, proteases have been classified as endo- or exoenzymes as shown in Table 2.1 (Rao *et al.*, 1998). Depending upon their catalytic mechanism, they have further been categorized as serine proteases, aspartic proteases, cysteine proteases, or metallo proteases. Further classification into different families and clans was done on the basis of their amino acid sequences and evolutionary relationships. They are referred to as acidic, neutral, or alkaline proteases based on the pH of their optimal activity (Rao *et al.*, 1998).

2.3 EXOPROTEASES

The exopeptidases act only near the ends of polypeptide chains. They are classified as amino- and carboxypeptidases on their site of action at the N or C terminus (Rao *et al.*, 1998).

Table: 2.1 Classification of proteases (Rao *et al.*, 1998)

Protease	EC no.
Exopeptidases	3.4.11
Aminopeptidases	3.4.14
Dipeptidyl peptidase	3.4.14
Tripeptidyl peptidase	3.4.16-3.4.18
Carboxypeptidase	3.4.16
Serine type protease	3.4.17
Metalloprotease	3.4.18
Cysteine type protease	3.4.15
Peptidyl dipeptidase	3.4.13
Dipeptidases	3.4.19
Omega peptidases	3.4.19
Endopeptidases	3.4.21-3.4.34
Serine protease	3.4.21
Cysteine protease	3.4.22
Aspartic protease	3.4.23
Metallo protease	3.4.24
Endopeptidases of unknown catalytic mechanism	3.4.99

2.3.1 Aminopeptidases

A single amino acid residue either a dipeptide or a tripeptide is liberated when aminopeptidases act at a free N terminus of the polypeptide chain. They are known to remove the N terminal Met that may be found in heterologously expressed proteins but not in many naturally occurring mature proteins. Aminopeptidases are present in a wide variety of microbial species including bacteria and fungi. Aminopeptidases can be considered as intracellular enzymes, but Rao and coworkers in 1998 reported an extracellular peptidase produced by *A. oryzae*.

2.3.2 Carboxypeptidases

The carboxypeptidases are characterized by their action at C terminals of the polypeptide chain and they liberate a single amino acid or a dipeptide. Based on the nature of the amino acid residue at the active site of the enzymes carboxypeptidases have been divided into three major groups, serine carboxypeptidases, metallo carboxypeptidases, and cysteine carboxypeptidases (Rao *et al.*, 1998).

2.4 ENDOPEPTIDASES

Endopeptidases attack on the inner peptide bonds of the polypeptide chain away from the N and C termini. There is a negative influence on the activity of enzyme due to the presence of the free amino or carboxyl group. The endopeptidases can be divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine and, (iv) metalloproteases (Rao *et al.*, 1998).

2.4.1 Serine proteases

Serine proteases contain a serine group in their active site and their presence is common in viruses, bacteria, and eukaryotes validating their importance to the organisms (Rao *et al.*, 1998). Serine proteases are recognized by their irreversible inhibition by 3, 4-dichloroisocoumarin (3, 4-DCl), L-3 carboxytrans 2, 3-epoxypropyl-leucylamido (4-guanidine) butane (E-64), diisopropylfluorophosphate (DFP), phenyl methyl sulfonyl fluoride (PMSF) and tosyl-L-lysine chloromethyl ketone (TLCK). Serine proteases show their optimum activity between pH 7 to 11. They show higher degree of substrate specificity. Their molecular masses range between 18 and 35 kDa. The isoelectric points of serine proteases are generally between pH 4 and 6. Serine alkaline proteases having greater activity at highly alkaline pH represent the largest subgroup of serine proteases (Rao *et al.*, 1998).

Serine alkaline proteases are produced by several bacteria, molds, yeast, and fungi. They can hydrolyze a peptide bond containing tyrosine, phenylalanine, or leucine at the carboxyl site of the splitting bond. The optimal pH of alkaline proteases is around pH 10,

and their isoelectric point is around pH 9. Their molecular weights are recorded in the range at 15 and 30 kDa. Serine proteases are produced by several bacteria such as *Arthrobacter*, *Streptomyces*, and *Flavobacterium* sp.but subtilisins produced by *Bacillus* sp. are the best known ones (Rao *et al.*, 1998).

Subtilisins of *Bacillus* origin represent the second largest family of serine proteases. Two different types of alkaline proteases, subtilisin Carlsberg, and subtilisin Novo or bacterial proteases Nagase (BPN'), have been identified.

Bacillus amyloliquefaciens is responsible for the production of Substilisin Novo or BPN which is commercially less important. The molecular weight of both substilisins is 27.5 kDa while they differ from each other by 58 amino acids. They have similar properties such as an optimal temperature of 60 °C and an optimal pH of 10 (Rao *et al.*, 1998).

2.4.2 Aspartic Proteases

Aspartic acid proteases are commonly known as acidic proteases. The catalytic activity of these endopeptidases depends up on the aspartic acid residues. Acidic proteases have been divided into three groups namely pepsin, retropepsin, and enzymes from pararetroviruses. Most aspartic proteases show maximal activity at low pH and have isoelectric points in the range of pH 3 to 4.5. Their molecular weights are in the range of 30 to 45 kDa. The aspartic proteases are inhibited by pepstatin (Rao *et al.*, 1998).

These proteases are widely distributed in animal cells, moulds and yeast but are seldom found in bacteria. Many of them contain aspartate residues at their active site and they show specificity towards aromatic or bulky amino acid residues at both sides of splitting point (Matsubara and Feder, 1971).

2.4.3 Cysteine / thiol Proteases

Cysteine proteases are observed in both prokaryotes and eukaryotes. Most of the time the activity of cysteine proteases are observed only in the presence of reducing agents such as HCN or cysteine. In catalytic mechanism of cysteine peptidases the thiol group of a single cysteine residue plays a key role. This group can undergo oxidation and can react with a variety of reagents; heavy metals, iodoacetate, N-ethyl-maleimide etc. They have

been divided into four groups based on their side-chain specificity (i) papain-like, (ii) trypsin-like (iii) specific to glutamic acid and, (iv) others. Papain is the best-known cysteine proteases. They show optimum activity under neutral condition (Kenny, 1999).

2.4.4 Metalloproteases

These are commercially important proteases containing a metal ion, which is involved in the catalytic activity. There are two major groups of metallo proteases, which are neutral and alkaline metallo protease. Neutral proteases contain an essential metal atom, usually zinc, and have pH optima near neutral. They are widely distributed among microorganisms and have specificity towards hydrophobic amino acid on the amino side of the peptide linkage being attacked. These enzymes are produced with particular abundance by *Bacillus* species (Malathi and Chakraborty, 1991).

2.5 COMMERCIAL APPLICATION OF MICROBIAL PROTEASES

2.5.1 Detergent Industry

Proteases have a wide range of applications in detergent industry as they are one of the standard ingredients of all kind of detergents. The use of proteases in laundry detergents accounts for approximately 25% of the total worldwide sales of enzymes (Godfrey and West, 1996). Rohm Company in Germany isolated the first enzyme preparation for industrial use, in 1914 (Lesney, 2003). They used a trypsin enzyme isolated from animal's pancreas that degraded proteins. It proved to be so powerful compared to traditional washing powders that the original small package size "made the German housewives suspicious; the product had to be reformulated and sold in larger packages" (Leisola *et al.*, 2001). The real breakthrough of enzymes occurred with the introduction of microbial proteases into washing powders. The first commercial bacterial *Bacillus* protease was marketed in 1956, under the trade name BIO-40 by the Swiss Company Gebrüder Schnyder. In 1962, Novo Nordisk, in Denmark, was the first company to mass-produce an alkaline protease suitable for wash conditions; they introduced Alcalase, produced by *Bacillus licheniformis* and commercially named BIOTEX. This was followed by Maxatase, a detergent made by Gist-Brocades (Lesney, 2003). The biggest

market for detergents is in the laundry industry, amounting to a worldwide production of 13 billion tons per year.

An ideal detergent protease must have broad substrate specificity to facilitate the removal of a large variety of stains (food, blood, grass, and body secretions). The proteases are used in detergent industry if they have excellent activity and stability at high pH and temperature, and compatibility with other chelating and oxidizing agents. The performance of a protease in a detergent depends hugely on the pI (ionic strength) value. For the best performance of a protease in a detergent, its pI (ionic strength) should coincide with the pH of the detergent solution. Esperase and Savinase T (Novo Industry) produced by alkalophilic *Bacillus* sp., are two commercial preparations with very high isoelectric points (pI 11.0): hence, they can withstand higher pH ranges. Since proteases are active at low temperatures they aid in energy conservation. A notable enhancement in the performance of protease in laundery detergents is expected if a combination of lipase, amylase, and cellulose is employed (Anwar and Saleemuddin, 1998).

Most of the detergent proteases utilized in industry serine proteases produced by *Bacillus* strains. Fungal alkaline proteases are advantageous due to the ease of downstream processing to prepare a microbe-free enzyme. An alkaline protease from *Conidiobolus coronatus* was also used as it is found to be compatible with commercial laundry detergents used in India (Phadatare *et al.*, 1993). A significant percentage (43%) of its activity was achieved at 50 °C for 50 min., in the presence of 25 mM Ca^{2+} and 1 M glycine (Bhosale *et al.*, 1995).

2.5.2 Food Industry

Proteases had been used in the food industry since ancient times. They served various purposes such as cheese making, baking, preparation of soy hydrolysates, and meat tenderization.

2.5.3 Dairy Industry

Law and Wigmore (1982) tested a range of protease for their ability to accelerate cheese ripening and demonstrated that, neutral proteases enhance flavor significantly without producing bitterness. The major application of proteases in diary industry is in cheese making, which accounts for 10% of the total industrial enzyme market (Burgess and Shaw, 1983).

The microbial enzymes showed two major disadvantages, *i.e.*, (a) the bitterness in cheese, after storage because of the presence of high levels of nonspecific and heat-stable proteases; and (b) a low yield. An extensive research on this matter has resulted in the production of enzymes that are completely inactivated at normal pasteurization temperatures and contain very low levels of nonspecific proteases (Godfrey and West, 1996).

2.5.4 Baking Industry

An important consideration in the safety assessment for recombinant enzymes is the safety of the source organism. Enzymes from A*spergillus oryzae* are used in production of drinks, juices, syrups, alcohols, chocolates, chocolate syrups, brewing, and baking because of the availability of *Aspergillus oryzae* in nature and its non-pathogenicity (Cheevadhanarak *et al.*, 1991). The enzyme is used in the baking industry as a processing aid to strengthen gluten in dough systems. It results into making of a more elastic and stronger gluten network similar to that resulted by traditional oxidizing agents such as potassium bromate. The enzyme is active in the dough and the leavening of the unbaked bread, but normally it gets inactivated at elevated temperatures during the baking. The enzyme is present during the processing, but is not expected to be present in the final food. Any residue left during the making of the food would be in the form of inactivated enzyme, which would be metabolized like any other protein (Tatsumi *et al.*, 1991).

2.5.5 Brewing Industry

The introduction of commercial enzymes, including proteases to the brewing process has contributed to the overall economics and flexibility of the brewing operation. Proteases have two major applications in brewing. They may be used during the cereal mashing process to increase the yield of extract and the level of α-amino nitrogen of the wort produced. Proteases are also used during the finishing stages of beer production to remove chill haze from beer (Felix and Villettaz, 1983).

2.5.6 Manufacture of Soy Products

Soybean protein constitutes about 46 percent of soybean meal and is used as an ingredient in animal feed, human food, and industrial products. About 95 percent of soybean meal is used to supply protein in livestock (poultry, swine, and cattle) feeds. For special foods, such as those destined for children, old people or athletes, food proteins are hydrolyzed. In general, food proteins are hydrolyzed for many reasons ranging from the improvement of nutritional and functional properties, texture characteristics to the removal of odor, flavor, and toxic or anti nutritive components. The most commonly used proteins in hydrolysis treatments are casein, whey and soy protein (Periago *et al.*, 1998). Seed of soybean contains high concentrations of protein and oil which makes it an important crop (Liang, 1999).

Compared to acid or alkali hydrolysis, enzymatic hydrolysis of protein, using selective proteases, provides more moderate conditions of the process and few or no undesirable side reactions or products. In addition, the final hydrolysate after neutralization contains fewer salts and the functionality of the final product can be controlled by selection of specific enzymes and reaction factors (Chiang *et al.*, 1999; Madsen *et al.*, 1997). Enzymatic modification of proteins, using selected proteases to split specific peptide bonds, is widely used (Turgeon *et al.*, 1992).

Soy protein hydrolysates have found applications in specialized adult nutritional formula usually in combination with other protein hydrolysates or intact proteins (Mahnoud, 1994). Enzyme modifications of soy proteins could offer the second or third generation of

products that might allow an even broader range of utilization. Actually, in one area this is already achieved by using a pepsin-digested soy protein product to make a whipping protein for egg white replacement (Hrčková *et al.*, 2002).

2.5.7 Medicine

Use of protease is also encouraging in the medicine such as urokinase, which is used for treatment of clotting disorders .*Aspergillus* and plant proteases are used as digestive aid in gastrointestinal disorders such as dyspepsia. Proteases have been claimed to dissolve films of material which prevent the healing of gastric ulcer and in the debridement of wounds, burns and blood clots. The protease of *Bacillus* and *Aspergillus* origin are being used in tooth pastes and mouth washes and are effective in the removal of plaque deposition on surface of teeth, retard dental calculus or tartar formation.(Gupta *et al.*, 2002b). Kudrya and Simonenko (1994) exploited the elastolytic activity of *B. subtilis* 316M for the preparation of elastoterase, which was applied for the treatment of burns, purulent wounds, carbuncles, furuncles and deep abscesses. Kim *et al.*, (2001) reported the use of alkaline protease from *Bacillus* sp. strain CK 11-4 as a thrombolytic agent having fibrinolytic activity (Gupta *et al.*, 2002b). Furthermore, *Bacillus* sp. has been recognized as being safe to human (Kumar and Takagi, 1999).

2.5.8 Nitrogen Recycle

Proteases play an important role in the nitrogen recycle that contributes to soil fertility. In paddy field soil, most of the nitrogen source is stored as biomass of protein and decomposes slowly to low molecular weight amino acids by the activity of soil protease (Sato and Omura, 1989).

2.5.9 Photographic Industry

An important role of alkaline proteases is reported in the bioprocessing of used X-ray or photographic films for silver recovery. 1.5-2.0% silver by weight is present in the gelatin layer of these waste films. The recovered silver from waste films can be reused. The conventional way of recovering silver from waste film is burning these waste films but

this causes a huge environmental pollution. Furthermore, base film made of polyester cannot be recovered by burning the waste films. A proteolytic treatment can be used to extract the silver which is bound to gelatin. By employing the enzymatic hydrolysis we can extract silver in addition to recovering the polyester film base that can be recycled (Gupta *et al.*, 2002b). Fujiwara and co-workers (1991) studied on this interesting application of alkaline proteases. According to them, an alkaline protease can be used to decompose the gelatinous coating of X-ray films to recover silver. Protease B18' shows it optimum activity at higher pH and temperature, around 13.0 and 85 °C. The enzyme was most active toward gelatin on film at pH 10.

An obligate alkaliphilic *Bacillus sphaericus* strain was isolated from alkaline soils in the Himalayas by Singh *et al.*, (1999) which produced an extracellular alkaline protease. The gelatin layer of X-ray films was hydrolyzed by using the strain of this alkaline protease within 12 min at 50 °C and at pH 11.0 (Singh *et al.*, 1999).

2.5.10 Silk Degumming

Silk industry is one of the least explored industries as far as application of protease is concerned. This is quite evident by the low number of patents filed describing the use of proteases for the degumming process of silk. The conventional method of silk degumming process is generally expensive and therefore an alternative method was much needed. An idea suggested was the use of protease preparations for degumming the silk prior to dyeing. An alkaline protease from *Bacillus* sp. RGR-14 was studied for silk degumming efficiently. A weight loss of 7.5% was observed after 5h of incubation of silk fiber with protease from *Bacillus* sp. The clusters of silk fibers had fallen apart as compared with the smooth and compacted structure of untreated fiber which was investigated by using the Scanning electron microscopy (Gupta *et al.*, 2002b).

2.6 SOURCES OF PROTEASES

Proteases execute a large variety of complex physiological functions. They are really important in conducting the essential metabolic and regulatory functions which can be understood by their presence in all forms of living organisms (Wandersman, 1989).

2.6.1 Plant Proteases

Plants are used as source of protease which depends on a number of factors such as the availability of land cultivation and the climatic conditions for growth. The production of protease from plant is genuinely cheap but it is too much time consuming. Papain, bromelain, keratinases, and ficin represent some of the well-known proteases of plant origin (Rao *et al.*, 1998).

2.6.1a Papain

Papain, a traditional plant protease with a long history of its usage, is extracted from the latex of papaya fruits (*Carica papaya*) which are grown in subtropical areas of west and central Africa and Asia (Tanzania, Uganda, Zaire, Sri Lanka, Thailand and India). The crude preparation of the enzyme has a wide specificity due to the presence of various proteinase and peptidase isozymes. The performance of the enzyme depends on various factors like the plant source, the climatic conditions for growth, the methods used in its extraction and purification; for example, the greener the fruit, the more active is the enzyme. It has been investigated that papain is active between pH 5.0 and 9.0 and is stable up to 80 or 90 °C, in the presence of substrates. It is thoroughly used in industry as a meat tenderizer. It has also other applications in the pharmaceutical, detergent, and veterinary industries, as well as other sectors of the food industry (Ward, 1985).

2.6.1b Bromelain

Bromelain is a cysteine protease which can be prepared from the juice of pineapple (*Ananas comosus*), supplied by Thailand and Africa. This enzyme is also used to tenderize meat like papain. It is also a stabilizer for latex paints and is an efficient additive in leather-tanning chemical mixture. In modern therapy, bromelain is taken as a digestive and used for its anti-inflammatory action after surgery (Yamada *et al.*, 1976). This enzyme is found to be active between pH 5 to 9 and its activation temperature is 70 °C which is lower than that of Papain.

2.6.1c Keratinases

Keratinases are produced by some insects and microorganisms. They are mainly serine proteases except the keratinase from yeasts which belong to the aspartic protease. They show higher activity in an alkaline environment and their optimal activity is reported up to 50 °C.

2.6.2 Animal Proteases

Pancreatic trypsin, chymotrypsin, pepsin and rennin are examples of animal proteases. These can be prepared in pure form in bulk quantities. Production of these proteases depends on the availability of livestock for slaughter, which in turn is governed by political and agricultural policies (Rao *et al.*, 1998).

2.6.2 a Trypsin

Trypsin is one of the three principal digestive proteinases which act with the other proteinases to break down dietary protein molecules to their component peptides and amino acids. Trypsin continues the process of digestion (began in the stomach) in the small intestine where a slightly alkaline environment (about pH 8.0) promotes its maximal enzymatic activity. Trypsin, produced in an inactive form by the pancreas, is found to be similar in chemical composition and in structure to the other chief pancreatic proteinase, chymotrypsin. Both enzymes have similar mechanisms of action; residues of histidine and serine are found in the active sites of both enzymes (The Columbia Encyclopedia, Sixth Edition). This enzyme is active only against the peptide bonds in protein molecules that have carboxyl groups donated by arginine and lysine (Baptista *et al.*, 1998). Trypsin is the most discriminating of all the proteolytic enzymes in terms of the restricted number of chemical bonds that it will attack. Good use of this fact has been exploited by chemists interested in the determination of the amino acid sequence of proteins; trypsin is widely employed as a reagent for the orderly and unambiguous cleavage of such molecules (Murphy and Fagain, 1996).

2.6.2 b Chymotrypsin

Chymotrypsin is a proteolytic or protein-digesting enzyme which is active in the mammalian intestinal tract. It catalyzes the hydrolysis of peptide bonds in which the carboxyl groups are provided by one of the three aromatic amino acids (phenylalanine, tyrosine or tryptophan). Chymotrypsin is produced in the pancreas as inactive, or zymogen, form chymotrypsinogen, and is activated by trypsin in a multistep process. Partly because it was one of the first enzymes available commercially in crystalline form, chymotrypsin has been studied extensively (Free online encyclopedia). Pure chymotrypsin is an inexpensive enzyme and is used only for diagnostic and analytical application (Binnie *et al.*, 1995).

2.6.2 c Pepsin

Pepsin is an enzyme produced in the mucosal lining of the stomach that acts to degrade protein. In the laboratory studies, pepsin is the most efficient in cleaving bonds involving the aromatic amino acids, phenylalanine, tryptophan, and tyrosine. Pepsin is synthesized in an inactive form by the stomach lining; hydrochloric acid, also produced by the gastric mucosa, is necessary to convert the inactive enzyme and to maintain the optimum acidity (pH 1.0–3.0) for pepsin function (Columbia encyclopedia). Pepsin is an aspartyl protease and resembles human immunodeficiency virus type 1 (HIV-protease, responsible for the maturation of HIV-1 (Blundell *et al.*, 1991). Pepsin was used in laundry detergents as early as 1913, but is now being replaced by a mixture of serine and metal microbial proteases that appear to be less degradable by soaps in alkaline conditions and at high temperatures (Adinarayana and Ellaiah, 2002).

2.6.2d Rennin

Rennet is a pepsin-like protease having the property of clotting, or curdling milk. Rennet is obtained from the stomach of young mammals living on milk, especially from the inner lining of the fourth, or true, stomach (abomasum) of milk-fed calves [encyclopedia webpage]. Rennet is produced as an inactive precursor prorennin and is converted to active rennin by the action of pepsin or by its autocatalysis. It is used extensively in the

making of cheese and junket. The specialized nature of the enzyme is due to its specificity in cleaving a single peptide bond in κ-casein to generate insoluble *para*-κ-casein and C-terminal glycopeptides (Rao *et al.*, 1998).

2.6.3 Microbial Proteases

The plant and animal proteases could not meet up with the world demands and this led to an increased interest and extensive studies towards the microbial proteases. Proteases from microbial sources have all essential characteristics required for their biotechnological applications (Rao *et al.*, 1998). Approximately 40% of the total worldwide enzyme sales are of microbial proteases and 90% of all enzymes materials produced for industrial processing are enzymes produced by the fermentation of microorganisms (Godfrey and West, 1996). Despite the fact that proteases are widespread in nature, microbes are preferred as the source of these enzymes. Characteristics such as rapid growth, limited space required for their cultivation, and the ease with which they can genetically be manipulated to generate new enzymes with specific altered properties, made these enzymes desirable for various applications in biotechnology (Beynon and Bond, 1989). Microbial proteases are extracellular in nature. They are directly secreted into the fermentation broth by the producer, thus simplifying downstream processing of the enzyme as compared to proteases recovered from plants and animals (Gupta *et al.*, 2002).

2.6.3 a Fungi

Fungi produce a broader variety of enzymes than do bacteria. Some fungi, as *Aspergillusoryzae*, can produce acid, neutral, and alkaline proteases. The fungal proteases evidence wide substrate specificity and are active over a broad pH range (4.0 to 11.0). But, they present a lower thermal tolerance and reaction rate than do bacterial enzymes (Cheevadhanarak *et al.*, 1991). Fungal acid proteases are stable between pH 2.5 and 6.0 and have an optimal pH between 4.0 and 4.5. Due to their temperature specificity and narrow pH, they became particularly useful in the cheese making industry. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating

agents. The action of plant, animal, and bacterial proteases in reducing the astringency of food protein hydrolysates is supplemented by the fungal neutral proteases. Fungal alkaline proteases are also used in food protein modifications (Berka *et al.*, 1990).

2.6.3 b Viruses

The cleavage of large proteins into smaller fragments by proteases is important for every form of life including viruses. Many human viruses such as herpes simplex virus, hepatitis C virus and HIV need proteases for efficient growth and replication inside the infected host cells. Serine, aspartic, and cysteine peptidases are found in various viruses (Rawlings and Barrett, 1993). All of the virus encoded peptidases are endopeptidases; there are no metallopeptidases. Human immunodeficiency virus (HIV), a causative of AIDS, presents an aspartic protease essential for the retroviral life cycle and it has been a good target for the chemotherapy with specific inhibitors. An extensive research had been carried out on the expression, purification, and enzymatic analysis of retroviral aspartic protease and its mutants (Kuo and Shafer, 1994).

2.6.3 c Bacteria

Among bacteria, the *Bacillus sp.* is specific producers of extracellular proteases (Spinosaa, 2000). *Bacillus subtilis* proteases have wider specificity than trypsin and chymotrypsin. They possess a number of industrially valuable properties including their ability to excrete several different hydrolytic enzymes into culture medium. The lack of Pathogeneicity and the ability to grow in simple culture medium can also be accounted for its application in industry (Daniel, 1984). *Bacillus* genus produces mainly neutral and alkaline proteases. The optimal activity of bacterial neutral proteases is reported in a pH range of 5.0–8.0 with relatively low thermal tolerance. This characteristic permits the control of their reactivity during food hydrolysates production with a low degree of hydrolysis. Owing to their intermediate rate of reaction, neutral proteases are valuable in the food industry as they generate less astringency in hydrolyzed food proteins than do the animal proteinases (Brueckner *et al.*, 1990). The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acids pairs. Some of the

neutral proteases are serine proteinases, which are not affected by chelating agents, while others are metalloprotease type and require divalent metal ions for their activity (Akimkina *et al.*, 1992).

Bacterial alkaline proteases are characterized by their high activity at alkaline pH, *e.g.*, pH 10.0, their wide substrate specificity and their optimal temperature over 60 °C. These properties make them appropriate for use in the detergent industry (Anwar and Saleemuddin, 1998). Alkaline proteases produced by thermophilic and alkaliphilic bacilli have a high tolerance for extreme temperature, pH, and chemical denaturing agents in non-aqueous environments (Johnvesly and Naik, 2001).

Bacteria belonging to *Bacillus* sp. are the most important source of several commercial microbial enzymes. They can be cultivated under extreme temperature and pH conditions to give products which can withstand a wide range of harsh environments. *Bacillus* is a rod-shaped, gram positive, spore forming, aerobic, usually Catalase positive, chemoorganotropic bacterium. Alkaliphilic *Bacillus* can be found mostly in alkaline environments such as soda soils, soda lakes, neutral environments and deep-sea sediments. Animal manure, man-made alkaline environments such as effluents from food, textile, tannery, and potato processing units, paper manufacturing units, calcium carbonate kilns and detergent industry are also good sources (Debabov, 1982).

2.7 ALKALINE PROTEASES OF *BACILLUS* STRAINS

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.*, 2002b).

Alkaline proteases are important group of enzymes physiologically and commercially and are used primarily as detergent additives. They have broad substrate specificities and will act to some extent under the extreme conditions encountered in domestic washing temperatures of 20 to 70 °C, a pH up to 11 and at high concentrations of detergents,

polyphosphates, chelating agents such as EDTA and oxidizing agents such as sodium perborate (Cowan, 1996).

Alkaline proteases of microbial origin dominate the worldwide enzyme market. They have a huge industrial potential because of their biochemical diversity and wide applications in tannery and food industries. In addition to food industry they are also helpful in medicinal formulations, detergents and processes like waste treatment, silver recovery and resolution of amino acid mixtures (Agrawal *et al.*, 2004; Gupta *et al.*, 2002a).

A wide range of microorganisms including bacteria, molds, yeasts and also mammalian tissues are responsible for the production of alkaline proteases (Singh *et al.*, 2001a). Despite a renewed interest in other microbial sources, a number of studies indicate that bacteria are by far the most popular source of commercial alkaline proteases to date. Bacterial alkaline proteases have high activity at alkaline pH, e.g., pH 10 and their broad substrate specificity. The optimal temperature is reported be around 60 °C. All these properties of bacterial alkaline proteases make them suitable for use in the detergent industry (Rao *et al.*, 1998).

From all the alkaliphilic bacteria that have been screened for use in various industrial applications, 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).

The most dominant group of alkaline protease producers are bacteria with the genus *Bacillus* being the most prominent source. *Bacillus* species from many different exotic environments have been explored and exploited extensively for alkaline protease production but most potential alkaline protease producing bacilli are strains of *B. licheniformis*, *B. subtilis*, *B. amyloliquefaciens*, and *B. majovensis* (Gupta *et al.*, 2002a). High temperature, pH, chemical denaturing agents and in non-aqueous environments tolerance is reported for the alkaline proteases produced by thermophilic and alkaliphilic bacilli (Johnvesly and Naik, 2001).

To understand the potential industrial applications of different proteases, various types of alkaline protease have been characterized. These enzymes can be used in detergent formulation, the food industry, leather processing, chemical synthesis and waste management (Gupta *et al.*, 2002a). In Table 2.2 some industrially important alkaline proteases produced from various *Bacillus* sp. are tabulated.

Table 2.2: Some industrially important alkaline proteases produced from various *Bacillus* sp. Anwar *et al.*, 1998

Species	optimum/stability	application(s)
Bacillus stearothermophilus	9.5	Detergents and heavy duty laundry powders
Bacillus sp. Y. (BYA)	10.0-12.5	Detergent formulations
Bacillus licheniformis	8.2	amino acids Catalyst for N-protected
Bacillus sp. (AH-101)	12.0-13.0	Dehairing / leather industry
Bacillus firmus	8	Detergent industry
Bacillus sp.(P-001A)	9.5	Production of biomass from natural waste
Bacillus licheniformis(Alcalase)	8.2	Active peptides
Bacillus subtilis	8.5	Bating agent in leather industry

2.8 EXTRACELLULAR ALKALINE PROTEASE PRODUCTION BY BACTERIA

2.8.1 Screening and selection of Strains

During the primary stage of development of an industrial fermentation, strain (s) capable of producing the target product in commercial yields is isolated. A large number of strains have to be screened intensively to identify high producers with novel properties. A conventional practice with many extracellular microbial products is to grow a large number of organisms on agar plate media and to compare each organism's production capability to the radius of the product's zone of diffusion around the colony. In the course

of designing a medium for screening proteases, it is essential that the medium should contain likely inducers of the product and be devoid of constituents that may repress enzyme synthesis (Kumar and Takagi, 1999).

The proteolytic bacteria when grown on casein agar or milk agar can produce a zone of clearance around their colonies which distinguished them from non proteolytic bacteria. It has been reported that *B. licheniformis* produces very narrow zones of hydrolysis on casein-agar despite being very good protease producers in submerged cultures. (Mao *et al.*, 1992) A similar observation was made of *Aspergillus oryzae* and *A. sojae* by Nasuno and Ohara *et al.*, 1971. So, no significant correlation has been reported between the size of clearance zone and quantity of protease produced under fermentation conditions.

2.8.2 OPTIMIZATION OF FERMENTATION MEDIUM

Submerged fermentation is employed for the production of alkaline proteases. Solid state fermentation has been used less frequently for the production of these enzymes (Chakraborty and Srinivasan 1993; Malathi and Chakraborty 1991; George *et al.*, 1992). The optimization of medium composition is done to maintain a balance between the various medium components so that the amount of unutilized components can be minimized. An extensive research has been carried out towards: (i) evaluation of the effect of various carbon and nitrogenous nutrients as cost-effective substrates on the yield of enzymes; (ii) requirement of divalent metal ions in the fermentation medium; and (iii) optimization of environmental and fermentation parameters such as pH, temperature, aeration, and agitation. Each organism or strain has its own special conditions for maximum enzyme production (Kumar and Takagi, 1999).

2.8.2 a Nitrogen source

Both inorganic and organic forms of nitrogen are metabolized, in most microorganisms to produce amino acids, nucleic acids, proteins, and cell wall components. An alkaline protease contains 15.6% nitrogen (Kole *et al.*, 1988) and its production depends on the availability of both carbon and nitrogen sources in the medium (Kole *et al.*, 1988). The requirement for a specific nitrogen supplement for the production of alkaline protease

differs from one organism to another organism. A lower amount of alkaline protease production was produced when an inorganic nitrogen source was used in the production medium (Sen and Satyanarayana, 1993, Chandrasekaran and Dhar, 1983, Chaphalkar andDey, 1994). Enzyme synthesis was found to be repressed by rapidly metabolizable nitrogen sources such as amino acids or ammonium ion concentrations in the medium (Frankena *et al.*, 1986, Giesecke *et al.*, 1991). On the contrary, several reports have demonstrated the use of organic nitrogen sources leading to higher enzyme production than the inorganic nitrogen sources. Fujiwara and Yamamoto, 1987 recorded maximum enzyme yields using a combination of 3% soybean meal and 1.5% bonito extract. Soybean meal was also reported to be a suitable nitrogen source for protease production (Sen and Satyanarayana, 1993; Chandrasekaran and Dhar, 1983; Tsai *et al.*, 1988; Cheng *et al.*, 1995).

It has been observed that the addition of certain amino compounds was effective in the production of extracellular enzymes by alkalophilic *Bacillus* sp. (Ikura and Horikoshi, 1987). Glycine was reported to inhibit the production of both amylase and protease. Casamino acids showed an inhibitory effect on the production of protease (Ong and Gaucher, 1976). Applying oil cakes as a nitrogen source did not turn out to be advantageous in enzyme production (Sen and Satyanarayana, 1993; Sinha and Satyanarayana, 1991).

2.8.2 b Carbon source

A reduction in protease production is reported because of the catabolite repression by glucose (Kole *et al.*, 1988, Frankena *et al.*, 1986, Hanlon *et al.*, 1982). Zamost *et al.* (1990) observed that the yields of protease production decreases with the decrease in pH brought about by the rapid growth of the organism. A high carbohydrate concentration shows an inhibitory effect on the enzyme production. Hence, 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. An increase in yields of alkaline proteases by using different sugars such as lactose (Malathi and Chakraborty, 1991), maltose (Tsuchiya *et al.*, 1991), sucrose

(Phadatare *et al.*, 1993) and fructose (Sen and Satyanarayana, 1993) were reported by several workers.

2.8.2 c pH and temperature

A strong dependence on the extracellular pH for cell growth and enzyme production is one of the most important characteristics of microorganisms. Many enzymatic processes and transport of various components across the cell membrane are affected strongly by the culture pH (Moon and Parulekar, 1991). A medium with neutral initial pH has been reported for alkaline protease production by *Bacillus pumilus* c172 (pB×96) transformants (Yaoyu *et al.*, 1997) while media with acidic pH have been reported for alkaline protease production by bacteria such as *Bacillus coagulans* (Gajju *et al.*, 1996), *Bacillus licheniformis* (Mao *et al.*, 1992), slightly alkaline media have been reported for production by *Bacillus alcalophilus* sub sp.*halodurans* KP1239 (Takii *et al.*, 1990) and *Bacillus licheniformis* N3 (Sen and Satyanarayana, 1993).

Temperature is another crucial parameter for the optimization of fermentation medium that has to be controlled and varied from organism to organism. The mechanism of temperature control on enzyme production is not well documented (Chaloupka, 1985). However, studies by Frankena *et al.*, 1986 showed that there is a link between enzyme synthesis and energy metabolism in *Bacilli*, which was controlled by temperature and oxygen uptake. The optimum temperature values reported for maximum protease production are given in Table 2.3.

Table 2.3: Optimum temperature values for maximum protease production

Optimum Temperature(°C)	Organism	[Reference]
28	*Penicillum griseofulvin*	Dixit and Verma,1993
30	*Bacillus* sp. B21-2 *Streptomyces diastaticus*	Fujiwara and Yamamoto, 1987 Chaphalkar and Dey,1994
32	*Aspergillus flavus*	Malathi and Chakraborty,1991
35	*Bacillus* sp. Y	Shimogaki *et al.*,1991
36	*Bacillus licheniformis* *Bacillus* sp. no. AH-101	Mao *et al.*,1992 Takami *et al.*,1989
37	*B. firmus*	Moon and Parulekar,1991
39.5	*Bacillus licheniformis*	Hübner *et al.*,1993
40	*Bacillus* sp. strain B189	Fujiwara *et al.*,1987
45	*Bacillus lichenformis*	Sen and Satyanarayana.,1993
55	*B. stearothermophilu*AP 4	Dhandapani and Vijayaragavan.,1994
60	*B. stearothermophilus* F1	Rahman *et al.*,1994

2.8.2 d Aeration and agitation

The aeration rate indirectly indicates the dissolved oxygen level in the fermentation broth during fermentation. Different dissolved oxygen profiles can be obtained by: (i) variations in the aeration rate; (ii) variations in the agitation speed of the bioreactor; or (iii) use of oxygen-rich or oxygen-deficient gas phase (appropriate air-oxygen or air-nitrogen mixtures) as the oxygen source (Moon and Parulekar, 1991). The variation in the agitation speed influences the extent of mixing in the shake flasks or the bioreactor and consequently affects the nutrient availability.

Although the cultures for bacterial alkaline protease production are generally agitated but its specific effect on the production has been studied rarely. The enhancing effect of agitation on alkaline protease production has been reported in *Bacillus licheniformis* S40 which required agitation at 200 rpm (Sen and Satyanarayana, 1993). The agitation rates reported in case of *Bacillus* sp.IS-3 (Purva *et al.*, 1998) and *Bacillus* sp. (Takami *et al.*, 1989) were 200 and 300 r.p.m respectively.

The effect of aeration in enhancing alkaline protease production has been reported in *Bacillus subtilis* (Hameed *et al.*, 1996) *Bacillus* sp.B21-2 (Fujiwara and Yamamoto, 1987).

2.8.2 e Metal Ion Requirement of Alkaline Proteases

Alkaline proteases require a divalent cation like Ca^{2+}, Mg^{2+} and Mn^{2+} or a combination of these cations, for maximum activity. These cations were also found to enhance the thermal stability of a *Bacillus* alkaline protease. It is believed that these cations protect the enzyme against thermal denaturation and play a vital role in maintaining the active conformation of the enzyme at high temperatures (Kumar and Takagi, 1999).

2.9 PROPERTIES OF ALKALINE PROTEASES

2.9.1 Optimum pH and stability of Alkaline Proteases

Enzymes are amphoteric in nature as they contain a large number of acidic and basic groups, mainly located on their surface. The charge on these groups depends on their acid dissociation constants which vary with the pH of their environment. Finally, the total net charge of the enzymes and the distribution of charges on their exterior surfaces got affected, in addition to the reactivity of the catalytically active groups. These effects have a huge impact on the neighborhood of the active sites, which will eventually affect the activity, structural stability and solubility of the enzyme (Chaplin and Bucke, 1990).

Alkaline proteases of the genus *Bacillus* exhibited an optimal activity and a good stability at high pH values (Margesin *et al.*, 1992). The optimum pH range of *Bacillus* alkaline

proteases exhibits optimum activity in the pH range from 9 to 11, with a few exceptions of higher pH optima of 11.5 (Fujiwara and Yamamoto, 1987), 11-12 (Kumar *et al.*, 1999), 12-13 (Takami *et al.*, 1989, Fujiwara *et al.*, 1991, Ferrero *et al.*, 1996) (Kumar and Takagi, 1999). The pH optima of some alkaline proteases produced from *Bacillus* sp. are given in Table 2.4.

Table 2.4: pH optimum of various alkaline proteases produced from *Bacillus* sp.

Source	Optimal pH	References
Bacillus sp. No. AH-101	12-13	Takami *et al.*, 1989
Bacillus cereus	10.5	Prakash *et al.*,2005
Bacillus mojavensis	10.5	Gupta and Beg, 2003
Bacillus sp. JB-99	6–12	Johnevelsy and Naik ,2001
Bacillus brevis	10.5	Banerjee *et al.*,1999
Bacillus stearothermophilus TP26	6–12	Gey and Unger ,1995
Bacillus thermoruber	9	Manchini *et al.*,1988
Bacillus sp. B21-2	11.5	Fujiwara and Yamamoto, 1987
Bacillus sp. SB5	10	Gupta *et al.*, 1999
Bacillus sp. B18'	13	Fujiwara *et al.*, 1991

The pH stability exhibited by different bacterial alkaline proteases differs widely. The alkaline proteases reported with high pH stability are AS protease of *Bacillus* sp.GX 6638 (Durham *et al.*, 1987), Vap k protease of Vibrio metschnikovii RH 530 (Kwon *et al.*, 1994) and the proteases of *Bacillus subtilis* RM 615 (Moon *et al.*, 1994) .These enzymes could retain 88%, 80% and 85% activity respectively, after incubation in buffers with pH 12.0 at 25 °C for 24 hours. But subtilisin Carlsberg, the most predominately used detergent protease was retaining only 20% of its original activity after incubation in buffers with pH 11.5 at 50 °C for one hour.

2.9.2 Optimum Temperature and Thermo stability of Alkaline Proteases

The optimum temperatures of alkaline proteases range from 50 to 70 °C. In addition, the enzyme from an alkalophilic *Bacillus* sp. B189 showed an exceptionally high optimum temperature of 85 °C. Alkaline proteases from *Bacillus* sp., *Streptomyces* sp. and *Thermus* sp. are quite stable at high temperatures, and the addition of $CaCl_2$ further enhanced enzyme thermostability (Kumar and Takagi, 1999).

Alkaliphilic bacilli, among all the alkaline protease, are found to be active and are highly stable in highly alkaline conditions (Rahman *et al.*, 1994). *B. stearothermophilus* strain F1 was the first thermophilic and alkaliphilic *Bacillus* sp. isolated by Salleh and friends in 1977. They were reported to be stable at 60 °C (Haki and Rakshit, 2003).

The optimum temperatures of some alkaline proteases from Bacillus sp. are summarized in Table 2.5.

Table 2.5: Optimum temperature of various alkaline proteases produced from *Bacillus* sp.

Source	Optimal temperature (°C)	References
Bacillus brevis	60	Banerjee *et al.* (1999)
Bacillus licheniformis	70	Manchini and Foretina (1998)
Bacillus stearothermophilus	60	Salleh *et al.* (1977)
Bacillus stearothermophilus	85	Rahman *et al.* (1994)
Bacillus sp. JB-99	80	Johnevelsy and Naik (2001)
Bacillus sp. no. AH-101	80	Takami *et al.* (1989)
Bacillus thermoruber	45	Manchini *et al.* (1988)
Bacillus sphaericus	55	Singh *et al.*, 1999
Bacillus mojavensis	60	Gupta and Beg, 2003
Bacillus sp. SB5	70	Gupta *et al.*, 1999

2.9.3 Inhibitors of Alkaline Proteases

The nature of the enzyme, its cofactor requirements, and the nature of the active site can be explored by their inhibition studies. Alkaline proteases found to be inhibited completely by phenylmethylsulfonyl fluoride (PMSF) and diisopropyl fluorophosphates (DFP). In this regard, PMSF sulfonates the essential serine residue in the active site, results in the complete loss of activity. This inhibition profile matches nicely and qualifies them to be considered as serine hydrolases. Some of the alkaline proteases exhibit sensitivity towards metal chelating agent, such as EDTA. Thiol inhibitors affect the alkaline enzymes produced by *Streptomyces* sp. but they have less impact on alkaline proteases of *Bacillus* sp. (Kumar and Takagi, 1999).

2.10 COMPOSITION OF HIDES AND SKINS

Water, proteins, fatty materials, some mineral salts and other substances (pigment, etc.) are found to present in fresh hides and skins. Among these, proteins are the most important components. Fig. 2.1 depicts the composition of a freshly flayed hide.

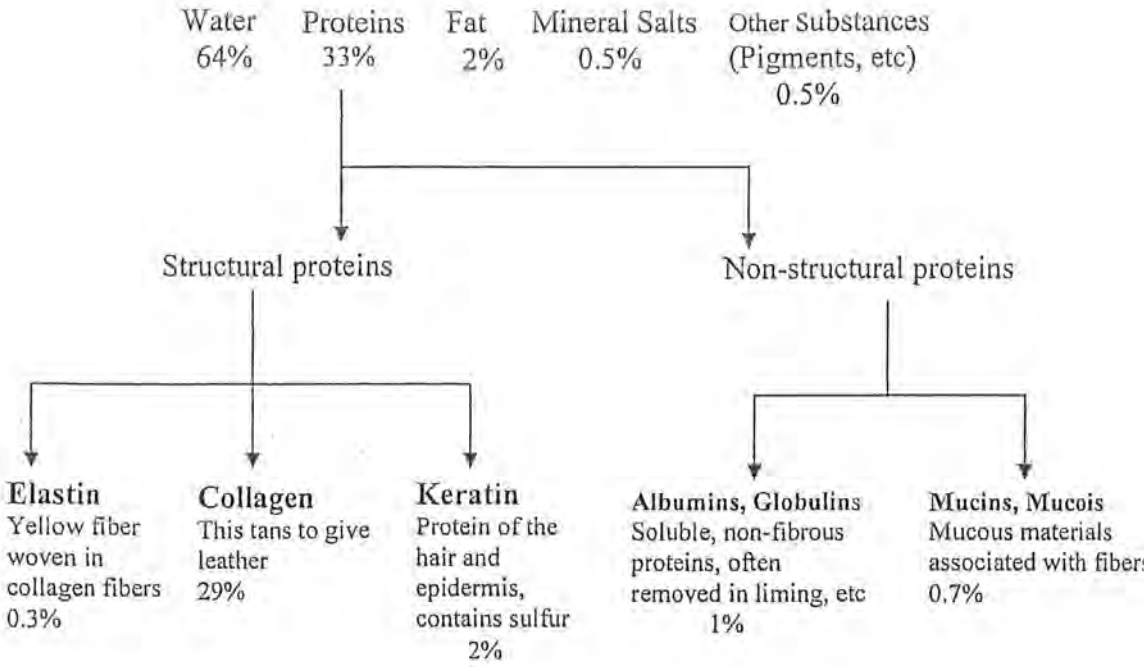


Fig. 2.1: Composition of hides and skins

An extensive research and detailed study revealed that approximately 80% of the dry matter of the hide and skin is made up of proteins such as structural (fibrous) and non-structural (globular) proteins. The corium is made up of an interwoven network of fiber bundles of collagen. This structural protein is thus the more important not only for the quantity but also for its reaction with tanning agent, which is the basis of leather manufacture. Other structural proteins found in the skin are the keratins of the epidermal system and the elastin of the elastic fibers and blood vessels. These proteins are present in small amounts. On other hand, the non-structural proteins are usually intimately involved in the vital processes of the tissue. Non-structural proteins are normally soluble in water or dilute salt solutions which are essential for their functions. Structural proteins are not soluble in water or dilute salt solution. Albumins, globulins, and other less well-defined proteins are present in skin and they are examples of non-structural proteins.

2.10.1 Structure of Collagen

Collagen is a major structural protein. There are 20 different types of collagen of which type I is predominant in skin. 35 Collagen has a rod-like structure about 2800 Å in length and 15 Å in diameter with molecular weight slightly less than 300,000. The primary sequence of collagen consists of Glycine (Gly)-X-Y with predominantly proline in the X position and hydroxyproline in the Y position. The distribution of amino acids among positions in the collagen triplet Gly-X-Y is given in Table 2.6.

Table 2.6: The distribution of amino acids among positions in the collagen triplet Gly-X-Y.

Amino acid	Position1	Position2	Position3	Total
Glycine	337	1	0	338
Alanine	0	60	61	121
Leucine	0	18	1	19
Isoleucine	0	3	4	7
Valine	0	9	8	17
Serine	0	17	18	35
Threonine	0	3	13	16
Methionine	0	2	5	7
Cystine	0	0	0	0
Proline	0	116	3	119
Hydroxy	0	1	113	114
Phenyl	0	12	0	12
Tyrosine	0	0	0	0
Tryptophan	0	0	0	0
Arginine	0	9	42	51
Asparagine	0	7	5	12
Glutamine	0	8	19	27
Histidine	0	2	0	2
Lysine	0	12	20	32
Hydroxylysine	0	0	4	4
Aspartic acid	0	16	15	31
Glutamic acid	0	41	6	47
Total	337	337	337	1011

2.11 Leather Dust Degradation

Collagenases are enzymes capable of inducing hydrolytic cleavages of the peptide bonds of collagen. Maschmann in 1937 described a collagenase activity for the first time in *Clostridium welchii* capable of proteolyzing gelatin (a denatured form of collagen) but having no effect on other common proteins.

Jennifer *et al.*, (1972) studied the collagenolytic activity of 110 aerobic halophilic and halotolerant bacterial strains isolated from crude hides. Three of these strains were identified as *Bacillus* species and eight as *Achromobacter* species. A collagenase active against native, insoluble collagen was isolated from the culture filtrate of *streptomyces* species C-51 (Akira *et al.*, 1987).

Extracellular collagenase was produced by the strain *Vibrio alignolyticus* in a peptone medium during the stationary growth phase. These cultures are also found to have extracellular protease activity. The production of alkaline protease activity in tryptone and minimal media was stimulated by Histidine and urocanic acid. Other compounds associated with histidine utilization pathway did not increase activity. Histidine shows a reverse effect on the repression of protease activity by glucose in minimal media. (Long *et al.*, 1981).

Collagenolytic enzymes produced by two *Bacillus* strains, *Bacillus Soc67* and *ply, 19* isolated from cases of periodontal diseases have been reported by Soederling and Paunio, 1981. These enzymes hydrolyzed native collagen, reconstituted collagen but had no activity for casein.

Six collagenases (alpha, beta, gamma, delta, epsilon and) from *Clostridium histolyticum* were isolated and characterized. All of these collagens were active against collagen (Bond and Van Wart, 1984). Petsushkara *et al.*, investigated the collagenolytic activity of bacterial strain *Bacillus subtilis, Bacillus licheniformis, Bacillus megaterium* and *Bacillus pumilus* isolated from manuscripts on parchment and museum leather exhibits. It was confirmed that these *Bacillus* strains had the ability to hydrolyze native collagen.

Berry and Shuttleworth (1988) studied the effect of three commercially available preparations of bacterial collagenase on insoluble collagen in the presence of sodium dodecyl sulfate and urea. These results showed that the most efficient degradation of collagen under these conditions was done by the enzymes isolated from *Achromobacter isophagus.*

Kaminishi *et al.*, 1988 cultured *Candida albicans* in a medium containing insoluble bovine Achilles tendon as a source of nitrogen and produced a collagen degrading protease. The degradation of Achilles tendon by the protease was verified by morphological changes and release of hydroxyproline.

Collagenolytic strain *Bacillus licheniformis* produced enzyme in a medium containing gelatin and purified to apparent homogeneity on the basis of SDS polyacrylamide gel electrophoresis. The purified enzyme had a molecular weight of approx. 29000 Da and hydrolyzed native insoluble collagen (Asdorrnnithee *et al.*, 1994).

In the optimization of aerobic digestion of calf skin collagenous waste, an analysis with respect to changes in levels of volatile solid, collagen, amino sugar, and hydroxyproline was done. High rate of collagenolysis was observed for first 4 days with 50% degradation of collagen and was measured with release of hydroxyproline (Lalitha *et al.*, 1994).

Jackson *et al., (*1995) monitored the hydrolysis of synthetic peptide 2-furanacryloyl –L-butylglycol-L-prolyl-L-alanine (FALGPA) by both bacterial and vertebral collagenase. A protocol was developed to adopt that method to the measurement of cell associated FALGPA hydrolytic activity in bacteria. The bacteria tested include *Bacillus cereus, Streptococcus agalactiae, streptococcus mutants,* and *Escherichia coli* and various levels of activity were identified. They presented a method that allows the detection of FALGPA hydrolysis using a small quantity of cells without the need for prior purification of collagenolytic enzyme or collection and concentration of a large volume of culture supernatant fluid.

2.12 WASTES GENERATED BY THE LEATHER INDUSTRY

The leather industry contributes significant quantities of wastes to the environment (Mu *et al.*, 2003). It has been observed that only 20% of the wet salted hides are good enough to be converted into commercial leather. 25% of the wet salted hides become chromium containing leather waste, and the remainder of the hides is lost in wastewater as fat, soluble protein, and solid suspended pollutants (Alexander *et al.*, 2000).

Tanning is a process which converts the raw, green hides into sanitized hides or leather. This operation is done to produce strong flexible leather, which can resist decay and spoilage. The raw hides undergo several steps during the tanning process which includes soaking, fleshing, hair removal, deliming, tanning, post tanning, and finishing (Thorstensen, 1993).

The processing of the raw animal hides starts with curing which is a process that involves salting and drying the hide. This process is done immediately on the hide just after stripping it from the animal. Salt and disinfectant are used to soften the hides before leaving it to cure for several hours. Curing is conducted to preserve the hides. After curing, the hides are sent to the tannery and soaked in water for several hours to few days to remove salt, dirt, blood, and debris from the skin. After the soaking, hides become smooth and are ready for a fleshing machine that strips the flesh from the hide's surface. A mixture of lime and water is employed to loosen the hair from the skin's surface. The hair is mechanically removed from the hide after soaking in limewater and sodium sulfide mixture for 1 to 10 days. The hides are delimed in a vat of weak acid or acid salts. Enzymes are used to smooth the grain layer of the hide which affords a soft and flexible product (Thorstensen, 1993).

After the deliming process, the hides are subjected to either vegetable or mineral tanning, depending on the type of hide and the intended product. In vegetable tanning, the hides are exposed to tannin, a natural product found in the bark, leaves, and fruits of chestnut, oak, and hemlock trees. Vegetable tanning is done to produce stiff leathers used in the manufacture of luggage, furniture, belts, etc. In mineral tanning, the hides are soaked in

chromium sulfate solution after being pickled in the acid and salt mixture. Mineral tanning is done to produce soft, stretchable leathers used in the manufacture of purses, bags, jackets, shoes, gloves, etc. After the tanning process, following by neutralization and washing, the hides are dyed and soaked in oils, soaps, greases, and waxes to make them more pliable. The hides are then put through a staking machine to make the leather soft and flexible. The final step in the tanning process is finishing, which is done by covering the grain surface with a polymer and then brushing it (Thorstensen, 1993). All the above processes generate considerable quantities of wastes, which find their way into the environment if not handled properly.

After the tanning process, skins are shaved to the required thickness by the shaving machine. This operation produces waste chrome shavings, also known as wet blue shavings. The average composition of the chrome shaving material is 2 to 4% Cr_2O_3, 60 to 70% moisture, and the remaining (38 to 48%) is protein content (Cabeza *et al.*, 1998).

2.13 REUSE AND RECYCLE TECHNOLOGIES OF THE LEATHER INDUSTRY

The increasing environmental awareness led leather industry to develop cleaner technologies by minimizing the amount of wastes generated and converting the reusable material into useful, finished products (Taylor *et al.*, 1998). The by-products obtained from the leather making process are chrome shavings or trimmings, hair sludge, fleshing grease, wastewater, and waste biosolids. The pollution due to chromium is one of the major concerns of the leather industry (Rao *et al.*, 2002).

In past decades, leather researchers have made a notable effort to study the re-use of leather waste. Recycling and reuse of the wastes generated from the leather industry not only reduces the discharge of wastes into the environment but also helps in the production of useful products, thereby increasing economic benefits of tanning. Before 1970, extensive pretreatment of the tanned wastes, including the manufacture of insulators, building materials, fibrous sheets and shoe soles was not studied too much. In addition, the method of making paper was introduced to produce both leather and paper substitutes. Between 1970 and 1993, a lot of publications and patents stressed on

hydrolyzing chromium containing leather waste so that the amino acids and peptides can be further recycled and used in feeds and fertilizers (Alves Dos Reis and Beleza, 1991a, b; Ohtsuka, 1973; Taylor *et al.*, 1992b, 1993a).

The tedious research during this period results into the development of various treatment methods. Basic hydrolysis, using $Ca(OH)_2$ with steam (Guardini, 1983; Holloway, 1978) or NaOH (Galatik *et al.*, 1988) at higher temperature and/or pressure (Maire and Lipsett, 1980), acid hydrolysis (Wojciech, 1998), and enzymatic hydrolysis (Sivaparvathi *et al.*, 1986a,b) were used for chrome recovery and the isolation of protein fractions. Peroxide treatments (Cot *et al.*, 1991; Cot and Aramon, 1986) can get collagen fiber and Cr (VI) by oxidization of leather scraps. Imai and Okamura reported the application of wet air oxidation and incineration of CCLW to recycle chromium (VI). An additional reductive step was required due to the generation of strongly toxic Cr (VI) as byproducts in these reactions. The removal of chromium never reaches to completion and it needs many repetitions, which is very expensive. Moreover, the more often the procedure is repeated, the more the decomposition of the collagen proceeds and collagen goes into solution, resulting in a mixture of Cr (VI) with protein (Mu *et al.*, 2003).

Acid hydrolysis was also done by Toshev *et al.*, (1980). They hydrolyzed the waste products from chrome tanning with sulfuric acid and used chromium containing hydrolysate as retanning agents. After precipitation of chromium, isolated amino acids could be used as animal supplement. Ohtsyka (1973) have hydrolyzed the waste with organic acids to obtain oligopeptides.

Brown *et al.* was credited for doing systematical laboratory (Cabeza *et al.*, 1999a; Taylor *et al.*, 1993b, 1997, 1998a) and pilot scale (Cabeza *et al.*, 1998c, 1999b; Taylor *et al.*, 1998b) studies on the treatment of CCLW in the past 10 years. The initial one-step process involved the use of alkaline proteolytic enzymes to isolate a chrome-free, hydrolysate product that can be used as feed or fertilizer (Taylor *et al.*, 1990, 1992b). A newer two-step process was also reported that can be used to obtain a gelable protein, with potential uses in adhesives, cosmetics, films, encapsulation or emulsifying, etc. in the first step, and a hydrolysate in the second step. In this process the CCLW were

digested with an initial alkali step and with an alkaline protease second step (Taylor *et al.*, 1991, 1992a, 1993a, 1994), or using two consecutive enzymes (Cabeza *et al.*, 1997, 1998b, 1999c) in the two-step process.

Cabeza *et al.*, 1997 applied two consecutive enzymes for isolation of protein products from solid tannery wastes. Chrome shavings were first treated with pepsin to isolate a high quality gelatin, and then a second extraction was performed using trypsin or alkaline protease to clear the chrome sludge and allow chrome recycling and isolation of low molecular weight hydrolysate.

It is well documented that chromium containing leather waste can be treated with enzymes but only after pretreatment to denature the collagen. Collagens denature in the presence of alkali at moderate temperature, such that enzyme may be added directly to the alkali treated shavings (Pravathi *et al.*, 1987).

Taylor (1995) investigated that when Ca $(OH)_2$ was used as an alkali for pretreatment, then 6% (based on wet weight of shavings) of an alkaline proteolytic enzyme was needed to solubilize 78% of shavings. While when MgO was used, higher the solubilization of shavings was achieved with less amount of enzyme, thus making the treatment more cost effective. The quality of the protein products isolated, that is, gelatin and collagen hydrolysate, has been studied and functional properties have been described (Taylor *et al.*, 1997, 1998).

Chemical and material properties such as moisture, chrome content, total Kjeldahl nitrogen, fat content, gel strengths, viscosities, (Taylor *et al.*, 1998) calcium, magnesium and amino acid analysis (Taylor *et al.*,1992) of the collagen hydrolysate have been studied. To investigate if the protein hydrolysate (extracted from chrome shavings) can be utilized in the preparation of adhesives, cosmetics, paints, flocculating agents and fireproofing agents, their ability to form gels, adhesiveness, capacity to form emulsions, foamability and oil and water absorption properties have been analyzed minutely. Those properties compare favorably with commercial gelatin samples (Taylor *et al.*, 1998).

MATERIALS AND METHODS

3.1 ISOLATION AND IDENTIFICATION OF MICROORGANISMS

Four strains of *Bacillus* sp. (SAL1, SAL2, SAL3, and SAL4) were isolated in the Microbiology Research Laboratory, Department of Microbiology, Quaid-I-Azam University, Islamabad, Pakistan. These strains were isolated from soil and waste materials of different tanneries (table 3.1). Isolates were identified based upon their colony morphology (shape, color, margin, elevation, transparency); gram's staining and biochemical characteristics. Results were interpreted according to Bergey's Manual of Determinative Bacteriology 8th edition (The Williams & Wilkins Company/Baltimore).

Table 3.1: Isolation of different strains of *Bacillus subtilis* from various sources

S.No.	*Bacillus subtilis* strain	Sources
1	SAL1	Tannery Waste
2	SAL2	Soil
3	SAL3	Soil
4	SAL4	Soil

The cultures were routinely maintained on nutrient agar slants having following composition:

Peptone	5.0 g
Meat extracts	1.0 g
Yeast extracts	2.0g
Sodium chloride	5 g
Agar	20.0 g
Distilled water	1000 ml
pH	7.0

The bacterial cultures were maintained at 37 °C.

3.2 SCREENING FOR PROTEOLYTIC ACTIVITY

The alkaline protease activity was observed by using a sample plate technique in which the zones of hydrolysis around the bacterial colonies were observed. Luria casein agar plates (1%) were used for this purpose. The medium contained the following composition:

Tryptone	10.0 g
Yeas extract	5.0 g
NaCl	10.0 g
Casein	10.0 g
Agar	18.0 g
Distilled water	1000 ml
pH	7.2

The purified bacterial isolates were inoculated on sterilized casein-agar plates and were incubated at 37 °C for 24 hours. The microbes showing a clear zone of casein hydrolysis were identified as protease producers. Depending on the maximum relative diameter of the zones, one strain was selected for further experimental studies. Species level identification and molecular level characterization of selected strain (16s rRNA sequencing and estimation of percentage of similarity to the most similar type stains) was also done.

3.3 SPECIES LEVEL IDENTIFICATION

Specie level Identification and carbon source utilization pattern was tested with the help of Analytical Profile Index (API 50CH) Strep System REF 50 300 (bioMérieux® sa 69280 Marcy l'Etoile-France) according to user's instructions manual. The biochemical and sugar fermentation tests of this system allowed presumptive identification of isolates up to species level. Results from the biochemical and the sugar fermentation test were recorded after the completion of 5 and 24 hours respectively. The eight digit numerical code was derived and the identification was done with the user's profile. Purified 24 hrs

fresh colonies (2-3) were mixed in CHB 50 media of Analytical Profile Index (API® 50CHB) Strep System REF 50 300 (bioMérieux® sa 69280 Marcy l'Etoile-France). Media was shaken well to homogenize the cells equally. Each ampule of the test try was filled with respective media containing cells up to neck. Tray wells were filled with distilled water to keep test tray wet at the bottom. Finally, the test tray was covered with transparent cover supplied with kit and incubated at 25 °C. Readings were recorded after every 24 hours and was recorded up to 96 hours.

3.4 MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF ISOLATES

Bacterial genomic DNA was extracted according to the protocol adapted from (Janarthanan and Vincent, 2007) (Appendix 3).

3.4.1 PCR amplification for 16S rRNA of isolated strains

Nucleic acids extracted from microorganisms were amplified by polymerase chain reaction in biometra® thermocycler.

3.4.2 PCR Reaction

Following PCR, conditions were used to amplify 16S rRNA from bacterial strains. Total reaction volume was 50 µL for each reaction.

Table 3.2: Optimized PCR Conditions

No.	Ingredients	Concentration	Volume
1	Template (0.2ng/ µL)	1ng	5 µL
2	primers (10 µM) each	1µL	1µL
3	dNTPs Mix (10mM)	1 µL	1 µL
4	PCR Standard Buffer	5 µL	5 µL
5	*Taq* DNA Polymerase	1 Unit	0.25 µL
6	ddH_2O	35.75 µL	35.75 µL
7	$MgCl_2$(50nM)	1 µl	1µl

PCR Amplification Primers, forward (fd1) primer having nucleotide sequence AGAGTTTGATCCTGGCTCAG and reverse (rd1) primer (AAGGAGGTGATCCAGCC) were used to amplify 16S rRNA gene fragments with *Taq* polymerase [Fermentas Cat. Nos. (EP0281and EP0402)].

3.4.3 Amplification

Amplification was carried out in Biometra® PCR machine with following conditions:

Table 3.3: Polymerase Chain Reaction Cycle

No.	Step	Temperature °C	Time (Min)	Cycles
1	Denaturation	94	3	30
		94	1	30
2	Annealing	56	1	30
3	Extension	72	2	30
	Final Extension	72	10	1
	Hold	4	∞	1

3.4.4 Visualization

The PCR products were analyzed on 0.9% agarose gel and subsequent staining with Ethidium Bromide (1μg ml^{-1} in TAE buffer)

3.4.5 PCR product purification

Purification of amplicons was done by using QIAquick PCR Purification Kit (Qiagen Ltd., Crawley, United Kingdom) as per user's instruction manual. This column removes primers and other reagents from the PCR product. The concentration of 15 ng/100 bp was

made as required for DNA sequencing of these PCR purified amplicons. Visualization was performed by 0.9% (w/v) agarose gel for 35 minutes (80 V & 400 mA) using Hyperladder I and IV (Bioline Ltd., London, United Kingdom).

3.4.6 PCR Product quantification

Isolated Genomic DNA from these bacteria was quantified on Nano-Drop spectrophotometer (Nanodrop™ 1000) by finding concentration at 230, 260 and 280 nm and checking contamination by 260:280 nm ratio and analyzed on 0.9% agarose gel.

3.4.7 DNA sequencing and identification of bacterial strains

Both DNA strands of 16S rRNA for bacterial strain SAL1 were sequenced. Determination of nucleotide sequence of the PCR purified fragments was performed using the ABI PRISM® BigDye™ Terminator cycle sequencing kit (Applied Biosystems Inc., Warrington, United Kingdom) at Texas Tech University, Lubbock, USA. Cycle-sequencing conditions were denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, and extension at 60 °C for 4 min for 25 cycles, with a final extension at 60 °C for 4 min. The sequencing primers used were same as amplification primers. After interpretation, respective sequences of strains were submitted to NCBI gene bank using *Sequin*, online software and accession numbers were assigned to each partial sequence.

3.5 PRODUCTION OF CRUDE ENZYME

The production of extracellular crude enzyme was done in two steps:

1. Inoculum preparation
2. Fermentation for protease production

3.5.1 Inoculum preparation

The Nutrient broth medium enriched with 1% of casein hydrolysate was used for inoculum preparation having following composition.

Nutrient broth	0.8 g

Casein hydrolysate	1.0 g
Distilled water	100 ml
pH	8.0

100 ml of the medium was prepared in 250 ml of Erlenmeyer flask and the pH of the medium was adjusted at 8 by using 0.1N NaOH and 0.1N HCl. The medium was then autoclaved at a temperature of 121 °C under pressure of 15 psi for 20 min. Then the medium was inoculated with *Bacillus subtilis* strain SAL1 under sterilized conditions followed by keeping in an incubator shaker at 37 °C and 150 rpm for 24 hours. This inoculum was used for further studies.

3.5.2 Fermentation for protease production

Batch culturing was done in shake flask and biofermentor. Various parameters were optimized for the production of protease in shake flask as well as in biofermentor. Production media having different composition were used to determine the optimum activity. The medium giving the maximum production of enzyme was selected for further optimization studies in shake flask and biofermentor. All experiments were performed in triplicate.

3.6 Selection of Media for Protease production

Effect of composition of medium on protease production was studied. Four media having following composition were used for this purpose.

Medium 1

Peptone	2%
Yeast extract	1%
Dextrose	2%
Distilled water	100 ml

(Lee *et al.*, 1992)

Medium 2

Gelatin	1%
Glucose	1%
Yeast extract	0.2%
K_2HPO_4	0.3%
KH_2PO_4	0.1%
$MgSO_4.7H_20$	trace
Distilled water	100 ml

(Shalinisen and T.Satyanarayana, 1993)

Medium 3

Gelatin	2%
Casein hydrolysate	0.48%
Glycerol 20%	1.2 ml
Distilled water	100 ml

(Hameed *et al.*, 1996)

Medium 4

Starch	5%
Soybean	1.5%
$Mgcl_2$	0.5%
Na2HCO3	2%
Corn steep liquor	1%
Distilled water	100ml

(Ming chu *et al.*, 1992)

The medium that showed the best results in relation to enzyme production was used for optimization studies.

3.7 BATCH CULTURING IN SHAKE FLASK

100 ml of casein gelatin medium was poured in a 250 ml of Erlenmeyer flask. The medium was autoclaved at 121 °C under pressure of 15 lbs for 20 min. Then glycerol (20%) which was sterilized separately in a hot air oven at 100 °C for 30 minutes was added aseptically in the medium. 10 ml of inoculum was added to it and was placed in a shaking incubator at 37 °C and 150 rpm for 120 hours. Samples were collected after every 12 hours. The supernatant or the crude enzyme extract was filtered and stored at – 20 °C until the assay for proteolytic activity is performed. Protease activity and protein estimation of each sample was done.

3.8 OPTIMIZATION OF VARIOUS PARAMETERS FOR PRODUCTION OF PROTEASE IN SHAKE FLASK

3.8.1 Effect of incubation period on the production of protease

Growth of *Bacillus* and the production of protease were carried out for 120 hours at 37 °C, pH 8.0 and 150 rpm. The samples were collected after every 12 hours to check the production of protease. Proteolytic activity, change in pH and total protein concentration was measured.

3.8.2 Effect of pH on enzyme production of Protease

Effect of pH on the growth of *Bacillus* and enzyme production was carried out at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0, 9.5, 10.0 by keeping temperature and speed at 37 °C and 150 rpm. Proteolytic activity change in pH and total protein concentration was observed.

3.8.3 Effect of Temperature on Protease production

The production of enzyme was carried out at 25, 30, 35, 37, 40, 45, 50 °C by keeping the speed 150 rpm and pH 8.5. Proteolytic activity, change in pH and the total protein concentration was measured.

3.8.4 Effect of Age of inoculum on Protease production

The inoculum aging 12, 18, 24, 30, 36 and 48 hours was used to observe the effect of age of inoculum at pH 8, temperature 37 °C and agitation speed 150 rpm. Proteolytic activity, change in pH and total protein concentration was determined.

3.8.5 Effect of size of inoculum on Protease production

Effect of inoculum size of 1%, 2.5%, 5%, 7.5%, 10%, 12.5%, 15%, 17.5% and 20% (percentages were calculated on the basis of volume of media) was also studied at pH, temperature and speed as 8.5, 37 °C and 150 rpm respectively. Proteolytic activity, change in pH and total protein concentration was observed.

3.8.6 Effect of agitation speed on Protease production

The effect of agitation speed was observed on the production of protease. The agitation speed was maintained at 100, 150, 200, 250, 300 and 350 rpm at pH, temperature of 8.0 and 37 °C respectively. Proteolytic activity, change in pH and total protein concentration was measured.

3.8.7 Effect of Carbon sources on Protease production

Effect of carbon sources on enzyme production was studied. Different carbon sources sucrose, fructose, manitol, dextrose, sorbitol, glycerol, glucose, maltose and lactose were used as 1% w/v solution at pH, temperature and agitation speed as 8.5, 37 °C and 150 rpm. Proteolytic activity, change in pH and total protein concentration was determined.

3.8.8 Effect of Nitrogen sources on Protease production

Effect of nitrogen sources on enzyme production was studied by using different nitrogen sources such as tryptone, peptone, beef extract, yeast extract and gelatin (1% w/v solution) at pH, temperature and agitation speed as 8.5, 37 °C and 150 rpm. Proteolytic activity, change in pH and total protein concentration was observed.

3.8.9 Effect of divalent cations on protease production

The effect of different metal ions on protease activity was determined by the addition of the corresponding ion at a final concentration of 1.0 mM to the medium at pH, temperature and agitation speed as 8.0, 37 °C and 150 rpm. Proteolytic activity, change in pH and total protein concentration was observed.

3.10 Protease Assay

The enzyme activity was assayed using azocasein as the substrate, according to the method of Sarath *et al.*, (1989) with some modifications.

3.10.1 Reagents

0.02M Tris-HCl buffer, pH 8.5
5% Trichloroacetic acid (TCA).
1% Azocasein in Tris-HCl buffer (1 gram of azocasein was dissolved in 100 ml of 0.02 M Tris-HCl and pH was adjusted to 8.5).

3.10.2 Assay Procedure

1ml of crude enzyme was mixed with 1 ml of 1% azocasein solution dissolved in 0.02 M Tris-HCl (trihydroxyaminomethane-hydrochloride) buffer (pH 8.5), and was incubated in a water bath at 55 °C for 10 minutes. The reaction was quenched by adding 1 ml of 5% Trichloroacetic acid (TCA). The mixture was then kept at 4 °C for 15 minutes.
After centrifugation at 4000 rpm for 20 minutes, 1 ml of supernatant was mixed with 1 ml of 0.4N NaOH and the absorbance was read at 440 nm. The blank was prepared by adding TCA before incubation at 55 °C. All assays were made in triplicate, and the average of three was taken to evaluate the activity units.

3.10.3 Unit of activity

One unit of protease activity is defined as the amount of enzyme that produces an increase in the absorbance of 0.01 under assay conditions.

3.10.4 Protein Estimation

Protein was estimated by bicinchoninic acid protein assay kit, with bovine serum albumin (BSA) as standard.

3.11 PURIFICATION OF ALKALINE PROTEASE

Following steps were carried out for purification of alkaline protease from *Bacillus subtilis* strain:

- Separation of bacteria from fermentation medium
- Ammonium Sulfate Precipitation
- Dialysis
- DEAE-Sephacryl anion exchange Chromatographic Purification
- Phenyl sepharose Hydrophobic Interaction Chromatography

3.11.1 Separation of bacteria from fermentation Medium

Separation of bacteria from fermentation media was facilitated by using the centrifugation technique. The culture medium was centrifuged at 10,000 rpm for 30 minutes at temperature 4 °C in a Kakusan Model H-251 centrifuge. The supernatant was filtered through Whatmann No.1 filter paper and kept at 4 °C. The pH of filtrate was adjusted at 8.5 by using 0.1N sodium hydroxide solution and the filtrate was placed in the cold room at -4° C for 60 minutes. Proteolytic activity as then determined by the method of Kunitz with some modifications.

3.11.2 Protease Precipitation by using ammonium sulfate

Precipitation of protease was carried out by using ammonium sulfate. Ammonium sulfate was added to the 100 ml of cell free culture suspension up to saturation of 30%. These

suspensions were stirred for half an hour at 4 °C in cold room. After sufficient shaking, precipitates were collected by centrifugation at 16000 rpm and 4 °C for 15 minutes. The pellets were suspended in 20 mM Tris-HCl buffer (pH = 8.5). Similarly, enzyme solution was then treated with 40, 50, 60, 70, 80, 90 and finally 100 % saturation of ammonium sulfate. Each time pellets were suspended in 20 mM Tris-HCl buffer (pH = 8.5). Proteolytic activity was determined in the supernatant after each treatment and precipitates were collected for further purification.

3.11.3 Dialysis

The precipitates obtained by ammonium sulfate precipitation were dialyzed by using 12000 molecular weight cut off dialyzing tube, which was placed in 2 liters of 20 mM Tris-HCl buffer (pH = 8.5) for 24 hours at 4 °C against three changes of buffer. The proteolytic activity of the dialyzed material was determined before further purification.

3.11.4 DEAE-Sephacryl anion exchange Chromatographic Purification

Anion exchange chromatography was performed in batch mode on DEAE-Sephacryl. 2 mL of DEAE-Sephacryl suspension was placed into a 15 mL plastic centrifuge tube. 10 mL of 0.02M Tris-HCl (pH 7.7) buffer was added to it and Vortexed for a few seconds before placing on a rotary table. It was rotated for 5 minutes. The suspension was centrifuged at 300 rpm for 10 minutes. The supernatant was decanted and discarded. Another 10 mL of buffer was added and vortexed for a few seconds and rotated for another 5 minutes. This procedure was repeated two more times. To the pelleted Q-Sepharose matrix, 2 mL of precipitated protease solution (from Dialysis) was added. It was Vortexed for a few seconds and then rotated for one hour in an orbital shaker (100 rpm) at 25 °C. The shaking was stopped after 1 hour, followed by filtration under vacuum. Before filtration, the filter disk was conditioned by applying suction, as by pipeting 2 mL of isopropanol and then 2 mL of water and then 2 mL of 0.02 M Tris-HCl (pH 8.5) buffer. These washings were discarded. The matrix and supernatant were thus separated. The separated matrix was repeatedly washed with the equilibration buffer until

no enzyme activity could be detected in the washings. The proteolytic fractions were combined and were subjected to hydrophobic interaction chromatography.

3.11.5 Phenyl sepharose Hydrophobic Interaction Chromatography

The pooled fractions from DEAE Sephacryl fractionation were applied to a Phenyl Sepharose column, which was equilibrated with 0.02M sodium hydrogen phosphate buffer containing 2.5M NaCl. The liquid was collected in a receiving tube. The column was washed with 0.02 M Tris-HCl buffer and then bound proteins were eluted with 50% ethylene glycol in 20 mM buffer Tris-HCl (pH = 8.5). Fractions were collected and analyzed for protease activity. Fractions with highest enzyme activity were pooled and used for further characterization.

3.12 CHARACTERIZATION

The enzyme purified from culture supernatant through various steps was then characterized.

3.12.1 Effect of Temperature on the activity and stability of the protease

The effect of temperature on pure enzyme was studied by assaying enzyme at different temperatures in the range of (30-70 °C), at pH 8 using azocasein as substrate. The thermo-stability of enzyme was studied by incubating the enzyme preparation at varying temperatures ranging from (40-90 °C) for 1 hr. Subsequently, enzyme activity was measured as above.

3.12.2 Effect of pH on the activity and stability of the protease

The effect of pH on protease activity was determined by incubating the reaction mixture at pH values ranging from 5.0 to 12.0, in the following buffer systems: 0.1 M sodium acetate (pH 4.0-5.5); 0.1 M sodium phosphate (pH 6.0–7.5); 0.1 M Tris–HCl (pH 8.0–9.0); 0.1 M glycine–NaOH (pH 9.5–12.0). To check the pH stability, the enzyme solution (50 µl) was mixed with 150 µl of each buffer solution and protease activity was measured under standard assay conditions after incubation for 1 h at 25 °C.

3.12.3 Effect of inhibitors on the activity of purified protease

Effect of inhibitors such as EDTA, ß-mercapto ethanol and PMSF was determined. Protease activity was measured after incubating the enzyme with different concentration of these inhibitors for 1 hour at 37 °C.

3.12.4 Effect of different surfactants on protease activity

The effect of different oxidizing agents and surfactants (Tween 20, Tween 45, Tween 65, SDS,and Triton X-100) on proteolytic activity of purified enzyme was studied by incubating the enzyme with these reagents (1%) for one hour at room temperature. The residual activity was then measured according to the standard assay conditions

3.13 MOLECULAR WEIGHT DETERMINATION BY SODIUM DODECYL SULFATE-POLYACRYLAMIDE GEL ELECTROPHORESIS (SDS-PAGE)

Molecular weight determination and confirmation for the purification of the protein obtained through different purification steps, was done using the method of SDS-PAGE (Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis) by the protocol followed by Laemmli (1970) with slight modifications. Electrophoresis is the movement of charged molecules in an electric field. Sodium dodecyl sulfate-Polyacrylamide gel Electrophoresis (SDS-PAGE) is used to assess the homogeneity of purified protease fractions through molecular weight determination.

3.14 MASS SPECTROMETRY (MALDI-TOF MS)

Molecular weight was also determined by MALDI-TOF mass spectrometer. The Voyager-DE MALDI-TOF mass spectrometer is a linear time-of-flight mass analyzer equipped with a nitrogen laser (337 nm) which ionizes the sample, and delays the extraction for improved resolution and mass accuracy. This can be used for a mass range

in excess of 300 kDa. This instrument utilizes a gold-coated stainless steel sample plate (up to 100 samples). Each sample position is precisely accessed using a computer controlled XY manipulator. The GRAMS software package allows for facile mass calibration, resolution, and mass measurements. This instrument is ideally suited for peptides, proteins, and oligonucleotides.

MALDI-TOF mass spectrometer is equipped with a nitrogen laser used for acquiring mass spectra. A saturated solution of sinapinic acid in 30% acetonitrile was used as matrix. The sample and matrix were mixed in appropriate ratio and 1 µl was applied as a spot on MALDI sample plate. The sample spot was allowed to dry at room temperature. Upon drying, the matrix forms crystals incorporating the sample. The experiment was performed in the positive ion mode. The accelerating voltage was 25 kv. The laser power and delay time were adjusted to generate a reasonable signal. Sample preparation and matrix solution was done as follows:

Sample preparation

Select matrix

↓

Prepare matrix

↓

Prepare sample

↓

Mix sample and matrix

↓

Load sample on clean sample plate

↓

Dry

3.15 CAPILLARY ELECTROPHORESIS

Purification of enzyme was also checked by capillary electrophoresis. A 65 cm (54 cm to the detector window) 50-µm inner diameter, deactivated silica capillary was used for CZE. The following procedure was followed for the deactivation of the silica capillary. The capillary was washed successively with 2 ml each of DI water, 1M NaOH, DI water, 1M HCl, DI water, isopropanol, toluene, and 5% trimethylchlorosilane in toluene.

Toluene was passed through magnesium sulfate to remove moisture. The capillary was incubated at 80 °C overnight with continuous flushing of nitrogen gas. The running buffer was 100 mM phosphate buffer, pH 2.5. The samples were prepared at a concentration of 4 µg/ µl in the run buffer. Electro-kinetic injection was performed at 15 kV for 30 s. Separation was done at 15 kV for 60 min and the current was 40 µA. The CE used here was a homemade instrument. A Spellman CZE 1000 R high voltage power supply and a Spectra 100 variable UV-VIS wavelength detector were used. The UV detection was done at 220 nm.

3.16 ZYMOGRAPHY

SDS-PAGE zymograms were performed as described by (Schmidt *et al.*, 1988) with some modifications. 10% polyacrylamide gels were copolymerized with 0.05% gelatin. Samples were dissolved in non-reducing sample buffer without heat denaturation and run at 100 V. Following electrophoresis, the gels were washed for 30 min in 50 mM Tris-HCl buffer (pH 7.6) containing 2.5% Triton X-100, with gentle agitation, in order to remove the excess of SDS. Then the gels were incubated for an additional 4 h with several changes in a solution of 50 mM Tris-HCl, pH 7.6, containing 0.2M NaCl and 5mM $CaCl_2$. After this, gels were stained with 0.1% Coomassie blue R-250 in methanol–acetic acid–water (40:10:50) followed by destaining with methanol–acetic acid–water (5:10:85) until clear bands appeared against the blue background.

3.17 PROCESSING OF LEATHER SOLID WASTE

3.17.1 Leather Dust Degradation

Leather dust (which is a solid waste of leather industry) was obtained from Institute of Leather Technology, Gujranwala, which was then washed, and dried. Proteolytic enzyme from *Bacillus subtilis* was used for degradation studies. The pH of leather dust was adjusted to 8.5 by using 0.1 g NaOH. Then 3000 PU of proteolytic enzyme were added and incubated for 24 hours at room temperature. The release of hydroxyproline was noted.

3.18 OPTIMIZATION OF DIFFERENT PARAMETERS FOR THE DEGRADATION OF LEATHER DUST

3.18.1 Effect of Incubation period on Dust Degradation

The effect of time on the degradation of leather dust was studied. To 1 g of leather dust, 3000 PU of enzyme was added and incubated for 144 hours at room temperature. Leather dust degradation was measured after every 24 hours by measuring the hydroxyproline released at room temperature.

3.18.2 Effect of pH on the degradation of leather dust

The effect of pH on 1 g of leather waste was studied at room temperature when treated with 3000 units per gram. Hydroxyproline released was calculated after 84 hours.

3.18.3 Effect of temperature on the degradation of leather dust

1 g of leather waste was treated with 3000 units per gram at different temperatures keeping pH at 9 for 144 hours. Degradation was calculated by the detection of hydroxyproline.

3.18.4 Effect of Proteolytic units on Leather Dust Degradation

Effect of proteolytic units on leather dust degradation was carried out. To 1 g of leather dust, 2000 PU, 3000 PU, 4000 PU, 5000 PU, 6000 PU and 7000 PU of enzyme were added and incubated for 144 hours at room temperature. Hydroxyproline release was noted.

3.18.5. HYDROXYPROLINE ASSAY

Hydroxyproline was assayed using the method of Leach (1951). Following reagents were used

1) 0.05M copper sulfate in water.

2) 2.5N sodium hydroxide (100 g NaOH pellets dissolved in 1 liter of water).

3) 6% hydrogen peroxide. Diluting 30%, (w/v) hydrogen peroxide made this. This dilution is not reliable for more than a day and is made fresh immediately before use.

4) 3N sulfuric acid. This solution contained 83 ml of conc.H_2SO_4/L.

5) 5% p-Dimethylaminobenzaldehyde. The reagent was dissolved in propanol. This solution was kept in dark.

Standard L-hydroxyproline solution: Hydroxyproline (0.5 g) was dissolved in water and diluted up to 400 ml. The 100 µg/ml solution is diluted to give concentration of 5, 10, 15, 20 µg of hydroxyproline/ml.

Procedure

To 1ml of sample, 1ml of 0.05M $CuSO_4$ was added, followed by the addition of 1ml of 2.5N NaOH. Gentle swirling of the liquid mixes the tube contents. The mixture was incubated in a water bath at 40 °C for 3-5 minutes. Then 1 ml of 6% hydrogen peroxide was added, which was immediately mixed by swirling the contents of the tube. During the swirling of the contents, no solution should remain on the walls of the tube above the level reached by the water bath. Otherwise, the destruction of hydrogen peroxide will be incomplete. The mixture was then incubated in water bath for 10 minutes. The tube was cooled with tap water, then 4 ml of 3N H_2SO_4 and finally, 2 ml of 5% paradimethyl aminobenzaldehyde solutions were added. The contents of the tubes were mixed by swirling after each addition and tubes were kept in water bath at 70 °C for 16 minutes. The solutions were then cooled, mixed and measured against the blank solutions at a

wavelength of 555 nm in 4 cm glass cuvet. The assays were made in triplicate and average readings were calculated.

3.19 BIOTREATMENT OF CHROME SHAVINGS WIT MICROBIAL ENZYME

3.19.1 Analysis of Chrome shaving

Chrome shavings were obtained from a commercial tannery and kept at room temperature. It was analyzed for pH, moisture, ash, Total Kjeldahl Nitrogen (TKN). Moisture was determined by heating the sample at 110 °C for 12 h. Ash in the dried products was determined by heating the sample at 600 °C for 4 hours.TKN was determined by the semi-micro Kjeldahl method. Chromium was determined using a Perkin-Elmer model AA7003 atomic absorption spectrophotometer.

3.20 STUDIES ON HYDROLYSIS METHODS

3.20.1 Enzymatic hydrolysis of chrome shaving (One-step Process)

100 grams of chrome shavings were shaken in 1 L of water containing 3 g MgO and 0.1 ml of surfactant at 60 °C for 4 h. This pretreatment step is necessary to obtain the optimal pH for the enzymatic digestion. Then 1 ml (4250 PU/mg) of enzyme solution was added to it for a period of 3 hours. The pH was maintained at 8.5-9.0 with small additions of magnesium oxide. After the enzymatic digestion (60 °C for 3 hours), the warm solution was filtered through Whatmann No. 1 filter paper in porcelain funnels under vacuum conditions. The filtered protein solutions and remained chrome sludge were stored at 4 °C for analysis.

3.20.2 Alkali hydrolysis of chrome shaving followed by enzymatic hydrolysis (Two-step Process)

In the first step of this process, 100 g of chrome shavings were mixed in 1 L of water. Then 0.1 ml of surfactant and 6.0 g of alkali was added to it. The flask containing the media was placed in a shaker at 70 °C for 6 hours. Then the warm solution was filtered through Whatmann No. 1 filter paper in porcelain funnels under vacuum condition. The

filtered protein solutions and the remained chrome sludge were stored at 4 °C for further analysis. In the second step of this process, additional water (200 ml), surfactant (0.1 ml), and alkali (2.0 g) were added to this remained chrome sludge and was shaken for 1.5 hrs. Then 1 ml (4250 PU/mg) of enzyme was added to it. The pH of medium was adjusted to 8.5 which was required to maintain the optimum alkalinity for promoting enzyme activity after which, it was shaken at 60 °C for 3.5 hours. The warm solution was filtered through Whatmann No. 1 filter paper in porcelain funnels under vacuum conditions. The mixed hydrolyzed protein solutions and the final chrome cake (containing protein residues and chromium) were stored at 4 °C for further use and analysis.

3.20.3 Analysis of recycled products

The recovered samples from above stated processes were analyzed for pH, ash, moisture, protein as TKN. The molecular weight range of protein fractions were estimated by SDS–PAGE (polyacrylamide gel electrophoresis in sodium sulfate).

3.20.4 SDS-PAGE

SDS-PAGE was performed on 12%gel according to Laemmli's protocol as well as by the PhastSystem™ electrophoresis unit.2 μg/μl of sample was prepared in the sample buffer (10 mM Tris/HCl, 1 Mm EDTA pH 8.0). The sample was then denatured with 2.5% SDS and 5% β-mercaptoethanol by heating at 100 °C for 5 min. Bromophenol blue indicator is added to a concentration of approximately 0.01%. SDS-PAGES was carried out by both methods.12% and 8-25% gradient homogeneous PAGE gels were used. Phastgel™ SDS-PAGE buffer strips were used. A Sigma wide range marker kit was used for calibration. The separation was carried out at 250 V, 10 mA, 3 W for 60 Vh at 15° C. The gels were stained with 0.1% Phastgel™ Blue R solution.

3.21 HYDROLYSIS OF GROUND FINISHED LEATHER

Scraps of ground-finished leather were obtained from a commercial tannery and were grounded to fine powder.100 g of this powder was mixed in 1 L of water. The pH of medium was adjusted to 12 with the help of potassium hydroxide and was shaken at 60

°C for 1.5 hrs. Next, pH was decreased to 8.5 with nitric acid before the addition of enzyme. Then 1ml (4250 PU/mg) of enzyme was added for two hours for the hydrolysis. Near the end of hydrolysis, pH was further decreased and the product is filtered through Whatmann No. 1 filter paper in porcelain funnels under vacuum conditions. The process produced a water-soluble organic nitrogen (peptide) mixture and a mineral containing sludge. They were stored at 4 °C for analysis.

3.21.1 Analysis of recycled products

The recovered samples from above stated processes were analyzed for pH, ash, moisture, protein as TKN. The molecular weight range of protein fractions were estimated by SDS–PAGE (polyacrylamide gel electrophoresis in sodium sulfate).

RESULTS

4.1 Identification of bacterial strains

Four strains of *Bacillus subtilis* (SAL1, SAL2, SAL3 and SAL4) isolated from different sources, such as tannery waste and soil were identified on the basis of standard microbiological, morphological and biochemical methods. The various morphological, biochemical and physiological characteristics of organisms are listed in Tables 4.1 and 4.2 and 4.3.

Table 4.1: Morphological characteristics of *Bacillus subtilis*

Gram's staining	+ve
Shape	Cocii bacilli
Spore formation	+ve

Table 4.2: Biochemical Tests for identification of *Bacillus subtilis*

Starch hydrolysis	+
Casein hydrolysis	+
Lipid hydrolysis	+
Indole production	–
Methyl red test	–
Voges-Proskauer	+
Citrate utilization	+
Nitrate reduction	+
Acid production	+
Gas production	–
Hemolysis on blood agar	+
Catalase test	+

Table 4.3: Physiological characteristics of *Bacillus subtilis*

Motility	+
Growth pH range	6-10
Growth temperature range (°C)	20-55

4.2 Qualitative Test/Protease producing ability

The ability of four bacterial isolates of *Bacillus subtilis* to produce protease was determined by measuring the diameter of zone of hydrolysis on 1% Luria casein agar plates around each colony. The results (table 12) showed that maximum zone of hydrolysis (4.3cm) was shown by *Bacillus subtilis* strain SAL1, isolated from tanneries waste, followed by *Bacillus* strain SAL4 (2.7cm), SAL2 (1.9cm) and SAL3 (1.1cm).

Table 4.4: Zones of hydrolysis of various strains of *Bacillus subtilis*

S.No.	*Bacillus subtilis* strain	Zone of hydrolysis(cm)
1	SAL1	4.3
2	SAL2	1.9
3	SAL3	1.1
4	SAL4	2.7

It is evident from the results that among the four strains of *Bacillus subtilis* maximum proteolytic activity was observed in case of *Bacillus* strain SAL1, therefore it was selected for further studies. Species level identification and molecular level characterization of selected strain (16s rDNA sequencing and estimation of percentage of similarity to the most similar type stains) was also done.

4.3 Species Level Identification

Analytical Profile Index (API 50CH) Strep System REF 50 300 (bioMérieux® sa 69280 Marcy l'Etoile-France) was used for species level identification (table 4.5).

Table 4.5: Tests of API system used to identify the species of strain SAL1

Tests	Results	Tests	Results	Tests	Results
Glycerol	+	Inositol	_	Inuline	+
Erythritol	_	D-Mannitol	+	D-Melezitose	_
D-Arabinose	+	D-Sorbitol	+	D-Rafinose	+
L-Arabinose	+	Methyl α-D Mannpyrunoside	_	Amidon	_
D-Ribose	+	Methyl α-D Glucopyranoside	+	Glycogen	+
D-Xylose	_	N-Acetyl Glucosamine	_	Xylitol	_
L-Xylose	_	Amygdaline	+	Gentiobiose	+
D-Addonitol	_	Arabutin	+	D-Turanose	+
Methyl β-Dxylpyranoside	_	Asculin	+	D-Lyxose	_
D-Glactose	+	Salicin	+	D-Tagatose	_
D-Glucose	+	D-Cellobiose	+	D-Fucose	_
D-Fructose	+	D-Maltose	+	L-Fucose	_
D-Mannose	+	D-Lactose	+	D-Arabitol	+
L-Sarbose	_	D-Mallobiose	_	L-Arabitol	+
L-Ramnose	_	D-Sucrose	+	Potassium Gluconate	+
Dulcitol	_	D-Trehalose	+	Potassium 2-Ketoglutarate	_
				Potassium 5-Ketogluconate	_

4.4 Identification of *Bacillus* strain SAL1 by 16SrRNA sequence analysis

The selected bacterial strain previously identified on the basis of morphology (table 4.1) and biochemical tests (Table 4.2) were further identified on the basis of molecular characterization, sequence analysis of 16S rRNA .The identification of this strain SAL1 was confirmed as *Bacillus subtilis* as sequence analysis of 16S rRNA showed 100% homologies with respective species in NCBI data bank (Table 4.6). The 16S rRNA sequence of this strain was deposited in NCBI with accession number as GQ267463 for SAL1 (table4.7).

Table 4.6: Molecular Identification of bacterial isolates showing similarity with SAL1

Accession Nos.	Score of homology	Query coverage	% homology	Identification
GQ148816.1	361	95%	100%	*Bacillus subtilis*
GQ148815.1	361	95%	100%	*Bacillus subtilis*
GQ148814.1	361	95%	100%	*Bacillus subtilis*
GQ148813.1	361	95%	100%	*Bacillus subtilis*
GQ148812.1	361	95%	100%	*Bacillus subtilis*

Table 4.7: 16S rRNA sequences of strain used in the study

Strain	Accession No.	Identified Bacterial strain
SAL1	GQ267463	*Bacillus subtilis*

4.5 Selection of Media for Protease Production

The protease production was carried out using four different media and the results (Fig. 4.2) show that the maximum specific activity was observed in case of medium M3 which contains gelatin, glycerol and casein hydrolysate. Protease activity was 101 PU/mg, 146 PU/mg and 167 PU/mg in case of M1, M2, and M4 respectively. Protease activity in case of M3 was 215PU/mg.

4.6 Effect of growth phase

Alkaline protease production by the strain *Bacillus subtilis* SAL1 as a function of growth at 37 ^{o}C is shown in Fig. 4.3. It is clear from the fig. that the production of enzyme by the strain could be noticed from the early exponential phase of growth. However it was producing only small quantity of enzyme in the early stages. A steady increase in production of enzyme was observed as the growth progressed from early exponential to early stationary phase. Maximum enzyme activity was observed in early stationary phase.

4.7 Optimization of parameters for protease production in shake flask culture

4.7.1 Effect of incubation period on the enzyme production

The cultures of *Bacillus* strain SAL1 were incubated for 96 hours and samples were taken after every 12 hours to check the growth and protease production. The results showed that the maximum specific proteolytic activity (263 PU/mg) was observed in the cell free supernatant of the culture taken after 72 hours of incubation (Fig. 4.4).

4.7.2 Effect of pH on enzyme production

It is evident from fig that the optimum pH for maximum production of protease was 8.5 after 72 hours of incubation. The maximum specific activity (276 PU/mg) was observed at pH 8.5 and there was decrease in specific activity with increase in pH (Fig.4.5). The specific activity at pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 9.0, 9.5 and 10 were 132 PU/mg, 139 PU/mg, 143 PU/mg, 145 PU/mg, 238 PU/mg, 257 PU/mg, 265 PU/mg, 269 PU/mg, 231 PU/mg and 187 PU/mg.

4.7.3 Effect of temperature on enzyme production

It is evident from the results that maximum specific activity was observed after 72hours of incubation at 37 °C (278 PU/mg). At the lower temperature i.e. 25, 30 and 35 °C, the specific activities were 128 PU/mg, 244 PU/mg, and 269 PU/mg respectively. While at the temperature higher than 37 °C i.e. 40, 45, 50 °C, the specific activities were 238 PU/mg, 204 PU/mg and 121 PU/mg respectively. It was observed that with further increase in temperature, production of protease decreased (Fig. 4.6).

4.7.4 Effect of age of inoculum on the enzyme production

It is evident from the results that maximum specific activity (352 PU/mg) was observed after 72 hours of incubation when 24 hours old broth culture of *Bacillus* strain SAL1 was used as an inoculum for the production of protease. In case of 12, 18, 30, 36, 48, 60, 72 hours old inoculum, the specific activities observed were 177 PU/mg, 246 PU/mg, 272 PU/mg, 241 PU/mg, 163 PU/mg, 97 PU/mg, and 80 PU/mg respectively (Fig. 4.7).

4.7.5 Effect of size of inoculum on the enzyme production

The inoculum was added to the production medium in varying concentration (1, 2.5, 5, 7.5, 10, 12.5, 15, 17.5, and 20). The results showed that optimum protease production was observed in case of 10% inoculum (294 PU/mg) where as in case of 1, 2.5, 5, 7.5, 10, 12.5, 17.5 and 20% inoculum sizes, the specific activities observed were 162 PU/mg, 248 PU/mg, 277 PU/mg, 290 PU/mg, 279 PU/mg, 248 PU/mg, 235 PU/mg and 224 PU/mg respectively (Fig. 4.8).

4.7.6 Effect of agitation speed on the enzyme production

The production of protease was observed by varying the agitation speed of shaker incubator at 100, 150, 200, 250 and 300 rpm. The results showed that maximum production of enzyme was observed after 72 hrs at 150 rpm (297 PU/mg). In case of 100, 200, 250, 300 and 350 rpm the specific activities were 241 PU/mg, 257 PU/mg, 248 PU/mg, 233 PU/mg, and 228 PU/mg respectively (Fig. 4.9).

4.7.7 Effect of various carbon sources on the production of protease

Effect of various carbon sources was observed on the production of protease. The different carbon sources (1% w/v) were added to the medium. It was observed that glycerol gave the maximum production of protease (296 PU/mg) followed by mannitol (268 PU/mg). The specific activities of other sugars such as sucrose, fructose, dextrose, sorbitol, lactose, glactose, maltose and glucose were 154 PU/mg, 246 PU/mg, 263 PU/mg, 206 PU/mg, 257 PU/mg, 228 PU/mg, 182 PU/mg, and 133 PU/mg (Fig. 4.10).

4.7.8 Effect of nitrogen sources on the production of protease

When different nitrogen sources (1% w/v) were used to check for the maximum production of protease, it was observed that yeast extract gave the optimum production of protease (355 PU/mg) when incubated for 72hrs.Where as the specific activities observed in case of other nitrogen sources were 149 PU/mg, 275 PU/mg, 249 PU/mg and 298 PU/mg for Tryptone, Peptone, Beef extract and Gelatin respectively (Fig. 4.11).

4.7.9 Effect of Divalent Cations on enzyme production

Effect of different metal cations (1 mM concentration) was observed on production of protease. The highest level of protease activity (387 PU/mg) was observed in the presence of Mn^{2+}. Addition of $Ca^{2+}$and Mg^{+2} also resulted in high protease production but less as compared to Mn^{2+} where as Cu^{2+} and Zn^{+2} caused inhibition of protease activity (Fig. 4.12).

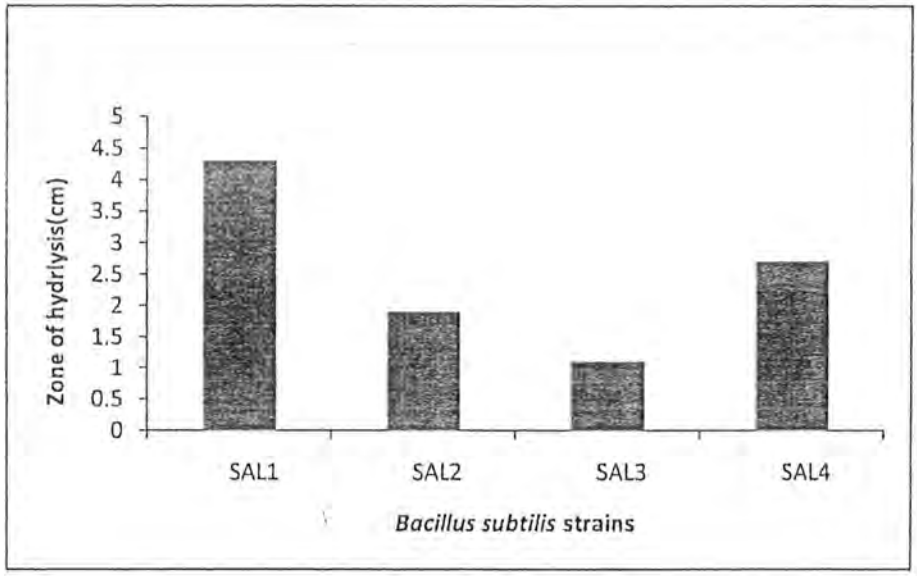


Fig. 4.1: Zone of hydrolysis of various isolates of *Bacillus subtilis* strains.

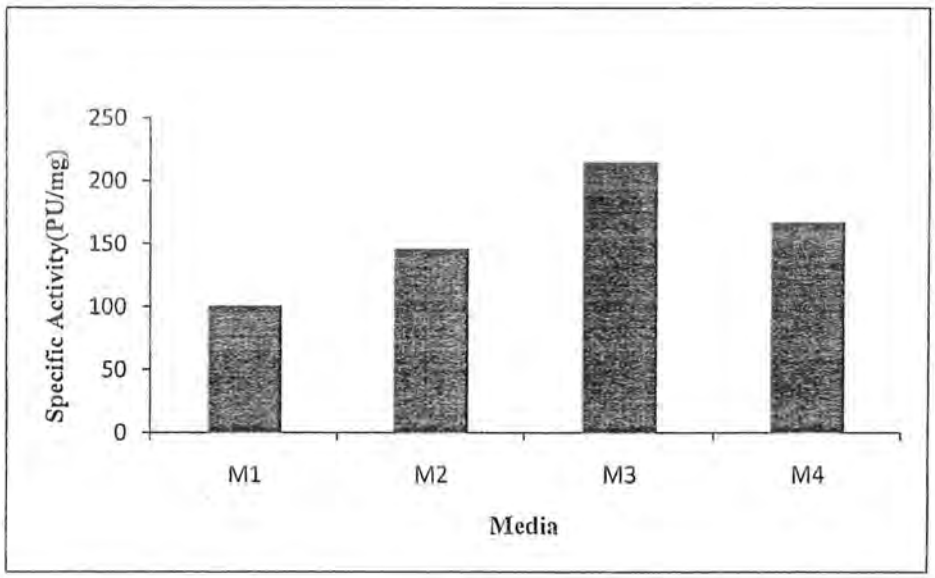


Fig. 4.2: Effect of different media used for the production of protease from *Bacillus subtilis* strain SAL1 after 48 hours of incubation, at 37 °C, 150 rpm and pH 8.0.

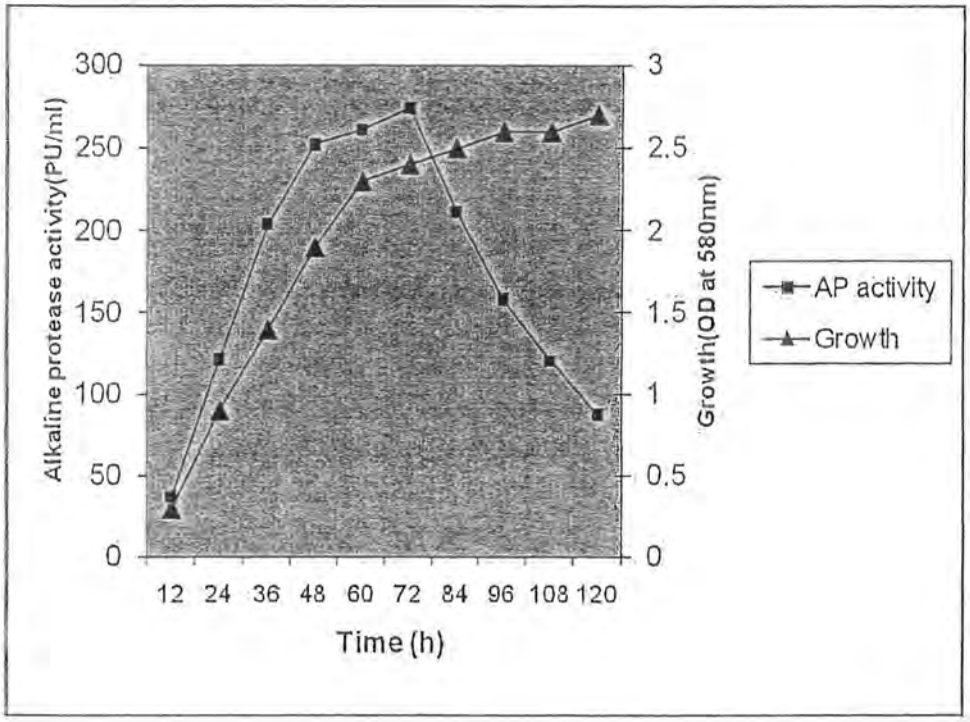


Fig. 4.3: Time course of growth and protease production by *Bacillus subtilis* SAL1

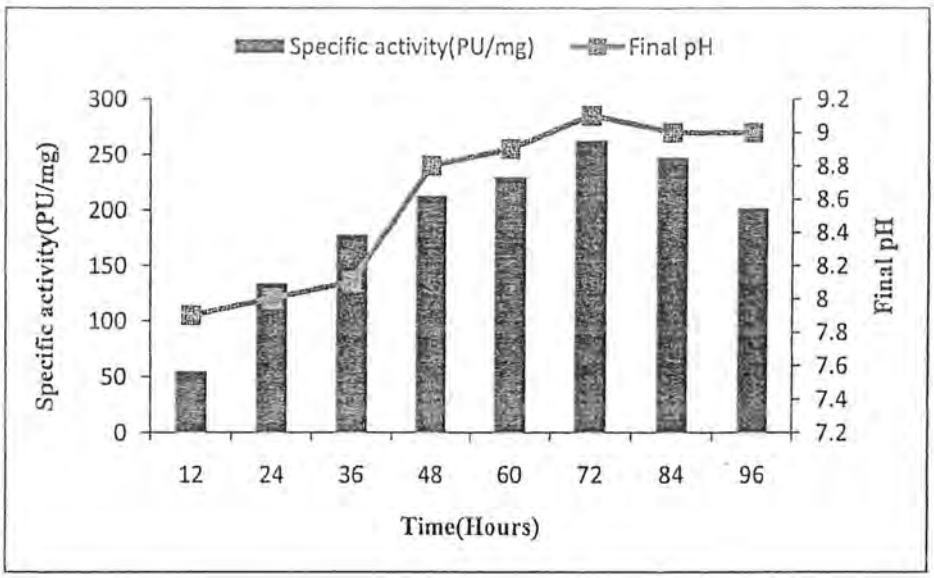


Fig. 4.4: Effect of incubation period on the production of protease from *Bacillus* sp. at 37 °C, 150 rpm and pH 8.0.

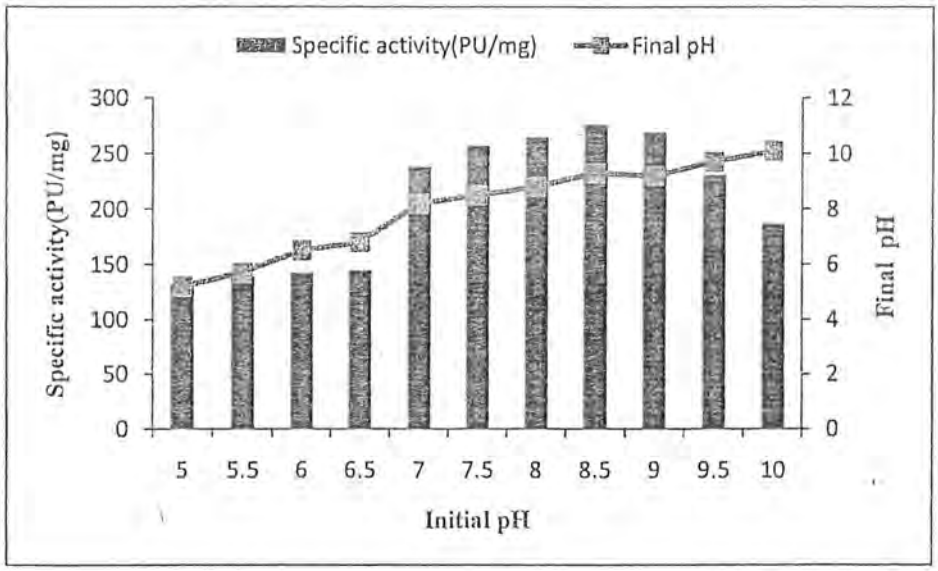


Fig. 4.5: Effect of initial pH of medium on the production of protease from *Bacillus subtilis* strain SAL1 at 37 °C, 150 rpm.

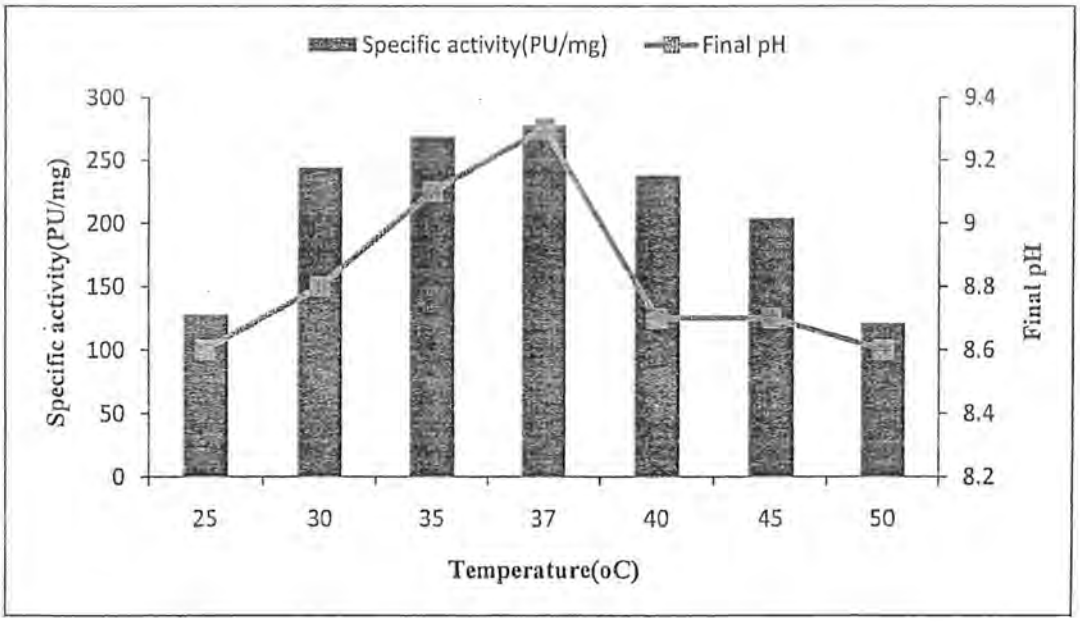


Fig. 4.6: Effect of different incubation temperatures on protease production from *Bacillus subtilis* strain SAL1 at 37 °C, 150 rpm and pH 8.5.

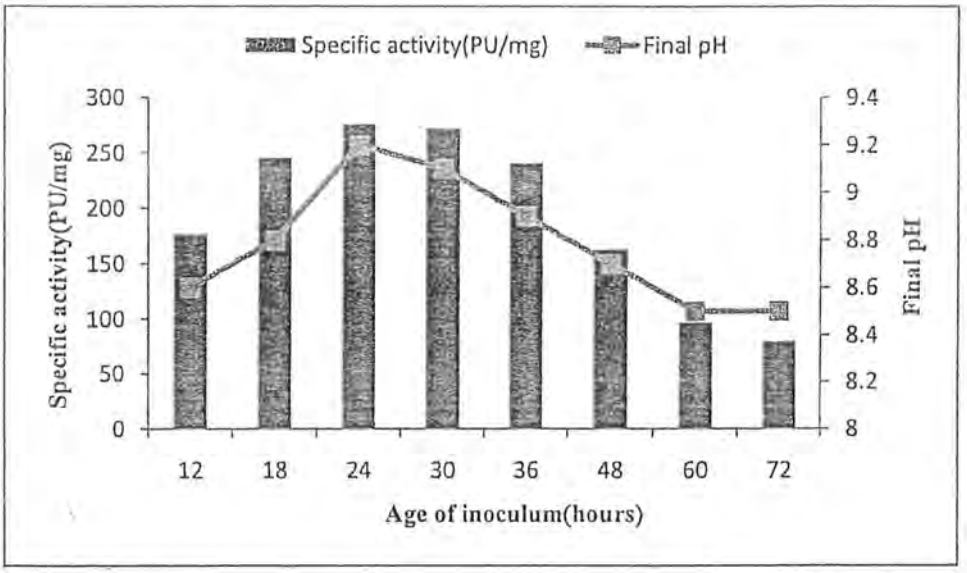


Fig. 4.7: Effect of age of inoculum on protease production from *Bacillus subtilis* strain SAL1 after 72 hrs, at 37 °C, 150 rpm and pH 8.5.

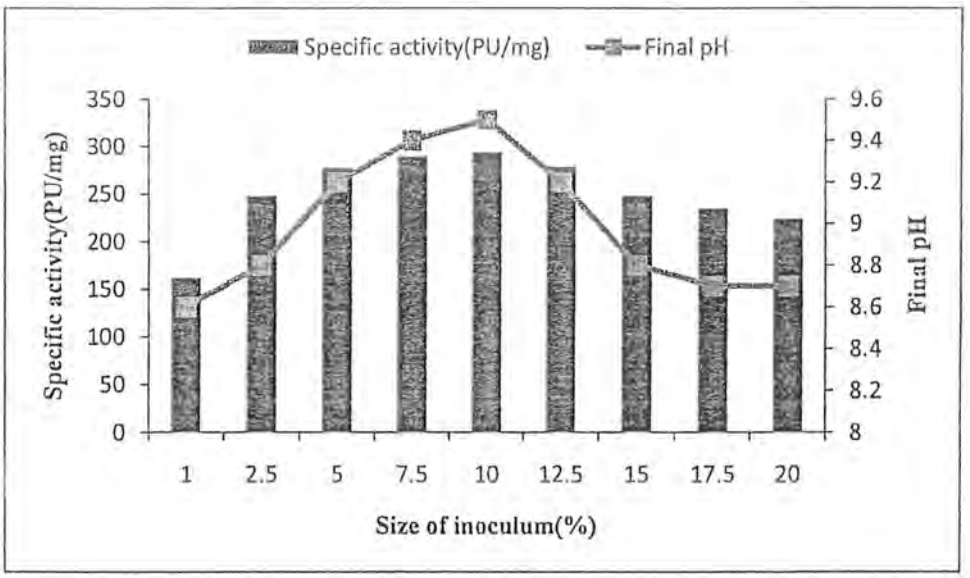


Fig. 4.8: Effect of size of inoculum on protease production from *Bacillus subtilis* strain SAL1 after 72 hrs, at 37 °C, 150 rpm and pH 8.5.

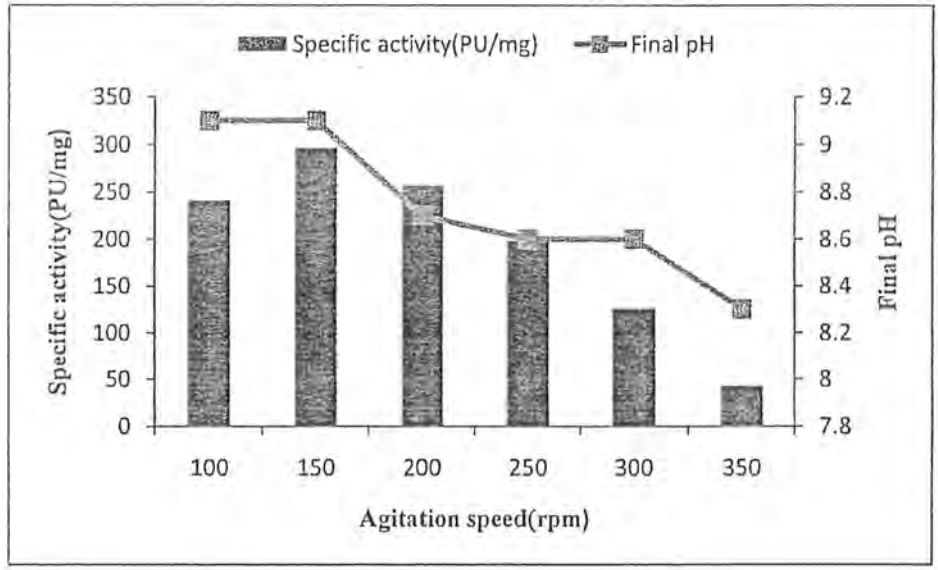


Fig. 4.9: Effect of agitation speed on protease production from *Bacillus subtilis* strain SAL1 after 72 hrs, at 37 °C and pH 8.5.

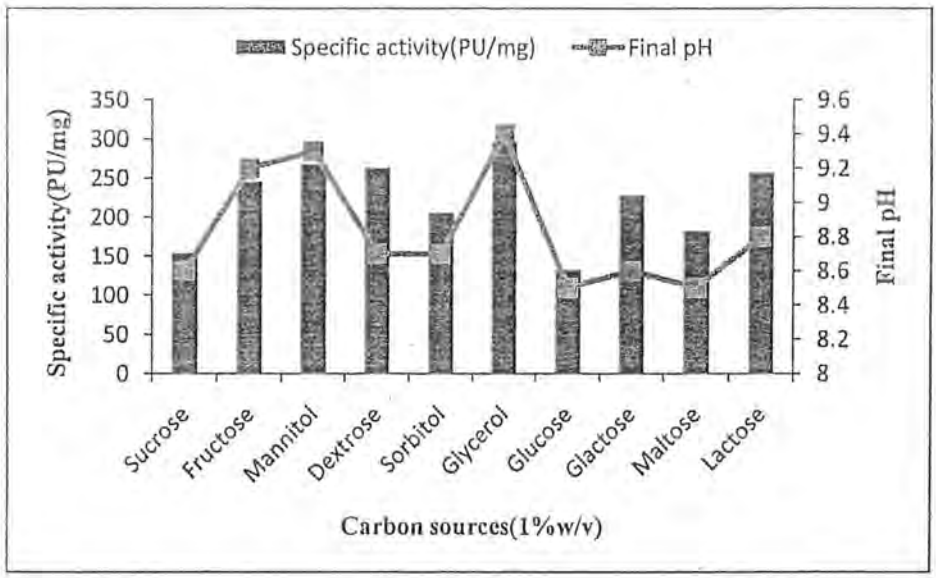


Fig.4.10: Effect of various carbon sources (1%w/v) on protease production from *Bacillus subtilis* strain SAL1 after 72 hrs, at 37 °C, 150 rpm and pH 8.5.

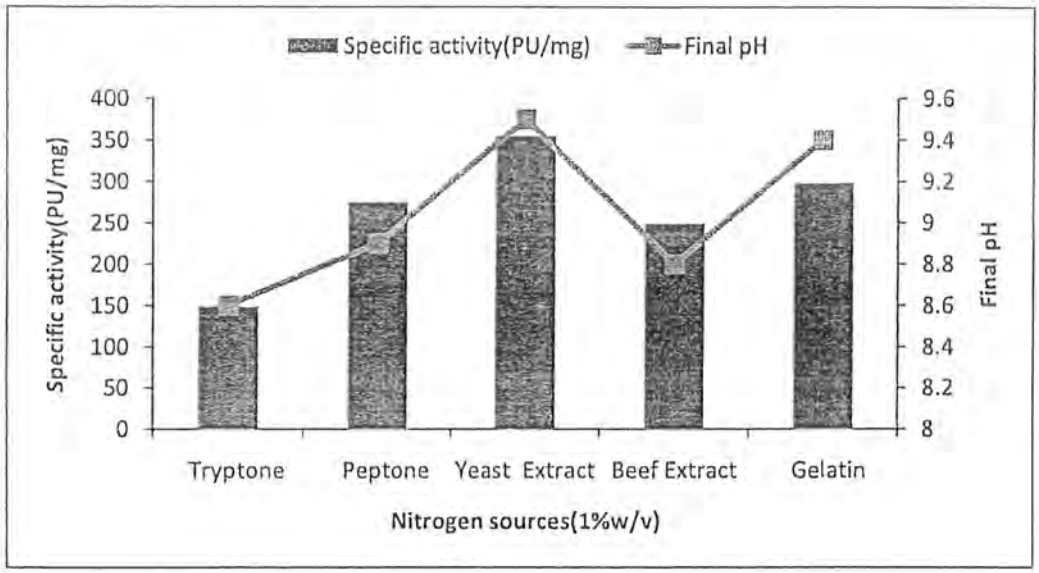


Fig. 4.11: Effect of different nitrogen sources (1%w/v) on protease production from *Bacillus subtilis* strain SAL1 after 72 hrs, at 37 °C, 150 rpm and pH 8.5.

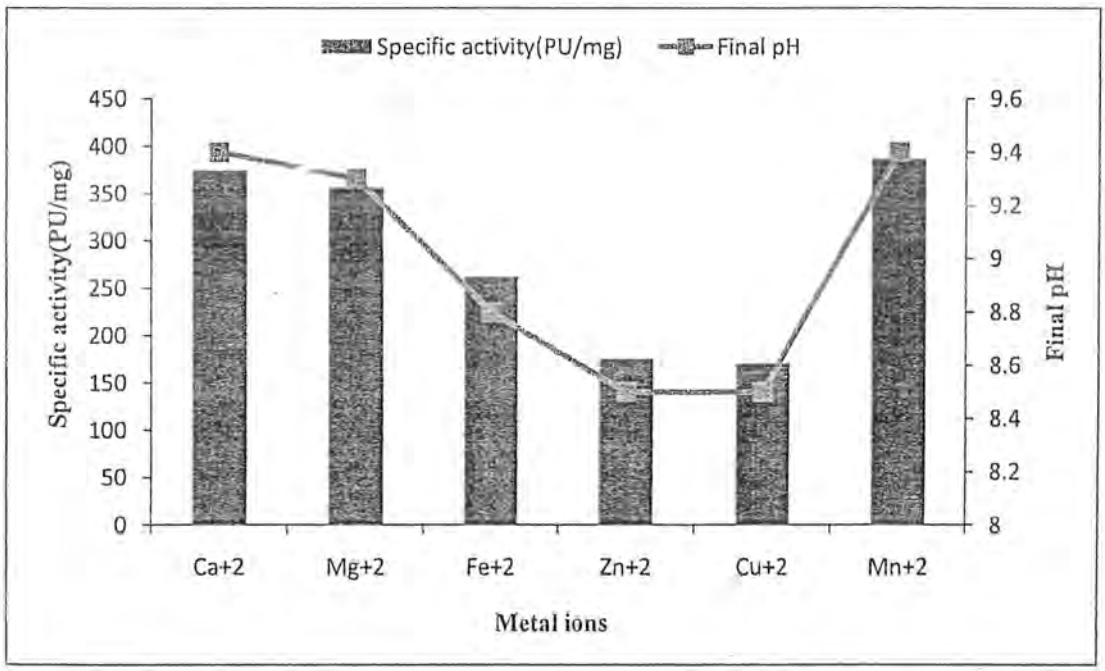


Fig. 4.12: Effect of different metal ions on protease production from *Bacillus subtilis* strain SAL1 after 72 hrs, at 37 °C, 150 rpm and pH 8.5.

4.8 Purification of protease

Purification of proteins is vital in the study of their function and expression. Separation involves removing any contaminants that are present in the mixture, these may be other proteins or completely different molecules altogether.

4.8.1 Ammonium sulfate precipitation

Ammonium sulfate precipitation was performed as the first step of protease purification. Different levels of saturation of ammonium sulfate were applied to the fermentation medium or cell free supernatant, which significantly affected the precipitation of protease as shown in fig.4.13. Ammonium sulfate fractions collected at levels of saturation of 10% (w/v) were collected and assayed for specific protease activity. It is clear from the fig. (4.13) that the maximum percentage of the alkaline protease can be precipitated by addition of 70% saturation of $(NH_4)_2SO_4$ to the culture filtrate. Hence 70% saturation of $(NH_4)_2SO_4$ was found suitable for the extraction of alkaline protease and resulted in 1.7 fold increase in specific activity compared to the unconcentrated supernatant.

4.8.2 Dialysis

Dialysis was employed to remove excess of salts from the precipitates and to elute out the molecules less than 12,000 D molecular weight. The precipitates collected after ammonium sulfate precipitation were subjected to dialyzing bag (12,000 cut off) against the three change of tris buffer (pH 8.5).

4.8.3 DEAE-Sephacryl anion exchange chromatography

Dialyzed ammonium sulfate precipitates were applied to DEAE-Sephacryl matrix. All the other contaminating proteins other than protease were bound to the matrix and protease was recovered in flow through and washings. These washings were collected and assayed for protease activity. This step resulted in 4.7 fold increase in specific activity as compared to the unconcentrated supernatant and 2.7 fold increase compared to ammonium sulfate precipitation. DEAE-Sephacryl

anion exchange chromatography effectively removed the considerable amount of impurities in crude enzyme extract and protease activity was raised.

4.8.4 Phenyl sepharose hydrophobic interaction chromatography

DEAE-Sephacryl purified sample was loaded on phenyl sepharose 6fast flow matrix. The enzyme with proteolytic activity was completely adsorbed on the matrix and all other impurities went into washing. The bound enzyme was eluted with 50% ethylene glycol in 0.02M Tris HCl buffer (pH 8). As it is clear from the figure that basic proteins were eluted first and after them protease was eluted. About 15 fractions each of 2ml were collected and assayed for protease activity. Those fractions with the highest enzyme activity were pooled and refrigerated. This step removed all proteins other than protease and resulted in 11.17 fold increase in specific activity as compared to the crude enzyme (table 4.8).

Table 4.8: A summary of purification steps of protease produced by *Bacillus subtilis*

Purification stage	Volume (ml)	Protein (mg/ ml)	Total Protein (mg)	Activity (PU / ml)	Total Activity (PU)	Specific Activity (PU/mg Protein)
Culture supernatant	150	0.66	99	251	37650	380
Ammonium sulfate Precipitation	45	0.42	18.9	283	12735	673
DEAE-Sephacryl chromatography	32	0.17	5.44	310	9920	1823
Phenyl sepharose chromatography	12	0.08	0.96	340	4080	4250

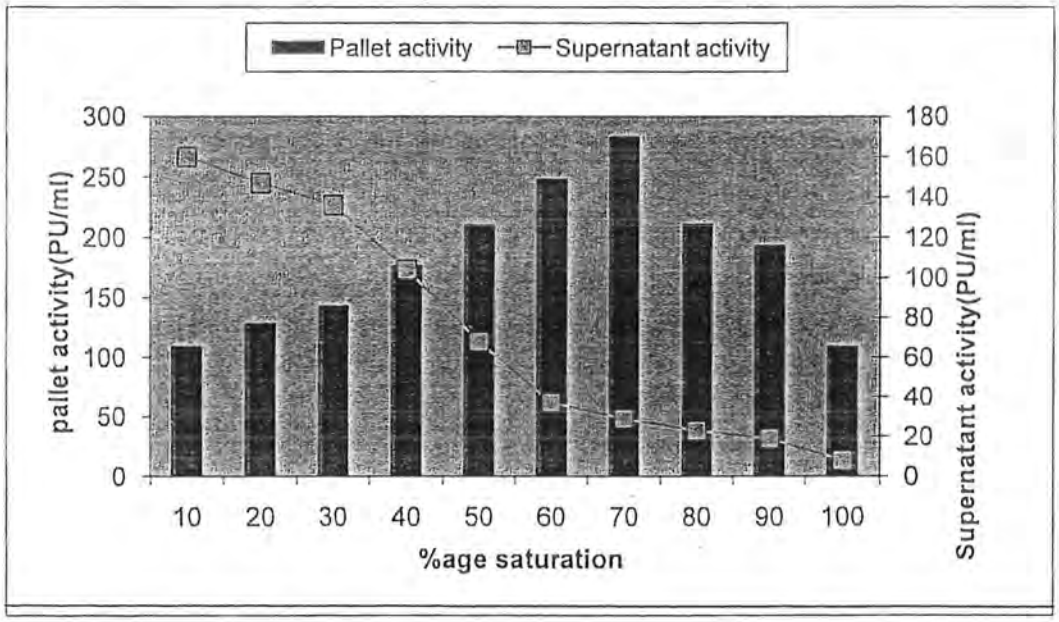
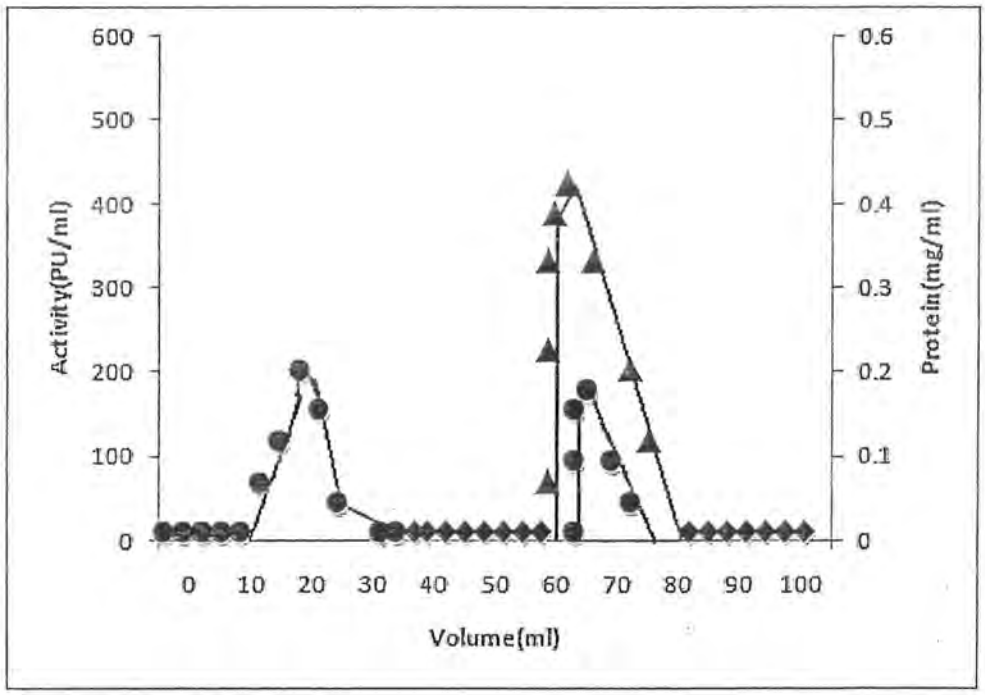


Fig.4.13: Activities of Precipitates from Ammonium Sulfate



▲ Protease activity ●Protein ◆ Elution buffer

Fig.4.14: Hydrophobic interaction chromatography of *Bacillus* protease on Phenyl Sepharose 6 Fast Flow

4.9 SDS-Polyacrylamide Gel electrophoresis and Zymography

Samples from protease purification steps (100µg eluted protein from Phenyl sepharose hydrophobic interaction chromatography) were pooled, dialyzed against distilled water, lyophilized and stored at -20 °C and run under denaturing condition on a 10% SDS-Polyacrylamide gel stained with silver (Fig. 20). The purified band showed a single band corresponding to molecular mass of 27000 Da by comparison to molecular weight standards. (Fig. 4.14)

Purified enzyme preparations were run under non-denaturing condition on a 10% SDS-Polyacrylamide gel in a 10% gel containing 0.05% gelatin. The gel was incubated overnight to allow hydrolysis of gelatin; the gels were stained with Coomassie blue. A clear zone of proteolytic activity against the blue back ground was observed as shown in fig.4.15. The zone of clearing corresponded to a migration distance which is consistent with an approximate molecular weight of 27,000 by comparison to molecular weight standards.

4.10 Capillary Electrophoresis and Mass Spectrometry (MALDI-TOF MS)

The homogeneity of enzyme was also checked by capillary electrophoresis which gave one peak confirming the purity of enzyme (Fig.4.17). The homogeneity and molecular weight of purified protease was also determined by mass spectrometer (MALDI-TOF). A single peak of protease activity was eluted and corresponded to a molecular mass of 27168 DA as shown in fig. 4.16. All these results indicate that the enzyme is a monomeric protein.

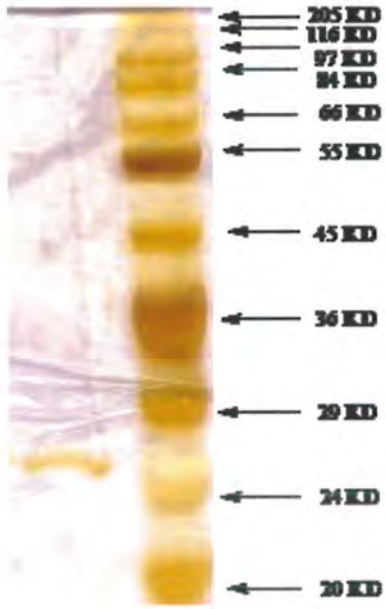


Fig. 4.15: Polyacrylamide gel electrophoresis of purified sample: Purified PAGE analysis was conducted on 10% polyacrylamide gel. (Lane 1: Purified enzyme Lane 2: Molecular weight markers Purified enzyme)

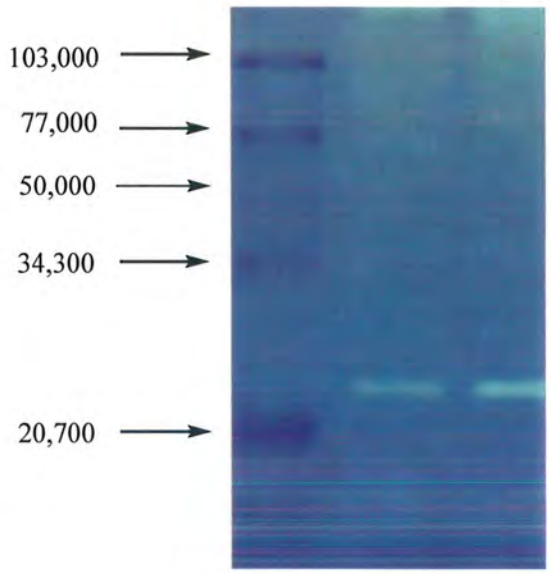


Fig. 4.16: Casein zymogram showing bands of protein cleavage on 10% SDS-PAGE. Lanes (left to right). Lane 1: molecular mass marker; lane 2: purified protease.

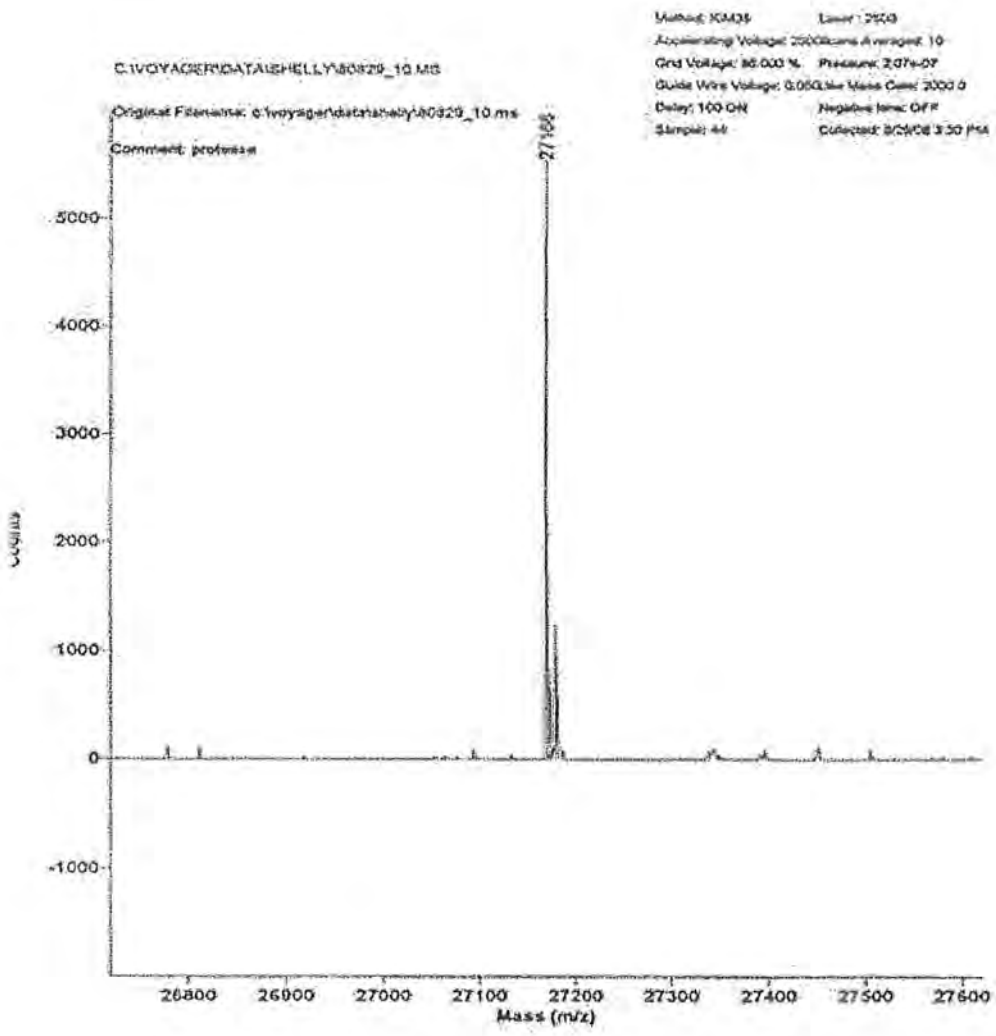


Fig. 4.17: MALDI spectrum of purified enzyme with sinapinic acid as matrix

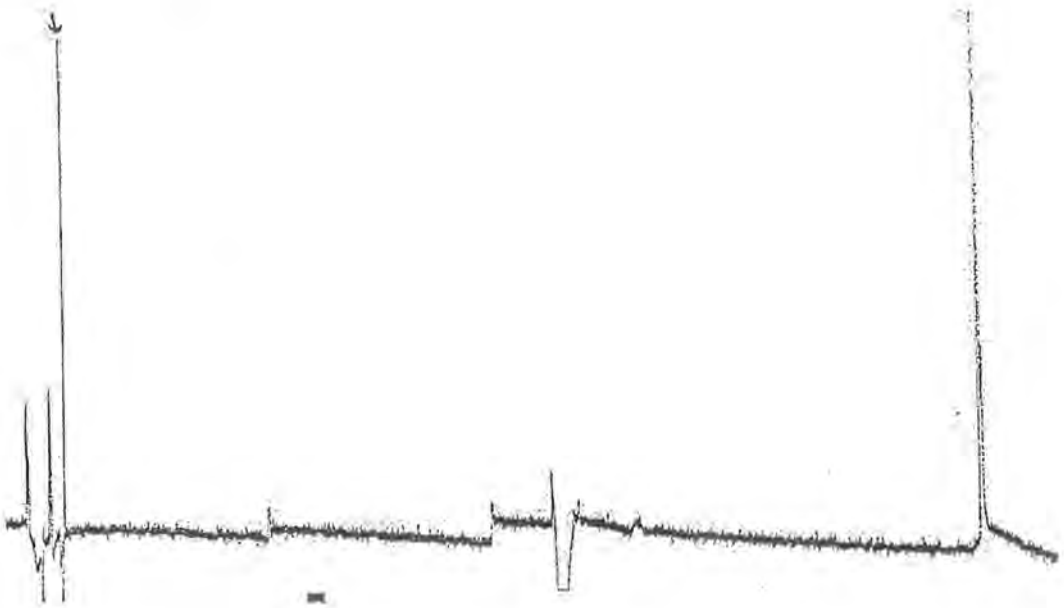


Fig. 4.18: Capillary electropherogram of purified protease showing a single peak. Conditions: 50mM phosphate buffer pH 2.5, electrokinetic injection 10 s @ 20 kV

4.11 Characterization of Purified Protease

4.11.1 Effect of pH on activity

The effect of pH on protease activity was determined by incubating the reaction mixture at pH values ranging from 5.0 to 12.0, in different buffer systems. Relative activities of protease at different pH are shown in fig.4.19. The maximum activity shown by enzyme has been taken as 100%.

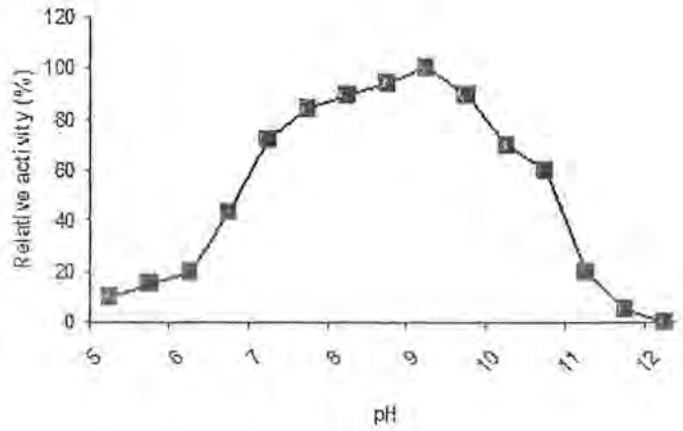


Fig. 4.19: pH optimum of purified protease

The optimum pH for caseinolysis by protease was found to be 9.0.

4.11.2 Effect of temperature on activity

The effect of temperature on pure enzyme was studied by assaying enzyme at different temperature in the range of (30-70 °C), at pH 8.5 using azocasein as substrate. The relative activities of purified enzyme at different temperatures are shown in fig.4.20. The maximum activity shown by enzyme has been taken as 100%

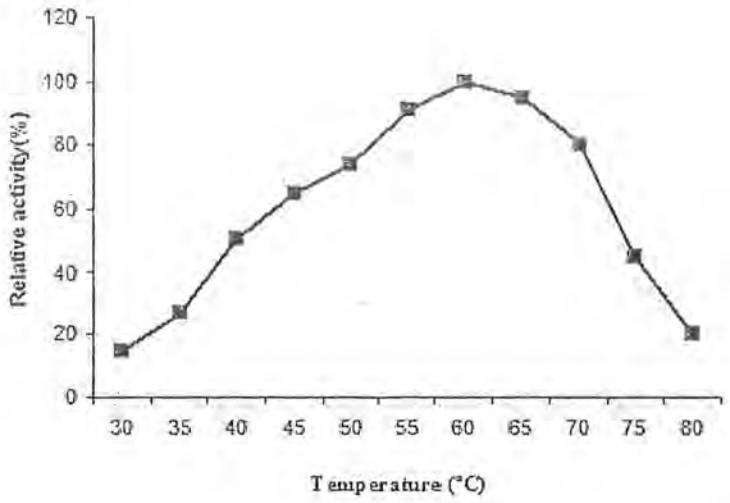


Fig.4.20: Effect of temperature on caseinolysis by purified protease

The optimum temperature for caseinolysis by protease was found to be 60 °C.

4.11.3 Effect of inhibitors on activity

Effects of different inhibitors on activity of enzyme are shown in fig.4.21. Activities after incubation with different inhibitors have been expressed relative to the controls. Activities shown by controls are taken as 100%. The enzyme was strongly inhibited by PMSF. A slight inhibition of activity was also noticed in case of EDTA.

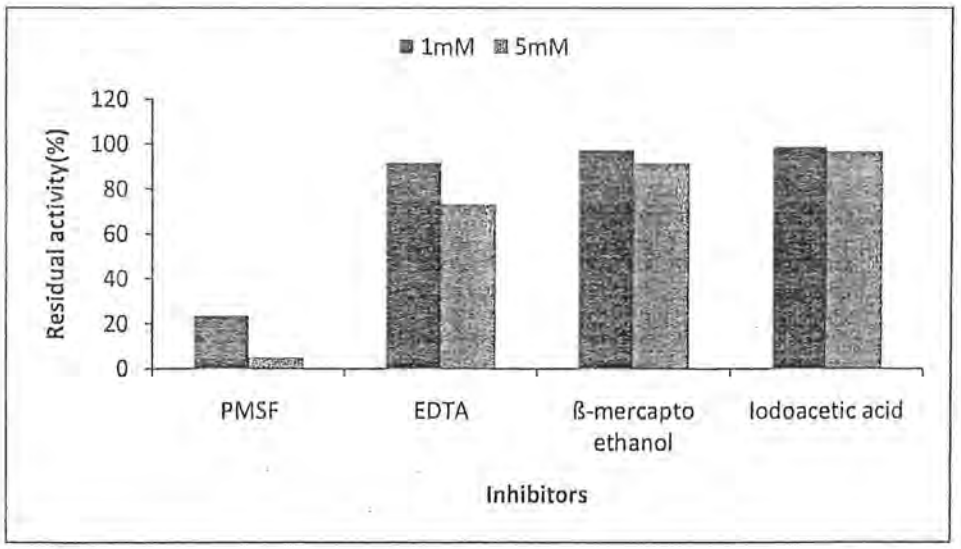


Fig. 4.21: Effect of different inhibitors on activity.

4.11.4 Effect of temperature on stability

The thermo stability of enzyme was studied by incubating the enzyme preparation at varying temperatures ranging from (40-90 °C) for 1 hour. The percentages of activity remaining after heat treatment at different temperatures are shown in fig. 4.22.

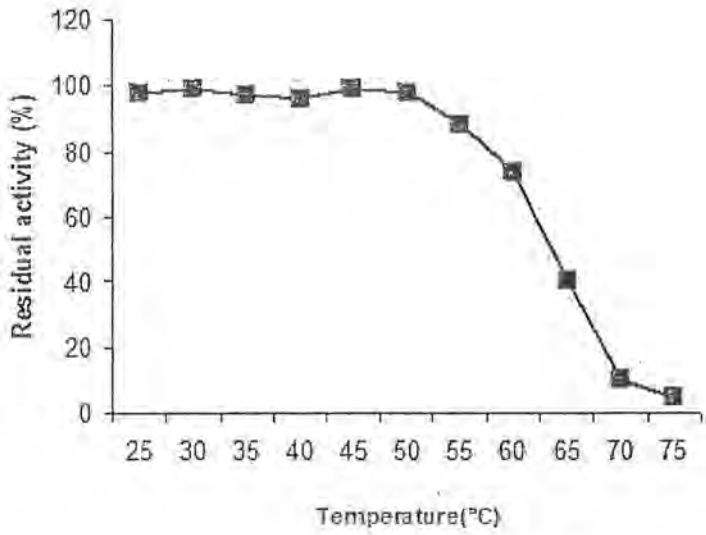


Fig. 4.22: Thermal stability of the purified protease at various temperatures.

Thermal stability of the purified protease at various temperatures (25–75 °C) was examined. The enzyme solution was incubated in 20 mM Tris–HCl (pH 8.5) at different temperatures for one hour and remaining activity was measured under standard assay conditions

The results showed that protease retained 100, 99.5, 98.6, 96.8, 96.3, 95.7, 92.8, 87.8, 74, 40 and only 11.5% of its activity when incubated for one hour at 25, 30, 35, 40, 45, 50, 55, 60, 65, 70 and 75 °C respectively.

4.11.5 Effect of pH on stability

The effect of different pH values on proteolytic activity of purified enzyme were studied by incubating the enzyme in various buffers with pH ranges from 4-12. The percentage of activity remaining after incubation was measured under standard assay conditions.

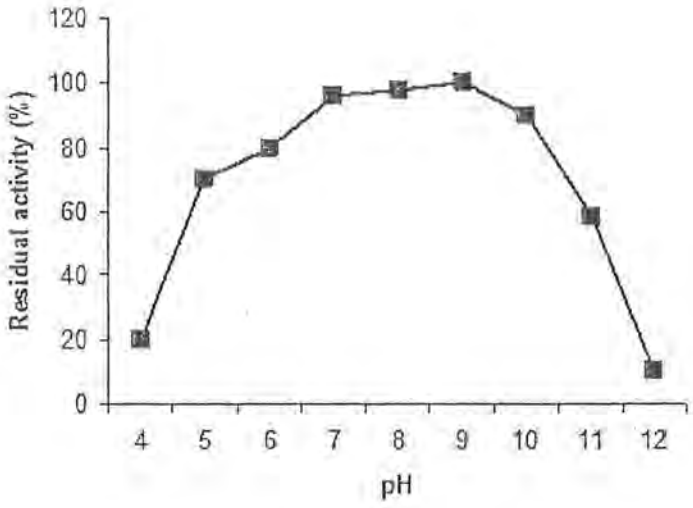


Fig.4.23: Effect of pH on the stability of protease

It is evident from the results that maximum proteolytic activity (380 PU/mg) was observed in the reaction mixture of pH 9. As the pH level is increased from 9 to 10, the enzyme activity decreases to (342 PU/mg) and the percentage stability was 90%. With the further increase in pH, there was decrease in enzyme activity. At pH 8, the enzyme is 98% stable with the activity of 372 PU/mg. At pH 4, 5, 6, and 7 the proteolytic activities are 152, 266, 304, and 365 with percentage stabilities as 40, 70, 80 and 96% respectively. From the results it is clear that enzyme is active in pH range of 7-10.

4.11.6 Effect of different surfactants on protease activity

The effect of different oxidizing agents and surfactants on proteolytic activity of purified enzyme was studied by incubating the enzyme with these reagents (1%) for one hour at room temperature. The residual activity was then measured according to the standard assay conditions.

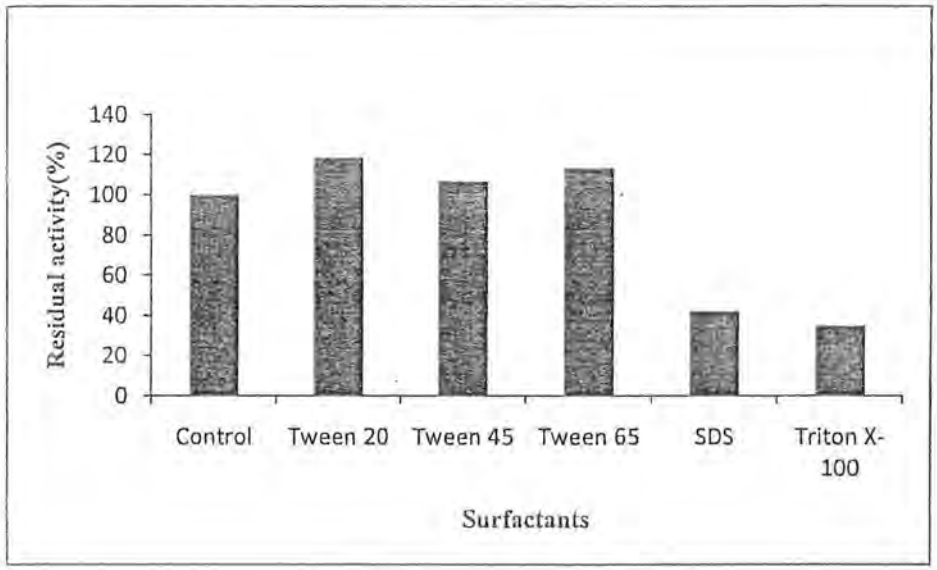


Fig.4.24: Effect of different surfactants on protease activity

The enzyme showed 118, 106 and 113% activity in the presence of Tween 20, Tween 45 and Tween 65 respectively, each at 1% concentration (by volume). However, the enzyme lost its activity in the presence of SDS and Triton X-100.These results indicate that this enzyme is compatible with most of the surfactants.

4.12 Leather Dust Degradation Studies

Proteolytic enzyme from *Bacillus subtilis* was used for degradation studies. The pH of leather dust was adjusted to 8.5 by using 0.1g NaOH. Then 3000 PU of proteolytic enzyme were added and incubated for 24 hours at room temperature. 2.7 µg/ml of hydroxyproline release was observed. To get the maximum degradation then parameters like time duration, pH, temperature and enzyme concentration were optimized.

4.13 Optimization of Different Parameters for Degradation of Leather Dust

4.13.1 Effect of time period

The maximum degradation of leather dust was observed after 84 hours as hydroxyproline released from leather dust is 7.4 µg/ml. Reasonable amount of leather waste is also degraded at 72 and 96 hours. The amount of hydroxyproline released at 12, 24, 36, 48, 60, 72, 96, 108, 120 and 144 hours was 1.9 µg/ml, 2.7 µg/ml, 3.4 µg/ml, 4.9 µg/ml, 5.7 µg/ml, 6.8 µg/ml, 6.5 µg/ml, 5.2 µg/ml, 5.1 µg/ml, and 5.1 µg/ml respectively (Fig. 4.25).

4.13.2 Effect of pH

It is evident from the result that maximum leather dust degradation was observed at pH 9.0. However the amount of hydroxyproline released were 6.7 µg/ml, 7.3 µg/ml, 8.1 µg/ml, 7.9 µg/ml, 4.2 µg/ml and 1.9 µg/ml at pH 7, 8, 9, 10, 11 and 12 respectively (Fig. 4.26).

4.13.3 Effect of temperature

The results show that although at temperature 45 °C maximum degradation of leather dust was observed as hydroxyproline released was 8.3 µg/ml, but it also worked well at temperature range from 25-60 °C with very little difference (Fig. 4.27).

4.13.4 Effect of proteolytic units

It is evident from the result that maximum leather dust degradation was observed when it was treated with 5000 PU of enzyme. There was no further increase in leather dust degradation when enzyme concentration was increased to 6000 PU, 7000 PU and 8000 PU(Fig. 4.28).

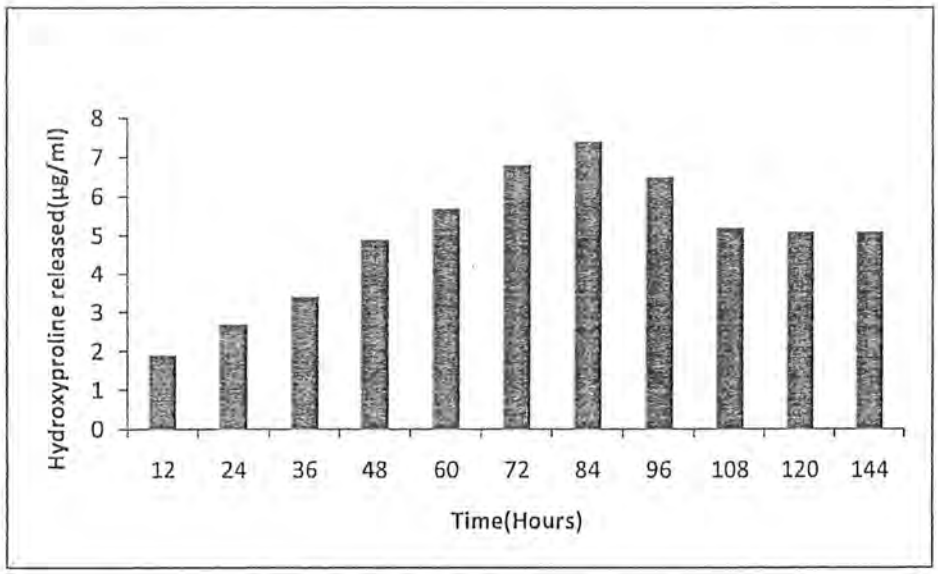


Fig. 4.25: Optimization of Time for maximum leather dust degradation with 3000 PU/mg of bacterial protease.

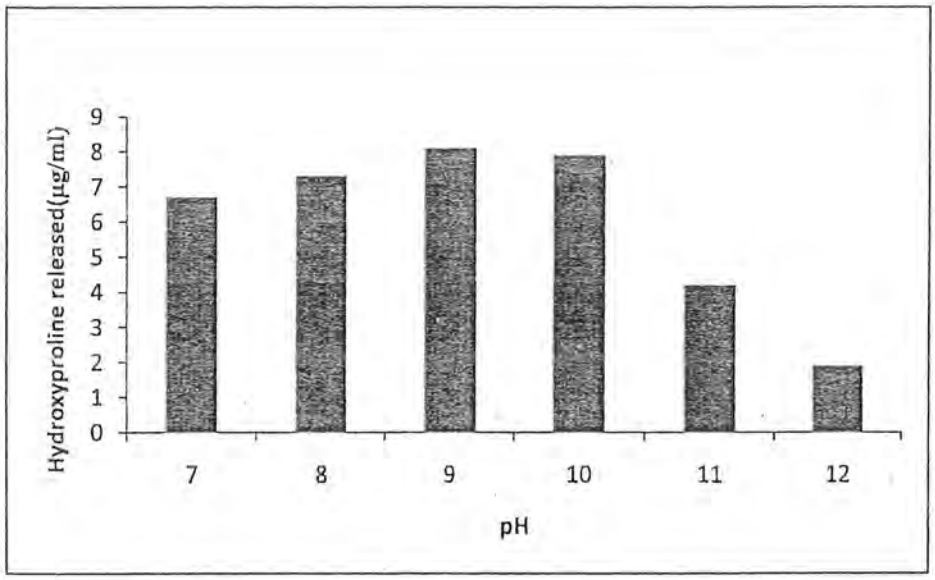


Fig.4.26: Effect of pH on the degradation of leather dust treated with 3000 PU/mg of bacterial protease after 84 hours of incubation.

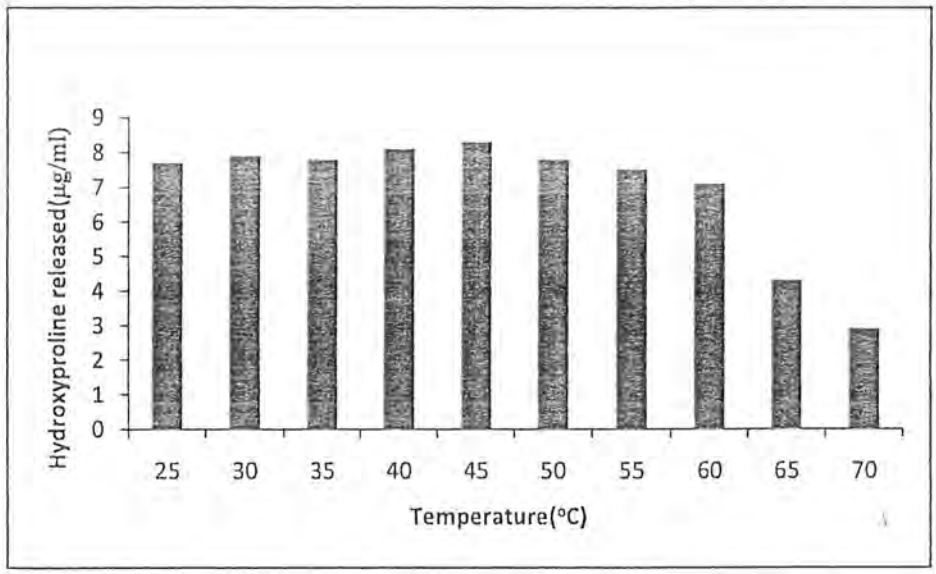


Fig. 4.27: Effect of temperature on the degradation of leather dust treated with 3000 PU/mg of bacterial protease after 84 hours of incubation at pH 9.0.

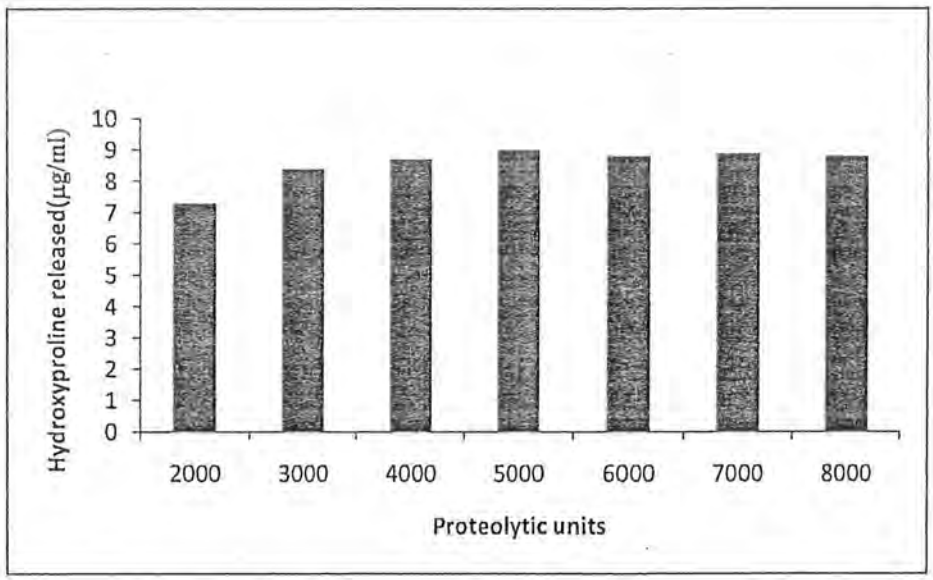


Fig. 4.28: Effect of proteolytic units on leather dust degradation at pH 9, temperature 45 °C for 84 hours.

4.14 BIOTREATMENT OF CHROME SHAVINGS WITH MICROBIAL ENZYME

4.14.1Analysis of Chrome shaving

The chrome shavings used in these experiments were obtained from a commercial tannery. They were analyzed for pH, moisture, ash and TKN. Analysis of leather waste indicates that it contains about 5% chromic oxide and about 17.42%TKN, as shown in Table 4.9.

Table 4.9 Analysis of Chrome Shavings

Parameter	Value
pH	4.19
Moisture (%)	50.19
Ash (%)	11.51
TKN (%)	17.42
Cr_2O_3 (%)	5.41

4.14.2 One-step Process

In the one step process of hydrolysis of chrome shavings, the shavings were pretreated at 60 °C for three hours with alkali (MgO). The enzyme was added and the samples were agitated for three more hours at this temperature. The products (hydrolyzed protein & chrome cake) were isolated and analyses were run on them.

Table 4.10: Chemical Analysis of Chrome Cake products from One-step process

Parameter	Value
Moisture (%)	89.5
Ash (%)	25.13
TKN (%)	10.38
Cr_2O_3 (%)	6.57

Table 4.11: Chemical Analysis of Protein products from One-step process

Parameter	Value
Total solids (%)	2.62
Ash (%)	7.41
TKN (%)	18.83
Cr (%)	0.031

The chemical analysis of chrome cake and Protein products is presented in Table 4.10 and 4.11. Chromium and ash contents of chrome cake are higher than found in protein products where as TKN values of protein products are higher as compare to chrome cake. It shows the efficient reaction of enzyme. The protein products were yellow in color and were not completely like gel. Ash and chromium contents protein products were also less.

The molecular weight distribution of the hydrolysate protein as obtained from SDS-PAGE is shown in fig.4.29&4.30.12% gel shows two intense bands at MWs~ 58 and 84 kDa. The 8–25% gradient gel shows a wide distribution of MW with prominent bands 97 000, 84 000, 55 000, 45 000 Da.

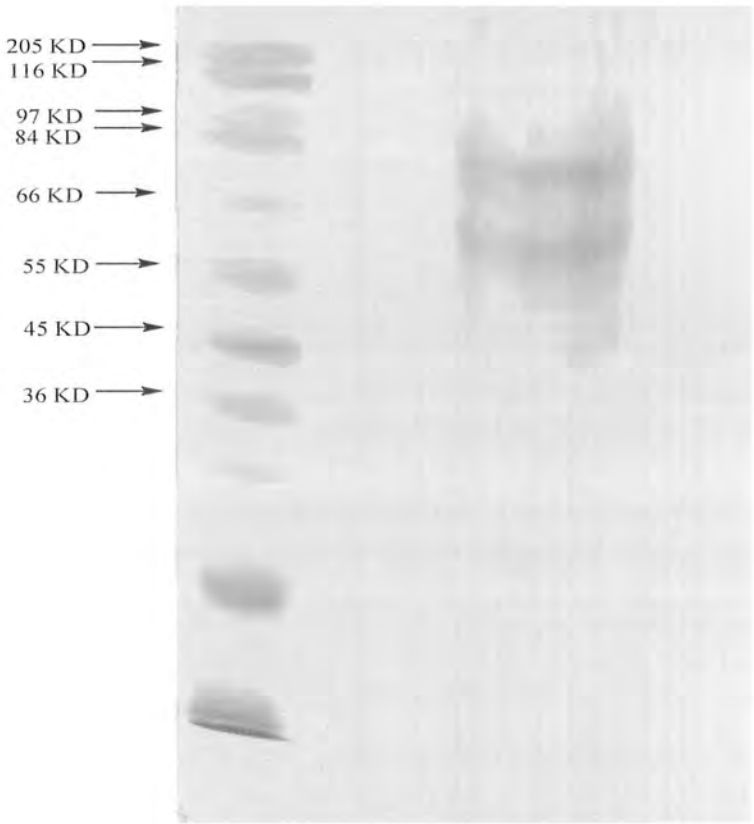


Fig.4.29: 12% SDS-PAGE gel for molecular weight distribution evaluations of protein products showing intense bands at MWs, 58 kDa and 84 kDa. (Lane 1: Molecular weight markers, Lane 2: hydrolysate from one step process).

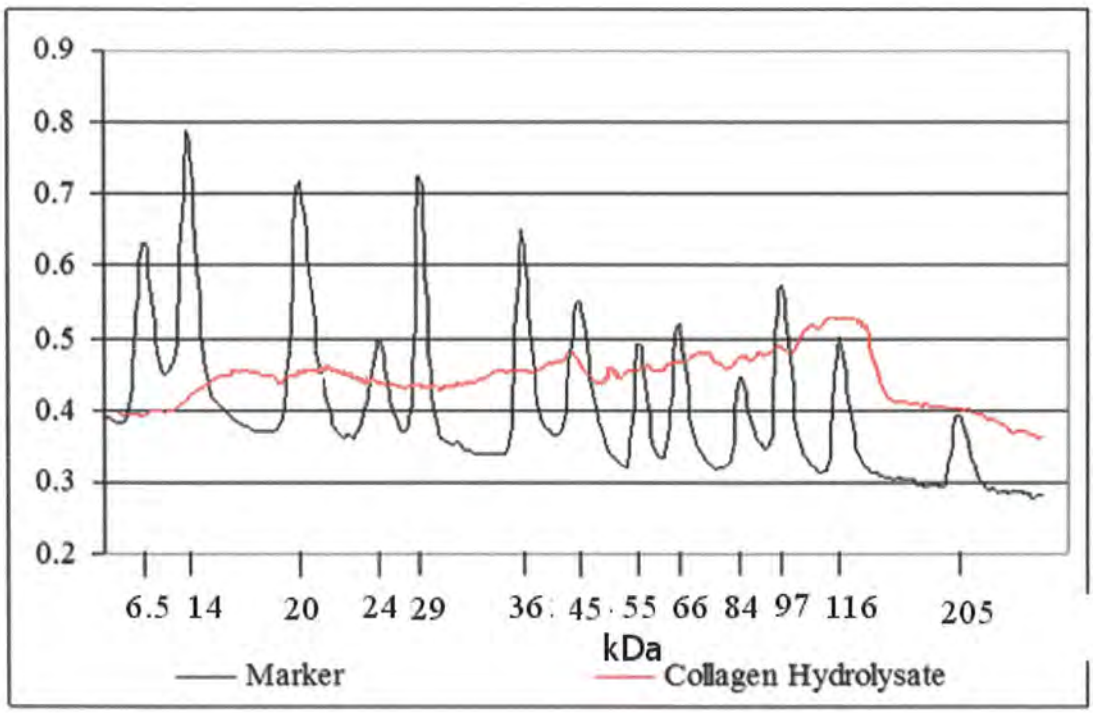


Fig. 4.30: Line graph of SDS-PAGE electropherogram of collagen Hydrolysate from one step process in 8-25% gradient gel. (Gel scanned using Computing Densitometer Scan v 5.0 and graph obtained from MD Image Quant Software v 3.3)

4.14.3 Two-step Process

In the two step process of hydrolysis of chrome shaving, it was first treated with MgO to extract gelatin in the first step and polypeptide in the second step, respectively. Magnesium oxide was used at 70 °C for 6 hours to isolate gelatin. In the second step of this process, chrome sludge was treated with enzyme at 60 °C for 3.5 hours. The protein products (gelatin&hydrolysate) and solid products (chrome sludge & chrome cake) were analyzed.

Table 4.12: Chemical Analysis of Gelable Protein Products

Parameter	Value
Total solids (%)	3.12
Ash (%)	13.71
TKN (%)	12.32
Cr (%)	0.027

Table 4.13: Chemical Analysis of Hydrolyzed Protein Products

Parameter	Value
Total solids (%)	3.51
Ash (%)	6.42
TKN (%)	17.98
Cr (%)	0.00

The chemical analysis of Gelable Protein Products (extracted by alkali) and Hydrolyzed Protein Products (extracted by enzyme) are presented in Tables 4.12& 4.13.TKN values of Hydrolyzed Protein Products are more as compare to the TKN values of Gelable Protein Products showing more protein contents. The ash and chrome contents of Hydrolyzed Protein Products are less as compare to Gelable Protein Products. It indicates more efficient reaction of enzyme.

Table 4.14: Chemical properties of Solid Products isolated from chrome shaving biotreatment

Parameter	Chrome sludge	Chrome cake
Moisture (%)	76.54	81.23
Ash (%)	23.18	31.33
TKN (%)	14.59	6.33
Cr_2O_3	5.52	7.83

The Chemical properties of the chrome sludge isolated after the extraction of gelatin and the chrome cake isolated after the hydrolysis step are summarized in Table 4.14. Chrome cake retained more moisture contents then did the chrome sludge because due to fibrous nature of chrome sludge, it was easy to filter. Ash contents of chrome cake are increased as compare to chrome sludge where as TKN of chrome cake has been decreased as compare to chrome sludge.
The molecular weight distribution of Hydrolyzed Protein Products obtained from SDS-PAGE is shown in fig.4.31&4.32. 12% gel shows two intense bands at MWs ~ 22 and 32 kDa. The 8–25% gradient gel shows a wide distribution of MW with prominent bands at 84 000, 66000, 55 000, 45 000, 24 000, 20 000 and 16, 000.

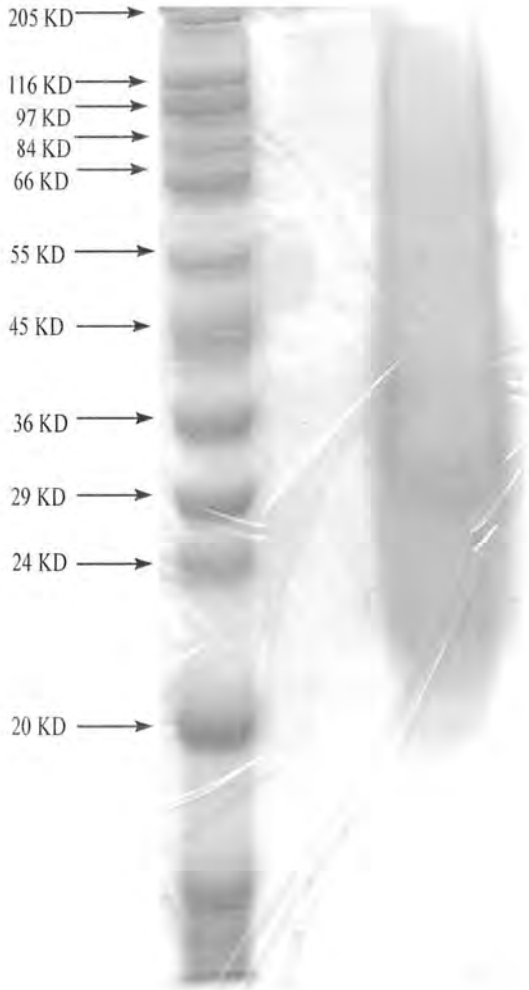


Fig.4.31:12%SDS-PAGE gel for molecular weight distribution evolutions of protein products showing intense bands at MWs, 22 kDa and 32 kDa. (Lane 1: Molecular weight markers, Lane 2: hydrolysate from two step process).

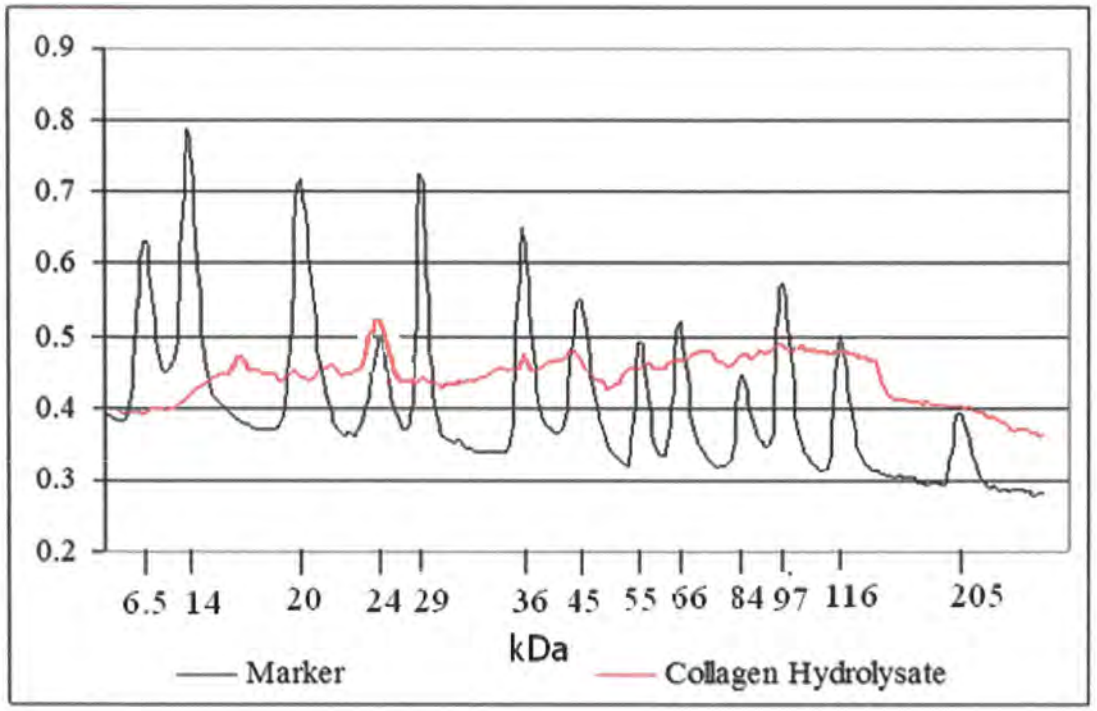


Fig. 4.32: Line graph of SDS-PAGE electropherogram of collagen Hydrolysate from two step process in 8-25% gradient gel. (Gel scanned using Computing Densitometer Scan v 5.0 and graph obtained from MD Image Quant Software v 3.3)

4.15 Hydrolysis of Ground finished Leather

Hydrolysis of ground finished leather began with potassium hydroxide treatment at pH12 and 60°C. Then the pH was decreased to 9 with nitric acid and protease was added. Near the end of hydrolysis the pH was further decreased to 5 and the product is filtered with the aid of spontaneous flocculation at this lower pH.

This process produced hydrolysate protein and chrome sludge which were stored for further analysis.

Table 4.15: Chemical properties of Chrome sludge

Parameter	Value
Moisture (%)	73.48
Total Ash (%)	15.7
TKN (%)	3.41
Cr_2O_3 (%)	6.96

Table 4.16: Chemical properties of Hydrolyzed Protein Products

Parameter	Value
Total solids (%)	3.12
Ash (%)	1.73
TKN (%)	15.87
Cr (%)	0.00

The Chemical properties of the chrome sludge and Hydrolyzed Protein Products show that TKN values of hydrolyzed protein products are much greater than the TKN values of chrome sludge where as chromium and ash contents of hydrolyzed protein products are much lower than those found in chrome sludge. (Table 4.16). It indicates the effectiveness of enzyme in the process of hydrolysis.

The molecular weight distribution of the hydrolysate protein was obtained by SDS-PAGE. 12% gel shows intense band at MWs 45 kDa (Fig.4.33). The 8–25% gradient gel shows a wide

distribution of MW with prominent bands at116000, 97000, 84 000, 55 000, 45 000, 29000, 24 000, 20 000 and 16, 000. (Fig.4.34).This data confirms the effectiveness of protease in hydrolyzing the collagen hydrolysate into smaller molecular weight peptides, however the presence of fractions with large molecular weights indicate that some of collagen fibrils have not been hydrolyzed by protease.

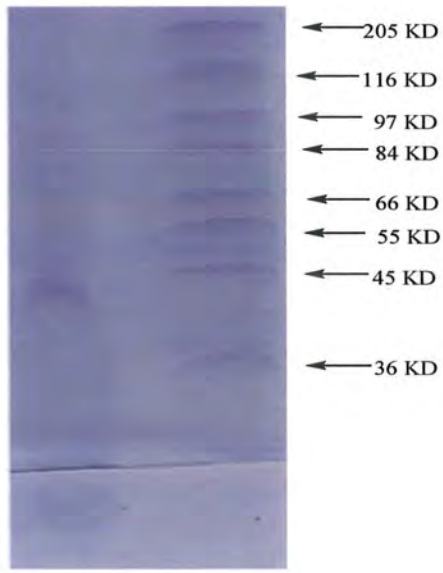


Fig. 4.33: 12%SDS-PAGE gel for molecular weight distribution evaluations of protein products)

(Lane 1: hydrolysate protein from upper shoe leather Lane2: Molecular weight markers)

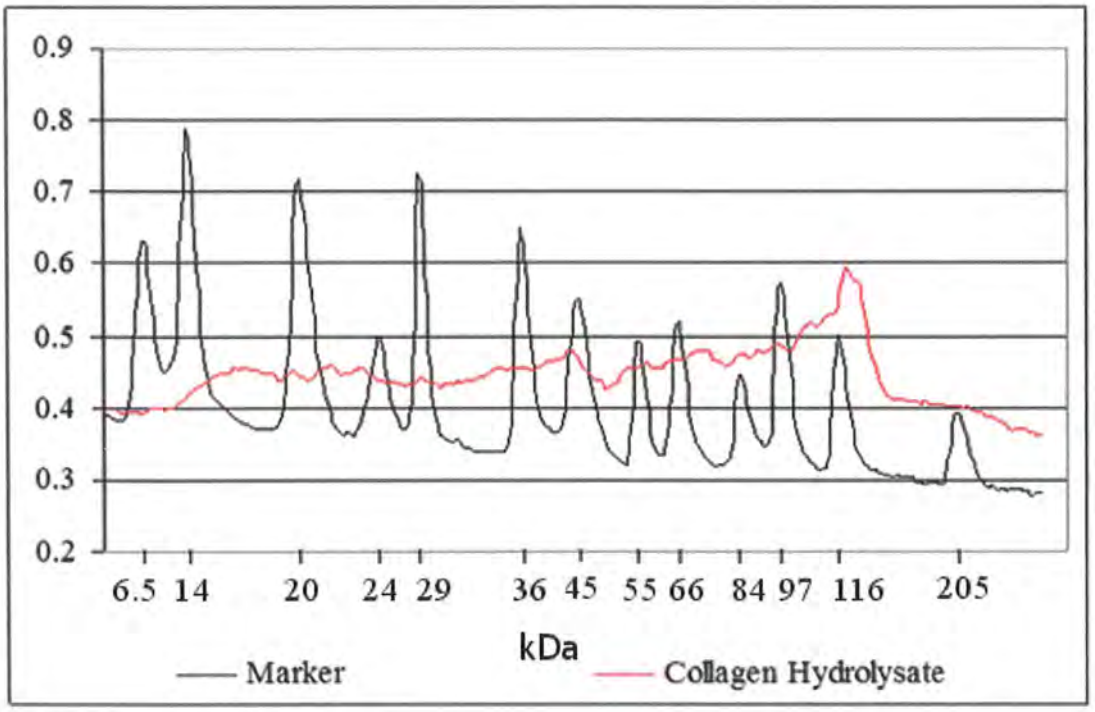


Fig. 4.34: Line graph of SDS-PAGE electropherogram of collagen Hydrolysate from upper shoe leather in 8-25% gradient gel. (Gel scanned using Computing Densitometer Scan v 5.0 and graph obtained from MD Image Quant Software v 3.3)

DISCUSSION

Proteases account for 60-65% of the global enzyme market. (Banerjee *et al.*, 1999; Laxman *et al.*, 2005; Genckel and Tari, 2006).An extensive studied was carried out on microbial proteases ever since the advent of enzymology as they are among the most important hydrolytic enzymes. Recently, a lot of studies and research has been conducted on proteolytic enzymes as they play a major role in the cellular metabolic processes in addition to their applicability in industries (Gupta *et al.*, 2002b).

As many as 3000 different enzymes have been identified and most of them have been used in industries for different purposes. Although, the application of proteases in biotechnological and industrial processes have been studied extensively but still a lot of things are yet to be achieved. There are so many available enzymes which cannot withstand the extreme industrial reaction conditions. This all led to a shift in attention towards the characterization of microorganisms which can thrive in extreme environments encountered often during industrial processes. Microorganisms also account for a two-third share of commercial protease's production in the world (Kumar and Takagi 1999).

With increasing industrial demands for the biocatalysts which can cope with industrial processes at harsh conditions, the isolation and characterization of new promising strains gained a lot of scientific attention to increase the yield of such enzymes (Gupta *et al.*, 2002b).

Most of the commercially important alkaline proteases are derived from *Bacillus* species. These bacteria are very important as they secrete large amounts of alkaline proteases having significant proteolytic activity and stability at high pH and temperatures (Kumar *et al.*, 1999; Pastor *et al.*, 2001).

In the present study, the ability of four strains of *Bacillus subtilis* (SAL1, SAL2, SAL3, and SAL4) was tested for the production of extracellular protease on Luria casein agar plates. Proteases have a wide range of applicability in different industries. Keeping in view the industrial importance of protease, an attempt was made to investigate different optimal culture conditions for maximum production of protease from *Bacillus subtilis* strain SAL1. The ecological sources play important role in ability of microorganism to produce certain products because the specific substrate and conditions are available and it is evident from the result that

Bacillus subtilis strain SAL1, isolated from tannery waste had more potential toward protease production because leather waste is rich in pretentious contents.

5.1 Isolation and Production

The primary stage in the development of an industrial fermentation process is to isolate strain(s) capable of producing the target product in commercial yields. Being the most important sources for enzyme production, the selection of suitable microorganism plays a key role in high yield of desirable enzyme. Keeping in view the industrial importance of protease, our great concern was to isolate and identify *Bacillus* sp. having a vital tendency to secrete extra-cellular proteolytic enzymes.

Qualitative test was performed for the initial screening of protease producing ability of *Bacillus* strains. The four isolates were inoculated on the sterilized casein-agar plates. Zone of hydrolysis were measured and the maximum zone correlated to the maximum protease producing isolate. The clear zone on those agar plates, around each bacterial colony was due to the hydrolysis of casein by proteolytic enzyme. The use of casein or milk agar for the isolation of protease producing bacteria has been reported by some other groups (Gessesse and Ghase, 1997). Proteolytic activity was shown by all strains; however, maximum activity was found in the case of *Bacillus subtilis* strain SAL1, isolated from tannery waste which was found to have 4.3 cm zone of hydrolysis.

Microbial proteases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. Most of the well studied microbial proteases are inducible extracellular enzymes. They are synthesized with in cell and exported to its external surface or environment. So, the biosynthesis of protease was done by submerged culture fermentation on shake flask and biofermenter. The ability of selected strain to maintain the high yielding nature was studied by sub culturing and testing the yields at monthly intervals. Repeated sub culturing was not having an effect on the stability of selected strain so this strain was used for further studies. The periodical testing of the yield by this strain was continued till the end of this study, at an interval of 3 months. Results were suggestive of the stable high yielding nature of the strain.

5.2 Optimization

Protease production is greatly influenced by the nutritional factors (carbon and nitrogen sources, metal ions, etc.) and physical factors (pH, temperature, dissolved oxygen, inoculation volume and incubation time). These factors play an important role in the growth of bacteria vis-'a-vis enzyme synthesis and secretion (Dutta *et al.,* 2004, Joo *et al,* 2005). With the objective of obtaining high yield of alkaline protease, the fermentation conditions (temperature, pH, agitation speed, inoculum concentration, incubation time and initial media pH) were optimized.

The study of the medium required for the best production of protease from different microbial sources or processes could not be finalized. The requirements for maximum enzyme production differs widely from one organism or strain to another. Four different media were tested for the best production of proteolytic enzyme. The best media that produced the optimal protease contained casein hydrolysate, gelatin and glycerol. Complex nitrogen sources are generally used for alkaline protease production but each organism demands a specific nitrogen supplement. Gelatin which acts as organic nitrogen source also enhances protease synthesis and bacterial growth. In this medium, glycerol acts as organic carbon source and it enhances the production of alkaline protease. Similar observations were made by (Qadeer *et al.,* 1990) while using xylose, manitol, fructose, sucrose and glycerol as carbon sources. In microorganisms, both organic and inorganic forms of nitrogen are metabolized to produce primarily amino acids, nucleic acids, proteins and cell wall components. The availability of both carbon and nitrogen sources in the medium matters a lot for the production of alkaline protease. Both have regulatory effects on the enzyme synthesis (Chu *et al.,* 1992, Moon *et al.,* 1991). Casein hydrolysate is a source of readymade amino acids which also encourages the foam formation to remove spores and cellular debris from the culture medium (Thomas and Winkler, 1977). It also enhances the extracellular alkaline protease synthesis.

The production of protease from *Bacillus* species can be correlated with growth. The production could be seen from early exponential phase onward. It was very low during the early stage of exponential phase. There was a steady increase in the protease production with the progression of growth from early exponential phase to early stationary phase. Maximum production was observed in early stationary phase. Different *Bacillus* species have been reported to be producing

maximum enzyme during the late exponential (Atalo and Gashe, 1993) post exponential (Manachini *et al.*, 1988) and stationary (Purva *et al.*, 1998) phases of growth. The exact reason for the increased production of protease during the later stages of growth is not known. There might be a relationship between protease production and sporulation, the event occurring mainly during the later stages of growth as observed by Debabov (1982).

The fermentation conditions (temperature, agitation speed, inoculum concentration, incubation time and initial media pH) were optimized in order to produce alkaline protease with maximum activity.

The optimum temperature for alkaline protease production by strain was found to be 37 °C. It has been observed that any change in the temperature decreases the proteolytic activity. This might be due to the reason that Growth temperature strongly affects the protease synthesis either by influencing the rate of biochemical reactions or inducing or repressing their production (Frankena *et al.*, 1986 and Ray *et al.*, 1992). Protease production have been reported at or around 37 °C by *B. alcalophilus* subsp. *halodurans* KP1239 (Takii *et al.*, 1990) and *B. firmus* (Moon and Parulekar, 1991).

The effect of initial pH of the medium on alkaline protease production was studied. The most optimum initial pH of the medium was 8.5. Alkaline protease production using media with alkaline pH have been reported by Takii *et al.*,1990, Manachini *et al.*, 1988, Sen and Satyanarayana, 1993 and Purva *et al.*, 1998.

Results of study on the effect of carbon sources on alkaline protease production show glycerol to be the best carbon source followed by mannitol. Glycerol is not easily degradable and needs a longer period of time to be exhausted from culture medium; hence it keeps the level of catabolite repressor lower and promotes protease production. Compared to glycerol, glucose and other easily metabolizable carbon sources were not so useful for alkaline protease production. Sen and Satyanarayana, (1993) who studied alkaline protease production by *Bacillus licheniformis* S40 have also reported similar observations.

Results of study on the effect of nitrogen sources on alkaline protease production showed yeast extract to be the best nitrogen source. It has been reported as a suitable nitrogen source for alkaline protease production by Prakasham *et al.*, 2006 and Rahman *et al.*, 2005.

In the present study protease production from *Bacillus* strain SAL1 was observed by adding different metal ions to the medium. These metal ions included Ca^{2+}, Mg^{2+}, Fe^{2+}, Zn^{2+}, Cu^{2+} and Mn^{2+}. Maximum specific activity (397 PU/mg) was observed in the medium containing Mn^{2+}. Stimulatory effect of Mn^{2+} on the protease production has been reported by Shafee *et al.*, 2005. This observation strongly suggested the requirement of some metal ions for protease production by this organism. Addition of metal ion to the growth media has shown an increase in the protease activity and is found to be mandatory for the production and stability of some proteases (Liao *et al.*, 1998; Kohlman *et al.*, 1991; Olson *et al.*, 1992). Addition of Ca^{2+} and Mg^{2+} also resulted in higher protease production however addition of Cu^{2+} and Fe^{2+} had an adverse effect on protease production. This observation is corroborated by previous studies which suggested inhibitory effect of Cu^{2+} on proteases (Adinarayana *et al.*, 2003, Palmieri *et al.*, 2001, Rice and Sartorelli, 2001).

It is evident from the results (Fig.4.7&4.8) that size and age of inoculum play an important role in the biosynthesis of proteolytic enzyme. It appears that the most suitable cells for use in system are those from 24 hours old culture. The initial suspension density has a considerable effect on the production of enzyme. The optimum level of inoculum for alkaline protease production by the strain was found to be 10%. This observation is in conformity with the reports by Sinha and Satyanarayana (1991) and Gaju *et al.*, (1996) who studied the alkaline protease production by *Bacillus licheniformis* N3, *Bacillus licheniformis* S40 and *Bacillus coagulans* PB77 respectively.

Agitation of culture was found to be essential for the high production of alkaline protease by *Bacillus* strain SAL1. In the present investigation, *Bacillus strain* showed maximum protease activity at 150 rpm agitation speed after 72 h of incubation. At this speed, aeration of the culture medium was increased which could lead to sufficient supply of dissolved oxygen in the media (Kumar *et al.*, 1999). Nutrient uptake by bacteria was also increased which resulted an increase in the protease production. At agitation speed greater than 200 rpm, protease activity was found to be reduced. This was perhaps due to the denaturation of enzymes caused by high agitation

speed (Lee *et al.*, 2002). At 100 rpm, an insufficient aeration and nutrient uptake perhaps caused the inability of bacteria to grow efficiently. At agitation speed greater than 200 rpm, however, excessive aeration and agitation could occur which led to cell lysis and increased cell permeability due to the abrasion by shear forces. Based on this finding, agitation speed of 150 rpm was used throughout the study.

As a result of optimization studies, the yield of alkaline protease by the strain could be increased approximately up to 80%.

5.3 Purification of alkaline proteases

Crude preparations of alkaline proteases are generally employed for commercial use. Nevertheless, the purification of alkaline proteases is important from the perspective of developing a better understanding of the functioning of the enzyme (Takagi 1993, Tsai *et al.*, 1988).

This protease was purified to homogeneity by a combination of ammonium sulfate precipitation, DEAE Sephacryl ion exchange and Phenyl Sepharose hydrophobic interaction chromatography.

The two liter culture grown for 72 hours was removed from the biofermentor and cell free supernatant was obtained by centrifugation (10,000 xg, 15 min) and filtered. The cell free solution obtained is then concentrated by ammonium sulfate precipitation. Ammonium sulfate purification increased the protease activity 20 fold as compared to the activity present in the non-concentrated culture supernatant. This precipitation step also decreased the overall protein concentration in the precipitation fraction containing the highest protease activity. Ammonium sulfate precipitation increased the protease activity by concentrating the enzyme. The increase in protease activity by using ammonium sulfate is consistent with available precedence (McKevitt *et al.*, 1989; Sexton *et al.*, 1994). The precipitate so obtained was subjected to dialysis for further removal of excessive salts and impurities.

The dialysis was followed by DEAE Sephacryl. DEAE Sephacryl played a selective permeable role for protease. All the proteins other than protease were bound to the matrix and protease was recovered in washing. Similar kind of behavior was also seen in the case of *Bacillus* sp. protease

(Shimogaki *et al.*, 1991) and *Pseudomonas* sp. protease (Gupta *et al.*, 2005). During the last step of purification, the specific activity was further enhanced. In this last step of purification, phenyl sepharose role was just opposite to the role of DEAE-sephacryl. Protease was completely retained by the matrix and all other impurities went into washing. The resultant bound protease was further eluted with 50% ethylene glycol. Almost similar behavior was reported for protease from *Pseudomonas* sp. (Gupta *et al.*, 2005).

SDS-PAGE of purified enzyme preparation is shown in fig.4.14. In silver stained gel, the purified preparation showed a single band corresponding to the molecular mass of 27000 Da. In literature, the alkaline protease with molecular weight ranging from 16-32 kDa is reported from *Bacillus* sp. (Adinarayana *et al.*, 2004; Kaur *et al.*, 1998; Jaswal *et al.*, 2007). Molecular mass of the purified protease was also confirmed by mass spectrometer (MALDI-TOF). The apparent molecular weight in SDS-PAGE was found to be 27 kDa, while the molecular weight in mass spectrometry (MALDI-TOF) analysis was found to be 27.168 kDa. The molecular mass of protease by mass spectrometry (MALDI-TOF) was also determined by Devaraja *et al.*, 2008 and the apparent molecular weight of their protease was found to be 16 kDa while the molecular weight in mass spectrometry (MALDI-TOF) analysis was found to be 16.350 kDa. The homogeneity of enzyme was also checked by capillary electrophoresis (fig.4.17) which gave one peak confirming the purity of the enzyme. Capillary electrophoresis for the analysis of proteolytic activity of protease has also been used by Albillos *et al.*, 2007.

The cell-free, filtered culture supernatant of *Bacillus* strain SAL1 exhibited a band of hydrolysis at a molecular weight of 27,000 Da on a 10 % casein zymogram (Fig.4.15), indicating that there was one protease within the extracellular supernatant. This band of hydrolysis at 27,000 Da corresponded to a band present in purified protease sample on a 10 % SDS-polyacrylamide gel.

5.4 Characterization

Some characteristics of purified protease were studied. The optimum pH for protease activity was 9, although enzyme was active in the pH range of 7-10. From a survey of literature it can be seen that the optimum pH range of alkaline proteases is generally between pH 9 and 11. Alkaline proteases of *Bacillus subtilis* PE-11 with similar properties have been reported by

Adinarayana *et al.*, (2003). These findings are in accordance with several earlier reports showing pH optima of 9.0-10.5 for protease from *Bacillus* sp. by Durham *et al.*, (1987), from *Xanthomonas maltophila* by Debette *et al.*, (1991), and *Vibrio metscnikovii* by Kwon *et al.*, (1994). The effect of pH on the stability of protease was studied. Protease was found to be stable in the pH range 7-10. Such highly alkali stable protease obtained from *Bacillus* sp. has been reported by Moon *et al.*, (1994).

The optimum temperature for protease activity was found to be 60 °C; however, it was not stable under its optimum temperature. Alkaline proteases of *Bacillus* sp. with similar temperature optima have been reported by Adinarayana *et al.*, (2003), Dhandapani *et al.*, (1994), Fujiwara *et al.*, (1987),Takii *et al.*, (1990) and Ferrero *et al.*, (1996). Thermal stability studies have indicated that protease retained 88% of its activity when exposed to 55 °C for 1 hr, however the percentage stability of enzyme declined to 74% at 60 °C and there was a sharp decrease in proteolytic activity with further increase in temperature. This inactivation of enzyme shows the destruction of enzyme at higher temperature incubation .The results showed that protease appeared to be heat labile at temperatures between 40-60 °C, when incubated for more than 1 hour.

The effects of various inhibitors on the activity of protease were studied. The enzyme was inhibited by PMSF indicating the presence of active serine residue in the catalytic site of enzyme. PMSF is a serine protease inhibitor which results in complete loss of the enzyme activity after inhibition (Jeong *et al.*, 2000, Adinarayana *et al.*, 2003, Feng *et al.*, 2001). So, these enzymes could be classified under serine proteases. A slight inhibition of activity by EDTA showed that alkaline protease in the present study had a contamination of neutral protease. ß-mercapto ethanol and Iodoacetic acid, the inhibitors of Cysteine and Sulphydryl proteases were having no inhibitory effect on the activity of protease.

Effects of various surfactants on the activity of protease were examined and then the stability of enzyme in terms of residual activity was determined. The enzyme showed 118, 106 and 113% activity in the presence of Tween 20, Tween 45 and Tween 65 respectively. This stimulatory effect of non-ionic surfactants on protease activity was also reported by Kamande *et al.*, 1999 and Nadeem *et al.*, 2008. However there was loss in the activity of enzyme in the presence of

SDS and Triton X-100. Loss of protease activity in the presence of SDS and Triton X-100 was also reported by Gupta *et al.*, (2005).

5.5 Role of Proteases in Leather Dust Degradation

Collagenolytic activity of protease was then checked by incubating it with leather dust under certain conditions. Solid wastes obtained during tanning are high in protein contents and contain mainly collagen. Collagen is composed of triple helix formed from three helically wound α-chains constructed from repeating Gly–X–Y triplets where X and Y can be any amino acid but are frequently the amino acids proline and hydroxyproline (Steinmann *et al.*, 1993). It was reported that leather dust when degraded by bacterial collagenolytic enzyme released hydroxyproline in the medium. Degradation of collagen and leather waste was reported previously by Lalitha *et al.*, 1994, Barthomeuf *et al.*, 1992 and Asdornnithe *et al.*, 1994.

Biodegradation of Leather Waste by Enzymatic Treatment using crude protease has been reported by Aftab *et al.*, 2006. But we used purified enzyme and observed that purified enzyme is more efficient in degradation of leather dust as compare to crude enzyme and purification increases the efficiency of enzyme.

Results show that the maximum collagen degradation of leather dust was observed at 84 hours of incubation. Reasonable amount of leather waste is also degraded at 72 and 96 hours of incubation. Similar effect of time period on degradation of leather dust has been reported by Lalitha *et al.*, 1994 and Aftab *et al.*, 2006. It is clear from table that maximum amount of hydroxyproline was released at pH 10 and temperature 45 °C. This may be due to the fact that our protease was most active in pH range of 7-10 and optimum temperature of our enzyme was 40-60 °C.

Figure 4.27 shows that the maximum degradation occurred when 5000 units per gram were used. Almost same degradation was observed when more than 5000 proteolytic units were used. It means that there is specific amount of degradable collagen present in leather dust, which is not effected by increasing the proteolytic units of enzyme.

In the light of present study, we can say that protease produced by *Bacillus subtilis* strain SAL1 had a collagenolytic activity and can be used to degrade leather dust which is a solid waste of leather goods manufacturing factories. Release of hydroxyproline indicates that amino acids are also produced in the degradation process, which can be purified and used in poultry feed and other applications. Also we can claim that purified enzyme is more efficient in degradation of leather waste as compare to the crude enzyme.

5.6 Processing Of Leather Waste

The objective of the present study was to reduce the solid wastes in tanning industry by treating chrome shavings with bacterial protease having collagenolytic activity to get high molecular weight gelable collagen protein and low molecular weight hydrolysate collagen protein.

The process used in this work for the treatment of chrome shaving has been studied for several years and several patents have been issued Taylor *et al.*, (1993a); Taylor *et al.*, (1996). We used the same protocol for the treatment of chrome shaving with slight modification of temperature. Our protease was stable at 60 °C for 1 hour of incubation. So temperature was maintained at 60 °C for enzyme reaction. The effect of pH on enzyme activity showed that optimum pH was 9, however, enzyme was active with in the range of pH 7-10. The stability of enzyme activity in alkaline condition is best for the extraction of small peptide hydrolysate protein (Cabeza *et al.*, 1998b).

Malathi and Chhakarborthy, 1991 reported that proteolytic enzyme played an important role in the tanning of hides and skins. Proteolytic enzymes from *Bacillus subtilis* have been used widely as bating and depilating agents. Brown *et al.*, 1996 studies showed that broad specificity of the enzyme and its stability at high temperature and in alkaline conditions helped this enzyme to use in the biotreatment of solid leather waste especially chrome shavings.

5.7 Biotreatment of chrome shaving

Analysis of chrome shaving indicates that it contains more than 80% of proteins. In this work, hydrolysis of chrome shaving was done by two ways. First process involved the use of alkaline proteolytic enzyme at moderate temperature for short period of time to isolate a hydrolysate

product. In the second set of experiment, chrome shavings were digested with alkali in the first step and with an alkaline protease in the second step, to isolate two different products: gelatin and hydrolysate.

Chemical analysis of Protein products from One-step process indicate that its TKN value is 18.83% which means that high yield of protein products is obtained. TKN content of Chrome Cake is less than the TKN value of protein products showing the effectiveness of enzyme. Ash contents of Protein products from One-step process are 7.41% where as chrome sludge from one-step process has 25.13% ash contents and 89.5% moisture. Ash contents of chrome cake are increased due to the oxidation of chromium to chromium oxide. Lower TKN content and higher ash contents of chrome cake is due to the reaction of protease with the protein in the shavings. The protein products obtained from this process are gel-like in appearance. Almost similar results were reported by Cabeza *et al.*, 1999c while using tryptec enzyme for treatment of chrome shavings. They reported that ash contents of hydrolysate protein were in the range of 9.7-18.9% whereas chrome sludge remained of one-step process had 36% ash contents and 82% moisture contents.

The second set of experiment was performed in two steps. For the two step process, in the first step, chrome shavings were treated with MgO and water to produce gelatin in the filtrate and a residue of chrome sludge.

$$MgO + H_2O \rightarrow Mg^{2+} + 2OH^- \qquad \text{Equation (3.1)}$$

The two hydroxide ions produced, react with chrome shavings to produce gelable protein, chromic hydroxide, and water.

$$2OH^- + (C_3H_5NO)_{18}\ Cr(OH).(H_2O)_4.26H_2O \rightarrow (C_3H_5NO)_{18} + Cr(OH)_3 + 30H_2O$$

Equation (3.2)

In the second step of this process, the chrome sludge was treated with the enzyme and mixed hydrolyzed protein solutions and the final chrome cake were obtained. Higher TKN contents of hydrolysate proteins and lower TKN content of chrome cake shows the effectiveness of enzyme in this process.

Ash contents of gelable proteins are 13.71% where as ash contents of hydrolysate proteins are 6.42%. Study conducted by Taylor *et al.*, 1998 show 8-20% ash contents in gelable proteins and 3-7% ash contents in hydrolysate proteins.

Similar experiment on pilot scale was performed by Taylor *et al.*, 1998. They demonstrated that the reproducibility of this process, the isolation of proteinous products from chrome shavings, in pilot plant scale is very good and better yields and product purity was obtained than the lab scale results. They concluded that due to low protein contents and the high chrome contents of chrome cake which is the final product of this process, it can be treated chemically to provide a chrome product able to be reused in tannery.

The molecular weight distribution of proteins (from one-step process) obtained from SDS-PAGE is shown in Fig.4.28&4.29. The 12% gel shows two intense bands at MWs ~ 58 and 84 kDa where as 8-25% showed a range of MWs with well-defined bands in some regions. Gradient gels are more effective in separating a mixture containing wide MW range fractions. The 8–25% gradient gel shows a wide distribution of MW with prominent bands at 97 000, 84 000, 55 000, 45 000 Da.

Similarly, the molecular weight distribution of proteins (from two-step process) obtained from SDS-PAGE is shown in Fig.12% gel shows two intense bands at MWs ~ 22 and 32 kDa (Fig. 4.30). Gradient gels are more effective in separating a mixture containing wide MW range fractions The 8–25% gradient gel shows a wide distribution of MW with prominent bands at 84 000, 66000, 55 000, 45 000, 24 000, 20 000 and 16, 000 (Fig. 4.31). These results show that two step process is more effective in hydrolysis of collagen into smaller molecular weight peptides.
Zhongkai *et al.*, 2005 have explored that collagen displays one β band (200kDa) and two α bands (100 kDa for α1 α2), for the unfolding polypeptide chains of the triple helix. It has been reported by Langmaier, 2005 that the molecular weight usually ranges within limits of 15-50 kDa for Hydrolysates, similarly molecular weight range of 50- 200 kDa for gelatin has been reported by Brown *et al.*,1994. In the light of these studies we can conclude that most proteins extracted from one step process were gelatins not hydrolysate.
In the year 1992, Taylor *et al.* reported the molecular weight of the protein fractions collected from the hydrolysis of chrome shavings. According to their investigations 8% of the fractions

were in the MW above 200 kDa, 32% in 100–200 kDa range, 28% in 50–100 kDa range and the rest (~32%) below 50 kDa.

In order to find the effect of protease on the finished leather, we applied it on the scraps of shoe upper leather. When these scraps were treated with above stated method, we did not get good results. So we treated them in a different way. First they were treated at high alkaline condition at 60 °C at pH 12, and then the pH was decreased for the enzyme reaction. This process produced hydrolysate protein and chrome sludge. It gave a high yield of protein in case of upper shoe leather. Hydrolysate protein from upper shoe leather showed intense band at MWs ~45 kDa (Fig. 4.32).where as 8–25% gradient gel shows a wide distribution of MWs (Fig. 4.33).

In the light of present work, we can claim that *Bacillus subtilis* strain SAL1 produces protease enzyme which because of its stability at high temperature and in alkaline conditions can be used for the biotreatment of solid waste released from leather industry. We can get good quality Gelable and hydrolysate protein from cheep cost processes that make leather industry as Eco friendly. These proteins have wide application in different industries. In addition, this process can control the environmental pollution as well.

- We are aware of the wide applicability and huge demand for protease in so many different types of industries. Hence, understanding the mechanism of production and different factors which affect its activity, stability can help us in improving the production and applicability of protease.
- Kinetic and stoichiometric parameters need to be studied extensively not only for the improvement of microbial growth but also for the development of enzyme.
- There is a great need for the development of immobilized thermophilic and alkalophilic proteases which can be used under typical industrial condition where reactor temperature can be as high as 70 °C.
- There is a need of thorough investigation of the protein structure and sequence analysis of genes involved in secretion of enzyme to create protease with novel properties.
- In past, health and environmental hazards have not been addressed properly in Pakistan. Hence, the application of enzyme like proteases, lipases and amylases should be promoted in the process of leather making.
- An extensive research to discover new microbial enzymes and their persistent screening can be helpful in opening new, simple routes for synthetic processes and, consequently, new way to solve environmental problems.
- Feasibility and study for the commercial production of protease and its marketing.
- Reutilization of solid waste for the production of protein and gelatin can be a good way to minimize the amount of pollutants coming out of industry. Therefore, optimization of methodologies for the reutilization of solid waste should be studied extensively.
- Molecular characterization of high grade gelatin for use in pharmaceuticals.
- Further studies on the utility of these enzymes for other commercial applications.

LITERATURE CITED

- Adinarayana, K, and P. Ellaiah. 2002. Response Surface Optimization of the Critical Medium Components for the Production of Alkaline Protease by a Newly Isolated *Bacillus* sp. J. Pharm. Pharmaceut. Sci. 5: 272 – 278.
- Adinarayana, K, and P. Ellaiah.2004.Investigation on alkaline protease production with *B. subtilis* PE-11 immobilized in calcium alginate gel beads. Process Biochem. 39: 1331-1339.
- Adinarayana, K., P.Ellaiah, and D.S.Prasad.2003. Purification and partial characterization of thermo stable serine alkaline protease from a newly isolated *Bacillus subtilis* PE-11. Pharm.Sci.Technol. 4: 1-9.
- Aftab, M. N., A. Hameed, I.U.Haq, and Sheng. C. R. 2006. Biodegradation of Leather Waste by Enzymatic Treatment. The Chinese J. of Process Engineering. 6: 462-465
- Agrawal, D., P. Patidar, T. Banerje, and S. Patil. 2004. Production of alkaline protease by *Penicillum* sp. under SSF conditions and its application to soy protein hydrolysis. Process Biochemistry. 39:977-981.
- Akimkina, T. V., E. A. Nosovskaya, and S. V. Kostrov. 1992. Cloning and Expression of the Neutral Proteinase Gene of *Bacillus cereus* in *Bacillus subtilis* Cells. J. Mol. Biol. 26: 418 – 423.
- Akira, E., S. Murakawa, and H. Shimizu. 1987. Purification and properties of collagenase from a *Streptomyces* species. J. Biochem. 102: 163-170.
- Albillosa, M., A. Mari, D. Bustob, P. M. Manuel, and O. Natividad. 2007. Analysis by capillary electrophoresis of the proteolytic activity of a Bacillus subtilis neutral protease on bovine caseins Silvia. Int. Dairy J. 17: 1195–1200.
- Alexander, K. T. W., D. R. Corning, N. J. Cory, V. J. Donohue, and R. L. Sykes. 1991. Environmental and safety issues - clean technology and environmental auditing. J. Soc. Leather Technol. Chem. 76:17-23.
- Alves, D. R. M, and V. Beleza.1991a. Utilization of leather waste: animal feed stuff from chrome shavings, part I. J. Soc. Leather Technol. Chem. 75: 15–19.
- Anwar, A, and M. Saleemuddin. 1998. Alkaline proteases, a review. J. Bioresour. Technol. 64: 175-183.

- Anwar, A, and M. Saleemuddin. 1998. Alkaline proteases: A review. Bioresour Technol. 64: 175–183.
- Asdornnithee, S., K.Akiyama, T.Sasaki, and R.Takata.1994.Isolation and characterization of a collagenolytic enzyme from Bacillus licheniformis N22.J.Ferment.Bioeng.78:283-287.
- Atalo, K., and B. A. Gashe. 1993. Protease production by a thermophilic *Bacillus* sp. (P-001A) which degrades various kinds of fibrous proteins. Biotechnology Letter. 15:1151-1156.
- Banerjee, R, and B.C.Bhattachyaryya. 1992. Optimization of extracellular protease biosynthesis by a newly isolated *Rhizopus orzae*. Bioprocess Eng.7:225-228.
- Banerjee, U. C., R. K. Sani, W. Azmi, and R. Soni. 1999. Thermostable alkaline protease from *Bacillus brevis* and its characterization as a laundry detergent additives. Process Biochem.35: 213-219.
- Baptista, A. M., P. H. Jonson, E. Hough, and S. B. Petersen. 1998. The Origin of Trypsin, Evidence for Multiple Gene Duplications in Trypsin. Springer – Verlag New York. J. Mol. Evol. 47:353 –362.
- Barthomeuf, C., H.Pourrat, and A. Pourrat.1992.Collagenolytic activity of a new semi-alkaline protease from *Aspergillus niger*.J.Ferment.Bioeng.73:233-236.
- 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.
- Berka, R. M., M. Ward, L. J. Wilson, K. J. Hayenga, K. H. Kodama, L. P. Carlomagno, and S. A. Thompson. 1990. Molecular Cloning and Deletion of the Gene Encoding Aspergillopepsin A from *Aspergillus awamori*. Gene. 86: 153 – 162.
- Berry,L, and C. A. Shuttleworth. 1988. Bacterial collagenase and collagen identification. Meat Sci. 18: 201-214.
- Beynon, R. J, and J. S. Bond. 1989. Proteolytic Enzymes,A Practical Approach. Oxford University Press. 105 – 124.

- Bhosale, S. H., M. B. Rao, V. V. Deshpande, and M. C. Srinivasan. 1995. Thermostability of High Activity Alkaline Protease from *Conidiobolus coronatus* (NCL 86.8.20). Enzyme Microb. Technol. 17: 136 – 139.
- Binnie, C., M. J. Butler, J. S. Aphale, M. A. Bourgault, M. A. Dizonno, P. Krygsman, L. Liao, E. Walczyz, and L. T. Malek. 1995. Isolation and Characterization of Two Genes Encoding Proteases associated with the Mycelium of *Streptomyces lividans 66*. J. Bacteriol. 177: 6033 – 6040.
- Bond, M.D, and H.E.Van Wart1984.Characterization of the individual collagenase from *Clostridium histolyticum*.Biochem.Wash.23:3085-3091.
- Brown, E. M., M. M. Taylor, and W. N. Marmer. 1996. Production and potential uses of co-products from solid tannery waste. J. Am. Leather Chem. Assoc.91: 270–276.
- Brown, E., C. Thompson, and M.M. Taylor.1994. Molecular size and conformation of protein recovered from chrome shavings. J.Am. Leather Chem. Assoc. 93: 215-220.
- Brown, E.M., M. M. Taylor, and W.N. Marmer.1996. Production and potential uses of co-products from solid tannery waste. J. Am. Leather Chem. *Assoc.*91: 270–276.
- Brueckner, R., O.Shoseyor, and R. H. Doi.1990. Multiple Active Forms of a Novel serine Protease from *Bacillus subtilis*. Mol.Gen. Genet. 221: 486 – 490.
- Burgess, K,and M.Shaw.1983. Dairy. In Industrial Enzymology (edited by T.Godfrey and J.Reichelt).London,Macmillan.pp 260-383.
- Cabeza, L. F., M. M. Taylor, E. M. Brown, and W. N. Marmer. 1997. Influence of pepsin and trypsin on chemical and physical properties of isolated gelatin from chrome shavings. J. Amer. Leather Chem. Assoc. 92: 200-208.
- Cabeza, L.F., A.J. Mcaloon, and W.C .Yee.1998b. Process simulation and cost estimation of treatment of chromium-containing leather waste. J. Am. Leather Chem. Assoc. 93: 2990–3135.
- Cabeza, L.F., M. M. Taylor, E. M. Brown, and W. N. Marmer. 1998a. Chemical and physical properties of protein products isolated from chromium-containing leather waste using two consecutive enzymes. J. Soc. Leather Technol. Chem. 82: 173–179.

- Cabeza, L.F., M.M. Taylor, E.M.Brown, and W.N. Marmer.1999b.Isolation of protein products from chrome-containing leather waste using two consecutive enzymes and purification of final chromium product: pilot plant studies. J. Soc. Leather Technol. Chem. 83:14–19.
- Cabeza, L.F., M.M.Taylor, E.M. Brown, and W.N.Marmer. 1999c. Use of tryptec enzyme preparations in treatment of chrome shavings. J. Am. Leather Chem. Assoc. 94: 268–287.
- Cabeza, L.F., S.M.Clauson, and M.M. Taylor.1999a. The effect of surfactant on isolation of protein products from chromium-containing leather waste. Influence on the process and on the chemical, physical and functional properties of the resultant gelatin. J. Am. Leather.94:190-198.
- Cabeza,L.F., M.M.Taylor,G.L.DiMai,E.M.Brown,W.N.Marmer, R.Carrio, P.J.Celma,and J,Cot.1998c. Processing of leather waste: pilot scale studies on chrome shavings. Part II. Purification of chrome cake and tanning trials. J. Am. Leather Chem. Assoc. 93:83–98.
- Chakraborty, R, and M. Srinivasan.1993. Production of a thermostable alkaline protease by a new *Pseudomonas* sp. by solid substrate fermentation. J. Microb. Biotechnol. 8: 7–16.
- Chaloupka, J.1985.Temperature as a factor regulating the synthesis of microbial enzymes. Microbiol. Sci. 2: 86–90.
- Chandrasekaran, S, and S.C. Dhar.1983. A low cost method for the production of extracellular alkaline proteinase using tapioca starch. J. Ferment. Technol. 61: 511–514.
- Changdao, M., W. Lin, M. Zhang, and. Zhu.2003.Towards zeros discharge of chromium-containing leather waste through improved alkali hydrolysis. Waste Management. 23:835-843.
- Chaphalkar, S,and S.Dey.1994. Some aspects of production of extracellular protease from *Streptomyces diastaticus*. J. Microb. Biotechnol. 9: 85–100.
- Chaplin M and Bucke C.1990. "The large-scale use of enzymes in solution", In *Enzyme Technology*, edited by M. Chaplin and C. Bucke, Cambridge University Press.

- Cheevadhanarak, S., D. V. Renno, G. Saunders, and G. Holt. 1991. Cloning and Selective Overexpression of an Alkaline Protease Encoding Gene from *Aspergillus oryzae*. Gene. 108: 151 – 155.
- Chen, W., P. H. Cooke, G. L. Dimiao, M. M. Taylor, and E. M. Brown. 2001. Modified collagen hydrolysate, potential for use as a filler for leather. J. Am. Leather Chem. Assoc. 96: 262-267.
- Cheng, S. W., H. M. Hu, S. W. Shen, H. Takagi, M. Asano, and Y. C. Tsai.1995. Production and characterization of keratinase of a feather-degrading *Bacillus licheniformis*. Biosci. Biotechnol. Biochem. 59:2239–2243.
- Chiang, W .D., C. H. J. Shin, and Y. H. Chu. 1999. Functional Properties of Soy Protein Hydrolysate Produced from a Continuous Membrane Reactor System. Food Chem. 65: 189 – 194.
- Christner, J.1995.Modern enzyme applications in the beam house. World Leather.10:60-64.
- Chu, I. M., C. Lee, and T. S. Li. 1992. Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATCC 14416. Enzyme Microb. Technol. 14: 755–61.
- Cot, J., A.M.Manich, C.Aramon.1991. Design of a pilot plant for complete processing of by-products of the tanning industry: preparation of a collagenic material with "zero" chrome content. J. Am. Leather Chem. Assoc. 86: 141–157.
- Cot, J., C.Aramon.1986. Waste processing in the tannery. J. Soc. Leather Technol. Chem. 70: 69–76.
- Cot, J.2004. An imaginary journey to the collagen molecule for a better understanding of leather waste treatments. J. Am. Leather Chem. Assoc. 99:322-349.
- Cowan, D., 1996. Industrial enzyme technology. Trends Biotechnol. 14:177–178.
- Daniel, C.S., L.K.Kopec, R.E.Yasbein, E.F.Young .1984.Characterization of *Bacillus subtilis*. DSM704 and its production of 1- Deoxynojiirimycin.Appl. Environ.Microbiol. 281:280-284.
- Debabov, V.G., 1982.The industrial use of *Bacilli*. In: Dubnau, D.A. (ed.), the molecular Biology of *Bacilli*, Academic press, New York, 331-370.

- Debette, J. 1991. Isolation and characterization of an extracellular proteinase produced by a soil strain of *Xanthomonas maltophila.* Curr. Microbiol. 22: 85–90.
- Devaraja, S., S. Nagaraju, Y. H. K. S. Mahadeswaraswamy, K. Girish. And Kemparaju. 2008. A low molecular weight serine protease: Purification and characterization from Hippasa agelenoides (funnel web) spider venom gland extract. Toxicon 52: 130–138.
- Dhandapani, R, and R.Vijayaragavan.1994. Production of a thermophilic extracellular alkaline protease by *Bacillus stearothermophilus* AP-4.World J Microbial Biotechnol.10: 33–35.
- Dixit, G,and S. C. Verma.1993.Production of alkaline proteases by *Penicillium griseofulvin.* Indian J. Microbiol. 33: 257–260.
- Durham, D.R.1987. Utility of subtilisins GX as a detergent additive. J. App. Bacteriolo. 63: 381-386.
- Dutta, J. R., P. K Dutta, and R.Banerjee.2004.Optimization of culture parameters for extracellular protease production from a newly isolated *Pseudomonas* sp. using response surface and artificial neural network models. Process Biochem. 39: 2193–2198.
- F.J. Berry., N. Costantini, and L.E. Smart.2002. Synthesis of chromium-containing pigments from chromium recovered from leather waste. Waste Manage.22:761–772.
- Felix,R,and J.C.Villettaz.1983.Wine. In industrial Enzymology (edited by T.Godfrey and J.Reichelt).London,MacMillan.410-421.
- Feng, Y. Y., W. B. Yang, S. L. Ong, and W.J. Ng. 2001. Fermentation of starch for enhanced alkaline protease production by constructing an alkalophilic *Bacillus pumilus* strain. Appl Microbiol Biotechnol. 57: 153–160.
- Ferrero M.A., G. R. Castro, C.M. Abate, M. D. Biagori, and F. Siňeriz.1996. Thermostable alkaline proteases of *Bacillus licheniformis* MIR 29: isolation, production and characterization. App. Microbiol. and Biotechnol. 45:327-332.
- Ferrero, M.A., G.R.Castro, C.M.Abate, M.D. Biagori, and F. Siňeriz.1996. Thermostable alkaline proteases of *Bacillus licheniformis* MIR 29: isolation, production and characterization. App. Microbiol. and Biotechnol. 45:327-332.

- Frankena, J., G. M. Koningstein, H. W. Verseveld.,and A. H. Stouthamer.1986. Effect of different limitations in chemostat cultures on growth and production of exocellular protease by *Bacillus licheniformis.* Appl Microbiol Biotechnol. 24: 106–112.
- Frankena, J., G.M. Koningstein, H. W.Verseveld, and A. H.Stouthamer. 1986. Effect of different limitations in chemostat cultures on growth and production of extracellular protease by *Bacillus licheniformis.* Appl. Microbiol. Biotechnol. 1986. 24:106–112.
- Fujiwara, N, and K. Yamamoto. 1987. Decomposition of gelatin layers on X-ray films by the alkaline protease from *Bacillus* sp. Hakkokogaku. 65:531–34.
- Fujiwara, N., K. Yamamoto, and A. Masui. 1991. Utilization of a thermostable alkaline protease from an alkalophilic thermophile for the recovery of silver from used X-ray film. J. Ferment. Bioeng. 72: 306-308.
- Gajju, H., T. C. Bhalla, and H. O. Agarwal.1996. Thermostable alkaline protease from thermophilic *Bacillus coagulans* PB-77. Indian J. Microbiol. 36:153–155..
- Galatik, A., J. Duda, and L. Minarik. 1988. Pressure hydrolysis of leather waste with sodium hydroxide. Czech. Patent CS 252,382 (CA 110:120688s).
- Genckal, H,and C.Tari .2006. Alkaline protease production from alkalophilic *acillus* sp. isolated from natural habitats. Enzyme and Microbial Technol. 39: 703 – 710.
- George, S., V. Raju, M.R.V.Krishnan, T.V.Subramanian, and K.Jayaraman. Production of protease by *Bacillus amyloliquefaciens* in solid-state fermentation and its application in the unhairing of hides and skins. Process Biochem 1995; 30:457–462.
- Gessesse, A, and A.B.Ghase. 1997. Production of alkaline protease by alkalophilic bacteria isolated from an alkaline soda lake.Biotechnol.Lett.19:479-481.
- Gey,M,and K. Unger.1995.Calculation of the molecular masses of two newly synthesized thermostable enzymes isolated from thermophilic microorganisms. J. Chromatogr.166: 188–193.
- Giesecke, U. E., G. Bierbaum, H. Rudde, U. Spohn,and C.Wandrey. 1991. Production of alkaline protease with *Bacillus licheniformis* in a controlled fed-batch process. Appl. Microbiol. Biotechnol. 35: 720–24.

- Godfrey, T, and S. West. 1996. Industrial Enzymology. 2nd Ed. MacMillan Press Ltd. London, UK.
- Guardini, G.1983. Extraction of proteins and chromium sulfate from chromium- tanned skin wastes. U.S. Patent 4, 483, 829.
- Gupta, R., and Q. K Beg. 2003. Purification and characterization of an oxidation-stable, thioldependent serine alkaline protease from *Bacillus mojavensis*. J. Enzy. Microbiol Technol. 32: 294-304.
- Gupta, R., K. Gupta, R. K. Saxena, and S. Khan. 1999. Bleach-stable, alkaline protease from *Bacillus* sp. Biotechnol. Letters, 21: 135-138.
- Gupta, R., Q. K. Beg, and P.Lorenz. 2002b. Bacterial alkaline proteases: molecular approaches and industrial applications. App. Microbiol. Biotechnol. 59:15-32.
- Gupta, R., Q. K.Beg, and B. Chauhan. 2002a.An overview on fermentation, downstream processing and properties of microbial alkaline proteases. App.Microbiol. Biotechnol. 60: 381-395.
- Gupta,A., R. Ipsita, S. K. Khare,and M. N. Gupta. 2005. Purification and characterization of a solvent stable protease from *Pseudomona aeruginosa* PseA. J of Chromatography A. 1069:155–161.
- Hafner, B., B.Rockstroh, E.Sommerfield, R.Neumann, and A. Ingeborg.1987. German Patent (East) DD 243,715.
- Haki,G. D., and S. K Rakshit. 2003. Developments in industrially important thermostable enzymes, a review. Bioresour.Technol.89:17-34.
- Hameed, A., M. A. Natt, and C. S. Evans. 1996. Production of alkaline protease by a new *Bacillus subtilis* isolate for use as a bating enzyme in leather treatment. World J. Microbiol. Biotechnol. 12: 289–291.
- Hanlon, G. W., N. A., Hodges, and A. D. Russel. 1987. The influence of glucose, ammonium and magnesium availability on the production of protease and bacitracin by *Bacillus licheniformis*. J. Gen. Microbiol. 128: 845–851.
- Headon, D.R, andG.Walsh. 1994. The industrial production of enzymes. J. Biotechnol. Adv. 12: 635-646.

- Heidemann, E.1991. Disposal and recycling of chrome-tanned materials. J. Am. Leather Chem. Assoc. 86: 331–333.
- Herbert, R. A., R. J. Sharp, andC.A. Fewson. 1992. Molecular Biology and Biotechnology of Extremophiles. Trends Biotechnol. 10: 324-330.
- Holloway, D.F.1978. Recovery and Separation of Nutritious Protein Hydrolysate and Chromium from Chrome Leather Scrap. US, Patent 4,100,154.
- Hrčková, M., M. Rusňáková, and J. Zemanovič. 2002. Enzymatic Hydrolysis of Defatted Soy Flour by Three Different Proteases and Their Effect on the Functional Properties of resulting Protein Hydrolysates. J. Food Sci. 20:7 -14.
- Hübner, U., U. Bock and K. Schügerl. 1993. Production of alkaline serine protease subtilisin Carlsberg by *Bacillus licheniformis* on complex medium in a stirred tank reactor. Appl Microbiol Biotechnol.40: 182–88.
- Ikura, Y, and K. Horikoshi. 1987. Effect of amino compounds on alkaline amylase production by alkaliphilic *Bacillus* sp. J. Ferment. Technol. 65:707–709.
- Jackson,R. J., M. L.Dao, and D.V.Lim.1995.Modified FALGPA assay for cell-associated collagenolytic activity. J. Microbiol. Methods.21: 209-215.
- Jaswal, R. K, and G. S. Kocher.2007. Partial characterization of a crude alkaline protease from Bacillus circulans and its detergent compatibility. The Internet Journal of Microbiology. 4:1-9.
- Jennifer, A., D. R. Woods, and R .L. Welton. 1972. Collagenolytic activity of aerobic halophiles from hides. J.Gen.Microbial.70: 315-319.
- Jeong,Y.H.,C.I.Wei,J.F.Preston,andM.R.Marshall. 2000. Purification and characterization of proteases from hepatopancreas of crawfish (*Procambarus clarkii*). J Food Biochem. 24: 311-332.
- Johnevelsy, B., and G. Naik.2001. Studies on production of thermostable alkaline protease from thermophilic and alkaliphilic Bacillus sp. JB-99 in a chemically defined medium. Proc. Biochem. 37: 139–144.

- Joo, H. S, and C. S Chang. 2005. Production of protease from a new alkalophilic *Bacillus* sp, I-312 grown on soybean meal: optimization and some properties. Process Biochem. 40: 1263–1270.
- Kamande, G.M., J. Baah, K. J. Cheng, T. A. McAllister, and J. A. Shelford.2000. Effects of Tween 60 and Tween 80 on Protease Activity, Thiol Group Reactivity, Protein Adsorption, and Cellulose Degradation by Rumen Microbial Enzymes .J. Dairy Sci. 83: 536–542.
- Kaminishi, H., Y. Hagihara, M. Tanaka, and T. Cho .1988 .Degradation of bovine achilles tendon collagen by *Candida albicans* proteinase.J .Med. Vet. Mycol. 26:315-318.
- Kaur,M., S. Dhillon, K. Chaudhary, and R. Singh. 1998. Production, purification and characterization of thermostable alkaline protease from *Bacillus polymyxa*. Indian J Microbiol, 38: 63-67.
- Kenny, A. J. 1999. "Nomenclature and Classes of Peptidases", In *Proteolytic Enzymes Tools and Targets*, edited by E.E. Sterchi and W. Stöcker, (Springer-Verlag, Germany), pp. 1-8.
- Kim, S. S., Y. J. Kim, and I. Rhee. 2001. Purification and characterization of a novel extracellular protease from *Bacillus cereus* KCTC 3674. J. *Archieves in Microbiology*, 175: 458-467.
- Kirk, O., T. V. Borchert, and C. C. Fuglsang. 2002. Industrial Enzyme Applications. J. Current Opinion Biotechnol. 13: 345 – 351.
- Kohlmann, K.L., S. S. Nielson, and M.R. Ladisch. 1991. Purification and characterization of an extracellular protease produced by *Pseudomonas florescens* M3/6. J. Dairy Sci. 74: 4125-4136.
- Kole, M.M., I. Draper, D. F. Gerson.1988. Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. J. Chem. Technol. Biotechnol. 41:197–206.
- Kolomaznik, K., M. Mladek, F. Langmaier, D. Janacova, and M. M. Taylor. 2000. Experience in industrial practice of enzymatic dechromation of chrome shavings. J. Am.Leather Chem. Assoc. 95: 55-63.

- Kresalkova, M., L. Hnanickova, J. Kupec, K. Kolomaznik, and P. Alexy. 2000. Application of protein hydrolysate from chrome shavings for polyvinyl alcohol-based biodegradable material. J. Am. Leather Chem. Assoc. 97: 143-149.
- Kudrya, V .A, and I. A. Simonenko. 1994. Alkaline serine proteinase and lectin isolation from the culture fluid of *Bacillus subtilis*. J. App. Microbiol. and Biotechnol. 41: 505-509.
- Kumar, C .G, and H. Takagi. 1999. Microbial alkaline proteases from a bioindustrial viewpoint J.Biotechnol. *Ad.* 17: 561-594.
- Kumar, C. G., M. P. Tiwari, and K. D. Jany. 1999. Novel alkaline serine proteases from alkaliphilic *Bacillus* sp. purification and some properties. Process Biochem. 34: 441-449.
- Kuo, L. C, and J. A. Shafer. 1994. Retroviral Proteases. Methods Enzymol. 241: 3 – 178.
- Kwon, Y. T., J. O. Kim, S. Y. Moon, H. H. Lee, and H. M. Rho.1994. Extracellular alkaline protease from alkalophilic *Vibrio metschnikovii* strain RH530. J. Biotechnol. Lett. 16: 413–18.
- Laemmli, U.K. 1970.Cleavage associated with the maturation of the head of bacteriophage T4. *Nature* 227: 680-685.
- Lalitha, K., K. R. Swaminathan, and R. P. Bai.1994. Kinetics of biomethanation of solid tannery waste and the concept of interactive metabolic control. J. Appl. Biochem. Biotechnol. 47: 73-78.
- Lane,D.j.1991.16/23S sequencing.In E. Stackebrandt and M.Goodfellow (ed.): Nucleic acids techniques in bacterial systematic.John Wiley and sons,New York. NY.115-175.
- Langmaier, F.2005. Modification of chrome-tanned leather waste hydrolysate with epichlorhydrin.J.Soc.Leather Tech. &Chem.90: 29-34.
- Law, B. A, and A. S. Wigmore.1982. Microbial proteinases as agents for accelerated cheese ripening. J. Soc.Dairy Technol. 35: 75-76.
- Laxman, R.S., A.P. Sonawane, S.V. More, B.S. Rao, M.V. Rele, V.V. Jogdand, V.V. Deshpande, and M.B. Rao. 2005. Optimization and scale up of production of alkaline protease from *Conidiobolus coronatus* . Process Biochem. 40: 3152 – 3158.

- Lee, P.G., C.N.A. Razak, R. Rahman, M.Basri, and A.B. Salleh.2002. Isolation and screening of an extracellular organic solvent-tolerant protease producer. Biochem. Eng. J. 13:73-77.
- Leisola, M., J. Jokela, O. Pastineu, O. Turunen, and H. Schoemaker. 2001. Industrial Use of Enzymes.www.hut.fi/Units/Biotechnology/Kem-70.415
- Lesney, M. 2003. For more and more Industrial Applications, Enzymes, Natural and Engineered, are Replacing Traditional Chemistry. Today's Chemist at Work. www.tcawonline.org
- Liang, J. H. 1999. Fluorescence due to Interactions of Oxidizing Soybean oil and Soy Proteins. J. Food Chem. 66: 103 – 106.
- Liao, C. H, and D. E. McCallus. 1998. Biochemical and genetic characterization of an extracellular protease from *Pseudomonas fluorescens* CY091. Appl Environ Microbiol. 64: 914–21.
- Long, S., M. A. Mothibeli, F. T. Robb, and D. R. Wood.1981. Regulation of extracellular protease activity by histidine in a collagenolytic *Vibrio alaginolyticus* strain.J.Gen. Microbiol.127: 193-199.
- Madsen, J. S., T. O. Ahmt, J. Otte, T. Halkier, and K. B. Qvist. 1997. Hydrolysis of β–lactoglobulin by Four Different Proteinases Monitored by Capillary Electrophoresis and High Performance Liquid Chromatography. Int. Dairy J. 7: 399 – 409.
- Mahnoud,M. I. 1994. Physicochemical and Functional Properties of Protein Hydrolysates in Nutritional Products. J. Food Technol.10: 89 – 94.
- Maire, M. S, and V.A. Lipsett. 1980. Offal enhancement. J. Am. Leather Chem. Assoc. 75: 16–33.
- Malathi, S, and R. Chakraborty.1991.Production of alkaline protease by a new *Aspergillus flavus* isolate under solid substrate fermentation conditions for use as a depilation agent. J. Appl. Environ. Microbiol. 57:712–716.
- Manachini, P. L., M. G. Fortina,and C. Parini .1988. Thermostable alkaline protease produced by *Bacillus thermoruber*. A new species of *Bacillus*. Appl Microbiol Biotechnol. 28: 409–413.

- Manchini, P., and M. Foretina. 1998. Production in sea–water of thermostable alkaline protease by a halotolerant strain of Bacillus licheniformis. J. Biotechnol. Lett. 20: 565–568.
- Mao, W., R. Pan and D. Freedman. 1992. High production of alkaline protease by *Bacillus licheniformis* in a fed-batch fermentation using a synthetic medium. J. Ind. Microbiol. 11:1–6.
- Margesin, R., N, Palma, F. Knauseder, and F. Schinner. 1992. Purification and characterization of an alkaline serine protease produced by a psychrotrophic *Bacillus* sp. J. Biotechnol.24: 203-206.
- Maschmann, E. 1937. Collagenase isolated from *Clostridium welchii* J.Biochem.Z. 295: 1-4.
- Matsubara, H, and J. Feder.1971. Other bacterial, mould and yeast proteases. In the Enzymes, ed.Boyer.Academic, New York.pp 721-795.
- Mckevitt,A.I.,S.Bajaksouzian,J.D.Klinger,andD.E.Woods.1989.Purificationand characterization of an extracellular protease from *Pseudomonas cepacia*. Infection and Immunity. 57: 771-778.
- Ming, C., L.Champion, and L.Tsu-Shun.1992. Production and degradation of alkaline protease in batch cultures of *Bacillus subtilis* ATTC 14416.Enzyme Microb.Technol.14:755-761.
- Moon, S. H, and S. J. Parulekar. 1991. A parametric study of protease production in batch and fed-batch cultures of *Bacillus firmus*. J. Biotechnol. Bioeng. 37: 467–483.
- Moon, S. Y., T. K. Oh, and H. M. Rho.1994. Purification and characterization of extracellular protease from Bacillus subtilis RM 615.Korean Biochem.J. 27: 323-329.
- Moon, S.H.,and S.J. Parulekar.1991. A parametric study of protease production in batch and fed-batch cultures of *Bacillus firmus*. Biotechnol. Bioeng. 37:467–483.
- Mu, C., W. Lin, M. Zhang, and Q .Zhu. (2003). Towards zero discharge of chromium-containing leather waste through improved alkali hydrolysis. Waste Management 23:835–843.

- Murphy, A, and O. C. Fagain. 1996. Stability Characteristics of Chemically Modified Soluble Trypsin. J. biotechnol. 49: 163 –171.
- Nadeem, M., I. Q. Javed, B. Shahjahan,and S. Qurat-ul-Ain. 2008. Effect of Medium Composition on Commercially Important Alkaline Protease Production by *Bacillus licheniformis* N-2. Food Technol. Biotechnol. 46: 388–394.
- Nasuno, S, andT.Ohara.1971. Hyper production of proteinase and some hydrolytic enzymes by mutants of *Aspergillus sojae*.J. Agric. Biol. Chem. 35: 829–835.
- Neurath, H. 1999. Proteolytic Enzymes, Past and Future. J. Proc. Natl. Acad. Sci. 96: 10962 – 10963.
- Noreau, J, and G. R. Drapeau. 1979 .Isolation and properties from the wild type and mutant strains of *Pseudomonas fragi*. J. Bacteriol.140: 911-916.
- Ohtsyka, K.1973. Amino acid seasoning produced from scraps obtained from chrome tanned leather. Japan Patent 7,329,145.
- Okamura, H,and k.Shirai.1976. Chrome shavings and its products. J. Am. Leather Chem. Assoc. 7: 173-179.
- Olson, J.C,and D.E. Ohman. 1992. Efficient production and processing of elastase and LasA by *Pseudomonas aeruginosa* require zinc and calcium ions. J. Bacteriol. 174: 4140-4147.
- Ong,P.S.,M.Gaucher.1976.Production,purification and characterization of thermomycolase, the extracellular serine protease of the thermophilic fungus *Malbranchea pulchella* var. *sulfurea*. Can. J. Microbiol. 22: 165–176.
- Palmieri, G., C. Bianco, G. Cennamo, P. Giardina, G. Marino, M. Monti, and G. Sannia. 2001. Purification,characterization, and functionalr role of a novel extracellular protease from *Pleurotus ostreatus*. Appl. Environ. Microbiol. 67: 2754-2759.
- Pastor, M.D., G.S. Lorda.,and A. Balatti. 2001. Protease obtention using *Bacillussubtilis* 3411 and amaranth seed meal medium at different aeration rates. Braz. J. Microbiol. 32: 1-8.
- Periago, M. J., M. L. Vidal, and G. Ros. 1998. Influence of Enzymatic Treatment on the Nutritional and Functional Properties of Pea Flour. J. Food. Chem. 63: 71 – 78.

- Phadatare, S. U., V. V. Deshpande, and M. C. Srinivasan. 1993. High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): Enzyme production and compatibility with commercial detergents. J. Enzyme Microb. Technol. 15: 72–76.
- Prakash,M., M.R.Banik, and C.B.koch.2005.Purification and Characterization of Bacillus cereus Protease Suitable for Detergent Industry.J.Applied Biochem.and Biotechnol.127:143-156.
- Prakasham, R.S., C. S. Rao, P.N. Sarma, and G. G. 2006. Husk. An inexpensive substrate for alkaline protease production by *Bacillus* sp. in solid-state fermentation, *Bioresour. Technol.* 97: 1449–1454.
- Priest, F.G.1988.Extracellular enzymes.In:cole J A,Knowles C J.Schlessinger D(eds).Aspects ofMicrobiology,9.Van Nostrand Reinhold,Workingham(UK).245-253.
- Purva,S.K.,L.K.Gupta,andJ.K.Gupta.1998.Thermostable alkaline protease from alkalophilic *Bacillus* sp.IS-3.Ind.J.Microbiol.38:149-152.
- Puvanakrishnan, R, and S. C. Dhar. 1985. J. Ind.S Leather Technol. Assoc. 33: 19 – 22.
- Qadeer, M.A., M. Rahman, J. Iqbal,and A. Ahmad. 1990. Studies on the production of extracellular proteolytic enzymes by *Bacillus subtilis*. J. Pure Appl. Sci. 1: 11-17.
- Rahman, R., L. P. Geok, M. Basri. and A. B. Salleh. 2005. An organic solvent-tolerant protease from *Pseudomonas aeruginosa* strain K. Nutritional factors affecting protease production. Enzyme Microb Technol. 36: 749–57..
- Rahman, R.N.Z.A., C. N. Razak, K. Ampon, M. Basri, W. M. Z. W., Yunus, and A. B. Salleh. 1994. Purification and characterization of a heat-stable alkaline protease from *Bacillus stearothermophilus* F1.J. App. Microbiol. Biotechnol. 40: 822-826.
- Rao, M. B., A. M. Tanksale, M. S. Ghatge, and V. V. Deshpande. 1998. Molecular and Biotechnological Aspects of Microbial Proteases. J. Microb. Molec. Biol. Rev. 62: 594 – 635.
- Rao,J.R., P. Thanikaivelan, K.J. Sreeram, and B.U. Nair.2002.Green route for the utilization of chrome shavings (chromium-containing solid waste) in tanning industry, Environ. Sci. Technol. 36: 1372–1376.

- Rawlings, N. D, and A. J. Barrett. 1993. Evolutionary Families of Peptidases. Biochem. J. 290: 205 – 218.
- Ray, M. K., K. U. Devi, G. S. Kumar,and S. Shivaji. 1992. Extracellular protease from the antarctic yeast *Candida humicola*. Appl Environ Microbiol. 58:1918–19123.
- Rice, A.M,and A.C. Sartorelli. 2001. Inhibition of 20 S and 26 S proteasome activities by lithium chloride. Impact on the differentiation of leukemia cells by alltrans- retinoic acid. J. Biol. Chem. 276: 42722-42727.
- Rittman, B. E, and P. L. McCarty. 2001. Environmental Biotechnology: Principles and Applications." 1st" edition.
- Rose, P.I. 1992."Inedible gelatin and glue"In Inedible Meat By-products, Advances in Meat Research, **8**, Pearson, A.M. and Dutson, T.R. Eds.; Elsevier Applied Science, London. 217-263.
- Salleh, A.B., M. Basri, and C. Razak. 1977. The effect of temperature on the protease from Bacillus stearothermophilus strain F1. Mal. J. Biochem. Mol. Biol. 2: 37–41.
- Sarath, G., R.S.D.L. Motte, and F.W. Wagner. 1989. Protease assay methods.*In* R.J. Beynon and J.S. Bond (eds.), Proteolytic enzymes: A practical approach. IRL Press, Oxford,New York, Tokyo.25-55.
- Sato, F, and H.omura.1989 .Soil enzymes activities in andosal paddy fields. Relationship between soil enzymes (acetylglucosaminidase, protease and adenosine deaminase) activity and microbial counts. Japan. J. Soil Sci. Plant Nutr.60: 34-40.
- Sen,S,andT.Satyanarayana.1993.Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. Ind. J. Microbiol. 33: 43–47.
- Sexton, M.M., A. L. Jones, W. Chaowagul, and D.E. Woods. 1994. Purification and characterization of a protease from *Pseudomonas pseudomallei*. Can. J. Microbiol. 40: 903-910.
- Shafee,N.,S.N.Aris,R.Rahman,M.Basri,andA.B.Salleh.2005.Optimization of environmental and nutritional conditions for the production of alkaline protease by newly isolated bacterium *Bacillus cereus* strain. J. Appl. Sci. Res. 146: 1–8.

- Shelly, D.C., L.F.Cabeza, and S. A. Ortkiese. 1999. Operational, Regulatory and Economic Considerations for Recovering a U.S.P.-Grade Gelatin from Wet-Blue Wastes. J. Am. Leather Chem. Assoc. 94: 48-54.
- Shimogaki, H., K. Takeuchi, T. Nishino, M .Ohdera, T .Kudo, K .Ohba, M. Iwama, and M. Irie.1991. Purification and properties of a novel surface-active agent and alkaline-resistant protease from *Bacillus* sp. Y. Agric. Biol.Chem .55:2251–58.
- Singh, J., N. Batra, and R.C. Sobti. 2001. Serine alkaline protease from a newly isolated *Bacillus* sp. SSR1. J. Proc. Biochem. 36: 781-785.
- Singh, J., R. M. Voohra, and D. K. Sahoo.1999. Alkaline protease from a new obligate alkaliphilic isolate of *Bacillus sphaericus*. J. Biotechnol. Lett. 21: 921-924.
- Sinha, N, and T. Satyanarayana. 1991. Alkaline protease production by thermophilic *Bacillus licheniformis*. Ind. J Microbial. 31:425–30.
- Sivaparvathi, M., K .Suseela, S.C. Nanda.1986b. Purification and properties of *Pseudomonas Aeruginosa* protease causing hydrolysis of chrome shavings. Leather Sci. 33: 303–307.
- Sivaparvathi, M., Suseela, K., Nanda, S.C., 1986a. Hydrolytic action of *Pseudomonas Aeruginosa* on chrome shavings. Leather Sci. 33: 8–11.
- Soderling, E, and K.U. Paunio.1981.Conditions of production and properties of the collagenolytic enzymes by two *Bacillus* strains from dental plaque.J.Peridont.Res.16:513-523.
- Sookkheo, B.,S. Sinchaikul, S. Phutrakul, andS.Chen.2000.Purification and characterization of the highly thermostable proteases from *Bacillus stearothermophilus* TLS33. J. Prot. Exp. Pur. 20:142-151.
- Spinosaa, M. R., T. Braccinia, E. Ricca, M. De Felice, L. Morellic, and G, Pozzia. 2000. On the fate of ingested *Bacillus* spores. J. Res. Microbiol. 151:361–368.
- Steinmann, B., P.M.Royce, and A.S. Furga.1993.The Ehlers Danlos syndrom,In: Connective Tissues and its heritable disorders:Molecular,Genetics and Medical aspects.Wiley-Liss Inc.351-407.
- Takagi, H.1993.Protein engineering on subtilisins. Int. J. Biochem. 25:307–312.

- Takami, H., T.Akiba, and K. Horikoshi.1989.Production of extremely thermostable alkaline protease from *Bacillus sp.* J. Appl. Microbiol. Biotechnol. 30:120–124.
- Takii Y., N .Kuriyama and Y.Suzuki.1990. Alkaline serine protease produced from citric acid by *Bacillus alcalophilus* subsp. *halodurans* KP1239.Appl.Microbiol Biotechnol.34:57–62.
- Tancous, J.J., R. Bellingham, W. Kllenberger, and A. McDonnel.1981. Conservation of chromium in the tanning industry. J. Am. Leather Chem. Assoc. 76:164-175.
- Tatsumi H., S. Murakami, Y. Ogawa, A. Masaki, Y. Ishida, K. Murakami, H. Kuwabe, H. Arimura. E. Nakamo, and H. Motai. 1991. Autoproteolytic Processing and Processing Dependent Secretion of *Aspergillus oryzae* protease in yeast. J. Agric. Biol.Chem. 55: 3099 – 3101.
- Taylor, M. M., E. J. Diefendorf, and G. C. Na. 1989. Enzymatic treatment of solid waste generated in the tanning industry. Proceedings of the 20th Congress of the International Union of Leather Technologists and Chemists Societies. Philadelphia, Pennsylvania.
- Taylor, M. M., E. J. Diefendorf, C. J. Thompson, E.M. Brown, W. N. Marmer, and L.F. Cabeza.1996. Extraction of value added byproducts from the treatment of chromium containing collagenous leather industry waste. J. Soc. Leather Technol. Chem. 81: 5-13.
- Taylor, M.M., Cabeza, C.F., DiMaio, G.L., Brown, E.M., Marmer, W.N., Carrio, R.,Celma, P.J. and Cot, J. 1998(b). Processing of leather waste: pilot scale studies on chrome shavings. Part I. Isolation and characterization of protein products and separation of chrome cake. J.Am. Leather Chem. Assoc. **93**, 61.
- Taylor, M.M., Diefendorf, E.J., Brown, E.M., Marmer, W.N. 1993a.Enzymatic processing of materials containing chromium and protein. US Patent 5,271,912.
- Taylor, M.M., Diefendorf, E.J., Na, G.C., Marmer, W.N.1992a. Enzymatic processing of materials containing chromium and protein. US Patent 5,094,946.
- Taylor, M.M., E.J. Diefendorf, and W.N. Marmen.1991. Efficiency of enzyme solubility of chrome shaving as influenced by choice of alkalinity-inducing agent. J. Am. Leather Chem. Assoc 86: 199–208.
- Taylor, M.M., E.J. Diefendorf, C.J .Thompson, E.M.Brown, W.N., Marmer, and L.F.

Cabeza.1997.Extraction of value added byproducts from the treatment of chromium containing collagenous leather industry waste. J. Soc. Leather Technol. Chem. 81: 5–13.

- Taylor, M.M., E.J. Diefendorf,G.C. Na,and W.N.Marmer.1992b. Enzymatic processing of materials containing chromium and protein. US Patent 5,094,946.
- Taylor, M.M., E.J.Diefendorf, C.J. Thompson, E.M.Brown, and W.N.Marmer.1993b. Effect of processing variables on ash content of gelable and hydrolyzed protein products isolated from treatment of chromium leather waste. J. Am. Leather Chem. Assoc. 88:358–367.
- Taylor, M.M., E.J.Diefendorf, W.N.Marmen, and E.M.Brown.1994. Effect of various alkalinity-inducing agents on chemical and physical properties of protein isolated from chromium-containing leather waste. J. Am. Leather Chem. Assoc. 89: 221–228.
- Taylor, M.M.,E.J. Diefendorf, and G.C. Na.1990. Enzymic treatment of chrome shavings. J. Am. Leather Chem. Assoc. 85: 261–282.
- Taylor,M.M.,E.J.Diefendorf,C.J.Thompson,E.M.Brown,W.N.Marmer,andL.F.Cabeza. 1997. Extraction of value added byproducts from the treatment of chromium containing collagenous leather industry waste. J. Soc. Leather Technol. Chem. 81: 5–13.
- Taylor,M.M.,L.F.Cabeza,W.N.Marmer,E.M.Brown,and.Kolomaznik.1998a. Functional properties of hydrolysis products from collagen. J. Am. Leather Chem. Assoc. 93: 40–50.
- Thomas, A., and M.A.Winkler.1977.Foam separation of biological materials. In: Wise A. and M.I.Biol. (ed.).Topics in enzyme and fermentation biotechnology.Inc.New York.
- Thorstensen, T.C.1993. In Practical Leather Technology. 4th Ed. Krieger Publishing, Malabar, FL, pp 64-126.
- Thorstensen, T.C.1993. In *Practical Leather Technology*; 4th edition, Krieger Publishing, Malabar, FL. pp 64,126.
- Toshevc,K.,M.Pesheva,and K.Milyanova.1980.Methods for the production of protein hydrolysate from chrome leather waste.Kosh.Obuvna Prom-dt.21:8-10.
- Tsai, Y. C., R. Y. Juang, S. F. Lin, S. W. Chen, M. Yamasaki, and G. Tamura. 1988. Production and characterization of an alkaline elastase produced by alkalophilic *Bacillus*

strain Ya-B. Appl Environ Microbiol. 54: 3156–61.

- Tsuchiya, K., H. Sakashita, Y. Nakamura, and T. Kimura.1991. Production of thermostable alkaline protease by alkaliphilic *Thermoactinomyces* sp. HS682. J. Agric. Bio. Chem. 55:3125–3127.
- Turgeon, S. L., S. F. Gauthier, and P. Paquin. 1992. Emulsifying Properties of Whey Peptide Fractions as a Function of pH and Ionic Strength. J. Food Sci. 57: 601 – 604.
- Uhlig, H.1998. Industrial Enzymes and their Applications. Trans. Elfriede M. Linsmaier-Bednar. New York: John Wiley and Sons.
- Ulrich, R.L, and T.A.Hughes.2001.A rapid procedure for isolating chromosomal DNA from Lactobacillus species and other Gram-positive bacteria. Letters in Appl.aMicro.32:52-56.
- Veeger, L.1994.Chrome recovery from incinerated tannery waste, World Leather. 7: 82-88.
- Wandersman, C.1989.Secretion, processing and activation of bacterial extracellular proteases. Mol. Microbiol. 3: 1825-1831.
- Ward, O.P. 1991. "Enzyme Bioprocessing Applications" In *Bioprocessing*, edited by O.P Ward, (Open University Press, Buckingham).151-170.
- Ward, O.P.1985.Proteolytic Enzymes" In Comprehensive Biotechnology, edited by M. Moo-Young, (Pergamon Press, Great Britain).789-818.
- Wilford, A. 1999. Waste in the Shoe Industry-Consequences for the Leather Industry. J. of the Society of Leather Technol. and Chem.83:84-86
- Wojciech, L., G, Mieczyslzw, M. Urszula .1998. Leather treatment to remove chromium. PCT Int. Appl. WO. 9803685.
- Yamada, F., N. Takahaski, and T. Murachi. 1976. Purification and Characterization of a Proteinase from Pineapple Fruit, Fruit Bromelain FA2. J. Biochem. 79: 1223 – 1234.
- Yaoyu, F., Y.Wenbo, and H.Zhimin.1997.Fermentation conditions of engineering strain utilizing starch for production of alkaline protease.Weishengwu Xuebao.37:473-476.
- Zamost, B. L., Q. I. Brantley, D. D. Elm, and C. M. Beck. 1990. Production and characterization of a thermostable protease produced by an asporogenous mutant of

Bacillus stearothermophilus. J. Ind. Microbiol. 5: 303–512.

- Zhongkai, A., L. Guoying, and S. Bi.2005.Physicochemical properties of collagen, gelatin and collagen hydrolysate derived from bovine limed split wastes.J.Soc.Leather Tech.&Chem. 90: 23-28.

Chromosomal DNA Isolation:

An overnight culture (1.5ml) was pelleted at 14000 rev/min at 4° C for 2 minutes and resuspended in 500µl of 5 mmol EDTA, pH8.0.A volume (100 µl) of a 30mg/ml of lysozyme was added to the cell suspension and incubated for 30-60min at 37° C. The suspension was centrifuged at 14000 rev/min at 4° C for 1 minute and pellet was gently resuspended in 850 µl of nuclei lysis solution. After through suspension, three freeze-thaw steps were performed using -60° C freezer and an 80° C water bath(4-6minutes in freezer followed by 1-2 minutes in water bath).Once the lysate cleared,4 µl(4mg/ml)RNase (promega) was added to the mixture and incubated for 30 minutes at 37° C.200-300 µl protein precipitation solution (promega) was added to the mixture and vortexed at medium speed for 10s.The lysate was centrifuged at 14000 rev/min at 4° C for 10 minutes and the supernatant was transferred to a clean 1.5ml Eppendorf tube. One additional centrifuge (14000 rev/min at 25° C for 5 minutes) was performed to remove any residual protein from the extract.DNA was precipitated by the addition of 600 µl isopropanol at room temperature.DNA was pelleted at 14000 rev/min at 4° C for 8-10 minutes and washed twice with 70%ethanol.DNA was resuspended in 50-100 µl of 10 mmol Tris HCl.DNA was analyzed on a 1% agarose gel (80v for 40 min) containing 0.5µgml^{-1} ethidium bromide. Concentration of DNA was measured by nano drop method.

Buffers/Reagents for SDS-PAGE

4X Resolving gel buffer

1.5M Tris (pH 8.8)

0.8%SDS

(Adjust pH with conc.HCl)

4X stacking buffer

0.5M Tris (pH 6.8)

0.8%SDS

(Adjust pH with conc.HCl)

Acrylamide/Bis-acrylamide solution

29 % Acrylamide

1% Bis-acrylamide

SDS (Sodium dodecylsulfate) solution

10% SDS

Ammonium per sulfate (APS) solution

10%APS

Can be stored 1 week at 4 °C

TEMED

Available already prepared

Fixative solution

Methanol	400ml
Glacial acetic acid	100ml
Distilled H_2O	500ml

Staining reagent

Coomassie Brilliant Blue	2.5g
Fixative solution	1000ml

De -staining reagent

Methanol	400ml
Glacial acetic acid	100ml
Distilled H_2O	500ml

4X Sample buffer

0.250 M Tris (pH 6.8)

8 %SDS

40 % glycerol

0.05%bromophenol blue

20% 2-mercaptoethanol

Reservoir/Running, buffer (10X)

0.025M Tris (pH 8.3)

0.192M glycine

0.1%SDS

Table: 6.1 Effect of different media on the production of protease at temperature 37°C, agitation speed 150rpm, pH 8.0 and 48 hours of incubation in shake flask.

Medium	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
M1	56	0.55	101
M2	98	0.67	146
M3	176	0.82	215
M4	141	0.84	167

Table: 6.2 Effect of incubation period on the production of protease from *Bacillus* sp. at 37 °C, pH 8.0 and 150rpm.

Time (hours)	Final pH	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
12	7.9	37	0.67	55
24	8	98	0.73	134
36	8.1	141	0.79	178
48	8.8	173	0.81	213
60	8.9	191	0.83	230
72	9.1	221	0.84	263
84	9	198	0.8	248
96	9	158	0.78	202

Table: 6.3 Effect of pH on the production of protease after 72 hours of incubation, at 37°C, and 150rpm.

pH Values	Final pH	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
5	5.2	41	0.31	132
5.5	5.7	53	0.38	139
6	6.5	79	0.55	143
6.5	6.8	102	0.7	145
7	8.2	172	0.72	238
7.5	8.5	198	0.77	257
8	8.8	223	0.84	265
8.5	9.3	235	0.85	276
9	9.2	218	0.81	269
9.5	9.7	178	0.77	231
10	10.1	88	0.47	187

Table: 6.4 Effect of temperature on the production of protease at agitation speed 150rpm, pH 8.5 and 72 hours of incubation in shake flask culture.

Temperature (°C)	Final pH	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
25	8.6	68	0.53	128
30	8.8	183	0.75	244
35	9.1	224	0.83	269
37	9.3	236	0.85	278
40	8.7	174	0.73	238
45	8.7	98	0.48	204
50	8.6	40	0.33	121

Table: 6.5 Effect of age of inoculum on the production of protease at temperature 37 °C, agitation speed 150rpm, pH 8.5 and 72 hours of incubation in shake flask culture.

Age of inoculum (hours)	Final pH	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
12	8.6	142	0.8	177
18	8.8	199	0.81	246
24	9.2	235	0.85	276
30	9.1	226	0.83	272
36	8.9	183	0.76	241
48	8.7	116	0.71	163
60	8.5	67	0.69	97
72	8.5	52	0.65	80

Table: 6.6 Effect of size of inoculum on the production of protease at temperature 37 °C, agitation speed 150rpm, pH 8.5 and 72 hours of incubation in shake flask culture.

Size of inoculum (%)	Final pH	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
1	8.6	117	0.72	162
2.5	8.8	191	0.77	248
5	9.2	233	0.84	277
7.5	9.4	241	0.83	290
10	9.5	247	0.84	294
12.5	9.2	221	0.79	279
15	8.8	191	0.77	248
17.5	8.7	174	0.74	235
20	8.7	161	0.72	224

Table: 6.7 Effect of speed on the production of protease from *Bacillus Subtilis* at temperature 37 °C, agitation speed 150rpm, pH 8.0 and 72 hours of incubation in shake flask culture.

Speed	Final pH	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
100	9.1	164	0.68	241
150	9.1	247	0.83	297
200	8.7	211	0.82	257
250	8.6	152	0.77	198
300	8.6	112	0.63	177
350	8.3	83	0.58	143

Table: 6.8 Effect of various carbon sources (1%w/v) on protease production from *Bacillus subtilis* strain SAL1 after 72hrs, at 37°C 150rpm and pH 8.5.

Carbon Source	Final pH	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
Sucrose	8.6	150	0.97	154
Fructose	9.2	197	0.8	246
Mannitol	9.3	247	0.92	268
Dextrose	8.7	171	0.65	263
Sorbitol	8.7	136	0.66	206
Glycerol	9.4	246	0.83	296
Glucose	8.5	140	1.05	133
Glactose	8.6	192	0.84	228
Maltose	8.5	204	1.12	182
Lactose	8.8	162	0.63	257

Table: 6.9 Effect of different nitrogen sources (1%w/v) on protease production from *Bacillus subtilis* strain SAL1 after 72hrs, at 37°C 150rpm and pH 8.5.

Nitrogen Sources	Final pH	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
Tryptone	8.6	100	0.67	149
Peptone	8.9	198	0.72	275
Yeast Extract	9.5	252	0.71	355
Beef Extract	8.8	232	0.93	249
Gelatin	9.4	245	0.82	298

Table: 6.10 Effect of different metal ions on protease production from *Bacillus subtilis* strain SAL1 after 72hrs, at 37°C 150rpm and pH 8.5.

Divalent Cations	Final pH	Proteolytic Activity (PU/ml)	Total Protein (mg/ml)	Specific Activity (PU/mg)
CaCl2	9.4	244	0.65	375
MgCl2	9.3	239	0.67	356
FeCl2	8.8	210	0.8	262
ZnCl2	8.5	148	0.84	176
CuCl2	8.5	118	0.69	171
MnCl2	9.4	256	0.66	387

Table: 6.11 Activities of Precipitates from Ammonium Sulfate

%age saturation	Pallet activity(PU/ml)	Supernatant activity(PU/ml)
10	109	160
20	128	147
30	143	136
40	176	104
50	210	68
60	248	37
70	283	29
80	211	23
90	193	19
100	109	8

Table: 6.12 pH optimum of purified protease.

pH	Proteolytic activity(PU/mg	Relative activity (%)
5	38	10
5.5	57	15
6	76	20
6.5	163	43
7	273	72
7.5	319	84
8	342	90
8.5	357	94
9	380	100
9.5	342	90
10	266	70
10.5	228	60
11	76	20
11.5	19	5
12	0	0

Table: 6.13 Effect of temperature on caseinolysis by purified protease

Temperature (^{0}C)	Proteolytic activity(PU/mg)	Relative activity (%)
30	57	15
35	102	27
40	190	50
45	247	65
50	281	74
55	349	91
60	380	100
65	361	95
70	304	80
75	171	45
80	76	20

Table: 6.14 Effect of temperature on the activity of purified protease

Temperature (^{0}C)	Proteolytic activity(PU/mg)	% Stability
25	380	100
30	378	99.5
35	375	98.6
40	368	96.8
45	366	96.3
50	364	95.7
55	353	92.8
60	334	87.8
65	281	74
70	152	40
75	44	11.5

Table: 6.15 Effect of pH on the activity of purified protease

pH Value	% Stability	Proteolytic activity (PU/ml)
4	40	152
5	70	266
6	80	304
7	96	365
8	98	372
9	100	380
10	90	342
11	58	220
12	10	38

Table: 6.16 Effect of inhibitors on activity of purified Protease

Inhibitors used	Specificity	Concentration (mM)	Residual activity (%)
None	-	-	100
PMSF	Serine protease inhibitor	1 5	23.75 5.09
EDTA	Metalloprotease inhibitor	1 5	91.7 73.21
ß-mercapto ethanol	Cysteine protease inhibitor	1 5	97.40 91.64
Iodoacetic acid	Sulphydryl protease inhibitor	1 5	98.9 96.7

Table: 6.17 Effect of different surfactants on protease activity

Surfactants/oxidizing agents	Proteolytic activity (PU/mg)	Residual activity (%)
Control	380	100
Tween 20	450	118.5
Tween 45	405	106.73
Tween 65	430	113.21
SDS	160	42.12
Triton X-100	132	34.9

Table: 6.17 Effect of time period on the degradation of 1g of leather waste at room temperature.

Time duration (Hours)	Hydroxyproline release (µg/ml)
12	1.9
24	2.7
36	3.4
48	4.9
60	5.7
72	6.8
84	7.4
96	6.5
108	5.2
120	5.1
144	5.1

Table: 6.18 Effect of pH on the degradation of 1g of leather waste for 84 hours of incubation

pH	Hydroxyproline release (µg/ml)
7	6.7
8	7.3
9	8.1
10	7.9
11	4.2
12	1.9

Table: 6.19 Effect of temperature at pH 9 on the degradation of 1g of leather waste on 84 hours of incubation.

Temperature (^{0}C)	Hydroxyproline release (µg/ml)
25	7.7
30	7.9
35	7.8
40	8.1
45	8.3
50	7.8
55	7.5
60	7.1
65	4.3
70	2.9

Table: 6.20 Effect of proteolytic units at pH 10 on degradation of 1g of leather waste at 45^0C temperature for 84 hours.

Proteolytic units	Hydroxyproline release (μg/ml)
2000	7.3
3000	8.4
4000	8.7
5000	9.0
6000	8.8
7000	8.9
8000	8.8