PRODUCTION OF ALKALINE PROTEASE WITH POTENTIAL APPLICATION IN LEATHER INDUSTRY

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MAY-2007

Submitted in partial fulfillment of the Degree of Bachelor of Technology

DEPARTMENT OF BIOTECHNOLOGY AND BIOINFORMATICS

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CERTIFICATE

This is to certify that the work entitled, "Production of Alkaline Protease with Potential Application in Leather Industry" submitted by Sweta Srivastava and Garima Bhadauria in partial fulfillment for the award of degree of Bachelor of Technology in Bioinformatics of Jaypee University of Information Technology has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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CANDIDATE'S DECLARATION

We hereby certify that the work, which is being presented in the thesis, entitled, "Production of Alkaline Protease with Potential Application in Leather Industry", is partial fulfillment of the requirements for the award of degree of Bachelor of Technology in Bioinformatics and submitted in Department of Biotechnology & Bioinformatics, Jaypee University of Information Technology, Waknaghat ,Distt. Solan, is an authentic record of our own work carried out under the supervision of Dr. Gargi Dey.

The matter presented in this thesis has not been submitted by us for the award of degree of any other degree of this or any other University.

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This is to certify that the above statement made by the candidates is correct and true to the best of my knowledge.

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ACKNOWLEDEMENT

We wish to express our sincere gratitude to **Dr. Gargi Dey**, for providing us invaluable guidance and suggestions. She was always there to listen and to give advices.

She taught us how to ask questions and express our ideas. She showed us different ways to approach a research problem and the need to be persistent to accomplish any goal.

We would also like to thank our Head of the Department, **Dr. R. S. Chauhan**, who was always there to meet and talk about our ideas, to proofread and mark up our papers and chapters, and to ask us good questions to help us think through our problems (whether analytical or computational)

We would also like to thank all the staff members of Bioinformatics Department of **Jaypee University Of Information Technology**, Solan, for providing us all the facilities required for the completion of this thesis.

We would also like to give our vote of thanks to Mr. Sudhir Tyagi, B-Tech in Leather Technology, for helping us out for testing our crude enzyme extract in the leather industry.

We wish to thank all our classmates and friends for their timely suggestions and cooperation during the period of our thesis.

Last, but not least, we thank our families for educating us with aspects from sciences, for unconditional support and encouragement to pursue our interests, even when the interests went beyond boundaries of language, field and geography, for listening to our complaints and frustrations, and for believing in us, for reminding us that our research should always be useful and serve good purposes for all humankind.

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ABSTRACT

As we move towards saving the environment by searching the alternatives to chemicals that are environmental pollutants, the latest answer is enzymes that have got as good properties and mode of action as chemicals, and are also biodegradable. Out of all the industries that are causing the environmental pollution, the major shareholder is the leather industry. One of the main processes of leather industry is dehairing, which contributes to the major part of pollution. The enzyme alkaline protease is an alternative to this. It will digest the hair from the animal skin. Many industries are still not using these enzymes because of the economic aspects. So the aim of the project was to set up an economically feasible methodology for production of alkaline protease and to test its applicability in the local tannery.

The strains *Aspergillus flavus* and *Bacillus subtilis* were chosen as the source for producing alkaline protease. The media constituents and the fermentation conditions were standardized to optimize the yield. As the emphasis was on economy of the whole production process, the media constituents chosen were mainly cheap raw materials which are generally used for industrial production of enzymes. The mode of fermentation chosen was solid state fermentation (SSF) which has some advantages over submerged state fermentation. In an SSF the water content of the substrate is greatly influenced by the absorbing capacity and the capillary forces of the substrate, the growth temperature, the amount of heat generated, the quantity of moisture evolved and the growth requirements of the organism.

Aspergillus flavus gave the best activity at 32°C. The media was enriched with various carbohydrate and organic nitrogen sources, and the further increase in the alkaline protease activity in the crude enzyme extract was observed. Lactose and yeast extract enhanced the production of alkaline protease in the solid state fermentation with wheat bran as the substrate.

Introduction of mutagenesis in the two strains was also performed using U-V light lamp. After mutagenesis, the microorganisms showed a considerable increase in the alkaline protease yield. The enzyme activity greatly improved up to 58.8 U/g of wheat bran in the case of *Aspergillus flavus*, from solid state fermentation, and up to 2.66 U/ml in the case of

Bacillus subtilis, from submerged phase fermentation, in comparison with its parent strain of 44.6 U/g of wheat bran in the case of *Aspergillus flavus* and 1.5 U/ml in the case of *Bacillus subtilis*.

The enzyme was tested for stability under various pH ranges and showed maximum stability in the pH range 10-12. The crude enzyme extract was further characterized for the temperature stability and it was found to be stable in a wide temperature range. The applicability of the enzyme in the dehairing process during the tanning of hide was tested with collaboration with Asia Tanneries, Kanpur. After the dehairing and the soaking process in the atmospheric conditions, the result was good. All the hair and the hair roots were removed from the skin and it was perfect to open the fiber.

CHAPTER 1

INTRODUCTION

1.1 Conventional Leather Processing

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

The principal leather making protein, collagen, exists in hides and skins in association with various globular proteins, viz. albumin, globulin, mucoids, and fibrous proteins such as elastin, keratin, and reticulin. During leather manufacture, the noncollagenous constituents are removed partially or completely in the various tanning operations; the extent of removal of these constituents decides the characteristics of the final leather. Besides chemical treatment, certain enzymatic treatments are also necessary to get optimum results. One such treatment, bating, is the only step in leather processing where enzymatic process cannot be substituted by chemical processes. The process of bating gives certain desired characteristics to the finished leather. Earlier, the process was carried out using dog dung or manure[2]. The use of this was not only unhygienic but process could also not be controlled.

In tanning operations, the hides and skins are first subjected to a water soak. For loosening the hair[3], the oldest method is the 'sweating' process – a natural autolysis or breakdown process. It is a mild putrefaction process induced at random. Since the type and quantity of the putrefying bacteria cannot be controlled, the process itself eludes control. Moreover, since the sensitivity to attack the epidermal proteins and the fibrous proteins of the corium by the proteolytic enzymes is more or less the same, the sweating may result in serious damage to the hide surface. Dehairing is followed by

opening up of fibre structure in 'liming'. The dehaired hide is transferred to an alkaline solution of lime milk where swelling occurs and the nonfibrillar proteins are dissolved. After mechanical removal of the subcutaneous tissue, deliming is performed in order to remove the adsorbed lime from the hide and to eliminate the lime swell.

The fat present in the hide skins is removed either as soluble lime soap or hydrolysis products like fatty acids. Kerosene, chlorinated hydrocarbons, and white spirit are used in the degreasing system which adds to the toxicity of the environment and effluents[4].

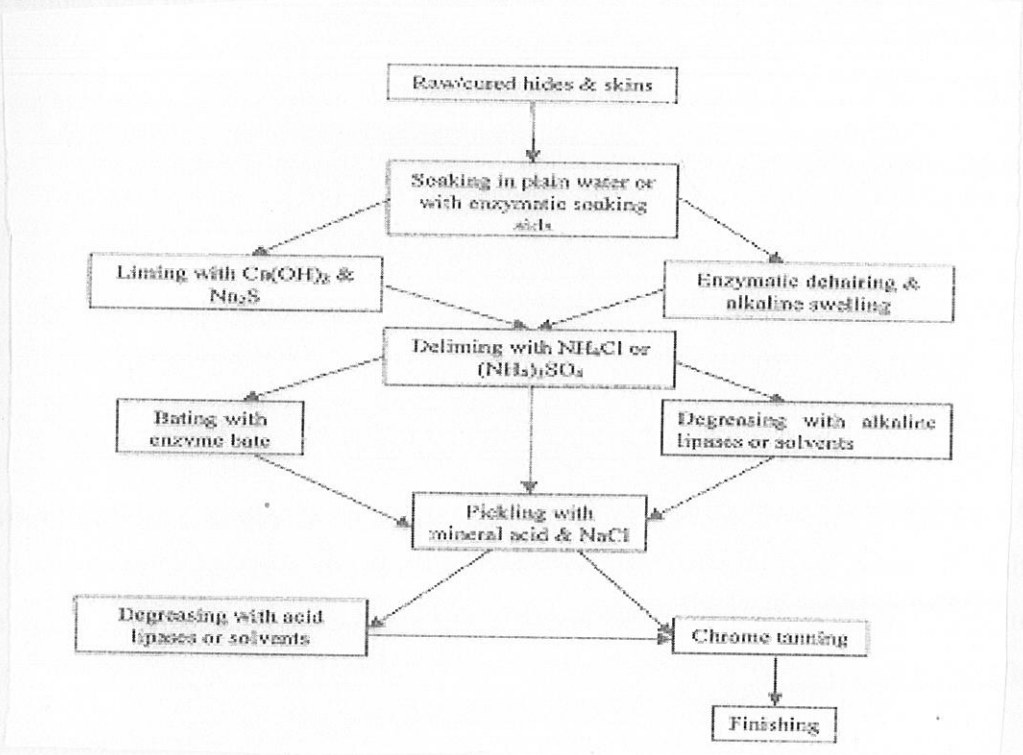


Fig. 1: Protocol currently followed by most leather industries.

1.2 What is the Problem?

Environmental pollution has been a major deterrent to the leather industrial development. Chemical and chemical-based industries are the prime targets of the environmentalists for their crusade against pollution, and leather industry has also not been left out of the reckoning. The generation of pollution is significantly high in the

tanning operations compared to the post-tanning operations[5]. Leather industry contributes to one of the major industrial pollution problems facing the country, and the pollution causing chemicals, viz. lime, sodium sulphide, salt, solvents, etc. arise mainly from the tanning processes of leather treating. The chemicals mainly responsible for pollution in tanning processes are lime, sodium sulphide, and caustic soda apart from common salt and degreasing chemicals. In fact, one third of the pollution caused by the leather industries results from the wastes generated during dehairing operations[6]. The wastes from the tanneries are let out into the drains which in turn empty into the main sewerage causing hazard to those who use this water. Many tanneries have been forced to close down because of their noncompliance with the standards laid down examples. The attention of tanners is focused towards revamping the processing methods, recovery systems, and effluent treatment techniques to make leather processing eco-friendly.

1.3 What is the Alternative?

Intensive efforts are being directed towards using a viable alternative technology for tanning processes using enzymes. This could be one of the ways of solving the industrial pollution problems resulting from tannery effluents. The hazards caused by the tannery effluents can be overcome by the use of enzymes, hence a viable alternative has been resorted to in tanning operations such as soaking, dehairing, bating, degreasing and offal (Viscera and trimmings of a butchered animal often considered inedible by humans) treatment.

1.4 Enzymes in Tanning

An important enzyme used in tanning processes belongs to the group of proteolytic enzymes, proteases. Obtained by microbial fermentation, the proteases are meant for use in the leather industry for dehairing, bating and soaking processes, and in the detergent industry for breaking down proteinaceous matter caused by body secretions, food stuffs, and blood[7]. The main advantages of the use of enzymes are specificity,

stereospecificity, activity under mild conditions, possibility of producing 'natural' products, nonpollutants, and biodegradability.

Although enzymes from plants, animals, and microbial sources have been used for decades, large-scale use of microbial enzymes received a boost only in 1960s following the introduction of fermentation technology. The enzymes or enzymatic formulations need not be pure but must be cheap compared to that of commercial chemicals used in leather industry.

Animal proteases and microbial proteases from bacteria and fungi are used in the tanning processes of leather manufacture. The most important criteria for their selection are their specificity, pH activity range as well as pH and thermal stability. If an enzyme is to act uniformly, it must be able to diffuse into the hide and this is obviously achieved with skins rather than with hides. In the latter case, an accumulation of enzyme at the surface of the grain occurs. A pronounced difference between the pH value of the solution and that of the hide is also possible[8].

The animal proteases are mixtures of trypsin, chymotrypsin, and various peptidases which may contain amylase or lipase as secondary enzymes. Mainly for economic reasons, enzymes from microorganisms have come to play a significant role in recent years and enzyme products of microbial origin are already being produced on a wide scale[9].

Since microorganisms can be made to propagate rapidly and profusely, they are an ideal source for enzymes[10]. Mainly, neutral and alkaline proteases are obtained from bacteria, which differ in their pH activity range[11]. Fungal proteases are also classified according to the pH activity range, fungal acid proteases[12]act between pH 2.5 and 6.0 and can be derived from *A. satoi*. These are used for bating prior to pickling and serve to open up the fibre structure. Fungal alkaline proteases[13] belong to the same group of serine proteases as alkaline bacterial proteases. However, these are more heat sensitive and are quickly deactivated above 60° C.

Table 1. Enzymes used in pretanning operations

Process	Enzyme	Microorganism
Soaking	Protease	*Aspergillus parasiticus*, *A. flavus*, *A. oryzae*, and *Bacillus subtilis*[19], *Rhizopus rhizopodiformis*[24]
	Carbohydrases	*Aspergillus awamori*[20]
Dehairing	Protease	*Aspergillus flavus*[19,59], *Aspergillus* sp.[24], *Bacillus subtilis*[61], *Lactobacillus* sp.[62], *Coniothecium* sp.[36], *B. amyloliquefaciens*[64], *Streptomyces griseus*, *S. fradiae*[63], *S. moderatus*[65]
Bating	Protease	*A. parasiticus*[67,68], *S. rimosus* and *B. licheniformis*[48], *B. subtilis*[69], *Penicillium janthinellum*[70]
Degreasing	Lipase	*Rhizopus nodosus*[46], *A. oryzae* and *A. flavus*[71]

Apart from bacterial and fungal proteases, specific proteases like keratinases[14] are known. Keratinases which hydrolyse keratins, are obtained from *Streptomyces fradiae* and can be used for dehairing[15]. Some of the important lipase-producing microorganisms used in degreasing are shown in Table. Lipases are used (i) in the oil and fat industry to modify fats for use in foods; (ii) in detergent compositions; (iii) for fatty acid production, lipid synthesis via reversal of hydrolysis and lipid modification by interesterification, and (iv) in degreasing of hides and skins[16].

1.4.1 Enzymes in Soaking

Soaking is the first operation in the tannery wherein the hides and skins are cleaned and softened with water[17]. Wet-salted or freshly slaughtered hides and skins do not require any chemical agent for their proper soaking[18]. Soaking is necessary for solubilization and elimination of salts and globular proteins contained within the fibrous structure of hides and skins. It is carried out under alkaline conditions at low temperature between 10° C and 20° C in water treated with antiseptics such as sodium hypochlorite, sodium pentachlorophenate, formic acid, etc.[19]. It is accelerated by some of the nonionic detergents and additives such as sodium sulphide or sodium tetra-sulphide.

The advantages of enzymatic soaking include loosening of the scud, initiation of the opening of the fibre structure, and production of leather with less wrinkled grain when used at an alkaline pH of less than 10.5. Use of enzyme preparation in soaking of

rabbit skins improves the softness and elasticity, and increases the area yield of the fur by 3.3% while reducing the processing time by 10–20 h.

Grimm[20] has described a soaking method using proteolytic enzymes and carbohydrases in the pH range of 5.5 to 10.0. Enzymes from *Aspergillus parasiticus*, *A. flavus*, *A. oryzae*, and *Bacillus subtilis* have been used alone or in mixtures. Rokhvarger and Zubin[21] suggested the use of carbohydrase from the mold culture *A. awamori* in soaking. Botev *et al.*[22] have reported the use of bacterial amylase for soaking dried wool lamb skins. Alkaline proteases of bacterial and fungal origin have been used for soaking which reduces the need for the liming chemicals by 30–60%. Soaking of dried furs in an aqueous bath containing 1% acid proteinase from *Rhizopus rhizopodiformis* and sodium bisulphite at 25° C for about 20 h has been reported by Asbeck *et al*[23]. Orlita and Beseda[24] have tested three commercial bacterial alkaline protease preparations for the soaking of salted cow hides. Thus, use of enzyme preparations results in a decrease in soaking time.

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

1.4.2 Enzymes in Dehairing

Dehairing is one of the main operations in the beam house. Five methods of dehairing are generally adopted, viz. (i) clipping process, (ii) scalding process, (iii) chemical process, (iv) sweating process, and (v) enzymatic process[25]. Of these, the most commonly practiced method of dehairing of hides and skins is the chemical process using lime and sodium sulphide. However, the use of high concentrations of lime and sodium sulphide creates an extremely alkaline environment resulting in the pulping of hair and its subsequent removal. While one cannot question the efficacy of this process, its inherent disadvantages have to be taken note of. Significant amongst these are:

i. It contributes in no small measure to the pollution load. Beam house processes generally account for 70–80% of the total COD of effluent from all leather making processes. About 75% of the organic waste from a tannery is from the beam house and 70% of this waste is from hair which is rich in nitrogen. These figures clearly illustrate the contribution made by the lime and sulphide process towards pollution[26].

ii. Sulphide is highly toxic with obnoxious odor. If left untreated, it can cause major problems in the sewers.

iii. The severe alkaline condition is a health hazard for the workers.

Enzymatic dehairing is suggested as an environmentally friendly alternative to the conventional chemical process[27]. The enzyme digests the basal cells of the hair bulb and the cells of the malphigian layer. This is followed by loosening of hair with an attack on the outermost sheath and subsequent swelling and breakdown of the inner root sheath and parts of the hair that are not keratinized[28]. Advantages of enzymatic dehairing are:

i. Significant reduction or even complete elimination of the use of sodium sulphide.

ii. Recovery of hair of good quality and strength with a good saleable value.

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

iv. Enzymatically dehaired leathers have shown better strength properties and greater surface area.

v. Simplification of tanning processes by cutting down one step, viz. bating.

vi. A significant nature of the enzymatic dehairing process is the time factor involved. The lime-sulphide process takes about 16 h, whereas the enzymatic dehairing would be also completed between 12 and 20 h [29].

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

Microbial proteases are derived from a wide variety of yeasts, molds, and bacteria. Yeast proteases are mainly intracellular in nature and therefore these enzymes have not

gained significant commercial interest. The protease from *A. flavus* was earlier being used for dehairing, and later it was reported that simultaneous dehairing and bating is possible with the protease of *A. flavus*[32]. Gillespie[33] has observed that the enzyme preparation from cultures of *A. oryzae*, *A. parasiticus*, *A. fumigatus*, *A. effusus*, *A. ochraceus*, *A. wentii*, and *P. griseofulvum* exhibit marked depilatory activity on sheep skins.

CLRI, India has developed Clarizyme, an alkaline serine protease, produced by *A. flavus* used for the dehairing of skins and hides[34]. *A. flavus* grows rapidly on wheat bran and produces large amounts of extracellular proteases. Extensive trials carried out in CLRI tannery have confirmed the successful use of this enzyme as a depilatory agent. The use of this enzymatic depilation process completely eliminates the use of sulphide, a toxic pollutant.

The production process employs solid state fermentation for the production of clarizyme by a new Aspergillus flavus strain using wheat bran as substrate. The enzyme loosens hair from skins and eliminates bating step in leather processing. Enzyme has good caseinolytic activity over a pH range of 6.0 to 10.0 and temperature range of 20-50 degree C with no effect on the collagen or elastin.

The fungal culture, *Conidiobolus* sp., isolated at NCL, Pune, produces high yields of extracellular alkaline protease[35]. The enzyme is active at pH 10.0 and is being tried for many industrial applications. Enzymes derived from bacteria have gained much commercial interest[36] because of their easy production capabilities by submerged cultivation, high yield of enzyme, short duration for production, and easy recovery of the enzyme.

Proteolytic enzymes derived from a large number of *Bacillus* sp. and *Streptomyces* sp. have been used in dehairing of hides and skins[37]. A lime and sulphide-free process of dehairing has been developed for the manufacture of suede from sheep skins using protease from *B. subtilis*[38]. Schlosser *et al.*[39] have reported a method of depilation in an acid medium containing *Lactobacillus* culture.

In dehairing, the hair loosening is effected at pH 10.0 using fungal or bacterial enzymes; the treatment period being approximately 12–16 h, followed by hair removal using mechanical means[40]. The treatment period can be substantially reduced if the

enzyme solution is fed in from the flesh side under pressure[41]. Enzymatic hair loosening processes play a role wherever high-quality hair, wool or bristles are to be recovered.

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

1. paint method,
2. dip method, and
3. spray method.

In the paint method, the enzyme solution is mixed with an inert material like kaolin, made into a thin paste, adjusted to the required pH, applied on the flesh side of hides and skins, piled flesh to flesh, covered with polythene sheets and kept till dehairing takes place. In the dip method of enzymatic unhairing, the hides or skins are kept immersed in the enzyme solution at the required pH in a pit or tub. The disadvantage encountered in this method is the unavoidable dilution of the enzyme solution. Even though enzyme penetration is observed to be uniform, dehairing at backbone and neck is not up to the mark. A novel spraying technique has been adopted for the application of multienzyme concentrate in depilation[42]. The advantages of this method over the painting and dip methods are that (i) even concentrated solutions can be sprayed, (ii) when the enzyme solution is sprayed on the flesh side with force, entry becomes easier, (iii) backbone and neck can be sprayed with more amount of enzyme, thereby making the process quicker, (iv) there is no effluent arising out of this method, and (v) after depilation, hair will be almost free from all the adhering skin tissues. Of late, dehairing by drumming is being practiced, and industrially this should be feasible.

1.4.3 Enzymes in Bating

Bating is a very important process in which enzymes have been successfully employed for centuries. The concept of softening hides by treating them in a warm infusion of animal dung has been termed as 'bating' and the product used for such process is known as a bate. The main object of bating is to remove some of the nonleather-forming proteinous materials like albumins, globulin, and mucoids from hides and skins, and to allow splitting up of collagen fibres to facilitate the penetration of tanning

materials and other processing chemicals, thereby giving the finished leather the desired characteristic properties like feel, softness, pliability, etc.[43].

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

The comparatively richer source for the proteolytic enzyme is the pancreas from bovine and pig. The proteolytic enzymes in the pancreas are present in inactive forms; chymotrypsin as chymotrypsinogen, trypsin as trypsinogen, and carboxypeptidase as procarboxypeptidase. A process has been patented for the activation of pancreatic enzymes by the use of acid protease from *A. fumigatus*[44].

Trabitzch[45] have reported the use of enzymes from *Aspergillus* species in bating and dehairing. A procedure has been developed for bating pig skins, using an enzyme preparation from *B. subtilis*, and bated skins exhibit good physicochemical properties[46]. Bacterial preparation from *S. rimosus* and *B. licheniformis* have been tested for their bating action and it is found that solubilization of collagen has been less pronounced under the influence of microbial proteases than under the influence of pancreatic protease[47]. A combination of both mold and pancreatic enzymes in suitable proportions will be an ideal bate for different types of leather.

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

1.4.4 Enzymes in Degreasing

Degreasing is an essential step in the production of glove and clothing leather. In this process there is removal of excess natural fats from greasy skins. The presence of natural grease in raw hides and skins, especially woolly sheep skins, results in various defects, viz. fatty spues, uneven dyeing and finishing, waxy patches in alum-tanned

leathers, and pink stain on wet blues[48]. During the degreasing operation in the tanning process, the fat or grease is removed from the interfibrillary spaces of the skins to facilitate the even penetration of tanning materials, fat liquors, and dyes, etc. Degreasing helps to obtain soft and piiable leather for garment manufacture.

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

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

In 1966, Trabitzsch[50] described the potential for lipases in degreasing skins. Baldano and Shestakova[51] compared the enzymatic and solvent degreasing of pig skin and have shown that both these methods remove approximately 50% of the grease. Yeshoda *et al*[52] used a fungal lipase for the degreasing of woolly sheep skins, pH range of 3.2–3.6 at 37° C for 1 h. Subsequently, Yeshoda *et al.* observed that degreasing and bating could be carried out simultaneously in the pH range of 7.8–8.0. An acid lipase from *Rhizopus nodosus* has been noticed to be very effective in the degreasing of sheep skins[53]. Zhang reported use of alkaline lipase in combination with the proteinase and pancreatin in softening pig skin to improve the degreasing effect[54]. Pfleiderer *et al*[55]. carried out degreasing of hides by soaking in an acidic bath containing a proteolytic enzyme (0.01–3.0%), and a nonionic surfactant (0.2–1.5%) or its mixture with anionic emulsifiers. A combination of proteolytic enzymes and emulsifiers gives optimum results in wet degreasing of sheep skins[56].

CLRI has developed a potent fungal lipase from *A. niger*[56] and a potent bacterial lipase[58]. Comparative studies on degreasing of sheep skins using the bacterial lipase and commercial detergent-based degreasing agent Gelon-PK have been carried out. Improved degreasing results with the bacterial lipase with added advantages of better softness, smoothness, and improvement in other physical properties[59]. Furthermore, the lipase without detergent is observed to show 70% degreasing in 2 h, with the effluent showing minimal pollution load.

Enzymatic degreasing can be carried out with acidic or alkaline lipases of fungal or bacterial origin. For degreasing, pickled pelts are kept immersed in an enzyme bath containing microbial lipase and water pH of 3.6, and left in the same bath overnight at a temperature of 28–32° C. The degreased pelts are then removed from the bath and subjected to salt wash twice with water and common salt for 40 min. The washed pelts are repickled, chrome tanned and taken for further processing[60]. The use of an alkaline lipase at a pH of 9.0 to 9.3 in the degreasing of pig skin results in short degreasing time and high degreasing efficiency[61].

1.4.5 Enzymes in By-Products Utilization and Effluent Treatment

Enzymes could be used in the treatment of fleshings and effluent from tannery processes. A combination of hydrolytic enzymes, viz. proteases, carbohydrases, and lipases would be required. The advantages to be realized include a protein by-product suitable for animal feed as well as energy conservation and fat recovery. Again, the major disadvantage would be the cost[62].

When raw hides are processed to leather, a number of by-products such as native hide material (claws, tails, necks, fleshings), pelt waste (trimmings, machine fleshings, gluestock, pelt cuts), and tanned material (shavings, leather cuts, buffing dust, chrome cuttings) are obtained[63].

Braeumer *et al.*[64] have described the enzymatic conversion of glue stock and other hide offal to technically useful byproducts by hydrolysing the pulverised hide wastes with an alkaline protease, pH 9.0–13.0, in the presence of urea, and then at pH 2.0–5.0 in the presence of a strong acid. Bronowski *et al.*[65] have shown that treating fleshings with pancreatic enzymes instead of heat treatment for separating the fat from the

proteinaceous matter requires much less energy, and the yield is increased from 60–65% to over 90%. Sauer[66] has described a process for the utilization of fleshings which consists of the enzymatic hydrolysis of the proteins, conditioning of the resulting liquid, and separating the fats and solids present in the hydrolysate. The outstanding feature of the process is a recovery of 91% of the fat in the fleshings and the application of the hydrolysate directly to the soil, as a fertilizer. Iliskovic and Mersed[67] have described the separation of fats from the fleshy wastes from cattle hide processing by treatment with enzymes.

The problem of waste treatment can be approached (i) by getting rid of the pollution by proper effluent treatment, and (ii) by controlling pollution occurring at different stages of leather manufacture[68]. Biotechnology plays an important role in tannery effluent treatment. The secondary treatment of tannery effluents, which relies on living organisms, is normally by anaerobic lagoons and aerobic lagoons. Open waste-ponds or anaerobic lagoons are installed in few south Indian tanneries where the atmospheric temperature (20–40° C) is suitable for this operation. In these ponds, microorganisms which thrive in oxygen-less environments are allowed to digest the waste. Anaerobic lagoons can be used for cleaning wastes coming from both the vegetable tanning and chrome tanning procedures. Closed type anaerobic systems are useful for tanneries situated in cold temperatures (5–10° C). Aerobic lagoon is a shallow water-tight pond of about 2–3 m depth. The wastes are kept for about a week. Fixed or floating type surface aerators blow oxygen or air into these for helping growth of organisms. This system requires less land and is economical for larger tanneries located in urban areas.

The necessity for chromium removal in tannery waste water is another area of waste management[69]. Microorganisms such as *A. fumigatus* and species of *Pseudomonas* when grown on chrome waste can 'leach' out chromium. Pentachlorophenol[68], a preservative used for raw as well as semi-processed skins, creates problems during handling and also during biological effluent treatment. *P. aeruginosa* could be used successfully to degrade pentachlorophenol. Other potential techniques for reduction of pollution load are recycling of immobilized enzymes to hydrolyse the solid waste, and recycling of immobilized whole cells to absorb or detoxify toxic metals in the effluent.

The tanneries in future will use a combination of chemical and enzymatic processes. The potential for use of microbial enzymes in leather processing lies mainly in areas in which pollution-causing chemicals, such as sodium sulphide, lime and solvents, are being used and conversion of waste products into potentially saleable by-products is possible. Future may witness eco-labelled leather/leather products emerging as niche products, and the experience gained by the Indian leather industry in this area might greatly help India to emerge as a global leader in leather industry.

After the analyzing the above enzymatic treatments for various steps of leather processing our objective focuses on the use of microbial enzymes as an alternate technology to the conventional methods, and investigates the economic feasibility of the project.

1.4.6 Dehairing Process by the Biotechnological Way

As we know the hair are made up of keratin, protein. So we can use enzymes to digest the hair protein. Here we are using the protein digesting enzyme alkaline protease. We searched for the microorganisms that are potential source for alkaline protease.

These are the enzymes that "digest" long protein chains to shorter fragments. Some of them can detach the terminal amino acids from the protein chain (exopeptidases– like aminopeptidases, carboxipeptidase A); the others "attack" internal peptide bonds of a protein (endopeptidases- like trypsin, chymotrypsin, pepsin, papain, elastase).

Proteases are divided into five major groups according to the character of their active site (catalytic site) and conditions of action: Serine, cysteine (thiol), aspartic (acid), metallo and mixed depending on the principal amino acid participating in catalysis. Proteases occur naturally in all organisms and constitute 1-5% of the gene content. These enzymes are involved in a multitude of physiological reactions from simple digestion of food proteins to highly regulated cascades (e.g., the blood clotting cascade, the complement system, apoptosis pathways, and the invertebrate prophenoloxidase activating cascade). Peptidases can break either specific peptide bonds (*limited proteolysis*), depending on the amino acid sequence of a protein, or break down a complete peptide to amino acids (*unlimited proteolysis*). The activity can

be a destructive change abolishing a protein's function or digesting it to its principal components; it can be an activation of a function or it can be a signal in a signaling pathway. Proteases are also a type of exotoxin, which is a virulence factor in bacteria pathogenesis. Bacteria exotoxic proteases destroy extracellular structures.

Proteases (proteinases) are large group of enzymes. Enzymes are divided into classes, one of which is the class of Hydrolases - these enzymes catalyze the reaction of hydrolysis of various bonds (peptide bonds, ester bonds etc.) with the participation of a water molecule. Proteolytic enzymes (proteases) belong to the class of Hydrolases. Proteases are involved in splitting the peptide bonds which link the amino acid residues (elementar units of proteins). Proteases are everywhere and they are involved in various metabolic processes. Acid proteases secreted into the stomach (such as Pepsin) and serine proteases present in duodenum (Trypsin, Chymotrypsin), enable us to digest the protein in food, proteases present in blood serum (Thrombin, Plasmin, etc.) play important role in blood clotting, as well as lysis of the clots, and the correct action of the immune system. Other proteases are present in leukocytes (Elastase, Cathepsin G) and play several different roles in metabolic control. Proteases determine the lifetime of other proteins playing important physiological role like hormones, antibodies, or other enzymes - this is one of the fastest "switching on" and "switching off" regulatory mechanisms in the physiology of an organism. By complex cooperative action the proteases may proceed as "cascade" reactions which result in amplification of the organism response to the physiological signal, and make this response very fast[69].

Commercially they are extremely important as more than 60% of the total enzyme market is made up of proteases, they are isolated from plants, animals, bacteria and fungi and there are many of them available commercially. They all catalyze the hydrolysis of proteins but there are many differences in the method of catalysis.

Inhibitors: The function of peptidases is inhibited by protease inhibitor enzymes. Examples of protease inhibitors are the class of serpins (*ser*ine *p*rotease or *p*eptidase *in*hibitors), incorporating alpha 1-antitrypsin. Other serpins are complement 1-inhibitor, antithrombin, alpha 1-antichymotrypsin, plasminogen activator inhibitor 1 (coagulation, fibrinolysis) and the recently discovered neuroserpin.

Natural protease inhibitors include the family of lipocalin proteins, which play a role in cell regulation and differentiation. Lipophilic ligands, attached to lipocalin proteins, have been found to possess tumor protease inhibiting properties[69].

Degradation: Proteases, being themselves proteins, are known to be cleaved by other protease molecules, sometimes of the same variety. This may be an important method of regulation of peptidase activity. Protease refers to a group of enzymes whose catalytic function is to hydrolyze (breakdown) peptide bonds of proteins. They are also called proteolytic enzymes or proteinases. Proteases differ in their ability to hydrolyze various peptide bonds. Each type of protease has a specific kind of peptide bonds it breaks. Proteolytic enzymes are very important in digestion as they breakdown the protein foods to liberate the amino acids needed by the body. Additionally, proteolytic enzymes have been used for a long time in various forms of therapy. Their use in medicine is gaining more and more attention as several clinical studies are indicating their benefits in oncology, inflammatory conditions, blood rheology control, and immune regulation[71].

CHAPTER 2

MATERIALS AND METHODS

2.1 Strains and Culture Conditions

1. *Bacillus subtilis* (Bacterial source)
2. *Aspergillus flavus* (Fungal source)

2.1.1 *Aspergillus flavus*

Kingdom :Fungi

Phylum:Ascomycota

Class: Eurotiomycetes

Order:Eurotiales

Family:Trichocomaceae

Genus:*Aspergillus*

Species:*flavus*

The organism used is *A. flavus* (MTCC No-277). The culture was routinely maintained on potato dextrose agar slants. The culture was routinely maintained on potato dextrose agar slants at 28°C

Solid state fermentation- The spore suspension of *A. flavus* was used for inoculation was prepared by adding 10ml of sterile distilled water to each slant and vigorously shaking the slant for 1 min.

Fermentation Conditions- Wheat bran (20 g) was thoroughly mixed with 30 ml of a salt solution containing, per liter, $NaNO_3$ (2 g), KH_2PO_4 (1 g), $MgSO_4$- $7H_20$ (0.5 g), KCl (0.5 g), $FeSO_4.7H_20$ (trace), and $ZnSO_4$. $7H_20$ (trace), adjusted to pH 7.0 in 250-ml flasks, and sterilized at 15 lb/in2 for 20 min[72]. The flasks were inoculated with 1 ml of the spore suspension aseptically. The contents were mixed thoroughly, incubated at 32°C for 48 hrs, and assayed for enzyme activity.

Extraction of the Enzyme- At the end of the fermentation period, the entire quantity of moldy bran was homogenized with 200 ml of distilled water and filtered with the help of a muslin cloth. The filtrate was used as the enzyme source.

2.1.2 *Bacillus subtilis*

Kingdom: Bacteria

Phylum: Firmicutes

Class:Bacilli

Order:Bacillales

Family:Bacillaceae

Genus:*Bacillus*

Species:*subtilis*

An alkaline protease– producing strain of *B subtilis* (MTCC No. – 1790) was obtained from IMTech Chandigarh. It was maintained on nutrient agar slants at 4°C and was subcultured.

Submerged state fermentation- Five milliliters of sterile distilled water was added to a 48-hours old slant of *B subtilis*. The cells were scraped from the slant into sterile distilled water and the resulting cell suspension was transferred, aseptically into 250-mL flasks containing production medium. The composition of the inoculum medium (delete is) : glucose 0.5%(w/v), peptone 0.75%(w/v), and salt solution 5%(v/v)- [KH_2PO_4 0.5%(w/v), MgSO4.$7H_2O$ 0.5%(w/v), $FeSO_4.7H_2O$ 0.01%(w/v)] with a pH of 7.0[73]. The production was carried out with shaking at 220 rpm at 37°C, for 48 hours. The content of the flasks were centrifuged at 5000 rpm for 15 minutes and the supernatant was taken as crude enzyme extract.

2.2 Mutagenesis

Mutagenesis with ultraviolet irradiation was carried out with a UV tube. The organisms were subjected to the UV radiation for variable time lengths (2, 4, 6, 8 and 10 minutes). After exposure the media (*Bacillus subtilis*- glucose 0.5%(w/v), peptone 0.75%(w/v), and salt solution 5%(v/v)- [KH_2PO_4 0.5%(w/v), MgSO4.$7H_2O$ 0.5%(w/v), $FeSO_4.7H_2O$ 0.01%(w/v)] with a pH of 7.0 and *Aspergillus flavus* wheat

bran (20 g) mixed with 30 ml of a salt solution containing per liter $NaNO_3$ (2 g), KH_2PO_4 (1 g), $MgSO_4$- $7H_20$ (0.5 g), KCl (0.5 g), $FeSO_4.7H_20$ (trace), and $ZnSO_4$. $7H_20$ (trace), adjusted to pH 7.0) was added and they were kept in the dark phase for 2 hours and then were grown for 48 hours at 37°C(*B. subtilis*) and 32 °C(*A. flavus*) in the same media.

2.2 Analytical Methods

2.2.1 Qualitative Assay

For qualitative analysis for the activity of alkaline protease in the crude enzyme extract casein-agar plates (composition:-1% casein, 1.5% agar) were made. In the two plates, standard enzyme was added (50 µl) on the top. The test enzyme of same amount from *Aspergillus flavus* and *Bacillus subtilis* was added below the standard enzyme. The plates were incubated for half hour at 32-37°C. They were then flooded with 1% $HgCl_2$. The $HgCl_2$ was poured off after 5 minutes and the plates were observed for zones of clearance. The standard results were compared with that of the test enzyme results.

2.2.2 Quantitative Assay

Conditions:-

Substrate: 1 ml of casein solution (1%)

Buffer: 1.9 ml of glycine-sodium hydroxide buffer (0.1 M, pH 9)

Enzyme: 0.1 ml of enzyme

The mixture was incubated at 32°C (assay temp.) for 30 min. After that the inhibitor was added,

Inhibitor: 2 ml of 5% Trichloroacetic Acid.

After adding the inhibitor it was allowed to rest at room temp and then centrifuged at 5000 rpm for 10 minutes. Supernatant was taken for calculating the optical density. An enzyme blank was always included[6]. The optical densities of the Trichloroacetic acid-soluble materials were read at 280 nm and compared with the standard plot. One unit of enzyme activity is defined as that amount of enzyme required to liberate 1 mg of tyrosine under standard assay conditions.

2.4 Preparation of Stocks

Stocks of the two organisms were prepared for the long term storage.

2.4.1 Preparing Stock of B. subtilis

Bacillus subtilis was grown overnight in the Nutrient Broth (10ml). After 24 hours, the culture (0.85ml) was transferred aseptically to the two sterilized vials which were already containing 0.15ml of 10% glycerol. The vials were sealed with the paraffin wax paper. The contents of the vials were mixed thoroughly. One vial was stored at -20°C and the other was stored at -80°C.

2.4.2 Preparing Stock of A. flavus

Method 1: *A. flavus* was grown on potato dextrose agar plates. When the growth was optimum on the plate, a part is scraped off with the sterilized inoculation loop and transferred to the two vials containing 10 ml of 10% glycerol aseptically. The vials were sealed with the paraffin wax paper. The contents of the vials were mixed thoroughly. One vial was stored at -20°C and the other was stored at -80°C.

Method 2: *A. flavus* was grown on potato dextrose agar plates. When the growth was optimum the plates were flooded with 10% glycerol. 10ml of the spore suspension was transferred to the 2 sterilized vials aseptically. The contents of the vials were mixed thoroughly. One vial was stored at -20°C and the other was stored at -80°C.

2.5 Protocol for Dehairing of the Hides Followed in the Tannery

This part of the project is done in collaboration with local tanneries of Kanpur. Alkaline protease was submitted to a tannery, Asia Tanners Pvt. Ltd, Kanpur who agreed to test our enzyme as a depilation agent in their laboratories. It was tested on goat hides. A pair of goat skin was cut into halves. The left half (control) was painted with a homogenous paste of 7% lime, 4% china clay, 0.2% wetting agent, 3% sulfide and 80% water (conventional depilation method). The right half was painted with enzyme paste -1.0% enzyme, 7.0% kaolin, and 20% 10-5 M NaOH (pH 9.0) solution [wet weight]). The paste was applied on the flesh side and the hides were piled

flesh to flesh. They were incubated for 4 hours. After incubation flooding was performed. The method of flooding includes keeping the hides in mixture 1(water 200%, balance paste, lime 1%) for few hours. Hair from both sets was removed with a blunt knife.

CHAPTER 3

RESULTS

3.1 Qualitative Assay

Alkaline protease showed more activity (Better zone of clearance) in the petridish with crude enzyme extract from *A. flavus* (Figure 2) than in the petridish with crude enzyme extract from *B. subtilis* (Figure 3).

Fig.2: Qualitative assay of *A. flavus*

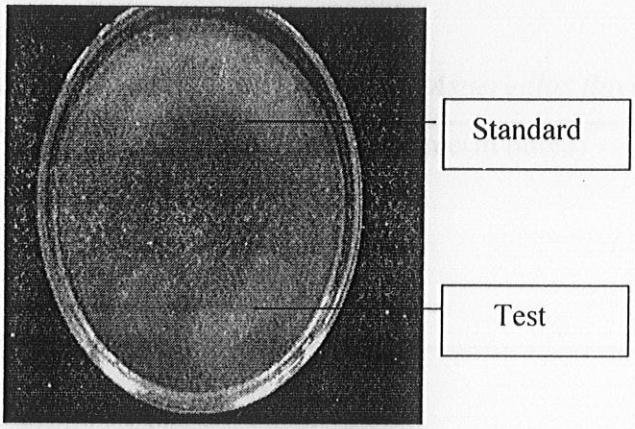
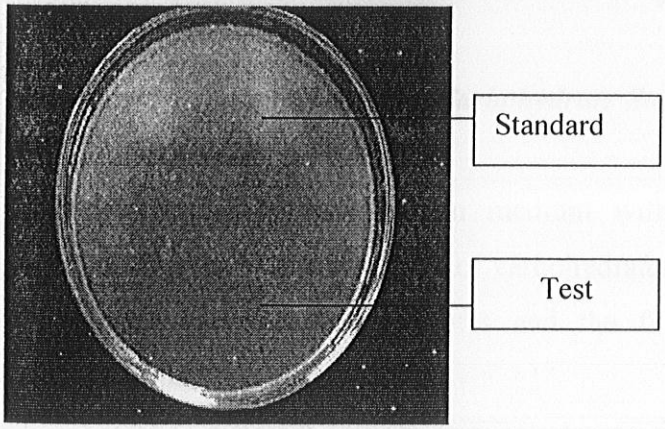


Fig.3: Qualitative assay of *B. subtilis*



3.2 Quantitative Assay

Bacillus subtilis and *A. flavus* are known to be good sources of alkaline protease. The bacterium, *B. subtilis* requires a lot of care in handling as it is very prone to contamination. This strain did not give a satisfactory result for alkaline protease at the time of quantitative assay (Table 2). It gave a very minimal reading for alkaline protease.

Table 2. *Bacillus subtilis* quantitative assay

Fermentation Time(in hours)	Enzyme activity(U/ml)
24	0.6
48	1.5
72	0.9

The fungal strain gave a good enzyme activity (Table 3) and was also not very prone to the contaminations.

Table 3. *Aspergillus flavus* quantitative assay:

Fermentation Time(in hours)	Enzyme activity(U/g)
24	12.66
48	44.6
72	31.33

3.3 Production Media Enrichment for *A. flavus*

3.3.1 Effect of Different Carbohydrate Sources on Alkaline Protease Production by A. flavus

Enrichment of fermentation medium with different carbohydrate sources was achieved by addition of 2g of carbohydrates per 10 g of wheat bran. Experiments were conducted in duplicates and the final consensus was the average of 2 independent trials.

Effect of different carbohydrate sources on alkaline protease production by *A. flavus* (Table 4) showed that lactose had a positive influence on the productivity of alkaline protease. All other sugars severely repressed the synthesis.

Table 4. Effect of different carbohydrate sources on alkaline protease production by *A. flavus*

Carbohydrate	Enzyme activity(U/g)
Control	44.6
Lactose	63.3
Sucrose	15.3
Starch	17.2

3.3.2 Effect of Different Organic Nitrogen Sources on Alkaline Protease Production by A. flavus.

Enhancement of protease yield with organic nitrogen sources was done by 1 g of organic nitrogen source per 10 g of wheat bran. Experiments were conducted in duplicates and the final consensus was the average of 2 independent trials.

Effect of different organic nitrogen sources on alkaline protease production by *A. flavus* (Table 5) showed that the yeast extract increased the enzyme synthesis considerably. Casein had no effect and peptone reduced the synthesis considerably.

Table 5. Effect of different organic nitrogen sources on alkaline protease production by *A. flavus*

Organic nitrogen source	Enzyme activity(U/g)
Control	44.6
Yeast extract	65.3
Casein	46.0
Peptone	17.3

3.4 Mutagenesis

Mutagenesis with ultraviolet irradiation was carried out using U-V tube. As a strategy for strain improvement, mutagenesis was performed to have an increased yield of alkaline protease. UV irradiation was performed on the *A. flavus and B. subtilis.* It gave a good increase in the production of alkaline protease. Effect of UV irradiation on alkaline protease production by *B. subtilis* showed that the

bacterial cells (Table 6) and fungal cells (Table 7) showed increase in the ability to produce alkaline protease.

There was an increase in activity as the time of exposure increased to 8 minutes but further exposure did not lead to much enhancement of yield of protease. The activity increased 1.6 times in the case of B. subtilis and 1.3 times in the case of A. flavus.

Table 6. Effect of UV irradiation on alkaline protease production by *B. subtilis*

Time of exposure(in minutes)	Enzyme activity(U/ml)
2	1.3
4	1.4
6	2.13
8	2.40
10	2.46

Table 7. Effect of UV irradiation on alkaline protease production by *A. flavus*

Time of exposure(in minutes)	Enzyme activity(U/g)
2	54.3
4	55.6
6	57.2
8	59.33
10	58.8

3.5 Stability Studies

3.5.1 Effect of pH on activity of Alkaline Protease produced by A. flavus

The pH stability was checked by characterization assay done with different pH conditions -pH 12- Glycine-NaOH buffer

pH 10- Glycine-NaOH buffer

pH 9.2- Standard buffer

pH 7- Standard buffer

pH 6- Tris-HCl buffer.

In this case crude enzyme extract was incubated with different pH conditions at 37 °C for 30 minutes. Assaying was done by the usual assay procedure followed.

Effect of pH on alkaline protease production by *A. flavus* (Table 8) showed that the enzyme gave a satisfactory activity within a wide pH range, hence it can be used for both soaking (pH 5.5-10) and dehairing (pH 6-12) processes involved in the leather processing, though the activity was affected in extremely basic and acidic ranges.

Table 8. Effect of pH on alkaline protease production by *A. flavus*

pH	*Enzyme activity(U/g)*	*% Deactivation*
Control	22	0
6	16	72.7
7	14	63.3
9.2	33.3	151.3
10	20	90.9
12	21	95.4

3.5.2 Effect of Temperature on Alkaline Protease Produced by A. flavus

Temperature stability was checked by incubating the enzyme at different temperature. The temperatures were: 20 °C, 32°C, 45°C, and 70 °C. Incubation time for these temperatures was 30 minutes. Assay temperature was 32 °C.

Effect of temperature on alkaline protease produced by *A. flavus* (Table 9) showed that the enzyme was stable at 32°C and the activity was reduced at 45°C. The activity was considerably reduced at 70°C.

Table 9. Effect of temperature on alkaline protease produced by *A. flavus*

Temperature(°C)	*Enzyme activity(U/g)*	*% Deactivation*
Control	44.3	0
32	44	99.3
45	27	60.9
70	0.6	0.01

3.5.3 Effect of Various Chemicals on Activity of Alkaline Protease produced by A. flavus

The stability of the enzyme was checked by assaying the enzyme along with the chemicals used at the time of dehairing and bating. The chemicals used in the two processes were incubated with the enzyme and the assay was done to observe effects, if any on the enzyme activity. Percentage of deactivation for the enzyme in

the crude extract from *A. flavus* was calculated to check the above. The test includes enzyme solution, glycine-NaOH buffer (pH 9), chemical and substrate(casein). The control is assayed with double distilled water instead of the chemical.

There are various chemicals used while performing dehairing and bating in the leather industry. The test of various chemicals on alkaline protease production by *A. flavus* (Table 10) showed that all the chemicals, to some extent, suppressed the enzyme activity.

Table 10. Effect of various chemicals on alkaline protease produced by *A. flavus.*

Chemical or compound	Activity(U/g)
Lime 7%	44.66
Na_2S 3%	45.33
Ammonium Sulphate 95%	46.6
Kaolin 4%	42.8
Mixture(lime, Na_2S , Ammonium Sulphate)	36.66
Control	56.66

Table11. Percentage deactivation due to the effect of various chemicals on alkaline protease produced by *A. flavus*

Chemical / Compound	% Deactivation
Lime	78.82
Na_2S	80.01
Ammonium Sulphate	82.35
Kaolin	75.67
Mixture	64.70

3.6 Application in Tannery

Cost estimation.

Bacillus subtilis

- Salt solution
  1. Glucose (20g)- Rs. 4.2
  2. Peptone (35g)- Rs. 41.65
  3. $MgSO_4.7H_2O$- Rs. 0.50
  4. KH_2PO_4- Rs. 0.50
  5. $FeSO_4$- trace

  Total- Rs. 46.85
- pH tablets- Rs. 30
- NaOH pellets- Rs. 7.5
- Assay chemicals
  1. Glycine(7.5g)- Rs. 9.75
  2. Trichloroacetic acid(TCA)(12.5g)- Rs. 9.5
  3. Casein (10g)- Rs. 9

  Total- Rs. 28.25
- HCl- Rs. 6.5

Gross Total- Rs. 119.1

Aspergillus flavus

- Salt solution
  1. Nitrate (1g)- Re.1
  2. KCl (1g)- Re.1
  3. $MgSO_4.7H_2O$- Rs. 0.50
  4. KH_2PO_4- Rs. 0.50
  5. $FeSO_4.7H_2O/ZnSO_4.7H_2O$ - trace

  Total – Rs. 4
- pH tablets- Rs. 30
- NaOH pellets- Rs. 7.5
- HCl- Rs. 6.5
- Wheat bran (3 Kgs)- Rs 18
- Assay chemicals
  4. Glycine(7.5g)- Rs. 9.75
  5. Trichloroacetic acid(TCA)(12.5g)- Rs. 9.5
  6. Casein (10g)- Rs. 9

  Total- Rs. 28.25

Gross Total- Rs. 94.25

Table 12. Comparison of various strains

B. subtilis MTCC No.:1790	Glucose 0.5%(w/v), peptone 0.75%(w/v), and salt solution 5%(v/v)- [KH_2PO_4 0.5%(w/v), $MgSO4.7H_2O$ 0.5%(w/v), $FeSO_4.7H_2O$ 0.01%(w/v)] [1]	1.5 U/ml	1. 1-Kunamneni Adinarayana, Bezawada Jyothi, and Poluri Ellaiah Pharmaceutica Biotechnology Division, Department of Pharmaceutical Sciences, Andhra University, Visakhapatnam 530 003, India.
A. flavus MTCC No.:277	Wheat bran (20 g), $NaNO_3$ (2 g), 1 ml salt solution- KH_2PO_4 (1 g), $MgSO_4.7H_20$ (0.5 g), KCl (0.5 g), $FeSO_4.7H_20$ (trace), and $ZnSO_4$. $7H_20$ (trace) [2]	44.6 U/g of wheat bran	2. S. Malathi* and R. Chakraborty, "Production of Alkaline Protease by a New Aspergillus flavus Isolate under Solid-Substrate Fermentation Conditions for Use as a Depilation Agent", Applied and Environmental Microbiology Mar. (1991), p. 712-716.
B subtilis PE-11.	Glucose, 0.5% (wt/vol); peptone, 0.75% (wt/vol); and salt solution, 5% (vol/vol) ($MgSO_4.7H_2O$, 0.5% [wt/vol]; KH_2PO_4, 0.5% [wt/vol]; and $FeSO_4.7H_2O$, 0.01% [wt/vol])[3]	10.2 U/mg	3. Kunamneni Adinarayana,[1] Poluri Ellaiah,[1] and Davuluri Siva Prasad[2], "Purification and Partial Characterization of Thermostable Serine Alkaline Protease from a Newly Isolated Bacillus subtilis PE-11", August 19, 2003
B. pumulis BA-06	2.5% wheat bran, 2% alkali-extracted soybean meal, 0.3% yeast extract, 0.4% KH_2PO_4, 0.04% NaH_2PO_4 and 0.3% $CaCO_3$ [4]	1200 U/ml	4. H.Y. Wang1, D.M. Liu1, Y. Liu2 Screening and mutagenesis of a novel Bacillus pumilus strain producing alkaline protease for dehairing", College of Life Science, Sichuan University, Chengdu 610064, P.R. China
Thermus aquaticus Y T-1	0.4% polypeptone, 0.2% yeast extract and a basal salt mixture[5]	5.020 U/ml	5. Hiroshi MATSUZAWA, Kazuhis TOKUGAWA, Masaru HAMAOKI, "Purification and characterization of aqualysin I (a thermophilic alkaline serine protease) produced by *Thermus aquaticus* Y T-1", Department of Agricultural Chemistry, The University of Tokyo

CONCLUSION

In the present study an attempt was made to set up an economically feasible fermentation process for production of alkaline protease and to test its applicability in the local tannery. The strains *Aspergillus flavus* and *Bacillus subtilis* were chosen as the source for producing alkaline protease.

The mode of fermentation for production of alkaline protease was solid state in case of *A. flavus* and submerged phase in case of *B. subtilis*. *A. flavus* showed maximum activity (44.6 U/g) at fermentation temperature 32°C with wheat bran and salt solution as a media.

Solid state fermentation, with wheat bran, was adopted for growth of *A. flavus* which gave better results than the submerged state fermentation adopted in the case of *B. subtilis*. It was also noted that the loss of substrate was less by the use of wheat bran(coarser variety), probably because it did not form a compact mass depletion and permitted better air circulation, heat dissipation, and penetration by mycelia. Also, it was a better prospect economically since it is cheaper than the finer variety of wheat brans. We have attempted to combine the cheap bran extract with advantages of a solid state fermentation system with the capacity of the *A. flavus* to grow very rapidly and produce prolific amounts of extra cellular proteases such that the final crude enzyme product is available at a commercial price attractive to the tanners.

The production media in case of *A. flavus* was further enriched with rich organic nitrogen and carbohydrate sources which in turn gave an increased yield on addition of lactose and yeast extract. (63-65 U/g)

With the help of mutagenesis, *B. subtilis*, which was giving poor yield (1.5 U/ml), gave an improved result.

Mutagenesis, as a strategy for strain improvement, increased the activity of the enzyme in the crude enzyme extract from both the strains (*A. flavus*-59.33 U/g, *B. subtilis*-2.46 U/ml)

As the enzyme was being produced for dehairing process in the tanneries the effect of temperature, pH and various salts used during the dehairing process was also investigated.

The enzyme showed stability in the alkaline pH and at temperature of 32°C to 40°C. This gave an upper hand to use the enzyme not only for dehairing but for soaking also. In soaking the pH range is 5.5-10 for which our enzyme is stable. The enzyme

activity tested in presence of various reagents used in dehairing process showed a 64% deactivation. This could be due to the fact that the enzyme was not formulated with any stabilizers. However, the results after dehairing process showed promising results with the present protease.

The final outcome of the present study is that the UV mutagenized strain of *A. flavus,* was found to be a potential source for commercial production of alkaline protease. The alkaline protease from *A. flavus* was found to be stable under various pH and temperature ranges and fulfills all the requirements of the tannery industry that they seek in an industrial enzyme. Further improvements in yield by strain improvement with recombinant DNA technology or by improving fermentation process through media engineering would also help.

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