OPTIMIZATION OF CONDITIONS FOR ALPHA GALACTOSIDASE PRODUCTION FROM *Aspergillus terreus* FOR APPLICATION IN LEATHER INDUSTRY (FIBER OPENING)

PROJECT REPORT

Submitted by

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BONAFIDE CERTIFICATE

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PROJECT WORK -PHASE II

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This is to certify that the project entitled

OPTIMIZATION OF CONDITIONS FOR ALPHA GALACTOSIDASE PRODUCTION FROM *Aspergillus terreus* FOR APPLICATION IN LEATHER INDUSTRY (FIBER OPENING)

is the bonafide record of project work done by

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of M.Tech during the year 2011-2012.

Dr. R.Baskar **Dr. A. Manickam**

Submitted for the Project Viva-Voce examination held on .....

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Internal Examiner External Examiner

DECLARATION

I affirm that the project work titled '**Optimization of conditions for alpha galactosidase production from *Aspergillus terreus* for application in leather industry (Fiber Opening)'** being submitted in partial fulfillment for the award of M.Tech is the original work carried out by me. It has not formed the part of any other project work submitted for award of any degree or diploma, either in this or any other University.

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

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ABSTRACT

Production of alpha-galactosidase or melibiase (α-D-galactosidase galacto hydrolase, EC 3.2.1.22) by *Aspergillus terreus*, a filamentous fungus isolated from saline environment was investigated in SSF. There are several advantages in employing SSF processes over the conventional submerged fermentation, like higher yields of enzymes and the ease with which the enzyme can purified. The impact of different parameters on enzyme production, enzyme stability and enzyme activity were studied. Several production parameters like pH, temperature, substrate, salinity, incubation time, moisture content were optimized. Enzyme activity at various pH and temperatures were optimized along with optimal temperature for enzyme stability. Of all the parameters optimized highest enzyme activity was obtained for wheat bran at 120 hours incubated at 30ºC, 1:1 moisture content and 2.5% salinity. Maximum enzyme activity was obtained with salinity as the organism was isolated from saline environment. In order to make this process cost effective, the enzyme was produced from wheat bran, with optimized moisture content, 10 gm of substrate: 10 ml water (1:1). The optimum enzyme activity parameters were determined and the optimum temperature and pH were found to be 55˚C and 5 respectively. Histological analysis of the enzyme treated samples reveal complete removal of hair and epidermis with good opening up of fibre structures in the both dermis and corium. Moreover, the collagen is not damaged and resulting in a leather of good quality. The developed process has resulted in a remarkable reduction of effluent loads in terms of biochemical oxygen demand, chemical oxygen demand, total dissolved solids.

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1. INTRODUCTION

1.1 LEATHER INDUSTRY

Leather industry is one of the oldest industries in India. It involves the conversion of animal skin into end products useful for making leather goods such as shoes, bags, belts and clothing. Animal skin has to undergo a series of operations in the making of these leather goods. The conventional method involves a complex combination of mechanical and chemical processes. The heart of these processes is the tanning operation in which inorganic materials become chemically bound to the protein structure of the hide and preserve it from deterioration. The pre-tanning operations involve soaking, fleshing, dehairing and bating.

Despite making significant contribution to the economy, the leather industry causes severe environmental pollution owing to the conventional use of toxic chemicals like lime-sodium sulphide in the industry. The pollution is so much that it has become a common occurrence that tanneries are forced to close down not only in developed countries, but also in developing countries like India. The amount of pollution is significantly high in the pre-tanning operations compared to the post-tanning operations. Therefore attention of tanners is now focused towards revamping the processing methods to make leather processing eco-friendly. Intensive efforts are being directed towards using viable alternative technology for pre-tanning processes using enzymes**.**

1.2 CONVENTIONAL LEATHER PROCESS

The raw skin has to undergo a series of chemical treatments before it turns into leather. The principal leather making involves proteins, collagen exists in hides and skins in association with various globular proteins, viz. albumin, globulin, mucoids; fibrous proteins such as elastin, keratin, and reticulin. During leather processing, the non-collagenous constituents are removed partially or completely in the various pre-tanning operations like soaking, liming, dehairing, deliming, bating, degreasing, and pickling. The extent of removal of these constituents decides the characteristics of the final leather. (Kamini *et al.,* 2007)

Curing is the process of preserving the hides from getting spoiled. Addition of salt is the most prevalent method of preserving the raw skin and hides from slaughter industry.

Soaking of the raw hide is done in water. It leads to rehydration of the skin. Better the rehydration better superior the leather. Soaking cleans hides and skins by removing dirt, blood, flesh, grease and dung.

Liming of the skin and hides is carried out before dehairing, as it causes the swelling of collagen structures which helps to open up the fibers. In liming the skin is treated with milk of lime. The conventional and most widely used way to remove hair from hides is to use sodium sulphide. They dissolve the hair and open up the fibre structure. It depends largely upon the phenomenon of hair loosening. This loosening is due to the breakdown of the disulphide links of the amino acid cystine. (Choudry *et al.,* 2004)

Bating is the process of beating the leather cruelly with sudden heavy strokes using iron or wooden rods. The purpose of bating is loosening and peptization of non-collagenous skin structure through the removal of residues of interfibrillary proteins, epidermis and scuds. Bating makes the leather soft and supple and prepares them for tanning (Choudry *et al.,* 2004). It removes swelling and makes the grain surface of the finished leather clean, smooth and fine.

Deliming is carried out using acid salts like ammonium sulphate or ammonium chloride. It is carried out at a pH of 9-10. Pickling is the process of acidification of the skin using salts and sulphuric acid until it is at or near pH value of 3.0 to 3.5.

Tanning is the last stage of leather manufacturing. It is the process of converting the unstable raw hides into leather, with adequate strength properties and resistant to physical and biological attacks. The tanning process can either be vegetable tanning or chromium tanning. Vegetable tanning makes use of tannins from tree barks and it produces thick and heavy leather. The importance of vegetable tanning has decreased since chrome tanning was introduced which makes use of chrome salts and produces light, inexpensive and bacterial resistance leather. Tanning improves the color and appearance of the finished leather. The disadvantages of conventional processes are discharge of large amount of waste water with high organic, inorganic contents which forces us to restore more eco friendly methods like using enzymes in various pre-tanning and tanning operations.

1.3 ENZYMES IN LEATHER INDUSTRY

According to Thanikaivelan *et al.,*(2004), biotechnology has been used in the tanning industry for several years. Currently, the majority of enzymatic preparations for the tanning industry do not have sufficient specificity. The principal advantage of an enzymatic method is that it has a shorter wetting time, better fiber opening and solubility, removal of proteins, fat and carbohydrates. Enzymes can be used for dehairing processes on hides/skin for removal of residual components, removal/dispersion of adipose components, and reduction on effluent load. The commonly used enzymes for the process include proteases and lipases which remove the hair and the flesh present in the skin and hides. Enzymes like proteases, lipases and amylases have important role in soaking, dehairing, degreasing and bating operations of leather manufacturing. Proteases are the most commonly used enzymes in leather production. The criteria for selection of the best protease are that it should be non-collagenolytic and non-keratinolytic in nature. It has the property to hydrolyze casein, elastin, albumin and globulin like proteins. Lipases are used in degreasing operation to hydrolyze fat materials of skin/ hide flesh and greases/oils produced by the glands in the grain.

Amylases are also used in bating of animal skin/hide. Elastases act on elastin (a protein) in elastic body tissue such as the skin and internal membranes. Proteases act on protein such as hair, the epidermis and soluble proteins within the corium.

Proteases have applications in various steps of leather processing, e.g., neutral proteases in soaking (Deshpande *et al.,* 2004), alkaline proteases in dehairing (Dayananda *et al.,* 2003).The pollution causing chemicals, viz. lime, sodium sulphide , salt, solvents, etc arise mainly from the pre-tanning processes of leather industries. In order to overcome the hazards caused by the tannery effluents, use of enzymes as a viable alternative has been reported in pre-tanning operation such as soaking, dehairing, bating, degreasing and treatment. This review focuses on the use of microbial enzymes as a technology to the conventional methods, and highlights the importance of these enzymes in minimizing the pollution load.

1.4 LEATHER TANNING WITH ENZYMES

1.4.1 DEHARING AND BATING

Hides and skins have hair attached to them that must be removed for their use as leather. The conventional way to remove hair from hides is to use harsh chemicals such as lime and sodium sulfide. These chemicals completely dissolve the hair and open up the fiber structure. With enzyme-assisted dehairing, it is possible to reduce the chemical requirements and obtain a cleaner product and a higher area yield with fewer chemicals in the wastewater. Since the enzyme does not dissolve the hair as the chemicals do, it is possible to filter out the hair, thus reducing the chemical and biological oxygen demand of the wastewater. Additionally, the hides and skins contain proteins and fat between the collagen fibers that must be all or partially removed before the hides can be tanned. To make the leather pliable, it is necessary to subject the hide to an enzymatic treatment before tanning to selectively dissolve certain protein and polysaccharide components. This is called bating. Traditionally, dog or pigeon dung was used as the bating agent. This was a difficult, unreliable and foul smelling process. Since "dung bates" owed their softening effect to the action of different enzymes, during the 20th century, the leather industry has switched over to using bacterial enzymes and pancreatic enzymes.

1.4.2 DEGREASING OF LEATHER

Traditionally, the degreasing of sheepskins is done by solvent-extraction using paraffin solvent systems. A new process based on the enzymatic breakdown of fats by a lipase enzyme has been introduced to the leather industry. The enzymatic degreasing process replaces the solvent-based process. Since the enzyme interferes less with the skin structure, the enzymatic process also results in a product with improved quality, for example, improved tear strength and more uniform color.

1.4.3 ENVIRONMENTAL BENEFITS

- Lower the amount of waste that enters the environment, lowers odor during processing, replaces solvent-based system, lowers volatile organic chemical load.

1.4.4 Consumer Benefits: Better leather quality- high durability, increased tear strength and long life of the finished goods.

1.5 ALPHA GALACTOSIDASES

1.5.1 DESCRIPTION

Alpha -Galactosidase or melibiase (α-D-galactoside galacto hydrolase, EC 3.2.1.22) is an exo-galactoside that cleaves and catalyses the terminal non-reducing α-1→6-linked galactose residues from α-D-galactosides, including galacto-oligosaccharides such as melibiase, raffinose and stachyose, and branched polysacharrides such as galactomannans and galactoglucomannans. The suitability of a particular enzyme is determined by its characteristics. The thermo stability of the enzyme makes it desirable for biotechnological and medicinal applications. They play a crucial role in improving the nutritional value of legume based food (Prema *et al.,* 2007). They can be used for the removal of anti-nutritive oligosaccharides. They can be obtained from a variety of sources including plants, animals and microorganisms such as fungi, yeast, bacteria and actinomycetes.

1.5.2 ACTIVATORS, COFACTORS AND INHIBITORS:

No activators or cofactors are required for the complete activity of Alpha-galactosidase.

1.5.3 USAGE LEVEL:

Enzyme requirements are generally dictated by processing conditions. They will vary with substrate concentration, degree of desired hydrolysis, pH, temperature and time of hydrolysis.

1.5.4 APPLICATIONS:

- Food Industry: Beet Sugar and General
- Dietary Industry: Digestive Aids

1.5.5 STORAGE:

In sealed containers, under cool, dry conditions, the product will maintain the declared activity for atleast 12 months. Storage life can be extended by storing under refrigeration at 5˚C.

1.6 *Aspergillus terreus*

Aspergillus is a genus consisting of several hundred mold species found in various climates worldwide. *Aspergillus* was first catalogued in 1729 by the Italian priest and biologist Pier Antonio Micheli. Viewing the fungi under a microscope, Micheli was reminded of the shape of an aspergillum (holy water sprinkler), from Latin *spargere* (to sprinkle), and named the genus accordingly. Today "aspergillum" is also the name of an asexual spore-forming structure common to all Aspergilli; around one-third of species are also known to have a sexual stage. *Aspergillus* species are highly aerobic and are found in almost all oxygen rich environments, where they commonly grow as molds on the surface of a substrate, as a result of the high oxygen tension. Commonly, fungi grow on carbon-rich substrates such as monosaccharides (such as glucose) and polysaccharides (such as amylase).

Aspergillus species are common contaminants of starchy foods (such as bread and potatoes), and grow in or on many plants and trees. In addition to growth on carbon sources, many species of *Aspergillus* demonstrate oligotrophy where they are capable of growing in nutrient-depleted environments, or environments in which there is a complete lack of key nutrients. *A. niger* is a prime example of this; it can be found growing on damp walls, as a major component of mildew.

Aspergillus is the name used for a genus of moulds that reproduce only by asexual means. The morphology of the conidiophore, the structure that bears asexual spores, is the most important Taxonomic character used in *Aspergillus* taxonomy. *Aspergillus* species are common and widespread. They are among the most successful groups of moulds with important roles in natural ecosystems and the human economy (Bennett *et al.,* 2004).

Aspergillus terreus can cause invasive infections in humans, which are often refractory to therapy with the antifungal drug amphotericin B and have a propensity to dissemination. The organism has diverse colony morphology, and sub-typing studies have demonstrated that isolates of *A. terreus* are remarkably diverse in their genotypes. However, detailed phylogenetic studies of section Terrei employing sequence information from protein coding regions have not been thus far attempted. Interestingly, *A. terreus* produces unicellular forms called accessory conidia *in vitro* and during infection; the clinical relevance of these structures are not well understood.(Arunmozhi Balajee, 2009).

1.7 SOLID STATE FERMENTATION

In contrast to Submerged (liquid state) Fermentation, Solid State Fermentation (SSF) is the cultivation of micro organisms under controlled conditions in the absence of free water. Examples of products of Solid State Fermentation include industrial enzymes, fuels and nutrient enriched animal feeds. The application of modern biotechnical knowledge and process control technologies can lead to significant productivity increases from this ancient process.

1.7.1 Advantages of Solid State Fermentation over Submerged Fermentation

- Higher volumetric productivity
- Usually simpler with lower energy requirements
- Might be easier to meet aeration requirements
- Resembles the natural habitat of some fungi and bacteria
- Easier downstream processing

Solid State Fermentation (SSF) has gained importance for the production of microbial enzymes due to economic advantages over conventional submerged fermentation and due to the possibility of using cheap and abundant agro-industrial wastes as substrate. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source (Pandey *et al.,* 1999). There has been considerable interest to produce alpha-Galactosidase in SSF process among various groups of microorganisms.

Solid state fermentation stimulates the growth of microorganism in nature of optimum moisture level. Solid substrates has been credited to be responsible for the beginning of fermentation technique to an ancient time (Mitchell *et al*., 1990).Microorganisms utilize various substrate as the source of the nutrient for growth and metabolic activities. The microorganism secretes the necessary enzymes for the degradation of the available substrate molecule in order to meet their nutritional requirements (Tunga *et al*., 1999). The SSF process are usually simpler and can use waste or agro industrial substrate, such as defatted soygean cake, wheat bran, gram bran, banana waste etc, for enzyme production (Germano *et al*., 2003; Krishna *et al*.,1996;Kashyap *et al*.,2003)

In leather industry, the usage of α-Galactosidase reduces the COD, BOD and TDS loads in the effluents, thus making it environmental friendly. This study reports the optimized production of the enzyme α-galactosidase from the fungal source, *A. terreus* by solid state fermentation and its effect in the fiber opening (de-swelling) of the skin that had undergone conventional dehairing process.

2. OBJECTIVES

- To optimize the production of α-galactosidase from *Aspergillus terreus*
- Study the enzyme characteristics.
- Application of the enzyme for pre-tanning operation (Fiber opening) in leather industry.

3. REVIEW OF LITERATURE

3.1 LEATHER INDUSTRY

Leather production uses raw material in the form of cow and buffalo hides and goat and sheep skins and a number of chemicals such as sodium chloride, lime, sodium sulphide etc. Leather manufacturing involves following major steps:

- Pre-Process
- Pre-Tanning Process
- Tanning Processes
- Wet Finishing Process
- Dry Machining
- Finishing

3.1.1 PRE- PROCESSING

In pre-processing skins/hides are received from slaughter house and salt is applied on the flesh side of the skins/hides for preservation of the skin/hide. Skin trimming is done to remove unwanted flesh surrounding the skin/hide.

3.1.2 PRE- TANNING PROCESS

Pre-tanning process starts with the soaking in which skin are made flaccid by soaking them in water. After soaking, hair is removed using lime and sodium sulphide. Unwanted flesh is removed with the help of fleshing machines after liming process. To prepare limed skin for tanning, the skins are delimed using ammonium sulphate and then skins are washed. Bating is done for further softening of hide/skin. Pickling, the process of acidification is done for the bated skins/hides with sulphuric acid and salts to preserve the skins before tanning.

3.1.3 TANNING PROCESS

Tanning is the process of converting a degradable material in to a non degradable material. Chrome tanning uses chromium sulphate as tanning agent. Tanning process stabilizes the collagen network of skin. After tanning, skins are called wet blue and are stored for some time and then they are sorted out according to quality. If hides of cows or buffaloes are being used for leather manufacturing, then after this they are sliced to give desired thickness. This process is not carried out on the skins of goats or sheep. After this the hair side of the wet blue is shaved to give the desired thickness (Javad *et al*., 2000).

3.1.4 WET FINISHING PROCESS

In order to give desired softness, color, strength, and quality to the leather, wet blue skins are processed further through wet finishing process. Fat liquoring process is carried out to impart desired softness and dyeing is to give it a color.

3.1.5 DRY MACHINE PROCESS AND FINISHING

Drying processes are carried out to dry the processed leather. These processes consist of smaying/setting, vaccum drying, stacking/toggling, buffing/shaving, trimming, pressing, and segregation of the leather. Finally finishing processes are carried out to impart durability and beauty to the leather.

The chemicals used in the leather industry can be divided into three broad categories:

1. Pre-tanning Chemicals
2. Tanning Chemicals
3. Finishing Chemicals

Pre-tanning chemicals are used to clean and prepare skins for the tanning process and they are mostly washed away with the waste water. Tanning chemicals react with the collagen fibers of the skin to convert them into leather. These chemicals are retained in the skin, but a good amount of these is discharged into waste water. Chrome sulphate is the basic tanning chemical. Apart from being expensive, chrome sulphate is also a serious pollutant. Finishing chemicals are used to impart certain properties to the leather like softness, color, appearance etc. Like tanning chemicals, finishing chemicals also get discharged into wastewater. Only those chemicals are fully retained which are applied as surface coating. A large amount of

water is used in whole manufacturing process.

The collected data shows 50-150 litres water is used for conversion of one kg of raw skin into leather. In the tanning process, water is used as carrier to facilitate different chemical reactions and after completion of process, the water leaves the system as wastewater in the same quantity as added to the system. Ground water is mainly used as processing water (Javad *et al.,* 2000).

3.2 POLLUTION LOADS

Conventional methods of pre-tanning, tanning and post-tanning processes discharge enormous amount of pollutants, which cause 98% of the pollution load from tannery. Conventional leather processing employs 25-30 liters of water per kg of hide processed and subsequently discharges them as waste water along with pollutants.

The pre-tanning operation alone contributes to 70-80% of the pollution load. Conventional leather processing subjects the skin to wide variations in pH. Such changes in pH due to the use of acid and alkali lead to the generation of salts resulting in a net increase in the chemical oxygen demand (COD), total dissolved solids (TDS), chlorides and sulphates in the tannery wastes. The wide variations in the pH also impair the bulk as well as the surface properties of the hides/skin. Also toxic gases like hydrogen sulphide are evolved during the process (Rao *et al.,* 2007).

The soaking operation is the prime contributor to the high TDS in waste water which account for nearly 40% in tannery waste water. This is due to the common salt used in curing. The dehairing is one of the most polluting operations due to the use of sodium sulphite. Lime has the potential to drive swelling gradually but the formation of lime sludge is the major drawback. The role of salt in pickling is to reduce swelling, but it contributes to total dissolved solids (TDS). Post tanning employs a wide range of pH and contribute to heavy metal pollution (Rao *et al.,* 2007).

Solid wastes containing protein and fat that constitute more than 60% of rawhide weight are disposed to the environment by leather factories without turning them to good use (Qzgunay *et al.*, 2007).

All these pose a serious threat to the ecological balance and therefore more efforts are made to develop an eco-efficient rationalized leather process using enzymes.

3.3 ENZYMES

Enzymes are well known biocatalysts that perform a multitude of biochemical reactions and are commercially exploited in the detergent, food, pharmaceutical, diagnostics and fine chemical industries. The desire to use enzymes is their catalytic potential and high specificity (Jha *et al.,* 2004). More than 3000 different enzymes are described to date; the majority has been isolated from mesophilic organisms.

These enzymes mainly function in a narrow range of pH, temperature and ionic strength. Moreover, the technological application of enzymes under demanding industrial conditions makes the currently known enzymes not recommendable. Thus, the search for new microbial sources is a continual exercise, where one must respect biodiversity. The microorganisms from diverse and exotic environments called as extremophiles, are an important source of enzymes, whose specific properties are expected to result in novel process applications (Kumar & Takagi *et al.,* 1999).

Enzymes play an important role in effluent treatment, petroleum sludge degradation, crude oil spill treatment. Typical applications include dissolution of blot clots, mould release agent in building and construction, turbidity removal in beverages (Jha *et al.,* 2004).

3.4 ROLE OF ENZYMES IN LEATHER INDUSTRY

The enzymatic action in leather production was reported to have started at research level in the early of 20th century and the first pattern was taken by Rohm in 1910 for the use of enzymes in bating. It took more than seventy years to apply them on industrial scale. Later on enzymes were successfully employed for the better quality leather production and also for waste treatment in leather industry. (Jha *et al.,*2004).

Enzymes can also be used for dehairing processes, on epidermis, removal of residual components, removal/dispersion of adipose components, and reduction on effluent load Macedo *et al.,*(2005), studied the capacity of dehairing from a keratinase obtained from *Bacillus subtilis*. It was observed that the enzyme does not hydrolyze collagen and has efficient hair removal, maintaining the same conditions (pH, time) of traditional dehairing process, but without using sulphide.(Dayanandan *et al.*, 2003) studied the dehairing of hides using alkaline protease, isolated from *Aspergillus tamarii*. The physical proprieties of the experimental leather in comparison with the control sets (traditional process) gave better results with respect to tensile strength and elongation at break.

Three methods of application are commonly used in the enzymatic dehairing process: (i) paint method, (ii) dip method, and (iii) 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 dehairing, 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. 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 (Kamini *et al.,* 2007). CLRI has developed a potent fungal lipase from *A. niger* and a potent bacterial lipase. 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 is obtained with the bacterial lipase and has added advantage of better softness, smoothness, and improvement in other physical properties. Furthermore, the lipase without detergent is observed to show 70% degreasing in 2 hours, with the effluent showing minimal pollution load (Kamini *et al.,* 2007).

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. 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.

During the bating process, enzymes can act on removal of degraded hairs and epidermis, promote the removal of non structural proteins and help on carbohydrates removal. 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*.

Trabitzch(1966) 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. 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. 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. (Kanth *et al.*, 2008) studied the application of a bacterial collagenase in leather dyeing. They obtained results, showing dye uptake as high as 99% by treatment with collagenase. Using the conventional process, the exhaustion of dyes was found to be 85%. The utilization of enzymes improved the softness of leather, while the strength characteristics are not significantly altered.

Enzymatic treatment can also be used on wastes generated during the leather process. The untanned wastes can be used upon by proteolyses enzymes, neutral and alkaline,

generating hydrolysates rich in fat and proteins, in temperature about 50°C. To tanned wastes, containing chromium, can be obtained three fractions: the cake containing chromium, proteins and hydrolysated collagen. Kumar *et al.,* (2008) studied the obtaining of an alkaline protease from *Pseudomonas aeruginosa* using proteinaceous wastes from tanneries. The authors say that the microbiological method to hydrolysate proteinaceous waste is an interesting alternative to other methods, like chemical and thermal, used these days for treatment of solid wastes.

Enzymes were used during soaking and liming operations. Additionally, a study was done on the bacterial decomposition of tanned leather wastes and the possibility to remove chromium contained in these wastes, using the bacteria, *Pseudomonas aeruginosa.* According to Jana *et al.,*(2004) alkaline and pancreatic proteases were used to remove non-fibrillary proteins during soaking. Neutral proteases are using in dehairing to remove the hair. Trypsin is used to make the leather soft and pliable in bating.

Degreasing is done with the help of lipases. In 1966, Trabitzsch described the potential for lipases in degreasing skins. They 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.,*(2004) used a fungal lipase for the degreasing of woolly sheep skins, pH range of 3.2–3.6 at 37°C for 1h. Subsequently, (Yeshoda *et al.,* 1999) observed that degreasing and bating could be carried out simultaneously in the pH range of 7.8–8.0. An acid lipase from *Rhizopusnodosus* has been noticed to be very effective in the degreasing of sheep skins.

Zhang reported use of alkaline lipase in combination with the proteinase and pancreatin in softening pig skin to improve the degreasing effect. Pfleiderer *et al*. (1999) carried out degreasing of hides by soaking in an acidic bath containing a proteolytic enzyme (0.01–3.0%), and a non-ionic 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.

Tryptec enzyme preparations are used in the treatment and recovery of chrome shavings used in the process of chrome tanning. Fiber opening process removes all the interfibrous materials especially proteoglycans and produces a system of fibers and fibrils of collagen which are clean. Sodium metasilicate was chosen to open the fiber bundles that were dehaired using enzyme based dehairing method and the extent of fiber opening was analyzed histology studies.

3.5 GALACTOSIDASES

Galactosidase is an enzyme that catalyzes the cleavage of terminal galactose residues from a variety of into monosaccharide's. It occurs in two forms: α-galactosidase (melibiose) and β-galactosidase (lactase).

3.6 ALPHA GALACTOSIDASE

Alpha galactosidase or melibiose (EC3.2.1.22) is an exogalactosidase that cleaves the terminal non-reducing α-1→6-linked galactose residues from α-D-galactosides, including galactose oligosaccharides such as melibiose, raffinose and stachyose and branched polysaccharides such as galactomannans and galactoglucomannans**.** They also act on glycoconjugates, glycoproteins and glycospingolipids. They are also known to catalyse transgalactosidases reactions especially at high substrate concentrations. They have potential technological and industrial applications.

3.6.1 SOURCES

Alpha galactosidase exists in various organisms like plants, animal and micro-organisms like fungi and bacteria. Mangrove actinomycetes have immense potential as a source of exo-enzymes. (Prema *et al.,*2007). A-galactosidases have been recently isolated from hemophilic and hyperthermophilic organisms (Immamura *et al.,* 2003; Mattess *et al.,* 2001). *Streptomucus greciolalbus* produces α-galactosidase through submerged fermentation (Prema *et al.,* 2007)

3.6.1.1 PLANT ALPHA GALACTOSIDASES

It can be derived from various parts of plant i.e., a fruit, a leaf, a seed, a stalk, a root, and a flower.The plant Galacto-ligosaccharides (GO) is responsible for intestinal disturbances. Enzymatic reduction of GO level in this product is highly undesirable to improve their acceptance. For this purpose, plant semi purified Alpha-galactosidases were used for GO hydrolysis in soybean flour and soy molasses. Alpha-Galactosidase from soybean germinating seeds presented maximal activity at pH 4.0-5.0 and 45-65^{0}C. The enzyme was completely inhibited by Ag^+ and Hg^{2+}, whereas only soybean enzyme was inhibited by galactose (Daniel *et al.,* 2006).

Alpha-galactosidases can be classified into two broad groups, acid or alkaline, according to the pH at which they show optimal activity (Keller & Pharr, 1996). Alpha-galactosidase shows proffered activity to melibiase.

The galactosyl sucrose sugars, stachyose and raffinose together with sucrose are primary translocated sugars in the phloem of cucurbits. The low concentrations of raffinose and stachyose in fruit tissues of muskmelon suggest that galactosyl sucrose unloded from phloem is rapidly metabolized, with initial hydrolyses by Alpha galactosidase as described in "cucurbits" (Schaffer *et al.,* 1996).

3.6.1.2 MICROBIAL ALPHA GALACTOSIDASES

Alpha- galactosidase occurs widely in microorganisms. However, despite the wide occurrence of this enzyme, only a small number have been purified and extensively studied. As might be expected, research in this area has centered on sources of Alpha-galactosidase that offer the greatest economic potential. Filamentous fungi have been extensively employed on an industrial scale for many decades in the production of a variety of enzymes. Alpha-galactosidase is among glycosyl hydrolyses (EC .3.2.1 – 3.2.3) that are a widespread group of enzymes hydrolyzing the glycosidic bonds between two carbohydrate residues (Murphy and Power, 2002). Microorganisms have the advantage of high production yields, and among them, fungal galactosidase are the most suitable for technological applications mainly due to their extracellular localization, acidic optima, and broad stability profiles.

The presence of this enzyme has been reported in *bifid bacterium* genera, whereas in lactic acid bacteria, it is produced only by some strains of *Lactobacillus, Leuconostoc,* and *Pediococcus.* The method developed to study α-gal from several sources is based on the absorbance measurement at 400 nm of the *p*-nitro phenol (PNP) released by the action of the enzyme (α-gal) upon its specific substrate, *p*-nitro phenyl-α-D-galactosidase (PNPG).

P-nitro phenyl-α-D-galactosidase + H_2O → D-galactose + *p*-nitro phenol

Bacterial enzyme producing species include *L.fumigatus, Bacillus stearothermophilus*and the fungal sources include *Aspergillus Niger, Aspergillus fumigatus, and Hum cola sp.* Among the various sources of the enzyme, the filamentous fungi has been the most exploited source, because of their ability to grow on complete solid substrate and production of extracellular enzyme (Krishna *et al.,* 2005).

The fungal species used here is *A. terreus* belonging to the genus Aspergillus and sub-genus Nidulantes and can be identified in the laboratory by morphological methods. Members of this species are diverse in their colonial morphology, and can grow as bright orange colonies to colonies that appear as various shades of cinnamon brown. On Capek Dox agar, *A. terreus* grows rapidly with very variable colony appearance ranging from heavily sporulating colonies to fluffy, poorly sporulating phenotypes.

Microscopically, conidiophores are typically long, columnar, colorless (hyaline) and smooth giving rise to sub-spherical vesicles that are biseriate. Conidia range in size from 1.5-2.4 mm in diameter, are smooth walled, globulus to slightly elliptical and striate. In addition, *A. terreus* produces heavy walled hyaline cells laterally on the hyphae, which are called accessory conidia or are sometimes referred to as aleurioconidia.

A. terreus is the only member of the genus Aspergillus that produces such structures. These accessory conidia can be produced singly or in clusters in submerged vegetative mycelium in vitro and have been observed in vivo during infection. Identification of accessory conidia in tissues of infected patients is a strong indication that the infecting organism is *A. terreus*. This facilitates early delineation of this organism from other *Aspergilli*, as well as from Fusarium, Paecilomyces and Acremonium species.

3.6.2 PRODUCTION OF ENZYME

The production of the enzyme can be carried out either submerged fermentation or solid state fermentation. The production and characterization of the enzyme is of economical importance.

SSF (Solid state fermentation) is defined as the fermentation involving solids in absence (or near absence) of free water; however, substrate must possess enough moisture to support metabolism and growth. (Pandey *et al.*,1994; Pandey *et al.,* 2000; *Pandey et al.,* 2001). Solid State Fermentation (SSF) stimulates the growth of microorganism in nature of optimum moisture level. Solids substrates have been credited to be responsible for the beginning of fermentation technique to an ancient time (Mitchell *et al.,* 1990). Microorganisms utilize various substrates as the source of the nutrient for the growth and metabolic activities. In SSF, the microorganism secretes the necessary enzymes for the degradation of the available substrate molecule in order to meet their nutritional requirements (Tunga *et al*., 1999). The SSF process are usually simpler and can use waste or agro industrial substrate, such as defatted soybean cake, wheat bran, gram bran, banana waste etc, for enzyme production (German *et al.,* 2003; Krishna *et al*., 1996; Kashyap *et al.,* 2003). In Solid state fermentation the solid material is insoluble that acts as both physical support and source of nutrients. SSF holds tremendous potential for the production of the enzyme. (Chalal *et al.,* 1985; Kotwala *et al.,* 1997; Wang *et al.,* 2007)

3.6.2.1 ADVANTAGES OF SOLID STATE FERMENTATION

SSF appears to possess several biotechnological advantages though at present on a laboratory scale only,

- Higher fermentation productivity
- Higher end-concentration of products
- Higher product stability
- Lower catabolic repression cultivation of microorganisms specialized for water-insoluble substrates or mixed cultivation of various fungi
- Lower demand on sterility due to the low water activity used in SSF.

Here, wheat bran is used as solid substrate and also as the support medium .Wheat bran refers to the tough outer shell of wheat that is often removed during processing.

TABLE 3.1 NUTRITIONAL VALUE OF WHEAT BRAN

CONTENT	AMOUNT IN g PER 100 g OF WHEAT
WATER	9.89 g
ENERGY	216 kcal
FAT	4.25 g
PROTEIN	15.55 g
CARBOHYDRATES	64.51 g
FIBER	42.8 g
SODIUM	2 mg
POTASSIUM	1182 mg
MEGNESIUM	1082 mg
VIT B_3	13.5 mg
VIT B_6	10103mg
VIT E	2,320 mg
MANGANESE	611 mgs

3.6.3 APPLICATION OF α GALACTOSIDASE

The enzyme that has been produced through various sources has been characterized. The enzyme that was produced by *A.terreus* GR was found to be 108 kD a protein which is on par with the galactosidase from white rot fungus. The molecular weight was determined using native and SDS PAGE. α-Galactosidase from *A. terreus GR* is thermos table, as it was not inactivated by heating at 65 °C for 40 min and also it has higher temperature optima and pH stabilities (Shankar *et al.,* 2008). α-galactosidase has a number of commercial applications such as processing of food products, animal feed processing, pulp and paper industry, sugar producing industry and medicine.

3.6.3.1. FOOD INDUSTRY

Because of lack of α-galactosidase in the human and animal intestinal track, the microbial flora metabolises raffinose family sugars in soya bean and leguminous plant seeds to induce gastric distress. The application of alpha galactosidase is to hydrolyze these compounds prior to ingestion which helps in the use of legumes as supplement with low sugar diets. This also helps to remove the flatulence (Soccol *et al.,* 2009)

Nutritional studies using commercial preparations of α galactosidase as supplements in the feed of monogastric animals demonstrate increase weight profit along with improved digestibility in swine and chickens. (Pandey *et al.,* 2007). Immobilized in calcium alginate/gelatin, α galactosidase can be used in the hydrolysis on soya bean milk components which causes flatulence (Mulimaniet *al.,* 2010). They have reported that it has reduced the raffinose and stachyose content by 90% and 92% respectively.

In sugar producing industry sucrose yield can be improved by α-galactosidase mediated elimination of raffinose and prevention of sugar beet crystallization (Linden *et al*., 1982).

3.3.6.2 PAPER AND PULP INDUSTRY

The enzyme α-galactosidase has got interesting application in pulp and paper industry, where biobleaching can be improved by adding the enzyme alpha galactosidase along with xylanase (Ramalingal *et al*., 1995)

3.3.6.3 MEDICINE

In the medical field, some α-galactosidases are able to cleave off terminal alpha 1,3 – linked D-galactosyl residues from blood B-type cell surface glycoprotein, thus causing the conversion of B-type blood to O-type (Goldstein and others 1982).Alpha galactosidases which are able to hydrolyse terminal glycolipid alphas-galactosyl residues might be used in the treatment of Fabry disease (Utsumia *et al.,* 2005) which is am X-linked error in glycospingolipid metabolism resulting from mutations in α-galactosidase A gene.

4. MATERIALS AND METHODS

4.1 SOURCE

The source of the enzyme Aspergillus *terreus* was isolated from marine sample collected from East Coast of India in the region of Tamil Nadu, The lyophilized culture was retrieved on Czapek Dox agar plates. The inoculum was obtained from the Czapek Dox agar slants. The slants were disturbed using sterile loop by the addition of water to get the spore water (inoculum).

4.2 CULTIVATION

The organism was grown on 250 ml Erlenmeyer flasks. 10% inoculum was added to 10g of substrate and the parameters such as moisture, incubation period, salinity, pH, temperature were the subjected for optimization**.**

4.3 ENZYME EXTRACTION

The enzyme was extracted by grinding 1g of mouldy substarte (along with the spores) in a mortar and pestle using about 10ml of acetate buffer (pH 5.0). Then it was filtered using a filter cloth and the filtrate was centrifuged at 10,000 rpm. The supernatant was collected and used as the enzyme extract (crude enzyme) .

4.4 ESTIMATION OF ALPHA GALACTOSIDASE

4.4.1 Materials required:

1.) Substrate - 2 mM para-nitro phenol-α-D-galactopyronoside (PNPG) prepared by dissolving 50 mg in 50 ml of acetate buffer ph-5.

2.) Enzyme - 0.1 ml of the crude extracted enzyme was taken and made up to 1ml by addition of water. (Dilution factor =10).

3.) 0.2 M sodium carbonate-2.12g of sodium carbonate was weighed and dissolved in 100ml of distilled water.

4.5 ASSAY PROTOCOL

Enzyme activity of the crude extract was determined by galactosidase assay (Garroa *et al.,* 2004) of Dey and Pridham method. Assay was carried out in test tubes.Substrate PNPG (0.8.ml) was added to test and control, The diluted enzyme was used for the assay by taking 0.1ml of the supernatant and adding 0.9 ml of water (10 times dilution), from which 0.1ml of enzyme was added to the test sample. Incubate at 55^{o}C for 15min in water bath. The reaction was stopped by adding Na_2CO_3 (3ml) in test and control which inhibits the activity of enzyme and then 0.1ml of enzyme was added to the control. Then the samples were read spectrophotometrically at 405nm. One enzyme unit (U) was defined as the amount of enzyme that released 0.1ml of PNP from its substrate PNPG per min under the given assay conditions. The results were expressed as U/g of substrate. O.D of the standard graph for PNP was 0.600 O.D at 405 nm.

TABLE 4.1 Estimation of Alpha Galactosidase

Reagents	TEST	Control
Substrate (PNPG)	0.8 ml	0.8 ml
Enzyme	0.1 ml	
----------	Incubate at 50 C for 15 min	----------
Sodium carbonate	3ml	3ml
Enzyme	---	0.1ml
Total volume	3.9 ml	3.9 ml

Absorbance was measured at 405nm.

Enzyme activity =

(O.D. of unknown) (Concentration of known) (Reaction volume) (D.F) (enzyme volume)

---------- ---------- ---------- -------

(O.D of Known) (Incubation time) (Volume of enzyme for assay) (g of substrate)

4.6 OPTIMIZATION OF ENZYME PRODUCTION

4.6.1 OPTIMIZATION OF SUBSTRATES

The nature of substrates employed in microbial enzyme production by Solid state fermentation is one of the most important factors. Substrate for SSF must me easily available, must have desired nutrition level for the organism to grow and must be economic. Hence, the organism was grown on various substrates like wheat bran, gingely oil cake, coconut oil cake and groundnut oil cake. All the above substrates used are a form of waste from wheat and oil mills and costs Rs 10-20/kg. The growth was assayed for every 24 hours, until the activity of the enzyme decreases.

4.6.2 OPTIMIZATION OF INCUBATION PERIOD

The organism was grown on a wheat bran and the assay was performed every 24 hours until a depletion in growth was observed to determine the maximum growth.

.4.6.3 OPTIMIZATION OF MOISTURE

Moisture percentage optimization was carried out to ascertain the effect of increasing moisture percentage on the growth of organism (measured through maximum enzyme activity).

Procedure:

- Six 250ml Erlenmeyer flasks were washed thoroughly and were kept in hot air oven for drying.
- The dried flasks were labeled 1 to 6 and 10g of wheat bran was added to each flask.
- To flask 1, 5ml water was added to 10 g of wheat bran.(wheat bran : water:: 1:0.5)
- Similarly to flask 2, 3, 4, 5 and 6(1:1.1:1.5, 1: 2, 1:2.5 and 1:3) were prepared respectively.
- All the flasks were inoculated using 1ml of spore water.
- The flasks were kept at 30◦C and assay was performed for every 24 hours.

4.6.4 OPTIMIZATION OF pH

The organism was grown with different pH using buffers ranging from 3-10 with acetate, phosphate and carbonate buffers accordingly, along with optimal saline content and moisture and temperature, the activity was assayed for every 24 hours.

4.6.5 OPTIMIZATION OF TEMPERATURE

The samples were incubated at different temperatures (25, 27, 30^{o}C), to optimize the temperature required for enzymatic production and growth with optimal moisture content and salinity.

4.6.6 OPTIMIZATION OF SALINE CONTENT

Cultivation was carried out with water differing in salt (NaCl) concentration (1.5, 2.5, 3.5, 5%) at optimal moisture content and the enzyme activity was assayed for every 24 hours to study the effect of salinity in the production of α-galactosidase.

4.7 OPTMIZATION OF ENZYME ACTIVITY

4.7.1 OPTIMIZATION OF THERMOSTABILITY

The enzyme added to substrate (PNPG) is incubated at various temperatures (25, 30, 40, 50, 55, 65^{0}C) for test and control respectively to determine the optimal temperature for enzyme activity.

4.7 2 OPTIMIZATION OF pH

The substrate (PNPG) is dissolved in acetate buffer to maintain the pH at 3, 4, 5, 6, in phosphate buffer for pH 7, 8 and carbonate buffer to maintain at pH 9 and 10. The above samples were kept at the optimum temperature obtained from 4.7.1.

4.8 CHARACTERIZATION OF ALPHA GALACTOSIDASE BY SDS-PAGE

Molecular weight and protein profile of α – galactosidase was determined by 10% SDS-PAGE. 8% stacking gel and 10% separating gel was used to caste the gel. About 10µl of crude enzyme was electrophoresed in the polyacrylamide slab gel under denaturing conditions in the presence of standard molecular weight marker (29KD to 205KD).The proteins were detected by Coomassie Brilliant Blue- 250.

4.9 ENZYMATIC FIBRE OPENING USING α-GALACTOSIDASE

The raw goat skin (back bone) about 500g was collected from slaughter house. The pre-fibre opening stages in leather process dehairing were carried out by using enzymatic procedure. The 300g of dehaired skin was treated with 100ml of crude enzyme (α-galactosidase) and about 250ml of distilled water was added to it and allowed to run in a rotary shaker for an hour. Then a sample piece of batted leather was taken and stored in 10 % formalin after which it was studied using histological methods.

The purpose of bating (fibre opening) is for loosening and peptization of non-collagenous skin structure through the removal of residues of interfibrillary proteins (galactosyl- residues found between the collagen fibres). This makes the leather soft and supple and prepares them for tanning and it removes swelling and makes the grain surface of the finished leather clean, smooth and fine.

4.10 CONFIRMATION OF FIBRE OPENING USING CARBOHYDRATE ASSAY

After fibre opening, the fibre opened solution was added in the range of 0.05-3 ml, to this 3ml of DNS (dinitrosalicyclic acid) was added and kept at 95ºC boiling water bath until the colour changes. To this 1ml of Rochelle salt (sodium potassium tartarate) was added and the absorbance was read at 575nm spectophotometrically.

4.11 EFFECT OF INHIBITORS ON ENZYME ACTIVITY

The effects of the inhibitors were studied by adding 200 µl of inhibitors (urea, EDTA, β-mercaptoethanol, bromosuccinimide) to 1.0 ml of the diluted enzyme at final concentration of 10mM. The mixture was incubated for 30 min and after which 0.1 ml of the diluted enzyme from the incubated mixture was taken and the assay was performed by Dey and Pridham method.

4.12 EFFECT OF METAL IONS ON ENZYME ACTIVITY

The effects of metal ions were studied by adding 200 µl of metal ions (Ag^{2+} ,Ca^{2+}, Cu^{2+}, Hg^{2+}, Cl^-) to 1.0 ml of the enzyme at final concentration of 10mM. The mixture was incubated for 30 min and after which 0.1 ml of the enzyme from the incubated mixture was taken and the assay was performed by Dey and Pridham method.

5. RESULTS AND DISCUSSION

The project was aimed at the production of alpha galactosidase from *Aspergillus terreus* by using Solid State Fermentation, optimization of production parameters, study of the enzyme characteristics and applying alpha galactosidase for pre-tanning operation of fiber opening. The confirmation of fibre opening was determined by Histological reports and Carbohydrate assay. The maximum activity exhibited by alpha galactosidase was determined in all the optimized parameters.

The use of microbial enzyme in leather industry was focused at reducing the use of chemical waste which is obtained in and as effluent. Various factors were optimized for the production of Alpha galactosidase in cheaper and efficient source and materials.

5.1 RETRIEVATION OF ASPERGILLUS TERREUS FROM MOTHER CULTURE

The organism *Aspergillus terreus* was obtained from Central Leather Research Institute in the Department of Biotechnology which was retrieved by producing sub culture in the form of slants. The slants were produced using Zapak dox agar and the inoculums was streaked and incubated at 30ºC for about 5 to 6 days. After incubation the slants were observed white with moldy *Aspergillus terreus* (fig.5.1).

(a) **(b)**

Figure 5.1 (a) Slant culture of Aspergillus terreus (b) Plate culture of Aspergillus terreus.

5.2 CULTIVATION OF ASPERGILLUS TERREUS

The organism was grown on 250 ml Erlenmeyer flasks u by adding 10% inoculum to 10g of substrate and the parameters such as moisture, incubation period, salinity, pH, temperature were optimized**.** The flask containing fully grown *Aspergillus terreus* was observed after 120th hour (fig.2)

(a) **(b)**

Figure 5.2 (a) 10g of wheat bran (b) 120th hour culture of Aspergillus terreus

5.3 EXTRACTION OF ALPHA GALACTOSIDASE

The enzyme was extracted by grinding 1g of mouldy substrate (along with the spores) in a mortar and pestle using about 10ml of acetate buffer (pH 5.0). Then it was filtered using a filter cloth and the filtrate collected was used as the enzyme extract.

5.4 STANDARDIZATION OF PNP

1mM stock of para nitro phenol was prepared and it was serialy diluted to get different concentrations say 5-50µmol. The absorbance was measured at 405 nm and the readings were tabulated.

TABLE 5.1 Standard concentration of PNP Vs O.D at 405

S.No	Concentration of PNP µmol	OD at 405
1	5	0.144
2	10	0.195
3	15	0.266
4	20	0.388
5	25	0.496
6	30	0.616
7	35	0.658
8	40	0.835
9	45	0.913
10	50	1.063

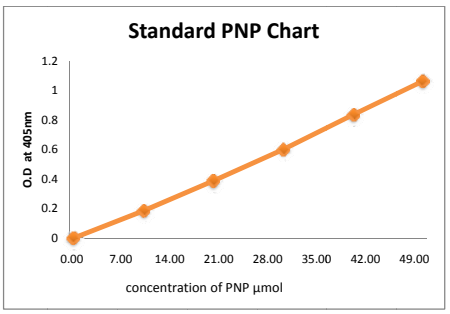


FIGURE 5. 3 Standard graph showing the variation in colour change of PNP with respect to concentration

5.5 ESTIMATION OF ALPHA GALACTOSIDASE

The crude enzyme was estimated using PNPG (Para Nitro Phenol α-D-Galactopyronoside) as substrate and the reaction was determined by the change in colour which was measured at 405nm spectrophoyometrically (fig.4).The maximum enzyme activity was calculated.

P-nitro phenyl-α-D-galactosidase + H_2O → D-galactose + *p*-nitro phenol

Figure 5.4 Reaction mixture showing Test and Control

Enzyme activity =

(O.D. of unknown) (Concentration of known) (Reaction volume) (D.F) (enzyme volume)

(O.D of Known) (Incubation time) (Volume of enzyme for assay) (g of substrate **ENZYME ACTIVITY= U/g of substrate**

5.6 OPTIMIZATION OF ENZYME PRODUCTION

5.6.1 OPTIMIZATION OF SUBSTRATE

The organism was grown on various substrates like wheat bran, gingely oil cake, coconut oil cake and groundnut oil cake and assay for enzyme activity was performed for every 24 hour. The maximum enzyme activity was observed when wheat bran was used as substrate (20560±253.46 U/g of substrate). A reduction in the enzyme activity was found when substrate other than wheat bran was used. Thus, further studies were carried out using wheat bran as substrate.

TABLE 5.2 Effect of the solid substrates on alpha galactosidase production within conditions: (10g of substrate, 10%inoculum, 50% moisture content, temperature at 30°C)

	Enzyme Activity in (U/g) with increasing incubation					
Substrate	24	48	72	96	120	144
Wheat bran	260	7124	11440	15340	20560	14508
Gingley oil cake	104	416	598	1014	1274	845
Groundnut oil cake	1157	1352	3159	4758	6370	3068
Coconut oil cake	13	52	130	156	325	143

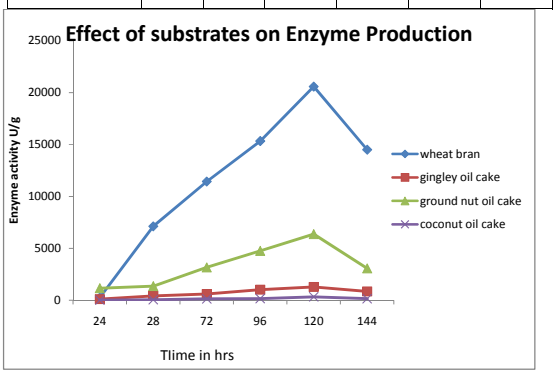


Figure 5.5 Graphical representation of effect of substrate on growth and alpha galactosidase production.

5.6.2 OPTIMIZATION OF INCUBATION PERIOD

The incubation period is governed by characteristics of the culture and is based on growth rate and the enzyme production. The incubation period for the alpha galactosidase production using wheat bran is given in the Table 5.3 and Fig. 5.6 showed that the optimal incubation time for alpha galactosidase was at 120th hour (21578±244.8 U/g of substrate). The reduction in the enzyme production was observed at 144th hour. Based on these results, incubation period at 120th hour was used for the further studies

TABLE 5.3 The effect of incubation period on alpha galactosidase production within conditions :(10g of wheat bran, 10%inoculum, 50% moisture content, temperature at 30°c)

	Enzyme activity in (U/g) with increasing incubation period					
Time (hrs)	24	48	72	96	120	144
Enzyme activity(U/g of substrate)	195	7280	11960	15977	21578	14027

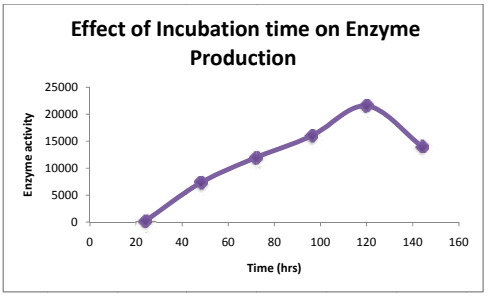


Figure 5.6 Graphical representation of effect of incubation period on alpha galactosidase production

5.6.3 OPTIMIZATION OF MOISTURE CONTENT

Initial moisture content is a critical factor in SSF since the moisture of the medium determines the microbial growth and the product yield. The table 5.4 shows that the optimal moisture level for alpha galactosidase was at 50% with (21554±200 U/g of substrate). The moisture content for the growth and substrate utilization depends on the organisms and the substrate used for the cultivation. A reduction in the enzyme production was observed at high moisture level and also below 1:1 (wheat bran: water) moisture content as shown in table. Based on these results, the moisture level at 50% was used for the further studies.

TABLE 5.4 The effect of moisture content on alpha galactosidase production within conditions (10 g of wheat bran, 10%inoculum)

Moisture	Enzyme activity in (U/g) with increasing incubation period					
	24	48	72	96	120	144
1:0.5	91	1560	5590	7930	7280	3640
1:1	195	7293	11963	15927	21554	140232
1:1.5	130	4186	5785	6643	10218	8658
1:2	78	2496	2912	3419	6201	4589
1:2.5	78	2132	2496	3068	4849	3965
1:3	52	1716	2249	2587	3224	2561

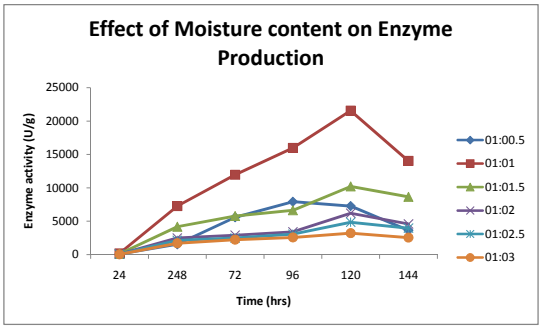


Figure 5.7 Graphical representation of effect of moisture content on alpha galactosidase production

5.6.4 OPTIMIZATION OF pH

The optimization of pH of the enzyme is shown in Table 5.5 and Fig 5.8. pH range between 3 and 10 was used to study the effect of pH on alpha galactosidase activity. From pH 3.0 (91U/g at 24 hours) enzyme activity increases linearly upto pH 6 after which it decreases. Maximum enzyme activity was observed at pH 6(11455±93.98 U/g of substrate) at 120th hour. This is found to be much lower than that of water. Therefore, water was used for further experiments.

TABLE 5.5 The Effect of pH on alpha galactosidase activity within conditions :(10g of wheat bran, 10%inoculum, 50% moisture content and temperature at 30 °c)

Enzyme activity in U/g with increasing incubation period						
pH	24	48	72	96	120	144
3	91	2652	6292	7605	1274	1274
4	65	2964	2730	2249	819	449
5	52	520	2652	1937	546	65
6	234	3926	6370	8450	11455	9334
7	52	2444	2821	1586	338	338
8	26	1456	2691	1391	143	559
9	26	858	1807	1417	91	364
10	26	585	1547	1274	52	546

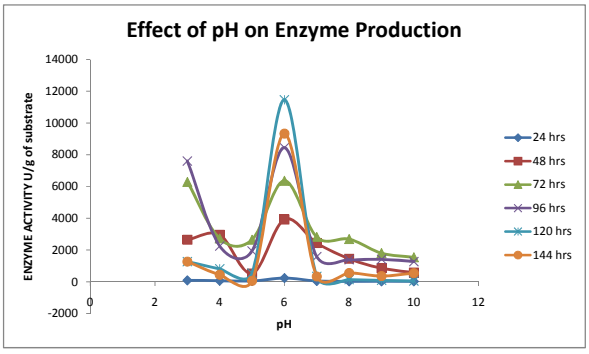


Figure 5.8 Graphical representation of effect of pH on alphagalactosidase production

5.6.5 OPTMIZATION OF TEMPERATURE

Alpha galactosidase activity was assayed at different temperatures 25ºC, 30ºC and 37ºC. The maximum enzyme activity was observed at 30ºC (21158±215.13 U/g of substrate). A reduction in enzyme activity was found above or below 30ºC. Based on these results, the temperature at 30ºC was used for the further studies.

TABLE 5.6 The Effect Of Temperature of the solid substrate on alpha galactosidase production within conditions :(10g of wheat bran, 10%inoculum, 50% moisture content, incubation till 144th hour)

	Enzyme activity in U/g with increasing incubation period					
TEMPERATURE ° C	24	48	72	96	120	144
25	117	663	3159	10517	13312	10322
30	221	7124	11622	17212	21158	16640
37	52	832	1937	5577	4290	2951

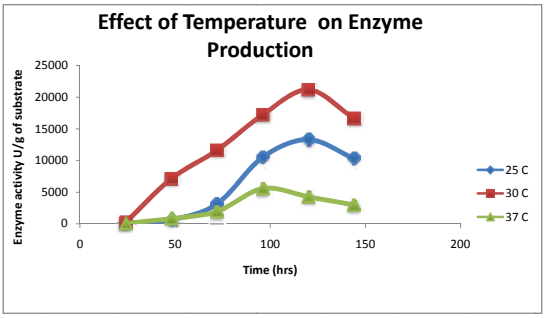


Figure 5.9 Graphical representation of effect of temperature on alpha galactosidase production.

5.6.6 OPTIMIZATION OF SALINE CONTENT

The enzyme activity was assayed in different saline concentrations (1.5, 2.5, 3.5, and 5%). The maximum enzyme activity was observed at 2.5% saline (25634±106.0 U/g of substrate). A reduction in the enzyme activity was found above or below 2.5% saline. Based on these results the saline concentration of 2.5% was used for further studies.

TABLE 5.7 The effect of saline content of the solid substrate on alpha galactosidase production within conditions :(10g of wheat bran, 10%inoculum, 1:1 moisture content, incubation till 144th hour and temperature at 30ºc).

Enzyme activity in U/g with increasing incubation period						
Salinity	24	48	72	96	120	144
1.50%	273	5005	9412	13559	20449	18460
2.50%	221	6227	12844	19994	25634	20332
3.50%	117	3042	8554	12844	16614	13442
5.00%	78	2184	7735	11336	13481	10959

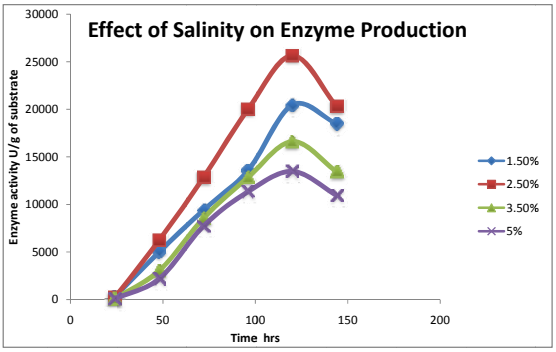


Figure 5.10 Graphical representation of effect of saline content on alpha galactosidase activity.

5.7 OPTIMIZATION OF ENZYME ACTIVITY

5.7.1 THERMOSTABILITY OF ENZYME

The temperature of the enzyme assay was altered in the range of (25, 30, 40, 50, 55, 65^{0}C) to determine the thermo-stability of the enzyme. The maximum activity was observed at temperature 55ºC (25334±241.23 U/g of substrate). A reduction in the enzyme activity was observed above 55ºC. Thus, it is found that alpha galactosidase is thermostable at 55ºC. (Fig 5.11)

TABLE 5.8 Thermostability of alpha galactosidase

Temp (ºC)	**25**	**30**	**40**	**50**	**55**	**65**
Enzyme activity (U/g of substrate	3627	2925	7085	19343	25334	11921

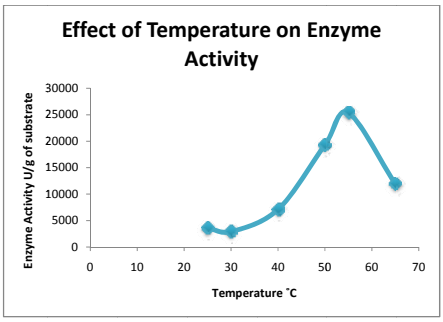


Figure 5.11 Graphical representation of effect of thermostability on alpha galactosidase activity.

5.7.2 THE EFFECT OF pH ON ENZYME ACTIVITY

The substrate (PNPG) is dissolved in acetate buffer to maintain the pH at 3, 4, 5, 6, in phosphate buffer for pH 7, 8 and carbonate buffer to maintain at pH 9 and 10. The assay was performed and the pH with maximum enzyme activity was found to be pH 5(25787±147.42 U/g of substrate). Table.9 and Fig.12 depicts the reduction in the enzyme activity with increase in pH.

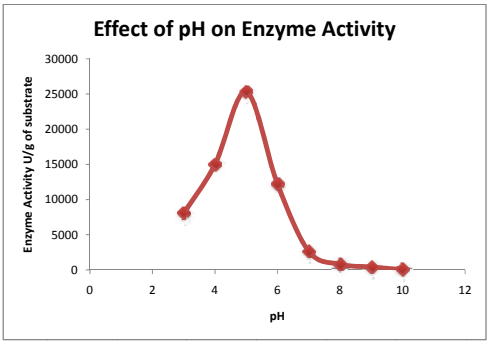


Figure 5.12 Graphical representation of effect of pH on alpha galactosidase activity.

TABLE 5.9 Effect of pH on alpha galactosidase activity within conditions: (10g of wheat bran, 10%inoculum, 50% moisture content, pH at 6, incubation till 120th hour and temperature at 30 ˚C)

pH	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
ENZYME ACTIVITY	8034	14989	25787	9489	2652	802	416	52

5.8. CHARACTERIZATION OF ALPHA GALACTOSIDASE BY SDS-PAGE

The electrophoretic patterns of crude enzyme and standard marker of different proteins in the range of 29KD - 205KDa is shown in the figure. The molecular weight of the crude enzyme was assumed to be in the range of 66KDa- 97KDa. (Fig 5.13)Further studies are to be carried out to determine the exact molecular weight by Elution method.

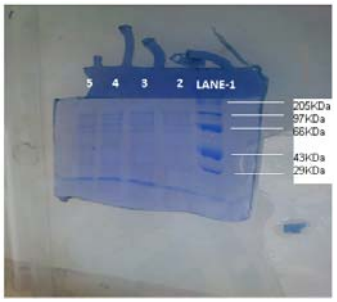
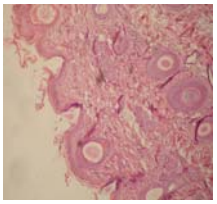


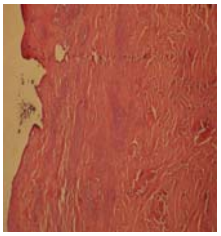
Figure 5.13 represents electrophoretic patterns of alpha galactosidase, lane 1: standard molecular weight markers, lane 2-5: crude alpha galactosidase.

5.9. ENZYMATIC FIBRE OPENING USING ALPHA-GALACTOSIDASE

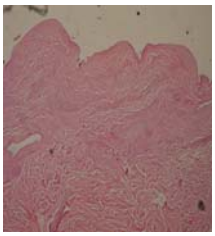
Crude alpha galactosidase obtained from *Aspergillus terreus* was used for fibre opening of dehaired goat skin. The goat skin was dehaired using protease enzyme. Only enzymatically dehaired skin can be used for enzymatic fiber opening as alternate use of chemical and enzymatic process can result in the degradation of leather quality. After shaking for one hour in rotary shaker the loosening and peptization of non-collagenous skin structure through the removal of residues of interfibrillary proteins (galactosyl- residues found between the collagen fibres) were observed through histological studies (Fig.5.14). This makes the leather soft and supple and prepares them for suitable for tanning and it removes swelling and makes the grain surface of the finished leather clean, smooth and fine.



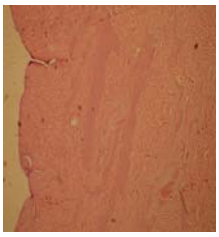
5.14 GOAT SKIN



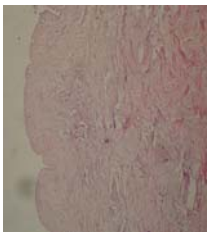
1A. CHEMICAL DEHARING



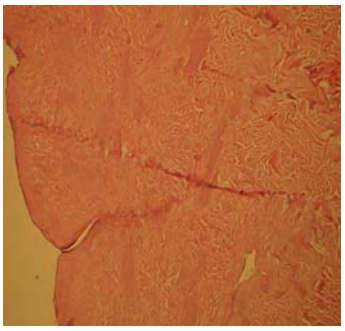
2A. ENZYMATIC DEHARING



1B. CHEMICAL BATING



2B. ENZYMATIC BATING

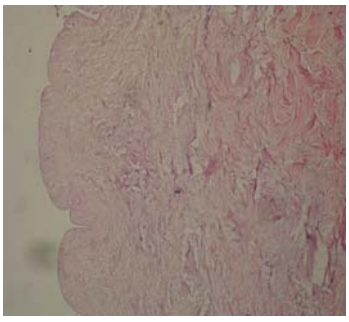


Keratinous Appendages still remained.

Incomplete fiber opening

Remains of hair follicles

Figure 5.15 (a): Conventional chemical bating process.



Keratinous Appendages completely removed

Complete fiber opening

No remains of hair follicles

Figure 5.15 (b) Enzymatic bating process by Alpha-galactosidase

5.10. Confirmation of Fibre Opening Using Carbohydrate Assay

After fibre opening, the fibre opened solution was taken in the range of 1-3ml and to this 3ml of DNS (dinitrosalicyclic acid) was added and kept at 95ºC boiling water bath until the colour change. To this 1ml of Rochelle salt (sodium potassium tartarate) was added and the absorbance was read at 575nm spectophotometrically (Fig.15) and the standard graph plotted was represented in Fig. 16.

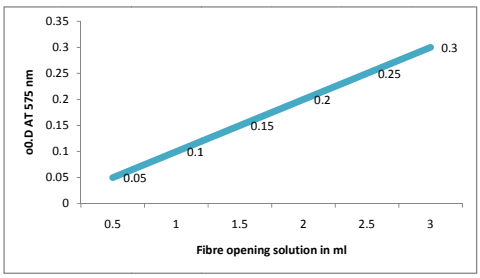


FIG 5.16 Conformation of fiber opening using carbohydrate assay

5.11 EFFECT OF INHIBITORS

The effects of the inhibitors were studied and of the maximum inhibition of galactosidase activity was found at 10mM concentration of bromosuccnimide. The relative activity was found to be only 0.3 % indicating that bromosuccnimide is an effective inhibitor of the enzyme. (fig 5. 17)

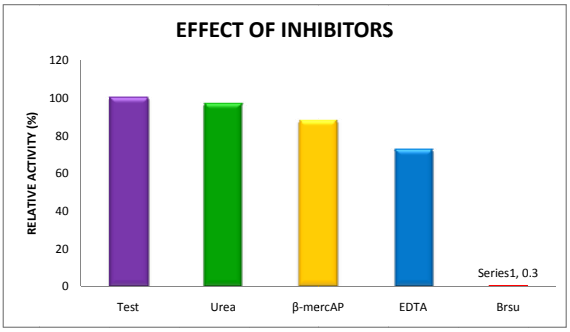


Figure 5.17 Graphical representation of the effect of inhibitors on enzyme activity

5.12 EFFECT OF METAL IONS

The effects of metal ions on the enzyme activity were studied. Copper, Silver and Mercury were found have pronounced effect on the activity of the enzyme galactosidase. Maximum enzyme inhibition occurred in the samples containing mercury ions. The relative activity was 2.9 % at final concentration 10mM.

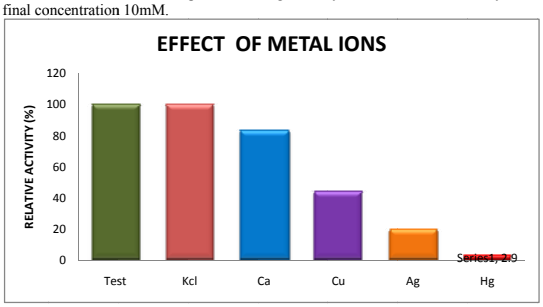


Figure 5.18 Graphical representation of the effect of metal ions of enzyme activity

6. CONCLUSION

In this study, the improvement of alpha-galactosidase production in solid state fermentation has proven to have potential effects with enhanced α-galactosidase activity. The production parameters like pH, temperature, incubation period, salinity, moisture content, substrate, thermo stability and pH stability were determined as

- Temperature - 37ºc
- Incubation period - 120th hour
- Salinity - 2.5%
- Moisture content - 50%
- Substrate - Wheat bran
- Thermo stability - 55ºc
- pH stability - 5

The results revealed that, inexpensive and simple medium compositions under efficient cultivation can be manipulated to increase the enzyme yield.

Eco-efficiency is the current highlight global industries activities to reduce the environmental impact of productions process.

The leather industry is under pressure to develop environmentally efficient leather-making processes to comply with modern pollution and discharge legislation. Conventional leather-processing methods are known to contribute significant pollution loads in tannery wastewaters. Thus the use of microbial enzymes in leather industry executes better results. The use of α-galactosidase in leather processing has been explored to achieve eco-efficiency as well as better quality of leather production. The process of fibre opening in raw goat skin was efficiently carried out using alpha galactosidase.

Therefore, this investigation has led to the development of a rationalized leather processing method for eco-efficiency.

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