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CHEMICAL AND BIOENGINEERING DEPARTMENT

LEATHER TECHNOLOGY STERAM

STUDIES ON OPTIMIZATION OF AMYLASE EXTRACTION USING FABRICATED EXTRACTOR COLUMN FOR FIBER OPENING AND CHARACTERIZATION OF ENZYME

By Biniyam Abdissa Gemeda

A Thesis Submitted to

The School of Chemical and Bio Engineering

Presented in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemical and Bio Engineering under Leather Technology Stream

Advisor: Dr. Solomon Kiros

Co-advisor: Dr. MK. Gowthaman

Addis Ababa Institute of Technology,

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Addis Ababa, Ethiopia

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Advised by:

Name____________________

Signature_________________

Date_____________________

Examined by:

1. Name_____________________ Signature________________ Date______
2. Name_____________________ Signature________________ Date______

DECLARATION

I hereby declare that the report of the post graduate Project Work entitled **"STUDIES ON OPTIMIZATION OF AMYLASE EXTRACTION USING FABRICATED EXTRACTOR COLUMN FOR FIBER OPENING AND CHARACTERIZATION OF ENZYME"** which is being submitted to the Addis Ababa Institute of Technology, AAiT, in partial fulfillment of the requirements for the award of the Degree of Master in leather technology in the department of Chemical Engineering, is a bonafide report of the work carried out by me. The material contained in this report has not been submitted to any University or Institution for the award of any degree.

Biniyam Abdissa Gemeda

Signature ___________________

Date_______________________

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Lists of Abbreviations

%	Percent
°C	Degree Celsius
µmol	Micromole
^{0}Be	Baume scale
BCS	Basic Chromium Sulfate
BOD	Biological Oxygen Demand
CLA	Cross linked amylose
COD	Chemical Oxygen Demand
CWB	Coarse Wheat Bran
D/W/D/P	Drain wash drain pile
D/W/D	Drain wash drain
DNS	Dinitrosalicylic acid
FWB	Fine Wheat Bran
Gm	Gram
GRAS	Generally Recognized as Safe
Hr	Hour
IU	International Unit
KDa	Kilo Dalton
L	Liter
LB	Laura bertani broth
L/O/N	Leave over night

Ml	Milli liter
Mm	Millimeter
N/D	Next day
OD	Optical Density
PG	Proteoglycan
Rpm	Revolution per minute
SEM	Scanning Electron Microscope
SmF	Submerged fermentation
SSF	Solid State Fermentation
TDS	Total Dissolved Solid
T_i	Absorbance
TS	Total Solid
U/gds	Unit per gram of dry substrate
U/l	Unit per liter
WB	Wheat Bran
α –amylase	Alpha amylase

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ABSTRACT

Amylases are important hydrolase enzymes which have been widely used since many decades. α-Amylase is in maximum demand due to its wide range of applications and plays a pivotal role such as leather making process. Due to absence of cheap and effective extraction method, it mostly leads to loss of enzyme activity as well as waste of crude enzyme extract.

This present work is concerned with characterization and studies to maximize and optimize the extraction process using fabricated extractor column from bacterial culture under solid state fermentation (SSF) using agro industrial residue for fiber opening. Various process parameters are optimized for maximum extraction of enzyme. The finding of the study suggests that, the optimum condition for maximum amylase extraction with medium sized wheat bran at pH of 7.1 and temperature of 30 ^{0}C yields 1332.31 U/gds and 2374.51 U/gds in solid state fermentation media respectively. In continuous column extraction process with optimum buffer flowing at rate of 100ml/min and optimum bed volume of 200gm provides a maximum activity of 1081.8 U/gds. Comparing batch with column system of extraction, the combined activity shows 114.89 and 551.3 U/gds respectively. Moreover, by comparing the conventional with experimental method of extraction, it shows a combined activity of 347.7 and 450.5 U/gds respectively. Hence the total yield of column extraction process shows 75% higher as compared with batch system and conventional method of enzyme extraction process. In order to check the benefits of α-amylase, the crude enzyme extract is partially purified before applying it on leather. In an attempt to reduce the pollution load from pre tanning operation, a concentrated amylase is applied on goat skin. Hence, studying the effect of fiber opening between enzymatic and chemical process by various tests were carried out. The finding revealed that the extracted enzyme releases 7.3 mg/gm of proteoglycans after enzyme treatment. The effect of fiber splitting on the strength properties related morphological changes of the crust leather samples were thoroughly investigated. SEM analysis was also carried out. According to the result an economical enzyme produced in this study shows a significant release of inter fibrillary materials. The strength property of crust leather shows tensile strength of 216.4 Kg/cm^{3}, tear strength of 42.76 Kg/cm and 62.17% elongation at break.

Keywords: α-amylase, bacillus subtillis, column extraction system, crust leather, enzyme extraction optimization, proteoglycan release, solid state fermentation

CHAPTER ONE

1. INTRODUCTION

1.1 Background

Ethiopian leather industry is relatively older industry with more than 90 years of involvement in processing leather and producing leather products. The leather industry made its strong foundation on the country's enormous population of livestock which provides ample opportunity for the development of the country's leather sub-sector industry. (LIDI et. al. 2017)

There are 32 tanneries presently processing hides and skins to different types of finished leather. Every year, the country produces about 5 million ton of Bovine hides-pieces, 8.1 million ton of sheep and 7.5 million ton of goat skins-pieces. Moreover, the country has about 2.5 percent of the world livestock population with about 57.83 million cattle; 28.04 million sheep and 28.61 million head of goat. (LIDI et. al. 2016)

Despite the significances in terms of economic gain through export market and local market opportunity from the sale of hides and skins; tanning industries have considerable adverse impacts on the health of workers, local community and the ambient environment. Leather industry has been categorized as one of the highly polluting industries and it has adverse impact on environment because of the generation of toxic liquid, solid and gaseous wastes. Solid and liquid wastes generated from leather making industries contain various chemicals which are used in tanneries. (Ramachandran *et al.*,2004)

As tannery can be an extremely risky workplace involving multiple hazards, the workers are facing different health hazards. For the tanning process, the epidermis of the hide is first removed and only the dermis transformed into leather. During leather making process, since the hide serves as a medium for numerous microorganisms, infection and bacterial contamination are major hazards for the workers. Tetanus, anthrax, leptospirosis, epizootic aphtha/mouth ulcer, fever, and brucellosis are also the possible diseases that workers could be contracted, during the tanning process, due to infected hides. For the workers of tanneries in Ethiopia, although injury (for example cutting of fingers) and skin irritation are the mentioned as major risks and due to the bad odor within the working environment, workers frequently suffer from headache, cold and asthma.

A large amount of Chemicals is used to convert raw cattle hides, goat and sheep skins in to leather. The chemical reagents consumption is very high. For 1000kg of hides about 400kg of chemicals is needed, including hazardous chemicals namely; sodium chloride, lime, sodium sulphide, sulphuric acid, basic chromium and others. A considerable amount of these chemicals are not absorbed in the production process and is discharged in to the environment. The capacity of world leather process is 15 million tons of hides and skins per year. The waste water discharge from world tanneries is about 600 million cubic meter per annum. On average 45 -50 m^3 of wastewater is discharged from tanning industry per ton of raw hide processed. (ELIA et. al. 2012)

Despite the adverse impacts of the effluents discharged from tanneries across the country, the surrounding communities are also arguing against the bad odor emitted from the tannery. Unlike to the problem related with water contamination, the problem related to the bad odor of the tannery is commonly shared by the nearby residents of all directions. As to the report of USAID, strong smells can damage the quality of life around the tannery site and may reduce or destroy community support for further production or expansion. Thus, as the surrounding community is complaining about the waste discharging systems of tanning industries. Hence, the tanneries are highly recommended to design and implement some more efficient waste treatment and discharge mechanisms. (USAID report, 2015)

In recent times, leather processing, especially the pre-tanning operations have become synonymous with environmental pollution due to the extensive use of a variety of substituting chemicals, a practice of several decades, since the inception of modern tanning. Recently developments in industrial biotechnology, defined as the use of enzymes or microorganisms for industrial processes, has offered a viable option to decrease or avoid environmental pollution from such industrial activities. Environmental safeguards have therefore become paramount and are now gradually being recognized in almost all stages of leather manufacturing. However, this is easier said than done. Systems that find favor as alternatives to chemical processing, require performance levels that either should match or be better. One such alternative is the use of microbial enzymes. Enzymes are biochemical that catalyze specific reactions and have myriad uses in food, textile, pharmaceutical, paper, leather, and other industries. Quite a few enzymes exist in the market for processes like depilation, degreasing, fiber opening etc. However, The factors that hindered from using enzyme instead of chemical in Ethiopian tanneries for soaking, dehairing and fiber opening applications, mainly is due to the cost and lack of availability of such enzymes with affordable price. Moreover, invariably all these products have some limitation or other; for instance, they are mostly chemical assisted, mixed with organic additives, expensive etc. (Thanikaivelan, P et. al.., 2004)

Production of enzymes is commonly done by fermentation and quite a few companies across the world are major players. In Ethiopia, research into enzyme fermentation has not been going on for several years and limited number of enzymes have been developed and some were up scaled in-house. One of the challenges in enzyme process/product development is in achieving optimal concentration from natural raw materials especially by solid state fermentation (SSF). The advantages of SSF over conventional submerged fermentation are quite a few and well documented. (Doelle H. W., 1991)

Downstream processing, namely; separation and purification of an enzyme produced at large scale from an organism require disruption of cells, removal of cell debris and nucleic acids, precipitation of proteins, ultrafiltration of the desired enzyme, chromatographic separations (optional), crystallization, and drying. In some cases, it may be more advantageous to use inactive (dead or resting) cells with the desired enzyme activity in immobilized form. This approach eliminates costly enzyme separation and purification steps and is therefore economically more feasible. The modern factory system, along with its large scope for the use of machinery and division of labor, could be mentioned as the technological gaps that makes it difficult for the expansion of enzyme production in large scale. (Smits JP., 1996)

Solid-liquid extraction (leaching) is a key-step in SSF and should be performed carefully to ensure maximal recovery of the product with minimal loss in activity and time. Bench scale extraction is usually carried out in flasks of sizes up to 3 L beyond which such a system becomes cumbersome. This therefore, restricts the amount of fermented material to be processed in a given length of time. On a larger scale it is necessary to employ other designs for extraction. Simplest of these are columns of cylindrical geometry.

1.2 Problem of statement

At present enzymes find increasing application in many industrial processes. As a result the global industrial market is growing very fast with a current estimated value of US$7 billion. Although enzymes are found in all living organisms, most industrial enzymes currently in use are obtained from microorganisms.

Ethiopia has a huge potential for the discovery of novel enzymes that could prove highly useful in different industrial processes. The East African region is endowed with unique microbial biodiversity which could serve as a source of novel enzymes for industrial application. Furthermore, because of the availability of extremely unique habitats, such as alkaline environments, hot springs, etc with huge microbial diversity, the region could be, in the long term highly competitive in the global industrial enzyme market.

Already some enzymes with an attractive potential for industrial application have been discovered from the region (Pennisi 1997, Gessesse, 1998; Mamo and Gessesse 1999; Gessesse et al. 2003; Hashim et al 2005, Yihun, 2007; Kebede 2008;; Damte, 2011;; Seid, 2011). Despite its tremendous potential for biotechnology innovation, to date the region make no use of this resource. Some of the reasons for the lack of growth in this sector could be limited capacity in research and development, lack of technology along with skilled human resources and others.

The production of leather is carried out in three stages namely; the beam house stage, tanning stage and crusting stage. These stages uses a wide variety of chemicals and surfactants which being non- biodegradable such that pollute the environment. Hence, three enzymes viz., amylase, lipase and protease have been known for quite some time to serve as ecofriendly, pre tanning alternatives to chemicals due to their specificity and biodegradability.

Amylase is the most common enzyme used worldwide in many industries for various purposes. Present production quantity in the world is not sufficient to fulfill the need of amylase enzyme. Lab scale extraction of enzyme from the fermented bacterial wheat bran involves the addition of appropriate buffer into the biomass. Mix the solution thoroughly with the help of mortar and pestle or mixer. This was then squeezed and filtered using a muslin cloth. The Filtrate obtained was then cold centrifuged in order to separate from smaller particles. The supernatant obtained was kept as crude for enzymatic assay, whereas the pellet gets discharged. Conventional extraction of amylase enzyme which are now used for commercial production do not fulfill the quality and quantity of enzyme as per need. From this investigation new extraction method will be investigated which can produce high amount of stable amylase enzyme. Proper optimized condition which increases the extraction of amylase enzyme from a given substrate and other optimizing factors. A novel technique will be developed for the amylase enzyme extraction which will be cheap and easier.

Amylase enzymes will be produced by known bacterial systems and extraction investigated in specially designed columns. Further concentration if needed shall be done before evaluating in pre tanning application. This experiment will help to provide in efficient extraction of concentrated amylase from agricultural waste by the column system. Hence it plays a vital role in showing an alternative and effective way in extraction process of an enzyme using SSF.

1.3 Objectives

1.3.1 General objective

The general objective of this study was to extract amylase from wheat bran by specially designed extractor column and characterization of enzyme.

1.3.2 Specific objectives

The specific objective of the study were:

- ✓ Specify key design parameters for an extractor column for efficient extraction of amylase
- ✓ Producing scaling up of amylase by SSF using wheat bran
- ✓ Characterization of enzymes and their effects on pH, temperature and particle size

- ✓ To optimize parameters (flow rate and bed volume) for extraction of amylase using single and dual columns
- ✓ Compare conventional with column type of extraction methods
- ✓ Correlate enzyme total yield
- ✓ Study the effect of using amylase on fiber opening or splitting application in leather processing.
- ✓ Analysis of spent fiber liquor for fiber opening

1.4 Scope of study

This research proposal is designed to develop a method to maximize and optimize the extraction process of amylase using a specially designed column. In this study a substrate is subjected to mechanical and biological decomposition in the presence of inoculums. Initially flask level (50 g) preparation for the enzymes with established conditions would be carried out and enzyme activities evaluated. Trays of capacity 1 kg shall be used to produce these enzymes. Extraction columns of suitable capacity shall be designed and fabricated in the glass blowing section. Extraction shall be carried out using pump / manual feeding and parameters viz., feed rate, recycling, packing density, etc. optimized. Extraction may be followed by a second step of concentration (if needed). The product obtained will be tested in the tannery for dehairing, fiber opening and defleshing/degreasing. Extraction efficiency will be determined for all the experiments.

1.5 Significance of the Study

Solid-liquid extraction at a larger scale is not much reported, it however is a subject of great significance. Many enzyme systems by SSF have been developed in this laboratory for efficient application in leather processing but no larger scale extraction systems have been investigated except to a minor extent. This study therefore, is expected to provide a lead and thrust to develop further extraction and other downstream processing strategies.

CHAPTER TWO

2. LITERATURE REVIEW

Biotechnology are considered as a useful alternative to conventional process technology in the industrial and analytical fields due to the fact that biological systems can accomplish complex chemical conversions under mild environmental conditions with high specificity, accuracy and efficiency unlike the chemical catalysis. Moreover biological systems help in ingredient substitution, less undesirable products, increased plant capacity, increased product yields and at the same time they are less energy intensive and less polluting. The variety of chemical transformations catalyzed by biocatalysts i.e. enzymes, which are now a prime target of exploitation by the emerging biotechnology based industries.

As enzymes being an organic catalyst and produced by living organisms, they occur in every living cell, and in all microorganisms. Enzymes are also biocatalysts that bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. Enzymes are highly specific in their action on substrates and often many different enzymes are required to bring concerted action, the sequence of metabolic reactions performed by the living cell. Almost all processes in a biological cell need enzymes to occur at significant rates. Since enzymes are selective for their substrates and only speed up a few reactions from among many possibilities, the set of enzymes made in a cell determines which metabolic pathways occur in that cell. Almost all enzymes which have been purified are protein in nature, and may or may not possess a non-protein prosthetic group for their biological activity.

2.4 Amylase

Amylases are one of the most important industrial enzymes that have a wide variety of applications ranging from conversion of starch to multi-dimensional purposes. Amylases are glycosyl hydrolases (GHs) as they cleave the glycosidic linkages between the glucose units in starch. The term amylase was used originally to designate enzymes capable of hydrolyzing α-1,4 glucosidic bonds of amylose, amylopectin, glycogen and their degradation products (Bernfeld,1955, Fisher and Stein, 1960). A number of enzymes associated with degradation of starch and related polysaccharides structures have been reported and studied (Boyer and Ingle, 1972; Griffin and Fogarty, 1973; Fogarty and Griffin, 1975). The most common classification of the glycosyl hydrolases has been to group them into different GH families, based on their amino acid sequence and structural similarities. The starch degrading enzymes are found mainly in GH family 13, 14 and 15 (Henrissat,1991; Henrissat and Davies, 1997; Coutinho and Henrissat, 1999).

The α-amylases of family GH 13 have a common three-dimensional structure comprising of three structural domains A, B and C .The A domain is the catalytic domain and has a $(\beta/\alpha)_8$ barrell structure. This domain carries the active site as well as an array of subsites each of which interacts with the individual glucose units in the substrate. The specificity of the α-amylases of this family varies and is governed mainly by the position of the catalytic site, the amino acid composition of the catalytic site, the number of sub-sites and the affinity of each of these sub-sites to the glucose residues in the substrate (MacGregor, 1993, Davies et al, 1997). Most of the α-amylases are metalloenzymes, which require calcium ions (Ca^{2+}) for their activity, structural integrity and stability The B domain which is involved Ca^{2+} in substrate and binding protrudes at the third β strand of the A domain. The C domain follows the A domain and it has been suggested to play a role in enzyme activity (Jespersen et al, 1991, van der Maarel et al, 2002).

Figure 2.1: 3D image of α-amylase

With the retention of α-anomeric configuration alpha-amylases (endo-1,4-α-D glucan glucanohydrolase [E.C.3.2.1.1]) catalyze the hydrolysis of alpha-1, 4-glycosidic linkages of polysaccharides of starch to yield in low molecular weight products; such as glucose, maltose, maltotriose units (i.e maltotrioses, maltotetrose and mixture of malto-oligosaccharides) (Anupama and Jayaraman 2011; Akcan 2011; Bakri *et al*., 2012).

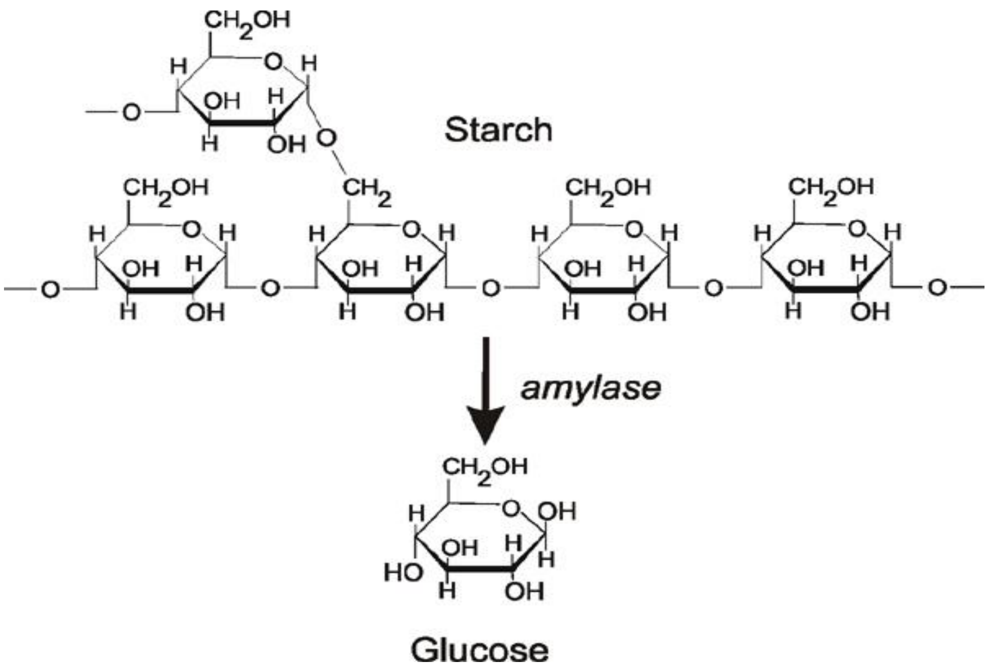


Figure 2.2. Action of α-amylase on starch molecules

Amylase enzymes have molecular weights between 10 and 210 KDa smaller than the macromolecules of starch they attack (Gupta et al. 2003). The structure of the α-amylase enzyme includes $(\beta/\alpha)_8$ or triosephosphate isomerase (TIM) barrel that contains the catalytic site residues. This conserves the glycolytic activity that enables the α-amylase enzyme's catalysis of the hydrolytic degradation of starch (van der Maarel et al. 2002). The catalytic mechanism of the α-amylase enzyme is a double displacement reaction that involves the formation of a covalent intermediate. Since the reaction catalyzed by the α-amylase does not consume it, the enzyme will still remain after the starch substrate has been degraded.

These α-amylase enzymes account for about 30 % of the world's enzyme production (Akcan 2011; Malle et al., 2012; Deb et al., 2013) and have a great significance with extensive biotechnological applications in bread and baking, food, textile, and paper industries (Sivaramakrishnan S. et al., 2006; Gupta R, Gigras P. et al. 2003). Today, amylases are available commercially in the large number and they have almost completely replaced chemical hydrolysis of starch processing and reduce the production of chemicals used in carbohydrate hydrolysis. Microorganisms produce different kinds of industrial enzymes. Bacteria which can produce the amylase are widely present in nature can easily be screened and tested for the production of amylase (Pokhrel et al., 2013). Commercially used alpha-amylase obtained mostly from different types of *Bacillus sp*. (Pokhrel et al.and Kumar et al, 2013; Deb et al., 2013).

2.5 Organisms producing α-amylase

Alpha-amylase are universally distributed throughout the animal, plant and microbial kingdom like several fungi, yeasts, bacteria and actinomycetes (Windish and Mhatre, 1965; Fogarty and Griffin, 1975 and Mantsala, 1989; Lonsane and Ramesh, 1990). However, enzymes from fungal and bacterial sources have dominated applications in industrial sectors. The major advantage of using microorganisms for the production of amylases is for economical bulk production capacity and the fact that microbes are easy to manipulate to obtain enzymes of desired characteristics (Lonsane and Ramesh, 1990).

Fungal sources are confined to terrestrial isolates, mostly to *Aspergillus* species and to only one species of *Penicillium*, *P.brunneum.* The *Aspergillus* species produce a large variety of extracellular enzymes, and amylases are the ones with most significant industrial importance. Filamentous fungi, such as *Aspergillus oryzae* and *Aspergillus niger*, produce considerable quantities of enzymes that are used extensively in the industry. *A. oryzae* has received increased attention as a favorable host for the production of heterologous proteins because of its ability to secrete a vast amount of high value proteins and industrial enzymes, e.g. α-amylase. *Aspergillus niger* has important hydrolytic capacities in the α-amylase production and due to its tolerance of acidity (pH <3), it allows the avoidance of bacterial contamination. Filamentous fungi are suitable microorganisms for solid-state fermentation (SSF), especially because their morphology allows them to colonize and penetrate the solid substrate. The fungal α-amylases are preferred over other microbial sources due to their more accepted GRAS (Generally Recognized as Safe) status. The thermophilic fungus *Thermomyces lanuginosus* is an excellent producer of amylase. A large variety of bacteria employ extracellular or intracellular enzymes able to convert starch or glycogen that can thus serve as energy and carbon sources. For commercial applications α-amylase is mainly derived from the genus *Bacillus.*

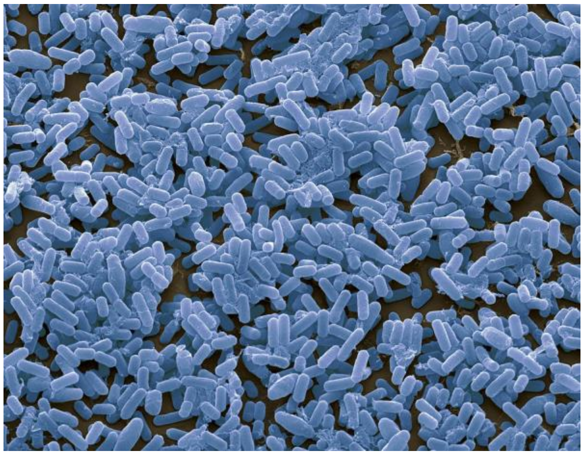


Figure 2.3. *Bacillus subtilis*

Bacillus subtilis is one of the most widely used bacteria for the production of specific chemicals and industrial enzymes and also a major source of amylase and protease enzymes. The major advantage of using microorganisms for production of amylases is in economical bulk production capacity and microbes are also easy to manipulate to obtain enzymes of desired characteristics. There are some factors, which influence the nature of their metabolic process and the enzyme production. The composition and concentration of media and physical parameters greatly affect the growth and production of extracellular amylase in bacteria. Optimization of cultural conditions is important for maximum production of microbial strains. *Bacillus* species and other forms of microorganisms grow at different rates with specificity to different substrates in the culture medium. The growth conditions also influence their enzymatic activities.

B.subtilis is also known for as an efficient expression host for production and secretion of proteins, and is one of the expression hosts used for the industrial production of enzymes. It is used in areas like production of interferon (Palva, 1983), insulin (Olmos-Soto *et al*, 2003) pathogenic antigens (Airaksinen *et al*, 2003) and toxins (Taira *et al*, 1989) and enzymes of great industrial use like proteases (Ho *et al*, 2003), α-amylases (Huang *et al*, 2004) and lipases (Ho *et al*, 2003). The most significant benefit of *B.subtilis* when compared with the other expression host production systems are (i) high cell density growth and (ii) secretion of the synthesized protein in the cultivation medium.

It is estimated that *Bacillus sp*. enzymes make up about 50% of the total global enzyme market. Amylases, biosynthesized by the bacteria, show unique characteristics such as thermophilic, thermotolerant, alkaline and acidophilic properties. Thermostability is a desired characteristic of most of the industrial enzymes. Thermostable enzymes isolated from thermophilic organisms have found a number of commercial applications because of their stability. As enzymatic liquefaction and saccharification of starch are performed at high temperatures (100–110°C), thermostable amylolytic enzymes have been currently investigated to improve industrial processes of starch degradation and are of great interest for the production of valuable products like glucose, crystalline dextrose, dextrose syrup, maltose and maltodextrins.*B. subtilis, B.stearothermophilus, B. licheniformis, B. amyloliquifaciens* are known to be good producers of α-amylase.

2.6 Microbial α-amylase production

Commercial sources of enzymes are obtained from three primary sources, i.e., animal tissue, plants and microbes. These naturally occurring enzymes are quite often not readily available in sufficient quantities for food applications or industrial use. However, by isolating microbial strains that produce the desired enzyme and optimizing the conditions for growth; commercial quantities can be obtained. Several methods, such as submerged fermentation (SmF) and solid-state fermentation (SSF) have been successfully used for α-amylase production from various microorganisms. Agro-industrial residues such as wheat bran, spent brewing grain, maize bran, rice bran, rice husk, coconut oil cake, mustard oil cake, corn bran, etc., are generally considered the best substrates for processes. In addition, the utilization of these agro industrial wastes, on one hand, provides alternative substrates and, on the other, helps in solving pollution problems, which otherwise may cause their disposal (Pandey et al. 1999).

Among plant and animal enzymes, microbial amylases have immense applications in various fields in world market because of their wide application in starch based industries especially food, textile, paper, detergent, pharmaceutical and baking industries (Anupama and Jayaraman 2011; Amutha and Priya 2011; Akcan 2011; Kaur *et al*., 2012). Example; in food industry amylase use in bread making, to break down complex sugars, such as starch (found in flour), into simple sugars. Modern bread making techniques have included amylases (often in the form of malted barley) into bread improver, thereby making the process faster and more practical for commercial use (Maton *et al.*1993).

2.7 Optimization of parameters for enzyme production and extraction

SSF processes are usually simpler and can use wastes or agro-industrial substrates, such as defatted soybean cake, wheat and rice bran for enzyme production. The minimal amount of water allows the production of metabolites in a more concentrated form, making the downstream processing less time consuming and less expensive (Germano *et al*., 2003).

Unfortunately, SSF is usually slower because of the diffusion barriers imposed by the solid nature of the fermented mass. However, the metabolic processes of the microorganisms are influenced to a great extent by the change of pH, temperature, substrate, water content, inoculum concentration, etc. Optimization of the various parameters and manipulations of media are one of the most important techniques used for the over production of amylase in large quantities (Balasubramanien et al, 2011). Various physical and chemical factors have been known to effect the production of alpha amylase such as temperature, pH, Incubation period, carbon, nitrogen sources, surfactants, phosphate, different metal ions, moisture and agitation with respect to SSF and SmF (Ellaiah et al, 2002). These conditions vary widely from species to species for each organism. Therefore, it becomes very important to know the environmental conditions of the microorganism for maximum production (Elibol and Moreira, 2005).

Nevertheless, research about SSF had been neglected for a long time not only because of the popularity of the submerged culture process but also for the difficulties associated with the measurement of parameters in SSF, such as microbial biomass, substrate consumption, concentration of products formed as well as the measurement of the physical properties of the system (Hesseltine, 1972). Alpha amylases are important enzymes and can be used for a variety of processes such as in detergents, leather processing, silver recovery, medical purposes, food processing, feeds and chemical industrial, as well as waste treatment (Zamost *et al*., 1991; Wiseman, 1993)

2.9 Method of Fermentation

Fermentation is the technique of biological conversion of complex substrates into simple compounds by various microorganisms such as bacteria and fungi. Fermentation has been classified into submerged fermentation (SmF) and Solid state fermentation (SSF) mainly based on the type of substrate used during fermentation.

The development of techniques such as Submerged Fermentation (SmF) and Solid State Fermentation (SSF) has led to industrial-level production of bioactive compounds. These techniques have been further refined based on various parameters such as the substrates used, environmental parameters and the organisms used for fermentation. Based on research, certain bioactive compounds have found to be produced in higher quantities in SSF, whereas other compounds have been extracted using SmF.

2.9.1 Submerged Fermentation

Submerged fermentation is the cultivation of microorganisms in liquid nutrient broth. Industrial enzymes can be produced using this process. This involves growing carefully selected microorganism (bacteria and fungi) in closed vessels containing a rich broth of nutrients (the fermentation medium) and a high concentration of oxygen. As the microorganisms break down the nutrients, they release the desired enzymes into solution.

Due to the development of large-scale fermentation technologies, the production of microbial enzymes accounts for a significant proportion of the biotechnology industries total output. Fermentation takes place in large vessels (fermenter) with volumes of up to 1,000 cubic meters.

2.9.2 Solid State Fermentation

Solid-state fermentation is another method used for the production of enzymes. Solid-state fermentation can be defined as the cultivation of microorganisms in the absence or near absence of free water in the substrate. However, there must be enough moisture present to support cell growth In addition to the conventional applications in food and fermentation industries, microbial enzymes have attained significant role in biotransformation’s involving organic solvent media, mainly for bioactive compounds.

This method is an alternative to the production of enzymes in liquid by submerged fermentation. SSF has many advantages over submerged fermentation. These include, high volumetric productivity, relatively high concentration of product, less effluent generated and simple fermentation equipment.

There are many substrates that can be utilized for the production of enzymes by SSF. These include wheat bran, rice bran, sugar beet pulp and wheat and corn flour. The selection of substrate depends on many factors, which is mainly related to the cost and the availability of the substrate. Other factors include particle size and the level of moisture. Smaller substrate particles have a larger surface area for the proliferation of the microorganisms, but if too small the efficiency of respiration will be impeded and poor growth and hence poor production of enzymes will result. Larger particles provide more efficient aeration and respiration, but there is a reduction in the surface area.

2.9.2.1 Substrate for SSF

The selection of a substrate for enzyme production in a SSF process depends upon several factors, mainly related with cost and availability of the substrate, and thus may involve screening of several agro-industrial residues. In a SSF process, the solid substrate not only supplies the nutrients to the microbial culture growing in it but also serves as an anchorage for the cells. SSF processes are distinct from SmF culturing, since microbial growth and product formation occurs at or near the surface of the solid substrate particle having low moisture contents. Moreover, water has profound impact on the physico-chemical properties of the solids and this, in turn, affects the overall process productivity.

A number of agro-industrial residues have been employed for the cultivation of microorganisms to produce host of enzymes. Some of the substrates that have been used included sugar cane bagasse, wheat bran, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soyhull, sago hampas, grapevine trimmings dust, saw dust, corncobs, coconut coir pith, banana waste, tea waste, cassava waste, palm oil mill waste, aspen pulp, sugar beet pulp, sweet sorghum pulp, apple pomace, peanut meal, rapeseed cake, coconut oil cake, mustard oil cake, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch, etc. Wheat bran however holds the key, and has most commonly been used, in various processes. (Doelle H W., 1991)

2.9.2.2 Factors affecting enzyme production in SSF

A number of environmental and physicochemical factors can drastically influence the growth and metabolic activities of microorganisms during solid substrate fermentation, including temperature, the moisture content of substrate, aeration, inoculum size, pH, carbon and nitrogen sources, metal ion supplement, and the particle size of the substrate (Pandey *et al.,* 2003). However according to Kalogeris *et al.,* (2003), three independent process parameters, namely incubation temperature, initial moisture content of substrate, and aeration rate, are key operating variables that decisively influence the microbial growth and metabolism-mediated product formation in solid substrate fermentation. Initial moisture content of the solid substrate is an important factor which dictates the growth of the organism and enzyme production; in the case of fungi a wider moisture range (20-70 %) supports better growth and metabolic activities, but for bacteria only a higher moisture content of the solid matrix can yield better performance **(**Gowthaman *et al*., 2001). So in SSF, it is quite important to maintain an optimum moisture range (Sabu *et al*., 2006).

2.9.2.3 Solid state fermentation using wheat bran

Solid state fermentation can be explained as the microbial cultivation process conducted basically by culturing microorganisms on a solid support in either complete absence or near absence of free flowing water. The moisture content retained by the solid support is the only water source for the process.

The production of α-amylase by submerged fermentation (SmF) using synthetic media has been used for many decades (Hamilton *et al.,* 1999). It works with synthetic media which are very expensive and uneconomical. Solid-state fermentation involves the cultivation of microorganisms on a solid substrate.

The use of agricultural and industrial wastes makes solid state fermentation attractive alternative method. SSF is better than SmF due to its simple techniques, low capital investment, less effluent generated, lower levels of catabolic repression, high volumetric productivity, better product recovery, best suited for fermentation technique involving fungi and bacteria that requires less moisture content and also it resembles the natural habitat of some fungi and bacteria, easier downstream processing and value addition of wastes. The metabolites so produced are concentrated and purification procedures are less costly.

2.10 Composition of wheat bran

Bran is created as a by-product in milling industries and one million tons of wheat can produce up to a quarter million tons of wheat bran (WB). Wheat (*Triticum aestivum*) is an ancient known food crop, cultivated since the beginning of human civilization and ranks first among world cereal crops. Production of wheat is closely related to the supply of irrigation water and amount of rain fed water (Ahmad et al., 2010).

WB is rich in carbohydrates (60%), protein (12%), fat (0.5%), minerals (2%), bioactive compounds and vitamins (Slavin, 2003). Along with these wheat bran also contains several important compounds such as phenolic acids, carotenoids, lignans, phytosterols, flavonoids, tochopherol and phytic acid which are distributed unequally in different WB tissues. Precise composition of macro and micronutrients may vary from cultivar to cultivar and the extraction technique of these compounds from bran. Wheat ash content is usually measured for quantification of bran. Amount of ash (mineral content) present is a true reflective of the bran quantity in wheat (Safdar et al., 2009).

Wheat kernel is made up of three major parts; seed coat or pericarp (bran), endosperm and germ (Hoseney, 1994). Detailed study by Safdar (2005) about the structure of wheat kernel shows that it contains about 68 to 80% endosperm, 14 to 19% bran and 2 to 3% germ. (Antoine et al. ,2002) have found that WB is a composite material made up of three discrete layers that are formed from numerous histological tissues. These tissue layers are divided into outer and inner pericarp (tube cells and cross cells), testa or seed coat, hyaline layer and aleurone layer.

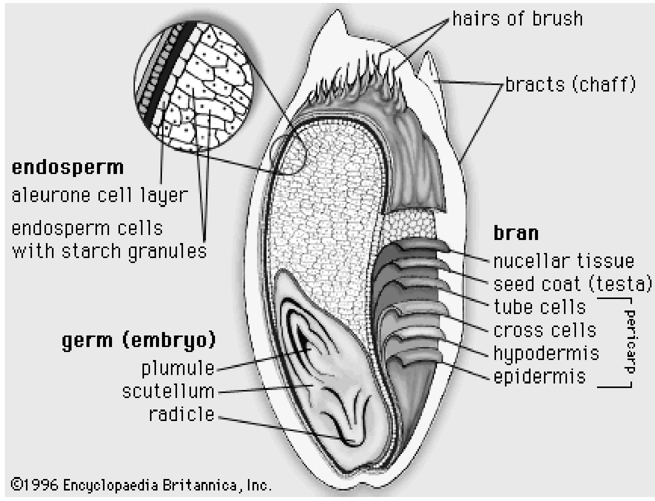


Figure 2.4: Histological composition of wheat grain. Adopted from surget and barron (2005)

2.11 Industrial application of amylase

The practical application and industrial use of enzymes to accomplish certain reactions apart from the cell, dates back many centuries and practiced long before the nature or function of enzymes was understood. Use of barley malt for starch conversion in brewing and treatment of hides in leather making are examples of ancient use of enzymes. It was not until nearly the turn of this century that the causative agents or enzymes responsible for bringing about such biochemical reactions became known.

Alpha amylase (1, 4-α-D glucan-glucanohydrolase) is one of the most important enzymes which can be used in number of industrial processes including brewing, baking, textile, detergent and paper industries. Because of low pH stability, raw starch digestibility and utilization of high concentration of starch, in starch processing, desizing of textiles, paper sizing, as detergent additive, and bread improvement, pharmaceutical industries, ethanol, and other fermentation processes (Haki and Rakshit 2003; Lowe 2002; Gomes et al.2005).

The global market for enzymes was about $2billion in 2004. It is expected to have an average annual growth rate of 3.3%. The share of carbohydrase's comprising amylases, isomerases, pectinases and cellulase is about 40 % (Riegal and Bissinger 2003). The food and beverage sectors utilize 90 % of the carbohydrates produced. Today, amylases have the major world market share of enzymes. In this light, microbial amylases have completely replaced chemical hydrolysis in the starch processing industry. Some of amylase applications are listed below:

2.11.1 Fermentation industry

Many agro-industrial by-products are replacing the synthetic and expensive substrates for the production of biotechnological products. Among the agro-industrial substrates, WB is one of the most attractive alternatives to synthetic medium in fermentation processes (Pandey, 1992). The coarse variety of WB is an efficient substrate due to its heat dissipation, better air circulation, loose particle binding and efficient penetration by mycelia and it is cheaper than fine bran so it is a better prospect economically in fermentation industry (Malathi and Chakraborty, 1991). Wheat bran is a potential candidate in fermentation industry because of its unique properties listed below:

✓ *Water retaining ability*

Apart from the presence of important nutritional components, physical characteristics of wheat bran also play vital role in fermentation process. WB has the ability to retain high moisture content in SSF. This ability of wheat bran promotes the fungal growth just as in the natural environmental conditions.

✓ *Complex substrate*

Complex nature of WB lies in its unique nutrient composition. The higher starch content of WB i.e., 75.6% as compared to other agro-industrial wastes such as rice bran (coarse waste 71.1%> rice powder 55.8%> medium waste 48.6%> fine waste 34.2%) can be correlated for higher amylase production (Ellaiah et al., 2002).

WB can be used as an inducer for a multitude of enzymes such as CMCase, xylosidase, glucosidase, L-arabinofuranosidase, amylase, protease, pectinolytic enzymes, rennet, alpha galactosidase, lipase, invertase and phytase (Maheswari and Chandra, 2000; Sindhu et al., 2009; Soarse et al., 2010; Javed et al., 2011). Xylanase production on commercial scale can also be achieved by using WB as a substrate as it is an agro-economical inducer due to its high xylan content (12.65% of dry material) (Kulkarni et al., 2011).

✓ *Nitrogen source*

Naturally, higher amount of the nitrogen also requires no or little addition of other nitrogen supplements in WB containing medium. Elevated nitrogen content of WB makes it suitable for the production of enzymes such as protease, amylase and glucoamylase. Increase in the production of acid protease with an increase in the nitrogen content of WB has been reported by Vishwanatha et al., 2009. Supplementation of WB with additional protein sources such as soy flour, defatted sesame flour, casein and peptone facilitate acid protease production. Although WB alone can be used as an efficient nitrogen source but supplementation of WB with glucose, peptone, yeast extract, KH2PO4 and CaO resulted in the highest spore production 1.7 × 1011 spore/g dry substrate (Vishwanatha et al., 2009).

2.11.2 Starch processing and food industries

The major market for α-amylases is in the starch industry, which is used for starch hydrolysis in the starch liquefaction process that converts starch into fructose and glucose syrups. The enzymatic conversion of all starch includes: gelatinization, which involves the dissolution of starch granules, thereby forming a viscous suspension; liquefaction, which involves partial hydrolysis and loss in viscosity; and finally saccharification, involving the production of glucose and maltose via further hydrolysis. This process requires the use of a highly thermostable a-amylase for starch liquefaction, which can act at temperatures around 70-100°C depending upon the temperature. Thermostable *a*-amylases are generally preferred as their application minimizes contamination risk and reduces reaction time, thus providing considerable energy saving. Hydrolysis carried out at higher temperatures also minimizes polymerization of D-glucose to isomaltose. The addition of α-amylase to the dough results in enhancing the rate of fermentation and the reduction of the viscosity of dough, resulting in improvements in the volume and texture of the product.

2.11.3 Production of high conversion syrup

They are produced from liquefied starch by hydrolysis with α-amylase and glucoamylase. This syrup comprises about 40% glucose, 45% maltose and the remainder maltotriose. They are used extensively in the brewing, baking, confectionary and soft drink industries.

2.11.4 Alcohol and biofuel industry

Bacterial amylase find a wide spread application for the hot liquification of starch containing materials before fermentation. This saves the purchase and storage of malt, which moreover may contain undesirable microorganisms that could cause an excessive pH drop during fermentation. Alcohol yields are increased by 1.0-3.0%.

For the bioethanol production, starch is the most used substrate due to its low price and easily available raw material in most regions of the world. In this production, starch has to be solubilized and then submitted to two enzymatic steps in order to obtain fermentable sugars. The conventional process for the bioconversion of starch into ethanol involves saccharification, where starch is converted into sugar using an amylolytic microorganism or enzymes such as gluco-amylase and α-amylase, followed by fermentation, where sugar is converted into ethanol using an ethanol fermenting microorganism such as yeast *Saccharomyces cerevisiae*.

2.11.5 Detergent industries

The use of enzymes in detergents formulations enhances the detergents ability to remove tough stains and making the detergent environmentally safe. Amylases are the second type of enzymes used in the formulation of enzymatic detergent, and 90% of all liquid detergents contain these enzymes. These enzymes catalyze the hydrolysis of glucosidic linkages in starch polymers, commonly found in foods such as pasta, fruit, chocolate, baby food, barbecue sauce and gravy. Examples of amylases used in the detergent industry are derived from *Bacillus or Aspergillus.*

2.11.6 Textile industries

Amylase are now widely used to remove starch which is used as an adhesive or size on threads of certain fabrics to prevent damage during weaving. Currently in the textile industry, there is a wide spread demand for faded jeans. This involves subjecting such clothes to amylases, a process commonly referred to as biowashing or biobleaching, an alternative to the term enzyme fade. This allows elegant softness and unique shades to be given to the cloth which overcomes the traditional methods of bleaching by sodium hypochlorite or trembling with pumice stones and also offer better safety as well as economy. It also randomly cleaves the starch into dextrins that are water soluble and can be removed by washing. α- amylases is used in warp sizing of textile fiber for manufacturing fibers with great strength

2.11.7 Clinical and medical applications

Amylases would be potentially useful in the pharmaceutical and fine chemicals industries if enzymes with suitable properties could be prepared. Interestingly, the first enzyme produced industrially was an amylase from a fungal source in 1894, which was used as a pharmaceutical aid for the treatment of digestive disorders. Synthetic and natural biodegradable polymers have been a major focus of interest in pharmaceutical research. The biodegradable polymers are used to control the drug release rate from parenteral controlled delivery systems.

2.11.8 Leather processing

In the manufacture of leather, the hide is made free from hair. This is done by employing pancreatic enzymes which hydrolyze the proteins of the hair follicles, thus freeing the hair so that it may be easily scraped off from the hide. Enzymes are currently applied at various stages of leather processing, from pre-tanning (i.e. including soaking, dehairing, bating and degreasing) operations until the final stages. Moreover, for effluent treatment that's generated during the leather making process.

The leather processing involves various operations in a cascade manner from raw hide to processed leather. The complete leather manufacturing process is divided into three fundamental sub-processes: preparatory stages, tanning, and crusting which runs in hand to hand manner.

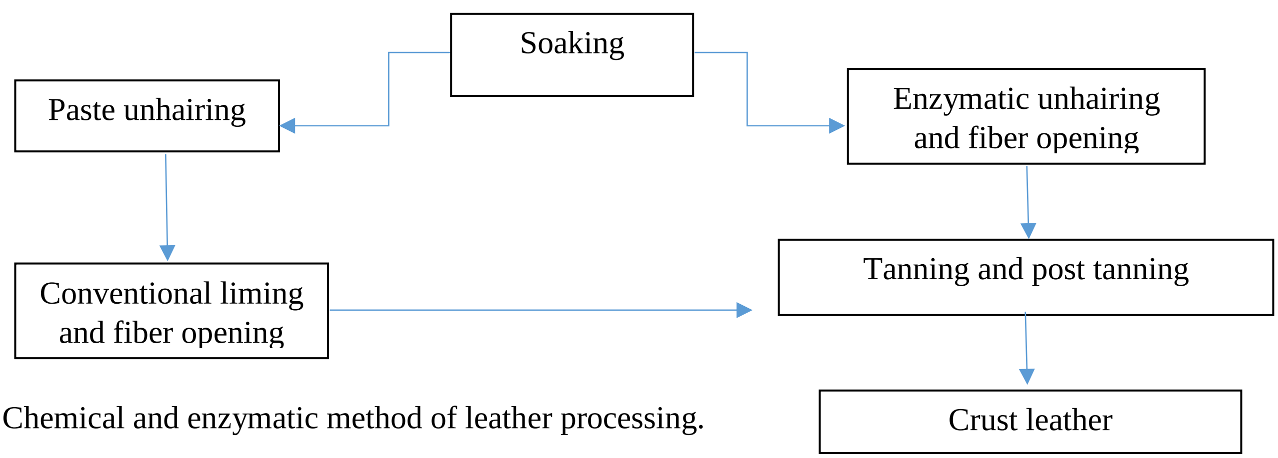


Fig 2.5: Chemical and enzymatic method of leather processing.

The raw hide has to undergo many of chemical treatments in cascade manner before it turns into flattering leathers which is comprised of soaking, liming, unhairing, deliming, bating, degreasing, and pickling. For most of these steps the chemicals used are quite toxic and used in large amount in these pre-tanning operations which results the leather processing industry one of the worst offenders of the environment.

2.12 Mechanism Involved In Enzymatic Pre-Tanning Operations

2.12.1 Un-Haring

Un- haring is the process of removing hairs and furs from the skin or hides without causing any grain damage. The enzyme which is used for un-haring is protease. Unhairing involves the digestion of basal cells of hair bulb and cells of malphigian layer. This is followed by loosening of hair with an attack on outermost sheath and breakdown of inner root sheath and parts of hair that are not keratinized. With this enzymatic process, it is possible to reduce the chemical exposure and enhance the softness and area yield. The use of chemicals will completely dissolve the hair but the enzyme helps in filtering out the hair thus reducing the BOD and COD demand of the waste water. This current process helps in improving the strength properties of the leather with greater surface area and provide ecofriendly environment to the workers. Enzymatic hair loosening processes play a role wherever high-quality hair, wool or bristles are to be recovered

2.12.2 Fiber Opening Mechanism

Fiber opening enzymes are provided for the mechanism which seems to reduce the pollution load and need for water treatment thus proving cost effective. The inter fibrillary proteins, which are mostly mucoids that contain carbohydrate as prosthetic groups, are removed during fiber opening. These non-collagenous proteins are known as proteoglycans are non-collagenous proteins and it has been shown that α-amylase has specific activity on carbohydrate-containing proteins (Thanikailvelan et al, 2004) have developed the enzyme-based fibre opening for cow hides using α-amylase without using lime at pH 8.0. The collagen is bound by proteoglycans which contain protein and glucose linked by the glycosidic linkages. α-amylase is substrate-specific enzyme engaged for this purpose to degrade these cementing substances made of glycoproteins and proteoglycans which opens the fiber matrix and induces swelling.

2.12.3 Conventional chemical Fiber opening and Unhairing

Conventionally chemicals have been used for these pre-tanning operations. The conventional unhairing method involves the use of high proportions of lime and sulfide, which contributes to 80–90% of the total pollution load in the leather industry and generates noxious gases as well as solid wastes, e.g. Hydrogen sulfide and lime. Since these chemicals are becoming problem for the environmental pollutions hence use of enzymatic treatments are also necessary to get optimum results without affecting environments. A class of enzyme, proteases has been used successfully in last two decades. One such treatment, bating, is the only step in leather processing where enzymatic process cannot be substituted by chemical processes. The unhairing by enzyme offers certain desired characteristics to the finished leather. Earlier, the process was carried out using dog dung or manure. The use of this was not only unhygienic but fermentation could also not be controlled.

2.12.4 Conventional Process vs Enzymatic Process

Conventional leather processing results in causing environmental pollution by discharging significant amount of environmental contaminants. But for the same process, the enzymatic method reduces the discharge of effluents by 90%. The emission of gaseous and aqueous discharge is large in the conventional process which is mainly responsible for global warming and climatic change. This is due to the high contribution of conventional process towards biological oxygen demand (BOD), chemical oxygen demand (COD), total dissolved solids (TDS), chlorides, sulphides, sulphates, lime, chromium, etc.

This is not in the case of the current invention since the enzymes not only efficient but also biodegradable. Also the conventional process is very tedious and time consuming since it involves many steps comprising soaking, liming, reliming, deliming, bating, pickling, chrome, tanning, basification, rechroming, utralization, washing, retanning, dyeing, fixing. In enzymatic process, these steps are replaced through the biocatalytic process in which the uptake of various chemicals is achieved by process innovations.

CHAPTER THREE

3 MATERIALS AND METHODS

3.1 Materials Required

An agro industrial residue called wheat bran was used as a solid support for solid state fermentation process. Glass was used for making an extractor column and also goat skin for application of enzyme. Laboratory equipment's (Centrifuge, hot air Oven, incubator, spectrophotometer, humidity chamber, sample collection containers (plastic), pH meter, rotary shaker, Laminar Air Flow Chamber etc.) used for the production, extraction and characterization of enzyme.

3.2 Methods

In this study, various experiments were designed and carried out in order to produce optimum amount of alpha amylase from *Bacillus subtillis* by column extractor under SSF. The methods used to reach at each specific objectives of this study are described as in the following sub-titles.

3.2.1 Sample collection

A good quality wheat bran is obtained from a local shop near Adyar city (India). And two goat skin are taken from tannery for enzyme application purposes.

3.2.2 Design of column

Assuming the extractor column will be packed by a WB without clogging or clumped together and assume that two extractor columns exhibit identical volume, bulk densities and total retention time for single and dual column extraction system is the same. And assume the total volume of extraction buffer before and after column extraction process is equal. Moreover in dual column extraction process, assume the input flow rate of an extractor buffer is equal to the output flow rate of the extractor column. Assume there is a negligible instrumental and human error in performing this experiment.

The design of the extraction column is based on the 1 liter of measuring cylinder. And hence calculating the volume and bulk density on the basis of dimensions of the 1 liter measuring cylinder and the wet wheat bran holding capacity of cylinder respectively was done. The design calculations are as follows;

Given: 1 liter measuring cylinder having a diameter of 6 cm and height of 57 cm.

Volume of cylinder:

$$Vol = \pi r^2 h$$

$$= 1{,}610.82 \text{ cm}^3$$

Calculating bulk density (BD):

Given;

- 1 liter of measuring cylinder weights 618 grams.
- Cylinder holding capacity measures of wet wheat bran alone to be 547 grams.

Therefore:

BD = Holding capacity of cylinder/ Volume of cylinder

$$= 0.34 \text{ gm/cm}^3$$

According to the bulk density of the cylinder, the extractor column was fabricated with dimensions of inner diameter and outer diameter of 6 cm and 7 cm respectively. Total Heights of 85 cm and stacking bed height of 52 cm. More over the mesh size is designed to have an aperture of 0.2 cm openings and a total diameter of 67mm.

Real aspects to consider during column extraction process are like; not only freshly prepared buffer solutions are used but also equal volume of fermented WB should be used for extraction procedure. And also the extraction process should be performed in favorable environmental conditions. After the extraction process completed, assays should be performed as soon as possible.

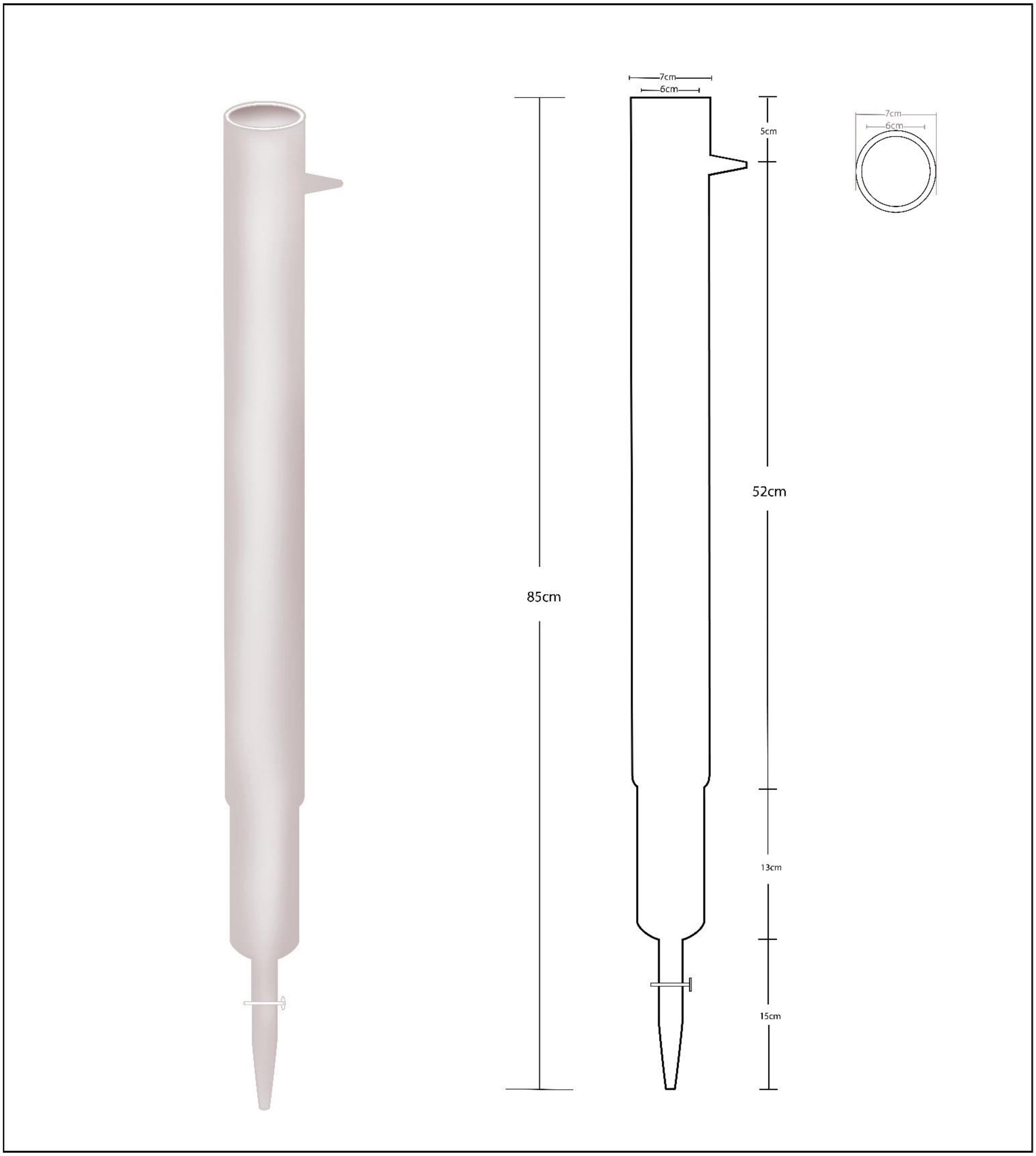


Figure 3.1 Extractor column, (designed by ArchiCAD 19.0 and Lumino pro 6.0)

3.2.3 Micro organism

A bacterial strain *Bacillus subtilis* Cultures were obtained from Department of Biotechnology (CSIR-CLRI) grown on agar broth. The culture was sub cultured in every month and maintained on LB agar at 4°C. Confirmation analysis was carried out before sub culturing procedure. Pass the slide through the flame of a Bunsen burner 3 times to heat fix the sample. Place the drops of crystal violet dye on the heat-fixed culture for 1 minute. After rinsing with water, add 5 drops of a solution of gram iodine. Wash the sample with alcohol and acetone. Hence by viewing the sample with microscope at 1000x magnification; the bacteria appear purple. Thus our bacteria is gram positive strain.

3.2.4 Sub culturing

A 100 ml of LB Agar was prepared with the following reagents namely; Yeast Extract, NaCl, Tryptone and agar with the composition of 0.5%, 1%, 1%, and 2.5% respectively and it was melted on a hot plate for the agar to dissolve completely. After which, it was poured into the slant test tubes 10-15 ml each. The slant test tubes were each then cover with cotton plug and kept for autoclaving. After autoclaving was done, the slant tubes were kept slanting on an L-rod under the laminar flow chamber for the agar to solidify in a slant position.

Figure 3.2. Bacillus S. culture on agar plate

After solidification of agar in the slant tubes, they were inoculated in zig-zag streak with a loop of *B.subtilis* from previously prepared agar slant. The inoculated slant test tubes are then kept in incubator at 30^0C. After *B.subtilis* growth was observed, the slant tubes were stored in refrigerator for future use.

3.2.5 Pre pre inoculum preparation

In the preparation of the Pre pre inoculum, the following procedures are followed.

3.2.5.1 LB broth preparation

LB Broth Composition: 0.5% Yeast Extract, 1% NaCl and Tryptone. A 100 ml of LB Broth was prepared according to the above composition in a 250ml Erlenmeyer flask and wound with a cotton plug and autoclaved at 121°C and 15 lbs. for 20 minutes. After autoclaving is done, the flask containing the LB broth was inoculated with a loop of *B.subtilis* from LB agar slants, under laminar air flow chamber. After inoculation the flask was kept in shaker at 120 rpm for 24 hours at 30°C.

3.2.5.2 Wheat bran autoclaving

- A good quality wheat bran obtained from local market was used as a substrate for α-amylase production.

- Mix thoroughly raw wheat bran with distilled water in the ratio of 1:1. Then autoclave it under 121 °C at 15 bar pressure for 20 minute.

3.2.6 Pre inoculation

Bacillus subtilis Cultures were grown on LB broth. The culture was sub cultured in every month & maintained at 4°C. The *Bacillus subtilis* culture slants maintained at NMITLI facility of CSIR-CLRI, Chennai, was taken as the microbial source for the study. A loopful of microbes from the slant were taken and inoculated into 50ml LB broth aseptically and incubated in rotary shaker at 120 rpm overnight (18h) at 30^0C.

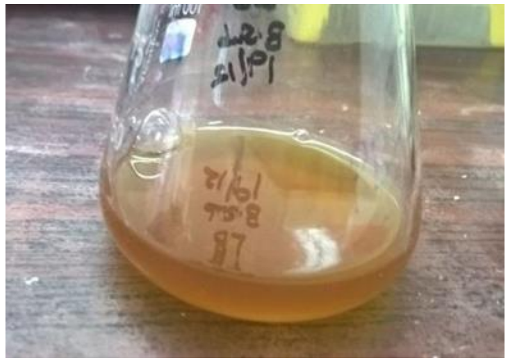


Figure 3.3. B. subtilis Cultures were grown on LB broth

3.2.7 Scale up production

A 10 gram of solid substrate were taken in a series of 250mL Erlenmeyer flasks containing wheat bran. The substrates were moistened with 10 mL distilled water (initial moisture content of 60%) and it was sterilized by autoclaving at standard conditions (121°C, 15 lbs/in^2 for 20 minutes). Once it is cooled, the flasks were inoculated with 250µL of spore suspension and the contents were thoroughly mixed using a sterile spatula and incubated at 30°C for 5 days. Samples were taken at 24 h intervals and extraction of enzyme was carried out accordingly.

The scale up process was carried out from 10gm flask level to 1kg. A 24hr old inoculum (4ml) was used as the pre inoculum for 10gm flask in 250ml Erlenmeyer conical flasks and kept in an incubator at 30^{0}C.

For 300gm, substrate inoculum of 30gm (incubated for 24hr) was inoculated into 270gm of substrate in a series of sterilized perforated steel trays (43x 22 cm) in a laminar air flow chamber. And its incubated at 30 ^{0}C in BOD incubator for a period of 96hr.

Similarly, tray level production was carried out using 1kg tray in sterilized perforated steel trays (40 x 60 cm) using 300 g of substrate inoculum per tray and assayed for enzyme activities at 24 hr time intervals. Samples were assayed for amylase activity at 24 hr interval.

Figure 3.4: Perforated tray

3.2.8 Enzyme extraction

Lab scale extraction of extra cellular enzyme from the fermented bacterial wheat bran involves the addition of 100ml phosphate buffer. Mix the solution thoroughly with the help of mortar and pestle or mixer. This was then squeezed and filtered using a muslin cloth. The entire mass of enzyme was thus extracted using the above procedure and biomass was kept aside. The Filtrate obtained was then cold centrifuged at 10000 rpm for 10 min to separate from smaller particles. The supernatant obtained was kept as crude for enzymatic assay, whereas the pellet gets discharged.

3.2.9 Enzyme assay

Amylase activity was assayed based on the amount of reducing sugar released following the modified dinitrosalicylic acid (DNS) method of *miller*. The DNS reagent was composed of: 2g/L phenol; 0.5g/L sodium sulfite; 200g/L sodium potassium tartarate (Rochelle salt); 10g/L sodium hydroxide (NaOH) pellet; and 10g/L dinitrosalicylic acid (DNS).

The enzyme extract were transferred to micro centrifuge tubes and centrifuged at 10000 rpm for 10 min. Supernatant was separated from cell debris and used as the crude enzyme. Bernfield miller procedure was used to determine amylase activity.

$$\text{Amylase activity, U/gds} = \frac{\text{Amount of glucose released (mg) x Dilution factor}}{\text{Reaction time (15 min) x Volume of enzyme (0.1 ml)}}$$

One unit of amylase activity was defined as the amount of enzyme releasing one µmol of glucose per ml per min at pH 6.5 and 50 ^{o}C and the activity was expressed as U/g of dry substrate (U/gds). Moisture content was estimated in the fermented substrate using a moisture analyzer. ***Unit = OD*1/Slope*volume of buffer*DF / Volume of enzyme*incubation time* Dry weight of substrate (U/gds).***

Table 3.1 Amylase assay protocol

Reagents	**Blank(ml)**	**Test 1(ml)**	**Test 2(ml)**	**Test 3(ml)**
1% starch	1	1	1	1
0.1 M phosphate buffer, pH 6.5	1.9	1.9	1.9	1.9
	Pre incubated @ 50^{0}C for 10 minutes			
Enzyme	----	0.1	0.1	0.1
	Incubation @ 87^{0}C for 10 minutes			
DNS	3	3	3	3
	Kept @ 87^{0}C for 10 minutes			
Potassium sodium tartarate	1	1	1	1

3.3 Characterization of enzymes

3.3.1 Optimization of substrate along with their particle size

It's well acknowledged that starch and starchy material are idle for the production of α-amylase. Since wheat bran have a high amount of starch material of nearly 20%, it makes it an outstanding substrate for α-amylase production and extraction process. Wheat bran (WB) with different particle size were analyzed for amylase extraction. The substrates was subjected to sieving employing mesh size of 1.41 mm. Any particle that passes through the sieve were called fine particles whereas the retained ones were called course particles. A 300 g of substrate were used for screening studies. Samples were collected at every 24 hrs and analyzed for amylase activity.

3.3.2 Effect of PH

Extraction of the fermented substrate was carried out with different buffer solution to find out the best extracting buffer. Enzyme sample was taken and mixed with equal proportions of buffers ranging from pH 3-10.

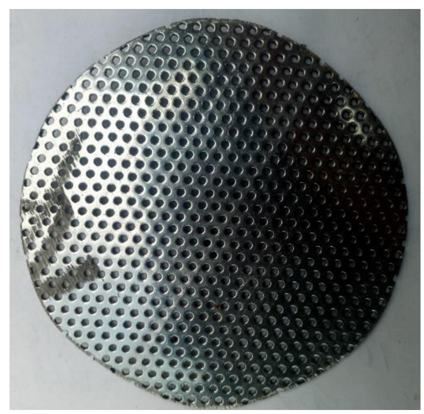
3.3.3 Effect of temperature

In order to determine the effect of temperature on amylase activity, the enzyme sample was taken and assay was performed for the enzyme at different temperatures ranging from 30-80˚C. The absorbance was then measured using a UV spectrophotometer.

3.4 Column extraction system

3.4.1 Single column extraction system

It consists of a mesh filter of having a pore size of 0.2-0.3mm. In single column extraction process, both batch and continuous extraction processes were carried out.



A B

Fig 3.6. Glass made extraction column (A), and Filter Mesh (B)

3.4.2 Dual column extraction system

In dual column extraction system, two columns of the same size and design are used for extraction process in such a way that the buffer is fed to the first column in a continuous manner by the help of peristatic pump. And the eluent of the first column is fed to the second column and finally the crude enzyme was obtained and analyzed for the activity. In this type of extraction system, a particular bed volume was equally divided into two separate columns. This process is carried out while other parameters kept constant.

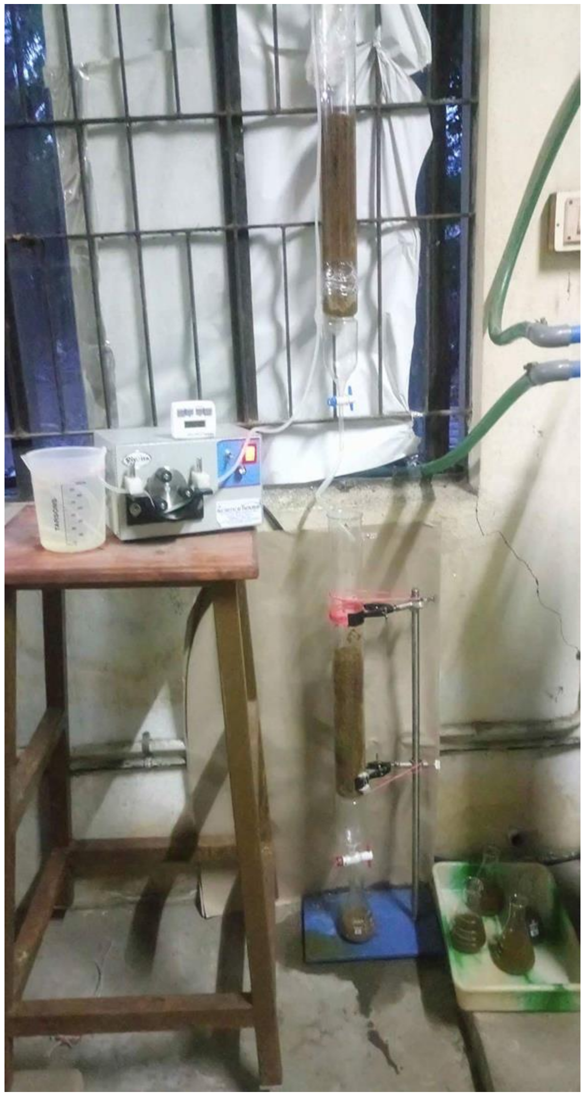


Figure 3.6. Dual column extraction.

3.5 Optimization of extraction parameters

Extraction shall be carried out using pump / manual feeding and parameters viz., feed rate, recycling, packing density, etc. optimized. The list of parameters optimized are mentioned below.

3.5.1 Optimization of flow rate

Various flow rates are taken at a constant bed volume in order to study their influence on the final activity of the enzyme. Here 1 litter of buffer solution at flow rates ranging from 50 to 150ml/min is allowed to pass through the column. Hence the flow rates are optimized to yield the peak activity of enzyme extract. A fraction is an eluent that contains a crude enzyme extract from the fermented WB. The concentration of the enzyme drops as the number of fraction increase. First 100gm of bed volume was taken with the respective flow rates. The eluent was collected at five fractions of 250 ml conical flask.

3.5.2 Optimization of bed volume

The extraction column was packed with different bed volume of height ranging from 100gram to 400gram fermented wheat bran and activity was studied at a constant flow rate of 100ml/min in a continuous process. And finally the eluent are collected in fractions and their activities were analyzed.

3.5.3 Batch vs continuous extraction process

A 100 gm of previously fermented WB was added to the column. Then 1 liter of buffer was poured slowly in to the column three times. The eluents were collected after 15 minutes of retention time. The collected eluent was taken in three distinct flasks at equal time interval and the activity was measured for each fractions as well as the combined one.

Unlike batch process, continuous system of column extraction involves a continuous flow of buffer by using peristatic pump. The column is filled with 100gm of fermented WB and the buffer solution is allowed to pass at a controlled flow rate ranging from 50 to 150ml/min. And finally the activity of the eluent is checked.

3.6 Partial purification of enzyme

Once the fermentation was over, the fermented broth was centrifuged to remove the biomass and the resulting cell-free supernatant was subjected to different purification steps like micro and ultra-filtration for partial purification, which would thereby help in increasing the shell life and stability of the enzymes. The Ultra filtered samples were estimated by Lowry's method

3.6.1 Microfiltration and Ultrafiltration

Ultrafiltration is one of the downstream processing steps by which the enzymes are concentrated based on their molecular weight. The equipment contains a pressure monitor and the membrane is inserted at the right as shown above. Initially a micro filter membrane of 0.2μ pore size is placed vertically in the right tube. And the left container is initially filled with water and the apparatus is switched on. This is done to wash the membrane and the apparatus of retained dust or impurity.

Afterwards, the supernatant collected from centrifugation is passed through the container on the left side which passes through a 10 kDa hollow-fibre membrane with a surface area of 420 cm^2 (GE Healthcare) at a certain flow rate and pressure using a peristaltic pump. And particles lesser than 0.2μ size are collected as filtrate. The desired enzymes which of molecular weight greater than the pore size will hence be rejected by the membrane and will be collected as retentate while those lesser than the pore size would be in the filtrate. The retentate thus obtained was concentrated and partially purified.

Figure 3.7. Micro and Ultra filtering equipment

After filtration, washing process needs to be carried out before removing the membrane. This is done so as to remove any residual particles settled within the membrane and also to maintain the membrane's efficiency and longevity.

Ultrafiltration membrane of 10 KDa pore size was placed in a similar manner and was allowed to undergo a water wash. Since the molecular weights of amylase is higher than 10 KDa, they are collected in the retentate while the filtrate is discarded. After the filtration process, the membranes were washed thoroughly and then stored at 4°C till further use

3.7 Application of amylase for fiber opening

This particular section has been attempted to focus on the pre-tanning operations especially on fiber opening by comparing the conventional method of fiber opening and experimental enzyme application on leather.

3.7.1 Drum Method

Two wet salted goat skins were used for this study. After the skins were cut in to two equal parts, one half were used for enzymatic treatment and the other half for conventional enzyme assisted chemical treatment. The initial weights of all the samples namely; sample C, BF-I, BF-II, AF-I and AF-II were noted after which all the experiments where done with enzyme concentration ranging from 2 to 5% amylase enzyme respectively, 100% Distilled Water.

For enzymatic opening up of fiber of the skin by crude amylase enzyme extract is used as an experimental method. The commercial enzyme product were used for control. The drumming method also employed to accelerate the process. The pH of the float adjusted to 6.5-7.0 before addition of enzyme for effective fiber opening. The wet weight of each skin after dehairing was noted. The control sample C, was treated with 10% (w/w) lime and 100% water for 48 hours followed by deliming and pickling. The experimental samples BF-I, BF-II, AF-I and AF-II were treated with crude enzyme having a volume ranging from 2% to 5% each of in-house amylase concentrate, and commercial enzyme product respectively along with 100% water in a drum for 4 h, followed by pickling process.

The spent enzyme liquor was collected from each sample bath at every 1 hour and taken for estimation of protein spectrophotometrically using standard procedures.

Table 3.2 Soaking, unhairing and liming of goat skin

Process	**%**	**Chemicals**	**Time, min**	**pH, ^{0}Be, etc**	**Remarks**
Wash, 3x	100	Water	10	^{0}Be<1.0	drained
Soaking	100	Water			
	0.5	Sodium carbonate			
	0.1	Wetting agents		pH 10	Run 2min every hour
Paint solution	2	Sodium sulfide		^{0}Be 14-16	
	6	Lime		^{0}Be 28-30	
					Enzymatic unharing
Add unhaired skins in separate drums	0.1	Wetting agents	20	pH 12	Run 10min

3.7.2 Dehairing, Fleshing and Fiber-opening Process

Hair-Loosening, opening of fibers and extent of fleshing was observed every hour. The skin is soaked in the enzyme solution in the drum overnight and observed until complete hair-loosening is observed. Final weights of the skins were noted and compared, after which samples from each drum were taken for further analysis.

Table 3.3; Deliming, bating and pickling

Process	%	Chemicals	Duration (min)	Remarks
Deliming	30	Water	30	
	0.1	Sodium metabisulphite		
		Ammonium sulphate	60	pH 7.5- 8.0
Bating	50	Water		
	0.5	Bating enzyme(basozyme CM)	60	For control
	2-5	*Crude enzyme*	60	*For experiment*
				D/W/D
Degreasing	3	Salt		
	0.5	Degreasing agent		
Pickling	100	Water		
	10	Common salt	10	
	0.3	Formic acid		
	0.2	Sulphuric acid	90	pH `2.8-3.0

3.7.3 Evaluation of using enzyme on fiber opening/splitting application

Wet-salted goatskins were chosen as the raw material for this study. Two soaked goatskins were de haired using standardized dehairing process as developed earlier. For fiber opening evaluation, the methodology reported by Thanikaivelan et al. was followed. Each skin was cut along and across into four pieces and labelled as C, BF-I, BF-II, AF-I and AF-II. The wet weight of each skin after dehairing was noted. The control sample was treated with 10% (w/w) lime and 100% water for 2 days followed by deliming and pickling. The experimental samples BF-I, BF-II, AF-I and AF-II were treated with 2% to 5% of the experimental product concentrated amylase based on soaked weight respectively, and the control done with commercial enzyme products respectively along with 100% water in a drum for 4 hrs followed by pickling process.

3.7.4 Analysis of spent fiber opening liquor

The spent liquor from fiber opening process was collected from control and experimental samples and analyzed for the liberation of proteoglycans spectrophotometrically using standard procedures. The results are expressed in mg^{-1} of the sample.

3.7.5 Tanning and Post Tanning

The enzyme treated skins were washed thoroughly and taken for conventional pickling process. The deliming and bating process were eliminated in the enzyme based process as the pH is around 8-9 and also lime was not used. The pickled pelt was subsequently processed for conventional chrome tanning using 8% BCS as given in following Table.

Table 3.4. Process recipe- tanning

<table>
<tr><th>Chemicals</th><th>% offer</th><th>Time(min)</th><th>Remarks</th></tr>
<tr><td>Pickle liquor</td><td>50</td><td rowspan="2">60</td><td rowspan="2">check pH 2.8-3.0</td></tr>
<tr><td>BCS</td><td>8</td></tr>
<tr><td>Water</td><td>50</td><td>30</td><td></td></tr>
<tr><td>Sodium formate</td><td>1</td><td>30</td><td></td></tr>
<tr><td>Sodium bicarbonate</td><td>1-1.5</td><td rowspan="2">4*10+60</td><td rowspan="2">check pH3.8-4.0 Drain: Aged for 24 h</td></tr>
<tr><td>Water</td><td>50</td></tr>
</table>

Process/chemicals	%	Duration(min)	Remarks
Wetting back			
Water	200	60	D/W/D
Wetting agent	0.5		
Rechroming			
Water	100		Check pH 2.8-3
Acetic acid	1	3*10'+ 30'	
BCS	3	40	
Chrome syntan	2		
Sodium formate	1	30	Check pH 3.8-4.2, L/O/N
Sodium bicarbonate	0.7	3*10'+30'	
Neutralization			
Water	150	10	
Sodium formate	0.5		
Sodium bicarbonate	0.3	(3*10)+30'	pH 5.0-5.2, Drained
Washing			
Water	200	20	D/W/D
RS-40	3		
Retanning			
Water	100	40	
DI	4		
FBb	4		
Tara	4		
Fatliqouring			
Synthetic fatliqour	2	60	
Semi synthetic	2		
Fish oil	1		
Neats foot oil	1		
FBb	3	20	
Formic acid	2	(2*10')+30'	D/W/D/P. N/D setting, hook to dry, staked and buffed

Table 3.5. Process recipe- post tanning crust leather.

3.7.6 Scanning Electron Microscope (SEM) Analysis

Samples from enzymatic as well as conventional de-haired pelts of goat skin were cut, washed properly with distilled water, fixed in buffered formalin, dehydrated with a series of methanol and then finally with acetone. After that samples were flushed with nitrogen gas to remove the acetone completely and freeze dried. The freeze dried samples were cut into 3-4 mm thickness, mounted vertically or horizontally on copper stubs, coated with platinum. Cross and surface view of samples was examined in SEM unit operated at an accelerating voltage of 5KV.

3.7.7 Physical characteristics of crust leather

The physical properties such as tear strength, tensile strength, elongation at break and grain crack for control and experimental crust leather were performed by standard procedures.

CHAPTER FOUR

4 RESULTS AND DISCUSSION

4.1 Effect of pH on Amylase Activity

Enzyme sample was taken and mixed with equal proportions of buffers ranging from pH 3-10 (using citric buffer pH 3, acetate buffer pH 4&5, Phosphate Buffer pH 6, 6.5, 7& 8, TRIS Buffer for pH 9 and carbonate buffer for pH 10) with 0.1M concentration; the assays were performed based on standard conditions.

As observed from pH vs activity graph, the activity of amylase enzyme is the highest (1332.31 U/gds) at pH 7.1, after which there is a significant drop in activity. According to the result α-amylase activity was considerably reduced at pH range of low acidic(citrate buffers) as well as at high basic pHs(Tris HCL buffers).

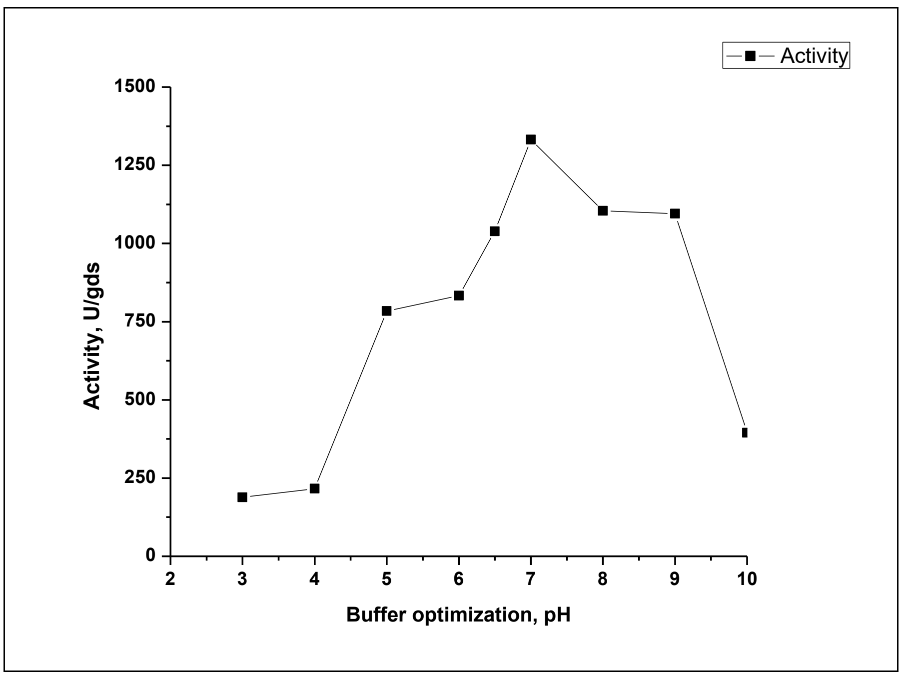


Fig. 4.1. Effect of pH on enzyme activity.

It was observed that the activity remained stable with little to no change in activity in a particular pH specific for enzyme amylase at pH 7.1. Therefore, it can be inferred that the maximum activity of the enzyme obtained is at these specific pH.

4.2 Effect of Temperature on Amylase Activity

The effect of varying incubation temperature (30-50°C) on the production of amylase was studied using SSF. Maximal amylase production of (2374.51 U/gds) was obtained in fermentation flask which was incubated at 30°C and the least amylase production of (450.16 U/gds) was showed at 40°C. Whereas, there was almost none amylase production that was incubated at 50°C. This is mainly due to the denaturation of the enzyme or protein at high temperature scale.

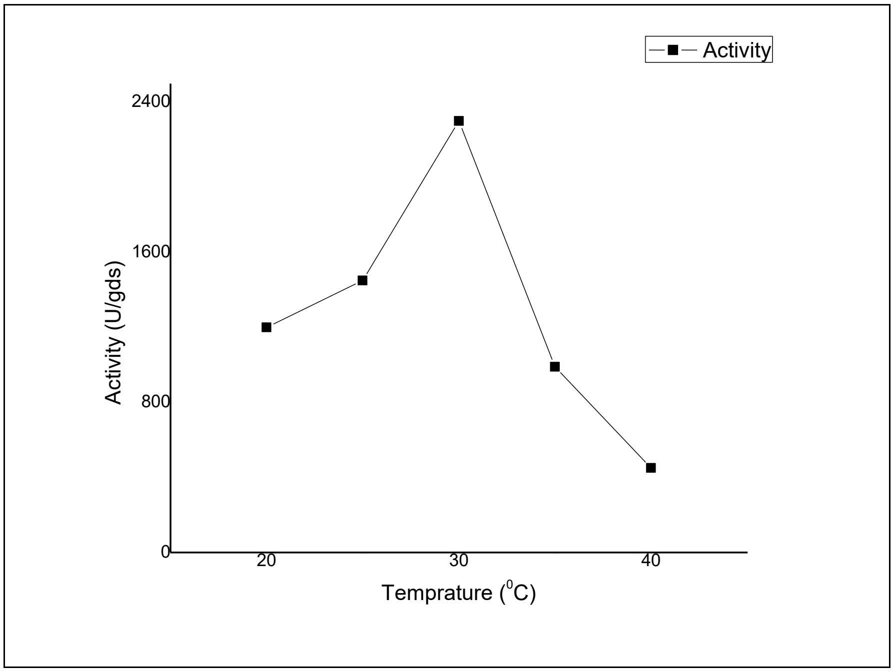


Fig 4.2. Effect of temperature on enzyme production

4.3 Scale up of enzyme production

The scale up experiments on enzyme production from flask to tray level fermentation were carried out. Scale up amylase production at 300 g, 500g and 1kg tray resulted in activity of 1472.6 U/gds, 973.18 U/gds and 525.25 U/gds respectively.

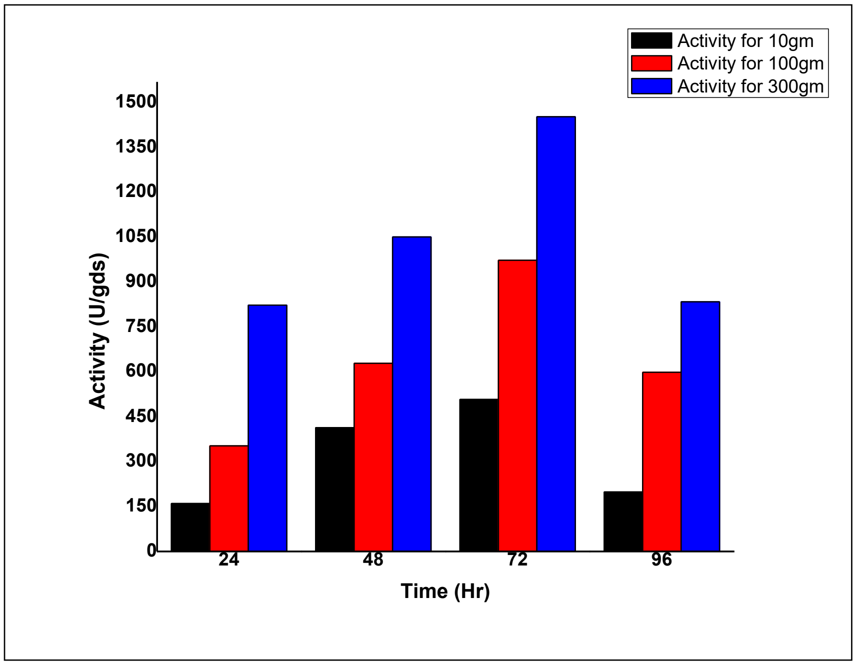


Fig 4.3 Scale up production for 300gm

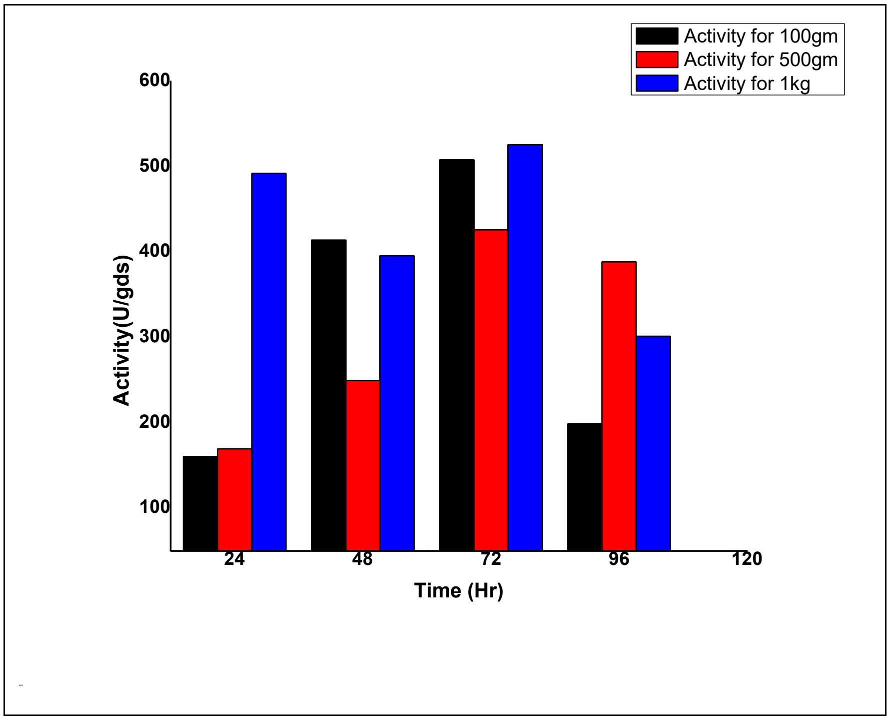


Figure 4.4: Scale up production for 1kg.

The decrease in activity at the tray level could be attributed to the increase in temperature due to the growth of bacterial culture during the fermentation process. Since SSF process typically produces high levels of heat and increased moisture content, thus the heat evolved and moisture developed during the process directly not only adversely affects the metabolic activities of the microorganisms but also the productivity of the desired product by naturally diluting its activity. (Ghildyal *et al* 1994).

4.4 Optimization of extraction parameters

Various physical and chemical parameters are optimized such as pH, temperature and particle size of WB along with optimizing the flow rate of buffer solution flowing through the column, packed with fermented WB as a bed volume. Assessment of batch and continuous column extraction system carried out for maximum extraction capability of amylase enzyme.

4.4.1 Optimizing substrate with their particle size, flow rate and bed volume

An agricultural waste like wheat bran which was used in this study was the best substrate for amylase extraction. Using wheat bran as a substrate for enzyme extraction is one way of value addition of wastes as well as a valuable input for amylase production. Next, in order to study the relationship between particle size and activity; substrates were crushed and sieved accordingly to coarse and fine particles. Then their activity was analyzed in every 24 hour.

The Wheat bran (WB) were subjected to sieving employing mesh size of 1.41 mm. Any particle that passes through the sieve were called fine particles; Fine wheat bran (FWB), whereas the retained ones were called course particles; Coarse wheat bran (CWB). A 100 g of substrate were used for screening studies. Samples were collected every 24 h and analyzed for amylase activity. Based on various particle size of the substrate namely; coarse and fine sized brans, the extraction experiment was carried out.

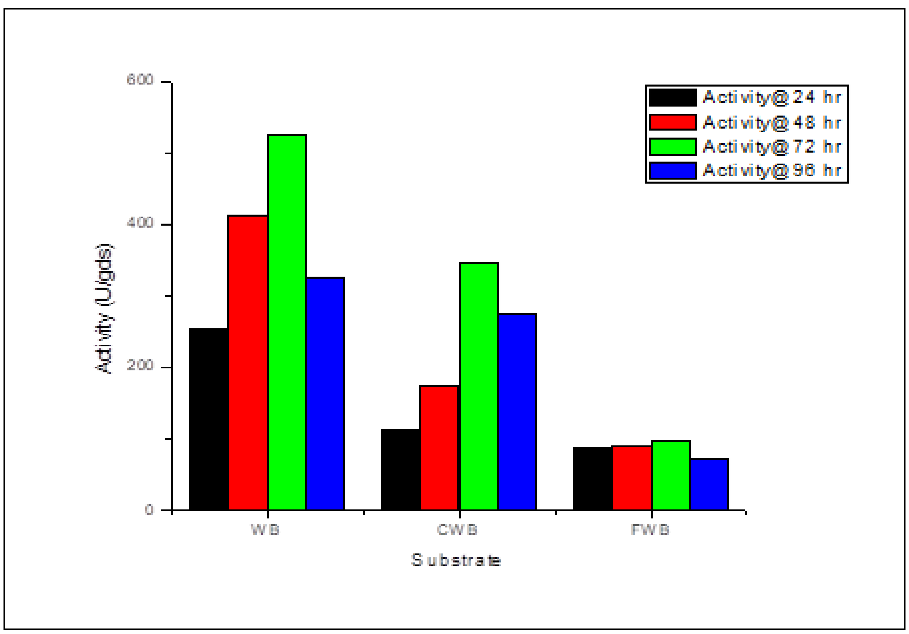


Fig. 4.5 Enzyme activity compared with particle size

The medium sized wheat bran (WB) has by far the highest activity (525.78 U/gds) at 72hrs of incubation period as compared to the other subdue particles of WB. This is due to the availability of larger surface area for the sustainability of enzymatic action. Whereas coarse and fine sized

WB have lower surface area for the enzyme to act, hence their potential for extraction of enzyme would be lesser respectively.

4.4.2 Optimization of flow rate

Various flow rates are taken at a constant bed volume to study their influence on the final activity of the enzyme. Here 1litter buffer solution flows at rate ranging from 50 to 150ml/min are optimized to check the peak activity. First 100gm of bed volume was taken with the respective flow rates. The eluent was collected at five fractions of 250 ml conical flask.

As it's shown in the graph below, the enzyme activity is much higher at a flow rate of 100ml/min compared with other flow rates. In addition to this fractional comparison, activity was studied with a combined enzyme activity with respect to each of the flow rates. According to the result the combined activity of 280.7 U/gds, 245.2 U/gds, 343.5 U/gds, 250.3 U/gds, and 229.6 U/gds was recorded by flow rates of 50, 75, 100, 125 and 150 ml/min respectively

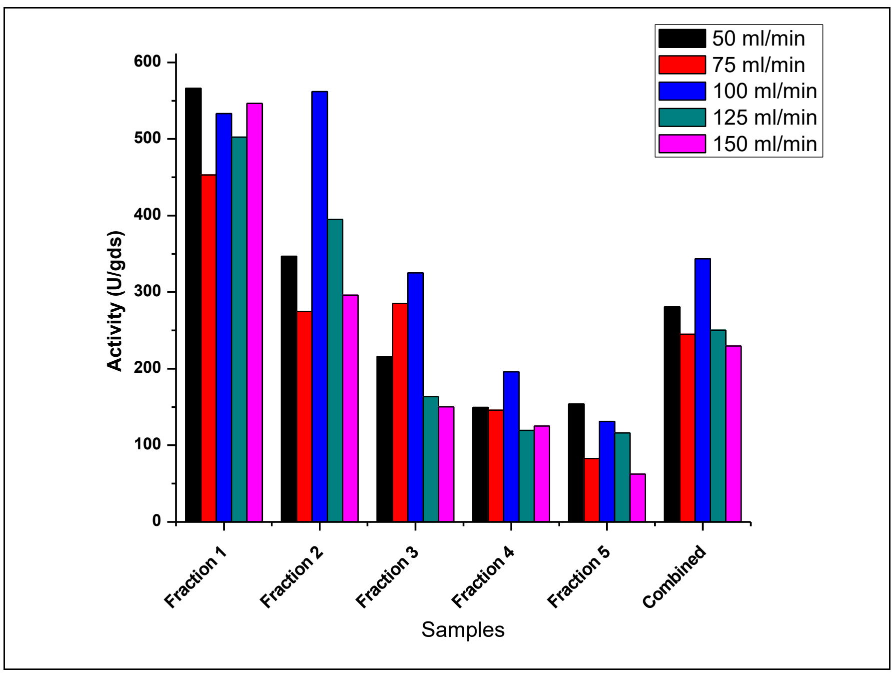


Fig. 4.6 Optimization of flow rate

4.4.3 Optimization of bed volume

An already optimized flow rate of 100ml/min were used for the extraction process. Different packing bed volume was extracted and activity was checked and presented as follows.

It's recorded that there is highest enzyme activity at when the column is packed with 200gm of fermented wheat bran. The combined activity for each bed volume suggests that 343.50 U/gds, 554.09 U/gds, 437.8 U/gds and 421.64 U/gds respectively. Moreover, the residual activity of bed volumes ranging from 100 to 400gms are 67.29 U/gds, 63.07 U/gds, 72.38 U/gds and 82.51 U/gds respectively. Hence for the specified column, the activity and the amount of enzyme extracted depends on the bed volume and design of the column.

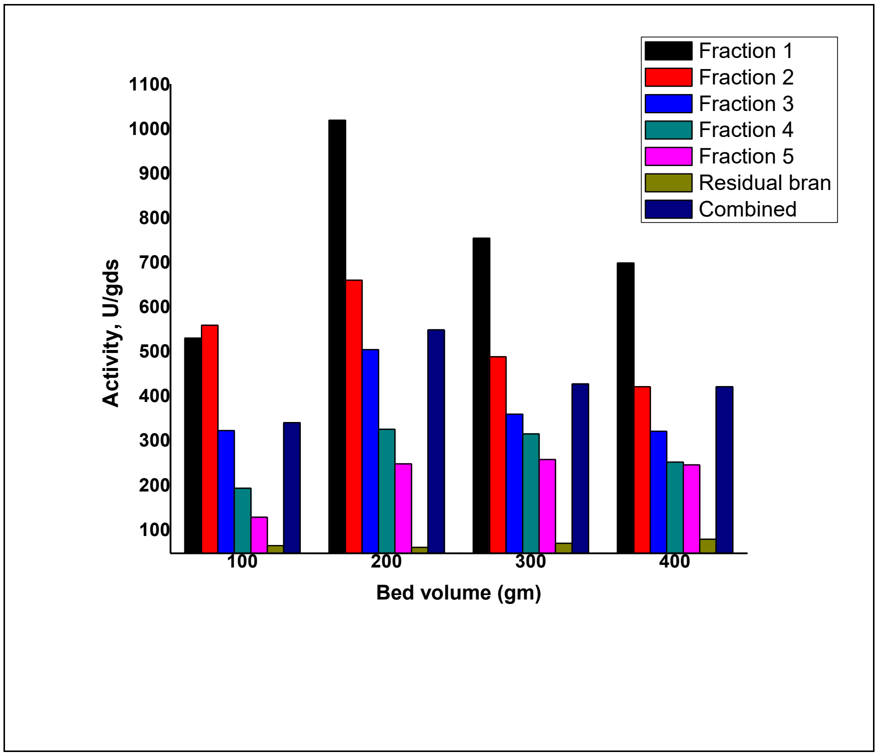


Fig 4.7 Optimization of bed volume

4.6 Dual column extraction

In single glass column extractor were used for extraction process where the whole volume of fermented WB was used and analyzed for extraction capability. Now by doubling the extractor column, the bed volume is divided in two halves for each columns and hence checking the activity for each of the fraction and as well as the combined enzyme solution along with residual activity gets analyzed.

For 100gm:

In dual column system involving 100gram of fermented WB, each of the columns carries 50 gram of WB having 1 liter volume of buffer continuously flowing at a rate of 100ml/min. Finally the eluent gets collected in five fractions each containing 200ml of crude enzyme in 250ml conical flask.

Whereas in single column system, undivided 100gm fermented WB as bed volume was used for extraction process. A volume of 1lit of extracting buffer flow at a rate of 100ml/min. The extracted enzyme collected in five fractions. (see appendix 4A)

Comparison between single (SC) and dual column (DC) extractor system was employed for the same total bed volume. Hence, according to the result obtained the activity on each of the fraction gets nearly doubled. The same is true for the combined activity of SC and DC resulting in 343.5 U/gds and 515.25 U/gds respectively.

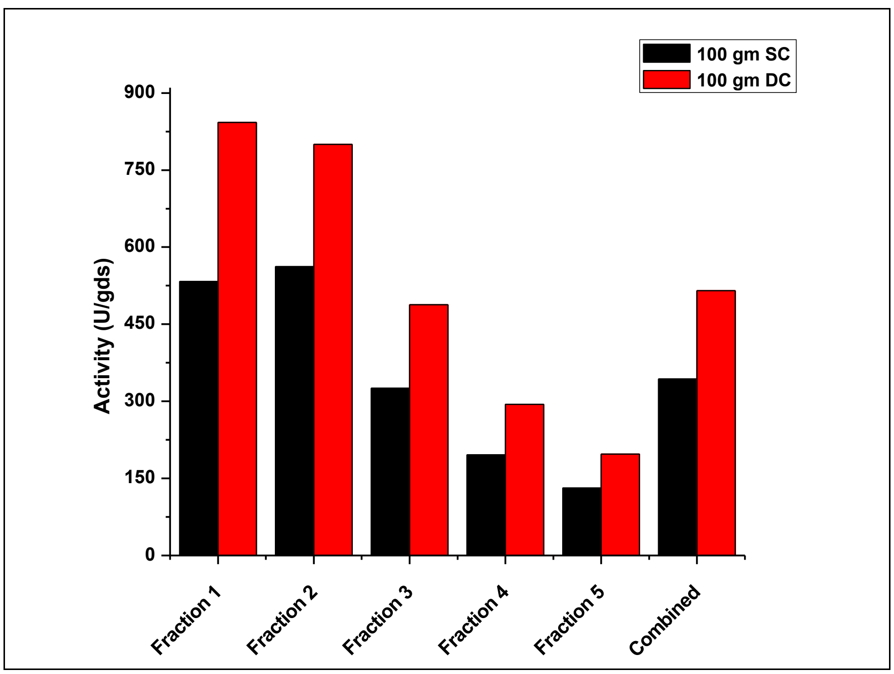


Fig. 4.8: Activity for 100gm in DC and SC

For 200gm:

Dual column extractor system involving 200 gram WB, both extractors will conceive 100 gm each and a buffer volume of 1 liter will flow continuously at a rate of 100ml/min. Comparison was made between dual column and single column extractor; where the whole 200gm extracted at once. The eluent was collected at five fractions. (see appendix 4B)

As shown below, not only the fraction but also the combined activity doubles in dual column system compared with single column type. Comparing with all other bed volume practiced in dual column extraction system, 200 gm shows the ultimate increment in activity compared with SC system. The combined activity of 200 gm bed volume in DC and SC was found out to be 554.09 U/gds and 1081.8 U/gds respectively.

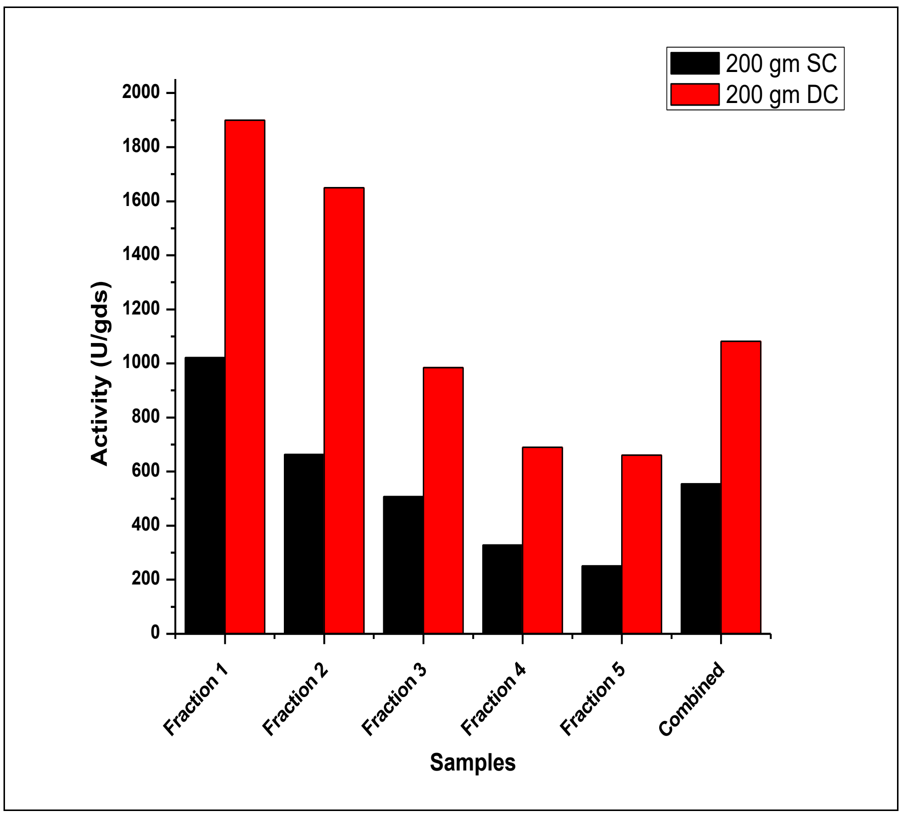


Fig. 4.9: Activity for 200gm in DC and SC

For 300 gm:

In case of DC system each column carries 150 gm of fermented WB each with a total of 300 gm. Thus for SC whole 300 gm was taken for extraction process. And the activity of the final eluent gets measured in combined solutions as well as in fractions. (see appendix 4C)

The activity for not only fractions but also the combined solutions have near doubled in their activity. The combined activity of SC and DC for 300 gm bed volume is 437.8 U/gds and 940.2 U/gds respectively.

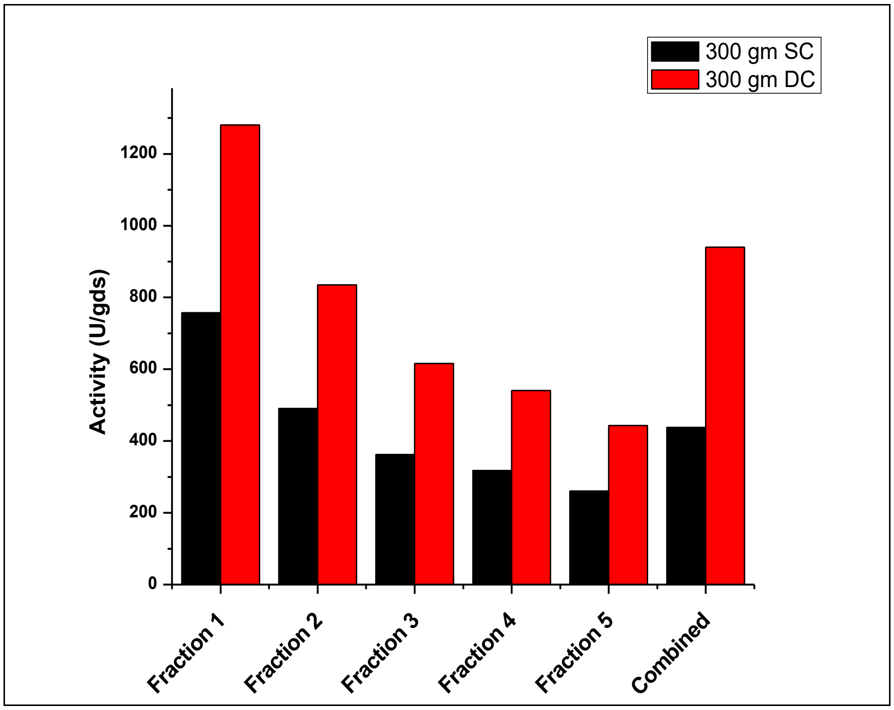


Fig. 4.10: Activity for 300gm in DC and SC

4.7 Comparison between batch and continuous extraction process

Examining the extraction capability for both systems were carried out. The combined activity of all the eluents from batch and continuous system are being analyzed and the following results were made.

According to the data obtained, continuous process yields a far greater extraction potential than the batch process of column extraction mechanism. The combined activity of batch and continuous are 114.89 and 551.3 U/gds respectively. Hence, continuous column extraction process allow for as maximum as possible yield compared with batch extraction.

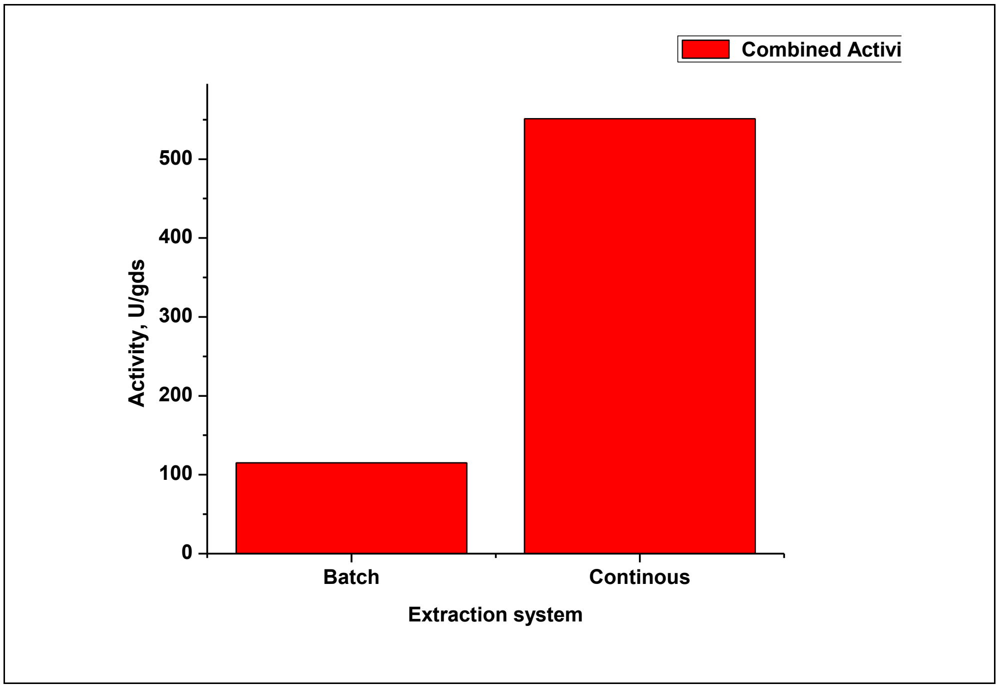


Fig. 4.11. Activity difference between batch and continuous process.

4.8 Comparison of conventional and column system

Comparisons between conventional and column systems were investigated. Based on the finding, the experimental method of extraction produces a higher amount of enzyme when compared to the conventional system.

According to the result, all fractional and combined activity of experimental indicates the highest enzymatic activity than the conventional method. Whereas the residual activity of the conventional method has the higher activity (86.13 U/gds) compared to column method of extraction. Since great amount of enzymes reside in the residual biomass, the conventional method shows the lower activity on the filtrate. On the other hand, the residual activity of the experimental system of extraction shows lower activity (65.12 U/gds) than the filtrate. Hence most of the enzyme have been extracted successfully.

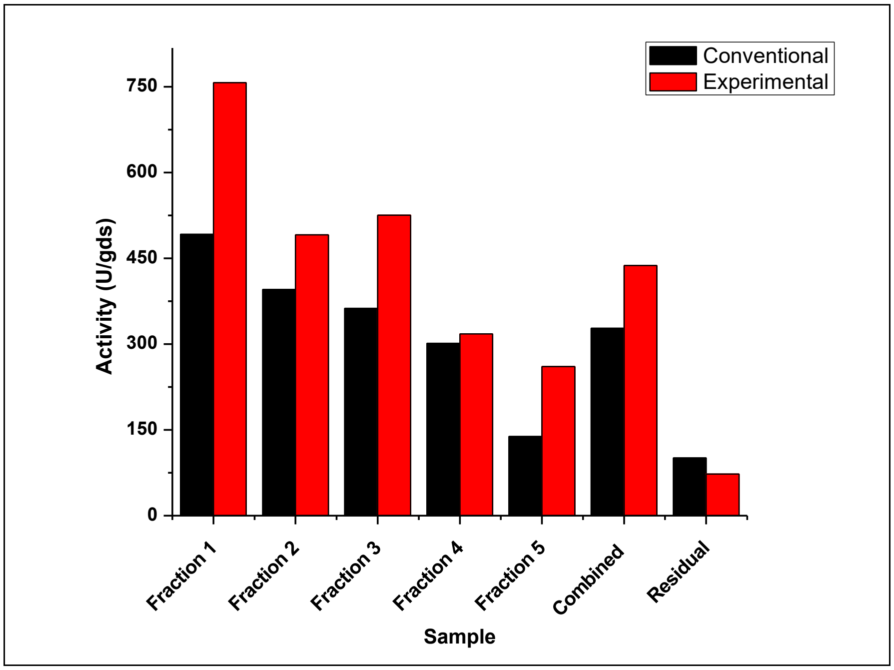


Fig. 4.12. Comparison among conventional and experimental methods

4.9 Correlating the total yield

Analyzing and correlating various extraction mechanisms, such as; conventional method, batch and continuous (DC and SC) enzyme extraction are in use for comparing the total yield on 200 gm WB based extraction system.

The continuous method of column extraction has the highest enzymatic yield (i.e. DC having 49.39% and SC to be 25.29%). Thus continuous column extraction system possess the overall yield of 74.68% than the conventional and batch system of extraction mechanisms. Hence their respective total yield obtained can be summarized and presented as follows;

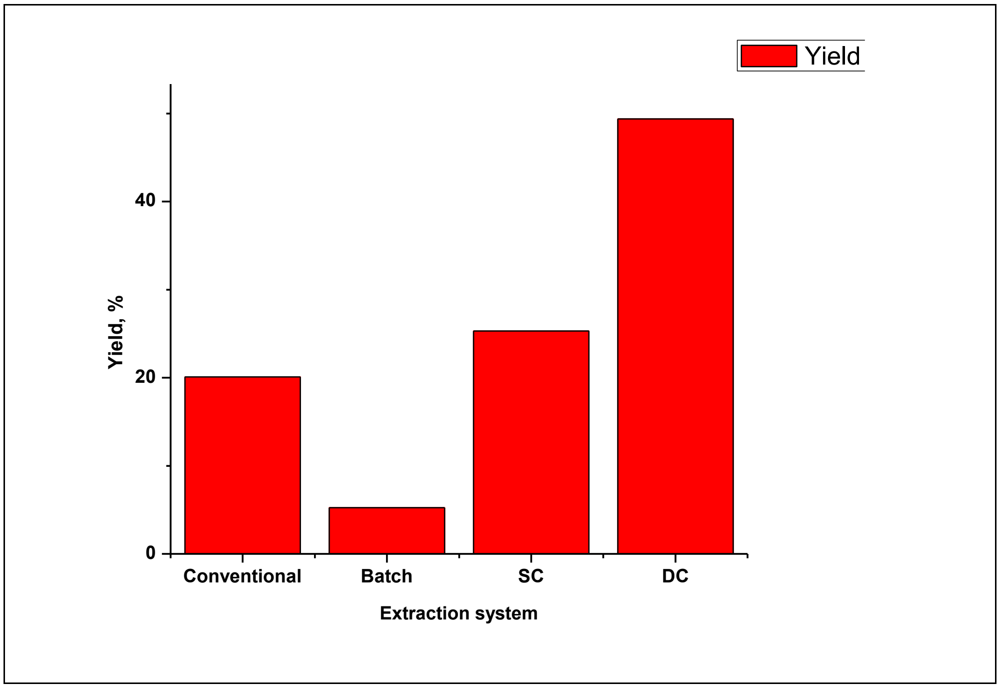


Fig. 4.13: Total yield

4.10 Ultrafiltration

The crude enzyme with amylase activity of 1146U/gds, was concentrated first via microfiltration and the filtrate is taken to ultrafiltration and concentrated to 2 fold. Hence the activity increased to 1649.45 U/gds. The ultra-filtered amylase enzyme was further used for application studies in leather industry.

4.11 Application of Amylase in Pre-Tanning Operations

The crude enzyme extract amylase activity of 1649.45 U/gds, produced by solid state fermentation of column extraction system was used for pre-tanning operations like fiber opening of goat skin. Enzymatic unhairing and fiber opening was carried out with 2 to 5% enzyme concentration offered based on raw weight of goat. Single pot unhairing and fiber opening process followed as given in Table 3.2 and 3.3. The process involves two soak with 100% water and followed by enzyme treatment by drum method with 100%water and 2-5% enzyme concentration. The process includes 9h duration with 10mins intermediate resting followed by resting overnight in drum (12h) (Madhumathi et al.,2007). In early studies it was reported as 24h for complete unhairing (Dayanandhan et al.,2003). This single pot, simultaneous unhairing and fiber opening is shown in fig below.

Figure 4.14: Dry salted goat skin

Fig. 4.15: Enzymatic method of unhairing and fiber opening (Left); conventional method of unhairing and fiber opening (Right).

Fig. 4.16: Enzyme assisted skin for fiber opening

4.11.1 Assessment for fiber opening

Conventional lime based fiber opening is a result of osmosis based fiber splitting, whereas a-amylase based fiber opening is a result of removal of PG. In both conventional as well as experimental methods, the net result is increased weight is due to water uptake of skin matrix. The weights of the skins before and after fiber opening were recorded.

As shown below the percentage increase in weight after swelling was evaluated from the difference in weights before and after fiber opening. The 2% (w/w) BFI and 3% (w/w) BFII sample is comparable with the control sample. The percentage increase in weights of AFII is higher than the other experiments. Hence it shows an enhanced fiber splitting than the conventional method. Since the fibers are more opened up in AFII sample, there is not only a well abstraction of hair but also an easily removal of flesh from the flesh layer of skin.

$$\% \text{ Increase in weight} = \frac{\text{Weight after enzyme treatment} - \text{Dehaired weight}}{\text{Dehaired weight}} \times 100$$

Table. 4.1 Assessment for fiber opening

SAMPLE	Weight of soaked skin (g)	Weight of de haired skin (g)	Weight after enzyme treatment(g)	%increase after treatment	Weight of flesh removed(g)
BF I	848.6	736.6	762.4	3.5	9.5
BF II	891.4	712.4	748	5	10.7
AF I	764.5	572.5	631.5	10.3	15.2
AF II	775.5	555.5	635.5	14.4	22
Control	767	616.1	651.5	5.8	12.2

4.11.2 Proteoglycan, PG release after enzyme treatment

During conventional reliming process, the release of the proteoglycan is considered as a bench mark for fiber opening. In order to examine the fiber opening efficiency; the release of proteins, proteoglycans and carbohydrates were estimated in the effluent liquor (Cantera et al.,1998). Amylase is highly specific enzyme in catalyzing the hydrolytic scission of a specific glycosidic bond. Amylases when treated with the skin matrix would initially affect the glycosidic linkages of the proteoglycan predominantly conjugated with skin matrix, especially collagen. The dilution factor at which the calculation carried out was 10 folds. And the concentration of proteoglycan are estimated and calculated using their respective standard graphs and the data is give in table below.

PG = OD*DF/1.175

Table 4.2 PG release from skin matrix

SAMPLE	T1	T2	PG (mg/gm)
AF II	0.449	0.403	7.3
AF I	0.380	0.361	6.35
BF II	0.259	0.169	3.67
BF I	0.226	0.072	2.55
Control	0.171	0.154	2.78

According to the above result, the skin treated with enzyme especially sample AF II, having an enzyme concentration of 5% (w/w), releases higher amount of PG as compared with the other enzyme treated skin samples including the control. Hence compared to conventional process, the enzyme shows a better opening up of fibers in the skin matrix.

4.11.3 Physical strength characteristics

After conditioning the crust leather at room temperature over a period of 48hrs, the properties like tear and tensile strength, elongation at break trials were carried out for all the crust leathers at both along and across line. The grain crack strength of crust leather for both control and experimental methods were also evaluated. The values of the crust leathers corresponding to each experiment are given in table 4.4. It is seen that both control and experimental leathers exhibit tensile, tear, grain crack and bursting strength values.

Table 4.3 Physical characteristics of goat crust leather

Sample	Tensile strength (Kg cm^{-2})		Tear strength (Kg cm^{-1})		Elongation at break (%)		Grain crack	
	Along	Across	Along	Across	Along	Across	Load, kg	Distention, mm
C	237.15	169.90	48.21	38.12	48.12	71.23	22	9.13
BF I	215	160.61	41.06	36.26	45.67	70.14	20	7.88
BF II	239.70	162.75	43.57	36.25	46.54	73.34	21	8.88
AF I	233.67	165.20	44.70	37.73	48.64	74.17	22	8.98
AF II	240.45	192.36	46.66	38.87	49.04	75.26	24	9.21

4.11.4 SEM Analysis

Morphological characterizations of crust leather goat skin was performed by means of scanning electron microscope. Scanning electron micrographs at grain and cross sectional surface of control and experimental crust leather samples with (×500) magnification power was performed.

Gain surface SEM analysis:

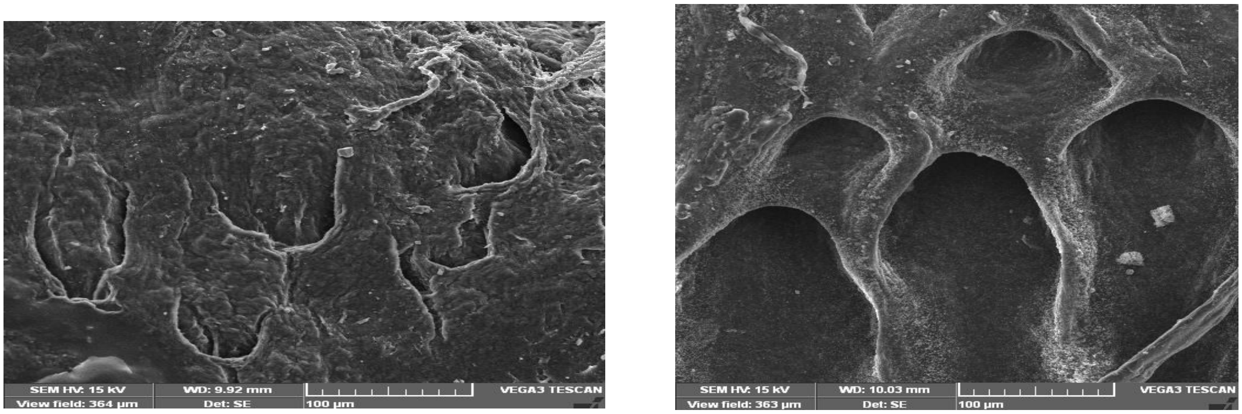


Figure 4.17: SEM analysis on grain surface of crust leather treated with 5% enzyme concentration and control respectively.

Cross section SEM analysis:

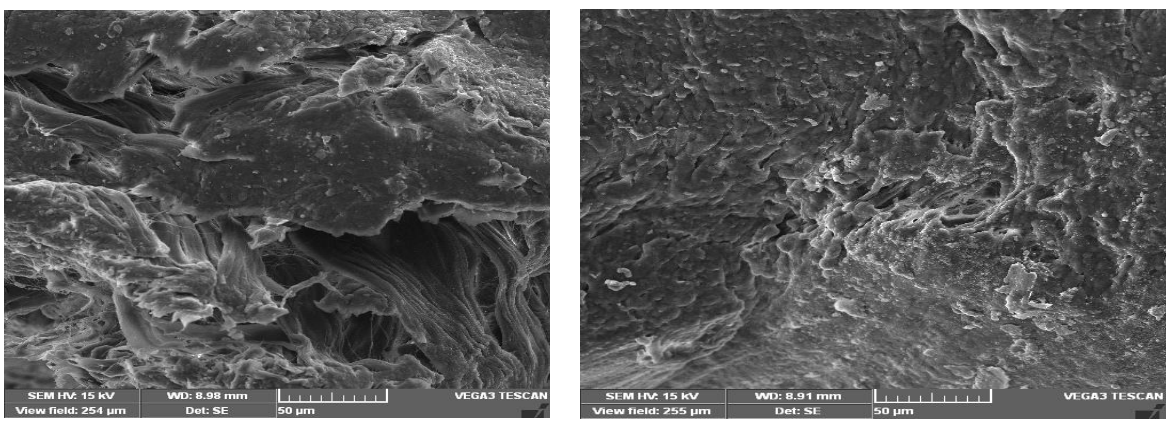


Figure 4.18: SEM analysis of crust leather with 5% enzyme and control respectively.

CHAPTER FIVE

5. CONCLUSION AND RECOMMENDATION

5.1 Conclusion

This present investigation on the extraction of amylase enzyme in a column system symbolizes an importance in the field of biotechnology, owing to the role of efficient extraction of enzymes in various biochemical and industrial processes.

In this study, optimization of extraction parameters for amylase from *Bacillus subtilis* was carried out. Production of the enzymes were done through Solid state fermentation, as it was observed from the previous studies that *Bacillus subtilis* produced higher activity of amylase under SSF using Wheat Bran as a substrate. Different physiochemical parameters influencing amylase production was optimized by one at a time approach.

Screening of agro-industrial residue as a solid support and source of nutrients for amylase production suggested that medium sized wheat bran particles enhances amylase production. The other optimum conditions for amylase extraction includes optimization of pH, incubation of temperature, particle size, flow rate, bed volume at constant flow rate, single and dual column setups were all studied. Hence by comparing the single and double extraction systems, the highest is observed and noted in the double extraction systems by nearly 2 folds.

Scale up studies showed that maximum amylase production increases with moderate amount of substrate level in the flasks. In tray system maximum amylase production was 65.1% higher when compared to flask level (250 mL). Since the medium contains only wheat bran which is an agro-industrial residue easily available throughout year and as a cheap substrate.

Generally speaking from the above mentioned methods of extraction namely; conventional, batch and continuous extraction systems. From these extraction processes the continuous system have by far the highest overall yield of 74.68% than the rest of extraction methods. Moreover, by comparing the experimental and conventional extracting system through residual activity, the conventional system of extraction possesses the highest residual enzyme activity than the experimental method.

The crude enzyme extract of amylase enzyme produced by SSF of column extraction system becomes partially purified before taken to pre tanning operation. A goat skin obtained from the tannery were cut into two pieces of almost equal size and weight and different enzyme formulations ranging from 2% - 5% w/w were applied to observe the dehairing, fiber opening and fleshing of goat skin. One of the parts were taken for conventional method of leather processing and is kept as control. It was observed that the experimental sample (AF II) which was tested with 5% (w/w) enzyme concentration, gives a better result in fiber opening and highest release of proteoglycans than the rest of the samples. But hair removal was efficient as compared to that of control. The strength property of the crust leather shows that the enzymatic method has a better strength property as compared with the chemical based fiber opening and unhairing system.

Future work may be focused on studies for commercialization of the process.

- ✓ Future work will focus on studying the characteristics of wheat bran.
- ✓ Utilization of mixed substrate in column extraction method of enzymes for effective practice in leather processing.
- ✓ Check for enzyme stability for further applications.
- ✓ Study on the other optimizing parameters in column extraction systems.

5.2 Recommendation

Leather processing industries cause adverse changes in the immediate environment and necessitated the development of enzyme-based processes as alternatives to currently employed chemical processes. There effective production, extraction and application will greatly affect their role as an alternative process to combat environmentally hazardous chemicals employed in leather manufacturing processes. Enzymatic based unhairing, fiber opening and resulted in removal of hair, interfibrillary substances and flesh which was better than to that of the conventional (non-ecofriendly) systems.

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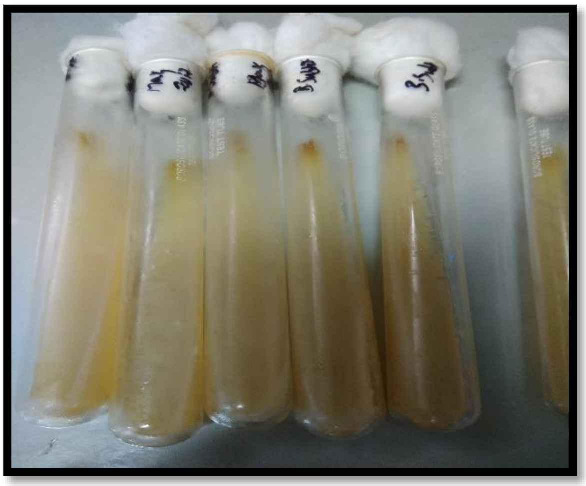
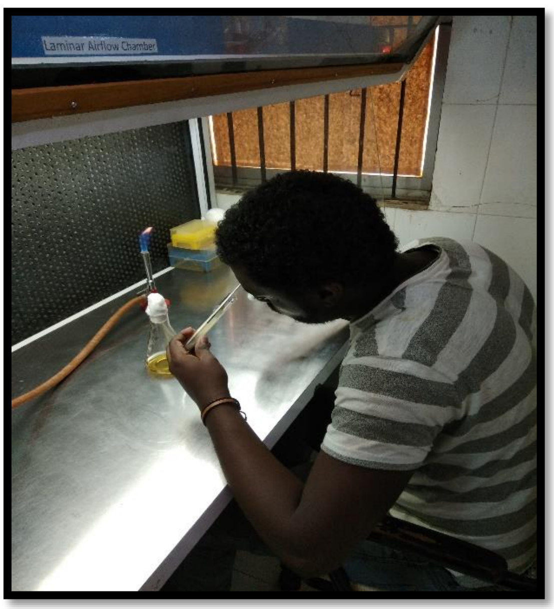
Appendix

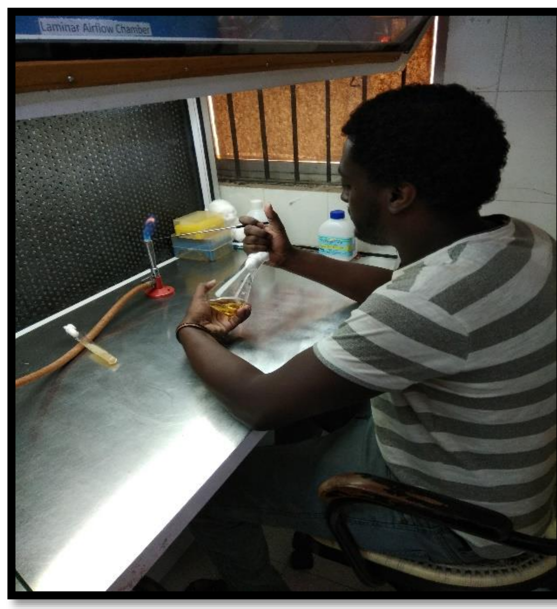


A

B



C



D

Appendix 1. Sub culturing; Bacillus subtillis culture growth on a LB agar (A), Pre pre inoculum preparation; an autoclaved LB broth in a 250ml Erlenmeyer flask (B), the researcher inoculating a flask containing the LB broth with a loop of *B.subtilis* from LB agar slant (C & D)

A B

C

Appendix 2. Inoculation; transferring the pre inoculated culture into the inoculum medium in a laminar air flow chamber and kept in incubator for 48 hrs (A), the tray level production medium kept in humidity chamber and assay done in every 24hrs. (B & C).

A

B

Appendix 3. Enzyme extraction using extractor column, extraction of enzyme in a continuous system viz single and dual column arrangement respectively (A & B).


MERCK
250 ml
BOROSIL
250 ml
2
4980
MERCK
250 ml
Qualigens
250 ml
MERCK
250 ml
5

A


Sh
250ml
BOROSILICATE GLASS
1
BOROSIL
250 ml
4980
MERCK
250 ml
Qualigens
250 ml
BOROSIL
250ml
4980

B


Sh
250ml
BOROSILICATE GLASS
1
BOROSIL
250 ml
4980
MERCK
Qualigens
250 ml
BOROSIL
250ml
4980

C

Appendix 4. Five fraction of crude enzyme extract of 100gm fermented WB (A), 200gm fermented wheat bran (B), 300gm fermented wheat bran (C) in 250ml Erlenmeyer flask from column extraction system prior assay was carried out.

A

B

C

D

Appendix 5. Application of amylase on leather; wet salted goat skin cut in the middle used for studying enzymatic treatment and other half for conventional enzyme assisted chemical treatment (A), unhairing of the conventional and experimental method (B), Fiber opening of the experimental method (C) and, conventional method of fiber opening (D).

A

B

C

D

Appendix 6. Some of equipment used during the research work, Hot plate dissolving starch in a buffer solution (A), Moisture analyzer, (HG63 Halogen moisture analyzer, Mettler Toledo (Pvt) Ltd., India) analyzing the moisture content of the WB during calculation of enzyme activity (B), Spectrophotometer measuring the absorbance at 540nm (C), and Specially designed extractor column for enzyme extraction (D)