P-1593

PURIFICATION AND CHARACTERIZATION OF α-AMYLASE FROM *Aspergillus fumigatus* AND ITS APPLICATION IN TEXTILE INDUSTRY

A PROJECT REPORT

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in partial fulfillment for the award of the degree of

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During recent years many new and extended uses have been developed for fungal enzymes.Most of these enzymes are used in textile processing,food manufacturing and in the manufacture of malt beverages and industrial alchohol.

Although certain micro-organisms are capable to secrete amylases when grown under submerged conditions either aerobically or anaerobically. Industrial production methods generally involve cultivation on the surface of unagitated liquid or semisolid substrates.The properties of enzymes depend on the source from which it is isolated.The enzymes from eukaryotes are considered to be more stable and biocompatible than these from prokaryotes.

In textile industry, the starch is applied on the raw cloth during sizing process to withstand the tension. During desizing process the starch is not completely removed by chemicals at the same time the applied chemicals may damage the cloth. If the starch is present after desizing process it hinders the dyeing process, so it should be removed . Since amylase acts on starch it can be used in desizing process.

This report deals with

- Production of amylase from *Aspergilus fumigatus* in submerged fermention.
- Characterization and purification of ∝-amylase.
- Application of ∝-amylase in textile industry for the removel of starch sizes, and it is proved that the enzyme efficiency is three times greater than commercially available chemical method.

CHAPTER-1

INTRODUCTION

An **enzyme** is a protein that catalyzes, or speeds up, a chemical reaction. The word comes from the Greek word *énsymo*, which comes from *én* ("at" or "in") and *simo* ("leaven" or "yeast"). Certain RNAs also have catalytic activity, but to differentiate them from protein enzymes, they are referred to as RNA enzymes or ribozymes.

Enzymes are essential to sustain life because most chemical reactions in biological cells would occur too slowly, or would lead to different products without enzymes.

Like all catalysts, enzymes work by providing an alternate pathway of lower activation energy of a reaction, thus allowing the reaction to proceed much faster. Enzymes may speed up reactions by a factor of many millions. An enzyme, like any catalyst, remains unaltered by the completed reaction and can therefore continue to function. Because enzymes do not affect the relative energy between the products and reagents, they do not affect equilibrium of a reaction. However, the advantage of enzymes compared to most other catalysts is their sterio-, regio- and chemoselectivity and specificity.

Enzyme activity can be affected by other molecules. Inhibitors are naturally occurring or synthetic molecules that decrease or abolish enzyme activity; activators are molecules that increase activity. Some irreversible inhibitors bind enzymes very tightly, effectively inactivating them. Many drugs and poisons act by inhibiting enzymes.

While all enzymes have a biological role, some enzymes are used commercially for other purposes. Many household cleaners use enzymes to speed up chemical reactions (*e.g.*, breaking down protein or starch stains in clothes).

More than 5,000 enzymes are known. Typically the suffix *-ase* is added to the name of the substrate (*e.g.*, amylase is the enzyme that catalyzes the cleavage of amylose) or the type of reaction (*e.g.*, DNA polymerase catalyzes the formation of DNA polymers). However, this is not always the case, especially when enzymes modify multiple substrates. For this reason Enzyme Commission or EC numbers are used to classify enzymes based on the reactions they catalyze. Even this is not a perfect solution, as enzymes from different species or even very similar enzymes in the same species may have identical EC numbers.

α- AMYLASE:

α-Amylase belongs to the glycosyl hydrolase 13 family of enzymes (EC 3.2.1.- and EC 2.4.1.-), a large group of enzymes that act to hydrolyse the glycosidic bonds between two or more carbohydrates, or between a carbohydrate and a non-carbohydrate moiety. The classification of these enzymes from CAZy (Carbohydrate-Active EnZymes) lists 85 different families based on sequence similarity. In addition to α-amylase, family 13 of glycosyl hydrolases includes a enzymes with a variety of known activities: pullulanase (EC 3.2.1.41), cyclomaltodextrin glucanotransferase (EC 2.4.1.19), cyclomaltodextrinase (EC 3.2.1.54), trehalose-6-phosphate hydrolase (EC 3.2.1.93), α-glucosidase (EC 3.2.1.20), isoamylase (EC 3.2.1.68), and amylosucrase (EC 2.4.1.4), amongst others. These enzymes use an aspartic acid residue as the catalytic nucleophile/base, and a glutamic acid residue as the catalytic proton donor.

Alpha-amylase acts upon large linear polymers at internal bonds. The hydrolytic products have alpha-configuration. The activity is present in all living organisms, however the enzymes vary remarkably even from tissue to tissue within a single species. The α–amylase act on polysaccharides such as starch, amylose, amylopectin.

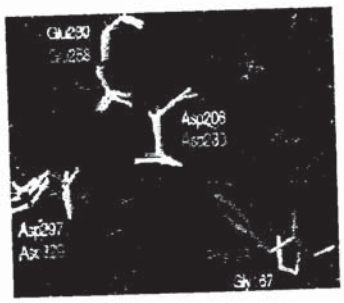


FIG.1.1. ACTIVE SITE OF α-AMYLASE

STARCH:

Starch is a complex carbohydrate which is insoluble in water. Starch (in particular cornstarch) is used in cooking for thickening sauces. In industry, it is used in the manufacture of adhesives, paper, and textiles. It is a white powder, and is tasteless and odorless.

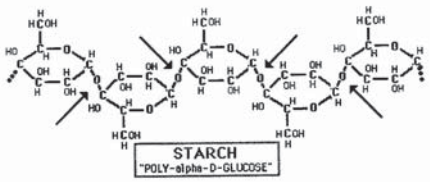


FIG.1.2. STARCH

Starchy substances constitute the major part of the human diet for most of the people in the world, as well as many other animals. They are synthesized naturally in a variety of plants. Some plant examples with high starch content are corn, potato, rice, sorghum, wheat, and cassava. It is no surprise that all of these are part of what we consume to derive carbohydrates. Similar to cellulose, starch molecules are glucose polymers linked together by the alpha-1,4 and alpha-1,6 glucosidic bonds, as opposed to the beta-1,4 glucosidic bonds for cellulose. In order to make use of the carbon and energy stored in starch, the human digestive system, with the help of the enzyme amylases, must first break down the polymer to smaller assimilable sugars, which is eventually converted to the individual basic glucose units.

Because of the existence of two types of linkages, the alpha-1,4 and the alpha-1,6, different structures are possible for starch molecules. An unbranched, single chain polymer of 500 to 2000 glucose subunits with only the alpha-1,4 glucosidic bonds is called *amylose*. On the other hand, the presence of alpha-1,6 glucosidic linkages results in a branched glucose polymer called *amylopectin*. The degree of branching in amylopectin is approximately one per twenty-five glucose units in the unbranched segments. Another closely related compound functioning as the glucose storage in animal cells is called *glycogen*, which has one branching per 12 glucose units. The degree of branching and the side chain length vary from source to source, but in general the more the chains are branched, the more the starch is soluble.

Starch is generally insoluble in water at room temperature. Because of this, starch in nature is stored in cells as small granules which can be seen under a microscope. Starch granules are quite resistant to penetration by both water and hydrolytic enzymes due to the formation of hydrogen bonds within the same molecule and with other neighboring molecules. However, these inter- and intra-hydrogen bonds can become weak as the temperature of the suspension is raised. When an aqueous suspension of starch is heated, the hydrogen bonds weaken, water is absorbed, and the starch granules swell. This process is commonly called *gelatinization* because the solution formed has a gelatinous, highly viscous consistency. The same process has long been employed to thicken broth in food preparation.

Depending on the relative location of the bond under attack as counted from the end of the chain, the products of this digestive process are dextrin, maltotriose, maltose, and glucose, etc. Dextrins are shorter, broken starch segments that form as the result of the random hydrolysis of internal glucosidic bonds. A molecule of maltotriose is formed if the third bond from the end of a starch molecule is cleaved; a molecule of maltose is formed if the point of attack is the second bond; a molecule of glucose results if the bond being cleaved is the terminal one; and so on. The initial step in random depolymerization is the splitting of large chains into various smaller sized segments. The breakdown of large particles drastically reduces the viscosity of gelatinized starch solution, resulting in a process called *liquefaction* because of the thinning of the solution. The final stages of depolymerization are mainly the formation of mono-, di-, and tri-saccharides. This process is called *saccharification*, due to the formation of saccharides.

Since a wide variety of organisms, including humans, can digest starch, alpha-amylase is obviously widely synthesized in nature, as opposed to cellulase. For example, human saliva and pancreatic secretion contain a large amount of alpha-amylase for starch digestion. The specificity of the bond attacked by alpha-amylases depends on the sources of the enzymes. Currently, two major classes of alpha-amylases are commercially produced through microbial fermentation. Based on the points of attack in the glucose polymer chain, they can be classified into two categories, liquefying and saccharifying.

Because the bacterial alpha-amylase to be used in this experiment randomly attacks only the alpha-1,4 bonds, it belongs to the liquefying category. The hydrolysis reaction catalyzed by this class of enzymes is usually carried out only to the extent that, for example, the starch is rendered soluble enough to allow easy removal from starch-sized fabrics in the textile industry. The paper industry also uses liquefying amylases on the starch used in paper coating where breakage into the smallest glucose subunits is actually undesirable. (One cannot bind cellulose fibers together with sugar!)

On the other hand, the fungal alpha-amylase belongs to the saccharifying category and attacks the second linkage from the non-reducing terminals (i.e. C4 end) of the straight segment, resulting in the splitting off of two glucose units at a time. Of course, the product is a disaccharide called maltose. The bond breakage is thus more extensive in saccharifying enzymes than in liquefying enzymes. The starch chains are literally chopped into small bits and pieces. Finally, the amyloglucosidase (also called glucoamylase) component of an amylase preparation selectively attacks the last bond on the non-reducing terminals. The type to be used in this experiment can act on both the alpha-1,4 and the alpha-1,6 glucosidic linkages at a relative rate of 1:20, resulting in the splitting off of simple glucose units into the solution. Fungal amylase and amyloglucosidase may be used together to convert starch to simple sugars. The practical applications of this type of enzyme mixture include the production of corn syrup and the conversion of cereal mashes to sugars in brewing.

HOUSEHOLD

Clothing starch or laundry starch is a liquid that is prepared by mixing a vegetable starch in water (earlier preparations also had to be boiled), and is used in the laundering of clothes. During the 19th century and early 20th century, it was stylish to stiffen the collars and sleeves of men's shirts and the ruffles of girls' petticoats by applying starch to them as the clean clothes were being ironed.

Aside from the smooth, crisp edges it gave to clothing, it served a practical purpose as well. Dirt and sweat from a person's neck and wrists would stick to the starch rather than fibers of the clothing, and would easily wash away along with the starch. Then, after each laundering, the starch would be reapplied.

AMYLOSE

Amylose is a linear polymer of glucose linked with mainly a(1? 4) bonds. It can be made of several thousands glucose units. It is one of the two components of starch, the other being amylopectin.

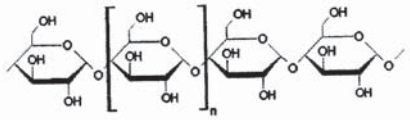
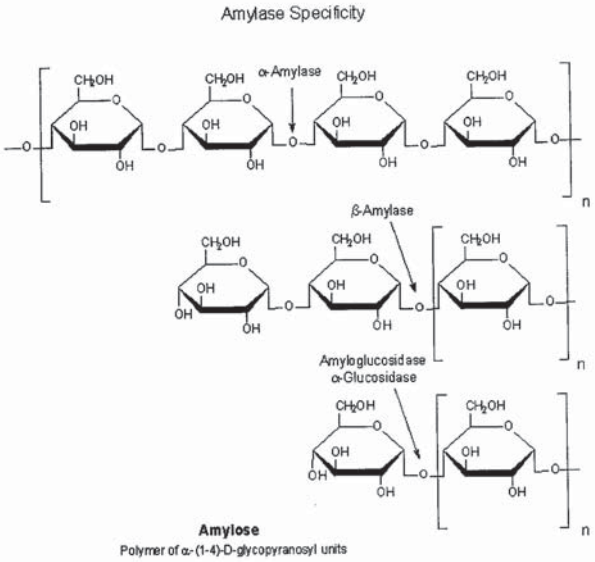


FIG.1.3. Amylose structure

The a(1? 4) bonds promote the formation of a helix structure. The structural formula of amylose is pictured at right. The number of repeated glucose subunits (n) can be many thousands.

Amylose starch is less readily digested than amylopectin. However it takes up less space so is preferred for storage in plants; it is how about 80% of the starch in plants is stored. The digestive enzyme amylase works on the ends of the starch molecule, breaking it down into sugars.

Iodine molecules fit neatly inside the helical structure of amylose, binding with the starch polymer, causing it to absorb certain wavelengths of light. Hence, a common test for starch is to mix it with a small amount of yellow iodine solution. In the presence of amylose a blue-black colour will be observed. The intensity of the colour can be tested with a colorimeter using a red filter, to indicate the concentration of starch present in the solution.



1.1. ASPERGILLUS FUMIGATUS

BIOLOGY AND PATHOLOGY

The molds such as *Aspergillus fumigatus* are filamentous fungi. They are especially prevalent growing on the nonliving organic materials in the soil. They disperse their non-sexual spores called conidia in the air. Most *Aspergilli* are harmless to humans and *A. fumigatus* in particular is harmless to humans whose immune system has not been compromised by disease,

ENZYME COMMISSION NUMBER

EC 3.2.1.1

Common name: α-amylase

Reaction: Endohydrolysis of 1,4-α-D-glucosidic linkages in polysaccharides containing three or more 1,4-α-linked D-glucose units

Other name(s): glycogenase; α-amylase, alpha-amylase; endoamylase; Taka-amylase A

Systematic name: 1,4-α-D-glucan glucanohydrolase

Comments: Acts on starch, glycogen and related polysaccharides and oligosaccharides in a random manner; reducing groups are liberated in the α-configuration. The term 'α' relates to the initial anomeric configuration of the free sugar group released and not to the configuration of the linkage hydrolysed.

FIG.1.4. Amylase structure

drug therapy or genetic conditions. Exposure to *A. fumigatus* can cause an allergic response in sensitive individuals. More importantly, *A. fumigatus* is an opportunistic pathogen of bone marrow transplant patients, AIDS patients, and other immune compromised individuals.

FIG.1.5. ***Aspergillus fumigatus***

Aspergillus fumigatus is the most common mold causing infection worldwide. The first infection described in man, an aspergilloma, was reported in Edinburgh in 1842 (Bennett, 1842) and many cases of invasive disease in non-immunocompromised patients were reported from the UK between 1890 (Wheaton, 1890) and 1947 (Cawley, 1947). These cases and more recent epidemiological data emphasize that *A. fumigatus* is a primary, albeit rare, pathogen of man. Allergic disease due to *Aspergillus* was first described in London in 1952 (Hinson *et al.*, 1952) and the first invasive (and fatal) infection in an immuno compromised patient was described in 1953 in the British Medical Journal in a patient from Gloucester (Rankin, 1953).

The frequency of invasive disease has risen approximately 14-fold over the 12 years to 1992, as judged after death in unselected autopsies. Invasive *Aspergillosis* has overtaken candidiasis as the most frequent fungal pathogen detected post mortem in tertiary care hospitals in Europe. Thus 4% of all patients dying had invasive *Aspergillosis,* compared with about 2% with

invasive candidiasis (Groll *et al.*, 1996; Vogeser *et al.*, 1999). Patients at risk based on the disease frequency include those with chronic granulomatous disease (25-40%), lung transplant recipients (17-26%), allogeneic bone marrow transplant patients (4-30%), neutropenic patients with leukemia (5-25%), heart transplant recipients (2-13%), pancreas transplant recipients (1-4%), renal transplant patients in Europe and the USA (~1%) and in India (~10%), and patients with AIDS, multiple myeloma and severe combined immunodeficiency (~4%) (Denning, 1998). Over 500,000 transplants are performed annually in the world. Acute leukemia affects about 3/100,000 of the population and on average each patient receives 3 cycles of chemotherapy, with each cycle defining a major risk period. Similar incidence is observed for high grade lymphoma patients who are also at high risk of invasive *Aspergillosis*. In the industrialised nations alone these treatment protocols generate about 250,000 periods of major risk per year. AIDS cases are predicted to exceed 40 million by the end of the year 2000 which would result in about 1.4 million cases of invasive *Aspergillosis*, although in developing countries most patients will not live long enough to get this disease.

The crude mortality from invasive *Aspergillosis* is around 85% and falls to around 50% if treated (Denning, 1996). The new drugs in trial (voriconazole, etc.) may reduce the mortality slightly (~10%) (Denning *et al.*, 1997a), but patients in trials tend to do better than those treated in clinical practice.

In addition to invasive disease, *Aspergillus* causes a number of other diseases in man. These include aspergilloma ("colonisation" of existing pulmonary cavities), sinusitis in normal people, allergic bronchopulmonary and sinus infections, keratitis (which usually leads to blindness in that eye and is common in the developing world) and postoperative infections in immunocompetent patients. Aspergilloma numbers are set to rise dramatically due to the increasing incidence of tuberculosis and such aspergilloma cases are notoriously difficult to treat. Cavities of 2 cm or larger after tuberculosis subsequently develop aspergillomas in 15-20% of patients (in the UK). The 5 year survival of patients with aspergillomas is about 40%. Allergic bronchopulmonary aspergillosis occurs in patients with cystic fibrosis and asthmatics (an increasing number) causing pulmonary fibrosis and death usually within 10 years of diagnosis.

CULTURE AND MORPHOLOGICAL CHARACTERISTICS

Identification of *A. fumigatus* is based predominantly upon the morphology of the conidia and conidiophores. The organism is characterized by green echinulate conidia, 2.5 to 3 micrometer in diameter, produced in chains basipetally from greenish phialides. 6 to 8 by 2 to 3 micrometer in size. A few isolates of *A. fumigatus* are pigmentless and produce white conidia (1). The chains of conidia are borne directly on broadly clavate vesicles (20 to 30 micrometer in diameter) in the absence of metulae No sexual stage is known for this species. *A. fumigatus* is a fast grower: the colony size can reach 4 ± 1 cm within a week when grown on Czapek-Dox agar at 25°C (2). *A. fumigatus* is a thermophilic species, with -growth occurring at temperatures as high as 55°C and survival maintained at temperatures up to 70°C (3, 4, 5, 6).

A.. fumigatus is morphologically more variable (6, 7, 8) than was originally described by Raper and Fennell (2). These variations have led to the description of several varieties of *A. fumigatus,* including *acolumnaris, phialiseptus, ellipticus, and sclerotiorum,* with the distinctions being based on only slight morphological differences. *A. fumigatus, A. brevipes, A.. duricaulis, A.. unilateralis, A.. viridinutans.* together with anamorphs of species within the perfect genus *Neosartorya,* a genus in which morphologically related species have been grouped, are classified as *Aspergillus* sect. fumigati. The search for a sexual state of *A.. fumigatus* has been attempted among *Neosartorya* species, since it would allow classical genetics to be pursued in *A. fumigatus.* To date, no such stage has been discovered.

PATHOGENESIS OF A. FUMIGATUS

Aspergillus fumigatus is the causative agent of invasive aspergillosis, which represents the second cause of death resulting from fungal infections in hospitals. Invasive aspergillosis is associated with a high mortality. This is mostly due to the poor sensitivity of the currently available diagnostic tests and the major use of amphotericin B therapy, which has deleterious side effects. Analysis of candidate virulence factors by reverse genetics has not been successful for the understanding of the pathogenic processes. We have therefore focused our interest towards the development of novel molecular tools that could be used to identify through insertional mutagenesis genes that are essential for growth of *A. fumigatus* under laboratory conditions and/or during invasion of the host and may hence serve as antifungal targets.

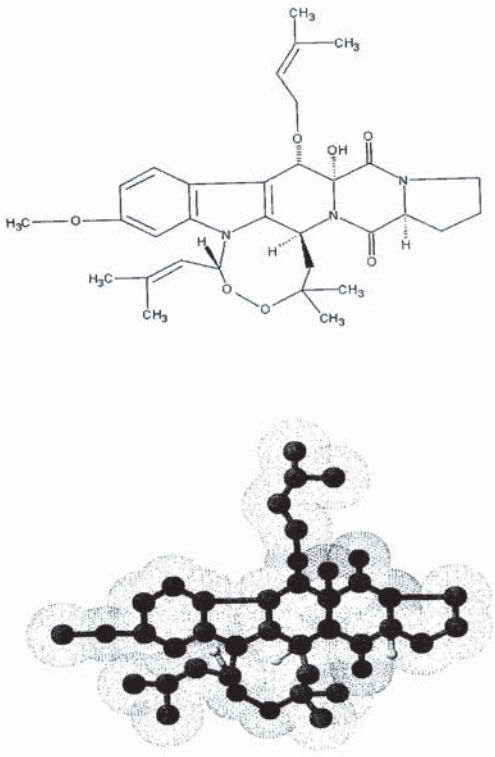


FIG.1.7. Fumetrimorgin-A 3-dimensional structure

oxic metabolites trivial name - fumitremorgin A

pecies	*A. caespitosus, A. fumigatus, N. fischeri*
ystematic ıame	5H-12H-3,4-Dioxa-5a,11a,15a-triazacyclooct(lm)indeno(5,6-b)fluorene-11,15(2H,13H)-dione, 1,10,10a,14,14a,15b-hexahydro-10a-hydroxy-7-methoxy-2,2-dimethyl-10-((3-methyl-2-butenyl)oxy)-5-(2-methyl-1-propenyl)-, (5R,10S,10aR,14aS,15bS)-
Molecular formulae	$C_{32}H_{41}N_3O_7$
Molecular weight	579.684
Selected references	Yamazaki, Mikio; Fujimoto, Haruhiro; Kawasaki, Takao (Res. Inst. Chemobiodyn., Chiba Univ., Chiba 280, Japan). Chem. Pharm. Bull., 28(1), 245-254 (English) 1980.
Toxicity	A tremorgenic mycotoxin. Intraperitoneal injection of 1 mg in mice caused visible tremors and a dose of 5 mg caused both sustained tremors and 70% mortality.

1.2. APPLICATIONS OF AMYLASE IN TEXTILE INDUSTRY

INTRODUCTION – SIZING & DESIZING

Size is the name given to the substance or mixture of substances that is applied to the warp thread before weaving. The size forms a coating around the surface of the thread before weaving. This coating provides the lubrication & prevents the breakage of warp thread during the weaving operation.

Desizing process removes the size after weaving. The method of desizing depends on the composition of the size. This article mainly deals with enzymatic desizing, which means the use of μ - amylase enzymes to remove starch size.

SIZING AGENTS

Sizing agents are selected on the basis of type of fabric, environmental friendliness, ease of removal, cost considerations, effluent treatment, etc.

NATURAL SIZING AGENTS

Natural sizing agents are based on natural substances and their derivatives:

- Starch and starch derivatives; native starch, degradation starch and chemical modified starch products
- Cellulosic derivatives; carboxymethlycellulose (CMC), methyl cellulose and oxyethlycellulose
- Protein-based starches; glue, gelatin, albumen

SYNTHETIC SIZING AGENTS

- Polyacrylates
- Modified polyesters
- Polyvinyl alcohols (PVA)
- Styrol/maleic acid copolymers

SIZE COMPOSITION

The choice of size components not only depends on the type of fibre, but also on factors such as economics, weaving technique and ease of size removal. It is important that the size should give a strong and even film and should adhere to the property of fibre. As the surface property of different fibres is different, it is understandable that the composition of size will not be the same for all fibre types. For e.g., a good size for cotton will not be a good size for polyester and vice versa.

Some common chemicals used to prepare sizes are Polyacrylic Acid (PA), Polyvinyl Alcohol (PVA), Starch, Modified Starch, Carboxymethyl cellulose (CMC) & Paraffin Waxes. As a general rule, cellulosic fibres including cotton, rayon and blend of these with synthetic fibres such as polyester, is sized with starch based sizes. Starch has good sizing properties on cellulosics and is economical. Thus, in the following discussion of Enzymatic Desizing, the main fibre type involved is cellulosic.

1.3. DESIZING PROCESSES

Desizing, irrespective of what the desizing agent is, involves impregnation of the fabric with the desizing agent, allowing the desizing agent to degrade or solubilise the size material, and finally to wash out the degradation products. The major desizing processes are:

- Enzymatic desizing of starches on cotton fabrics
- Oxidative desizing
- Acid desizing
- Removal of water-soluble sizes

OXIDATIVE DESIZING

In oxidative desizing, the risk of damage to the cellulose fiber is very high, and its use for desizing is increasingly rare. Oxidative desizing uses sodium or potassium persulphates or sodium bromite as an oxidizing agent.

ACID DESIZING

Cold solutions of dilute sulphuric or hydrochloric acids are used to hydrolyze the starch, however, this has the disadvantage of also affecting the cellulose fiber in cotton fabrics.

REMOVAL OF WATER-SOLUBLE SIZES

Fabrics containing water soluble sizes can be desized by washing using hot water, perhaps containing wetting agents and a mild alkali. The water replaces the size on the outer surface of the fiber, and absorbs within the fiber to remove any size residue.

DESIZING – PART OF THE FINISHING OPERATIONS

Cloth as it comes from the loom is not a finished product. It has to be treated in a series of processes before the final article is obtained. These processes are collectively known as finishing operations. There are basically two types of processes involved:-

- Removal of impurities (this includes desizing)
- Application of various chemicals e. g. dyes, softeners, etc.

In order to choose good methods for removing the impurities, it is very important to have an idea of what the impurities are. We must consider both the impurities that are present in the fibre itself and the impurities that have been deliberately applied during the processing, i.e., the size.

REMOVAL OF STARCH SIZES

Starch based size can not be removed simply by washing the cloth with hot water. This is because starch is not soluble in water. Starch can be made soluble by a hydrolysis reaction in which the starch polymer is broken down into small soluble fragments. Complete hydrolysis of starch leads mainly to glucose since starch is a polymer made up of glucose. However, complete hydrolysis is not needed: partial hydrolysis gives sufficient solubility.

Here it is important to remember that cellulose is also a polymer of glucose. The only chemical difference between starch and cellulose is the way in which the glucose units are joined together. It is important to find a way of hydrolyzing the starch with out hydrolyzing the cellulose and this can be a little tricky as both are chemically so similar. For e.g., use of hot acid as an efficient way of hydrolyzing the starch would not be a good idea because there would also be a lot of breakdown of cellulose.

This problem can be solved using enzymes. Enzymes are selective catalyst which only act on specific chemical compounds. The α-amylases are enzymes that catalyse the hydrolysis of starch. These α-amylase enzymes have no effect on cellulose.

Amylase enzymes have been used for many years in textile industry for removal of starch sizes. Originally enzymes from plant or animal sources were used but these have long since been completely replaced by enzymes of bacterial origin obtained by fermentation processes. The first bacterial enzymes made available for this purpose were the *Bacillus subtilis* type of α-amylase. This product has already been used for more than 30 years. During the last decade, the superior α-amylase produced by *Bacillus licheniformis* is becoming increasingly popular with the textile industry.

CHAPTER-2
REVIEW OF LITERATURE

- Upton and Fogarty (1976) have reported on Production and Purification of Thermostable Amylase and Protease of *Thermomonospora viridis*.
- Kundu and Das have reported on Production of Amylase in Liquid Culture by a Strain of *Aspergillus oryzae*.
- Onitsuka and Nakano have reported on Purification and Characterization of Periplasmic α-Amylase from *Xanthomonas campestris* K-1 1 151.
- Hayashida and Yuji have reported on Production and Characteristics of Raw-Starch-Digesting α-Amylase from a Protease-Negative *Aspergillus ficum* Mutant.
- Tanaka, and Sekine have reported on Acid-stable amylase from *Aspergillus aureus*.
- Hockenhull and Herbert have reported on the amylase and maltase of *Clostridium acetobutylicum*.
- Asai and Minoda have reported on The amylase production by submerged culture of *Aspergillus*.
- Mense and Langlykke have reported on Production of mold amylases in submerged culture.
- Lulla, Johar, and Subrahmanyan have reported on Developmentof amylase under submerged cultivation *Aspergillus oryzae*.
- Vallee, Stein, Sumerwell, and Edmond have reported on Metal Content of a-Amylases of Various Origins.
- Mense, Corman, Van lanen and Langlykke have reported on production of mold amylases in submerged culture.

- Yabuki and Fukui have reported on Presence of Binding Site for a-Amylase and of Masking Protein for This Site on Mycelial Cell Wall of *Aspergillus oryzae.*
- Fuwa, has reported on A new method for microdetermination amylase activity by the use of amylose as the substrate.
- Guoqiang, Claire, Alexei and Gregory have reported on Cloning, Sequencing, and Expression of the Gene Encoding Extracellular a-Amylase from *Pyrococcus furiosus* and Biochemical Characterization of the Recombinant Enzyme.
- Chung, Kobayashi, Kanai, Akiba, and Kudo Purification and properties of extracellular amylase from the hyperthermophilic archaeon *Thermococcus profundus*
- Fukusumi, Kamizono, Horinouchi, and Beppu. Cloning and nucleotide sequence of a heat-stable amylase gene from an anaerobic thermophile,*Dictyoglomus thermophilum.*
- Yoshino, and Hayashida have reported on Enzymatic modification of glucoamylase of *Aspergillus awamori.*
- Minoda, Arai, Torigoe, and Yamadahave reported on Acid-stable a-amylase of black *Aspergilli.*
- BhelIa, and Altosaar have reported on Purification and some properties of the extracellular α-Amylase from *Aspergillus awamori.*
- Jonsson and Martin have reported on Protease production by *Aspergillus fumigatus.*
- Toyama, has reported on Amylolytic action of *Aspergillus oryzae* in submerged culture. I. Selection of strains and study of culture conditions.
- Tsuchiya, Corman, and Koepsell have reported on Production on mold amylases in submerged culture. II. Factors affecting the production of alpha amylases and maltase by certain *Aspergilli.*

CHAPTER 3

OBJECTIVE

OBJECTIVE

The main objective of this project is

- Production of ∝-amylase from *Aspergilus fumigatus* in submerged fermention.
- Characterization and purification of the enzyme ∝-amylase.
- Application of ∝-amylase in textile industry for the removal of starch sizes, and it is proved that the enzyme efficiency is three times greater than commercially available chemical method.

CHAPTER- 4

MATERIALS AND METHODS

4.1 MATERIALS

The fungi was isolated from soil and identified in dept. of Plant Pathology, IARI (Indian Agricultural Research Institute) as *Aspergillus fumigatus*. The chemicals such as raffinose, stachyose, p-nitrophenyl-a-galactopyranoside (α-PNPG) were purchased from Sigma chemicals. The DEAE-Sepharose used was also obtained from Sigma chemicals. The galactose, fructose, glucose, sucrose, maltose and PMSF were purchased from Hi-Media, Mumbai. The protein molecular weight marker used for SDS-PAGE was purchased from Bangalore Genei. All other reagents used were of analytical grade.

4.2 METHODS

4.2.1 PREPARATION OF BUFFERS

The following buffers were used:

- Acetate buffer: Stock solution of A, 0.2 M Acetic acid (7.55 ml of Acetic acid in 100 ml) and stock solution of B, 0.2 M Sodium acetate (16.4 g of Sodium acetate in 100 ml) was prepared. Acetate buffer pH (4 to 5.5) was prepared by mixing appropriate proportions of A and B (Table 3.1), diluted to 100 mL and pH of the solution was adjusted and checked with pH meter.
- Glycine–HCl buffer: Stock solution A, 0.2M glycine (15.01 g in 1 L) and stock solution B 0.2 N HCl was prepared. Glycine-HCl buffer pH (2.6 to3.6) was prepared by taking 25 mL of solution A and amount of B as given (Table 3.2), diluted to a total of 100mL and the pH was checked with a pH meter.
- Sodium Phosphate buffer: Stock solution of A, 0.2 M monobasic sodium phosphate (27.89 g in 1000 ml) and stock solution of B, 0.2 M dibasic sodium phosphate (95.365 g in 1000 ml) was prepared. Sodium phosphate buffer pH (6 to 8.5) was prepared by mixing appropriate proportions of A and B (Table 3.3), diluted to 100 mL and pH of the solution was adjusted and checked with pH meter.
- Tris-HCl buffer: Stock solution of A 0.2 M Tris hydroxy methyl aminomethane, 24.2 g in 100ml. Solution B 0.2 N HCl was used to adjust the pH to 9.

TABLE 4.1: ACETATE BUFFER

A	B	pH
46.3	3.7	3.6
41.0	9.0	4.0
25.5	24.5	4.6
14.8	35.2	5.0
4.8	45.2	5.6

TABLE 4.2: GLYCINE-HCl BUFFER

B	pH
12.1	2.6
5.7	3.0
2.5	3.6

TABLE 4.3: PHOSPHATE BUFFER

A	B	pH
87.7	12.3	6.0
73.5	26.5	6.4
39.0	61.0	7.0
16.0	84.0	7.5
5.3	94.7	8.0

4.2.2: PREPARATION OF ENZYME EXTRACT

The enzyme extract was prepared by growing *Aspergillus fumigatus* in Czapek-dox broth with starch as carbon source. It was then filtered and the filtrate was used as enzyme source.

4.3 CHARACTERIZATION

4.3.1 SCREENING FOR PRESENCE OF DIFFERENT ENZYMES:

4.3.1.1 INVERTASE ASSAY

Invertase activity in the crude enzyme extract from *Aspergillus fumigatus* was carried out according to the method of Ricardo and Rees (1970). The reaction mixture contained 0.2 ml of enzyme extract, 0.3 mL of 1% sucrose and 1.5mL acetate buffer (0.2 M, pH 5.5). the tubes were incubated at 40°C water bath for 15 min. 1 mL of DNS was added and incubated in boiling water bath for 5 min. 1mL of 40% Rochelle salt (Sodium Potassium tartarate) solution was added and the color developed was measured spectrophotometrically at 540nm.

4.3.1.2 XYLANASE ASSAY

Xylanase activity in the crude enzyme extract from *Aspergillus fumigatus* was carried out. The reaction mixture contained 0.5 ml of enzyme extract, 1.0mL of Xylan and 1.5mL acetate buffer (0.2 M, pH 7.5). The tubes were incubated at 37°C water bath for 15 min. 1 mL of DNS was added and incubated in boiling water bath for 5 min (Dubois et al, 1951). 1mL of 40% Rochelle salt (sodium potassium tartarate) solution was added and the color developed was measured spectrophotometrically at 540nm.

4.3.1.3 PROTEASE ASSAY

Protease activity in the crude enzyme extract from *Aspergillus fumigatus* was carried out. The reaction mixture contained 0.1 mL enzyme, 0.5 mL of 1% casein and 1.5 mL of phosphate buffer (0.2M, pH 6.5). The tubes were incubated at 40°C water bath for 15 min. 1mL of 10% TCA was added and the contents were filtered and filtrate was used for protein estimation by Folin Lowry method (Lowry *et al.*, 1951).

4.3.1.4. β -GALACTOSIDASE ASSAY

ß-galactosidase activity was assayed by spectrometric measurement of the release of p-nitrophenol from p-nitrophenyl ß-D-galactopyranoside (ß-PNPG) at 405nm by modified method of Dey and Pridham (1969). The reaction mixture consists of 100 µL of 10mM PNPG in water, 800 µL of acetate buffer (0.2M pH 4.6) and 100 µL of appropriately diluted enzyme. After incubation at 37°C for 15 minutes, the reaction was stopped by the addition of 3 mL of 0.2 M sodium carbonate and the color developed was measured spectrophotometrically at 405nm.

4.3.1.5 LIPASE ASSAY

Lipase activity in the crude enzyme extract from *Aspergillus fumigatus* was carried out 0.7mL of emulsifying reagent and 0.3mL of olive oil was sonicated and to this mixture 0.2mL of enzyme extract, 0.8mL of phosphate buffer (0.2M, pH 8) was added and incubated at 50°C for 30min. To this 2mL of acetone and ethanol (1:1) mixture was added and titrated against 0.01N NaOH with phenolphthalein as indicator. The titre value was noted.

EMULSIFYING AGENT

1.79g of NaCl, 0.014 g of KH_2PO_4, 54mL of glycerol, 0.3g guargum was mixed and the solution was made upto 100 mL with distilled water.

4.3.1.6 α-GALACTOSIDASE ASSAY

α-Galactosidase activity was assayed by spectrometric measurement of the release of p-nitrophenol from p-nitrophenyl-a-D-galactopyranoside(a-PNPG) at 405nm by modified method of Dey and Pridham. The reaction mixture consists of 100µL of 10mM PNPG in water, 800µL of acetate buffer (0.2M pH 4.6) and 100µL of appropriately diluted enzyme. After incubation at 37°C for 15 minutes, the reaction was stopped by the addition of 3mL of 0.2M sodium carbonate. One unit of enzyme activity is defined as the amount of enzyme releasing 1µmol of p-nitrophenol per minute under assay conditions.

PREPARATION OF PNPG

30 mg of p-nitrophenyl α-D-galactopyranoside was dissolved in distilled water and the volume was made upto 10 mL. This was used as substrate for α-galactosidase assay.

4.3.2 EFFECT OF DIFFERENT FACTORS ON α-AMYLASE ACTIVITY

4.3.2.1 OPTIMUM pH

The effect of pH on the enzyme was measured at 37°C in the pH range from 3 to 9.0. The following buffers were used: 100mM acetate buffer (pH 3.6 to 5.5), 100mM phosphate buffer (pH 6 to 7) and 100mM Tris-Hcl buffer (pH 7.5 to 9.0).

4.3.2.2 OPTIMUM TEMPERATURE

The influence of the temperature on a-amylase activity was determined by incubating the assay mixture for 15 min at temperature from 20-70°C.

4.3.2.3 pH STABILITY

pH stability was determined by incubating the enzyme extract at pH- 4.0, 5.0 and 6.0 for two hour and the residual activity was determined, every 30 min.

4.3.2.4 TEMPERATURE STABILITY

The cell free extracts were heated to 50°C, 60°C for 2 hours in a water bath and enzyme activity was determined every 30 min, and assayed for remaining a-amylase activity.

4.3.3 EFFECT OF METAL IONS, SUGARS AND SOME REAGENTS

A reaction mixture consisting of 0.2mL of enzyme solution, 1.45mL of 0.2M acetate buffer (pH 4.6) and 0.05mL of metal ions (Ag^{2+}, Hg^{2+}, K^+, Fe^{2+}, Zn^{2+}, Ca^{2+}, Cu^{2+} and Mg^{2+}) or sugars (xylose, glucose, galactose, fructose, maltose, lactose, melibiose, raffinose) or some reagents (sodium azide, EDTA, 1,10-phenanthroline, N-bromosuccinamide and PMSF, final concentration indicated) were incubated for 15 min at 40°C and a-amylase activity was determined. The activity was expressed as a percentage of the activity level in the absence of the compound.

4.3.4 EFFECT OF SUGARS IN INDUCTION OF α-AMYLASE

The Czapek-dox broth was prepared with different carbon source such as sucrose, fructose, arabinose, galactose, xylan, glucose, guargum, starch, lactose, maltose, and xylose.

The Czapek-dox medium composition is as follows:

Components	Amount (g/L)
$NaNO_3$	2.0
KH_2PO_4	1.0
KCl	0.5
$MgSO_4.7H_2O$	0.5
$FeSO_4.7H_2O$	0.5
Carbon source	20

25mL of broth with different carbon source was prepared and *Aspergillus fumigatus* was inoculated and allowed to grow for a period of seven days. It was then filtered and the filtrate was checked for α-amylase activity.

4.3.5 EFFECT OF VARYING SUBSTRATE CONCENTRATION

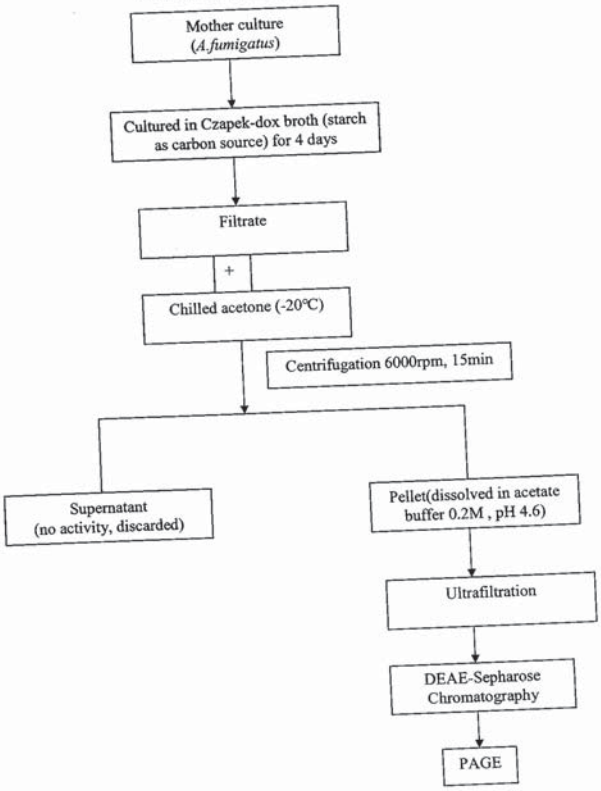
The α-amylase assay was performed by varying the concentrations of starch from 25µL to 500µL. The absorbance was measured at 540nm and readings were tabulated.

The starch solution was prepared (1g in 100mL). The degradation of starch by α-amylase was checked by DNS method. Varying concentrations of starch solution (25µL to 500µL) was treated with 0.2mL of appropriately diluted enzyme. This was incubated at 40°C for 15 min in a water bath. 1mL of DNS reagent was added and kept in boiling water bath for 5 min (Dubois et. al., 1951) and to that 1mL of 40% of Rochelle salt (sodium potassium tartarate) solution was added and the color developed was measured spectrophotometrically at 540nm.

DNS REAGENT

1g DNSA (Dinitrosalicylic Acid), 200mg crystalline phenol and 50 mg sodium sulphite was dissolved in 100mL of 1% sodium hydroxide solution.

FIG.4.1. PURIFICATION CHART



4.4. PURIFICATION

4.4.1 ACETONE PRECIPITATION

To the enzyme extract (600ml), equal volume of chilled acetone (-20°C) was added slowly with constant shaking and the mixture was kept at 4°C for 6h. At the end of 6h, the mixture was centrifuged at 6000rpm for 15min and the supernatant was discarded. The precipitate was dissolved in acetate buffer (0.2M, pH 4.6).

4.4.2 ULTRAFILTRATION

The acetone precipitated enzyme was filtered for 30 minutes with a 50 kDa Omega Ultra filtration membrane unit (PALL Corporation, USA). The retentate and filtrate was collected separately and was checked for α-amylase activity.

4.4.3. ION-EXCHANGE CHROMATOGRAPHY

4.4.3.1 REGENERATION OF THE DEAE-SEPHAROSE COLUMN

- The column was washed with 150mL of Tris-HCl (0.1M,pH 8.5) containing 0.5M NaCl.
- The column was washed with 0.1M sodium acetate buffer pH 4.6 containing 0.5M NaCl.

4.4.3.2 DEAE-SEPHAROSE CHROMATOGRAPHY

The enzyme obtained from ultra filtration step was loaded on to a DEAE-Sepharose column equilibrated with acetate buffer (25mM, pH 4.6) and the column was washed with same buffer. The enzyme bound to DEAE-Sepharose was eluted with acetate buffer (25mM, pH 4.6). 3mL fractions were collected. All fractions were checked for a-amylase activity (A_{540}) and protein at 280nm.The fractions (50-60) had maximum enzyme activity were pooled.

4.4.4 PAGE

- **Monomer solution**

The solution contained 29.2g of acrylamide and 0.8g of N-N- methlyene bis acrylamide in final volume of 100mL made in distilled water. The solution was filtered through Whatman number 1 filter paper.

- **1.875M Tris buffer pH 8.8**

22.76g of Tris was dissolved in distilled water. The pH was adjusted to 8.8 with HCl and the volume was made up to 100mL. The solution was stored at refrigerator.

- **1.25M Tris-HCl buffer pH 6.8**

15.125g of Tris was dissolved in distilled water. The pH was adjusted to 6.8 with 1N HCl and the volume of the solution was made up to 100mL. The solution was stored at refrigerator.

- **Sodium dodecyl sulphate (SDS) 10% w/v**

10g of SDS was dissolved in distilled water and final volume was made up to 100mL and stored at room temperature.

- **Ammonium persulfate (APS), (1% w/v)**

Ammonium persulfate 100mg was dissolved in distilled water and the volume was made up to 10mL. Freshly prepared APS was used every time.

- **Electrophoresis buffer (10X)**

Tris base 6g, glycine 14.4g and SDS 10g was dissolved in distilled water and the volume was made up to 1000mL and stored at room temperature.

- Seperating gel mixture (10%)

Stock acrylamide solution	3.71mL
Tris HCl (pH 8.8)	2mL
Distilled Water	4.5mL
APS	100µL
TEMED	20 µL

The mixture was poured into cassette kept in the gel casting apparatus and was allowed to polymerize.

- Stacking gel mixture (4%)

Stock acrylamide solution	340 µL
Tris HCl (pH 6.8)	250 µL
Distilled Water	1.87 mL
APS	50µL
TEMED	10 µL

The mixture was poured into the cassette above the separating gel. A comb was introduced into the spacer gel and was allowed to polymerize in presence of light. After polymerization the comb was removed from the cassette and the gel was inserted into the gasket of electrophoretic apparatus. The bromophenol blue containing sample buffer was mixed with the protein sample and was loaded in the wells. Electrophoresis was performed at room temperature and at constant current of 100 volts. When the bromophenol blue dye stopped 1 cm from the bottom of the gel, electrophoresis was stopped. The gel was removed from glass plate and was stained.

4.4.4.1 ASSAY

One part of the gel was equilibrated with acetate buffer (0.2M, pH 4.6) and was cut into uniform pieces. Each piece was checked for a-amylase activity.

4.4.4.2 COOMASSIE BRILLIANT BLUE STAINING

The gel was soaked in staining solution. After allowing it to stain for 3 hour the gel was immersed in the destaining solution. Destaining was stopped when the gel background was colorless.

- Staining solution

1g Coomassie brilliant blue in a solution containing 40ml methanol, 10ml glacial acetic acid and 50ml distilled water.

- Destaining solution

Solution containing 40ml methanol, 10ml glacial acetic acid and 50ml distilled water.

4.4.4.3 SILVER STAINING

- The gel was transferred into a container containing washing solution and was washed for ten minutes.
- The washing solution was then discarded and the gel was rinsed with distilled water for two minutes.
- The gel was soaked in sodium thiosulphate for 1 to 2 minutes.
- Then it was washed twice with distilled water for 2 minutes.
- The gel was silver nitrate solution for 10 minutes with accompanied shaking.
- Then it was washed twice distilled with water for 2 minutes.
- Then it was soaked in developing solution with intermittent shaking till dark brown color band appears.
- When sufficient intensity of band developed, the reaction is stopped by adding stopper solution.

REAGENTS

WASHING SOLUTION

1mL of formaldehyde, 40mL methanol and 60mL of distilled water was mixed together.

Sodium thiosulphate:

200mg of sodium thiosulphate was dissolved in 1000mL of distilled water.

Silver nitrate solution (0.1%):

0.1g of silver nitrate was dissolved and the volume was made up to 100mL with distilled water.

DEVELOPING SOLUTION

3g of sodium carbonate was dissolved in 80mL of distilled water and to this 1mL of above sodium thiosulphate solution and 1mL of formaldehyde was added and the volume was made up to 100mL with distilled water.

Stopper solution:

5% acetic acid solution (5 mL of glacial acetic acid made up to 100mL with distilled water).

4.4.5 BSA CALIBRATION CURVE

A standard protein curve was determined by the method described by Lowry *et al.*, (1951). Bovine serum albumin (BSA, 0.1mg/ml) was used as standard protein. Different concentrations of BSA solutions were pipetted out in the test tube and the total volume was made up to 1mL with distilled water. 2.1mL of alkaline copper reagent was added to each tube, mixed thoroughly and allowed to stand at room temperature for10min. Then to each tube 0.2mL of 1N Folins-Ciocalteau's reagent was added with immediate mixing. The extinction was read after 20min incubation against appropriate reagent blank at 660 nm in an ELICO spectrophotometer. A standard graph was constructed.

ALKALINE COPPER REAGENT

Reagent A was prepared by dissolving 2g sodium carbonate in 0.1N sodium hydroxide.

Reagent B was prepared by dissolving 1g copper sulfate in 100mL of distilled water.

Reagent C was prepared by dissolving 1g sodium potassium tartarate in100mL of distilled water.

98mL of reagent A was mixed with 1mL of reagent B and 1mL of reagent C just before use.

FOLIN-CIOCALTEAU'S REAGENT

2N Folin-Ciocalteau reagent commercially available was diluted with distilled water in the ratio 1:1(v/v) just before use.

4.4.6 MALTOSE CALIBRATION CURVE

A standard calibration curve of maltose was constructed according to the method of Tanaka *et al.*, A standard maltose solution was prepared with a concentration of 1mg/2ml of distilled water, different volumes of maltose solution 0.2, 0.4, 0.6, 0.8 and 1 ml was taken in a series of test tubes and the volume was made up to 2ml with distilled water to each tube. One ml of DNS was added and tubes were kept in boiling water bath for exactly 5 minutes. Then, 1ml of 40% Rochelle salt was added to the test

tubes. The optical density of yellow color formed was measured at 540nm against blank in spectrophotometer. A graph was drawn.

Table 4.4: BSA CALIBRATION TABLE

Reagents	B	S_1	S_2	S_3	S_4	S_5
Volume of working std (mL)	0.0	0.2	0.4	0.6	0.8	1.0
Concentration(µg)	0	20	40	60	80	100
Volume of distilled water (mL)	1.0	0.8	0.6	0.4	0.2	0.0
Volume of alkaline copper reagent (mL)	2.1	2.1	2.1	2.1	2.1	2.1
Incubate at room temperature for 10 min						
Volume of Folin's reagent (mL)	0.2	0.2	0.2	0.2	0.2	0.2
Incubate at room temperature for 20 min						
Absorbance at 660nm.	0.0	0.139	0.199	0.261	0.345	0.411

Table 4.5 MALTOSE CALIBRATION TABLE

Reagents	B	S_1	S_2	S_3	S_4	S_5
Volume of maltose (mL)	0.0	0.2	0.4	0.6	0.8	1.0
Concentration (µg)	0.0	20	40	60	80	100
Volume of distilled water (mL)	1.0	1.8	1.6	1.4	1.2	1.0
Volume of DNS (ml)	1.0	1.0	1.0	1.0	1.0	1.0
Incubate in boiling water bath for 5 min						
OD at 540nm	0.0	0.015	0.031	0.045	0.057	0.065

4.5. IMMOBILIZATION

4.5.1 IMMOBILIZATION IN CALCIUM ALGINATE

2.5% (w/v) sodium alginate solution was prepared and to that 2ml of crude enzyme extract was added and mixed well. It was left undisturbed for 30 minutes. The calcium alginate beads developed when sodium alginate solution was added to excess of 0.2M calcium chloride solution. The beads were used as enzyme source for α-amylase assay.

4.5.2 IMMOBILIZATION IN AGAR

2.5% agar solution was prepared and to that 2mL of crude enzyme extract was added and mixed well. It was poured onto a glass plate and left undisturbed for 20 minutes, after which it was cut into pieces of uniform size and used as enzyme source for α-amylase assay.

CHAPTER-5

RESULTS AND DISCUSSION

5.1 Characterization:

The α-amylase activity was determined in the filtrate of *Aspergillus fumigatus*. It was also found that a very little amount of hydrolytic enzymes such as invertase, protease, xylanase, ß-galactosidase and lipase were produced extracellularly by *Aspergillus fumigatus*.

As expected, α-amylase activity was found to vary with pH .The effect of pH on enzyme activity is shown in figure 4.1. The cell free extracts exhibited α-amylase activity in a pH range between 2.6 to 6.0, the optimum pH being 4.6.

α-amylase from *Aspergillus fumigatus* had an optimum temperature at 45°C.

The α-amylase enzyme was found to be stable at pH- 5.0and 6.0.

The α-amylase activity was found to decrease very slightly at temperatures 50°C and 60°C in 2 h.

Table 4.5 shows the effect of metal ions on α-amylase activity. Among the cations tested, heavy metal ions such as Ag^{+} and Hg^{2+} strongly inhibited the enzyme activity indicated that tryptophan is present at or near active site.

Table 4.6 shows the effect of reagents on α-amylase activity. Among the reagents tested N-bromosuccinamide inhibited the enzyme activity to about 84.2%. The above result indicated the presence of tryptophan at or near active site.

The reagents such as PMSF and 1, 10-phenanthroline slightly inhibited the enzyme activity indicating that it is not a metallo-enzyme.

Table5.1 DETERMINATION OF OPTIMUM pH

pH	Absorbance (540nm)	Activity (U/ml)
2.6	0.368	0.533
3.0	0.180	0.233
3.6	0.018	0.04
4.0	0.020	0.046
4.6	0.070	0.156
5.0	0.065	0.146
5.5	0.024	0.036
6.0	0.006	0.016
7.0	0.000	0.000
8.0	0.028	0.063
9.0	0.000	0.000

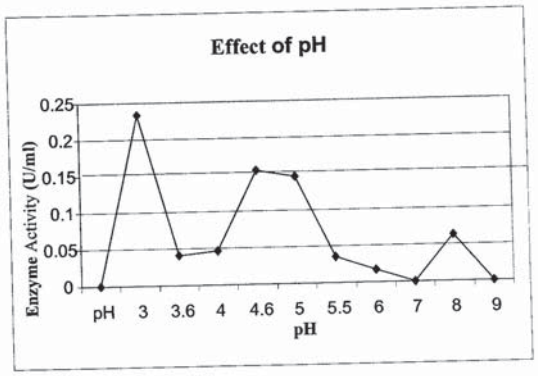


Figure 5.1. Effect of pH on α-amylase activity

Table5.2. DETERMINATION OF OPTIMUM TEMPERATURE

TEMPERATURE	ABSORBANCE (540nm)	ACTIVITY (U/ml)
20	0.014	0.033
25	0.024	0.053
30	0.000	0.00
35	0.022	0.05
40	0.132	0.288
45	0.135	0.305
50	0.141	0.31
55	0.009	0.023
60	0.087	0.176
70	0.045	0.10

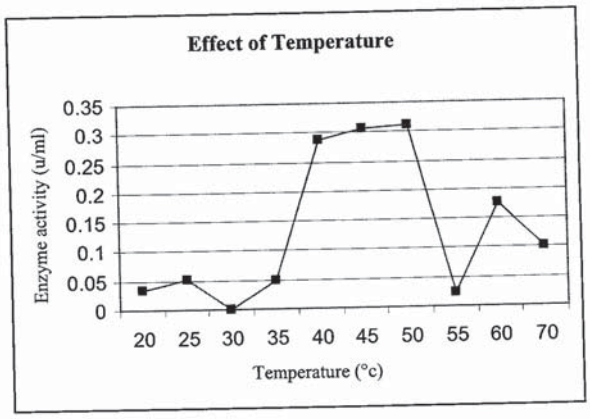


Figure.5.2: Effect of temperature on α-amylase activity

Table.5.3: Effect of pH on stability of α-amylase

Activity (U/ml) 0mins	Activity (U/ml) 30mins	Activity (U/ml) 60mins	Activity (U/ml) 90mins	Activity (U/ml) 120mins
0.633	0.583	0.55	0.533	0.483
0.516	0.50	0.45	0.40	0.366
0.483	0.466	0.40	0.316	0.350

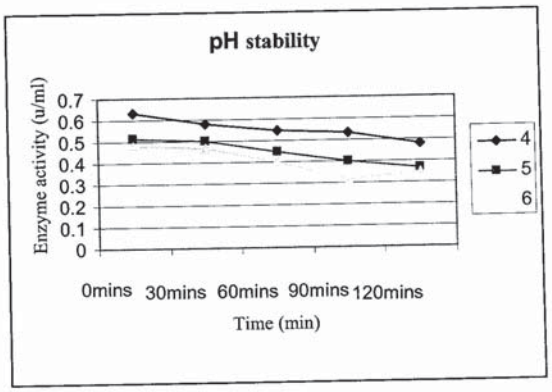


FIGURE 5.3. Effect of pH on stability of α-amylase

Table5.4 Effect of temperature on stability of α-amylase

Temperature (°C)	Activity (U/mL) 0min	Activity (U/mL) 30 min	Activity (U/mL) 60 min	Activity (U/mL) 90 min	Activity (U/mL) 120 min
50	0.583	0.733	0.916	1.35	1.316
60	0.716	0.866	1.183	1.416	1.45

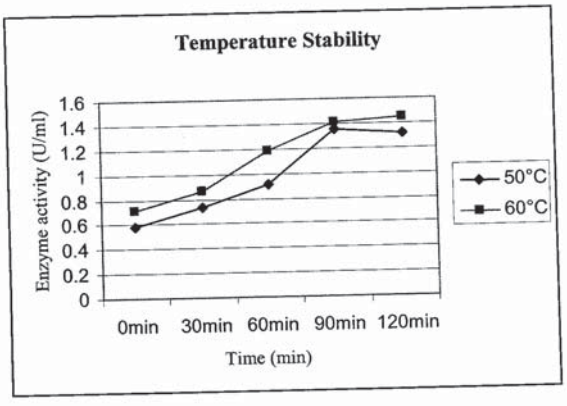


FIGURE 5.4 Effect of temperature on stability of α-amylase

TABLE 5.5. EFFECT OF METAL IONS ON α-AMYLASE ACTIVITY

METAL ION	ABSORBANCE (540nm)	ACTIVITY (U/ml)
Control (without metal ion)	0.060	0.133
Ag^{2+}	0.00	0.00
Hg^{2+}	0.00	0.00
K^{+}	0.026	0.0566
Fe^{2+}	0.061	0.133
Zn^{2+}	0.058	0.13
Ca^{2+}	0.064	0.143
Mg^{2+}	0.043	0.096
Cu^{2+}	0.011	0.023

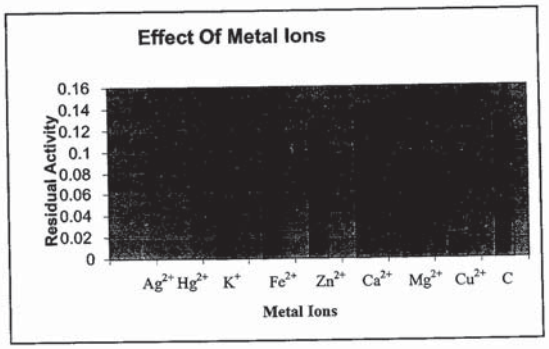


FIGURE 5.5 Effect of metal ions on α-amylase activity

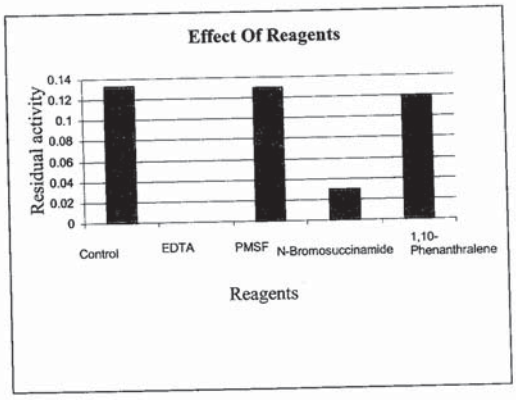


FIGURE 5.6 Effect of reagents on α-amylase activity

Table 5.6 EFFECT OF REAGENTS ON α-AMYLASE ACTIVITY

REAGENT	ABSORBANCE(540nm)	ACTIVITY(U/ml)
Control	0.06	0.133
EDTA	0.00	0.00
PMSF	0.058	0.13
N-Bromosuccinamide	0.013	0.03
1,10-Phenanthralene	0.054	0.120

Table.5.7. EFFECT OF SUBSTRATE (K_m) CONCENTRATION (STARCH)

SUBSTRATE CONCENTRATION S (µL)	ACTIVITY, V (U/ML)	1/S (X 10^3)	1/V
25	0	40	0
50	0	20	0
100	0	10	0
200	0	5	0
250	0.053	4	18.86
300	0.15	3.3	6.6
350	0.2	2.8	5
400	0.35	2.5	2.85
450	0.40	2.22	2.5
500	0.433	2	2.309

5.2 PURIFICATION

The purification steps are shown in flow chart (Flow chart.)

The a-amylase was partially purified; this was achieved by combination of acetone precipitation, ultrafiltration, and DEAE-Sepharose chromatography.

Native PAGE analysis of acetone precipitated protein indicated that the molecular weight of a-amylase is greater than 55kDa.

ULTRAFILTRATION

The filtrate and retentate collected after ultrafiltration of the acetone precipitated enzyme was subjected to a-amylase assay. The a-amylase activity was found only in the retentate and not in the filtrate confirms the results obtained in native PAGE.

ION EXCHANGE CHROMATOGRAPHY

The a-amylase activity was observed in fraction numbers 50 to 60. The fraction number 54, 55, and 56 showed higher enzyme titres and were pooled.

PAGE

After three purification steps, native PAGE and SDS-PAGE of the final enzyme preparation showed a single band. The molecular weight of the purified a-amylase by native PAGE was estimated to be 55 kDa. Cozzone *et al.*, (1970). These showed that the molecular weight of the purified enzymes was around 51,000-54,000 Da. Granger *et al.*, (1975) who indicate the core weight to be 50,000.A single band in SDS-PAGE confirmed that the enzyme is a monomer.

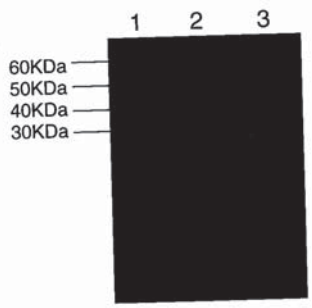


FIG.5.7. NATIVE PAGE of the α-amylase. The gel electrophoresis was done as described by Dr.Sadasivam and A.Manickam.

Legend
1- Marker
2- Sample 1
3- Sample 2

Table5.8. Purification table

Steps	Volume (mL)	Protein (mg)	Activity (Units)	Specific activity (U/mg)	Purification (-fold)	Yield (%)
Crude	600	1.120	7.38	6.58	1	100
Acetone precipitation	50	0.750	6.58	8.77	1.332	7.42
Ultrafiltration	15	0.532	10	18.80	2.14	4.55
Ion-exchange chromatography	6	0.058	4.05	69.8	3.71	1.62

5.3 IMMOBILIZATION

The a-amylase immobilized in 2.5% (w/v) calcium alginate beads retained 80% and activity of free enzyme. The a-amylase immobilized in 2.5% of agar retained 88% of free enzyme activity.

5.4 APPLICATION IN TEXTILE INDUSTRY

The starch is added to clothes in order to withstand the tension. Thus addition of starch is done to increase the tensile strength of the cloth. But the same starch hinders the uniform colouring of the cloth, which is the subsequent step in textile manufacturing. This applied starch is known as size. Usually the starch is removed from clothes by chemical methods. This process is called desizing.

In this process we have used a-amylase for effective removal of starch from grey cloth. The process include the following steps

i. Starch was added to the grey cloth and the initial weight was noted
ii. The enzyme a-amylase which was collected from the purification process was added to the fabric to remove the starch
iii. The final weight of the fabric was noted and result was tabulated.

TABLE 5.9.DESIZING PROCESS USING α-AMYLASE

Y TH ^{2})	INITIAL CLOTH WEIGHT	BUFFER pH-4.5	Incubation 30 mins	ENZYMES	Incubation 2hrs	FINAL CLOTH WEIGHT	SAMPLE+ IODINE SOLUTION
rol	180mg	3ml		Nil		178mg	Dark blue
	180mg	3ml		100µl		162mg	Pale blue

$\frac{(178-162)}{178} \times 100 = 8.9$ $\frac{162}{178} \times 100 = 91$

$\frac{178}{162} \times 100$

$\frac{180-178}{180} = \frac{2}{180} \times 100 = 1.1\%$

$\frac{180-162}{180} = \frac{18}{180} \times 100 = 10\%$

Significant starch removal was observed. This method was three times more effective than the normal chemical methods. Weight loss was attributed to the starch concentration by treating with iodine solution. The amount of blue colour formed corresponds directly to the starch concentration in the cloth.

The advantages of a-amylase over chemical methods are:

1. Less time
2. High efficiency
3. Eco-friendly method

CHAPTER- 6
CONCLUSION

CONCLUSION

The enzyme α-amylase was produced from *Aspergillus fumigatus*. The optimum pH and temperature was found to be 4.5 and 45°C respectively. Enzyme from crude was precipitated by using acetone , then it was purified by ultra filtration and ion exchange chromatography. Then the molecular weight of the enzyme was determined by running native PAGE. Approximately it was around 55Kda. The enzyme purified from crude was used for textile application. It was observed that this enzymatic method was three times more effective than the normal chemical methods. It was also much effective in removing starch sizes from cloth. This enzyme won't cause any damage to cloth, at the same time it won't affect the strength of the cloth.

CHAPTER-7
REFERENCES

REFERENCES

1. Baird, S. D., Hefford, M. A., Johnson, D. A., Sung, W. L., Yaguchi, M.Seligy, V. L. (1990) .Biochem. Biophys. Res. Commun. 169,1035-1039
2. Bennett JH. On the parasitic vegetable structures found growing in living animals. *Trans Roy Soc Edinburgh* (1842)15:277-279.
3. Cawley EP.,(1947). *Aspergillosis* and the *Aspergilli*. *Arch Intern Med* 80:423-434.
4. Denning DW, del Favero A, Gluckman E, Rhunke M, Yonren S, Troke P, Sarantis N. The efficacy and tolerability of UK 109-196 (Voriconazole) in the treatment of invasive aspergillosis (IA). *Abstr F552 ISHAM* 1997; Parma, Italy 8-13 .
5. Denning DW. (1996). Therapeutic outcome of invasive *Aspergillosis*. *Clin Infect Dis* 23:608-615.
6. Denning DW. (1998). Invasive aspergillosis (State-of-the-art clinical article). *Clin Infect Dis* 26:781-805.
7. Groll AH, Shah PM, Mentzel C, Schneider M, Just-Neubling G, Huebling G, Huebner K. (1996). Trends in the postmortem epidemiology of invasive fungal infections at a university hospital. *J Infect* 33:23-32.

8. Haines, J. (1995). *Aspergillus* in compost: straw man or fatal flaw. Biocycle 6: 32-35.

9. Henrissat, B. (1990). Protein Sequences Data Anal. 3, 523-526.

10. Henrissat, B., Claeyssens, M., Tomme, P., Lemesle, L. & Mornon, J. P.(1989) Gene 81, 83-95.

11. Kwon-Chung, K.J. & Bennett, J.E. (1992). Medical mycology. Lea & Febiger. Philadelphia, Pa.

12. Leslie, C.E., Flannigan, B. & Milne, L.J.R. (1988). Morphological studies on clinical isolates of *Aspergillus fumigatus*. J. Med. Vet. Mycol, 26: 335-341.

13. Lowry.H.,Nira J Rosebrough.,Lewis Farr and Rose J.(1951).Protein measurement with the folin phenol reagent.

14. Py, B., Bortoli-German, I., Haiech, J., Chippaux, M. & Barras, F. (1991).Protein Eng. 4, 325-333.

15. Rankin NE. (1953). Disseminated *Aspergillosis* and *Moniliasis* associated with agranulocytosis and antibiotic therapy. Br Med J April 25:918-919.

16. Raper, K.B. & Fennel, D.I. (1965). *Aspergillus fumigatus* group. p. 238-268. *In* K.B.Raper and D.I.Fennel (ed.), the genus *Aspergillus*. The William & Wilkins Co., Baltimore, Md.

17. Samson, R.A. & Van Reenen-Hoekstra, E.S. (1988). Introduction to food-borne fungi. Centraalbureau voor Schimmel cultures, Baarn, Delft, The Netherlands.

18. Samson, R.A. (1994). Current systematics of the genus *Aspergillus*, p. 261-276. *In* K.A.Powell, A. Renwick & J.F.Peberdy (ed.), The genus *Aspergillus*: from taxonomy and genetics to industrial application. Plenum Press, London, United Kingdom.

19. Schmidt, A. & Wolff, M.H. (1997). Morphological characteristics of *Aspergillus fumigatus* strains isolated from patients samples. Mycoses 40: 347-351.

20. Sadasivam, S.and Manickam, A.(1991) Biochemical methods.,Second edition:59-63.

21. Sinnott, M. L. (1990). Chem. Rev. 90, 1171-1202.

22. Vogeser M, Wanders A, Haas A, Ruckdeschel G. (1999). A four-year review of fatal aspergillosis. *Eur J Clin Microbiol Infect Dis* 18:42-45.

23. Wheaton SW. (1890) Case primarily of tubercle, in which a fungus (*Aspergillus*) grew in the bronchi and lung, stimulating actinomycosis. *Path Trans* 41:34-37.

24. Withers, S. G., Warren, R. A. J., Street, I. P., Rupitz, K., Kempton, J. B.Aebersold, R. (1990) J. Am. Chem. Soc. 112, 5887-5889.

25. Zvelebil, M. J. J. & Sternberg, M. J. E. (1988) Protein Eng. 2, 127-138.