Optimization and Production of Fungal Laccase and its Effect on Denim Fabric

By

S. MURUGANANDHI
(REG. NO : 04MP39)

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Master of Philosophy in Biochemistry

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Certificate

CERTIFICATE

This is to certify that the dissertation entitled **"Optimization and Production of Fungal Laccase and its Effect on Denim Fabric"** *submitted to the Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore in partial fulfillment of the requirement for the degree of* ***Master of Philosophy in Biochemistry*** *is record of original research work done by* ***S. Muruganandhi*** *during the period of her study in the Department of Biochemistry, Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore under my supervision and guidance and the dissertation has not formed the basis for the award of any Degree / Diploma / Associateship / Fellowship or other similar title to any candidate of any University.*

Parvathami 28/9/05
Signature of the guide

Forwarded

Parvathami 28/9/05
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I hereby declare that the dissertation entitled **"Optimization and Production of Fungal Laccase and its Effect on Denim Fabric"** *submitted to the Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore in partial fulfillment of the requirement for the degree of* ***Master of Philosophy in Biochemistry*** *is record of original research work done by me under the supervision and guidance of* ***Dr. R. Parvatham,*** *Professor and Head, Department of Biochemistry & Biotechnology, Avinashilingam Institute for Home Science and Higher Education for Women (Deemed University), Coimbatore and that it has not formed the basis for the award of any Degree / Diploma / Associateship / Fellowship or other similar title to any candidate of any University.*

Signature of Candidate

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Introduction

1.0 INTRODUCTION

Microbial enzymes are widely used in a great variety of industrial applications such as brewing, detergent formulation, baking, food processing, paper and pulp industry and textile industry. Enzymes acting on natural substrate can improve the fabric quality and value.

Textile industry has a wide potential for adaptation of enzyme technology in its processes like desizing, biowashing, biopolishing, degumming, wool finishing, effluent treatment, denim bleaching and finishing. The important classes of enzymes used in textile application include majority of hydrolases and few oxidoreductases (Gowda *et al.*, 2004).

Enzymes have been used in textile processing industry, the important aspects to be considered are minimicing the pollution of water and atmosphere during production below specified limits, minimics the usage of harmful chemicals and minimicing the discharge of waste water volume of the possible extent (Natarajan, 1998).

Denim is the most popular fabric worldwide and among the all age groups. Denim fabric is made from 100% cotton yarns and dyed with indigo dye. The speciality of the denim is that only warp yarns are dyed and weft yarns are white. The processing of denim fabrics has become more less simple and routine. The processing includes wetting, desizing, biofading, bleaching and softening. The general aim of processing the denim garments is to remove size from the fabric and also to remove indigo dye. This gives a used look to denims which is very popular (Shelke, 2001).

The bleaching of denim fabrics creates different shades of blues. The purpose of the bleaching to decolorize the indigo dye to a certain level for getting the desired shade. This was achieved by the denim manufacturer by employing denim stone washing. Process of stone washing utilizes pumice

stones, which are put together with in the washing machine, and they provide abrasion and removal of colours from the garment. Though pumice stone washing gives the desired result, it has got several disadvantages like damage to the machine and garments (Nikhil and Anita, 2003). Enzymes have replaced the use of volcanic lawa stones in the preparation of Denim from all indigo-dyed cotton fibre to achieve a high degree of dye fading. Laccase is a new candidate in this field.

Laccases (bezenediol :oxygenoxidoreductase; EC1.10.3.2), multicopper enzymes belonging to the blue oxidases,catalyse the one-electron abstraction from a wide variety of organic and inorganic substrates. Laccases are found in plants, insects and bacteria but the most important sources of this enzymes are fungi. Laccases are attractive and industrially relevant enzymes that can be used for a number of applications, e.g. for biocatalytic purposes such as delignification of lignocellulosics and cross-linking of polysaccharides, for bioremediation such as waste detoxification and textile industry transformation (Christiane *et al.*, 2002).

New laccase based bleaching technique affects only the indigo. The natural raw white of weft yarn is retained, giving the woven fabric a darker shade, which is not implicitly achieved with chemical bleaching. In case of chemical bleaching the fabric is adversly affected by loss of the tear and tensile strength. Laccases opens up the door to bleach the denim with out losing the strength of the fabric (Ashok and Maheshwari, 2004).

The present trend in the textile industry is towards eco-friendliness. Enzymes have facilitated textile processing and proven to be favourable alternative to many finishing chemicals. This is because the enzymes are biodegradable, work under mild conditions and save the precious energy (Doshi and Shelke, 2001).

Hence the present investigation "**Optimization and production of fungal laccase and its effect on denim fabric**"has been selected with following objectives.

- Exploitation of agro soil as microbial source
- Isolation and identification of fungi from the selected source
- Subculturing of identified fungi
- Determination of laccase activity in selected substrate and selection of the microbial strain which produce the highest enzyme activity
- Purification of laccase
- Application of laccase in textile industry

Review of Literature

2.0 REVIEW OF LITERATURE

Enzymes are used 'now-a-days' in every aspect of chemical processing not as an agent, but in the whole process like detergent, animal feed, food processing and textiles. Textile production is characterized by high quality requirements, high productivity and high flexibility to meet the basic requirement and quality effective production (Vigneswaran, 2004).

Enzymes impart novel finishes to textiles, garments and fabrics. World over textile processing is undergoing a sea change in traditional time consuming and often polluting process are being replaced with efficient quick and green technology. Some enzyme modifies the surface properties of textiles, which acts favourably on dye uptake and other finishes (Monika and Alka, 2004). They are readily biodegradable and can be absorbed back into the environment after use (Thomas and Stig, 2003).

In today's world of fashion, denim occupies a special place. The spread of denim culture, brought a rapid changing of fashion. The review of literature pertaining to the study **"Optimization and production of fungal laccase and its effect on denim fabric"** is discussed under the following headings.

2.1 ENZYMES IN TEXTILE INDUSTRY

- 2.1.1 Desizing
- 2.1.2 Bioscouring
- 2.1.3 Degumming
- 2.1.4 Biopolising

2.2 LACCASE - AN OVERVIEW

- 2.2.1 Mode of action

2.3 LACCASE IN BIOBLEACHING

- 2.3.1 Denim washing
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2.4 OTHER AREAS OF LACCASE

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2.4.2 Laccase in pulp bleaching

2.5 ENZYME PRODUCTION BY MICROBIAL FERMENTATION

2.5.1 Substrates for enzyme production

2.5.2 Microbes for enzyme production

2.6 OPTIMIZATION OF CULTURE CONDITIONS

2.7 COMMERCIALLY AVAILABLE ENZYMES

2.8 SCOPE OF ENZYMES IN TEXTILE INDUSTRY

2.1 ENZYMES IN TEXTILE INDUSTRY

Enzymatic processes have been increasingly incorporated in textiles over the last years. Cotton wool, flax or starches are natural materials used in textiles that can be processed with enzymes. Enzymes have been used for desizing, scouring, polishing, washing, degumming, peroxide degradation in bleaching baths, decolourisation of dye house waste waters, bleaching of released dyestuff and inhibiting dye transfer.

2.1.1 Desizing

Desizing is one of the oldest applications in textile industry. Sizing is a necessary process in weaing to prevent the breakage of cotton warp threads. The size material in general consists of starch either alone or in combination with other polymers. Sizing materials are coated on the surface of the thread and causes insufficient absorption of the water, which results in poor dyeing and finishing. Therefore, removal of the size is necessary for obtaining absorbent fabric to make it suitable for further processing. Removal of the size is called desizing (Shrivastava, 1998).

Conventional desizing may be carried out by treating the fabric with strong chemicals. The chemicals can damage the fabric, and are often

hazardous to handle. Starch degrading enzymes (amylases) are a more environmentally friendly method of desizing. Amylases can bring complete removal of the starch size without harming the fabric (Moses, 2003).

2.1.2 Bioscouring

The non-cellulosic components present in matured cotton fibres are found in cuticle and in the primary cell wall, which the outermost concentric layer that makes up the cotton fibre.The surface layers which contain, proteins, non-cellulosic polysaccharides and other unidentified substances constitute approximately 10% of the total fibre weight. Scouring process removes non-cellulosic impurities,which is necessary for dyeing and finishing (Bohringer and Rupp, 2002).

Traditionally large amount of chemicals are used in the treatment of fabrics. Boiling in sodium hydroxide is the most widely practiced conventional processing to remove such impurities and to improve the wetting and penetration of aqueous dyeing and finishing (Lawson and Duarrant, 2000). The draw back of this process is the use of large amounts of caustic soda (upto 80 kg/ton fibre material) as well as large quantities of rinse water from once scouring processes is complete. The waste water that comes out from their processes has high pH that necessitates neutralization with H_2SO_4 or CO_2, in either case adding to the salt load of the effluent (Li and Hardin,1998). There would be a high chemical oxygen demand (COD) and biochemical oxygen demand (BOD).Efforts made in the recent past to develop a mild and environment friendly process. Environmentally friendly bioscouring using suitable enzymes instead of alkali is gaining more and more acceptance (Traore and Diller, 2000).

A lot of research activities have been directed to find the ability of enzymes such as pectinases, proteases, cellulases and lipases to remove the non–cellulosic material (Ramasamy and Kandsamy,2004).

2.1.3 Degumming

Degumming is basically removal of sericin from raw silk by treatment with soap or alkali (Gulrajani *et al.*, 1997). The ability of proteolytic enzymes to hydrolyse peptide bonds formed by specific aminoacid has been effectively exploited to develop degumming processes. Proteolytic enzymes such as trypsin and papain have been used for silk deguming (Sonwalkar *et al.,*1992).

Degumming of silk with proteolytic enzyme like degummase improves the whiteness of the fabric with no adverse effect on the wettability and strength (Gulrajani *et al.*, 1996).

2.1.4 Biopolising

Removal of protruded fibre is called polising. In conventional process protruded fibers are removed by polising and smoothness is imparted by chemical treatment. The conventional methods are temporary. The fibers return on the surface of the fabric and chemicals are removed after a few washings and fuzz is formed (Duran and Duran, 2000).

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Biopolising is a biological process in which the specific cellulase enzyme acts on the yarn surface of cellulosic fabric to improve their surface characteristics (Menezes, 2002). Cellulase is the one successfully identified for carrying out enzyme biopolishing of textile (Ravichandran, 2000). Pilling and fuzziness would not appear after use of celluase. It gives clear look to the fabric and imparts softness to the fabric.

2.2 LACCASE - AN OVERVIEW

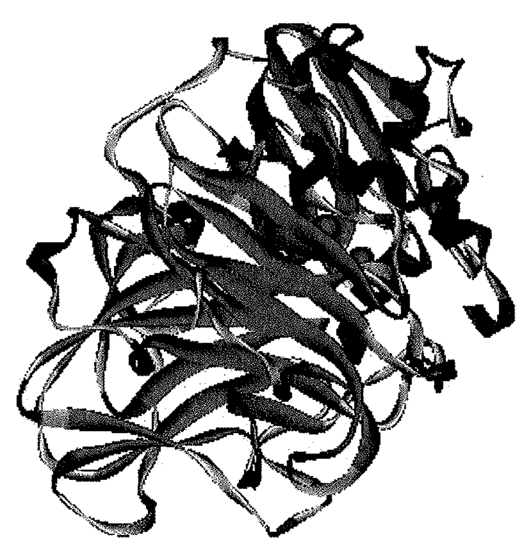
Laccases are a family of multicopper oxidases that bear a close resemblance particularly in aminoacid sequence, to ascorbate oxidase (found in cucumbers) and mammalian plasma protein ceruloplasmin. First isolated in

1883 from Rhusvenicifera, the Japanese lacquer tree, laccases are also commonly found in fungi such as the lignolytic white-rot fungus *Trametes versicolor.* They have also recently been discovered in bacteria. Their physiological function is still under intense investigation although they are implicated in the syuthesis and degradation of the biopolymer lignin, wound response mechanisms and the morphogenesis of microorganisms.

Figure 1 shows the ribbon representation of the X-Ray crystallographic structure of laccase from *Trametes versicolor* (Piontek *et al.*, 2002).

FIGURE 1

RIBBON REPRESENTATION OF THE X-RAY CRYSTALLOGRAPHIC STRUCTURE OF LACCASE



2.2.1 Mode of action

Laccases (p-diphenol :dioxygen oxidoreductase; EC 1.10.3.2) are blue multi copper oxidoreductases and are widespread and found in numerous plants, bacteria and fungi (Sigoillot *et al.,* 2004) .

Laccases have a broad specificity that enable them to catalyse the one electron oxidation of a range of substrates. These substrates, usually phenolic in nature, provide electrons for the concomitant four electron reduction of

atmospheric O_2 to water. Catalysis takes place via the four copper centres which are classified according to their electronic properties.

The four copper atoms of the laccases are distributed in one mononuclear (T1) center and one trinuclear (T2-T3) center. T1 copper, responsible for the characteristic blue color of the enzyme, is the primary electron acceptor during oxidation of a substrate (Ducros *et al.,* 1998). Electron capture by this copper is enhanced by a favourable electronic environment, due to the amino acid residues involved in the copper binding (Solomen *et al.*, 2001). Electrons are transferred to the two-electron acceptor, the T2-T3 center. This trinuclear center accepts these electrons with the simultaneous reduction of a molecule of dioxygen (Thurston, 1994). This reaction allows the oxidation of phenolic compounds, like the polyphenols or methoxy substituted mono phenols (Call and Mucke, 1997). Non-phenolic compounds can also be oxidized in the presence of a redox mediator, such as 2, 2^1-azino-bis-(3-ethyltiazoline-6-sulfonate) (ABTS), which allows the oxidation of high redox potential substrates (Bourbannais *et al.,* 1998; Johannes and Majcherczyk, 2000).

2.3 LACCASE IN BIOBLEACHING

Enzymes are used in the textile industry because they can replace harsh chemicals, accelerate reaction, act only on specific substrates, operate under specific conditions, easy to control and biodegradable.

2.3.1 Denim washing

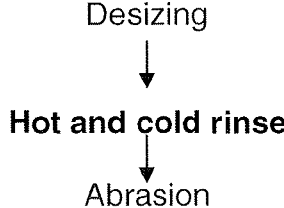
Denim industry is driven by fashion trends. Classic blue denim consists of a warp dyed with indigo and a white weft. Garment washing plays an important role to provide fashion and functionality elements. Various washing techniques of denim are stone washing, acid washing, chemical washing and enzyme washing. Out of which stone washing and enzyme washing are predominant (Suman and Khambra, 2005). In conventional bleaching with sodium hypochlorite, the white yarn becomes whiter and the blue yarn

becomes lighter. Figure 2 shows the steps involved in conventional denim wet processing (Ashok and Maheswari, 2004).

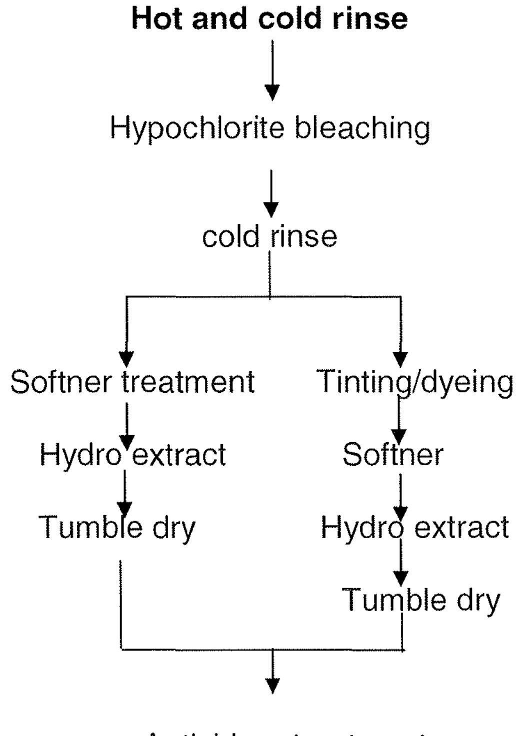
The aged look of denim is obtained by the non-homogenous removal of the indigo dye trapped inside the fibres by the co-operative action of enzymatic hydrolysis and mechanical stress such as beating and fiction. During enzymatic treatment of denim with cellulases the removal of denim was found to backstain the cotton fabric, where as no such behaviour was observed with the traditional stone wash with pumice stones and without enzymes.

FIGURE 2

CONVENTIONAL DENIM WET PROCESS



(Stone wash/acid enzyme wash/neutral enzyme wash/stone+ enzyme/mechanical dry abrasion)



The redeposition of inigo dye on the cotton fibre diminishes the desired contrast between white and blue yarns and can be easily noted on the reverse side of the fibers. Due to commercial interest the phenomenon was studied extensively in the last years and it was found that beside dye cellulose interactions (Andreaus *et al.*, 2000) mainly dye-cellulase interactions (Campos *et al.*, 2000; Gusakov *et al.*, 2001) are the major cause for Indigo backstaining.

When using the new laccase-based technology, the bleaching action is specific to indigo. The natural off-white colour of the white yarns are retained, giving a darker look that is impossible to achieve with hypochlorite.

2.3.2 Indigo decolourisation

Laccase is an enzyme that decolourise Indigo. It is an important step in the treatment and finishing of denim fabrics. The bleaching system is combination of an enzyme and a mediator. Enzyme alone is not capable of breaking the indigo. However, in the presence of an aqueous medium, the enzyme gets oxidized, which attacks the mediator to convert it into free radicals. These free radicals attack indigo and convert it into oxidized products (Anon, 2000).

Figures 3 and 4 show the mechanism of Indigo decolourisation.

FIGURE 3

MECHANISM OF INDIGO DECOLORISATION

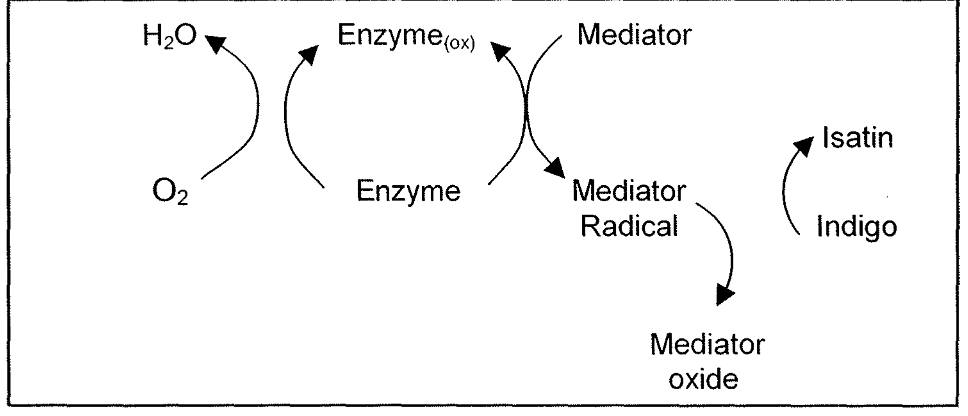


FIGURE 4

OXIDATION OF INDIGO

Indigo

Isatin

Isatic acid

Anthranilic acid

This enzyme system has made it possible to get rid off the chlorine based bleaching agents. Also, the process is very much controlled than with hypochlorite.

2.4 LACCASE IN OTHER AREAS

2.4.1 Laccase in effluent treatment

Waste water from textile industry, especially process houses, is characterized by high COD and BOD, suspended solids and intense color due to the extensive use of dyes. This type of water must be treated before discharging it into the environment. The water must be decolourised an harmful chemicals must be converted into harmless chemicals.

Biological treatments have been used to reduce the COD of textile effluents. Physical and chemical treatments are effective for color removal but require more energy and chemicals than biological processes. They also concentrate the pollution into solid or liquid side streams which require additional treatments or disposal. Biological processes completely mineralize pollutants and are cheaper (Shaw, 2002).

Laccases have highest potential to transform the textile dyes into colorless products. The rate of the laccase catalyzed decolourization of the dyes increases with the increase in temperature upto certain degree above which dye decolourisation decreases or does not take place at all. The optimum pH for laccase catalyzed decolourisation depends on the type of the dye used. Laccase of *Trametes modesta* may be used for decolourization of textile dye stuffs, effluent treatments and bioremediation or as a bleaching agent (Abadulla , 2000).

The use of lignin degrading white-rot fungi has attracted increasing scientific attention as these organisms are able to degrade a wide range of recalcitrant organic compounds such as polycyclic aromatic hydrocarbons, chlorophenol and various azo, heterocyclic and polymeric dyes. The major enzymes associated with the lignin degradation are laccase, lignin peroxidase and manganese peroxidase. The laccases are the important enzymes which catalyzes the oxidation of phenolic and non-phenolic compounds (Nyanhongo , 2002).

2.4.2 Laccase in pulp bleaching

Enzymes have high potential for improving traditional pulp and paper manufacturing processes due to their high specificity and environmental friendliness.Fungal laccases in the presence of mediators are the most promising bleaching enzymes due to their ability to degrade lignin. The laccase mediator-system (LMS) allows the development of totally cholrine free (TCF) bleaching sequences for various types of pulp (Camarero *et al.*, 2002).

O.Garcia *et al.* (2003) reported the joint use of *Pycnoporus cinnabarinus* laccase and the mediator 1-Hydroxy benzotrizole (HBT). They were found to be a highly efficient choice for delignifying flax pulp. Considering a two stage sequence, the results were better than those obtained in multi-stage TCF sequences using oxygen, hydrogen peroxide, ozone or some other chlorine-free chemical agents.

Treatment of wheat straw kraft pulp using laccases expressed in *P.cinnabarinus* or *A.niger* with 1-hydroxy benzotriazole as redox mediator achieved a delignification close to 75% (Sigoillet *et al.*, 2004).

2.5 ENZYME PRODUCTION BY MICROBIAL FERMENTATION

2.5.1 Substrates for enzyme production

Enzymes are the most important products obtained for human needs through microbial sources. Solid state fermentation (SSF) holds tremendous potential for the production of enzymes. It can be of special interest in those processes where the crude fermented product may be used directly as the enzyme source (Tengerdy, 1998).

Solid state fermentation (SSF) is a process where an insoluble substrate is fermented with sufficient moisture but without free water. SSF offers several advantage over liquid (submerged) fermentation mainly in the areas of biomass energy conservation and solid waster management (Mukherjee *et al.*,1998).

A large number of microorganisms, including bacteria, yeast and fungi are employed to produce different groups of enzymes (Pandey and Ashok, 1992).

Agro-industrial residues are generally considered as the best substrates for the SSF processes. A number of such substrates have been employed for the cultivation of microorganisms to produce host of enzymes. Some of the substrates that have been used included wheat bran, sugarcane bagasse, rice bran, maize bran, gram bran, wheat straw, rice straw, rice husk, soyhull, sago hampus, saw dust, corn cobs, grape vine trimmings dust, 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, coconut oil cake, mustard oil cake, wheat floor, corn floor, steamed rice, steam pre-treated willow and starch (Benjamin and Pandey, 1997;Selvakumar *et al.*, 1998;). Wheat bran is most commonly been used in various processes.

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 serve as anchorage for the cells. Among the several factors that are important for microbial growth and enzyme production using a particular substrate, particle size, moisture level and water activity are the most critical. Generally, smaller substrate particles provide larger surface area for microbial attack (Zadrazil and Puniya, 1995).

Wheat straw was used for cultivating several fungal strains to produce laccase, lignin peroxidase and manganese peroxidase (Vares *et al.*,1995; Gupte *et al.*,1998).

Homolka *et al.*(1997) studied laccase production from three strains of *Pleurotus sp.*(Obtained after protoplast regeneration of the control strain) and found a comparable activity in two strains and the other exhibited a lower activity.

Verma and Madamwar (2002) studied lignocellulosic wastes and found the substrates such as neem hull, wheat bran and sugarcane bagasse were excellent substrates for the production of ligninolytic enzymes under solid state fermentation by white-rot fungi. A ligninolytic enzyme system with high activity showing enhanced decomposition was obtained by cocultivation of *Pleurotus ostreatus* and *Phanerochaete chrysosporium* on combinations of lignocellulosic waste. Among the various substrate combinations examined, neem hull and wheat bran wastes gave the highest lignolytic activity. A maximum production of laccases of 772 U/g was obtained on the 20th day at 28 ±1°C under solid-state fermentation.

Kahraman and Yesilada(2001) reported the induction of laccase by white-rot fungi using olive-oil mill wastewaters and spent cotton stalks as medium components. Moldes *et al.* (2003) used grape seeds as substratum for solid state culture to produce laccase by *Tramates hirsute.*

An acidic laccase producing *Ganoderma sp.* KK-02 produced 16.0 U/ml and 365 U/g of laccase activity, when grown under submerged (SmF) and solid state (SSF) fermentation conditions, respectively. Modification of the substrate (wheat bran) molecular architecture by *in vivo* enzymatic digestion (IVED) and *in vitro* xylanase digestion (IVXD) increased subsequent laccase production from *Ganoderma sp.* KK02 by 1.31-fold (21.0 U/ml) (SmF); 2.21-fold (810 U/g) (SSF) and 1.10-fold (18.0 U/g) (SmF); 1.78-fold (650.0 U/g) respectively, when compared with untreated wheat bran. Further enhancement in laccase yield under SmF and SSF was obtained when IVED treated wheat bran was used in conjugation with aminoacids, vitamins, surfactants and PEG (Sharma *et al.,* 2005).

2.5.2 Microbes for production of enzyme

Enzymes can be isolated from animals, plants and microbial origins. The increasing range of commercial applications of enzymes has always encouraged exploitation of newer sources. Microbial cells can provide excellent supply of enzymes (Rohson and Sandeep, 1999).

Various fungi like *Trametes modesta, Trametes versicolor, Trametes hirsuta and Sclerotium rolfsii were* used for laccase production.Several investigations indicated that laccase is widely distributed in many genera of white rot fungi (de Jong,1994 ; Palaez,1995).

The highest redox potential laccases were produced by white rot fungi, such as *Trametes versicolor* and *Pycnoporus cinnbarinus* (Kanbi *et al.*,2002).

Sigoillet *et al.* (2004) reported the production of laccase from *Pycnoporus cinnabarinus* (a well known lignolytic fungus) and their corresponding recombinant laccases in *Aspergillus oryzae* and *A.niger* hosts without using the extensive laccase inducers required by the native source. The natural laccase exhibited a higher redox potential (around 810 mV) compared with *Aspergillus niger* (760 mV) and *Aspergillus oryzae* (735 mV).

2.6 OPTIMIZATION OF CULTURE CONDITIONS

Fungal laccases have been detected and purified from many species, some of which produce multiple isoenzymes (Solomen *et al.,* 1996). It has been reported that laccase production by various fungi increased after supplementing the fungal cultures with different phenolic substrates (Platt *et al.*,1994).

Srinivasan *et al.* (1995) showed that *Phanerochaete chrysosporium* 13Km-F1767 produces extracellular laccase in a defined culture medium containing cellulose (10g/L) and 24mM ammonium tartarate.

Laccase from *Coriolus hirstus* was characterised and found that the enzyme is more pH stable and thermostable over a wide range of pH values (3.5-6.5) and temperature (20°C-50°C)(O.Koroljova-Skorobogat'ko .,1998).

Boghos *et al.* (2000) observed the optimum pH 4.5 and tempterature 75°C for production of laccase from *Marasmius quercophilus* .

2.7 COMMERCIALLY AVAILABLE ENZYMES

Cellusoft L of Novo Nordisk A/S is the enzyme used for biopolishing (Ravichandran, 2000). Lumis offers range of enzymes like Remsize, Lumizyme and Sizeclear for desizing products in various stage. These products can be used on woven cotton fabrics, denims and on other silk cottons.

Lumis offers cellulase in varied strength and formulations to impart faded abraded look on denims. Products are available under brand names like Denlumizyme Nc, Lumizyme Ac,Lumibrade and Lumiwash. It also offers Denase classic to decolourise Indigo enzymatically.

Lumis offers oxkil and lumizyme CA for hydrogen peroxide decomposition. Lumis has introduced Lumizyme pcx stable protease in solid form fiber. The same product can be used for enzymatic degumming of silk fiber.

Amylolytic enzymes in their natural form or modified state can destroy any type of starch turning it into water-soluble product without affecting the cellulose. Eg : Aquazim, Aquazim ultra and Termamyl (Noro Nordisk) (Subhendu Das *et al.*, 2000).

2.8 SCOPE OF ENZYMES IN TEXTILE INDUSTRY

Enzymes play an out standing role in textile industry. The major area of application of enzymes in textile industry are

- Replacement of harsh and energy demanding chemicals.
- Environment friendly routes to textile auxillaries such as dyestuffs.
- Novel uses for enzymes in textile finishing.
- Development of low energy enzyme based detergents.
- Waste management using environment friendly processes.

Experimental Procedure

3.0 EXPERIMENTAL PROCEDURE

Development in biotechnology, particularly in areas as such protein engineering and directed evolution have provided important tools for the efficient development of new enzymes (Kirk *et al.*, 2002). Microbial enzymes find potential application in a number of industrial processes. Hence the present study was aimed at isolating fungi and optimizing the culture conditions for maximum enzyme production.

The experimental procedure for the study "**Optimization and production of fungal laccase and its effect on denim fabric**" was carried out under the following headings

3.1 SELECTION OF SOURCE

3.2 ISOLATION OF FUNGI

3.3 IDENTIFICATION OF FUNGI

3.3.1 Identification by morphological observation

3.3.2 Identification by staining

3.4 CULTURING OF FUNGAL SPECIES FOR ENZYME PRODUCTION

3.4.1 Substrates used for enzyme production

3.5 EXTRACTION OF ENZYME FROM FUNGAL CULTURES

3.6 DETERMINATION OF PROTEIN CONTENT

3.7 ENZYME ASSAY

3.7.1 Laccase

3.8 OPTIMIZATION OF GROWTH CONDITIONS OF FUNGI

3.8.1 Effect of pH on enzyme production

3.8.2 Effect of temperature on enzyme production

3.9 PURIFICATION OF ENZYME

3.9.1 Ammonium sulphate precipitation

3.9.2 Dialysis

3.9.3 Purification by gel filtration chromatography

3.10 APPLICATION OF LACCASE ON DENIM FABRIC

3.10.1 Fabric selection and treatment

3.10.1.1 Conventional treatment

3.10.1.2 Enzymatic treatment

3.10.2 Evaluation of the treated fabric

3.10.2.1 Weight of fabric

3.10.2.2 Tensile strength

3.10.2.3 Thickness

3.10.2.4 Stiffness

3.10.2.5 Abrasive Resistance

3.1 SELECTION OF SOURCE

Various sources are being exploited by the scientist from which the microbes produce scientifically and industrially important enzymes. Soil collected from agricultural land was used as the microbial source for the present study. The soil were serially diluted from 10^{-4} dilution to 10^{-8} and inoculated into suitable media for the isolation of fungi.

3.2 ISOLATION OF FUNGI

To isolate fungi, Rose Bengal Chloramphenicol Agar medium (Appendix I) was used. The serially diluted soil sample were grown on Rose Bengal Chloramphenicol agar medium. These plates were incubated at room temperature for 7 days and observed for colonies.

3.3 IDENTIFICATION OF FUNGI

3.3.1 Identification by morphological observation

Fungal colonies were identified based on its morphological characteristics.

3.3.2 Identification by staining

The fungal colonies were identified by staining with Lactophenol cotton blue. The procedure is elaborated in Appendix II.

3.4 CULTURING OF FUNGAL SPECIES FOR ENZYME PRODUCTION

Identified fungal colonies were stored at 4°C until they were used. Fungal colonies were inoculated using various substrates in 250ml conical flask with 70% moisture content at room temperature for 7 days of incubation period.

3.4.1 Substrates used for enzyme production

The fungal species and substrates used for present study are given below.

Selected fungal species were,

- *Aspergillus niger*
- *Penicillium sp.*
- *Fusarium sp.*
- *Rhizopus sp.*

Substrates used were,

- Wheat bran
- Rice bran
- Sorghum flour
- Sugarcane bagasse

3.5 EXTRACTION OF ENZYME FROM FUNGAL CULTURES

The substrates were inoculated with selected fungal species and incubated for 7 days and used for extraction of enzymes. The extraction was done with phosphate buffer (pH 7.0) by mechanical grinding. The contents

were centrifuged and the supernatant was used for enzyme assay. The procedure is explained in Appendix III.

3.6 ENZYME ASSAY

3.6.1 Laccase assay

The laccase activity was determined based on the amount of tetraguaiacol formed (Sarkanen *et al.*, 1999). One unit of laccase activity is defined as the amount of tetraguaiacol formed per minute per litre of enzyme extract. The procedure is explained in Appendix V.

3.7 DETERMINATION OF PROTEIN CONTENT

The protein concentration of the crude fungal extracts were determined by Lowry's method. The method is described in Appendix IV.

3.8 OPTIMIZATION OF GROWTH CONDITIONS OF FUNGI

The effect of pH and temperature on enzyme production were studied with selected fungal species and substrates.

3.8.1 Effect of pH on enzyme production

In order to optimize the pH for enzyme production, the pH of the growth medium was adjusted to varying pH such as 4.0,5.0,6.0,7.0 and 8.0 and allowed to grow at room temperature with selected fungal cultures.

3.8.2 Effect of temperature on enzyme production

To optimize the temperature for enzyme production, the culture flasks were incubated at different temperature such as 17°C, 27°C, 37°C, 47°C and 57°C and the initial medium pH was kept neutral. The procedure is described in Appendix VI.

3.9 PURIFICATION OF ENZYME

It has been reported that sugarcane bagasse could enhance the production of laccase by 15 - 32 fold in *Pleurotus ostreatus* (Sen *et al.*, 2004). Therefore sugarcane bagasse was used as an additional substrate for the production of enzyme to compare the activity of the enzyme produced from *Aspergillus niger* and *Penicillium sp*. cultured in wheat bran and rice bran.

3.9.1 Ammonium sulphate precipitation

The crude extracts from *Aspergillus niger* and *Penicillium sp*. cultured in wheat bran, rice bran and sugarcane bagasse were precipitated with 60% ammonium sulphate. This was done by the method of Jayaraman (1981) as given in Appendix VII.

3.9.2 Dialysis

The ammonium sulphate precipitated samples were subjected to dialysis. Dialysis selectively removes the small molecules from a sample containing mixture of both small and large molecules. The procedure was elaborated in Appendix VIII.

3.9.3 Purification by gel filtration chromotography

The dialysed sample from *Aspergillus niger* cultured in sugarcane bagasse were loaded onto sephadex G-50 column and equilibrated with 10mM sodium phosphate buffer. Elution was done with same buffer at a flow rate of 2ml per minute and 20 fractions were collected. The protein estimation and enzyme assay were carried out with each of the fractions collected. The procedure is explained in Appendix IX.

3.10 EFFECT OF LACCASE ON DENIM FABRIC

3.10.1 Fabric selection and treatment

The denim fabrics used for the present study were light weight denim and heavy weight denim. The fabrics were treated by conventional (Hypochlorite bleaching) and enzymatic bleaching methods.

3.10.1.1 Conventional treatment

Conventional bleaching was performed with 3% of aqueous sodium hypochlorite. The procedure is given in Appendix X.

3.10.1.2 Enzymatic treatment

Two enzyme samples, one ammonium sulphate precipitated sample and other dialyzed sample containing laccase enzyme were prepared from *Aspergillus niger* cultured in sugarcane baggasse. The procedure is described in Appendix X.

3.10.2 Evaluation of the treated fabric

3.10.2.1 Weight of fabric

The fabric weight may be determined by measuring the weight as yards per pound for given widths. Fabric weight is also expressed as mass per unit area and weight per unit length (Booth, 1996). The procedure is elaborated in Appendix XI.

3.10.2.2 Tensile strength

According to Angappan and Gopalakrishnan (1993), the breaking strength is the measure of resistance of the fabric to a tensile load or stress in warp and weft direction. The procedure is depicted in Appendix XII.

3.10.2.3 Thickness of fabric

Thickness is defined as the distance between two parallel surfaces, while entering a specified presence on a material (Shrinkle, 1972). The procedure is described in Appendix XIII.

3.10.2.4 Stiffness of fabric

Fabric stiffness is defined as the resistance of a fabric to bending slates ASTM (1983). It also adds that bending length is a measure of the interaction between fabric weight and fabric stiffness as shown by the way in

Results and Discussion

4.0 RESULTS AND DISCUSSION

Laccases are oxidoreductases. Laccases have a special application in denim industry in which they attack and degrade the indigo pigments thereby uniformly bleaching the denim goods to impart a faded look to the garment or fabric. In the present study, laccases were produced from the fungus isolated from the agricultural soil. Growth conditions, namely initial medium pH and temperature for maximum production of enzyme were optimized. The produced enzymes were partially purified and their effect on denim fabric was analysed.

The findings of the study entitled "**Optimization and production of fungal laccase and its effect on denim fabric**" are discussed under the following headings.

4.1 ISOLATION AND IDENTIFICATION OF FUNGUS

4.2 CULTURING OF FUNGI IN SELECTED SUBSTRATES

4.2.1 Laccase activity in fungal extracts

4.3 OPTIMIZATION OF CULTURE CONDITIONS FOR LACCASE PRODUCTION

4.3.1 Effect of initial medium pH on laccase production from selected fungal cultures

4.3.2 Effect of temperature on laccase production from selected fungal cultures

4.4 PURIFICATION OF ENZYME

4.4.1 Purification by ammonium sulphate precipitation

4.4.2 Purification by dialysis

4.4.3 Purification by gel filtration chromatography

4.5 EFFECT OF ENZYME ON DENIM FABRIC

4.5.1 Weight loss

4.5.2 Tensile strength

4.5.3 Thickness

4.5.4 Stiffness

4.5.5 Abrasive resistant

4.1 ISOLATION AND IDENTIFICATION OF FUNGUS

Fungal colonies were isolated from the soil (Plate I) on Rose Bengal Chloramphenicol Agar medium by standard dilution technique. Based on the colony morphology and Lactophenol cotton blue staining, a total of four fungal isolates namely, *Aspergillus niger, Penicillium sp., Rhizopus sp.* and *Fusarium sp.* were isolated.

Table 1 illustrates the colony morphology and the microscopic appearance of selected fungal isolates. Plates II and III show the colony morphology and microscopic view of isolated fungi.

TABLE 1

COLONY MORPHOLOGY AND MICROSCOPIC APPEARANCE OF FUNGAL ISOLATES

Colony morphology	Microscopic appearance	Identified Fungi
1. Mature cultures usually black or brownish colour	Single-celled spores (conidia) in chains developing at the end of sterigma arising from the terminal bulb of conidiophore; the vesicle: long conidiophore arise from septate mycelium	*Aspergillus niger*
2. Rapidly growing colonies in shades of green.	Chains of single-celled conidia are produced from a specialized condiogenous cell called phiadide.	*Penicillium sp.*
3. Rapidly growing white coloured fungus swarms over entire plate; aerial mycelium cottony and fuzzy.	Spores are oval, colourless or brown; non septate mycelium gives to straight sporingiophores that terminate with black sporangium containing a columeela; root like hyphae (rhizoids) penetrate the medium	*Rhizopus sp.*
4. Wooly, white, fuzzy colonies changing colour to pink, purple or yellow	Multicelled spores (Conidia) are oval or cresent-shaped and attached to conidiospores arising from a septate mycellium	*Fusarium sp.*

4.2 CULTURING OF FUNGI IN SELECTED SUBSTRATES

The identified fungi were subcultured in various substrates namely wheat bran, rice bran and sorghum flour. The protein content and laccase activity of fungal extracts were determined.

4.2.1 Laccase activity in fungal extracts

Table 2 and Fig. 5a and 5b illustrate the protein content and specific activity of laccase obtained from crude extracts of fungal cultures (Plate IV).

TABLE 2

PROTEIN CONTENT (mg/ml) AND LACCASE ACTIVITY ($U\ mg^{-1}$) OF FUNGAL EXTRACTS

Fungi	Wheat bran		Rice bran		Sorghum flour	
	Protein	Laccase activity	Protein	Laccase activity	Protein	Laccase activity
Aspergillus niger	11.294	**0.192**	5.172	0.063	4.393	0.029
Penicillium sp.	8.714	0.025	4.845	0.041	0.925	**0.121**
Fusarium sp.	5.923	0.028	1.760	**0.067**	2.604	0.033
Rhizopus sp.	1.872	0.026	1.568	**0.035**	2.173	0.028

The highest laccase activity (0.192 $U\ mg^{-1}$) was noticed from *Aspergillus niger* cultured in wheat bran followed by *Penicillium sp.* (0.121 $U\ mg^{-1}$) cultured in sorghum flour. The highest laccase activity produced from *Fusarium sp.* and *Rhizopus sp.* in rice bran was 0.067 and 0.035 $U\ mg^{-1}$ respectively. The growth of *Aspergillus niger,* the protein content and the activity of laccase were higher in wheat bran when compared with other substrates.

The trend of laccase activity of fungal extracts using different substrates are as follows:

Aspergillus niger	: Wheat bran > Rice bran > Sorghum flour
Penicillium sp.	: Sorghum flour > Rice bran > wheat bran
Fusarium sp.	: Rice bran > Sorghum flour > wheat bran
Rhizopus sp.	: Rice bran > Sorghum flour > wheat bran

4.3 OPTIMIZATION OF CULTURE CONDITIONS FOR LACCASE PRODUCTION

The effect of initial medium pH and temperature for optimum laccase production for all the isolated fungal species on selected substrates were studied.

4.3.1 Effect of initial medium pH on laccase production from the selected fungal cultures

The substrates namely wheat bran, rice bran and sorghum flour were inoculated with selected fungal species at different pH 4.0, 5.0, 6.0, 7.0 and 8.0 and incubated at room temperature. The activity of the enzyme in the extracts are presented in Table 3 and Fig. 6a, 6b, 6c and 6d.

Aspergillus niger exhibited the optimum initial medium pH at 6.0 in wheat bran, rice bran and sorghum flour and the laccase activity was found to be 0.144, 0.099 and 0.055 (U mg^{-1}) respectively.

The highest laccase activity was obtained from *Penicillium sp.* at pH 7.0 (0.192 U mg^{-1}) cultured in sorghum flour. In wheat bran the optimum initial medium pH was found to be 6.0 (0.069 U mg^{-1}) and in rice bran it was at pH 4.0 (0.103 U mg^{-1}).

The optimum initial medium pH was observed at 6.0 from *Fusarium sp.* cultured in sorghum flour (0.074 U mg^{-1}). In rice bran, the optimum activity was obtained at pH 7.0 (0.067 U mg^{-1}), whereas, in wheat bran, the optimum activity was noticed at pH 8.0.

TABLE 3

EFFECT OF INITIAL MEDIUM pH ON LACCASE PRODUCTION FROM SELECTED FUNGAL CULTURES

Fungi	Initial medium pH	Laccase activity ($U\ mg^{-1}$)		
		Wheat bran	Rice bran	Sorghum flour
Aspergilus niger	4.0	0.120	0.081	0.031
	5.0	0.008	0.045	0.035
	6.0	**0.144**	**0.099**	**0.055**
	7.0	0.121	0.063	0.029
	8.0	0.095	0.055	0.047
Penicillium sp.	4.0	0.057	**0.103**	0.038
	5.0	0.042	0.053	0.075
	6.0	**0.069**	0.061	0.124
	7.0	0.025	0.041	**0.192**
	8.0	0.045	0.059	0.105
Fusarium sp.	4.0	0.021	0.044	0.049
	5.0	0.031	0.056	0.043
	6.0	0.044	0.045	**0.074**
	7.0	0.028	**0.067**	0.033
	8.0	**0.051**	0.053	0.052
Rhizopus sp.	4.0	0.018	0.030	0.004
	5.0	0.018	0.019	0.017
	6.0	0.018	**0.039**	0.015
	7.0	**0.026**	0.035	**0.028**
	8.0	0.015	0.023	0.022

Rhizopus sp. produced the maximum laccase activity at pH 6.0 (0.039 $U\ mg^{-1}$) in rice bran, whereas, wheat bran and sorghum flour produced the optimum laccase activity at pH 7.0.

Of the four species and the three substrates selected, the highest laccase activity was obtained at pH 7.0 from *Pencillium sp.* in sorghum flour when incubated at room temperature. The optimum initial medium pH for the selected fungal species in different substrates ranged from 4.0 to 8.0.

S.Timur *et al.* (2004) reported the development of thick film electrode based biosensors containing *Tramates versicolor* (TvL) laccases and *Aspergillus niger* (AnL) laccases and the activity was optimum at pH 4.5.

FIGURE 6

EFFECT OF INITIAL MEDIUM pH ON LACCASE PRODUCTION FROM SELECTED FUNGAL CULTURES

Fig.6a. Aspergillus niger

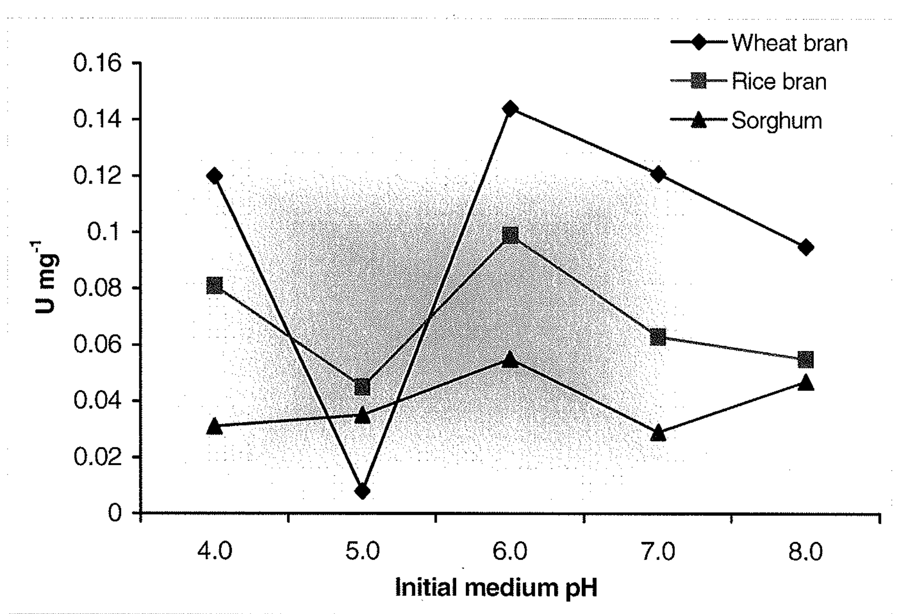
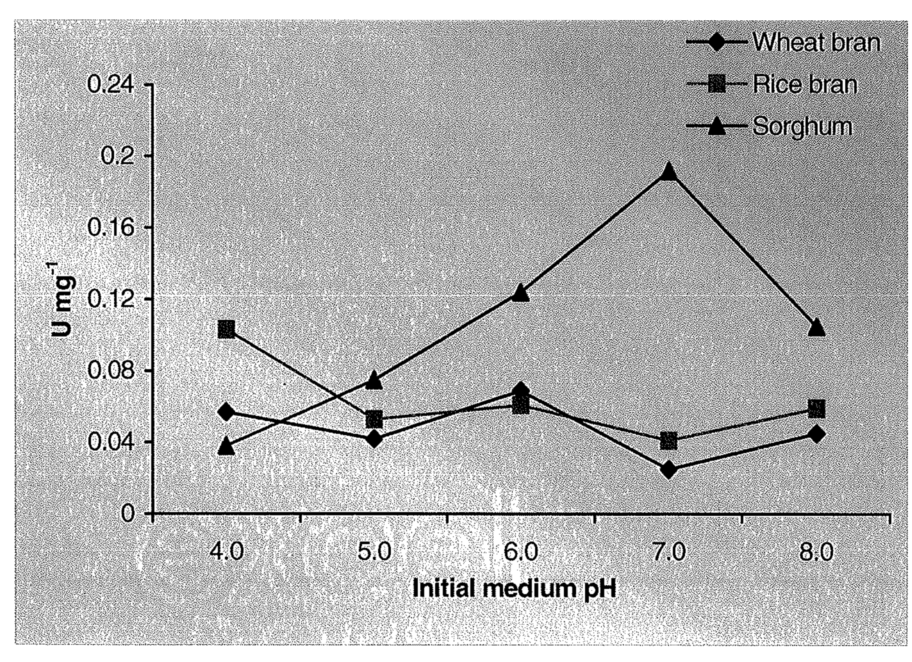


Fig. 6b. *Penicillium sp.*



EFFECT OF INITIAL MEDIUM pH ON LACCASE PRODUCTION FROM SELECTED FUNGAL CULTURES

Fig.6c. ***Fusarium sp.***

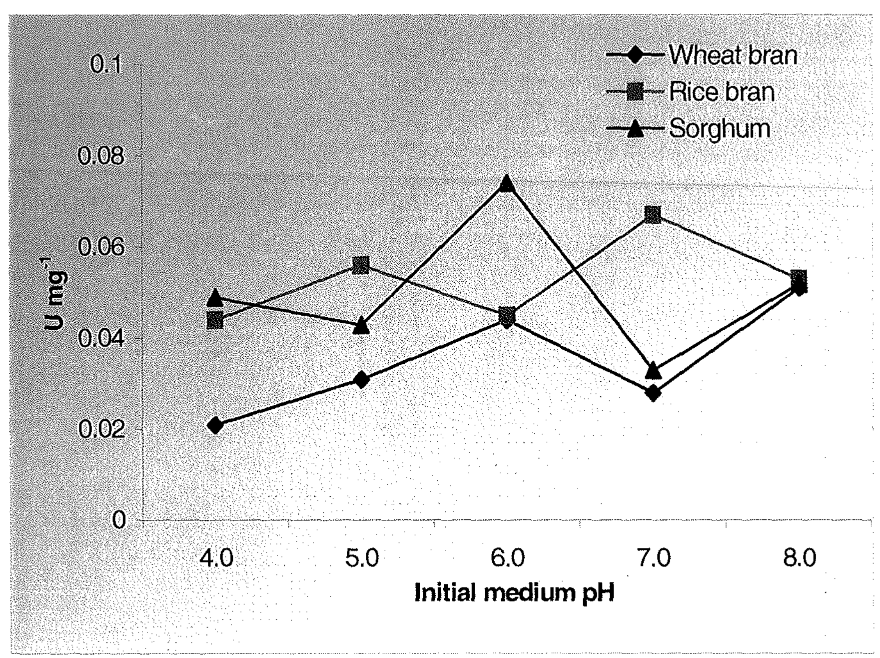
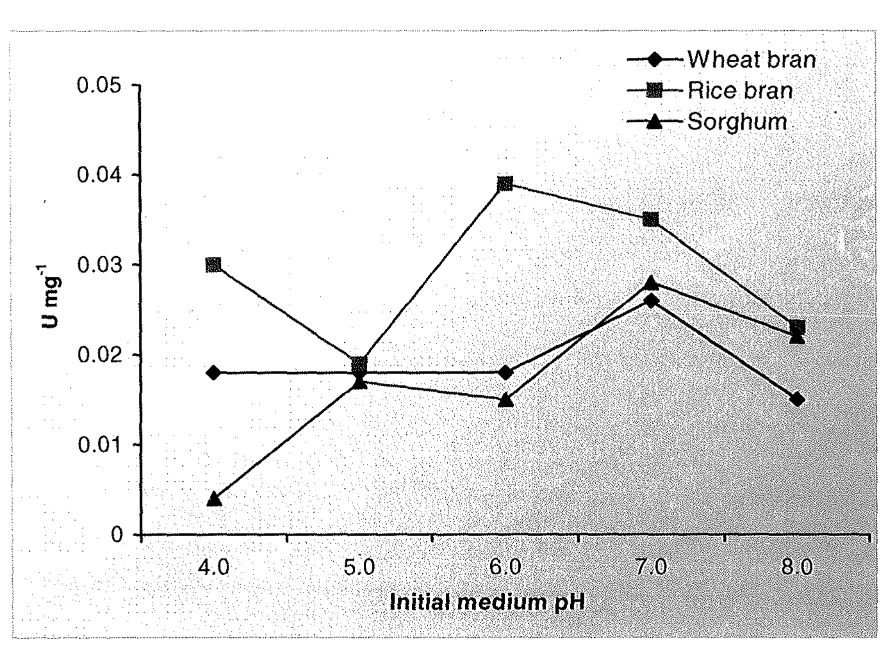


Fig.6d. ***Rhizopus sp.***



4.3.2 Effect of temperature on laccase production from the selected fungal cultures

The selected substrates were inoculated with *Aspergillus niger, Penicillium sp., Fusarium sp.* and *Rhizopus sp.* and incubated at different temperatures 17°C, 27°C, 37°C, 47°C and 57°C at neutral pH and the results are presented in Table 4 and Fig. 7a, 7b, 7c and 7d.

TABLE 4

EFFECT OF TEMPERATURE ON LACCASE PRODUCTION FROM SELECTED FUNGAL CULTURES

Fungi	Incubation temperature (°C)	Laccase activity ($U\ mg^{-1}$)		
		Wheat bran	Rice bran	Sorghum flour
Aspergillus niger	17	0.045	0.041	0.060
	27	**0.209**	0.031	0.097
	37	0.178	0.034	0.069
	47	0.042	**0.144**	**0.127**
	57	0.073	0.057	0.068
Penicillium sp.	17	0.038	0.059	0.042
	27	**0.278**	0.029	0.137
	37	0.034	0.038	0.040
	47	0.080	**0.083**	**0.250**
	57	0.053	0.073	0.074
Fusarium sp.	17	**0.042**	**0.039**	**0.057**
	27	0.028	0.032	0.031
	37	0.011	0.019	0.007
	47	0.018	0.029	0.027
	57	0.012	0.012	0.004
Rhizopus sp.	17	**0.042**	**0.045**	**0.053**
	27	0.016	0.025	0.007
	37	0.020	0.018	0.004
	47	0.034	0.028	0.041
	57	0.016	0.008	0.011

The optimum temperature for maximum production of laccase from *Aspergillus niger* (0.209 $U\ mg^{-1}$) and *Penicillium sp.* (0.278 $U\ mg^{-1}$) was found to be at 27°C in wheat bran, whereas, rice bran and sorghum flour gave the highest activity at 47°C.

FIGURE 7

EFFECT OF TEMPERATURE ON LACCASE PRODUCTION FROM SELECTED FUNGAL CULTURES

Fig.7a. Aspergillus niger

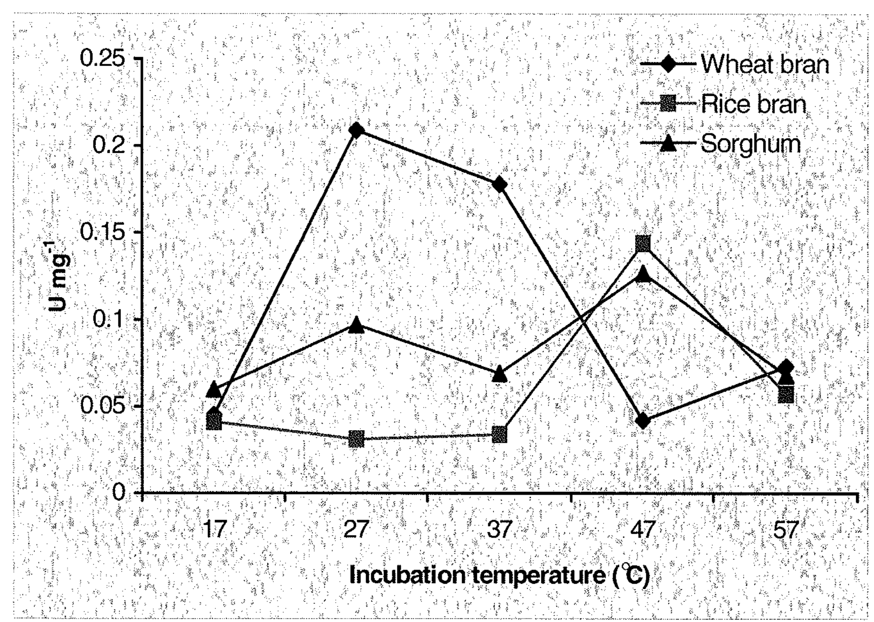
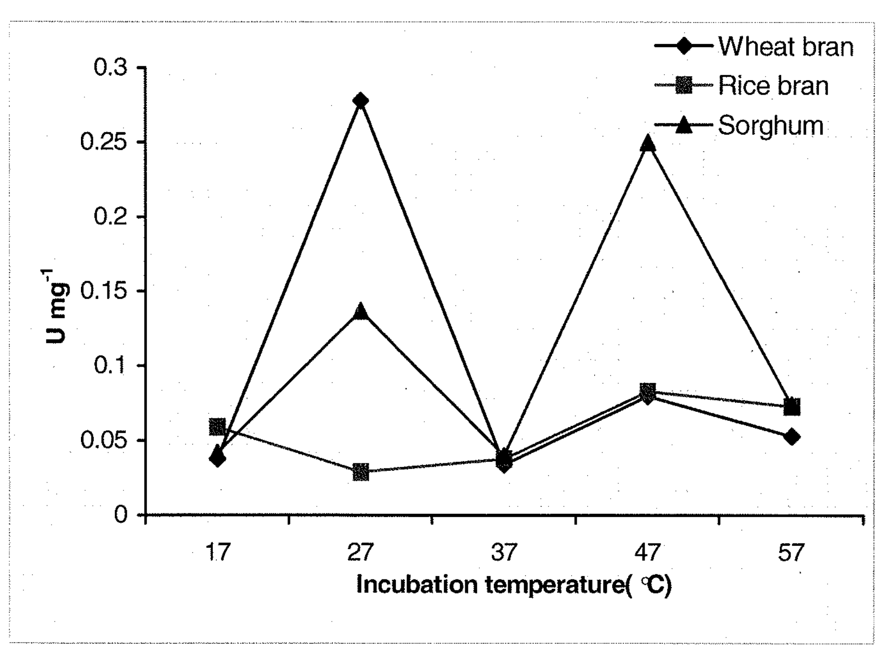


Fig.7b. Penicillium sp.



EFFECT OF TEMPERATURE ON LACCASE PRODUCTION FROM SELECTED FUNGAL CULTURES

Fig.7c. *Fusarium sp.*

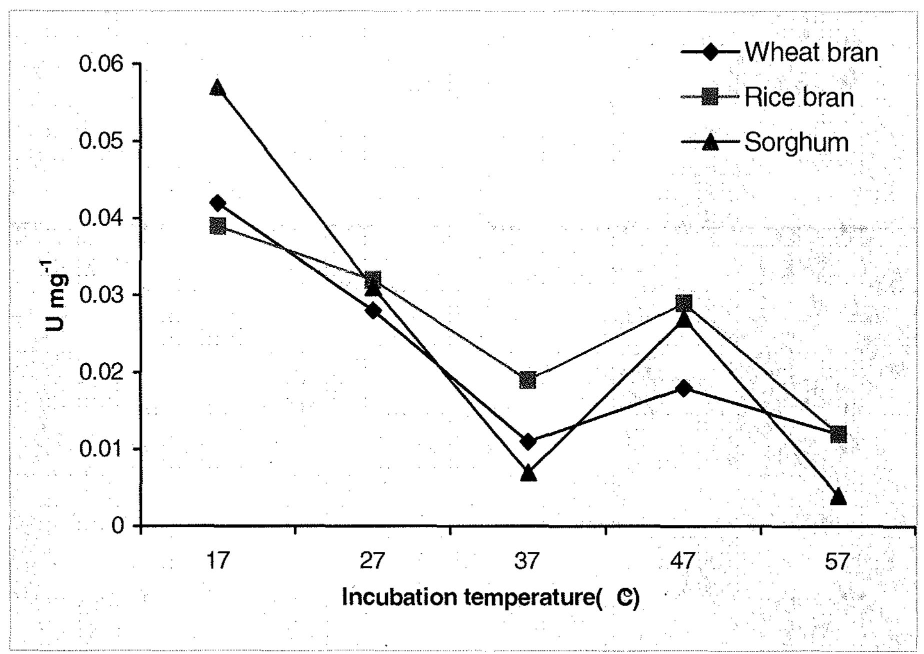
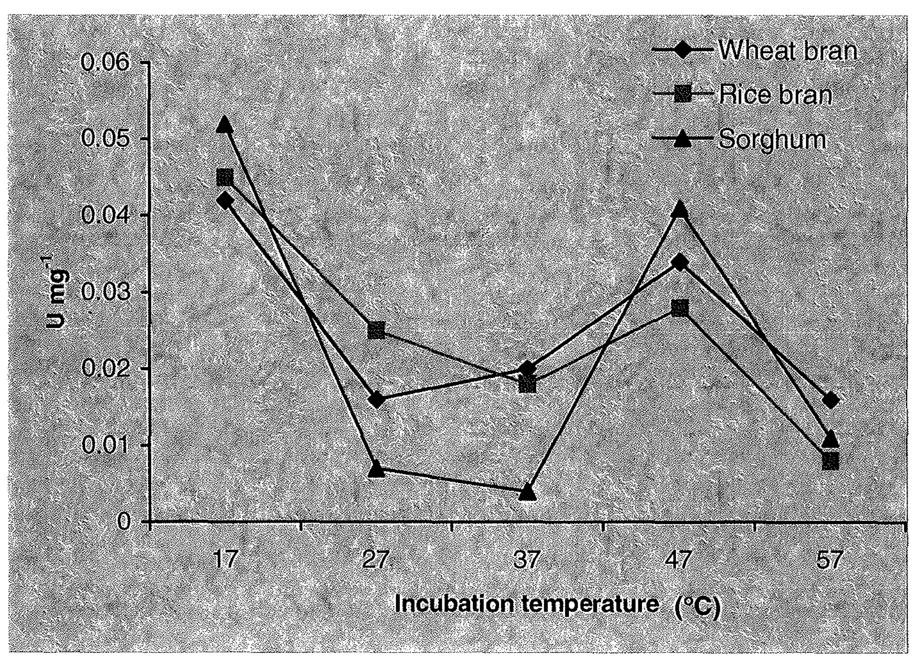


Fig.7d. Rhizopus sp.



The optimum temperature for laccase activity from *Fusarium sp.* and *Rhizopus sp.* was found to be 17°C in all the selected substrates, namely, wheat bran, rice bran and sorghum flour.

The selected fungal cultures exhibited the optimum temperature which ranged from 17°C to 47°C in the selected substrates. The highest laccase activity (0.278 U mg^{-1}) was obtained at temperature 27°C from *Penicillium sp.*in wheat bran.

Pointing *et al.*(2000) reported that the activity of laccase in crude culture filtrate of *Pycnoporus sanguineus* was stable at temperature 35°C and pH 3.0.

Marques *et al.* (2002) studied the condition for production of laccase from *Pleurotus pulmonarius* in solid state fermentation with initial moisture content of 75% and cultivation period of 5 days at 30°C. The optimum pH and temperature for laccase activity were reported to be 6.5 and 50°C, respectively.

4.4 PURIFICATION OF ENZYME

The enzymes extracted from *Aspergillus niger* and *Penicillium sp.*cultured in wheat bran, rice bran and sugarcane bagasse were subjected to purification by ammonium sulphate precipitation, dialysis and gel filtration chromatography.

4.4.1 Ammonium sulphate precipitation

The crude extracts from fungal cultures were precipitated with 60% ammonium sulphate and the resulting precipitate was dissolved in phosphate buffer (pH 7.0) and subjected to dialysis.

4.4.2 Dialysis

The precipitated enzyme samples were dialysed against phosphate buffer at pH 7.0. The protein content and enzyme activity of the dialysed samples were determined.

The activity of laccase from *Aspergillus niger* and *Penicillium sp.* in the selected substrates after ammonium sulphate precipitation and dialysis are depicted in Tables 5a and 5b and Figures 8a and 8b.

TABLE 5a

LACCASE ACTIVITY FROM *Aspergillus niger* AFTER PARTIAL PURIFICATION

Substrate	Purification step	Protein content mg/ml	Total activity IU/ml	Specific activity Umg^{-1}	Purification fold
Wheat bran	Crude extract	11.294	0.703	0.062	-
	60% ammonium sulphate	5.726	0.374	0.065	1.1
	Dialysis	3.195	0.264	0.083	1.3
Rice bran	Crude extract	5.172	0.110	0.021	-
	60% ammonium sulphate	4.793	0.193	0.040	1.9
	Dialysis	0.159	0.055	0.346	16.5
Sugarcane bagasse	Crude extract	4.203	0.046	0.011	-
	60% ammonium sulplhate	3.879	0.141	0.036	3.2
	Dialysis	0.087	0.064	0.436	39.6

TABLE 5b

LACCASE ACTIVITY FROM *Penicillium sp.* AFTER PARTIAL PURIFICATION

Substrate	Purification step	Protein content mg/ml	Total activity IU/ml	Specific activity Umg^{-1}	Purification fold
Wheat bran	Crude extract	8.714	0.160	0.018	-
	60% ammonium sulphate	4.302	0.109	0.025	1.3
	Dialysis	0.412	0.038	0.093	5.1
Rice bran	Crude extract	4.845	0.124	0.026	-
	60% ammonium sulphate	1.997	0.081	0.041	1.6
	Dialysis	0.356	0.035	0.098	3.8
Sugarcane bagasse	Crude extract	3.538	0.125	0.035	-
	60% ammonium sulplhate	1.865	0.083	0.044	1.2
	Dialysis	0.097	0.037	0.383	10.9

It is evident from Tables 5a and 5b and Figures 8a and 8b that purification has increased the specific activity of laccase after ammonium sulphate precipitation and dialysis in all the samples. The specific activity of enzyme produced from *Aspergillus niger* cultured in sugarcane bagasse was the highest (0.436 U mg^{-1}) after dialysis and the increase was found to be 39.6 fold when compared to the activity of the crude extract.

The dialyzed enzyme sample from *Aspergillus niger* cultured in sugarcane bagasse was taken for further purification by gel filtration chromatography.

FIGURE 8a

LACCASE ACTIVITY FROM *Aspergillus niger* AFTER PARTIAL PURIFICATION

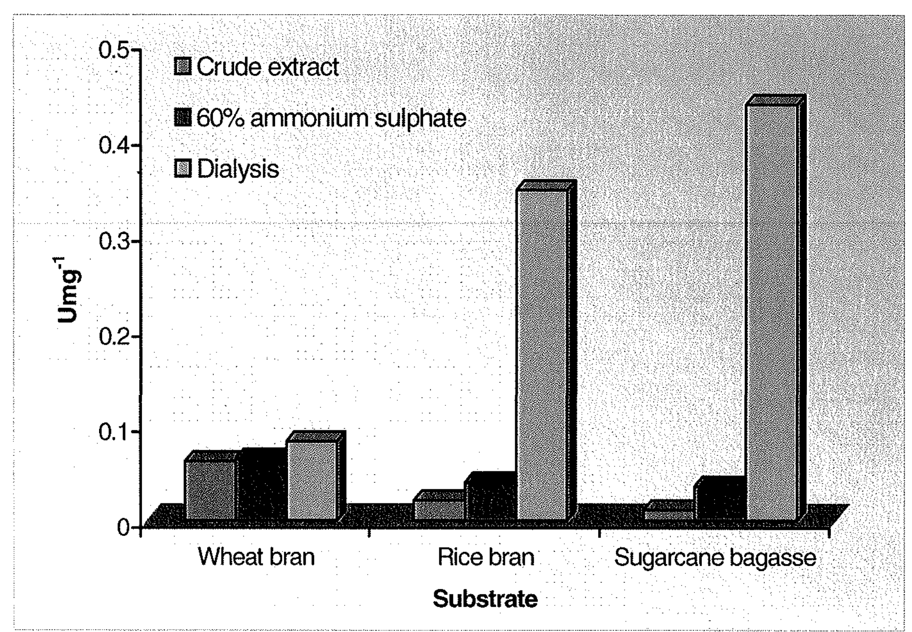
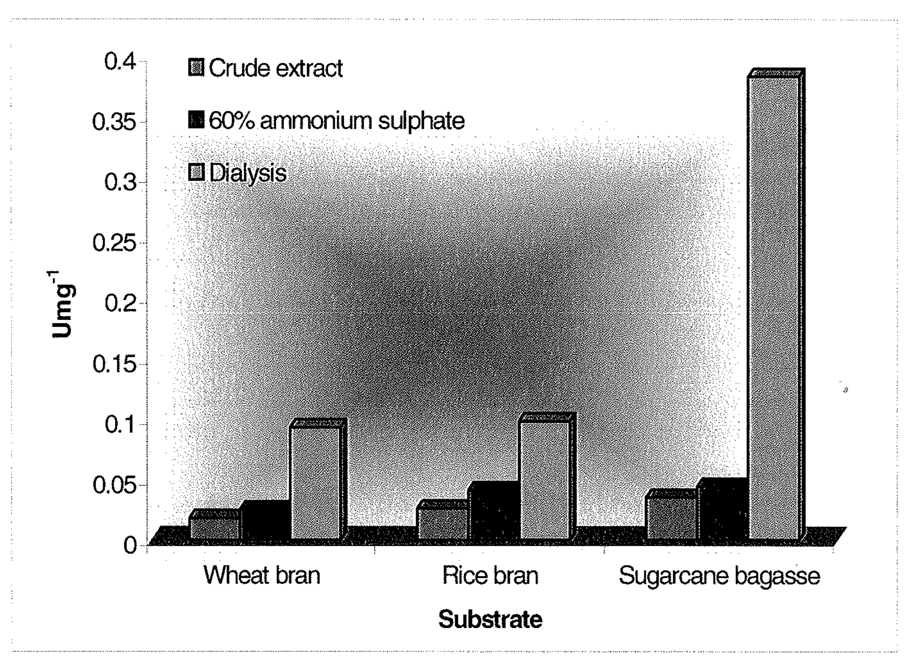


FIGURE 8b

LACCASE ACTIVITY FROM *Penicillium sp.* AFTER PARTIAL PURIFICATION



4.4.3 Purification by gel filtration chromatography

The dialysed sample from *Aspergillus niger* cultured in sugarcane bagasse was applied on sephadex G-50 column. Elution was done with a linear gradiant buffer at a flow rate of 2.0ml/min. 20 fractions were collected. The collected fractions were pooled into 10 tubes by combining 2 subsequent fractions (1+2, 3+4... and so on) to get a volume of 4.0 ml for spectrophotometric measurement. The protein content and laccase activity were measured in each of the 10 fractions and the results are depicted in Table 6 and Fig.9.

TABLE 6

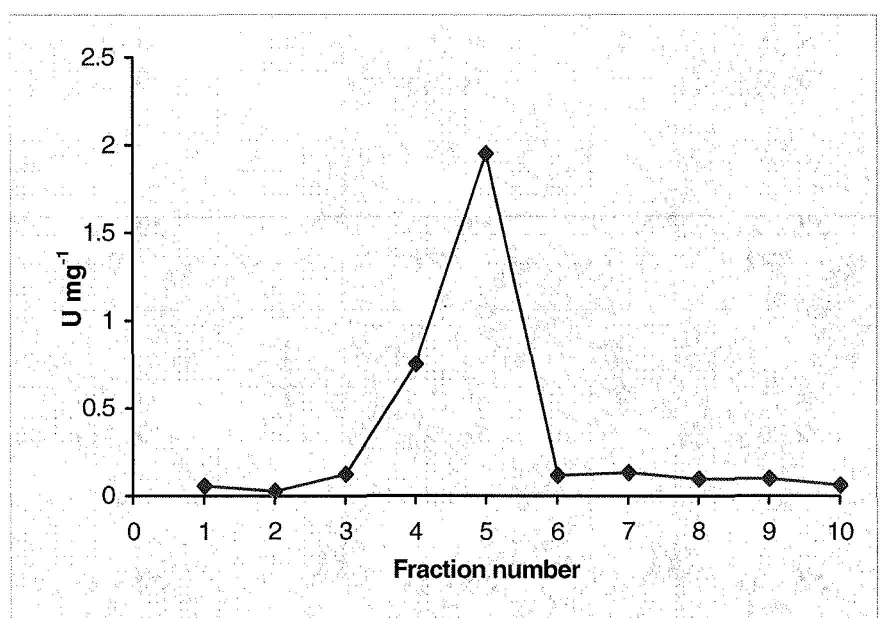
PURIFICATION BY GEL FILTRATION CHROMATOGRAPHY

Fraction No.	Protein (mg / ml)	Laccase activity U mg^{-1}
1	0.072	0.056
2	0.085	0.027
3	0.099	0.125
4	0.021	0.753
5	0.004	**1.951**
6	0.004	0.117
7	0.003	0.132
8	0.008	0.097
9	0.006	0.098
10	0.004	0.061

Laccase activity was maximum in fraction 5 followed by fraction 4.

FIGURE 9

PURIFICATION BY GEL FILTRATION CHROMATOGRAPHY



4.5 EFFECT OF LACCASE ON DENIM FABRIC

To study the effect of laccases on denim fabric, ammonium sulphate precipitated sample (Sample I) and dialyzed sample (Sample II) prepared from *Aspergillus niger* cultured in sugarcane bagasse were taken. The laccase activity in sample I and II was analysed to be 0.036 and 0.436 U mg^{-1} respectively.

The denim fabrics selected were light weight denim and heavy weight denim.The fabrics were treated conventionally (Hypochlorite bleaching) and enzymatically and the results are presented under the following headings:

4.5.1 Weight loss

The percentage weight loss was determined by measuring the difference in the weight of the treated fabric and the untreated fabric and the results are depicted in Table 7 and Fig. 10.

TABLE 7

PERCENTAGE WEIGHT LOSS OF DENIM FABRICS

Type of fabric	Treatment of fabric	Mean weight (gm)	Effect of treatment	
			Loss/Gain	% Loss/Gain
Light weight denim	U	2.05	-	-
	C	1.80	0.25	12.2
	E_1	1.74	0.31	15.1
	E_2	1.78	0.27	13.2
Heavy weight denim	U	2.72	-	-
	C	1.95	0.77	28.0
	E_1	1.74	0.98	36.02
	E_2	1.74	0.98	36.02

U – Untreated sample (Control)

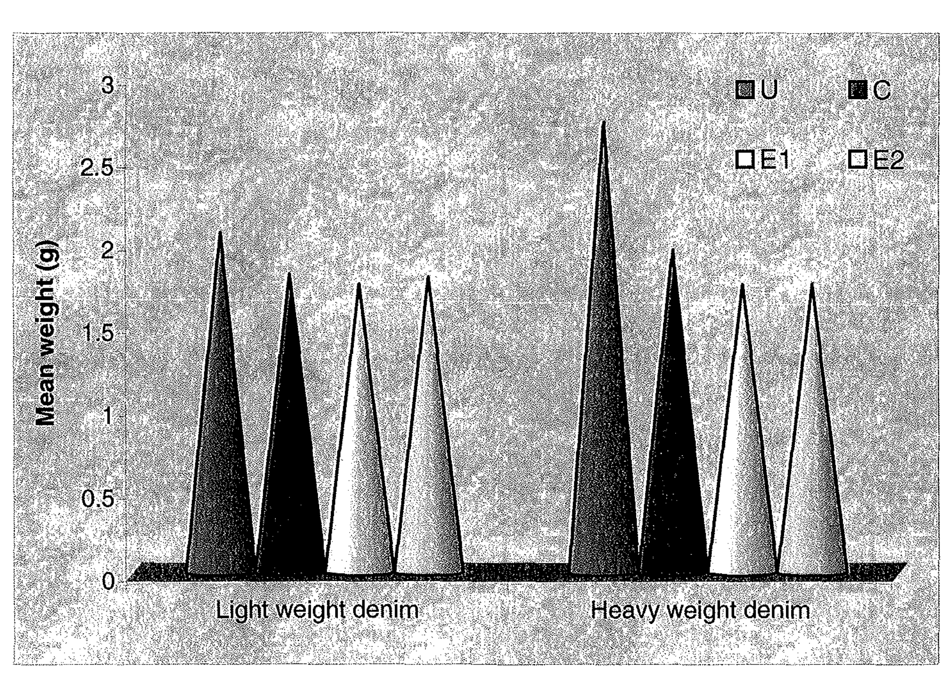
C – Conventionally treated

E_1 – Sample I treated

E_2 – Sample II treated

FIGURE 10

PERCENTAGE WEIGHT LOSS OF DENIM FABRICS



It is observed that the weight loss was greater in the enzyme treated fabrics than the conventionally treated fabrics. The maximum weight loss (36.02%) was obtained in heavy weight denim when treated with both enzyme samples I and II. In case of light weight denim the maximum weight loss (15.1%) was reported in sample I.

Enzyme treatment showed the weight loss in denim fabrics which attributed to removal of the protruding fibers which were present on the surface. The presence of other enzymes namely cellulase, pectinase, lipase, amylase and protease in the enzyme samples might have removed the cellulosic and non-cellulosic substances of fabric. Hence the cumulative effect of enzyme treatment has resulted in reduction in the weight of the fibre.

4.5.2 Tensile strength

The tensile strength of the conventionally and enzymatically treated fabrics were tested along warp and weft directions and the mean values are presented in Table 8 and Fig.11.

TABLE 8

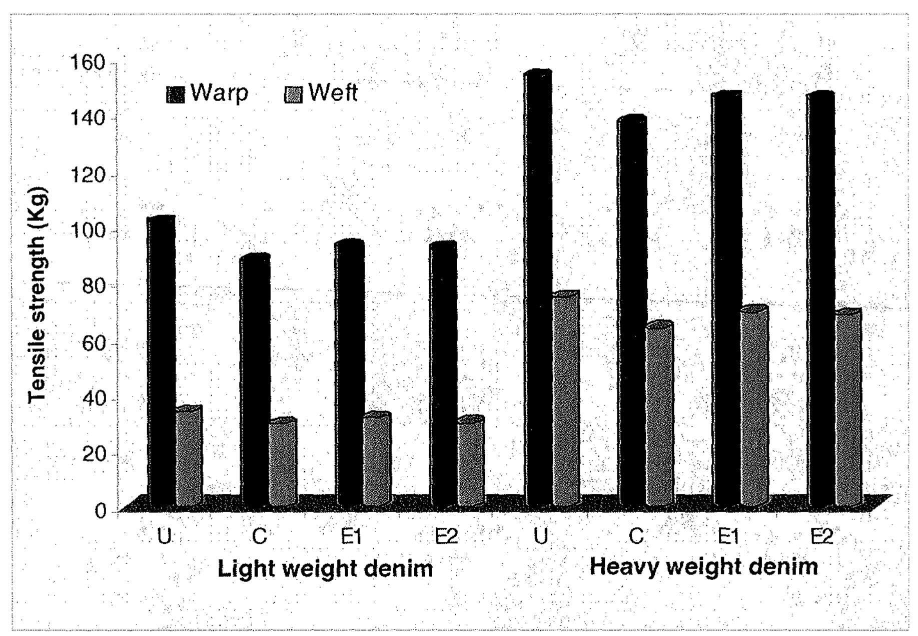
TENSILE STRENGTH OF FABRIC

Type of fabric	Treatment of fabric	Tensile strength (Kg)		Effect of treatment			
				Loss/Gain		% Loss / Gain	
		Warp	Weft	Warp	Weft	Warp	Weft
Light weight denim	U	101.6	33.8	-	-	-	-
	C	87.7	29.4	13.9	4.4	13.7	13.0
	E_1	93.0	31.5	8.6	2.3	9.4	11.4
	E_2	92.0	30.0	9.6	3.8	8.4	6.8
Heavy weight denim	U	153.6	74.8	-	-	-	-
	C	137.2	63.8	16.4	11.8	10.7	15.8
	E_1	146.0	69.5	7.6	5.3	5.3	8.4
	E_2	145.5	68.5	8.1	6.3	4.9	7.08

U – Untreated sample (Control)
C – Conventionally treated
E_1– Sample I treated
E_2– Sample II treated

FIGURE 11

TENSILE STRENGTH OF FABRIC



The tensile strength losses were lesser in enzymatically treated fabrics than conventionally treated fabrics. In light weight denim the highest tensile loss 9.4% and 11.4% were obtained in warp and weft directions respectively when treated with ammonium sulphate precipitated sample. In case of heavy weight denim, the tensile strength loss was found to be 5.3% in warpwise and 8.4% in weftwise when treated with ammonium sulphate precipitated sample followed by dialyzed sample.

In conventional treatment a strong oxidizing agent attacks cotton and reduces the strength whereas laccase does not attack the cotton and retains its strength. Akin *et al.* (2000) reported that different enzyme formulations can modify fiber strength properties.

4.5.3 Fabric thickness

The fabric thickness is an indicator of warmth and heaviness of the fabric in use. The mean of the fabric thickness after the treatment are presented in Table 9 and Fig.12.

TABLE 9

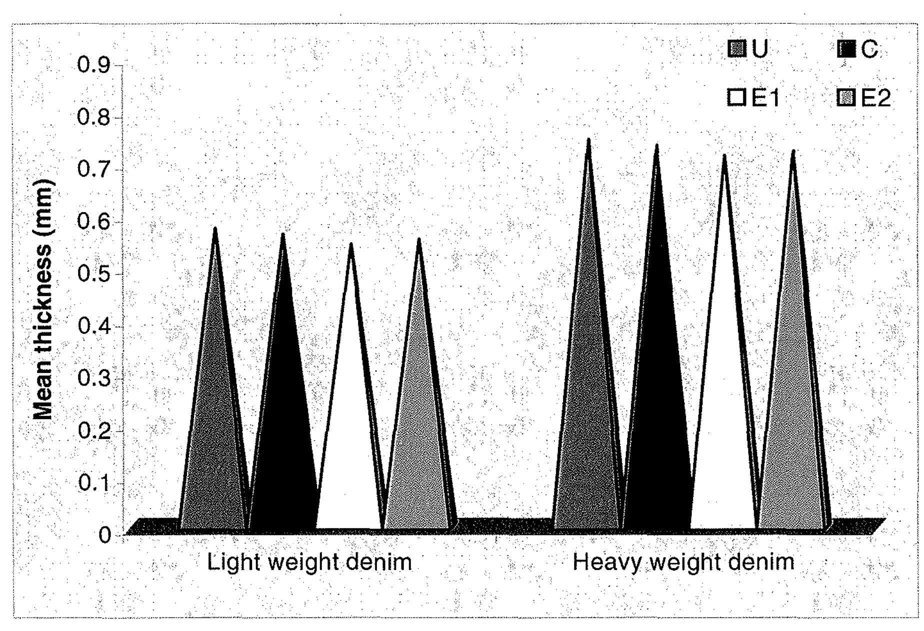
FABRIC THICKNESS AFTER ENZYME TREATMENT

Type of fabric	Treatment of fabric	Mean thickness (mm)	Effect of treatment	
			Loss/Gain	% Loss/Gain
Light weight Denim	U	0.57	-	-
	C	0.56	0.01	1.7
	E_1	0.54	0.03	5.3
	E_2	0.55	0.02	3.5
Heavy weight denim	U	0.74	-	-
	C	0.73	0.01	1.4
	E_1	0.71	0.03	4.1
	E_2	0.72	0.02	2.7

U- Untreated sample (Control)
C - Conventionally treated
E_1 - Sample I treated
E_2 - Sample II treated

FIGURE 12

FABRIC THICKNESS AFTER ENZYME TREATMENT



From Table 9 it is observed that there is a loss in fabric thickness. The loss was higher in enzyme treated fabrics than conventionally treated fabrics. The fabric thickness loss was greater in denim fabrics when treated with enzyme from ammonium sulphate precipitated sample (Sample I) when compared with dialyzed sample (Sample II).

Among the enzyme treated sample the presence of other enzymes in ammonium sulphate precipitated sample could have resulted in the loss of fabric thickness.

4.5.4 Stiffness of fabric

The fabric stiffness was determined by shirley stiffness tester. The results are indicated in Table 10 and Fig. 13.

TABLE 10

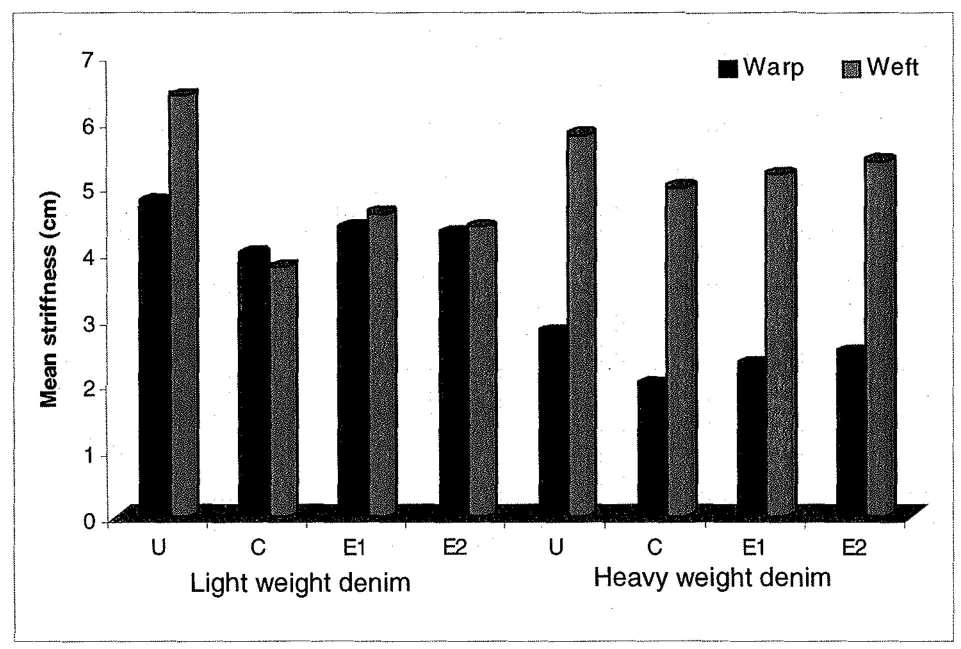
STIFFNESS OF FABRIC

Type of fabric	Treatment of fabric	Mean (cm)		Effect of treatment			
				Loss/Gain		% loss / Gain	
		Warp	Weft	Warp	Weft	Warp	Weft
Light weight denim	U	4.8	6.4	-	-	-	-
	C	4.0	3.8	0.8	2.6	16.7	40.6
	E_1	4.4	4.6	0.4	1.8	8.3	31.2
	E_2	4.3	4.4	0.5	2.0	10.4	28.1
Heavy weight denim	U	2.8	5.8	-	-	-	-
	C	2.0	5.0	0.8	0.8	28.6	13.8
	E_1	2.3	5.2	0.5	0.6	17.9	10.3
	E_2	2.5	5.4	0.3	0.4	10.7	6.9

U - Untreated sample (Control)
C - Conventionally treated
E_1 - Sample I treated
E_2 - Sample II treated

FIGURE 13

STIFFNESS OF FABRIC



In general, the stiffness loss was lesser in enzyme treated fabric than conventionally treated fabric. However, the stiffness loss in denim fabrics treated with ammonium sulphate precipitated samples were higher than the denim fabrics treated with dialyzed sample. This may be due to the presence of more cellulase enzyme in ammonium sulphate precipitated sample than dialyzed sample.

Traore and Diller (2000) reported that cellulase treatment basically reduce the tensile properties.The enzyme treatment has improved the softness of fabric which gets reflected in form of decrease in stiffness loss.

4.5.5 Abrasive resistance of fabric

Abrasive resistance were determined in untreated, conventional and enzyme treated fabrics. The results are depicted in Table 11.

TABLE 11

ABRASIVE RESISTANCE OF FABRIC

Type of fabric	Treatment of fabric	Abrasive resistance	Effect of treatment	
			Loss/Gain	% Loss/gain
Light weight denim	U	0.29	-	-
	C	0.25	0.04	13.8
	E_1	0.27	0.02	6.8
	E_2	0.26	0.03	10.8
Heavy weight denim	U	0.49	-	-
	C	0.43	0.06	12.2
	E_1	0.46	0.03	6.1
	E_2	0.45	0.04	8.2

U - Untreated sample (Control)
C - Conventionally treated
E_1 - Sample I treated
E_2 - Sample II treated

The loss in abrasive resistance was lesser in enzymatically treated fabrics than conventionally treated fabrics. The loss in abrasive resistance in denim fabrics treated with dialyzed sample were greater than the fabrics treated with ammonium sulphate precipitated sample. It is observed that the increasing concentration of laccase has produced more abrasion to the fabric.

Summary and Conclusion

5.0 SUMMARY AND CONCLUSION

Today enzymes have become an integral part of the textile processing with the increase in awareness and regulation about environment concerns, enzymes are the obvious choice. Hence the present study was taken upto understand the enzymes needed in textile processing by culturing micro organisms. Agricultural soil was used as a source for the isolation of fungi. The isolated fungi were subcultured in Rose Bengal Chloramphenicol Agar medium to identify fungi.

The fungal species, namely, *Aspergillus niger, Penicillium sp.,Fusarium sp.* and *Rhizopus sp.* were inoculated in different substrates and the extracts were assayed for laccase activity.

The effect of culture conditions namely the incubation temperature and initial medium pH for maximum enzyme production were studied with all the selected fungal species and substrates. To study the effect of enzyme on denim fabrics, the enzyme samples from *Aspergillus niger* cultured in sugarcane bagasse were preferred. Two enzyme samples, one ammonium sulphate precipitated sample dissolved in phosphate buffer and the other dialysed sample were prepared. The light weight and heavy weight denim fabrics were treated conventionally with hypochlorite and enzymatically with the two prepared enzyme samples. Weight loss, tensile strength, thickness, stiffness and abrasive resistance were tested to study the effect of enzymatic treatment.

The findings of the study "**Optimization and production of fungal laccase and its effect on denim fabric**" are summarized as follows.

The selected fungal species exhibited optimum initial medium pH which varied from 4.0 to 8.0 in different substrates. *Penicillium sp.* produced

the highest amount of laccase activity (0.192 U mg^{-1}) at the initial medium pH 7.0 with sorghum flour as substrate at room temperature when compared with *Aspergillus niger, Fusarium sp.* and *Rhizopus sp.*

The optimum temperature for enzyme production was found to vary from 17°C to 47°C for the selected fungal species inoculated with different substrates. Among the fungi and the substrates, wheat bran with *Penicillium sp.* gave the highest laccase activity (0.278 U mg^{-1}) at temperature 27°C at neutral pH.

The enzymes extracted from *Aspergillus niger* and *Penicillium sp.* cultured in wheat bran, rice bran and sugarcane bagasse were subjected to purification by ammonium sulphate precipitation followed by dialysis.The specific activity of laccase produced from *Aspergillus niger* cultured in sugarcane bagasse was the highest both in ammonium sulphate precipitated sample and dialysed sample and the increase was found to be 3.2 and 39.6 fold respectively.

For further purification by gel filtration chromatography, the dialysed sample from *Aspergillus niger* cultured in sugarcane bagasse was applied on sephadex G-50 column. The highest laccase activity (1.951 U mg^{-1}) was obtained in fraction 5 followed by fraction 4.

In both light weight denim and heavy weight denim fabrics, the weight loss and thickness loss were higher in enzyme treated samples than conventionally treated samples. Loss of tensile strength, stiffness and abrasion were lesser in enzyme treated samples than conventionally treated samples.

Among the two enzyme samples, the weight of fabric, tensile strength, thickness and stiffness were reduced greater in ammonium sulphate precipitated sample than dialysed sample. The dialysed sample produced the highest reduction in abrasive reistance in denim fabrics.

In conclusion, the present study contributes to

- Identification of fungi that produce enzymes useful in textile industry from soil source.
- Optimization of initial medium pH and incubation temperature for maximum enzyme production.
- Purification of enzyme.
- Understand the effect of enzyme on denim fabrics.

SCOPE OF FURTHER RESEARCH

- Screening of laccase producing microorganisms
- Large scale production of enzymes using cheaper substrates and high efficiency mutants.
- Screening and production of mutants for increased enzyme production
- Effect of enzymatic treatment on different fabrics.
- Efficiency of novel enzymes in different textile processing, pulp bleaching and effluent treatment.
- Role of laccase in dye decolourisation.

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Appendices

APPENDIX I

COMPOSITION OF ROSE BENGAL CHLORAMPHENICOL AGAR MEDIUM

Composition	(g/L)
Peptone	5.0
Glucose	10.0
Potassium dihydrogen phosphate	1.0
Magnesium sulphate	0.5
Rose Bengal	0.5
Chloramphenicol	0.1
Agar	15.5
pH	7.2 ±0.2 at 25°C

APPENDIX II

LACTOPHENOL COTTON BLUE STAINING (Kannan, 1996)

PRINCIPLE

Lacto phenol cotton blue stains the fungal cytoplasm and provides a light blue background against which the walls of hyphae can readily be seen. It contains four constituents: Phenol which serves as fungicide, lactic acid which stains cytoplasm of fungus, glycerol which gives a semi permanent preparation and cotton blue.

REAGENTS

Phenol Crystals	20g
Lactic acid	20ml
Glycerol	40ml
Cotton Blue (1% aqueous)	0.05(2ml)

Made up the volume to 100ml. Dissolved the phenol crystals and the other ingredients by heating the mixture gently.

PROCEDURE

Placed a drop of lactophenol cotton blue on a clean glass slide. Transferred a small amount of fungus with spores and spore bearing structure into the drop using flamed needle. Mixed gently and placed a cover slip over the preparation. Examined under the microscope.

APPENDIX III

EXTRACTION OF ENZYMES FROM FUNGAL CULTURES

After 7 days of incubation period the fermented cultures were subjected to mechanical grinding using 0.2M phosphate buffer (pH 7.0).The culture was filtered through whatmann No.1 filter paper and the filterate was centrifuged at 10,000g for 30 minutes at 4°C. The supernatant was used for the enzyme assays.

APPENDIX IV

DETERMINATION OF PROTEIN CONTENT
(Lowry *et al.*, 1951)

PRINCIPLE

The blue colour developed by the reduction of phosphomolybdic phosphotungstic components in the Folinciocalteau reagent by the biuret reaction of the protein with alkaline cupric tartarate are measured in Lowry's method.

REAGENTS

1. Solution A : 1% Copper sulphate
2. Solution B : 2% Sodium potassium tartarate
3. Solution C : 2% Sodium carbonate in 0.1N NaOH.

4. Solution D : Mixed just before use, 1ml of solution A, 1ml of solution B and 100ml of solution C.
5. Solution E : 1N Folin ciocalteau reagent C.
   Mixed equal volumes of commercially available reagent and diluted with water just prior to use stored protected from light.
6. Standard BSA: 50mg of BSA in 50ml of 0.1 N NaOH. Diluted to 1:10 for working standard.

PROCEDURE

Aliquots of standard protein solution (0-1000µg) were taken and the enzyme sample were made upto 1ml with 0.1N NaOH. Shook well to treat the protein with alkali. Added 5ml of solution D, mixed well and incubated at 37^0C for 3 minutes. Added 0.5ml of solution E to each tube, mixed well and incubated for 30 minutes in the dark. Read the colour developed at 660nm against a reagent blank. Fit a linear regression in a scientific calculator and read the protein concentration in the aliquot taken.

APPENDIX V

DETERMINATION OF LACCASE ACTIVITY
(Sarkanen *et al.*, 1999)

PRINCIPLE

Guaiacol is used a is substrate for the assay of laccase. The resulting oxidized guaiacol is probably more than one compound and depends on the reaction conditions. The rate of formation of tetraguaiacol product is a measure of laccase activity and can be assayed spectrophotometrically at 440nm.

REAGENTS

(1) Sodium acetate buffer 50mM (pH 4.5)
Soln A: 0.05 M acetic acid (11.55g/L)
Soln B: 0.05 M sodium acetate (16.4g/L)

Mixed 25.2ml of solution A and 24.5ml of solution B and diluted to 100 ml.

(2) Guaiacol (2mM)

0.02ml of Guaiacol solution was made upto 100 ml with buffer

PROCEDURE

Pipetted out into a clean 1cm cuvette of 2.0ml of 0.05M guaiacol substrate. The reaction is initiated by the addition of 0.5ml of enzyme extract and immediately note down the absorbance at 440nm in a spectrophotometer.

Calculation

One unit of enzyme activity was expressed as the amount of tetraguaiacol formed per minute per litre of enzyme extract.

APPENDIX VI

OPTIMIZATION OF INCUBATION TEMPERATURE FOR MAXIMUM ENZYME PRODUCTION BY FUNGI

In 250ml sterile conical flasks, 10g of different substrates were taken and autoclaved. The media was then inoculated with two loopful of fungal cultures and incubated at selected temperatures for 3-4 days.

APPENDIX VII

AMMONIUM SULPHATE PRECIPITATION OF PROTEIN (Jayaraman, 1981)

PRINCIPLE

Ammonium sulfate is a highly water soluble salt that is valuable for producing differential precipitation of proteins. The high ionic strength of ammonium sulfate solution causes the more hydrophobic proteins to associate resulting in precipitation. Proteins will usually precipitate with in a small range of ammonium sulfate concentration depending upon the particular

protein. The ammonium sulfate is often used to concentrate enzymes by first precipitating them by adding of an appropriate concentration and then resolubilizing them in a minimum volume of buffer.

REAGENT

Ammonium sulfate.
60% =36.6gm of ammonium sulphate in 100ml aliquot.

PROCEDURE

To the homogenate ammonium sulfate was added in small amount with stirring to give to percent concentration. This was kept for 15-20mts for full precipitation and the precipitate contains considerable amounts of ammonium sulfate that could be removed by dialysis.

APPENDIX VIII

DIALYSIS
(Ninfa and Ballog, 1980)

PRINCIPLE

Dialysis is a process, by which small molecules selectively removed from a sample containing mixture of both small and large molecules. Dialysis is used to separate molecules on the basis of size. The pore of the dialysis membrane allow small molecules such as solvents, salts products and substrate to diffuse freely through the membrane, whereas protein and other large molecules are retained.

PROCEDURE

The sample solution was filled in the dialysis bag and the open end was closed by using dialysis clips. The bag was then placed in buffer solution for 2 hours with 2-3 changes of buffer. The retentate was collected, and centrifuged at 12,000 rpm for 50 minutes. The precipitate was dissolved in the same buffer and used for further step.

APPENDIX IX

GEL FILTRATION CHROMATOGRAPHY
(Andrews, 1964)

PRINCIPLE

Separation is based on the fact that proteins are macromolecules whereas the salts are low molecular weight substances. When the sample is passed through a column packed with G-25 (or) G-50, protein remain totally excluded from the gel and eluted.

REAGENTS

(1) Glass column
(2) Sephadex G - 50
(3) Sodium phosphate buffer pH 4.5 (10mM)

PROCEDURE

1. Suspended 5 g of sephadex G-50 in 10 mm sodium phosphate buffer (pH 4.5) and allow to swell by keeping it for 3-4 hours.
2. Decant the excess of buffer along with any suspended fine particles to obtain slurry of reasonable thickness.
3. Fixed the column upright on a burette stand with the help of clamps.
4. Kept the outlet of the column closed placed a plug of glass wool at the base of the column and pour a small volume of buffer or distilled water into the column.
5. Gradually pour the slurry into it along the inner side of its wall and if necessary with gentle tapping to expel any air bubbles.
6. Allow the chromatographic media to settle down evenly and then opened the outlet to drain excess liquid from the column.
7. The sample was applied over the chromatographic column by the following method.

The mobile phase at the top of the packing was drained off till the bed surface gets exposed closed the outlet and gently apply the sample uniformly over the bed surface using micropipette and loaded sample was then allowed to just entire into the column by opening the outlet. A small amount of mobile phase (as buffer) was added to wash the trace of the sample into the column.

(1) Added sufficient amount of buffer on the top of the column and connected it to the buffer reservoir.

(2) Collected fractions (2ml each) were determined for its protein content either by maintaining absorbance at 280nm or by Lowry's method and enzyme assay.

(3) Plot the graph of concentration of protein and enzyme in y axis and fraction number (or) elution volume in x axis.

APPENDIX X

BLEACHING
(Menezes, 2002)

CONVENTIONAL BLEACHING

Bleaching of denim fabrics were done at room temperature for 20 min, keeping the materials with 3% sodium hypochlorite solution. The pH of the bath was maintained at 4 - 5.

ENZYMATIC BLEACHING

The denim fabrics were treated with 1% of two partially purified enzyme samples and 0.2% of hydrogen peroxide solution at 60°C for 1 hour. The pH of the bath was maintained at 4.0 - 4.5.

APPENDIX XI

WEIGHT OF FABRIC

About 10 samples were cut from the material by using a template and readings were taken in mg per square yard. The same procedure was

followed for the other samples. The mean fabric weight of each samples were calculated and recorded.

APPENDIX XII

TENSILE STRENGTH OF FABRIC

The Eureka Pendulum Type Tensile Strength Tester was used to determine the breaking strength of the samples. Ten test samples with a minimum length of 33.02 and a width of 3.81cm were cut from each of the sample materials. Each sample was raveled to 2.54cm in the width of and 340.08cm long by drawing the yarns from the sides. The sample was clamped firmly between the two jaws. Care was taken to see that the sample was perpendicular to the load. The load was applied until the sample was broken. The dial reading in kilograms were noted. Ten readings were taken for each material and the mean value was calculated.

APPENDIX XIII

THICKNESS OF FABRIC

The difference in thickness of the samples was found out using the Baty Thickness Tester to measure upto an accuracy of 0.01mm. The fabric was placed into the pressure foot and annual reading was noted from a clock type dial guage. The readings were taken at 10 different places in each sample and the mean value was obtained. The same procedure was followed for all the other samples.

APPENDIX XIV

STIFFNESS OF FABRIC

Shirley Stiffness Tester was used to determine the stiffness of the samples. The sample was cut according to the template size and then both template and sample were transferred to the plat form with material underneath. Both were slowly pushed forward. The strip of the material commenced to droop over the edge of the plat form and the movement of the template and the sample was viewed until the tip of the sample viewed in the mirror cuts both index lines. The bending length was immediately read from

the scale opposite a zero line, engraved on the side of the plat from. Each sample was tested 4 times, at each end and again with carried out for all the other samples. Mean values for the bending length in warp and weft directions were calculated.

APPENDIX XV

ABRASIVE RESISTANCE OF FABRIC

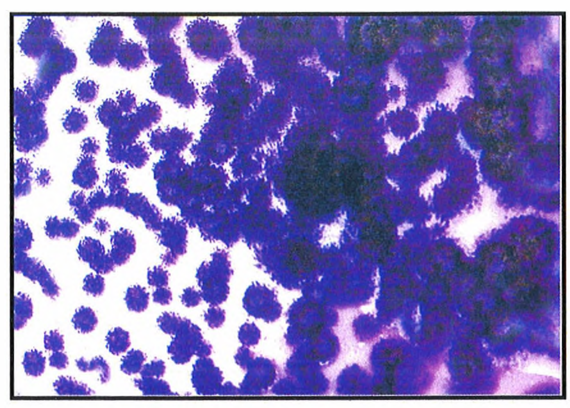
Martindale Abrasion Tester was used to determine. The abrasive resistance of the fabric. Four specimens of test sample with a minimum diameter of 38mm were cut circularly and fixed on the sample holders. The sample holders were able to more vertically in the clamp bushes. The sample holders touch the table surface and it was flatted and more in the same phase in which the top plate slides. Because of this movement the cloth was rubbed against the cloth surface in harmonic pattern. At one stage it was circular and their changes to a curve of an ellipse until the line becomes a straight line along the diagonal of the circle. This type of motion has the advantage that the specimen was rubbed in all portions i.e., both in warp and weft ways. The same procedure was carried out for all the samples. End point was determined by plotting weight loss against the number of cycles and the graph was analysed.

PLATE I

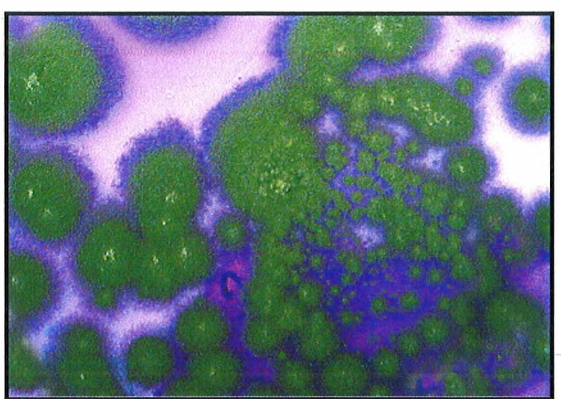
SOIL

PLATE II

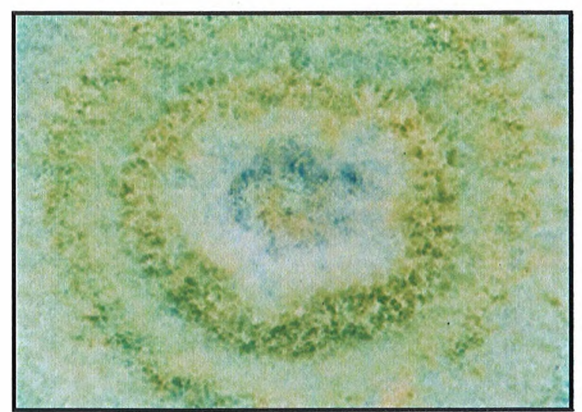
FUNGAL COLONIES



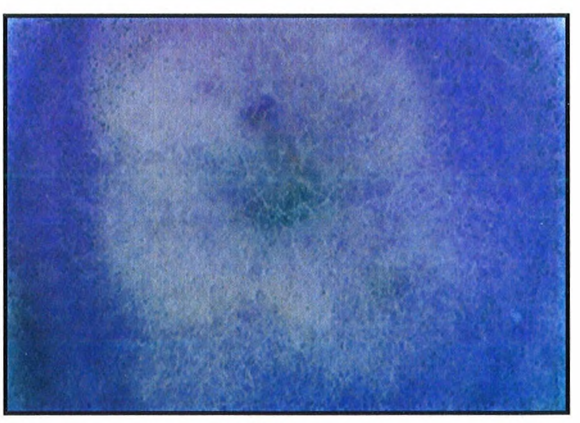
Aspergillus niger



Penicillium sp.



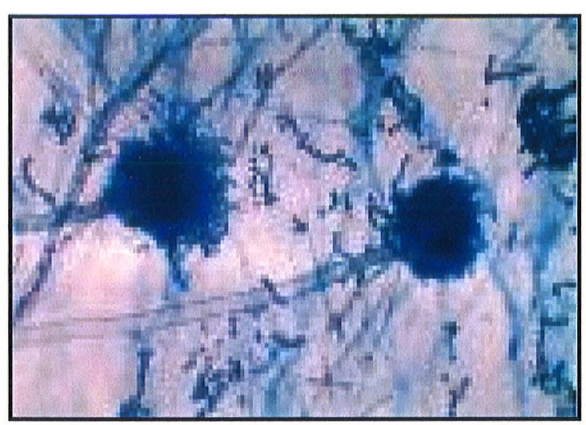
Fusarium sp.



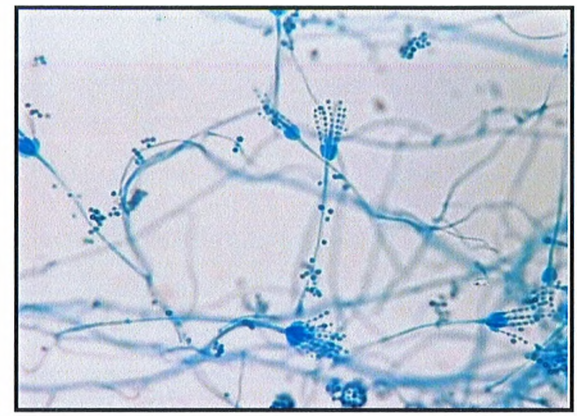
Rhizopus sp.

PLATE III

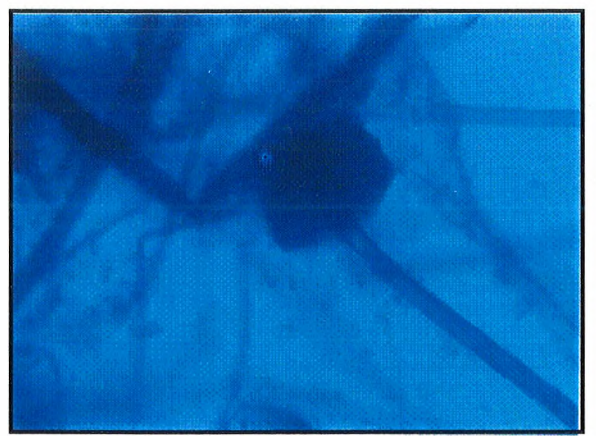
MICROSCOPIC VIEW OF FUNGI



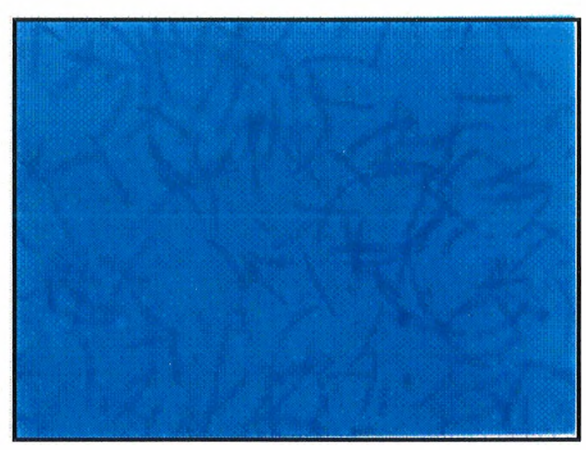
Aspergillus niger



Penicillium sp.



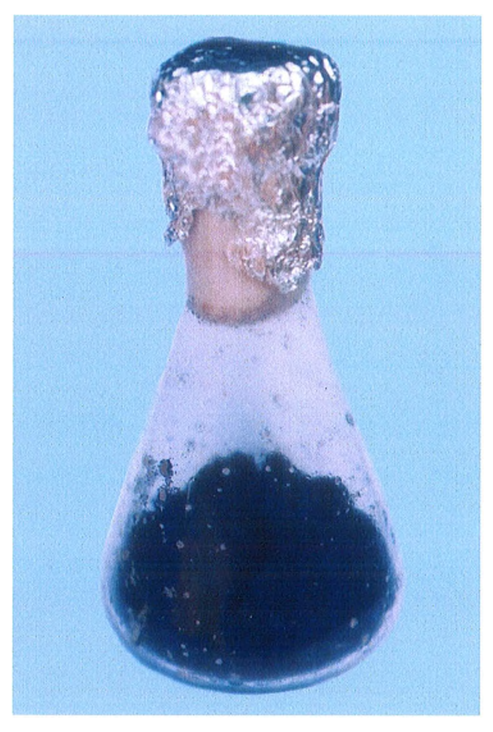
Fusarium sp.



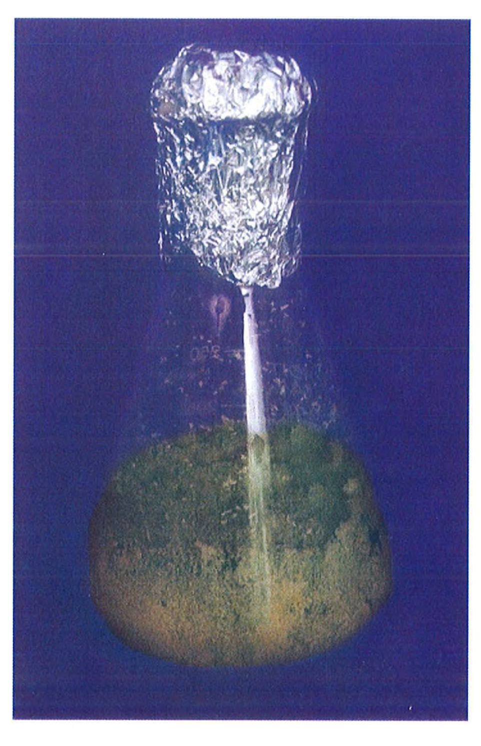
Rhizopus sp.

PLATE IV

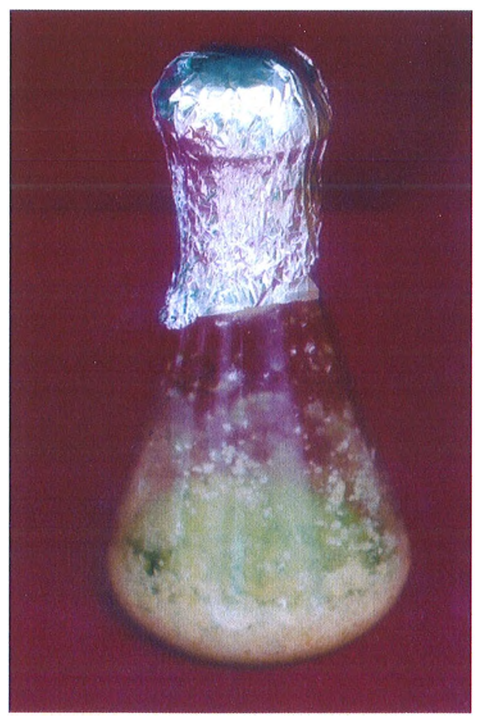
SUBSTRATES INOCULATED WITH SELECTED SPECIES



Aspergillus niger



Penicillium sp.



Fusarium sp.

Rhizopus sp.

FIGURE 5

PROTEIN CONTENT (mg/ml) AND LACCASE ACTIVITY (U mg^{-1}) OF FUNGAL EXTRACTS

Fig. 5a. PROTEIN CONTENT

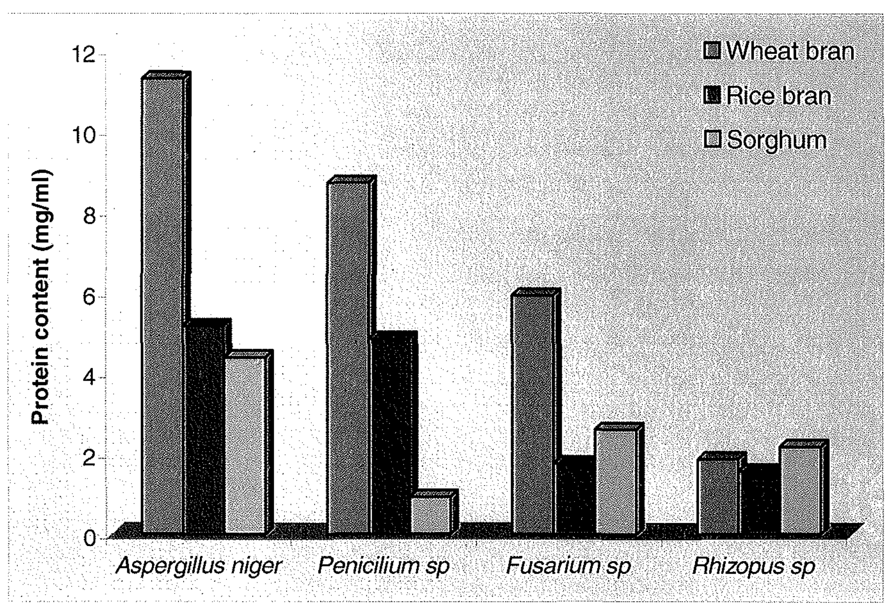


Fig.5b. LACCASE ACTIVITY