

***Production and Optimization of Xylanases
and Cellulases from Fungi and their
effects on Paper Pulp***

By

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Master of Philosophy in Biochemistry

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Declaration

DECLARATION

*I hereby declare that the dissertation entitled “**Production and Optimization of Xylanases and Cellulases from fungi and their effects on Paper Pulp**” 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.*

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*This is to certify that the dissertation entitled “**Production and Optimization of Xylanases and Cellulases from fungi and their effects on Paper Pulp**” 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 **Kalpana. K** 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.*

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Introduction

1.0 INTRODUCTION

Plant biomass is comprised of three major polymeric constituents such as celluloses, hemicelluloses and lignins. Hemicelluloses are a composite of different non-cellulosic polysaccharide in which xylan being the major polysaccharide (Collins *et al.*, 2005).

Xylan, which has a backbone of β -1, 4-linked xylopyranosyl residues contain various substituted side groups such as acetyl L-arabino-furonosyl and O-methyl glucuronyl residues. Xylanase is a key enzyme for xylan depolymerization. The enzymes involved in hydrolysis of the main chain of xylans are endo xylanase (1,4- β -D xylan xylano hydrolase; E.C.3.2.1.8) and β -xylosidase (β -D-xyloside xylohydrolase; EC.3.2.1.34) (Biely, 1985).

An extensive isolation and screening programme were initiated to select a variety of microorganisms that produce enzymes of potential use in industrial application and pollution control. Filamentous fungi have long been used for the production of metabolites and enzymes. A number of xylanases have been purified from a wide variety of microorganisms such as *Bacillus sp.*, *Clostridium sp.*, *Aspergillus sp.*, *Fusarium sp.* and *Trichoderma sp.* (Saha and Bothast., 1999).

White rot fungi are generally known to produce from their cellular metabolic processes, both lignin biodegrading and cellulase hydrolyzing enzymes, namely; cellulases, laccases, lignin peroxidases, manganese peroxidases and xylanases. Some thermophilic fungi have been shown to produce thermostable xylanases (Kalogeris *et al.*, 1998) and cellulase free xylanase (Gomes *et al.*, 1993) and several thermophilic anaerobic bacteria have been shown to produce xylanolytic enzymes that convert xylan and hemicellulose wastes to ethanol (Arhing *et al.*, 1996).

Utilization of whole cell biocatalyst is effective in improving cost efficiency. A number of microorganism, including bacteria, yeast and fungi have been used as whole cell biocatalysts. But fungi, which are eukaryotic cells, are advantageous in repeated use because of their rigid cell wall (Lee *et.al.*, 2002).

Fungal xylanase can be produced using two main methods, solid state cultivation system and submerged liquid systems. Solid state fermentation [SSF] has gained renewed interest from researchers in recent years and has often been employed for the production of xylanases, because of number of economic and engineering advantages (Pandey, 1999).

Cellulases and hemicellulases have numerous applications in various industries including chemicals, fuels, food ,brewery and wine,animal feed, textile and laundry,paper and agriculture (Beauchemin *et al.*,2001, Sun and Cheng, 2002 ; Beauchemin *et al.*,2003). These enzymes are used commercially in scandinavia, Canada, the United states and chile. It is estimated that approximately 20% of the >1 billion US dollars of the world's sale of industrial enzymes consists of cellulases, hemicellulases and pectinases.The world market for industrial enzymes will increase in the range of 1.7 - 2.0 billion US dollars by the year 2005 (Bhat, 2000).

The catalytic actions of lignolytic and xylanolytic enzyme, makes them potentially good tools for pulp and paper industry. They can be used either for direct biodegradation of chloroorganics, hemicellulase and cellulase sludge pollutants (Honda.,1998). Xylanases act as environment friendly biobleaching agents to replace chlorine-containing reagents of chemical bleaching (Christov *et al.*, 1998). The treatment of pulps with xylanases leads to significant reduction in chemical consumption with almost no loss in pulp yield or quality.

The most important industrial application of xylanase is prebleaching of pulp so as to minimize the use of toxic chlorine - containing chemicals in the subsequent bleaching step (Beg *et al.*, 2001).

Lignocellulose biotechnology offers significant opportunities to developing countries for addressing some of the issues highlighted since most of the technology is based on the utilisation of readily available residual plant biomass considered as “waste” to produce numerous value-added products (Howard *et al.*, 2003).

Hence the present investigation “**Production and Optimization of xylanases and cellulases from fungi and their effects on paper pulp**” has been selected with following objectives:

- Selection of natural source for enzyme production
- Isolation and identification of fungi from the selected source
- Subculturing of fungi
- Production of enzymes from fungi using low cost substrates
- Optimization of culture conditions for enzyme production
- Purification of enzymes
- To study the effects of enzyme on paper and pulp bleaching process.

Review of Literature

2.0 REVIEW OF LITERATURE

The paper and pulp industry is a potential source of major pollution, generating large volumes of intensely coloured effluent for each metric ton of paper produced. In the production of paper, residual lignin from wood pulp is chemically liberated by using chlorine bleaching. Elemental chlorine reacts with lignin and other organic matter in the pulp forming chlorinated compounds that extracted with alkali (Ali and Sreekrishnan, 2001). The effluents have high biological oxygen demand (BOD) and chemical oxygen demand (COD). Chlorinated compounds are measured as adsorbable organic halides and chlorinated lignin derivatives such as chlorolignols, dioxins and sulfur compounds. Some of these compounds are carcinogenic, toxic and recalcitrant to degradation and are known as persistent organic pollutants. Enzymes are preferred to reduce the use of chlorine and chlorine compounds in the bleaching process (Raghukumar *et al.*, 2004).

The use of enzyme is an ecofriendly technology that effectively addresses the concern over the environmental hazards of conventionally used chlorine in pulp and paper industries (Kohli *et al.*, 2001). Considering these views, the present study **“Production and Optimization of xylanases and cellulases from fungi and their effects on paper pulp”** was taken up and the review of literature pertaining to this was discussed as follows:

2.1 XYLAN

2.2 XYLANOLYTIC ENZYMES-AN OVERVIEW

2.2.1 Xylanase

2.2.2 β -D xylosidases

2.2.3 Mechanism of action

2.2.4 Xylanases from mesophilic and thermophilic sources.

2.3 CELLULOLYTIC ENZYMES

2.3.1 cellulase producing microorganisms

2.4 APPLICATION OF ENZYMES

2.4.1 Biobleaching paper pulp

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2.4.3 Xylanase in food processing

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2.5 MICROBIAL PRODUCTION OF ENZYMES

2.4.1 Enzyme production by solid state fermentation using different substrates

2.4.2 Optimization of culture conditions.

2.6 COMMERCIALY AVAILABLE ENZYMES

2.7 OTHER ENZYMES IN PULP INDUSTRY

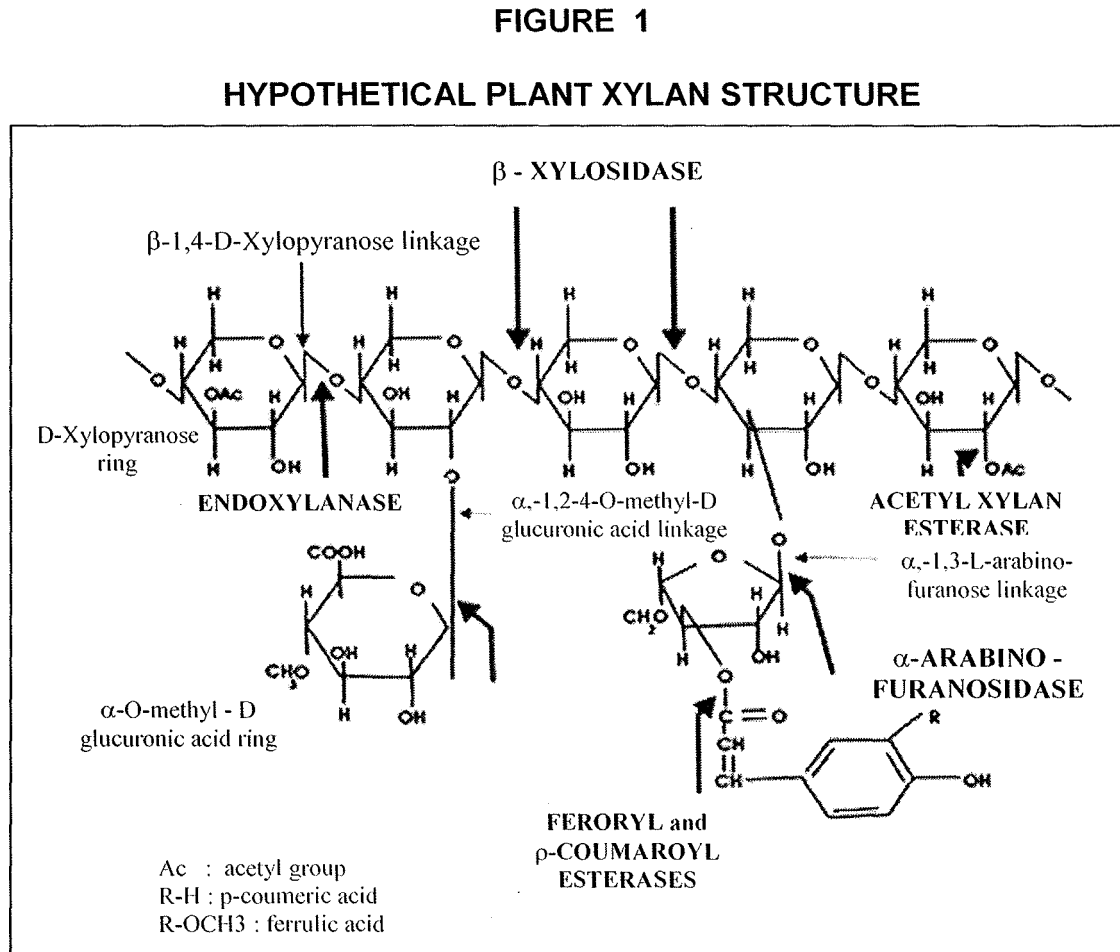
2.1 XYLAN

Xylan, a major structural component of plant cell walls and the most abundant renewable hemicellulose, represents a vast reserve of reduced carbon on earth. Xylan constitutes 20-40% of total plant biomass and its efficient utilization could enhance the economic competitiveness of bioconversion processes intended to compete with conventional industrial processes. Therefore, a significant interest in xylan degradation has been generated because of its possible applications in ruminal digestion, waste treatment, energy generation, production of chemicals and paper manufacture (Sunna and Antranikian., 1997).

xylan is a linear polymer of β -D xylano pyranosyl units linked by β -(1,4) glycosidic bonds. In nature, the polysaccharide backbone may be added to 4-O-methyl α -D-glucuronopyranosyl units, acetyl

groups and α -L-arabinofuranosyl, in variable proportions (Coughlan and Hazlewood, 1993).

Figure 1 shows a hypothetical plant xylan structure and site of attack by microbial xylanases.



2.2 XYLANOLYTIC ENZYMES-AN OVERVIEW

2.2.1 Xylanases

Xylanases catalyze the hydrolysis of xylans. These enzymes are produced mainly by microorganisms and take part in the breakdown of plant cell wall along with other enzymes that hydrolyze polysaccharides. Xylanases are distributed in marine algae, protozoans, crustaceans, insects, snails and seeds of land plants (Sunna and Antranikian, 1997). Among microbial sources, filamentous fungi are especially important as they secrete enzymes at higher

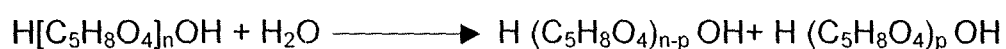
level than those found in yeast and bacteria. Xylanases are enzymatic complex responsible for the hydrolysis of xylan, which include endo β -xylanase and β -xylosidase (Dalboge, 1997).

2.2.2 β -D-xylosidases

β -D-xylosidases (1,4- β -D xylan xylohydrolase; EC.3.2.1.37) can be classified according to their relative affinities for xylobiose and larger xylooligosaccharides. Xylobiase and exo 1,4- β -xylanases can be recognized as distinct entities (Biely, 1993). Xylosidases hydrolyze small xylooligosaccharides and xylobiose releasing β -D-xylopyranosyl residues from the non-reducing terminus. Purified β -xylosidases usually do not hydrolyze xylan; their best substrate is xylobiose and their affinity for xylooligosaccharides is inversely proportional to its degree of polymerization. They are able to cleave artificial substrates such as p-nitro phenyl, o-nitrophenyl and β -D-xylopyranoside (Andrade *et al*, 2004 ; Zanoelo *et al.*, 2004).

2.2.3 Mechanism of action

Endo-1, 4- β -xylanase (1,4- β -D-xylan xylanohydrolase); (EC.3.2.1.8) cleaves the glycosidic bonds in the xylan backbone, bringing about a reduction in the degree of polymerization of the substrate. Xylan is not attacked randomly, but the bonds selected for hydrolysis depend on the nature of the substrate molecule, i.e., on the chain length, the degree of branching and the presence of substituents (Li *et al.*, 2000). Initially, β -D-xylopyranosyl is produced as the main hydrolytic product. The hydrolysis of xylan by an endoxylanase is



This equation shows the stoichiometry of a single hydrolytic event in a xylan molecule and such a reaction might occur at many points in the chain (Polizeli *et al.*, 2005).

2.2.4 Xylanases from mesophilic and thermophilic source

Research on microorganisms that utilize xylan is becoming more and more relevant in ecological and economic terms. Xylanases are synthesized by mesophiles and thermophiles (Smith *et al.*, 1991). Among the mesophilic fungi, the genera *Aspergillus* and *Trichoderma* are pre-eminent in xylanase production. In recent years, a lot of effort has been taken for the isolation of thermophilic and even extremophilic microorganisms as they produce enzymes of greater stability (Brunis *et al.*, 2001; Monti *et al.*, 2003).

Thermophilic and thermotolerant fungi are important sources of thermostable enzymes, which they degrade plant residues at elevated temperatures. Therefore, higher enzyme yields would result owing to greater enzyme stability, which is important during enzyme recovery and purification. Thermostable enzymes such as proteases, lipases, α -amylase, glucoamylase, cellulase and xylanase have been characterized and reported from thermophilic fungi especially endo β -1,4-xylanase (EC 3.2.1.8)(Maheshwari *et al.*, 2000).

2.3 CELLULOLYTIC ENZYMES

Cellulases catalyse the hydrolysis of cellulosic material. They are composed of a complex mixture of enzyme proteins with different specificities to hydrolyse glycosidic bonds. Cellulases can be divided into three major enzyme activity classes (Rabinovich *et al.*, 2002).

These are endoglucanases or endo-1,4 β glucanases (EC 3.2.1.4), Cellobiohydrolase (EC 3.2.1.91) and β -glucosidase (EC 3.2.1.21). Endoglucanases, often called carboxymethylcellulase are proposed to initiate attack randomly at multiple internal sites in the amorphous regions of the cellulose fibre opening-up sites for subsequent attack by the cellobiohydrolases. Cellobiohydrolase, often called as exoglucanase is the major component of the fungal cellulase system accounting for 40-70% of the

total cellulase proteins and can hydrolyse highly crystalline cellulose. Cellulobiohydrolases remove mono- and dimers from the end of the glucose chain. β -glucosidase hydrolyse glucose dimers and in some cases cellooligosaccharides to glucose (Howard *et al.*, 2003).

2.3.1 Cellulase producing microorganisms

Fungi are the main cellulase producing microorganisms, though a few bacteria and actinomycetes have also been reported to yield cellulase activity. Microorganisms of the genera *Trichoderma* and *Aspergillus* are the cellulase producers and crude enzymes produced by these microorganisms are commercially available for agricultural use (Sibtain *et al.*, 2003).

2.4 APPLICATION OF ENZYMES

2.4.1 Bio bleaching of paper pulp

Biobleaching is an alternative method to reduce the use of chlorine in the bleaching process. Biological bleaching is carried out using fungal/bacterial species to degrade residual lignin in the pulp by using hemicellulolytic enzyme such as xylanase or by lignocellulolytic enzymes such as manganese peroxidase and laccase (Carimir schenkel *et al.*, 1995).

2.4.1.1 Xylanase in paper Industry

Pulp bleaching technology is changing due to environmental public concerns. Technologies have been developed in order to replace chlorine compounds as bleaching agents. The use of xylanases in pulp bleaching sequences is a technical alternative and many research projects are being developed. The most significant effect of xylanase on pulp bleaching were reported to be a reduction in the requirement for elemental chlorine and a decrease in the amount of organochlorine compounds in bleach plant effluents (Medeiros *et al.*, 2000).

Cellulase free xylanases have potential application in the selective removal of hemicellulose from pulp to give brightness to the paper. This technology effectively addresses the concern over the environmental hazards of conventionally used chlorine in paper and pulp industries, leading to release of organochlorines toxic to all forms of life (Beg *et al.*, 2001 and Kohli *et al.*, 2001).

Medeiros *et al.* (2002) reported the use of cellulase free xylan degrading enzyme preparations from *Acrophialophora nainiana*, *Humicola grisea*, *Var thermoidea* and two *Trichoderma hazianum* strains as bleaching agents for eucalyptus kraft pulp prior to a chlorine dioxide and alkaline bleaching sequence. Overall, the enzyme preparations from *Trichoderma hazianum* were marginally more effective in reducing pulp viscosity and improved the brightness of the kraft pulp.

Kansoh *et al.* (2004) used enzymatic pretreatment of softwood kraft pulp using xylanase and mannase, single or in combination, either sequentially or simultaneously. Enzymes were obtained from *Streptomyces galbus* NR that has been cultivated in a medium containing either xylan of sugarcane bagasse or galactomannan of palm seeds as carbon sources. The partially purified enzyme preparation of xylanase and mannase showed high bleaching activity. Xylanase was found to be more effective for paper bleaching than mannase. When xylanase and mannase were dosed together, both enzymes were able to enhance the liberation of reducing sugars and improve pulp bleachability.

Hemicellulase which include β -mannase and xylanase activities had shown considerable potential in fiber modification and bleach boosting of pulps (Gubitz *et al.*, 1996).

Several fungal isolates obtained from marine habitats exhibited alkaline xylanase activity. The crude enzyme from *Aspergillus niger* N10cc was cellulase free and with high xylanase activity of 580U/L. It was found to bleach sugarcane bagasse pulp by 60 min treatment at 55°C (Raghukumar *et al.*, 2004).

2.4.1.2 Cellulase in paper industry

Enzymes have been used to improve physical properties of pulp fibres and have a commercial role. Cellulases enhance pulp fibrillation and there by improve paper strength. They reduce fibre coarseness and increase paper density and smoothness.

The treatment with a mixture of cellulases and xylanases prior to peroxide bleaching was found to enhance significantly the brightness of jute fibres, thereby reducing the peroxide requirement for bleaching (Gulrajani *et al.*, 1998)

2.4.2 Xylanase in animal feed

The second rapidly emerging thrust area for xylanase application is its use as an animal feed additive for mono gastrics like poultry and pigs which do not have xylanolytic microflora in their digestive tracts. Xylanase reduce viscosity and increase absorption by breaking down the non starch polysaccharides (NSPs) in high fibre rye and barley based feeds (Bed ford and Classon., 1992). Phosphorous addition to the feed and its extinction into the environment as a pollutant can be reduced by treating with phytase (Wyss., 1999). Pretreatment of agricultural silage and grain feed by xylanase has also been reported to improve its nutritional value (Beg *et al.*, 2001).

2.4.3 Xylanase in food processing

Enzymatic hydrolysis of xylan in agricultural and industrial substances are used to obtain sugar supplements for producing speciality product such as the artificial sweetener, xylitol. xylanases have potential applications in the food industry, such as those concerned with baking and production of starch and juices (Nigam and Singh., 1995).

Xylanases may be employed in bread making, together with α -amylase, malting amylase, glucose oxidase and proteases. The xylanases, like the other hemicellulases, break down the hemicellulose in wheat flour, helping in the redistribution of water and leaving the dough softer and easier to knead. During the bread making process, they delay crumb formation, and allows the dough to grow. With the use of xylanases, there has been an increase in bread volumes, greater absorption of water and improved resistance to fermentation (Camacho and Aguilar, 2003).

In biscuit-baking process, xylanase is recommended for making cream crackers lighter, improves the texture, palatability and uniformity of the wafers (Harbak and Thygesen, 2002).

Xylanases and xylan are used in the pharmaceutical industry. Xylanases are sometime added in combination with a complex of enzymes (hemicellulases, proteases and other) as a dietary supplement or to treat poor digestion. Hydrolytic products of xylan, such as β -D-xylopyronosyl residues, can be converted into combustible liquids (ethanol), solvents and artificial low calorie sweetners (Screenath and Jeffries, 2000).

In fuel-alcohol production, xylanases decrease the viscosity of the mash and prevents fouling problems in distilling equipment. It improves the extraction oil from oil-rich plant material such as corn oil from corn embryos.

2.4.4 Cellulase in Textile industry

The cellulase modifies the properties of cotton material, surface area and fabric structure (Ibrahim and Elzairy, 1999). A combined treatment of pectinase and xylanase with reduced amounts of cellulases in the removal of protruding superficial fibres make the fabric surface smoother (Sundar and Nalankilli, 2002). Cellulases are being used more and more in denim washing to achieve a stone washed look without the need of stones (Menezes, 2002).

2.5 MICROBIAL PRODUCTION OF ENZYMES

2.5.1 Enzyme production by solid state fermentation (SSF) using different substrates

Solid state fermentation refers to the growth of microorganisms on solid materials without the presence of free liquid (Cannel and Mooyoung., 1980). The solid state Fermentation (SSF) technique can improve the yield and economy of enzyme production (Ghildyal *et al.*, 1985).

Filamentous fungi are the most commonly used microorganisms in SSF because they are able to grow on solid materials with low water content (Pandey.,1992). Solid state fermentation typically uses agricultural residues such as wheat straw, wheat bran, rice straw and sugarcane baggase (Biswas *et al.*,1998). The advantages of SSF are simpler product recovery and reduction of bacterial contamination due to low moisture level (Shuler and Kargi, 1992).

The production of high yields of an enzyme using SSF requires optimal environmental conditions to promote maximum growth of the microorganism and increased production of the enzyme. The environmental conditions include incubation temperature, moisture content, depth of substrate and length of time for fermentation as well as presence of surfactant (Smits *et al.*, 1996).

A large number of mesophilic and thermophilic fungi, eg. *Aspergillus* strains (Yinbo *et al.*, 1996; Kadowaki *et al.*, 1997). *Trichoderma* strains (Ridder *et al.*, 1997), *Chaetomium cellulyticum* (Dubeau *et al.*, 1997), *Thermomyces lanugionsus* (Puchart *et al.*, 1999) and *Penicillium* strains (Cortez *et al.*, 1998) can produce xylanase.

Gawande and Kamat (1999) had studied the two strains of *Aspergillus terreus* (*Aspergillus* sp. 5) and *Aspergillus niger* (*Aspergillus* sp. 44) known to produce xylanase (EC 3.2.1.8) with undetectable amounts of cellulase on

various lignocellulosic substrates using solid state fermentation. Among the lignocellulosic substrates used, wheat bran was reported to be the best for xylanase production.

Judith and Nei (2002) had evaluated the use of milled sugarcane bagasse as carbon source and the influence of xylose and glucose on the production of endoxylanase and β -xylosidase enzymes by *Aspergillus awamori* in solid state fermentation.

Malarvizhi *et al.* (2003) had reported the production of xylanase employing shake and solid-state culture conditions with *Ganoderma lucidum*, a white rot fungus. Among the different agrowastes used, wheat bran was found to be the best substrate for the test fungus production of xylanase than sugarcane bagasse and rice bran in solid-state fermentation.

Bakri *et al.* (2003) reported that *Penicillium canescens* 10-10c filamentous fungi produce xylanase by solid state fermentation. Among the different agricultural wastes used, wheat straw was found to be best substrate than wheat bran, beet pulp and soja meal.

Fungal, bacterial and actinomycete xylanolytic enzymes are generally induced by xylan, xylobiose, xylose and lignocellulosic residues that contain xylan (Kuhad *et al.*, 1998).

Gupta *et al.* (2001) had shown the enhancement in the production of an alkali stable xylanase, with dual pH optima, from haloalkalophilic *Staphylococcus sp.* SG-13 using agro-residues in submerged fermentation and a biphasic system. The agro-residues such as wheat bran, sugarcane bagasse, corncorbs and popular wood, when used as sole carbon source, improved the xylanase yield by five-fold as compared to xylose and xylan.

Microbial xylanases that are thermostable, active at alkaline pH and cellulase free are generally preferred for biobleaching of paper pulp (Raghukumar *et al.*, 2004)

Xylanase production was performed by growing a *Bacillus* isolate on agricultural by-products wheat straw, wheat bran, corncobs and cotton bagasse. A maximum xylanase activity of 180 U/ml was obtained together with a cellulase activity of 0.03 U/ml on 4 (w/v) corncobs (Banu Avcioglu *et al.*, 2005).

Solomon *et al.* (1999) produced cellulase from *Aspergillus flavus* on bagasse pretreated with ballmilling and caustic soda and the production was reported as 0.054 IU / ml.

Zhiyou (2002) studied the production of cellulase from *Trichoderma reesei* using dairy manure as a substrate and found 1.74 IU / ml.

2.5.2 OPTIMIZATION OF CULTURE CONDITIONS

Optimization for xylanase and cellulase production from *Aspergillus niger* ATCC 6275 under both submerged and solid substrate cultivation showed that optimal initial moisture content was 60% for xylanase and carboxy methylcellulase activities with temperature optima of 30°C and 35°C respectively (Prasertsan *et al.*, 1997).

Kango *et al.* (2003) had isolated *Emericella nidulans* NK-62 from bird nesting material and tested for xylanase production. The fungi grown on medium containing wheatbran supplemented with czapek's mineral salt solution was found be optimum at 60° C with pH range of 6.5.

The xyn A gene encoding a xylanase from the recently isolated *Bacillus* sp.strain BP-7 has been cloned and expressed in *E.Coil*. Recombinant xylanase A showed high activity on xylans from hard woods and cereals and

exhibited maximum activity at pH 6 and temperature of 60°C (Gallardo *et al.*, 2004).

Streptomyces sp. QG-11-3, produced a cellulase free thermostable xylanase (96 IU/ml) and a pectinase (46 IU/ml) in the Horikoshi medium supplemented with 1% w/v wheatbran. Carbon sources that favoured xylanase production were rice bran and birch wood xylan. The optimum pH values for xylanase and pectinase were 8.6 and 3.0 respectively at 60°C (Beg *et al.*, 2000).

Charain *et al.* (2002) reported the production of cellulase free xylanase by *Streptomyces sp.* Ab 106 on finely ground cane bagasse at 55°C. The highest xylanase activity (10.6 IU) was obtained after 6 days of fermentation in shaken flask at 100 rpm of pH 7.0.

Andreas *et al.* (2001) reported that most of the xylanase preparations were active at slightly acidic or neutral pH in pulp bleaching.

Gomez *et al.* (1992) reported that optimum temperature for the maximum production of cellulases and xylanases was 31.1°C with *Trichoderma viridie Bt 2169*.

Sibtain *et al.* (2003) reported the maximal cellulase activity at pH 5.5 and temperature of 28°C using different carbon sources with *Trichoderma reesei*.

Rajasekhar *et al.* (1998) isolated four fungal cultures, namely, *Aspergillus niger*, *Penicillium species*, *Aspergillus species* and *Myrothecium roridum* from polluted soil sample and *Aspergillus niger* exhibited the highest cellulolytic activity.

2.6 COMMERCIALY AVAILABLE ENZYMES

Novo Nordisk A/S produced the pulpzyme HA, the first commercially available xylanase from *Trichoderma reesi* to be used in the biobleaching of

wood pulps. They achieved a 20% decrease in kappa number of oxygen delignified birch kraft pulp. Cartazyme (Clariant) improved the brightness of kraft pulps (Garg, 1998).

Cellusoft L liquid enzyme consists of cellulases obtained from *Trichoderma reesei* and it is used for biopolishing of fabrics (Duzhak *et al.*, 2000). Biotouch L is the commercial cellulase for biofinishing (Song *et al.*, 2001).

2.7 OTHER ENZYMES IN PULP INDUSTRY

Montiel *et al.* (2002) had reported that purified mannases from *Trichoderma reesei* has significantly increased the bleachability of pulps without xylanase activity.

Mannases can act with xylanases as biological prebleaching agent for pulp. This treatment leads to higher final brightness values or in the consumption of bleaching chemicals and decreased environmental loadings. (Vikari *et al.*, 1994; Clarke *et al.*, 2000).

Natural and recombinant fungal laccases were produced in *Aspergillus oryzae* and *Aspergillus niger* hosts using the *lacl* gene from *Pycnoporus cinnabarinus*. Treatment of pulp using laccases expressed in *Pycnoporus cinnabarinus* or *Aspergillus niger* achieved a delignification up to 75% whereas recombinant laccase from *Aspergillus oryzae* was not able to delignify pulp (Sigolliot *et al.*, 2004).

Experimental Procedure

3.0 EXPERIMENTAL PROCEDURE

Enzymes have high potential for improving traditional pulp of paper manufacturing processes due to their high specificity and environmental friendliness. So they are used successfully in place of chemicals for bleaching. The present study was aimed to isolate and optimize the conditions for maximum production of xylanases and cellulases from microbial cultures using different substrates.

The experimental procedure pertaining to the study “**Production and Optimization of xylanases and cellulases from fungi and their effects on paper pulp**” is given under the following heading.

3.1 COLLECTION AND PROCESSING OF SOURCE

3.2 ISOLATION OF FUNGI

3.3 IDENTIFICATION OF FUNGI

3.3.1 Morphological observation

3.3.2 Staining

3.4 SUBCULTURE OF IDENTIFIED STRAINS

3.5 SUBSTRATES USED FOR ENZYME PRODUCTION

3.6 CULTURING OF FUNGAL SPECIES FROM THE SOURCE

3.6.1 Extraction of enzymes

3.7 DETERMINATION OF PROTEIN CONTENT

3.8 ENZYME ASSAYS

3.8.1 Xylanase

3.8.2 Cellulase

3.9 OPTIMIZATION OF CULTURE CONDITIONS FOR ENZYME PRODUCTION

3.9.1 Effect of initial medium pH on enzyme production from fungi

3.9.2 Effect of Temperature on enzyme production by fungi

3.10 PURIFICATION OF ENZYME

3.10.1 Ethanol precipitation

3.10.2 Purification by chromatographic method

3.10.2.1 Gel filtration chromatography

3.11 EFFECT OF ENZYMES ON PAPER PULP

3.11.1 Selection of paper pulp sample

3.11.2 Enzyme pretreatment of pulp

3.11.3 Determination of reducing sugar

3.11.4 Determination of carbohydrate

3.11.5 Determination of kappa number

3.11.6 Determination of brightness index.

3.1 COLLECTION AND PROCESSING OF SOURCE

The microbial source used in the present study was soil from agricultural land. The soil sample was serially diluted from 10^{-2} to 10^{-8} and inoculated into Rose Bengal Chloramphenicol media for the isolation of microbes.

3.2 ISOLATION OF FUNGI

Rose Bengal Chloramphenicol agar medium (Appendix I) was used for the isolation of fungi. The serially diluted soil samples were spread plated on Rose Bengal Chloramphenicol medium. The plates were incubated at room temperature for 7 days and observed for colonies.

3.3 IDENTIFICATION OF FUNGI

3.3.1 Morphological observation

Fungal colonies were identified based on their morphological characteristics.

3.3.2 Staining

The fungal colonies were identified by staining with lactophenolcotton blue. The method is explained in Appendix II.

3.4 SUBCULTURING OF IDENTIFIED STRAINS

The fungal strains were subcultured in Rose Bengal Chloramphenicol agar medium and allowed to grow for 7 days at room temperature. The colonies were stored at 4°C until they were used.

3.5 SUBSTRATES USED FOR ENZYME PRODUCTION

Various cheap substrates were used for enzyme production using the isolated fungal species. The fungal species and substrates used for the 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

3.6 CULTURING OF FUNGAL SPECIES FOR ENZYME PRODUCTION

The substrates were taken in 250ml conical flask and adjusted the moisture content to 70%. The flasks were sterilized at 121°C for 30 minutes and allowed to cool. After cooling, the flasks were inoculated with selected fungal colonies and allowed to grow at room temperature for 7 days.

3.6.1 Enzyme extraction

10g of fermented substrates were subjected to mechanical grinding using 50ml of 0.2M phosphate buffer of pH 7.0. The material was filtered through

Whatman filter paper and the filtrate was centrifuged at 10000 g for 30 minutes at 4°C. The supernatant was used for the enzyme assays.

3.7 DETERMINATION OF PROTEIN CONTENT

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

3.8 ENZYME ASSAY

3.8.1 Xylanase assay

Xylanase activity was determined based on the total reducing sugar released which was estimated spectrometrically by the method of Bailey *et al* (1992). One unit of xylanase activity is defined as the amount of enzyme which produces one micromole of xylose per minute under assay conditions per gram of substrate. The procedure is given in Appendix IV.

3.8.2 Cellulase assay

Cellulase activity was determined by spectrophotometric method (Danison and koehn, 1977). One unit of cellulase activity was defined as the amount of enzyme that releases 1mg of reducing sugar equivalent to glucose per minute under the assay conditions. The procedure is elaborated in Appendix V.

3.9 OPTIMIZATION OF CULTURAL CONDITIONS FOR ENZYME PRODUCTION

The effect of initial medium pH and temperature on enzyme production of fungal species in selected substrates were carried out.

3.9.1 Effect of initial medium pH on enzyme production by fungi

Fungal cultures were inoculated into the selected substrates. The initial pH of the moistening agent (phosphate buffer) was adjusted to 4.0, 5.0, 6.0, 7.0 and 8.0 and allowed to grow in room temperature.

3.9.2 Effect of Temperature on enzyme production by fungi

The Effect of temperature on the enzyme activity was studied by varying the temperature such as 17°C, 27°C, 37°C, 47°C & 57°C of the reaction mixture. The procedure is depicted in Appendix VI.

3.10 ENZYME PURIFICATION

Of the four species selected for the study, *Aspergillus niger* and *Fusarium sp.* produced the enzyme with higher activity than *Penicillium sp.* and *Rhizopus sp.* Hence for purification of enzymes, the crude enzyme extract from *Aspergillus niger* and *Fusarium sp.* cultured in all the selected substrates were taken.

3.10.1 Ethanol precipitation

The crude enzyme was partially purified by precipitating with equal volume of chilled ethanol by the method proposed by Keskar *et al.* (1989) which is explained in Appendix VII. The precipitate was dissolved in 15 ml of sodium citrate buffer (pH 5.0) and the activities of xylanase and cellulase were estimated.

3.10.2 Purification by chromatographic method

To further purify the enzyme, the ethanol precipitated sample from *Aspergillus niger* cultured with wheat bran was taken.

3.10.2.1 Gel filtration chromatography

The precipitate was dissolved in citrate buffer and loaded into sephadex G-50 column and equilibrated with 50 mM sodium citrate buffer (pH 5.0). The enzyme was eluted with a linear gradient buffer at a flow rate of 2.0 ml/min. 20 fractions were collected and pooled into 10 tubes by combining subsequent 2 fractions (1+2,3+4,5+6,7+8..... and so on), to get a volume of 4.0 ml to determine enzyme activity. The protein content, activities of xylanase and

cellulase were measured in each of the pooled fractions. The procedure is described in Appendix VIII.

3.11 EFFECT OF ENZYMES ON PAPER PULP

3.11.1 Selection of paper pulp sample

Ink paper pulp and photocopy paper pulp samples were selected for the study. The samples were obtained from Amaravathi Sri Venkateswara paper and board mills Ltd, Swaminathapuram, udumalpet, Tamilnadu.

3.11.2 Treatment of pulp with enzyme

The pulp was treated with 1000 IU of enzyme per gram of the dry pulp sample and was incubated at 50°C for 24 hrs and 48 hrs. After the treatment, the pulp was filtered and the residues were air dried. The filtrate was used to determine the reducing sugar and the carbohydrate content. The residue was used for the preparation of handsheets. The handsheets were used to determine the kappa number and brightness.

3.11.3 Determination of reducing sugar

The filtrate obtained is used for determining reducing sugar by the method of Nelson-somogyi. The method is explained in Appendix IX.

3.11.4 Determination of carbohydrate

The carbohydrate content of the filtrate was determined by the method of Hodge and Hofreiter (1962). The procedure is elaborated in Appendix X.

3.11.5 Determination of kappa number

The kappa number is used as a criteria for the lignin content of pulps. The kappa number is determined as the volume of 0.1N potassium permanganate solution consumed by 1 gram of moisture free residue under the

conditions described in the standard procedure of TAPPI test method. The procedure is described in Appendix XI.

3.11.6 Determination of brightness

Degree of brightness (%) is a measure of the 'whiteness' of the tested paper. Brightness of the sample were measured at 457nm in a spectrophotometer equipped with a reflection sphere.

Results and Discussion

4.0 RESULTS AND DISCUSSION

The production of xylanolytic enzymes has received considerable attention because of their potential applications in the pulp and paper industry. Hence a study was carried out by isolating the fungi from the soil that produces xylanases and cellulases. The isolated fungal species were subcultured with selected substrates and the enzymes were extracted and assayed for their activities and their effect on pulp bleaching was determined.

The results of the study entitled “**Production and Optimization of xylanases and cellulases from fungi and their effects on paper pulp**” are discussed under the following headings:

4.1 ISOLATION AND IDENTIFICATION OF FUNGI FROM THE SOIL

4.2 ACTIVITY OF XYLANASES AND CELLULASES FROM SELECTED FUNGI IN VARIOUS SUBSTRATES

4.3 OPTIMIZATION OF GROWTH CONDITIONS FOR MAXIMUM ENZYME PRODUCTION

4.3.1 Effect of Initial medium pH on xylanase production.

4.3.2 Effect of Temperature on xylanase production.

4.3.3 Effect of Initial medium pH on cellulase production.

4.3.4 Effect of Temperature on cellulase production.

4.4 PURIFICATION OF ENZYMES

4.4.1 Purification by ethanol precipitation.

4.4.2 Purification by gel filtration chromatography using sephadex G-50 column.

4.5 EFFECT OF ENZYMES ON PULP TREATMENT

4.5.1 Effect of enzymes on the release of reducing sugar from pulp samples.

4.5.2 Effect of enzymes on the release of carbohydrate content from pulp samples.

4.5.3 Effect of enzymes on the kappa number of pulp samples.

4.5.4 Effect of enzymes on the brightness (%) of pulp samples.

4.2 ACTIVITY OF XYLANASES AND CELLULASES FROM THE EXTRACTS OF SELECTED FUNGI CULTURED IN VARIOUS SUBSTRATES

All the four isolated fungal species were inoculated in the selected substrates, namely, wheat bran, rice bran and sorghum flour. They were allowed to grow in solid state with initial moisture content of 70% for seven days at room temperature with an initial pH of 7.0 (plate IV).

The protein content and the enzyme activity of the extracts from four fungal cultures in three substrates are given in Table 2.

TABLE 2
PROTEIN CONTENT (mg/ml) AND THE ENZYME ACTIVITY (U mg⁻¹) FROM THE EXTRACTS OF SELECTED FUNGI CULTURED IN VARIOUS SUBSTRATES

Substrates	Enzyme	Fungal species			
		<i>Aspergillus niger</i>	<i>Penicillium Sp.</i>	<i>Fusarium sp.</i>	<i>Rhizopus sp.</i>
Wheat bran	Protein	5.816	4.302	11.872	5.923
	Xylanase	0.080	0.009	0.050	0.029
	Cellulase	0.041	0.011	0.019	0.009
Rice bran	Protein	1.734	1.997	1.568	1.760
	Xylanase	0.024	0.024	0.045	0.042
	Cellulase	0.036	0.006	0.037	0.018
Sorghum flour	Protein	4.393	0.925	8.024	12.604
	Xylanase	0.023	0.010	0.089	0.009
	Cellulase	0.035	0.005	0.075	0.002

It is observed from Table 2 and Fig. 2 that *Fusarium sp.* produced the highest xylanase activity (0.089 U mg⁻¹) in sorghum flour followed by *Aspergillus niger* (0.080 U mg⁻¹) in wheat bran when compared to *Penicillium sp.* and *Rhizopus sp.*

Fusarium sp. favoured the maximum cellulase activity (0.075 U mg⁻¹) in sorghum flour. The next highest activity (0.041 U mg⁻¹) of the cellulase was found in wheat bran cultured with *Aspergillus niger* (Fig.3).

FIGURE 2
ACTIVITY OF XYLANASES ($U\ mg^{-1}$) FROM FUNGAL ISOLATES
WITH SELECTED SUBSTRATES

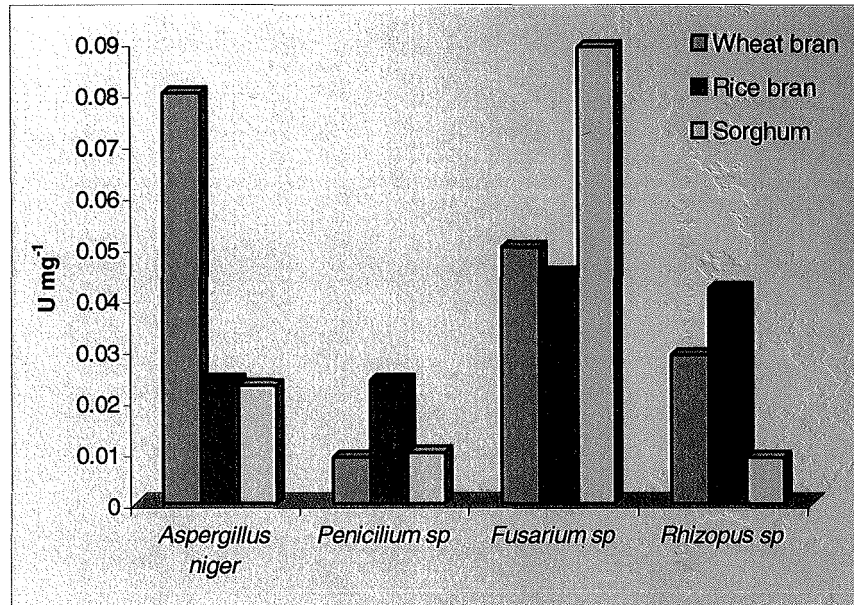
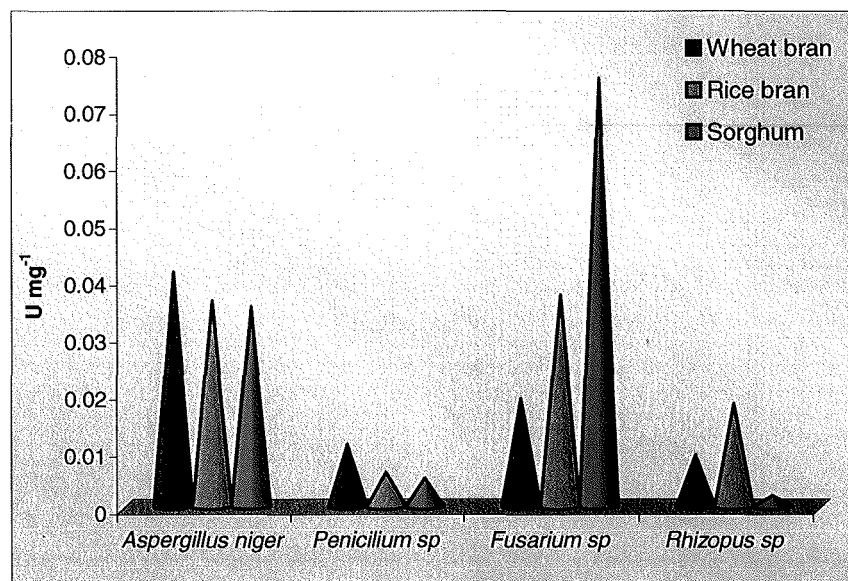


FIGURE 3
ACTIVITY OF CELLULASES ($U\ mg^{-1}$) FROM FUNGAL ISOLATES
WITH SELECTED SUBSTRATES



A high yield of xylanase with wheat bran in solid state fermentation with *Aspergillus ochraceus* was reported by Biswas *et al.*, 1998.

4.3 OPTIMIZATION OF GROWTH CONDITIONS FOR MAXIMUM ENZYME PRODUCTION

To determine the optimum growth conditions for maximum enzyme production, the activity of the enzymes were assayed after an incubation period of 7 days in varying initial medium pH (4.0-8.0) and temperature (17°C, 27°C, 37°C, 47°C and 57°C) with selected substrates and fungal species.

4.3.1 Effect of Initial medium pH on xylanase production

Table 3 and Fig. 4 describe the effect of various initial medium pH on xylanase production at room temperature.

TABLE 3
EFFECT OF INITIAL MEDIUM pH ON XYLANASE PRODUCTION FROM
SELECTED SPECIES WITH SELECTED SUBSTRATES

Initial medium pH	Xylanase activity (U mg ⁻¹)		
	Substrates		
	Wheat bran	Rice bran	Sorghum flour
<i>Aspergillus niger</i>			
4.0	0.083	0.020	0.025
5.0	0.115	0.048	0.033
6.0	0.110	0.030	0.040
7.0	0.080	0.024	0.023
8.0	0.050	0.021	0.015
<i>Fusarium sp.</i>			
4.0	0.045	0.041	0.051
5.0	0.072	0.052	0.071
6.0	0.060	0.061	0.076
7.0	0.050	0.045	0.089
8.0	0.026	0.032	0.013
<i>Penicillium sp.</i>			
4.0	0.014	0.017	0.006
5.0	0.018	0.025	0.009
6.0	0.012	0.033	0.011
7.0	0.009	0.024	0.010
8.0	0.035	0.017	0.008
<i>Rhizopus sp.</i>			
4.0	0.020	0.010	0.004
5.0	0.015	0.024	0.001
6.0	0.018	0.027	0.007
7.0	0.029	0.042	0.009
8.0	0.023	0.029	0.005

The optimum initial medium pH for the production of xylanase from *Aspergillus niger* was found to be 5.0. Xylanases produced by *Aspergillus niger* in wheat bran was higher (0.115 U mg⁻¹) when compared with the production of the enzyme in rice bran and sorghum flour (Fig.4a). Krisana *et al.*(2005) reported an optimum pH of 5.0 for xylanase production from *Aspergillus niger* BCC 14405 in wheat straw. Carmona *et al.* (2004) observed from *Aspergillus versicolor*, an optimum pH of 6.5 for xylanase production using wheat bran as carbon source.

Fusarium sp. exhibited the optimum initial medium pH for xylanase production as 7.0 (0.089 U mg⁻¹) in sorghum flour whereas, in wheat bran the optimum initial medium pH (0.072 U mg⁻¹) was 5.0 and in rice bran it was 6.0 (0.061 U mg⁻¹).

Saha (2001) isolated and purified xylanase from *Fusarium verticillioides* (NRRL 26518) by screening soil samples using corn fiber xylan as a carbon source. The optimum pH and temperature was found to be 5.0 and 50°C respectively.

The optimum initial medium pH for maximum xylanase production (0.035 U mg⁻¹) from *Penicillium sp.* in wheatbran was 8.0. However, the maximum xylanase production for rice bran was found to be at pH 6.0 (0.033 U mg⁻¹) and pH 8.0 (0.011 U mg⁻¹) for sorghum flour (Fig.4c).

The optimum initial medium pH in *Rhizopus sp.* for the highest xylanase activity was 7.0 (0.042 U mg⁻¹) in rice bran (Fig.4d).

The production of xylanase from *Penicillium janthinellum* 87m - 115 from different agricultural wastes showed that corncob (55.3 U/ml) and oat husk (54.8 U/ml) were the best inducers of xylanase at neutral pH (Oliveria *et al.*, 2004). Ryan *et al.* (2003) had observed the optimum pH for xylanase as 3.8 for *Penicillium capsulatum*.

FIGURE 4

EFFECT OF INITIAL MEDIUM pH ON XYLANASE PRODUCTION FROM SELECTED SPECIES WITH SELECTED SUBSTRATES

Fig. 4a. *Aspergillus niger*

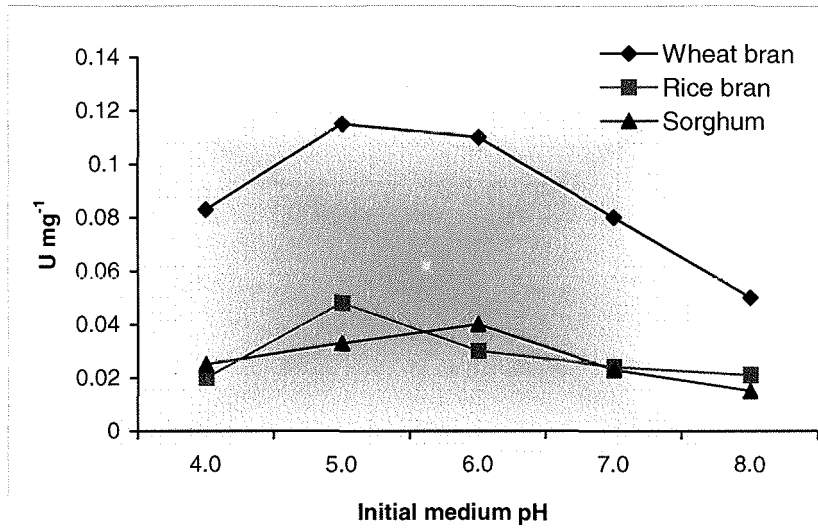
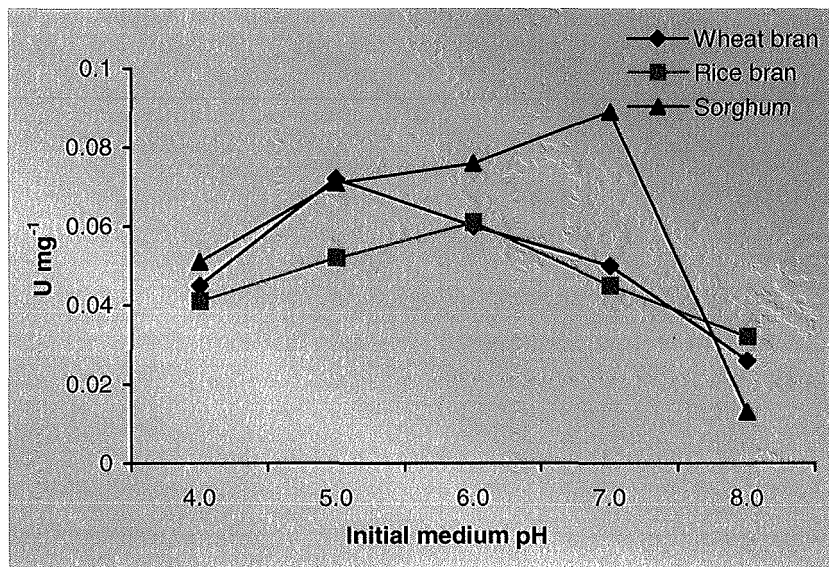


Fig. 4b. *Fusarium sp.*



EFFECT OF INITIAL MEDIUM pH ON XYLANASE PRODUCTION FROM SELECTED SPECIES WITH SELECTED SUBSTRATES

Fig. 4c. *Penicillium sp.*

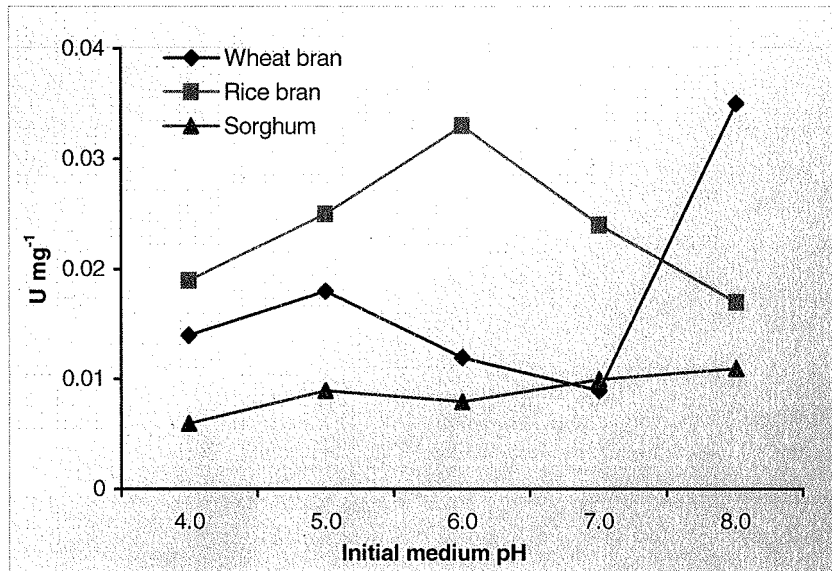
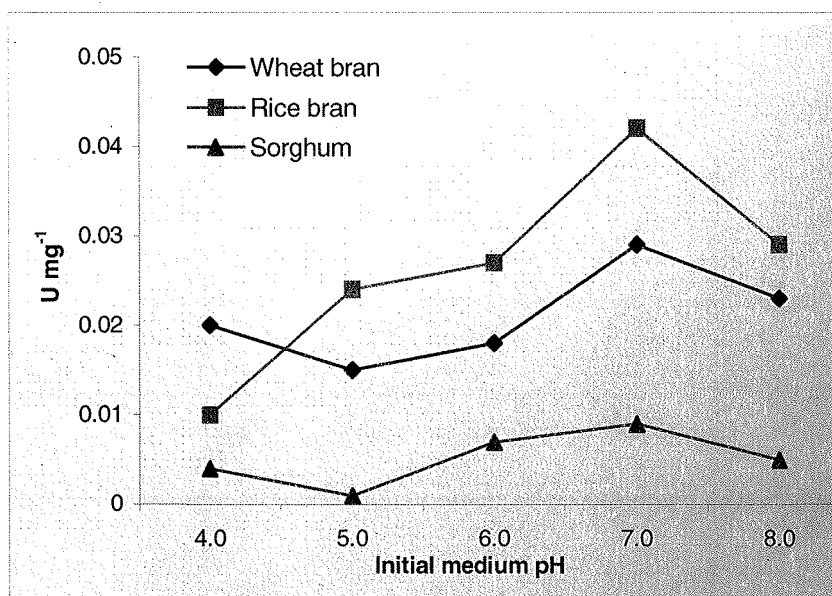


Fig.4d. *Rhizopus sp.*



Among the microbial isolates, the optimum initial medium pH for xylanase production was found to range between pH 4.0 and pH 8.0 depending upon the selected species and substrates. It can be inferred that of the four species and three substrates, *Aspergillus niger* with wheat bran was found to be suitable for high xylanase production at pH 5.0.

4.3.2 Effect of Temperature on xylanase production

Table 4 and Fig. 5 reveal the effect of temperature on xylanase production by selected species using various substrates at pH 7.0.

TABLE 4
EFFECT OF TEMPERATURE ON XYLANASE PRODUCTION FROM
SELECTED SPECIES WITH SELECTED SUBSTRATES

Incubation Temperature (°C)	Xylanase activity (U mg ⁻¹)		
	Substrates		
	Wheat bran	Rice bran	Sorghum flour
<i>Aspergillus niger</i>			
17	0.020	0.015	0.011
27	0.759	0.019	0.199
37	0.089	0.030	0.087
47	0.027	0.011	0.021
57	0.021	0.006	0.010
<i>Fusarium sp.</i>			
17	0.017	0.015	0.025
27	0.020	0.021	0.029
37	0.038	0.046	0.060
47	0.063	0.059	0.119
57	0.006	0.041	0.060
<i>Penicillium sp.</i>			
17	0.004	0.014	0.022
27	0.027	0.061	0.014
37	0.029	0.129	0.040
47	0.047	0.026	0.055
57	0.076	0.027	0.123
<i>Rhizopus sp.</i>			
17	0.005	0.026	0.033
27	0.061	0.080	0.017
37	0.041	0.099	0.064
47	0.038	0.021	0.035
57	0.020	0.021	0.024

FIGURE 5

EFFECT OF TEMPERATURE ON XYLANASE PRODUCTION FROM SELECTED SPECIES WITH SELECTED SUBSTRATES

Fig.5a. *Aspergillus niger*

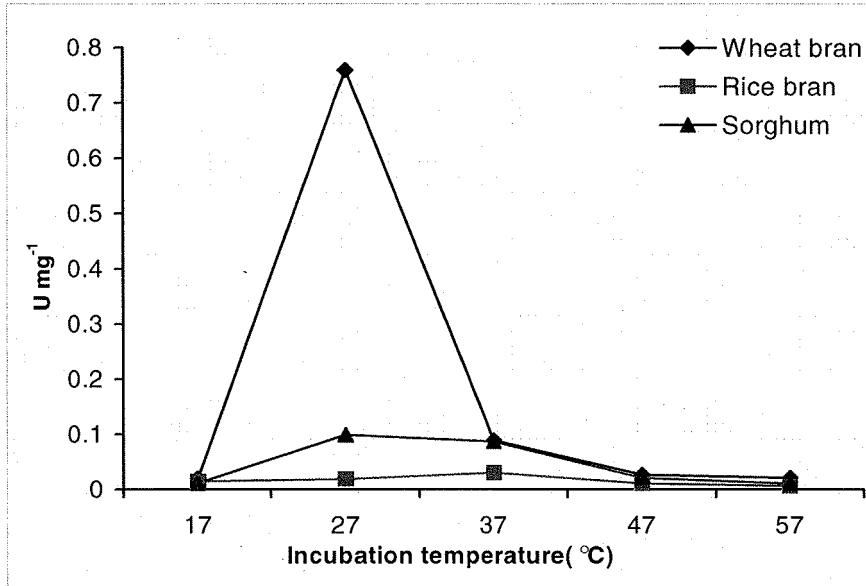
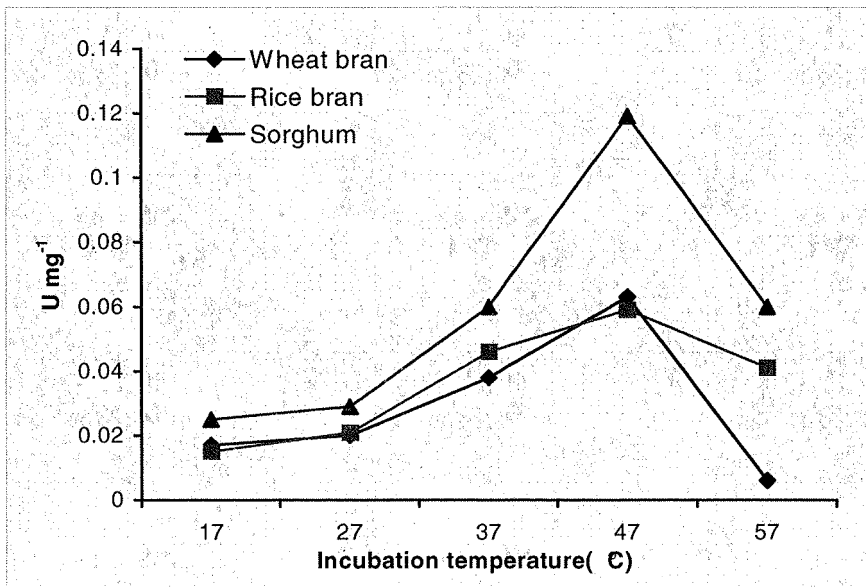


Fig. 5b. *Fusarium sp.*



**EFFECT OF TEMPERATURE ON XYLANASE PRODUCTION FROM
SELECTED SPECIES WITH SELECTED SUBSTRATES .**

Fig.5c. *Penicillium sp.*

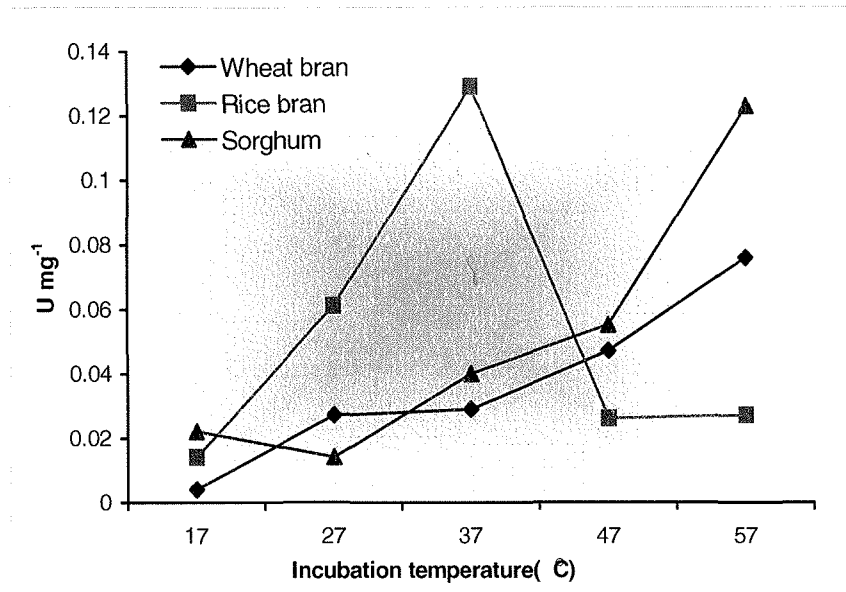
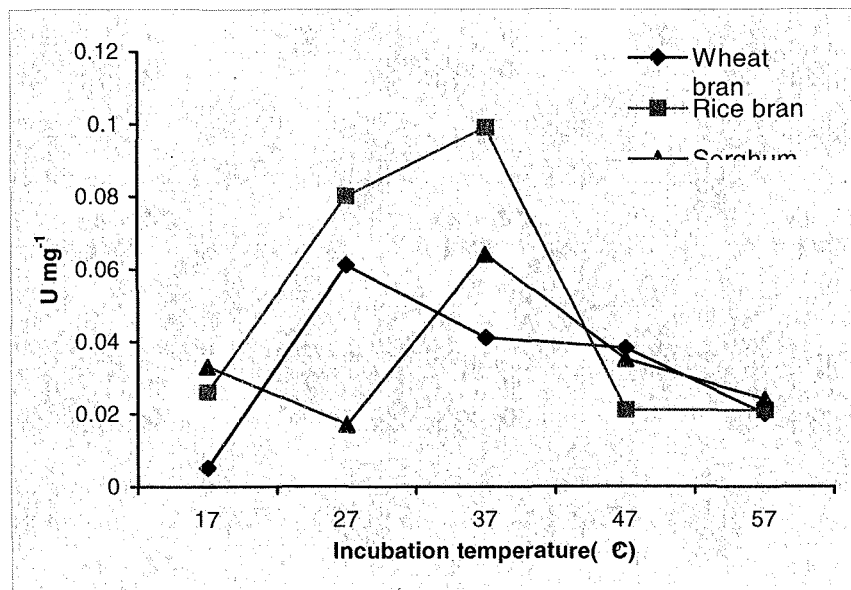


Fig.5d. *Rhizopus sp.*



The optimum temperature for the maximum production of xylanase from *Aspergillus niger* was found to be 27°C with wheat bran (0.759 U mg⁻¹) and sorghum flour (0.199 U mg⁻¹), whereas, for rice bran the optimum temperature was 37°C (0.030 U mg⁻¹).

According to Lenastovicz *et al.* (2005) the production of multiple xylanases (isoenzymes I, II and III) from *Aspergillus fumigatus* was greatly increased at 30°C, the growth temperature.

It is noted from Table 4, the optimum temperature for maximum xylanase production from *Fusarium sp.* was 47°C with all the selected substrates. Jorge *et al.* (2005) had shown that the optimum temperature as 55°C at pH 5.0 for the purified extracellular xylanases from two pathogenic races of *Fusarium oxysporum f.sp ciceris*.

The high xylanase production (0.129 U mg⁻¹) from *Penicillium sp.* was found to be at an optimum temperature of 37°C in rice bran, whereas, the optimum temperature for wheat bran (0.076 U mg⁻¹) and sorghum flour (0.123 U mg⁻¹) was found to be 57°C (Fig.5b).

It is observed from Table 4 that the optimum temperature for xylanase production from *Rhizopus sp.* was found to be 37°C with rice bran and sorghum flour. However, in wheat bran the xylanase production was optimum at 57°C (Fig.5c).

The new xylanase (pg xynA) from *Penicillium griseofulvum* A160 showed an optimum temperature of 50°C at pH 5.0 (Johniaux *et al.*, 2005). Adsul *et al.* (2004) showed in their study that xylanase production from *Penicillium jathinellum* was high in bagasse sample at 37°C.

The results showed a variation in the optimum temperature between 27°C and 57°C with selected fungi and selected substrates. The highest xylanase production (0.759 U mg⁻¹) was noticed at 27°C from *Aspergillus niger* with wheat bran.

The present study is comparable with the results of Kheng and Ibrahim (2005) who observed that the production of xylanase was maximum at the ambient temperature (28 ± 3°C).

4.3.3 Effect of Initial medium pH on cellulase production

The effect of initial medium pH on cellulase production at room temperature is shown in Table 5 and Fig. 6.

TABLE 5
EFFECT OF INITIAL MEDIUM pH ON CELLULASE PRODUCTION FROM SELECTED SPECIES WITH SELECTED SUBSTRATES

Initial medium pH	Cellulase activity (U mg ⁻¹)		
	Substrates		
	Wheat bran	Rice bran	Sorghum flour
<i>Aspergillus niger</i>			
4.0	0.058	0.026	0.032
5.0	0.068	0.049	0.046
6.0	0.086	0.069	0.034
7.0	0.051	0.036	0.035
8.0	0.046	0.020	0.022
<i>Fusarium sp.</i>			
4.0	0.015	0.025	0.057
5.0	0.016	0.029	0.021
6.0	0.018	0.038	0.080
7.0	0.019	0.037	0.075
8.0	0.012	0.035	0.031
<i>Penicillium sp.</i>			
4.0	0.006	0.009	0.006
5.0	0.006	0.011	0.007
6.0	0.006	0.012	0.004
7.0	0.011	0.006	0.005
8.0	0.004	0.005	0.003
<i>Rhizopus sp.</i>			
4.0	0.001	0.014	0.001
5.0	0.003	0.008	0.001
6.0	0.005	0.012	0.001
7.0	0.004	0.018	0.002
8.0	0.004	0.004	0.001

FIGURE 6

EFFECT OF INITIAL MEDIUM pH ON CELLULASE PRODUCTION FROM SELECTED SPECIES WITH SELECTED SUBSTRATES

Fig. 6a. *Aspergillus niger*

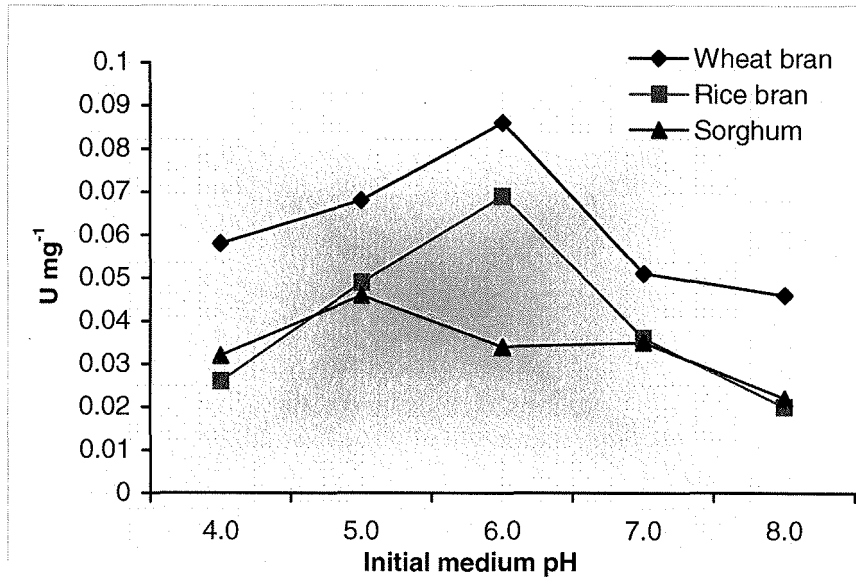
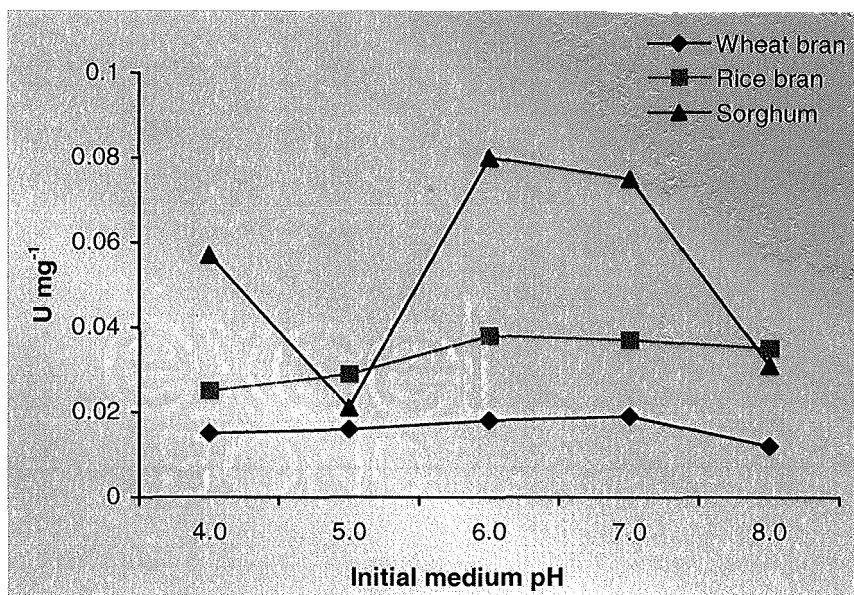


Fig. 6b. *Fusarium sp.*



EFFECT OF INITIAL MEDIUM pH ON CELLULASE PRODUCTION FROM SELECTED SPECIES WITH SELECTED SUBSTRATES

Fig. 6c. *Penicillium sp.*

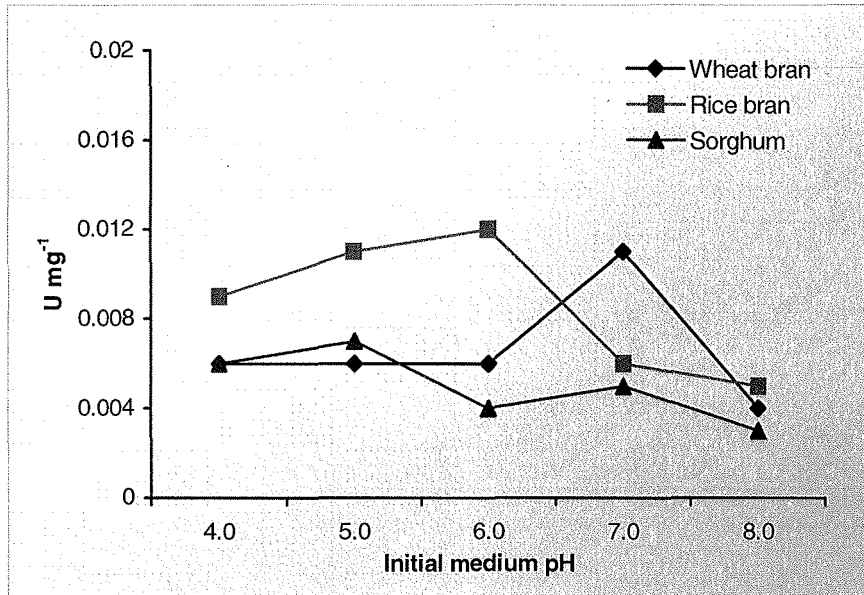
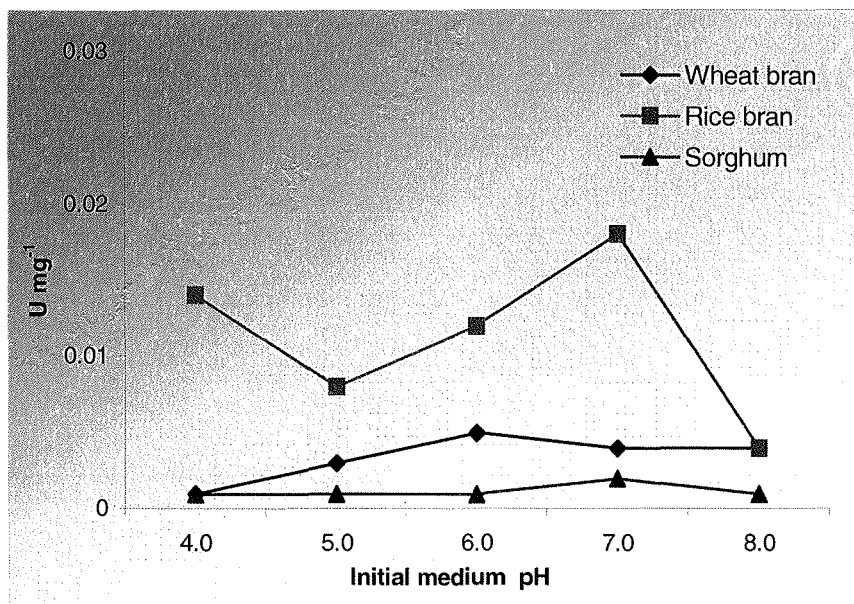


Fig. 6d. *Rhizopus sp.*



It is noted from Table 5 and Fig.6 *Aspergillus niger* showed the maximum cellulase activity (0.086 U mg^{-1}) in wheat bran at an optimum initial medium pH 6.0 whereas, in rice bran and sorghum flour, the optimum initial medium pH was 6.0 (0.096 U mg^{-1}) and 5.0 (0.046 U mg^{-1}) respectively (Fig.6a).

Bagasse, corncob and sawdust were used as lignocellulosic substrates for the production of cellulase enzyme using *Aspergillus flavus* by Ojumu *et al.* (2003) who had reported that sawdust was the best substrate with an enzyme activity of 0.073 U mg^{-1} .

The optimum initial medium pH for peak cellulase production from *Fusarium sp.* was 6.0 in sorghum flour (0.080 U mg^{-1}) and rice bran (0.038 U mg^{-1}). However, it was pH7.0 (0.019 U mg^{-1}) for wheat bran.

The high cellulase production (0.012 U mg^{-1}) from *Penicillium sp.* was found at an initial medium pH of 6.0 in rice bran, pH 7.0 in wheat bran (0.011 U mg^{-1}) and pH 5.0 in sorghum flour. The activity in sorghum flour was lower than that produced in rice bran and wheat bran (Fig.6c).

Rhizopus sp. produced the maximum cellulase activity in ricebran (0.018 U mg^{-1}) at an optimum initial medium pH of 7. The cellulase activity was much lower in wheat bran (0.005 U mg^{-1}) and sorghum flour (0.002 U mg^{-1}) with an optimum initial medium pH of 6 and 7 respectively when compared with the enzyme activity in rice bran.

Antony *et al.*(2004) reported the optimum conditions for cellulase activity as pH 4.8 and temperature 50°C for *Trichoderma reesi* and *Tricoderma hazarium*.

Kotchoni *et al.* (2003) observed that cellulase yield by *Bacillus pumilus* (BpCRI 6) was four times higher than wild type under optimum growth conditions of pH 6.5 and temperature 25°C .

4.3.4 Effect of Temperature on cellulase production.

The effect of Temperature on cellulase production at pH 7.0 are given in Table 6 and Fig. 7.

TABLE 6
EFFECT OF TEMPERATURE ON CELLULASE PRODUCTION FROM
SELECTED SPECIES WITH SELECTED SUBSTRATES

Incubation temperature (°C)	Cellulase activity (U mg ⁻¹)		
	Substrates		
	Wheat bran	Rice bran	Sorghum flour
<i>Aspergillus niger</i>			
17	0.059	0.065	0.019
27	0.385	0.253	0.196
37	0.398	0.214	0.157
47	0.107	0.137	0.088
57	0.093	0.074	0.032
<i>Fusarium sp.</i>			
17	0.063	0.017	0.020
27	0.069	0.063	0.036
37	0.093	0.082	0.064
47	0.126	0.098	0.079
57	0.016	0.075	0.042
<i>Penicillium sp.</i>			
17	0.010	0.018	0.009
27	0.012	0.013	0.012
37	0.014	0.034	0.043
47	0.008	0.020	0.025
57	0.017	0.015	0.024
<i>Rhizopus sp.</i>			
17	0.010	0.022	0.008
27	0.012	0.013	0.009
37	0.023	0.014	0.011
47	0.021	0.014	0.008
57	0.017	0.013	0.003

The optimum temperature for the production of cellulase from *Aspergillus niger* with wheat bran (0.398 U mg⁻¹) was found to be 37°C, whereas, for rice bran (0.253 U mg⁻¹) and sorghum flour (0.196 U mg⁻¹) the optimum temperature was found to be 27°C (Fig.7a).

FIGURE 7

EFFECT OF TEMPERATURE ON CELLULASE PRODUCTION FROM
SELECTED SPECIES WITH SELECTED SUBSTRATES

Fig. 7a. *Aspergillus niger*

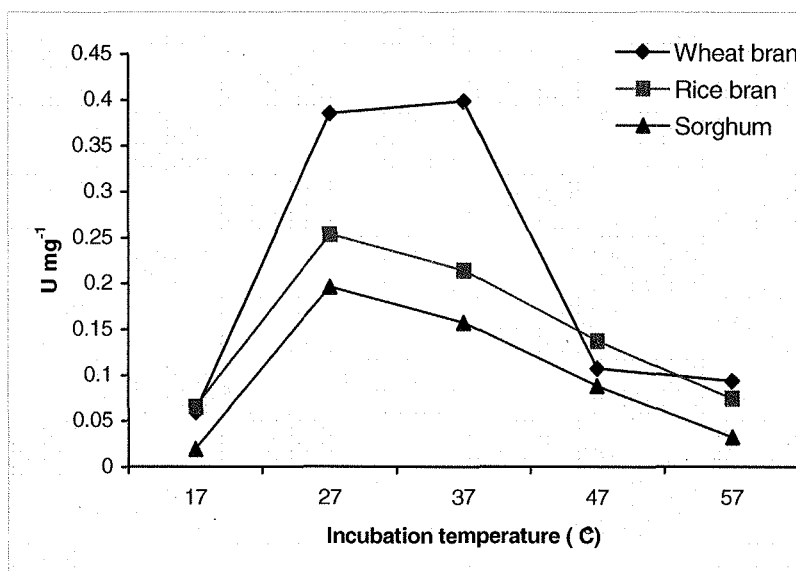
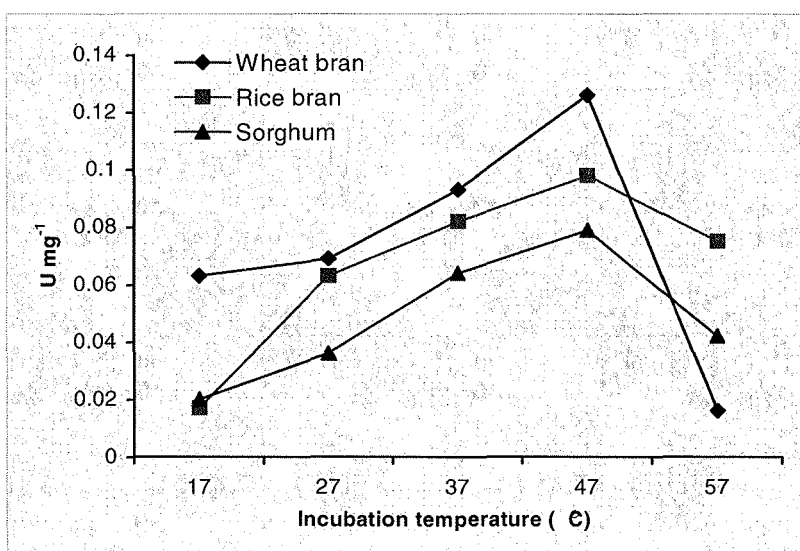


Fig. 7b. *Fusarium sp.*



EFFECT OF TEMPERATURE ON CELLULASE PRODUCTION FROM SELECTED SPECIES WITH SELECTED SUBSTRATES

Fig.7c. Penicillium sp.

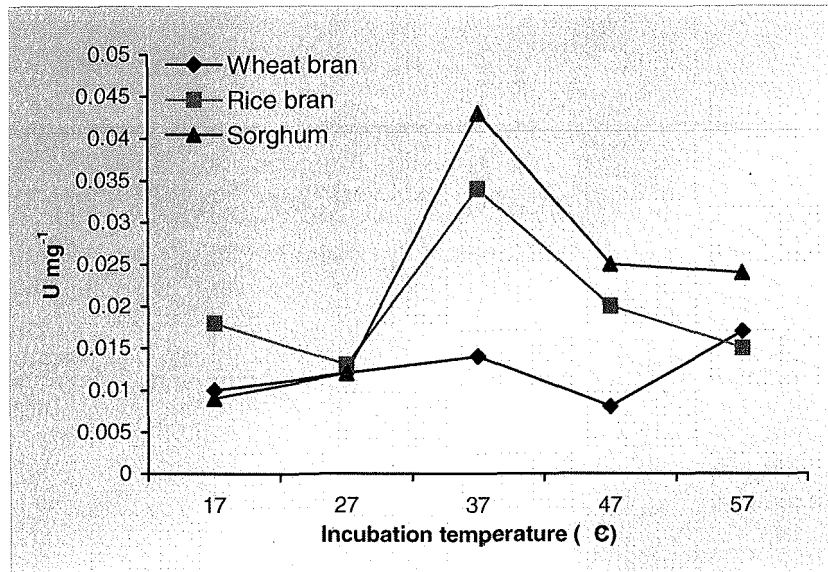
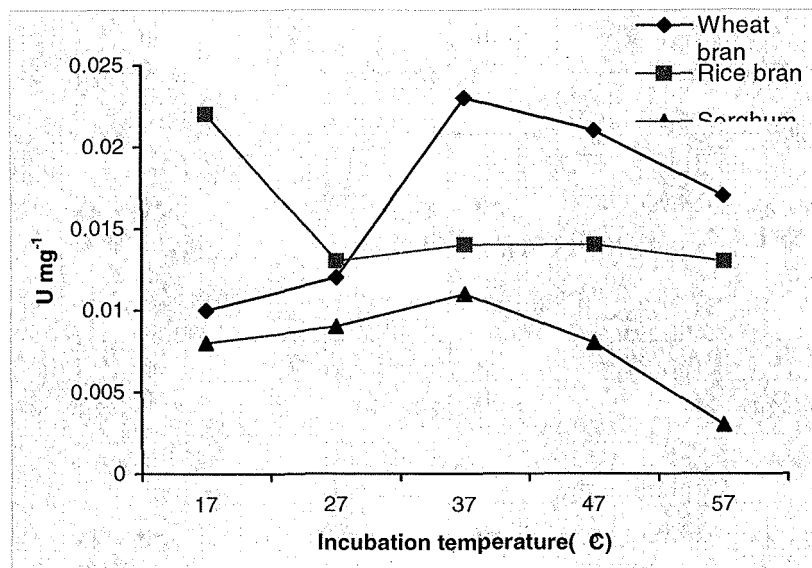


Fig.7d. Rhizopus sp.



The increased activity of cellulase can be obtained by cultivating *Aspergillus flavus* in solid state fermentation at room temperature (Nigam, 1996).

The production of cellulase from *Fusarium sp.* was optimum at 47°C with all the selected substrates (Fig.7b).

The optimum temperature for cellulase production from *Penicillium sp.* was found to be 37°C with all the selected substrates (Fig. 7c).

The optimum temperature for cellulase production from *Rhizopus sp.* was 47°C with wheatbran (0.021 U mg⁻¹) and rice bran (0.014 U mg⁻¹), whereas, it was 37°C with sorghum flour (0.011 U mg⁻¹).

Chaabouni *et al.* (2005) studied the two endoglucanases (EGA and EGB) purified from *Penicillium occitanis* and showed the optimal temperature of 60°C and 50°C respectively for EGA and EGB.

Of all the fungal isolates and selected substrates, the highest cellulase activity was observed from *Aspergillus niger* at 37°C (0.398 U mg⁻¹) with wheat bran.

4.4 PURIFICATION OF ENZYMES

Of the four species selected for the study, *Aspergillus niger* and *Fusarium sp.* produced the enzyme with higher activity than *Penicillium sp.* and *Rhizopus sp.* Hence for purification of enzymes, the crude enzyme extract from *Aspergillus niger* and *Fusarium sp.* cultured in all the selected substrates were taken. The crude enzyme extracts were precipitated with equal volumes of chilled ethanol and the precipitate was dissolved in 15 ml of sodium citrate buffer (pH 5.0) and the activities of xylanases and cellulases were estimated.

4.4.1 Purification by ethanol precipitation.

The activities of xylanase and cellulase after ethanol precipitation are given in Table 7 and Figs.8 and 9.

TABLE 7
SPECIFIC ACTIVITY OF XYLANASE AND CELLULASE (U mg⁻¹) AFTER ETHANOL PRECIPITATION

Substrates	Step fractions	<i>Aspergillus niger</i>		<i>Fusarium sp</i>	
		Xylanase	Cellulase	Xylanase	Cellulase
Wheat bran	Crude extract	0.080	0.041	0.050	0.019
	Ethanol precipitation	0.11	0.037	0.048	0.023
	Purification fold	1.4	-	-	1.2
Rice bran	Crude extract	0.024	0.036	0.045	0.037
	Ethanol precipitation	0.032	0.030	0.052	0.029
	Purification fold	1.3	-	1.1	-
Sorghum flour	Crude extract	0.023	0.035	0.069	0.075
	Ethanol precipitation	0.032	0.037	0.073	0.042
	Purification fold	1.3	1.0	1.05	-

Ethanol precipitation has increased the specific activity of xylanase. The xylanase activity from *Aspergillus niger* cultured in wheat bran exhibited highest activity (0.11 U mg⁻¹) with 1.4 fold increase when compared with the activity of the enzyme from other substrates and from *Fusarium sp.* (Fig.8). In general, cellulase activity was decreased after ethanol precipitation (Fig.9).

FIGURE 8

SPECIFIC ACTIVITY OF ENZYMES AFTER ETHANOL PRECIPITATION
FROM *Aspergillus niger*

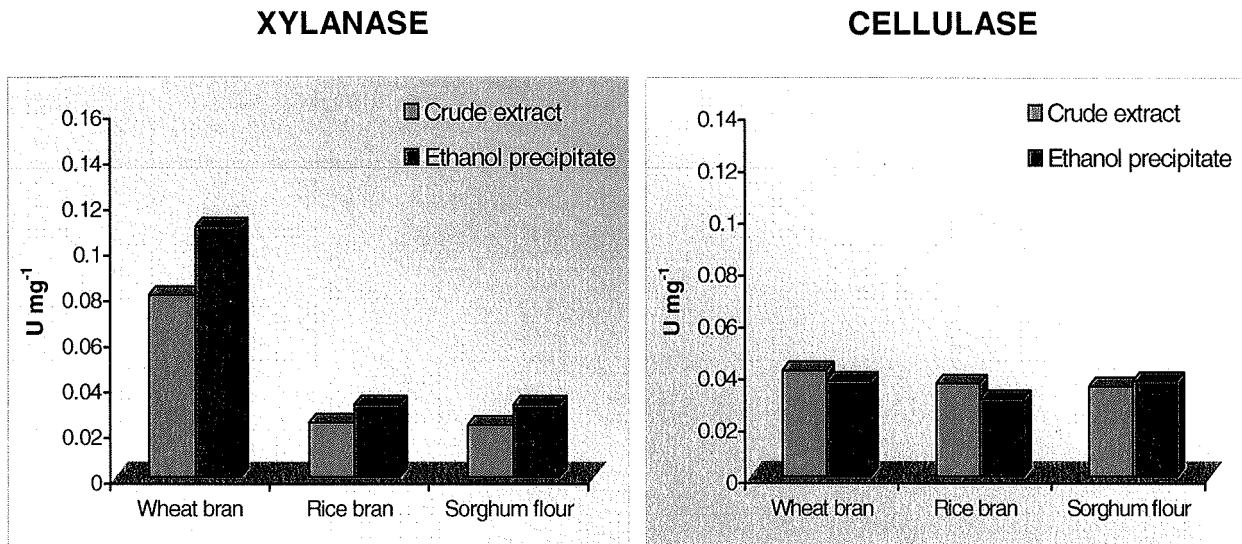
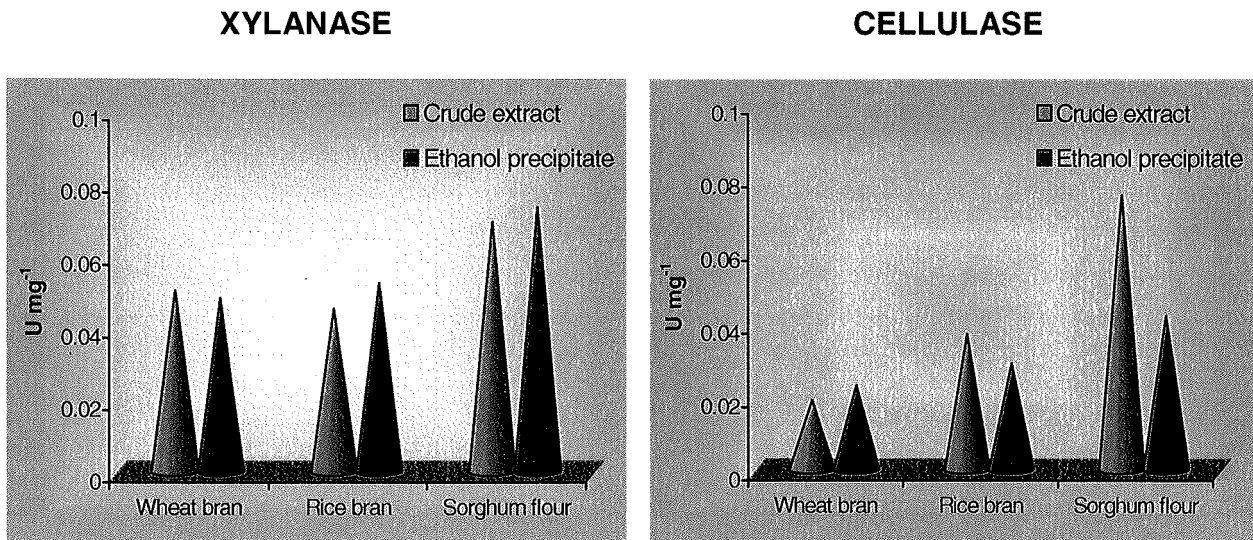


FIGURE 9

SPECIFIC ACTIVITY OF ENZYMES AFTER ETHANOL PRECIPITATION
FROM *Fusarium sp.*



The cellulase activity was lower than the xylanase activity in all the samples.

This is in agreement with the studies of Fadel (2001) who had shown that 80% of cold ethanol precipitates 97% of total xylanase activity with low cellulase activity.

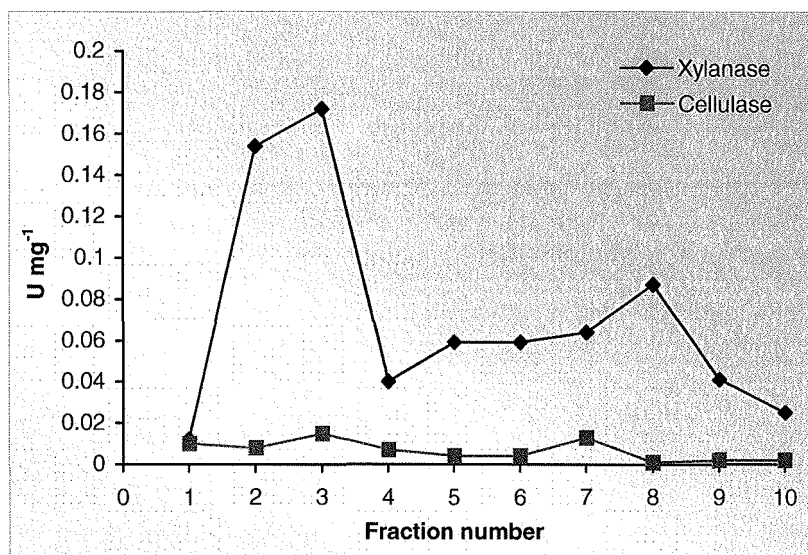
4.4.2 Purification by gelfiltration chromatography using sephadex G-50 column

The ethanol precipitated sample from *Aspergillus niger* cultured with wheatbran was applied on sephadex G-50 column. The enzyme was eluted with a linear gradient buffer at a flow rate of 2.0 ml/min. 20 fractions were collected and pooled into 10 tubes by combining subsequent 2 fractions (1+2,3+4,5+6,7+8..... and so on), to get a volume of 4.0 ml to determine enzyme activity. The protein content, activities of xylanase and cellulase were measured in each of the pooled fractions and the results are depicted in Table 8 and Figure 10.

TABLE 8
PROTEIN CONTENT AND ENZYME ACTIVITY OF DIFFERENT FRACTIONS FROM SEPHADEX G-50 COLUMN

Fraction No.	Protein mg/ml	Xylanase activity (U mg ⁻¹)	Cellulase activity (U mg ⁻¹)
1	5.959	0.012	0.010
2	0.853	0.154	0.008
3	0.763	0.172	0.015
4	2.898	0.040	0.007
5	1.601	0.059	0.004
6	3.276	0.059	0.004
7	2.745	0.064	0.013
8	0.965	0.087	0.001
9	1.787	0.041	0.002
10	0.782	0.025	0.002

FIGURE 10
ENZYME ACTIVITY OF DIFFERENT FRACTIONS FROM
SEPHADEX G-50 COLUMN



The highest xylanase activity was shown by the fraction 3 followed by fraction 2. The cellulase activity was also maximum in fraction 3. However, the xylanase activity was 11.4 times higher than cellulase activity.

4.5 EFFECT OF ENZYMES ON PULP TREATMENT

Treatment of pulp with enzyme preparation, at an early stage of pulp bleaching reduces the amount of the chlorine chemicals required for subsequent bleaching and enhanced brightness (Gaosheng *et al.*, 2003). Enzymes have been successfully used in paper industry for increasing the brightness of the pulp and in the development of totally chlorine free bleaching sequence.

The crude extract from fungal species namely *Aspergillus niger* and *Fusarium sp.* cultured in wheat bran, rice bran and sorghum flour were partially purified by ethanol precipitation. The ethanol precipitated enzyme dissolved in citrate buffer was used to treat the pulp samples.

Two types of pulp samples, namely, ink paper pulp and photocopy paper pulp were treated with 1000 IU of enzyme per gram of dry pulp. Control samples were also treated similarly without the enzyme. After incubation with enzyme, the pulp was filtered and the supernatant was assayed for reducing sugar and carbohydrate content. The residue was used for the preparation of handsheets and the brightness and kappa number of the hand sheets were determined.

4.5.1 Effect of enzymes on the release of reducing sugar from pulp samples

The percentage of reducing sugar released from the pulp samples are presented in Table 9.

TABLE 9
EFFECT OF ENZYME TREATMENT ON THE RELEASE OF REDUCING SUGAR FROM PULP SAMPLES

Samples	Reducing sugar			
	Ink paper pulp		Photocopy paper pulp	
	24 hrs	48 hrs	24 hrs	48 hrs
Control	1.51	1.34	3.14	2.58
Sample I				
Wheat bran	3.10	2.82	2.63	3.51
Rice bran	1.70	1.45	2.24	1.54
Sorghum flour	3.10	2.25	1.49	2.40
Sample II				
Wheat bran	2.02	1.51	1.89	1.51
Rice bran	1.86	1.31	1.54	1.37
Sorghum flour	2.32	1.51	1.42	1.14

Sample I : The pulp treated with the enzyme from *Aspergillus niger*

Sample II : The pulp treated with the enzyme from *Fusarium sp.*

Control : The pulp treated with buffer without the enzyme.

The percentage of reducing sugar increased after enzyme treatment when compared with control. Among the ink paper pulp samples, the samples treated with the enzyme for 24 hrs released higher amount of reducing sugar than the samples treated with 48 hrs.

The enzyme from wheat bran and sorghum flour inoculated with *Aspergillus niger* released a higher amount of reducing sugar when compared with the enzyme obtained from rice bran. The release of reducing sugar by the enzyme from *Fusarium sp.* inoculated in different substrates was found to be lower than that released by the enzyme from *Aspergillus niger*.

The photocopy paper pulp samples treated with the enzyme from *Aspergillus niger* and *Fusarium sp.* inoculated with various substrates for 24 hrs and 48 hrs exhibited a reduction in the release of reducing sugar when compared with the control sample except the enzyme from wheat bran inoculated with *Aspergillus niger*, which released the highest amount of reducing sugar in 48 hrs.

The results correlated with the observations of Archana and Satyanarayan (2003) who had reported that reducing sugars released by enzyme cultured with wheat bran was significantly higher than the other selected substrates.

4.5.2 Effect of enzyme on the release of Carbohydrate from the pulp samples

The amount of carbohydrate released from the pulp samples after treatment was given in Table 10.

There was an over all reduction in carbohydrate content in the enzyme treated ink paper pulp and photocopy paper pulp with few exceptions. The samples treated with the enzyme from wheat bran showed an increased release of carbohydrate than the control.

TABLE 10

EFFECT OF ENZYME TREATMENT ON THE RELEASE OF CARBOHYDRATE CONTENT FROM PULP SAMPLES

Samples	Carbohydrate content			
	Ink paper pulp		Photocopy paper pulp	
	24 hrs	48 hrs	24 hrs	48 hrs
Control	4.82	4.50	4.01	3.98
Sample I				
Wheat bran	5.01	4.98	4.33	4.72
Rice bran	2.70	2.45	2.86	2.31
Sorghum flour	3.10	2.70	2.51	2.32
Sample II				
Wheat bran	3.95	4.17	3.69	3.49
Rice bran	3.83	2.84	3.52	3.52
Sorghum flour	3.72	3.54	3.49	3.34

Sample I : The pulp treated with the enzyme from *Aspergillus niger*

Sample II : The pulp treated with the enzyme from *Fusarium sp.*

Control : The pulp treated with buffer without the enzyme

4.5.3 Effect of enzyme treatment on kappa number of pulp samples

The enzyme treated pulps were analysed for kappa number using the TAPPI test method (1998). The kappa number of pulp samples treated with the enzyme preparation are presented in Table 11. kappa number decreased in enzyme treated pulps. The reduction in the kappa number is an indicative of delignification and bleachability of pulp samples.

The ink paper pulp and photocopy paper pulp treated for 48 hrs with the enzyme from *Aspergillus niger* cultured with wheat bran showed the highest decrease in kappa number when compared with other samples. The reduction in kappa number indicates the improvement in the bleachability of pulp samples.

TABLE 11
EFFECT OF ENZYME TREATMENT ON KAPPA NUMBER
OF PULP SAMPLES

Samples	kappa number			
	Ink paper pulp		Photocopy paper pulp	
	24 hrs	48 hrs	24 hrs	48 hrs
Control	3.12	2.53	3.41	2.89
Sample I				
Wheat bran	1.46	1.50	1.53	1.26
Rice bran	1.70	1.52	1.54	1.74
Sorghum flour	2.18	2.06	2.15	1.40
Sample II				
Wheat bran	1.12	1.04	1.96	1.88
Rice bran	1.82	1.54	1.10	1.12
Sorghum flour	1.96	1.16	1.50	1.32

Sample I : The pulp treated with the enzyme from *Aspergillus niger*

Sample II : The pulp treated with the enzyme from *Fusarium sp.*

Control : The pulp treated with buffer without the enzyme.

The findings of the present study are in agreement with the report of Madlala *et al.* (2001) who observed a decrease in kappa number of different pulp samples treated with xylanase enzyme from *Thermomyces lanuginosus*.

Medeiros *et al.* (2002) reported a reduction in kappa number with xylanase preparations from *Trichoderma harzianum* strain 6.

Raghukumar *et al.* (2004) reported a decrease in kappa number in pulp sample treated with xylanase from *Aspergillus niger* cultured with different substrates.

4.5.4 Effect of enzyme on the brightness (%) of pulp samples

The brightness (%) of pulp samples after treatment with enzymes are depicted in Table 12.

TABLE 12

EFFECT OF ENZYME TREATMENT ON THE BRIGHTNESS OF PULP SAMPLES

Samples	Degree of brightness (%)			
	Ink paper pulp		Photocopy paper pulp	
	24 hrs	48 hrs	24 hrs	48 hrs
Control	55	56	56	56
Sample I				
Wheat bran	60	61	59	61
Rice bran	57	58	56	57
Sorghum flour	57	59	57	58
Sample II				
Wheat bran	56	57	56	58
Rice bran	57	57	57	58
Sorghum flour	56	56	57	59

Sample I : The pulp treated with the enzyme from *Aspergillus niger*

Sample II : The pulp treated with the enzyme from *Fusarium sp.*

Control : The pulp treated with buffer without the enzyme.

An increase in brightness percentage was noticed in all the samples treated with the enzyme from *Aspergillus niger* when compared with control. The increase was high with samples treated with the enzyme extracted from wheat bran.

5.0 SUMMARY AND CONCLUSION

Enzymes are used in paper and pulp industries to avoid the usage of chlorine for bleaching the pulps in order to reduce the energy consumption and protect the environment from pollution.

The study entitled “**Production and Optimization of xylanases and cellulases from fungi and their effects on paper pulp**” was aimed to isolate the fungi from the soil and culture them to produce enzymes namely xylanases and cellulases which have industrial applications. The prepared enzymes were studied for their effect on ink paper pulp and photocopy paper pulp.

Fungi, namely, *Aspergillus niger*, *Penicillium species*, *Fusarium species* and *Rhizopus species* were isolated from the soil. The isolated fungi were cultured at different initial medium pH(4.0-8.0) and selected temperature(17°C,27°C,37°C,47°C and 57°C) using various cheap substrates, namely, wheat bran, rice bran and sorghum flour to optimize the maximum enzyme production.

Among the four species and three substrates used, *Aspergillus niger* with wheat bran was found to be suitable for high xylanase production (0.115 U mg^{-1}) at the initial medium pH 5.0 and room temperature.

Of all the selected fungal isolates and three substrates, the highest cellulase production was observed from *Aspergillus niger* at neutral pH and temperature 37°C (0.398 U mg^{-1}) with wheat bran.

The crude enzyme extracts from *Aspergillus niger* and *Fusarium sp.* cultured in all the selected substrates at optimum conditions were taken for

further studies. The crude extracts were precipitated with ethanol and assayed the enzyme activities.

Ethanol precipitation increased the specific activity of xylanases. The increase in the activity of the enzyme from *Aspergillus niger* cultured in wheat bran was 1.4 fold compared with the activity of enzyme from other selected substrates and also from *Fusarium sp.* cultured in selected substrates.

The precipitated enzyme was dissolved in phosphate buffer and purified through sephadex G-50 column. Fraction 3 showed the highest specific activity of xylanase (0.172 U mg^{-1}) followed by fraction 2 (0.154 U mg^{-1}). The activity of cellulase was also maximum in fraction 3, however, 11.4 fold lower than that of xylanase (0.015 U mg^{-1}).

The ink paper pulp and photocopy paper pulp samples treated with enzyme samples showed the release of reducing sugar and carbohydrate content. Reduction in kappa number was noticed indicating the increased bleachability of pulp samples. The brightness of the pulp samples were higher in enzyme treated samples when compared with control treatment.

From the above study, it is concluded that enzymes has been successfully used in increasing the brightness of pulps, which reduces the energy consumption and protect the environment from pollution.










Scope for further studies










1. Identification of micro organisms that produce cellulase free xylanase which are mostly preferred in paper industry.
2. Optimization of enzyme production using various carbon sources, nitrogen sources, metal ions and inhibitors at various concentration.










3. To explore other species of fungi, for higher xylanase production, especially white rot fungi
4. Modification of organisms by random mutagenesis and selection of hyper-enzyme producing mutants.
5. Isolation of xylanase from marine fungi which has potential use for biobleaching.










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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	0.05
Chloramphenicol	0.1
Agar	15.5
pH	7.2 ± 0.2 at 25°C

APPENDIX II

LACTOPHENOL COTTON BLUE STAINING (Kannan,1996)

PRINCIPLE

Lactophenol 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 the cytoplasm of fungus, glycerol which gives a semi permanent preparation and cotton blue.

REAGENTS

Lacto phenol cotton Blue

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

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

PRINCIPLE

The blue colour developed by the reduction of phosphomolybdic phosphotungstic compounds plus the colour developed by the Biuret reaction of the protein with alkaline cupric tartarate, are measured spectrophotometrically.

REAGENTS

1. Solution A: 1% Copper sulphate
2. Solution B: 2% sodium potassium tartarate.
3. Solution C: 2% sodium carbonate in 0.1N sodium hydroxide
4. Solution D:
Mix 1ml of solution A, 1ml of solution B and 10ml of solution C just before use.
5. Solution E: 1N Folin ciocalteau reagent (Mixed equal volumes of commercially available reagent and diluted with water just prior to use) stored protected from light.
6. Standard BSA: 50 mg of BSA in 50ml of 0.1 NaoH. Diluted 1:10 for working standard.

PROCEDURE

Aliquots of standard protein solution (100µg-1000µg) and the sample solutions were made upto 1ml with 0.1N NaOH and shook well to treat the protein with alkali. Added 5 ml of solution D, mixed well and incubated at 37°C for 3 minutes. Added 0.5ml of solution E to each tube, mixed well and incubated for further 3 minutes at 37°C. Measure the colour developed at 610nm against reagent blank.

CALCULATION

Amount of protein content was calculated from the standard curve of BSA

APPENDIX IV

DETERMINATION OF XYLANASE (Bailey et al, 1992)

PRINCIPLE

The xylanase activity was determined by measuring the release of reducing sugar using the dinitrosalicylic acid method (Miller, 1956).

REAGENTS

1. Xylan suspension:

2.5 g of xylan (oat spelt xylan, Himedia), suspended in 80 ml of 0.05M sodium citrate buffer, pH 5.3 at 60°C and heated until boiling. The suspension was mixed overnight and brought to a volume of 100 ml.

2. Dinitrosalicylic acid Reagent

3. 40% Rochelle salt solution (potassium sodium tartarate)

4. Xylose standard:

Dissolved 1g xylose in 100ml distilled water and diluted 1:50 so that 1ml contains 200 µg of xylose.

PROCEDURE

Pipetted out 0.9 ml of the xylan suspension, preheated at 50°C and incubated for 10 minutes with 0.1ml of crude enzyme. The reaction was stopped by adding 2ml of dinitrosalicylic reagent. The colour formed was measured spectrophotometrically at 540nm, Prepared a standard with xylose in the concentration range of 100-1000µg.

CALCULATION

Xylanase activity was expressed in international units (IU), where one unit is the amount of enzyme which produces one micromole of product (xylose) per ml of culture per minute under assay conditions per mg of protein.

APPENDIX V

DETERMINATION OF CELLULASE ACTIVITY (Denison and koehn, 1977)

PRINCIPLE

The production of reducing sugar (glucose) due to cellulolytic activity is measured by dinitro-salicylic acid method.

REAGENTS

1. Filter paper disc: Cut the what mann filter paper No:1 with a paper punch (7mm diameter) to ensure the same surface area of substrate in the reaction tube
2. Dinitrosalicylic acid reagent.
Dissolved by stirring 1g dinitrosalicylic acid, 200 mg crystalline phenol and 50 mg sodium sulphite in 100ml of 1% NaOH stored at 4°C .
3. 40% Rochelle salt solution (Potassium sodium tartarate)
4. Standard glucose:
5. Dissolved 50mg glucose in 50ml distilled water

PROCEDURE

Pipetted out 0.5ml of enzyme extract to 32 mg of dry what man No.1 filter paper. Incubated the mixture for 1 hour at 50°C. Stopped the reaction by the addition of 0.5ml of dinitrosalicylic acid reagent. Heated the mixture in a boiling water bath for 5 minutes. While the tubes were warm, added 1 ml of potassium sodium tartarate solution and cooled the tubes at room temperature. Made up the volume to 5ml in all the tubes. Measured the absorbance at 540 nm. Prepared a standard with glucose in the concentration range 50-1000 µg.

CALCULATION

One unit of enzyme activity was expressed as the milligram of glucose released per minute per milligram of protein.

APPENDIX VI

EFFECT OF TEMPERATURE ON ENZYME PRODUCTION BY FUNGI

In 250 ml sterile conical flask, 10g of various substrates were taken and autoclaved. The media was then inoculated with 2 loopful of fungal cultures and incubated at selected temperatures for 7 days.

APPENDIX VII

ETHANOL PRECIPITATION (keskar *et al.*, 1989)

The culture filtrate was concentrated by precipitating the enzyme with equal volumes of chilled ethanol and kept undisturbed for overnight at 4°C. This was then centrifuged at 10,000rpm for 30min at 4°C. The resulting precipitate was then dissolved in 15ml of sodium citrate buffer (0.05m) pH5 and is used for further purification.

APPENDIX VIII

GEL FILTRATION CHROMATOGRAPHY (Andrews, 1964)

PRINCIPLE

Gel filtration chromatography was first introduced by A.J. Porth and D. Flodin in 1959. The gel filtration chromatography separates molecules according to their size and shapes. If the size of molecule is larger than the pore size of the gel bead, it is completely retarded.

MATERIALS & REAGENTS

1. Glass wool
2. Sephadex G-50
3. 50mM sodium citrate buffer (pH 5.0)

PROCEDURE

1. Suspended 3g of sephadex G-50 in 50mM sodium citrate buffer (pH5.0) and allow to swell by keeping it for 3-4 hrs.
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 poured a small volume of buffer or distilled water into the column.
5. Gradually pour the slurry into it along the innerside 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 enter into the column by opening the outlet. A small amount of mobile phase(as buffer) was added to wash the traces of the sample into the column.

8. Added sufficient amount of buffer on the top of the column and connected it to the buffer reservoir.
9. Collected fractions (2ml each) were determined for its protein content either by maintaining absorbance at 280 nm or by Lowry's method and enzyme assay.
10. Plot the graph, of concentration of protein and enzyme in y axis and the fraction number (or) elution volume in x axis.

APPENDIX IX

DETERMINATION OF REDUCING SUGAR (Nelson, 1944; Somogyi, 1952)

PRINCIPLE

The reducing sugar content was estimated by Nelson-somogyi method.

REAGENTS

1. Copper reagent:

Anhydrous sodium carbonate (2.4g) and sodium potassium tartarate (2.4g) was dissolved in about 25ml of water. To this carbonate solution copper sulphate solution (0.4g in 4ml of water) was added with stirring and then 1.6 g of sodium bicarbonbate was added. The two solutions were mixed and diluted to 100ml.

2. Nelson's chromogenic reagent (arsenomolybdate reagent)

Ammonium molybdate (2.5g) was dissolved in 45ml of water and 2.5ml of concentrated sulphuric acid was added and mixed. To this 0.3g of sodium hydrogen arsenate dissolved in 25ml of water was added. After mixing, the

solution was incubated at 37°C for 48 hours. The reagent was stored in brown bottle.

PROCEDURE

To 1ml of filtrate, 1ml of alkaline copper reagent was added and boiled for 10 minutes. After cooling down for 1 minute, 1ml of arsenomolybdate reagent was added and the solution was made upto 5ml with distilled water. The intensity of the colour developed was read at 540nm in a spectrophotometer. Quantities of sugar present in the samples were determined with reference to a standard graph prepared with known quantities of glucose.

APPENDIX X

DETERMINATION OF CARBOHYDRATE (Hodge and Hofreiter, 1962)

PRINCIPLE

The carbohydrate content of the enzyme sample was estimated by the method of Hodge and Hofreiter (1962)

REAGENTS

1. 2.5N Hydrochloric acid
2. Anthrone reagent:

Anthrone (200mg) dissolved in 100ml of ice cold 95% sulphuric acid.

PROCEDURE

The dissolved sample (100 mg) was hydrolysed in 5ml of 2.5 N hydrochloric acid in boiling water bath and then cooled. To 0.5 ml aliquots, 4ml of anthrone reagent was added and boiled for 8 minutes. The sample was cooled rapidly and the green colour developed was read in spectrophotometer at 630 nm. Quantities of carbohydrates present in the sample were determined with reference to a standard graph prepared by using known quantities of glucose.

APPENDIX XI
DETERMINATION OF KAPPA NUMBER
(TAPPI,1998)

PRINCIPLE

Kappa number is used as a criterion for the lignin content of pulp and is determined as the volume of 0.1N KMnO₄ consumed by 1g of moisture free pulp.

PROCEDURE

A portion of the cut piece of hand sheet that could consume approximately 50% of KMnO₄ solution (0.1%) was weighed out and disintegrated in 500ml of water until free of fibre clots or bundles. The disintegrated suspension was made upto 800ml. To 100ml of KMnO₄ solution (0.1N), 100ml of H₂SO₄ (4N) was added, cooled at 25°C and immediately added to disintegrated hand sheet suspension. After 10 minutes, the reaction was stopped by the addition of 20ml of potassium iodide solution (1N) and titrated against sodium thiosulphate (0.2N). Starch solution (0.2%) was used as the indicator. A blank titration was carried out in the same manner, but without pulp.

The kappa number was calculated by the formula

$$k = \frac{p \times f}{w} \quad \&$$
$$P = \frac{(b-a)N}{0.1}$$

where k=kappa number

f= factor for correction to the 50% permanganate consumption depending on the volume of P

w= weight of moisture free pulp sample used for estimation(g).

P= amount of 0.1N Permanganate consumed.

a= amount of thiosulphate consumed by sample(ml).

b= amount of thiosulphate consumed in blank determination(ml).

N= Normality of thiosulphate.

correction of reaction temperature:

$$k = \left(\frac{p \times f}{w}\right) \times 0.1 + 0.031 (25-t)$$

where t= actual reaction temperature in c.

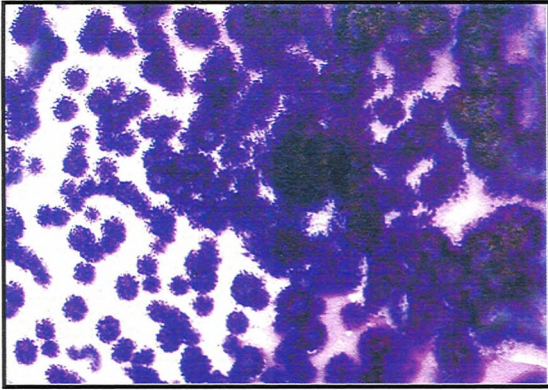
PLATE I

SOIL

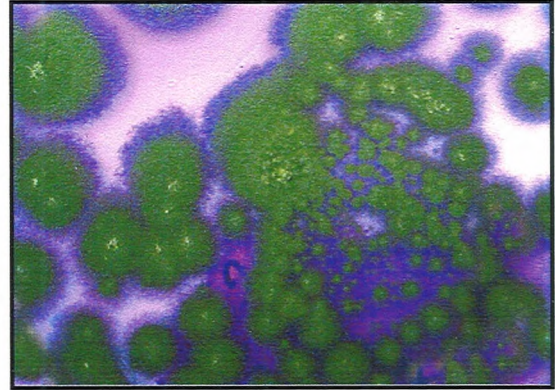


PLATE II

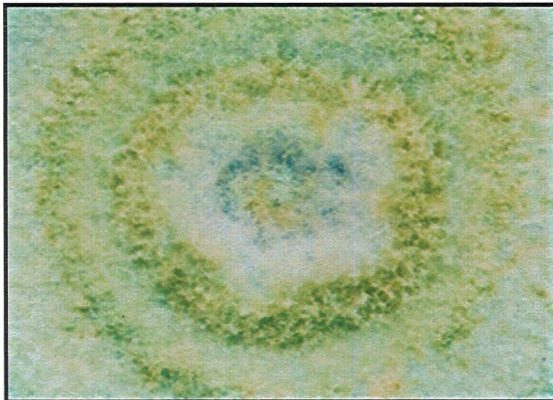
FUNGAL COLONIES



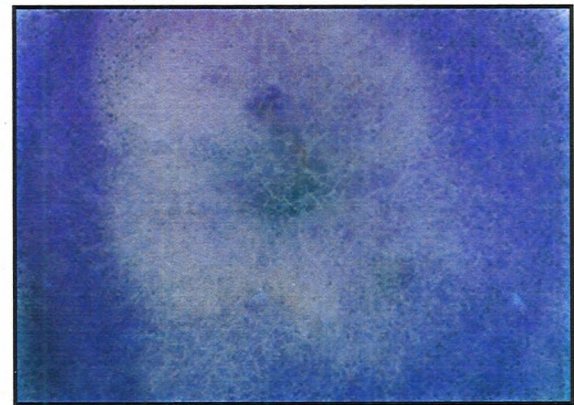
Aspergillus niger



Penicillium sp.



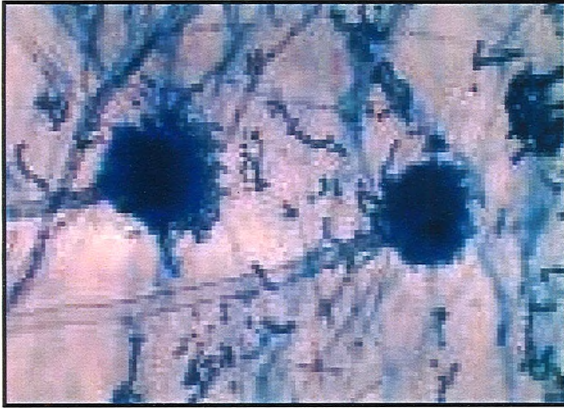
Fusarium sp.



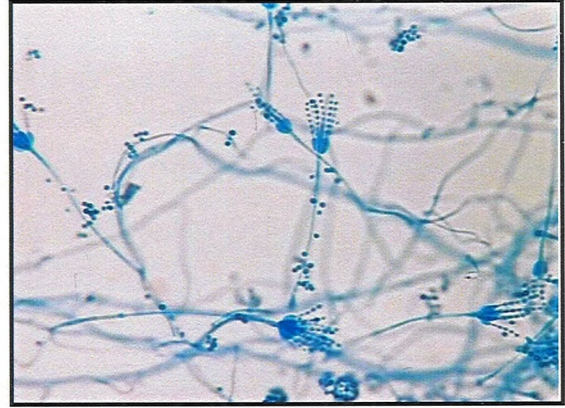
Rhizopus sp.

PLATE III

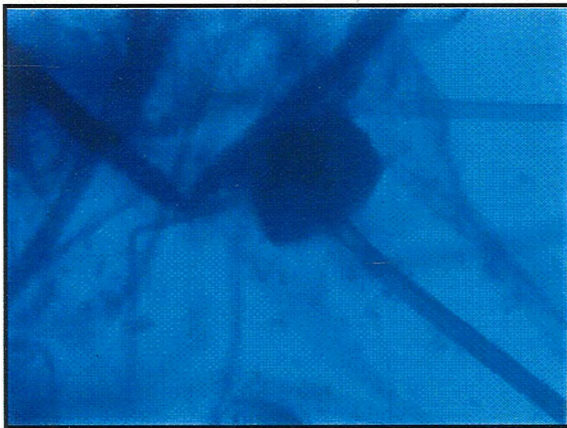
MICROSCOPIC VIEW OF FUNGI



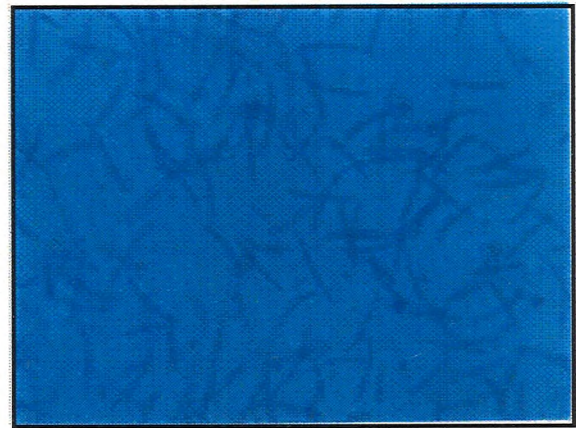
Aspergillus niger



Penicillium sp.



Fusarium sp.

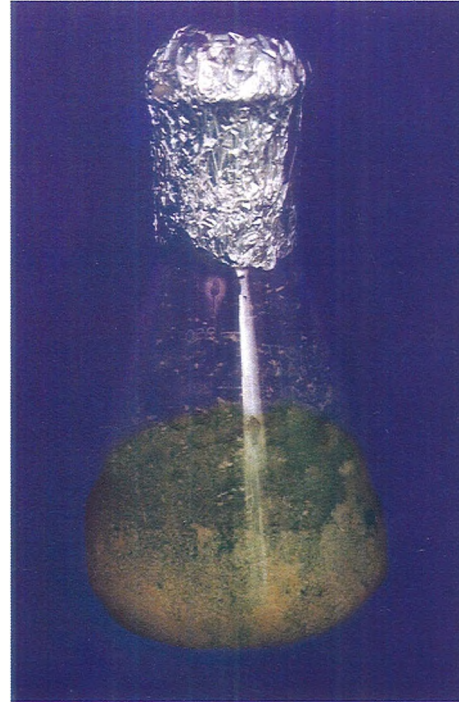


Rhizopus sp.

PLATE IV
SUBSTRATES INOCULATED WITH
SELECTED SPECIES



Aspergillus niger



Penicillium sp.



Fusarium sp.



Rhizopus sp.