ENHANCED CELLULASE PRODUCTION BY SOLID STATE FERMENTATION AND ITS APPLICATION IN BIOSTONING AND BIOCONVERSION PROCESSES

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CERTIFICATE

This is to certify that the research work entitled **"Enhanced Cellulase Production by Solid State Fermentation and its Application in Biostoning and Bioconversion Processes"** is a bonafide record of the work done by **Mr. M. VIJAY ANTONY**, Department of Botany and Microbiology under my supervision for the degree of Doctor of Philosophy in Microbiology to be awarded by Bharathidasan University and no part has been presented for any other degree or diploma.

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DECLARATION

I do hereby declare that this research work has been originally carried out by me in the Department of Botany and Microbiology, A.V.V.M. Sri Pushpam College (Autonomous), Poondi, Thanjavur, and no part of this research work has been submitted elsewhere for any other degree.

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Dedicated

to my

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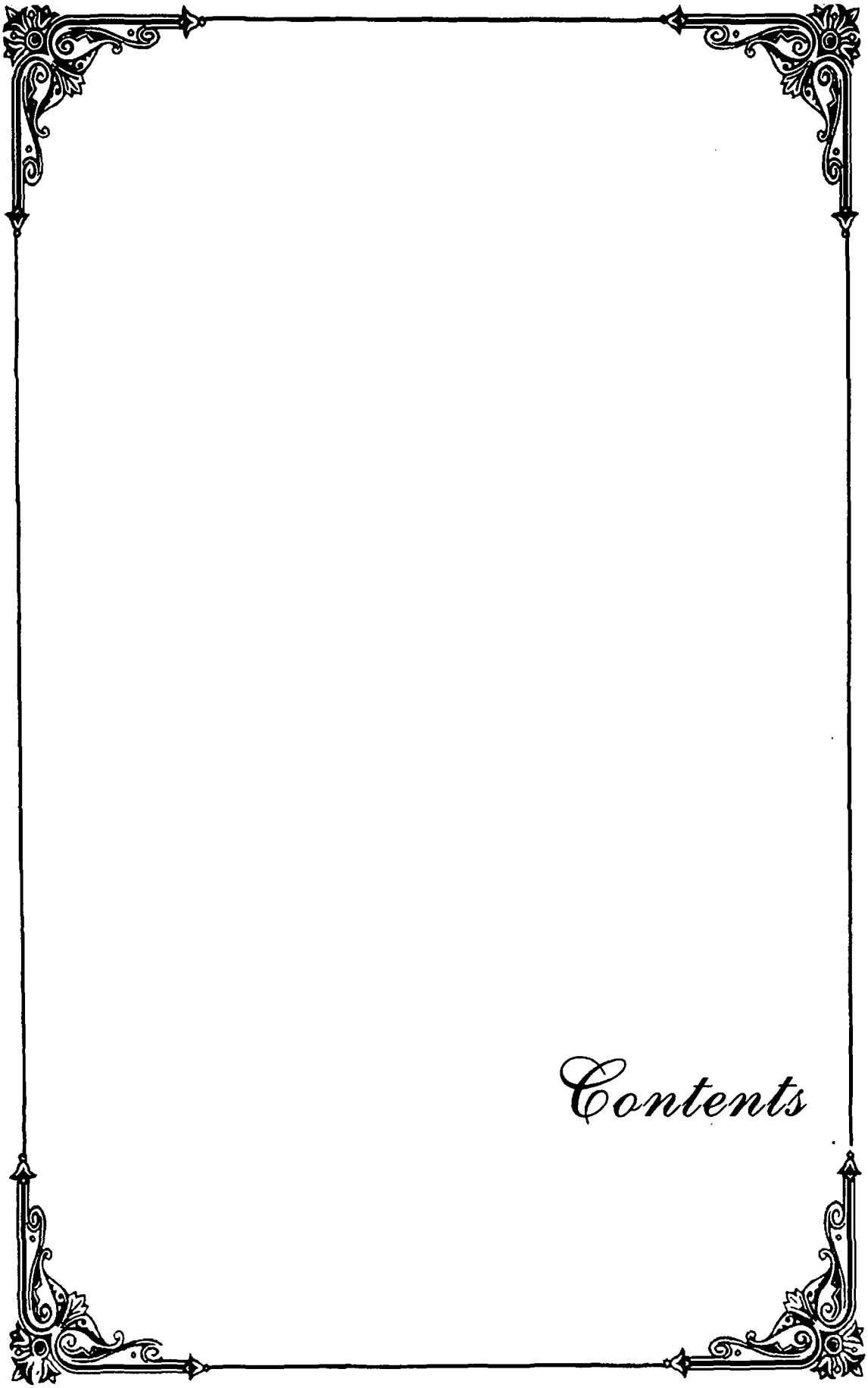

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LIST OF ABBREVIATIONS

α	-	Alpha
β	-	Beta
CMC	-	Carboxymethyl cellulose
CMCase	-	Carboxymethyl cellulase
DEAE	-	Diethyl amino ethyl
DNS	-	Dinitro salicylic acid
FPase	-	Filter paper activity
IU	-	International unit
KDa	-	Kilo daltons
MCC	-	Microcrystalline cellulose
PAGE	-	Polyacrylamide gel electrophoresis
PDA	-	Potato dextrose agar
pNPG	-	Para-Nitrophenyl-β-D-glycopyranoside
SDS	-	Sodium dodecyl sulphate
SmF	-	Submerged fermentation
SSF	-	Solid state
TCA	-	Trichloro acetic acid
Tris	-	Tris (hydroxymethyl) amino methane
UV	-	Ultraviolet

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Introduction

1. INTRODUCTION

Biotechnology is currently considered as a useful alternative to conventional process technology in industry and analytical fields. This is mainly because unlike chemical catalysts, enzymes have the advantage of accomplishing complex chemical conversions under mild environmental conditions with high specificity and efficiency. The variety of biochemical transformations catalyzed by enzymes has made these biocatalysts a prime target of exploitation by the emerging biotech industries and cellulase enzyme is no exception.

Cellulase is a hydrolytic multi component enzyme complex consisting of three enzymes acting synergistically for the complete hydrolysis of cellulose to glucose units. Endo β - 1,4, glucanase (Carboxymethyl cellulase - E.C 3.2.1.4) attacks the glycosidic bonds at random position of cellulose chain. Exo β - 1,4 glucanase (Filter paperase - E.C. 3.2.1.74) splits cellobiose residues or less commonly glucose molecules from cellulose chain ends. β - glucosidase (Cellobiase - E.C. 3.2.1.21) hydrolyses cellobiose to yield glucose molecules (Wood and McCare, 1979).

The most abundant carbohydrate polymer in the biosphere is cellulose, which is synthesized at the rate of 4×10^7 tons per year (Singh and Hayashi, 1995). These cellulosic materials, occurring as agriculture forestry residues, and municipal solid wastes etc. are the only renewable raw material available in large quantities, which need to be properly utilized to meet our needs of energy, chemicals, food and feed for long-range solutions using microbial cellulase mainly from fungi (Klysov, 1990). Cellulosic materials can be hydrolyzed using

acid or enzymes. Acid hydrolysis involves high temperature in which undesirable byproducts are formed (Tiwary *et al.*, 1988). Enzymatic hydrolysis by cellulase has attracted much interest in recent years.

The available information, on the utilization of the cellulosic materials, indicates that proper utilization and enzymatic conversion of these materials into products of interest are not fully achieved due to high cost of cellulase, low specific activity of the enzyme, inactivation of cellulase at operational conditions and product inhibition of cellulases (Mandels, 1985). These factors restrict the enzymatic bioconversion of cellulose to useful products. Hence more attention is still focussed on the isolation of new strains and raising mutants of the available strains that can produce more cellulases with greater specific activity and stability to product inhibition.

Cellulase has a very broad range of applications in industries either directly or indirectly. Cellulase is employed in the clarification of fruits juices along with pectinase and finds application in the saccharification of cellulosic wastes. Despite the fact that research on cellulase was prompted by a need to prevent their hydrolytic attack on cotton clothing (Reese, 1950), today cellulase research has gained momentum on the enzymatic modification (Biostoning) of cotton fibres to increase their spinning quality in textile mills. ''Stonewashing'' of denim garments like blue jeans using cellulases instead of pumice stones is an example of environmental benefits.

In textile and paper industries, cellulases are expected to be effective in eliminating biological oxygen demand (BOD) and decolourising dyes in textile waste streams (Stork and Pulls, 1995). Cellulases are very effective catalysts, acting under mild conditions, and do not require high-energy input which is often associated with chemical processes. Also synergistic action of

cellulase complex on cotton fibre will cause severe problem by decreasing the fibre strength. As individual component enzymes of cellulase have unique features, it is essential to achieve the desired performance benefits with little loss of fabric strength (Mehta *et al.*, 1990).

Recently the interest in using cellulase in detergents has picked up with high utilization rate. The current demand of 55 tonnes per annum of cellulases is expected to cross 330 tonnes per annum in the next few years (Pandey *et al.*, 1999). However the cost, inadequate availability of indigenous enzyme and the absence of desired enzyme characteristics in available enzymes has resulted in investigation to develop an economically viable process for the production of cellulase having all the desired characteristics.

Currently the enzyme is being produced largely by submerged fermentation. A few reports are available in literature on the enzyme production by solid substrate fermentation. Owing to numerous potential advantages of solid substrate fermentation over submerged fermentation, investigations were carried out to develop a suitable bioprocess for the production of cellulase under solid substrate fermentation. The scope of the present work has been targeted as follows:

- Screening of fungal strains for highly potent cellulase producers.
- Optimization of various submerged and solid state fermentation parameters for cellulase production.
- Enhanced cellulase production by mutation.
- Purification and characterization of FPase enzyme.
- Role of FPase on cotton fibre.
- Role of cellulase in bioconversion of waste paper.

Review of Literature

2. REVIEW OF LITERATURE

Microbial enzymes bring about most of the beneficial processes. One such important process was the degradation of cellulose by cellulase enzyme. Organic wastes, from renewable agricultural and forest remains, comprise cellulose, hemicellulose and lignin at the rate of 4: 3: 1 (Eriksson and Wood, 1985). Though mankind has utilized cellulose for centuries, its utility has been recognized only after the identification of cellulose degrading enzyme called cellulase (Selleire, 1906).

During the World War II, the US army was alarmed at the rate of deterioration of cellulosic materials including clothing, tents and sand bags in South Pacific. This led to the initiation of cellulase research. Several organizations within the army setup laboratories to find an immediate solution to the problem. Reese at the US Natick laboratories made serious efforts and found that microorganisms were responsible for the cotton deterioration by secreting extracellular enzymes, which could degrade cellulose.

A parent strain, QM6A, was isolated from a shelter remains of the army and identified as *Trichoderma viride* and later recognized as *Trichoderma reesei* (Reese, 1976). The immediate benefits of the army program led to further research on selection and characterization of hyper cellulolytic strains of *Trichoderma reesei*. This project not only improved the production of cellulase by *T. reesei*, but also aroused worldwide interest in

this area of research. The work at the Natick army Laboratory and in other places quickly led to the recognition that cellulosic wastes could be converted into glucose, soluble sugars, alcohols, single cell protein and other industrially useful chemicals by using cellulases (Eriksson, 1982).

The milestones in the past fifty years of cellulase research were

- Isolation of microorganisms producing cellulases (Dasari *et al.,* 1990; Marchessuault and Sundarajan, 1993;),
- Improving the yield of cellulases by mutation and protoplast fusion (Avgerinos and Wang, 1993),
- Purification and characterization the cellulase components (Fierobe *et al.,* 1993; Fillingham *et al.,* 1999; Aro *et al.,* 2001; Fierobe *et al.,* 2001),
- Understanding the mechanism of cellulose degradation (Fillingham *et al.,* 1999; Fierobe *et al.,* 2001),
- Isolation of genes responsible for the production of cellulase, cloning and expression of cellulase genes (Amartey *et al.,* 1991; Begiun, 1990),
- Determination of the 3-D structures of cellulase components (Beguin and Aubert, 1994; Desai and Papoutsakis, 1999),
- Understanding the structural and functional relationships in cellulases (Beguin and Aubert, 1994; Baskaran Ahn and Lynd, 1995; Ding *et al.,* 2001), and

- Demonstrating the potential industrial use of cellulases (Alfredsson *et al.*, 1988; Bayer *et al.*, 1994).

Several reviews have covered the cellulase research on many aspects (Beguin, 1990; Beguin and Aubert 1994; Coughlan, 1985; Esterbauer *et al.*, 1991; Gilkes *et al.*, 1992; Hungate, 1950; Kuibek, 1992; Lamed and Bayer, 1988; Ljungdahl and Eriksson, 1985; Mandels, 1985; Wood, 1992). This review summarizes the present state of the art on cellulases, from both applied and fundamental viewpoints.

2.1 OCCURRENCE OF CELLULASE

Though a few plant and animal sources of cellulase were known, overwhelming importance was given to the microbial sources because of the economic feasibility of its production by fermentation techniques (Reese, 1976). Many species of bacteria, fungi and yeasts were known to produce cellulolytic enzymes.

2.1.1 Cellulolytic bacteria

Cellulolytic enzymes were produced by a wide variety of aerobic and anaerobic, mesophilic and thermophilic bacteria. However, relatively a few bacteria produce high levels of extracellular cellulase capable of solublizing the crystalline cellulose extensively (Chauvet and Merce, 1988). Among the cellulose degraders, cellulolytic thermophilic microorganisms such as, *Clostridium thermocellum, Thermonospora fusca, Thermoascus auarantiascus, Sporotrichum thermophile, Humicola insolens* and

Chaetomium thermophile were of particular interest, because of their ability to produce thermostable cellulase. Most of the bacteria produce mainly endoglucanase, and were able to ferment a wide range of substrates (El-Nawwi and El-Kader, 1996).

The anaerobic bacteria *Clostridium thermocellum* (Chang *et al.*, 1997), degrade the highly crystalline cellulose such as cotton by producing a high molecular mass enzyme complex called cellulosomes. In the case of *C. thermocellum*, cellulosomes consist of several endoglucanases and at least three exoglucanases (Aurilia *et al.*, 2000). It has been suggested that these anaerobic bacteria must attach themselves with cellulosic substrate in order to effect cellulolysis (Dombek and Ingram, 1986; Bhat *et al.*, 1997).

The cellulolytic system of *Clostridium thermocellum* has gained considerable interest in recent years, since it produces a multicomponent cellulolytic complex termed cellulosomes (Lamed and Bayer, 1988).

2.1.2 Cellulolytic Fungi

Among a large number of cellulolytic microorganisms, mostly fungi were able to utilize cellulose for their life processes and could produce the complete set of cellulases for the hydrolysis of cellulose into soluble sugar (Beguin and Aubert, 1994; Coughlan, 1985).

However, only some fungi have been reported to produce high titres of cellulase and most of them belonged to white rot and soft rot fungi group. The white rot fungi had the capability to degrade lignin and

lignocellulosic substrates (Esterbauer *et al.*, 1991). The first white rot fungus isolated was *Chrysosporium lignorrum*, which was later, given the name of *Sporotrichum lignorum* and subsequently *Phanerochate chrysosporium.* Soft rot fungi were capable of degrading both lignin and polysaccharides. The capability of these fungi to degrade cellulose varies. The best-known example in this category is *Trichoderma reesei. Penicillium funiculosum, Fusraium* spp and *Aspergillus* spp (Alfredsson *et al.*, 1988; Amartey, 1991; Wood, 1992).

Several fungi were isolated, screened for cellulase production and studied for the conversion of cellulose to glucose (Gomes *et al.*, 1990). Fungi capable of hydrolyzing cellulose under aerobic condition include *Phanerochaete chrysosporium, Fusarium solani, Penicillium* spp, *Pleurotus florida, Trichoderna reese, Trichoderma harzianum, Aspergillus* spp (Wood and Ma Crae, 1972; Olutiola, 1977; Hayashid and Yoshika, 1980; Saddler *et al.*, 1985; Ganju *et al.*, 1990). Most active cellulose degrading fungal genera are *Aspergillus, Penicillium, Trichoderma,* and *Sporotrichum* (Erikson and Peterson, 1975; Wood, 1992; Irwin *et al.*, 1993; Yu and Saddler, 1995).

Studies on rumen anaerobic fungi suggest that these microorganisms have definite role in colonization and degradation of lingocellulose in the rumen (Alexander, 1972). Anaerobic fungi have been shown to produce extracellular cellulases and xylanases, which are important in the breakdown of lingocellulose (Chen *et al.*, 1992). Several studies have been carried out on the production and characterization of cellulolytic enzymes from these microorganisms and indicated their importance in the

degradation of cellulosic materials and subsequent fermentation in the rumen (Ljungdahl and Eriksson, 1985). Anaerobic cellulolytic fungi belonging to the genera *Neocallimastix, Cacomyces, Orpinomyces, Piromyces* and *Ruminomyces*, have been described and classified (Fan *et al.,* 1980). At present it is a well-established fact that obligate anaerobic fungi are found in the saliva, alimentary tracks and faces of number of animals such as ruminants and herbivorous non-ruminant mammals (Chen *et al.,* 1997). Among these species, *Neocallimastix frontalis* has been studied in great detail. The *N. frontalis* RK21 was the most effective degrader of crystalline cellulose so far known and plays an important role in the digestion of cellulose in rumen (Correa *et al.,* 1992).

Cellulose digestion capabilities of various thermophilic fungi such as *Thiotherestris* spp, *Aspergillus terreus, Thermoascus aurantiacus, Myceliopthora fergusii* and *Trichoderma harzianum* were comparatively studied (Wood, 1992).

2.1.3 Hemicellulose degrading fungi

Seal and Eggins (1976) studied the role of fungi for recycling agricultural wastes rich in hemicellulose. They also reported that mesophilic fungi produced the important xylanases, which convert xylan into simple sugars.

Xylanase, from a large number of fungi, have been reported and studied in detail. They include *Aspergillus niger* (Costa-Ferreira *et al.*, 1994), *Penicillium janthinellum* (Takenashi and Tsujisaka, 1973), *Trichoderma*

koningi (Wood and McCrae, 1986), *T. reesei* QM9414 (Dekker, 1985), *Trichoderma viride* (Toda *et al.,* 1970), *Trichoderma. longibrachiatum* (Royer and Nakas, 1989), and *Humicola lanuginose* (Anand and Vithayathil, 1990). Hussein *et al.,* (1992) isolated twelve fungi which convert lack liquor hemicellulose into microbial biomass and single cell protein and classified them into high biomass producers like *Aspergillus terreus* and *Paecilomyces simplicissima*, moderate biomass producers such as *Actinopolyspora* spp, *Penicillium funiculosus, Paecilomyces divaricata* and *Peacilomyces silvatic,* low biomass producers such as *Trichoderma album, Aspergillus fumiculosis, Alternaria humicolo, Penicillium janthinellum, Aspergillus versicolor, Aspergillus nidulans* and *Chaetomium magnum.* Although numerous microorganisms have been reported to hydrolyse xylan, only a few can produce high level of xylanases (Bhat *et al.,* 1992). Chen *et al.,* (1986) reported that a strain of *Aspergillus terreus* A- 07 isolated from soil was suitable for the production of xylanases in commercial scale.

2.1.4 Xylanases

The enzyme, which breaks down xylan called as xylanases. Three different types of xylanases were involved in xylan degradation (Dekker, 1985). They are endo β- (1-4) - D-xylanases [β- (1- 4 D – xylan - xylano hydrolase (EC 3.2.1.8)], exo - β (1-4) – D - xylanases (β- 1 (1-4) D- xylan xylohydrolase) and β- xylosidsae or xylobiase (EC 3.2.1.37)

Endo - β - xylanases act randomly on xylan to produce large amount of xylooligosaccharides, of various chain length. These enzymes are of four types.

Non arabinose liberating endo xylanases: these enzymes cannot act on L-arabinosyl initiated branch points at β- (1-4) linkages. These can breakdown xylooligosaccharides as small as xylose. Non-arabinose liberating exoxylanases: these enzymes cannot cleave branch points at α- (1-2) and α- (1-3). They produce mainly oligosaccharides larger than xylose but do not have any action of xylotriose and xylobiose. Arabinose liberating endo xylanases: these enzymes could cleave the xylan at the branch point and produce mainly xylobiose, xylose and arabinose. Arabinose liberating exo xylanases: these could hydrolyze the branch points and produce intermediate size xylooligosaccharides and arabinoses.

Exo - β (1-4) – D - xylanases remove the single xylose unit from the non-reducing end of the xylan chain. This does not form xylo oligosaccharides.

β - xylosidase or xylobiase (EC 3.2.1.37), hydrolyse disaccharides like xylobiose and the higher xylooligosaccharides with decreasing specific affinity.

2.2 SUBSTRATES FOR CELLULOLYTIC ENZYMES

a) Cellulose

Most of the cellulose was produced as a component of plant cell walls and occurs in varying amounts. Natural cellulose is crystalline, insoluble and occurs as fibres of densely packed with anhydrous glucose chains of 15 to 10, 000 glucose units by β-1,4- glycosidic bonds. It gives structural strength to plant cell wall. It was evident that naturally synthesized cellulose was nearly pure and was associated with other polysaccharides such as hemicellulose and lignin (Kennedy and White, 1983). The linear chains of cellulose glucosyl residues of cellulose undergo self-assembly at the site of biosynthesis and the regulation of co-aggregation process by the association of hemicellulose (Aho and Paloheimo, 1990).

b) Hemicellulose

Hemicellulose was the second most abundant fraction available in nature next to cellulose. The term hemicellulose, was first introduced by Schulze (1891). The hardwood plant species contain up to 35% hemicellulose. The hemicelluloses were other wise called as xylan, present in both primary and secondary layers of the plant cell wall, where they are closely associated with lignin and cellulose (Dekker, 1985). Also, it occurs as a storage polymer in seeds Xylan consists of D- xylose as its monomeric unit (Taiz and Honigman, 1976), and it forms the structural component in cell wall of all woody plants (Erikson, 1990). The hemicellulose includes the heteropolymers, xylan, mannan, galatan and arabnan. The major monomers

present in most of the hemicelluloses are D- xylose, D- mannose, D- galatose and L- arabinose (Costa-Ferreira, 1994).

2.3 MECHANISM OF CELLULOSE DEGRADATION BY FUNGI

In the beginning it was difficult to predict the mode of action of cellulase. Reese *et al.*, (1950), classified cellulase as C_1 and C_x types. According to this concept, C_1 could attack native cellulose of higher crystallinity, while, C_x doesn't attack, but it could split the cellulose fragments produced by the action of C1. The mode of action of C_1 has long been questioned. Later it was established that the cellulase was a multi enzyme complex, which brings out the complete degradation of cellulose to monosaccharide units. Coughlan (1985) termed the initial stage of cellulose breakdown as a morphogenesis. According to Koennig and Cochran (1994), initial attack on cellulose takes place *via* H_2O_2 /Fe^{++} system, where C_2, C_3, or C_6 carbon of cellulose are oxidized and thereby the ordered packing of cellulose chain gets disrupted and thus endoglucanase starts hydrolysis. The other possibilities for substrate degradation by hydrolytic enzymes was unaided by oxidative or other non-hydrolytic process.

Further degradation takes place by the extension of morphogensis by the formation of short fibres due to the sequential removal of cellobiose units from non reducing end. This was followed by simultaneous multi point attack by enzymes on substrate on many chains (Coughlan and Ljungdahal, 1998; Henrissat *et al.*, 1985). Further possibility was desertification in which

without the release of reducing sugar, microfibrils or layers are stripped from the cellulose. The endo and exo enzymes were involved in further degradation. Cellobiose and cellodextrins were formed as a result of synergistic action of these enzymes. In cellulose digestion by fungi, catalytic activity of β - glucosidase on cellobiose to release glucose residues from the non-reducing ends. Exoglucanase if present may also participate in the process (Henrissat *et al.*, 1985).

The 1,4 - β - D - xylosidic linkages in xylan are randomly hydrolysed by endo - 1,4 - β - D - xylanases. These enzymes have a higher hydrolysis rate which cuts polymeric xylan than xylo-oligomers. Their main hydrolytic products were being xylobiose, xylotriose and substituted oligomers that contained two to four xylose residues. Endoxylanase from several sources have been extensively studied. Especially cellulase produced by filamentous fungi such as, *Trichoderma* and *Aspergillus* spp. They could remove single xylose residues specificities some β – xylosidases have been reported to be active against xylobiose but inactive towards polymeric xylan. The β - xylosidase purified from *T. reesei* could release different length and form polymeric xylan (Biely *et al.*, 1985).

2.3.1 Assay methods employed for cellulolytic enzymes

The complexity of cellulolytic enzymes and their substrates was reflected on the assay methods used by different workers (Mandels *et al.*, 1976; Enari and Markkanen, 1977). Though cellulolytic enzymes attack the same D – 1,4 - glucosidase linkages and they differ only in specificity

regarding the structure surrounding the linkage. Thus it may be hard to differentiate between the activities of different groups of cellulolytic enzymes. The situation is more complicated by the fact that the activities are usually determined in solutions containing mixtures of various cellulolytic enzymes. Because the cellulolytic enzymes not only hydrolyze the same substrate but also act synergistically and there activity is greatly influenced by the amount of individual enzymes present in the complex.

The purpose of determination of cellulolytic activity is different and this should be taken into account while choosing the assay methods. The activity of individual enzymes should be monitored for biochemical studies. For these purposes specific substrates are needed and the initial reaction rates or selection of mutants where the number of determinations required are usually large, the methods employed should be simple and less time consuming. In technical process development, where the aim is to produce fermentable sugars, quantitative determination of glucose in grade cellulose as substrate is essential.

2.3.1 Total cellulolytic activity

The important commercial application of cellulolytic enzymes depends on total hydrolysis of cellulose to glucose. In such cases for activity measurements the substrate as such is an insoluble cellulolsic material. The important fact in this is the time factor. Since the substrate is insoluble, time is required for the enzyme to diffuse into cellulose fibres and for the hydrolyzed product to diffuse out of the fibre. In order to get meaningful results, the assay

procedure requires a long reaction time. The activity of the complete cellulases complex (Cellobiohydrolase carboxymethyl cellulase and D-glucosidase) can be measured by using crystalline cellulose such as filter paper, cotton fibres or avicel. The most generally used assay for cellulolytic enzyme is that of Mandels and Weber (1969) in which the filter paper in the substrate and the reducing sugar formed under standard condition is measured, quantified and expressed as μ- moles of glucose equivalents per min generally known as filter paper activity units (FPA).

2.3.2 Solubilizing activity

The most important activity of mixture cellulolytic enzymes is its solubilizing effect. Solubilization of native cellulose is caused by the synergistic action of cellobiohydrolase and endoglucanase for determining the solubilizing activity. Cotton fibres, microcrystalline cellulose and hydrocellulose have been used as substrates. These methods are based on the determination of reducing sugar (Mandels and Weber, 1969) loss of weight (Halliwell and Riaz, 1970), decrease in optical density of cellulose suspension (Nummi *et al.*, 1981) and on the solubilization of dyed oligosaccharides from dyed insoluble cellulose (Reese, 1976).

a) Endoglucanase

Endoglucanase enzyme attacks β-1,4 - glucosidic linkages randomly in swollen hydrocellulose or substituted cellulose. The most commonly used substrate for endoglucanase activity is the carboxymethyl cellulose. This substrate has been used by many workers for determining

endoglucanase activity by measuring reduction in viscosity because even a few random cleavages in the cellulose chain by the action of cellulase will cause a marked decrease in chain length. The activity could also be measured by estimating reducing sugar (Mandels and Weber, 1969). In certain cases hydroxyl methylcellulose is preferred as substrate over carboxy methylcellulose for quantification of preparations having low endoglucanase activity (Eriksson, 1978).

b) Exoglucanase

The substrate of choice for the measurement of cellobiohydrolase activity was microcrystalline cellulose or avicel. The activity was determined by measuring the reducing sugar formed. If reducing sugar measuring was the index for quantification of cellobiohydrolases activity, the presence of β-glucosidase will interfere in the assay system due to the hydrolysis of cellobiose to glucose resulting in higher values. Thus it is not possible to get a true estimation of cellobiohydrolase activity in the presence of other cellulolytic enzymes. However interference of β-glucosidase could be overcome by including D - glucono - 1,5 lactone which inhibits β-glucosidase in the assay system (Henryissat *et al.,* 1985).

c) β - glucosidase

β - glucosidase was a hydrolytic enzyme which catalyses various compounds with β - glucosidic linkages. It has been shown to play a key role in the cellulase complex of enzymes by hydrolyzing cellobiose to glucose. It

is the only cellulolytic enzyme for which specific substrates are available. Cellobiose p - Nitrophenyl β - D - glucopyranoside and salicin have been used commonly as substrates for the estimation of β - glucosidase activity (Selby and Maitland, 1967).

2.4 METHODS OF CELLULASE PRODUCTION

Technics of both submerged and solid state fermentation have been applied for fungal cellulase production.

2.4.1 Cellulase production under submerged fermentation

Submerged fermentation is defined as the growth of microorganisms in liquid medium in which various nutrients are either dissolved to suspend as particulate solids as in many cases of commercial medium in shake flask (Desrochers *et al.*, 1980; Arima, 1964). Various factors that affect the production of cellulases by submerged fermentation are carbon and nitrogen sources, pH and temperature of the fermentation medium (Bailey *et al.*, 1993).

a) Fermentation medium

Research in US army laboratories at Natick led to the formulation of medium containing cellulose with trace elements, which could yield high titers of cellulase in the fermenting broth (Mandels and Reese, 1957; Sternberg, 1976). Generally for cellulase production, medium developed by Sternberg (1976) has been employed without any change (Warzywoda *et al.*, 1983; Srinivasan and Seeta Laxman, 1988). Further it has also been observed

that the yields of cellulases could be increased with increase in cellulose content of the medium up to a certain level (Durand *et al.,* 1988). Another medium, which has been used frequently for cellulase production, is the modified Vogel's salt medium of Montenecourt and Eveleigh (1977).

b) Carbon source

The nature of carbon sources usually determines the effectiveness of cellulases production (Ryu and Mandels, 1980). The pure form of cellulose like solka floc, cotton, avicel or commercial cellulose pulp was considered as good carbon sources for getting higher yields of the enzymes (Ryu and Mandels, 1980; Coughlan, 1985). So far industrial cellulase fermentation chiefly used any of these defined substrates. However the use of the above carbon sources is cost intensive and causes operational problems in bioreactors due to their insoluble nature (Coughlan, 1985; Srinivasan and Seeta Laxman, 1988).

Lignocellulosic wastes were tried as an alternative carbon sources because of their inexpensiveness and abundant availability for cellulase production (Kawamori *et al.,* 1986; Bhat *et al.,* 1992). Though the lignocellulosic wastes are inexpensive it would create lot of operational problems in bioreactors due to its insoluble nature (Mandels *et al.,* 1978; Srinivasan and Seeta Laxman, 1988).

c) Nitrogen source

Like carbon sources, nitrogen source is also an important factor for the growth and production of cellulases. The nitrogen sources like ammonium sulphate, urea peptone are most commonly used in the production medium.

Among the nitrogen sources, urea inhibited the synthesis of enzymes in various microorganisms like *Trichoderma viride* (Reese and Mandels, 1963); *Penicillium pinophillum* (Brown, 1985); *Aspergillus nidulans* (Bagga and Sandhu, 1987); Though urea was found to inhibit the cellulases in the above organisms, it was reported to increase the cellulases activity in *Penicillium funiculosum* and *Ghocladium virens* (Gomes *et al.*, 1990).

The cellulase synthesis in *Aspergillus fumigatus* (Trivedy and Rao, 1980) was inhibited by ammonium sulphate. The type of inhibition varies from one species to the other. Lizak (1975), reported that the inhibition of cellulase synthesis was due to the ammonium ions of ammonium sulphate. Even though the ammonium ions are known to increase cellulases synthesis in many organisms, they inhibit the cellulase activity in certain organisms (Trivedi and Rao, 1980; Ali and Sayed, 1992). The increase in enzyme titers by incorporation of peptone in the medium has been reported (Mandels and Weber, 1969). In *Batryodiplodia theobromae* the growth and enzyme productivity is reported to be better, when sodium nitrate is used as nitrogen sources (Gong and Tsao, 1979). The incorporation of mixed nitrogen sources like ammonium sulphate, peptone and urea have shown to be effective

compared to single nitrogen source in many cases (Feniksova *et al.*, 1960; Gupta *et al.*, 1972).

2.4.2 Cellulase production under solid state fermentation (SSF)

Currently industrial demand for cellulases is met by production methods using submerged fermentation SmF processes. The cost of production in SmF systems is however high and it is uneconomic to use them in many of the above mentioned processes. This therefore necessitates reduction in production cost by developing alternative method, for example the SSF systems. Over the past 15-20 years, SSF has gained renewed attention in producing food, feed, pharmaceutical and agricultural industries in industrialized nations (Pandey *et al.*, 1999).

Pandey (1992) described a SSF process for the production of thermostable xylanase by thermophilic *Bacillus licheniformis*. The enzyme production was found to be higher in SSF system than in SmF system. Also reported that the enzyme produced in SSF system, was more thermostable than in SmF.

a) Factors affecting SSF

The efficiency of productivity and economy of solid substrate fermentation are affected by a number of factors such as,type of solid substrate, supplementation of nitrogen source, nitrogen source concentration, moisture content and water activity, pH of the fermentation medium,

fermentation temperature, fermentation time and the nature of inoculum (Kumar and Singh, 1990).

In solid substrate fermentations generally unrefined materials of agricultural origin were used, which not only supply the nutrients to the culture but also serve as anchorage to the microbial cells (Feniksova *et al.*, 1960). Particle size and shape is extremely important since it affects the surface area to volume ratio of the particles and the packing density within the substrate mass (Lonsane *et al.*, 1985). The availability of nutrients to the microorganisms from the solid matrix depends on the structure of solid substrate, porosity, shape, size, internal surface area and adsorption behaviour of the solid substrate (Moo Young *et al.*, 1983). In some cases it is necessary to improve susceptibility of the solid substrate to microbial attack and the properties of the solid substrate could be modified by pretreatment (autoclaving) to improve the performance of the process (Lonsane *et al.*, 1985; Barrios and Meja, 1996).

The single most important factor that makes solid substrate fermentation different from submerged fermentation was moisture content of the medium. The moisture levels in SSF processes, has a msrked effect on growth kinetics. The optimum moisture content for the organism and the substrate utilization depends on the organism and the substrate used for cultivation (Lonsane *et al.*, 1985; Zadrazil and Burnnert, 1981).

The initial pH of the medium plays a significant role in the growth and metabolism of the organism, which is difficult to control in solid substrate

fermentation. Hence it is desirable to maintain a broad pH range and pH optima during the growth of the organism (Raimbault and Alazard, 1980; Cooney, 1981). Some of the filamentous fungi grow well in the pH range of 4.0 – 6.0 (Mitchell *et al.,* 1992).

The maintenance of constant temperature during the entire period of fermentation is generally difficult because of the large amount of metabolic heat generated and mass transfer effects (Tengerdy, 1985). The rate of heat generation generally varies from 70 – 340 KJ/Kg/h (Arima, 1964). Effective removal of the metabolic heat because of the poor thermal conductivity of moist solid substrates leading to deep temperature gradients. External cooling or evaporative devices have to be employed for efficient heat removal from the fermentation system (Raimbault and Alazard, 1980).

b) Advantages and economics of SSF over SmF

The listing an array of advantage of solid substrate fermentation might not be sufficient enough to indicate better economics of solid substrate over submerged fermentation process. Direct and indirect comparative data available in the literature could be used as an index to determine the economic advantage of solid substrate fermentation over submerged fermentation (Ghildyal *et al.,* 1985). The comparison based on product titres in fermentation medium indicates higher product titres in solid substrate fermentation (Tengerdy, 1985). The high product concentration in the fermented solid in the case of solid substrate fermentation leads to lower expenses on downstream processing and effluent treatment. The absence of

undesirable compounds formation in solid substrate fermentation when compared to submerged fermentation not only allows higher selling cost but also attracts more demand for the product (Deschamps *et al.*, 1985). On the whole solid substrate fermentation appears to be comparatively more economical than the conventional submerged fermentation process for the production of some industrial enzymes (Pandey, 1992).

Cellulases are inducible enzymes, formed only in the presence of cellulose and its derivatives. Thus induction of cellulase requires cost intensive pure substrates, which could act as inducers and media constituents in submerged fermentation. Usually agro-industrial wastes as substrates for the production of cellulase can overcome this by solid substrate fermentation system. Almost all the agro industrial wastes contain cellulose, which could induce the biosynthesis of cellulases in filamentous fungi (Ramesh and Lonsane, 1990).

Glucose, the end product of cellulose hydrolysis is known to inhibit the production of cellulases. This type of catabolic repression is a severe problem in submerged fermentation system; which can be eliminated to a large extent when switched over to solid substrate fermentation system (Kumar and Singh, 1990).

In cellulase production by submerged fermentation, normally 8 - 10% solids were used in batch processes. This results in the requirement of large volume of fermentors, which in turn reflects on higher capital investment, whereas it is possible to use 30 - 35% solid at much lower capital

investment in solid substrate fermentation process. The foam problem, which is frequently encountered in submerged fermentation system, is totally absent in solid substrate fermentation (Arima, 1964). All the above factors make solid substrate fermentation process more economic for the production of cellulase over submerged fermentation.

2.5 STRAIN IMPROVEMENT STUDIES

To enhance cellulase production by microorganisms and to reduce production costs, several efforts such as mutation (Gallo *et al.*, 1978) gene cloning (Beguin, 1990), Protoplast fusion (Ogawa *et al.*, 1987) were made. Intense research effort resulting in the isolation of fungal mutant strains over producing the cellulase has improved the economics of enzymatic conversion of the cellulose component of lignocellulose (Montenecort and Eveleigh, 1979, Mandels, 1981).

Both physical and chemical methods have been employed for inducing mutations in cellulolytic organisms with a view to obtain hyper production of cellulase. Though the mutant strains have been developed for other cellulolytic organisms, *Trichoderma reesei* is given much importance, which was isolated at US army laboratories Natick USA (Mandels, 1985). The mutant strains like *Trichoderma reesei*QM6 (Reese, 1976), *Trichoderma reesei*NG14 (Montenecourt and Eveleigh, 1977), *Trichoderma reesei* MCG77 (Gallo *et al.,* 1978). *Trichoderma reesei* L27 (Shoemaker *et al.,* 1983) have been developed from the parent strain *Trichoderma reesei*QM9414 by subjecting it to different mutational methods.

VTT-D strains were also developed at the Technical Research Centre, Finland from the parent *Trichoderma reesei* QM9414. Between VTT-D series, VTT D 80133 was found to be most stable (Nevalainen, 1985) *Trichoderma reesei* CL847. Warzywoda *et al.*, (1983) also developed a mutant *Trichoderma reesei* MCG77 from *Trichoderma reesei* QM9414 that showed higher cellulolytiic activity and some morphological changes such as intensified conidiation and pigmentation. Mutant strains from other organisms like *Clostridium thermocellum* (Bhat *et al.*, 1997); *Phanerochate chrysosporium /Sporotrichum purverulentum* (Erikson, 1978) were found to be hyper cellulase producers compared to their parent strain.

2.6 Purification of cellulase component

A highly purified enzyme was a prerequisite for the study of the properties of the enzyme. All the usual purification techniques could be applied to purify the cellulase components. The various purification methods include the adsorption chromatography using avicel or cellulose (Emert *et al.*, 1974; Shoemaker *et al.*, 1983) and ion exchange chromatography (Eriksson and Petterson, 1975) and aqueous two-phase system (Sivers and Tejernald, 2000).

Generally a combination of ion exchange chromatography along with filtration, isoelectric focusing methods (Selby and Maitland, 1967) were employed for purifying cellulase components (Eriksson, 1978). Affinity chromatography using conconavalin -A (Gong *et al.*, 1979), cross liked cellulose (Wood and McCrae, 1986) and high performance liquid

chromatography have been employed for the purification of cellulase components (Fan *et al.*, 1980). The immunological properties for identifying the individual enzyme have also been developed (Nummi *et al.*, 1981).

The exo and endo glucosidases have been purified from different fungal strains like *Trichoderma reesei* (Shoemaker *et al.*, 1983); *Trichoderma koningii* (Wood and McCrae, 1979); *Phanerochaete chrysosporium* (Eriksson and Wood, 1985); *Fusarium solani* (Wood and McCrae, 1979) and *Penicillium filamentosa* (Tanaka *et al.*, 1995). The cellobiohydrolases is usually glycoprotein except the one obtained from *Sporotrichum* spp (Eriksson and Pettersson, 1975). The molecular weight usually varies from 42000 dalton (Berghem *et al.*, 1975) to 72000 (Gong *et al.*, 1979). The cellobiohydrolase exists both in mono and multi molecular forms (Wood, 1992). The multiplicity of the enzyme might be due to the varied sugar moiety in the carbohydrate chain of the glycoprotein (Beguin, 1990). The multimolecular form of cellobiohydrolase has been dealt in detail in the case of *Trichoderma reesei* and *Penicillium pinophilium* (Wood and McCrae, 1986).

The endoglucanase from *Trichoderma reesei* (Emert *et al.*, 1974; Beguin and Aubert, 1994), *Trichoderma koningii* (Wood and McCrae, 1972), *Fusarium* spp (Wood, 1969) and *Sporotrichum* (Erikkson and Pettersson, 1975) have been well characterized. The multiplicity is more profound among endoglucanase as compared to exoglucanase (Eriksson and Pettersson, 1975). The molecular weight endoglucanase isolated from *Trichoderma reesei* varied from 5000 -15000 (Selby and Maitland, 1967).

The purification of β - glucosidase has been reported by many workers (Berghem *et al.,* 1975; Reese, 1976; Gong *et al.,* 1977 and Wood, 1992) β - glucosidase has a broad molecular weight ranging from 47000 - 340000 (Beguin and Aubert, 1994). The β-glucosidase obtained from *Clostridium thermocellum* has been shown to be heat stable containing molecular weight of 50 kDa (Alzari *et al.*, 1996).

2.7 APPLICATIONS OF CELLULASE

Biotechnology of cellulases and hemicellulases began in the early 1980's; first in animal feed followed by food applications (Mandels, 1985). Potential applications of cellulases was in the production edible sugar from biomass for human consumption, production of alcohol (El-Nawawi and El-Karer, 1996). Single cell protein SCP and organic acids (Garg and Neelakantan 1982).

Cellulase, along with pectinase and hemicellulases, plays an important role in clarifying fruit juices. In baking industry, the combined use of cellulase with amylase helps in reducing the average size of polymers present in dough preparation (Voragen and Pilnik, 1989).

Cellulases were being employed for the extraction of oils. Emert *et al.,* (1974) reported that cellulases when incorporated along with pectinase have shown to increase the efficiency of oil extraction from sunflower kernels when compared with traditional process. Reports of Wood, (1992) convey that cellulases could increase the resistance for rancidity formation in olive oil extraction. Cellulase and hemicellulases have been used in pulp and paper

industry. It helps in solubilizing the fine, floarelary particles by decaying the small fibrils on the fiber surface (Jackson *et al.*, 1986).

The other potential application of cellulase includes (Mandels, 1985)

- Isolation of plants protoplast in association with xylanase and pectinase.
- Recovering agar agar from seaweed.
- Improving the solubility of raw materials in brewing.
- Saccharification of de-lignified woody waste in combination with xylanases.
- Using cellulase as pharmaceutical medicine for animals and human being
- Manufacturing diagnostic kit and ornamental things.

2.7.1 Role of cellulase in biostoning

In the late 1980's, commercial application of cellulases has been introduced in detergent industries and has gained general appreciation and increased use in early 1990s due to its function in brightening the colour and enhancing the softeners of cotton fabrics. Enzymes have been used in textile industry for desizing, scouring, polishing, washing, degumming, peroxide degradation in bleaching baths as well as for decolourisation of dyehouse wastewaters, bleaching of released dyestuff and inhibiting dye transfer (Gusakov *et al.*, 2001).

The use of cellulase, particularly for biostoning of denim jean and cotton fabrics in textile industry has increased considerably. In traditional stonewashing the abrasion process gives them a slightly worn look. This is mainly due to the presence of partially detached microfibrils. Accelerated abrasion done using acidic cellulase results in loosing of unwanted fibrils and gives smoother surface, original colour and brightness to the garment (Montegut *et al.*, 1991).

Before the advent of stone washing, breaking in a new pair of blue jeans meant weeks of stiffness and discomfort, since new denim feels more like a suit of shiny blue armor than the centerpiece of the casual wardrobe. The introduction of stonewashed jeans changed all that. By pre-washing the jeans with highly abrasive stones, the stiff denim fabric could be weakened and softened. Stone washing provided an instant, off-the-shelf, comfortable fit and a slightly worn look that could be paid for with cash rather than prolonged suffering (Cavaco-paulo *et al.*, 1998).

Washing with stones seems simple enough, but it is actually fraught with problems for the manufacturer. The stones actually small pebbles are pumice, which is highly abrasive volcanic rock. One to two kilograms of stones are needed for each pair of jeans, occupying up to half the washing machine load and limiting the number of pairs that can be treated in each cycle. The stones abrade the machinery as well as the denim, leading to significant repair and replacement costs. It is difficult to precisely control the abrasive action, and up to several percent of the clothes in each load are ruined by excess wear. At the end of the treatment, pebbles must be removed

from the jeans by hand, further increasing costs. And finally, pumice stones lose up to half their weight during one wash cycle, creating grit that clogs drains and generates mountains of sludge requiring disposal (Mehta *et al.*, 1990).

The use of cellulase, particularly used for biostoning of denim jean and cotton fabrics in textile industry has increased considerably. In traditional stonewashing the abrasion process gives them a slightly worn look. This is mainly due to the presence of partially detached microfibrils. Accelerated abrasion done using acidic cellulase results in loosing of unwanted fibrils and gives smoother surface, original colour and brightness to the garment (Clarkson *et al.*, 1994).

Jeans are made from cotton, of course, which is virtually pure cellulose. Cellulase digests cellulose into simpler carbohydrates. Treating a new pair of jeans with cellulase breaks down the stiff cellulose fibers, and loosens some of the indigo dye as well.The result is the same soft, slightly faded look of "stone-washed," without the stones. "Biostoning," as it is called, was introduced in Europe in 1989 and was first used in the United States in 1990 (Heikinheimo *et al.*, 1998).

Cellulases have been developed for a range of temperatures and pH's, and they are currently used not only for biostoning but also for achieving other types of finishes in a variety of fabrics. "Pilling," the raising of little balls of fabric on the surface of a garment after repeated washing, can be largely prevented by pre-treatment with cellulase. Clumping of many fine

microfibrils left on the fabric after final treatment causes pills. These microfibrils are perfect targets for rapid cellulase treatment, since their very high surface area allows the enzyme to attack quickly from all sides, while leaving the base fabric relatively untouched. With their ability to break down stubborn plant fibers, cellulases also have applications in waste processing (Cavaco-Paulo and Almedia, 1994).

2.7.2 Role of cellulase in bioconversion

Biodegradation is one method that is employed by nature to reduce the accumulation of biomass in the environment, which imparts toxicity to the environment. Home (1757) was the first to carry out research in the way, which has developed into present day biomass degradation studies. it has long been suggested that cellulose containing biomass represents a vast renewable source that can function as substrate for ethanol production which, in turn, may be used as an alternative source for energy (Mandels, 1981).

Organic waste mainly waste paper material is a major component of the solid waste produced daily by households, offices, industry, and the agricultural sector. Typical examples of organic waste are grass and tree cuttings, vegetable peels, used paper materials, straw, and residues produced during the harvesting of agricultural products. In most cases, these waste materials are burnt or dumped, thus aggravating environmental pollution. It would, therefore, be of great environmental benefit if these waste materials could be recycled into other useful products relieving the strain on natural resources (Way man *et al.*, 1992).

Interest in the production of ethanol, fuel cleaner blended gasoline is increasing world over. Over the past 25 years, intensive research has been carried out on the production of ethanol from biomass occurring in the form of lignocellulosic residues. Ethanol could be used as a substitute for fossil liquid fuels, such as gasoline and diesel. Ethanol from the biomass has the advantage of being produced from a renewable resource and, further more give no net contribution to global warming (Christakopoulous *et al.*, 1993).

Enzymatic hydrolysis of lignocellulosic agro wastes to get net fermentable sugars and ethanol involves less engineering problems than acidic hydrolysis. There fore, intrest in enzymatic hydrolysis of lignocellulosic wastes have increased lately. (Eriksson and Wood, 1985). The sugars obtained by hydrolysis of cellulosic wastes, represent an intresting substrate for ethanol production in terms of availability and cost (Ryu and Mandels, 1980).

Materials & Methods

3. MATERIALS AND METHODS

3.1 ISOLATION AND SCREENING OF CELLULOLYTIC FUNGAL STRAINS

3.1.1 Site of material collection

The study area belongs to the Cauvery delta regions of Thanjavur, Trichy, Thiruvaroor, Nagapattinam, Perambalur and Pudukottai districts. This delta is situated in South India, which is one of the oldest delta areas in Asia. The holy river Cauvery starts its origin in Medikere of Karnataka and flows through Tamilnadu and completes the course by joining the Bay of Bengal forming a wide delta from Thanjavur onwards. The study area, starting with an apex at Grand anaicut spreads over 70 kilometers and gradually widens to a base of about 250 kms at the sea coast and lies between latitudes 9° 50' and 11° 25' and longitudes between 78° 50' and 80°, formed by the river Cauvery with its five distributaries (Fig. 1).

3.1.2 Collection of samples

From different locations such as Adhanur, Budalur, Thiruvaiyaru, Papanasam, Alangudy, Nedumbalam, Uppiliyapuram, Aduthurai, kalathur, Vallam, Irugur, Musiri, Omanur, Pilamedu, Solagampatti, Alathur, Mannachanallur, Vayalogam, Srirangam, Perambalur, Manaparai, Madhukkur, Kallivayal, Padugai, Kivalur, Kodavasal, Musiri, Peravurani, Pudukottai, Arantangi, Nagapattinam, Kallayam, Sethi, Palaviduthi, Sirkali,

Sikar, Thirvengadu and Kohur samples like field soil, decayed wastes, garden soil, and effluents from paper mill premises, were collected during the summer season (April – May 2001) using aseptical techniques (Parkinson *et al.*, 1971).

3.1.3 Isolation of mycoflora

Mycoflora were isolated by dilution plate technique (Waksman, 1922) at 10^{-4} dilution on Czapex dox agar (Purivis *et al*, 1964). The culture plates were incubated at room temperature for seven days. After the incubation period, micro fungal population was enumerated and purified by hypal tip method (Ricker and Ricker, 1936) and stored in Czapex dox agar slants under refrigeration.

The semi permanent slides for the isolated fungal strains were prepared using lactophenol cotton blue stains (Rajendran *et al.*, 1994). Some of the fungal species were photographed using photomicrographic unit Leitz Diaplan (Germany). The fungal strains were identified with the help of Dr. M.N. Abubacker, Professor and Head, Department of Botany, National College, Trichy, Tamilnadu, India, as per the guidelines given in the manual compendium of soil fungal strains (Domsch *et al.*, 1980).

3.1.4 Screening of fungal strains for cellulase production

Isolated fungal strains were screened for cellulase activity by growing on Czapek Dox agar supplemented with 1% microcrystalline cellulose powder, instead of sucrose, and incubated at room temperature for

7 days. Cellulolytic strains were identified on the basis of clear zone formation (Rajendran *et al.*, 1994).

To screen maximum cellulase producers, cellulolytic fungal strains were cultivated in 20 ml of screening broth medium in a 100 ml Erlenmeyer flask and incubated on an orbital shaker at 30°C for 7 days at 150 rpm. Spent samples from duplicate flasks were taken daily and monitored for analytical studies (Rajendran *et al.*, 1994).

3.1.5 Analytical studies

Analytical studies such as assays for cellulase and xylanase enzymes, soluble protein content, and fungal biomass were performed as per the method described below.

a) Enzyme assay

Assay of cellulase and xylanase was done by measuring the release of reducing sugars by dinitrosalicylic acid (DNS) method of Miller, (1959) with glucose as standard.

Endoglucanase

Carboxymethyl cellulase activity was determined according to the method of Mandels *et al.*, (1976). The assay mixture contained 0.5 ml of 0.05 M citrate buffer (pH 4.8), 0.5 ml of enzyme and 0.5 ml of 2% carboxymethyl cellulose. The mixture was incubated for 30 min at 50°C along with enzyme blank and heat inactivated enzymes as controls. The reaction

was terminated, by adding 3.0 ml of DNS reagent. The tubes were kept in boiling water bath for 5 min cooled and 20 ml of distilled water was added. The reducing sugar liberated during the reaction was measured at 540 nm. One international unit (IU) is defined as μ moles of glucose liberated per min under assay conditions.

Exoglucanase

Filter paper activity was assayed according to the methodology adopted by Mandels *et al.,* (1976). The assay mixture contained 50 mg filter paper strip (Whatman No.1, 60mm x 40mm), 0.5ml of suitably diluted enzyme and 1.0 ml of 0.05 M citrate buffer (pH 4.8). The mixture was incubated for 1 h at 50°C along with enzyme blank and heat inactivated enzyme as controls. The reaction was terminated, by adding 3.0 ml of DNS reagent. The tubes were kept in boiling water bath for 5 min, cooled and 20 ml of diluted water was added. The reducing sugar liberated was measured at 540 nm and quantified by using standard curve for glucose. One international unit is defined as μ moles of glucose liberated per min under assay conditions.

β - glucosidase

The assay was carried out as per the procedure described by Wood and Bhat, (1988). The reaction mixture contained 0.7 ml of 0.1 M sodium acetate buffer (pH 4.8), 0.2 ml of 1 mM p – Nitrophenyl-β-D–glucopyranoside and 0.1 M ml of enzyme. The reaction mixture was

incubated at 50°C for 30 min and terminated by adding 3 ml of glycine NaOH buffer (pH 10.8). The liberated para nitrophenol was measured at 420 nm. The enzyme activity is defined as μ moles of pare nitrophenol liberated per min under assay conditions.

Xylanase

Xylanase activity was assayed as per the method described by Shamala and Sreekantiah, (1985). To 0.5 ml of sodium acetate buffer (0.05M, pH 5.5), 1.0 ml of 1% larch wood xylan and 0.5 ml of enzyme was added and incubated at 50°C for 30 min. The reducing sugar liberated was estimated by using DNS method. One international unit of xylanase activity was defined as μ moles of reducing sugar liberated per min under assay conditions.

b) Protein content

Lowry *et al.*, (1951) method was used for estimating the soluble protein content of the filterate taking bovine serum albumin as standard.

Reagent A

2% Sodium carbonate in 0.1 % sodium hydroxide.

Reagent B

0.5% Copper sulphate in 1% sodium tartarate.

Reagent C

To 50 ml of solution A, 1 ml of solution B was added.

Folin's phenol reagent

The commercial Folin's phenol reagent was diluted in the 1: 2 ratio with glass-distilled water. This reagent was freshly prepared.

To 0.5 ml of the supernatant, 2 ml of 10% trichoroacetic acid was added and incubated for 60 min. After the incubation period, the sample was centrifuged at 15,000 rpm for 30 min. The supernatant was discarded and to the pellet 5 ml of solution C was added. After 10 min, 0.5 ml of diluted Folin's phenol reagent was added and the tubes were shaken vigorously in a cyclomixer and incubated in the dark for 30 min for colour development. The intensity of colour developed was read at 500 nm in a spectrophotometer.

c) Biomass

Dry weight of the mycelium was determined by filtering the culture broth through a pre-weighed Whatman. No.1 filter paper, and washed with distilled water. The filter paper along with mycelia was dried over night at 60°C and weighed to a constant weight. The growth was expressed as mycelial dry weight (g/l).

3.2 PRODUCTION OF CELLULASE AND XYLANASE

3.2.1 Submerged fermentation

Submerged fermentation was carried out under shake flask cultivation (Rajendran *et al.*, 1994). To enhance cellulase and xylanase production various parameters such as carbon, nitrogen, pH of the medium and fermentation temperature were optimized as follows.

a) Effect of various carbon sources on cellulase production

To find out the suitable carbon source for maximum cellulase production, 0.5g of 10 defined carbon sources such as microcrystalline cellulose, carboxymethyl cellulose, solka floc, glycerol, pectin, cellobiose, xylan, filter paper, cotton, cellodextrin and 7 natural cellulosics namely wheat bran, rice bran, paddy straw, sugarcane bagasse, cotton seed, paper sludge and black gram husk, were added to 20 ml of Czapek's Dox mineral medium, instead of sucrose in separate 100 ml flasks and sterilized by autoclaving. Inoculation was performed with the sporulating agar block (5 X 5 mm) and incubated at 30°C and shaken at 160 rpm/h on an orbital shaker for seven days. A set of flasks was taken at every 1 d interval and supernatant obtained by centrifugation was used for analytical studies following the methods described above.

b) Effect of concentration of carbon source on cellulase production

Experiments were conducted to determine the optimum substrate level by supplementing the selected carbon source at varied concentrations (0.5 – 7%) to Czapek's Dox mineral medium and inoculated for enzyme production. Enzyme assays were performed at every 1 d interval for 7 days.

c) Effect of various nitrogen sources on cellulase production

To study the effect of various nitrogen sources on the production of cellulolytic enzymes, instead of sodium nitrate 0.2 g of seven different

nitrogen sources such as yeast extract, peptone, triptone, ammonium sulphate, ammonium nitrate, ammonium phosphate and urea were added to Czapek Dox broth medium containing optimized carbon source. Enzyme production and enzyme assay methods were followed as described above.

d) Effect of concentration of nitrogen source on cellulase production

To investigate the effect of different concentration of nitrogen source on the production of cellulase and xylanase enzymes, selected nitrogen source at varied concentration (0.1 - 1%) was added to the 20 ml of Czapek's Dox mineral medium that contained optimized carbon source. Inoculation, incubation and enzyme activity measurements were made as described above.

e) Effect of pH on enzyme production

20 ml of Czapek's Dox medium containing the selected carbon and nitrogen, with initial pH ranging from 3.0-8.0 were taken in 100 ml ErlenMeyer flasks. Inoculation, incubation and enzyme assays were carried out as mentioned in previous experiments.

f) Effect of temperature on cellulase production

At various temperatures *viz.* 20 - 45°C assays studies were executed using the selected fungal strains on optimized medium. The optimum incubation temperature for enzyme production was determined by incubating the inoculated flask from the results of enzyme assays.

g) Estimation of co produced enzymes

In addtion to cellulase and xylanase assay supernatants were also assayed to find out the activity of co-produced enzymes such as amylase, pectinase, phytase, α - galactosidase, amyloglucosidase, protease, and chitinase.

Amylase

The amylase activity was estimated as per the method of Smith and Row (1949), 0.9 ml of 1% soluble starch was incubated with 0.1 ml of enzyme extract at 50°C for 10 minutes. After incubation, 1 ml of 1N hydrochloric acid and 0.1 ml of iodine solution (3%) were added and the volume was up to 25 ml with distilled water and the optical density was read at 620 nm in a spectrophotometer. The blank was prepared using the buffer instead of 0.1 ml of enzyme. One IU of amylase activity was defined as the amount of enzyme, which will produce 10 % fall in the intensity of the blue colour of starch-iodine complex under the assay condition.

Pectinase

Pectinase activity was measured using reducing sugar method (Miller, 1959). In pectinase assay, 1% pectin was used as substrate. Galactouronic acid standard was used for determinate of pectinase activity. One IU of pectinase activity was defined as the amount of enzyme that liberates 1μ mole of reducing sugar per minute under the assay condition.

Phytase

The phytase activity of the enzyme extracts was measured using the method of Heinonen and Lahti (1981). The enzyme extract (0.02 ml) was mixed with 0.1 ml of 15 mM sodium phytate (Sigma) and 0.35 ml of 0.2 M acetate buffer (pH 5.0). The mixture was incubated at 42°C for 30 min on a water bath. After incubation 4 ml of AAM (Acetone – Acid – Molybdate) solution was added. The contents were vortexed and 0.4 ml of 1 M Citric acid was pippetted into each tube to arrest the reaction. After mixing again, the yellow colour developed was measured at 420 nm in a 2000Z double beam spectrometer. The blank was run simultaneously without enzyme under identical conditions. A standard was prepared by using potassium dihydrogen orthophosphate (KH_2PO_4), from which the quantity of enzyme was calculated.

One IU of phytase activity was defined as the amount of enzyme that liberates 1μ mole of inorganic phosphorus per minute under standard assay conditions.

α - galactosidase

α - galactosidase activity was assayed as per the methodology of (Srinivas, 1996). The assay mixture contained 0.6 ml of para-nitrophenyl-α - D-galactosidase (8 μ moles), 0.3 ml of citrate – phosphate buffer (0.4 M, pH 4.5) and 0.1 ml of suitably diluted enzyme. The mixture was incubated at 50°C for 10 min and the reaction was stopped by the adding 5 ml of 0.1 M sodium carbonate. The release of para nitrophynol was measured at 405 nm.

One unit of enzyme activity is defined as the amount of enzymes required to liberate 1μ mole of para nitro phenol per minute under assay conditions.

Amyloglucosidase

Amyloglucosidase activity was determined based on its action on soluble starch and the reducing sugar was estimated by DNS method (Miller, 1959). 0.2 ml of the culture filtrate was added to 5 ml of 4% soluble starch (0.2 M acetate buffer, pH 4.2) and was incubated at 60°C for 60 min. The reaction was stopped, by keeping the test tubes in a boiling water bath for 10 min. An aliquot was taken for reducing sugar determination. One IU of amyloglucosidase activity was defined as that quantity of enzyme which releases one mg of glucose liberated under the above assay conditions (Ramakrishna *et al.,* 1990).

Protease

The reactants, 2 ml of haemoglobin (1% in 0.05M sodium acetate buffer, pH 5.0) and 2 ml of enzyme were incubated for 30 min at 50°C. The reaction was terminated by the addition of tricholoroacetic acid (2 ml, 10%). After mixing on a cyclomixer, the tubes were centrifuged and the protein content of the supernatants was assayed by Lowry *et al.,* (1951) method. The unit activity is defined as the absorption measured at 450 nm by the action of one μ g L-tyrosine under the assay conditions used (Srinivas, 1996).

Chitinase

Culture supernatants were incubated with p-nitrophenyl-N-acetyl-glucosaminide (130 $\mu g/\mu L$) for 3 min at 30°C. Reaction was stopped by the addition of 1 M NaOH. The released p-nitrophenol was measured spectrophotometrically at 410 nm. The enzymatic activity is expressed as μM of p-nitrophenol released per minute. Blanks were carried out using boiled enzyme, and sterile culture medium (Pan *et al.*, 1991).

3.2.2 Solid state fermentation

A solid state fermentation system for cellulase and xylanase production was developed and its culture conditions were optimized (Ray *et al.*, 1993; Raimbault and Alazard, 1980)

a) Effect of substrates on cellulase and xylanase production

To find out the suitable substrate for maximum enzyme production, 5 g of various agricultural residues such as wheat bran, rice bran, cotton seed husks, sugarcane bagasse, corn cob, paper sludge, black gram husk, saw dust and paddy straw substrates were taken in a 250 ml Erlen meyer flask separately and autoclaved, moistened to 40% using sterile distilled water and inoculated with 1 ml of 3 day old shaken culture. Spent medium samples were drawn every day and 1g of the dried substrate was used for biomass measurements in terms of chitin content and the rest of the sample was used for enzyme and soluble protein assays.

Extraction of enzyme was performed by mixing the fermented solid substrate with 20 ml of distilled water and shaken for 1 h to liberate the adsorbed enzyme from the substrate. Then the aqueous solution was filtered thoroughly with a Whatman No.1 filter paper and centrifuged at 15,000 rpm for 10 min at 4°C. The brownish clear supernatant solution was stored under refrigeration for further analysis (Babu and Satyanarayana, 1995).

Biomass

As biomass in SSF cannot be determined directly as mycelial dry weight, chitin, the principal component of fungal cell wall was measured

Chitin content

For chitin estimation fungal spent medium was hydrolyzed with 6N HCl for 4 h at 100°C (Phillips and Gordon, 1989). The acid water was filtered and evoprated to dryness at 45 - 50°C. The dry hydrolysates were then redissolved in 10 ml of distilled water. The resultant aminosugar in the hydrolysate solution was estimated by the method of Chen and Johnson (1983).

Reagents

- Acetyl – acetone reagent – 4% acetyl acetone in 1.25 N sodium carbonate (v/v).

- Ehrlich reagent – 1.6 g of N – N dimethyl para aminobenzadehyde in 60 ml mixture of ethanol and Conc. HCl (1 : 1 v/v).

One ml of the hydrolysate in a teflon lined screw cap tube. 1 ml of acetylacetone reagent was added and heated at 90°C for 10 min. After cooling, 4 ml of 96% ethanol and 1 ml of Ehrlich reagent were added and left undisturbed for 45 min. Then the sample was read at 530 nm. D – glucosamine was used as standard.

b) Effect of nitrogen source

The effect of various nitrogen sources on enzyme production, by selected fungal strains, was studied by adding different nitrogen sources such as, soya bean meal, alfa alfa, yeast extract, peptone, urea, triptone, ammonium chloride, ammonium nitrate, ammonium phosphate and sodium nitrate with 40% moistened substrate at 0.5% level and incubated at room temperature after inoculation. Enzyme activities were measured at every 1 d interval, from the day of incubation, over a period of 7 days.

c) Effect of moisture level

To find out the optimum moisture content for the production of cellulolytic enzymes, moisture level of the solid substrate was kept varied between 20 – 80% and fungal cultivations were made in optimized carbon and nitrogen medium and enzyme activities were measured at every 1 day interval from the day of inoculation over a period of 7 days.

d) Effect of pH on cellulase and xylanase production

The effect of pH on the production of cellulolytic enzymes was studied by growing the fungal strains on the solid substrate in the pH range of 3.0 - 7.0. Enzyme activities were measured at every day interval from the day of inoculation as per the procedure described above.

f) Effect of temperature on enzyme production

The optimum temperature for maximum production of cellulolytic enzymes was determined, by performing the enzyme production studies by the selected fungal strains at different temperatures *viz* 25, 30, 40, 45 and 50°C. The pH and moisture content of the substrate were adjusted to its optimal level. The enzyme extraction was carried out from the next day of inoculation, and enzyme activities were estimated.

g) Estimation of co produced enzymes

The crude enzyme extract from cellulolytic fungal strains fermented solid substrate was assayed for the co produced enzymes such as amylase, pectinase, phytase, α - galactosidase, amyloglucosidase, Protease assay, and chitinase activities over a period of 7 days.

h) Effect of solvents on cellulase and xylanase extraction

From the fermented substrate, crude enzyme was extracted with different solvents like 2% calcium chloride ($CaCl_2$ solution), 0.1% Tween 80, 0.1% Triton X 100, 5% glycerol, 10 mM Phosphate buffer, 40% ethanol,

distilled water and tap water in 1: 2 solid to solvent ratio (Shamala and Sreekantiah, 1985). The enzyme was extracted twice and pooled for assaying enzyme activities.

i) Effect of different levels of water on cellulase and xylanase extraction

Enzymes from the spent substrate were extracted with selected solvent in different ratio (solid solvent at the ratio 1: 5, 1: 10, 1: 15, 1: 20 and 1: 25). The enzyme was extracted twice and the pooled extract was assayed for cellulolytic activities.

3.3 STRAIN IMPROVEMENT BY MUTATION

In order to increase the cellulase yield, mutation studies were tried with both physical (UV) and chemical mutagens (NTG) as described by Vipul and Sheela, (1997).

3.3.1 Collection of spores

The maximum cellulase producing strain was selected for mutation studies. The organism was grown on potato dextrose agar for 7 days and the spores were harvested in sterile saline.

3.3.2 Mutation by NTG

2 ml of spore suspension (10^8 spores/ ml) was treated with different levels of NTG (Sigma) (50, 100, 150 and 200 μg/ml) for 1 h at room temperature, with intermittent shaking. The spores were then washed and

suspended in sterile distilled water, and plated on potato dextrose agar medium containing 1% Triton x100 as colony restrictor. To determine the killing pattern, serial dilution was made and plated on to potato dextrose agar medium with. The number of survivors was plotted against the concentration of NTG used.

3.3.3 Mutation by UV

5 ml of spore suspension was subjected to UV irradiation for different time periods *viz.* 15, 30, 45, 60 min, in an open sterile dish using a germicidal lamp (UV – 260 nm, 8W lamp) at a distance of 25 cm. After irradiation 0.1 ml of the irradiated spores were spread plated on cellulose agar medium. The kill pattern was obtained by plotting the number of survivors was plotted against the UV exposure periods (min).

3.3.4 Selection and screening of mutants

The single colonies obtained on potato dextrose agar medium, were transferred to screening cellulose agar medium and incubated for 4 days. Duplicate plates were stained with congo red for the development of clear zone (Tansey, 1971) and halo zone diameter/colony diameter ratio was measured. Hyper cellulolytic mutants were initially selected on the basis of the diameter of the hydrolysis zone surrounding the colonies.

3.3.5 Enzyme production by mutants

To study the enzyme production, mutant strains were grown in optimized solid state fermentation conditions for 7 days. Spent samples from

duplicate flasks were drawn every day for assaying enzyme activity, soluble protein and fungal biomass.

3.4 DETERMINATION OF ENZYME PROPERTIES

To study the effect of pH and temperature on cellulolytic activity and stability for that supernatant in both fermented submerged and solid medium were assayed.

a) Effect of pH on enzyme activity

To study the effect of pH on cellulolytic enzymes activity supernatants obtained from both submerged and solid media were taken as enzyme source and enzyme assays were performed in varied pH range of 4.0 – 6.0 using acetate buffer (0.2 M).

b) Effect of temperature on enzyme activity

The effect of temperature on enzyme activity was studied by assaying cellulase and xylanase activity at various temperatures (30 - 70°C).

c) Metal ions on cellulase and xylanase activity

20 different metal ions at 1 – 10 mM concentration were incubated with the reaction mixture for 30 min. After the incubation period, cellulase and xylanase assays were performed to determine the effect of metal ions in enzyme activities.

d) Effect of pH on cellulase and xylanase enzyme stability

To study the effect of pH on the stability of cellulase and xylanase enzymes, reaction mixtures containing 0.5 ml of enzyme solution and 0.5 ml of acetate buffer at various pH levels (4.0 – 7.0) various buffers – were incubated at room temperature for 60 min.

e) Effect of temperature on cellulase and xylanase stability

To study the influence of temperature on the stability of cellulolytic enzymes, the enzyme solution was kept incubated at various temperatures from 40 - 70°C for 60 min. the activity of the enzyme was assayed by DNS method.

3.5 PURIFICATION OF FPase

Cellulase enzyme produced by the hyper cellulolytic mutant strain, under SSF, was extracted from the fermented solid substrate, and centrifuged at 10,000 rpm for 20 min at 4°C. The clear supernatant was taken as enzyme source. The crude enzyme was purified by the method proposed by Ulger and Saglam, (2001) in a stepwise process.

Step – 1

Filtrate (3: 1, v/v). The mixture was stirred and kept at 4°C for 12 h, then centrifuged at 8000 rpm under cooling for 20 min. The supernatant was discharged and the precipitated enzymes was washed with acetone and then dried to an acetone free preparation. The precipitation was dissolved in

20 mM acetate duffer (pH 6.3) and dialysed overnight against 20 mM phosphate buffer (pH 7.2) and purified by the following DEAE chromatographic method.

Step –2 : Ion exchange chromatography on DEAE Sephadex A - 50

The dialysed ammonium sulphate fraction was carefully layered on the top of a pre-equilibrated DEAE Sephadex A - 50 column (50 x 2.5 cm) and protein were eluted with 600 ml 20 mM phosphate buffer (pH 7.2) containing a linear gradient of sodium chloride (0.0 – 0.5 M) at a flow rate of 1.0 ml /min at 4°C. Fractions (0.5 ml) were collected. The fractions showing maximum enzyme activity were concentrated to a minimum volume and dialysed against 20 mM phosphate buffer (pH 6.5) at 4°C.

3.6 Characterization of purified FPase enzyme

a) Effect of pH, temperature and metal ions on purified FPase

The pH, temperature and metal ion optima for the FPase enzyme was determined as per the method described above.

b) Determination of Km and Vmax

A constant volume of enzyme (0.2 mg/ml) was incubated with different concentrations of avicel (0 - 32 mg/ml in 0.05 sodium citrate buffer; pH - 5.0), at 50°C for 30 min. Cellulase activity was determined for the samples at different concentrations of the substrate. K_m and V_{max} values were determined from Lineweaver – Burke plot, (Lineweaver, 1934).

c) Carbohydrate estimation

In order to estimate the carbohydrate content of the purified enzyme, the method of Dubbois *et al.*, (1956), was employed. To 0.5 ml of sample, 0.3 ml of 5% phenol and 1.8 ml of concentrated sulphuric acid was added and the content were mixed immediately. After the tubes were cooled to room temperature the absorption of the coloured solution was measured at 485 nm. Glucose was used as standard.

d) Determination of molecular weight

The molecular weight of the purified cellulase enzyme was determined by sodium dedecyl sulphate - polyacrylamide gel electrophoresis (SDS - PAGE) (Laemmli, 1970).

Reagents

Stock acrylamide solution		
Acrylamide	-	30 g
Bisacrylamide	-	0.8 g
Water to	-	100 ml
Separating gel buffer		
Tris HCl	-	22.7 g
Water to	-	100 ml
pH	-	8.8

Seperating gel (10%)

Stock acrylamide solution	-	13.3 ml
Separating gel buffer	-	8.0 ml
Water	-	18.1 ml

The gel mixture was degassed on a water pump for 3 - 5 min and then following were added.

Ammonium persulphate solution (5%)	-	0.2 ml
Sodium dodecyl sulphate (SDS)	-	0.4 ml
Solution (10%)		
N,N,N,N- tetramethylene diamine	-	20µl
(TEMED)		
Stacking gel buffer		
Tris- HCl	-	7.6 g
Water to	-	100 ml
pH	-	6.8

Stacking gel mixture (4%)

Stocking acrylamide solution	-	1.35 ml
Separating gel buffer	-	1.0 ml
Water	-	7.5 ml

The gel mixture was degassed for 3 - 5 min on a water pump and added with the following solutions

Ammonium persulphate solution	-	50 µl
SDS solution (10%)	-	100 µl
TEMED	-	10 µl
Electrode buffer		
Tris- HCl	-	12 g
Glycine	-	28.8 g
SDS	-	2 g
Distilled water	-	2 g
pH	-	8.2 – 8.4

Sample buffer (5X concentration)		
Stacking gel buffer	-	5 ml
SDS	-	0.5 g
Sucrose	-	5 g
Mercaptoethanol	-	0.25 ml
(0.5 w/v solution in water)		
Water	-	10 ml

Standard marker protein	Molecular weight (kDa)
Bovine serum albumin	- 66
Egg albumin	- 45
Glyceraldehyde –3- phosphate dehydrogenase	- 36
Carbonic anhydrase	- 29
Trypsinogen	- 24

The above marker protein samples were dissolved (1 mg/ml) in sample buffer for loading the well 25 µl was used.

Staining solution

Coomassie brilliant blue - 0.1%

Developer

Sodium carbonate solution - 80 ml

(3 g in 80 ml)

Sodium thiosulphate solution - 1.0 ml

Formaldehyde - 1.0 ml

Water - 100 ml

Stopper

Acetic acid solution

Thoroughly cleaned and dried glass plates and spacers were assembled properly and held together with bulldog clips in an upright position. White petroleum jelly or 2% agar (melted in a boiling water bath) was applied around the edge of the spacers to hold them in place and to seal the chamber between the glass plates.

The separating gel solution was poured in the chamber between the glass plates, layered on top with distilled water and left undisturbed for 30 - 60 min, for polymerization. After polymerization, the water layer was removed and the surface was washed with a little stacking gel solution. Then the stacking gel mixture was poured on the top and comb was placed in the stacking gel, the gel was left for setting (30 - 60 min). Once the stacking gel

was polymerized, the comb was removed without the disturbing the well and the gel was installed in the electrophoresis apparatus after removing the chip and agar. The tanks were filled sufficiently with electrode buffer and any air trapped at the bottom of the gel was removed.

The protein content of the sample was made uniform by using 5 X concentration of sample buffer. The samples were heated in a boiling water bath for 2 - 3 min. After cooling, 10 µl of sample was injected into the sample well using a micro syringe through the stacking gel and then run at 30 mA until the bromophenol blue (marker dye) reaches the bottom of the gel. After the run was complete, the gel was removed carefully from the plates, transferred to a clean plastic container and washed with washing solution with slow shaking for 10 min.

The wash solution was discarded and the gel was rinsed with plenty of water for 2 min and soaked in sodium thio sulphate solution (0.2%) for 1 - 2 min. Then the gel was washed twice with water, each time 1 - 2 min, drained and socked in the staining solution for 10 min with gently shaking and then again washed twice in water. When sufficient intensity of band developed, adding acetic acid solution stopped the reaction. The gel was photographed and stored in polyethylene bags.

e) Zymogram

Non denaturing alkaline polyacrylamide gel electrophoresis (PAGE) was performed by the standard method with 3.75% staking gel at pH - 6.8 and a 5% separating gel at pH - 8.8. For development of zymogram

the gels were electrophoresed at 4°C. The gels were flooded with the reaction mixture containing 10 ml of hydroxylammonium sulphate (neutralized with 1 M, KOH and pH adjusted to 6.3), 30 ml of 50 mM sodium [phosphate buffer, pH - 6.3 and 50 ml of distilled water and incubated at 37°C for 30 min. The gels were developed, by flooding them with the ferric chloride reagent (Mc Hale and Coughlan, 1981).

f) Spectral analysis

UV-absorbtion spectra

The absorption spectrum of purified enzyme sample in sodium acetate buffer, pH – 5.0 was recorded in the range of 200 – 300 nm using UV – 240 Spectrophotometer, Shimadzu.

Fluoresent spectra

The excitation and emission spectra of the purified FPase were taken in an Aminco-Bowman spectro fluorimeter. The emission spectrum was recorded after excitation of sample at 282 nm. The relative fluorescence intensities of the enzyme were recorded.

3.7 BIOSTONING BY PURIFIED FPASE

In this study the efficiency of purified FPase was tested for its usage in biostoning processes. Various parameters such as effect of pretreatment and microscopic observation were studied.

a) Effect of cellulase activity on cotton fiber

Enzymatic treatment of cotton fibre was experimented with a mono and multi component cellulases such as purified FPase and a commercial cellulolytic (Sigma) enzymes. 50 ml of 2% enzyme solution at pH 5.0 (50 mM acetate buffer) was added to 1 g of cotton fiber in a 250 ml Erlen mayer flask. After incubation for 4 h at 50°C, the reaction was stopped by increasing the temperature to 90°C for 10 min liquid portion was analyzed for reducing sugar released due to enzymatic hydrolysis by DNS method (Miller, 1959), and the cotton fibres were rinsed with distilled water air dried and weighed.

b) Effect of pretreatment

To increase cellulase activity, cotton fibres were subjected pretreatment with steaming at 100°C for 5 min, oxidation with 0.5% H_2O_2, alkaline treatment with 2N KOH at 30°C and strong alkaline treatment was performed with 25% NaOH at 5°C (Buchert *et al*, 2000). After pretreatments, cotton fibres were washed thoroughly with distilled water and exposed to cellulolytic activity.

c) Effect of cellulase on cotton fiber structure

After enzyme treatments, cotton fibers were washed thoroughly with distilled water. The fibrillation effect of cellulases on cotton fiber was examined under light microscope (60 x magnification). After staining with 0.1% congo red dye photomicrographs were taken.

3.8 BIOCONVERSION BY CELLULASE COMPLEX

In this experiment, the influence of various parameters such as different waste paper, concentration of waste paper, incubation hour, pH, incubation temperature and concentration of enzyme on bioconversion were investigated.

a) Bioconversion of different waste paper by cellulase

Bioconversion of different waste paper materials such as manuscript paper (unglazed fool scap paper), copier paper (used for photocopying), filter paper (Whatman No. 1), cardboard (used for packing) and newspaper were collected from a local paper industry. Reaction mixture containing pieces (40 mg) of each used paper and 1 ml of enzyme solution was incubated at 50°C for 30 min and then amount of reducing sugar released was estimated by DNS method (Miller, 1959).

b) Effect of cellulase on various concentration of waste paper

The substrate that was maximally bio converted was taken in different amounts (5 – 70 mg) and 1 ml of cellulase enzyme extract was added. After incubation for 30 min at 50°C, DNS method was followed to determine the optimum substrate concentration for maximum bioconversion in terms of amount of reducing sugar liberated during the reaction.

c) Effect of different concentration of cellulase on fixed amount of waste paper

Selected waste paper at optimum level was incubated with various enzyme concentration (0.01 – 0.3 mg/ml) and reducing sugar measurements were performed to find out the optimum enzyme concentration for maximum bioconversion.

d) Effect of incubation temperature on bioconversion

Reaction mixture containing, optimum substrate and enzyme levels, was incubated at different temperatures *viz* 10 - 80°C. Reducing sugar content in the assay mixture was measured after 1 h incubation.

e) Effect of pH on bioconversion

pH of the reaction mixture was varied between 2.0 – 7.5. The amount of reducing sugar released was examined after 1 h incubation.

f) Effect of incubation period

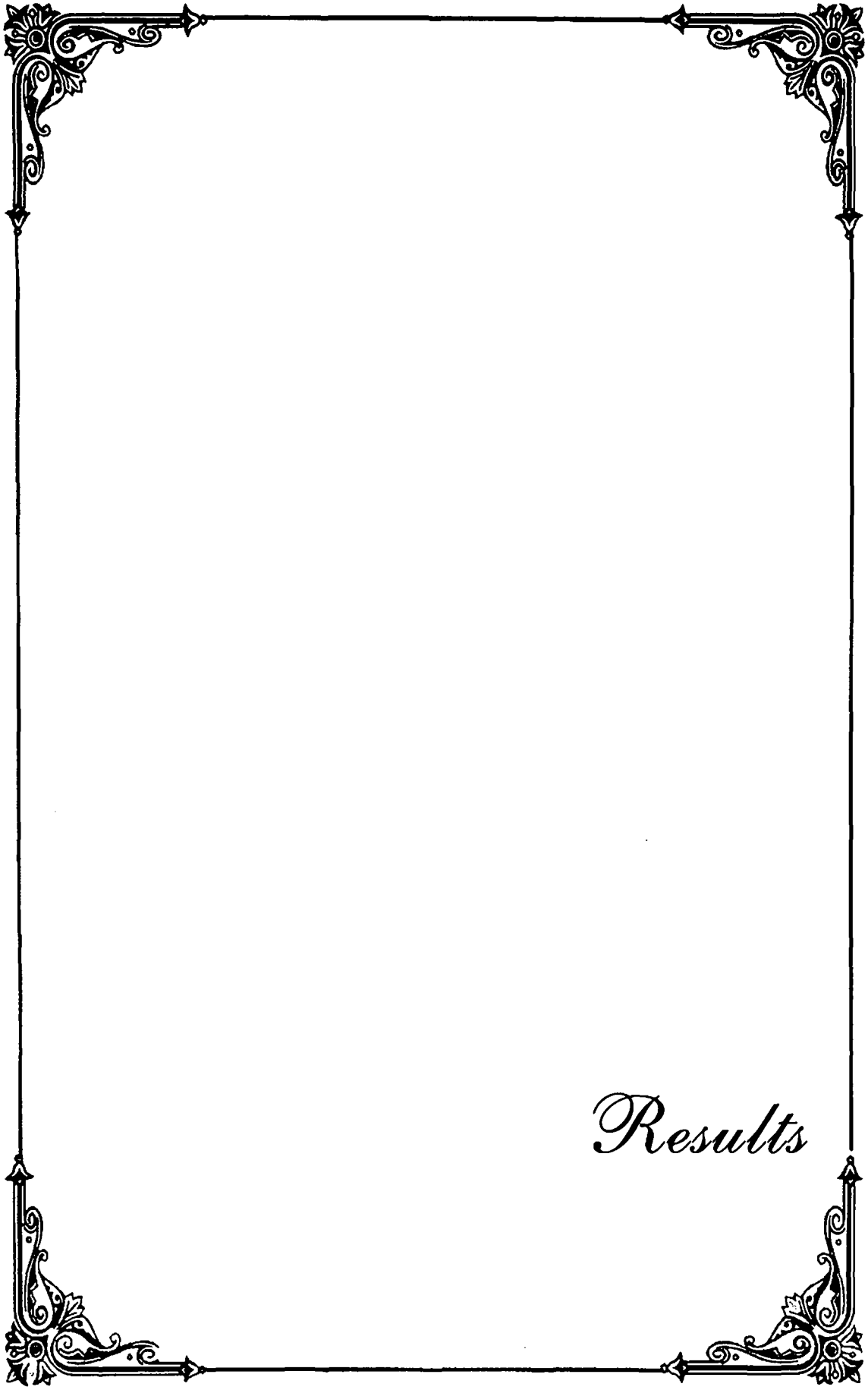
To find out the optimum incubation period, selected waste paper was subjected to bioconversion at optimum condition by the cellulase enzyme. The reaction mixture containing optimum concentrations of substrate and enzyme at pH 5.5 was incubated at 50°C for 1 – 10 h. The optimum incubation period for maximum bioconversion was determined by measuring the reducing sugar released during bioconversion.

3.9 STATISTICAL ANALYSIS

Data obtained in the present study when ever necessary was subjected to statically analysis such as ANOVA and T-Test were carried out with the help of SPSS (Statical Package for Social Sciences).

3.10 LITERATURE SURVEY

Necessary literatures were collected from Journals available in Libraries of Bharathidasan, Madurai Kamaraj, Bharathiyar, Madras, and Mysore universities. By accessing internet (PUBMED, NCBI, Sciencedirect.com and many other search engines) latest electronic publications have been gathered. Abstracts were obtained by CD-ROM search from National center for Science information (NISCOM) at Indian Institute of Science, Bangalore.


Results

4. RESULTS

In the present study, experiments were carried out for the selection of cellulolytic fungal strains by screening, optimization of submerged and solid state fermentations parameters for cellulase production, improvement of cellulase yield by mutation, purification and characterization of cellulase enzyme, evaluation of using cellulase in biostoning and bioconversion processes. The results are presented here.

4.1 SCREENING OF FUNGAL STRAINS FOR CELLULASE AND XYLANASE PRODUCTION

A total of 310 fungal strains were isolated from 49 samples *viz.* compost heap, field soil, garden soil, decayed agro waste, effluents from paper mills etc. collected from 38 sampling centres in Cauvery delta regions of Thanjavur, Trichy, Thiruvaroor, Nagapattinam and Pudukottai districts (Fig. 1).

All the 310 strains belong to the following 17 genera, with 33 species such as, *Aspergillus*, *Alternaria*, *Acremonium*, *Bipolaris*, *Cunninghamella*, *Chaetomium*, *Curvularia*, *Fusarium*, *Helminthosporium*, *Penicillium*, *Rhizopus*, *Sclerotium*, *Verticillium*, *Trichoderma*, *Trichosporium*, *Mucor* and *Umbellula*.

Aspergillus acculeautus (2 strains), *Aspergillus americana* (5), *A. fumigatus* (19), *A.flavus* (21), *A. niger* (44), *A. ustus* (6), *A. oryzae* (19), *A. terreus* (18), *A. versicolar* (4), *Alternaria alternata* (2), *Cunninghamella* spp (3), *Bipolaris* spp (2), *Chetomium* spp (2), *Curvularia* spp (4), *Acremonium* spp (3), *Fusarium oxysporum* (12), *Helminthosporium oryzae* (6), *Penicillium chrysogenum* (14), *P. citrinum* (6), *P. digitatum* (8), *P. japanicum* (10), *P. lanosum* (3), *P. notatum* (5), *Rhizopus nigricans* (2), *Sclerotium* spp (1 strain), *Trichoderma longibrachiatum* (18), *T. harzianum* (16), *T. koningii* (9), *T. viride* (38), *Verticillium* spp (1), *Trichosporium* spp (2), *Mucor* spp (4) and *Umbellula* spp (1).

Ability of these 310 strains, to produce cellulase enzyme was screened first on Czapek Dox agar containing cellulsose powder (1% w/v) as carbon source. Among 310 strains screened, only 140 strains showed clear zone formation (Fig. 28 & Table 1).

These 140 cellulolytic strains belong to the following 6 genera *Aspergillus*, *Fusarium*, *Helminthosporium*, *Penicillium*, *Rhizopus*, and *Trichoderma*, with 20 species as follows, *Aspergillus acculeutus* (1 strain), *A. americana* (2 strains), *A. fumigatus* (4), *A. flavus* (3), *A. niger* (25), *Aspergillus ustus* (2), *A. oryzae* (4), *A. terreus* (5), *A. versicolar* (2), *Fusarium oxysporum* (1), *Helminthosporium oryzae* (1), *Penicillium chrysogenum* (3), *P. digitatum* (5), *P. japanicum* (1), *P. lanosum* (2), *Rhizopus nigricans* (1), *Trichoderma longibrachiatum* (18), *T. harzianum* (17), *T. koningii* (9), *T. viride* (34) (Figs. 2 – 21).

Further screening of these 140 strains, for enzyme production, was carried out under shake flask cultivation, over a period of 7 days. From the results, following 9 strains, belonging to 2 genera and 3 species namely, *Aspergillus niger*AVCM1, *A. niger* SPCV93, *Trichoderma harzianum* AVCM 7, *T. harzianum* SPCV56 *T. harzianum* SPCV127, *T. viride* AVCM4, *T. viride* SPCV3, *T. viride* SPCV11 and *T. viride* SPCV41 (Figs. 22 – 27), showed maximum cellulase and xylanase production on 5th day (Table 1) Soluble protein and fungal biomass production by the 140 cellulolytic strains were also found to be maximum on 5th day. Finally, the above-mentioned 9 fungal strains were selected for enzyme production studies.

4.2 PRODUCTION OF CELLULASE AND XYLANASE

Production of cellulase and xylanase by 9 fungal strains was optimized in both submerged and solid state fermentations.

4.2.1 Optimization of parameters of Submerged fermentation

Industrial cellulase production has been done mainly through submerged fermentation. Optimized physico-chemical parameters enhanced the enzyme yield. In the present study, the effect of various submerged fermentation parameters such as, carbon source, nitrogen source, pH of the medium fermentation temperature and fermentation period on cellulase and xylanase production, by 9 fungal strains, was investigated over a period of 7 days. Results are presented in tables 2 – 13.

The effect of various defined substrates such as, avicel, carboxy methyl cellulose (CMC), solka floc, pectin, glycerol, cellobiose, xylan, filter paper, cotton and cellodextrin and natural cellulosic substrates like, wheat bran, rice bran, paddy straw, sugarcane bagasse, cotton seed, paper sludge and black gram seed husk, on cellulase and xylanase production, by the 9 fungal strains was studied.

Among the 10 defined substrates, *T. viride* SPCV3 produced high levels of CMCase (5.516 IU/ml), FPase (1.587 IU/ml), β - glucosidase (0.168 IU/ml) and xylanase (0.523 IU/ml) enzymes, when avicel was used as the carbon source, on 5th day. Next to avicel, substrates such as CMC, solka floc, cotton and filter paper induced moderate quantity of cellulase and xylanase enzymes in *T. viride* SPCV3. On the other hand, cellobiose and xylan substrates, induced specifically β - glucosidase and xylanase enzymes respectively. In glycerol, pectin and cellodextrin substrates, *T. viride* SPCV3 showed poor enzyme production. Similar enzyme inducing tendency of these defined substrates was observed in other fungal strains also (Tables 2 - 4).

The effect of 8 different natural cellulosic substrates such as wheat bran, rice bran, paddy straw, sugarcane bagasse, cotton seed, paper sludge and black gram husk, on enzyme production by 9 fungal strains was investigated. Results indicated that all the strains, showed the highest release of cellulase and xylanase enzymes on 5th day, when wheat bran was used as the carbon source (Tables 5 - 7). Enzyme production by all the fungal strains increased progressively up to 5 days of fermentation and then decreased (Fig. 29).

Among the 9 strains employed for cellulase production, *T. viride*SPCV3 ranked first with a maximum secretion of CMCase (10.319 IU/ml) FPase (2.523 IU/ml), β - glucosidase (0.150 IU/ml) and xylanase (1.318 IU/ml) enzymes. This was followed by other strains of *Trichoderma* and *Aspergillus* spp. Rice bran was the second carbon source that induced high enzyme yield by all the strains. In the other six substrates, enzyme production was comparatively less (Tables 5 - 7).

In the present study, soluble protein accumulation in the medium gradually increased till 5th day in all the substrates tested, with a maximum in wheat bran (Fig. 30), and afterwards a declination was observed. Among the 18 substrates tested, fungal growth was high in wheat bran on 5th day, and decreased thereafter (Fig. 31)

The influence of wheat bran at different percentage (0.5 – 8%) in the medium, on enzyme production was studied. The 9 fungal strains showed an increase in cellulase and xylanase yield with the increase wheat bran concentration up to 2%, on 5th day of fermentation (Figs. 32 - 34). Further increase in wheat bran concentration, decreased the enzyme yield.

In this study, the effect of 7 different nitrogen sources, such as yeast extract, peptone, urea, soya bean meal, sodium nitrate, ammonium phosphate and ammonium nitrate on cellulase and xylanase production by 9 fungal strains was investigated. Results revealed that, maximum enzyme production was achieved by these strains when yeast extract was used as the source of nitrogen. *T. viride* SPCV3, showed the maximum synthesis of

CMCase (10.458 IU/ml), FPase (3.000 IU/ml), β - glucosidase (0.180 IU/ml) and xylanase (1.608 IU/ml) enzymes on 5th day of incubation. Peptone, soya bean meal and urea showed moderate enzyme yield. The enzyme secretion was comparatively less, with other nitrogen sources tested (Tables 8 - 10).

The effect of yeast extract at different concentrations (0.1 – 1%) on enzyme production was investigated. Result showed that, with the increase in yeast extract concentration from 0.1 to 0.6%; there was a concomitant increase in cellulase and xylanase release by all the 9 fungal strains (Figs 35 - 37). Enzyme production decreased with further increase in yeast extract concentration. *T. viride* SPCV3, showed the highest yield of CMCase (10.551 IU/ml), FPase (4.038 IU/ml), β - glucosidase (0.183 IU/ml)and xylanase (1.634 IU/ml) enzymes on 5th day (Fig. 35). Hence 0.6% yeast extract was used in further experiments on enzyme production.

The effect of initial pH of the medium (3.0 – 8.0), on cellulase and xylanase production, by the 9 fungal strains was studied. Results indicated that, among the pH levels experimented, pH 5.0 was optimum for *T.viride* SPCV3 for the luxurious growth as well as maximum production of CMCase (10.617 IU/ml), FPase (4.064 IU/ml), β-glucosidase (0.244 IU/ml) and xylanase (2.179 IU/ml) enzymes, on 5th day (Figs 38 - 40). Similarly, in other fungal strains also, pH 5.0 was found to be optimum for the production of cellulase and xylanase enzymes (Figs. 38 - 40).

Enzyme production studied at different incubation temperatures revealed that, the 9 fungal strains yielded maximum cellulase and xylanase

enzymes, when grown at 30°C for 5 days (Tables 11 – 13). Among the 9 fungal strains investigated, *T. viride* SPCV3 had shown the maximum production of CMCase (10.618 IU/ml), FPase (4.065 IU/ml), β - glucosidase (0.230 IU/ml) and xylanase (2.038 IU/ml) enzymes. The profile also revealed that further increase in fermentation temperature above 30°C, relatively decreased the enzyme production. The same kind of relation was also observed with other fungal strains also (Tables 11 – 13).

In this work, the production of associated enzymes such as amylase, pectinase, phytase, α - galactosidase, amyloglucosidase, protease and chitinase by the 9 fungal strains were assayed. However, the data indicated that the above enzymes were produced only in negligible concentration.

4.2.2 Optimization of parameters of Solid state fermentation

Solid state fermentation plays an important role in commercial enzyme production from filamentous fungal sources. In the present investigation, for the production of maximum cellulase and xylanase by 9 fungal strains, following parameters of solid state fermentation such as, selection of substrate and nitrogen source; moisture level and pH of the medium; incubation temperature and extraction of enzymes from the fermented substrate were optimized. The results are presented in tables 14 – 23.

It is most convenient to use natural cellulosics as substrates for solid state fermentation. In the present study, 9 different and easily available agro industrial substrates namely, wheat bran, sugarcane bagasse, rice bran, paddy straw, paper sludge, saw dust, corn cob, cotton seed and black gram seed husk were investigated for maximum enzyme production. Out of 9 substrates tested, with wheat bran *T. viride*SPCV3 showed maximum of cellulolytic enzymes such as, CMCase (44.155 IU/g), FPase (12.080 IU/g), β - glucosidase (0.659 IU/g) and xylanase (8.635 IU/g) enzymes on 5th day of fermentation. The enzyme secretion was less in other strains tested with wheat bran (Table 14.). Studies on the fermentation time revealed that enzyme production by all the strains inoculated on wheat bran showed a steady increase up to 5 days, and quickly decreased there after (Fig. 41).

Next to wheat bran, a substantial amount of enzyme production was observed with paper sludge substrate (Table 14). However, the production was less with other substrates tested for enzyme production by all fungal strains (Tables 14 – 16).

The accumulation of soluble protein and fungal biomass on the wheat bran medium reached a maximum on 5th day and subsequently decreased with further incubations (Figs. 42 & 43). Based on the results obtained in the present study, for further experiments wheat bran was selected as the ideal substrate for cellulase production.

Nitrogen source in media is an important factor that affects enzyme production under solid state fermentation. In this investigation, various

nitrogen sources such as, soya bean meal, yeast extract, peptone, urea, triptone, caesin, ammonium chloride, ammonium nitrate, ammonium phosphate and sodium nitrate were added at 5% level to the wheat bran substrate. Results revealed that, among the 10 different nitrogen sources, maximum production of cellulase and xylanase by the 9 fungal strains was obtained with soya bean meal, on 5th day of fermentation (Tables 17 - 19). Next to soya bean meal, higher enzyme synthesis was observed when using yeast extract was as the source of nitrogen. Enzyme production was comparatively less with other nitrogen sources tested. Based on this study, 5% soya bean meal was selected as the best nitrogen additive for the simple wheat bran substrate to increase cellulase and xylanase production.

Moisture content of the substrate was an important factor that affected enzyme production under solid state fermentation. In the present study, the effect of different levels of moisture from 20 to 80%on enzyme production was studied. Results revealed that, with the increase in moisture content from 20 to 55%, there was a parallel raise in cellulase and xylanase production by all the 9 fungal strains. This 55% moisture level of the substrate was found to be optimum for maximum production of cellulolytic enzymes by the 9 fungal strains on 5th day of fermentation (Tables 20 – 22).

Among the 9 fungal strains tested, *T. viride* SPCV3 showed the highest production of CMCase (63.142 IU/g), FPase (17.274 IU/g), β-glucosidase (0.942 IU/g) and xylanase (12.348 IU/g) enzymes, on 55% moistened wheat bran – soya bean meal substrate. Further increase in

moisture level, gradually decreased the enzyme yield in all the fungal cultivations.

While studying the enzyme production by *Trichoderma* and *Aspergillus* strains at different hydrogen ion levels (3.0 – 8.0), all strains responded well for enzyme production at pH 5.0. Above and below this optimum pH, enzyme synthesis was found to be less. Among the 9 fungal strains, *T. viride* SPCV3, showed maximum yield of CMCase (71.350 IU/g), FPase (19.519 IU/g), β-glucosidase (1.065 IU/g) and xylanase (13.953 IU/g) enzymes, on 5th day of fermentation (Figs. 44 – 46).

In order to establish the optimum incubation temperature for cellulase and xylanase production under solid state fermentation, experiments were carried out at different incubation temperatures *viz* 25, 30, 35, 40, 45, 50 and 55°C. At 30°C incubation, *T. viride* SPCV3 showed the highest secretion of CMCase (83.480 IU/g), FPase (22.838 IU/g), β-glucosidase (1.106 IU/g) and xylanase (15.325 IU/g) enzymes on 5th day. Further increase in incubation temperature beyond 30°C, a rapid fall in enzyme yield was observed. Similarly, 30°C was also found to be the optimum incubation temperature for maximum enzyme production by other fungal strains used in this study (Figs. 47 – 49).

Cellulase producing fungal strains were also tested for the production of some hydrolytic enzymes such as amylase, pectinase, phytase, lignase, α- galactosidase, protease, amyloglucosidase and chitinase under optimized solid state fermentation medium. Results indicated that, among the

seven associated enzymes studied, amylase production was maximum in all the strains, whereas chitinase and phytase production was maximum in *T. harzianum* and *A. niger* strains. However, protease production by all the strains was negligible. *T. viride*SPCV3 showed the optimum release of amylase (4.055 IU/g), pectinase (3.345 IU/g), phytase (0.935 IU/g), α-galactosidase (1.006 IU/g), protease (0.023 IU/g), amyloglucosidase (2.867 IU/g) and chitinase (1.584 IU/g) on third day (Table 23).

Extraction of cellulase and xylanase enzymes using different solvents such as 0.1% Tween80, 20% ethanol, sterile water, acetate buffer (pH 4.8, 5.0 and 5.5), 2% calcium chloride, 0.1% Triton X 100 and 5% glycerol was studied on 5^{th} day of incubation. Among the 9 different solvents used, maximum enzyme extraction from all the spent media was achieved with 0.1% Tween 80 (Figs. 50 - 52). Hence 0.1% Tween80 was found to be the suitable solvent for enzyme extraction.

The effect of different ratio (1: 5 – 1: 25) of 0.1% Tween80 to substrate on cellulase and xylanase extraction was studied. Maximum extraction was achieved at 1: 15 level from the spent medium of 9 fungal strains (Figs. 53 – 55).

4.3 MUTATION STUDIES FOR STRAIN IMPROVEMENT

Strain improvement by mutation, is an essential and important part of any economic bioprocess development. Production costs of cellulase could be reduced by developing strains with increased productivity on cheaper substrates and with other unique and desirable characteristics.

In the present study, attempts were made to obtain hyper cellulolytic mutant from the wild type strain *T. viride* SPCV3 that showed maximum cellulase production under SSF. *T. viride* SPCV3 was subjected to both physical (UV) and chemical (NTG) mutagenic treatments (Figs. 56 – 57). The enzyme production by mutants was monitored over a period of 7 days and the results were presented.

Based on plate assay method 4 UV mutants of *T. viride* SPCV3 namely, SPCV3M1, SPCV3M2, SPCV3M3 and SPCV3M4 were isolated. They were grown on optimized solid state fermentation conditions used for the wild type. Among them, the mutant *Trichoderma viride* SPCV3M1 (Figs. 58 – 59) showed maximum production of CMCase (108.523 IU/g), FPase (29.689 IU/g), β-glucosidase (1.620 IU/g) and xylanase (21.222 IU/g) enzymes on 4^{th} day of incubation, whereas, in other mutants, cellulase yield was not enhanced (Fig. 60).

In the present study, fungal growth and soluble protein production by the mutants was investigated. Results revealed that, there was no significant difference in fungal biomass and soluble protein production was observed between the wild type and mutant strains.

Production of associated enzymes such as pectinase, phytase, protease, acid phosphatase and chitinase by *T.viride* SPCV3M1 were found to be decreased by 3 times, when compared with the wild type. However amylase production was not altered. Whereas, in other mutants the level of associated enzymes production remained unchanged.

4.4 PROPERTIES OF CELLULASE AND XYLANASE ENZYMES PRODUCED UNDER SmF AND SSF

In the present study, the properties of various fungal cellulase and xylanase enzymes produced under submerged and solid state fermentations were determined and are presented in tables 24 - 26.

pH optima for the activity of CMCase, FPase, β - glucosidase and xylanase enzymes was 4.8, 5.0, 5.0 and 5.0 respectively. Optimum temperature for cellulase and xylanase activities was 50°C.

Cellulolytic enzymes from wild type and mutant strains were most stable in the pH range of 4.5 – 6.0. Thermo stability of cellulase and xylanase enzymes was investigated between 20 and 30°C. Results revealed that cellulolytic enzymes of wild type fungal strains were 50% stable at 60°C. On the other hand, cellulase and xylanase produced by the mutant strain *T.viride* SPCV3M1 showed 75% stability even at 60 – 70°C, when compared with the wild type enzymes.

The effect of 14 different metal ions on cellulase and xylanase activity was investigated. Results denoted that, Na^{2+}, K^{2+}, Mg^{2+}, ions had neither stimulatory nor inhibitory effect on cellulolytic activities. Whereas, Hg^{2+}, Pb^{2+}, Zn^{2+}, Cu^{2+}, Cd^{2+}, Ag^{2+} and Fe^{2+} ions showed complete inhibition of all the cellulase and xylanase activities. However, Mn^{2+}, Co^{2+} and Ca^{2+} ions increased the activities of CMCase, β - glucosidase and xylanase enzymes respectively. Also, FPase enzyme from four wild type and four mutant strains

of *Trichoderma viride* were stimulated by Mn^{2+}, Ba^{2+} and Ca^{2+} ions (Tables 24 - 26).

4.5 PURIFICATION AND CHARACTERIZATION OF CELLULASE

In the present study, FPase, a component of cellulase complex was purified from the solid state culture supernatant of *T. viride* SPCV3M1, by following step wise procedures such as, precipitation by ice cold ethanol, followed by Sephadex A -50 gel filtration.

Crude enzyme in the supernatant had a specific activity of 62.71(IU/mg of protein). After ethanol precipitation, the specific activity increased to 144.24 (IU/mg of protein) with a purification fold of 2.48 and the yield was 17.3%. After gel filtration through Sephadex A - 50 coloumn, specific activity and purification fold were 356.83 (IU/mg) of protein and 2.9 respectively. The yield at this step was 0.58% (Table 27).

In the present study, the purified FPase enzyme was characterized by both biochemical and biophysical methods. The results are given in Tables and Figures.

Biochemical characterization of the purified FPase, was carried out by determining pH and temperature optima; effect of various metal ions and sugars on enzyme activity; estimation of molecular weight by SDS PAGE and zymogram, and calculation of Km and Vmax values.

The optimum pH and temperature for FPase activity was found to be 5.0 and 50°C respectively. FPase enzyme was found to be 75% stable between pH 4.5 – 6.0 and at temperatures 50 - 70°C (Table 28). The Km and Vmax values of purified FPase at 50°C calculated from Line Weaver Burk plot was found to be 0.28 mM and 238.09 μ moles/min/mg of protein on avicel substrate (Fig. 61).

The effect of various metal ions (10 mM) on purified FPase indicated that, Na^{2+}, K^{2+}, Mg^{2+}, ions had neither inhibited nor stimulated the enzyme activity. Al^{2+}, Pb^{2+}, Hg^{2+}, Ag^{2+}, Cu^{2+}, and Co^{2+} ions showed complete inactivation of the enzyme. Mn^{2+}, Ba^{2+} and Ca^{2+} ions stimulated FPase activity considerably (Table 27).

Among different sugars, fructose and sucrose did not show any significant inhibition, whereas sugars like arabinose, lactose, and 2 - deoxy glucomannose, maltose and galactose showed inhibition (Table 28).

The purified FPase, when analyzed for carbohydrate content, it did not show any carbohydrate content, indicating that the enzyme was not a glycoprotein.

The purified enzyme protein showed a single band on SDS PAGE (Fig. 65). Determination of molecular weight by gel docking indicated that the protein was a monomer with a molecular weight of 47 kDa. Purified FPase when subjected to zymogram analysis, showed a single clear zone

corresponding to 50 kDa, indicating the homogeneity of the purified enzyme (Fig. 66).

Biophysical characterization of purified FPase, was performed by UV and fluorescence spectroscopic studies. The UV absorption spectra of purified enzyme showed two peaks one at 235 nm and the other at 280 nm, corresponding to the absorption of peptide bond and aromatic amino acids respectively (Fig. 62). The excitation and emission fluorescent spectra of the purified FPase showed a maximum and minimum peaks at 292 and 340 nm respectively (Figs. 63 – 64).

4.6 ROLE OF CELLULASE IN BIOSTONING

Biostoning is the process of using cellulase to remove micro fibrils from the cotton fibre surface, for getting textiles with brighter and polished appearance. Most of the available cellulases, can cause fibre damage during the reaction. Hence, it is essential, to use a suitable cellulase, for the treatment of cotton fibres before spinning. In the present study, the role of purified FPase in fibrillation of cotton fibre was investigated. For a comparing the activity of the purified FPase, a commercial cellulase complex enzyme was used.

In the present investigation, the activities of mono and multi component cellulases on cotton fibre was evaluated as reduction in dry weight in terms of reducing sugars released during the reaction. Results indicated that, reducing sugar yield was higher, when cotton fibres were treated with cellulase complex (0.27%), when compared with FPase (0.21%). This

indicates the higher hydrolytic activity of the cellulase complex on cotton fibre, as against the FPase enzyme.

In order to increase the accessibility of the cotton fibres towards the activity of cellulase enzymes, cotton fibres were subjected to different pretreatments such as, steaming, oxidation with Fenton's reagent, and mild and strong alkaline washings. Out of four pretreatments tried in this study, steaming prior to enzymatic treatment increased the activities of both mono and multi component cellulases by 5 and 3 folds respectively (Table 29), whereas, in the other pretreatments, the enzyme activity was comparatively less.

Steam treated cotton fibres were subjected to enzymatic modification for 30 min using mono and multi component cellulases. Microscopic observations illustrated that the cotton fibre treated with FPase enzyme had a smooth and polished surface due to the specific and clear fibrillation activity of the enzyme, whereas, cotton fibre treated with multi component cellulase showed erosion and deep cracks on the fibre wall (Figs. 67 - 69).

4.7 ROLE OF CELLULASE IN BIOCONVERSION

Used paper materials, could be bioconverted by cellulase enzyme complex. In this study, cellulase produced by *Trichoderma viride* SPCV3M1, under solid state fermentation was applied in the hydrolysis of waste paper. To improve bioconversion, parameters such as different waste papers;

concentration of waste paper and enzyme; pH and incubation temperature were optimized. The results are presented in tables 30 - 31 and Figs. 70 – 71.

In the present study, enzymatic hydrolysis of various waste paper materials such as cardboard, news paper, copier paper, filter paper, paper pulp, paper sludge and office paper with cellulase was investigated. Results showed that, among the 7 different paper sources, cardboard produced highest glucose release during saccharification. This was followed by office paper, and copier paper and filter paper (Table 30). The other paper materials exhibited low levels of bioconversion, while newspaper showed resistance to enzyme activity.

Studies on the effect of different levels (5 – 70 mg) ofcardboard on enzyme activity was measured in terms of reducing sugar accumulation during the reaction. Results showed an increasing trend up to 30 mg of cardboard. Beyond this optimum substrate concentration, a decline in biodegradation was observed (Table 30).

In the present investigation, at different pH levels (2.0 – 7.5), biodegradation of cardboard by cellulase was studied. A gradual increase in reducing sugar release was noticed from pH 2.0 – 5.5. Further increase in pH, decreased the reducing sugar release by the enzyme. From this study, pH 5.5 was considered as the optimum pH for maximum bioconversion of cardboard (Table 31).

Investigations were performed to study the effect of different incubation temperatures (20 - 80°C) on bioconversion efficiency of cellulase.

Results indicated that, 55°C was the optimum temperature for the cellulolytic enzymes for maximum hydrolysis of cardboard (Table 31).

As changing enzyme concentration could influence bioconversion, a fixed amount of cardboard (35 mg) was subjected to hydrolysis at various enzyme concentrations (0.1 – 3 mg/ml), at pH 5.5 and incubated at 55°C (Fig. 71). Results indicated that, maximum reducing sugar formation was observed at an enzyme concentration of 0.16 mg/ml.

In the present study, under optimum conditions, bioconversion was studied at different incubation periods (1 – 10h). Cardboard was increasingly degraded up to 7h incubation. Beyond 7 h period degradation was feable (Fig. 70).

Discussion

5. DISCUSSION

Cellulase is an important multi component enzyme capable of splitting β, 1,4 linkages in cellulose molecules, which finds application in food, feed and chemical industries (Mandels, 1985). Recently, use of cellulase in textile and paper industries, has increased the demand for this enzyme. However, the low yield and high cost of the enzyme limits its versatile role. Thus, for the successful application of cellulase, it is necessary to find a suitable enzyme source for developing a cost-effective process for cellulase production. At present cellulase is produced by Submerged fermentation (SmF). An alternative technology to SmF is Solid State Fermentation (SSF), which has many potential advantages over SmF (Pandey *et al*, 1999). Hence, the present investigation focused on the isolation of effective strains for cellulase production by SSF optimization of various fermentation parameters, characterization of enzyme and its role in biostoning and bioconversion process. The major findings of the present investigation are discussed here.

5.1 SCREENING OF FUNGAL STRAINS FOR CELLULASE AND XYLANASE PRODUCTION

The production of any microbial metabolite by fermentation begins with the selection of appropriate microorganisms, which could grow on an inexpensive and simple medium, with out the requirement of any expensive growth promoters, and were capable of producing high titres of the desired

product in short time. This was achieved by screening large number of microorganisms, and it continues to be an important aspect of biotechnology (Brown, 1985).

It is essential to select the appropriate natural sources during the isolation process of cellulase producing microorganisms (Bergman *et al.*, 1988). The screening of cellulolytic microorganisms by conventional process, primarily involves the visual inspection of each isolated microorganisms for their growth, and formation of clear zone on the cellulose agar medium (Acebal *et al.*, 1986). Filamentous fungi are sources for industrial cellulases (Klysov, 1990). In order to obtain suitable source for cellulase production, several cellulolytic fungal strains were isolated from different biotics, and screened by many workers in the past (Gomes *et al*, 1992). *Trichoderma reesei* QM9414 isolated by the US army was considered as the best strain to produce high levels of cellulase (Reese, 1976).

In addition to *Trichoderma reesei* QM9414, *Aspergillus clavatus* (Olutiola, 1977); *Aspergillus* spp and *Trichoderma* spp (Fugio and Moo Young, 1980); thermophillic fungi *Humicola insolens*YH - 8 (Hayashid and Yoshika, 1980); the phycomycetus fungi *Neocalimastix frontalis* (Orpin, 1981); *Penicillium pinophillum*, *Aspergillus quadranticus* and *Gliomastix*, *Murorum* (Sethi and Rawala, 1987); and *Aspergillus terreus* GN1, (Yadav *et al.*, 1988) were also screened for cellulase production.

In the present study, a total of 310 fungal strains belonging to 17 genera with 33 species were isolated from Cauvery delta ecosystem. Based on

plate screening method, 140 strains comprising of 6 genera with 20 species were identified to be cellulolytic. Kadar and Omar, (1999) screened Malaysian highlands and isolated 16 cellulolytic fungi, and found that most of them were *Trichoderma* spp. In the present investigation, among the 140 strains found to be cellulolytic , *Trichoderma* and *Aspergillus* spp were more in number.

These 140 cellulolytic strains were further screened for the production of saccharifying enzymes such as cellulase and xylanase enzymes. Results showed, that out of 140 strains screened, 9 fungal strains such, as *Aspergillus niger* AVCM1, *A.niger* SPCV93, *Trichoderma harzianum* AVCM7, *T.harzianum* SPCV56 *T.harzianum* SPCV127, *T. viride* AVCM4, *T.viride* SPCV3, *T.viride* SPCV11 and *T.viride* SPCV41 produced maximum cellulolytic enzymes, and were selected for further studies.

5.2 PRODUCTION OF CELLULASE AND XYLANASE

Production of cellulase and xylanase by 9 fungal strains was optimized in both submerged and solid state fermentations.

5.2.1 Submerged fermentation

Submerged fermentation (SmF), technique has a wide range of industrial application for the production of value-aided products. According to Olutiola and Cole, (1977) the amount and properties of the cellulase produced by microorganisms depends on the growth conditions of submerged fermentation system. In this work, to increase the production of cellulolytic

enzymes by the 9 fungal strains, different factors like carbon and nitrogen sources, pH and temperature have been altered.

Various parameters of SmF process, were optimized for maximum cellulase production by many workers using, *Aspergillus fumigatus* (Trivedy and Rao, 1980); *Fusarium oxysporum* (Narania and Reddy, 1978); *Trichoderma viride* (Gupta *et al.,* 1972); *Pyrenochaeta terrestris* (Horton and Keen, 1966) in *Trichoderma viride* (Ismailova and Loginova, 1975), a thermotolerant *Aspergillus terreus* 17P (Ismailova and Loginova, 1975); *Schizophillum commune* (Desrochers *et al.*, 1980); *Penicillium pinophilum*, *Aspergillus quadricinctus* and *Glimoastix murorum* (Sethi and Rawala, 1987); *A.terreus* N1 (Garg and Neelkantan, 1982a) and *T.reesei* (Warzywoda *et al.*, 1983).

Nature of substrate could significantly influence cellulase production (Bahkali, 1992). In the present study, cellulase and xylanase production by the selected fungal strains on different substrates was investigated. Results revealed that, among the 10 defined substrates tested, all the 9 fungal strains showed manifested production of cellulase and xylanase with avicel substrate. Rajendran *et al.*, (1994) stated that *Humicola fuscotra* produced maximum cellulase in the presence of avicel, which is in accordance with the present study Also, moderate enzyme production observed with carboxy methyl cellulose, solka floc, cotton, filter paper and paper sludge substrates supports the earlier reports obtained with *Penicillium pinophilum*, *Aspergillus quadricinctus* and *Glimoastix murorum* (Sethi and Rawala, 1987) and *A.terreus* (Ali and Sayeed, 1992).

In the earlier studies, pectin and xylan were known to be efficient inducers for cellulases in *Aspergillus nidulans* (Bagga and Sandhu, 1987), *Streptomyces flavogriseus* (Kluepfel and Ishaque, 1982) and *Streptomyces lividsans* (Kluepfel *et al.,* 1986). But they could not induce appreciable cellulolytic activity in *Trichoderma and Aseprgillus* strains tested in this study. This result may be due to the inability of these fungal strains to utilize xylan and pectin for cellulase production as reported by Godden *et al.,* (1989) and Anand and Vidhyathil, (1990) using *Streptomyces* spp strains EC1 and *Humicola lanuginosa* respectively.

Poor cellulase production in glycerol, and cellodextrin substrates by the nine strains observed in the present study, supports the earlier reports of Bagga *et al.*, (1989) and Royar and Nakas (1990). The inability of cellobiose to induce higher exoglucanase, by the nine fungal strains, obsreved in this study is in accordance with the results obtained for *Thermonospora curvata* (Stutzehberger, 1972) and *Trichoderma reesei* (Ilmen *et al.,* 1997).

Saddler *et al.*, (1985) reported that *Trichoderma harzianum* E58, produced relatively higher xylanase in cellulose substrate than xylan, which can be favourably compared with the present study regarding xylanase production by strains of *A niger*, *T.harizianum* and *T.viride*. In the present study, the specific induction of β - glucosidase and xylanase enzymes, by cellobiose and xylan substrates, in 9 fungal strains showed a positive correlation with the results of *Streptomyces* spp (Godden *et al.*, 1989) and *T.reesei* QM9414 (Hermova *et al.*, 1989).

Most works aimed at the production of cellulase by using pure cellulose. Though pure cellulose gives good cellulase productivity, its cost is very high. Agricultural wastes like wheat straw, rice bran, paddy straw, saw dust, etc. were tried to substitute pure cellulose (Millet *et al.*, 1976; Ghedalia and Miron, 1981; Kawamori *et al.*, 1986). In the present study, among the 8 natural cellulosic substrates such as wheat bran, rice bran, paddy straw, sugarcane bagasse, cotton seed, paper sludge and black gram seed husk tested, all the 9 fungal strains produced maximum cellulase and xylanase enzymes with wheat bran, on 5^{th} day of fermentation. Maximum production of cellulase and xylanase with wheat bran substrate might have resulted due to slow metabolism of the fungi on this substrate, which could not be hydrolyzed easily as reported by MacKenzie *et al.*, (1987).

Higher induction of cellulase by lignocellulosic substrates has been reported in *Aspergillus terreus* (Okunev *et al.*, 1983), *Streptomyces lividsans* (Kluepfel *et al.*, 1986), *S. flavogriseus* and *S. olivochromogenes* (MacKenzie *et al.*, 1987), *A. nidulans* (Bagga and sandhu, 1987), *Streptomyces* spp EC1 (Godden *et al.*, 1989) and *A. niger* and *A. phoenicis* (Reczey *et al.*, 1998). In the present study also, natural lignocellulosic substrates induced higher levels of cellulolytic enzymes in 9 fungal strains, when compared with the defined substrates.

Relatively low yield of cellulolytic enzymes on black gram husk and paper sludge substrates recorded in this study might be due to their exclusion along with unutilized substrates, since these enzymes more strongly get adsorbed on cellulose particles, (Bagga and Sandhu, 1987; Linder *et al.*,

1996). Hence, based on the results obtained in the present investigation, wheat bran could be successfully utilized as a renewable carbon source, for the development of economically feasible technology for cellulase production.

Studies on the effect of different levels (0.5 - 8%) of wheat bran on cellulase and xylanase production, by the 9 fungal strains, revealed that, enzyme production increased up to 2% wheat bran concentration, and afterwards gradually decreased. This decrease in cellulase in production with increasing substrate concentration beyond the optimum may be attributed either due to a physical adsorption of the enzyme on the substrate (Reese and Mandels, 1963) or due to a repression of cellulase synthesis due to accumulation of products of hydrolysis in the medium (Horton and Keen, 1966) or to the acid conditions that may be developed at high substrate levels (Strenberg, 1976).

Optimum cellulase synthesis by *Humicola fuscotra* (Rajendran *et al.*, 1994) and *Aspergillus ochraceous* MTCC 1877 (Ruckmani *et al.*, 2002) was noticed at 2% concentrations of cellulose powder and wheat bran respectively. In the present study also, 2% wheat bran was the affirmative carbon source for maximum enzyme production.

Nitrogen source in the medium is an important nutrient that affects enzyme production (Lizak, 1975). In the present study, among the 7 different nitrogen sources tested, 9 fungal strains, showed optimum production of cellulase and xylanase enzymes with yeast extract followed by peptone.

Ganju *et al.*, (1990), reported that addition of peptone or yeast extract increased celulase production by *Chaetomium thermophille*. According to Mandels and Webber, (1969), the beneficial effect of peptone could be due to the fact that the organism more readily assimilated it. These results are in good correlation with the present study in using yeast extract as nitrogen source for enzyme production by the strains of *Trichoderma* spp and *Aspergillus* spp. In the present investigation, moderate enzyme yields recorded by the fungal strains with glycine and urea shows agreement with the results obtained with *Aspergillus quadranticus* (Sethi and Rawala, 1987).

Nitrogen source giving the optimum yield of cellulolytic enzymes by 9 fungal strains was taken and the effect of varying nitrogen concentration on cellulase and xylanase production was examined. Yeast extract at 0.6% (w/v) was found to be optimum for maximum enzyme yields by these fungal strains. Optimum cellulase production was obtained at 0.1% (w/v) yeast extract concentration in *Chaetomium thermophille* (Ganju *et al.*, 1990). The present work shows variations with the work of Ganju *et al.*, (1990) with respect to cellulase production.

pH of the medium plays an important role in the production of cellulolytic enzymes by several organisms such as *A. fumigatus* (Trivedi and Rao, 1980), *Sporotrichum thermophille* Cautts and Smith, (1976) and *T. viride* (Sternberg, 1976). Bailey *et al*, (1993) proposed that, *T.reesei* produced maximum cellulolytic enzymes in the pH range of 5.0 – 5.5. In this study also, among the pH levels tested, pH 5.0 was found to be optimum for maximum enzyme production by *Aspergillus* and *Trichoderma* strains.

It has been reported that the incubation temperature directly affected cellulase production (Saddler *et al.*, 1985). In the present study, production of cellulase and xylanase was studied at different temperature. It was observed that, *Trichoderma* and *Aspergillus* strains produced maximum enzyme, when incubated at 30 ± 2°c on 5th day. Similar results were also reported by various authors using *A. niger* Van Tieghem (Azizan *et al.*, 1993); *A. niger* (Deschamps *et al.*, 1985); *A.terreus* (Ali and Sayeed, 1992) and *T. reesei* QM9414 (Acebal *et al.*, 1986).

Studies on the production of some associated enzymes such as amylase, pectinase, phytase, α - galactosidase, amyloglucosidase, protease and chitinase by the 9 fungal strains revealed that, the above enzymes were produced at negligible concentration. This result was due to the increased assimilation of cellulose when compared to other polysaccharides in wheat bran, as documented by Ramakrishna *et al.*, (1990).

5.2.2 Solid state fermentation (SSF)

Solid state fermentation (SSF) involves the growth of microorganism (typically fungi) on moist solid substrates, have considerable economic potential in producing enzymes and other chemicals. Recently, SSF has gained renewed interest because, it exhibits certain advantages over submerged fermentation (Srinivasan and Seeta laxman, 1988) Nutritional and physicochemical factors like carbon and nitrogen sources, moisture content, pH of the fermentation medium and incubation temperature in solid-state fermentation system have a considerable effect on the fungal growth and

product formation (Han *et al.*, 1987; Asha kumari *et al.*, 1999; Pandey *et al.*, 1999).

The optimization plays a critical role in the development of an economic and efficient SSF process. In SSF, the product yield was not only governed by the ability of the strain, but also by the nutritional and physical environment in which the organism was cultivated (Kim *et al.*, 1985). The various parameters optimized, should be able to provide the nutrients for efficient growth and metabolism of the organism for higher product formation (Olutiola and Cole, 1977; Pandey *et al.*, 1999). Literature survey revealed that detailed reports on the optimization of fermentation parameters for cellualse production by solid state fermentation system is inadequate, when compared with submerged fermentation.

Optimization of the SSF process starts with the selection of carbon source, which is a key parameter that determines the economy of the process. Hence, in the present investigation, enzyme production studies were carried out using different agro industrial residues as substrates namely, wheat bran, rice bran, cotton seed husks, sugarcane bagasse, corn cob, paper sludge, black gram husk, saw dust and paddy straw. Results revealed that among the 9 carbon sources, wheat bran was found to be the appropriate substrate for maximum cellulase and xylanase production. The reason for substantial production of cellulolytic enzymes, by 9 fungal strains, reported in the present study, may be attributed to the various components present in the wheat bran, which helped in growth, metabolism and product formation by the organisms as suggested by Mitchell and Lonsane, (1992) and Feniksova *et al.*, (1960).

The selection of nitrogen source plays an important role in the development of an efficient and economic fermentation process (Kargi *et al.*, 1985). In this study, strains of *Trichoderma viride, Trichoderma harzianum* and *Aspergillus niger*, were able to produce high titres of cellulase on fifth day of fermentation in simple wheat bran medium under SSF. It was of interest to determine, whether it was possible to increase cellulase productivity by incorporating additional nitrogen sources in the wheat bran medium.

In this investigation, various nitrogen sources such as yeast extract, peptone, urea, soya bean meal, sodium nitrate, ammonium phosphate and ammonium nitrate were added to wheat bran substrate at 5% level. Cellulase and xylanase production by the 9 fungal strains revealed that, the addition of soya bean meal showed significant increase in enzyme production when soya bean meal was added at 5% level. Hence, use of soya bean meal, as nitrogen amendment will enable for cost effective bioprocess development as suggested by (Han *et al.*, 1987).

In this study, inclusion of ammonium phosphate and sodium nitrate to the wheat bran substrate was found to inhibit cellulase production by 50% and 75% respectively. This type of inhibition by certain phosphate ions and urea has been observed in submerged fermentation for cellulase production (Lizak, 1975; Ray, *et al.*, 1993). The other added nitrogen sources did not show any significant effect on enzyme production.

Moisture content of the substrate is one of the most important factors, which affects the productivity of solid state fermentation system due to its influence on the physical properties of solid and formation of products (Mitchell and Lonsane, 1992). The water present in the solid substrate fermentation system exists in a complex form within solid matrix (Moo Young *et al.*, 1983) or as a thin layer adsorbed to the surface of the particle or less tightly bound within the capillary region of the solid (Mudgett, 1986).

In the present study, to determine the optimal moisture content for maximum production of cellulolytic enzymes, the moisture content of the wheat bran medium was varied between 35 – 85% and the enzyme production profile by the 9 fungal strains was monitored. The production of cellulase and xylanase enzyme increased with increase in initial moisture content up to 50%, after which the productivity decreased gradually and diminished at 80% moisture content. In contrast, Ramesh and Lonsane, (1990) reported the highest enzyme production in basal wheat bran medium with 65% moisture content.

In SSF higher moisture level decreases porosity, changes the particle structure, promotes stickiness development, reduces gas volume exchange and decreases diffusion, which results in lowered oxygen transfer (Lonsane *et al.*, 1985). The report of Zadrazil and Burnnert, (1981) showed that lower moisture content causes a reduction in the solubility of nutrients of the solid substrate. This causes a low degree of swelling and high water tension. Thus from the results of this study, 55% moisture content was found to be best suited for maximum cellulase production. These earlier reports,

agreed with the results for cellulase and xylanase production recorded in the present study, at moisture levels above and below the optimum.

pH of the fermentation medium may change in response to metabolic activity of the microorganism. The most obvious reason is the secretion of several metabolites into the medium, and also due to the utilization of nitrogen sources in the medium by microorganisms (Raimbault and Alazard, 1980; Cooney, 1981). Usually the filamentous fungi will have broad range of pH for their optimal growth. This is also true in the case of cellulase production by fungi under solid substrate fermentation system as reported by earlier workers (Desschamps *et al.,* 1985; Shamala and Sreekantiah, 1985).

In the present study the initial pH of the medium was kept varied between 3.5 and 7.5, and the enzyme production profile by the 9 strains of *Trichoderma* and *Aspergillus* spp was monitored. At pH 3.5, absolutely there was no growth of fungal strains on the solid substrate. The organisms were able to grow well and could produce maximum cellulase at pH 5.0. This result might be due to the buffering action of the wheat bran as pointed out by Raimbault, (1980). Enzyme production decreased, when the initial pH of the medium was increased beyond pH 5.0. Similar results were also recorded for xylanase production by *Aspergillus fischeri* (Chandra Raj and Chandra, 1995), and *A.ochraceus* (Ruckmani *et al.,* 2002) under submerged fermentation.

The effect of different incubation temperature on the level of enzyme production showed that, the optimum temperature for maximum production of cellulolytic enzymes was 30°C. Enzyme production siginificantly decreased at incubations above 30°C. Ray *et al.*, (1993), found that 30 ± 2°C was found to be the optimum temperature for enzyme production under SSF which could be positively connected with the present study.

Thus, an incubation temperature of 30°C and pH 5.0 was found to be most favourable for the production of cellulase and xylanase enzymes by *Trichoderma viride, Trichoderma harzianum,* and *Aspergillus niger* strains under solid substrate fermentation system. The above values were almost related to the optimum temperature and pH values reported in literature for SmF (Gokhale *et al.,* 1991) and SSF (Ray *et al.*, 1993).

Leaching or extraction is an important unit operation in SSF system involving the recovery of product from the fermented substrate in the form of crude extract. The desired product may be present in thin film of water over the solid surface, or in solid form itself if its concentration crosses the super saturation limit. The efficiency of leaching and the selection of leaching technique are critical for maximum recovery of the product. In the extraction of enzyme from the fermented solid, it is necessary to select the suitable solvent (Eriksson *et al.*, 2002). An ideal solvent is one, which should be able to leach the enzyme selectively and completely at room temperature, with minimal contact time, and preferably at optimal pH from the fermented

substrates (Treybal, 1981; Ramesh and Lone sane, 1990; Mitchell and Lonsane, 1992).

In general, solvents used for the leaching of enzyme from the fermented substrates usually include water (Tengerdy, 1985), salt solution (Srinivas, 1996) and buffer (Ramesh and Lonsane, 1990). In the present study, solvents used for leaching the enzyme from the fermented substrates were, water, 0.5% sodium chloride, 5% glycerol, 2% calcium chloride, 0.1% Triton X 100, 0.1% of Tween80 and Sodium acetate buffer (0.1M, pH 5.0).

Results revealed that, maximum amount of cellulase and xylanase could be extracted from the fermented solid using 0.1% Tween80 as solvent. Helle *et al.*, (1993), suggested that surfactant adsorbs at air – liquid interface and may thus prevent enzyme denaturations in the hydrolysis mixture. Also, adsorption of enzymes to substrate has been shown to decrease in the presence of surfactants (Castanon and Wike, 1981; Helle *et al.*, 1993). The addition of non-ionic surfactant increased the accumulation cellulase enzymes in the medium due to the dominating effect of the surfactant on lignin (Erikson *et al.*, 2002). These reports were in good relation with the results obtained in this study.

Leaching efficiency and concentration of product in leachate depends mainly on solid to solvent ratio, which ultimately governs the economy of the process (Pandey *et al.*, 1999). The ratio of solid to solvent is critical during the leaching of enzyme. It is always desirable to use minimum amount of solvent to get the product in concentrated form. In the present,

work experiments were conducted at various solid – solvent ratio ranging from 1: 5 to 1: 25 for leaching cellulase from the fermented substrates using 0.1% Tween 80 as solvent. Results showed that, the leaching efficiency increased from 1:5 to 1:15 ratio, and decreased there after. Hence 1:15 solid to solvent ratio was selected which could avoid more dilution of the enzyme in the final leachate. Maximum filter paper activity was obtained in 1:10 (w/v) extract of mouldy bran with distilled water extaction (Shamala and Sreekantiah, 1985). But in the present study, highest enzyme was recovered by using 0.1% of Tween 80 in 1:15 ratio.

For the fungal strains to grow on wheat bran particles, it should penetrate into the micro pores of the substrate by producing some hydrolytic enzymes such as amylase, pectinase, phytase, α - galactosidase, amyloglucosidase, protease and chitinase (Mitchell *et al.*, 1992). In this study also, the fungal strains produce these associated enzymes up to 3rd day of fermentation. However the data on co production of above enzymes indicated that all the enzymes were produced at low concentration. The results of the present work, supports the earlier report of Han and Wilfred (1988).

It is very difficult to make a true comparison of the enzyme titers obtained by SmF and SSF techniques because of the physical nature of the fermentation medium involved. However, for all practical purposes, the method employed by Tengerdy, (1985) was followed. This was based on the approximation that one liter of extract obtained by extracting one kilo gram of fermented solid by counter current extraction will be equivalent to the amount of enzyme present in one litre of fermentation broth obtained by SmF is equal

to the amount generally employed for the enzyme titers. In the present study, cellulase production was found to be 8.098 times higher in solid state fermentation when compared with submerged fermentation, which makes SSF to be the most ideal system for cellulase production.

5.3 STRAIN IMPROVEMENT

Among the microorganisms identified as suitable for the production of cellulase, *T. reesei* was regarded as most potent cellulolytic fungus. There fore, in recent years studies have been intensified on further improvement of the organism for increased cellulase production (Durand *et al.*, 1988; Beguin, 1990; Nevalainen and Penttila, 1995). Traditional techniques like, induced random mutations followed by selection is still a major tool for strain improvement programs, and thus has not been eliminated by new procedures of molecular biology (Vipul and Sheela, 1997). Such attempts have been made in the past with *T. reesei* for hyper secretion of cellulase. However despite these efforts, the production of glucose from waste cellulose was not commercially feasible due to high cost of cellulase production at industrial level and the lack of enzyme with desirable characteristics (Szengyel *et al.*, 2000).

In the present work, the wild type strain *T. viride*SPCV3 was subjected to mutagenic treatments with UV and NTG. As a result of 10 min UV exposure, 4 colonies that showed large clearing zone were isolated, and were screened for enzyme production using optimized SSF medium used for the wild type.

Among them, the mutant strain *T. viride* SPCV3M1 secreted approximately 3 times more cellulase and xylanase enzyme than the wild type on 4th day of incubation. Results of fungal growth, measured in terms of glucosamine content of the mycelia, showed no significant difference between the mutant and wild type fungal strains This result indicates, that the enhancement of enzyme production by the mutant strains was not due to an increase in growth but due to the enhanced secretion of the enzyme (Bhat *et al.*, 1992), whereas, the unaltered soluble protein production observed in this study, indicates high specific activity of the enzymes as reported with *Aspergillus fischeri* (Chandra Raj and Chandra, 1995 & 1996 and *A.ochraceus* Ruckmani *et al.*, 2002)

Since mutation in fungi, may often result in the improvement of a desired character of economic importance (Queener and Lively, 1986; Ruckmani *et al.*, 2002), in this work, a significant enhancement in cellulolytic activity was obtained by UV mutation. This could be the result of single mutation, which leads to the increased secretion of enzymes. A similar relation was also observed with the hyper cellulolytic strain of *T. reesei* namely, MCG77 by Gallo *et al.*, (1978) respectively.

According to Eriksson (1982), *T. reesei* is inherently poor producer of β - glucosidase and improvement in the synthesis, and could bring out a distinct improvement in the efficiency of total cellulase activity of the fungus. In the present study also, the mutant strain *T. viride* SPCV3M1 showed hyper cellulase production. Also the production of associated enzymes such as

pectinase, chitinase, phytase decreased by 40 – 60%. The production of protease was negligible. But the production of amylase was unaffected.

Additionally, the fermentation time required for maximum enzyme production by the mutant strain was 4 days. Further this strain was found to be very stable, with little or no reversion and grows rapidly in fermentation solid medium, and its enhanced levels of cellulase production have remained enhanced over 2 years. Earlier observation made by Sternberg (1976) and Vipul and Sheela (1997) using the mutant *T. reesei* QM9414 showed that maximum cellulase was produced after 5th day. But in the present study, the fermentation time was earlier when compared with the previous reports.

5.4 CHARACTERIZATION OF CELLULASE

Cellulase activity was strongly influenced by physicochemical parameters like pH, temperature and metal ions (Elisashivili, 1993). In the present study, the optimal pH and temperature for cellulase and xylanases obtained from the wild type, and mutant strains were found to be in the range of 4.5 – 5.5 and 45°C - 50°C. Cellulases obtained from *T. reesei* (Ishaque and Kluepful, 1980; Gomes *et al.*, 1992; Kaar and Holtzapple, 2000), *T. reesei* C30 and *T. harzianum* E58 (Saddler *et al.*, 1985), *Humocola fuscotra* (Rajendran *et al.*, 1994), and xylanases from *Humicola insolens* (Dubey and Johri, 1987; *Aspergillus fischeri* (Chandra Raj and Chandra, 1995 & 1996 and *A.ochraceus* (Ruckmani *et al.*, 2002) were active in the pH and temperature range of 4.5-6.0 and 45 - 50°C.

Cellulolytic enzymes from wild type and mutant strains were found to be stable in the pH range of 4.0 – 6.5 and at temperatures 50 - 70°C. More over cellulase from the mutant *T. viride* SPCV3M1 retained 75% activity at temperature 70 - 80°C, when compared with the enzymes from the wild type *T. viride* SPCV3. Thus, the mutagenesis has also increased the thermostability of cellulolytic enzymes to some extent.

Kanamoto *et al.*, (1979) reported that Cu^{++} and Hg^{2+} inhibited enzyme activity, while Ca^{2+} and Mg^{2+} was found to be stimulatory. In the present investigation, the addition of Mn^{2+}, Ba^{2+} and Ca^{2+}, and Co^{2+} ions (at 10mM concentration) increased the hydrolytic efficiency of CMCase, FPase and β-glucosidase enzymes. On the other hand enzyme activities were completely inhibited in the presence of Cu^{2+} and Hg^{2+}.

This increase or decrease by various divalent cations, observed in this work, was due to the increase or decrease in adsorption affinity and tightness, and or change in tightness and adsorption affinity during metal ion - enzyme complex formation, that might have affected cellulose degradation, as confirmed by Kim *et al.*, (2001).

As cellulase is a multi component enzyme, the isolation of individual components from the complex involves certain specific purification procedures. In the past, several workers have purified the cellulase components. Wood and McCrae, (1986), purified the exoglucanase from the *Penicillium pinophillum* cellulase complex. Exo glucnanses namely CBH1 and CBHII from the commercial *T. reesei* cellulase preparation was isolated

by a series of chromatography procedures involving DEAE sepharose CL6B and Sephadex G – 50 by Claeyssens *et al.*, (1990), Rahkamo *et al.*, (1996) and Kim *et al.*, (2001). Two exoglucanases from the brown rot fungi was detected after Sephadex G – 50 purification, by Schimidhalter and Canevascani, (1993).

An exoglucanase called as avicelase was separated from the cellulase complex of *Aspergillus niger* (Amano *et al.*, 1996). Zhang *et al.*, (1999) purified the FPase enzyme from *Thermonospora fusca*, by using stepwise chromatographic procedures. Ulger and Salgam, (2001) followed ammounim sulphate fractionation and ion exchange charamatography on DEAE – Sephadex A – 50 column for the isolation of cellobiohydrolase.

In this study, FPase, an exoglucanase enzyme component of cellulase complex from the mutant strain *Trichoderma viride*SPCV3M1 was purified, as according to the method described for isolating FPase enzyme, from the mutant strain *T.viride* QM9414 (Ulger and Salgam, 2001). The fold of purification and specific activity of the purified FPase observed in this study, agreed with the reports obtained with the purification experiments of cellulase obtained from organisms like *Aspergillus niger* (Amano *et al.*, 1996) and *T.viride* QM9414 (Ulger and Salgam, 2001).

The purified enzyme when subjected to electrophoresis in SDS polyacrylamide gel electrophoresis, exhibited a single protein band corresponding to the molecular weight of 47 kDa indicating the homogeneity of the purified enzyme. Molecular weight ranging from 37 – 50 kDa has been

reported for exoglucanase from various fungal sources (Wood, 1992 and Amano *et al.*, 1996). Zymogram analysis of the native poly acrylamide gel showed clear zone development in the molecular weight regions of 50 kDa from the lane loaded with the purified FPase. This shows that the enzyme is purely FPase as confirmed by Kim *et al.*, (2001); Amano *et al.*, (1996) and Ulger and Salgam, (2001) for the FPase isolated from *Clostridium* spp, *A.niger* and *T.viride* QM9414 respectively.

The purified FPase did not show any carbohydrate content indicating that the enzyme was not a glycoprotein. Most of the fungal exoglucanases are glycoproteins (Pearce and Bauchop, 1985), except the one obtained from *Sporotrichum thermophillum* that was also not a glycoprotein (Erikson and Peterson, 1975).

The low K_m and V_{max} values estimated from Line Weaver Burk plot (Line Weaver and Burk, 1934), reflects the highest affinity of the enzyme towards Whatman No.1 fiter paper as substrate. This result indicates that the enzyme was truly an exoglucanase especially the FPase. Similarly other reports with the exoglucanase from *T. reesei*, *A. niger* and *Irpex lacteus* (Amano *et al.*, 1996), *T. viride* QM9414 and (Ulger and Salgam, 2001) correlated well with present investigation.

The UV absorption spectra of purified exoglucanase showed two peaks at 235 and 280 nm corresponding to the absorption of peptide bonds and aromatic amino acids respectively. The maximum and minimum peaks for excitation and emission fluorescence spectra of the purified FPase was obtained at 292 and 340 nm respectively. Cheng *et al.*, (1996) reported that,

the fluorescence of protein originates mainly due to the aromatic amino acids, the present study agrees with the earlier observations of Cheng *et al.*, (1996)

5.0 BIOSTONING

Cotton fibres used in the manufacture of popular garments such as blue jeans and other denim garments have the tendency for fuzz formation (short fibres protruding from the fibre surface) and pilling (fluffy / loosened fuzz attached to fibre surface). In these fabrics the indigo dye was mostly attached to the short cotton fibres. Due to repeated washings these fabrics show aged look (Heikinheimo *et al.*, 2000). These phenomena were considered negative features for the quality of cellulosic fabrics. Hence, removal of micro fibres will increase the commercial value of fabrics. This was accomplished by using cellulases in the process called bio stoning (Mehta *et al.*, 1990; Balanchard and Graves, 1995), which is a key step in textile industry for producing high quality garments (Liu *et al.*, 2000).

The mode of action of purified endo and exo glucanases as well as cellulase mixtures on different types of cotton fabrics have been evaluated to some extent (Cavaco-Paulo and Almedia, 1994; Cavaco-Paulo *et al.*, 1996; Heikinheimo *et al.*, 1998 & 2000; Miettinen-Oinonen *et al.*, 2001). In the present study, purified FPase, a component of cellulase enzyme produced by the mutant *Trichoderma viride*SPCV3M1 was used in the enzymatic removal of micro fibres of cotton, was investigated in comparison with a commercial cellulase complex.

Results of, hydrolytic activities of mono and multi component enzymes, measured in terms of reducing sugar released, showed that lesser hydrolysis yield of 0.21% of the original dry weight of cotton fibre was obtained with FPase treatment. Whereas, with the multi component cellulase, a higher hydrolysis yield of 0.27% was observed. Similar observations were made by Miettinen Oinonen *et al.*, (2000), using a cellulase preparation with low endoglucanase activity.

Because of high crystallinity (Benedict *et al.*, 1994), and the presence of waxy compounds on the outer most layers of cotton fibre, makes it less accessible to enzymatic modification by cellulase (Hartzell and Hsieh, 2000). Hence, in the present investigation, different pretreatments such as steaming, oxidation with Fenton's reagents and alkaline pretreatments either with mild or strong alkaline were performed to increase the hydrolytic activity of cellulase enzymes. Results of reducing sugar measurements revealed that, of the four pretreatments, steaming of cotton fibres was found to enhance hydrolytic actvity by 3 and 5 folds for the two types of cellulases such as, FPase, and cellulase mixture respectively. Yu *et al.*, (1995), also observed a similar result in the hydrolysis of lignocellulosics.

According to Grethlein, (1985) the efficiency of cellulase action on cotton fibre, was related with the specific surface area. It could be expected that the accessibility of cotton for enzymatic modification would increase by pre treatment of cotton fibre. In this study steaming increased accessibility of the fibres for the enzyme due to the partial removal of the waxy layer and or swelling less ordered or semi crystalline cellulose (Kim *et al.*, 1985). Also,

oxidation of cotton cellulose by Fenton's reagent, strong and mild alkaline treatments, improved the hydrolytic activity of the enzymes to some extent. This effect might be due to decreased crystalinity and concomitant changes in cellulose structure (Benedict *et al.*, 1994).

Thus, the accessibility of cotton cellulose to enzymatic hydrolysis could be increased by steaming pretreatment, which could subsequently may offer economic benefits by decreasing enzyme dosages needed for biostoning and biofinishing of cotton fabrics in textile industries.

In this work, microscopic examination of the effects of the enzymatic treatments on steam treated cotton fibre structure illustrated different modes of action of mono and multi component cellulases. Micro fibres present in the surface of cotton fibre were clearly fibrillated, giving a polished surface to the cotton fibre. This was due to the specific action of the Fpase enzyme. On the other hand, cellulase mixture acted more locally giving rise to erosion, defects and deep cracks on fibre wall. The fibre damage in cotton fibre treated with multicomponent cellulase, observed in this study might be due to the activity of endoglucanase enzyme present in the cellulase complex. In biostoning experiments using cotton fabrics, modified *T.reesei* cellulase preparation lacking endoglucanase II activity, performed better during biostoning and bio finishing of cotton and caused low fibre damages (Miettinen Oinonen *et al.*, 2000). Rahkamo *et al.*, (1996) and Stork and Pulls, (1995), observed a similar effect in cellulose fibres of kraft and dissolving pulps. These reports agree with the results obtained in the present study.

Based on the results of the present study, it could be concluded that the performance of whole cellulase preparations was quite different from the

FPase enzyme and the latter offered better performance in removing the micro fibres from the cotton fibre surface. There fore, in textile industries for obtaining soft and brighter fabrics it could be recommended to use the FPase enzyme for biostoning and biofinishing of cotton fibres.

6.0 BIOCONVERSION

Currently, environmental issues such as the depletion of non-renewable energy resources and pollution are topics of interest. Cellulose, the most abundant organic compound represents 40 - 60% of municipal solid wastes such as bio waste originating from house hold and industries with waste paper being a major component of these materials (Yu, 1996). Bioconversion of cellulose component of organic solid waste to glucose, which can be subsequently fermented into ethanol, provides a valuable fuel source (Way man *et al.*, 1992). In addition, the disposal of these organic solids is one of the most significant challenges facing environmental scientists. If these materials can be used as a substrate for the production of ethanol or other bio products, then two environmental concerns are addressed at one time. Increasing knowledge in the mode of action of cellulases and their recent applications have greatly increased the prospects of enzymatic hydrolysis over chemical processes, because of its potentially high saccharification efficiency and avoidance of pollution (Kubicek *et al.*, 1993).

In the present study, enzymatic degradation of various waste paper materials such as cardboard, news paper, copier paper, filter paper, paper pulp, paper sludge and office paper was investigated by incubating with

cellulase from *Trichoderma viride* SPCV3M1. Among the different waste paper materials tried, cardboard was maximally bioconverted, followed by office and copier papers. Whereas, Cellulases from *Penicillium funiculosum* and *Aspergillus niger* showed the highest bioconversion with office paper (van Wyk, 1997).

Beguin (1994) stated that *Trichoderma reesei* produces the most effective cellulase enzyme system for the hydrolysis of highly ordered cellulose forms. In this work also, the cellulase complex of *T.viride* SPCV3M1, was found to be competent in saccharifying the paper products. Rajendran, (1994) reported that, less amount of xylanase enzyme was essential for treating paper wastes in effluents. In this study, *T.viride* SPCV3M1 produces a minimum quantity of xylanase, which might have supported optimum bioconversion of waste paper.

The amount of enzyme and waste paper material in the reaction mixture has a direct relationship with the speed of the reaction that produces glucose (Robinson *et al.*, 1994). In this study, experiments performed with various enzyme and substrate concentrations, for maximum bioconversion revealed that, maximum hydrolysis resulted with 30 mg and 0.2 mg/ml of cardboard and enzyme levels respectively. Hydrolysis of pretreated waste paper materials, by different concentrations of *Penicillium funiculosum* cellulase, showed differences in reducing sugar release (Van Wyk, 1999), which could be positively connected with the present study.

Investigations on the time course for enzymatic hydrolysis of card board by cellulase enzyme from *Trichoderma viride*SPCV3M1 showed a gradual increase in reducing sugar accumulation up to 6^{th} h and remained constant up to 7^{th} h. Way man *et al.,* (1992), also observed, a similar relation, with two different cellulases.

Considering the rate at which effluents pumped into rivers, and the volumes of solid waste dumped, for conserving the environment by limiting pollution through the bioconversion of organic waste into sugars, it may be suggested to use the cellulolytic enzymes produced by *T.viride* SPCV3M1. Thus it would assist for the development of alternative, economically feasible and renewable energy resources from bio wastes.

Table 1 Screening of cellulolytic fungi

S.No	Organism	CD/CZ ratio	Enzyme Activity (IU/ml)				Protein (mg/ml)	Biomass (mg/l)
			Endoglucanase	Exoglucanase	β - glucosidase	Xylanase		
1	*Aspergillus aculeatus* SPCV52	+	2.454	0.752	0.121	1.410	4.172	3.433
2	*A. americana* SPCV30	+	2.417	0.741	0.119	1.389	4.108	3.381
3	*A. americana* SPCV66	++	3.050	0.935	0.150	1.753	5.185	4.267
4	*A. flavus* SPCV12	+	2.190	0.671	0.108	1.259	3.723	3.064
5	*A. flavus* SPCV38	+	2.677	0.821	0.132	1.538	4.551	3.745
6	*A. flavus* SPCV53	+	2.024	0.627	0.101	1.104	3.441	2.832
7	*A. fumigatus* SPCV1	++	3.452	1.058	0.170	1.984	5.869	4.830
8	*A. fumigatus* SPCV109	++	2.750	0.843	0.135	1.580	4.675	3.847
9	*A. fumigatus* SPCV39	++	3.335	1.022	0.164	1.917	5.670	4.666
10	*A. fumigatus* SPCV77	++	3.050	0.935	0.150	1.753	5.185	4.267
11	*A. niger* SPCV112	+	1.933	0.593	0.095	1.111	3.287	2.705
12	*A. niger* SPCV13	++	2.900	0.889	0.143	1.667	4.930	4.057
13	*A. niger* SPCV133	++	2.998	0.919	0.148	1.723	5.097	4.195
14	*A. niger* SPCV19	+	2.403	0.737	0.118	1.381	4.085	3.362
15	*A. niger* SPCV2	+	3.335	1.022	0.164	1.917	5.670	4.666
16	*A. niger* SPCV35	+	2.143	0.657	0.106	1.232	3.644	2.999
17	*A. niger* SPCV4	++	3.351	1.027	0.165	1.926	5.697	4.688
18	*A. niger* SPCV42	+	2.713	0.832	0.134	1.559	4.612	3.795
19	*A. niger* SPCV43	++	3.427	1.051	0.169	1.970	5.826	4.795
20	*A. niger* SPCV49	+	2.500	0.766	0.123	1.437	4.250	3.498
21	*A. niger* SPCV50	+	2.500	0.766	0.123	1.437	4.250	3.498

Contd...

22	*A. niger* SPCV54	++	3.314	1.016	0.163	1.905	5.634	4.637
23	*A. niger* SPCV55	+	0.124	1.465	0.246	1.047	0.211	0.173
24	*A. niger* SPCV60	+++	4.382	1.343	0.216	2.519	7.450	6.131
25	*A. niger* SPCV61	+	2.598	0.796	0.128	1.493	4.416	3.634
26	*A. niger* SPCV63	+	2.589	0.794	0.128	1.488	4.402	3.622
27	*A. niger* SPCV67	+	2.689	0.824	0.132	1.545	4.571	3.762
28	*A. niger* SPCV68	++	3.274	1.004	0.161	1.882	5.566	4.581
29	*A. niger* SPCV69	++	2.729	0.837	0.134	1.569	4.640	3.818
30	*A. niger* SPCV84	+	2.584	0.792	0.127	1.485	4.392	3.615
31	*A. niger* SPCV10	++	2.736	0.839	0.135	1.572	4.651	3.827
32	**A. niger* SPCV93	++	2.236	1.078	0.102	1.005	3.954	3.124
33	*A. niger* SPCV95	+++	4.833	1.481	0.238	2.778	8.217	6.762
34	*A. niger* AVCM1	++	2.733	0.838	0.135	1.571	4.646	3.823
35	*A. oryzae* SPCV117	++	2.733	0.804	0.115	1.531	4.626	3.803
36	*A. oryzae* SPCV44	++	2.800	0.858	0.138	1.609	4.760	3.917
37	*A. oryzae* SPCV70	+	2.716	0.832	0.134	1.561	4.617	3.800
38	*A. oryzae* SPCV78	++	3.248	0.996	0.160	1.867	5.522	4.544
39	*A. terreus* SPCV5	++	2.836	0.869	0.140	1.630	4.820	3.967
40	*A. terreus* SPCV14	+	2.175	0.667	0.107	1.250	3.698	3.043
41	*A. terreus* SPCV64	+	1.513	0.464	0.075	0.870	2.572	2.117
42	*A. terreus* SPCV65	++	3.314	1.016	0.163	1.905	5.634	4.637
43	*A. terreus* SPCV66	+	2.552	0.782	0.126	1.467	4.338	3.570
44	*A. ustus* SPCV102	+	2.502	0.582	0.186	1.427	4.308	3.270
45	*A. ustus* SPCV73	+	2.071	0.635	0.102	1.190	3.521	2.898
46	*A. versicolar* SPCV20	+	2.507	0.768	0.123	1.441	4.262	3.507

Contd...

47	*A. versicolar* SPCV71	+	2.279	0.698	0.112	1.310	3.874	3.188
48	*Helminthosporium oryzae* SPCV130	+	2.231	0.684	0.110	1.282	3.792	3.121
49	*Fusarium oxysporam* SPCV85	+	1.624	0.498	0.080	0.933	2.761	2.272
50	*Penicillium chrysogenum* SPCV15	+	2.175	0.667	0.107	1.250	3.698	3.043
51	*P. chrysogenum* SPCV40	+	2.279	0.698	0.112	1.310	3.874	3.188
52	*P. chrysogenum* SPCV62	++	3.314	1.016	0.163	1.905	5.634	4.637
53	*P. digitatum* SPCV21	+	2.642	0.810	0.130	1.519	4.492	3.696
54	*P. digitatum* SPCV45	+	2.400	0.736	0.118	1.379	4.080	3.358
55	*P. digitatum* SPCV46	+	2.436	0.747	0.120	1.400	4.141	3.408
56	*P. digitatum* SPCV51	+	1.541	0.231	0.061	0.624	2.620	2.156
57	*P. digitatum* SPCV6	++	3.050	0.935	0.150	1.753	5.185	4.267
58	*P. japanicum* SPCV36	+	1.513	0.464	0.075	0.870	2.572	2.117
59	*P. lanosom* SPCV7	+	2.461	0.754	0.121	1.414	4.183	3.442
60	*P. lanosom* SPCV89	+	1.450	0.444	0.071	0.833	2.465	2.029
61	*Rhizopus nigricans*SPCV8	++	2.115	0.648	0.104	1.215	3.595	2.958
62	*Trichoderma harzianum* SPCV103	++	2.852	0.874	0.141	1.639	4.849	3.991
63	*T. harzianum* SPCV104	+++	3.955	1.212	0.195	2.273	6.723	5.532
64	**T. harzianum* SPCV127	+++	3.890	1.192	0.192	2.236	6.613	5.442
65	*T. harzianum* SPCV136	+++	4.984	1.528	0.246	2.865	8.473	6.973
66	*T. harzianum* SPCV22	++	2.900	0.889	0.143	1.667	4.930	4.057
67	*T. harzianum* SPCV23	+	2.320	0.711	0.114	1.333	3.944	3.246
68	*T. harzianum* SPCV24	++	3.176	0.974	0.156	1.825	5.400	4.443
69	*T. harzianum* SPCV25	++	3.176	0.974	0.156	1.825	5.400	4.443
70	*T. harzianum* SPCV26	+++	4.408	1.351	0.217	2.533	7.494	6.167
71	*T. harzianum* SPCV27	++	2.788	0.855	0.137	1.603	4.740	3.901

Contd...

72	*T. harzianum* SPCV309	++	2.852	0.874	0.141	1.639	4.849	3.991
73	*T. harzianum* SPCV16	++	3.176	0.974	0.156	1.825	5.400	4.443
74	**T. harzianum* SPCV56	+++	5.110	1.566	0.252	2.937	8.686	7.148
75	*T. harzianum* SPCV79	++	2.762	0.847	0.136	1.587	4.695	3.864
76	*T. harzianum* SPCV80	++	2.806	0.860	0.138	1.613	4.771	3.926
77	*T. harzianum* SPCV81	+++	4.350	1.333	0.214	2.500	7.395	6.086
78	*T. harzianum* SPCV82	++	2.739	0.840	0.135	1.574	4.656	3.832
79	*T. harzianum* AVCM7	++	2.107	0.439	0.119	1.349	4.194	3.139
80	*T. koningii* SPCV101	++	2.774	0.850	0.137	1.594	4.716	3.881
81	*T. koningii* SPCV107	+++	3.783	1.159	0.186	2.174	6.430	5.292
82	*T. koningii* SPCV108	++	2.736	0.839	0.135	1.572	4.651	3.827
83	*T. koningii* SPCV28	++	2.850	0.874	0.140	1.638	4.845	3.987
84	*T. koningii* SPCV86	++	1.856	0.569	0.091	1.067	3.155	2.597
85	*T. koningii* SPCV87	++	1.740	0.533	0.086	1.000	2.958	2.434
86	*T. koningii* SPCV88	+++	4.309	1.321	0.212	2.476	7.325	6.028
87	*T. koningii* SPCV9	++	3.511	1.076	0.173	2.018	5.968	4.911
88	*T. koningii* SPCV94	+++	4.218	1.293	0.208	2.424	7.171	5.901
89	*T. longibrachatum* SPCV110	+++	3.770	1.156	0.186	2.167	6.409	5.274
90	*T. longibrachatum* SPCV111	+++	3.625	1.111	0.179	2.083	6.163	5.071
91	*T. longibrachatum* SPCV121	+++	3.356	1.029	0.165	1.929	5.706	4.696
92	*T. longibrachatum* SPCV17	++	3.176	0.974	0.156	1.825	5.400	4.443
93	*T. longibrachatum* SPCV18	++	3.176	0.974	0.156	1.825	5.400	4.443
94	*T. longibrachatum* SPCV29	++	3.107	0.952	0.153	1.786	5.282	4.347
95	*T. longibrachatum* SPCV57	++	3.190	0.978	0.157	1.833	5.423	4.463
96	*T. longibrachatum* SPCV58	++	3.176	0.974	0.156	1.825	5.400	4.443

Contd...

97	*T. longibrachatum* SPCV59	++	3.176	0.974	0.156	1.825	5.400	4.443
98	*T. longibrachatum* SPCV74	++	3.050	0.935	0.150	1.753	5.185	4.267
99	*T. longibrachatum* SPCV75	++	3.049	0.934	0.150	1.752	5.183	4.265
100	*T. longibrachatum* SPCV90	++	3.222	0.988	0.159	1.852	5.478	4.508
101	*T. longibrachatum* SPCV91	+	2.210	0.677	0.109	1.270	3.756	3.091
102	*T. longibrachatum* SPCV92	+	2.175	0.667	0.107	1.250	3.698	3.043
103	*T. longibrachatum* SPCV96	+++	4.210	1.290	0.207	2.419	7.156	5.889
104	*T. longibrachatum* SPCV97	+++	4.045	1.240	0.199	2.325	6.876	5.659
105	*T. longibrachatum* SPCV98	+++	4.094	1.255	0.202	2.353	6.960	5.728
106	*T. longibrachatum* SPCV99	+++	4.005	1.228	0.197	2.302	6.808	5.603
107	*T. viride* SPCV105	+++	3.792	1.162	0.187	2.179	6.447	5.305
108	*T. viride* SPCV106	+++	4.143	1.270	0.204	2.381	7.043	5.796
109	**T. viride* SPCV11	+++	5.203	1.595	0.256	2.990	8.845	7.279
110	*T. viride* SPCV113	++	2.951	1.113	0.124	1.764	5.017	4.128
111	*T. viride* SPCV114	+++	3.712	1.138	0.183	2.133	6.310	5.193
112	*T. viride* SPCV115	+++	3.663	1.123	0.180	2.105	6.227	5.125
113	*T. viride* SPCV116	+++	3.639	1.115	0.179	2.092	6.187	5.091
114	*T. viride* SPCV118	+++	3.673	1.126	0.181	2.111	6.245	5.139
115	*T. viride* SPCV119	+++	3.729	1.143	0.184	2.143	6.339	5.216
116	*T. viride* SPCV120	+++	3.544	1.086	0.175	2.037	6.026	4.959
117	*T. viride* SPCV122	+++	3.609	1.106	0.178	2.074	6.135	5.049
118	*T. viride* SPCV123	++	2.803	0.859	0.138	1.611	4.766	3.922
119	*T. viride* SPCV124	+++	4.579	1.404	0.226	2.632	7.784	6.406
120	*T. viride* SPCV125	+++	4.579	1.404	0.226	2.632	7.784	6.406
121	*T. viride* SPCV126	+++	4.640	1.422	0.229	2.667	7.888	6.491

Contd...

122	*T. viride* SPCV128	+++	3.492	1.070	0.172	2.007	5.936	4.885
123	*T. viride* SPCV129	++	3.045	0.933	0.150	1.750	5.177	4.260
124	*T. viride* SPCV131	+++	4.524	1.387	0.223	2.600	7.691	6.329
125	*T. viride* SPCV132	++	4.462	1.368	0.220	2.564	7.585	6.242
126	*T. viride* SPCV134	+++	4.447	1.363	0.219	2.556	7.559	6.221
127	*T. viride* SPCV135	+++	4.397	1.348	0.217	2.527	7.475	6.151
128	*T. viride* SPCV137	+++	4.419	1.354	0.218	2.540	7.512	6.182
129	**T. viride* SPCV41	+++	5.118	1.569	0.252	2.941	8.700	7.160
130	**T. viride* SPCV3	+++	5.386	1.651	0.265	3.095	9.156	7.535
131	*T. viride* SPCV31	+	2.417	0.741	0.119	1.389	4.108	3.381
132	*T. viride* SPCV32	++	3.152	0.966	0.155	1.812	5.359	4.410
133	*T. viride* SPCV33	++	3.115	0.955	0.153	1.790	5.295	4.358
134	*T. viride* SPCV34	+++	4.238	1.299	0.209	2.436	7.205	5.930
135	*T. viride* SPCV37	++	3.335	1.022	0.164	1.917	5.670	4.666
136	*T. viride* SPCV47	++	3.050	0.935	0.150	1.753	5.185	4.267
137	*T. viride* SPCV48	+	2.505	0.768	0.123	1.439	4.258	3.504
138	*T. viride* SPCV76	++	3.050	0.935	0.150	1.753	5.185	4.267
139	*T. viride* SPCV83	+++	3.544	1.086	0.175	2.037	6.026	4.959
140	*T. vriide* AVCM4	++	2.427	0.731	0.119	1.359	4.094	3.390

CD/CZ - Colony diameter / clear zone diameter ratio

\+ - 0.05 mm

++ - 0.05 - 0.10 mm

+++ - 0.10 - 0.15 mm

Cellulolytic enzymes

Endoglucanase, Exoglucanase, b- glucosidase and Xylanase.

* Represents maximum producing fungal strains

Table 2 Effect of defined carbon substrates on cellulase and xylanase production by strains of *Trichoderma viride*

Substrate	Cellulase												Xylanase (IU/ml)			
	CMCase (IU/ml)				FPase (IU/ml)				β- glucosidase (IU/ml)							
	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41
Avicel	4.378	5.516	5.324	5.117	1.443	1.587	1.532	1.097	0.153	0.168	0.162	0.116	0.475	0.523	0.505	0.362
CMC	5.015	4.816	4.648	4.467	1.337	1.471	1.420	1.017	0.162	0.178	0.172	0.123	0.466	0.513	0.495	0.355
Solka floc	2.801	3.081	2.974	2.858	0.930	1.023	0.987	0.707	0.009	0.010	0.010	0.007	0.464	0.510	0.492	0.353
Pectin	0.033	0.036	0.035	0.033	0.027	0.030	0.029	0.021	0.001	0.001	0.001	0.001	0.008	0.009	0.009	0.006
Glycerol	0.025	0.027	0.026	0.025	0.032	0.035	0.034	0.024	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Cellobiose	0.289	0.318	0.307	0.295	0.305	0.335	0.323	0.232	1.224	1.346	1.299	0.930	0.001	0.001	0.001	0.001
Xylan	0.698	0.768	0.741	0.712	0.217	0.239	0.231	0.165	0.016	0.018	0.017	0.012	0.729	1.802	0.774	0.554
Filter Paper	2.745	3.020	2.915	2.801	0.498	0.548	0.529	0.379	0.107	0.118	0.114	0.082	0.077	0.085	0.082	0.059
Cotton	0.987	1.086	1.048	1.007	0.325	0.357	0.345	0.247	0.097	0.107	0.103	0.074	0.009	0.010	0.010	0.007
Cellodextrin	0.082	0.090	0.087	0.083	0.015	0.017	0.016	0.012	0.006	0.007	0.007	0.005	0.008	0.009	0.009	0.006
F	1436.920**				668.680**				13.578**				131.796**			

Values are mean of three assays of cultures in replicates.

** (P< 0.001), * maximum enzyme producing strain.

Trichoderma viride strains (*T. viride* AVCM4, *T. viride* SPCV3*, *T. viride* SPCV11 and *T. viride* SPCV41).

Table 3 Effect of defined carbon substrates on cellulase and xylanase production by strains of *Trichoderma harzianum*

Substrate	Cellulase									Xylanase (IU/ml)		
	CMCase (IU/ml)			FPase (IU/ml)			β- glucosidase (IU/ml)					
	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127
Avicel	2.172	5.183	4.716	0.625	1.505	1.369	0.066	0.129	0.142	0.206	0.496	0.545
CMC	1.596	4.525	4.118	0.579	1.395	1.269	0.070	0.137	0.151	0.202	0.486	0.534
Solka floc	1.213	2.895	2.634	0.403	0.970	0.883	0.044	0.008	0.008	0.201	0.484	0.531
Pectin	0.014	0.034	0.031	0.011	0.028	0.026	0.000	0.001	0.001	0.004	0.009	0.009
Glycerol	0.011	0.025	0.023	0.010	0.033	0.030	0.000	0.000	0.000	0.000	0.000	0.000
Cellobiose	0.125	0.299	0.272	0.132	0.318	0.289	0.053	1.036	1.138	0.002	0.001	0.001
Xylan	0.302	0.722	0.657	0.094	0.227	0.206	0.027	0.014	0.015	1.604	0.760	0.836
Filter Paper	1.189	2.838	2.582	0.216	0.520	0.473	0.046	0.091	0.100	0.033	0.081	0.089
Cotton	0.328	1.020	0.929	0.912	0.338	0.308	0.042	0.082	0.090	0.096	0.009	0.010
Cellodextrin	0.035	0.085	0.077	0.007	0.016	0.015	0.003	0.005	0.006	0.004	0.009	0.009
F	93918.940**			8406.698**			53.273**			824.355**		

Values are mean of three assays of cultures in replicates.

** (P< 0.001), * maximum enzyme producing strain.

Trichoderma harzianum strains (*T. harzianum* AVCM7, *T. harzianum* SPCV56* and *T. harzianum* SPCV127).

Table 4 Effect of defined carbon substrates on cellulase and xylanase production by strains of *Aspergillis niger*

Substrate	Cellulase						Xylanase (IU/ml)	
	CMCase (IU/ml)		FPase (IU/ml)		β-glucosidase(IU/ml)			
	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93
Avicel	1.974	4.288	0.571	1.245	0.060	0.129	0.187	0.495
CMC	1.451	3.743	0.529	1.154	0.064	0.137	0.184	0.486
Solka floc	1.103	2.395	0.368	0.802	0.040	0.008	0.183	0.483
Pectin	0.013	0.028	0.010	0.024	0.000	0.001	0.003	0.009
Glycerol	0.010	0.021	0.009	0.027	0.000	0.000	0.000	0.000
Cellobiose	0.114	0.247	0.120	0.263	0.048	1.035	0.002	0.001
Xylan	0.275	0.597	0.086	0.187	0.025	0.014	1.458	0.760
Filter Paper	1.081	2.347	0.197	0.430	0.042	0.091	0.030	0.081
Cotton	0.298	0.844	0.833	0.280	0.038	0.082	0.087	0.009
Cellodextrin	0.032	0.070	0.006	0.013	0.003	0.005	0.003	0.009
t	286.469**		86.544**		7.529**		34.423**	

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Aspergillus niger strains (*A. niger* AVCM1 and *A. niger* SPCV93*).

Table 5 Effect of natural cellulosic substrates on cellulase and xylanase production by strains of *Trichoderma viride*

Substrate	Cellulase												Xylanase (IU/ml)			
	CMCase (IU/ml)				FPase (IU/ml)				β- glucosidase (IU/ml)							
	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41
Wheat bran	5.381	10.319	9.055	9.572	2.166	2.523	2.477	1.951	0.106	0.150	0.135	0.106	1.134	1.318	1.271	1.395
Rice bran	3.164	6.780	5.949	6.289	1.995	2.194	1.925	1.517	0.096	0.106	0.093	0.073	1.005	1.226	1.163	0.916
Paddy straw	2.478	7.126	6.253	6.610	1.297	1.427	1.252	0.986	0.080	0.088	0.077	0.061	1.067	1.294	0.923	0.963
Bagasse	3.973	9.870	8.661	9.156	1.185	2.891	2.537	1.998	0.153	0.183	0.161	0.126	1.755	1.730	0.794	1.334
Cotton seed	3.067	4.474	3.926	4.150	0.615	0.676	0.593	0.467	0.080	0.088	0.077	0.061	0.795	0.775	0.768	0.605
Corncob	3.799	4.179	3.667	3.876	1.410	1.551	1.361	1.072	0.057	0.063	0.055	0.044	1.119	0.617	0.717	0.565
Paper sludge	2.208	3.529	3.097	3.274	0.535	0.589	0.517	0.407	0.038	0.042	0.037	0.029	0.627	0.590	0.606	0.477
Black gram husk	1.994	3.293	2.890	3.055	0.712	0.783	0.687	0.541	0.072	0.079	0.069	0.055	0.585	0.444	0.565	0.445
F	190176.8**				1796.577**				18.824**				312.886**			

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Trichoderma viride strains (*T. viride* AVCM4, *T. viride* SPCV3*, *T. viride* SPCV11 and *T. viride* SPCV41).

Table 6 Effect of natural cellulosic substrates on cellulase and xylanase production by strains of *Trichoderma harzianum*

Substrate	Cellulase									Xylanase (IU/ml)		
	CMCase (IU/ml)			FPase (IU/ml)			β- glucosidase (IU/ml)					
	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127
Wheat bran	4.063	9.696	8.823	1.111	2.677	2.436	0.061	0.119	0.130	0.794	1.813	2.103
Rice bran	2.669	6.370	5.797	0.864	2.080	1.893	0.042	0.082	0.090	0.522	1.257	1.381
Paddy straw	2.806	6.696	6.093	0.562	1.353	1.231	0.035	0.068	0.074	0.549	1.321	1.452
Bagasse	3.886	9.274	8.439	1.138	2.741	2.494	0.072	0.141	0.155	0.760	1.830	2.011
Cotton seed	1.761	4.204	3.825	0.266	0.641	0.583	0.035	0.068	0.074	0.344	0.830	0.912
Corncob	1.645	3.927	3.573	0.611	1.471	1.338	0.025	0.048	0.053	0.322	0.775	0.851
Paper sludge	1.389	3.316	3.017	0.232	0.558	0.508	0.017	0.032	0.036	0.272	0.654	0.719
Black gram husk	1.296	3.094	2.816	0.308	0.742	0.676	0.031	0.061	0.067	0.254	0.611	0.671
F	78814.778**			11575.860**			20.144**			10714.841**		

Values are mean of three assays of cultures in replicates.

** (P< 0.001), * maximum enzyme producing strain.

Trichoderma harzianum strains (*T. harzianum* AVCM7, *T. harzianum* SPCV56* and *T. harzianum* SPCV127).

Table 7 Effect of natural cellulosic substrates on cellulase and xylanase production by strains of *Aspergillis niger*

Substrate	Cellulase						Xylanase (IU/ml)	
	CMCase (IU/ml)		FPase (IU/ml)		β- glucosidase (IU/ml)			
	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93
Wheat bran	3.693	8.021	1.015	2.214	0.045	2.214	0.722	1.911
Rice bran	2.427	5.270	0.789	1.721	0.038	1.721	0.475	1.256
Paddy straw	2.550	5.539	0.513	1.119	0.031	1.119	0.499	1.320
Bagasse	3.533	7.672	1.039	2.268	0.065	2.268	0.691	1.828
Cotton seed	1.601	3.478	0.243	0.530	0.031	0.530	0.313	0.829
Corncob	1.496	3.248	0.558	1.217	0.023	1.217	0.292	0.774
Paper sludge	1.263	2.743	0.212	0.462	0.015	0.462	0.247	0.654
Black gram husk	1.179	2.560	0.282	0.614	0.028	0.614	0.230	0.610
t	537.018**		142.935**		270.355**		188.606**	

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Aspergillus niger strains (*A. niger* AVCM1 and *A. niger* SPCV93*).

Table 8 Effect of nitrogen sources on cellulase and xylanase production by strains of *Trichoderma viride*

Nitrogen	Cellulase												Xylanase (IU/ml)			
	CMCase (IU/ml)				FPase (IU/ml)				β- glucosidase (IU/ml)							
	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41
Yeast extract	8.045	10.458	10.094	9.701	2.307	3.000	2.895	2.073	0.139	0.180	0.174	0.125	1.237	1.608	1.452	1.349
Peptone	7.585	9.860	9.517	9.146	2.175	2.828	2.730	1.955	0.131	0.170	0.164	0.117	1.166	1.516	1.414	1.048
Soya bean meal	7.163	9.312	8.988	8.638	2.055	2.671	2.578	1.846	0.123	0.160	0.155	0.111	1.102	1.432	1.382	0.990
Urea	4.952	6.437	6.213	5.971	1.420	1.846	1.782	1.276	0.085	0.111	0.107	0.077	0.761	0.990	0.955	0.684
Sodium nitrate	4.632	6.021	5.812	5.585	1.328	1.727	1.667	1.194	0.080	0.104	0.100	0.072	0.712	0.926	0.894	0.640
Ammonium phosphate	4.472	5.813	5.611	5.392	1.283	1.667	1.609	1.152	0.077	0.100	0.097	0.069	0.688	0.894	0.863	0.618
Ammonium nitrate	5.179	6.733	6.499	6.246	1.486	1.931	1.864	1.335	0.089	0.116	0.112	0.080	0.796	1.035	0.999	0.716
F	29308.039**				4697.510**				11.939**				801.359**			

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Trichoderma viride strains (*T. viride* AVCM4, *T. viride* SPCV3*, *T. viride* SPCV11 and *T. viride* SPCV41).

Table 9 Effect of nitrogen sources on cellulase and xylanase production by strains of *Trichoderma harzianum*

Nitrogen	Cellulase									Xylanase (IU/ml)		
	CMCase (IU/ml)			FPase (IU/ml)			β- glucosidase (IU/ml)					
	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127
Yeast extract	4.117	9.826	8.942	1.281	2.814	2.588	0.071	0.139	0.152	0.933	1.825	2.676
Peptone	3.882	9.264	8.431	1.113	2.681	2.450	0.067	0.131	0.144	0.597	1.438	1.580
Soya bean meal	3.666	8.749	7.962	1.052	2.532	2.304	0.063	0.123	0.136	0.564	1.358	1.492
Urea	2.534	6.048	5.504	0.727	1.750	1.593	0.044	0.085	0.094	0.390	0.939	1.031
Sodium nitrate	2.370	5.657	5.148	0.680	1.637	1.490	0.041	0.080	0.088	0.365	0.878	0.965
Ammonium phosphate	2.289	5.462	4.970	0.656	1.581	1.439	0.039	0.077	0.085	0.352	0.848	0.931
Ammonium nitrate	2.651	6.326	5.757	0.760	1.831	1.666	0.046	0.089	0.098	0.408	0.982	1.079
F	229413.8**			21118.063**			51.014**			17989.344**		

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Trichoderma harzianum strains (*T. harzianum* AVCM7, *T. harzianum* SPCV56* and *T. harzianum* SPCV127).

Table 10 Effect of nitrogen sources on cellulase and xylanase production by strains of *Aspergillis niger*

Nitrogen	Cellulase						Xylanase (IU/ml)	
	CMCase (IU/ml)		FPase (IU/ml)		β- glucosidase(IU/ml)			
	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93
Yeast extract	3.743	8.129	1.078	2.353	0.064	0.138	0.976	1.523
Peptone	3.529	7.664	1.017	2.218	0.061	0.131	0.543	1.436
Soya bean meal	3.333	7.238	0.960	2.095	0.057	0.123	0.513	1.356
Urea	2.304	5.003	0.664	1.448	0.040	0.085	0.354	0.938
Sodium nitrate	2.155	4.680	0.621	1.355	0.037	0.080	0.331	0.877
Ammonium phosphate	2.081	4.518	0.599	1.308	0.036	0.077	0.320	0.847
Ammonium nitrate	2.410	5.234	0.694	1.515	0.042	0.089	0.371	0.981
t	532.779**		191.569**		11.488**		68.986**	

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Aspergillus niger strains (*A. niger* AVCM1 and *A. niger* SPCV93*)

Table 11 Effect of temperature on cellulase and xylanase production by strains of *Trichoderma viride*

Temperature°C	Cellulase												Xylanase (IU/ml)			
	CMCase (IU/ml)				FPase (IU/ml)				β- glucosidase (IU/ml)							
	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41
20	6.101	7.930	7.653	7.353	2.336	3.036	2.930	2.815	0.142	0.184	0.178	0.171	1.253	1.628	1.571	1.510
25	7.931	10.309	9.949	9.558	3.036	3.947	3.809	3.659	0.184	0.239	0.231	0.222	1.629	2.117	2.043	1.903
30	8.169	10.618	10.247	9.845	3.127	4.065	3.923	3.769	0.190	0.246	0.238	0.228	1.677	2.180	2.109	1.929
35	7.635	9.923	9.577	9.201	2.923	3.799	3.666	3.523	0.177	0.230	0.222	0.213	1.568	2.038	1.966	1.889
40	6.756	8.782	8.475	8.142	2.587	3.362	3.245	3.117	0.157	0.204	0.197	0.189	1.387	1.803	1.740	1.672
50	4.504	5.854	5.650	5.428	1.724	2.241	2.163	2.078	0.105	0.136	0.131	0.126	0.925	1.202	1.160	1.115
F	42269.594**				7616.185**				27.330**				1954.365**			

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Trichoderma viride strains (*T. viride* AVCM4, *T. viride* SPCV3*, *T. viride* SPCV11 and *T. viride* SPCV41).

Table 12 Effect of temperature on cellulase and xylanase production by strains of *Trichoderma harzianum*

Temperature°C	Cellulase									Xylanase (IU/ml)		
	CMCase (IU/ml)			FPase (IU/ml)			β- glucosidase (IU/ml)					
	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127
20	3.125	7.453	6.780	1.197	2.853	2.596	0.073	0.173	0.157	0.642	1.530	1.392
25	4.063	9.688	8.815	1.556	3.709	3.375	0.094	0.225	0.205	0.834	1.989	1.810
30	4.185	9.979	9.079	1.602	3.820	3.576	0.097	1.932	0.211	0.959	2.049	2.864
35	3.911	9.326	8.485	1.497	3.571	3.248	0.091	0.216	0.197	0.803	1.915	1.742
40	3.461	8.253	7.509	1.325	3.160	2.875	0.080	0.191	0.174	0.711	1.695	1.542
45	2.308	5.502	5.006	0.883	2.106	1.917	0.054	0.128	0.116	0.474	1.130	1.028
F	352118.8**			36289.940**			34470.385**			21495.575**		

Values are mean of three assays of cultures in replicates.

** (P< 0.001), * maximum enzyme producing strain.

Trichoderma harzianum strains (*T. harzianum* AVCM7, *T. harzianum* SPCV56* and *T. harzianum* SPCV127).

Table 13 Effect of temperature on cellulase and xylanase production by strains of *Aspergillis niger*

Temperature°C	Cellulase						Xylanase (IU/ml)	
	CMCase (IU/ml)		FPase (IU/ml)		β- glucosidase (IU/ml)			
	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93
20	2.842	6.168	1.088	2.361	0.066	0.143	0.584	1.267
25	3.695	8.018	1.415	3.070	0.086	0.186	0.759	1.646
30	3.806	8.259	1.457	3.162	0.088	0.192	1.002	1.696
35	3.557	7.719	1.362	2.955	0.083	0.179	0.730	1.585
40	3.148	6.831	1.205	2.615	0.073	0.158	0.646	1.403
50	2.099	4.554	0.803	1.743	0.049	0.106	0.431	0.935
t	657.954**		202.386**		10.919**		77.195**	

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Aspergillus niger strains (*A. niger* AVCM1 and *A. niger* SPCV93*).

Table 14 Effect of natural cellulosic substrates on cellulase and xylanase production by strains of *Trichoderma viride* under SSF

Substrate	Cellulase												Xylanase (IU/ml)			
	CMCase (IU/ml)				FPase (IU/ml)				β- glucosidase (IU/ml)							
	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41
Wheat bran	7.364	44.155	35.607	31.798	2.818	12.080	9.741	6.482	0.169	0.659	0.531	0.354	1.511	8.635	6.963	4.634
Paper sludge	6.694	40.141	32.370	28.908	2.562	10.981	8.856	5.893	0.154	0.599	0.483	0.321	1.374	7.850	6.330	4.212
Rice bran	3.927	26.374	21.269	18.993	2.364	8.535	6.882	4.580	0.111	0.412	0.333	0.221	1.275	5.158	4.159	2.768
Paddy straw	2.851	27.720	22.354	19.963	1.443	5.551	4.476	2.979	0.133	0.342	0.276	0.184	1.041	5.421	4.371	2.909
Bagasse	5.654	38.394	30.962	27.650	2.060	11.246	9.069	6.035	0.016	0.712	0.574	0.382	1.284	7.508	6.055	4.029
Saw dust	3.383	17.404	14.035	12.533	0.309	2.630	2.121	1.411	0.011	0.342	0.276	0.184	0.725	3.403	2.745	1.826
Corncob	5.043	16.256	13.109	11.707	1.462	6.033	4.865	3.238	0.009	0.245	0.198	0.132	1.757	3.179	2.564	1.706
Cotton seed	1.820	13.728	11.070	9.886	0.100	2.291	1.848	1.230	0.002	0.163	0.132	0.088	0.141	2.685	2.165	1.441
Black gram husk	1.826	12.810	10.330	9.225	1.129	3.046	2.456	1.635	0.011	0.307	0.248	0.165	0.162	2.505	2.020	1.344
F	6801628.0**				499751.8**				855.188**				127437.7**			

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Trichoderma viride strains (*T. viride* AVCM4, *T. viride* SPCV3*, *T. viride* SPCV11 and *T. viride* SPCV41).

Table 15 Effect of natural cellulosic substrates on cellulase and xylanase production by strains of *Trichoderma harzianum* under SSF

Substrate	Cellulase									Xylanase (IU/ml)		
	CMCase (IU/ml)			FPase (IU/ml)			β- glucosidase (IU/ml)					
	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127
Wheat bran	5.854	35.409	27.563	1.602	9.775	7.609	0.087	0.433	0.407	1.145	6.987	6.568
Paper sludge	5.322	32.190	25.057	1.456	8.886	6.917	0.079	0.393	0.370	1.041	6.352	5.971
Rice bran	3.497	21.150	16.464	1.132	6.906	5.376	0.055	0.271	0.255	0.684	4.174	3.923
Paddy straw	3.675	22.229	17.304	0.736	4.492	3.497	0.045	0.225	0.211	0.719	4.387	4.123
Bagasse	5.090	30.789	23.967	1.491	9.100	7.084	0.094	0.468	0.439	0.995	6.076	5.711
Saw dust	2.307	13.956	10.864	0.349	2.128	1.656	0.045	0.225	0.211	0.451	2.754	2.589
Corncob	2.155	13.036	10.148	0.800	4.882	3.801	0.032	0.161	0.151	0.421	2.572	2.418
Cotton seed	1.820	11.009	8.569	0.304	1.854	1.443	0.022	0.107	0.101	0.356	2.172	2.042
Black gram husk	1.698	10.272	7.996	0.404	2.465	1.919	0.041	0.202	0.190	0.332	2.027	1.905
F	8336880**			499269.4**			739.489**			328504.7**		

Values are mean of three assays of cultures in replicates.

** ($P< 0.001$), * maximum enzyme producing strain.

Trichoderma harzianum strains (*T. harzianum* AVCM7, *T. harzianum* SPCV56* and *T. harzianum* SPCV127).

Table 16 Effect of natural cellulosic substrates on cellulase and xylanase production by strains of *Aspergillis niger* under SSF

Substrate	Cellulase						Xylanase (IU/ml)	
	CMCase (IU/ml)		FPase (IU/ml)		β- glucosidase(IU/ml)			
	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93
Wheat bran	4.794	14.911	1.317	4.116	0.072	0.220	0.937	3.553
Paper sludge	4.358	13.555	1.198	3.742	0.065	0.200	0.852	3.230
Rice bran	2.863	8.906	0.931	2.908	0.045	0.138	0.560	2.122
Paddy straw	3.010	9.361	0.605	1.892	0.037	0.114	0.589	2.231
Bagasse	4.168	12.966	1.227	3.832	0.077	0.238	0.815	3.090
Saw dust	1.890	5.877	0.287	0.896	0.037	0.114	0.370	1.401
Corncob	1.765	5.490	0.658	2.056	0.027	0.082	0.345	1.308
Cotton seed	1.490	4.636	0.250	0.781	0.018	0.055	0.291	1.105
Black gram husk	1.391	4.326	0.332	1.038	0.033	0.103	0.272	1.031
t	430.930**		365.075**		15.101**		257.541**	

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Aspergillus niger strains (*A. niger* AVCM1 and *A. niger* SPCV93*)

Table 17 Effect of nitrogen sources on cellulase and xylanase production by strains of *Trichoderma viride*

Nitrogen	Cellulase												Xylanase (IU/ml)			
	CMCase (IU/ml)				FPase (IU/ml)				β- glucosidase (IU/ml)							
	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41
Soya been meal	10.015	57.402	46.289	41.338	3.664	15.704	12.664	8.427	0.220	0.857	0.691	0.460	1.964	11.225	9.052	6.024
Yeast extract	9.100	48.571	39.168	34.978	3.100	12.288	10.715	7.130	0.186	0.725	0.585	0.389	1.662	9.498	7.660	5.097
Peptone	7.806	46.804	37.744	33.706	2.988	12.704	10.326	6.871	0.179	0.699	0.563	0.375	1.602	9.153	7.381	4.912
Urea	9.630	40.682	32.806	29.297	3.623	11.668	9.409	6.262	0.218	0.701	0.565	0.376	1.942	6.256	5.045	3.357
Triptone	9.608	38.355	30.930	27.622	3.415	11.001	8.871	5.903	0.205	0.661	0.533	0.354	1.831	5.898	4.756	3.165
Caesin	8.246	36.224	29.211	26.087	3.226	10.390	8.378	5.575	0.194	0.624	0.503	0.335	1.729	5.570	4.492	2.989
Ammonium chloride	7.774	25.040	20.193	18.033	2.230	7.182	5.792	3.854	0.134	0.431	0.348	0.231	1.195	3.851	3.105	2.066
Ammonium nitrate	7.272	23.422	18.888	16.867	2.086	6.718	5.417	3.605	0.125	0.403	0.325	0.216	1.118	3.602	2.905	1.933
Sodium nitrate	7.020	22.613	18.235	16.284	2.014	6.486	5.230	3.480	0.121	0.389	0.314	0.209	1.080	3.477	2.804	1.866
Ammonium phosphate	8.131	26.191	21.121	18.862	2.332	7.512	6.058	4.031	0.140	0.451	0.364	0.242	1.250	4.028	3.248	2.161
F	1.1E+07**				767504.7**				2096.800**				403592.9**			

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Trichoderma viride strains (*T. viride* AVCM4, *T. viride* SPCV3*, *T. viride* SPCV11 and *T. viride* SPCV41).

Table 18 Effect of nitrogen sources on cellulase and xylanase production by strains of *Trichoderma harzianum*

Nitrogen	Cellulase									Xylanase (IU/ml)		
	CMCase (IU/ml)			FPase (IU/ml)			β- glucosidase (IU/ml)					
	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127
Soya been meal	7.610	46.031	35.832	2.082	12.707	9.892	0.194	0.563	0.529	1.488	9.083	8.539
Yeast extract	6.440	38.949	30.320	1.762	10.752	8.370	0.096	0.476	0.448	1.259	7.686	7.225
Peptone	6.205	37.533	29.217	1.698	10.361	8.066	0.093	0.459	0.431	1.214	7.407	6.962
Urea	5.394	32.623	25.395	1.547	9.442	7.350	0.093	0.460	0.433	0.829	5.062	4.759
Triptone	5.085	30.758	23.943	1.459	8.902	6.930	0.088	0.434	0.408	0.782	4.773	4.487
Caesin	4.803	29.048	22.612	1.377	8.407	6.545	0.083	0.410	0.385	0.739	4.508	4.237
Ammonium chloride	3.320	20.080	15.631	0.952	5.812	4.524	0.057	0.283	0.266	0.511	3.116	2.929
Ammonium nitrate	3.105	18.782	14.621	0.891	5.436	4.232	0.053	0.265	0.249	0.478	2.915	2.740
Sodium nitrate	2.998	18.133	14.116	0.860	5.248	4.085	0.052	0.256	0.240	0.461	2.814	2.645
Ammonium phosphate	3.473	21.003	16.350	0.996	6.079	4.732	0.060	0.296	0.278	0.534	3.259	3.064
F	8549308**			101550**			1573.522**			525727.8**		

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Trichoderma harzianum strains (*T. harzianum* AVCM7, *T. harzianum* SPCV56* and *T. harzianum* SPCV127).

Table 19 Effect of nitrogen sources on cellulase and xylanase production by strains of *Aspergillis niger*

Nitrogen	Cellulase						Xylanase (IU/ml)	
	CMCase (IU/ml)		FPase (IU/ml)		β- glucosidase(IU/ml)			
	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93
Soya been meal	6.232	19.384	1.713	5.351	0.093	0.286	1.219	4.619
Yeast extract	5.273	16.402	1.449	4.528	0.079	0.242	1.031	3.909
Peptone	5.081	15.806	1.397	4.363	0.076	0.233	0.994	3.766
Urea	4.417	13.738	1.273	3.976	0.076	0.234	0.679	2.574
Triptone	4.164	12.952	1.200	3.749	0.072	0.221	0.640	2.427
Caesin	3.933	12.233	1.133	3.540	0.068	0.208	0.605	2.292
Ammonium chloride	2.719	8.456	0.783	2.447	0.047	0.144	0.418	1.585
Ammonium nitrate	2.543	7.909	0.733	2.289	0.044	0.135	0.391	1.482
Sodium nitrate	2.455	7.636	0.707	2.210	0.042	0.130	0.378	1.431
Ammonium phosphate	2.844	8.845	0.819	2.560	0.049	0.151	0.437	1.657
t	1386.756**		364.678**		25.716**		509.613**	

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Aspergillus niger strains (*A. niger* AVCM1 and *A. niger* SPCV93*).

Table 20 Effect of moisture content on cellulase and xylanase production by strains of *Trichoderma viride* under SSF

Moisture (%)	Cellulase												Xylanase (IU/ml)			
	CMCase (IU/ml)				FPase (IU/ml)				β- glucosidase (IU/ml)							
	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41	AVCM4	SPCV3	SPCV11	SPCV41
20	9.669	57.976	46.752	41.751	3.701	15.861	12.790	8.511	0.222	0.865	0.698	0.464	1.984	11.337	9.143	6.084
30	9.765	58.550	47.215	42.164	3.737	16.018	12.917	8.595	0.224	0.874	0.705	0.469	2.004	11.450	9.233	6.144
35	9.860	59.124	47.678	42.578	3.774	16.175	13.043	8.680	0.227	0.882	0.712	0.473	2.023	11.562	9.324	6.204
40	9.956	59.698	48.141	42.991	3.811	16.332	13.170	8.764	0.229	0.891	0.718	0.478	2.043	11.674	9.414	6.265
45	10.042	60.214	48.558	43.363	3.844	16.473	13.284	8.840	0.231	0.899	0.725	0.482	2.061	11.775	9.496	6.319
50	10.128	60.731	48.974	43.735	3.877	16.614	13.398	8.916	0.233	0.906	0.731	0.486	2.078	11.876	9.577	6.373
55	10.530	63.142	50.918	45.472	4.030	17.274	13.930	9.270	0.242	0.942	0.760	0.506	2.161	12.348	9.957	6.626
60	8.464	50.752	40.928	36.549	3.240	13.885	11.197	7.451	0.195	0.757	0.611	0.406	1.737	9.925	8.004	5.326
65	5.885	35.285	28.454	25.411	2.252	9.653	7.784	5.180	0.135	0.527	0.425	0.283	1.208	6.900	5.564	3.703
70	3.324	19.932	16.073	14.354	1.272	5.453	4.397	2.926	0.076	0.297	0.240	0.160	0.682	3.898	3.143	2.092
80	2.541	15.233	12.285	10.970	0.972	4.167	3.361	2.236	0.058	0.227	0.183	0.122	0.521	2.979	2.402	1.599
F	1.3E+07**				672932.3**				2150.76**				705836.6**			

Values are mean of three assays of cultures in replicates.

** (P< 0.001), * maximum enzyme producing strain.

Trichoderma viride strains (*T. viride* AVCM4, *T. viride* SPCV3*, *T. viride* SPCV11 and *T. viride* SPCV41).

Table 21 Effect of moisture content on cellulase and xylanase production by strains of *Trichoderma harzianum* under SSF

Moisture (%)	Cellulase									Xylanase (IU/ml)		
	CMCase (IU/ml)			FPase (IU/ml)			β- glucosidase (IU/ml)					
	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127	AVCM7	SPCV56	SPCV127
20	7.687	46.491	36.190	2.103	12.834	9.991	0.115	0.568	0.534	1.503	9.174	8.624
30	7.763	46.952	36.549	2.124	12.961	10.090	0.116	0.574	0.539	1.518	9.265	8.709
35	7.839	47.412	36.907	2.144	13.089	10.189	0.117	0.580	0.545	1.533	9.356	8.795
40	7.915	47.872	37.265	2.165	13.216	10.287	0.118	0.585	0.550	1.548	9.447	8.880
45	7.983	48.287	37.588	2.184	13.330	10.377	0.119	0.590	0.555	1.561	9.529	8.957
50	8.052	48.701	37.910	2.203	13.444	10.466	0.120	0.595	0.560	1.575	9.610	9.034
55	8.372	50.634	39.415	2.290	13.978	10.881	0.125	0.619	0.582	1.637	9.992	9.393
60	6.729	40.699	31.682	1.841	11.235	8.746	0.100	0.497	0.468	1.316	8.031	7.550
65	4.678	28.296	22.026	1.280	7.811	6.081	0.070	0.346	0.325	0.915	5.584	5.249
70	2.643	15.983	12.442	0.723	4.412	3.435	0.039	0.195	0.184	0.517	3.154	2.965
80	2.020	12.216	9.509	0.553	3.372	2.625	0.030	0.149	0.140	0.395	2.411	2.266
F	8899100**			1297258**			2022.384**			682893.0**		

Values are mean of three assays of cultures in replicates.

** (P< 0.001), * maximum enzyme producing strain.

Trichoderma harzianum strains (*T. harzianum* AVCM7, *T. harzianum* SPCV56* and *T. harzianum* SPCV127).

Table 22 Effect of moisture content on cellulase and xylanase production by strains of *Aspergillis niger* under SSF

Moisture (%)	Cellulase						Xylanase (IU/ml)	
	CMCase (IU/ml)		FPase (IU/ml)		β- glucosidase (IU/ml)			
	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93	AVCM1	SPCV93
20	6.294	19.578	1.730	5.405	0.094	0.289	1.231	4.665
30	6.357	19.772	1.747	5.458	0.095	0.292	1.243	4.712
35	6.419	19.966	1.764	5.512	0.096	0.295	1.255	4.758
40	6.481	20.160	1.781	5.565	0.097	0.298	1.267	4.804
45	6.537	20.334	1.797	5.613	0.098	0.300	1.278	4.846
50	6.593	20.509	1.812	5.662	0.098	0.303	1.289	4.887
55	6.855	21.323	1.884	5.886	0.102	0.315	1.341	5.081
60	5.510	17.139	1.514	4.731	0.082	0.253	1.078	4.084
65	3.831	11.916	1.053	3.289	0.057	0.176	0.749	2.839
70	2.164	6.731	0.595	1.858	0.032	0.099	0.423	1.604
80	1.654	5.144	0.455	1.420	0.025	0.076	0.323	1.226
t	1442.197**		543.305**		18.854**		363.496**	

Values are mean of three assays of cultures in replicates.

** ($P < 0.001$), * maximum enzyme producing strain.

Aspergillus niger strains (*A. niger* AVCM1 and *A. niger* SPCV93*).

Table 23 Production of associated enzymes by strains of *Trichoderma* and *Aspergillus* under SSF

Enzyme	*Trichoderma viride*				*Trichoderma harzianum*			*Aspergillus niger*	
	AVCM4	SPCV3	SPCV11	SPCV41	AVCM 7	SPCV 56	SPCV 127	AVCM 1	SPCV 93
Amylase	0.676	4.055	3.270	2.920	0.538	3.251	2.531	0.440	1.369
Pectinase	0.558	3.345	2.698	2.409	0.444	2.682	2.088	0.363	1.130
Phytase	1.656	0.935	1.174	1.834	1.522	3.156	2.457	2.427	2.329
α - galactosidase	0.168	1.006	0.811	0.724	0.133	0.807	0.628	0.109	0.340
Protease	0.070	0.023	0.041	0.004	0.056	0.039	0.064	0.046	0.043
Amyloglucosidase	0.478	2.867	2.312	2.065	0.380	2.299	1.790	0.311	0.968
Chitinase	1.598	1.584	1.890	1.581	1.475	2.874	2.237	2.389	2.210
F	F - 52675.255**				F - 60856334**			t - 106.266**	

Values are mean of three assays of cultures in replicates.

** (P< 0.001), * maximum enzyme producing strains.

Trichoderma and *Aspergillus* strains (*Trichoderma viride* AVCM4, *T. viride* SPCV3, *T. viride* SPCV11*, *T. viride* SPCV41, *Trichoderma harzianum* AVCM7, *T. harzianum* SPCV56*, *T. harzianum* SPCV127, *Aspergillus niger* AVCM1 and *A. niger* SPCV93*)

Table 24 Enzymatic properties of cellulase and xylanase produced under submerged frementation

Enzyme properties	Enzyme activity (IU/ml)								
	Trichoderma viride				*Trichoderma harzianum*			*Aspergillus niger*	
CMCase	**AVCM4**	**SPCV3**	**SPCV11**	**SPCV41**	**AVCM7**	**SPCV56**	**SPCV127**	**AVCM1**	**SPCV93**
Optimal Temperature - 50°C	4.144	6.934	6.420	5.945	2.889	3.536	3.214	2.922	2.656
Optimal pH - 4.8	6.445	10.769	8.403	8.073	3.657	3.668	6.069	6.166	5.606
Thermo stability (75%) - 50°C	4.776	11.557	11.157	10.721	4.550	10.859	9.881	4.137	8.983
pH stability (75%) – 5.5	6.850	8.905	8.534	8.178	3.715	4.032	6.166	4.061	5.196
80% inhibitory metal ions	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}
FPase									
Optimal Temperature - 50°C	1.746	2.358	1.998	1.950	0.850	1.873	1.612	1.602	1.457
Optimal pH - 4.8	2.022	3.628	2.519	2.414	1.027	2.201	3.366	3.060	2.782
Thermo stability (75%) - 50°C	1.828	4.423	4.270	4.103	1.741	4.156	3.782	1.583	3.438
pH stability (75%) – 5.5	1.684	2.181	1.974	1.892	0.933	2.001	3.006	2.898	2.180
80% inhibitory metal ions	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}

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Enzyme properties	Enzyme activity (IU/ml)								
	Trichoderma viride				*Trichoderma harzianum*			*Aspergillus niger*	
β- glucosidase	**AVCM4**	**SPCV3**	**SPCV11**	**SPCV41**	**AVCM7**	**SPCV56**	**SPCV127**	**AVCM1**	**SPCV93**
Optimal Temperature - 50°C	0.025	0.230	0.213	0.197	0.096	0.133	0.121	0.110	0.101
Optimal pH - 4.8	0.212	0.266	0.214	0.228	0.148	0.207	0.311	0.344	0.313
Thermo stability (75%) - 50°C	0.110	0.266	0.257	0.247	0.105	0.25	0.228	0.095	0.207
pH stability (75%) – 5.5	0.134	0.162	0.155	0.148	0.123	0.322	0.159	0.181	0.283
80% inhibitory metal ions	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}
Xylanase									
Optimal Temperature - 50°C	0.198	1.195	1.106	1.024	0.161	1.169	1.063	1.134	1.031
Optimal pH - 4.8	1.491	1.939	1.858	1.781	0.261	1.904	2.913	2.648	2.407
Thermo stability (75%) - 50°C	0.980	2.371	2.289	2.199	0.933	2.227	2.027	0.849	1.843
pH stability (75%) – 5.5	1.114	1.448	1.387	1.330	0.255	1.922	1.551	1.707	1.981
80% inhibitory metal ions	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}

Values are mean of three assays of cultures in replicates.
Trichoderma viride AVCM4, *T. viride* SPCV3*, *T. viride* SPCV11, *T. viride* SPCV41, *Trichoderma harzianum* AVCM7, *T. harzianum* SPCV56*, *T. harzianum* SPCV127, *Aspergillus niger* AVCM1 and *A. niger* SPCV93*.
* Strains producing cellulase with optimum activity under various parameters.

Table 25 Enzymatic properties of cellulase and xylanase by strains of *Trichoderma viride*

Enzyme properties	Emzyme activity (IU/ml)								
	Trichoderma viride				*Trichoderma harzianum*			*Aspergillus niger*	
CMCase	**AVCM4**	**SPCV3**	**SPCV11**	**SPCV41**	**AVCM7**	**SPCV56**	**SPCV127**	**AVCM1**	**SPCV93**
Optimal Temperature - 50°C	6.507	26.972	20.865	17.953	3.784	11.735	9.128	3.448	4.489
Optimal pH - 4.8	11.829	38.103	30.507	27.167	5.354	14.724	19.263	7.276	9.474
Thermo stability (75%) - 50°C	9.049	29.311	23.467	20.881	4.118	11.326	14.818	5.597	7.287
pH stability (75%) – 5.5	10.009	32.241	23.112	20.579	4.531	12.459	14.581	6.157	8.016
80% inhibitory metal ions	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Pb^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}
FPase									
Optimal Temperature - 50°C	2.507	9.183	7.495	6.588	1.114	6.437	5.006	1.891	2.251
Optimal pH – 4.8	3.174	10.224	8.186	7.289	1.345	7.308	9.561	3.611	4.702
Thermo stability (75%) - 50°C	2.443	7.781	6.428	5.834	1.035	5.621	7.354	2.778	3.617
pH stability (75%) – 5.5	2.686	8.651	6.971	6.169	1.138	6.183	8.09	3.056	3.978
80% inhibitory metal ions	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}

Contd...

Enzyme properties	Emzyme activity (IU/ml)								
	Trichoderma viride				*Trichoderma harzianum*			*Aspergillus niger*	
β- glucosidase	**AVCM4**	**SPCV3**	**SPCV11**	**SPCV41**	**AVCM7**	**SPCV56**	**SPCV127**	**AVCM1**	**SPCV93**
Optimal Temperature - 50°C	0.045	0.894	0.692	0.595	0.125	0.441	0.343	0.129	0.169
Optimal pH - 4.8	0.334	1.073	0.859	0.765	0.221	0.821	1.079	0.406	0.528
Thermo stability (75%) - 50°C	0.165	0.825	0.626	0.588	0.139	0.631	0.826	0.311	0.406
pH stability (75%) – 5.5	0.282	0.908	0.727	0.647	0.186	0.695	0.909	0.343	0.367
80% inhibitory metal ions	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}
Xylanase									
Optimal Temperature - 50°C	0.311	4.648	4.596	3.394	0.211	4.556	3.543	1.142	1.743
Optimal pH - 4.8	2.341	7.542	6.038	5.377	0.342	6.323	8.221	3.125	4.068
Thermo stability (75%) - 50°C	1.801	5.765	4.648	4.398	0.263	4.864	6.383	2.403	2.906
pH stability (75%) – 5.5	1.981	6.381	5.109	4.551	0.292	5.351	6.911	2.644	2.828
80% inhibitory metal ions	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}

Values are mean of three assays of cultures in replicates.
Trichoderma viride AVCM4, *T. viride* SPCV3*, *T. viride* SPCV11, *T. viride* SPCV41, *Trichoderma harzianum* AVCM7, *T. harzianum* SPCV56*, *T. harzianum* SPCV127, *Aspergillus niger* AVCM1 and *A. niger* SPCV93*.
* Strains producing cellulase with optimum activity under various parameters.

Table 26 Enzymatic properties of cellulase and xylanase by strains of mutant under SSF

Enzyme properties	Enzyme activity (IU/ml)			
	Mutant strains			
CMCase	**SPCV3M1**	**SPCV3M2**	**SPCV3M3**	**SPCV3M4**
Optimal Temperature - 50°C	35.064	17.981	24.725	35.064
Optimal pH - 4.8	49.533	25.402	34.927	49.533
Thermo stability (75%) - 50°C	38.103	17.540	26.867	38.103
pH stability (75%) – 5.5	41.913	21.494	29.554	41.913
80% inhibitory metal ions	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}
FPase				
Optimal Temperature - 50°C	10.915	5.597	7.696	10.915
Optimal pH - 4.8	11.458	5.876	8.079	11.458
Thermo stability (75%) - 50°C	10.813	4.520	6.215	8.813
pH stability (75%) – 5.5	11.246	5.767	7.930	10.246
80% inhibitory metal ions	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}

Contd...

Enzyme properties	Enzyme activity (IU/ml)			
	Mutant strains			
β- glucosidase	**SPCV3M1**	**SPCV3M2**	**SPCV3M3**	**SPCV3M4**
Optimal Temperature - 50°C	1.163	0.596	0.820	1.163
Optimal pH - 4.8	0.899	0.461	0.634	0.899
Thermo stability (75%) - 50°C	1.091	0.354	0.487	0.691
pH stability (75%) – 5.5	1.180	0.605	0.832	1.180
80% inhibitory metal ions	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}	Fe^{2+}, Ba^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}
Xylanase				
Optimal Temperature - 50°C	6.043	3.099	4.261	6.043
Optimal pH - 4.8	8.053	4.130	5.679	8.053
Thermo stability (75%) - 50°C	7.495	3.177	4.368	6.195
pH stability (75%) – 5.5	8.296	4.254	5.850	8.296
80% inhibitory metal ions	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}	Fe^{2+}, Cu^{2+}
80% stimulatory metal ions	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}	Co^{2+}, Mg^{2+}

Values are mean of three assays of cultures in replicates.
T. viride SPCV3M1*, *T. viride* SPCV3M2, *T. viride* SPCV3M3 and *T. viride* SPCV3M4.
* Strain producing cellulase with optimum activity under various parameters

Table 27 Purification steps of cellulase from culture filterate of *Trichoderma viride* SPCV3M1

Fraction	volume ml	Protein mg/ml	Total protein mg	FPU activity U	Total FPU activity U	Specif activity U/mg	Yield	Purification fraction
Culture supernatant	1000	6.46	6460.00	1.98	1979.27	0.307	100.00	1.00
Ammonium sulphate	100	20.72	2072.00	15.78	1578.00	0.762	79.70	2.48
DEAE-Sephadex	10	1.05	10.50	20.18	201.80	19.220	12.79	25.22

Table 28 Characterization of purified FPase enzyme

Properties	FPase activity
Optimum Temperature	50°C
Optimum pH	4.8
pH stability (75%)	5.5
Thermo stability (75%)	70°C
80% inhibitory metal ions	Fe^{2+} & Cu^{2+}
80% stimulatory metal ions	Co^{2+} & Ba^{2+}
Inhibitory sugar (90%)	Sucrose & Fructose
Stimulatory sugar (36%)	Araabinose & Lactose
Vmax (μ moles/min/mg of protein)	238.09
Km (mM)	0.28
Molecular weight (SDS PAGE)	47
Molecular weight (Zymogram)	50
Carbohydrate content	Nil

Table 29 Effect of different pretreatments of cotton fiber and cotton fiber dry weight on the action of purified cellulases

Treatments	Reducing sugar (mg/ml)		Dry weight 10 mg (w/v)	
	FPase	Cellulase complex	FPase	Cellulase complex
Steaming	4.35	3.91	9.014	9.408
Fentons	3.67	3.48	9.157	9.518
Slight alkaline	3.11	3.16	9.547	9.764
Strong alkaline	2.01	2.63	9.671	9.781
Control	0.42	0.49	9.974	9.998

Table 30 Effect of different waste papers and their concentrations on bioconversion by cellulolytic enzymes

	Waste Paper	Reducing sugar (mg/ml)								
		Concentration of waste paper (mg)								
		5	10	15	20	30*	40	50	60	70
F = 35.881**	Cardboard*	0.400	0.412	0.424	0.476	0.570	0.415	0.434	0.384	0.382
	News paper	0.395	0.407	0.419	0.471	0.563	0.430	0.410	0.379	0.378
	Copier paper	0.199	0.205	0.210	0.237	0.283	0.216	0.206	0.191	0.190
	Filter paper	0.138	0.143	0.147	0.165	0.197	0.141	0.153	0.133	0.132
	Paper Pulp	0.009	0.010	0.010	0.011	0.013	0.010	0.010	0.009	0.009
	Paper sludge	0.076	0.078	0.080	0.090	0.108	0.082	0.078	0.073	0.072
	Office paper	0.397	0.409	0.421	0.473	0.567	0.433	0.412	0.381	0.380
		F = 12.617**								

Values are mean of three experiments.

** ($P < 0.001$), * Maximum bioconverted substrate.

Table 31 Effect of various pH and incubation temperature on bioconversion by cellulase

	pH	Reducing sugar (mg/ml)							
		Incubation temperature°C							
		20	30	40	50	55*	60	70	80
F = 652.982**	2.00	0.236	0.278	0.284	0.309	0.310	0.260	0.222	0.219
	2.50	0.283	0.334	0.341	0.371	0.372	0.312	0.267	0.310
	3.00	0.345	0.311	0.409	0.446	0.446	0.375	0.320	0.372
	3.50	0.408	0.481	0.490	0.535	0.535	0.450	0.384	0.447
	4.00	0.489	0.567	0.589	0.642	0.642	0.540	0.461	0.536
	4.50	0.499	0.589	0.600	0.654	0.655	0.550	0.470	0.547
	5.00	0.501	0.592	0.603	0.658	0.658	0.553	0.473	0.550
	5.50*	0.602	0.710	0.727	0.789	0.790	0.664	0.567	0.660
	6.00	0.447	0.545	0.658	0.707	0.718	0.604	0.517	0.510
	6.50	0.411	0.439	0.552	0.610	0.611	0.498	0.411	0.405
	7.00	0.211	0.226	0.239	0.286	0.297	0.286	0.205	0.202
	F = 1818.724**								

Values are mean of three assays of cultures in replicates.

** (P< 0.001), * Optimum pH and temperature for maximum bioconversion of cardboard

Fig. 2 *Aspergillus acculeutus* (100 x)

Fig. 3 *Aspergillus Americana* (100 x)

Fig. 4 *Aspergillus fumigatus* (100 x)

Fig. 5 *Aspergillus flavus* (100 x)

Fig. 6 *Aspergillus niger* (100 x)

Fig. 7 *Aspergillus ustus* (100 x)

Fig. 8 *Aspergillus oryzae* (100 x)

Fig. 9 *Aspergillus terreus* (100 x)

Fig. 10 *Aspergillus versicolar* (100 x)

Fig. 11 *Fusarium oxysporum* (100 x)

Fig. 12 *Helminthosporium oryzae* (100 x)

Fig. 13 *Penicillium chrysogenum* (100 x)

Fig. 14 *Penicillium digitatum* (100 x)

Fig. 15 *Penicillium japanicum* (100 x)

Fig. 16 *Penicillium lanosum* (100 x)

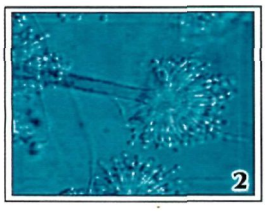
Fig. 17 *Rhizopus nigricans* (100 x)

Fig. 18 *Trichoderma longibrachiatum* (100 x)

Fig. 19 *Trichoderma harzianum* (100 x)

Fig. 20 *Trichoderma koningii* (100 x)

Fig. 21 *Trichoderma viride* (100 x)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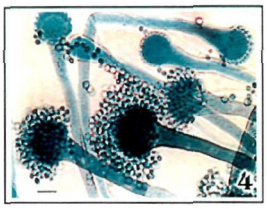
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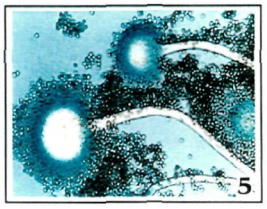
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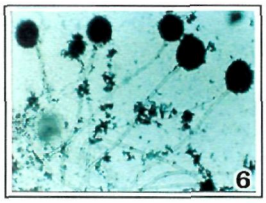
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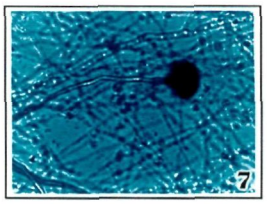
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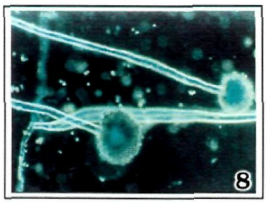
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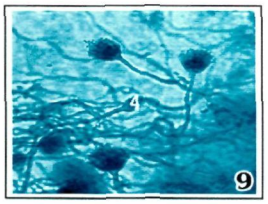
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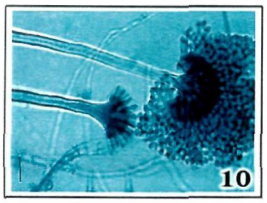
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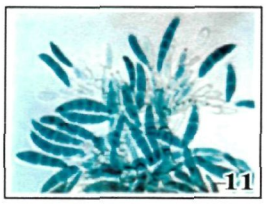
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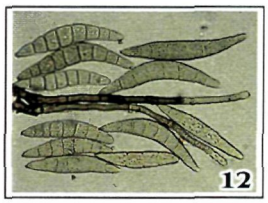
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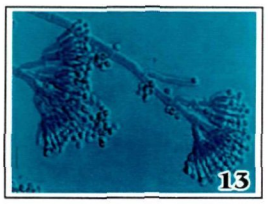
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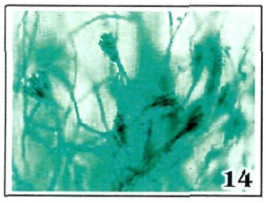
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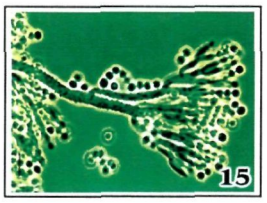
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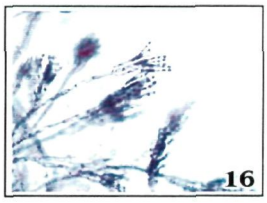
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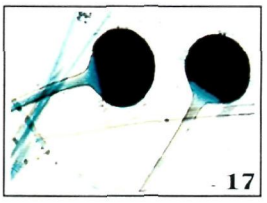
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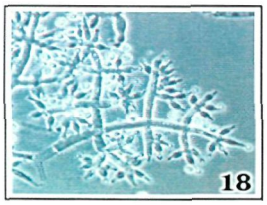
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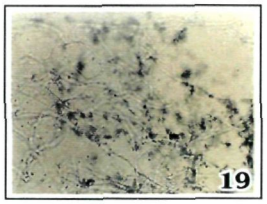
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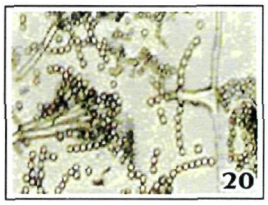
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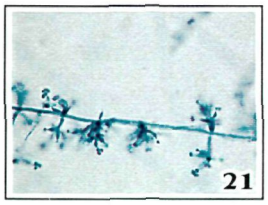
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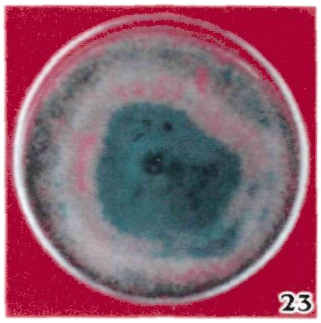
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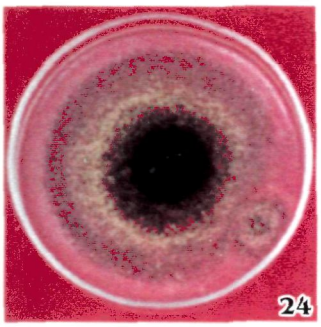
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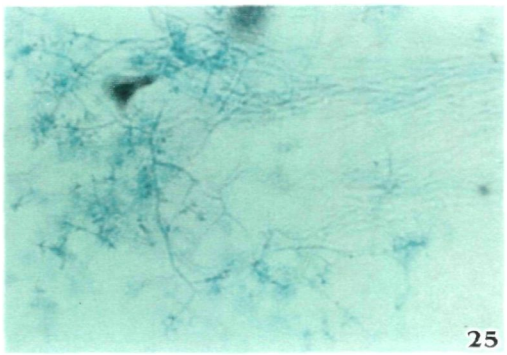
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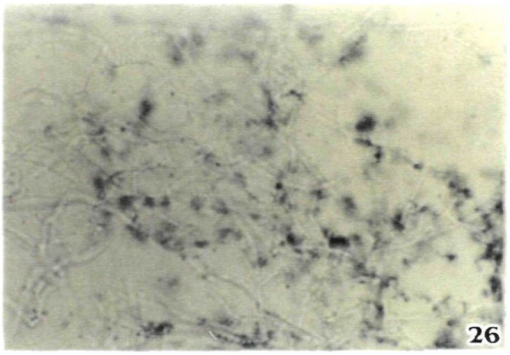
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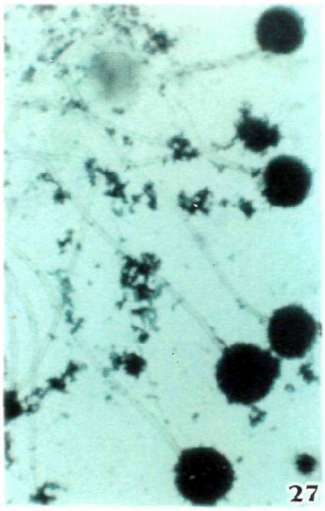
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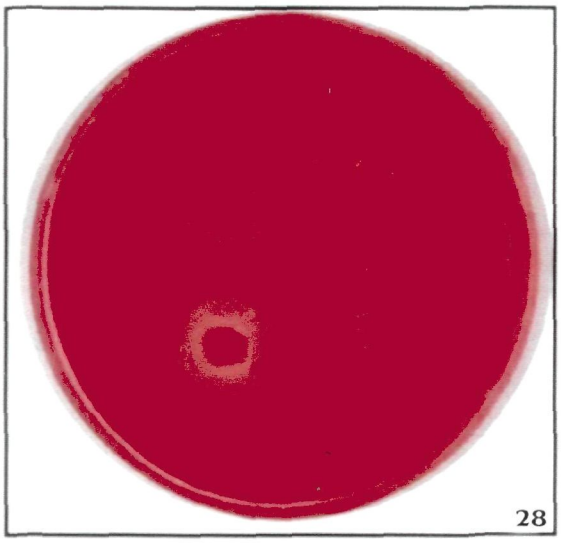
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Fig. 58 Plate culture of the mutant *Trichoderma viride* SPCV3M1

Fig. 59 Solid state fermentation of mutant *Trichoderma viride* SPCV3M1.

Fig. 62 SDS gel image purified cellulase.

Lan 1 – Standard molecular weight protein.

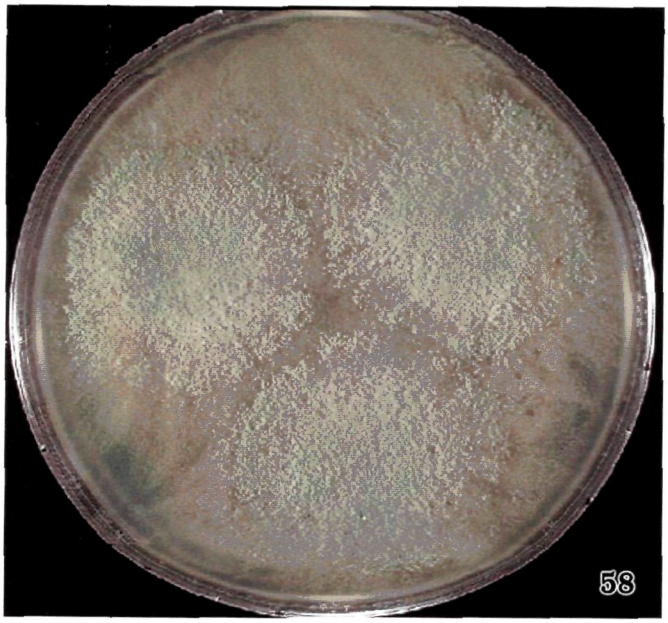
Lan 2 - Ethanol precipitated protein.

Lan 3 - Purified cellulase (FPase).

Fig. 63 Zymogram for purified FPase.

Lan 1 - Standard cellulase (Sigma).

Lan 2 - Purified FPase.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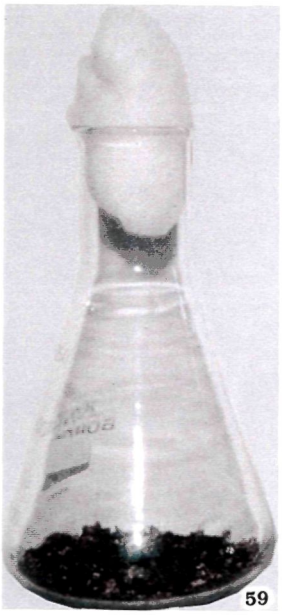
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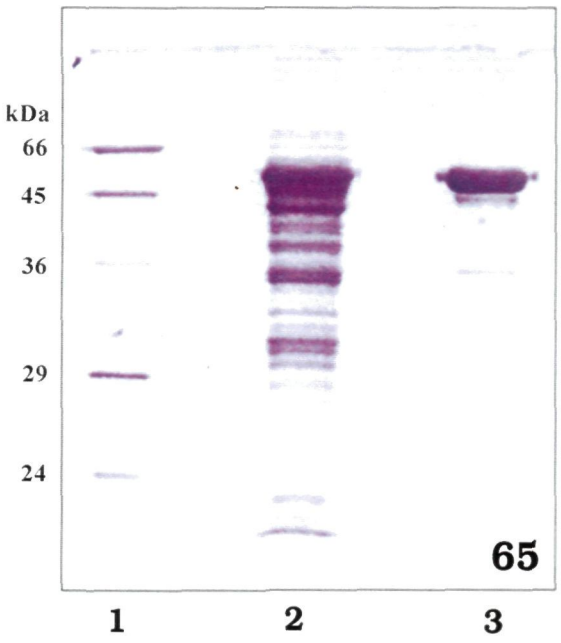
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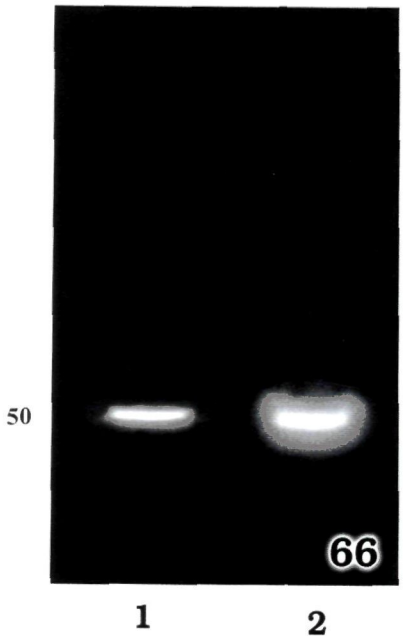
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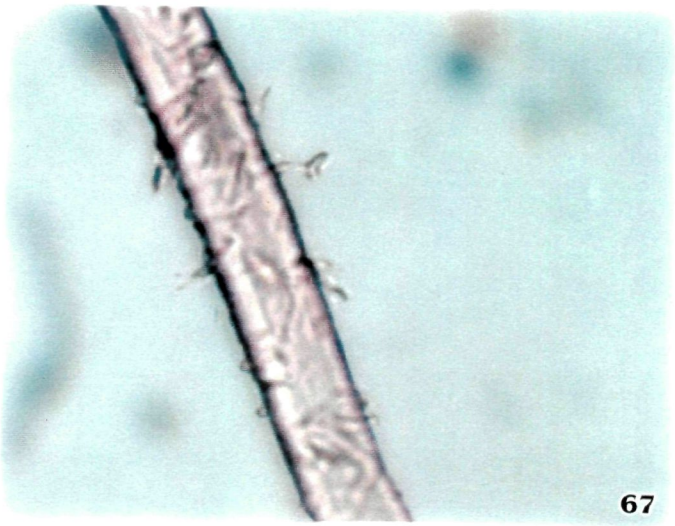
Fig. 67 Light microscopic photo of cotton fiber before enzymatic modification by FPase (100 x).

Fig. 68 Light microscopic photo of cotton fiber after enzymatic modification by FPase (100 x).

Fig. 69 Light microscopic photo of cotton fiber after enzymatic modification by cellulase (Sigma) (100 x).



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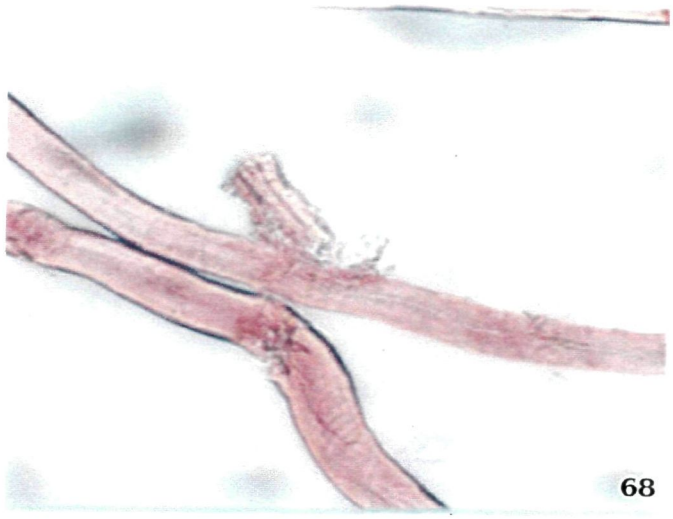

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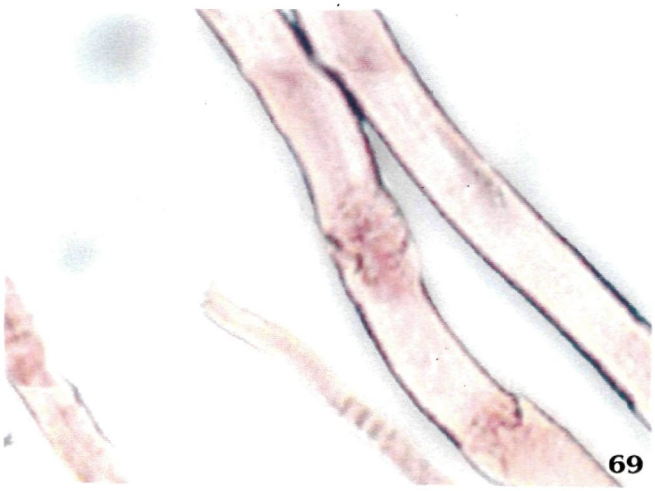


69

Fig. 1 Site of sample collection.

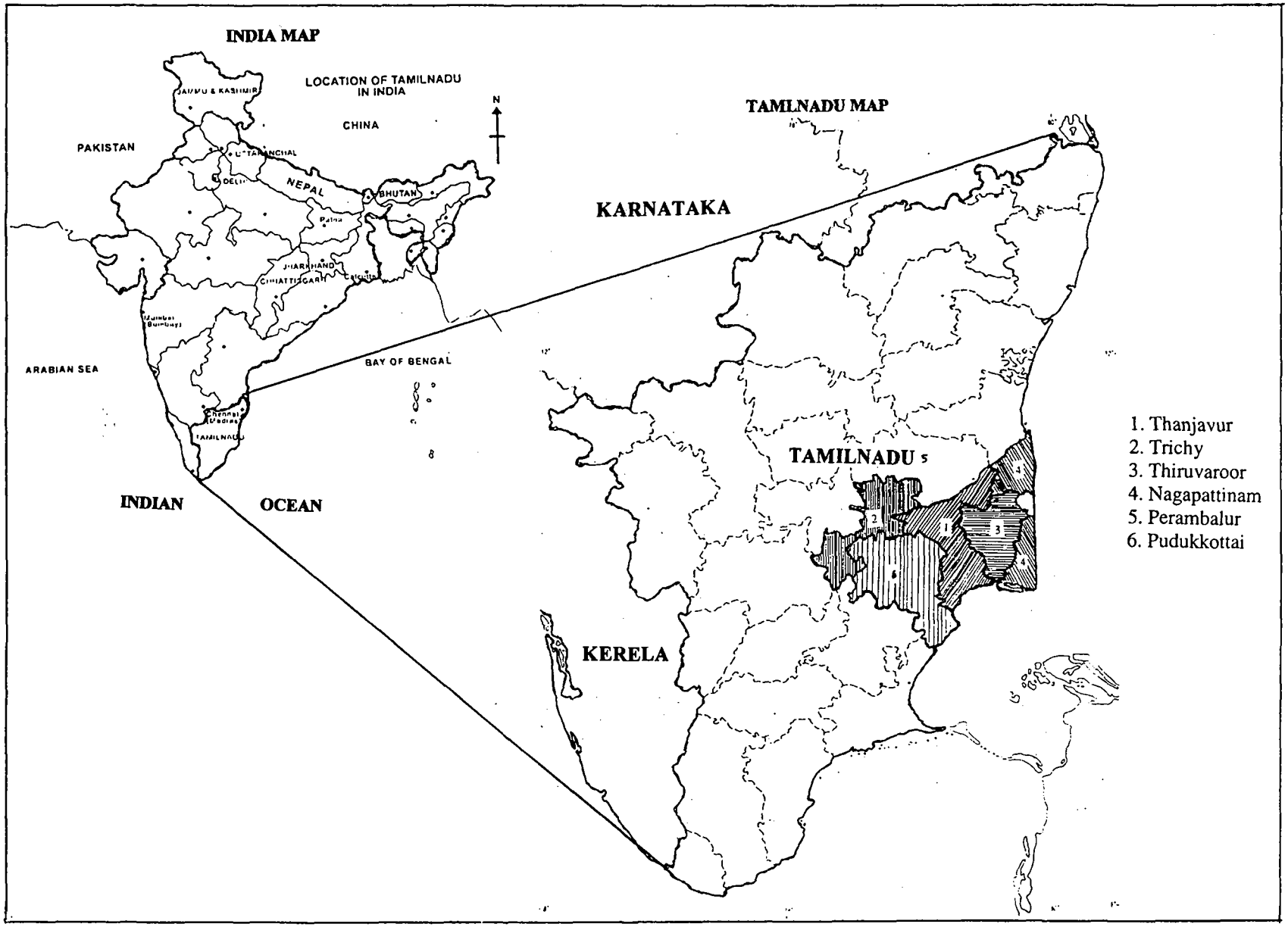


Fig. 29 Effect of incubation period on cellulase and xylanase production.

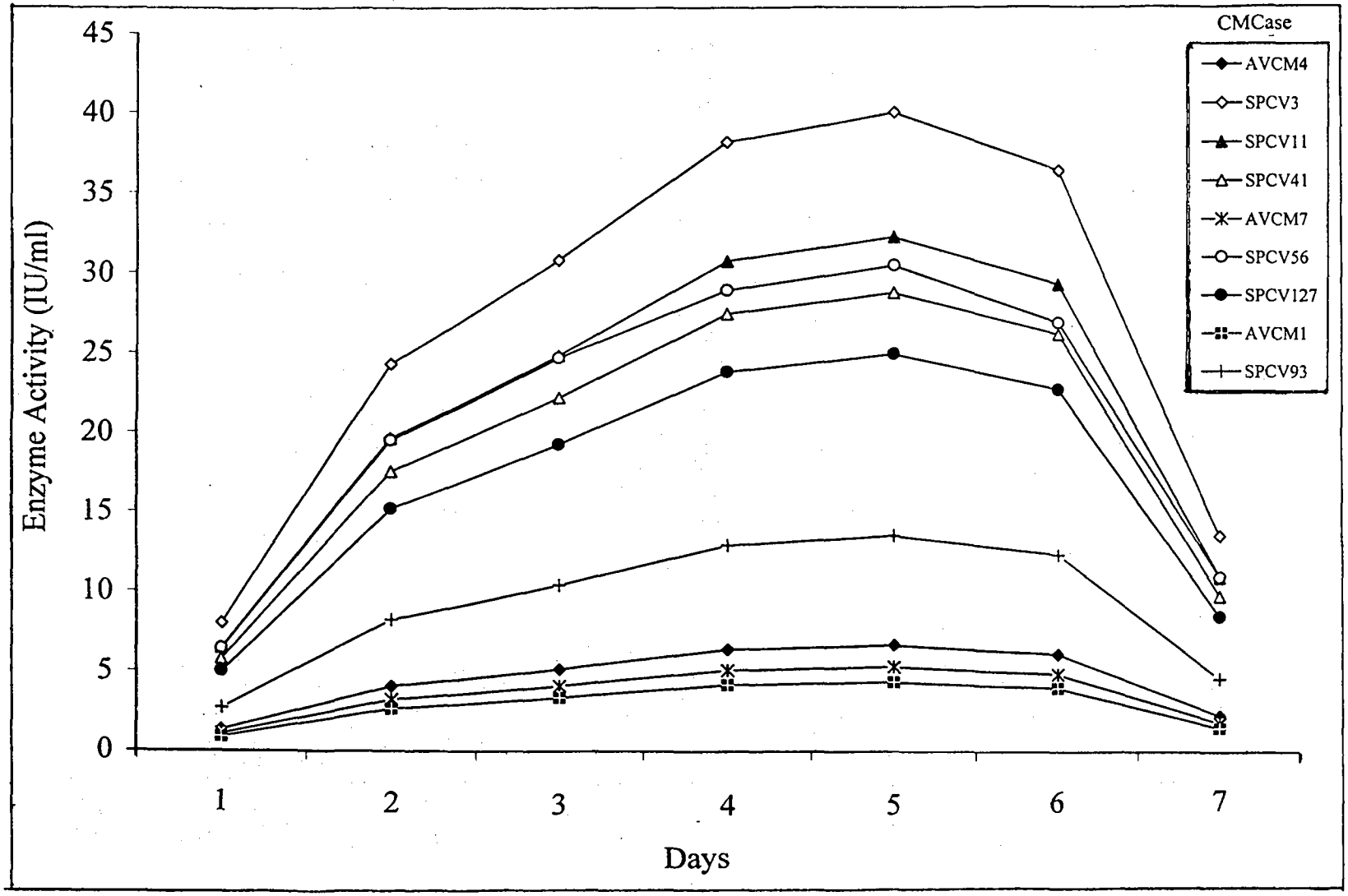


Fig. 30 Effect of incubation period on soluble protein production.

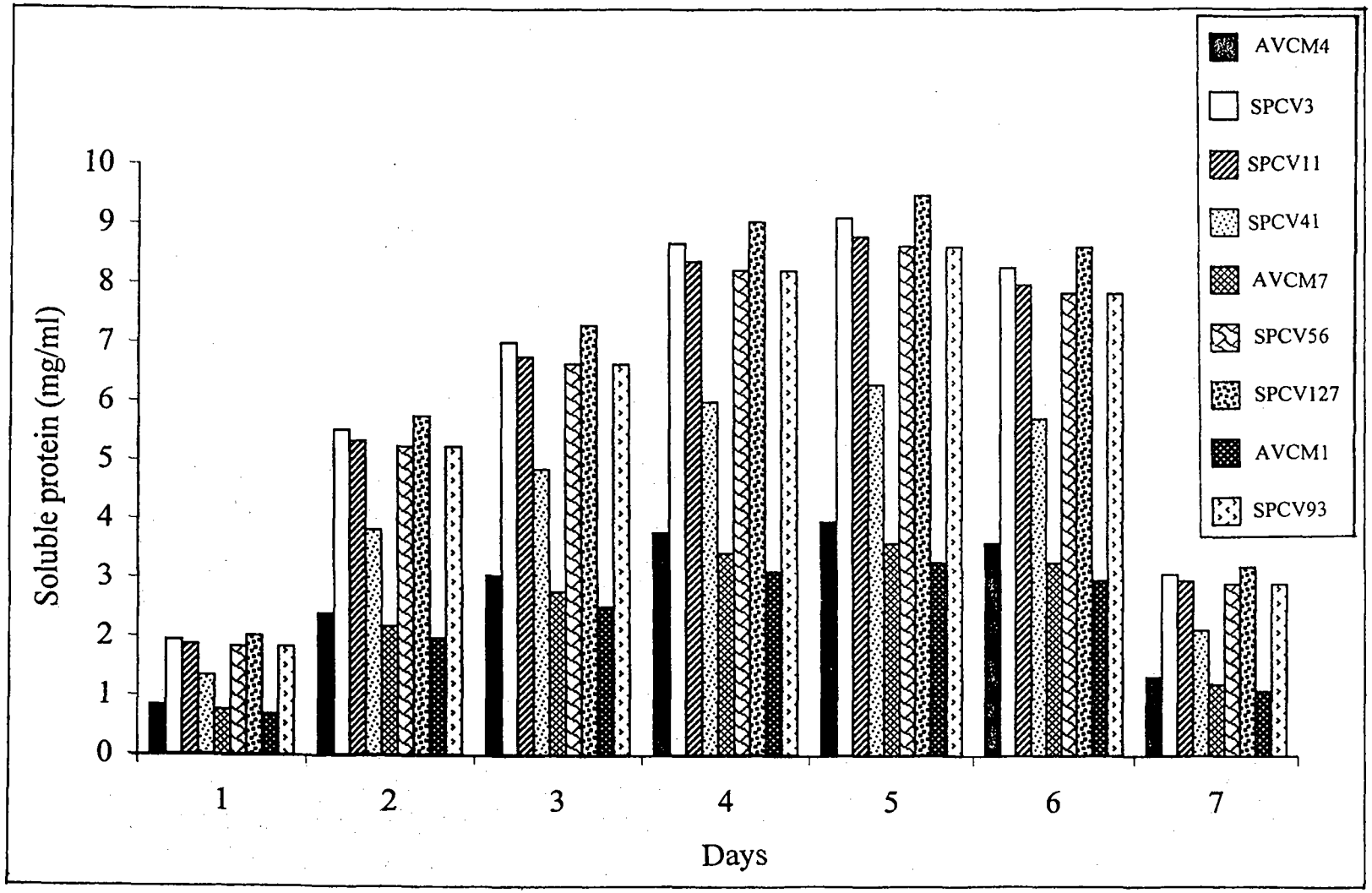


Fig. 31 Effect of incubation period on biomass production.

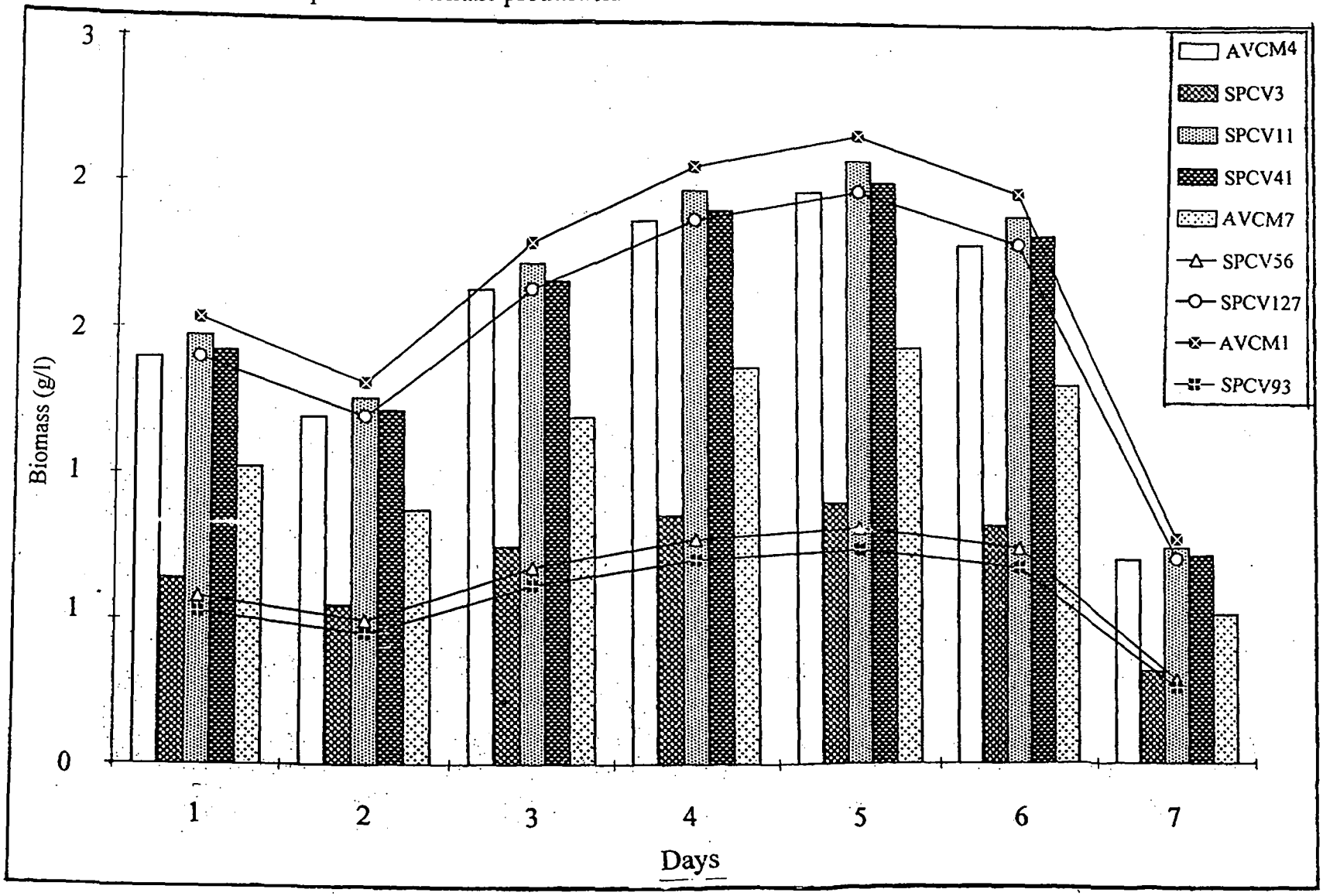
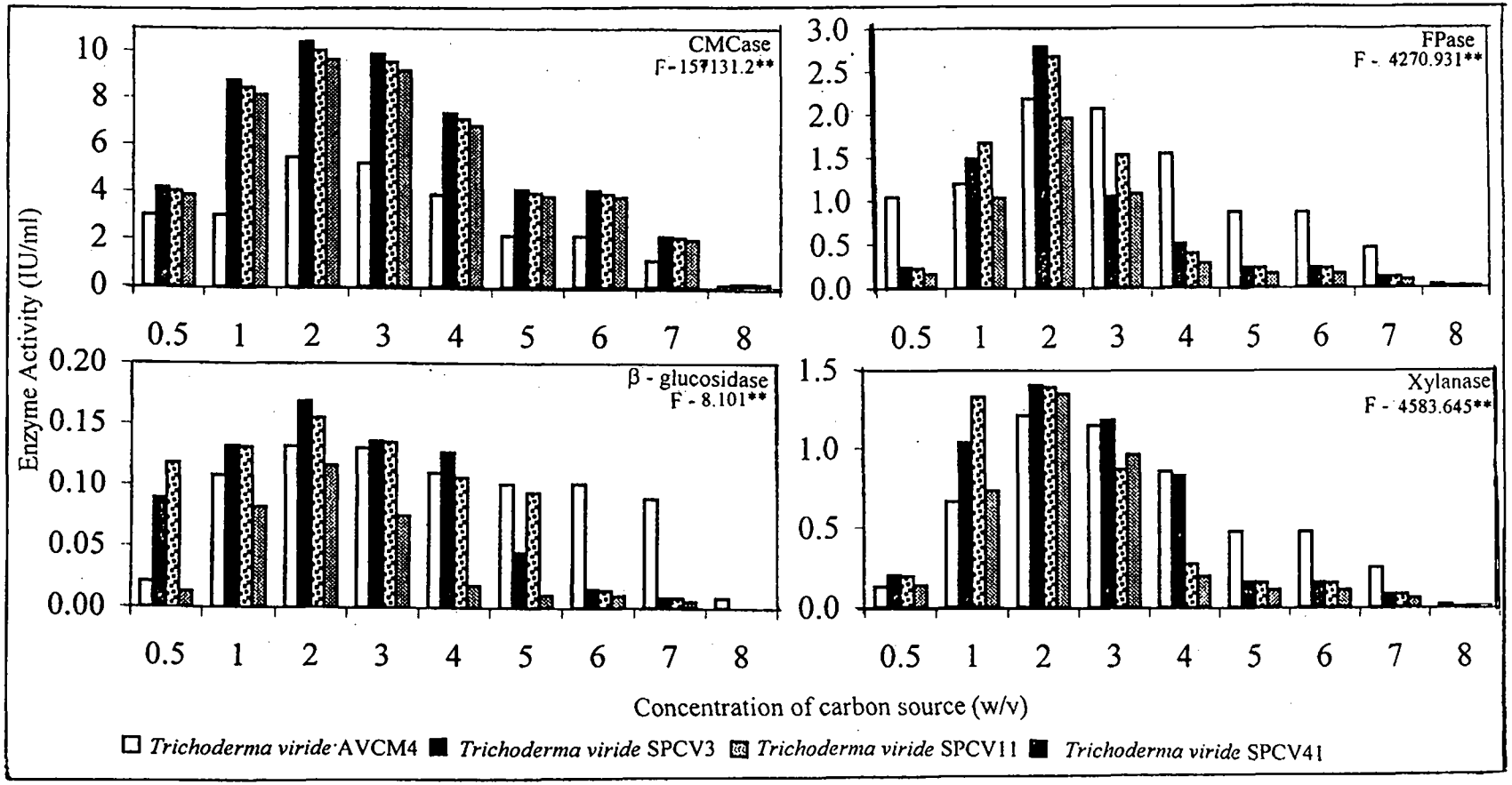


Fig. 32 Effect of wheat bran concentration on cellulase and xylanase production by strains of *Trichoderma viride*.



Values are mean of three assays of cultures in replicates.

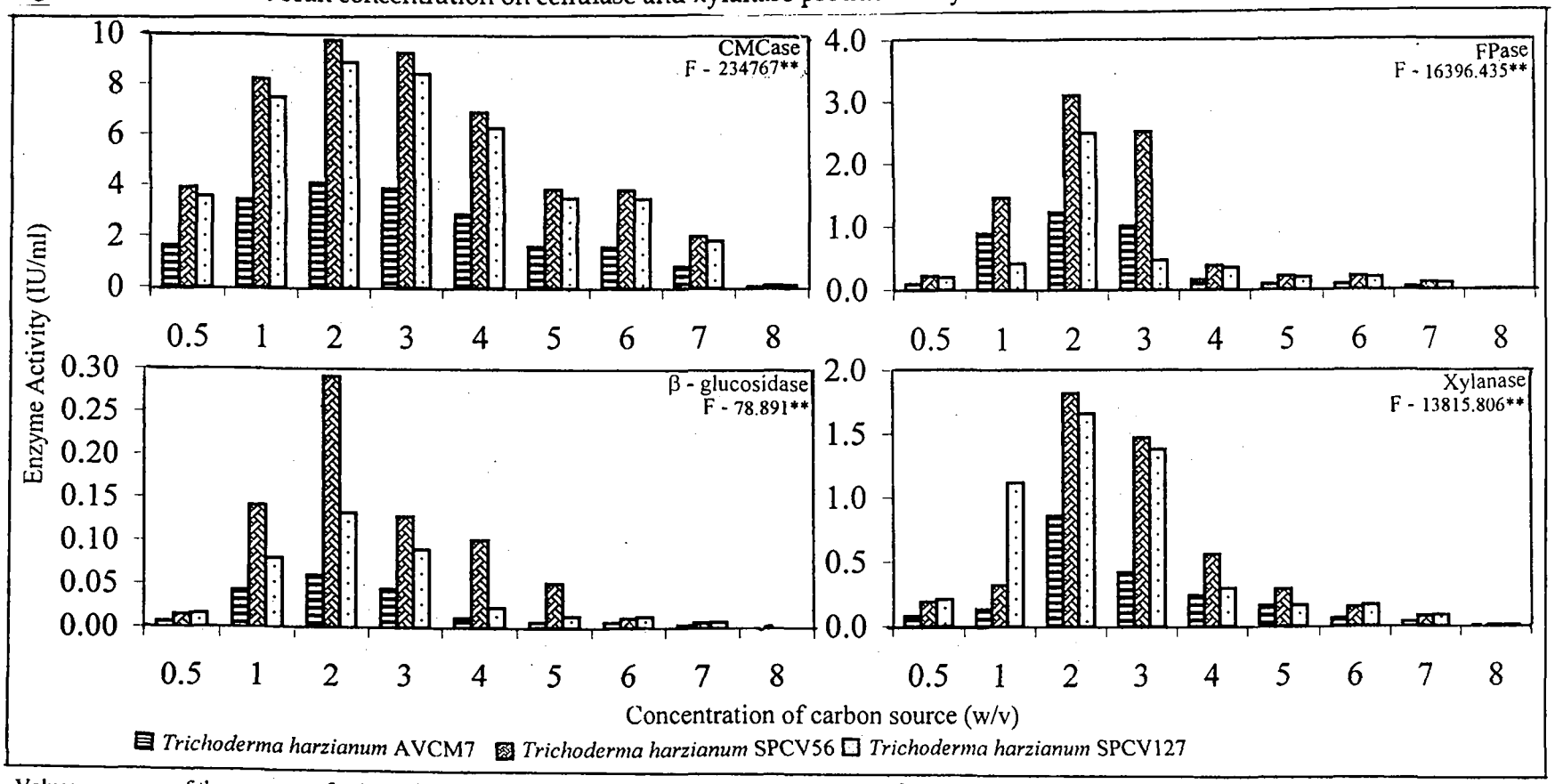
** - (P< 0.001).

Trichoderma viride strains.

Trichoderma viride AVCM4, ***Trichoderma viride*** SPCV3*, ***Trichoderma viride*** SPCV11, ***Trichoderma viride*** SPCV41.

* Represent maximum enzyme producing *Trichoderma viride* strain.

Fig. 33 Effect of wheat bran concentration on cellulase and xylanase production by strains of *Trichoderma harzianum*.



Values are mean of three assays of cultures in replicates.

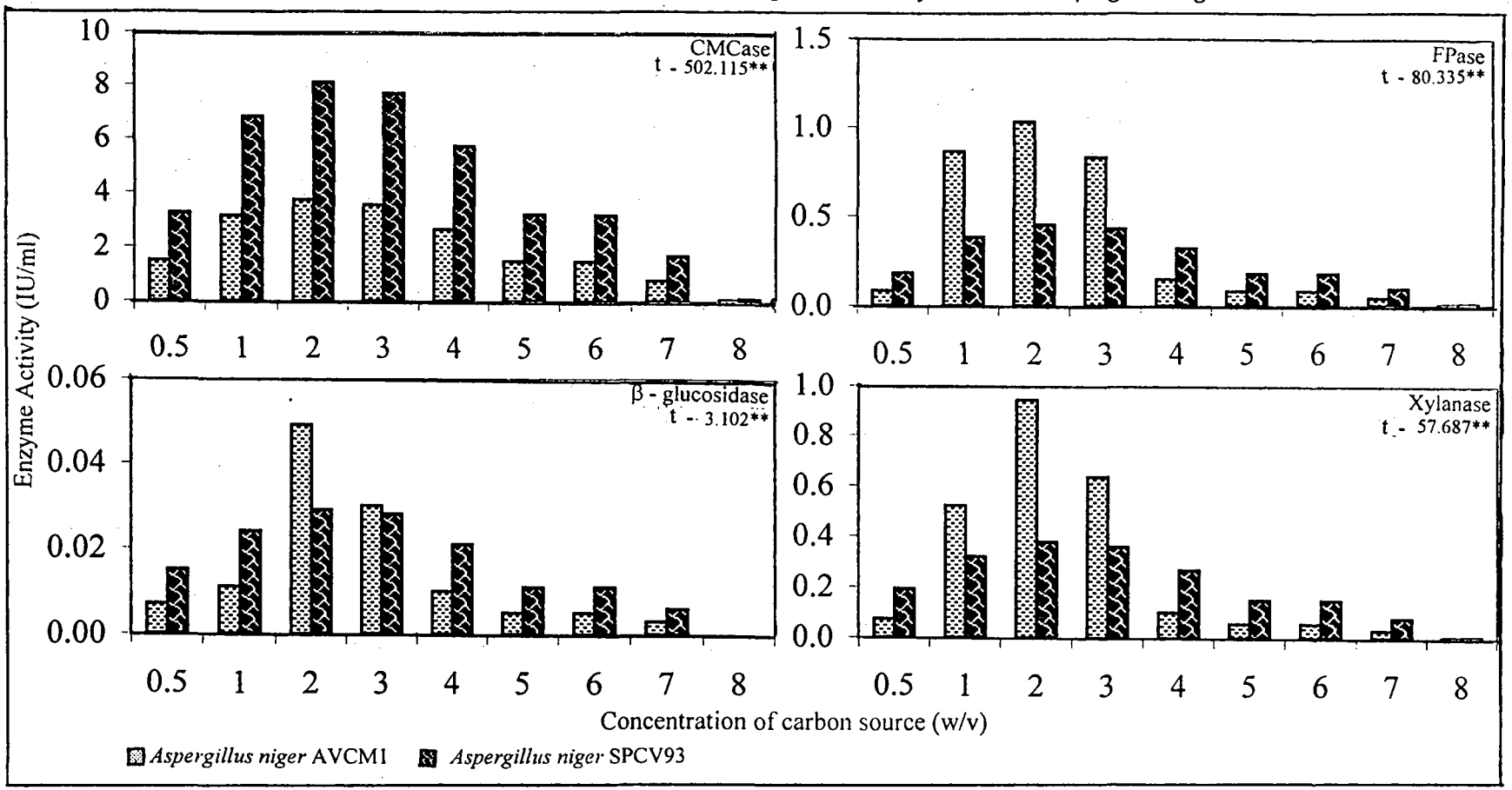
** - (P< 0.001).

Trichoderma harzianum strains.

Trichoderma harzianum AVCM7, ***Trichoderma harzianum*** SPCV56*, ***Trichoderma harzianum*** SPCV127.

* Represent maximum enzyme producing *Trichoderma harzianum* strain.

Fig. 34 Effect of wheat bran concentration on cellulase and xylanase production by strains of *Aspergillus niger*.



Values are mean of three assays of cultures in replicates.

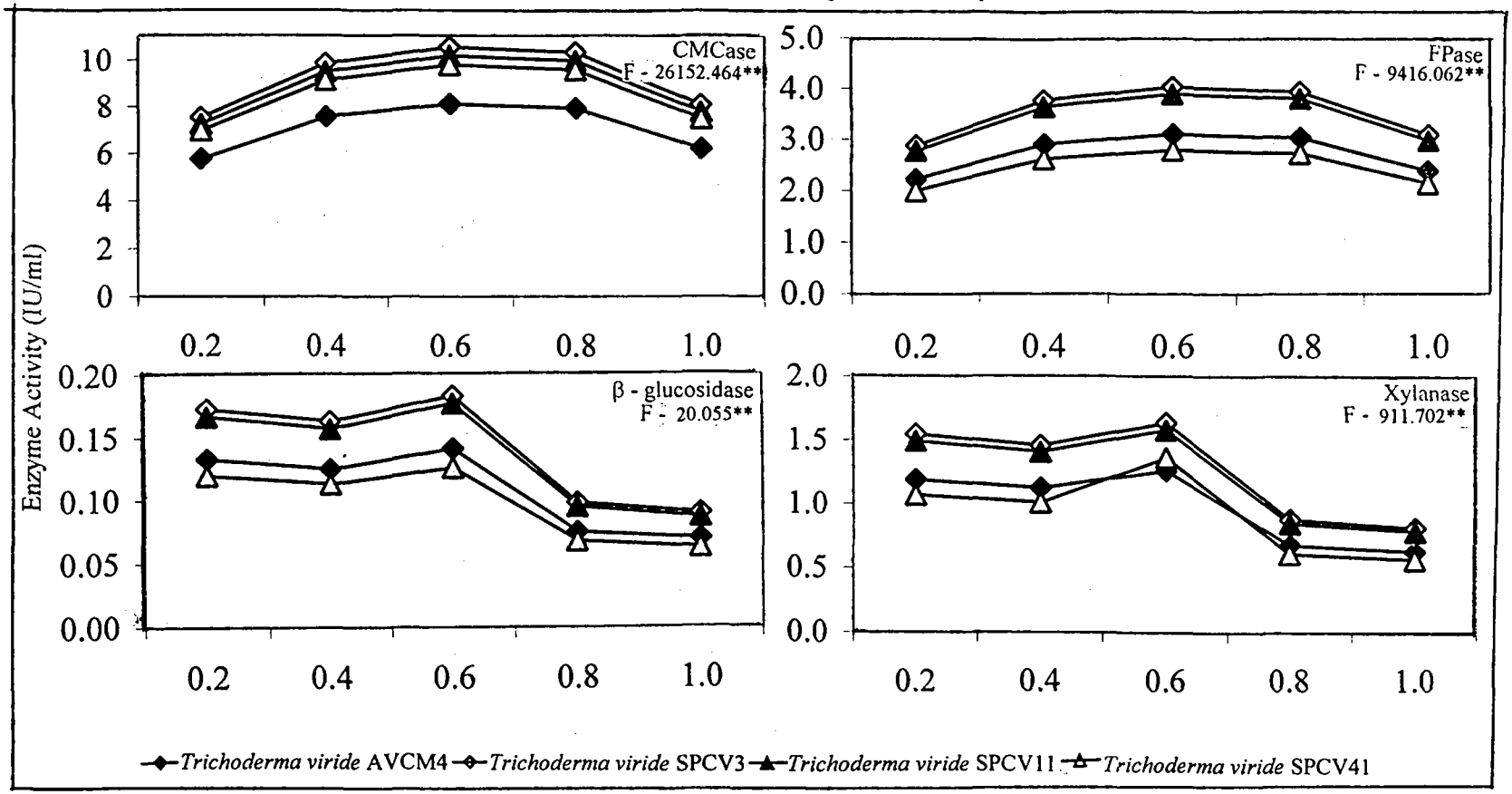
** - (P< 0.001).

Aspergillus niger strains.

***Aspergillus niger* AVCM1, *Aspergillus niger* SPCV93*.**

* Represent maximum enzyme producing *Aspergillus niger* strain.

Fig. 35 Effect of yeast extract concentration on cellulase and xylanase production by strains of *Trichoderma vride.*



Values are mean of three assays of cultures in replicates.

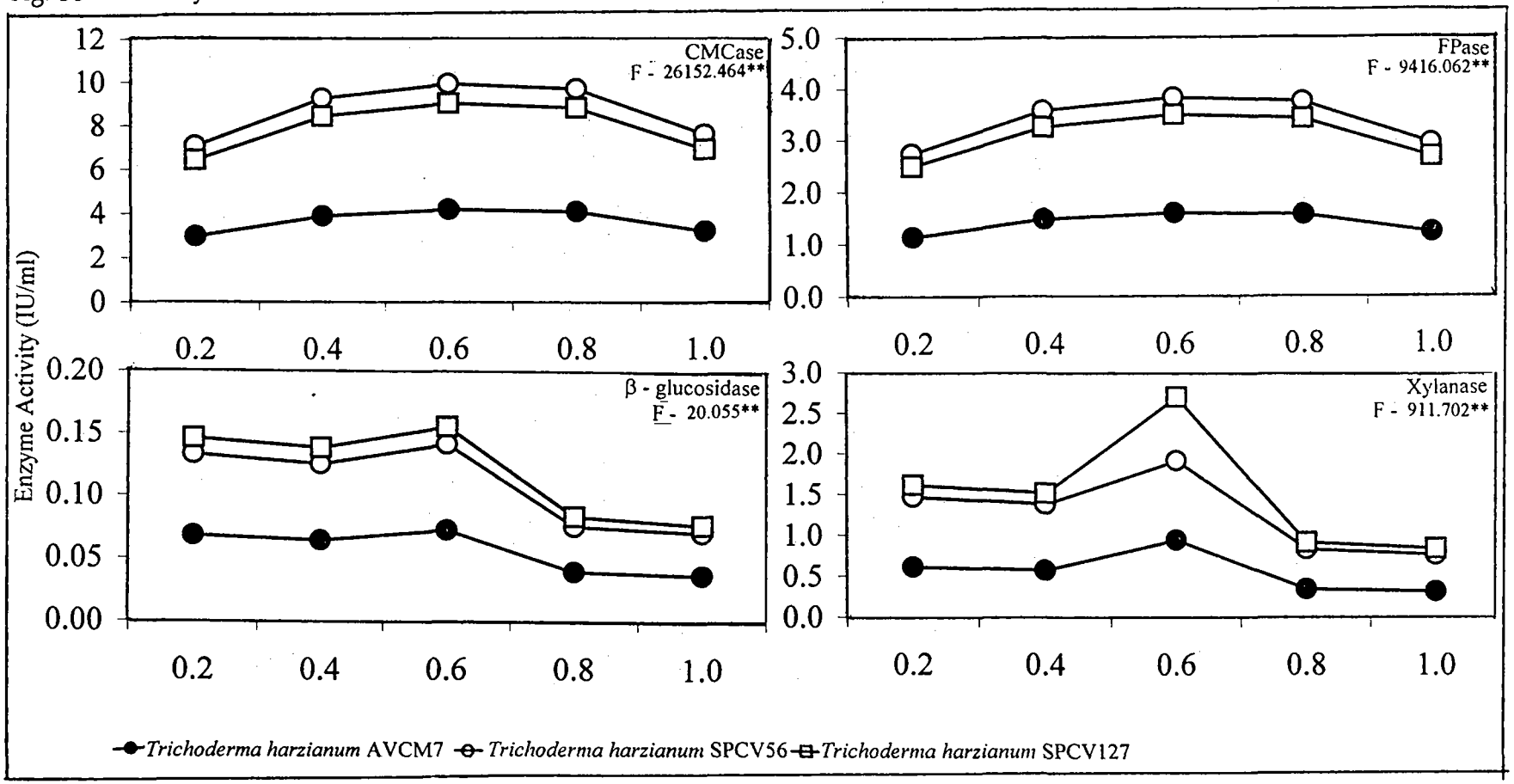
** - (P< 0.001).

Trichoderma viride strains.

Trichoderma viride AVCM4, *Trichoderma viride* SPCV3*, *Trichoderma viride* SPCV11, *Trichoderma viride* SPCV41.

* Represent maximum enzyme producing *Trichoderma viride* strain.

Fig. 36 Effect of yeast extract concentration on cellulase and xylanase production by strains of *Trichoderma harzianum.*



Values are mean of three assays of cultures in replicates.

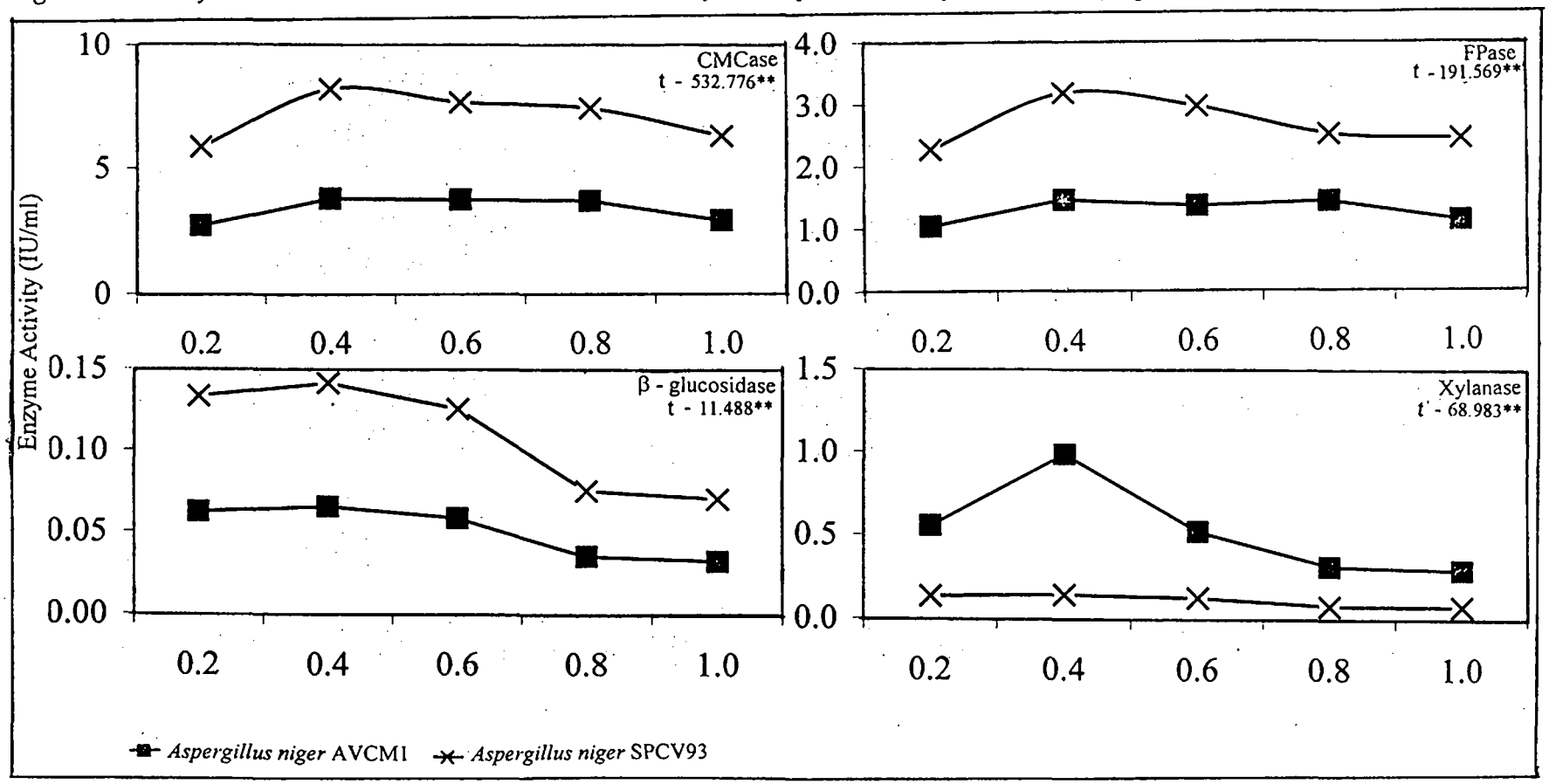
** - (P< 0.001).

Trichoderma harzianum strains.

Trichoderma harzianum **AVCM7, *Trichoderma harzianum* SPCV56*, *Trichoderma harzianum* SPCV127.**

* Represent maximum enzyme producing *Trichoderma harzianum* strain.

Fig. 37 Effect of yeast extract concentration on cellulase and xylanase production by strains of *Aspergillus niger*.



Values are mean of three assays of cultures in replicates.

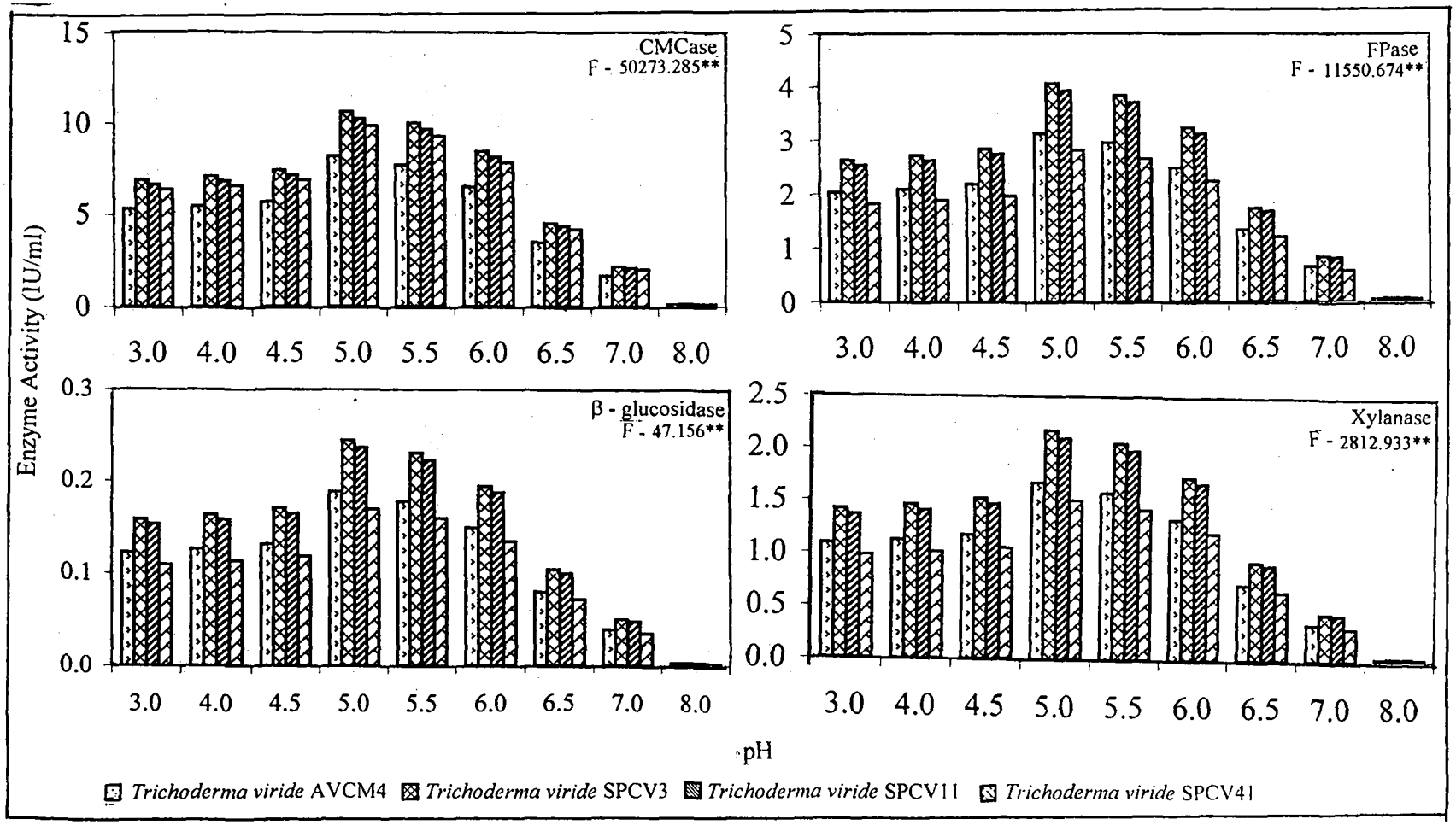
** - (P< 0.001).

Aspergillus niger strains.

***Aspergillus niger* AVCM1, *Aspergillus niger* SPCV93*.**

* Represent maximum enzyme producing *Aspergillus niger* strain.

Fig. 38 Effect of pH on cellulase and xylanase production by strains of *Trichoderma viride*.



Values are mean of three assays of cultures in replicates.

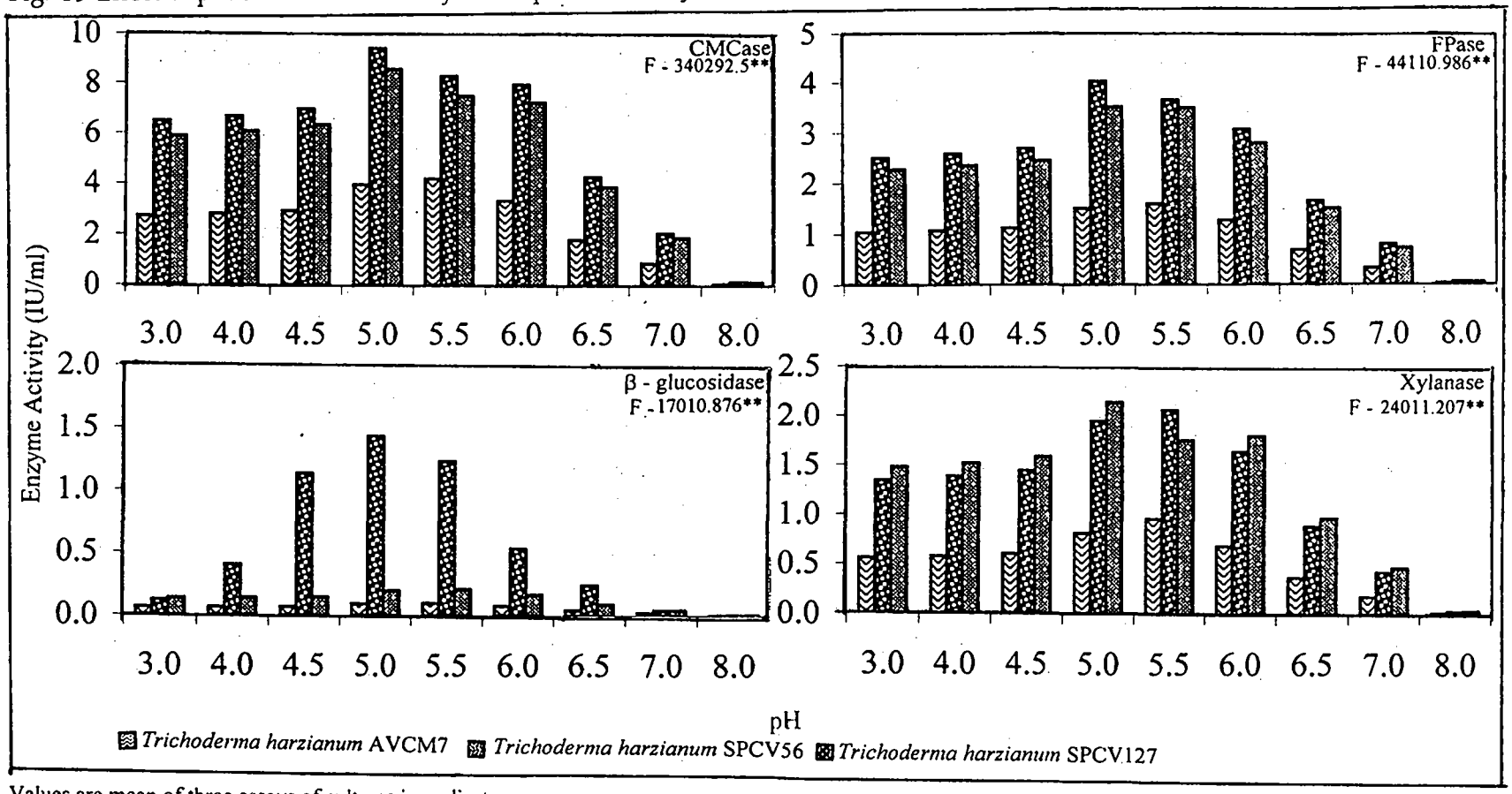
** - (P< 0.001).

Trichoderma viride strains.

Trichoderma viride **AVCM4, *Trichoderma viride* SPCV3*, *Trichoderma viride* SPCV11, *Trichoderma viride* SPCV41.**

* Represent maximum enzyme producing *Trichoderma viride* strain.

Fig. 39 Effect of pH on cellulase and xylanase production by strains of *Trichoderma harzianum*.



Values are mean of three assays of cultures in replicates.

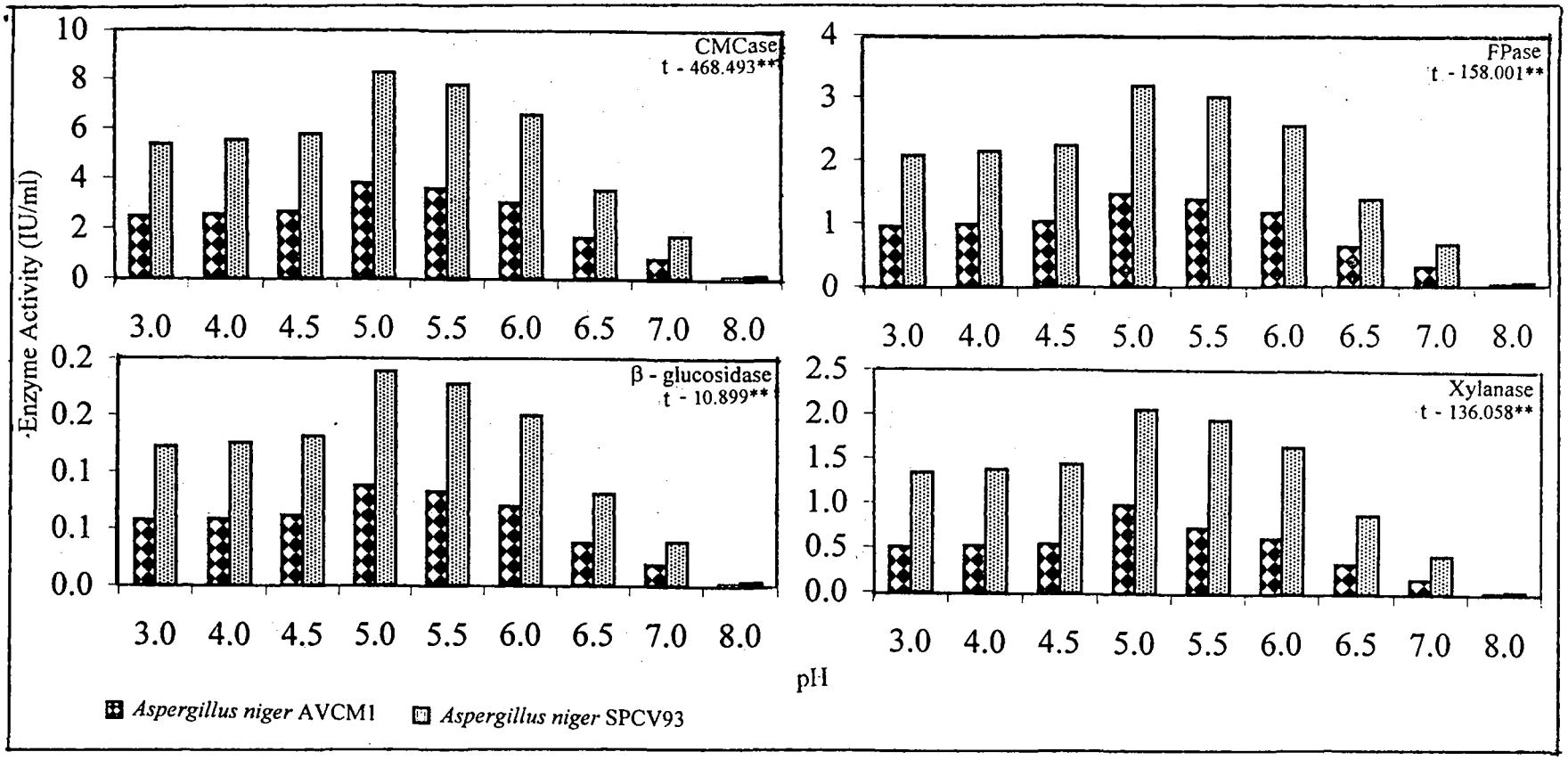
** - (P< 0.001).

Trichoderma harzianum strains.

Trichoderma harzianum AVCM7, ***Trichoderma harzianum*** SPCV56*, ***Trichoderma harzianum*** SPCV127.

* Represent maximum enzyme producing *Trichoderma harzianum* strain.

Fig. 40 Effect of pH on cellulase and xylanase by production strains of *Aspergillus niger*.



Values are mean of three assays of cultures in replicates.

** - (P< 0.001).

Aspergillus niger strains.

Aspergillus niger AVCM1, ***Aspergillus niger*** SPCV93*.

* Represent maximum enzyme producing *Aspergillus niger* strain.

Fig. 41 Effect of incubation period on cellulase and xylanase production *via* SSF.

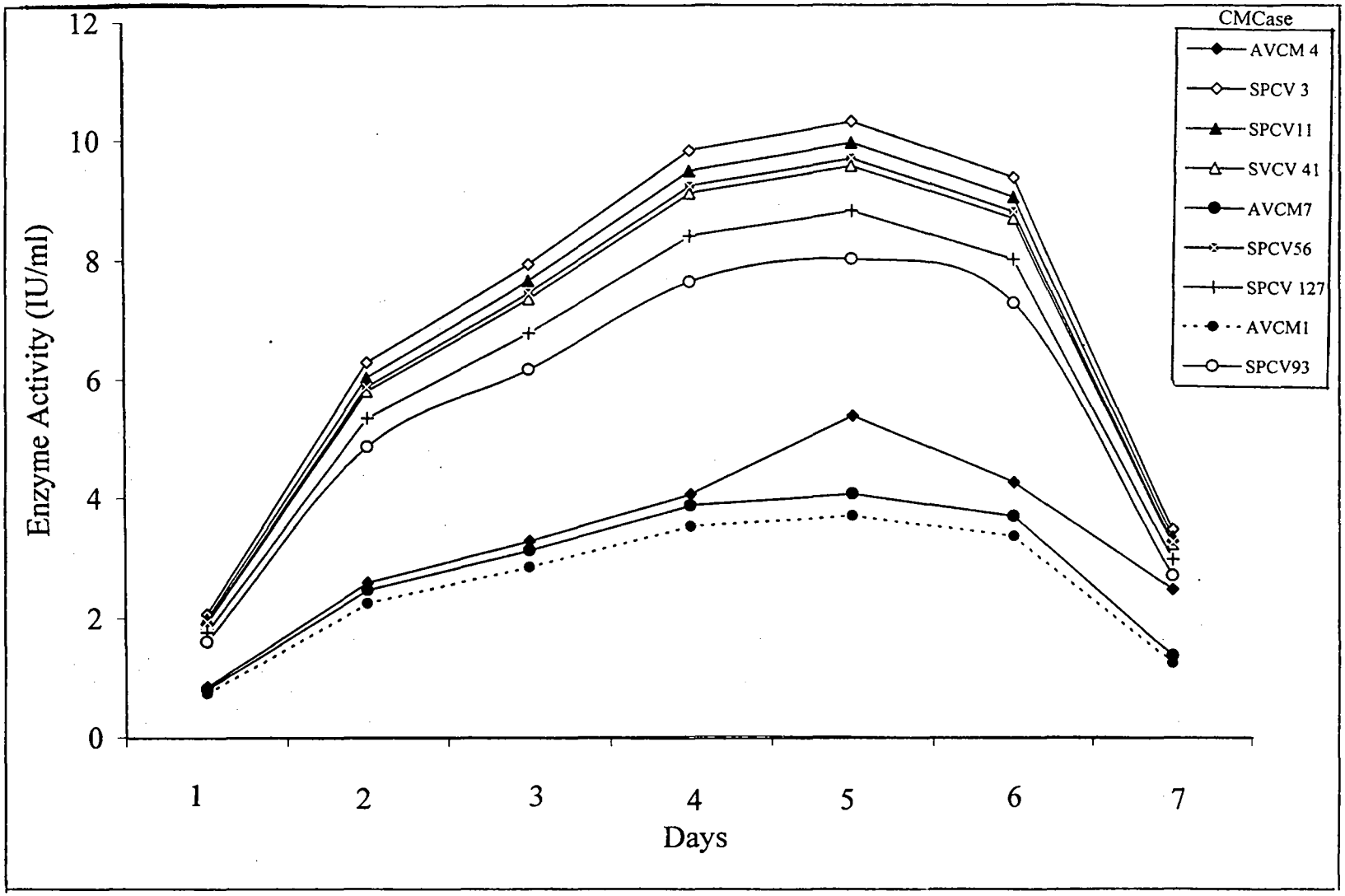


Fig. 42 Effect of incubation period on soluble protein production *via* SSF.

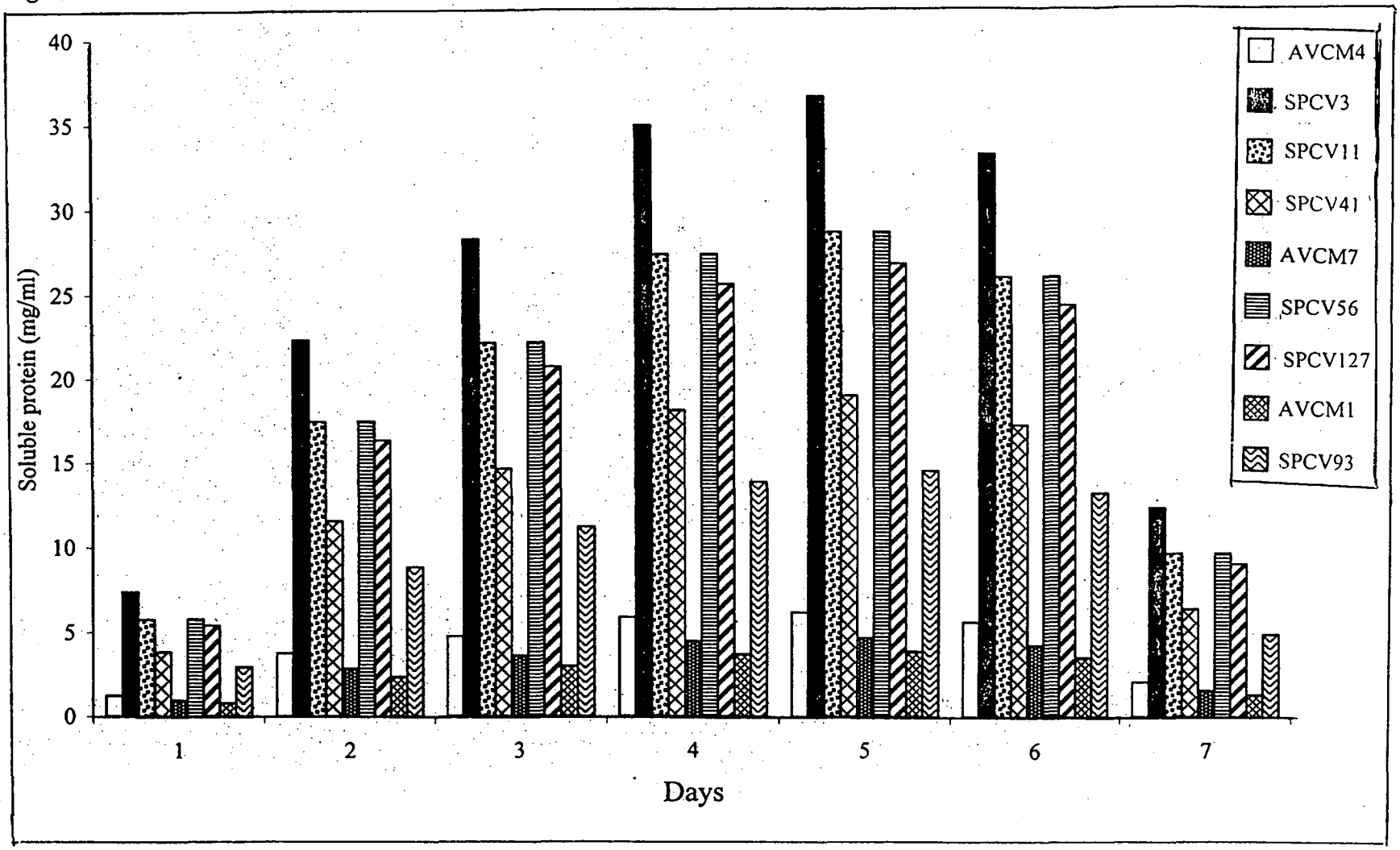


Fig. 43 Effect of incubation period on Biomass *via* SSF.

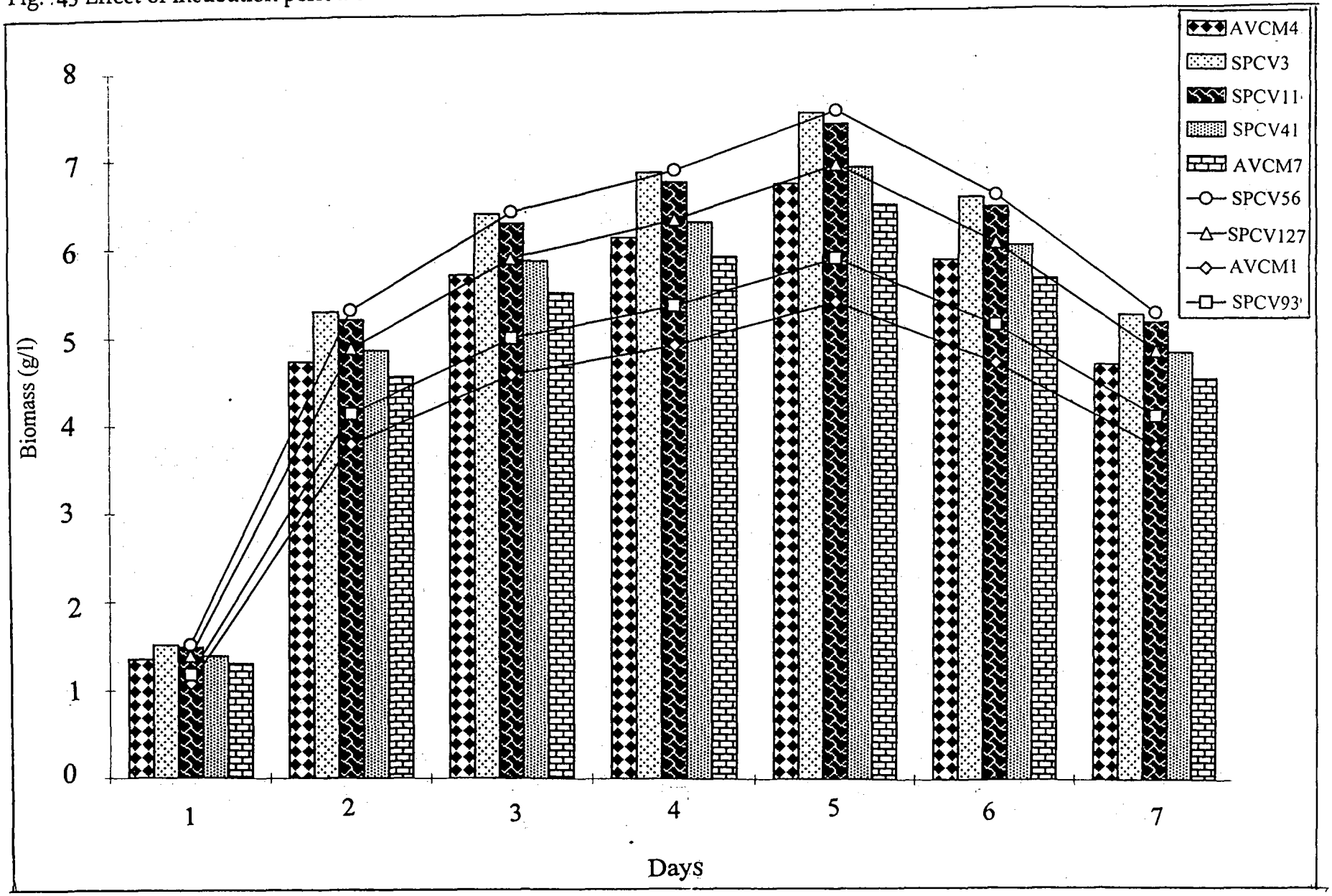
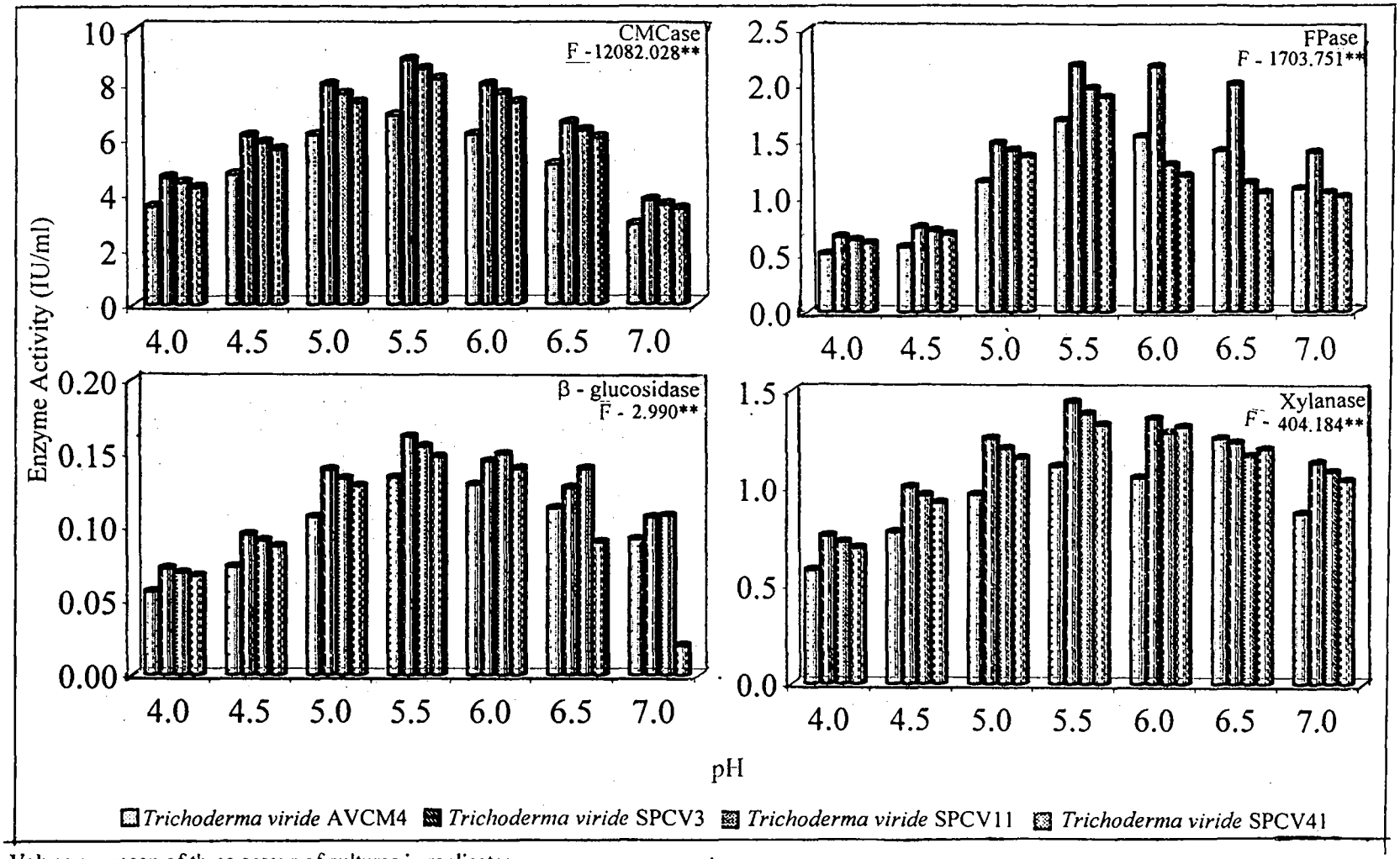


Fig. 44 Effect of pH on cellulase and xylanase production by strains of *Trichoderma viride*.



Values are mean of three assays of cultures in replicates.

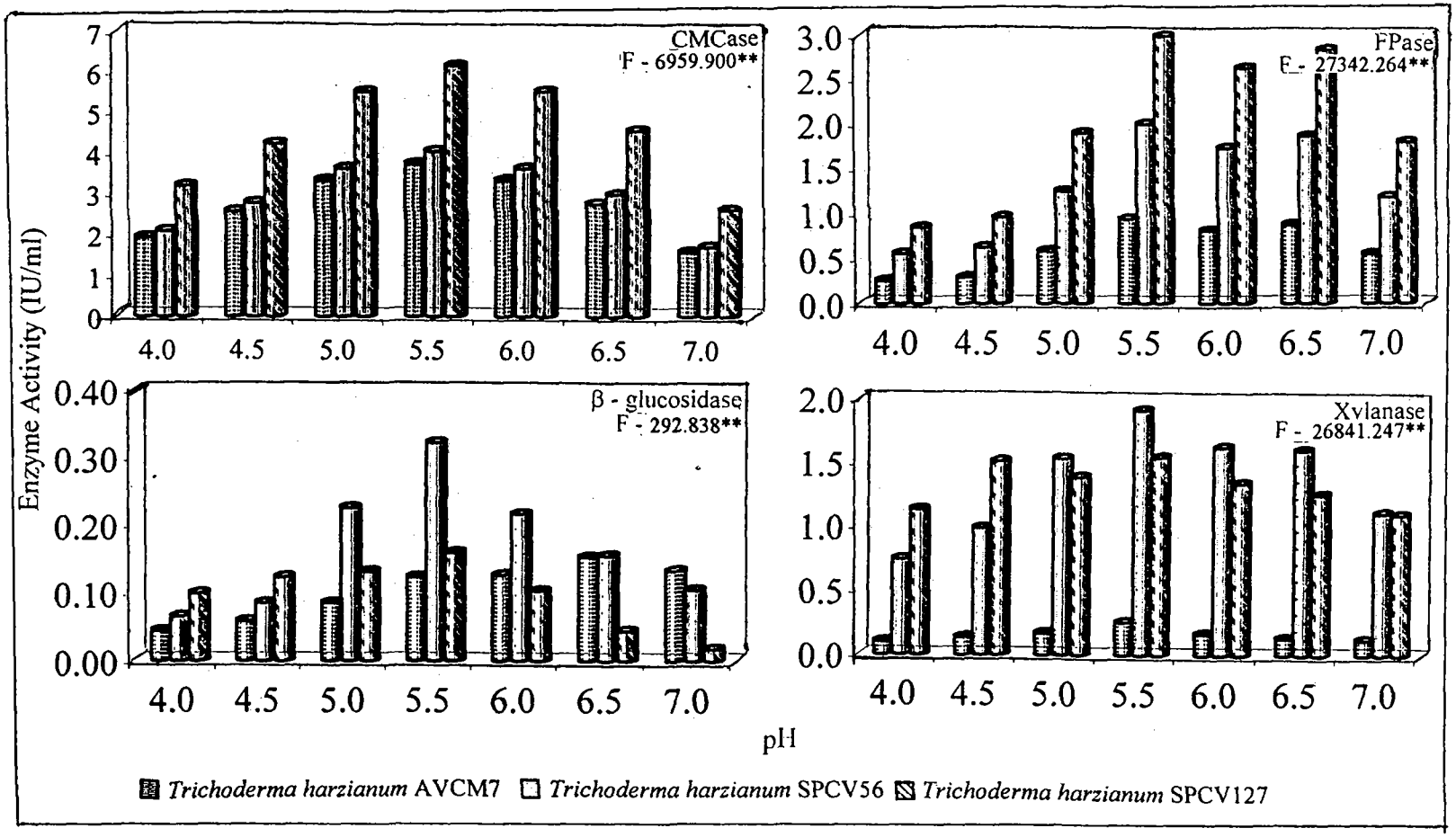
** - (P< 0.001).

Trichoderma viride strains.

Trichoderma viride AVCM4, *Trichoderma viride* SPCV3*, *Trichoderma viride* SPCV11, *Trichoderma viride* SPCV41.

* Represent maximum enzyme producing *Trichoderma viride* strain.

Fig. 45 Effect of pH on cellulase and xylanase production by strains of *Trichoderma harzianum*.



Values are mean of three assays of cultures in replicates.

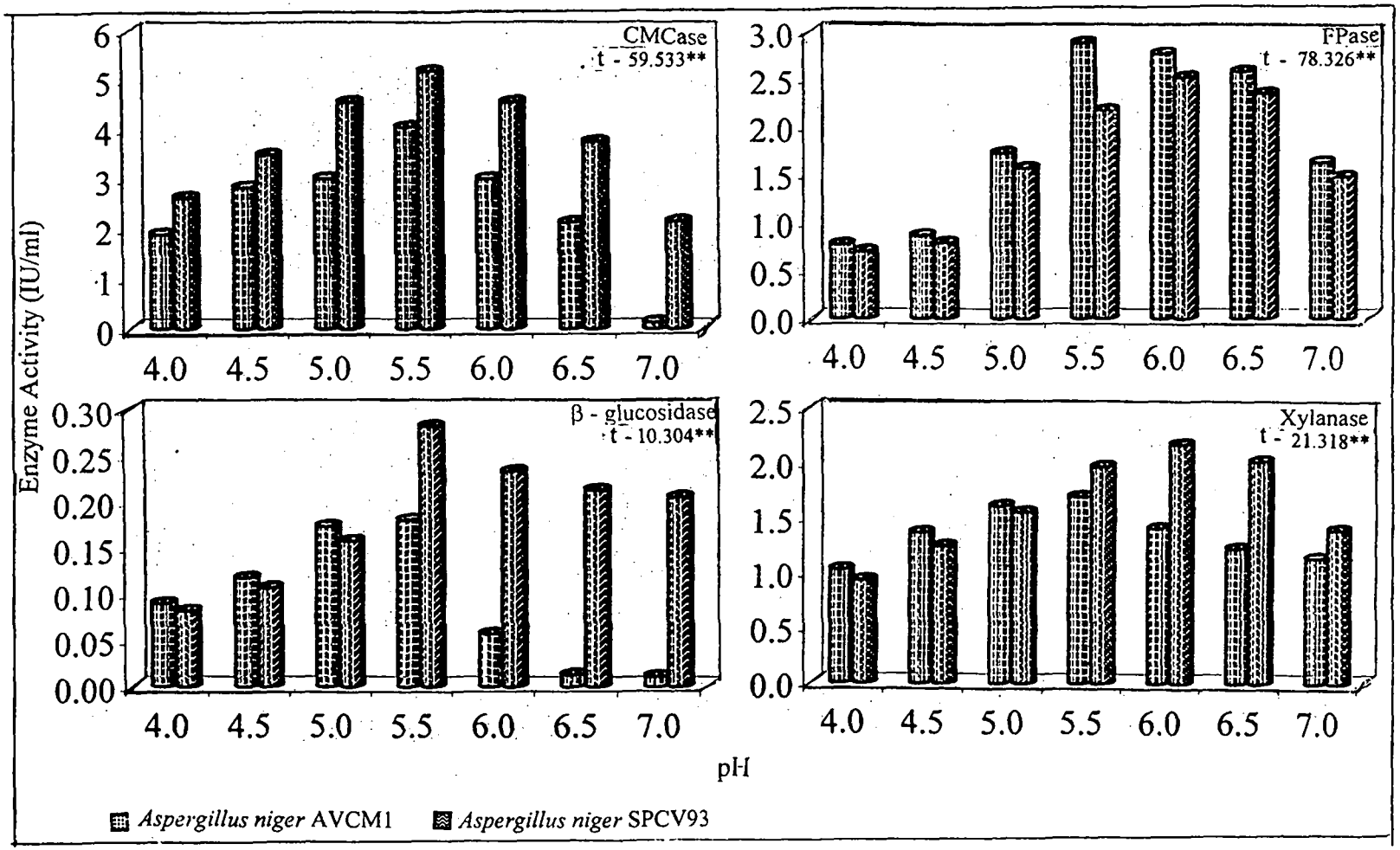
** - ($P < 0.001$).

Trichoderma harzianum strains.

Trichoderma harzianum AVCM7, ***Trichoderma harzianum*** SPCV56*, ***Trichoderma harzianum*** SPCV127.

* Represent maximum enzyme producing *Trichoderma harzianum* strain.

Fig. 46 Effect of pH on cellulase and xylanase production by strains of *Aspergillus niger*.



Values are mean of three assays of cultures in replicates.

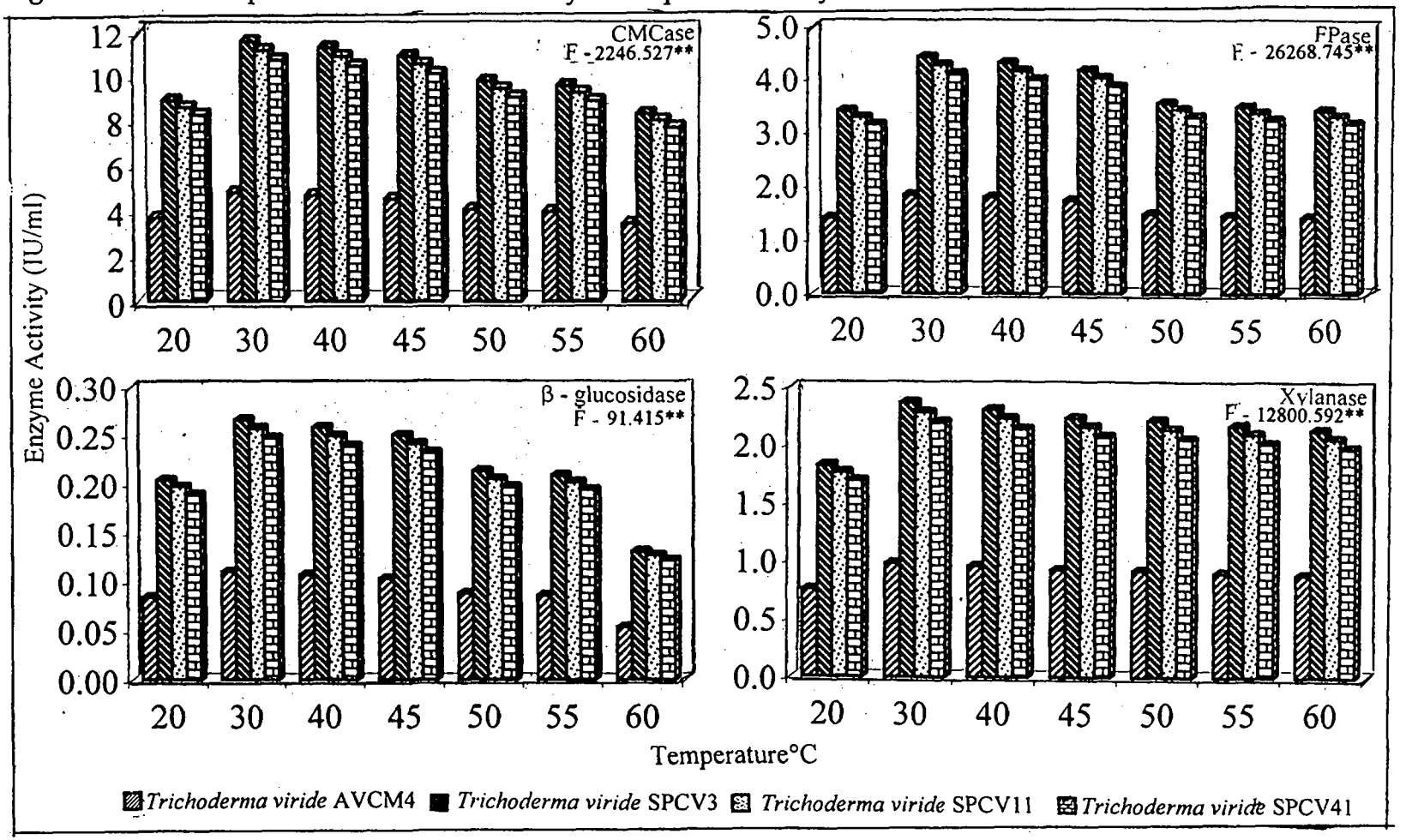
** - (P< 0.001).

Aspergillus niger strains.

Aspergillus niger* AVCM1, *Aspergillus niger* SPCV93.

* Represent maximum enzyme producing *Aspergillus niger* strain.

Fig. 47 Effect of temperature on cellulase and xylanase production by strains of *Trichoderma viride*.



Values are mean of three assays of cultures in replicates.

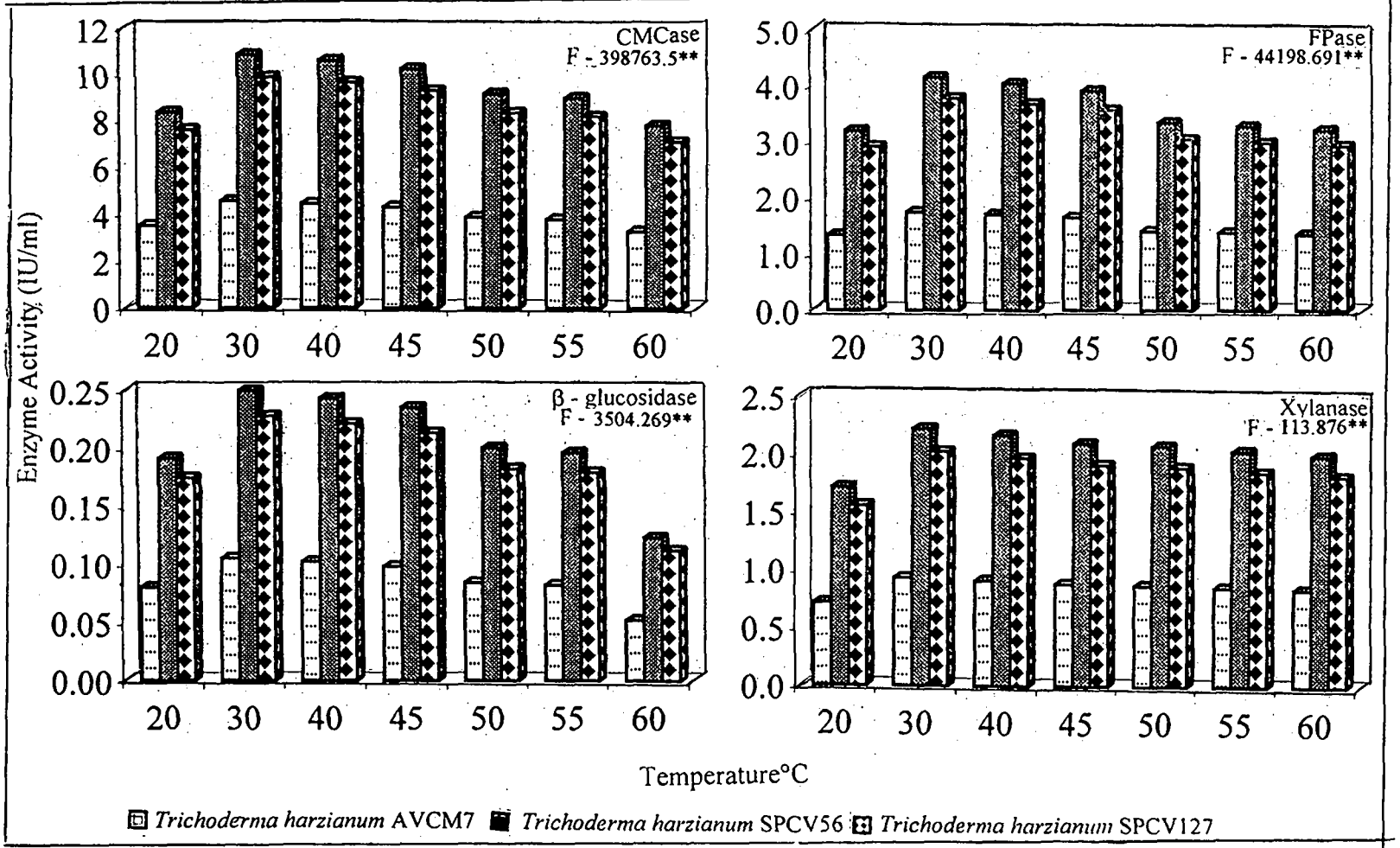
** - (P< 0.001).

Trichoderma viride strains.

Trichoderma viride AVCM4, *Trichoderma viride* SPCV3*, *Trichoderma viride* SPCV11, *Trichoderma viride* SPCV41.

* Represent maximum enzyme producing *Trichoderma viride* strain.

Fig. 48 Effect of temperature on cellulase and xylanase production by strains of *Trichoderma harzianum.*



Values are mean of three assays of cultures in replicates.

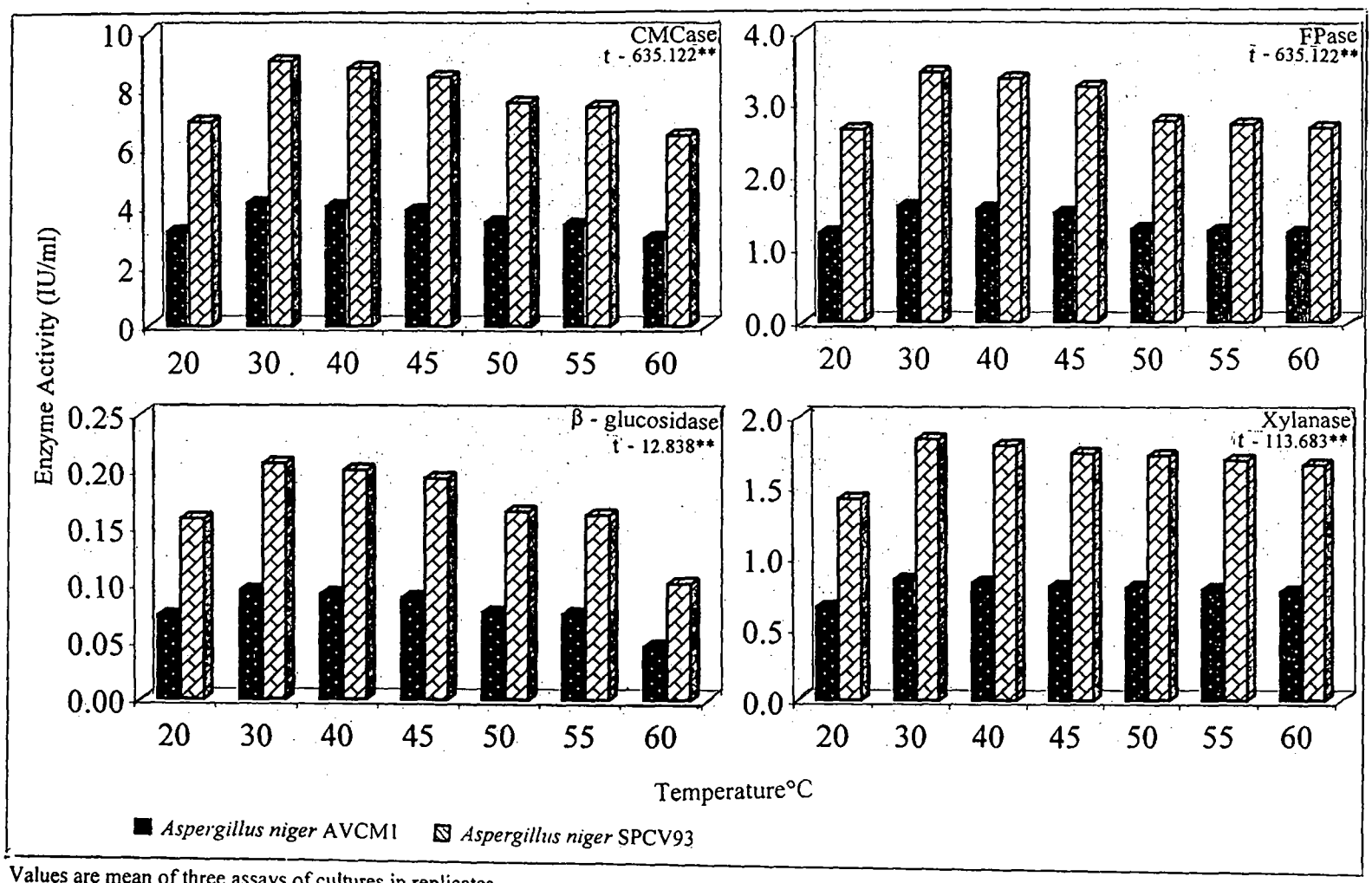
** - (P< 0.001).

Trichoderma harzianum strains.

Trichoderma harzianum AVCM7, ***Trichoderma harzianum*** SPCV56*, ***Trichoderma harzianum*** SPCV127.

* Represent maximum enzyme producing *Trichoderma harzianum* strain.

Fig. 49 Effect of temperature on cellulase and xylanase production by strains of *Aspergillus niger*.



Values are mean of three assays of cultures in replicates.

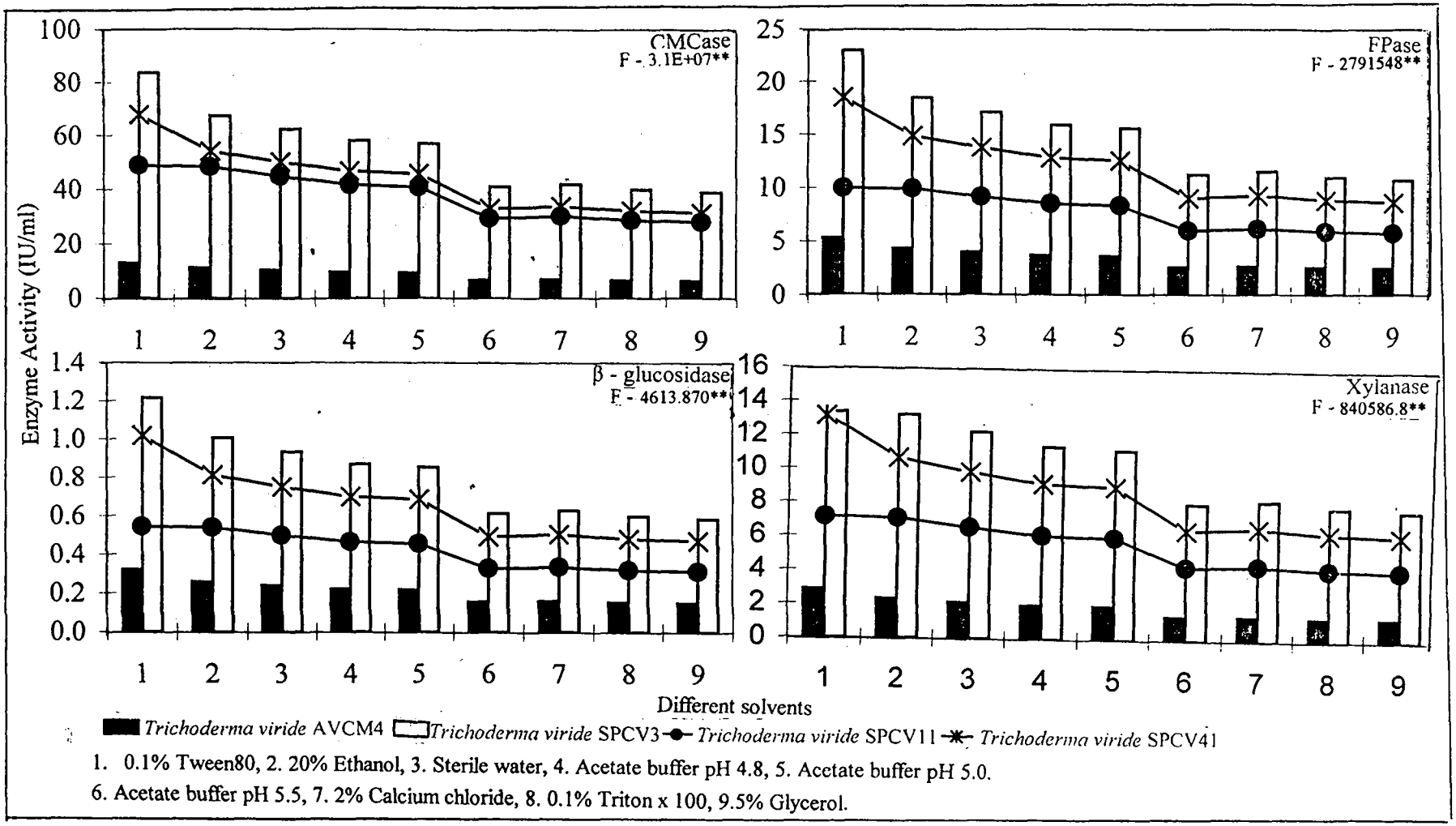
** - (P< 0.001).

Aspergillus niger strains.

Aspergillus niger AVCM1, *Aspergillus niger* SPCV93*.

* Represent maximum enzyme producing *Aspergillus niger* strain.

Fig. 50 Effect of different solvents on cellulase and xylanase recovery by strains of *Trichoderma viride*.



Values are mean of three assays of cultures in replicates.

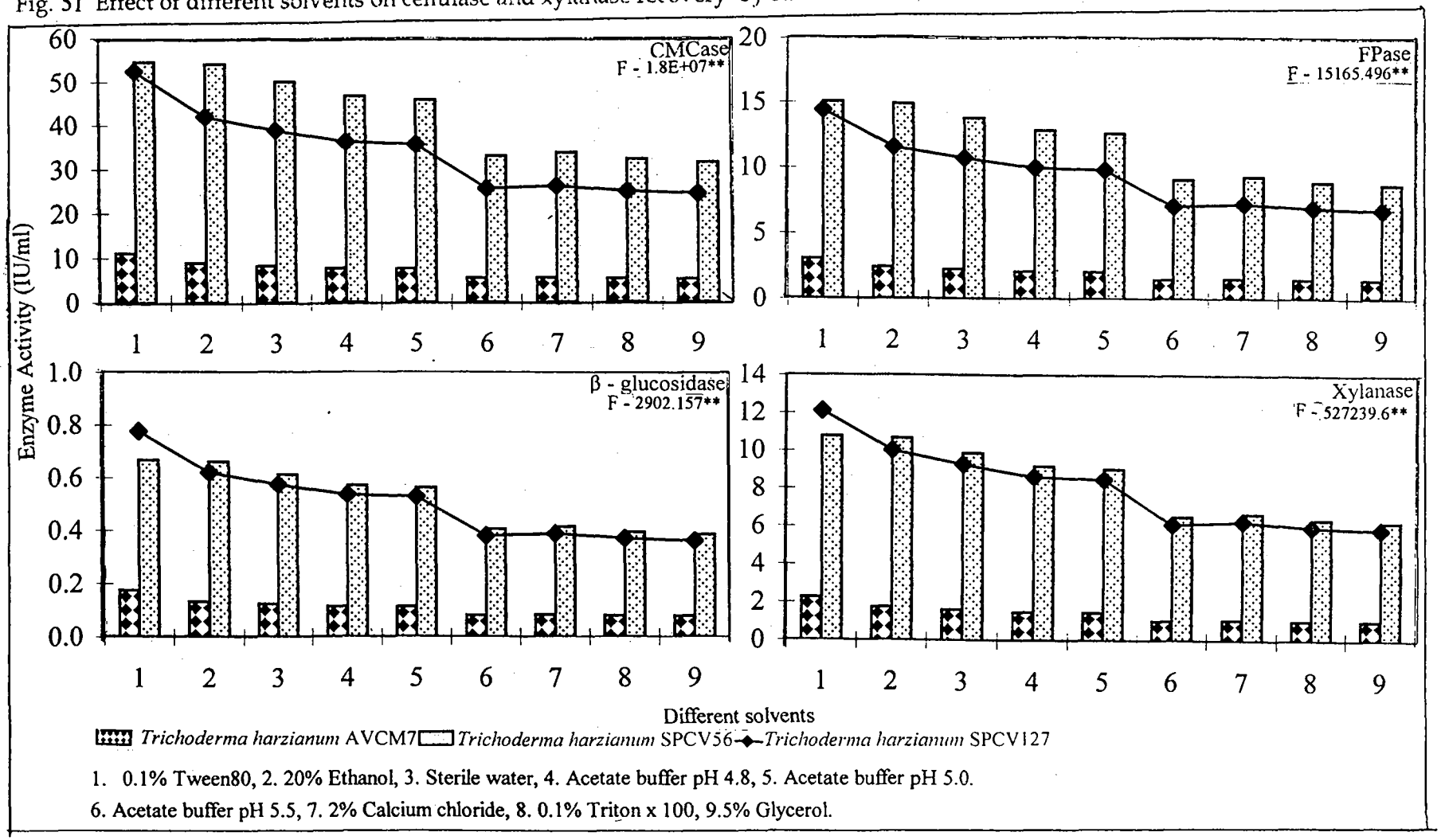
** - (P< 0.001).

Trichoderma viride strains.

Trichoderma viride AVCM4, ***Trichoderma viride*** SPCV3*, ***Trichoderma viride*** SPCV11, ***Trichoderma viride*** SPCV41.

* Represent maximum enzyme producing *Trichoderma viride* strain.

Fig. 51 Effect of different solvents on cellulase and xylanase recovery by strains of *Trichoderma harzianum*.



Values are mean of three assays of cultures in replicates.

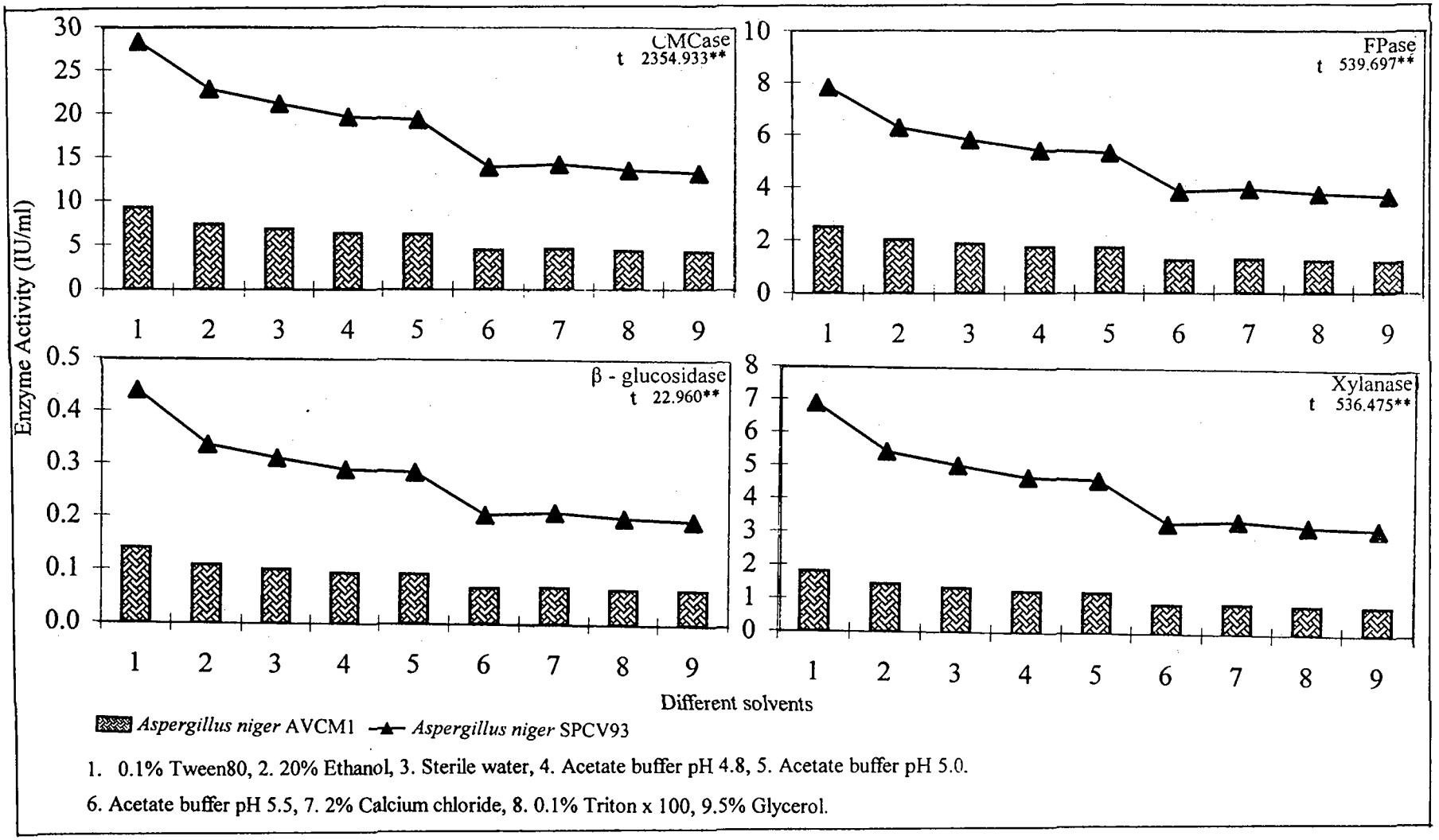
** - (P< 0.001).

Trichoderma harzianum strains.

Trichoderma harzianum AVCM7, ***Trichoderma harzianum*** SPCV56*, ***Trichoderma harzianum*** SPCV127.

* Represent maximum enzyme producing *Trichoderma harzianum* strain.

Fig. 52 Effect of different solvents on cellulase and xylanase recovery by strains of *Aspergillus niger*.



Values are mean of three assays of cultures in replicates.

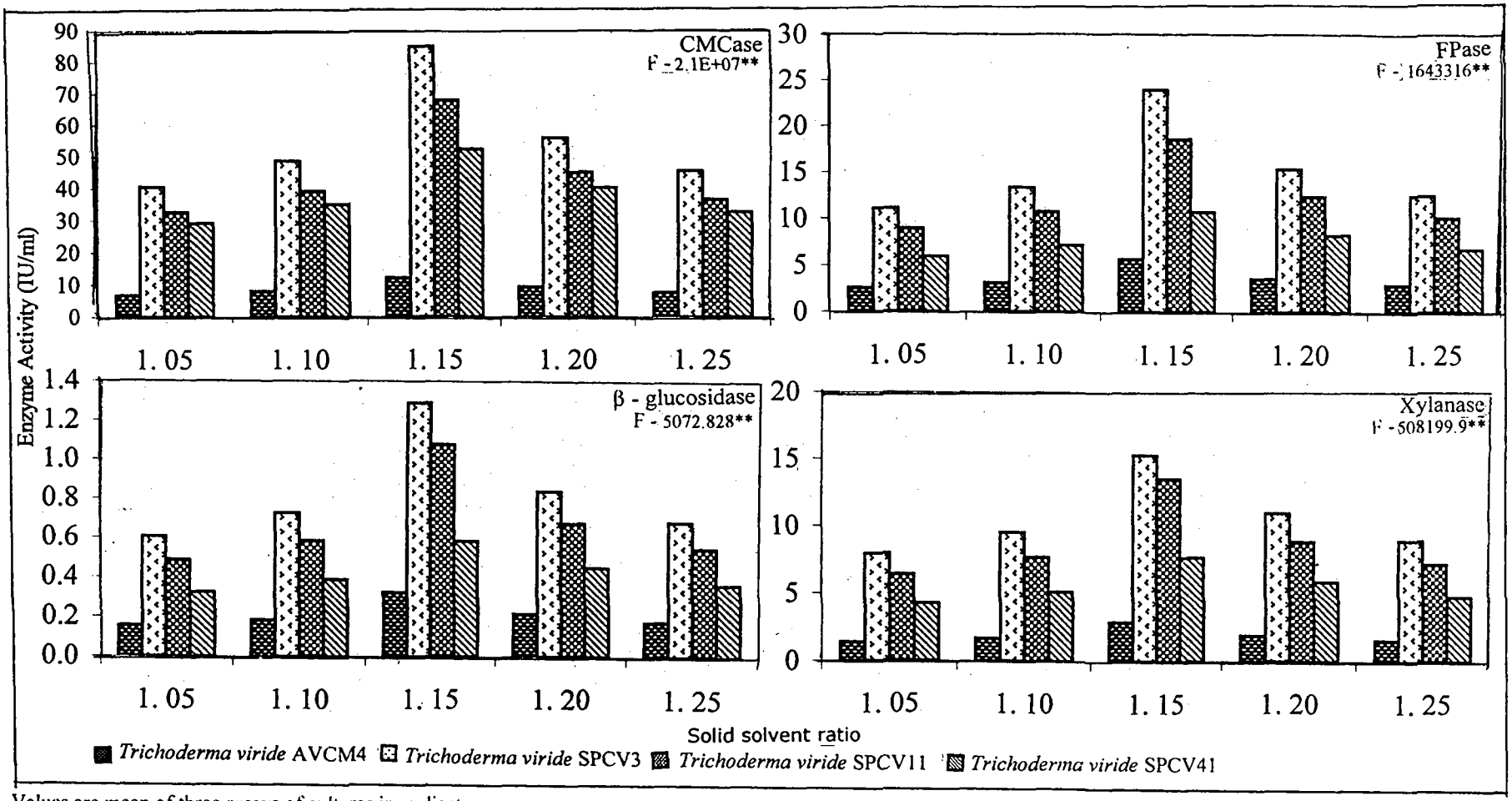
** - (P< 0.001).

Aspergillus niger strains.

***Aspergillus niger* AVCM1, *Aspergillus niger* SPCV93*.**

* Represent maximum enzyme producing *Aspergillus niger* strain.

Fig. 53 Effect of different solid- solvent ratios on cellulase and xylanase recovery by strains of *Trichoderma viride*.



Values are mean of three assays of cultures in replicates.

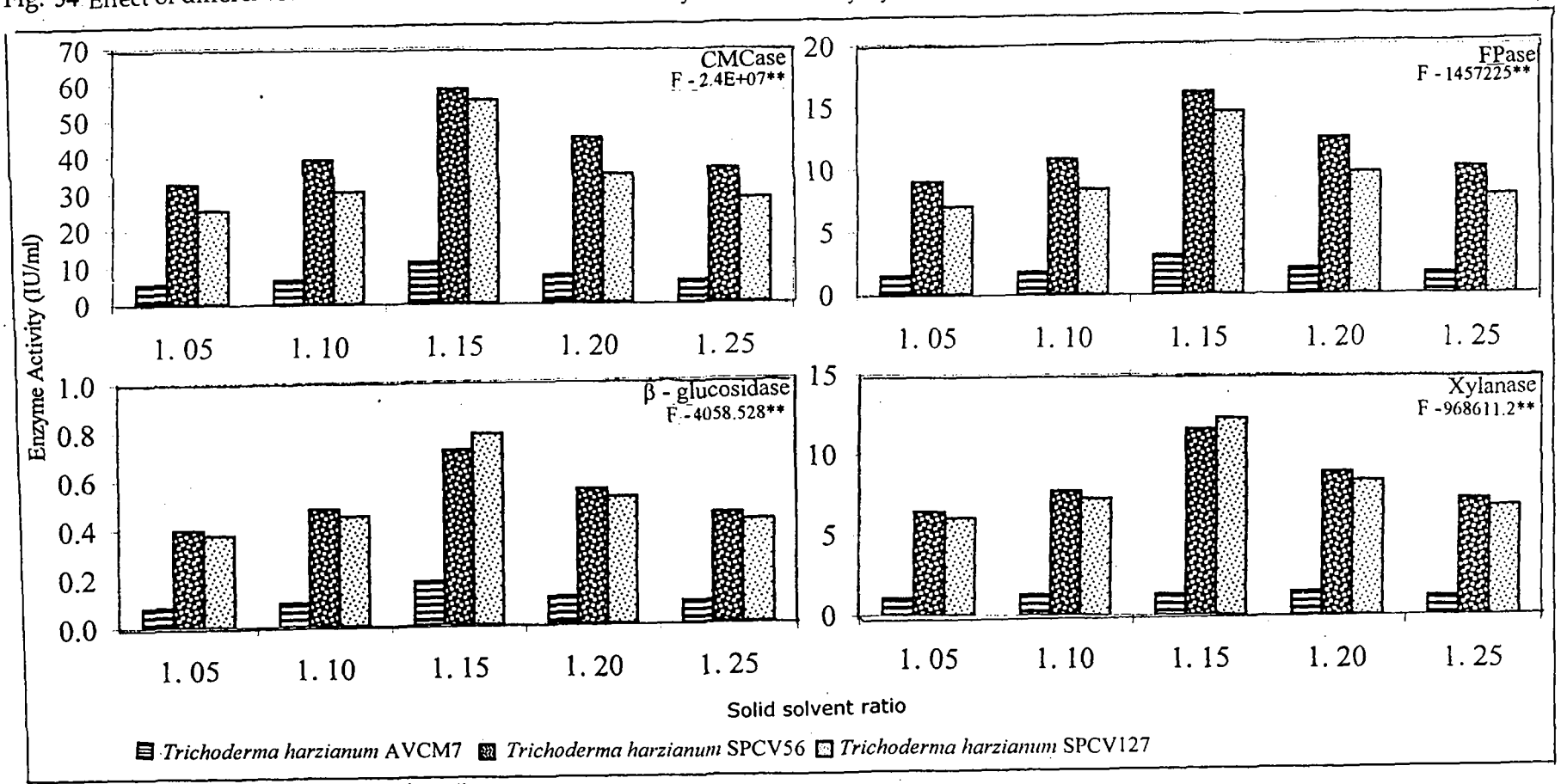
** - (P< 0.001).

Trichoderma viride strains.

***Trichoderma viride* AVCM4, *Trichoderma viride* SPCV3*, *Trichoderma viride* SPCV11, *Trichoderma viride* SPCV41.**

* Represent maximum enzyme producing *Trichoderma viride* strain.

Fig. 54 Effect of different solid- solvent ratios on cellulase and xylanase recovery by strains of *Trichoderma harzianum*.



Values are mean of three assays of cultures in replicates.

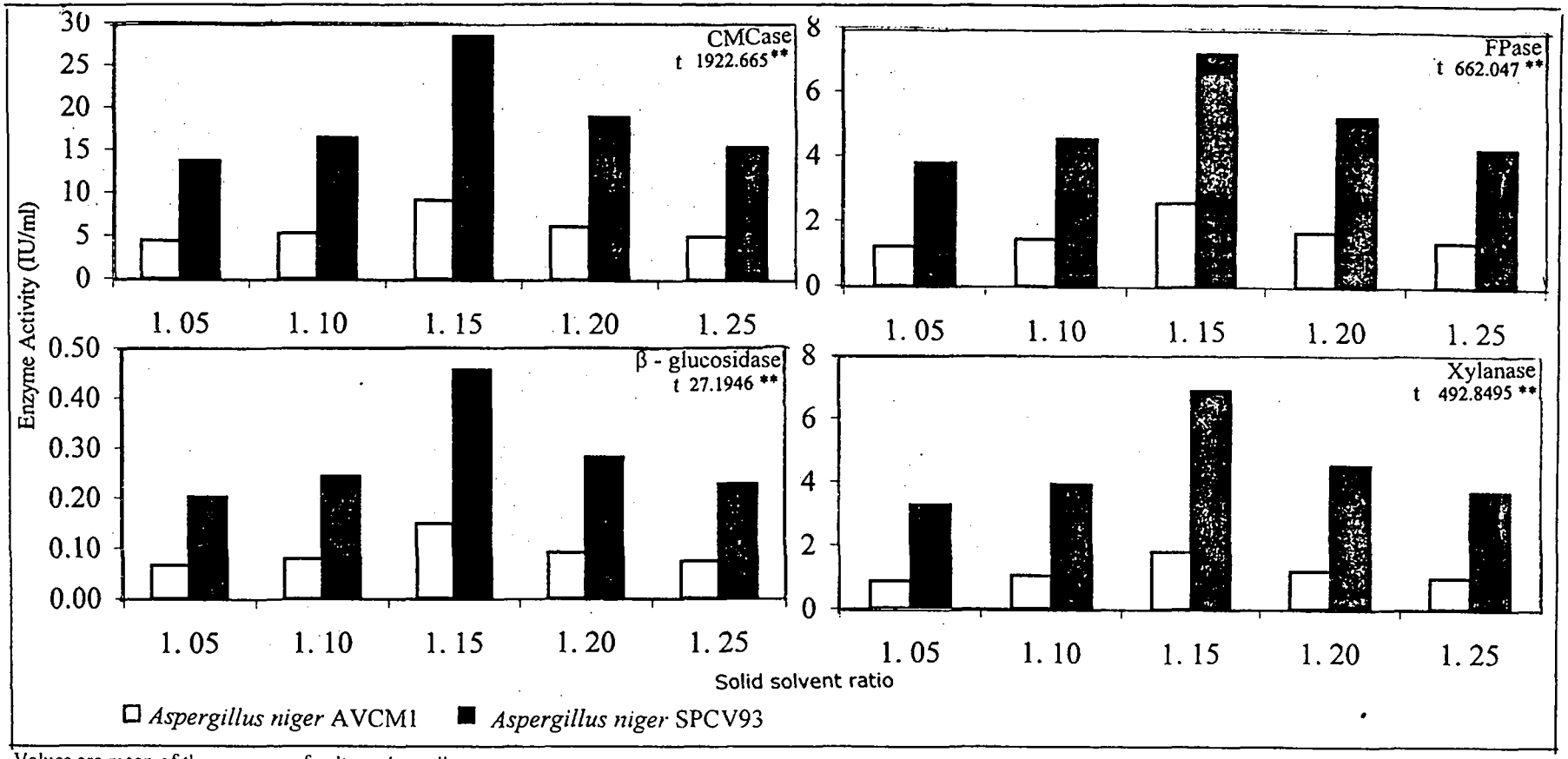
** - (P< 0.001).

Trichoderma harzianum strains.

Trichoderma harzianum AVCM7, ***Trichoderma harzianum*** SPCV56*, ***Trichoderma harzianum*** SPCV127.

* Represent maximum enzyme producing *Trichoderma harzianum* strain.

Fig. 55 Effect of different solvents on cellulase and xylanase recovery by strains of *Aspergillus niger*.



Values are mean of three assays of cultures in replicates.

** - (P< 0.001).

Aspergillus niger strains.

Aspergillus niger AVCM1, *Aspergillus niger* SPCV93*.

* Represent maximum enzyme producing *Aspergillus niger* strain.

Fig. 56 Effect of time of UV exposure on the survival of Trichoderma viride SPCV3

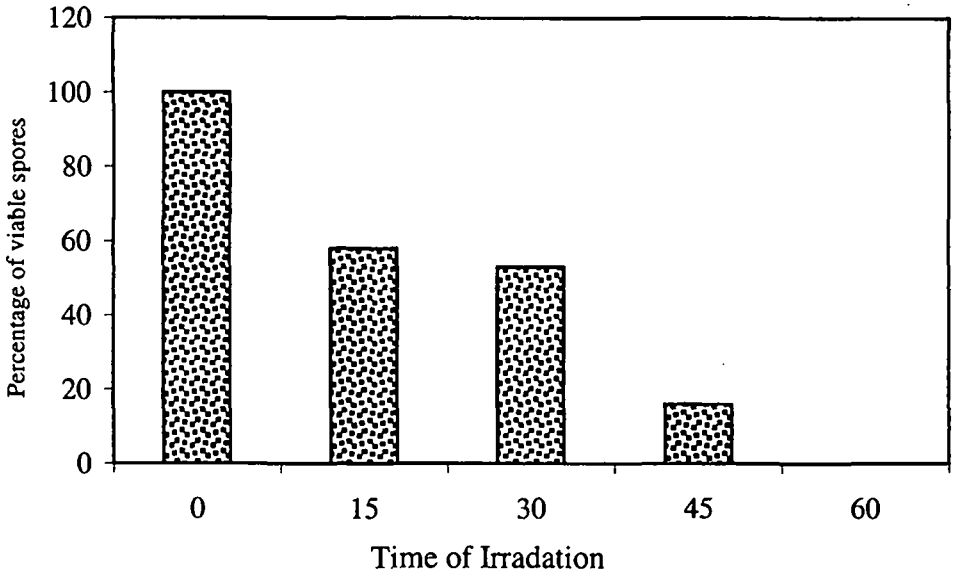


Fig.57 Effect of NTG concentration on the survival of Trichoderma viride SPCV3

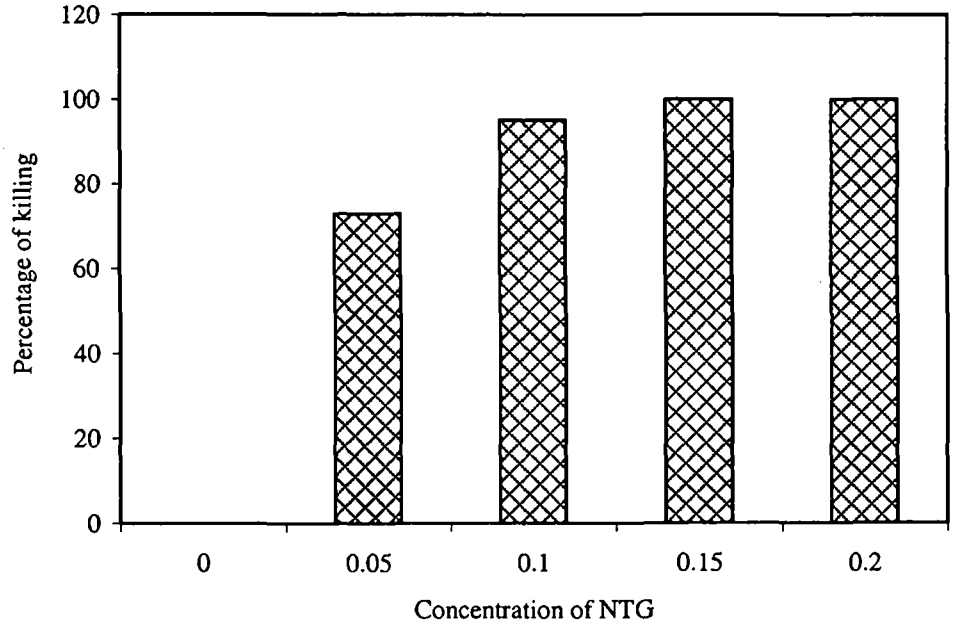
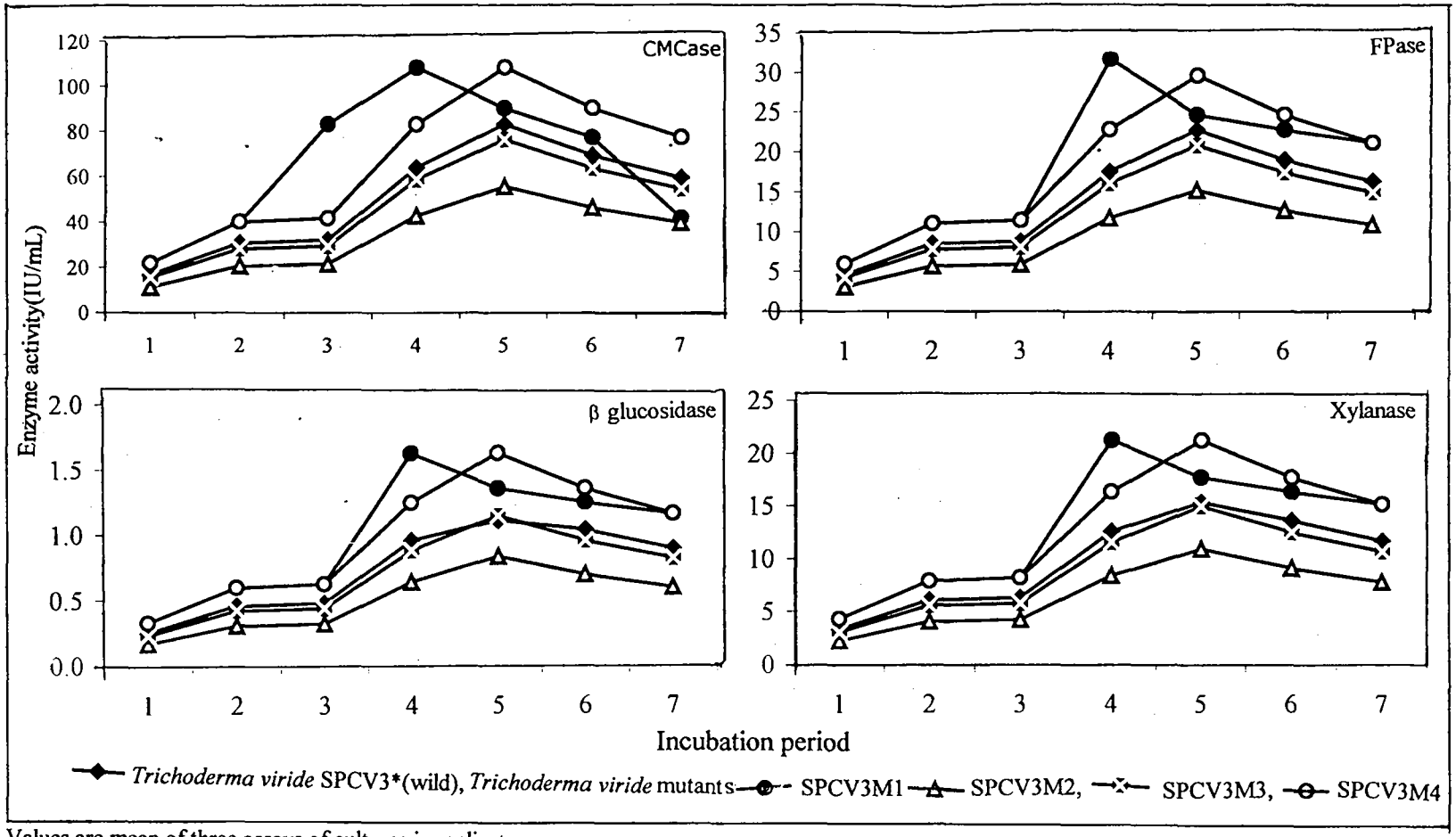


Fig. 60 Effect of incubation period on cellulase and xylanse production by mutant strains of *Trichoderma viride* under SSF.



Values are mean of three assays of cultures in replicates.

** - (P< 0.001).

Trichoderma viride mutant strains.

Trichoderma viride SPCV3*(wild), *Trichoderma viride* SPCV3M1, *Trichoderma viride* SPCV3M2,

Trichoderma viride SPCV3M3, *Trichoderma viride* SPCV3M4.

* Represent maximum enzyme producing *Trichoderma viride* strain.

Fig. 61 Kinetic characteristics of purified FPase (Line Weaver Burk plot).

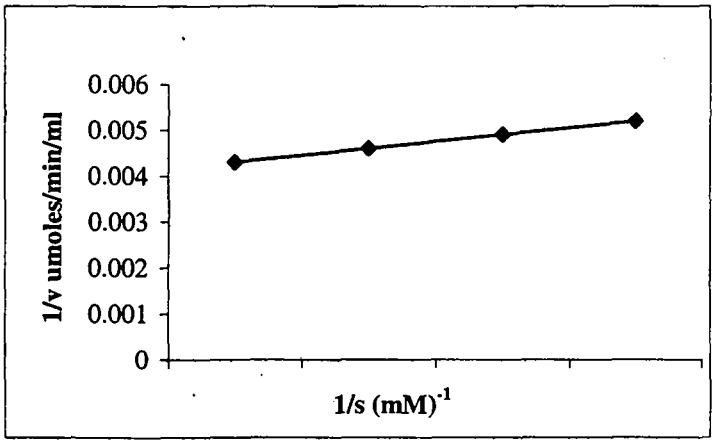


Fig.62 UV spectral analysis of Purified FPase.

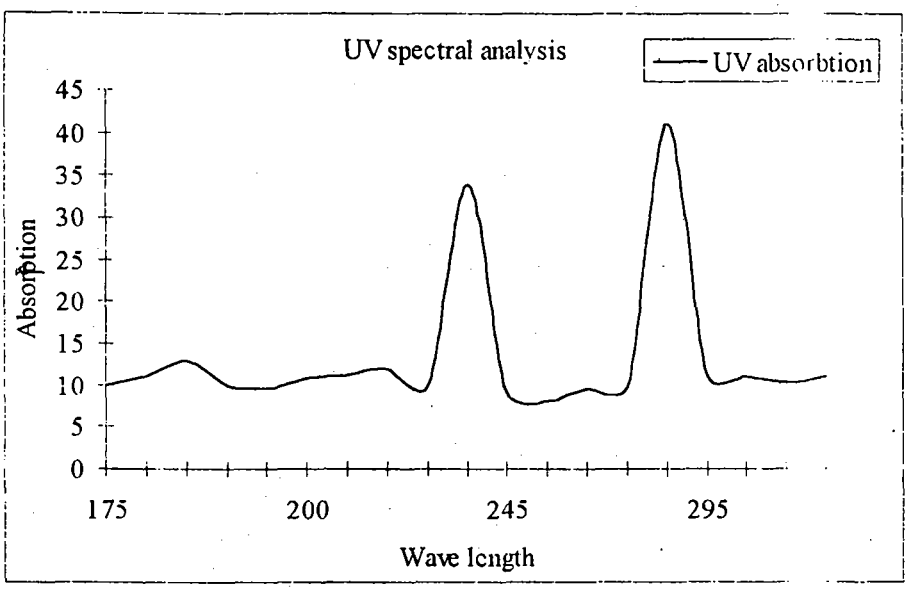


Fig. 63 Absorption spectrum of Purified FPase fluorescent spectra.

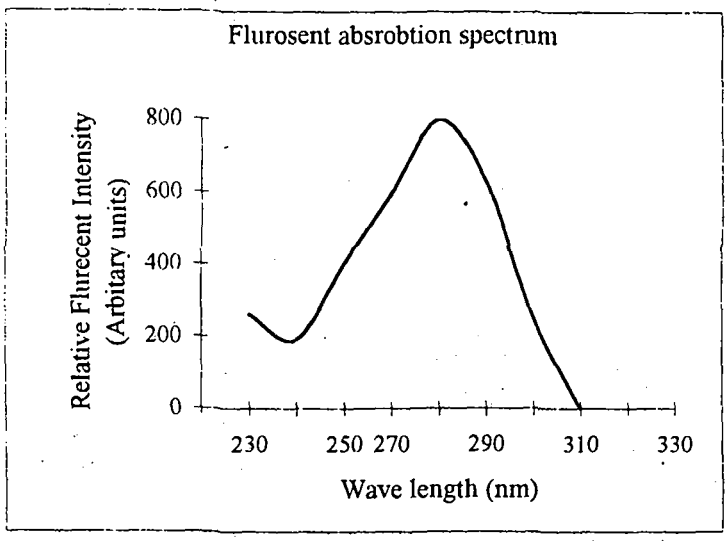


Fig. 64 Emission spectrum of Purified FPase fluorescent spectra.

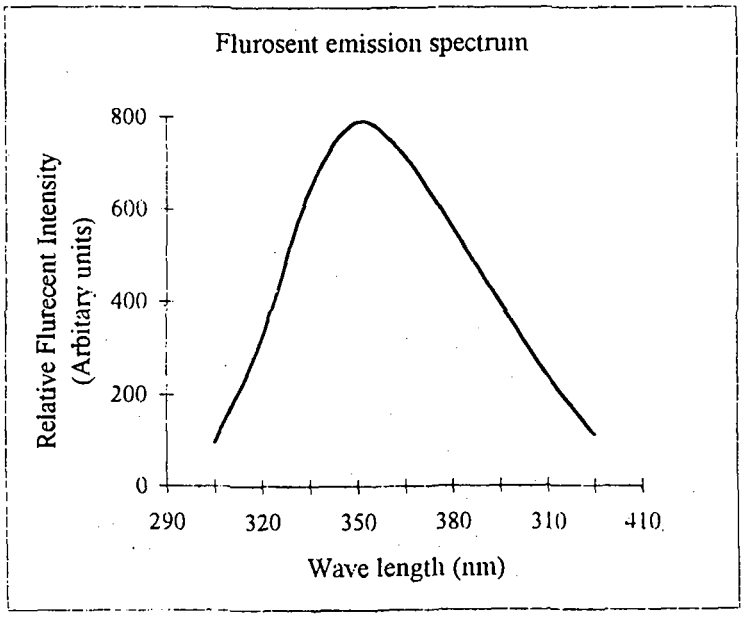


Fig. 70 Effect of incubation period on bioconversion.

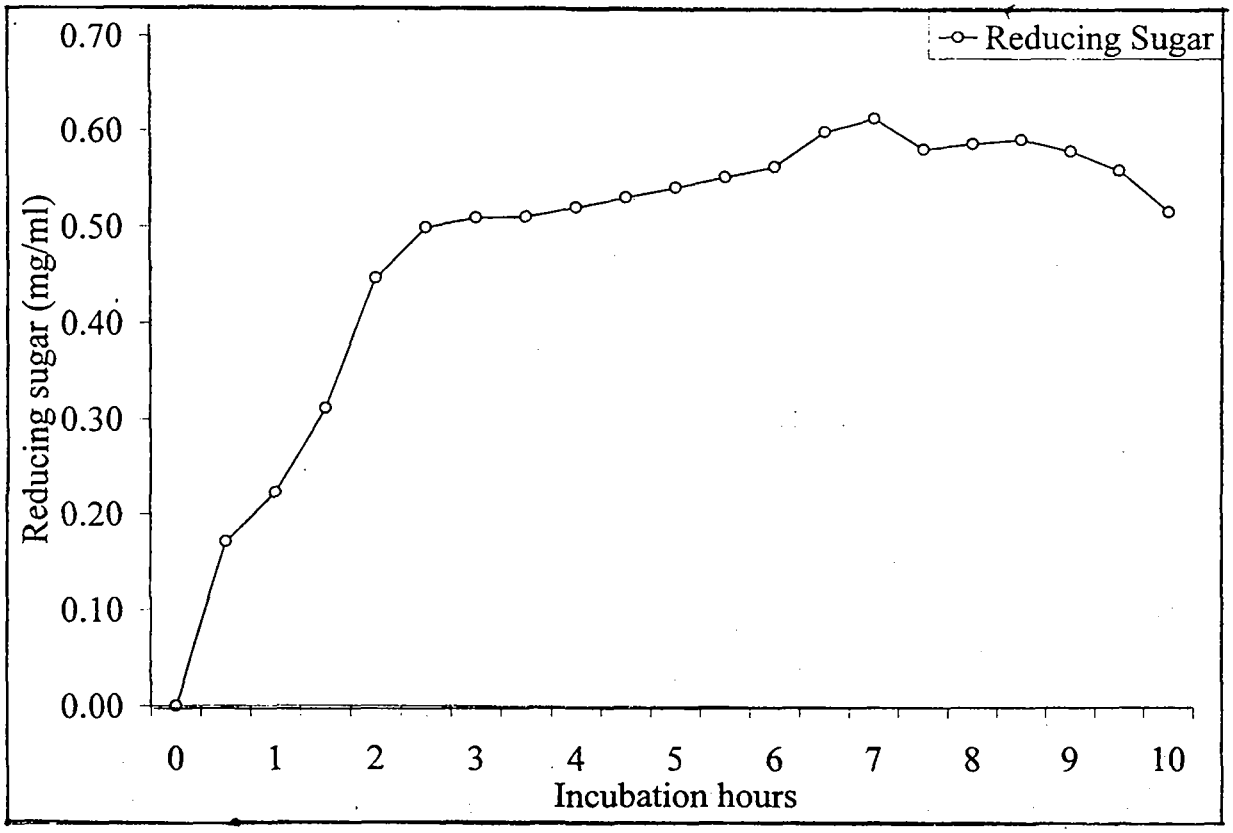
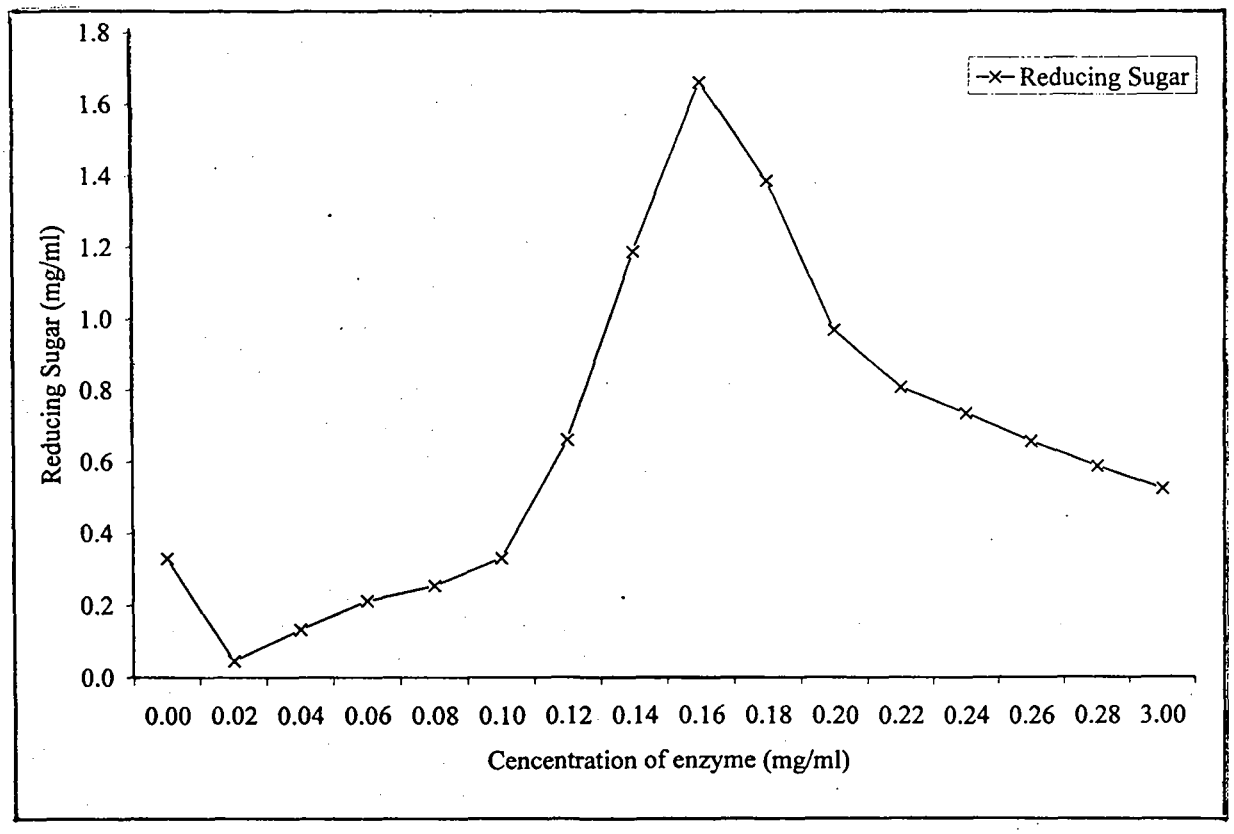
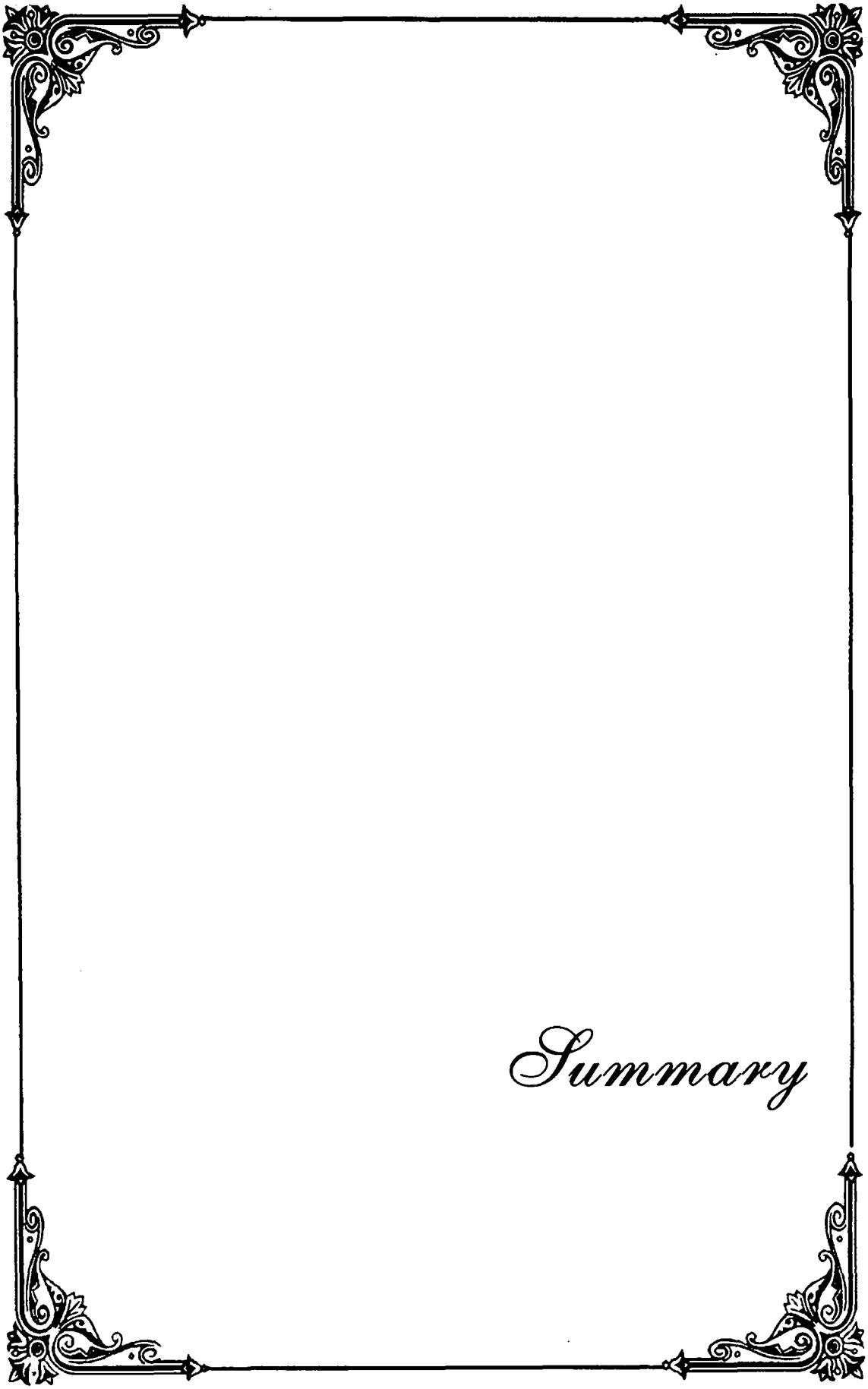


Fig. 71 Effect of concentration of cellulase on bioconversion.





Summary

6. SUMMARY

- Among 310 fungal strains isolated from various sources screened for cellulase production, only 9 strains namely *Aspergillus niger* AVCM1, *A.niger* SPCV93, *Trichoderma harzianum* AVCM7, *T.harzianum* SPCV56 *T.harzianum* SPCV127, *T. viride* AVCM4, *T.viride* SPCV3, *T.viride* SPCV11 and *T.viride* SPCV41, were selected for further studies.

- Enzyme production studies were performed under both submerged (SmF) and solid state (SSF) fermentations. In submerged fermentation, cellulase and xylanase production was maximum under the optimized cultural conditions: 2% wheat bran (carbon source); 0.6% yeast extract (nitrogen source); at 5.0, and 30°C incubation for 5 days.

- Cellulase and xylanase production under SSF was optimum on a simple wheat bran medium – 5% soya bean meal medium under the following conditions: moisture content 55%, pH 5.0, incubation temperature 30°C and fermentation period 5 days. Maximum enzyme from the fermented substrate was extracted by using 0.1% Tween 80 as solvent in 1: 15 substrate to solvent ratio. Enzyme yield under SSF was 8.098 times higher than SmF. Among the fungal strains tested, *T.viride* SPCV3 ranked first in enzyme production under both SmF and SSF.

- In addition to high titres of cellulolytic enzymes, these strains produced associated enzymes such as amylase, pectinase, phytase, α- galactosidase, amyloglucosidase, chitinase, and negligible level of protease, up to 3rd day under solid state fermentation.

- *T. viride* SPCV3 was mutated with UV light to increase cellulase production. The mutant strain SPCV3M1 with dark pigmentation and faster growth showed 27% improvement in cellulolytic yield under optimized solid state growth conditions on 4th day when compared with the wild type. Further co-production of pectinase, phytase, lignase, α- galactosidase, protease, amyloglucosidase and chitinase were decreased by 72 – 18%.

- The curde cellulase and xylanase enzymes had a pH and temperature optima of 4.8 - 5.2 and 50°C. Enzymes were stable in the pH range of 4.0 - 6.5 and 50 - 70°C respectively.

- Subjecting the supernatant to ethanol precipitation and ion exchange chromatography on DEAE Sephadex A 50 coloumn purified FPase, a component of cellulase complex from the mutant T.viride SPCV3M1. The purification fold and specific activity were 2.9 and 356.83 IU/mg respectively; FPase enzyme had optimum activity in the pH range of 4.5 – 6.0; and temperature 50 – 70°C; the Km and Vmax values of the FPase for crystalline cellulose were 0.28 mM and 238.09 μ moles/min/ mg of protein respectively and molecular weight was 47kDa (SDS PAGE and zymogram). The excitation and emission, for fluorescence

spectra showed a maximum and minimum at 292 and 340 nm respectively.

- The effect of treating cotton fibres with mono and multi component cellulases was investigated. Results revealed that the FPase enzyme showed lower hydrolysis when compared with the cellulase complex. Among pretreatment methods used, steaming of cotton fibre increased the activity of the cellulases. Enzymatic treatments were examined microscopically, and found that, fibrillation activity of FPase enzyme on cotton fibre gave a highly polished surface to the cotton fibre. But the activity of cellulase complex, showed erosion, deep cracks and partial cutting of fibres.

- The crude cellulase produced from the mutant strain *T.viride* SPCV3M1 under SSF was applied in the bioconversion of waste paper materials. Maximum saccharification was attained with 50 mg of cardboard and 0.16 mg/ml of enzyme concentration, at optimum pH and temperature of 5.0 and 50°C respectively, on 7^{th} h of incubation.

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2. Vijay Antony, M., Kasinathan, P. and Thajuddin, N. (2004). Biodegradation of waste paper by cellulase from a mutant *Trichoderma viride.* Proceedings of National Symposium on Recent Trends in Biological Research, A.V.V.M. Sri. Pushpam College (Autonomous), Poondi, Thanjavur.

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THERMOSTABLE CELLULASE FROM *TRICHODERMA VIRIDE* AND *TRICHODERMA HARZIANUM*

Vijay Antony M*, Mohan S and Kasinathan P.

Abstract

Cellulolytic and thermotolerant fungi *Trichoderma viride and Trichoderma harzianum* were isolated from irrigated paddy field soil of Thanjavur district, grown at wide pH range 4-8. Maximum production of CMCase 10.319, 9. 746 U/mL; FPase 2.823, 1.521 U/mL; and β glucosidase 0.154, 0.153 U/mL; were obtained in wheat bran yeast extract medium at pH 5.0 on 5 days of incubation. The maximum enzyme activity occurred at pH 5.0, 5.2, and 5.6 in T. viride and 5.0, 5.5, 5.3 in *T harzianum* endo, exo and β glucosidase respectively. The optimum temperature for cellulase activity was 40-45 C. *T.viride* cellulase was stable at room temperature without any dedectable loss of activity than *T.harzianum* which is stable for only two days.

Key words: Thermostable cellulase, *Trichoderma viride, Trichoderma harzianum*, Agricultural residues, Induction.

Introduction

Cellulose is the most abundant biopolymer on earth and has gaining momentum as potential substrate for the production and processing of food, chemicals, fuel ethanol and manufactured good through enzymatic bioconversion by microbial cellulases. (1) Enzymes that hydrolyze complex cellulose polymer to simple monomeric glucose unit are called cellulases. Cellulolytic enzymes are generally induced as multienzyme system, composed of different enzymes. *viz*, Endoglucanase, Exoglucanase and 2-glucosidase (2). In fungi these enzymes are extra cellular and inductive in nature (3).

A synergistic action between these activities is required for complete cellulose hydrolysis. endo and exoglucanase hydrolyze native cellulose producing cellobiose and glucose. 2-glucosidase subsequently cleaves cellobiose to give glucose (4).

Though cellulolytic microorganism are abundant in nature fungi are the most extensively studied with respect to cellulose degradations and cellulase production. In fungi, *Trichoderma spp.* is the best-known source for extra cellular cellulase producer, capable of solublizing crystalline cellulose (5). This enlightens its versatility in cellulase production.

The production of cellulase from lignocellulosic substrates has its own lacunae such as its cost effective production due to high pretreatment expense of lignocellulose and low specific activity of cellulase at operational temperature and pH and product inhibition of cellulase (6). For the efficient complete hydrolysis of lignocellulose residues it would be desirable to have a thermophilic and thermo stable cellulase system. By operating at elevated temperatures, we could limit the incidence of contamination while the thermo stable enzyme would maintain their activity and could be recycled for addition to fresh substrate. In comparison to the intensive investigation carried out on mesophilic fungi such as *T reesei* and *A.niger* search for higher cellulolytic and thermophilic fungi has been relatively limited.

Hence more attention is still focused on the isolation of new strains and mutants of the available strains that can produce more cellulase with greater specific activity, stability and resistance to product inhibition (7).

Though microorganisms are ubiquitous, soil is the repository of microbes. The number and variety of microbes in soil depends on the nature and composition of soil type (8). This study explains the cellulase activity of two thermo tolerant fungi *T viride* and *T harzianum* isolated from irrigated paddy field soil of Thanjavur district. So far there is no such report on thermo stable cellulase in this soil.

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Table 1: Cellulase production by *T. viride* on Agricultural residues at pH 5.5 after 5d incubation

Agricultural Residues	Soluble protein mgmL^{-1}	CMCase UmL^{-1}	FPase UmL^{-1}	2-glucosidase UmL^{-1}
Wheat bran	2.322	10.319	2.823	0.154
Bagasse	2.615	9.870	2.891	0.183
Rice bran	1.246	6,780	2.194	0.045
Kambu	2.210	4.474	0.676	0.106
Rice straw	1.369	4.126	1.427	0.088
Ragi	1.794	3.529	0.589	0.102
Avicel	1.726	5.293	0.516	0.071

Table 2: Cellulase production by *T. harzianum* on Agricultural residues at pH 5.5 after 5d incubation

Agricultural Residues	Soluble protein mgmL^{-1}	CMCase UmL^{-1}	FPase UmL^{-1}	2-glucosidase UmL^{-1}
Wheat bran	1.021	9.746	1.521	0.153
Bagasse	1.611	6.650	1.631	0.120
Rice bran	1.643	4.813	1.732	0.144
Kambu	1.572	4.114	0.170	0.126
Rice straw	1.840	3.172	0.978	0.169
Ragi	1.301	3.107	0.366	0.134
Avicel	1.543	3.212	0.710	0.035

Materials And Methods

The fungi *T viride* and *T harzianum* were isolated from irrigated paddy field soil and maintained on potato dextrose agar (PDA) medium. And screening for effective cellulolysis was made using selective medium (9) and observed for clearing zone formation.

Samples were withdrawn in every 24h interval and spindown at 4 C for 10 min. Supernatants were used as enzyme source. Cellulase assay was done by measuring released reducing sugars (RS) by dinitrosalicylic acid (DNS) method of Miller et al (10) with glucose as standard. Protein was determined by the method of Lowry (11). CMCase, Fpase were determined by the method of (7) using DNS where one IU is defined as the amount of enzyme releasing 1/μ mol glucose per minute. β-glucosidase assay was performed using the method of (12). The unit of β-glucosidase activity IU is defined as the amount of enzyme liberating one AI mol p-nitrophenol per minute. The influence of various carbon and nitrogen sources and initial pH, temperature, on the enzyme production was investigated. The time course of maximum enzyme production and thermo stability of cellulase was also examined.

Results and Discussion

Identification and screening for cellulolysis

Based on the cultural and morphological characters fungal (13) isolated were identified to be *Trichoderma viride* and *Trichoderma harzianum*. In cellulose agar, *T viride* produced large clear zone (4 mm) around the colony than *T harzianum* (2.8 mm) and thus isolates were initially screened for visible and efficient cellulolysis.

Cellulase production in different cellulosic substrates.

The effect of different cellulose substrates on cellulase production by the two fungi was investigated (Table 1 & 2). The production of endo and exo cellulase enzyme components were maximum in *T. viride* and while. β-glucosidase was maximum in *T. harzianum*. β glucosidase activity in sugarcane bagasse was optimum for *T. viride* and in rice straw for *T. harzianum*. Considering the overall maximum yield, wheat bran ranks first for both the fungi. These differences in cellulase yield probably result from the variation in surface area, degree of hydration and availability of substrate to organism or physical adsorption of enzyme from culture filtrate (4).

As wheat bran exemplified utmost cellulase production, the effect of different wheat bran concentration on enzyme productivity by the two fungi was examined. Optimum production of all the three components of cellulase occurred with 2% (w/v) wheat bran in *T. viride* and with 1.5% (w/v) wheat bran in *T. harzianum*. Where as cellulase production in CD medium containing Avicel as carbon source was little less.

The decrease in cellulase production with the increase in substrate concentration beyond optimum may be attributed either to a physical adsorption of the enzyme on substrate of to a repression of cellulase synthesis due to end products of hydrolysis in the medium or due to the acid concentration that may develop at high cellulase levels (7).

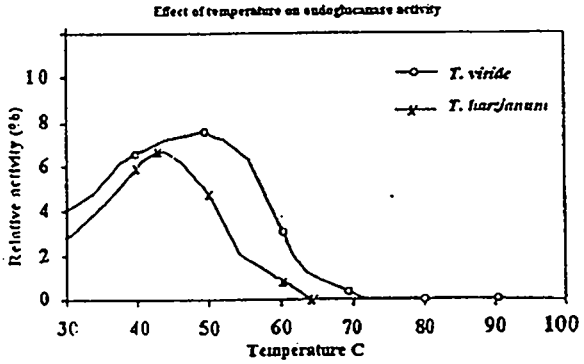
Cellulase production with selected Nitrogen concentration

The effect of various nitrogen sources giving optimum cellulase yield by these fungi (Yeast extract, Peptone, Urea and Glycine) was studied from 0.1% - 1.0% (w/v). Maximum endoglucanase yield was noticed at 0.5% yeast extract by the two fungi. Though CD medium contains mineral supplements, it is found that this medium did not support higher production of enzyme than simple wheat bran medium. This may be due to complex nature of wheat bran and the induction of multiple enzymes by wheat bran.

Characterization of cellulase of *T. viride and T. harzianum*

From the results these two fungi produced most active exocellulase profile, which is comparable to other fungal cellulases (5). The optimal pH and temperature cellulase activity were 5.6-6.0 and 45°C. The pH optima for CMCase, FPase, 2-glucosidase for *T. viride* were 5, 5.2, 5.6 and for *T. harzianum* were 5, 5.5, 5.3 respectively. This indicates that this *T. Viride* produces a thermostable cellulase with high specific activity (48 U/mg of protein) and than *T. harzianum* (36 U/mg of protein). In alkaline conditions a pH change to 6.0 was noticed in two fungi and cellulase productivity was comparatively less in alkaline pH. As initial pH directly influence cellulase production (14) increase in pH decreased cellulase production by 50% in the two fungi. Both the isolates were shown to be true thermophilous, exhibiting optimal growth (78 and 62 mg/flask) temperature between 45°C – 50°C and little or no growth at 20°C.

Cellulase produced by *T. viride* was most active at pH 5.0 and stable up to 50°C where as cellulase of *T. harzianum* was most active at pH 5.0 - 5.5 and stable upto 45°C (fig I) above this temperature the enzyme rapidly loss its activity more over *T. viride* cellulase at room temperature for a week at pH 5.0-6.5 without any detectable loss of activity. Thus these isolates produced a complete set of cellulases. More over *T. viride* produces high levels of endoglucanase and exoglucanase than *T. harzianum*. However further studies in strain improvement and other parameters involving culture conditions enhanced cellulase production by these isolates may be achieved.



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PRODUCTION AND PROPERTIES OF CELLULOLYTIC ENZYMES BY *TRICHODERMA VIRIDE* AND *TRICHODERMA HARZIANUM* IN SUBMERGED FERMENTATION

M. VIJAY ANTONY[1]* S. MOHAN[1], P. KASINATHAN[1] AND N. THAJUDDIN[2]

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Key words : Cellulase, Submerged fermentation, Trichoderma harzianum, Trichoderma viride, Xylanase.

Abstract–A complete set of cellulase comprising of endo glucanase, exo glucanase, β-glucosidase and xylanase was produced by *Trichoderma viride* and *Trichoderma harzianum* by submerged fermentation. Enzymes production was maximum in 1% Avicel as carbon source and a combination of 0.6% yeast extract as nitrogen source at pH 5.0 on 5th day of incubation. Cellulase and xylanase of both the Spp. were optimally active at pH 4.8-5.2 and at 50°C. Stability of these enzymes was more than 60% at pH 4.0 -6.5 and at 45°C - 55°C.

INTRODUCTION

Cellulase and xylanases are important extra cellular microbial enzymes which hydrolyze cellulose and xylan. They are the two most inexpensive source of biomass utilized for the production of valuable components such as sugar protein and other chemicals via enzymatic bioconversion. Hence these enzymes have high industrial value. Variety of cellulolytic fungi generates high level of cellulase and xylanase (Rokar and Nakas 1990). Commercial production of cellulase by submerged fermentation appears to be less productive. The production of these enzymes is particularly dependent on cultural conditions. (Ganju *et al.* 1990). The present study was aimed at optimization of submerged fermentation parameters for stable land enhance production of cellulase and xylanase enzyme.

MATERIALS AND METHODS

The fungi *Trichoderma viride* SPCV11 and *Trichoderma harzianum* SPCV127 were isolated from paddy field soils of Cauvery delta and maintained in Potato dextrose agar (PDA) medium. The control organism *Trichoderma viride* AVCM4 and *Trichoderma harzianum* AVCM11 were obtained from A.V.C. College Mayiladuthurai.

Using spore inoculum, enzyme productoin studies was carried out in Czapek Dox broth for 7 days at 30°C on a rotary shaker at 180 rpm (Rajendran *et al.* 1994).

Analytical methods : Enzyme assay

Enzyme assays were performed in 5th day culture filtrate of shaker flask cultures. Endoglucanase activity was measured by the method of Mandels (1985). Xylanase assay was performed according to Mandels *et al.*, (1976). Where one IU is defined as the amount of enzyme liberating 1μ mole of glucose per minute. Under standard assay conditions. β-glucosidase activity was determined using Theodorou *et al.*, (1980) method. One unit of β-glucosidase activity is defined as the amount of enzyme that liberates 1μ mole of P-nitro phenol per minute. Soluble protein content was determined by Folin-phenol method (Lowry *et al.* 1951). Biomass measurements were made a described by Rajendran *et al.* (1994). All the data presented here represents the average of 3 assays of cultures in duplicate.

RESULTS AND DISCUSSION

The results of cellulase and xylanase production by *Trichoderma viride* and *Trichoderma harzianum* in various substrates, effect of Avicel and yeast extract concentration on enzyme production, influence of initial pH on production of cellulase and xylanase,

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pH an temperature optimal for activity and stability of enzymes were given in Tables 1 and 2 and Figs. 1-7.

Enzymes production in different substrates

The two fungi namely *Trichoderma viride* and *Trichoderma harzianum* tested for enzyme production in various defined substrates revealed that the nature of substrate significantly influenced cellulase productivity. (Table 1). Avicel induced maximum endoglucanase production (i.e.). 5.516 (IU/ml) for 2.502 (IU/ml) and exoglucanase 1.587 (IU/ml) and 1.340 (IU/ml) for *T. viride* SPCV11 and *T. harzianum* SPCV127 as against control 3.116 (IU/ml), 2.172(IU/ml) and 1.174 (IU/ml), 0.625 (IU/ml) for endo exo glucanase of *T. viride* ACM4 and *T. harzianum* AVCM7 respectively. β-glucosidase secretion varied from 0.384 (IU/ml) to 0.237 (IU/ml) in cellobiose.

Both the fungi produced maximum xylanase in xylan (i.e.) 1.302 (IU/ml) for *T. viride* SPCV11 and 1.86 (IU/ml) for *T harzianum* SPCV 127 when compared with control 0.895 (IU/ml) and 1.604 (IU/ml). Saddler *et al.*, (1987) proposed that *T harzianum* E58 produced relatively high xylanase in cellulosic substrate than xylan. The present study shows some variations in xylanase production with respect of *T. harzianum* E58. The poor enzyme production in glycerol, pectin and cellodextrin by these strains substantiate earlier reports of Bagga *et al.*, (1989) and Rokar Nakas (1990).

The solubel protein and biomass content was maximum in avicel 90 µg/ml and 820.35 (mg/20ml) for *T. viride* SPCV11 than *T. harzianum* SPCV127, which produced 82.7 µg/ml and 639.44 (mg/20 ml) 611.72 (mg/20 ml). These results can be positively correlated with Rajendran *et al.* (1994) Study with *Humicola fuscotra*.

Effect of concentration of Avicel as carbon sources on enzyme production

Maximum cellulase and xylanase production occurred at 1% Avicel concentration (Fig. 1). The endoglucanase production varied from 5.957(IU/ml) to 5.543(IU/ml). The optimum 5.597 (IU/ml) was recorded in *T. viride* SPCV11. At higher concentration cellulase and xylanase production decreases. The same trend was reported by Mandels (1985) with reference to *T. viride*.

Effect of various nitrogen sources on cellulase and xylanase production

Nitrogen source is an important amendment that affects enzyme production. In the present study the seven nitrogen sources both the fungi revealed higher production of enzymes in organic nitrogen sources (i.e.) yeast extract followed by peptone (Table 2). *T. viride*, SPCV11 produced the maximum of 5.990(IU/ml), 1.728 (IU/ml), 0.182(IU/ml) and 0.568(IU/ml) and the control 4.608(IU/ml) 1.326 (IU/ml), 0.140(IU/ml), and 0.437(IU/ml), whereas *T. harzianum* SPCV 127 produced 5.629 (IU/ml), 1.634 (IU/ml), 0.140 (IU/ml) and 0.539 (IU/ml) when compared with control 2.538(IU/ml), 0.679 (IU/ml), 0.072 (IU/ml) and 0.224(IU/ml) for endoglucanase, exoglucanase, β-glucosidase and xylanase respectively. Influence of yeast extract as an affirmative nitrogen source in the production of cellulase the present work can be compared with (Gangu *et al.*, 1990) with reference to *Chaetomium thermophille*.

Effect of concentration of yeast extract as nitrogen on cellulase and xylanase production

Yeast extract at 0.6% (w/v) was found to be optimum for maximum cellulase and xylanase production (Fig. 2) *T. viride* SPCV11 secreted 6.001 (IU/ml), 1.727 (IU/ml), 0.193 (IU/ml) and 0.269 (IU/ml) as against control 4.616 (IU/ml), 1.328 (IU/ml), 0.149 (IU/ml) and 0.438 (IU/ml). Whereas *T. harzianum* SPCV127 produced 5.369 (IU/ml), 1.634 (IU/ml), 0.141 (IU/ml) and 0.077 (IU/ml) and 0.224 (IU/ml) for endoglucanase, exoglucanase, β-glucosidase and xylanase respectively. Optimum cellulase production was obtained at 0.1% yeast extract concentration in *Chaetomium thermophille* (Ganju *et al.* 1990). But the present work shows variations with the work of Ganju *et al.*, (1990) with respect to cellulase production.

Effect of pH on the production of cellulase and xylanase

The pH of the medium play an important role in the production of cellulolytic enzymes. pH 5.0 was found to be optimum of enzymes production by these fungi in Avicel yeast extract medium (Fig. 3). *T. viride* SPCV11 produced 6.233 (IU/ml), 1.793 (IU/ml), 0.201 (IU/ml), and 0.591 (IU/ml), for endoglucanase exoglucanase, β. glucosidase and xylanase respectively. The control *T. viride* AVCM4 produced 4.795 (IU/ml), 1.379(IU/ml), 0.146 (IU/ml) and 0.155 (IU/ml) for *T. harzianum* SPCV127 it was 5.857 (IU/ml), 1.700 (IU/ml), 0.174(IU/ml) and 0.592 (IU/ml) as against control. Ganju *et al.*, (1990) stated the maximum cellulase production results when the

Table 1. Cellulase and Xylanase production (IU/ml culture filtrate) by the 2 fungi with different carbon sources.

Carbon source	*T. viride* AVCM4 (control)				*T. viride* SPCV11				*T. harzianum* AVCM7 (control)				*T. harzianum* SPCV1227			
	Endo glucanase	Exo glucanase	β-gluco-sidase	Xylanase	Endo glucanase	Exo glucanase	β-gluco sidase	Xylanse	Endo glucanase	Exo glucanase	β-gluco-sidase	Xylanase	Endo glucanase	Exo glucanase	β-gluco-sidase	Xylanase
Avicel	3.116	1.174	0.130	0.253	5.516	1.587	0.168	1.528	2.172	0.625	0.066	0.206	2.502	1.340	0.097	1.051
CMC	4.258	0.127	0.141	0.106	4.816	1.471	0.177	1.513	1.596	0.579	0.070	0.202	1.707	1.218	0.085	0.101
Solka floc	2.311	0.757	0.072	0.376	3.081	1.023	0.078	1.510	1.213	0.403	0.044	0.201	2.241	0.216	0.093	1.488
Pectin	0.098	0.020	0.001	0.001	0.036	0.030	0.001	1.009	0.014	0.011	0.002	0.004	0.116	0.010	0.001	0.001
Glycerol	0.034	0.003	0.001	0.002	0.027	0.035	0.001	0.002	0.011	0.010	0.007	0.001	0.011	0.031	0.002	0.003
Cellobiose	0.210	0.115	0.235	0.001	0.318	0.085	0.346	0.007	0.125	0.032	0.053	0.002	0.293	0.038	0.237	0.004
Xylan	0.531	0.143	0.034	0.895	0.768	0.239	0.018	1.302	0.302	0.094	0.027	1.604	0.461	0.156	0.010	1.816
Filter paper	2.141	0.947	0.068	0.008	3.020	1.048	0.118	0.085	1.189	0.216	0.046	0.033	1.764	1.004	0.069	0.041
Cotton	2.112	0.626	0.043	0.019	1.086	1.857	0.107	0.090	0.328	0.912	0.042	0.096	0.622	1.067	0.012	0.023
Cellodextrin	0.074	0.021	0.002	0.007	0.070	0.017	0.007	0.009	0.035	0.007	0.003	0.004	0.085	0.016	0.005	0.009

Table 2. Cellulase and Xylanase production (IU/ml culture filtrate) by the 2 fungi with different nitrogen sources.

Nitrogen source	*T. viride* AVCM4 (control)				*T. viride* SPCV11				*T. harzianum* AVCM7 (control)				*T. harzianum* SPCV1227			
	Endo giucanase	Exo glucanase	β-gluco-sidase	Xylanase	Endo glucanase	Exo glucanase	β-gluco sidase	Xylanse	Endo glucanase	Exo glucanase	β-gluco-sidase	Xylanase	Endo glucanase	Exo glucanase	β-gluco-sidase	Xylanase
Yeast extract	4.068	1.326	0.140	0.437	5.990	1.728	0.182	0.538	2.358	0.679	0.072	0.224	5.629	1.634	0.140	0.539
Peptone	4.023	1.229	0.149	0.429	5.230	1.590	0.193	0.557	2.059	0.627	0.076	0.219	4.914	1.515	0.139	0.528
Glycine	2.523	0.458	0.199	0.071	3.280	0.545	0.128	0.092	1.291	0.734	0.004	0.036	3.082	0.564	0.009	0.688
Urea	2.574	0.855	0.008	0.426	3.346	1.112	0.011	0.554	1.317	0.437	0.002	0.218	3.144	1.053	0.008	0.525
Ammonium chloride	0.030	0.025	0.001	0.008	0.039	0.033	0.001	0.010	0.015	0.013	0.001	0.004	0.037	0.031	0.001	0.009
Sodium nitrate	0.023	0.029	0.002	0.005	0.029	0.038	0.002	0.008	0.012	0.005	0.005	0.003	0.028	0.006	0.001	0.005
Magnesium nitrate	0.866	0.080	0.024	0.001	0.845	0.364	0.062	0.001	0.786	0.143	0.008	0.001	0.324	0.172	0.025	0.001
Ammonium nitrate	0.642	0.200	0.015	0.670	0.834	0.260	0.020	0.871	0.328	0.102	0.052	0.343	0.784	0.246	0.015	0.826

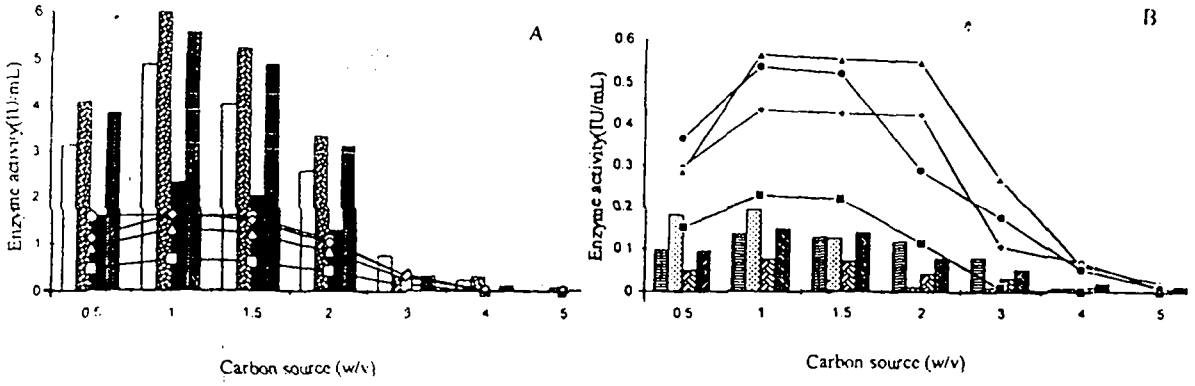

Fig. 1. Effect of concentration of Avicel on enzymes production.
A (Endo and Exo glucanases) B (ß-glucosidase and Xylanase)

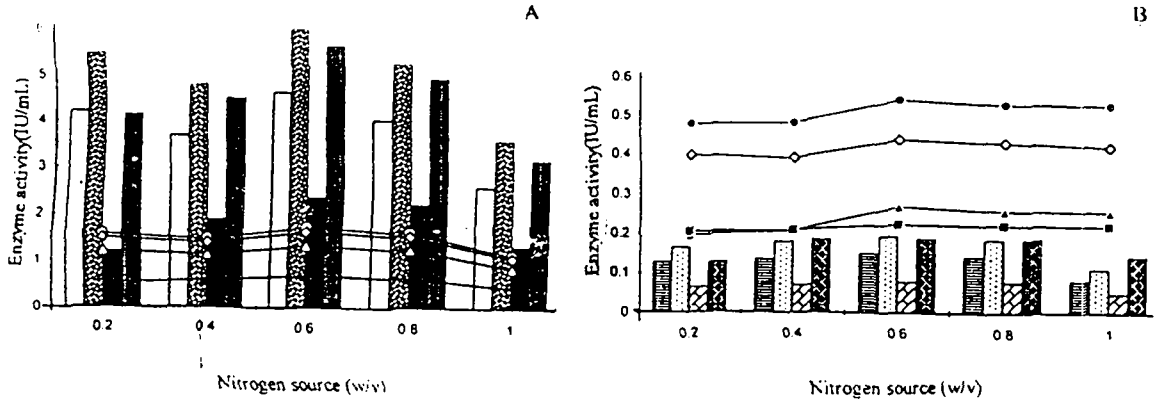

Fig. 2. Effect of concentration of Yeast extract on the accumulation of A (Endo and Exo glucanases) B (ß-glucosidase and Xylanase)

Endo glucanase
T. viride AVCM 4
T. viride SPCV 11
T. harzianum AVCM 7
T. harzianum SPCV 127

ß- glucosidase
T. viride AVCM 4
T. viride SPCV 11
T. harzianum AVCM 7
T. harzianum SPCV 127

Exo glucanase
T. viride AVCM 4
T. viride SPCV 11
T. harzianum AVCM 7
T. harzianum SPCV 127

Xylanase
T. viride AVCM 4
T. viride SPCV 11
T. harzianum AVCM 7
T. harzianum SPCV 127

initial pH of the medium maintained between 5 and 6 for *Chaetomium thermophille*. The present study also agrees with the results of Ganju *et al.* (1990).

Effect of pH and temperature on cellulase and xylanase activity

The endoglucanase exoglucanase, *ß. glucosidase* and xylanase activity of both the fungi were measured over a pH range of 4.0 to 5.6 and at temperature 25°C to 70°C (Figs. 4 and 5).

The endoglucanase activity attained its maximum at 4.8 and for exoglucanase activity it was 4.8. The β-glucosidase activity was optimum at pH 5.2 then dropped. The optimal temperature for

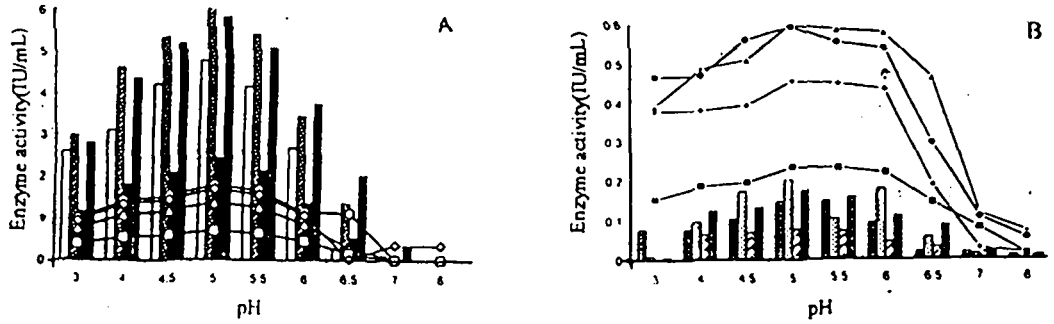

Fig. 3. Effect of pH on the production of A (Endo and Exo glucanases) B (β-glucosidase and Xylanase)

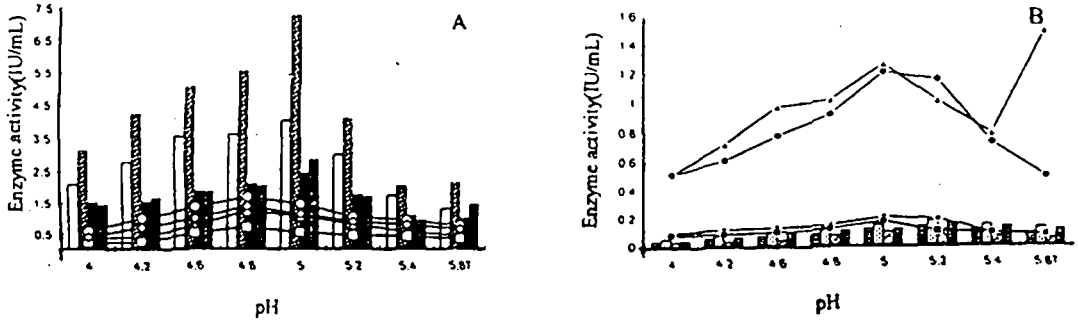

Fig. 4. Effect of pH on the enzyme activity of A (Endo and Exo glucanases) B (β-glucosidase and Xylanase)

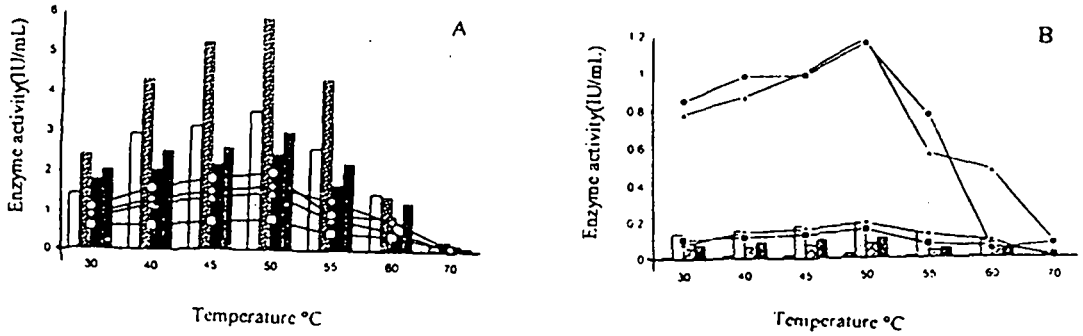

Fig. 5. Effect of temperature on the enzyme activity of A (Endo and Exo glucanases) B (β-glucosidase and Xylanase)

Endo glucanase
- *T. viride* AVCM 4
- *T. viride* SPCV 11
- *T. harzianum* AVCM 7
- *T. harzianum* SPCV 127

β- glucosidase
- *T. viride* AVCM 4
- *T. viride* SPCV 11
- *T. harzianum* AVCM 7
- *T. harzianum* SPCV 127

Exo glucanase
- *T. viride* AVCM 4
- *T. viride* SPCV 11
- *T. harzianum* AVCM 7
- *T. harzianum* SPCV 127

Xylanase
- *T. viride* AVCM 4
- *T. viride* SPCV 11
- *T. harzianum* AVCM 7
- *T. harzianum* SPCV 127

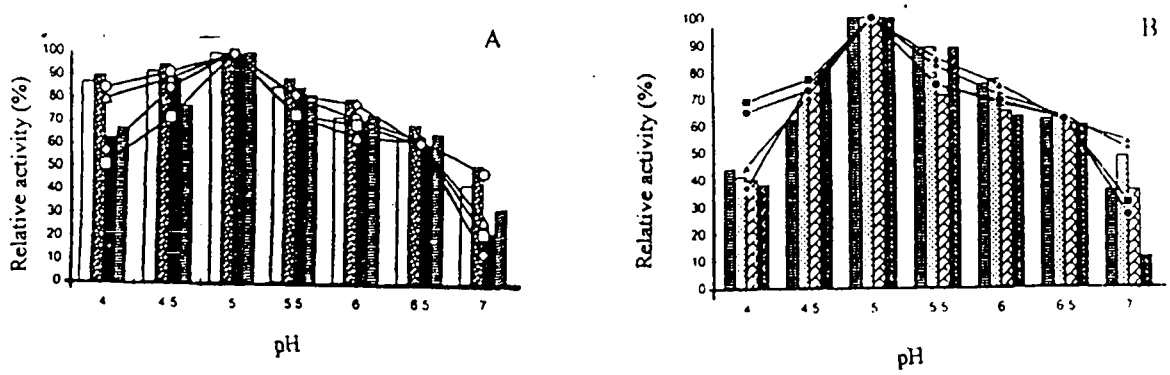

Fig. 6. Effect of pH on the enzyme stability of A (Endo and Exo glucanases) B (β-glucosidase and Xylanase)

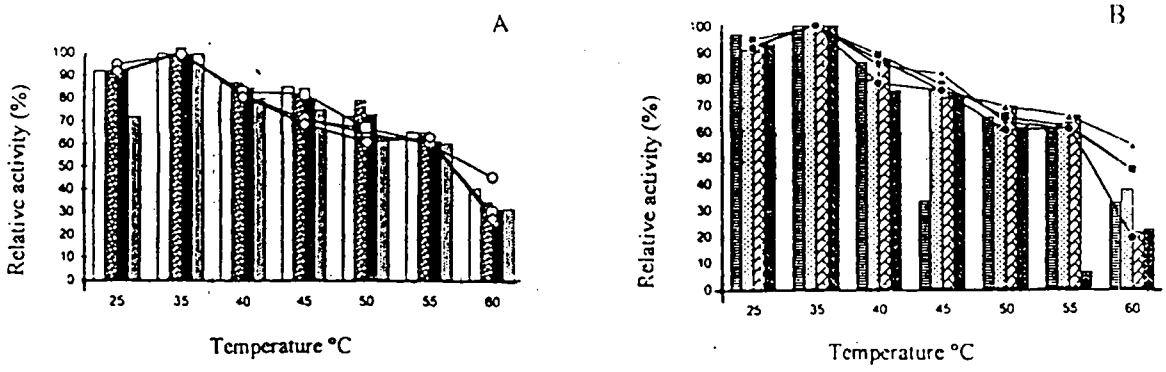

Fig. 7. Effect of temperature on the enzyme stability of A (Endo and Exo glucanases) B (β-glucosidase and Xylanase)

Endo glucanase
- *T. viride* AVCM 4
- *T. viride* SPCV 11
- *T. harzianum* AVCM 7
- *T. harzianum* SPCV 127

β- glucosidase
- *T. viride* AVCM 4
- *T. viride* SPCV 11
- *T. harzianum* AVCM 7
- *T. harzianum* SPCV 127

Exo glucanase
- *T. viride* AVCM 4
- *T. viride* SPCV 11
- *T. harzianum* AVCM 7
- *T. harzianum* SPCV 127

Xylanase
- *T. viride* AVCM 4
- *T. viride* SPCV 11
- *T. harzianum* AVCM 7
- *T. harzianum* SPCV 127

maximum cellulase activity in both the Spp. was at 45°C-50°C. According to Saddler *et al.*, (1990) cellulase of *T. reesei* C 30 and *T. harzianum* E 58 showed stable activity at pH 4.8 and at 50°C. The present study lies parallel with the report of Saddler *et al.* (1990).

The optimum pH and temperature of xylanase activity of these two fungi were 5.0 and 5.5 and at 50°C. Dubey and Johri (1987) pointed out the optimum pH and temperature for fungal xylanase activity were 4.5 to 5.0 and 45°C to 50°C. The present study shows some differences with that of Dubey and Johri (1987).

Effect of pH and temperature on cellulase and xylanase stability

Cellulase of both the fungi pronounced maximum stability up to pH 6.5 and at 50°C with more than 70°C activity (Fig. 6 and 7). Beyond this optimum rapid loss of activity was noticed. These results agrees with Ganju *et al.*, (1990).

Xylanase of *T. viride* and *T. harzianum* had optimum stability over a pH range of 4.0 – 7.0 at 50°C with more than 60% of xylanase activity. This manifests the results of Dubey and Johri (1987).

CONCLUSION

From the above results in can be concluded that the thermotolerant fungi *T. viride* and *T. harzianum* produced thermostable cellulase and xylanase using avicel under optimized condition.

There is a scope for improvement of the enzymes for many commercial applications with respect to its activity and cost of production using cheaper carbon and nitrogen sources.

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UTILIZATION AND OPTIMIZATION OF RENEWABLE RESOURCES FOR THE STABLE PRODUCTION OF CELLULOSE DEGRADING FUNGAL ENZYMES

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Key words : Cellulase, Submerged fermentation, Trichoderma harzianum, Trichoderma viride, *Xylanase.*

Abstract–Studies on fungal cellulase production via solid-state fermentation revealed that maximum enzyme production a mixture of wheat bran + rice straw 1:1 at 55% moisture content was observed on 4th day of incubation. 0.4% yeast extract, pH 5.0 were found to be optimum for high enzyme production. Using water as solvent maximum enzyme recovery was gained. The optimal pH and temperature for enzyme activity were 4.6 - 5.0 and 50 - 55°C. The crude enzyme was stable at pH 4.0-7.0 and at 40 - 60°C with more than 50% of activity when compared with control. Cellulase and xylanase synthesized in solid fermentation was little more thermostable than that from liquid culture.

INTRODUCTION

In plants cellulose usually found in association with heme cellulose and lignin as a component of lignocellulose. The enzymatic hydrolysis of cellulose takes place under the action of cellulase complex containing endoglucanase [EC 3.2, 1.4], exoglucanase [EC3.2.1.91] and glucosidase [EC 3.2.1.21]. Xylanases are responsible for hemi cellulose degradation. These enzymes comprise together a system to convert cellulose to glucose.

Most of the commercial cellulases from *Trichoderma* spp. are produced by submerged fermentation (SmF) (Tolan and foody, 1999). Solid-state fermentation (SSF) is advantageous over SmF to obtain concentrated metabolites and subsequent purification procedures are economical. Agro industrial residues are generally considered to be the best substrates for SSF process. Production of cellulase and simple sugars on agricultural residues by cellulolytic fungi have received considerable interest in recent times. SSF is successfully used for large scale production of fungal metabolites and bioconversion of plant, animal wastes into useful products. (Aido and Henry 1982). In this paper we report the establishment of cultural conditions for SSF optimal production cellulose hydrolyzing enzyme.

MATERIALS AND METHODS

To generate cellulase and xylanase enzymes by solid-state fermentation, 5 gm of each substrate was taken in a 250 ml conical flask at 60% water holding capacity. After sterilization 3ml of homogenized 4-day-old fungal culture was grown in potato dextrose broth, inoculated over the substrate and incubated at room temperature for 7 day. The moisture content of the substrate was monitored periodically and sterilized water spray was given to avoid drying and to maintain 60% moisture on the substrate. Samples were with drawn at every 24 hrs interval. Crude enzyme was extracted with 20ml of sterile water and supernatant was used for cellulase activity measurements. (Ray *et al.*, 1993). Xylanase assay was performed by the method of Mandels *et al.* (1976). Soluble protein content was analyzed according to Lowry *et al.* (1951). Biomass in SSF was determined by estimating glucosidase contents. (Aido and Henry 1982).

RESULTS AND DISCUSSION

Studies on various process parameters revealed that cultural conditions play an important role in cellulase production.

Screening of agricultural residues for cellulase

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and xylanase production in solid-state fermentation showed that wheat bran amended with rice straw (1:1) is the suitable substrate. (Table 1) maximum soluble protein content 6.195, 36.829, 4.702, 28.859 (mg/ml). Biomass 6.774, 7.589, 6.540, 7.618 (g/l) was noticed on 4 the day of incubation. Increased Production of cellulase in wheat bran owing to the fact that it contains ample nutrients and able or remain loose in moist conditions, thus providing a large surface area Smith *et al.* (1996), Which can be auspiciously compared with the reports of the present study.

Nitrogen in media is an important factor that affects enzyme production. Among the different nitrogen sources experimented, yeast extract was found to be the best nitrogen source. Optimum concentrations of yeast extract was determined as 0.4% for maximum enzyme activity of *T. viride* SPCV 11 40.682, 11.668, 0.701, 6.256 (IU/ml) in opposition to control 12.630, 3.623, 0.218, 1.942 (IU/ml) and for *T. harzianum* SPCV 127, 32.623, 9.442, 0.460, 5.062 (IU/ml) compared to its control 5.394, 1.547, 0.093, 0.829 (IU/ml) respectively for cellulase and xylanase activities.

Influence of moisture content on cellulase production was evaluated by varying the levels of moisture (%) on the substrate from 20-80%. Enzyme accumulation was found to be high in 55% moisture content of fourth day of incubation in the four cultures. *T. viride* SPCV11 produced 63.142, 17.247, 0.942, 12.348 (IU/ml) and the control 10.530, 4.030, 0.242, 2.161 (IU/ml) of *T. harzianum* SPCV127 it was 50.634, 13.978, 0.619, 9.992 (IU/ml) when compared with the control 8.372, 2.290, 0.125, 1.637 (IU/ml) for endoglucanase, exoglucanase, β-glucosidase and xylanase respectively. In the present study, high moistly content changes the geometry of the solid particles, promotes stickiness, reduces gas exchange and decreases diffusion ensuing low enzyme production. Zadrazi and Burnet (1981) reported that low moisture content decreased solubility of nutrients, low degree of swelling and high water tension. Our study reveals that moisture content has some effects on cellulase production.

pH of the medium played an imperative role in cellulase production. In the present study among the pH levels both the fungi produced significant level of cellulolytic enzyme at pH 5.0 (Table 2). The germane temperature was found to be 35°C for optimum secretion of *T. viride* SPCV 11.83.480, 22.838, 1.246, 16.325 (IU/ml) and the content had 13.922, 5.329, 0.320, 2.857 (IU/ml) by for endoglucanase, exoglucanase, β-glucosidase and xylanase activities correspondingly. *T harzianum* SPCV 127 had shown 66.943, 18.480, 0.878, and 13.120 (IU/ml) with respect to its control 11.068, 3.028, 0.165, 2.640 (IU/ml) Possible reasons attributed for lesser production of cellulases at unfavourable pH and temperatures were reduced metabolism and growth rate of the fungus. Wang *et al. (1994)* accounted that optimum cellulase and xylanase production by *Aspergillus sp.* G393 was at pH 5.0 and at 35°C. the reports of the present study parallels with that of *Aspergillus* sp. G 393.

From the fermented substrate different solvents like 2% $CaCl_2$, 10 mM phosphate buffer, 85% ethanol and distilled water, sterile water were used to extort the enzyme on 4 day of incubation. Crude enzyme extracted with water displayed maximum activity of 68.079, 18.625, 1.016, and 13.313 (IU/ml) for *T. viride* SPCV 11 and *T. harzianum* SPCV127 54.593, 15.071, 0.667, 10.773 (IU/ml). For the control *T. viride* AVCM411. 354, 4.346, 0.261, 2.330 (IU/ml) and *T. harzianum* AVCM7 produced 9.026, 2.469, 0.135, 1.765 (IU/ml).

Among the different ratio of water to substrate viz., 1:5, 1:10, 1:15 and 1:20 the maximum extraction was arrived at 1:10 level on 4 day from all the specified fungi. The corresponding enzyme activities were 69.983, 19.966, 1.089, 14.012 (IU/ml) for *T. viride* Spcv11 and 11.572, 4.659, 0.280, 2.498 (IU/ml) of the control. Regarding *T. harzianum* SPCV 127 enzyme activities were 58.526, 16.157, 0.715, 11.509 (IU/ml) and corresponding control cultures 9.676, 2.647, 0.144, 1.892 (IU/ml) endoglucanase, exoglucanase, β. glucosidase and xylanase activities similarly. Maximum cellulases activity acquired in 1:10 ratio extract of mouldy bran with distilled water by Shamala and Sreekantiah (1985). Hence the optimum enzyme recovery recorded by water in the present study validate the above results.

Cellulase and xylanase activity of both the fungi was determined by over a pH range of 4.0 to 5.6 and at temperature 25°C to 70°C (Fig. 1 and 2). pH optima for maximum endoglucanase activity was 4.8 and for exoglucanase activity it was 5.0. The β/glucosidase activity was optimum at pH 5.2. The optimal temperature for maximum cellulase activity in both the Spp. was at 45°C.-50°C. The optimum pH and temperature of xylanase activity of these two fungi were 5.0 and 5.5 at 50°C. A good number of fungal cellulases and xylanases had shown their

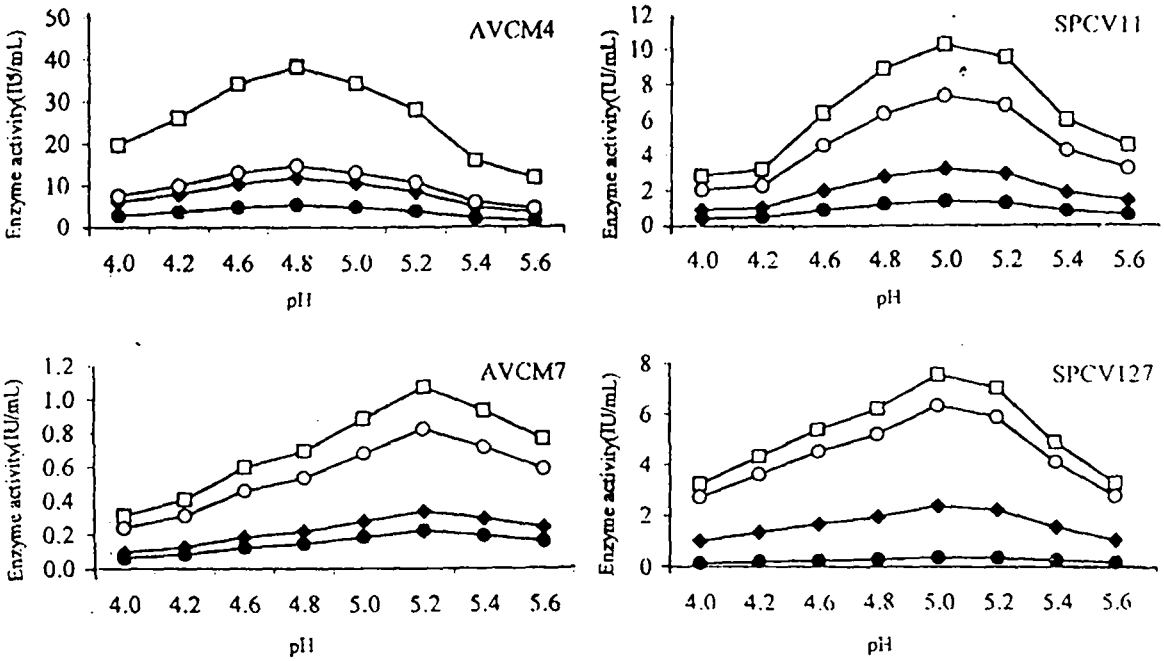

Fig. 1. Effect of pH on cellulase and xylanase activity

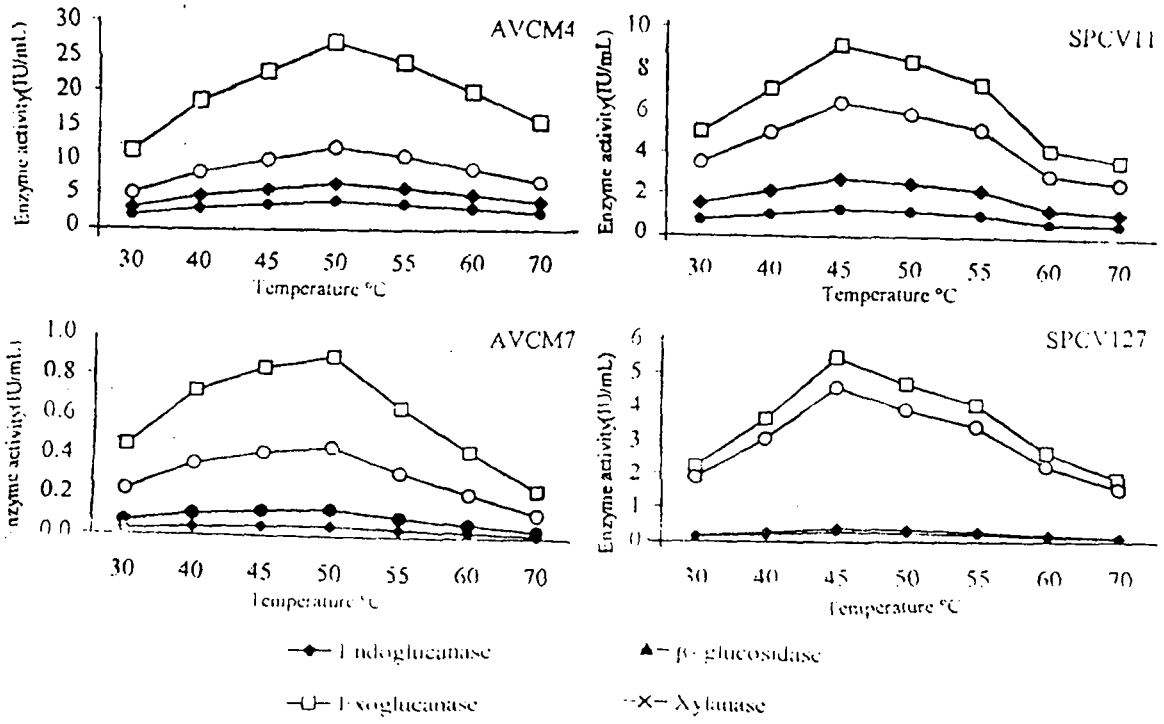

Fig. 2. Effect of temperature on cellulase and xylanase activity

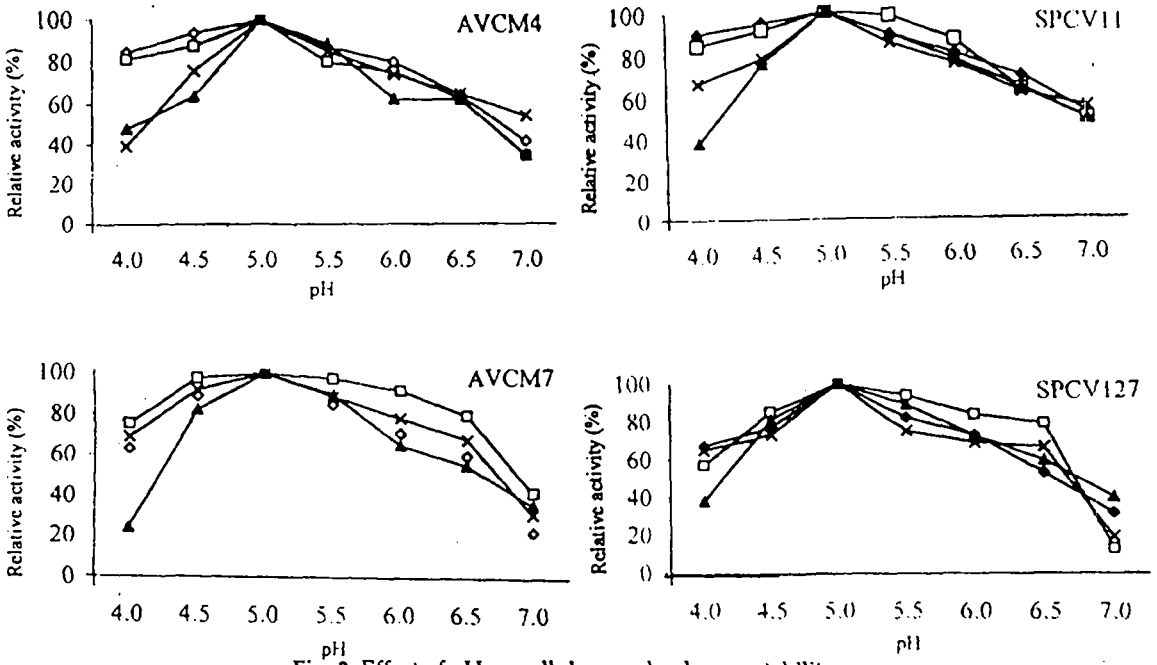

Fig. 3. Effect of pH on cellulase and xylanase stability

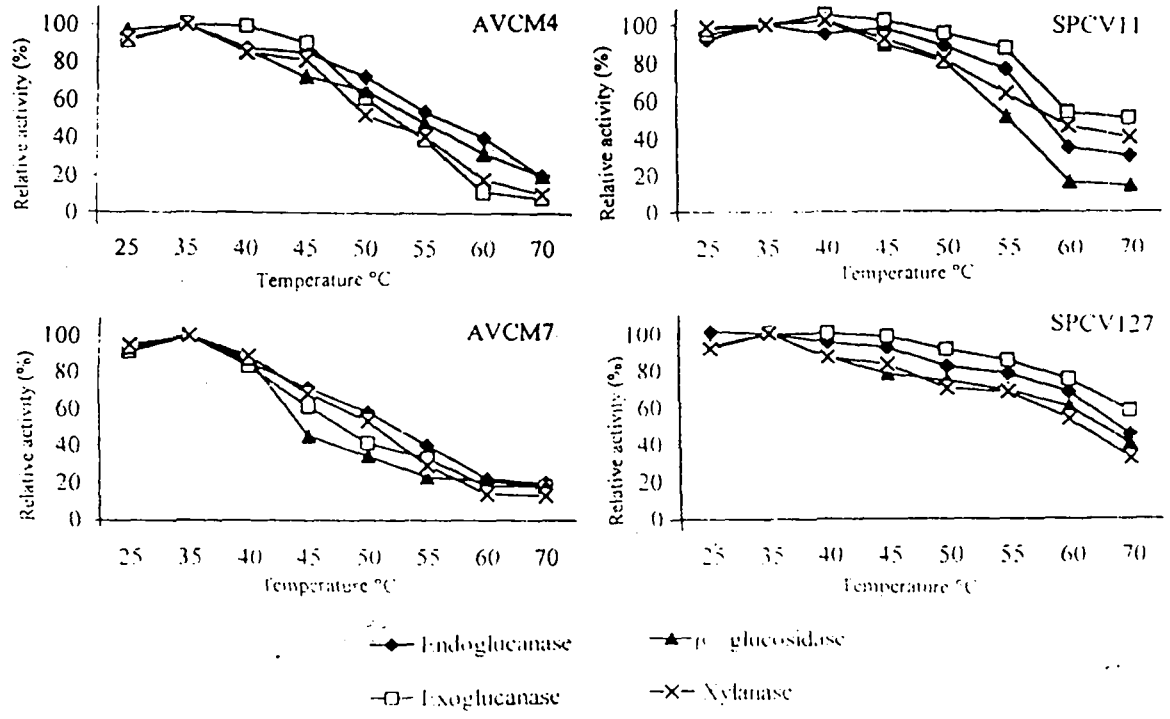

Fig. 4. Effect of temperature on cellulase and xylanase stability

maximum activity at pH 5.0 and at 50°C.

To study the effect of pH cellulase and xylanase stability the enzyme solutions with different buffers were incubated at room temperature for 30 min and enzyme assays were performed in standard assay conditions. As shown in (Figs. 3 and 4) Cellulase and Xylanase of *T. viride* and *T. harzianum* were stable a pH range 4.0-6.5 with more than 60% of enzyme activity. Beyond this optimum rapid loss of activity was noticed. Thermostability cellulase and xylanase were determined by incubating the enzyme source at various temperatures form 25-90°C. Enzyme activity measurements convey that 60% of enzyme activity were maintained at 55°C and swiftly declined there after.

CONCLUSION

In the present study cellulolytic activities encountered in the culture filtrate of *T. viride* and *T. harzianum* Showed the presence of all individual cellulolytic components viz., endoglucanase exo glucanase β-glucosidase and xylanase in the medium suggesting the presence of completer cellulolytic system. The stable and cost effective cellulase produced by these fungi would facilitate a number of commercial applications.

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