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THE QUANTIFICATION OF FUNGAL BIOMASS AND CELLULASE ACTIVITY OF *Thielavia terrestris*.

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ABSTRACT

A range of thermophilic fungi were screened for their ability to produce an effective extracellular cellulase enzyme system. Thielavia terrestris 255B was shown to be a promising organism as it produced an efficient, thermostable cellulolytic enzyme complex. The quantification of fungal growth was also studied using different biomass estimating procedures, such as the dry weight, biuret, Kjeldahl, ergosterol and ELISA methods. The ergosterol and Kjeldahl procedures gave the most reproducible values for biomass estimation. Although the ELISA method resulted in values fairly representative of cell growth it still requires further modification before it can be used routinely. It was apparent that fungal biomass was not directly related to cellulase production and that the latter peaked after fungal growth was completed.

Although crude thermophilic enzymes can operate effectively at elevated temperatures, their activities were found to be comparable to commercial mesophilic cellulases. However, as thermophilic cellulases are much more thermostable, their half-lives are considerably longer and the enzymes can be used for prolonged reaction periods. A comparison of standard assay methods and the cellulase complexes overall hydrolytic potential was made. This showed that the cellulase assays were not representative of the enzyme mixtures ability to hydrolyze various cellulosic substrates.

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TABLE OF CONTENTS page

LIST OF FIGURES

LIST OF TABLES

I. INTRODUCTION

Background

With the present rate of population growth and the decreasing availability of coal, gas and oil, it is probable that the demand for traditional energy resources will far exceed the supply by the end of the next century. Bioenergy technologies are examples of what Lovins (1977) calls a "soft energy path". Biomass in the form of agricultural and forest wastes accumulates every year in large quantities, resulting in the deterioration of the environment and the loss of a potentially valuable resource (Bisara and Ghose 1981, Coughlan 1985).

Biomass in the form of cellulose, hemicellulose and lignin provides a means of collecting and storing solar energy and hence represents an important energy and material resource. The high moisture content of biomass makes it less suitable for most chemical treatments and more suitable for biological processing. In nature it takes over fifty years to decompose a dead tree. A variety of strategies have therefore been explored to develop processes which can bring about the degradation and conversion of biomass. They range from direct thermal methods such as pyrolysis, to chemical methods such as acid hydrolysis and biological methods such as enzymatic hydrolysis.
There is considerable debate regarding the relative economic advantages of the enzyme- versus acid- hydrolysis approach. Dilute acid hydrolysis generally results in a maximum recovery of 62 % of the original cellulose as glucose, with excessive by-product formation including the production of microbial growth

inhibitors (Bungay 1981) detracting from the overall process. Concentrated acid processes, while resulting in greater conversion efficiencies, involve extremely high capital cost equipment, as well as considerable maintenance, acid recovery and pollution abatement costs. In addition acid hydrolysis does not always result in the sole production of the glucose monomer. It often further degrades this valuable product to decomposition products such as hydroxymethylfurfural. In contrast, enzymatic hydrolysis is considerably more efficient (95 % conversion), yielding simple sugars which can be used as substrates for fermentation to a variety of products such as ethanol, butanediol, acetone and citric acid.

Hydrolytic enzymes have already been applied in other fields. Bacterial and fungal amylases and proteases are among the most commercialized enzymes and have found applications in the food and laundry detergent industries (Fogarty and Kelly 1979, Tilburg 1984). A significant portion of commercial enzymes produced world-wide are used in the detergent (35 %) and starch based (30 %) industries (Aunstrup 1980). Over the years these enzymes have been changed to become more thermostable, to enhance their longevity and the rate of reaction. One of the objectives of the work reported in this thesis is to determine whether thermophilic cellulases have desirable characteristics which make them more commercially attractive than the many mesophilic cellulases which are currently marketed.

The wood decay fungi have proven to be among the most cellulolytic organisms, with relatively few cellulolytic bacteria so far identified (Saddler 1986). The fungi are generally

50-1000 times more hydrolytic than the most active cellulolytic bacteria (Saddler et al 1984). This higher activity is mostly due to the greater amount of extracellular protein secreted by the fungi. In fact, the cellulolytic fungi are probably among the greatest producers of extracellular protein identified to date. Most of the cellulolytic bacteria secrete an incomplete cellulase complex which can only hydrolyze highly modified substrates such as filter paper or carboxymethyl cellulose. When the overall hydrolytic activity of the fungal and bacterial systems were compared it was found that the majority of the complexes were deficient in one or more of the endoglucanase, exoglucanase or B-glucosidase components (Saddler 1986).

Examples of the type of materials utilised as solid substrates by fungi for growth include plant tissue such as wood and pulp and paper products (Matcham et al 1984). The ability of fungi to secrete large amounts of cellulases combined with their filamentous mode of growth indicate that the fungi are better suited than the bacteria for efficient cellulose degradation. Despite the considerable amount of research devoted to cellulolytic fungi some areas such as, the development of representative enzymatic assay methods for the quantification of both the enzyme specific activities and the hydrolytic potential of the enzyme complex, remain to be fully resolved. A similar problem exists with other commercial enzymes. Although amylases are better studied, the exact determination of individual enzyme activities is still a problem (Taufel 1988). As cellulases tend to occur as complexes containing various enzymes, it is difficult to standarize assays for specific enzyme activities. It is also apparent that the specific activities are not always representative of

the hydrolytic potential of the enzyme complex. The cellulase assays for specific activity are normally conducted over a short time period (5-60 min) and are often not representative of a long term hydrolysis where enzymes may lose their activities or end product inhibition may influence the rate of the reaction (Saddler 1986).

As cellulolytic fungi are usually grown on an insoluble substrate a further problem is how to differentiate the increase in the weight of mycelia and the decrease in the amount of initial substrate used for growth. This information is required if we wish to make a correlation between fungal growth and enzyme production. It is known that an increase in mycelial growth on soluble substrates does not necessarly parallel the increase in enzyme production (Ghose et al 1975). When quantifying the amount of extracellular enzymes produced by bacteria, enzyme activity is often correlated with microbial biomass by monitoring extracellular amounts of protein and determining the dry weight of the culture (Schmidel and Fernandes 1976, Moreira et al 1981a, Canevascini et al 1983, Bratbak and Dundas 1984, Shaker et al 1984 and 1985). This is a much more complicated issue when filamentous organisms grown on solid substrates are assayed. A review of the literature indicates the absence of papers quantifying cellulase activity on the basis of the corresponding fungal biomass. One of the goals of this study was to determine the relationship between fungal growth and cellulase production.

In the work reported in this thesis we used a commercial cellulose (Avicel) and steam treated aspenwood as the lignocellulosic substrates for studying the growth and cellulase production of various thermophilic fungi. The major objectives of

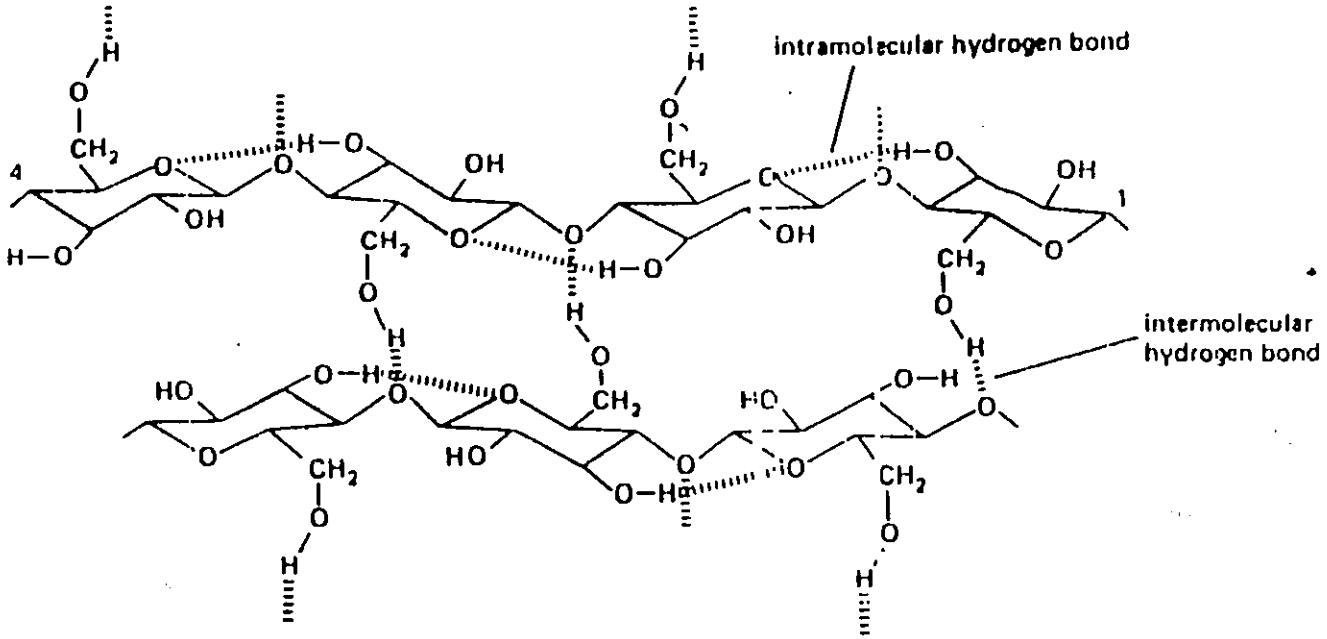
this work were to identify thermophilic fungi with high cellulase activities, correlate enzyme production with changes in fungal growth and determine if the cellulase assays were representative of the hydrolytic potential of the enzyme complex.

Lignocellulose structure

Cellulose is the most plentiful organic compound on earth. Approximately 1.8 x 10^{12} tons of cellulose are biosynthetized every year (Henrissat 1985). With its skeletal function it is widely distributed in the plant kingdom. Cellulose constitutes the bulk of the cell wall and varies in its purity according to its source. For example the seed hair of the cotton plant contains 92-96 % cellulose, the flax fibre 80 %, the jute fibre 65-75 % and the wood cell wall contains 40-50 %. Cellulose is also found in the cell wall of algae, mosses and several species of fungi (Wood 1970).

Cellulose is a linear polymer of up to 14, 000 anhydroglucose residues in a chain configuration, held together by B-1,4 linkages. Each residue is rotated 180 degree about the main axis with respect to its neighbouring residues (fig. 1). Thus, the basic recurring unit is cellobiose. Cellulose chains, orientated in parallel and staggered with respect to their partners, associate to form insoluble fibrils in which the chains are held together by hydrogen bonding. It has been deduced that the hydrogen-bonding network consists of inter- and intramolecular bonds between successive and adjacent dextrose (i.e. -D(+) glucose) residues (Gardner and Blackwell 1974,

FIGURE 1. STRUCTURE OF CELLULOSE



Cellulose chains showing the β-1, 4-linked residues rotated through 180 degrees with respect to their neighbours in the chain. Intermolecular hydrogen bonds tightly crosslink adiacent chains within a microfibril. Intramolecular hydrogen bonds stabilize each chain (Coughlan 1985).

Winterburn 1974, Rees et al 1982). The intramolecular bonds help to maintain the rigidity of the cellulose chain. Within cellulose fibres there are areas of complete order, i.e. crystalline areas, and also less well-ordered or amorphous regions. The degree of crystallinity within fibres varies with the source of the cellulose and the treatment to which it has been subjected. In the native state, cellulose fibres are associated with other polysaccharides, viz. hemicellulose and pectin and also with lignin (Rees 1977, Alberts et al 1983). The crystallinity of the native material and its association with lignin are the major factors limiting the cellulase action during enzymatic hydrolysis of cellulose.

As well as substrate complexity the cellulase enzyme mixture itself is equally as complex. Although cellulase enzymes have high specificity and activity, an enzymatic hydrolysis process remains very expensive due to the relatively slow reaction rate. As the rate is very dependent on the reaction temperature (within the limits of enzyme stability), the application of thermophilic cellulolytic microbes can potentially reduce the cost of an enzyme hydrolysis process and make this process more efficient. This progressive development has occurred with the thermophilic amylases which have replaced their mesophilic counterparts at most commercial plants (Aunstrup 1980, Ruttloff 1988). Almost all the past work reported in the literature on cellulase production has been done with mesophilic organisms. One of the objectives of the work reported in this thesis was to study the relative activities of thermophilic cellulase systems and compare them with one mesophilic strain.

Cellulase production

The recent history of cellulase production owes a lot to Mary Mandels and Elwyn Reese who isolated fungal strains from cotton clothing during the Korean war. They subsequently mutated these strains to obtain various highly hydrolytic strains of Trichoderma reesei (Mandels and Reese 1964). Virtually all of the fungi which are being assessed for commercial applications in countries such as Finland, France, Japan, etc. owe their origins to the mutants originally isolated by the Natick group. T.reesei mutants are among the most prolific producers of cellulase enzymes. Other fungi such as T.koningii, Fusarium solani, Penicillium funiculosum, Sporotrichum pulverulentum are also being considered for commercial exploitation, while Thermomonospora spp, Talaromyces emersonii and others show promise in this regard (Coughlan 1985).

Mesophilic cellulases have been produced by cultivation of the appropriate organisms under various conditions such as, shake flasks, batch reactors (Moreira et al 1981b), batch reactors with temperature and pH profiling (Tangnu et al 1981), fed-batch fermentations (Allen and Mortensen 1981, Hendy et al 1984), continous cultivation on glucose (Brown and Zainudeen 1977), two-stage (growth at 32^{o}C, pH 4.5 in first stage and enzyme production at 28^{o}C, pH 3.5 in second stage) continous production in a aqueous two-phase system (Persson et al 1984), by immobilized T.reesei cells (Kumakura et al 1984) and by the solid-state Koji process in Japan. In order to increase both the rate and the yield of cellulose hydrolysis a variety of

research approches have been taken. The regulation of cellulase enzymes is one of them.

Cellulase regulation

In virtually all the microorganisms examined to date, the synthesis of cellulases is "induced" by the presence of cellulose and repressed by the presence of dextrose or other readily metabolized sugars in the growth medium. Evidence supporting this conclusion has been obtained for bacteria, e.g. Acetivibrio cellulolyticus (Saddler et al 1980) or Cellulomonas uda (Stoppok et al 1982), actimomycetes, e.g. Thermomonospora (Moreira et al 1981a, Fennington et al 1984) and fungi including Penicillium janthinellum (Rapp et al 1982), Sporotrichum pulverulentum (Eriksson and Hamp 1978) and Trichoderma reesei (Sternberg 1976). However, the nature of the actual "inducer" has yet to be determined. The most generally accepted view of the "induction" process, but one that remains to be tested experimentally, is that the organisms in question produces low basal or constitutive levels of cellulase. This results in some breakdown of cellulose in the medium and soluble products including cellobiose, are taken into the cell where they are converted to the actual "inducer" of enzyme synthesis (Gong and Tsao 1979). Cellobiose is known to "induce" cellulase synthesis by T.reesei (Mandels and Reese 1960) and by S.pulverulentum (Eriksson and Hamp 1978). However, it is effective only at high concentrations (10mg/L). Lactose is also an "inducer" in some species, including T.reesei, but again only at high concentrations (Gong et al 1979). In contrast, sophorose (2-O-B-glucopyranosyl-D-glucose)

used at low concentrations (1 mg/L) was reported as being a powerful inducer of endo- and exocellulase synthesis by T.reesei (Sternberg and Mandels 1979) and by S.pulverulentum (Eriksson and Hamp 1978). A thorough examination of the literature indicates that very little work in this area has been done with the thermophilic fungi (Coughlan 1985).

Enzyme yield and productivity

Considerable enhancement of cellulase productivity, especially by Trichoderma sp., has been achieved in the last twenty years by optimization of the growth media and by various innovations in process engineering. Further improvements have been achieved by isolation of new species or the production of mutants. Some of the objectives of the various mutation programmes carried out by other researchers were to improve the enzyme productivity and/or intrinsic properties of the enzymes. This was done by either, increasing the rate and extent of enzyme synthesis and secretion, development of catabolite resistant or constitutive mutants, isolation of mutants producing enzymes with greater specific activity, or increased operational stability and resistance to product inhibition. Considerable success has been achieved in enhancing enzyme productivity especially with T.reesei mutants such as the hypercellulolytic mutants T.reesei Rut-C30 and CL-847. Over the last 15 years cellulase production has been increased by mutation from productivity values of 1 FPU/mL to approximately 20 FPU/mL (Montenecourt and Eveleigh 1978, Warzywoda et al 1983, Watson and Nelligan 1983, Durand et al 1988).

Catabolite repression has also been greatly overcome in the VTT

series of T.reesei mutants (particularly strain VTT-D-79125) where they produce considerable quantities of cellulase using mutants grown on dextrose (Bailey and Oksanen 1984). However this organism still requires cellulose to stimulate high levels of cellulase. It appears that no one has yet isolated a strain which produces cellulase enzymes with a higher specific activity. Although high specific activity of each component is important, the synergistic action of all enzymes in the cellulase complex is needed for effective cellulose hydrolysis.

Mechanism of cellulose hydrolysis

The insoluble and polymeric nature of cellulose determines the nature of the initial enzymatic attack of the substrate. The organism must either produce extracellular enzymes which diffuse to and break the cellulose chains or the microorganism must be in direct contact with the cellulose fibrils and its enzyme present on the outer surface of the cell wall. To be effective a cellulase system must be able to gain access to the cellulose fibrils. Oligosaccharides small enough to be transported into the cell are the product of hydrolysis. These oligosaccharides or monosaccharides may also be involved in the regulation and synthesis of the cellulase system. Cellulases tend to function as a system or complex of enzymes of which the precise activities and subcellular location of the individual components varies with the identity, nutritional state and growth phase of the organism. A cellulase system capable of hydrolysing native cellulose to glucose in generally thought to require the following enzymes (fig. 2): Endo-1,4-B-D-glucanase (1,4-B-D-glucan 4-gluconohydrolase, E.C. 3.2.14). This group of enzymes causes

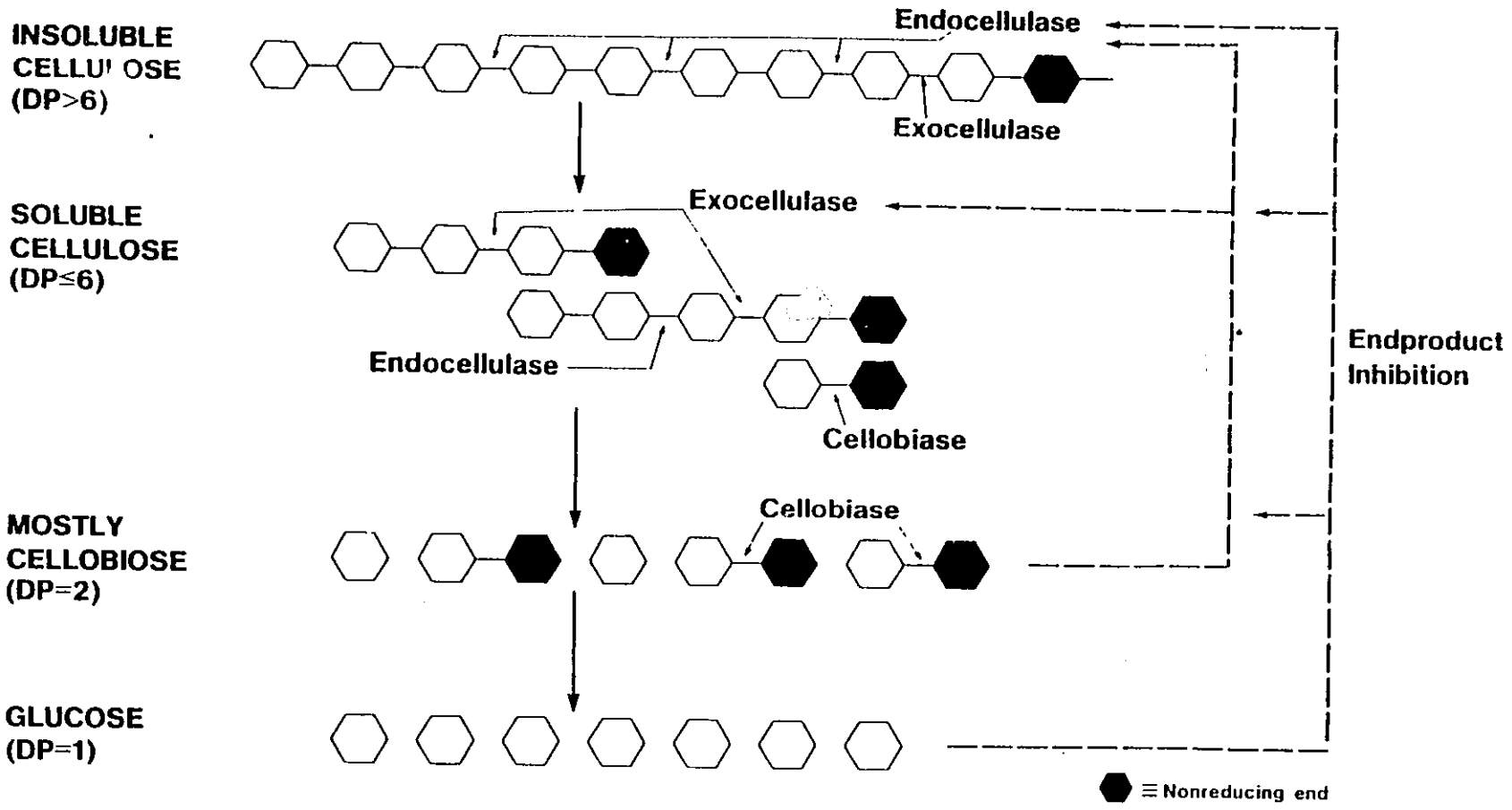
internal hydrolytic cleavage of the cellulose chain. There may be several components varing in degree of randomness, one of which may be the enzyme which first acts on crystalline or highly ordered cellulose.

Exo-1,4-B-D-glucanase. Usually of two types with both acting from the non-reducing ends of the major chain or shorter chains produced by the action of endo-glucanase. Exo-cellulases may be exo-cellobiohydrolase, removing cellobiose units, or exo-1,4-B-D-glucosidase, removing glucose units.

Cellobiases or B-D-glucosidases (E.C. 3.2.1.21). These enzymes convert cellobiose and other cellodextrins into glucose, depending on which activity is being assayed.

The original hyphothesis of how cellulose hydrolysis occurs was proposed by Mandels and Reese (1964). They suggested that two types of cellulolytic enzymes were involved in the hydrolysis of crystalline cellulose. The C1 enzyme initiates hydrolysis by rendering cellulose accessible to the Cx enzymes. This group consists of enzymes acting by exo- and endo-actions. This simple hypothesis has been modified by later results (Streamer et al 1975, Berghem et al 1975, Shoemaker and Brown 1978, Klyosov et al 1980). Although some authors have proposed that the first attack is carried out by an oxidative, nonhydrolytic "hydrogen bondase" or "swelling enzyme" (Eriksson et al 1974, Vaheri 1983, Griffin et al 1984), it is now generally believed that cellulose hydrolysis is initiated by the synergistic activity of various hydrolytic enzymes. Some authors believe that the initiating enzyme is an endo-B-glucanase while others consider it to be a cellobiohydrolase, which liberates cellobiose from the chain ends by an exomechanism. Another exoenzyme, exoglucohydrolase, has

FIGURE 2. SCHEMATIC REPRESENTATION OF THE SYNERGISTIC ENZYME HYDROLYSIS OF CELLULOSE.



also been described (Klyosov et al 1980), however its role in the hydrolysis of cellulose has not yet been clarified. B-glucosidase (Reese and Mandels 1971) and a second cellobiohydrolase (Fagerstam and Petterson 1980) have also been suggested as being important in the initial stages of hydrolysis.

It is apparent that there is no clear agreement as to whether the initiating enzyme in the hydrolysis is an exo- or an endo-type enzyme. However, it is commonly accepted that for an exhaustive hydrolysis of crystalline cellulose to glucose the synergistic interaction of the various endoglucanase, exoglucanase and B-glucosidase components is required (Ladisch et al 1983, McHale and Coughlan 1980, Wood 1968). This is described schematicaly in fig. 2. The separate enzyme components acting alone result in little or incomplete hydrolysis. The synergistic effect, where cooperative action is greater than the sum of the action of the separate enzyme components, is considered a characteristic of an efficient cellulolytic enzyme complex (Wood 1969, Enari and Niku-Paavola 1987). Although the individual activities of the various strains of thermophilic fungi can be very high, a better equilibrium of enzymes is more important than individually thermostable components. For example, if one of the enzyme components is temperature labile the hydrolytic activity of the cellulase complex is greatly reduced to the extent that it may be unable to hydrolyze any cellulosic substrates. This phenomenom will be described in the work reported in this thesis. The difficulty in obtaining a clear picture of the sequence of events which occur during the hydrolysis of crystalline cellulose is a result of the complex protein properties of the cellulolytic enzymes as well as the inherent difficulty of analyzing the various insoluble,

cellulose hydrolysis products. All cellulolytic enzymes share similar physiochemical properties and are thus difficult to purify. On the other hand, the hydrolysis of insoluble cellulose is usually analyzed only by the soluble hydrolysis products. Thus, insoluble reaction products are not always studied. A representative, reproducible assay method for quantitating the hydrolytic potential of various cellulase systems has yet to be developed.

Measurement of cellulase activity

In qualitative assays a number of methods have been suggested for the detection and measurement of an effective cellulase system (Mullings 1985). The qualitative assays are essentially methods for detecting cellulolytic activity in screening programmes or used for detecting gross differences in activity between organisms. Some of the common plate assay methods rely on the incorporation of solid cellulose in an opaque agar growth medium. Kaufman et al (1976) used finely ground cellulose in agar plates and detected zones of clearing by cellulolytic isolates. Cellulose in an overlay medium can also be used and gives more rapid results. Swelling of cellulose with phosphoric acid produces a more open structure and when incorporated into growth media, zones of different hydrolysis sizes can be seen (Tansey 1971a, Sternberg 1976). There have been several attempts to devise rapid, sensitive, quantitative assays using this overlay procedure (Wabneg et al 1980), however these methods are very subjective in interpretation.

Cellulase components from a number of organisms have been purified and separated using disc gels incorporated with dyed

cellulose (Beguin 1983, Bartley et al 1984). Well established methods such as thin layer chromatography (Stahl and Kaltenbach 1965) or high performance liquid chromatography (HPLC) can be used for the qualitative detection and characterization of the intermediates of the hydrolysis reaction.

The quantitative assays can be divided into three types. Alteration of some physical property of the cellulose substrate, reduction in substrate quantity, or accumulation of some product of the degradative process. In the latter category there are various methods which allow a distinction to be made between the various components of the cellulase complex.

Assays involving measurement of physical changes are mostly of historical interest because of their insensitivity. These include the "swelling factor" measured by alkali uptake of cotton and the reduction of tensile strength of thread (Wood 1975). Total loss of substrate can be measured by several chemical means. These assays are not very sensitive, do not relate to the actions of an individual enzyme and there is such a wide variety of substrates to choose from that comparison of results in the literature is difficult.

The measurement of products accumulating during hydrolysis has involved a bewildering range of substrates, assays and units. The types of cellulosic material which would probably be used as substrates on an industrial or pilot-scale process are totally unsuitable as substrates for any systematic mechanistic studies. The confusion over units complicates matters. The "filter paper units" of Mandels et al (1976) are often used but are dependent on dilutions of a culture suspension and an arbitrary amount of

glucose equivalents assayed by non-specific colorimetric methods. The carboxymethylcellulose (CMC) assay used for assaying endoglucanase activity can often be misleading as some enzyme preparations will not hydrolyse insoluble cellulose, but readily break down soluble derivatives, such as CMC, which requires only endo-1,4-B-D-glucanases (Mandels et al 1976).

For measurement of total cellulase activity, substrates vary in crystallinity, from highly crystalline cotton to non-crystalline, soluble forms of cellulose such as cellotetraose. Only a complete cellulase system will hydrolyse cotton. However when this substrate is used, the units of activity determined are usually low and assays have to be lengthy, usually taking hours or even days (Haggett et al 1982). Avicel and Solka floc are two milled microcrystalline substrates used as alternatives to filter paper and have short fibres of more uniform length. Walseth cellulose is cellulose swollen with phosphoric acid and thus has a more open structure for enzyme attack and units of activity are correspondingly higher. Shredded newspaper would be a good substrate for industrial fermentation, however it is unsuited as a standard substrate as it has a high content of short chain length oligomers as well as a relatively high hemicellulose content.

Hemicelluloses are less recalcitrant to enzyme attack than cellulose and crude cellulase preparations may often contain hemicellulase activity. The short oligomers and less crystalline structure will distort the picture of enzyme-substrate interaction. Even substrates such as Avicel and cellulose powder contain at least 10 % (w/w) non-glucose sugars, particularly arabinose, galactose and

xylose (Lynch et al 1981). Cellotetraose was first advocated by Reese (1969) as a substrate for distinguishing exo-glucanase activity. It is possible to separate enzyme components from the complex but we may endup removing a functional unit of importance to the complex. Therefore there is a problem of how to measure the enzymatic activity of each cellulase enzyme. Usually the hydrolytic potential of the complete cellulase complex is studied. The specific activity of a particular cellulase enzyme or group of enzymes is assayed using distinct substrates to quantitate activity.

Hydrolysis

The rate of saccharification of cellulose fibres apparently falls off after an initial high rate and it is argued that initial rates should not be measured (Bacon 1979) as they are not representative of the overall rate of the hydrolysis reaction. The products of saccharification are usually measured as a specific sugar or as total reducing sugars. Reducing sugars are the product of all cellulose degradation schemes, their rate of production reflecting overall cellulase activity. If an insoluble cellulose substrate is used then the enzyme complex will almost certainly be saturated with substrate. If hydrolysis is not allowed to go to completion the remainder is usually removed by centrifugation. The Nelson-Somogyi (Nelson 1944) and the dinitrosalicylate (DNS) (Miller 1959) methods were by far the most popular procedures used for estimating the reducing sugars released on hydrolysis of purified cellulose or lignocellulosic substrates. Neither of the methods are specific for sugars, with the result that other reducing substances present

can cause significant interference. Moreover, the response to either reagent varies considerably from sugar to sugar. These drawbacks do not matter too much when saccharification of purified cellulosic substrates are studied. However, as pointed out by Rivers et al (1984), and Breuil and Saddler (1985), the DNS method can seriously overestimate the extent of hydrolysis (i.e. reducing sugars released) when impure lignocellulosic materials are used. Furthermore the release of coloured material such as tannins, during hydrolysis of pretreated lignocellulosic substrates seriously interfere with these assays. We have found high-pressure liquid chromatography to be a reliable procedure for the analysis of the sugar content and composition of hydrolysates.

There have been several attempts in recent years to find acceptable methods for expressing activity in cellulolytic enzymes and to compare the results of different assay procedures (Galas et al 1981, Canevascini and Gattlen 1981). It seems that often the conditions defined for assaying the various cellulase activities are not fully representative of the hydrolytic potential of the enzyme complex (Wojtczak et al 1987).

As was already mentioned, the rate of enzymatic hydrolysis of cellulose is greatly influenced by the reaction temperature. All of the commercial cellulase enzymes that are available are currently obtained from mesophilic fungi. It is probable that cellulases which operate at elevated temperatures would greatly increase the efficiency and the economics of an enzymatic hydrolysis process.

Thermophilic cellulase system

The first description of a thermophilic fungus is attributed to Tsiklinski with her discovery of Thermomyces (Humicola) lanuginosa in 1899 (Tsiklinski 1899). Presently, over 50 thermophilic fungi have been described (Cooney and Emerson 1964, Fergus 1969, Kane and Mullins 1973, Romanelli et al 1975, Thakre and Johri 1980, Deacon 1985). Thermophiles are classified as organisms having a maximum growth temperature at or above 50°C and a minimum growth temperature at or above 20°C (Cooney and Emerson 1964).

Possible advantages in using the thermophiles are as follows: high metabolic activity leading to enhanced product formation rates; pathogenic organisms are killed or at least repressed in growth; no intensive cooling necessary during fermentation; for stopping a reaction, no chilling below 20°C is necessary; increased diffusion rates, ionization and solubility of chemicals; metabolic heat energy from biomass breakdown and from stirring can be used for the fermentation process (energy efficient process); decreased density, surface tension, and viscosity of culture broth; easy recovery of volatile products direct from the culture and during the fermentation process; production of heat stable proteins; low biomass production leads to high product substrate ratios (Wiegel and Ljungdahl 1984). The application of thermophilic fungal enzymes has already been proven in the food and laundry detergent industries using both starch and protein hydrolysing enzymes (Fogarty and Kelly 1979, Aunstrup 1980, Hollo and Laszlo 1988, Ruttloff 1988, Vyskocil et al 1988). Also thermophilic enzymes from bacteria have been widely applied in ethanol production, food and beverage industry and the waste management (Zeikus

et al 1981, Wiegel and Ljungdahl 1984, Malik et al 1983). Some bacteria have already been patented (Ljungdahl and Wiegel 1981, Wiegel and Ljungdahl 1983, Zeikus et al 1983).

Thermophilic fungi are frequently found in self-heating compost piles of organic matter, largely made up of cellulose. It is therefore not surprising that some thermophilic fungi are reported to produce cellulolytic enzymes. A number of review articles on cellulolytic and thermophilic fungi are currently available (Rosenberg 1975, Jain et al 1979, Sen et al 1981, Moreira et al 1981a, Shepherd et al 1981, Bilai et al 1985 a, Waldron and Eveleigh 1986). The search for highly cellulolytic and thermophilic fungi has been undertaken by several groups. Two species have been already patented: Thielavia terrestris (Skinner and Tokuyama 1978) and Sporotrichum cellulophilum (Komura et al 1978).

The use of thermostable cellulases would require less frequent replacement of the enzymes and permit cellulose hydrolysis to operate at higher temperatures, with expected increases in efficiency. The high temperature tolerance associated with greater stability also reduces the risk of microbial contamination during hydrolysis. Such stable cellulases are frequently produced by thermophilic decay fungi.

Quantification of fungal biomass and its correlation to cellulase production

One objective of the work reported in this thesis was to select a thermophilic, cellulolytic fungus and to evaluate its enzyme production as well as enzyme thermostability. In order to actively improve cellulase production and secretion it is desirable to be able to correlate enzyme production with fungal growth. Although there are reliable procedures for the estimation of bacterial biomass (Bratbak and Dundas 1984) many of these methods cannot be applied to filamentous organisms. These options are further complicated when an insoluble substrate is used for growth.

As mentioned before, there are various solid cellulosic substrates which are utilised by filamentous fungi. Fungi are sometimes present as a monoculture. However, in most natural environments, one or more fungi species will be found associated with other classes of microorganisms, e.g. in compost or solids. There, fungal growth assay methods have attempted to utilise fungal specific compounds as the basis of growth assesment . Increased interest in biotechnology and the use of bioconversion processes means that questions concering growth rate measurement, total fungal biomass yield and the quantity of extracellular products in solid substrates will have to be more accurately assessed.

The growth kinetics of filamentous fungi in liquid media have been explored (Righelato 1975), and many methods are available for quantitatively determining fungal growth in stirred or static cultures or determining growth on the surface of agar media (Calam 1969). Such methods include wet or dry weight determinations, packed cell volume, absorbance measurements, total protein, total nitrogen,

substrate consumption, CO_2 production and O_2 consumption (Calam 1969). Many of these methods are impractical when fungi are grown on solid substrates with the fungal mycelium tightly intermingled with the substrate.

One of the most commonly measured components present in fungal cells is protein (Herbert et al 1971). However, there is no truly specific method for the analysis of protein. The biuret procedure has been shown to be less subjected to interference and to be the most representative method of evaluating fungal proteins (Jernejc et al 1986). The total nitrogen determination is also very often used to evaluate the protein content of the cells. However this method may result in inaccurate values due to the non protein nitrogen present in many other cell components i.e. amino acids, nucleic acids, chitin, lipids as well as the nitrogen that is often included in the culture media.

Assays for either DNA or RNA can be made after using appropriate acid treatments (Mallette 1969). The method is often accurate, assuming that the quantity of non-fungal nucleic acid in the initial substrate is very low, e.g. plant cell nucleic acid associated with cellulosic substrates or other carbohydrate polymers. More chemically complex substrates may contain material which will interfere with the colorimetric estimations for DNA or RNA.

Assays for determining the quantity of extractable ATP have been extensively used for determining total biomass in

various complex ecological environments such as sediments or soils (Jenkinson et al 1979). The estimation of ATP can be used to determine fungal biomass in solid substrates in the absence of other living organisms. However, the ATP content can vary markedly between various species.

The substrate consumption/weight loss method has been extensively used in biodegradation studies of solid materials, e.g. wood or textiles (Bravery 1975). By making certain assumptions about fungal growth in the substrate it is possible to arrive at estimates of fungal biomass. The type of assumptions made would include; an estimate of the yield coefficient; weight of fungus produced/ weight of substrate consumed (Solomons 1975); that the bulk volatilised substrate is CO_2; and that there is little diffusion of soluble materials from the mycelium into the substrate. This method will give only an approximate estimate of biomass and is also a relatively insensitive assay.

Chitin estimation. Chitin is an insoluble linear polymer of B-1,4-linked N-acetylglucosamine units produced by most fungi and all insects. It is found in variable quantities in the cell walls of most fungi (Rosenberger 1976), but not in green plants, most animals or other microorganisms. The method was first used for estimating fungal biomass by Arima and Uozomi (1967) to measure the amount of Aspergillus oryzae in fermented rice (rice-koji). It has now been used to estimate fungal biomass in living plant tissue (Ride and Drysdale 1972),

decaying wood (Swift 1973), leaf litter (Frankland et al 1978), wood (Braid and Line 1981) and cereal grains (Nandi 1978). One of the problems of the assay was observed by its first users, Arima and Uozomi (1967), i.e. chitin content of fungal cells does not remain in direct proportion to fungal growth. Other workers have observed similar effects (Sharma et al 1977, Whipps and Lewis 1980). The other sources of error are the tendency for the chitin content of mycelium to vary with age and the presence of extraneous hexosamines within the substrate which cause interference with the assay (Matcham et al 1985).

Ergosterol is the predominant sterol of most fungi (Weete 1974). It is not found to any significant extent in most green plants. Its use as a measure of fungal biomass was described by Seitz et al (1977, 1979) to measure the extent of fungal colonization of cereal grains. Ergosterol can be quantitatively measured by gas chromatography, HPLC or UV spectrophotometry. As with other fungal products the ergosterol content may change with the physiological status of the fungus (Seitz et al 1979). In addition, fungi may vary markedly between species in their ergosterol content. A further complication of this spectral assay might arise if the fungus produces other sterols which have similar spectral properties (Matcham et al 1984).

Serological methods, primarly the enzyme linked immunosorbent assay (ELISA) have been succesfuly used for detecting and quantifying viruses (Bar-Joseph and Garnsey 1981),

bacteria (Cambra and Lopez 1978, Cother and Vruggink 1980) and fungi (Casper and Mendgen 1979, Johnson et al 1982, Ageulon and Dunez 1984) in herbaceous plants, but in most cases no quantification of fungal biomass has been reported. More recently Breuil et al (1988), used ELISA to quantitate fungal biomass on solid wood. The benefit of the assay methods is that by linking enzymes or radiolabels to specific antibodies, the "signal" produced by antibody-antigen interactions can be greatly amplified and thus produce a highly sensitive assay.

Respiration rate ($\underline{CO_2}$ $\underline{\text{evolution}}$) has previously been used to measure total fungal biomass in soils (Anderson and Domsch 1975). In order to separate fungal and bacterial contributions to total respiration, inhibitors of prokaryote or eukaryote specificity were used. $\underline{\text{Oxygen}}$ $\underline{\text{consumption}}$ has also been utilised for biomass estimation in koji fermentation studies (Okazaki and Sugama 1979). Consumption of oxygen was measured by a manometric method coupled to a pressure detector.

The work reported in this thesis compares five methods of estimating different cell components in order to determine the most reliable and efficient procedure for quantifying biomass. A comparison between dry weight, biuret, Kjeldahl, ergosterol and ELISA was undertaken in the presence of soluble as well solid substrates (Avicel and steam treated aspen wood, SEAW). Although the perfect method for biomass estimation does not presently exist it was important to find the most reliable among the available

procedures.

This work describes a search for a thermophilic fungus which produces an efficient cellulase enzyme system. The major objectives of this study were, to initially screen for thermophilic and cellulolytic fungi, to compare their enzyme activities and thermostabilities, to search for a reliable procedure to measure fungal biomass and to correlate fungal growth with cellulase enzyme production.

II. SCREENING FOR HIGHLY CELLULOLYTIC AND THERMOPHILIC FUNGI

II.I. MATERIALS AND METHODS

Microorganisms

Aspergillus fumigatus 179D, *A.niger* 207G, *A.terreus* C304D, *Acremonium sp* 630A, and *Humicola sp.* 631A were isolated from wood chip piles. *Sporotrichum thermophile* C419, *Melanocarpus albomyces* C433, *Thermoascus aurantiacus* C412, 235E and 235F, *Thielavia terrestris* 255B, *Phanerochaete chrysosporium* C435 were obtained from the Forintek culture collection.

The patented strain of *Thielavia terrestris* 255C was obtained from American Type Culture Collection (Skinner and Tokuyama 1978). A mesophilic fungus *Trichoderma harzianum* E58, which is commonly used at Forintek was included as a representative of the mesophilic fungi.

All of the isolated species as well as *S.thermophile* C419 are Imperfect Fungi. *P.chrysosporium* C435 belongs to the Basidiomycete and all the other species belong to the Ascomycete.

II.I.I. Isolation of fungi.

A 5 g sample of 4 months old hard wood chips obtained from a wood chip pile located at MacLaren Pulp and Paper, Thurso, Quebec was put into 60 ml of Tansey's medium (Tansey 1971b)(in 300 ml volume Erlenmeyer flasks) and shaken at 45^{o}C, 200 rpm for 2 h. The suspension at different dilutions, (1/10, 1/100, 1/1000) was used to

inoculate Tansey's solid medium plates. The Petri dish plates were prepared by putting one layer of agar on the bottom and Tansey's medium supplemented with 0.1 % Triton x 100 and 0.5 % $CaCO_3$ on the top. Different substrates were applied. These included; a) 1 % acid swollen cellulose (ASC), prepared according to Rautela and Cowling (1966), b) 1 % Avicel, c) 0.5 % acid swollen cellulose (ASC) combined with 0.5 % glycerol and d) 1 % ASC with 0.4 % deoxyglucose. One set of samples was incubated at 37°C and the other at 44°C. At the latter temperature moisture saturated plastic bags were used to minimize dessication. Also Tansey's liquid medium supplemented with 0.3 % $CaCO_3$ (pH 6.35) was used for fungal isolation. Filter paper strips (Whatman #1), 1 % steam exploded aspen wood (240°C, 80 sec.) and sawdust (black spruce extracted by ethanol-benzin 1:2) were used as substrates. Malt agar medium plates had previously been used for the isolation of these strains.

A tube clearing method was also used. The organisms were grown on Tansey's solid medium supplemented with gelrite and the various substrates already described. The depth of clearing was analyzed and used to indicate the hydrolytic potential of the fungi.

The cultures of fungi were maintained on malt agar and corn-yeast-malt media.

II.I.II. Optimum temperature for growth and enzyme activities.

To estimate the optimal growth temperatures, the cultures were grown in peptone yeast glucose medium containing ; 0.2 % yeast extract, 0.1 % peptone, 0.02 % $MgSO_4x7H_2O$, 0.01 % $CaCl_2x2H_2O$, 2 % glucose, and shaken (200 rpm), at various temperatures (40,44,45,

47 and 50°C) for 48 hours. Fungal biomass was evaluated by the dry weight method.

The optimum temperatures for cellulase activities were determined by growing the fungi in cellulolytic media at various temperatures. The basic medium used contained; 0.2 % $(NH_4)_2SO_4$, 0.27 % KH_2PO_4, 0.039% $CaCl_2x2H_2O$, 0.03 % $MgSO_4x7H_2O$, 0.1 % peptone, 0.1 % yeast extract, 0.0005 % $FeSO_4x7H_2O$, 0.0002 % $MnSO_4xH_2O$, 0.0001 % $ZnSO_4x\ 7H_2O$ and 0.0004 % $CoCl_2x6H_2O$ (Mandels et al 1971). The medium was buffered with 3 g/L potassium phthalate and supplemented with 2 % cellulose (Avicel or Solka floc).

The second medium was Vogel's medium (Montenecourt and Eveleigh 1978) containing 0.25 % sodium citrate x $2H_2O$, 0.5 % KH_2PO_4, 0.2 % NH_4NO_3, 0.02 % $MgSO_4$ x $7H_2O$, 0.01 % $CaCl_2$ x $2H_2O$, 0.05 % biotin, 0.2 % myoinositol, 0.02 % Ca panthonate, 0.02 % pyridoxine-HCl, 0.02 % thymine-HCl, 0.5 % citric acid x H_2O, 0.5 % $ZnSO_4x7H_2O$, 0.1 % $Fe(NH_4)_2(SO_4)_2x5H_2O$, 0.025 % $CuSO_4x5H_2O$, 0.005 % $MnSO_4x2H_2O$, 0.005 % H_3BO_3, 0.005 % Na_2Mo_2 x $2H_2O$, 0.1 % peptone and 0.2 % Tween 80. The medium was supplemented with 2 % cellulose as previously described.

Cultures were assayed every 24 h for cellulase activity using the Nelson and Somogyi procedure (Nelson 1944, Somogyi 1952) to quantitate the liberated sugars. The sampling was carried out by asceptically withdrawing a portion of the fungal cultures and filtering them through a Whatman glass filter. The intra- and extra-cellular enzyme activities were assayed. To prepare the intracellular fraction the mycelium was first harvested by centrifuging (10000 rpm, 30 min., 4°C). The pellets were then washed with 0.05 M acetate buffer, pH 4.8, then centrifuged again. The cells were reconstituted in

the same buffer to the equivalent amount of original culture fluid, then sonified and assayed for cellulolytic activity. The original supernate was used for the determination of extracellular cellulase activity.

II.I.III. Inoculum and test media.

The fungi grown on malt agar or corn yeast malt slants for 3-4 days (44°C) were used as a source of inoculum. Spores, mycelium and agar were blended with distilled water for a few seconds in a sterile blender. Ten milliliters of this suspension was used to inoculate peptone yeast glucose medium containing; 0.2 % yeast extract, 0.1 % peptone, 0.02 % $MgSO_4x7H_2O$, 0.01 % $CaCl_2x2H_2O$ and 2 % glucose. A 1:5 ratio of the medium volume and to the flask volume was used to give optimal aeration. This medium was always used to pre-grow the cultures to provide the inoculum. The fungi were grown at their optimum growth temperatures. After 48 h the mycelium was harvested, resuspended in distilled water and used as an inoculum for different media (see II.I.II.).

II.I.IV. Enzymatic assays.

In our preliminary studies on cellulase production the enzyme activity was usually assayed by the 3,5-dinitrosalicylic acid (DNS) assay of Miller (1959). When the optimum medium and substrate were established for each of the fungi the experiment was repeated using the more precise Nelson (1944) and Somogyi (1952) method (Breuil and Saddler 1985). One millilitre of appropriately diluted crude filtrate was added to 1.0 mL 0.05 M

acetate buffer, pH 4.8, containing various substrates. After incubation for various times the reactions were terminated by the addition of 1.0 ml Nelson copper reagent. The solution was placed in boiling water for 10 min., cooled in running water, then 1.0 ml of Nelson reagent was added. The absorbance was measured at 500 nm.

Filter paper activity was determined by the method of Mandels et al (1976). Culture filtrate (1 ml) was added to 1.0 ml of 0.05 M acetate buffer, pH 4.8, containing a 1 cm x 6 cm strip (50 mg) of Whatman #1 filter paper and incubated for 30 min. Endo-1,4-B-D-glucanase activity was determined by incubating 1 ml culture filtrate with 20 mg carboxymethyl cellulose (Sigma) in 1 ml acetate buffer for 5 min. B-D-glucosidase activity was determined by incubating 1 ml culture filtrate with 10 ml salicin (Sigma) in 1 ml acetate buffer for 5 min.

One unit of filter paper, endo-1,4-B-D-glucanase and B-D-glucosidase activity was defined as one micromole D-glucose equivalent released per minute.

II.I.V. Measurement of enzyme thermostability.

The pH of crude culture filtrates were adjusted to 4.8 and stored at 50, 60 and 70^{o}C for various time periods prior to assaying for residual activity. The samples which were incubated at 50^{o}C were assayed after 6, 12, 24 h and every subsequent 24 h for seven days. The samples incubated at 60^{o}C were assayed after 30 min, 1, 3, 6, 12, 24 and every subsequent 24 h period. The samples incubated at 70^{o}C

were assayed after 30 min, 1, 3, 6, 12, 24 and 48 h. All of the assays for residual activities were conducted using the Nelson-Somogyi procedure at the optimal temperatures for the particular cellulase assay.

II.I.VI. Preliminary studies on hydrolysis.

T.terrestris strains were grown on the cellulolytic medium previously described by Mandels et al (1971), using 2 % Avicel as the substrate. T.aurantiacus strains were grown on Vogel's medium (Montenecourt and Eveleigh 1978) using 2 % Solka floc as the substrate. When the maximum cellulase activities were detected the cultures were harvested and the supernatants were used to quantify the hydrolytic potential of the strains. Both Avicel and Solka floc were used, at a 2 % concentration, to assay for hydrolytic activity.

These hydrolysis assays were carried out at 60^{o}C. The reactions were stopped after 6,12,24 and 48 h by boiling the samples for 10 min. One millilitre amounts of each suspension were centrifuged and the clear supernates were analyzed for reducing sugar by the Nelson and Somogyi procedure.

II.II. RESULTS AND DISCUSSION

After reviewing the scientific literature and any background information available in culture collection catalogues, several thermophilic fungi were selected as strains with the potential to be highly cellulolytic. These strains were also supplemented with several other strains which had been isolated from wood chip piles located in the Ottawa area. These included Aspergillus fumigatus 179D, A.niger 207G, A.terreus C304D, Acremonium sp. 630A and Humicola sp. 631A. The initial screening work using an agar diffusion method (Rautela and Cowling 1966) did not give any clear results because of the difficulty of accurately measuring the depth of clearing. We therefore decided to directly grow the isolates in liquid culture using 2 % w/v cellulose (Avicel or Solka floc) as the substrate and assaying the extracellular enzyme activities. The various cellulase activities were assayed at 24 h intervals using the DNS method to measure the reducing sugars released (table 1). Both T.terrestris 255B and A.terreus C304D showed similar activity to that obtained with the mesophilic fungus Trichoderma harzianum E58. All the reported data for T.harzianum E58 has been taken from the literature. Although the growth conditions were not the same as for the thermophiles, the reported values had previously been found to be optimal for T.harzianum E58 strain.

Good B-glucosidase yields were obtained with S.thermophile C419, A.terreus C304D and T.terrestris 255B. The latter two strains were the only ones which showed high filter paper activity values. Although Thermoascus aurantiacus 235F did not show high levels of activity, it had previously been reported to be highly cellulolytic

TABLE 1: Peak cellulase activities detected from 11 thermophilic fungi and one mesophilic fungus grown on 2% cellulose at 44°C.

FUNGUS	MEDIA a)	ASSAY TEMPERATURE (°C)	DAYS OF GROWTH	B-GLUCOSIDASE	ENDOGLUCANASE ACTIVITY IU/mL	FILTER PAPER
Thielavia terrestris 255B	D+A	50	5	0.64	13.20	0.41
Sporotrichum thermophile C419	D+A	50	5	1.24	7.26	0.19
Aspergillus terreus C304D	D+A	50	5	0.99	12.30	0.33
Thermoascus aurantiacus 235F	V+SF	50	7	0.10	1.54	<0.1
Thermoascus aurantiacus 235E	V+SF	50	7	0.05	<0.1	<0.1
Phanerochaete chrysosporium C435	D+A	50	7	0.02	1.70	0.08
Melanocarpus albomyces C433	D+A	50	7	0.01	<0.1	<0.1
Acremonium sp 630A	D+A	60	6	0.05	4.90	0.17
Aspergillus fumigatus 179D	D+A	60	6	0.13	1.75	0.09
Aspergillus niger 207G	V+SF	60	3	0.65	3.15	0.06
Humicola sp 631A	D+A	60	3	0.03	0.29	<0.1
Trichoderma harzianum E58 b)	V+SF	50	3	0.6-0.8	15-20	0.6-0.8

a) Media used included; D+A - basic medium described by Mandels et al (1971) with 40 mM KH_2PO_4, buffered with 3 G/L potassium phthalate and with urea omitted. Media supplemented with 2 % Avicel, V+S - Vogel's medium (Montenecourt and Eveleigh 1978). Media supplemented with 2% Solka floc.
b) Values taken from Saddler et al (1985), growth temp. was 28°C.

(Thakre and Johri 1980, Deacon 1985). For this reason we decided to assay the cellulase activity of this strain in more detail as well as those exhibiting high activity in the initial screening work. Although Aspergillus fumigatus showed relatively high cellulase activity, it was not further evaluated because of its known pathogenic properties (Shaker et al 1984).

Each of the strains was initially grown on peptone yeast glucose medium at a variety of temperatures. In this way we hoped to determine optimum temperature for growth by monitoring the dry weight of mycelia (Table 2). Both strains of Thermoascus aurantiacus as well as Sporotrichum thermophile exhibited the highest growth temperatures. This was similar to the results of Romanelli et al (1975) who reported T.aurantiacus as having an optimum growth temperature of 46-51^{o}C. The optimum growth temperature for T.terrestris was also similar to that reported by Margaritis and Merchant (1986) who found that temperatures between 44-52^{o}C were optimal for the growth of this organism. All of these fungi could grow at considerably higher temperatures than that of T.harzianum and could be classified as truly thermophilic. Other workers (Eriksen and Goksoyr 1976 , Skinner and Tokuyama 1978, Grajek 1986) have claimed that the prefered temperatures for the growth of thermophilic fungi are in the range of 40^{o} to 50^{o}C. Previously Rosenberg (1975) had studied temperature optima for 21 species of thermophilic and thermotolerant fungi and reported temperatures between 40-50^{o}C as the optima for the growth of S.thermophile, 40-45^{o}C for T.aurantiacus and 45^{o}C for Thielavia thermophila.

TABLE 2. OPTIMUM GROWTH TEMPERATURES FOR SIX THERMOPHILIC FUNGI GROWN IN PEPTONE YEAST GLUCOSE MEDIUM DETERMINED BY MEASURING THE DRY WEIGHT (mg) OF THE MYCELIA.

ORGANISM	DRY WEIGHT (mg) OF MYCELIA AFTER GROWTH AT TEMPERATURE °C					
	40	44	45	47	50	55
Thermoascus aurantiacus 235E	5.08	5.93	6.02	6.74	6.86[a]	6.80
Thermoascus aurantiacus 235F	1.98	2.20	2.50	2.68	3.31	3.25
Sporotrichum thermophile C419	4.24	4.94	5.14	6.70	6.80	6.40
Thielavia terrestris 255B	6.04	6.23	6.38	6.28	0.26	0.19
Thielavia terrestris 255C	1.56	1.90	1.87	0.16	0.13	0.08
Aspergillus terreus C304	6.69	6.74	6.85	4.06	0.83	0.02

a - underline value indicates the highest weight value obtained

Once we had established the best temperature for growing the different cellulolytic fungi we next wanted to determine the optimum conditions for assaying cellulase activity (fig. 3 A-C). The optimal temperature for endoglucanase activity was between 65-75^{o}C. Only T.terrestris 255B and A.terreus C304D exhibited a lower optimum temperature for this activity. Four of the thermophilic fungi exhibited optimum temperatures at 70^{o}C or more for both endoglucanase and B-glucosidase activities. They included both of the T.aurantiacus strains, S.thermophile and T.terrestris. The B-glucosidase activity showed the widest temperature range between 60-78^{o}C. T.aurantiacus 235E exhibited the highest optimum temperatures for all cellulase enzymes and A.terreus C304D the lowest.

Although all of the thermophilic strains demonstrated higher temperature optima for endoglucanase and B-glucosidase activities, the optimum temperature for assaying the filter paper activity of T.harzianum was only slightly lower than that of some of the thermophiles. There was not such a large difference in the temperature profiles of the cellulase activities of the thermophilic and mesophilic strains, compared to the sharp contrast in temperature optima for growth (Wojtczak et al 1987). The optimum temperatures for cellulase activities were greater than those for optimal growth.

Although these results are in agreement with the results of Grajek (1986) who studied T.aurantiacus and T.terrestris, they contradict the results of Eriksen and Goksoyr (1976) who reported that the optimum temperature for cellulase activity of Chaetomium thermophile var.dissitum was slightly lower than the optimum temperature for growth. The values obtained for cellulase activities of T.terrestris 255C were slightly higher than those reported

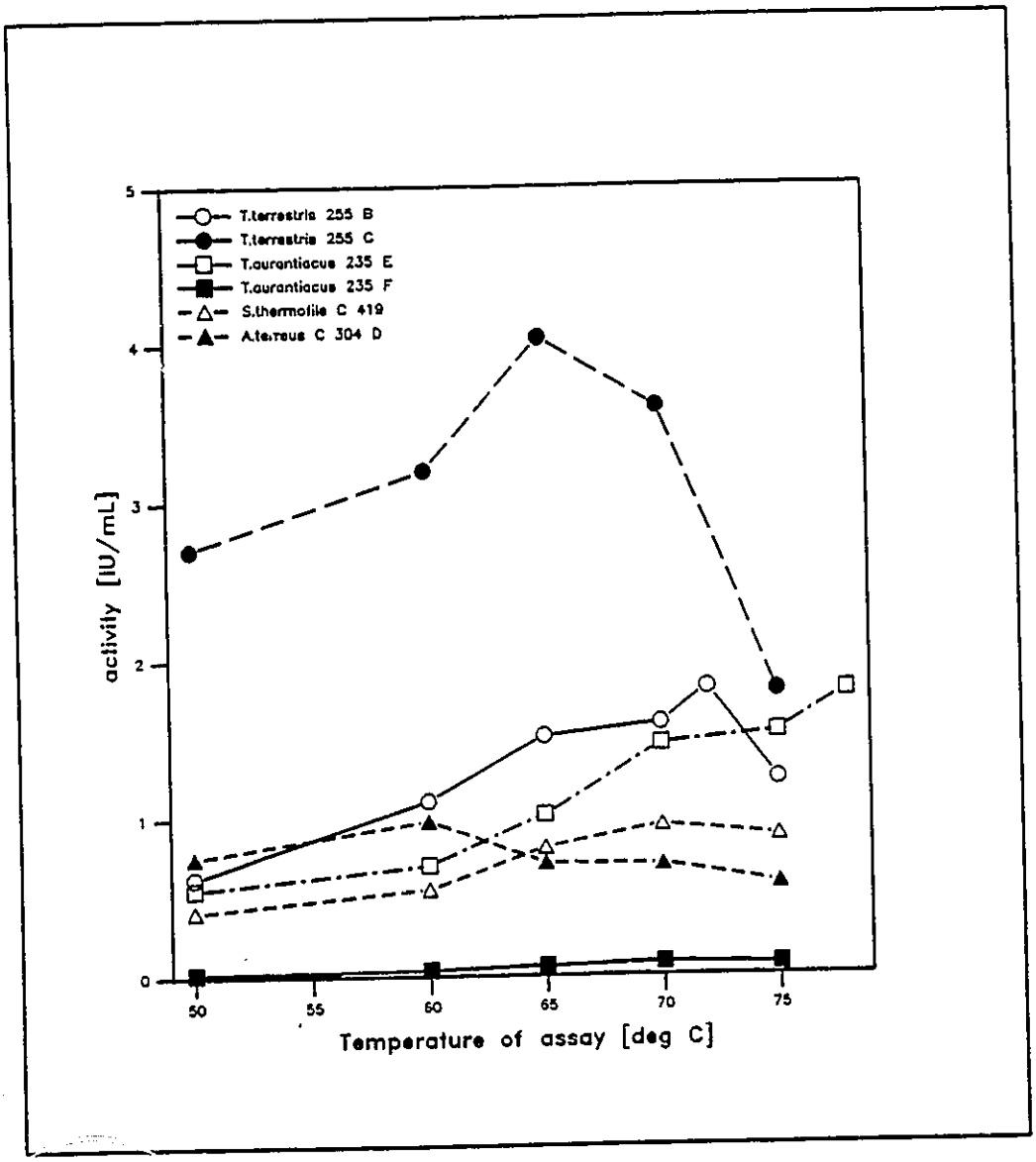


FIGURE 3A. Comparison of optimum temperatures for assaying Beta-glucosidase

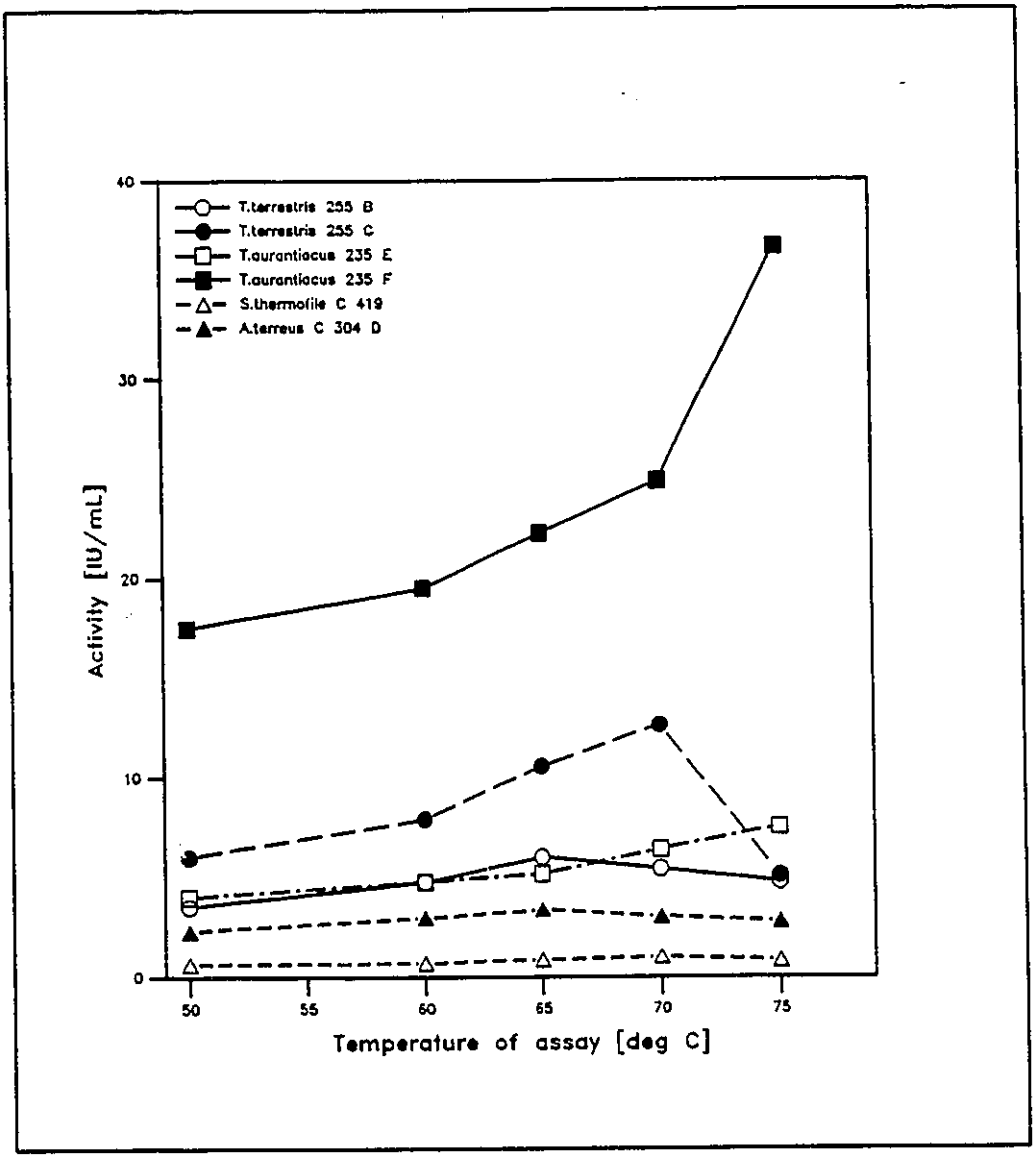


FIGURE 3B. Comparison of optimum temperatures for assaying Endoglucanase

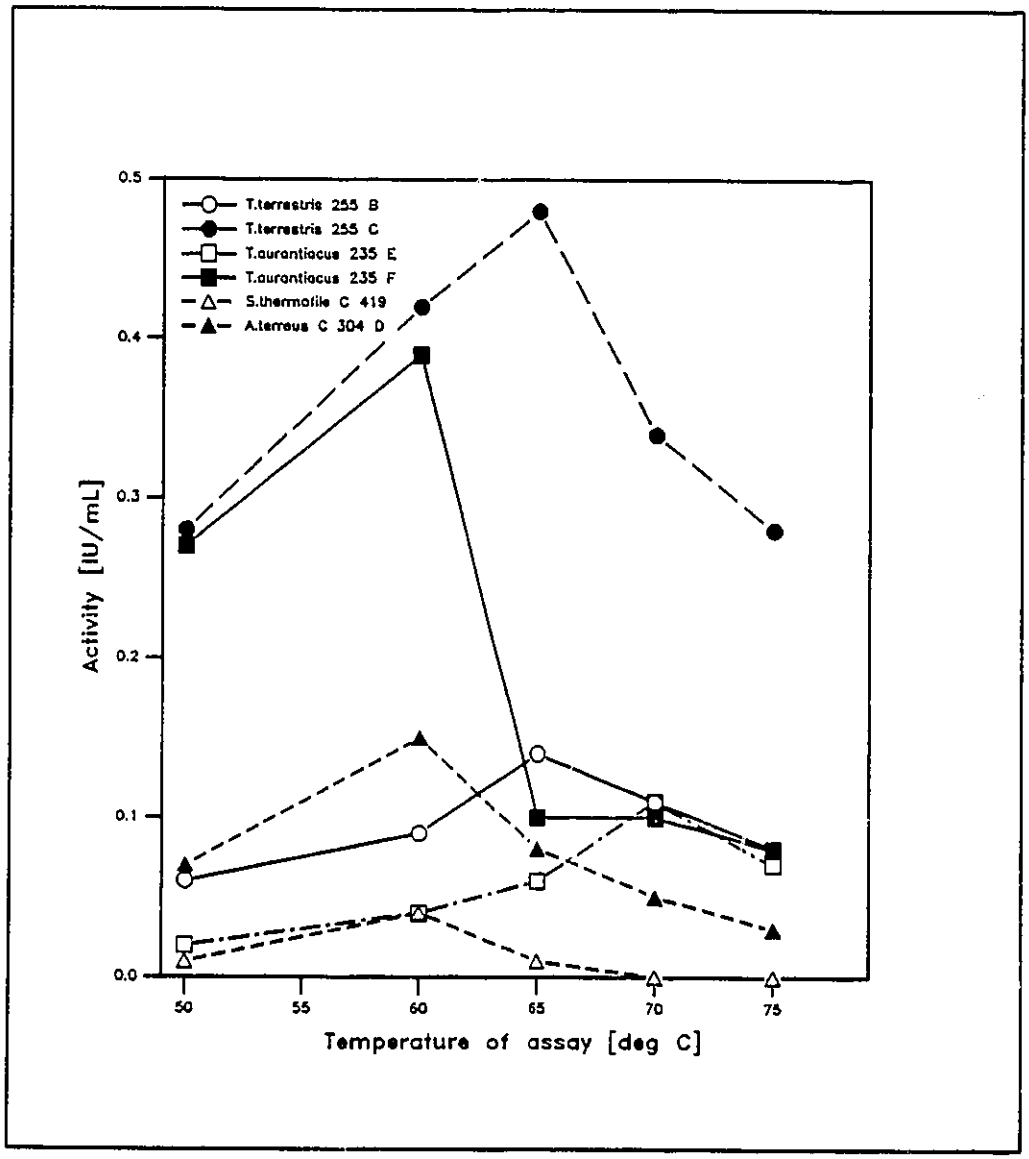


FIGURE 3C. Comparison of optimum temperatures for assaying Filter Paper activity

for this organism by other workers (Skinner and Tokuyama 1978, Margaritis and Marchant 1983, Durand et al 1984).

As other workers had shown that there was as much as a 20^{o} to 30^{o}C difference in the temperature optima of extracellular and cell associated cellulase activities (Araujo et al 1983, McHale and Coughlan 1980) we compared the temperature profiles of the extracellular and cell associated activities from the two Thielavia terrestris strains (fig.4A). We did not find a great difference in optimal temperatures between the extracellular and cell associated fractions of cellulase enzymes from strain 255C (fig.4B). When these values were compared with those reported by most other workers (table 3) they were found to be in general agreement. However, these results differ from those of Araujo et al (1983) and McHale and Coughlan (1980) as the optimum temperature for growth was only slightly lower than those for the enzyme assay. The pH optima for both extracellular and cell associated B-glucosidase activities were around 4.8, similar to most other fungal B-glucosidases (Araujo et al 1983, Rodionova et al 1987).

Once the temperature optima had been established, the cellulase production of the six thermophilic strains were followed over a seven day period (fig. 5-10). The highest levels of cellulase activity were achieved after 5-6 days of growth when most of the fungi went from an actively growing phase to a more stationary phase of growth. A similar phenomenom had been reported for the mesophile Trichoderma harzianum E58 which produced highest cellulase levels at the time of sporulation. Both T.terrestris strains, 255B and 255C produced twice the amount of endoglucanase extracellularly than was found to be cell associated. Strain 255B produced five ti[illegible] more cell

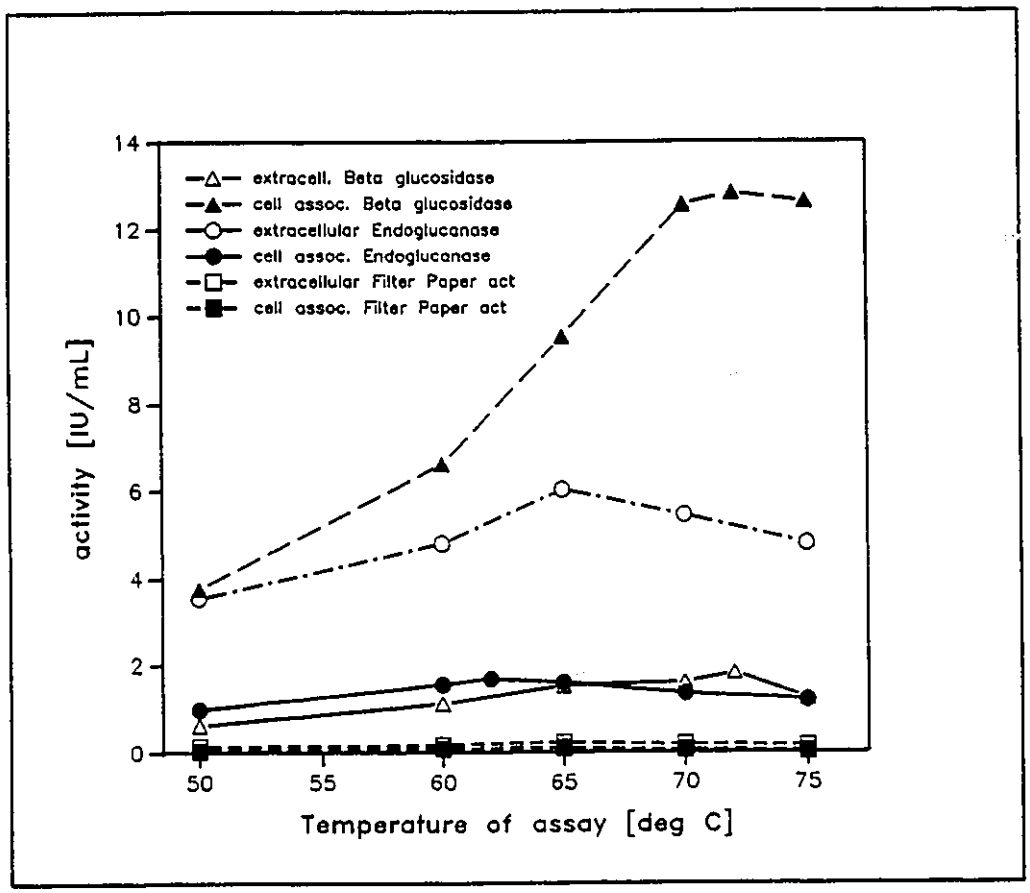


FIGURE 4A. Comparison of optimum temperatures for assaying extracellular and cell associated cellulase activities of T.terrestris 255B.

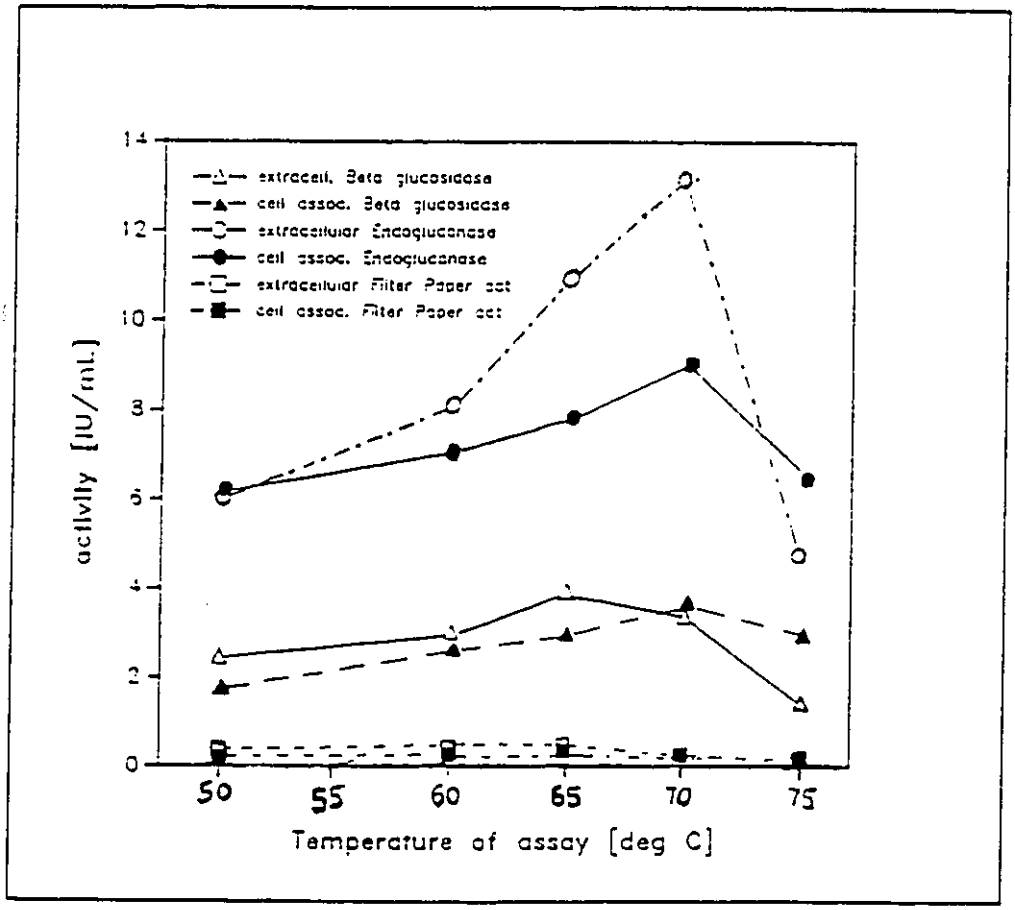


FIGURE 48. Comparison of optimum temperatures for assaying extra-

TABLE 3. OPTIMUM TEMPERATURE (^{o}C) FOR DIFFERENT CELLULASE ACTIVITIES FROM VARIOUS STRAINS OF *Thielavia terrestris*.

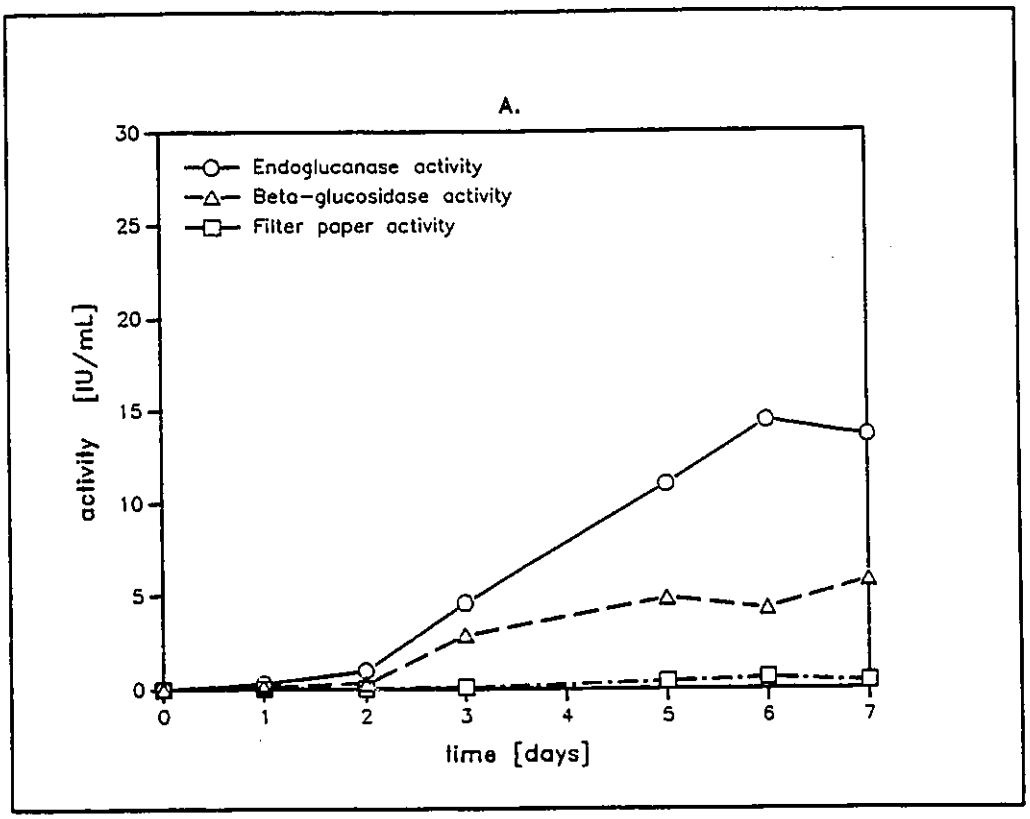
T.TERRESTRIS STRAIN NO.		B-GLUCOSIDASE ACTIVITY		ENDOGLUCANASE ACTIVITY		FILTER PAPER ACTIVITY	
		extra cell	cell assoc	extra cell	cell assoc	extra cell	cell assoc
255B		72	72	65	62	65	64
NRRL8126	(a)	65	70	70	70	65	65
	(b)	67	nd	60	nd	60	nd
ATCC 26917	(c)	70	nd	70	nd	nd	nd

(a) present work

(b) data from Durand et al (1984)

(c) data from Margaritis and Merchant (1983)

nd - not determined



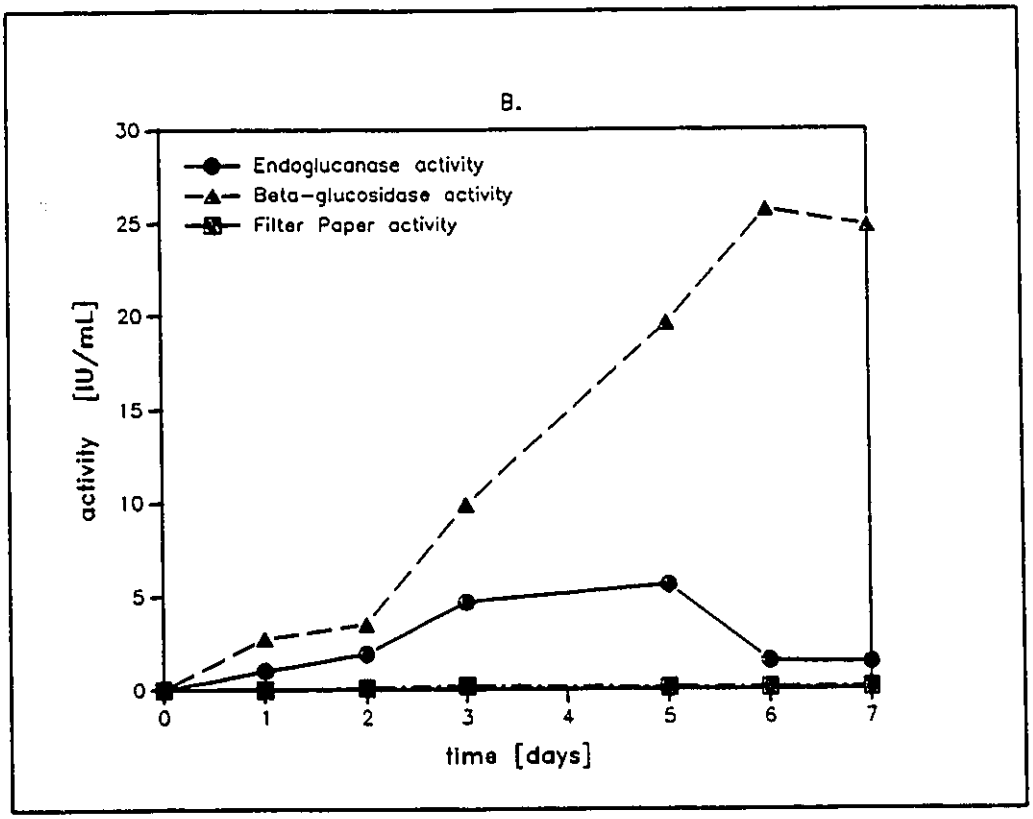
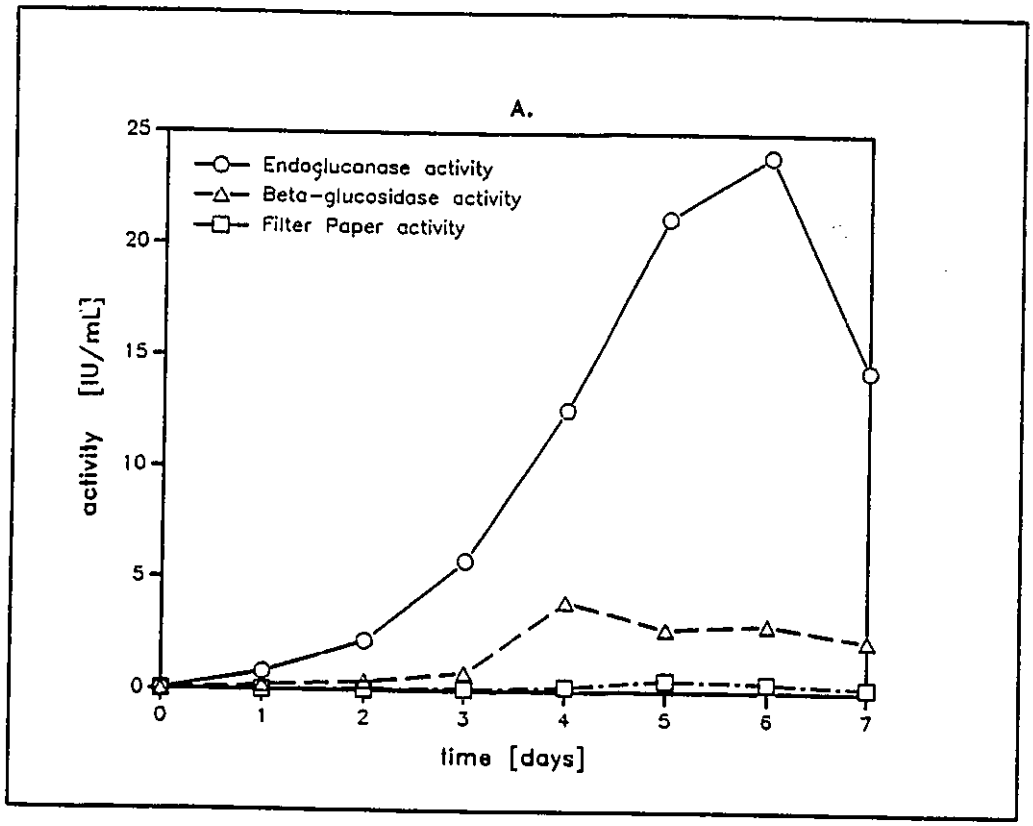


FIGURE 5. The A. extracellular and B. cell associated cellulase production of Thielavia terrestris 255B grown on 2% Avicel at 44 deg C.



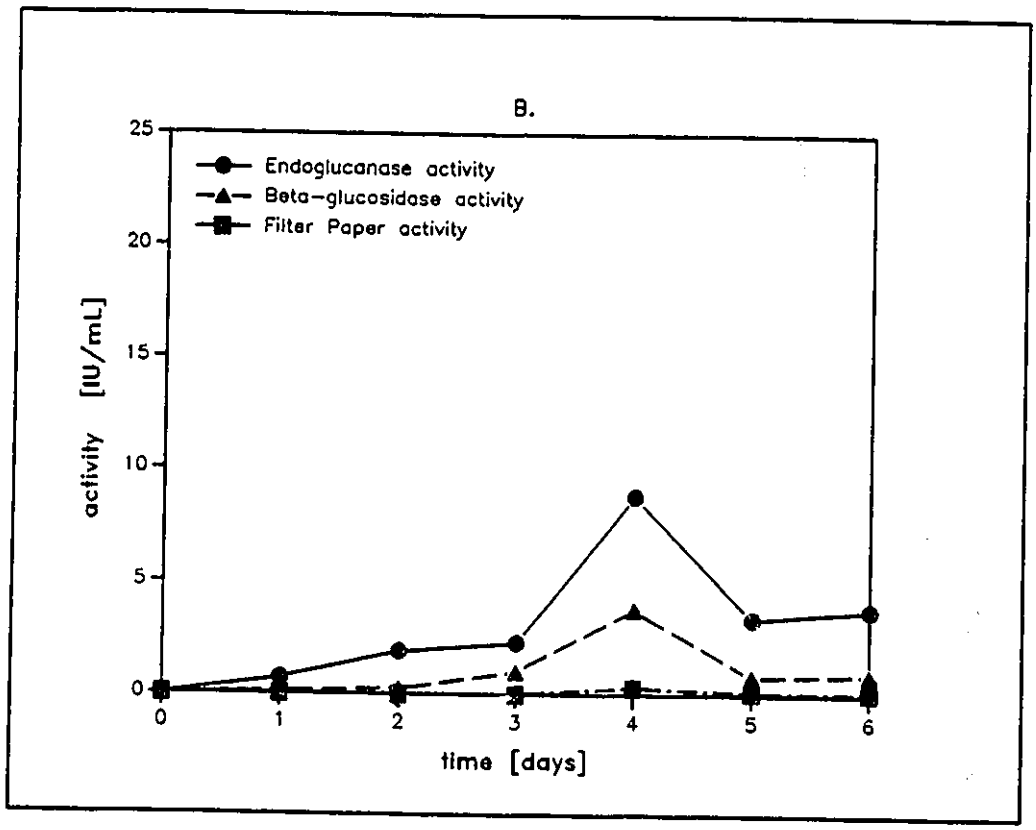
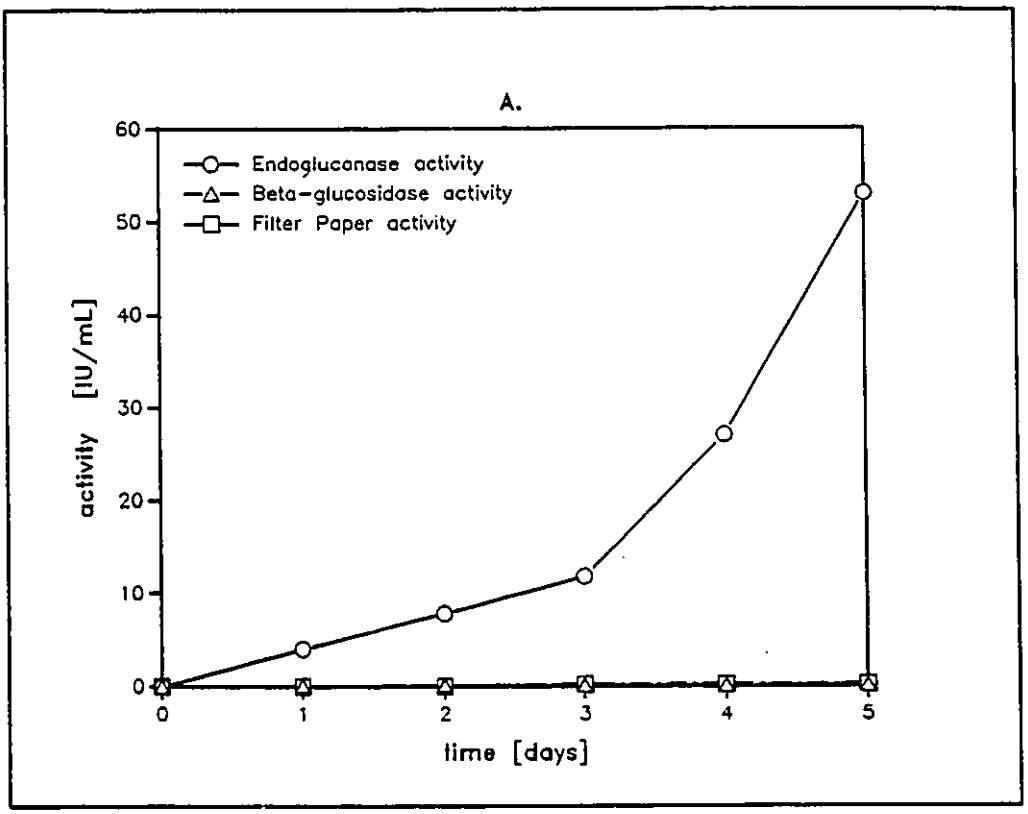


FIGURE 6. The A. extracellular and B. cell associated cellulase production of Thielavia terrestris 255C grown on 2% Avicel at 44 deg C.



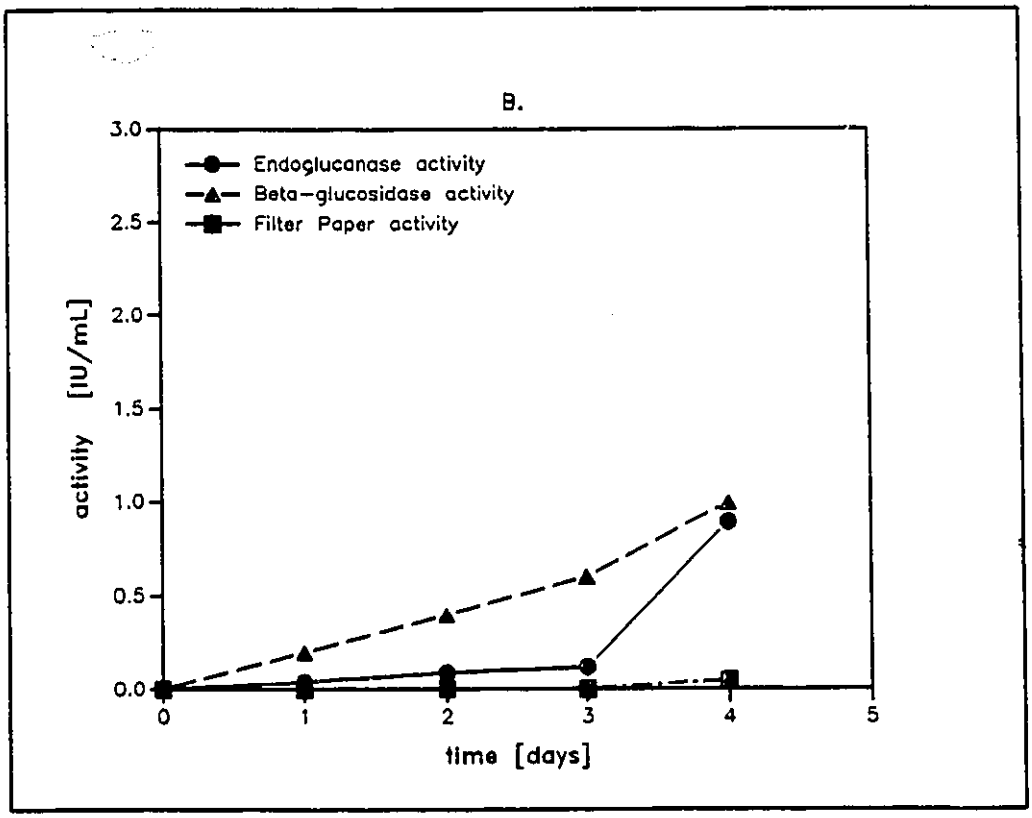


FIGURE 7. The A. extracellular and B. cell associated cellulase production of Thermoascus aurantiacus 235F grown on 2% Solka floc at 50 deg C.

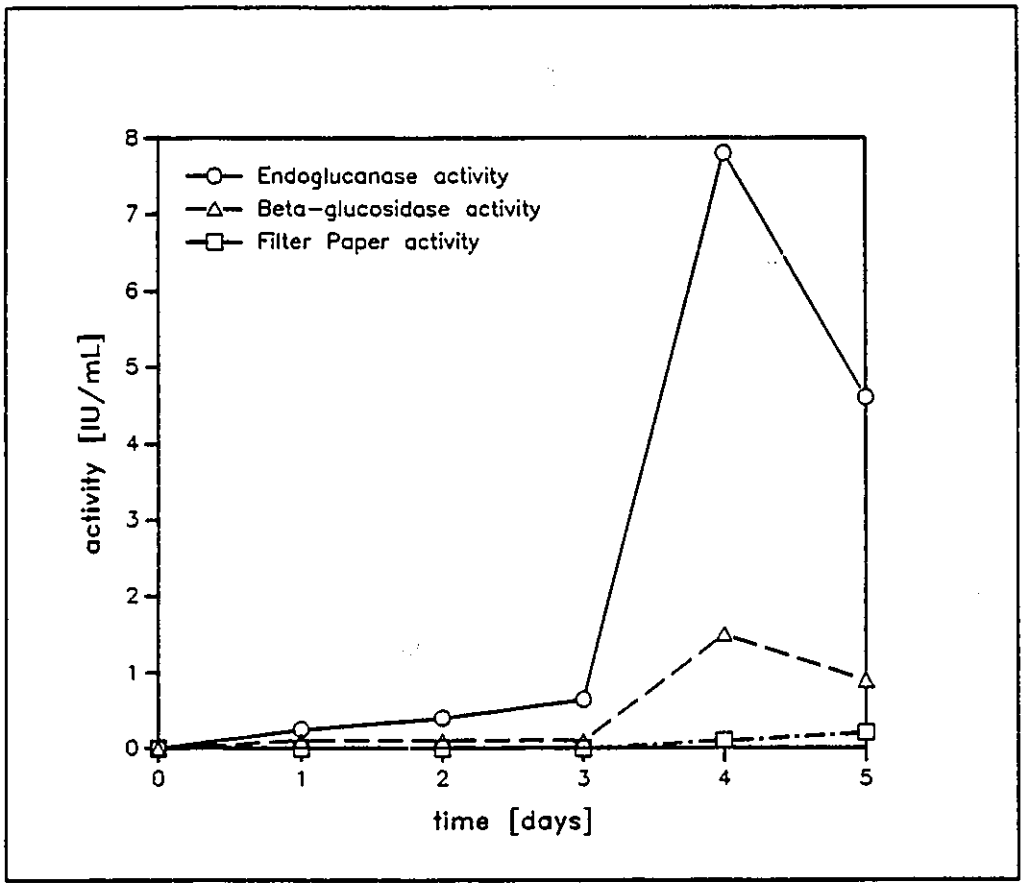


FIGURE 8. Extracellular cellulase production of Thermoascus aurantiacus 235E grown on 2% Solka floc at 50 deg C.

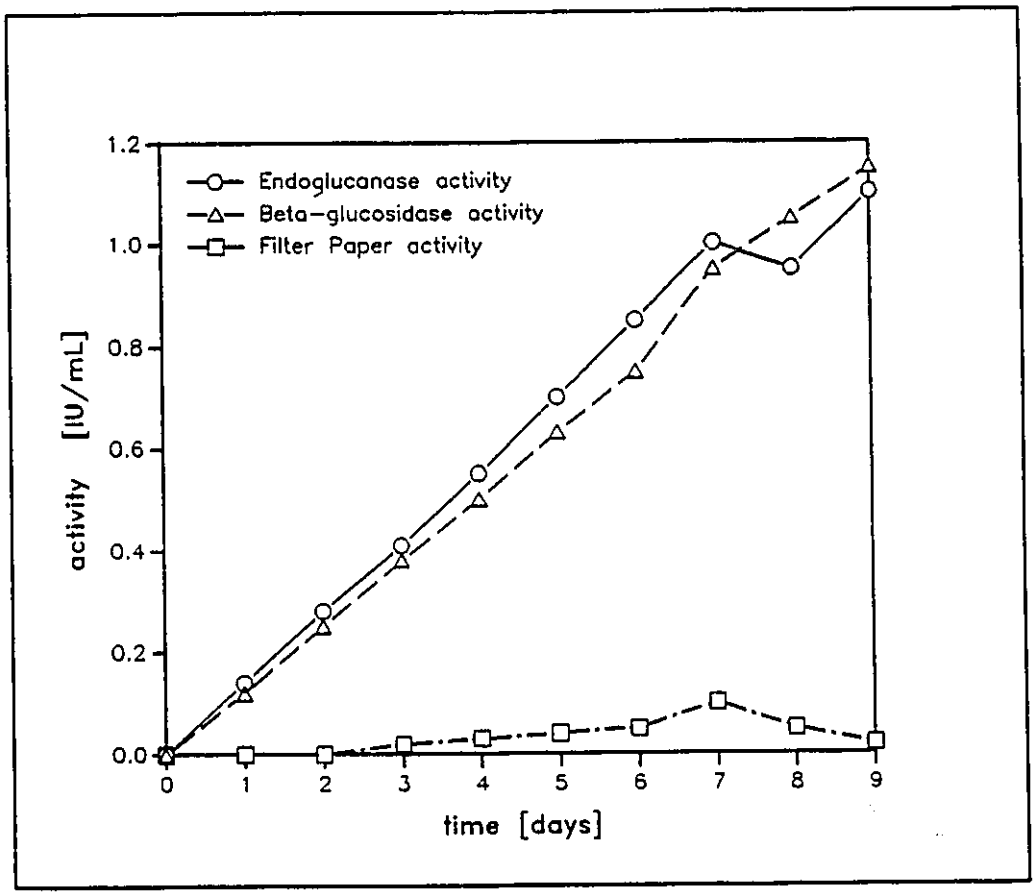
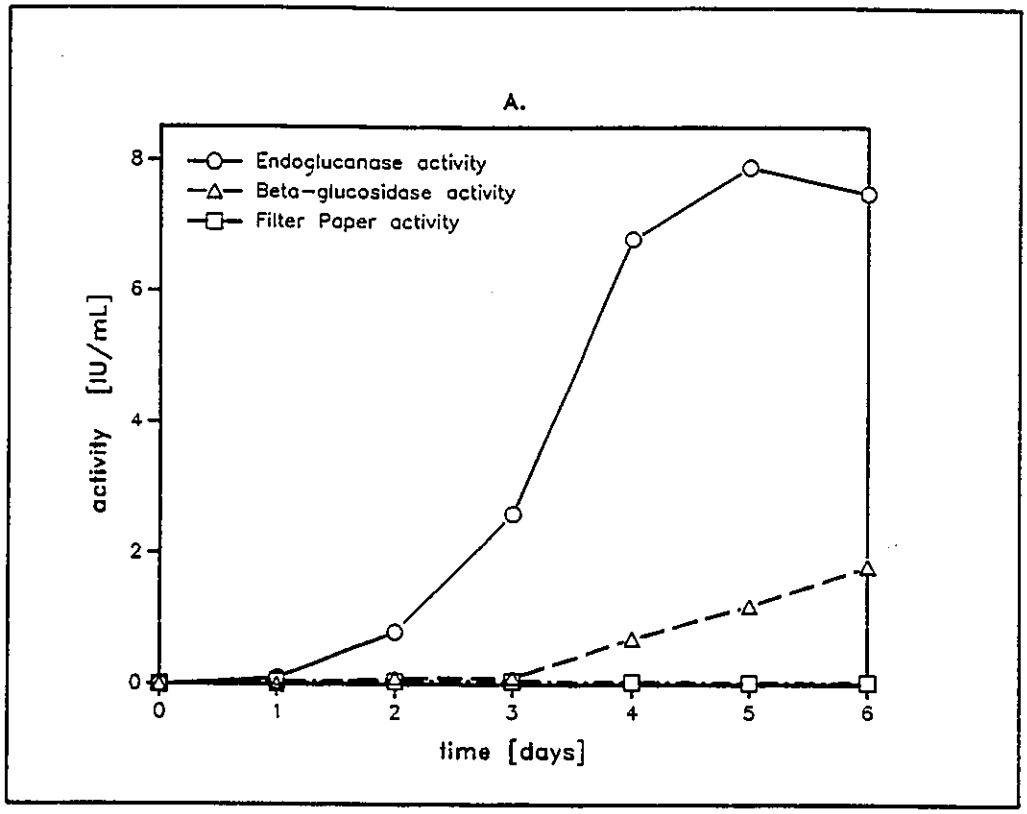


FIGURE 9. Extracellular cellulase production of Sporotrichum thermofile C419 grown on 2% Avicel at 50 deg C.



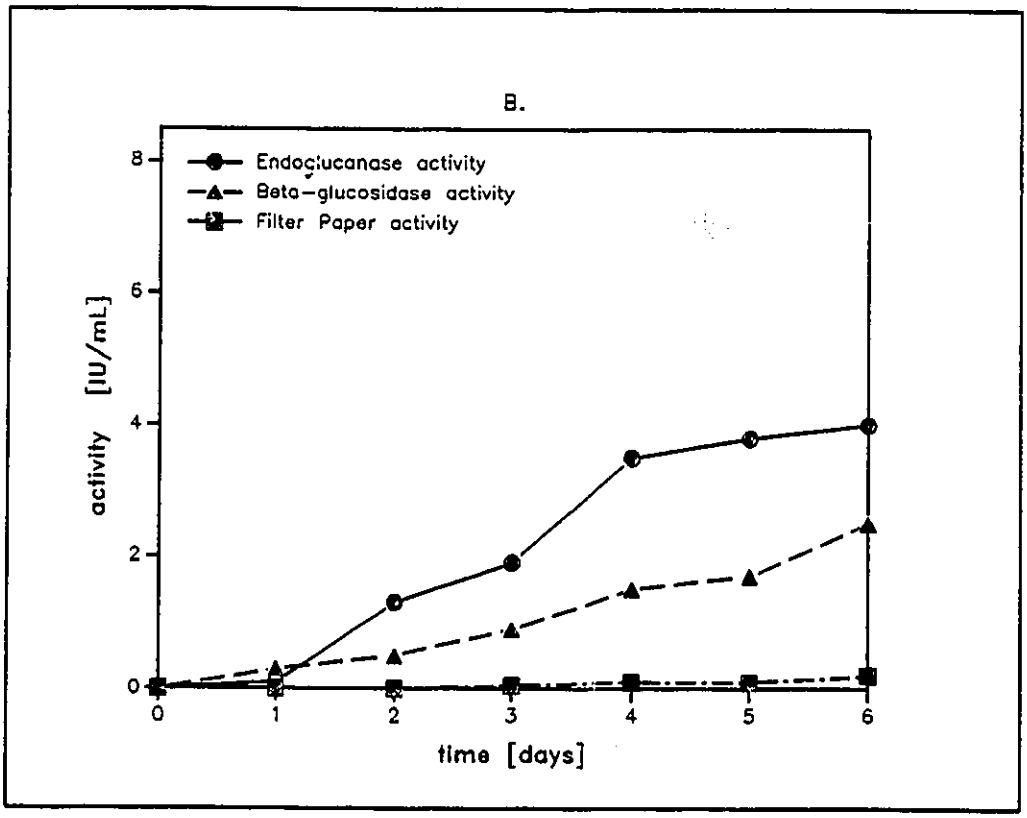


FIGURE 10. The A. extracellular and B. cell associated cellulase production of Aspergillus terreus C304D grown on 2% Avicel at 45 deg C.

associated B-glucosidase than extracellular enzyme. Although T.aurantiacus 235F produced high levels of extracellular endoglucanase activity it only produced very low levels of the other activities, while the cell associated activity was negligable (fig.7). Strain 235E also produced very low cell associated activities and the extracellular endoglucanase values (fig.8) were considerably lower than those obtained with the strain 235F. A sharp peak of endoglucanase activity was observed after 4 days growth of T.aurantiacus 235E (fig.8). This peak activity was obtained in duplicated experiments. A similar, but not so drastic peak was observed for the extracellular B-glucosidase activity (fig.8). S.thermophile C419 produced low cell associated activities and the extracellular endoglucanase and B-glucosidase activities were also very low (fig. 9). A.terreus C304D produced twice the amount of endoglucanase activity in the extracellular fraction as compared to its cell associated counterpart. However, it produced about the same amount of B-glucosidase activity in both fractions (fig. 10). All of the thermophilic fungi generally produced very low levels of filter paper activity. Although all the fungi released a complete complex of cellulase enzymes, capable of hydrolysing a cellulosic substrate such as filter paper, it appeared that these enzymes were not always in good equilibrium.

When the enzyme activities were measured using an incubation temperature of 50^{o}C, which is routinely used with mesophilic fungi such as T.harzianum, the B-glucosidase, endoglucanase and filter paper activities of culture filtrates from T.terrestris 255B were only 35 %, 50 % and 40 % respectively of values detected at the optimum temperature (Breuil et al 1986).

We had also monitored the pH of the media at the same time as the enzymatic assays were carried out (table 4). The pH values did not change appreciably, indicating that the phthalate and $CaCO_3$ contained in the media had an adequate buffering capacity.

Our preliminary study of the thermophiles indicated that cellulase production was not considerably greater than that reported for mesophiles such as T.harzianum E58. Although cellulase production was comparable we next wanted to find out if the thermophilic enzymes would be more thermostable than their mesophilic counterparts. The half lives of the various enzyme activities present in the culture filtrates of thermophilic fungi were determined after prolonged incubation at 60°C (table 5). T.aurantiacus was shown to have the most thermostable cellulase complex for all three of the filter paper, endoglucanase and B-glucosidase activities. The thermostability values for T.harzianum E58 were considerably lower than those from the thermophiles. The values reported for mesophilic strains were taken from the literature (Saddler et al 1985).

When the culture filtrates of the thermophiles were assayed at 50°C nearly all of the cellulase activities had a half-life greater than 72 h (table 6). The exceptions were the B-glucosidase activities of T.terrestris 255B and A.terreus C304D which had half-lives of 45 h and 19 h respectively. As the cellulases of T.aurantiacus 235F and T.terrestris 255B were stable at 60°C these activities were followed over prolonged incubation at 70°C (table 7). The cellulases of the strain 235F were the most thermostable with the endoglucanase activity demonstrating the longest half-life of approximately 12 h. Athough the B-glucosidase activity of both strains appeared to be

TABLE 4. THE pH OF THE MEDIA OF VARIOUS THERMOPHILIC FUNGI GROWN ON 2 % CELLULOSE.

TIME OF GROWTH (days)	pH OF CULTURE FILTRATES					
	T.terrestris		T.aurantiacus		A.terreus	S.thermophile
	255B	255C	235E	235F	C304D	C419
0	5.80	5.80	5.60	5.60	5.80	5.60
1	4.34	nd	nd	nd	6.45	nd
2	4.44	nd	7.03	nd	6.13	nd
3	4.94	3.95	6.84	6.08	5.64	5.16
4	4.18	4.60	6.98	6.18	5.40	nd
5	5.42	4.80	6.90	6.39	5.40	nd
6	5.75	4.95	6.83	6.35	5.43	5.33
7	5.65	5.03	6.84	nd	nd	nd

TABLE 5. HALF-LIVES OF THE CELLULASE ACTIVITIES FROM CULTURE FILTRATES OF SIX THERMOPHILIC FUNGI AND T.harzianum INCUBATED AT 60°C.

ORGANISM	HALF LIFE (hrs)		
	ENDOGLUCANASE ACTIVITY	B-GLUCOSIDASE ACTIVITY	FILTER PAPER ACTIVITY
Thielavia terrestris 255B	> 96	10.8	32
Thielavia terrestris 255C	72	3.5	96
Thermoascus aurantiacus 235E	> 264	96	> 264
Thermoascus aurantiacus 235F	> 241	26.4	48
Aspergillus terreus C304D	> 96	1.0	5.0
Sporotrichum thermophile C419	9.0	3.0	2.0
Trichoderma harzianum[a] E58	0.9	0.75	0.9

a) Values taken from Saddler et al 1985

TABLE 6. HALF-LIVES OF THE CELLULASE ACTIVITIES FROM CULTURE FILTRATES OF SIX THERMOPHILIC FUNGI AND *T.harzianum* INCUBATED AT 50°C.

ORGANISM	HALF LIFE (hrs)		
	ENDOGLUCANASE ACTIVITY	B-GLUCOSIDASE ACTIVITY	FILTER PAPER ACTIVITY
Thielavia terrestris 255B	115	45	192
Thielavia terrestris 255C	> 72	> 72	> 72
Thermoascus aurantiacus 235E	> 264	> 264	> 264
Thermoascus aurantiacus 235F	> 192	> 96	> 96
Aspergillus terreus 304A	> 100	19	> 96
Sporotrichum thermophile C419	> 264	> 264	> 264
Trichoderma harzianum E58 [a]	20	8	20

a) values taken from Saddler et al 1985.

TABLE 7. HALF-LIVES OF THE CELLULASE ACTIVITIES FROM TWO THERMOPHILIC FUNGI INCUBATED AT 70°C.

ORGANISM	HALF LIFE (hrs)		
	ENDOGLUCANASE ACTIVITY	B-GLUCOSIDASE ACTIVITY	FILTER PAPER ACTIVITY
Thielavia terrestris 255B	1.0	0.5	1.0
Thermoascus aurantiacus 235E	12.0	1.9	4.7

the most temperature labile component it was the most thermostable enzyme among the known B-glucosidases. T.terrestris 255B produced a high level of cell associated B-glucosidase which was considerably more thermostable than the mesophilic counterpart.

Previously we had shown (Breuil et al 1986) that the majority of the B-glucosidase activity of T.terrestris 255B was cell associated rather than being released into the culture filtrate. As the pH and temperature of incubation are known to influence the activities of the various enzymes we compared the profiles of both the cell associated and extracellular B-glucosidase activities over a range of temperatures and pH (fig. 11). This was carried out using typical assay conditions with normal enzyme and substrate concentrations. It was apparent that the extracellular and cell associated activities had similar pH (4.5-5.5) and temperature (72^{o}C) optima. A similar optimum pH range between 4.5 - 6.0 has been previously reported for T.terrestris (Skinner and Tokuyama 1978, Margaritis and Merchant 1986), Cheatomium thermophile var.dissitum (Eriksen and Goksoyr 1976), and for T.aurantiacus (Grajek 1987). There was a significant difference however when the thermostability of the two fractions were compared (fig. 12). In this case, the enzymes were preincubated for the indicated times and temperatures prior to carrying out the normal cellulase assay.

Although our interest was in enzyme thermostability in unbuffered medium, information in the literature (Sadana and Henley 1986, Stutzenberger and Lupo 1986) indicated that the presence of buffer can improve enzyme stability. As it was probable that the buffering capacity of the medium could influence the long term stability of various enzymes, the cellulase activities of T.terrestris

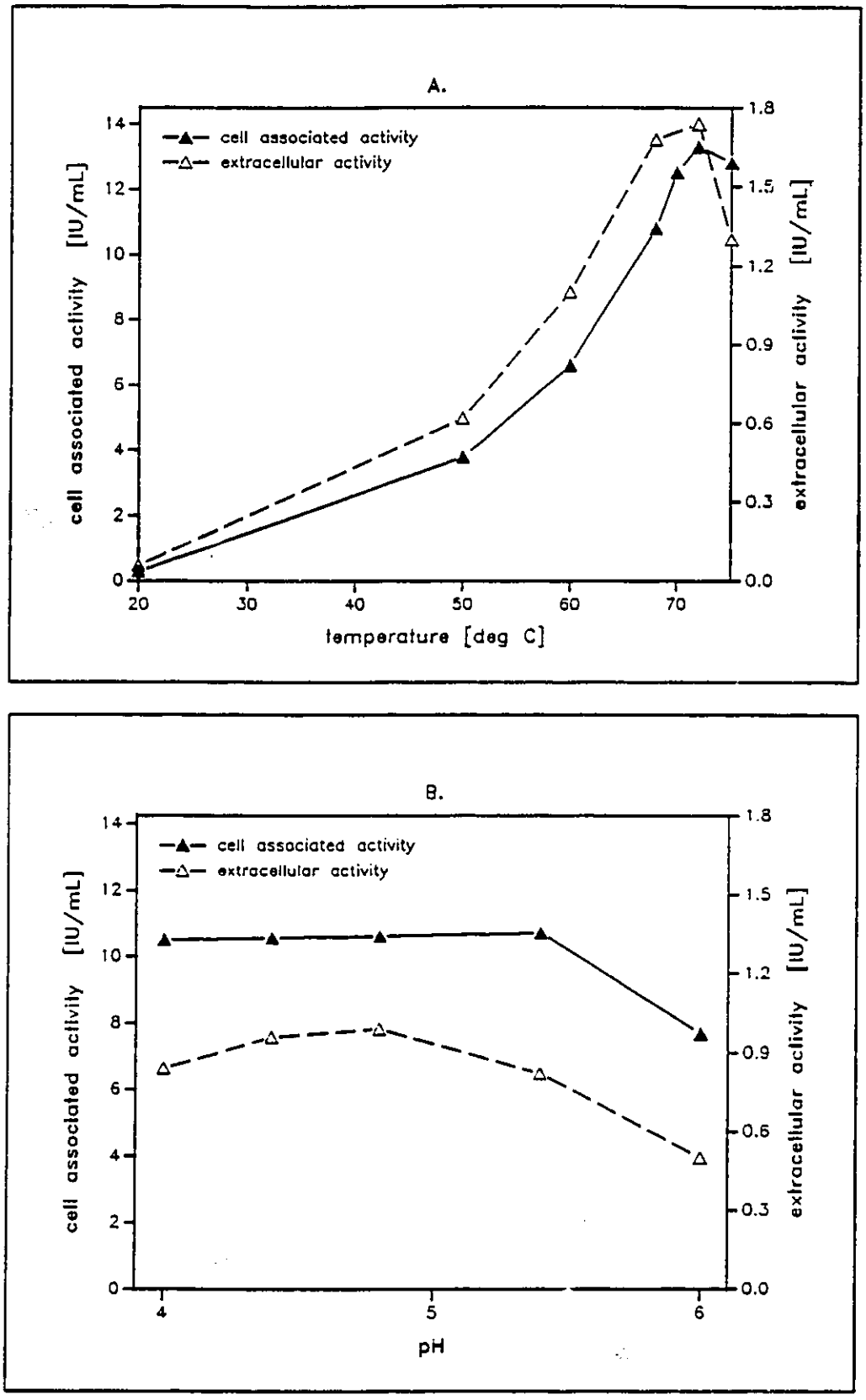
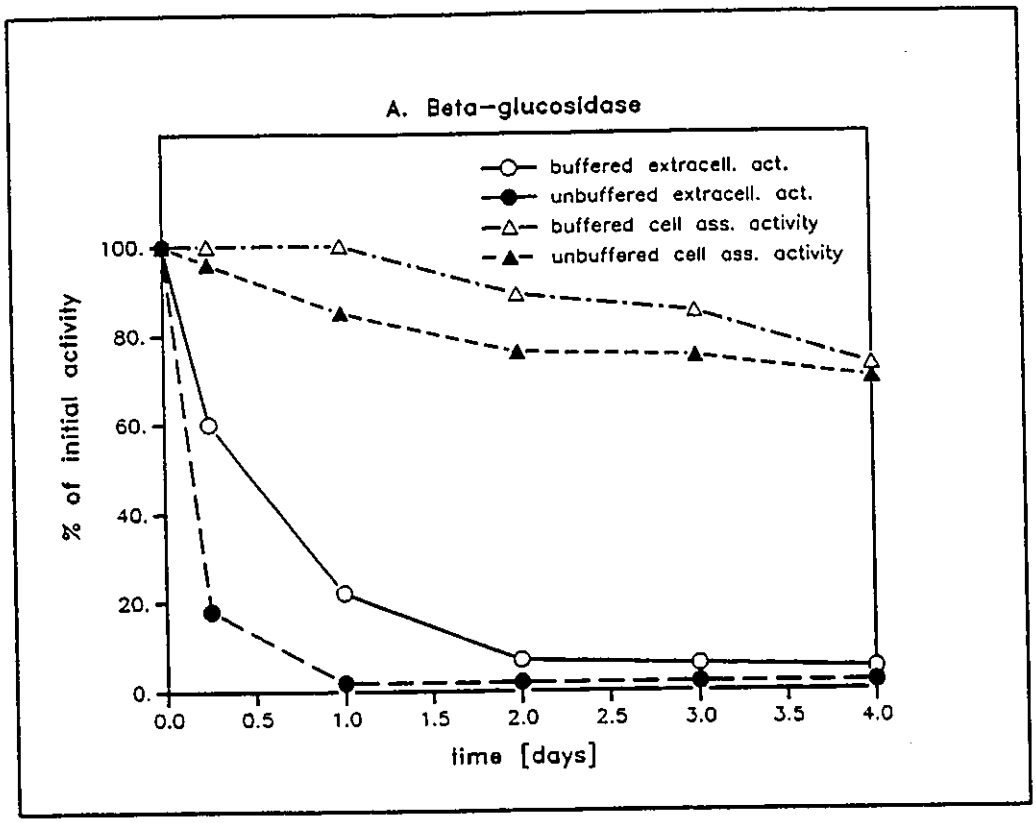


FIGURE 11. The effect of A. temperature and B. pH, on cell associated and extracellular Beta-glucosidase activity of Thielavia terrestris 255B.



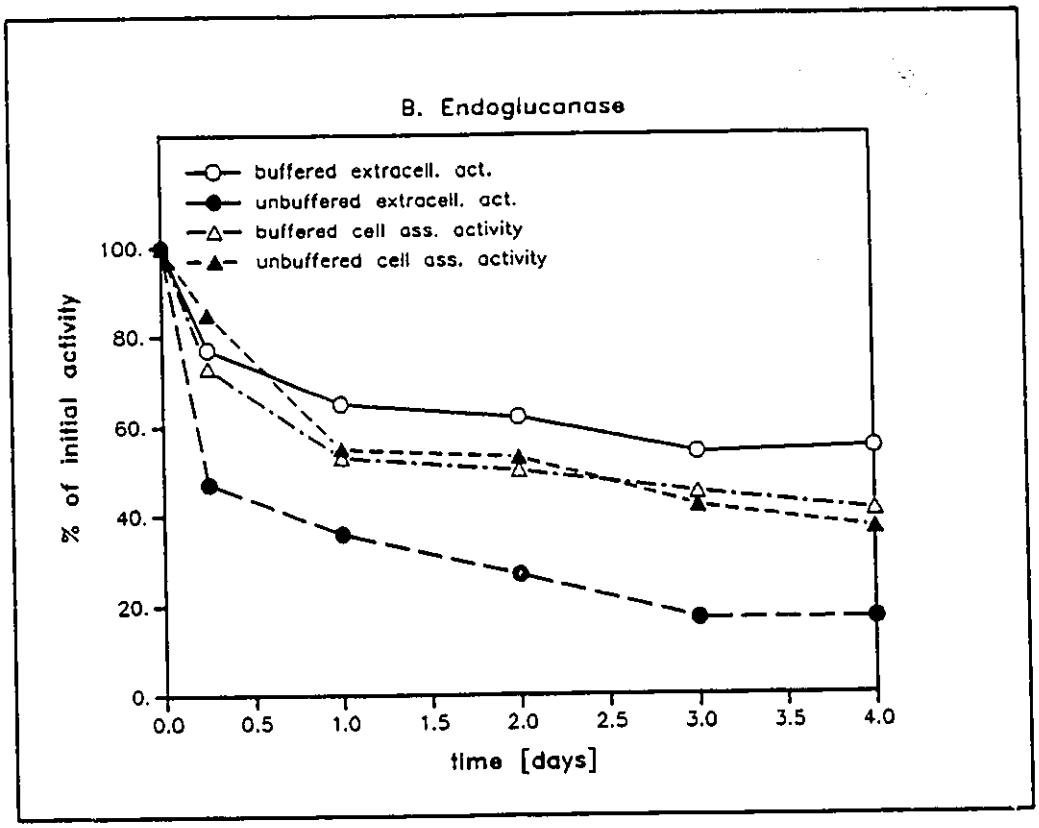
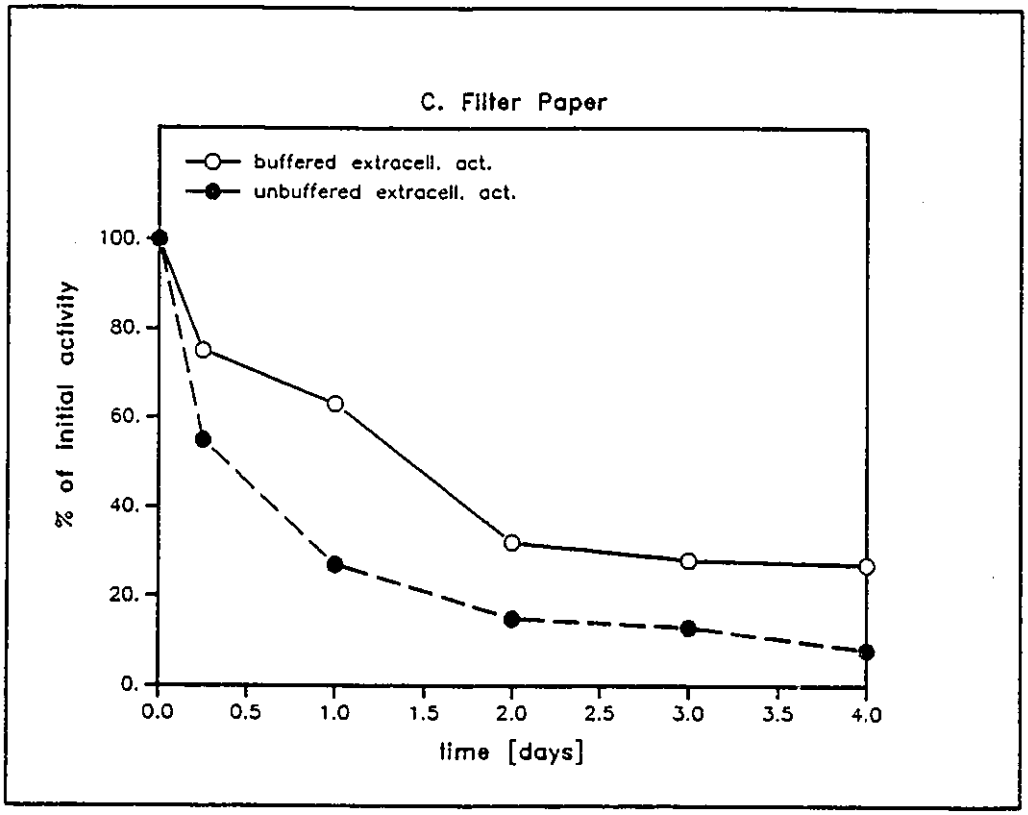
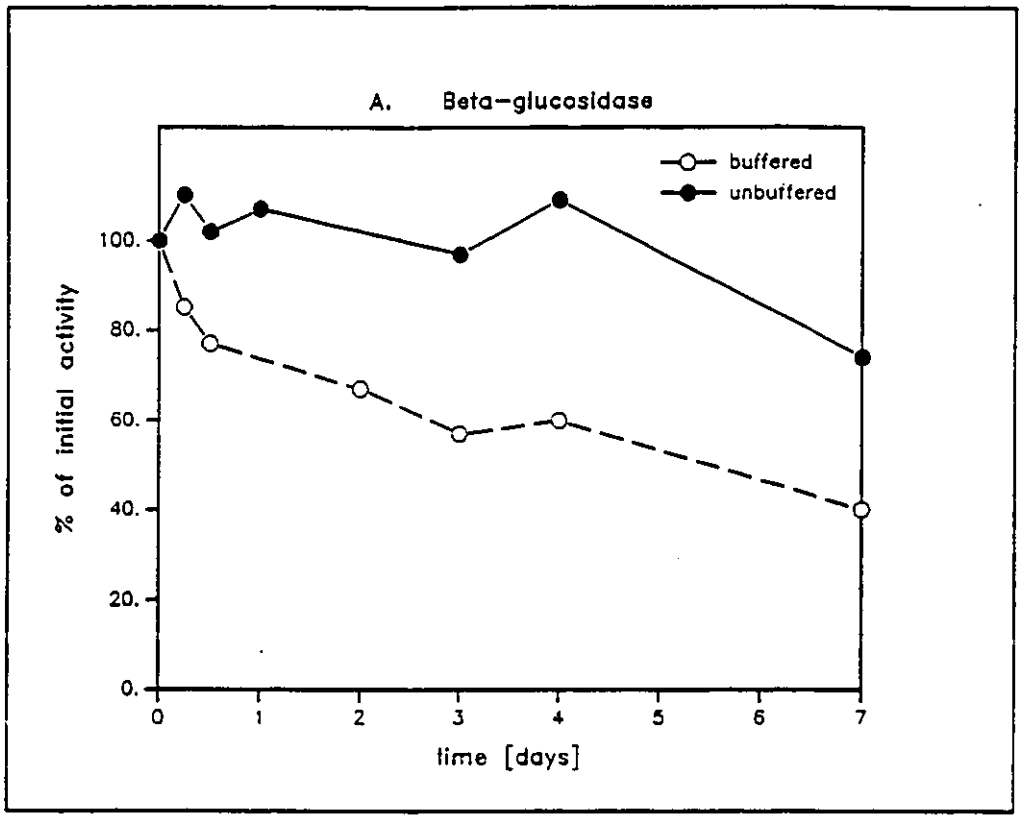


FIGURE 12. Temperature-Time stability of T.terrestris 255B extracellular and cell associated A) Beta-glucosidase, B) Endoglucanase and C) Filter Paper activities at 60 deg C.


C. Filter Paper
buffered extracell. act.
unbuffered extracell. act.
% of initial activity
100.
80.
60.
40.
20.
0.
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
time [days]

255B (fig. 12) and T.aurantiacus 235F (fig. 13) were assayed in the presence and absence of acetate buffer (pH 4.8), over prolonged incubation at 60°C. The cellulase activities of A.terreus were assayed at 50°C (fig. 14). We had previously determined that acetate buffer and pH 4.8 gave the highest enzyme activities as determined by the various cellulase assay methods. Approximately 95 % of the unbuffered extracellular B-glucosidase activity of T.terrestris 255B was lost after 24 h incubation at 60°C while the same conditions of incubation only reduced the cell associated activity by 20 % (fig. 12A). The cell associated endoglucanase activity of T.terrestris 255B was also shown to be more thermostable at 60°C than its extracellular counterpart (fig. 12B). We only assayed the extracellular filter paper activity as it was probable that there would only be low cell associated values (fig.12C). A similar profile was obtained with the B-glucosidase of A.terreus, with the cell associated enzyme being more thermostable than the extracellular enzyme (fig. 15). This may indicate that the mycelium contains stabilizing factors, such as an extra protein associated with the cell associated fraction, that inhibits thermal inactivation of the enzyme. In nearly all cases the stability of the cellulase activities of T.terrestris and A.terreus were enhanced by the presence of buffer. The greatest improvement was observed with extracellular endoglucanase of T.terrestris where only 18 % of original activity was detected after 3 days incubation with non-buffered enzyme and 58 % with buffered enzyme.

The thermostability of T.terrestris B-glucosidase is higher than the B-glucosidase thermostability of various Aspergillus



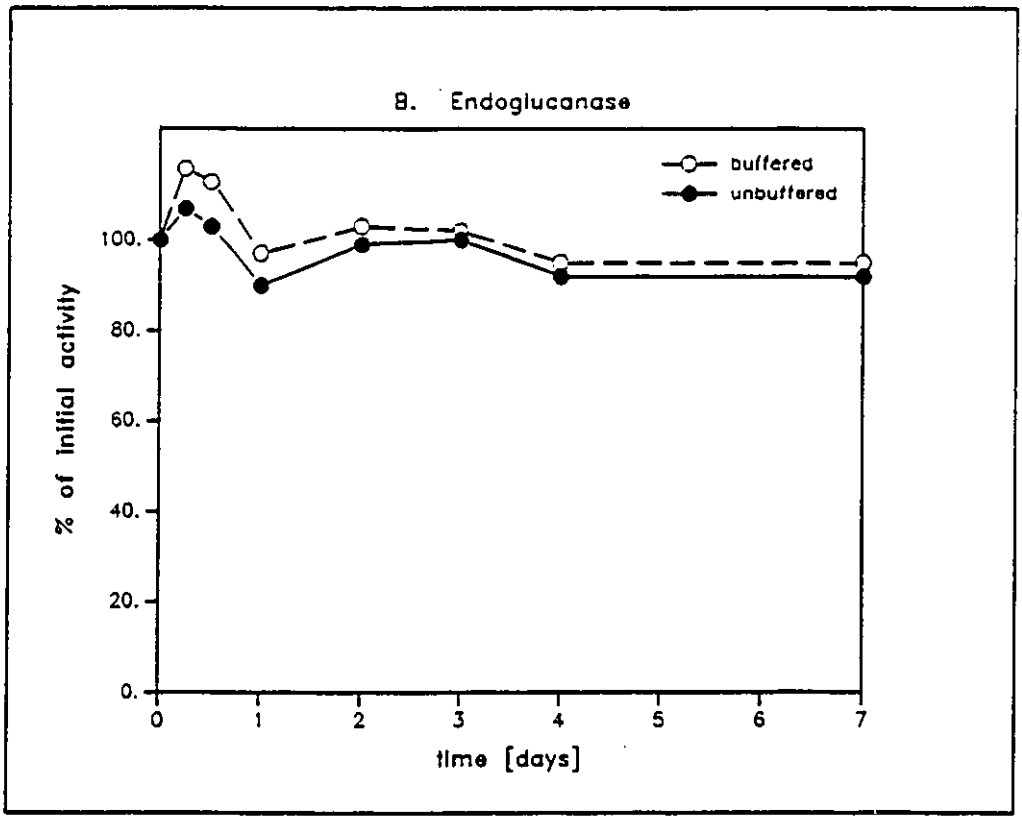
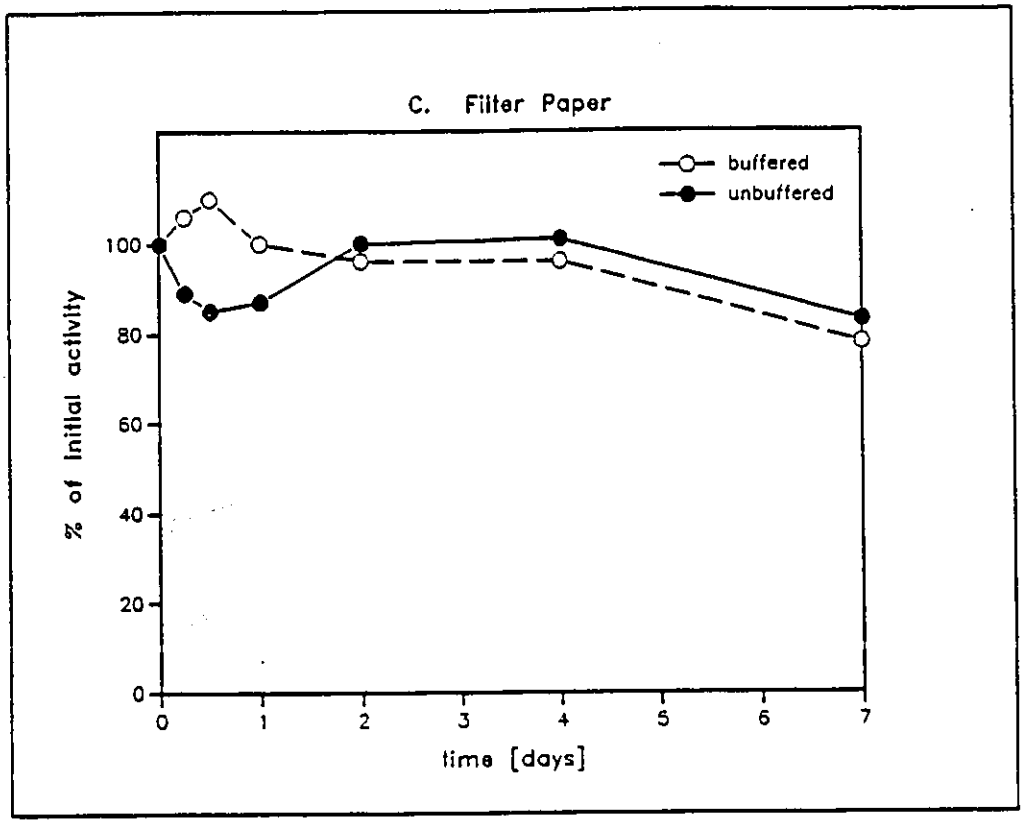
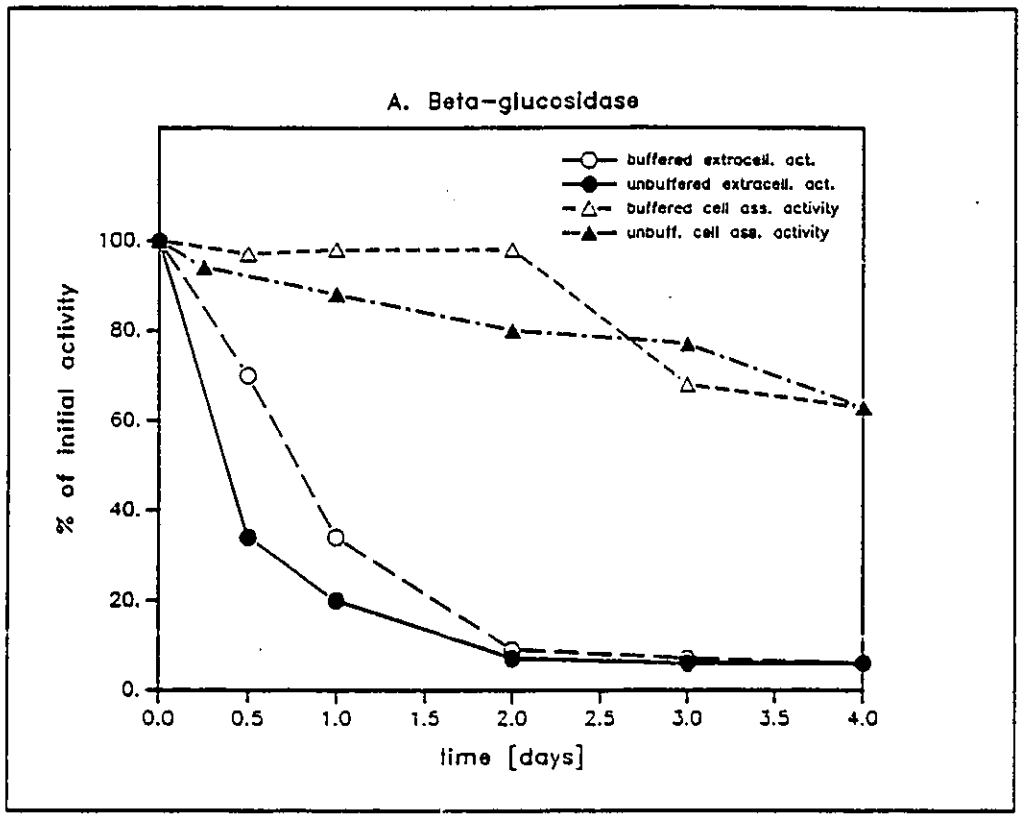


FIGURE 13. Thermostability of buffered and unbuffered extracellular A. Beta-glucosidase, B. Endoglucanase and C. Filter Paper activities of Thermoascus aurantiacus 235F incubated at 60 deg C.


C. Filter Paper
buffered
unbuffered
% of initial activity
100
80
60
40
20
0
0
1
2
3
4
5
6
7
time [days]



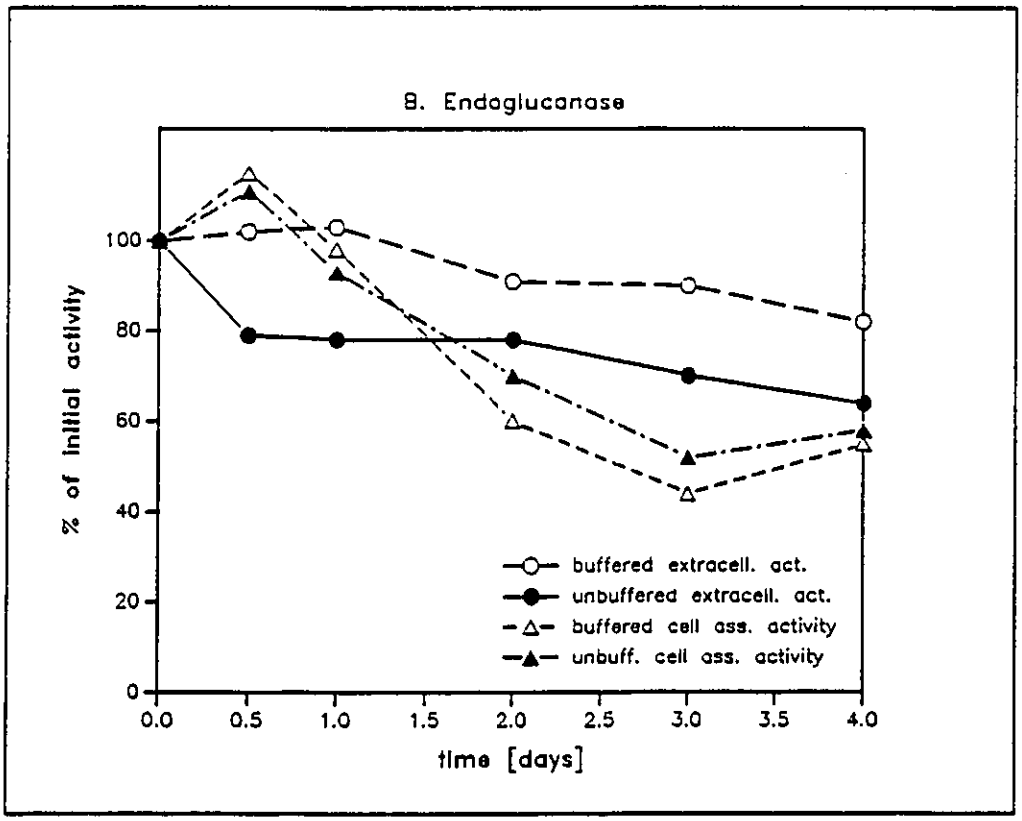
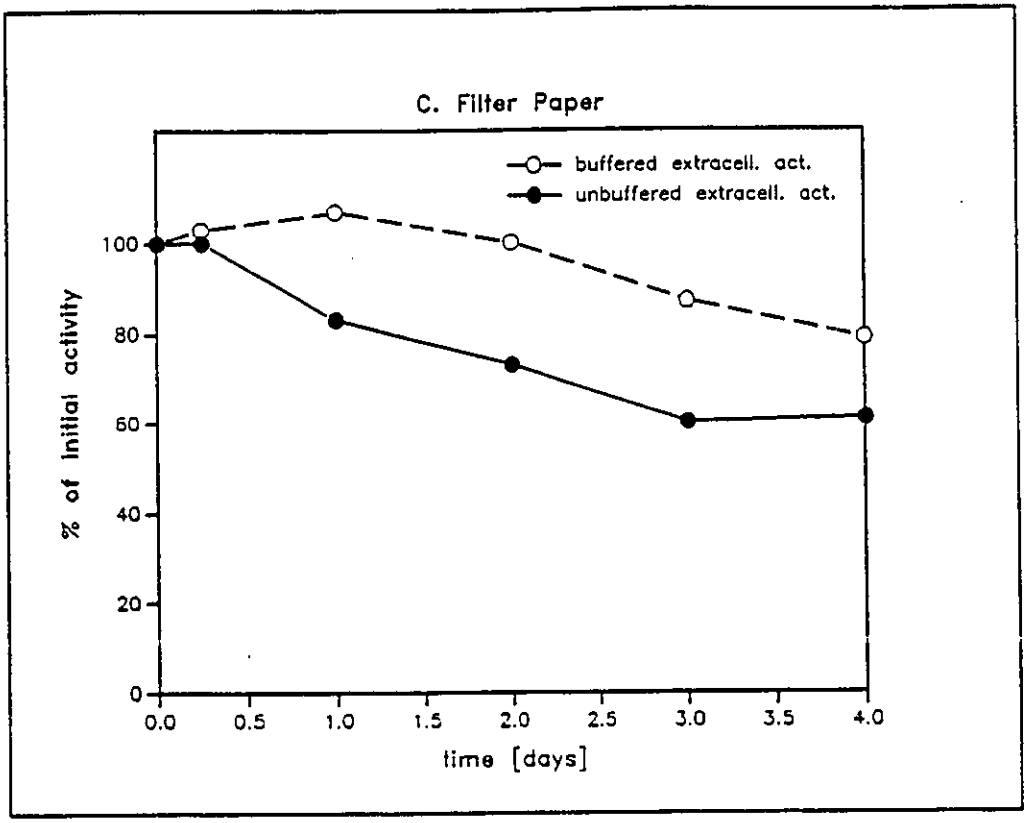
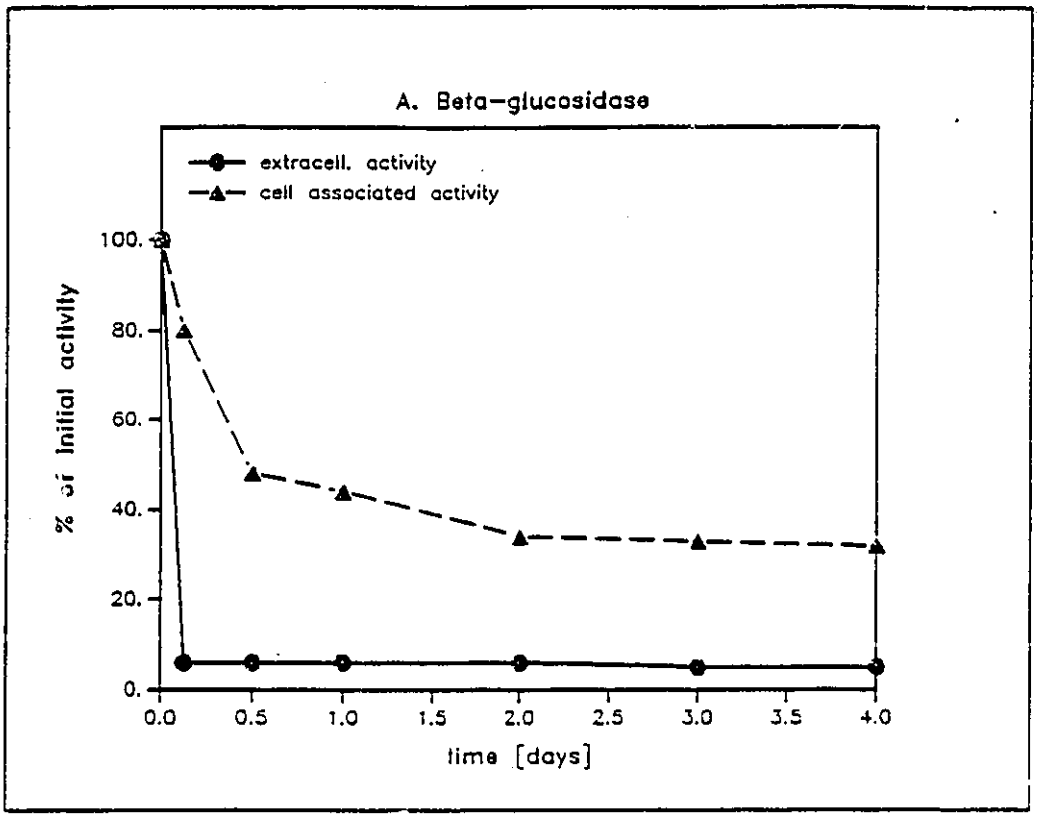


FIGURE 14. Temperature–Time stability of A.terreus C304D extracellular and cell associated A) Beta-glucosidase, B) Endoglucanase and C) filter paper activities at 50 deg C. Both culture filtrates and cell assoc fractions were buffered or unbuffered


C. Filter Paper
buffered extracell. act.
unbuffered extracell. act.
% of initial activity
100
80
60
40
20
0
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
time [days]



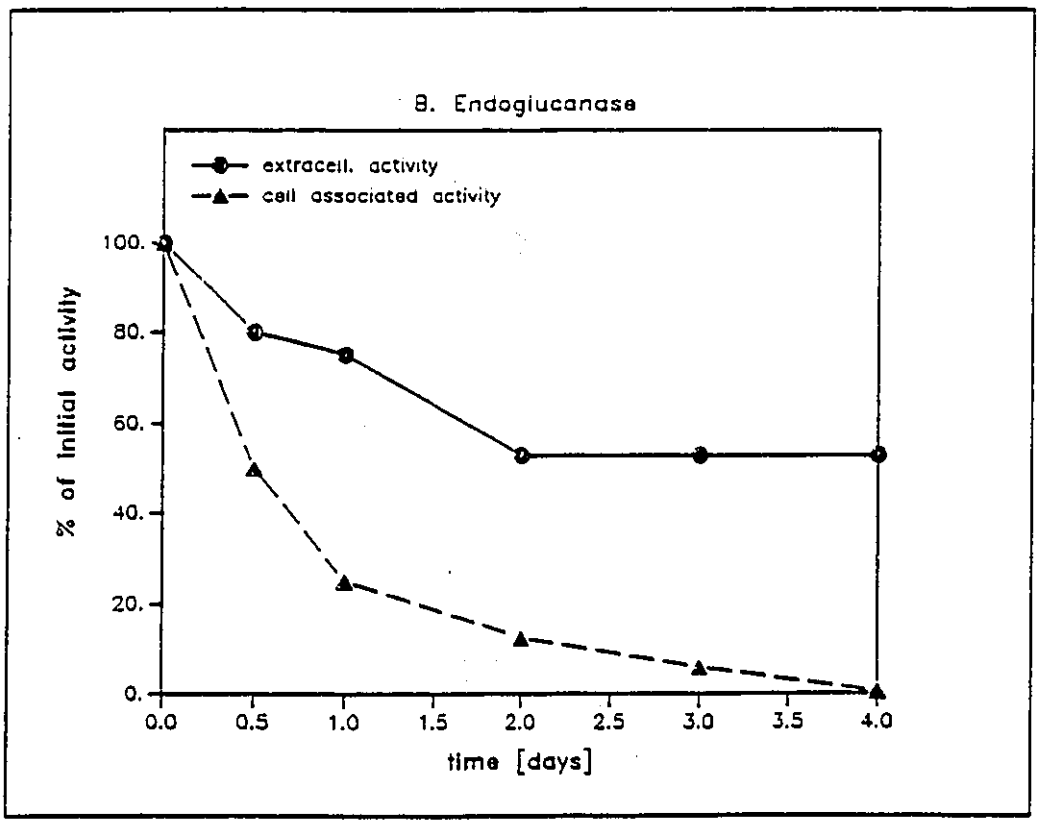


FIGURE 15. Thermostability of the extracellular and cell associated A. Beta-glucosidase and B. Endoglucanse activities of Aspergillus terreus C304D at 60 deg C.

this enzyme (Woodward and Wiseman 1982, Gokhale et al 1984, Shamala and Sreekantiah 1986). It is probable that B-glucosidase preparations from T.terrestris could also be used in the same manner as filtrates from Aspergillus which are currently used to supplement B-glucosidase deficient cellulase preparations. When the endoglucanase activity of A.terreus was examined, the cell associated activity proved to be more temperature labile (fig. 13). The extracellular endoglucanase maintained 50 % of its original activity while only low levels of activity were detected in the cell associated fraction after incubation at 60°C for 4 days. It is possible that the cell associated activity, once isolated from the more controlled environment within the cell, is less stable. Another factor which should be considered is the possible influence of proteases present in the crude enzyme mixture which could either decrease or increase the activity over prolonged incubation.

Although the buffered and unbuffered filter paper and endoglucanase activities of culture filtrates from T.aurantiacus 235F generally showed similar thermostability profiles, the buffered B-glucosidase fraction showed considerably lower activity than the unbuffered enzyme (fig. 15). When the half-lives of the various cellulase and B-glucosidase activities of T.terrestris culture filtrates were assayed at 60°C (table 8), strain 255B was shown to have lost more than 40 % of its original activity after 6 h incubation in comparison to the more than 70 % loss of B-glucosidase activity of strain NRRL 8126. When the thermostability of the different cellulase and B-glucosidase activities were compared to the values reported in the literature (Durand et al 1984), the patented NRRL 8126 strain was found to be significantly less thermostable than had been stated

TABLE 8. HALF-LIVES OF VARIOUS CELLULASE ACTIVITIES OF CULTURE FILTRATES FROM THREE STRAINS OF *Thielavia terrestris* INCUBATED AT 60°C.

STRAIN OF *T.terrestris*		HALF-LIFE (HOURS) B-GLUCOSIDASE ACTIVITY	ENDOGLUCANASE ACTIVITY	FILTER PAPER ACTIVITY
255B		10	>96	34
NRRL 8126	a)	4	72	96
	b)	8	>72	56
ATTC 26917	c)	>40	5	5

a) values determined in this work (Breuil et al 1986)

b) values taken from Durand et al (1984)

c) values taken from Margaritis and Merchant (1983)

in the patent (Skinner and Tokuyama 1978). Although the enzymes of T.terrestris 255B were less thermostable than those of T.aurantiacus, they were still considerably more stable than enzymes from mesophilic fungi such as T.harzianum.

Previously we had demonstrated that the cellulase activity, as determined by incubation with substrates such as filter paper or carboxymethylcellulose, was not representative of the long term hydrolysis that is invariably required to obtain the best possible hydrolysis of native cellulosic substrates (Saddler et al 1985). It could be expected therefore that cellulase systems which are more thermostable would demonstrate a higher hydrolytic potential over prolonged incubation time. Batch hydrolysis of pure cellulose (2 % Avicel and Solka floc) was conducted at 60^{o}C for various time periods (table 9). Solka floc was probably more readily hydrolyzed than Avicel because of its less crystalline structure. Both strains of T.terrestris showed good hydrolytic activity resulting in about 40-50 % saccharification of the Solka floc within 12 h. Neither of the T.aurantiacus strains exhibited good hydrolytic activity, probably due to their low B-glucosidase and filter paper activities. It has been previously shown that a good equilibrium of all cellulase components is essential for an effective hydrolytic cellulase complex (Beldman et al 1988). It is possible that if either strain 235F or 235E were combined with, for example, T.aurantiacus Miehe which is a high B-glucosidase activity producer, (Bedino et al 1985), the hydrolytic activity of the mixed culture would be greatly enhanced. Mixed cultures with improved cellulolytic characteristics have already been reported (Trivedi and Ray 1985).

TABLE 9. COMPARISON OF THE HYDROLYTIC ACTIVITY OF THERMOPHILIC FUNGI OVER PROLONGED INCUBATION WITH TWO CELLULOSIC SUBSTRATES.

ORGANISM	SUBSTRATE (2%)	INCUBATION TIME (HOURS)	%HYDROLYSIS	GLUCOSE RELEASED (mg/mL)	TOTAL REDUCING SUGARS RELEASED
Thielavia terrestris 255B FP=7.5[a]	Solka floc	6	38.5	4.66	7.70
		12	48.2	6.38	9.64
		24	62.6	7.72	12.52
	Avicel	6	19.8	2.82	3.96
		12	27.6	3.78	5.52
		24	35.9	4.90	7.18
		48	45.8	6.36	9.16
Thielavia terrestris 255C FP=15[a]	Solka floc	6	31.2	4.16	6.24
		12	40.6	5.70	8.12
		24	53.7	7.16	10.75
		48	56.1	8.20	11.22
	Avicel	6	18.1	2.58	3.62
		12	25.1	3.72	5.02
		24	31.0	4.28	6.20
		48	31.7	4.20	6.34
Thermoascus aurantiacus 235F FP=15[a]	Solka floc	6	20.0	1.66	4.00
		12	33.5	2.72	6.70
		24	37.1	4.32	7.42
		48	45.2	5.48	9.04
	Avicel	6	2.9	0.52	0.58
		12	8.9	0.88	1.78
		24	11.0	1.40	2.20
Thermoascus aurantiacus 235E FP=2.6[a]	Solka floc	6	1.2	0.38	0.42
		12	2.0	0.58	0.60
		24	9.1	1.12	1.82
		48	11.9	1.64	2.38
	Avicel	6	1.0	0.14	0.20
		12	1.4	0.12	0.28
		24	2.3	0.28	0.46
		48	4.0	0.56	0.80

[a] - Filter paper activity per gram of substrate used in the hydrolysis experiment

II.III CONCLUSIONS

Although several thermophilic fungi were succesfully isolated from wood chip piles the isolates from the Forintek culture collection were shown to produce higher levels of the various cellulase activities. All of the strains released a complete cellulase system capable of hydrolysing cellulose efficiently at high temperatures. Although there were considerable differences in the temperatures at which the mesophilic and thermophilic fungi could optimally grow, there was little difference when optimum temperatures for cellulase activities were compared. The level of enzyme production by the thermophilic fungi was not significantly higher than that of the mesophiles with the exception of high B-glucosidase levels produced by Thielavia terrestris 255B. Highest levels of all cellulase activities were obtained after the active growth phase, during the stationary phase and the onset of sporulation.

As previous work on the enzymatic hydrolysis of starch had utilised thermophilic bacteria and actinomycetes to obtain thermotolerant amylases capable of fast, efficient hydrolysis of starch at elevated temperatures, we had hoped that a similar strategy could be followed to obtain a more efficient cellulase system. Although the thermophilic cellulases had considerably longer half-lives than the mesophilic enzymes, using the traditional cellulase assays, there was no apparent differences in the activities of the two types of cellulases. However when a longer term hydrolysis was carried out, the benefit of utilizing a thermotolerant cellulase system was more apparent. As any bioconversion process is likely to involve hydrolysis at high substrate concentrations over prolonged

times it is probable that a thermophilic cellulase system would prove to be of as much value as the thermophilic amylases presently used in the starch conversion industry.

It was apparent that the composition of the cellulase system was probably at least as important as the relative thermostabilities of the various enzyme components. All of the components of the thermophilic cellulase mixtures retained most of their activity at elevated temperatures and were more hydrolytic than T.harzianum.

After the initial screening and assesment of the various thermophiles, Thielavia terrestris 255B and Thermoascus aurantiacus 235F were found to produce effective cellulases with good thermal stability characteristics. As the next objective of this thesis was to relate cellulase production with mycelial biomass, T.terrestris was subsequently used to compare various methods of estimating fungal biomass.

III. QUANTIFICATION OF FUNGAL GROWTH AND ITS CORRELATION WITH ENZYME PRODUCTION

Biomass concentration is one of the most fundamental fermentation process variables. There are many methods available for quantifying fungal growth in liquid cultures (Calam 1969). They include: wet or dry weight measurements, absorbance, total protein or nitrogen content measurements, substrate consumption and a range of alternatives based on various physiological and metabolic activities. When filamentous cellulolytic fungi are grown on insoluble cellulosic substrates, a practically inseparable mixture of the fungal cell mass and the substrate is formed, thus excluding the possibility of making quantitative determinations of the biomass of the microorganism based on weight measurements.

Although it would appear easier to measure bacterial biomass, there is still no routine, reliable method for the direct determination of bacterial cell biomass other than direct weighing of the cells (Bratbak and Dundas 1984). Usually the estimates are made by converting bacterial biovolume into inorganic carbon (Fuhrman and Azam 1980).

The standard method of monitoring cell concentration is direct determination of cell weight or volume after separation from the culture fluid and drying at temperatures of about 100^{o}C. However, this is a time consuming and impractical method for quantifying cells present in fermentation media containing insoluble material. Several techniques measure optical properties of the cell suspension. However these methods are influenced by several factors such as growth

on optical surfaces, air bubbles in the sensing device, changes in culture morphology, nonhomogeneity of culture samples, high levels of colouration of media or the influence of suspended solids. In the last decade, attention has focused on procedures providing indirect estimates by measuring process variables associated with culture growth. These include the measurement of culture viscosity (Shimmons et al 1976), carbon dioxide evolution (Park et al 1983) or oxygen uptake and mass balance (Zabriskie and Humprey 1978). Indirect methods include quantification of typical cellular compounds such as proteins, sterols, nucleotides (ATP, NAD) or nucleic acids.

In many cases extracellular microbial enzyme activities are reported as corresponding to the volume of media or the amount of cell growth required to achieve the indicated enzyme activity. Unfortunately, when filamentous fungi are grown on cellulosic substrates, there are few methods that can provide representative and reproducible values for fungal biomass. For this reason most studies with cellulolytic fungi do not indicate the corresponding fungal growth associated with the cellulase production values that are reported. Methods such as total nitrogen, chitin, protein, or ergosterol content provide indirect ways of measuring fungal biomass.

Immunological methods have been used to both detect and quantify pathogenic fungi on plants (Casper and Mendgen 1979) and in wood (Breuil et al 1988). We have examined the feasibility of using an enzyme-linked immunosorbent assay (ELISA) for quantifying the growth of T.terrestris 255B on soluble and insoluble substrates as well as the reliability of several indirect chemical indices. In this part of the work we have tried to determine the most

reliable procedure for quantifying fungal growth and relate the calculated biomass values to the corresponding cellulase activities.

III.1. MATERIALS AND METHODS

Microorganism and cultivation conditions

T.terrestris 255B was obtained from the Forintek Culture Collection. This thermophilic ascomycete was pregrown in shake cultures (200 rpm) for 48 h at 45°C, in a complex medium containing; 0.2 % yeast extract, 0.1 % peptone, 0.02 % $MgSO_4x7H_2O$, 0.01 % $CaCl_2$ x $2H_2O$ and 2 % glucose (Breuil et al 1986). After 2 days of growth the cells were harvested, washed in acetate buffer (pH 4.8), macerated in a blender and a mycelium inoculum of 5 mL (approx. 5 mg/mL dry weight) was used to initiate growth. The medium used for cellulase production contained 0.2 % $(NH_4)_2SO_4$, 0.27 % KH_2PO_4, 0.039 % $CaCl_2x2H_2O$, 0.03 % $MgSO_4x7H_2O$, 0.1 % peptone, 0.1 % yeast extract, 0.0005 % $FeSO_4x7H_2O$, 0.0002 % $MnSO_4xH_2O$, 0.0001 % $ZnSO_4x7H_2O$, and 0.0004 % $CoCl_2x6H_2O$ (Mandels et al 1971). The medium was buffered with 3 g/L potassium phthalate and supplemented with 2 % cellulose, using either Avicel or steam exploded aspenwood, which had been water extracted (SEAW) as the substrate (Saddler et al 1982). T.terrestris 255B was incubated for 4 days in the complex medium and a week in the cellulolytic media at 45° and 44°C respectively (Breuil et al 1986). The samples from peptone yeast glucose medium were harvested at 12,24,48,72 and 96 h. Samples from the cellulolytic media were harvested every 24 h over a 7 day period. Each sample was harvested in 4 replicates. All samples were freeze-dried for storage

to ensure that the same material was available for all biomass estimating procedures.

Methods for determining fungal biomass

The dry weight method. As fungal growth is not very homogeneous, the contents of a whole flask were always harvested. The total volume was measured, then the culture was filtered under vacuum through Whatman #1 filter paper and dried in an oven at 105°C, for 4-6 h. The dried samples were cooled in a dessicator and the dry weight was measured.

The biuret method for protein determination (Herbert et al 1971). Sodium hydroxide was first added to the protein, followed by an aqueous $CuSO_4$ solution. The mixture was centrifuged and excess copper was removed as the precipitated hydroxide leaving the coloured Cu-protein complex in solution. This method allows a large excess of $CuSO_4$ to be added while still giving an almost colourless blank solution. The biuret colour is strictly proportional to the amount of protein added. Crude protein was calculated as described by Bailey and Ollis (1977).

The Kjeldahl method for determination of protein (Kjeldahl 1883, Pomeranz and Moore 1975) consisted of three steps: 1. The organic nitrogen was converted to an ammonium salt by digestion (45 min at 450°C) with 5 mL concentrated (95.9 %) sulphuric acid. 2. The ammonium salt was converted in 1 % boric acid with bromocresol green and methyl

red indicator solution to ammonia, which is distilled and recovered quantitatively in an acid solution (0.1 N HCl). 3. The ammonia in the distillate was determined by titration of the excess acid with standard base (40 % NaOH). This analysis was made in the Kjeltek Auto 1030 Analyzer. The organic nitrogen content was obtained for each sample by subtracting the inorganic nitrogen from the total nitrogen content. The total protein was calculated as total N x 6.25 (Herbert et al 1971, Trivedi and Ray 1985, Royer and Nakas 1987).

For ergosterol quantification the method of Seitz et al (1977) was used. A sample of 200 mg on a dry weight basis was blended (Bronson sonifier, cell disruptor 200) with 30 mL of methanol for 2 min. The probe used for sonification was rinsed with 5 mL of methanol and the combined mixture was centrifuged for 25 min (10000 rpm). The supernate was collected in 250 mL flat bottom flasks. The pellet (cake) was resuspended in 15 mL of methanol and blended for 1 min, the probe was rinced again with 5 mL of methanol and the sample was centrifuged for 25 min (10000 rpm). When 0.8 g of potasium hydroxide and 18 mL of 95 % ethanol had been added to the combined supernates, the mixture was refluxed for 30 min., cooled, diluted with 20 mL of water, and extracted three times with pentane (3 x 50 mL). The combined pentane extracts were evaporated to dryness, the residues were taken up in methylene chloride-isopropanol (99:1), dried under nitrogen and made up to 1 mL with methanol-methylene chloride (1:1). High performance liquid chromatography (HPLC) analysis was carried out with ergosterol eluted at 3 min. at

When the biuret, Kjeldahl and ergosterol methods were used, the total biomass was calculated for the fungal mycelium grown on lignocellulosic substrates. These chemical indices measure only a part of the fungal mycelium such as the protein or ergosterol content. To determine the protein and ergosterol content of fungi grown on lignocellulosic substrates, controls of the fungi grown on an equivalent amount of glucose were also assayed. The values for both protein and ergosterol content in the mycelium obtained from the glucose medium were applied in the calculation of total fungal biomass on solid substrates. The protein and ergosterol values obtained on the solid substrates were divided by the "factors" of 17 % and 0.62 % respectively (the latter values had been previously determined after growth on soluble substrates). The total biomass values were expressed as a percentage.

The enzyme-linked immunosorbent assay (ELISA). The procedure of Breuil et al (1988) was followed. T.terrestris 255B was grown in a liquid medium containing malt extract 10 g/L, yeast extract 4 g/L and glucose 4 g/L. Cultures were grown for 2 or 3 days on a rotary shaker at 44^{o}C. The mycelium was removed by centrifugation, washed 3 times with 0.01 M sodium phosphate buffered saline (PBS) pH 7.4 and broken in a hand homogenizer with PBS. Broken mycelium was resuspended in 0.3 % formalin for 2-3 hours at room temperature. This preparation was then washed 3 times with PBS (10,000rpm, 15 min, 10^{o}C), resuspended to its original level in PBS (dry weight of 20 mg/mL) and frozen in small aliquots. Frozen aliquots were transferred on the peptone yeast glucose medium plates and left at 37^{o}C for 3 days

to see if formalin killed the fungus (no fungal growth was observed). When antigens from the mycelia were required for immunization, 0.25 mL mycelium in PBS (5 mg dry weight) of a frozen aliquot was emulsified with an equal volume of Freud's complete adjuvant. This antigen was then injected into the biceps femoris and deltoid muscles of a New Zealand white rabbit. After 2 to 3 weeks the rabbits were challenged with a single injection of fungi into the biceps femoris muscle using incomplete Freud's adjuvant. The rabbits were bled out two weeks after the final injection. As a control, pre-immune serum was taken from the marginal ear vein of each rabbit before inoculation. Antiserum was stored at -20^{o}C. The method followed for the enzyme linked immunosorbent assay (ELISA) was that of Voller et al (1979), using polystyrene microtiter plates (Dynatech Immulon 1). In the ELISA, plates were coated with hand homogenized mycelia (standard curve) or with freeze dried preparations of fungal mat (cells grown in peptone yeast glucose medium, on cellulose and wood), ground by a mortar and pestle in sodium carbonate at pH 9.6. Dilution series of each sample were replicated 5 to 6 times. Antiserum and conjugate (goat antirabbit IgG labelled with horseradish peroxidase from BioRad) were used routinely at 1/1000 and 1/2000 dilutions respectively.

The antigen was generally used at an optimum and minimum concentration of 15 ug/mL and 0.5 ug/mL dry weight respectively. Casein, 0.5 % (w/v) in sodium phosphate buffered saline (PBS) pH 7.4, was used as a diluent for sera and conjugates. The microtiter plates were coated with 100 uL antigen in 0.1 M sodium carbonate buffer pH 9.6 and stored overnight at 37^{o}C. The wells were washed 3 times with PBS containing 0.5 % casein (Kenna et al 1985).

Plates were then incubated with the anti-Thielavia serum, diluted 1/1000 for 2 hours at 37^{o}C. After washing, goat antibody to rabbit immunoglobulin G, conjugated with horseradish peroxidase (Bio Rad) and diluted 1/1000 was added and the plates were incubated for a further 2 h at room temperature. The wells were washed with 0.2 % Tween 20 in PBS and incubated with 100 uL substrate solution (50 mL citric acid / Na_2HPO_4 buffer at pH 5.0 with 17 mg o-phenylenediamine and 20 uL 30 % H_2O_2). The reaction was stopped after 5 minutes by adding 100 uL 1 N H_2SO_4. The absorbance values were read on a BIOTEK EL310 autoreader (Mandel Scientific) at 490 nm.

At the time of harvesting the corresponding cellulase values were determined using the Nelson (1944) and Somogyi (1952) assay. Each experiment was done in duplicate. All results from the biomass determination were statistically analyzed using the Statistical Analysis System (SAS) available on the Vax computer system.

III. RESULTS AND DISCUSSION

When filamentous fungi are grown in submerged culture, the type of growth patterns observed varies from the "pellet" form, consisting of compact discrete masses of hyphae, to the "filamentous" form, in which the hyphae form a homogenous suspension dispersed through the medium (Camici et al 1952, Pirt 1966, Whitaker and Long 1973, Metz and Kossen 1977). T.terrestris 255B represents an intermediate form of growth consisting of a light pellet mass of hyphae dispersed in the medium.

The first growth pattern presentation of filamentous fungi was reported in 1924 when an individual hyphae was shown to grow exponentially. Since that time it has been established that the mass of a fungal colony follows the exponential law during colonial growth (Trinci 1970, 1972, 1974 and 1983, Koch 1975, Granade et al 1985). The growth of T.terrestris 255B was followed for four days on glucose. The growth curve measured by the dry weight showed rapid growth during the first day, followed by a slower growth phase reaching a maximum at day 3 (fig.17A). Thereafter, there is a stationary phase where there is no further glucose available for further growth and the mycelium is observed to start to autolyse. The curves of the total cell protein measured by the biuret and Kjeldahl methods indicated that the highest protein values were also reached after 3 days growth. Maximum values of 1.9 mg and 1.7 mg protein/mL culture were obtained by the biuret and Kjeldahl methods respectively (fig. 17A). The amount of protein detected was not constant during growth (table 10), with the Kjeldahl method showing more variability. At 12 h a high figure of 30 % protein content was detected. This abnormally high value was

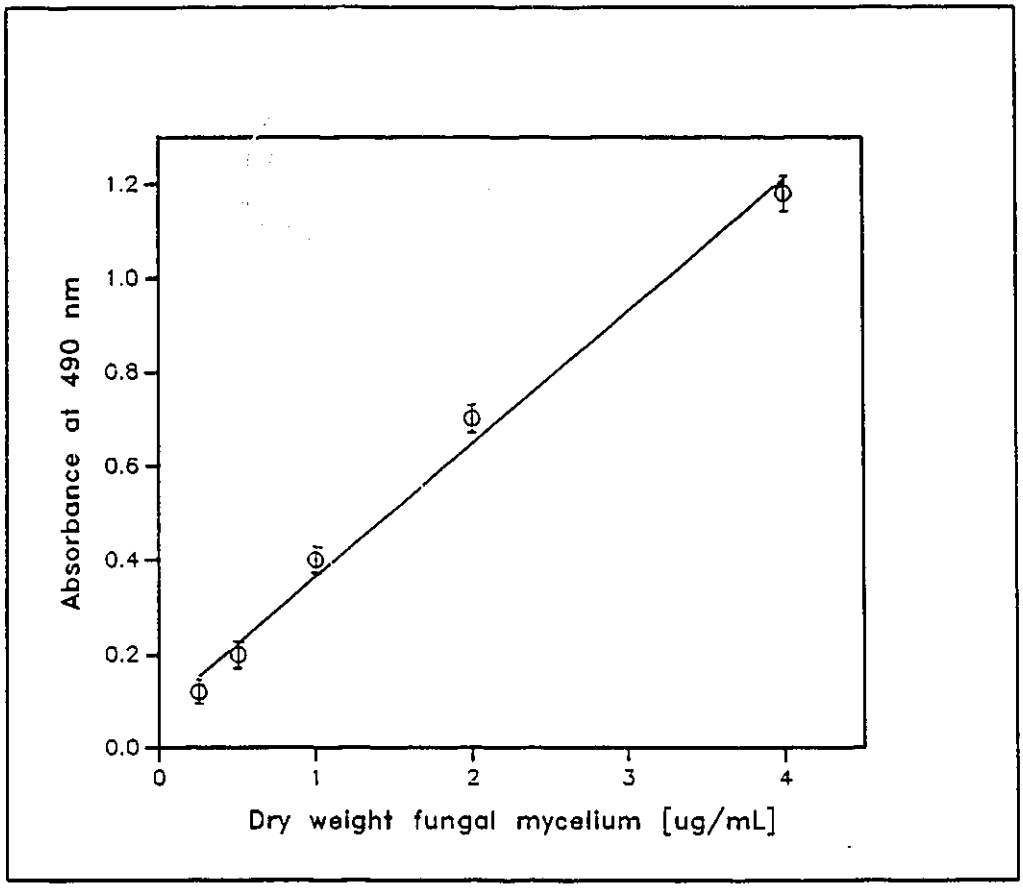
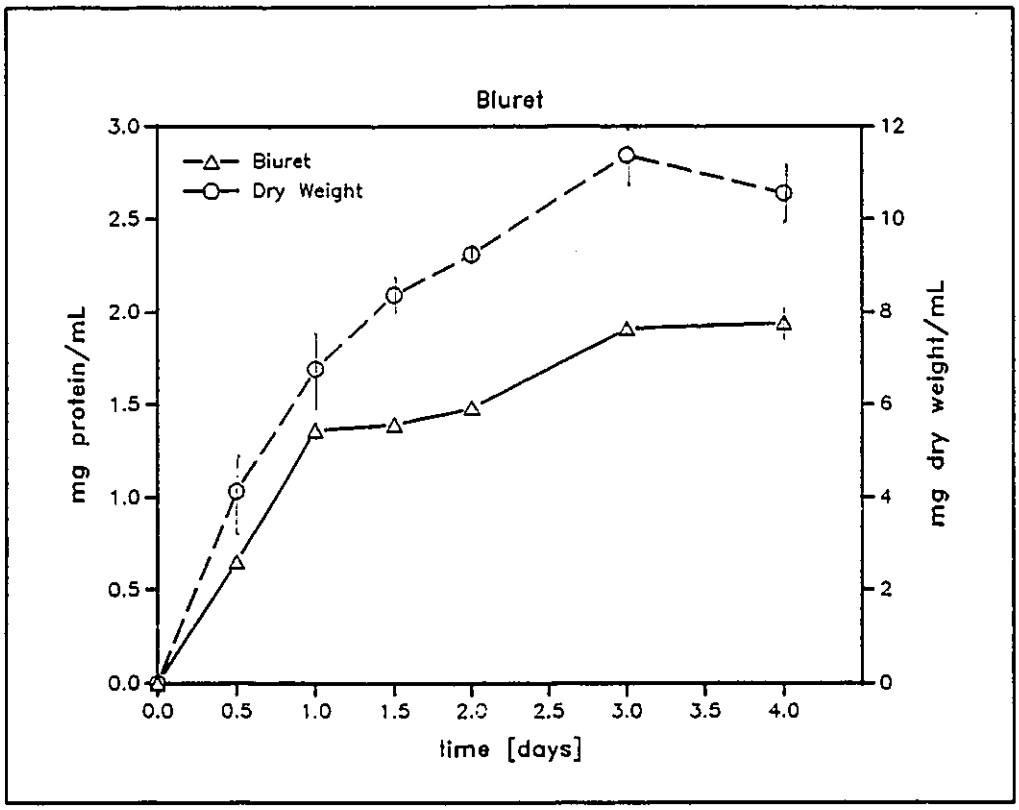


FIGURE 16. Standard curve for quantitation of mycelium using ELISA method, calculated from a dilution of cell fragments of Thielavia terrestris 255B. Coefficient of regression r=0.99



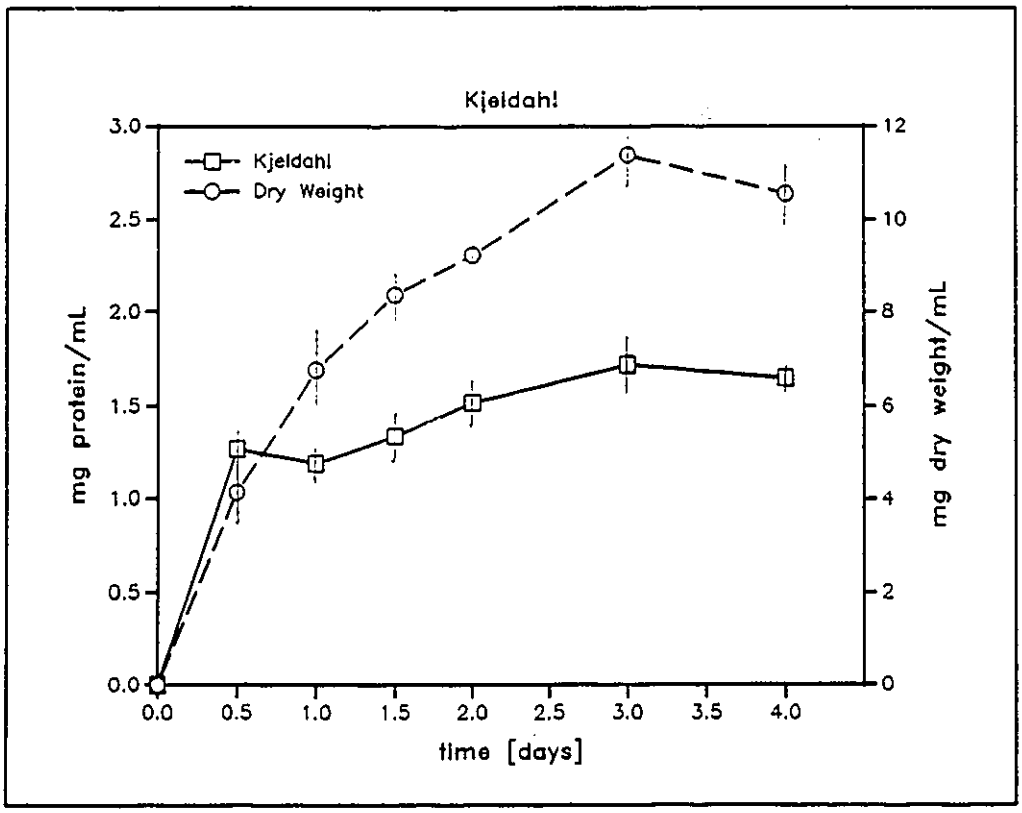
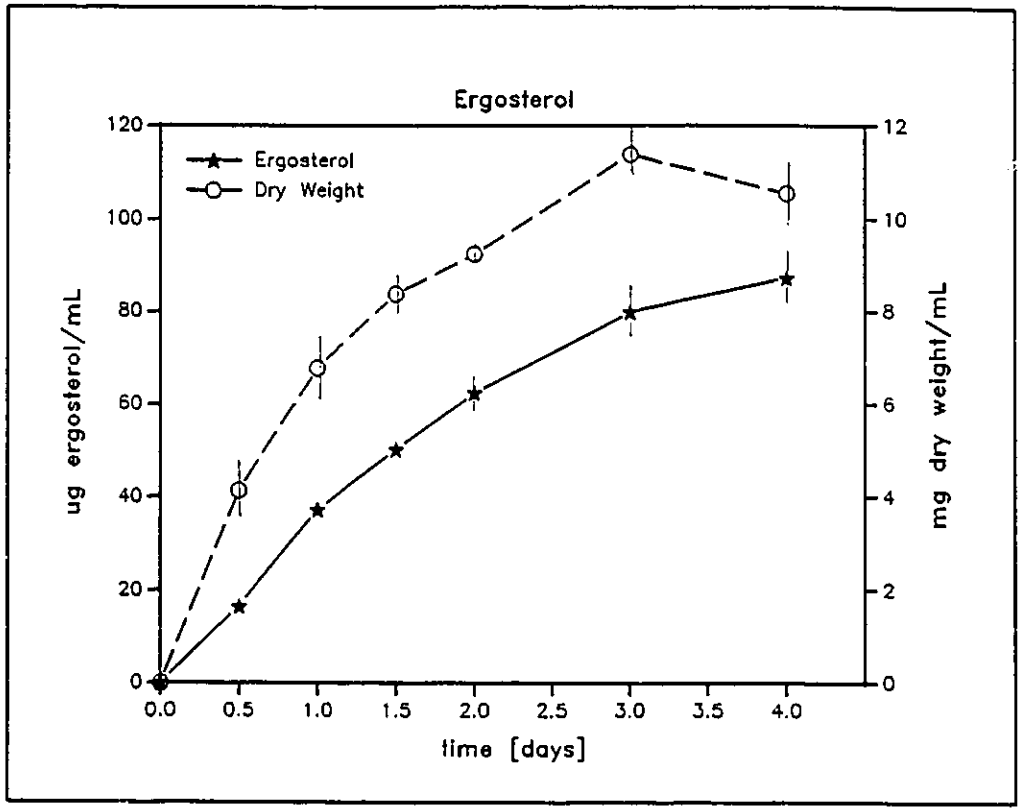


FIGURE 17A. Comparison of the Biuret and Kjeldahl methods of determining the fungal biomass of Thielavia terrestris 255B grown on peptone yeast glucose medium.



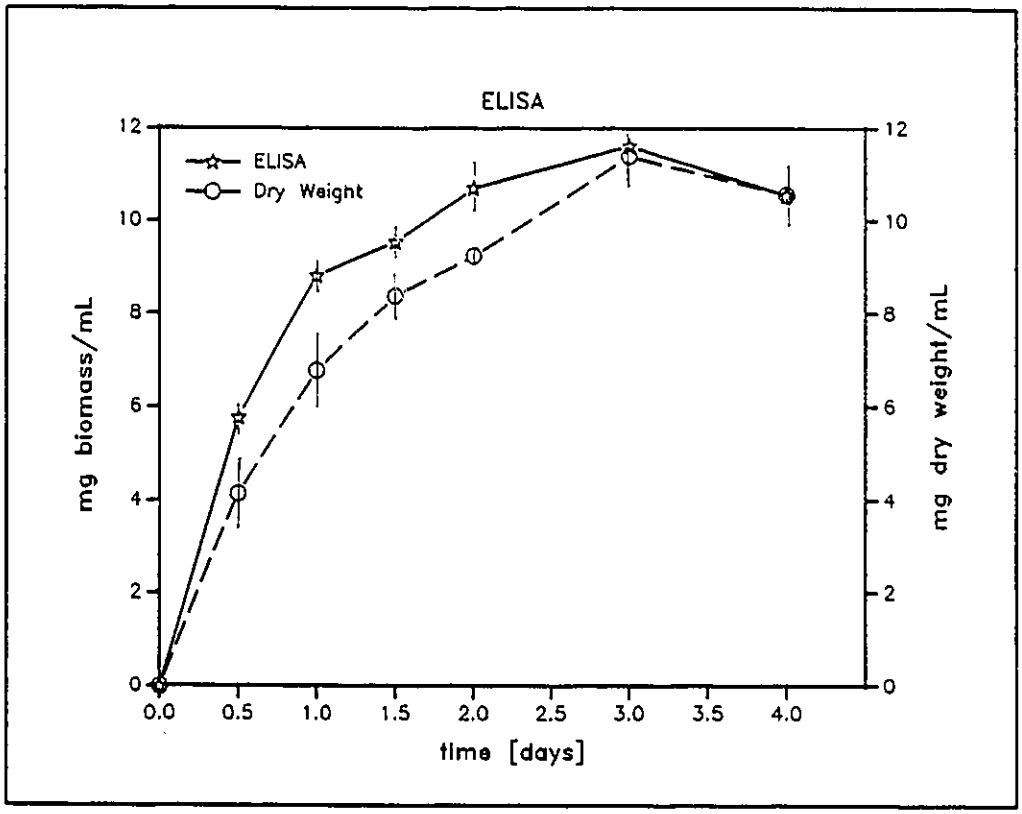


FIGURE 17.B. Comparison of the Ergosterol and ELISA methods of determining the fungal biomass of Thielavia terrestris 255B grown on peptone yeast glucose medium.

TABLE 10. COMPARISON OF DIFFERENT METHODS FOR DETERMINING PROTEIN CONTENT OF T.terrestris 255B GROWN ON GLUCOSE.

	% PROTEIN	
TIME (days)	BIURET	KJELDAHL
0.5	17	(30)[a]
1.0	19	18
1.5	17	16
2.0	16	16
3.0	17	14
4.0	18	15
Average value	17	16

a) - although this exceptionally high value was obtained in duplicate experiments it was not included in the calculation of the mean

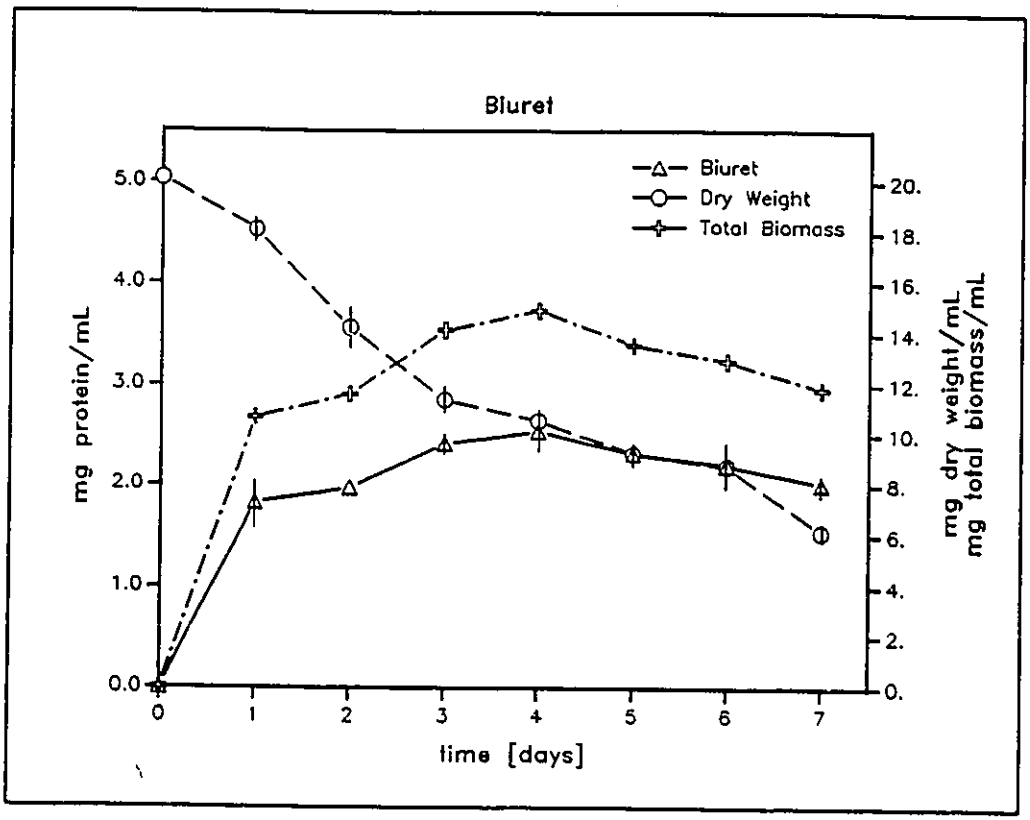
obtained in duplicate experiments. This high value could be due to non protein nitrogen such as nucleic acids, especially RNA (McGetric and Bull 1979, Koliander et al 1984). As this phenomenom did not persist over time this artificially high value was not considered in the average value calculations. The presence of chitin could also influence the Kjeldahl values determined during growth. Its content in the cell wall varies from 1-25 % depending on the species of fungi which is assayed (Johnson and Chen 1983, Kanazawa 1987). The chitin content not only differs between species but also changes within species depending on the age of the mycelium (Sharma et al 1977, Aidoo et al 1981, Matcham et al 1984, Ruperez and Leal 1986). A similar figure for the average protein content of the fungal cells was determined by both the biuret and Kjeldahl methods (17 % and 16 % respectively). Although the mycelium of some fungi show an extremely high (40 %) protein content (Chahal et al 1987), most of the mycelia from celluolytic organisms have a protein content in the range of 16-25 % (Gorkina et al 1982, Rao et al 1984, Jernejc et al 1986, Royer and Nakas 1987). The protein content of the mycelium of T.terrestris grown on a soluble carbon source fluctuated between 16 to 19 %. The average value of 16-17 % is comparable to that observed with other species. The variability in the protein content we observed during growth of the fungi was in agreement with the results of Jernejc et al (1986). They are however contradictory to the results of Schmidell and Fernandes (1976) or Gorkina et al (1982) who claimed that a constant amount of protein was produced in the mycelium of Aspergillus niger or A.terreus. The protein content of fungal mycelia is considerably lower than the protein content of bacteria and

Although the amount of protein detected was much lower than the equivalent dry weight values obtained, the pattern of growth measured by the biuret and Kjeldahl methods were similar (fig. 17A). The growth pattern obtained by the ergosterol method was similar to the dry weight profile (fig. 17B). The ergosterol content which represents less than 1 % of the dry weight of the mycelium, was found to fluctuate from 0.42 % to 0.89 % over the period of growth. These results are similar to the results of Nout et al (1987) who showed that the ergosterol content of Rhizopus oligosporus biomass was influenced by the age of the fungus. Our results differ from the results of Elliot et al (1974) who reported relatively small changes in total ergosterol content with mycelial age. The ergosterol content has also been reported to be influenced by the composition of the substrate (Newell et al 1987, Nout et al 1987). The spectral assay method might also be influenced if the fungus produces other sterols which have similar spectral properties to ergosterol (Woods 1971). On average, the ergosterol content was equivalent to 0.62 % of the dry weight of mycelia.

A mycelial suspension from the frozen stock was serialy diluted and a standard curve was constructed to establish a correlation between the ELISA absorbance readings and the fungal biomass (fig. 16). Consequently a mycelial concentration could be calculated from the ELISA readings, if a standard curve was included in each assay. In the assay using plates coated with cells of T.terrestris 255B grown in peptone yeast glucose medium (fig. 16), the ELISA readings were found to increase in direct proportion to the mycelial dry weight. The ELISA growth curve

followed the same pattern as the dry weight, although the values were slightly higher. With the ELISA there is no requirement for a conversion factor as compared to other chemical indices such as protein or ergosterol. All of the assayed methods detected maximum growth at day 3. Thereafter sporulation and autolysis seem to occur

When insoluble substrates were present in the medium the dry weight curve could not be used for fungal growth estimation as the dry weight measurement gave the combined mycelial weight and the amount of solid substrate still present in the medium. The fungi grown on solid substrate have to use some of the substrate as energy to produce extracellular enzymes. We expected therefore lower growth values on solid substrates than was obtained with growth on glucose. On Avicel, the biuret protein curve showed that the fungus was growing actively during the first day (fig. 18A). Thereafter the protein values slowly increase until day 4 when a maximum value of 2.5 mg protein/mL was reached. The protein curve determined by the Kjeldahl method showed an actively growing phase during the first 2 days followed by gradual decrease in protein values (fig. 18A). The total fungal biomass was calculated using protein estimates obtained from the yeast glucose medium (see p.79 for details). Comparable peak protein values (2.5 and 2.4 mg/mL) were obtained by both the biuret and the Kjeldahl measurements, however these values were detected at days 4 and 2 respectively. In addition the calculated values for both measurements overestimated the total biomass as the residual dry weight values (the fungus + residual cellulose) were lower than the biomass values determined by the protein content (fig. 18A). A possible explanation is that the percentage of protein present in the mycelium grown on Avicel is higher than that obtained



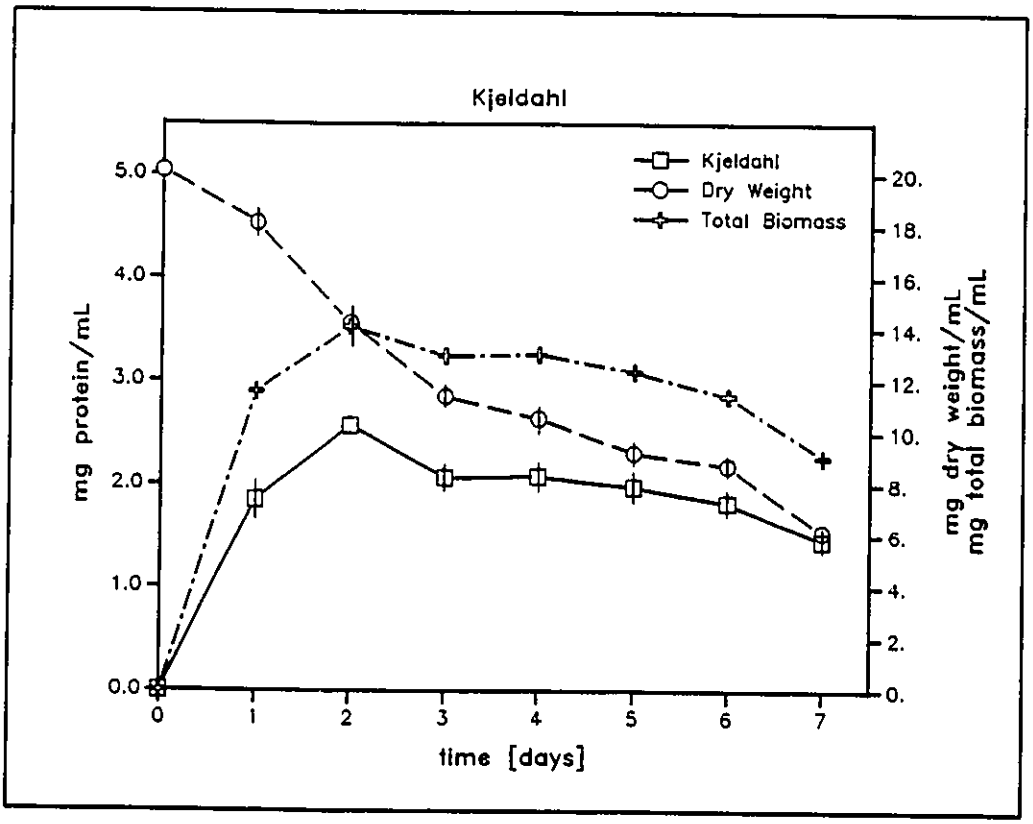
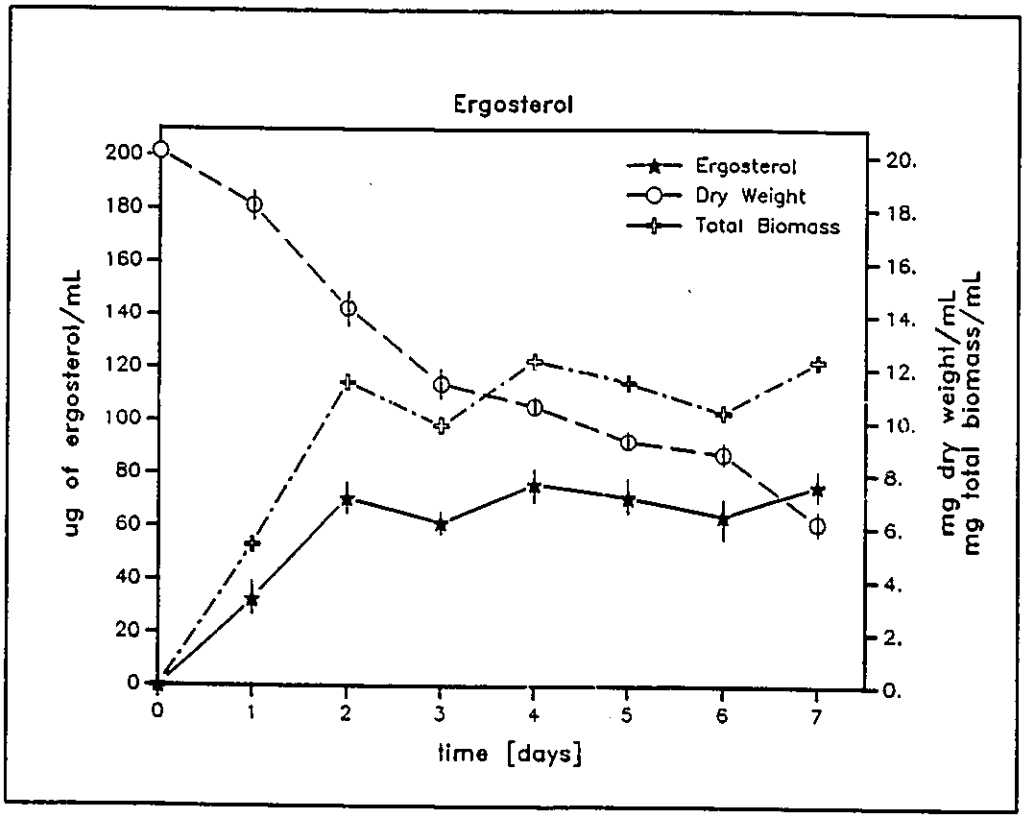


FIGURE 18A. Estimation of biomass of Thielavia terrestris 255B grown on 2% Avicel by the Biuret and Kjeldahl methods.



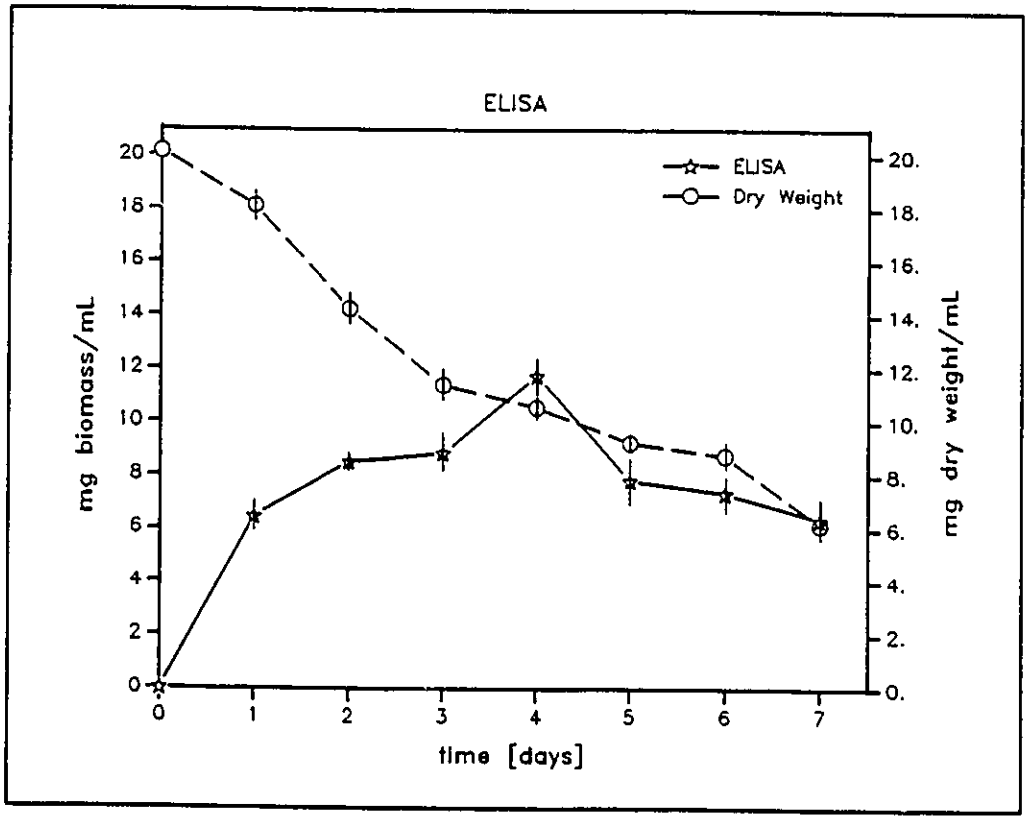


FIGURE 18B. Estimation of biomass of Thielavia terrestris 255B grown on 2% Avicel by the Ergosterol and ELISA methods

when the fungus is grown on a soluble carbon source. Previously it had been shown that the protein content of fungal mycelium varied with the carbon source used for growth (Shaker et al 1985, Kassim and Ghazi 1987). This was the most probable explanation as the morphology of the mycelium was observed to be different in the two media. The hypha was much thicker in the peptone yeast glucose medium than when it was grown in the cellulolytic medium.

The ergosterol curve increased during the first 2 days (reaching 90 % of its maximum value) then levelled off. The highest ergosterol content (76.16 ug/mL) was detected at day 4. The amount of this sterol was not constant during the growth (fig. 18A). When the total biomass was calculated using a figure of 0.62 % as the ergosterol content of the mycelium, it also appeared that the ergosterol method was overestimating the biomass.

With ELISA, it should be noted that the point of maximum growth (11.5 mg/mL) was the only place where the biomass was overestimated since the dry weight, which includes the fungus and the substrate, is only 10.5 mg/mL. However, all the other points are below the residual dry weight and could reflect the actual growth of the fungus. Maximum growth was detected at day 4 by each of the biuret, ergosterol and ELISA methods (Breuil et al 1989).

It was apparent that determining accurate fungal biomass values using a pure cellulosic substrate was more difficult than when using soluble substrates such as glucose. When realistic substrates such as steam exploded aspenwood are used, the components in the wood, such as lignin, extractives, etc, can further complicate the assay by interfering with the reagents or detection methods. When steam treated aspenwood was used as the growth substrate

material within the substrate interfered with the colorimetric biuret assay. As we could not develop a way to eliminate this interference the protein determination of biomass was not measured by the biuret. When the Kjeldahl method was used, the growth of T.terrestris 255B on steam treated aspen wood was generally found to be slower than that obtained when Avicel was used as the substrate (fig. 19A).

The protein values determined by the Kjeldahl method showed a steady increase in protein content until day 6. The total biomass values were calculated using the previously calculated estimate determined after growth on glucose, that 17 % of the fungal dry weight is protein (fig. 19A). The ergosterol profile also showed that growth increased until day 6, followed by a quick drop at day 7 (fig. 19B). With the ergosterol assay, the total biomass values were calculated from the ergosterol content which was previously calculated to be 0.62 % of the dry weight. The total biomass curve showed a similar pattern to the one obtained for ergosterol content.

The fungus was shown to grow more slowly on steam treated wood than on Avicel and the highest ELISA values were observed after 5 days growth (fig. 19B). It was apparent that the ELISA method overestimated the fungal biomass as the dry weight values measured after day 5 were lower then those calculated from the ELISA values. Using the ELISA method, fungal biomass increased until day 5 when a maximum value of 14 mg/mL was observed. The values dropped slightly at day 6 and 7. This tendency to overestimate the values after growth on lignocellulosic substrates could be the result of the antigen chosen to represent the fungi, as the organism was originaly grown in liquid

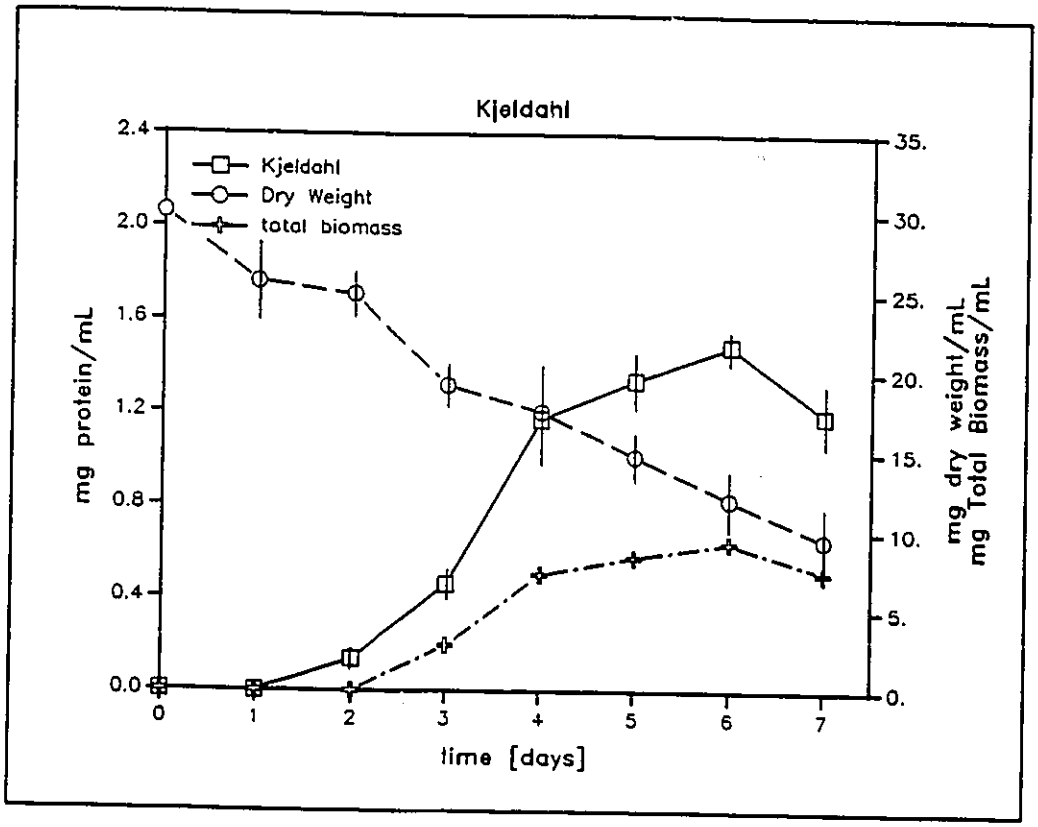
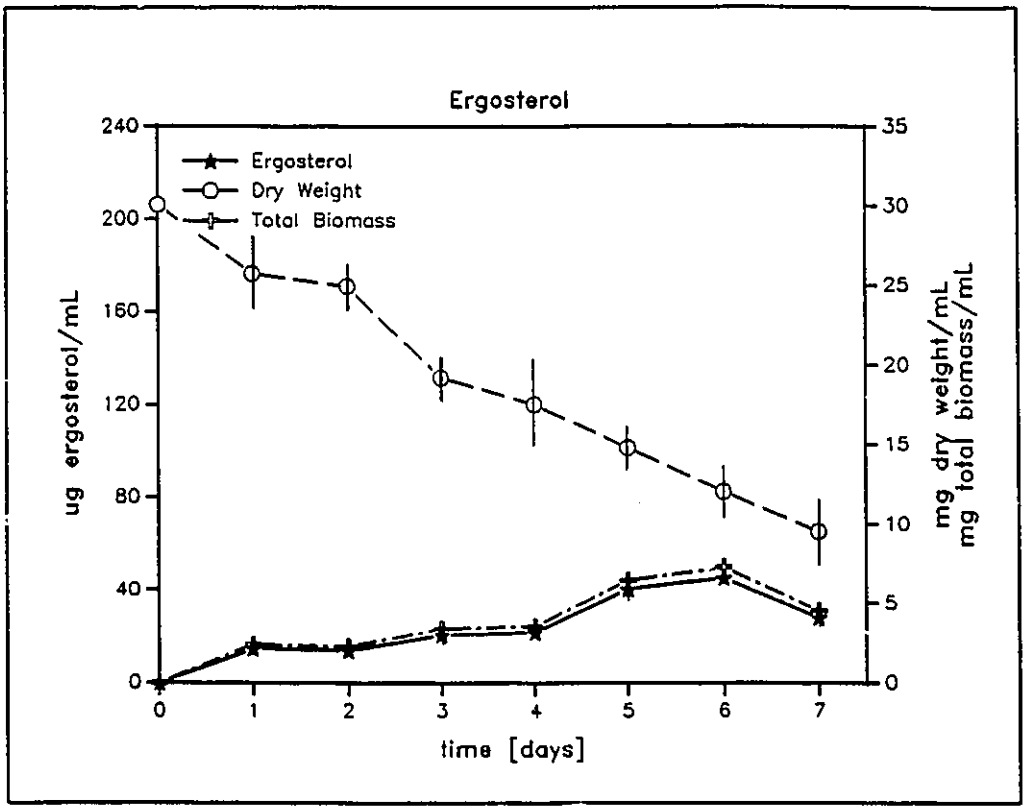


FIGURE 19A. The protein and estimated biomass values of T.terrestris 255B grown on steam treated, water extracted wood (2% cellulose content) by the Kjeldahl method.



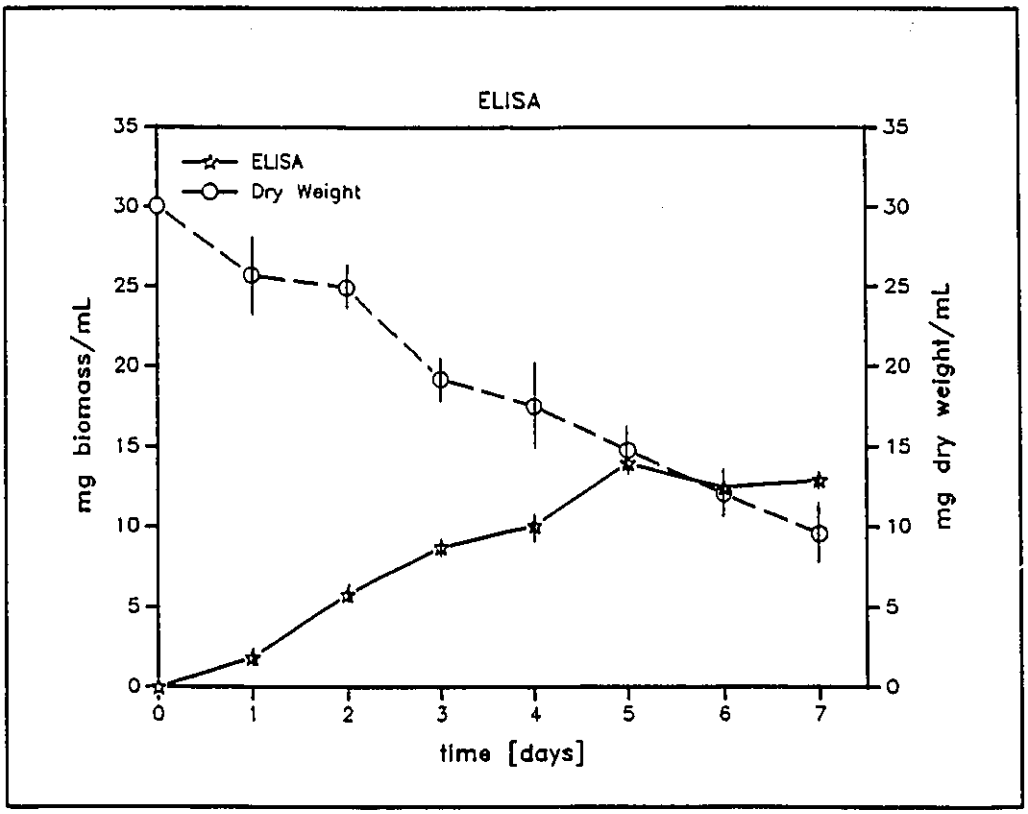


FIGURE 19B. The protein and estimated biomass values of T.terrestris 255B grown on steam treated, water extracted wood (2% cellulose content) by the Ergosterol and ELISA methods.

medium, or the possibility that the antigen was not evenly distributed within the fungal structure (Lloyd et al 1978). It was also likely that the amount of antigen changes with the age and growth rate of the the fungi and the environmental conditions. A part of the problem could also be related to the extraction procedure used to remove the antigens when the fungus was grown on the lignocellulosic substrate. In order to get representative samples the whole contents of each flask was removed, washed, lyophilized and ground. The homogeneous powder was further ground by a mortar and pestle, releasing both cell wall and cytoplasmic antigens. This material reacted with the antibodies giving an enhanced reaction in comparison to the washed sonified mycelium used on the soluble carbon source. Although all of the assay procedures showed an actively growing phase when wood was used as the substrate, different methods expressed different lengths of this phase.

Statistical analysis. The precision of each of the procedures was compared in order to establish their reliability. The precision was defined as an agreement among repeated measurements of the same parameter (Pomeranz et al 1977). Statistically, no significant difference was found between repeated experiments when soluble substrates were used. This, therefore enabled us to combine all replicates and calculate the coefficient of variation values (table 11). An average value between 4.8 - 5.8 were found for the dry weight, biuret, Kjeldahl and ergosterol methods. Only the ELISA method showed low variation. The same parameters were calculated for both insoluble substrates (table 12 and 13). A significant

TABLE 11. THE COEFFICIENT OF VARIATION (%) VALUES FOR FIVE METHODS OF MEASURING FUNGAL GROWTH ON SOLUBLE CARBON SUBSTRATES.

TIME (day)	DRY WEIGHT	BIURET	KJELDAHL	ERGOSTEROL	ELISA
0.5	9.66	2.67	3.57	4.47	3.03
1.0	6.76	4.61	2.24	3.58	2.65
1.5	2.78	7.37	6.82	1.82	2.55
2.0	0.66	5.27	9.33	4.92	3.03
3.0	5.90	9.89	9.41	7.26	2.58
4.0	3.76	3.19	3.73	6.80	5.45
average	4.90	5.50	5.80	4.80	3.20

difference was found between repeated experiments with insoluble substrates which prevented the data from being pooled. Fortunately, similar growth patterns were observed for each method studied in all experiments. Intrinsic variations of between 5.6 and 6.1 % were found for the biuret, Kjeldahl, ergosterol and ELISA methods and 1.6 % for the dry weight method. As was mentioned before, the latter method could not be used when an insoluble substrate was used. All of the methods had similar coefficient of variation values within the range of 3 to 6 %. The biuret and Kjeldahl methods had the same precision on both cellulosic substrates.

An assumption was made that for a precise analytical method the confidence intervals should not be greater than 10 % of the mean. For each method a number of required replicates was calculated (from available data) in order to keep the confidence intervals as narrow as possible (table 14). It seems that the precision of the various procedures was changing with the medium. More replicates are required for the values determined on the complex insoluble substrates. It was also apparent that fewer replicates than actually taken could be used for study on peptone yeast glucose medium.

A statistical comparison between the methods studied showed that none of them was perfect or superior. The precision of the various methods was similar and did not allow us to make any final conclusions. The biuret method, which detects peptide bonds, was found to be as good as the Kjeldahl method for determining protein after growth on soluble substrates and on Avicel. As the biuret method is easy and fast (150 samples can be assayed per day), and does not involve any hazardous chemicals, it may be the method of choice.

TABLE 12. THE COEFFICIENT OF VARIATION VALUES (%) FOR FIVE METHODS OF MEASURING FUNGAL GROWTH ON AVICEL.

TIME (day)	DRY WEIGHT	BIURET	KJELDAHL	ERGOSTEROL	ELISA
1	0.22	5.68	7.50	1.28	6.09
2	2.10	nd	4.45	8.27	5.43
3	2.16	5.52	0.88	8.04	5.25
4	1.41	6.31	6.36	4.97	5.04
5	1.58	7.29	8.76	7.81	7.24
6	2.57	5.15	6.89	9.14	6.42
7	1.73	3.86	nd	3.53	5.05
average	1.60	5.60	5.80	6.10	5.70

TABLE 13. THE COEFFICIENT OF VARIATION VALUES (%) FOR FOUR METHODS OF MEASURING FUNGAL GROWTH ON PRETREATED WOOD.

TIME (day)	DRY WEIGHT	KJELDAHL	ERGOSTEROL	ELISA
1	4.45	nd	nd	3.06
2	1.36	4.69	0.43	2.84
3	3.50	3.24	4.56	3.70
4	6.73	0.81	4.70	3.42
5	4.40	2.95	5.64	2.57
6	7.52	4.90	1.09	5.86
8	4.32	7.90	2.56	8.66
average	4.60	4.10	3.10	4.30

TABLE 14. CALCULATED NUMBER OF REPLICATES REQUIRED TO KEEP THE CONFIDENCE INTERVALS WITHIN 10 % OF THE MEAN.

METHOD	NUMBER OF REPLICATES FOR DIFFERENT MEDIA		
	PYE	AVICEL	SEAW
DRY WEIGHT	2-3 (4)	1-2 (4)	6 (4)
BIURET	4 (8)	4-6 (4)	6 (4)
KJELDAHL	7 (8)	2-3 (4)	3 (4)
ERGOSTEROL	4 (8)	10 (4)	4 (4)
ELISA	2-3 (12)	5-9 (6)	3 (6)

In brackets, a number of replicates applied in our experiments.

PYE - peptone yeast glucose extract

SEAW - steam exploded aspen wood

However, its main disadvantage is that, being a colorimetric assay, it is affected by the colour of the organism and substrates. The Kjeldahl method was also fast as it could assay 150 samples per day. Although the reagents are slightly corrosive it provided a relatively accurate method of estimating fungal growth on Avicel and lignocellulosic substrates. However, as it measured the total nitrogen content, the assay may be affected by nitrogen of non-protein origin present in the organism or media.

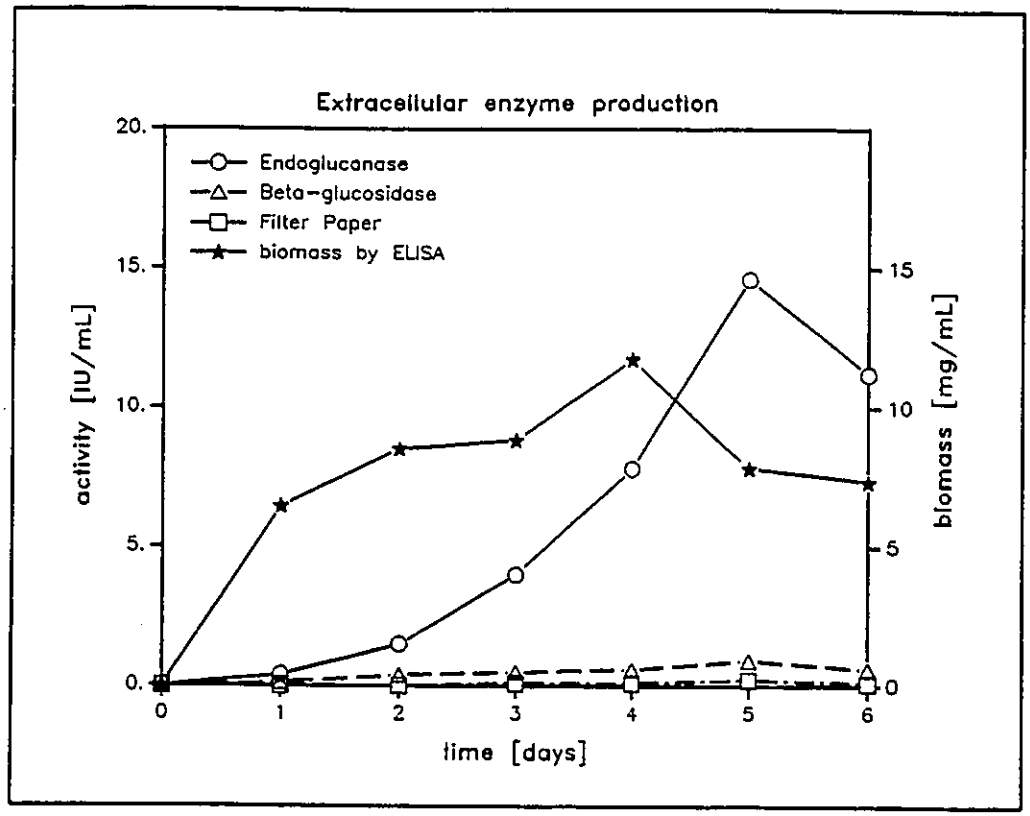
Although the ergosterol method of Seitz et al (1977) was found to accurately reflect the growth of the fungi grown on lignocellulosic substrates, it is a long and moderately toxic procedure. This chemical method has been succesfully used for the measurement of fungal growth in samples containing mixtures of bacteria and fungi. However, this method does not differentiate between fungal species and the ergosterol present in microorganisms of green-microalgae decomposition systems (Newell et al 1987) can also contribute to the ergosterol values.

It seems that all of the methods which measure only a part of the biomass are not completely satisfactory. The dry weight method of determining total biomass does not give quantitative values, carries some practical problems with the sampling of substrates which are not perfectly homogenous and is not suitable for biomass quantification when solid substrates are used. The ELISA is a sensitive, fast procedure capable of detecting microgram quantities of antigen. However, as this is still a relatively new technique which has not yet been routinely applied to measure fungal growth on lignocellulosic substrates it still requires further research before it can become a routine procedure. The choice of any method for biomass determination

should largely depend on the purpose of the biomass quantitation and the availability of equipment.

At the same time that the growth of T.terrestris 255B was measured on solid substrates, the cellulase production was determined using the Nelson and Somogyi reducing sugar assay (Nelson 1944, Somogyi 1952). Cellulase activities in both the extracellular and cell associated fractions were followed. A 2 % cellulose substrate concentration proved to be the most appropriate concentration for comparing cellulase production and fungal growth.
This was also reported recently by Kassim and Ghazi (1987). Although most of the growth on Avicel was obtained after 48 h, only low levels of activity were detected in both the extracellular and cell associated fractions. The highest enzyme levels were obtained at day 5 with extracellular and cell associated endoglucanase values of 14.6 and 5.0 IU/mL and extracellular and cell associated B-glucosidase values of 0.9 and 17.6 IU/mL obtained respectively (fig. 20). Very low levels of filter paper activity were obtained with a maximum value of 0.2 IU/mL obtained for both fractions. After day 5 a drop in enzyme production values was observed. Maximum cellulase activities were not detected as the same time as maximum fungal growth was obtained.

When T.terrestris 255B was grown on steam exploded aspen wood it produced significantly lower amounts of cellulases than when it was grown on Avicel. Although the steam treated aspen wood was water washed, residual material such as lignin products probably inhibited growth. Cellulase production increased constantly during growth, reaching a maximum value at day 8, two or three days after



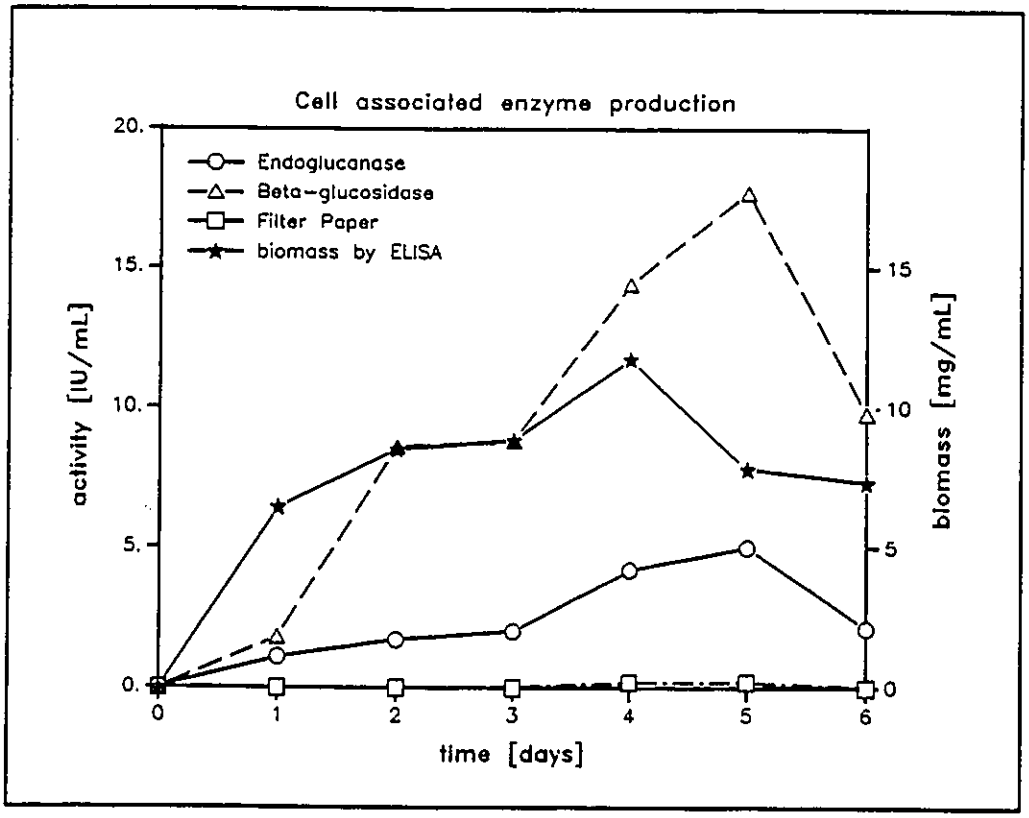


FIGURE 20. Extracellular and cell associated enzyme production of Thielavia terrestris 255B grown on 2% Avicel at 44 deg C.

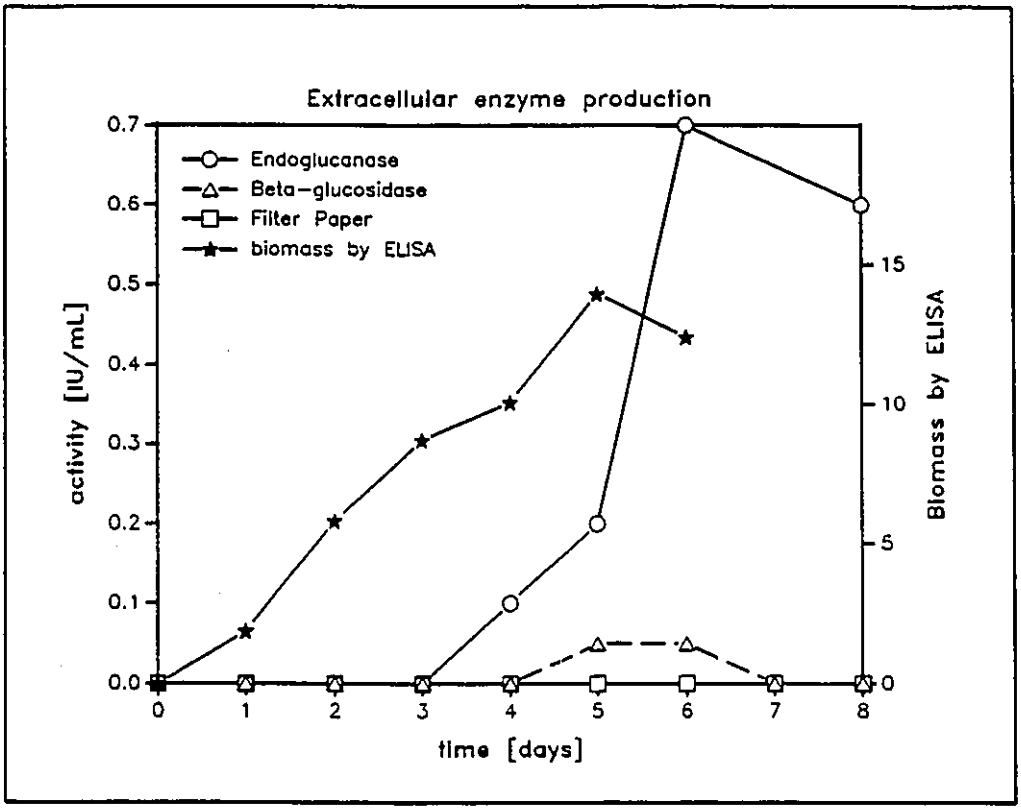


FIGURE 21. Extracellular enzyme production of Thielavia terrestris 255B grown on SEAW (2% cellulose) at 44 deg C.

maximum growth was detected. Peak values of 30.8, 3.5 and 0.2 IU/mL were obtained for the cell associated B-glucosidase, endoglucanase and filter paper activities respectively (fig. 21). Maximum values of 0.1, 0.7 and 0.2 IU/mL were obtained for the extracellular B-glucosidase, endoglucanase and filter paper activities respectively. Other workers had previously claimed (Ghose et al 1975, Feldman et al 1988) that the process of cellulase biosynthesis in fungi was non-growth associated. We have found that this is also true for T.terrestris growth on Avicel where maximum enzyme production was obtained after 5 days incubation, when the growth of T.terrestris was already completed. The same trend was also observed after growth on wood as the highest cellulase activities were detected at day 8, several days after maximum fungal biomass values were detected. Both the extracellular and cell associated enzyme activities showed the same behavior.

III.III CONCLUSIONS

One of the major objectives of this work was to identify a reliable procedure for mycelial biomass quantification when the fungi were grown on insoluble cellulosic substrates. It was apparent that there was a significant morphological difference between the fungus grown on soluble and insoluble substrates with lower biomass values obtained after growth on the latter substrate. The observation was not unexpected as a substantial amount of the cell energy was used for producing and secreting the extracellular enzymes necessary for the hydrolysis of the lignocellulosic substrates.

Although there are several methods for estimating biomass, many of these procedures are impractical when the fungi are grown on insoluble substrates. None of the methods appeared to be significantly better than any of the others with the ELISA providing more accurate values when Avicel was used as the substrate and the ergosterol method appearing more accurate on steam exploded wood.

As each of the biuret, Kjeldahl and ergosterol methods measure only one component of the mycelia, they suffered from the disadvantage that substrate, metabolic and fermentation conditions can influence the relative amounts of the various components and affect the relative proportions of the various components with respect to both fungal biomass and cellulase secretion. Although the ELISA provided a method of directly measuring the amount of fungal biomass present, it still requires considerable research both to assess the actual representation of the measured antigens between the glucose and cellulose grown mycelia and the influence of the substrate on the assay itself.

These preliminary results indicate that the desirability of a particular method of determining fungal biomass will depend on both the major reason for measuring the biomass and the availability of equipment. All of the biomass measurement methods indicated that maximum cellulase production occurred after fungal growth was completed. Future work should compare the relative growth rates of the mesophilic and thermophilic fungi in comparison to their cellulase productivities.

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APPENIDIX

DESCRIPTION OF *Thielavia terrestris*

T.terrestris 255B was isolated by Evans (1971) from abandoned coal spoil tips in United Kingdom. It is a thermophilic ascomycete (Malloch and Cain 1973) which is common in heated habitats (Samson et al 1977). The taxonomy and the picture of this organism is presented in fig. 22.

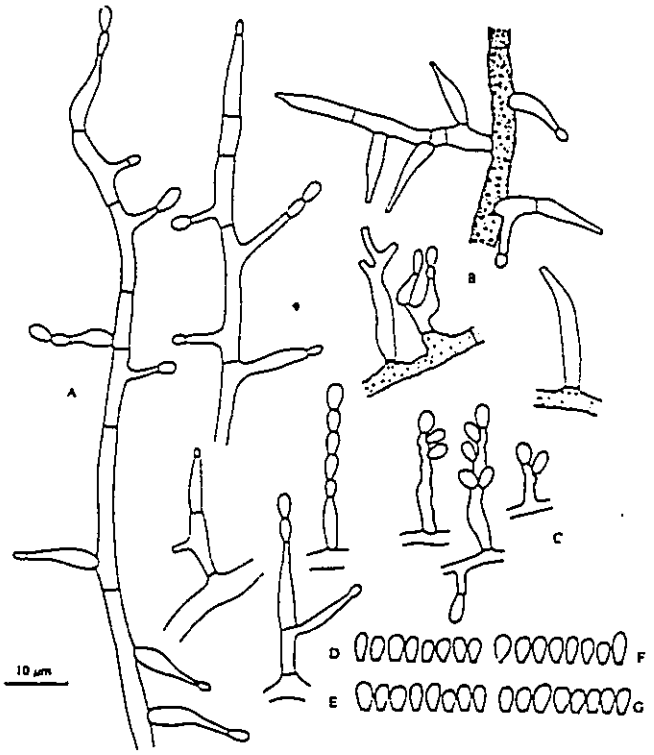

Fig. 1. *Thielavia terrestris* conidial state. (A–C) Conidiophores, (D–G) conidia. (D) *Arrhenomyces alabamensis* CBS 466.74, (E) CBS 355.66, (F) CBS 492.74, (G) strain von Klopotek A 84.

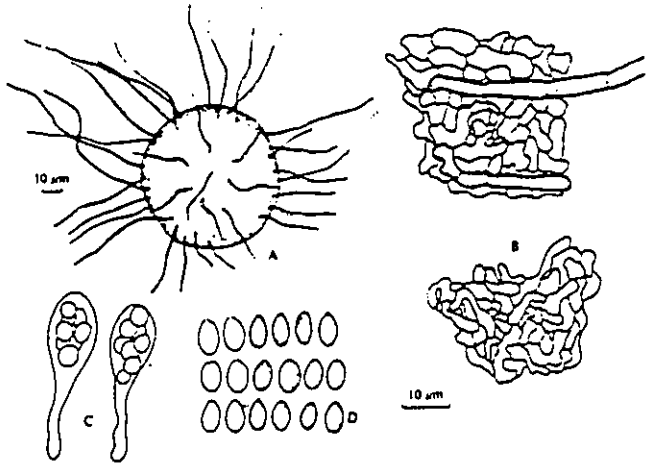

Fig. 2. *Thielavia terrestris*. (A) Ascoma, (B) ascomatal wall, (C) asci, (D) ascospores.

taxonomy by J.A. von Arx

KINGDOM:	MYCOTA
DIVISION:	EUMYCOTA
SUBDIVISION:	ASCOMYCOTINA
ORDER:	SPHAERIALES
FAMILY:	SORDARIACEAE
SPECIES:	THIELAVIA TERRESTRIS

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A comparison of the thermostability of cellulases from various thermophilic fungi

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Summary. The cellulase activities of six thermophilic fungi were compared. Although the thermophilic fungi grew at relatively high temperatures (>45° C) the optimum temperatures for assaying the various cellulase activities were only slightly higher than the optimum temperatures for the mesophilic fungi, *Trichoderma harzianum*. Over prolonged incubation (>24 h) the thermophilic strains demonstrated a higher hydrolytic potential as a result of the greater thermostability of the cellulase components. Although the extracellular cellulase activities had similar pH and temperature optima, in some cases the thermostability of the extracellular components were considerably lower.

Introduction

Most commercial sources of cellulase enzymes utilize the extracellular enzymes produced by mesophilic fungi such as *Trichoderma reesei* or *Aspergillus niger*. Although these fungi exhibit very high hydrolytic activity, as measured by filter paper or β-glucosidase activities, we have found that an assay carried out over 30–60 minutes is not representative of the prolonged incubation that is required for high or complete hydrolysis of various pretreated lignocellulosic residues (Saddler et al. 1985). For the efficient, complete hydrolysis of lignocellulosic residues it would be desirable to have a thermophilic and thermostable cellulase system. By operating at elevated temperatures, we could limit the incidence of contamination while the thermostable enzymes would maintain their activity and could be recycled for addition to fresh substrate. In comparison to the intensive investigation carried out on mesophilic fungi, the search for highly cellulolytic and thermophilic fungi has been relatively limited. Any work carried out has mainly emphasized the isolation and identification of new species and the purification of some of the individual, extracellular cellulases. Very little information is available on the productivities and temperature stabilities of thermophilic fungi such as the patented strains *Thielavia terrestris* (Skinner and Tokuyama 1978) and *Sporotrichum cellulophilum* (Komura et al. 1978). The objective of the present study was to evaluate, under identical laboratory conditions, the thermostability and productivities of various thermophilic fungi and to compare their hydrolytic potential with a highly cellulolytic, mesophilic strain, *Trichoderma harzianum*.

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Materials and methods

Organisms and culture conditions

The thermophilic strains *Thielavia terrestris*, (255B) *Thermoascus aurantiacus* 235F, *Aspergillus terreus* Thom. (304A) and *Myceliophthora fergussi* 264C were obtained from the Forintek culture collection. The patented strain of *T. terrestris* NRRL 8126 (255C) was obtained from the Northern Regional Research Laboratory, USDA.

The organisms were pregrown in a medium containing: 0.2% yeast extract, 0.1% peptone, 0.02% $MgSO_4 \cdot 7H_2O$, 0.01% $CaCl_2 \cdot 2H_2O$ and 2% glucose. After 2 to 3 days growth at 44° C, the cells were harvested, washed, macerated in a blender and a mycelium inoculum of 5 ml (approx. 5 mg/ml dry weight) was used to initiate the growth on a cellulolytic medium. The production media for cellulases were, 1. the basic medium described by Mandels et al. (1971) with 40 mM $\cdot KH_2PO_4$, buffered with 3 g/l potassium hydrogen phthalate, and with urea omitted, 2. Vogel's medium (Montenecourt and Eveleigh 1978). The media were supplemented with 2% cellulose (Avicel or Solka floc).

Table 1. Enzyme production of several thermophilic fungi and one mesophilic fungus (measured by the Nelson-Somogyi method at optimum temperature)

Organism	Media[a]	Duration of growth (days)	Optimum growth temp. (°C)	Enzyme activity (IU/ml)		
				β-Glucosidase	Endoglucanase	Filter paper activity
Thielavia terrestris 255B	D+A	6	45	4.24	14.40	0.606
Thielavia terrestris 255C	D+A	6	45	2.00	24.00	0.480
Aspergillus terreus Thom. 304A	D+A	5	45	1.10	8.40	0.062
Thermoascus aurantiacus 235E	V+SF	6	50	2.36	4.22	0.076
Thermoascus aurantiacus 235F	V+SF	5	50	0.20	50.40	0.367
Myceliophthora fergusii 264C	D+A	6	50	1.05	0.95	0.020
Trichoderma harzianum E58	V+SF	3—4	28	0.95	12.00	0.700

Cultures were grown for various times until maximum activities were detected in the culture filtrates

[a] D+A — basic medium described by Mandels et al. (1971) with 40 mM KH_2PO_4, buffered with 3 g/l potassium phthalate and with urea omitted. Media supplemented with 2% Avicel
V+SF — Vogel's medium (Montenecourt and Eveleigh 1978) Media supplemented with 2% Solka floc

Enzyme preparation and assays

The mycelium was harvested at different times by centrifuging (8000 rpm, 30 min, 4°C) and the supernatant was used for determination of extracellular enzyme activity. The pellets were first washed with 0.05 M acetate buffer pH 4.8, then sonified. Before breakage, the cells were reconstituted in buffer to the equivalent amount of original culture fluid. In this way extracellular activities reported in IU/ml of culture filtrate were compared with intracellular activities and reported as IU/ml of sonicated mycelium in buffer. Reducing sugars were measured by the Nelson and Somogyi procedure (Somogyi 1952). Endoglucanase, filter paper and β-glucosidase activities were determined as previously described (Breuil and Saddler 1985).

One unit of activity was defined as 1 μmole of glucose equivalents released per minute.

Results

Initially we looked at six different thermophilic fungi and compared the various enzyme activities produced by the fungi after incubation for various times and temperatures (Table 1). We had previously determined the media and cellulosic substrate which seemed to result in the most active extracellular cellulase profile. All of the strains were shown to be true thermophiles, exhibiting temperature optima for growth between 45°C—50°C and little or no growth at temperatures below 20°C. In contrast, the mesophilic strain, *T. harzianum*, grew well at 20°C, with optimum growth occurring at 28°C, while no growth was detected at temperatures above 35°C. The thermophiles took 5—6 days to produce the highest levels of extracellular activity while *T. harzianum* produced maximum cellulase activity after 3—4 days growth. *T. harzianum* also produced slightly higher levels of filter paper activity than the thermophiles, although some of these strains produced considerably higher β-glucosidase and endoglucanase activities.

We next examined the optimum temperatures for assaying the various cellulase components of the extracellular enzymes obtained after growing the fungi for the previously indicated times (Table 2). There was not such a dramatic difference in the temperature profiles of the thermophilic and mesophilic strains, compared to the sharp contrast in temperature optima for growth. Although all of the thermophilic strains demonstrated higher temperature optima for endoglucanase and β-glucosidase activities, the filter paper activity of *T. harzianum* was only slightly lower than some of the thermophiles.

Previously we had demonstrated that the cellulase activity, as determined by incubation with substrates such as filter paper or carboxymethylcellulose, was not representative of the long term hydrolysis that is invariably required to obtain the best possible hydrolysis of the substrate (Saddler et al. 1985). It could be expected therefore that

Table 2. Optimum temperatures for growth and cellulase activities of several thermophilic fungi and one mesophilic fungus

Organism	Growth temp.	Optimum temperature for enzyme activity (°C)		
		Endo-glucanase	β-Gluco-sidase	FP activity
Thermoascus aurantiacus 235E	50	75	78	70
Thermoascus aurantiacus 235F	50	75	70	60
Myceliophthora fergusii 264C	50	70	70	60
Thielavia terrestris 255B	46–47	65	72	65
Thielavia terrestris 255C	45	70	65	65
Aspergillus terreus Thom. 304A	45	65	60	60
Trichoderma harzianum E58	28	50	50	50

those cellulase systems which are more thermostable would demonstrate a higher hydrolytic potential over a prolonged incubation. The thermostability of the cellulase complexes from the various fungi were determined after prolonged incubation at 60°C (Table 3). *T. aurantiacus* was shown to have the most thermostable cellulase complex for all three of the filter paper, endoglucanase and β-glucosidase activities. Nearly all of the cellulase activities of the various thermophiles had a half-life greater than 72 h, when they were incubated at 50°C. The exceptions were the β-glucosidase activities of *T. terrestris* 255B and *A. terreus* 304A which had half-lives of 45 h and 19 h, respectively.

Table 3. Half-lives (in hours) of extracellular enzymes of several thermophilic fungi and *T. harzianum*, incubated at 60°C

Organism	Endo-glucanase activity	β-Gluco-sidase activity	Filter paper activity
Thielavia terrestris 255B	> 96	~ 10.8	~ 32
Thielavia terrestris 255C	~ 72	~ 3.5	~ 96
Thermoascus aurantiacus 235E	> 264	96	> 264
Thermoascus aurantiacus 235F	> 241	26.4	48
Aspergillus terreus Thom 304A	> 96	1	~ 5
Myceliophthora fergusii 264C	9	~ 3	~ 2
Trichoderma harzianum E58	0.9	0.75	0.9

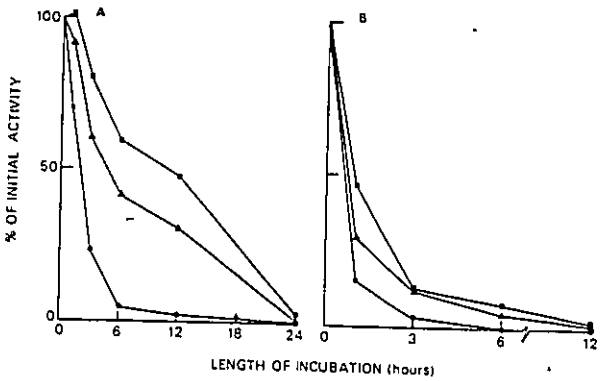

Fig. 1. Stability of β-glucosidase (●—●), endoglucanase (■—■) and filter paper (▲—▲) activity of crude filtrate from *T. aurantiacus* 235F (A) and from *T. terrestris* 255B (B) incubated at 70°C

As the cellulases of *T. aurantiacus* 235F and *T. terrestris* 255B were very stable at 60°C these activities were followed over prolonged incubation at 70°C (Fig. 1). The cellulases of strain 235F were the most thermostable with the endoglucanase activity demonstrating the longest half-life of approximately 10 h. The β-glucosidase activity of both strains appeared to be the most temperature labile component. Less than 15% of the original β-glucosidase activity of strain 255B was detected after 1 h incubation at 70°C.

Previously we had shown (Breuil et al. 1986)

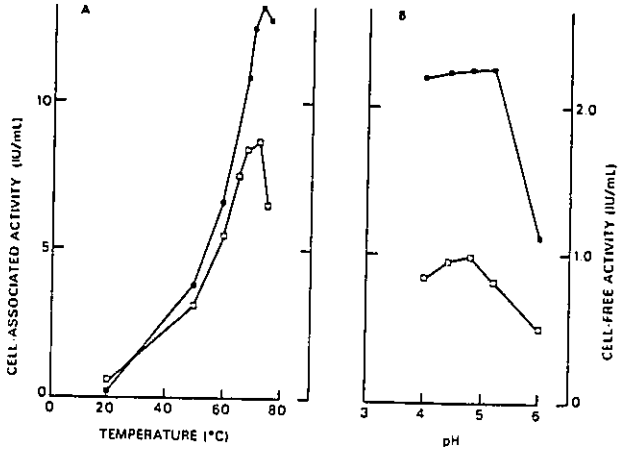

Fig. 2. Effect of (A) temperature and (B) pH on the activities of cell-associated (■—■) and cell-free (□—□) β-glucosidase of *T. terrestris* 255B

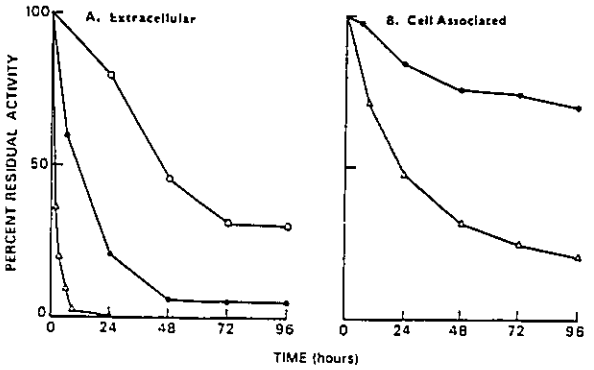

Fig. 3. Thermostability of β-glucosidase activity of *T. terrestris* 255B in (A) culture filtrates and (B) cell associated fractions. Incubation temperatures were 65°C (Δ—Δ), 60°C (●—●) and 50°C (○—○)

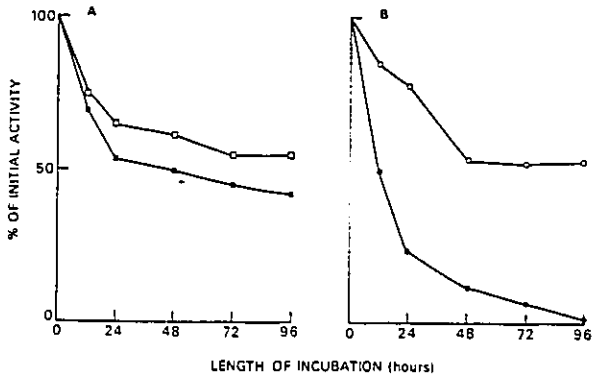

Fig. 4. Thermostability of endoglucanase activity of A. *T. terrestris* 255B. B. *A. terreus* Thom. in culture filtrates (□—□, ○—○) and cell associated fractions (■—■, ●—●). Incubation temperature was 60°C

that the cell associated β-glucosidase activity of *T. terrestris* 255B was the major component when compared to the extracellular activity detected in the culture filtrate. As the pH and temperature of incubation are known to influence the activities of the various enzymes we compared the profiles of both the cell associated and extracellular β-glucosidase activities over a range of temperatures and pH (Fig. 2). This was carried out using typical assay conditions with normal enzyme and substrate concentrations. It was apparent that the extracellular and intracellular activities had similar pH and temperature optimum. There was a significant difference however when the thermostability of the two fractions were compared (Fig. 3). In this case, the enzymes were preincubated for the indicated times and temperatures prior to carrying out the β-glucosidase assay. Approximately 95% of the extracellular activity was lost after 48 h incubation at 60°C while the same conditions of incubation only reduced the cell associated activity by 25%. After incubation at 65°C for 24 h, trace amounts of extracellular activity were obtained while approximately half of the original cell associated activity could still be detected. A similar profile was obtained with the β-glucosidase of *A. terreus* with the cell-associated enzyme being more thermostable than the extracellular enzyme. However, when the endoglucanase activities of *T. terrestris* and *A. terreus* were examined (Fig. 4) the cell associated activities proved to be more temperature labile. The extracellular endoglucanase of *A. terreus* maintained 50% of its original activity while only low levels of activity were detected with the cell associated fraction after incubation at 60°C for 96 h.

As it was probable that the buffering capacity of the medium could influence the long term stability of the various enzymes, the extracellular activities of both *T. terrestris* and *T. aurantiacus* were assayed in the presence and absence of acetate buffer (pH 4.8), after prolonged incubation at

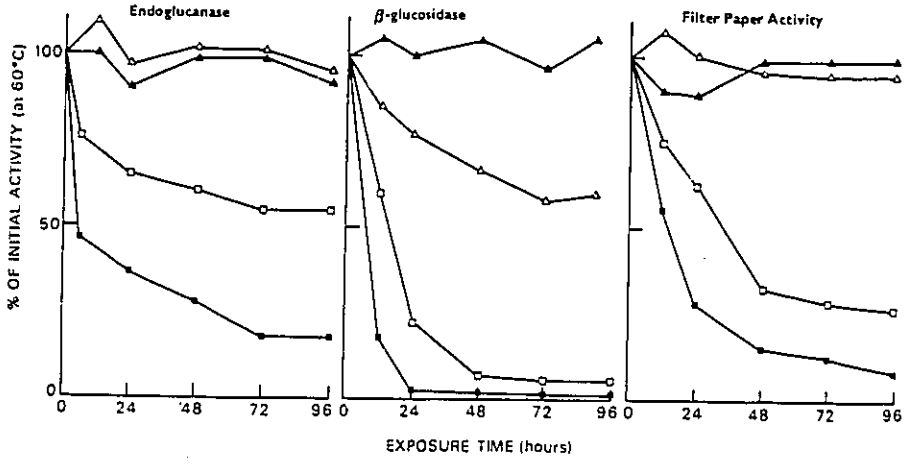

Fig. 5. Stability of the exoenzymes in crude filtrate of *T. terrestris* 255B (□—□, ■—■) and *T. aurantiacus* 235F (Δ—Δ, ▲—▲); in presence (□—□, Δ—Δ) or in absence (▲—▲, ■—■) of buffer

60° C (Fig. 5). We had previously determined that this buffer and pH gave the highest enzyme activities as determined by the various cellulase assay methods. The stability of all of the cellulase activities of *T. terrestris* were enhanced by the presence of buffer with the endoglucanase activity showing the greatest improvement. The filter paper and endoglucanase values of *T. aurantiacus* were similar for both buffered and unbuffered media. However, the buffer decreased the thermostability of the β-glucosidase component.

Discussion

Thermophilic fungi are classified as organisms that have maximum growth temperatures at or above 50° C and minimum growth temperatures at or above 20° C (Cooney and Emerson 1964). As expected, enzymes produced by thermophilic fungi exhibited optimum activity at temperatures higher than their mesophilic counterparts. However, in general, fungal cellulases have higher temperature optima than other enzyme systems (Reese 1975). The optimum temperatures for determining cellulase activity, between 60° C and 78° C, were similar to those found for other thermophilic, cellulolytic fungi (Sen et al. 1982; McHale and Coughlan 1980; Durand et al. 1984). The thermophilic fungi used in this work all produced a complete extracellular cellulase system. We had previously shown (Breuil et al. 1986) that the high level of filter paper, β-glucosidase and endoglucanase activities of *T. terrestris* 255B were representative of this strain's ability to hydrolyse various cellulosic substrates such as Avicel and filter paper at elevated temperatures. *T. aurantiacus* 235F looked promising as it produced significantly higher levels of endoglucanase. Generally, it seems that thermophilic fungi produce lower levels of filter paper activity in comparison to mesophilic fungi. This observation is similar to previous reports (Jain et al. 1979) that some thermophilic fungi did not produce filter paper activity when cellulose or filter paper were used as the only carbon source. Our studies also agreed with previous observations that a significant amount of the β-glucosidase activity was cell associated.

The extracellular β-glucosidase component of the thermophilic strains was the most temperature labile of all the cellulase activities. The cell associated β-glucosidase component of *T. terrestris* was shown to be considerably more stable than the extracellular components, in contrast to previous reports (Araujo et al. 1983; McHale and Coughlan 1980) which showed that the intracellular β-glucosidase activities of *Humicola* sp. and *Talaromyces emersonii* were considerably less stable than the extracellular activity. The higher stability could be the result of extra protein associated with this fraction enhancing the stability of the β-glucosidase activity. It might be expected that the intracellular activity, once isolated from the more controlled environment within the cell, would be less stable as the addition of buffer to the medium significantly enhanced the thermostability of the extracellular β-glucosidase. Another factor which should also be considered is the possible influence of proteases present in the crude enzyme mixtures which could either decrease or increase the activity over prolonged incubation.

It has been recognized that the synergistic interaction of the various endoglucanase, exoglucanase and β-glucosidase components are required for efficient hydrolysis (Ladisch et al. 1983; McHale and Coughlan 1980; Wood 1968). Although the individual activities of the various strains of thermophilic fungi can be very high, a comparison of the overall hydrolytic activities of *T. terrestris* 255B and *T. aurantiacus* 235F indicated that a better equilibrium of enzymes is more important than individually thermostable components. It is also apparent that, even for the thermophilic fungi, a more efficient long term hydrolysis is obtained when the cellulase complex is incubated in the 60° C to 65° C range, at temperatures below the optima determined by the cellulase assays. This seems to indicate that the conditions defined for assaying the various cellulase activities are not fully representative of the hydrolytic potential of the enzyme complex.

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Production and localization of cellulases and β-glucosidase from the thermophilic fungus Thielavia terrestris

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SUMMARY

The enzyme production and localization of Thielavia terrestris strains C464 and NRRL 8126 were compared to determine their optimum temperature and pH for cellulase activity. High levels of intracellular β-glucosidase activity were detected in the former strain. The intracellular β-glucosidase of both strains were more thermostable than the extracellular enzyme; the half life of T. terrestris (C464) endoglucanase activity at 60°C was greater than 96 hrs.

INTRODUCTION

In the bioconversion of lignocellulosic residues it would be desirable to have a thermophilic and thermostable cellulase system. By operating at elevated temperatures, we could limit the incidence of contamination while the thermostable enzymes maintained their activity and could be recycled for addition to fresh substrate. During the screening of thermophilic fungi obtained from various culture collections including the Forintek culture collection, we found that various strains of Thielavia terrestris were among the most cellulolytic. Other workers (Skinner and Tokuyama, 1978; Margaritis and Merchant, 1983; Durand et al, 1984) had also used highly active strains of T. terrestris and strain NRRL 8126 had been patented because of this high activity. We have made a comparison between this strain and the most active T. terrestris strain that we have isolated, with regard to the activity, location and half life of the cellulase components.

MATERIALS AND METHODS

Organisms and culture conditions. Thielavia terrestris C464 was from the Forintek collection. The patented strain of T. terrestris NRRL 8126 was obtained from the American Type Culture Collection. The organisms were pregrown in a medium containing; 0.2% yeast extract, 0.1% peptone, 0.02% $MgSO_4.7H_2O$), 0.01% $CaCl_2 2H_2O$ and 2% glucose. After 2 to 3 days growth at 44°C, the cells were harvested, washed and a mycelium inoculum of 5 mL (5 mg/mL dry weight) was used to initiate the growth on a cellulolytic medium. The production medium for cellulases was the basic medium described by Mandels et al (1971) with 40 mM KH2PO4, buffered with 3 g/L potassium phthalate, and with urea omitted. The medium was supplemented with 2% cellulose (Avicel), 0.1% peptone and 0.1% yeast extract.

Enzyme preparation. The mycelium was harvested at different times by centrifuging (8000 RPM, 30 min., 4°C) and the supernatant was used for determination of extracellular enzyme activity. The pellets were first washed with 0.05M acetate buffer, pH 4.8, then homogenized and sonified. Dry weight and cell protein concentration (Herbert et al, 1971) were determined on the washed mycelium. The sonicated cells were reconstituted

way extracellular activities reported for IU/mL of culture filtrate were compared with intracellular activities reported per IU/mL of sonified mycelia in buffer.

Reducing sugar was measured by the Nelson and Somogyi procedure (Somogyi, 1952). Endoglucanase, filter paper and β-glucosidase activities were determined as previously described (Breuil and Saddler 1985a). When salicin was used as the substrate one unit of activity was defined as 1 µmole of glucose equivalents released per minute. As cellobiose contains twice as much glucose as salicin one unit of activity was defined as 2 µmole glucose equivalents released per minute to make the cellobiose values comparable to those obtained with salicin (Breuil and Saddler, 1985b)

RESULTS AND DISCUSSION

Enzyme pH and temperature optima

The optimum temperatures for the various cellulase activities (Table 1) were slightly higher than those reported by other workers (Skinner and Tokuyama, 1978; Margaritis and Merchant, 1983; Durand et al, 1984). At an incubation temperature of 50°C, which is routinely used with mesophilic fungi, the β-glucosidase, endoglucanase and filter paper activities of culture filtrates from T. terrestris C464 were only 35%, 50% and 40% respectively of the values measured at the optimum temperature. The intracellular and extracellular activities had a similar temperature profile. This differs from the results of some other workers (Araujo et al, 1983; McHale and Coughlan, 1981) who reported a 20° to 30°C difference in the temperature optima of extracellular and intracellular cellulolytic enzymes from the thermophilic fungi Humicola sp. and Talaromyces emersonii. The pH optima for both extracellular and intracellular β-glucosidase activities were around 4.8, similar to most other fungal β-glucosidases (Araujo, 1983).

Enzyme production and localization

Enzyme activities in both the extracellular and cell associated fraction were followed over a period of 7 days. There was a rapid increase in fungal biomass, as represented by the cell associated protein, with 68% of the maximum values obtained after only 24 hrs growth. The pH of the medium showed only a slight variation during growth which indicated that the phthalate in the medium had adequate buffering capacity. Although 70% of the final growth was achieved after 48 hrs, at this time there was only low levels of activity detected in both the intracellular and extracellular fractions. However, after this time, the cell associated β-glucosidase activity increased rapidly, to a maximum value of 25 IU/mL after 6 days growth. The extracellular β-glucosidase activity also increased after 48 hrs growth, reaching a maximum value of 7 IU/mL after 7 days growth. The higher values obtained when salicin was used in place of cellobiose to assay for β-glucosidase activity probably reflect more than one β-glucosidase being present with the cellobiose being a more specific substrate. The β-glucosidase values for T. terrestris NRRL 8126 grown under the same conditions showed a peak extracellular activity of 1.3 IU/mL at day 6 and optimum cell associated activity of 1.2 IU/mL at day 4. T. terrestris NRRL 8126 released more endoglucanase activity

however, with a value of 30 IU/mL after 6 days growth. The profile of the filter paper activity was similar for both strains with the cell associated activity reaching a maximum around day 3 while the extracellular activity reached a peak at day 6. The NRRL 8126 and C464 strains had peak activities of 0.35 IU/mL and 0.55 IU/mL respectively. The combined intracellular and extracellular β-glucosidase activity of strain C464 appears to be among the highest reported values for filamentous fungi. Gokhale et al, (1984) have reported an Aspergillus sp. which could produce 11.3 IU/mL of β-glucosidase activity, as defined in this paper, after 12-14 days growth while a mutated strain of Sclerotium rolfsi could produce 7-12 IU/mL of β-glucosidase after 14 days of growth (Sadana et al 1980).

Influence of temperature and duration of incubation on cellulase and β-glucosidase activities

The pH of the culture filtrates and reconstituted, sonicated mycelia were adjusted to 4.8 prior to assessing the thermostability of the β-glucosidase component. Strain C464 lost more than 40% of its original activity after 6 hrs incubation at 60°C in comparison to the more than a 70% loss of β-glucosidase activity of strain NRRL 8126 under the same conditions. The cell-associated activity of both strains appeared to be more thermostable than the extracellular activity. These results contrast with those of Araujo et al (1983) and McHale and Coughlan (1981) who found that the intracellular β-glucosidase activity of both Humicola sp. and Talaromyces emersoni were less stable than the extracellular activity. When the thermostability of the different cellulase and β-glucosidase activities were compared to the values reported in the literature (Table 2) (Durand et al, 1984) the patented NRRL 8126 strain was significantly less thermostable than had been stated in the patent (Skinner and Tokuyama, 1978).

Thus the thermostability of the cellulase sytems from both the T. terrestris strains was considerably higher than the enzymes from mesophilic fungi used by most groups working on the bioconversion of cellulose. T. terrestris has a β-glucosidase activity which was comparable to that from various Aspergillus, which are acknowledged as being among the most prolific producers of this enzyme. It is probable that T. terrestris has the potential to replace culture filtrates of Aspergillus as the supplement to β-glucosidase deficient cellulase preparations. If strains or mutants with higher cellulase activity can be isolated, these may provide the highly active, thermostable enzyme systems which could make the recycle of cellulolytic enzymes a feasible proposition.

ACKNOWLEDGEMENTS

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Table 1 Optimum temperature (°C) for different cellulase activities from 3 strains of Thielavia terrestris [EC = extracellular; IC = intracellular]

Strain no.	/B-glucosidase		endoglucanase		filter-paper activity	
	EC	IC	EC	IC	EC	IC
C 464	72	72	65	62	65	64
NRRL 8126 (a)	65	70	70	70	65	65
(b)	67	ns	60	ns	60	ns
ATCC 26917(c)	70	ns	70	ns	ns	ns

(a) present work
(b) from Durand et al. (1984)
(c) from Margaritis & Merchant (1983)
ns = not stated in published data

Table 2 Half-life (hours at 60°C) for different cellulase activities from 3 strains of Thielavia terrestris

Strain no.	/B-glucosidase	endoglucanase	filter-paper activity
C 464	10.3	>96	34
NRRL 8126 (a)	3.7	72	96
(b)	8	>72	56
ATCC 26917(c)	>40	5	5

(a),(b),(c) as Table 1.

ESTIMATION OF FUNGAL GROWTH ON LIGNOCELLULOSIC SUBSTRATES USING AN ENZYME-LINKED IMMUNOSORBENT ASSAY

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SUMMARY

The feasibility of using an enzyme-linked immunosorbent assay (ELISA) for quantifying the growth of Thielavia terrestris on cellulosic substrates was studied. When T. terrestris was grown, using glucose as the substrate, the ELISA readings correlated well with the mycelial dry weight values. When lignocellulosic substrates were used, the ELISA appeared to slightly overestimate fungal growth. The ELISA values indicated that the fungi grew more slowly on steam-treated aspenwood than on commercial sources of cellulose (Avicel).

INTRODUCTION

In many cases extracellular microbial enzyme activities are reported as corresponding to the volume of media or the amount of cell growth required to achieve the indicated enzyme activity. Unfortunately, when filamentous fungi are grown on cellulosic substrates, there are few methods that can provide representative and reproducible values for fungal biomass. For this reason most studies with cellulolytic fungi do not indicate the corresponding fungal growth associated with the cellulase production values that are reported. Methods such as total nitrogen, chitin, protein, or ergosterol content provide indirect ways of measuring fungal biomass. However, these methods have proved to be unsatisfactory when complex substrates such as lignocellulosic material are used as the growth substrate. Immunological methods have been used to both detect and quantify pathogenic fungi on plants (Casper and Mendgen, 1979; Johnson et al, 1983, Mendgen 1987), in soils (Aldwell and Hall, 1986), and in wood (Breuil et al, 1988; Goodell et al, 1988). In the work reported in this paper, we have examined the feasibility of using an enzyme-linked immunosorbent assay (ELISA) for quantifying the growth of Thielavia terrestris on soluble and insoluble substrates.

MATERIALS & METHODS

Organisms and Culture Conditions. Thielavia terrestris 255B is a thermophilic and cellulolytic fungus. The cultures were maintained on 2% malt extract agar at room temperature. This species and the other fungal species used in this study were obtained from the Forintek culture collection. The organism was pregrown in a medium containing 0.1% peptone, 0.2% yeast extract, and 2% glucose for 48 h at its optimum growth temperature 45°C (Breuil et al, 1986). A series of 500 mL Erlenmeyer flasks containing 100 mL of medium (Breuil et al, 1986) with 2% cellulose as Avicel or steam-exploded aspenwood (240°/80 sec.,

each inoculated with a 5 mL suspension of T. terrestris hyphae fragments (Breuil et al, 1986) and incubated on a rotary shaker at 44°C for a week. At daily intervals following incubation, replicate flasks were harvested by centrifugation. The pellets were washed and lyophilized to determine the total dry weight (fungus + substrate). Biomass was determined using the ELISA method while protein was determined by the biuret method (Herbert et al, 1971).

Antigen and Serum Production. Antigenic material was obtained from the mycelium of T. terrestris 255B by growing the fungi in a liquid medium containing malt extract 10 g/L, yeast extract 4 g/L, and glucose 4 g/L. Cultures were grown for 2 or 3 days on a rotary shaker at 44°C. The mycelium was removed by centrifugation, washed three times with 0.01M sodium phosphate buffered saline (PBS), pH 7.4, and broken in a hand homogenizer with PBS. This preparation was washed three times with PBS, resuspended in a volume of PBS, giving a dry weight of 20 mg/mL; and frozen in small aliquots. When fungal antigens were required for immunization, 0.25 mL (5 mg dry weight) of a frozen aliquot was diluted with 0.25 mL PBS and emulsified with an equal volume of Freund's complete adjuvant. This antigen was then injected into the biceps femoris and deltoid muscles of a New Zealand white rabbit. After 2-3 weeks, the rabbits were challenged with a single injection of fungi into the biceps femoris muscle, using incomplete Freund's adjuvant. The rabbits were bled out 2 weeks after the final injection. For control, pre-immune serum was taken from the marginal ear vein of each rabbit before inoculation. Antiserum was stored at -20°C.

Enzyme-Linked Immunosorbent Assay (ELISA). The assays were carried out as described by Breuil et al, (1988). In the ELISA, plates were coated with mycelia that had been hand homogenized (standard curve) or sonified (cells grown in peptone yeast glucose medium); or with freeze-dried preparations of fungal mat (cellulose and wood media), ground by mortar and pestle in sodium carbonate at pH 9.6. Dilution series of each sample were replicated five to six times. Antiserum and conjugate (goat antirabbit IgG labelled with horseradish peroxidase from BioRad) were used routinely at 1:1000 and 1:2000 dilution, respectively.

RESULTS AND DISCUSSION

Antibodies were prepared using homogenized mycelium from T. terrestris. A dilution end point of 1/48000 was determined by indirect ELISA after the plates were coated with 4 μg/mL of fungal mycelium. The antiserum raised against T. terrestris 255B had the greatest degree of specificity against its own antigens and the assay could accurately detect dilutions as low as 0.25 μg/mL of fungal mycelia. The highest degree of cross-reactivity was obtained with T. terrestris strain 255C, which gave a value of about 40% of that obtained with strain 255B. The anti T. terrestris showed less than 4% cross reactivity when mycelia from two strains of Thermoascus aurantiacus (253E and 235F) and Trichoderma harzianum were assayed.

We next wanted to determine whether a correlation could be established between the ELISA absorbance readings and the corresponding fungal

biomass values. A mycelial suspension from the frozen stock was serially diluted and a standard curve was constructed (Figure 1). When T. terrestris was grown on a soluble glucose, yeast peptone media, the ELISA readings were very similar to the mycelial dry weight values (Figure 2). The dry weight growth curve showed an initially rapid 24 h growth, followed by a slow down. After three days, the reduction in dry weight was probably due to autolysis of the mycelia. The ELISA growth curve followed the same pattern as the dry weight determination, although the values were slightly higher. A maximum total cell protein value of 1.9 mg protein/mL of culture was obtained after three days growth, using the biuret method for determination.

When Avicel was used as the growth substrate (Figure 3), a similar total cell protein value was obtained with a maximum of 1.7 mg protein/mL of culture obtained at day four. The organism started to sporulate at this time and many of the mycelia underwent autolysis, resulting in a progressive decrease in detected biomass. The dry weight values progressively decreased, reflecting the utilization of the substrate and effectively masking the contribution that the increase in mycelial biomass added to the dry weight value.

Previously we and other workers had found that the total protein values were approximately equivalent to 17% of the total biomass values. When the corresponding mycelial biomass value was plotted (Figure 3) it was apparent that, after 2 days growth, the values were considerably higher than the actual dry weight values that were determined directly. The biomass values determined by the ELISA method appeared to better quantify the true amount of mycelia present. The similar dry weight and ELISA values detected after 7 days growth reflected the complete utilization of the cellulose substrate, as the fungus represented the only source of available biomass. The slightly higher value obtained after 4 days growth was within experimental error.

The growth of T. terrestris was then followed using steam-treated aspenwood as the cellulosic substrate (Figure 4). The protein content could not be determined when this substrate was used as coloured agents present in the pretreated wood interfered with the assay. The ELISA values indicated that the fungus initially grow more slowly on this substrate, with highest biomass values detected after 5 days' growth. The ELISA values after 6 days' growth were slightly higher than the biomass values determined by direct dry weight measurement.

These initial results showed that an ELISA assay can provide a reproducible method for quantifying the amount of mycelial biomass produced when fungi are grown on various cellulosic substrates. Traditional methods such as ergosterol, chitin, or protein determination all require some form of conversion factor which assumes that these chemical indices are evenly distributed within the fungal structure. This assumption has not proved to be true, as researchers (Matcham et al, 1985; Jernejc et al, 1986; Ruperez and Leal, 1986; Nout et al, 1987) have shown that the amounts of the various parameters change with age, growth rate, carbon source and environmental conditions. Although the same reservations may be true of the ELISA method, initial studies with the quantification of fungal pathogens present in soil and plants (Mendgen, 1987; Aldwell and Hall, 1986) have shown good reproducibility. We have also shown (Breuil et al, 1988) that the ELISA assay does not seem to be greatly influenced by agents present within the wood.

If an ELISA based method proves to be as good as these initial studies suggest, it should be possible to follow the growth of cellulolytic fungi and correlate enzyme production with equivalent cell growth. This method can provide a way of determining whether the type of cellulosic substrate influences the growth and enzyme production of cellulolytic fungi.

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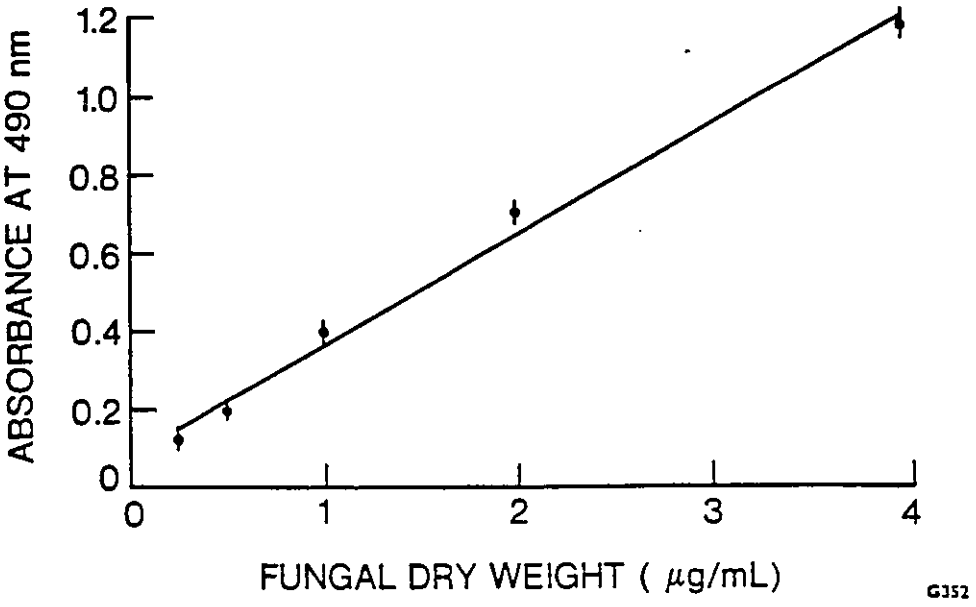
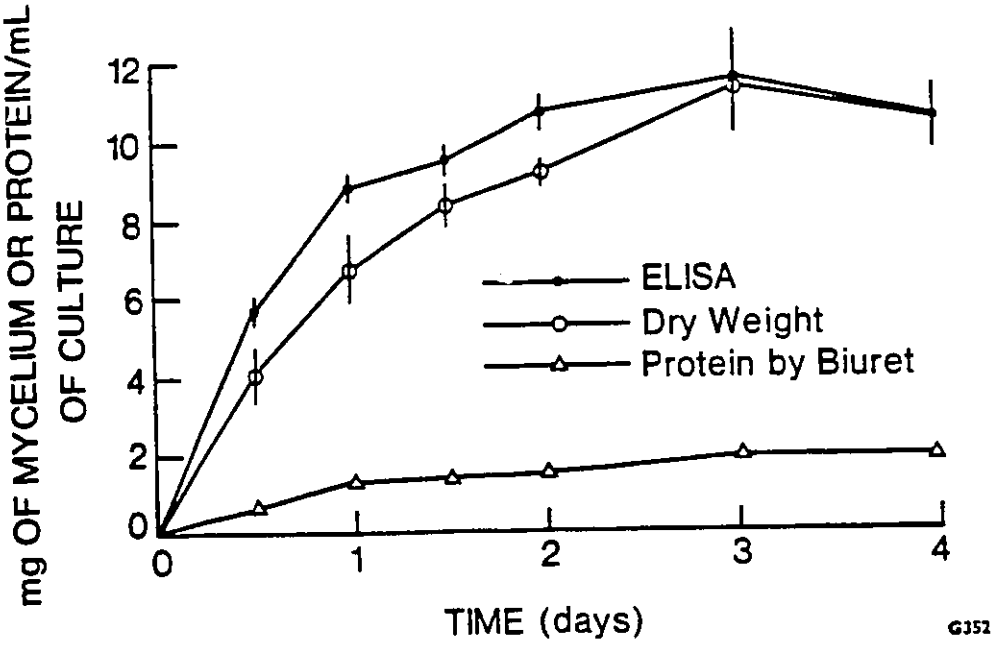


FIGURE 1. Standard curve for quantification of mycelium using ELISA, calculated from a dilution of cell fragments of Thielavia terrestris 255B. Standard deviation is shown. Coeff of regr. = 0.99



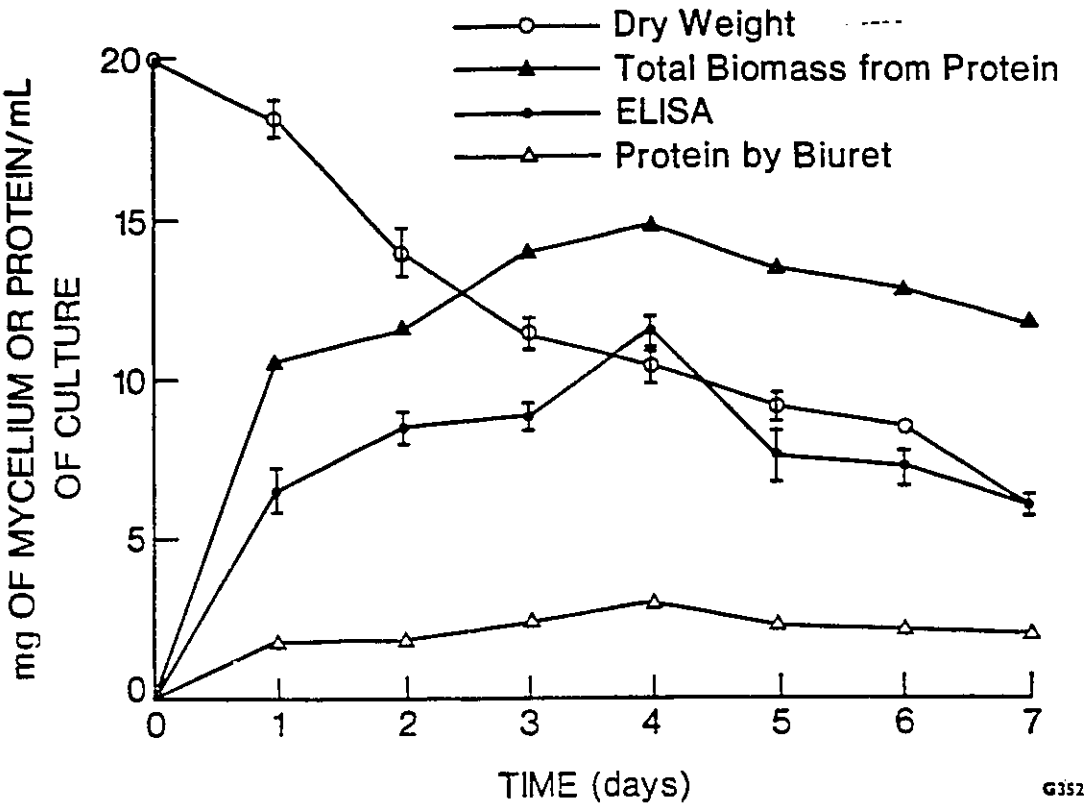


FIGURE 3. Growth of Thielavia terrestris 255B on 2% Avicel measured by the ELISA and protein methods.