THE CHARACTERISATION OF A CELLULOLYTIC MICROBIAL COMMUNITY ISOLATED FROM SOIL

Lesley Ann Harrison B.Sc (Hons)

A thesis submitted for the Degree of Doctor of Philosophy

University of Warwick
Environmental Sciences Department

June 1985

To

My Parents

TABLE OF CONTENTS

List of Figures

List of Tables

List of Tables

List of Appendices

Acknowledgements

I would like to express my thanks to:

Professor J H Slater and **Dr J M Lynch** for their guidance and constructive criticism of this work.

Dr J B Parry for her helpful supervision.

Dr J C Stewart for his practical advice and interest in the project.

Professor D P Kelly and **Dr M J MacDonald** for their critical reading of the manuscript.

The **technical staff** of the Department of Environmental Sciences.

Ann Branch for the excellent typing of this thesis.

The Science and Engineering Research Council for financial support during the course of this research.

Declaration

I declare that this thesis is a report of the research undertaken by myself in the Department of Environmental Sciences (1980 - 1983) under the supervision of Professor J H Slater, University of Warwick and Dr J M Lynch, Letcombe Laboratory, ARC. It is my own original work and has not been presented before.

Summary

A cellulolytic microbial community consisting of eight fungi and two bacteria was isolated from soil using a continuous flow enrichment technique. Each member of the community was grown in axenic culture and their levels of cellulase activity and rates of cellulose hydrolysis were determined. The strongly cellulolytic organisms were Aspergillus fumigatus and Penicillium simplicissimum. Results suggested that the other community members used the intermediates of cellulose hydrolysis produced by the primary cellulose degraders. The major β-glucosidase producing organisms were three Fusarium strains.

Mixed and axenic culture studies indicated that there was no apparent synergism between members of the community during the degradation of either cellulose (Sigmacell type 20), hay or straw. Synergism was also lacking when the fungi were grown under different cultural conditions. The community degraded cellulose at a slower rate than did P. simplicissimum alone, and a lag in cellulase activity was evident. With lignocellulose, the community showed similar degradation rates to those of A. fumigatus and P. simplicissimum, although its levels of enzyme activity, with the exception of β-glucosidase, were lower.

Culture conditions were found to have a strong influence on cellulase activity. With A. fumigatus, P. simplicissimum and the community grown in an unbuffered medium, increasing the cellulose concentration resulted in a decrease in **cellulase** activity, although endoglucanase and β-glucosidase levels were unaffected. Increasing the cellulose concentration in cultures grown in a buffered medium resulted in a dramatic increase in β-glucosidase activity. These levels were increased further by increasing the buffer molarity. Increasing the cellulose concentration in the buffered medium also resulted in an increase in the cellulase activity of P. simplicissimum and the community.

Increased levels of β-glucosidase were produced by P.simplicissimum grown in a buffered medium with cellobiose as the carbon source. Studies of β-glucosidase location indicated that P. simplicissimum produced predominantly cell-associated enzyme activity which was not released into the growth medium until the latter stages of growth.

CHAPTER ONE

INTRODUCTION

CHAPTER ONE

INTRODUCTION

Lignocellulose is widely distributed in the plant kingdom, giving protection, support and strength to plants. It consists of three major groups of polymers: cellulose, hemicellulose and lignin. Cellulose forms the bulk of lignocellulosic material, its content varying from 40-60%. Hemicellulose and lignin are present in smaller amounts, their contents varying from 15-50% and 15-25%, respectively (Dekker & Lindner, 1979). These chemical constituents and their relative ratios vary from source to source, typical compositions of lignocellulosic materials being given in Table 1.

Morphology of the plant cell wall - Cellulose is the major structural component of the cell wall, forming an outer supportive matrix for the membrane (Wenzl, 1970). Hemicellulose is found in close association with the cellulose, within the spaces created by the network of cellulose strands. As the plant cell ages, secondary products are secreted, in particular lignin which forms a bond between the cell walls.

The cell wall of the cotton fibre consists of approximately 94% cellulose, 1% protein, traces of hemicellulose and pectin but no lignin. In contrast, wood cell walls are rich in cellulose, hemicellulose, pectin and lignin. Both the single celled cotton fibre and the multicellular wood have very similar molecular structures. The cotton fibre cell wall consists of an outer layer, the cuticle, consisting of pectin and wax. The equivalent layer in wood tissue consists of intercellular substances together with the primary wall and is known as the middle lamella. Both structures have a thin primary wall composed of a loose random network of microfibrils (i.e. aggregates of cellulose fibres) surrounding a relatively thick secondary wall (Fig. 1). The secondary wall consists of three layers, designated S_1 S_2 and S_3. The microfibrils in the S_1 and S_3

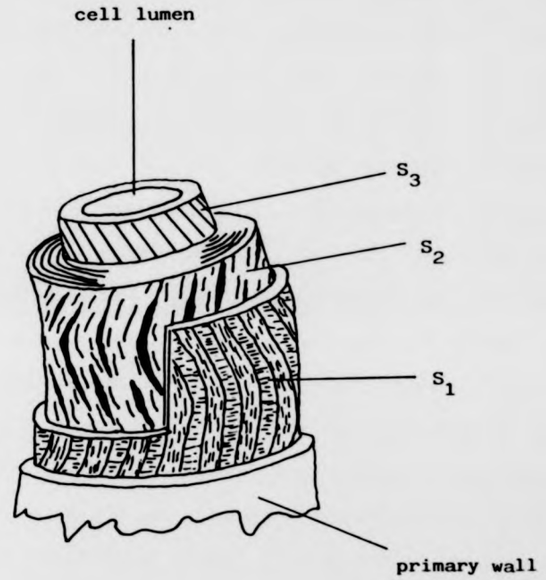
Table 1 Typical compositions of lignocellulosic materials

Raw material	Cellulose (%)	Hemicellulose (%)	Lignin (%)	Extractives (%)	Ash (%)	Reference
spruce wood	43.0	27.0	28.6	1.8	0.4	Rydholm (1965)
pine wood	44.0	26.0	27.8	5.3	0.4	"
birch wood	40.0	39.0	19.5	3.1	0.3	"
pine kraft pulp	77.0	18.0	5.0	0.2	0.4	"
bagasse	33.4	30.0	18.9	6.0	2.4	Clark (1969)
wheat straw	30.5	28.4	18.0	3.5	11.0	"
rice straw	32.1	24.0	12.5	4.6	17.5	"
bamboo	-	19.6	20.1	1.2	3.3	"
cotton	80-95	5-20	-	-	-	Cowling & Kirk (1976)

- denotes no data given

Fig. 1

Morphology of the secondary wall of a cotton fibre (Rogers and Perkins, 1968).



layer are deposited in a flat helix with respect to the fibre axis whereas in the S_2 layer they are deposited almost parallel to the fibre. Although the S_3 layer is prominent in wood cell walls it is not always evident in cotton fibres.

Lignin

Structure of lignin - Lignin is a complex three dimensional phenolic polymer composed of three different units; p-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol. The basic molecular structure is given in Fig. 2. These units are covalently bonded by a number of different links (Higuchi et al., 1981) to form a molecule which is very recalcitrant due to its resistance to chemical and biological degradation (Kirk et al., 1978). There are three major groups of lignins: (1) coniferous lignin formed mainly from coniferyl alcohol units, (2) hardwood lignin containing a mixture of coniferyl and sinapyl alcohol units and (3) grass lignin formed from coniferyl, sinapyl and p-coumaryl alcohol units.

Microorganisms degrading lignin - The most efficient degraders of lignin are the fungi, the best studied being Phanerochaete chrysosporium (McCarthy et al., 1984), Sporotrichum pulverulentum (Eriksson, 1978) and Polyporus versicolor (Leatham & Kirk, 1983), which are all able to degrade lignin to CO_2 and H_2O. Efficient lignin degrading bacteria such as Gram positive Streptomyces (Phellan et al., 1979) and Gram negative Pseudomonas sp. (Kawakani, 1980) are able to solubilise lignin to large molecular weight products but are less efficient at mineralising the substrate than the fungi.

Mechanism of breakdown - In the past, phenoloxidases such as laccase and peroxidase have been implicated in lignin degradation (Ander & Eriksson, 1976). Recently, the first ligninase was described (Tien & Kirk, 1983) as an extracellular oxidative H_2O_2-requiring enzyme involved in lignin degradation.

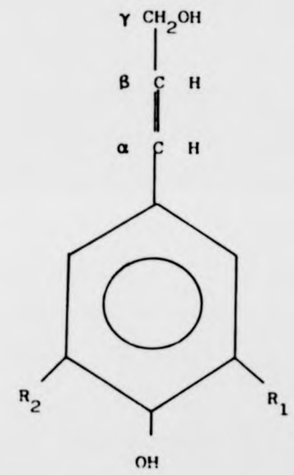


Fig. 2

Basic molecular structure of lignin (Higuchi et al., 1981).

p-coumaryl alcohol; R_1 and R_2 = H.

sinapyl alcohol; R_1 and R_2 = OCH_3.

coniferyl alcohol; R_1 = OCH_3, R_2 = H.

Hemicellulose

Structure of hemicellulose - Hemicellulose is a heterogeneous polymer composed of three hexoses (glucose, mannose and galactose), and two pentoses (xylose and arabinose) together with their uronic acids. Figure 3 shows the structure of a commonly occurring hemicellulose (Dekker, 1979).

Hemicelluloses can be classified into three groups (Wenzl, 1970). (1) D-Xylans, with a backbone of poly-β-1,4-xylan linked laterally to arabinose, glucuronic acid and arabinoglucuronic acid, (2) Mannans, composed of galactomannans and glucomannans and (3) galactans, composed of arabinogalactans.

Microorganisms degrading hemicellulose - Most of the work on hemicellulases has been concerned with xylanases because their substrate, xylan, forms a large proportion of hemicellulose in plants. Xylanase-producing microorganisms include bacteria such as Cellulomonas (Rickard & Laughlin, 1980), Bacillus subtilis (Viikari et al., 1978), actinomycetes such as Saccharomonospora viridis (McCarthy & Broda, 1984) and fungi such as Trichoderma reesei (Dekker, 1983) and T. longibrachiatum (Sandhu & Kalra, 1982).

Mechanism of breakdown - Several types of hemicellulases have been identified; D-galactonases, D-mannanases and D-xylanases. Each type occurs in two basic forms, as exo-and endo- enzymes. Purified xylanases have been obtained using the culture filtrates of T. reesei (Bisaria & Ghose, 1977), Trichoderma roseum (Siwinska & Galas, 1977) and Aspergillus niger (Gorbacheva & Rodjonova, 1977). Endo-xylanases (β-1,4-D-xylan xylanohydrolase, EC 3.2.1.8) are the only types to have been characterised. They have been found to exist in two forms, those which can release L-arabinose from arabinoxylans and arabinoglucuronoxylans and those which cannot. Both types are capable of hydrolysing glucuronoxylans and β-1, 4 -D-xylans. Although exo-xylanases have also been reported, there is

Fig. 3

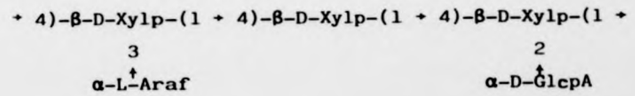
L-Arabino-D-glucurono-D-xylan

(Grass; Dekker, 1979).

Araf = arabinofuranose

Xylp = xylopyranoside

GlcpA = glucopyronosyluronide



no literature on their purification or mode of action.

β-Xylosidases (β-D-xyloside xylohydrolase, EC 3.2.1.37) have been characterised; they hydrolyse β(1-4) links at the non-reducing ends of xylooligosaccharides and β(1-4)-aryl xylopyranosides to produce xylose.

Ghose and Bisaria (1979) have studied the role of xylanase in the degradation of bagasse. Their results suggest that the xylanase helped to create more accessible regions which could be acted upon by exo- and endoglucanases. Furthermore, xylanase action was found to be most effective when acting synergistically with the cellulase complex.

Cellulose

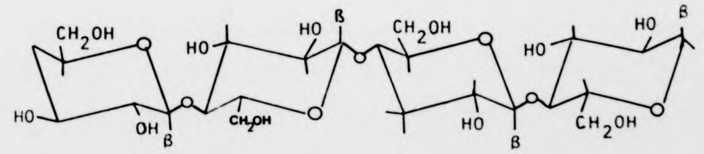
Chemical structure - Cellulose is a linear polymer composed of anhydroglucose units joined together by β(1-4)-glucosidic bonds. Each unit has 3 hydroxyl groups; 1 primary group in the 6-position and 2 secondary groups in the 2- and 3-positions (Fig. 4).

Molecular size - Estimates of the molecular size of cellulose, based on the degree of polymerisation (D.P, number of glucose units) can be determined by physical or chemical methods. Physical methods are more commonly used and include osmometry, viscosity, light scattering and ultra-centrifugal sedimentation. Chemical methods include complete methylation of the cellulose with alkali to calculate the ratio of non-reducing end groups to the rest of the structure. This method, however, can lead to depolymerisation and consequently is open to error due to the size of the cellulose. Therefore, physical methods are generally used in preference to chemical methods.

Variations in the reported molecular weight of cellulose are most likely a result of the different methods used, e.g. treatment with cuprammonium hydroxide can lead to an increase in the D.P. value whereas dry grinding and acid extraction can decrease the value. Ränby (1958) estimated wood cellulose to have a D.P. value of 6,000-8,000, whereas

Fig. 4

Conformational formula of cellulose



Goring and Timell (1962) cited values of 8,000-10,000. In a survey given by Cowling and Brown (1969) D.P. values of cellulose were found to vary between 15.0 and 14,000 with an average value of 3,000. According to Mandels and Reese (1965) and Siegel (1963) D.P. values as high as 100,000 were obtained with carefully purified cellulose.

Crystalline structure - The structure of cellulose was studied as early as 1937 by Meyer and Misch using x-ray crystallography. Diffraction patterns revealed that the structure consisted of repeating units, two glucose residues in length. The chains of glucose units so formed were coupled together with hydrogen bonds resulting in larger units ordered in a crystalline manner.

There appears to be some disagreement as to how the chains of glucose units are arranged. Gardner and Blackwell (1974), using x-ray crystallography of highly crystalline cellulose from the cell walls of the alga Valonia ventricosa, proposed a parallel chain model for cellulose. It was suggested that all the units were lying in the same direction, with a network of hydrogen bonding. One intermolecular bond (O6-H-O3) and two intramolecular bonds (O3-H-O5) and (O6-H-O2) existed with each glucose residue.

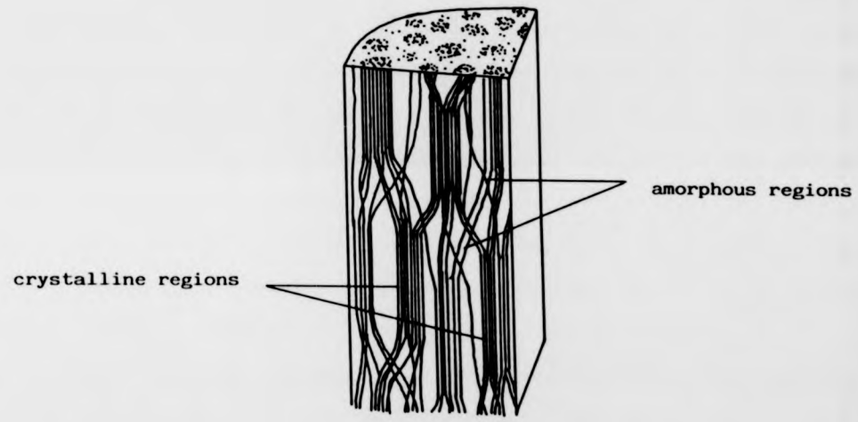
Crystalline and amorphous cellulose - X-ray diffraction patterns have revealed that cellulose is not made up entirely of crystalline units but has some areas showing less order (Dunlap & Chiang, 1980). These areas, known as amorphous regions, are free from hydrogen bonding.

Sihtola and Neimo (1975) have suggested that cellulose chains consist of a number of crystalline regions, with an average length of 100 ± 20 nm, which are separated by partially parallel and disorganised regions, 30-40 nm in length (Fig. 5). These cellulose chains are joined together by covalent bonds to form an elementary fibre.

Electron microscopy studies of purified cellulose have revealed an

Fig. 5

Arrangement of elementary fibrils (Sihtola and Neimo, 1975).



orderly arrangement of strands called microfibrils (Preston et al., 1948 and Frey-Wyssling et al., 1948). These microfibrils are reported to be 5-40 nm in diameter, depending upon the source. They have been described as cylindrical (Ranby, 1952), as flattened ribbons (Bayley & Bishop, 1958) and as square or rectangular in cross section (Mutlethaler, 1963). The dimensions vary depending upon the aggregation of the smaller units of cellulose, the elementary fibre.

The structure of cellulose is still a subject of considerable controversy. Over the years, several models of the molecular arrangement of elementary fibrils have been proposed and at present five are under consideration (Fig. 6). Chang (1971) stated that the most acceptable model is that summarised in Fig. 6C. In this model the cellulose chain folds back and forth on itself within a single plane. Chang (1971) suggested that the β(1-4) linkages were deflected in the region of folding, giving rise to more susceptible amorphous regions. Several of these structures are linked together to form an elementary fibre, 16 of which make up a fibril. Four of these fibrils then form a microfibril.

Microorganisms degrading cellulose - Cellulolytic bacteria are widespread in both aerobic and anaerobic environments. They are commonly found associated with the gut of herbivores and the rumen of cattle as well as being free-living microorganisms in soil and water. Typical cellulolytic bacteria belong to the following genera: Clostridium (Lee & Blackburn, 1975), Cellulomonas (Stewart & Leatherwood, 1976), Streptomyces (Kluepfel et al., 1980), Pseudomonas (formerly Cellvibrio; Hofsten & Berg, 1972).

The present state of knowledge of the degradation of cellulose is based mainly on the studies of cellulolytic fungi. Although many fungi have been reported to be cellulolytic to some extent only a few are capable of extensive degradation of insoluble cellulose. These include Trichoderma reesei (Ryu & Mandels, 1980), T. koningii (Halliwell & Riaz,

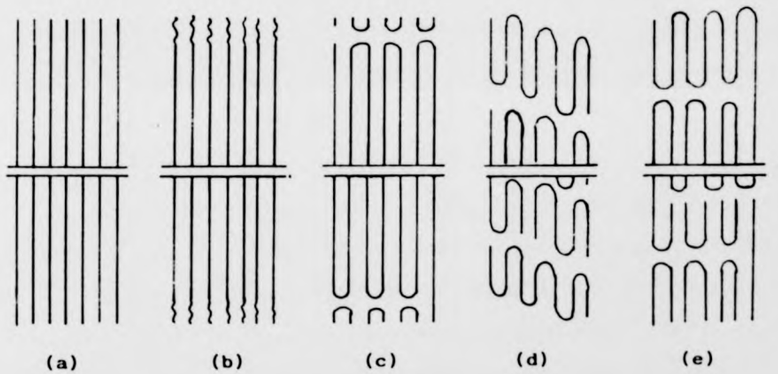


Fig. 6

Molecular arrangement of the elementary fibril (Chang, 1971).

1971), Sporotrichum pulverulentum (Eriksson, 1975), Penicillium funiculosum (Selby, 1969), Fusarium solani (Wood, 1972) and Chaetomium thermophilum var. dissitum (Gøksøyr et al., 1975).

Mechanism of cellulose degradation - It is now generally accepted that at least three types of enzymes are involved in the degradation of crystalline cellulose:

1. Endo-β-1,4-glucanases (EC 3.2.1.4)

These form a group of enzymes sometimes referred to as carboxy - methyl (CM-) cellulases or the Cx component. The enzymes are capable of hydrolysing soluble cellulose derivatives such as carboxymethyl cellulose (CMC), amorphous cellulose such as "Walseth" and cellodextrins, but show very little activity against crystalline cellulose. The cellulose chain is randomly hydrolysed at the β (1-4) linkages to produce glucose, cellobiose and cellodextrins.

2. Exo-β-1,4-glucanase (EC 3.2.1.91).

This enzyme is known as cellobiohydrolase or the C_1 component. It is capable of hydrolysing amorphous and microcrystalline cellulose, with almost no activity on cotton and very limited activity on CMC. It hydrolyses the cellulose chain by removing cellobiose units from the non-reducing ends to give cellobiose as the sole product.

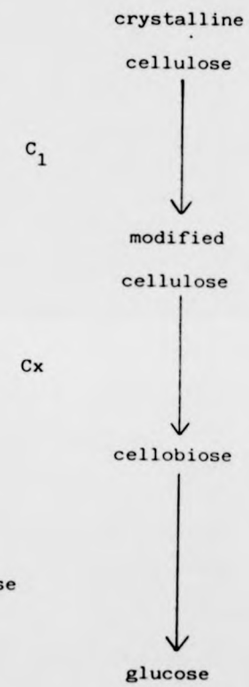
3. β-Glucosidase (EC 3.2.1.21).

This enzyme is also known as cellobiase and hydrolyses cellobiose and cellodextrins by cleaving the β (1-4)-glucosidic bond to produce glucose. It is not capable of hydrolysing cellulose.

A major advance in the understanding of cellulose degradation was based on the observation by Reese et al. (1950) that some microorganisms were able to hydrolyse crystalline cellulose whilst others were only able to hydrolyse soluble cellulose. It was suggested that the crystalline cellulose was converted to soluble sugars by a two-step sequential process

Fig. 7

Sequence of enzymatic reactions in the degradation of crystalline cellulose (Reese et al., 1950).



(Fig. 7). The first step was carried out by a C_1 factor which converted the crystalline cellulose to reactive cellulose. The C_1 factor was thought to act by increasing moisture uptake such that the cellulose chains became separated. The reactive cellulose was then attacked by the Cx enzyme. This is known as the (C_1-Cx) concept where x denotes the multicomponent nature of the system. Reese suggested that microorganisms growing on soluble cellulose produced only Cx enzymes, whereas those growing on crystalline cellulose produced a C_1 factor and Cx enzymes.

Further studies of cellulose degradation indicated that although cellulolytic microorganisms themselves were capable of extensive hydrolysis of crystalline cellulose, the cell-free filtrates were only able to degrade the cellulose to a very limited extent. As a result of the difficulty in preparing filtrates which were active against crystalline cellulose, most of the work in the period 1950-1964 concentrated on Cx activity. However, it was later discovered that the culture filtrates of T. reesei and T. koningii were capable of extensive hydrolysis of crystalline cellulose. As a result, an extensive study of the C_1 component was carried out.

Several workers (King & Vessal, 1969; Li et al., 1965; Mandels & Reese, 1964) supported the idea of the (C_1-Cx) concept and that the C_1 component had a non-hydrolytic function. On the other hand, some experimental results appeared to contradict the (C_1-Cx) concept. Several attempts were made to determine the mode of action of the C_1 component. Wood (1972) examined crystalline cellulose before and after treatment with the C_1 component using electron microscopy, differential thermal analysis, and infrared spectroscopy to determine whether any physical changes in the substrate could be detected as a result of attack by the C_1 component. There was no evidence of the disaggregation of the cellulose chains in preparation for attack by Cx and

the action of C_1 still remained unclear.

As a result of the work carried out by Selby and Maitland (1967), it was suggested that the (C_1-C_x) concept proposed by Reese should be abandoned and the mechanism of cellulose degradation redefined in different terms. Selby and Maitland (1967) suggested that crystalline cellulose was hydrolysed by the synergistic action of the cellulase components. These authors separated the culture filtrate of Trichoderma viride into its various components; carboxylmethyl-cellulase, cellobiase and C_1. The cellulase activities of the separated components were reduced considerably compared with the activity of the unfractionated culture filtrate (Table 2). Reconstitution experiments indicated that all of the original activity could only be obtained when all components were recombined in their original proportion. This suggested that the cotton solubilising activity of the cellulase complex was dependent upon the synergistic action of the C_1, CM-cellulase and cellobiase components.

Synergistic effects have also been reported with Penicillium funiculosum (Selby 1969), Trichoderma reesei (Pettersson et al. 1972), T. koningii (Wood & McCrae, 1975) and Fusarium solani (Wood and McCrae, 1977). Investigations of synergism continued and revealed that C_1 acted synergistically only with certain Cx components. Wood and McCrae (1979) separated six Cx components from T. koningii and found that a low molecular weight component (Cx_1) showed the most random attack and the greatest solubilising capability but did not show any synergism with C_1. It was suggested by these workers that extensive hydrolysis of crystalline cellulose is accomplished only by those pairs of enzymes which can act synergistically and which form a loose complex on the surface of the crystalline region of the cellulose.

There is now substantial evidence that the C_1 component is a cellobiohydrolase, removing cellobiose units from the non-reducing ends of

TABLE 2 Solubilization of cotton by components of Trichoderma viride. alone and in combination (Selby & Maitland, 1967).

Component(s) (0.5 ml of each component, total volume made up to 1.5 ml with buffer solution, pH 4.5)	Solubilization	Relative cellulase activity	Recovery of cellulase activity (%)
Reference solution diluted 4 times	46.0	0.24	100
$C_1 + C_x$	47.7	0.25	104
C_1	1.0	<0.01	1
CM-cellulase	3.2	0.01	4
Cellobiase	0.8	<0.01	<1
CM-cellulase + cellobiase	1.8	<0.01	2
C_1 + CM-cellulase	21.0	<0.08	35
C_1 + cellobiase	14.0	0.05	20
C_1 + cellobiase + CM-cellulase	47.2	0.25	104

the cellulose chains (Halliwell & Riaz, 1971; Halliwell et al, 1972; Halliwell & Griffin, 1973; Pettersson et al, 1972). However, there is some disagreement between authors as to the extent to which highly ordered cellulose is hydrolysed by the C_1 component. The C_1 components from Trichoderma koningii (Wood & McCrae, 1972), Fusarium solani (Wood & Phillips, 1969) and Penicillium funiculosum (Selby, 1969) were all unable to attack crystalline cellulose to any significant extent. In contrast, the C_1 components from Trichoderma reesei (Mandels & Reese, 1964; Nisizawa et al., 1972) were able to attack crystalline cellulose. These differences were observed despite the fact that highly purified C_1 components, free from contaminating Cx components were used.

Since crystalline cellulose has few ends available for attack by a cellobiohydrolase, it is now thought that one of the Cx components attacks the cellulose first to provide free ends for the cellobiohydrolase. To avoid confusion, the terms C_1 and Cx should no longer be used as the C_1 component is not responsible for the initiation of cellulose degradation. Instead, the words exoglucanase (cellobiohydrolase) and endoglucanase should be used.

Pettersson (1975) suggested that the mechanism of enzymatic cellulose degradation was as follows (Fig.8). In the first stage of the reaction, regions of low crystallinity in the cellulose are attacked by endoglucanases and free chain ends are created. Exoglucanases start the degradation from the chain ends, hydrolytically removing cellobiose. The cellobiose is hydrolysed to glucose through the action of β-glucosidase. In support of this new theory, Streamer et al. (1975) observed that if dewaxed cotton was pretreated with endoglucanase, the exoglucanase released a greater amount of degradation products than from untreated cotton. Further support has been provided by the work of Wood and McCrae (1972) and Pettersson et al. (1972).

native cellulose —endoglucanase→ modified cellulose

modified cellulose —exoglucanase→ cellobiose

cellobiose —β-glucosidase→ glucose

Fig. 8

A mechanism for enzymatic cellulose degradation. Modified cellulose formed from native cellulose by the action of the endoglucanase on non-crystalline regions of the cellulose fibre. Free chain ends are created (Pettersson,1975).

Precise details of the initiation reaction for cellulose degradation are still not clear. It is possible that the enzymes involved have not yet been identified. Eriksson et al. (1975) have demonstrated that an oxidative enzyme (cellobiose oxidase) may be involved in cellulose degradation. Working with Sporotrichum pulverulentum, Eriksson et al. (1975) showed that the percentage degradation of cellulose was reduced considerably in the absence of oxygen (Table 3) suggesting that an additional oxidising enzyme may be necessary. Similar observations have been made with Myrothecium verrucaria, Polyporus adustus, and Trichoderma reesei (Eriksson et al., 1975). They suggested that this enzyme may insert uronic acid moieties into cellulose thereby breaking the hydrogen bonds between the chains.

According to Wood and McCrae (1979), the absence of oxygen had a much less dramatic effect on the extent of cellulose hydrolysis by Trichoderma koningii, Fusarium solani and Penicillium funiculosum. This indicated that the presence of an oxidising enzyme seems unlikely with these organisms.

Since it is now generally accepted that endoglucanases act randomly through the cellulose chain allowing subsequent action by exoglucanases, Reese (1977) has now modified his original (C_1-Cx) concept. This new concept states that C_1 is capable of disrupting both hydrogen and covalent bonds in the cellulose chain. Thus C_1 is a very special endoglucanase, having properties not possessed by other endoglucanases including disruption of hydrogen bonds, lack of activity on CMC and inability to act on its own products. Other randomly acting components are capable of hydrolysing the C_1 component products.

Effect of cellulase on cellulose fibres

In the initial stages of cellulose degradation, attack by cellulolytic enzymes results in extensive changes in the physical

Precise details of the initiation reaction for cellulose degradation are still not clear. It is possible that the enzymes involved have not yet been identified. Eriksson et al. (1975) have demonstrated that an oxidative enzyme (cellobiose oxidase) may be involved in cellulose degradation. Working with Sporotrichum pulverulentum, Eriksson et al. (1975) showed that the percentage degradation of cellulose was reduced considerably in the absence of oxygen (Table 3) suggesting that an additional oxidising enzyme may be necessary. Similar observations have been made with Myrothecium verrucaria, Polyporus adustus, and Trichoderma reesei (Eriksson et al., 1975). They suggested that this enzyme may insert uronic acid moieties into cellulose thereby breaking the hydrogen bonds between the chains.

According to Wood and McCrae (1979), the absence of oxygen had a much less dramatic effect on the extent of cellulose hydrolysis by Trichoderma koningii, Fusarium solani and Penicillium funiculosum. This indicated that the presence of an oxidising enzyme seems unlikely with these organisms.

Since it is now generally accepted that endoglucanases act randomly through the cellulose chain allowing subsequent action by exoglucanases, Reese (1977) has now modified his original (C_1-Cx) concept. This new concept states that C_1 is capable of disrupting both hydrogen and covalent bonds in the cellulose chain. Thus C_1 is a very special endoglucanase, having properties not possessed by other endoglucanases including disruption of hydrogen bonds, lack of activity on CMC and inability to act on its own products. Other randomly acting components are capable of hydrolysing the C_1 component products.

Effect of cellulase on cellulose fibres

In the initial stages of cellulose degradation, attack by cellulolytic enzymes results in extensive changes in the physical

TABLE 3 Degradation of cotton by cell-free, concentrated culture solutions of four different cellulose-degrading fungi in the presence and absence of oxygen (Eriksson, 1975).

Organism	Cellulose degradation (wt loss %)	
	O_2 atmosphere	N_2 atmosphere
Sporotrichum pulverulentum[a]	52.1	21.5
Polyporus adustus[b]	42.6	18.0
Myrothecium verrucaria[b]	33.6	17.0
Trichoderma reesei[c]	20.0	10.0

[a] Culture solution concentrated 50 times

[b] Culture solution concentrated 30 times

[c] Culture solution concentrated 20 times

properties of the cellulose fibres prior to reducing sugar production. These changes include transverse cracking, loss in tensile strength, lowering of the degree of polymerisation, increased capacity for moisture or alkali uptake, and fragmentation to short separable fibres. The latter two are the most significant phenomena.

1. Swelling of cellulose fibres.

This is sometimes known as the swelling factor or S-factor and refers to the increased capacity of the cellulose fibres for moisture or alkali uptake occurring in the initial stages of enzymatic attack. The precise action of the S-factor is unknown. It has been suggested that it is an enzyme-like component causing damage to the primary wall possibly attacking either atypical linkages in the cellulose fibre or non-cellulosic components (Reese & Gilligan, 1954).

Most reports have suggested that the S-factor is associated with the Cx component (Reese & Gilligan, 1954; Youatt, 1962) but possible connections with the C_1 component as yet cannot be excluded. In a study made by Wood (1968) using Trichoderma koningii cellulase, it was found that although the C_1 component alone did not show any S-factor activity, synergistic effects were observed when the C_1 component was mixed with the Cx component. It therefore appeared that the overall S-factor activity resulted from several enzymes acting in concert.

2. Short fibre formation.

The breakdown of cellulose fibres into smaller segments prior to the production of reducing sugars was first observed by Toyama (1960) working with T. koningii. A similar observation was made by Halliwell (1965) also working with the same fungus. This author found that in the initial stages of degradation the majority of the substrate was converted to insoluble short fibres with only a very small amount of reducing sugars being produced. The presence of oxygen appeared to be important as

disintegration was increased when the cellulose fibres were shaken.

Several workers investigating the formation of short fibres (Selby & Maitland, 1967; Liu & King, 1967) suggested that the C_1 component was involved. The fragmentation of cellulose fibres has been extensively studied by Halliwell and Riaz (1970, 1971). Working with cultures of T. koningii, these authors separated the cellulase complex into various components and found that the ability to form short fibres was associated with a CM-cellulase and a C_2 component. These two components acted synergistically and their action was unaffected by other components. Similarly, Wood (1975) and Pettersson (1975) have found short fibre forming activity to be associated with the CM-cellulase components.

Structural features of cellulose in relation to enzymatic degradation

Microorganisms degrading cellulose can be found in close association with either the exterior surface of the cellulose fibre, as often is the case with cotton, or within the fibre lumina, as with wood. Cell wall-bound or cell-free cellulases produced by these microorganisms hydrolyse the cellulose to soluble products. Direct physical contact between the cellulase and the cellulose fibre is a prerequisite of cellulose hydrolysis. Any structural features that limit enzyme accessibility or diffusion will have a profound influence on the susceptibility of the fibre to degradation (Cowling, 1975). Such features include:

1. Moisture content - the presence of water is essential for the degradation of cellulose fibres as it provides a medium for the diffusion of enzymes between the organism and the cellulose fibres. In addition, the water causes swelling of the cellulose fibres such that the structure is more accessible to the cellulolytic enzymes and is also necessary for the hydrolytic cleavage of the glucosidic links in the cellulose chain. As little as 10% moisture is adequate for the degradation of cotton fibres

(Siu, 1951) and when the water content falls below this value the cellulose fibres become more resistant to enzymatic attack.

2. Size and diffusibility of the cellulases - cellulolytic enzymes must be able to diffuse from the organism to the accessible surface of the cellulose fibre. This movement can be restricted depending upon the size and shape of the cellulases, capillary spaces, i.e. lumina of cells, spaces between microfibrils and the ability of cellulases to penetrate the capillaries.

3. Degree of crystallinity - highly crystalline cellulose preparations have been found to be more resistant to enzymatic attack than those cellulose preparations with lower crystallinity (Norkrans, 1950). Any treatment of cellulose that alters the proportion of crystalline material, such as reprecipitation from solution or mechanical disruption, will alter the susceptibility of the cellulose to enzymatic attack.

4. Unit cell dimensions - cellulose occurs in four different crystal structures (designated cellulose I, II, III and IV; Bellegsen & Tonnensen, 1971) which can be distinguished by their characteristic x-ray diffraction patterns. Cellulose I is found in native cellulosic materials, cellulose II in regenerated materials such as viscose, cellulose III is formed after treatment with anhydrous ethylamine and form IV after treatment with high temperatures. These four structures differ in the dimensions of the repeating 3-D unit in the crystalline regions. Rautela (1967) showed that Trichoderma reesei grown on each of the crystal forms of cellulose exhibited a lower activation energy for the substrate on which it had been grown compared with the other three. This indicated that the cellulases adopted a shape which could accommodate a specific crystal form of cellulose.

5. Conformation and steric rigidity of the anhydroglucose units - greater resistance of the crystalline regions of cellulose as compared

with amorphous regions may be due to differences in conformation and steric rigidity of the anhydroglucose units and not merely due to physical inaccessibility.

6. Associated non-cellulosic material - cellulose is often associated with a variety of other non-cellulosic material which may influence the susceptibility of the cellulose fibre to enzymatic attack. Growth of cellulolytic organisms may be stimulated by the presence of soluble carbohydrates or vitamins, or inhibited by toxic substances such as phenolic compounds. The accessibility of the cellulose may be reduced by the presence of substances deposited within the fine capillary structure of the cell wall. In addition, specific enzyme inhibitors may be present which reduce the rate or extent of hydrolysis. Crystalline cellulose associated with lignin forms a very resistant material, apparently because the cellulases are unable to diffuse to the accessible regions.

7. Nature, concentration and distribution of substituent groups - cellulose derivatives containing substituent groups such as methyl, ethyl, carboxymethyl are very stable since these groups are not readily removed by enzymatic cleavage.

Thus, the susceptibility of cellulose to enzyme degradation is determined mostly by its accessibility to cellulases. Any structural features that limit accessibility of the cellulose to the enzymes diminish the susceptibility of the cellulose fibre to enzymatic degradation.

Cellulase synthesis

In many cellulolytic organisms synthesis of the cellulase complex is regulated by an induction-repression mechanism. Several carbon sources such as cellobiose, sophorose, lactose, soluble cellulose derivatives and, in particular, insoluble cellulose have been found to act as inducers. Mandels and Reese (1960) reported that the cellulolytic enzymes produced by *Trichoderma reesei* were induced during growth with cellulose as the

sole carbon source. Since cellulose is an insoluble substrate, unable to enter the cell and induce cellulase synthesis, Mandels and Reese proposed that small amounts of cellulase were produced constitutively and released into the medium. Constitutive production of this type is known as basal synthesis. Soluble end products of cellulolysis, able to enter the cell, then acted as inducers of cellulase synthesis. This proposal has been supported by Eriksson and Hamp (1978) working with Sporotrichum pulverulentum.

Mandels et al. (1962) also found that sophorose was a powerful cellulase inducer in T. reesei, similar observations were later made by Nisizawa et al. (1971). Other studies by Sternberg and Mandels (1979) and Loewenberg and Chapman (1977) revealed that a considerably lower sophorose concentration induced cellulase synthesis, in comparison with cellulose and other inducers. Although sophorose has been shown to be a potent inducer of cellulase synthesis, there is no evidence that it would be involved in the natural environment. Cellobiose at low concentrations induces cellulase synthesis (Ghose et al., 1975) and this is assumed to be the natural inducer.

Cellulase synthesis is repressed in the presence of glucose (Nisizawa et al., 1971; Ohki, 1975 and Goldberger et al., 1976). Addition of glucose to a culture of Trichoderma reesei growing on cellulose results in the immediate cessation of cellulase synthesis until the glucose is exhausted or its residual level falls below a critical value (Gallo et al., 1979; Pettersen, 1977). This effect is presumably due to catabolite repression.

Although there is evidence that the synthesis of cellulase is regulated by induction and repression, the exact mechanism remains unknown. Mechanisms regulating other inducible-catabolic enzymes, such as β-galactosidase in Escherichia coli, have been well studied and could serve

as models for cellulases (Fennington et al., 1983). Thus cyclic adenosine 3'5' monophosphate (c AMP) may play an important role in the regulation of cellulase synthesis and has been implicated in the regulation of lignin degradation in fungi (MacDonald et al., 1984). With glucose readily available as an energy source, c AMP levels in the cell would be low, thus preventing the synthesis of enzymes capable of utilising other carbon sources.

Location of cellulases

Cellulose, being insoluble in water and highly polymeric, cannot penetrate microbial cell walls. Initial attack must therefore take place outside the cell by cellulolytic enzymes located on the surface of the cell wall or in the medium surrounding the microbial cell.

The terminology used in the literature to describe the location of cellulolytic enzymes in microbial cultures is confusing as words such as exoenzyme and extracellular are used. Henceforth, cell-free will be used to describe enzymes which are not associated with the cell, cell-bound for enzymes which are bound to the cell wall, and intracellular for enzymes located inside the cell.

Cellulases have been detected both as cell-free and cell-bound enzymes. Berg (1975) found that cell-free endoglucanases were produced during the growth of Cellvibrio fulvus on cellulose. A similar observation was made with Cellulomonas uda (Stoppok et al., 1982), although very small amounts of intracellular endoglucanase were also produced. Stoppok et al. (1982) suggested that this low level was the basal level of constitutive endoglucanase; such basal levels have been confirmed for Sporotrichum pulverulentum (Eriksson & Hamp, 1978), and Trichoderma reesei (Gong & Tsao, 1979). Cell-free cellulases have also been detected during the growth of fungi such as Penicillium janthinellum (Rapp et al., 1981).

In contrast, Berg and Pettersson (1977) reported that much of the cellulose of Trichoderma reesei was cell-bound during active growth and only released into the medium following cell lysis. Analysis of the cell wall carbohydrates of this fungus revealed the presence of glucose, mannose and galactose which were similar to the carbohydrate content of C_1 cellulase of T. reesei. This suggested that the cellulases may have been bound to the cell wall. In support of this, electron microscopy studies showed the close association of T. reesei hyphae and cellulose fibres (Berg & Hofsten, 1976), suggesting that cellulases could be located on the cell surface. Similar studies also revealed that many lysed cells were present when the cell-free cellulases were detected.

Kyslíková and Volfová (1981) also found that T. reesei produced cell-bound endoglucanase, although cell-free endoglucanase was detected during active growth. Another fungus reported to produce both cell-free and cell bound cellulases was Dendryphiella arenaria (MacDonald & Speedie, 1982). However, in this case, the endoglucanase was predominantly cell-free and the **cellulase (exoglucanase) cell-bound**

Berg (1975) demonstrated that the location of cellulases was dependent upon the carbon source for growth. When grown on cellulose, Cellvibrio fulvus produced cell-free endoglucanase. On cellobiose, the endoglucanase was partially cell-bound and intracellular. In addition, Trichoderma reesei produced cell-bound endoglucanase when growing on cellulose but mainly cell-free endoglucanase on CMC (Berg & Pettersson, 1977).

The location of β-glucosidase is not restricted to the cell wall surface or to the outside of the cell as with cellulases since low molecular weight products of cellulase action are able to enter the cell. β-Glucosidase has been found to be cell-bound, cell-free and intracellular. Berg and Pettersson (1977) found that β-glucosidase from

T. reesei was essentially cell-bound and not released unless the cells were autolysing. In cultures of Penicillium janthinellum grown on cellulose, β-glucosidases were predomonantly cell-free, with only a small amount associated with the mycelium (Rapp et al., 1981). Intracellular β-glucosidase was found with cultures of Sporotrichum thermophile, only very small amounts were associated with the cell wall and no significant amounts of cell-free β-glucosidase were ever found (Canevascini & Meyer, 1979).

Assays for cellulolytic activity

A wide variety of methods can be used for the determination of cellulolytic activity. These differ greatly in sensitivity and in the cellulase components detected, depending upon the substrate used, the effect measured and the length and conditions of the assay.

Table 4 gives a list of substrates which are commonly used in the determination of cellulolytic activity. The most highly resistant forms of cellulose include cotton lint and dewaxed cotton, whereas the least resistant include CMC. The susceptibility of the other substrates lies between these two extremes.

Assay methods are normally based on the following criteria:

1. Decrease in viscosity of cellulose derivatives (Almin & Eriksson, 1967).

The viscosity of cellulose is dependent upon its chain length. A few random breaks in the cellulose chain can cause a marked decrease in the chain length, hence making this a very sensitive assay.

Although CMC is a soluble cellulose derivative it is generally not used in this assay since its viscosity is greatly dependent upon pH, ionic strength and polyvalent cations. Hence its use requires a thorough

Table 4 Substrates used in cellulase assays (Wood, 1970)

Substrate	Example
native cotton	cotton lint
mechanically modified cellulose	ball-milled cellulose
purified cellulose	filter paper; cellulose powder
alkali-treated cellulose	alkali swollen cellulose
acid-treated cellulose	hydrocellulose; Avicel
regenerated cellulose	Cellophane; viscose
soluble cellulose derivatives	carboxymethylcellulose

knowledge of its properties. Non-ionic substituted celluloses, such as hydroxyethylcellulose are usually used, particularly when determining low levels of enzyme activity. This assay measures endoglucanase activity independent of exoglucanase.

2. Production of reducing sugars from soluble cellulose derivatives (Mandels & Weber, 1969).

Production of reducing sugars can be determined either by the dinitrosalicylic acid method (Miller, 1959), the Somogyi-Nelson method (Somogyi, 1952; Nelson, 1944), the orcinol method (Vasseur, 1948) or the modified Hoffman ferricyanide method described by Grady and Lamar (1959).

This assay also measures endoglucanase activity independent of exoglucanase activity.

3. Loss in weight or production of reducing sugars from an insoluble substrate (Halliwell, 1965).

This assay measures the combined action of exo- and endoglucanases. Cotton is generally used as a substrate because of its resistance to enzyme attack making it the best indicator of exoglucanase activity. More extensive hydrolysis is necessary if weight loss is measured than if the production of reducing sugars is used.

4. Change in turbidity of cellulose suspension (King, 1965).

Using hydrocellulose or Avicel as a substrate, this method can be used to measure the combined action of exo-and endoglucanases. It is often a method used to screen for cellulase production.

5. Measurement of clearance zones in agar (Eriksson & Goodell, 1974).

Solid media containing phosphoric acid-swollen cellulose can be used to detect the production of cellulases by the formation of clear zones around the organism.

6. Release of dye from dyed insoluble substrates.

Dyed filter paper (Poincelot & Day, 1972), solka floc (Leisola *et*

al., 1975) and Avicel (Leisola et al., 1975) have been used for this purpose and the release of dye is measured colorimetrically.

7. Measurement of less clearly defined activities such as reduction in breaking strength of thread (Selby, 1963), swelling of cotton (Reese & Gilligan, 1954) and microfragmentation of cellulose micelles (Halliwell, 1966).

Most assays used for determining cellulolytic activity are carried out with culture filtrates containing a mixture of enzymes. The overall cellulolytic activity, endoglucanase activity and β-glucosidase activity can be determined. Direct measurements of exoglucanase activity can only be made with preparations of purified enzyme since there is no specific substrate available for exoglucanase.

Overall cellulolytic activity - in determining the overall cellulolytic activity, the synergistic action of exoglucanase and endoglucanase is measured. The type of substrate used in the assay is important. It must be an insoluble cellulosic material which is not readily hydrolysed, e.g. cotton, Avicel, solka floc. In addition, the reaction time is important and it should be long enough for the enzyme to diffuse into the insoluble substrate and for the hydrolysis products to diffuse out of the fibre. Also, it should allow for an appreciable fraction of the less accessible bonds to be hydrolysed so that measurement is not based on enzyme action on the most susceptible regions of the substrate. Wood and McCrae (1978) use an 18hr and 7d cotton assay which overcomes the problems of measuring the hydrolysis of the amorphous cellulose only. Assays which are generally used include Sections 3-7 described above .

When the production of reducing sugars from an insoluble substrate is measured, the activity determined is the sum of the different cellulolytic activities. Hence the results obtained depend upon the

relative proportions of the different enzymes. β-Glucosidase activity, producing glucose can also greatly influence the results. Determination of the loss in weight of an insoluble substrate generally gives more reliable results but this method is tedious and impractical when many samples are used. Methods following the release of dye from insoluble substrates are frequently used and have proved convenient and rapid. However, owing to the impermeable nature of the crystalline regions of the cellulose, it is possible that the dye will not have penetrated these areas. In this case, the dye release is a measure of the hydrolysis of amorphous cellulose. When using this method, possible effects of the dye on the growth of the organism and on the cellulolytic enzymes must be considered. Measurement of clearance zones in agar is also used to measure the overall cellulolytic activity. However, this method is invalid if only cell-bound, non diffusable enzymes are produced.

Endoglucanase activity - this can be determined by measuring either the decrease in viscosity or the production of reducing sugars using CMC as a substrate (Sections 1 and 2 described above). Decrease in viscosity is frequently used as it is the most sensitive of the two methods and requires a short incubation time. Both assays measure endoglucanase activity independent of exoglucanase, as the substrate is not attacked by glucanases other than endoglucanase. The presence of β-glucosidase influences the production of reducing sugars as this enzyme is capable of hydrolysing small oligosaccharides produced by the action of endoglucanases.

β-Glucosidase activity - fewest problems arise in the determination of β-glucosidase activity. Standard assay methods use cellobiose (Selby & Maitland, 1967), or aryl β-glucosides such as p-nitrophenyl β-D glucoside (Wood, 1968) or o-nitrophenyl β-D-glucopyranoside as substrates.

Applications of Cellulases

Early work on cellulose focused its attention on preventing the degradation of cellulosic material. Today this work is expanding to exploit and further develop biotechnological applications of cellulolytic microorganisms.

Current and potential uses of cellulases fall into two main categories (Coughlan & Folan, 1979):-

1. Hydrolysis of cellulose to provide substrates for a variety of chemical and fermentation industries. End products from such industries could include methane, ethanol, citric acid, antibiotics, single cell protein, etc.
2. Direct uses of cellulases in food processing, preparation of animal foodstuffs, pharmaceutical processes and pollution control, e.g. improving the digestibility of forage, extraction of flavours, oils or juices from fruit and vegetables. Some of these processes are currently being used commercially, particularly in Japan. The rest must remain as potential uses for the present time as the technology is still undergoing development.

Microbial Communities Degrading Cellulose

Microorganisms rarely occur alone in the natural environment but are generally found in communities composed of different populations with various metabolic activities. The types of interactions which can occur within a community are listed in Table 5 (based on definitions given by Alexander, 1971).

Although the degradation of cellulose by microorganisms in axenic culture has been extensively studied, it is likely that in natural environments, cellulose is degraded by communities of organisms. Most of the evidence for the involvement of microbial communities in cellulose degradation has come from investigations of anaerobic environments such as

TABLE 5 Types of interactions between two species

1)	Mutualism	Both species benefit
2)	Neutralism	Neither species benefit
3)	Antagonism	One of the interacting species suffers because of the activity of a second species.
	i) Competition	Two species in rivalry for a limiting factor in the environment, both become affected.
	ii) Amensalism	One species is suppressed due to the release of toxins by another.
	iii) Predation	Ingestion of one species by another.
	iv) Parasitism	Attack and feeding by one species on another.
4)	Synergism	Association of two species having complementary activities resulting in greater product formation than by one species alone.
5)	Commensalism	One species benefits from the presence of a second which itself does not derive any advantage or disadvantage from the activity of the first species.

activated sludge (Weimer & Zeikus, 1977; Laube & Martin, 1981) and the rumen (Latham & Wolin, 1977; Wolin & Miller, 1983). Several types of interactions have been demonstrated and some of these are described below.

1. Mutualism

A mutualistic association has been demonstrated between the cellulolytic Ruminococcus albus and the non-cellulolytic Bacillus ruminicola (Bryant & Wolin, 1975). In cultures growing in a defined medium with cellulose as the carbon source the following interactions occurred. Ruminococcus albus requires both straight chain and branched fatty acids for the synthesis of amino acids. Although it provides itself with the straight chain fatty acids from cellulose fermentation, it relies on B. ruminicola to supply the branched fatty acids. In addition, R. albus is supplied with a nitrogen source in the form of ammonium ions. In return, B. ruminicola is provided with cellulose degradation products which it uses as an energy source.

Another example of mutualism exists between the cellulolytic bacterium R. albus and the methanogenic Methanobacterium ruminantium found in the rumen. The interactions between these two species have been reviewed by Wolin and Miller (1983) and are shown in Fig. 9. R. albus ferments carbohydrates formed from the degradation of cellulose, via the Embden-Meyerhof-Parnas pathway. When grown in pure culture the major end product is ethanol. At an intermediate step in the pathway, glyceraldehyde-3-phosphate is oxidised with the formation of NADH. As NADH is reoxidised, this leads to the accumulation of H_2 which prevents further reoxidation. The bacterium therefore has to rely on the formation of electron sink products such as ethanol for the reoxidation of NADH. However, in the presence of methanogenic bacteria such as M. ruminantium, the fermentation pathway becomes altered and acetate becomes the major end product. The methanogenic bacterium reduces CO_2 using H_2 generated from

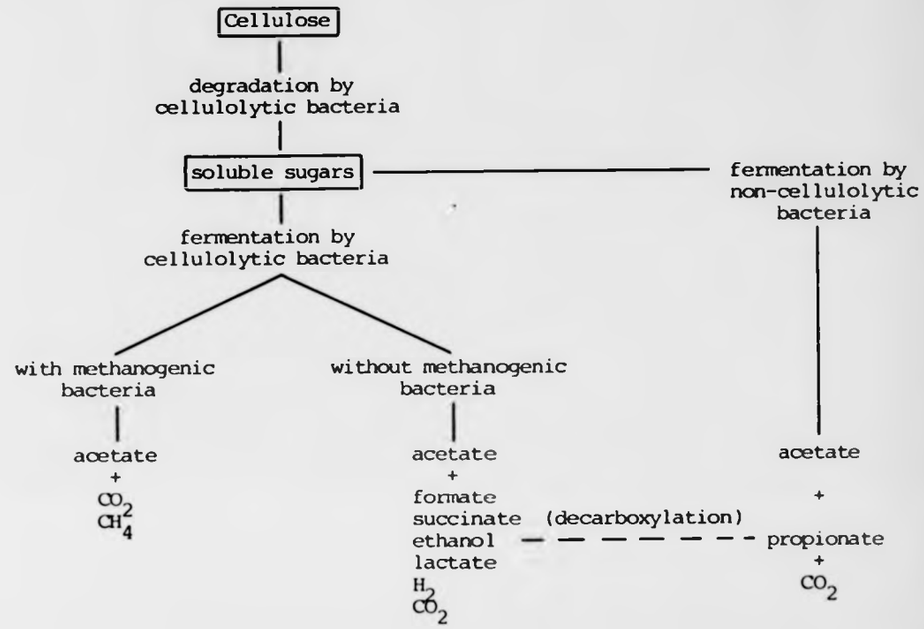


Fig. 9

Interactions between some cellulolytic and non-cellulolytic rumen bacteria during fermentation of cellulose.

Cellulolytic bacteria represented by: Ruminococcus flavefaciens
R. albus
Bacteroides succinogenes

Non-cellulolytic carbohydrate fermenting bacteria represented by : Selenomonas ruminantium

Methanogenic bacteria represented by: Methanobacterium ruminantium

the reoxidation of NADH and this results in the formation of methane. This is often referred to as the interspecies hydrogen transfer reaction. In the mixed culture, both the cellulolytic and methanogenic bacteria benefit; the methanogen receives an energy source and the cellulolytic bacterium gains ATP when acetyl-CoA is converted to acetate.

Similar types of reaction are known to occur with the cellulolytic bacterium Ruminococcus flavefaciens which normally produces succinate as the major end product (Wolin & Miller, 1983). In the presence of methanogenic bacteria the fermentation pathway is altered such that acetate is the major fermentation product.

2. Neutralism

The association of the cellulolytic bacterium Bacteroides succinogenes and the non-cellulolytic bacterium Selenomonas ruminantium (Scheifinger & Wolin, 1973) is one of the few examples of neutralism which occurs in the degradation of cellulose. Scheifinger and Wolin (1973) demonstrated that these two organisms grew well in mixed culture with cellulose as the carbon source. Products of cellulose degradation released by B. succinogenes support the growth of S. ruminantium. The two species compete for these degradation products and this results in a decrease in the number of B. succinogenes. In addition to producing propionate from the carbohydrates, S. ruminantium also decarboxylates succinate produced by B. succinogenes to propionate. However, this does not provide an energetic advantage for either species.

3. Synergism

In many cases, microbial populations have been found to act synergistically in the degradation of cellulose. As a result of the interactions between the different microbial populations, the community shows an increased rate of cellulolysis compared with the microorganisms in monoculture. Some examples of synergism and factors contributing to

this effect are given below.

Enebo (1949) demonstrated the synergistic effects of a microbial community consisting of the cellulolytic bacterium Clostridium thermocellulaseum and two non-cellulolytic bacteria Clostridium thermobutyricum and Bacillus thermolacticus. This community converted up to twice the amount of cellulose in considerably less time than the cellulolytic bacterium alone. The interactions among the three organisms in the mixed culture were assumed to take place as follows. Bacillus thermolacticus lowered the redox potential and this enabled the anaerobic bacteria C. thermocellulaseum and C. thermobutyricum to grow. As C.thermobutyricum and B. thermolacticus were incapable of utilising cellulose they were dependent upon the cellulose degradation products as carbon sources. It was suggested that the removal of these products prevented the inhibition of cellulase activity, thus permitting further cellulolysis.

Synergistic effects have also been observed in communities, possibly as a result of the removal of metabolic inhibitors. Some of the major end products of cellulose fermentation include volatile fatty acids such as acetate and propionate which are often inhibitory to the growth of anaerobic cellulolytic microorganisms (Khan, 1980). The utilisation of these fatty acids by methanogenic bacteria such as Methanosarcina barkeri removes the inhibitory effect on the cellulolytic microorganisms and allows further cellulolysis to occur.

Hofsten et al. (1971) suggested that one possible reason for the increased growth rate observed with a mixed culture containing a cellulolytic Sporocytophaga species and a non-cellulolytic eubacterium was the supply of growth factors essential for cellulose hydrolysis.

Recently, Veal and Lynch (1984) demonstrated synergism between the cellulolytic fungus Trichoderma harzianum and the anaerobic N_2-fixing

bacterium Clostridium butyricum. These authors found that the two organisms in co-culture decomposed cellulose at a substantially increased rate compared with the fungus alone. It was suggested that C. butyricum, unable to grow on the cellulose, relied on the end products of fungal cellulolysis for growth. As a result, any end product inhibition of fungal cellulases was removed. In addition, C. butyricum made N_2 available to T. harzianum. The anaerobic bacterium was able to grow under aerobic conditions, presumably as T. harzianum intercepted the available oxygen. As a consequence the rate of cellulose hydrolysis increased.

The possibility also exists that synergism occurs as a result of the concerted action of cellulolytic enzymes produced by different microorganisms. Evidence for this has been provided by Wood and McCrae (1979). These authors found that by combining cellulase components produced by a number of different fungi, the percentage solubilisation of cellulose was increased compared with that by the components alone.

Aims of the present study

The purpose of this work was to characterise a cellulolytic microbial community isolated from soil in order to determine whether the concept that "microbial communities are more efficient at degrading cellulose than pure cultures" is valid.

The approach taken was to characterise the individual members of the community in terms of their cellulase production and their rates of cellulose breakdown and thereby make a direct comparison with that of the community.

Attempts were made to determine the role of the individual members in the degradation of cellulose and to examine whether synergistic interactions occurred within the community during this process.

To achieve these aims, a study was made of the community grown on cellulose (Sigmacell type 20) and the lignocellulosic substrates, hay and straw, under different cultural conditions. The ultimate experimental procedures included the following:

1. Estimation of cellulase , endoglucanase and β-glucosidase activity in culture filtrates of the community and its individual members grown on crystalline cellulose.
2. Measurement of the rate and extent of cellulose (Sigmacell type 20) degradation by each of the cultures.
3. Determination of the influence of cultural conditions (pH and cellulose concentration) on enzyme production by the community.
4. Measurement of enzyme production and rates of lignocellulose degradation by the community.

CHAPTER TWO

MATERIALS AND METHODS

CHAPTER TWO

MATERIALS AND METHODS

1. ENRICHMENT AND ISOLATION OF CELLULOLYTIC ORGANISMS

1.1 Design and Maintenance of continuous flow chemostat

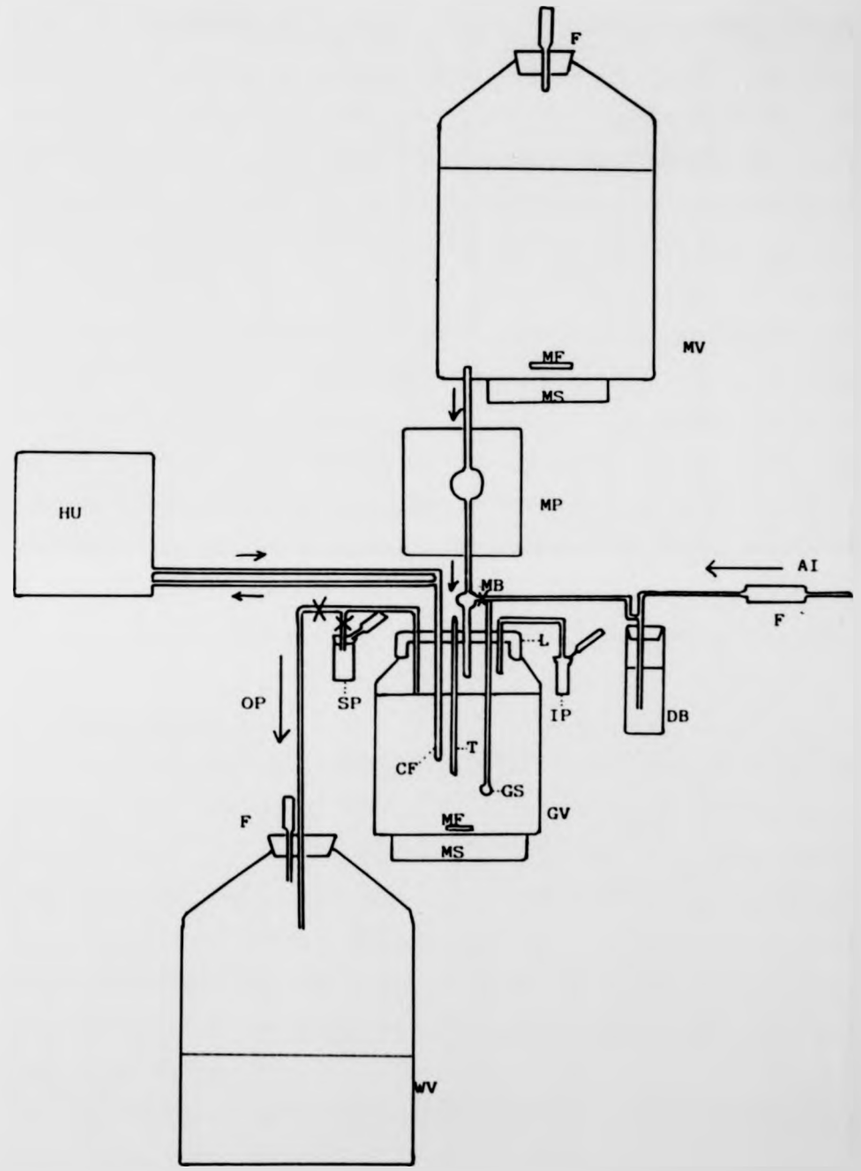
The design of the continuous flow chemostat used for the enrichment of cellulolytic organisms from soil is shown in Fig. 10. It was designed specifically for use with an insoluble cellulosic substrate, since blockage of medium lines occurred when more conventional peristaltically pumped chemostats were used.

The 1 litre capacity growth vessel (G.V.; Quickfit, L.H. Engineering, Stoke) had an operating volume of 0.8 litres. Growth medium temperature was maintained at a constant 30°C (± 2°C) using a water circulating cooling finger (C.F., Corning) and a heating unit (H.U; Conair-Churchill). The medium was aerated with air (0.5 l/min) using a glass sparger (G.S.). The air was sterilised by passing it through a filter (F.) packed with cotton wool and glass wool, the latter to allow a faster rate of aeration than can be obtained with cotton wool alone. The efficiency of the filter unit was predetermined by passing air through sterile medium for 48h, after which time no bacterial or fungal contamination was evident when aliquots of the medium were inoculated onto nutrient agar plates. A follower (M.F; 3 cm) within the medium and a magnetic stirrer (M.S; Gallenkamp) were used to maintain an agitation rate of 1,000 rpm.

The volume of growth medium in the culture vessel was kept constant using an overflow pipe (O.P.). A 20 litre Pyrex vessel (WV, Fisons) was used to collect the medium overflow and could be aseptically replaced with an empty vessel when necessary. Cellulose was kept in suspension in the fresh growth medium (initially 8 litres), contained in a 10 litre glass aspirator (MV, Fisons), by using a magnetic stirrer (M.S) and follower

Fig. 10

Diagrammatic representation of continuous flow chemostat MV; medium vessel, WV: waste vessel, GV; growth vessel, L; lid, MP; membrane pump, HU; heating unit, CF; cooling finger, T; thermometer, GS; glass sparger, MB; media break IP; inoculum port, SP; sample port, MS; magnetic stirrer, MF; magnetic follower, F; filter, AI; air in, OP; overflow pipe, DB; Dreschlerbottle.



(M.F). Fresh growth medium was continuously supplied through silicone tubing (6 mm diameter) connected to the base of the medium vessel by a Braun membrane pump (M.P; model FE211, Scientific Instruments Ltd). The length of the flow line to and from the pump was minimised to prevent settling of the cellulose particles. Contamination of fresh growth medium by microorganisms from the growth vessel was prevented by the use of a glass media break (M.B). All tubing was secured in place by plastic Schuco clips. An inoculation port (IP), consisting of a sterile bottle (250 ml) connected to the top of the growth vessel, was used to inoculate the chemostat. A sample port (SP) in the overflow line allowed samples of medium (25 ml) to be withdrawn into a Universal bottle, after first closing the line at the entrance to the waste vessel with a Hoffman clip.

Growth medium was initially sterilised in the medium vessel by autoclaving at 15 psi for 40 min, all other glassware together with the silicone tubing and the membrane pump head were sterilised at 15 psi for 15 min.

1.2 Growth medium

For the growth of microorganisms in the chemostat, a modified Czapek-Dox (C D) medium was used. The medium contained, per litre of distilled water: cellulose (Sigmacell type 20), 5g; $(NH_4)_2SO_4$, 2.5g; KH_2PO_4, 1g; $MgSO_4.7H_2O$, 0.5g; KCl, 0.5g; $FeSO_4.7H_2O$, 5 mg; $MnSO_4.H_2O$, 1.5mg; $ZnSO_4.7H_2O$, 1.4 mg; $CoCl_2.6H_2O$, 2mg. The trace metals were sterilised separately from the growth medium by autoclaving at 10 psi for 10 min and added when the medium was cool. After autoclaving, the growth medium was at pH 4.8.

Small volumes of growth medium (100 or 500 ml) used in subsequent flask cultures were autoclaved at 15 psi for 15 min.

1.3 Source of inoculum and chemostat operation

As a source of inoculum, 10g of soil (supplied by ARC Letcombe Laboratory) taken from a field in which wheat had been cultivated for a number of years, were added to the cooled medium in the growth vessel via the inoculation port.

The chemostat was operated as a batch system for 18d before continuous flow culture was started. Initially the dilution rate was $0.165h^{-1}$, but after 7d it was adjusted to $0.05h^{-1}$ and maintained at this rate for 72 d.

1.4 Isolation of microorganisms

For the isolation of microorganisms from the chemostat, 1ml aliquots of culture medium, collected as described, were serially diluted and plated onto the following media:

(i) Luria agar - tryptone, 1% (w/v); NaCl, 1% (w/v); yeast extract, 0.5% (w/v); agar, 2% (w/v); pH adjusted to 7.0.

(ii) 4% (w/v) malt extract agar (MEA; Oxoid).

(iii) 4% MEA containing crystal violet, 0.015 µg/ml and streptomycin sulphate, 30 µg/ml.

(iv) Modified Czapek-Dox medium (described above) with the addition of 2% (w/v) Lab M agar.

After inoculation, the plates were incubated at 30°C and examined for growth every day for 5d. Resulting colonies of either bacterial or fungal origin were transferred to fresh plates of the medium on which they were originally isolated using a sterile needle. This procedure was repeated several times before a pure culture of the isolate was obtained.

2. GROWTH OF BACTERIA ON CELLULOSE AND CELLOBIOSE

Bacteria were maintained on 2% (w/v) nutrient agar slopes at 4°C, following growth at 30°C for 2d. Conical flasks (250 ml) containing 0.5% (w/v)cellulose (Sigmacell type 20), CMC or cellobiose in 100 ml modified Czapek-

Dox medium (Section 1.2) were inoculated with the coryneform bacterium and the Alcaligenes/Pseudomonas sp. Flasks were incubated at 30°C in an orbital shaker (Gallenkamp) shaking at 150 rpm, for the required length of time.

Growth medium (5 ml) was aseptically removed from the culture flasks at regular intervals and the insoluble cellulose (Sigmacell type 20) was removed by centrifuging the sample in an MSE Super Minor centrifuge (5,000xg for 5 min at room temperature). The absorbance of the supernatant and the growth medium containing the soluble carbon source was measured at 560 nm in a Pye Unicam spectrophotometer (Model SP1700) against a distilled water blank.

The bacteria were also plated onto the following solid media:

1. Modified Czapek-Dox medium (Section 1.2) containing 0.5% (w/v) cellulose (Sigmacell type 20), with the addition of 2% (w/v) Lab M agar.
2. The medium described in 1 above, with Whatman No.1 filter paper (5.0 cm) as the carbon source. After inoculation, plates were incubated at 30°C and examined for growth every day for 14d.

3. GROWTH OF THE FUNGI ON CELLULOSE (SIGMACELL TYPE 20)

3.1 Preparation of Inoculum

Fungi were maintained on 4% (w/v) MEA slopes at 4°C following growth and sporulation at 30°C for 4d. To prepare inocula, slopes were flooded with 10 ml of distilled water with 0.2% (v/v) Triton X-100 and 4 glass beads (4.0 mm diameter) added. After gentle shaking to dislodge the spores, 1 ml of the resulting suspension was added to 100 ml of sterile growth medium.

For the community, the inoculum was prepared by combining 1 ml of spore suspension from each of the individual fungal species in a Universal bottle. The suspension thus formed was gently mixed by inverting the bottle several times, and 1 ml was added to 100 ml of sterile growth

medium.

3.2 Viable spore counts

Serial dilutions of the spore suspension, in 1 ml distilled water, were prepared and 0.1 ml aliquots were plated onto 4% (w/v) MEA plates containing 0.2% (w/v) ox gall to restrict fungal growth. After inoculation, plates were incubated at 30°C and examined for fungal growth every day. The number of viable spores was counted and the original number in the spore suspension determined.

3.3 Growth media and culture conditions

Fungi were grown on the following media:

i) 0.5% (w/v) cellulose (Sigmacell type 20) in modified CD medium.

ii) 0.5% - 5% (w/v) cellulose (Sigmacell type 20) in modified CD medium.

iii) 0.5% - 5% (w/v) cellulose (Sigmacell type 20) in 0.1M and 0.4M phosphate-buffered medium.

Modified CD medium was prepared and autoclaved as described in Section 1.2. Phosphate-buffered medium contained per litre of 0.1M or 0.4M phosphate buffer, pH 5.7 and 6.2 respectively: $(NH_4)_2SO_4$, 2.5g; $MgSO_4.7H_2O$, 0.5g; KCl, 0.5g; $FeSO_4.7H_2O$, 5mg; $MnSO_4.H_2O$, 1.5mg; $ZnSO_4.7H_2O$, 1.4mg; $CoCl_2.6H_2O$, 2mg; and was autoclaved as described for the modified CD medium.

Flasks (250 ml) containing 100 ml growth medium were inoculated with 1 ml of a spore suspension (Section 3.1) and incubated at 30°C in an orbital shaker (Gallenkamp) with shaking at 150 rpm. Two flasks were used for each cultural condition and each fungus. To prepare culture filtrates for enzyme assays, 5 ml samples were aseptically removed from the culture flasks and filtered through Whatman glass fibre filter papers (GF/C, 2.5cm) to remove the residual cellulose and fungal propagules. Efficiency of the filter system was determined by microscopic examination of the culture filtrates, and the filtrates were stored at -20°C.

3.4 Assay for cellulase activity

Measurements of cellulolytic activity were based on reducing sugars released from either cellulose (Avicel; Fluka) or carboxymethyl cellulose (CMC; Sigma) which were estimated colorimetrically using the dinitrosalicylic acid (DNS) method of Miller (1959). 1,4-β-D Glucan cellobiohydrolase (EC 3.2.1.91) was estimated from the cellulose (Avicel) assay and is referred to here as **cellulase**. 1,4-(1,3;1,4)-β-D Glucan 4-glucanohydrolase (EC 3.2.1.4) was estimated using the CMC assay and is referred to here as endoglucanase.

3.4.1 Preparation of DNS reagent

1g DNS, 20g potassium sodium tartrate, 0.2g phenol and 0.05g Na_2SO_3 were dissolved in 50 ml of 2% (w/v) NaOH. The final volume was then adjusted to 100 ml with distilled water.

3.4.2 Glucose standard curve

A range of glucose concentrations (0-0.2 mg/ml) distilled water) was prepared and 2 ml aliquots were added to 2 ml DNS reagent. For colour development, the tubes were placed in a boiling water bath for 15 min, cooled to room temperature and the absorbance measured at 575 nm in a Pye Unicam spectrophotometer (Model SP1700) against a reagent blank. A calibration curve was constructed of glucose concentration against absorbance at 575 nm (Appendix 1).

3.4.3 **Cellulase** activity

Samples of culture filtrates (0.2 ml) were incubated with 1.8 ml of 1% (w/v) Avicel in 0.05M sodium acetate buffer, pH 5, and 0.05% (w/v) NaN_3 added to prevent microbial contamination. The mixtures were incubated at 30°C for 5d, after which time the residual cellulose was removed by centrifugation and 2 ml DNS reagent added to the supernatant. After colour development using the procedure described above, the absorbance was measured at 575 nm. Reducing sugars were estimated from a previously constructed standard curve.

Enzyme units were expressed as micromoles of glucose equivalents released per day per ml of culture filtrate under the assay conditions stated.

3.4.4 Endoglucanase activity

Samples of culture filtrate (1 ml) were added to 1 ml 1% (w/v) CMC in 0.1M sodium acetate buffer, pH 4.8, and incubated at 50°C for 30 min. The reaction was stopped by the addition of 2 ml DNS reagent. Reducing sugars were estimated as described above.

With this assay, the rate of release of reducing sugars was only linear in the range of 0-0.2 mg/ml. Therefore, culture filtrates were diluted in 0.1M sodium acetate buffer, pH 4.8, such that the production of reducing sugars did not exceed 0.2 mg/ml under the standard assay conditions.

Culture filtrates from the 0.1M and 0.4M phosphate-buffered media were found to interfere with the DNS reagent such that a colour change no longer occurred in the presence of reducing sugars. To overcome this problem, MNaOH was added to the culture filtrates to adjust the pH back to the working range of the reagent. Samples were then placed in a boiling water bath and reducing sugars determined as previously described.

Enzyme units were expressed as micromoles of glucose equivalents released per min per ml of culture filtrate under the assay conditions stated.

3.4.5 PNP standard curve

A range of concentrations of para-nitrophenol (PNP , 0-0.25 mg/ml distilled water) were prepared and 0.5 ml aliquots were added to 10 ml 0.1M Na_2CO_3. Absorbance was measured at 420 nm in a Pye Unicam spectrophotometer (Model SP1700) against a 0.1M Na_2CO_3 blank. A calibration curve was constructed of PNP (mg/ml) against absorbance at 420 nm (Appendix 2).

3.4.6 β-Glucosidase activity

β-Glucosidase activity (β-D-glucoside glucohydrolase (EC 3.2.1.21) was determined colorimetrically by the method of Okada et al. (1968). The incubation mixture contained 0.25 ml 1.7 mM para-nitrophenyl-β-D-glucopyranoside (PNG) in 0.1M sodium acetate buffer, pH 4.8, 0.5 ml 0.1M sodium acetate buffer, pH 4.8, and 0.25 ml culture filtrate. The mixtures were incubated at 30°C for 20 min, after which time 10 ml 0.1M $NaCO_3$ were added. The release of PNP was estimated by measuring the absorbance at 420 nm, as previously described. PNP was estimated from a PNP standard curve.

The rate of release of PNP was linear in the range 0-0.07 mg/ml. Therefore, samples of culture filtrates were diluted in 0.1M sodium acetate buffer, pH 4.8, such that the amount of PNP released did not exceed 0.07 mg/ml under the given assay conditions.

Unless otherwise stated, enzyme units were expressed as nanomoles of glucose equivalents released per min per ml culture filtrate under the assay conditions.

3.5 Determination of soluble protein

The concentration of protein released into the growth medium was measured by the method of Lowry et al., (1951). Samples of culture filtrate were dialysed overnight at 4°C against distilled water using Visking tubing (31/32, Scientific Instruments Ltd) to remove salts which interfered with the protein assay. An alkaline copper reagent with the following composition was prepared: 1 ml 0.5% (w/v) $CuSO_4$ in 1% (w/v) sodium tartrate added to 50 ml 2% (w/v) Na_2CO_3 in 0.1M NaOH, and 5 ml were added to 1 ml culture filtrate (dialysed). The sample was mixed well and left at room temperature for 10 min. Folin-Ciocalteau reagent was diluted as follows: 1·5ml reagent added to 1 ml water, and 0.5 ml was added to the sample, mixed immediately and allowed to stand for 30 min. The

absorbance was measured at 600 nm using a Pye Unicam spectrophotometer (Model SP1700) against a reagent blank. The protein was estimated from a previously constructed standard curve.

3.5.1 Protein standard curve

A range of concentrations of bovine serum albumin (BSA, Sigma, 0-200 µg/ml) was prepared and the procedure described above was followed, replacing 1 ml culture filtrate with 1 ml BSA.

A standard curve of BSA (mg/ml) against absorbance at 600 nm was constructed (Appendix 3).

3.6 Dry weight measurements

Residual cellulose and mycelium were separated from the growth medium by filtration through a preweighed glass filter crucible (porosity 1). The samples were dried at 108°C to constant weight and the dry weight of the material determined. Losses in dry weight were then calculated.

4. GROWTH OF FUNGI ON LIGNOCELLULOSE

Conical flasks (250 ml) containing 0.5% (w/v) hay or straw (2 cm in length) in 100 ml modified CD medium (Section 1.2) were inoculated with 1 ml of spore suspension and incubated at 30°C in an orbital shaker at 150 rpm. Two flasks were used for each substrate and each fungus. After the desired incubation time, flasks were harvested, dry weights determined and culture filtrates assayed for cellulolytic activity as previously described. In addition, culture filtrates were assayed for xylanase activity (Section 4.1) and changes in the composition of the hay were determined (Section 4.2).

4.1 Assay for xylanase activity

Measurements of xylanase activity were based on the determination of reducing sugars released from xylan, estimated using the DNS method of Miller (1959).

A sample of culture filtrate (1 ml) was incubated with 1 ml 2% (w/v) xylan in 0.1M sodium acetate buffer, pH 4.8, at 40°C for 15 min. At the

end of the reaction time, 2 ml DNS reagent were added and the xylan removed by centrifugation using an MSE Super Minor centrifuge (5,000 xg, at room temperature for 5 min). Reducing sugars were determined by the method described in Section 3.4.2 and estimated from a standard curve using xylose (0-0.2 mg/ml) as the standard (Appendix 1).

Enzyme units were expressed as millimoles of xylose equivalents released per min per ml of culture filtrate under the standard assay conditions.

4.2 Chemical composition of lignocellulosic material

Estimates of the chemical composition of straw were based on dry weight losses after the sequential chemical extraction of the straw to remove methanol soluble material, hemicellulose and cellulose.

4.2.1 Removal of methanol soluble material

Straw (1-2g) was extracted with 50 ml methanol for 2h at 50°C, with refluxing in a soxhlet apparatus. Following this, the methanol was evaporated to dryness and the weight of the residue representing the methanol soluble extractives was determined.

4.2.2 Removal of hemicellulose

After extraction of the methanol soluble material from the straw, hemicellulose was removed by the addition of 50 ml of 24% (w/v) KOH, with continuous stirring for 4h. To separate the solid material from the KOH containing the dissolved hemicellulose, the sample was centrifuged in a Beckman centrifuge (Model J2-21, 10,000 xg at room temperature for 5 min). Following this, the solid material was washed twice with 100 ml distilled water, filtered through a preweighed glass filter crucible (porosity 1) and dried to constant weight at 108°C. The weight of residual material, presumed to consist of cellulose and lignin, together with the methanol soluble material were subtracted from the original weight of the straw to give an estimate of the hemicellulose.

4.2.3 Removal of cellulose

To remove the cellulose, the residual material was ground in a mortar using a pestle, 2 ml of 70% (v/v) H_2SO_4 were added, and the sample left at room temperature for 2h. The acid was then diluted to 5% (v/v) with distilled water and refluxed for 2½h. Solid material was removed by filtration through a glass filter crucible (porosity 1), washed twice with 100 ml distilled water, refiltered through a glass filter crucible (porosity 1) and dried at 108°C to constant weight. This material represented the acid insoluble lignin component. The weight of cellulose was determined by subtraction of this fraction from the weight of the cellulose and lignin fraction.

5. LOCATION OF β-GLUCOSIDASE ACTIVITY

Conical flasks (250 ml) containing 100 ml modified CD medium (Section 1.2) or 0.1M phosphate-buffered medium, pH 5.7 (Section 2.3), were prepared without a carbon source. After sterilising at 15 psi for 15 min, 0.5g cellobiose was added and the flasks resterilised at 10 psi for 10 min. To ensure this was a suitable procedure for sterilising cellobiose the medium was assayed, after autoclaving, for the presence of glucose using a diagnostic kit based on glucose oxidase (Sigma).

Flasks were inoculated with 1 ml of spore suspension and incubated at 30°C in an orbital shaker at 150 rpm. Two flasks were prepared for each type of medium and each fungus. After the required time period the mycelium was separated from the medium by filtration through a glass filter crucible (porosity 1). The culture filtrate, representing the extracellular fraction was assayed for β-glucosidase activity (Section 3.4.6). The mycelium was washed twice using 20 ml aliquots of 0.1M sodium acetate buffer, pH 4.8, to remove traces of the culture filtrate, and resuspended in 20 ml 0.1M sodium acetate buffer, pH 4.8. The intracellular β-glucosidase was released by disrupting the mycelium in a

French pressure cell at 12,000 psi. The lysate was centrifuged in a Beckman centrifuge (Model J2-21; 12,000 xg at 4°C, for 30 min) to remove cell debris. The pellet was resuspended in 20 ml 0.1M sodium acetate buffer, pH 4.8, and assayed for β-glucosidase activity. The supernatant, representing the soluble intracellular fraction, was also assayed for β-glucosidase activity.

The concentration of cellobiose in the culture filtrate was estimated colorimetrically by the DNS method described previously. Reducing sugars were estimated from a standard curve using cellobiose (0-0.2 mg/ml) as the standard (Appendix 1).

Plate assay for β-glucosidase activity

The PNG assay of Okada et al. (1968) was modified such that fungi could be rapidly screened for β-glucosidase activity. Fungi were grown on modified CD medium containing 2% (w/v) agar with 1g/l PNG as the carbon source and β-glucosidase activity was detected by the appearance of yellow zones surrounding the fungal colonies.

The following types of media were prepared:

i) modified CD medium, containing 2% (w/v) Lab M agar, 0.2% (w/v) ox gall and 0.05 - 1g/l PNG, at pH 4.8.

ii) modified CD medium, containing 2% (w/v) Lab M agar, 0.2% (w/v) ox gall, 1g/l PNG and 5g/l cellobiose, at pH 4.8.

iii) modified CD medium, containing 2% (w/v) Lab M agar, 0.2% (w/v) ox gall and 1g/l PNG at pH 2.5-4.8.

Medium (i) was prepared by combining all constituents, except PNG and autoclaving at 15 psi for 15 min. After this time, PNG was filter sterilised and added to the medium at final concentrations of 0.05-1g/l. Filter sterilisation of the PNG was necessary since the glucoside is unstable under heat sterilising conditions. Medium (ii) was prepared in a similar way except that filter sterilised PNG and cellobiose were added to concentrations of 1 g/l and 5 g/l respectively. With medium

(iii), the agar was also added separately after autoclaving and cooling to 45°C, since it is hydrolysed by autoclaving under acidic conditions.

Sterilised medium was dispensed into Petri plates and each was inoculated with a 10 µl spore suspension. The plates were incubated at 30°C until fungal growth was visible, and colonies were flooded with 10 ml of 2% (w/v) Lab M agar with 0.1M Na_2CO_3 added when the agar had cooled to 45°C. After 15 min, β-glucosidase activity was detected by the appearance of yellow zones surrounding the fungal colonies and was quantified by measuring the diameter of these zones.

CHAPTER THREE

PROPERTIES OF A CELLULOLYTIC COMMUNITY GROWING ON CELLULOSE (SIGMACELL TYPE 20)

CHAPTER THREE

PROPERTIES OF A CELLULOLYTIC COMMUNITY GROWING ON CELLULOSE (SIGMACELL TYPE 20)

Introduction

In natural environments microbial populations with different metabolic activities interact and combine to form microbial communities (Slater & Bull, 1978). It has often been reported that these communities are more efficient at degrading their substrates than are individual populations. For example, the herbicide dalapon was degraded by a microbial community consisting of bacteria and fungi, at a faster rate than by the bacteria alone (Senior et al., 1976). Also, orcinol was degraded by a community consisting of three bacteria at a faster rate than by one of its members alone (Osman et al., 1976).

Microbial communities have been reported to be more efficient at degrading naturally occurring substrates such as cellulose (Enebo, 1949). This author isolated a microbial community consisting of three thermophilic bacteria; Bacillus thermolacticus, Clostridium thermobutyricum and C. thermocellulaseum. He found that the community degraded cellulose at a considerably faster rate than the cellulolytic bacterium C. thermocellulaseum, alone.

Endo- and exoglucanases from different microorganisms have been shown to act synergistically in the degradation of insoluble cellulose (Wood, 1969a). It is possible that a well balanced cellulase complex, consisting of components produced by a variety of organisms, will be supplied by a microbial community. Under these conditions the rate of cellulose degradation may be increased. In addition, interactions may occur between the different species in the community such that the growth of the cellulolytic organisms is stimulated.

In the past, studies of cellulose degradation by microbial

communities have examined the contribution made by anaerobic microorganisms. It was the purpose of this study to isolate and characterise an aerobic cellulolytic community, with the aim of improving cellulolysis.

Results

1. ISOLATION

Table 6 lists the fungi and bacteria enriched from soil using continuous culture enrichment. Each fungal isolate was identified by the Commonwealth Mycological Institute, Kew, Surrey and each bacterial isolate by the National Collection of Industrial Bacteria, Torry Research Station, Aberdeen.

2. ESTABLISHMENT OF THE COMMUNITY

Table 7 shows the establishment of the microbial community in the chemostat over 51d. After inoculation, the chemostat was maintained in batch culture for 18d to allow sufficient time for the proliferation of organisms present in the inoculum. For the first 7d (batch culture), several different types of bacteria were isolated from the chemostat and one fungal species. Following the initiation of continuous culture, the number of different types of bacteria dropped, possibly due to the elimination of some species which were unable to use either the cellulosic substrate or fungal breakdown products. After 33d (continuous culture), only two species of bacteria were isolated and were subsequently found to be non-cellulolytic; it seems reasonable to assume that these organisms relied upon fungal degradation products of cellulose for their carbon source.

Although the number of different fungi apparently increased over the period of continuous culture, this may have been due to variations in the number of viable propagules plated onto the isolation media. If the

TABLE 6 Fungi and bacteria isolated from soil using continuous culture enrichment

Malt extract[1]	Modified malt extract[2]	Luria[3]	Cellulose[4]
Penicillium nigricans Bainier ex Thom IMI 267962			
Paecilomyces lilacinus (Thom) Samson IMI 267963			
Fusarium oxysporum Schlect IMI 267964	*Fusarium oxysporum* Schlect IMI 267964	*Fusarium oxysporum* Schlect IMI 267964	
		Fusarium oxysporum Schlect IMI 267968	
	Fusarium oxysporum Schlect IMI 267966		
	Penicillium simplicissimum (Oudemans) Thom IMI 267965	*Penicillium simplicissimum* (Oudemans) Thom IMI 267965	
	Aspergillus fumigatus Fresenius IMI 267967		
		Alcaligenes/ *Pseudomonas* sp. Coryneform bacterium	Alcaligenes/ *Pseudomonas* sp. Coryneform bacterium
			Gliocladium roseum Bainier IMI 267969

[1] malt extract agar (MEA), 4%(w/v)

[2] MEA (4% w/v) containing 15 ng/ml crystal violet, and 30 µg/ml streptomycin sulphate

[3] tryptone, 1% (w/v); NaCl, 1% (w/v); yeast extract, 0.5% (w/v); agar, 2% (w/v), pH 7.0

[4] modified CD medium containing cellulose (Sigmacell type 20)(0.5% w/v), and agar (2% w/v)

TABLE 7 Establishment of a cellulolytic microbial community

	Time (days)	Bacteria number of types	Bacteria number of viable cells /ml	Fungi number of species
batch culture	3	6	ND	0
	7	4	ND	1
	18	ND	ND	ND
continuous culture	4	3	9×10^6	2
	10	3	4×10^6	3
	13	1	1×10^6	7
	33	2	4×10^6	8

ND = not determined.

The chemostat was maintained in batch culture for 18d before the initiation of continuous culture, at a dilution rate of $0.165h^{-1}$, reduced to $0.05h^{-1}$ after 7d.

number of viable propagules in the culture was low initially then those species which grew from aliquots (0.1 ml) of the culture (800 ml) may not have been truly representative of the fungal population.

3. CELLULASE PRODUCTION UNDER CONTINUOUS CULTURE CONDITIONS

The production of cellulases was investigated after the microbial community had become established in the chemostat. Although cellulase (0-1.73 EU) and endoglucanase (0.66-2.4 EU) activities (Table 8) were recorded between 27d and 62d, both activities decreased sharply thereafter. Throughout this period, β-glucosidase activity could not be detected.

Acidic conditions (pH 2.5) recorded in the medium could have been responsible for the loss of cellulase activity. Mandels et al. (1975) reported that there was almost 100% inactivation of β-glucosidase at pH 3.0. As the pH dropped to 2.5, filter paper activity decreased by 45% and endoglucanase by 20%.

As a result, it was decided to control the pH of the medium in the chemostat between 4.5 and 5.0, in an attempt to prevent the loss of cellulase activity. This proved unsuccessful, as did further trials keeping the pH at 4.5 from the start of the culture. In view of these problems which were arising, it was decided to use flask cultures for further studies.

4. GROWTH OF BACTERIA

None of the bacteria isolated (Coryneform and Pseudomonas/ Alcaligenes sp.) were capable of using cellulose (Sigmacell type 20), CMC, or cellobiose as a carbon source. They were also unable to grow on agar containing filter paper or cellulose as the sole carbon source. Since the bacteria were non-cellulolytic, producing neither cellulases nor β-glucosidase, it was decided not to include them in the community studies which followed. Consequently, further identification tests to determine the genera and species of the bacteria were not carried out by Torry Research Station.

TABLE 8 Production of extracellular cellulase and endoglucanase by the community growing on 0.5% (w/v)cellulose (Sigmacell type 20)in continuous culture

Time (days) in continuous culture	Endoglucanase activity EU	Cellulase activity EU
27	2.40	1.73
32	2.34	1.21
37	2.01	0.04
42	1.20	0.72
47	0.66	1.56
52	1.20	0
57	1.16	0
62	0.79	0.88
67	0	0
72	0	0

5. PROPERTIES OF THE FUNGAL COMMUNITY

Figures 11-13 show cellulase production by the community and its individual members during growth on 0.5% (w/v)cellulose (Sigmacell type 20).

Highest **cellulase** activity (Fig. 11) was produced by the community, Aspergillus fumigatus and Penicillium simplicissimum, activity being more than 5-fold greater than that produced by the other fungi. Activity produced by these three cultures rapidly increased to reach a maximum level (5.0-7.9 EU) between 10 and 20d, decreasing thereafter. All three Fusarium oxysporum strains and Gliocladium roseum produced less **cellulase** activity than the community, A. fumigatus and P.simplicissimum. With G. roseum, activity was not detected until day 40. No detectable levels of exoglucanase activities were obtained with cultures of Paecilomyces lilacinus and Penicillium nigricans.

Figure 12 shows endoglucanase activity detected during growth of the fungi on cellulose. Maximum levels of endoglucanase activity produced by the community (7.4 EU) and P. simplicissimum (7.3 EU) were almost 4-fold greater than those of A. fumigatus and at least 15-fold greater than those of the other fungi. Activity produced by P. nigricans was very low (0.15 EU) in comparison with the other fungi and was only detected on day 40. No endoglucanase activity was detected in cultures of P. lilacinus.

Cultures of P. simplicissimum and the community showed a rapid increase in endoglucanase activity up to 20d and 30d, respectively, decreasing thereafter. Although endoglucanase activity in A. fumigatus also increased during the first 7d of growth, activity fell slightly and then continued to increase at a slower rate. Very low levels of activity ($\leq$ 0.6 EU) were detected throughout the growth of the remaining cultures.

Although β-glucosidase was produced by all fungi (Fig. 13), activity varied considerably between cultures. In contrast to cellulase and endoglucanase, the highest levels of β-glucosidase were produced by the

Fig. 11

Cellulase activity in cultures of the community and its members grown on 0.5% (w/v) cellulose

(■—■) Community

(▲—▲) A. fumigatus

(△—△) F. oxysporum (strain 1)

(□··□) F. oxysporum (strain 2)

(●··●) F. oxysporum (strain 3)

(▲··▲) G. roseum

(□—□) P. lilacinus

(○—○) P. nigricans

(●—●) P. simplicissimum

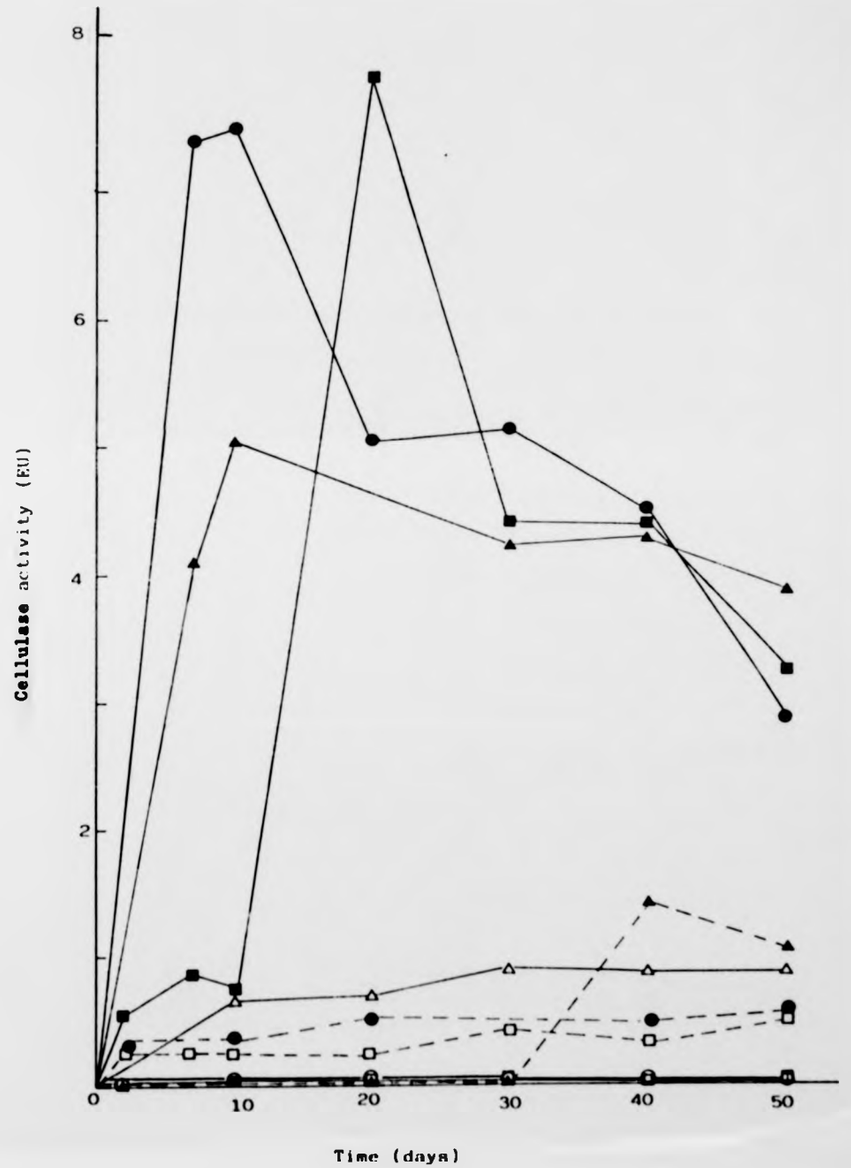


Fig. 12

Endoglucanase activity in cultures of the community and its members grown on 0.5% (w/v) cellulose.

(■—■) Community

(▲—▲) A. fumigatus

(△—△) F. oxysporum (strain 1)

(□-□) F. oxysporum (strain 2)

(●-●) F. oxysporum (strain 3)

(▲-▲) G. roseum

(□—□) P. lilacinus

(○—○) P. nigricans

(●—●) P. simplicissimum

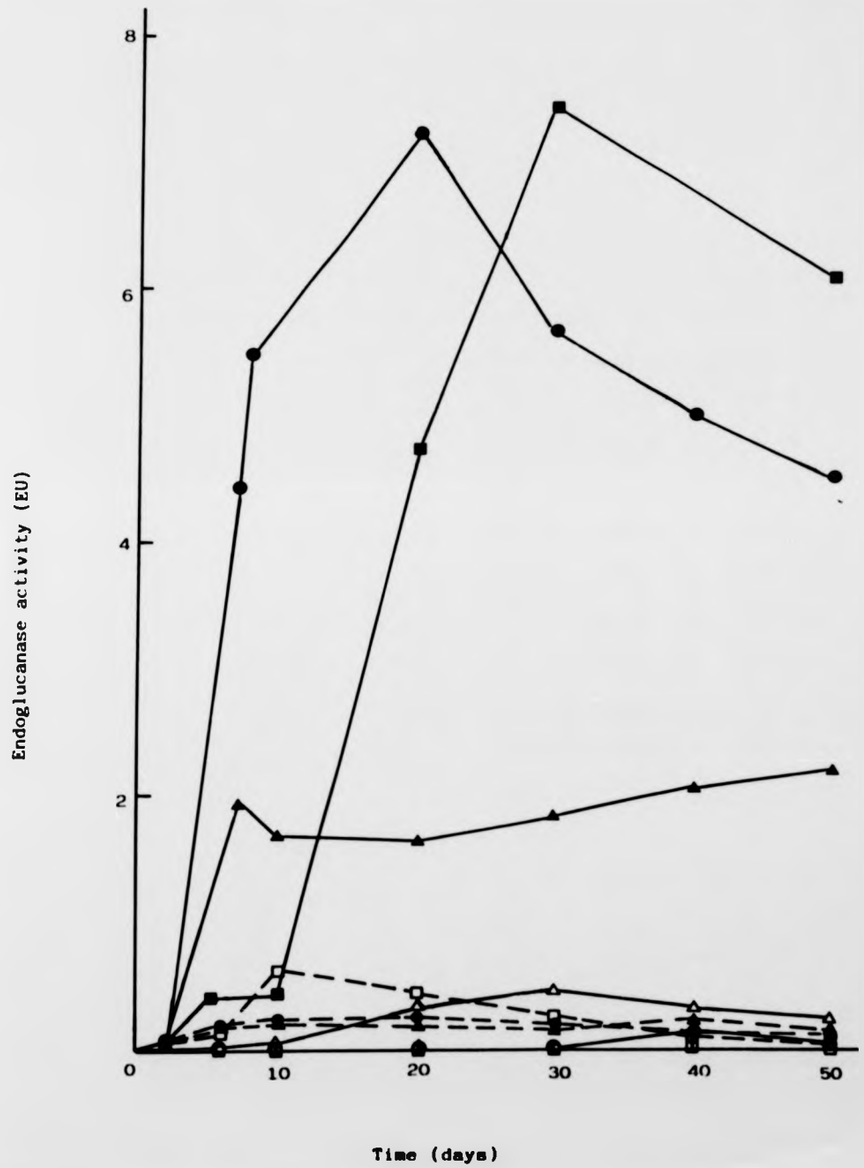


Fig. 13

β-Glucosidase activity in cultures of the community and its members grown on 0.5% (w/v) cellulose.

(■—■) Community

(▲—▲) A. fumigatus

(△—△) F. oxysporum (strain 1)

(□--□) F. oxysporum (strain 2)

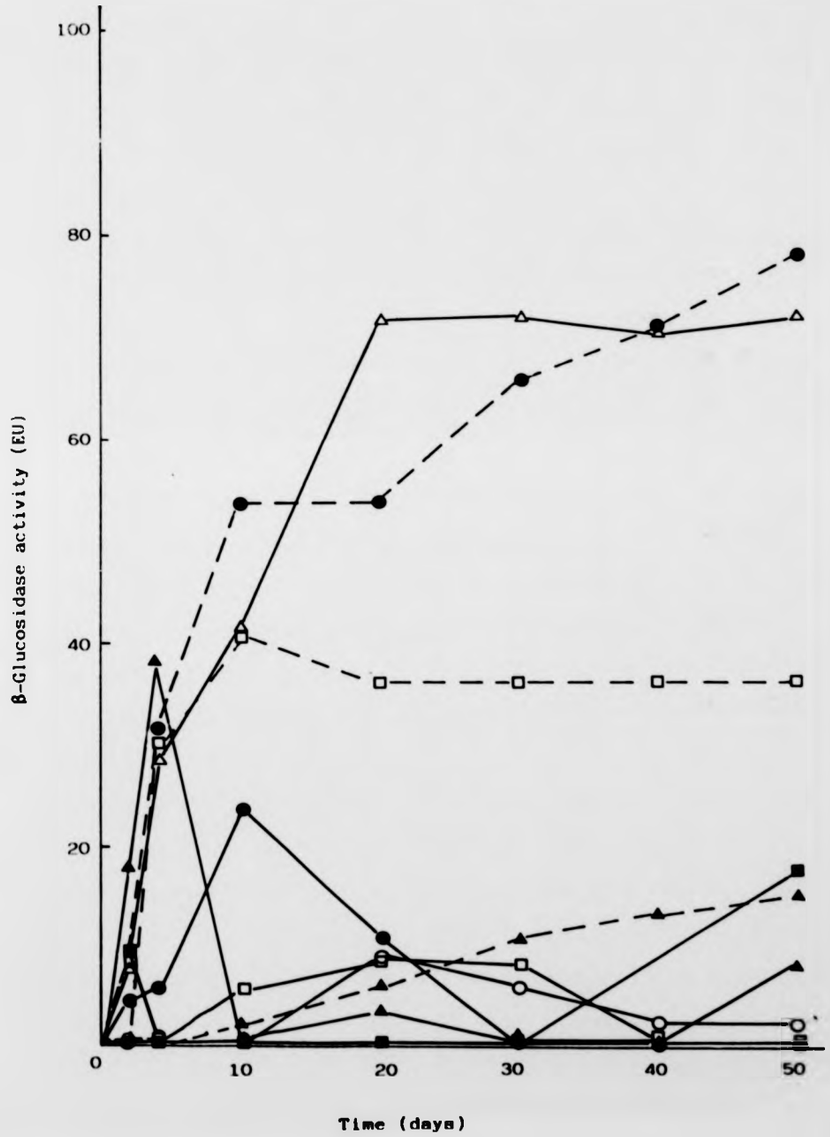
(●--●) F. oxysporum (strain 3)

(▲--▲) G. roseum

(□—□) P. lilacinus

(○—○) P. nigricans

(●—●) P. simplicissimum



three F. oxysporum strains, with maximum levels between 40 and 78 EU. Levels produced by strains 2 and 1 increased to day 10 and 20, respectively, and remained constant thereafter, whereas that produced by strain 3 continued to rise throughout growth. Production of β-glucosidase by A. fumigatus, P. simplicissimum and the community was considerably less than that of the three F. oxysporum strains with maximum levels of 38, 24 and 17 EU, respectively. In addition, activity could only be detected during the initial and final stages of growth. Activity produced by G.roseum, P. lilacinus and P. nigricans was also low in comparison with the three F. oxysporum strains, maximum levels being 15, 8 and 8 EU, respectively.

Extracellular soluble protein detected during growth on cellulose is shown in Figure 14. Highest levels produced by A. fumigatus, P.simplicissimum and the community (190-243 µg/ml) were more than 3-fold greater than those produced by the other fungi. Extremely low levels (< 20 µg/ml) were produced by Fusarium strains 2 and 3, G. roseum and P.lilacinus, with slightly higher levels (30-60 µg/ml) produced by F.oxysporum (strain 1) and P. nigricans. In general, protein production increased throughout growth, the exceptions being F. oxysporum (strains 2 and 3), G. roseum and P. lilacinus where levels remained constant after an initial increase.

Figure 15 shows the pH of the medium during growth of the fungi on cellulose. In all cases, the pH of the medium dropped following inoculation, although the rate and extent of the drop varied with each culture. An initial rapid decrease in pH was recorded with A. fumigatus, P. simplicissimum and the community, the pH dropping from 4.8 to between 3.3 and 2.9 within 5d and remaining at this value thereafter. Although a rapid drop in pH was also recorded with the three F. oxysporum strains, the pH did not drop below 3.3. The remaining cultures showed either a

Fig. 14

Extracellular soluble protein in cultures of the community and its members grown on 0.5% (w/v) cellulose.

(■—■) Community

(▲—▲) A. fumigatus

(△—△) F. oxysporum (strain 1)

(□-□) F. oxysporum (strain 2)

(●-●) F. oxysporum (strain 3)

(▲-▲) G. roseum

(□—□) P. lilacinus

(○—○) P. nigricans

(●—●) P. simplicissimum

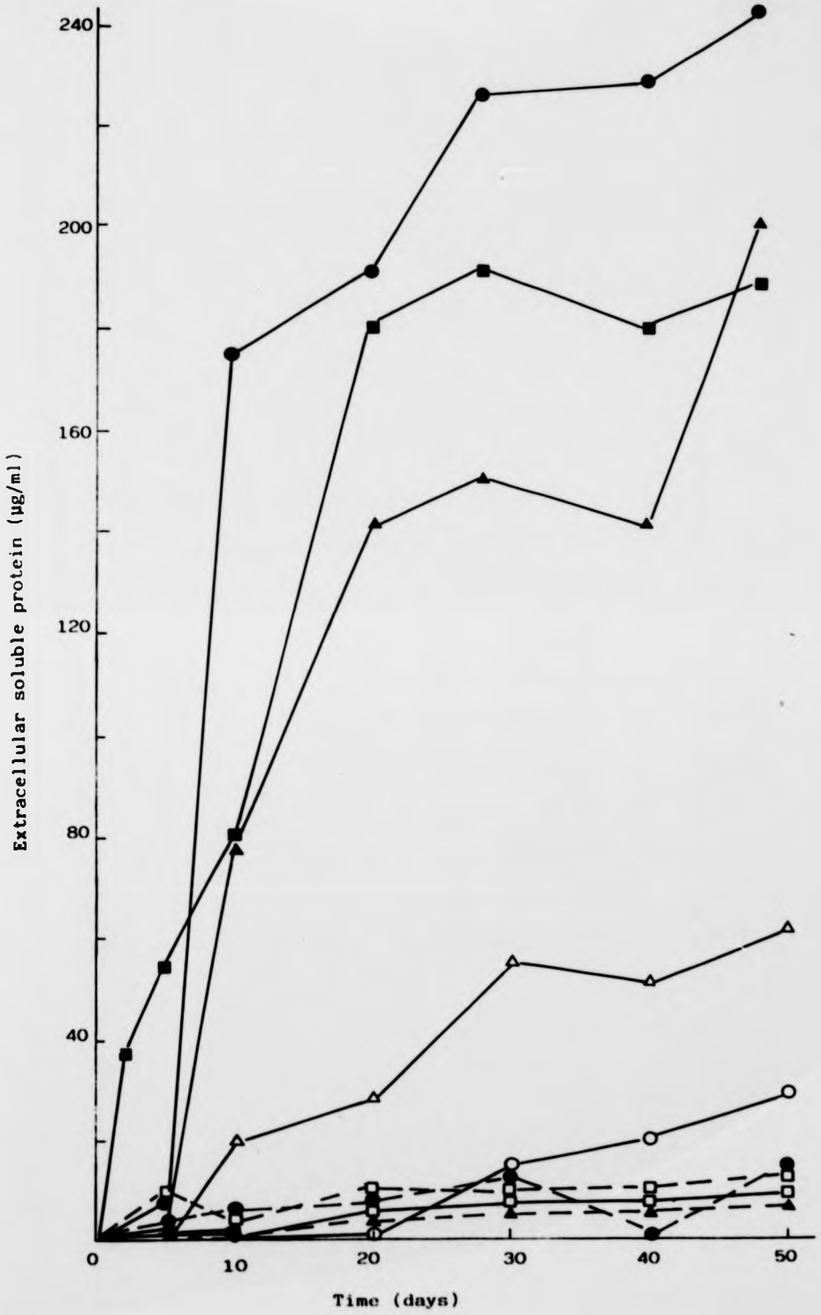


Fig. 15

Changes in the pH of the medium during growth of the community and its members on 0.5% (w/v) cellulose.

(■—■) Community

(▲—▲) A. fumigatus

(△—△) F. oxysporum (strain 1)

(□--□) F. oxysporum (strain 2)

(●--●) F. oxysporum (strain 3)

(▲--▲) G. roseum

(□—□) P. lilacinus

(○—○) P. nigricans

(●—●) P. simplicissimum

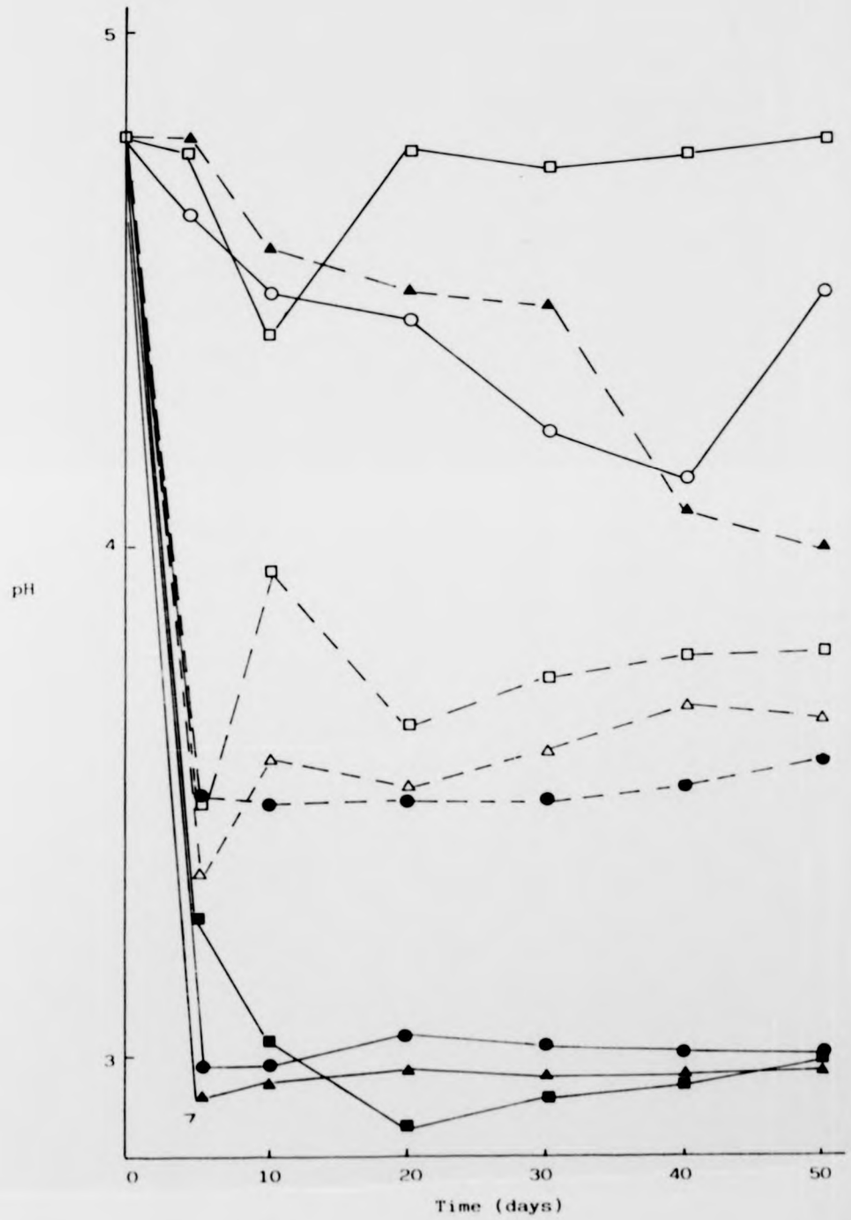
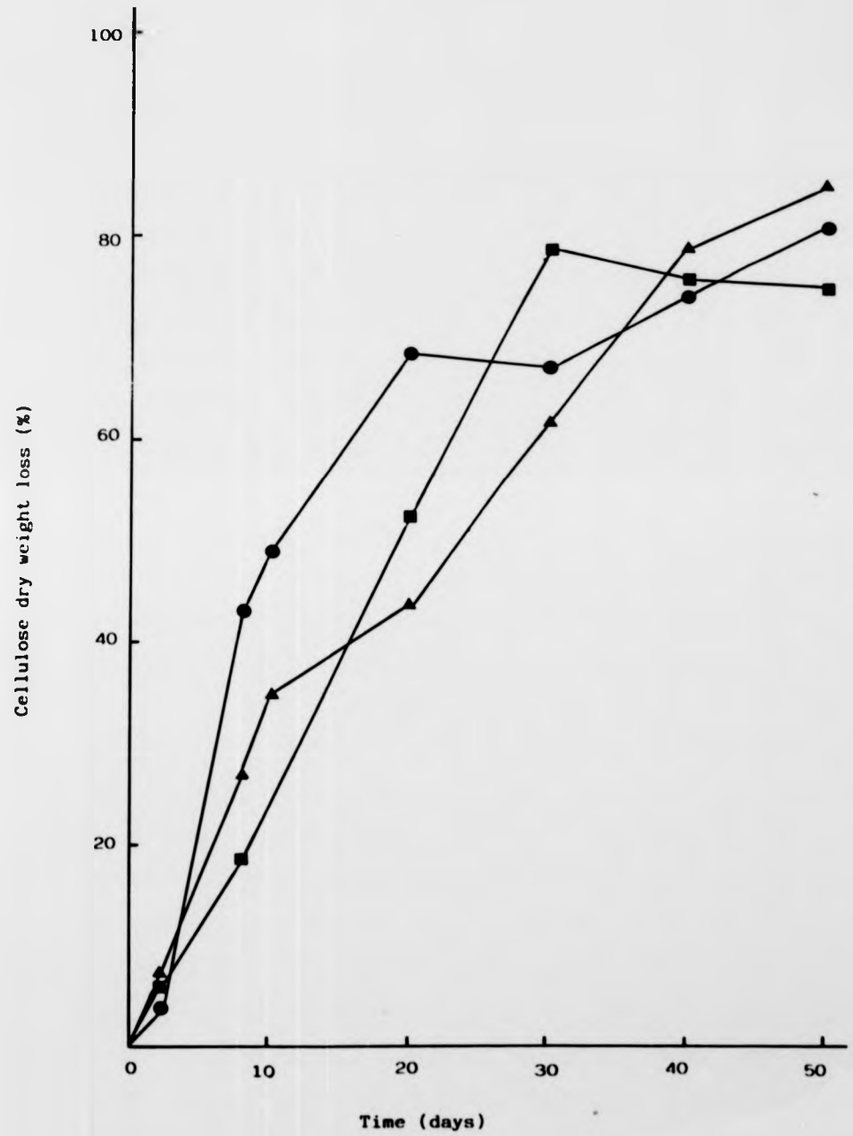


TABLE 9 Loss in dry weight of 0.5% (w/v) cellulose (Sigmacell) by the community and its individual members after 30d

Organism	cellulose (Sigmacell type 20) % (w/v) dry weight loss
community	58
P. simplicissimum	64
A. fumigatus	65
F. oxysporum 1	14
2	28
3	28
P. nigricans	14
G. roseum	13
P. lilacinus	5

Fig. 16

A comparison of cellulose dry weight loss in cultures of A. fumigatus (▲—▲), P. simplicissimum (●—●), and the community (■—■).



slight drop in pH, followed by an increase as with P. lilacinus or a gradual drop as with G. roseum and P. nigricans.

Table 9 shows cellulose dry weight loss recorded after growth of the fungi for 30d. Greatest weight losses due to the community, A. fumigatus and P. simplicissimum (58-65%) were at least 2-fold greater than those produced by the other fungi. The smallest weight loss was recorded with P. lilacinus (4.6%), the remaining fungi giving weight losses between approximately 14% - 29%.

A. fumigatus, P. simplicissimum and the community (cultures giving the highest weight losses) were further investigated to compare their rates and extents of cellulose degradation. Figure 16 shows the loss in dry weight of cellulose during growth of these organisms for 50d. Although the three cultures gave similar dry weight losses (75-85%) after 50d, the rate of weight loss varied with each culture. The fastest initial rate was produced by P. simplicissimum, with a 68% weight loss in 20d, followed by an additional 13% weight loss over the next 30d. In contrast, the community produced a maximum weight loss of 79% after 30d, whilst A.fumigatus gave a gradual loss in weight over the 50d period.

Discussion

In the present study a microbial community was isolated from a continuous culture system after prolonged enrichment with insoluble cellulose. Under such culture conditions it is possible that the manifestation of those microorganisms unable to synthesise enzymes of the cellulase complex did not occur due to competition from the primary cellulose degraders. This situation could result if (a) the rate of glucose production from extracellular cellulose hydrolysis and its uptake by the primary degraders was equal, or (b) if the final reactions of cellulolysis (e.g. oligosaccharides containing three glucose units or less, to glucose) were primarily intracellular. Alternatively, non-cellulolytic

slight drop in pH, followed by an increase as with P. lilacinus or a gradual drop as with G. roseum and P. nigricans.

Table 9 shows cellulose dry weight loss recorded after growth of the fungi for 30d. Greatest weight losses due to the community, A. fumigatus and P. simplicissimum (58-65%) were at least 2-fold greater than those produced by the other fungi. The smallest weight loss was recorded with P. lilacinus (4.6%), the remaining fungi giving weight losses between approximately 14% - 29%.

A. fumigatus, P. simplicissimum and the community (cultures giving the highest weight losses) were further investigated to compare their rates and extents of cellulose degradation. Figure 16 shows the loss in dry weight of cellulose during growth of these organisms for 50d. Although the three cultures gave similar dry weight losses (75-85%) after 50d, the rate of weight loss varied with each culture. The fastest initial rate was produced by P. simplicissimum, with a 68% weight loss in 20d, followed by an additional 13% weight loss over the next 30d. In contrast, the community produced a maximum weight loss of 79% after 30d, whilst A.fumigatus gave a gradual loss in weight over the 50d period.

Discussion

In the present study a microbial community was isolated from a continuous culture system after prolonged enrichment with insoluble cellulose. Under such culture conditions it is possible that the manifestation of those microorganisms unable to synthesise enzymes of the cellulase complex did not occur due to competition from the primary cellulose degraders. This situation could result if (a) the rate of glucose production from extracellular cellulose hydrolysis and its uptake by the primary degraders was equal, or (b) if the final reactions of cellulolysis (e.g. oligosaccharides containing three glucose units or less, to glucose) were primarily intracellular. Alternatively, non-cellulolytic

organisms could persist in the community if sugars produced by the primary degraders were available to other organisms and the rate of sugar uptake by the non-cellulolytic organisms exceeded that of the primary degraders.

Studies of the fungi growing in axenic culture indicated that A.fumigatus and P. simplicissimum were strongly cellulolytic, F. oxysporum (3 strains), G. roseum and P. nigricans degraded cellulose to a limited extent whilst P. lilacinus and both types of bacteria were non-cellulolytic. It is likely that growing as a mixed culture, A.fumigatus and P. simplicissimum would be the primary cellulose degraders, with the other organisms using the cellulose breakdown products supplied by these two fungi.

There are several other reports on the cellulolytic activity of the fungi isolated in the present study. Marsh et al. (1949) reported on the cellulolytic activity of A. fumigatus and P. simplicissimum, both fungi being found to be good cellulose degraders. More recently, the cellulolytic activity of A. fumigatus has been reported by Stewart and Parry (1981), Thakre and Johri (1980), and Trivedi and Rao (1979). P.nigricans has been shown to degrade cellulose to a limited extent (Marsh et al., 1949) and several species of Paecilomyces have been found to be non-cellulolytic. These observations are in agreement with those obtained here.

There are also reports on the cellulolytic activity of F. oxysporum (Marsh et al., 1949; Olutiola, 1978) and G. roseum (Marsh et al., 1949), both organisms being capable of hydrolysing insoluble cellulose.

It is interesting to note that of the organisms tested for cellulolytic activity by Marsh et al. (1949), P. simplicissimum and A.fumigatus compared favourably with Trichoderma reesei, an organism now considered to be one of the best cellulose degraders.

It was surprising to find that Trichoderma was not isolated in the present study as it is a cellulolytic fungus frequently isolated from soil. It is unlikely that culture conditions in the chemostat prevented the growth of this organism since it has been shown to grow and hydrolyse cellulose at 30°C (Berg & Pettersson, 1977). In addition, Trichoderma can grow at pH 4.5 (Peitersen, 1975) and at pH 5.0 (Ghose et al., 1975) in a medium containing $(NH_4)_2SO_4$ as the nitrogen source (Mandels et al., 1975). The possibility exists that antagonism from other species in the chemostat prevented its growth, although in the absence of a more detailed investigation this can only be conjecture.

Low levels of cellulase activity were detected during continuous culture of the community on cellulose. Similarly, Mandels and Andreotti (1978) found that low levels of cellulase were produced by Trichoderma reesei growing on cellulose in continuous culture. Due to the physicochemical properties of cellulose, different patterns of substrate degradation from those found in more conventional chemostat cultures were evident. Firstly, because the amorphous regions of the cellulose were more easily hydrolysed than the crystalline areas, an excess of the substrate was prevalent and its complete breakdown never occurred. In addition, with the production of extracellular cellulases, enzyme/substrate rather than substrate/organism associations were formed. It is possible that in this study low levels of cellulases were detected in the chemostat because the continuous supply of cellulose to the system provided binding sites for cellulase adsorption. If high cellulase levels were produced one could speculate that the amorphous regions of the cellulose would be rapidly hydrolysed, producing reducing sugars which accumulated in the medium. This accumulation would result in repression of cellulase synthesis and/or inactivation of already formed cellulases.

Results from the studies of the community and its members grown in flask cultures indicated that there was no obvious synergism between the members of the community. To the contrary, cellulose breakdown by the community was slower than that achieved by P. simplicissimum in axenic culture. This suggested that P. simplicissimum was able to grow better in axenic culture, possibly because it was not deprived of cellulose hydrolysis products which were used by other opportunistic members of the community. Alternatively, other members of the community may have had an antagonistic effect on P. simplicissimum, possibly as a result of the production of primary or secondary metabolites. This suggestion is supported by the observations made by Basu (1948) who studied the growth of several cellulolytic organisms in axenic and co-culture. This author found that some of the strongly cellulolytic fungi were partially inhibited by organisms which had little ability to attack cellulose by themselves. As a result, Basu (1948) suggested that isolated organisms showing strong cellulolytic activity may not be so important in the natural environment.

In the present study further evidence that P. simplicissimum grows better alone is provided by the results of cellulase production. Both cellulase and endoglucanase production by the community showed a lag phase which was not observed when P. simplicissimum was grown in axenic culture.

Studies of the community and its members grown on cellulose indicated that a rapid drop in medium pH occurred in some of the cultures. A similar drop in medium pH has been reported in cultures of Aspergillus fumigatus (Stewart & Parry, 1981), Talaromyces emersonii (McHale & Coughlan, 1981) and Trichoderma reesei (Andreotti et al., 1977) growing on cellulose or other carbohydrates when ammonium was present as the nitrogen source. It has been suggested that the drop in observed pH resulted from the uptake of glucose and assimilation of nitrogen (Sternberg, 1976).

Another explanation may be the release of metabolic acids into the medium, causing a drop in pH (McHale & Coughlin, 1981).

The pH of the medium is reported to increase after all the cellulose has been used (Mandels et al., 1975). These authors suggested that the increase in pH in cultures of Trichoderma was due to the organisms' ability to neutralise or reverse the acidic conditions, possibly due to the secretion of NH_4^+.

Acidic conditions in the medium appear to have a profound effect on cellulase and β-glucosidase activity (Mandels et al., 1975). These authors found that if the pH of the medium was adjusted during growth on cellulose, from pH 4.0 to 3.0, most of the β-glucosidase activity was lost. Filter paper activity decreased by 45% as the pH dropped to 2.5; endoglucanase activity was more stable with only a 20% loss of activity. Similar results were obtained when 0.5% glucose was added to cultures growing on cellulose, the pH of the medium rapidly dropped to 2.5 as the glucose was utilised.

In the present study, the pH of the medium dropped from 4.8 to 2.5 in cultures of A. fumigatus, P. simplicissimum and the community. This drop in pH may explain their loss of β-glucosidase activity.

Mandels et al. (1975) stated that acid production regulated the uptake of glucose into the cell. They suggested that an indirect inhibition of β-glucosidase activity by glucose was caused by the lowering of the extracellular pH as discussed above.

Decreases in enzyme activity were frequently observed in cultures of A. fumigatus, P. simplicissimum and the community. This may have been due to the secretion of proteases which were specific to cellulases. Proteases have been postulated to be involved in the specific inactivation or modification of biologically active proteins (Switzer, 1977) and, therefore, it seems reasonable to suggest that they were responsible for

the declines in enzyme activity observed here. Extracellular proteases have been demonstrated in cultures of Penicillium janthinellum but there was no evidence to suggest they were specific to cellulases (Rapp et al., 1981).

Another possible explanation for the decrease in enzyme activity is that binding sites became available to the cellulases as the cellulose was hydrolysed. Castonan and Wilke (1980) found that cellulase (exoglucanase) adsorbed to newspaper as the substrate was degraded. Alternatively, cellobiose may have accumulated in the medium as a result of the loss of β-glucosidase activity, and acted as an inhibitor of cellulases. This explanation is unlikely as reducing sugars were not detected in the medium in the present study.

There are several methods available to determine cellulolytic activity and these have been described in Chapter 1. Here, cellulase and endoglucanase activity was determined by measuring reducing sugar production from Avicel and CMC respectively, and β-glucosidase activity by measuring PNP release from PNG. These assays were selected for the following reasons. Measurement of reducing sugar production from insoluble cellulose is one of the most commonly used methods for determining exoglucanase activity. Several different substrates can be used, including Avicel (Wood & McCrae, 1977), dewaxed cotton (Wood, 1969b), filter paper (Mandels et al., 1975) and H_3PO_4 - swollen cellulose (Shoemaker & Brown, 1978). Canevascini and Gattlen (1981) compared reducing sugar production using these four substrates and found that higher levels of reducing sugar were produced from filter paper than Avicel and dewaxed cotton. Presumably the presence of both amorphous and crystalline regions in the filter paper made it more susceptible to attack than the other substrates. Under the standard assay conditions, the enzyme is incubated with the filter paper for a very short time in

comparison with other assays. It is therefore probable that only the most accessible regions are hydrolysed during this time. Similarly, high levels of reducing sugar were produced from H_3PO_4 - swollen cellulose (Canevascini and Gattlen, 1981). These two substrates were therefore considered unsuitable for use in this study.

In a review of the various substrates available for detecting cellulolytic activity (Wood, 1970), Avicel is claimed to be a highly crystalline substrate and may therefore be a good substrate for assay of true cellulase activity[1]. Avicel was therefore used as a substrate for the determination of exoglucanase activity in this study.

Endoglucanase activity can be estimated from viscometric (Almin & Eriksson, 1967) or reducing sugar determinations (Mandels & Weber, 1969) using carboxymethyl cellulose as a substrate. Although the viscometric method is very sensitive, the most commonly used method is that of reducing sugar determinations. This is mainly due to the fact that the viscosity of CMC is affected by a number of factors such as pH, and ionic strength, making the viscometric method more difficult to carry out. Consequently, the method used in this study was that of reducing sugar determinations.

The various methods which are available to measure β-glucosidase activity include the use of different substrates such as salicin (Hirayama et al., 1976), cellobiose (Canevascini and Meyer, 1979) and PNG (Okada et al., 1968). Of these, the PNG method was selected for use in this study as it is a well documented, simple colorimetric assay.

Measurements of cellulose and lignocellulose degradation are made difficult by the close association formed between the microorganisms and their substrate. One method which has been commonly used measures substrate weight loss. Although this method is convenient, the estimation of weight loss is unavoidably affected by the presence of microbial

[1] Recently however, Avicel has been shown to contain up to 30% amorphous cellulose (β-γ- cellulose, Johnson et al. 1982).

biomass and this must be taken into consideration. There are several reports of cellulose and lignocellulose degradation as measured by substrate weight loss. Trivedi and Rao (1980, 1981) used this method to determine cellulose degradation by Aspergillus fumigatus and Fusarium oxysporum. Kapoor et al. (1982) measured the loss in weight of wheat straw caused by several different fungi including A. fumigatus. Loss in weight of yellow birch and red pine blocks resulting from their colonisation by Phanerochaete chrysosporium and Trichoderma reesei has also been reported (Saddler, 1982). An alternative method which can be used to measure substrate degradation is the release of $^{14}CO_2$ from ^{14}C-labelled cellulose or lignocellulose substrates (Maccubbin & Hodson, 1980).

Microbial biomass measurements are also made difficult because of the close association between the microorganisms and their substrate. Methods which are used are often time-consuming, require several replicates and may not be very reproducible. Recently, Matcham et al. (1984) reviewed the methods available for estimating fungal growth in solid substrates and pointed out that it is essential to compare data from two or more different assays. Biomass can be estimated by measuring total nitrogen (Kjeldahl), nucleic acids, ATP, CO_2/O_2 evolution, ^{15}N incorporation, chitin, ergosterol, fluorescent antibody, enzyme linked immunoassay and radioimmunoassay, and growth linked enzymes. The most commonly used assays to measure fungal biomass appear to be those which estimate ergosterol and chitin. As with all the other methods, these two have their limitations since both compounds vary with species, age and physiological state of the organism.

In summary:

1. A cellulolytic microbial community was isolated from soil and characterised in terms of cellulase production and cellulose degradation.

2. Flask culture studies indicated that there was no obvious synergism between the fungi in the community. Penicillium simplicissimum appeared to grow better in axenic culture than in a mixed culture. It was suggested that the growth of this fungus was affected by other organisms in the community, possibly as a result of their removal of cellulose breakdown products or their production of inhibitory metabolites.

3. Growing in continuous culture, the community produced low levels of cellulase activity. Possible reasons for this are discussed.

CHAPTER FOUR

PRODUCTION AND LOCATION OF β-GLUCOSIDASE ACTIVITY

CHAPTER FOUR

PRODUCTION AND LOCATION OF β-GLUCOSIDASE ACTIVITY

INTRODUCTION

The extracellular β-glucosidases of A. fumigatus, P. simplicissimum and the community were generally present at low levels when these cultures were grown on cellulose (Chapter 3). Low levels of β-glucosidase have also been detected in cultures of Trichoderma reesei by Sternberg (1976a) and it was suggested that this enzyme was inactivated under the acidic conditions which developed in the medium. β-Glucosidase inactivation was prevented by controlling the pH of the medium during growth (Sternberg, 1976a). One of the purposes of this study was to determine whether β-glucosidase levels could be increased using a buffered medium.

Since the cellulolytic organisms used in the present study produced low levels of extracellular β-glucosidase, it was possible that β-glucosidase activity was predominantly intracellular. Several cellulolytic microorganisms have been shown to produce intracellular β-glucosidases (Canevascini & Meyer, 1979). It was therefore decided to study the production and intracellular location of β-glucosidase activity in one of the cultures.

In this study, cellobiose was used as a substrate in preference to cellulose for several reasons. Firstly, since cellulases have been shown to bind to insoluble cellulose (Mandels et al., 1971), the solubility of cellobiose eliminated enzyme adsorption problems which may have arisen using cellulose. Secondly, microorganisms have also been shown to bind cellulose (Binder & Ghose, 1978). The use of cellobiose as a substrate eliminated this problem.

RESULTS

1. β-GLUCOSIDASE PRODUCTION ON 0.5% (W/V) CELLOBIOSE IN AN UNBUFFERED MEDIUM

Figure 17 shows the change in cellobiose concentration in cultures of the community and its individual members grown in an unbuffered medium. All of the fungi were able to use cellobiose as a carbon source, the fastest rate of breakdown being achieved by F. oxysporum (strain 1), P. simplicissimum and the community. The rates of cellobiose hydrolysis by G. roseum and P. lilacinus were less than those of the other fungi.

Fig. 18 shows the change in the pH of the unbuffered medium. With the exception of P. nigricans, all cultures showed a drop in medium pH from 4.8 initially to between 2.3 and 2.7 within 4d. However, with P.nigricans the pH dropped at a slower rate, decreasing from 4.8 to 2.6 after 7d.

Despite the fungi being able to use cellobiose, no detectable levels of extracellular β-glucosidase were recorded in any of the cultures.

2. β-GLUCOSIDASE PRODUCTION ON 0.5% (W/V) CELLOBIOSE IN A MEDIUM BUFFERED WITH 0.1M PHOSPHATE

Representatives of the community were selected for further study based on their cellulolytic activities. Aspergillus fumigatus and P.simplicissimum were capable of extensive hydrolysis of cellulose whilst the two F. oxysporum strains were only capable of hydrolysing cellulose to a limited extent.

Figure 19 shows the cellobiose concentration, medium pH and β-glucosidase activity in four cultures grown on cellobiose in a buffered medium. Extracellular β-glucosidase activity was detected in all of the cultures, with maximum levels ranging from 29-64 EU. In general, enzyme activity increased throughout growth, the exception being F.oxysporum (strain 1) which showed an increase to day 5 followed by a decrease.

Fig. 17

Changes in cellobiose concentration during growth of the community and its members in an unbuffered medium.

(■—■) Community

(▲—▲) A. fumigatus

(△—△) F. oxysporum (strain 1)

(□-□) F. oxysporum (strain 2)

(●-●) F. oxysporum (strain 3)

(▲-▲) G. roseum

(□—□) P. lilacinus

(○—○) P. nigricans

(●—●) P. simplicissimum

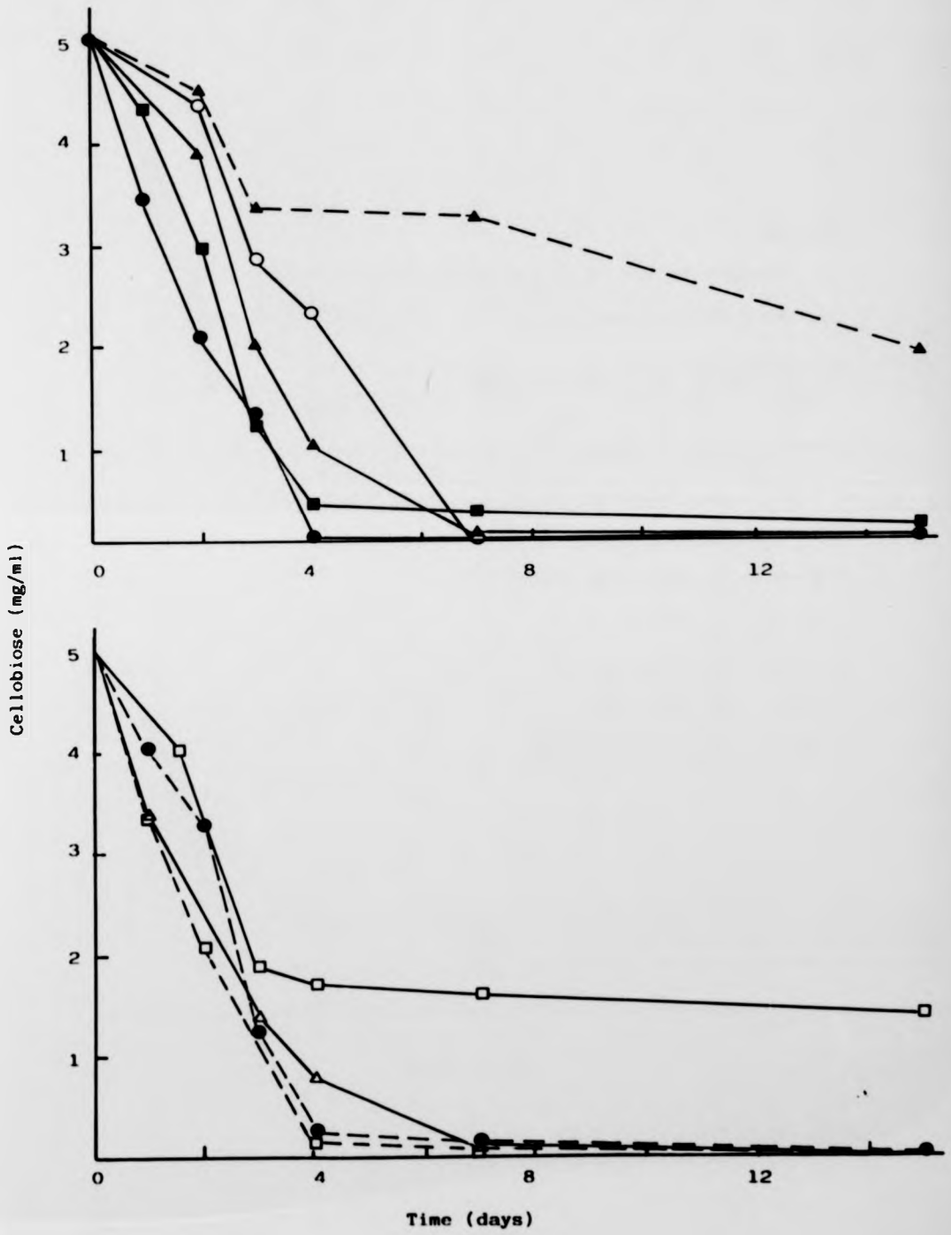


Fig. 18

Changes in the pH of the medium during growth of the community and its members on 0.5% (w/v) cellobiose in an unbuffered medium.

(■—■) Community

(▲—▲) A. fumigatus

(△—△) F. oxysporum (strain 1)

(□--□) F. oxysporum (strain 2)

(●--●) F. oxysporum (strain 3)

(▲--▲) G. roseum

(□—□) P. lilacinus

(○—○) P. nigricans

(●—●) P. simplicissimum

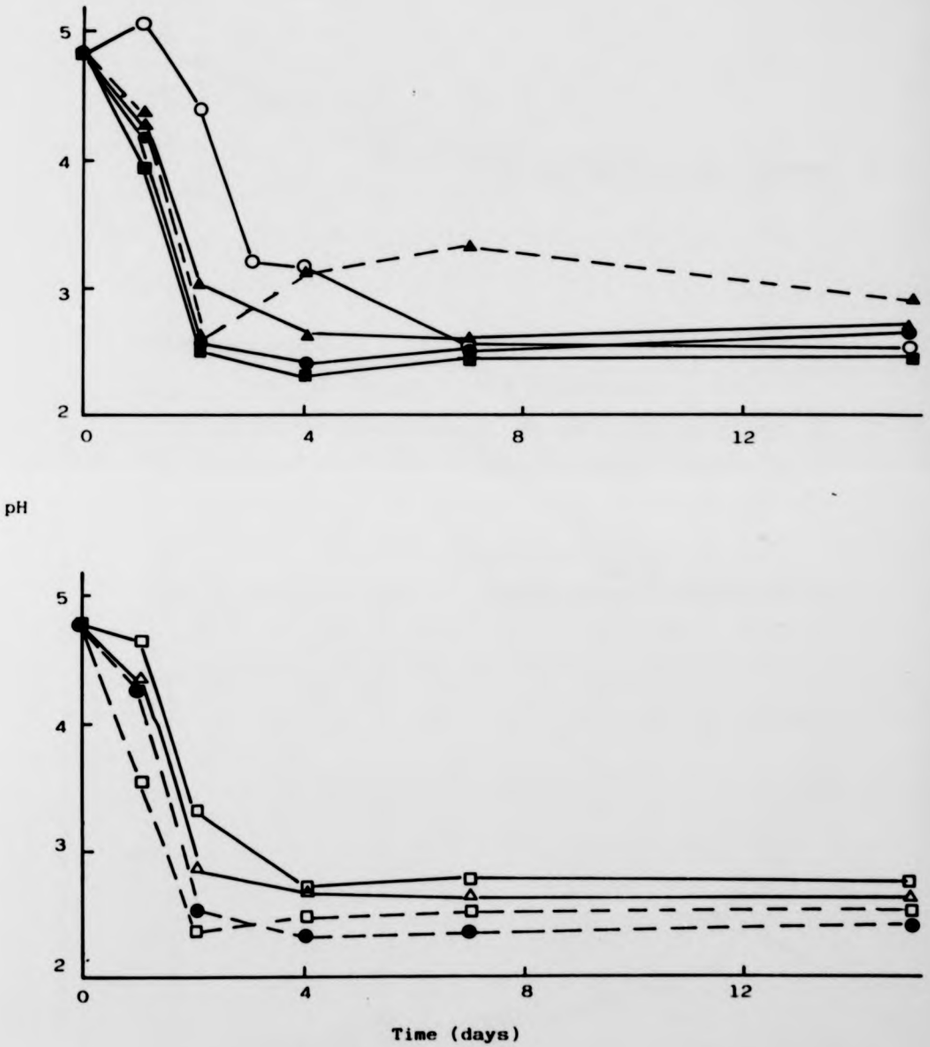


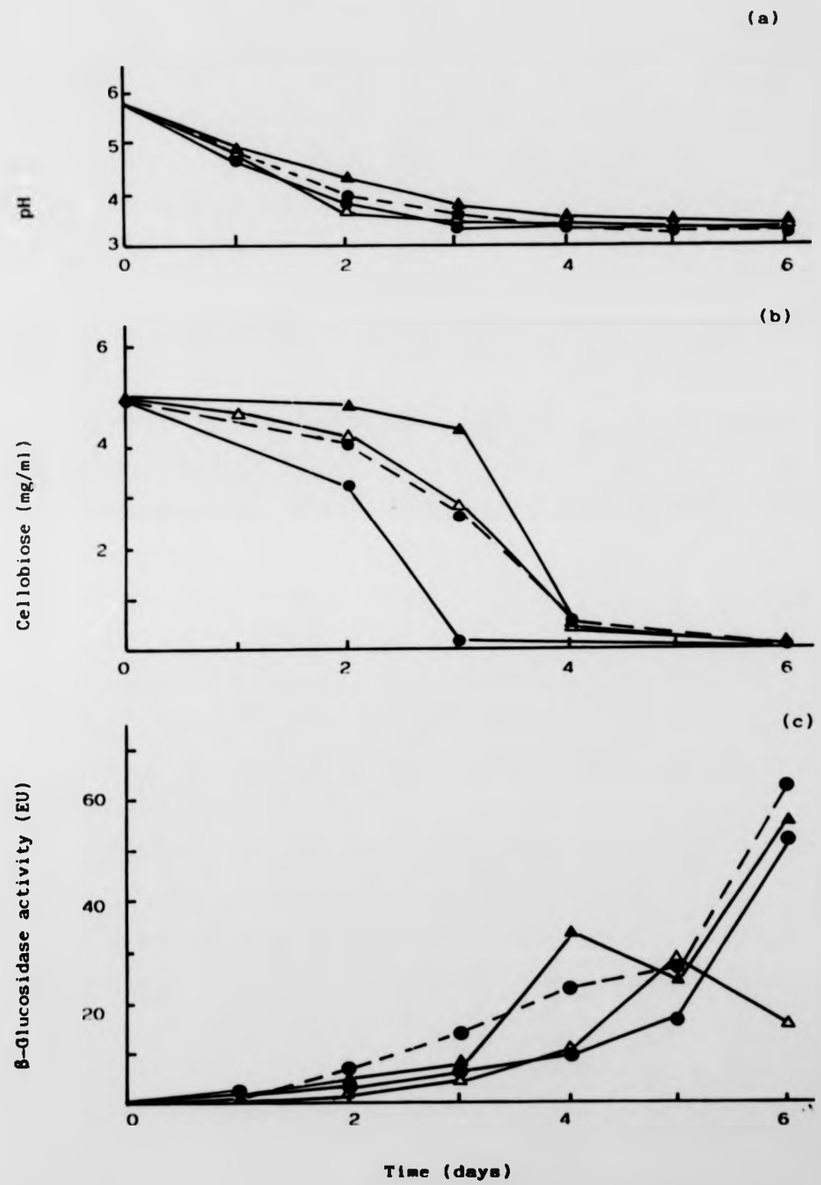
Fig. 19

Aspergillus fumigatus (▲——▲), *F. oxysporum* strain 1 (△——△), *F. oxysporum* strain 3 (●- -●) and *P. simplicissimum* (●——●) grown on 0.5% cellobiose in 0.1M phosphate-buffered medium

(a) pH

(b) Cellobiose concentration

(c) β-Glucosidase activity



Extracellular β-glucosidase in cultures of A. fumigatus and F.oxysporum (strains 1 & 3) showed a rapid increase in activity whilst cellobiose concentrations in the medium remained relatively high. This differs from the results obtained with P. simplicissimum as levels of extracellular β-glucosidase activity did not increase until most of the cellobiose had been consumed.

With all four cultures, the pH of the medium dropped from an initial pH of 5.7 to approximately 3.3 during the first 3-4d, remaining constant thereafter.

3. PRODUCTION AND LOCATION OF β-GLUCOSIDASE BY P. SIMPLICISSIMUM IN A BUFFERED AND UNBUFFERED MEDIUM

P. simplicissimum was selected for this study since previous experiments indicated that it grew rapidly on cellobiose and produced β-glucosidase. Fig. 20 shows the cellobiose concentration and medium pH in cultures of P. simplicissimum grown on 0.5% (w/v) cellobiose in a buffered and unbuffered medium. In the unbuffered medium, the pH dropped from 4.8 to 2.5 within 2d. A drop was also observed in the buffered medium although in this case it fell to only 3.4 after 2d, and remained constant thereafter.

Measurements of cellobiose concentration indicated that the substrate was used at a similar rate in both types of media.

The production and location of β-glucosidase in cultures of P.simplicissimum grown in a buffered and unbuffered medium are shown in Fig. 21. In the buffered medium (Fig. 21a), β-glucosidase activity was detected in the extracellular and cell-associated (particulate and soluble intracellular) fractions. In the initial stages of growth (3d), the sum of the activities in the cell-associated fractions was more than 3-fold greater than that in the extracellular fraction. However, after 4d, the

Fig. 20

P. simplicissimum grown on 0.5% (w/v) cellobiose in a buffered and unbuffered medium

(a) pH: (O—O) buffered medium

(●—●) unbuffered medium

(b) cellobiose concentration (□—□) buffered medium

(■—■) unbuffered medium

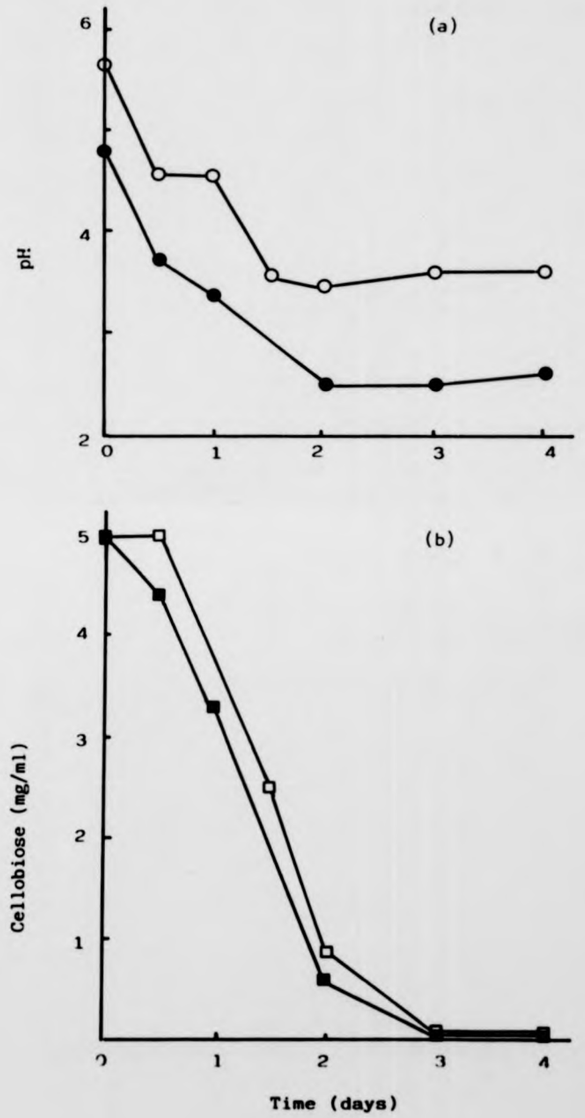


Fig. 21

A comparison of β-glucosidase production and location in cultures of P. simplicissimum grown on 0.5% (w/v) cellobiose in (a) buffered medium, (b) unbuffered medium.

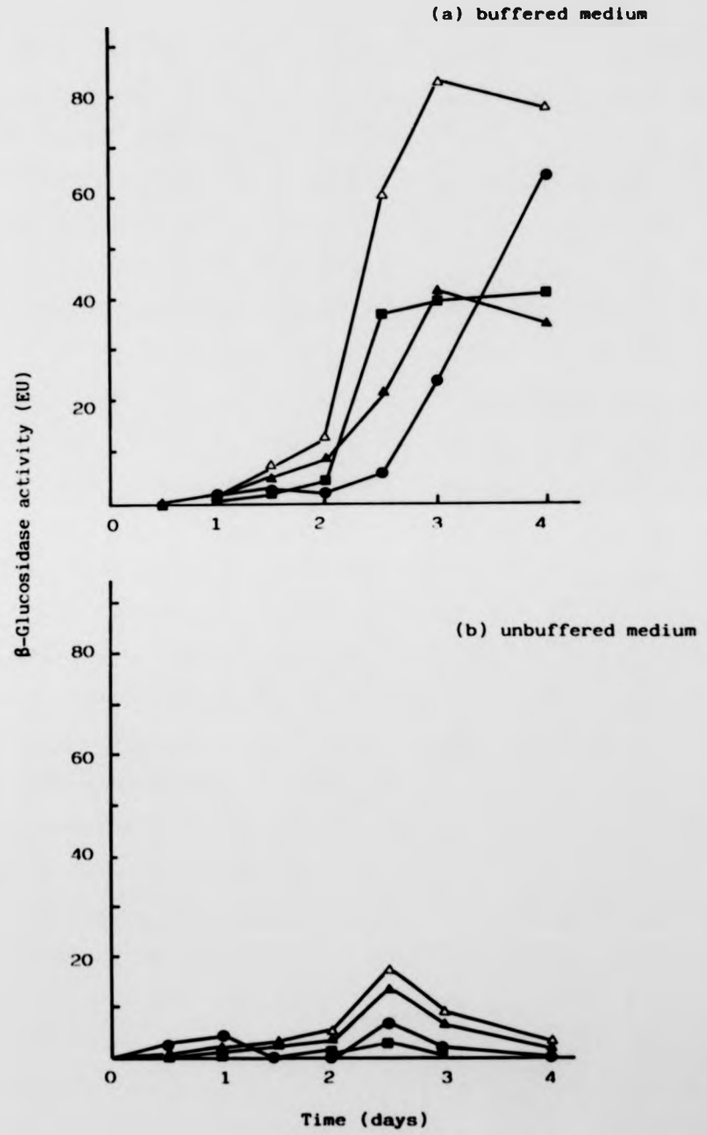
β-Glucosidase activity:

(●—●) extracellular

(▲—▲) soluble intracellular

(■—■) particulate

(△—△) sum of intracellular



activities in the total cell-associated and extracellular fractions were similar. The total cell-associated activity increased rapidly to 83 EU after 3d and decreased thereafter. The extracellular activity, on the other hand, generally increased throughout growth with a rapid increase (to 65 EU) in the latter stages (2.5-4d).

Levels of β-glucosidase activity in the soluble intracellular and particulate fractions were generally similar throughout growth, maximum levels (42 EU) being detected after 3 and 4d, respectively.

In the unbuffered medium (Fig. 21b), β-glucosidase activity was also detected in the cell-associated and extracellular fractions. Throughout the 4d growth period, the total activity in the cell-associated fractions was greater than that in the extracellular fraction, activity being more than 2-fold greater between days 2 and 3. β-Glucosidase activity in the soluble intracellular fraction was more than 3-fold greater than in the particulate fraction after 2.5d. Activity in all three fractions reached a maximum level between days 2 and 3, with a rapid decrease thereafter.

A comparison of β-glucosidase activity in the two types of media showed that levels in the buffered medium were substantially higher than those in the unbuffered medium. In the buffered medium, maximum activity in the extracellular fraction was approximately 8-fold greater, and in the cell-associated fraction 5-fold greater, than that in the unbuffered medium.

Figure 22 shows the production and location of β-glucosidase in cultures of P. simplicissimum grown in a buffered medium for 13d. These results indicated that during the latter stages of growth (7-13d), the activity in the extracellular fraction was drastically higher than in the cell-associated fraction, with a 13-fold increase after 13d. Activity in the extracellular fraction increased throughout the growth period, whereas that in the cell-associated fractions increased to day 7 and decreased thereafter.

Fig. 22

β-Glucosidase production and location in cultures of P. simplicissimum grown on 0.5% (w/v) cellobiose in a buffered medium.

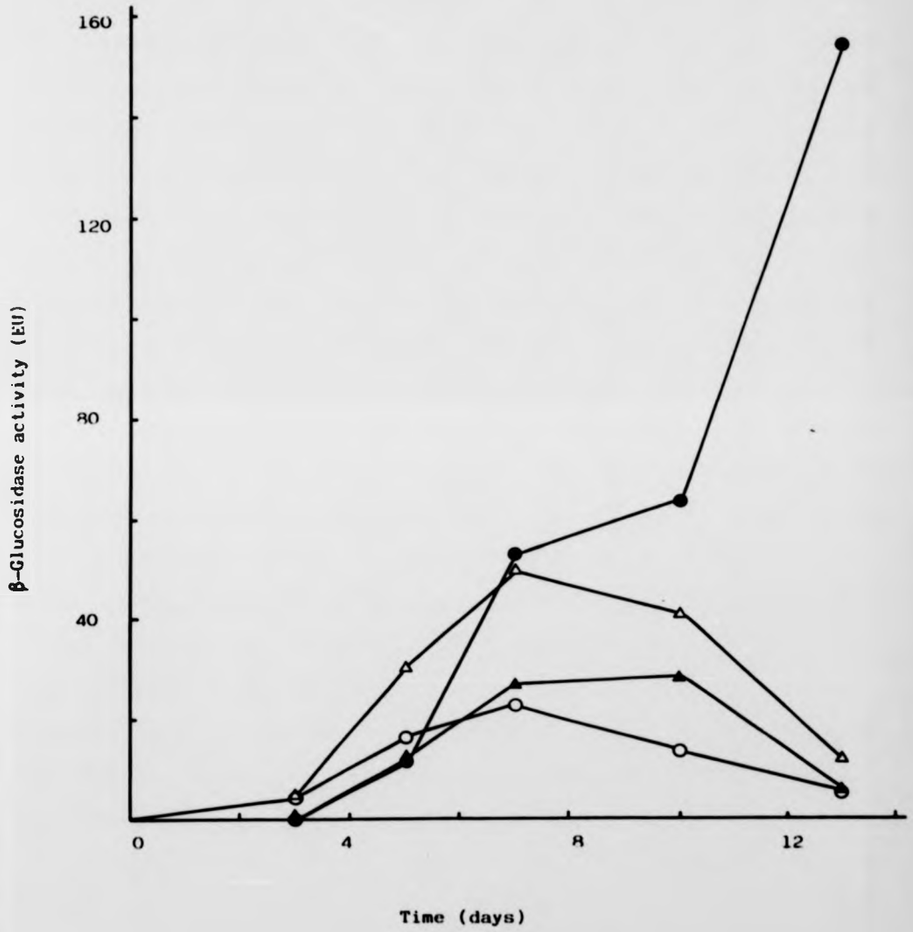
β-Glucosidase activity:

(●—●) extracellular

(▲—▲) soluble intracellular

(○—○) particulate

(△—△) sum of intracellular



4. PLATE ASSAY TO DETECT β-GLUCOSIDASE ACTIVITY

Standard techniques employed to detect β-glucosidase activity generally measure activity in the culture filtrate. However in this study it was shown that extracellular β-glucosidase was not detected in the culture medium when acidic conditions had developed during growth. Therefore, a method was developed which enabled fungi to be screened for β-glucosidase production. The method was based on the β-glucosidase assay of Okada et al. (1968) using PNG as a substrate. Fungi were grown on a solid growth medium containing PNG as the carbon source, and then the cultures were flooded with Na_2CO_3. β-Glucosidase produced by the fungi hydrolysed PNG with the release of PNP which could be detected visually after colour development with Na_2CO_3. This resulted in the formation of yellow zones around colonies producing β-glucosidase.

It was necessary to find a suitable concentration of PNG to incorporate into the solid growth medium. The concentration had to be high enough to allow β-glucosidase activity to be detected without being toxic to the fungi. Table 10 shows the effect of PNG concentration on growth and β-glucosidase production by cultures of P. simplicissimum. Results indicated that although the higher PNG concentrations (0.1-1g/l) were inhibitory to growth, β-glucosidase activity was increased. Media containing 1g/l PNG without cellobiose showed the highest β-glucosidase activity; therefore this concentration was incorporated in the solid media thereafter.

It was also necessary to determine the most suitable method to estimate PNG production. Several techniques were tried and included flooding the cultures with a solution of 0.1M Na_2CO_3. In addition, a two layer method was used with a layer of solid growth medium on top of a layer of solid medium containing Na_2CO_3. The most successful method producing clearly defined yellow regions around the fungal colonies was

TABLE 10 Effect of PNG concentration on β-glucosidase production by *P. simplicissimum* grown on a solid medium

Cellobiose (g/l)	PNG (g/l)	Growth	β-Glucosidase activity
5	0.00	++	-
5	0.05	++	+
5	0.10	+	++
5	0.50	+	+++
5	1.00	+	+++
0	1.00	++	++++

Growth: ++ good

+ sparse

β-Glucosidase activity: ++++ high

- none

TABLE 11 Growth and β-glucosidase production by fungi grown on a solid medium containing PNG (lg/l)

Organism	Growth	β-Glucosidase
Community	++	++++
A. fumigatus	++++	++
F. oxysporum (strain 1)	+++	+++
F. oxysporum (strain 2)	++	++++
F. oxysporum (strain 3)	+++	+++++
G. roseum	+++	+
P. lilacinus	+	+
P. nigricans	+++	++++
P. simplicissimum	+++	++++

Growth: ++++ abundant

\+ sparse

β-Glucosidase activity: ++++ high

\+ low

TABLE 12 **Effect of pH on β-glucosidase production by** P. simplicissimum **grown on a solid medium**

pH	Growth	β-Glucosidase activity
4.8	++++	+++++
4.0	+++	+++
3.5	++	+
3.0	+	+
2.5	+	+

Growth: ++++ abundant

\+ sparse

β-Glucosidase activity: +++++ high

\+ low

one in which the culture was flooded with Na_2CO_3 in molten agar. This method was used thereafter.

Table 11 shows the production of β-glucosidase by the community and its individual members growing on a solid medium containing PNG. All of the fungi produced β-glucosidase, greatest activity being produced by F.oxysporum (strain 3) and the lowest by G. roseum and P. lilacinus. These results were obtained within 48h; hence this is a rapid method for the detection of β-glucosidase producing organisms.

Factors affecting growth and β-glucosidase production, such as pH were also studied using this method, as shown in Table 12. These results indicated that growth and β-glucosidase production by P. simplicissimum were considerably reduced at pH 3.0 and below in comparison with pH 4.8.

DISCUSSION

In cultures grown on cellobiose in an unbuffered medium no significant levels of extracellular β-glucosidase were produced, and during the growth period a drop in medium pH occurred. Similar observations were made by Schewale and Sadana (1978) with an unidentified basidiomycete, and Mandels et al. (1975) during the growth of Trichoderma reesei on cellulose. Mandels et al. (1975) concluded that β-glucosidase was inactivated under the acidic conditions which developed during cellulose fermentation. It is reasonable to suggest, therefore, that the absence of extracellular β-glucosidase in this study resulted from its inactivation under the low pH conditions which developed in the unbuffered medium. In support of this, it was found that higher levels of extracellular β-glucosidase were produced by the fungi when grown on cellobiose in a buffered medium than on the same substrate in an unbuffered medium.

A similar observation was made by Sternberg (1976a), who reported a 4-fold increase in β-glucosidase activity in cultures of Trichoderma

growing on cellobiose in a citrate-buffered medium (pH5-8), compared with that in an unbuffered medium. He stated that the increase in β-glucosidase activity was due to the prevention of pH inactivation in the buffered medium. The prevention of β-glucosidase inactivation may also have accounted for the high enzyme levels recorded in the present study. Although the pH of the buffered medium decreased at a much slower rate than the unbuffered medium, it is possible that some β-glucosidase inactivation still occurred in the buffered medium.

Higher levels of activity in the buffered medium may have also resulted from increased growth, and therefore enzyme production, due to the slow decrease in medium pH or from the increased phosphate concentration used in the buffered medium. Desai et al. (1982) found that the phosphate concentration in cultures of Scytalidium lignicola had a marked effect on extracellular cellulase activity. As the concentration of phosphate increased from 0 to 0.4% cellulase activity and, in particular, β-glucosidase activity, rapidly increased.

The absence of extracellular β-glucosidase in the unbuffered medium may also have been because the fungi produced only cell-associated β-glucosidase under conditions of low pH. β-Glucosidase has been detected as an intracellular, cell-bound and extracellular enzyme in different organisms, or in the same organism depending upon the carbon source. Intracellular β-glucosidase was recorded in cultures of Sporotrichum thermophile, with only very small amounts associated with the cell wall and with no significant amounts of β-glucosidase detected extracellularly (Canevascini & Meyer, 1979). In contrast, Rapp et al. (1981) found that Penicillium janthinellum produced primarily extracellular β-glucosidase, with only very small amounts located intracellularly and in association with the cell wall.

Studies of β-glucosidase location in the buffered and unbuffered media revealed the presence of extracellular and cell-associated β-glucosidase in cultures of P. simplicissimum grown on cellobiose. Throughout the growth period, β-glucosidase was predominantly cell-associated in both types of media. However, the pattern in the buffered medium was notably different from the unbuffered medium since high levels of extracellular β-glucosidase activity were detected in the later stages of growth. It appears that cultures of P. simplicissimum grown on cellobiose produced a β-glucosidase which was predominantly cell-associated and not released into the medium until most of the cellobiose had been utilised and cell lysis was likely to have occurred. Since the unbuffered medium did not show an increase in extracellular β-glucosidase in the later stages of growth, it is possible that the enzyme was released into the medium but became inactivated under the acidic conditions.

A similar explanation for the appearance of extracellular β-glucosidase in cultures of Trichoderma reesei was postulated by Berg and Pettersson (1977). During its active growth on cellobiose, this fungus produced only cell-associated β-glucosidase. When the stationary phase was reached the enzyme was released into the medium, most likely as a result of cell lysis (Berg & Pettersson, 1977).

Results obtained from the location studies also indicated that levels of β-glucosidase activity in all three fractions from the buffered medium were considerably higher than those in the unbuffered medium. Despite this, P. simplicissimum hydrolysed cellobiose at a similar rate in both types of media. This suggests that β-glucosidase was over produced in the buffered medium and that the rate of cellobiose hydrolysis was maximal in each case.

Studies of extracellular β-glucosidase production by A. fumigatus and F. oxysporum (strains 1 and 3) growing in a buffered medium suggested that these fungi produced "truly extracellular" β-glucosidases. Extracellular β-glucosidase activity was found to increase in the initial stages of growth when less than 50% of the cellobiose had been utilised. It is therefore unlikely that the appearance of extracellular β-glucosidase resulted from cell lysis.

Using the plate assay for β-glucosidase activity, it was found that the community and all the individual members were able to produce β-glucosidase. This is unlike the results obtained using the conventional β-glucosidase assay since, in this case, all of the fungi produced negligible levels of activity.

There appear to be several differences between the two β-glucosidase assays which could account for the results obtained. With the plate assay, β-glucosidase activity is measured during growth. It therefore seems reasonable to suggest that cell-bound activity is measured in addition to extracellular activity. The plate assay may also measure intracellular activity but this would be dependent on the uptake of PNG across the cell membrane and the subsequent release of PNP. The conventional β-glucosidase assay measures only extracellular activity.

In addition, the plate assay unlike the conventional assay, measures activity as soon as the enzyme and PNG come into contact. In the case of the conventional assay, the enzyme is subjected to the various conditions in the environment before its activity is measured.

In summary:-

1. Negligible levels of extracellular β-glucosidase were detected in cultures grown in an unbuffered medium with cellobiose as the sole carbon source. It is possible that this was a result of pH inactivation or that the fungi produced predominantly cell-associated β-glucosidases.

2. Higher levels of β-glucosidase activity were obtained using a buffered medium, suggesting that pH inactivation of β-glucosidase was prevented. Alternatively, the increased levels may have been due to increased microbial biomass resulting from the difference in cultural conditions.

3. Studies of β-glucosidase location indicated that P simplicissimum produced a predominantly cell-associated β-glucosidase. The enzyme was not released into the medium until the latter stages of growth, probably as a result of cell lysis. An increase in extracellular activity was observed in the buffered medium but not in the unbuffered presumably as pH inactivation occurred in the latter case.

4. P. simplicissimum hydrolysed cellobiose at a similar rate in both types of media. Differences in levels of β-glucosidase activity suggested that the enzyme was over produced in the buffered medium and that cellobiose was hydrolysed at a maximum rate in both situations.

CHAPTER FIVE

CELLULASE PRODUCTION UNDER DIFFERENT CULTURAL CONDITIONS

CHAPTER FIVE

CELLULASE PRODUCTION UNDER DIFFERENT CULTURAL CONDITIONS

INTRODUCTION

Results presented and discussed in Chapter 3 indicated that there was no synergism between members of the community in the degradation of cellulose. It was decided to extend this study by investigating cellulase production under different cultural conditions.

Growth medium pH and cellulose concentration are known to be critical factors affecting cellulase production by several microorganisms (Linko et al. 1977; Mandels & Andreotti, 1978; Mandels & Sternberg, 1976 and Sternberg, 1976b). Results obtained in Chapter 4 indicated that β-glucosidase production by P. simplicissimum was affected by the pH of the medium.

If the key enzymes involved in cellulolysis are affected by cultural conditions, the possibility existed that the growth of other organisms in the community could become affected. With complex interactions between members of the community such a situation could result in the organisms acting synergistically in the hydrolysis of cellulose.

RESULTS

1. A COMPARISON OF CELLULASE ACTIVITY IN CULTURES OF A. FUMIGATUS, P.SIMPLICISSIMUM AND THE COMMUNITY GROWN IN A PHOSPHATE-BUFFERED MEDIUM

i. Cellulase

Figure 23a-c shows **cellulase** activity detected in the three cultures grown on 0.5-5% cellulose in a phosphate-buffered medium. Levels of enzyme activity produced by the community were similar to those produced by P. simplicissimum after 30d. However, the cellulose concentration giving the highest activity varied with the two cultures; with the community the highest activity was obtained on 1% cellulose, whereas with P. simplicissimum it was obtained on 5% cellulose.

In the initial stages of growth (10d), **cellulase** activity produced by the community was 2-5 fold greater than that produced by P.simplicissimum. Levels produced by the community remained constant or decreased somewhat thereafter, whilst those produced by P. simplicissimum continued to increase to day 20.

Throughout most of the 30d growth period A. fumigatus produced lower levels of **cellulase** activity than P. simplicissimum and the community, the highest level of activity being about 50% less than the highest level produced by the other two cultures.

ii. Endoglucanase

Endoglucanase activity detected in cultures grown on 0.5-5% cellulose is shown in Fig. 24a-c. In general, the highest levels of enzyme activity were produced by P. simplicissimum, activity ranging from 8-30 EU after 30d. A. fumigatus and the community produced similar levels of endoglucanase activity (2-5 EU after 30d), with the exception of that produced on 1% cellulose. Activity produced by the community on 1% cellulose was greater than that produced by A. fumigatus, being 5-fold greater after 30d.

iii. β-Glucosidase

Figure 25a-c shows β-glucosidase activity in cultures grown on 0.5-5% cellulose. Unlike the situation with cellulase and endoglucanase, levels of β-glucosidase produced by the three cultures on varying cellulose concentrations were generally similar. One exception was with 5% cellulose where the level of enzyme produced by A. fumigatus was greater than that produced by the other two cultures, being at least 15-fold greater after 30d.

These results indicated that the community did not show an increase in cellulase production compared with P. simplicissimum when grown in a

Fig. 23

A comparison of **cellulase** activity in cultures of A. fumigatus, P. simplicissimum and the community grown on cellulose in a buffered and unbuffered medium.

(●—●) 0.5% cellulose

(▲—▲) 1.0% cellulose

(■—■) 2.0% cellulose

(□—□) 5.0% cellulose

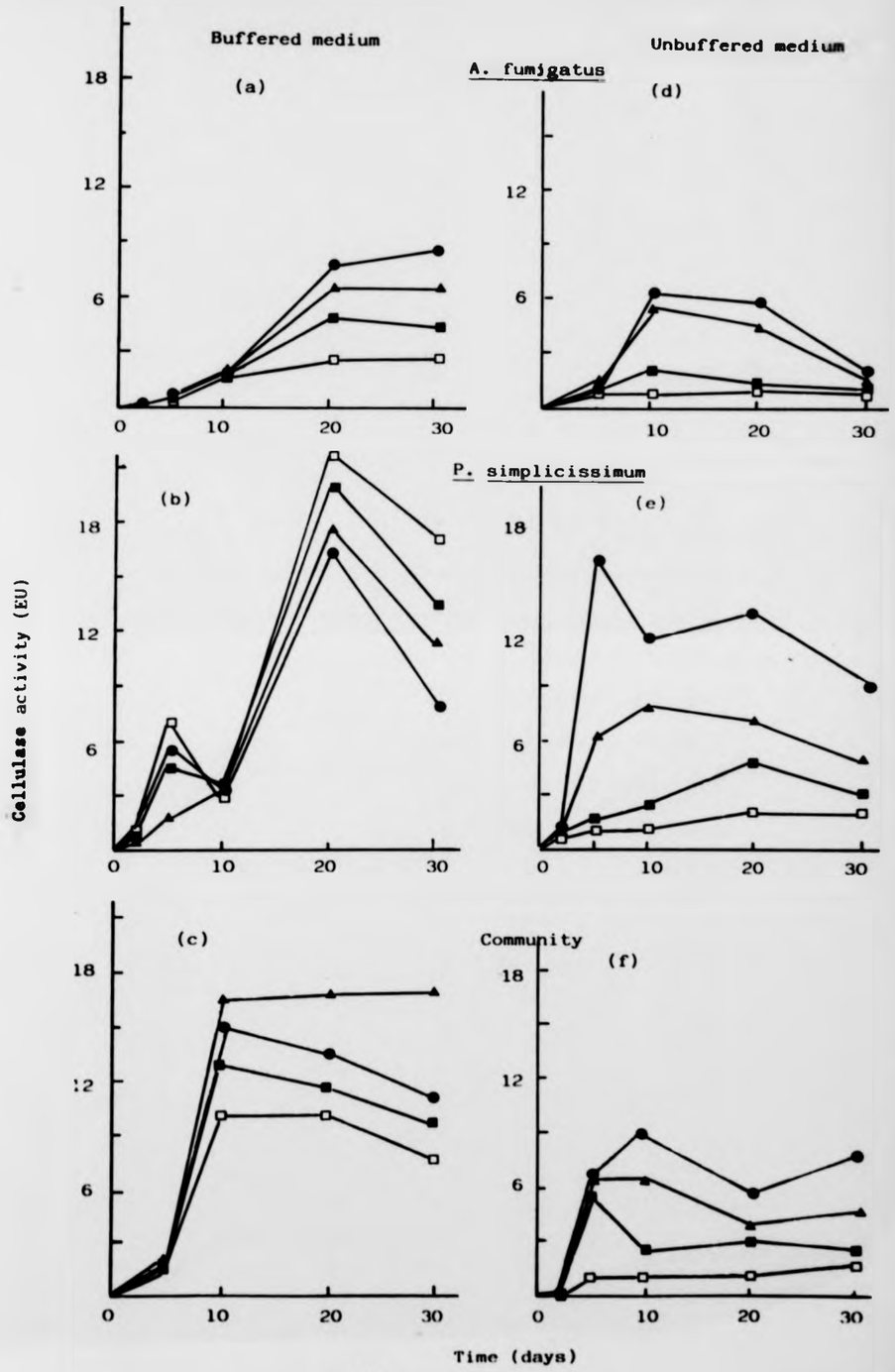


Fig. 24

A comparison of endoglucanase activity in cultures of A. fumigatus, P. simplicissimum and the community grown on cellulose in a buffered and unbuffered medium.

(●—●) 0.5% cellulose

(▲—▲) 1.0% cellulose

(■—■) 2.0% cellulose

(□—□) 5.0% cellulose

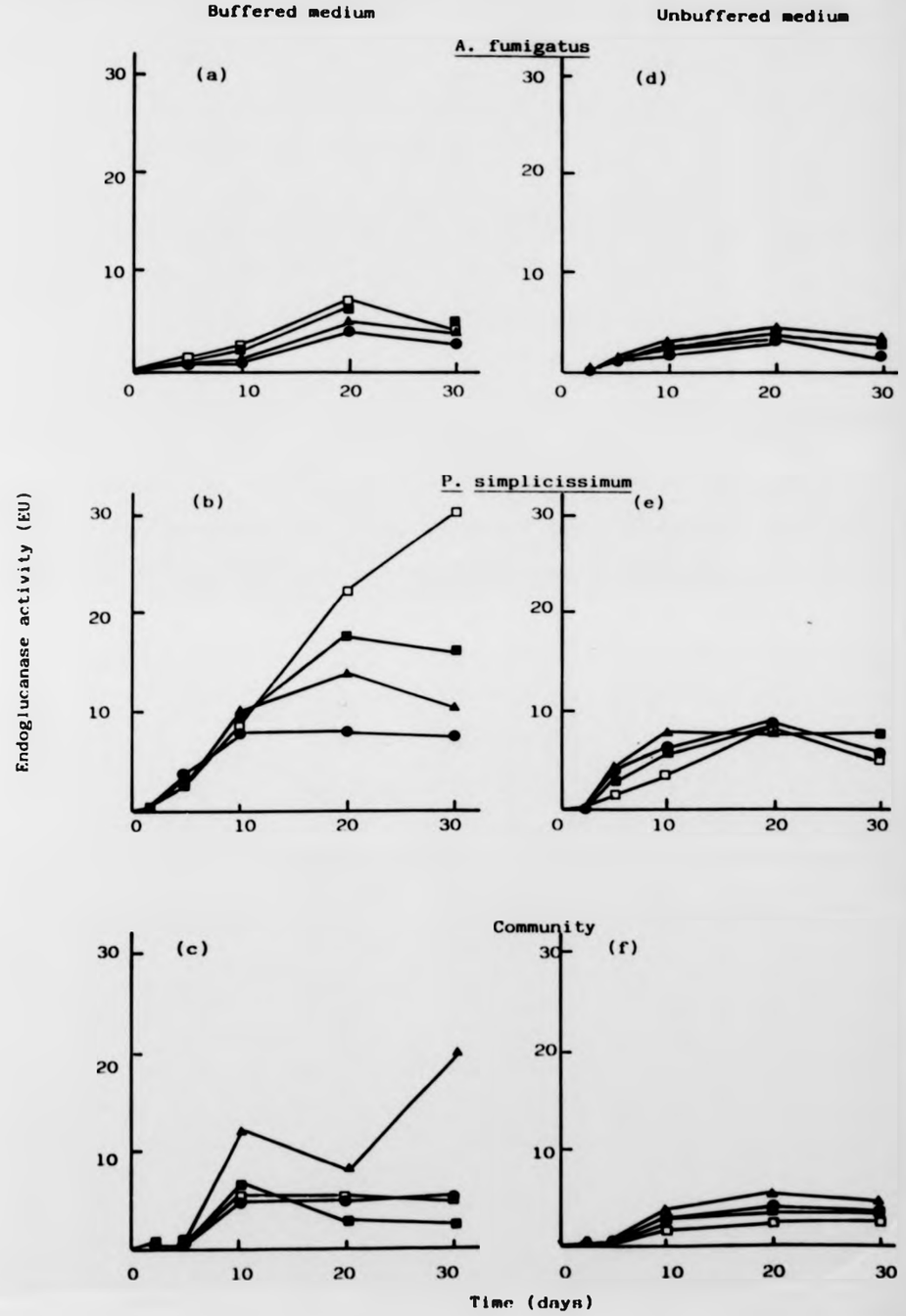


Fig. 25

A comparison of β-Glucosidase activity in cultures of A. fumigatus, P. simplicissimum and the community grown on cellulose in a buffered and unbuffered medium.

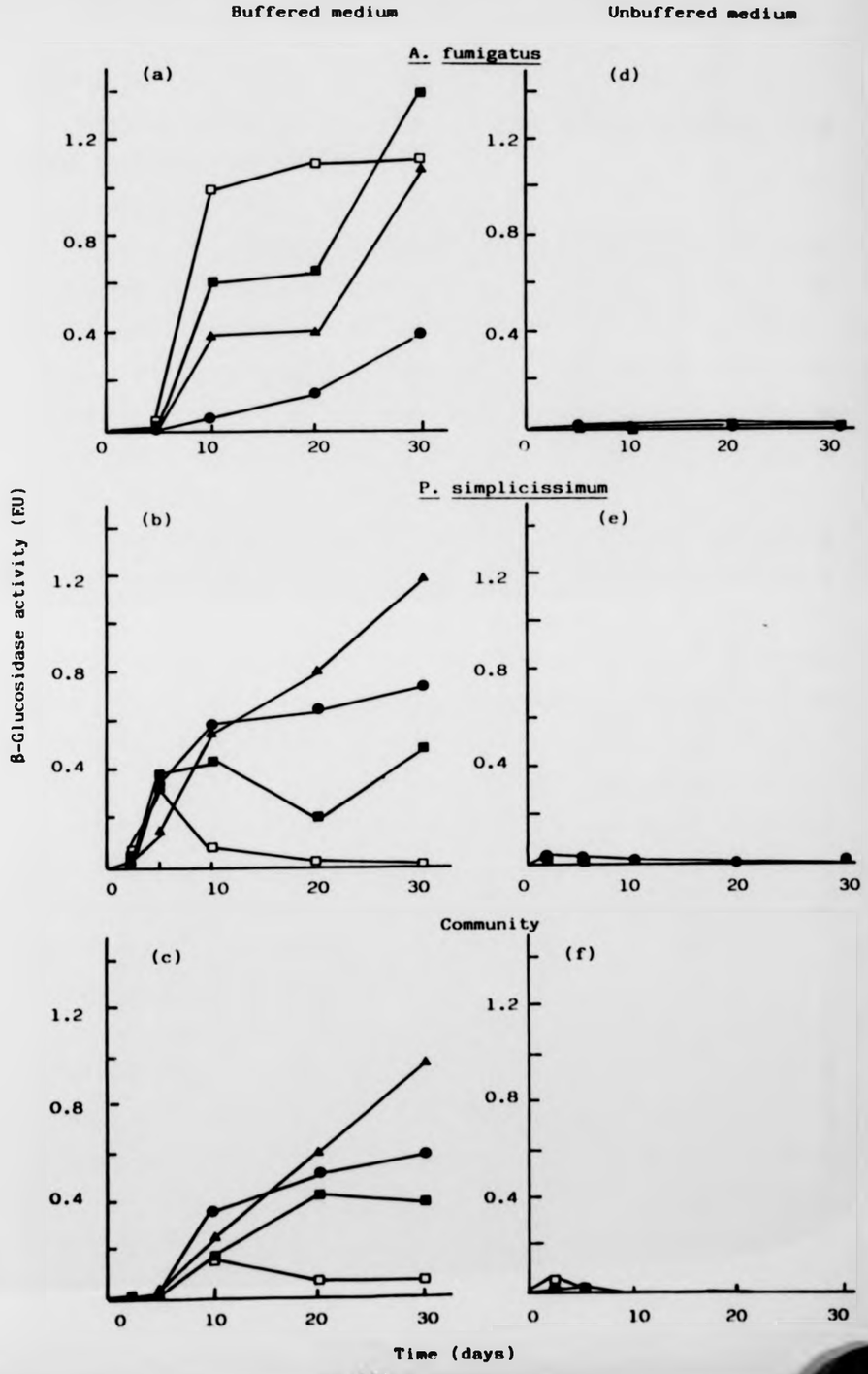
(●—●) 0.5% cellulose

(▲—▲) 1.0% cellulose

(■—■) 2.0% cellulose

(□—□) 5.0% cellulose

Enzyme units (EU) are expressed as μmoles glucose equivalents released per minute per ml culture filtrate.



phosphate-buffered medium with increasing cellulose concentration.

2 EFFECT OF CELLULOSE CONCENTRATION ON CELLULASE ACTIVITY IN A BUFFERED AND UNBUFFERED MEDIUM

i. Aspergillus fumigatus

The highest **cellulase** activity in the buffered medium (Fig. 23a) was obtained from cultures grown on 0.5% cellulose. Increasing the cellulose concentration resulted in a direct decrease in **cellulase** activity, with a 3-fold decrease being observed on 5% cellulose after 30d. The effect of cellulose concentration on **cellulase** activity became apparent after 20d, and with all substrate concentrations the activity increased to day 20, remaining constant thereafter.

In the unbuffered medium (Fig. 23d) the highest **cellulase** activity was obtained on 0.5 and 1% cellulose between 10 and 20d with considerably lower levels being obtained on 2 and 5% cellulose. After 30d the level of activity on the lower cellulose concentrations decreased rapidly with the result that similar levels of activity were obtained with all cellulose concentrations.

These results indicated that cultures of A. fumigatus produced higher levels of **cellulase** activity in the buffered medium compared with the unbuffered medium, particularly with 2 and 5% cellulose, where maximum levels were approximately 3-fold greater.

ii. Penicillium simplicissimum

In the buffered medium (Fig. 23b) maximum **cellulase** activity was obtained from cultures grown on 5% cellulose and activity increased as the cellulose concentration increased. After 30d the activity obtained on 5% cellulose was 2-fold greater than that on 0.5% cellulose, the effect becoming more pronounced between 20 and 30d. Maximum activity on all cellulose concentrations occurred after 20d, with levels decreasing there-

after.

In the unbuffered medium (Fig. 23e) maximum **cellulase** activity was obtained from cultures grown on 0.5% cellulose. Increasing the cellulose concentration resulted in a direct decrease in **cellulase** activity such that after 30d, activity obtained from cultures grown on 5% cellulose was 4-fold less than that on 0.5% cellulose. The effect of cellulose concentration on **cellulase** activity became apparent during the early stages of growth (5d). Maximum activity on all cellulose concentrations occurred during the first 20d, levels generally decreasing thereafter.

A comparison of **cellulase** activity obtained from cultures of P.simplicissimum grown on the two types of media indicated that, overall, enzyme activity in the buffered medium was greater than that in the unbuffered medium.

iii. Community

Using the buffered medium (Fig. 23c), maximum **cellulase** activity was obtained from cultures grown on 1% cellulose; increasing the cellulose concentration above this caused a decrease in activity. After 30d activity obtained on 5% cellulose was about half that on 1% cellulose. Enzyme levels produced on all concentrations of cellulose increased to day 10 and declined thereafter, with the exception of those on 1% cellulose which remained constant. The effect of cellulose concentration on **cellulase** activity became more pronounced as time proceeded.

Maximum **cellulase** activity in the unbuffered medium (Fig. 23f) was obtained from cultures grown on 0.5% cellulose, activity decreasing as the cellulose concentration increased. The activity obtained on 0.5% cellulose was 4-fold greater than that on 5% cellulose after 30d. After the initial increase in activity which occurred with all substrate

concentrations, levels decreased slightly or remained constant.

A comparison of **cellulase** activity in cultures of the community grown in the two types of media indicated that levels were higher in the buffered medium, maximum activity (on 1% cellulose) being 2-fold greater than that obtained in the unbuffered medium.

3 **EFFECT OF CELLULOSE CONCENTRATION ON ENDOGLUCANASE ACTIVITY IN A BUFFERED AND UNBUFFERED MEDIUM**

i. Aspergillus fumigatus

The effect of cellulose concentration on endoglucanase activity in the buffered medium is shown in Fig. 24a. In general, cellulose concentration had little effect on endoglucanase activity, although after 20d, activity obtained on 5% cellulose was almost 2-fold greater than that on 0.5% cellulose. With all substrate concentrations activity increased to 20d and decreased thereafter.

With the unbuffered medium (Fig. 24d) cellulose concentration similarly had no effect on endoglucanase activity. Maximum levels of enzyme activity were obtained after 20d.

A comparison of endoglucanase activity in cultures of A. fumigatus indicated that similar levels were produced on the two types of media.

ii. Penicillium simplicissimum

In the buffered medium (Fig. 24b) there was a marked effect on endoglucanase activity with increasing cellulose concentration resulting in an increase in enzyme activity. Maximum levels obtained on 5% cellulose were 4-fold greater than those on 0.5% cellulose. The effect of cellulose concentration on endoglucanase activity became apparent after 20d. Activity obtained from cultures grown on 5% cellulose increased throughout the 30d growth period whereas activity on the other cellulose

concentrations remained constant or decreased after 20d.

In the unbuffered medium (Fig. 24e) cellulose concentration had no effect on endoglucanase activity. Enzyme activity generally increased to 10 or 20d and remained constant thereafter.

A comparison of endoglucanase activity in cultures of P.simplicissimum indicated that maximum activity was obtained using the buffered medium, levels being 3-fold greater than those obtained in the unbuffered medium.

iii. Community

In the buffered medium (Fig. 24c) endoglucanase activity obtained on 1% cellulose was considerably higher (4-fold after 30d) than that obtained with the other substrate concentrations. The effect of cellulose concentration on endoglucanase activity became apparent after 10d.

Enzyme activity in the cultures grown on varying cellulose concentrations increased to day 10. Activity on 0.5 and 5% cellulose remained constant thereafter whilst that on 1 and 2% cellulose decreased to day 20 and then either increased or remained constant.

Endoglucanase activity in the unbuffered medium (Fig. 24f) was unaffected by the cellulose concentration. Levels increased slowly during the first 10 to 20d and remained constant thereafter.

These results indicated that in general, endoglucanase levels produced by the community in the buffered and unbuffered media were similar throughout the growth period, with the exception of those on 1% cellulose where levels were higher in the buffered than in the unbuffered medium.

4 EFFECT OF CELLULOSE CONCENTRATION ON β-GLUCOSIDASE ACTIVITY IN A BUFFERED AND UNBUFFERED MEDIUM

i. Aspergillus fumigatus

In the buffered medium (Fig. 25a) an increase in cellulose concentration generally resulted in an increase in β-glucosidase activity. After 20d the activity obtained on 5% cellulose was more than 7-fold greater than that on 0.5% cellulose. At a later stage of growth (30d), however, increasing the cellulose concentration above 2% resulted in a decrease in β-glucosidase activity.

With the exception of 5% cellulose, β-glucosidase activity obtained with all substrate concentrations increased throughout the 30d growth period. In contrast, 5% cellulose showed a rapid increase to 10d, with only a slight increase thereafter.

In the unbuffered medium (Fig. 25d), negligible levels of β-glucosidase activity were obtained with all substrate concentrations.

A comparison of β-glucosidase activity in cultures of A. fumigatus indicated that with all cellulose concentrations, considerably higher levels were obtained in the buffered medium than in the unbuffered medium.

ii. Penicillium simplicissimum

In the buffered medium (Fig. 25b) maximum β-glucosidase activity was obtained in cultures grown on 1% cellulose. Activity was considerably reduced when the initial cellulose concentration exceeded this value, negligible levels being detected on 5% cellulose after 30d. The effect of cellulose concentration on β-glucosidase activity became more apparent as time progressed.

With the exception of 1% cellulose, all substrate concentrations showed a rapid increase in activity to 5 or 10d, levels decreasing or increasing only slightly thereafter. Levels of activity obtained on 1%

cellulose increased throughout the incubation period.

Negligible levels of β-glucosidase activity were detected in the unbuffered medium (Fig. 25e) with all substrate concentrations.

Results indicated that P. simplicissimum grown on all cellulose concentrations produced considerably higher levels of β-glucosidase in the buffered medium than in the unbuffered medium.

iii. Community

Maximum β-glucosidase activity in the buffered medium (Fig. 25c) was obtained from cultures grown on 1% cellulose, with greatly reduced levels of activity being obtained when the initial cellulose concentration exceeded this value. The maximum activity on 1% cellulose was 12-fold greater than that on 5% cellulose after 30d. Although the activity on 0.5% and 1% cellulose increased throughout growth, that on 2% cellulose increased to day 20, remaining constant thereafter, whilst that on 5% cellulose declined after 10d.

Negligible levels of β-glucosidase activity were obtained in the unbuffered medium (Fig. 25f) on all cellulose concentrations.

A comparison of β-glucosidase production by the community grown in the two types of media indicated that levels were greatly increased in buffered medium.

5 pH OF THE BUFFERED AND UNBUFFERED GROWTH MEDIUM

Figure 26 shows the change in the pH of the buffered and unbuffered medium in cultures of A. fumigatus, P. simplicissimum and the community grown on varying cellulose concentrations. In the buffered medium (Fig. 26a-c) the pH decreased with increasing cellulose concentration, similar observations being made with all three cultures. With 5% cellulose the pH dropped from 5.7 to between 2.9 and 3.4, whereas with 0.5% cellulose the

Fig. 26

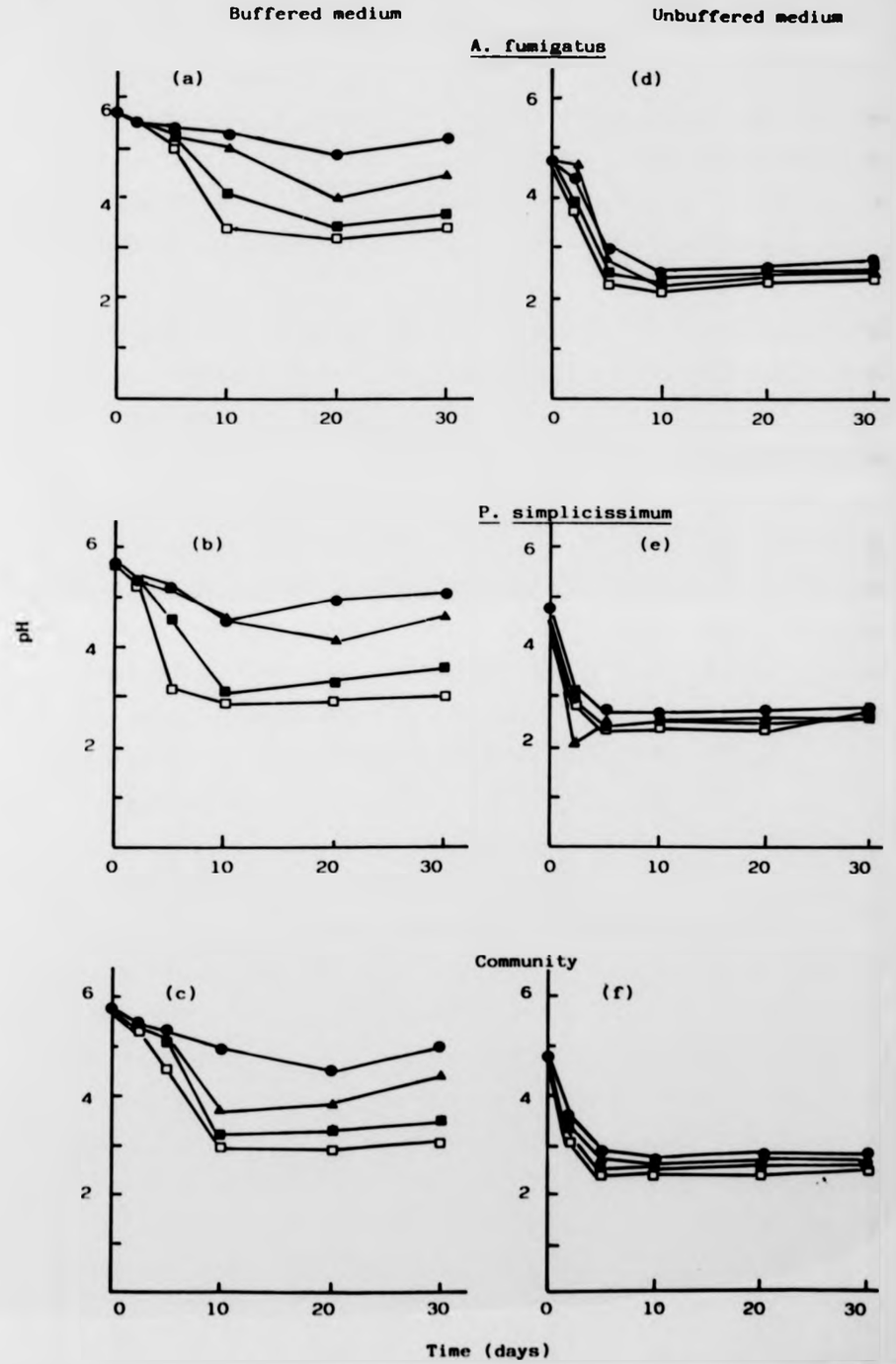
A comparison of the changes in the pH of a buffered and unbuffered medium in cultures of A. fumigatus, P. simplicissimum and the community grown on cellulose.

(●—●) 0.5% cellulose

(▲—▲) 1.0% cellulose

(■—■) 2.0% cellulose

(□—□) 5.0% cellulose



pH dropped to between 4.5 and 5.0.

In contrast, in the unbuffered medium (Fig. 26d-f), all cultures showed an initial rapid drop in pH (from 4.8 to between 2.0 and 2.8) within the first 10d, the pH remaining at this low value thereafter. A similar drop was observed with each of the cellulose concentrations.

A comparison of the two types of media indicated that in general the pH of the buffered medium was higher and decreased at a slower rate than that of the unbuffered medium.

6 EFFECT OF CELLULOSE CONCENTRATION ON CELLULASE PRODUCTION IN A 0.4M PHOSPHATE-BUFFERED MEDIUM

Results from Section 5 indicated that the buffering capacity of the medium (0.1M phosphate) was insufficient under these cultural conditions and hence the pH dropped below 3.5 with the higher cellulose concentrations. Thus it became difficult to determine the effect of pH control on cellulase production. This experiment was therefore repeated using a buffered medium with an increase in molarity.

i. Cellulase

With increasing cellulose concentration in cultures of A. fumigatus (Fig. 27a) there was an increase in **cellulase** activity up to 1% cellulose. Activity was considerably reduced when the initial cellulose concentration exceeded this value. The effect of cellulose concentration on **cellulase** activity was most apparent after 30d, the highest activity (on 1% cellulose) was 2-4-fold greater than that on 2 and 5% cellulose.

Cellulose concentration appeared to have little effect on **cellulase** activity in cultures of P. simplicissimum (Fig. 27b) with the exception of 5% cellulose. In this case very low levels of **cellulase** activity detected in the initial stages of growth (up to 20d) rapidly increased to produce the highest level after 30d.

Fig. 27

Cellulase activity in cultures of A. fumigatus, P. simplicissimum and the community grown on cellulose in a 0.4M phosphate-buffered medium.

(●—●) 0.5% cellulose

(▲—▲) 1.0% cellulose

(■—■) 2.0% cellulose

(□—□) 5.0% cellulose

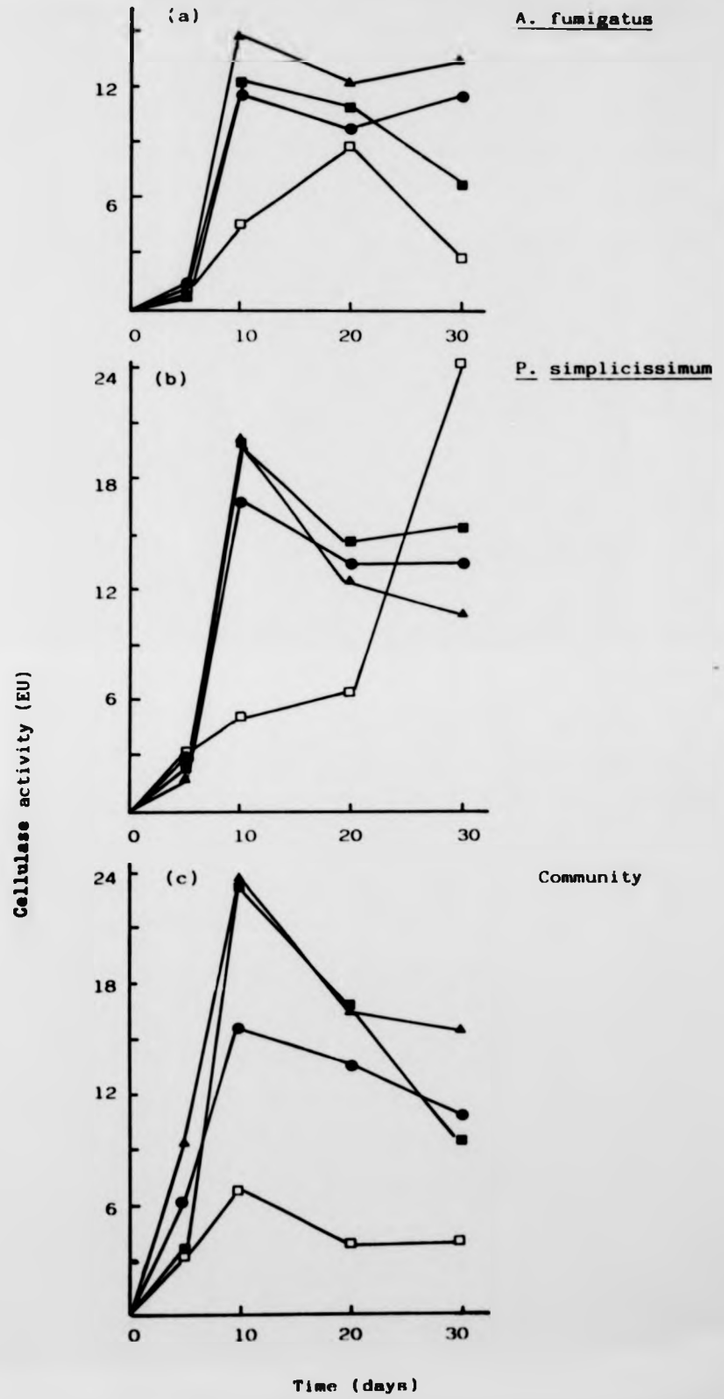


Figure 27c shows the effect of cellulose concentration on **cellulase** produced by the community. Activity generally increased with increasing cellulose concentration up to 1%. Levels produced on 2% cellulose were either similar to or less than those obtained on 1% cellulose. With the highest cellulose concentration (5%) activity was greatly reduced, levels being almost 4-fold less than those on 1% cellulose. All substrate concentrations showed an increase in **cellulase** activity to day 10 with activity declining thereafter.

ii. Endoglucanase

Endoglucanase activity detected in cultures of A. fumigatus (Fig. 28a) was unaffected by cellulose concentration, activity increasing throughout the 30d growth period.

With cultures of P. simplicissimum (Fig. 28b), increasing the cellulose concentration (up to 2%) resulted in a direct increase in endoglucanase activity after 30d, but increasing the concentration above this did not result in a further increase in activity. The highest level of activity (on 2 and 5% cellulose) was 4-fold greater than that obtained on 0.5% cellulose. Enzyme levels detected in cultures grown on 5% cellulose, although similar to those on 2% cellulose after 30d, were considerably lower between days10 and 20.

Highest levels of endoglucanase activity produced by the community (Fig. 28c) were obtained with cultures grown on 2% cellulose. Considerably lower levels of activity were produced by cultures grown on 5% cellulose, with a 5-fold decrease after 30d. Endoglucanase activity generally increased throughout the growth period, with the exception of 5% cellulose, levels increasing to 10d and remaining constant thereafter.

iii. β-Glucosidase

With cultures of A. fumigatus (Fig. 29a) the highest level of β-glucosidase was obtained in cultures grown on 2 and 5% cellulose after

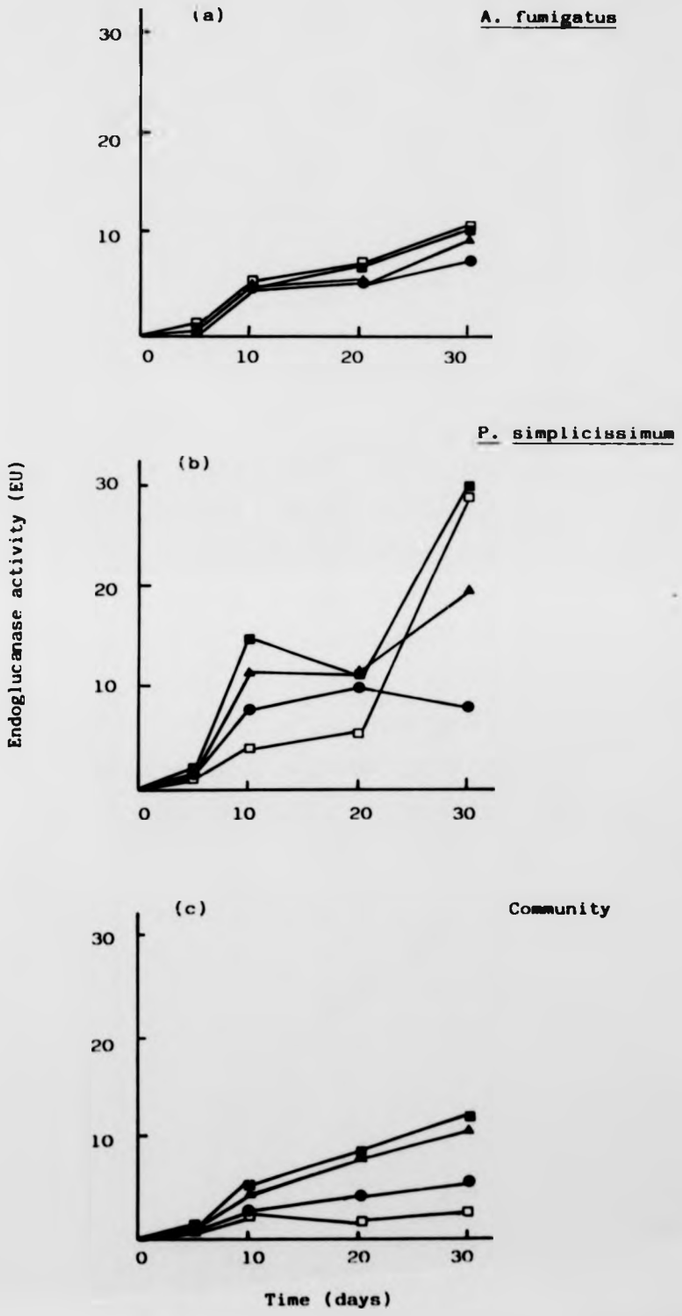


Fig. 28

Endoglucanase activity in cultures of A. fumigatus, P. simplicissimum and the community grown on cellulose in a 0.4M phosphate-buffered medium.

(●—●) 0.5% cellulose

(▲—▲) 1.0% cellulose

(■—■) 2.0% cellulose

(□—□) 5.0% cellulose

Fig. 29

β-Glucosidase activity in cultures of A. fumigatus, P. simplicissimum and the community grown on cellulose in a 0.4M phosphate-buffered medium.

(●—●) 0.5% cellulose

(▲—▲) 1.0% cellulose

(■—■) 2.0% cellulose

(□—□) 5.0% cellulose

Enzymes units (EU) expressed as μmoles glucose equivalents released per minute per ml culture filtrate.

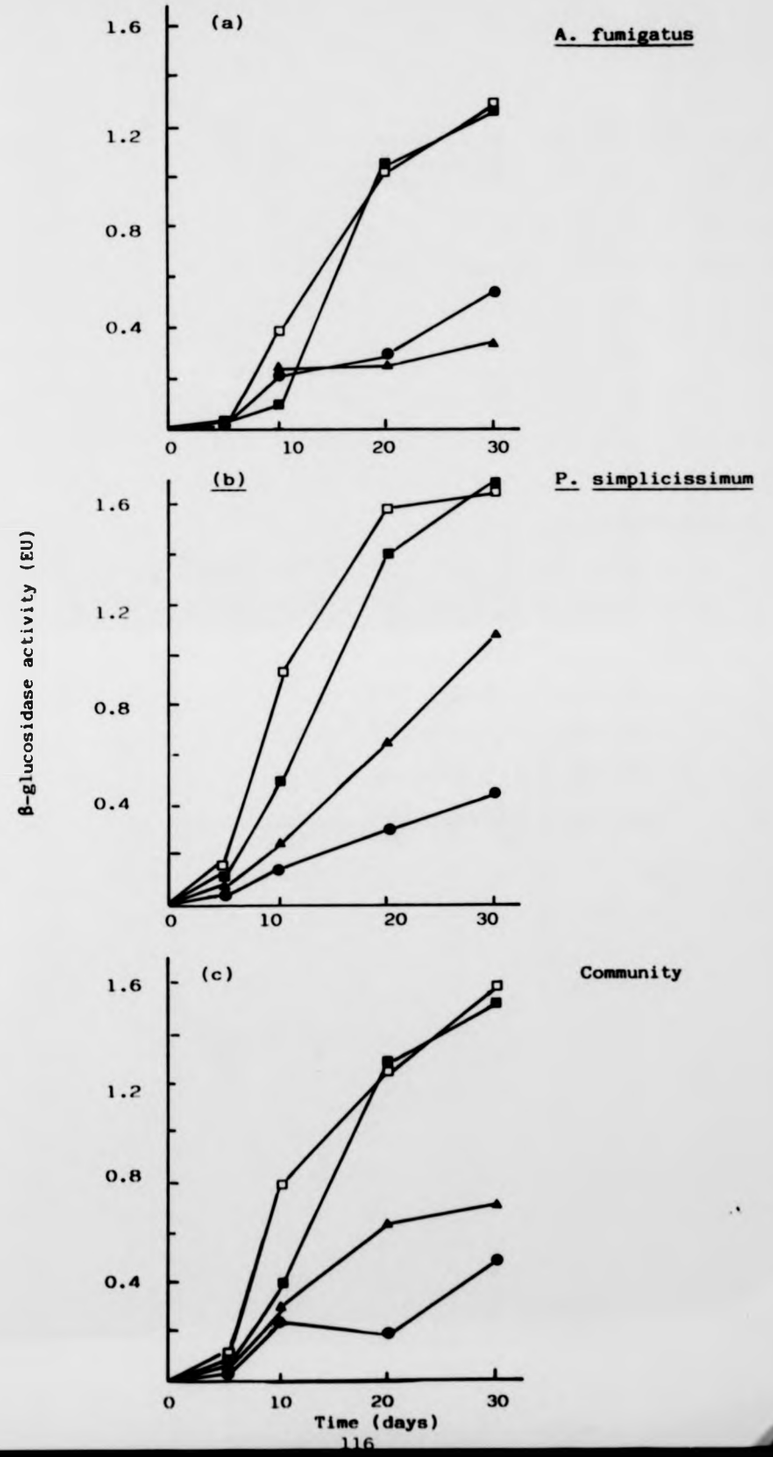


Fig. 30

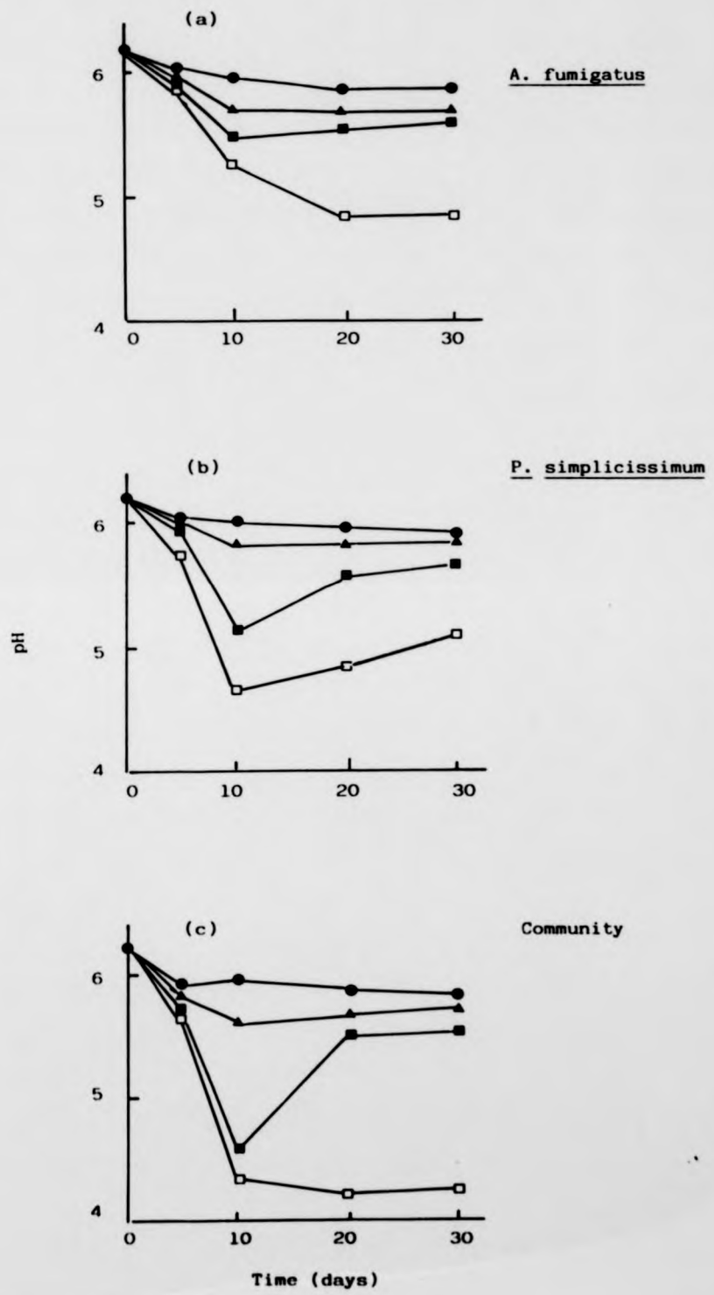
Changes in the pH of a 0.4M phosphate-buffered medium during growth of A. fumigatus, P. simplicissimum and the community on cellulose.

(●—●) 0.5% cellulose

(▲—▲) 1.0% cellulose

(■—■) 2.0% cellulose

(□—□) 5.0% cellulose



30d, activity being 2-4 fold greater than that obtained on 0.5 and 5% cellulose. β-Glucosidase activity detected in cultures grown on 2 and 5% cellulose rapidly increased throughout the growth period, whilst on 0.5 and 1% cellulose, activity increased steadily over the 30d growth period.

Figure 29b shows that β-glucosidase produced by P. simplicissimum generally increased with increasing cellulose concentration, the highest levels being obtained on 2 and 5% cellulose after 30d. At these concentrations, enzyme levels were almost 4-fold greater than those obtained on 0.5% cellulose after 30d. Activity increased throughout growth on all cellulose concentrations.

β-Glucosidase production by the community (Fig. 29c) was similar to that of P. simplicissimum on all substrate concentrations.

7 **pH OF THE BUFFERED MEDIUM**

Figure 30 shows the change in the pH of the medium during the growth of A. fumigatus, P. simplicissimum and the community on 0.5-5% cellulose. In general the pH dropped during the first 10d, remaining constant or increasing thereafter. With all three cultures, as the cellulose concentration increased the drop in the pH of the medium increased such that on 0.5% cellulose the pH dropped to 5.8, whereas on 5% cellulose the pH dropped to between 4.2 and 4.8. In all cases the pH of the medium did not drop below 4.0.

DISCUSSION

Results from this study indicated that the community did not produce higher levels of cellulase activity than A. fumigatus and P.simplicissimum grown in axenic culture. It appears that under different cultural conditions (i.e. cellulose concentration and growth medium pH) synergism was lacking between members of the community in the degradation of cellulose.

It is apparent from this work that cultural conditions have a strong

influence on cellulase activity. Several observations which were made are discussed below:

(a) β-Glucosidase production was greatly increased using a buffered medium and increasing the cellulose concentration to 1 or 2% (w/v). Negligible levels of β-glucosidase activity detected in the unbuffered medium were presumably a result of pH inactivation since the pH of the growth medium dropped to between pH 2 and 2.7. Using the buffered medium, however, conditions in the flasks were less acidic, the pH dropping to between 2.9 and 4.9. This may have accounted for the higher levels of β-glucosidase activity detected in the buffered medium since it is likely that β-glucosidase inactivation did not occur to the same extent as in the unbuffered medium.

Increasing the cellulose concentration above 2% (w/v) in the buffered medium resulted in lower levels of β-glucosidase activity. Since the growth medium appeared to have a weak buffering capacity and acidic conditions (pH 2.9-3.2) developed in those cultures grown on 5% cellulose, pH inactivation presumably accounted for the reduced levels of activity.

Using a buffered medium with an increase in molarity high levels of β-glucosidase were obtained on 5% cellulose. In this case, the pH of the medium did not drop below 4.0, so presumably β-glucosidase inactivation was prevented.

In agreement with this work, Sternberg (1976b) demonstrated that β-glucosidase production by Trichoderma reesei was increased using a buffered medium and increasing the cellulose concentration from 0.75 to 2% (w/v). Linko et al. (1977) similarly found that higher levels of β-glucosidase were obtained using a buffered medium in which the pH dropped to between 3.4 and 3.8 compared with an unbuffered medium where the pH dropped to between 2.6 and 2.9.

(b) In general, increased levels of cellulase and endoglucanase activity were obtained using a buffered medium and increasing the cellulose concentration. The exception to this was **cellulase** activity detected in cultures of A. fumigatus which showed a decrease with increasing cellulose concentration. It seems reasonable to suggest that higher levels of cellulase were produced in the buffered medium because pH inactivation was either prevented or reduced compared with that in the unbuffered medium. The initial pH of the buffered medium and its composition (increased phosphate) may have also been more favourable for growth and hence cellulase production was increased.

Increasing the cellulose concentration above 1% (w/v) in the buffered medium resulted in the production of lower levels of cellulase and endoglucanase activity by the community. This may be explained as follows. Firstly, repression of cellulase synthesis or end-product inhibition may have occurred. In support of this, cellulose degradation, as indicated by the rate of acid production (Mandels et al., 1975), was more rapid on the higher cellulose concentrations. This may have led to the accumulation of end-products resulting in cellulase inhibition or repression of cellulase synthesis. Secondly, the pH of the medium became more acidic as the cellulose concentration increased and this may have resulted in cellulase inactivation. To determine whether pH inactivation was responsible for the reduced levels of cellulase activity, the fungi were grown in a medium with a stronger buffering capacity. Using this type of medium reduced levels of cellulase and endoglucanase were still produced on the highest cellulose concentrations. Since the pH of this buffered medium did not drop below 4, pH inactivation is unlikely to have accounted for the low levels of enzyme activity.

The results obtained from this study are in agreement with those reported by Sternberg (1976b). This author found that by controlling the

pH of the medium so that it did not drop below 3.5, and increasing the cellulose concentration from 0.75 to 2% (w/v) enzymes able to hydrolyze filter paper and CMC were greatly increased.

(c) Increasing the cellulose concentration in the unbuffered medium resulted in a decrease in **cellulase** activity with all three cultures. This is consistent with the observations made by Saddler and Khan (1980) with cultures of Acetivibrio cellulolyticus. Low levels of cellulase (exoglucanase) activity were also obtained on the higher cellulose concentrations in cultures of Sporotrichum thermophile (Coutts & Smith, 1976). These authors postulated that at low cellulose concentrations free enzyme accumulated in the medium after all the binding sites were saturated. With high cellulose concentrations, however, all the cellulase adsorbed to the cellulose such that no free enzyme remained. It was suggested that the cellulases were bound tightly to the cellulose since they could neither be removed by prolonged washing of the cellulose nor by disruption in a Waring blender at high speeds. Saddler and Khan (1980) demonstrated that despite the lower levels of cellulase activity on the highest cellulose concentration (7.5% w/v) twice as much cellulose was degraded at this concentration compared with that at a lower concentration of 0.1% (w/v). This provides further support for cellulase adsorption at the higher cellulose concentrations.

An additional reason to explain the low levels of enzyme activity on the higher cellulose concentrations was given by Coutts and Smith (1976). It was suggested that with the high cellulose concentrations, end-products were generated at a fast rate and their accumulation led to cellulase repression.

With the present study, it is possible that, in addition to enzyme adsorption and repression, pH inactivation accounted for the low levels of

activity with the high cellulose concentrations. As the cellulose concentration increased, a greater drop was observed in the pH of the medium with very acidic conditions (pH2) developing in cultures growing on 5% (w/v) cellulose.

(d) In the unbuffered medium endoglucanase activity unlike **cellulase** activity, was not affected by the concentration of cellulose. Coutts and Smith (1976) found that cellulose concentration had a more dramatic effect on cellulase (exoglucanase) than endoglucanase activity. At levels of 2% (w/v) cellulose or higher, **cellulase** activity was almost undetected whilst endoglucanase showed only a moderate decline in activity.

In summary:

1. There was no apparent synergism between members of the community during the degradation of cellulose under different cultural conditions.

2. Cellulose concentration and pH had a strong influence on cellulase activity.

(i) Higher levels of β-glucosidase activity were obtained in cultures grown in a buffered medium compared with an unbuffered medium. With increasing cellulose concentration (up to 2%) in the buffered medium there was an increase in β-glucosidase activity. Higher levels of activity were obtained on 5% cellulose using a buffered medium with increased molarity.

(ii) Higher levels of cellulase were also obtained using a buffered medium and increasing the cellulose concentration.

(iii) Increasing the cellulose concentration in the unbuffered medium resulted in a direct decrease in **cellulase** activity whilst endoglucanase and β-glucosidase activity remained unaffected.

CHAPTER SIX

PROPERTIES OF A MICROBIAL COMMUNITY DEGRADING LIGNOCELLULOSE

CHAPTER SIX

PROPERTIES OF A MICROBIAL COMMUNITY DEGRADING LIGNOCELLULOSE

INTRODUCTION

For the complete degradation of lignocellulosic material a wide variety of enzymes is required. These enzymes include, in addition to the enzymes forming the cellulase complex, several types of hemicellulases and ligninases. A microbial community, consisting of several microorganisms with different metabolic activities, may provide all the enzymes necessary for complete hydrolysis. The enzymes produced by the community could act synergistically to increase the rate of lignocellulose degradation compared with that achieved by single cultures. This contention is supported by the observation made by Mishra *et al*. (1981) that mixed cultures containing two or three fungi increased the loss in weight of lignocellulose compared with that by one fungus alone.

The purpose of the present work was to determine whether the fungal community isolated could degrade lignocellulose at a faster rate than its individual members.

Since xylan constitutes a large proportion of hemicellulose in plants, xylanase activity was measured in addition to cellulase activity in cultures growing on lignocellulose.

RESULTS

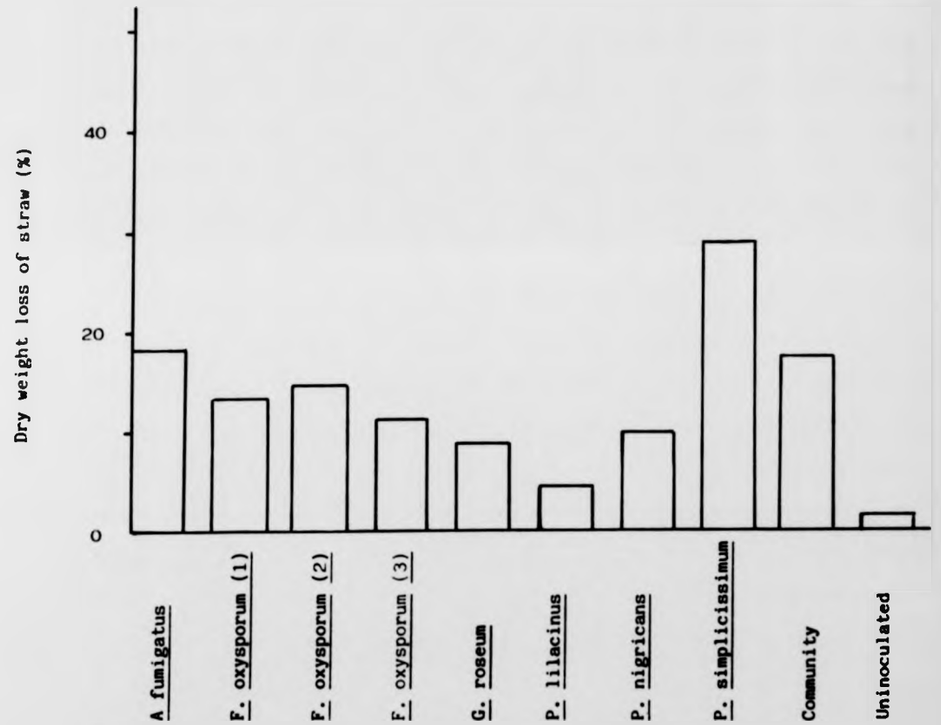
1. Growth of a microbial community and its members on straw

i. Loss in dry weight of straw

Figure 31 shows the loss in dry weight of straw after incubation with each fungal species and the community for 15d. The greatest loss in dry weight (29%) was achieved by *P. simplicissimum*, and with the exception of *P. lilacinus*, all of the other fungi showed weight losses ranging from 9 to 18%. *P. lilacinus* appeared to be incapable of degrading straw to any

Fig. 31

Loss in dry weight of straw following growth of the community and its members for 15d.



significant extent (4%) and the uninoculated flasks showed a weight loss of 1.2%.

ii. Enzyme production

Extracellular cellulase and xylanase produced by the fungi after 15d are shown in Fig. 32. **Cellulase** activity (0.89-1.93 EU) was detected in cultures of A. fumigatus and P. simplicissimum and the community (Fig. 32a), activity produced by the community being half that produced by the other two fungi.

Endoglucanase activity (Fig. 32b) was produced by all of the cultures, with the exception of P. lilacinus. Highest endoglucanase levels (2.5 EU) were found with A. fumigatus and P. simplicissimum, the other cultures producing only very low levels of activity (< 0.1 EU). No detectable levels of endoglucanase activity were found in cultures of P.lilacinus after 15d.

β-Glucosidase (Fig. 32c) was produced by all of the fungi, with the exception of P. lilacinus. Highest levels of activity (7.8-13.5 EU) were produced by the three F. oxysporum strains and the community. With the other fungi, β-glucosidase activity varied from between 2.8 and 5.0 EU.

Xylanase was the only enzyme of those studied to be produced by all of the cultures, highest levels of activity being detected in cultures of A. fumigatus (15 EU) and P. simplicissimum (17.3 EU). All of the other cultures, with the exception of P. nigricans (3.2 EU) and the community (6 EU), produced very low levels of activity of <0.8 EU (Fig. 32d).

iii. pH of growth medium

Figure 33 shows the change in medium pH in cultures grown on 0.5% (w/v) straw. In general, the pH dropped during the initial stages of growth (4d) and either increased or remained constant thereafter, the greatest initial drop (from 5.2 to 3.35) being produced by A. fumigatus. The exceptions to this were G. roseum, P. lilacinus and P. nigricans, all

Fig. 32

Cellulase and xylanase activity in cultures of the community and its members grown on 0.5% (w/v) straw for 15d.

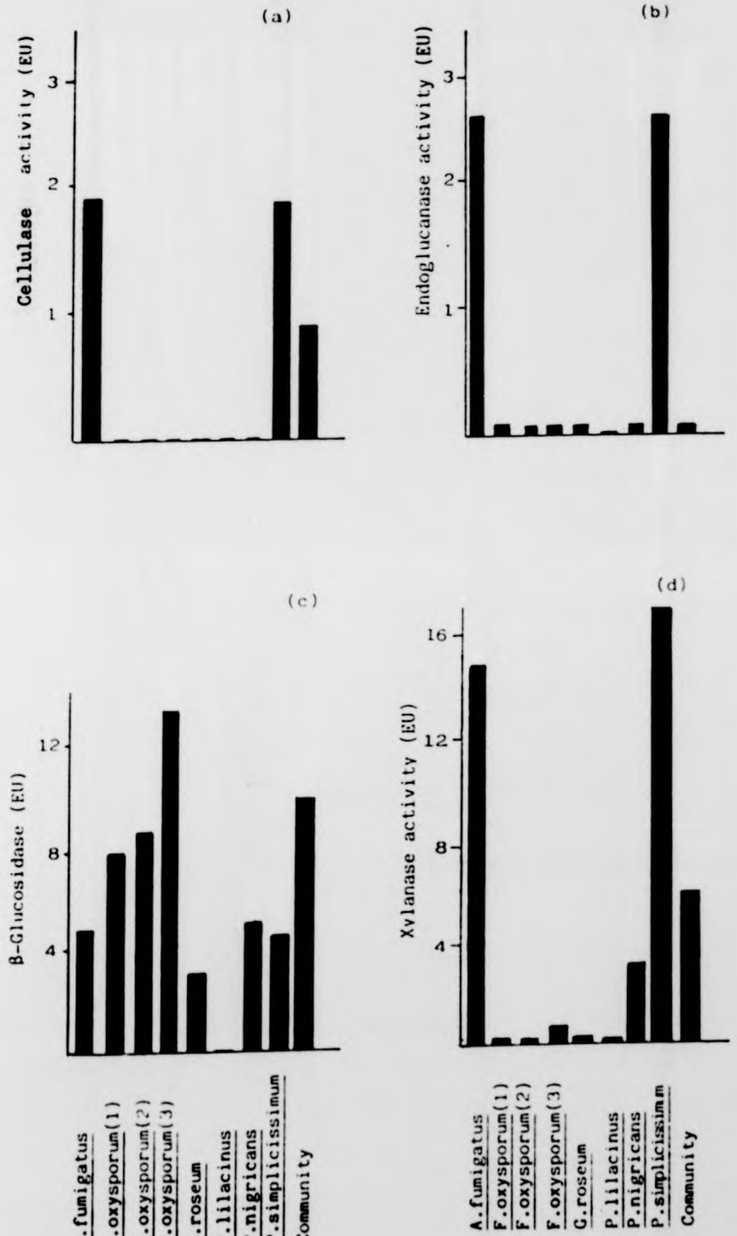


Fig. 33

Changes in the pH of the medium during growth of the community and its members on 0.5% (w/v) straw.

(■—■) Community

(▲—▲) A. fumigatus

(△—△) F. oxysporum (strain 1)

(□- -□) F. oxysporum (strain 2)

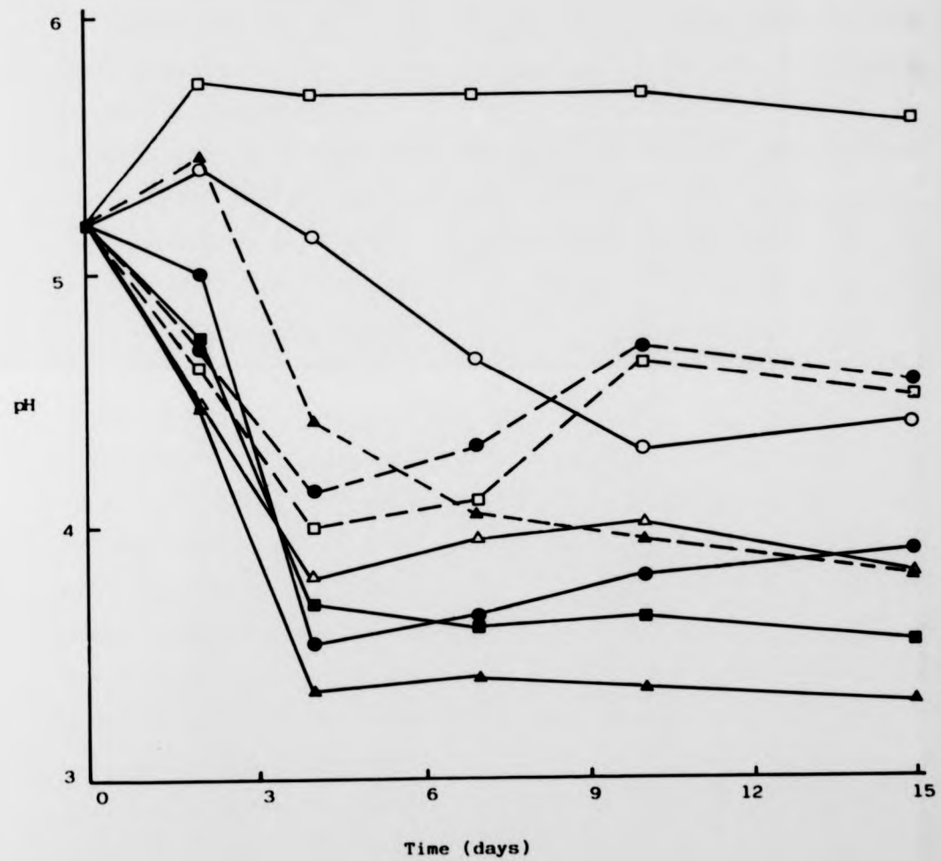
(●- -●) F. oxysporum (strain 3)

(▲- -▲) G. roseum

(□—□) P. lilacinus

(○—○) P. nigricans

(●—●) P. simplicissimum



three cultures showing an initial rise in pH. Although in P. lilacinus the pH remained constant after the initial rise, the other two fungi then showed a steady drop in pH until 15d.

2. **Growth of a microbial community and its members on hay.**

i. Loss in dry weight of hay

Figure 34 shows the loss in dry weight of hay following growth of the fungal species and the community on the substrate for 15d. A. fumigatus (23.5%), P. simplicissimum (32.5%) and the community (28.5%) produced the greatest dry weight losses, the other fungi causing weight losses between 14 and 19.5%. Uninoculated flasks indicated a loss in dry weight of 4.5%, this possibly being due to the leakage of water soluble materials from the hay into the medium.

ii. Enzyme production

Cellulase and xylanase activity (Fig. 35) were measured in those flasks showing the highest percentage loss in dry weight of hay (A.fumigatus, P. simplicissimum and the community). In general, enzyme levels produced by the community were 3-7 fold less than those produced by the other two fungi, with the exception of β-glucosidase activity which was 2-fold greater. A. fumigatus and P. simplicissimum produced similar levels of β-glucosidase and xylanase, but differences were observed in levels of cellulase and endoglucanase, levels produced by A. fumigatus being almost 2-fold greater than those of P. simplicissimum.

iii. pH of growth medium

Figure 36 shows the change in the pH of the medium during growth of each of the fungal species and the community on hay for 15d. With the exception of P. lilacinus, all cultures showed an initial drop in the pH of the medium, with the pH remaining constant or increasing thereafter. The greatest drop from 4.8 to between 3.4 and 3.7, in cultures of A.fumigatus, P. simplicissimum and the community, occurred after 4d. With

Fig. 34

Loss in dry weight of hay following growth of the community and its members for 15d.

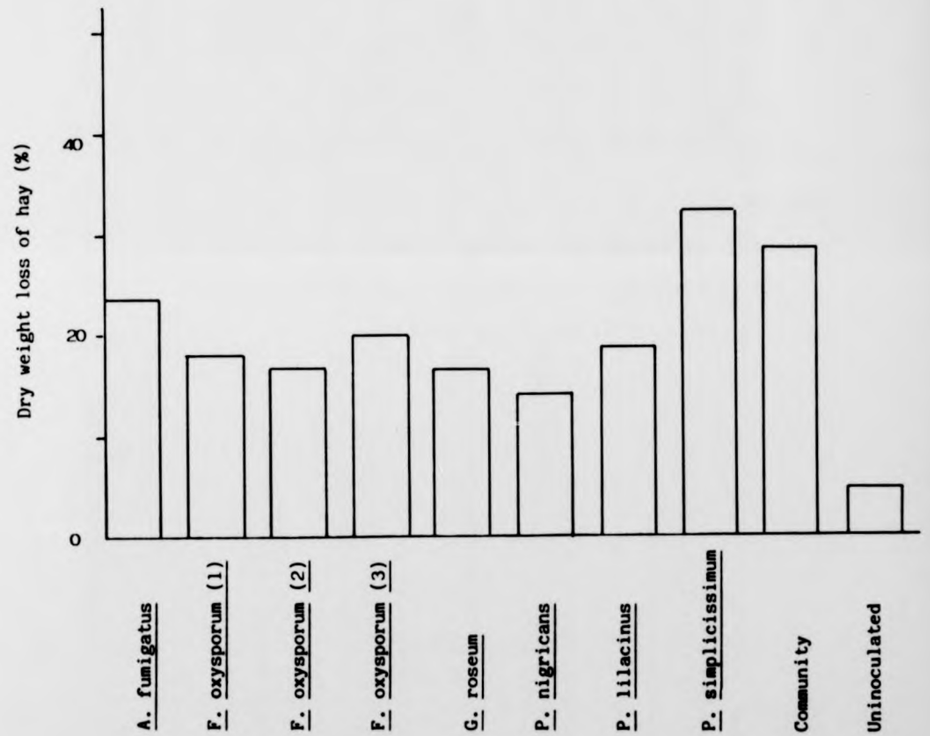
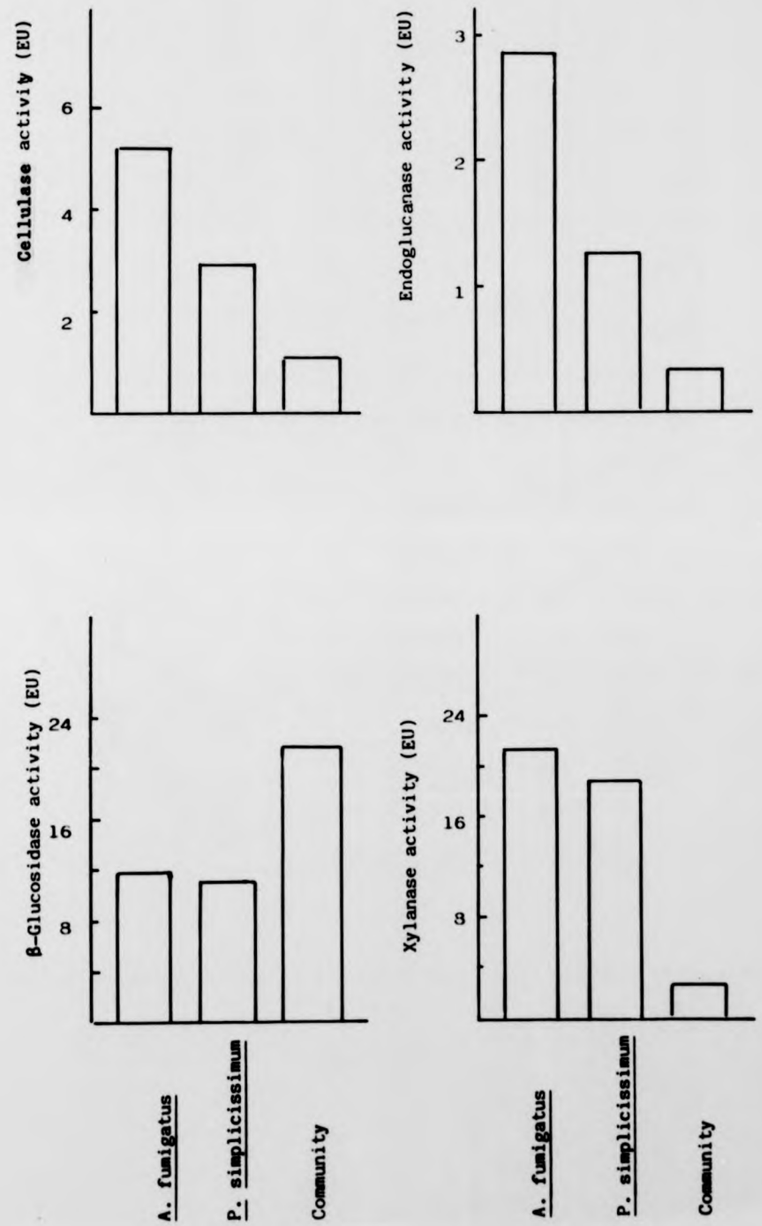


Fig. 35

Cellulase and xylanase activity in cultures of A. fumigatus, P. simplicissimum and the community grown on 0.5% (w/v) hay for 15d.



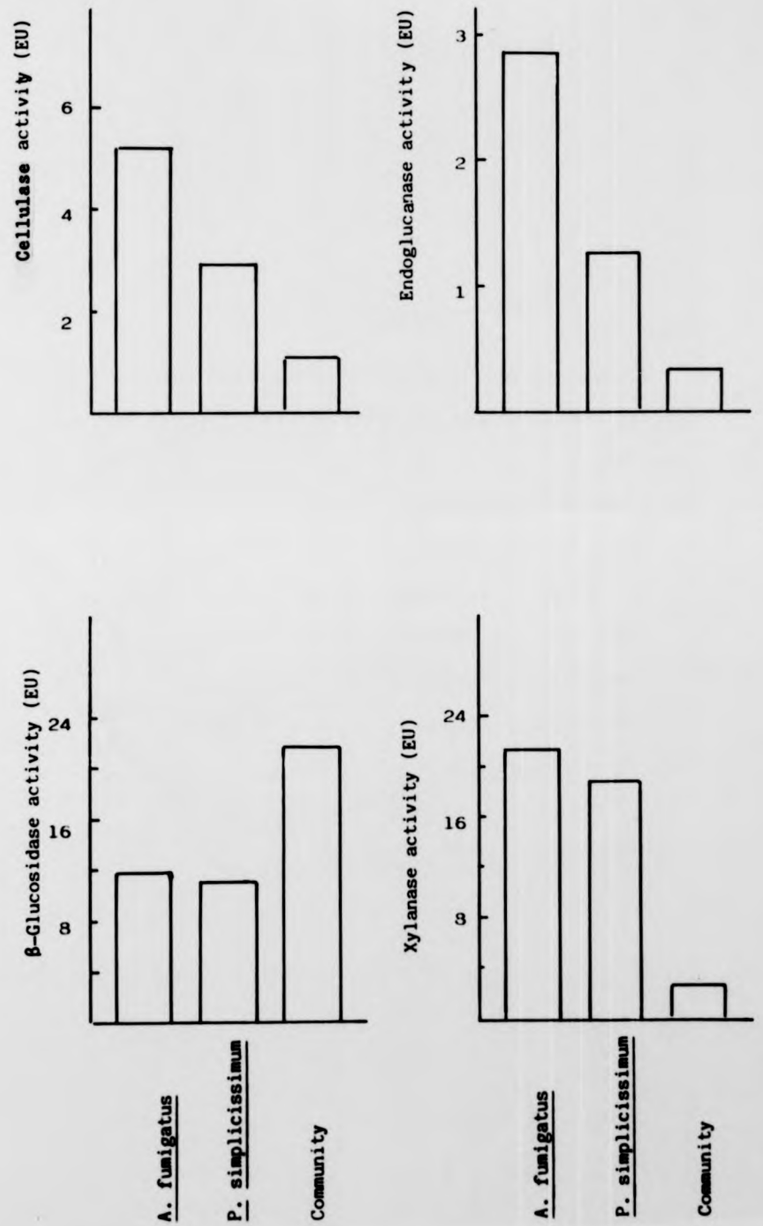


Fig. 35

Cellulase and xylanase activity in cultures of A. fumigatus, P. simplicissimum and the community grown on 0.5% (w/v) hay for 15d.

Fig. 36

Changes in the pH of the medium during the growth of the community and its members on 0.5% (w/v) hay for 15d.

(■—■) community

(▲—▲) A. fumigatus

(△—△) F. oxysporum (strain 1)

(□---□) F. oxysporum (strain 2)

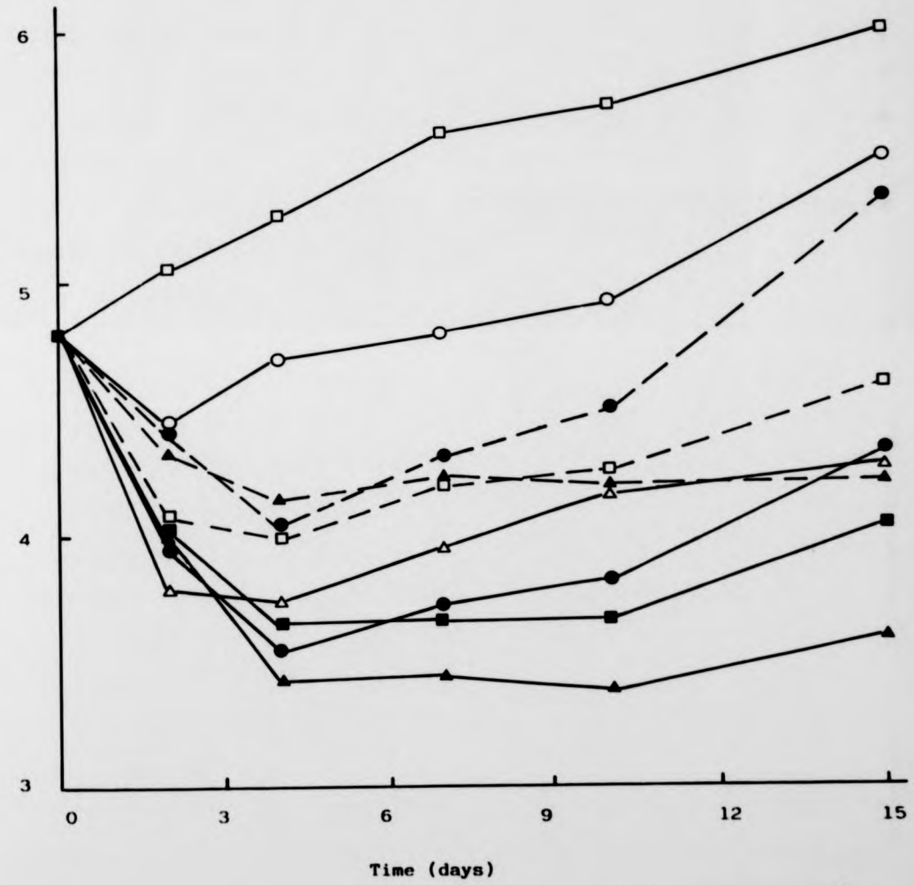
(●---●) F. oxysporum (strain 3)

(▲---▲) G. roseum

(□—□) P. lilacinus

(○—○) P. nigricans

(●—●) P. simplicissimum



cultures of P. lilacinus, the pH of the medium increased throughout growth to pH6 after 15d.

Results from these preliminary experiments indicated that after 15d the highest enzyme levels and greatest loss in dry weight of hay and straw were achieved by A. fumigatus, P. simplicissimum and the community. Studies using these three cultures were extended to determine whether there was any significant difference between their levels of enzyme activity over a period of 60d. In addition, the rate and extent of lignocellulose degradation by these two fungi and the community were compared.

3. **A comparison of lignocellulose degradation by a microbial community and two of its members in axenic culture.**

i. Loss in dry weight

Figure 37 shows the loss in dry weight of hay and straw after inoculation with A. fumigatus, P. simplicissimum and the community and incubation for 60d. Results indicated that hay was degraded at a similar rate by the three cultures, with 39-43% degradation after 60d. The rate of straw degradation by the three cultures was similar over the 60d incubation period, with a maximum of 32-36% degradation. With both substrates, the degradation rate was rapid in the initial stages of growth but decreased thereafter. Within the first 10d of growth, 50% of the final loss in dry weight had occurred.

ii. Enzyme production

Cellulase

Figure 38a shows **cellulase** activity detected in cultures of A.fumigatus, P. simplicissimum and the community grown on straw. A.fumigatus and P. simplicissimum gave similar patterns of activity, although levels produced by P. simplicissimum were higher than those of A.fumigatus, 40% higher during the initial stages. With both fungi, after

Fig. 37

Loss in dry weight of (a) straw and (b) hay in cultures of A. fumigatus (▲——▲), P. simplicissimum (●——●), and the community (■——■).

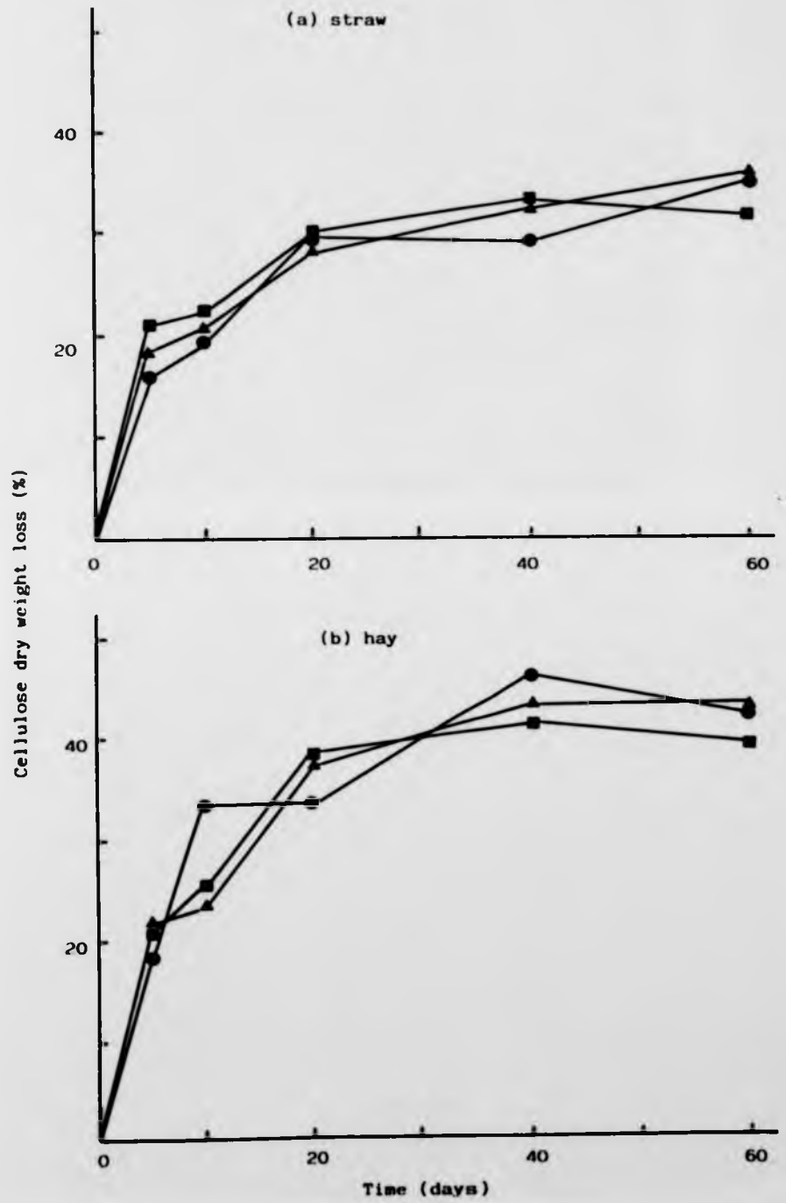
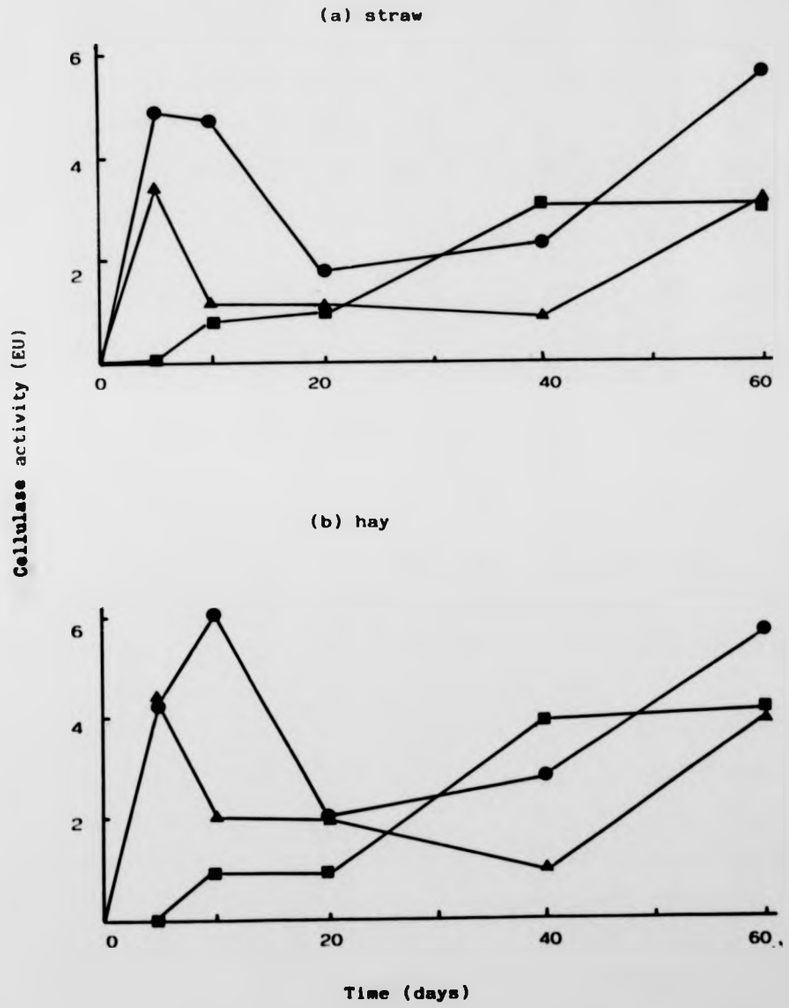


Fig. 38

A comparison of **cellulase** activity in cultures of A. fumigatus (▲——▲), P. simplicissimum (●——●) and the community (■——■) grown on (a) 0.5% (w/v) straw and (b) 0.5% (w/v) hay.



the initial rapid increase in activity (5d), a 3-fold decrease was observed, followed by a further rapid increase (between days40 and 60).

In contrast to these two fungi the community did not produce **cellulase** activity until day 10, levels increasing to day 40 and remaining constant thereafter. After 60d, the level of activity produced by the community was similar to that of A. fumigatus and almost 2-fold less than that of P. simplicissimum.

The production of **cellulase** in cultures growing on hay (Fig. 38b) followed a similar pattern to that observed on straw and, in general, levels were approximately 25% greater.

Endoglucanase

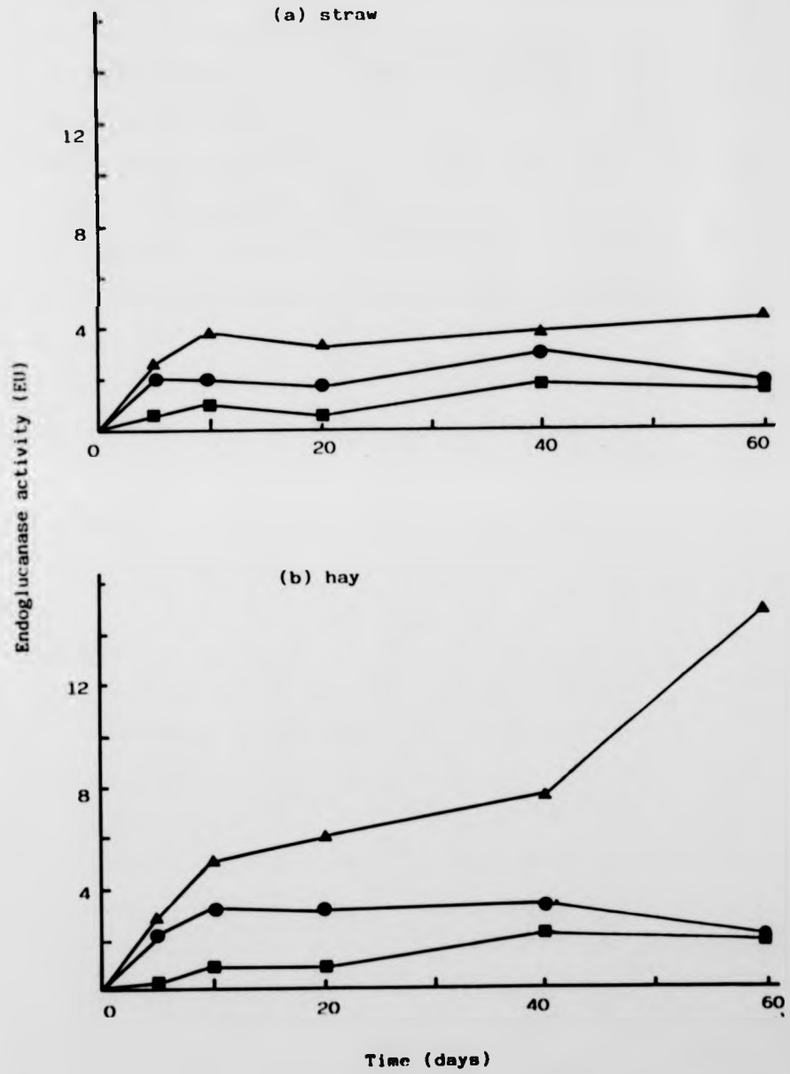
Endoglucanase production by the fungi growing on straw (Fig. 39a) followed similar patterns, activity increasing during the initial stages of growth (5-10d) and generally remaining constant thereafter. Highest enzyme levels produced by A. fumigatus were approximately 2-fold greater than those detected in cultures of P. simplicissimum and the community after 60d.

Growing on hay (Fig. 39b) endoglucanase production by A. fumigatus was considerably greater than that by P. simplicissimum and the community, activity being more than 7-fold greater after 60d. A. fumigatus showed an increase in endoglucanase activity throughout the 60d growth period, with a particularly rapid increase occurring in the latter stages. In contrast, P. simplicissimum showed an increase in activity until day 10, the activity remaining constant until day 40 and decreasing thereafter. The community produced the lowest level of endoglucanase activity throughout the growth period and showed a slow increase in activity.

A comparison of levels of endoglucanase activity produced on hay and straw indicated that P. simplicissimum and the community produced similar levels on the two substrates. With A. fumigatus, however, endoglucanase

Fig. 39

A comparison of endoglucanase activity in cultures of A. fumigatus (▲——▲), P. simplicissimum (●——●) and the community (■——■) grown on (a) 0.5% (w/v) straw and (b) 0.5% (w/v) hay.



levels on hay were higher than those on straw, approximately 3-fold greater after 60d.

β-Glucosidase

Figure 40a shows β-glucosidase activity detected in cultures grown on straw. Highest enzyme levels were produced by the community, activity being 3-fold greater than that produced by the other two cultures after 60d. β-Glucosidase production by the community increased throughout the growth period. P. simplicissimum showed an increase in activity to day 20, levels remaining constant therefore. A. fumigatus, on the other hand, produced fluctuating levels of β-glucosidase activity throughout the incubation period.

Cultures grown on hay (Fig. 40b) produced similar patterns of activity to those observed on straw, although levels of activity in general were higher. Lowest levels of activity were produced by P.simplicissimum. Activity produced by A. fumigatus fluctuated but overall showed an increase which was similar to that produced by the community.

Xylanase

Xylanase production by the three cultures grown on straw is given in Fig. 41a. Throughout the 60d growth period, the community produced very low levels of xylanase activity (0.02 EU). Enzyme levels produced by P. simplicissimum were slightly higher (0.06 EU) than those produced by the community. Highest levels were produced by A. fumigatus, particularly in the final stages, the activity increasing more than 10-fold such that after 60d levels were 12-fold and 37-fold greater than P. simplicissimum and the community, respectively.

Similar observations were made with cultures growing on hay (Fig.41b) although in general, levels were approximately 25% higher.

Fig. 40

A comparison of ß-glucosidase activity in cultures of A. fumigatus (▲——▲), P simplicissimum (●——●) and the community (■——■) grown on (a) 0.5% (w/v) straw and (b) 0.5% (w/v) hay.

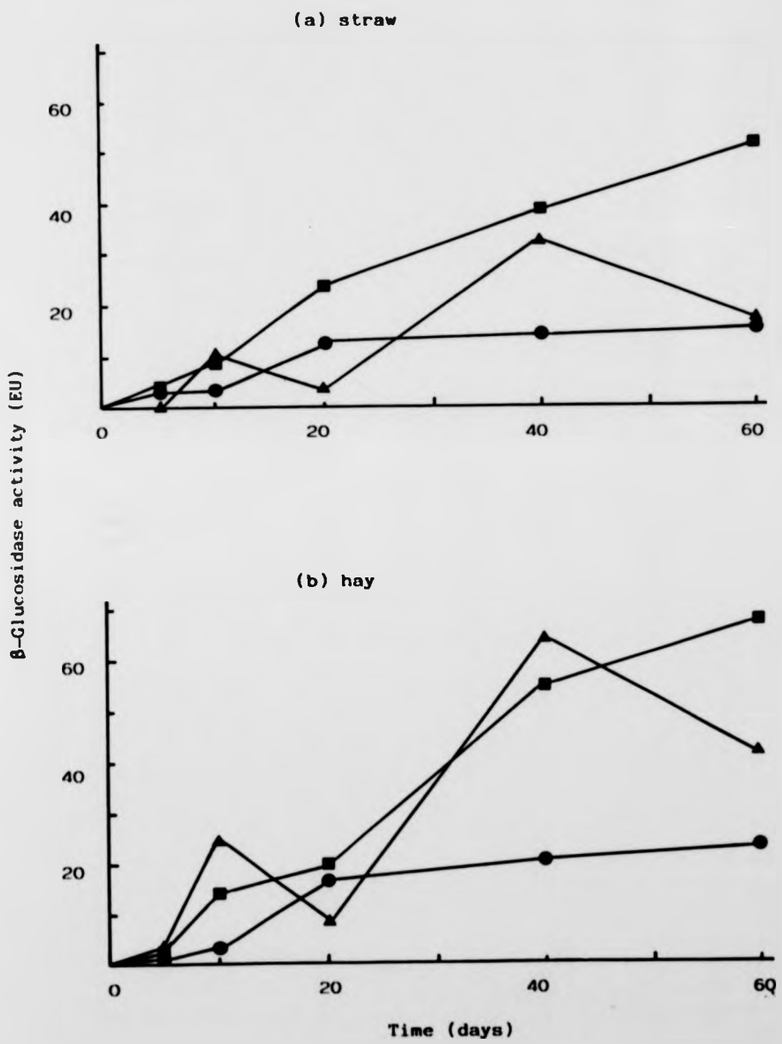
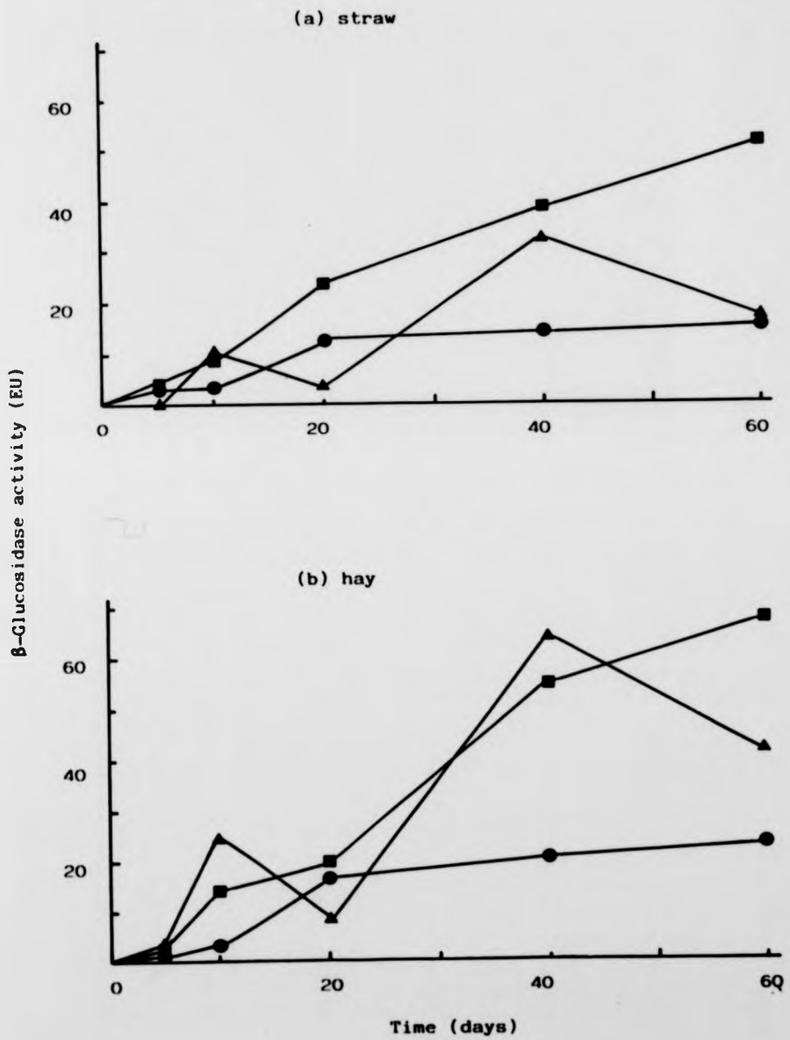


Fig. 40

A comparison of β-glucosidase activity in cultures of A. fumigatus (▲——▲), P simplicissimum (●——●) and the community (■——■) grown on (a) 0.5% (w/v) straw and (b) 0.5% (w/v) hay.



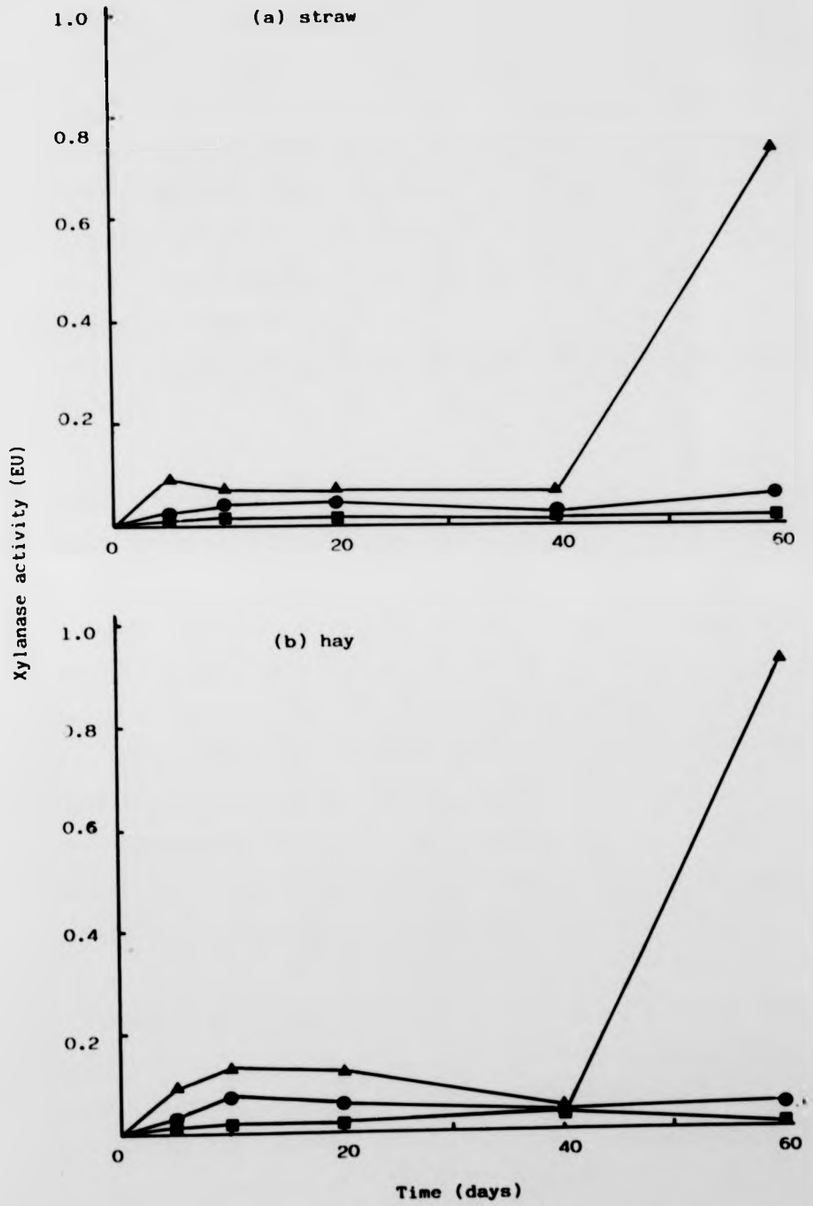


Fig. 41

A comparison of xylanase activity in cultures of *A. fumigatus* (▲——▲), *P. simplicissimum* (●——●) and the community (■——■) grown on (a) 0.5% (w/v) straw and (b) 0.5% (w/v) hay.

iii. pH of growth medium

Changes in the pH of the medium observed in cultures growing on straw are shown in Fig. 42a. In general, the cultures showed a drop in medium pH from 5.2 to between 3.3 and 3.6 in the initial stages of growth (5d), the pH increasing thereafter. The exception to this was A.fumigatus, the pH increasing after the initial drop, then decreasing further to 2.55 before the final increase. In general, the greatest drop in medium pH was observed in A. fumigatus cultures and the smallest drop in P.simplicissimum cultures.

Similar observations were made with the three cultures growing on hay (Fig. 42b).

These results indicated that although the three cultures produced drastically different levels of cellulase and xylanase activity, they were able to degrade lignocellulose at a similar rate. Due to the multicomponent nature of the plant material, it was possible that the three cultures degraded the various components, (cellulose, hemicellulose and lignin) at different rates but the overall rate could still have been similar. It was therefore decided to examine the rate and extent of degradation of the chemical constituents of hay.

iv. Changes in the chemical composition of hay

Figure 43a shows the amount of hemicellulose in hay following inoculation with A. fumigatus, P. simplicissimum and the community. With all three cultures, values rapidly decreased (40-50%) during the initial stages of the incubation period (20d). The amount of hemicellulose in A.fumigatus cultures remained constant thereafter, P. simplicissimum and the community showing a further decrease of approximately 10% after 60d.

The cellulose content of the hay (Fig. 43b) decreased at a slower rate than hemicellulose, with a 50% loss after 40d. In general, the cellulose content decreased at a constant rate until day 40, remaining

Fig. 42

Changes in the pH of the medium during growth of A. fumigatus (▲——▲), P. simplicissimum (●——●) and the community (■——■) on (a) 0.5% (w/v) straw and (b) 0.5% (w/v) hay.

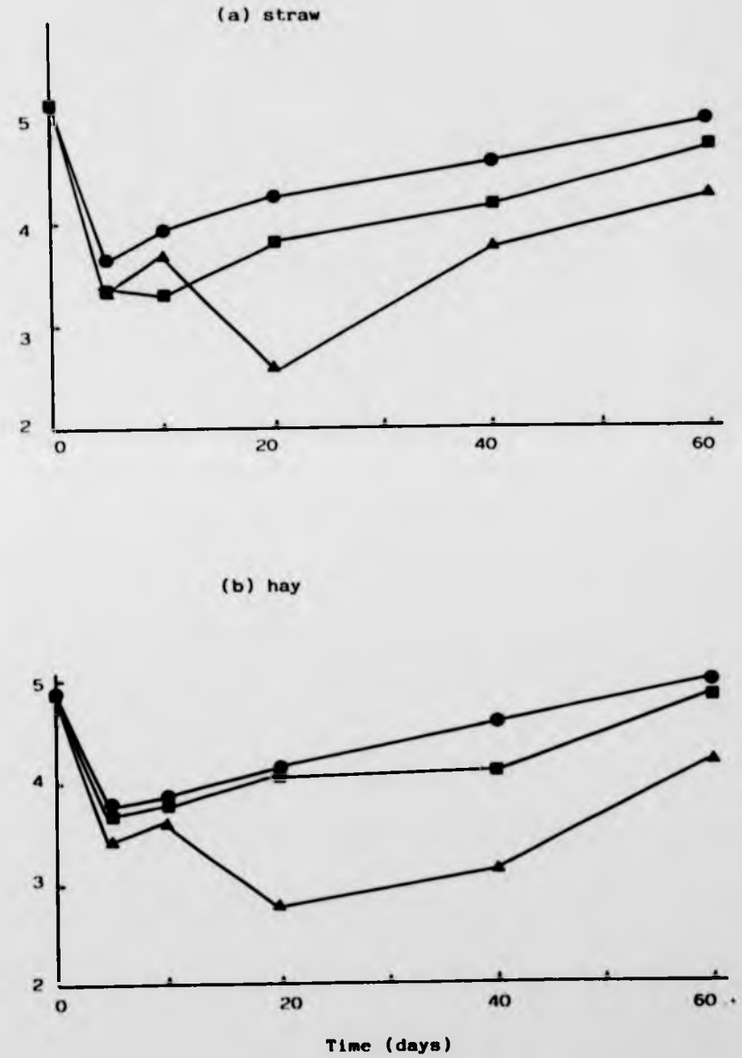
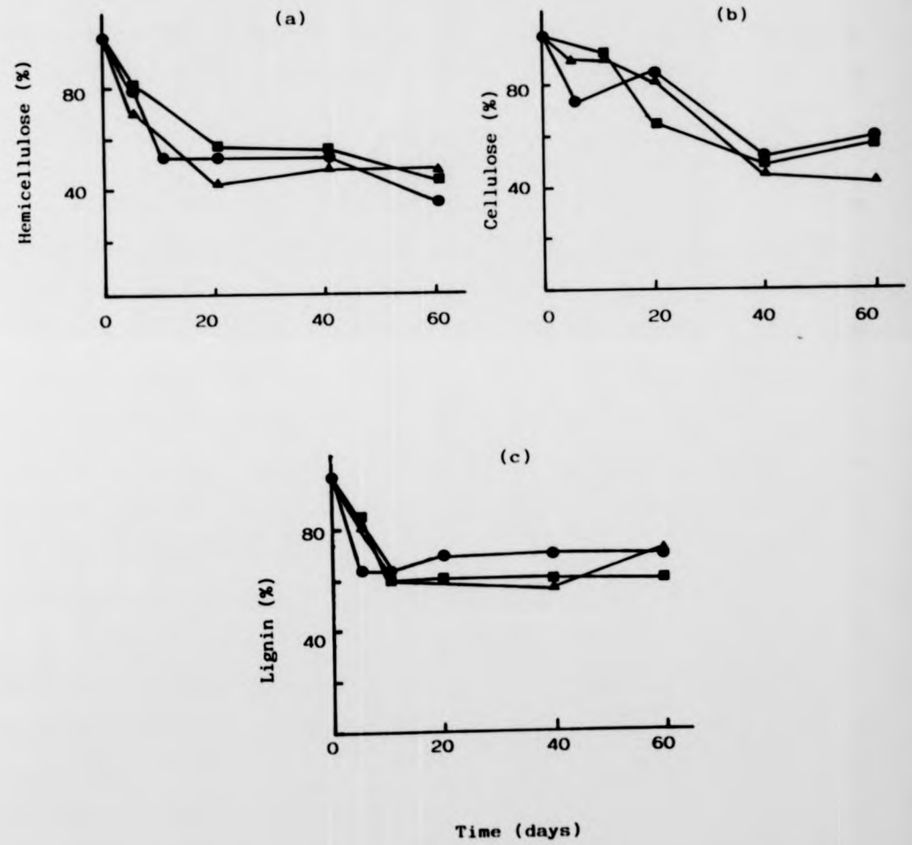


Fig. 43

Changes in the chemical composition of hay following growth of A. fumigatus (▲——▲), P. simplicissimum (●——●) and the community (■——■).



constant thereafter.

The lignin content of the hay (Fig. 43c) decreased during the initial incubation period (5-10d), with no further decrease occurring thereafter. Similar observations were made with all three cultures. Decreases in the lignin content of hay may have been a result of the modification of lignin structure by the microorganisms, producing low molecular weight fragments which were removed during the extraction procedure. On the other hand, actual lignin degradation may have occurred.

Discussion

Results from this study indicated that there was no obvious synergism between the members of the community in the breakdown of lignocellulosic material. With the exception of β-glucosidase, enzyme production by the community was lower than that by both A. fumigatus and P. simplicissimum growing in axenic culture. As discussed in Chapter 3, the growth of the primary degraders in the community may have been reduced compared with that in axenic culture due to the removal of breakdown products by other members, and hence cellulase and xylanase production was reduced. Antagonistic interactions between the members of the community could also have reduced the growth of the primary degraders.

Levels of β-glucosidase produced by the community were higher than those produced by P. simplicissimum and in some instances were higher than those produced by A. fumigatus growing in axenic culture. This situation could arise if organisms other than the primary cellulose degraders were the major β-glucosidase producers.

Despite the community producing reduced enzyme levels, lignocellulose was degraded at a similar rate to that achieved by A.fumigatus and P. simplicissimum. Kapoor et al. (1982) similarly found that the rate of degradation of wheat straw by a mixed culture of fungi

was equal to that by one fungus alone.

In the present study, the possibility existed that the degradation rates were maximal and that A. fumigatus and P. simplicissimum over-produced their enzymes. If the breakdown of cellulose and hemicellulose was limited by their association with the more recalcitrant lignin component, this would also tend to equalise the degradation rates in the mixed and axenic cultures.

Considerable differences were observed in levels of cellulase and xylanase produced by A. fumigatus and P. simplicissimum growing on both hay and straw in axenic culture. Since cellulases have been found to be intracellular, cell-bound and extracellular, it is possible that the enzymes produced by these two fungi were located at different sites, rather than being totally extracellular. A. fumigatus and P.simplicissimum may also differ in the extent to which they attack the various chemical constituents of hay and straw, resulting in the synthesis of different enzyme levels. Since cellulases act synergistically with other cellulase components (Wood & McCrae, 1975) and with hemicellulases (Ghose & Bisaria, 1979), the overall activity of the cellulase complex may be more important than the activity of individual enzymes.

Measurements of cellulase and xylanase activity revealed that in some instances there was a decrease in enzyme activity. This observation can be accounted for if (a) the cellulases adsorbed to the substrate. Evidence for the adsorption of cellulases on lignocellulosic material is provided by the work of Ghose and Bisaria (1979), and Goel and Ramachandran (1983). Using rice straw, Goel and Ramachandran (1983) found that nearly 55% of the activity towards filter paper, 50% of the activity towards CM-cellulose and 60% of the β-glucosidase activity was adsorbed. Ghose and Bisaria (1979) similarly reported cellulase and endoglucanase activity adsorbed to lignocellulosic substrates but they suggested

ß-glucosidase was not adsorbed.

(b) the cellulases were inactivated due to the acidic conditions in the medium. In cultures of A. fumigatus the pH of the medium dropped to 2.6 and 2.8 on straw and hay, respectively, and this may have been responsible for the decrease observed in ß-glucosidase activity.

(c) reducing sugars accumulated in the medium and inhibited the cellulases, or proteolytic enzymes were produced as discussed in Chapter 3.

There is evidence from the present work to suggest that the xylanase produced by A. fumigatus was predominantly cell-bound and released into the medium in the latter stages of growth, probably as a result of cell lysis. This is indicated by the rapid increase in xylanase activity recorded in A. fumigatus cultures in the final growth stages. In support of this, Berg and Pettersson (1977) found that extracellular cellulase activity in cultures of Trichoderma reesi (formerly T. viride) rapidly increased in the latter stages of growth and suggested that this was due to the release of cell-bound cellulases following cell lysis.

Less dramatic increases in cellulase and endoglucanase activity were observed in cultures during the final stages of growth. Adsorbed cellulases released into the medium as the substrate was hydrolysed may have been unable to readsorb if binding sites were unavailable, and hence extracellular levels of enzyme increased. In the final stages of growth it is unlikely that reducing sugars accumulated in the medium since the rate of lignocellulose degradation slowed down as the more recalcitrant molecules were encountered. Consequently, end-product inhibition of cellulase and xylanase activity may have been removed.

Several methods are available to determine the chemical composition of lignocellulosic material, all of which involve a sequential extraction of the various components: soluble material, cellulose, hemicellulose and

lignin. The major difference between the methods is that either the hemicellulose and lignin components are extracted to leave the cellulose, or the hemicellulose and cellulose are extracted so that the lignin remains. For example, the method of van Soest and Wine (1968) involves treating lignocellulosic material with an acid detergent to remove the water soluble substrate, fats and hemicellulose. The residual material is then treated with acetic acid-buffered potassium permanganate to oxidise and solubilise the lignin. Alcoholic solutions of oxalic and hydrochloric acids are used to wash the lignin to remove manganese and iron oxides. After washing, the residual material is cellulose.

With the second method, the hemicellulose is solubilised with sodium hydroxide and the remaining material treated with cadoxen (Tris (ethylenediamine) cadmium hydroxide) or sulphuric acid to extract the cellulose (Tsao et al., 1978). Lignin is the insoluble material which remains.

Studies of the changes in the chemical composition of hay, following inoculation with A. fumigatus, P. simplicissimum and the community indicated that the three cultures degraded hemicellulose and cellulose. There was no apparent difference between the cultures in the rate and extent of degradation of the components. Although it appeared that the fungi degraded or modified the structure of lignin, a more detailed study using ^{14}C-labelled lignocellulose is necessary to determine if the fungi are lignin degraders.

In summary:

1. Results indicated that there was no obvious synergism between members of the community in the breakdown of lignocellulose; rates of degradation by A. fumigatus, P. simplicissimum and the community were similar.

2. With the exception of β-glucosidase, enzyme production by the community was less than that of A. fumigatus and P. simplicissimum grown in axenic culture. This situation could arise if:

(a) growth of the primary cellulose degraders in the community was reduced as a result of competition for available nutrients or antagonism between members.

(b) microorganisms other than the primary cellulose degraders were the major β-glucosidase producers in the community.

3. Levels of cellulase and xylanase activity were discussed in relation to the rate of lignocellulose hydrolysis.

4. There was no apparent difference between the three cultures in the degradation of the chemical constituents of hay.

CHAPTER SEVEN

GENERAL DISCUSSION

CHAPTER SEVEN

GENERAL DISCUSSION

1. CELLULOSE BREAKDOWN: A PURE OR MIXED CULTURE PROCESS?

In the present study it was expected that synergism would occur between different members of the community for the following reasons:

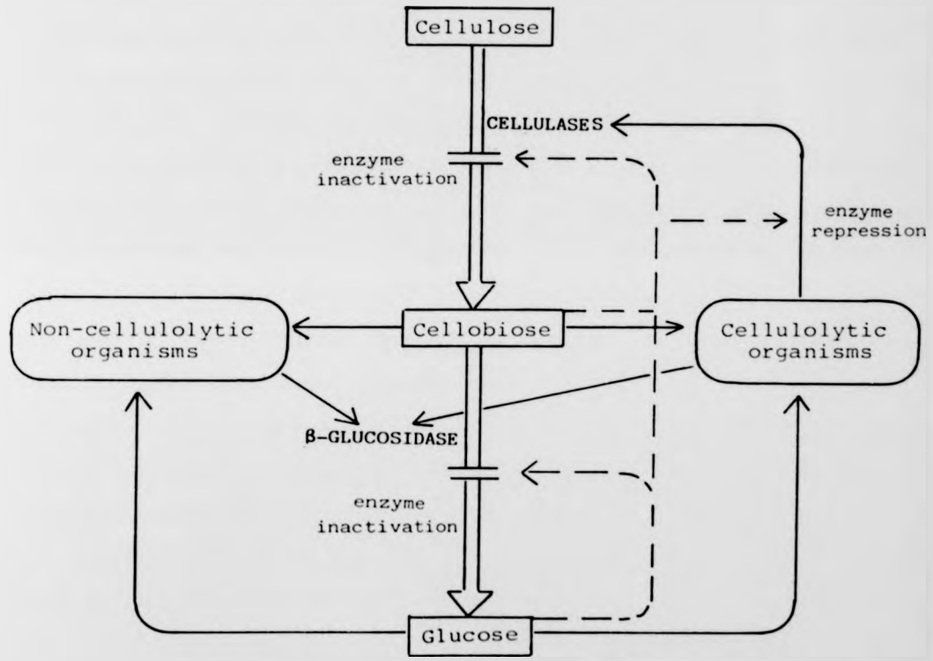
(a) Cellulose, being an insoluble substrate, is hydrolysed by extracellular cellulases to produce a number of intermediates available to other microorganisms. If glucose or cellobiose were taken up by other organisms this would prevent end-product inhibition of cellulases and thereby increase the rate of cellulolysis. Other organisms present in the community could also have a stimulatory effect by supplying the primary cellulose degraders with growth factors.

(b) Cellulose is a complex polymer requiring several enzymes for its breakdown but few organisms are able to produce the complete enzyme system and hydrolyse cellulose extensively. It therefore seemed likely that a microbial community consisting of members with different metabolic activities, would act cooperatively and hydrolyse cellulose at a faster rate than its members in pure culture.

A possible explanation for the lack of synergism between the organisms studied is that the community was an artefact composed of organisms fortuitously selected by the enrichment technique. The situation arising in the continuous enrichment system where cellulose was never substrate limiting, may have resulted in the accumulation of oligosaccharides which encouraged other non-helpful commensals to become established. The various organisms could have then persisted in the community, perhaps in a non-steady state, by way of complex interactions (Fig. 44). There is the possibility that competition occurred for available nutrients. In the culture system, whether batch or continuous, the non-cellulolytic organisms may have had an advantage, possibly having

Fig. 44

Possible interactions between microorganisms during cellulose degradation.



a better system for the uptake of glucose than the cellulolytic organisms such as A. fumigatus. As a result of the partial removal of the sugars by the non-cellulolytic organisms, the cellulolytic organisms would be inhibited, and subsequently would decline in population size. In turn, this would limit the population size of the non-cellulolytic organisms.

Interactions of this type could also lead to fluctuations in levels of enzyme activity. For example, if reducing sugars accumulated due to a decline in the non-cellulose degraders, this would result in inhibition of cellulase activity and/or repression of cellulase synthesis. This would then reduce the level of reducing sugars in the medium.

Another possible type of interaction that could have occurred between members of the community is antagonism. This can result from the production of extracellular primary and secondary metabolites by microorganisms affecting other organisms in the surrounding environment. An example of such a metabolite is the antibiotic griseofulvin, produced by several organisms including Penicillium species. Of the fungi isolated in this study, most have been reported to produce antibiotics and show antagonism towards other organisms (Table 13). Of particular interest are the reports that F. oxysporum can be antagonised by P. lilacinus and G.roseum (Domsch et al. 1980) and may also be inhibited by P. simplicissimum since Penicillium janthinellum (a very closely related species) shows antagonism towards F. oxysporum.

The lack of synergism within the community could also have been the result of a non-cooperating community becoming established, i.e. organisms did not have a stimulatory effect on other members. In this case, the concept that cellulose is degraded more efficiently in the natural environment by mixed cultures of microorganisms is not true. It is possible that in this system the rate limiting step, hydrolysing cellulose to cellobiose, was achieved by one cellulolytic organism such as

Table 13 Antagonistic activity of fungi (Domsch et al., 1980)

Fungus	Examples of organisms antagonised	Antagonistic metabolites produced
Aspergillus fumigatus	Gram-positive and Gram-negative bacteria	gliotoxin; ferricrocin; fumigacin
Fusarium oxysporum	various fungi	—
Gliocladium roseum	*Fusarium oxysporum*; *Bacillus subtilis*	—
Paecilomyces lilacinus	*Fusarium oxysporum*; various bacteria	lilacinin; leucinostatin
Penicillium nigricans	*Fusarium* spp.; *Pythium ultinum*; various bacteria	griseofulvin

P.simplicissimum, and was unaffected by other organisms in a positive way. If the other organisms had a positive effect, for example by removing end-products, this could have had a stimulatory effect on cellulase production.

2. THE POSSIBLE ROLE OF BACTERIA IN CELLULOSE DEGRADATION

It is possible that the exclusion of the non-cellulolytic bacteria from the reconstituted mixed culture used in this study affected the interactions between the individual members and subsequently altered the properties of the community. Bacteria similar to those found associated with this cellulolytic community (e.g. Pseudomonas) have been found to enhance cellulose degradation by cellulolytic organisms (Srinivasan & Han, 1969). These authors studied the growth kinetics of a cellulolytic Cellulomonas, a non-cellulolytic Pseudomonas, and a mixed culture, and found that the growth rate was increased when both organisms were present. Several explanations were given to account for the behaviour of the two organisms in the mixed culture. It was suggested that Cellulomonas hydrolysed cellulose to cellobiose which was then assimilated at a slow rate. This led to an accumulation of cellobiose in the medium inhibiting cellulase activity. Continuous removal of part of the cellobiose by Pseudomonas removed the limiting factor of the cellulolytic organism and thereby enhanced its growth. Pseudomonas could also have supplied Cellulomonas with a necessary growth factor. Further work is needed with the cellulolytic community isolated in the present study to determine the effect of including the non-cellulolytic bacteria in the community.

3. INFLUENCE OF PHYSICOCHEMICAL CONDITIONS AND THE BREAKDOWN OF LIGNOCELLULOSIC SUBSTRATES

The strong influence of pH on the levels of cellulase and endoglucanase observed in this work (Chapter 6), suggested that local variations in the natural environment could greatly affect the composition of the microbial

communities. Consequently, the overall rate of cellulose breakdown would be affected. A similar effect is also likely with changes in temperature and oxygen supply. In the latter case, the role of anaerobic or microaerophilic bacteria could be enhanced.

It is also likely that the variation in levels of β-glucosidase resulting from pH (Chapter 6) would influence the extent of extracellular production of free glucose and consequently the relative population sizes of the different members of the community.

Breakdown of lignocellulosic substrates (i.e. hay and straw) also indicated a lack of synergism between the members of the community. It seemed reasonable to expect the fungi to cooperate in the breakdown of lignocellulose because of the nature of this substrate together with the greater number of enzymes required for its breakdown. The results obtained with lignocellulose were not unlike those with pure cellulose and could simply reflect the 'loose' nature of the association of the organisms in the mixture.

4. FURTHER INVESTIGATIONS WITH MICROBIAL COMMUNITIES

Although the microbial community used in this study did not enhance cellulase production or cellulose breakdown, mixed cultures may prove useful in the future. Further investigations are required in the following areas:

(a) other enriched cellulolytic communities, with particular attention being paid to the non-cellulolytic organisms.

(b) combinations of organisms with different metabolic activities.

Several authors have demonstrated that mixed cultures (containing two or three organisms) can increase microbial biomass (Peitersen, 1975a), growth rates (Srinivasan & Han, 1969; Hofsten et al., 1971) and cellulose breakdown (Maki, 1954; Enebo, 1949). All reports suggested that the increase observed was due to the presence of

non-cellulolytic organisms. It is possible that microbial communities enriched from natural environments will improve cellulolysis still further.

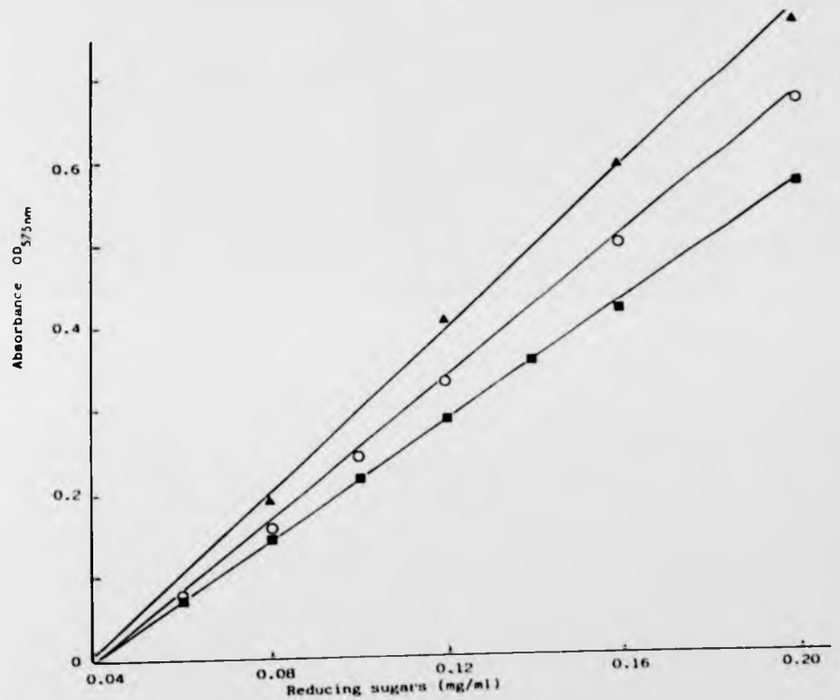
Mixed cultures containing microorganisms with different metabolic activities have also been shown to increase the rate of cellulolysis. Sternberg et al., (1976) found that cellulase from Trichoderma and β-glucosidase from Aspergillus acted synergistically to increase the rate of cellulose hydrolysis. More recently, Duff et al. (1985) demonstrated that the hydrolysis of lignocellulosic material was greatly improved using enzymes produced by a mixed culture of Trichoderma reesei and Aspergillus phoenicis. There is the possibility that lignocellulose hydrolysis would be increased if an efficient cellulose degrader was combined with good hemicellulose and lignin degraders. This seems reasonable to suggest since lignin and hemicellulose act as barriers to the degradation of cellulose, and their removal by organisms present in the mixed culture would probably increase the susceptibility of the cellulose to enzymatic attack. In support of this, Ghose and Bisaria (1979) demonstrated that hemicellulases, such as xylanases, acted synergistically with cellulases to increase the rate of cellulose degradation. A hemicellulolytic bacterium (Bacteroides ruminicola) has also been shown to increase cellulose breakdown by a cellulolytic bacterium as a result of a synergistic relationship (Dehority & Scott, 1967).

In conclusion, it is clear from my studies, however, that synergism and community cooperativity is not inevitable in cellulose degrading cultures.

A P P E N D I C E S

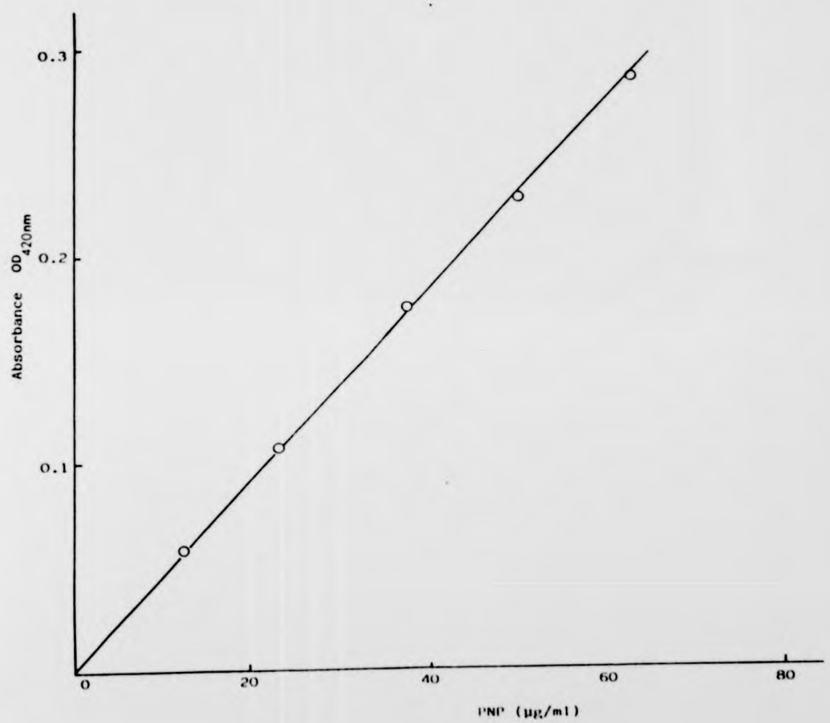
APPENDIX 1

Standard curve of reducing sugars using cellobiose (■—■), glucose (O—O), and xylose (▲—▲).



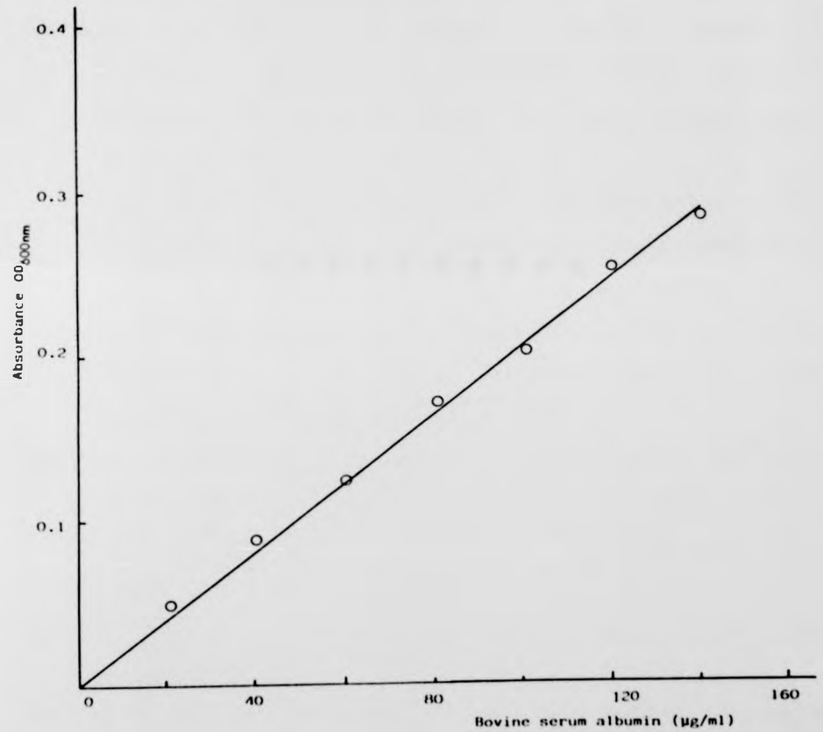
APPENDIX 2

Standard curve of p-nitrophenol.



APPENDIX 3

Standard curve of protein using bovine serum albumin.



REFERENCES

ALEXANDER, M. (1971). Microbial Ecology, John Wiley, London.

ALMIN, K.E. & ERIKSSON, K.-E. (1967). Enzymatic degradation of polymers. I. Viscometric method for the determination of enzymatic activity. Biochim. Biophys. Acta 139: 238-247.

ANDER, P. & ERIKSSON, K.-E. (1976). The importance of phenol oxidase activity in lignin degradation by the white-rot fungus Sporotrichum pulverulentum. Arch. Microbiol. 109: 1-8.

ANDREOTTI, R.E., MANDELS, M. & ROCHE, C. (1977). Effect of some fermentation variables on growth and cellulase production by Trichoderma QM9414. (T.K. Ghose, ed). Proc. Biocon. Symp. 11T, Delhi, p249-267.

BASU, S.N. (1948). Fungal decomposition of jute fibre and cellulose. Part 1 - A preliminary survey of commonly occurring species. Part 2 - The effect of some environment factors. J. Tex. Ind. 34: 1232-1248.

BAYLEY, S.T. & BISHOP, C.T. (1958). The role of non-glucose residues in determining the crystallinity of native cellulose. Biochim. Biophys. Acta 29: 209-211.

BELLEGSEN, O. & TONNENSEN, B.A. (1971). Cited by Bisaria, V.S. and Ghose, T.K. (1981). Biodegradation of cellulosic materials: substrates, microorganisms, enzymes and products. Enzyme Microb. Technol. 3: 90-104.

BERG, B. (1975). Cellulase location in Cellvibrio fulvus. Can. J. Microbiol. 21: 51-57.

BERG, B. & HOFSTEN, B. (1976). The ultrastructure of the fungus Trichoderma viride and investigation of its growth on cellulose. J. Appl. Bact. 41: 395-399.

BERG, B. & PETTERSSON, G. (1977). Location and formation of cellulases in Trichoderma viride. J. Appl. Bacteriol. 42: 65-75.

BINDER, A. & GHOSE, T.K. (1978). Adsorption of cellulose by Trichoderma

viride. Biotechnol. Bioeng. 20: 1187-1199.

BISARIA, V.S. & GHOSE, T.K. (1977). Cited by Bisaria, V.S. and Ghose, T.K. (1981). Biodegradation of cellulosic materials. Enzyme Microb. Technol. 3: 90-104.

BRYANT, M.P. & WOLIN, M.J. (1975). Rumen bacteria and their metabolic interactions. In Proceedings of the First Internatial Congress of the International Association of Microbiology Societies Vol 2, Developmental Microbiology, Ecology, p297 (Hasegawa, T, ed), Science Council of Japan, Tokyo.

CANEVASCINI, G. & GATTLEN, C. (1981). A comparative investigation of various cellulase assay procedures. Biotechnol. Bioeng. 23: 1573-1590.

CANEVASCINI, G. & MEYER, H.-P. (1979). β-Glucosidase in the cellulolytic fungus Sporotrichum thermophile Apinis. Exp. Mycol. 3: 203-214.

CASTONAN, M. & WILKE, C.R. (1980). Adsorption and recovery of cellulases during hydrolysis of newspaper. Biotechnol. Bioeng. 22: 1037-1053.

CHANG, M. (1971). Folding chain model and annealing of cellulose. Polymer Sci. 36: 343-362.

CLARK, T.F. (1969). Cited by Brown, D.E. (1983). Lignocellulose hydrolysis. Phil.Trans.R.Soc.Lond. B300, 305-322.

COUGHLAN, M.P. & FOLAN, M.A. (1979). Cellulose and cellulases: Food for thought, food for the future. Int. J. Biochem. 10: 103-108.

COUTTS, A.D. & SMITH, R.E. (1976). Factors influencing the production of cellulases by Sporotrichum thermophile. Appl.Environ.Microbiol. 31: 819-825.

COWLING, E.B. (1975). Physical and chemical constraints in the hydrolysis of cellulose and lignocellulosic materials. Biotechnol. Bioeng. Symp. 5: 163-181.

COWLING, E.B. & BROWN, W. (1969). Structural features of cellulosic materials in relation to enzymatic hydrolysis. In Cellulases and their applications. Adv. Chem. Ser. 95 (G.J. Hajny and E. Reese, eds), p152-187. Amer. Chem. Soc. Washington, D.C.

COWLING, E.B. & KIRK, T.K. (1976). Properties of cellulose and lignocellulosic materials as substrates for enzymatic conversion processes. Biotechnol. Bioeng. Symp. 6: 95-123.

DEHORITY, B.A. & SCOTT, H.W. (1967). Extent of cellulose and hemicellulose digestion in various forages by pure cultures of rumen bacteria. J. Dairy Sci. 50: 1136-1141.

DEKKER, R.F.H. (1979). The hemicellulase group of enzymes. In: Polysaccharides in Foods, p93-108 (Blanchard, J.M.V. and Mitchell, J.R., eds), Butterworths, London.

DEKKER, R.F.H. (1983). Bioconversion of hemicellulose: aspects of hemicellulase production by Trichoderma reesei QM9414 and enzymatic saccharification of hemicellulose. Biotechnol. Bioeng. 25: 1127-1146.

DEKKER, R.F.H. & LINDNER, W.A. (1979). Bioutilisation of lignocellulosic waste materials: a review. South African J. Sci. 75: 65-71.

DESAI, J.D., DESAI, A.J. & PATEL, N.P. (1982). Production of cellulases and β-glucosidase by shake culture of Scytalidium lignicola. J. Ferment. Technol. 60: 117-124.

DOMSCH, K.H., GAMS, W. & ANDERSON, T.H. (1980). Compendium of soil fungi. I and II. Academic Press.

DUFF,S.J.B., COOPER, D.G. & FULLER, O.M. (1985). Cellulase and beta-glucosidase production by mixed culture of Trichoderma reesei Rut C30 and Aspergillus phoenicis. Biotechnol. Lett. 7: 185-190.

DUNLAP, C.E. & CHIANG, L.H. (1980). Cellulose degradation - a common link. In: Utilisation and recycle of agricultural wastes and

residues (Shuler, M.L., ed), p19-65. Florida: C.R.P. Press.

ENEBO, L. (1949). Symbiosis in thermophilic cellulose fermentation. Nature, 163: 805.

ERIKSSON, K.-E. (1975). Enzyme mechanisms involved in the degradation of wood components. In: Symposium on enzymatic hydrolysis of cellulose (Bailey, M., Enari, T.M., Linko, H., eds). Aulanko, Finland, 12-14 March 1975. SITRA, Helsinki 1975, p263-280.

ERIKSSON, K.-E. (1978). Enzyme mechanisms involved in cellulose hydrolysis by the rot fungus Sporotrichum pulverulentum. Biotechnol. Bioeng. 20: 317-332.

ERIKSSON, K.-E. & GOODELL, E.W. (1974). Pleiotropic mutants of the wood-rotting fungus Polyporus adustus lacking cellulase, mannanase and xylanase. Can. J. Microbiol. 20: 371-378.

ERIKSSON, K.-E. & HAMP, S.G. (1978). Regulation of endo-1,4-β-glucanase production in Sporotrichum pulverulentum. Eur. J. Biochem. 90: 183-190.

ERIKSSON, K.-E., PETTERSSON, B. & WESTERMARK, U. (1975). Oxidation: an important enzyme reaction in fungal degradation of cellulose. FEBS. Lett. 49: 282-285.

FENNINGTON, G., NEUBAUER, D. & STUTZENBERGER, F. (1983). Adenosine 3',5'-cyclic monophosphate levels in Thermonospora curvata during cellulase biosynthesis. Biotechnol. Bioeng. 25: 2271-2276.

FREY-WYSSLING, A., MUHLETHALER, K. & WYCKOFF, R.W.G. (1948). Cited by Wood, T.M. (1970). Cellulose and cellulolysis. World Rev.Nutri.Diet. 12: 227-265.

GALLO, B.J., ANDREOTTI, R., ROCHE, C., RYU, D. & MANDELS, M. (1979). Utilisation of agricultural crop residues (Han. Y.W. and Smith, S.K., eds),USDA Agricultural Research Service, ARS W-53, p189.

GARDNER, K.H. & BLACKWELL, J. (1974). The hydrogen bonding in native cellulose. Biochim. Biophys. Acta. 343: 232-237.

GHOSE, T.K. & BISARIA, V.S. (1979). Studies on the mechanism of enzymatic hydrolysis of cellulosic substances. Biotechnol. Bioeng. 21: 131-146.

GHOSE, T.K., PATHAK, A.N. & BISARIA, V.S. (1975). Kinetic and dynamic studies of Trichoderma viride cellulase production. In: Symposium on enzymatic hydrolysis of cellulose (Bailey, M., Enari, T.M., Linko, H., eds). Aulanko, Finland, 12-14 March, 1975. SITRA, Helsinki, p111-136.

GOEL, S.C. & RAMACHANDRAN, K.B. (1983). Studies on the adsorption of cellulase on lignocellulosics. J.Ferment.Technol. 61: 281-286.

GØKSØYR, J., EIDSA, G., ERIKSEN, J. & OSMUNDSVAG, K. (1975). A comparison of cellulases from different microorganisms. In: Symposium on enzymatic hydrolysis of cellulose (Bailey, M., Enari, T.M., Linko, H., eds). Aulanko, Finland, 12-14 March 1975. SITRA, Helsinki, pp217-230.

GOLDBERGER, R.F., DELEY, R.G. & MULLINIX, K.P. (1976). Regulation of gene expression in prokaryotic organisms. Adv. Genet. 18: 1-67.

GONG, C.S. & TSAO, G.T. (1979). Cellulase biosynthesis and regulation. In Ann. Rep. Ferm. Proc. 3, p111-140 (D. Perlman & G.T. Tsao, eds), Academic Press Inc., New York.

GORBACHEVA, I.V. & RODJONOVA, N.A. (1977). Purification and characterisation of endo-1,4-β-xylanase from Aspergillus niger strain (T.K. Ghose, ed), Proc.Biocon.Symp, ITT Dehli, p227-248.

GORING, D.A.I. & TIMELL, T.E. (1962). Molecular weight of native celluloses. Tappi. 45: 454-460.

GRADY, H.J. & LAMAR, M.A. (1959). Glucose determination by automatic chemical analysis. Clin. Chem. 5: 542-550.

HALLIWELL, G. (1965). Hydrolysis of fibrous cotton and reprecipitated cellulose by cellulolytic enzymes from soil microorganisms. Biochem.J. 95: 270-281.

HALLIWELL, G. (1966). Solubilisation of native and derived forms of cellulose by cell-free microbial enzymes. Biochem. J. 100: 315-320.

HALLIWELL, G. & GRIFFIN, M. (1973). The nature and mode of action of the cellulolytic component C_1 of Trichoderma koningii on native cellulose. Biochem. J. 135: 587-594.

HALLIWELL, G. & RIAZ, M. (1970). The formation of short fibres from native cellulose by components of Trichoderma koningii cellulase. Biochem. J. 116: 35-42.

HALLIWELL, G. & RIAZ, M. (1971). Interactions between components of the cellulase complex of Trichoderma koningii on native substrates. Arch.Mikrobiol. 78, 295-309.

HIGUCHI, T., SHIMADA, M., NAKATSUBO, F. & KIRK, T.K. (1981). Some recent advances in lignin biodegradation research as related to potential applications. Proc. Bioconv. & Biochem. Eng. Symp. ITT, Delhi, 1: 205-231.

HIRAYAMA, T., SUDO, T., NAGAYAMA, H., MATSUDA, K. & TAMARI, K. (1976). Studies on cellulases of phytopathogenic fungus Pyricularia oryzae cavara. 1. Number and interrelation of components of Cx enzyme from Pyricularia oryzae. Agric. Biol. Chem. 40: 2137-2142.

HOFSTEN, B.V., BERG, B. & BESKAW, S. (1971). Observations on bacteria occurring together with Sporocytophaga in aerobic enrichment cultures on cellulose. Arch. Mikrobiol. 79: 69-79.

HOFSTEN, B.V. & BERG, B. (1972). Cellulase formation in Cellvibrio fulvus. Proc. IV IFS: Ferment. Technol. Today. p731-734.

KAPOOR, K.K., MISHRA, M.M. & YADAV, K.S. (1982). Degradation of lignocellulose by single and mixed cultures of thermophilic

cellulolytic fungi. ZBl Mikrobiol. 137: 91-96.

KAWAKANI, H. (1980). Degradation of lignin-related aromatics and lignins by several Pseudomonads. Lignin biodegradation: microbiology, chemistry and potential applications. II. Kirk, T.K., Higuchi, T. and Chang, H.M. (eds), CRC Press.

KHAN, A.W. (1980). Degradation of cellulose to methane by a coculture of Acetivibrio cellulolyticus and Methanosarcina barkeri. FEMS Microbiol. Lett. 9: 233-235.

KING, K.W. (1965). Enzymatic attack on highly crystalline hydrocellulose. J. Ferment.Technol. 43: 79-94.

KING, K.W. & VESSAL, M.I. (1969). The fractionation of Myrothecium verrucaria cellulase by gel filtration. Adv. Chem. Ser. 95: 7-25.

KIRK, T.K., YANG, H.H. & KEYSER, P. (1978). The chemistry and physiology of the fungal degradation of lignin. Devel. Ind. Microbiol. 19: 51-61.

KLUEPFEL, D., BIRON, L. & ISHAQUE, M. (1980). Simultaneous production of cellulase complex and glucose isomerase by Streptomyces flavogriseus. Biotechnol. Lett. 2: 309-314.

KYSLÍKOVÁ, E. & VOLFOVÁ, O. (1981). Cell growth and cellulase production in Trichoderma viride on microcrystalline cellulose. Folia Microbiol. 26: 303-308.

LATHAM, M.J. & WOLIN, M.J. (1977). Fermentation of cellulose by Ruminococcus flavefaciens in the presence and absence of Methanobacterium ruminantium. Appl. Environ. Microbiol. 34: 297-301.

LAUBE, V.M. & MARTIN, S.M. (1981). Conversion of cellulose to methane and carbon dioxide by triculture of Acetivibrio cellulolyticus, Desulfovibrio sp., and Methanosarcina barkeri. Appl. Environ. Microbiol. 42: 413-420.

LEATHAM, G.F. & KIRK, T.K. (1983). Regulation of ligninolytic activity by

nutrient nitrogen in white-rot basidiomycetes. FEMS Microbiol. Lett. 16: 65-67.

LEE, B.H. & BLACKBURN, T.H. (1975). Cellulase production by a thermophilic Clostridium species. Appl. Microbiol. 30: 346-353.

LEISOLA, M., LINKO, M. & KARVONEN, E. (1975). Determination of the activities of a cellulase complex. In: Symposium on enzymatic hydrolysis of cellulose (Bailey, M., Enari, T.M., Linko, H., eds). Aulanko, Finland, 12-14 March 1975. SITRA, Helsinki, p297-313.

LI, L.H., FLORA, R.M. & KING, K.W. (1965). Individual roles of cellulase components derived from Trichoderma viride. Arch. Biochem. Biophys. 111: 439-447.

LINKO, M., MARKKANEN, P. & BAILEY, M. (1977). Production of cellulases and hemicellulases by Trichoderma viride (T.K. Ghose, ed). Proc.Biocon.Symp. IIT, Dehli, p329-350.

LIU, T.H. & KING, K.W. (1967). Fragmentation during enzymatic degradation of cellulose. Arch. Biochem. Biophys. 120: 462-464.

LOEWENBERG, J.R. & CHAPMAN, C.M. (1977). Sophorose metabolism and cellulase induction in Trichoderma. Arch. Microbiol. 113: 61-64.

LOWRY, O.H., ROSENBROUGH, N.J., FARR, A.L. & ROSE, R.J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem.. 193: 265-275.

MacDONALD, M.J., PATERSON, A. & BRODA, P. (1984). Possible relationship between cyclic AMP and idiophasic metabolism in the white-rot fungus Phanerochaete chrysosporium J. Bacteriol. 160: 470-472.

MacDONALD, M.J. & SPEEDIE, M.K. (1982). Cell-associated and extracellular cellulolytic enzyme activity in the marine fungus Dendryphiella arenaria. Can. J. Bot. 60: 838-844.

McCARTHY, A.J. & BRODA, P. (1984). Screening for lignin-degrading actinomycetes and characterisation of their activity against (^{14}C) lignin-labelled wheat lignocellulose. J. Gen. Microbiol. 130: 2905-2913.

McCARTHY, A.J., MacDONALD, M.J., PATERSON, A. & BRODA, P. (1984). Degradation of (^{14}C) lignin-labelled wheat lignocellulose by white-rot fungi. J. Gen. Microbiol. 130: 1023-1030.

McHALE, A. & COUGHLAN, M.P. (1981). The cellulolytic system of Talaromyces emersonii. Identification of the various components produced during growth on cellulosic media. Biochim. Biophys. Acta. 662: 145-151.

MACCUBBIN, A.E. & HODSON, R.E. (1980). Mineralization of detrital lignocelluloses by salt marsh sediment microflora. Appl. Environ. Microbiol. 40: 735-740.

MAKI, L.R. (1954). Experiments on the microbiology of cellulose decomposition in a municipal sewage plant. Antonie van Leeuwenhoek 20: 185-200.

MANDELS,M.& ANDREOTTI, R.E. (1978). Problems and challenges in the cellulose to cellulase fermentation. Process Biochem. p6-13.

MANDELS, M., KOSTICK, J. & PARIZEK, R. (1971). The use of adsorbed cellulase in the continuous conversion of cellulose to glucose. J. Polymer Sci. 36: 445-459.

MANDELS, M., PARRISH, F.W. & REESE, E.T. (1962). Sophorose as an inducer of cellulase in Trichoderma viride. J. Bacteriol. 83: 400-408.

MANDELS, M. & REESE, E.T. (1960). Induction of cellulase in fungi by cellobiose. J. Bacteriol. 79: 816-826.

MANDELS, M. & REESE, E.T. (1964). Fungal cellulases and the microbial decomposition of cellulosic fabric. Develop. Indust. Microbiol. 5: 5-20.

MANDELS, M. & REESE, E.T. (1965). Inhibition of cellulases. Ann. Rev. Phytopath. 3: 85-102.

MANDELS, M., STERNBERG, D. & ANDREOTTI, R.E. (1975). Growth and cellulase

production by Trichoderma. In: Symposium on enzymatic hydrolysis of cellulose (Bailey, M., Enari, T.M., Linko, H., ed). Aulanko, Finland, 12-14 March 1975. SITRA, Helsinki, p81-109.

MANDELS, M. & STERNBERG, D. (1976). Recent advances in cellulase technology. J. Ferment. Technol. 54: 267-286.

MANDELS, M. & WEBER, J. (1969). The production of cellulases, In: Cellulases and their applications. G.J. Hajny and E.T. Reese (eds), p391-414. American Chemical Society, Washington, D.C.

MARSH, P.B., BOLLENBACHER, K., BUTLER, M.L. & RAPER, K.B. (1949). The fungi concerned in fiber deterioration. II: their ability to decompose cellulose. Tex. Res. J., 19: 462-484.

MATCHAM, S.E., JORDAN, B.R. & WOOD, D.A. (1984). Methods for the assessment of fungal growth on solid substrates. In Microbiological methods for environmental biotechnology. Society for Applied Bacteriology, Technical Series 19. J.M. Grainger, J.M. Lynch (eds), p5-18. Academic Press, London.

MEYER, K.H. & MISCH, L. (1937). Cited by Wood, T.M. (1970). Cellulose and cellulolysis. World Rev.Nutri.Diet. 12: 227-265.

MILLER, G.L. (1959). Use of dinitrosalicylic acid for the determination of reducing sugars. Anal. Chem. 31: 426-428.

MISHRA, M.M., YADAV, K.S. & KAPOOR, K.K. (1981). Degradation of lignocellulose by mixed cultures of cellulolytic fungi and their competitive ability. Zbl.Bakt.II.Abt. 136: 603-608.

MUTLETHALER, K. (1963). Cited by Wood, T.M. (1970). Cellulose and cellulolysis. World Rev.Nutr.Diet. 12: 227-265.

NELSON, N. (1944). A photometric adaptation of the Somogyi method for the determination of glucose. J. Biochem. 153: 375-380.

NISIZAWA, T., SUZUKI, H., NAKAYAMA, M. & NISIZAWA, K. (1971). Inductive formation of cellulase by sophorose in Trichoderma viride. J.

Biochem. 70: 375-385.

NISIZAWA, K., TOMITA, Y., KANDA, T., SUZUKI, H. & WAKABAYASHI, K. (1972). Substrate specificity of C_1 and Cx cellulase components from fungi. Proc. IV IFS: Ferment. Technol. Today, p719-725.

NORKRANS, B. (1950). Cited by Cowling, E.B. & Brown, W. (1969). Structural features of cellulosic materials in relation to enzymatic hydrolysis. In Cellulases and their applications. Adv.Chem.Ser. 95, G.J. Hajny, E. Reese (eds), p152-187. Amer. Chem. Soc. Washington, D.C.

OHKI, R. (1975). Transient repression of catabolite-sensitive enzyme synthesis elicited by 2,4-dichlorophenol. J. Bacteriol. 123: 815-823.

OKADA, G., NISIZAWA, K. & SUZUKI, H. (1968). Cellulase components from Trichoderma viride. J. Biochem 63: 591-607.

OLUTIOLA, P.O., (1978). Growth, sporulation and production of pectic and cellulolytic enzymes in Fusarium oxysporum. Trans. Br. Mycol. Soc. 70: 109-114.

OSMAN, A., BULL, A.T. & SLATER, J.H. (1976). Cited by Bull, A.T. (1980). Biodegradation: some attitudes and strategies of microorganisms and microbiologists. In Contemporary Microbial Ecology. Ellwood, D.C., Hedger, J.N., Latham, M.J., Lynch, J.M. and Slater, J.H. (eds), p107-136. Academic Press.

PEITERSEN, N. (1975). Production of cellulase and protein from barley straw by Trichoderma viride. Biotechnol. Bioeng. 17: 361-374.

PEITERSEN, N. (1975a). Cellulase and protein production from mixed cultures of Trichoderma viride and a yeast. Biotechnol.Bioeng. 17: 1291-1299.

PEITERSEN, N. (1977). Transient response of Trichoderma growing on cellulose in a continuous flow culture. (T.K. Ghose, ed).

Proc.Bioconv.Symp., ITT, Dehli, p281-290.

PETTERSSON, L.G. (1975). Mechanism of enzymatic hydrolysis of cellulose by Trichoderma viride. In Symposium on enzymatic hydrolysis of cellulose (Bailey, M., Enari, T.M., Linko, H., eds). Aulanko, Finland, 12-14 March 1975. SITRA, Helsinki, p255.

PETTERSSON, L.G., AXIO-FREDRICKSSON, U.B. & BERGHEN, L.E.R. (1972). Mechanism of enzymatic cellulose degradation. Proc. IV. IFS: Ferment.Technol. Today. p723.

PHELLAN, M.B., CRAWFORD, D.L. & POMETTO, A.L. III (1979). Isolation of lignocellulose-decomposing actinomycetes and degradation of specifically ^{14}C-labelled lignocellulose by six selected Streptomyces strains. Can. J. Microbiol. 25: 1270-1276.

POINCELOT, R.P. & DAY, P.R. (1972). Simple dye release assay for determining cellulolytic activity of fungi. Appl. Microbiol. 23: 875-879.

PRESTON, R.D., NICOLAI, E., REED, R. & MILLARD, A. (1948). An electron microscope study of cellulose in the wall of Valonia ventricosa. Nature 162: 665-667.

RÅNBY, B.G. (1952). The cellulose micelles. Tappi. 35: 53-58.

RÅNBY, B.G. (1958). Cited by Wood, T.M. (1970). Cellulose and cellulolysis. World Rev.Nutr.Diet. 12: 227-265.

RAPP, P., GROTE, E. & WAGNER, F. (1981). Formation and location of 1,4-β-glucanases and 1,4-β-glucosidases from Penicillium janthinellum. Appl. Environ. Microbiol. 41: 857-866.

RAUTELA, G.S. (1967). Cited by Cowling, E.B. and Brown, W. (1969). Structural features of cellulosic materials in relation to enzymatic hydrolysis. In Cellulases and their applications. Adv.Chem.Ser. 95 (G.J. Hajny and E.T. Reese, eds), p152-187. Amer Chem. Soc, Washington, D.C.

REESE, E.T. (1977). Degradation of polymeric carbohydrates by microbial enzymes. Recent Adv. Phytochem. 11: 311-365.

REESE, E.T. & GILLIGAN, W. (1954). The swelling factor in cellulose hydrolysis. Text. Res. J.. 24: 663-669.

REESE, E.T., SIU, R.G.H. & LEVINSON, H.S. (1950). The biological degradation of soluble cellulose derivatives and its relation to the mechanism of cellulose hydrolysis. J. Bacteriol. 59: 485-497.

RICKARD, P.A.D. & LAUGHLIN, T.A. (1980). Detection and assay of xylanolytic enzymes in a Cellulomonas isolate. Biotechnol. Lett. 2: 363-368.

ROGERS, H.J. & PERKINS, H.R. (1968). Cited by Wood, T.M. (1970). Cellulose and cellulolysis. World Rev.Nutr.Diet. 12: 227-265.

RYDHOLM, S.A. (1965). Cited by Brown, D.E. (1983). Lignocellulose hydrolysis. Phil.Trans.R.Soc.Lond. B300: 305-322.

RYU, D.D.Y. & MANDELS, M. (1980). Cellulase biosynthesis and applications. Enzyme Microb. Technol. 2: 91-102.

SADDLER, J.N. (1982). Screening of highly cellulolytic fungi and the action of their cellulase enzyme systems. Enzyme Microb. Technol. 4: 414-418.

SADDLER, J.N. & KHAN, A.W. (1980). Cellulase production by Acetivibrio cellulolyticus. Can. J. Microbiol. 26: 760-765.

SANDHU, D.K. & KALRA, M.K. (1982). Production of cellulase, xylanase and pectinase by Trichoderma longibrachiatum on different substrates. Trans. Br. Mycol. Soc. 79: 409-413.

SCHEIFINGER, C.C. & WOLIN, M.J. (1973). Propionate formation from cellulose and soluble sugars by combined cultures of Bacteroides succinogenes and Selenomonas ruminantium. Appl. Microbiol. 26: 789-795.

SCHEWALE, J.G. & SADANA, J.C. (1978). Cellulase and β-glucosidase

production by a basidiomycete species. Can.J.Microbiol. 24: 1204-1216.

SELBY, K. (1963). Advances in Enzymatic Hydrolysis of Cellulose and Related Materials, p33. E.T. Reese (ed.). Pergamon Press Ltd., London.

SELBY, K. (1969). The purification and properties of the C_1-component of the cellulase complex. Adv. Chem. Series. 95: 34-50.

SELBY, K. & MAITLAND, C.C. (1967). The cellulase complex. Separation of the components involved in the solubilization of cotton. Biochem. J. 104: 716-724.

SENIOR, E., BULL, A.T. & SLATER, J.H. (1976). Enzyme evolution in a microbial community growing on the herbicide dalapon. Nature 263: 476-479.

SHOEMAKER, S.P. & BROWN, R.D. (1978). Characterization of endo-1,4-beta-D-glucanases purified from Trichoderma viride. Biochim. Biophys. Acta. 523: 147-161.

SIEGEL, S.M. (1963). Cited by Wood, T.M. (1970). Cellulose and cellulolysis. World Rev.Nutr.Diet. 12: 227-265.

SIHTOLA, H. & NEIMO, L. (1975). The structure and properties of cellulose. In Symposium on Enzymatic Hydrolysis of Cellulose, (Bailey, M., Enari, T.-M., and Linko, M., eds). Aulanko, Finland, 12-14 March, 1975. SITRA, Helsinki, p9-21.

SIU, R.G.H. (1951). Cited by Cowling, E.B. & Brown, W. (1969). Structural features of cellulosic materials in relation to enzymatic hydrolysis. In Cellulases and their applications. Adv.Chem.Ser 95 (G.J. Hajny and E. Reese, eds), p152-187. Amer.Chem.Soc, Washington, D.C.

SIWINSKA, K. & GALAS, E. (1977). Cellulases and hemicellulases from Trichoderma viride and Trichoderma roseum, T.K. Ghose (ed). Proc. Int. Bioconv. Symp. IIT Dehli, p89-103.

SLATER, J.H. & BULL, A.T. (1978). Interactions between microbial populations. In Companion to Microbiology. Bull, A.T. & Meadow, P.M. (eds), p181-206. Longman, London.

SOEST, P.J. van & WINE, R.H. (1968). Determination of lignin and cellulose in acid detergent fibre with permanganate. J. Assoc. Off. Anal. Chem. 51: 780-785.

SOMOGYI, M. (1952). Notes on sugar determination. J. Biol. Chem. 195: 19-23.

SRINIVASAN, V.R. & HAN, Y.W. (1969). Utilization of bagasse. In Cellulases and their applications. Adv.Chem.Ser 95 G.J. Hajny and E.T. Reese, (eds), p447-460. Amer.Chem.Soc, Washington, D.C.

STERNBERG, D. (1976). Production of cellulase by Trichoderma. Biotechnol.Bioeng.Symp. No.6. E.L. Gaden, M. Mandels, E. Reese and L.A. Spano, (eds), p35-53. Wiley.

STERNBERG, D. (1976a). β-Glucosidase of Trichoderma: its biosynthesis and role in saccharification of cellulose. Appl.Environ.Microbiol. 31: 648-654.

STERNBERG, D. (1976b). A method for increasing cellulase production by Trichoderma viride. Biotechnol.Bioeng. 18: 1751-1760.

STERNBERG, D. & MANDELS, G.R. (1979). Induction of cellulolytic enzymes in Trichoderma reesei by sophorose. J. Bacteriol. 139: 761-769.

STERNBERG, D., VIJAYAKUMAR, P. & REESE, E.T. (1976). β-Glucosidase: microbial production and effect on enzymatic hydrolysis of cellulose. Can.J.Microbiol. 23: 139-147.

STEWART, B.J. & LEATHERWOOD, J.M. (1976). Depressed synthesis of cellulase by Cellulomonas. J. Bacteriol. 128: 609-615.

STEWART, J.C. & PARRY, J.B. (1981). Factors influencing the production of cellulase by Aspergillus fumigatus Fresenius. J. Gen. Microbiol. 125: 33-39.

STOPPOK, W., RAPP, P. & WAGNER, F. (1982). Formation, location and regulation of endo-1,4-β-glucanases and β-glucosidases from Cellulomonas uda. Appl. Env. Microbiol. 44: 44-53.

STREAMER, M., ERIKSSON, K.-E. & PETTERSSON, B. (1975). Extracellular enzyme system utilized by the fungus Sporotrichum pulverulentum (Chrysosporium lignorum) for the breakdown of cellulose. Functional characterization of five endo-1,4-β-glucanases and one exo-1,4-β-glucanase. Eur. J. Biochem. 59: 607-613.

SWITZER, R. (1977). Cited by Wolf, D.H. (1980). Control of metabolism in yeast and other lower eukaryotes through action of proteinases. Adv. Microbial. Physiol. 21: 267-293.

THAKRE, R.P. & JOHRI, B.N. (1980). Degradation of natural cellulose by thermophilic and thermotolerant fungi. Acta. Botanica Indica. 8: 45-49.

TIEN, M., & KIRK, T.K. (1983). Lignin degrading enzyme from the hymenomycete Phanerochaete chrysosporium. Burds. Science 221: 661-663.

TOYAMA, N. (1960). Cited by Wood, T.M. (1970). Cellulose and cellulolysis. World Rev.Nutr.Diet. 12: 227-265.

TRIVEDI, L.S. & RAO, K.K. (1979). Production of cellulolytic enzymes by Aspergillus fumigatus. Ind. J. Exp. Biol. 17: 671-674.

TRIVEDI, L.S. & RAO, K.K. (1980). Cellulase induction in Aspergillus fumigatus M216. Ind. J. Exp. Biol. 18: 240-242.

TRIVEDI, L.S. & RAO, K.K. (1981). Production of cellulolytic enzymes by Fusarium species. Biotechnol. Letts. 3: 281-284.

TSAO, G.T., LADISCH, M., LADISCH, C., HSU, T.A., DALE, B. & CHOU, T. (1978). Fermentation substrates from cellulosic materials: production of fermentable sugars from cellulosic materials. Ann. Rep. Ferm. Proc. 2: 1-21.

VASSEUR, E. (1948). Spectrophotometric study on the orcinol reaction with carbohydrates. Acta. Chem. Scandinavica. 2: 693-701.

VEAL, D.A. & LYNCH, J.M. (1984). Associative cellulolysis and dinitrogen fixation by co-cultures of Trichoderma harzianum and Clostridium butyricum. Nature 310: 695-697.

VIIKARI, L., KLEMOLA, M. & LINKO, M. (1978). Production of xylanases. Proc. Symp. Bioconversion Food Technol., Helsinki, 8-10 February, 1978, p115-118.

WEIMER, P.J. & ZEIKUS, J.G. (1977). Fermentation of cellulose and cellobiose by Clostridium thermocellum in the absence and presence of Methanobacterium thermoautotrophicum. Appl. Environ. Microbiol. 33: 289-297.

WENZL, H.F.J. (1970). Cited by Brown, D.E. (1983). Lignocellulose hydrolysis. Phil.Trans.R.Soc.Lond. B300: 305-322.

WOLIN, M.J. & MILLER, T.L. (1983). Interactions of microbial populations in cellulose fermentation. Fed. Proc. Fed. Am. Soc. Exp. Biol. 42: 109-113.

WOOD, T.M. (1968). Cellulolytic enzyme system of Trichoderma koningii. Separation of components attacking native cellulose. Biochem. J. 109: 217-227.

WOOD, T.M. (1969a). The relationship between cellulolytic and pseudo-cellulolytic microorganisms. Biochim. Biophys. Acta. 192: 531-534.

WOOD, T.M. (1969b). The cellulase of Fusarium solani. Resolution of the enzyme complex. Biochem. J. 115: 457-464.

WOOD, T.M. (1970). Cellulose and cellulolysis. World Rev. Nutr. Diet. 12: 227-265.

WOOD, T.M. (1972). The C_1 component of the cellulase complex. Proc. 4th Int. Ferment. Symp: Ferment. Technol. Today, p711-718.

WOOD, T.M. (1975). Properties and mode of action of cellulases. In

Cellulose as a chemical and energy source, plll. C.R. Wilke (ed.). Wiley, New York.

WOOD, T.M. & McCRAE, S.I. (1972). The purification and properties of the C_1 component of Trichoderma koningii cellulase. Biochem. J. 128: 1183-1192.

WOOD, T.M. & McCRAE, S.I. (1975). The cellulase complex of Trichoderma koningii. In Symposium on enzymatic hydrolysis of cellulose (Bailey, M., Enari, T.-M., Linko, H., eds). Aulanko, Finland, 12-14 March 1975. SITRA, Helsinki, p231-254.

WOOD, T.M. & McCRAE, S.I. (1977). Cellulase from Fusarium solani: purification and properties of the C_1 component. Carbohydrate Res. 57: 117-133.

WOOD, T.M. & McCRAE, S.I. (1978). The cellulase of Trichoderma koningii. Purification and properties of some endoglucanase components with special reference to their action on cellulose when acting alone or in synergism with the cellobiohydrolase. Biochem. J. 171: 61-72.

WOOD, T.M. & McCRAE, S.I. (1979). Synergism between enzymes involved in the solubilisation of native cellulose. Adv. Chem. Ser. 181: 181-209.

WOOD, T.M. & PHILLIPS, D.R. (1969). Another source of cellulase. Nature 222: 986-987.

YOUATT, G. (1962). The S factor in the enzymatic hydrolysis of cellulose. Text. Res. J. 32: 158-160.

Additional References

Halliwell, G., Griffin, M., & Vincent, R. (1972). The role of component C_1 in cellulolytic systems. Biochem. J. 127 : 43p.

Johnson, E. A., Sakajoh, M., Halliwell, G. & Madia, A. (1982). Saccharification of complex substrates by the cellulase system from Clostridium thermocellum. Appl. Env. Microbiol. 43 : 1125-1132.