# TOWER CONTINUOUS FERMENTATION OF CELLULOSIC WASTES

by

DIONYSIOS ANTZOULATOS, B.Sc.,M.Sc.

A Thesis submitted to the University of Aston in Birmingham in partial fulfilment for the award of the Degree of Doctor of Philosophy

January 1981

The University of Aston in Birmingham

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The aim of this work was the study of the direct bioconversion of cellulose to biomass in a novel Tower fermentation system.

Ball milled a-cellulose and newspaper were used as model substrates. Five cellulolytic fungi were selected from the literature for their bioconversion abilities. Their optimum pH and temperature for growth were determined on agar fermentation media by their linear growth rate.

Batch cultures at their pH and temperature optima (controlled) were performed in a five litre Tower fermenter to assess their cellulose upgrading ability. The criteria were: substrate degradation, biomass productivity and protein content of biomass.

A strain of Sporotrichum thermophile was selected and cultured continuously. The operational characteristics of the system were studied with respect to dilution rate, substrate concentration, substrate degradation, extracellular cellulase activity, biomass and protein productivity. The results confirmed the ability of the system to operate at dilution rates higher than the specific growth rate of the organism and demonstrated the differences in the retention properties of the Tower between soluble and insoluble carbohydrates fermentations.

The fermentation was affected by the morphology of the organism and suspended solids concentration. Biomass concentration, cellulase activity and substrate degradation efficiency were decreasing with dilution rate whereas the total solids concentration increased. Maximum productivity was obtained well below the critical dilution rate. The most important factor which controlled the fermentation was the hydrolysis of the substrate. The biodegradability of the organism was not inhibited by the products of hydrolysis. The negative effect of lignin was also confirmed.

The continuous Tower fermentation of cellulosic materials is biotechnically feasible. It is best suited for substrates with low lignin and low crystalline cellulose content as chemical waste fibres and waste water from fibre board mills.

Key words: Continuous Fermentation Cellulosic Fungi

Dedicated to my parents

Amalia and Nicolas Antzoulatos

CERTIFICATE

I hereby certify that the work which constitutes this Thesis has neither been, nor is being submitted in application for any other degree.

Signed _____

Date 18 - 1 - 81

## DECLARATION

I hereby declare that the work submitted in this Thesis is the result of my own investigations except where reference is made to published literature or assistance acknowledged.

Signed ---------- Candidate

---------- Supervisor

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# LITERATURE SURVEY

## 1.1 General Introduction

The last decade has witnessed widespread global efforts for the more efficient utilization of the non renewable resources, for the recycling of by-products disposed of as wastes and the utilization of renewable materials as sources of food and energy.

The main reasons which have prompted these efforts are: the increasing cost and the perspective of an eventual depletion of the non renewable resources (especially oil and natural gas which are the basic industrial feedstock), the economical and environmental problems associated with the disposal of wastes, and the enlarging gap between world protein production and protein need (Rogers 1976) (Gray 1962).

One increasingly attractive source of protein produced by unconventional agricultural methods is cultured microbial biomass, generally referred to as single cell protein (SCP), optimized for product quality and quantity in controlled environments by fermentation processes (Litchfield 1977).

Several types of fermentation processes for SCP production have been commercially realized as the American (Amoco), British (BP, ICI), Cuban, Finish (Pekilo), Russian and Swedish (Symba). The major disadvantages of these processes are the level of the processing technologies and the capital required for installation operation and maintenance of the equipment which are unattractively high, especially for the underdeveloped countries where protein is needed most. In addition these processes utilize materials which are becoming increasingly expensive (methanol, ethanol, molasses, wood hydrolysates, hydrocarbons) or wastes which are rapidly becoming scarce as

waste sulphite liquor or potato wastes (Moo-Young et al, 1979).

Cellulose is the most abundant organic compound and cellulosic materials are extensively used for their physical and chemical properties. Cellulosic by-products generated from wood processing industries along with agricultural and food processing residues, feedlot wastes and the cellulosic portion of municipal wastes if appropriately exploited instead of a potential pollutant they could represent a valuable substrate either for conversion to food and energy or as a substitute for the virgin material (Tilak, 1977).

Recycling is practiced in a variety of cellulosic by-products as utilization of wood and agricultural residues by the pulping industry, recovery of waste paper and waste fibres for repulping, use of food processing and agricultural residues as animal feed. Still huge quantities of cellulosic materials remain which are considered and disposed of as wastes by methods which are either expensive or represent a pollution hazard and which in either cases do not fully exploit the energy potential of the substrate.

Theoretically all cellulosic wastes can be recycled or transformed to useful products by chemical or biological methods ( see Figures 1 and 2). However, there are economical and biological factors which play a decisive role both in the further utilization of a cellulosic waste and in the process to be used. Economical factors include the availability of the waste (quantity, cost of collection transportation and storage, seasonal production), alternative uses of the waste, available market and value of the final product, cost of disposal of the initial waste and cost of disposal of the secondary waste, capital investment

Figure 1. A simplified scheme of some processes and products based on the bioconversion of cellulose

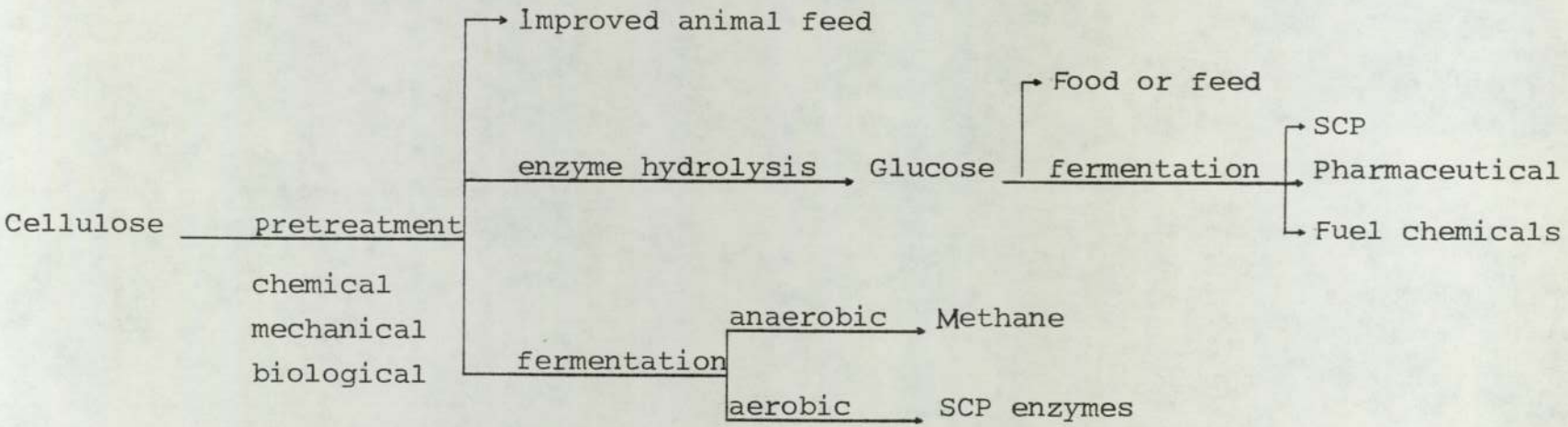
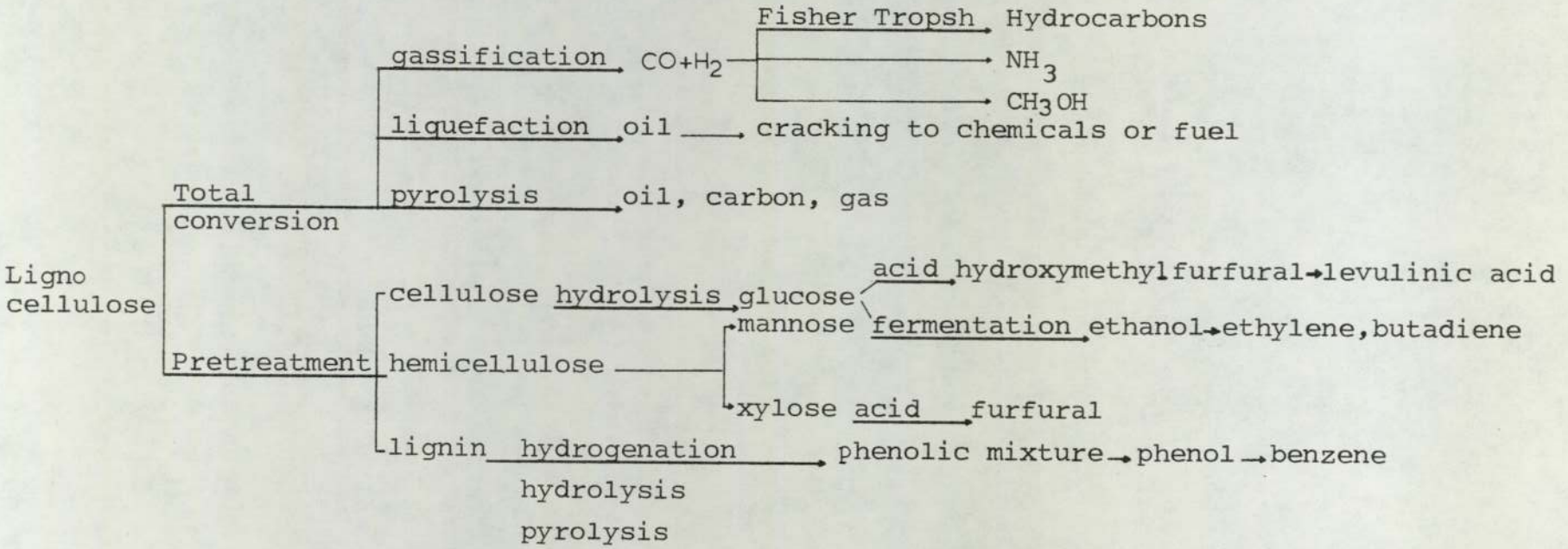


Figure 2. Chemical conversion processes applicable to total lignocellulose, cellulose, hemicellulose and lignin. Goldstein 1976, modified.



and operational costs, competition with similar products derived from other sources. Biological factors include the nutritional value of the final product, the presence of toxic compounds which may inhibit the process or render the final product unacceptable, and the biodegradability of the waste which is mainly determined by the nature of cellulose and the presence of lignin (Callihan and Dunlap, 1969, 1971, 1973) (Wimpenny, 1974) (Ghose and Ghosh, 1978) (Ladish, 1979).

Chemical transformation of cellulosic materials can produce a wide variety of products. Plastics and polymers can be produced without the degradation of macromolecules to simple compounds and repolymerization (Goldstein, 1975). Chemical methods are used for pretreatment of cellulosic materials (lignin removal, increase of cellulose reactivity) before further chemical or biological treatment of cellulose. The hydrolysis of cellulose to its monomer glucose is also receiving much attention. The hydrolysis can be achieved either chemically (acid hydrolysis) or biologically, enzymatic hydrolysis. The glucose produced can be used either directly as food or it can be fermented to a variety of products including microbial biomass (Reese et al, 1972).

Acid hydrolysis is carried out at high temperature and pressure and needs expensive anticorrosive equipment. The catalysis is non specific and during hydrolysis two consecutive reactions take place: Cellulose → Fermentable sugars → Decomposed sugars. The decomposed sugars must be separated because they are toxic. The acid is usually recovered by vacuum evaporation, (if Hydrochloric acid is used) or by electrodialysis, if Sulphuric acid is used. Enzymatic hydrolysis requires almost always a pretreatment

Table (1) Comparison of the main features of Acid and Enzymatic hydrolysis of cellulosic materials. (Linko, 1977, modified)

| | HYDROLYSIS | |
|---|---|---|
| | Acid | Enzymatic |
| Pretreatment | May be necessary | Necessary |
| Rate of hydrolysis | Minutes | Hours |
| pH | Very acid | Around 5 |
| Temperature | High e.g. 200°C | Low e.g. 45°C |
| Pressure | High | Atmospheric |
| Yield | Depends on Pre-treatment, Material, Process details | As in acid |
| Catalysis | Non specific | Specific |
| Decomposed sugars | Present | Absent |
| Industrial application | Yes, USSR | No. Pilot only |
| Main capital investment | Equipment Recovery | Cellulases Pretreatment |

of the substrate for the disruption of cellulose structure and removal of lignin seal. Because the catalysis is specific there is no formation of decomposed sugars and the process requires mild temperature and atmospheric pressure. The rate of hydrolysis however is very slow compared with acid hydrolysis and the enzymes used are still more expensive than the acid catalysts. Acid hydrolysis of cellulosic materials is practiced in industrial scale in U.S.S.R. but data of economical feasibility have not been published (Linko, 1977). A comparison of the main features of the two processes is presented in Table (1).

The conversion of cellulosic material to food suitable for human consumption is accomplished mainly by ruminants through the activity of rumen microorganisms. As in the case of enzymatic hydrolysis the crystallinity of cellulose and the presence of lignin inhibit the complete digestion of the substrate and thus considerable efforts have been made and several processes have been proposed to increase the fodder nutritive value of materials such as agricultural and wood residues. Among these processes are the American (Jelk), Canadian (Stake) and Danish (Rexen). These processes however produce only feed carbohydrate without converting any of the raw material into protein (Moo-Young et al, 1979).

A novel approach in the bioconversion of cellulose is the aerobic submerged growth of microorganisms able to utilize cellulose or a suitably pretreated cellulosic substrate and the utilization of the protein enriched final product as animal feed. By this method, which is optimized towards biomass production, the three-step process of cellulase production, substrate hydrolysis and microbial growth are carried out in the same vessel at the same time. This process can alleviate environmental pollution and could find appropriate application on feedlots where the protein enriched substrate could replace the increasingly expensive soymeal or fishmeat animal-feed protein supplements. This process which is receiving increasing attention (Ek and Eriksson, 1977) (Janus, 1977) (Moo-Young et al, 1977) (Forage and Righelato, 1978) was chosen for the fermentation of cellulosic materials presented in this work.

## 1.2. The Substrate

Cellulose is a structural polysaccharide synthesized and found in close association with other substances. In a particular cellulosic material, the structure of cellulose and the degree of association with non cellulosic compounds largely determine its resistance to microbial attack and thus its selection as substrate for biological conversion. A description of the structure of cellulose, the associated substances, sources of cellulosic substrates, the features affecting their degradation and the pretreatments used to enhance cellulose degradation is given in this sub-section.

### 1.2.1. The chemical structure of cellulose

Cellulose is a high molecular weight linear polymer. Its repeating unit is the β-D-anhydroglucopyranose in the chair configuration coupled at the 1- and 4-carbon atoms by a covalent β-glucosidic bond. The hydroxyl groups are in the equatorial and the hydrogen atoms are in the axial position. The structural unit of the polymer is cellobiose because every other repeating unit is rotated $180^{o}$ around the main axis. This structure results in an unstrained linear configuration with minimum steric hindrance (Ghose, 1977), Figure (3). The conformation and steric rigidity of the anhydroglucose units is decreasing as the crystallinity of cellulose decreases (King, 1961).

The two terminal glucose units are different from the other molecule units and also differ from each other. One contains a reducing hemiacetal group (-CHO) whereas the other contains an extra secondary hydroxyl group and has

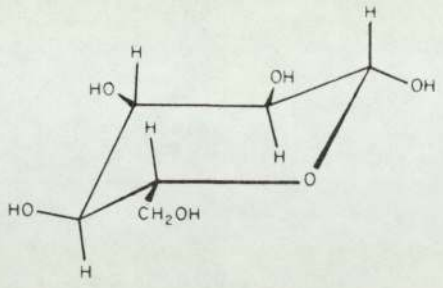
no reducing power. The acetal β-1,4-bond of cellulose is sensitive to acids and during hydrolysis, the reducing and non reducing glucose residues appear at each point of the cleavage (McBurney, 1954), Figure (3). Acid hydrolysis of soluble cellulose is of first order and going essentially to completion. When the substrate is insoluble the reaction proceeds rapidly at first and then changes to a slow rate. The X-ray pattern during the first rapid stage of hydrolysis shows no change but as the second stage proceeds it becomes gradually diffuse. Davidson (1943) quoted in McBurney (1954) explained this phenomenon on the basis of two cellulose states. The easily penetrable being the amorphous and the more resistant being the crystalline state which produced the stable X-ray pattern. Cellulose hydrolysis could then proceed first rapidly through the amorphous accessible part and then slowly as the reagent acted on the crystallites. The same course of reaction is followed during enzymatic hydrolysis of cellulose (Norkrans, 1950) (Walseth, 1952 a,b).

The hydroxyl groups in each anhydroglucose unit, one primary in carbon 6 and two secondary in carbon 2 and 3, have the same properties as the primary and secondary groups in low molecular weight aliphatic alcohols. The hydroxyl groups and the glucosidic bond mainly determine the chemical properties of cellulose because all significant chemical reactions occur at these locations.

Cellulose derivatives are formed by replacing the hydrogen of the hydroxyl groups with such groups as methyl ethyl, hydroxymethyl, carboxymethyl, etc. The average number of substituent groups attached to each anhydroglucose unit is termed Degree of Substitution (D.S). The D.S. for

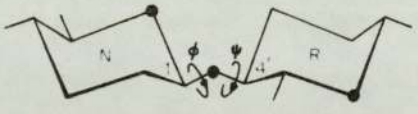
Figure 3. The Chemical structure of cellulose.

a. β-D-glucose in the C1 conformation



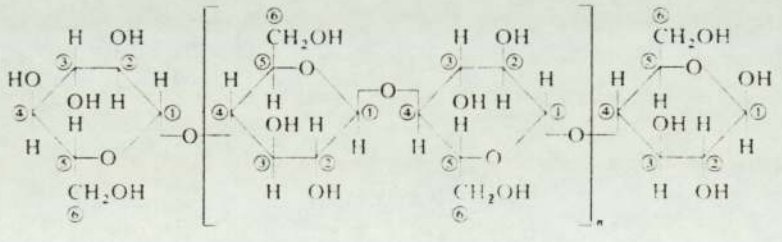

The -OHs lie almost in the plane of the ring and the -H lie almost normal to this plane. From Preston (1974).

b. Cellobiose (β-D-glucopyranosyl-(1-4)-β-D-glucopyranoside)




●: oxygen, $CH_2$-OH marked by lines, -OH and -H omitted. Φ and ψ show the angles of rotation. R = the residue nearer to the reducing end of the chain, N = the residue nearer to the non reducing end. From Preston (1974).

c. The structural (non conformational) formula of cellulose. From Reese et al (1972)




non reducing end

reducing end

a particular sample does not indicate the real number of substituent groups which has been added to each unit because the substituting reagents react most readily with the most accessible parts of the substrate. The addition of these groups makes cellulose non crystalline and soluble in water in proportion to its D.S. Complete solubility is usually attained at D.S. from 0.5 to 0.7 depending on the solvating capacity of the substituents (Cowling,1958)(Cowling and Brown,1969).Cellulose is insoluble in water because the hydroxyl groups of glucose residues have a stronger tendency to form hydrogen bonds with each other than with water molecules (Berghem, 1974). Intrachain hydrogen bonds occur between hydroxyl in carbon 3 of one anhydroglucose unit and the ring oxygen atom of the next one (Sihtola and Neimo, 1975) (Ranby, 1969). Swelling of cellulose in water occurs in the amorphous regions where the cellulose chains are loosely packed and results in a structure of large internal surface. Upon drying, the internal surfaces are brought together and hydrogen bonding occurs. Addition of water on dried cellulose fibres usually results in rehydration of the amorphous regions. However, it is possible that drying at elevated temperatures, above 150$^{o}$C, may inhibit rehydration (Ghose and Kostick, 1969).

The number of repeating units or degree of polymerization (D.P.) of cellulose preparations varies, according to the method of treatment, from 15< to >10000 (Ghose, 1977). In native cellulose the cellulose chains are of widely varying lengths and so the D.P. varies throughout the sample. Moreover, the methods of purification and solubilization used,depolymerize the molecule to a certain

degree (Norkrans, 1967). The reactivity of the cellulose molecule depends mainly on the availability of the hydroxyl groups and of the glucosidic linkages to different reagents. The chemical structure of cellulose determines both chemical and physical properties of cellulose but the forces between the cellulose chains produce a supermolecular structure that profoundly influences most properties of the material (Mark, 1954). The reactivity of native cellulose is further complicated by the intimate association of cellulose aggregates with non cellulosic substances, mainly hemicelluloses and lignin.

### 1.2.2 The physical structure of cellulose

Native cellulose is polymolecular. Cellulose chains of different D.P. are bound by intermolecular hydrogen bonds and van der Waals forces into cohesive aggregates, Siu (1951). When the molecules lie parallel to each other and spaced regularly they form ordered or crystalline regions spatially separated, except at the surface, from other substances. When the regularity of parallelism is less, amorphous regions appear in assocation with other substances, (Jurasek et al, 1967) (Siu and Reese, 1953).

There are no sharp boundaries between the amorphous and the crystalline regions and long cellulose molecules can run through a large number of areas of different degrees of crystallinity (Preston, 1974) (Sihtola and Neimo, 1975). Depending on the treatment to which it has been subjected, cellulose occurs in four distinct crystal structures designated as cellulose I (native cellulose), cellulose II (regenerated cellulose as viscose, cellophane, mercerized cotton) and cellulose III and IV (treated with anhydrous

ethylamine and certain high temperatures respectively). They differ in the dimensions of their unit cells and in the amount of amorphous material present (Cowling and Brown, 1969). Figure 4 shows the molecular arrangement and the hydrogen bonding in the unit cell of native cellulose as determined by X-ray diffraction techniques. The unit cell (i.e. the repeating three dimensional unit within the crystalline regions) accommodates two cellobiose units, one at the centre of the cell and one fourth of each of the four units which are placed at the corners of the monoclinic cell. The corner units are shared by each of the four unit cells which meet at the corners. The cellulose chain in the centre of the cell runs in an anti-parallel direction to those at the corners. Due to the different distances between atoms of neighbouring cellulose chains, Table 2 , hydrogen bonding between adjacent chains occurs along the a-axis and van der Waals forces along the c-axis (Mark, 1954) (Sihtola and Neimo, 1975) (Fan et al, 1980). Parallel arrangement of cellulose chains has been proposed by Gardner and Blackwell (1971) and Sarko and Muggli quoted in Albersheim (1975).

The cellulose aggregates and the associated substances form long slender bundles of non-definite length called microfibrils. The microfibrils are distinct structural entities in that few cellulose molecules, if any, link one microfibril to another, (Cowling and Kirk , 1976) (Preston , 1974), Figure 5.

Elementary fibrils or protofibrils, (bodies 3.5 nm wide) have been proposed by several workers as subunits of the microfibrils (Frey Wyssling and Muhlethaler, 1963) (Heyn, 1965, 1966, 1969) (Ohad and Danon, 1964) .The existence of elementary

Figure 4. The unit cell of native cellulose. From Meyer and Mish (1937)

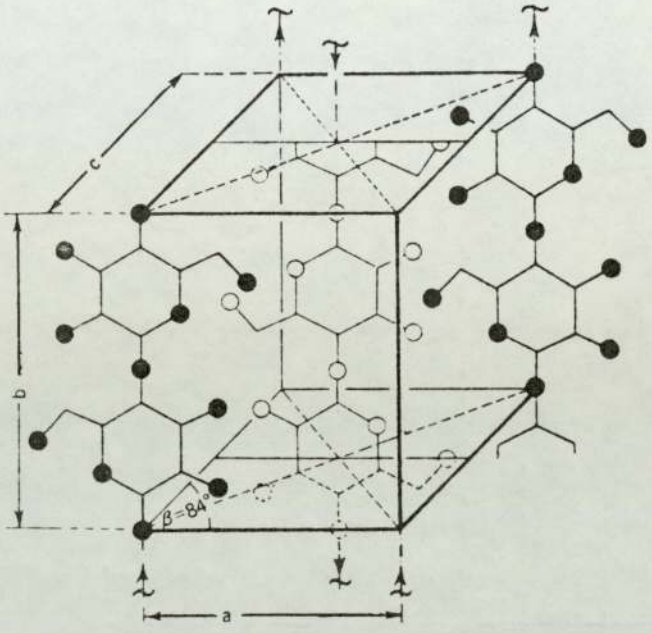
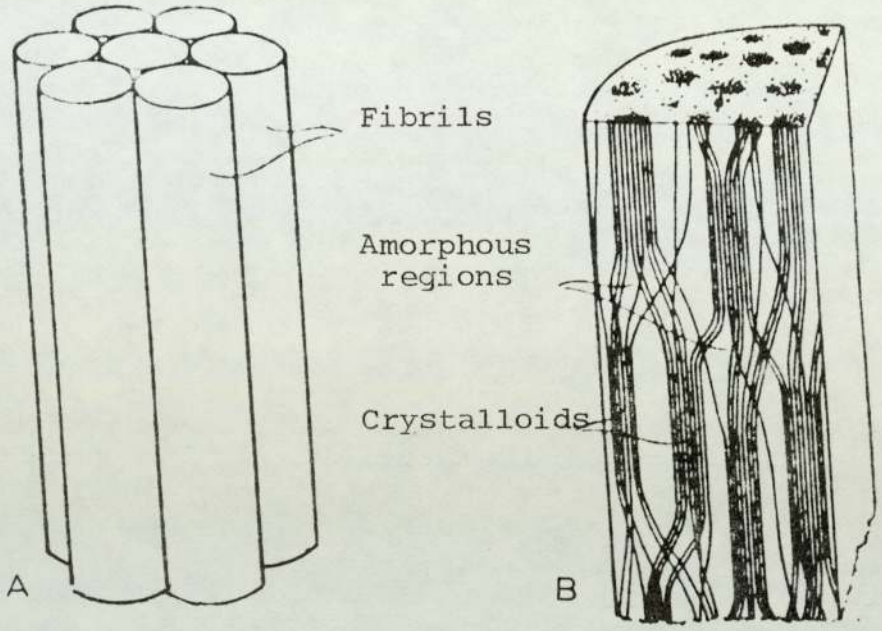



Table 2. Stability of bonds in native cellulose (Sihtola and Neimo, 1975).

| Dimensions | Length nm | Stability kcal/mole | Nature of bond |
|---|---|---|---|
| a | 0.817 | 15 | Hydrogen |
| b | 1.031 | 50 | Covalent |
| c | 0.784 | 8 | van der Waals |

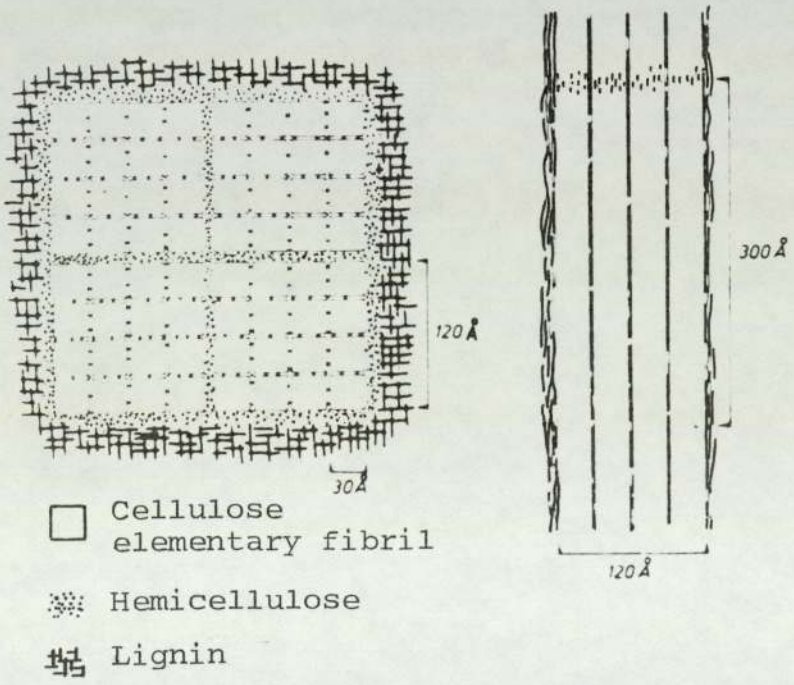
Figure 5. Alignment and composition of cellulose microfibrils. From Sihtola and Neimo (1975)




A Bundle of parallel fibrils (held together crosswise by hydrogen bonds).

B Lateral sectional view of one fibril

Figure 6. Model of the ultrastructural organization of the cell wall components of wood. (From Fengel, 1971)



fibrils has been criticized by Preston (1974).

Another controversial subject is the shape of the cellulose molecule in the cellulose aggregates. There are two main concepts. The folded chain concept (Marx-Figini and Schulz 1966) Manley (1964,71) and the straight line concept (Muggli et al, 1969) (Frey-Wyssling and Muhlethaler, 1963) (Preston and Cronshaw, 1958) (Preston,1974).

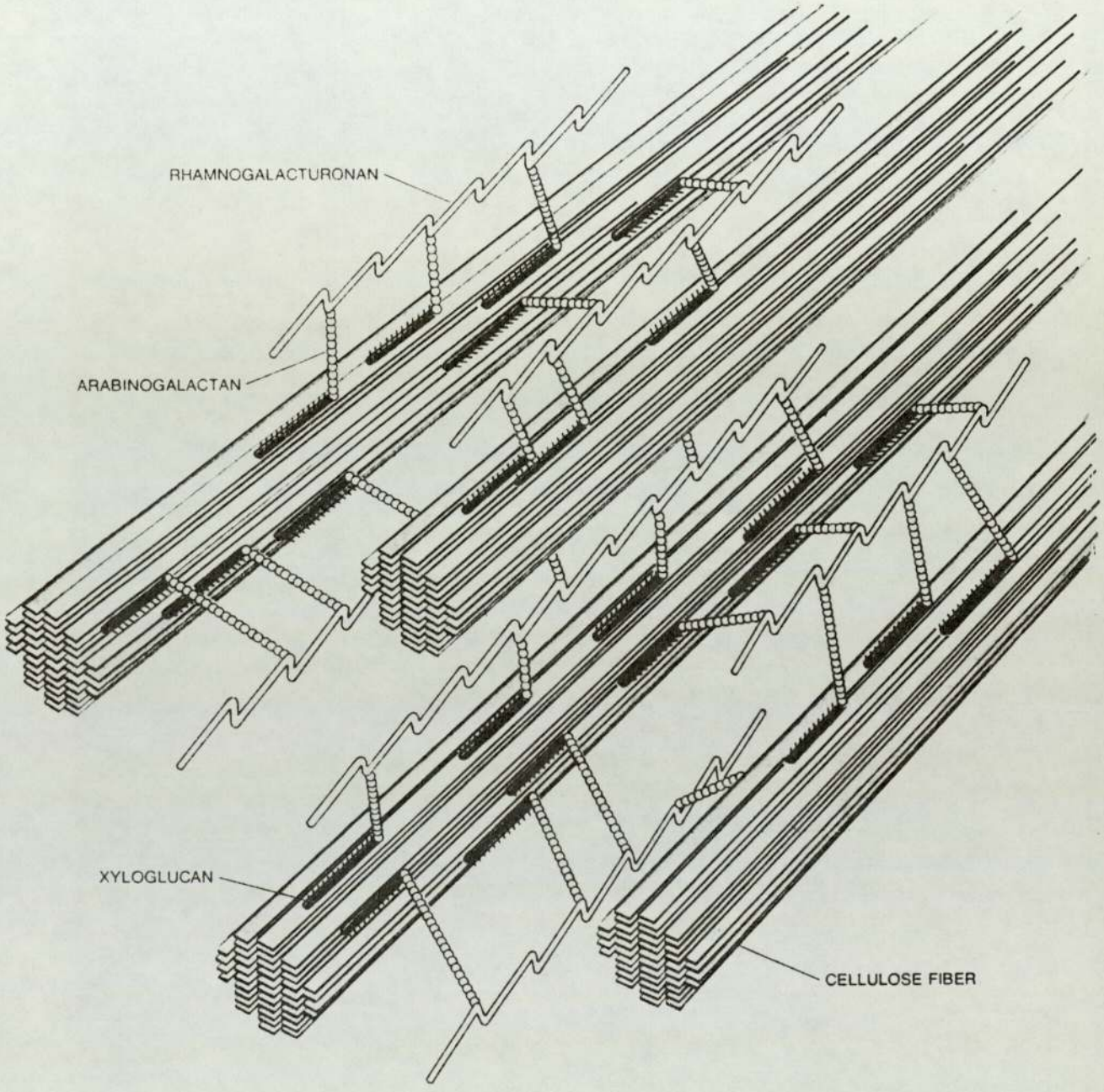
The macromolecular physical structure of native cellulose is a complex function of molecular forces within and between individual cellulose molecules, between microfibrils in fibres, and between microfibrils and the cellulose associated substances mainly hemicelluloses and lignin, (Callihan and Dunlap, 1971).

### 1.2.3 Cellulose associated substances

The major constituents of plant cells are cellulose hemicelluloses and lignin. Their occurrence varies according to plant species and age of cell and their relative proportion is different in the middle lamella and the different layers of cell wall (Norkrans, 1967). Hemicelluloses and lignin form a matrix surrounding the cellulose. Lignin and hemicelluloses may penetrate the spaces between cellulose molecules in the amorphous regions of cellulose aggregates (Cowling and Brown, 1969), Fig. 6.

Hemicelluloses is a generic name of a biochemically heterogeneous group of polysaccharides mainly short chain polymers of glucose, galactose, mannose, xylose, arabinose and uronic acids of glucose and galactose. They mainly form branched heteropolymers by 1-3, 1-4 and 1-6 glucosidic bonds and their D.P. seldom exceeds 200, (Cowling and Kirk,

Figure 7. Cellulose-hemicellulose association.
From Albersheim et al (1975)*




**MODEL OF THE CELL WALL** devised by the author and his colleagues* assumes that cellulose fibers are linked together by three other polysaccharides. Many xyloglucan molecules adhere to the surface of the fibers. Each xyloglucan molecule binds to a single arabinogalactan chain, which in turn binds to a single rhamnogalacturonan molecule. Each rhamnogalacturonan chain can receive several arabinogalactan molecules, radiating from different cellulose fibers. Similarly, each cellulose fiber can be connected to several rhamnogalacturonan chains. As a result of this extensive cross-linking the fibers are immobilized in a seemingly rigid matrix.

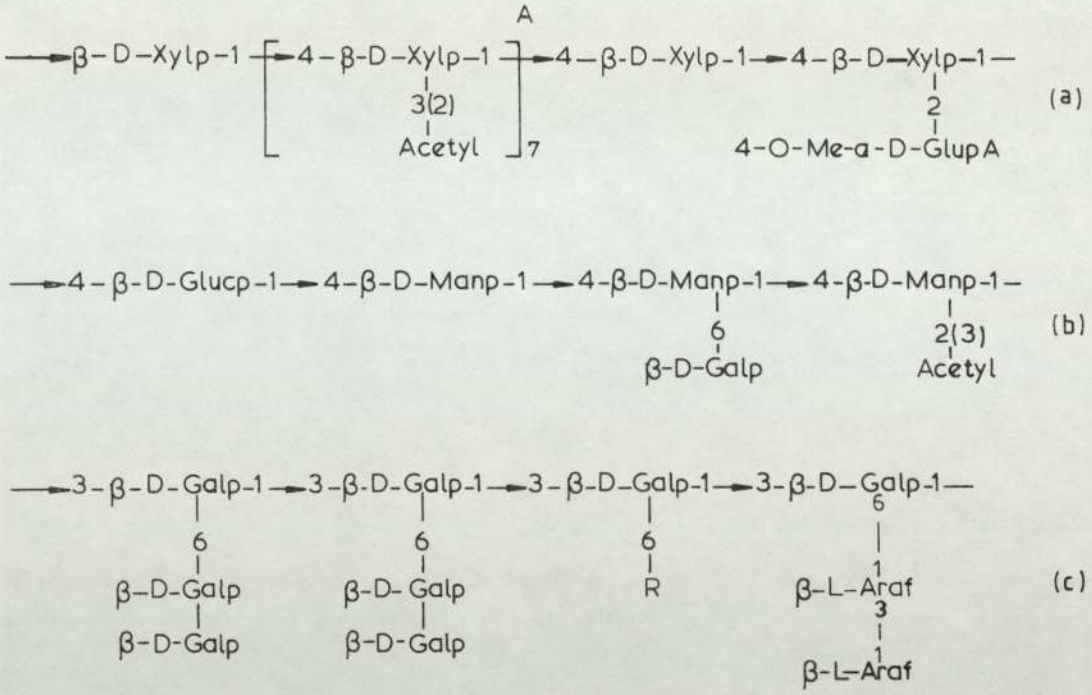
1976). The composition of the hemicelluloses varies with plant species. Xylose is the main hemicellulose in forages and hardwood, mannan is the main hemicellulose in soft woods (Pigden and Heaney, 1969) (Preston, 1974). Hemicelluloses are less resistant to microbial attack than cellulose and lignin. They have a broad range of response to various solvents and they are generally separated from the lignocellulosic complex by extraction with dilute alkali or acid (Pigden and Heaney, 1969). Hemicelluloses are closely associated or occluded by cellulose and play an important role in the chemical and physical properties of cellulosic materials. A model of cellulose-hemicellulose association and sections of hemicellulose chains are shown in Figs 7 and 8a respectively.

Lignin is a polymer formed in situ by the oxidative free radical polymerization of coniferyl, coumaryl and sinapyl alcohols. The structure of lignin is shown in Fig 8b.

The relative amounts of the alcohols present in a plant sample depends on the plant species (Cowling and Kirk, 1976). The condensation of the hydroxycinnamyl alcohols into the lignin polymer structure is catalyzed by enzymes which dehydrogenate the alcohols forming highly reactive quinone methide radicals (Freudenberg, 1965). The condensation of the radicals to form the lignin polymer is not an enzymatic but a random irreversible chemical process. Because there is a high diversity of intermonomer bonds there is no one specific bond to be attacked by the enzymes and as a result lignin is very resistant to microbial degradation. In wood lignin is concentrated between the cellulose microfibrils and in the amorphous regions between cellulose crystallites.

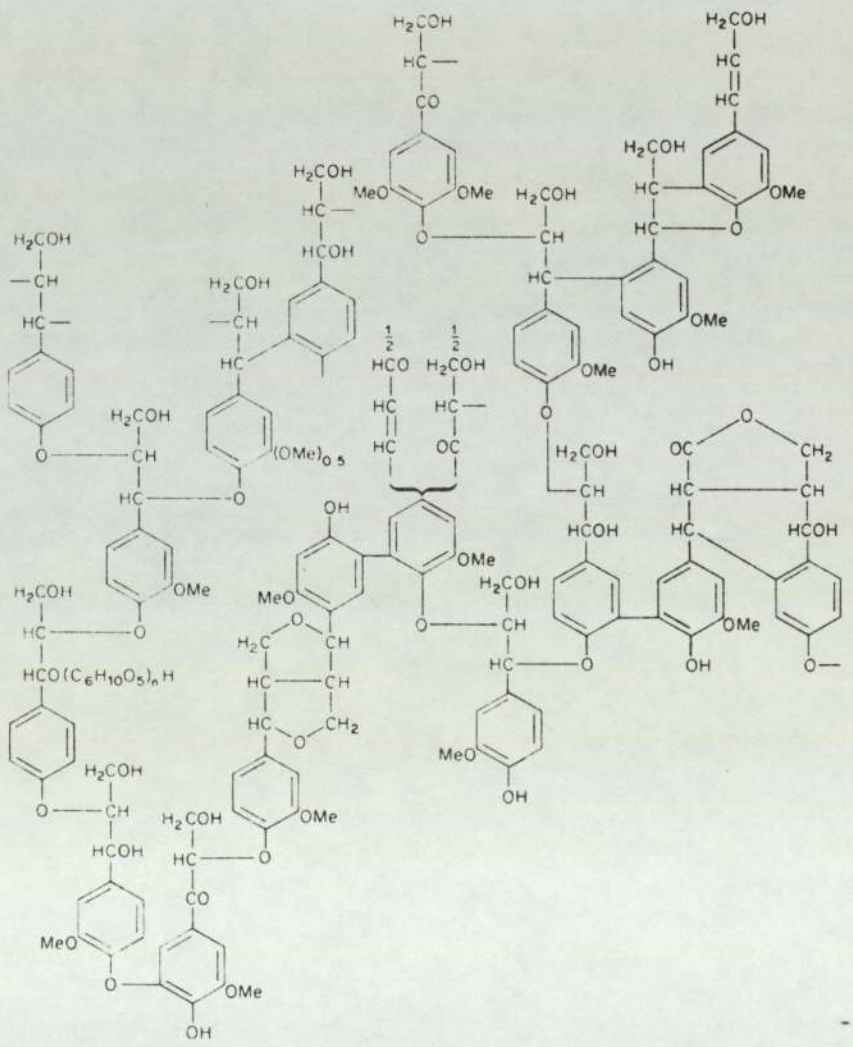
Figure 8. The chemical structure of hemicellulose (A) and lignin (B). (Preston, 1974)

A




R = β-D-Galp or L-Araf or D-Glucopyranosyluronic acid Xyl=xylose Glu=glucose Man=mannose, Gal=galactose, Me=methyl, Ara=arabinose, p=pyranosyl, f=furanosyl. a=hardwood glucuronoxylan, b=O-acetyl-galacto-glucomannan, c=arabinogalactan.

B



The nature of association, chemical or physical, between lignin and cellulose is not certain. An accepted model is that lignin and wood carbohydrates form a mutually interpenetrating system of polymers the nature of association being largely physical with a portion of lignin being linked to hemicelluloses by covalent bonding. The presence of lignin prevents cellulases and hemicellulases from contacting the glucosidic linkages in cellulose and hemicellulose and before any enzymatic activity is possible to any appreciable extent either the physical structure of lignin must be disrupted or the material must be delignified (Cowling and Kirk, 1976) (Cowling and Brown, 1969) (Pew and Weyna,1962) (Acharya,1935) (Fontaine,1941).

### 1.2.4 Cellulosic substrates

Cellulose is the major constituent of plant cell wall comprising from one third to one half of dry plant material (Hajny and Reese, 1969). Unlike other resources such as petroleum coal and minerals, cellulose is constantly replenished by the process of photosynthesis (Spano et al, 1975). As such, cellulose is the most plentiful natural resource and it has been one of the most readily available and useful material to man since the beginning of civilization (Cowling and Brown, 1969). Natural cellulose is almost always associated with a variety of other substances, the most important being hemicelluloses and lignin. According to Cowling and Kirk (1976) the main natural sources of cellulose are:

1) The stems of hardwoods: 40-55% cellulose, 24-40% hemicellulose, 18-25% lignin.

2) The stems of softwoods: 45-50% cellulose, 25-35% hemicellulose, 25-35% lignin.

3) The stems of monocotyledons: 25-40% cellulose, 25-50% hemicellulose, 10-30% lignin.

4) Parenchyma cells of leaves: 15-20% cellulose, 80-85% pectin + hemicellulose, little or no lignin.

5) Certain seed hairs: 80-95% cellulose, 5-20% hemicellulose, little or no lignin.

The greater quantities of cellulose are derived from the sources 1-3 which are heavily lignified (Cowling and Kirk, 1976). The abundance of cellulosic materials, their extensive use and their recalcitrant nature has resulted in the generation of large amounts of cellulosic by-products in agriculture, forestry , animal farming, and wood pulp and food processing industries. The high consumption of disposable cellulosic materials in developed countries has resulted in the major part of municipal wastes being cellulosic (Cowling and Brown, 1969). The cellulose content of some major types of wastes in the U.K., according to Porteous (1977) is given in Table 3.

Table 3 Sources of cellulosic wastes in UK

| | Cellulose content (% d.w.) | Nett cellulose ($10^6$ tonnes) |
|---|---|---|
| Domestic refuse | 25 | 4.5 |
| Straw | 50 | 1.74 - 4.5 |
| Sawmill wastes | 45 | 0.9 |
| Forestry wastes | 45 | 0.45 |
| Wood processing | 45 | 0.45 |

The abundance of cellulosic wastes coupled with the increasing need for protein and the increasing prices of oil derived proteins have markedly increased the interest in the utilization of cellulosic wastes by cellulolytic microorganisms for S.C.P. production (Moo-Young et al, 1978a).

On the basis of their acceptability, availability, alternative uses and composition, the types of cellulosic wastes which stand a reasonable chance for economical conversion to S.C.P. fall in the following categories: Agricultural and feedlot wastes, waste paper, wood processing wastes including paper and pulp mill effluents, food processing wastes (Wimpenny, 1974) (Stone, 1976) (Sloneker, 1976) (Cooper, 1976).

The harvesting and processing of crops generate huge amounts of residues in the form of leaves, stalks, stubble, straw, vines, hulls, bagasse, etc. which by nature are mostly cellulosic (Mantell, 1975). The major quantities of agricultural residues are disposed of as waste by ploughing or burning (Sloneker, 1976). Agricultural residues are found in their native state. Their cellulose content varies from 20-50% and their hemicellulose and lignin content from 50% to 80% (Cowling and Kirk, 1976). Table 4 shows the cellulosic composition of some common agricultural residues.

Although the impact of the continuous removal of agricultural residues remains to be examined, they are considered a suitable substrate for S.C.P. production, especially in areas of intense cultivation (Sloneker, 1976). Other novel biological uses include enzymatic hydrolysis to sugars, methane generation, improvement of

their fodder value by enzyme treatment. A pretreatment to enhance their reactivity is almost always necessary but agricultural residues are usually easily amenable to chemical modification (Ghose, 1977).

Table 4 Composition of some important Agricultural residues, % D.W.

| Plant residue | Non cellulose Carbohydrate | Cellulose | Lignin | References |
|---|---|---|---|---|
| Bagasse | 20-30 | 35-40 | 10-15 | Porteous (1977) |
| Corn stalks | 56.8 | 29.3 | 31 | Sloneker (1976) |
| Kenaf stalks | 58.6 | 41.9 | 12.3 | Sloneker (1976) |
| Rice straw | 24 | 32 | 12.5 | Porteous (1977) |
| Oat straw | 35.5 | 36.9 | 7.4 | Dunlap (1969) |
| Wheat straw | 35.6 | 46 | 8 | Dunlap (1969) |

Feedlot wastes present a serious pollution problem in areas where intensive livestock rearing is practiced. They are expensive to transport and dispose of in large areas. When dried, they contain lower quantities of P,N,K, than the commercial fertilizers and so they cannot compete economically with them (Mantell, 1975). The methods of their treatment and disposal are basically the same as those used in domestic sewage. Alternative biological methods for their utilization include direct feeding or cross feeding of the manures after a suitable treatment as drying or sterilizing (Knapp and Howell, 1979). They are a potential substrate for S.C.P. production by fermentation of their extractable and/or their solid portion which is cellulosic (Reddy and Erdman, 1977), (Bellamy, 1974). Table 5 shows the composition of the solid portion of

a steer feedlot waste fed a ration of shell corn and corn silage (straw and wasted feed particles included) filtered through a two-layer cheese cloth.

Table 5 Composition of the solid portion of a feedlot waste (Reddy and Erdman, 1977)

| Component | % dry matter |
|---|---|
| Cellulose | 24.5 |
| Non cellulose carbohydrate | 25.0 |
| Lignin | 8.5 |
| Ash | 11.5 |
| Nitrogen | 2.0 |

Vegetable and fruit processing solid residues are a suitable substrate for cellulose fermentation only if their cellulose to extractable carbohydrate ratio is high. Their fermentation presents problems because they are highly putrescible. They are extensively used as animal feed or they find other uses in some ancillary product or industry. The usual method of disposal is dumping (Cooper, 1976) (Waldemeyer,1974).

Wood processing generates large amounts of cellulosic residues as shavings, trimmings, sawdust, which are considered potential substrates for S.C.P. production. Their main alternative uses are burning as fuel and production of pulp or other fibre products. A large proportion of the residues is disposed of as waste by burning or dumping (Stone, 1976).

Pulp and paper making, including waste paper repulping, generates the largest amounts of industrial cellulosic

wastes (Andren et al, 1976). Mechanical pulping reduces wood to fibre and mechanical pulps have the same cellulosic content as the original material. Chemical pulps have higher biodegradability than mechanical pulps, because the chemical processing separates cellulose from hemicellulose and lignin (Andren et al, 1976) (Stone, 1976). Table 6 shows the amount of suspended solids (s.s.) generated from virgin pulp mills for different pulping processes.

Table 6 Cellulosic wastes from pulp mills (per tonne of product) (Franklin and Hunt, 1972).

| | s.s (kg) | Waste water ($m^3$) |
|---|---|---|
| Kraft pulp bleached | 63.6 | 170 |
| Kraft pulp unbleached | 22.7 | 83.2 |
| Groundwood pulp | 11.3 | 38 |
| Neutral sulphite semi-chemical pulp | 22.7 | 52 |
| Sulphite pulp | 28 | 181 |

Recycling of the waste fibres is extensively practiced in pulp mills and only the fibres which cannot be recycled, mainly due to unsuitable size reduction, are characterized as waste fibres. Even if they usually contain various amounts of clay they are considered as one of the most suitable substrates for biological conversion because they have already undergone a substantial pretreatment. They are found in large quantities in small areas and they usually have zero or negative value to the paper companies (Eriksson and Larsson, 1975) (Andren et al, 1976)(Spano, 1976). Because pulp and papermaking residues have undergone at least partial disruption of the lignin-cellulose

complex, they are considered as good feedstuffs for ruminants (Millett et al, 1973). The usual method of disposal is dumping or burning in the plant boilers for steam raising.

Waste paper is of particular interest because it is available in large quantities on a continuous basis and it constitutes the major component of the solid municipal wastes. Table 7.

Table 7 Composition of U.K. domestic refuse

% W/W 1974 dateline (Porteous, 1977)

| | |
|---|---|
| Paper and cardboard | 36 |
| Vegetables and foodstuffs | 18 |
| Dust and ashes | 17 |
| Rags | 3 |
| Glass | 10 |
| Metals | 10 |
| Plastics | 3 |
| Unclassified | 3 |

The recovery of the waste paper, especially before reaching the main municipal waste stream, is highly organized in countries which import newsprint or pulp as in the U.K. (Porteous, 1977). Waste paper is used in the manufacture of lower grade papers or card boards. In the U.K. de-inked waste paper is used up to 30% in newsprint manufacture. Apart from the costs involved and secondary pollution generated during de-inking and repulping, there are technical limitations in the repulping of waste paper due to continuous size reduction of the cellulose fibres

which makes them unsuitable for reuse. Thus, up to 50% of input fibre to de-inking and repulping plants are washed in the waste effluent (Porteous, 1977). Another factor which affects its reuse is the fluctuating market demands for waste paper which result in the disposal of collected paper as municipal garbage.

One form of waste paper which is most easily kept apart from refuse and can be easily collected by local authorities or other organizations is newspaper. Large quantities are also stocked as undistributed and recollected newspapers.

The composition of newspaper depends on the type of wood, type of pulp and ratio of pulps used. Newspaper has an ink content of less than 1% by weight (Dinius and Oltjen, 1972) which usually consists of 86% mineral oil,13% carbon black, 1% tonnes (Askew, 1969). Feeding trials using newspaper as part of the diet of beef dairy cattles have shown that it is nutritious and non toxic (Dinious and Oltjen, 1972). Waste newspaper is considered a good substrate for enzymatic hydrolysis, the ink and other impurities present do not cause any special problems (Spano et al, 1975).

In the present work, newsprint was chosen as the model lignocellulosic substrate for continuous fermentation.

### 1.2.5 Physical and chemical features of cellulose affecting its biological degradation

The direct contact between cellulose and cellulolytic enzymes is the most important factor for the hydrolysis of the substrate. Since native cellulose is insoluble and structurally complex, its susceptibility to biodegradation is determined by its accessibility to cellulolytic enzymes

excreted by or bound on the surface of cellulolytic microorganisms.

According to Cowling and Brown (1969), the main physical and chemical features of native cellulose which affect its susceptibility are:

Moisture content of the fibre

Increases the accessibility by hydrating the spaces between microfibrils and between the cellulose molecules (within the amorphous regions) and thus opening up the fine structure of fibres. It provides a medium for the diffusion of the enzymes into the fibre, for the removal of the products of hydrolysis and also provides the elements of water which are added to the cellulose during the cleavage of the glucosidic bonds.

Size and diffusibility of cellulolytic enzymes in relation to the capillary structure in cellulose

The capillary voids permit the diffusion of cellulase molecules of compatible shape and size. The presence of non cellulosic substances around molecule aggregates or between cellulose molecules reduces the diffusibility of the enzymes by reducing their affinity for cellulose. Stone et al (1969) reported that the enzymatic degradation of various cellulose samples of different degrees of swelling (by means of Phosphoric acid) is proportional to the accessibility of cellulose molecules which in turn is controlled by the porosity of the material.

Degree of crystallinity

The resistance of the crystalline cellulose to enzymatic hydrolysis is well established (Norkrans, 1950) (Walseth, 1952 a,b). According to Norkrans (1950) as the

more accessible parts of cellulose are hydrolysed the disaggregated cellulose chain ends show a tendency to re-crystallize with one another thus making the residue more resistant to further hydrolysis.

Unit cell dimensions of the crystallites

The different crystalline forms of cellulose, cellulose II, III, and IV, which arise upon chemical or physical treatment of cellulose I (native cellulose), show different reactivities to cellulolytic enzymes. However, it is difficult to determine if this is only due to the changes in the unit cell dimensions or also due to the differences in the amount of the amorphous cellulose present.

Conformation and steric rigidity of the anhydroglucose units

According to King (1961) the increasing susceptibility of cellulose to enzymatic hydrolysis in the order: crystalline cellulose, amorphous cellulose, water insoluble cellodextrins, water soluble cellodextrins, may not only be due to the increasing physical accessibility of the cellulases to their site of action but also due to the increasing molecular flexibility which could permit more frequent assumptions of chain conformations suitable for the optimum positioning of the enzyme on cellulose. Experimental verification of this hypothesis remains to be investigated (Cowling and Kirk, 1976).

Degree of polymerization of the cellulose

The D.P. is of significance in the case of endwise acting components. Below D.P.6 the molecules loose their structural relationship and become soluble with a con-

siderable increase in susceptibility to enzymatic hydrolysis which however is mainly due to the solubilization than to shortening of cellulose molecules.

Nature of substances with which the cellulose is associated and the nature of that association

. The main substances are: lignin, hemicellulose (see 1.2.3 ) mineral constituents and extraneous materials. Mineral constituents are essential for the growth and development of cellulolytic microorganisms. Extraneous materials include a variety of organic substances soluble in neutral solvents as alcohol, acetone, benzene, ether, methanol and water. Soluble carbohydrates and vitamins promote the growth of cellulolytic microorganisms. Cellulase inhibitors have been found in many plant families and in various parts of the plants. They usually are more effective against the $C_1$ than the $C_x$ component, many of these substances are phenolic in nature (Mandels and Reese, 1965). The deposition of various materials within the fibre structure decrease the accessibility of cellulose to cellulolytic enzymes.

The nature concentration and distribution of substituent groups

Soluble cellulose derivatives circumvent the action of $C_1$ component by providing isolated chain molecules in solution. The susceptibility of cellulose derivatives increases with increasing solubility and D.S. up to the point of complete solubilization, usually at D.S. from 0.5 to 0.7. After this point susceptibility decreases with increasing D.S. and at D.S. about 1.0 the substrate is unaccessible to cellulolytic enzymes.

Generally the close contact between cellulolytic enzymes and cellulose is determined by the substituent group's size, number of glucose units and affinity for water.

1.2.6 Pretreatment of cellulosic materials

The features of cellulosic materials which inhibit the action of cellulolytic enzymes can be modified by various methods, alone or in combination, by using cellulose solvents, swelling agents, steaming, heating, radiation, grinding (Millett et al, 1975). Because the effectiveness of various pretreatments on the enzymatic or microbial digestion varies depending on the substrate, the optimum pretreatment must be established for each type of cellulosic material (Han and Callihan, 1974).

The application of high pressure saturated steam to fibre fraction of cattle manure (Nesse et al,1977) or to aspen wood (Macdonald and Mathews, 1979) promoted their enzyme hydrolysis. Han and Callihan (1974) reported that this treatment promoted the fermentation of rice straw or sugarcane bagasse by cellulolytic microorganisms only when the substrate was treated by Sodium hydroxide.

Heat treatment alone has negative effects on cellulose hydrolysis because it leads to a decrease in porosity (Stone et al, 1969) (Ghose and Kostick, 1969). When heat treatment is followed by ball milling the enzymatic hydrolysis is increased (Ghose and Kostick, 1969). Improvement in hydrolysis of wood cellulose by high temperature milling has also been reported by Katz and Reese (1968).

Mandels et al (1974) do not recommend heating of cellulosic materials because of the possibility of undesirable cross reactions with the non-cellulosic

substances.

Irradiation can degrade cellulose especially in the presence of a sensitizer agent (Phillips and Arthur, 1964). Reese et al (1956) reported that γ-rays in high doses increased the cellulose susceptibility to degradation whereas low doses produced the opposite effect. The fermentation of cellulosic wastes by Aspergillus fumigatus was improved when the substrate was subjected to nitrite accelerated photodegradation. This pretreatment was more effective than alkali swelling, electron radiation or high temperature hydrolysis, (Rogers et al, 1972). However, Fookson and Frohnsdorff (1973) reported that nitrate accelerated photodegradation was a less effective pretreatment than alkali swelling for the growth of Akaligenes faecalis and Cellulomonas sp. on pure cellulose.

The fine structure of cellulose can be altered by cellulose solvents as concentrated acids or alkalis, liquid ammonia, solutions of cuprammonium or cupriethylene salts, cadoxene,or by swelling agents as dilute solutions of cellulose solvents, dimethyl formamide, dioxane, (Warwicker et al, 1966).

Alkali swelling or dissolving and regenerating cellulose has been used as pretreatment to enhance enzymatic hydrolysis (Sasaki et al, 1979) (Mandels et al, 1974) or microbial fermentation of cellulosic materials by Cellulomonas sp.(Han and Callihan, 1974) (Callihan and Dunlap, 1971). Swan (1977), Peitersen (1975), Moo-Young et al (1978) reported improvement in bioconversion of wood sawdust by acid or alkali pretreatments. Increased susceptibility to microbial or enzymic conversion has been

reported for cellulosic substrates subjected to chemical pulping and bleaching (Baker et al 1973) (Andren et al 1976).

Caulfield and More (1974) measured the degree of crystallinity of mechanically treated (ball-milled) cellulose before and after partial hydrolysis. They reported that ball milling increased the enzyme reactivity of both the crystalline and amorphous parts of the substrate. They also reported that the reactivity of the crystalline component of cellulose was increased to a greater extent than the reactivity of the amorphous component.

They concluded that the overall increase in reactivity is due more to the decrease in the size of cellulose particles and increase in their available for enzyme contact surface area, than to the reduction in their crystallinity.

Mandels et al (1974) evaluated the effect of different chemical and mechanical pretreatments on the enzymatic hydrolysis of waste newspaper and found that ball milling was the most effective pretreatment. A new milling technique increasing newspaper susceptibility by 125% over the untreated control has been reported by Tassimari and Macy (1977). In this work, the substrates used for the microbial fermentation of cellulosic wastes were pretreated by ball milling. Pretreatments used by other workers for the production of biomass from cellulosic substrates are given in the 'Results and Discussion' section (Tables 14, 15).

## 1.3 Biological degradation of cellulose

The ability of microorganisms to ferment diverse materials into biomass and a variety of by-products has long been recognized and exploited. Substrates as carbohydrates, alcohols, lipids, hydrocarbons, organic acids have been found suitable for their growth. Production of alcohols, organic acids, antibiotics, vitamins, enzymes, microbial protein and steroids has gained industrial importance (Hospodka, 1966)(Righelato and Elsworth, 1970) (Moo-Young, 1975).

Cellulose viewed as fermentable carbohydrate presents special problems. It is insoluble, it can be utilized only by microorganisms producing cellulolytic enzymes and it is resistant to the action of these enzymes because of its crystallinity and association with lignin. Cellulose is hydrolysed to metabolizable products before entering cell metabolic pathways (Callihan and Dunlap, 1971). The role of microorganisms in the decomposition of cellulosic materials was recognized as early as 1883 by Hoppe-Seyler. Omelianskii (1902) pioneered much of the early work on the fermentation of cellulose by bacteria to form gases, acids and humus. Van Iterson (1904) presented experimental evidence of cellulose decomposition by fungi. Pringshein and Lichtenstein (1920) used Aspergillus fumigatus to upgrade the nutritional value of straw. However, the slow growth of microorganisms on cellulosic materials prevented their economical exploitation.

More recently the isolation of new cellulolytic microorganisms or the development of mutants with higher

growth rates and higher cellulolytic activities and the improvement in pretreatment methods and fermentation processes has increased the prospect of a profitable cellulose fermentation (Callihan and Dunlap, 1971), (Mandels, 1975) (Chahal and Hawksworth 1976) (Kristiansen, 1978).

The cellulolytic enzymes involved in cellulose hydrolysis, their mode of action and factors which affect their production and activity are discussed in parts 1.3.1 to 1.3.4. The production of SCP from cellulolytic microorganisms and the type of reactor used in this work are discussed in parts 1.3.4 and 1.3.5.

### 1.3.1 Cellulolytic enzymes

The degeneration of β-1,4-glucan, cellulose is a complex process requiring the synergistic action of a group of enzymes, collectively called cellulases, which have the ability not only to hydrolyse the β-1,4 glucosidic bond which forms the individual cellulose chains but also to disrupt the crystalline cellulose structure. A significant step in the research on the enzymatic degradation of cellulose was the observation that culture filtrates from the cellulolytic fungi Trichoderma reesei and Trichoderma koningii could extensively hydrolyse native cellulose (Mandels and Reese, 1964) (Halliwell, 1965a). The subsequent numerous studies on the purification and activity of the purified components established that at least three types of cellulolytic components, with different acting patterns and narrow range of substrate specificity, are required for any appreciable hydrolysis of native cellulose: the $C_1$ component, the $C_x$ component and β- glucosidases. (Mandels and Reese, 1964) (Li et al, 1965) (Halliwell, 1965b)(Selby and Maitland, 1965,1967)(Reese,1975)

(Berghem, 1974). The components are not single proteins since several isoenzymes differing in properties have been fractionated (Enari and Markkanen, 1977) (Halliwell, 1979).

β-glucosidase or cellobiase, E.C. 3.2.1.21

Cellobiase acts on soluble cellodextrins from cellobiose to celloexose liberating glucose units (King and Vessal, 1969) (Reese, 1975). Three cellobiase fractions have been reported in T.reesei by Gong et al (1977). Glucose has been reported as an inhibitor of cellobiase at 5 mM (Gong et al, 1977) in contrast Maguire (1977) reported that glucose at 4.85 mM acted as activator during the hydrolysis of cellobiose by cellobiase. Cellobiase is usually measured from the glucose produced by its action on cellobiose (Selby and Maitland, 1967).

Exo-glucosidase E.C.3.2.1.74

It has been called and exo-glucanase and is considered by some workers as a member of the $C_x$ component. (Mandels and Reese, 1964) (King and Vessal, 1969). Exo-glucosidase acts by an endwise mechanism liberating glucose units from the non reducing end of the cellulose chain. It acts by inversion of configuration, i.e. produces a-glucose units. According to Halliwell (1979), a highly purified exo-glucosidase from T.koningii did not show any activity either on CMC or on insoluble cellodextrins.

Cellobiase and exo-glucosidase have common substrates from cellobiose to celloexaose. Cellobiase hydrolyses more rapidly the dimers and trimers and it is less

effective on longer chains, exo-glucosidase shows the opposit effect (Reese, 1969) (Reese et al, 1968)(King and Vessal 1969).

Glucose has been reported as a competitive inhibitor of exo-glucosidase. At 50 μM the hydrolysis of smaller oligomers cellobiose and cellotriose was particularly inhibited. The affinity of exo-glucosidase for the higher cellodextrins may be due to the formation of inhibitory hydrolytic products (Halliwell, 1979).

Exo-glucosidase is usually measured from the glucose produced by its action on soluble cellodextrins. In the presence of cellobiose it can be differentiated by its preference for the higher oligomers (Reese, 1969).

CM-cellulase or $C_x$ component or endo-glucanase E.C.3.2.1.4

Endo-glucanase acts on soluble cellulose derivatives or on cellulose which has been rendered susceptible to enzymatic attack by physical or chemical treatment as Phosphoric acid-swollen cellulose, but not on crystalline cellulose (Wood, 1972). Its action is of random nature, the terminal linkages being less susceptible to hydrolysis (King and Vessal, 1969)(Bergham, 1974). A CM-cellulase with β-glucosidase activity derived from T.viride was reported by Shikata and Nisizawa (1975). This enzyme was able to hydrolyse CM-cellulose to cellobiose and cellobiose to glucose but was inactive on crystalline cellulose. Okada (1976) reported a CM-cellulase, from T.viride, able to hydrolyse oligosaccharides mainly to cellobiose and glucose, similar attack was observed on CMC. Avicel, cotton and filter paper produced reducing sugars. Kanda

et al (1976) reported three types of CM-cellulase activity in Irpex lacteus culture filtrates. The purified CM-cellulases were able to hydrolyse celloexaose and CMC to cellobiose and cellotriose. Phosphoric acid-swollen cellulose and Avicel were hydrolysed likewise but they produced also smaller amounts of cellotetraose and glucose. The activity of the three CM-cellulases differed on the relative degree of hydrolysis of CMC and Avicel.

Nissizawa et al (1972) reported CM-cellulase activity ($C_x$) from I.lacteus, on cellodextrins above cellotriose, CM-cellulose, Avicel and cellulose powder. The hydrolysis products being mainly cellobiose and smaller amounts of glucose, cellotriose and cellotetraose. The CM-cellulase, from a commercial Trichoderma viride cellulase was slightly active on cellulose in producing reducing sugars, but it was active in reducing its degree of polymerization. Synergism between $C_x$ and $C_1$ was observed towards cellulose but not towards CM-cellulose.

CM-cellulases with the ability to produce short fibres from crystalline cellulose have been reported by Berghem et al (1976), Halliwell and Riaz (1970). However, further purification of the short-fibre forming CM-cellulase revealed the presence of two components, one with CM-cellulase activity and no short-fibre forming activity and another (called $C_2$) with ability to form small amounts of short fibres from native cellulose. The activity of $C_2$ increased considerably in the presence of the CM-cellulase component from which it has been separated (Halliwell and Riaz, 1971).

Various CM-cellulase fractions have been reported

in enzyme preparations obtained from the same microorganism. Halliwell and Riaz (1970) reported two fractions in T. koningii whereas Wood (1968) reported four fractions. Up to twelve fractions have been reported in Myrothecium verrucaria (Halliwell, 1979). Nissisawa (1972) reported one fraction in I.lacteus whereas Kanda et al (1976) reported three fractions. CM-cellulase is measured on soluble cellulose derivatives as CM-cellulose either from the decrease in viscosity (Eriksson, 1969) or from the production of reducing sugars (Mandels and Weber, 1969). Measurement of viscosity decrease is a very sensitive assay because even a few random breaks in a chain cause a considerable decrease in the average DP. Measurement of the reducing sugars is less sensitive especially in the presence of other cellulolytic components.

$C_1$ component or exo-glucanase or cellobiohydrolase, E.C. 3.2.1.91

Acts by an endwise mechanism liberating cellobiose units from the non reducing end of cellulose chains which have been liberated by the action of $C_x$. Its cellulolytic activity when acting alone is very low. The full potential of $C_1$ is attained in the presence of $C_x$ and cellobiase (Halliwell, 1979) (Halliwell et al, 1972) (Halliwell and Riaz, 1971) (Petterson, et al 1972) (Berghem and Petterson, 1973) (Halliwell andGriffin, 1973) (Wood and McCrae, 1972). Purified $C_1$ component from T.viride showed no action on cellobiose or CMC and it could solubilize cotton to 1-6%. A significant increase, 3 to 5-fold, in cotton solubilization occurred when $C_1$ acted

along with the $C_x$ component (Wood, 1968) (Selby, 1968). According to Berghem and Pettersson (1973), $C_1$ purified from T.viride acted on native cellulose, microcrystalline cellulose, Phosphoric acid-swollen cellulose and cellote traose by succesively releasing terminal cellobiose units. It was, however, sensitive to substitution on the cellulose chain and was inactive towards CMC. A purified $C_1$ component from Sporotrichum pulverulentum acted in a different way. Acting on Avicel, it could release glucose,cellobiose,cellotriose and cellotetraose in a relative ratio at 1:9:1:1 respectively. A similar pattern was observed in the hydrolysis of soluble cellodextrins (Streamer et al, 1975).

Nissizawa et al (1972) reported $C_1$ (Avicelace) activity from I.lacteus on cellodextrins above cellotriose, CM-cellulose, Avicel, and cellulose powder. The hydrolysis products being mainly cellobiose and smaller amounts of glucose,cellotriose and cellotetrose. The $C_1$ activity from a commercial T.viride cellulase was active on Avicel, cellobiose being the main product. The activity of $C_1$ towards Avicel degradation was enhanced in the presence of $C_x$.

Cellobiose is a competitive inhibitor of $C_1$ component. The degradation of microcrystalline cellulose by $C_1$ increased considerably when cellobiose was continuously removed or when cellobiase was added (Berghem et al, 1976).

Various $C_1$ fractions have been reported from the culture filtrates of different microorganisms. Gum and Brown (1977) reported four fractions in T.reesei which were always associated with weak CM-cellulase activity. Wood and

McCrae (1972) reported two fractions in T.koningii and four $C_1$ fractions were reported by Wood and McCrae (1977) in Fusarium solani.

The characterization of $C_1$ as cellobiohydrolase is not generally accepted. Reese (1975 and 1976) considers $C_1$ as a member of the $C_x$ random acting enzymes which is able to initiate the enzymatic attack on crystalline cellose by splitting both β-1,4 linkages and hydrogen bonds but unable to act on CMC or on products of its own action. The combined activity of $C_1$ and β-glucanase is usually measured by the production of reducing sugars or loss of residue weight from solid cellulose. The role of $C_1$ becomes more important as the resistance of substrate increases. Cotton is considered to be the most resistant cellulose and the best indicator of $C_1$ activity (Mandels and Reese, 1964) (Mandels and Weber, 1969). The activity of $C_1$ component is also affected by the presence of cellobiase (Enari and Markkanen, 1977).

Other cellulolytic enzymes

In 1974, Westermark and Eriksson detectedin the culture filtrate of Sporotrichum pulverulentum a new wood-degrading enzyme, cellobiose: quinone oxidoreductase, which is most likely to be of importance in cellulose degradation. Eriksson et al (1974) reported the existence of a cellulose oxidizing enzyme in the cell-free culture filtrates of S. pulverulentum, Polyporus adustus, M.verrucaria and T.viride. This enzyme is believed to participate in the degradation of crystalline cellulose by inserting uronic acid moieties into the cellulose, breaking the hydrogen bonds between the

cellulose molecules. However, this enzyme has not yet been purified in sufficient amounts to verify this hypothesis (Enari and Markkanen , 1977).

The stability and activity of cellulolytic enzymes is affected by pH and temperature. The optimum temperature for cellulase activity for reactions of 1 to 24 h duration is about 50$^{o}$ (Mandels and Reese, 1965). The thermal stability of the different cellulolytic components varies. Wood (1975) reported a 10% loss of the original $C_x$ activity of T.koningii after 4 hours at 60$^{o}$C and pH5 whereas the $C_1$ and β-glucosidase activity loss was 80%. The optimum pH and temperatures for cellulolytic activity of some cellulases obtained from various microorganisms are given in Table 8.

Table 8 pH and temperature activity optima of various cellulases

| Microorganism | pH | $^{o}$C | Reference |
|---|---|---|---|
| Chaetomium thermophile | 5 | 58 | Eriksen and Goksoyr, 1976 |
| Fusarium moniliforme | 4.5 | 60 | Matsumoto et al, 1974 |
| Myrothecium verrucaria | 5.0 | | Halliwell, 1961 |
| Sporotrichum thermophile | 5.0 | | Couts and Smith, 1976 |
| Thermomonospora fusca | 6.0 | 65 | Stutzenberger, 1971 |
| Trichoderma koningii | 3.5-5 | 55 | Iwasaki et al, 1965 |
| Trichoderma viride | 4.5-5 | 60 | Okada, 1975 |
| Sporotrichum pulverulentum | 5 | | Eriksson and Larsson, 1975 |

1.3.2. Mode of action of cellulases

In 1950, Reese and co-workers proposed that a multi enzyme cellulolytic system isnecessary for the conversion of native cellulose to soluble sugars (Figure 9a). This hypothesis was based on the observation that many microorganisms were able to utilize modified cellulose or cellodextrins for growth but were unable to utilize native cellulose, these organisms were called non-cellulolytic. Other microorganisms called cellulolytic, were able to utilize both types of substrate. It was suggested that both groups possessed cellulolytic enzymes, called $C_x$, which could hydrolyse the β-1,4 glucosidic bonds in a series of steps, but only the cellulolytic organisms possessed an additional factor called $C_1$ which could act in an undefined way, possibly by disrupting hydrogen bonding, and could convert the structure of cellulose in a way that made available the β-1,4 linkages to $C_x$.

The purification and fractionation of the cellulase components from culture filtrates of different micro-organisms (Reese,1975) and the study of the action of purified components alone and in different combinations (Flora,1965)led to modification of the original $C_1$-$C_x$ concept and in the development of other hypotheses about the mode of action of cellulase complex, Figs 9(bc) and 10.

In 1975, Reese proposed a modification of the $C_1$-$C_x$ concept, Figure 9b. According to this concept, $C_1$ is a member of the $C_x$ enzymes, a random acting endo-glucanase, active on crystalline cellulose by hydrolysing β-1,4 linkages and disrupting the hydrogen bonding between

Figure 9. Proposed mechanisms for the enzymatic degradation of cellulose

a. According to Reese et al (1950)

Crystalline cellulose $\xrightarrow{C_1}$ Amorphous cellulose $\xrightarrow{Cx}$ Soluble products capable of diffusion into the cells

Non cellulolytic microorganisms

Cellulolytic microorganisms

b. According to Reese (1975)

Crystalline cellulose $\xrightarrow{C_1}$ Modified cellulose

- Endo-enzymes → Oligomers
- Cellobiohydrolase → Cellobiose
- Glucohydrolase → Glucose

and/or Cellobiase

c. According to Petterson (1975)
from Enari and Markkanen (1977)

Crystalline cellulose $\xrightarrow{\text{Endoglucanase}}$ Cellulose$^r$

Cellulose$^r$ $\xrightarrow{\text{Exoglucanase}}$ Cellobiose

Cellobiose $\xrightarrow{\beta\text{-glucosidase}}$ 2 Gluose

---

Cellulose$^r$: Formed by the action of Endoglucanase on the amorphous regions. Free chain ends are created.

cellulose chains. $C_1$ is not able to act on CMC, or on products of its own action, since it cannot produce soluble products from crystalline cellulose. The $C_x$ enzymes hydrolyse the liberated chains making thus possible further action of $C_1$ on the underlying crystalline structure.

According to Humphrey et al (1976) cellulose hydrolysis takes place at selective sites which disappear as the hydrolysis proceeds. These sites are thought to be edges due to surface roughness and are called active sites in contrast to smooth areas considered as inactive sites.

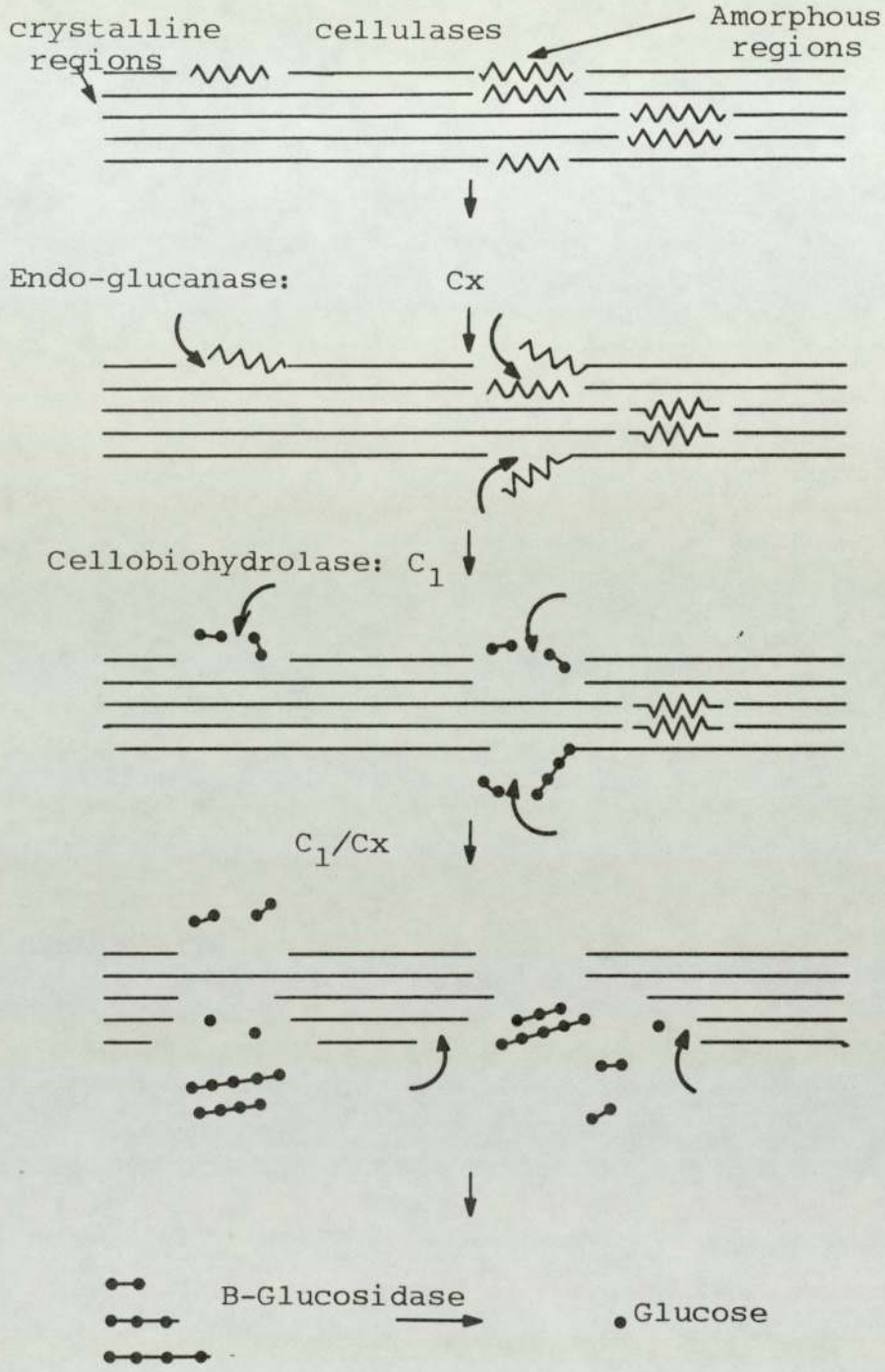
Chahal et al (1976) by examining the degradation pattern of cellulose pulp and crystalline cellulose by the cellulases of T.reesei and Chaetomium cellulolyticum found evidence that during the initial stage of hydrolysis the $C_x$ shortens the fibres by attacking the originally present transverse fissures of the fibres and the amorphous regions. In the later stages the $C_1$ disrupts the hydrogen bonding and van der Waals forces and splits the fibrils along the longitudinal fissures preferably at the fibre ends where the $C_x$ enzymes are already active.

According to Halliwell and Riaz (1970, 1971), the enzymatic degradation of cellulose is initiated by the action of $C_x$ component (CM cellulase + $C_2$) which produces short fibres further hydrolysed by cellobiase and cellobiohydrolace. Wood and McCrae (1972), Wood (1972), Eriksson and Petterson (1972), Berghem (1974), Petterson et al (1972) proposed a similar mechanism according to which $C_x$ initiates the hydrolysis of native cellulose by attacking less ordered regions and the hydrolysis is proceeding through the action of $C_1$ which removes cellobiose units

from the free-end chains, (Figure 9c).

Reese (1975, 1976) criticised the new concept mentioned above and the view that $C_1$ and cellobiohydrolase are identical on the grounds that: 1) The fact that cellobiohydrolase co-chromatograms with $C_1$ is not sufficient evidence that $C_1$ and cellobiohydrolase are identical. [However until fractionation of the $C_1$ component as characterized by Reese (1975, 1976) its existence remains tentative]. 2) Purified cellobiohydrolase and $C_x$ from the cellulolytic bacterium Cellvibrio gilvus (Storvick and King, 1960) acting together on native cellulose produced a negligible hydrolysis. [There are however a number of reports in the literature showing strong action on crystalline cellulose]. 3) If $C_x$ acts first the reaction could easily go to completion without the participation of cellobiohydrolase ($C_1$). 4) Before the formation of soluble products there is a number of changes in crystalline cellulose which are not likely to be mediated by the properties of $C_x$. These changes are: 4a) Increased swelling in Sodium hydroxide (Reese and Gilligan, 1954). [However, in Mandels and Reese (1964) this change is reported to be associated more with the $C_x$ than the $C_1$]. 4b) Trasnverse cracking and spiral fissures (Marsh, 1957). [However, Chahal et al (1976) suggest that fissures and cracking in cellulose fibres reported as a result of enzymatic action may well have been there before the addition of the enzyme. Although they support the concept of $C_1$ as able to split hydrogen and van der Waals bonds they suggest that the action of $C_1$ follows the action of $C_x$]. 4c) Loss in tensile strength [However, according to Selby (1963) this change may be due

Figure 10. Shcematic representation of sequential stages in cellulolysis. Flickinger (1980).



to a factor isolated from the $C_x$ component]. 4d) Decrease in D.P. and increase in the alkali solubility. 4e) Fragmentation. [However short-fiber forming activity has been reported either as due to a separate factor $C_2$ (Halliwell, 1969) or due to $C_x$ (Berghem, 1974)]. 4f) Decrease in moisture regain. 5) When $C_1$ and $C_x$ are acting on native cellulose in the presence of methocel, which inhibits the action of $C_x$, a build-up of alkali soluble cellulose occured (Reese 1975). This indicates that $C_1$ acts first and the alkali soluble products accumulate because of the inability of $C_x$ to hydrolyse them to soluble oligomers.

Although much is known about the nature of the purified cellulolytic components the mechanism of cellulose degradation is not yet clearly understood especially the initiation of crystalline cellulose degradation. The cellulolytic mechanisms proposed vary with substrate type, enzyme source, degree of enzyme purification and conditions of the assays. Moreover, the possibility of still undetected enzymes which participate in cellulose degradation cannot be ruled out (Enari and Markkanen, 1977).

### 1.3.3. Cellulases production

The production of the different cellulase components during growth of cellulolytic microorganisms is affected by the type of substrate and the conditions of growth.

The crystallinity of cellulose affects the relative production of $C_1$ and $C_x$ enzymes (Mandels and Weber, 1969) (Mandels et al, 1974). Rautela and King (1968) found that T.viride grown on various crystal forms of cellulose (Cellulose I, II, II/IV, IV) produced enzymes which were preferentially capable of solubilizing the homologous forms.

Cellulolytic enzymes are strongly adsorbed by insoluble cellulose. Huang (1975) reported that half of the cellulase activity was rapidly adsorbed by cellulose and released in the liquid phase as the substrate was hydrolysed. Strong adsorption of cellulase ($C_x$) has been also reported by Eriksen and Goksoyr (1976). Prolonged disruption of the mycelium and residual cellulose did not liberate $C_1$ component in S.thermophile fermentations, (Coutts and Smith, 1976). Berg and Petterson (1977) in T.viride fermentations found cellulase activity in the filtrate only after cellulose has been degraded although differential analysis of bound cellulase activity and cell protein indicated that enzyme production was growth associated and no repression had occurred. A linear increase in cellulase adsorption with increasing cellulose concentration has been reported by Mandels et al (1971), Wilke and Yang (1975), Eriksen and Goksoyr (1976).

The production of cellulolytic enzymes is affected by the concentration of the insoluble cellulose present. According to Coutts and Smith (1976), $C_1$ activity decreased in S.thermophile fermentations when cellulose concentration was higher than 2%. $C_x$ activity was not affected to the same extent. Mandels and Weber (1969) found that the optimum cellulose concentration for cellulase production by T. reesei batch fermentations was from 0.5 to 1%. Increased cellulase yields at higher cellulose concentrations, when the pH of the fementation was controlled above 3.0, have been reported (Sternberg, 1976a).

Mandels and Reese (1960) reported that during growth of T.reesei on easily fermentable carbon sources, cellulase

formation was repressed and it was detected in the medium only after most of the carbon source had been consumed. They found that the repression could be prevented by slowing the growth rate of the fungus using suboptimum growth conditions as excess Cobalt, deficiencies of mineral nutrients as Calcium, Magnesium or trace metals, suboptimum temperature and aeration. Rapid metabolism of sugars also caused disappearance of preformed cellulases. This effect has been related to the developed low pH which below 3.0 causes enzyme inactivation (Mandels and Andreotti, 1978). β-glucosidase is more sensitive to low pH than saccharifying cellulase ($C_1$ + $C_x$) (Mandels et al, 1975).

Addition of easily assimilated substances as glucose glycerol, peptone and surfactants as Tween 80 or Sodium oleate affect cellulase production. Their positive or negative effect depends on their concentration (Mandels et al, 1974) (Griffin et al, 1974) (Reese and Maguire 1971). The mechanism of enhancement of cellulase production by surfactants may be related to increased permeability of the cell membrane which allows for more rapid cellulase secretion which in turn leads to greater enzyme synthesis (Sternberg, 1976b). Surfactants usually inhibit cellulase action. This may be to their detergent activity which prevents the absorption of cellulases by cellulose (Romanelli et al, 1975). According to Mandels and Weber (1969), cellulases in fungi are adaptive enzymes. For a fungus growing on cellulose the true inducers are the soluble products of hydrolysis especially cellobiose (Mandels and Reese, 1960). T.viride produced cellulases only when grown on cellulose, on glucans with β-1,4 linkage

and on a few oligosaccharides (Mandels and Reese, 1957, 1960 and 1962). Sophorose a β-1,2 glucoside is a powerful inducer for T.reesei (Mandels and Weber, 1969). Nisizawa et al (1971 a, b) found that sophorose at low concentration ($10^{-5}$ - $10^{-3}$M) was a strong cellulase ($C_x$) inducer in T.viride acting independently of mycelium age. Glucose,maltose,fructose and glycerol inihited cellulase formation by sophorose in vivo but at the same concentration ($10^{-2}$M), they were not inhibitory to the cellulase activity in vitro. Their effect was suggested to be a catabolite repression of enzyme formation. Sophorose above a certain concentration (>$10^{-3}$M) strongly inhibited cellulase formation. It was suggested that some amounts of sophorose are split by β-glucosidase to produce glucose which eventually accumulates and represses cellulase formation. Cellobiose and cellooligosaccharides were found to possess little or no effect on the inductive formation of cellulase. Nissizawa et al (1971 a, b) suggested that the formation of cellulase in T.viride is controlled by a repressor-inducer mechanism in which sophorose participates as an inducer.

Sternberg (1976b) also suggests that the reported induction of cellulases by cellobiose may not be affected by cellobiose directly but by formation of transfer products.

Higley,(1973) reported $C_x$ production by the brown-rot fungi Poria monticola, Lentinus lepideus and Lenzites trabea when grown on simple sugars or non cellulosic polysaccharides and suggests that in these funi $C_x$ is constitutive. In the white-rot fungi Polyporous versicolor, Ganoderma applanatum and Peniophora sp. $C_1$ and $C_x$ enzymes

were induced only on cellulose containing media and their production was repressed by the addition of glucose or cellobiose.

Hulme and Stranks (1970, 1971) reported cellulase production by M.verrucaria irrespectively of the carbohydrate source in the medium by reducing the growth rate of the fungus. They observed however an apparent need for some rapid growth of the cultures before any significant cellulase production. Cultures restricted to a low growth produced low enzyme yields. They suggested that cellulase is a constitutive enzyme and its formation simply required the removal of catabolite repression.

Many workers have not found a direct relationship between microbial protein and cellulase activity in the culture filtrate (Chahal and Dhaliwal, 1973) (Eriksson and Larsson, 1975). This may occur if cellulases are cell bound or because absorption of the enzyme on the substrate is so strong that a significant part of its activity cannot be measured in the filtrate. Crawford and McCoy (1972) Yoshikawa et al (1974), Eriksen and Goksoyr (1976), Humphrey et al (1976) observed growth associated cellulase production. Several reports have appeared recently on continuous cellulase production by T.reesei in STR. Single stage processes with cellulose as carbon source have been reported by Mandels et al (1975) and Peitersen (1977). Ghose and Sahai (1979) used cellulose as substrate in a single-stage process with cell recycle. Brown et al (1975) reported a single-stage continuous production on commercial glucose containing traces of sophorose and Ryu et al (1979) reported continuous production in a two-stage process using

lactose as the sole carbon source..Mitra and Wilke (1975) used a two-stage system for continuous cellulase production by T.reesei. The first stage was utilizing glucose for growth only and the second stage utilizing pure cellulose for enzyme induction. They developed a model which relates enzyme production, dilution rate and biomass in the induction stage:

$$P = \frac{k_E X\ f(\theta)}{D_2}$$

where P = enzyme production, filter paper units/ml

$k_E$ = specific enzyme production rate, $\frac{F.P.U}{mg\ biomass}\ h^{-1}$

X = biomass in the induction stage, mg/ml

$f(\theta)$ = fraction of cells remaining in the induction stage for a time greater than the adaptation time required for enzyme synthesis

$D_2$ = dilution rate in the cellulose stage, $h^{-1}$

This model however is highly speculative and further research is needed for a more comprehensive interpretation of this type of enzyme induction (Mitra and Wilke, 1975).

The complexity of any model describing cellulases production is further increased because of the possibility of still undetected enzymes which participate in cellulose degradation (Enari and Markkanen, 1977) and because the known cellulase components are not easily measured and have strong synergistic effect on each other.

1.3.4. Fermentation of cellulosic materials

Many microorganisms although they are able to degrade cellulose, they have not been found suitable for SCP production (Chahal and Gray, 1969) (Updegraff, 1971) (Mandels et al, 1975). Thus one of the main factors which determine the efficiency of a process producing SCP from cellulose is the selection of a suitable microorganism which must be also able to grow on simple inexpensive media, is non toxic and can be easily harvested. Bacteria have a potential advantage over fungi because of their faster growth rate and higher crude protein content. The advantages of fungi are that they have simpler nutritional requirements, the mycelia have a natural occuring texture, are easier to harvest and utilize as feed supplement, and they have a lower nucleic acid content (Rolf and Spicer, 1973) (Worgan, 1973).

According to their oxygen and temperature requirements for growth, cellulolytic microorganisms for SCP production can be divided into aerobic or anaerobic, mesophilic (operating range $20^{o}$-$45^{o}$C) or thermophilic (operating range $45^{o}$-$60^{o}$C) (Bellamy, 1974).

Anaerobic mesophilic

The fermentation of cellulosic materials by the rumen microorganisms is the oldest, largest and until now the most effective method for conversion of cellulose to human food. Anaerobic bacteria including Bacterioides succinogens, Butirivibrio fibrisolvens, Clostridium locheandii, Cillobacterium cellosolvens, Cellulomonas fimi, Ruminococcus flavefaciens have been found as the major agents of cellulose digestion (Bellamy, 1969). However, the cellulosic materials are not

utilized effectively due to the inability of anaerobic microorganisms to utilize lignin and the inhibitory effect of lignin seal on cellulose degradation (Bellamy, 1974,1977).

The use of bacteria for direct production of SCP suitable for cattle feed has been proposed by Coe and Turk (1973). In this case the microbial protein is a by-product of methane production from feedlot wastes. Continuous cellulose degradation by rumen microorganisms has been reported by Gray (1978).

Anaerobic thermophilic

Anaerobic thermophilic bacteria play an active role in composting. They cannot utilize lignin. Cellulose fermentation by mixed bacterial population in batch cultures has been reported by Olson et al (1937), Hajny et al (1951) and in continuous culture by Hall (1960). Cellulose hydrolysis and ethanol production by Clostridium thermocellum has been reported by Cooney et al (1978). The author is not aware of any programme of anaerobic thermophilic fermentation for SCP production.

Aerobic mesophilic

Most of cellulosic materials in nature are recycled by aerobic mesophilic microorganisms (Molina, 1968). Most of the known cellulolytic fungi belong to this group. All filamentous fungi appear to be aerobic although oxygen requirements vary with species and growth conditions (Berry, 1975). Cellulolytic microorganisms which have been used for SCP production include, bacteria Cellulomonas sp (Han and Callihan, 1974), Brevibacterium sp (Fu and Thayer, 1975),

and fungi A.fumigatus (Rogers et al, 1972), Chaetomium cellulolyticum (Moo-Young et al, 1977), M.verrucaria (Chahal and Gray, 1970), Trichoderma spp (Chahal and Gray, 1970) (Peitersen, 1975), Sporotrichum spp (Eriksson and Larsson, 1975) (Romanelli et al, 1975) Rhizoctonia solani (Chahal et al, 1975). Continuous fermentation of cellulosic materials has been reported by Callihan and Dunlap (1973) and by Peitersen (1977).

Aerobic thermophilic

Cellulolytic activity has been reported in bacteria (Bellamy, 1969), fungi (Cooney and Emerson, 1964) (Tansey, 1971ab) and actinomycetes (Fergus, 1969) (Bellamy, 1974). Cellulolytic microorganisms used in SCP production from cellulosic materials include Bacillus spp (Bellamy, 1969) Thermoactinomyces spp (Bellamy, 1974) (Humprey et al 1977) Thermomonspora fusca (Crawford et al, 1973) S.thermophile Chaetomium thermophile (Barnes 1972, 1974).

Substrate degradation and SCP production is also influenced by the type of process (liquid or solid-based) selected for fermentation of cellulosic materials. In solid fermentations only part of the assimilable carbohydrate is immediately available to the microorganisms. Size reduction and moisture content of the substrate and even dispersion of inoculum and nutrients are affecting growth and substrate utilisation. The filamentous form of cellulolytic fungi and actinomycetes makes them the preferred microorganisms for solid fermentation ( Forage , 1979). Compared with liquid

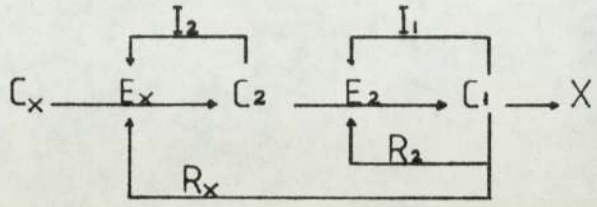
fermentations, solid-based processes represent an increase in terms of substrate concentration per unit volume of reactor and usually are simpler and require less energy input. However, protein yields and productivities are usually lower (Forage, 1979) (Pamment et al, 1978ab, 1979) (Barnes, 1974) (Barnes et al, 1972).

In liquid-based fermentations mass transfer of oxygen and nutrients from the liquid phase to the microorganism are more efficient. When fungi are used the efficiency of the process is affected by their morphology (pellet, floculant, filamentous), which mainly depends on the physical conditions within the reactor, the type and size of inoculum, the growth medium and the fungal strain (Whitaker and Long, 1973) (Metz and Kossen, 1977). Control of the fungal morphology is important if the mycelium is to be used as protein source. Large pellets although easier to harvest usually contain highly vacuolized degenerated cells with a lower protein content than cells in the filamentous form (Hofsten and Ryden, 1975).

Cellulose viewed as fermentable substrate involves not only the problems related to soluble carbohydrate fermentations but also problems related to the insolubility of the substrate and multiplicity of cellulolytic enzymes (Mandels and Andreotti, 1976). Optimum conditions for biomass production have been found different from those required for cellulase production (Mandels et al, 1975)(Sternberg, 1976) (Andreotti et al, 1977) (Gallo et al, 1978). Cellulose is only a precursor to the substrate (glucose) which is finally metabolized (Humphrey et al, 1977). Glucose production is affected by the amount and type of cellulolytic enzymes produced, inhibition/repression of cellulase synthesis,

product inhibition, substrate type and growth conditions (Mandels and Andreotti, 1976) (Mukkopadhyay and Ghose, 1977) (Peitersen and Ross, 1979) (Vohra et al, 1980).

A model for cellulose fermentation by Thermoactinomyces sp has been proposed by Armiger et al, 1976, and Humphrey et al, 1977:




According to this model the crystalline cellulose ($C_x$) is hydrolysed to cellobiose ($C_2$) by the synergistic action of endo-glucanase and cellobiohydrolase ($E_x$). $C_2$ is hydrolysed to glucose ($C_1$) by cellobiase ($E_2$). $C_1$ is utilized by the microorganism (X) for growth and production of new enzymes $E_x$ and $E_2$. $C_1$ inhibits $E_2$ and also represses the synthesis of $E_x$ and $E_2$. $C_2$ inhibits $E_x$. The following equations were obtained for cellulose degradation, growth rate and enzyme production:

CELLULOSE: $$\frac{dC_x}{dt} = KS\left|\frac{K'S\ E_x}{a + E_x}\right| \frac{I_2}{I_2 + C_2}$$

CELLOBIOSE: $$\frac{dC_2}{dt} = \frac{dC_x}{dt} - \frac{K_2\ E_2\ C_2}{K_M + C_2 + C_1(K_M/I_1)}$$

GLUCOSE: $$\frac{dC_1}{dt} = \frac{K_2\ E_2\ C_2}{K_M + C_2 + C_1\ (K_M/I_1)} - \left(\frac{1}{Y_{x/c_1}}\ \frac{dX}{dt} + mX\right)$$

CELLS: $$\frac{dX}{dt} = \frac{\mu_{max}\ C_1\ X}{K_s + C_1}$$

$E_x$: $$\frac{dE_x}{dt} = Y_{E_{x/x}}\left(1 - \frac{C_1}{R_x + C_1}\right)\frac{dX}{dt}$$

$E_2$: $$\frac{dE_2}{dt} = Y_{E_{2/x}}\left(1 - \frac{C_1}{R_2 + C_1}\right)\frac{dX}{dt}$$

where: $C_1$ = glucose conc$^n$, $gl^{-1}$

$C_2$ = cellobiose conc$^n$, $gl^{-1}$

$C_x$ = cellulose conc$^n$, $gl^{-1}$

$E_2$ = cellobiase conc$^n$, units activity $l^{-1}$

$E_x$ = cellulase conc$^n$, units activity $l^{-1}$

$I_1$ = glucose formation inhibition constant, $gl^{-1}$

$I_z$ = cellobiose formation inhibition constant, $gl^{-1}$

K = constant

K' = absorption coefficient

$K_z$ = turnover number, g/unit activity hr

$K_M$ = Michaelis constant, $gl^{-1}$

$K_s$ = saturation constant, $gl^{-1}$

Y = yield constant

m = maintenance constant $gl^{-1}$ $h^{-1}$

R = repression constant $gl^{-1}$

S = surface area/unit cellulose mass

$\alpha$ = enzyme absorption constant, units

$\mu$ = cell specific growth rate, $h^{-1}$

This model used Monod kinetics to express growth and it is based on the assumption that enzyme production is growth related, cellulose can be represented as spherical particles and it is hydrolysed at selective sites, enzyme activity is inhibited by the products of its reaction and the enzyme generation system is repressed by glucose.

However, this model was useful in predicting general trends in cellulose fermentation and it was not found satisfactory for process control (Armiger et al, 1976).

Although the fermentation of cellulosic materials has been extensively studied its mechanism has not yet been fully

understood. More studies are required especially on the structure and hydrolysis of the substrate, the enzymes involved and regulation of their synthesis which are subjects still debated.

### 1.3.5. The Tower fermenter

The efficiency of a fermentation is influenced by reactor design and operation. It is desirable to have a reactor which is easy to operate, simple to construct and yet gives high productivity.

The Tower or Tubular fermenter has been described by Greenshields et al (1971) as an elongated non-mechanically stirred fermenter through which there is a unidirectional flow of medium or gases. For most purposes the Tower fermenter is operated in a well-mixed mode and it is analogous to the conventional stirred tank reactors, the main differences being in the methods of agitation and aeration. The use of Tower fermenters in the production of microbial biomass and metabolites has been reviewed by Greenshields (1972), Greenshields and Smith (1974), Smith and Greenshields (1974).

Tower fermenters have been used for the production of fungal biomass in batch culture and on media containing either cellulosic materials as carbon source (Barnes, 1974) or soluble carbon sources (Daunter, 1972) (Morris, 1972). The Tower fermenter was further developed for the continuous fermentation of biodegradable materials by filamentous microorganisms or floculent yeasts by Greenshields and Pannell (1974). Pannell (1976) studied extensively the continuous culture of the filamentous fungus A.niger on

sucrose based media and developed the design of the Tower fermenter which has been used in our laboratory to study the continuous culture of filamentous fungi on starch wastes (Spensley, 1977) palm oil wastes (Stockbridge, 1979), whey (Ewen, 1980).

Pannell (1976) reported that the operating characteristics of the Tower fermenter under conditions of excess nutrients differed from those of a stirred tank reactor in that the specific growth rate ($\mu$) of the fungus was found to be equal or less than the dilution rate (D). Steady states were reported at dilution rates up to $7.0h^{-1}$. Complete washout of the organism occured only when the dilution rate was so high that the contents of the fermenter became heterogenous. The reason for the biomass retention has been attributed to the sedimentary nature of the mycelial flocks (Pannell, 1976) and to the formation of air slugs in the fermenter which resulted in a biomass deficient broth in the fermenter effluent, Stockbridge (1979).

The Tower fermenter was chosen for this project because of its simplicity in design and construction and because it had shown good performance in continuous fermentations with media containing insoluble particles (R.N. Greenshields, personal communication). It was also felt that the additional information produced during this project would lead to a better understanding of the operating characteristics of the new fermenter, and to the assessment of its potential value for the fermentation of cellulosic materials.

# SECTION 2

# MATERIALS AND METHODS

## 2.1. Microorganisms used

Five cellulolytic fungi were used in this project. Two strains of Trichoderma viride designated as T.viride 1 and T.viride 2. Two strains of Sporotrichum thermophile designated as S.thermophile 1 and S.thermophile 2. One strain of Sporotrichum pulverulentum.

T.viride 1 and 2 were obtained from the fungal culture collection of our laboratory (culture Nos. D40-1-2 and D40-5-5 respectively). S.thermophile 1 was supplied by Dr. M.R. Tansey (Dept. of Plant Sicence, Indiana University). S.thermophile 2 was supplied by Dr. G. Barnes (N.C.B. Yorkshire Regional Laboratory) and S.pulverulentum was supplied by Dr. A.N. Emery , (Dept. of Chemical Engineering, Birmingham University).

These species have been shown to produce a complete cellulase system active against crystalline cellulose and have been used in submerged fermentations to produce SCP from cellulosic materials (Barnes, 1974) (Chahal and Gray, 1969) (Eriksson and Larsson, 1975). The cultures were maintained on Agar medium slants and subcultured to fresh homologus medium monthly. The composition of Agar medium is given in Table 9 .

## 2.2 Media used

The media used in this study were based on the medium devised by Chahal and Gray (1969) for the production of SCP from cellulosic materials. The composition of the media is given in Table 9.. The newspaper used (Birmingham Evening Mail) contained (W/W): 52.7% Cellulose, 23.1% Lignin, 21% Hemicellulose (Hehir, 1977).

Table 9 Media Composition

| | | Agar | Inoculum | Batch | | Continuous | | | |
|---|---|---|---|---|---|---|---|---|---|
| Agar | $g.l^{-1}$ | 15.0 | - | - | - | - | - | - | - |
| Cellulose[1] | " | 8.0* | 8.0* | 10.0* | - | 7.5** | 10.0** | 15.0** | - |
| Newspaper[2] | " | - | - | - | 15.0* | - | - | - | 15.0** |
| Urea | " | 0.8 | 1.2 | 1.2 | 1.2 | 0.9 | 1.2 | 1.8 | 1.2 |
| Glucose | " | - | 3.0 | - | - | - | - | - | - |
| Peptone | " | - | 0.3 | - | - | - | - | - | - |
| Potassium di-hydrogen phosphate | " | 3.5 | 3.5 | 0.4 | 0.4 | 0.3 | 0.4 | 0.6 | 0.4 |
| Magnesium sulphate | " | 1.0 | 1.0 | 1.0 | 1.0 | 0.75 | 1.0 | 1.5 | 1.0 |
| Zinc sulphate solution | $ml.l^{-1}$ | 1.0 | 1.0 | 1.0 | 1.0 | 0.75 | 1.0 | 1.5 | 1.0 |
| Ferric chloride " | " | 1.0 | 1.0 | 1.0 | 1.0 | 0.75 | 1.0 | 1.5 | 1.0 |
| Vitamin " | " | 1.0 | 1.0 | 1.0 | 1.0 | 0.75 | 1.0 | 1.5 | 1.0 |
| Trace elements " | " | 1.0 | 1.0 | 1.0 | 1.0 | 0.75 | 1.0 | 1.5 | 1.0 |

Vitamin solution $g.l^{-1}$

| | | | |
|---|---|---|---|
| Thiamine HCl | 0.05 | Pyridoxine HCl | 0.02 |
| Ca-pantothenate | 0.10 | Nicotinamide | 0.10 |
| p-aminobenzoic acid | 0.02 | Inositol | 0.20 |
| Riboflavin | 0.02 | | |

Trace elements solution $g.l^{-1}$

| | |
|---|---|
| Boric acid | 0.12 |
| Ammonium molybdate | 0.48 |
| Cupric sulphate | 0.78 |
| Manganese chloride | 0.15 |

Distilled water was used to make up the media to the required volume.

1 Whatman a-cellulose ball milled as 6% w/v suspension for 12(*) or 6(**) hours

2 First shredded to a fluffy mass in a small hammer mill and then ball milled as 5% w/v suspension for 12(*) or 6(**) hours.

## 2.3. Growth of fungi on cellulose agar at different pH and temperature values

### 2.3.1. Preparation of the medium

A modified Chahal and Gray (1969) cellulose based medium was used. The composition of the agar medium is given in Table 9 . The medium except for urea and vitamin solution was dispensed in 150 ml screw cap bottles (100 ml medium per bottle) and autoclaved at 120°C for 15 minutes. Urea and vitamin solutions were membrane filter sterilized and added to the medium after autoclaving. The medium was kept at 55°C in a water bath with frequent shaking. The pH of the complete medium was adjusted to the required value with predetermined amounts of sterile IN Sodium hydroxide or Hydrocloric acid. Twenty five ml medium were dispersed into each of a set of 9 cm diameter plastic petri dishes and dried overnight at 35°C before inoculation.

### 2.3.2. Inoculation and incubation

Agar discs 7 mm in diameter were cut with their outer edge about 2 mm inside the growth front of a 2-3 days old colony grown on the same medium. Each disc was inverted and placed at the centre of the test agar. The cultures were incubated at different temperatures for up to 80 hours. Incubation temperatures were checked frequently and were held within 0.5°C. Each incubator was supplied with a dish filled with water in order to prevent desiccation. The fungi were first incubated at different temperatures at pH5 in order to determine the optimum temperature for growth and they were incubated at their optimum temperature at different pH values for optimum pH determination.

2.3.3. Growth measurement

Five replicates were used for each experiment. At frequent time intervals the agar dishes were viewed against a light background and the diameter of the fungal colony was measured to the nearest mm with the aid of a ruler. Two measurements of each culture were taken at right angles to one another. The data obtained for each set of plates and at each observation were treated statistically to find the effect of the treatment in the linear growth of the colony at 95% confidence limits (Parker, 1973). The rate of growth was calculated during the linear phase of growth which was attained during the first day of incubation. Final pH values were determined at the end of each incubation by cutting off an agar block, from which the mycelium has been removed, homogenizing with an equal amount of distilled water and measuring the pH with a Pye temperature compensated pH meter with glass electrode. An average reading from five replicates from each test was considered as the final pH value. Temperature and pH values leading to the maximum linear growth under the conditions of the experiment were taken as optimum for the growth of the fungus.

2.4. Fermentation of cellulosic materials

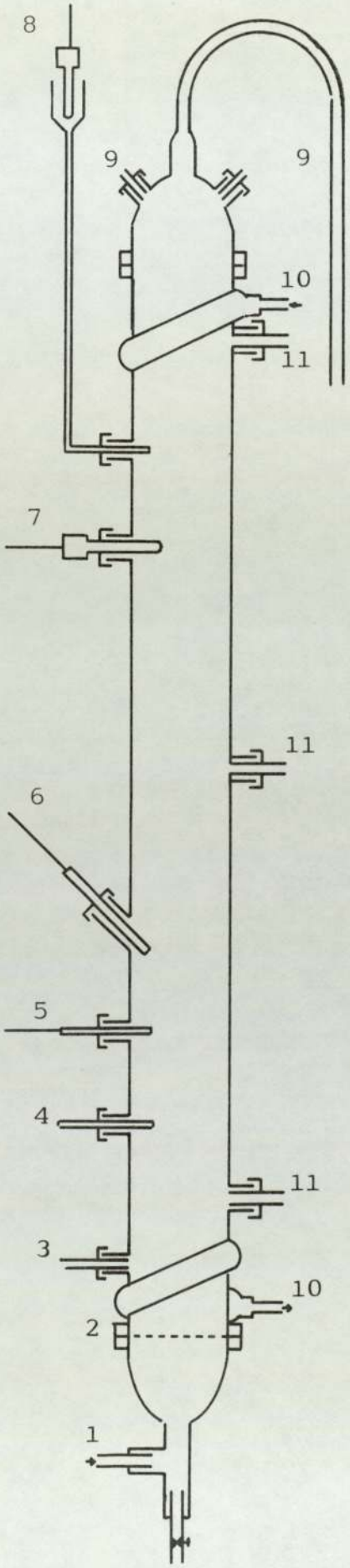
2.4.1. The Tower fermenter system

The Tower fermenter system used in batch and continuous fermentations consisted of the following.

2.4.1.1 The Tower fermenter

The Tower fermenter used (5 l working volume) was based on that described by Greenshields and Pannell (1974). It consisted of four sections connected with P.T.F.E. gaskets

Figure 11. Diagram of the 5 lit Tower fermenter.




1. Air inlet
2. Perforated plate
3. Medium inlet
4. Thermometer
5. Thermistor
6. D.O. Probe
7. pH Probe
8. pH reference probe
9. Acid/Alkali addition ports
10. Attemperation coil
11. Sample ports

and it was constructed by the University glassblowers.

The main section of the fermenter consisted of one 97 cm long/8.2 cm internal diameter tubular section with SQ glass screw-capped ports for medium inlet, thermometer, fermenter sampling, dissolved oxygen probe, thermistor probe, pH and pH reference probes. The upper section was constructed from a 8.2/2.5 cm pipe reducer on the top of which was connected a 2.5 cm end fitting leading to a 0.8 cm internal diameter swan neck which served as effluent and exhaust gas outlet. Two ports were fitted on the pipe reducer for acid or alkali addition. The lower section was made from another 8.2/2.5 cm pipe reducer leading to a 2.5 cm internal diameter T piece. The horizontal arm served as air inlet the vertical being led to a plastic tube sealed with a Hoffman clip. Between the base and the main body of the fermenter was fitted the air distributor which was a P.T.F.E. plate 2 mm thick with 1 mm diameter holes drilled in a regular pattern 1 cm apart. This air distributor was used because it was cheap and easy to construct. Other air distributors were not used because it was observed that bubble coalescence always occured in the presence of fungal mycelium, forming air slugs within a few centimetres of the distributor and thus other forms of air distribution would probably have been no more effective, (Stockbridge , 1979). Morris (1972) also reported that sintered glass plates rapidly became blocked by mycelial growth.

The diagram of the 5 litre Tower fementer is shown in Figure 11 .

2.4.1.2. The air supply

Compressed air was fed from the laboratory air system

via a pressure reducer and air/oil filter unit (Air Power Minett Ltd., Birmingham), to a second pressure reducer and oil filter (Spirax Monnier,Cheltenham) mounted to the fermenter framework. The air supply rate was monitored by a 0-50 l $min^{-1}$ flow meter (Gapmeter Ltd.) from which it was led via a plastic tube fitted with a porous stone to the bottom of a 20 l aspirator filled with distilled water. The vapour saturated air was led, via a Gamma 12 filter sterilizer fitted with a Grade 0.3 filter (Gallenkamp Ltd.), to the air inlet of the fermenter via a non return valve in order to prevent back flow of the fermenter contents in the event of an air supply failure. The air flow rate used throughout this study was 6.25 l $min^{-1}$ corresponding to a superficial gas velocity of 2 cm $sec^{-1}$ in the 8.2 cm dia. section.

2.4.1.3. The medium supply

The medium flow rate, F, is given by the ratio $\frac{V}{t}$ where V in litres is the volume of the inflowing medium at a time interval t (in hours). The medium dilution rate, D, is given by the ratio $\frac{F}{v}$ where F is the feed rate in l $h^{-1}$ and v is the active volume of the fermenter (which at the aeration rate used in this work was 5.025 lit).

To ensure good mixing and constant inflow rate of the suspended solids, Pyrex glass aspirators of different volumetric capacity (20 l and 10 l ) were used depending on the concentration of the suspended solids. The medium supply vessels were equipped with inlet air filters and magnetic stirrer followers and were being constantly mixed by means of a magnetic stirrer (L.H. Engineering, Bucks). Medium was withdrawn from the base of the aspirator and fed to the

fermenter through a 3 mm internal diameter silicone rubber tubing by a Baron Yemm BYO 800 peristaltic pump (Norris Industries, Rushton, Northants). The part of the tubing which was in touch with the head of the pump was changed frequently to avoid deformation of the tube. When changing aspirators, 'steri-connectors' (L.H. Engineering) were used to reduce the chances of contamination.

Low feed rates were achieved with the use of an electric timer which was set to supply power to the pump for 15 seconds per minute.

2.4.1.4. The temperature control

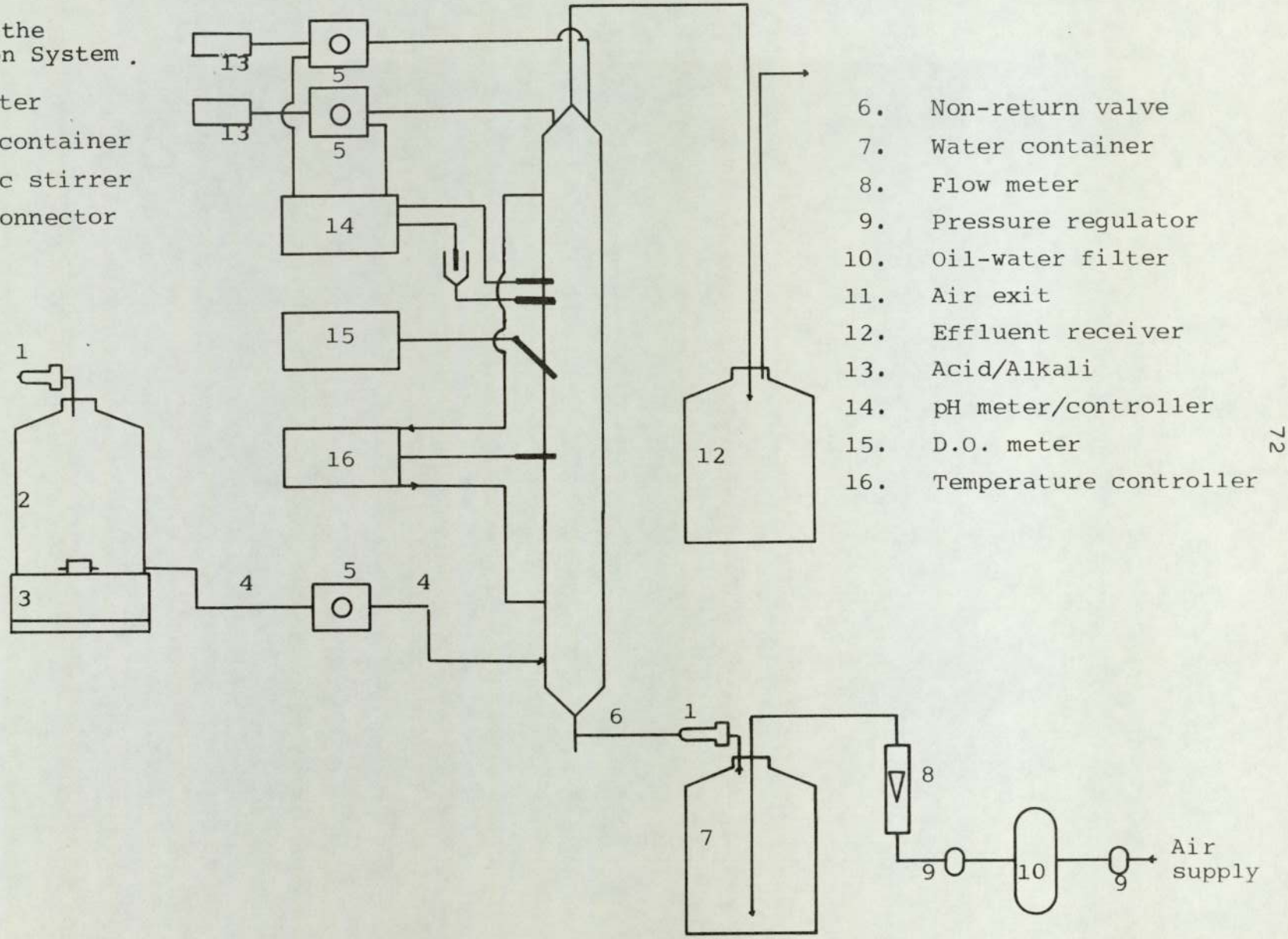
The temperature of the fementation was controlled with the aid of a thermistor probe, a thermocirculator (Churchill Instruments Co. Middlesex) and vinyl tubing through which the water was circulated. A thermometer ($-10^{o}$ to $110^{o}$C) was also used to check the temperature of the fermentation. The temperature was maintained within $0.5^{o}$C of the required value.

2.4.1.5. Dissolved oxygen

The dissolved oxygen concentration in the fermenter was measured by the use of a steam sterilizable silver-lead electrode with a replaceable polypropylene membrane (Uniprobe Instruments Ltd., Cardiff) connected to a New Brunswick, Model D.O.50 oxygen meter-recorder. Entry of the probe was through a port mounted at $45^{o}$C to the fermenter wall. Dissolved oxygen measurements were affected by rapid fungal growth over the end of the probe and thus the system gave only a rough indication of the state of the fermentation.

Figure 12.
Diagram of the
Fermentation System.

1. Air filter
2. Medium container
3. Magnetic stirrer
4. Steri-connector
5. Pump




6. Non-return valve
7. Water container
8. Flow meter
9. Pressure regulator
10. Oil-water filter
11. Air exit
12. Effluent receiver
13. Acid/Alkali
14. pH meter/controller
15. D.O. meter
16. Temperature controller

2.4.1.6. pH measurement and control

pH was measured with a pH meter controller (E.I.L918) using a steam sterilizable probe in conjunction with a remotely positioned reference probe connected by a KCl bridge to the fermenter contents (both electrodes were supplied by EIL, Richmond, Surrey). pH control solutions (concentrated Phosphoric acid or 10N Sodium hydroxide) were pumped to the fermenter by two peristaltic pumps (Watson Marlow, Bucks) via silicone rubber tubing with 1 mm bore. The required pH value was maintained to within ± 0.2 pH units.

The complete 5 litre fermenter system is shown in Figure 12.

2.5. Fermentation procedures

2.5.1. Preparation of inoculum

Mycelial inoculum was used in all fermentations. The inoculum medium is given in Table 9. 125 ml portions of the medium were dispersed into six 500 ml, conical flasks which were sealed with cotton-wool plugs and autoclaved at 120°C for 15 min. Urea and vitamins were added after autoclaving from filter sterilized solutions. The pH of the medium was adjusted to the required value with predetermined amounts of Sodium hydroxide or Hydrochloric acid. Each flask was inoculated with mycelium removed from four agar discs (7 cm dia.) taken from a 2-3 days old culture of the desired fungus grown on Agar medium. The inoculated flasks were incubated in a Gallenkamp shaking incubator at the required temperatures until good growth had occured. The contents of four flasks were collected in a sterile 1 l conical flask and used as inoculum. In batch fermentations the contents of

the rest two flasks were analysed for mycelium, cellulose, and protein content.

2.5.2. Preparation of fermenter medium

In batch fermentations media were prepared in 5 l batches in 10 l aspirator jars. In the continuous fermentations they were prepared in 20 l or 10 l batches depending on the concentration of cellulosic material. They were autoclaved at 125°C for 30 minutes. Urea, vitamins and glucose were sterilized separately and added to the medium after autoclaving. The composition of the media used is given in Table 9.

2.5.3. Preparation of the fermenter, start-up and running of fermentations

At the end of each fermentation the fermenter was emptied, cleaned with "Pyroneg" detergent (Diversey Chemicals Ltd.) and rinsed thoroughly with tap water. The fermenter was sterilized by free steaming. Provision was made for the condensate to be continuously drained. All probes were sterilized *in situ*. Medium and air inlet lines were autoclaved separately, and were connected aseptically to the fermenter. After steaming (for at least 24 hours) sterile air was passed through the fermenter together with steam. The air and steam supplies were slowly increased and decreased respectively in order to maintain a positive pressure in the fermenter and avoid sudden cooling which could create a partial vacuum. About 3 l of the medium was quickly pumped into the fermenter and the pH and temperature controllers were set to the required values. The inoculum was injected through a port and the aeration increased to the required level. In the continuous

fermentations the culture was left to grow for 10 to 20 hours before pumping began at the dilution rate to be investigated. In the case of batch fermentations the culture volume was immediately made up to 5 l.

2.5.4. Fermenter sampling

a) Fermenter contents

The first 5 to 10 ml discarded, a second sample was collected from the sampling port of the fermenter in a measuring cylinder. The sample volume was 80-100 ml in the case of batch fermentations and 60 to 90 ml in the case of continuous fermentations depending on the dilution rate and the frequency of sampling. An aliquot of the sample was centrifuged at high speed for 5 minutes and the clear supernatant transfered to a screw-cap bottle and stored at 1°C for later analysis. The solid material in the centrifuge tube was resuspended in water and filtered with the remainder of the sample through a glass-fibre filter paper. The solids were peeled off the filter paper and dried at 105°C to constant weight.

b) Fermenter effluent

A known volume was collected in a measuring cylinder from the effluent line and treated as above.

## 2.6 Analytical Methods

### 2.6.1 Cellulose determination

The semi-micro method of Updegraff (1969) was used as modified by Halsted (1973). According to this method lignin hemicellulose and xylosans are extracted with Acetic acid/ Nitric acid reagent and the remaining cellulose is dissolved in 67% Sulphuric acid and determined by the anthrone method of Scott and Melvin (1953). The step by step procedure is given in Appendix 1.

### 2.6.2 Reducing sugars determination

Reducing sugars in the supernatant or liberated during enzyme assays were measured as glucose equivalent using the reduction of 3,5-dinitrosalicylic acid to 3-amino-5-nitrocalicylic acid (Miller, 1959). Details of the method and preparation of the reagent are given in Appendix 1.

### 2.6.3 Filter paper activity (F.P.A.) determination

The extracellular cellulolytic activity was assayed on a Whatman No. 1 filter paper as substrate according to the method developed by Mandels and Weber (1969). F.P.A. is expressed as units.$ml^{-1}$ and indicates the mg of reducing sugars as glucose produced under the assay conditions by 1 ml of undiluted culture supernatant. Corrections were made for reducing sugars already present in the assayed sample. (F.P.A. indicates both $C_1$ and $C_x$ extracellular cellulolytic activity.) Details of the method are given in Appendix 1.

2.6.4. Nitrogen determination

Tests for nitrogen content were performed on dried and ground samples of mycelium plus remaining solids.

Nitrogen determination was based upon the micro-kjeldahl method (Greenshields, 1976).

. Accurately weighed samples (25-50 mg) were incinerated in micro-kjeldahl digestion flasks with 2 ml of concentrated Sulphuric acid and 20 mg catalyst (Potasium sulphate. Copper sulphate, Selenium powder in ratio 32:5:1 respectively). The sample was transferred in a Markham still and the distillation of ammonia was effected by the addition of 5 ml 10 N Sodium hydroxide. The first 15 ml of distillate were collected into 10 ml of saturated Boric acid solution which was back titrated with 0.01 N Hydrochloric acid using Tashiros indicator. From the amount of acid required to complete the neutralisation the quantity of ammonia and hence nitrogen in the original sample coule be readily calculated. The crude protein content was estimated as (N x 6.25).

# SECTION 3

# RESULTS AND DISCUSSION

## 3.1. Growth of cellulolytic fungi on cellulose agar at different temperature and pH values.

### 3.1.1. Results

The fungi tested grew in the form of radial colonies. After a lag period, which differed with the microorganism and the conditionsof the experiment, the rate of increase in colony diameter was essentially linear indicating that the experimental conditions were reasonably constant. (Bull and Trinci, 1977) (Tansey, 1970). In each plate the difference between the shortest and longest diameter was always less than 4 mm and frequently less than 1 mm. The difference between the longest and shortest diameter in each set of measurements was always less than 6 mm and frequently less than 4 mm. Variation in growth between replicates may be due to minor differences in incubation temperature, relative humidity or depth of agar layer (Gundersen, 1962).

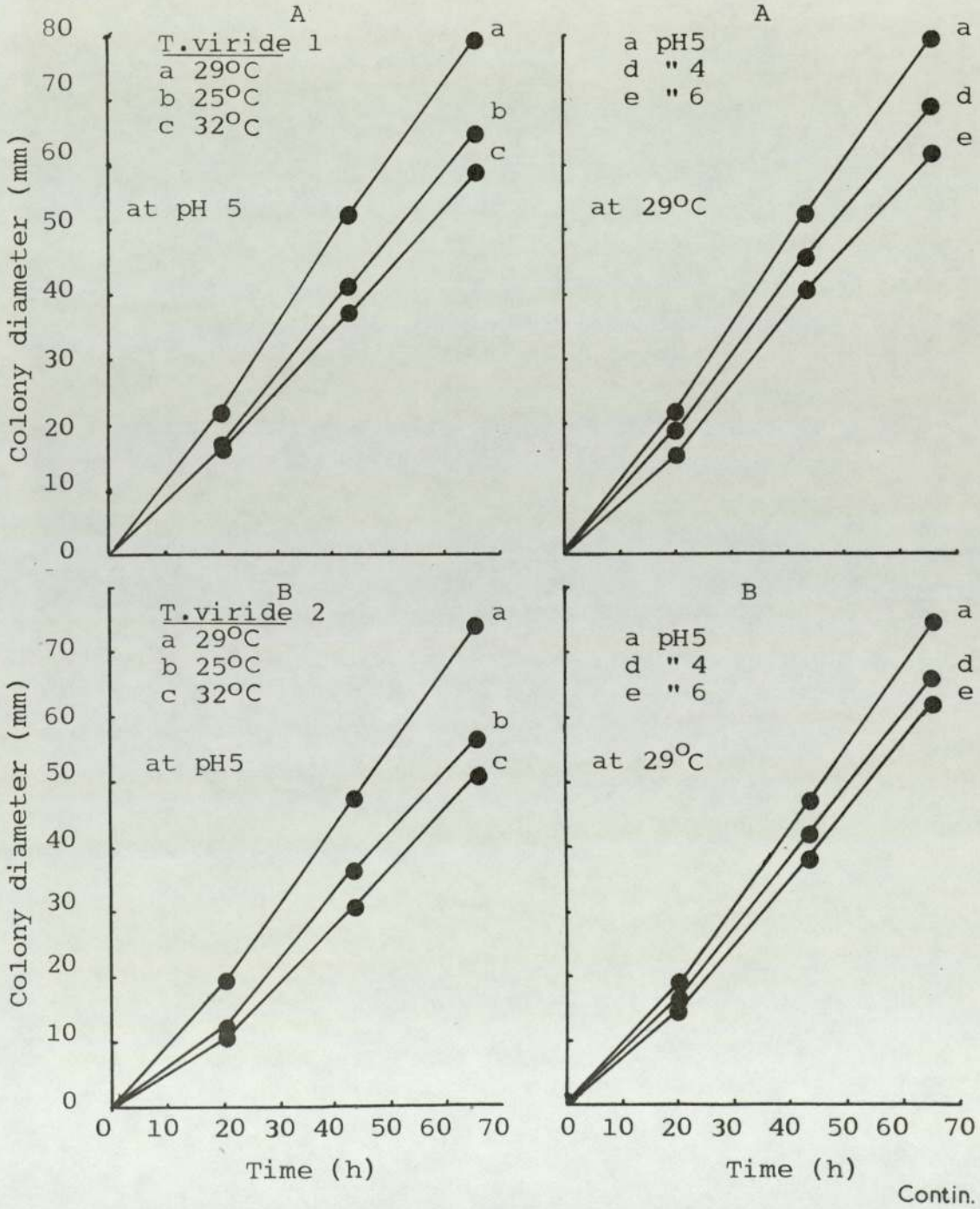
The relations between temperature, pH and growth measured as increase in colony diameter are presented in Figure 13(A-E). The linear growth rates along with the final pH values are given in Table 10.

From the results it can be seen that within the range tested pH and temperature had a definite effect on the growth of cellulolytic fungi. The two strains of T.viride showed the same temperature (29°C) and pH (5) optima whereas the two strains of S.thermophile showed the same pH (6) but different temperature optima, 42° and 45°C. S. pulverulentum grew better at 35°C and pH 5.

### 3.1.2. Discussion

The importance of temperature and pH in fermentation

Figure 13. Linear growth of cellulolytic fungi on cellulose agar at different pH and temperature values.




Contin.

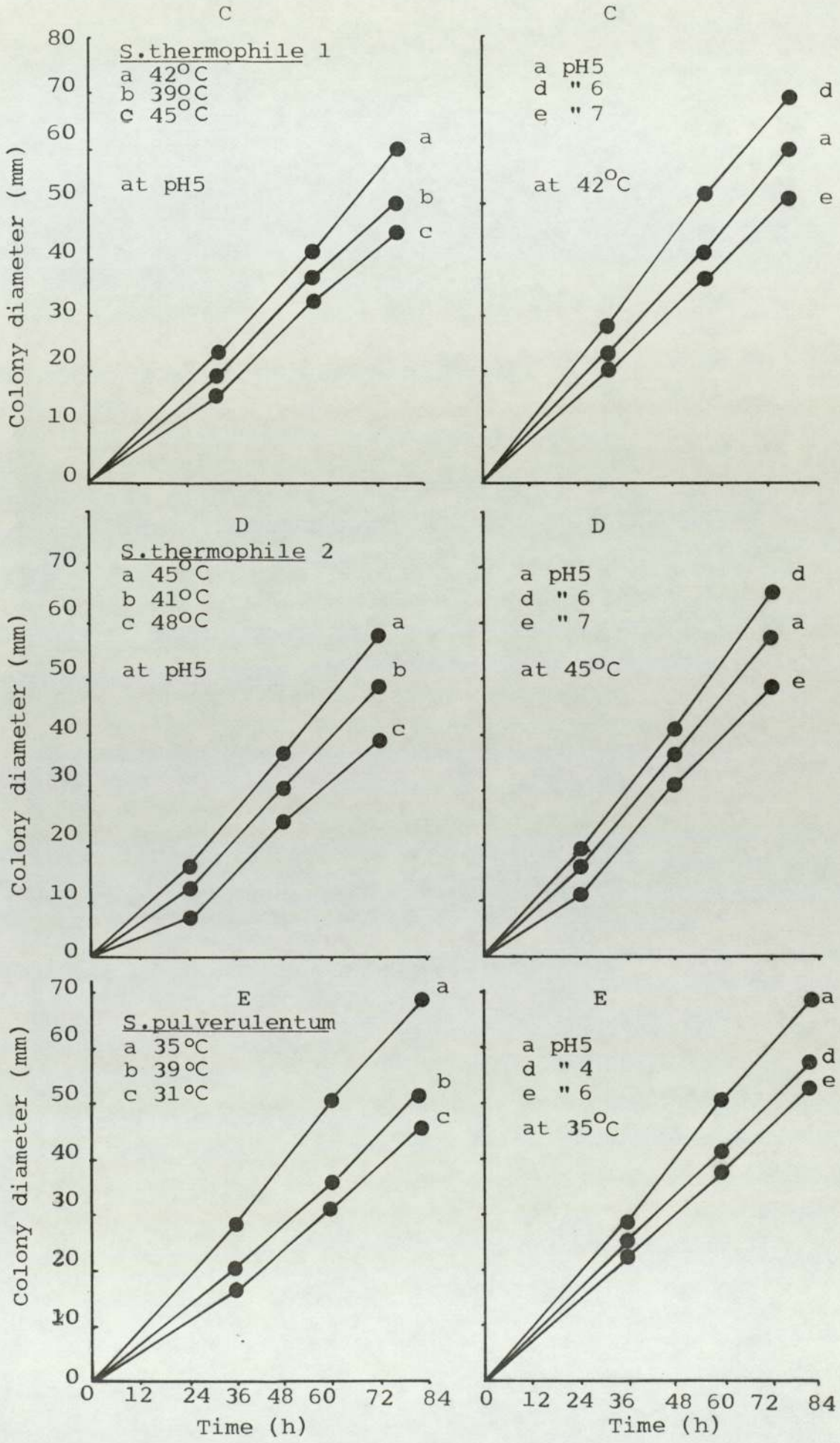

C
S.thermophile 1
a 42°C
b 39°C
c 45°C
at pH5
a
b
c
C
a pH5
d " 6
e " 7
at 42°C
d
a
e
D
S.thermophile 2
a 45°C
b 41°C
c 48°C
at pH5
a
b
c
D
a pH5
d " 6
e " 7
at 45°C
d
a
e
E
S.pulverulentum
a 35°C
b 39°C
c 31°C
a
b
c
E
a pH5
d " 4
e " 6
at 35°C
a
d
e
Colony diameter (mm)
0 10 20 30 40 50 60 70 80
0 12 24 36 48 60 72 84
Time (h)

Table 10

Linear growth rates (L.g.r) of cellulolytic fungi at different pH and Temperature values

| T. viride 1 | | | T.viride 2 | | | S.pulverulentum | | | S.thermophile 1 | | | S.thermophile 2 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $^{o}C$ | pH | L.g.r $mm.h^{-1}$ | $^{o}C$ | pH | L.g.r $mm.h^{-1}$ | $^{o}C$ | pH | L.g.r $mm.h^{-1}$ | $^{o}C$ | pH | L.g.r $mm.h^{-1}$ | $^{o}C$ | pH | L.g.r $mm.h^{-1}$ |
| 25 | 5 (4.7) | 0.86 | 25 | 5 (4.8) | 0.81 | 31 | 5 (5.1) | 0.63 | 39 | 5 (5.3) | 0.69 | 41 | 5 (4.8) | 0.75 |
| 29 | 5 (4.7) | 1.04 | 29 | 5 (4.8) | 1.00 | 35 | 5 (5.2) | 0.87 | 42 | 5 (5.2) | 0.81 | 45 | 5 (5.3) | 0.86 |
| 32 | 5 (4.9) | 0.77 | 32 | 5 (4.8) | 0.73 | 39 | 5 (4.9) | 0.68 | 45 | 5 (5.1) | 0.65 | 48 | 5 (5.2) | 0.66 |
| 29 | 4 (3.8) | 0.91 | 29 | 4 (3.7 | 0.89 | 35 | 4 (4.2) | 0.69 | 42 | 6 (5.9) | 0.91 | 45 | 6 (6.2) | 0.97 |
| 29 | 6 (5.8) | 0.84 | 29 | 6 (5.7) | 0.86 | 35 | 6 (6.1) | 0.61 | 42 | 7 (7.0) | 0.69 | 45 | 7 (7.1) | 0.78 |

Figures in parentheses relate to the final pH values.

processes has long been recognised. Temperature is a factor which affects directly the microorganism since the cell temperature must become equal to the temperature of the environment (Pirt, 1975). Temperature affects the growth rate, the nutrient requirements and the nature of metabolism since it affects the enzyme synthesis and specificity of enzyme reactions, the structure of cell components especially lipids and proteins,and cell permeability and composition (Pirt, 1975). The pH of the medium also affects the growth rate (Pirt and Callow, 1960), the composition of biomass, the nature of metabolism and the production and activity of enzymes (Pirt, 1975) (Harrison, 1978). However, the effects of pH at sub-cellular level are not clearly understood. Most fungi have an optimum pH for growth between 5-7. The internal pH of the cells is well-buffered and controlled independently of the medium at around pH 5-6, and it is not easy to establish if changes in the pH of the medium affect the microorganism directly or indirectly (Berry, 1975).

According to Andreeva et al (1970) and Andreyeva et al (1973), the pH effect on the growth rate is due to enzyme non competitive inhibition by the hydrogen ions. Brown and Halsted (1975) studied the effect of pH on the specific growth rate of T.reesei QM9123 grown on glucose and supported the postulation expressed by Muzychenko et al (1973) that the effect of pH on growth rate is associated with a change in the rate of diffusional transfer of the substrate/permease complex due to locally high charge densities within the pores of the cell membrane.

The growth of cellulolytic fungi on cellulose media is strongly influenced by the temperature and pH of the medium

and it has been frequently reported that optimum pH and temperature values for growth do not necessarily coincide with the values required for optimum production of cellulolytic enzymes (El-Kersh, et al 1973) (Mandels and Andreotti, 1978).

In our experiments the rate of linear growth was chosen as criterion for estimation of the optimum pH and temperature values for growth because it is relatively simple and it has been found reliable and related to the specific growth rate of the tested microorganisms in submerged cultures (Trinci, 1971) (Righelato, 1975) (Bull and Trinci, 1977). This method has been frequently used to determine the optimum temperature and/or pH values of fungi to be used in submerged fermentation of cellulosic materials (Rosenberg, 1975, 1978) (Chang, 1967) (Barnes, 1974) or effluents from the food and drink processing industries (Tomlinson, 1976).

Optimum temperatures and pH values for cellulolytic fungi grown on agar media have frequently been reported and include the species tested in this work (Table 11). However, because of the difference in media, environmental conditions, and possible differences between the strains, the reported values served as a guide of the ranges tested.

The differences observed may be attributed to the above mentioned factors which have been shown to affect the temperature and pH requirements of microorganisms (Allsopp, 1973) (McShane, 1976) and their growth rates (Bull and Trinci, 1977) (Trinci, 1969).

In our experiments, T.viride 1 and 2 were the faster growing fungi. However, since observations based on the linear growth rates between different species or strains cannot be used to assess their relative growth in submerged culture

Table 11. Reported Linear Growth Rates (L.g.r, $mm.h^{-1}$) and temperature and pH optima of cellulolytic fungi

| Organism | Agar medium | °C | pH | L.g.r. | References |
|---|---|---|---|---|---|
| T.viride | Cellulose | 25 | 5.5 | 0.75 | Penn, 1977 |
| T.viride | Cellulose | 25 | 4.5 | - | Sharp and Eggins 1970 |
| T.viride | Newsprint | 25 | 5.5 | 1.03* | Barnes,1974 |
| T.viride | Cellulose | 25 | 5.5 | 1.08* | Barnes,1974 |
| T.viride | Cellulose | 29 | 5 | 1.04 | This work |
| T.viride | Cellulose | 29 | 5 | 1.00 | This work |
| S.pulverulentum | Glucose | 40 | 3.9-5.2 | 0.66* | Rosenberg 1975 |
| S.pulverulentum | Cellulose | 35 | 5 | 0.87 | This work |
| S.thermophile | Glucose | 42.2-45 | 6.1-6.8 | 0.99* | Rosenberg 1975 |
| S.thermophile | Czapek-Dox | 35 | 6.5 | - | Chang, 1967 |
| S.thermophile | Yp Ss | 40 | 7.3 | 1.20 | Chapman,1974 |
| S.thermophile | Yp Ss | 45 | 7.3 | 0.71* | Kuthubutheen 1977 |
| S.thermophile | Newspaper | 45 | 5.5 | 0.71* | Barnes,1974 |
| S.thermophile | Cellulose | 45 | 5.5 | 0.76* | Barnes,1974 |
| S.thermophile 1 | Cellulose | 42 | 6 | 0.91 | This work |
| S.thermophile 2 | Cellulose | 45 | 6 | 0.97 | This work |

* Calculated from published data

(Trinci, 1969) (Bull and Trinci, 1977) the fungi were grown and assessed in batch cultures at the temperatures and pH values which let to the maximum linear growth rates as determined in this study.

## 3.2. Batch culture of cellulolytic fungi on cellulosic substrates in a 5 l Tower fermenter

### 3.2.1. Results

In order to assess their ability to degrade and transform cellulose into biomass the selected cellulolytic fungi were grown in batch cultures in the 5 l Tower fermenter at their optimum pH and temperature for growth as determined in (3.1). The growth medium was based on that devised by Chahal and Gray (1969) with omission of glucose so that cellulose was the only carbon source. Antifoam at a concentration of 0.01 $ml.l^{-1}$ was incorporated in the medium before autoclaving. Mycelium grown in shake flasks was used as inoculum at 10% v/v basis. The composition of inocula and the obtained fermentation data are given in Appendix 2 a-f.

In all fermentations, the fungi grew in the form of diffuse mycelial flocks with the insoluble material entrapped in themycelium. Foaming occured in all fermentations and it was more pronounced in the beginning of each fermentation, however not to an extent which would require additional antifoam. The mycelium and the insoluble particles showed a tendency to accumulate in the wall of the fermenter above the liquid surface. Whenever cell wall growth was observed the aeration was increased for a few seconds so that the mycelium and substrate were washed back into the fermentation broth.

The course of batch fermentation by each fungus is presented in Figs 13 to 19 in terms of substrate degradation biomass, reducing sugar, filter paper activity, and crude protein production. Table 12 presents growth data obtained

near the beginning of stationary phase at each fermentation. Table 13 presents these data as percentage of the results obtained with S.thermophile 2. Table 14ab presents a summary of the obtained and reported batch fermentation data using various cellulolytic microorganisms and cellulosic substrates.

T.viride 1 (Figure 14) degraded 85.4% of the initial substrate concentration ($C_o$) in 45 hours and produced 2.90 $g.l^{-1}$ mycelium with 33.9% crude protein (C.P). The filter paper activity (F.P.A.) at this time was 1.8 $units.ml^{-1}$.

T.viride 2 (Figure 15) degraded 92.5% of Co in 48 hours and produced 3.3 $g.l^{-1}$ mycelium with 30.3% C.P. content. The F.P.A. was 2.2 $units.ml^{-1}$. S.pulverulentum (Figure 16) degraded 64.4% of Co in 49 hours and the mycelium concentration observed was 2.5 $g.l^{-1}$ with 32.4% crude protein content. The F.P.A. was 1.0 $unit.ml^{-1}$. S.thermophile 1 (Figure 17) degraded 78.1% of Co in 48 hours producing 3.10 $g.l^{-1}$ mycelium with 33.9% C.P. content. The F.P.A. was 1.2 $units.ml^{-1}$. S.thermophile 2 (Figure 18) was the fastest growing fungus and gave the best results degrading 78.2% of the initial cellulose in 39 hours and producing 4.50 $g l^{-1}$ mycelium with 42.6% C.P. content. The F.P.A. was 1.1 $units.ml^{-1}$. S.thermophile 2 showed also good growth on newspaper degrading 70.2% of the contained cellulose and giving a final product with 18.8% C.P. content. The F.P.A. was 0.8 $units.ml^{-1}$ (Fig 19).

In all fermentations the low level of reducing sugars observed ($0.2 < g.l^{-1}$) indicated that the products of hydrolysis did not repress the production of cellulases and that the degradation of the substrate depended on the cellulolytic ability of the microorganism.

Figure 14. Batch culture of T.viride 1 on ball milled cellulose at 29°C,pH5,1.6 v/v/m.

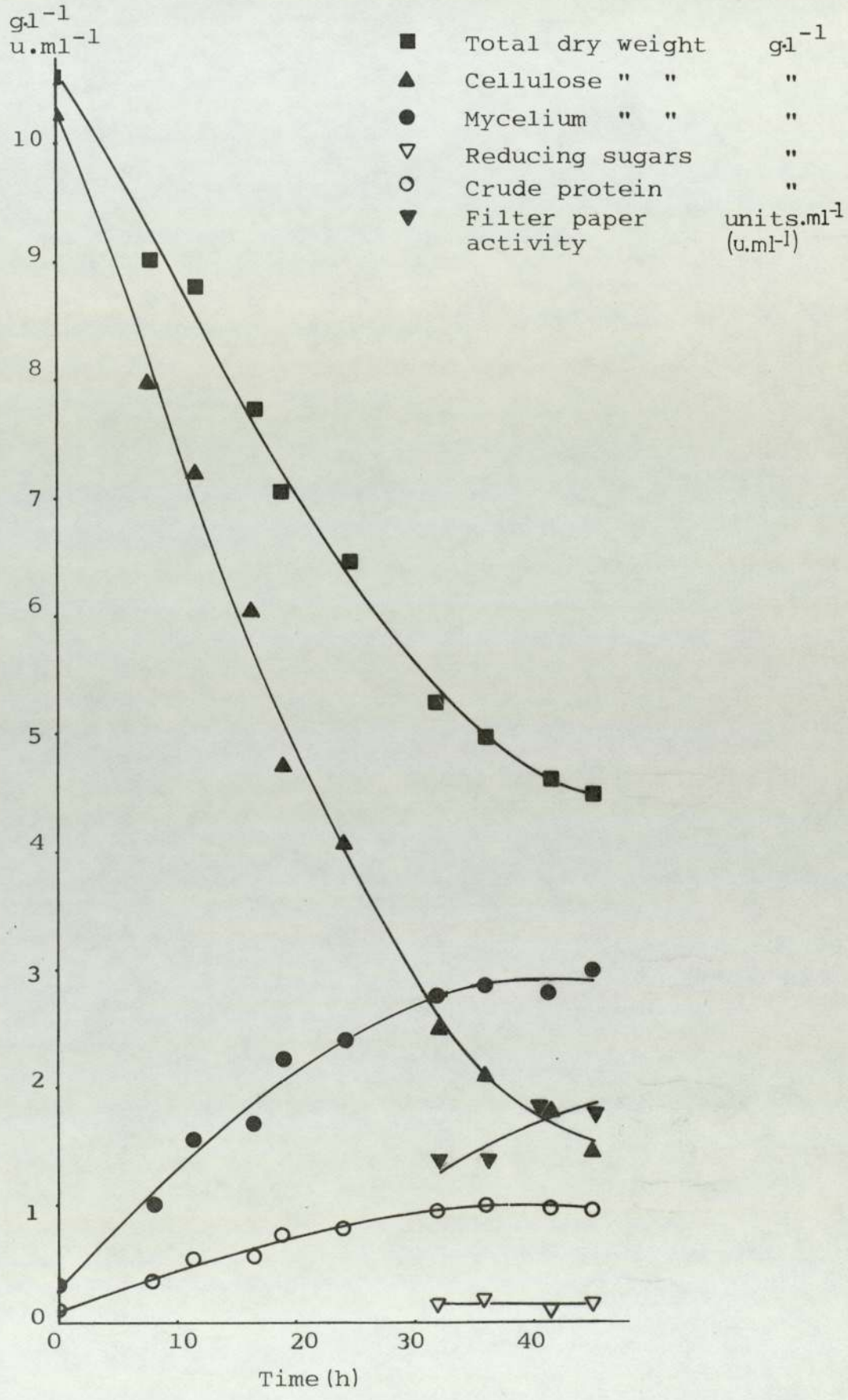


Figure 15. Batch culture of T.viride 2 on ball milled cellulose at 29°C, pH 5, 1.6 v/v/m.

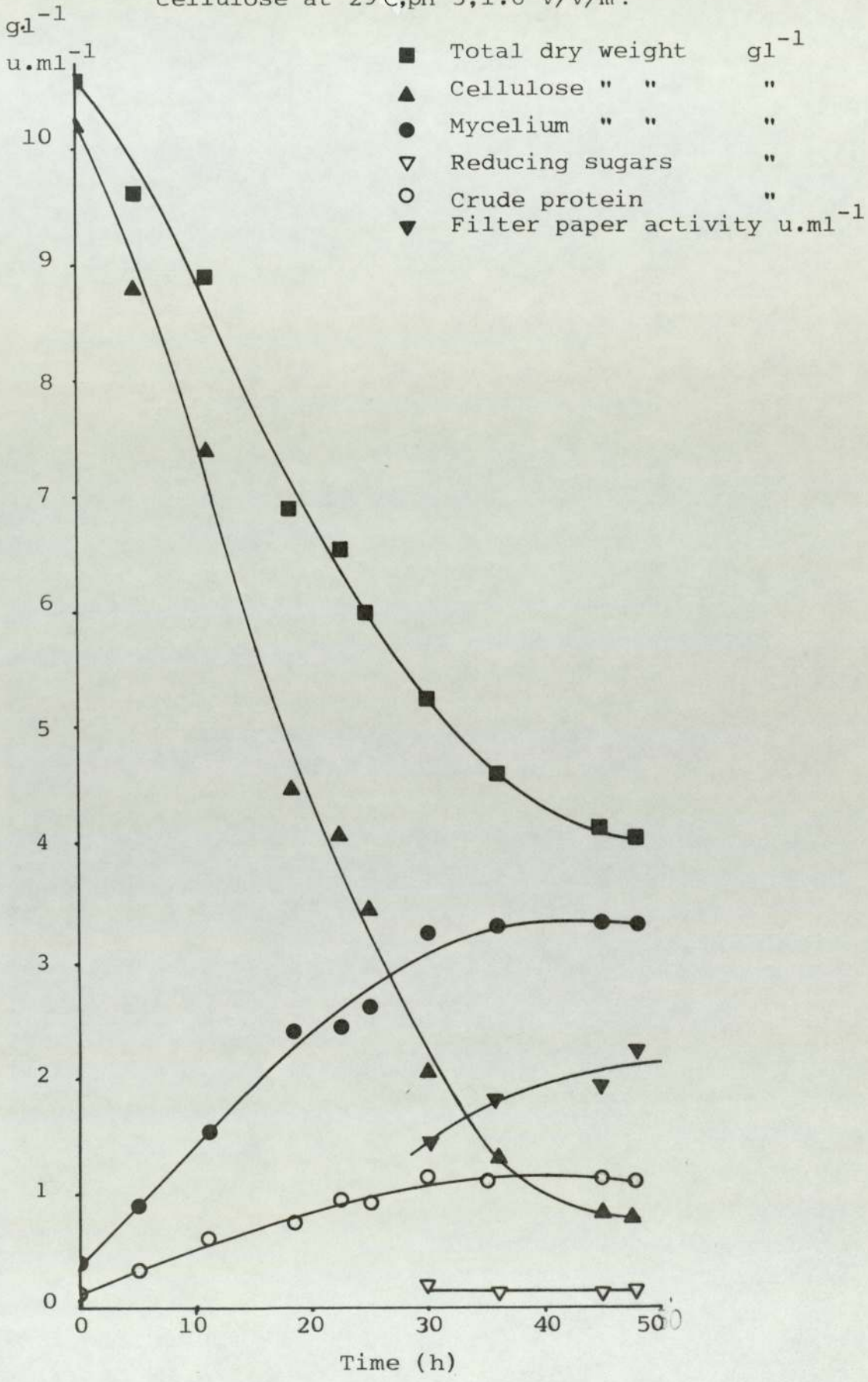


Figure 16. Batch culture of S.pulverulentum on ball milled cellulose at 35°C, pH 5, 1.6 v/v/m .

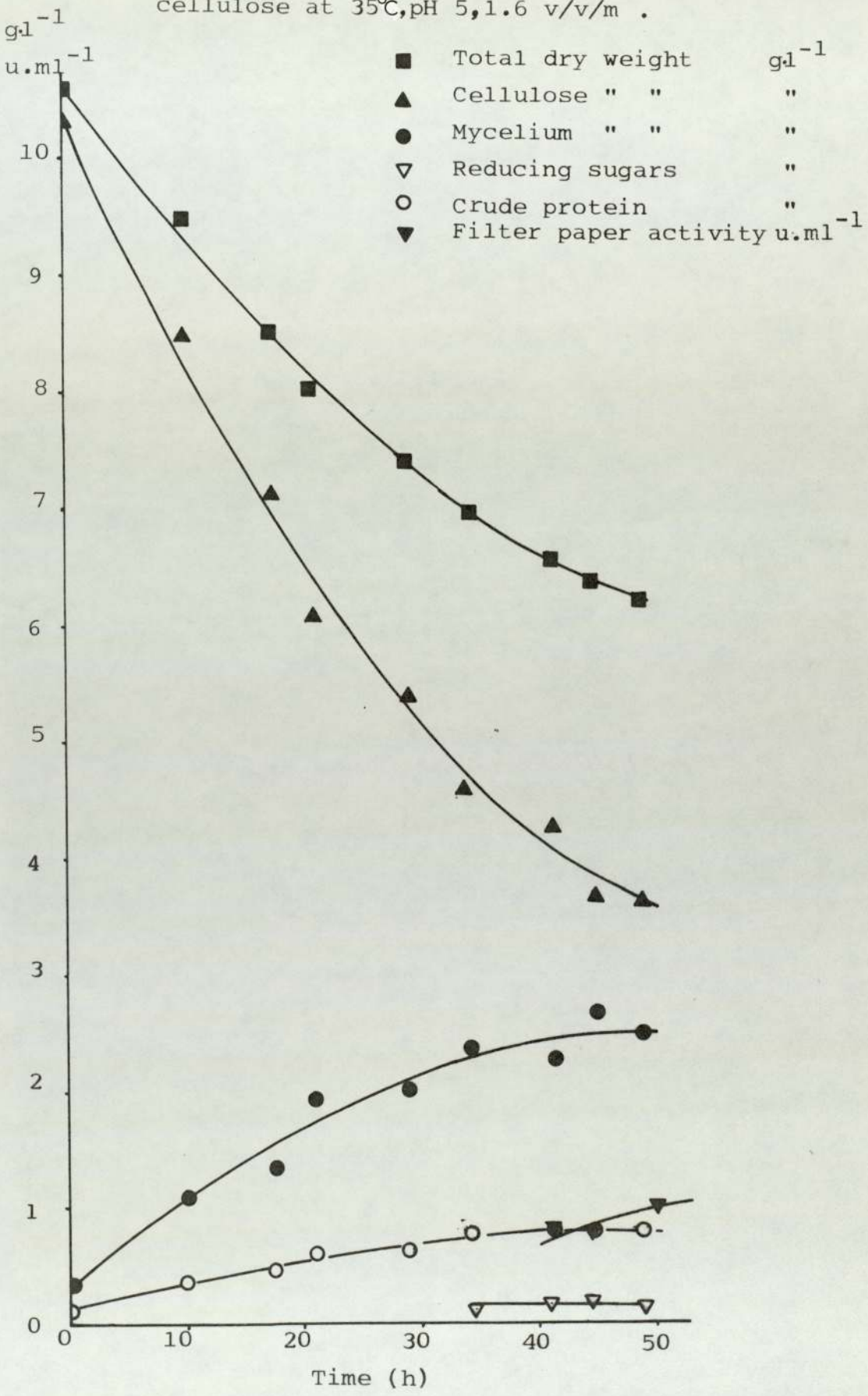


Figure 17. Batch culture of S.thermophile 1 on ball milled cellulose at 42°C, pH 6, 1.6 v/v/m.

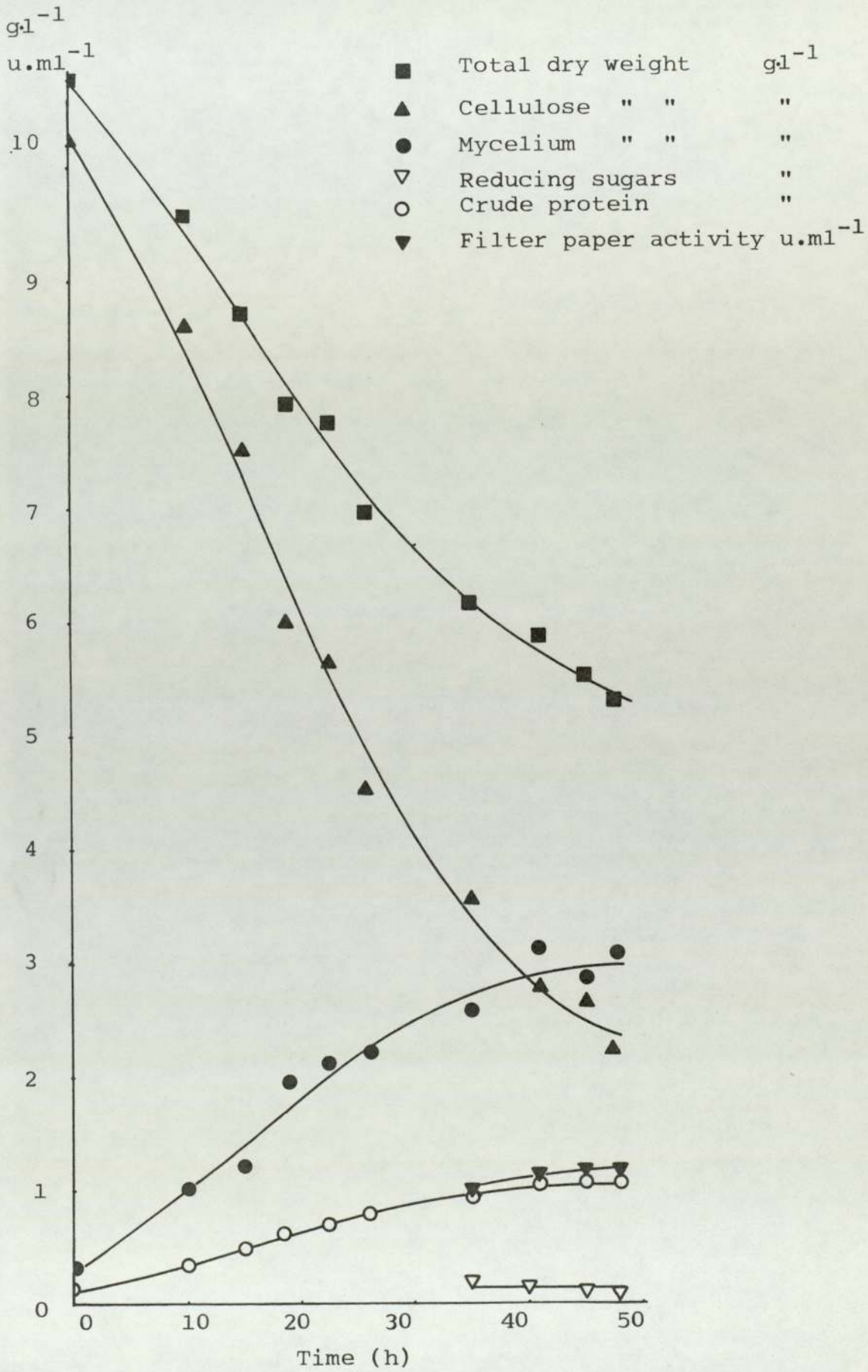


Figure 18. Batch culture of S.thermophile 2 on ball milled cellulose at 45°C, pH 6, 1.6 v/v/m.

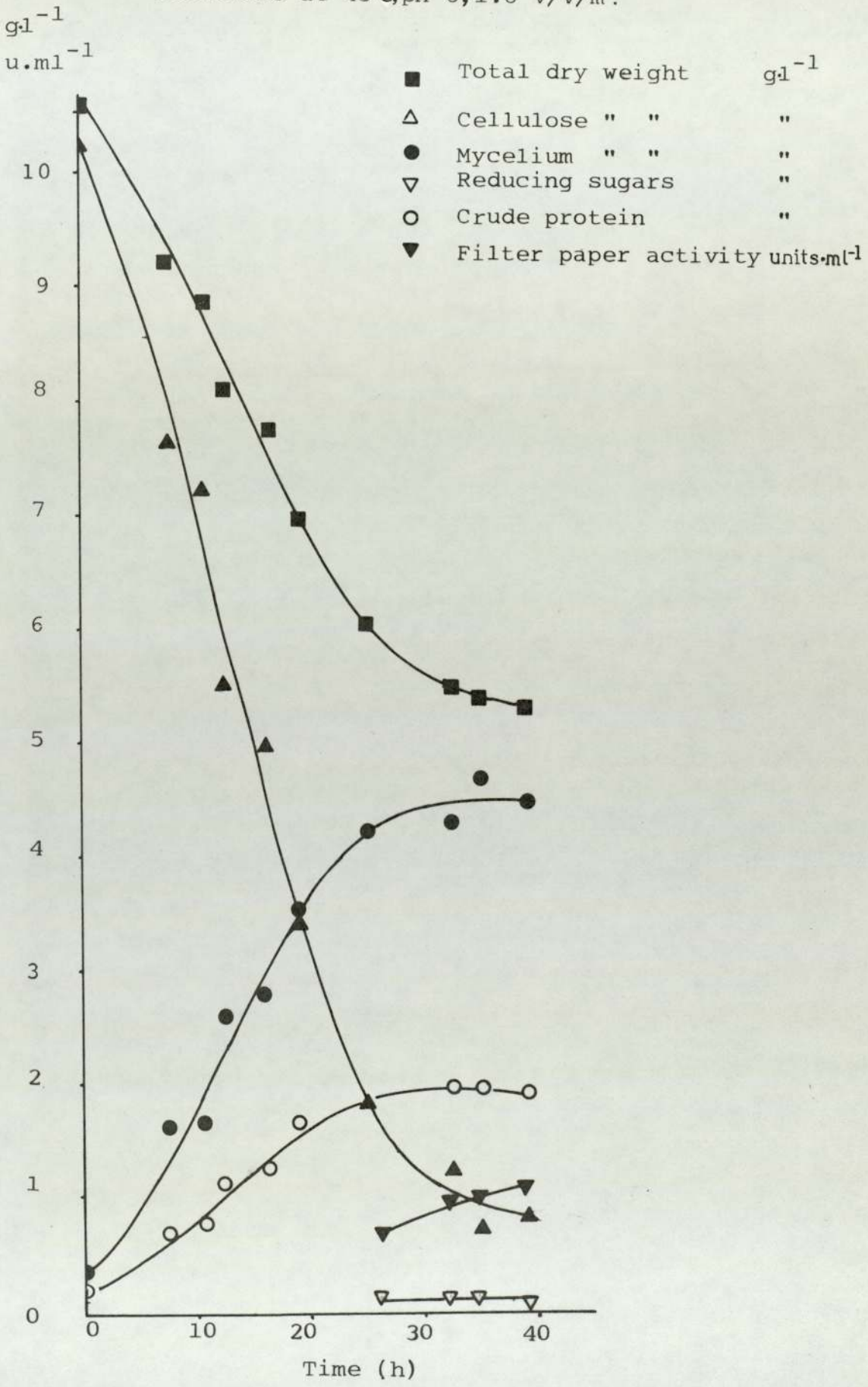
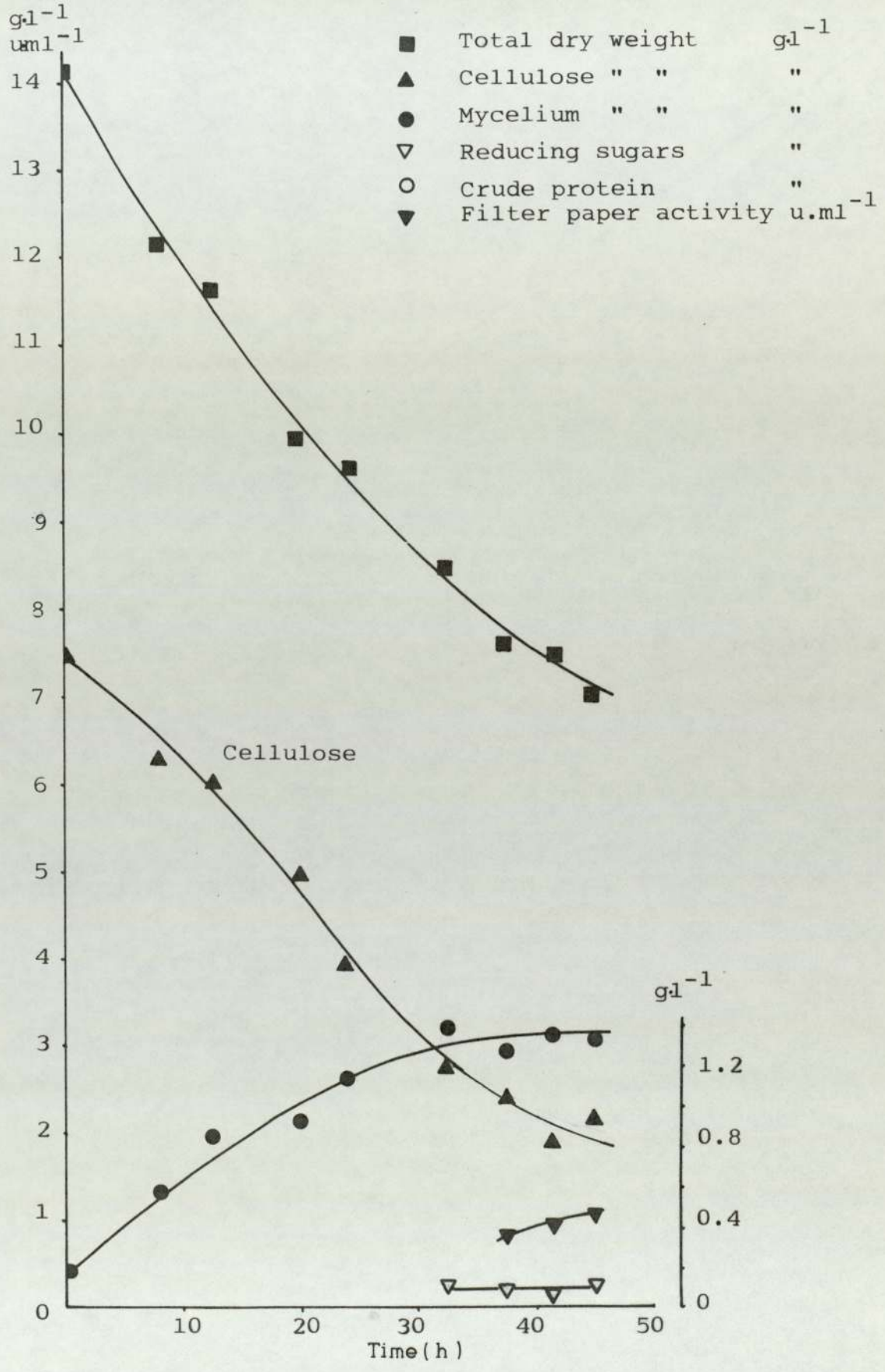


Figure 19. Batch culture of S.thermophile 2 on ball milled newspaper at 45°C, pH6, 1.6 v/v/m.



## 3.2.2. Discussion

### 3.2.2.1. Cellulose fermentation by T.viride 1 and T.viride 2

Many species of the genus Trichoderma are active towards cellulose with strains which produce a complete cellulose system able to degrade crystalline cellulose (Simmons, 1977). T.viride is a standard test organism used in measurements of biodeterioration of cellulosic materials (Mandels and Reese, 1964). It is also one of the main microorganisms used for the industrial production of cellulolytic enzymes (Toyama, 1969, 1976) (Toyama and Ogawa, 1972).

Trichoderma species and especially T.viride and T. reesei have frequently been used to study the feasibility of bioconversion of cellulosic materials (Peitersen, 1975ab), see also Table 14ab, feedlot wastes (Morrison et al, 1977) (Griffin et al, 1974) and wastes from food and drink processing industries (Church et al, 1969, 1972) (Updegraff et al,1973) (Tomlinson, 1976).

In our experiments, Figures 14 and 15, Tables 12 and 13). T.viride 1 and 2 required the same time to produce their maximum biomass and protein and showed the same efficiency in the conversion of substrate to protein. The fermentation by T.viride 2 produced better results in terms of F.P.activity substrate degradation and mycelium and protein productivity.

Compared with the other cellulolytic fungi tested,the fermentations by T.viride 1 and 2 were characterized by high substrate degradation, F.P. activity and low mycelium and protein yields. High substrate degradation and low bioconversion has frequently been observed in cellulose fermentations where Trichoderma spp were compared with other cellulolytic

Table 12 Fermentation data of the cellulolytic fungi tested in batch cultures

| | | Trichoderma viride 1 | Trichoderma viride 2 | Sporotrichum pulverulentum | Sporotrichum thermophile 1 | Sporotrichum thermophile 2 | Sporotrichum thermophile 2* |
|---|---|---|---|---|---|---|---|
| Initial Cellulose Conc$^{n}$ (Co) | $g.l^{-1}$ | 10.23 | 10.23 | 10.31 | 10.31 | 10.23 | 7.40 |
| Fermentation Time | h | 36 | 36 | 41 | 41 | 32 | 40 |
| Total Dry Weight (T.D.W) | $g.l^{-1}$ | 4.94 | 4 55 | 6.55 | 5.70 | 5.50 | 7.15 |
| Mycelium Dry Weight (M.D.W) | $g.l^{-1}$ | 2.90 | 3.30 | 2.45 | 2.90 | 4.50 | 3.30[1] |
| Filter paper activity (FPA) | u/ml | 1.4 | 1.8 | 0.8 | 1.1 | 1.0 | 0.7 |
| Reducing Sugars (R.S) | $g.l^{-1}$ | 0.19 | 0.12 | 0.16 | 0.16 | 0.16 | 0.16 |
| Cellulose Degraded (C.D.) | $g.l^{-1}$ | 8.19 | 8.98 | 6.21 | 7.61 | 9.18 | 5.27 |
| Cellulose Degraded as % Co | | 80.1 | 87.8 | 60.2 | 73.8 | 89.7 | 71.2 |
| Crude Protein (C.P) | $g.l^{-1}$ | 1.02 | 1.12 | 0.80 | 1.05 | 1.96 | 1.36 |
| Crude Protein as % MDW | | 35.2 | 33.9 | 32.7 | 36.2 | 43.5 | 43.5[1] |
| Crude protein as % TDW | | 20.6 | 24.6 | 12.2 | 18.4 | 35.6 | 19.0 |
| C.P. Productivity | $mg.l^{-1}.h^{-1}$ | 28.3 | 31.1 | 19.5 | 25.6 | 61.2 | 34.0 |
| Mycelium Productivity | $mg.l^{-1}.h^{-1}$ | 80.5 | 91.6 | 59.7 | 70.7 | 140.0 | 82.5[1] |
| C.P. Yield g (C.P)/g(C.D) | | 0.12 | 0.12 | 0.13 | 0.14 | 0.21 | 0.17[2] |
| Mycelium Yield g(M.D.W)/g (C.D) | | 0.35 | 0.37 | 0.39 | 0.38 | 0.49 | 0.41[2] |

* Newspaper fermentation

1. Based on the protein content of S.thermophile 2 grown on cellulose
2. Based on the cellulose and hemicellulose content and assuming total degradation of hemicellulose

Table 13. Fermentation data as percentage of the *S.thermophile* 2 batch cellulose fermentation.

| | *Trichoderma viride* 1 | *Trichoderma viride* 2 | *Sporotrichum pulverulentum* | *Sporotrichum thermophile* 1 | *Sporotrichum thermophile* 2* |
|---|---|---|---|---|---|
| Total Dry Weight (T.D.W.) | 89.8 | 82.7 | 119.0 | 103.6 | 130.0 |
| Mycelium Dry Weight (M.D.W.) | 64.4 | 73.3 | 54.4 | 64.4 | 73.3 |
| Cellulose Degraded (C.D.) | 89.2 | 97.8 | 67.6 | 82.9 | - |
| Crude Protein (C.P.) | 52.0 | 57.1 | 40.8 | 53.6 | 69.4 |
| F.P.A. | 140.0 | 180.0 | 80.0 | 110.0 | 70.0 |
| C.P. content of M.D.W. | 80.9 | 77.9 | 83.2 | 83.2 | 100 |
| C.P. content of T.D.W. | 57.9 | 69.1 | 34.3 | 51.7 | 53.4 |
| C.P. Productivity | 46.2 | 50.8 | 31.9 | 41.8 | 55.5 |
| M.D.W. Productivity | 57.5 | 65.4 | 42.6 | 50.5 | 58.9 |
| C.P. Yield | 57.1 | 57.1 | 61.9 | 66.7 | 80.9 |
| M.D.W. Yield | 71.4 | 75.5 | 79.6 | 77.6 | 83.7 |

* Ball milled newspaper fermentation

fungi. (Chahal and Gray, 1969) (Moo-Young et al, 1977) (Chahal et al, 1977). This has been attributed to the high production of cellulolytic enzymes which were synthesized at the expense of mycelial protein (Moo-Young et al, 1977). Comparing the results obtained by T.viride 1 and 2 with reported results (Table 14 a,b) it can be seen that T.viride 1 and 2 compare favourably in terms of substrate degradation but not in terms of biomass or protein productivity.

Peitersen (1975) reported improved fermentations when mixed cultures of T.reesei QM 9123 and a yeast were grown on chemically pretreated straw. However, the obtained productivities (e.g. 22 mg protein $l.h^{-1}$) do not compare favourably with other reported results, Table 14 a,b.

3.2.2.2. Cellulose fermentation by S.pulverulentum

S.pulverulentum has been reported as possessing a wide enzymic adaptability and as able to degrade and convert to fungal protein many types of cellulosic wastes (Eriksson,1977) (Ander and Eriksson,1978)(v.Hofsten and v.Hofsten 1974). In batch semicontinuous and continuous fermentations, of agricultural by products or chemically pretreated cellulose,specific growth rates ($\mu$) up to $1.173h^{-1}$ have been reported and biomass yields from 30 to 60% with a crude protein content of 30 to 44% were obtained depending on the substrate and fermentation conditions (v.Hofsten and Ryden, 1975) (v.Hofsten, 1976) (Janus, 1977) (Ek and Eriksson, 1977).

S.pulverulentum is able to cause extensive deterioration on stored wood (Nilsson, 1965) and has been described as one of the most efficient microorganisms for the degradation of cellulosic and lignocellulosic materials (Eriksson and Larsson,

1975). However, Rosenberg (1978) reported that S.pulverulentum required a specific substrate moisture content for degradation of lignocellulosic materials. No obvious lignocellulose degradation was observed under submerged conditions.

Long fermentation times (6 days) and biomass with low crude protein content (6% on powdered cellulose, 13.8 on waste fibres) have been reported by Eriksson and Larsson (1975). The slow growth was attributed to the inaccessibility of the glucosidic bonds due to the crystallinity of the substrate or the presence of lignin, and the low protein content to the high amount of cellulases required for the degradation of the substrate which were produced at the expense of mycelial protein. Another factor which may have contributed to the slow growth of the fungus is the temperature of the medium 30°C which is lower than the range 35°-40°C which has been reported as optimum for growth (vHofsten and Ryden, 1975) (vHofsten,1976) (Nilsson, 1965) (Rosenberg, 1975) (Janus, 1977).

In our experiments,(Figure 16, Tables 12 and 13) S.pulverulentum did not compare favourably with the other cellulolytic fungi in terms of substrate degradation, F.P. activity and biomass and protein productivity. However, it was more efficient than T.viride 1 and 2 in converting the degraded substrate to mycelium and protein, and the low F.P.A. observed may be due to the absorption of the cellulolytic enzymes on the non degraded cellulose. The fermentation by S.pulverulentum also does not compare favourably with most of the reported results (Table 14a,b).

3.2.2.3. Cellulose fermentation by S.thermophile 1 and 2 and newspaper, fermentation by S.thermophile 2

S.thermophile is a relatively newly discovered species (Henssen, 1957) able to grow over a wide range of temperatures 24°-55°C and according to Cooney and Emerson (1964), is near the definition limit of a true thermophile. S.thermophile is active in the decomposition of plant materials able to degrade wood in pure culture (Ofosu-Asiedu and Smith, 1973) and it is one of the main agents of biodeterioration of stored wood chips in the hot area of the pile (Shields and Unligil, 1968). It is able to ferment starchy materials (Reade and Gregory 1975) (Khor et al 1976) and it has been shown to utilize cellobiose and hemicellulose almost as well as glucose (Chang, 1967). When pure cellulose was used as the main carbon source high substrate degradation (91%) was reported by Barnes (1974) and production of mycelium with 40% biuret protein was reported by Romanelli (1975), see Table 14b.

The results obtained from the two varieties of S. thermophile are shown in Figures 17,18 and Tables 12,13. It can be clearly seen that although the F.P. activities were the same, the cellulose fermentation by S.thermophile 2 was superior to S.thermophile 1 and to the other fungi tested in terms of substrate degradation and mycelium and protein productivities and yields. S.thermophile 2 compares also favourably with most of the cellulolytic microorganisms used in cellulose fermentations, Table 14b. The batch fermentation of ball milled newspaper by S.thermophile 2 is presented in Figure 19 and Table 12. Because of the presence of non cellulose substances, the mycelium dry weight was not

estimated as, total d.w - cellulose d.w, but it was estimated from the crude protein produced assuming that the mycelium grown on newspaper contained as much protein as when grown on cellulose. In Figure 19 the protein scale is adjusted so that the mycelium d.w. can ne read directly on the dryweight scale. In fermentation of cellulosic materials, mycelium d.w. determination based on the protein produced may lead to overestimation because of the absorption of cellulases on the remaining cellulose (Moreira et al, 1978) or non cellulose solids (Andren et al, 1976). This may be also the cause of the reduced F.P. activity observed.

From the presented results it can be seen that S. thermophile 2 was able to convert the lignocellulosic substrate to biomass satisfactorily compared with the other cellulolytic fungi tested on pure cellulose and also compared with reported results on the fermentation of lignocellulosic materials. Tables 13, 14a.

Comparing the fermentations of ball milled cellulose and newspaper by S.thermophile it can be seen that although the degradable substrate, cellulose and cellulose plus hemicellulose, was quantitatively the same, the F.P. activity, the percentage of degraded cellulose, and mycelium and protein productivities and yields were lower in the newspaper fermentation. Similar observations have frequently been reported in the literature when pure cellulose and lignocellulose have been used as substrate by the same microorganism. The reduction in fermentation efficiency has been attributed to the presence of lignin which acts as barrier between the cellulose and the cellulolytic enzymes. (Moo-Young et al, 1977), (Chahal et al, 1977). See also Table 14a,b.

Table 14a Reported data on batch fermentation of cellulosic materials

| Microorganism | Substrate (Co) | Co $g.l^{-1}$ | Time h | Co degraded % | Biomass $g.l^{-1}$ | Protein $g.l^{-1}$ | %TDW | Productivity $mg.l^{-1}.h^{-1}$ Biomass | Protein | References |
|---|---|---|---|---|---|---|---|---|---|---|
| T.reesei QM9414 | Alkali treated sawdust§ | 10.0(9.0) | 36 | (81*) | - | 1.3b | 25.4 | - | 36.1 | Moo-Young et al (1977) |
| T.reesei QM9123 | Alkali treated straw§ | 10.0(4.0) | 48 | - | 3.68* | 1.84c | 23.8 | 76.6* | 28.3* | Peitersen (1975) |
| T.longibranchiatum | Delignified bagasse | 10.0(3.58) | 144 | 54.5* | 0.86 | 0.60 | 27.0 | 5.9* | 4.2* | Sidhu & Sandhu(1980) |
| Brevibacterium sp | Ground wood | 10.0(5.9) | 72 | 82* | - | 0.53b | - | - | 7.3 | Fu & Thayer (1973) |
| C.cellulolyticum | Alkali treated sawdust§ | 10.0(9.0) | 36 | (78*) | - | 1.80b | 40.0 | - | 50.0 | Moo-Young et al (1977) |
| C.cellulolyticum | Acid treated sawdust | 10.0(4.7) | 43 | (42) | - | 1.30b | 15.0 | - | 30.0 | Moo-Young et al (1978) |
| C.cellulolyticum | Alkali treated sawdust | 10.0(4.7) | 28 | (44) | - | 0.75c | 11.0 | - | 27.0 | as above |
| C.cellulolyticum | Bleached kraft pulp fines | 5.0(2.3) | 23 | (84*) | - | 0.65c* | 22.0* | - | 28.3* | Pamment et al (1979) |
| C.cellulolyticum | Ground wood/ sulphite fines | 10.0(4.8) | 112 | (41.3) | - | 0.34c* | 4.02 | - | 3.0* | as above |
| C.cellulolyticum | Ground straw | 10.0 | 48 | 16 | - | 0.43 | 4.6 | - | 8.9 | Chachal et al (1977) |
| Thermomonospora fusca | Pulping fines§ | 5.0(3.2) | 96 | - | 0.67 | 0.40c | 30.0 | 7.0* | 4.2* | Crawford et al (1973) |
| C.cellulolyticum | Kraft pulp fines | 5.0(3.2) | 24 | (80*) | - | 1.0*c | 27.5* | - | 41.6* | Pamment et al (1979) |
| S.pulverulentum | Pulping fines | 10.0 | 144 | 56 | 3.5* | 0.40*c | 6.1* | 24.3* | 3.4* | Eriksson & Larsson(1975) |
| M.verrucaria | Ball milled newspaper§ | 40.0(20.4) | 144 | (56) | - | 3.30c | 9.8* | - | 22.9* | Updegraff (1971) |
| S.thermophile | Ball milled " § | 8.0(3.4) | 11 | 90 | 4.1 | 1.03c | 19.2 | 372.7 | 93.0 | Barnes (1974) |
| S.thermophile 2 | Ball milled " | 13.7(3.5) | 40 | (71.2) | 3.3 | 1.36c | 19.0 | 82.5 | 34.0 | This work |

Table 14b Reported data on batch fermentation of cellulose

| Microorganism | Substrate (Co) | Co $g.l^{-1}$ | Time h | Co degraded % | Biomass $g.l^{-1}$ | Protein $g.l^{-1}$ | Protein %T.D.W | Productivity $mg.l^{-1}.h^{-1}$ Biomass | Productivity $mg.l^{-1}.h^{-1}$ Protein | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| Trichoderma sp | Delignified pulp§ | 15.0 | 120 | - | - | 1.49c | 23.8 | - | 12.4* | Chahal & Gray (1970) |
| T.reesei QM9414 | Amorphous cellulose | 10.8* | 36 | 82.4* | 3.0* | 1.10b | 22.4 | 83.3* | 30.6 | Moo-Young et al(1977) |
| T.reesei QM9414 | Amorphous cellulose§ | 10.0 | 43 | 81.0 | 4.9 | 1.96b | 35.5 | 114.0* | 45.5* | Andreotti et al(1977) |
| T.reesei QM9414 | Ball milled " § | 7.5 | 48 | 80.0 | - | 1.85b | - | - | 38.5* | Peitersen (1977) |
| T.reesei QM9414 | Powdered cellulose | 10.0 | 50 | 70.0* | 4.5 | 2.77b | 36.9* | 90.0* | 55.4* | Hendy & Gray(1979) |
| T.longibranch-iatum | Powdered cellulose | 5.0 | 144 | 15.0* | - | 0.15c | 3.1 | - | 1.0* | Sidhu & Sandhu(1980) |
| T.viride 2 | Ball milled " | 10.2 | 36 | 87.8 | 3.3 | 1.12c | 24.6 | 91.6 | 31.1 | This work |
| T.viride 1 | Ball milled " | 10.2 | 36 | 80.1 | 2.9 | 1.02c | 20.6 | 80.5 | 28.3 | This work |
| M.verrucaria | Delignified pulp§ | 15.0 | 120 | - | - | 1.49c | 17.85 | - | 12.4* | Chahal & Gray(1970) |
| C.globosum | Delignified pulp§ | 15.0 | 120 | - | - | 1.20c | 10.30 | - | 10.0* | Chahal & Gray(1970) |
| R.solani | Delignified pulp§ | 15.0 | 72 | - | - | 2.16c | 22.70 | - | 30.0* | Chahal et al (1975) |
| S.pulverulentum | Powdered cellulose§ | 10.0 | 168 | - | - | 0.34c | 4.30 | - | 2.0* | Eriksson & Larsson(1975) |
| S.pulverulentum | Ball milled " | 10.3 | 41 | 60.2 | 2.4 | 0.80c | 12.2 | 59.7 | 19.5 | This work |
| Thermoactinomyces sp. | Powdered cellulose | 8.0 | 20 | 78.0 | 2.4 | 1.32c | 31.80 | 120.0* | 66.0* | Humphrey et al (1977) |
| C.cellulolyticum | Amorphous cellulose§ | 10.0 | 36 | 73.0* | 4.0* | 2.70b | 40.30 | 111.0* | 75.0 | Moo-Young et al(1977) |

continued.....

Table 14b Continued

| Microorganism | Substrate (Co) | Co $g.l^{-1}$ | Time h | Co degraded % | Biomass $g.l^{-1}$ | Protein $g.l^{-1}$ | Protein % TDW | Productivity $mg.l^{-1}.h^{-1}$ Biomass | Productivity $mg.l^{-1}.h^{-1}$ Protein | Reference |
|---|---|---|---|---|---|---|---|---|---|---|
| C.cellulolyticum | Amorphous cellulose§ | 9.5 | 24 | - | - | 1.85b | 46.20 | - | 77.0* | Chahal & Wang (1978) |
| S.thermophile | Powdered cellulose§ | 10.0 | 72 | 56.0 | 2.1 | 0.82b | 12.70* | 29.0* | 11.3* | Romanelli et al (1975) |
| S.thermophile | Ball-milled " § | 8.0 | 10 | 91.2* | 5.7 | 1.43c | 17.0* | 570.0* | 143.0* | Barnes (1974) |
| S.thermophile 1 | Ball-milled " | 10.3 | 41 | 73.8 | 2.9 | 1.05c | 18.4 | 70.7 | 25.6 | This work |

c = Kjeldahl b = biuret * calculated from published data

§ = supplemented with easily metabolized substances

Numbers in parehtneses indicate cellulose content or % of cellulose degradation

The aim of batch fermentation of ball milled cellulose by the selected cellulolytic fungi was to assess their ability in the conversion of substrate to mycelium and protein. The tested microorganisms showed different fermentation abilities. It was apparent, however, that S.thermophile 2 was the best fungus in terms of substrate degradation and biomass and protein productivity and yield. It was also able to ferment satisfactory lignocellulosic materials compared with the fungi tested and other microorganisms reported in the literature. S.thermophile 2 was then selected for further experiments on the continuous fermentation of pure cellulose and lignocellulosic materials.

### 3.3 Continuous culture of S.thermophile 2 on cellulosic materials in a 5 l Tower fermenter.

The experimental work on the continuous fermentation of cellulosic materials was carried out in two parts.

In the first part S.thermophile 2 was cultured continuously on media with different initial cellulose concentrations (Co) and at different dilution rates (D). The purpose of these experiments was to assess the ability of the microorganism to grow continuously in Tower fermenters and to examine the effects of dilution rate, initial cellulose concentration and non fermented cellulose on the fermentation.

In the second part the experimental work was of a more applied nature. A lignocellulosic material (newspaper) which may be considered as waste was used as model substrate in order to examine the effects of dilution rate and non fermented solids on the fermentation.

Each continuous run started from a batch culture in order to avoid build up of mycelium and non degraded solids in the fermenter which could affect the kinetic characteristics of the fermentation (Greenshields, 1977, personal communication). After the beginning of each continuous operation, samples were collected from the effluent line and their cellulose content was determined. A steady state was assumed to have been achieved when three consecutive measurements of the effluent total dry weight ($X_{ET}$) taken at two hour intervals agreed within 4% and showed no upward or downward trend in terms of mycelium ($X_E$) and cellulose ($C_E$) content. In the case of newspaper fermentation the mycelium could not be determined and the criterion was based on the cellulose content of $X_{ET}$. Steady states were achieved after about four residence times and maintained for about five residence times.

Once a steady state was achieved samples from both the fermenter and effluent line were taken and analysed as described in section 2. The fermenter samples were collected from the middle port. Samples taken periodically from the upper and lower ports of the fermenter showed no significant difference indicating that the fermenter contents were well mixed.

### 3.3.1 Continuous culture of S.thermophile 2 on pure cellulose

#### 3.3.1.1 Fermentation profile

S.thermophile 2 was grown continuously at 45°C, pH6, 1.6 v/v/m, and at different dilution rates (D) on media containing the following cellulose concentrations (Co):

Co = 7.5 $g.l^{-1}$ D from 0.036 to 0.062 $h^{-1}$ (run 1)

Co = 10.0 $g.l^{-1}$ D from 0.034 to 0.060 $h^{-1}$ (run 2)

Co = 15.0 $g.l^{-1}$ D from 0.030 to 0.058 $h^{-1}$ (run 3)

The data obtained at steady states are tabulated in Appendice 3 a,b and illustrated in Figs. 20 to 28. Fig. 20 illustrates the fermentation profile of run 1. The same trend was observed in runs 2 and 3.

The continuous fermentation of cellulose was characterized by an increase in both the fermenter and effluent dry weight when both the initial cellulose concentration and the dilution rate were increased.

Determination of the cellulose content of the fermenter and effluent samples showed that the increase was due to the non degraded cellulose solids whereas the mycelium in both the fermenter and effluent stream decreased when the dilution rate was increased.

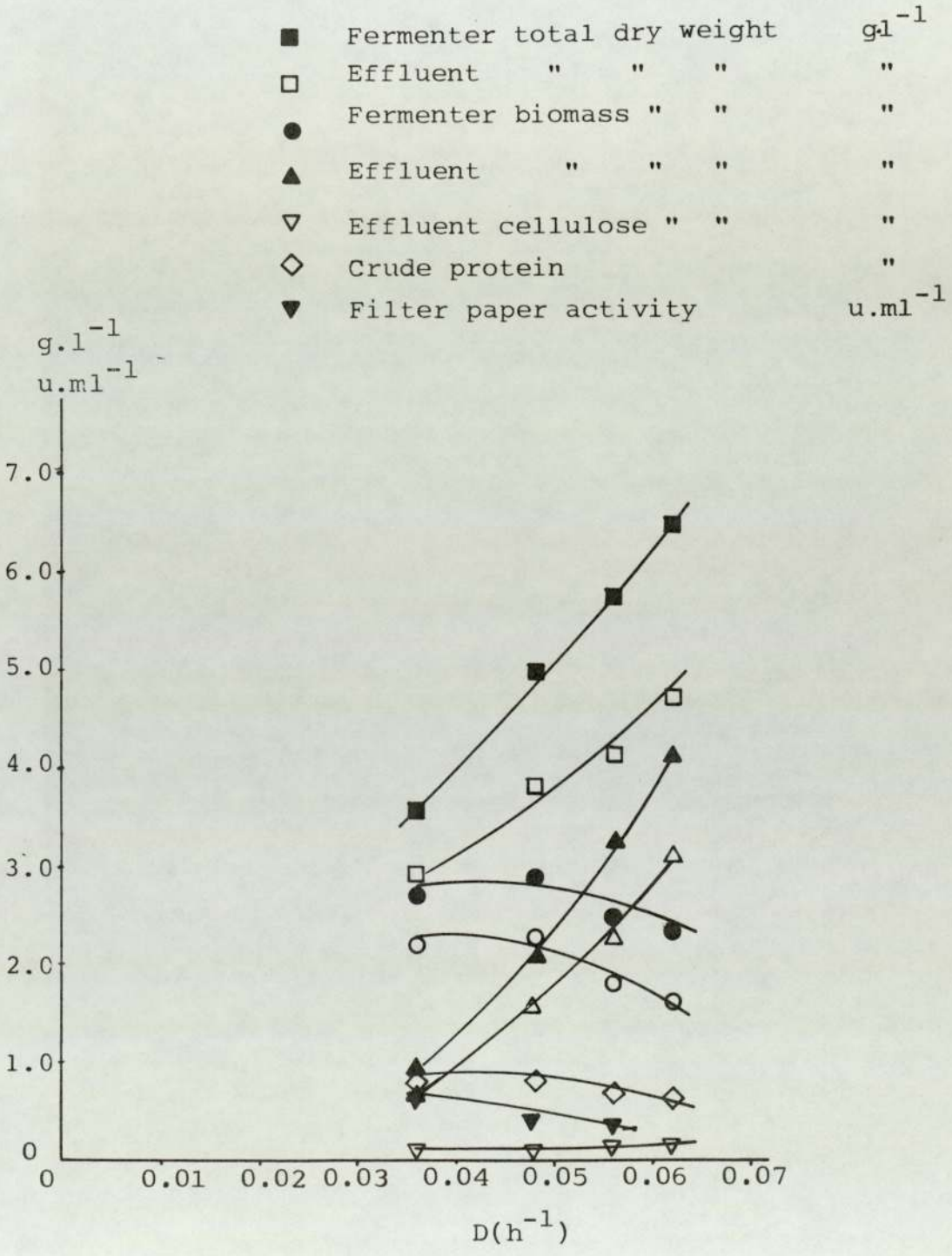
Increase in the initial cellulose concentration did not result in a proportional increase in the degradation of the substrate (Fig. 21) and this affected both the concentration of biomass in the fermenter and effluent stream and the composition of the obtained product.

The crude protein content of the mycelium showed a tendency to decrease with dilution rate and was relatively unaffected by the initial substrate concentration.

The biomass yield (Y) expresses the relation between the biomass produced and the substrate utilized by the microorganism. It may be used to describe the biomass formed from any of the utilized components of the medium. In this work it is used to describe the conversion of cellulose into

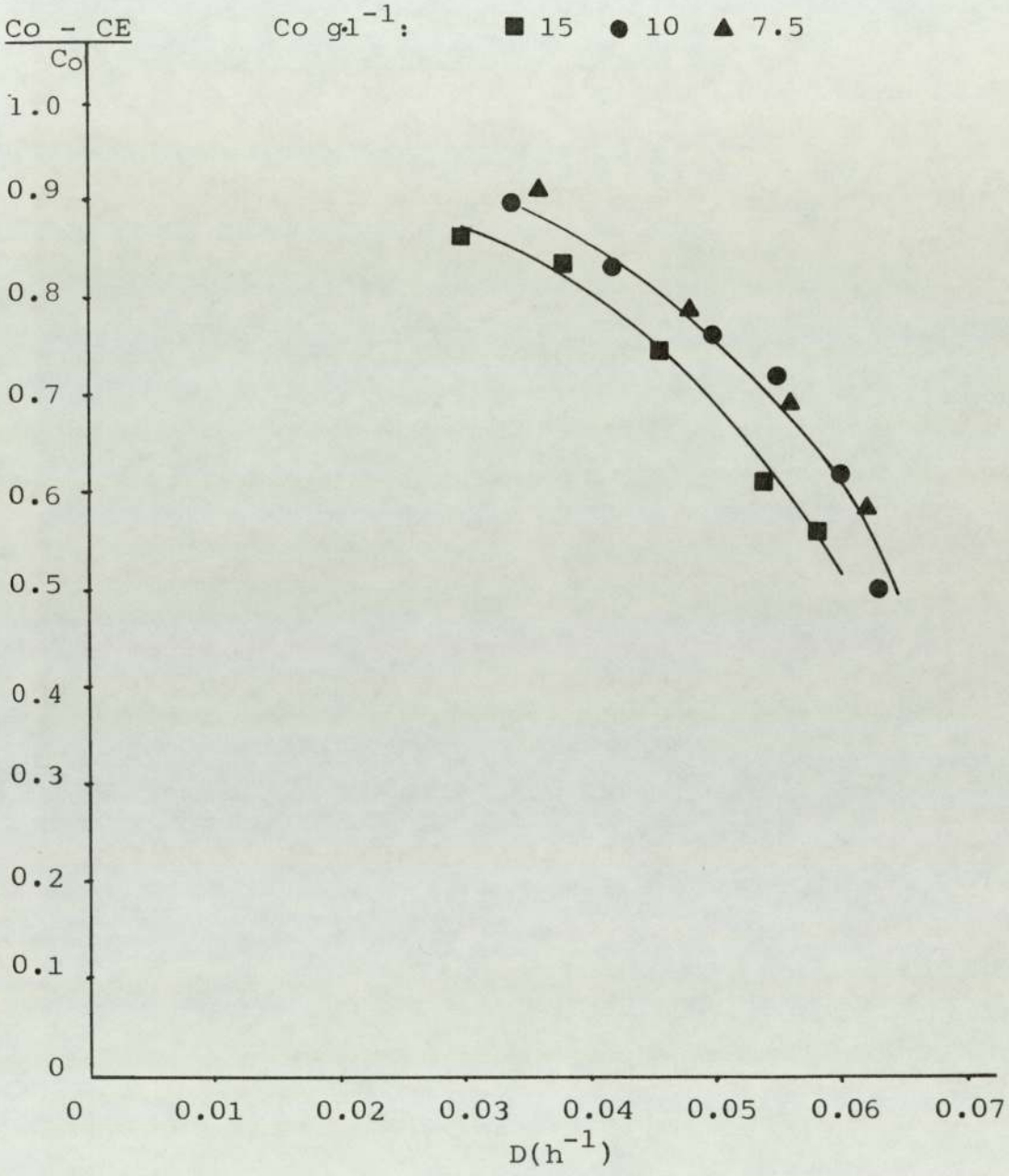
Continuous culture of S.thermophile 2 on ball milled cellulose at 45°C, pH6, 1.6 v/v/m. Steady state values.

Figure 20.Fermentation profile of run 1 (Co = 7.5 $gl^{-1}$).



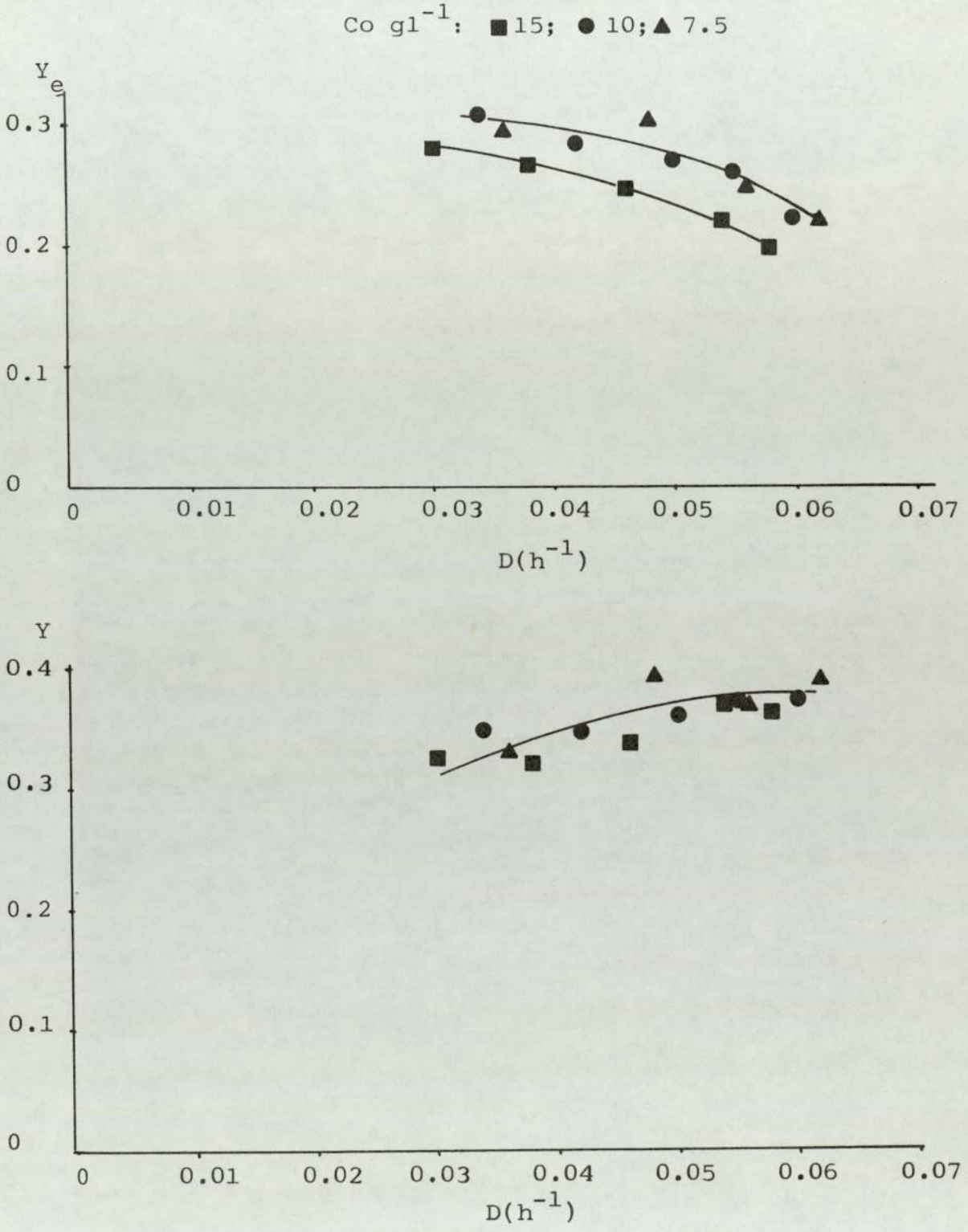
Continuous culture of S.thermophile 2 on ball milled cellulose at 45°C, pH6, 1.6 v/v/m. Steady state values.

Figure 21. Relation between cellulose degradation efficiency $\left(\frac{Co - C_E}{Co}\right)$ and dilution rate (D).



Continuous culture of S.thermophile 2 on ball milled cellulose at 45°C,pH6,1.6 v/v/m. Steady state values.

Fig 22.Relation between biomass yield (Y), biomass effective yield (Ye) and dilution rate (D).



mycelium and it was calculated as $Y = \frac{D.X_E}{D.C}$, where C is the amount of hydrolysed and consumed cellulose. The biomass yield showed a tendency to increase with dilution rate whereas the effective biomass yield $Y_e = \frac{D.X_E}{D.C_o}$ decreased ( Fig. 22 ).

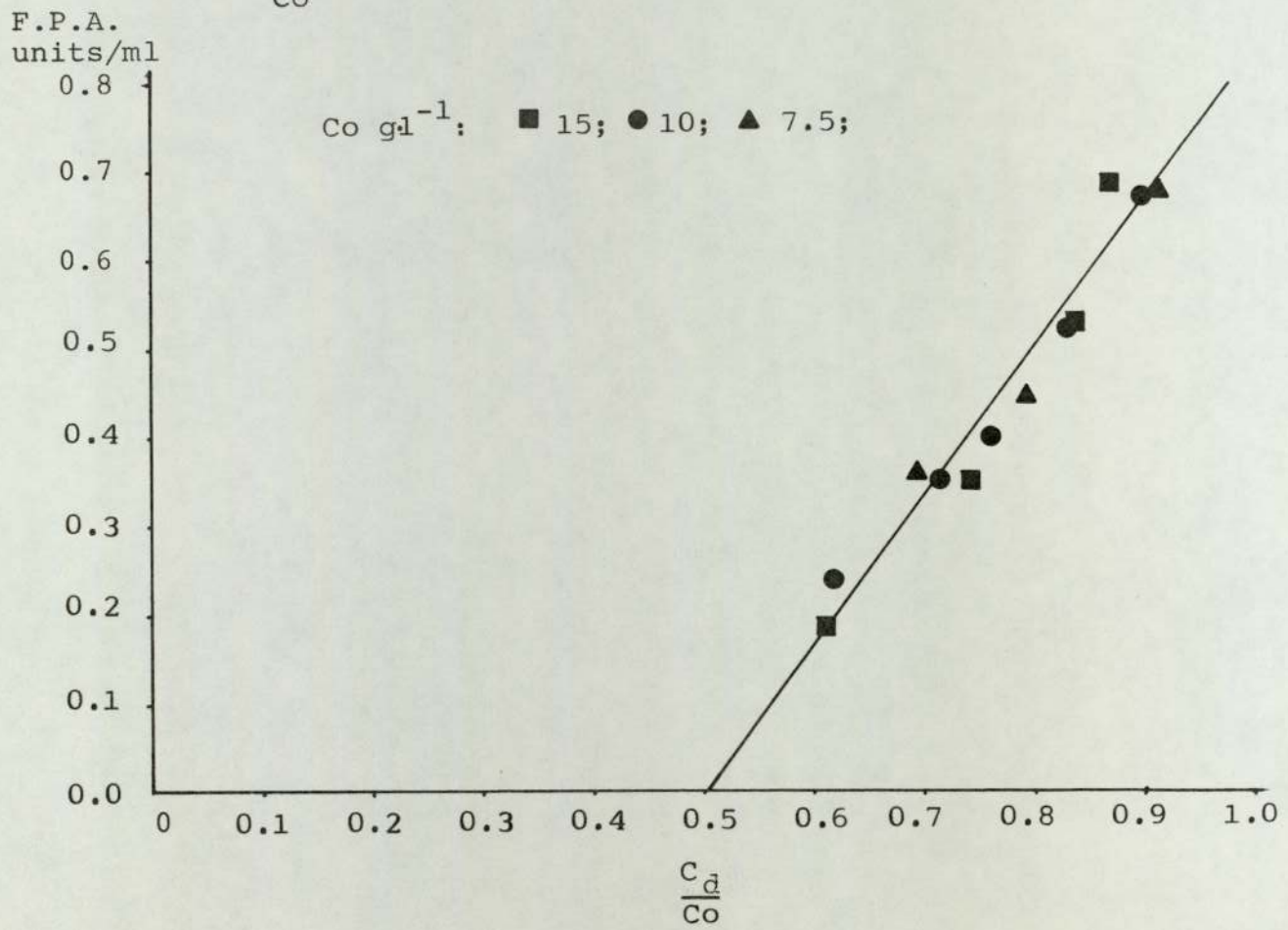
The reducing sugars concentration showed a tendency to increase when the initial substrate concentration and dilution rate increased. However it remained well below the concentration (10mM) which is considered as inhibitory to the cellulase system (Peitersen, 1975a). The low level of reducing sugars also suggests that the degraded cellulose was almost completely consumed by the microorganism and the hydrolysis of the substrate was the factor which controlled the fermentation. The extracellular cellulase activity (F.P.A.) decreased with dilution rate. Fig. 23 shows the relation between the efficiency of cellulose degradation $(\frac{C_d}{C_o})$ and the detected F.P.A. The increased concentration of non degraded cellulose affected the detection of cellulolytic enzymes which show a strong tendency to adsorb on cellulose solids. From Fig. 23 it can also be seen that little extracellular cellulase activity can be expected when less than 50% of the initial substrate is degraded.

Another characteristic of the fermentation was the difference observed between the concentration of cellulose and mycelium in the fermenter and in the effluent stream. In all fermentations the non degraded cellulose and the mycelium concentration in the fermenter were higher than the corresponding concentrations in the effluent stream.

Microscopical examination of fermenter and effluent samples showed, apart from the mycelium-cellulose aggregates,

Continuous culture of S.thermophile 2 on ball milled cellulose at 45°C, pH6, 1.6 v/v/m. Steady state values.

Figure 23. Relation between Filter paper activity (F.P.A.) and relative cellulose degradation $(\frac{C_d}{Co})$.



and free particles of cellulose and fragments of mycelium. The mycelial fragments and cellulose particles appeared more abundant at the higher dilution rates ($D > 0.042\ h^{-1}$) suggesting that the increased cellulose solids affected the morphology of the mycelium.

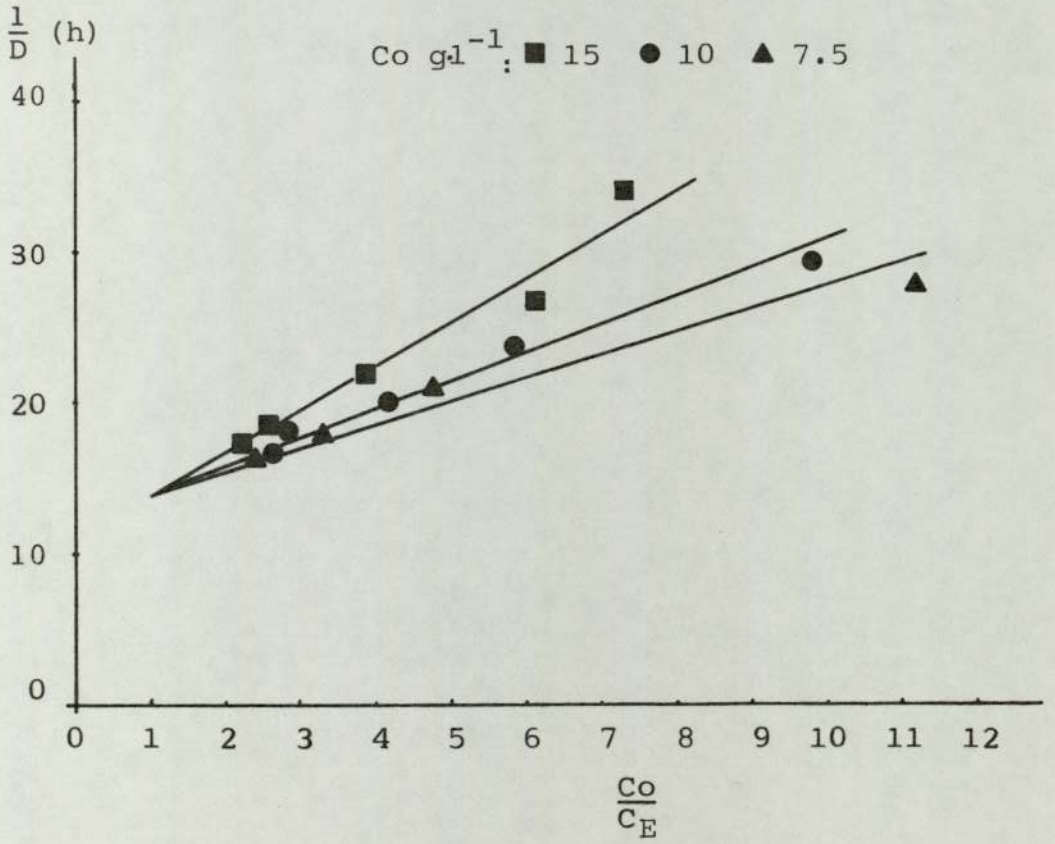
3.3.1.2 Cellulose degradation

The degraded cellulose ($C_d$) was calculated from the cellulose content of the effluent total dry weight ($C_E$) as $C_o = Co - C_E$. The effect of the dilution rate on the efficiency of substrate degradation $\frac{C_d}{C_o}$ is shown in Fig. 21. High degradation efficiency ($\frac{C_d}{C_o}$ 0.8) was achieved at dilution rates below $0.045\ h^{-1}$ and it was rapidly reduced as the dilution rate increased. The decrease in the efficiency of substrate degradation resulted in an increase of the cellulose in the effluent stream which was rapidly approaching the value of the initial cellulose concentration as the dilution rate was increased.

The relation between cellulose degradation and dilution rate can also be seen in Fig. 24 which is a reciprocal plot of D vs. the relative cellulose concentration, expressed as $\frac{C_E}{C_o}$. Assuming that wash out occurs when the concentration of cellulose in the effluent becomes equal to the initial substrate concentration (i.e. $\frac{C_o}{C_E} = 1$) then, under the experimental conditions, the fermenter could not operate at D higher than about $0.073\ h^{-1}$.

Continuous culture of S.thermophile 2 on ball milled cellulose at 45°C,pH6,1.6 v/v/m. Steady state values.

Figure 24. Reciprocal plot of dilution rate (D) versus the relative cellulose concentration ($C_E/Co$). D crit ( ~0.073) obtained at $C_E$ = Co



3.3.1.3 Specific growth rate ($\mu$) of S.thermophile 2 and productivity of the Tower fermentation system

The specific growth rate of a microorganism and the productivity of a continuous culture system are represented by the equations:

Growth rate $\frac{dx}{dt} = \mu . X_F$

Productivity $\frac{dx}{dt} = D . X_E$

and at steady state

$$\mu X_F = D X_E$$

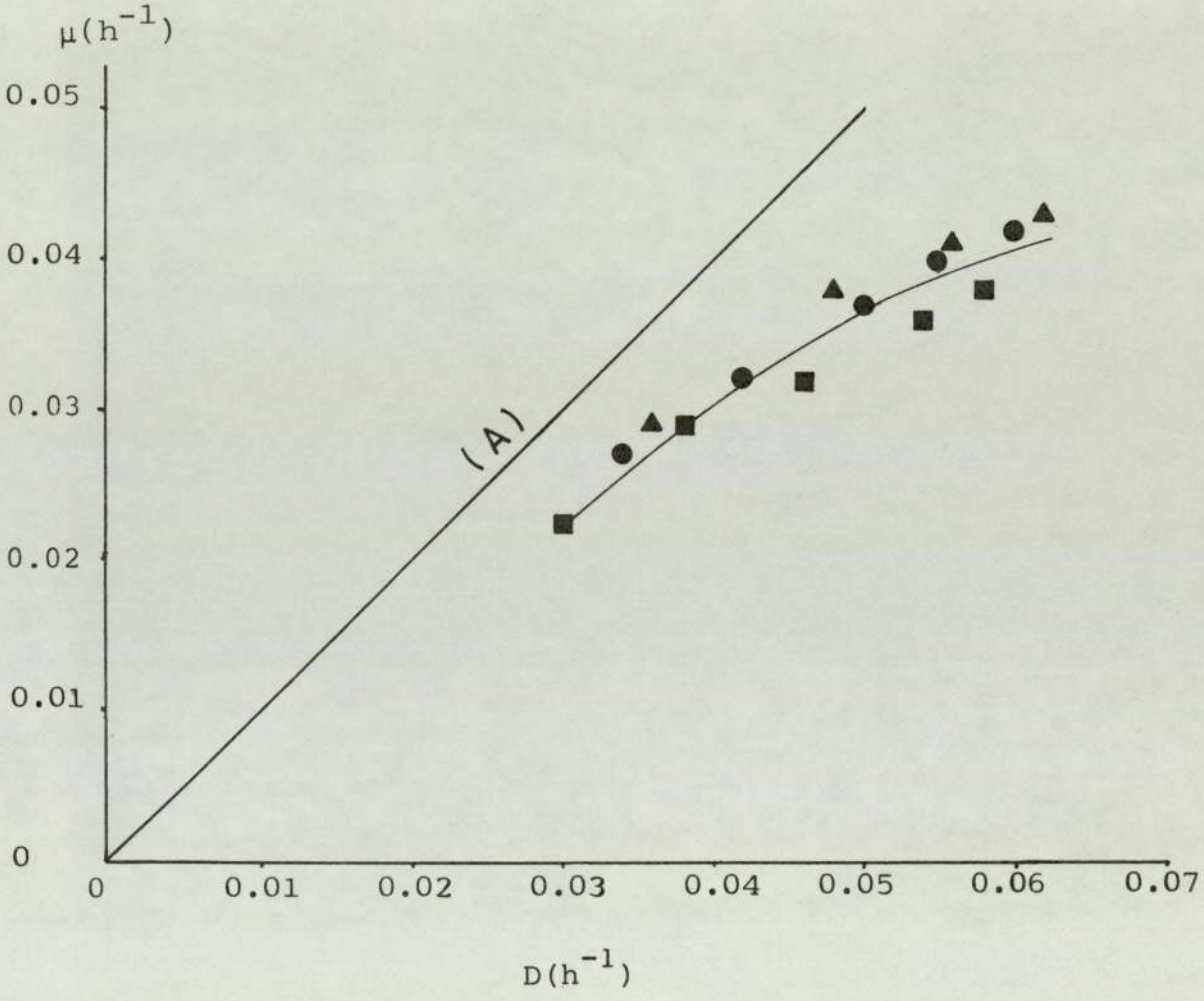
In fermentation systems where $X_E = X_F$ the specific growth rate is numerically equal to the dilution rate. In Tower fermenter, due to the retention properties of the system which result in $X_F > X_E$ this simplification cannot be made and the specific growth rate is calculated as $\mu = D \frac{X_E}{X_F}$ (Spensley, 1977) (Pannell, 1976) (Nerantzis, 1978). The relation between $\mu$ and D in this series of experiments is shown in Fig. 24. The specific growth rate increased with dilution rate, the increase however was non linear due to the variation in $\frac{X_E}{X_F}$ ratio. The effects of dilution rate on the productivity of the system are illustrated in Fig. 25. Due to the presence of non degraded cellulose solids the total dry weight output ($D.X_{ET}$) increased continuously with increasing dilution rate. The biomass productivity ($DX_E$) closely followed the trend of the substrate utilization rate, increasing up to $D \approx 0.055\ h^{-1}$ and then decreased. The same trend was observed in the crude protein productivity (D.P), however the effect of the dilution rate was less pronounced possibly because the crude protein content of the total product included the cellulolytic enzymes adsorbed

Continuous culture of S.thermophile 2 on ball milled cellulose at 45°C,pH6,1.6 v/v/m. Steady state values.

Figure 25.Relation between specific growth rate (μ) and dilution rate (D)

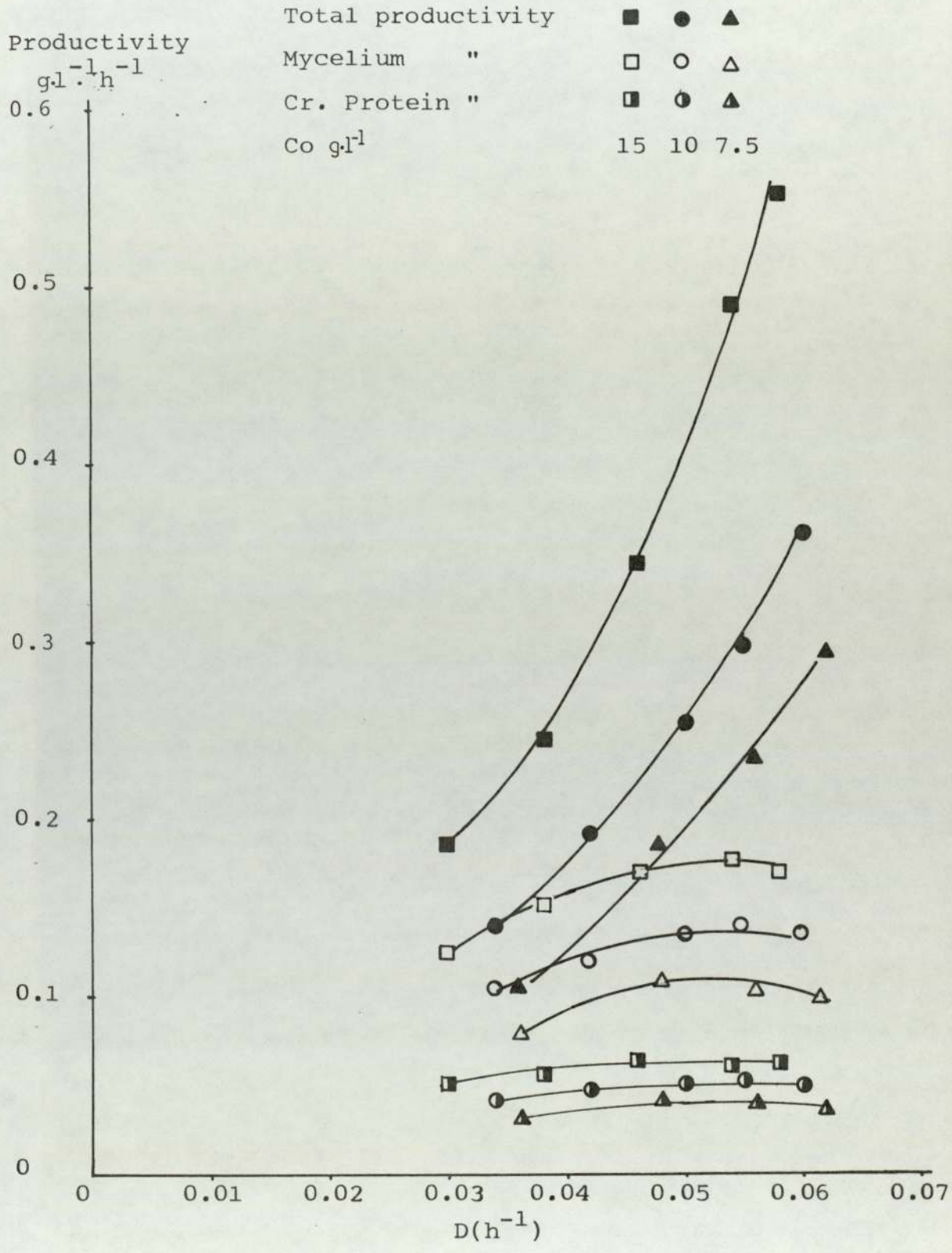
Co $g.l^{-1}$: ■ 15; ● 10; ▲ 7.5

Line A: case for μ = D



Continuous culture of S.thermophile 2 on ball milled cellulose at 45°C, pH 6, 1.6 v/v/m. Steady state values.

Figure 26. Relation between productivity and dilution rate (D)



on the cellulose solids. This also may be the cause of the continuous decrease in the productivity of extracellular cellulolytic enzymes.

### 3.3.1.4 Cellulose degradation rate ($DC_d$) and cellulose utilization rate (DC)

The effect of dilution rate on cellulose utilization rate is illustrated in Fig. 26. The straight lines represent the cellulose supply rate (DCo). The graph shows that only at low dilution rates ($D < 0.04\ h^{-1}$) the consumption rate was close to the substrate supply rate. Maximum substrate utilization rates were obtained at $D \approx 0.055\ h^{-1}$. Higher dilution rates resulted in an increase of (DC). Because of the small amount of reducing sugars in the fermenter broth the same trend was observed in the variation of cellulose degradation rate.

### 3.3.1.5 Determination of the true growth yield ($Y_G$) and maintenance energy coefficient (m)

Cellulolytic microorganisms grown on cellulose utilize the hydrolysed substrate for their maintenance and the production of new cell material. The overall rate of substrate consumption at steady state is given by the equation (Pirt, 1975)

total rate of consumption = rate of consumption for growth + rate of consumption for maintenance

or $$\frac{ds}{dt} = \frac{\mu \cdot x}{Y_G} + mx$$

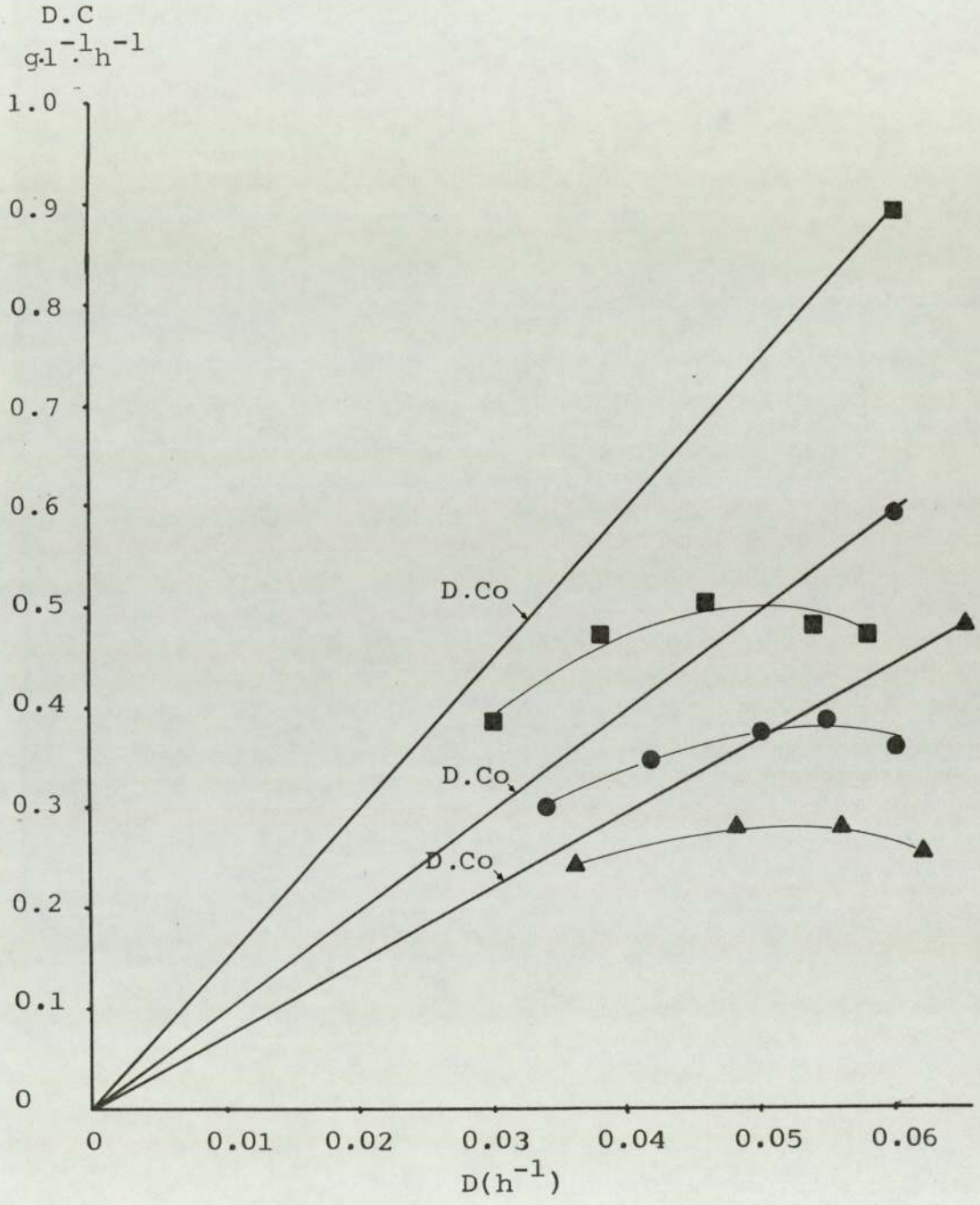
where:

Continuous culture of S.thermophile 2 on ball milled cellulose at 45°C, pH 6, 1.6 v/v/m. Steady state values.

Figure 27. Relation between cellulose utilization rate (D.C) and dilution rate (D)

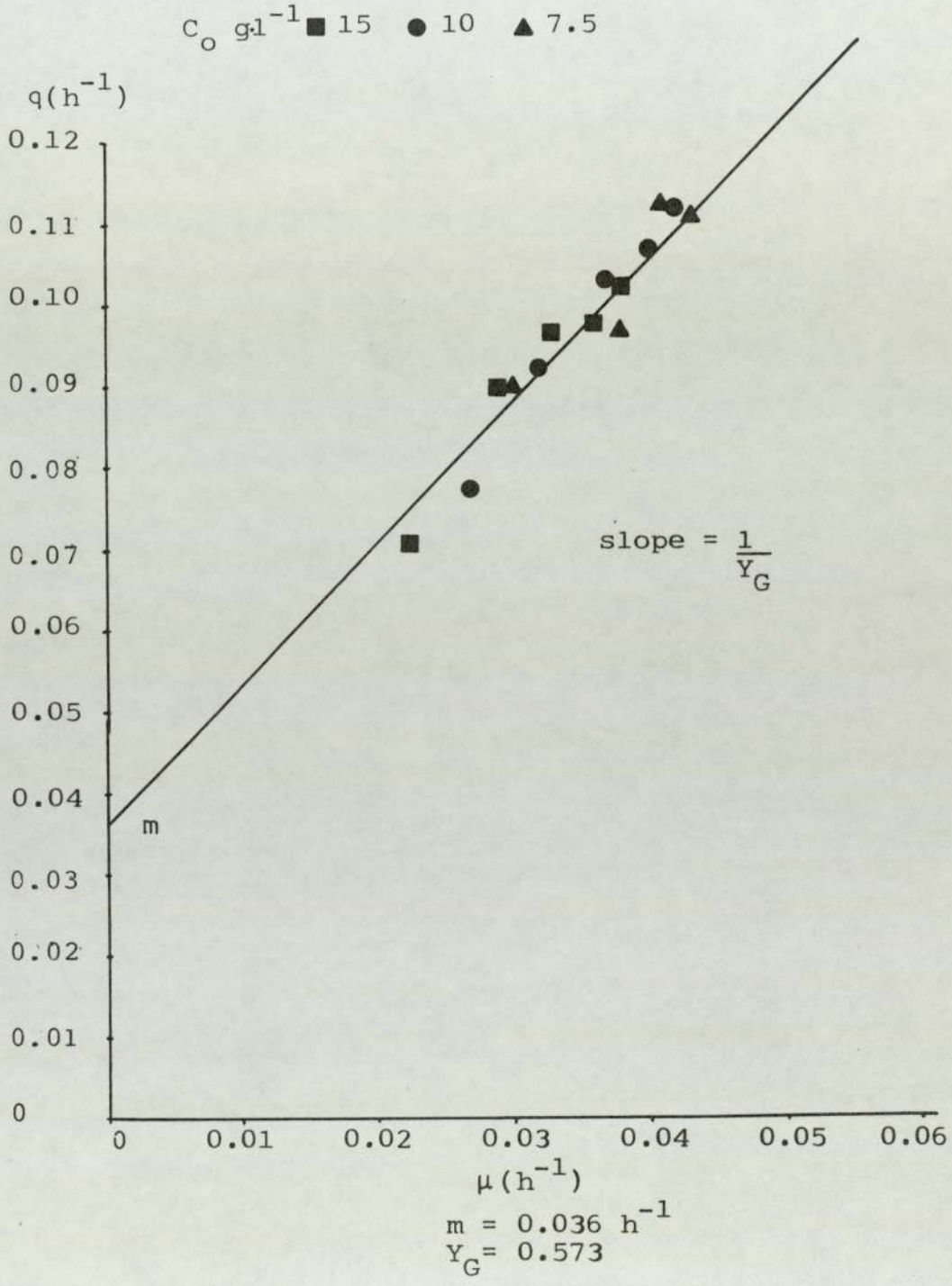
Co $g.l^{-1}$: ■ 15 ● 10 ▲ 7.5

D.Co = Cellulose supply rate



Continuous culture of S.thermophile 2 on ball milled cellulose at 45°C, pH 6, 1.6 v/v/m. Steady state values.

Figure 28. Relation between the specific substrate utilization rate (q) and specific growth rate (μ)



| | | | |
|---|---|---|---|
| $x$ | = | biomass concentration in the fermenter | $g.l^{-1}$ |
| $\mu$ | = | specific growth rate of microorganism | $h^{-1}$ |
| $s$ | = | concentration of substrate | $g.l^{-1}$ |
| $Y_G$ | = | true growth yield | - |
| $m$ | = | maintenance energy coefficient | $h^{-1}$ |

The maintenance energy coefficient is a measure of the energy used independently of growth. It is used in the turnover of macromolecules, osmotic regulation, cellular organisation and for the production of secondary metabolites (Righelato et al, 1968). The true growth yield is a measure of the substrate utilized purely for growth of the organism. The maintenance coefficient is therefore the specific substrate consumption rate (q) at zero specific growth rate and the true growth yield is the substrate consumed as $\mu$ tends to infinity.

In order to determine $Y_G$ and m the specific substrate utilization rate (estimated as $q = \frac{DC}{X_F}$) was plotted against the specific growth rate ($\mu$) of the microorganism (Fig. 27). The slope of the line gives the reciprocal of $Y_G$ and the intersept of the ordinate gives the value of m (Pirt, 1975).

The values obtained under the conditions of our experiments were m = 0.036 g cellulose/g mycel./h and $Y_G$ = 0.573 g mycel./g cellulose.

### 3.3.2 Discussion of the results

#### 3.3.2.1 The total solids concentration in the fermenter ($X_{FT}$) and effluent stream ($X_{ET}$)

Comparison between the fermenter and effluent stream dry weights showed that at each steady state the fermenter

concentration, in terms of both mycelium and cellulose solids, was higher than that in the effluent stream. This indicated the existence of a concentration mechanism which resulted in a partial retention of the fermenter contents.

This internal recycling has been frequently reported by investigators working on the Tower fermentation of soluble carbohydrates by yeasts or bacteria (Greenshields and Smith, 1971) (Nerantzis, 1978) and filamentous fungi (Pannell, 1976) (Spensley, 1977) (Stockbridge, 1979) (Pace and Righelato, 1976).

Greenshields and Smith (1971) attributed this phenomenon to the interaction between fluidization and sedimentation forces and the colonial structure of the microorganism. The same opinion was expressed by Pannell (1976) and Spensley (1977).

In fermentation media containing both soluble carbohydrates and inert non fermentable solids, the presence of the latter was found to be an additional factor which affected both the morphology of the microorganism and the retention properties of the fermenter (Spensley, 1977) (Stockbridge, 1979). A 'channelling mechanism' was postulated as another factor affecting the retention properties of the Tower fermenter by Stockbridge (1979). This mechanism depended upon the formation of air slugs in the fermenter and 'biomass deficient' channels formed behind the air slugs and ejected from the fermenter. This mechanism however cannot explain steady state results reported by Spensley (1977) where a higher solids concentration was observed in

the effluent than in the fermenter. Spensley attributed this negative retention effect to minute air bubbles adsorbed or entrapped within the mycelial colonies which reduced their overall density. Foaming of the culture also affected the retention properties of the fermenter especially with respect to free non fermentable solid particles, mycelial fragments or monocellular microorganisms (Pannell, 1976) (Stockbridge, 1979).

Pace and Righelato (1976) reported that two factors affected the mycelium retention properties of the Tower fermenter. A concentration gradient which exists in the upper part of the fermenter body and which is determined by a sedimentation constant ($K_{sed}$) and a proportional separation in the effluent line ($K_o$). They related the biomass concentration in the fermenter ($X_F$) to the biomass concentration in the effluent ($X_E$) as $X_E = X_F \cdot K_o \cdot K_{sed}$. However the existence of a concentration gradient in the fermenter has not been generally accepted. Pannell (1974) reported that no concentration gradient was observed in the Tower fermenter at dilution rates up to 0.25 $h^{-1}$ with the fermenter containing up to 20 $gl^{-1}$ biomass. Stockbridge (1979) working with soluble carbohydrates containing non fermentable cellulose solids up to 15 $gl^{-1}$ reported that no concentration gradient of the mycelium-cellulose aggregates was observed along the length of the fermenter. The same opinion was expressed by Spensley (1977).

Our results (Figs. 25,26) suggest that the increased concentration of non fermented cellulose solids with dilution rate increased the biomass retention properties of the

fermenter. This was attributed mainly to the increase in the overall density of the mycelium-cellulose aggregates. The free mycelial fragments and cellulose particles may have been fully fluidized or subjected to a preferential wash out due to the foaming of the fermenter medium as suggested by Pannell(1976) and Stockbridge (1979).

With regard to the existence of a concentration gradient, samples taken periodically from different ports of the fermenter did not show either inconsistency or difference higher than $\pm$ 4% indicating that the retention mechanism was effective mainly in the outflow of the fermenter. However because the upper sampling port was about 16 cm below the beginning of the outflow, operation of the concentration mechanism in the area between the upper port and below the outflow cannot be excluded.

### 3.3.2.2 Performance assessment of the Tower continuous fermentation of cellulose by S.thermophile 2

Cellulolytic microorganisms are unable to metabolize cellulose directly. The substrate must be first hydrolysed to glucose by extracellular or cell bound cellulases and then utilized by the microorganism for maintenance and production of new cell material (Humphrey et al, 1977).

Viewed as a conversion process, cellulose → biomass, the fermentation was assessed by the efficiency of substrate degradation and biomass productivity.

### 3.3.2.3 Cellulose degradation

Cellulose can be considered as a multiple substrate

because the particles are composed of regions with different physical and chemical properties which not only respond differently to the action of cellulolytic enzymes but also can affect the production of a specific component of the cellulase complex (Rautela and King, 1968).

In continuous fermentations of easily metabolized substances the substrate available for growth is proportional to the dilution rate. In cellulose fermentations an increase in the cellulose supply rate represents only an increase in the potential carbon source, and the degradation and consumption of cellulose will be determined by a complex interaction between growth,cellulase production and cellulase activity.

The hydrolysis of the substrate was the most important factor which controlled the fermentation. The hydrolysed cellulose served as the growth substrate and determined the growth rate of the microorganism. The product of hydrolysis has also been reported to affect the production and activity of the cellulase components (Mandels et al, 1975). The non degraded cellulose solids affected the fermentation because they increased the washing out of the cellulases due to their adsorption properties. They also affected the morphology and sedimentation of mycelium and thus the retention characteristics of the fermenter.

At low dilution rates ($D < 0.04\ h^{-1}$) both the extracellular cellulolytic activity and the substrate degradation efficiency were high (Figs 20,21). This was attributed to the higher biomass concentration in the fermenter and the higher residence times which permitted a prolongued action of cellulolytic enzymes and thus more efficient degradation of

the substrate. The non degraded cellulose solids did not affect the enzyme activity in the fermenter because there were highly hydrolysed and thus they had fewer active sites for adsorption and removal of cellulases.

At higher dilution rates both the substrate degradation efficiency and the extracellular cellulolytic activity were rapidly reduced. The calculated dilution rate at which zero degradation would occur was about 0.074 $h^{-1}$. Rapid decrease of substrate utilization and extracellular cellulase activity at dilution rates well below the critical value have also been reported in the continuous culture of T.reesei QM 9414 in stirred tank reactors. The reason was attributed to catabolite repression of the remaining cellulose on the biosynthesis of cellulolytic enzymes (Ghose and Sahai, 1979) (Peitersen 1977). Another possible reason may be the increased removal of cellulolytic enzymes adsorbed on the non degraded cellulose particles.

Reported data on the continuous fermentation of cellulosic materials are given in Table (15). It can be seen that S.thermophile compares well in terms of substrate degradation with T.reesei (Sahai and Ghose, 1977) (Peitersen, 1977). The high substrate degradation by S.pulverulentum can be attributed to the chemical treatment which renders the cellulose fibres very susceptible to biodegradation.

### 3.3.2.4 The productivity of the system

The biomass productivity in any fermentation system ($DX_E$) is a direct measure of the growth which has occured and it is determined by the quantity of biomass in the fermenter ($X_F$),

Table 15 Reported data on the continuous fermentation of cellulosic materials

| Microorganism | | | T.reesei | T.reesei | S.pulverulentum | S.thermophile 2 |
|---|---|---|---|---|---|---|
| Dilution rate | D | $h^{-1}$ | 0.012-0.028 | 0.033-0.080 | 0.042 | 0.030-0.062 |
| D crit | | $h^{-1}$ | 0.031 | 0.170 | - | 0.074 |
| Pretreatment | | | ball milling | ball milling | chemical | ball milling |
| Cellulose conc$^{n}$ | Co | $g.l^{-1}$ | 5.0 | 4-11.0 | 20.0 | 7.5-15.0 |
| " degradation | % | | 99-82 | 75-50 | 100 | 90-56 |
| Biomass productivity | $D_{XE}$ | $g.l^{-1}h^{-1}$ | 0.013-0.033 | 0.053-0.198 | 0.415 | 0.08-0.18 |
| True growth yield | $Y_G$ | | 0.45 | 0.83 | - | 0.57 |
| Maintenance coeff. | m | $h^{-1}$ | 0.031 | 0.034 | - | 0.036 |
| Overall biomass Yield | Y | | 0.21-0.29 | 0.39-0.68 | 0.5 | 0.32-0.39 |
| Effective " | $Y_e$ | | 0.21-0.27 | 0.38-0.28 | 0.5 | 0.31-0.20 |
| Product composition | | | | | | |
| Mycelium | $\%X_{ET}$ | | 94-57 | 60-38 | 100 | 77-31 |
| Protein | $\%X_{ET}$ | | - | 32-16 | 50 | 31-12 |
| Cellulose | $\%X_{ET}$ | | 6-43 | 40-62 | 0 | 33-69 |
| Operation | | | STR | STR | STR | Tower |
| pH/Temperature | | | 5.0/28$^{o}$ | 5.0/30$^{o}$ | 4.6/38$^{o}$ | 6.0/45$^{o}$ |
| References | | | Sahai and Ghose, 1977. | Peitersen, 1977. | Ek and Eriksson, 1977. | This work |

and the specific growth rate ($\mu$) of the microorganism (Pannell, 1976).

In Tower fermentations, due to the biomass retention, the productivity of the system can be represented by the relation

$\mu X_F = DX_E = Y_G [ D(Co - C_E - RS) - m.X_F ]$ (Spensley 1977)

The low amount of reducing sugars ( RS ) in the fermenter indicates that the factors which determined the biomass productivity were the substrate degradation rate, the biomass retention in the fermenter (which increases the substrate consumed for maintenance $m.X_F$) and the true growth yield ($Y_G$) which determines the amount of biomass that will be produced from the remaining substrate. The values of m and $Y_G$ for S.thermophile 2 were 0.036 $h^{-1}$ and 0.573 respectively and compare well with values reported for other fungi (Humphrey et al,1977) (Righelato,1975), see also Table 15.

The biomass retention in the fermenter and the decrease in substrate utilization efficiency resulted in a non linear increase in productivity with dilution rate. The same factors also resulted in the small increase of the biomass Yield ($Y_{XE}$) with dilution rate and in the continuous decrease of the effective yield (Ye).

In this work the protein content of the mycelium was estimated from the nitrogen content of the total dry weight (mycelium plus non degraded cellulose) and it is possible that it has been affected both by the cellulolytic enzymes adsorbed on the cellulose solids and by the non protein nitrogen of the mycelium (Moreira et al, 1978)(Spensley, 1977).

The crude protein content of the mycelium was reduced with dilution rate and the protein productivity showed little variation with this parameter. Reduction in the protein content of mycelium with D in cellulose fermentation has also been reported by Peitersen (1977) and it is attributed to the production of cellulolytic enzymes (Eriksson and Larsson,1975).

Reported continuous cellulose fermentation data are given in Table 15. The different parameters obtained by T.reesei QM 9414 have been attributed mainly to the difference in the nature and source of the substrate (Sahai and Ghose, 1977). The same can be attributed to the differences observed between T.reesei(Peitersen,1977),S.pulverulentum (Ek and Eriksson, 1977) and S.thermophile 2. The pretreatment of the substrate is of primary importance in bioconversion processes because it largely determines the biodegradability of the substrate and thus the overall performance of the fermentation system.

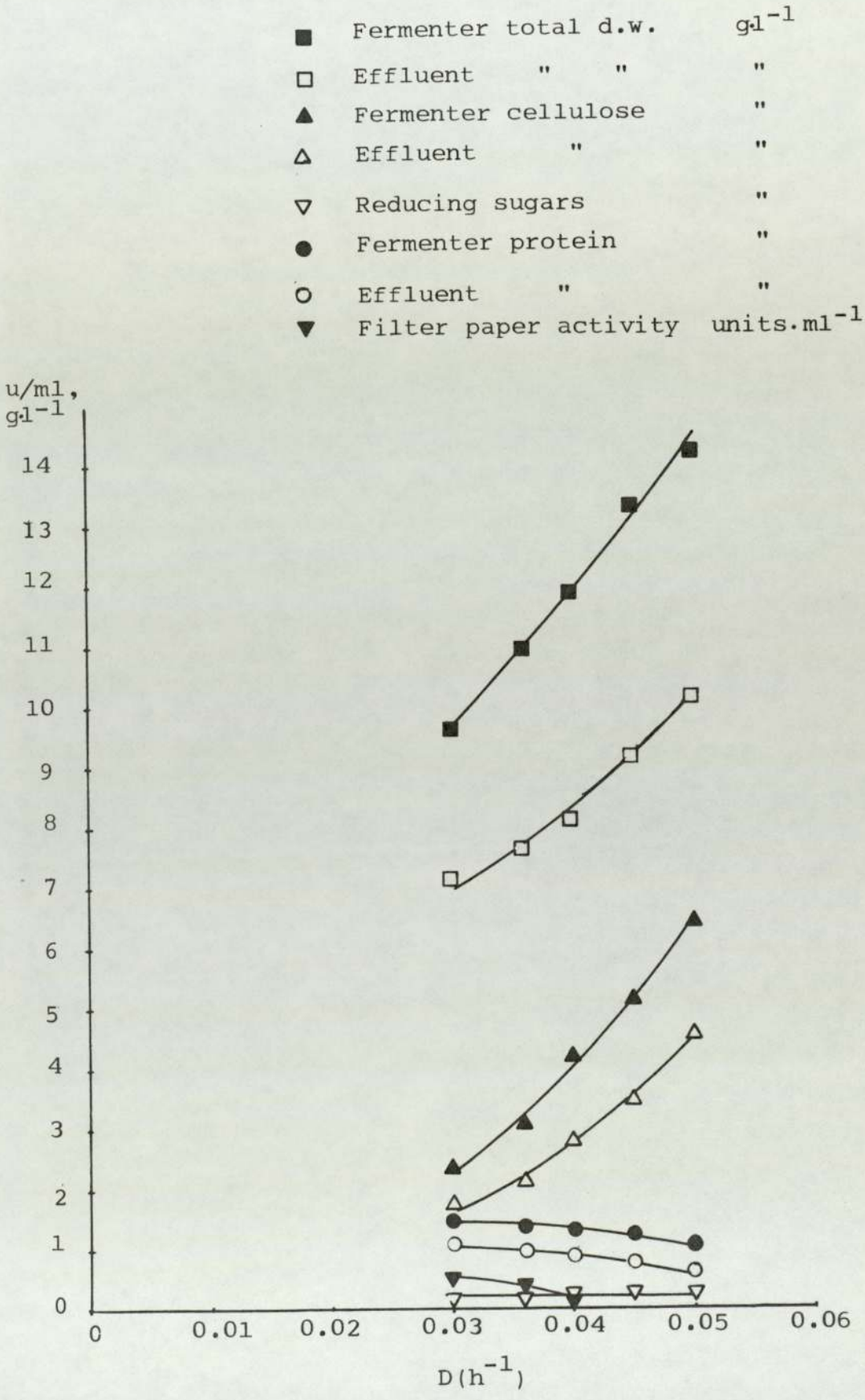
### 3.3.3 Continuous culture of S.thermophile 2 on newspaper

#### 3.3.3.1 Fermentation profile

S.thermophile 2 was grown continuously at 45°C, pH6, 1.6 v/v/m and at dilution rates from 0.030 to 0.050 $h^{-1}$ on a medium containing 15 $g.l^{-1}$ ball milled newspaper. The composition of the growth medium has been given in Table 9. The fermentation data obtained at steady states are tabulated in Appendice 3 c,d and illustrated in Figs 28 to 33. From Fig. 28 it can be seen that the newspaper fermentation showed a profile similar to that observed in the fermentation of

Continuous culture of S.thermophile 2 on ball milled newspaper at 45°C, pH 6, 1.6 v/v/m. Steady state values.

Figure 29. Fermentation profile (Co = 1 5 $g.l^{-1}$)



pure cellulose. The total dry weight, in both the fermenter and effluent stream, increased with dilution rate. Determination of the cellulose and crude protein content of the fermenter and effluent samples showed that the cellulose content increased with D whereas the protein content decreased. The reducing sugars remained at low level ($<0.26\ g.l^{-1}$) and showed little variation with D whereas the filter paper activity rapidly decreased and at $D = 0.045\ h^{-1}$ could not be detected.

The concentration of the non degraded cellulose in the fermenter was higher than the corresponding concentration in the effluent stream and the same relation was assumed for the non cellulose components of the samples.

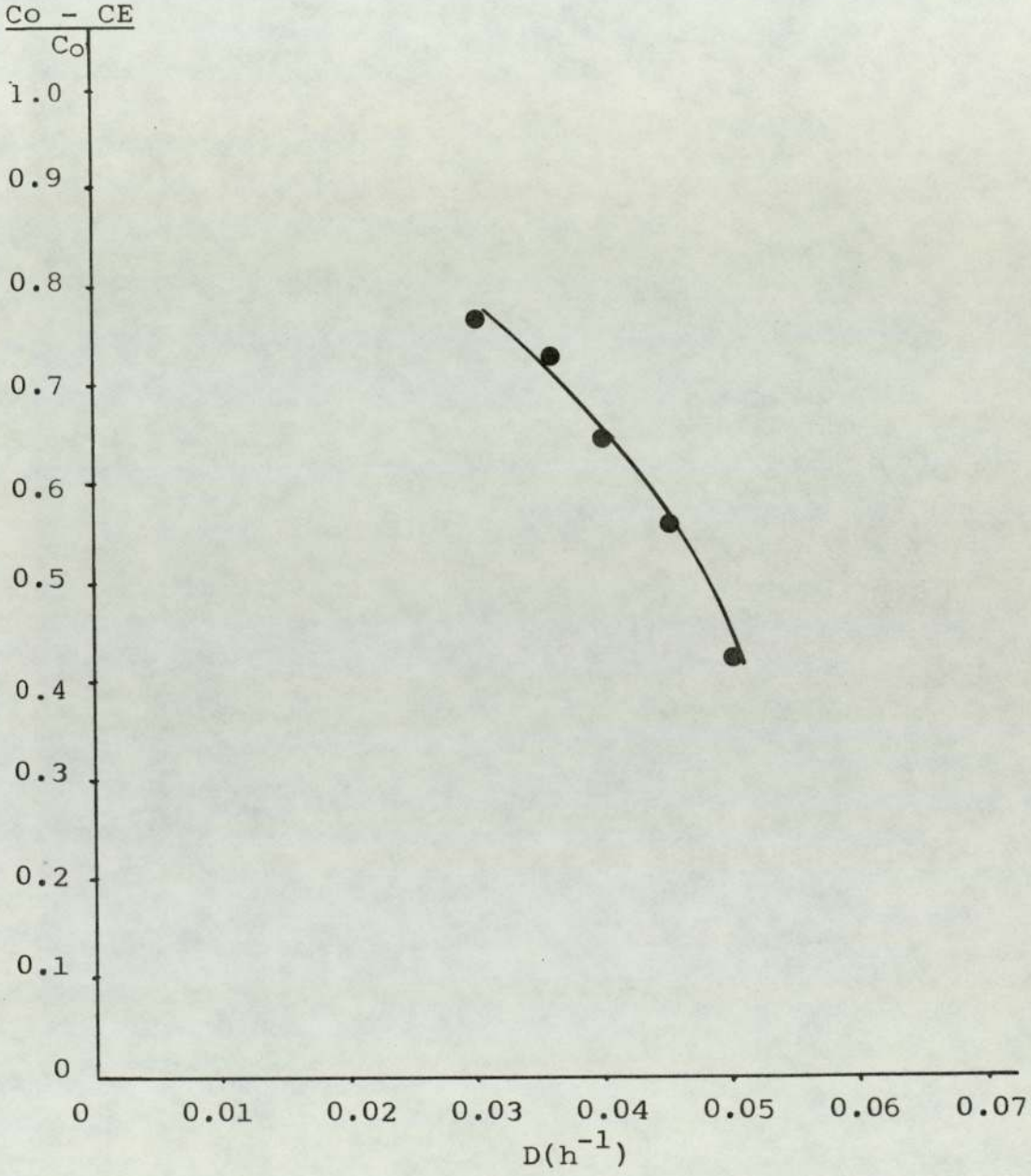
As in cellulose fermentation, microscopical examination of samples taken from both the fermenter and the effluent line showed mycelium-substrate aggregates and mycelium and substrate fragments. The frequency of the later increased with dilution rate.

3.3.3.2 Cellulose degradation

The degraded cellulose ($C_d$) was calculated from the cellulose content of the effluent stream ($C_E$) as $C_d = C_o - C_E$. The degraded cellulose represents only part of the degraded substrate as S.thermophile 2 is also able to ferment hemicellulose and possibly to degrade lignin (Barnes 1974). From Fig. 29 it can be seen that high degradation efficiency ($>0.78$) was achieved at $D < 0.036\ h^{-1}$ and rapidly decreased as D increased. The reciprocal plot of D vs. the relative

Continuous culture of S.thermophile 2 on ball milled newspaper at45°C,pH6,1.6 v/v/m. Steady state values.

Figure 30.Relation between cellulose degradation efficiency $\left(\frac{Co - C_E}{Co}\right)$ and dilution rate (D).



Continuous culture of S.thermophile 2 on ball milled newspaper at 45°C,pH 6,1.6 v/v/m. Steady state values.

Figure 31. Reciprocal plot of dilution rate D versus the relative cellulose degradation ($C_E$/Co). D crit ( ~ 0.06 $h^{-1}$) obtained at $C_E$ = Co

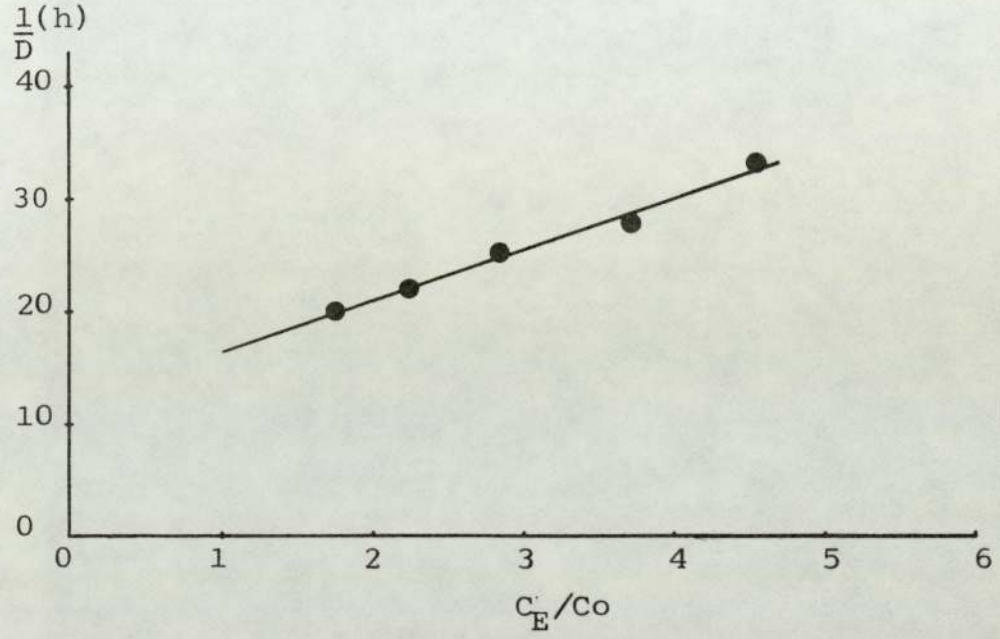
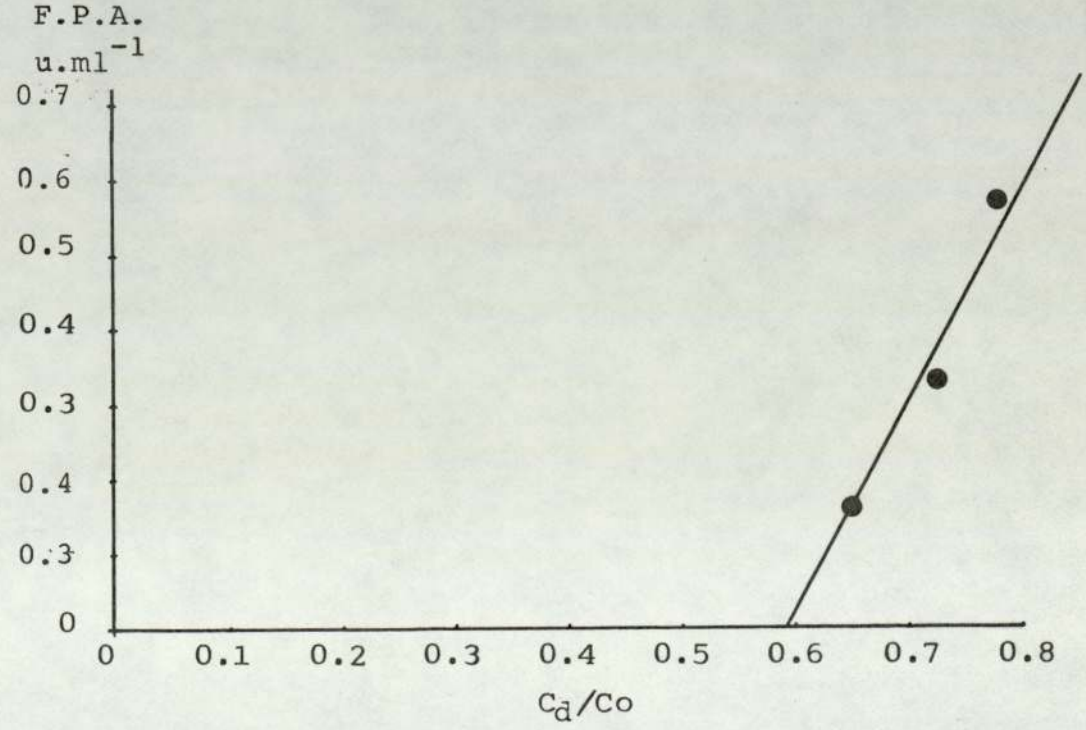


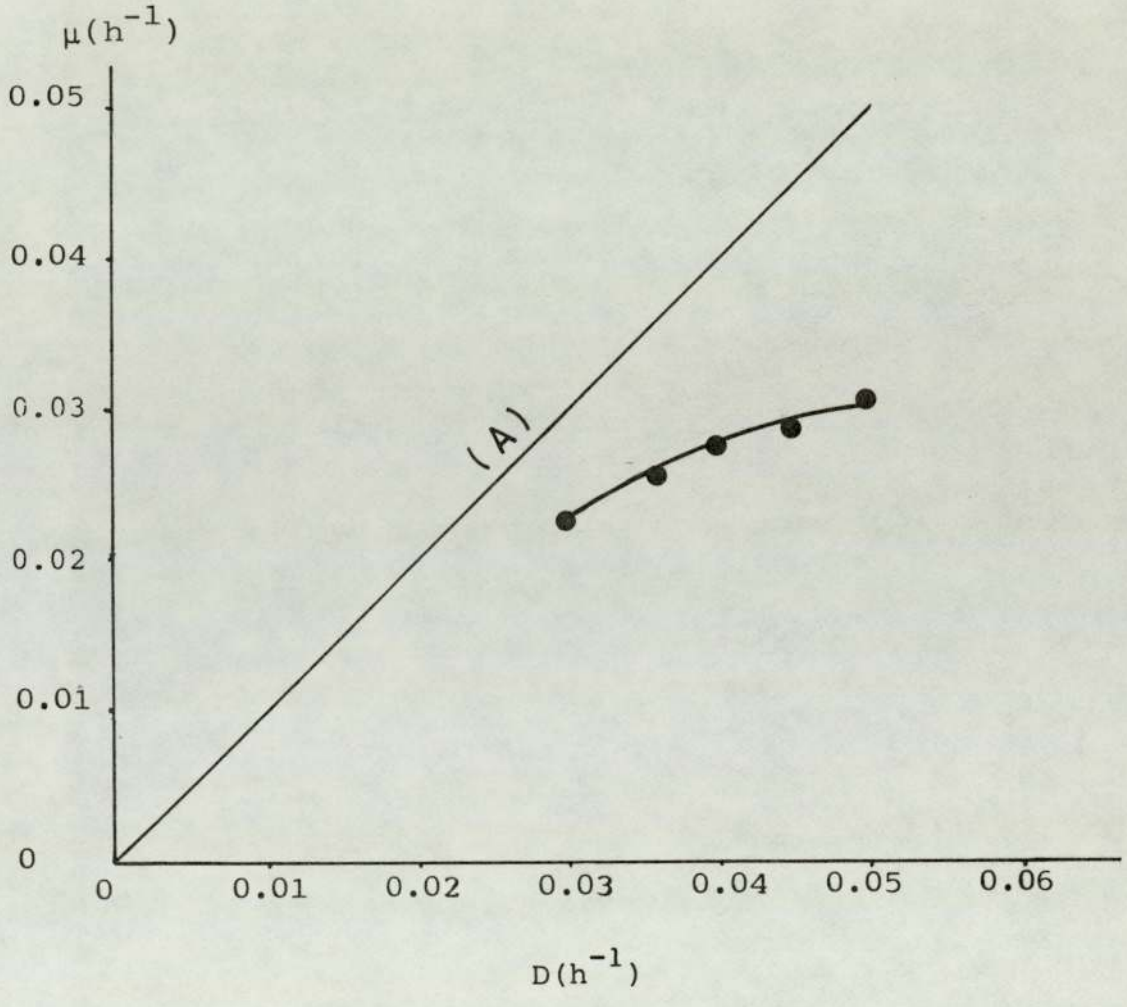

Figure 32. Relation between filter paper activity (F.P.A.) and relative cellulose concentration ($C_d$/Co)



Continuous culture of S.thermophile 2 on ball milled newspaper at 45°C,pH6,1.6 v/v/m. Steady state values.

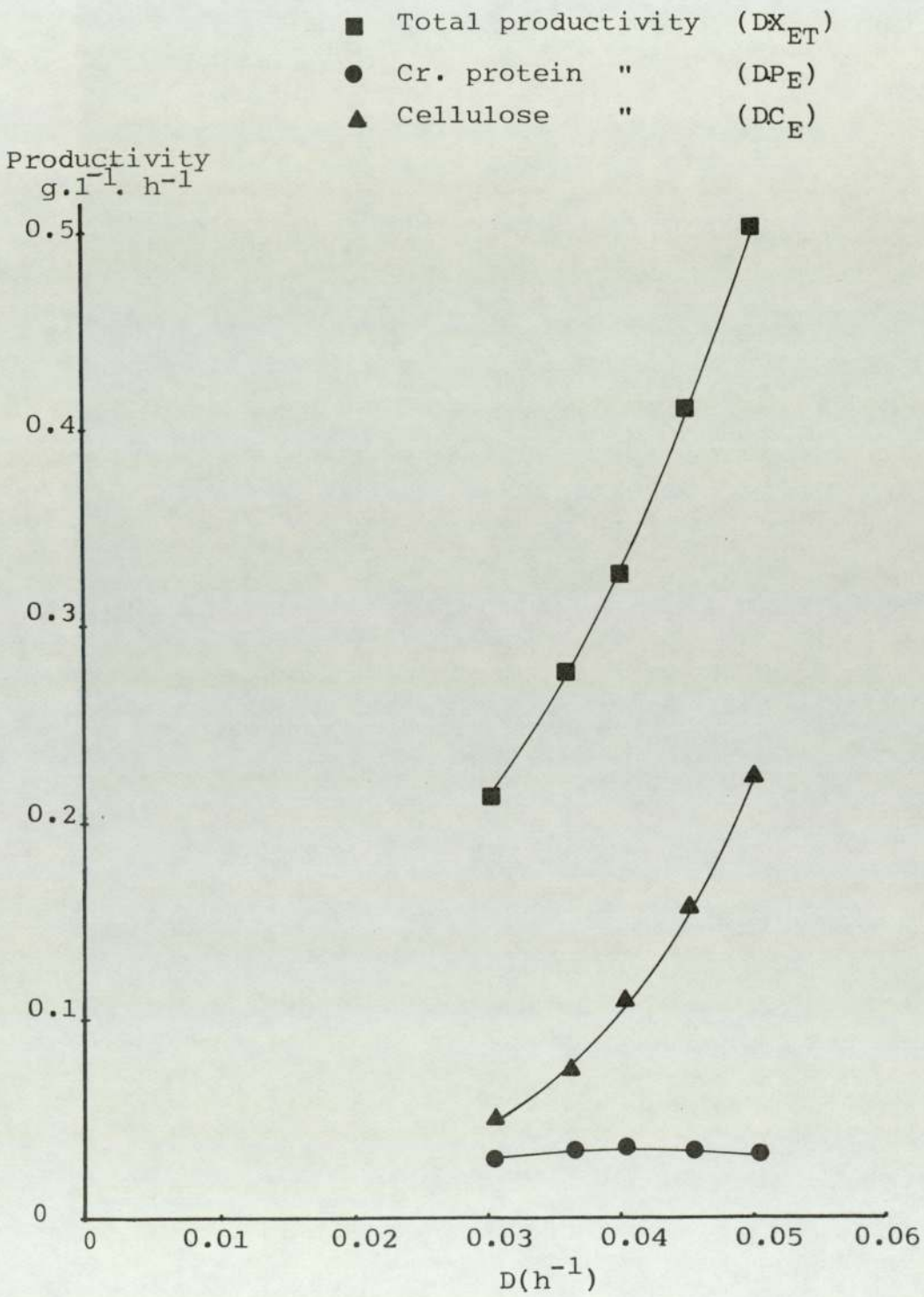
Figure 33. Relation between specific growth rate ($\mu$) and dilution rate (D)

Line A: case for $\mu = D$



Continuous culture of S.thermophile 2 on ball milled newspaper at 45°C,pH 6,1.6 v/v/m. Steady state values.

Figure 34.Relation between productivity and dilution rate (D)



cellulose concentration in Fig.(30) indicates zero degradation at $D \approx 0.060\ h^{-1}$. The relation between cellulose degradation efficiency and F.P.A. in Fig.(31) indicates that little activity can be expected when less than 60% of cellulose is degraded.

### 3.3.3.3 Specific growth rate (μ) of S.thermophile 2 and productivity of the Tower fermentation system

The variation of the specific growth rate of micro-organism (calculated as $D.P_E/P_F$) with D is shown in Fig. 32. The specific growth rate increased with D. As in cellulose fermentation the values of μ were lower than the corresponding values of D and their relation was not linear.

The total dry weight output and the cellulose output increased with dilution rate whereas the max. crude protein productivity was obtained at $D \approx 0.040\ h^{-1}$ (Fig. 33).

## 3.3.4 Discussion of the results

### 3.3.4.1 The total solids concentration in the fermenter and effluent stream

In the experiments with pure cellulose as carbon source the mycelium concentration was determined by subtracting the non degraded cellulose from the total dry weight (e.g. $X_E = X_{ET} - C_E$). The presence of non cellulose substances in the newspaper led to additional problems in the determination of fermentation parameters. The total dry weight of the fermenter and effluent stream was composed of mycelium, cellulose and non cellulose solids which were difficult to determine.

Comparison between the fermenter and effluent dry weights showed that at each steady state the fermenter concentration was higher in terms of both crude protein (and presumably mycelium) and cellulose solids. As in cellulose fermentation, this indicated the existence of a concentration mechanism in the fermenter, which is discussed in more detail in 3.3.2.

3.3.4.2 Performance assessment of the Tower continuous fermentation of newspaper by S.thermophile 2

The presence of cellulose associated substances increased the multiplicity of the substrate. The microorganism encountered all the difficulties associated with the degradation of cellulose and additionally it had to overcome the lignin barrier in order to degrade the cellulose and hemicellulose.

The low level of reducing sugars suggests that, as in the fermentation of cellulose, the hydrolysed part of cellulose and hemicellulose was rapidly consumed and the fermentation was controlled by the cellulose and hemicellulose degradation rate.

Although hemicellulose is degraded easier than cellulose the overall effect of lignin resulted in a decrease of the fermentation efficiency.

This can be clearly seen by comparison of cellulose and newspaper fermentation data presented in Table 16.

The further reduction in cellulose degradation efficiency with dilution rate was attributed mainly to the presence of lignin which both prevented the cellulases-cellulose contact

Table 16 Comparison of fermentation data obtained in continuous Tower fermentation of ball milled cellulose and newspaper by S.thermophile 2 at 45°, pH6, 1.6 v/v/m. steady state values

| | | | cellulose | newspaper |
|---|---|---|---|---|
| Substrate conc$^{n}$ | So | $gl^{-1}$ | 7.5-15 | 15.00 |
| Fermentable So | | $gl^{-1}$ | 7.5-15 | 10.04(1) |
| Dilution rate | D | $h^{-1}$ | 0.030-0.062 | 0.03-0.05 |
| Dcrit | | $h^{-1}$ | 0.074 | 0.060(2) |
| Specific growth rate | | $h^{-1}$ | 0.029-0.042 | 0.023-0.031(3) |
| Cellulose degradation | % | | 90-56 | 78-43 |
| Crude protein productivity | $DP_E$ | $gl^{-1}h^{-1}$ | 0.032-0.064 | 0.032-0.037 |
| Effective c.protein yield | $Y_{ep}$ | | 0.12-0.07 | 0.07-0.04(4) |
| Product composition | % | | | |
| Crude protein | $\%X_{ET}$ | | 30.2-11.5 | 15.2-6.3 |
| Cellulose | $\%X_{ET}$ | | 23.5-68.8 | 24.2-44.4 |

(1) based on the cellulose and hemicellulose content of newspaper

(2) based on cellulose degradation

(3) calculated from the crude protein content of $X_{ET}$ and $X_{FT}$

(4) based on the initial substrate conc$^{n}$

and removed cellulases from the fermenter by adsorption. The same effect was assumed on the hemicellulose. The presence of lignin was also considered as the cause of the faster disappearance of the F.P. activity from the fermentation broth ( Fig. 32 ). The presence of lignin has been reported to reduce both the growth of the cellulolytic microorganism T.reesei and the production of cellulolytic enzymes (Vohra et al,1980), and this may be another reason for the lower performance of the newspaper fermentation. The reduction in the effective protein yield with dilution rate can be attributed to the decrease both in the efficiency of cellulose degradation and (based on the cellulose fermentation) in the protein content of mycelium. The increase in the concentration of non degraded solids affected also both the composition of the final product and the retention properties of the fermenter. The later can be seen from the variation of the specific growth rate with dilution rate. The specific growth rate ($\mu$) was determined from the dilution rate (D) and the ratio $\frac{P_E}{P_F}$ $\frac{(\text{c. protein effl.})}{(\text{c. protein ferm.})}$ which was assumed to represent the ratio $X_E:X_F$, i.e. $\mu = D.\frac{P_E}{P_F}$ .

However the values of $\mu$ can be considered as tentative as the protein content of the samples is affected by the cellulases adsorbed on the solids.

# SECTION 4

# GENERAL DISCUSSION AND CONCLUSIONS

The aim of this work was to study the bioconversion of cellulosic materials to biomass in a novel Tower fermentation system. The Tower fermenter has been reported as having an internal recycling potential realized when microorganisms of a suitable morphology are cultured continuously (Greenshields and Pannell, 1975). This property enabled soluble carbohydrate fermentations to run at high dilution rates and it was decided that an investigation should be carried out to verify and assess this potential in the case of cellulosic materials. The micro-organisms used in this study were chosen from the literature. They belonged to species characterized by their ability of extensive attack on all forms of cellulose and they have been used for biomass production from cellulosic materials ( Table 14 a,b ). The experimental work was carried out in three stages. In the first stage the linear growth of fungi was measured on cellulose agar media in order to establish the optimum pH and temperature values for growth. This method was chosen because it was easy to perform and has been found reliable and related to the specific growth rate of the tested microorganisms in batch submerged cultures. However the optimum pH and temperature values as determined by this method may have been affected by the multiplicity of the substrate and may correspond more closely to the optimum values for growth on the amorphous part of cellulose. The linear growth test also did not give information about the morphology of the fungus which has been shown to play an important role in continuous Tower fermentations (Spensley, 1977). It is thus suggested that

in future investigations the optimum pH and temperature fermentation conditions be assayed in continuous culture and on the substrate to be fermented where the overall effect of the pH and temperature on the morphology, substrate degradation and productivity could be estimated more accurately.

In the second stage the cellulolytic fungi were cultured batchwise in a 5 litre Tower fermenter on cellulose media at their optimum pH and temperature. Their bioconversion potential was assessed in terms of substrate degradation, biomass production and protein (N x 6.25) content of biomass. The growth data obtained compare well with reported cellulose fermentations (Table 14 a,b). This can be attributed to the experimental conditions and to the efficiency of the Tower fermenter to provide good mixing and aeration (Pannell, 1976). The low level of reducing sugars observed indicated that the microorganisms metabolized the growth substrate as rapidly as it was hydrolysed and that their growth depended on their cellulose degradation ability. Among the fungi tested S.thermophile 2 was found to be the most potent agent of bioconversion. The results obtained from both cellulose and news paper fermentations compared well with data reported in the literature. The difference in the fermentation efficiency observed between cellulose and newspaper fermentation confirmed the negative effect of lignin in cellulose bioconversion processes and the need for a more efficient substrate pretreatment in order to overcome the lignin barrier.

In the third stage S.thermophile 2 was grown continuously in a 5 litre Tower fermenter on cellulose and newspaper

media at different substrate concentrations and dilution rates. An understanding of the continuous operation of the system hinged upon the cellulolytic ability of the micro-organism and upon the effects of dilution rate as a fluidising force, as a means of potential carbon source supply, and also as a means of removal of mycelium, non degraded solids, and cellulolytic enzymes from the fermenter.

The biomass concentration in the fermenter and the productivity of the system were determined mainly by the substrate available for growth and the retention properties of the fermenter. This resulted in a considerable degree of independence of the specific growth rate from the dilution rate.

The retention properties of the fermenter and its ability to operate at dilution rates higher to the specific growth rate were confirmed. However the fermentation profile differed considerably with respect to biomass concentration in the fermenter from the Tower fermentation of soluble carbohydrates.

In soluble carbohydrate fermentations by filamentous fungi the biomass concentration in the Tower fermenter has been reported as independent of the initial substrate concentration, unstable at low (up to $\approx 0.08\ h^{-1}$) dilution rates and stable at high dilution rates ($D > 0.2\ h^{-1}$) where the biomass concentration tended to be independent of the dilution rate (Pannell,1976).

However our results indicated that the biomass concentration in the fermenter was affected by the initial substrate concentration and it was more stable at the lower dilution

rates employed.

These differences in the fermentation profile between soluble and insoluble carbohydrates was attributed to the effects of the non-utilized substrate. In the case of soluble carbohydrates the remaining substrate does not affect physically the fermentation. In the case of cellulosic materials the non-degraded particles, apart from the adverse effect on the concentration of extracellular cellulolytic enzymes, affected the morphology and sedimentation of the mycelial flocks and also "diluted" the biomass content of the fermenter. The continuous fermentation of newspaper, due to the lignin effect, was less efficient than the cellulose fermentation as judged by the decrease in productivity and efficiency of substrate utilization. The growth of the microorganism on both substrates was controlled mainly by its ability to hydrolyse and subsequently utilize the supplied potential carbon source. The low level of reducing sugars observed indicated that the hydrolysis of the substrate was the most crucial factor and that the fermentation was not repressed by the products of hydrolysis.

The rapid decrease in substrate degradation efficiency with dilution rate implies that for maximum utilization of the substrate the dilution rate should be low. This however is incompatible with a high biomass productivity which requires both high substrate utilization at high (as near to D crit) dilution rates. High substrate concentrations could partially overcome the productivity restrictions imposed by the dilution rate. However the results obtained in the pure

cellulose fermentations showed that an increase in substrate concentration did not result in a proportional increase in the substrate utilization efficiency. This indicates the adverse effect of the suspended solids in the overall performance of the system.

It thus seems that despite the abundance of cellulosic materials their direct bioconversion is severely limited by the low biodegradability of the substrate. It can be expected however that the efficiency of the process will be improved when cellulosic materials which have undergone a more efficient pretreatment are used as substrates. Industrial effluents from chemical pulp and board mills contain cellulosics which have undergone extensive pretreatment and at the present they are considered wastes and represent pollution hazards.

The fermentation of chemical fibres by S.pulverulentum showed promising results and the fermentation by the same microorganism of waste water from a fibre board mill proved to be economically feasible (Ek and Eriksson, 1977). The high efficiency of the Tower fermenter in the treatment of effluents containing soluble oligo- and poly-saccharides (Spensley, 1977) (Stockbridge, 1979) coupled with the low cost of construction, installation, maintenance and operation (Greenshields 1978, personal communication) make it a favourable candidate for the treatment of such effluents. The use of S.thermophile 2, which in the batch experiments performed better than S.pulverulentum, may also improve further the efficiency of the fermentation.

Experimental work with any novel fermentation process especially on one where both the fermenter and the fermentation

is radical in so many aspects, raises many problem areas worthy of further investigation. One of the most important factors is the biodegradability of the substrate which affects the balance between the rates of substrate supply and substrate hydrolysis. Natural cellulosic materials are very resistant to microbial attack. A more efficient pretreatment could increase the degradation of the substrate. Ball milling is becoming prohibitively expensive. Chemical pretreatment is effective especially in the case of cellulosics with high lignin content. However it creates problems associated with the treatment of the resulting effluent and the loss of the easily removed polysaccharides, especially hemicelluloses. A short duration mechanical pretreatment followed by the pretreatment proposed by Worgan (1973a) could be worthy of further investigation. The Tower fermentation of cellulosic materials in a continuous mode of operation presents special problems associated with the continuous pumping of a solids containing medium and the continuous removal of the cellulolytic enzymes adsorbed on the non degraded substrate. A semi continuous mode of operation could avoid this problem and also permit an investigation on the effect of high substrate concentration on the efficiency of the process. An integral part of any high substrate concentration experiments should be the study of the oxygen regime in the fermenter. Humphrey et al (1977) expressed the opinion that high solids concentrations could probably result in oxygen limited fermentation. The use of a probe which does not permit mycelial growth could provide information about the oxygen requirements of the

microorganism and also about the state of the fermentation. If the oxygen is found to be a limiting factor a further investigation is needed on both the fermenter design and methods of aeration for improvement of the oxygen transfer rate.

A possible modification is the addition of an external recirculation pipe similar to that proposed by v.Hofsten and Ryden (1975) which could create a downward flow reducing the foaming of the culture. This could make unnecessary the addition of antifoam thus improving the oxygenation of the fermenter broth.

The use of newly discovered cellulolytic microorganisms as Thermoactinomyces sp., C.cellulolyticum or mutants of T.reesei as the strain QM 9414 could also improve the efficiency of the process especially if their morphology is found suitable for continuous Tower fermentation. These microorganisms have been reported to be actively cellulolytic and to contain a higher amount of mycelial protein than S.thermophile 2. The use of T.reesei QM 9414 and Thermoactinomyces sp. are of particular interest. The former has been cultured continuously with very good yield coefficient (Peitersen, 1977), the latter is able to grow at high temperatures $\sim 55^{o}$ (Humphrey et al, 1977) and it would be of interest to study its performance in non sterile media.

The fermentation of cellulosic materials with process target the production of cellulolytic enzymes is another way by which the potential of these materials can be exploited. Recent works have shown that the cellulases productivity of the fermentation can be increased by external recycling of

the fermenter solids content (Ghose and Sahai, 1979). The retention properties of the Tower fermenter give to this system a built-in advantage for such processes and thus the use of Tower fermenter for continuous production of cellulolytic enzymes is also recommended for further investigation. In this case the use of T.reesei mutants and reconsideration of the growth conditions are also suggested.

The observations made during the course of this work confirmed the biotechnical feasibility of the Tower continuous fermentation of cellulosic materials and gave a more detailed insight into the problems concerning both the process and the mould behaviour in a Tower fermenter. The Tower fermenter was very simple in terms of design and construction indicating its potential for low level technology fermentations. Viewed as a tool for cellulose bioconversion to biomass the system suffered from a low productivity and low yield coefficient because of the nature of the substrate and the extra burden imposed on the microorganism for its hydrolysis. However the fermentation of cellulosic materials is still in its infancy and it is reasonable to expect that further research will improve the efficiency of the process and enable the economical exploit of the enormous energy potential of the substrate. It is hoped that the work contained in this thesis may to a small extent further this aim.

# APPENDICES

Appendix 1

ANALYTICAL METHODS

Cellulose Determination: Procedure

1. 0.2g of powdered sample is placed in a centrifuge tube, 10 ml of distilled water added and then centrifuged at 3,000 r.p.m. for 5 minutes. The supernatant is discarded.
2. 1 ml of Acetic acid/Nitric acid reagent are added, mixed well, and 2 ml more added.
3. With a marble on top, to reduce evaporation and create a refluxing action, the tubes are placed in a boiling water bath for 30 minutes, centrifuged and the supernatant discarded.
4. 10 ml of distilled water are added, mixed, centrifuged and the supernatant discarded.
5. 2 ml 67.0% Sulphuric acid (v/v) are added, mixed and a further 8 ml added. The mixture is allowed to stand for 1 hour.
6. 1 ml of the liquid (in step5)is taken in a measuring cylinder and diluted to 100 ml with distilled water. It was centrifuged when cloudy.
7. 1 ml of the diluted sample (in step 6) is taken, 4 ml of distilled water are added and the sample is cooled in an ice bath.
8. 10 ml of cold Anthrone reagent are added by layering with a pipette mixed well and the sample returned to ice bath until all tubes are mixed.
9. With a marble on top, the tubes are placed in a boiling water bath for 16 minutes, cooled in an ice bath for 2-3 minutes, and allowed to stand at room temperature for 10 minutes.

10. A portion of the sample is placed in a 1 cm quartz cell and its optical density read at 620 nm.

Reagent blank and standards of 50,100,150 and 200 mg cellulose are done similarly from step (1).

Standard

50 mg of pure cellulose (dried for 6 hours at 105$^{o}$C and cooled over anhydrous alumina) is dissolved in 10 ml 67% Sulphuric acid (v/v) with warming. This was diluted to 250 ml with distilled water to contain 200 µg cellulose ml$^{-1}$. 0.5, 1.0, 1.5 and 2.0 ml stock standard is analysed from step (7) by adding the appropriate quantity of distilled water to each to bring the final volume to 5 ml.

Acetic acid/Nitric acid reagent

It is prepared by mixing 150 ml 80% Acetic acid and 15 ml of concentrated Nitric acid.

Anthrone reagent

It is prepared by mixing 0.2g of Anthrone in 100 ml 95% Sulphuric acid. The reagent was prepared about 4 hours in advance and stored in an ice water bath.

Reducing sugars determination

Assay procedure

1. 1 ml of the sample to be analysed is placed in a test tube and 3 ml of DNS reagent added.
2. The tube is placed in a boiling water bath for 15 minutes.
3. The tube is removed from the water bath and 2 ml of Rochelle

salt are immediately added to prevent loss of colour formed. The tube is then cooled under cold tap water.

4. The final volume of the tube is made up to 10 ml with distilled water to compensate for any evaporation losses.
5. A portion of the sample is placed in a 1 cm quartz cell and its optical density read at 575 nm.

DNS reagent

The final composition of the reagent was:

| | |
|---|---|
| A. 3-5 dinitrocalicylic acid | 10.0 $g.l^{-1}$ |
| B. Phenol | 2.0 $g.l^{-1}$ |
| C. Sodium sulphite | 0.5 $g.l^{-1}$ |
| D. Sodium hydroxide | 10.0 $g.l^{-1}$ |

To prevent deterioration of the reagent because of atmospheric oxidation of Sodium sulphite the components A and B were placed in a container and dissolved by stirring with the required volume of Sodium hydroxide solution. Sodium sulphite was added to aliquots before the reagent was to be used.

Rochelle salt solution was a 40% solution of Sodium potassium tartrate (Rochelle salt). D-glucose was used as standard in concentration O-2 $g.l^{-1}$.

Filter Paper activity determination

1. A Whatman No. 1 filter paper strip measuring 1x6 cm (approx weight 50 mg) was ruled up andplaced in a 10 $cm^3$ centrifuge tube.
2. 1 ml of Citrate buffer solution was added to the tube followed by 1 ml of supernatant.

3. The tube was placed in a 50$^{o}$C water bath for 1 hour.
4. At the end of the incubation period, the sample was centrifuged at 5000 r.p.m. for 5 minutes causing any insoluble matter to settle to the bottom of the tube.
5. 1 ml of the clear solution was placed in a test tube followed by 3 cm$^{3}$ of D.N.S. reagent. The rest of the assay procedure was the same as for the reducing sugars assay described earlier.

To correct for any reducing sugars already present a blank tube was prepared with the omission of filter paper.

D-glucose was used as standard.

Filter paper activity is expressed as mg of reducing sugars as glucose produced from 1 ml of supernatant under the assay conditions.

Appendix 2 (a)

BATCH FERMENTATION DATA

T.viride 1 Ball milled cellulose fermentation at 29°, pH5. Inoculum 500 ml, 10% v/v, grown in shake flasks (see 2.2) for 62 h. Composition of inoculum: cellulose1.12g, Mycelium 1.73g, Cr. Protein 0.63g.

Experimental Results*

| Time h | Cellulose $g.l^{-1}$ | TDW $g.l^{-1}$ | MDW $g.l^{-1}$ | Cr.Protein $g.l^{-1}$ | | F.P.A units. $ml^{-1}$ |
|---|---|---|---|---|---|---|
| 0 | 10.23 | 10.58 | 0.35 | 0.13 | | - |
| 8 | 8.00 | 9.05 | 1.05 | 0.39 | | - |
| 11.5 | 7.22 | 8.82 | 1.6 | 0.57 | | - |
| 16.5 | 6.05 | 7.76 | 1.71 | 0.60 | | - |
| 19 | 4.77 | 7.06 | 2.29 | 0.78 | | - |
| 24 | 4.05 | 6.48 | 2.43 | 0.82 | | - |
| 32 | 2.48 | 5.29 | 2.81 | 0.97 | (0.16) | 1.4 |
| 36 | 2.04 | 4.94 | 2.90 | 1.02 | (0.19) | 1.4 |
| 41.5 | 1.83 | 4.65 | 2.82 | 0.99 | (0.12) | 1.8 |
| 45 | 1.49 | 4.50 | 3.01 | 1.02 | (0.15) | 1.8 |

* Figures in parentheses refer to Reducing sugars $conc^{n}$ $g.l^{-1}$

Appendix 2 b

T.viride 2. Ball milled cellulose fermentation at 29°, pH5. Inoculum 500 ml, 10% v/v, grown in shake flasks (see 2.2) for 62 h. Composition of inoculum: Cellulose 1.18g; Mycelium 1.80 g; C.Protein 0.63g

Experimental Results*

| Time h | Cellulose $g.l^{-1}$ | TDW $g.l^{-1}$ | MDW $g.l^{-1}$ | C.Protein $g.l^{-1}$ | F.P.A units. $ml^{-1}$ |
|---|---|---|---|---|---|
| 0 | 10.23 | 10.59 | 0.36 | 0.13 | - |
| 5 | 8.80 | 9.64 | 0.84 | 0.31 | - |
| 11 | 7.40 | 8.92 | 1.52 | 0.60 | - |
| 18.5 | 4.49 | 6.90 | 2.41 | 0.78 | - |
| 22.5 | 4.10 | 6.55 | 2.45 | 0.91 | - |
| 25 | 3.43 | 6.03 | 2.60 | 0.92 | - |
| 30 | 2.05 | 5.30 | 3.25 | 1.0 (0.18) | 1.4 |
| 35 | 1.28 | 4.58 | 3.30 | 1.12 (0.12) | 1.8 |
| 45 | 0.83 | 4.13 | 3.30 | 1.10 (0.12) | 1.9 |
| 48 | 0.77 | 4.07 | 3.30 | 1.00 (0.14) | 2.2 |

* Figures in parentheses refer to Reducing sugars $conc^{n}$ $g.l^{-1}$

Appendix 2 c

S.pulverulentum. Ball milled cellulose fermentation at 35° pH5. Inoculum 500 ml, 10% v/v, grown in shake flasks (see 2.2 ) for 62 h. Composition of inoculum: Cellulose 1.6g; Mycelium 1.76g; C.Protein 0.53g

Experimental Results*

| Time h | Cellulose $g.l^{-1}$ | T.D.W. $g.l^{-1}$ | M.D.W. $g.l^{-1}$ | C.Protein $g.l^{-1}$ | F.P.A |
|---|---|---|---|---|---|
| 0 | 10.31 | 10.66 | 0.35 | 0.11 | - |
| 10 | 8.45 | 9.50 | 1.05 | 0.37 | - |
| 17.5 | 7.13 | 8.49 | 1.36 | 0.48 | - |
| 21 | 6.08 | 8.03 | 1.94 | 0.62 | - |
| 29 | 5.37 | 7.39 | 2.02 | 0.66 | - |
| 34.5 | 4.56 | 6.91 | 2.35 | 0.78 (0.14) | - |
| 41.5 | 4.25 | 6.52 | 2.27 | 0.80 (0.16) | 0.8 |
| 45 | 3.63 | 6.31 | 2.68 | 0.81 (0.18) | 0.8 |
| 49 | 3.67 | 6.17 | 2.50 | 0.81 (0.14) | 1.0 |

* Figures in parentheses refer to Reducing sugars $conc^{n}$ $g.l^{-1}$

Appendix 2d

S.thermophile 1. Ball milled cellulose fermentation at 42° pH6. Inoculum 500, 10% v/v, grown in shake flasks (see 2.2) for 48h. Composition of inoculum: Cellulose 1.58g; Mycelium 1.68g; C.Protein 0.55g.

Experimental Results*

| Time h | Cellulose $g.l^{-1}$ | T.D.W. $g.l^{-1}$ | M.D.W. $g.l^{-1}$ | C. Protein $g.l^{-1}$ | | F.P.A units. $ml^{-1}$ |
|---|---|---|---|---|---|---|
| 0 | 10.31 | 10.65 | 0.34 | 0.11 | | - |
| 10 | 8.58 | 9.58 | 1.00 | 0.35 | | - |
| 15 | 7.49 | 8.70 | 1.21 | 0.43 | | - |
| 19 | 5.97 | 7.90 | 1.93 | 0.63 | | - |
| 22.5 | 5.61 | 7.72 | 2.11 | 0.69 | | - |
| 26 | 4.51 | 6.95 | 2.24 | 0.78 | | - |
| 35 | 3.57 | 6.15 | 2.58 | 0.94 | (0.18) | 1.0 |
| 41 | 2.70 | 5.80 | 3.10 | 1.05 | (0.16) | 1.1 |
| 45 | 2.64 | 5.50 | 2.86 | 1.05 | (0.12) | 1.2 |
| 48 | 2.26 | 5.36 | 3.10 | 1.05 | (0.12) | 1.2 |

* Figures in parentheses refer to Reducing sugars $conc^{n}$ $g.l^{-1}$

Appendix 2e

S.thermophile 2. Ball milled cellulose fermentation at 45° pH6. Inoculum 500 ml, 10% v/v, grown in shake flasks (see 2.2) for 48 hours. Composition of inoculum: Cellulose 1.22g, Mycelium 1.89g, C.Protein 0.85g.

Experimental Results*

| Time h | Cellulose $g.l^{-1}$ | T.D.W. $g.l^{-1}$ | M.D.W. $g.l^{-1}$ | C.Protein $g.l^{-1}$ | F.P.A units. $ml^{-1}$ |
|---|---|---|---|---|---|
| 0 | 10.23 | 10.61 | 0.38 | 0.17 | - |
| 7.5 | 7.61 | 9.24 | 1.63 | 0.69 | - |
| 10.5 | 7.21 | 8.86 | 1.65 | 0.80 | - |
| 12.5 | 5.50 | 8.11 | 2.61 | 1.12 | - |
| 16 | 4.96 | 7.75 | 2.79 | 1.28 | - |
| 19 | 3.45 | 6.97 | 3.52 | 1.66 | - |
| 25 | 1.85 | 6.05 | 4.20 | 1.86 (0.16) | 0.7 |
| 32.5 | 1.20 | 5.50 | 4.30 | 1.99 (0.16) | 1.0 |
| 35 | 0.72 | 5.36 | 4.68 | 1.96 (0.16) | 1.0 |
| 39 | 0.80 | 5.30 | 4.50 | 1.92 (0.12) | 1.1 |

* Figures in parentheses refer to Reducing sugars $conc^{n}$ $g.l^{-1}$

Appendix 2f

S.thermophile 2. Bill milled newspaper fermentation at 45° pH 6. Inoculum 500 ml, 10% v/v, grown in shake flasks (see 2.2) for 50 h. Composition of Inoculum: Cellulose $0.92_g$, Mycelium $1.83_g$, C.Protein $0.83_g$.

Experimental Results*

| Time h | Cellulose $g.l^{-1}$ | T.D.W. $g.l^{-1}$ | Cr. Protein $g.l^{-1}$ | F.P.A units. $ml^{-1}$ |
|---|---|---|---|---|
| 0 | 7.40 | 14.23 | 0.17 | - |
| 8 | 6.27 | 12.14 | 0.57 | - |
| 12.5 | 6.00 | 11.61 | 0.84 | - |
| 20 | 4.93 | 9.94 | 0.92 | - |
| 24 | 3.93 | 9.60 | 1.13 | - |
| 32.5 | 2.72 | 8.46 | 1.39 (0.15) | - |
| 37.5 | 2.41 | 7.59 | 1.27 (0.15) | 0.6 |
| 41.5 | 1.87 | 7.52 | 1.36 (0.16) | 0.7 |
| 45 | 2.2 | 7.01 | 1.32 (0.13) | 0.8 |

* Figures in parentheses refer to Reducing sugars $conc^n$ $g.l^{-1}$

Appendix 3 (a)

CONTINUOUS CULTURE DATA

Continuous culture of S.thermophile 2 on ball milled cellulose at 45°, pH6, 1.6v/v/m. Steady state values

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial cellulose conc$^{n}$ | Co | $g.l^{-1}$ | 7.5 | 7.5 | 7.5 | 7.5 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| Dilution rate | D | $h^{-1}$ | 0.036 | 0.048 | 0.056 | 0.062 | 0.034 | 0.042 | 0.050 | 0.055 | 0.060 |
| Total d.w.(fermenter) | $X_{FT}$ | $g.l^{-1}$ | 3.64 | 5.00 | 5.75 | 6.48 | 5.39 | 5.91 | 7.12 | 7.49 | 8.34 |
| Cellulose d.w. " | $C_F$ | $g.l^{-1}$ | 0.88 | 2.12 | 3.23 | 4.12 | 1.49 | 2.16 | 3.49 | 3.92 | 5.15 |
| Biomass d.w. " | $X_F$ | $g.l^{-1}$ | 2.62 | 2.88 | 2.52 | 2.36 | 3.90 | 3.75 | 3.63 | 3.57 | 3.19 |
| Total d.w.(effluent) | $X_{ET}$ | $g.l^{-1}$ | 2.85 | 3.85 | 4.17 | 4.76 | 4.11 | 4.55 | 5.09 | 5.42 | 6.05 |
| Cellulose d.w. " | $C_E$ | $g.l^{-1}$ | 0.67 | 1.57 | 2.32 | 3.12 | 1.02 | 1.70 | 2.40 | 2.82 | 3.81 |
| Biomass d.w. " | $X_E$ | $g.l^{-1}$ | 2.18 | 2.28 | 1.85 | 1.64 | 3.09 | 2.85 | 2.69 | 2.60 | 2.24 |
| Cr. protein | P | $g.l^{-1}$ | 0.90 | 0.88 | 0.71 | 0.62 | 1.24 | 1.09 | 1.03 | 0.97 | 0.85 |
| Reducing sugars | R.S. | $g.l^{-1}$ | 0.11 | 0.11 | 0.15 | 0.18 | 0.12 | 0.12 | 0.10 | 0.14 | 0.22 |
| Filter paper activity | E | units.$ml^{-1}$ | 0.68 | 0.44 | 0.36 | - | 0.67 | 0.52 | 0.40 | 0.35 | 0.24 |



Continued

3 a

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Initial cellulose conc$^{n}$ | Co | $g.l^{-1}$ | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Dilution rate | D | $h^{-1}$ | 0.030 | 0.038 | 0.046 | 0.054 | 0.058 |
| Total d.w.(fermenter) | $X_{FT}$ | $g.l^{-1}$ | 8.13 | 8.70 | 10.59 | 12.64 | 14.18 |
| Cellulose d.w. " | $C_F$ | $g.l^{-1}$ | 2.62 | 3.45 | 5.40 | 7.45 | 8.40 |
| Biomass d.w. " | $X_F$ | $g.l^{-1}$ | 5.51 | 5.25 | 5.19 | 4.94 | 4.58 |
| Total d.w.(effluent) | $X_{ET}$ | $g.l^{-1}$ | 6.16 | 6.48 | 7.46 | 9.10 | 9.57 |
| Cellulose d.w. " | $C_E$ | $g.l^{-1}$ | 2.03 | 2.46 | 3.85 | 5.80 | 6.59 |
| Biomass d.w. " | $X_E$ | $g.l^{-1}$ | 4.13 | 4.00 | 3.61 | 3.30 | 2.98 |
| Cr. protein " | P | $g.l^{-1}$ | 1.69 | 1.51 | 1.39 | 1.24 | 1.10 |
| Reducing sugars | R.S. | $g.l^{-1}$ | 0.12 | 0.11 | 0.21 | 0.26 | 0.26 |
| Filter paper activity | E | $u.ml^{-1}$ | 0.69 | 0.53 | 0.35 | 0.19 | - |

Appendix 3 b

Continuous culture of S.thermophile 2 on ball milled cellulose at 45° pH6 1.6 v/v/m.
Steady state values

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Initial cellulose conc$^{n}$ | Co | $g.l^{-1}$ | 7.5 | 7.5 | 7.5 | 7.5 | 10.0 | 10.0 | 10.0 | 10.0 | 10.0 |
| Dilution rate | D | $h^{-1}$ | 0.036 | 0.048 | 0.056 | 0.062 | 0.034 | 0.042 | 0.050 | 0.055 | 0.060 |
| Cellul. supply rate | DCo | $g.l^{-1}.h^{-1}$ | 0.270 | 0.360 | 0.420 | 0.465 | 0.340 | 0.420 | 0.500 | 0.550 | 0.600 |
| Cellul. output " | $DC_E$ | $g.l^{-1}.h^{-1}$ | 0.024 | 0.075 | 0.130 | 0.194 | 0.035 | 0.071 | 0.120 | 0.155 | 0.229 |
| Biomass productivity | $DX_E$ | $g.l^{-1}.h^{-1}$ | 0.080 | 0.109 | 0.104 | 0.102 | 0.105 | 0.120 | 0.135 | 0.143 | 0.134 |
| Cr. protein " | DP | $g.l^{-1}.h^{-1}$ | 0.032 | 0.042 | 0.040 | 0.038 | 0.042 | 0.047 | 0.052 | 0.053 | 0.051 |
| Cellulase (FPA)" | DE | $u.ml^{-1}.h^{-1}$ | 0.025 | 0.021 | 0.020 | - | 0.028 | 0.022 | 0.02 | 0.019 | 0.014 |
| Total " | $DX_{ET}$ | $g.l^{-1}.h^{-1}$ | 0.104 | 0.185 | 0.234 | 0.295 | 0.140 | 0.191 | 0.255 | 0.298 | 0.363 |
| Cellulose consumption rate | DC | $g.l^{-1}.h^{-1}$ | 0.242 | 0.280 | 0.282 | 0.262 | 0.302 | 0.345 | 0.374 | 0.384 | 0.359 |
| Specific cellulose consumption rate | | $h^{-1}$ | 0.089 | 0.097 | 0.112 | 0.111 | 0.077 | 0.092 | 0.103 | 0.107 | 0.113 |
| Specific growth rate | | $h^{-1}$ | 0.029 | 0.038 | 0.041 | 0.043 | 0.027 | 0.032 | 0.037 | 0.040 | 0.042 |
| Cellulose degradation rate | DCd | $g.l^{-1}.h^{-1}$ | 0.246 | 0.285 | 0.290 | 0.271 | 0.305 | 0.349 | 0.380 | 0.369 | 0.371 |



Continued

3 b

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Initial cellulose conc$^{n}$ | Co | $g.l^{-1}$ | 15.0 | 15.0 | 15.0 | 15.0 | 15.0 |
| Dilution rate | D | $h^{-1}$ | 0.030 | 0.038 | 0.046 | 0.054 | 0.058 |
| Cellulose supply rate | DCo | $g.l^{-1}.h^{-1}$ | 0.450 | 0.570 | 0.690 | 0.810 | 0.870 |
| Cellulose output " | $DC_E$ | $g.l^{-1}.h^{-1}$ | 0.061 | 0.094 | 0.177 | 0.313 | 0.382 |
| Biomass productivity | $DX_E$ | $g.l^{-1}.h^{-1}$ | 0.124 | 0.152 | 0.170 | 0.178 | 0.173 |
| Cr. protein " | DP | $g.l^{-1}.h^{-1}$ | 0.051 | 0.057 | 0.064 | 0.062 | 0.064 |
| Cellulase (FPA)" | DE | $u.ml^{-1}h^{-1}$ | 0.021 | 0.020 | 0.016 | 0.010 | - |
| Total " | $DX_{ET}$ | $g.l^{-1}.h^{-1}$ | 0.185 | 0.245 | 0.347 | 0.491 | 0.555 |
| Cellulose consumption rate | DC | $g.l^{-1}.h^{-1}$ | 0.386 | 0.473 | 0.504 | 0.484 | 0.474 |
| Specific cellulose consumption rate | | $h^{-1}$ | 0.070 | 0.09 | 0.097 | 0.098 | 0.103 |
| Specific growth rate | | $h^{-1}$ | 0.023 | 0.029 | 0.033 | 0.036 | 0.038 |
| Cellulose degradation rate | DCd | $g.l^{-1}.h^{-1}$ | 0.389 | 0.476 | 0.513 | 0.497 | 0.488 |

Appendix 3 c

Continuous culture of S.thermophile 2 on ball milled newspaper at 45° pH6 1.6 v/v/m.
Steady state values

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Newspaper Conc$^{n}$ | No | $g.l^{-1}$ | 15.00 | 15.00 | 15.00 | 15.00 | 15.00 |
| Cellulose " | Co | $g.l^{-1}$ | 7.91 | 7.91 | 7.91 | 7.91 | 7.91 |
| Dilution rate | D | $h^{-1}$ | 0.030 | 0.036 | 0.040 | 0.045 | 0.050 |
| Total d.w. fermenter | $X_{FT}$ | $g.l^{-1}$ | 9.63 | 10.99 | 11.88 | 13.38 | 14.25 |
| Cellulose d.w. fermenter | $C_F$ | $g.l^{-1}$ | 2.35 | 3.05 | 4.18 | 5.23 | 6.44 |
| Crude protein " | $P_F$ | $g.l^{-1}$ | 1.43 | 1.38 | 1.34 | 1.21 | 1.03 |
| Total d.w. effluent | $X_{ET}$ | $g.l^{-1}$ | 7.17 | 7.69 | 8.17 | 9.12 | 10.16 |
| Cellulose d.w. " | $C_E$ | $g.l^{-1}$ | 1.74 | 2.12 | 2.77 | 3.49 | 4.51 |
| Crude protein " | $P_E$ | $g.l^{-1}$ | 1.09 | 0.99 | 0.93 | 0.77 | 0.64 |
| Reducing sugars | R.S. | $g.l^{-1}$ | 0.18 | 0.18 | 0.26 | 0.22 | 0.20 |
| Filter Paper Activity | E | $\frac{\text{units}}{\text{ml}}$ | 0.56 | 0.33 | 0.17 | - | - |

Appendix 3 d

Continuous culture of S.thermophile 2 on ball milled newspaper at 45° pH6 1.6 v/v/m
Steady state values

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Dilution rate | D | $h^{-1}$ | 0.030 | 0.036 | 0.040 | 0.045 | 0.050 |
| Newspaper supply rate | $DN_o$ | $g.l^{-1}.h^{-1}$ | 0.450 | 0.540 | 0.600 | 0.675 | 0.750 |
| Cellulose supply rate | DCo | $g.l^{-1}.h^{-1}$ | 0.237 | 0.285 | 0.316 | 0.356 | 0.395 |
| Cellulose output rate | $DC_E$ | $g.l^{-1}.h^{-1}$ | 0.052 | 0.076 | 0.111 | 0.157 | 0.225 |
| Crude protein productivity | $DP_E$ | $g.l^{-1}.h^{-1}$ | 0.033 | 0.036 | 0.037 | 0.035 | 0.032 |
| Cellulase productivity | DE | $\frac{\text{units}}{\text{ml.h}}$ | 0.017 | 0.012 | 0.007 | - | - |
| Total solids output rate | $DX_{ET}$ | $g.l^{-1}.h^{-1}$ | 0.216 | 0.277 | 0.327 | 0.410 | 0.508 |
| Cellulose degradation rate | DCd | $g.l^{-1}.h^{-1}$ | 0.185 | 0.209 | 0.205 | 0.199 | 0.170 |
| Specific growth rate | $\mu$ | $h^{-1}$ | 0.023 | 0.026 | 0.028 | 0.029 | 0.031 |

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