STUDIES ON CELLULASE PRODUCTION WITH PURE AND MIXED FUNGAL FERMENTATIONS

by

Sheldon Joseph Blaine Duff
Department of Chemical Engineering
McGill University, Montreal
May, 1986

A thesis submitted to the Faculty of Graduate Studies and Research
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

ISBN 0-315-34465-2

ACKNOWLEDGEMENTS

I owe thanks to many people all of whom have helped me in one way or another throughout the course of this work. Firstly I would like to express my thanks to David Cooper for his heroic rescue six years ago and for all of his help since. I would also like to thank Dr. Maynard Fuller for always trying to make the thesis the best that it could be. As well my appreciation is expressed to Bert van den Berg for clearing the administrative channels so that I could do the degree and for his suggestions throughout the work.

I would like to thank all of the summer and co-op students who have provided help and a lot of laughs over the past four years: Joey Milner, Liz Pason, Gillian Grieg, Kim Wong, Chris Perrin, Linda Cornfield, Pat Watson, and Joanna Brooke.

I would like to express special thanks to Lenore, my sister, who first suggested that I do a PhD, for all of the emotional support which she has given me. Thanks to my brother Des, who has all the brains in the family, for his support. My thanks too, to my parents for their encouragement. Finally to my wife Carrie, I would like to express my appreciation for her patience, good humour, and support throughout the last two years.

ABSTRACT

Studies were undertaken to increase the productivity and yield of cellulase from fermentations with Trichoderma reesei Rut C30. When positively-charged colloidal materials were added to the growth medium, final enzyme titers were improved by two fold in cultures grown using lactose as the principal carbon source. The colloids bound lactose, and subsequently released it to the organism. Retarded uptake of carbon source is known to induce cellulase production by Trichoderma. In cellulose-based fermentations, colloid addition resulted in a smaller relative improvement in enzyme titer. The low sugar levels present in these fermentations confirmed that a second mechanism was present by which the colloids improved enzyme yield.

Trichoderma cellulase is deficient in beta-glucosidase activity. In an effort to overcome this deficiency, Trichoderma was cultured with another fungus Aspergillus phoenicis, a known producer of beta-glucosidase. The cellulase produced by the mixed culture showed a greater hydrolytic potential than cellulase produced in pure cultures of Trichoderma. The improved hydrolytic potential was due mainly to the increased resistance to end-product inhibition which was afforded by the increased beta-glucosidase activity of the mixed culture cellulase.

RESUME

Des études ont été entreprises pour améliorer le rendement de la production en cellulase par Trichoderma reesei Rut C 30. Dans un milieu contenant du lactose comme source de carbone principale, une concentration finale en enzyme deux fois supérieure a été obtenue lorsque des substances colloïdales chargées positivement ont été ajoutées au milieu de culture. Les colloïdes se sont liées au lactose et ont libere ce dernier au microorganisme par la suite. Un retard dans l'assimilation de la source de carbone induit la production de cellulase par Trichoderma. En ajoutant des colloïdes a des fermentations à base de cellulose, l'augmentation du titre en enzyme était relativement plus faible. Les faibles quantités de sucre présentes dans ces fermentations confirment qu'il y avait un deuxième mécanisme par lequel les colloïdes augmentaient le rendement en enzyme.

La cellulase de Trichoderma contient peu de bêta-glucosidase. Pour remédier à cette déficience, Trichoderma a été cultivé avec un autre moisissure Aspergillus phoenicis, un producteur connu de bêta-glucosidase. La cellulase produite en culture mixte a montré un potentiel hydrolytique plus grand que celui obtenu dans les cultures pures de Trichoderma. Cet accroissement dans le potentiel hydrolytique était principalement dû à l'augmentation de la résistance à l'inhibition par le produit final, et ce grâce à l'accroissement de l'activité bêta-glucosidasique de la cellulase provenant de la culture mixte.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

1. The use of inorganic colloidal materials in a biological process has not been previously reported. The production of cellulase by Trichoderma reesei Rut C30 was improved through the addition of small, positively-charged colloidal particles. The mechanism of the colloid effect appears to be related to its ability to bind, and subsequently release, soluble sugars.

2. Mixed cultivation of Trichoderma reesei Rut C30 and Aspergillus phoenicis was carried out in an effort to improve the beta-glucosidase activity of the cellulase produced over that which could be produced using Trichoderma alone. The cellulase produced using the mixed culture had up to a four-fold improvement in beta-glucosidase activity. This resulted in a much greater hydrolytic potential as compared to cellulase produced in pure cultures of Trichoderma.

3. A thorough study of the effect of a variety of surfactants on enzymatic hydrolysis of cellulose was undertaken in this work. It had been previously known that when Tween 80 was used in enzymatic hydrolysis reactors, it was able to improve the efficiency of the cellulose degradation. In this work, it was found that the surfactant effect was a general one, and was independent of the characteristics of the surfactant used. The surfactants used exerted a two-fold effect: they improved the interaction of the enzyme with the solid substrate (cellulose) as well as reducing the effects of interfacial denaturation on the cellulase.

3. The use of a nitrogen source (specifically $(NH_4)_2SO_4$) in the dilute HCL used for fermenter pH control has not been previously reported. By using this additional nitrogen source, an adequate nitrogen supply was maintained through additions of both acid and base (NH_4OH) during automatic pH adjustments.

1. INTRODUCTION

Declining reserves of fossil fuels have spurred interest in the development of methods by which renewable resources, such as cellulose, may be utilized. Cellulose is an abundant resource, the development of which is especially pertinent to Canada because of this country's large annual production of forest biomass.

Cellulose is a linear, long chain polymer of beta-D glucose linked together by 1,4' glycosidic bonds. The dimer cellobiose is the repeating unit of the cellulose polymer. Individual cellulose molecules are linked together in a parallel arrangement to form elementary fibrils, aggregates of which form microfibrils, which are visible under a light microscope. Hemicelluloses, polymers of sugars other than glucose, are closely associated with cellulose in the plant cell wall. The remaining 25 % of the plant cell wall consists of lignins. Lignins are three dimensional network polymers which act as a physical barrier to the degradation of cellulose.

There are a number of different approaches by which cellulosic materials can be degraded. These methods include:

combustion

pyrolysis

gasification

acid hydrolysis

enzymatic hydrolysis

Enzymatic hydrolysis has a number of advantages including:

1. The reaction conditions used are mild (low temperature and pressure).

2. Equipment costs are low because there are no toxic or corrosive by-products.

3. The sugars produced are usable by virtually all microorganisms and can be converted to a variety of useful products.

Before lignocellulosic material can be attacked by cellulose-degrading (cellulase) enzymes, it is necessary to perform a pretreatment on it in order to increase the accessibility of the cellulose. Lignin acts as a physical barrier to cellulose degradation. Because of this, pretreatments which remove lignin are able to increase the accessibility of the lignocellulosic material to enzymatic degradation. Regardless of which pretreatment method is chosen, the desirable features of a such a pretreatment process are the same. These include:

1. Lignin should be removed in a recoverable form that can be used for other applications.

2. The hemicellulose portion of the plant should be solublized and recovered. Wood consists of approximately 30% hemicelluloses, and the utilization of this component is an essential part of an economic enzymatic hydrolysis scheme.

3. The accessibility of the cellulose should be increased to promote enzymic attack.

4. The pretreatment should result in minimal loss of the cellulosic substrate.

The main methods of pretreatment are summarized in Table 1.1.

Although the structure of cellulose is simple, the synergistic action of at least three enzymes is required to degrade it. The first, endo-1,4-beta-D-glucanase (E.C. 3.2.1.4), attacks the intact cellulose chain and produces "nicks" in the glucan polymer. These sites are then subject to attack by a second enzyme exoglucanase or 1,4-beta-D-glucancellobiohydrolase (E.C.

3.2.1.91). this enzyme successively cleaves cellobiose units from the chain. A third enzyme, beta-glucosidase (E.C. 3.2.1.21), cleaves the cellobiose units into two glucose monomers.

Although it is possible to effect the conversion of lignocellulosic material to fermentable sugars using known technology, there are two problem areas which render the economics of the process unfavourable at present. The first is the expense of cellulase enzyme production. Even with hypercellulolytic mutants of cellulose-degrading fungi such as Trichoderma reesei, the cost of producing cellulase has remained high. This can be mainly attributed to the low productivity of the fermentation (Mandels and Andreotti, 1978). The second problem area becomes apparent when cellulase enzymes are used to hydrolyse cellulose. The end products of the hydrolysis inhibit the cellulase enzymes. Cellobiose is a strong inhibitor of endoglucanase, but the product of beta-glucosidase activtiy, glucose, is a less potent inhibitor of endoglucanase activity. For this reason, it is desirable to have an enzyme complex which is high in beta-glucosidase activity.

The focus of this work is on two main problem areas. Firstly, methods were examined by which the yield of cellulase using Trichoderma could be improved. This area is examined in Chapter 4.1. The other problem area addressed is the low beta-glucosidase activity of the cellulase. In an effort to increase the betaglucosidase activity of the Trichoderma enzyme, mixed cultivation of Trichoderma and Aspergillus was undertaken. This work is described in Chapter 4.3 and 4.4.

PRETREATMENT	ADVANTAGE	DISADVANTAGE
Biological	-no corrosion -no disposal problems	-slow -significant cellulose consumption
Mechanical	-reduces crystal structure	-slow -species selective -delignification required
Alkali/Acid	-can be very effective under carefully controlled conditions	-degradation (loss) of cellulose -expense of recovery/ neutralization -corrosion
Steam explosion	-hemicellulose recovered -increased accessibility -high yields	-toxic by-products

Table 1.1. Summary of pretreatment technologies.

2. BACKGROUND AND LITERATURE REVIEW

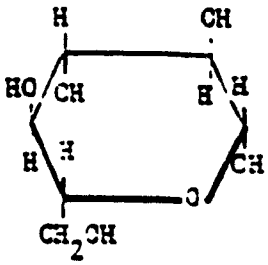
2.1. Introduction

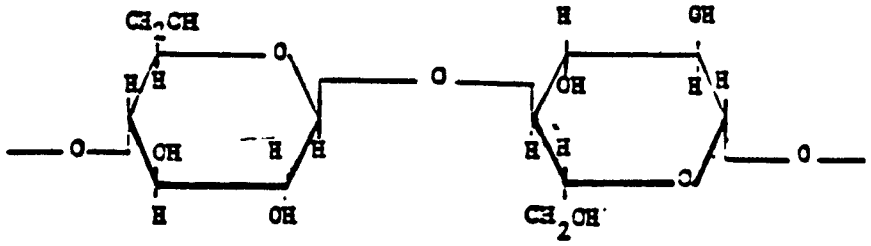
2.1.1. Cellulose structure

Cellulose is the most abundant organic chemical on earth making up approximately 50 % of the total biomass. About a hundred billion tons are produced each year. The structure of cellulose is not complex. It is a long chain polymer of beta-D-glucose in the pyranose form, linked together by 1,4' glycosidic bonds to form cellobiose residues, the repeating unit of cellulose. Because the linkage is in the beta form, alternate glucose units must be rotated through 180°. As a result, individual chains of cellulose tend to form organised crystalline bundles held together by hydrogen bonding (Figure 2.1). Because the conformation of the pyranose ring is one of minimum energy, the cellulose crystal is highly stable.

Cellulose is completely insoluble in water. The hydrogen bonding described above is not disrupted by water. Cellulose can be swollen, dispersed, or dissolved by strong acids, alkalies, concentrated salt solutions and various complexing agents. The degree of crystallinity of different kinds of cellulose varies with their origin and treatment. Cotton is approximately 70 % crystalline, while regenerated cellulose is about 40 % crystalline.

Closely associated with cellulose in plant cell walls are hemicellulose, another group of structural polysaccharides. These molecules are polymers of sugars other than glucose, both hexoses and pentoses, and are branched, with degrees of polymerization (DP) ranging from less than 100, to about





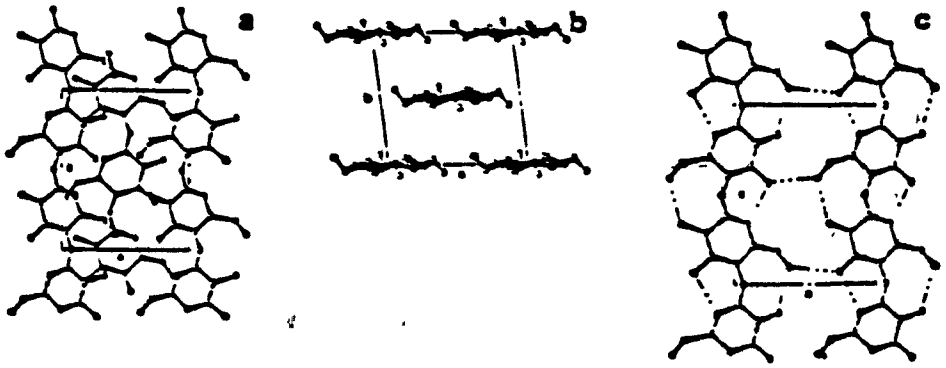


Figure 2.1. Projections of the parallel chain model for cellulose I. (a) Projection perpendicular to the ac plane. (b) Projection perpendicular to the ab plane looking along the fibre axis. (c) Hydrogen bonding network. From Blackwell et al., 1977. With permission.

200 sugar units. They are insoluble in water, but can be dissolved in strong alkali, thus providing a mechanism by which they can be removed from holocellulose, leaving pure crystalline (alpha) cellulose behind. Hemicellulose exists in only a few forms which are common to all plants. The structure of hemicellulose is modified in different plant species and within one species, in different tissues. Softwoods contain about 25% hemicellulose comprised of mannose, galactose, xylose, glucose and arabinose in decreasing abundance. Hardwoods, which contain about 30% hemicelluloses, have as major hemicellulose sugars: xylose, galactose and mannose, with minor amounts of rhamnose and arabinose. Both hardwood and softwood hemicelluloses contain 4-O-methylglucuronic acid. Generally annual plants and hardwoods contain more pentosans and softwoods contain predominantly hexosans. The most predominant hemicelluloses of hardwoods are glucuronoxylans in which a linear or singly branched 1,4 -linked beta-D xylopyranose backbone has pyranose forms of 4-O-methyl-D glucuronic acid attached by an alpha link to the xylose.

Arabinoglucuronoxylans, containing both arabinose and uronic acids appear in softwoods, wheat straw and food crops. Arabinogalactans, water soluble polysaccharides that are highly branched, have been isolated from a number of softwoods.

In conifers, the predominant hemicellulose is glucomannan of low molecular weight. The ratio of glucose to mannose varies from 1:1 to 1:4 and the structure is essentially 1,4'-beta pyranose in nature.

Lignin is the third major component of plant cell walls in woody plants,

making up the remaining 25% of the cell wall material. Lignin serves to cement adjoining wood fibres, stiffen individual fibres and act as a barrier to enzymatic degradation of the cell wall.

Lignin is a three dimensional network polymer consisting of phenylpropane units in a variety of linkages (Sarkanen and Ludwig, 1971). Although lignins from divergent sources (grasses, softwoods, hardwoods) differ in methoxyl substitution and degree of carbon-carbon linkage between phenyl groups, their common sturctural features predominate. The schematic structure of a conifer lignin, shown in Figure 2.2, shows the important features for conversion to chemicals.

Lignins are formed by an enzymatically initiated free radical polymerization of precursers in the form of parahydroxy-cinnamyl alcohol. Precursers again vary between hard and softwoods and grasses. Hardwood lignins, by virtue of their smaller degree of cross-linking, are more easily dissolved.

The molecular weight of a lignin sample will strongly depend on the method by which it was isolated. Dissolved lignin fractions may be small enough to behave as simple compounds or large enough to exhibit high polymer behavior. In breaking native lignin structure, fragments of varying size are produced.

2.1.2. Cell wall organization

Cellulose occurs in the cell wall in microfibrils that possess a crystalline core surrounded by an amorphous region. Evidence indicates that the structure of cellulose is an elementary fibril 3.5 nm wide with a

microfibril consisting of an assembly of four crystalline elementary fibres. For cellulose D.P. 10000 the minimum microfibril length would be 500 nm (0.5 micrometers). Microfibril widths vary from 10-30 nm depending upon the number of elementary fibers aggregated, as well as the amount of amorphous material which surrounds the crystalline core.

Hemicelluloses and lignin are matrix polymers that surround the microfibrils as well as the constituent elementary fibrils. In addition, amorphous regions of the cellulose may also be penetrated by matrix polymers. Thus the cell wall is analogous to a fibre reinforced plastic, with cellulose fibres embedded in an amorphous matrix of hemicelluloses and lignin.

The above structure is not uniform across the cell wall. Regions such as the middle lamella have higher proportions of lignin especially in the cell corners.

In wood, the primary wall and adjacent intercellular substance between contiguous cells is referred to as the compound middle lamella. In both wood and cotton the secondary wall usually consists of 3 layers designated S1 S2, and S3. The S1 and S3 layers are quite thin; the S2 layer is of variable thickness but usually forms the bulk of the cell wall substance. In the S1 and S3 layers, cellulose molecules are deposited in a flat helix with respect to the fibre axis, whereas they are parallel to it in the S2. Within each layer of the secondary wall the cellulose fibres are arranged in microfibrils (Figure 2.3).

2.1.3. Requirements for cellulose degradation: organism, enzyme, substrate relationships

Microorganisms that degrade material fibres live either on the surface of the fibre or in the case of wood, in the fibre lumina. The organisms secrete extracellular enzymes which degrade the polymeric constituents of the fibre into metabolizable and soluble products. The enzymes themselves can be cell bound or, as is more often the case, they can be excreted into their environment, diffuse the required distance, and act upon the accessible portions of the microfibril. Because direct contact between enzyme and substrate is required before the hydrolytic reaction can occur, any chemical or physical feature of the natural fibre which limits the ability of an organism to synthesize the enzymes profoundly affects the degradability of the fibre. A similar influence will be exerted if the fibre or its constituents limit enzyme access via some structural aberration.

2.1.4. Physical and chemical constraints on cellulose degradability

The physical and chemical features of cellulosic materials that influence their susceptability to enzymatic degradation include:

a. The moisture content of the fibril.

b. The size and diffusibility of reagent molecules in relation to size and surface properties of the gross capillaries and other accessible surface area of the material.

c. The degree of crystallinity of a particular cellulose.

d. The unit cell dimensions of the cellulose.

e. The conformation and steric rigidity of the anhydroglucose units.

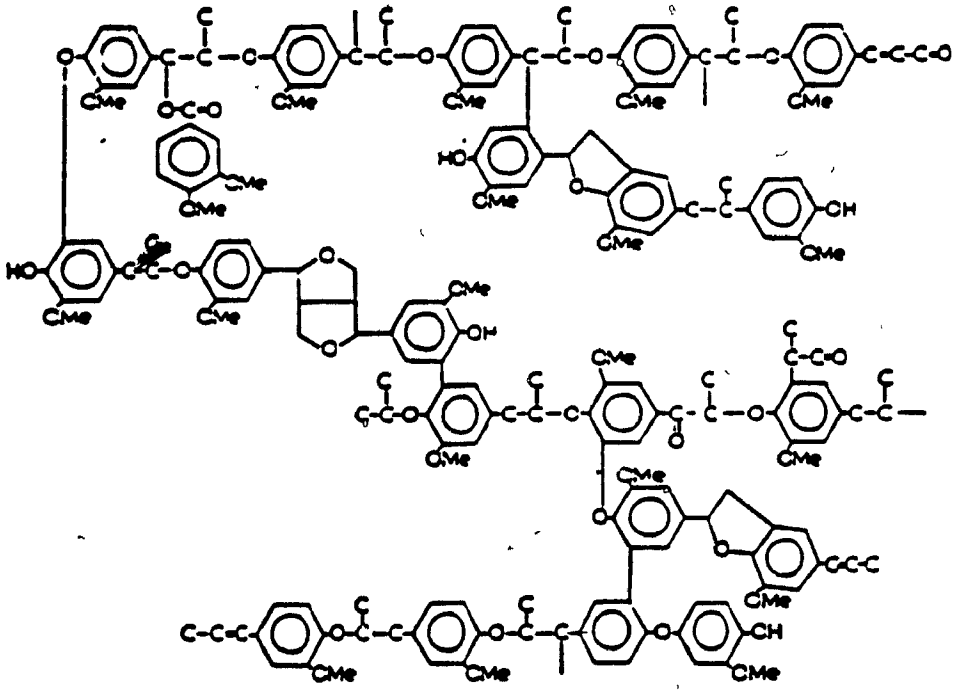


Figure 2.2. Abbreviated skeletal schematic structure of conifer lignin. From Goldstein, 1981b. With permission.

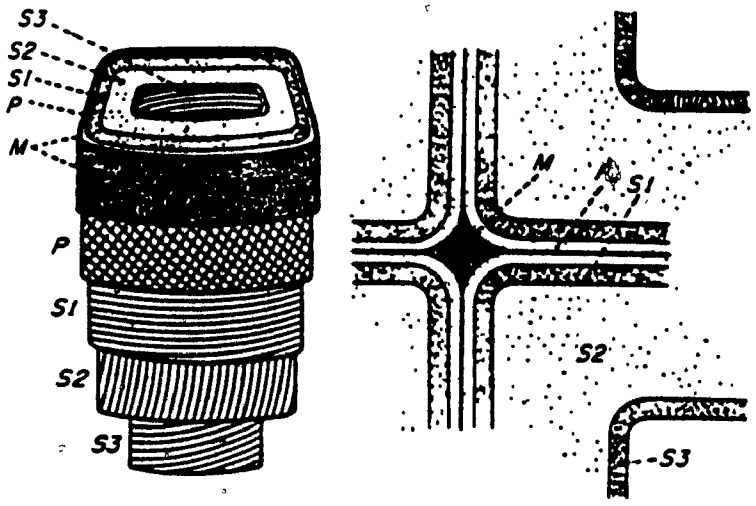


Figure 2.3. The various layers of the cell wall of cellulose fibres. The true intercellular substance or middle lamella (M) and adjacent primary walls (P) comprise the compound middle lamella of wood cells. In cotton, no true intercellular material is present but a layer of cutin coats the exterior surfaces of the primary wall. The secondary walls are composed of outer (S1), middle (S2), and inner (S3) layers. From Hagglund, 1949. With permission.

f. The degree of polymerization of cellulose.

g. The nature of substances with which cellulose is associated.

h. The nature, concentration and distribution of substituents.

a) Moisture content of the fibril

Cellulose materials are protected from deterioration if their moisture level is held below a certain critical value that is characteristic of the material and organism involved. For wood, this value is slightly above the fibre saturation point.

Moisture swells the fibre by hydrating the cellulose molecules and thereby opening the fine structure and making the substrate more accessible to cellulolytic enzymes and other reagents.

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

Accessible surface is determined by the size, shape and surface properties of the microscopic and submicroscopic capillaries within the fibre in relation to size, shape and diffusibility of the cellulolytic agents themselves.

Capillary voids in wood range from gross capillaries such as the cell lumina, which range from 20 nm to 10 micrometers in diameter, to cell wall capillaries which are below 20 nm. The surface area of the gross capillaries is approximately 2000 cm^2/gram of cotton or wood and the surface area of cell wall capillaries $3 \times 10^6 cm^2$/gram. Thus, if cellulolytic agents can penetrate the cell wall capillaries, a

substantially faster rate of reaction should result (Cowling and Kirk, 1976). The effect of pore size on the rate of cellulose hydrolysis has recently been studied (Grethlein, 1985).

The cellulolytic enzymes of microorganisms are water soluble proteins of high molecular weight. Estimates of dimensions are given in Table 2.1 for cellulolytic enzymes from a variety of organisms. As can be seen from the table, only the fraction of the cell wall capillaries which are close to 20 nm in diameter are large enough to allow penetration of the cellulolytic enzymes.

c) Degree of crystallinity

Cellulolytic enzymes readily degrade amorphous cellulose but are unable to attack highly crystalline cellulose. As a consequence, as enzymatic hydrolysis proceeds, an increase in crystallinity of the substrate is observed. Pretreatment methods, described briefly below, alter the proportion of crystalline material and thus modify the susceptibility of the cellulose to degradation.

d) Unit cell dimensions of the crystallites present

Cellulose exists in four recognized crystal structures, cellulose I, II, III, and IV. Cellulose I is the crystal form in native cellulosic materials. Cellulose II is found in regenerated cellulose such as viscose filaments, cellophane and mercerized cotton. Cellulose III and IV are formed by treatment with anhydrous ethylamine and certain high temperatures respectively. It is not possible to form cellulose II, III or IV without altering the degree of crystallinity of the material, and thus it is difficult to attribute a change in degree of susceptibility to enzymatic

Organism	Molecular Weight	Equivalent	
		Sphere Diameter (nm)	Ellipsoid W x L (nm)
Aspergillus niger	--	5.8	3.2 x 19.2
Chrysosporium lignorum	--	3.7	2.0 x 12.0
	--	3.8	1.5 x 9.0
Fomes annosus	--	4.2	2.3 x 14.0
	--	3.8	2.1 x 12.5
Myrothecium verrucaria	63000	7.7	4.2 x 25.2
	49000	6.3	3.5 x 20.8
	55000	6.8	3.7 x 22.4
	30000	5.1	2.8 x 16.8
	30000	5.6	3.1 x 18.6
	5300	2.4	1.3 x 7.9
Penicillium notatum	35000	6.4	3.5 x 21.0
	35000	5.5	3.0 x 18.2
Polyporus versicolor	51000	6.4	3.5 x 21.0
	11400	3.3	1.8 x 10.8
Stereum sanguinolentum	20500	6.2	3.4 x 20.4
	--	4.3	204 x 14.2
Trichoderma koningi	50000	6.4	3.5 x 21.0
	26000	4.6	2.5 x 15.2
Trichoderma reesei	76000	7.6	4.2 x 25.0
	49000	6.3	3.5 x 20.8
Average for all enzymes taken together		5.9	3.3 x 20.0

Table 2.I. Estimated sizes of fungal cellulases. After Cowling and Kirk, 1976.

degradation to a change in unit cell dimensions only. A study involving all 4 types of cellulose indicated that T. viride can adapt, at the synthesis level, the structure of the active site of cellulase, so as to accomodate the specific crystal lattice upon which it is growing (Rautala, 1967; Ghose, 1977).

e) Conformation and steric rigidity of the anhydroglucose units

Within the crystalline regions, the glucose units may occur in the so called "chair" conformation with alternate glucopyranose units oriented in opposite directions within the lattice. These speculations have not been confirmed experimentally.

f) Degree of polymerization of the cellulose

The length of cellulose molecules in a fibre vary over a range from 15 to 14000 glucose units. Most isolated cellulases studied to date appear to hydrolyse cellulose at random sites along the length of molecules thus rendering the degree of polymerization of less importance than other constraints such as pore size.

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

i. Constituent minerals:

Cellulose fibres contain about 1% ash which includes all of the elements essential for the growth and development of cellulolytic microorganisms.

ii. Extraneous materials

In cellulosic substrates the extraneous materials include a wide

variety of organic substances that are soluble in neutral solvents such as acetone, ether, methanol, ethanol, benzene, and water. Included in these materials are vitamins, soluble carbohydrates, toxic substances such as phenolics, capillary deposits which block enzyme access, specific enzyme inhibitors, and very small amounts of nitrogen and phosphorus.

iii. Lignin

Crystalline cellulose when combined with lignin is one of nature's most resistant materials. Many enzymes and microorganisms are unable to degrade lignin. The precise chemical nature of the association between lignin and cellulose fibres is not clear. Although chemical bonding has been suggested, it seems likely that the association between cellulose and lignin is a physical mutual interpenetration.

In order for wood to be degraded by cellulolytic organisms it must be partially delignified. (Vohra et al., 1980). Various pre-treatments result in varying degrees of delignification, as will be discussed in a later section.

h) The nature, concentration, and distribution of substituent groups

Replacement of the hydrogen of the primary and secondary hydroxyl groups of cellulose by certain substituents, such as a carboxymethyl group, reduces cellulose crystallinity and increases its water solubility. Degree of substitution (DS) refers to the average number of substituent groups attached to the hydroxyls of each glucose unit in the polymer. For carboxymethyl cellulose, a DS of 0.5 to 0.7 ensures complete water

solubility at concentrations normally used (< 5%). Up until the point of complete solubility, as the cellulose becomes more soluble and less crystalline, its susceptability to enzymatic attack increases. After this point the addition of more substituent groups decreases susceptibility to attack until complete immunity results at a DS slightly greater than 1. Large substituent groups are more effective in contributing to resistance than smaller ones (Millet et al., 1970).

2.1.5. Pretreatment

As alluded to in the above discussion, lignocellulosic material must undergo pretreatment to increase its susceptibiltiy to hydrolytic enzymes. The methods include: biological degradation of lignin, grinding of the substrate in a vibratory ball mill, chemical pretreatment including alkali and acid, and steam explosion.

a) Biological degradation of lignin

White rot fungi (Basidiomycetes) are able to utilize lignin as well as cellulose. A summary of the organisms involved is given in Table 2.2. Biological degradation holds little promise, however, because of the long incubation times involved and because of considerable amount of cellulose and other carbohydrate is consumed by the organism. Recent attempts with different bacterial species have met with little success (Janshekar and Fiechter, 1982).

b) Grinding/ball milling

The effectiveness of grinding lignocellulosic materials is species selective (Millet et al., 1970). Within about 2 hours of ball milling, all woods reach a plateau of degradability. The plateau

varies considerably, however, ranging from 80% for aspen, to 20 % for red alder. Ball milling requires a long period of time to decrease crystallinity. Sigmacell, a commercial cellulose preparation, was subjected to ball milling in an effort to reduce its crystallinity. For a decrease in crystallinity index from 88.8 to 36.5, 95 hours of ball milling was required (Table 2.3) (Fan et al., 1980). A comprehensive report on the effect of compression milling on crystallinity, accessibility, specific surface area and degree of polymerization of cellulose has been published (Ryu et al., 1982).

c) Alkali

Sodium hydroxide and ammonia cause extensive swelling and separation of the structural elements of cellulose, leading to the formation of cellulose II from cellulose I and enhancing degradability (Theodorou et al, 1981). Alkali treatments proved to be useful in increasing the digestibility of lignocellulosics (Millet et al., 1975) as well as increasing their utilization as a carbon source for SCP production (Chahal and MooYoung, 1981).

d) Acid

A selective hydrolysis process has been developed to separate hemicellulose sugars, such as xylose, from holocellulose. The residual material, when hydrolysed, gives mainly glucose (Knappert et al., 1980; Lee et al., 1978). The result is a 90% yield of xyloses from hemicellulose after acid hydrolysis. As well, a 5 fold increase in the degradability of the milled residual material was observed.

e) Sodium chlorite ($NaClO_2$) hydrolysis

Sodium chlorite, a strong oxidizing agent has long been used to prepare holocellulose, the total carbohydrate portion of lignocellulose (Green, 1963). Digestibility was improved (Goering and van Soet, 1968), and an increase in protein production using a Cochliobolus species, were noted using sodium chlorite-treated straw (Chahal et al., 1979). This chemical treatment offers an advantage in that the hemicellulose remains intact along with the cellulose.

Comparisons of various methods of chemical pretreatment have recently been published (Taniguchi et al., 1982; Detroy et al., 1980). The chemical methods, although very effective, suffer from the high cost of the chemicals involved. Also, with the acid and alkali methods, the residue must be neutralized, requiring large amounts of chemicals per ton of cellulose.

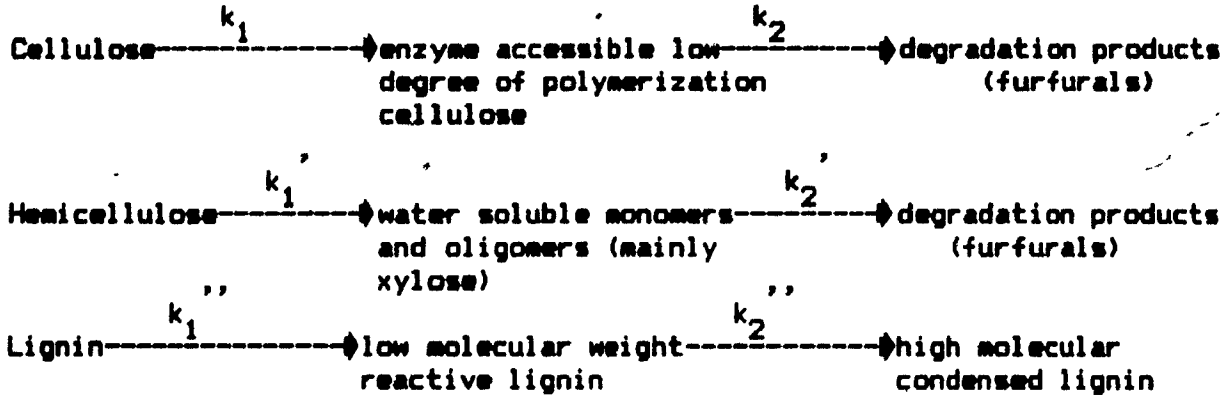
f) Steam explosion processes

Masonite Incorporated, a lumber company, first developed the steam explosion process in 1975 (Gallowy, 1975). Wood chips were exploded into wool-like fibers which were then washed to remove the solublized hemicellulose fraction. In the process, the hemicelluloses were concentrated and sold as animal feed, while the fibers were used to fabricate "press board".

Steam treatment under high pressure makes the lignocellulosic materials easily accessible to hydrolytic enzymes (Casebier et al., 1969; Alcohols newsletter, 1980; Bender, 1979; Lora and Wayman, 1978; Noble, 1980). There are two other processes (Stake and Ictech) that

are based on the same principle of steam explosion. Differences exist only in pressure, time, starting moisture content, and method of extrusion (Alcohol newsletter, 1980).

The effect of steam explosion on lignocellulosics can be represented by the following first order reactions:



It has been reported that phenolic compounds increased from 0.43 to 5.3 % after treatment of bagasse with 500 psig steam for 45 minutes (Campbell et al., 1973). Such phenolics and furfurals are toxic to most microroganisms, and the resultant hydrolysate may not be suitable for subsequent fermentations.

Stake and Iotech have claimed excellent results for their processes (Taylor, 1980; Noble, 1980). Iotech claims a yield of 21% reducing sugars from 20 % cellulose when their product was hydrolysed with commercial cellulase. There remains some uncertainty over the amount of toxic substances in an exploded product with such a degree of degradability.

2.2. The cellulase enzyme system

2.2.1. History

The ability of a variety of microorganisms to degrade cellulose has been recognized since the turn of the century. Table 2.4 lists some of the known cellulolytic microorganisms (Mandels and Andreotti, 1978). In 1912, Pringsheim suggested the involvement of two enzymes in cellulose degradation, based on the observation that glucose and cellobiose were produced. He postulated that one enzyme (cellulase) cleaved cellobiose from cellulose, leaving a second enzyme (cellobiase) to hydrolyse the cellobiose into two glucose monomers.

In the early 1950's, Reese and coworkers (Reese et al., 1950; Reese and Levinson, 1952; Reese, 1956) noted that large numbers of organisms were capable of hydrolysing water soluble cellulose derivatives, but that few could attack crystalline cellulose. They postulated that truly cellulolytic organisms produced enzymes that the others lacked. C_1 was the name given to the enzyme which could attack cellulose and reduce its degree of crystallinity, yielding short chains. The second enzyme, C_x, was the hydrolytic component, attacking and solublizing the short chains. In the ensuing years, many attempts were made to purify the C_1 and C_x enzymes (Li et al., 1965; Selby and Maitland, 1967; Wood, 1968; Eriksson and Rzedowski, 1969; Wood and MacRae, 1972; Halliwell and Griffin, 1973). There is now general agreement that the cellulase system consists of three major types of enzymes, each of which is necessary for the efficient degradation of cellulose. The first enzyme, endo-1,4 beta-D glucanase (EC 3.2.1.4) randomly cleaves internal glucosidic bonds within an unbroken glucan chain.

This creates non-reducing chain ends upon which the second enzyme, 1,4 beta-D glucan cellobiohydrolase (EC 3.2.1.91), acts. This enzyme cleaves cellobiose dimers from the glucan, and releases them into solution. There, the third enzyme beta-glucosidase (EC 3.2.1.21), completes the hydrolysis by breaking the cellobiose into two glucose monomers. Figure 2.4 illustrates the overall process. Table 2.5 describes the variety of assays which have been developed to characterize the various component activities. The cellulases of Trichoderma reesei QM 9414 have been well characterized. There are at least four endoglucanases and two cellobiohydrolases. Gritzali and Brown (1979) and Halliwell (1979) have published reviews of the cellulase system. An excellent electron microscopic study of enzymatic degradation of cellulose has also been published (White, 1982).

2.2.2. Regulation of cellulase

a) Biosynthesis of the enzymes

The regulation of cellulase enzymes is somewhat unusual in that it involves an insoluble substrate. Although the substrate is not soluble and as such, is not taken up by Trichoderma, it is involved in the induction and repression of cellulase production and with the repression of cellulase synthesis. How it is that cellulose is involved in this process is still the subject of some controversy. Cellulose has long been known to be the most effective carbon source for induction of endoglucanase (CMCase) activity (Mandels and Reese, 1960). Since cellulose itself is insoluble, it seemed likely that the soluble degradation products such as cellobiose were the actual inducers. Other known inducers include lactose, salicin, trioses of glucosyl-cellobiose type and sophorose. The latter compound was

ORGANISM	RESULT	REFERENCE
Phanerochaete chrysosporium	50% delignification of aspen wood in 30 days	Eriksson, 1977 Reid, 1979
Chrysosporium pruinosum	40% delignification of manure in 30-60 days	Rosenberg, 1980
Pleurotus ostreatus	40% delignification of wheat straw in 30-60 days	Detroy and Rhodes 1980
Phanerochaete chrysosporium	Nitrogen and oxygen additions increased delignification 2-5 fold	Yang et al., 1980

Table 2.2. Effectiveness of white rot fungi used for biological delignification.

Solvent-dried cellulose	Specific surface area (m^2/g)	Crystallinity index (CrI)
Microcrystalline cellulose (Sigmacell)	1.97	88.8
Sieved Solka Floc from 270-400 mesh	3.90	77.1
Solka Floc ball milled for varied times		
12 hr.	1.54	65.1
24 hr.	1.59	59.4
48 hr.	1.56	58.1
96 hr.	1.15	36.5

Table 2.3. Specific surface area and crystallinity indices of solvent-dried cellulose.

Bacteria

Cellovibrio fulvus, aerobe, mesophile
Cellovibrio gilvus, aerobe, mesophile
Cellovibrio vulgaris, aerobe, mesophile
Cellulomonas, aerobe, mesophile
Pseudomonas fluorescens, aerobe, mesophile
Ruminococcus, anaerobe, Rumen
Clostridium thermocellum, anaerobe, thermophile

Actinomycetes (aerobic)

Steptomyces CMB 814, mesophile
Thermoactinomycete, thermophile
Thermomonospora curvata, thermophile
Thermomonospora fusca, thermophile

Fungi (aerobic)

Mesophilic	Thermophilic
Aspergillus niger	Sporotrichum pulverulentum
Trametes sanguinea	Sporotrichum pruinosum
Poria	Sporotrichum dimorphosporum
Myrothecium verrucaria	Sporotrichum thermophilium
Pestalotiopsis westerdiikii	Chaetomium thermophilium
Penicillium iriensis	Thermoascus aurantiacus
Penicillium funiculosum	
Penicillium variabile	
Polyporus versicolor	
Polyporus tulipferae	
Fusarium solani	
Trichoderma reesei	
Trichoderma koningii	

Table 2.4. Partial list of known cellulolytic microorganisms.

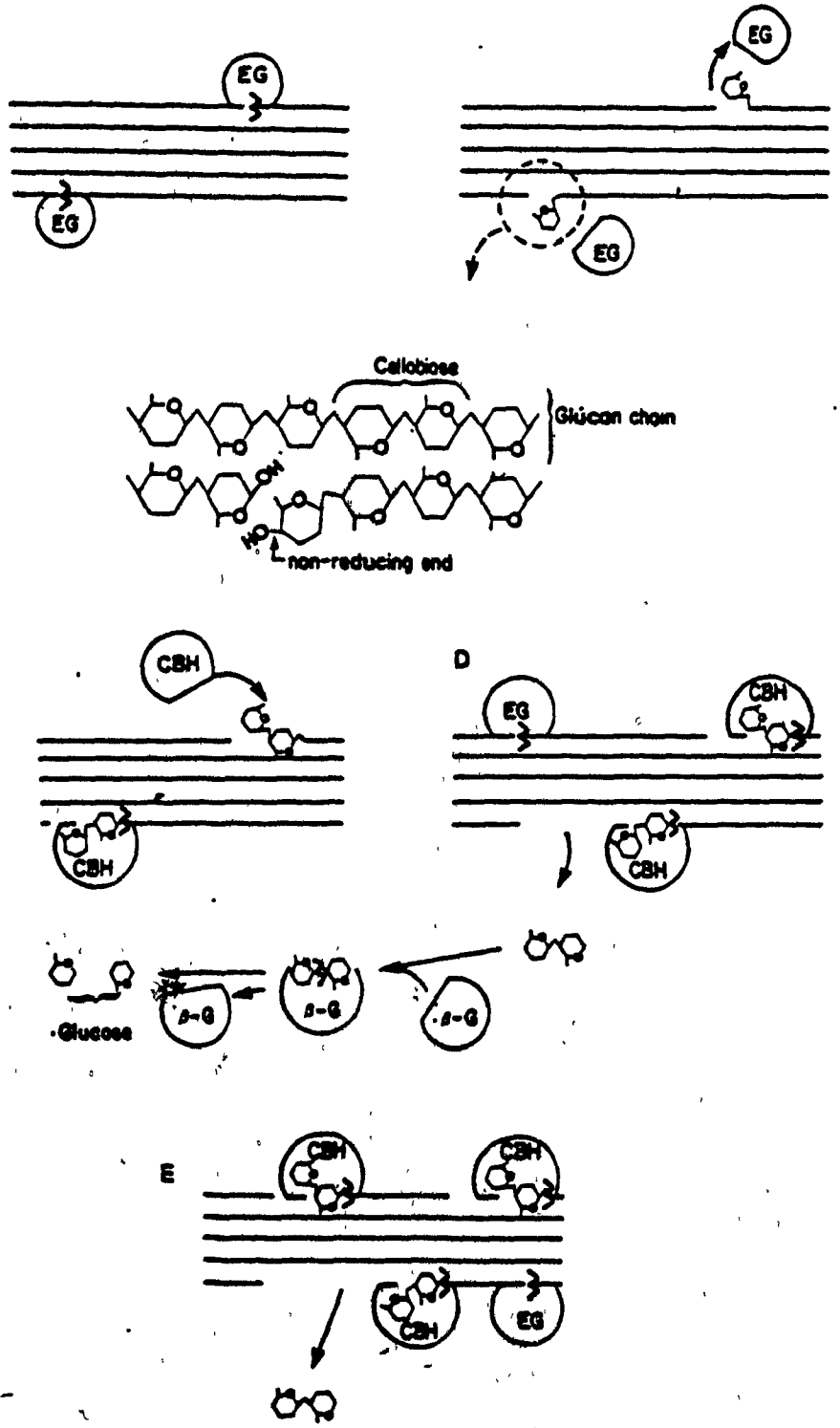


Figure 2.4. Schematic representation of cellulase-catalysed degradation of crystalline cellulose. White, 1982. With permission.

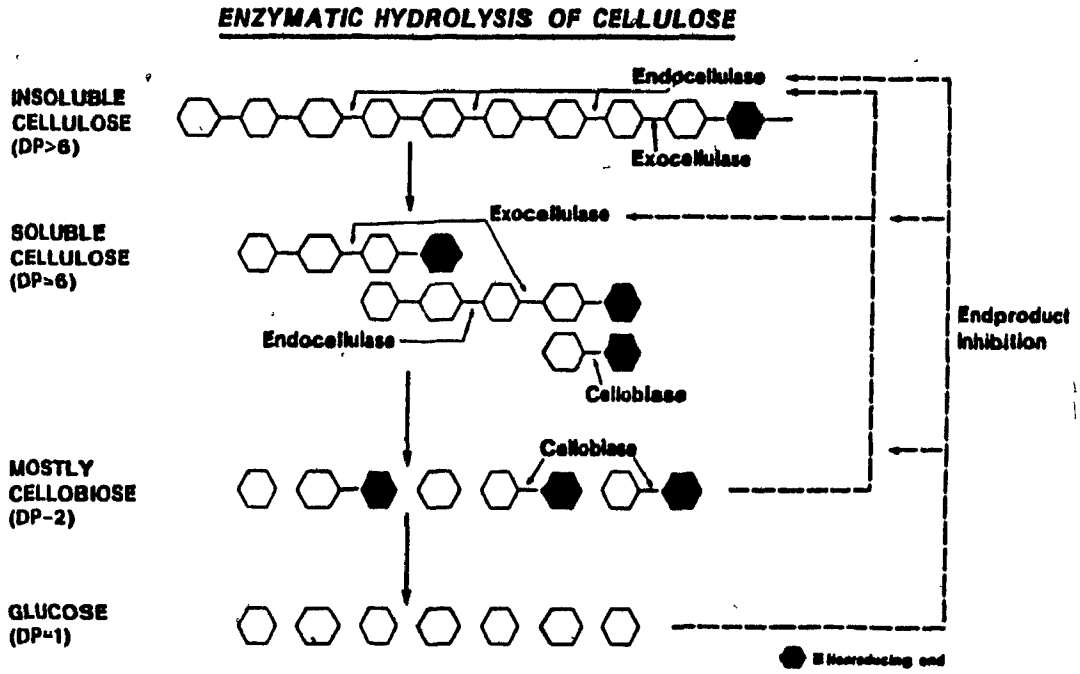


Figure 2.5. Schematic view of end-product inhibition of cellulase activity.

Enzyme assayed	Test substrate	Product measured
Endo-1,4 beta-D glucanase (CMCase or C_x)	carboxy-methyl cellulose	total reducing sugars
1,4 beta-D glucan cellobiohydrolase (exoglucanase, C_1)	avicel (crystalline cellulose)	total reducing sugars
beta-glucosidase (cellobiase)	cellobiose	glucose
Overall cellulase activity (filter paper activity)	filter paper	total reducing sugars

Table 2.5. Summary of cellulase assays.

by far the most potent inducer of CMCase activity in T. reesei. Sophorose was over 2000 times more effective as an inducer than cellobiose, however this powerful inducing capacity was limited to T. reesei alone (Mandels and Weber, 1969). Even so, the levels of cellulase produced were still markedly lower than could be achieved using cellulose as a carbon source. In general, a slow supply of inducer to a growing culture reduces catabolite repression and enhances enzyme yield. Good enzyme yields can be attained above optimum concentrations of inducer by decreasing the degradability of the substrate or by adverse modification of the growth conditions (Faith et al., 1971; Reese et al., 1969; Reese, 1972; Mandels et al., 1975).

The action of cellobiose is complex in that it induces endo-beta 1,4-glucanase at low concentrations (less than 60mM) but it can also act as an inhibitor at higher concentrations. High concentrations, (0.5-1.0%) of cellobiose or another readily metabolizable carbon source, repress endoglucanase production so that the enzyme does not appear until after the carbohydrate has been consumed (Mandels and Weber, 1969; Halliwell, 1979). When glucose is added to a rapidly growing culture, the pH drops to 2.5 and cellulase activity in the culture broth is sharply reduced. Addition of glucose to cultures where cellulose supplies have already been depleted does not cause the enzymes present to lose their activity. That the glucose effect is, in essence, a pH effect was confirmed by the addition of acid to growing cultures (Mandels et al., 1975.). Beta-glucosidase activity decreased below pH 4 and ceased at pH 3. This drop in pH is believed to be a control mechanism whereby the activity of enzymes already synthesized is curtailed in the presence of glucose.

The addition of sophorose (1mM) to washed mycelia of T. reesei induced cellulase production if the glucose concentration was below 1 mM. Glucose concentrations of greater than 100 mM repressed the sophorose induction completely. Other carbohydrates such as maltose, fructose, glucanate, glycerol, glutamic acid and pyruvic acid were also able to eliminate the effect of sophorose (Nisizawa et al., 1972). Exoglucanase activity increased in the mycelium and was released to the medium upon replacement of the glucose by sophorose. For this reason the monomer could not have been acting to prevent the release of the enzyme.

Mandels and Weber (1969) reported that, if the metabolism of soluble substrates is slowed down by suboptimum conditions of temperature, aeration, or nutrients, T. reesei produced as much cellulase on these substrates as it did when grown on cellulose. The same does not appear to be true for the hypercellulolytic mutant Rut C30 (Chahal et al., 1982).

b) Control of enzyme activity

In addition to regulation of enzyme synthesis, the cellulase complex is subject to fine control through inhibition of the activities of various components of the multi enzyme complex (Halliwell,1979). As glucose concentration increases, it progressively inhibits beta-glucosidase via a competitive inhibition mechanism. This inhibition first appears at glucose concentrations higher than 90 mM (Maguire, 1977a). The kinetic constants, pH dependance , and structural effects of the inhibition have been described (Bisset and Sternberg, 1978; Maguire, 1977a; Woodward and Arnold, 1981). Cellobiose also exerts a competitive inhibiton on exoglucanase activity (Halliwell, 1975; Howell and Stuck, 1975; Maguire, 1977b; Wood and McRae, 1975). Figure 2.5 illustrates the overall endproduct inhibition

scheme.

2.2.3. Cellulolytic organisms

a) General

A wide variety of organisms, both aerobic and anaerobic have been shown to produce cellulolytic enzymes (Table 2.4) (Sternberg, 1976). The most active organism found is Trichoderma reesei (formerly Trichoderma viride). A major contribution to enhanced enzyme productivity has been made through the development and use of hypercellulolytic mutants of T. reesei. Much effort has gone toward the production and characterization of a number of mutant strains of T. reesei (Farkas and Labudova, 1981; Gallo et al., 1978; Ghose, 1981; Ghosh et al., 1982; Labudova, 1981; Mandels et al., 1976; Mandels et al., 1978; Mishra et al., 1982; Montenecourt and Eveleigh, 1977, 1978; Nevelainen et al., 1980, Gallo et al., 1978, Warzywoda et al., 1983; Saddler, 1982). The result has been a great improvement in cellulase activity and productivity, over the original strain QM 6a (Mandels and Weber, 1969) (Table 2.6).

b) Growth conditions

Most of the studies using T. reesei for the production of cellulolytic enzymes has been on a small scale. A brief overview of the process development for enzyme production is described below. A standardized commercial process for the production of cellulase does not exist at this time. Improvements to growth medium and conditions are being reported rapidly and are summarized below.

i. Medium

The basic medium for cellulase production is shown in Table 2.7. There have been a variety of changes in medium constituents such as protein source, and surfactant type and concentration in an effort to reduce medium cost and increase cellulase yield.

Peptone replacement

Protease peptone, although necessary to obtain high yields of cellulase, is a very costly component of the medium. It can be replaced with cottonseed flour, phyton, casein hydrolysate, yeast extract, or corn steep liquor with only slight decreases in enzyme production (Mandels and Weber, 1969).

Surfactants

Addition of Tween 80 and Tween 40 doubled the yield of cellulase in T. reesei (Reese and Maguire, 1971). The mechanism by which surfactants exert their effect is not well understood, but may be related to an increase in cell wall permeability. An increase in permeability of the cell membrane has been observed when the organism is grown with the surfactant (Reese and Maguire, 1969, 1970; Yakovleva, 1982). This increase in permeability is of a general nature, and is correlated with a loss or leakage of a variety of intracellular components.

pH

The initial pH of the medium is adjusted to pH 5.5-6. The pH starts to fall due to the utilization of the NH_4^+ cations from $(NH_4)_2SO_4$. Growth of the organism is best at pH 5 and decreases in rate down to pH 3.5. The specific yield of protein, however, follows the reverse

sequence, with optimum yield occurring when the pH was controlled at 3.5 (Androetti et al., 1977). Cycling of the medium pH during the fermentation has been reported to improve cellulase yields (Mukhopadhyay and Malik, 1980).

ii. Age and volume of inoculum

For cellulase production using T. reesei, the amount of inoculum (1-5% v/v) produced little effect on the final enzyme concentration. The physiological state of the inoculum did, however, influence the kinetics of enzyme production. The use of spore inoculation resulted in longer lag phases as well as a decrease in the final concentration of enzymes produced (Andreotti et al, 1977). An inoculum ratio of greater than 5% (v/v) has been widely used, however the actual amount of biomass contained within the inoculum is by no means standardized.

iii. Temperature

The rate of growth of T. reesei continues to increase up to a temperature of 35° C, however the maximum rate of enzyme production is observed at 29° C. For Trichoderma fermentations the use of temperature cycling has resulted in a considerable increase in final enzyme yield. This technique involves the use of a higher temperature (33-34° C.) during the initial stages of the fermentation, to promote a rapid accumulation of biomass during the logarithmic growth phase of the organism. Subsequently, the temperature is reduced to a value close to the optimum for enzyme production (Nystrom and DiLuca, 1977; Mukhopadyay and Malik, 1980).

iv. Substrate

The amount of cellulase produced by Trichoderma varies considerably depending upon the nature of the cellulosic substrate and on the pretreatment which the substrate has undergone. The use of different substrates such as Avicel, absorbant cotton, newsprint, and Solka Floc, resulted in varied rates of cellulase production. Substrate-dependant differences in the proportions of the various components of the cellulase produced were also observed (Andren et al., 1975; Herr, 1979).

A cellulose concentration of 0.75% was initially reported to be optimal for cellulase production by T. reesei (Mandels and Weber, 1969). By increasing the concentration of carbon and nitrogen, increases were achieved in the yield of cellulase from batch cultures (Sternberg, 1976; Sternberg et al., 1979). The supply of nitrogen was found to be critical, and a carbon:nitrogen ratio of ca. 8 is required for optimum growth. Control of pH through the addition of NH_4OH is in wide use as a means to ensure that Trichoderma fermentations are not nitrogen limited.

Other substrates which have been evaluated for their ability to induce cellulase production include: agricultural residues (Demain, 1972; Ferrer, et al., 1977), whey (Lobanok et al., 1977), high cellulose concentrations (Nystrom and DiLuca, 1978), and hemicelluloses (Highley, 1976; Linko et al., 1975). The effect of ammonia assimilation on cellulase production has been studied (Sternberg and Dorval, 1979). A number of review articles dealing with substrates for cellulase production have appeared (Brown, 1976; Korculanin et al., 1975; Ryu and Mandels, 1980).

2.2.4. Kinetics of cellulase production

The kinetics of cellulase synthesis by Trichoderma are poorly understood, chiefly due to a lack of information concerning the growth parameters of the organism. There exists some controversy as to whether the production of cellulase is a growth related function (Sternberg, 1976; Berg and Pettersson, 1977; Ghose et al., 1975). The following brief description of the kinetics of cellulose utilization is based upon data from a number of investigators (Andreotti et al., 1976; Chahal et al., 1981, 1982; Gallo et al., 1978; Peitersen, 1975; Ryu and Mandels, 1980; Sternberg, 1976; Volfova and Kyslikova, 1981).

i. Initial growth of the organism starts immediately on the soluble sugars and proteins present in the medium. After 12-24 hours, these soluble compounds are depleted. No cellulose has been used up until this point.

ii. Once the soluble nutrients are depleted, metabolic pathways are shifted toward the utilization of cellulose.

iii. Mycelium biomass synthesized is difficult to estimate in the presence of cellulose. Based on available data it appears that there are four phases: a short lag phase, followed by a fast exponential growth on the soluble constituents of the medium, a slow growth phase during which the cellulose is consumed, and finally, a stationary phase during which biomass is dying as fast as it is being synthesized.

iv) Cellulase activity is not initiated until all of the soluble sugars etc. are consumed. After the phase described in (i), cellulase

production increases parallelling the exponential growth phase. Cellulase production levels off when the cellulose is completely consumed. A sharp increase in cellulase concentration has been reported at the end of the fermentation which has been attributed to de novo synthesis (Ghose, 1977) and to autolysis and release of the intracellular cellulase (Chahal et al., 1982). Evidence pointing to the latter includes a decline in biomass and increase in soluble sugars released by disintegrating biomass.

2.2.5. Cellulase production: state of the art.

A number of researchers have reported significant gains in the effort to produce cellulase enzymes. Pilot scale studies have been reported in which fermentation parameters have been studied in larger scale (30-400 liter) vessels (Andren and Nystrom, 1976; Nystrom and Allen, 1976; Nystrom and DiLuca, 1977). Continuous production of cellulase enzymes has been attempted in several reactor types including: a continuous flow column (Theodorou et al., 1981), a 2 stage reactor (Ryu et al., 1979), a CSTR (Peitersen, 1977; Mitra and Wilke, 1975; Sahai and Ghose, 1977; Ghose and Sahai, 1979). Fed batch studies have been reported using: cellulose (Hendy et al., 1984; Watson et al., 1984), soluble carbon sources (Ghose and Sahai, 1979; Allen and Mortensen, 1981), and a carbon nitrogen double fed batch technique (Bottvaldova et al., 1982). Attempts to immobilize *T. reesei* (Frein et al., 1982) and cellulase (Woodward and Zachry, 1982) have recently been reported.

Strain	FP activity (shake fl.) FPU/ml.	FP productivity (shake flask) FPU/l./hr.	FP activity (fermenter) FPU/ml.	FP productivity (fermenter) FPU/l/hr.	Ref.
QM 6a	0.5	3			a
QM 9414	10	12		30-46	b
NG 74	40-50				c,d
MCG 77			5	72	c,d
Rut C30	15		57	200	e
CL 847			17.5	125	f

Table 2.6. Improvements in Trichoderma reesei over original strain QM 6a. References are: a Mandels et al., 1976

b Montenecourt and Eveleigh, 1978

c Gallo et al., 1978

d Mandels et al., 1978

e Watson et al., 1984

f Warzywoda et al, 1983

Component	Concentration (g/l)	Component	Concentration (mg/l)
$(NH_4)_2SO_4$	1.4	$FeSO_4.7H_2O$	5.0
KH_2PO_4	2.0	$MnSO_4.H_2O$	1.6
$CaCl_2.2H_2O$	0.3	$CoCl_2$	
$MgSO_4.7H_2O$	0.3	$ZnSO_4.7H_2O$	1.4
Urea	0.3		
Cellulose	7.5-10		
Peptone	0.75-1.0		
Tween 80	1-2		

Table 2.7. Trichoderma reesei medium for cellulase production. (Mandels medium) (Mandels and Weber, 1969)

Substrate concn. (wt %)	Enzyme concn. (FPU/ml)	Temperature (^{o}C)	Reducing sugar at 24 h. (g/l)	% Hydrolysis at 24 h.
2.5	0.5	50	16	64
5.0	0.5	50	23	43
7.5	0.5	50	26	33
2.5	1.0	50	20	77
5.0	1.0	50	33	60
7.5	1.0	50	39	47
2.5	1.5	50	21	79
5.0	1.5	50	39	67
7.5	1.5	50	46	53
5.0	1.0	45	28	54
5.0	1.5	45	33	59
5.0	1.0	55	30	54
5.0	1.5	55	33	61

$$\% \text{ Hydrolysis} = \frac{\text{glucose (g/l)} \times 0.9}{\text{Substrate (g/l)}}$$

Table 2.8. Effect of temperature and enzyme loading on enzymatic hydrolysis of cellulose. (After Mandels and Sternberg, 1976; and Reese and Mandels, 1980).

2.3. Cellulose hydrolysis

2.3.1. General

The broth obtained from cellulase fermentation can be concentrated by ultrafiltration or precipitation or it can be used directly as a source of enzyme for the hydrolysis of cellulosic material. The measurement and expression of cellulase activity has been a source of confusion because of the array of substrates, methods of determining activity, and units of activity that have been applied to cellulase. The filter paper assay (Mandels et al., 1976) has been widely used by many workers because it provides a simple and easily reproducible measure of the complete cellulase activity. Filter paper activity is expressed simply as the milligrams of glucose released when 0.5 ml. of enzyme solution acts on 50 mg. of Whatman No. 1 filter paper at 50 C for 1 hour. Other common assays include: measurement of the activity of endo-beta 1,4 glucanase (Cx or CMCase) on carboxymethylcellulose, exo-beta 1,4 glucanase (C_1, CBH, Cellobiohydrolase, glucocellulase) activity on crystalline cellulose, and beta-glucosidase (cellobiase) activity on cellobiose. Most of the mutants of T. reesei produce a cellulase complex which is deficient in beta-glucosidase. As a result, cellobiose accumulates in the hydrolysate and inhibits the activity or represses the formation of the components of the cellulase complex.

2.3.2. Factors affecting hydrolysis

The efficiency of an enzymatic hydrolysis process is affected by many factors including: time of reaction, temperature, pH, substrate concentration, the nature of the substrate, substrate pretreatment, the presence of inhibitors, the balance of individual enzyme components, and agitation of the hydrolysis vessel.

a) pH

The pH optimum for cellulose hydrolysis is 4.8 (Mandels and Weber, 1969).

b) Temperature

There are two effects of temperature on the enzymatic hydrolysis of cellulose. As with any chemical reaction, there is the effect of increasing reaction rate with increasing temperature. At higher temperatures, however, there is an increased rate of thermal deactivation of the cellulase enzymes (Palmer, 1975). At 45° C. the hydrolysis reaction is slow and can achieve the levels of hydrolysis achieved at 50° C. only after extended hydrolysis times. At 55 and 60° C, initial rates were higher but enzyme deactivation accounted for a decrease in reaction rate after 6 hours. Fifty degrees was found to be the optimum temperature for hydrolysis. Similar temperature effects were also noted in other studies (Table 2.8) (Mandels and Sternberg, 1976; Reese and Mandels, 1980).

c) Concentration of substrate and enzyme

As indicated in Table 2.8, actual yields of sugar increase as the concentration of the substrate increases, however the conversion efficiency of the substrate to soluble sugars, is reduced. A similar relationship is observed with increasing enzyme concentration, that is, with increasing enzyme concentration, total sugar yield is increased while the yield per unit enzyme decreases. Sternberg (1976) reported that the highest hydrolysis rates were obtained with high substrate and enzyme concentrations and short hydrolysis times. However under such conditions, only 20-30% hydrolysis of the substrate is achieved. The resultant dilute syrups are produced at the expense of large quantities

of enzyme. In these studies, however, enzyme to substrate ratios varied with increasing substrate concentration, making the results difficult to interpret. Similarly in another study (Andren et al., 1975), 20 units of enzyme were used per gram of substrate for a 10% slurry of newspaper. The enzyme concentration was reduced to below 15 units/gram when a 15% slurry of newsprint was used. In more recent work (Mandels et al., 1981), a cellulase concentration of 20 units/gram cellulose, gave over 90% hydrolysis of 5-15% slurry of cellulose in 48 hours.

d) Effect of structural features and pretreatments

There are a number of constraints on the enzymatic conversion of cellulose. They include: insolubility of the substrate, the highly crystalline nature of the substrate, and the physical and chemical links between the cellulose and lignins present. These characteristics, previously described, make the pretreatment of the lignocellulosic materials essential.

i) Crystallinity

The influence of the degree of crystallinity of a cellulosic substrate upon its susceptibility to enzymatic hydrolysis has been known for some time (Reese et al., 1957; Walseth, 1952). Cellulases readily degrade the more accessible amorphous regions of cellulose, but are less effective in degrading highly crystalline areas. The crystallinity of cellulose appears to be the main factor limiting enzymatic digestibility, while the degree of polymerization is less significant (Cowling and Kirk, 1976). It has been reported that hydrolysis rate is mainly dependant on the fine structural order of

cellulose which can be best represented by crystallinity, rather than by simple surface area measurements (Fan et al., 1980). Mandels and Sternberg (1976) reported that various cellulosic substrates were more susceptible to enzymatic hydrolysis when they were first ball milled to reduce crystallinity. In a recent study Grethlein (1985) found no relationship between crystallinity and hydrolysis rate. Detailed studies on the effect of the physicochemical properties of cellulose on the rate of enzyme adsorption and hydrolysis rate have been reported (Lee et al., 1982). The mechanism of cellulose hydrolysis has been studied from the perspective of: cellulase adsorption (Wilke and Mitra, 1975; Huang, 1975b; Mandels et al., 1971, Peitersen et al., 1977), reaction mechanism (Lee and Fan, 1982; Sasaki et al., 1979). Included have been studies on inhibition (Howell and Stuck, 1975), deactivation (Howell and Mangat, 1978), and enzyme and substrate concentration (Huang, 1975a). There have been a number of attempts to model the hydrolysis reactions (Lee et al,1978, Lee and Fan, 1982, 1983; Fan et al., 1980; Fan and Lee, 1983; Huang, 1975b; Peitersen and Ross, 1979; Holtzapple et al., 1984a, 1984b; Wald et al., 1984).

ii. Effect of lignin

Lignin reduces the susceptibility of cellulosic material to enzymatic degradation. Many attempts have been made to develop an understanding of the effect of lignin on cellulose hydrolysis (Crawford, 1975, 1976; Kirk, 1975, Riaz et al., 1977). The effect of delignification is evident in Table 2.9 (Data from Mandels and Sternberg, 1976; Toyama and Ogawa, 1975). Ball milling reduces the size and crystallinity index of the substrate, but large proportions

Substrate	Percent Decomposition	
	24 h.	48 h.
Bagasse	6	10
Bagasse (ball milled)	42	48
Bagasse (delignified)	87	88

Table 2.9. Effect of delignification on enzymatic hydrolysis of bagasse.

of cellulose remain bonded to lignin and are not easily accessible to the enzyme complex.

iii. Type of enzyme: ratio of component activities

As described previously, most preparations of T. reesei cellulase are deficient in beta-glucosidase. Cellulase preparations from this organism have from 0.02 to 0.1 units of beta-glucosidase activity per unit of filter paper activity. As a result, the cellobiose levels in the hydrolysate vary between 10 and 70% of the total sugars, depending on the nature of the enzyme and on the concentration of the substrate (Andren et al, 1975; Palmer, 1975). There have been efforts to increase the beta-glucosidase activity of cellulase from T. reesei by pH and temperature profiling (Tangnu et al., 1981). It has been reported that 1-1.5 beta glucosidase units per filter paper unit is sufficient for complete hydrolysis (Chahal et al., 1982, Ryu and Mandels, 1980). However the addition of beta-glucosidase to Trichoderma cellulase markedly increases the hydrolytic potential of the enzyme (Sternberg et al., 1977). Aspergillus phoenicis (Allen and Sternberg, 1980) and Schizophyllum commune (Desrochers et al., 1981a,b) are two promising sources for beta-glucosidase. Although S. commune produces 31.4 IU/ml of beta-glucosidase, the enzyme is not stable under the conditions used for practical hydrolyses (Durocher et al., 1981a,b) Also, the high ratio of S. commune beta-glucosidase to Trichoderma cellulase required for hydrolysis (6.8:1) makes the organism an impractical choice. Beta-glucosidase from A. phoenicis shows a much higher degree of synergism with Trichoderma cellulase (Mandels, 1981). A recent study indicated that beta-

glucosidase from a bacteria (Cellulomonas) could be used in combination with Trichoderma cellulase (Choudhury et al., 1981). A review of the role of beta-glucosidase in cellulose hydrolysis has been published (Shewale, 1982).

e) Effect of surfactant addition on enzymatic hydrolysis of cellulose

Surface active agents, or surfactants, are molecules which contain a hydrophilic and a lipophilic (hydrophobic) moeity within the same molecule. In aqueous solutions or suspensions, surfactants tend to aggregate at phase interfaces. In this way, they are best able to "accomodate" both moeities which exist within them. In aqueous solutions for example, surfactants tend to accumulate at the interface between the liquid phase and the gas phase (air). The hydrophilic portion of the molecule is oriented so as to project into the bulk liquid phase. The lipophilic portion of the surfactant molecules are aligned along the surface so as to be oriented into the gas phase as much as possible. By aggregating at the interface (or surface) the surface free energy per unit area is reduced. This is the reduction in so-called "surface tension" which is associated with surface active agents.

In solid suspensions, where there are three distinct phases, the effect of surfactants becomes more complex. While the same phenomenon is occurring at the gas-liquid, a second surfactant-related effect is present at the solid-liquid interface. Surfactants accumulate at this interface as well. This can result in an increase, or decrease in the wetting of the solid by the liquid phase.

In the cellulose hydrolysis system, the extent of hydrolysis is increased by the addition of Tween 80, a non-ionic surfactant (Castanon and Wilke, 1981). These workers correlated the use of the surfactant with an increase in the amount of cellulase present in the hydrolysis broth. They concluded that the surfactant influenced the enzyme-substrate interaction in such a way as to facilitate the release of the enzyme from its "bound" state into the hydrolysis broth. The hydrolytic efficiency was improved because the surfactant was able to increase the efficiency of the enzyme mass transfer from the solid to liquid phase.

Cellulase has been shown to be denatured by shear (Reese, 1980). This inactivation was found to be lessened, to a degree, by surfactants. In another study, exposure to phase interfaces was found to result in a rapid inactivation of cellulase (Kim et al., 1982). The air-water interface was found to have a much greater denaturing effect than did simple shear stress. Surfactants were again able to lessen the extent of interfacial denaturation by displacing enzyme protein from the interfacial area. Thus the proportion of the cellulase at the interface was decreased and the percent inactivation was proportionally decreased. There has been no work in which the denaturing effect of the solid-liquid interface was examined.

2.3.3 Continuous saccharification

Early attempts were made to run continuous hydrolysis reactors. In stirred tank reactor, a yield of 4-6% glucose has been achieved from 10% cellulose feed for 200 hours. By comparison, batch reactors produced 6%

sugar (Ghose and Kostack, 1970). In a membrane reactor, the same workers achieved 75% hydrolysis in 8 hours running time. More recent work using membrane reactors (Ohlson et al., 1984) and countercurrent plug flow reactors (Fox et al., 1983) has resulted in further process improvements. An attempt has recently been made to re-utilize enzymes for the saccharification of lignocellulosic materials (Deshpande and Eriksson, 1984).

2.4 Mixed microbial fermentations

2.4.1. General considerations

It has long been recognized that the "growth range" of different microorganisms overlap, despite evolutionary pressure upon the microorganism to occupy a characteristic niche. This overlap can lead to a variety of types of interaction between microorganisms which share a particular niche. These types of interactions range from synergism, through mutual tolerance, to competition. Most microbial communities can be classified into one of seven types (Slater and Bull, 1978).

Class one communities are usually commensual or mutualistic relationships in which one member of the community depends on another for the provision of certain growth factors.

Class two communities are those in which the toxic metabolites of one organism or of one group of organisms, are consumed by another member of the community. There are many examples of such relationships in the literature, and a number have been well documented (Slater, 1978). There is some evidence which indicates that such such cultures show an increased tolerance to physiological stress (Wilkinson et al., 1974; Cremieux et al.,

1977).

Class three microbial communities may be the consequences of a number of nutritional interactions that affect the overall growth kinetics of the population. In these communities, the interaction between members results in a modification of individual population growth parameters. These modifications yield a population which is more competitive.

Class four communities are generally mutualistic; the survival of the community depends on the concerted activity of two or more populations.

Class five communities are a special case of class four whereby a primary population, growing on a rapidly metabolizable carbon source, simultaneously co-metabolizes another compound. The product of this second organism supports the growth of the first organism (Slater, 1981).

Class six organisms are usually anaerobic. Under anoxic conditions, fermentative organisms require a sink to dispose of excess reducing power. A number of examples exist where a second organism acts as an electron sink, enabling the first organism to rid itself of excess electrons which it acquired during its metabolic activities (Wolin, 1977; Murray and Khan, 1983).

Class seven microbial communities are characterized by the fact that they possess two or more species which are competing for a primary carbon source. The interaction among the members of these communities is not well understood.

2.4.2 Mixed cultivation of cellulolytic microorganisms

Most of the work describing the fermentation of lignocellulosics by mixed cultures has utilized anaerobic bacteria (Khan, 1977; Hungate, 1944; Leschine and Canale, 1984). These relationships are generally class two interactions. The first organism, the true cellulolytic member of the association, degrades cellulose to its constituent monomer sugars. In close association with this organism is a second organism which utilizes the liberated sugars to form ethanol, acetic acid, and butyric acid. Although the overall conversion of cellulose to ethanol is efficient (80%), the slow growth rates and the technical difficulties associated with anaerobic fermentations, limit the practical value of such systems.

Recent advances in genetic engineering (Montenecourt and Eveleigh, 1977; Warzywoda et al., 1983) and fermentation technology (Hendy et al., 1984; Persson et al., 1984; McLean and Podruzny, 1985) have dramatically increased the yield of cellulase produced by fungi such as Trichoderma reesei. However, mutants such as Trichoderma reesei Rut C30 produce an enzyme complex which is deficient in beta-glucosidase activity. Use of this enzyme under practical hydrolysis conditions results in an accumulation of cellobiose, a strong competitive inhibitor of exoglucanase activity. The result is a decrease in the rate of sugar production and in the final concentration of sugar produced. Several species of Aspergillus produce beta-glucosidase in large quantities. (Sternberg et al., 1977; Scrivastava et al, 1981). Although beta-glucosidase from Aspergillus shows a high degree of synergism with Trichoderma cellulase, a separate fermentation step to produce it is not practical.

To overcome this obstacle, attention has recently been focussed on mixed cultivation of aerobic cellulolytic organisms. Trichoderma longbriatum CD 172 and Scatylidium lignicola have been grown in mixed culture (Trevidi and Desai, 1984). The cellulase produced by the mixed culture had approximately the same endo and exoglucanase activities as the cellulase produced by Trichoderma longbriatum in pure culture. However, the beta-glucosidase activity of the cellulase was more than doubled through the contribution of the Scatylidium component. The cellulase produced was tested against a variety of substrates (Trevidi and Ray, 1985). Trichoderma reesei D1-6 and Aspergillus wentii PT2804 have been cultivated for the production of cellulase and xylanase (Panda et al., 1983). As well, Memnoniella echinata and Fusarium roseum have been grown in mixed cultures as a means of improving the feed value of citrus waste (Clementi et al. 1985).

Although the principle has been demonstrated, much work remains to be carried out before the application of mixed cultivation of microorganisms can be applied to the cellulase fermentation field.

3. MATERIALS AND METHODS

3.1. Growth of Trichoderma reesei Rut C30

Trichoderma reesei Rut C30 (Montenecourt and Eveleigh, 1979) was obtained from the Agricultural Research Service Patent Collection, Peoria, Illinois. The organism was maintained on slants which consisted of Mandels medium (Table 2.6) supplemented with 20 grams/liter Bacto agar and 10 g/l CF11 cellulose (Whatman Ltd., England). The same medium without Bacto agar, was used for growth in shake flasks. The carbon source used was either lactose or CF11 cellulose, and was varied up to 30 g/l. At higher carbon source concentrations, the concentrations of other media components was varied as indicated in Table 3.1. Colloidal materials were added to some media. Sodium citrate (1.5 g/l) was added as a buffering agent as indicated. The initial pH was adjusted with 3 Molar HCL or NaOH to between 5.5 and 5.7 in all cases. The medium was dispensed into 250 ml. (100 ml. working volume) or 500 ml. (200 ml. working volume) Erlenmeyer flasks and inoculated with 3 day old cultures grown in the same medium but without additives such as colloids or metal salts. Inoculum volume was 5 % of the working volume of the flask. Cultures were incubated at 27 ^{o} C with shaking (175 RPM).

3.1.1. Addition of colloidal materials

The following colloidal materials were used in the growth medium: ALON (Cabot Corp., Boston, Mass.), a positively-charged alumina-based colloid; CAB-O-SIL M5,MS7, and EH5 (Cabot Corp.), silica-based colloids of three different size ranges; WESOL-P (Wesolite Co., Wilmington Del.), a positively-charged alumina silica sol; and the hydrolysis products of salts of aluminum, ($AlK(SO_4)_2$) and iron ($FeCl_3$, $Fe_2(SO_4)_2$), positively charged

colloids. Manganese sulphate, a metal salt which does not form a colloid was used as a control.

3.2. Binding Studies

To assess the ability of the colloids to influence the concentration of lactose in solution, binding studies were carried out with lactose, ALON, trace elements, and potassium phosphate in various combinations. In each test, a solution of lactose was made up (10 g/l), and the test substances were added to it. The tubes were then shaken gently at room temperature for 10 minutes, centifuged (4000 x g, 20 min.), and the lactose concentration in the supernatant was assessed by high performance liquid chromatography (Spectra Physics model SP 8100). The column used, a polypore carbohydrate column (Brownlee Laboratories Inc., Santa Clara, California) was kept at 80^{0} C. Water was used as the mobile phase at a flow rate of 0.3 ml./min.

In some cases a loss of binding capacity occured with ALON and it became necessary to remove surface contamination. This was done as follows: ALON was stirred with 10 % (v/v) HCl for 1 hour at room temperature. The ALON was then washed by filtration until the pH of the wash water was above that of ALON in water (ca. 4.4). The ALON was then dried at 105 ^{0}C. overnight and used in the binding studies.

3.3. Growth of Aspergillus phoenicis ATCC 329

Aspergillus phoenicis was obtained from the U.S. Army (Natick) laboratories and was maintained on potato dextrose agar (PDA) slants (Becton Dickinson and Co., Cockeysville, MD.). The medium used for growth of Aspergillus is shown in Table 3.2. Amygdalin was added to increase the

production of beta-glucosidase (Sternberg et al., 1977). After approximately 10 days of growth the biomass was removed by centrifugation (8000 x g, 30 min.), and the crude broth was clarified by filtration (0.45 micron pore size, Millipore Corp., Bedford, MA). The clarified broth was then assayed for beta-glucosidase activity.

3.4. Growth of the Mixed Culture

3.4.1. pH control during shake flask cultivation of mixed culture

The pH of shake flasks was controlled within a range of 0.5 pH units through the use of Amberlite cationic exchange resins. The resins were prepared using the method of Styer and Durbin (1982). Only the $Ca(OH)_2$-treated resin was used. The concentration of Amberlite routinely used was 50 g/l.

3.4.2. Media optimization

The growth medium used for shake flask cultivation of the mixed culture is shown in Table 3.2. Colloidal materials (ALON and $FeCl_3$) and amygdalin were added to the medium in some cases as indicated. The effect of potassium, magnesium, and calcium salts concentration was examined by varying their concentrations, while maintaining the same weight proportions of each salt. The effect of variations in starch concentration at constant cellulose concentration were examined.

Mixed cultures were inoculated from 3 day old pure cultures of Trichoderma and Aspergillus cultures which were grown on 10 g/l cellulose and 10g/l starch respectively. Unless otherwise indicated, the volumetric inoculum ratio of Trichoderma to Aspergillus was 3:1. The mixed cultures were

incubated at 27° C., unless otherwise indicated.

3.4.3. Effect of temperature on enzyme production by mixed culture

The effect of incubation temperature on enzyme production by the mixed culture was investigated in two ways. Initially, the cultures were grown at four different temperatures, 27, 30, 33 and 36°C. and enzyme activity in the culture broth was monitored. In a second set of runs, the effect of temperature cycling was determined. The cultures were incubated at the same four temperatures (27, 30, 33, and 36 ° C.) during the initial 48 hours of growth, after which all cultures were incubated at 27°C.

3.4.4. Growth kinetics of mixed cultures in lab-scale fermenters.

All fermentations were performed in Chemap CF or LF model fermenters equiped with 14 liter vessels. The working volume was 10 liters. The fermenters were equipped with a paddle-type stirring system (Fundaspin ^{R}) to reduce shear stress on the mycelium. Impeller rotational speed was 250 RPM unless otherwise stated. Fermenter temperature was controlled at 27°C., and pH was controlled at 4.6, unless otherwise indicated. In order to supply an adequate amount of nitrogen to the fermenter, 1 M NH_4OH and a solution of 1 M HCl which also contained 1M NH_4Cl, were used to control pH. Foam control was effected through the use of Dow Corning Antifoam A. The antifoam agent was added at a level of 0.02 % (v/v) initially, and as required througout the fermentation. Inoculum volume was 4 % v/v. The volumetric inoculum ratio of Trichoderma to Aspergillus was maintained at 3. Samples were removed via a steam sterilizable sampling port on the bottom of the fermenter vessel. The port was sterilized for 10 minutes prior to, and after sampling.

3.5. Hydrolyses

3.5.1. Evaluation of the hydrolytic potential of Trichoderma versus mixed culture cellulase

The enzymes produced through mixed cultivation of Trichoderma reesei and Aspergillus phoenicis, and in pure cultures of Trichoderma were tested for their hydrolytic potential with Solka Floc unless otherwise indicated. Whole broth from culture flasks was centrifuged (4000 x g, 20 min) to remove biomass, and clarified by Millipore filtration (0.45 micron). Crude cellulase preparations were stored at -20°C until use. The enzyme loading ratio (IU cellulase activity/gram Solka Floc), and enzyme activity ratio (IU beta-glucosidase/ IU cellulase activity) were varied as indicated. The enzyme activity ratio was varied through the addition of crude beta-glucosidase from Aspergillus or from almonds (Sigma Chemical Company, St. Louis. Mo.). At high enzyme loading ratios (40-50), it was necessary to concentrate the beta-glucosidase from Aspergillus to avoid overdilution of the hydrolysis mixture. This was achieved through precipitation of the enzyme with 4 volumes of acetone at -20°C. The crude protein was recovered by centrifugation (8000 x g, 35 min.) and resuspended in a minimal volume of 0.05 M citrate buffer (pH 5). Substrate (Solka Floc) concentration was either 100 or 150 g/l. Unless otherwise indicated, hydrolyses were carried out at 50 °C., in 125 ml. screw-capped Erlenmeyer flasks in a temperature controlled incubator. Rotational speed was maintained at 175 RPM. Each flask contained 5-50 IU FPA/ gram Solka Floc, 0.02 % sodium azide (for control of contamination), 0.05 M citrate buffer (pH 4.8), Solka Floc, and in some cases supplemental beta-glucosidase . Samples were removed and clarified by centrifugation (4000 x g, 20 min.). The supernatant was then assayed for reducing glucose and for total reducing sugars.

In some hydrolyses, the effect of surfactant additions were tested. For these tests the cellulase came from Trichoderma cultures which had been grown using the normal medium (Table 3.1) but without added Tween 80. The hydrolysis flasks were prepared exactly as described above except that surfactants were added at a concentration of 0.2 % (w/v).

3.5.2. Kinetic characterization of crude cellulase from mixed culture

In order to determine some of the kinetic parameters of the crude cellulase produced in the mixted cultures, it was necessary to change some of the standard hydrolysis conditions described above. Temperature was varied from 40-60 ^{o} C. The pH of the hydrolysis mixture was also varied over the range 3-6.5. The effect of end product inhibition was determined through the addition of glucose to the hydrolysis mixture. In order to determine the stability of the individual enzymes of the cellulase complex under the conditions used for hydrolysis, experiments were carried out under the standard hydrolysis conditions without substrate. In these experiments, samples were assayed for FPA, beta-glucosidase activity, Avicelase activity, and a carboxymethylcellulase (CMCase) activity. In some experiments, substate concentration was varied as indicated.

3.6. Elution of cellulase from cellulose using surfactant solutions

To determine the ability of surfactant solutions to elute cellulase from cellulose, cellulose slurries (10% w/v) were prepared by adding Whatman cellulose powder (CF11) to acetate buffer (0.2 M. pH 5) or to water. Rut C30 broth was added and the adsorption of cellulase to cellulose was allowed to take place at room temperature with gentle agitation for 20 minutes. The cellulose-enzyme complex was then removed by centrifugation, and resuspended in water, buffer, or aqueous surfactant solution (0.2%

w/v). The enzyme was eluted for 1 hour by gentle agitation at room temperature. Filter paper activity and soluble protein measurements were made on the original crude cellulase broth, the supernatant liquid after adsorption, and on the supernatant liquid following elution.

3.7. Analyses

Crude broth was assayed for its activity against a variety of substrates. The assays are summarized in Table 3.3. All enzyme activities were expressed in International Units (IU). Reducing sugar was estimated with dinitrosalicylic acid reagent (Miller, 1959). Glucose was estimated enzymatically by the glucostat method (Raabo and Terkildsen, 1960). Soluble protein was determined by the method of Lowry et. al. (1951). Phosphate was assayed as described in Standard Methods (APHA, 1975).

Biomass was determined by dry weight when lactose was used as the principle carbon source. In cellulose-based fermentations, 2 samples were filtered (0.45 micron), and washed 3 times with hot (50^{o} C.) distilled water. One sample was dried overnight at 105^{o}C. and weighed after cooling in a dessicator. This gave a value for biomass plus residual cellulose. The nitrogen content of the second sample was determined by performing a Kjeldhal determination. Kjeldhal nitrogen was determined using an automatic Kjeldhal titrator (Buchi, Swtitzerland). Biomass was estimated using a conversion factor (biomass (g/l)= Nitrogen x 7.14). Residual cellulose was determined by difference.

Starch was estimated as follows: fermenter samples were filtered and washed with a known volume of hot (50^{o} C.) distilled water. The filtrate was

diluted such that the starch concentration was in the range 10-70 micrograms/ ml. To 0.1 ml. samples of filtrate, 0.2 ml. of amyloglucosidase (Boehringer Mannheim Canada, Dorval, Quebec.) was added. The mixture was incubated at 60°C. for 15 minutes. The tubes were then boiled for 10 minutes to stop the reaction. After cooling, 0.2 ml. of water was added, and the contents of the tube were mixed. The glucose concentration in the tube was then estimated as described above. The absorbance at 450 nm from the glucostat assay was compared to a standard solution of soluble starch (BDH Chemicals). A sample standard curve is shown in Figure 3.1.

Initial rates of production of both glucose and total reducing sugars were determined for some hydrolyses. The initial (0-8 hr.) sugar concentration data were fitted using a polynomial fitting program on a Digital PDP 11 computer. The data were weighted to favour the observations taken early in the hydrolysis reaction. The weighting factor used was $(0.5 + C_s)^{-2}$, where C_s = the sugar concentration at any given time. Inital rate values were obtained by taking the derivative of the polynomial at time 0.

The kinetic parameters of batch growth were defined as follows:

$$G = \frac{dx}{dt} = \text{volumetric rate of biomass generation (Maximum) } gl^{-1}h^{-1}$$

$$Y_{x/s} = \frac{\Delta X}{\Delta S} \quad \frac{\text{Total change in biomass concn.}}{\text{Total change in substrate concn.}}$$

$$\frac{dS}{dt} = \text{Rate of substrate utilization}$$

Medium component	Concn for the following concn of carbon source:		
	10	20	30
KH_2PO_4	2	4	4
$CaCl_2$	0.3	0.6	0.6
$MgSO_4$	0.3	0.6	0.6
Urea	0.3	0.6	0.9
$(NH_4)_2SO_4$	1.4	2.8	4.2
Proteose peptone	1.0	2.0	3.0
Tween 80	2.0	2.0	2.0
$FeSO_4$*	5.0	10.0	10.0
$MnSO_4$*	1.6	3.2	3.2
$CoCl_2$*	2.0	4.0	4.0
$ZnSO_4$*	1.4	2.8	2.8

Table 3.1. Medium used for growth of Trichoderma reesei Rut C30. Quantities are in grams per liter except for those compounds marked with an asterisk (*). For these compounds the quantities shown are in mg/liter.

ORGANISM	TRICHODERMA	ASPERGILLUS	MIXED
Component			
KH_2PO_4	2.0	2.0	4.0
$CaCl_2.2H_2O$	0.4	0.4	0.8
$MgSO_4.7H_2O$	0.3	0.3	0.6
$(NH_4)_2SO_4$	1.4	1.4	2.8
UREA	0.3	--	0.3
BACTOPEPTONE	1.0	1.0	2.0
TWEEN 80	2.0	2.0	2.0
$FeSO_4.7H_2O$	5×10^{-3}	5×10^{-3}	1×10^{-2}
$MnSO_4.H_2O$	1.6×10^{-3}	1.6×10^{-3}	3.2×10^{-3}
$ZnSO_4.7H_2O$	1.4×10^{-3}	6.4×10^{-3}	7.8×10^{-3}
$CoCl_2$	2.0×10^{-3}	2.0×10^{-3}	4.0×10^{-3}
CITRIC ACID.H_2O	--	5×10^{-3}	5×10^{-3}
$Fe(NH_4)_2(SO_4)_2.5H_2O$	--	1×10^{-3}	1×10^{-3}
$CuSO_4.5H_2O$	--	2.5×10^{-4}	2.5×10^{-4}
H_3BO_3	--	5×10^{-5}	5.0×10^{-5}
$Na_2Mo_4.2H_2O$	--	5×10^{-5}	5×10^{-5}

TABLE 3.2. Medium for growth of Trichoderma, Aspergillus, and mixed culture. All values are in grams/liter.

ASSAY	SUBSTRATE	ENZYME MEASURED	PRODUCT MEASURED	REF.
FILTER PAPER	FILTER PAPER	TOTAL CELLULASE	REDUCING SUGAR	a
β-GLUCOSIDASE	CELLOBIOSE	β-GLUCOSIDASE	GLUCOSE	b
CMCase	CMC	ENDOGLUCANASE	REDUCING SUGAR	a
AVICELase	AVICEL	EXOGLUCANASE	REDUCING SUGAR	a

TABLE 3.3. Summary of cellulase assays. References: a. (Mandels et al., 1976); b. (Tangnu et al., 1981).

3.8. Presentation of data and error analysis

The experimental data presented in the work can be divided into three main catagories:

i. Results from growth experiments

These results include the following data: pH, enzyme activity, biomass concentration, substrate concentration, extracellular protein concentration.

ii. Results from surfactant elution studies

iii. Results from enzymatic hydrolyses in which no actual growth occurs. In these cases, the data presented are: total sugar concentration, glucose concentration or percent hydrolysis (weight total sugar / weight cellulose originally added).

Each group will be discussed in some detail.

i. Data from growth experiments

Shake flask-scale growth studies were performed in triplicate on at least two separate occasions. Larger-scale fermentations (5 and 10 liter) were performed in duplicate only.

A sample of the data from a shake flask-scale growth experiment is given in Table 3.4. The mean and standard deviation for these data were calculated according to the following formulae:

$$\text{Mean} = \bar{y} = \frac{\sum_{i=1}^{N} y_i}{N} \qquad i = 1,2,3,\ldots N$$

$$\text{Standard deviation} = \left[\frac{\sum y_i^2}{N} - \frac{(\sum y_i)^2}{N^2} \times \frac{N}{N-1} \right]^{\frac{1}{2}}$$

These data can be used for two purposes: to assess quantitatively whether there is an acceptable level of uncertainty (variability) in the data; and to determine whether there is a "real" effect present i.e. whether two means are significantly different from each other.

Two mean values were judged to be significantly different from each other if their values plus or minus one standard deviation, did not "overlap".

It is possible to say that growth of the mixed culture using starch as a carbon source produces less filter paper activity than using cellulose or starch plus cellulose (Table 3.4). However, growth using starch plus cellulose cannot be said to be better than cellulose alone for the production of filter paper activity. Similarly, for the production of filter paper activity, Trichoderma alone can not be said to be better than the mixed culture grown on starch plus cellulose. This type of semi-quantitative analysis of data is often adequate to draw qualitative conclusions for complex biological systems.

ii. Results from surfactant elution studies

In studies to determine the effect of surfactants on elution of cellulase from solka floc, there was a considerable degree of variability between values observed in replicate trials. For example, in four trials using Pluronic L61 surfactant, the values for percent of filter paper activity

eluted were: 27.8, 27.7, 32.2 and 17.6. The standard deviation for these values is 6.18. Applying the same analysis as described in section i. above, it is possible to conclude that Pluronic L61 improves the recovery of filter paper activity over that achieved with water or buffers (Table 4.8, page 98). However, because of the high degree of variability in the data, it is not possible to conclude that any one of the surfactants tested is superior to another. Although the data in Table 4.8 are presented in a quantitative form, they again, must be interpreted in a semi-quantitiative manner. The conclusions drawn from this work are not qualified by the variability in the data.

iii. Results from enzymatic hydrolysis

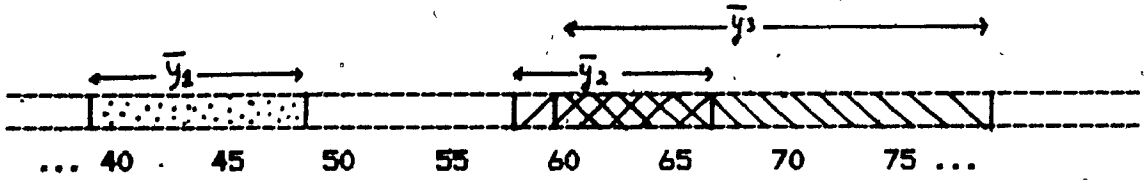
Enzymatic hydrolysis of cellulose was carried out in a buffered system containing crude cellulase (catalyst) and solka floc or steam exploded aspen wood (substrate). It has been shown that the hydrolysis reaction, under defined conditions, can be modelled and fitted with a smooth curve (Ryu and Mandels, 1980). For this reason, the data obtained in hydrolysis experiments have been illustrated in Chapter 4 using a smooth curve. Lack of fit to the curve indicates the degree of random error in the analysis.

Sample hydrolysis data are given below. The same type of analysis can be applied to these data as was described in the preceeding sections i. and ii. For example:

Mean value 1 (From Table 3.5) = 44 ($\pm$ 4.7)

Mean value 2 = 62 ($\pm$ 4.2)

Mean value 3 = 69 ($\pm$ 9.4)



The value for Mean 1 is significantly different from the other two mean values, since its Mean plus standard deviation (48.7) does not bring it up to the value of the other Means minus their respective standard deviations (57.8 and 59.6 respectively). Mean 2 and Mean 3 however, cannot be judged to be significantly different from each other, since Mean 2 plus its standard deviation ($62 \pm 4.2 = 66.2$) is greater than (overlaps with) the value for Mean 3 minus its standard deviation (59.6).

In all cases the calculated standard deviations for this type of experiment were small when compared to the mean values. For this reason there is judged to be an acceptible level of variability in the data. Since all hydrolyses were carried out in the same manner, the degree of uncertainity is likely to be of similar magnitude in each case.

Cellulose concn. (g/l)	Starch concn. (g/l)	Inoculum	FPA (mean) (IU/ml)	Range (Mean - SD) (IU/ml)	Range (Mean + SD) (IU/ml)
0	10	Mixed	1.5	1.36	1.64
10	0	Mixed	2.8	2.6	3.0
10	10	Mixed	3.0	2.72	3.28
10	0	*T. reesei*	3.5	3.02	3.92

Table 3.4. Sample data for enzyme production by mixed culture grown on different carbon sources. (SD = standard deviation).

Enzyme loading ratio (IU FPA/g Solka Floc)	Final sugar concentration produced (grams/liter)
10	44 (± 4.7)
20	62 (± 4.2)
30	69 (± 9.4)

Table 3.5. Sample data for enzymatic hydrolysis of cellulose.

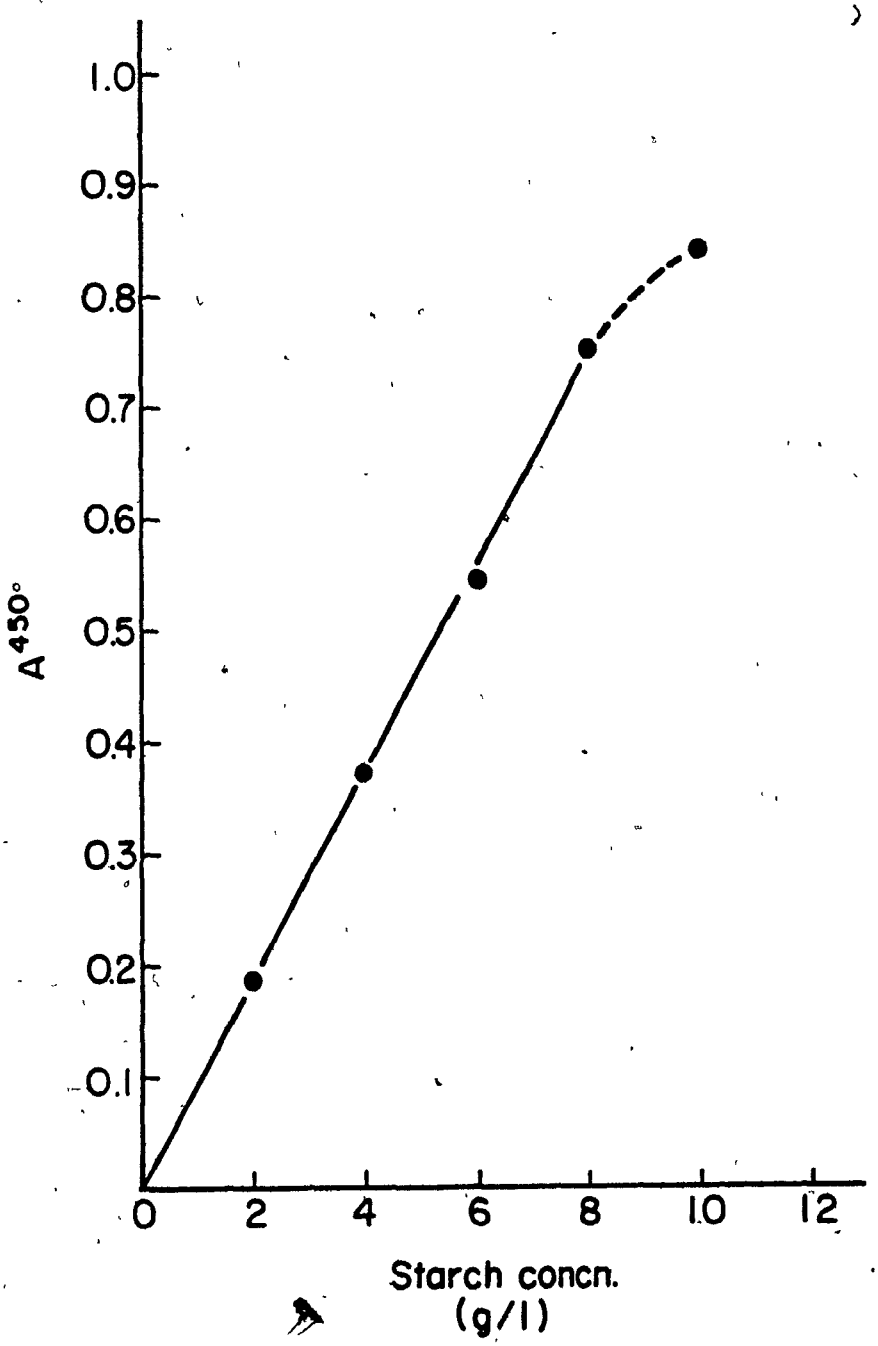


Figure 3.1. Typical standard curve for amyloglucosidase-based starch assay.

4. RESULTS AND DISCUSSION

4.1. Effect of Addition of Colloidal Materials on Cellulase Production by *Trichoderma reesei* Rut C30

4.1.1. Effect of carbon source on enzyme production by *Trichoderma*

The final concentration of cellulase produced by *Trichoderma reesei* Rut C30 was much lower when lactose was used as a carbon source than when cellulose was used. Typical cellulase activities were 1.5 IU/ml for lactose-grown cultures and 2.5 IU/ml for cellulose-grown cultures (Table 4.1). The specific activity (IU FPA/gram soluble protein) of the cellulase produced by *Trichoderma reesei* Rut C30 did not change when the carbon source was changed from cellulose to lactose. In both types of fermentation the specific activity of the cellulase produced was approximately 1 IU filter paper activity per gram of soluble extracellular protein. The maximum value for extracellular protein and for cellulase activity was reached more rapidly (2-4 days) in cultures grown on lactose than in those grown with cellulose (8-10 days) as a carbon source. The overall productivity of the two fermentations were comparable because of the compressed time scale of the lactose-based fermentations.

4.1.2. Effect of colloid addition on cellulase production by *Trichoderma*

Addition of Alon (5 g/l) to growth media increased the final cellulase concentration by up to two fold for cellulose- and lactose-grown cultures (Table 4.1). Higher concentrations of lactose resulted in only slightly higher enzyme concentrations. The cellulase production profile for

lactose-Alon fermentations was similar to cellulose-based fermentations (Figure 4.1). It should be noted that solid lines have been inserted in the figure to facilitate interpretation of the data, and are not the result of any curve fitting or theoretical interpretation. Other positively-charged colloidal materials such as Wesol-P, and trivalent salts of aluminum and iron produced an effect similar to Alon but slightly smaller in magnitude (Figure 4.2, Table 4.4). The aluminum potassium sulphate and ferric chloride formed extensive precipitates when added to flasks containing the Trichoderma growth medium. These salts are well known to form positively-charged colloids in aqueous solutions (Cotton and Wilkinson, 1972). Manganese sulphate, a metal salt which does not form colloidal particles in aqueous solutions, did not result in an increase in the concentration of cellulase produced. The addition of uncharged silica-based colloids (Cabosil M5, MS7 and EH5) did not cause an increase in the amount of cellulase produced. The effect was dependent upon the addition of positively charged particles of colloidal dimensions.

The concentration of colloidal materials which was optimum for cellulase production varied depending upon which type of colloid was used. Addition of Alon in concentrations up to 5 g/l resulted in a linear increase in cellulase production. Further increases in the concentration of Alon resulted in a slight decrease in the amount of enzyme produced. The optimum concentration of Wesol-P was 3 g/l, while the iron and aluminum salts had their greatest effect when added in concentrations between 0.5 and 1.5 g/l.

The colloid-dependant variation in optimum concentration which was observed

Carbon source	Carbon source concn. (g/l)	Alon concn. (g/l)	Maximum FPA (IU/ml)	Maximum protein concn. (g/l)
Lactose	10	0	1.5	1.6
	10	5	3.0	2.7
Cellulose	10	0	2.5	2.6
	10	5	4.5	3.7
	20	0	5.9	5.0
	20	5	7.6	7.0
	30	0	7.1	6.1
	30	5	9.5	9.0

Table 4.1. Effect of carbon source concentration and colloid addition on enzyme production.

can be explained in terms of the colloid characteristics. Alon and Wesol-P are "stable" colloids. Although they may become associated with some components of the medium, their particulate nature is not modified by hydration when placed in aqueous solutions. The differences in optimum concentration between Alon and Wesol-P can be explained by differences in their specific gravity (3.6 for Alon, 1.2 for Wesol-P), surface area (100 m^2/g for Alon, 220 m^2/g for Wesol-P), and charge density. Little information is available concerning the magnitude of the positive charge which is present on commercial colloids.

The relatively low (0.5-1.5 g/l.) optimum concentration of the aluminum and iron salts reflects the high degree of hydration which they undergo in aqueous solutions. The colloidal species which are formed are dictated by the following three equilibria (Cotton and Wilkinson, 1972):

$$Fe(H_2O)_6^{3+} = Fe(H_2O)_5(OH)^{2+} + H^+ \qquad K = 10^{-3.05}$$

$$Fe(H_2O)_5(OH)^{2+} = Fe(H_2O)_4(OH)_2^{+} + H^+ \qquad K = 10^{-6.31}$$

$$2\,Fe(H_2O)_6^{3+} = Fe(H_2O)_4(OH)_2Fe(H_2O)_4^{4+} + 2H^+ \qquad K = 10^{-2.91}$$

Which species predominates depends upon the pH of the system. At approximately pH 3, colloidal gels are formed and ultimately hydrous ferric oxide is precipitated as a red-brown gelatinous mass. Thus the surface area and actual mass of colloidal material formed are not directly dependent upon the amount of salt originally added to the solution.

Increases in the colloid concentration above the optimum values resulted in slight decreases in the concentration of cellulase produced. Increases in viscosity associated with high concentrations of colloid may be responsible

for the decrease in cellulase produced. Problems with mixing, oxygen and other nutrient transfer asociated with high solids fermentations have been documented (Mandels and Andreotti, 1978).

4.1.3. Mechanism of the colloid effect

The mechanism by which the positively-charged particles exert their effect appears to be related to their ability to bind sugars such as lactose. When added to an aqueous solution of 10 g/l. lactose, a concentration of 5 g/l. Alon bound ca. 15% of the lactose from a 10 g/l solution (Table 4.2). The addition of phosphate (KH_2PO_4, 2 g/l) increased the amount of lactose bound to 30% of the total. Approximately one-third of the phosphate in solution was removed with the lactose. The addition of metal salts normally supplied as trace elements in the growth medium appeared to have a negative effect on the binding capacity of the colloid. Individually, or when added in combination, the salts reduced the binding to 15% of the lactose bound in the presence of phosphate, and eliminated binding in the absence of phosphate.

Transition metal ions such as iron (III) are also known to have a high affinity for ligands which coordinate through oxygen, such as phosphate ions and sugars (Cotton and Wilkinson, 1972). In Trichoderma reesei growth media, then, there is an equilibrium established between "free" lactose and lactose which is associated with the positively charged colloid through a phosphate ligand. During the initial growth phase, the "free" lactose is used by the organism. As it becomes depleted, the equilibrium shifts towards the "free" lactose, resulting in a slow release of colloid-associated lactose. The result is that after the initial growth phase is

complete, there is a constant low concentration of soluble carbon available to the organism. Improvements in enzyme titers achieved by this reduction in the effective carbon source concentration are mechanistically similar to those recently achieved using fed batch fermentations.

Early work on the production of cellulases showed that it was environmental stress, such as a depleted carbon source that triggered the induction of cellulase enzymes (Mandels and Reese, 1957). In soluble carbon source-based systems, growth of the microorganism is not dependent upon hydrolysis of the degradation-resistant polymer cellulose. The high concentration of available carbon source initially present, results in a rapid initial growth phase. In batch fermentations, the carbon source concentration is rapidly reduced to low levels, and the enzyme-making mechanism of the cell is turned on. The lack of an available carbon source to supply the organism with maintenance energy after this initial growth period, results in a rapid decline in the microbial population, and a relatively low final concentration of cellulase produced.

In fed batch fermentations, a rapid initial growth phase is desirable to increase the biomass concentration in the fermenter. In this system, the initial growth period is a batch fermentation and the concentration of carbon source is again depleted. This induces enzyme synthesis by Trichoderma. However, in the fed batch fermentation, the induction phase which follows the initial growth phase, is prolonged through the addition of low levels of utilizable carbon source. In this way, maintenance energy is supplied to the biomass, to enable it to continue synthesizing cellulase even though it is in a stationary growth phase. It is this type of situation which exists in batch fermentations in which colloidal materials

have been added to the medium. The initial rapid growth phase is supported by the "free" lactose present. Subsequently, a supply of carbon source made available by the controlled release of "bound" lactose, supplies the biomass with the maintenance energy needed to prolong the induction phase.

4.1.4. Colloid effect: Second mechanism

It should be noted that the relative increase in enzyme titers observed when colloid was added to cellulose-based fermentations was smaller than that observed for lactose-based media. In Trichoderma fermentations, the growth rate of the organism is dependent upon the hydrolysis rate of the insoluble substrate. As cellulose is solublized, the sugars produced are utilized. The net result is that for cellulose-grown Trichoderma cultures, very low levels of free sugars are present in a growing culture (< 0.2 g/l). The strong effect which Alon had on cellulase production in this system was not likely due to a sugar-colloid interaction, as was the case with the lactose-based fermentation. Rather, there exists a second mechanism by which colloids enhance enzyme production. Although the nature of the interaction is not clear, the effect is not limited to this system.

Microbial cells are usually found to be negatively-charged in aqueous systems. Thus, there is expected to be an association between the cells and the positively-charged colloidal particles. Because only the positively-charged colloids, Alon, Wesol-P, as well as the iron and aluminum hydrates, enhanced enzyme production, it is likely that the positive charge is necessary to bring about the close association which was observed to exist between the mycelium and the colloidal particles. It is

also possible that the presence of the colloids at the cell wall aids in the release of enzyme, thus lowering the internal concentration, and favouring an increase in enzyme synthesis.

The addition of colloidal materials to cellulose-based fermentations resulted in enzyme titers which are normally only achieved with higher concentrations of cellulose. In batch cultures with cellulose (30 g/l) and Alon (5 g/l), FPA values of 9-10 IU/ml were routinely observed. Maximum values of greater than 13 IU/ml were recorded in several batches. These results compared favourably with the results of other workers (Table 4.3). Both the productivity (60 IU/l/h) and the yield (433 IU FPA/g cellulose) of the fermentations with Alon incorporated into the growth medium, were improved over those reported by other workers for batch fermentations with Trichoderma reesei Rut C30 (Hendy et al., 1982, 1984).

Although fed batch fermentations have resulted in the highest yield and productivity of cellulase reported to date (Watson et al., 1984), continuous or semicontinuous fermentations have not gained widespread acceptance in the fermentation industry. Problems with contamination and feed handling which are associated with continuous processes are the main reasons for the continued use of batch fermentation. In this work, the yield of cellulase per gram of cellulose utilized, was approximately doubled over those reported for fed-batch work. The productivity of the colloid batch fermentations, however remains lower than the fed-batch values by a factor of approximately 3. If improved productivities can be achieved using this system, conventional batch fermentations, supplemented with colloid additions, may become more attractive than the fed batch systems.

4.1.5. Economics of enzyme production

The cost of cellulase enzyme production is the major expense in an process for the enzymatic conversion of cellulose to ethanol. The cost breakdown is approximately as follows (Ryu and Mandels, 1980):

	Unit cost (cents/ gal 95% ETOH)	Percent cost
Enzyme production	57.3	43.4
Pretreatment	30.38	23.0
Hydrolysis	13.03	10.0
Ethanol production	31.07	23.6

By improving the yield and productivity of cellulase production, the cost of the overall process can be reduced significantly, making the enzymatic degradation of cellulose more competitive with other technologies. In Section 4.2, the hydrolytic potential of Trichoderma cellulase will be examined.

Colloid	Additive (g/l)	% Lactose bound
5.0 g/l Alon	None	15
	KH_2PO_4 (2)	33
	KH_2PO_4 (4)	33
	$FeSO_4$ (5 x 10-3)	0
	$MnSO_4$ (1.6 x 10-3)	0
	$ZnSO_4$ (1.4 x 10-3)	0
	$CoCl_2$ (2.0 x 10-3)	0
10 g/l Alon	KH_2PO_4 (2)	6.3
	KH_2PO_4 (4)	5.7
1.0 g/l Alon	KH_2PO_4 (2)	40
	KH_2PO_4 (4)	37
5.0 g/l Cab-o-sil M5	None	0
	KH_2PO_4 (2)	0
5.0 g/l $FeCl_3$	KH_2PO_4 (2)	15
None	KH_2PO_4 (2)	0

Table 4.2. Effect of metal salts and phosphate on lactose-Alon binding

Mutant	Carbon source	Carbon source concn.	Maximum FPA (IU/ml)	Reference
Rut C30	Solka Floc	20	4.2	
		50	8.0	Hendy et al.
		75	8.4	1984
		100	8.0	
Rut C30	Hardwood pulp	50	12	Watson and Nelligan 1983
Rut C30	Solka Floc	50	14.4	Tangnu et al., 1981
CL847	Lactose	60	5	Warzywoda et al.,
	CC 41 Cellulose	50	13.7	1983
	+Wheat bran	20		
	Lactose + cotton	60	14.1	
	pulp	5.0		
Rut C30	Cellulose CF11	30	13.2	Duff et al., 1985a

Table 4.3. Comparison of published cellulase yields using Trichoderma reesei

Salt	Salt concn (g/liter)	% Increase in FPA	% Increase in soluble protein concn
$ALK(SO_4)_2$	0.5	11	23
	1.5	13	18
$FeCl_3$	0.5	23	17
	1.5	19	22
$Fe_2(SO_4)_3$	0.5	9	11
	1.5	1	8
$MnSO_4$	0.5	0	0
	1.5	1	3

Table 4.4. Effect of the addition of metal salts to citrate-buffered *Trichoderma reesei* growth medium.

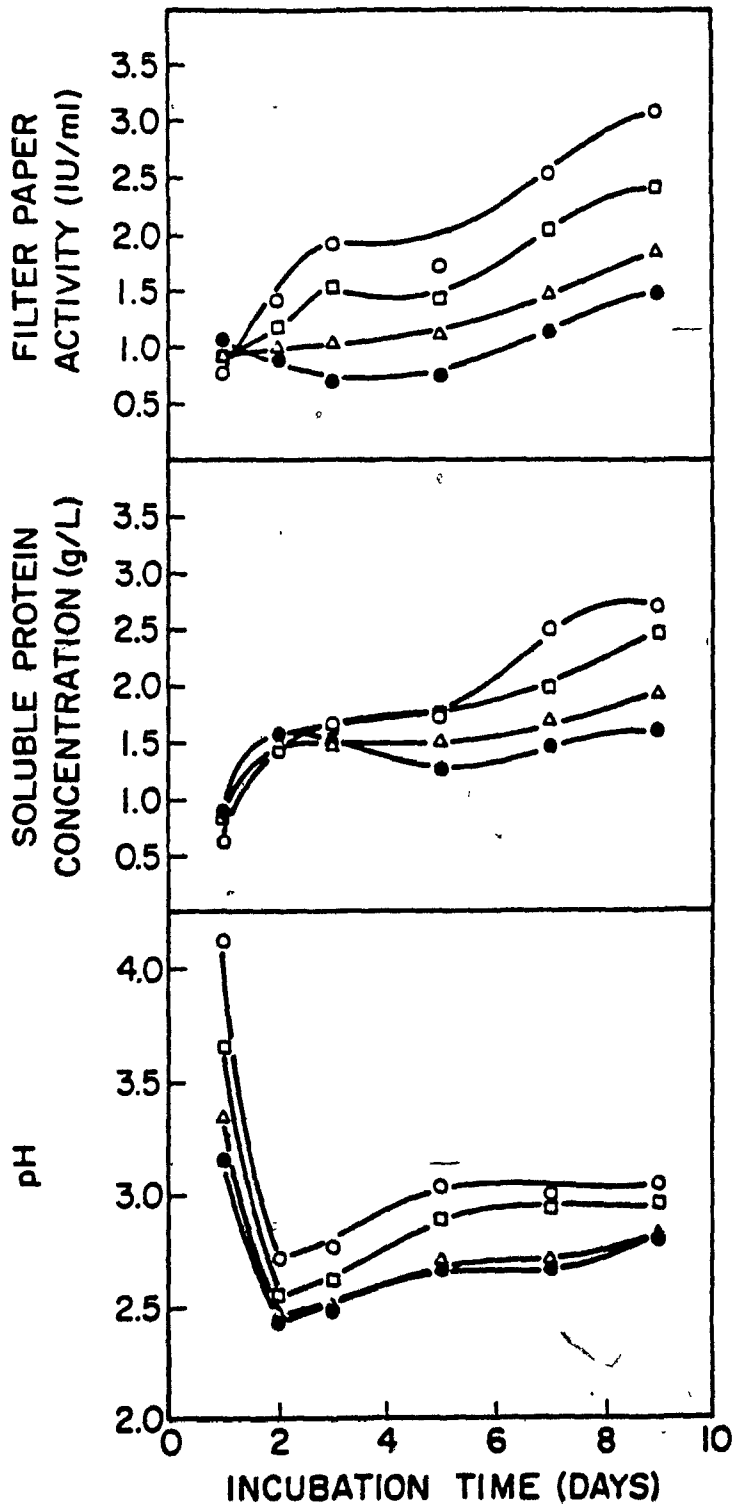


Figure 4.1. Effect of Alon addition on pH, extracellular protein concentration, and filter paper activity during growth of *T. reesei* Rut C30 in shake flasks containing 10 g/l lactose. Symbols: ○ 5 g/l Alon, □ 3 g/l Alon, △ 1 g/l Alon, ● Control.

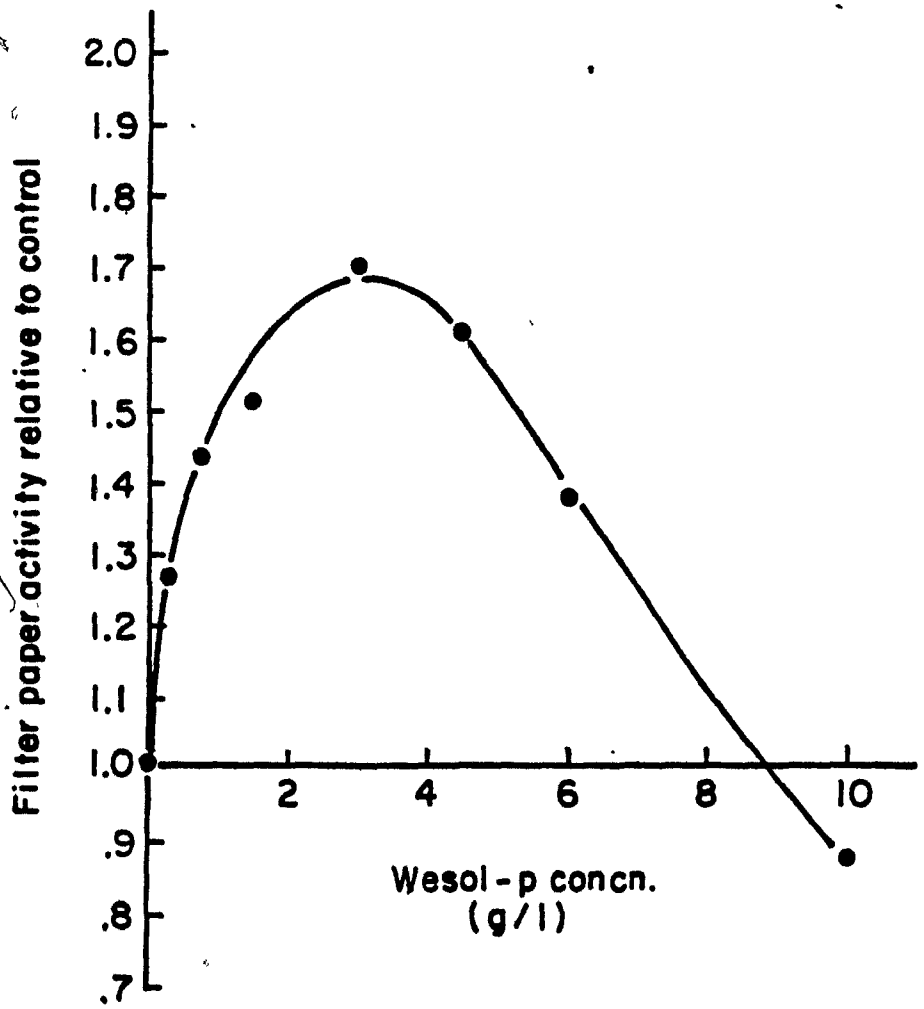


Figure 4.2. Effect of Wesol-P concentration on cellulase activity produced by shake flask cultures of *T. reesei*.

4.2. Application of Trichoderma cellulase to hydrolysis of Solka Floc

4.2.1 Effect of enzyme loading ratio on hydrolysis of cellulose by Trichoderma cellulase

The enzyme loading ratio for a given hydrolysis reaction is defined as the enzyme activity (filter paper activity, carboxymethylcellulase activity etc.) used per gram of substrate in the hydrolysis reaction. It has been reported by a number of researchers that the initial rate of hydrolysis is linearly dependant on the enzyme loading ratio applied. Lee and Fan (1982) found that the extent of soluble protein adsorption onto the substrate, as well as the initial rate of hydrolysis of the substrate, increased proportionately to the enzyme concentration, as the enzyme concentration was increased. The increase in initial hydrolysis rate slowed substantially when the initial enzyme concentration exceeded approximately 1 g/l. They postulated that this decrease was due to adsorption onto inactive sites, or to the formation of multiple layers of enzyme on the cellulose surface. Since only the enzyme adsorbed in the first layer is able to play a part in the hydrolysis, this is equivalent to surface area limitation.

The initial rate of hydrolysis is strongly affected by the adsorption of cellulase on the cellulosic substrate. This in turn is strongly dependant on the major structural feature of the cellulose such as specific surface area, and crystallinity index, as well as on enzyme and substrate concentration.

Initial hydrolysis rates have little bearing on practical hydrolysis systems. A typical sugar concentration versus time curve is shown in Figure 4.3. The rate of production of cellobiose and glucose falls off dramatically over extended hydrolysis times. This is due to a variety of factors including:

1. inactivation of enzymes
2. the higher crystallinity and lower specific surface area of the residual cellulose
3. end-product inhibition

All of these factors combine to make the analysis of enzymatic hydrolysis difficult. A mechanistic kinetic model has been developed which includes a variety of factors including: the effect of cellulose structure, the mode of action of cellulase, and the mode of interaction between cellulose and enzyme molecules (Fan and Lee, 1983). The data obtained in this work for the effect of loading ratio on enzymatic hydrolysis of cellulose (Table 4.5), do not correspond well with the data given in the aforementioned study (Fan and Lee, 1983). There are a number of reasons by which the discrepancy may be accounted for:

1. the organism used to produce the cellulase was different
2. the substrate used was different and in our work there was no attempt to obtain a standard particle size
3. the method of pretreatment of the substrate was different

The applicability of a given mechanistic model only to a well defined set of hydrolysis conditions question the utility of such modelling based on conditions other than those used in a production-scale reactor.

In this study, the final (72 hr.) concentration of sugar increased with increasing concentration of enzyme (Table 4.5). The overall productivity of the hydrolyses (enyme activity units produced per liter per hour)

increased from 0.6 $gl^{-1}h^{-1}$ at 10 IU FPA/g Solka Floc, to 1 $gl^{-1}h^{-1}$ at 50 IU FPA/g Solka Floc. However, the benefit to each subsequent increment in enzyme loading diminished. Thus the yield per unit enzyme added decreased from 62 g sugar/gram of enzyme added, to 18 g sugar/gram enzyme added. Since the cost of enzyme production is a major cost in the process, the yield is an important consideration.

One of the major factors which caused the diminishing yields at higher enzyme loading was inhibition of the enzyme activites by accumulated end products. The effectiveness of glucose and cellobiose as inhibitors of cellulase activity has been well documented (Ryu and Mandels, 1980). Cellobiose is a much more potent inhibitor than is glucose. Because beta-glucosidase is the enzyme which is responsible for this conversion, an active beta-glucosidase component in a cellulase preparation benefits both the yield and the productivity of the hydrolysis reaction.

Using Trichoderma cellulase, as the enzyme loading ratio increased there is a rapid increase in the initial rate at which the hydrolysis proceeded. Cellobiose rapidly accumulated and was able to reach inhibitory levels very early in the hydrolysis. The rate of conversion of cellobiose to glucose lagged behind because of the deficiency in beta-glucosidase activity. At high enzyme loading ratios, the actual amount of beta-glucosidase present increased, and the conversion of cellobiose to glucose was improved over that achieved at low loading ratios (Figure 4.4).

4.2.2. Effect of beta-glucosidase supplementation on hydrolysis reactions using Trichoderma cellulase

The addition of supplemental beta-glucosidase reduces end product inhibition by improving the conversion of cellobiose to glucose. The result is an increase in the final concentration of sugar produced in the hydrolysis. As described in section 4.2.2, the conversion of cellobiose to glucose by Trichoderma cellulase is dependent upon the enzyme loading ratio. By supplementing the Trichoderma cellulase with beta-glucosidase, the conversion efficiency of cellobiose to glucose becomes independant of the loading ratio (Figure 4.4). As a result, the productivity of hydrolyses supplemented with beta-glucosidase improves significantly over those with no added beta-glucosidase (Table 4.5).

The optimum concentration of beta-glucosidase is dependant on the type of substrate to be degraded, the conditions under which the hydrolysis is carried out, and the source of the enzyme preparations. Beta-glucosidase from different sources showed varying degrees of synergy with cellulase from Trichoderma (Sternberg et al, 1977). Beta-glucosidase from almonds was able to improve the conversion of cellobiose to glucose (as indicated by the glucose/reducing sugar ratio) up to a ratio of 2.5 IU beta-glucosidase: 1 IU filter paper activity (Figure 4.5). However a similar degree of conversion can be achieved using a much smaller amount of beta-glucosidase from Aspergillus phoenicis. Other workers have indicated that beta-glucosidase from other "black" Aspergillus species show a high degree of synergy with Trichoderma celluase (Enari et al., 1980; Scrivastava et al., 1981).

The value of improved cellulase production by Trichoderma reesei is limited, to some extent, by the fact that the enzyme produced by Trichoderma is deficient in beta-glucosidase. As described in section 2.4.2, this results in an accumulation of cellobiose during hydrolysis and a decrease in the hydrolytic efficiency of the cellulase. In an attempt to increase the beta-glucosidase concentration of the cellulase, mixed cultivation of Trichoderma reesei Rut C30 and Aspergillus phoenicis was attempted, and the results are described in Chapter 4.3.

4.2.3. Effect of surface active agents upon hydrolysis of Solka Floc by Trichoderma cellulase.

Surfactants can be classified according to a variety of characteristics including:

1. solubility in water and/or organic solvents
2. wetting capacity
3. ability to emulsify or demulsify
4. foaming capacity
5. gelation characteristics
6. viscosity
7. toxicity, odour, taste

In general, surfactants possess a hydrophobic and a hydrophilic moeity within the same molecule. They tend to align themselves at phase interfaces. By doing so, they are able to modify the surface characteristics of one or both of the phases. For example, in aqueous solutions, surfactants tend to accumulate at the interface between the

gas phase (air) and the bulk liquid phase. The hydrophilic part of the molecule orients into the aqueous phase while the hydrophobic (lipophilic) portion of the molecule is aligned at the surface. The result is a decrease in free energy at the surface and a reduction in surface tension. At a solid-liquid interface such as exists in the cellulose hydrolysis system, the accumulation at the surface can result in an improved wetting of the substrate. This can affect the enzyme-substrate interaction either positively or negatively, depending on the characteristics of a particular system.

It has been previously reported that the addition of Tween 80 (polyoxyethylene (220) sorbitan monooleate) to hydrolysis flasks resulted in an improvement in the efficiency of hydrolysis (Castanon and Wilke, 1981). In the cellulose hydrolysis systems, the hydrolysis mixture consists of an insoluble substrate immersed in a liquid solution. The adsorption of the cellulase is critical for the catalytic reaction to occur. It would be expected that the addition of surfactant molecules, which orient at solid-liquid interfaces, would affect the hydrolysis reaction. A variety of sufactants were, however, able to improve the hydrolysis to the same extent that Tween 80 did. These surfactants are:

Pluronic L 10, L 61 (polyoxypropylene-polyoxyethylene copolymers)

Atlas G-263 (N-cetyl-N-ethyl morpholinium ethosulphate)

Atlas G-3300 (alkyl aryl sulphonate)

Atlas G-3634A (quaternary ammonium compound)

A summary of the characteristics of these surfactants is given in Table 4.6. Although the surfacts tested varied greatly in wetting ability, molecular weight, and chemical characteristics, all except Atlas G-3300 improved the extent of hyrolysis by an amount equal to that produced by

Tween 80 (Figure 4.6). The negative effect of Atlas G3300 was due to a inactivation by that surfactant of the cellulase.

This type of general surfactant effect is unusual. It is possible that the accumulation of any surfactant at the solid-liquid interface improves the interaction between the enzyme and substrate. The surfactant may "improve" the interaction by simply decreasing the strength with which the enzyme is bound to cellulose, and thus improving the catalytic reaction by releasing active cellulase for another reaction.

To investigate further, Tween 80 was used as a model surfactant. The effect of the addition of Tween was examined on hydrolyses run at three different degrees of agitation. The results are shown in Table 4.7. The flasks which contained Tween 80 had more filter paper activity in the broth than the control flasks. The protein concentration in the liquid phase was also higher in the Tween 80 flasks, but not in proportion to the increased filter paper activity. Thus, the protein which was present in the liquid phase of the flasks which contained Tween 80 had a higher specific activity (IU enzyme activity/gram protein) than did the control flasks.

In the enzymatic hydrolysis system, there exists an equilibrium between bound and "free" cellulase. The presence of the Tween 80 shifted the equilibrium toward "free" cellulase, as evidenced by the higher filter paper activity and higher protein in the aqueous phase of those cultures. This was in agreement with the results of Castanon and Wilke (1982). What is possibly more important, is the enhanced specific activity of the cellulase which was present in the aqueous phase of the Tween 80

hydrolyses. This indicated that either Tween 80 is able to facilitate the release of active cellulase from the cellulose, or that it protects the activity of the cellulase from shear or interfacial denaturation when it is in the "free" state. There is some evidence to support both assertions (Castanon and Wilke, 1982; Basu and Pal, 1956; Kim et al., 1982; Reese, 1980). The specific activity of the extracellular protein (cellulase) is lowest in the flasks which were subjected to the highest level of agitation in both Tween 80 as well as control flasks. This indicates that some enzyme denaturation is occurring which is related to the higher agitation rate of these flasks. A mechanism by which surfactants stabilize cellulase has been described. Kim et al., (1982) have postulated that an air-liquid interface exerts a much greater denaturing stress on cellulase protein than does shear stress. They postulated that cellulase which is at the interface becomes unfolded and thereby inactivated. Agitation serves to increase the rate of denaturation, not by increasing the shear stress, but rather by replenishing the interfacial area with fresh, active protein. In static cultures, the proportion of the protein which is at the surface and therefore subject to the denaturing effects, is small in proportion to the total protein. When a culture is well mixed, however, the recharging of the interfacial boundary with bulk liquid, exposes a large proportion of the bulk liquid phase to the denaturing forces. Surface active agents are able to displace the enzyme molecules from the interfacial area. By decreasing the proportion of the protein at the surface, the surfactants are able to decrease the percent inactivation of the enzyme. In our work, hydrolyses with Tween 80 added showed a greater benefit of increased agitation than did the control flasks. Also, the control cultures produced approximately the same extent of hydrolysis when incubated with moderate (50 RPM) or high (150 RPM) agitation. These two facts indicate that there

is, indeed, some degree of interfacial stabilization in cultures with Tween 80 added. In static cultures as well, there is a strong increase in hydrolytic efficiency in flasks which contained Tween 80 as compared to controls without Tween 80. This cannot be explained by shear or interfacial stabilization. It must, rather, be due to an increase in efficiency of the reaction of cellulase with cellulose. By increasing the ease with which the cellulase is able to desorb from the surface of the cellulose, the hydrolytic efficiency is increased. Thus it would appear that there are two modes by which surfactants ehhance the hydrolysis reaction: by stabilizing "free" enzyme with respect to interfacial forces, and by increasing the efficiency of the enzyme-cellulose interaction at the cellulose surface. The fact that the surfactant effect is exhibited by a wide variety of surfactants indicates that the interfacial phenomenon may be more important. Since all surfactants aggregate at the interfaces, the effect, would not be dependent on chemical characteristics of the surfactant. It would be expected that if the effect was based upon a modification of the enzyme-substrate interacton, the chemical characteristics of the surfactant, such as molecular weight, hydrophobic-lipophilic balance, and charge would be important to the effect. As described previously, they did not appear to be important.

4.2.4. Elution of cellulase from cellulose using surfactant solutions

To determine if Tween 80 and other surfactants were able to remove active cellulase from cellulose, elution studies were undertaken. It was found that the surfactants did not significantly increase the amount of soluble protein which was eluted from cellulose. They did, however, protect the

activity of the protein which was released. In flasks with surfactant added, the protein which was released retained more of its activity than in those with no surfactant added. This observation supports the hypothesis that by protecting the activity of cellulase, surfactants are able to increase the efficiency of the hydrolysis reaction.

Enzyme loading ratio (IU FPA/g. S.F.)	Enzyme activity ratio (IU beta-g/IU FPA)	Final Reducing sugar concn. (g/l)	Final glucose concn. (g/l)	G/R
10	0	44	23	.52
	0.75	62	41	.66
20	0	62	35	.56
	0.75	82	56	.68
30	0	69	42	.60
	0.75	92	60	.65
40	0	69	41	.59
	0.75	88	59	.67
50	0	70	46	.65
	0.75	90	61	.67

Table 4.5. Effect of enzyme loading ratio and beta-glucosidase supplementation on hydrolysis of Solka Floc using Trichoderma cellulase.

Surfactant	HLB	Ionic nature	Specific gravity
Tween 80	15	non	NA
Pluronic L10	10	non	1.04
L61	10	non	1.01
Atlas G263	>30	non	1.0
Atlas G3300	117	anionic	1.0
Atlas G3634	NA	cationic	1.1

Table 4.6. Characteristics of surfactants tested. HLB refers to the hydrophilic-lipophilic balance in the surfactant molecule.

Tween 80 concn. (g/l)	Shaking speed (RPM)	Glucose produced (final) (g/l)	Average free FPA in liquid (g/l)	Average protein in liquid (g/l)	Specific activity (IU FPA per g protein)
0	0	39	1.6	0.7	2.3
	50	53	1.9	0.8	2.4
	150	53	1.7	0.9	2.1
1.5	0	47	3.0	1.0	3.0
	50	59	3.0	1.0	3.0
	150	64	2.8	1.1	2.5

Table 4.7. Effect of Tween 80 and shaking speed on hydrolysis of Solka Floc by Trichoderma cellulase.

Eluent	% FPA eluted	% Soluble protein eluted
Water	4	19
Citrate buffer	11	30
Acetate buffer	11	33
Pluronic L 10	28	21
L 35	23	22
L 61	26	40
Tween 80	25	24

Table 4.8. Elution of cellulase activity from Solka Floc using surfactant solutions. All buffers were 0.2 M, pH 4.8; surfactant solutions were 0.2 % w/v.

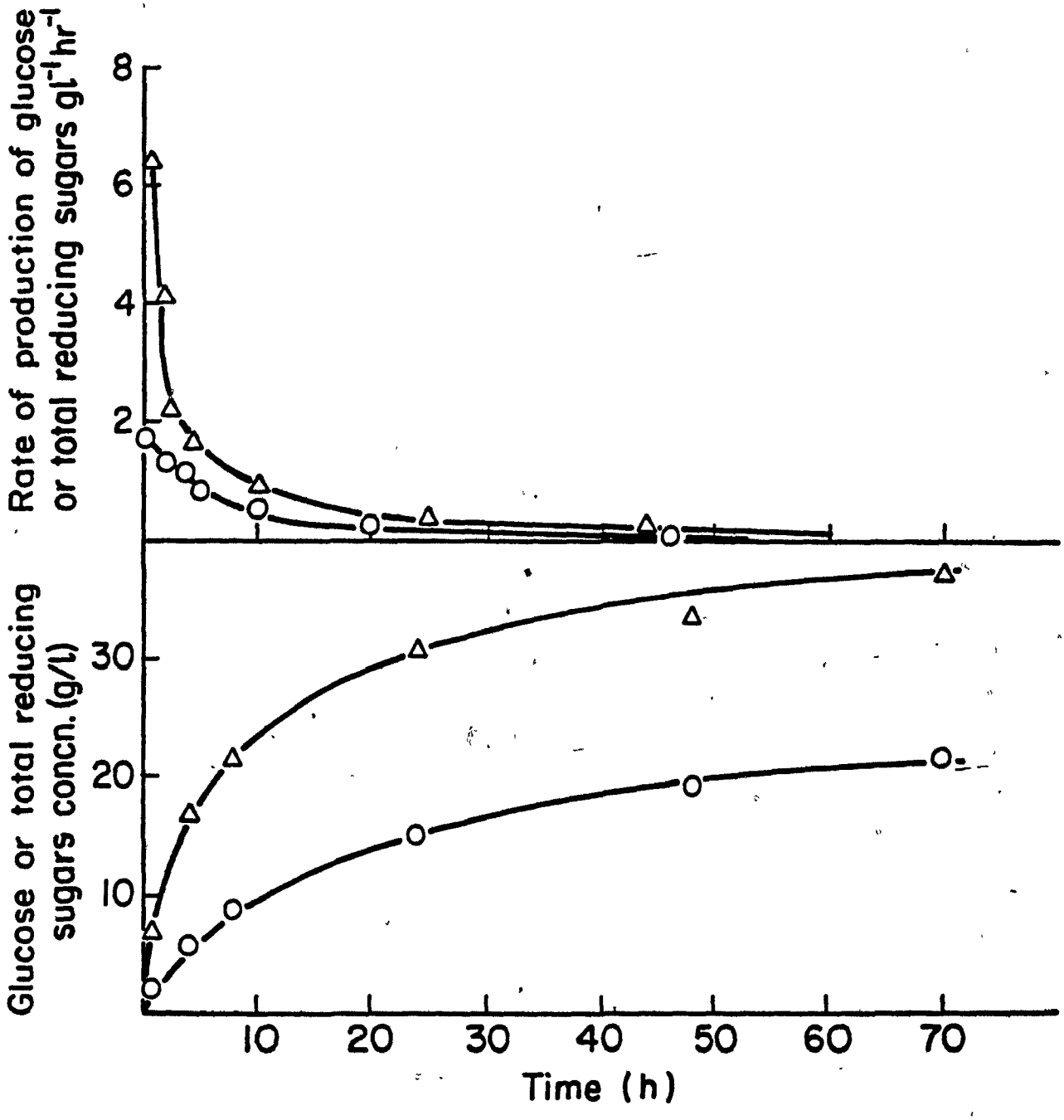


Figure 4.3. A typical hydrolysis curve using Trichoderma cellulase to hydrolyse Solka Floc. Symbols: ○ glucose, △ total reducing sugars.

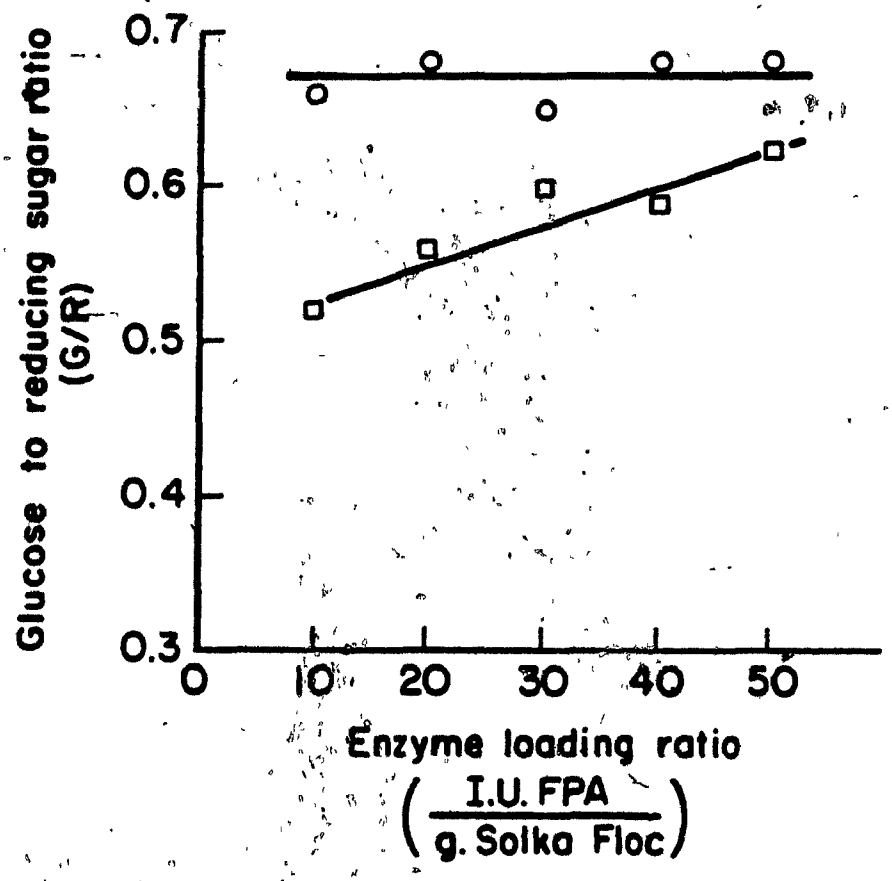


Figure 4.4. Effect of enzyme loading ratio on the conversion of cellobiose to glucose during hydrolysis of Solka Floc by Trichoderma cellulase alone □, and supplemented with beta-glucosidase ○

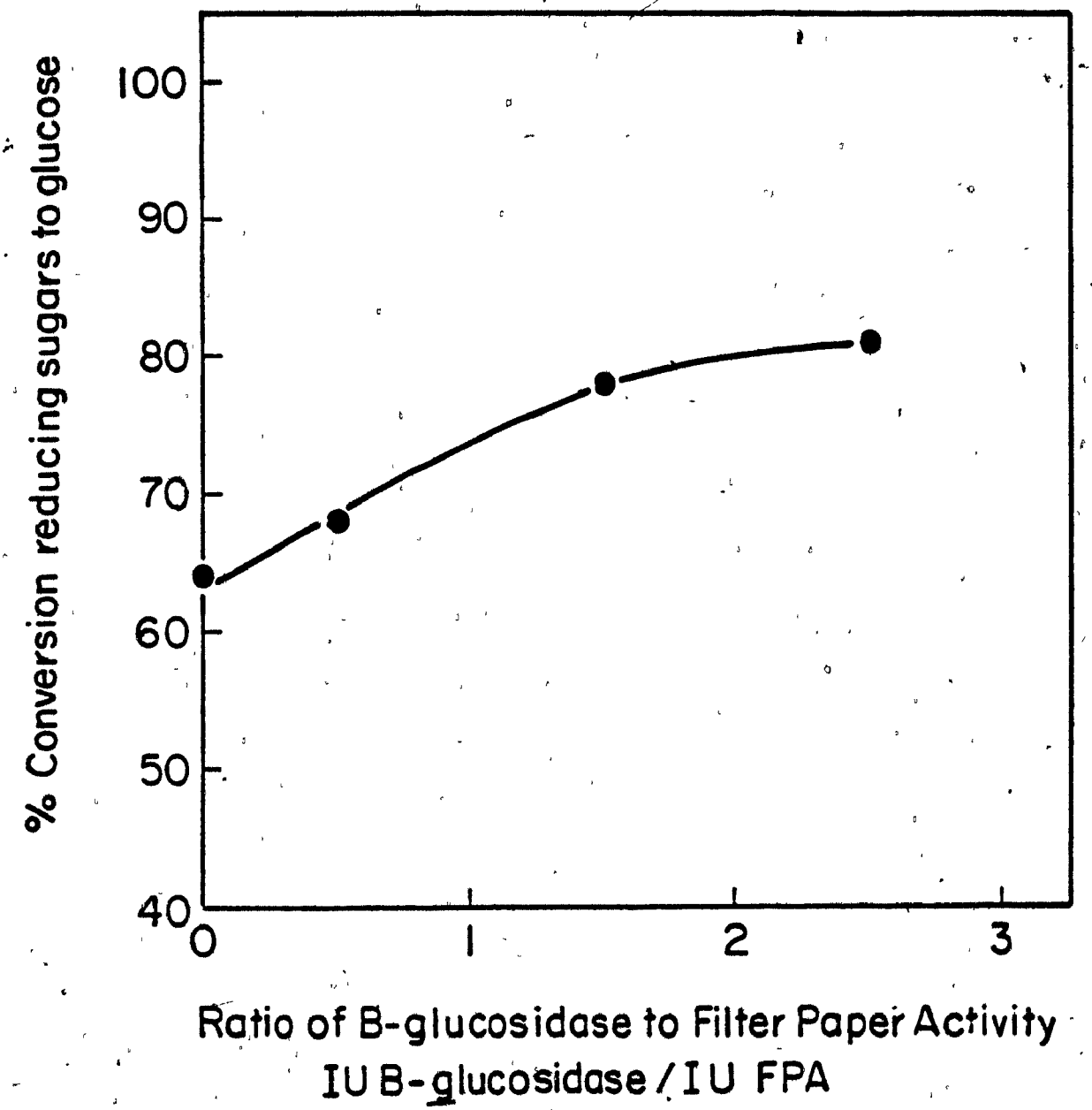


Figure 4.5. Effect of increasing beta-glucosidase:FPA ratio on the conversion of cellobiose to glucose during enzymatic hydrolysis of Solka Floc. Source of the beta-glucosidase was almonds.

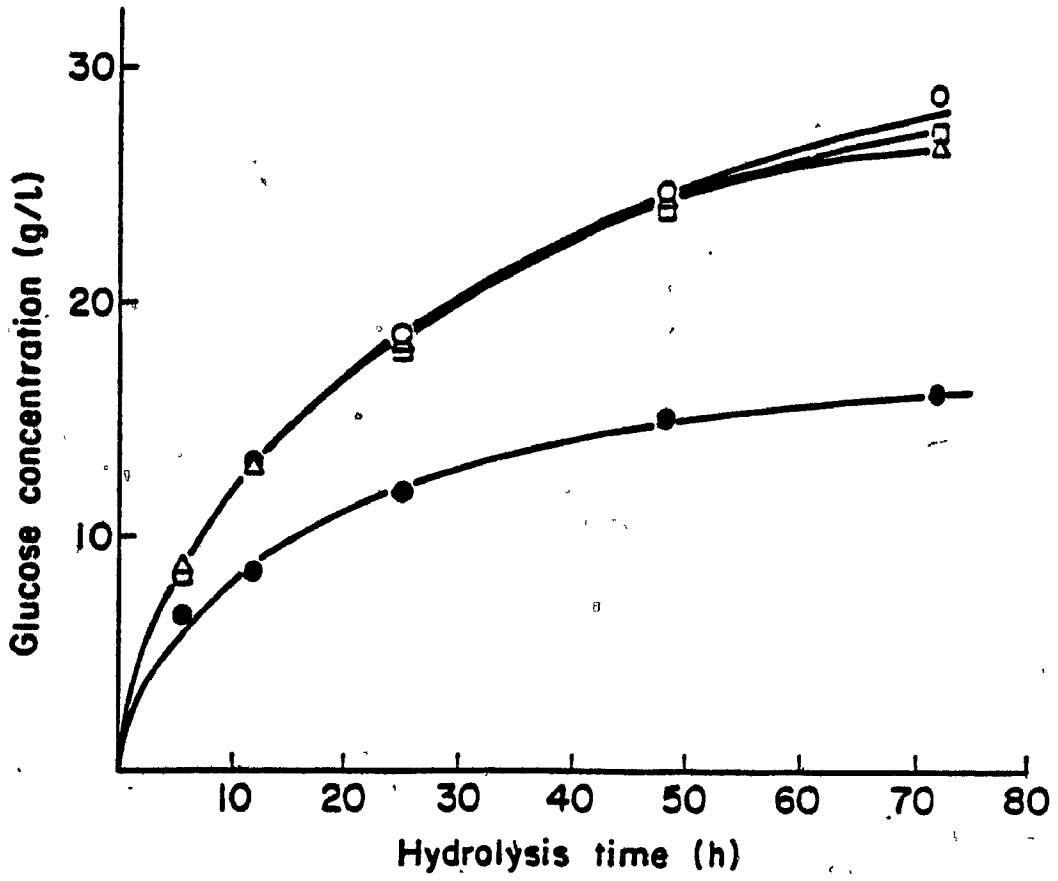


Figure 4.6. Effect of Tween 80 △, Pluronic L10 ○, and Pluronic L61 □ addition on hydrolysis of Solka Floc by *Trichoderma* cellulase. Control flasks ● had no added surfactant.

4.3. Mixed cultivation of T. reesei Rut C30 and Aspergillus phoenicis

4.3.1 Initial studies

The initial attempts to grow Trichoderma reesei and Aspergillus phoenicis in mixed culture used a medium which was essentially a combination of the two media used for growth of the two pure cultures (Table 3.2). Initial indications showed that it was possible to produce a beta-glucosidase-rich cellulase system using mixed cultivation of the two organisms (Table 4.9). This enhanced beta-glucosidase activity translated into an increased hydrolytic potential (Figure 4.7). Further study was undertaken, in an effort to optimize the production of cellulase from the mixed culture.

4.3.2. Optimum beta-glucosidase concentration required for hydrolysis

There exists some disagreement in the literature over the beta-glucosidase (beta-g) to CMCase ratio required for complete hydrolysis of cellulosic substrates (Chahal et al., 1982; Ryu and Mandels, 1980). Most researchers have placed the value of this ratio between 0.5-1.5 IU beta-glucosidase activity/ IU FPA. There are a number of reasons for this variation in reported values.

The filter paper activity of a cellulase preparation is strongly dependent on its beta-glucosidase activity. For this reason, an attempt to find an optimum ratio of beta-glucosidase:FPA ratio is difficult, since the two enzyme activities are not independant of each other.

A second reason is that the activity of a given type of cellulase system is dependent upon the nature of the substrate which is used to evaluate its hydrolytic potential. It has been well documented that a particular cellulosic substrate can show varied susceptibility to degradation by cellulases with different ratios of component activities (Ryu and Mandels, 1980). Because different substrates are used to evaluate the hydrolytic potential of the cellulases, different beta-glucosidase:FPA ratios were found to be optimum.

Finally, the term "optimum" is somewhat ambiguous. In a true sense, the optimum beta-glucosidase:filter paper activity ratio is that which gives the best (fastest) rate of hydrolysis. As will be discussed in section 4.3.4, the initial rate of hydrolysis varies directly with enzyme loading ratio (IU enzyme activity/gram of substrate) up to an enzyme concentration of 1 gram/l. From this it is obvious that as enzyme concentration is increased the time required for complete hydrolysis would be reduced. However enzyme loading ratios are limited in practical systems by the high cost of the enzymes involved. For this reason, the "optimum" beta-glucosidase:filter paper activity ratio is usually qualified by a practical time period over which the hydrolysis is allowed to proceed. The "optimum" ratio, then, must be qualified by expressing the substrate type and concentration, as well as the enzyme loading ratio.

The hydrolysis system which was used to evaluate the hydrolytic potential of the different enzyme preparations was designed to mimic practical hydrolysis conditions. For this reason, Solka Floc was used at a concentration of 100 g/l to ensure that the level of sugar which would result from the hydrolysis would be of the same order of magnitude as that

which would exist in a full-scale process. The system allows for a more "realistic" assessment of the hydrolytic potential of given enzyme preparation.

At a fixed enzyme loading ratio (20 IU carboxymethylcellulase activity/gram Solka Floc), both the initial rate of glucose production and the final (72 hr.) concentration of glucose produced, increased with increasing beta-g:CMCase ratio up to a ratio of 1:1 (Table 4.10). The total amount of reducing sugar produced over the hydrolysis period also increased up to a beta-glucosidase:carboxymethylcellulase ratio of 1. Because the rate of conversion of cellobiose to glucose was increased up to a ratio of 1, the overall efficiency of the hydrolysis varied in the same way. This reflects the control over the practical hydrolysis system which is exerted by glucose inhibition. The addition of beta-glucosidase to give a ratio greater than 1 did not have an appreciable additional effect on the final extent of hydrolysis.

In this determination, CMCase activity was used rather than filter paper activity, because the CMCase activity test was found to be more reproducible. Also it should be noted that the optimum ratio determined in this work applies only to this particular enzyme system under the conditions noted, and do not apply to systems which differ in substrate, or in the mode by which the enzyme was produced.

4.3.3. Effect of pH on cellulase production by mixed cultivation of *Trichoderma* and *Aspergillus*

In shake flasks, pH control is limited, usually, to the addition of buffering salts such as phosphate and citrate. However, the use of ammonium sulphate as one of the principal nitrogen sources in the medium makes a more potent buffering system necessary. Without further buffering, the pH drops rapidly, due to the utilization of the ammonia, and liberation of H^+ ions. It was found that the addition of Amberlite cation exchange resin provided sufficient buffering capacity for this system. With 50 g/l of the cation exchange resin present, pH was controlled within 1 pH unit of the starting pH, and the enzyme activity produced was markedly improved (Figure 4.8).

4.3.4. Effect of media modifications on enzyme production by mixed cultures of Trichoderma and Aspergillus

As described previously, the calcium, magnesium and potassium salts used in the mixed culture medium are present mainly to provide buffering capacity to the system. In practical (fermenter-scale) systems, this buffering capacity is, to a large extent, unnecessary because of the presence of automatic pH control. In an effort to reduce medium cost and complexity, the concentration of these salts was reduced and the effect on enzyme production determined. Although these tests were done on shake flask scale, pH was controlled by the addition of Amberlite. It was found that the salts concentration could be reduced by 50% with no adverse affect on enzyme activity produced in the broth (Table 4.11).

The type and concentration of carbon source was found to have a strong effect on the relative concentrations of the various components of the cellulase system produced (Table 4.9). This effect was studied further by

varying the starch concentration from 1 to 10 g/l at a fixed (10 g/l) concentration of cellulose. The beta-glucosidase activity of the resultant cellulase increased with increasing starch concentration, while the overall cellulase activity, as measured by filter paper activity, decreased (Figure 4.9). The increase in beta-glucosidase activity is not surprising, in that starch is a preferred substrate for Aspergillus. However the decrease in filter paper activity is less easily explained. This decrease may indicate that a competition exists between Trichoderma and Aspergillus for some component of the medium. It is also possible that the products of starch fermentation by Aspergillus inhibit enzyme production by Trichoderma, or the activity of those enzymes already produced. Recent evidence has indicated that Aspergillus wentii produces an enzyme, mannanase, which decreases the activity of cellulase from Trichoderma (Ghose et al., 1985). Cellulase has been reported to be a glycoprotein with a mannose-containing polysaccharide as the main carbohydrate moiety (Gum and Brown, 1977). The mannanase is thought to act upon the mannose moiety of the cellulase, and thereby rendering the enzyme inactive. It is unclear why destruction of the glycosidic bond would render cellulase inactive, and no evidence was given to suggest that the enzyme involved was not a protease. In this work, a concentration of 10 g/l cellulose and 7 g/l starch was found to yield a broth with the desired ratio of beta-glucosidase:CMCase activity.

4.3.5. Effect of growth temperature on enzyme production by mixed culture

The temperature at which the mixed cultures were incubated had a strong effect on enzyme production by the mixed culture. The optimum temperature for enzyme production by the mixed culture was 27^{o} C. Growth of the mixed

culture at higher temperatures (Table 4.12) resulted in a decrease in enzyme activity.

Temperature cycling, whereby the cultures were incubated at a higher temperature for 48 hours followed by a reduction to 27° C., resulted in no improvement in the final enzyme titers. This technique has been shown to be effective in pure culture of Trichoderma (Nystrom and DiLuca, 1977).

4.3.6. Fermenter-scale growth of the mixed culture

A typical batch fermentation profile diagram is shown in Figure 4.10. The lag phase was reduced in duration compared to that which existed in shake flask-scale cultures. Most of the production of FPA and beta-glucosidase activity occurred in the later stages of batch fermentation, after the concentration of the starch and cellulose had been reduced to low levels. It is interesting to note that there is an inflexion point in the cellulose concentration curve at the 24 hr. mark, a time which coincides with the inoculation of the second component, Aspergillus phoenicis. At this point, the first derivative of the cellulose concentration curve begins to decrease, indicating that the rate of cellulose utilization by the biomass present had slowed. Upon addition of the Aspergillus there was a increase in the concentration of glucose in the broth, due to the action of the beta-glucosidase in the seed culture upon the starch present. The presence of this preferred carbon source in concentrations up to 0.5 g/l, resulted in a decrease in the rate of cellulose utilization by the Trichoderma component. This non-segregated use of the starch resulted in a decrease in available carbon source for the Aspergillus component of the mixed culture.

The pH at which the fermenter was controlled proved to be the critical factor in determining the relative concentration of the various components of the cellulase complex produced. At pH 4.6, the final enzyme titers were: 4.25 IU FPA and 1.1 IU beta-g for a ratio of 4. When the pH was raised these ratios changed dramatically. At high pH (> 5.5), the Aspergillus component of the mixed culture was able to dominate, and the amount of FPA produced was negligible.

The method by which the pH of the broth was controlled in fermentations using pure cultures of Trichoderma, was different from that which was used for the mixed culture. In fermentations with T. reesei alone, the pH of the broth tends to decrease and remain low (ca. 3) until the end of the fermentation. For this reason it is possible to ensure an adequate nitrogen supply by using NH_4OH for pH control. In mixed cultures, however, the pH tends to decrease at first, followed by an increase after ca. 48 hr. To ensure an adequate nitrogen supply throughout the fermentation, it was necessary to make the 1M HCl solution used for pH control 1M in $(NH_4)_2SO_4$. Some of the kinetic parameters for growth of the mixed culture at pH 4.6 are:

Maximum volumetric growth rate = 0.68 $gl^{-1}h^{-1}$

Biomass yield coefficient ($Y_{x/s}$) = 0.13 g biomass/ gram substrate

Maximum rate of starch uptake = 0.13 $gl^{-1}h^{-1}$

Maximum rate of cellulose uptake = 0.31 $gl^{-1}h^{-1}$

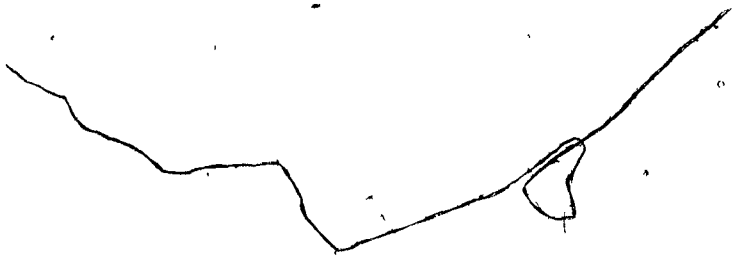
Overall productivity = 43 IU FPA $l^{-1}h^{-1}$

The mixed culture of Trichoderma and Aspergillus appears to be one in which a competition exists between the two species present, a class 7-type of interaction. By accurate control of the medium pH, it is possible to

ensure that neither organism dominates and that a cellulase is produced which has a much higher beta-glucosidase activity. The improved hydrolytic potential of this cellulase will be examined in section 4.4.

Cellulose Concn. (g/l)	Starch Concn. (g/l)	Inoculum	Final FPA (IU/ml)	Final beta-glucosidase (IU/ml)
0	10	Mixed	1.5	2.8
10	0	Mixed	2.8	1.1
10	10	Mixed	3.0	2.9
10	10	Trichoderma	3.5	0.7
10	10	Aspergillus	0	4.5

Table 4.9. Enzyme production by Trichoderma reesei and Aspergillus phoenicis grown separately and in mixed culture.



Beta-glucosidase / CMCase Ratio	Relative initial rate of glucose production	Relative final concn. of glucose produced
0	0.27	0.44
0.2	0.48	0.75
0.5	0.77	0.88
1.0	0.99	0.99
1.2	1.0	1.0
1.5	0.99	0.99

Table 4.10. Effect of beta-glucosidase:CMCase ratio on the relative inital rate of glucose production and on the relative final concentration of glucose produced.

Relative salts concentration	Relative final FPA (IU/ml)	Relative final beta-g activity (IU/ml)
0	0.4	0.5
0.25	0.9	0.9
0.5	1.0	1.0
0.75	0.9	0.9
1.0	1.0	1.0

Table 4.11. Effect of varied salts concentration on filter paper activity and beta-glucosidase activity produced by mixed culture.

Growth temperature (°C.)	Relative FPA	Relative beta-glucosidase	Relative Extracellular Protein
27	1.0	1.0	1.0
30	.85	1.0	.89
33	.66	.45	.64
36	.5	.22	.52

Table 4.12. Relative FPA, beta-glucosidase, and extracellular protein concentration in culture broths of mixed culture grown at various temperatures.

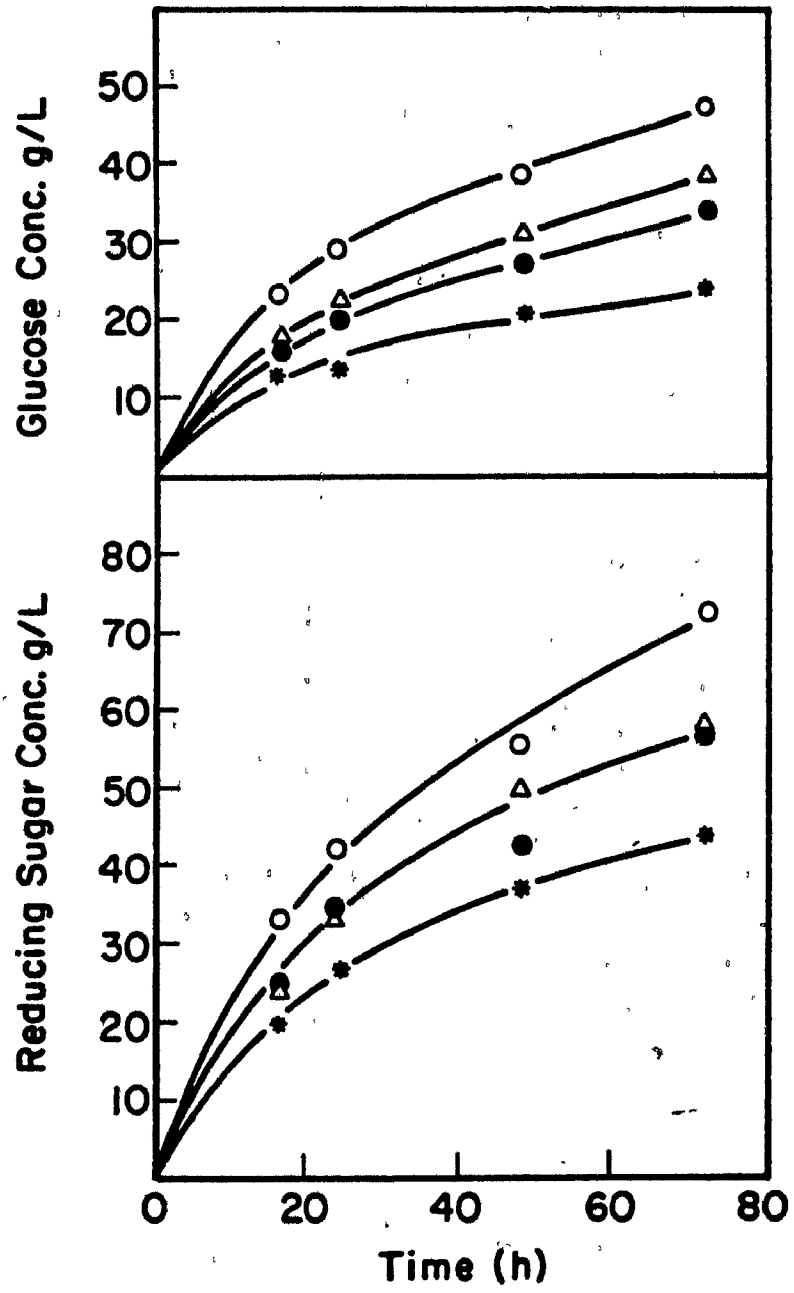


Figure 4.7. Hydrolytic potential of mixed culture cellulase produced on different carbon sources: Symbols: ● 10 g/l starch, △ 10 g/l cellulose, ○ 10 g/l starch + 10 g/l cellulose, ✱ Control (*T. reesei* alone).

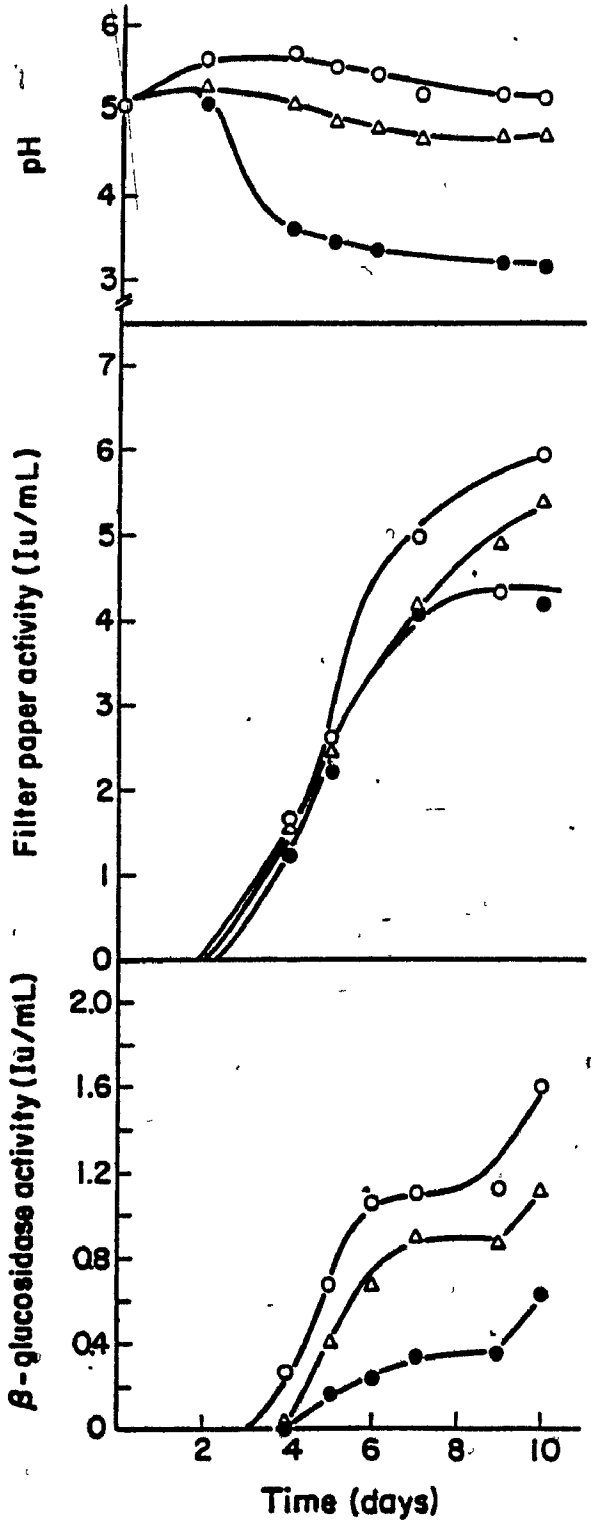


Figure 4.8. Effect of pH control with Amberlite on pH, FPA and beta-glucosidase activity in mixed cultures. Symbols: ○ 50 g/l Amberlite, △ 30 g/l Amberlite, ● Control (no Amberlite added).

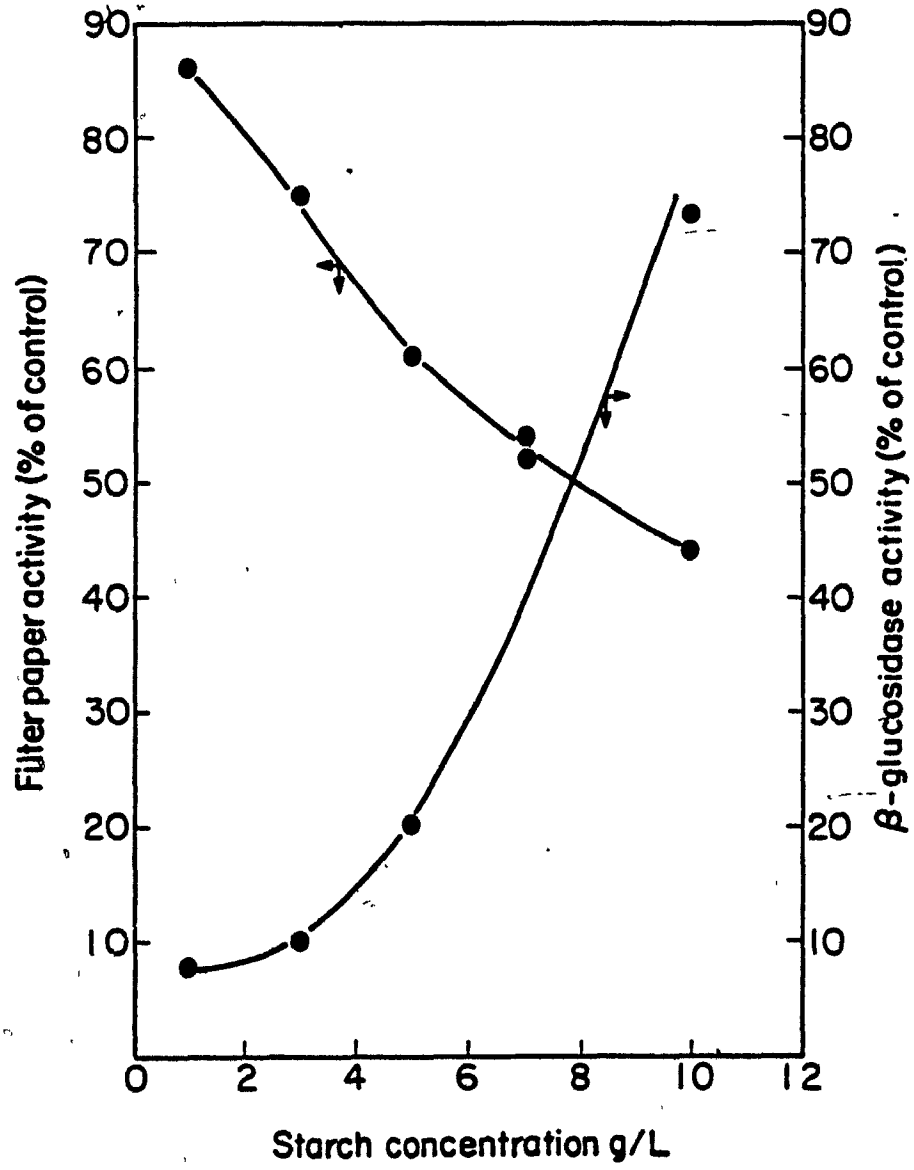


Figure 4.9. Effect of starch concentration on filter paper activity and beta-glucosidase activity produced in mixed cultures with fixed (10 g/l) cellulose concentrations. Percent of control refers to the enzyme activities produced by pure cultures of either Trichoderma grown on 1 % cellulose or Aspergillus grown on 1 % starch.

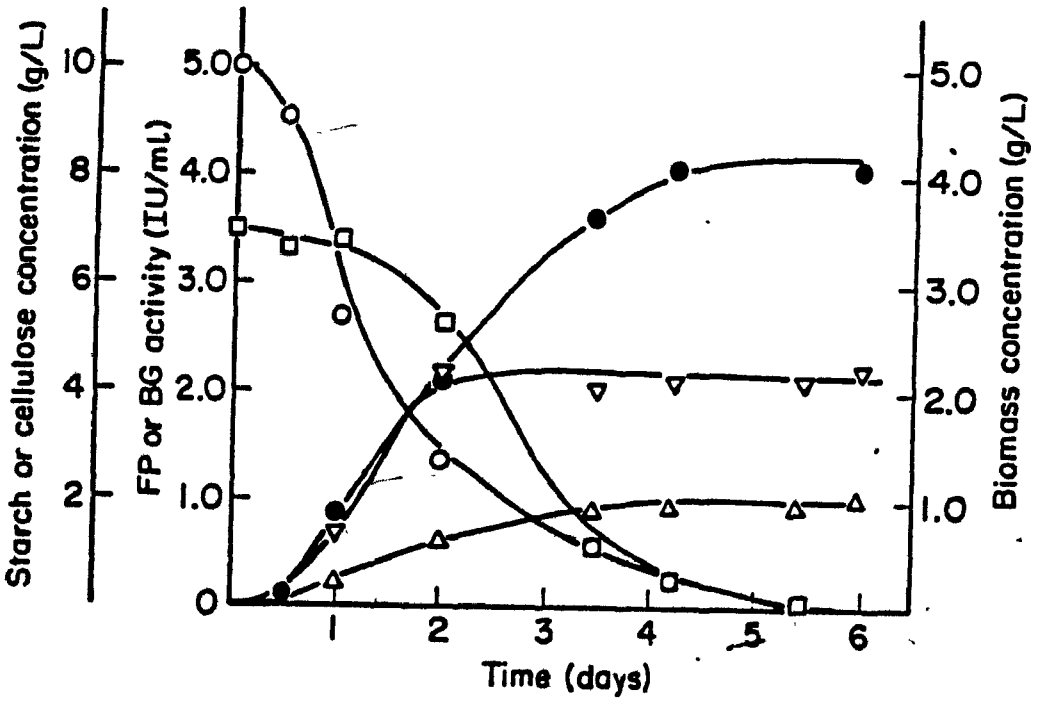


Figure 4.10. Batch growth fermentation profile for mixed culture grown at pH 4.6. Symbols: ● filter paper activity, △ beta-glucosidase activity, □ starch concentration, ○ cellulose concentration, ▽ biomass concentration.

4.4. Evaluation of the hydrolytic potential of a crude cellulase from mixed cultivation of Trichoderma reesei and Aspergillus phoenicis

4.4.1 Activity of cellulase from mixed culture versus Trichoderma cellulase

Cellulase produced by mixed cultivation of Trichoderma reesei Rut C30 and Aspergillus phoenicis was more effective at degrading Solka Floc than was cellulase produced by Trichoderma alone (Figure 4.11). The initial rate of reducing sugar production was increased approximately 10 percent while the rate of glucose production was more than doubled. Glucose:cellobiose ratios in the mixed culture hydrolysis were consistantly higher than those in the hydrolysis using the Trichoderma enzyme. Glucose is a less potent inhibitor of cellulase activity than cellobiose. As a result, it is advantageous to maximize the conversion of cellobiose to glucose. In this way the decrease in cellulase activity which is associated with high cellobiose concentrations is minimized. The enhanced beta-glucosidase activity of the mixed culture resulted in lower concentrations of cellobiose. For this reason, the rate of sugar production declined less rapidly in the hydrolyses using the mixed culture enzyme.

4.4.2 pH profile

The pH of the medium had a strong effect on the activity of cellulase produced in the mixed culture (Figure 4.12). Although a small amount of glucose is produced by the direct action of exoglucanase on cellulose, most of the glucose produced during enzymatic hydrolysis of cellulose is generated by the activity of beta-glucosidase on cellobiose. As a result,

the rate of glucose accumulation can be used as an indicator of beta-glucosidase activity of the cellulase complex. For the mixed culture cellulase, the rate of glucose accumulation reached a maximum at ca. pH 4.75. This is in agreement with optimum pH values which have been published for beta-glucosidase from Aspergillus phoenicis (Bisset and Sternberg, 1978) and others of the genus (Woodward and Wohlpart, 1982; Scrivastava et al., 1984). The sharp loss of activity observed when the pH was decreased below pH 3.5, reflects an inhibition of the Aspergillus beta-glucosidase activity which is related to the concentration of the basic carboxylate anion in the citrate molecule at that pH (Woodward and Wohlpart, 1982). The pH optimum for reducing sugar production was approximately 4-4.25. This value corresponds with the lower pH optima which have been previously reported for the other components of the cellulase from Trichoderma (Durand et al., 1984).

4.4.3 Temperature optimum and thermal stability

For mixed culture cellulase, as temperature was increased over the range from 40 to 60 ^{o} C., there was an increase in the initial rate of production of glucose and total reducing sugar (Table 4.13). There are a number of factors involved in this temperature effect.

The optimum temperature for beta-glucosidase activity has been reported to be in the range 60 to 65^{o} C (Woodward and Wohlpart, 1982; Bisset and Sternberg, 1978; Scrivastava et al., 1984). In this study, as the hydrolysis temperature was increased over the test range, there was an increase in the activity of the beta-glucosidase present in the system.

The beta-glucosidase converted cellobiose to glucose more efficiently and, as described in Section 4.2.2, this resulted in an increase in the overall efficiency of the hydrolysis. In any practical hydrolysis system, as temperature is increased, there is a balance between this enhanced enzyme activity and the increased rate of thermal deactivation of the enzymes which occurs at higher temperatures. In hydrolyses using the mixed culture cellulase, as temperature was increased from 50 to 60°C., the half-life of the beta-glucosidase component decreased from 48 to 10 hours (Table 4.14). The latter value corresponds to published half life data for Aspergillus (Scrivastava et al., 1984). The strong dependence of endo- and exo-glucanase activities upon the removal of cellobiose from the system makes it difficult to determine if the decreased activities observed for these enzymes were due to thermal deactivation or to the decrease in beta-glucosidase activity. If defined in terms of sugar produced over the entire hydrolysis period (72 hours), the optimum temperature was 55 degrees.

4.4.4 Enzyme loading ratio

Using mixed culture cellulase, there was linear increase in the rate of glucose and total reducing sugar production with increased enzyme loading ratios (Figure 4.13). The highest enzyme loading ratio (40 IU CMCase activity/ g Solka Floc) represented a soluble protein concentration of approximately 3 g/L. The increased beta-glucosidase activity of the mixed culture cellulase was able to partially offset the very rapid accumulation of cellobiose which is normally observed at high enzyme loading ratios (Duff et al., 1985b) (Section 4.2.2). Thus, when there is high beta-glucosidase activity associated with the cellulase complex, there is an

increased benefit to the use of higher loading ratios.

4.4.5 End-product inhibition

The effect of end-product inhibition on the activity of cellulase has been well documented (Ryu and Mandels, 1980; Okazaki and MooYoung, 1978). The increased beta-glucosidase activity of the mixed culture was reflected in an increased resistance to end product inhibition. In hydrolyses using mixed culture cellulase with added end product (30 g/l glucose), the concentration of glucose and total reducing sugar after 72 hours, were 80 and 87% respectively of the control flasks which contained no added glucose. The corresponding values for Trichoderma cellulase were 71 and 59 %. The initial rate of glucose and reducing sugar production for the mixed culture cellulase were not strongly affected by increasing the added glucose concentration from 30 to 50 g/l (Table 4.15). This ability to continue hydrolysing cellulose in the presence of high concentrations of sugar is essential in a practical hydrolysis reactor (Ryu and Mandels, 1980).

4.4.6 Hydrolysis of potential large-scale substrates

Steam exploded aspen wood (SEAW) has been described as a potential substrate for large-scale enzymatic hydrolysis (Sinitsyn et al., 1983). In order to determine the hydrolytic potential of the mixed culture cellulase using a substrate which could potentially be used on a large scale, the enzyme was tested with SEAW (Table 4.16). The mixed culture cellulase was able to hydrolyse the complex substrates more effectively than Trichoderma

cellulase. Using higher concentrations of SEAW, sugar solutions of greater than 100 g/l have been produced in 72 hours using the mixed culture enzyme.

Temperature (°C.)	Relative rate of sugar production (g/l/hr)	
	Glucose	Reducing sugar
40	0.59	0.53
45	0.84	0.69
50	1	1
55	1.3	1.3
60	1.7	1.6

Table 4.13. Effect of temperature on relative initial rate of glucose and reducing sugar production.

Activity	Avicelase	CMCase	FPase	Beta-glucosidase
Temperature (^{o}C)				
50	38	>72	>72	48
60	19	29	33	10

Table 4.14. Half-lives of mixed culture cellulase component activities. All values are in hours.

Glucose added (g/l)	Relative initial rate of sugar production (g/l/hr)	
	Glucose	Reducing sugar
0	1	1
30	0.47	0.59
50	0.47	0.48

Table 4.15. Relative initial rates of glucose and reducing sugar production in the presence of varied amounts of end-product (glucose).

	TRICHODERMA		MIXED CULTURE	
	% Hydrolysis	G/R	% Hydrolysis	G/R
SUBSTRATE				
Solka Floc	64	65	87	77
SEAW	51	50	72	69

Table 4.16. Hydrolysis of Solka Floc and Steam Exploded Aspen Wood (SEAW) using cellulase from Trichoderma and mixed culture. Enzyme loading ratio was 60 IU CMCase activity per gram of substrate.

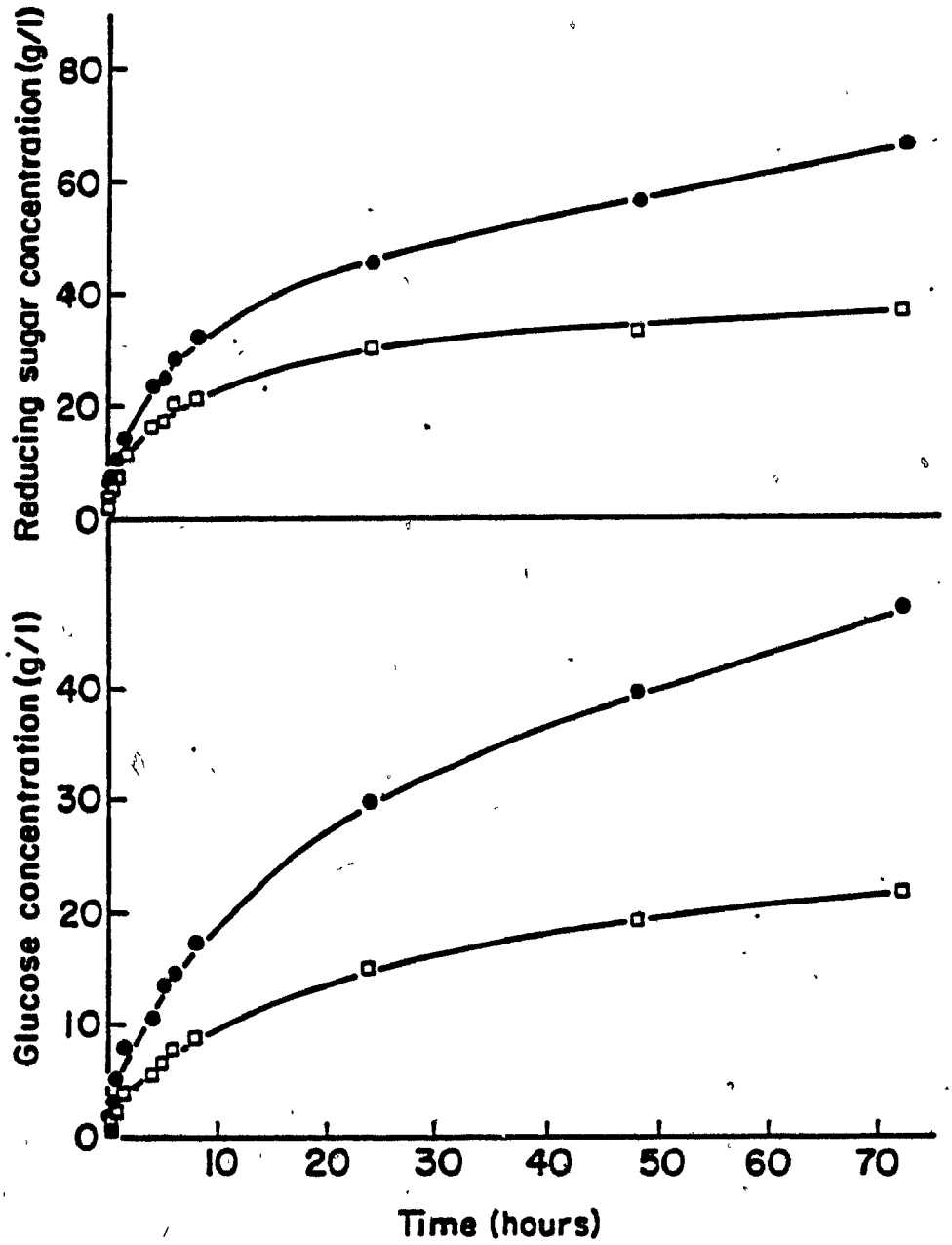


Figure 4.11. Glucose and reducing sugar production profiles for hydrolysis of solka floc with cellulase from □ Trichoderma and and ● mixed culture.

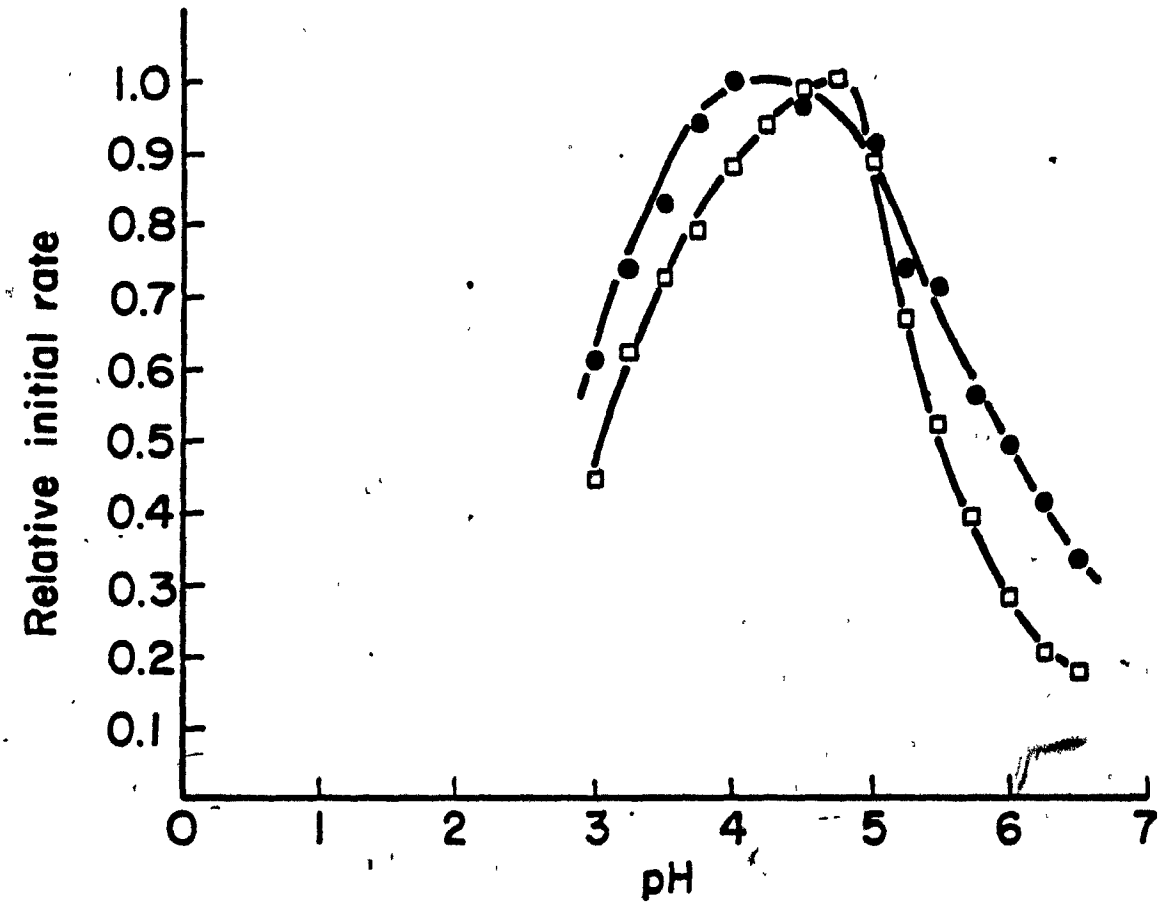


Figure 4.12. Effect of pH on the rate of production of reducing sugars ●, and glucose □ by mixed culture cellulase.

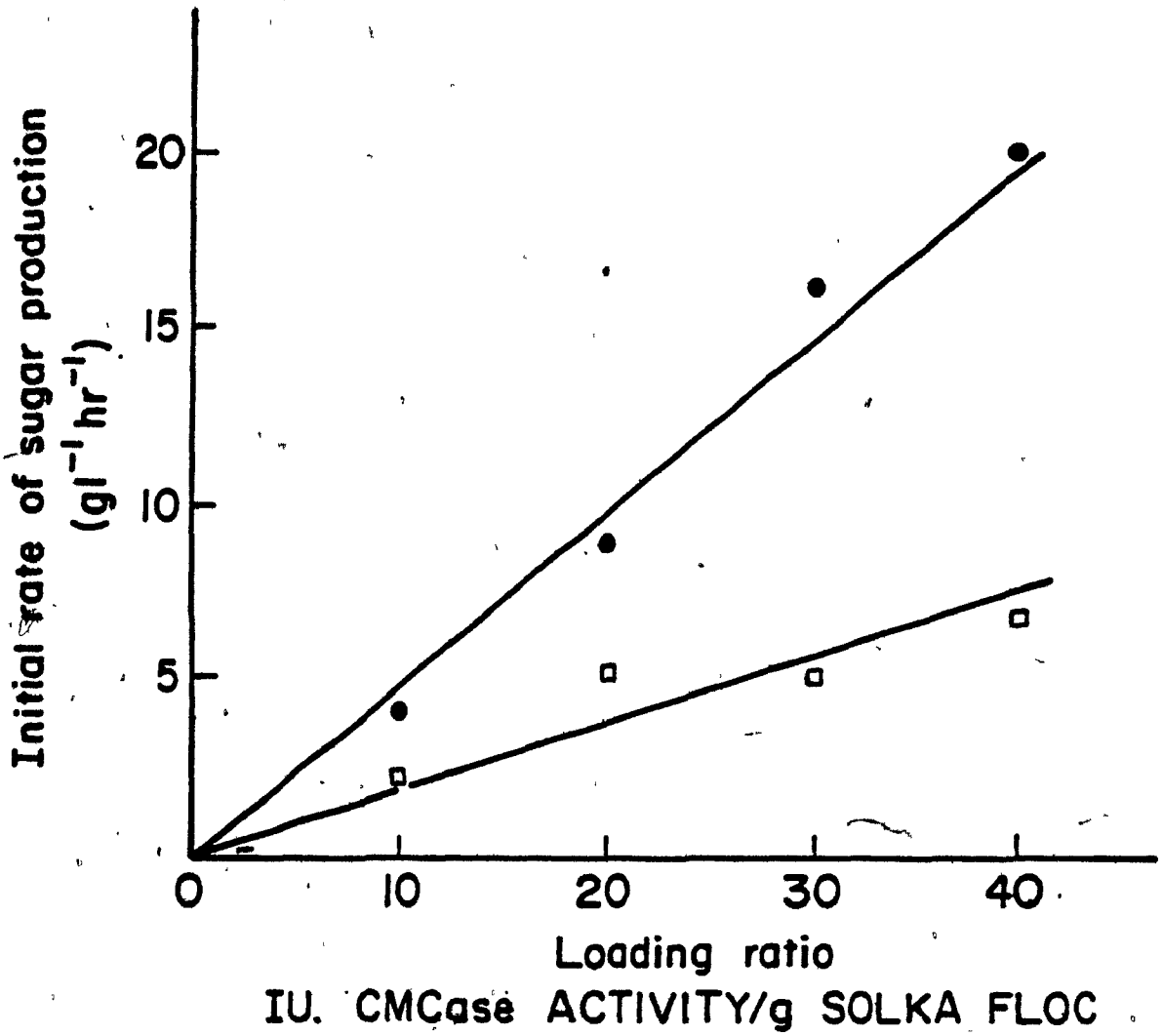


Figure 4.13. Effect of enzyme loading ratio on the rate of production of reducing sugars ● , and glucose □ by mixed culture cellulase.

5. CONCLUSIONS

1. The production of cellulase by Trichoderma reesei Rut C30 can be improved through the addition of small positively charged particles. The mechanism appears to be, in part, related to the ability of the colloid to bind, and subsequently release, soluble sugars to the organism. This mechanism does not explain the enhanced production of cellulase in cellulose fermentations, where the concentration of soluble sugars is very low. A second mechanism exists whereby the colloid influences the induction or secretion of the enzyme at the cell surface. This type of effect bears further investigation.

2. The cellulase produced by T. reesei is deficient in beta-glucosidase activity. The deficiency is particularly evident at low enzyme loading ratios (IU cellulase activity/ gram Solka Floc). At low enzyme loading ratios, the conversion of cellobiose to glucose is the rate limiting step in the hydrolysis reaction. Supplementation with beta-glucosidase removes the dependancy of the cellobiose to glucose reaction on loading ratio and improves the productivity and the yield of the hydrolysis reaction.

3. The effectiveness of Trichoderma cellulase when used to hydrolyse cellulose, is enhanced by the addition of surface active agents. This is due to two factors. Firstly, the surfactant affects the binding of the enzyme with substrate, so as to increase the ease with which the enzyme re-enters the bulk liquid phase. Secondly, the surfactant molecules displace the enzyme from the air-liquid interface. By doing so, a smaller portion of the total enzyme concentration is exposed to the strong denaturing

forces which exist at interfaces. The general nature of the surfactant effect indicates that the second mechanism is more important.

4. A cellulase with a higher beta-glucosidase activity can be produced through mixed cultivation of Trichoderma reesei with Aspergillus phoenicis The interaction between the two fungi appears to be competition for available nutrients. Medium pH is a critical factor in ensuring that one organism is unable to dominate over the other.

5. The cellulase produced by mixed cultivation of Trichoderma reesei Rut C30 and Aspergillus phoenicis has a much improved hydrolytic potential compared to that produced by Trichoderma alone, and shows a greater resistance to end product inhibition.

REFERENCES

American Public Health Association. 1975. Standard methods for the examination of water and wastewater, 14th ed. American Public Health Assoc., N.Y.

Alcohol Fuels R&D Newsletter. 1980. U.S. Dept. of Energy.

Allen, A. and D. Sternberg. 1980. Biotechnol. Bioeng. Symp. 10, p. 189.

Allen A. L., and R.E. Mortensen. 1981. Biotechnol. Bioeng. 23, p. 2641.

Andren, R.K., and J.M. Nystrom. 1976. A.I.Ch.E. Symp. Series 72, p. 91.

Andren, R.K., M.H. Mandels, and J.E. Madeiros. 1975. Appl. Polymer Symp. 28, p.205.

Andreotti, R.E., M. Mandels, and C. Roche. 1976. Proc. Bioconversion Symp. IIT, Delhi., p.249.

Basu, S.N. and P.N. Pal. 1956. Nature, p. 3121.

Bender R. 1979. U. S. Patent 4,136,207.

Berg, B., and G. Pettersson. 1977. J. Appl. Bacteriol. 42, p. 65.

Bisset, F., and D. Sternberg. 1978. Appl. Environ. Microbiol. 35, p.750.

Blackwell, J., F.J. Kolpak, and K.H. Gardner. 1977. In J.C. Arthur (ed.), Cellulose chemistry and technology, American Chemical Society, Washington, D.C., p. 49.

Brown, D.E. 1976. Octagon paper, 3, p.1.

Campbell, C.M., O. Wayman, R.W. Stanleg, G.D. Kamstra, S.E. Olbrich, E.B. Hoa, T. Nakayama, G.O. Kohler, H.G. Walker, and R. Graham. 1973. Proc. Western Section Amer. Soc. Animal Sci. 24, p. 173.

Casebier, R.L., J.K. Hamilton, and H.L. Hergert. 1969. TAPPI 52(12), p.2369.

Castanon M., and C. R. Wilke. 1981. Biotechnol. Bioeng. 23, p. 1365.

Chahal, D.S., S. McGuire, H. Pikon, and G. Noble. 1981. Paper presented at the symposium on Fuels and Chemicals from Biomass, ACS Ann. Meeting.

Chahal, D.S., S. McGuire, H. Pikon, G. Noble. 1982. Biomass 2, p.127.

Chahal, D.S. and M. Moo-Young. 1981. Dev. Indust. Microbiol. 22, p. 143.

Chahal, D.S., M. MooYoung, and G.S Dhillon. 1979. Can. J. Microbiol. 25, p. 793.

Choudhury, N., N.W. Dunn, and P.P. Gray. 1981. Biotech. Lett. 3, p. 493.

Clementi, F., M. Moresi, and J. Rossi. 1985. Appl. Microb. Biotechnol. 22, p. 26.

Cotton, F.A., and G. Wilkinson. 1972. Advanced Inorganic Chemistry, 3rd ed. Interscience Publishers, N.Y.

Cowling, E.B., and T.K. Kirk. 1976. Biotech. Bioeng. Symp. 5, p. 163.

Cowling, E.B., T.K. Kirk, and T. Kent. 1976. Biotech. Bioeng. Symp. 6, p. 95.

Crawford, D.L. 1975. Biotech. Bioeng. Symp. 5, p. 163.

Crawford, D.L., and R.L. Crawford. 1976. Appl. Environ. Microb. 31, p. 714.

Cremieux, A., J. Chevalier, M. Combert, G. Dumeuil, D. Parlouar, and D. Ballerini. 1977. Eur. J. Appl. Microbiol. 4, p. 1

Demain, A. L. 1972. J. Appl. Chem. Biotechnol. 22, p. 345.

Deshpande, M.V., and K.E. Eriksson. 1984. Enz. Microb. Technol. 5, p. 338

Desrochers, M., L. Jurasek, and M.G. Paice. 1981. Dev. Ind. Microbiol. 22, p. 675.

Desrochers, M., L. Jurasek, and M.G. Paice. 1981. Appl. Environ. Microbiol. 41, p. 222.

Detroy, R. W., L.A. Lindenfelser, G. St. Julian, and W.L. Orton. 1980. Biotech. Bioeng. Symp. 10, p. 135.

Detroy, R.W., and R.A. Rhodes. 1980. Paper presented at the organisation for economic cooperation and development cooperative research project on food production and preservation. Amersfoort, The Netherlands, Oct. 8-10.

Duff, S.J.B., D.G. Cooper, and O.M. Fuller. 1985a. Appl. Environ. Microbiol. 49, p. 934.

Duff, S.J.B., D.G. Cooper, and O.M. Fuller. 1985b. Biotech. Lett. 7, p. 185

Durand, H., P. Soucaille, and G. Tiraby. 1984. Enz. Microb. Technol. 6, p. 175

Enari, T-M., M.L. Niku-Paavola, and M. Nummi. 1980. Second Int. Symp. on Bioconv. and Biochem. Engineering. IIT.

Eriksson, K.E. 1977. Proc. Bioconv. Symp. IIT. Delhi.

Eriksson, K.E., and W. Rezdowski. 1969. Arch. Biochem. Biophys. 129, p. 683.

Faith, W.T. C.E. Neubeck, and E.T. Reese. 1971. Adv. Biochem. Eng. 1, p. 77.

Fan, L.T., and Y.H. Lee. 1983. Biotech. Bioeng. 25, p. 2707

Fan, L.T., Y.H. Lee, and D.H. Beardmore. 1980. Biotech. Bioeng. 22, p. 177.

Farkas, V., and I. Labudova. 1981. Folia Microbiol. 26, p.129.

Ferrer, A., Y. Alroy, and I. Britto. 1977. Proc. Ann. Biochem. Eng. Symp. 7, p. 83.

Fox, D., N.W. Dunn, P.P. Gray, and W.L. Marsden. 1983. J. Chem. Tech. Biotechnol. 33B, p. 114

Frein, E.M., B.S. Montenecourt, and D.E. Eveleigh. 1982. Biotech. Lett. 4, p. 287.

Gallo, B.J., R. Andreotti, C. Roche, D. Ryu, and M. Mandels. 1978. Biotech. Bioeng. Symp. 8, p.89.

Galloway, D. 1975. Pulp and paper, p. 104.

Ghose, T. K. 1977. Adv. in Biochem. Eng. 6., p.39.

Ghose, T.K. 1981. J. Cell Biology 91, p. 400.

Ghose, T.K., and J.A. Kostack. 1970. Biotech. Bioeng. 12, p.921.

Ghose, T.K., and V. Sahai. 1979. Biotech. Bioeng. 21, p.283.

Ghosh V.K., T.K. Ghose, and K.S. Gopalkrishnan. 1982. Biotech. Bioeng. 24, p. 241.

Ghose, T.K., A.N. Pathak, and U. S. Bisaria. 1975. Proc. Symp. Enz. Hydrol. of Cellulose. Auklando, Finland, M. Bailey, T.M. Linko, and M.P. Linko (eds.), p. 111.

Goering, H.K., and P.K. Van Soet. 1968. J. Dairy Sci. 51, p. 917.

Goldstein, I.S. 1981a. Chemicals from cellulose, p. 101 in I Golstein (ed.), Organic chemicals from biomass, CRC Press, Boca Raton, Fla.

Golstein, I.S. 1981b. Composition of biomass, p. 9 in I. Goldstein (ed.), Organic chemicals from biomass, CRC Press, Boca Raton, Fla.

Gottvaldova, M., J. Kucera, and U. Podrazky. 1982. Biotech. Lett. 4, p. 229.

Green, J.W. 1963. Methods in carbohydrate chemistry 3, p. 9.

Grethlein, H.E. 1985. Bio/technology 2, p.155.

Gritzali, M. and R.D. Brown. 1979. Mechanisms of enzymatic and acid catalysis, p. 237 in R.D. Brown and L. Jurasek (eds.) Hydrolysis of Cellulose. Adv. Chem. Ser. 181.

Hagglund, E. 1949. The Chemistry of Wood. Academic Press, Orlando, Florida.

Halliwell, G. 1975. Proc. Symp. Enzymatic Hydrol. of Cellulose, Helsinki, p. 319.

Halliwell, G. 1979. Prog. Ind. Microbiol. 15, p. 60.

Halliwell, G. and M. Griffin. 1973. Biochem. J. 135, p. 587.

Hendy, N.A., C.R. Wilke, and H.W. Blanch. 1982. Biotechnol. Lett. 4, p. 785.

Herr, D. 1979. Biotech. Bioeng. 21, p.1361.

Highly, T.L. 1976. Mater. Org. 11, p. 33.

Holtzapple, M.T., H.S. Caram, and A.E. Humphrey. 1984. Biotech. Bioeng. 26, p. 753.

Holtzapple, M.T., H.S. Caram, and A.E. Humphrey. 1984. Biotech. Bioeng. 26, p. 775.

Howell, J.A., and M. Mangat. 1978. Biotech. Bioeng. 20, p. 847.

Howell, J.A., and J.D. Stuck. 1975. Biotech. Bioeng. 17, p. 873.

Huang, A.A. 1975. Biotech. Bioeng. 17, p. 1421.

Huang, A.A. 1975. Biotech. Bioeng. Symp. 5, p. 245.

Hungate, R.E. 1944. J. Bacteriol. 48, p. 499.

Janshekar, H., and A. Fiechter. 1982. European. J. Appl. Microb. Biotech. 14, p. 47.

Khan, A.W. 1977. Can. J. Microbiol. 23, p. 1700.

Kim, M.H., S.B. Lee, D.D.Y. Ryu, and E.T. Reese. 1982. Enzyme Microb. Technol. 4, p. 99.

Kirk, T.K. 1975. Biotech. Bioeng. Symp..5, p. 139.

Knappert, D., H. Grethlein, and A. Converse. 1980. Biotech. Bioeng. 22, p.1449.

Korculanin, A., Z. Juric, O. Pospisil, and H. Mantanic. 1975. Microbiologija 12, p. 81.

Labudova, I. 1981. Eur. J. Appl. Microbiol. Biotech. 12, p. 16.

Lee, S.B., H.S. Shen, and D.D. Ryu. 1982. Biotech. Bioeng. 24, p. 2137.

Lee, S.E., W.B. Arminger, C.M. Watteeu, and A.E. Humphrey. 1978. Biotech. Bioeng. 20, p 141.

Lee, Y.H., and L.T. Fan. 1982. Biotech. Bioeng. 24, p. 2383.

Lee, Y.H., and L.T. Fan. 1983. Biotech. Bioeng. 25, p. 939.

Lee, Y.Y., C.M. Liu, T. Johnson, and A.P. Chambres. 1978. Biotech. Bioeng. Symp. 8, p. 75.

Leschine, S.B., and E. Canale-Parola. 1984. Curr. Microbiol. 11, p. 129.

Li, L.H., R.M. Flora, and K.W. King. 1965. Arch. Biochem. Biophys. 111, p. 439.

Linko, M., P. Markkanen, M. Bailey, and M. Leisola. 1975. Symp. on Enz. Hydrol. of Cellulose. Finnish Natl. Fund for Res. Dev., Helinski.

Lobanok, A.G. , I. Edzins, L. Bogomazova, V.V. Smetanin, R. Karhlins, and M. Bekers. 1977. Latv. PSR. Zinat. Akad. Vestis. p. 27.

Lora, J.H. and M. Wayman. 1978. Delignification of Hardwoods by Autohydrolysis and Extraction. Tappi 61(6), p. 47.

Lowry, O.H., N.J. Rosebrough, A.L. Farr, and R.J. Randall. 1951. J. Biol. Chem. 193, p. 265.

Maguire, R.J. 1977. Can. J. Biochem. 55, p. 19.

Maguire, J.R. 1977. Can. J. Biochem. 55, p. 644.

Mandels, M. 1981. Celluloses. Am. Rep. Ferm. Proc. Vol.5.

Mandels, M., and R.E. Andreotti. 1978. Process Biochem. 5, p. 6.

Mandels, M., R. Andreotti, and C. Roche. 1976. Biotech. Biog. Symp. 6, p. 21.

Mandels, M., S. Dorval, and J. Medeiros. 1978. Proc. 2nd Ann. Fuels from Biomass Symp., Renssalaer Polytechnic Inst. Troy NY.

Mandels, M., J. Kostuk, and R. Parizek. 1971. J. Polymer Sci. 36, p. 445.

Mandels, M., J.E. Medeiros, R.E. Andreotti, and F.H. Bisset. 1981. Biotech. Bioeng. 23, p. 2009.

Mandels, M., and E.T. Reese. 1960. J. Bacteriol. 79, p. 816.

Mandels, M., and E.T. Reese. 1957. J. Bacteriol. 73, p.269.

Mandels, M., and D. Sternberg. 1976. J. Farm Tech. 54(4), p. 267.

Mandels, M., D. Sternberg, and R.E. Andreotti. 1978. Proc. Symposium on Enzymatic Hydrol. of Cellulose. Finland, p. 81.

Mandels, M., and J. Weber. 1969. The Production of Cellulases. Adv. Chem. Series, 95, p. 391.

McLean, D., and M.F. Podrużny. Biotech. Lett. 7, p. 683.

Miller, G.L. 1959. Anal. Chem. 31, p. 426.

Millet, M.A., A.J. Baker, W.C. Feist, R.W. Millenberge, and L.D. Sattar. 1970. J. Animal Sci. 31(4), p. 981.

Millet, M.A., A.J. Baker, and L.D. Sattar. 1975. Biotech. Bioeng. Symp. 5, p. 193.

Mishra, S., K.S. Gopalkrishnan, and T.K. Ghose. 1982. Biotech. Bioeng. 24, p. 251.

Mitra, G., and C.R. Wilke. 1975. Biotech. Bioeng. 17, p. 1.

Montenecourt, B., and D.E.S. Eveleigh. 1978. Proc. 2nd Ann. Fuels from Biomass Symp. Rensselaer Polytechnic Inst., Troy, N.Y.

Montenecourt, B.S., and D.E.S. Eveleigh. 1977. Appl. Envir. Microbiol. 34, p. 777.

Montenecourt, B.S., and D.E. Eveleigh. 1979. TAPPI 28, p. 101.

Murray, W.D., and S.J.B. Duff. 1984. Proc. Sixth Int. Symp. on Alcohol. Fuels Technol. 2, p. 20.

Murray, W.D., and A.W. Khan. 1983. Can. J. Microbiol. 29, p. 342-347.

Mukhopadhyay, S.N., and R.K. Malik. 1980. Biotech. Bioeng. 22, p. 2237.

Nevalainen, J.M.H., E.T. Palva, and M.J. Bailey. 1980. Enz. Microl. Tech., Vol. 2, p. 59.

Nisizawa, T., H. Suzuki, and K. Nisizawa. 1972. J. Biochem. 71, p. 999.

Noble, G. 1980. Final report submitted to U.S. Dept. of Energy, Fuels from Biomass Program, Iotech Ltd.

Nystrom, J.M. , and A.L. Allen. 1976. Biotech. Bioeng. Symp. 6, p. 55.

Nystrom, J.M., and P.H. Diluca. 1977. Proc. Symp. Bioconversion. of Cellulosic Substances into Energy Chemicals Microbial Protein IIT. New Delhi, p. 293.

Ohlson, I., G. Tragardh, and B. Hahn-Haagerdal. 1984. Biotech. Bioeng. 26, p. 647.

Okazaki, M., and M. Moo-Young. 1978. Biotech. Bioeng. 20, p. 637.

Palmer, J.K. 1975. Applied Polymer Symp. 28, p. 237.

Panda, T., V.S. Bisaria, and T.K Ghose. 1983. Biotech. Lett. 5, p. 767.

Peitersen, N. 1975. Biotech. Bioeng. 17, p. 361.

Peitersen, N. 1977. Biotech. Bioeng. 19, p. 337.

Peitersen, N., J. Medeiros, and M. Mandels. 1977. Biotech. Bioeng. 19, p. 1091.

Peitersen, N., and E.W. Ross. 1979. Biotech. Bioeng. 21, p. 997.

Persson, I., F. Tjerneld, and H.W. Blanch. 1984. Enz. Microb. Technol. 6, p. 415.

Pringsheim, H. 1912. Z. Physiol. Chem. 78, p. 266.

Raabo, E., and T.C. Terkildsen. 1960. Scand. J. Clin. Lab. Invest. 122, p. 402.

Rautala. G.S. 1967. PhD Thesis, Virginia Polytech. Inst., Blakesburg, Va.

Reese, E.T. 1956. Appl. Microbiol. 4, p. 39.

Reese, E.T. 1972. Biotech. Bioeng. Symp. 3, p. 43.

Reese, E.T. 1980. J. Appl. Biochem. 2, p. 36.

Reese, E.T., J.E. Lola, and F.W. Parrish. 1969. J. Bacteriol. 100, p. 1151.

Reese, E.T., and M. Mandels. 1980. Biotech. Bioeng. 22, p. 323.

Reese, E.T., and A. Maguire. 1971. Dev. Ind. Microb. 12, p. 212.

Reese, E.T., and A. Maguire. 1969. Appl. Microb., p. 242.

Reese, E.T., L. Segel, and V.W. Tripp. 1957. Textile Res. J. 27, p. 626.

Reese, E.T., R.G. Siu, and H.S. Levinson. 1950. J. Bacteriol. 59, p. 185.

Reese, E.T. and H.S. Levinson. 1952. Physiol. Plant. 5, p. 345.

Reid, I.D. 1979. Can. J. Botany 57, p. 2050.

Riaz, M., C.R. Wilke, Y. Yamanaka, and P.A. Corroad. 1977. Proc. Symp. Res. Appl. Natl. Needs. 2, p. 108.

Rosenberg, S.L. 1980. Dev. Industrial. Microbiol. 20, p. 133.

Ryu, D., R. Andreotti, M. Mandels, and B. Gallo. 1979. Biotech. Bioeng. 24, p. 1047.

Ryu, D., S.B. Lee, T. Tassinaro, and C. Macy. 1982. Biotech. Bioeng. 24, p. 1047.

Ryu, D., and M. Mandels. 1980. Enz. Microb. Technol. 2, p. 91.

Saddler, J.N. 1982. Enz. Microb. Technol. 4, p. 414.

Sahai, V., and T.K. Ghose. 1977. Proc. Bioconv. Symp. IIT, Delhi, p. 269.

Sarkanen, K.U., and C.H. Ludwig. 1971. Lignins: Occurence Formation Structure and Reactions. Wiley Interscience, N.Y.

Sasaki, T., T. Tanaka, N. Nanbu, Y. Sato, and K. Kainuma. 1979. Biotech. Bioeng. 21, p. 1031.

Scrivastava, S.K., K.S. Gopalkrishnan, and K.B. Ramachandran. 1984. Enz. Microb. Technol. 6, p. 508.

Scrivastava, S.K., K.B. Ramachandran, and K. S. Gopalkrishnan. 1981. Biotech. Lett. 3, p. 477.

Selby, K., and C.C. Maitland. 1967. Biochem. J. 104, p. 716.

Shewale, J.G. 1982. Int. J. Biochem. 14, p. 435.

Sinitsyn, A.P., H.R. Bungay, and L.S. Clescerı. 1983. Biotech. Bioeng. 25, p. 1393.

Slater, J.H. 1978. The role of microbial communities in the natural environment, p. 137-154. In K.W.A. Chater and H.J. Somerville (eds.), The oil industry and microbial ecosystems. Heydon and Sons Ltd., London.

Slater, J.H. 1981. Mixed cultures and microbial communities, p. 1 in M.E Bushnell and J.H. Slater (eds.) Mixed Culture Fermentations. Academic Press, London.

Slater, J. H., and Bull, A.T. 1978. Interactions between microbial populations p. 181, in A.T Bull and P.M. Meadow (eds.) Companion to Microbiology. Longman Ltd. London.

Sternberg, D. 1976. Biotech. Bioeng. Symp. 6, p. 35.

Sternberg, D.,and S. Dorval. 1979. Biotech. Bioeng. 21, p. 181.

Sternberg, D., P. Vijayakumar, and E.T. Reese. 1977. Can. J. Microbiol. 23, p. 139.

Styer, D.J., and R.D. Durbin. 1982. Can. J. Microb. 28, p.986.

Tangnu, S.K., H.W. Blanch, and C.B. Wilke. 1981. Biotech. Bioeng. 23, p. 1837.

Taniguchi, M., M. Tanaka, R. Matsuno, and R. Kamikubo. 1982. European. J. Appl. Microbiol. Biotechnol. 14, p.35.

Tarkow, H. and W D. Feist. 1969. Adv. Chem. Ser. 95, p. 197.

Taylor, J.D. 1980. Paper presented at organisation for economic cooperation and development workshop, Amersfoort, The Netherlands.

Theodoroa, M.K., M.J. Bazin, and A.P.J. Trinci. 1981. Arch. Microbiol. 22, p. 37.

Toyama, N., and K. Ogawa. 1975. Biotech. Bioeng. Symp. 5, p. 225.

Trevida, S.M. and J.D. Desai. 1984. J. Ferm. Technol. 62, p.211.

Trevida, S.M., and Ray, R.M. 1985. J. Ferm. Technol. 63, p.299.

Vohra, R.M., C.K. Shirkot, S. Dhawan, and K.G. Gupta. 1980. Biotech. Bioeng. 22, p. 1497.

Volfova, O., and E. Kyslikova. 1981. Folia Microbiol. 26, p. 309.

Wald, S., C.R. Wilke, and H.W. Blanch. 1984. Biotech. Bioeng. 26, p. 221.

Walseth, C.S. 1952. TAPPI. 35, p. 233.

Warzywoda, M., V. Ferre, and J. Pourquie. 1983. Biotech. Bioeng. 25, p.3005.

Warzywoda, M., J.P. Vandecasteele, and J. Pourquie. 1983. Biotech. Lett. 4, p. 243.

Watson, T.G., and I. Nelligan. 1983. Biotechnol. Lett. 5, p. 25.

Watson, T.G., I. Nelligan, and L. Lessing. 1984. Biotech. Lett. 6, p. 667.

White, A.R. 1982. Visualization of cellulases and cellulose degradation. p. 489 in R.M. Brown (ed.) Cellulose and Other Natural Polymer Systems. N.Y.

Wilke, C.R., and G. Mitra. 1975. Biotech. Bioeng. Symp. 5, p. 253.

Wilkinson, T.G., H.H. Topiwala, and G. Hamer. (1974). Biotechnol. Bioeng. 16, p. 41-49.

Wolin, M.J. (1977). Adv. Microbial. Ecol. 3, p. 44.

Wood, T.M. 1968. Biochem. J. 109, p. 217.

Wood, T., and S.I. McCrae. 1975. The cellulase complex of Trichoderma koningii, p. 231 in M. Bailey, T.M. Enari, and M. Linko (eds.) Symposium on enzymatic hydrolysis of cellulose. The Finnish National Fund for Research and Development, Helsinki.

Wood, T.M., and S.I. McCrae. 1972. Biochem. J. 128, p. 1183.

Woodward, J., and S.L. Arnold. 1981. Biotech. Bioeng. 23, p. 1553.

Woodward, J., and G.S. Zachry. 1982. Enz. Microb. Technol. 4, p. 245.

Woodward, J., and D.L. Wohlpart. 1982. J. Chem. Tech. Biotech. 332. p. 245.

Yang, H.H., M.J. Effland, and T.K. Kirk. 1980. Biotech. Bioeng. 22, p. 165.

Yakovleva, M.B., A. Leuchenberger, L.G. Loginova, and H. Rutloff. 1982. Microbiology 51, p.59.