NYSERDA--97-6

Optimizing Cellulase Mixtures for Maximum Rate and Extent of Hydrolysis

Final Report 97-6
March 1997

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March 1997

OPTIMIZING CELLULASE MIXTURES FOR MAXIMUM RATE AND EXTENT OF HYDROLYSIS

Final Report

Prepared for

THE NEW YORK STATE
ENERGY RESEARCH AND DEVELOPMENT AUTHORITY

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1150-ERER-ER-89

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ABSTRACT

Pure *Thomomonospora fusca* and *Trichoderma reesei* cellulases and their mixtures were studied to determine the optimal set of cellulases for biomass hydrolysis. The objective was to reduce the cost of cellulase in order to help lower the overall processing cost of the enzymatic conversion of biomass cellulose to sugars, which can then be fermented into fuels and other energy-intensive chemicals. No cellulase mixture was obtained that was much better than the best commercially available preparations. However, the study has greatly increased knowledge of *T. fusca* cellulases, synergism, and cellulose binding, and provide evidence that future work will produce cellulases with higher activity in degrading crystalline cellulose. *T. fusca* cellulases may have good industrial potential because: 1) they are compatible with industrial processes that operate at elevated temperatures; 2) they retain 90% of their activity under neutral or basic conditions, which provides a great deal of flexibility in reactor design and operation; and 3) tools are now available to change specific amino acid residues in their catalytic domains and to assess how these changes influence catalysis.

Key Words (for NTIS): Thermomonospora fusca, Trichoderma reesei, cellulases, biomass hydrolysis, ethanol production.

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SUMMARY

There has been considerable interest in the enzymatic conversion of biomass cellulose to ethanol as steady improvements in the process have reduced the projected costs three-fold over the past 10 years. However, the cost of cellulase is a major factor in the current process (~40% of total cost). Our research has focused on studies of pure *Thermomonospora fusca* and *Trichoderma reesei* cellulases and their mixtures, trying to determine the optimal set of cellulases for biomass hydrolysis. *Thermomonospora fusca* secretes 6 cellulases. Three of these: E_1, E_2, and E_5 are endocellulases, two (E_3 and E_6) are exocellulases, while E_4 has properties from both classes.

We have developed procedures for purifying all 6 enzymes to homogeneity from the culture supernatant of a protease negative mutant of *T. fusca*, ER1. We also have cloned the structural genes for all of these enzymes. The six cloned genes have been expressed separately in *Streptomyces lividans*, strain TK24 and/or TKM 31 (a low protease mutant of TK24). We have developed procedures to purify each of these enzymes to homogeneity from the culture supernatant of the appropriate S. *lividans* clone. We have constructed vectors that increased the level of expression of the *T. fusca* cellulases.

T. fusca cellulases exhibit a much broader pH and temperature optima than do *T. reesei* cellulases. For example, *T. fusca* E_3 and *T. reesei* CBHII were incubated at pH 5.5 for 16 hours at temperatures from 30°C to 70°C. CBHII retained only 15% of its activity at 42°C; while E_3 retained full activity at 55°C, had 70% of its activity at 60°C, and about 20% of its activity at 65°C. E_3 had a broad pH optimum at pH 7.0, retaining 90% of its activity between pH 6 and 10 and 60% of its activity between pH 5 and 11.

We also examined the ability of these enzymes to fragment cellulose particles and only two of the five pure cellulases tested had significant fragmentation activity: endoglucanases E_2 and E_5 from *T. fusca*. Mixtures of pure cellulases and crude cellulases had much higher fragmentation activity than the pure enzymes, showing that there was strong synergism in fragmentation. Fragmentation of cellulose by *T. fusca* crude cellulase was inhibited by 1% cellobiose.

Studies of the effect of cellulase mass ratios on synergism in mixtures as well as the extent of hydrolysis were completed and showed there was a region where both synergism and the extent of hydrolysis were insensitive to changes in the ratio. A shipworm bacterial cellulase and two *Cellulomonase fimi* cellulases were tested for their ability to give synergism with our cellulases and none did. A ß-glucosidase from *Caldocellum saccharolyticum* that is compatible with our cellulases was identified and an *E. coli* strain that overproduces the enzyme was obtained. The *T. fusca* E_3 gene was cloned and sequenced. Overall these studies have greatly increased our knowledge of *T. fusca* cellulases, synergism, and cellulose binding, and support the possibility that future work will produce cellulases with higher activity in degrading crystalline cellulose.

Section 1

INTRODUCTION

Today there is a steady expansion in the industrial use of enzymes.[1] This expansion is stimulated by the progress made by molecular biologists, biochemists, and engineers in developing novel enzyme production and purification schemes. These schemes range from enzyme engineering by site-directed mutagenesis to technical refinements in chromatographic procedures. In addition, the need to obtain added value from agricultural products and the need to recycle waste is also stimulating much interest in the industrial use of enzymes.[2,3] For instance, in the US paper represents approximately 45% of the 180 million metric tons of waste generated annually. This cellulose source is receiving much attention because of the potential to enzymatically convert it to fermentable sugars for subsequent conversion to energy and chemicals.[4-6,7] NYSERDA support of this project is just one of several ligno-cellulose projects that they have funded over the last 15 years.

Enzymatic conversion of lignocellulose to fermentable sugars is one of the industrial applications of enzymes that has received considerable attention over the last 5 years. Much of this interest is driven by the large volume of cellulose in the US muncipal solid waste stream and in other industrial wastes such as paper mill sludges. Also, ethanol production from ligno-cellulosic biomass is seen as an attractive emerging technology for reducing our dependence on foreign oil. The enzymes that degrade cellulose into the fermentable sugar, glucose, are called cellulases.

For the last 7 years, our goal has been to determine whether the mix of cellulases from different cellulolytic microorganisms can be optimized to maximize the rate and extent of hydrolysis. Many of the enzymes we are studying are produced by the thermophilic bacterium, *Thermomonospora fusca,* which secretes six cellulases. In addition, we are also studying cellulases produced by *Trichoderma reesei,* the source of most commercial cellulase. To meet this objective we proposed a comprehensive series of experiments and kinetic studies with pure cellulases from these two microorganisms as well as with defined mixtures of these cellulases. The specific tasks to be accomplished were:

- Implement and refine purification protocols that have already been developed for *T. reesei* cellulases
- Conduct kinetic studies using the pure cellulase components from the two systems on different substrates
- Develop purification protocols for any remaining *T. fusca* cellulases that have not been purified and can enhance cellulose degradation
- Conduct synergistic/mixing studies within cellulase systems and across systems
- Assess the potential for this type of optimization

- Clone and sequence the *T. fusca* E_3 gene

- Look for additional classes of cellulases from other microbial systems that can increase the rate of cellulose hydrolysis by *T. reesei* crude cellulase

- Develop kinetic models of synergism in the enzymatic digestion of cellulosic substrate

- Assess the relative costs of producing the *T.reesei* Exo I cellulase by either purification or cloning; in addition, provide a brief technical and economic assessment of the potential for using cloned cellulases in industrial applications.

This report documents the activities that were carried out to fulfill these tasks and provides an overview and an assessment of future research activities.

Section 2

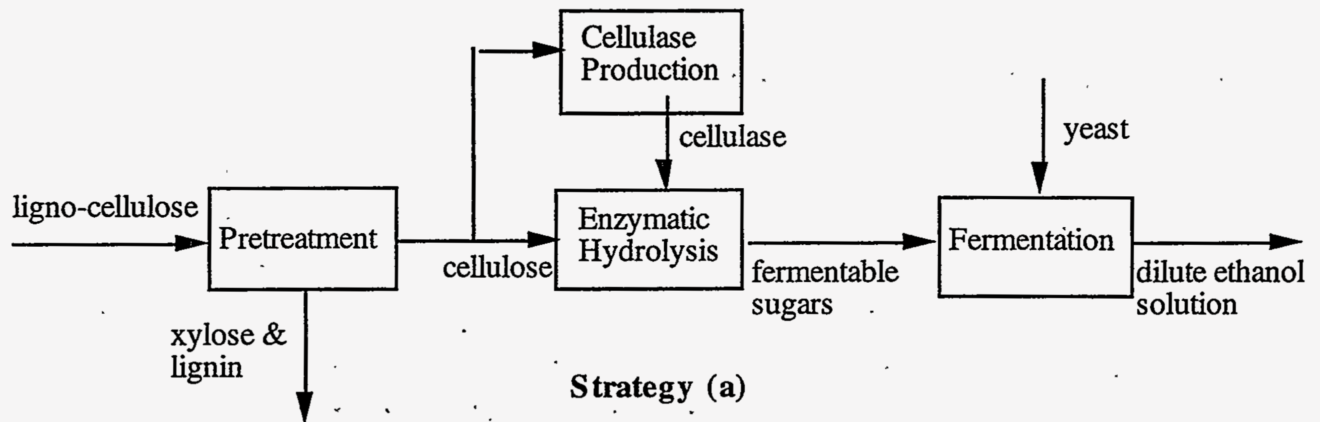
BACKGROUND

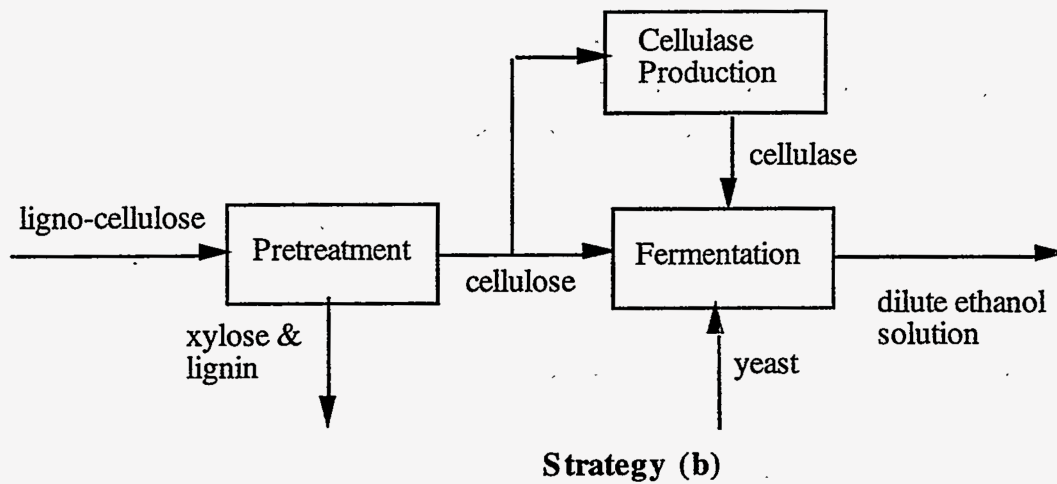
LIGNO-CELLULOSE CONVERSION TO ETHANOL

An editorial in the journal "*Science* " and a research article 2 years ago expressed much optimistism about the potential for commercialization of ethanol production from cellulosic biomass.[7] There are several factors that contributed to these positive assessments. First, cellulose is the most abundant carbohydrate produced by the biosphere, and tremendous gains have been made in improving the yield of cellulose from the nation's forests and farmlands by developing faster growing plants. Secondly, cellulose makes up a high percentage (40%) of the municipal solid waste stream generated across the country. Finally, through advances in enzymology and microbiology, the projected cost of ethanol production from ligno-cellulosic biomass has been reduced by a factor of 3 over the last 10 years.[1] Biochemical conversion technologies are of particular interest because of the many products that can be produced by fermentation and because these technologies tend to be environmentally benign.[2,3]

Several promising biochemical conversion strategies for converting cellulose to ethanol are currently under development in the US (see Figure 2-1): separate hydrolysis and fermentation (SHF), simultaneous saccharification and fermentation (SSF), and direct microbial conversion (DMC).[4,5] SHF is a sequential system where ligno-cellulosic feedstocks are first pretreated to remove the lignin and hemicellulose. The resulting cellulose slurry is enzymatically hydrolyzed to glucose, and finally the glucose is fermented to ethanol using yeast. The major disadvantage of this approach is the large amounts of expensive enzymes needed to achieve high levels of cellulose conversion to glucose. Since some of the need for a high level of enzymes is due to cellobiose and glucose inhibition of the enzymes, an approach that would remove these products from the reactor as they are produced would enhance the overall rate of hydrolysis.

In SSF, hydrolysis of cellulose to glucose and the fermentation of glucose to ethanol are carried out in the same vessel. This approach leads to a major reduction in the cost of enzyme per gal of ethanol. Wright[6] estimates that enzyme cost per gallon of ethanol for SHF and SSF are $0.65 and $0.13, respectively — an 80% reduction. DMC goes further by combining enzyme production, cellulose hydrolysis, and fermentation in a single process. The major proposed advantage of DMC processes is that there is no added cost for enzyme production.[5] The major disadvantage of this system is the difficulty of optimizing the microorganisms so that proper amounts of all the various enzymes needed for hydrolysis and fermentation are synthesized.[5] All of these strategies will benefit from efforts to improve cellulase activities.





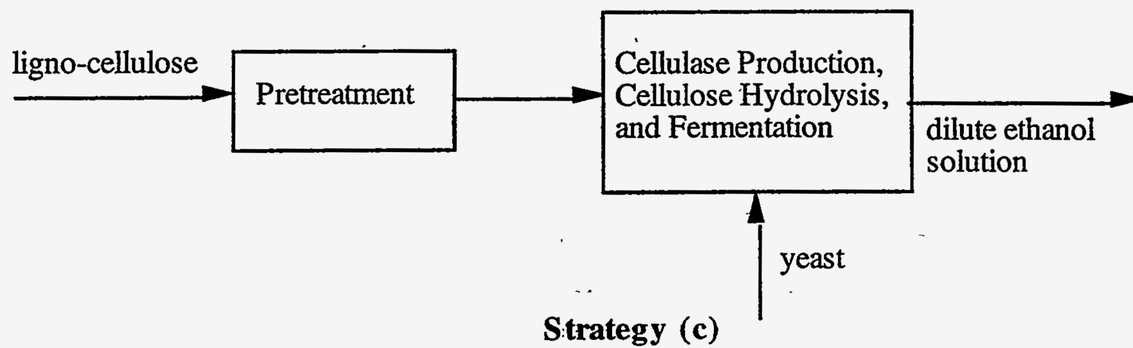


Figure 2-1. Process flow diagrams for (a) SHF, (b) SSF and (c) DMC.

SOURCES AND GENERAL CHARACTERISTICS OF CELLULASES

Both fungal and bacterial cellulolytic organisms produce multiple cellulases when grown on cellulose.[8-13] *T reesei* produces the most active crude cellulase mixture and is the source of most commercial cellulase. *T. reesei* cellulases are the most studied cellulases at this time. However, there are several other cellulase system that have received considerable attention; these are listed in Table 2-1 along with the characteristics that make them interesting sources of cellulases.

Traditionally cellulases were classified as either exoglucanases or endoglucanases.[14-16] Endoglucanases, often called CM-cellulases (CMCases), attack randomly along the cellulose fiber, resulting in a rapid decrease in the chain length of CMC or H_3PO_4 swollen cellulose yielding glucose, cellobiose, cellotriose, and other higher oligomers.[9,15] Exoglucanases, or cellobiohydrolases, cleave cellobiose units from the ends of celulose chains. They are a major constituent of crude fungal cellulases and are required for hydrolysis of highly crystalline cellulose.[9-15] Within each classification several cellulases are often present, each displaying a different affinity for cellulose and sometimes yielding different oligosaccarides as products.[17,18]

Binding Kinetics

Because enzymatic degradation of cellulose is a heterogeneous reaction, the characterization and modeling of cellulase hydrolytic mechanisms must involve an analysis of the binding kinetics. Investigation into the rate and extent of binding, binding reversibility, cellulose saturation, and cellobiose inhibition of binding are necessary. Our goal is to obtain this information and develop appropriate binding and hydrolytic kinetic models.

Early binding research work by Mandels, Kostick & Parizek[19] established the saturating binding behavior of cellulases, while work by Beldman et al.[17] offered insight into the differences in binding characteristics of several *Trichoderma reesei* cellulases. Early research by Stone et al.[20] and Grethelien[21] demonstrated how the pore structure of cellulosic materials influence the extent of binding and hydrolysis. Recently, work by Ooshima et al.[22] and Kurakae et al.[23] has focused on developing a kinetic framework for correlating the extent of binding to rates and extents of hydrolysis. Also, there has been much interest in the role that cellulase binding plays in defining what fraction of the cellulases in a mixture are available for synergism.[24]

Table 2-1. Cellulases to be investigated.

Organism	Source	Interesting Property
Shipworm bacterium	Dr. Richard Greene	high fragmentation low reducing sugar
Cellulomonas fimi	Dr. Tony Warren	bacterial exocellulase high specific activity
Clostridium thermocellum	Dr. Arnold Demain	anaerobic exocellulase
Acetivibrio cellulolyticus	Dr. J. N. Saddler	low molecular weight endoglucanase high specific activity
Thermomonospora fusca	Dr. David B. Wilson	bacterial exocellulase thermal stable celluases high fragmentation high specific activity

Fragmentation Activity

It has been known for some time that cellulose particles undergo fragmentation during enzymatic hydrolysis.[25-26] Several investigators have observed transverse cracks and complete transverse breakage of cellulose during enzymatic hydrolysis.[25-27] Others have observed an increase in the number of cellulose particles[28,29] and a shift in the volume fraction distribution during enzymatic hydrolysis.[30-31] The rate and extent of particle formation during hydrolysis increases linearly with increasing enzyme concentration.[30-35] Also, the rate and extent of fragmentation, as measured by changes in mean particle volume with time, decreases with decreasing initial particle size.[36]

Both endoglucanases and exoglucanases are capable of fragmenting cellulose.[33,37,38] However, it has been reported that the bulk of the fragmentation activity is associated with endoglucanases.[37,38]

Synergism

By themselves individual cellulases are not very effective in degrading crystalline cellulose. Significant hydrolysis can only be achieved through mixtures of cellulases (synergism).[11,13,15] Another enzyme that is needed is ß-glucosidase, which hydrolyzes cellobiose to glucose.

Given that synergistic interactions between cellulases are essential for significant cellulose hydrolysis, the challenge is to find ways to manipulate these interactions to maximize the rate and extent of hydrolysis. Beldman et al.[11] studying *T. reesei* cellulases observed that the maximum degree of synergism occurred at a molar ratio of Endo/Exo I much lower than 1. They also observed that combinations of endoglucanases with Exo II result in lower degrees of synergism than combinations of endoglucanases with Exo I. Woodward et al.[39] concluded that the greatest degree of synergism was obtained at total enzyme concentrations below that needed to saturate the available binding sites on the substrate. This finding suggests that a minimum amount of surface area is needed for an individual cellulase to be effective in hydrolyzing cellulose.

Our work has shown that the cellulases from the thermophilic aerobic actinomycete, *T. fusca*[40] can act synergistically with cellulases from *T. reesei*. In addition to *T. fusca* there are several other cellulase systems that are of interest to us (see Table 2-1). Each of these cellulolytic microorganisms produces cellulases that are reported to have characteristics that differ from those of the *T. reesei* cellulase system.

Synergistic mechanisms have been proposed by several investigators.[41-43] Wood and McCrae[42] reviewed the sequential hypothesis, where endoglucanases create new non-reducing ends for exoglucanases to

cleave to reducing sugars. They suggested that this hypothesis may be an oversimplification of the mechanism. They argued that if this hypothesis is correct, then the logical expectation is for C_1 from one system to act synergistically with C_x from another system. Culture filtrates of *Stachybotrys atra* and *M. verrucaria* mixed with C_1 of *T. koningii* or *P. funiculosum* only yield small increases in the rate and extent of cotton hydrolysis.[37,42] On the other hand, culture filtrates of some strains of *Memnoniella echinata* and *Gliocladium roseum* give variable results when mixed with the C_x components from *T. koningii* or *P. funiculosum*.[37] Instead of totally rejecting the sequential endo-exoglucanase hypothesis, Wood and McCrae[37] proposed that this model of synergism is one of two models of synergism that occur during the hydrolysis process. The second model, which Wood and McCrae[42] admit is highly speculative, is the formation of an endo-exoglucanase complex on the crystalline surface of the cellulose chains.

Ryu, Kim, and Mandels'[43] competitive adsorption model postulates three modes of synergism. In the first mode, the cellobiohydrolase speeds up the action of the adsorbed endoglucanase, accompanied by the desorption of endoglucanase. In the second mode, the two adsorbed enzyme components, endoglucanase and exoglucanase, simultaneously affect each other. In the third mode the adsorbed endoglucanase speeds up the desorption of cellobiohydrolase. The first mode is similar to the sequential endo-exoglucanase proposal presented previously. The second and third modes could conceivably be lumped together to represent the enzyme-enzyme complex proposed by Wood and McCrae.[42] Analysis of the adsorption and desorption process for different mixtures of cellobiohydrolases and endoglucanases and the addition of components at different time intervals could provide a means of identifying which model is most appropriate.

Wood[9] also presented a hypothesis for the synergism between cellobiohydrolases I and II. He speculated that the observed synergism could be explained if one considers that the two enzymes exhibit substrate stereospecificity and have been synthesized to attack the two different non-reducing end-groups. Removing cellobiose units successively from one type of non-reducing chain-end exposes a non-reducing end group on another chain with the correct configuration for attack by the other stereospecific cellobiohydrolase. A problem with this proposal is that there is no evidence for sterochemically different non-reducing ends of cellulose chains. At present the detailed mechanism responsible for synergism is not well known for any cellulase; nor is it known if the same mechanism is responsible for the synergisms seen with fungal and the synergism seem with bacterial cellulases.

Recently, we found that exocellulases can be divided into two classes, ones that attack the nonreducing ends of cellulose molecules and ones that attack the reducing ends. In this study, we showed that E_3 and CBHII attack the non-reducing end while E_6 and CBHI attack the reducing end. Finally, we showed that exo-exo synergism only occurs when the two exocellulases come from different classes.

Section 3

RESEARCH ACTIVITIES AND RESULTS

Early in this research project we conducted synergism studies with cellulases from the shipworm bacterium and from the *Clostridium thermocellum*. None of these cellulases exhibited significant synergism when mixed with cellulases from *T. fusca* and *T. reesei*. However, mixtures of endo- and exo-cellulases from *T. fusca* and *T. reesei* did exhibit synergistic behavior and, therefore, warranted further investigation. This investigation involved three major tasks (see Figure 3-1):

- Develop cloning, fermentation, and protein purification methods for the production of pure *T. fusca* and *T. reesei* cellulases
- Determine the chemical and physical properities of the individual cellulases, such as enzyme activity, binding kinetics, and cellulose fragmentation activity
- Engineer cellulase mixtures to obtain high rates of cellulose hydrolysis by mixing different cellulases in different combinations.

Thermomonospora fusca secretes 6 cellulases. Three of these: E_1, E_2, and E_5 are endocellulases, two (E_3 and E_6) are exocellulases, while E_4 has properties from both classes. We have developed procedures for purifying all 6 enzymes to homogeneity from the culture supernatant of a protease negative mutant of *T. fusca*, ER1. We also have cloned the structural genes for all of these enzymes. The six cloned genes have been expressed separately in *Streptomyces lividans*, strain TK24, and/or TKM 31 (a low protease mutant of TK24). We have developed an expression vector for *S. lividans* containing a promoter that gives higher levels of expression of the *T. fusca* cellulase genes. In addition, we have developed procedures to purify each of these enzymes to homogeneity from the culture supernatant of the appropriate S. *lividans* clone. We also have purified two exocellulases, CBHI and CBHII, from the culture supernatant of the fungus, *Trichoderma reesei*, strain L-27, as well as five endoglucanases. *T. reesei* produces the most active crude cellulase mixture and is the source of most commercial cellulase.

CLONING OF *T. FUSCA* CELLULASE GENES INTO *S. LIVIDANS*

The gene coding for the *T. fusca* cellulase E_3 was the only cellulase gene that was to be cloned into *S. lividans* as part of this research. However, as noted earlier all six genes for *T. fusca* cellulases, E_1 - E_6, have been cloned into *S. lividans*. Listed in Table 3-1 are a list of the six clones and references that describe the cloning methods. In this report we only provide details on the cloning of the E3 gene.

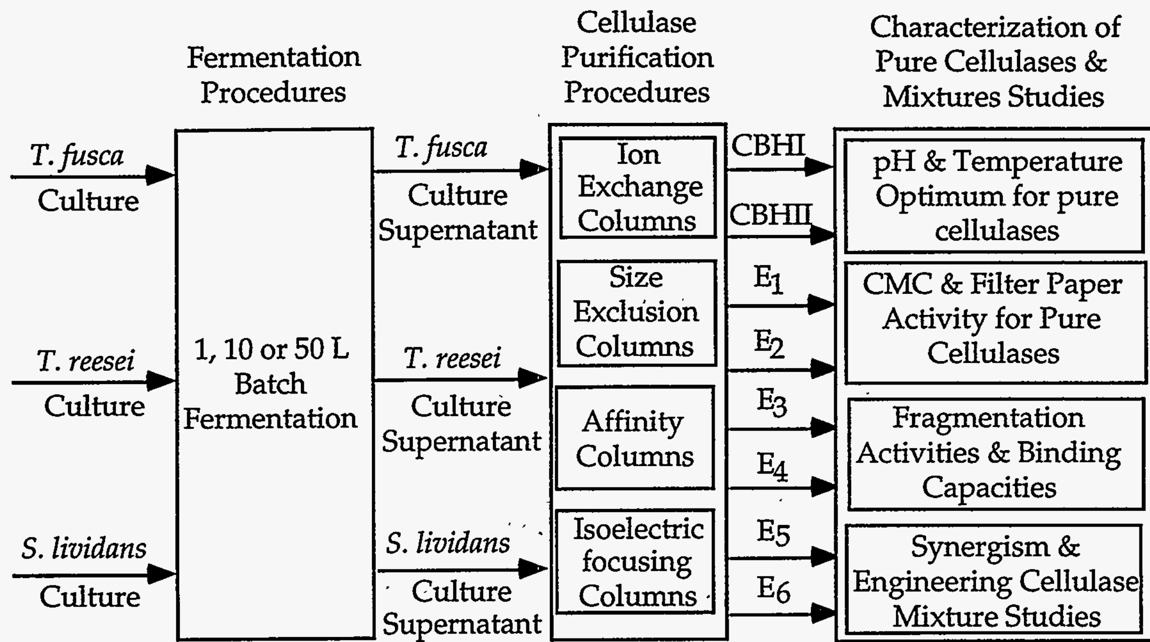


Figure 3-1. Summary of research and development activities.

Table 3-1. **Cloned cellulase genes and enzyme yield.**

T. fusca Cellulase	*S. lividans* Clone	Plasmid	Yield of Crude Cellulase (mg/L)	Yield of Pure Cellulase (mg/L)	Reference
E_1	S113	pSHE1	35	27	44
E_2	pGG85	PGG85	13	2.0	45
	S141	pBW2	87	26	46
E_3	S121	pSZ7A	57	20	47
E_4	S130	pE4T2A	39	24	44
E_5	pGG74	pGG74	73	7	45
E_6	S155	pE6	~1.0	0.7	unpublished

Cloning of the E_3 Gene

The method we used to clone the E_3 gene is shown in Figure 3-2.[47] Because E3 has low activity on all of the substrates we tested, it is not surprising that we were unable to find clones that contained the E_3 gene by screening *T. fusca* libraries in *E. coli* for CMCase activity. Therefore, we made two degenerate oligonucleotide probes based on E3 amino acid sequences. One (N E3) was designed to code for a sequence present in the N-terminal sequence of E_3 and the second (N12 E_3) was designed to code for the N-terminal sequence of a 12 kD peptide produced by cyanogen bromide cleavage of E_3. These oligonucleotides were used to probe *T. fusca* DNA that had been digested with the restriction enzyme, *Not*I and separated on an agarose gel (Southern blot). The N E_3 probe reacted with several bands including one of 7.1 kb while the N12 E_3 probe only reacted with the 7.1 kb band. A new library was prepared by isolating the DNA present in the 7 kb region from an agarose gel run on *Not*I digested *T. fusca* DNA and ligating it into the plasmid, pBluescript SK^{+}, that had been cut with *Not*I.

The ligation mixture was used to transform *E. coli* strain DH5a, selecting for ampicillin resistance. About 150 transformants were screened with the N12E3 probe by colony hybridization and four were positive. All of the positive colonies contained a 7.1 kb *NotI* insert in their plasmid DNA, were CMCase positive by the CMC overlay assay, and produced a 65 kd protein that reacted with E_3 specific antibodies (Western blot). The insert DNA was subcloned and a 3kb *Pst*I fragment was identified that encoded E_3, although it did not contain the E_3 promoter. The *Pst*I fragment was ligated into pUC18 cut with *Pst*I to give plasmid pSZ5, in which the E_3 gene is oriented in the opposite direction from the *lac* promoter, and pSZ6, in which it is oriented with the *lac* promoter (Figure 3-2).

DNA Sequencing

The sequence of the E_3 gene and its 3' untranslated region were determined using DNA from pSZ6 by the dideoxy-chain termination method with a Sequenase kit. The sequence of the 5' untranslated region was determined from the original clone since this region was not present on pSZ6. The sequence is given in Figure 3-3. It encodes a 596 residue protein that starts with a 38 residue signal sequence followed by the N-terminal sequence determined for E_3. The E_3 sequence also contains the sequence determined from the cyanogen bromide E_3 peptide. The amino acid composition of E_3 predicted from the DNA sequence agrees well with that determined for E_3 isolated from *T. fusca*.

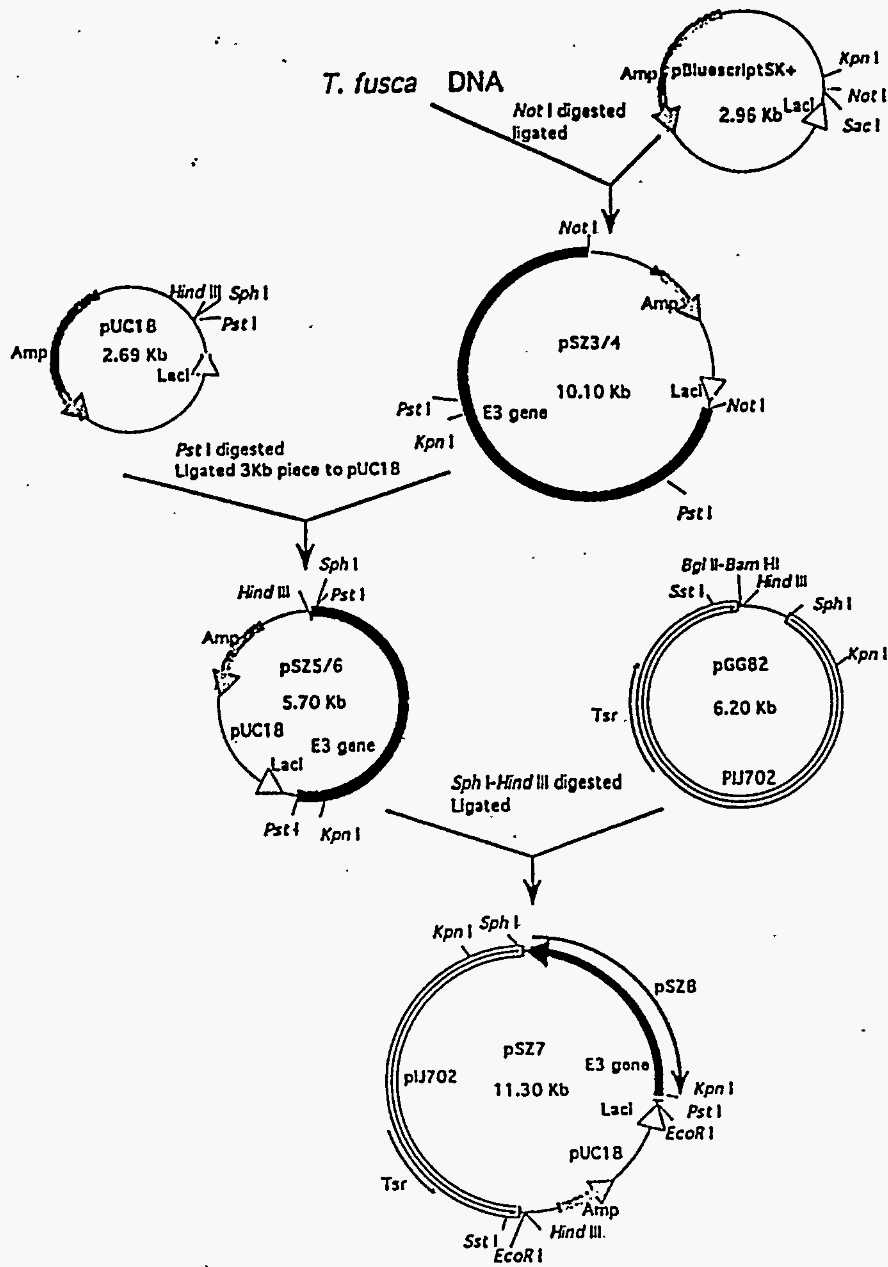


Figure 3-2. Cloning and subcloning of the E_3 gene from *T. fusca*.

Note: Thin black lines denote pUC18/pBluescript SK+ sequences, the open and lined curve indicates the PIJ&02 sequence, and thick black curves represent T. fusca DNA. Lac I is the promoter in the pUC plasmid.

TGTTCCGTTCCGTCACCATCCTTGCGCGTCCCGGCGGAGGGGGGAAGCACCCCGCGAGATGGCTCCGCCACGGCCTGTTTCCGACCCCCG 90

TCACAAAAGCCCATTTAACGCGGTATTTACAACCGGTCATGAAGTGGCTACTCTCTTTTGGGAGCGCTCCCGTGCCGCTAGTCACACTGG 180

13 bp repeat

GACGTGAATGGCGTCACGGTAGGGCTCGTCGTGTGACACGCATTTTCGACCCTGCTTTAAGTCCCTAAGTGGGAGCGCTCCCAGCCTTCG 270

14 bp repeat

GGAGAACTCCCACACAACCAACCGTCCGACGCCACTCTCCCAGCGCTCAAACGGAGGCAGCAGTGTTCACCATCCCCCGCTCCCCTCCGG 360

GGCGCCCGGCCGTCGTCCGCGCAACCACGCCGACCGGTCGGCTGAACACTGCAGCGTCCGGTTCTCGACCATCCCCTTGCGAGAGAACAT 450

CCTCCAACCAAGGAAGACACCGATATGAGTAAAGTTCGTGCCACGAACAGACGTTCGTGGATGCGGCGCGGCCTGGCAGCCGCCTCTGGA 540
Met Ser Lys Val Arg Ala Thr Asn Arg Arg Ser Trp Met Arg Arg Gly Leu Ala Ala Ala Ser Gly

CTGGCGCTTGGCGCCTCCATGGTGGCGTTCGCTGCTCCGGCCAACGCCGCCGGCTGCTCGGTGGACTACACGGTCAACTCCTGGGGTACC 630
Leu Ala Leu Gly Ala Ser Met Val Ala Phe Ala Ala Pro Ala Asn Ala Ala Gly Cys Ser Val Asp Tyr Thr Val Asn Ser Trp Gly Thr

Mature N terminal

GGGTTCACCGCCAACGTCACCATCACCAACCTCGGCAGTGCGATCAACGGCTGGACCCTGGAGTGGGACTTCCCCGGCAACCAGCAGGTG 720
Gly Phe Thr Ala Asn Val Thr Ile Thr Asn Leu Gly Ser Ala Ile Asn Gly Trp Thr Leu Glu Trp Asp Phe Pro Gly Asn Gln Gln Val

Mature N terminal

ACCAACCTGTGGAACGGGACCTACACCCAGTCCGGGCAGCACGTGTCGGTCAGCAACGCCCCGTACAACGCCTCCATCCCGGCCAACGGA 810
Thr Asn Leu Trp Asn Gly Thr Tyr Thr Gln Ser Gly Gln His Val Ser Val Ser Asn Ala Pro Tyr Asn Ala Ser Ile Pro Ala Asn Gly

ACGGTTGAGTTCGGGTTCAACGGCTCCTACTCGGGCAGCAACGACATCCCCTCCTCCTTCAAGCTGAACGGGGTTACCTGCGACGGCTCG 900
Thr Val Glu Phe Gly Phe Asn Gly Ser Tyr Ser Gly Ser Asn Asp Ile Pro Ser Ser Phe Lys Leu Asn Gly Val Thr Cys Asp Gly Ser

GACGACCCCGACCCCGAGCCCAGCCCCTCCCCCAGCCCTTCCCCCAGCCCCACAGACCCGGATGAGCCGGGCGGCCCGACCAACCCGCCC 990
Asp Asp Pro Asp Pro Glu Pro Ser Pro Ser Pro Ser Pro Ser Pro Ser Pro Thr Asp Pro Asp Glu Pro Gly Gly Pro Thr Asn Pro Pro

ACCAACCCCGGCGAGAAGGTCGACAACCCGTTCGAGGGCGCCAAGCTGTACGTGAACCCGGTCTGGTCGGCCAAGGCCGCCGCTGAGCCG 1080
Thr Asn Pro Gly Glu Lys Val Asp Asn Pro Phe Glu Gly Ala Lys Leu Tyr Val Asn Pro Val Trp Ser Ala Lys Ala Ala Ala Glu Pro

E3cd

GGCGGTTCCGCGGTCGCCAACGAGTCCACCGCTGTCTGGCTGGACCGTATCGGCGCCATCGAGGGCAACGACAGCCCGACCACCGGCTCC 1170
Gly Gly Ser Ala Val Ala Asn Glu Ser Thr Ala Val Trp Leu Asp Arg Ile Gly Ala Ile Glu Gly Asn Asp Ser Pro Thr Thr Gly Ser

ATGGGTCTGCGCGACCACCTGGAGGAGGCCGTCCGCCAGTCCGGTGGCGACCCGCTGACCATCCAGGTCGTCATCTACAACCTGCCCGGC 1260
Met Gly Leu Arg Asp His Leu Glu Glu Ala Val Arg Gln Ser Gly Gly Asp Pro Leu Thr Ile Gln Val Val Ile Tyr Asn Leu Pro Gly

CGCGACTGCGCCGCGCTGGCCTCCAACGGTGAGCTGGGTCCCGATGAACTCGACCGCTACAAGAGCGAGTACATCGACCCGATCGCCGAC 1350
Arg Asp Cys Ala Ala Leu Ala Ser Asn Gly Glu Leu Gly Pro Asp Glu Leu Asp Arg Tyr Lys Ser Glu Tyr Ile Asp Pro Ile Ala Asp

ATCATGTGGGACTTCGCAGACTACGAGAACCTGCGGATCGTCGCCATCATCGAGATCGACTCCCTGCCCAACCTCGTCACCAACGTGGGC 1440
Ile Met Trp Asp Phe Ala Asp Tyr Glu Asn Leu Arg Ile Val Ala Ile Ile Glu Ile Asp Ser Leu Pro Asn Leu Val Thr Asn Val Gly

GGGAACGGCGGCACCGAGCTCTGCGCCTACATGAAGCAGAACGGCGGCTACGTCAACGGTGTCGGCTACGCCCTCCGCAAGCTGGGCGAG 1530
Gly Asn Gly Gly Thr Glu Leu Cys Ala Tyr Met Lys Gln Asn Gly Gly Tyr Val Asn Gly Val Gly Tyr Ala Leu Arg Lys Leu Gly Glu

ATCCCGAACGTCTACAACTACATCGACGCCGCCCACCACGGCTGGATCGGCTGGGACTCCAACTTCGGCCCCTCGGTGGACATCTTCTAC 1620
Ile Pro Asn Val Tyr Asn Tyr Ile Asp Ala Ala His His Gly Trp Ile Gly Trp Asp Ser Asn Phe Gly Pro Ser Val Asp Ile Phe Tyr

GAGGCCGCCAACGCCTCCGGCTCCACCGTGGACTACGTGCACGGCTTCATCTCCAACACGGCCAACTACTCGGCCACTGTGGAGCCGTAC 1710
Glu Ala Ala Asn Ala Ser Gly Ser Thr Val Asp Tyr Val His Gly Phe Ile Ser Asn Thr Ala Asn Tyr Ser Ala Thr Val Glu Pro Tyr

CTGGACGTCAACGGCACCGTTAACGGCCAGCTCATCCGCCAGTCCAAGTGGGTTGACTGGAACCAGTACGTCGACGAGCTCTCCTTCGTC 1800
Leu Asp Val Asn Gly Thr Val Asn Gly Gln Leu Ile Arg Gln Ser Lys Trp Val Asp Trp Asn Gln Tyr Val Asp Glu Leu Ser Phe Val

CAGGACCTGCGTCAGGCCCTGATCGCCAAGGGCTTCCGGTCCGACATCGGTATGCTCATCGACACCTCCCGCAACGGCTGGGGTGGCCCG 1890
Gln Asp Leu Arg Gln Ala Leu Ile Ala Lys Gly Phe Arg Ser Asp Ile Gly Met Leu Ile Asp Thr Ser Arg Asn Gly Trp Gly Gly Pro

AACCGTCCGACCGGACCGAGCTCCTCCACCGACCTCAACACCTACGTTGACGAGAGCCGTATCGACCGCCGTATCCACCCCGGTAACTGG 1980
Asn Arg Pro Thr Gly Pro Ser Ser Ser Thr Asp Leu Asn Thr Tyr Val Asp Glu Ser Arg Ile Asp Arg Arg Ile His Pro Gly Asn Trp

TGCAACCAGGCCGGTGCGGGCCTCGGCGAGCGGCCCACGGTCAACCCGGCTCCCGGTGTTGACGCCTACGTCTGGGTGAAGCCCCCGGGT 2070
Cys Asn Gln Ala Gly Ala Gly Leu Gly Glu Arg Pro Thr Val Asn Pro Ala Pro Gly Val Asp Ala Tyr Val Trp Val Lys Pro Pro Gly

GAGTCCGACGGCGCCAGCGAGGAGATCCCGAACGACGAGGGCAAGGGCTTCGACCGCATGTGCGACCCGACCTACCAGGGCAACGCCCGC 2160
Glu Ser Asp Gly Ala Ser Glu Glu Ile Pro Asn Asp Glu Gly Lys Gly Phe Asp Arg Met Cys Asp Pro Thr Tyr Gln Gly Asn Ala Arg

AACGGCAACAACCCCTCGGGTGCGCTGCCCAACGCCCCCATCTCCGGCCACTGGTTCTCTGCCCAGTTCCGCGAGCTGCTGGCCAACGCC 2250
Asn Gly Asn Asn Pro Ser Gly Ala Leu Pro Asn Ala Pro Ile Ser Gly His Trp Phe Ser Ala Gln Phe Arg Glu Leu Leu Ala Asn Ala

TACCCGCCTCTGTAAAGCGGAGTGAGGCAACGGCTGACAGCCTCAACGAGGAACTGATCAGCACCTCCTAGCCGGAGACGGCGCCCGTCC 2340
Tyr Pro Pro Leu •

ACTCCCCGTGGGCGGGCGCCGCTTTTATGCCGACCCGTGCCCCAGCCGCAAGGGGCACGGGTCGGCCTATTCCGGCGATGTCGGTCACGT 2430

Figure 3-3. DNA and amino acid sequence of *T. fusca* cellulase E_3.

When the amino acid sequence of E_3 was used to scan the sequences present in various databases for similar sequences, it was found to contain two different domains. One, at the N-terminus, was similar to the sequence of a number of bacterial cellulose binding domains, including those present in the five other *T. fusca* cellulase genes that we have cloned and sequenced. This 104 residue sequence was followed by a 33 residue sequence rich in proline that appears to be a hinge region. The rest of the sequence was similar to the catalytic domains of cellulases in family B. It was closest to *T. reesei* CBHII (35% identity and 60% similarity), which explains why E_3 is so similar to CBHII in its enzymatic activity.

DNA sequences have been obtained for several other *T. fusca* cellulases and from these sequences we have been able to identify the structural families to which the catalytic domain of these enzymes belong. The structural family to which each of the *T. fusca* cellulases' catalytic domain belong to, and those for other important cellulase systems, are presented in Table 3-2. Also presented in this table is the anomeric bond configuration of the products for each enzyme. The *T. fusca* cellulases are found in some of the structural families that include some of the major *T. reesei* cellulases. Although it is apparent that belonging to a family does not mean that a cellulase will exhibit the same hydrolytic behavior, they do share some common features such as stereoselectivity.

<u>Expression of the E_3 Gene in *E. coli* and *S.. lividans*</u>

Plasmid DNA from pSZ6 and from pGG82, which is a multicopy *S. lividans* plasmid, were cut with *Hind* III and *Sph*I; the appropriate fragments were isolated from an agarose gel, ligated together, and transformed into *E. coli* to give pSZ7. Since pSZ6 lacks the E_3 promoter, a 4.1kb Pst I fragment that contained the E_3 promoter was isolated from the original E_3 plasmid by partial digestion and cloned into pUC18 to give pSZ10. pSZ10 was cloned into pGG82 as described to give pSZ12. pSZ7 and pSZ12 were transformed into *S. lividans* spheroplasts and transformants were selected with thiostrepton. Plasmid pS27 was subsequently digested with EcoRI and ligated to give pS27A, which contains only pIJ702 and E_3. This plasmid was transformed into *Streptomyces lividans* to give S121, a very stable, high producing strain.

E. coli containing pSZ6 produced about 0.1 mg of E_3 per gram of cells, while the *S. lividans* transformants produced about 30 mg of secreted E_3 per liter of culture. We have purified E3 from *T. fusca,* an *S. lividans* transformant and an *E. coli* transformant. The enzymatic properties of all three enzymes appear to be the same. However, E_3 from *T. fusca is* glycosylated and contains about 15 hexoses per molecule. E_3 from S. *lividans is* also glycosylated; however, it only contains about 6 hexoses per molecule. E_3 from *E. coli* is not glycosylated.

Table 3-2. Mode of attack, and catalytic and binding domains structural families for the four cellulases and $CBDE_3$ used in this study, and several other fungal and bacterial cellulase systems.

Enzyme	Mode of Attack	Catalytic Domain Structural Family*	Binding Domain Structural Family	References
T. fusca E_3	exo	6 (B)	II	47 & 50
T. fusca E_4	exo/endo	9 (E2)	II, III	44 & 50
T. fusca E_5	endo	5 (A2)	II	45 & 50
T. fusca $CBDE_3$	NA	NA	II	47 & 50
T. reesei CBHI	exo	7 (C)	I	48, 49 & 50
T. fusca E_1	endo	9 (E1)	II	47 & 50
T. fusca E_2	endo	6 (B)	II	45 & 50
T. fusca E_6	exo	48 (L)	II	Unpublished
T. reesei CBHII	exo	6 (B)	I	49 - 53
T. reesei EGI	endo	7 (C3)	I	49, 50, 54 & 55
C. fimi CenB	endo	9 (E2)	II	50 & 58
C. fimi CenC	endo	9 (E1)	II	50, 57 & 58
C. fimi CbhB	exo	48 (L)	II	50, 58 & 59
C. thermocellum CelC	endo	5 (A3)	IV	50, 58 & 60
C. thermocellum CelH	endo	5 (A4)		50, 58 & 61

*Using the classification scheme of Henrissat et al.[49]

CELLULASE CULTURES

T. fusca ER-1, producing all of the cellulases was grown in 10 liters of Hagerdal medium containing 1% Solka floc at 52°C with aeration for 26 hours.[30]

S. lividans TKM31 or TK24 strains containing *T. fusca* cellulase genes were grown in 10 liters of NMMP medium containing 0.5% cellobiose at 30° C for 48 hours or in 10 liters of TSB medium and 5 μg/ml thiostrepton.

T. reesei strain L-27 was grown in 10 liters of Mandels and Weber medium (pH 3.5) containing 1% Solka floc at 27° C for 4 days.[31]

Phenylmethylsulfonyl fluoride (0.1mM) was added to all culture supernatants to inhibit proteases after harvesting the cells.

FERMENTATION

The desired *Streptomyces* strain was grown for several days from a frozen stock until the cells were well grown but not too old. This culture was used to innoculate a 250-ml culture of TSB and thiostrepton medium. After overnight growth this culture was used to innoculate a 10-liter fermentor at 30 °C, with an air flow of 10 liters per minute and 150 rpm stirring. The pH was maintained at 7.3 with 5 M NH_4OH. It is critical that the cells be actively growing. The supernatant was harvested after 36-48 hours, usually after the pH goes to 8.5.

CELLULASE PURIFICATION

Purification of Cellulases from *T. reesei* Culture Supernatant

T. reesei exoglucanase CBHI and CBHII were obtained from the culture supernatant of *T. reesei* L27.[31] The culture supernatant was harvested after 4 days using a Sharples continuous flow centrifuge. Ammonium sulfate was added to a concentration of 1 M and the supernatant was applied to a 250-ml phenyl Sepharose column (see Figure 3-4). The column was eluted with 1 liter of 1 M $(NH_4)_2SO_4$, 2 liters of 10 mM KPi buffer pH 6.5, and finally 500 ml of water. A 150 ml head was left on the column each time the buffer was changed. The fractions from the 1 M ammonium sulfate wash contained CBHI and some CBHII and were combined, concentrated, and desalted. This material was applied to a DEAE Sephadex G50 anion exchange column equilibrated with 0.01M NaAc pH 5.0 and eluted with a NaCl gradient (0.18 to 0.30M). CBHI fractions, identified by activity assays and isoelectric focusing (IEF)

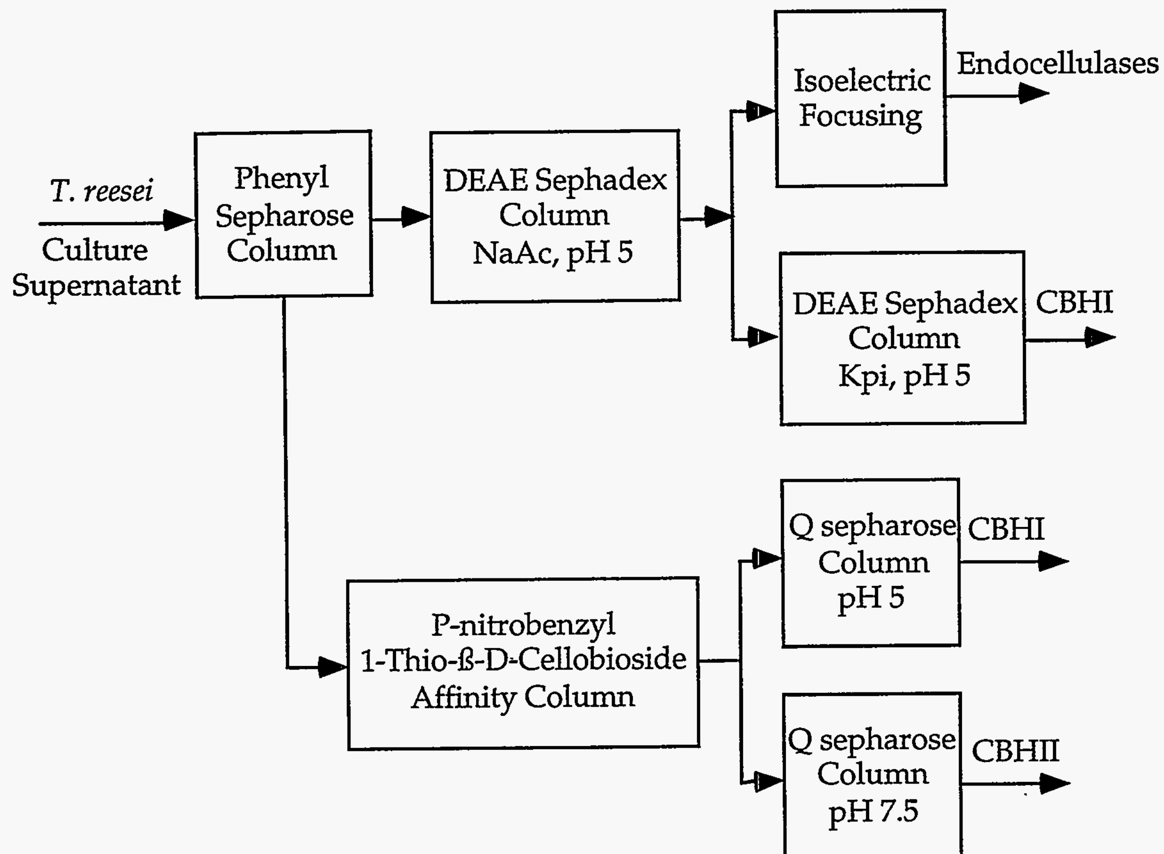


Figure 3-4. Procedures for the purification of *T. reesei* cellulases.

gels, were combined and applied to a second DEAE Sephadex G50 column using 0.01 MKpi pH 6.0 buffer and eluted with a NaCl gradient as before. IEF gels, commassie stained and overlayed with CMC[40], were used to identify the purest CBHI fractions. CBHII was obtained by loading the eluted enzyme solution from the phenyl sepharose column onto a p-nitrobenzyl 1-thio-B-D-cellobioside affinity column. Depending on whether CBHI or CBHII was to be recovered, the enzyme solution eluted from the affinity column was loaded on a Q Sepharose Column at pH 5 or pH 7.5, as illustrated in Figure 3-4.

Endocellulases were purified by taking a later eluting fraction from the phenyl Sepharose column that contained endocellulases as judged by an analytical IEF gel and chromatographing it on a DEAE Sephadex G50 column at pH 5.0. The column was eluted with a linear gradient of NaCl (0.06 - 0.12M). The fractions that contained endocellulases, as judged on an IEF gel, were combined concentrated, desalted using a Centricon-30 device, and fractionated by isoelectric focusing using a Bio-Lyte gel with ampholines from pH 3.0-10.0. The eluted IEF bands were desalted on a Pharmacia PD-10 column. Five different endocellulases were isolated as judged by their isoelectric points.

<u>Purification of Cellulases from *T. fusca* Culture Supernatant</u>

The various methods for obtaining cellulases from the *T. fusca* culture are depicted in Figure 3-5. All purification procedures were performed at 4 °C. Cellulases were concentrated using a PTTK 30,000 NMWL membrane (Millipore Filter Corp., Bedford, MA). Column fractions were monitored at 280 nm, and assayed for CMC and filter paper activity; selected fractions were electrophoresed on sodium dodecyl sulfate polyacrylamide gels (SDS-Page).

The purification steps for enzymes from *T. fusca* supernatant are shown in Figure 3-5. The first step uses 351 g 1^{-1} of ammonium sulfate, which was added to precipitate out the enzymes. After sitting for 50 min the culture supernatant was centrifuged and the resulting pellet was re-suspended in 0.005 M KPi buffer, pH 6.5. The re-suspended pellet was loaded onto an ACA 34 sizing column, which served to desalt and eliminate smaller proteins. The fractions containing activity were loaded onto a hydroxylapatite column, which gives a preliminary separation of cellulases. Purification of the different fractions was accomplished using Q Sepharose, cellobiose affinity, Con A Sepharose, and Porous II Q HPLC columns.

<u>Purification of Cellulases from *S. lividans* Culture Filtrate</u>

T. fusca E_2 was obtained by filtering the culture supernatant from *S. lividans* TK24 containing the *T. fusca* E_2 gene through a 0.22 microM Durapore filter (Millipore Filter Corp., Bedford, MA). The filtrate was loaded onto a phenyl Sepharose column, which partially purifies, desalts, and concentrates the enzyme (Figure 3-5b). Fractions containing at least 80% E_2 on SDS-PAGE were

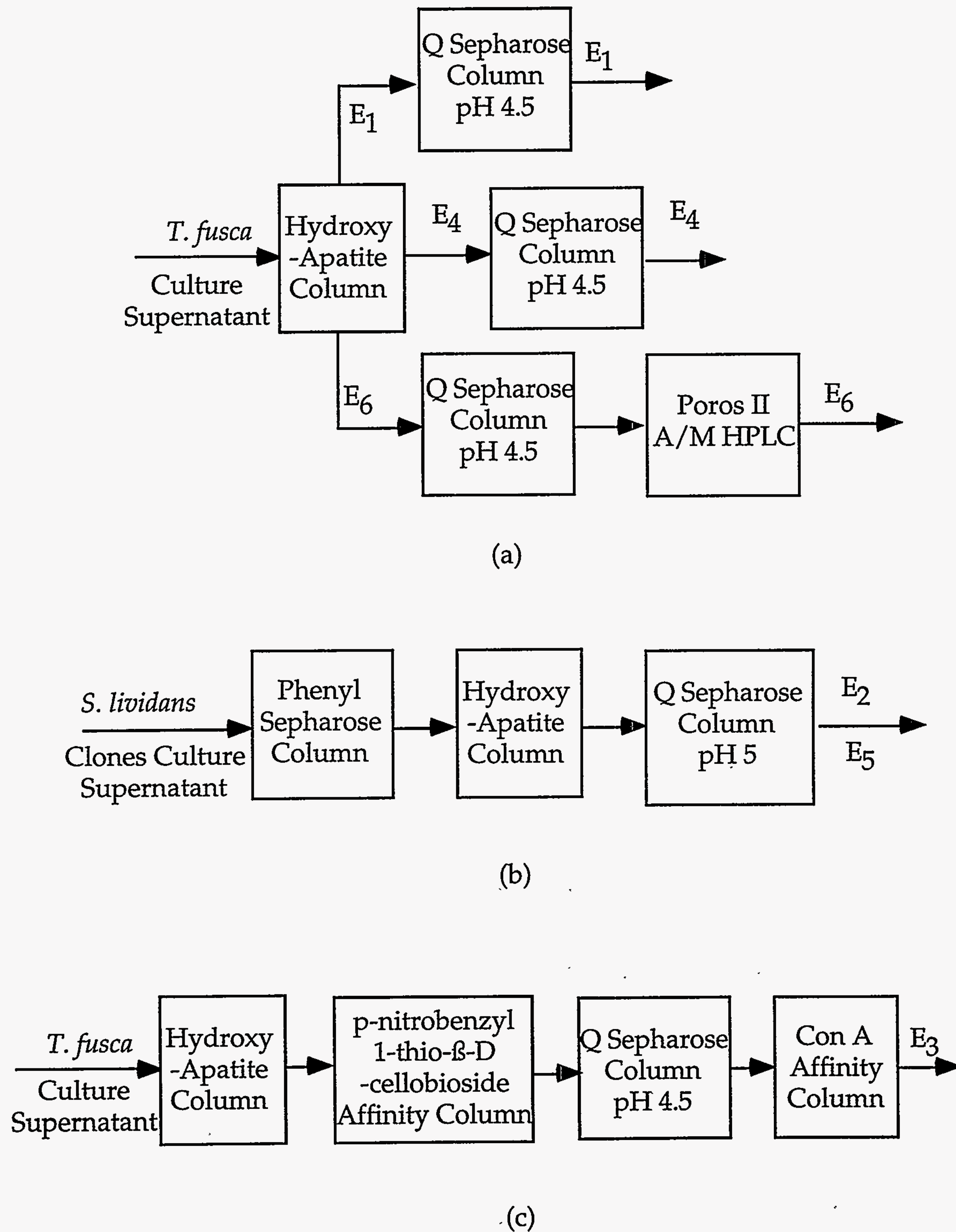


Figure 3-5. Initial procedures used to produce *T. fusca* cellulases.

combined, and loaded onto a small Hydroxyapatite C (Clarkson Chemical Co, Williamport, PA) column in 0.001M KPi pH 6 buffer. E_2 is not retained but many contaminating proteins are removed by this procedure.

The final purification step was a Q Sepharose Column equilibrated with 0.01 M Bis Tris buffer pH 6.0. E_2 was eluted using a 0 to 150 mM NaCl gradient in the same buffer. A 10 liter culture of pGG85 produced 21 mg of 95% pure E_2. We have a new plasmid, pBW2, which gave 258 mg of 93% pure E_2 from a 10-liter culture.

At this time E_1, E_3, E_4, E_5, and E_6 are all purfied from the culture supernatant of their respective *S. lividan* clones using the same basic procedure. The hydroxyapatite step is not effective for E_1, E_3, E_4, and E_5 and is not used. The yield for each cellulase in the crude supernatant, and as purified proteins, are given in Table 3-2. It is evident that the use of cloned enzymes provides a significant advantage in obtaining purified cellulase proteins. The use of a new promotor, pLTI, in the E_2 clone pBW2 gave a dramatic increase in the amount of E_2 produced. We are currently divising an *E. coli* - *S. lividans* shuttle plasmid that will contain pLT1 and are planning to use it to express the other *T. fusca* cellulase genes.

CELLULASE PURITY

For our investigation, SDS PAGE and IEF were used as methods to show the purity of each cellulase preparation. Presented in Figure 3-6 are SDS PAGE and IEF gels run on the cellulases used in this investigation. With the exception of E_1, all the enzymes exhibited a single band on the SDS gel. The E_2,E_3,E_5, and CBHI preparations gave single bands on the IEF gel. The *T. fusca* E_1 preparation had several bands on the IEF gel and a single major band on the SDS gel. The IEF gel of the CBHI preparation resulted in the characteristic isoenzyme pattern observed by Van Tilbeurgh et al.[62] while CBHII showed single bands on the IEF and SDS gels (data not shown). Isoelectric focusing was done using Pharmacia (Pistaway, NJ) precast IEF 3-9 gels.

CHARACTERIZATION AND SPECIFIC ACTIVITIES OF INDIVIDUAL CELLULASES

A comparison of the activities of the cellulases on different cellulose substrates demonstrates the variety of enzymes that are present. Table 3-2 lists the specific activities for the *T. fusca* enzymes and *T. reesei* CBHI and CBHII. The endocellulases (E_1, E_2, and E_5) have very high activity on CMC and quite high activity on swollen cellulose. In contrast, the exo cellulases have very low activity on all of the substrates and yet they are a very important component of any cellulase system, as will be demonstrated in a later section. E_4 is a hybrid cellulase with intermediate activity on CMC and swollen cellulose but comparatively high activity on bacterial microcrystalline cellulose (BMCC).

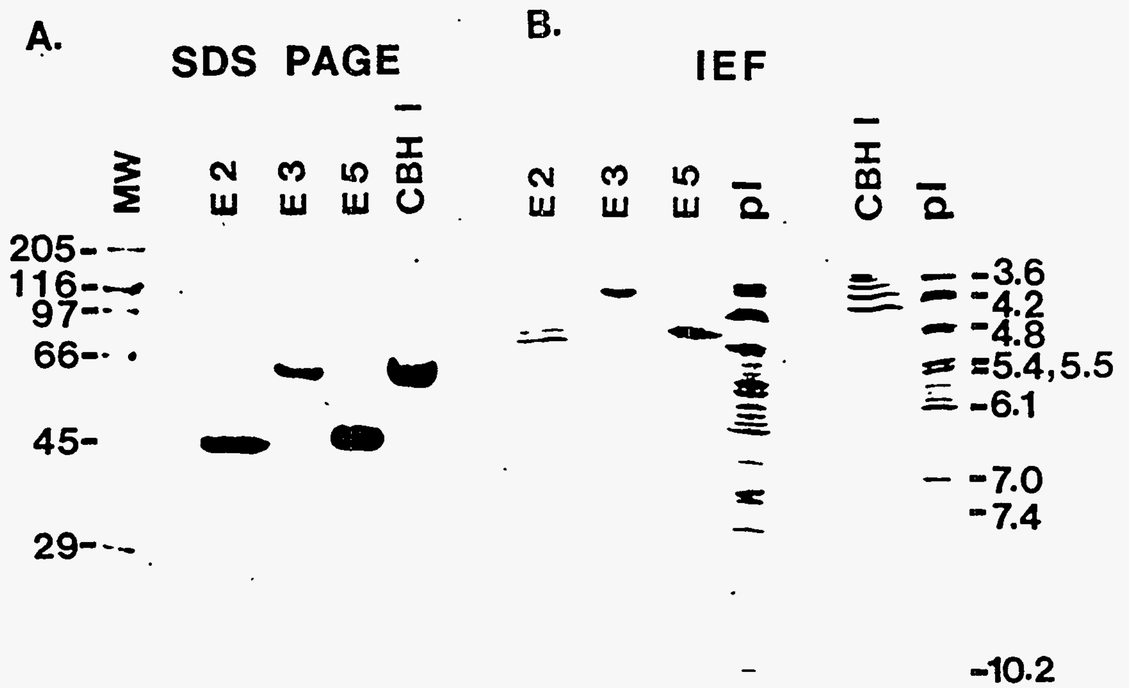


Figure 3-6. SDS gels of cellulases.

CHARACTERIZATION AND PERFORMANCE OF PURE CELLULASES

Thermostability and pH Optimum of E_3

E_3 and CBHII were incubated at pH 5.5 for 16 hours at temperatures from 30°C to 70°C. CBHII retained only 15% of its activity at 42°C while E_3 retained full activity at 55°C, had 70% of its activity at 60°C and about 20% of its activity at 65°C. E_3 had a broad pH optimum at pH 7.0, retaining 90% of its activity between pH 6 and 10 and 60% of its activity between pH 5 and 11.

CMC and Filter Paper Activity of Pure Cellulases

The methods that are most often used to assay cellulases are based on the ability of the cellulase to hydrolyze filter paper (Whatman #l) or CMC (Sigma, low viscosity). Uniform assay standards have not been adopted by all researchers, and this lack of standard assays complicates efforts to compare activities reported by different research groups. We have adopted the method of Ghose[63] to measure filter paper and CMC activities. This method was chosen because it set a minimum level of digestion, 4% digestion of filter paper and 5% digestion of CMC, for reliable and accurate measure of activity. The filter paper reactions used 3 mg of filter paper in a 0.4 ml reaction volume with a time of 16 hours at 50°C in 0.05M KPi pH buffer while CMC reactions contained 1 % CMC in a final volume of 0.4 ml.

The CMC and filter paper activities of most of the cellulases studied are listed in Table 3-3. *T. reesei* crude had the highest filter paper and CMC activities. *T. fusca* crude had the second highest filter paper activity but less CMC activity than E_1 and E_5. *T. fusca* E_3 and *T. reesei* CBHI have very low CMCase activities. At pH 6.0, *Trichoderma* crude and CBHI had 25% of the filter paper activity found at pH 4.5. Also, they retain 97% of their activity after incubation at 50°C for 21 hours. *T. reesei* CBHI and several *T. fusca* cellulases were assayed on BMCC for 4 hours using 2 mg of each enzyme and the results are given in Table 3-3.

Development of a Procedure to Characterize Cellulases

Even though cellulases are classified as being either endo or exocellulases there has not been a generally accepted method to determine whether an enzyme is an endo or an exocellulase. We have developed a simple assay that appears to distinguish endocellulases from exocellulases. This assay involves incubating the enzyme to be tested with a disc of filter paper in an appropriate buffer for sufficient time to get enough cleavage to accurately measure the amount of reducing sugar that is produced. At the end of the incubation, the filter paper is removed with forceps, rinsed two times with distilled water, and resuspended in buffer. Then the original sample from the incubation and

Table 3-3. Molecular weight and specific activities of the cellulases studied.

Protein	MW kD	CMC $\frac{\mu moe\ of\ cellobiose}{\mu mole\ min}$	Swollen Cellulose $\frac{\mu moe\ of\ cellobiose}{\mu mole\ min}$	Filter Paper $\frac{\mu moe\ of\ cellobiose}{\mu mole\ min}$	BMCC $\frac{\mu moe\ of\ cellobiose}{\mu mole\ min}$
E_1	101.2	5,410	363.0	0.18	
E_2	43.0	433	758	0.77	6.0
E_2cd	30.4	404	472	0.42	
E_3	59.6	0.3	3.9	0.13	2.0
E_3cd	45.7	0.5	1.1	0.05	
E_4	90.2	647	183	0.90	17.0
E_4cd	68.5	664	133	0.24	11.0
E_5	46.3	2,840	90.0	0.83	5.0
E_5cd	34.4	2,477	85.3	0.57	
E_6 (*T. fusca*)	106	4.3	12.2	0.39	
E_6 cloned	104	0.9	2.4	0.12	0.6
CBHI	52.0	2.1	9.3	0.89	3.0
CBHII	47.2	1.4	8.6	0.76	

the filter paper are reacted with the DNS reagent and the amount of reducing groups present in each sample are determined. An exocellulase produces primarily soluble products so that most of the reducing sugar (90 - 95%) is present in the original tube, while only 5 to 10% is present in the insoluble fraction. An endocellulase produces both soluble and insoluble reducing groups so that significant amounts of reducing groups are present in the tube with the filter paper (35 - 45%)(insoluble fraction). We have tested eight enzymes by this procedure and three behaved like endocellulases, E_1, E_2, and E_5, four behaved like exocellulases E_3, E_6 and CBHI and CBHII while E_4 gave an intermediate response. The results are given in Table 3-4. All of our other experiments on these enzymes are consistent with this assay so that we believe it is a valuable tool for evaluating cellulases. This conclusion was also supported by the workshop on the Nomenclature of Cellulases held June 2-5, 1994, where this assay was recommended as one of the four basic assays that should be used to characterize the activity of a cellulase.

We have also developed a method to determine whether an exocellulase attacks the reducing end or the nonreducing end of a cellulose molecule. Our procedure is to label the reducing sugar of cellopentose with O^{18} by exchange with O^{18} water. Then the enzyme to be tested is incubated with the labeled sugar and the cleavage products are run on a mass spectrometer to determine the amount of O^{18} cellobiose and O^{18} cellotriose that are produced. An enzyme that attacks the reducing end should produce more O^{18} cellobiose than O^{18} cellotriose, while the reverse is found for an enzyme that attacks the non-reducing end. Determination of the 3-dimensional structures of CBHI and CBHII have shown that CBHI attacks the reducing end of cellulose, while CBHII attacks the nonreducing end; and our results are consistent with these findings. By our assay, E_3 attacks the non reducing end as would be expected since E_3 is structurally and functionally very similar to CBHII. Our assay shows that E_4 and E_6 attack the reducing end of cellulose chains (see Table 3-4).

<u>Measurement of Fragmentation Activity</u>

Cellulose fragmentation was measured using an Elzone Model 180 XY particle counter manufactured by Particle Data, Inc. (Elhurst, IL) as described by Walker et al.[30]

Fragmentation studies were run on *T. fusca* E_1, E_2, E_3, E_5; *T. reesei* CBHI; and mixtures of these cellulases. The concentration of cellulases used for the different mixtures are listed in Table 3-5. The ratios of E_1 to E_3, E_2 to E_3, and E_5 to E_3 were based on an earlier study by Wilson.[9] The ratios of *T. fusca* endoglucanases to CBHI were determined from a parallel investigation where the ratios that yielded optimal DSE and rate of hydrolysis were determined experimentally.

Table 3-4. Distribution of reducing ends between filter paper and supernatant after 16 hr.

Enzyme	Percent digestion (%)	Filter paper reducing sugar (%)	Supernatant reducing sugar (%)	Ratio soluble/ insoluable reducing sugar
E_1	1.4	40	60	1.5
E_2	4.4	27	73	2.7
$E_{2,cd}$	2.7	43	57	1.3
E_3	4.6	8	92	12.1
E_4	4.0	13	87	7.0
$E_{4,90}$	2.8	14	86	14
$E_{4,68}$	1.1	28	72	28
E_5	5.7	31	69	2.2
E_5cd	2.9	48	52	1.1
E_6	3.6	4	96	23.4
CBHI	4.6	4	96	22.0

Table 3-5. Enzyme concentration used in fragmentation experiments.

Cellulase Mixture	Enzyme Concentration Tested (mg/ml)
E_2	0.020, 0.025 &0.10
E_3	0.040, 0.050, 0.075 & 0.10
E_5	0.025 & 0.10
CBHI	0.040, 0.050, 0.075 & 0.10

All digestions were run in 25-ml round bottom flasks containing 0.7 g of sieved Avicel in 14 ml of 0.05M KPi buffer pH 6.0 at 50 °C. To facilitate comparison to our earlier fragmentation studies using *T. fusca* crude cellulase, a pH of 6.0 was used as a compromise between the pH optimal of 4.5 for CBHI and a pH optimal of 6.5 for the *T. fusca* cellulases. At pH 6.0 *T. reesei* CBHI had 25% of its activity at pH 4.5. Incubation, hydrolysis, sampling, and sample processing were performed using the slightly modified procedure of Walker et al.[24] The modification involves using 200 ml of electrolyte in the particle counter sampling flask instead of 150 ml and performing pre-hydrolysis incubation and hydrolysis at 50 °C. All hydrolysis experiments were replicated two times to conserve pure cellulases. For all experiments the total protein concentration was 0.1 mg ml^{-1}.

Although an increase in the total number of particles is an indication that fragmentation has occurred, it does not provide insight in to how the particle sizes are changing with time. A more useful measure of fragmentation is the shift in the particle size distribution with time. In one of our earlier studies, we concluded that volume fraction distributions were preferable to population distributions for accurate measurement of the shift in the mass distribution during hydrolysis.[30] Volume fraction distributions where calculated using the following equation:

$$f_v(D_i) = \frac{n_i D_i^3}{\sum_{i=n_c+1}^{128} n_i D_i^3} \qquad (1)$$

where D_i is particle diameter (μm) and n_i is number of particles in size interval i.

Particle size distributions were measured using a particle counter. Measurements were made with a 190-μm orifice tube and a 2,000-μl volume section. The counter was calibrated to give a particle size measurement range of 8.03 to 130 μm; 0.7% NaCl was used as the electrolyte. Fragmentation studies were run on *T. fusca* E_1, E_2, E_3, E_5, *T. reesei* CBHI, and mixtures of these cellulases. All experiments were conducted using dry sieved Avicel PH 102 suspended in 0.05 KPi buffer (pH 6) and reactors containing 5% Avicel solution were incubated in a 50 °C water bath for 0.5 hr for the ratio experiments and 3 hr for the fragmentation experiments. Pure cellulases were added to the reactors to obtain the desired cellulase concentration and samples were removed from the flask every 20 minutes to 1 hr for immediate analysis on the particle counter or for reducing sugar and free enzyme concentration determination.

Cumulative volume fraction distributions at the start of hydrolysis and 4 hours later are presented in Figure 3-7 for pure E_1, E_2, E_3, E_5, CBHI and no enzyme. Reactors containing E_1, E_3, CBHI and no cellulase showed negligible shifts in the cellulose particle volume distribution. *T. fusca* E_5 exhibit a small but significant shift in the volume fraction distribution with time, while E_2 exhibited the highest shift in volume fraction distribution. These results show that *T. fusca* E_2 and E_5 are active endoglucanases which causes the cellulose fragmentation expected of cellulases attacking numerous sites within particles. For more details see Walker et al.[31]

Cellulase Binding Kinetics

Equilibrium binding of *Thermomonospora fusca* E_3, E_4, and E_5, the E_3 binding domain ($CBDE_3$), and *Trichoderma reesei* CBHI to Avicel PH102, and bacterial microcrystalline cellulose (BMCC) were studied. Cellulase binding has traditionally been analyzed using the Langmuir adsorption isotherm.[17,34,39,64]

$$E_b = \frac{E_{b,m} K_a E_f}{1.0 + K_a E_f} \qquad (2)$$

where

E_b =	bound cellulase concentration	(μmol g^{-1})
$E_{b,m}$ =	maximum adsorptive level of cellulase	(μmol g^{-1})
E_f =	free cellulase concentration	(μmol l^{-1})
K_a =	association constant	(l μmol $^{-1}$)

The critical assumptions that form the basis of this model are that the adsorption energy is uniform over the entire cellulose surface, and that the rates of adsorption and desorption are in equilibrium.

Binding results for E_3, $CBDE_3$, E_4, E_5, and CBHI on Avicel are presented in Figure 3-8. The data fit quite well to the Langmuir isotherm and the apparent $E_{b,m}$s, which were determined by visual approximation of the saturation portion of the curves, and range from 0.32 μmol g^{-1} for E_4 to 1.6 μmol g^{-1} for $CBDE_3$.[66] Each isotherm, with the exception of that for $CBDE_3$ contains a minimum of three data points in the saturation portion of the curve. This validation of saturation is important in generating accurate estimations of the $E_{b,m}$s. The binding results on BMCC are shown in Figure 3-9. These five isotherms demonstrate Langmuir-type binding, and have the same correlation between the standard deviation

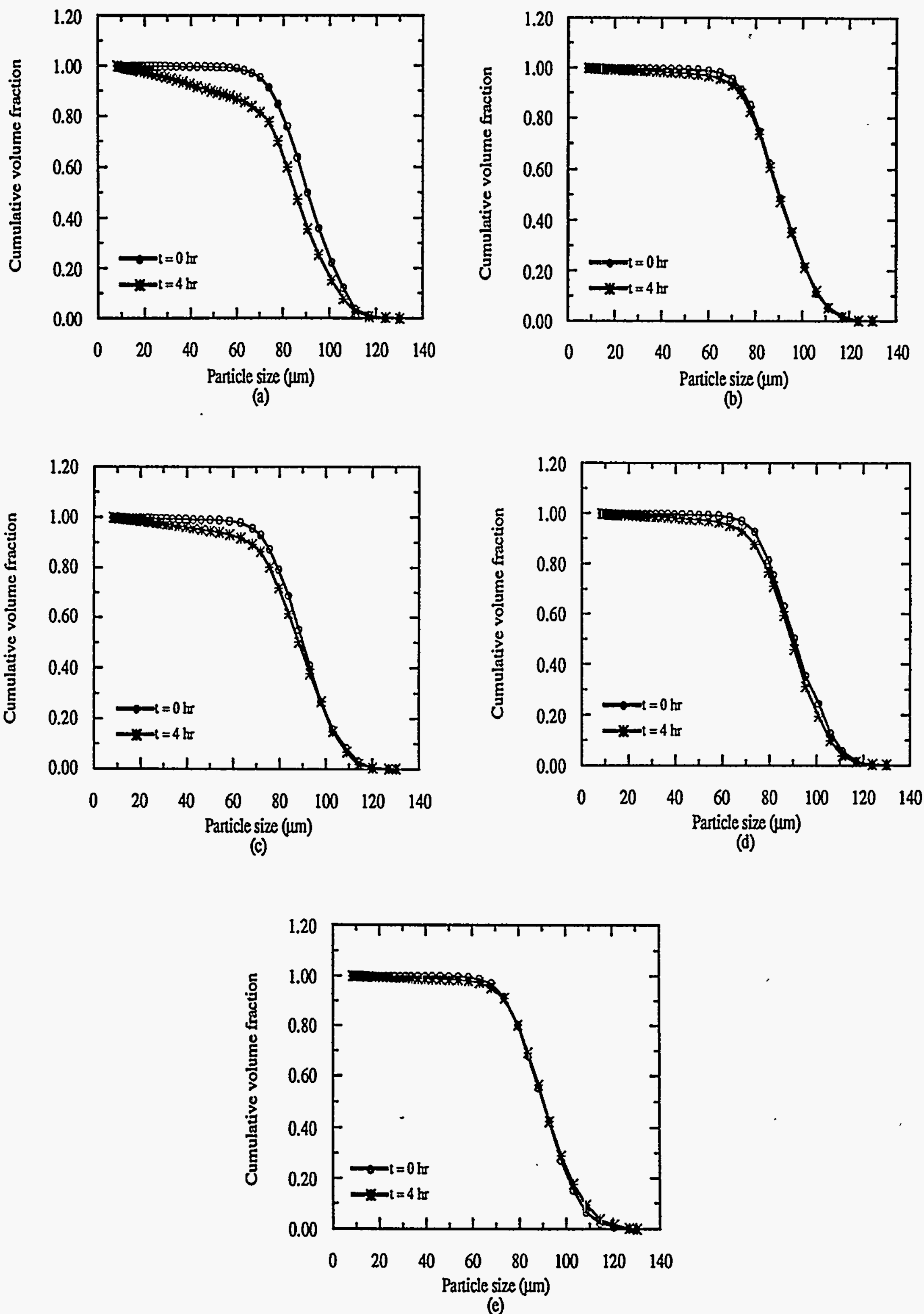


Figure 3-7. Cumulative volume fraction distritributions at the start and termination of hydrolysis for pure cellulases at a concentration of 0.1 mg ml^{-1}; (a) E_1, (b) E_2, (c) E_3, (d) E_5, (e) CBHI, and (f) no enzyme.

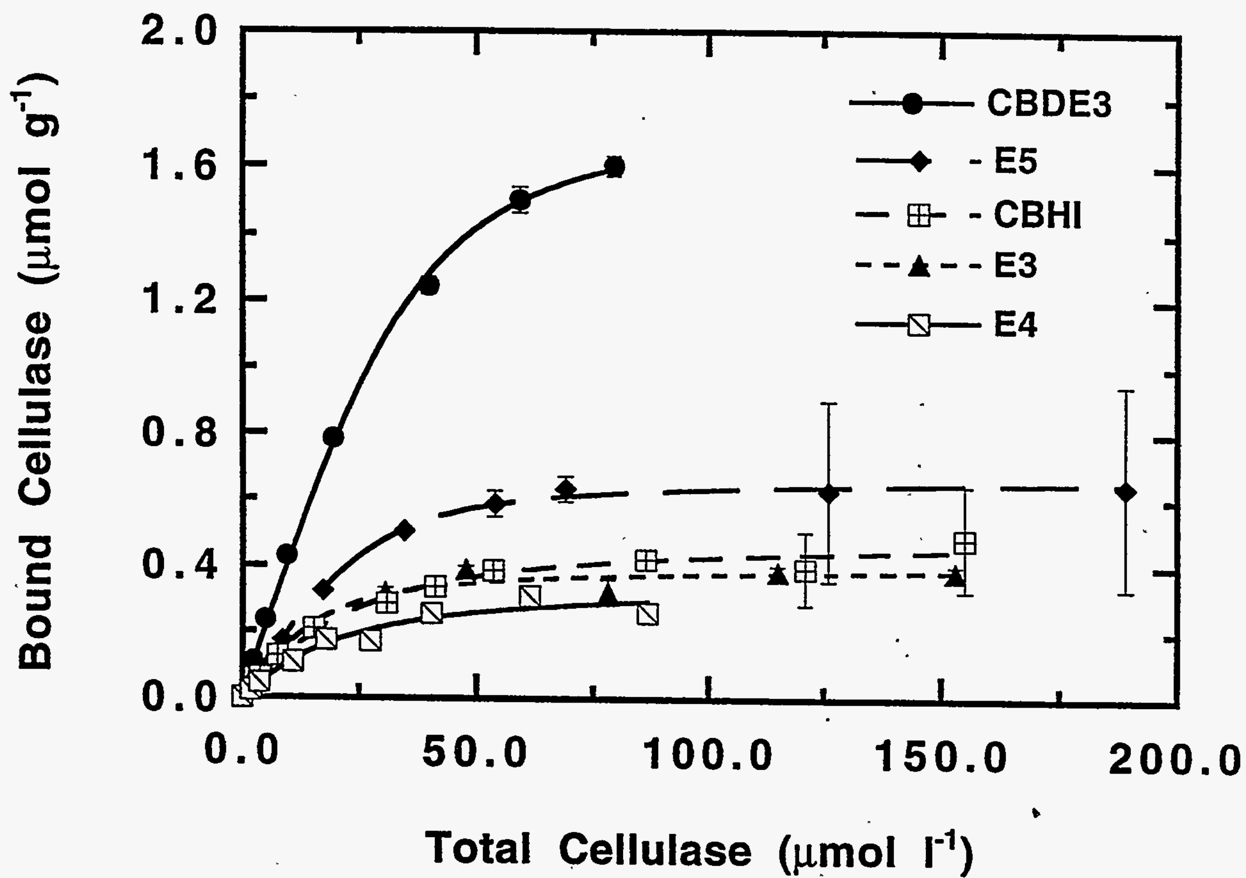


Figure 3-8. Binding isotherms of *T. fusca* E_3, $CBDE_3$, E_4, and E_5, and *T. reesei* CBHI on Avicel PH102.

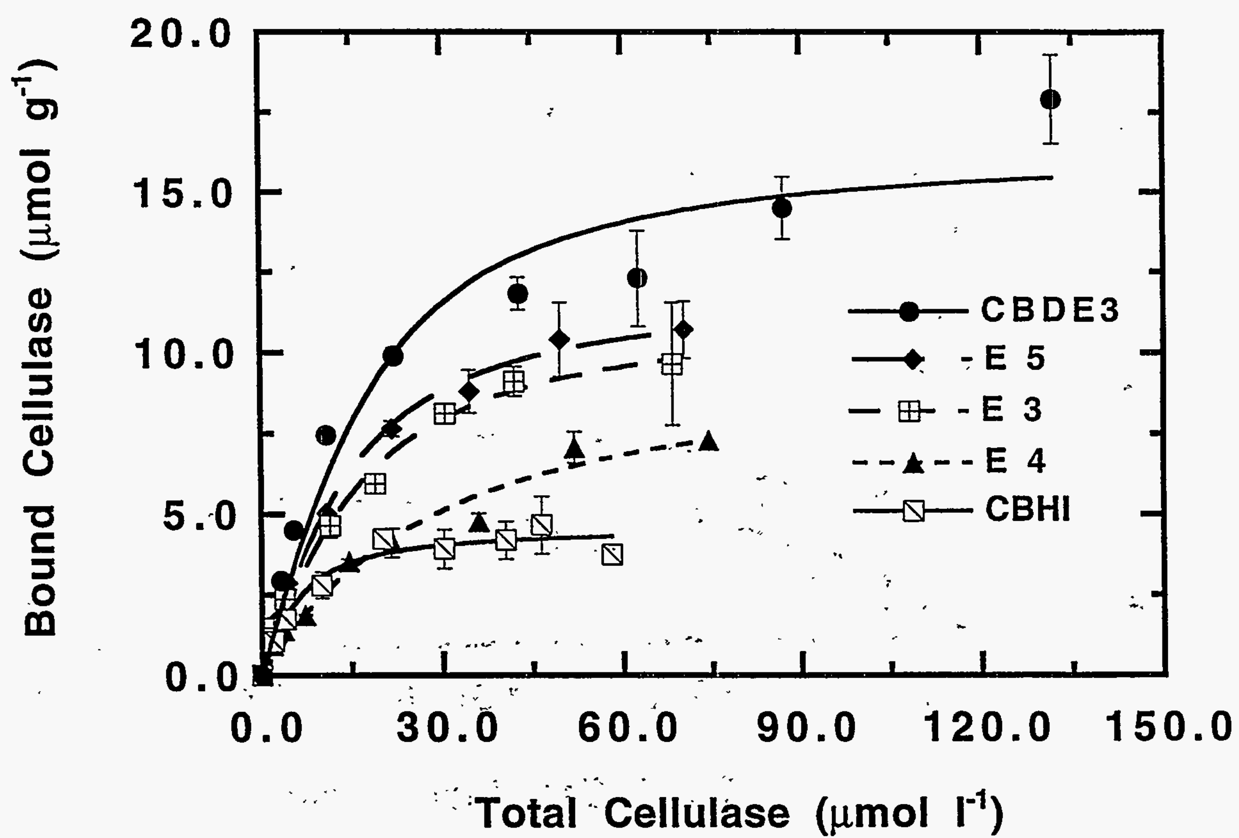


Figure 3-9. Binding isotherms of *T. fusca* E_3, $CBDE_3$, E_4, and E_5, and *T. reesei* CBHI on BMCC.

Table 3-6. Estimates of the Langmuir binding constants.

Cellulase	Avicel PH102		BMCC	
	$E_{b,m}$ ($\mu mol\ g^{-1}$)	K_a ($l\ \mu mol^{-1}$)	$E_{b,m}$ ($\mu mol\ g^{-1}$)	K_a ($l\ \mu mol^{-1}$)
E_3	0.40	0.20	11.4	0.10
$CBDE_3$	1.77	0.182	16.5	0.124
E_4	0.34	0.077	9.7	0.044
E_5	0.67	0.22	12.0	0.13
CBHI	0.48	0.09	4.6	0.28

Table 3-7. Summary of published cellulase binding constants.

Cellulase	Substrate	$E_{b,m}$		K_a		References
		$\mu mol\ g^{-1}$	$mg\ g^{-1*}$	$l\ \mu mol^{-1}$	$ml\ mg^{1*}$	
T. reesei CBHI	BMCC	4.2		0.43	6.9	65
C. fimi CenA	BMCC	3.1		0.40		67
C. fimi Cex		3.6		0.33		
T. reesei CBHI	Avicel	1.1		0.28		68
T. reesei CBHI	Avicel	0.29		na		69
T. reesei CBHII		0.24				
T. reesei CBHI	Avicel	0.10	5.2	na	na	39
T. reesei CBHII			8.6	na	na	
T. reesei EGI			5.1	na	na	
T. reesei EGII			4.1	na	na	
T. viride EndoI	Avicel	2.5	130	0.04	0.88	17
T. viride EndoIII		0.45	26	0.68	12	
T. viride EndoV		1.8	110	0.05	0.89	
T. viride EndoVI		0.08	4.1	0.18	3.4	
T. viride ExoII		0.11	6.6	0.30	5.0	
T. viride ExoIII		1.0	63	0.43	6.9	

na=Not available.

* Literature values reported in mass units; they were converted to molar units for comparison.

and independent variable that were found for the Avicel data. The apparent maximum binding capacity ranged from 4.5 μmol g^{-1} for *T. reesei* CBHI to15.2 μmol g^{-1} for $CBDE_3$.

Table 3-6 contains the estimates of the kinetic constants for each cellulase on both Avicel and BMCC. The differences between the apparent $E_{b,m}$s and the estimated $E_{b,m}$s are acceptable, ranging from 1-14%. In addition, the CBHI $E_{b,m}$s for Avicel and BMCC are comparable to values found in the literature (see Tables 3.6 and 3.7). The most striking feature of the estimates is the differences between the $E_{b,m}$s on Avicel and those on BMCC. The $E_{b,m}$s of E3, $CBDE_3$, E_4, E_5, and CBHI on BMCC are higher than those estimated for the Avicel systems by factors of 29, 9, 29, 18, and 10, respectively.[66] This major difference in the maximum extent of binding suggests that BMCC is considerably more accessibile to the cellulases than Avicel.

PERFORMANCE OF CELLULASE MIXTURES

<u>Filter Paper Activity of Cellulase Mixtures</u>

The major goal of this project was to develop cellulase mixtures with higher rates and extent of hydrolysis than those obtained with currently available cellulases. Most commercial cellulases are basically the culture supernatant obtained from growing a particular high yielding *T. reesei* strain. As noted earlier, the measurements of cellulase activity most often reported in the literature are CMC and filter paper activities. There are very few studies where filter paper or CMC activities are measured using the same method as employed by our research team. Fortunately, Beck, Johnson, and Baker[70] of the Tennessee Valley Authority, measured the filter paper activity of three commerical cellulases using the method of Ghose[63] employed by us. They reported specific filter paper activities that ranged from 168 - 285 FPU g^{-1}.

We have measured filter paper activity for several pure cellulases and cellulase mixtures and these are given in column 2 of Table 3-8.[71] As a measure of synergism, the filter paper activity was divided by the activity obtained for *T. fusca* E_3 (see column 3 of Table 3-8), which has the highest activity obtained for a pure cellulase. Several of our mixtures and our cellulase crudes have higher filter paper activities than those reported by Beck et al.[70] The mixture of *T. reesei* CBHI and *T. fusca* E3 and E5 and the mixture *T. reesei* CBHI and CBHII and *T. fusca* E_5 were the best engineered mixtures.[71] These two mixtures had filter paper activities comparable to that of our highly active *T. reesei* crude.

There are several factors that must be considered in evaluating the data presented in Table 3-8. First, commercial cellulases are culture filtrates. The culture filtrate is generally not further processed to separate the cellulases from other proteins. Our cellulase crudes have been subjected to some

Table 3-8. Filter paper activity of pure cellulases and mixtures of cellulase.

Cellulase	Specific Activity ($FPU\ g^{-1}$)	Ratio (SA/54)
E_1	5.0	
E_2	52.0	1.0
E_3	54.0	1.0
E_5	29.0	.5
CBHI	53.0	1.0
CBHII	10.0	.2
T.reesei crude	900.0	16.7
T. fusca crude	350.0	6.5
CBHII+E_3	60.0	1.1
E_1+E_2	77.0	1.4
E_1+E_3	104.0	1.9
CBHI+CBHII	140.0	2.6
CBHI+E1	180.0	3.3
E_2+E_3	210.0	3.9
E5+E_3	250.0	4.6
E_3+E_2+E_5	250.0	4.6
CBHI+CBHII+E_3	250.0	4.6
CBHI+CBHII+E_1	305.0	5.6
CBHI+E_2	310.0	5.7
CBHI+E_5	340.0	6.3
CBHI+CBHI+E_3	350.0	6.5
CBHI+E_3	430.0	8.0
CBHI+E_3+E_1	488.0	9.0
T. fusca crude+CBHI	540.0	10.0
E3+E_2+E_5+HAP3(E4+E?)	540.0	10.0
T.reesei crude+E_3	710.0	13.1
CBHI+E_3+E_2	710.0	13.1
CBHI+E_3+E_5	750.0	13.9
CBHI+CBHII+E_2	710.0	13.1
CBHI+CBHII+E_5	750.0	13.9

Table 3-9. Filter paper activities for cellulase mixtures at different pH.

Cellulases	Filter Paper Digested (%)	Filter Paper Activity pH 4.5 (FPU g^{-1})	pH 5.0 (FPU g^{-1})	pH 5.5 (FPU g^{-1})
T. fusca crude	4	100	190	300
T. reesei crude	4	1970	1970	1340
CBHI	6	160	140	120
E2	4	65	75	81
E3	4	53	61	61
E5	4	38	84	84
E3 + E2	4	350	460	810
E3 + E5	4	290	460	610
CBHI + E3	6	580	580	690
CBHI + E5	8	540	610	630
CBHI + E3 + E5	6	1,000	1,500	2,700
CBHI + CBHII + E5	6	2,200	2,200	2,200

purification which results in the higher activities reported in Table 3-8. Another factor to consider is that the mixtures reported in Table 3-8 have not been optimized. The mass fractions of the cellulases in the mixture can be altered so that the rate and extent of hydrolysis falls in an optimum range. This is discussed later in the report for three of the mixtures shown in Table 3-8. Finally, none of these mixtures contain a ß-glucosidase, which reduces the inhibitory effect of cellobiose on cellulase mixtures.

In addition to cellulase mass fraction, pH and the type of buffer influence the activities of our cellulase mixtures. An earlier study on the influence of pH on filter paper activity is shown in Table 3-9. *T. fusca* and *T. reesei* crudes, CBHI and the pure *T. fusca* endoglucanses are sensitive to pH. The *T. fusca* cellulases have comparable activities at pH 5.0 and 5.5. However, several mixtures containing both CBHI and *T. fusca* enzymes exhibit very little response to change in pH. Also, the filter paper activities reported in Table 3-9 are considerably higher than those reported in Table 3-8. The only difference between the two sets of data is that phosphate buffer was used in the measurements reported in Table 3-8 and sodium acetate buffer was used in the meaurements reported in Table 3-9. Given these results, we have decided to run our future experiments in sodium acetate buffer at pH 5.5.

<u>Fragmentation Activity of Cellulase Mixtures</u>

Fragmentation studies were conducted using the cellulase mixtures listed in Table 3-10 using the same particle size determination method presented earlier. All digestions were run in 25-ml round-bottomed flasks containing 0.7 g of sieved Avicel PH 102 in 14 ml of 0.05 M KPi buffer at pH 6.0 and 50 °C. Details of these experiments are presented in Walker at el.[31]

Cumulative volume fraction distributions at the start and termination of hydrolysis are presented in Figures 3-10a - 3-10f for mixtures of E_3 and the other *T. fusca* endoglucanases, and mixtures of *T. fusca* endoglucanases and CBHI, respectively. The data clearly show that the highest level of fragmentation was achieved by the three cellulase mixtures containing E_2, E_3, and CBHI. A significant shift in the cumulative volume fraction was also observed with mixtures of E_2 and E_3, E_2 and CBHI, E_3 and CBHI, and E_5 and CBHI.[31]

Mixtures of E_3 and *T. fusca* E_2 or E_5, as well as mixtures of these cellulases and CBHI, gave considerably higher rates of fragmentation than any pure cellulase. Cellulase mixtures containing CBHI and E_2 or E_5 had 23 and 74% more fragmentation activity than the mixtures of E_3 and E_2 or E_5. Also, the cellulase mixtures containing CBHI produced rates of fragmentation comparable to those obtained with *T. fusca* and *T. reesei* crude cellulases.[31] This would suggest that different synergistic mechanisms are at work when E_3 is mixed with these two cellulases than when CBHI is present.

Table 3-10. Enzyme concentration used in fragmentation experiments.

Cellulase mixture	E_2 (mg/ml)	E_3 (mg/ml)	E_5 (mg/ml)	CBHI (mg/ml)
E_2 & E_3	0.025	0.075		
E_5 & E_3		0.075	0.025	
E_2 & CBHI	0.025			0.075
E_3 & CBHI		0.050		0.050
E_5 & CBHI			0.025	0.075
E_2, E_3 & CBHI	0.020	0.040		0.040

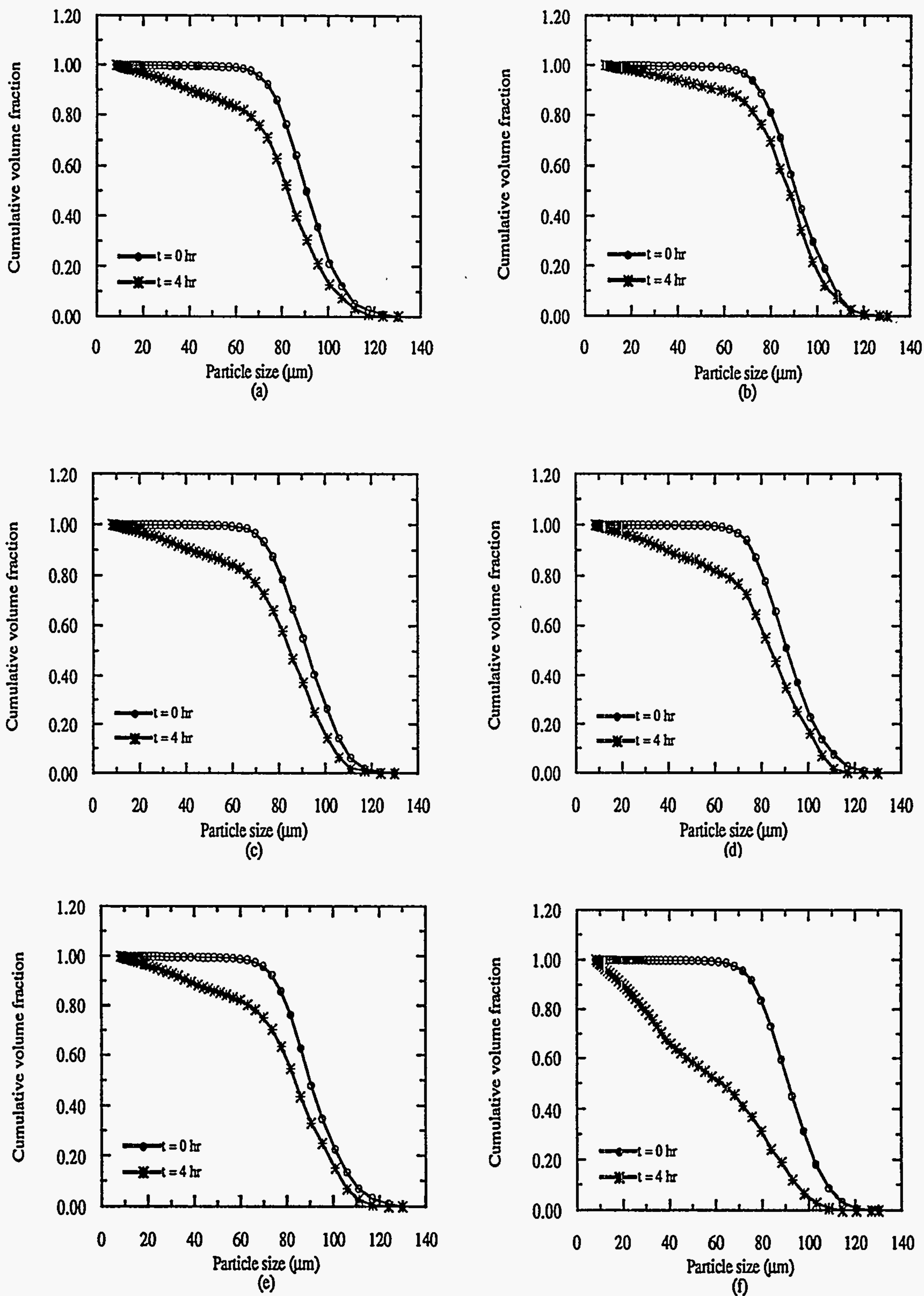


Figure 3-10. **Cumulative volume fraction distribution for mixtures of *T. fusca* endoglucanses with CBHI at the start and termination of hydrolysis; (a) E_2 and E_3, (b) E_5 and E_3, (c) E_2 and CBHI, (d) E_3 and CBHI, (e) E_5 and CBHI, and (f) E_2, E_3 and CBHI.**

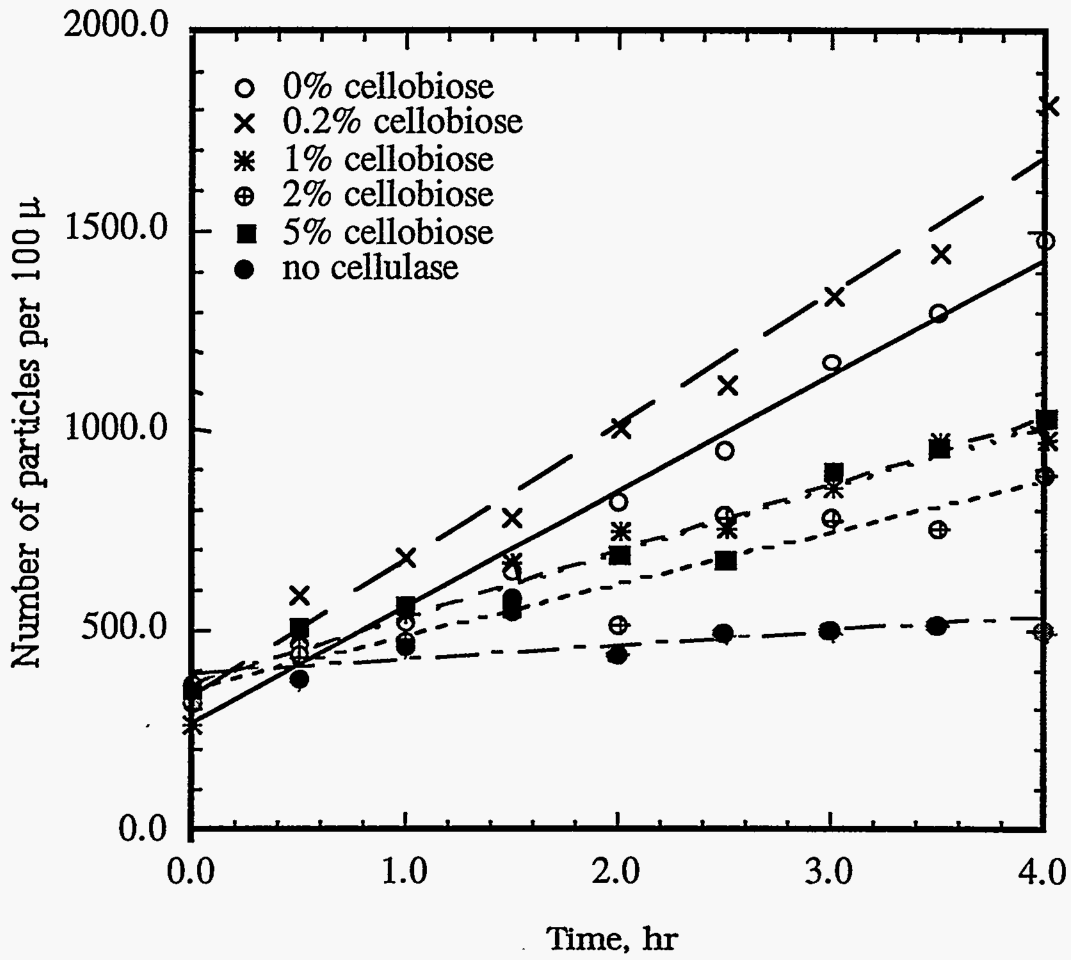


Figure 3-11. Cellobiose inhibition of fragmentation activity.

Inhibition of *T. fusca* Crude

The fragmentation assay also permitted a series of experiments to be conducted that verifed that *T. fusca* cellulases are strongly inhibited by cellobiose. These experiments were conducted using sieved Avicel PH 102 mixed with KPi buffer and cellobiose concentration ranging from 0 to 10%. The number of particles formed during hydrolysis was used to measure cellulase activity. As indicated in Figure 3-11, a cellobiose concentration of 1% reduces the number of particles produced in 4 hours by 50%, and even at 0.2% cellobiose we see a reduction of 17% in the number of particles produced in 4 hours. These results underscore the importance of identifying an active ß-glucosidase that is pH and temperature compatible with our cellulases to reduce the inhibitory effects of cellobiose.

Influence of Cellulase Mass Ratio on Maximum Extent and DSE

We investigated cross synergism between the major cellulases of *T. fusca*, and *T. reesei* CBHI and CBHII.[24] Our previous work has indicated that at least a tri-mixture of cellulases is necessary to effectively fragment and hydrolyze microcrystalline cellulase.[31,72] One mixture that looked particularly promising is a mixture of *T. fusca* E_5 and E_3, plus *T. reesei* CBHI. In this study, different mole fractions of these three cellulases were studied for reducing sugar production at 2 hr, degree of synergism, and cellulose binding. In addition, the effects of adding a ß-glucosidase into this cellulase system were studied.

Experiments using pure cellulases and mixtures of cellulases were incubated for 2 hr with a 4% Avicel solution. The cellulases were mixed together to achieve the desired mole fraction and then added to Avicel solution. All samples were in 2-ml screw-cap microcentrifuge tubes containing 20 mg of Avicel PH 102, the appropriate amount of enzyme, and enough 0.05 NaAc buffer, pH 5.5, to bring the total volume to 0.5 ml. At pH 5.5, CBHI retained 75% of its activity at pH 4.5. Hydrolysis was performed at 50°C. Two total cellulase concentrations, 8.3 and 12.2 μM, where investigated. The ratio of E_5 to the total cellulase concentration was varied from 0.00 to 0.72, while the ratio of CBHI to the total cellulase concentration was varied from 0.00 to 0.83.[24] In addition, these experiments were conducted with and without ß-glucosidase.

The percent conversions of cellulose to cellobiose are presented in Figure 3-12, with and without ß-glucosidase, for different $[E_5]_t/[E]_t$ and for $[E]_t$ equal to 8.3 and 12.2 μM. Without a ß-glucosidase, a maximum percent conversion of 9.8% was observed for an $[E_5]_t/[E]_t$ mole fraction of 0.21 and an $[E]_t$ equal to 8.3 μM. With the ß-glucosidase, a maximum percent conversion of 14% was observed for an $[E_5]_t/[E]_t$ mole fraction of 0.13 and an $[E]_t$ equal to 8.3 μM. The introduction of the ß-glucosidase resulted in a 40%

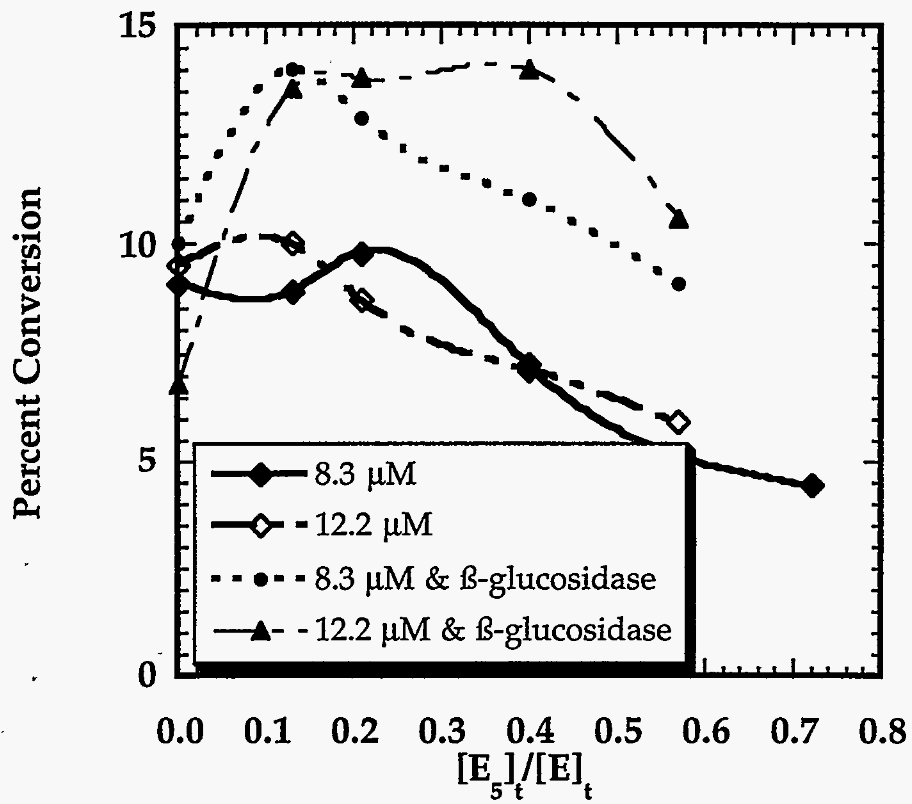


Figure 3-12. **Percent conversion for different $[E_5]_t/[E]_t$ mole fraction with and without a ß-glucosidase for two total cellulase concentations.**

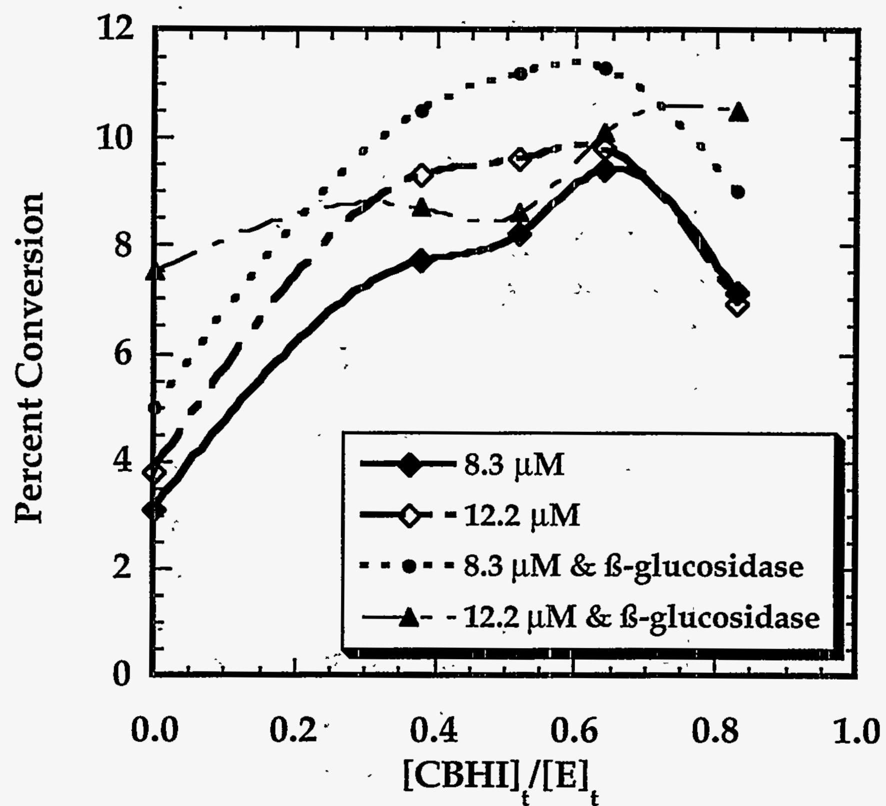


Figure 3-13. **Percent conversion for different [CBHI]$_t$/[E]$_t$ mole fraction with and without a ß-glucosidase for two total cellulase concentations.**

increase in the maximum percent conversion. Optimal endocellulase activity occurs in the $[E_5]_t/[E]_t$ mole fraction range 0.13 to 0.21, which is consistent with the endo fraction of *T.reesei* crude cellulase. Changing the component ratio on either side of this range causes a substantial decrease in extent. Finally, higher $[E]_t$ did not improve maximum percent conversion.

Presented in Figure 3-13 are the results obtain by fixing the $[E_5]_t/[E]_t$ ratio to 0.17 and varying the $[CBHI]_t/[E]_t$ mole fraction. Without a ß-glucosidase, a maximum percent of 9.4% was observed at $[CBHI]_t/[E]_t$ mole fraction of 0.64 and an $[E]_t$ equal to 12.2 μM. However, this was only a 4% increase over the maximum percent conversion observed with a $[E]_t$ equal to 8.3 μM. With the ß-glucosidase, a maximum percent of 14% was observed at a $[CBHI]_t/[E]_t$ mole fraction of 0.20 and $[E]_t$ equal to 8.3 μM.[24] The introduction of the ß-glucosidase resulted in a 45% increase in the maximum pecent conversion. These results confirm that at least three types of cellulases and a ß-glucosidase must be present in a mixutre to effectively hydrolyze microcrystalline cellulose. The three cellulases are an effective endoglucanase, such as *T. fusca* E_2 or E_5, and two types of exocellulase, such as *T. fusca* E_3 and E_4 or *T. reesei* CBHI and CBHII.

These results also suggest that the endo mole fraction is the most important one influencing the rate and extent of catalysis in cellulase-cellulose reactions.[24] With or without a ß-glucosidase, percent conversion values are relatively insensitive to change in the $[CBHI]_t/[E]_t$ ratio above 0.4 mole fraction as illustrated by Figure 3-13. Wood et al.[9] and Bothwell et al.[72] also demonstrated that exo-exo synergism is fairly insensitive to the fraction of the two exo enzymes in tri-mixtures.

Morphological Influence on the Performance of Cellulase Mixtures

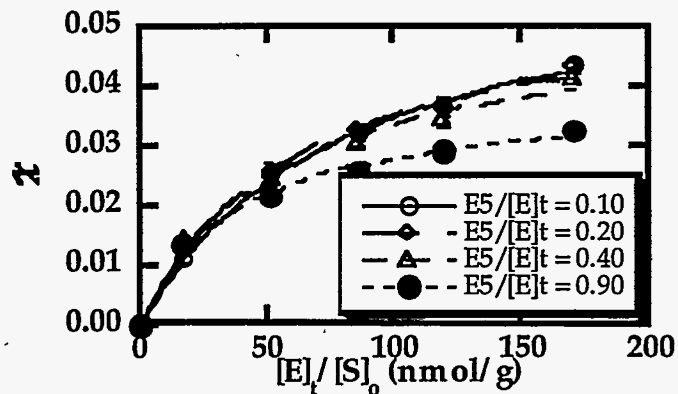
As reported earlier, Bothwell et al.[66] observed a much higher level of binding on BMCC compared to Avicel. The maximum adsorption levels, $E_{b,m}$, for all four cellulases and the $CBDE_3$ were 9 to 30 times higher on BMCC than on Avicel. The above observation is consistent with the results of studies that evaluated the pore structures of Avicel and BMCC using the nitrogen adsorption technique.[73,74] Using this method, Marshall and Sixsmith[74] and Lee et al.[73] reported a specific surface area of 10.0, and 5.4 $m^2\ g^{-1}$ for Avicel PH102, respectively, while the manufacturer of BMCC (Weyerhaeuser, WA) reported its specific surface area to be 200 $m^2\ g^{-1}$. This factor of 20-40 difference in reported surface areas compares favorably with the factor of 10-30 difference in the $E_{b,m}$s of the cellulases on Avicel as compared to their $E_{b,m}$s on BMCC. The binding data of Bothwell et al.[66] and the specific surface area results suggest that BMCC is more accessible to cellulase and thus should be more reactive to cellulases. This result has been confirmed by a recent set of experimental studies examining the role of cellulase concentration and mixture on the extent of hydrolysis.

These hydrolysis experiments were conducted with binary mixtures of E_3, E_4, and E_5. After enzyme addition, the reactors were immediately placed in a 50°C incubator and rotated end-over-end for 16 hours. For each binary mixture ratio, both the Avicel and bacterial microcystalline cellulose (BMCC) reactions were set up with the same enzyme stock solution and incubated at the same time. E_5/E_3 and E_5/E_4 mixtures were run at four different ratios, 10%, 20%, 40%, and 90% E_5. E_3/E_4 mixtures were run at 25%, 50%, and 75% E3. Triplicate reactors were set up for each ratio on Avicel and BMCC. All total cellulase concentrations were below the maximum binding capacity of E_4 on both Avicel and BMCC. Since E_4 has the lowest binding level on both substrates, competitive adsorption of cellulases in the synergistic mixtures should not have occurred.

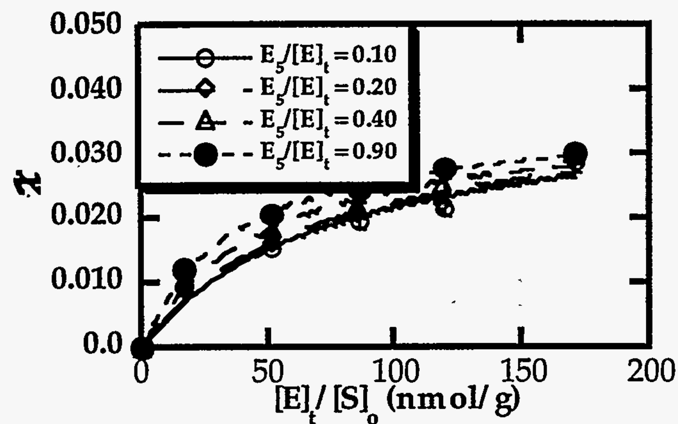
Presented in Figure 3-14a - 3-14c are the extent of conversion (mole of reducing sugar per mole of total reducing sugar represented by the cellulose), χs, for mixtures of E_3+E_5, E_4+E_5 and E_3+E_4, respectively. $[E]_t$s used in this experiment ranged from 0.68 to 6.80 nmol/ml. Each of the three mixtures exhibited a characteristically non-linear (saturating) behavior with increasing $[E]_t$ with χ_{max} of 0.044, 0.031, and 0.030 for E_3+E_5, E_4+E_5, and E_3+E_4 mixtures, respectively. In general, percent conversions were insensitive to changes in mole fraction, $[E_5]/[E]_t$ and $[E_3]/[E]_t$. This is particularly true for the E_3+E_4 mixture where varying $[E_3]/[E]_t$ from 0.25 to 0.75 resulted in no significant change in the extent of conversion. There was a little more sensitivity to molar fraction for the E_3+E_5 mixture where an E_5 mole fraction, $[E_5]/[E]_t$ of 0.90 resulted in approximately a 71% reduction in percent conversion at the higher $[E]_t$.

Presented in Figure 3-15a - 3-15c are the BMCC χs for mixtures of E_3+E_5, E_4+E_5, and E_3+E_4, respectively. $[E]_t$ for the three cellulase mixtures ranged from 0.25 to 2.44 nmol/ml (2.5-25% of the maximum binding of E_4). As was the case with Avicel, these mixtures exhibited a characteristically non-linear (saturating) behavior with increasing $[E]_t$ with χ_{max}s of 0.91, 0.84, and 0.98 for the E_3+E_5, E_4+E_5, and E_3+E_4 mixtures, respectively. In comparing these much higher χs on BMCC to the Avicel results, one must note that the cellulase binding capacity of the BMCC is much higher than that of Avicel and that the ratio $[E]_t/[S]_o$ is greater. However, what is more impressive is that these very high χ are obtained without the presence of a ß-glucosidase.

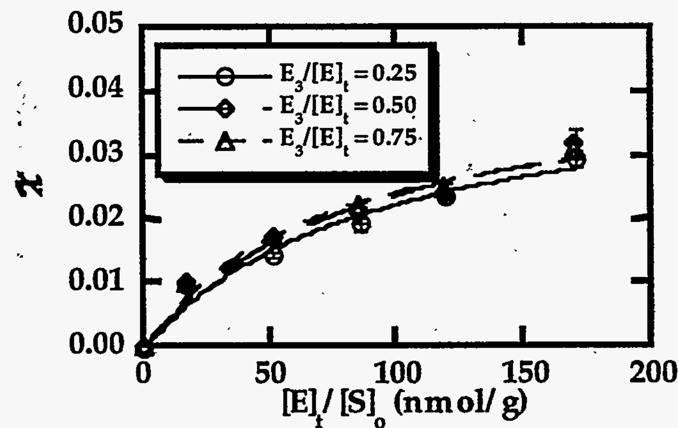
The BMCC experiments, in general, exhibited a greater senstivity of χ due to cellulase mole fraction as illustrated in Figure 3-15a and 3-15b. The one exception is the E_3+E_4 mixture, which exhibited the same insensitivity to changes in the mole fraction of the different cellulase components that was observed with this mixture on Avicel. Both the E_3+E_5 and E_4+E_5 mixtures exhibited a very significant reduction in χ when $[E_5]/[E]_t$ was 0.90. The χ_{max} observed for the E_3+E_5 mixture was 0.91 for a $[E_5]/[E]_t$ of 0.20. The E_3+E_5 mixture with a $[E_5]/[E]_t$ of 0.10 gave the second χ_{max} of 0.86. The χ_{max} by the E_4+E_5 mixtures was 0.84 with a $[E_5]/[E]_t$ of 0.9 and with a $[E_5]/[E]_t$ of 0.20.



(a)

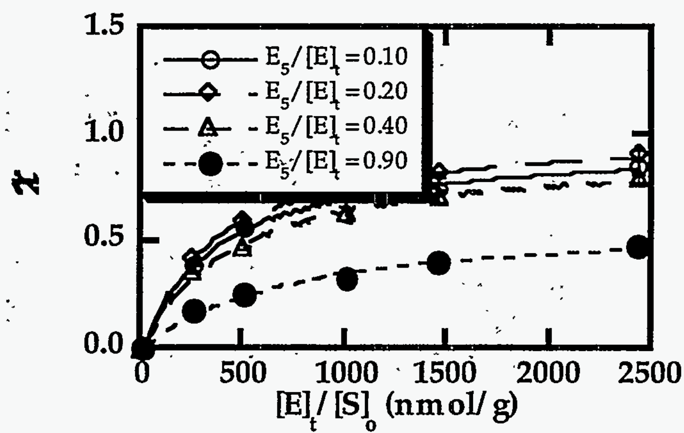


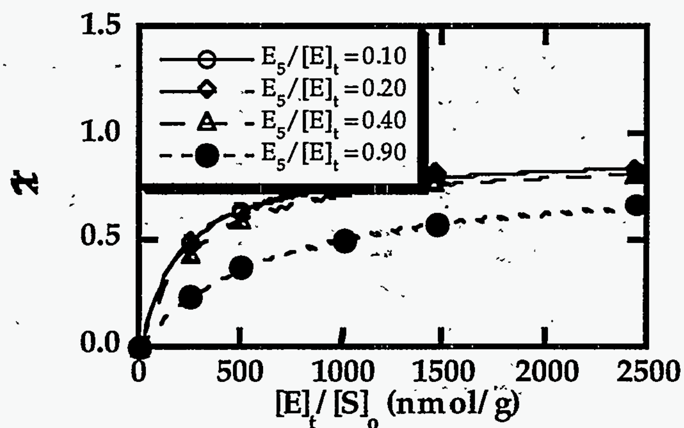
(b)



(c)

Figure 3-14. Avicel extent of conversions, χs, for mixture of (a) E_3+E_5, (b) E_4+E_5, and (c) E_3+E_4.





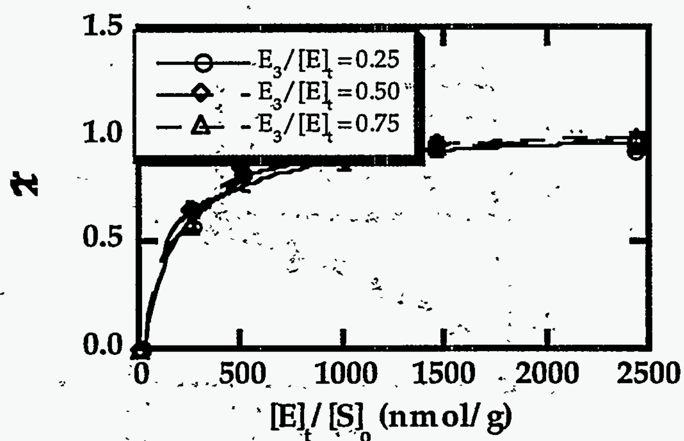


Figure 3-15. BMCC extent of conversions, χs, for mixture of (a) E_3+E_5, (b) E_4+E_5, and (c) E_3+E_4.

The observed increased in extent of hydrolysis due to level of binding is consistent with the earlier work by Stone et al.[20] and Grethlëin [21], and more recent work Kurakae et al.[23] There can be some enhancement gained from mixing cellulases, but the most important factor in determining extents and rates of hydrolysis is the accessibility of the cellulose and the digestability of the substrate.

Section 4

SUMMARY OF ACTIVITIES AND RESEARCH RESULTS

From studies with individual cellulases we know that the *T. fusca* cellulases E_2 and E_5 are very active endoglucanases that produce many random cuts in the cellulose chain. This is strongly supported by the fragmentation studies done with these two cellulases and the fraction of soluble versus insoluble reducing end obtained during cellulose hydrolysis.[31,71] *T. fusca* E_3 is an exoglucanase that attacks the cellulose from the nonreducing end of the polymer and has no fragmentation activity. This enzyme behaves like *T. reesei* CBHII in mixtures and its catalytic domain belongs to the same structural family as CBHII's catalytic domain. E_3 acts synergistically with E_2, E_5, and CBHI in both cellulose fragmentation and reducing sugar activities. E_4 is an active cellulase that gives synergism with all of the tested cellulases. It also acts synergistically with E_6, but this has only been shown in a reducing sugar assay. All of the *T. fusca* cellulases have a higher extent of binding on BMCC than on Avicel, and on BMCC they bind more tightly than CBHI.

From our mixing studies with Avicel we have learned that the endoglucanase mole fraction is the most important one influencing the rate and extent of catalysis.[24] With or without a ß-glucosidase, percent conversion values are relatively insensitive to change in the $[CBHI]_t/[E_3]_t$ (exo/exo) ratio. Wood et al.[9] and Bothwell et al.[72] also demonstrated that exo-exo synergism is fairly insensitive to the fraction of the two exoglucanases in bi-mixtures. In addition, these experiments demonstrated that increasing the total cellulase concentration, $[E]_t$, greater than 8.3 μM (0.21 μmol of cellulase per gram of cellulose), resulted in no significant change in the degree of synergistic effect or extent of hydrolysis.[24] More recent unpublished studies have confirmed this upper limit for Avicel. In addition, we have learned that the upper limit for BMCC is of the order of 1.0 μmol of cellulase per gram of cellulose -- approximately five times the upper limit for Avicel. The differences in the observed extent of conversions for BMCC and Avicel are consistent with the differences in the level of cellulase binding observed for these two substrates.

In the years since we started this project there have been considerable progress made in understanding the behavior of individual cellulases and mixtures of cellulases. We know from the DNA sequences and X-ray structures that there is considerable homology between the *T. fusca* cellulases and cellulases from other microbial systems, especially *Cellulomonas fimi*, which also produces six cellulases belonging to the the same set of families as the *T. fusca* cellulases. It appears that nature, through the process of evolution, has engineered a group of cellulases that are effective in meeting the energy needs of a broad spectrum of microorganisms that utilize cellulose as a carbon source. In hindsight, it is not surprising that we do not see large changes in the extent of hydrolysis by blending cellulases from different microbial systems. This result has been confirmed by the results obtained from many of our published

and unpublished cellulase mixture studies.[24,71,72] Although, there is considerable variability in the activity of commercial cellulases, mostly *Trichoderma reesei* crudes, we have yet to obtain a cellulase mixture that is much better than the best commercial preparation.

Despite our unsuccessful attempt to obtain better rates and extents of fermentable sugar production, we are still very optimistic about the industrial application of *T. fusca* cellulases. This optimism is driven by four factors.

- *T. fusca* cellulases are more temperature stable than cellulases from *T. reesei*, the microbial source for most commercial cellulases. This implies a great//er compatibility with industrial processes that operate at elevated temperatures

- The broad pH optimum of the *T.fusca* cellulases. In activity studies conducted at different pH, *T. fusca* E_3 retains 90% of its activity between pH 6 and 10, and 60% of its activity at pH 11. This retention of 90% of its activity under neutral or basic conditions provide a great deal of flexibility in reactor design and operation, and this characteristic opens up opportunities for other applications of these cellulases

- The cloning and site-direct mutagenesis expertise developed at Cornell that supports our efforts to engineer cellulases with higher specific activities. We have gained considerable insight into the structure of the E_2 catalytic domain and the mechanism of substrate binding and subsequent catalysis. We have the tools to change specific amino acid residues in the catalytic domain and to assess how these changes influence catalysis.

- There is considerable industrial interest in our enzymes. Several companies are testing our enzymes to assess potential commercial applications. These companies have not shared their perspective on the commercial applications of our enzymes, but the strong interest leads us to be optimistic.

It is difficult to assess the economics of our cellulases without a specific application. We know that for ligno-cellulose conversion to ethanol the advantage that our cellulases offer, relative to current commercial cellulase preparation, is higher temperature stability and broader pH optimum. Both of these can have a significant effect on the capital cost of the enzyme reactor and the effectiveness of the hydrolysis process. Unfortunately, we do not possess the plant design and engineering economic models to assess how big an impact our cellulases can have on capital and operating costs. The biotechnology companies producing enzymes for industrial use are in a much better position to perform this assessment.

Section 5

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