THE USE OF CELLULASE ADSORPTION AND RECYCLE AS A MEANS OF ASSESSING AND ENHANCING THE HYDROLYSIS OF CELLULOSIC SUBSTRATES

by

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ABSTRACT

In the past, two main approaches have been used to try to enhance the enzymatic hydrolysis step of a lignocellulosics residue-to-ethanol process. In one approach, cellulosic substrates can be pretreated to increase their susceptibility to cellulases. In an alternative approach, the cellulases are recovered and re-used in order to lower the cost of the enzymes required for the hydrolysis step.

The increased susceptibility of pretreated substrates has been attributed to an increase in the surface area accessible to cellulases, as the enzymes must first adsorb to the substrate in order to carry out hydrolysis. However, it has not been clearly demonstrated that the amount of cellulase adsorbed to different substrates is the primary factor determining the hydrolysis rate. In the first part of this study, CBD<sub>ceX</sub>, the nonhydrolytic cellulose-binding domain of a Cellulomonas fimi cellulase, was evaluated as a probe to measure the surface area available for cellulase adsorption. The adsorption isotherms of CBD<sub>ceX</sub> and Celluclast, a commercial cellulase mixture, were determined using various cellulosic substrates. It was found that the adsorption of both protein preparations could be represented by the Langmuir isotherm. P<sub>max</sub>, the maximal adsorbed protein, was calculated in order to compare the amount of surface area in each substrate that was available to CBD<sub>ceX</sub> or Celluclast. The surface areas of nine different substrates, as measured by CBD<sub>ceX</sub> and Celluclast adsorption, were found to be different in all cases except one.

In the second part of this study, the relationship between cellulase adsorption and the initial hydrolysis rate was examined. The accessibility of the various substrates to cellulases and the corresponding hydrolysis rates were measured. There was no apparent correlation between the amount of enzyme...
adsorbed and the initial hydrolysis rate. Specific hydrolysis rates were also found to differ among the various substrates. It was apparent that both accessibility to the cellulases and the specific hydrolysis rates were changed by chemical and physical pretreatment of the substrate.

The third part of this study focussed on cellulase recycling as a means of reducing the amount of enzyme required for cellulose hydrolysis. Three cellulase recycling strategies were evaluated to determine their efficiencies after 5 rounds of hydrolysis. The cellulases were recovered from the residual substrate containing adsorbed enzymes, the reaction mixture consisting of both the residual substrate and the supernatant, and the reaction mixture consisting of the supernatant and the non-cellulosic residue obtained after complete hydrolysis of the cellulose in each substrate. The effect of lignin on recycling was assessed by using both steam-exploded birch (WB, 32% lignin) and delignified steam-exploded birch (PB, 4% lignin) as the substrates. The activity of the recovered enzymes was assessed by measuring the amount of reducing sugars obtained after each hydrolysis round. The only strategy that resulted in the complete recovery of all of the cellulase activity for 4 hydrolysis rounds was when the cellulases from the supernatant and the non-cellulosic residue were recycled together after complete hydrolysis of the PB substrate. When either of the other two recycling strategies was used, the recovered cellulase activity decreased with each recycling step. Also, when these two recycling strategies were used, the recovered activities did not correspond to the activities expected from the amount of cellulase protein recovered during recycling. In all 3 recycling strategies studied, lower cellulase activity was recovered from the substrate with the higher lignin content (WB).
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1. INTRODUCTION

The enzymatic hydrolysis of cellulose to glucose not only yields valuable materials for short-term consumption, it should also yield long-term environmental benefits. Glucose, in its unrefined form, is the starting material for the production of single-cell protein, organic acids, and alcohols. The conversion of glucose to ethanol, in particular, is a topic of intensive research. Interest in the conversion of biomass into ethanol was originally sparked by rising oil prices in the 1970s, which led many countries to investigate alternative energy sources. Current interest, however, appears to be motivated by concerns over the harmful effects of fossil fuel combustion, which contribute significantly to global warming and air quality. The use of ethanol-blended fuels should result in the release of less harmful engine emissions than are currently released from engines using non-blended gasolines (Wyman and Hinman, 1990). There is also a growing awareness that the world's supply of fossil fuels is limited. An alternative energy source is necessary to replace this supply once it is depleted. Lignocellulosic materials present a reasonable alternative energy source, since these materials are both abundant and renewable. Furthermore, these substrates are readily available in the form of industrial wastes. Vast quantities of these wastes accumulate from the agricultural, food-processing, and the pulp and paper industries each year. Their utilization as feedstocks for the production of ethanol would take advantage of materials which would otherwise be burned or used as landfill.

The hydrolysis of cellulose to glucose is the rate-limiting step in the
production of ethanol from cellulosic substrates, since these materials are hydrolyzed only slowly by cellulases (Fan et al., 1987). Extensive research has been directed towards improving the efficiency of enzymatic cellulose hydrolysis processes (Walker and Wilson, 1991). However, despite many years of research, a cost-effective bioconversion process for the enzymatic conversion of lignocellulosics has not been developed. This is partly due to the recalcitrance of the substrate. Native cellulosic substrates are highly resistant to degradation, and therefore require pretreatment to increase their digestibility by cellulases (Hayn et al., 1993). The factors which influence the rate and extent of their degradation must be more clearly defined in order to develop effective pretreatment strategies for increasing the enzymatic susceptibility of these substrates. The complexity of the enzymatic hydrolysis reaction has also proven to be a major obstacle to various attempts to try to increase the rate of cellulose hydrolysis. The cellulases themselves are a complex mixture of many enzymes which interact in cellulose hydrolysis (Enari and Niku-Paavola, 1987; Wood, 1992). Their mode of action is still not fully understood. A better understanding of the action of cellulases would provide a basis for optimizing their efficiency in cellulose hydrolysis. The reaction is further complicated by changes which occur to the structure of the substrate as the reaction progresses (Lee and Fan, 1983). Therefore the influence that these changes have on the hydrolysis reaction must also be understood in order to optimize cellulose bioconversion processes.
1.1 THE HYDROLYTIC RESISTANCE OF LIGNOCELLULOSIC SUBSTRATES

A fundamental obstacle to efficient cellulose hydrolysis is the structure of the substrate. The purpose of wood in nature is to provide structure and support to trees, and as would be expected, wood is highly resistant to enzymatic breakdown. This is partly due to the close association of the highly ordered cellulose skeleton with the two other major components of wood, lignin and hemicellulose.

Cellulose is the most abundant component of wood, constituting between 40 and 45% of the dry weight of the wood (Sjostrom, 1993). A single cellulose molecule consists of about 10,000 glucopyranose units linked together by $\beta(1\to4)$ glycosidic bonds (Fan et al., 1987). Individual cellulose chains in parallel orientation with one another are held together by intermolecular hydrogen bonds to form the smallest unit of a cellulose fiber, the elementary fibril (Sjostrom, 1993; Fan et al., 1987). The hydrogen bonding may be extensive in the highly ordered, or crystalline, regions. Amorphous regions in cellulose are composed of less ordered regions where the cellulose chains are held by fewer hydrogen bonds. The crystallinity of cellulose, which ranges between 50% and 90% in different wood species (Sjostrom, 1993), has significant implications for the enzymatic hydrolysis of cellulose, as it has been proposed that highly crystalline materials are degraded much more slowly than amorphous materials (Sasaki et al., 1979, Fan et al., 1980, 1981). In the cell wall, elementary fibrils are organized into microfibrils. Each microfibril consists of a bundle of elementary fibrils cemented together by hemicellulose. The microfibrils, in turn, are
associated with hemicellulose and lignin to form a rigid, protected structure that is highly resistant to hydrolytic attack (Dekker, 1989).

Hemicellulose is a heteropolymer consisting of various linked sugars. At different sites, the linear sugar backbone is substituted with acetyl or glucuronic acid groups. In the wood cell, hemicellulose is dispersed around the cellulose microfibrils. The association of the hemicellulose with the cellulose plays a large role in maintaining the rigidity of wood in trees by holding together the individual microfibrils in the cellulose fiber.

Lignin is the other major non-cellulosic component found in wood. Lignin, a complex polymer, is composed of phenylpropane units linked together by carbon-carbon or ether bonds (Sjostrom, 1993), and it is generally found around the polysaccharide components in the cell wall. Most of the lignin is only physically associated with these components. However, some lignin may also be linked covalently to the hemicellulose component (Sjostrom, 1993). The lignin coating the cellulose fibers provides a further barrier to hydrolytic attack by cellulases Gharpuray et al., 1983; Sinitsyn et al., 1991).

1.2 THE COMPLEXITY OF CELLULASE SYSTEMS

1.2.1. Classification of Cellulases

It is well-known that the hydrolysis of crystalline cellulose requires the action of multiple cellulase components. In their C₁-Cₓ theory, Reese et al. (1950) originally proposed that a nonhydrolytic factor, C₁, was required to "activate" cellulose before the hydrolytic Cₓ factors could degrade it. Subsequent
work by other researchers could not demonstrate conclusively that $C_1$ was nonhydrolytic (Fan et al., 1987). Furthermore, as better methods for the purification of cellulases were developed, it became evident that previously isolated $C_1$ and $C_\alpha$ factors were composed of several enzyme components. Many of these components were found to differ with respect to their ability to hydrolyze different cellulosic substrates. This led to the development of the commonly used classification system based on the activity that cellulases exhibited on various substrates (Wood and Bhat, 1988).

In this system, cellulases which could hydrolyze the internal glycosidic linkages between the carboxymethyl substituents on carboxymethylcellulose (CMC) were classified as endoglucanases. Alternatively, exoglucanases should only degrade cellulose from the ends of cellulose chains, and should therefore exhibit only low activity on CMC, since the substituents on CMC prevent the exoglucanases from proceeding along the cellulose chain. However, exoglucanases should exhibit high activity on substrates such as Avicel. $\beta$-glucosidases, a third class of enzyme, show high activity towards cellobiose, which they degrade to glucose (Wood and Bhat, 1988).

It was subsequently found that many cellulases exhibited different degrees of activity towards different substrates (Wood, 1992). Thus, these enzymes could not be classified strictly as endoglucanases or exoglucanases. Nevertheless, many researchers continued to use the established classification system since the enzymes could usually be said to exhibit activities that were more characteristic of either endoglucanases or exoglucanases. Van Tilbeurgh and Claeyssens (1985) have also suggested that a series of small chromogenic
or fluorogenic substrates could be used to differentiate more clearly among the different enzymes based on the products they release from these substrates. This classification system should better distinguish between enzymes with overlapping specificities under the old classification scheme. However, because these substrates are soluble, they may not be representative of insoluble substrates such as cellulose. Thus, the data obtained using these model substrates should not be interpreted as directly indicative of the mode of action of cellulases on cellulose.

1.2.2 Structures and Enzymatic Properties of Cellulases

Cellulases are produced by bacteria, actinomycetes, and fungi. However, earlier studies focussed mainly on fungal cellulases. For many years, cellulolytic bacteria were largely ignored because their extracellular cellulases hydrolyzed crystalline cellulose poorly compared to the cellulases of cellulolytic fungi. This was attributed to the lack of exoglucanase in the cell-free culture filtrates of many cellulolytic bacteria, since effective hydrolysis of crystalline cellulose required both endoglucanases and exoglucanases (Wood and McCrae, 1972). However, it has been proposed that the exoglucanases of many bacteria may be cell-wall bound, as crystalline cellulose can be efficiently utilized by bacteria during cultivation (Coughlan and Ljungdahl, 1987).

Emphasis has also been placed on fungal cellulases because of the large amounts of extracellular protein produced by fungi, such as Trichoderma reesei, when compared to bacteria. More recently, however, interest in bacterial cellulases has grown, partly because techniques in molecular biology have made
it possible to produce large amounts of cellulases. The cloning of several cellulases from *Cellulomonas fimi* (Gilkes et al., 1991) has made the *C. fimi* cellulase system one of the best characterized bacterial cellulase systems.

### 1.2.2.1 *Trichoderma reesei* Cellulases

The exact number of cellulases produced by *T. reesei* is still not known because of the difficulties researchers have had in purifying the enzymes. Two cellobiohydrolases, CBH I and CBH II, and at least six endoglucanases have so far been identified (Enari and Niku-Paavola, 1987; Kubicek, 1992) from the *T. reesei* cellulase system. Only small amounts of β-glucosidase have been detected in the culture filtrate.

The different cellulases in this system range in size from about 30 kDa to about 80 kDa, and in pI from about 4 to 7.5. Several of the cellulases have been shown to possess a heavily O-glycosylated region, whose function is unknown. However, the non-glycosylated form of CBH I has been shown to retain its activity on soluble substrates (Knowles et al., 1988). Thus, glycosylation does not appear to be involved in catalysis. A role in enzyme secretion or in protection of the protein against proteolysis has been suggested (Kubicek, 1992).

Six of the *T. reesei* cellulases have been cloned: CBH I (Shoemaker et al., 1983), CBH II (Teeri et al., 1987), EG I (Penttila et al., 1986), EG II (Saloheimo et al., 1988), EGIII (Ward et al., 1993), and EG V (Saloheimo et al., 1993). Of these, four (CBH I, CBH II, EG I, EG II) have been characterized in some detail. In these four cellulases, a region of about 35 to 40 amino acids,
which are about 70% conserved, has been found (Knowles et al., 1989). This region may be found either at the C terminal (CBH I, EG I) or the N terminal (CBH II, EG II) of the proteins. The highly glycosylated region links this terminal peptide, which is thought to be involved in cellulose binding, to the rest of the protein (Knowles et al., 1989).

It is now generally accepted that at least these four enzymes are organized into discrete domains. X-ray diffraction studies have shown that CBH I has a large globular head connected to a flexible tail by a serine- and threonine-rich hinge (Schmuck et al., 1986). It has been suggested that the conserved tail corresponds to the cellulose-binding domain (CBD), and that the head contains the catalytic site of the cellulase. Removal of the tail by limited proteolysis results in reduced affinity of the CBH I and CBH II cores (heads) to Avicel, yet both cores retain their activities on soluble substrates (Tomme et al., 1988). The core protein of EG II (formerly called £G·III), isolated from the culture filtrate of T. reesei, has also been shown to have full activity on carboxymethylcellulose but had reduced binding and activity on Avicel compared to intact EG II (Stahlberg et al., 1988).

1.2.2.2 Cellulomonas fimii Cellulases

In the C. fimii cellulase system, an exoglucanase/xylanase (Cex) and three endoglucanases (CenA, CenB, and CenC) have been cloned and characterized. CenA and Cex have molecular weights of about 50 kDa (Gilkes et al., 1988), whereas CenB and CenC are about twice as large, with molecular weights of 110 kDa and 130 kDa, respectively (Meinke et al., 1991; Coutinho et al., 1993).
The organization of at least two Cellulomonas fimi cellulases closely resembles that of the four main Trichoderma reesei cellulases (CBH I, CBH II, EG I, EG II). Small Angle X-ray Scattering (SAXS) analysis has shown that the nonglycosylated form of CenA, produced in E. coli, is roughly tadpole-shaped, and is similar to the Trichoderma cellulases in appearance (Pilz et al., 1990). This enzyme has a compact catalytic head region, a linker region rich in proline and threonine, and an N-terminal cellulose-binding domain (CBD). A cellulose-binding region showing 50% amino acid homology to the CenA CBD has also been found at the C terminal of Cex. In Cex, this domain is also joined by a proline- and threonine-rich linker region to the rest of the enzyme. It is likely that Cex is also tadpole-shaped, since the organization of this enzyme is similar to that of CenA (Pilz et al., 1990).

The other cellulases of C. fimi are organized differently. Endoglucanase B (CenB) has five discrete domains, one of which is involved in cellulose binding, and one which is the catalytic domain. Endoglucanase C (CenC) is also unusual, in that its catalytic domain is flanked by two different amino acid repeat sequences at either end. While the N-terminal sequence mediates cellulose binding, the role of the C-terminal repeats is unknown (Coutinho et al., 1992).

Some studies have suggested another role for the CBDs in addition to their function in anchoring the enzyme to cellulose. CenA has been shown to disrupt cellulose structure, rendering it more accessible to subsequent binding by a fluorescently labelled CBD (Din et al., 1991). The authors suggested that the CBD of CenA may play the role of the C1 component in the theory proposed by Reese et al. (1950). In a recent study (Coutinho et al., 1993), it was shown
that the activity of the CenA catalytic domain (p30) was modulated by the cellulose-binding domain attached to it. When the CBD of CenA was covalently joined to p30, the activity of the whole enzyme on crystalline substrates such as bacterial microcrystalline cellulose (BMCC) and Avicel was increased compared to the activity of p30 alone. This was not observed on highly accessible substrates such as acid-swollen cellulose or carboxymethylcellulose. However, when the CBD from CenC was covalently joined to p30, the activity of p30 was increased on Avicel, but not on BMCC. These results also seemed to suggest a role other than substrate binding for cellulose-binding domains.

1.3 MODE OF CELLULASE ACTION DURING CELLULOSE HYDROLYSIS

For many years, it was thought that the hydrolysis of crystalline cellulose was initiated by endoglucanase attack (Wood and McCrae, 1972). In this model, the cleavage of internal glycosidic linkages along the cellulose chain exposed new chain ends on the cellulose for the action of exoglucanases. Hydrolysis at these reaction sites resulted in the removal of cellobiose residues which, in turn, generated new sites for endoglucanases by exposing previously inaccessible glycosidic linkages in the interior of the cellulose fiber. The cellobiose released into the reaction supernatant by these enzymes was subsequently degraded to glucose by the action of β-glucosidase. Thus, crystalline cellulose was efficiently degraded by the synergistic interaction of these cellulases.

Synergism occurs when the observed hydrolytic activity from a mixture of cellulases is greater than the sum of the activities expected from the individual
cellulases acting alone. The synergism that has been observed between endoglucanases and exoglucanases in many studies (Woodward, 1991) is consistent with the hydrolysis model described. However, synergism between CBH I and CBH II has also been observed (Fagerstam and Pettersson, 1980; Henrissat et al., 1985). The results of further studies suggested that CBH II showed endoglucanase activity (Niku-Paavola et al., 1986; Kyriacou et al., 1987). In light of this, Enari and Niku-Paavola (1987) have proposed a new model for the hydrolysis of crystalline cellulose.

In this model, it was suggested that the initial attack on crystalline cellulose involved synergistic action by CBH I and CBH II. The soluble cellodextrins released by these enzymes were then hydrolyzed by endoglucanases to cellobiose, which was subsequently degraded to glucose by β-glucosidases.

In subsequent work, however, Wood et al. (1989), proposed that at least three enzymes were required to hydrolyze crystalline cellulose. In their study, it was found that a CBH II preparation from P. pinophilum was contaminated with small amounts of an endoglucanase, which could be removed by affinity chromatography on a column of p-aminobenzyl-1-thio-β-D-cellobioside. After removal of the contaminating endoglucanase from CBH II, no synergism was observed between CBH I and CBH II. In fact, synergism was only observed when CBH I and CBH II were mixed with one of several endoglucanases. Thus, it is apparent that a generally accepted model for cellulose hydrolysis is far from being established.
1.4 DISTRIBUTION OF CELLULASES DURING CELLULOSE HYDROLYSIS

Adsorption of the cellulases to cellulosic substrates is a critical step in the hydrolysis of crystalline cellulose. Several studies have found that the cellulases adsorb quickly to the substrate, and then begin to desorb into the reaction supernatant once the hydrolysis begins (Lee and Fan, 1983; Nutor and Converse, 1991). The amount of cellulase enzyme initially adsorbed to the substrate has received considerable attention because the hydrolysis rate is thought to be dependent on the amount of cellulase adsorbed to the substrate (Lee and Fan, 1983; Wald et al., 1984; Converse et al., 1990). However, a decrease in the effectiveness of the adsorbed cellulases has been observed at high concentrations of adsorbed enzyme (logen Corp. Final Report, 1990; Converse et al., 1990).

As the hydrolysis reaction progresses, the cellulases are gradually desorbed from the substrate. A recent study has shown that the proportion of each adsorbed cellulase component relative to the others is maintained throughout the hydrolysis of a birch substrate (Yu et al., manuscript submitted). Thus, the composition of the cellulases involved in hydrolysis does not likely change during the reaction.

At the end of the hydrolysis reaction, a portion of the cellulases may remain adsorbed to the substrate residue left after complete hydrolysis. In several studies, it was found that about 30% to 60% of the initially added cellulase activity remained adsorbed to the lignin residue after complete hydrolysis (Deshpande and Eriksson, 1984; Ooshima et al., 1990). The
cellulases adsorbed to lignin residues has been shown to be only partly recoverable (Girard and Converse, 1993). Thus, the irreversible adsorption of cellulases to lignin may also have implications for cellulase recovery.

1.5 POSSIBLE FACTORS AFFECTING CELLULOSE HYDROLYSIS KINETICS

The initial phase of cellulose hydrolysis is characterized by the rapid release of soluble sugars. Then, as hydrolysis proceeds, the rate of sugar production decreases and eventually levels off. Several studies have shown that the specific hydrolysis rate declines rapidly with increased conversion of the substrate (Lee and Fan, 1983; Wang and Converse, 1991). Furthermore, the extent of substrate conversion varies with the substrate used for the reaction. These characteristics of a typical hydrolysis reaction are partly due to the intrinsic structural features of the substrate and may also reflect changes that occur to the substrate as the reaction progresses. They may also reflect the susceptibility of the cellulases to denaturation or inhibition (Dekker, 1989).

1.5.1 Substrate-related Factors Affecting Hydrolysis

The susceptibility of cellulosic substrates to enzymatic hydrolysis is thought to depend on various structural features of the substrate. These include the crystallinity of cellulose (Sasaki et al., 1979; Fan et al., 1980, 1981), the degree of cellulose polymerization (Puri, 1984), the lignin content (Gharpuray et al., 1983; Sinitsyn et al., 1991), and the surface area accessible to cellulases (Thompson et al., 1992). The importance of each of these factors in determining
the susceptibility of the substrate is still unclear. However, the surface area of
the substrate available to cellulases has frequently been suggested as the most
influential factor determining the hydrolysis rate because adsorption of the
cellulases to the cellulose is a critical step in the reaction. A strong correlation
between accessible surface area and the hydrolysis rate has been obtained in
several studies (Gharpuray et al., 1983; Sinitsyn et al., 1991; Thompson et al.,
1992), although these surface areas were determined under conditions that did
not accurately represent the adsorption of cellulases in hydrolysis reactions.
Similarly, the initial susceptibility of the substrates could not be inferred from the
hydrolysis rates determined in these studies because these rates were
determined only after an extended period of hydrolysis, when significant changes
had already occurred to the substrate, which could influence the hydrolysis rate.
Thus, the dependence of the hydrolysis rate on the surface area available to
cellulases has not been conclusively demonstrated.

Another factor that has been suggested to affect the hydrolysis of
cellulosic substrates is the degree of polymerization (DP) of the cellulose. A
substrate with a high degree of polymerization might be relatively inaccessible
to cellulase attack because of its low number of free ends. However, no clear
conclusion has been reached on the relationship between the DP of a substrate
and the corresponding hydrolysis rate (Puri, 1984; Sinitsyn, 1991).

The crystallinity of cellulose might also be expected to play a role in
determining the hydrolysis rate of a substrate. In a highly crystalline substrate,
the closely packed, hydrogen-bonded cellulose molecules might be less
accessible to cellulase attack than the more loosely organized amorphous
cellulose. A few studies have found a correlation between the hydrolysis rate of cellulose and its crystallinity (Sasaki et al., 1979; Fan et al., 1980, 1981) while others have not (Caulfield and Moore, 1974; Thompson et al., 1992). Sinitsyn (1991) suggested that the effect of reduced crystallinity on hydrolysis rate is actually due to the accompanying increase in surface area rather than a reduction in the proportion of the crystalline material in the substrate. Several studies have subsequently incorporated the effects of crystallinity and surface area together in empirical equations relating the structural features of the substrate to the enzymatic hydrolysis rate (Fan et al., 1981; Gharpuray et al., 1983).

The lignin content of a substrate is also considered to play a large role in determining its susceptibility. It is generally assumed that some form of pretreatment is required to remove the lignin barrier surrounding the cellulose microfibril. In fact, several studies have found that an increase in the enzymatic hydrolysis rate was related to a decrease in the lignin content of substrates (Gharpuray et al., 1983; Sinitsyn et al., 1991). There is some evidence that complete removal of the lignin may not be necessary. It has been suggested that lignin is "melted" during the steam pretreatment process. The subsequent formation of the melted lignin into globules may expose cellulose further to cellulase attack (Saddler et al., 1982; Wong et al., 1988) even though the lignin has not been removed.

However, it is also possible that the residual lignin in these substrates may adsorb cellulases. The adsorption of cellulyases to both isolated lignin (Chernaglazov et al., 1988) and the lignaceous residues left after complete
hydrolysis (Deshpande and Eriksson, 1984; Ooshima et al., 1990; Girard and Converse, 1993) has been reported. Cellulases that are immobilized through irreversible adsorption to lignin should be unavailable for further reaction with cellulose. Thus, the presence of lignin could decrease the hydrolysis rate of lignocellulosic substrates.

### 1.5.2 Enzyme-related Factors Affecting Hydrolysis

The hydrolysis kinetics may also be affected by the ability of the cellulases to hydrolyze the substrate. End-product inhibition of the cellulases is well-known. Cellobiose is thought to be a stronger inhibitor of the cellulases than glucose (Lee and Fan, 1983, Iogen Corp. Final Report, 1990). However, the effects of accumulated cellobiose can be easily relieved by the addition of β-glucosidase to convert the cellobiose to glucose (Breuil et al., 1992). High levels of glucose present in hydrolysis mixtures can also be a problem. This problem can be alleviated through the removal of glucose during hydrolysis by methods such as ultrafiltration (Ishihara et al., 1991) or simultaneous saccharification and fermentation (Mes-Hartree et al., 1987). Enzyme inactivation by agitation or prolonged incubation at the high temperatures (40°C-50°C) required for hydrolysis may also affect the hydrolysis rate. Evidence for the loss of cellulase activity at high temperatures has been observed (Hogan et al., 1987). However, Ramos et al. (1993) observed only a 10% decrease in cellulase activity after a 15 day incubation of the cellulases at 45°C.
1.6 APPROACHES TO IMPROVING THE HYDROLYSIS OF LIGNOCELLULOSIC MATERIALS

Improvements in the hydrolysis rates of lignocellulosic materials can be obtained by increasing, to a certain extent, the amount of cellulase used in the conversion process. However, the cost of the enzymes, which are already high, would increase significantly. Strategies for improving the hydrolysis rate without increasing the cost of the enzymes required for hydrolysis have followed two approaches. One approach focusses on increasing the susceptibility of the substrate to enzymatic hydrolysis. The other approach focusses on reducing or re-using the enzyme used in the hydrolysis reaction.

1.6.1 Substrate-related Approach

Pretreatment of the substrate is necessary to remove the lignin and disrupt the structure of the substrate before cellulose hydrolysis. The most widely used methods are either chemical or physical.

Chemical pretreatment methods are aimed primarily at extracting the lignin and hemicellulose from the substrate, or disrupting the crystallinity of the cellulose in the substrate (Fan et al., 1987). A variety of different chemicals have been shown to achieve different effects. Alkalis such as sodium hydroxide, ammonia, and ammonium sulfate are thought to increase the surface area of the substrate by swelling the substrate or by removing the non-cellulosic components associated with the cellulose. In their study, Sinitsyn et al. (1991) found that the surface areas of both cotton linters and bagasse were increased after treatment with sodium hydroxide. Similarly, Gharpuray et al. (1983) found
an increase in the surface area of wheat straw after treatment with sodium hydroxide; this was attributed primarily to the removal of approximately 30-50% of the lignin and 25% of the hemicellulose from the wheat straw.

Acids can also act as swelling agents. Stone et al. (1969) obtained a series of cellulose substrates with different pore volumes by swelling cotton linters with increasing concentrations of phosphoric acid. Treatment of wheat straw with peracetic acid was also found to drastically increase surface area by removing the lignin and reducing the crystallinity of the substrate (Gharpuray et al., 1983).

A variety of other chemicals have also been effective at increasing the digestibility of substrates by removing lignin and hemicellulose. These include oxidizing agents such as nitrogen oxides, sodium chlorite, ozone, and hydrogen peroxide (Puri, 1984; Gould, 1984; Sinitsyn et al., 1991), as well as cellulose dissolving agents such as cadoxen (Sinitsyn et al., 1991). The main disadvantage of many chemical treatments is the recovery of the chemicals, which can be expensive and/or toxic, after pretreatment.

Physical pretreatment methods are generally aimed at reducing the size of the substrate particles or at breaking up the crystalline structure of the cellulose. A variety of milling and grinding techniques have been explored. It is thought that grinding or milling increases the surface area to volume ratio of the substrate and results in an increase in the amount of the substrate exposed to the cellulases (Fan et al., 1987). While these methods can be highly effective, the increases in susceptibilities that are obtained may be dependent on the substrates used as well as the method of milling or grinding used (Fan et al.,
Irradiation of the substrate with gamma or X-ray radiation can also be effective in disrupting the substrate structure through decreases in the degree of polymerization of the cellulose (Sinitsyn et al., 1991). However, the effectiveness of this method again varies with the substrate (Fan et al., 1987).

One of the most commonly used pretreatment methods is steam pretreatment (Saddler et al., 1993). In this method, wood chips are treated with steam at high temperature and pressure for a short time. During this process, a portion of the hemicelluloses is hydrolyzed. Water-washing of the resultant substrate removes the hydrolyzed hemicellulose and a portion of the lignin, and leaves behind a substrate composed of cellulose, lignin, and residual hemicellulose. Further extraction of this substrate with chemicals such as sodium hydroxide and hydrogen peroxide effectively removes most of the residual lignin and hemicellulose (Gould et al., 1984). Even without this second pretreatment step, the susceptibility of the substrate is greatly enhanced by the steam pretreatment.

For many of these pretreatment methods, it appeared that the increase in digestibility of the substrate was a result of increased accessibility to the cellulases. Grethlein (1985) showed that the removal of lignin and hemicellulose by steam pretreatment resulted in an increase in the pore volume of the substrate. However, it has not been proven that this increase in pore volume is responsible for the increase in the substrate susceptibility to cellulases. Similarly, a change in surface area may not be the only change resulting from swelling of a substrate in phosphoric acid. Pretreatments may also cause other
changes to the substrate that result in a more easily hydrolyzed substrate for the cellulases.

1.6.2 Enzyme-related Approaches

The cost of cellulases accounts for a large portion of the overall cost of lignocellulosic bioconversion processes (Nguyen, 1993). One means of reducing the cost of the enzymes would be the development of improved strains of cellulase-producing fungi. It has been estimated that enzymatic conversion processes would be economically feasible if it were possible to produce cellulases at a concentration of 20 FPU/ml at a productivity of 200 FPUL\(^{-1}\)h\(^{-1}\) (Persson et al., 1991). Extremely high cellulase production has been obtained as a result of programs aimed at improving strains through mutation and variations in culture conditions. So far, the targeted yields have been obtained by fed-batch culture of *Trichoderma reesei* grown on pure cellulose as a carbon source. However, the cost of pure cellulose makes this uneconomical in large-scale processes. Current work is aimed at using lignocellulose as a cheaper substrate for cellulase production. Alternatively, a soluble carbon source, such as lactose, has been used (Pourquie et al., 1988). A fed-batch cultivation method using lactose as substrate has yielded enzyme concentrations and productivities comparable to those using pure cellulose as substrate. The soluble xylose or xylose oligomers as well as the lignocellulosic hydrolysates from enzymatic saccharification have also been proposed as substrates for cellulase production (Pourquie and Warzywoda, 1993).

Another approach to reduce the cost of cellulases would be the mass-
production of cellulases from cloned cellulase genes. A number of cellulases have been cloned and their products have also been shown to be active (Kubicek, 1992). However, the roles of the multiple enzymes in a typical cellulase mixture are still unclear. The identification of the key enzymes which are required for efficient hydrolysis of cellulose would permit the production of only those enzymes which are important for hydrolysis. Thus, the cost of cellulase production would be reduced as large amounts of only the necessary enzymes would be produced.

A third approach to reducing the cost of cellulases would be to recover and re-use the cellulases. A substantial amount of work has been carried out on the recovery of cellulases. From these studies, it is clear that the cellulase activity can be recovered from either the non-cellulosic residue (Girard and Converse, 1993), the residual substrate (Rao et al., 1983; Sinitsyn et al., 1983; Deshpande and Eriksson, 1984; Otter et al., 1989), or the supernatant (Ramos et al., 1993) of hydrolysis reactions. However, the ability of the recovered cellulases to hydrolyze a lignocellulosic substrate in a subsequent round of hydrolysis was not evaluated to any great extent. Similarly, most of these previous studies did not use multiple recycling steps in order to determine the possible savings obtained with one loading of cellulase. Thus, an effective method for cellulase recycling has not yet been established.

1.7 OBJECTIVES

Previous attempts at improving the economics of the enzymatic hydrolysis
component of various lignocellulosic bioconversion processes have generally focused on two aspects. One approach has been to pretreat the cellulosic substrates to improve their susceptibility to enzymatic hydrolysis. An alternative strategy has been to re-use the cellulase enzymes. In the former approach, the increased susceptibility of pretreated substrates has often been attributed to an increase in the surface area accessible to cellulases. Thus, the change in the surface area of a substrate may be a useful parameter for assessing the effectiveness of pretreatments. As described in Section 2 of this study, we have evaluated the adsorption characteristics of CBD_{cex}, the cellulose-binding domain from a C. fimii cellulase, added to various cellulosic substrates. The objective of this section was to determine if CBD_{cex} could be used to measure the accessible surface area of cellulosic substrates.

While the extent of substrate conversion has been found to depend on the amount of surface area available for cellulase adsorption (Lee and Fan, 1983), the relationship between the initial susceptibility of a substrate and the amount of enzyme adsorbed to it has not been extensively studied. As described in Section 3, we have studied the relationship between enzyme adsorption and the corresponding initial hydrolysis rate. The objective of this study was to gain a better understanding of the role of adsorption in determining the initial susceptibility of substrates.

As mentioned above, another strategy for improving the economics of lignocellulosic bioconversion processes is to reduce the amount of enzyme used in the enzymatic hydrolysis step. One means of achieving this reduction would be to recycle the cellulases after hydrolysis. In Section 4 of this study, three
cellulase recycling strategies were evaluated to determine their efficiencies in five rounds of hydrolysis. The objective of this study was to identify an effective strategy for the recovery and re-use of cellulases in the hydrolysis of lignocellulosic materials.
2. EVALUATION OF CBD_{ex} AS A PROBE FOR MEASURING THE ACCESSIBILITY OF CELLULOSIC SUBSTRATES TO CELLULASES

2.1 BACKGROUND

The surface area accessible to cellulases is believed to be an important factor influencing the hydrolysis of cellulosic materials (Converse, 1993). Several methods have been proposed as a means of measuring the surface area of cellulosic substrates to which the cellulase enzymes can adsorb. For example, the nitrogen adsorption method has been used to calculate the surface area from the number of nitrogen gas molecules which are adsorbed to the substrate (Fan et al., 1983; Gharpuray et al., 1991). However, this method requires drying of the substrate, which alters its structure by causing the collapse of pores within the cellulosic sample. It is highly probable that the surface area measured using this method results in an overestimate of the surface area accessible to cellulases, since a nitrogen molecule is much smaller than a cellulase enzyme.

A more representative accessible surface area is probably determined by the solute exclusion method (Stone et al., 1969). In this method, a series of dextran probes of different sizes are used to determine the fraction of the pores in a substrate which are large enough for cellulases to enter. From the estimated volume of these pores, the surface area accessible to the cellulases can be calculated. As the analysis is conducted in water, the structure of the substrate is maintained and is more representative of the substrate structure during hydrolysis. However, a major drawback to this method is that the external
surface area of the substrate particles tends to be neglected, since the probes would not adsorb to these external surfaces.

In another approach, Sinitsyn et al. (1991) used purified chymotrypsin and peroxidase to try to determine surface areas that included both the internal and external surfaces of the substrate. These enzymes were chosen because they were similar in size to the average Trichoderma cellulase (50 Å) and because they had the additional advantage of being nonhydrolytic toward cellulose. However, even these probes did not fully reflect the surface area available to cellulases because these nonspecifically adsorbing proteins did not take into account the affinity of cellulases for cellulose.

Cellulases from both Trichoderma reesei and Cellulomonas fimi are known to possess both cellulose-binding and catalytic domains (Gilkes et al., 1991). The cellulose-binding domain of Cex, an exoglucanase/xylanase from C. fimi (CBD \(_{\text{cex}}\)), retained its affinity for cellulose when it was separated from its catalytic domain (Gilkes et al., 1988). Therefore, CBD \(_{\text{cex}}\) had potential as a simple, nonhydrolytic probe for determining the accessible surface area of cellulosic substrates to cellulases. However, CBD \(_{\text{cex}}\) can only be used to measure the surface area covered by Trichoderma cellulases if the surface areas covered by CBD \(_{\text{cex}}\) and Trichoderma cellulases are the same. In this study, the adsorption isotherms of CBD \(_{\text{cex}}\) and Celluclast, a commercial cellulase mixture from Trichoderma reesei, were compared using nine cellulosic substrates. The maximum adsorbable protein, \(P_{\text{max}}\), was used to compare the amount of surface area in each substrate that was accessible to CBD \(_{\text{cex}}\) and Celluclast.
2.2 MATERIALS AND METHODS

2.2.1 Substrates

Avicel PH101 (AV), a microcrystalline cellulose, and Solka-floc BW300 (SF), a hammer-milled sulfite pulp, were purchased from Fluka (Switzerland) and James River Corp. (USA), respectively. Acid-swollen Avicel (ASA) was prepared by treating AV with 85% phosphoric acid (Walseth, 1952). Portions of ASA were either air-dried for four days to give the "AD" substrate or oven-dried overnight at 80°C to give the "OD" substrate. A sulfite pulp (SP) was provided by Q. Nguyen (Tembec, Canada) and water-washed steam-exploded birch (WB) by J. Tolan (Iogen, Canada). Alkaline extraction of the steam-exploded birch was carried out with 0.4% NaOH (Saddler et al., 1983) to give the "AB" substrate, and a portion of "AB" was treated with 1% hydrogen peroxide (Gould, 1984) to produce the "PB" substrate. The cellulose contents of these substrates (84% for the Avicel-derived substrates, 90% for PB, 84% for AB, 60% for WB, 93% for SP, and 89% for SF) were determined by measuring glucose content in their sulfuric acid hydrolysates (Clarke and Mackie, 1987). Klason lignin was determined to be 4% for PB, 11% for AB, and 32% for WB, using the TAPPI standard method T222 os-74. No Klason lignin was detected in any of the sulfite pulp, Solka floc, or Avicel-derived substrates. All the substrates were stored, without drying, at 4°C except for Avicel and Solka floc, which had been previously dried, and were stored at room temperature. The moisture contents of the substrates were determined in order to calculate the substrate loadings used in the experiments on the basis of the substrate dry weights.
2.2.2 Cellulases and Cellulose Binding Domain

Celluclast (80 IU/ml, 80 mg protein/ml; batch #CCN 3027, Novo-Nordisk A/S, Denmark) was the cellulase used in all experiments.

The cellulose binding domain of a C. fimii exoglucanase/xylanase (CBD$_{cex}$) was provided by D. Kilburn (University of BC, Canada). An absorbance coefficient of 2.3 absorbance units per mg of purified CBD$_{cex}$ protein was used to calculate the amount of CBD$_{cex}$ protein in the adsorption experiments (Edgar Ong, personal communication).

2.2.3 FITC-Labelling of CBD$_{cex}$

Fluorescein isothiocyanate (FITC; Sigma) was dissolved in dimethylsulfoxide to a final concentration of 5 mg/ml. The dissolved FITC was added to CBD$_{cex}$ (5mg/ml in 50 mM Tris-HCl, pH 8) at 0.05 mg dye/mg CBD$_{cex}$ and the reaction mixture was agitated at 4°C in the dark for 19 hours. FITC-labelled CBD$_{cex}$ was separated from the unlabelled CBD$_{cex}$ in the reaction mixture by gel filtration chromatography using a BioGel P6 column eluted with 50 mM Tris-HCl (pH 8). Fractions with a volume of 0.5 ml were collected and their absorbances at 280 nm and 495 nm were measured. Fractions with a high absorbance at both wavelengths were pooled and used for the determination of CBD$_{cex}$ adsorption isotherms.

2.2.4 Determination of Cellulase Adsorption Kinetics and Isotherms

Experiments were conducted to determine the time required for Celluclast to reach adsorption equilibrium using the various substrates. Celluclast at a final protein concentration of 3 mg/ml (i.e., 150 IU/(g substrate)) was added to tubes
containing a fixed amount of substrate. The final substrate concentration was 2% (dry weight, (d.w.)/v) in 50 mM sodium acetate (pH 4.8). The tubes were incubated at 4°C with agitation. Aliquots of the mixture were removed at 0, 0.5, 1, 5, 10, 30, 60, 90, 120, and 240 min during the incubation and centrifuged to remove the substrate. The amount of protein in the supernatants was measured by the Bradford assay (Bio-Rad, USA). The time required to attain adsorption equilibrium was determined to be: 10 minutes for ASA, 90 minutes for AV and SF, and 60 minutes for SP, AD, OD, and the birchwood derived substrates.

When determining the adsorption isotherms, different amounts of Celluclast were incubated with a 2% concentration of each substrate in acetate buffer at 4°C until adsorption equilibrium was reached. After the reactions were centrifuged to remove the substrate, the amount of protein in the supernatant was measured in order to determine the amount of unadsorbed Celluclast. Adsorbed Celluclast was calculated as the difference between the amount of protein initially added and the amount of unadsorbed protein in the supernatant.

The experimental data were fitted to the Langmuir adsorption isotherm using the following linearized form of the equation:

\[
\frac{P}{P_{ads}} = \frac{1}{P_{max}K_p} + \frac{1}{P_{max}}P
\]

where

- \( P \) = concentration of unadsorbed Celluclast (mg Celluclast/ml)
- \( P_{ads} \) = concentration of adsorbed Celluclast (mg Celluclast/mg cellulose)
- \( P_{max} \) = the maximal adsorbed Celluclast (mg Celluclast/mg cellulose)
- \( K_p \) = equilibrium constant (ml/mg Celluclast).
Regression analysis was performed to determine the slope and y-intercept, thus allowing calculations of $P_{\text{max}}$ as slope$^{-1}$, and $K_p$ as $(P_{\text{max}} \cdot \text{y-intercept})^{-1}$.

2.2.5 Determination of CBD<sub>cex</sub> Adsorption Kinetics and Isotherms

The CBD<sub>cex</sub> adsorption kinetics were determined as previously described for the Celluclast preparation, with the following exceptions. For these reactions, a mixture of FITC-labelled CBD<sub>cex</sub> (7.5% of the total mixture) and unlabelled CBD<sub>cex</sub> (92.5% of the total mixture) was added, at a final protein concentration of 0.05 mg/ml, to tubes containing each substrate at a final concentration of 0.1% ((d.w.)/v), in 50 mM sodium phosphate (pH 7). The amount of unadsorbed protein in the supernatant after adsorption at room temperature was determined using a fluorometer to measure the fluorescence of the supernatant at 495 nm using a fluorometer. The time required to attain adsorption equilibrium was shown to be the same as for the Celluclast preparation: 10 minutes for ASA, 90 minutes for both AV and SF, and 60 minutes for SP, AD, OD, WB, AB, and PB.

For the adsorption isotherms, different amounts of CBD<sub>cex</sub> or the FITC-labelled CBD<sub>cex</sub>/CBD<sub>cex</sub> mixture were incubated and mixed with 0.1% substrate in phosphate buffer at room temperature. After adsorption equilibrium was reached, the amount of CBD<sub>cex</sub> protein in the supernatant was measured in two ways. In the first method, for reactions containing initially added protein concentrations of 0.15 mg/ml to 0.8 mg/ml, the absorbance of the supernatant was measured at 280 nm ($A_{280}$). The amount of unadsorbed CBD<sub>cex</sub> protein in the supernatant was then calculated by dividing the $A_{280}$ by 2.3, the absorbance
coefficient of CBD<sub>cex</sub>. The second method was used at protein concentrations lower than 0.15 mg/ml, when the FITC-labelled CBD<sub>cex</sub>/CBD<sub>cex</sub> mixture was used for the adsorption reactions. At these concentrations, the amount of fluorescence in the supernatant after adsorption was measured at 495 nm using a fluorometer. The amount of unadsorbed CBD<sub>cex</sub> in these supernatants was then determined by comparison to a dose-response curve of the fluorescence obtained using known amounts of the FITC-labelled CBD<sub>cex</sub>/CBD<sub>cex</sub> mixture. For both sets of experiments, the amount of adsorbed CBD<sub>cex</sub> was calculated as the difference between the amount of CBD<sub>cex</sub> initially added and the amount of unadsorbed CBD<sub>cex</sub> in the supernatant. The values of unadsorbed CBD<sub>cex</sub> obtained by both methods was the same (data not shown).

The data were fitted to the Langmuir Equation as described previously when Celluclast was used as the enzyme source.

2.3. RESULTS AND DISCUSSION

The adsorption isotherms of CBD<sub>cex</sub> and Celluclast on nine different substrates were initially determined (Figure 1). The good fit of the experimental data to the model indicated that the adsorption of both of these protein preparations could be represented by the Langmuir isotherm. The equilibrium constant (K<sub>p</sub>) and the maximal adsorbed protein (P<sub>max</sub>) for all nine substrates studied are shown in Table I.

The P<sub>max</sub> of these substrates was the parameter we hoped to use to determine the accessibility of the various substrates to CBD<sub>cex</sub> or Celluclast.
Figure 1. Adsorption isotherms for Celluclast and CBD_{cex} on Avicel (AV), Water-washed steam-exploded birch (WB), and Alkali- and Peroxide-treated steam-exploded birch (PB). The theoretical curve was generated using the adsorption parameters $K_p$ and $P_{\text{max}}$ determined from the experimental data. The coefficient of determination ($r^2$) shows a good fit of the data to the Langmuir isotherm.
Table I. Adsorption Parameters for Celluclast and CBDcex on Various Cellulosic Substrates. The ranking of the substrates in order of decreasing $P_{\text{max}}$ is indicated in parentheses for both Celluclast and CBDcex.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$P_{\text{max}}$ (mg protein/mg cellulose$^{-1}$)</th>
<th>$K_n$ (ml/mg protein$^{-1}$)</th>
<th>$P_{\text{max}}$ (mg protein/mg cellulose$^{-1}$)</th>
<th>$K_n$ (ml/mg protein$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ASA</td>
<td>(1) 0.513 ± 0.015</td>
<td>1.0 ± 0.2</td>
<td>(2) 0.396 ± 0.014</td>
<td>12.9 ± 3.5</td>
</tr>
<tr>
<td>AB</td>
<td>(2) 0.237 ± 0.008</td>
<td>2.8 ± 1.8</td>
<td>(1) 0.434 ± 0.009</td>
<td>18.4 ± 2.8</td>
</tr>
<tr>
<td>PB</td>
<td>(3) 0.233 ± 0.007</td>
<td>1.9 ± 0.8</td>
<td>(4) 0.321 ± 0.013</td>
<td>43.8 ± 31.0</td>
</tr>
<tr>
<td>WB</td>
<td>(4) 0.214 ± 0.005</td>
<td>2.1 ± 1.1</td>
<td>(3) 0.374 ± 0.022</td>
<td>13.9 ± 5.7</td>
</tr>
<tr>
<td>AD</td>
<td>(5) 0.192 ± 0.002</td>
<td>14.1 ± 41.4</td>
<td>(6) 0.186 ± 0.008</td>
<td>22.4 ± 7.9</td>
</tr>
<tr>
<td>SP</td>
<td>(6) 0.119 ± 0.007</td>
<td>2.1 ± 2.1</td>
<td>(5) 0.218 ± 0.004</td>
<td>28.7 ± 6.6</td>
</tr>
<tr>
<td>OD</td>
<td>(7) 0.113 ± 0.005</td>
<td>3.5 ± 6.8</td>
<td>(7) 0.077 ± 0.006</td>
<td>29.5 ± 26.2</td>
</tr>
<tr>
<td>AV</td>
<td>(8) 0.111 ± 0.006</td>
<td>0.6 ± 0.3</td>
<td>(9) 0.050 ± 0.008</td>
<td>13.4 ± 20.1</td>
</tr>
<tr>
<td>SF</td>
<td>(9) 0.048 ± 0.003</td>
<td>1.3 ± 0.4</td>
<td>(8) 0.051 ± 0.008</td>
<td>14.8 ± 11.3</td>
</tr>
</tbody>
</table>
When the substrates were ranked in order of decreasing $P_{\text{max}}$, it was apparent that the order of the accessibilities measured in this way for these substrates was different (Table I). This suggested that the surface area of the different substrates available to CBD$_{\text{ex}}$ was not the same as the surface areas available to the Celluclast mixture. We had expected that the $P_{\text{max}}$ of the substrates would be consistently greater for CBD$_{\text{ex}}$ than for the Celluclast mixture because the bacterial binding domain is about three to seven times smaller than average molecular weight values that have been reported for the cellulases from *Trichoderma reesei* (Gilkes et al., 1992; Kubicek, 1992). However, it was found that some of the substrates studied had a $P_{\text{max}}$ for CBD$_{\text{ex}}$ that was smaller than the equivalent $P_{\text{max}}$ obtained with the Celluclast mixture (Table I). This suggested that the $P_{\text{max}}$ may not be solely dependent on the size of the protein used for adsorption. Previously, Kyriacou et al. (1989) showed that both common and distinct adsorption sites existed for *T. reesei* CBH I, EGI, EG II, and EG III. It is also possible that different adsorption sites exist for the cellulases of *C. fimii* and *T. reesei*. CBD$_{\text{ex}}$ is a purified single component whereas Celluclast consists of multiple cellulase proteins. Each cellulase may have some distinct adsorption sites on the substrate which are not available for the binding of other cellulases. Therefore, a complete mixture of cellulases such as Celluclast may fill all the available sites on the substrate. CBD$_{\text{ex}}$, on the other hand, may only fill a certain proportion of the sites to which the Celluclast cellulases can adsorb. Thus, the adsorption of a single cellulase component may not accurately represent the adsorption of a complete cellulase mixture.

The corresponding $K_p$s for the substrates are also shown in Table I. $K_p$
is a measure of the affinity of the proteins for each substrate. In general, the $K_p$ s of CBD$_\text{cex}$ were found to be an order of magnitude larger than those obtained with the Celluclast mixture. This result further emphasizes the adsorptive differences between CBD$_\text{cex}$ and Celluclast. The $K_p$ of Celluclast represents an "average" affinity of the individual cellulases in the mixture for a given substrate. In contrast, the $K_p$ of CBD$_\text{cex}$ represents the affinity of only one component. It is also possible that the adsorption affinities of CBD$_\text{cex}$ compared to Celluclast reflect some differences between the adsorption of a binding domain compared to the adsorption of an entire cellulase.

Initially, CBD$_\text{cex}$ had appeared to be an ideal probe to determine the accessibility of cellulosic substrates to cellulases, as several disadvantages associated with methods used in earlier studies (Fan et al. 1983; Gharpuray et al., 1991; Grethlein, 1985; Thompson et al., 1992) could be avoided. As the adsorption of CBD$_\text{cex}$ was carried out in buffer, the determinations could be conducted in an environment that closely resembled the conditions of cellulose hydrolysis. Also, the adsorption of CBD$_\text{cex}$ to both the external and internal surfaces of the substrates permitted the determination of the external surface area, which was not accounted for when nonadsorptive probes were used (Grethlein, 1985; Thompson et al., 1992). In addition, CBD$_\text{cex}$ had an affinity for cellulose, unlike the other proteins which have been used to determine surface area (Sinitsyn et al., 1991). It was hoped that the CBD would adsorb to the same sites as the cellulases in the Celluclast mixture, as similar binding domains have been found in several *Trichoderma reesei* cellulases (Gilkes et al., 1991). However, the results of this study indicated that the adsorption of a single,
purified cellulase fragment such as CBD$_{cex}$ did not represent the adsorption of the mixture of different cellulases in Celluclast. Thus, CBD$_{cex}$ could not be used to determine the accessibility of cellulosic substrates to cellulase mixtures such as Celluclast.
3. ASSESSMENT OF THE ENZYMATIC SUSCEPTIBILITY OF CELLULOSIC SUBSTRATES USING SPECIFIC HYDROLYSIS RATES AND ENZYME ADSORPTION

3.1 BACKGROUND

As mentioned previously, the susceptibility of cellulosic substrates to enzymatic hydrolysis may depend on various structural features of the substrate (Sasaki et al., 1979; Fan et al., 1980; Gharpuray et al., 1983; Puri, 1984; Sinitsyn et al., 1991; Thompson et al., 1992). In particular, the amount of surface area available for enzyme binding would be expected to be important in determining the hydrolysis rate because the cellulases must first adsorb onto the insoluble substrate in order to carry out hydrolysis. Several reports have suggested that the rate of hydrolysis is proportional to the amount of adsorbed protein (Lee and Fan, 1983; Wald et al., 1984; Converse et al., 1990), while others have suggested that it depends only on the surface area of the substrate (Grethlein, 1985; Thompson et al., 1992). However, most of these studies were carried out over an extensive period of hydrolysis during which the substrate might have undergone substantial changes. To study the relationship between the hydrolysis rate and the adsorbed enzyme, the hydrolysis rate should be measured at an early stage in the process, when product inhibition, enzyme inactivation, and substrate recalcitrance can be considered to be less influential.

It is expected that a better understanding of the relationship between enzyme adsorption and the corresponding hydrolysis rate would lead to a more efficient use of enzymes in cellulose hydrolysis. As described in the previous
section, the maximal extent of cellulase adsorption was used to measure the accessibility of a substrate. The dependence of the initial hydrolysis rate on the accessibility as well as on the actual amount of adsorbed enzyme was evaluated using a range of different substrates. The specific hydrolysis rate (i.e. the initial rate per amount of adsorbed cellulases) was also calculated, in order to measure the initial susceptibility, or reactivity, of the substrate. This also allowed us to assess the influence of different pretreatments on both the accessibility and the reactivity of the various cellulosic substrates.

3.2 MATERIALS AND METHODS

3.2.1 Substrates

The nine cellulosic substrates (AV, AD, OD, ASA, SP, SF, WB, AB, and PB) described in Section 2 were also used in these studies.

3.2.2 Cellulases

Celluclast (80 IU/ml, 80 mg protein/ml; batch #CCN 3027, Novo-Nordisk A/S, Denmark) was the cellulase used in all experiments. For cellulose hydrolysis, it was supplemented with a β-glucosidase, Novozym (792 CBU/ml, 73 mg protein/ml; batch #DCN 0012, Novo-Nordisk A/S) at 3.5 CBU/IU Celluclast to alleviate end-product inhibition by cellobiose (Breuil et al., 1992).

3.2.3 Measurement of Initial Hydrolysis Rates

The Celluclast/Novozym mixture was added to 2% ((d.w.)/v) of each substrate in 50 mM sodium acetate (pH 4.8) at a cellulase loading of 0.2 mg/ml
(10 IU/(g cellulose)). For each substrate, the hydrolysis reaction was allowed to proceed for 5 minutes at 50°C. The reaction was stopped by boiling for 5 minutes, and the tubes were centrifuged to remove the insoluble material. The release of glucose and cellobiose was determined by HPLC using the Biorad HPX-87H column. Hydrolysis was calculated as the amount of reducing sugar released per hour, with reducing sugar determined as (mg glucose) + (0.5 x mg cellobiose x 1.053).

3.2.4 Measurement of the Specific Hydrolysis Rate of Cellulases

For each substrate, the Celluclast/Novozym mixture was incubated with 2% substrate in acetate buffer at 4°C until adsorption equilibrium was reached. The substrate was recovered by centrifugation, washed, and resuspended in acetate buffer containing Novozym. The subsequent hydrolysis was carried out as described in the previous sub-section. Before hydrolysis was carried out, an aliquot of the reaction mixture was removed and the protein in the supernatant measured. The specific hydrolysis rate was calculated as the hydrolysis rate obtained after 5 minutes of reaction divided by the amount of protein adsorbed prior to the initiation of hydrolysis. All of the protein adsorbed onto the cellulosic substrate was assumed to originate from the Celluclast mixture as protein adsorption was not observed when Novozym alone was added to the substrates.

3.3 RESULTS AND DISCUSSION

3.3.1 Cellulase Adsorption and Hydrolysis of Different Substrates

As described in the previous section, when we determined the adsorption
isotherms of Celluclast (Figure 1), a good fit of the experimental data to the model indicated that adsorption of the proteins present in the Celluclast mixture could be represented by a Langmuir isotherm. We determined (Table II) the equilibrium constant ($K_p$) and maximal adsorbed protein ($P_{max}$) for all nine substrates studied, along with the corresponding initial hydrolysis rates. These initial hydrolysis rates were determined before more than 10% of the cellulose in each substrate was hydrolyzed. When the different substrates were arranged in the order of decreasing $P_{max}$, the corresponding initial rates did not decrease accordingly. This indicated a poor correlation between these two parameters (Figure 2(a)). It seems that the initial hydrolysis rate does not solely depend on the total surface area available for enzyme binding. A plot of the initial hydrolysis rate against the actual amount of adsorbed Celluclast (Figure 2(b)) also showed poor correlation between these two parameters. It was apparent that substrates with similar amount of adsorbed enzyme were not hydrolyzed at similar rates. This suggested that different specific hydrolysis rates could be expected when different cellulosic substrates were used.

### 3.3.2 Specific Hydrolysis Rate

As the specific hydrolysis rate is a measure of the ease with which a substrate can be hydrolysed by bound cellulases, the data in Table II indicates that the structural features of different substrates might affect this rate. The specific hydrolysis rates were found to be different for most of the substrates, with the birch-derived substrates showing relatively low specific hydrolysis rates. Since the surface coverage of a substrate by a given quantity of Celluclast can
Table II. Adsorption parameters for Celluclast on various cellulosic substrates and the corresponding initial hydrolysis rates and specific hydrolysis rates. Hydrolysis rates were determined at a low coverage of the substrate surface by Celluclast.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>(Abbreviation)</th>
<th>$P_{\text{max}}$ (mg Celluclast)</th>
<th>$K_p$ (ml • mg Celluclast$^{-1}$)</th>
<th>Initial Hydrolysis Rate (mg • h$^{-1}$)</th>
<th>Specific Hydrolysis Rate (mg • h$^{-1}$ • mg Celluclast$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acid Swollen Avicel</td>
<td>(ASA)</td>
<td>0.513 ± 0.015</td>
<td>1.0 ± 0.2</td>
<td>1.39 ± 0.15</td>
<td>100 ± 18</td>
</tr>
<tr>
<td>Air-Dried ASA</td>
<td>(AD)</td>
<td>0.192 ± 0.002</td>
<td>14.1 ± 1.4</td>
<td>1.25 ± 0.04</td>
<td>91 ± 11</td>
</tr>
<tr>
<td>Oven-Dried ASA</td>
<td>(OD)</td>
<td>0.113 ± 0.005</td>
<td>3.5 ± 6.8</td>
<td>1.02 ± 0.05</td>
<td>70 ± 8</td>
</tr>
<tr>
<td>Avicel</td>
<td>(AV)</td>
<td>0.111 ± 0.006</td>
<td>0.6 ± 0.3</td>
<td>0.56 ± 0.11</td>
<td>45 ± 14</td>
</tr>
<tr>
<td>Water-washed steam-exploded Birch</td>
<td>(WB)</td>
<td>0.214 ± 0.005</td>
<td>2.1 ± 1.1</td>
<td>0.76 ± 0.02</td>
<td>38 ± 4</td>
</tr>
<tr>
<td>Alkaline-washed WB</td>
<td>(AB)</td>
<td>0.237 ± 0.008</td>
<td>2.8 ± 1.8</td>
<td>0.54 ± 0.03</td>
<td>36 ± 4</td>
</tr>
<tr>
<td>Peroxide-treated AB</td>
<td>(PB)</td>
<td>0.233 ± 0.007</td>
<td>1.9 ± 0.8</td>
<td>0.61 ± 0.01</td>
<td>47 ± 4</td>
</tr>
<tr>
<td>Solka-Floc</td>
<td>(SF)</td>
<td>0.048 ± 0.003</td>
<td>1.3 ± 0.4</td>
<td>0.68 ± 0.03</td>
<td>58 ± 8</td>
</tr>
<tr>
<td>Sulfite Pulp</td>
<td>(SP)</td>
<td>0.119 ± 0.007</td>
<td>2.1 ± 2.1</td>
<td>0.87 ± 0.02</td>
<td>72 ± 10</td>
</tr>
</tbody>
</table>

*Means and standard deviations ($n=2-4$) are provided.*
Figure 2. Plots of the initial hydrolysis rates of different cellulosic substrates against (a) the maximal adsorption capacity for Celluclast ($P_{\text{max}}$) and (b) the Celluclast adsorbed ($P_{\text{ads}}$).
be expressed as a percent of the substrate's maximal binding capacity for Celluclast \( (P_{\text{max}}) \), the dependency of specific hydrolysis rate on the percentage of the surface covered by cellulases was assessed (Figure 3). It was apparent that the specific hydrolysis rate decreased as more of the substrate was covered by adsorbed enzymes, suggesting that the specific hydrolysis rate is a function of \( P_{\text{ads}} \). A power law relationship for these two parameters has been suggested previously by other workers (logen Corp., 1990). When the data obtained in our study was fitted to the power law equation \( v = a \cdot P_{\text{ads}}^b \), where \( v \) = specific rate, \( a \) & \( b \) = constants), the exponent, \( b \), for the WB, PB and AV substrates were found to be between 0.56 and 0.6. This is in agreement with the previous finding (logen Corp., 1990) that the reaction order is less than unity (0.66-0.68). Thus, it would appear that the initial specific hydrolysis rate drops when more enzymes are adsorbed on to the substrate. In addition, the specific hydrolysis rate of a particular substrate has been shown to decrease as hydrolysis progresses (Wang and Converse, 1991; Ooshima et al., 1991). This is most likely due to multiple factors, including substrate recalcitrance, enzyme inactivation, and product inhibition. For our substrates, it was found that there was a 10%-50% decrease in the specific rate after about 20% substrate conversion (data not shown).

### 3.3.3 Effect of Pretreatments

Chemical and physical pretreatments of substrates can result in increased hydrolysis rates and yields. One of the major benefits of pretreatment has been attributed to increases in the surface area available to cellulases (Thompson et
Figure 3. Effect of surface coverage by Cellulase on the specific hydrolysis rates of selected cellulosic substrates: AV (Avicel), PB (alkali- and peroxide extracted steam-exploded birch), and WB (water-washed, steam-exploded birch)
al, 1992). Stone et al. (1969) found that the glucose yield was linearly correlated with the available surface area when a single substrate, cotton linters, was swollen to increasing extents using phosphoric acid. However, they did not consider the effect that this treatment might have on the specific hydrolysis rate. Our results show that both the amount of bound cellulases and the specific hydrolysis rate were increased when Avicel was swollen with phosphoric acid to produce acid-swollen Avicel (ASA; Table II). It would seem that acid swelling increases the surface area of the substrate available for enzyme adsorption as well as disrupting the highly ordered state of the crystalline cellulose in Avicel. When the ASA substrate was subsequently dried, the swelling action of water was removed, and the accessibility of the substrate probably decreased as the spaces between and within the fibers collapsed. Thus, the OD and AD substrates bound less cellulases than did the ASA substrate. The collapse of many of the "pores" might also serve to bring neighbouring surfaces together resulting in their tighter alignment. This may be responsible for the lower specific hydrolysis rate of the OD substrate (Table II).

In the same way, pretreatment of the birch substrates probably resulted in changes in the specific hydrolysis rate and $P_{\text{max}}$. When alkali was used to extract the water-washed, steam exploded birch substrate (WB; containing 33% lignin) to produce the alkali washed substrate (AB; 10% lignin), a slight increase in the cellulase binding capacity, with no change in the specific hydrolysis rate was achieved. Peroxide treatment of the AB substrate further reduced the lignin content of the peroxide-treated (PB) substrate to 5%. This treatment resulted in an increase in the specific hydrolysis rate with no effect on the cellulase binding
capacity when compared to the AB substrate.

It is apparent that both substrate accessibility to cellulases and the specific hydrolysis rate have to be considered in any kinetic studies of cellulose hydrolysis. As has been suggested previously (Wang and Converse, 1991), both of these two parameters should be used to evaluate cellulose pretreatment methods in terms of their action. From our study of nine different substrates, it seemed that certain pretreatment methods might increase both the substrate accessibility and the specific hydrolysis rate (e.g. phosphoric acid treatment), while other methods might only change one of the two parameters (e.g. alkali extraction, peroxide treatment). By considering only the hydrolysis yield, as was used in previous studies, the potential effect of the pretreatment step cannot be fully determined.
4. ASSESSMENT OF ENZYME RECYCLING STRATEGIES FOR THE HYDROLYSIS OF LIGNOCELLULOSIC MATERIALS

4.1 BACKGROUND

As mentioned previously in the Introduction section, it should be possible to recycle the cellulases used in cellulose bioconversion processes, since the cellulases can be recovered from the reaction after the enzymatic hydrolysis step. It is generally acknowledged that the first step in cellulose hydrolysis involves adsorption of cellulases onto the cellulosic substrate. As cellulose hydrolysis proceeds, a portion of the adsorbed enzymes is gradually released back into the reaction supernatant (Lee and Fan, 1983; Ooshima et al., 1990). During the course of hydrolysis, the cellulases are distributed between the substrate and the supernatant. Therefore, cellulases should be recoverable from either phase of the reaction. The simplest method for cellulase recovery is to re-adsorb the enzymes in the supernatant onto fresh substrate (Ramos et al., 1993). However, it is probable that cellulase recovery would be incomplete, as a portion of the enzymes will remain adsorbed onto the residual substrate. In order to recover these adsorbed cellulases, many agents, including detergents (Rao et al., 1983; Otter et al., 1989), alkali (Otter et al., 1989), glycerol (Deshpande and Eriksson, 1984; Otter et al., 1989), urea (Deshpande and Eriksson, 1984), and phosphate or acetate buffers of varying pH (Sinitsyn et al., 1983; Deshpande and Eriksson, 1984) have been used in attempts to elute cellulase activity from various substrates. The highest reported results were obtained in a study which used a one-step extraction procedure with alkali and...
Tween 80 to recover adsorbed Avicelase activity. In this case, only about 65% of the originally added Avicelase activity was eluted from the Avicel (Otter et al., 1989). It has also been reported (Vallander and Eriksson, 1987; Singh et al., 1991; Ramos et al., 1993, 1994) that cellulases can be recovered by simple contact of the residual substrate with fresh substrate. Ramos et al. (1993, 1994) showed that residual eucalyptus substrate after partial hydrolysis retained cellulase activity which could be used to hydrolyze more substrate. In similar work, Singh et al. (1991) achieved sugar production by mixing residual potato pulp with fresh potato pulp, while Vallander and Eriksson (1987) also observed sugar production when fresh substrate was added to residual wheat straw or aspen. As these residual substrates still contained cellulose, it was not clear whether the adsorbed cellulases were able to hydrolyze the fresh substrate before all the residual substrate was first hydrolyzed.

During the hydrolysis of lignocellulosic substrates, the lignin content and distribution in the substrate will likely affect the recovery of cellulases during hydrolysis. It has been shown that cellulases adsorb to both isolated lignin (Chernaglazov et al., 1988), and the lignaceous residues left after complete hydrolysis (Deshpande and Eriksson, 1984; Girard and Converse, 1993; Ooshima et al., 1990). The rate of cellulose hydrolysis has been shown to decrease when lignin was added to the hydrolysis reaction (Chernaglazov et al., 1988). Girard and Converse (1993) observed sugar production in a mixture of fresh steam-exploded wood and lignaceous residue containing adsorbed cellulases. However, only about 15-25% of the cellulase activity was recovered from these lignaceous residues. This suggested that a large portion of the
cellulases might be irreversibly adsorbed onto the lignaceous residues.

It is apparent that cellulase activity can be recovered from either the residual substrates, the non-cellulosic residues, or the supernatants of the hydrolysis reactions. However, in none of these studies was the recovery of cellulases quantified in terms of their hydrolytic activity towards the substrate from which they were recovered. Cellulase recovery in previous studies (Rao et al., 1983; Sinitsyn et al., 1983; Deshpande and Eriksson, 1984; Otter et al., 1989) was often measured in terms of Avicelase, CMCase, or FPase activities. However, these activity measurements may not truly reflect the ability of the recovered cellulases to hydrolyze a given lignocellulosic substrate. Furthermore, most of the previous studies did not evaluate multiple recycling steps in order to determine the possible savings resulting from the use of one batch of cellulases to carry out multiple hydrolysis steps.

In this section, three cellulase recycling strategies were evaluated to determine their efficiencies based on enzyme savings. Each strategy was carried out for five successive hydrolysis rounds. The first strategy was to recycle the cellulases adsorbed onto the residual substrate after a fixed time of hydrolysis. The second strategy was to recycle the cellulases present in the complete reaction mixture (i.e., supernatant and residual substrate), also after a fixed time of hydrolysis. The third strategy was to recycle the cellulases present in the complete reaction after all of the cellulose had been hydrolyzed.

To determine if the lignin content of the substrate affected the recovery of the cellulases, two lignocellulosic substrates containing 32% and 4% lignin were used. These were, respectively, water-washed steam-exploded birch (WB), and
the same material which had been further extracted with alkali and peroxide (PB).

In this work, cellulase recovery was determined both in terms of the cellulase activity recovered, and the cellulase protein recovered. The recovered cellulase activity was quantitatively determined by comparing the sugar yield obtained after each recycling step to a set of standard hydrolysis curves obtained with different cellulase doses (Eklund et al., 1992). This approach circumvented the problem of the non-linearity of the relationship between the cellulase dose used and sugar yield obtained. By quantifying the amount of protein as well as the amount of activity recovered in each step, it was possible to evaluate each cellulase recycling strategy in terms of both the cellulase recovery yield and the effectiveness of the recovered enzymes for subsequent hydrolysis rounds.

4.2 MATERIALS AND METHODS

4.2.1 Substrates

Avicel PH101 (AV), a microcrystalline cellulose, was purchased from Fluka (Switzerland). Dyed Avicel was prepared according to Wood (1988) by treating AV with Remazol Brilliant Blue R (Sigma, USA). The water-washed steam-exploded birch (WB) and the alkaline- and peroxide-treated birch (PB) substrates were obtained from the same batch of substrates prepared for the work described in Sections 2 and 3. The cellulose content of these substrates was 90% and 60% for PB and WB respectively, and the Klason lignin content was
4% for PB and 32% for WB (Section 2). The steam-exploded and extracted substrates were stored without drying at 4°C. The moisture contents of the birch substrates were determined to be 79% and 73% for PB and WB, respectively.

### 4.2.2 Cellulases

The cellulases used in this section were described in Section 3.

### 4.2.3 Standard Hydrolysis Procedure

Hydrolysis by the Celluclast/Novozym mixture was carried out at a substrate concentration of 2% (dry weight (d.w.)/v) in 50 mM sodium acetate (pH 4.8). The reaction was incubated at 45°C, with agitation at 200 rpm on a platform incubator shaker. Samples were removed from the reaction at different times, chilled with ice to stop the reaction, and then centrifuged to remove the insoluble materials. The reducing sugar concentration in the supernatant was determined by the Nelson-Somogyi method (Wood and Bhat, 1988). As the hydrolysis reactions were always supplemented with Novozym, the reducing sugar content was always shown to contain >95% glucose, as determined by HPLC (data not shown). The percent conversion of the substrate was calculated from the reducing sugar content, as a percentage of the theoretical reducing sugar available in the substrate. Total protein in the supernatant was measured by the Bradford Assay (Biorad, USA) using IgG as the protein standard. Since the Novozym was previously shown not to adsorb onto the birch substrates (Section 2), the amount of Celluclast protein recovered was calculated by subtracting the amount of Novozym protein added from the total protein recovered.
4.2.4 Evaluation of Cellulase Activity Recovered Using Standard Hydrolysis Curves

For both the PB and WB substrates, a series of reactions containing cellulase loadings of 1 IU/(g cellulose) to 20 IU/(g cellulose) was carried out under standard conditions. The reducing sugars released were determined at 1, 4, 8, 12, and 24 h of hydrolysis and the percent conversion of the substrate was calculated. Thus, dose-response curves for each time point could be obtained for both the PB and WB substrates.

Data from our recycling experiments was evaluated as follows. The percent conversion was determined after a given time in each hydrolysis round. Using the dose-response curve for that particular time point, percent conversion was converted into the equivalent cellulase dose. This cellulase dose was termed the "observed activity" of each hydrolysis round. Since the amount of cellulase protein recovered after each recycling step was determined, the cellulase dose in each subsequent hydrolysis round can then be calculated using the specific activity of the Celluclast added (1 IU/mg). This cellulase dose, based on cellulase protein recovery, was defined as the "expected activity" of each hydrolysis round.

4.2.5 Celluclast Recycling Experiments

During the recycling experiments, hydrolysis was carried out under standard conditions. In each recycling step, Novozym, at the concentration used in a standard hydrolysis reaction, was added to the reaction in order to replace the Novozym lost in the supernatant. As mentioned previously, Novozym did not adsorb to the substrates.
Recycling of enzymes adsorbed to the residual substrate: When PB was used as the substrate, the reaction was chilled with ice to stop the reaction after 12h of hydrolysis. The residual substrate was recovered by filtration through a Whatman GF/A filter and washed with cold 50 mM sodium acetate (pH 4.8). Reducing sugars in the supernatant were measured to determine the percent conversion of the substrate. The amount of fresh substrate required to bring the total substrate concentration back to 2% could then be determined. The added fresh substrate and the recovered residual substrate were resuspended in acetate buffer containing Novozym. A second round of hydrolysis was then initiated, and the recycling was repeated after another 12 h of hydrolysis. Cellulase recycling was carried out four times for a total of five hydrolysis rounds. The protein in the supernatant was also measured after each hydrolysis round in order to calculate the amount of cellulase protein adsorbed onto the residual substrate. When WB was used as the substrate, cellulase recycling was carried out using the same procedure, except that each hydrolysis round was carried out for 24 h.

Recycling of enzymes adsorbed to the residual substrate and present in the supernatant: When the PB substrate was used, the reaction was cooled with ice after 12 h of hydrolysis, and the residual substrate was separated from the supernatant by filtration, as described before. The percentage of the substrate hydrolyzed was determined, and the amount of fresh substrate required to bring the total substrate concentration back to 2% was calculated. The fresh substrate was added to the recovered supernatant, and the cellulases in the supernatant were allowed to adsorb onto the fresh substrate at 4°C for 60 min until
adsorption equilibrium was reached. The suspension was then filtered to recover the fresh substrate, and the amount of protein in the filtrate was measured in order to calculate the amount of cellulase protein adsorbed onto the fresh substrate. The fresh substrate, together with acetate buffer and Novozym, was added to the residual substrate. A second round of hydrolysis was initiated, and recycling was repeated after 12 h of hydrolysis. Again, cellulase recycling was carried out four times in a total of five hydrolysis rounds. For WB, the recycling procedure was the same, except that recycling was carried out after 24 h of hydrolysis.

Recycling of enzymes associated with the non-cellulosic residue and the supernatant: The Celluclast/Novozym mixture was incubated with a 2% PB concentration in acetate buffer at 4°C for 60 min until adsorption equilibrium was reached. The substrate was recovered by filtration, washed, and resuspended in acetate buffer containing Novozym. The first round of hydrolysis was then initiated. After 24 h of hydrolysis, the percent conversion of the substrate was determined in order to obtain "observed activity". When complete hydrolysis of the substrate was achieved, the reaction was cooled with ice. Fresh substrate (see below for the amount added) was then added to the reaction to reach a final concentration of 2% and the mixture was incubated at 4°C for 60 min to allow for cellulase adsorption. The mixture of fresh substrate and the non-cellulosic residue was then recovered by filtration and resuspended in buffer containing Novozym. A second round of hydrolysis was carried out and recycling was repeated after complete hydrolysis of the substrate was achieved.

The protein in the filtrate was measured after each adsorption step to
determine the amount of unadsorbed cellulase protein lost in the filtrate. The amount of fresh substrate required to keep the enzyme loading constant in the next hydrolysis round was then calculated. The volume of the hydrolysis reaction was always adjusted to maintain a 2% substrate consistency for each hydrolysis round. A total of five hydrolysis rounds (4 recycling steps) was carried out. The same recycling procedure was used for the WB substrate.

4.2.6 Determination of the Specific Activity of Cellulases Towards Substrates Hydrolyzed to Different Extents

To determine the specific hydrolysis rates of previously hydrolyzed PB substrate by adsorbed cellulases, the PB substrate was hydrolyzed under standard conditions at a cellulase loading of 20 IU/(g cellulose). At different times during the reaction, aliquots of the reaction mixture were removed and chilled with ice. After centrifugation to recover the residual substrate, the protein in each supernatant was measured. The adsorbed cellulase protein was calculated as the difference between the amount of protein initially added and the amount of protein in the supernatant. The recovered substrates were washed and resuspended at a concentration of 2% in acetate buffer containing Novozym. For each substrate, cellulose hydrolysis by the adsorbed enzymes was carried out for a period of 5 min. Specific hydrolysis rate was calculated as the reducing sugars produced per hour divided by the amount of adsorbed protein.

4.2.7 Hydrolysis of Substrate Mixtures Containing Residual Birch Substrate and Dyed Avicel

Hydrolysis of the cellulose in dyed Avicel results in the release of Remazol
Brilliant Blue R dye into the reaction supernatant. Since the dye absorbs at 595 nm, the hydrolysis of dyed Avicel can be determined by measuring the absorbance of the supernatant at 595 nm ($A_{595}$). The percent conversion can also be determined by measuring the reducing sugars released during the reaction. The $A_{595}$ of the supernatant was shown to be proportional to the percent conversion of dyed Avicel, as determined from the reducing sugar values, and the ratio of $A_{595}$ to percent conversion was calculated to be 0.076. Using this ratio, the percent conversion of dyed Avicel could be determined by measuring only the $A_{595}$ of the supernatant. Therefore, dyed Avicel could be used to distinguish between the hydrolysis of fresh and residual substrates.

Reactions containing 2% PB or WB were hydrolyzed at 20 IU/(g cellulose) under standard conditions until 60-70% of the cellulose was converted to reducing sugars. The residual substrate was recovered by filtration, and the protein in the supernatant was measured so that the amount of cellulase protein adsorbed to the substrate could be determined. After fresh dyed Avicel was added to bring the final substrate concentration to 2%, the substrate mixture was resuspended in acetate buffer containing Novozym. A subsequent round of hydrolysis was then initiated. Reducing sugars as well as $A_{595}$ of the supernatant were measured at 0, 1, 4, 8, 12, 24, and 48 h of hydrolysis in order to calculate percent conversion due to each substrate. The percent conversion of dyed Avicel was determined by $A_{595}$ as described in the previous paragraph. Percent conversion of the residual substrate was calculated as the difference between the percent conversion of both substrates, which was measured as total reducing sugar yields, and the percent conversion of dyed Avicel.
4.3 RESULTS AND DISCUSSION

4.3.1 Distribution of Cellulases Between the Substrate and the Supernatant During Hydrolysis

Initially, the proportion of the added Celluclast proteins that were present in the reaction supernatant during hydrolysis of the PB and WB substrates was determined (Figure 4). As hydrolysis progressed, the adsorbed proteins were gradually released into the supernatant. When PB was used as the substrate, about 50% of the added protein was present in the supernatant after complete hydrolysis of PB at 24 h of incubation (Fig. 4(a)). The release of about 90% of the added protein into the supernatant was achieved in this reaction by incubating the reaction at 45°C for an additional 24 h. However, a comparison of the protein values detected in the supernatant after 48 h of hydrolysis in duplicate experiments showed that the amount of protein desorbed could vary from about 60% to 100%. When WB was used as the substrate, about 50% of the added protein was detected in the supernatant after complete hydrolysis of WB at 48 h (Fig. 4(b)). Further incubation of the reaction at 45°C for an additional 24 h did not increase the amount of protein released into the supernatant.

In an earlier study, Ooshima et al. (1990) found that only about 60-70% of the cellulases could be recovered in solution after complete hydrolysis of their lignocellulosic substrate. By measuring Avicelase activity and CMCase activity, Deshpande and Eriksson (1984) also observed that about 40-50% of these activities were recovered in the supernatant after 24 h hydrolysis of steam-exploded wheat straw and lignin-rich residue. The lower recoveries reported by
Hydrolysis of (a) PB (alkali- and peroxide-treated steam-exploded birch) and (b) WB (water-washed steam-exploded birch) by Cellulast/Novozym at a cellulase dose of 20 IU/(g cellulose) under standard hydrolysis conditions. Percent conversion (Δ) and percent cellulase protein in the supernatant (□) were determined at different times during hydrolysis.

Figure 4.
these workers after complete hydrolysis of their substrates might be due to the higher lignin contents of the substrates that they used compared to the relatively low lignin content of the PB substrate. It is possible that the cellulase proteins may be irreversibly adsorbed onto the lignin in the residues of these substrates, as well as in the residue of the WB substrate used in this study.

4.3.2 Recycling of Enzymes Adsorbed to the Residual Substrate During Hydrolysis

From the profile obtained in Figure 4, it was apparent that after 12h and 24h of hydrolysis respectively for the PB and WB substrates, most of the substrate had been hydrolyzed while the majority of the added cellulase protein was still associated with the residual substrate. To try to maximize both the hydrolysis yield and the cellulase protein recovery, enzyme recycling was carried out after 12 h of hydrolysis for the PB, and 24 h for the WB substrate. When PB was used as the substrate, the observed activity decreased from 18 IU/(g cellulose) to 11 IU/(g cellulose) after one recycling round (Table III). The observed activity continued to decrease with each subsequent recycling step. By the fifth round, only 28% of the originally added cellulase activity could be recovered. After each recycling round, the observed activity was lower than the activity expected based on the cellulase protein recovered. This suggested that the recovered cellulases were not as effective in hydrolyzing the substrate after recycling as were the original cellulases. With WB as substrate, the recovered activities were lower than those obtained with PB; this could have been a consequence of the higher lignin content of the WB substrate. As with the PB substrate, the observed activity decreased with each recycling round, and the
Table III. Recovery of Protein and Cellulase Activity During Recycling of Cellulases Adsorbed to Residual PB (Alkali- and Peroxide- treated Steam-exploded Birch and WB (Water-washed Steam-exploded Birch)

<table>
<thead>
<tr>
<th>Hydrolysis Round</th>
<th>Conversion(^a) (%)</th>
<th>Original Cellulase Protein Recovered (%)</th>
<th>Expected Activity(^b) (IU/g cellulose)</th>
<th>Observed Activity(^c) (IU/g cellulose)</th>
<th>Original Activity Recovered(^d) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>75.8 ± 2.3</td>
<td>100.0</td>
<td>18</td>
<td>18</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>54.5 ± 5.9</td>
<td>82.8 ± 3.4</td>
<td>15</td>
<td>11</td>
<td>61</td>
</tr>
<tr>
<td>3</td>
<td>45.1 ± 5.5</td>
<td>78.1 ± 0.5</td>
<td>14</td>
<td>8</td>
<td>44</td>
</tr>
<tr>
<td>4</td>
<td>40.8 ± 5.3</td>
<td>65.7 ± 11.5</td>
<td>12</td>
<td>7</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>33.2 ± 3.7</td>
<td>64.3 ± 9.3</td>
<td>12</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>WB</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>79.7 ± 4.2</td>
<td>100.0</td>
<td>19</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>52.9 ± 4.9</td>
<td>84.2 ± 5.3</td>
<td>16</td>
<td>9</td>
<td>47</td>
</tr>
<tr>
<td>3</td>
<td>41.0 ± 8.4</td>
<td>79.7 ± 1.1</td>
<td>15</td>
<td>6</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>34.8 ± 4.6</td>
<td>76.0 ± 5.1</td>
<td>14</td>
<td>4</td>
<td>21</td>
</tr>
<tr>
<td>5</td>
<td>27.9 ± 8.1</td>
<td>73.1 ± 0.9</td>
<td>14</td>
<td>3</td>
<td>16</td>
</tr>
</tbody>
</table>

\(^a\) percent conversion was determined after 12 h of hydrolysis for PB and 24 h of hydrolysis for WB.

\(^b\) expected activity was calculated based on protein recovery in each recycling round.

\(^c\) observed activity was determined using dose-response curves for WB and PB hydrolysis.

\(^d\) percent original activity recovered was calculated by dividing observed activity in each hydrolysis round by the observed activity in the first hydrolysis round.
observed activity was lower than the activity expected based on the cellulase protein recovered. These results again suggested that the recovered cellulases were not as effective in subsequent rounds of hydrolysis.

Previously, Vallander and Eriksson (1987) had shown that only 32-55% of the original sugar yields could be obtained when cellulases adsorbed onto the residual steam-exploded aspen and wheat straw substrates were used to hydrolyze a mixture of fresh and residual substrates. However, Ramos et al. (1993, 1994) found that the hydrolysis yield obtained with cellulases adsorbed onto the residual substrate of steam-exploded, peroxide-treated eucalyptus was similar to that obtained with fresh cellulases after one round of recycling. After 6 rounds of enzyme recycling, 54% of the hydrolysis yield could still be recovered. This difference was probably due to the low lignin content of the peroxide-treated eucalyptus substrate (0.7%).

4.3.3 Recycling of Enzymes Present in the Residual Substrate and the Hydrolysis Supernatant

To try to improve the recovery of cellulase activity, the enzymes present in the supernatant, in addition to those associated with the residual substrate, were also recycled. Approximately 30% of the initially added cellulase proteins were still in the supernatants during recycling of the residual substrates for both PB and WB (Figure 4). Recovery of additional activity should be possible if these enzymes in the supernatant are recycled together with the residual substrate. For both substrates, the activity recovered in each round improved by up to 24% (Table IV) compared to the values obtained when only the enzymes adsorbed onto residual substrate were recycled (Table III). As was
<table>
<thead>
<tr>
<th>Hydrolysis Round</th>
<th>Conversion&lt;sup&gt;a&lt;/sup&gt; (%)</th>
<th>Original Cellulase Protein Recovered (%)</th>
<th>Expected Activity&lt;sup&gt;b&lt;/sup&gt; (IU/g cellulose)</th>
<th>Observed Activity&lt;sup&gt;c&lt;/sup&gt; (IU/g cellulose)</th>
<th>Original Activity Recovered&lt;sup&gt;d&lt;/sup&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB 1</td>
<td>78.9 ± 6.1</td>
<td>100.0</td>
<td>19</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>68.1 ± 11.1</td>
<td>92.0 ± 3.3</td>
<td>17</td>
<td>16</td>
<td>84</td>
</tr>
<tr>
<td>3</td>
<td>59.2 ± 5.9</td>
<td>84.2 ± 2.9</td>
<td>16</td>
<td>13</td>
<td>68</td>
</tr>
<tr>
<td>4</td>
<td>47.6 ± 2.7</td>
<td>79.2 ± 5.2</td>
<td>15</td>
<td>9</td>
<td>47</td>
</tr>
<tr>
<td>5</td>
<td>41.0 ± 5.1</td>
<td>77.8 ± 3.3</td>
<td>15</td>
<td>7</td>
<td>37</td>
</tr>
<tr>
<td>WB 1</td>
<td>80.2 ± 0.2</td>
<td>100.0</td>
<td>19</td>
<td>19</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>65.5 ± 7.3</td>
<td>95.7 ± 0.1</td>
<td>18</td>
<td>13</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>57.6 ± 5.3</td>
<td>92.5 ± 2.6</td>
<td>18</td>
<td>10</td>
<td>53</td>
</tr>
<tr>
<td>4</td>
<td>47.6 ± 5.6</td>
<td>90.9 ± 3.8</td>
<td>17</td>
<td>7</td>
<td>37</td>
</tr>
<tr>
<td>5</td>
<td>30.8 ± 9.7</td>
<td>88.9 ± 4.5</td>
<td>17</td>
<td>3</td>
<td>16</td>
</tr>
</tbody>
</table>

<sup>a</sup> percent conversion was determined after 12 h of hydrolysis for PB and 24 h of hydrolysis for WB.

<sup>b</sup> expected activity was calculated based on protein recovery in each recycling round.

<sup>c</sup> observed activity was determined using dose-response curves for WB and PB hydrolysis.

<sup>d</sup> percent original activity recovered was calculated by dividing observed activity in each hydrolysis round by the observed activity in the first hydrolysis round.
found in the previous recycling strategy, the observed activity detected in each round was lower than the expected activity, and the values decreased with each recycling step. When PB was used as the substrate, only 37% of the original activity was recovered by the end of the fifth hydrolysis round, even though about 80% of the originally added protein was recycled. Again, lower activities were recovered when WB was used as the substrate compared to the values obtained using PB. In the former case, only 16% of the original activity was recovered by the fifth hydrolysis round, although almost 90% of the added protein was recycled.

4.3.4 Factors Contributing to the Low Observed Activity Recovered

These results indicate that additional activity can be recovered simply by recycling the cellulases present in the supernatant in addition to those adsorbed to the residual substrate. However, the observed activities were still lower than the activities expected on the basis of recovered cellulase protein. Although inactivation of the cellulases by prolonged incubation at 45°C might result in the low observed activities recovered in successive recycling steps, Ramos et al. (1993) had previously shown that there was only a 10% drop in the rate of substrate hydrolysis after a 15 day pre-incubation of cellulases at 45°C.

The decrease in effectiveness of the recovered cellulases in subsequent rounds of hydrolysis could be due to the loss of particular enzyme components during recycling. It is recognized that efficient hydrolysis of crystalline cellulose requires multiple cellulase components. In the first recycling method, only the cellulase components adsorbed to the residual substrate were recycled. It is
possible that particular enzyme components present in the original Celluclast mixture were no longer adsorbed onto the residual substrate after a certain period of hydrolysis. Thus, the loss of these cellulase components from the recycled residual substrate might have affected the synergistic interaction of the cellulases during the hydrolysis of the substrates. Even in the second recycling strategy, a small amount (10-20%) of cellulase protein was still not recovered from the supernatant. It is possible that particular enzyme components were also lost using this recycling strategy.

In order to determine if particular cellulase components were lost during recycling, the cellulase proteins that were not recycled by the two recycling strategies were analyzed by a previously described chromatofocusing method (Yu et al., 1993). It was found that all of the cellulase components that were present in the original Celluclast were found to be present in the filtrates containing non-recovered cellulase proteins (data not shown). Thus, there did not appear to be selective loss of particular Celluclast components during the recycling steps. In fact, the proportions of the cellulase components bound onto the substrates were similar to those in the original Celluclast, and these proportions are maintained throughout cellulose hydrolysis (manuscript submitted, Yu et al.).

During the first recycling strategy, where the enzymes associated with the residual substrate were recycled, both the fresh and residual substrates appeared to be hydrolyzed. However, it was unclear to what extent each substrate was being hydrolyzed. It was possible that the cellulases adsorbed onto the residual substrate might not be able to transfer to the fresh substrate
until the residual substrate was completely hydrolyzed. Alternatively, the cellulases adsorbed onto the residual substrate may have been able to partition themselves between the fresh substrate and the residual substrate when the substrates were mixed together.

In order to determine which substrate was being hydrolyzed during the first recycling strategy, one round of recycling was carried out using PB or WB as the residual substrate and dyed Avicel as the fresh substrate. Hydrolysis of the fresh substrate (dyed Avicel) could be determined by measuring the absorbance of the supernatant at 595 nm, while hydrolysis of the combined substrates could be determined by measuring the amount of reducing sugars released. Consequently, hydrolysis of the residual substrate could be distinguished from hydrolysis of the fresh substrate using this strategy.

It appeared that both the fresh and residual substrates were hydrolyzed throughout the reaction in this experiment (Figure 5), implying that the cellulases which had been adsorbed on the residual substrate were able to quickly partition themselves between the fresh substrate and the residual substrate. Using dose-response curves for the hydrolysis of dyed Avicel, PB, and WB as described previously, the observed activities of adsorbed cellulases on fresh substrate (Avicel) and residual substrate (PB or WB) were determined. The proportions of fresh substrate and residual substrate initially present in the substrate mixture were calculated. The fresh substrate (Avicel) constituted about 60% of the cellulose content of the total mixture, while the residual substrate (WB or PB substrates) made up the remaining 40% of the cellulose content. It appeared that most of the originally added cellulase activity remained associated with the
Hydrolysis of a substrate mixture of dyed Avicel and residual substrate obtained after one hydrolysis round. After one hydrolysis round, dyed Avicel was added to either residual PB (alkali- and peroxide-treated steam-exploded birch) or residual WB (water-washed steam-exploded birch) and the next round of hydrolysis was carried out under standard hydrolysis conditions. Reducing sugar and $A_{595}$ were measured at different times to calculate percent conversion of the residual substrate and the fresh substrate. (a) PB (□) as residual substrate (38% of total cellulose), dyed Avicel (▼) as fresh substrate (62% of total cellulose); (b) WB (□) as residual substrate (36% of total cellulose), dyed Avicel (▼) as fresh substrate (64% of total cellulose).
residual substrate. Only 30% (when PB was used as the residual substrate) and 40% (when WB was used as the residual substrate) of the observed activity was transferred to the fresh substrate (Avicel). This uneven distribution of cellulases between the fresh Avicel and the residual steam-exploded birch substrates might be attributed to the difference in adsorption affinity of cellulases to Avicel and these birch substrates. As shown previously, the adsorption affinity of Celluclast to Avicel was found to be about 3 times lower than the affinity of Celluclast to the PB and WB substrates (Table I). This could account for the distribution of cellulases observed in the above experiments. If the PB and WB substrates were used as the fresh substrates instead of Avicel, it is possible that the cellulases would be distributed more evenly between the fresh substrate and the residual substrate, since Celluclast would have the same affinity to both the fresh and residual substrates in this case.

Since both the residual and fresh substrates were hydrolyzed during enzyme recycling, the low observed activity recoveries could be due to the physical limitations imposed by the presence of the residual substrate. It has been reported in several studies that substrates become increasingly recalcitrant as hydrolysis progresses (Lee and Fan, 1983; Wang and Converse, 1991). The slower hydrolysis of the more recalcitrant residual substrate which accumulated after each recycling round could have resulted in the lower observed activities recovered during enzyme recycling.

To determine if the residual substrate recovered after each recycling round was more recalcitrant than fresh substrate, the specific hydrolysis rate of PB substrate that had been previously hydrolyzed to increasing extents was
measured. It was found that the specific hydrolysis rate of the PB substrate dropped 3 to 4 fold by the time the original material was about 40% hydrolyzed (Figure 6). This agrees with the previous observations obtained using Solka Floc (Lee and Fan, 1983) and steam-pretreated poplar wood (Wang and Converse, 1991), where similar declines in the specific hydrolysis rates were observed during substrate conversion. Since the residual substrate appears to be hydrolyzed more slowly than the fresh substrate, a lower observed activity would be obtained from a mixture of fresh and residual substrates compared to just fresh substrate alone, even when the same cellulase dose was present in both hydrolyses.

The results of the two experiments described above probably accounted for the low observed cellulase activity which was recovered in the recycling experiments. In both recycling strategies, the presence of residual substrate in the subsequent hydrolysis rounds caused an apparent drop in the observed activity due to the recalcitrant nature of the residual substrate. Therefore, the decrease in the recovery of observed activity does not actually reflect the loss of cellulases during recycling. This is supported by the fact that 65 to 90% of cellulase proteins could be recovered by these recycling strategies (Tables III and IV). In theory, the cellulose in both substrates can be completely hydrolyzed in each round of hydrolysis provided that the time of hydrolysis is extended.

4.3.5 Recycling of the Enzymes Present in the Non-cellulosic Substrate Residue and the Supernatant

A third strategy for recycling cellulases was designed to avoid the accumulation of residual substrate during recycling. Originally, we had hoped
Effect of cellulose conversion on the specific hydrolysis rate of PB (alkali- and peroxide-treated steam-exploded birch). The specific hydrolysis rates of PB hydrolyzed to different extents were determined after 5 minutes of hydrolysis by the adsorbed enzymes under standard hydrolysis conditions.

Figure 6.
to recycle the cellulases released into the supernatant after complete hydrolysis. However, as our earlier results had indicated that the amount of the enzymes released into the supernatant after complete hydrolysis was highly variable, a different approach was needed in order to recover all of the cellulases. In this third recycling strategy, fresh substrate was added to the reaction containing the non-cellulosic residue after complete hydrolysis of the cellulose. The cellulases present in the reaction were allowed to adsorb onto the fresh substrate by incubating the mixture at 4°C until adsorption equilibrium was reached. The fresh substrate and non-cellulosic residue were then recovered, and used in the subsequent round of hydrolysis.

When PB was used as the substrate, all of the original activity could be recovered after 4 consecutive hydrolysis rounds, as indicated by the complete hydrolysis obtained after each round (Table V). After the fifth hydrolysis round, 95% of the original activity could still be recovered. This indicated that the cellulases were able to transfer from the non-cellulosic residue, which was predominantly lignin, to the fresh substrate, as about 50% of the cellulase proteins was shown to be associated with the lignin residue before the addition of fresh substrate. This result also indicated that there was no inactivation of the cellulases for at least four days during recycling.

However, different results were obtained when WB was used as the substrate (Table V). The observed activity decreased with each successive recycling step. In the second hydrolysis round, only 71% of the original activity was recovered. Complete hydrolysis could only be achieved by extending the reaction time of each subsequent hydrolysis round. The time required for
Table V. Recovery of Cellulase Activity During Recycling of Cellulases in Non-Cellulosic Residue and Reaction Supernatant for PB (Alkali- and Peroxide-treated Steam-exploded Birch and WB (Water-washed Steam-exploded Birch)

<table>
<thead>
<tr>
<th>Hydrolysis Round</th>
<th>Conversion(^a) (%)</th>
<th>Observed Activity(^b) (IU/g cellulose)</th>
<th>Original Activity Recovered(^c) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>97.8 ± 3.7</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>96.0 ± 2.3</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>3</td>
<td>101.6 ± 3.5</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>4</td>
<td>103.0 ± 4.2</td>
<td>20</td>
<td>100</td>
</tr>
<tr>
<td>5</td>
<td>93.1 ± 0.7</td>
<td>19</td>
<td>95</td>
</tr>
<tr>
<td>WB</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>68.4 ± 0.0</td>
<td>14</td>
<td>100</td>
</tr>
<tr>
<td>2</td>
<td>57.5 ± 1.7</td>
<td>10</td>
<td>71</td>
</tr>
<tr>
<td>3</td>
<td>42.8 ± 2.0</td>
<td>6</td>
<td>43</td>
</tr>
<tr>
<td>4</td>
<td>33.4 ± 2.3</td>
<td>4</td>
<td>29</td>
</tr>
<tr>
<td>5</td>
<td>24.9 ± 0.0</td>
<td>2</td>
<td>14</td>
</tr>
</tbody>
</table>

\(^a\) Percent conversion was determined after 24 h of hydrolysis.

\(^b\) Observed activity was determined using dose-response curves for hydrolysis of PB and WB.

\(^c\) Percent original activity recovered was calculated by dividing observed activity in each hydrolysis round by the activity in the first round.
complete hydrolysis increased from 3 d in the first hydrolysis round to 16 d by the fifth hydrolysis round. Previously, Girard and Converse (1993) had observed a four-fold decrease in the specific conversion rate of fresh steam-pretreated wood when cellulases adsorbed onto the lignaceous residue were used instead of fresh cellulases. Their results suggested that a larger portion of the cellulases was irreversibly adsorbed to the lignaceous residue compared to the result obtained in this study. This was probably due to differences in the enzymes and substrates used.

In our third recycling strategy, the lignaceous residue was not removed from the reaction. Thus, the lignin content of the reaction mixture continued to increase with the increasing number of hydrolysis rounds carried out. When PB (4% lignin) was used as the substrate, the lignin constituted only about 17% of the weight of the substrate by the fifth round of hydrolysis. However, when WB (32% lignin) was used as the substrate, the lignin content was as high as 70% by the fifth round of hydrolysis. This probably accounted for the dramatic difference in the recovered activity which was observed between the PB and WB substrates.

Previously, Chernaglazov et al. (1988) showed that the rate of substrate hydrolysis decreased about three-fold when lignin was added to microcrystalline cellulose to a lignin content of 33%. It was suggested that the drop in the hydrolysis rate could be due to irreversible adsorption of cellulases onto lignin. In the present study, a 30% decrease of the original cellulase activity was observed after one round of recycling with WB as substrate. In this round of hydrolysis, the amount of lignin present in the reaction would have been
equivalent to the amount of lignin in a hydrolysis reaction using a lignocellulosic substrate containing 48% lignin. However, half of the lignin content was in the form of cellulose-free lignin which had accumulated after complete hydrolysis of the cellulose, while the other half was associated with the carbohydrate present in the freshly added substrate. It is possible that these two forms of lignin have different properties with respect to cellulase adsorption or entrapment. Cellulases might be more tightly associated with the cellulose-free lignin than they are with the carbohydrate-associated lignin. Thus, a large proportion of the cellulases may not have been recovered during recycling when using WB as the substrate because of the large amounts of cellulose-free lignin which had been accumulated during the five hydrolysis rounds. However, the low content of cellulose-free lignin that was present during PB recycling (17% after 4 recycling steps) might not be high enough to give a significant decrease in the observed cellulase activity. The difference in recoveries of observed activity between the PB and WB substrates in the first and second strategies was small (20%) compared to that in the third strategy. This could be due to the relatively low cellulose-free lignin content in the reactions when the first and second recycling strategies were used.

4.3.6 Cellulase Savings Obtained by Recycling

The potential cellulase savings projected for each of the three recycling strategies are summarized in Table VI. The savings were calculated by comparing the amount of cellulase activity required for 5 rounds of hydrolysis without recycling to the amount of cellulase activity required for 5 rounds of
Table VI. Cellulase Savings Obtained by Enzyme Recycling during 5 Hydrolysis Rounds$^a$

<table>
<thead>
<tr>
<th></th>
<th>PB</th>
<th>WB</th>
</tr>
</thead>
<tbody>
<tr>
<td>residual substrate alone</td>
<td>148%</td>
<td>130%</td>
</tr>
<tr>
<td>residual substrate + supernatant</td>
<td>189%</td>
<td>153%</td>
</tr>
<tr>
<td>non-cellulosic residue + supernatant</td>
<td>427%</td>
<td>146%</td>
</tr>
</tbody>
</table>

$^a$ percent savings was calculated by dividing the cellulase activity required for 5 rounds of hydrolysis without recycling by the cellulase activity required for 5 rounds of hydrolysis with recycling.
hydrolysis using each of our recycling strategies. The highest savings were achieved when the enzymes from the non-cellulosic PB residue and supernatant were recycled together. With this strategy, the same cellulases could be re-used at least four times, for a cellulase savings of 427%. It may be possible to continue recycling the enzymes for several more rounds. However, further recycling of the cellulases may depend on the removal of some of the lignin found in the starting substrate, as the results from this work suggest that a high lignin content in a substrate decreases the recovery of cellulase activity. It would be useful to examine enzyme recycling using substrates containing various amounts of lignin, in order to define the pretreatment required of lignocellulosic substrates that would permit efficient cellulase recycling. However, the cost of such pretreatment, as well as the cost of implementing the recycling strategy in a plant, must be considered before it can be assured that this recycling strategy will help improve the economics of the bioconversion process.
5. DISCUSSION AND CONCLUSIONS

It is apparent that the efficiency of enzymatic cellulose conversion processes can be improved by reducing the cost of the enzymatic hydrolysis step (Nguyen, 1993). One approach to reducing this cost is to increase the susceptibility of the substrate to hydrolysis, as it is known that various pretreatments of different substrates result in an increase in the rate and/or yield of hydrolysis. A second approach is to try to reduce the cost of the cellulases required. Strategies aimed at reducing this cost by increasing the specific activity of the enzymes, or by producing large quantities of the cloned cellulases have not yet been successful, partly because the exact combination of cellulases that is required for efficient hydrolysis has still not been defined. In the work reported here, we have examined cellulase recycling as a possible strategy for lowering the cost of the enzymes required for cellulose hydrolysis.

It was apparent that many different pretreatments have been used successfully to increase the susceptibility of substrates to cellulase attack (Fan et al., 1987). However, it is still not known whether a specific pretreatment applied to one substrate is the most effective method or set of conditions for treating another substrate. It seems that in order to determine the most efficient means of pretreatment for a substrate, the factors that affect its susceptibility must first be understood. In particular, the relationship between substrate susceptibility and enzyme adsorption has received considerable attention in the last ten years (Converse, 1993) as it is obvious that the cellulases must first be able to adsorb to their substrate before they can hydrolyze it. Several studies
have shown a strong correlation between the susceptibility to enzymatic hydrolysis of a substrate, as measured by the hydrolysis rate, and the surface area available for cellulase adsorption (Grethlein, 1985; Thompson et al., 1992). However, it is not yet safe to conclude that the hydrolysis rate depends primarily on the available surface area, as the current methods used to measure the surface areas of different substrates might not accurately represent the surface area available for cellulase adsorption. As described in the first section of this study, we used CBD_{cex}, the cellulose-binding domain from a cellulase of *C. fimi,* to evaluate its suitability as a probe to predict the surface area accessible to a mixture of cellulases. As the CBD_{cex} component is nonhydrolytic and possesses an affinity for cellulose, it initially appeared to have potential as a probe for investigating the adsorption of cellulases. However, we found that the accessible cellulose surface area, as measured by CBD_{cex}, was quite different from the area accessible to the enzymes in Celluclast. This probably reflected the differences that occur between the single component, CBD_{cex}, and the multi-component Celluclast mixture. As several studies have indicated (Kyriacou et al., 1989; Yu et al., submitted), both common and distinct adsorption sites exist for the different *T. reesei* cellulase components. Different adsorption sites may therefore also exist for the cellulases from *Trichoderma reesei* and *Cellulomonas fimi.* For studies examining a given multicomponent cellulase system, it appears that the best probes to represent the adsorption of the components would be the cellulases themselves.

Since our results indicated that CBD_{cex} could not be used to predict the accessibility of substrates to multicomponent cellulase mixtures, our subsequent
examination of the relationship between cellulase adsorption and the hydrolysis rate was conducted using the Celluclast mixture itself to measure the accessible surface area. As mentioned previously in Section 3, a good correlation between the hydrolysis rate and the accessible surface area has not been conclusively demonstrated because the hydrolysis rates in earlier studies were measured only after extensive hydrolysis had taken place. The results presented in Section 3 of our study showed that the initial hydrolysis rate of different substrates was not related to the $P_{\text{max}}$, which was calculated as the maximum amount of cellulase adsorbable to the substrate. This result, while contrary to those found in previous studies, was not unexpected, as in most cellulose hydrolysis processes, the amount of cellulase adsorbed to the substrate is much lower than $P_{\text{max}}$. Thus, the $P_{\text{max}}$ of a substrate would not reflect the hydrolysis of the substrate in these processes. This observation is also in agreement with an earlier study (Igen Corp., 1990), which showed that the hydrolysis rate decreased as more enzyme was adsorbed to the substrate. These authors suggested that this was because the cellulases could not interact effectively due to crowding at higher enzyme doses. Thus, even if the substrates were saturated with cellulase, in any studies which hoped to relate enzyme adsorption to the hydrolysis rate, the $P_{\text{max}}$ would not necessarily reflect the susceptibility of a substrate. Under these conditions, the hydrolysis rate may be limited by both the crowding of the enzymes, and the structural features of the substrate.

In our work, we have only determined the total amount of protein adsorbed to the various substrates, rather than the amount of the individual components. It is possible that the types of cellulase components adsorbed onto
different substrates can influence the efficiency with which they are hydrolyzed. It has been shown that efficient hydrolysis of crystalline cellulose requires the presence of exoglucanases as well as endoglucanases (Wood and McCrae, 1972). Thus, substrates which can adsorb all of the cellulase components required for efficient hydrolysis may be more susceptible to hydrolysis than substrates which are unable to adsorb the required enzymes due to structural features which may limit the accessibility of the substrates to some of these cellulases. However, in a separate study (Yu et al., 1993), we showed that all of the cellulase components were able to adsorb to several different substrates (Avicel, Acid-swollen Avicel, and steam-exploded birch substrates) even though all of the adsorbed components may not be necessary for efficient hydrolysis (Yu and Saddler, submitted). Therefore, it appears that the susceptibility of the cellulosic substrates may depend more on the structural features of the substrate than on the type of the adsorbed enzymes. This was also suggested by the finding in this study that different substrates had different specific hydrolysis rates. The specific hydrolysis rate, which is representative of the reactivity of a substrate, likely depends on a combination of the structural features (e.g., surface area, crystallinity, degree of polymerization) which had been suggested in earlier studies to affect hydrolysis rate (Converse, 1993).

As both the rate and the yield of hydrolysis should be maximized to obtain efficient hydrolysis, it was suggested that substrate pretreatments be evaluated by observing their effects on the specific hydrolysis rate and the $P_{\text{max}}$ values. Pretreatments have been observed to alter one or more properties of various lignocellulosic substrates. For example, a pretreatment such as steam explosion
may change the surface area of a substrate available to cellulases (which is represented by $P_{\text{max}}$), or the susceptibility of the cellulose (which is represented by the specific hydrolysis rate), or it may change both properties. By evaluating different pretreatments with respect to their effect on both $P_{\text{max}}$ and the specific hydrolysis rate, it might be possible to determine the mechanism by which each pretreatment increases the susceptibility of a substrate.

As mentioned previously, cellulase recycling appeared to be among the most feasible strategies for reducing the cost of the enzymes used in cellulose hydrolysis. Since none of the cellulase components were shown to be selectively adsorbed onto the birch substrates during hydrolysis (Yu et al., 1993), it was theoretically possible to recover substantial amounts of active enzyme by simply recycling the cellulases from either the substrate during hydrolysis, or from the supernatant after hydrolysis. However, successful recycling was only carried out when the enzymes present in the complete hydrolysis mixture were recycled after complete hydrolysis of the PB substrate. Using this strategy, the same cellulases could be recycled without any loss of activity for at least four rounds of hydrolysis. Only a small input (a total of 12% over 5 rounds) of cellulase protein would be required to compensate for the unadsorbed protein that was lost during the recycling process.

It appeared that successful recycling was dependent on two conditions. First, the substrate had to be completely hydrolyzed before the recycling was carried out. This was required to ensure that the presence of residual substrate did not result in a decrease in the observed cellulase activity recovered in the subsequent hydrolysis round. Second, a low lignin content in the substrate was
required because at higher lignin contents, a portion of the cellulases was either irreversibly adsorbed to the lignin residue, or trapped within the lignin residue, and could not be recovered. The results from this work imply that the efficiency of cellulase recycle would probably be greatest using substrates with a low lignin content. In future work, it would be interesting to determine how the lignin type and content affect the recovery and reuse of cellulases added to different substrates. It appeared, from this study, that the increasing bulk resulting from lignin accumulation during the recycling steps greatly reduces the efficiency of enzyme recycle.

By combining the two approaches discussed in this study, a significant improvement in the efficiency of enzymatic cellulose conversion processes could be achieved. We first showed that the adsorption of a multicomponent enzyme system such as Celluclast could not be represented by a single cellulase component such as CBDcex. Thus, for the next part of our study, we used the Celluclast mixture itself in order to study the relationship between the amount of enzyme adsorbed and the initial hydrolysis rate for different cellulosic substrates. It was concluded that the various cellulosic substrates differed substantially in their susceptibility to hydrolysis. This seemed to be more dependent on the structural features of the substrate than on the amount of enzyme adsorbed. As these structural features are probably influenced by the type of pretreatment required to result in optimal hydrolysis of a given substrate, further work is required to determine the most effective pretreatments for different cellulosic substrates. In the last section of this work, we assessed various strategies to try to identify a method for the efficient recycling of cellulases. It was apparent
that the effects of the lignin content and lignin distribution in different cellulosic substrates will influence any proposed recycling strategy. The best results were obtained by recycling the cellulases after the complete hydrolysis of substrates with low lignin contents.
6. REFERENCES


