AN ABSTRACT OF THE THESIS OF

John J. Fenske for the degree of Doctor of Philosophy in Food Science and Technology presented on June 30, 1998. Title: Utilization of Lignocellulosic Polysaccharides.

Abstract approved: ____________________

Michael H. Penner

Lignocellulosic biomass represents a vast supply of fermentable carbohydrates and functional aromatic compounds. Conversion of lignocellulosics to ethanol and other useful products would be of widespread economical and environmental benefit. Better understanding of the behavior of different lignicellulosic feedstocks in fermentation protocols as well as catalytic activites involved in lignocellulosic depolymerization will further enhance the commercial viability of biomass-to-ethanol conversion processes.

The relative toxicity of the combined non-xylose components in prehydrolysates derived from three different lignocellulosic biomass feedstocks (poplar, corn stover and switchgrass, or *Panicum virgatum L.*) was determined using a *Pichia stipits* fermentation assay. The relative toxicity of the prehydrolysates, in decreasing order, was poplar-derived prehydrolysates > switchgrass-derived prehydrolysates > corn stover-derived prehydrolysates. Ethanol yields averaged 74%, 83% and 88% of control values for poplar, switchgrass and corn stover prehydrolysates, respectively. Volumetric ethanol productivities (g ethanol l^{-1} h^{-1}) averaged 32%, 70% and 102% of control values for poplar, switchgrass and corn stover prehydrolysates, respectively. Ethanol productivities correlated closely with acetate concentrations in the prehydrolysates; however, regression

lines correlating acetate concentrations and ethanol productivities were found to be feedstock-dependent.

Differences in the relative toxicity of xylose-rich prehydrolysates derived from woody and herbaceous feedstocks are likely due to the relative abundance of a variety of inhibitory compounds, *e.g.* acetate and aromatic comounds. Fourteen aromatic monomers present in prehydrolysates prepared from corn stover, switchgrass, and poplar were tentatively identified by comparison with published mass spectra. The concentrations of the aromatic monomers totaled 112, 141 and 247 $mg(l)^{-1}$ for corn stover, switchgrass and poplar prehydrolysates, respectively. The woody and herbaceous feedstocks differed in both amount and type of aromatic monomers.

The cellulases of *Trichoderma reesei* are the most widely studied for use in the depolymerization of lignocellulosics. The *Trichoderma* cellobiohydrolases CBH1 and CBH2 are traditionally categorized as exo-acting cellulases. A simple individual-based model was created to explore the potential effects of native endo activity on substrate-velocity profiles. The model results indicate that an enzyme with a small amount of endo activity will show an apparent substrate inhibition as substrate levels are increased. Actual hydrolysis studies using affinity chromatography-purified CBH2 preparations from three laboratories indicate that CBH2 has native endo activity, while CBH1 does not.

Utilization of Lignocellulosic Polysaccharides

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Head of Department of Food Science And Technology

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

John J. Fenske, Author

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Contribution of Authors

Dr. Michael H. Penner was involved in the design, analysis and writing of each chapter. Dr. Andrew Hashimoto was involved in the experimental design of Chapter 3. Donald Griffin was involved in the data analysis of Chapter 4. Dr. John Bolte was involved in the design, analysis and writing of chapter 5.

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CHAPTER 1

Introduction

Lignocellulosic biomass (plant material such as wood, grasses and agricultural residues) represents a vast supply of fermentable carbohydrates and functional aromatic compounds. Fermenting these carbohydrates to ethanol for use as a liquid transportation fuel would be of great economic and ecological benefit. However, the conversion of lignocellulosics to more useful products presents a special challenge. Put simply, the primary components of plant material, *i.e.* cellulose, lignin and hemicellulose, are not intended for easy breakdown. In fact, the opposite is true. These polymers are the plant's physical and chemical defense against the environment, predators, and pathogens.

The difficulty in purifying the massive amounts of glucose polymerized in cellulose stems from the recalcitrant chemical and physical nature of cellulose, as well as the lignin and hemisellulose which surround it. Hemicellulose is more easily obtained, but along with the sugars comes a slew of compounds such as extractives and degradation products. Present among these nonsaccharide components are compounds known to inhibit fermentative micro-organisms.

This dissertation concerns itself with two aspects of lignocellulosic conversion. Chapter 3 deals with differences in the fermentability of the liquefied hemicellosic fraction (called the prehydrolysate) of three potential feedstocks, a hybrid poplar, corn stover, and switchgrass. Chapter 3 has been accepted for publication in *Applied Biochemistry and*

Biotechnology. Chapter 4, completed a year after Chapter 3, concerns itself with comparing levels of potential inhibitors in the three prehydrolysates. It has been accepted as a note in *The Journal of Industrial Microbiology*.

Chapters 5 and 6 deal with a different aspect of lignocellulosic polysaccharide utilization, namely the enzymatic hydrolysis of cellulose. Specifically, the third paper describes a simple, individual-based model which simulates the action of a certain class of cellulases on a cellulose crystal and predicts certain kinetic behavior based on different catalytic activities. Chapter 5 will be submitted as a letter to *The Journal of Theoretical Biology*. Chapter 6 presents cellulose hydrolysis data which, based on the model predictions of Chapter 5, gives evidence for a predicted but unproven action pattern for a cellulytic enzyme produced by *Trichoderma reesei*. Chapter 6 will be submitted to *Enzyme and Microbial Technology*.

CHAPTER 2

Literature Review

2.1 Lignocellulosic Biomass Conversion

Most plant biomass can be categorized as lignocellulosic, and the category includes material such as wood, agricultural residues and municipal solid waste. Lignocellulosics are renewable, under-utilized feedstocks which represent a huge source of fermentable sugars. Producing ethanol from these sugars and burning it in place of, or in conjuction with, gasoline could provide enormous economic and environmental benefits (Lynd *et al.*, 1991; von Sivers and Zacchi 1996). The advantages of lignocellulosic-derived fuel ethanol over gasoline include:

- Unlike petroleum, the lignocellulosic feedstocks are renewable in the short term.
- The feedstocks would be domestically produced, reducing the United State's dependence on imported oil. Oil imports account for approximately one half of the U.S. trade deficit.
- Ethanol could be readily integrated into the current gasoline and diesel distrubution network. This is not the case for other gasoline alternatives such as hydrogen or electricity.
- The burning of lignocellulosic-derived ethanol introduces no net carbon dioxide into the atmosphere, since the CO_2 released during combustion is equal to the amount fixed by the plant during growth.

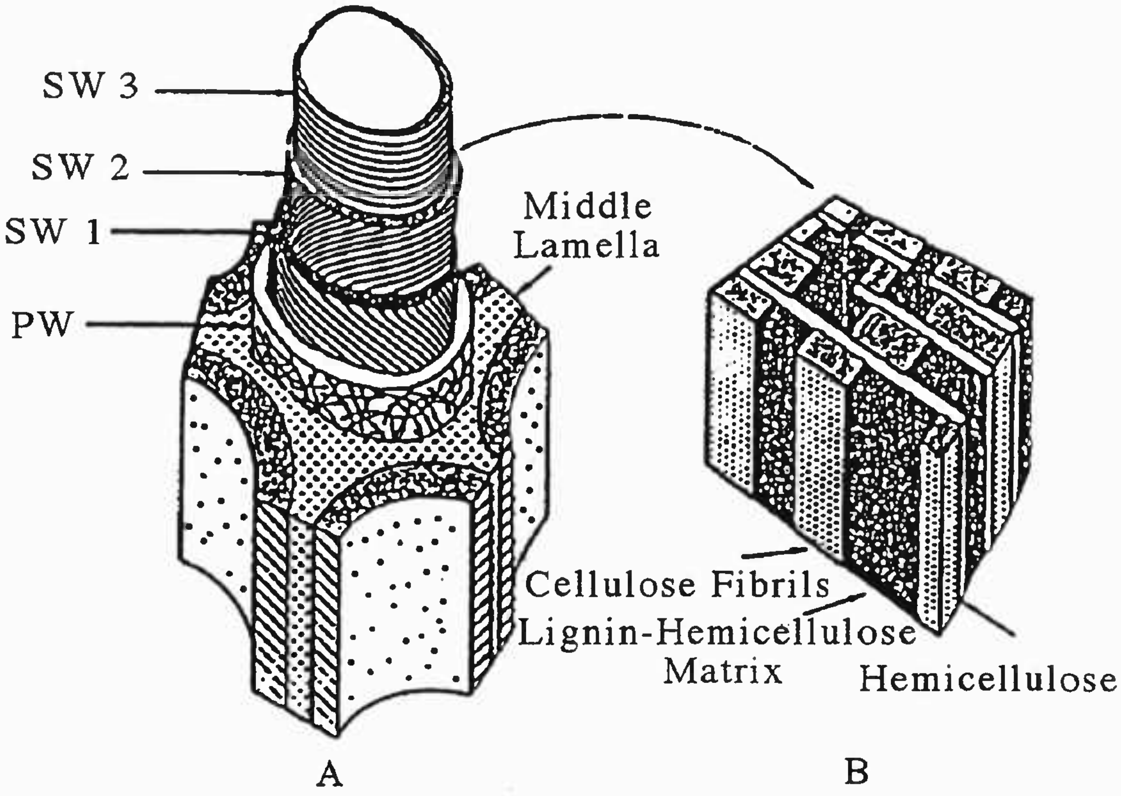
- Ethanol burns cleaner than gasoline, producing fewer pollutants.

2.2 Lignocellulosic Biomass

Lignocellulosic biomass consists primarily of cellulose, lignin and hemicellulose. Cellulose is the most (and lignin the second-most) abundant natural polymer on earth. To put the quantities in perspective, the amount of cellulose produced amounts to nearly 70 kg per person per day (Goodenough and Goodenough, 1993). The three components' proportions in lignocellulosics are different according to the source, but average 40-60% (w/w) cellulose, 10-25% lignin and 10-30% hemicellulose (Puls and Schuseil, 1993). The vast quantities of lignocellulosics make them important components of our biosphere and attractive feedstocks for conversion to useful products. Conversion schemes usually involve some form of depolymerization and subsequent transformation of the resultant monomers via fermentation, chemical or enzymatic processing. Although the abundance of lignocellulosic compounds presents a great opportunity, their natural state does not lend itself to utilization. In fact, the function of most of the lignocellulosic material is to provide physical support for the plant by resisting attack by the elements, herbivores and microbes. Contrast the lignocellulosic polysaccharide cellulose, to the energy storage polysaccharide starch, both of which are homopolymers of glucose. Starch is readily depolymerized by conditions which have little to no effect on cellulose. Thus starch is converted to high fructose corn syrup or ethanol with minimal energy inputs compared to what is necessary for cellulose conversion.

The recalcitrance of the lignocellulosic polysaccharides is explained both by the nature of the bonds present and the arrangement of the polymers in the plant. An analogy between the structure of reinforced concrete and lignocellulosic biomass is helpful in understanding the functions of the various components. Like reinforcing bars, cellulose provides strength. Like conrete, lignin provides strength and binding, while hemicellulose acts as a binding enhancer between lignin and cellulose. Figure 2.1 shows a wood cell and the arrangement of its components.

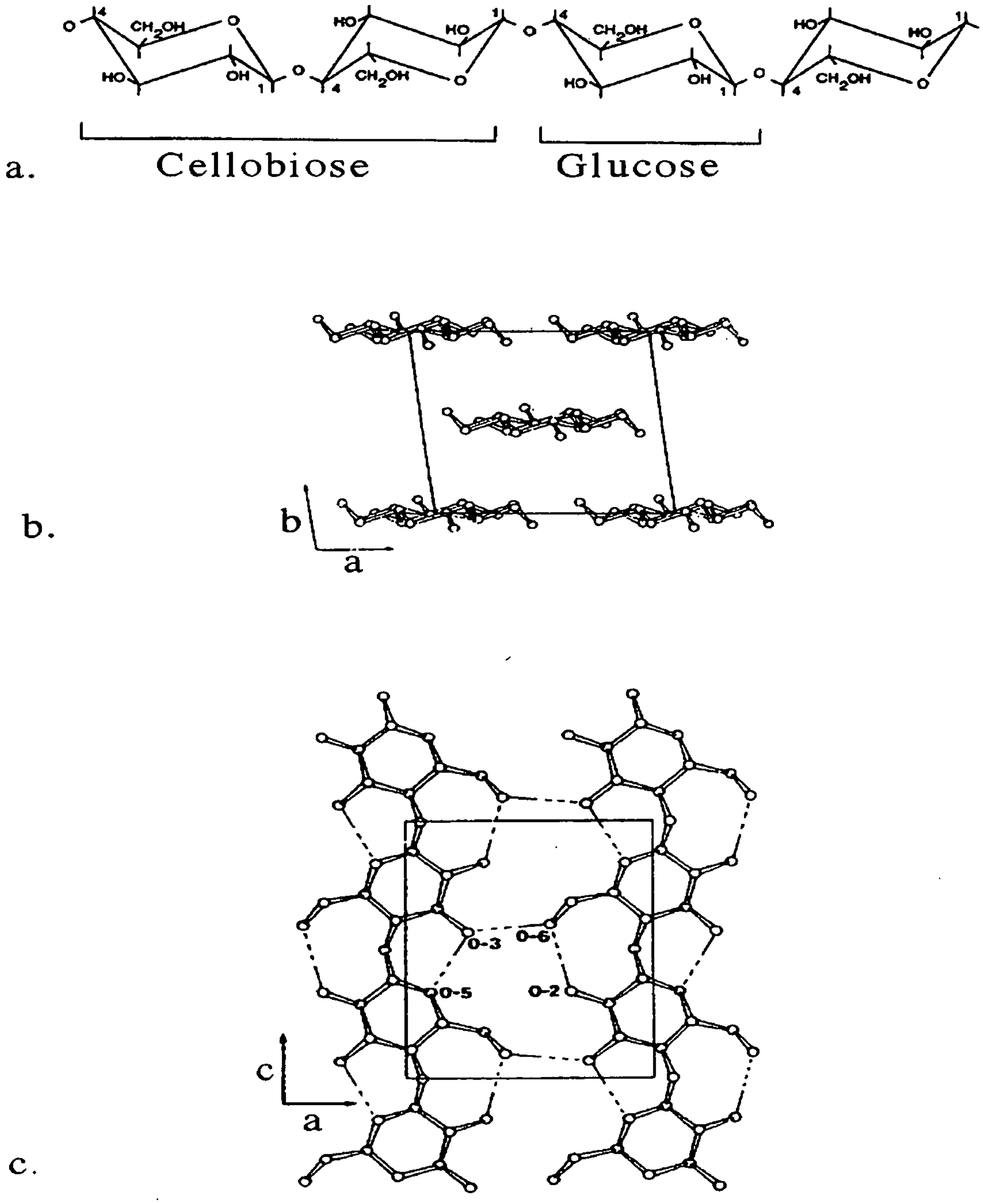
Figure 2.1. a) Organization of the cell wall layers of woody plants. b) Likely relationship of lignin and hemicellulose to cellulose microfibrils in the secondary cell wall. Taken from Béguin and Aubert, 1994.



2.2.1 Cellulose

Cellulose (Figure 2.2) is a nonbranched homopolymer of β 1-4 D-glucopyranose. Since the beta linkage inverts the neighboring glucose, the repeating unit of glucose is actually the disaccharide cellobiose. The degree of polymerization of the polymer chain in native wood has been estimated to be approximately 3,000 to 10,000 (Fan *et al.*, 1980). Cellulose chains form microfibrils, which are tightly packed bunches of cellulose chains, held together by hydrogen bonding, both intra- and intermolecular. The intramolecular bonding causes the long, rigid polymer to adopt a slight twist, forming a ribbon. The intermolecular hydrogen bonding, along with van der Waal's forces, cause the glucan chains to pack very tightly together, forming crystalline regions. The crystalline portions are interspersed with regions of less order, called amorphous regions. The close packing of the crystalline portions are what give cellulose its recalcitrant, insoluble and rigid character. A native wood microfibril, thought to be the smallest naturally occuring structural cellulose unit, is approximately 70% crystalline (Fan *et al.*, 1980). The size of microfibrils differs from source to source. The square cross section of a wood microfibril measures around 3-4 nm (Chanzy, 1990).

Figure 2.2 Cellulose Structure. From Gardner and Blackwell, 1974.



Based on chain packing, cellulose can be grouped into two major allomorphs, cellulose I and cellulose II. Cellulose I has the glucan ribbons which are layered, staggered and parallel (*i.e.* the reducing end-to-nonreducing end direction is consistent throughout). In nature, cellulose I predominates. For cellulose II, the glucan chains are antiparallel and not staggered. Cellulose II, also called regenerated cellulose, is produced via alkali treatments of cellulose I. Apparently, only a few organisms synthesize cellulose II (Lee *et al.*, 1994).

2.2.2 Hemicellulose

The word hemicellulose denotes not one particular molecule, but rather a class of polysaccharides with similar structures. Hemicelluloses are alkali-soluble cell wall polysaccharides associated with cellulose (Puls and Schuseil, 1993). A broad range of polysaccharides fit this definition. Hemicelluloses are heterogenous polymers, and are usually branched. Xylans, polymers in which the backbone is made up of β-1,4 linked xylopyranose residues, are the most abundant of the hemicelluloses in both woody and herbaceous lignocellulosics. Most of the branches in xylan originate at the number 2 or 3 carbon of the main chain xylose. In hardwoods, approximately one in ten backbone xylose units are substituted with 4-*o*-methyl-α-D-glucuronopyranosyl moieties bound at carbon 2 of the main chain xylose. These uronic acid side chains are also present in grass hemicellulose, however they are less common. Grass xylans are more heavily substituted than those of hardwoods. Approximately one in nine backbone monomers is substituted with an α arabinofuranose residue at the number 2 and/or 3 carbon. The arabinose sidechains may also be ester linked to phenolic compounds such as coumaric or ferulic acid. In barley straw, approximately 7% of the arabinose sidechains were esterified. Acetyl groups are another type of sidechain commonly found in woody and herbaceous lignocellulosics. It is believed that nearly half of the xylose units are acetylated (Puls and Schuseil, 1993). Figures 2.3 and 2.4 show partial structures of xylans from wood and grass, respectively.

Figure 2.3. Partial structure of arabinoxylan from wood. Taken from Puls and Schuseil, 1993.

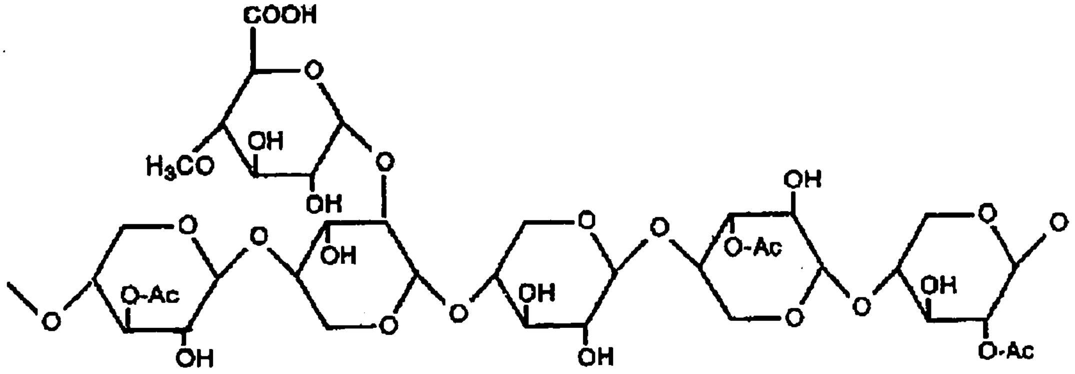
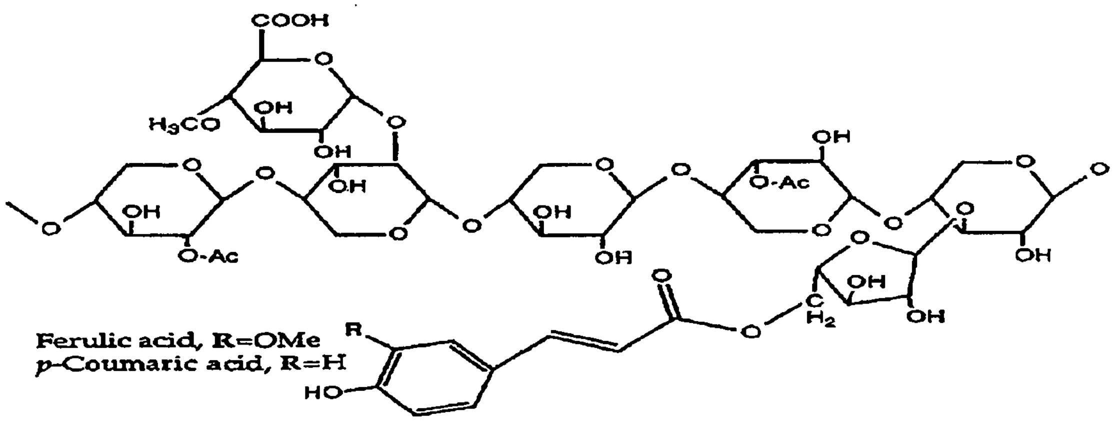


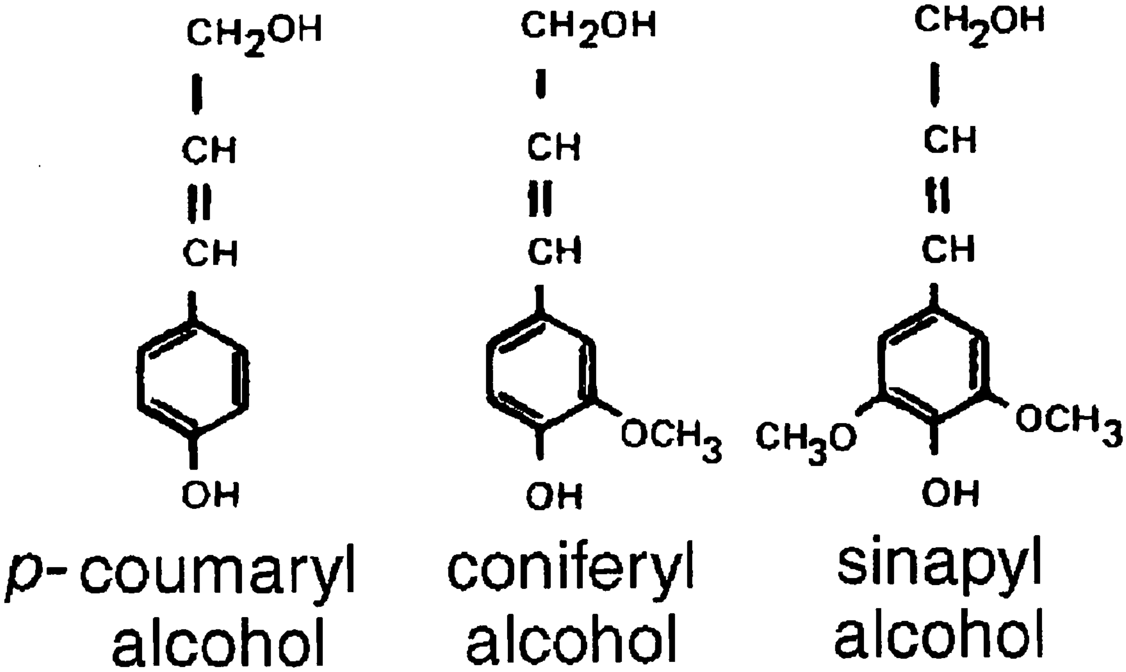
Figure 2.4. Proposed chemical structure for a typical grass arabinoxylan. Taken from Puls and Schuseil, 1993.



2.2.3 Lignin

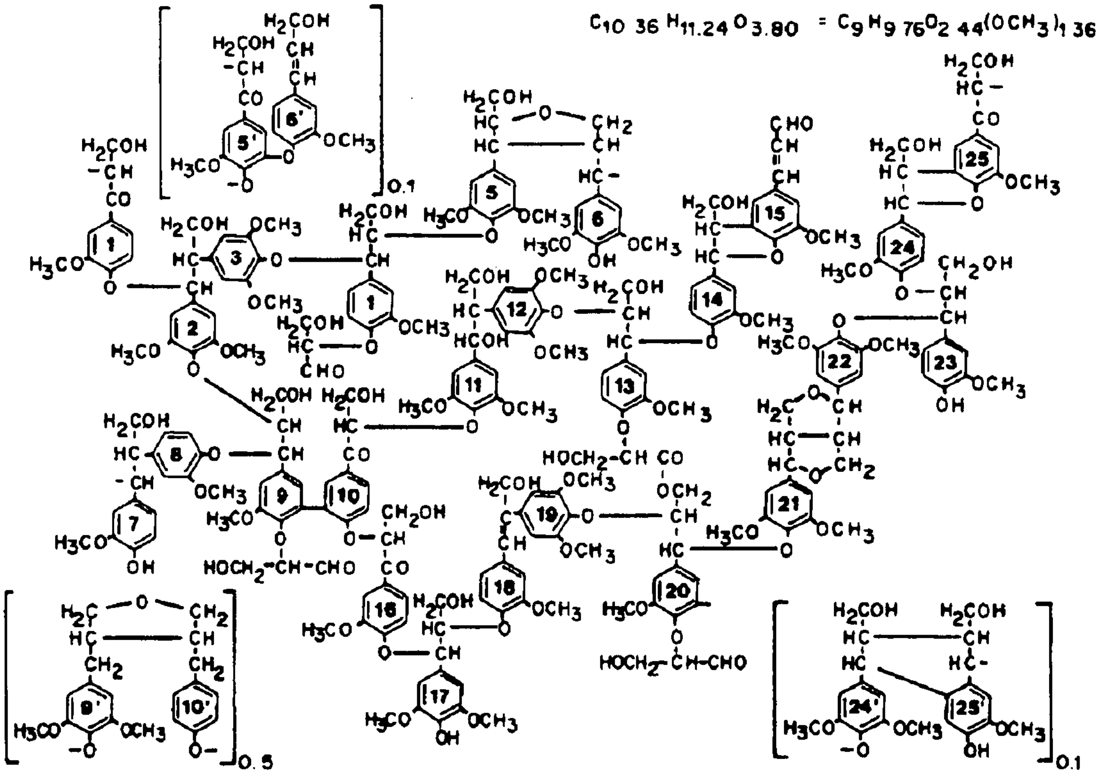
Lignin is a polymeric material derived from enzyme-intitiated and chemically-driven polymerization of phenyl-propenyl alcohol precursors. The three main precursors are shown in figure 2.5 (Crawford, 1981). The synthesis of lignin is thought to proceed by a random, free radical mechanism which is initiated by peroxidase enzymes (Sarkanen and Ludwig, 1971). The intermonomer bonds formed are largely alkyl-aryl ethers, as well as alkyl-aryl and aryl-aryl carbon-carbon bonds. Covalent linkages between hemicellulosic monosaccharides and lignin have been reported, and usually are ester or ether bonds. An hyposthesized, partial structure of lignin from beechwood is shown in Figure 2.6 (Nimz, 1974).

Figure 2.5. Monomeric precursors of lignin. Taken from Crawford, 1981.



There are three major groups of lignins which are classified according to the ratios of the various monomeric units in the bulk polymer. These lignin classes are guaiacyl, guaiacyl-syringyl, and guaiacyl-syringyl-*p*-hydroxyphenyl. Representative plant members are conifers, dicotyledonous angiosperms, and highly evolved grasses, repectively (Crawford, 1981). Having classified the lignins, it must be realized that these groupings are somewhat arbitrary, and there is overlap in the type of lignin found in plants (Adler, 1977). Much of the difficulty in describing lignin structure derives from its refractory nature, making isolation of native lignin extremely challenging.

Figure 2.6. Proposed partial structure of beech lignin. Taken from Nimz, 1974.

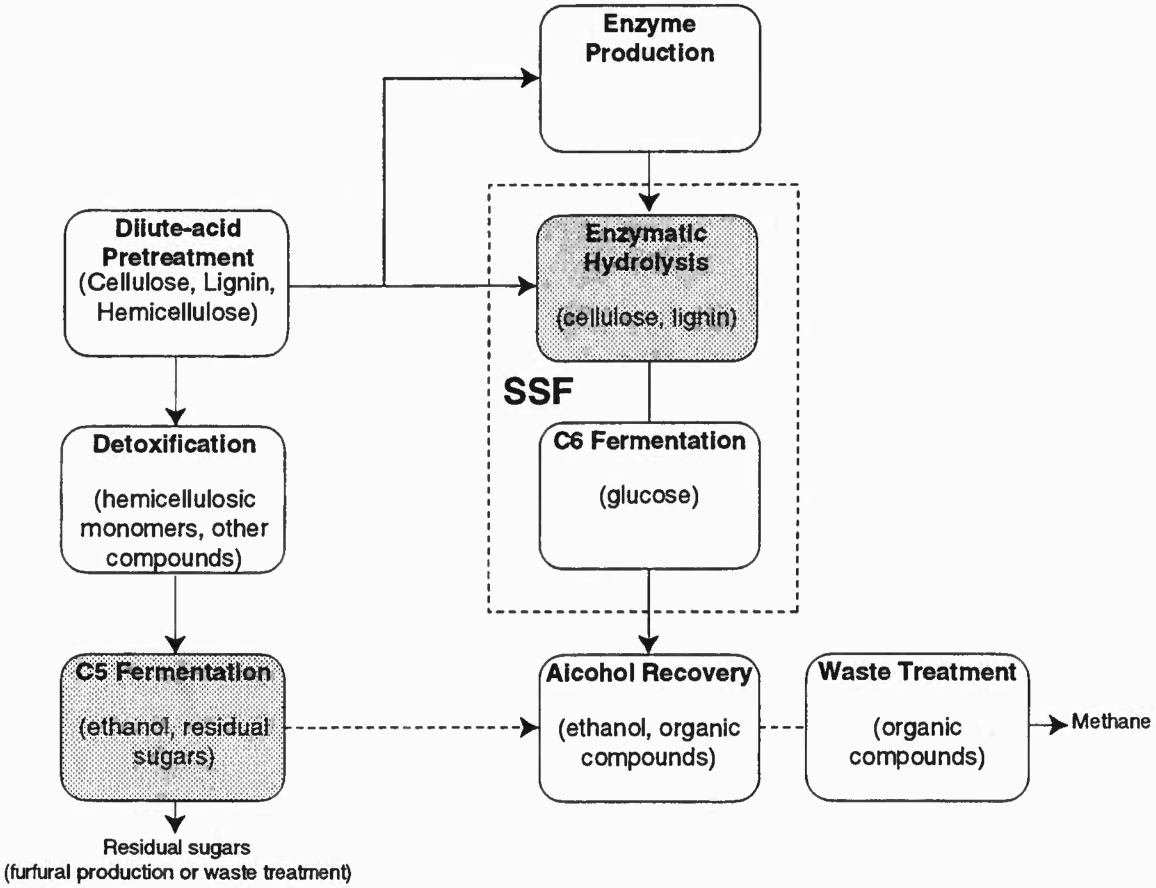


2.3 Conversion of Biomass to Ethanol

Potential biomass-to-ethanol conversion processes can be separated into three categories. These are the enzymatic, dilute-acid, and concentrated acid processes. It is generally accepted that the process which has the greatest potential for commercial viability is the enzymatic process (von Sivers and Zacchi, 1997). The enzymatic and dilute-acid process share a similar first step involving high temperature and dilute acid which solubilizes the hemicellulose fraction.

A summary of the enzymatic process is shown in Figure 2.7. The focus of this dissertation involves the two steps highlighted, *i.e.* the fermentation of the xylose stream and the enzymatic conversion of cellulose to glucose. In the enzymatic process, milled biomass is pretreated with dilute acid at high temperatures, yielding a solids stream, which contains mostly cellulose and lignin, and a liquid stream (called the prehydrolysate), which contains hydrolyzed hemicellulosic sugars (primarily xylose) along with a number of other compounds, such as acetic acid, degradation compounds of lignin and sugar, as well as extractable materials including fatty acids, pigments and waxes. The objective of the pretreatment is two-fold. First, the pretreatment renders the cellulose component of the lignocellulosic material susceptible to cellulase attack; without pretreatment, cellulase enzymes are very slow-acting (Grohmann *et al.*, 1985). The secondary objective of the pretreatment is to recover hemicellulosic sugars, mainly xylose. Harsh pretreatments,. *i.e.* those involving long times, high acid concentrations, and high temperatures, favor the degradation of xylose to furfural, thus reducing the overall sugar yield from lignocellulosic biomass (Esteghlian *et al.*, 1997).

Figure 2.7. Outline of enzymatic biomass-to-ethanol process . Adapted from von Sivers and Zacchi, 1997)



2.3.1 Liquid stream (prehydrolysate) utilization

Fermentation of the hemicellulosic sugars is essential in making an biomass-to-ethanol process economically viable (von Sivers and Zacchi, 1996; Hinman *et al.*, 1989). Bacteria, yeast and other fungi and are capable of fermenting xylose to ethanol (Skoog and Hahn-Hägerdahl, 1988) although *Saccharomyces cerevisiae* cannot (Barnett, 1976). Both bacteria and eukaryotes convert xylose to xyulose in the initial stages of metabolism. Bacteria do so using xylose isomerase, while yeast and fungi use a two-step oxidation and reduction in which NADPH and NAD are cofactors (Skoog and Hahn-Hägerdahl, 1988) .

Table 2.1 lists a number of xylose-fermenting microorganisms along with their fermentative capabilities. *Pichia stipitis*, compared to other xylose fermenting yeast, ferments xylose with a high ethanol yield (g ethanol produced per g sugar) and relatively high volumetric ethanol productivity (g ethanol produced per liter per hour) (Skoog and Hahn-Hägerdahl, 1988). Although *Pichia* has a relatively high ethanol productivity among the xylose-fermenting yeasts, its productivity is approximately 30 fold less than *Saccharomyces* and its specific ethanol productivity (ethanol productivity per gram cell mass) is approximately 8 fold lower than *Saccharomyces* (Jeppsson, 1996).

Table 2.1. Performance of xylose-fermenting microorganisms in laboratory media. Adapted from Jeppsson, 1996.

Strain	Xylose (g l^{-1})	Ethanol (g l^{-1})	Yield (g g^{-1})	Productivity (g l^{-1} h^{-1})	Reference
Bacteria, naturally occuring					
Bacteroides paypragmatus GP4	44	6.5	0.15	0.09	Patel, 1984
Thermoanaerobacter ethanolicus ATCC 31938	3.6	1.5	0.42	NA	Lacis and Lawford, 1991
T. ethanolicus ATCC 31938	19.5	4.6	0.24	NA	Lacis and Lawford, 1991
Bacteria, recombinant[c]					
Eschericia coli KO11 (pdc,adhB, frd-)	80	42	0.52	0.6	Alterhum and Ingram, 1989
Klebsiella planticola(pdc)	67[b]	24	0.36[a]	0.29	Tolan and Finn, 1987
Zymomonas mobilis CP4 (*p*ZCB5)	25	11	0.45	0.24	Zhang *et al.*, 1995
Filametous fungi, naturally occuring					
Fusarium sambicum VTT-D-77056	50	14	0.28	0.14	Ueng and Gong, 1982
F. oxysporum VTT-D-80134	50	13	0.26	0.08	Suihko and Enari, 1981
Yeasts, Naturally occurring					
Candida shehatae CBS 5813	20	6.6	0.33	0.03	Toivola *et al.*, 1984
C. shehatae NRRL Y-12856	50	24	0.48	0.29	Slininger *et al.*, 1985
Pachysolen tannophilus NRRL Y-2460	50	16	0.32	0.16	Schneider *et al.*, 1981
Pichia stipitis CSIR-Y567	50	21.3	0.42	0.85[d]	du Preez and Prior, 1985
P. stipitis NRRL Y-7124	50	20	0.40	0.28	Slininger *et al.*, 1985
Yeasts, recombinant[e]					
Saccharomyces cerevisiae TJ1 (XR,XDH)	50	2.7	0.05	0.04	Tantarungki *et al.*, 1993
Schizosaccharomyces pombe ATCC 38299 (XI)	50	21	0.42	0.13	Chan *et al.*, 1989

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[a] g ethanol g^{-1} consumed xylose

[b] g consumed xylose (g l^{-1})

[c] The relevant genotype is given in parentheses. *pdc*, pyruvate decarboxylase; *adh*B, alcohol dehydrogenase II; *frd*, fumarate reductase; pZCB5 contains the genes for xylose isomerase, xyulokinase, transaldolase and transketolase.

[d] maximumvolumetric productivity (g l^{-1} h^{-1})

[e] The relevant genotype is given in parentheses. XI, xylose isomerase; XDH, xylitol dehydrogenase; XR, xylose reductase

2.3.1.1 *Pichia characteristics*

Although *Pichia* is an efficient fermenter of xylose, the conditions under which it does so make it a challenging yeast to use industrially. *Pichia* is Crabtree negative (Passoth *et al.*, 1996), meaning that unlike *Saccharomyces,* respiration is not suppressed at high sugar concentrations in the media. The oxygen requirements of *Pichia* are such that if too much is present, cell mass is produced at the expense of alcohol and alcohol is reassimilated (Lohmeier *et al.*, 1989), whereas if too little oxygen is in solution, ethanol production is slowed and byproducts, *e.g.* xylitol, become predominant (Ligthelm *et al.*, 1988). Such finely tuned oxygen control is difficult and costly to maintain on the industrial scale.

In addition to the disadvantages inherent to *Pichia* (and other xylose-fermenting organisms) with respect to specific ethanol productivity and oxygen requirements when fermenting control xylose solutions, the challenge of fermenting the xylose present in the complex mixture that makes up prehydrolysate is considerable.

2.3.1.2 *Microbial inhibitors in prehydrolysates*

Lignocellulosic prehydrolysate contains a wide array of compounds other than carbohydrates, some of which are known inhibitors of microorganisms. The inhibitors can be grouped into classes according to their origin (Olsson and Hahn-Hägerdahl, 1996); 1) compounds released during pretreatment, *e.g.* acetic acid, 2) extractives, *e.g.* organic acids, 3) sugar degradation products, *e.g.* furfural, 4) lignin degradation products and phenolics, *e.g.* syringaldehyde and parahydroxy benzoic acid, 5) fermentation products, *e.g.* ethanol, and 6) contaminants released by processing equipment, *e.g.* chromium. In addition, the normal products of fermentation (ethanol and acetic acid) act as microbial inhibitors. The inhibitors may work additively or synergistically (Olsson and Hahn-Hägerdahl, 1996).

2.3.1.2.1 *Microbial inhibitor mechanisms*

With the exception of acetic acid, the exact mechanisms by which the inhibiting compounds work is not very well understood. Acetic acid exerts its influence at lower pH, where the protonated form dominates. The acid enters the cell matrix and dissociates, lowering the pH of the cell (van Zyl *et al.*, 1991). As a consequence of the lowered pH, the proton gradient is more difficult for the yeast to maintain and nutrient transport is impaired (Ko and Edwards, 1975; Herrero *et al.*, 1985). The inhibitory properties of other organic acids are probably exerted in a similar fashion to acetic acid.

The microbial inhibition by sugar degradation products and phenolics are less easily explained. The effects of the inhibitors (when measured by ethanol productivities) could be the result of either a specific blocking of the fermentation pathway, or due to a more general slowdown of yeast metabolism. In many cases, the latter explanation appears to better describe prehydrolysate fermentation results. Biochemical processes are the basis for life, and enzymes play a crucial role in these processes. Thus, many toxic effects are the result of an undesirable influence on enzymatic activity (Ariëns *et al.*, 1976). Tannins, or large phenolic multimers, are known to non-specifically bind and precipitate proteins (Salunkhe *et al.*, 1990), and although it seems unlikely that such a large molecule could enter the yeast cytoplasm, it is possible that the tannins could influence the availability of nutrients from the medium by a similar mechanism, thus effectively inhibiting metabolism. Heavy metals such as chromium can bind to free sulfhydryl groups, causing irreversible enzyme inhibition. Molecules which mimic normal substrates can also inhibit enzymatic activity. They may do so by competing with normal substrates for active sites. As a result normal, production is slowed, or worse yet for the microorganism, "lethal synthesis" may occur, in which the mimetic is incorporated into the product, resulting in an abnormal, even toxic product. Uncoupling of energy producing reactions is another mechanism of toxicity. An example of uncoupling was given above wherein acetic acid crossed into the yeast cytoplasm, destroying the proton gradient necessary for ATP synthesis. Chelation of essential metals by the carboxyl groups of organic acids is another potential mechanism of inhibition. Interference with DNA and RNA synthesis is another potential means of metabolic slowdown and general toxicity.

2.3.1.2.2 *Detoxification of prehydrolysates*

Due to inhibiting compounds, the fermentation of prehydrolysate-containing media is generally slower, less complete and results in lower final ethanol concentrations than do fermentations of defined laboratory media. Detoxification of prehydrolysates is often necessary before fermentation is possible. Several methods of toxicity reduction have been demonstrated in the literature, *e.g.* steam stripping (Yu *et al.* 1987), solvent extraction (Wilson *et al.*, 1989; Clark and Mackie, 1984), ion exchange (Tran and Chambers, 1986; Frazer and MacCaskey, 1989), ion exclusion (Buchert *et al.*, 1990) molecular sieving

(Tran and Chambers, 1986), use of activated charcoal (Roberto *et al.*, 1991) and overliming (van Zyl *et al.*, 1988; Frazer and MacCaskey, 1989).

Besides detoxification, efforts at adapting *Pichia* to lignocellulosic prehydrolysates have been made, resulting in strains that were able to more completely ferment neutralized woody prehydrolysates (Parekh *et al.*, 1988). The greater efficiency of the adapted strains was in part attributed to an increase in ploidy, which would result in gene dosage effects for the enzymes important in the xylose fermentation pathway, as well as increased resistance to harmful recessive mutations (Talbot and Wayman, 1989).

The ubiquity of acetate groups in hemicellulose-acetate which is released into the prehydrolysate upon pretreatment-means that acetate concentrations must be either low or that acetate must be in the deprotonated form in order for yeast to ferment xylose to ethanol efficiently. Raising the pH greatly reduces the toxic effects of acetic acid (van Zyl *et al.*, 1991), elevated pH also makes contamination by bacteria more likely.

2.3.2 Solids stream utilization

The cellulose fraction of the solids stream can be converted to glucose using fungal cellulases, such as those produced by *Trichoderma reesei.* This produces a hydrolysate liquid rich in glucose, along with a solids stream, which contains mostly lignin. The cellulases can be used in conjunction with glucose-fermenting yeast in a process called simultaneous saccharification and fermentation (SSF). SSF, by maintaining glucose concentrations lower than what is encountered in a separate hydrolysis and fermentation process, has the advantage of lowering end-product inhibition of the cellulase system. This lowering of inhibition results in a lower enzyme requirement. Enzyme production represents a large fraction of the total cost of biomass conversion (von Sivers and Zacchi, 1997).

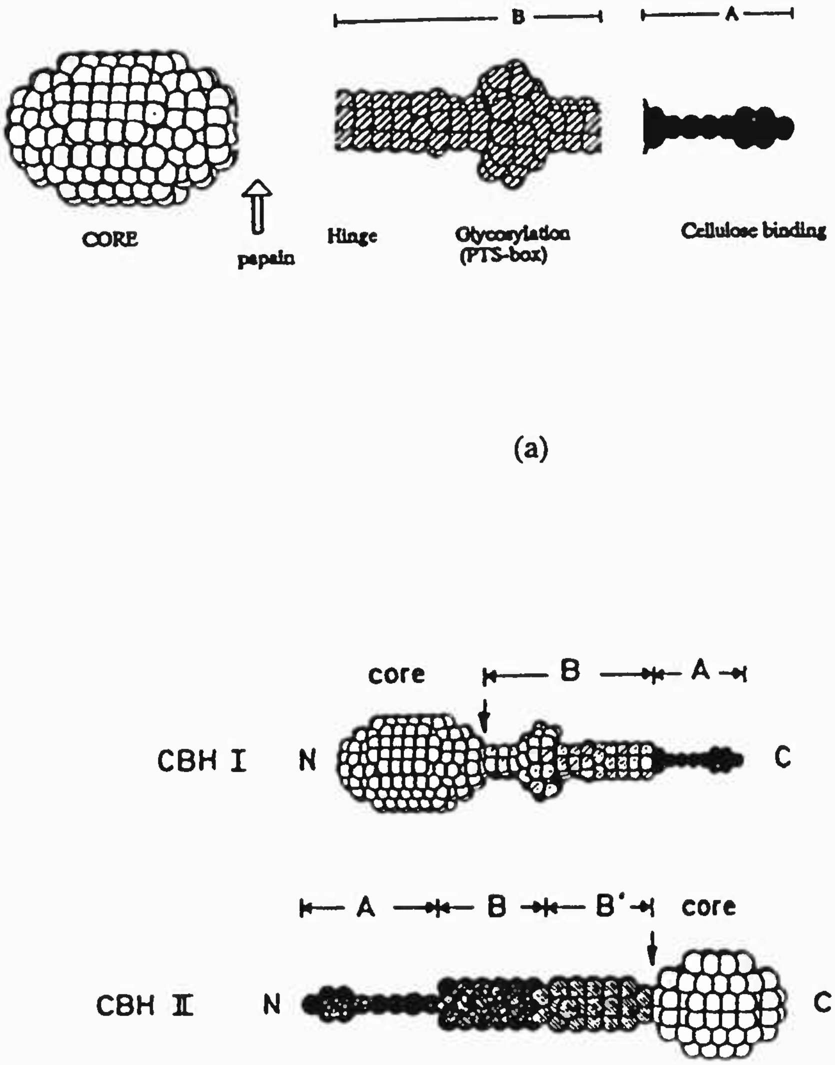
2.3.3 Cellulose hydrolysis

The vast quantities of cellulose available on the planet make its degradation an important step in carbon cycling. In nature, the degradation of cellulose is usually effected by microorganisms. Glucose, the end-product of cellulose hydrolysis, is a precursor for many potential products, e.g. ethanol and single-cell protein.

2.3.4 Cellulases

Cellulases normally consist of a mixture of three classes of glycosidic hydrolases (Wilson *et al.*, 1995). These are endoglucanases (1,4-β-D-Glucan-4 glucanohydrolase, EC 3.2.1.4), exoglucanases, or cellobiohydrolases, (1,4-β-D-Glucan-cellobiohydrolase or exoglucanase, EC 3.2.1.91) and β-glucosidases (β-D-Glucoside glucohydrolase or cellobiase, EC 3.2.1.21). The exo- and endo-cellulases typically have a three domain structure, *i.e.* a cellulose binding domain, a catalytic domain and a linker region (Wilson *et. al.,* 1995). The domain structure of two cellobiohydrolases produced by *Trichoderma reesei* are shown in Figure 2.8.

Figure 2.8. (a) The domain structure of *Trichoderma reesei* cellobiohydrolases. (b) Comparison between the tertiary structures of CBH1 and CBH2. Taken from Abuja *et al.*, 1988)



(a)

(b)

The primary producers of cellulases are microorganisms, in particular the fungi. *Trichoderma, Fusarium, Phanerochaete, Schizophyllum and Penicillum* are all cellulytic fungi. Bacteria with the ability to utilize cellulose as a carbon source include *Cellulomonas sp., Pseudomonas fluorescens, Clostridium thermocellum, Sporatrichum thermophilum* and others. Some termite-associated protozoa can digest cellulose as well. Also, endogenous cellulases are part of the ripening and morphogenesis processes of many plants (Béguin and Aubert, 1992). Cellulases are traditionally classified as endo- or exo-acting, although there is mounting evidence that a more realistic representation might be a continuum which allows for enzymes to have both endo and exo activity (Teeri, 1997).

Trichoderma reesei produces, at the least, four endoglucanases, two exoglucanases and β-glucosidase. Endoglucanase accounts for approximately 15% of the total cellulase mass, while the two cellobiohydrolases, CBH1 and CBH2 comprise an estimated 60 and 25% of the mass, respectively (Chirico and Brown, 1987). CBH1 hydrolyzes cellodextrins and cellulose with retention of anomeric configuration, while CBH2 does so with inversion (Claeyssens *et al.*, 1990; Knowles *et al.*, 1988; Konstantinidis *et al.*, 1993). All of the *T. reesei* enzymes belong to one of three hydrophobic cluster analysis families. Some of the properties of the cellulases of Trichoderma reesei are listed in Table 2.2

Table 2.2. Properties of *Trichoderma reesei* cellobiohydrolases.

Enzyme	MW (kDa)	pI	% Carbohydrate	Preferred chain end of attack	Catalytic mechanism	Reference
CBH1	64	3.9	5.6	reducing	retaining	1,2,4
CBH2	53	5.9	18	non-reducing	inverting	1,3,4

References. 1. Bhikhabhai *et al.*, 1984; 2. Divne *et al.*, 1994; 3. Rouvinen *et al.*, 1990; 4. Barr *et al.*, 1996

2.3.4.1 *Cellulase adsorption*

The cellulose\cellulase system is heterogenous, *i.e.* it consists of insoluble (cellulose) and soluble (cellulase) fractions. Adsorption of the cellulase is the first step in hydrolysis. The initial rate of hydrolysis is apparently related to the amount of enzyme adsorbed on the substrate surface (Steiner *et al.*, 1988; Nidetzky and Steiner, 1993). Langmuir isotherms describe the adsorption of cellulases onto celluloses well (Ooshima, 1990; Beldman et al., 1987), although conflicting reports appear in the literature, *e.g.* a modified Langmuir-Freundlich model was found to be far better than a simple Langmuir model by one group (Medve *et al.*, 1997). Deviation from Langmuir isotherms can probably be attributed to the heterogeneity of the cellulose surface (crystalline and amorphous regions) or to other nonspecific cellulase-cellulose interactions.

The adsorption of cellulytic enzymes onto the substrate surface is affected by parameters one would expect, *i.e.* the properties of the cellulose, pH, ionic stregth and temperature (Kyriacou *et al.*, 1988; 1989; Steiner *et al.*, 1988).

2.3.4.2 *Catalytic mechanisms*

Once the cellulases have attached to the cellulose surface, catalysis may begin. The cellulytic enzymes catalyze hydrolysis via two separate general acid/base mechanisms, one which results in retention of the original anomeric configuration, the other in inversion.

Inverting glycosidases act by way of a single-displacement, S_N2-type reaction. A water molecule adds at the anomeric carbon of the glycosidic bond. The two catalytic residues of the enzyme are usually aspartic or glutamic residues, one which acts as a base, the other as an acid. The base helps to deprotonate the water molecule, while the acid protonates the glycosidic oxygen, making it a better leaving group. Glycosidases which retain the configuration at the anomeric carbon usually operate via a double-displacement, S_N1-type reaction in which a covalent glycosyl-enyzyme intermediate is temporarily made and subsequently hydrolyzed via a oxocarbonium ion-like transition state. Retaining enzymes can also catalyze transglycosylation, in which another carbhydrate attacks in the place of water at the anomeric center. Inverting enzymes have also been shown to catalyze the reverse (condensation) reaction (Sinnot, 1990). Figure 2.9 shows inverting and retaining reaction mechanisms.

Endoglucanases cleave cellulose chains at internal linkages, producing two free chain ends, one reducing, one non-reducing. Cellobiohydrolases cleave cellobiose from the chain ends. The specificity of the CBHs for either nonreducing or reducing ends is still a matter of some debate, although it appears that for the T. reesei exocellulases, CBH1 works primarily from the reducing end, while CBH2 works from the nonreducing chain end (Barr *et al.*, 1996). β-Glucosidase cleaves cellobiose into two glucose units, thus the activities of the combined cellulases produce glucose. A schematic of cellulytic activity is shown in Figure 2.10.

Figure 2.9. Inverting and retaining mechanisms of enzymatic glycosidic hydrolysis. Modified from Withers and Aeversold, 1995.

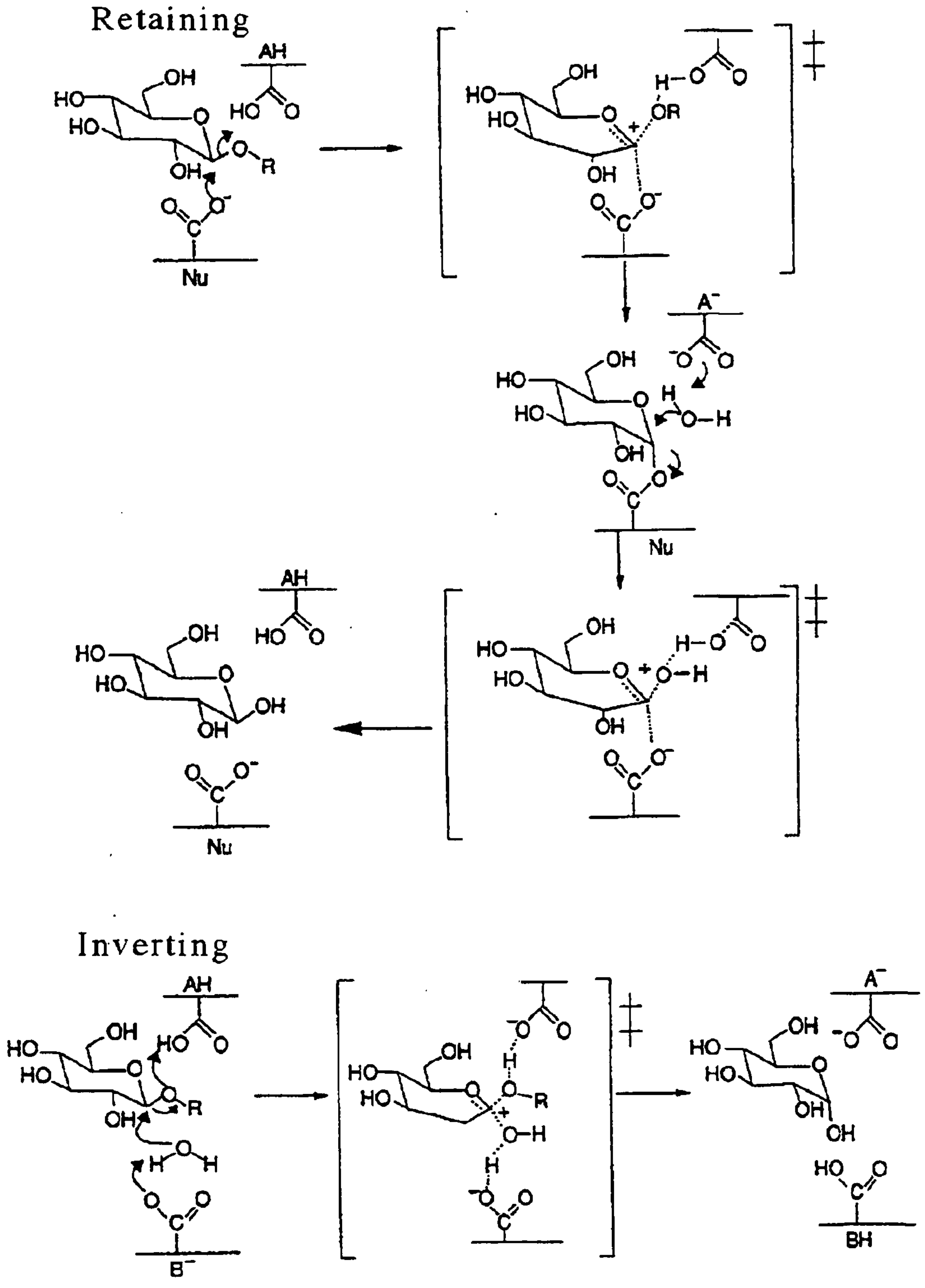
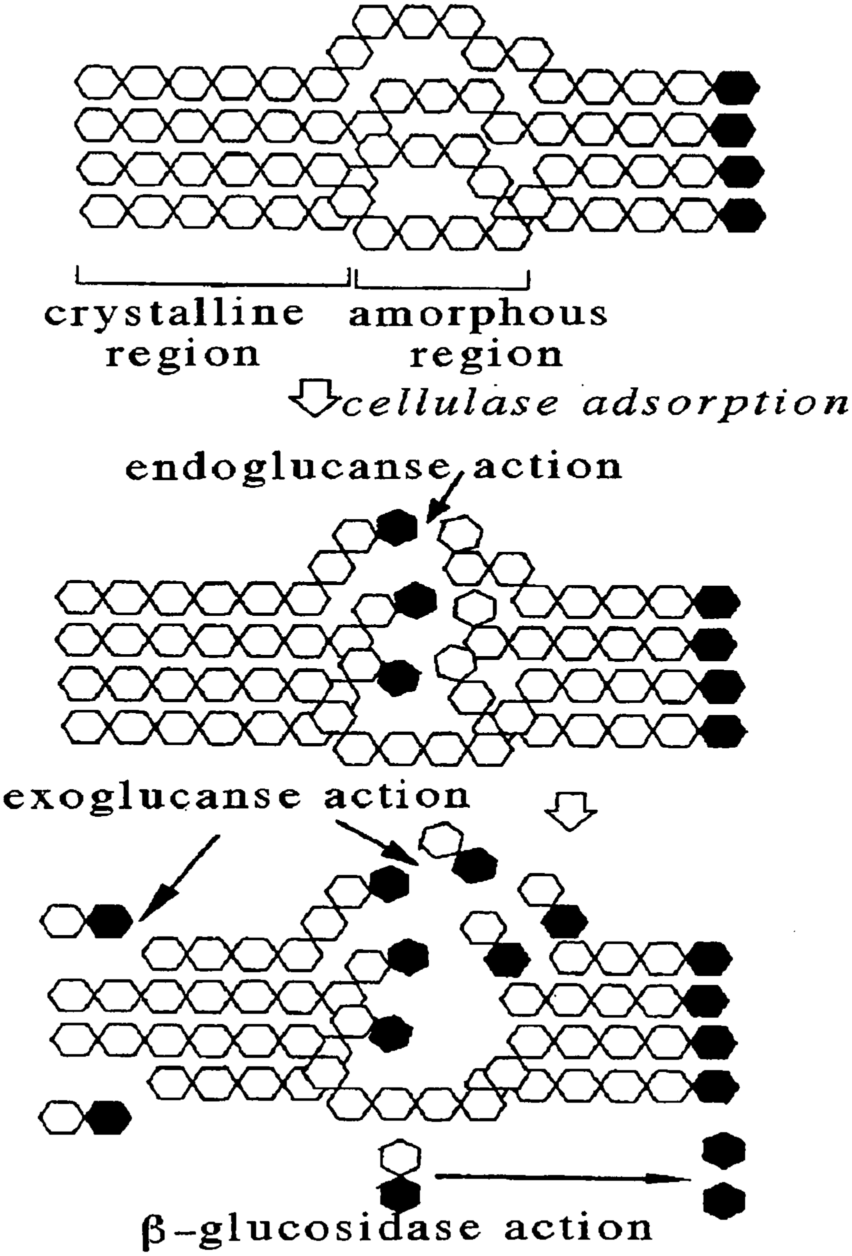


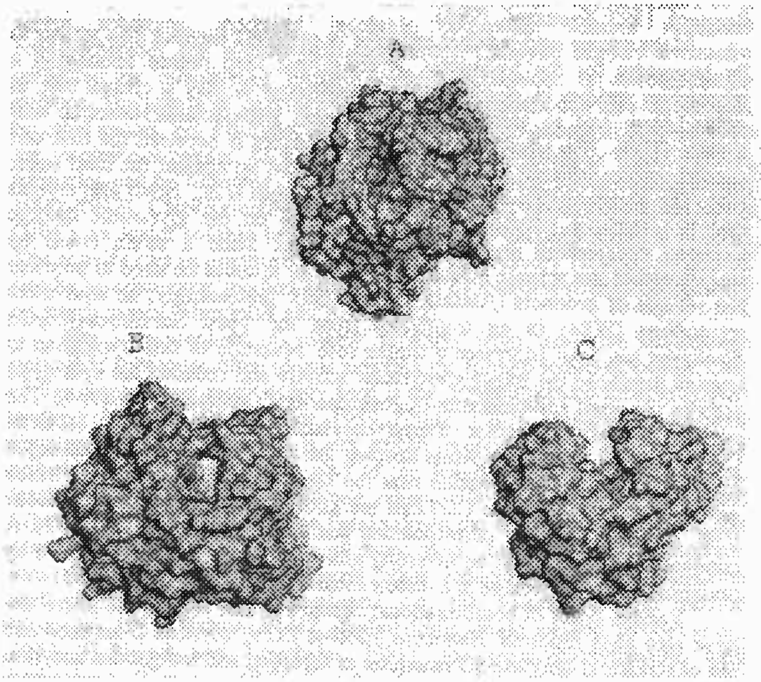
Figure 2.10. Schematic diagram of cellulase activity. Hexagons represent glucose residues, reducing ends are shown in black. Taken from Béguin and Aubert, 1992.



2.3.5 The continuum of exo-endo activity

A current theory holds that the endo- versus exo- action-pattern of naturally occuring polysaccharide-hydrolyzing enzymes most probably forms a continuum ranging from strict endo- to strict exo-type enzymes (Teeri, 1997). Recently, a *Cellulomonas fimi* cellulase expressed in *E. coli* was shown to have both endo and exo activity (Tomme *et al.*, 1996). Cellulases with a classic endo-action pattern have open cleft-type active sites while with exo-action pattern have enclosed, tunnel-like active sites. Figure 2.11 shows the three active site topologies of glycosyl hydrolases (Davies and Henrissat, 1995). Along the continuum are those enzymes which are primarily exo-acting, but which have a small amount of endo-activity. The active site of enzymes of this latter type may be characterized as tunnels with roofs that on rare occasion retract, *i.e.* as having active sites typically enclosed but which may be temporarily exposed due to fewer peptide loops enclosing the tunnel and/or loops of greater mobility (Warren, 1996; Kleywegt *et al.*, 1997).

Figure 2.11. Active site topologies of three different types of glycosyl hydrolases. a) pocket of a glucoamylase; b) tunnel of a cellobiohydrolase; c) cleft of an endoglucanase



2.3.5.1 *Synergism*

When the rate of reaction produced by a mixture of two enzymes is greater than what would be predicted by addition their individual activities, synergism is said to occur. Synergism is frequently seen in cellulase\cellulose systems (Nidetzky *et al.*, 1995, Woodward *et al.*, 1988; Woodward, 1991). Two types of synergism, endo-exo and exo-exo, have been frequently described in the literature. For example, synergism between both CBHs and EGs of *T. reesei* as well as between CBH1and CBH2 have been characterized (Nidetzky *et al.*, 1995). Also, cross-species synergism has been reported (Walker *et al.*, 1993). However, cross-species synergism is not universal, and thus synergism is specific to certain enzymes and not a general property of all enzyme combinations. The degree of synergism is dependent on enzyme-to-substrate ratios (Woodward *et al.*, 1988), type of enzyme and substrate as well as other factors. With regard to polysaccharide-degrading enzymes, synergism is most apparent when insoluble substrates are used, and negligible on soluble substrates (Fujii and Shimizu, 1986).

The classic rationale for endo\exo synergism is that endos create new chain ends for exos, in effect increasing the substrate concentration for the exo-enzyme. (Woodward, 1991). In this case, product formation is dependent on the activity of the exo-acting component. Increases in substrate concentrations lead to saturation of the exo-acting component, hence the exo-acting enzyme becomes less dependent on endo-generated chain ends. Consequently, increases in substrate concentration reduce the degree of synergism as the exo-enzyme approaches maximum velocity (Fujii and Kawamura, 1984). This rationale is normally considered in reference to soluble enzyme/soluble substrate systems.

The situation probably differs for glycosidases acting on insoluble polysaccharides. In this case, the enzymes are often comprised of two domains, with the catalytic site located in one domain and a separate substrate binding site located in the other (Wilson *et al.*, 1995). The binding domain serves to anchor the enzyme to the surface of the insoluble substrate. If exo-acting enzymes are randomly associated with the substrate surface, then a percentage of these enzymes will be at loci distant from their sites of optimal activity, *i.e.* the chain ends. Increasing substrate concentrations are expected to yield maximum amounts of adsorbed enzyme, yet only a fraction of the adsorbed enzyme may be anchored at a catalytically productive locus (in the case of exo-acting enzymes, a catalytically productive locus would be one in the vicinity of a chain end). In this scenario, synergism between endo- and exo-enzymes can result from the ability of endo-enzymes to create new chain ends in an exo-enzyme-occupied nonproductive loci. The net result, with respect to the activity of the exo-acting enzyme, is that a catalytically nonproductive locus would have been converted to a catalytically productive locus. This scenario is most easily envisioned for the case where the exo-acting enzyme has limited surface mobility (Jervis *et al.*, 1997; Claeyssens and Tomme, 1989)

2.3.5.2 *Substrate inhibition*

In the case of either exo-exo or endo-exo synergism, it seems intuitive that a requirement would be that the enzymes be physically near enough to one another that they can take advantage of hydrolysis brought about by neighboring enzymes. A natural consequence of this requirement is that, as cellulytic enzymes become more distant from one another on the substrate surface, *i.e.* as substrate concentrations are increased and the concentration of crude cellulase is held constant, an apparent substrate inhibition occurs. Such a phenomenon with *Trichoderma reesei* crude cellulase has been described (Huang and Penner, 1991). No such effect was observed for a purified CBH1 preparation. Substrate inhibition has implications regarding the activities of cellulytic enzymes. A phenomenon directly related to substrate inhibition is the decrease in synergism as the concentration of cellulase increases (Woodward *et al.*, 1988)

2.4 Individual-Based Models

Individual-based models track the behavior or performance of a collection of separate entities. The main advantage of individual-based models is that they do not use population averages to generate predictions (Haeffner, 1996). An individual-based model is of particular use when individual entities interact. Individual-based models have gained popularity in the field of ecology (Judson, 1994), *e.g.* to model predator\prey interactions or the effects of habitat destruction. In contrast to individual-based models, population-based models describe the average behavior of a large group. The Michaelis-Menten equation is an example of a population-based enzyme kinetics model.

Chance is not the exclusive domain of individual-based models, however it frequently plays a large role in deciding what occurs at a given simulation step. For example, a simulated roll of the dice is used to decide if a particular individual find a mate in a given timeframe or if a catalytic event will occur. The stochastic (or random) nature of individual based models means that the model must be run many times to generate a distribution of outputs. This is in contrast to deterministic models, in which a given set of inputs yields but one output.

The multiple runs required of stochastic models increases the computational burden, as does the number of individual entities being tracked in an individual based model. Thus, individual-based models, as do most models, involve a tradeoff between verisimilitude and computational burden.

CHAPTER 3

Relative Fermentability of Lignocellulosic Dilute-Acid Prehydrolysates: Application of a *Pichia stipitis*-Based Toxicity Assay[a,b]

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3.1 Abstract

The relative toxicity of the combined non-xylose components in prehydrolysates derived from three different lignocellulosic biomass feedstocks was determined. One woody (poplar) and two herbaceous (corn stover and switchgrass) feedstocks were dilute-acid pretreated under temperature and acid conditions chosen to optimize xylose recovery in the liquid prehydrolysate; xylose yields averaged 96%, 89% and 87% of theoretical for switchgrass, corn stover and poplar, respectively. Prehydrolysates from each feedstock were neutralized, adjusted to equivalent xylose concentrations, and bio-assayed for toxicity using a standardized fermentation protocol with *Pichia stipitis* NRRL 11545. Full time courses for ethanol production (30 to 60 hrs) clearly illustrate the distinct inhibitory effects of prehydrolysates from different feedstocks. The relative toxicity of the prehydrolysates, ranked in order of decreasing toxicity, is poplar-derived prehydrolysates > switchgrass-derived prehydrolysates > corn stover-derived prehydrolysates. The inhibition of ethanol production appeared to be the result of a general slow-down of yeast metabolism, rather than due to the production of alternative, non-ethanol end products. Ethanol yields averaged 74%, 83% and 88% of control values for poplar, switchgrass and corn stover prehydrolysates, respectively. Volumetric ethanol productivities (g ethanol $(l)^{-1}(\text{hour})^{-1}$) averaged 32%, 70% and 102% of control values for poplar, switchgrass and corn stover prehydrolysates, respectively. Ethanol productivities correlated closely with acetate concentrations in the prehydrolysates; however, regression lines correlating acetate concentrations and ethanol productivities were found to be feedstock-dependent.

3.2 Introduction

A widely studied process for converting lignocellulosic biomass to ethanol involves a pretreatment of the lignocellulosic biomass with dilute-acid (around 1.0 wt. %) at high temperatures (over 140°C) to catalyze the hydrolysis of lignocellulosic biomass hemicellulose (Penner *et al.*, 1996). Dilute-acid pretreatment yields a solids stream consisting of mostly lignin and cellulose. *Saccharomyces cerevisiae*, when used in conjunction with fungal cellulases, can ferment the cellulose fraction of the solids stream to ethanol (Spindler *et al.*, 1991). Dilute-acid pretreatment also yields an aqueous prehydrolysate stream containing hemicellulose-derived xylose, lesser amounts of other sugars, acetic acid, degradation products of lignin and carbohydrates, as well as other endogenous compounds (Olsson and Hahn-Hägerdahl, 1996).

Xylose constitutes a large fraction of the dry weight of most lignocellulosic biomass (Puls and Schuseil, 1993) and thus, fermentation of prehydrolysate xylose is essential for the economic viability of industrial biomass-to-ethanol processes (Hinman *et al.*, 1989). Bacteria, yeast and other fungi are capable of fermenting xylose to ethanol (Skoog and Hahn-Hägerdahl, 1988). *Pichia stipitis* is a yeast which does so with high ethanol yield (g ethanol produced per g sugar) and relatively high volumetric ethanol productivity (g ethanol produced per liter per hour) when compared to other xylose fermenting yeast (Skoog and Hahn-Hägerdahl, 1988, Jeppsson, 1996). Several papers describing ethanol yields from *Pichia* fermentation of lignocellulosic biomass prehydrolysates have been published, including studies with prehydrolysate derived from aspen wood (Wilson *et al.,* 1989, Parekh *et al.*, 1988), pine (Parekh *et al.*, 1988, Qureshi and Manderson, 1991), corn stover (Qureshi and Manderson, 1991), red oak (Tran and

Chambers, 1986), sugar cane bagasse (Roberto *et al.*, 1991), corn cob (Hahn-Hägerdahl *et al.*, 1994), mixed wood chips (Perego *et al.*, 1990), and eucalyptus (Ferrari *et al.*, 1992). Toxic compounds in these lignocellulosic biomass prehydrolysates tend to inhibit *Pichia* fermentations, causing lower ethanol yields and lower ethanol productivities compared to those attained in control fermentations. Relatively low ethanol yields and productivities are associated with prehydrolysate fermentations using other microorganisms as well (Olsson and Hahn-Hägerdahl, 1996). *Pichia* fermentations can be improved by conditioning the yeast to the particular prehydrolysate as well as by removing inhibitors. For a review of the fermentation of prehydrolysates and detoxification, see Olsson and Hahn-Hägerdahl (Olsson and Hahn-Hägerdahl, 1996).

When comparing the feasibility of different biomass sources for biomass-to-ethanol processes it is important to consider the relative toxicity of the respective prehydrolysates, since this will ultimately influence the ability of microbes to produce ethanol from hemicellulose. In general, literature comparisons of the relative toxicity of prehydrolysates derived from different biomass sources are difficult. This is because most papers concerning prehydrolysate fermentation focus on a single feedstock and there is little consistency between studies with respect to fermentation protocols. When comparing data from different papers, one finds that feedstock pretreatment conditions often do not correlate and fermentation parameters may vary considerably, including differences in yeast species (and strains) and inoculum levels.

The objective of this study was two-fold. The first objective was to determine the relative toxicity of three important prehydrolysates (obtained from a hybrid poplar, corn stover, and switchgrass) using a standardized fermentation bio-assay. A *Pichia* based bio-assay was chosen for this work due to the well-documented susceptibility of this yeast to prehydrolysate inhibition (Olsson and Hahn-Hägerdahl, 1996; Skoog and Hahn-Hägerdahl, 1988, Wilson *et al.*, 1989, Parekh *et al.*, 1988; Qureshi and Manderson, 1991; Tran and Chambers, 1986; Roberto *et al.*, 1991; Hahn-Hägerdahl *et al.*, 1994; Perego *et al.*, 1990; Ferrari *et al.*, 1992) this is a physiological characteristic expected to correlate with enhanced sensitivity in a bio-assay. The second objective was to provide information on *Pichia* fermentation of prehydrolysates obtained from dilute-acid pretreated switchgrass (*Panicum virgatum L.).* Switchgrass is of interest because it is currently considered a leading candidate for use as a biomass-to-ethanol crop (Downing *et al.*, 1995; Sanderson *et al.*, 1996) and yet, to our knowledge, representative fermentations of dilute-acid prepared switchgrass prehydrolysates have not been published. Information of this nature is obviously necessary when evaluating the economic potential of switchgrass-to-ethanol processes.

3.3 Materials and Methods

3.3.1 Media, organism, and seed culture preparation.

YM broth was prepared by adding 3g yeast extract (Difco), 3g malt extract (Sigma), 5g peptone (Difco), and 10g glucose (Sigma) to 1L distilled H_2O and autoclaving for 30 min. at 121°C. YM agar was prepared in the same manner as the YM broth, but with the addition of 20g agar (Difco). *Pichia stipitis* NRRL 11545 was supplied by the National Renewable Energy Laboratory as a lyophilized powder. The powder was dissolved in YM broth then spread on YM agar. An isolated colony obtained from the plate was used to inoculate 200 ml of filter-sterilized 30 $g(l)^{-1}$ xylose (Sigma) and 10 gl^{-1} yeast extract (Difco) solution in a baffled 500 ml flask. After 24 hours of incubation at 25°C and 200 rpm, the cells were centrifuged at 4,000 g, resuspended in yeast extract solution, and glycerol was added to a final concentration of 10% (w/v). The stock cultures were divided into 1 ml aliquots and stored at -70°C.

3.3.2 Prehydrolysate preparation.

Poplar, corn stover and switchgrass feedstocks were dried and milled (60 mesh) prior to shipment by the National Renewable Energy Laboratory. The composition of the original feedstocks is shown in Table 3.1. Feedstocks were dilute-acid pretreated at 10% solids (w/w) in a stainless steel Parr reactor with a Pyrex liner as described previously (Esteghlalian *et al.*, 1997). The prehydrolysate was stored at 4°C for 2 days, neutralized to pH 6.0 with $Ca(OH)_2$, and sterile-filtered through a 0.22 μm membrane prior to fermentations. No blockage of the membrane was observed. Filtration was chosen as a sterilization method in order to avoid a second heating regimen potentially more rigorous than the original pretreatment conditions.

3.3.3 Toxicity assay.

The fermentation-based toxicity assay used in this study was developed at the National Renewable Energy Laboratory and is similar to the fermentation protocol described by Boynton and McMillan (Boynton and McMillan, 1994).

Table 3.1. Percent composition of three lignocellulosic feedstocks on a dry-weight basis.

Component	Feed Stock		
	Corn Stover	Switchgrass	Poplar
Glucan	36.0[a] (0.4)[b]	32.2 (0.3)	39.8 (0.01)
Xylan	19.8 (0.3)	20.3 (0.3)	14.8 (0.03)
Galactan	1.3 (0.1)	-	-
Arabinan	2.8 (0.1)	3.7 (0.04)	1.2 (0.02)
Mannan	-	0.4 (0.02)	2.4 (0.04)
Total glycan	59.9	56.6	58.2
Klason lignin	26.9 (0.1)	19.5 (0.3)	26.9 (0.3)
Acid-soluble lignin	1.9 (0.03)	3.7 (0.03)	2.2 (0.06)
Ash	7.2 (0.01)	7.1 (0.06)	1.3 (0.02)
Uronic Acid	nd	1.1 (0.1)	2.4 (0.1)
Acetyl Groups	1.38 (0.1)	2.35 (0.1)	2.93 (0.1)
Other	2.7	9.7	6.1
Total	100	100	100

[a] all values are weight percents on a dry basis

[b] values in parentheses are standard errors of the means

3.3.4 Inoculum preparation.

One ml of previously prepared and frozen seed culture was added to 200 ml of 30 $g(l)^{-1}$ xylose (Sigma) and 10 $g(l)^{-1}$ yeast extract (Difco) in 500 ml baffled flasks equipped with Morton closures. The pH was maintained ≥5.0 via manual addition of 1*N* NaOH. Temperature was controlled at 30°C and the orbital shaker speed was 200 rpm. When the culture reached a dry cell mass (DCM) of approximately 5.0 $g(l)^{-1}$ (approximately 24 hours, determined using a optical density measurement previously calibrated with actual DCM measurements), the yeast were centrifuged, the supernatant discarded, and the cells resuspended in 1 $g(l)^{-1}$ yeast extract to make the DCM approximately 50 $g(l)^{-1}$.

3.3.5 Fermentation protocol.

The prehydrolysate resulting from each pretreatment condition was fermented in duplicate. Sterile prehydrolysate (prepared as described above), concentrated xylose solution, yeast extract solution, water and concentrated *Pichia* inoculum were added to 250 ml unbaffled flasks with Morton closures, yielding 100 ml of medium with the following composition: 80% (v/v) prehydrolysate, 30 $g(l)^{-1}$ xylose, 10 $g(l)^{-1}$ yeast extract, and 5 $g(l)^{-1}$dry cell mass. Xylose and yeast extract were added to the prehydrolysates in order to facilitate comparisons of the impact of non-xylose prehydrolysate components on the fermentation performance of *Pichia*. When starting with equal concentrations of xylose, yeast extract, and by inclusion of the proper controls, it can be inferred that differences in fermentation parameters are due to non-xylose compounds in the prehydrolysate, and not differences in initial carbohydrate or nutrient loads. Fermentations were conducted at 30°C and the agitation speed was 150 rpm. The pH was initially 6.0 ± 0.1 and maintained throughout the fermentation between 5.5 and 6.0 via manual addition of 1 *N* NaOH, which was added if the pH dropped below 5.5. Typically, NaOH additions were required at 3 and 6 hours post-inoculation for control fermentations, while the prehydrolysate-containing fermentations did not require NaOH addition until 12 hours post-inoculation.

3.3.6 Fermentation controls.

Duplicate control fermentations containing only *Pichia*, xylose, yeast extract and water was fermented alongside each run. To independently assess the influence of acetate on *Pichia* under the fermentation conditions described above, varying amounts of acetic acid were added to 1% H_2SO_4 (w/w) solution to simulate the aqueous prehydrolysate. Xylose and yeast extract were then added to the same concentrations as for the control fermentation above.

3.3.7 Analytical methods.

The composition of the feedstocks was done as described previously (Esteghlalian *et al.*, 1997; Fenske, 1994); lignin, as Klason lignin; acid-soluble lignin, as determined by absorbance at 205 nm; glycans, as the sum of monomers resulting from acid hydrolysis; uronic acid, as acids resulting from acid hydrolysis; and acetyl groups, as acetate liberated during acid hydrolysis. In the prehydrolysate and fermentation broths, glucose, xylose, xylitol, acetate and ethanol were measured via HPLC using an Aminex HPX-87H column (65°C, 0.6 ml 0.01*N* H_2SO_4 min.$^{-1}$) and a refractive index detector. Dry cell mass was measured gravimetrically on duplicate 10 ml samples after centrifuging, washing twice with distilled water, and drying at 90°C to constant weight.

3.3.8 Data analysis.

Simple linear regression analyses were performed using Microsoft Excel 5.0. Correlations were evaluated based on the corresponding *p*-values. A *t*-test was conducted to compare slope values of the different data sets, with *p*-values being used to judge the significance of differences.

3.4 Results and Discussion.

The different prehydrolysates were prepared by dilute-acid pretreatment under conditions favoring maximum xylose yields within the constraints of 140-180°C, 0.6-1.2% (w/w) H_2SO_4, and 0.5-5.0 minutes (Esteghlalian *et al.*, 1997). Xylose yields were high; percent of theoretical xylose yields for corn stover, switchgrass and poplar prehydrolysates averaged 89%, 96% and 87%, respectively. The composition of the prehydrolysates, along with the ethanol yields and productivities resulting from *Pichia* fermentation of those prehydrolysates, are summarized in Table 3.2. In addition to the monomeric xylose concentrations shown in Table 3.2, there was an additional 2-6% of soluble xylose oligomers present. The relative composition of the prehydrolysates are in general agreement with that which would be predicted based on the composition of the feedstocks (Table 3.1). The corn stover prehydrolysates contained the most xylose and the least acetic acid, while the poplar prehydrolysates had the least xylose and most acetic acid. All measurements were made after neutralization to 6.0 with $Ca(OH)_2$ and subsequent sterile filtration (0.22 μm). Neutralization with $Ca(OH)_2$ has been shown to have no effect on the acetate or furfural content of a pine prehydrolysate, while lowering the monomeric and polymeric phenolic concentrations by approximately 25% (Frazer and McCaskey, 1989).

Table 3.2. Pretreatment conditions, composition, and *Pichia stipitis* fermentation results of dilute-acid prehydrolysates prepared from lignocellulosic biomass.

Label	Feed stock	Time (min.)	Weight % H_2SO_4	Temp. (°C)	Glucose (gl^{-1})	Xylose (gl^{-1})	Acetate (gl^{-1})	Ethanol yield (gg^{-1})[a]	EtOH productivity (gl^{-1}h^{-1})[a]	Final Dry Cell Mass (percent relative to control)[b]
CS 1	Corn stover	2.1	1.1	170	3.5	20.8	1.2	0.41	0.80	+6
CS 2	Corn stover	1.4	0.9	180	3.5	20.7	1.2	0.41	0.85	+5
CS 3	Corn stover	1.1	1.0	180	3.9	23.3	1.4	0.39	0.74	+6
CS 4	Corn stover	3.9	1.2	160	3.7	23.3	1.4	0.41	0.75	+4
POP 1	poplar	1.1	1.1	170	2.2	16.6	3.0	0.35	0.25	-10
POP 2	poplar	1.1	1.2	170	2.4	17.4	3.2	0.31	0.20	-17
POP 3	poplar	0.5	1.0	180	1.9	14.3	2.5	0.38	0.32	-18
POP 4	poplar	0.5	1.1	180	2.3	15.6	2.9	0.32	0.22	-15
SG 1	Switch grass	0.5	1.1	180	5.7	24.7	2.3	0.37	0.47	+7
SG 2	Switch grass	0.5	1.0	180	5.7	25.1	2.1	0.36	0.46	+4
SG 3	Switch grass	0.5	1.0	180	5.8	22.6	1.7	0.42	0.68	+9
Average of Controls (n=5)		na[c]	na[c]	na[c]	0	30.65 (0.47)[d]	0	0.46 (0.004)[d]	0.77 (0.02)[d]	na[c]

[a] Calculated at maximum ethanol concentration. See methods section for fermentation parameters; xylose added to all prehydrolysates to a final concentration of 30 gl^{-1}

[b] Values were calculated using the formula = 100*(final DCM-control final DCM)/control DCM

[c] na = not applicable

[d] Values in parentheses are standard errors of the mean

Coefficients of variance for the sugar and ethanol concentrations of the duplicate fermentations were calculated and ranged from 2% to 9%. Ethanol yields (g ethanol per g sugar) were calculated at the point of maximum ethanol concentration (Table 3.2). As a point of reference, 0.51 g ethanol per g xylose is most commonly considered a maximum theoretical yield (Evans and Ratledge, 1984). However, yields are known to be strongly dependent on fermentation conditions. The control samples in this study, which are based on the use of an "optimum" semi-defined medium, had average ethanol yields equal to approximately 90% of theoretical (using 0.51 g ethanol (g sugar)$^{-1}$ as a reference value). Ethanol yields from all of the prehydrolysates were a minimum of 10% below that of the control samples. Ethanol yields from poplar prehydrolysates were the lowest, averaging ~67% of theoretical; those from corn stover prehydrolysate were the highest, averaging ~80% of theoretical. Yield calculations are based on total sugar in the prehydrolysate, which includes xylose, glucose and arabinose. Arabinose is included in these calculations by default, as arabinose co-elutes with xylose on the column typically employed for fermentation media analyses. Arabinose in a prehydrolysate will lower apparent ethanol yields, since under microaerobic conditions *Pichia* does not assimilate arabinose until xylose and glucose are consumed, and then it does so concurrently with ethanol (Delgenes et al., 1988). Arabinose measurements on prehydrolysates from each feedstock indicate that the ethanol yield was lowered approximately 6%, 8% and 2% for corn stover, switchgrass and poplar, respectively.

One measure of the rate of ethanol production is volumetric ethanol productivity (g ethanol(l)$^{-1}$(hour)$^{-1}$), in which the calculation is based on the time required to reach maximum ethanol concentration (Olsson and Hahn-Hägerdahl, 1996). The means of the relative ethanol productivities (i.e. the productivity of the prehydrolysate-based media divided by the productivity of the control media) were corn stover (102%) > switchgrass (70%) > poplar (32%) (Table 3.2). These values corresponded well with relative initial rates of ethanol production-corn stover (89%) > switchgrass (61%) > poplar (34%)-where "initial rate" is defined as the apparent linear rate of ethanol production in the early phase of fermentation (based on the 0, 6, and 12 hr time points of Figure 3.1). The complete time courses for ethanol production (Figure 3.1) clearly show that rates of ethanol production throughout the fermentation are feedstock specific, i.e. there was little to no overlap in the time courses corresponding to prehydrolysates derived from switchgrass, corn stover and poplar. Prehydrolysates derived from the same feedstock fermented similarly, with only poplar prehydrolysates showing noticeable variability. The reason for the greater variability between poplar prehydrolysates is not clear. This variability is not explained by differences in acetate or furfural concentrations. Furfural concentrations were less than 0.05 $g(l)^{-1}$ at the beginning of a typical fermentation, and less than 0.01 $g(l)^{-1}$ 6 hours after inoculation.

The time courses for xylose consumption in each of the prehydrolysates are presented in Figure 3.2. As expected, the prehydrolysates where ethanol was produced the fastest showed the highest rates of xylose consumption. Comparison of xylose utilization rates for the three feedstocks indicates an overall slowing of xylose consumption in poplar prehydrolysate fermentations, which was reflected in the slower production of ethanol.

Figure 3.1. Ethanol concentration vs. time during *P. stipitis* fermentations of lignocellulosic prehydrolysates. See Table 3.1 for legend key.

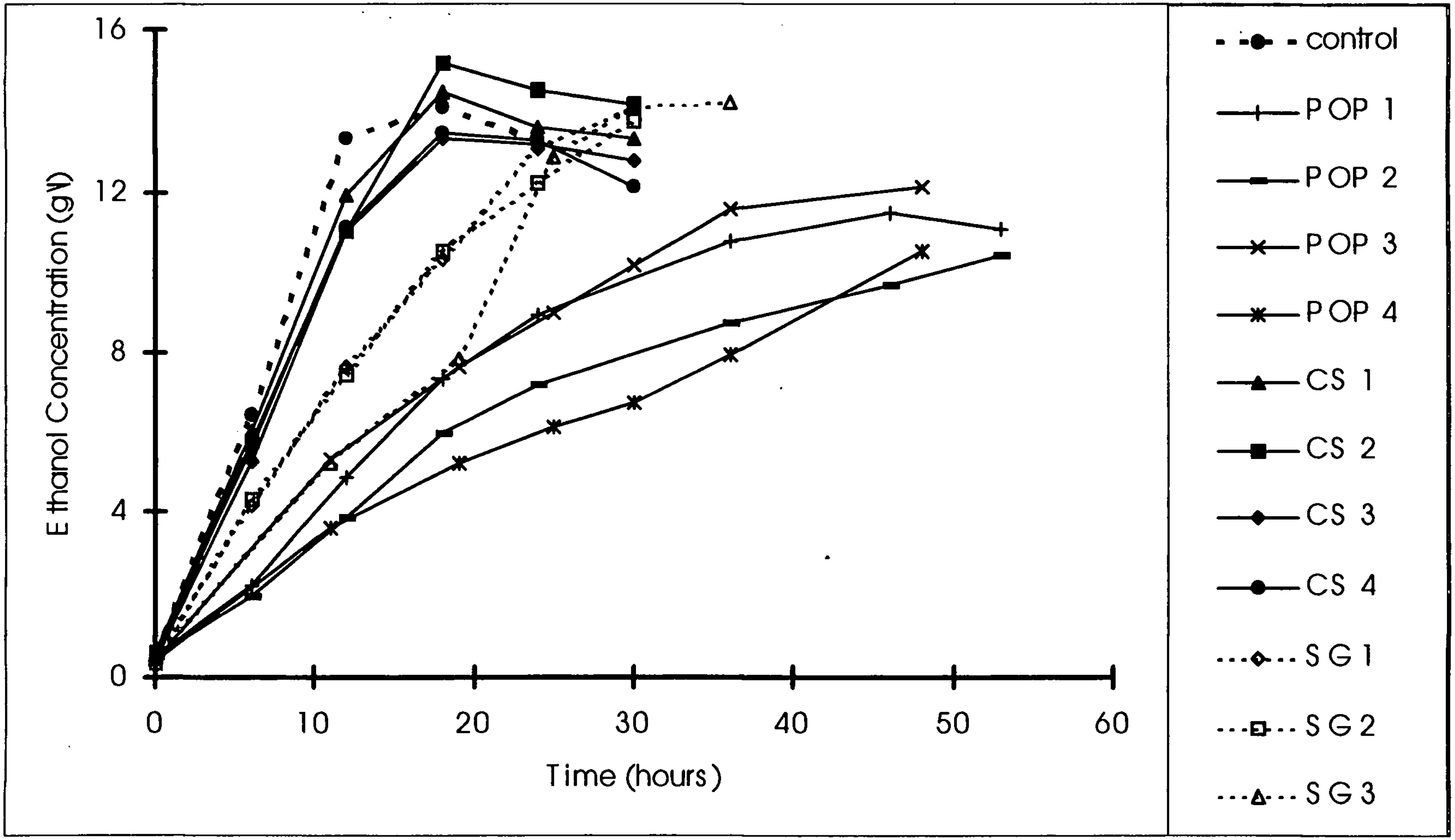
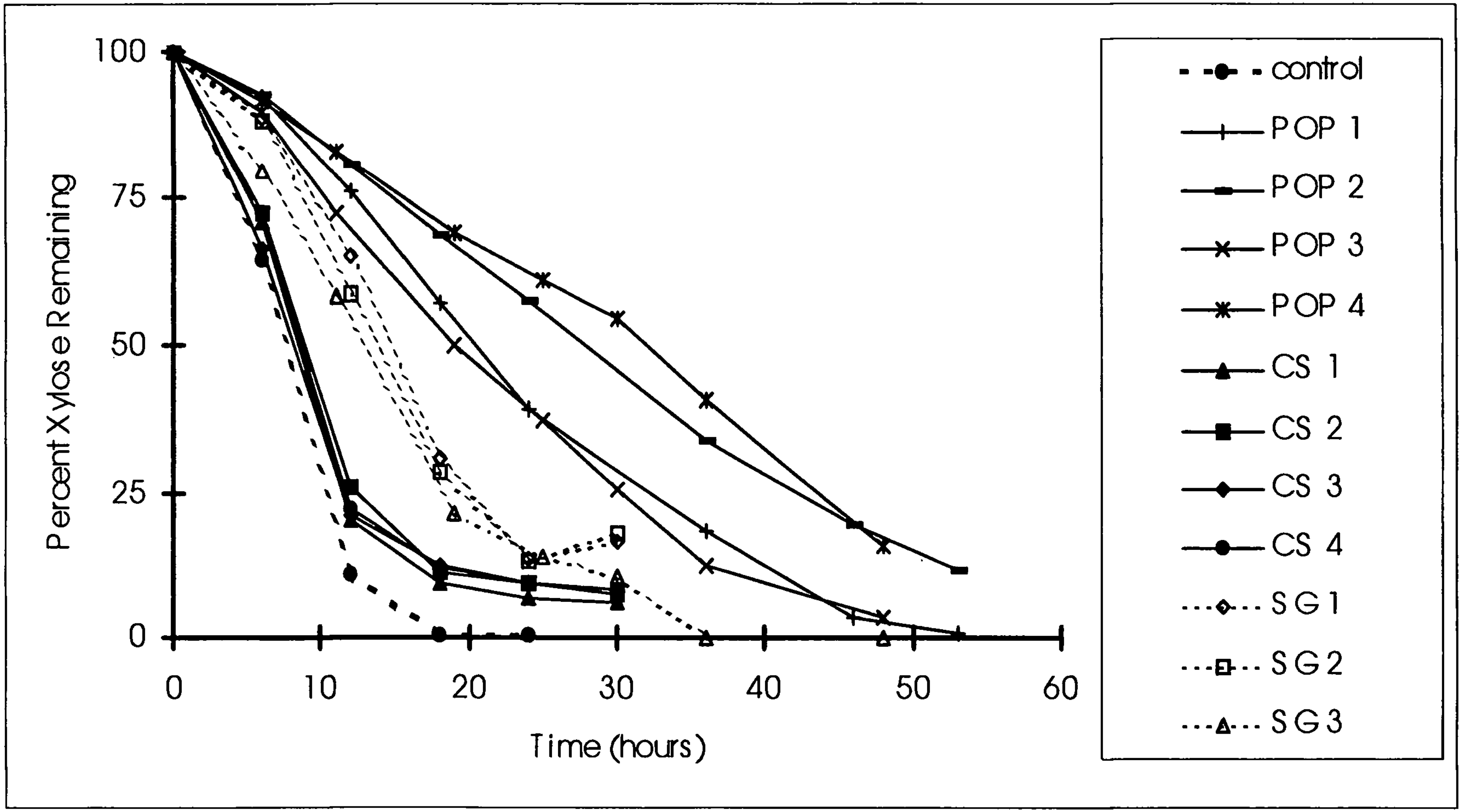


Figure 3.2. Percent xylose remaining vs. time during *P. stipitis* fermentations of lignocellulosic prehydrolysates. See Table 3.1 for legend key.



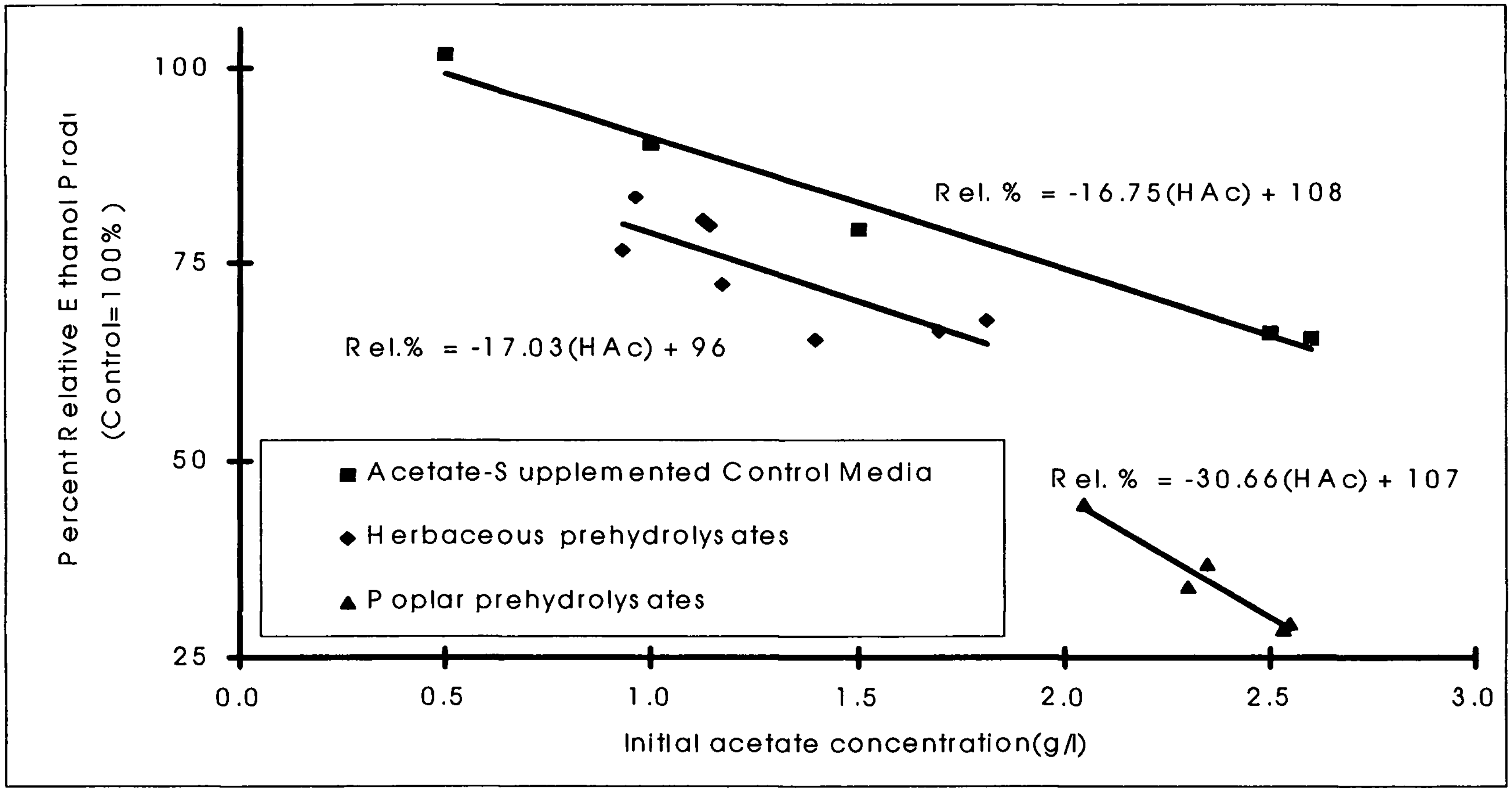
The similarity in the time courses of ethanol production and xylose uptake for a given prehydrolysate medium strongly suggest that the inhibition observed in this study is due to a general decrease in the rate of xylose metabolism. This mechanism is distinct from one in which alternative, non-ethanol end products, *e.g.* xylitol or glycerol, are produced. The idea that the observed inhibition in prehydrolysate-based media is due to a general slow-down in yeast metabolism is also consistent with the dry cell mass data of Table 3.2, which shows that in general, dry cell mass production was lowest in the fermentations with the lowest rates of ethanol production.

In a typical control fermentation the concentration of xylitol in the medium peaked between 0.5 and 1 $g(l)^{-1}$ at 12 hours and then slowly tapered off. In contrast, for corn stover and switchgrass fermentations, the xylitol concentration peaked near 4 $g(l)^{-1}$ at 12 to 18 hours, then dropped precipitously to approximately 0.5 $g(l)^{-1}$ within the next 6 hours. For the faster fermenting poplar prehydrolysates (poplar 1 and poplar 3) the xylitol concentration peaked near 1.4 $g(l)^{-1}$ at 24 hours and dropped to 0.8 $g(l)^{-1}$. The slower fermenting poplar prehydrolysates (poplar 2 and poplar 4), the xylitol concentration reached a maximum of 0.8 $g(l)^{-1}$ at 36 hours. Thus, a brief burst of xylitol in the media was associated with the faster fermenting, prehydrolysate-containing media, *i.e.* all herbaceous prehydrolysates and the two faster-fermenting poplar prehydrolysates. The secreted xylitol apparently was quickly assimilated and metabolized by *Pichia*, as evidenced by its rapid disappearance.

Acetate inhibition of fermentation is well-documented in *Pichia* systems (Tran and Chambers, 1986; Ferrari *et al.*, 1992; van Zyl *et al.*, 1991). Hence, correlations between prehydrolysate acetate concentrations and corresponding ethanol productivities were determined. A plot of acetate concentration vs. relative ethanol productivity for each of the prehydrolysates is shown in Figure 3.3. The data were grouped into three sets. The first set corresponds to those fermentations that were done with acetate-supplemented control media. These values represent the inhibition of *Pichia* due to acetate alone. The second and third data sets include fermentations done on the herbaceous (switchgrass and corn stover) and woody (poplar) prehydrolysates, respectively. These show the inhibition due to all the compounds in the prehydrolysates, including acetate. The best-fit line obtained using simple linear regression is included for each of the three data sets.

Simple linear regression analysis of ethanol productivity versus prehydrolysate acetate concentrations for the acetate-supplemented control media gives convincing evidence that, over the range of acetate concentrations shown, relative productivity is linearly correlated with acetate concentration (two-sided p-value, 0.001). Comparison of the best-fit regression lines describing the acetate-supplemented control media fermentations and the poplar prehydrolysate fermentations, which are significantly different (two-sided p-value = 0.03), strongly suggests that apparent acetate toxicity is dependent on the fermentation medium; *i.e.* the slopes of the lines correlating acetate concentration and ethanol productivity are different in the acetate-supplemented control media and poplar prehydrolysate media.

Figure 3.3. Percent relative volumetric ethanol productivity at 12 hours vs. initial acetate concentration for *P. stipitis* fermentations of dilute-acid lignocellulosic biomass prehydrolysate media and acetate-supplemented control media solutions. Initial acetate concentrations reflect dilution by addition of inocula and yeast extract.



The slope of the regression line of ethanol productivity on initial acetate concentration for the herbaceous prehydrolysate-based media was not significantly different from the corresponding slope for acetate-supplemented control media. Comparing the regression lines of the poplar prehydrolysate data and the herbaceous prehydrolysate data suggests, although inconclusively (two-sided p-value, 0.08), that correlations between ethanol productivity and initial acetate concentration are different for woody and herbaceous feedstocks. A cursory analysis of Figure 3.3 shows that non-acetate compounds in poplar prehydrolysate are associated with a higher degree of inhibition than that found in the corresponding herbaceous prehydrolysates. In this regard, prehydrolysate compounds other than acetate, such as lignin and carbohydrate degradation products, have been shown to be toxic to *Pichia* (Tran and Chambers, 1986; Delgenes *et al.*, 1996). The 1996 paper by Delgenes *et al.* illustrates that both the quantity and quality of phenolics are important factors in inhibition of *Pichia.* A linear correlation between ethanol productivities and acetate concentration may be expected for most lignocellulosic-derived prehydrolysates, since acetate liberation is one means of assessing the general severity of a dilute-acid pretreatment.

The relative toxicity of prehydrolysates derived from three important lignocellulosic feedstocks has been determined using a standardized bio-assay. The relative toxicity of the prehydrolysates, in order of decreasing toxicity, was poplar > switchgrass > corn stover. This trend agrees with work done by Parekh *et al.* (Parekh *et al.*, 1988), in which a herbaceous (corn stover) prehydrolysate was reported to be more completely fermented than were prehydrolysates prepared from woody feedstocks (pine and aspen). The differences in ethanol productivities observed in the present study are relatively large, ranging from 32% (poplar) to 102% (corn stover) of control. This broad range appears to reflect the relatively high sensitivity of the *Pichia*-based bioassay employed in this study. The diminished ethanol productivities associated with the fermentations of biomass-derived prehydrolysates was found to be closely related to prehydrolysate acetate concentrations. However, control fermentations with acetate-supplemented media indicate that inhibitors other than acetate are present in each of the prehydrolysates. Ethanol productivities, xylose utilization, non-ethanol end product levels and dry cell mass productions are all consistent with the reduction in ethanol productivity being a result of prehydrolysate inhibitors causing a general slow-down in yeast metabolism.

3.5 Acknowledgments

We thank Drs. Alan Bakalinsky (Oregon State University) and James McMillan (National Renewable Energy Laboratory) for their helpful discussions. This work was supported in part by the National Renewable Energy Laboratory, Golden, CO.

CHAPTER 4

Comparison of Aromatic Monomers in Lignocellulosic Biomass Prehydrolysates[1,2]

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4.1 Abstract

Differences in the relative toxicity of xylose-rich prehydrolysates derived from woody and herbaceous feedstocks are likely due to the relative abundance of a variety of inhibitory compounds. Acetate, as well as several aromatic monomers, has been shown to be inhibitors of the xylose-fermenting yeast, *Pichia stipitis*. Comparative information on the concentration of known and likely inhibitors, other than acetate, is lacking. The present study provides data on the aromatic monomer composition of representative herbaceous and woody prehydrolysates. Dilute-acid prehydrolysates were prepared from three feedstocks; two herbaceous, corn stover and switchgrass (*Panicum virgatum L.*), and one woody (poplar). The prehydrolysates were neutralized with $Ca(OH)_2$, extracted with ethyl acetate, trimethylsilylated, and analyzed by GC-MS. Fourteen aromatic monomers were tentatively identified by comparison with published mass spectra. The concentrations of the aromatic monomers totaled 112, 141 and 247 $mg(l)^{-1}$ for corn stover, switchgrass and poplar prehydrolysates, respectively. This is also the order of increasing inhibition of growth and ethanol productivity observed for *Pichia* fermentations. The woody prehydrolysate contained approximately four-fold more syringyl-based monomers than did the herbaceous prehydrolysates, while guaiacyl-containing compounds were more evenly distributed.

4.2 Introduction

The production of fuel ethanol from lignocellulosic biomass feedstocks has several benefits including domestic availability, pollution reduction and ease of introduction into existing gasoline and diesel distribution networks (von Sivers and Zacchi, 1996; Lynd *et al.*, 1991). One widely studied process for converting lignocellulosic biomass to ethanol involves a pretreatment of the feedstock with dilute acid (approximately 1.0% (w/w) sulfuric acid) at temperatures over 140°C, conditions which catalyze the hydrolysis of biomass hemicellulose, but leave the cellulose fraction largely intact (Penner *et al.*, 1996). This dilute-acid pretreatment yields a solids stream, consisting of mostly lignin and cellulose, and an aqueous stream, called the prehydrolysate, which contains hemicellulose-derived xylose, lesser amounts of other carbohydrates, sugar degradation products, lignin degradation products, acetic acid, and other compounds (Olsson and Hahn-Hägerdahl, 1996). Sugars (mostly xylose) in the prehydrolysate typically represent 15-30% of the original dry weight of the biomass (Esteghlalian *et al.*, 1997). Rapid and efficient fermentation of these sugars is essential for making biomass-to-ethanol conversion processes economically viable (von Sivers and Zacchi, 1996).

Dilute-acid prehydrolysates contain many compounds other than sugars, and some of these inhibit fermentation by and growth of microorganisms. These inhibitors can be divided into groups based on their origin (Olsson and Hahn-Hägerdahl, 1996); 1) compounds released during pretreatment, e.g. acetic acid, 2) sugar degradation products, e.g. furfural, 3) lignin degradation products, e.g. syringaldehyde, 4) fermentation products, e.g. ethanol, and 5) contaminants released by processing equipment, e.g. chromium. For a review of prehydrolysate fermentation and a discussion of the role of inhibitors, see Olsson and Hahn-Hägerdahl (Olsson and Hahn-Hägerdahl, 1996).

Several papers describing ethanol yields from *Pichia stipitis* fermentations of dilute-acid prepared lignocellulosic prehydrolysates have been published, including studies of prehydrolysate derived from aspen wood (Wilson *et al.*, 1989), red oak (Tran and Chambers, 1986), sugar cane bagasse (Roberto *et al.*, 1991), corn cob (Hahn-Hägerdahl *et al.*, 1994), mixed wood chips (Perego *et al.*, 1990), eucalyptus (Ferrrari *et al.*, 1992), switchgrass (Fenske *et al.*, 1998), corn stover (Fenske *et al.*, 1998, Parekh *et al.*, 1988), poplar (Fenske *et al.*, 1998; Parekh *et al.*, 1988), rice straw (Moniruzzaman, 1995), *Pinus radiata* (Qureshi and Manderson, 1991) and wheat straw (Delgenes *et al.*, 1988). *Pichia*, although an efficient fermenter of xylose, is susceptible to inhibition by toxic compounds in lignocellulosic prehydrolysates, leading to lower ethanol productivities (g ethanol $l^{-1}hr^{-1}$) and yields (g ethanol g sugar consumed^{-1}) compared to those achieved in fermentations of control media (Olsson and Hahn-Hägerdahl, 1996). Among the compounds identified in prehydrolysates which are known to inhibit *Pichia* fermentations are vanillin, syringealdehyde, acetic acid and furfural (Tran and Chambers, 1986; van Zyl *et al.*, 1991; Delgenes *et al.*, 1996). The lignin-derived compounds, *i.e.* syringealdehyde and vanillin, have been shown to be particularly potent inhibitors of ethanol production and cell growth (Tran and Chambers, 1986; Delgenes *et al.*, 1996), especially when compared on a concentration basis to acetic acid or sugar degradation compounds. In particular, vanillin, a guaiacyl- containing compound, was shown to be more inhibitory than representative syringyl- and hydroxybenzyl-containing compounds (Delgenes *et al.*, 1996).

Phenolic compounds in prehydrolysates prepared from woody substrates have been described (Wilson *et al.*, 1989; Tran and Chambers, 1986; Buchert *et al.*, 1990, Ando *et al.*, 1986). In contrast, relatively little is known about the lignin-derived compounds in prehydrolysate obtained from herbaceous lignocellulosics such as switchgrass (*Panicum virgatum L.*) and corn stover. Switchgrass and corn stover are considered to be excellent candidate feedstocks for conversion to ethanol (Sanderson *et al.*, 1996; Downing *et al.*, 1995), and thus are of particular interest.

The aim of the present study was to identify and quantify phenolics and lignin-derived compounds found in the prehydrolysate derived from two herbaceous feedstocks (switchgrass and corn stover) and one woody feedstock (poplar). These feedstocks were chosen based on their potential for use in biomass-to-ethanol processes and because the relative fermentability of their prehydrolysates has been established (Fenske *et al.*, 1998). The results provide analytical information that should be useful in determining the chemical basis for differences in rates and extents of prehydrolysate fermentations as well as for designing detoxification processes (Olsson and Hahn-Hägerdahl, 1996).

4.3 Materials and Methods

4.3.1 Sample preparation.

Milled and dried feedstocks (poplar, corn stover and switchgrass) were treated in a 0.6 l stainless steel Parr reactor at 10% solids (180°C, 1% (w/w) H_2SO_4, one minute) as described (Esteghlalian *et al.*, 1997). The resulting material was filtered through VWR 413 grade paper, and neutralized to pH 6.0 with $Ca(OH)_2$ as described (Fenske *et al.*, 1998). The pretreatment conditions were chosen to optimize the yield of xylose in the liquid prehydrolysate. One ml of 0.13 gl^{-1} o-vanillin (Sigma) internal standard solution was added to 10 ml of prehydrolysate. 10 ml of ethyl acetate was added and mixed by inversion. After separation of the two phases, the upper layer was transferred to a test tube and dried under N_2 at 40°C for 15 min. The residue was redissolved in 0.5 ml of ethyl acetate and transferred to a vial to which was added 50 µl pyridine and 300 µl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) from Sigma. The mixture was allowed to react at room temperature overnight.

4.3.2 GC-MS

Gas chromatography/mass spectrometry was performed on a 10 m SE-54 capillary column using a Finnigan GC/MS. Mass spectra were obtained at 70 eV. For quantification of tentatively identified phenolic compounds, a 1:1 response ratio with o-vanillin was assumed.

4.4 Results and Discussion

Partial total ion count (TIC) chromatograms obtained from TMS-derivatized ethyl acetate extracts from corn stover, switchgrass and poplar prehydrolysates are shown in Figure 4.1. In all cases, chromatograms reflect neutralized prehydrolysates, *i.e.* suitable for fermentation. This approach was taken to facilitate comparison of the GC-MS data with fermentation results. Ethyl acetate was used for extraction because it has been shown to remove phenolic compounds such as parahydroxybenzoic acid and vanillin from aspen prehydrolysates. Ethyl acetate was also shown to reduce inhibition of microbial growth and fermentation of prehydrolysates (Wilson *et al.*, 1989). Thus it can be expected that microbial inhibitors, along with non-inhibitory compounds, are to be found in an ethyl acetate extract.

By matching mass spectra with those in the literature (Lapierre *et al.*, 1983; Niemelä and Sjöström, 1986; Wittkowski, 1985), 14 compounds with structures indicative of lignin monomers or esterified phenolics were tentatively identified. Table 4.1 summarizes the identification and quantification of the chromatogram peaks from the three feed stocks. The concentrations of the identified lignin-derived components total 247, 141 and 112 mg(l)$^{-1}$ for poplar, switchgrass and corn stover prehydrolysates, respectively. This is also the order of decreasing microbial inhibition, as measured by ethanol yield, productivity, and cell mass increase in *Pichia* fermentations, as shown in Table 4.2. In addition, this is also the order of decreasing Klason lignin content for the feedstocks (Esteghlalian *et al.*, 1997). Similarly, the Klason lignin content of native woody feedstocks was found to be negatively associated with ethanol yields using *Saccharomyces* in simultaneous saccharification and fermentation experiments (Vinzant *et al.*, 1997). The acetate concentrations averaged 1.3, 2.0 and 2.9 gl^{-1} for corn stover, switchgrass and poplar prehydrolysates, respectively. Furfural and hydroxymethylfurfural concentrations were less than 0.01 g/l for all neutralized prehydrolysates.

The most striking difference in both the type and amount of aromatic compounds was found among the syringyl compounds. In poplar prehydrolysate, the concentration of syringyl-containing compounds was approximately four-fold higher than in switchgrass or corn stover prehydrolysates. The guaiacyl compounds were more evenly distributed among the three prehydrolysates, with corn stover having the lowest concentration of identified guaiacyl derivatives, and switchgrass the highest. Although other work indicates that guaiacyl-containing monomers are highly toxic to *Pichia* (Delgenes *et al.*, 1996), the concentrations used in that study were far higher than anything seen in the prehydrolysates examined here. The work currently described was conducted on native, unextracted biomass, so it is difficult to determine whether differences in the ratios of the three main phenyl derivatives are due to differences in the core lignin structures of the three feedstocks, or if they represent differences in more-easily solubilized non-core lignin materials.

The concentrations of syringealdehyde (S-CHO, Table 4.1) and vanillin (G-CHO, Table 4.1) in the poplar prehydrolysate are similar to those reported by Buchert *et al.* (1991) for birch wood prehydrolysate prepared by steam treatment and extracted with dimethylchloride without neutralization, although the concentrations of 1-guaiacyl acetol ($G\text{-}CH_2\text{-}CO\text{-}CH_2OH$, Table 4.1) and 1-syringyl acetol ($S\text{-}CH_2\text{-}CO\text{-}CH_2OH$, Table 4.1) in poplar prehydrolysate were found to be 20-30 fold higher. In comparison to red oak prehydrolysate pretreated under similar conditions (Tran and Chambers, 1986), poplar prehydrolysate contained 5-fold less vanillin and 10-fold less syringealdehyde, although the extraction and preparation regimens were different.

Compounds which were unique to specific prehydrolysates included vanillyl propanol ($G\text{-}CH_2\text{-}CH_2\text{-}CH_2OH$, Table 4.1) in switchgrass, syringyl methyl ketone ($S\text{-}CO\text{-}CH_3$, Table 4.1) in corn stover and para-hydroxybenzoic acid (H-COOH, Table 4.1) in poplar. Para-hydroxybenzoic acid has been shown to be a major component in poplar-derived hydrolysates (Ando *et al.*, 1986; Bardet and Robert, 1985).

In previous work (Fenske *et al.*, 1998) it was shown that prehydrolysates from herbaceous feedstocks were more easily fermentable than that from woody feedstocks, and that the discrepancies could be only partially attributed to acetate concentration differences. It is demonstrated here that prehydrolysates derived from herbaceous feedstocks (switchgrass and corn stover) contain much less phenolic monomers than poplar-derived prehydrolysate. When considered alongside the lower acetate concentrations of the herbaceous prehydrolysates, the evidence presented here provides a good rationale for the lower toxicity of herbaceous prehydrolysates. Whether this trend can be generalized to other feedstocks can only be determined by direct comparison. This data provides a basis for comparison in assessing the feasibility of feedstocks for biomass-to-ethanol processes.

4.5 Acknowlegements

Ths work was funded in part by the National Renewable Energy Laboratory, Golden, Colorado.

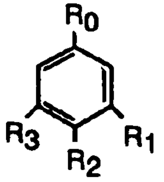
Table 4.1. Lignin-derived compounds tentatively identified in dilute-acid prehydrolysates prepared from poplar, switchgrass and corn stover.

Phenolic moietie[c]	R_0	Chromatogram peak labels	Poplar ($mg\ l^{-1}$)	Switchgrass ($mg\ l^{-1}$)	Corn Stover ($mg\ l^{-1}$)
H	-COOH	POP3	11.1 (0.8)	nd	nd
H	-C=CH-COOH	SG14, CS13	nd	7.7 (0.2)	10.9 (0.2)
S	-CHO	CS4, POP4	29.3 (2.0)	nd	10.0 (0.3)
S	$-CHOH-CO-CH_3$	SG13, POP11	35.3 (2.2)	9.1 (0.3)	10.0 (0.6)
S	$-CH_2-CO-CH2OH$	SG17,19; CS15,17; POP14,16	74.4 (4.0)	26.3 (1.5)	17.2 (0.8)
S	$-CO-CH_3$	CS8	nd	nd	5.2 (0.1)
S	$-CO-CO-CH_3$	SG16, CS14, POP13	17.0 (0.8)	2.6 (0.1)	9.5 (0.3)
G	$-CO-CHOH-CH_3$	SG12, CS11, POP10	1.0 (0.1)	2.0 (0.1)	nd
G	-CHO	CS2, SG2, POP2	15.1 (0. 4)[a]	16.5 (0. 6)	19.6 (0. 2)
G	$-CH_2-CO$	SG9, POP7	4.4 (0.4)	1.5 (0.1)	nd
G	$-CH_2-CH_2-CH_2OH$	SG11	nd	18.4 (0.8)	nd
G	-C=CH-COH	CS10, POP9	8.5 (0.5)	trace	4.0 (0.6)
G	$-CH_2-CO-CH_2OH$	SG15,18; CS13,16; POP12,15	51.2 (2.5)	56.7 (3.0)	26.0 (1.1)

[a] Values in parentheses are standard errors of the means

[b] nd = not detected

[c]



Moietie	R_1	R_2	R_3
Hydroxyphenyl (H)	H	OH	H
Guaiacyl (G)	OCH_3	OH	H
Syringyl (S)	OCH_3	OH	OCH_3

Table 4.2. Inhibitor concentrations and fermentation results obtained from poplar, switchgrass and corn stover prehydrolysates.

Prehydrolysate source	*Acetate (g/l)*[1]	*p-Hydroxy-phenyl monomers (mg/l)*	*Guaiacyl monomers (mg/l)*	*Syringyl monomers (mg/l)*	*Ethanol yield (g ethanol/ g sugar)*[1]	*Ethanol productivity (g ethanol/ l * hr)*[1]	*Final dry cell mass (percent relative to control)*[1]
Poplar	2.9	11.1	80.2	156.0	0.34	0.25	-15
Switchgrass	2.0	7.7	95.1	38.0	0.38	0.54	+5
Cornstover	1.3	10.9	30.0	51.9	0.41	0.79	+7

[1] From Fenske *et al.*, 1998

Figure 4.1. TIC Chromatograms obtained from TMS-derivatized ethyl acetate extracts of switchgrass (a), corn stover (b), and poplar (c) prehydrolysates. See Table 4.3 for explanation of abbreviations.

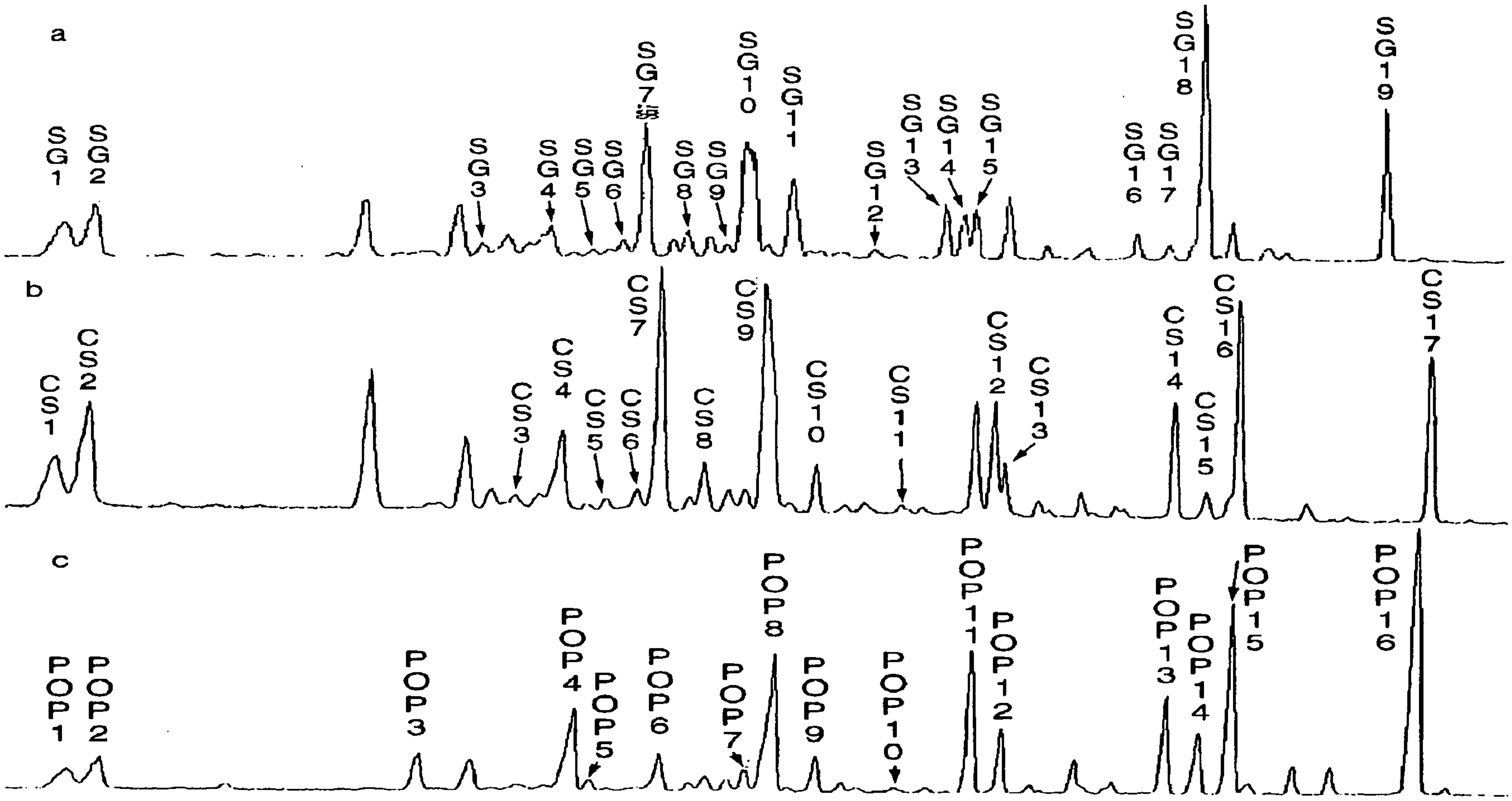


Table 4.3. Identifications of peaks label in Figure 4.1 See Table 4.1 for explanation of H, G, and S.

Peak label			Tentative identification
SG 1	CS 1	POP 1	ortho-vanillin (internal standard)
SG 2	CS 2	POP 2	G-CHO
		POP 3	H-COOH
SG 3			arabinose
	CS 3		arabinofuranose
	CS 4	POP 4	S-CHO
SG 4	CS 5		arabinose
SG 5			arabinose
SG 6			levo-glucosan
SG 7		POP 5	xylose
SG 8			arabinose
	CS 6	POP 6	xylopyranose
	CS 7		xylose
SG 9		POP 7	G-CH2-CO
	CS 8		S-CO-CH3
SG 10	CS 9	POP 8	xylose + aromatic moieties
	CS 10	POP 9	G-C=C-COH
SG 11			G-CH2-CH2-CH2OH
SG 12	CS 11	POP 10	G-CO-CHOH-CH3 (ketonic)
SG 13		POP 11	S-CHOH-CO-CH3
SG 14	CS 12		H-C=CH-COOH
SG 15	CS 13	POP 12	G-CH2-CO-CH2OH (ketonic)
SG 16	CS 14	POP 13	S-CO-CO-CH3
SG 17	CS 15	POP 14	S-CH2-CO-CH2OH (ketonic)
SG 18	CS 16	POP 15	G-CH2-CO-CH2OH (transenolic)
SG 19	CS 17	POP 16	S-CH2-CO-CH2OH (transenolic)

CHAPTER 5

A Simple Individual-Based Model of Insoluble Polysaccharide Hydrolysis: Evidence of Autosynergism for Dual-Activity Enzymes[1]

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Key Words: Individual-based model, polysaccharide hydrolysis, hydrolase

[1] For submission as a letter to the Journal of Theoretical Biology

Polysaccharide hydrolyzing glycosidases, such as amylases, cellulases and chitinases, are traditionally categorized as either endo- or exo-acting. The endo-acting enzymes randomly cleave glycosidic linkages internal to the polymer chain; the exo-acting enzymes specifically cleave linkages at the chain termini. Structurally, the major difference in the two classes of enzymes appears to be the accessibility of the active site (Teeri, 1997; Kleywegt *et al.*, 1997). The active site of endo-acting enzymes typically is in a surface cleft and that of exo-acting enzymes is in an enclosed tunnel or pocket. Structural data on homologous endo/exo cellulolytic enzyme pairs from the fungus *Trichoderma reesei* provide prototypical examples of cleft- and tunnel-shaped active sites.

A common kinetic phenomena observed with mixtures of endo- and exo-acting enzymes is synergism (Walker and Wilson, 1991; Fujii and Kuwamara, 1984; Henrissat *et al.*, 1985; Nidetzky *et al.*, 1995). Synergistic enzymes are ones whose combined activity, when working in combination, is greater than that of the sum of equal quantities of the two enzymes acting independently. The classic rationale for synergism between endo- and exo-acting enzymes is that endo-acting enzymes provide new chain ends for exo-acting enzymes, thus increasing the effective substrate concentration for the exo-acting enzyme (Woodward, 1991). In this rationale, product formation is highly dependent on the activity of the exo-acting component. Increases in reaction mixture substrate concentrations eventually lead to saturation of the exo-acting component and, hence, the exo-acting enzyme becomes less dependent on the presence of endo-generated chain ends. This means that increases in substrate concentration lead to a reduction in the degree of synergism as the enzyme approaches maximum velocity (Fujii and Kawamura, 1984).

This scenario is typically considered with reference to soluble enzyme/soluble substrate systems.

The situation may be somewhat different for glycosidases acting on insoluble polysaccharides. In this case, the enzymes are often comprised of two domains, with the catalytic site located in one domain and a separate substrate binding site located in the other (Wilson *et al.*, 1995). The binding domain serves to anchor the enzyme to the surface of the insoluble substrate. If exo-acting enzymes are randomly associated with the substrate surface, then a percentage of these enzymes will be at loci distant from their sites of optimal activity, *i.e.* the chain ends. Increasing substrate concentrations are expected to yield maximum amounts of adsorbed enzyme, yet only a fraction of the adsorbed enzyme may be anchored at a catalytically productive locus (in the case of exo-acting enzymes, a catalytically productive locus would be one in the vicinity of a chain end). In this scenario, synergism between endo- and exo-enzymes can result from the ability of endo-enzymes to create new chain ends in exo-enzyme-occupied nonproductive loci. The net result, with respect to the activity of the exo-acting enzyme, is that a catalytically nonproductive locus would have been converted to a catalytically productive locus. This scenario is most easily envisioned for the case where the exo-acting enzyme has limited surface mobility (Jervis *et al.*, 1997; Claeyssens and Tomme, 1989)

It follows from the above that, in order for enzymes which act on the surface of an insoluble substrate to be synergistic, they must work in close enough proximity to take advantage of each other's activity (Penner and Liaw, 1994). Furthermore, enzymes which are relatively close to one another at lower substrate:enzyme ratios will become distant at higher substrate:enzyme ratios. Thus, considering the case of randomly associated exo-and endo-acting enzymes, synergy will be reduced as substrate concentrations increase above that required for maximum enzyme adsorption. An example of such an enzyme system is found in the cellulases of *T. reesei*. The loss of synergy upon surface dilution would be visible as an apparent substrate inhibition, and such kinetic behavior has been observed with crude *Trichoderma reesei* cellulase systems (Huang and Penner, 1991). This apparent "substrate inhibition", which is a consequence of reduced synergism, is obviously the result of events distinct from those associated with substrate inhibition in classical soluble-enzyme, soluble-substrate systems (Cornish-Bowden, 1979).

A current theory holds that the endo- versus exo- action-pattern of naturally occuring polysaccharide-hydrolyzing enzymes most probably forms a continuum ranging from strict endo- to strict exo-type enzymes (Teeri, 1997). Those showing the classic endo-action pattern have open cleft-type active sites while those showing the classic exo-action pattern have enclosed, tunnel-like active sites. Along the continuum are those enzymes which are primarily exo-acting, but which have a small amount of endo-activity. An example of this type of glycosidase is a *Cellulomonas fimi* cellulase expressed in *E. coli*, which was shown to have both endo and exo activity (Tomme *et al.*, 1996). The active site of enzymes along the continuum may be characterized as tunnels with roofs that on rare occasion retract, *i.e.* as having active sites typically enclosed but which may be temporarily exposed due to relatively few peptide loops enclosing the active site tunnel and/or enclosing loops with mobility (Warren, 1996; Kleywegt *et al.*, 1997).

This note addresses the issue of whether a homogeneous enzyme preparation, having an action pattern along the exo/endo continuum, may display any of the synergistic properties typical of multiple enzyme systems. The potential for this type of "autosynergistic" behavior exists by virtue of the enzyme having both endo and exo activity. To our knowledge, this topic has not been explored and yet it is of direct relevance to the catabolism of major carbon sources in the bioshpere, including cellulose, starch and chitin. We have addressed this question by modeling a simple glycosidase/insoluble polysaccharide substrate reaction system.

A two-dimensional array, representing the polysaccharide surface, was created for the enzyme to act upon (see Figure 5.1). The matrix consisted of cells containing either 1 or 0, with 1 representing a internal chain linkage and 0 representing a terminal glycosidic linkage, or chain break. There was exactly one randomly placed break per chain. Substrate surface saturation with enzyme was modeled by allowing, on average, only a defined number of enzyme molecules per chain to bind. Increases in substrate concentration were simulated by adding more chains to the matrix, in order to keep the ratio of internal to terminal glycosidic linkages constant.

Figure 5.1 Partial matrix used to represent polysaccharide surface. 1 and 0 represent an internal and terminal glycosidic linkage, respectively.

<- Y axis ->

1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1
1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	1
1	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1
1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0
1	1	1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1
1	1	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	1

<- X axis -> (polysaccharide chains)

To start, the appropriate number of enzyme molecules was placed on the matrix. A certain proportion was put on randomly selected chain ends, and the remainder were randomly distributed across the entire matrix. After the distribution of enzymes on the matrix there were two populations, those enzymes located on chain termini, and those on internal linkages. For each individual enzyme molecule, the following steps were executed.

For an enzyme on a terminal glycosidic linkage, there was a defined probability that an "exo" hydrolysis would occur. That probability was compared to a number randomly selected from a normal distribution. If the probability was exceeded, an exo cut was made, *i.e.* the enzyme moved one cell to the right (the x dimension in Figure 1), and the product variable was incremented. The above steps were repeated until the number selected was less than the exo probability, at which point the next enzyme molecule was evaluated.

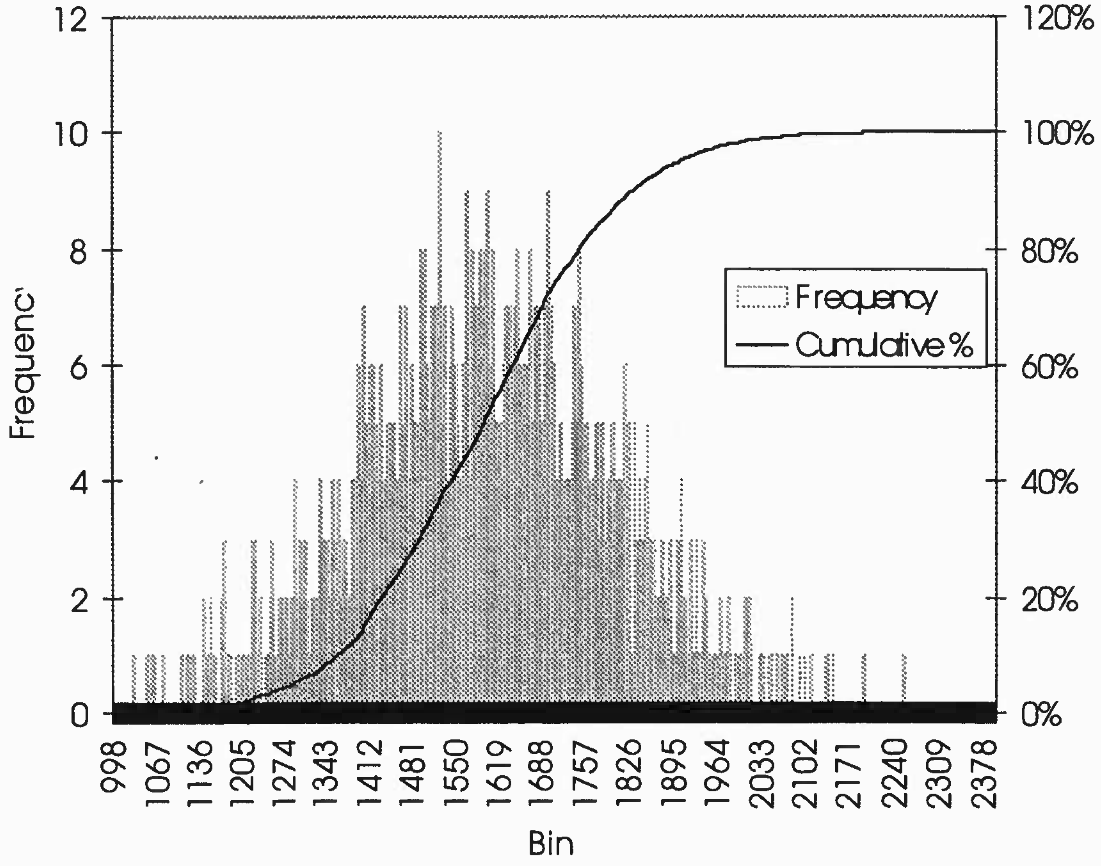
Similarly, for an enzyme on an internal glycosidic linkage, there was a probability that an "endo" hydrolysis would occur. Again, the probability was compared to a randomly generated number. If the number was large enough the cell was set equal to zero, but nothing was added to the product. At this point an endo cut was said to occur and thereafter, the enzyme was treated as though it had begun on a chain terminus. A new random number was generated, and the enzyme continued along the chain adding to the product until the probability of an exo cut was not met. In summary, exo cuts added to the product but created no new chain ends, while endo cuts created new chain ends but added nothing to the product.

After all the enzymes were evaluated, time was incremented and the enzymes were independently and randomly moved in the x and y dimensions. For each enzyme, the distance traveled (0, 1 or 2 cells) in the x and y dimension was chosen randomly and separately. Insoluble polysaccharide hydrolyzing enzymes often have two domains, a catalytic domain containing the active site and a binding domain which serves to anchor the enzyme to its insoluble substrate (Wilson *et al.*, 1995). It thus seems appropriate that the mobility of the enzymes is modeled in such a way that, on average, enzymes will not move far from starting points, unless processive catalytic activity drives it along a substrate chain.

A Monte Carlo simulation approach was used to analyze the stochastic nature of the system. For each run, the following values were tallied: (1) exo cuts made by enzymes starting on chain termini, (2) endo cuts, (3) exo cuts made immediately following an endo cut, and (4) product, *i.e.* the sum of exo cuts from (1) and (3). The impact of changes in certain parameters on predicted product levels was assessed. These parameters included the probabilities that an endo or exo catalytic event would occur and the fraction of enzyme that was initially bound to terminal glycosidic linkages.

The results analyzed consist of averages calculated from 2,000 runs performed consecutively during a single program execution. Standard deviations and coefficients of variation (100% * SD/mean) were used to determine an appropriate number of runs to ensure reproducibility. A typical histogram from a program execution is shown in Figure 5.2. The histograms all showed a normal Gaussian distribution.

Figure 5.2. Histogram of product predicted by 2000 runs at one set of conditions.



Interesting trends observed in the model-generated data include:

- Apparent substrate inhibition occurred only when endo activity was present
- Although endo activity was necessary for substrate inhibition to occur, substrate inhibition rose to a maximum and then decreased as the probablility of an endo cut increased.
- Apparent substrate inhibition was directly proportional to the probability of an exo cut
- Apparent substrate inhibition was inversely proportional to the fraction of total enzyme which was initially bound to terminal glycosidic linkages.

In Figures 5.3 and 5.4, the product versus substrate concentration curves for different exo and endo probabilities are shown. The maxima of curves correspond to cases where all of the available enzyme is bound on the least possible number of substrate chains. Substrate inhibition (as measured at a given concentration) varied between 15 and 40%. The number of exo cuts made immediately following an endo cut plateaued as substrate increased. In contrast, the number of "regular" exo cuts, *i.e.* those made by enzymes starting on already present terminal linkages, decreased with increasing substrate concentrations. Thus, the source of the apparent substrate inhibition was the decrease in "regular" exo cuts, a direct result of there being, on average, fewer endo-generated chain termini in the vicinity of any given enzyme.

The results suggest that in order for autosynergism to occur the enzymes have to be in relatively close proximity. For the model presented, this condition is met more frequently by increasing the number of time steps per run. This allows the enzymes to make more endo cuts and travel further to take advantage of endo cuts made by neighbors.

The decrease in substrate inhibition with the increase in proportion of enzyme initally bound to terminal linkages is due to the fact that more enzymes begin on productive loci. This makes them less dependent on nearby enzymes for help in creating a productive locus. The decrease in substrate inhibition at a given substrate concentration seen with an increase in endo activity is due to the increased overall activity of isolated enzymes. A enzyme which has a good chance of making its own chain break doesn't need to be near a neighboring endo-active enzyme to begin hydrolysis.

Figure 5.3 Model predictions for product at different substrate concentrations and different probabilities of exo activity.

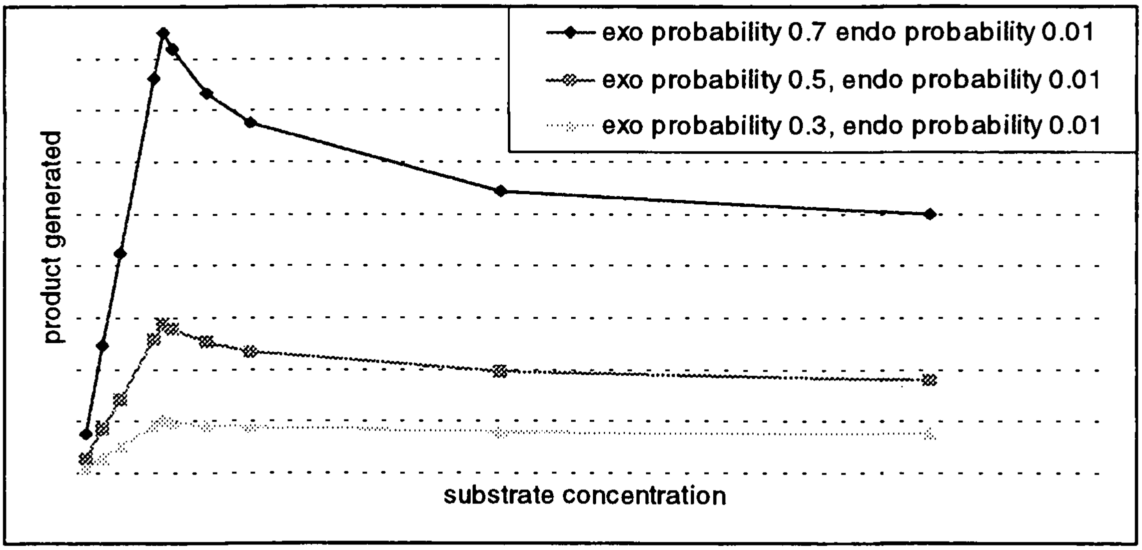
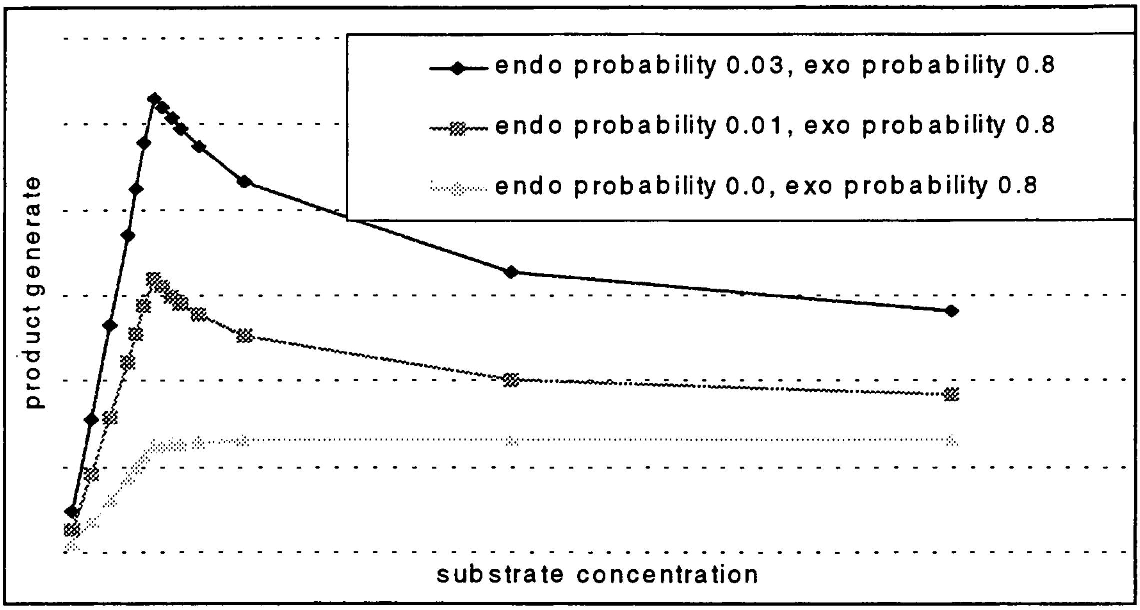


Figure 5.4. Model predictions for product at different substrate concentrations and different probabilities of endo activity.



This study demonstrates the potential of using individual-based models to explore mechanisms of polysaccharide hydrolysis. A model of this type has been used to invesitgate the effects of ligand shape on binding to a substrate (Sild et al., 1996). The directness of an individual-based model is appealing. Differences in individual-based model predictions are the immediate result of differences in the defined behavior of individual entities. In comparison, population-based models describe average behavior, and attaching a physical meaning to the parameter values generated can be difficult.

The current model shows that apparent substrate inhibition, a direct result of a decrease in autosynergism, to be possible in a single-enzyme system. This behavior is relevant to predominantly exo-acting enzymes which are randomly anchored to their insoluble substrates, *i.e.* which can associate with both internal and terminal chain linkages

(Wilson *et al.*, 1995). The concept of autosynergism with a supporting model does not appear to be in the glycosidase literature. This behavior is particularly important when considering synergistic enzyme pairs. For example, autosynergism may provide a rationale for the "exo-exo" synergism observed for selected cellulytic enzymes (Nidetzky *et al.*, 1995; Woodward, 1991); the rationale being that such synergism results from native endo activity on the part of one of the exo-enzymes. Similarly, autosynergism implies that two enzymes need not have opposite chain-end specificities in order be synergistic (Barr *et al.*, 1996).

Note: The computer model used to generate the data is listed in the Appendix.

CHAPTER 6

Kinetic Evidence for Endo Activity of CBH II Acting on Microcrystalline Cellulose[1]

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Key Words: *Trichoderma reesei*, cellobiohydrolase, microcrystalline cellulose, endo exo activity

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6.1 Abstract

The intent of this study was to determine if the substrate-velocity profiles for CBH I and CBH II acting on microcrystalline cellulose are consistent with enzymes having native endo activity. Recent simulation studies indicate that apparent substrate inhibition with homogeneous polysaccharide-degrading enzyme preparations is indicative of endo activity. The rates of microcrystalline cellulose hydrolysis by low concentrations of purified CBH2 prepared by three separate laboratories were determined. The activity of one CBH1 preapration was also measured. CBH1 showed typical saturation kinetics as the substrate concentration increased, while CBH2 showed an apparent substrate inhibition, which was also observed when using bacterial microcrystalline cellulose as the substrate. Based on the predictions made by an individual-based model, the behavior of CBH2 is consistent with that of an enzyme possessing both endo and exo activity. This behavior also has implications for "exo-exo" synergism.

6.2 Introduction

A great deal of effort has been put towards characterizing the two cellobiohydrolases produced by *Trichoderma reesei*, CBH1 and CBH2. At first glance, one might assume a high degreee of similarity between two enzymes made by the same organism which both possess a binding domain, linker region, and catalytic core (Claeyssens and Tomme, 1989) and hydrolyze crystalline cellulose to cellobiose. However, the two hydrolases are quite different with respect to many properties, including molecular weight (Bhikhabhai *et al.*, 1984), *p*I (Bhikhabhai *et al.*, 1984), catalytic mechanism (inversion vs. retention) (Knowles *et al.*, 1988), and preferred direction of attack on cellulose chains (Barr *et al.*, 1996). In addition, synergism between CBH1 and CBH2 has been reported by many researchers. For reviews of cellulases and synergism, see Walker and Wilson, (Walker and Wilson, 1991), Nidetzky *et al.* (Nidetzky *et al.*, 1995) and Woodward (Woodward, 1991).

In the classic model of endo-exo synergism, endo enzymes cleave cellulose chains in the middle, creating new ends for the exo enzymes, while the activity of the exo enzyme exposes new chains for the endo enzyme to act upon (Woodward, 1991). The distinguishing characteristic of endo- and exo-acting cellulases is the geometry of the substrate binding sites (Kleywegt *et al.*, 1997). The substrate binding site of endocellulases sits in a cleft, or groove, compared to the tunnel-enclosed binding site typical of exo-cellulases. A cleft-shaped binding site allows an enzyme to bind the substrate chain anywhere along its length, while a tunnel should force the substrate chain to enter in an end-wise fashion to reach the catalytic site (Rouvinen *et al.*, 1990). The manner of binding determines the site of chain hydrolysis, thus an endo enzyme can clip a chain anywhere along its length, while an exo is restricted to hydrolysis at the terminus.

The rigid categorization of cellulases as either exo or endo is being questioned (Teeri, 1997; Goyal *et al.*, 1991). The classification of cellulytic action is largely drawn from activities on certain substrates such as soluble, substituted celluloses, microcrystalline cellulose, as well as and enzyme's effect on the substrate's degree of polymerization. Those characterizations are by nature imprecise yet subsequently, as the three dimensional structures of the enzymes were elucidated, the classifications were validated when the cleft- and tunnel-shaped substrate binding sites of EGI (Kleywegt *et al.*, 1997), CBH1

(Divne *et al.*, 1994) and CBH2 (Rouvinen, 1990) were made known, Clear proof of the importance of the active site topology was given when deletion of one of the active site surface loops resulted in increased "endolytic" character (Meinke *et al.*, 1995).

The tunnels of CBH1 and CBH2 are formed by loops which fold over otherwise cleft-shaped binding sites. The behavior of the loops during enzyme action is not fully understood. It has been suggested that the loops forming the roof of the tunnels may, however infrequently, open and allow an otherwise exo-enzyme to have endo activity (Warren, 1996). The frequency with which an "exo" enzyme catalyzed an "endo" hydrolysis may be a function of the flexibility of the loops enclosing the active site. Consequently, if the number or mobility of the loops forming the roof of binding site tunnels were different, it might be expected that the enzymes would have different levels of endo activity.

To date, there is no good explanation for exo-exo synergism. The CBH1-CBH2 synergism described in the literature (Irwin *et al.*, 1993; Nidetzky *et al.*, 1995) could be a result of a minor amount of endo activity from one of the enzymes. Such differences in specificity are easily visualized, given the dissimilarities between CBH1 and CBH2 with respect to direction of attack. Exo-exo synergism might also result from the potentially disruptive action of the cellulose binding domain. Such activity has been reported for bacterial cellulose binding domains (Din *et al.*, 1991), however the binding domains of *T. reesei* cellulases have been shown to differ from fungal cellulases and probably serve the same function in different ways. (Tomme *et al.*, 1995). In the case of either exo-exo or endo-exo synergism, it seems intuitive that a requirement would be that enzymes act at the same loci, either simultaneously or sequentially. A natural consequence of this requirement is that, as cellulytic enzymes become more distant from one another on the substrate surface, *i.e.* as substrate concentrations are increased and the concentration of crude cellulase is held constant, an apparent substrate inhibition occurs. Such a phenomenon was described previously (Huang and Penner, 1991). No such effect was observed for a purified CBH1 preparation.

A similar investigation was undertaken with purified CBH2 and the results are discussed here. The primary objective of this study was to determine if CBH2 behaved similarly to CBH1 at low enzyme:substrate ratios. A secondary objective was to explain any differences observed, both in terms of what is known about the enzymes' structures and by means of an individual-based model. Differences in behavior and their impact on exo-exo synergism rationales are also examined.

6.3 Materials and Methods

6.3.1 Cellulase purification.

CBH1 and CBH2 were purified from commercial preparation of crude *Trichoderma reesei* cellulase (Spezyme-CP, Menlo Park, California) using DEAE, affinity chromatography, and hydrophobic interaction chromatography as previously described (Piyachomkwan and Penner, 1998) except the CBH was run through the affinity column twice, once with and once without 1 M NaCl. The CBH2 preparation showed one band on SDS-PAGE and no activity on *p*-nitrophenyl cellobioside.

Additional CBH2 samples were also generously provided by scientists from VTT (Espoo, Finland) and the National Renewable Energy Laboratory (NREL) in Golden, Colorado. The NREL CBH2 was also purified by affinity chromatography, alhough the affinity ligand differed slightly. The purity of the NREL preparation was assessed with monoclonal antibodies and found not to contain any of the two major endoglucanases. The VTT CBH2 was purified from a recombinant *Trichoderma reesei* strain which had the genes encoding for the major endoglucanse s (EGI and EGII) eliminated (Koivula *et al.*, 1996). The VTT preparation was also purified using affinity chromatography.

6.3.2 Bacterial microcrystalline cellulose preparation

Bacterial Microcrystalline Cellulose (BMCC) was prepared from a *Acetobacter xylinium* culture as described by Hestrin (Hestrin, 1963) and Gilkes *et al.* (Gilkes *et al.*, 1992).

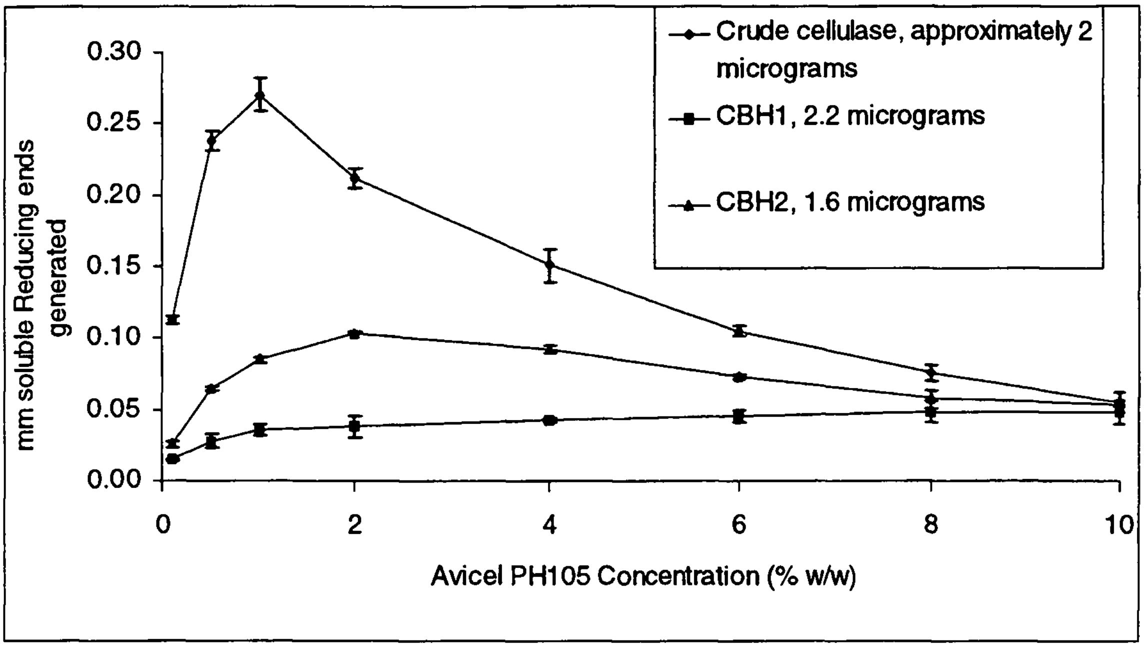
6.3.3 Enzymatic hydrolysis

A 2.0 ml-capacity microfuge tube containing Avicel PH105 suspended in 50 mM, pH 5.0 NaAc buffer (total volume, 0.9 ml) was thermostatted at 50°C. Enzyme (0.1 ml) in the same buffer was added. The vials were then rotated with inversion in a 50°C incubator for two hours. The reaction was terminated by raising the pH to 12 and placing the samples on ice. The samples were centrifuged and put through a 0.22 μm filter, and the filtrate assayed for reducing sugars by the BCA method (Doner and Irwin, 1992). Enzyme concentrations were estimated using A280 values and molar extinction coefficients of 75000 and 73000 M^{-1} cm^{-1} for CBH1 and CBH2, respectively (Tomme *et al.*, 1988)

6.4 Results and Discussion

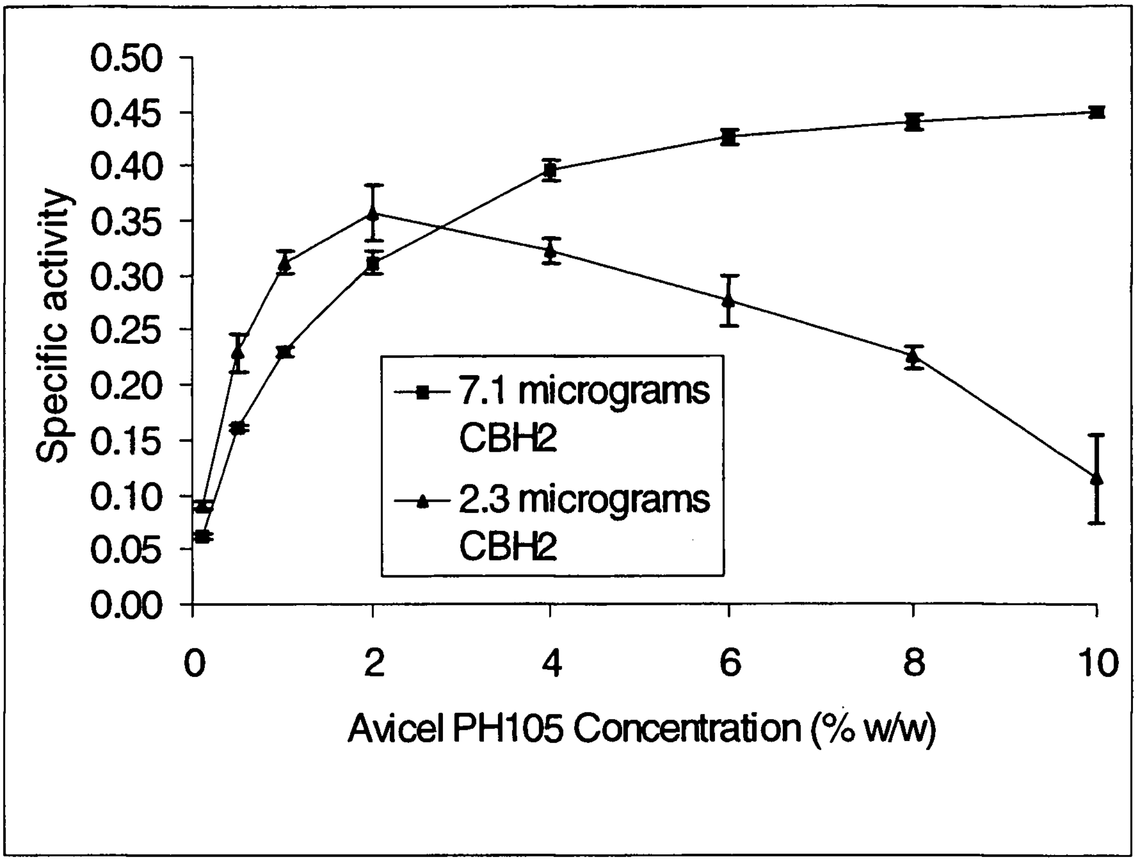
The results of the microcrystalline hydrolysis assays using crude cellulase, purified CBH1 and purified CBH2 (both prepared by our laboratory) are shown in Figure 6.1. Both crude cellulase and CBH2 showed significant apparent substrate inhibition. The inhibition was approximately 70% for crude cellulase and 40% for CBH2. Only CBH1 did not show an apparent substrate inhibition, even when the test was conducted at low enough enzyme concentrations that the product could no longer be detected by the sensitive BCA assay (data not shown). The apparent substrate inhibition is directly related to the amount of enzyme used, *i.e.* if more enzyme is added , the activity will plateau. This phenomenon is demonstrated in Figure 6.2, which shows the results obtained using different amounts of CBH2.

Figure 6.1. Reaction velocity vs. substrate concentration for *Trichoderma reesei* crude cellulase, purified CBH1 and purified CBH2.



In order to determine if the CBH2 results were an artifact of the purification methods, the same assays were conducted using CBH2 generously provided by researchers at the National Renewable Energy Laboratory (Golden, CO) and VTT (Espoo, Finland). See the Materials and Methods section for details. The data obtained using the three different CBH2 preparations are shown in Figure 6.3. All three enzymes showed similar apparent substrate inhibition. The VTT and OSU preparations showed a roughly 40% drop in activity, while the NREL sample lost nearly 55% of its peak activity. The concentration of the VTT and OSU preparations was nearly identical, while the NREL sample was approximately 20% less.

Figure 6.2. Reaction velocity vs. substrate concentration for purified CBH2 from *Trichoderma reesei* at different enzyme loads.



The question of whether the results were an artifact caused by Avicel was addressed by conducting the same assay using bacterial micro-crystalline cellulose (BMCC) as a substrate. The nature of BMCC does not allow the same amount of material to be used, and consequently, lower enzyme concentrations were employed. The results, showing clear apparent substrate inhibition, are shown in Figure 6.4. The loss of activity was approximately 30% relative to the maximum. Thus, the apparent substrate inhibition does not seem to be an artifact of Avicel, but appears to be a characteristic of CBH2, since no such effect was observed for CBH1.

Figure 6.3. Reaction velocity vs. substrate concentration for purified *Trichoderma reesei* CBH2 from three different laboratories. OSU, Oregon State University; VTT, VTT, Espoo, Finland; NREL, National Renewable Energy Laboratory, Golden Colorado.

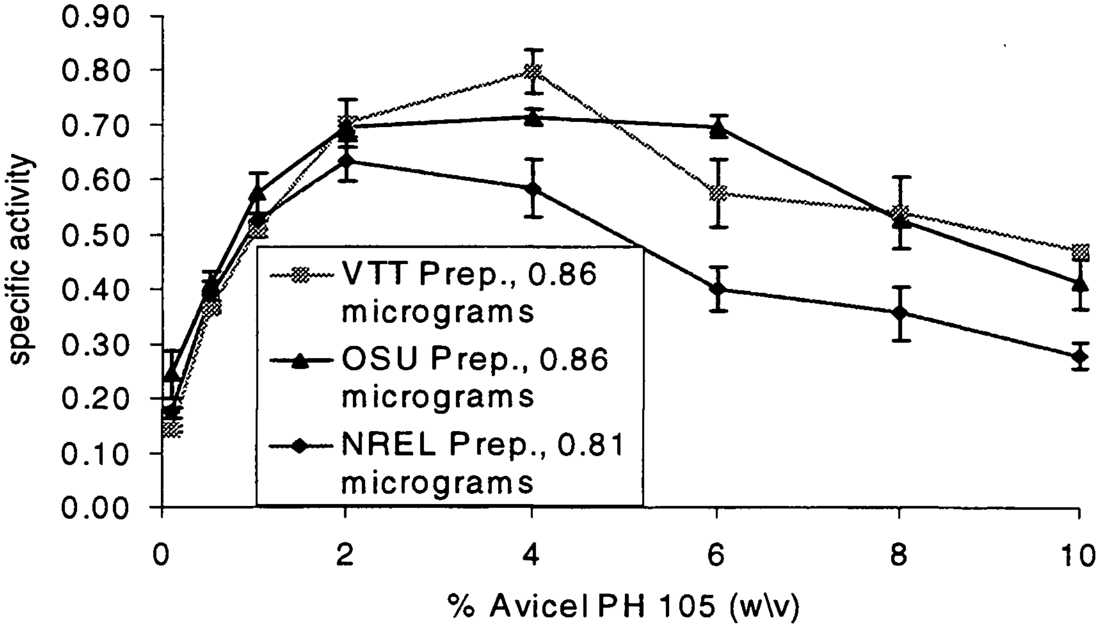
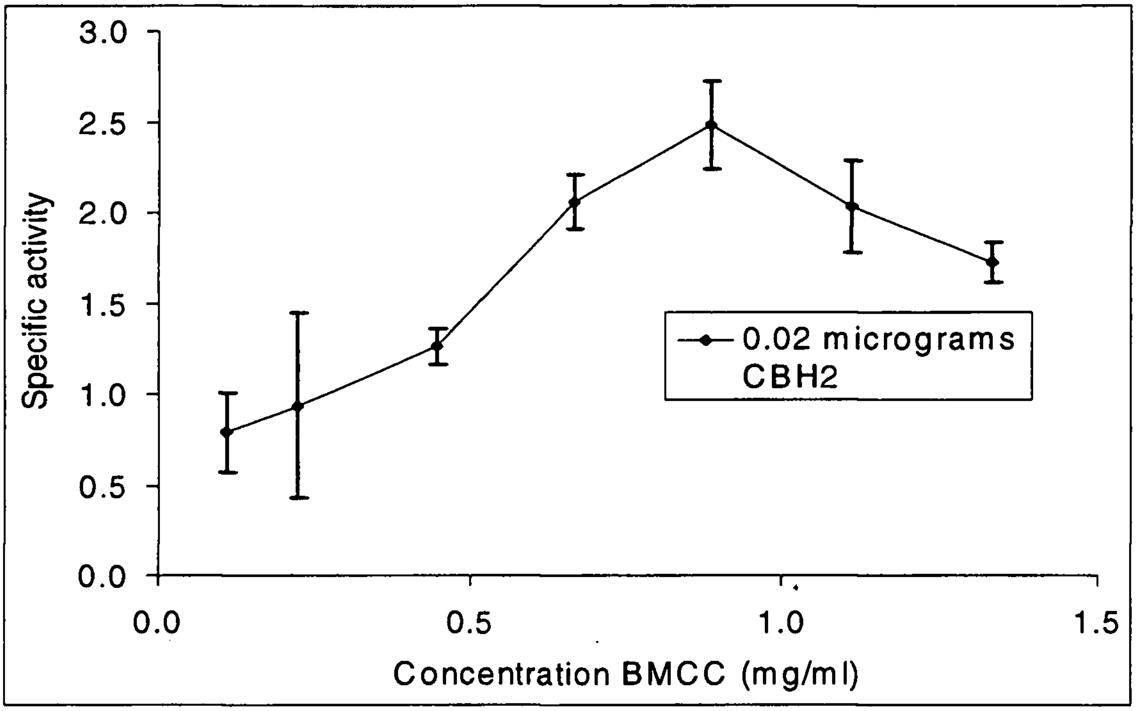


Figure 6.4. Reaction velocity vs. substrate concentration for purified *Trichoderma reesei* CBH2 (OSU prep) on bacterial microcrystalline cellulose.



When it became evident that a seemingly homogenous enzyme preparation of CBH2 gave an apparent substrate inhibition while CBH1 did not, potential rationales were sought. The first and most obvious is that there was an endo contaminant in the CBH2 preparations. Cellulases are notoriously difficult to isolate from one another (Reinkainen *et al.*, 1995). However, after observing the same behavior with preparations that had been put through an affinity column twice using vastly different ionic strengths (each time holding the enzyme on the column for more than 10 bed volumes) as well as with preparations which were pure according to antibody tests and one prepared from a *T.*

reesei strain devoid of the genes encoding for the EG2, alternative explanations were examined.

It has been suggested that instead of classifying cellulytic enzymes as either exo or endo, a more appropriate description of the cellulases might be a continuum ranging from exo to endo (Teeri, 1997). One possible rationale for the susceptibility of CBH2 to apparent substrate inhibition was the presence of two activities in the same enzyme, *i.e.* one which was primarily an exo enzyme but was able to make an endo cut on rare occasion. An individual-based model was created to explore this issue (Fenske *et al.*, 1998).

The cellulose polysaccharide surface was modeled by a two-dimensional matrix. In the matrix, 1 represented an internal chain bond, and 0 represented a chain end, of which each chain had one. Substrate saturation with enzyme was simulated by limiting the number of enzyme molecules per chain. Enzyme binding was modeled by distributing the appropriate number of enzyme molecules across the matrix. A set fraction was placed on randomly selected chain ends, while the remainder placed at random across the entire matrix. Then, activity of the enzymes was modeled as follows.

Separate probabilities of endo and exo hydrolysis were defined. If the enzyme was on a chain end, a number randomly selected from a normal distribution was compared to the probability of an exo cut occuring. If the number chosen exceeded the exo cut probablility, the enzyme moved one cell to the right, and the product total was incremented. This process continued until the probability was not met, and then the next enzyme was evaluated. If the enzyme was on an internal chain linkage, the random number was compared to the likelihood that an endo-cut would occur. If the endo cut was deemed to occur, a chain break was created, but nothing was added to the product. Subsequently the enzyme was handled as if it started at a chain break, *i.e.* it proceeded along the chain adding to the product until no exo cut occured. In summary, exo cuts created no new chain breaks, while endo cuts created new chain breaks. After all the enzymes were evaluated, time was incremented and the enzymes moved in the x and y dimensions. For each enzyme, the distance traveled in each dimension was chosen separately, and no assumption regarding the direction of movement was made.

The model predicted that an enzyme with no endo activity would not show apparent substrate inhibition. Similarly, the model predicted an enzyme with dual activity would show substrate inhibition. The model predictions indicate that an apparent substrate inhibition is possible with a homogenous enzyme with dual activity, and that a contaminating enzyme is not necessarily present if such kinetic behaivior is observed. Of course, the possibility of endo contaminants in the CBH2 preparations, cannot be completely ignored. However, the structures of the catalytic cores of CBH1 and CBH2 suggest that there may be reason to believe them to have different endo-exo properties.

The binding site tunnel of CBH1, measuring approximately 50 Å, is much longer than the 20 Å tunnel of CBH2 (Divne et al., 1998; Rouvinen et al., 1994). As might be expected, the CBH1 binding site has more loci for glucose moieties, ten compared to six for CBH2. The lower number of binding sites makes it likely that CBH2 is more likely to dissociate from the substrate surface, *i.e.* show a lower processivity than CBH1. The impact of the catalytic core on the binding of intact CBH1 has been shown by binding studies using purified cellulose binding domain (Linder and Teeri, 1996). Binding domains alone show readily reversible binding, while intact enzymes show tight binding (Linder and Teeri, 1996).

In addition to the differences in number of substrate binding sites, CBH1 has four loops forming the "roof" of the catalytic site tunnel, while CBH2 has two. It is not known if the loops possess mobility, and researchers have never observed a tunnel loop out of position. However, it does seem probable that the fewer loops forming the roof, the more likely it is that one may fold out, thus exposing the catalytic sites to the substrate.

In short, the result of a shorter binding site is more mobility, and the result of fewer, potentially more mobile loops is greater inherent endo activity. An individual-based model predicted that one way for substrate inhibition to occur was if endo activity were present. Thus, one explanation for the apparent substrate inhibition observed for CBH2 appears to be the presence of native endo activity. Native endo activity on the part of CBH2 would also explain the "exo-exo" synergism between CBH1 and CBH2. This type of synergism would also exist for enzymes with the same chain-end specificity working at the same loci in sequential order, so long as one of the enzymes has the ability to make endo cuts.

CHAPTER 7

Summary

This dissertation has focused on two different aspects of lignocellulosic biomass conversion. The first two papers dealt with hemicellulosic prehydrolysate fermentation by *Pichia stipitis*. From the results of those two studies, it is apparent that poplar-derived hydrolysates are significantly more toxic to *Pichia* than are herbaceous-derived prehydrolysates. Differences in concentration of known and suspected inhibitors were shown to account for the discrepancy in fermentability. This is valuable information to consider when considering potential feedstocks for commercialization of a biomass-to-ethanol process.

The third and fourth papers dealt with a different facet of lignocellulosic conversion, namely the enzymatic depolymerization of cellulose. An individual-based model of a soluble enzyme\insoluble substrate system was created and the impact of native endo activity on an otherwise exo-active enzyme was explored. The model results indicated that enzymes with different activity profiles should show different substrate velocity profiles. The fourth paper reported the different substrate\velocity profiles of *Trichoderma reesei* CBH1 and CBH2 on microcrystalline cellulose. The behavior of the enzymes indicated that CBH2 had native endo activity, while CBH1 did not.

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Appendix

Model of Soluble Enzyme-Insoluble Substrate System
(written for C\C++ environment)

```
#include <stdio.h>
#include <math.h>
#include <time.h>
#include <stdlib.h>


/*--Constants--*/

#define numChains           2
#define DP                  20            /*    DP is the degree of polymerization                       */
#define RE                  numChains     /*    RE are the number of reducing ends (chain breaks)        */

#define probNoExo           20            /*    rnd # must be larger than probNoExo for exo cut to occur  */
#define probNoEndo          99            /*    rnd # must be larger than probNoExo for endo cut to occur */

#define END                 100           /*    number of time steps per run                             */
#define numRuns             2000          /*    number of runs per execution                             */

#define CBHconc             40            /*    CBHconc must be an even number                           */
#define SitesAvail          numChains*4   /*    on average, 4 CBH per chain allowed                      */
#define ProbLimit           100           /*    rnd number 0->ProbLimit determines if next cut occurs    */
#define maxDist             4             /*    maxDist-1 is the max distance in any direction           */
                                          /*    CBH moves in one time step                               */

#define fracCBHrandBound 0.6              /*    # of CBH bound at random sites if CBH is non-saturating  */

#define rndBoundCBH         SitesAvail*fracCBHrandBound

                                          /*    # of CBH bound at random sites if CBH is saturating      */
/*--Function Prototypes--*/

int Travel (  int CBH_RowLoc [],  int CBH_ColLoc [CBHconc], int CBHads);

int sortFunction(const void *intOne, const void *intTwo);
```

```
/*--Main Program--*/

int main(void)
{
    FILE *AnOutPutFile;

    static short int cellulose  [numChains+2][DP+2];                    /*defines cellulose matrix */
    int CBH_RowLoc [CBHconc];
    int CBH_ColLoc [CBHconc];
    int rowSorter  [numChains];
    int row      [numChains];
    int tracker       [numChains];

    int randomRow=0, randomColumn=0;

    short int r=0, c=0,n=0,m=0, i=0, x=0, y=0, glucose=0, prob=0, numExoCuts=0,
    moverCounter=0, timeStep=0, run=0, numEndoCuts=0, numEndoExoCuts=0,
    REcounter=0,
    CBHads=0 ;

    srand(time(NULL));

    AnOutPutFile = fopen ( "AnOutPutFile", "w");

for  (run=0; run < numRuns; ++run)                                      /*    beginning of run loop       */
    {
    r=0 ;
    c=0 ;
    x=0 ;
    randomRow=0, randomColumn=0 ;
    glucose=0 ;
    numExoCuts=0 ;
    numEndoCuts=0 ;
    numEndoExoCuts=0 ;
    moverCounter=0, timeStep=0;
```

```
REcounter=0;
CBHads =0;

for (r=0; r<numChains; r++)                    /* generates cellulose matrix              */
    {for (c=0; c<DP; c++)
        {if ( (r >=numChains) || (c>= DP) )
            printf ("\n  cellulose [][] overwrite!");
        cellulose [r] [c] = 1 ;
        }
    }

while ( x< RE)                                 /*  generates chain breaks at random       */
    {                                          /*  places, one on EACH chain              */
    randomColumn= rand() %DP;

    if (cellulose [x][randomColumn] == 1)
        {
        if (x >= numChains || randomColumn>= DP)            /*  alarm if array overwrite */
            printf ("\n overwrite attempt in RE GEN!");
        else
            {cellulose [x][randomColumn] = 0;
            x++;
            }
        }
    }

if (CBHconc < SitesAvail)                         /* CBH locator for cases where CBHconc is less
                                                      than SitesAvail  i.e. non-saturating conditions        */
    {
    x=0;

    while ( x < CBHconc*fracCBHrandBound )               /*   places  part of CBH in random
                                                              locations on matrix                   */
        {randomRow = rand() %numChains;
        randomColumn= rand() %DP;
```

```
            if (randomRow >= numChains || randomColumn >= DP)       /*  alarm if array overwrite      */
             printf("CBH placed outside of cellulose matrix \n");

        if (x >= CBHconc)                                           /*  alarm if array overwrite         */
            printf("\n overwrite of CBHRowColLocs[] random x:%d", x);

        CBH_RowLoc [x] = randomRow;
        CBH_ColLoc [x] = randomColumn;

        CBHads++;
        x++;
        }

    x=CBHconc*fracCBHrandBound;
    REcounter=0;

    for (i=0; i<numChains; i++)                         /*  orders rows randomly for placement of CBH     */
    {tracker[i]= i;
    rowSorter[i]=i;
    rowSorter[i] = rand() %100000;
    tracker[i] = rowSorter[i];
    }

qsort((void*) rowSorter, numChains, sizeof (rowSorter[0]), sortFunction);

for (n=0; n<numChains; n++)
    {
    for (m=0; m<numChains; m++)
        {if (rowSorter[n] == tracker[m])
            {row[n]=m;
            }
        }                                                   /*  end of row sorting               */
    }

    i=0;
```

```
    n=0;

    while ( ( x< CBHconc) && ( REcounter< RE ))          /*  places remainder of CBH on chain breaks          */
        {
        if (x >= CBHconc)
            printf("\n overwrite of CBHRowColLocs[] endbound, x:%d",x);

        for (y=0; y<DP; ++y)
            {
                if (cellulose [row[n]] [y] ==0)
                    {
                    CBH_RowLoc [x] = row[n];
                    CBH_ColLoc [x] = y;
                    }
            }
        x++;
        CBHads++;
        REcounter++;
        n++;

        }
    }                       /* end CBH Locator for CBHconc< SitesAvail                              */

else                            /* CBH Locator for cases where CBHconc is greater than SitesAvail       */
    {
    x=0;

    while ( x < rndBoundCBH )               /* places part of CBH in random locations on matrix     */
        {randomRow = rand() %numChains;
        randomColumn= rand() %DP;

        if (randomRow >= numChains || randomColumn >= DP)          /*  alarm if array overwrite          */
          printf("CBH placed outside of cellulose matrix \n");

        if (x >= CBHconc)                                    /*  alarm if array overwrite                */
```

```
        printf("\n overwrite of CBHRowColLocs[] random x:%d", x);

    CBH_RowLoc [x] = randomRow;
    CBH_ColLoc [x] = randomColumn;
    x++;
    CBHads++;
    }

REcounter=0;
x = rndBoundCBH;
r=0;
while (  (x < CBHconc) && ( REcounter< RE ) )                 /*   places part of CBH on chain breaks          */
    {
    if (randomRow >= numChains || randomColumn >= DP)
        printf("CBH placed outside of cellulose matrix \n");

    if (x >= CBHconc)
        printf("\n overwrite of CBHRowColLocs[] endbound, x:%d",x);

    for (y=0; y<DP; ++y)
        {
            if (cellulose [r] [y] ==0)
                {
                CBH_RowLoc [x] = r;
                CBH_ColLoc [x] = y;
                }
        }
    x++;
    r++;
    REcounter++;
    CBHads++;
    }
while (CBHads <SitesAvail)                    /*       places remainder of CBH at random locations          */
    {randomRow = rand() %numChains;
    randomColumn= rand() %DP;
```

```
        if (randomRow >= numChains || randomColumn >= DP)       /*  alarm if array overwrite        */
          printf("CBH placed outside of cellulose matrix \n");

        if (x >= CBHconc)                                       /*  alarm if array overwrite        */
            printf("\n overwrite of CBHRowColLocs[] random x:%d", x);

        CBH_RowLoc [x] = randomRow;
        CBH_ColLoc [x] = randomColumn;
        x++;
        CBHads++;
        }
    }                               /*  End CBH locator for CBHconc >SitesAvail     */

while (timeStep < END)              /*  beginning of time step loop                 */
    {
    x=0;
    while ( x< CBHads)              /*  beginning of CBHconc loop                   */
        {
        prob = rand() %ProbLimit;

        if (x >= CBHconc)
            {                                   /*  warning for overstepping bounds     */
            printf(" \n x too high in start of timeStep loop");

            }
        /*------------beginning exo case           --------------------------------------*/
        if (cellulose [CBH_RowLoc [x]] [CBH_ColLoc [x]] == 0 && prob >= probNoExo)
            {
            moverCounter = 0;

            while ( prob >= probNoExo && moverCounter <DP )
                {
                if (cellulose [CBH_RowLoc [x]] [CBH_ColLoc [x]] == 1)
                    {
```

```
            if ( (CBH_RowLoc [x] >= numChains) || (CBH_ColLoc [x] >=DP) )
                {printf("\n overwrite cellulose in Exo cutter x:%d",x);

                }
            cellulose [CBH_RowLoc [x]] [CBH_ColLoc [x]] = 1;/* Exo Cut          */
            glucose = glucose +1;
            numExoCuts = numExoCuts +1;
            }

        else
            ;

        if ( CBH_ColLoc [x] < (DP-1) )          /* moves CBH[x] after Exo Cut      */
            CBH_ColLoc [x] = CBH_ColLoc [x] +1;
        else
            CBH_ColLoc [x] = 0;

        prob = rand() %ProbLimit;
        moverCounter++;
        }                                      /* end Exo Cut loop                */
    }                                          /* end Exo While Loop              */

/*--------- beginning endo case---------------------------------------------*/
if  (cellulose [CBH_RowLoc [x]] [CBH_ColLoc [x]] == 1 && prob >= probNoEndo)
    {
    if  ( (CBH_RowLoc [x] >= numChains) || (CBH_ColLoc [x] >=DP) )
            {printf("\n overwrite cellulose in Endo cutter, x:%d",x);

            }
    cellulose [CBH_RowLoc [x]] [CBH_ColLoc [x]] = 0;/* Endo Cut            */
    glucose = glucose +1 ;
    numEndoCuts = numEndoCuts+1  ;
    prob = rand() %ProbLimit;

    moverCounter=0;
```

```
            while ( prob >= probNoExo && moverCounter <DP )
                {
                if (cellulose [CBH_RowLoc [x]] [CBH_ColLoc [x]] == 1)
                    {
                    if  ( (CBH_RowLoc [x] >= numChains) || (CBH_ColLoc [x] >=DP) )
                        {printf("\n overwrite cellulose in EndoExo cutter, x:%d",x);

                        }
                    cellulose [CBH_RowLoc [x]] [CBH_ColLoc [x]] = 1;          /* EndoExoCut              */
                    glucose = glucose +1;
                    numEndoExoCuts = numEndoExoCuts +1;
                    }

                else
                    ;

                if ( CBH_ColLoc [x] < (DP-1) )            /* moves CBH[x] after EndoExo Cut          */
                    CBH_ColLoc [x] = CBH_ColLoc [x] +1;
                else
                    CBH_ColLoc [x] = 0;

                prob = rand() %ProbLimit;
                moverCounter++;
                }                                    /* end ENDO Exo loop                   */

            }                                        /* end Endo loop                          */

    if (x < SitesAvail -1 )             /*   keeps from counting  CBH greater than should be adsorbed        */
        x++;
    else
        x=CBHads+1;
    }                                                /* end CBHads while Loop               */

Travel ( CBH_RowLoc, CBH_ColLoc, CBHads);
```

```
    timeStep++;

    }                                               /*   end While (timeStep < END loop              */


    fprintf(AnOutPutFile," \n run# %d, glucose:%d, numExoCuts:%d, numEndoCuts:%d,numEndoExoCuts:%d ",
    run,  glucose, numExoCuts, numEndoCuts, numEndoExoCuts);

    }                                               /* end for (run < numRuns) loop           */
fclose (AnOutPutFile);
return 0;

}                                                   /*   end Main()                           */

 int Travel (  int CBH_RowLoc [],  int CBH_ColLoc [CBHconc], int CBHads)
    {

    int y=0, z=0, numMoves=0, n=0;
    int prob = 0;

    for (y=0; y < CBHads; ++y)            /*   determines distance to be moved in row direction       */
        {
        n= rand() %maxDist;

        if (n==0)
            numMoves =0;
        if (n==1)
            numMoves =1;
        if (n==2)
            numMoves =2;
        if (n==3)
            numMoves =3;

        prob = rand() %10;
```

```
for (z=0; z<numMoves; ++z)
    {

                                                        /*      row locator                         */
    if (prob >4 && CBH_RowLoc[y] < (numChains -1) )
        CBH_RowLoc[y] = CBH_RowLoc[y] +1;
    else
        if (prob >4 && CBH_RowLoc[y] == (numChains -1) )   /*  keeps with limits of matrix    */
            CBH_RowLoc[y] = 0 ;

    if    (prob <= 4 && CBH_RowLoc[y] > 0 )
        CBH_RowLoc[y] = CBH_RowLoc[y] -1;
    else
        if (prob <=4 && CBH_RowLoc[y] == 0 )          /*      keeps with limits of matrix    */
            CBH_RowLoc[y] = (numChains -1);
    }

n= rand() %maxDist;

if (n==0)
    numMoves =0;        /*      determines distance to be moved in column direction        */
if (n==1)
    numMoves =1;
if (n==2)
    numMoves =2;
if (n==3)
    numMoves =3;

prob = rand() %10;

for (z=0; z<numMoves; ++z)
    {


    if (prob >4 && CBH_ColLoc[y] < (DP -1) )           /*  Column locator                      */
```

```
                        CBH_ColLoc[y] = CBH_ColLoc[y] +1;
                    else
                        if (prob >4 && CBH_ColLoc[y] == (DP -1) )
                            CBH_ColLoc[y] =0;

                    if   (prob <= 4 && CBH_ColLoc[y] > 0 )
                        CBH_ColLoc[y] = CBH_ColLoc[y] -1;          /*   keeps with in limits of matrix                */
                    else
                        if (prob <=4 && CBH_ColLoc[y] == 0 )         /*   keeps within limits of matrix            */
                            CBH_ColLoc[y] = (DP -1);
                    }
                }                                                    /*   end of the for CBHconc Travel() loop      */

        return 1;
            }

        int sortFunction(const void *a, const void *b)          /*   sorts arrray of ints from low to high           */
            {
            int intOne = *( (int*)a);
            int intTwo = *( (int*)b);

            if (intOne <intTwo)
                return -1;

            if (intOne == intTwo)
                return 0;

            return 1;
            }
```