DISSERTATION

# ALKALINE/PERACETIC ACID AS A PRETREATMENT OF LIGNOCELLULOSIC BIOMASS FOR ETHANOL FUEL PRODUCTION

Submitted by

Lincoln Cambraia Teixeira

Department of Forest Sciences

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY LINCOLN CAMBRAIA TEIXEIRA ENTITLED ALKALINE/PERACETIC ACID AS A PRETREATMENT OF LIGNOCELLULOSIC BIOMASS FOR ETHANOL FUEL PRODUCTION BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Co-adviser

Adviser

Department Head

ABSTRACT OF DISSERTATION

ALKALINE/PERACETIC ACID AS A PRETREATMENT OF LIGNOCELLULOSIC BIOMASS FOR ETHANOL FUEL PRODUCTION

Peracetic acid is a lignin oxidation pretreatment with low energy input by which biomass can be treated in a silo type system for improving enzymatic digestibility of lignocellulosic materials for ethanol production. Experimentally, ground hybrid poplar wood and sugar cane bagasse are placed in plastic bags and a peracetic acid solution is added to the biomass in different concentrations based on oven-dry biomass. The ratio of solution to biomass is 6:1; after initial mixing of the resulting paste, a seven-day storage period at about 20 °C is used in this study.

As a complementary method, a series of pre-pretreatments using stoichiometric amounts of sodium hydroxide and ammonium hydroxide based on 4-methyl-glucuronic acid and acetyl content in the biomass is been performed before addition of peracetic acid. The alkaline solutions are added to the biomass in a ratio of 14:1 solution to biomass; the slurry is mixed for 24 hours at ambient temperature. The above procedures give high xylan content substrates. Consequently, xylanase/beta-glucosidase combinations are more effective than cellulase preparations in hydrolyzing these materials. The pretreatment effectiveness is evaluated using standard enzymatic hydrolysis and simultaneous saccharification and cofermentation (SSCF) procedures.

Hybrid poplar wood pretreated with 15 and 21% peracetic acid based on oven-dry weight of wood gives glucan conversion yields of 76.5 and 98.3%, respectively. Sugar cane bagasse pretreated with the same loadings gives corresponding yields of 85.9 and 93.1%. Raw wood and raw bagasse give corresponding yields of 6.8 and 28.8%, respectively. The combined 6% NaOH/15% peracetic acid pretreatments increase the glucan conversion yields from 76.5 to 100.0% for hybrid poplar wood and from 85.9 to 97.6% for sugar cane bagasse. Respective ethanol yields of 92.8 and 91.9% are obtained from 6% NaOH/15% peracetic acid pretreated materials using recombinant *Zymomonas mobilis* CP4/pZB5.

Peracetic acid pretreatment improves enzymatic digestibility of hybrid poplar wood and sugar cane bagasse. Based on reduction of acetyl groups in the two lignocellulosic materials, alkaline pre-pretreatments are helpful in reducing peracetic acid requirements in the pretreatment and consequently diminishing growth inhibition of the bacteria that was observed using higher peracetic acid loadings.

Lincoln Cambraia Teixeira
Department of Forest Sciences
Colorado State University
Fort Collins, CO 80523
Fall 1998

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# CHAPTER 1

# INTRODUCTION

The use of biomass (lignocellulosic residues from farm, forest and urban areas) has been a promising research field. For many years a production of fermentation-based commodities such as ethanol fuel, acetone-butanol, cell mass protein, and by-products from lactic acid chemistry has been studied. Two important lignocellulosic materials, hybrid poplar wood and sugar cane bagasse, are often cited in the literature as promising sources. Requirements such as, availability and production costs make their use viable. Hybrid poplar is recognized as an alternative woody crop considering its high productivity, 18 to 26 dry Mg $ha^{-1}$ $yr^{-1}$, in Pacific Northwest region (Wright, 1994) and constitutes an important biomass source to support large-scale production of fuels (Wyman, 1996). Sugar cane bagasse, a residue from sugar cane industry, is available at the sugar mill site at no additional cost because harvesting, transportation and storage costs are borne by the sugar production. In addition, Prine and Woodard (1993) have estimated the oven-dry biomass yield for perennial tall grasses, including sugar cane, varying from 20 to 45 Mg $ha^{-1}$ $yr^{-1}$ in the cooler subtropical to mild temperature locations to over 60 Mg $ha^{-1}$ $yr^{-1}$ in the lower portion of the Florida peninsula. For sugar cane grown on phosphoric clay, Stricker et al. (1993) have reported yields of fresh biomass (moisture content of 73%) varying from 137.6 to 198.6 Mg $ha^{-1}$ $yr^{-1}$ in central Florida.

Based on environmental reasons ethanol, a clean-burning liquid fuel, constitutes

the most important approach for using lignocellulosic residues. Ethanol is a better fuel than gasoline because of its excellent physical-chemistry characteristics. Adding 10 vol percent ethanol to gasoline increases octane number, improves engine efficiencies by excellent anti-knock properties, and oxygenates gasoline (Bailey, 1996).

Since older technologies such as acid hydrolysis for production of sugars followed by fermentation to ethanol are not considered anymore because of high losses in the overall process, enzymatic hydrolysis, despite its complexity, appears as an alternative environmentally friendly approach.

Biomass, by simple definition, is composed basically of cellulose microfibrils surrounded by a mix of complex polymeric carbohydrates including mostly xylan and glucomannan types that are to some extent linked with another polymeric structure called lignin. Extractives and mineral components are present in small amounts. This complex matrix makes cellulose inaccessible to hydrolytic enzymes. Several approaches have been proposed for improving enzymatic digestibility of lignocellulosic materials. Conventional methods for making biomass more accessible to enzymes target lignin and/or hemicellulose removal (Soltes, 1989; Grohmann et al.,1985; Holtzapple and Humphrey, 1984). Prehydrolysis using steam explosion and diluted acids constitute the most studied technologies. Both methods use high temperature and pressure reactors and have added costs for fractionation procedures. The use of peracetic acid at relatively high temperatures, about 100 °C, has been studied for improving biomass digestibility (Myung and Kennely, 1992; Farid et al., 1983; Gharpuray et al., 1983; Fan et al., 1981; Toyama and Ogawa, (1975), but little work has been done at ambient temperature (Rodríguez-Vázquez, 1993; Holtzapple, 1992). Based on a low temperature pretreatment approach, a silo type system designed for using peracetic acid constitutes a new alternative for pretreating biomass. This method

does not require expensive reactors, energy input or fractionation procedures. In addition, there are no significant carbohydrate losses, furfural is not produced during the process, and lignin is oxidized during the ensiling type pretreatment.

Peracetic acid has been recognized as a powerful oxidizing agent and it is very selective towards the lignin structure. It cleaves aromatic nuclei in lignin generating dicarboxylic acids and their lactones (Lai and Sarkanen, 1968). The aliphatic propane side-chain is displaced to some extent during the oxidative process (Sakai and Kishimoto, 1966). Another important aspect that should be considered is that peracetic acid is produced by reaction of peroxides and acetic acid. Large scale production of peracetic acid, 10,000 metric ton/year, began in 1996 in Finland (Anonymous, 1996; and 1995) using acetic acid and hydrogen peroxide raw materials supplied by the Oulu plants. In the laboratory, acetic anhydride was used only for obtaining better peracetic acid yield but it is far too expensive for even pilot scale evaluation of this process (Szmant, 1989). Conveniently prepared from ethanol by aerobic fermentation, and recycled from consumption of peracetic acid in the pretreatment, acetic acid is available and inexpensive, especially in countries with strong ethanol production capabilities. Because of the worldwide expansion of hydrogen peroxide production (Szmant, 1989) and since it has been recognized as an environmentally friendly chemical (Wilson, 1994), the cost of peracetic acid will probably decrease.

The feasibility of pretreating two biomass sources, hybrid poplar and sugar cane bagasse, with peracetic acid only, and also with a prior alkaline wash are evaluated. Because these pretreated materials contain most of the original hemicellulose content, combinations of commercial enzymes are investigated in this study. The effectiveness of various pretreatment conditions is evaluated by standard enzymatic hydrolysis and simultaneous saccharification and cofermentation procedures.

# CHAPTER 2

# LITERATURE REVIEW

## CHEMICAL AND PHYSICAL CHARACTERISTICS OF BIOMASS

### Elemental composition

Biomass, defined here as a lignocellulosic material, is composed primarily of the following chemical elements, with relative percent ratio given: carbon 50%, hydrogen 6% and oxygen 44%. Nitrogen and ash are very low in woody biomass (Tsoumis, 1991; Pettersen, 1984; Rydholm, 1965). Field crop residues differ from woody biomass by a relatively higher nitrogen and ash content, especially as silicates (Jones and Semrau, 1984).

### Chemical composition

Cellulose, hemicelluloses (polyoses) and lignin are the principal fractions in whole biomass. Minor polymeric substances include starch, pectin, and condensed tannins. Low-molecular-weight substances generically called extractives are aromatic phenolic compounds, terpenes, fats, and aliphatic acids, together with, inorganic matter commonly called ash, constitute the remaining fractions of biomass (Fengel and Weneger, 1984). Table 2.1 shows the chemical composition of some lignocellulosic materials.

Table 2.1. Chemical composition of selected lignocellulosic material (dry wt. basis, %).

| Component | Typical softwood | Typical hardwood | Typical field crop residue | Refuse derived fuel |
|---|---|---|---|---|
| Cellulose | 42.0 | 43.5 | 39.0 | 45.5 |
| Hemicellulose: | | | | |
| Pentosans | 8.0 | 19.0 | 21.0 | 2.5 |
| Hexosans | 12.0 | 5.0 | 6.5 | 3.0 |
| Others * | 4.0 | 5.0 | - | 3.0 |
| Sugars and starch | Neg.** | Neg. | Neg. | 8.5 |
| Lignin | 28.0 | 21.0 | 15.0 | 10.0 |
| Extractives | 4.0 | 6.0 | 2.5 | 6.7 |
| Protein | Neg. | Neg. | 5.0 | 3.3 |
| Ash | 1.0 | 0.5 | 10.5 | 2.5 |

Source: Jones and Semrau, 1984.
* Hydrolysis products including uronic acids and acetyl groups.
** Negligible amount

Hardwoods generally have less lignin than softwoods and the hemicellulose is defined as a xylan type because of the low amount of glucomannan (Jones and Semrau, 1984; Schroeder, 1978). The fraction of hemicelluloses in typical field crop residue is much higher than in woody biomass and the cellulose content tends to be much lower (Jones and Semrau, 1984). The composition of hybrid poplar clones and sugar cane bagasse, specifically for comparison with the biomasses studied here, are presented in Table 2.2.

Cell-wall structure and chemical distribution

Knowledge of the architecture of the cell wall as well as distribution of its chemical components represents an important field of study in biomass pretreatment. Côté (1967) has proposed schematically the layering of the cell wall (Fig. 2.1) and a transmission electron micrograph of a transverse section of tamarack (*Larix laricina*) tracheids has shown layering with sufficient detail (Fig. 2.2). Anatomically the mature cell wall is formed by a thin primary wall capable of increasing its surface area when the cell develops and a thick secondary wall. The middle lamella is between primary walls and together these three layers constitute the compound middle lamela. The secondary wall is divided in three layers: S1, S2, and S3, formed by deposition of additional wall material to the inside of the primary wall. The wall layers are incapable of further surface enlargement and are classified as secondary-wall layers (Panshin and De Zeeuw, 1980). Because of its lower microfibril angle to longitudinal cell axis, greater thickness and higher cellulose content, the S2 layer is primarily responsible for the strength of the woody cell (Panshin and De Zeeuw, 1980; Wangaard, 1979).

The chemical components of the cell wall are not uniformly distributed throughout the cell wall. The first analysis of the compound middle lamella was made

Table 2.2. Chemical composition of hybrid poplar clones and sugar cane bagasse (Dry weight basis, %).

| Component | H. poplar DN-17[1] | H. poplar DN-182[1] | H. poplar DN-34[1] | H. poplar NC5260[1] | H. poplar NE-388[2] | H. poplar (*P. delt.* x *P.nigra*)[3] | Sugar cane bagasse[4] | Sugar cane bagasse[1] |
|---|---|---|---|---|---|---|---|---|
| Cellulose | 43.7 | 43.9 | 43.4 | 46.2 | 53.1 | 44.2 | 33.0* | 40.2 |
| Pentosans | 18.1** | 17.7** | 18.3** | 16.9** | 17.3 | 18.8 | 30.0 | 23.0** |
| Lignin | 23.3 | 23.5 | 23.8 | 21.3 | 26.4 | 29.6 | 29.0 | 25.2 |
| Extractives | 3.6 | 2.9 | 2.6 | 4.1 | 2.0*** | 5.4 | - | 4.4 |
| Ash | 0.9 | 0.8 | 0.7 | 0.5 | 1.2 | 1.1 | 4.0 | 4.0 |

Source: [1] Wiselogel et al, (1996), [2] Holtzapple(1981), [3] Brito (1994), and [4] Kuhad et al. (1997)
* values reported as hexosans
** xylan and arabinan were summed to make the pentosan total
*** alcohol-benzene extraction only

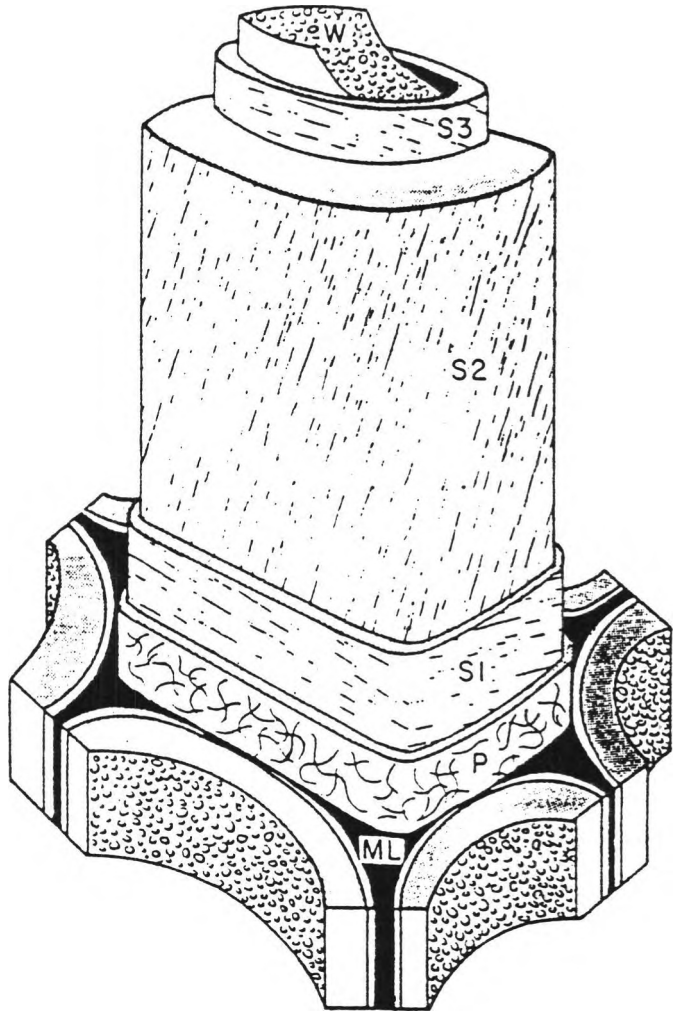



Fig. 2.1. Mature woody cell showing the middle lamela (ML), primary wall (P), layering of secondary wall (S1, S2, and S3), and warty layer (W) (Côté, 1967)

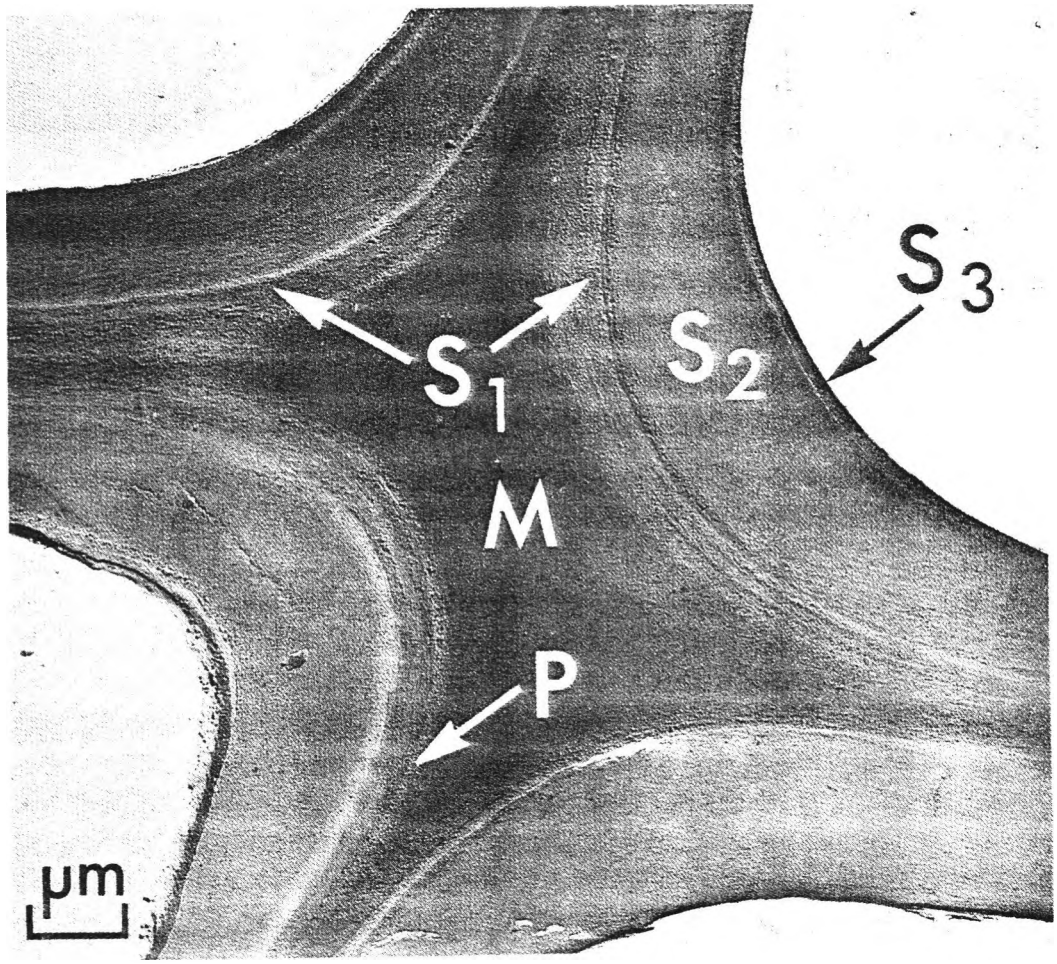



Fig. 2.2. Cross section of earlywood tracheids showing the middle lamela (M), primary wall (P), layers of secondary wall (S1, S2, and S3) ( Sjöström, 1991).

by Bailey (1936) using microdissection technique. Lignin distribution in the secondary wall has been investigated by Kutscha (1968) by ultraviolet light spectroscopy. Other methods such as chromatography, chemical analysis of isolated cells at various stages of developments, dissolution of non-lignin compounds, and fluorescence microscopy have been used for chemical analyses of the cell wall (Meier, 1957). The distribution of chemical constituents within the cell wall after compilation and inferring data is shown in Fig. 2.3.

Cellulose

Cellulose is the most important and abundant natural substance produced by living organisms (Fengel and Weneger, 1984). The chemistry of cellulose began in 1838 after Payen isolated, by use of chemicals, a uniform chemical substance from plant tissues. The elemental analysis revealed three major elements: carbon 44.4%, hydrogen 6.2% and oxygen 49.3%, that is equivalent to an empirical formula of $C_6H_{10}O_5$ which is exactly the molecular formula of glucose after losing a water molecule (Immergut, 1975; Janes, 1969). Payen later, defined cellulose as a carbohydrate polymer based on glucose and isomeric with starch, which coincides at some extent with the present-day concept. This concept was later modified and the term cellulose was employed for the more hydrolysis resistant fraction of the carbohydrates, sometimes with a prefix, indicating mode of isolation such as Cross and Bevan cellulose, alpha-celulose, etc (Rydholm, 1965). Alpha-cellulose is wood cellulose which is insoluble in strong (17.5%) sodium hydroxide solution. The fraction which is soluble in alkaline medium and precipitates with lowering the pH is called beta-cellulose. Gamma-cellulose is the designation for the fraction which remains in solution even in neutralized medium (Fengel and Weneger, 1984).

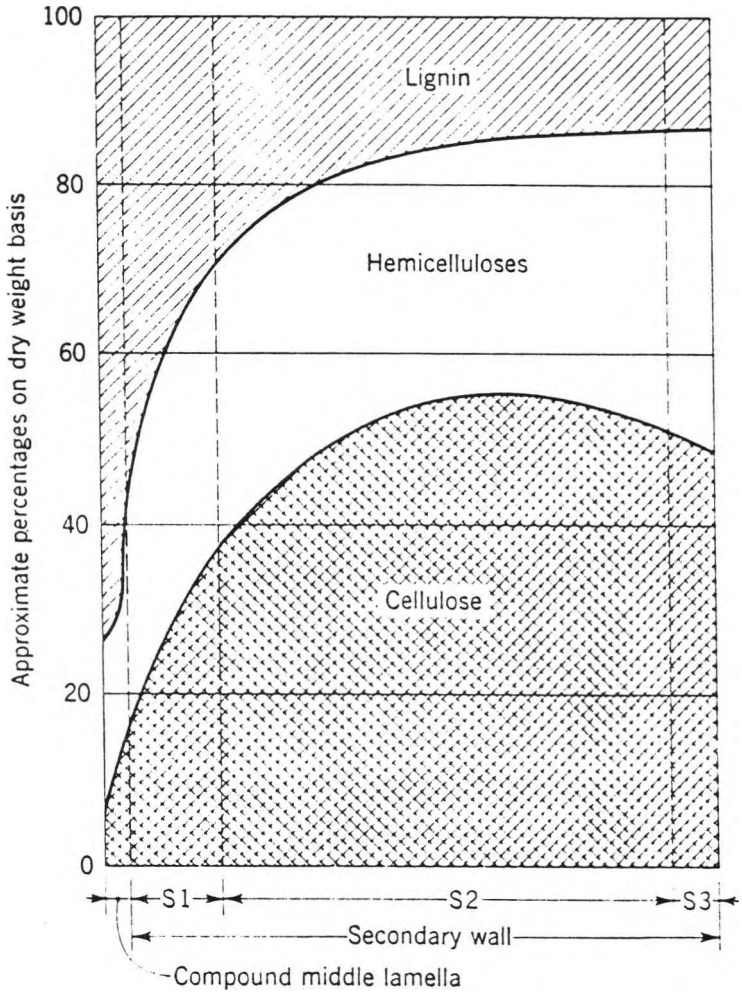



Fig. 2.3. Distribution of the major chemical constituents within the cell wall layering in softwoods (Panshin and De Zeeuw, 1980).

Cellulose is a linear macromolecule composed of (1→4)-β-D-anhydro-glucopyranose units (Fig. 2.4) and only the configuration of the C1 position is different from that of amylose (linear starch) which is made of (1→4)-α-D-anhydroglucopyranose units. The degree of polymerization of cellulose averages from 7 000 to 15 000 for woody plant cellulose (Fengel and Weneger, 1984). The strength of wood depends on these linear and moderately crystalline, cellulose molecules.

As is characteristic of all carbohydrates, the cellulose is also susceptible of chemical reactions through its hydroxyl groups, specifically the two secondary (OH at C2 and C3) and one primary (HO at C6). Ethers, esters, sulfate, nitrate, acetate, and xanthate are some of the industrial cellulose derivatives (Sjöström, 1993). The peeling reaction, cellulose hydrolysis and oxidation are important to the pulp and paper industry and to biomass conversion to sugars. The peeling reaction, under alkaline pulping, occurs at the reducing end of the cellulose chain or at any location where cellulose has been oxidized to a carbonyl or carboxyl group (McGinnis and Shafizadeh, 1991; Fengel and Weneger, 1984). The reaction is stopped by consequent formation of glucosaccharinic and glucometasaccharinic acids end monomers, and other alkali-stable end-groups (Sjöström, 1993; Fengel and Weneger, 1984). Acid, alkaline, and enzymatic hydrolysis are extensively discussed in the literature and are present in numerous chemical and enzymatic processes. Cellulose can be readily oxidized at all the functional groups, which is dependent on pH, temperature and reactants (Meller, 1960). The reactions between alkaline solutions and oxidized cellulose are very well understood. Hydrogen bonding formation is a well known characteristic of this straight polymer and some physical properties of wood such as swelling and shrinkage are related to cellulose-water affinity due to H-bonding (Sjöström, 1993; Fengel and Weneger, 1984).

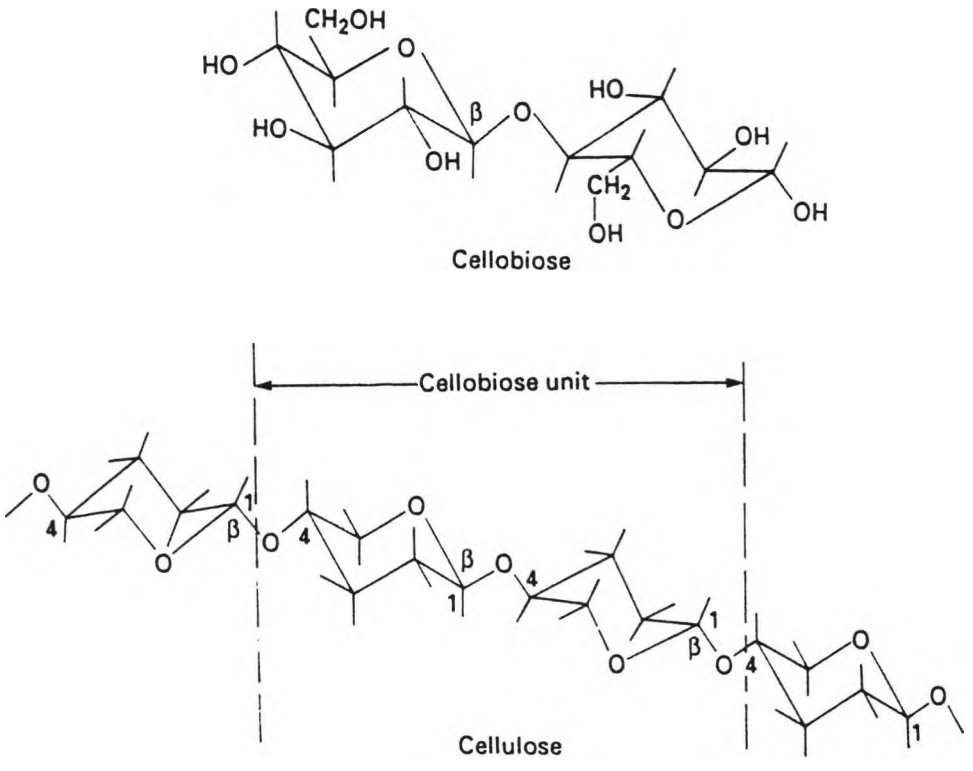



Fig. 2.4. Structure of cellulose showing cellobiose unit (Wayman and Parekh, 1990).

As shown in Fig. 2.5, cellulose in plant tissues is always found packed in microfibrils and is surrounded by hemicellulose and lignin. Around 50 cellulose units form a microfibril that appears to be roughly cylindrical and that measures 10-30 μm in diameter. The amorphous and crystalline regions in the microfibril are an important issue influencing wood physical properties and they are also illustrated in Fig. 2.5 (Tsoumis, 1991). Cellulose crystallites are considered one of the barriers to enzymatic degradation by cellulases.

For more than 40 years, the cellulose anti-parallel conformation theory was accepted. Recently, further work by computer assisted conformational analysis has shown that all cellulose chains within a microfibril are oriented in the same direction (Wangaard, 1979).

## Hemicelluloses

A significant fraction of biomasses is known as hemicelluloses and grasses contain relatively higher amounts than woody biomass. The inadequate name, hemicellulose, was at first suggested by Schulze, in 1891, in order to identify those polysaccharides easily extractable from plants by alkali (Fengel and Weneger, 1984; Timmel, 1964).

Hemicelluloses (or polyoses), heterogeneous branched polymers, are composed of sugar units such as glucose, mannose, galactose, xylose and arabinose. Acetyl groups and 4-O-methyl-glucuronic acid might also be part of the hemicellulose structure (Sjöström, 1991; Fengel and Weneger, 1984; Pettersen, 1984; Timmel, 1964). Xylose and mannose are the most important sugars found in plant hemicellulose, and are often indicated as xylans and mannans. In woody tissues mannans are correctly referred to as glucomannans (Fengel and weneger, 1984). Xylan is the major

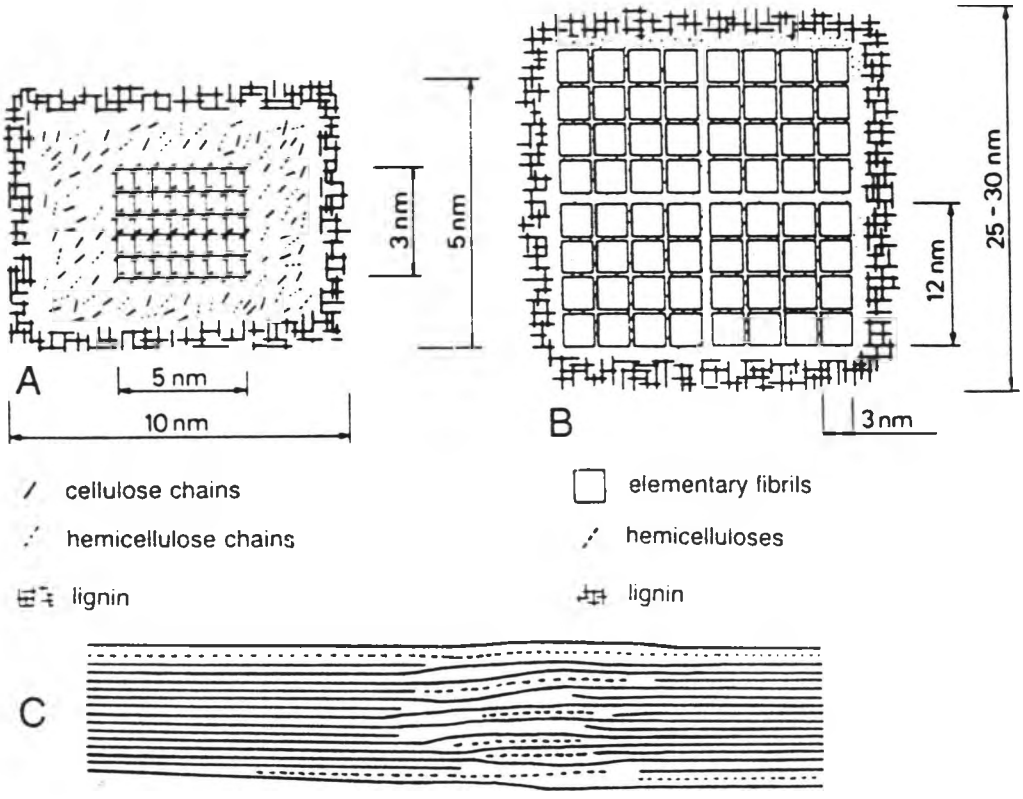



Fig. 2.5. Models for ultrastructural organization of a microfibril. (A, B) Cross sections, in A showing a crystalline core surrounded by amorphous cellulose, hemicellulose and lignin. In B, the core is formed of elementary fibrils. (C) Longitudinal section showing crystalline and amorphous regions (Tsoumis, 1991).

hemicellulose in hardwoods and grasses (Wilkie, 1979; Timmel, 1967) and it is extensively acetylated specially in hardwoods (Sjöström, 1993; Fengel and Weneger, 1984; Timmel, 1967).

Hardwood xylan is composed of a homopolymer backbone of xylose units linked by β-(1→4)-glycosidic bonds. Acetyl groups are present attached in C2 and C3 positions and 4-O-methylglucuronic acid is linked by an α-(1→2)-glycosidic bond (Fig. 2.6). The ratio of xylose, acetyl groups and 4-O-methylglucuronic acid varies considerably, but averages approximately 10:7:1. The degree of polymerization (DP) is from 100 to 200 xylose units (Fengel and Weneger, 1984; Timmel, 1964).

Softwood xylans differs from hardwood xylan by the presence of arabinose linked by an α-(1→3)-glycosidic bond (Fig. 2.7) and the lack of acetyl groups. The softwood xylan contains almost double the amount of 4-O-methylglucuronic acid residues when compared to hardwood xylan, and the approximate ratio is 8:1.6:1 respectively for xylose, uronic acid residue, and arabinose. The DP is shorter than that of hardwood xylans, being around 70-130. (Fengel and Weneger, 1984).

The three branch residues, arabinose, 4-O-methylglucuronic acid and acetyl groups are very common in hardwood and softwood xylans, however galactose has been reported in some grass xylans (Fengel and Weneger, 1984).

Hardwood mannans, actually glucomannans, are composed of a heteropolymer backbone consisting of mannose and glucose units. The ratio of mannose to glucose is 1.5-2:1, characteristic of several temperate hardwood species, but a ratio of 1:1 is found in birchwood (Fig. 2.8). These glucomannans are shorter than other hemicelluloses, with a DP of 60-70, and are less important because they represent 3-5% of the hardwood composition (Fengel and Weneger, 1984).

Softwood mannans are the most important hemicellulose found in softwoods.

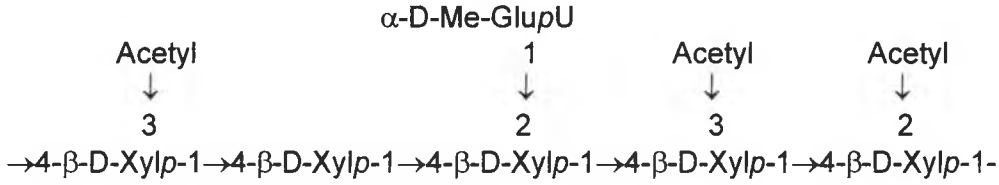



Fig. 2.6. Representation of the O-acetyl-4-O-methylglucuroroxylan segment in hardwood xylan (adapted from Fengel and Weneger, 1984).

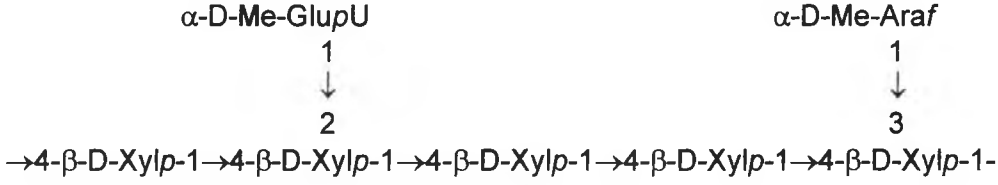



Fig. 2.7. Representation of the arabino-4-O-methylglucuroroxylan segment in softwood xylan (adapted from Fengel and Weneger, 1984).

→4-β-D-Glu*p*-1→4-β-D-Man*p*-1→4-β-D-Glu*p*-1→4-β-D-Man*p*-1

Fig. 2.8. Representation of the glucomannan segment in hardwoods.

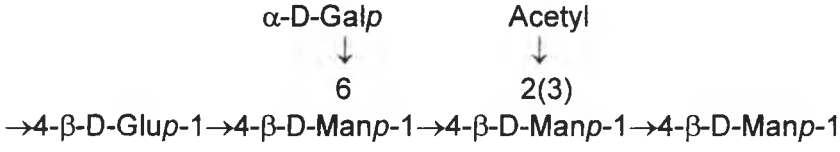



Fig. 2.9. Representation of the O-acetyl-galacto-glucomannan segment in softwoods (adapted from Timmel, 1965).

As in hardwood mannans, they are composed of a glucomannan backbone. Softwood mannans also contain galactose besides glucose, and are actually galactoglucomannans. In spite of their predominance in all softwoods, the galactoglucomannans were the last wood polysaccharides to be discovered (Timmel, 1967). They differ from hardwood glucomannan in that branches of acetyl groups and galactose residues are attached to it; also in that higher ratio of 3:1 mannose to glucose. The major fraction of the hexosans extracted with potassium hydroxide together with the xylan, consists of a water-soluble galactoglucomannan containing galactose, glucose, and mannose residues in a ratio of 1:1:3. Extraction of the residual holocellulose with sodium hydroxide-borate gives a second polysaccharide, often referred to as a glucomannan, where the ratio between the three hexose residues is 0.1:1:3 for galactose, glucose, and mannose (Timmel, 1965). The number of acetyl groups and galactose residues varies among different species. The acetyl groups are equally linked to the C2 and C3 of mannose and the galactose residues are linked by an $\alpha$-(1$\rightarrow$6)-glycosidic bond (Fig. 2.9). The DP varies from 35 to 142 among 18 softwood species (Fengel and Weneger, 1984).

The behavior of hemicelluloses during alkaline pulping has been extensively studied. Xylan hemicelluloses are less easily degraded than glucomannans. Deacetylation is fast and quantitative. The 4-O-methyl-glucuronic residues are partially or completely removed, depending on the severity of the conditions. The degree of polymerization of the hemicelluloses backbone is reduced either by the peeling reaction or randomly by cleavage (Grace et al., 1989).

Lignin

The most complex structure in plant tissue is lignin, which makes it a very interesting field of study and still the focus of much research as indicated by numerous publications throughout the world. The chemistry of lignin challenges scientists looking for answers about its structure and reactions. According to Sarkanen and Ludwig (1971), the word lignin is derived from the Latin word "lignun" which means wood. Lignin constitutes a very important natural material because it is found in representative amounts varying from 15 to 36% in plant tissue (Sarkanen and Ludwig, 1971; Rydholm, 1965).

Lignin is formed by dehydropolymerization of the three precursors, p-coumaryl, coniferyl and sinapyl alcohols generically known as phenyl propane units, Fig. 2.10 (Casey, 1980; Freudenberg and Neish,1968). Most of the lignin in softwoods is composed of coniferyl alcohol units. Figure 2.11 shows how coniferyl alcohol is activated for starting the polymerization. A more condensed lignin is expected in softwoods due to more overall carbon-carbon bonds. Hardwoods tend to have an equal mix of sinapyl and coniferyl alcohols. The p-coumaryl alcohol is present in the two types of wood in small amounts (Sjöström, 1993; Fengel and Weneger, 1984; Sarkanen and Ludwig, 1971). Sarkanen and Hergert (1971) define lignin in softwoods as guaiacyl lignin and in hardwoods as guaiacyl-syringyl lignins. Some proposed structures of hardwood and softwood lignins are presented in Figs. 2.12 and 2.13 respectively. The most common linkages, ether and carbon-carbon, between phenylpropane units are shown in Fig. 2.14.

The lignin in the secondary wall of sugarcane is composed of syringyl, guaiacyl, and p-hydroxyphenyl propane units in addition to phenolic acid residues, and

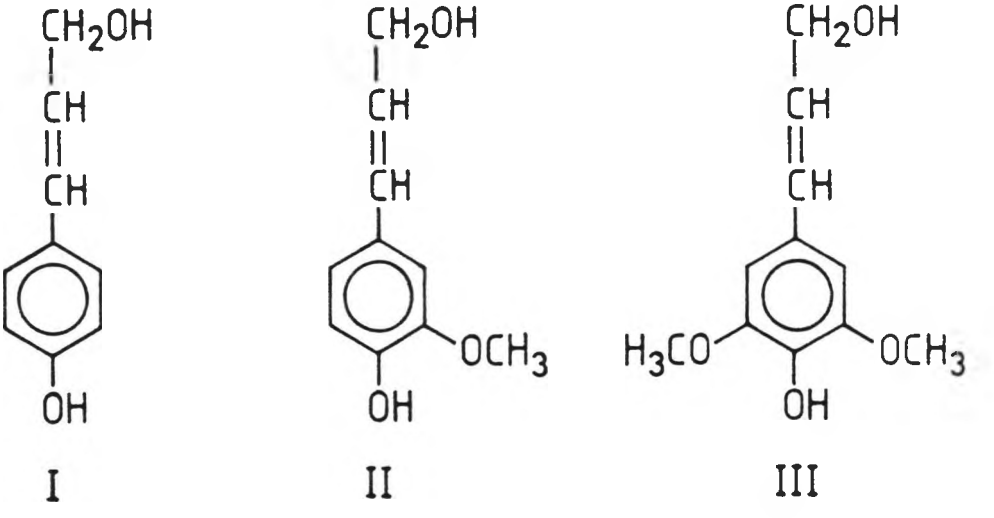



Fig. 2.10. The building units of lignin: p-coumaryl alcohol (I), coniferyl alcohol (II), sinapyl alcohol, (III) (Fengel and Weneger, 1984).

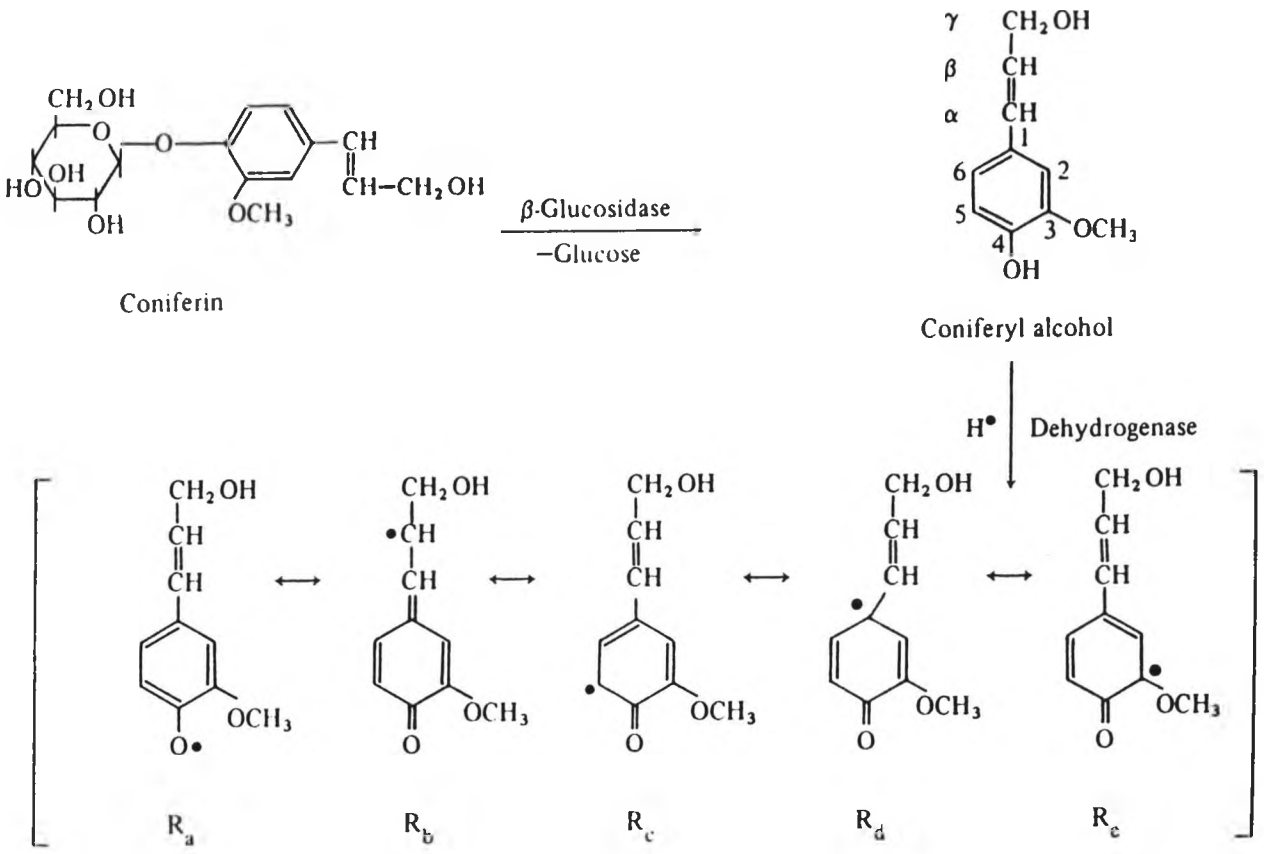



Fig. 2.11. Phenoxy radical formation from coniferin (Casey, 1980)

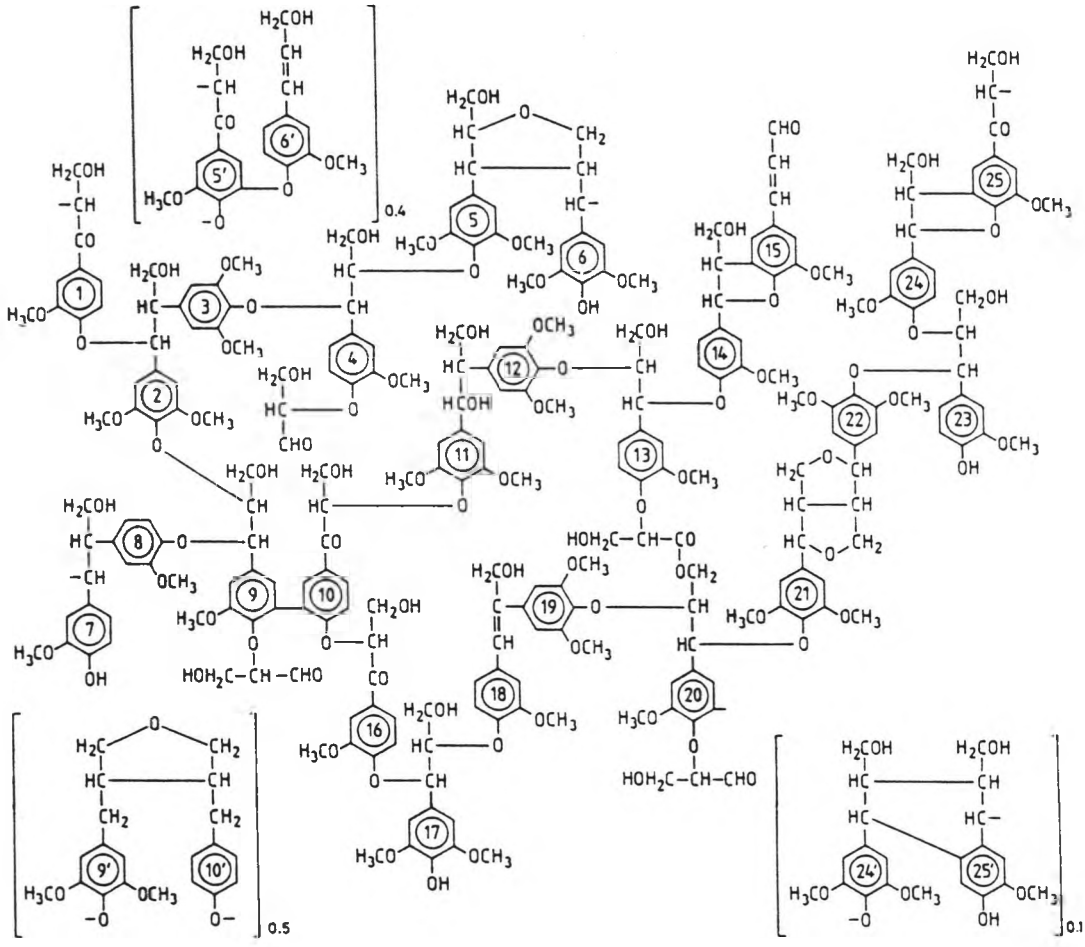


Fig. 2.12. Proposed structure of beech lignin (according to Nimz, 1974)

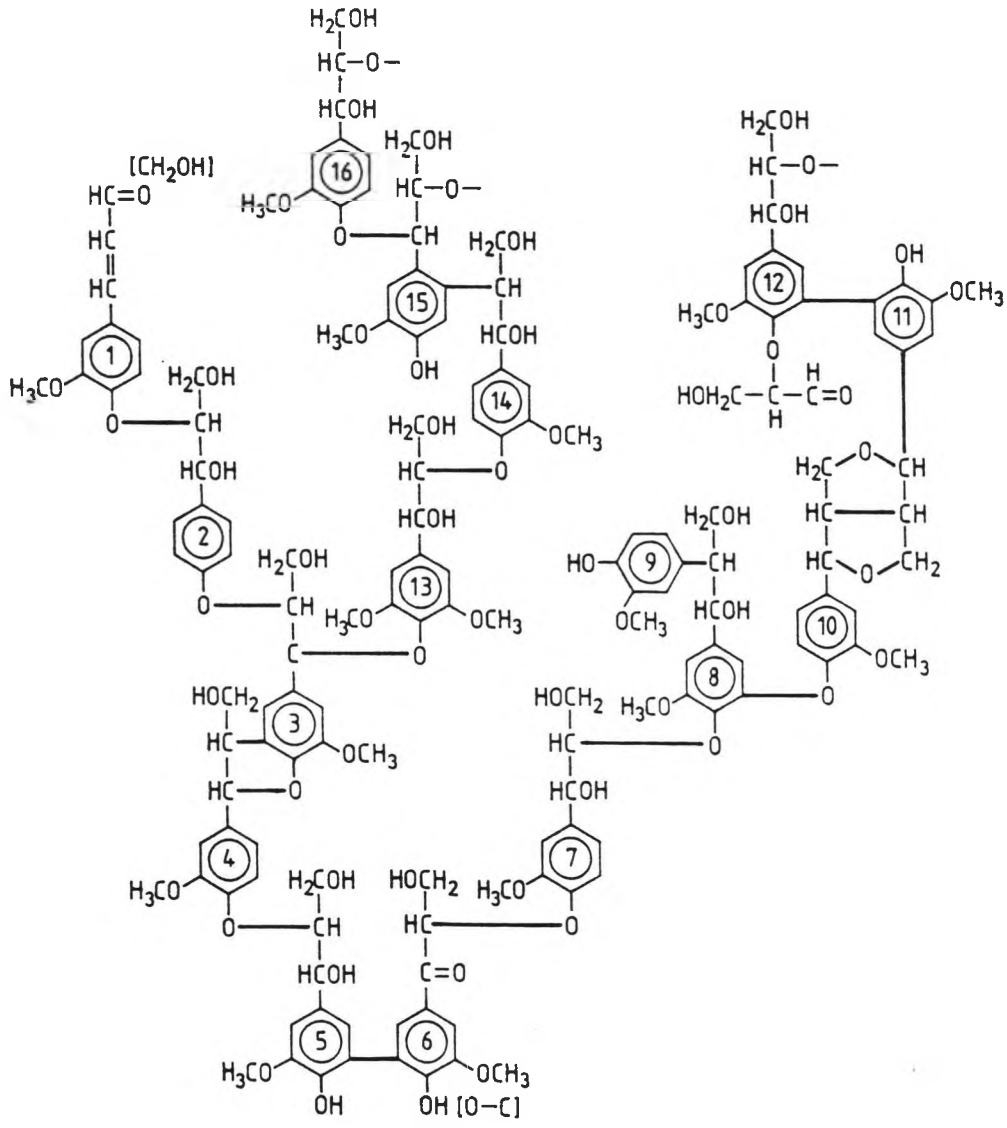


Fig 2.13. Proposed structure of spruce lignin (according to Adler, 1977).

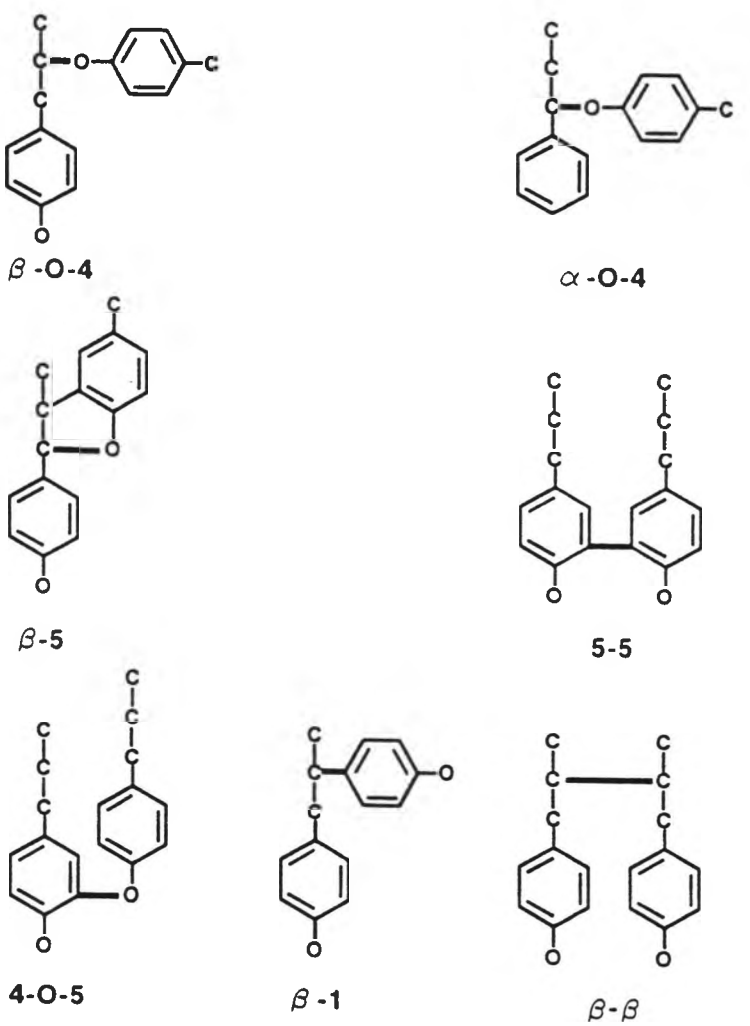


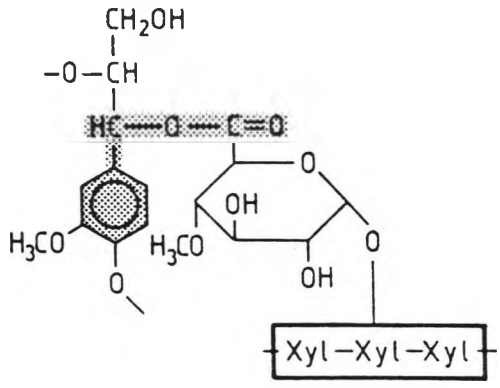

Fig. 2.14. Common linkages between the phenylpropane units (Sjöström, 1993).

the proportion of these monolignols is S > G > H. Sinaptic, p-coumaric, and ferulic acids are probably linked to the lignin structure by covalent ester bonds (He and Terashima, 1990). Smith (1955) has reported for bagasse lignin as much as 11% of p-coumaric acid and small amounts of ferulic, p-hydroxybenzoic, syringic and vanillic acids.

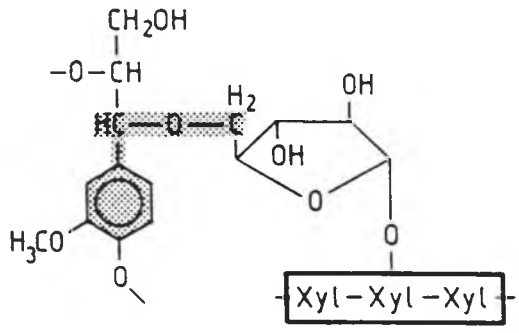
Lignin-hemicellulose complexes have been isolated suggesting the existence of covalent lignin-polysaccharides bonds (Joseleau et al., 1991; Fengel and Weneger, 1984; Glinski and Nicholls, 1977; Morrison, I. M., 1974; Lai and Sarkanen, 1971). Figure 2.15 shows the most frequent suggested linkage types.

As a result of numerous reactive groups such as carbonyl, phenolic and benzylic hydroxyl groups plus considerable unsaturation, lignin undergoes several types of chemical reactions. The most important chemical reactions known are those related to alkaline pulping, both Kraft and soda processes. Formation of quinone methide by alkaline cleavage of the alpha-aryl ether bond, sulfidolytic cleavage of beta-aryl ether bonds in the Kraft process, and formation of beta-aroxy styrene formation in soda pulping are the most important in alkaline pulping. Alkaline bleaching reactions include oxidation during the hypochlorite stage and oxidation with oxygen and peroxides. The chlorination of lignin targets destruction of resonance on the phenyl ring (Fengel and Weneger, 1984). Anthraquinone has been used as a sulfur-reducing additive in Kraft cooking and it cleaves the beta-aryl ether bonds in lignin (Sjöström, 1993).

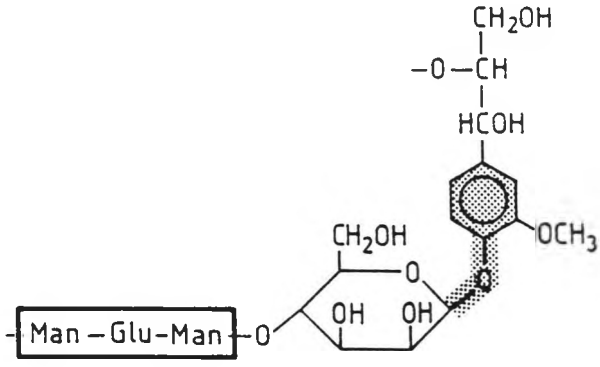
Strong mineral acids, such as hydrochloric or sulfuric acid, do not cause extensive lignin solubilization. However, they do affect the benzyl-carbon position of the basic phenylpropane unit, which is frequently occupied by either hydroxy or ether groups in the original lignin. As a consequence, possible products formed during




Benzyl ester linkage




Benzyl ether linkage




Phenyl glycosidic linkage

Fig. 2.15. Most frequent suggested types of lignin-polysaccharide linkages (Fengel and Weneger, 1984)

acidic reaction conditions are a result of new carbon-carbon bonds with an increase in molecular weight (Casey, 1980). Condensation of lignin can also occurs under alkaline media such as in the Kraft process (Rydholm, 1965).

Lignin oxidation by peracetic acid and hydrogen peroxide is of interest from the standpoint of high-yield pulp bleaching. Fig. 2.16 shows the oxidative degradation reactions of lignin with peracetic acid. Electrophilic ring hydroxylation, demethylation, ortho and para-quinone formation, and aromatic-ring cleavages are the more prominent mechanisms (Casey, 1980).

Pectins

The galacturonans, the galactans, and the arabinans compose the substance called pectin. Galacturonans of various composition are components of many plants. The content of galacturonans in soft and hardwood is less than 1%. They are predominantly deposited in the middle lamellae and the tori of bordered pit-membranes. A polysaccharide group of galactans, particularly the arabinogalactan from larchwood, can be isolated in amounts of 10-25% and in others species such as *Pinus, Araucaria, Acer, Fagus,* and *Betula* amounts of 0.5-3%. Arabinan has been isolated from *Pinus pinaster*, maritime pine, in a yield of 0.31% (Fendel and Weneger, 1984).

Extractives

Extractives cover a large number of different compounds which can be extracted from plants by means of polar and non-polar solvents. Organic solvent solubles as well as water-soluble carbohydrates and inorganic compounds also belong to the extractable substances. Terpenes, lignans, stilbenes, tannins, flavonoids, fats,

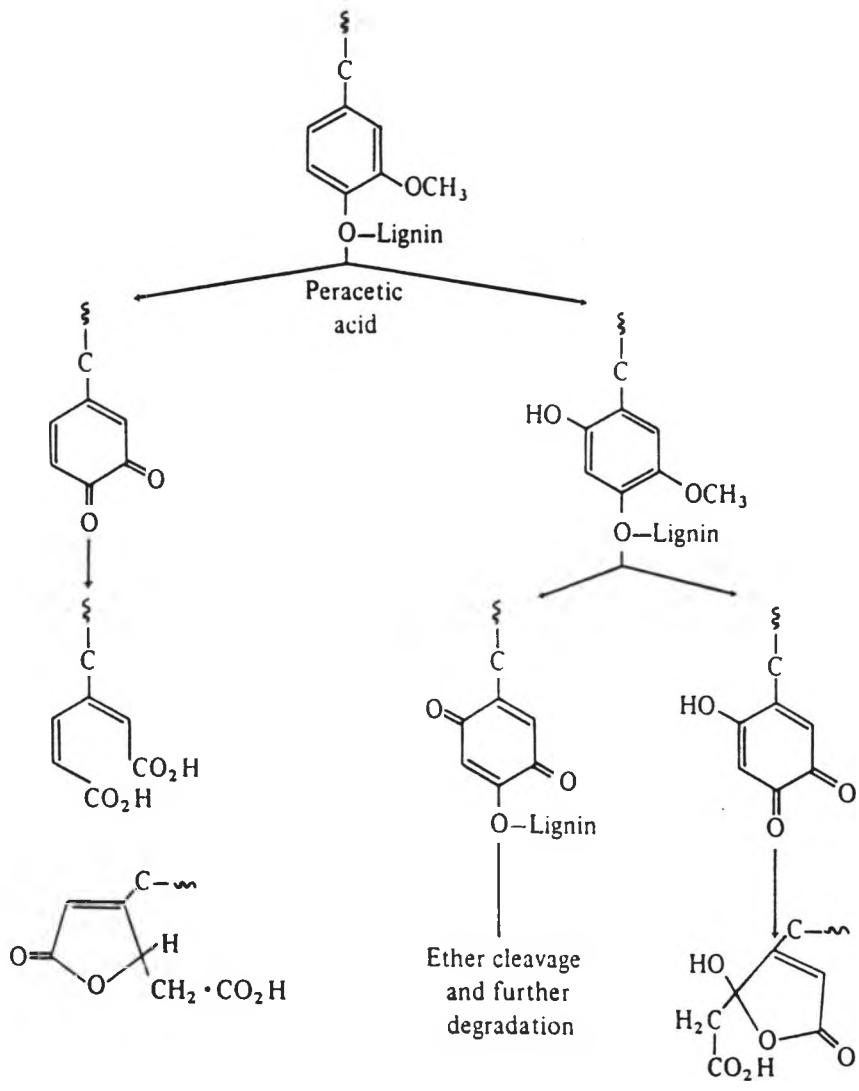



Fig. 2.16. Oxidative degradation reactions with peracetic acid (Casey, 1980).

waxes, fatty acids, alcohols, steroids, and higher hydrocarbons constitute the extractives.

The extractive content varies from less than 1%, as in poplar, to more than 10%, as in redwood (Fengel and Grosser, 1975). Tropical woods can have as much as 19.8% (Yatagai and Takahashi, 1980). The heartwood natural resistance to fungal attack has been attributed to the presence of extractives (Wangaard, 1992; Panshin and De Zeeuw, 1980). Specific gravity, hardness, and compressive stress of wood as well as permeability can be affected by presence of extractives (Panshin and De Zeeuw, 1980).

### Ash

The ash content, oxidized inorganic fraction, for domestic woods varies from 0.1 to 0.5% of the oven-dry weight of wood. Calcium, potassium, and magnesium, usually account for 70 percent of the total ash present (Panshin and de Zeeuw, 1980). Field crop residues such as sugar cane bagasse, rice and wheat straws, have a high ash content mainly because of higher silica content in addition to alkaline metal salts. (Anonymous, 1991; Shafizadeh, 1983). Chatters (1963) discussed the presence of mineral constituents in wood fibers and one conclusion was that they are dispersed at random in the cell walls.

## BIOMASS PRETREATMENTS, PHYSICAL AND CHEMICAL MODIFICATIONS

The structure of cell the wall as previous described resembles that of a reinforced concrete with cellulose fibers being the metal rods and lignin the cement. When untreated native biomass is submitted to enzymatic degradation a low extent of

degradation is expected, often under 20 percent. For obtaining higher degradation rates and satisfactory bioconversion yields, physical, chemical or biological pretreatments should be performed on the biomass. According to Table 2.3, many different pretreatments have been proposed in the literature. Most are effective in disrupting the lignin-carbohydrate complex, and others in disrupting the highly ordered cellulose structure (Fan et al., 1987). The biomass pretreatments as well as their physical and chemical modifications will be discussed.

Physical pretreatments

Physical pretreatments are classified in two general categories, mechanical and non-mechanical pretreatments.

Ball-milling is an effective mechanical method for enhancing enzymatic hydrolysis by causing reduction in crystallinity, increase in bulk density, and decrease of degree of polymerization in addition to particle size reduction (Fan et al., 1987; Chang, 1981; Millet et al, 1976). In spite of being a good pretreating method, its application has declined because of the required energy input to mill biomass into particles bellow 150 μm that represents more than 25% of total energy content of the substrate (McMillan, 1994). Further mechanical comminution methods as two-roll, hammer, colloid and vibro energy milling are as costly as the ball-milling process (Fan et al., 1987).

Thermal pretreatments are based on use of direct high pressure steam or external thermal source as in the pyrolysis process. Crystallinity and degree of polymerization are reduced as in ball-milling. According to Fan et al (1981), a pyrolysis pretreatment at 170 °C of Solka Floc had a marked increase in enzymatic hydrolysis mainly because of depolymerization of cellulose. Delignification and hemicellulose

Table 2.3. Methods used for pretreatment of lignocellulosics (Fan et al., 1987)

| Physical | Chemical | Biological |
|---|---|---|
| Ball-milling | Alkali: | Fungi |
| Two-roll milling | Sodium hydroxide | |
| Hammer milling | Ammonia | |
| Colloid milling | Ammonium sulfite | |
| Vibro energy milling | Acid: | |
| High pressure steaming | Sulfuric acid | |
| Extrusion | Hydrochloric acid | |
| Expansion | Phosphoric acid | |
| Pyrolysis | Nitric acid | |
| | Hydrofluoric acid | |
| | Gas: | |
| High energy radiation | Chlorine dioxide | |
| | Nitrogen dioxide | |
| | Sulfur dioxide | |
| | Oxidizing agents: | |
| | Hydrogen peroxide | |
| | Ozone | |
| | Cellulose solvents: | |
| | Cadoxen* | |
| | CMCS** | |
| | Solvent extraction of lignin: | |
| | Ethanol-water extraction | |
| | Benzene-ethanol extracton | |
| | Ethylene glycol extraction | |
| | Butanol-water extraction | |
| | Swelling agents | |

* Alkaline solution of ethylene diamine.

** Solution composed of sodium tartrate, ferric chlorite, sodium sulfite, and sodium hydroxide.

degradation generally occur in thermal treatments using high pressure steam as in the industrial Masonite process (Koran et al. 1978; Millet et al, 1975).

Irradiation by gamma rays or high-velocity electrons improves enzymatic digestibility but losses of nearly 45% of original carbohydrate as usable occurs depending on the magnitude of irradiation. High-intensity ultraviolet light (3650 Å) has been used and patented for inducing deep-seated structural alterations. A twenty-four hour irradiation pretreatment gave up to a fourfold increase in the rate of biodegradation in various substrates (Millet et al., 1975).

Chemical pretreatments

Chemical pretreatments are most important and extensively studied because of process effectiveness (Gharpuray et al., 1983). The use of alkalis, inorganic acids, oxidizing agents, and organic solvents has been proposed from ambient to relatively high temperatures (100-240 °C). Acids serve primarily for hydrolysis of cellulose rather than as reagents for pretreatment. Acid hydrolysis of cellulose on an industrial scale was started during the First World War and has been used for a long period in the former USSR (Fan et al., 1987). Acid processes for cellulose hydrolysis and their mechanisms have been discussed (Goldstein, 1983).

Prehydrolysis using dilute sulfuric acid (Linden and Schroeder, 1996; Grohmann et al., 1985) and steam explosion with $SO_2$ or sulfuric acid (Saddler et al., 1993) constitute the most promising methods because of high bioconversion yields and low cost of catalysts. Technical and economical viability studies of prehydrolysis using dilute acid was performed at the pilot plant level (Hinman et al., 1992). Very efficient tubular high pressure continuous steam explosion equipment (Stake Technology Ltd.), that operates at temperatures of 200-240 °C and for a variety of different retention

times, has been developed and commercialized (Heitz, 1991; Taylor, 1986 and1980). The IOGEN Corporation, in Ottawa, has developed other equipment based on the Masonite gun in which the wood chips, after steaming at about 200-250 °C for varying times from 20 to 100 seconds, are exploded by pressure release (DeLong, 1981). Despite their great acceptance these thermal-chemical treatments are considered costly because of the energy input, equipment corrosion, and fractionation steps. In addition, they present chemical disposal problems because of the sulfuric acid catalyst used. With the intent of diminishing disposal problems, Brito (1994) has tested dilute phosphoric acid at the same concentration level as phosphate required in the medium for yeast growth. In both methods, hemicelluloses are almost completely removed from the substrate with significant losses, which are dependent on treatment residence time (Schwald et al., 1989; Grohmann et al., 1985; Holtzapple, 1981). Lignin is more resistant under the process conditions with significant retention and recondensation. Dilute hydrochloric and nitric acids have also been investigated (Brink, 1994 and 1993; Fan et al., 1987).

The use of concentrated sulfuric acid at ambient temperatures has been investigated for enhancing enzymatic hydrolysis of rice hulls. Cellulose is dissolved in a short period of time and precipitated with acetone. Ninety five percent of recovered cellulose was converted to glucose by commercial enzymes (Tsao et al, 1978).

Alkaline treatments constitute the second most tested method. Alkaline effects on enhancing enzymatic hydrolysis of biomass are related to swelling, delignification, and xylan deacetylation (Kong, 1992; Kong, 1990; Mitchell, 1989; Soltes, 1989; Grohmann et al, 1989; Millet, 1976 and 1975). A significant amount of hemicellulose is solubilized as well. Generally, alkaline pretreatment is more effective on agricultural residues and herbaceous crops than on woody biomass (Hsu, 1996). The use of

sodium hydroxide by itself has been investigated in numerous studies (Playne, 1984; Fan et al., 1981; Millet, 1976 and 1975) and also in combination with peroxides (Gould, 1987, 1985 and 1984). Baker et al. (1974) treated aspen wood at ambient temperature with 0.5 and 1.0% solutions of NaOH at various liquid-to-solid ratios. The results for a maximum effect on *in vitro* digestibility show that 5 to 6 g of alkali should be added to 100g of oven dry weight of wood. The minimum quantity of sodium hydroxide needed for attaining maximum digestibility is roughly equivalent stoichiometrically to the combined acetyl and carboxyl content of the aspen. The digestibility was improved by approximately 50% suggesting that alkaline treatment at ambient temperature should be combined with a second treatment. Kong et al. (1992) obtained an increase of more than four-fold on reducing sugar yields when ground aspen was treated with KOH causing a complete removal of acetyl groups from the xylan backbone.

Aqueous ammonia at 160°C has been used for improving enzymatic digestibility of straws. The bioconversion yields were below 70% of total carbohydrates (Waiss et al., 1972). Ammonia recycled percolation pretreatment at temperatures of 145-170 °C has also been tested by itself or as a complementary pretreatment to the dilute-acid process (Wu and Lee, 1997; Yoon et al., 1994).

Dale (1986) has patented a process called AFEX, an ammonia freeze-explosion method, where biomass and liquid ammonia are pressurized at low temperature and after a few minutes the pressure is released causing fiber explosion. The physical and chemical effects increase the biomass surface area, decrease crystallinity, cause lignin alteration, and the hemicellulose is prehydrolyzed. Several papers has been published using the AFEX technology (Holzapple et al., 1991; Dale, 1985 and 1982). This

process seems to be effective for treating agricultural residues and herbaceous crops but has not proven as effective on hardwoods and softwoods (McMillan, 1994).

Boiling peracetic acid for 1 hour, has been tested on woody biomass and agricultural residues prior to enzymatic degradation as a lignin-oxidative pretreatment approach (Myung and Kennelly, 1992; Farid et al., 1983; Gharpuray et al., 1983; Fan et al., 1981; Toyama and Ogawa, 1975). Ando et al. (1988) have soaked Japanese cedar chips in different concentrations of peracetic acid solutions before steam explosion pretreatment. The saccharification yields increase with higher residence time and higher acid concentration. Lignocellulosic municipal solid waste, MSW, has been treated with peracetic acid only, at ambient temperatue for 1 day and concentrations varying from 3 to 20% of acid based on the oven-dry weight of MSW, or as a pre-pretreatment prior to ammonia fiber explosion pretreatment (AFEX). According to Holzapple (1992), when peracetic acid was used alone no significant sugar yields were verified but when it was used before the AFEX pretreatment the digestibility significantly increased in a synergistic, not additive, manner. Rodríguez-Vázquez (1993) has performed peracetic acid pretreatments, at ambient temperature during 15 days and concentrations varying from 2 to11% based on oven-dry weight of wood, in aspen wood, ponderosa pine and wheat straw. There is a direct correlation between the quantity of peracetic acid used in the pretreatment and the increase in enzyme accessibility. Pretreatment of woody biomass and sugar cane bagasse with peracetic acid, at ambient temperature for a seven-day period and concentrations varying from a mild 6% to a more severe 60% based on oven-dry weight of biomass, was evaluated recently and is an integral part of this dissertation (Teixeira et al., 1998a and 1998b in press). Toyama and Ogawa, 1975, have studied the effects of peracetic acid in combination with alkali. The sequence peracetic acid-NaOH or NaOH-peracetic acid

does not affect the sugar yields when hardwoods are used, but for softwoods the peracetic acid steps should be performed before alkaline treatment.

According to Lai and Sarkanen (1968), peracetic acid cleaves aromatic nuclei in lignin generating dicarboxylic acids and their lactones (Fig. 2.17). The aliphatic propane side-chain is displaced to some extent during the oxidative process (Sakai and Kishimoto, 1966). At pH 7, peracetic acid is a lignin-preserving bleaching agent in oxidizing mainly carbonyl as well as olefinic groups, while beta-O-4 ether bonds and aromatic rings are relatively stable. Under acid conditions, oxidation of aromatic rings to quinone and quinonemethide structures as well as splitting of beta-O-4 ethers occurs, leading to delignification (Nimz et al., 1992).

Wheat straw was irradiated by an electron beam in presence of peracetic acid as a new pretreatment approach. The carbohydrate bioconversion increases with increases in the magnitude of irradiation (Zhaoxin and Kumakura, 1995).

In addition to enhancing of enzymatic digestibility of biomass, the use of peracetic acid as a delignifying and bleaching agent has been extensively studied (Kawamoto et al., 1994; Nimz et al. 1992; Glinski and Nicholls, 1977; Albrecht and Nicholls, 1976; Marchall, 1976; France, 1974; Sakai et al., 1972; Farrand and Johnson, 1971; Davidage et al., 1958). Preparation of chemical cellulose from steam-exploded wood by peracetic acid has also been reported (Van Winkle and Glasser, 1995).

Other oxidizing agents, such as ozone, chlorine dioxide and hydrogen peroxide have been suggested for treating biomass. Ozone might be effective in degrading lignin without excessive amounts of pollutants (Fan et al., 1987).

Organic solvents such as ethanol, methanol, and acetone have been used to solubilize lignin from various substrates in processes commonly termed organosolv (Bennani et al., 1991; Chum et al, 1988; Chum et al, 1985) . The technology is similar

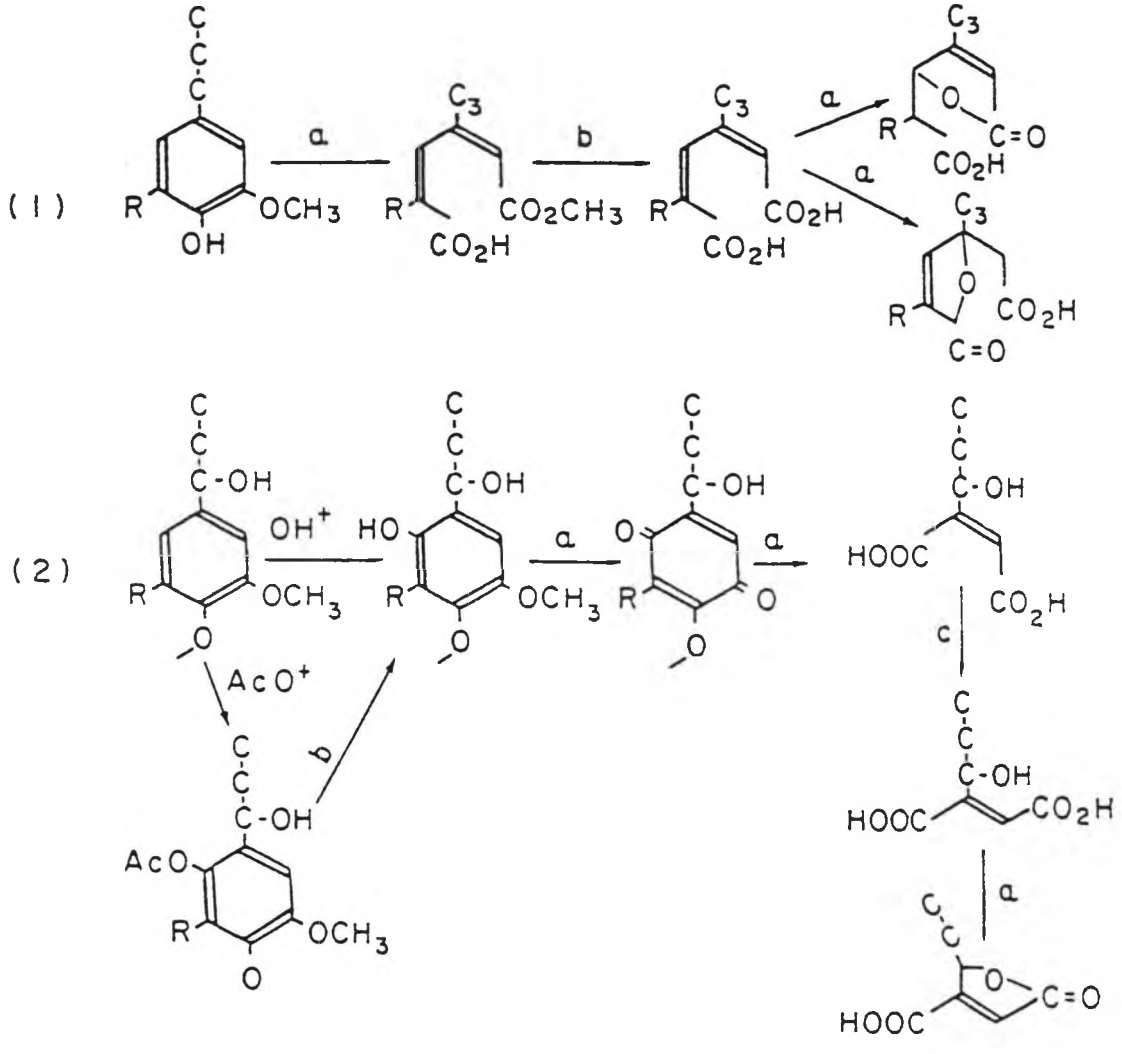



Fig. 2.17 Proposed oxidation mechanism of lignin by peracetic acid . Pathway (1) is predominant for free phenolic units of the guaiacyl type; Pathway (2) is likely to predominate the reactions of syringyl and etherified guaiacyl propane units. (a) Oxidative ring opening by peracetic acid, (b) Hydrolysis, (c) Isomerization. R = H, $OCH_3$ or alkyl group (Lai and Sarkanen, 1968).

to the proposed industrial ALCELL process approach for the production of chemical cellulose (Pye and Lora, 1991). Organosolv processes catalyzed by acids or alkalis for pretreatment of biomass have been reported (Chum et al., 1990; Paszner and Cho, 1988). In spite of being effective, the organosolv process is costly even for making pulp (Aziz and Sarkanen, 1989).

Another group of pretreatments based on cellulose-dissolving solvents such as cadoxen, an alkaline ethylene diamine solution, and CMCS, composed of sodium tartarate, ferric chlorite, sodium sulfite and sodium hydroxide solution, have been used to dissolve cellulose at room temperature. They have obtained from 90 to 95% glucose yields based on the total amount of cellulose reprecipitated by adding excess water (Tsao et al., 1981; Tsao et al., 1978). These solvents, however, must be used in conjunction with a hemicellulose removal process (Hsu, 1996). Cost and solution toxicity, especially for cadoxen, represent some disadvantages for these cellulose-dissolving processes (Fan et al., 1987).

Biological pretreatment

The biological pretreatments are based on the application of lignin-solubilizing microorganisms to render the carbohydrates in biomass amenable to enzymatic hydrolysis. The microorganisms can be classified into two categories, brown rots and white rots. Brown rots attack primarily the cell wall carbohydrates, leaving behind a network consisting of modified lignin, with small amounts of more resistant crystalline cellulose. The white rots attack both lignin and cellulose, leaving behind a spongy or stringy mass (Fan et al., 1987; Panshin and De Zeeuw, 1980).

Rodríguez-Vázquez (1993) has compared chemical versus biological pretreatments using the brown rot fungus *Poria monticola*. In addition to weight losses,

the depolymerization of lignin was verified in the decayed biomasses: aspen wood, ponderosa pine and wheat straw.

White-rot fungi as mentioned above decompose lignin as well as the cellulose and hemicellulose in wood. Some remove lignin faster than cellulose resulting in decayed wood with a lower lignin content. Rumen digestibility tests have been performed on white-rot decayed aspen and birch wood. Aspen digestibility was improved from 48 to 78% because lignin content was reduced from 17 to 10% by fungal attack (Baker et al., 1974).

Biological delignification appears to be a promising approach for pretreating biomass, but its slow rate has prevented its usage in large scale industrial processes. Genetic modifications of lignin degrading microorganisms or partial physical/chemical processing prior to biological pretreatment can be a key for improving overall process rates (Fan et al., 1987).

# CHAPTER 3

# METHODOLOGY

This methodology covers description of the biomasses including milling operations, chemical composition analysis, pretreatment conditions, peracetic acid preparation and analyses, substrate analyses such as carbohydrate, Klason lignin and acetyl content, and finally the enzymatic hydrolysis and simultaneous saccharification and cofermentation standard (SSCF) tests.

## BIOMASS PREPARATION

Hybrid poplar (*Populus deltoides x Populus nigra*) provided by the National Renewable Energy Laboratory (NREL), Golden, Colorado and sugar cane bagasse provided by Cajun Sugar Co-op, Inc., New Iberia, Louisiana were milled and screened to 20-80 mesh. The moisture content of the two air-dried biomasses were 5.6% and 7.0%, respectively.

## BIOMASS CHEMICAL COMPOSITION

### Extractive-free biomass

Based on Tappi standard method T 264 om-82 (1988), duplicate biomass meal samples were weighted to 0.01 g in tared labeled fritted-glass thimbles. The thimbles were filled with biomass to within 1/2 inch of the siphon tube on the extraction apparatus. A paper cone was set on top of the thimble to prevent loss of sample due to splashing. The lignocellulosic materials were extracted with 400 mL of ethanol-benzene (1:2). Porcelain chips were added to prevent superheating and bumping of extracting solvent. The samples were extracted for 8 hours, until the siphoning liquid was colorless.

After cooling, the thimbles were placed in evaporating dishes. The partial extracted biomass was washed with 250 mL of ethyl alcohol, and extracted with another portion of 250 mL of ethanol for 6 hours. After cooling the thimbles were placed in evaporating dishes again. The samples were rinsed under water aspirator vacuum with three portions of boiling water. After rinsing, the biomass was spread and air dried to constant weight. The extractive content was calculated by difference in weight due to extraction, based both on original biomass weight and extractive-free weight.

### Holocellulose

Following Browning (1967), triplicate samples of extractive-free, air-dry biomass meal were weighted to 5.0 g and transferred to 250 mL Erlenmeyer flasks. Distilled water (160 mL), glacial acetic acid (0.5 mL), and sodium chlorite (1.5 g), were added in

that order. A smaller Erlenmeyer flask was inverted in the neck of the reaction flask and the flask was placed on a hot plate. The holocellulose preparations were performed in a hood because of the high toxicity of the orange fumes of chlorine dioxide. 10 times as poisonous as chlorine gas. The hot plate was adjusted to produce a temperature of 70-80 ºC. After one hour at reaction temperature, during which the contents were mixed by occasional swirling, additional portions of glacial acetic acid (0.5 mL), and sodium chlorite (1.5 g), were added to each flask. The heating was continued at 70-80 ºC for an additional hour. At the end of second hour the additions of acetic acid and sodium chlorite were repeated.

At the end of the reaction time, the flask was cooled with cold tap water and the holocellulose filtered on a coarse-porosity fritted glass crucible with sufficient distilled water to transfer all the holocellulose and to remove the color and odor of chlorine dioxide. The holocellulose was finally washed with hot water, acetone, and partially dried by aspiration of air through the crucible. After partial drying, the holocellulose was spread in an evaporating dish and air dried to constant weight. The final weight was determined and the holocellulose yield was calculated according to the expression:

$$\% \text{ Holocellulose} = (W_2 / W_1) \times 100$$

Where $W_1$ is the weight (g) of extractive-free biomass and $W_2$ is the weight (g) of holocellulose.

Pentosans

Based on Tappi standard method T 223 cm-84 (1988) and Browning (1967), triplicate air-dried extractive-free biomass meal (1.0 g weighed to the nearest 0.1 mg)

were placed in the 500 mL Erlenmeyer reaction flasks and 100 mL of 12% hydrochloric acid was added to each flask, washing all the material to the bottom of the flask. The flask was marked to indicate the liquid level. Porcelain chips to avoid solution bumping were added and the flask attached to a distillation apparatus. Hydrochloric acid, 300 mL, was added to a dropping funnel and 50 mL of distilled water was also added to the receiving Erlenmeyer flask together with a magnetic stirring bar. The condenser cooling water and the "Stir-kool" were turned on. When the water in the receiving flask formed an ice coating around the flask the distillation was started. A distillation rate of 50 mL every 15 min was maintained and a total of 300 mL of distillate was collected in a period of 90 min. The temperature of the vapor was maintained in the range of 105-110 °C and the stopcock in the dropping funnel was adjusted for maintaining the original 100 mL level in the reaction flask.

To the Erlenmeyer receiving flask containing the condensate, at temperature of 0 °C, was added a bromate-bromide solution (20 mL, 0.2N) using a pipet and with minimum agitation. The flask was stopped promptly after addition, shaken, and allowed to stand for exactly 5 min. The stopper was removed and potassium iodide solution (10 mL, 10%) was added from a graduated cylinder and the stopper quickly replaced. The solution was shaken again to allow absorption of the bromine vapor, and then titrated with 0.1N standard sodium thiosulfate solution using a starch indicator near the end-point of the titration.

A blank titration was performed in the same manner, including all reagents, except that the hydrochloric acid (270 mL of 12% HCl or 3.5N solution) which was dilluted to 350 mL, instead of 300 mL of distillate plus 50 mL of water.

The pentosan content was calculated according to the following equation:

$$\% \text{ Pentosan} = \frac{7.5N\,(V2 - V1) - 1.0}{W}$$

Where N is 0.1, the normality of thiosulfate solution, V1 is the titration in mL for the solution being tested, V2 is the titration in mL for the blank, and W is the weight in grams of the sample. The factor 7.5 is the product of 0.048 x 1.375 x 100 / 0.88, where: 1.375 is the ratio of pentose and furfural molecular weights, and 0.88 is an empirical factor to compensate for incomplete conversion of pentosans to furfural. The number 1.0 is subtracted to compensate for the hydroxymethylfurfural and other possible distillable aldehydes formed during the acidic treatment of wood.

Seifert cellulose

Following the procedure given by Browning (1967) and Seifert (1956), triplicate samples of extractive-free air-dried biomass were weighted (1.0 g) to the nearest 0.1 mg on an analytical baance. The samples were transferred to a 100 mL round reaction flask, adapted to a condenser cooling water and refluxed with a mixture of acetylacetone (6 mL), dioxane (2 mL), and hydrochloric acid (1.5 mL, density 1.19) for 30 min on a boiling water-bath. The mixture was filtered on a fritted-glass crucible and washed successively with methanol, dioxane, hot water, dioxane, methanol, and ether, and dried at 105 ºC.

The pure cellulose content was calculated according to the equation:

$$\% \text{ Cellulose} = (W_2 / W_1) \times 100$$

Where $W_1$ is the weight (g) of extractive-free biomass and $W_2$ is the weight (g) of residue after the treatment. The moisture content of extractive-free biomass must be

taken into account because the cellulose has been dried at 105 ºC and extractive-free biomass was only air dried.

Klason lignin

According to TAPPI standard method T 222 om-83 (1988) and Browning (1967), triplicate samples of extractive-free air-dried biomass meal were weighted (1.0 g) to the nearest 0.1 mg on an analytical balance. The biomass was transferred to a 100 mL beaker and 72% sulfuric acid (15 mL) was added slowly with very gentle stirring. The mixture was allowed to stand for two hours in a water bath at 20±1 ºC with frequent stirring. The prehydrolysed material was washed into a 1 L Erlenmeyer flask, and water (560 mL) was added for dilution of the sulfuric acid to 3%. An Erlenmeyer flask, 100 mL, was inverted in the neck of the larger flask and, after addition of porcelain chips to avoid solution bumping, the solution was boiled for 4 hours. Before boiling, the liquid level was noted on the flask and hourly additions of hot water to the flask maintained the original volume.

The insoluble lignin was allowed to settle overnight, and the liquor was filtered through a fritted-glass crucible which was previously dried at 105 ºC, kept in a desiccator, and weighted to the nearest 0.1 mg. Finally, the lignin residue was transferred completely to the crucible and washed free of acid and dissolved carbohydrates with hot water (500 mL minimum). The crucible and contents were dried at 105 ºC for two hours, cooled in desiccator, and weighted. The drying and weighing were repeated until constant weight, and yield was calculated according to the following equation:

$$\% \text{ Klason lignin} = (W_2 / W_1) \times 100$$

Where $W_1$ is the weight (g) of extractive-free biomass and $W_2$ is the weight (g) of condensed lignin. The moisture content of extractive-free biomass must be taken into account because lignin was dried at 105 ºC and extractive-free biomass was air dried.

Acid-soluble lignin

The acid-soluble lignin was determined based on Schöning and Johansson (1965). The filtrate from the Klason lignin (560 mL) was diluted to 1000 mL with 3% sulfuric acid. The absorption of the soluble lignin was measured at 205 nm in a differential U.V Perkin-Elmer spectrometer, model Coleman 124, using a 3% sulfuric acid solution as a reference solution. The cell path length was 1 cm. The filtrate was diluted 10 fold with 3% sulfuric acid to obtain absorbance in the range of 0.2 to 0.8. The acid-soluble lignin was calculated by the following equation:

$$\% \text{ Acid-soluble lignin} = \frac{(A / \varepsilon) \times D \times V}{1000 \times W} \times 100$$

Where: A = sample absorbance; $\varepsilon$ = 110 L $g^{-1}$ $cm^{-1}$ (average extinction coefficient for natural lignins isolated from different woods); D = dilution factor of the filtrate (expressed as $V_d / V_o$ where $V_d$ is the volume (mL) of the diluted filtrate and $V_o$ is the volume (mL) of the original amount of filtrate diluted); V = total volume of the filtrate (mL); W = oven-dry weight of biomass (g). The cell path length parameter has been omitted in the equation because it was the standard 1 cm.

Ash content

Using TAPPI standard method T 211 om-85 (1988), duplicate samples of extractive-free biomass were weighted ( 5.0 g) directly in porcelain crucibles that had

been previously heated at 575±25 °C, cooled in a desiccator and tared to the nearest 0.1 mg on an analytical balance. A lid was used to prevent loss during carbonization of biomass. The lignocellulosic material was burned before being placed in the muffle furnace. After this initial carbonization the crucible was placed in the muffle furnace at the same temperature (575±25 °C) to complete carbonization of the biomass to ash. The crucible was removed from the furnace and cooled in a desiccator to ambient temperature. The crucible was weighted to the nearest 0.1 mg and the percent of ash in the sample was calculated by the following expression:

$$\% \text{ Ash} = (W_2 / W_1) \times 100$$

Where $W_1$ is the weight (g) of extractive-free biomass and $W_2$ is the weight (g) of ash. The moisture content of extractive-free biomass must be taken into account because the ash was fully carbonized and desiccated, and extractive-free biomass was air dried.

## PERACETIC ACID PREPARATION AND ANALYSIS

Peracetic acid was prepared by reacting stabilized hydrogen peroxide, concentration of 35%, and reagent grade acetic anhydride in a 2:3 ratio respectively, according to Schroeder (1994). The reaction vessel (2 L Erlenmeyer flask) was placed into an ice bath and hydrogen peroxide (400 mL) was added to the reaction flask, cooled to approximately 10 °C while being mixed by a magnetic stirring bar. After equalizing the temperature, the acetic anhydride was added to the reaction flask dropwise or as a fine stream while stirring the contents of the flask. The reaction is

highly exothermic and the reaction temperature was maintained between 60-70 °C for obtaining the maximum concentration of peracetic acid. Temperatures above 80 °C must be avoided and always add the anhydride to the aqueous peroxide. The controlling of the temperature and the order of addition are important to avoid the risk of an explosion. The reaction solution was stirred overnight to allow further peracetic acid formation and equilibrium. The final concentration of peracetic acid is around 27-30% and the decomposition rate is approximately 0.2-1% per day at ambient temperature.

Peracetic acid solutions were analyzed in the presence of hydrogen peroxide by first titrating the hydrogen peroxide with potassium permanganate and then a separate iodometric titration of peracetic acid (Anonymous, 1965; Reichert et al., 1939). A 5 mL sample was first diluted to 250 mL in a volumetric flask. Three aliquots of 5 mL of the dilute peracetic acid were added to 125 mL Erlenmeyer flasks along with 1-2 mL of a saturated solution of $MnSO_4$ in 50% glacial acetic acid, and 1-2 mL of pure glacial acetic acid was also added. The solution was titrated with 0.1N $KMnO_4$ to a pale pink endpoint. To another three Erlenmeyer flasks, 10 mL of 10% KI solution and 50 mL of distilled water were added and stirred. An aliquot 5 mL of the same diluted peracetic acid was added to each flask followed by an addition of 1-2 mL of glacial acetic acid. The solution was stirred and titrated with $Na_2S_2O_4$ to yellow, then starch indicator was added and titrated to colorless. The residual hydrogen peroxide and peracetic acid concentrations were determined by the following equation:

$$\% H_2O_2 = \frac{(\text{mL of } KMnO_4)(\text{Normality of } KMnO_4)(1.7)}{(\text{dilution factor})}$$

$$\% \text{ Peracetic acid} = \frac{(\text{mL of } Na_2S_2O_4 - \text{mL of } KMnO_4)(\text{Normality of } Na_2S_2O_4)(3.8)}{(\text{dilution factor})}$$

## PRETREATMENTS

The ground biomasses were placed in plastic bags and peracetic acid solutions at loading of 0, 6, 9, 15, 21, 30, and 60% peracetic acid based on oven-dry biomass weight were added. The solutions were added at a ratio of 6:1 liquor to wood, mixed, and stored for a seven-day period at ambient temperature. The samples were washed with distilled water on Buchner funnels until the filtrate was pH 5. The wet samples were bagged in plastic and stored in a freezer for later analysis.

As an auxiliary method, a series of pre-pretreatments using 3 and 6% of sodium hydroxide and 2.63, 5.25 and 14.00 % of ammonium hydroxide based on oven-dry weight of biomass were performed. These alkaline solutions were mixed with ground hybrid poplar at ratio of 14:1 and a ratio of 17:1 for sugar cane bagasse. After 24 hours at room temperature the biomass was washed to neutral pH with distilled water after transferring to Buchner funnels. Subsequent 6, 9 and 15% of peracetic acid loadings based on oven-dry biomass were added to this material using the conditions for peracetic acid treatment described above.

## SUBSTRATE ANALYSIS (PRETREATED BIOMASS)

### Carbohydrate composition

Two stage acid hydrolysis was performed for determining carbohydrate composition on the peracetic acid and the alkaline/peracetic acid pretreated samples, and the raw hybrid poplar and sugar cane bagasse according to a standard procedure of the National Renewable Energy Laboratory, Golden, CO (Ehrman, 1992). Triplicate samples of pretreated biomass, fully dried under vacuum at 45 ºC, were weighted

(0.3 g) into 25 mL borosilicate glass sample vials to the nearest 0.1 mg on an analytical balance. Sulfuric acid 72% (3 mL) was added and mixed carefully using a small glass rod. The vials were placed in a water bath at 30 ± 1 °C for 2 hours with frequent stirring. The prehydrolyzed material was quantitatively transferred to 100 mL serum bottles, using 84.0 mL of distilled water, resulting in a 3% acid solution. The serum bottles were tightly sealed with crimp cap and silicone septum and autoclaved for 1 hour in wet cycle at 121 °C. The autoclaved bottles were removed from the autoclave and cooled for 30 min before being placed in a water bath for further cooling. The resulting solution was transferred to a 100 mL volumetric flask and made to volume with distilled water. The volumetric flask contents were mixed vigorously for homogenization and a 20 mL aliquot pipeted into a 250 mL Erlenmeyer flask. Calcium carbonate (0.7 g) was added slowly to the flask for acid neutralization and let stand for 10 min. The pH was checked with pH indicator strips and a desired pH = 6 was attained. Additional calcium carbonate might be used if the pH is still lower than 6. The neutralized solution was mixed and passed through a 0.45 μm filter, and using a 5 mL syringe transferred directly to an autosampler vial. The vial was sealed and the hydrolyzed sugars were analyzed using HPLC equipped with a 300 x 7.8 mm HPX-87P Bio-Rad column, specific for isolation of sugar obtained by hydrolysis of wood, coupled to a Waters Associates refractive index (RI) detector. Sugar degradation was corrected by running separately glucose, xylose, arabinose, mannose, and galactose along with pretreated biomass during the hydrolysis steps. A series of standards of the sugars mentioned was needed for preparing calibration curves starting at the detection limit of the instrument and extending up to 4.0 mg/mL. Sugar content was calculated according to the following equations:

$$\% \text{ Sugar recovered} = \frac{\text{concentration detected by HPLC, mg/mL}}{\text{known conc. of sugar before hydrolysis, mg/mL}} \times 100$$

$$\text{Corrected sugar conc., mg/mL} = \frac{\text{sugar conc. obtained by HPLC, mg/mL}}{\frac{\text{\% sugar recovered}}{100}}$$

$$\% \text{ Sugar} = \frac{\text{corrected sugar conc., mg/mL} \times (1 \text{ g}/1000 \text{ mg}) \times \text{volume filtrate, mL}}{\text{sample weight, g}} \times 100$$

Klason and acid-soluble lignin

Klason and acid-soluble lignin determinations were performed on pretreated biomasses according to previously described methods in chemical composition of biomass section without residual extractives being removed by any solvent extraction method.

Acetyl content

The acetyl content in the pretreated biomasses was performed according to a modified procedure for acetylated carbohydrate polymers described by Browning (1967) and by McComb and McCready (1957). The reaction time between hydroxylamine and acetyl groups present in the biomass was increased from 15 min to 150 min as a result of empirical experiments performed with raw hybrid poplar(Fig. 3.1).

Triplicate samples of pretreated hybrid poplar and sugar cane bagasse, estimated to contain 2-11 mg of acetyl, were weighed and placed in a 100 mL beaker. While the mixture was stirred with a magnetic stirrer, 25 mL of a 3.75% hydroxylamine solution was added. Then 25 mL of a 9.4% sodium hydroxide solution was added dropwise during the first 15 min. After 150 min of mixing at ambient temperature, the

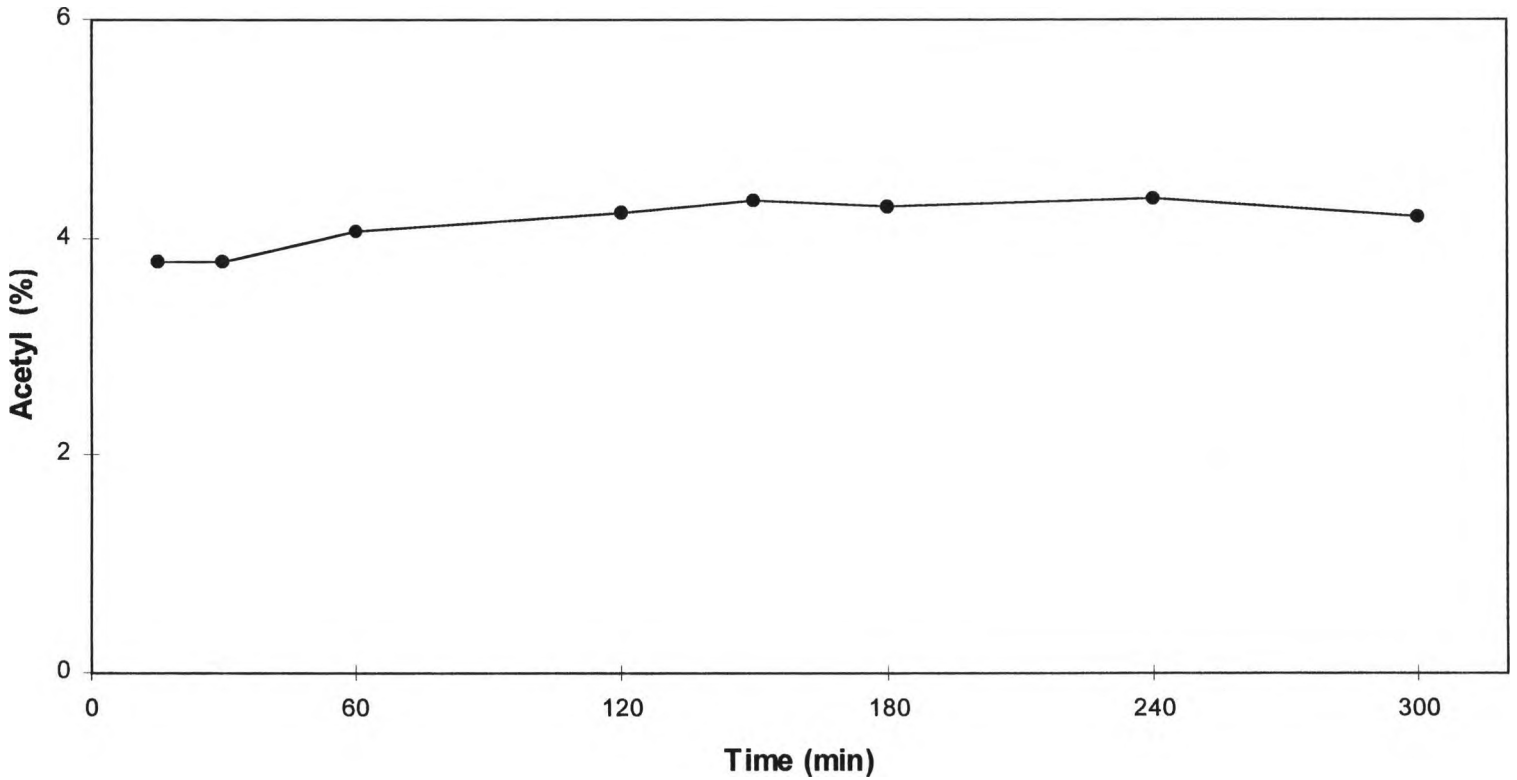



Fig. 3.1. Empirical optimization of reaction time between hydroxilamine and acetyl groups present in hybrid poplar. Acetyl content is based on oven-dry weight of wood.

slurry was coarsely filtered through glass wool, and an aliquot of 2 mL of solution pipeted into a 25 mL volumetric flask. Five mL of water and 5 mL acid-methanol solution (a) were added and mixed. The solution was made to volume with ferric perchlorate solution (b) added in small increments with thorough mixing after each addition. After 5 min, the solution was passed through a Whatman No. 12 filter paper to remove the precipitate of pectin hydroxamic acid-ferric complex and the absorbance, of the acetohydroxamic acid formed, was measured at 520 nm on a spectrophotometer Bausch & Lomb, model Spectronic 20. The acetyl content was calculated by use of a standard curve obtained using four different weighed glucose pentaacetate reference samples ran in parallel to the lignocellulosic samples. A blank was also performed for zeroing the spectrophotometer.

(a). Acid-methanol solution: 35.2 mL of perchloric acid 70% was chilled and made to 500 mL with chilled reagent grade absolute methanol.

(b). Ferric perchlorate: 1.93 g of $FeCl_3 \cdot 6H_2O$ was dissolved in concentrated hydrochroric acid (5 mL), perchloric acid (5 mL, 70%) was added, and the solution was evaporated almost to dryness. The residue was diluted to 100 mL with water for use as a stock solution. Perchloric acid (8.3 mL, 70%) was added to 60 mL of the stock ferric perchlorate solution, cooled in ice, and made to 500 mL with chilled reagent grade absolute methanol. Caution! Normal precaution should be taken in mixing, handling, and storing reagents containing perchloric acid.

ENZYMATIC HYDROLYSIS STANDARD TESTS

The commercial enzymes, Novo SP431 (xylanase), Celluclast (cellulase), and Novozym 188, (β-glucosidase), used in the enzymatic hydrolysis tests were provided by Novo Nordisk Biochem North America, Inc., Franklinton, North Carolina. The enzyme Spezyme (cellulase) was produced by Environmental Biotechnologies, Inc., Santa Rosa, California. The cellulase activities were found to be 39.2 IFPU/mL for SP431, 90.6 IFPU/mL for Celluclast, 91.4 IFPU/mL for Spezyme and less than 1 IFPU/mL for Novozym 188 using IUPAC procedure (Ghose, 1987) . The xylanase activity was 5,452.0 IU/mL for SP431, 826.0 IU/mL for Celluclast, 3,567.0 IU/mL for Spezyme and 560.0 IU/mL for Novozym 188 based on an interlaboratory testing method (Bailey, 1992). The β-glucosidase activity was 27.0 pNPGU/mL for SP431, 37.0 pNPGU/mL for Celluclast, 40.0 pNPGU/mL for Spezyme and 226.0 pNPGU/mL for Novozym 188 (Burden and Whitney, 1995).

The enzymatic hydrolysis tests according to an NREL procedure (Philippidis. 1993) modified without addition of YP (yeast-peptone) medium, were performed in 250 mL Erlenmeyer flasks with a biomass loading based of 1 g cellulose content to 100 mL of 0.05 M acetate buffer (pH 5). Flasks were autoclaved for 20 min and after cooling, sterile distilled water was added to obtain the original weight. Enzyme loading was calculated to be 25 IFPU/g of cellulose. Depending on the combination of enzymes used, xylanase and total β-glucosidase loadings vary as shown in Table 4.4. on page 71. The temperature was maintained at 37 °C in an incubator shaker operating at 125 rpm. Note: According to a product data sheet from Novo Nordisk Biochem North America, Inc., Franklinton, NC, the optimum temperature for cellulases is around 50-60°C but enzymatic tests were performed at a lower temperature because

37 °C is the maximum temperature tolerated by most microorganisms used in simultaneous saccharification and cofermentation tests (Saddler et al, 1982; Takagi et al, 1977). Samples for sugar analyses were collected in a laminar flow hood with sterile plastic pipets and directly transferred to 1 mL capped plastic tubes at 0, 5, 10, 24, 48, and 120 hours of enzymatic hydrolysis. The plastic tubes were boiled for five minutes to inactivate the enzymes, cooled and centrifuged. Supernatant was passed through a 45 µm filter, and using a 5 mL plastic syringe transferred directly to a 1 mL HPLC plastic vial. The hydrolyzed sugars were analyzed using the same equipment described previously in the carbohydrate composition section. The glucan and xylan conversion yields were calculated from results of sugar analyses as described previously in the carbohydrate composition section using the following equations:

$$\text{Glucan convesion yield (\%)} = \frac{[\text{Glucose}] + 1.053\,[\text{Cellobiose}]}{1.111\ f\,[\text{Biomass}]} \times 100$$

Where:

[Glucose] Residual glucose concentration (g/L)

[Cellobiose] Residual cellobiose (g/L)

[Biomass] Dry biomass concentration at the beginning of the hydrolysis (g/L)

f Cellulose fraction in dry biomass (g/g)

$$\text{Apparent xylan conversion yield (\%)} = \frac{[\text{Xylose}]}{1.136\ f\,[\text{Biomass}]} \times 100$$

Where:

| | |
|---|---|
| [Xylose] | Residual glucose concentration (g/L) |
| [Biomass] | Dry biomass concentration at the beginning of the hydrolysis (g/L) |
| f | Apparent xylan fraction in dry biomass (g/g) |

## SIMULTANEOUS SACCHARIFICATION AND CO-FERMENTAION TESTS - SSCF

The SSCF tests were performed according to a modification of standard procedures (Philippids et al, 1993). A combination of the enzymes Novozym 188 and SP431 (Novo Nordisk, Franklinton, NC) was used at a loading of 8.33 IFPU/g of cellulose. Enzyme loading was reduced three-fold compared to the loading used in the enzymatic hydrolysis tests with the intent of avoiding high accumulation of sugars in the first hours of the process. Farid et al. (1983), have obtained a satisfactory and linear sugar release, almost 0.5 g of glucose per hour, using a cellulase loading of 8 IFPU/g in sugar cane bagasse pretreated with peracetic acid. Complete enzymatic hydrolysis conversion of steam-pretreated aspen post-treated with hydrogen peroxide was achieved in three days using a loading of 10 IFPU/g cellulose. Using higher loading of 16 IFPU/g cellulose the same yield was obtained with the only difference being a time reduction, from 3 to 2 days (Breuil et al., 1990). Economy using less enzyme was also considered based on a statement that the production of enzymes is the most expensive step in ethanol production from lignocellulosic materials (Wilke et al., 1976). In spite of enzyme loading being lowered three-fold, each flask still had a total of 25 IFPU.

The optimum temperature growth for *Z. mobilis* has been demonstrated 30 °C (Grohmann, 1993; Rogers et al, 1982; Swings and De Ley, 1977) but the temperature

of 37 °C, common in SSF tests, was maintained in order to avoid decrease in the enzyme activity. Two cultures collections strains CP3 and CP4 were reported to grow at 42 °C (Montenecourt, 1985; Golçalves de Lima et al., 1970). Saddler et al. (1982) obtained good ethanol yields from steaming exploded poplar with excellent glucose consumption in six-days of simultaneous sacchrification and fermentation experiments performed at 37 °C using *Z. mobilis* Z2 (ATCC 29191). According to Spangler et al (1986), the glucose was completely utilized in 48 hours of fermentation using *Z. mobilis* strain ATCC 29501 at 37 °C and at 38 °C only 10% of glucose was not utilized. In addition, the specific growth rate was the same at both temperatures of 30 and 37 °C and the specific glucose up-take rate was even higher at 37 °C than at 30 °C for *Z. mobilis* ZM4 (CP4), (Rogers et al, 1982; Lee et al, 1981)

The amount of biomass was calculated based on 3g cellulose and weighted directly into 250 mL an Erlenmeyer flask to the nearest 0.01 g. Xylan content varied for each sample depending on its content in the biomass. Distilled water (approximatelly 60 mL) was added to each flask, and the pH was adjusted to 5. A rubber stopper gas trap was placed onto the Erlenmeyer flask. Weight of flask and contents was recorded and then the flask was autoclaved for 20min. After cooling, sterile distilled water was added to achieve the initial weight. One mL of sterile tetracycline solution containing 1mg of the antibiotic was added to each flask. Following addition of 10mL of sterile 10X yeast extract medium, 10mL of inoculum prepared as described below was added. Flasks were placed in the shaker at 37 °C and 150rpm. Samples were collected at 0, 1, 2, 3, 5, 7, and 10 days as described in the previous section for later HPLC analysis and the only difference was that the capped plastic tube was not boiled for inactivating the enzymes because of ethanol volatility. The formation of ethanol, cellobiose, glucose, xylose, and acetate was

measured by HPLC using the same equipment described in carbohydrate composition section, using a 300 x 7.8 mm HPX-87H Bio-Rad column, specific for isolation of organic acids . The ethanol yields were calculated according to the following equation:

$$\text{Ethanol yield (\%)} = \frac{[EtOH]_f - [EtOH]_o}{0.51\,[(\text{Glucan} \times 1.111) + (\text{Xylan} \times 1.136)]} \times 100$$

Where:

$[EtOH]_f$ Ethanol concentration at the end of cofermentation (g/L)

$[EtOH]_o$ Ethanol concentration at the beginning of the cofermentation (g/L)

[Glucan] Glucan loading (g/L)

[Xylan] Xylan loading (g/L)

The practical conversion factor of 0.51, xylose to ethanol, is the same as determined for glucose (McMillan, 1996).

Frozen cultures of recombinant *Zymomonas mobilis* CP4/pZB5 that was supplied by NREL was used for inoculum. The inoculum preparation was performed using a medium containing 0.7% of glucose, 0.3% of xylose, 1.0% of yeast extract, 0.2% of $HK_2PO_4$, and 1mg of tetracycline. The frozen culture first was transferred to a test tube containing 10mL of sterile medium and placed in the incubator at 37°C with no shaking for 12 hours. This was then transferred to a 500mL Erlenmeyer flask with 300mL of the same medium. The inoculum was ready for use within 12-16 hours after the second transfer.

# CHAPTER 4

# RESULTS AND DISCUSSION

## Chemical composition of the biomasses

The chemical compositions of the hybrid poplar wood and sugar cane bagasse used in this studies are shown in Table 4.1. The extractive content in sugar cane bagasse is greater than in hybrid poplar due to residual sucrose from industrial processing. The main components of the two biomasses: cellulose, hemicellulose, and lignin are in accord with literature data in Table 2.2. Seifert cellulose content in hybrid poplar *(P. deltoides x P. nigra)* is slightly lower compared to previous analysis for the same hybrid poplar clone, Brito (1994).

## Pretreatment time effect

Since peracetic acid pretreatment is being evaluated at ambient temperature, a series of pretreatments were performed to define the minimum reaction time necessary for completing the reaction. Hybrid poplar was used. Two concentrations of peracetic acid were evaluated, 6 and 9% based on oven-dry weight of wood, through 3, 7, 14, and 28 days of contact time. According to Fig. 4.1 and Appendix I, Table 1, as indicated by the release of glucose from the substrate, a seven-day period was found to be a reasonable period for use in further pretreatments. As shown in Appendix I, Table 1, three-day samples contain slightly more lignin than samples from other

Table 4.1 Chemical composition of hybrid poplar and sugar cane bagasse

| COMPONENT | HYBRID POPLAR WOOD AMOUNT (%) | SUGAR CANE BAGASSE AMOUNT (%) |
|---|---|---|
| Cellulose* | 41.7 | 39.6 |
| Pentosans* | 18.4 | 29.7 |
| Lignin: Klason* | 25.0 | 22.2 |
| Soluble* | 3.4 | 2.5 |
| Holocellulose* | 70.4 | 74.5 |
| Ash* | 0.8 | 4.1 |
| Extractives | 4.8 | 14.3 |

* Results based on extractive free biomass

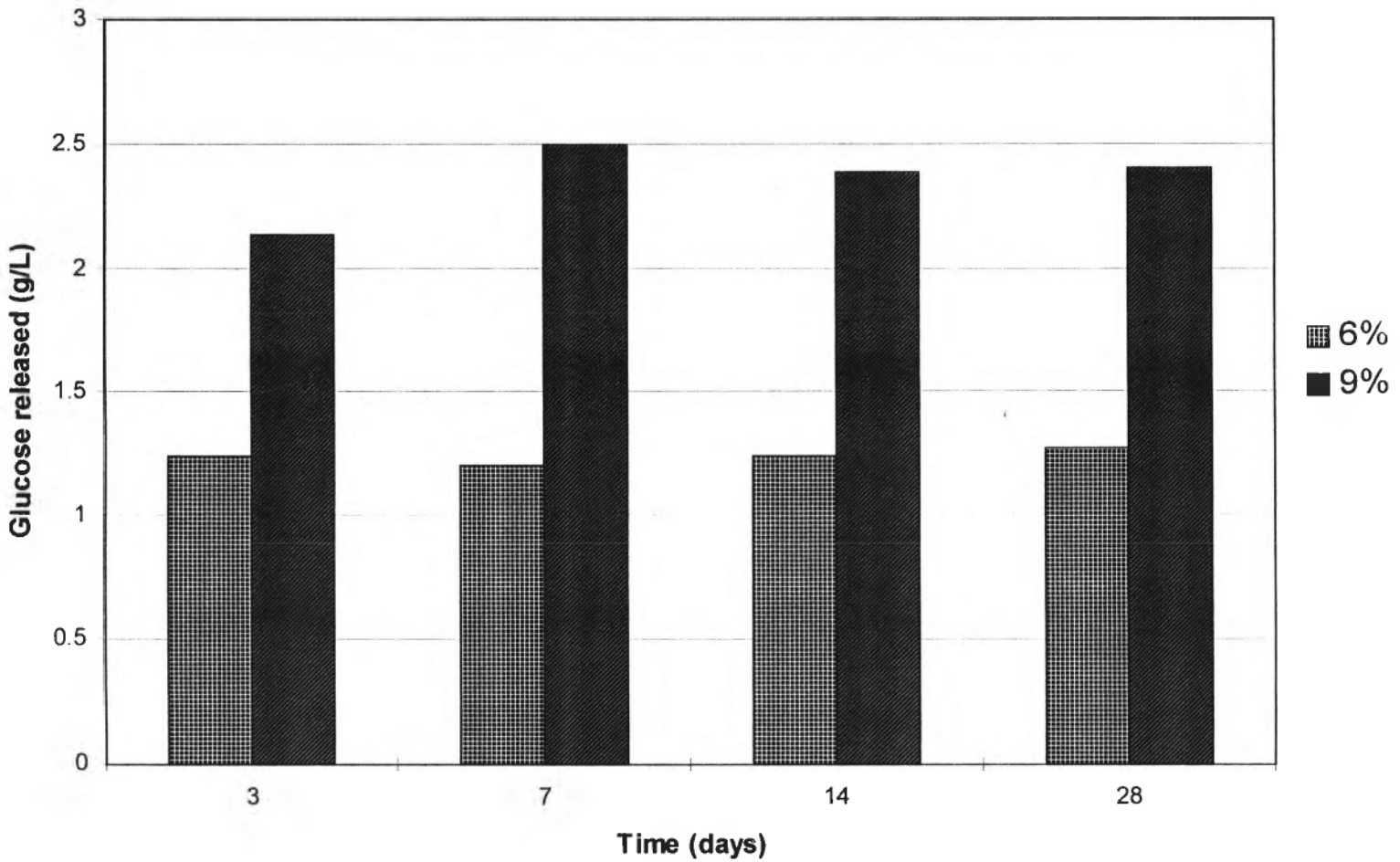



Fig. 4.1. Time effect of peracetic acid on enzymatic hydrolysis of hybrid poplar. Concentrations of peracetic acid are based on oven-dry weight of wood. The combination of Spezyme/Novozym 188 has been used with a total loading of 25 IFPU/g cellulose.

conditions. This indicates that the peracetic acid still has some effect after the three-day period.

Pretreatment overall yields

Peracetic acid and alkaline/peracetic acid pretreatments are compared to conventional pretreatments. Brito (1994) obtained corresponding yields from dilute sulfuric acid prehydrolysis of hybrid poplar of less than 70 percent. A similar average yield has been by published by Grohmann et al. (1985). Schwald et al. (1989) reported recovery yields that varied from 49.9 to 63.0%, depending on temperatures used, from steam explosion pretreatment of aspenwood. Mackie et al. (1985) have presented recovery yields from 65.7 to 70.8% for steam explosion of the same hardwood. Dekker and Wallis (1983) presented overall solid yield of approximately 60% from steaming exploded bagasse. The losses from other dilute acid pretreatment methods are relatively great compared to the average 85 percent yield obtained here (Table 4.2). In Table 4.3 the yields of pretreated bagasse are less than the yields noted in Table 4.2 because approximately 10% sucrose remains in the extracted bagasse used as substrate for these pretreatments (unpublished data). Peracetic acid when used at higher temperatures, 100°C, and higher concentrations than used here gave lower overall yields, from 38 to 70%, depending on acid concentration, for woody biomasses (Toyama and Ogawa,1975).

With both substrates treated with the higher concentrations of peracetic acid, lignin fragmentation reduces the Klason lignin values and pretreatment yields. Soluble lignin concentration increases with the loading concentrations except at the 60% peracetic acid loading. At this loading, the soluble lignin content is probably reduced because the oxidative fragmentation of the phenyl ring structures which decreases the

Table 4.2. Partial composition and yield of pretreated hybrid poplar. The xylan content is reported as a linear polymer not considering the side groups of 4-methyl-glucuronic acid and acetyl.

| Pretreatment condition | Glucan (%) | Xylan (%) | Klason lignin (%) | Soluble lignin (%) | Pretreatment overall yield(%) |
|---|---|---|---|---|---|
| 0% peracetic acid (raw wood) | 39.8 | 13.4 | 25.4 | 2.5 | 99.0 |
| 6% peracetic acid | 41.5 | 13.7 | 22.0 | 3.0 | 97.2 |
| 9% peracetic acid | 42.0 | 14.2 | 20.5 | 3.6 | 94.8 |
| 15% peracetic acid | 44.0 | 15.4 | 18.1 | 3.9 | 91.5 |
| 21% peracetic acid | 46.2 | 16.5 | 12.5 | 4.4 | 87.3 |
| 30% peracetic acid | 52.2 | 17.4 | 7.0 | 4.7 | 81.7 |
| 60% peracetic acid | 55.6 | 18.5 | 1.3 | 3.4 | 71.4 |
| A 6% NaOH - 15% peracetic acid | 50.0 | 17.1 | 15.1 | 3.6 | 77.9 |
| B 6% NaOH - 9% peracetic acid | 46.8 | 15.7 | 12.0 | 3.9 | 84.0 |
| C 3% NaOH - 15% peracetic acid | 47.3 | 16.7 | 17.6 | 3.4 | 84.8 |
| D 3% NaOH - 9% peracetic acid | 45.5 | 15.7 | 12.0 | 5.7 | 87.6 |
| 14% $NH_4OH$ - 15% peracetic acid | 45.9 | 16.5 | 17.3 | 4.7 | 84.8 |
| E 5.25% $NH_4OH$ - 15% peracetic acid | 45.7 | 15.7 | 13.1 | 5.0 | 85.4 |
| F 5.25% $NH_4OH$ - 9% peracetic acid | 43.9 | 14.5 | 17.1 | 4.8 | 88.9 |
| G 2.63% $NH_4OH$ - 15% peracetic acid | 44.9 | 15.5 | 13.9 | 4.9 | 88.2 |
| H 2.63% $NH_4OH$ - 9% peracetic acid | 43.6 | 14.3 | 18.7 | 4.4 | 89.8 |

Table 4.3. Partial composition and yield of pretreated sugar cane bagasse. The xylan content is reported as a linear polymer not considering any side groups.

| Pretreatment condition | Glucan (%) | Xylan (%) | Klason lignin (%) | Soluble lignin (%) | Pretreatment overall yield (%) |
|---|---|---|---|---|---|
| 0% peracetic acid (raw bagasse) | 39.9 | 23.4 | 22.0 | 2.1 | 88.2 |
| 6% peracetic acid | 42.0 | 23.9 | 20.5 | 2.6 | 85.8 |
| 9% peracetic acid | 43.3 | 24.7 | 18.6 | 3.4 | 84.6 |
| 15% peracetic acid | 44.2 | 25.2 | 14.1 | 4.3 | 81.6 |
| 21% peracetic acid | 49.1 | 26.5 | 10.8 | 5.1 | 78.4 |
| 30% peracetic acid | 53.4 | 27.4 | 6.3 | 5.1 | 74.6 |
| 60% peracetic acid | 55.9 | 27.7 | 4.3 | 3.3 | 68.6 |
| 6% NaOH - 15% peracetic acid | 50.1 | 26.6 | 11.4 | 4.1 | 74.6 |
| 6% NaOH - 9% peracetic acid | 47.9 | 25.5 | 16.5 | 3.5 | 78.0 |
| 6% NaOH - 6% peracetic acid | 46.9 | 25.0 | 17.2 | 3.0 | 78.0 |
| 3% NaOH - 15% peracetic acid | 47.7 | 25.6 | 15.6 | 4.9 | 77.8 |
| 3% NaOH - 9% peracetic acid | 45.4 | 24.9 | 12.9 | 3.6 | 80.8 |
| 5.25% $NH_4OH$ - 15% peracetic acid | 46.5 | 25.4 | 13.0 | 4.9 | 77.2 |
| 5.25% $NH_4OH$ - 9% peracetic acid | 45.1 | 24.7 | 16.0 | 4.2 | 80.1 |
| 5.25% $NH_4OH$ - 6% peracetic acid | 44.1 | 24.0 | 19.8 | 4.0 | 81.4 |
| 2.63% $NH_4OH$ - 15% peracetic acid | 45.6 | 25.3 | 14.1 | 5.3 | 79.3 |
| 2.63% $NH_4OH$ - 9% peracetic acid | 44.6 | 24.6 | 17.2 | 3.9 | 81.3 |

absorption at 205 nm. The carbohydrate contents are greater at the higher loading values because of lignin solubilization. However, the relative carbohydrate losses are low as demonstrated by the constant ratio of glucan to xylan independent of peracetic acid loading. If carbohydrate loss were a significant factor, the xylan content would be expected to decrease with severity of the pretreatment. In Tables 4.2 and 4.3, the xylan is reported based on the HPLC analysis of xylose in the two-stage acid hydrolysis procedure. Hence, 4-methyl-glucuronic acid and acetyl contents are not included in the xylan composition. The reported values consider only the linear polymer without attached functional groups, and therefore are lower than actual compositions.

Lignin retention as a function of the increase of peracetic acid loading

According to Figs. 4.2 and 4.3, the increase of peracetic acid loading diminishes the amount of lignin in both biomasses, hybrid poplar and sugar cane bagasse. The lignin retention diminishes as a function of the concentration of the oxidative acid and the carbohydrate hydrolysis conversion increases. The latter is inversely related to lignin removal. The retained lignin in hybrid poplar is a little lower than in sugar cane bagasse after peracetic acid pretreatment, especially for higher concentrations of peracetic acid. Hybrid poplar delignifies more easily than sugar cane bagasse under the pretreatment conditions used. In both biomasses, the loading of 21% of peracetic acid is the minimum amount required for satisfactory sugar yields, which are respectively 100% for glucose and 90% for xylose.

Enzyme screening tests

Considering that the substrates still have high amounts of xylan after the pretreatments (Tables 4.2 and 4.3), a series of tests using two cellulases containing

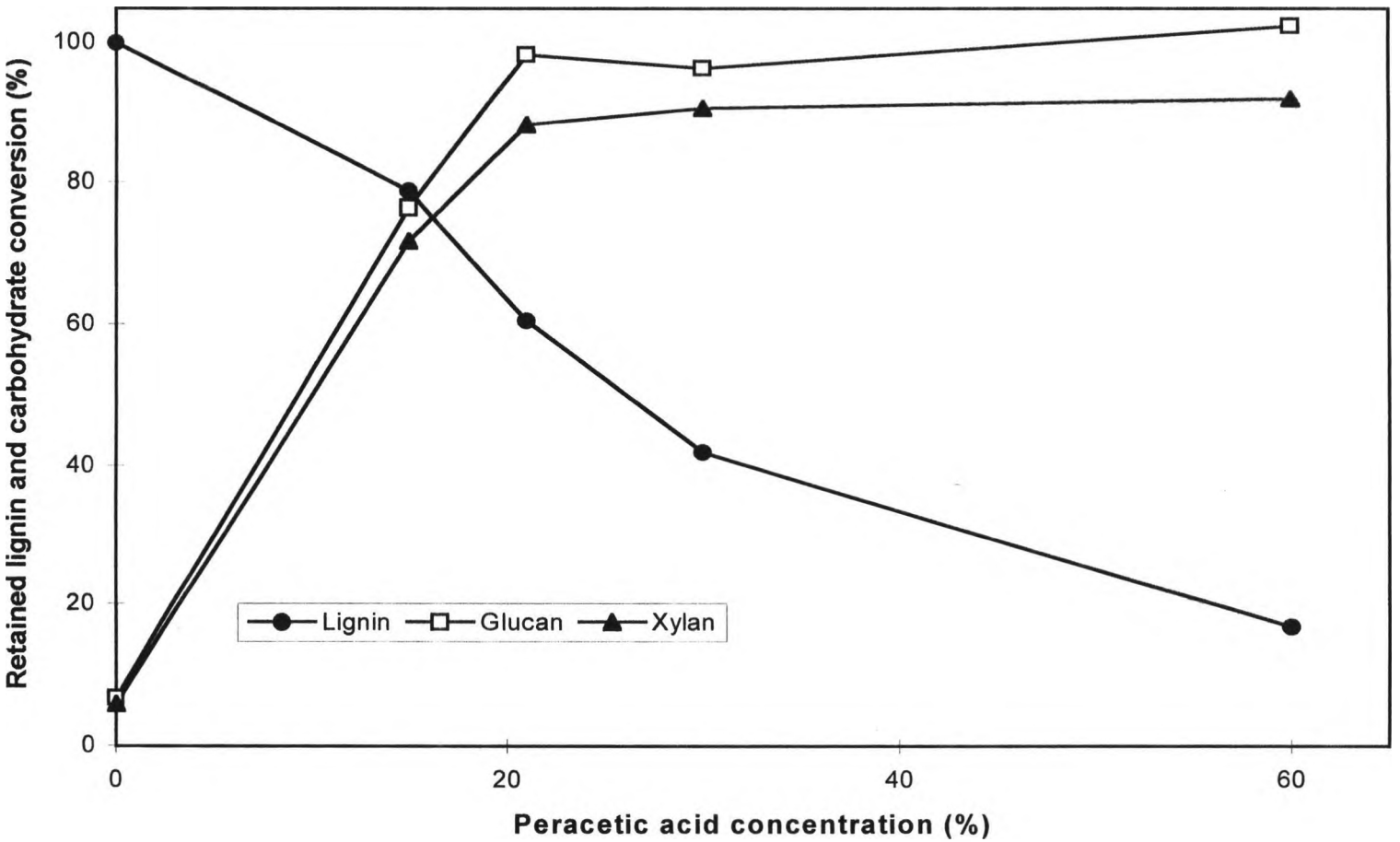



Fig. 4.2. Lignin retention and carbohydrate hydrolysis conversion as a function of peracetic acid concentration for hybrid poplar. Amount of acid is based on oven-dry weight of wood in a ratio of 1:6 biomass to water.

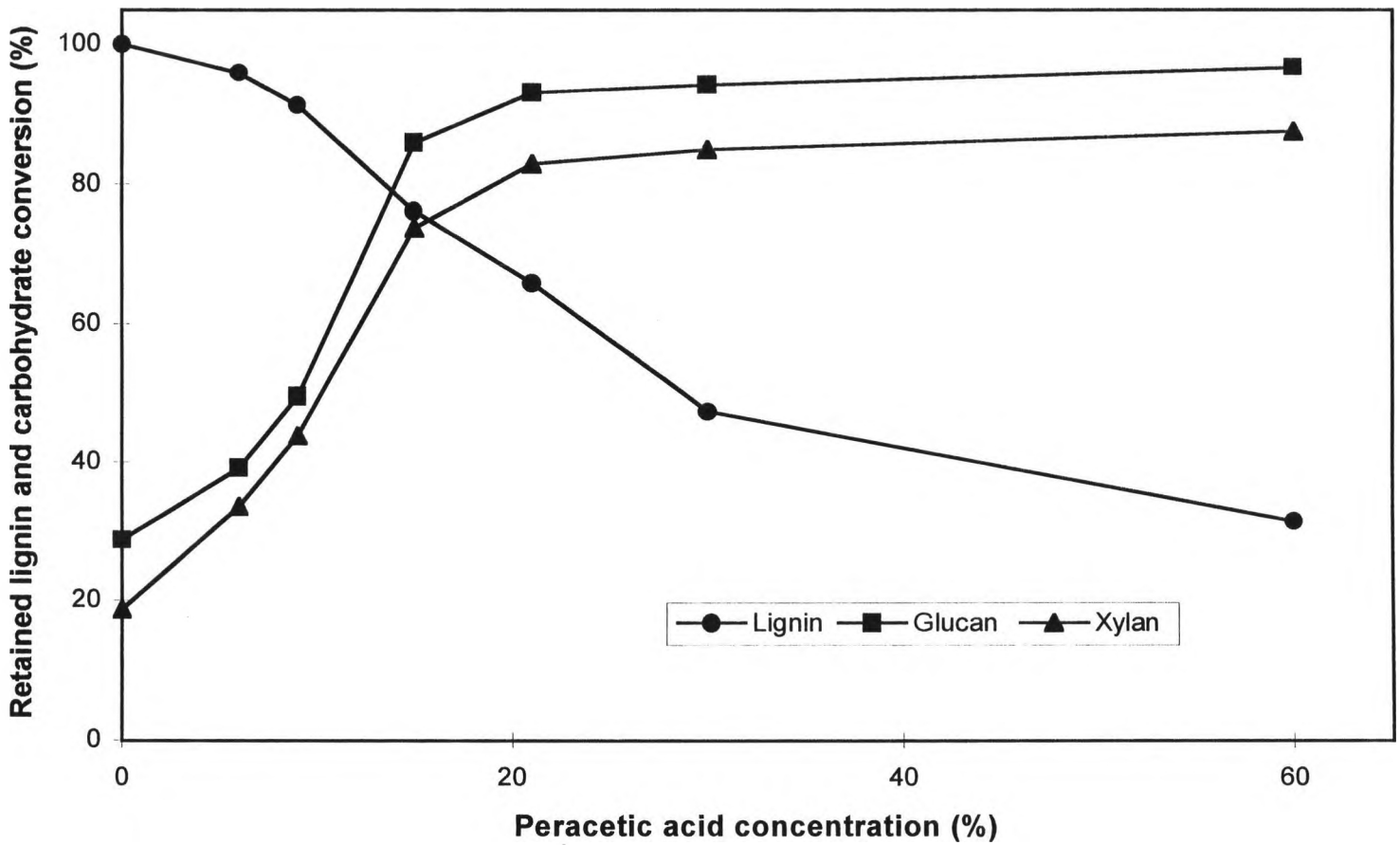



Fig. 4.3. Lignin retention and carbohydrate hydrolysis conversion as a function of peracetic acid concentration for sugar cane bagasse. Amount of acid is based on oven-dry weight of biomass in a ratio of 1:6 biomass to water.

xylanases (Celluclast and Spezyme), one xylanase (SP431) and a β-glucosidase (Novozym 188) were performed to define and choose the best enzyme, or combination between enzymes, to promote better yields in the enzymatic conversion of peracetic acid pretreated substrates. Preliminary results showed that the combination of the xylanase SP431 and Novozym 188 was more efficient in the enzymatic hydrolysis tests than other combinations. Figure 4.4 shows the behavior of 15% peracetic acid treated hybrid poplar with different enzymes and their combinations. When using different cellulase combinations, for example, Celluclast/Novozym 188 or Spezyme/Novozym 188, the results were less than satisfactory. The difference can be explained by the higher amount of xylanase activities present in the SP431/ Novozym 188 combination than in the others (Table 4.4). In addition, these results show that the addition of Novozym 188 caused significant improvement when compared to the use of single cellulases. Synergetic effects of β-glucosidase in cellulases as verified here have been reported in the literature (Breuil, 1992; Dekker and Wallis, 1983). The SP431, when used alone, gives a good xylose yield on xylan hydrolysis when compared to cellulases carrying xylanases reinforcing the decision of using it as the enzyme of choice for further tests (Fig. 4.4B). Celluclast when used alone or in combination with Novozym 188 showed poor glucan and xylan conversion yields because they contained low amounts of xylanases (Fig. 4.4).

Enzymatic hydrolysis tests of peracetic acid treated biomass

As stated in the methodology, the enzymatic hydrolysis tests were performed on both substrates, pretreated hybrid poplar and sugar cane bagasse, with the intent of evaluating the pretreatment efficiency on biomass conversion to sugars. Furthermore,

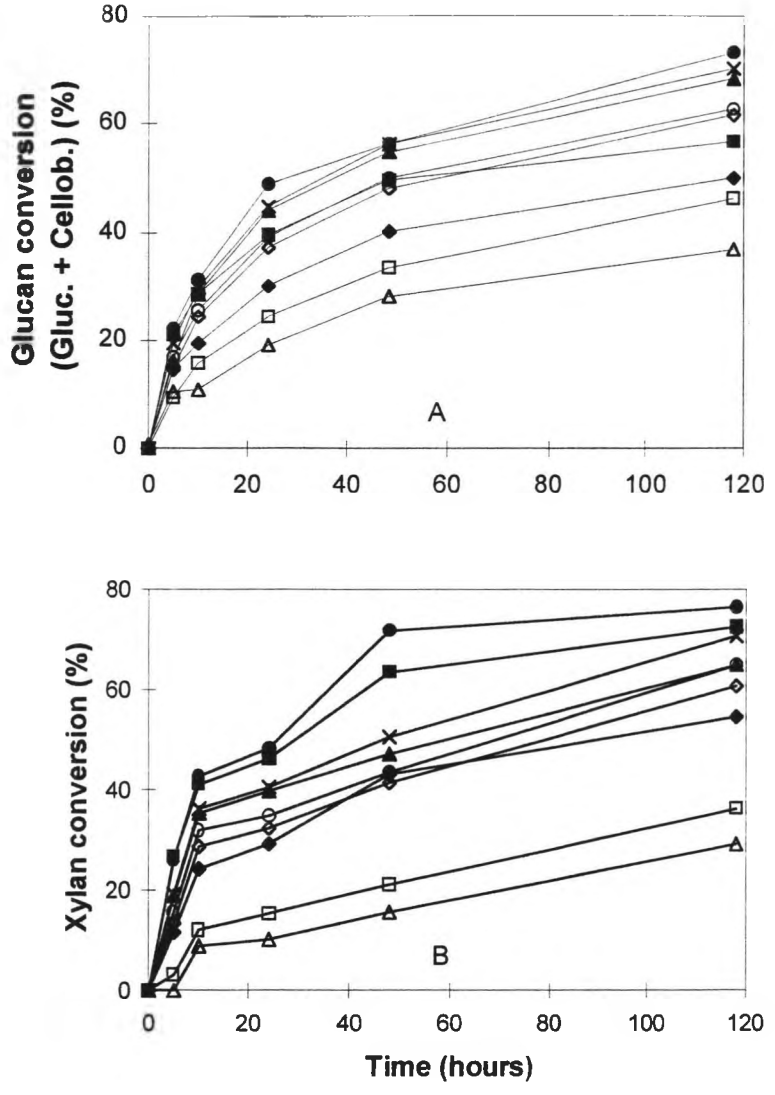



Fig. 4.4. Enzyme pool test for identifying the best enzyme combination using the 15% peracetic acid hybrid poplar treated sample. An equal loading of 25.0 IFPU per gram of cellulose was added to each sample. A. glucan conversion as percent of total glucan. B. xylan conversion as percent of apparent xylan. The xylanase varied in the amounts present in the various enzyme preparations (see Table 4.4). Legend: ● SP431/Novozym 118 combination, ○ Celluclast/SP431/Novozym 188 combination, ■ SP431 only, □ Celluclast/Novozym 188 combination, △ Celluclast only, x Spezyme/SP431/Novozym 188 combination, ◆ Spezyme only, ▲ Celluclast/ Spezyme/SP431/Novozym 188 combination, ◇ Spezyme/Novozym 188 combination.

Table 4.4. Enzymatic loading and activities in various combinations of industrial enzymes used to hydrolyze peracetic acid pretreated hybrid poplar.

| Enzyme combinations | % of enzyme | Total cellulase loading IFPU/g cellulose | Total xylanase loading IU/g cellulose | Total β-glucosidase loading IU/g cellulose |
|---|---|---|---|---|
| SP431 | 76.2 | 25.0 | 3,601.3 | 62.5 |
| Novozym 188 | 23.8 | | | |
| SP431 | 100.0 | 25.0 | 3,489.3 | 17.3 |
| Spezyme | 57.9 | 25.0 | 1,096.2 | 56.2 |
| Novozym 188 | 42.1 | | | |
| Spezyme | 100.0 | 25.0 | 984.2 | 11.0 |
| Spezyme | 39.1 | 25.0 | 1,617.5 | 76.7 |
| SP431 | 24.5 | | | |
| Novozym 188 | 36.4 | | | |
| Celluclast | 100.0 | 25.0 | 227.2 | 10.2 |
| Celluclast | 57.9 | 25.0 | 339.2 | 55.4 |
| Novozym 188 | 42.1 | | | |
| Celluclast | 39.1 | 25.0 | 1,025.6 | 56.8 |
| SP431 | 24.5 | | | |
| Novozym 188 | 36.4 | | | |
| Celluclast | 19.5 | 25.0 | 1,321.5 | 57.1 |
| Spezyme | 19.5 | | | |
| SP431 | 24.6 | | | |
| Novozym188 | 36.4 | | | |

the best conditions for Simultaneous Saccharification and Co-Fermentation tests (SSCF) were defined.

The combination of SP431 and Novozym 188 was used with the 0, 15, 21, 30, and 60% treated peracetic acid poplar samples as described in the methodology and the results are shown in Fig. 4.5 and 4.6. The 60% treated hybrid poplar sample was readily converted to sugars in only 24 hours (Fig. 4.5 and 4.6). Considering the good releasing sugar pattern, the 21% loading pretreated hybrid poplar is acceptable for attaining the requirements for Simultaneous Saccharification and Co-Fermentation (Philippidis 1996) using the recombinant *Zymomonas mobilis* developed by Zhang et al. (1995). The data indicate 98.3% of the cellulose and 88.2% of the apparent xylan in the 21% loading pretreated hybrid poplar are converted to respective simple sugars in 120 hours (Fig. 4.5 and 4.6). The 0% treated sample, raw wood, had a very poor conversion of only 6.8% of cellulose and 6.1% of apparent xylan to simple sugars at the end of the hydrolysis period (Fig. 4.5 and Fig. 4.6). Sugar yields are higher here than found in the literature, for similar concentrations of 14 and 28% of peracetic acid based on oven-dry weight of wood and higher temperature, 100°C, with Toyama and Ogawa (1975) reporting respective sugar yields of 30.2 and 66.9%. These lower results can be a consequence of using only cellulase instead of also adding xylanase activity, which has been not presented in the reference. When peracetic acid was used at higher concentrations of 70 and 140% based on oven-dry weight of wood, yields of 92.3 and 90.6% were achieved (Toyama and Ogawa, 1975).

For peracetic acid treated sugar cane bagasse , the 21, 30, and 60% peracetic pretreated samples show the best results on biomass conversion to simple sugars (Fig. 4.7 and 4.8). The 21% treated bagasse looks very acceptable for attaining the SSCF requirements with a conversion of 93.1 and 82.9% respectively for glucan and

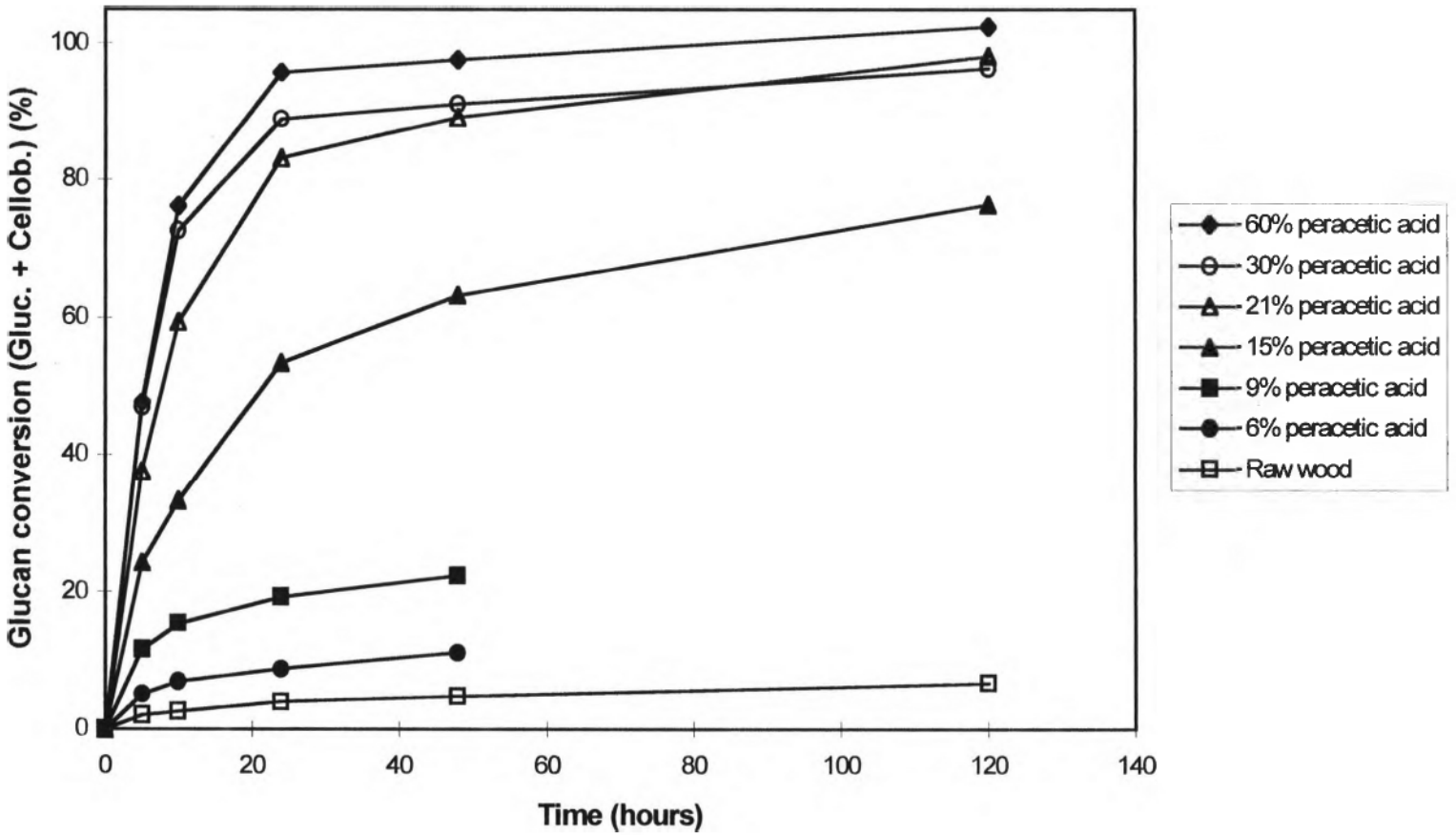



Fig. 4.5. Enzymatic hydrolysis of hybrid poplar (glucan fraction) pretreated with different concentrations of peracetic acid. Amount of acid is based on oven dry-weight of wood in a ratio of 1:6 biomass to water. Six and 9% peracetic acid pretreated samples were hydrolyzed using the Spezyme/Novozym 188 combination. The others were performed using the SP431/Novozym 188 combination as described in the methodology.

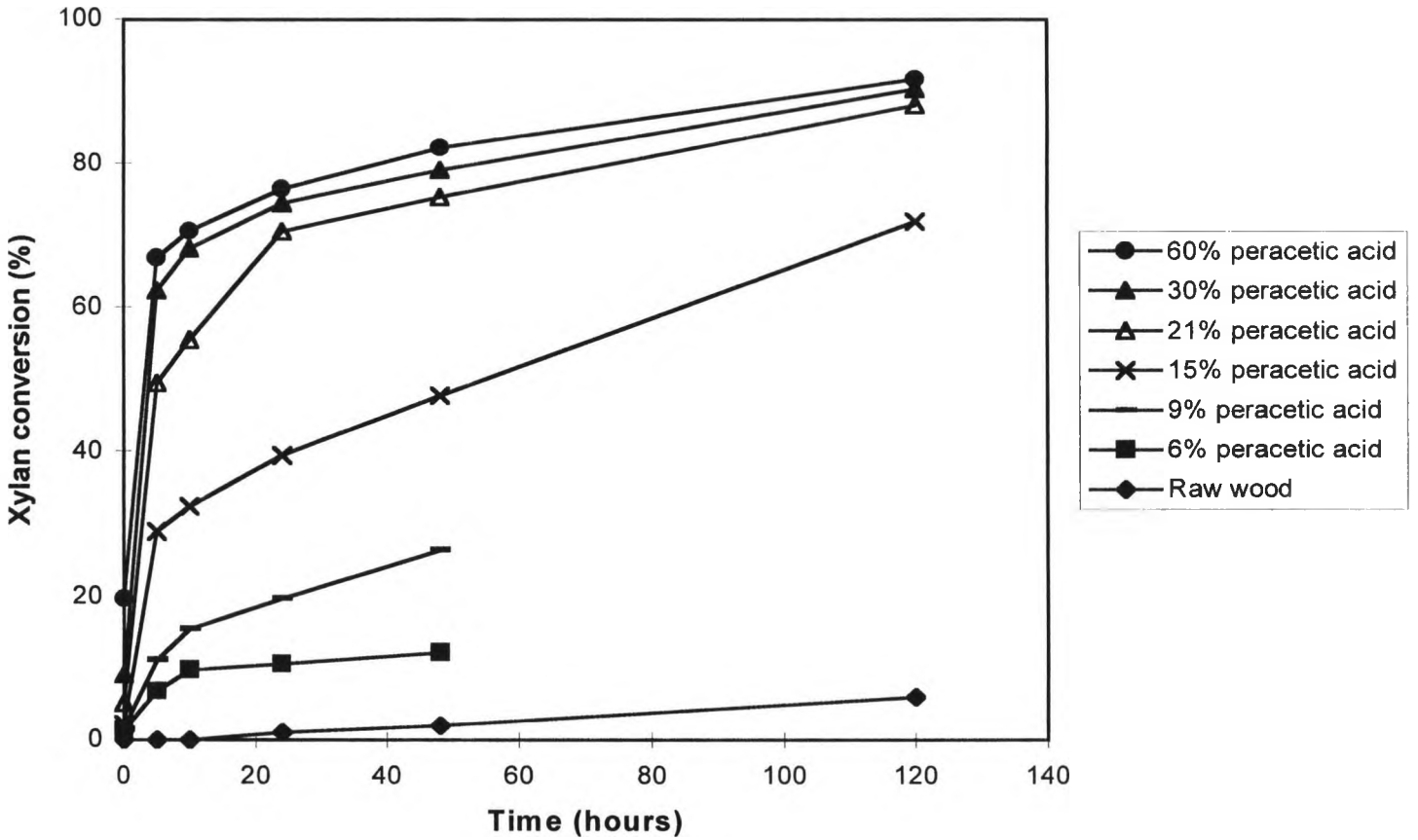



Fig. 4.6. Enzymatic hydrolysis of hybrid poplar (xylan fraction) pretreated with different concentrations of peracetic acid. Amount of acid is based on oven dry-weight of wood in a ratio of 1:6 biomass to water. Six and 9% peracetic acid pretreated samples were hydrolyzed using the Spezyme/Novozym 188 combination. The others were performed using the SP431/Novozym 188 combination as described in the methodology.

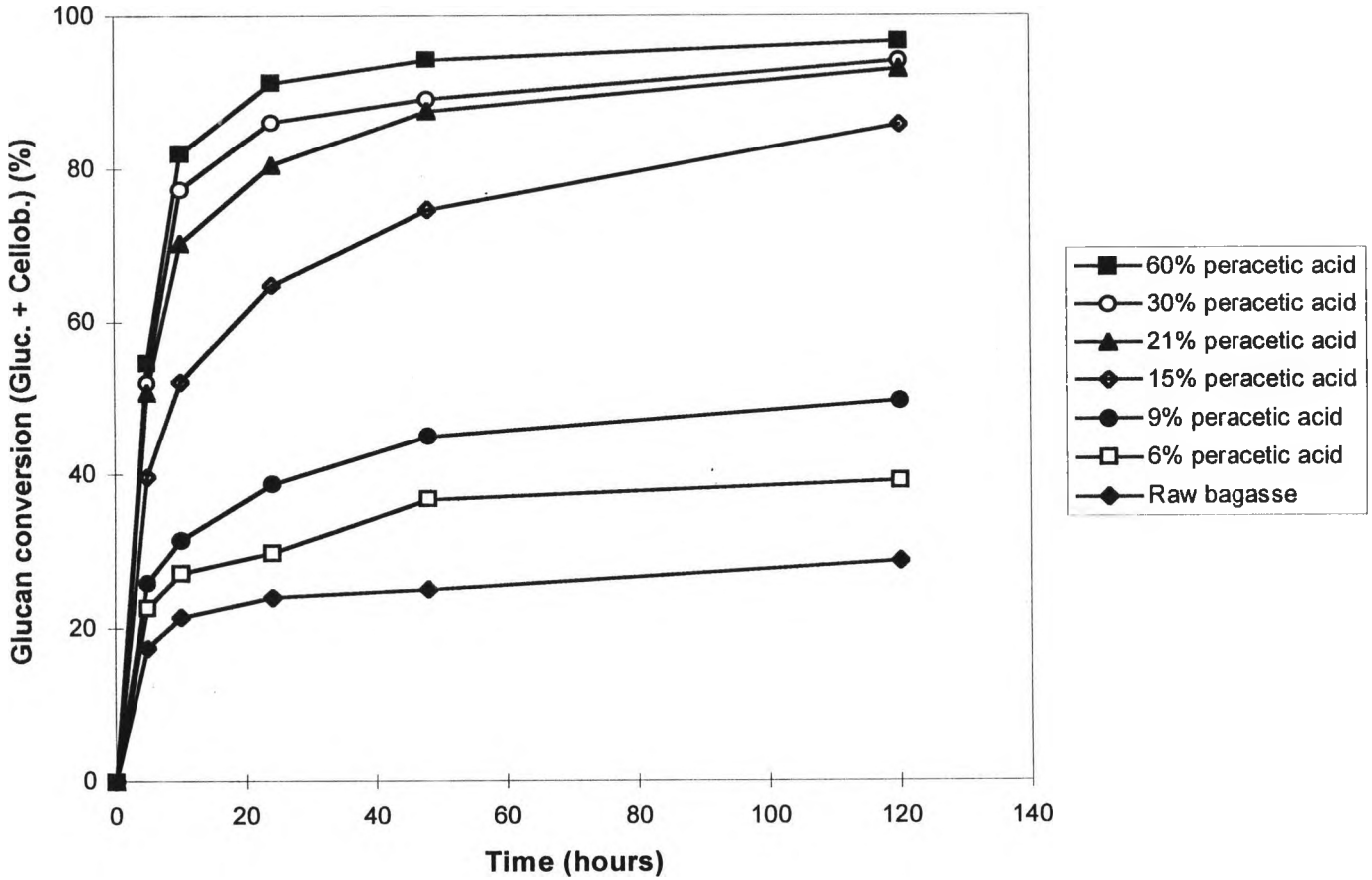



Fig. 4.7. Enzymatic hydrolysis of sugar cane bagasse (glucan fraction) pretreated with different concentrations of peracetic acid. Amount of acid is based on oven-dry weight of biomass in a ratio of 1:6 biomass to water.

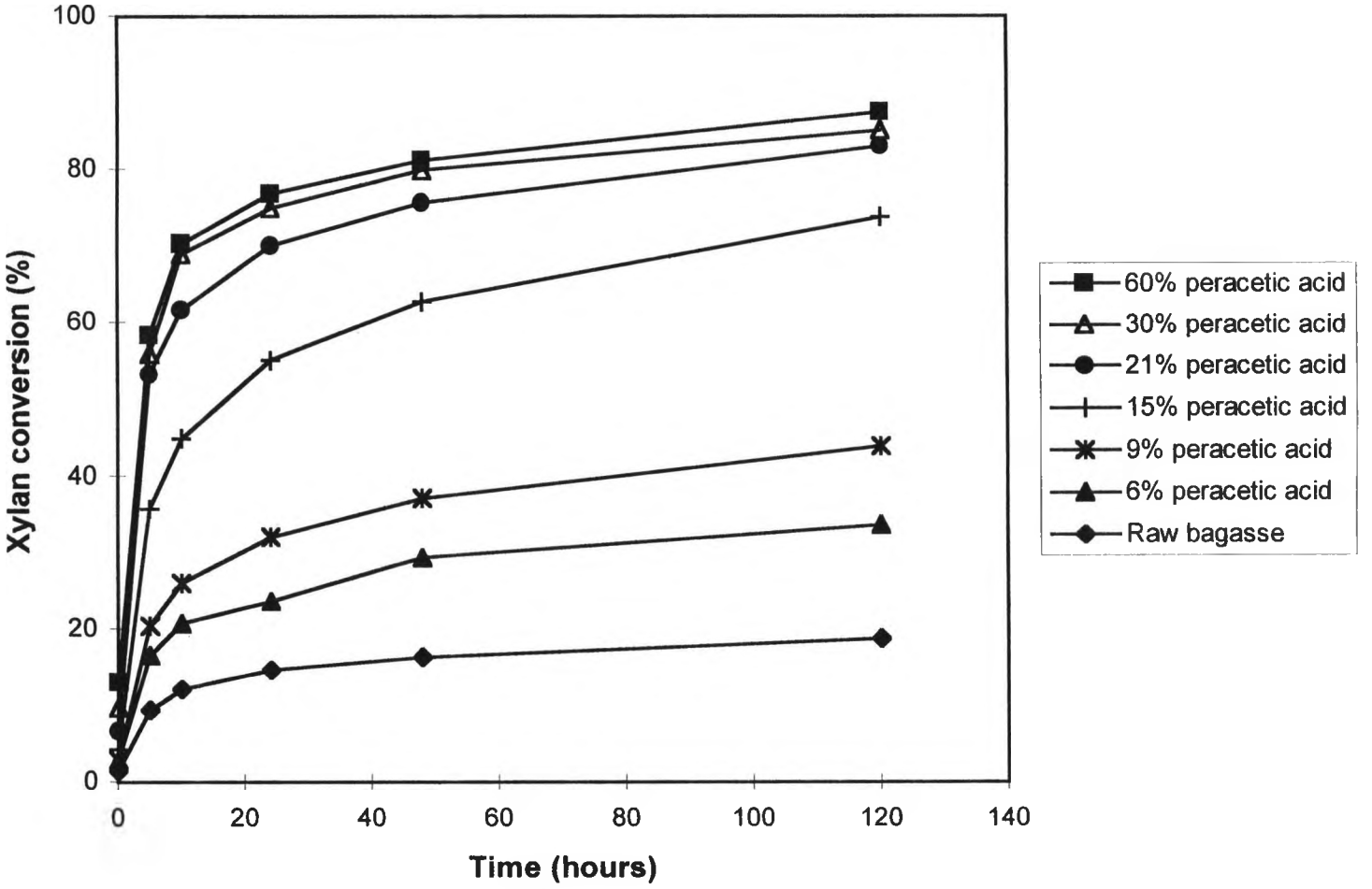



Fig. 4.8. Enzymatic hydrolysis of sugar cane bagasse (xylan fraction) pretreated with different concentrations of peracetic acid. Amount of acid is based on oven-dry weight of biomass in a ratio of 1:6 biomass to water.

apparent xylan (Fig. 4.7 and 4.8). Similar sugar yields have been reported for sugar cane bagasse and other farm residues when they are treated with peracetic acid under the more severe conditions of higher temperature and higher peracetic acid concentration (Farid et al. 1983). The raw (non-treated) bagasse had poor conversion of only 28.8% of glucan and 18.8% of apparent xylan to simple sugars at the end of the hydrolysis period.

Enzymatic hydrolysis tests of alkaline/peracetic acid treated biomass

As mentioned in the literature review chapter, the use of diluted alkaline solutions has been studied for improving biomass digestibility for different purposes. The stereo chemical impediments caused by acetyl groups to action of enzymes in the xylan backbone are a very important issue in the enzymatic hydrolysis of biomass.

With the intent of reducing the acetyl content in the substrates, alkaline pre-pretreatments were performed on hybrid poplar using 3 and 6% sodium hydroxide, and 2.63, 5.25 and 14.00% ammonium hydroxide, based on dry wood weight, and a ratio of 14:1 solution to biomass. Only the 9 and 15% peracetic acid pretreatments were evaluated. This procedure, as shown in Figures 4.9 and 4.10, improved the simple sugar yield when comparisons were made at 9 and 15% peracetic acid treatment without the prior use of base suggesting economy in the pretreatment because of reduction in peracetic acid loading (Figs. 4.5 and 4.6). Respective conversions were estimated to be 40 and 75% higher at the end of 120 hours of enzymatic hydrolysis when compared to pretreatments without base. The sodium hydroxide pre-pretreated samples also showed better conversion in the course of 120 hour enzymatic hydrolysis. Specifically, the sample treated with 6% NaOH / 9% peracetic acid (95% yield) showed better conversion than the sample treated only with 15% peracetic acid (75% yield).

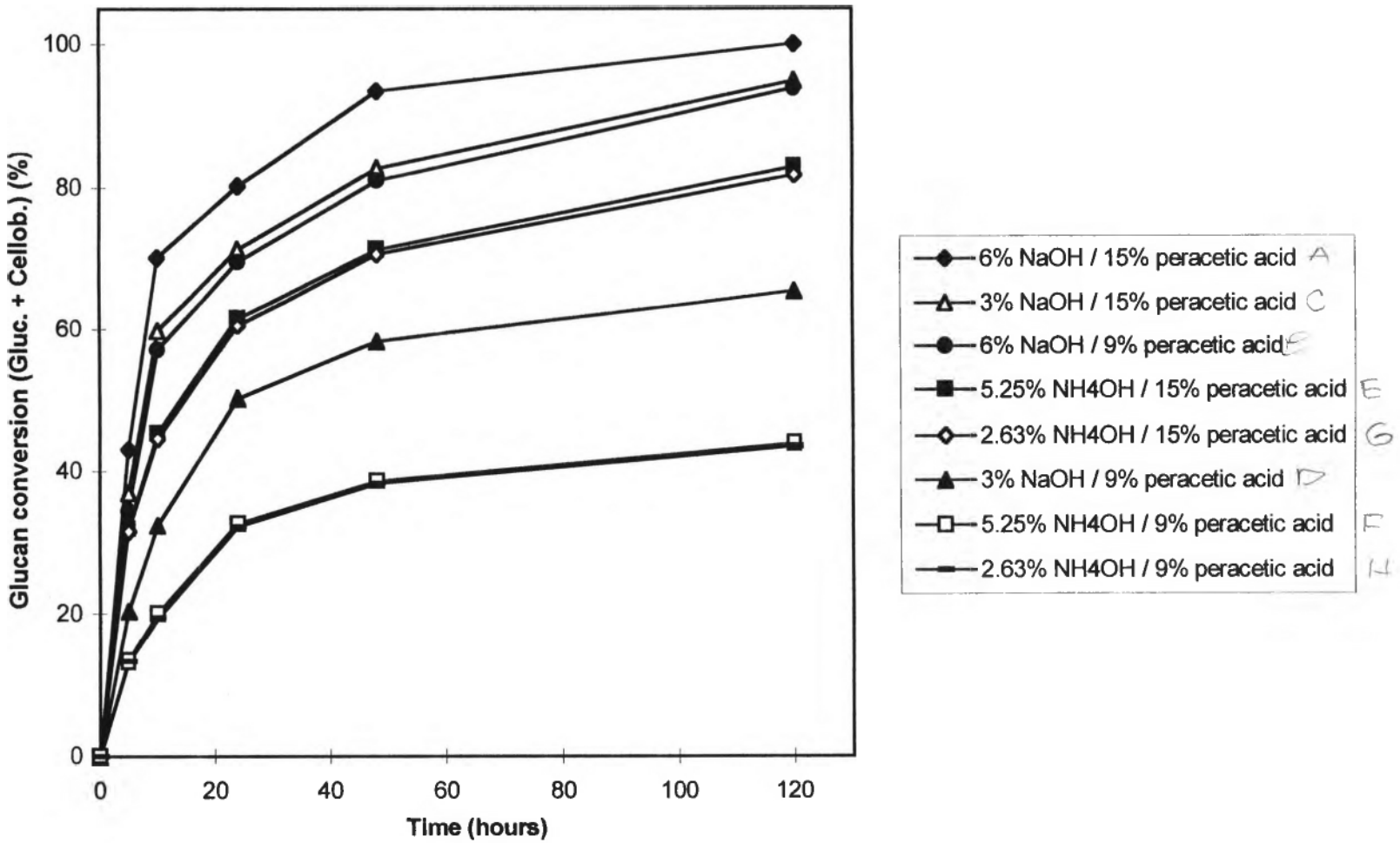



Fig. 4.9. Enzymatic hydrolysis of hybrid poplar (glucan fraction) pretreated with different concentrations of alkalis and peracetic acid. Amounts of base and acid are based on oven-dry weight of wood. Ratios of biomass to water are 1:14 for alkaline and 1:6 for acidic treatments.

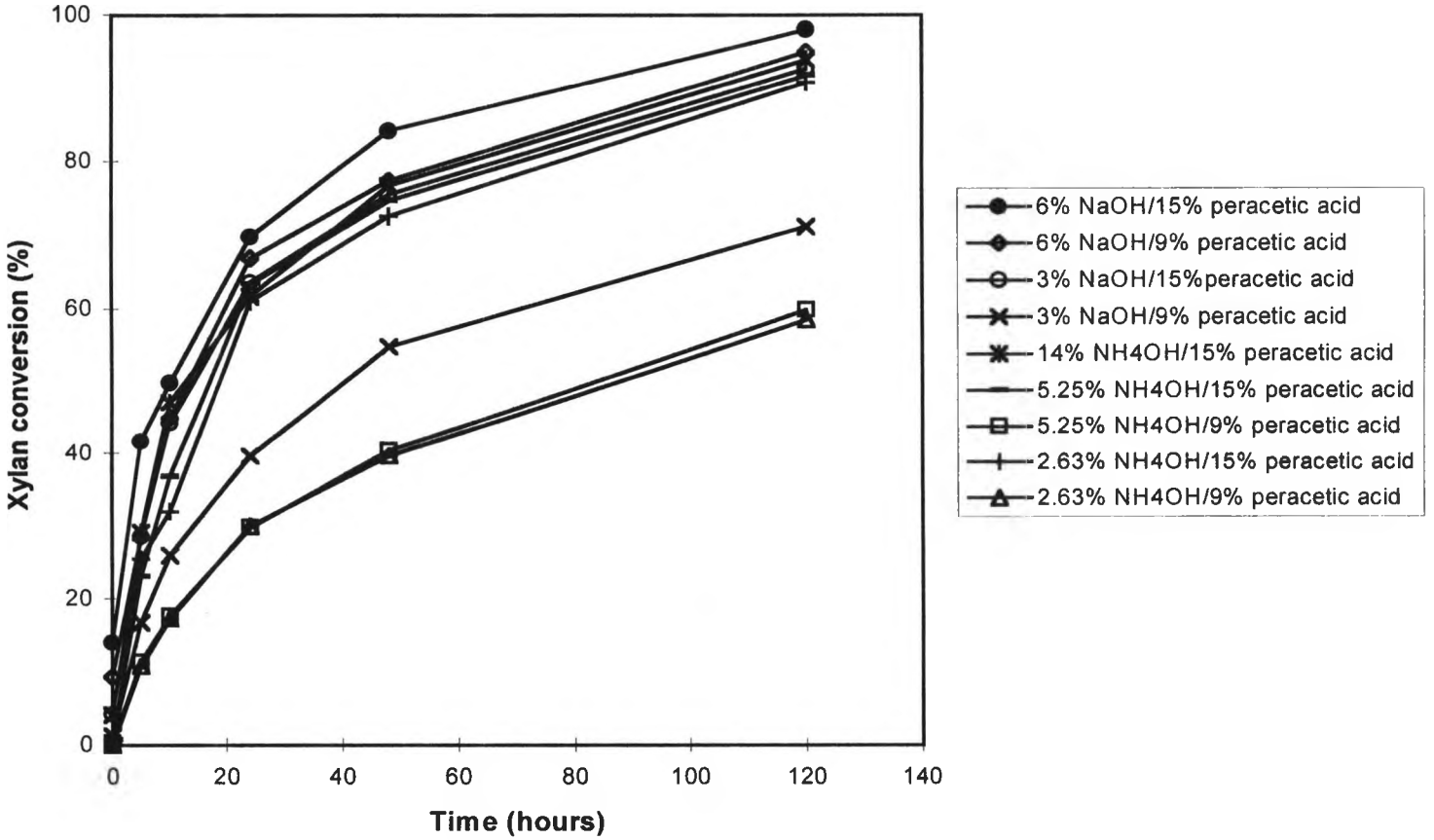



Fig. 4.10. Enzymatic hydrolysis of hybrid poplar (xylan fraction) pretreated with different concentrations of alkalis and peracetic acid. Amounts of base and acid are based on oven-dry weight of wood. Ratios of biomass to water are 1:14 for alkaline and 1:6 for acidic treatments.

A similar conversion yield of 93.7% for enzymatic digestibility (and 99.9% of the potentially degradable fraction) measured as percentage of disappearance on ruminal sacco, have been reported in the literature for the hardwoods, *Machilus thumbergii* and hybrid poplar, when treated with NaOH followed by peracetic acid under more severity conditions (Toyama and Ogawa, 1975; Myung and Kennelly, 1992). The order of using the two chemicals, NaOH and peracetic acid, has no effect on sugar yields for hardwoods, but when the biomass is a softwood different sugar yields are relevant, especially if the alkaline treatment is performed before the oxidative step, which resulted in lowering the yield (Toyama and Ogawa, 1975)

According to data in Figures 4.9 and 4.10, the ammonium hydroxide pre-pretreatment using 2.63 and 5.25% based on oven dry weight of woody biomass causes little improvement in the conversion to sugars compared to the use of no base at all. Ammonium hydroxide, being a weak base, requires the use of that base at the higher concentration of 14% based on oven dry weight of biomass to improve conversion.

As with poplar, the sugar cane bagasse was submitted to an alkaline pre-pretreatment prior to peracetic acid addition (see Figures 4.11 and 4.12). Alkaline concentrations were the same as used for poplar. Due to mixing difficulty the ratio had to be increased to 17:1 solution to biomass. Only the 6, 9 and 15% peracetic acid pretreatments were evaluated. The sodium hydroxide pre-pretreated samples show satisfactory conversion in the course of 120 hours of enzymatic hydrolysis. Specifically, the sample treated with 6% NaOH/15% peracetic acid shows a complete conversion very similar to the sample treated with only 21% peracetic acid. Sugar conversions found here are very similar to steam-exploded bagasse (Dekker and Wallis, 1983).

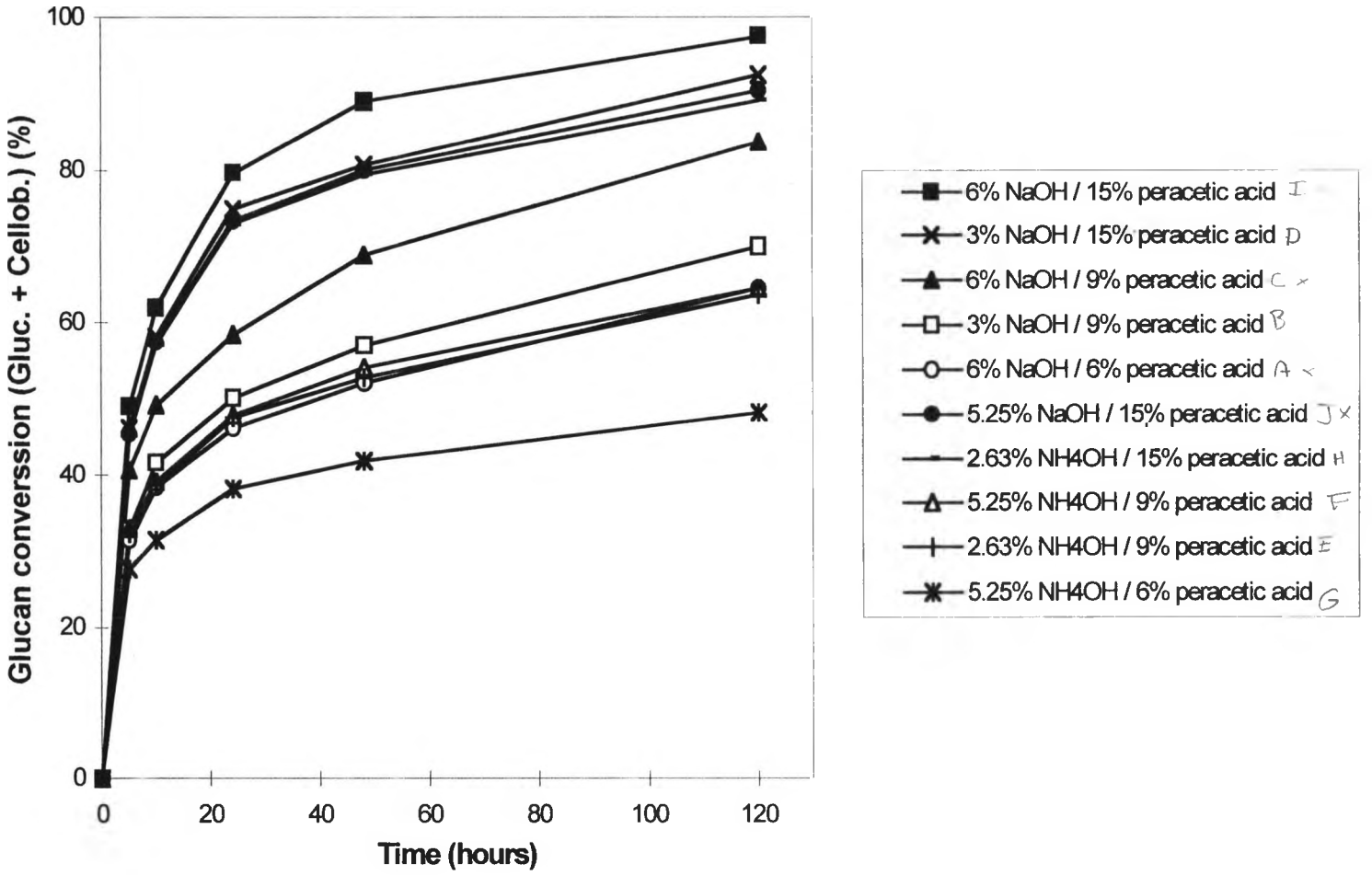



Fig. 4.11. Enzymatic hydrolysis of sugar cane bagasse (glucan fraction) pretreated with different concentrations of alkalis and peracetic acid. Amounts of base and acid are based on oven-dry weight of biomass. Ratios of biomass to water are 1:17 for alkaline and 1:6 for acidic treatments.

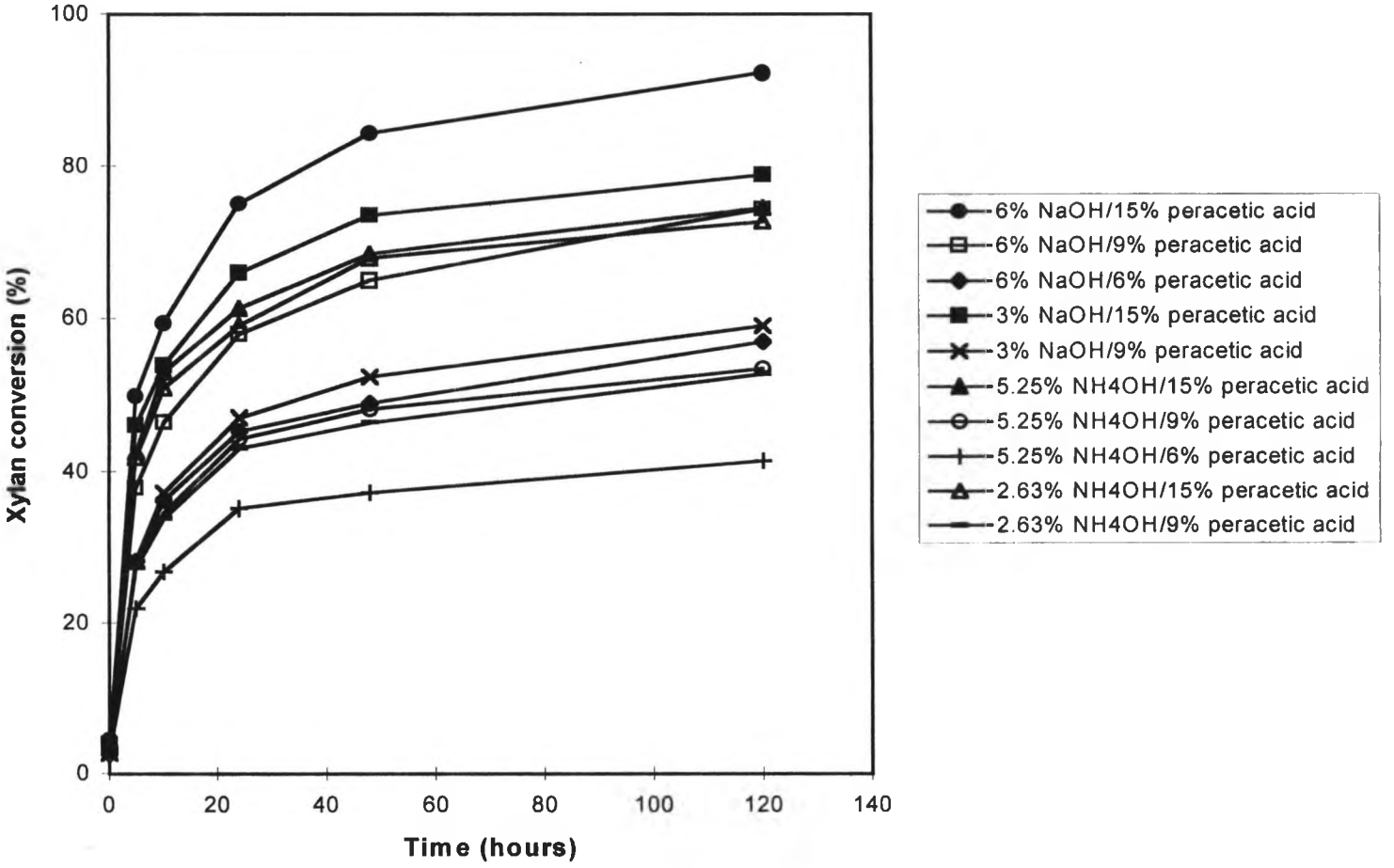



Fig. 4.12. Enzymatic hydrolysis of sugar cane bagasse (xylan fraction) pretreated with different concentrations of alkalis and peracetic acid. Amounts of base and acid are based on oven-dry weight of biomass. Ratios of biomass to water are 1:17 for alkaline and 1:6 for acidic treatments.

The added effect of using alkaline treatment followed by peracetic acid has been reported by Farid et al. (1983). A combined pretreatment in wheat straw using 2.0% NaOH at the boiling point and subsequent reaction with also a boiling solution of peracetic acid gave a sugar yield of 90.0%. Using only a 2% NaOH solution a conversion of 36.0% was verified and when only peracetic acid was used a yield of 67.3% was reported. Untreated biomass gave 1.5% conversion.

Tables 4.5 and 4.6 show the acetyl content and retention after peracetic acid and alkaline/peracetic acid pretreatments in hybrid poplar and sugar cane bagasse. Pretreatment with peracetic acid only appears ineffective in acetyl removal in hybrid poplar, and only in higher concentrations of 30 and 60% are some acetyl groups removed from the xylan backbone (Table 4.5). The acetyl removal from use of peracetic acid only appears more significant in sugar cane bagasse for concentrations from 15 to 60% peracetic acid, Table 4.6. As shown in Table 4.5 and 4.6, after treating hybrid poplar with 6% NaOH prior to peracetic acid, about-one quarter of acetyl groups still remain in the xylan backbone of hybrid poplar and approximately two-fifths remain in the bagasse hemicellulose. Even though the ratio of acetyl groups to xylan is higher in hybrid poplar, 0.35, than in sugar cane bagasse, 0.25, the later biomass has 26% more acetyl groups per oven-dry weight of biomass. This difference of 26%, in the amount of acetyl groups, could explain the higher acetyl retention in sugar cane bagasse. The Figures 4.13 and 4.14 show the influence of presence of acetyl groups on enzymatic hydrolysis of the biomasses. The use of alkali prior to peracetic acid causes little improvement on enzymatic hydrolysis in samples treated with successive 15% peracetic acid. The effect of alkaline treatment is more significant in samples treated with successive 9% and 6% peracetic acid, as can be verified by the slope increase in those curves. Specifically for sugar cane bagasse, projections from the

Table 4.5. Acetyl content and retention after pretreatment of hybrid poplar. Values for the retained acetyl have been corrected to raw wood considering variations in the amount of xylan in the treated substrate.

| Pretreatment condition | Acetyl content (%) | Retained acetyl (%) |
|---|---|---|
| 0% peracetic acid (raw wood) | 4.68 | 100.0 |
| 6% peracetic acid | 4.86 | 101.6 |
| 9% peracetic acid | 5.04 | 101.6 |
| 15% peracetic acid | 5.50 | 102.3 |
| 21% peracetic acid | 5.74 | 99.6 |
| 30% peracetic acid | 5.76 | 94.8 |
| 60% peracetic acid | 5.86 | 90.0 |
| 6% NaOH/15% peracetic acid | 1.42 | 23.8 |
| 6% NaOH/9% peracetic acid | 1.40 | 25.5 |
| 3% NaOH/15% peracetic acid | 3.00 | 51.4 |
| 3% NaOH/9% peracetic acid | 2.86 | 52.2 |
| 14% $NH_4OH$/15% peracetic acid | 4.15 | 72.0 |
| 5.25% $NH_4OH$/15% peracetic acid | 5.03 | 91.7 |
| 5.25% $NH_4OH$/9% peracetic acid | 4.97 | 98.1 |
| 2.63% $NH_4OH$/15% peracetic acid | 5.14 | 94.8 |
| 2.63% $NH_4OH$/9% peracetic acid | 5.05 | 101.1 |

Table 4.6. Acetyl content and retention after pretreatment of sugar cane bagasse. Values for the retained acetyl have been corrected to raw biomass considering variations in the amount of xylan in the treated substrate.

| Pretreatment condition | Acetate content (%) | Retained acetyl (%) |
|---|---|---|
| 0% peracetic acid (raw bagasse) | 5.92 | 100.0 |
| 6% peracetic acid | 6.16 | 101.9 |
| 9% peracetic acid | 6.16 | 98.6 |
| 15% peracetic acid | 5.99 | 94.0 |
| 21% peracetic acid | 5.84 | 87.1 |
| 30% peracetic acid | 5.53 | 79.8 |
| 60% peracetic acid | 4.88 | 69.6 |
| 6% NaOH/15% peracetic acid | 2.95 | 43.8 |
| 6% NaOH/9% peracetic acid | 3.07 | 47.6 |
| 6% NaOH/6% peracetic acid | 3.20 | 50.8 |
| 3% NaOH/15% peracetic acid | 4.51 | 69.6 |
| 3% NaOH/9% peracetic acid | 4.76 | 75.6 |
| 5.25% $NH_4OH$/15% peracetic acid | 5.68 | 88.4 |
| 5.25% $NH_4OH$/9% peracetic acid | 5.78 | 92.5 |
| 5.25% $NH_4OH$/6% peracetic acid | 5.73 | 94.4 |
| 2.63% $NH_4OH$/15% peracetic acid | 5.85 | 91.4 |
| 2.63% $NH_4OH$/9% peracetic acid | 5.87 | 94.3 |

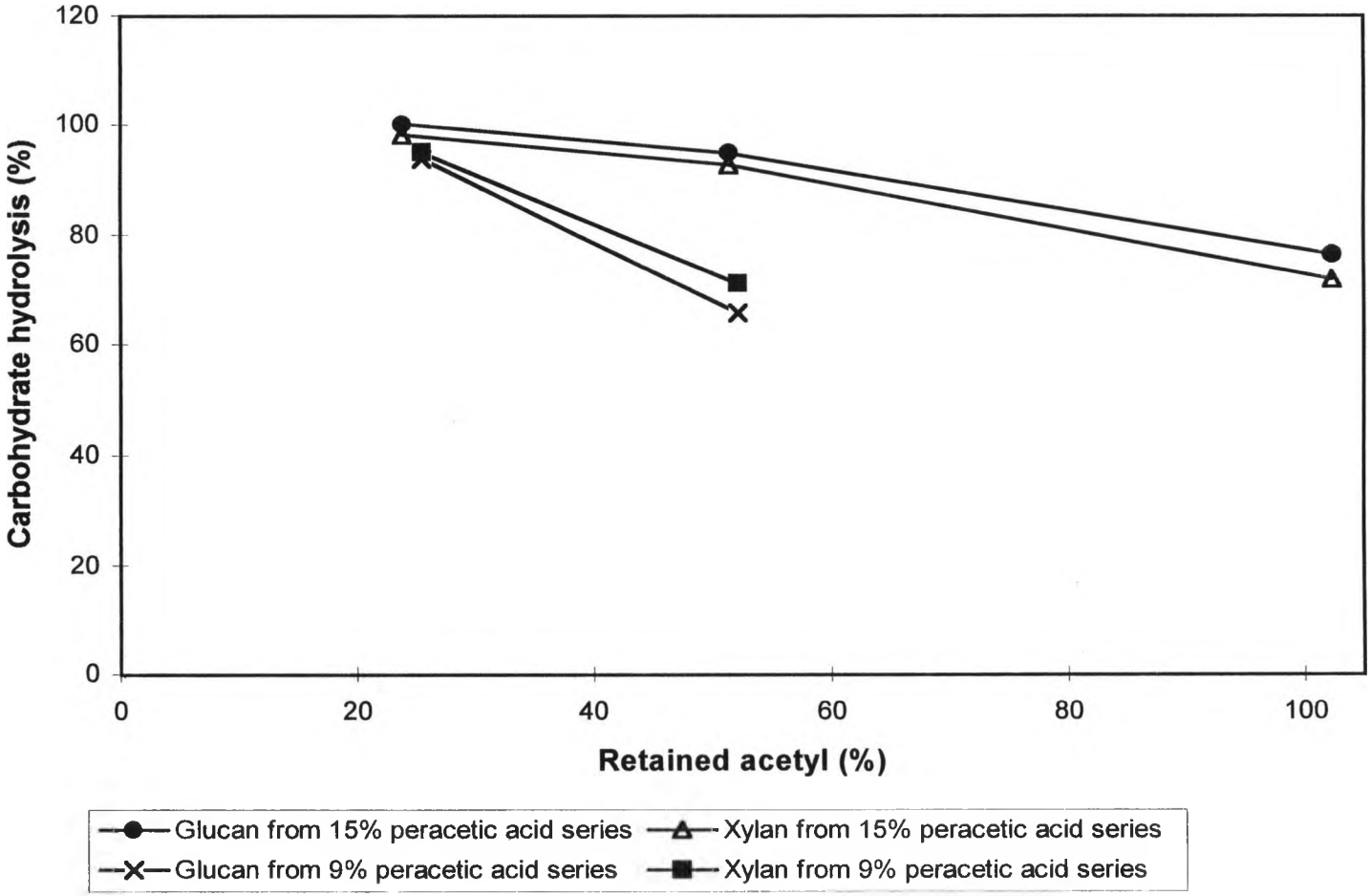



Fig. 4.13. Carbohydrate hydrolysis as a function of retained acetyl groups in pretreated hybrid poplar.

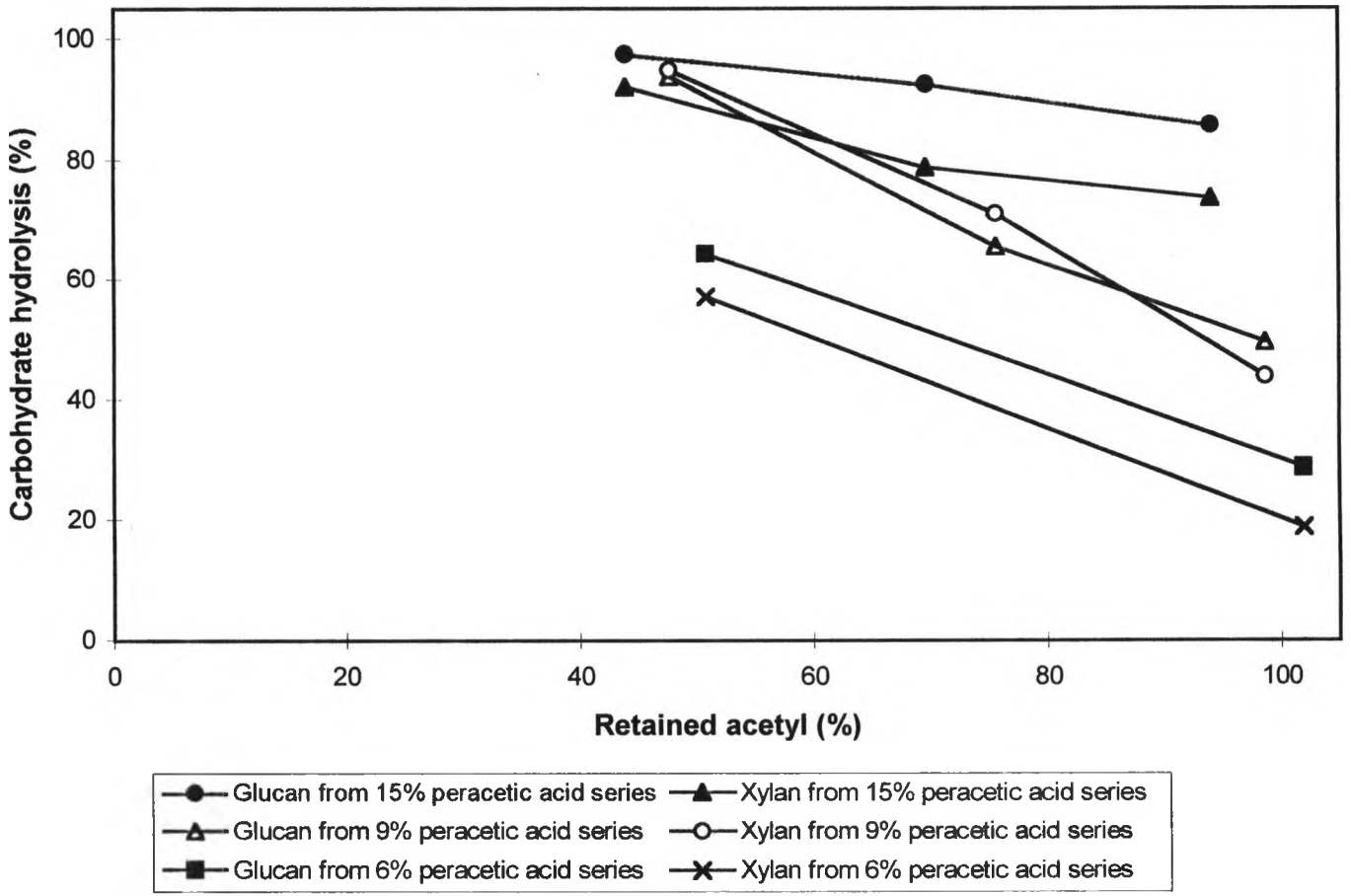



Fig. 4.14. Carbohydrate hydrolysis as a function of retained acetyl groups in pretreated sugar cane bagasse.

6% peracetic acid curves suggest that if the 50% of the remaining acetyl groups are removed from the biomass, similar yields close to 100% conversion could be obtained. The survey of studies with the alkaline pre-pretreatments was not exhaustive, especially the use of the less expensive ammonium hydroxide. Considerably more work is required to establish optimal conditions in combination with peracetic acid pretreatments.

## Simultaneous Saccharification and Co-Fermentation Tests - SSCF

### Hybrid Poplar

The Figure 4.15 shows the ethanol yields of hybrid poplar samples from different pretreatment conditions using the recombinant *Z. mobilis* CP4/pZB5 as described in the methodology. The maximum average ethanol yield, corresponding to 21.3g/L or 92.8% yield, was reached in 10 days of fermentation in the sample pretreated in two steps with 6% NaOH/15% peracetic acid. A period of 7 days was enough for achieving a yield of 92.6%. This result is slightly lower than found in the literature, 95.0% yield, for the same recombinant microorganism using a medium containing a mix of glucose and xylose (Zang et al., 1995). The slight difference in yield can be assumed to be due to a concentrated medium containing monosaccharides ready to be consumed by the recombinant bacteria. Besides medium concentration, temperature effects should be considered in decreasing yields. As stated in the methodology, *Z. mobilis* grows better under 30 °C than at 37 °C, the temperature used, at which this species can also grows satisfactorily (Grohmann, 1993; Saddler et al., 1982; Swings and De Ley, 1977). Significant decreases in both cell mass and ethanol formation has been verified for

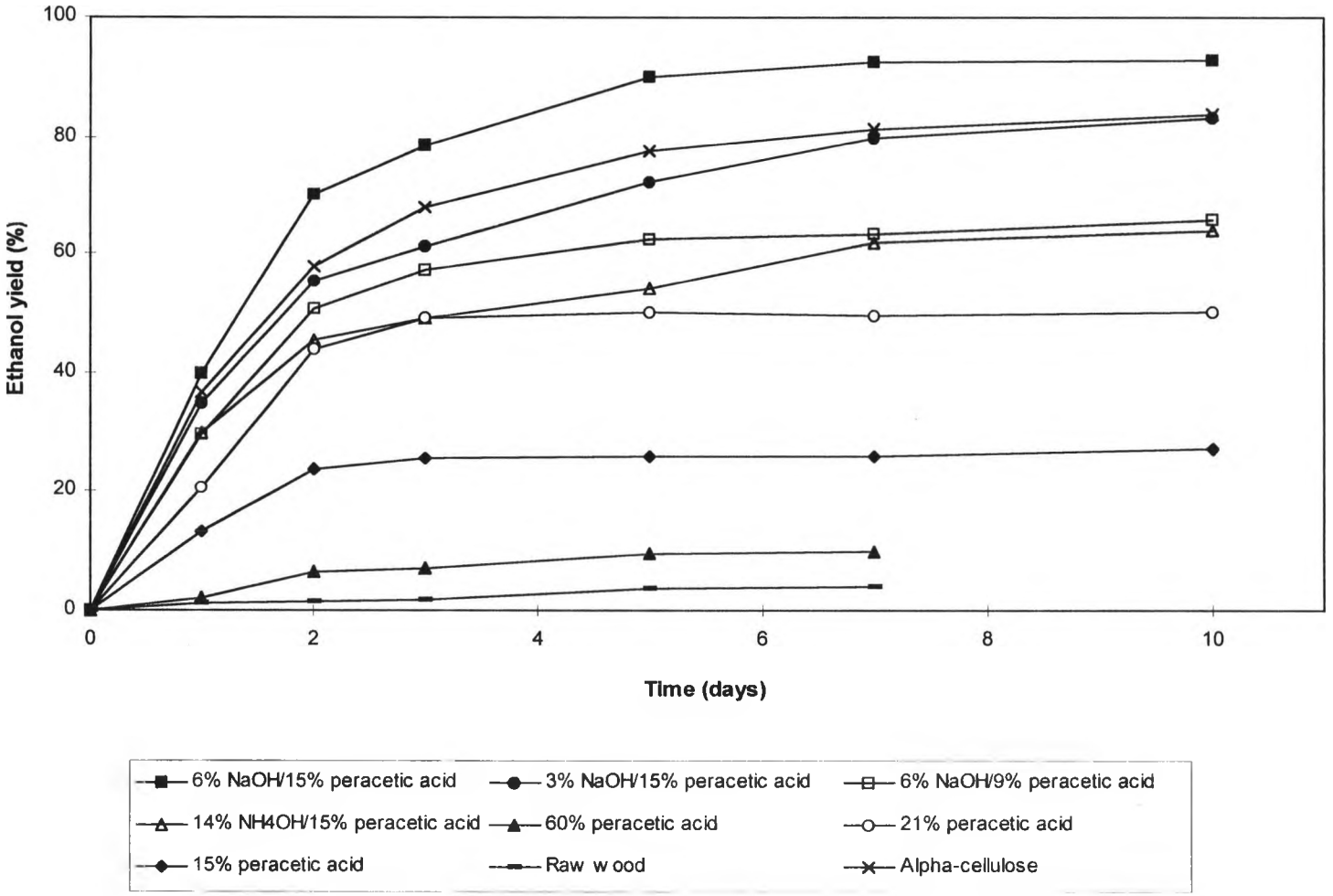



Fig. 4.15. Ethanol yields from SSCF of samples from different pretreatment conditions for hybrid poplar, raw wood and alpha-cellulose.

temperatures higher than 37 °C in simultaneous saccharification and fermentation tests using *Z. mobilis* ATCC 29501 (Spangler and Emert, 1986). According to Gonçalves de Lima et al. (1970) *Z. mobilis* var. *recifencis* strains CP1, CP2, and CP3 grow well between 25 and 42 °C in a liquid medium. The concentration of xylose was higher than glucose during the process indicating that glucose is consumed before xylose by the recombinant bacteria. The concentration of glucose and xylose were very low after 7 days indicating a satisfactory sugar utilization during the course of the cofermentation (Fig 4.16). Saddler et al. (1982) have presented satisfactory ethanol yields from steam exploded poplar with excellent glucose consumption in six-day simultaneous saccharification and fermentation experiments performed at 37 °C and using *Z. mobilis* Z2 (ATCC 29191). The significant ethanol yield found for the alkaline/peracetic acid pretreated hybrid poplar experiment presented here and a satisfactory xylose utilization (Figs. 4.15 and 4.16) confirm the high temperature tolerance by *Z. mobilis* and oppose the data indicating decreases in number of tetracycline resistant colonies and ethanol tolerance when the fermentation temperature is increased from 30 to 37 °C (Ingram et al., 1984; Carey et al., 1983). Barbosa et al. (1992) have shown that yields varying from 85 to 91% from pine hemicellulose hydrolysates using a recombinant *E. coli* K011 are improved when using commercial sugar mixture simulating hemicellulose hydrolysate. Toon et al. (1997) have reported much lower conversion: 78.4% of the available glucose and 56.1% of available xylose from corn biomass using a recombinant *Saccharomyces*; also acetic acid at a concentration of 2.52 g/L was the predominant inhibitor present in the pretreated biomass slurry. Lawford and Rousseau (1992) have reported an one-third decrease in growth yield of *Z. mobilis* ATCC 29191 when acetic acid, concentration of 3.37 g/L, is added to a glucose medium. The low acetyl content in the 6% NaOH/15% peracetic acid pretreated hybrid poplar contributed

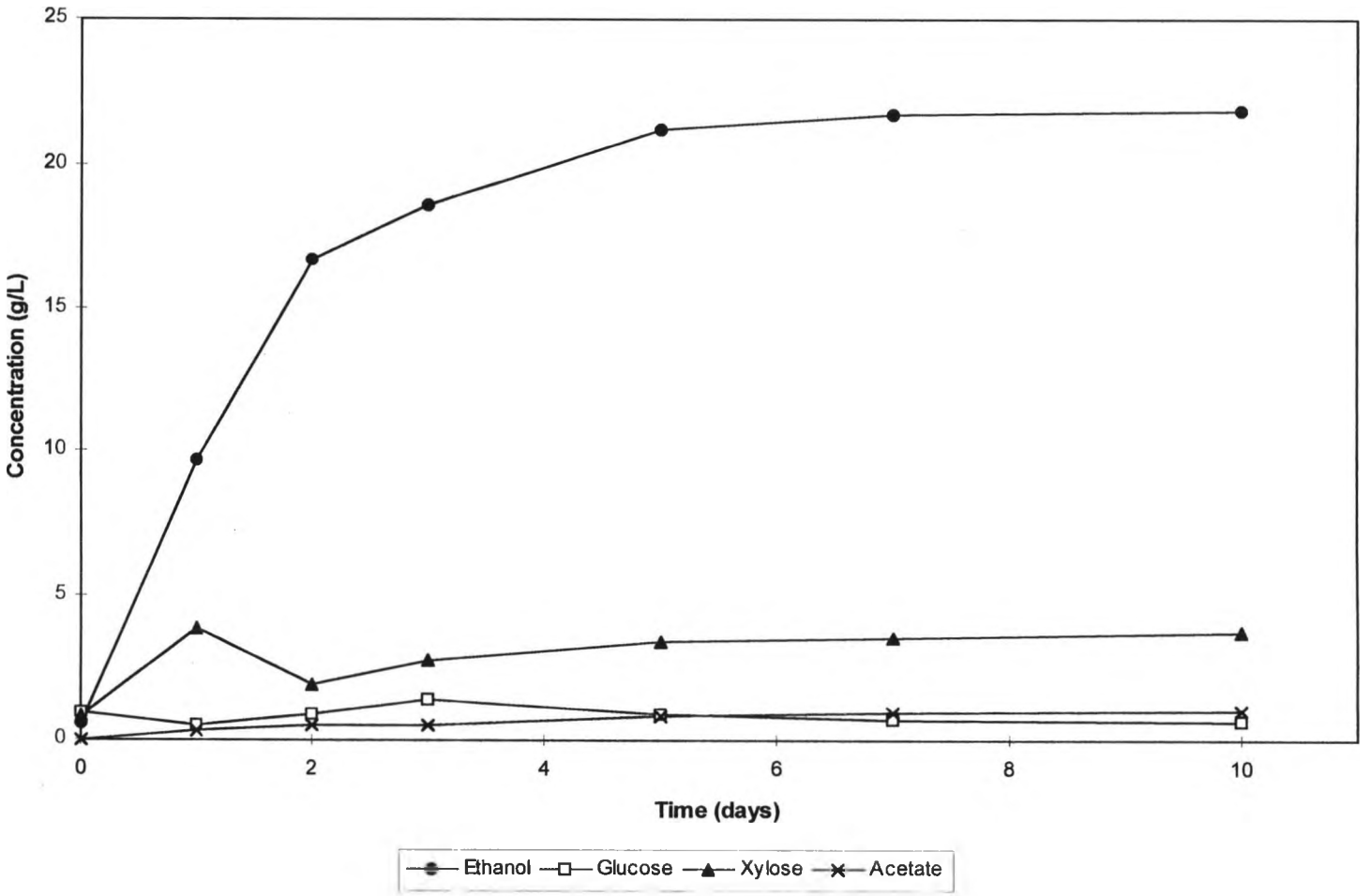



Fig. 4.16. SSCF kinetics for 6% NaOH/15% peracetic acid pretreated hybrid poplar.

to a low level of acetate during the cofermentation and consequently lowering growth inhibition. Also, the acetic acid by-product of peracetic acid reaction has been removed efficiently by washing the pretreated biomass to pH 5. Because that inhibitor metabolite was formed at concentrations below 1g/L, efficiency of ethanol conversion by the *Z. mobilis* with this practical substrate is quite satisfactory.

The recombinant *Z. mobilis* does not demonstrate good ethanol conversion when hybrid poplar is treated only with peracetic acid. The use of 15% peracetic acid based on oven-dry wood does not improve biomass digestibility (Figs. 4.5 and 4.6) and consequently the substrate treated at that loading does not fit the simultaneous saccharification and cofermentation tests (Fig. 4.15). When peracetic acid is used in concentrations of 21% or above a good biomass digestibility is verified (Fig. 4.5 and 4.6) but a satisfactory ethanol yield is not achieved (Fig. 4.15).

The best explanation found for these results could be the presence of inhibitor generated during the oxidative pretreatment step using peracetic acid, possibly a lignin by-product fragment. According to Fig. 4.17 and 4.18, the release of sugar has been verified but unsatisfactory sugar uptake by the recombinant bacteria has been observed, which is suggestive of product inhibition. With the intent of diminishing the level of inhibitor in the woody biomass, a second alkaline wash, using a solution of $NH_4OH$ calculated to be 5.25% based on oven-dry weight of wood, was performed in two pretreatment samples, 21% peracetic acid and 6% NaOH/15% peracetic acid pretreated hybrid poplar, before enzyme loading and inoculation. The biomass was mixed overnight and filtered to remove possible growth inhibitors. A better ethanol yield (80.9%) was observed for the alkaline post-washed substrate when compared with unwashed 21% peracetic acid pretreated hybrid poplar (50.2%). (Figs. 4.17 and 4.19; and Appendix VI, Tables 6 and 10). No improvement in the final ethanol

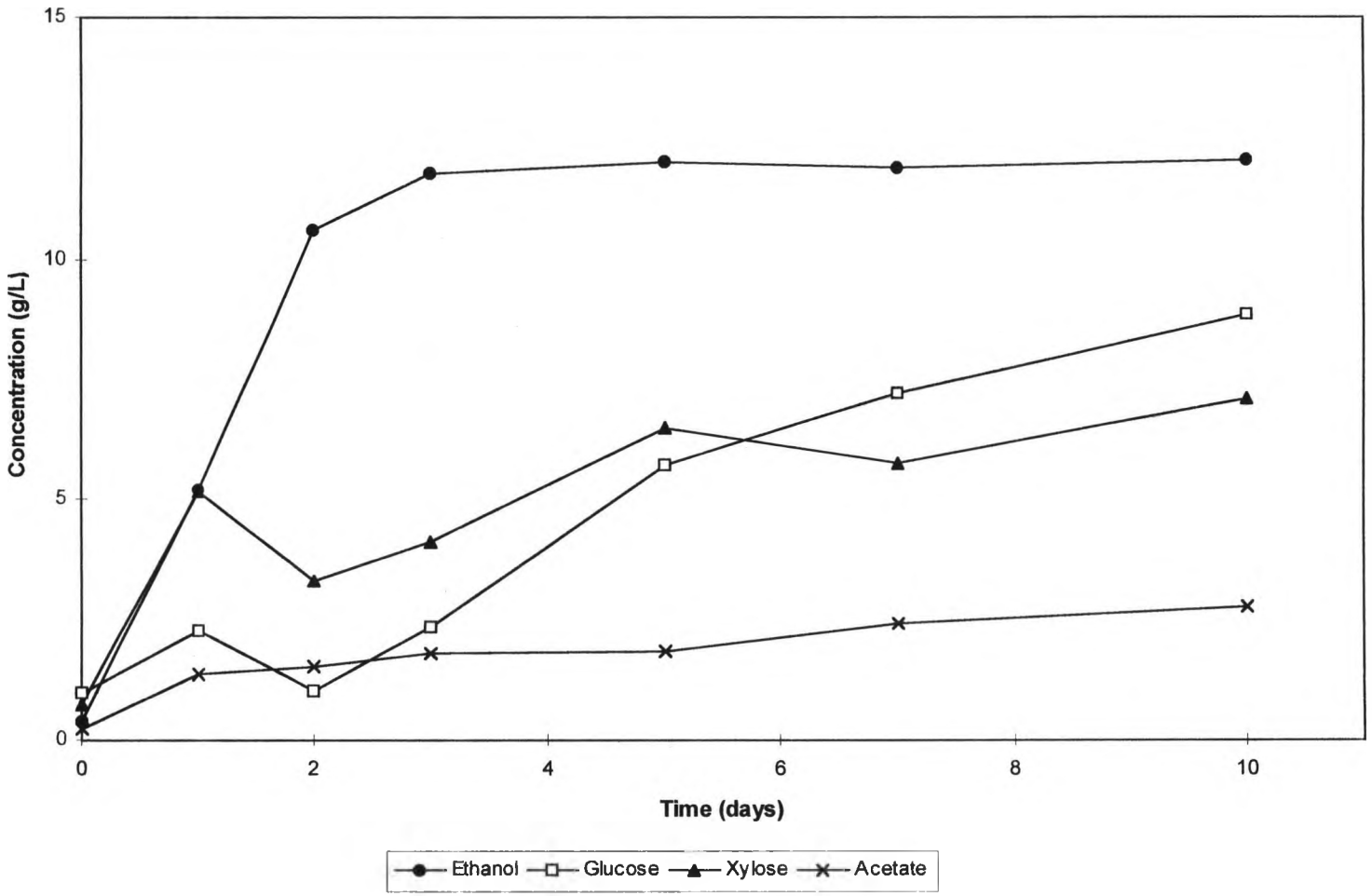



Fig. 4.17. SSCF kinetics for 21% peracetic acid pretreated hybrid poplar.

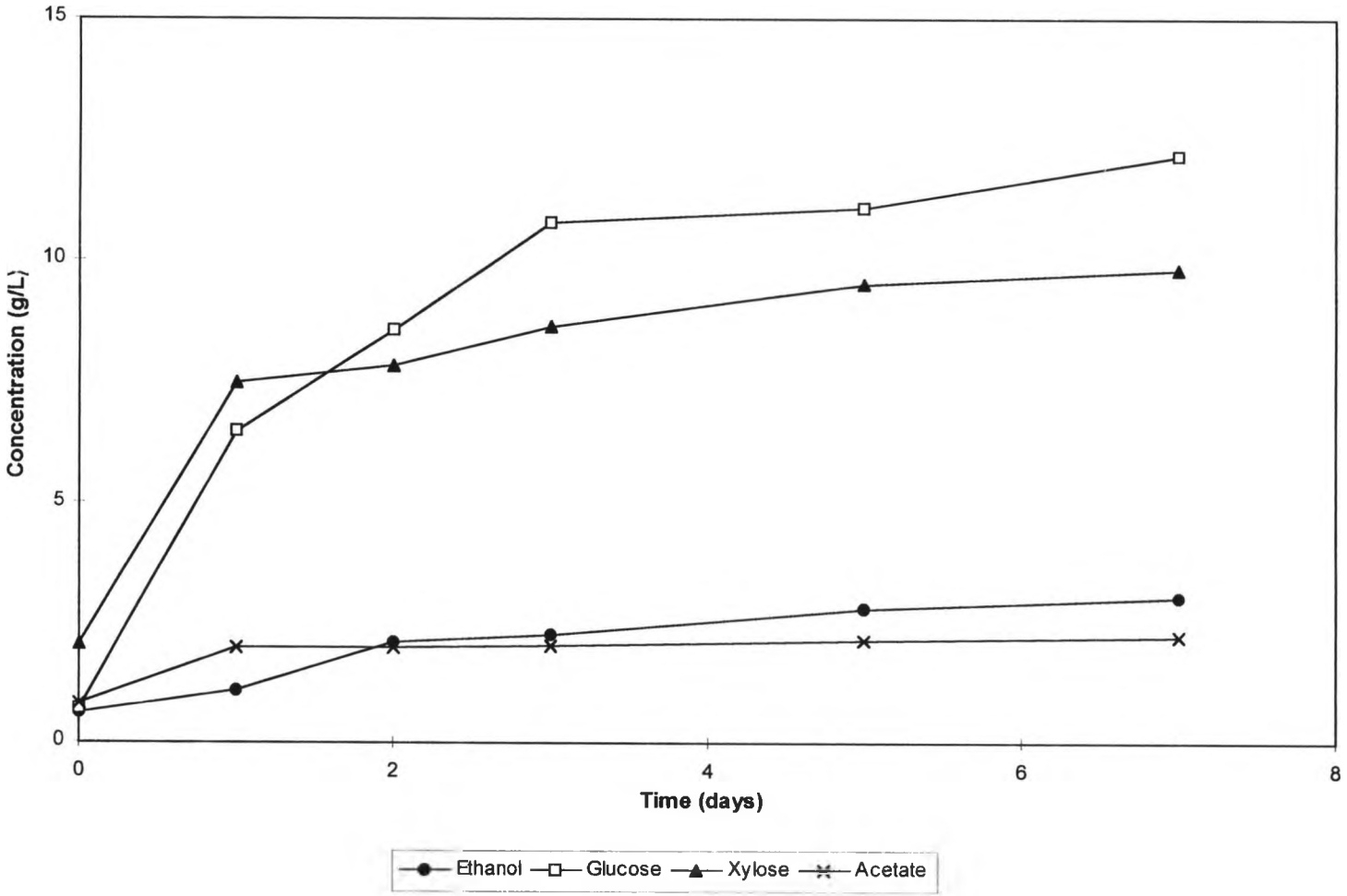



Fig. 4.18. SSCF kinetics for 60% peracetic acid pretreated hybrid poplar.

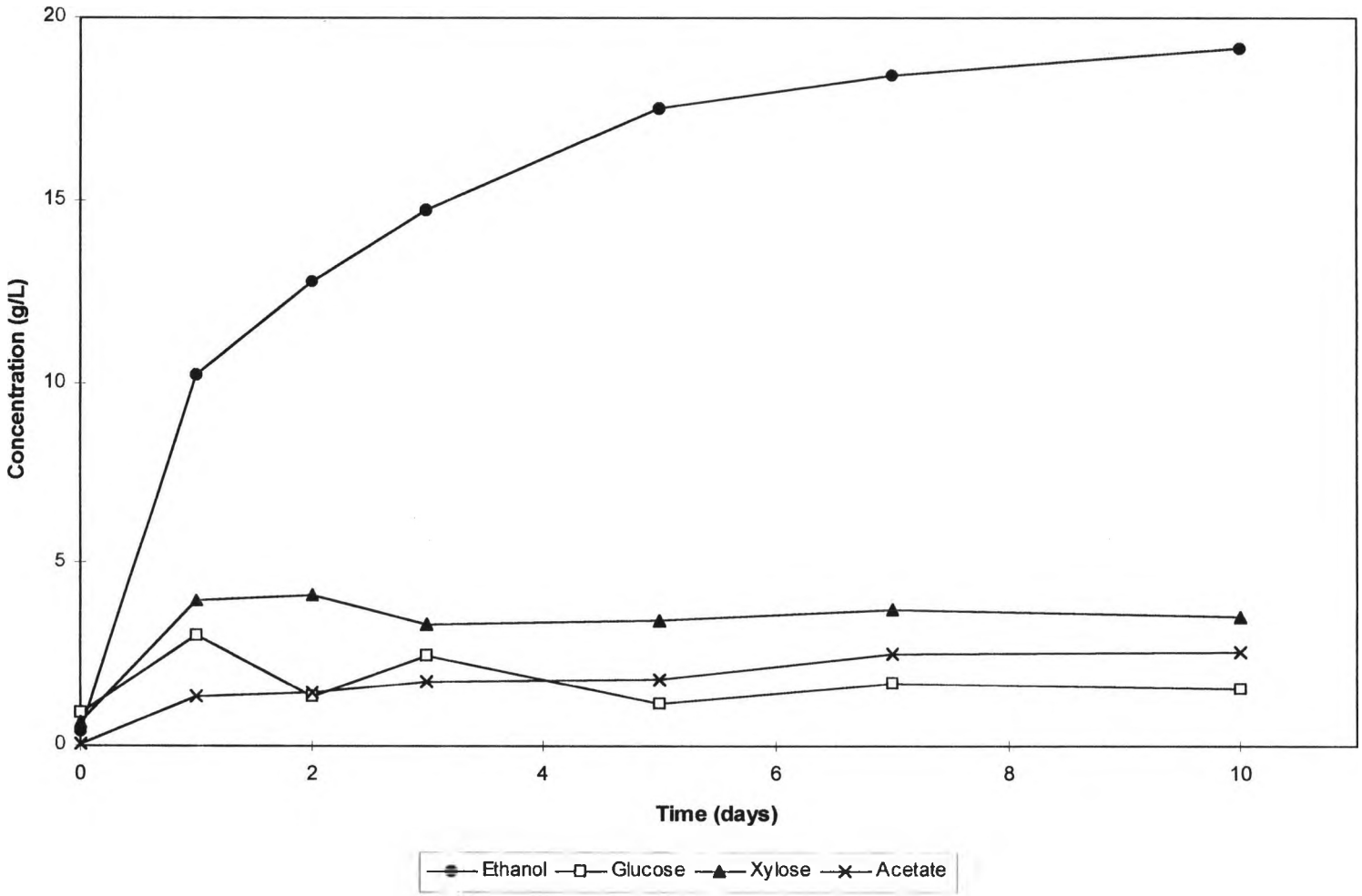



Fig. 4.19. SSCF kinetics for alkaline post-washed 21% peracetic acid pretreated hybrid poplar.

concentration has been observed for the alkaline post-washed 6% NaOH/15% peracetic acid pretreated sample. The yield has decreased slightly from 92.8%, for unwashed, to 90.4% for alkaline post-washed sample, probably from some carbohydrate losses during washing (Figs. 4.16 and 4.20; and Appendix VII, Tables 1 and 9). Only a better initial ethanol formation rate and xylose uptake has been noted for the alkaline post-washed sample.

Confirming the results of enzymatic hydrolysis of raw hybrid poplar, untreated substrate gives very low and unsatisfactory ethanol yields under the SSCF process using recombinant *Z. mobilis* (Figs. 4.15 and 4.21).

High yields from the ambient temperature pretreatments are greater than those from severe high temperature pretreatments as used for the production of the chemical grade pulp, alpha-cellulose. Possibly, precipitation of the hemicellulose and/or condensation of lignin on the cellulose microfibrils prevents more complete enzymatic conversion, as verified with relatively lower ethanol yields using alpha-cellulose (Fig. 4.15 and 4.22), from which xylan hydrolysis products are found by HPLC (data not presented). Crystallinity might be a factor as well. The crystallinity index for wood pulps and chemical grade pulp varies from 60 to 70% (Fengel and Weneger 1984). Brito (1994) has reported crystallinity varying from 76 to 85% for hybrid poplar treated with phosphoric acid under pressure and temperature. The crystallinity index for peracetic acid treated wheat straw is reduced drastically from 69.6 to 28.2% when 100 g of the biomass is boiled with 1000 mL of an acid solution prepared by equal volumes of acetic anhydride and 35% hydrogen peroxide (Gharpuray et al. 1983). Fermentation kinetics for alpha-cellulose, a reference substrate, are shown in Fig. 4.22.

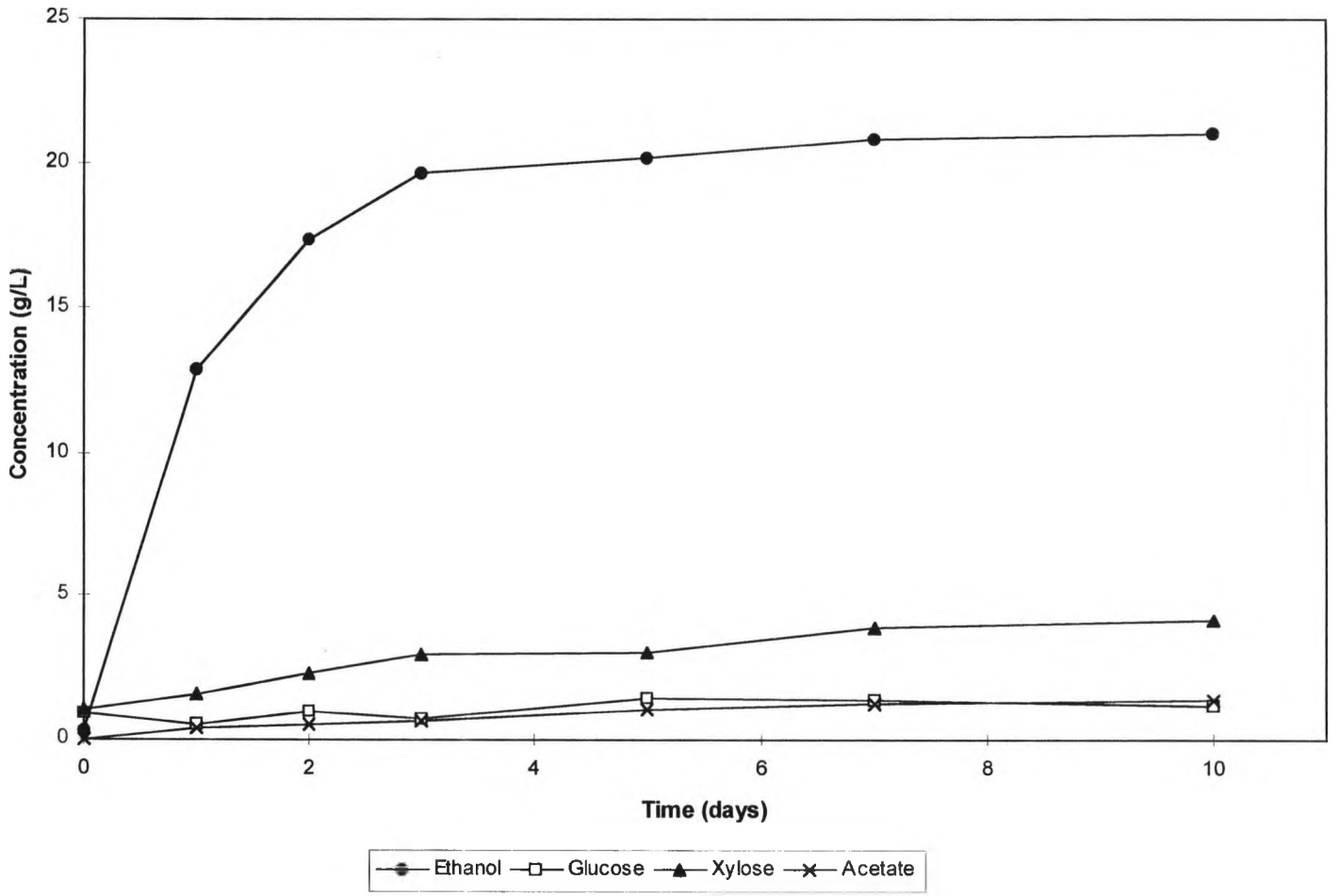



Fig. 4.20. SSCF kinetics for alkaline post-washed 6% NaOH/15% peracetic acid pretreated hybrid poplar.

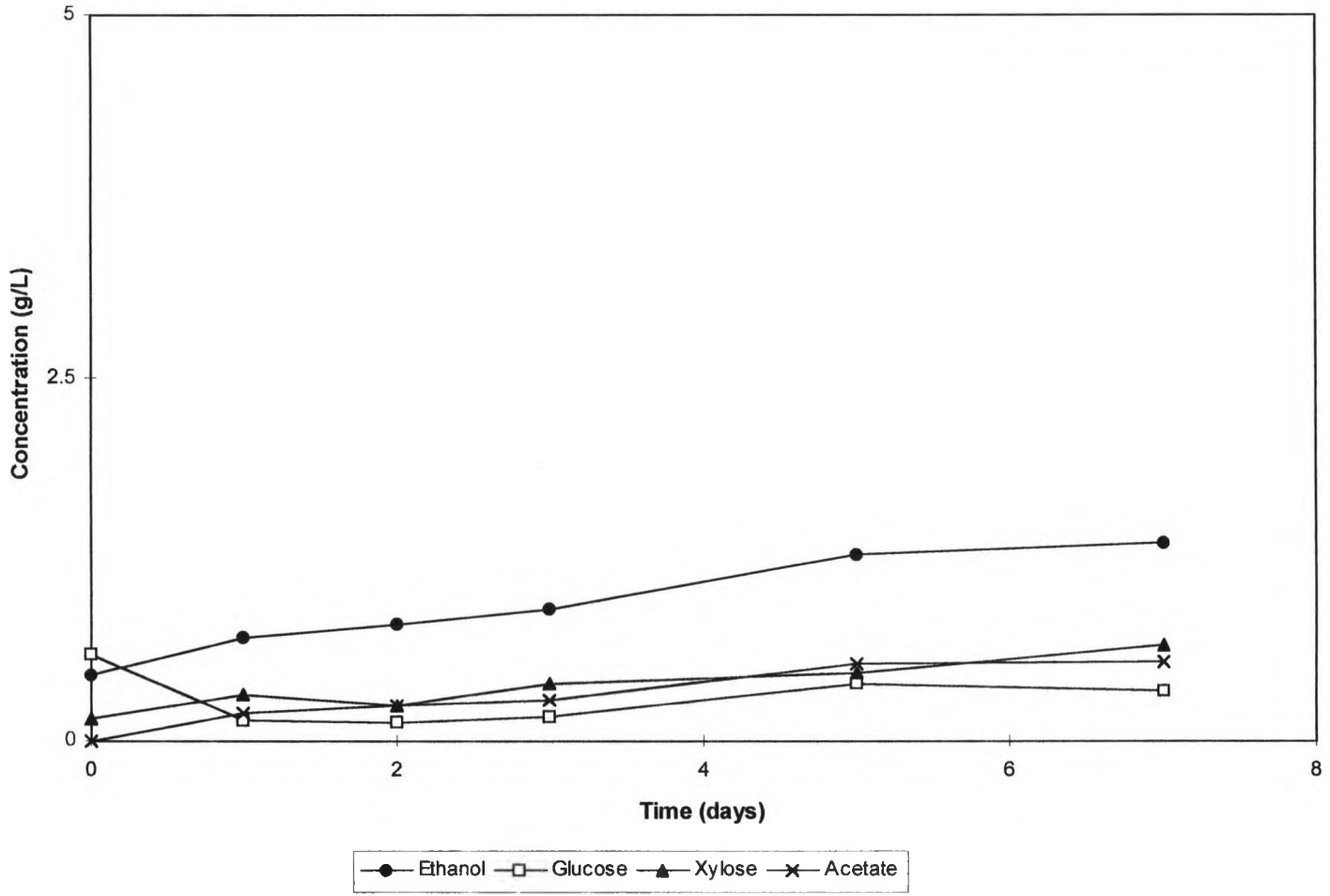



Fig. 4.21. SSCF kinetics for raw hybrid poplar.

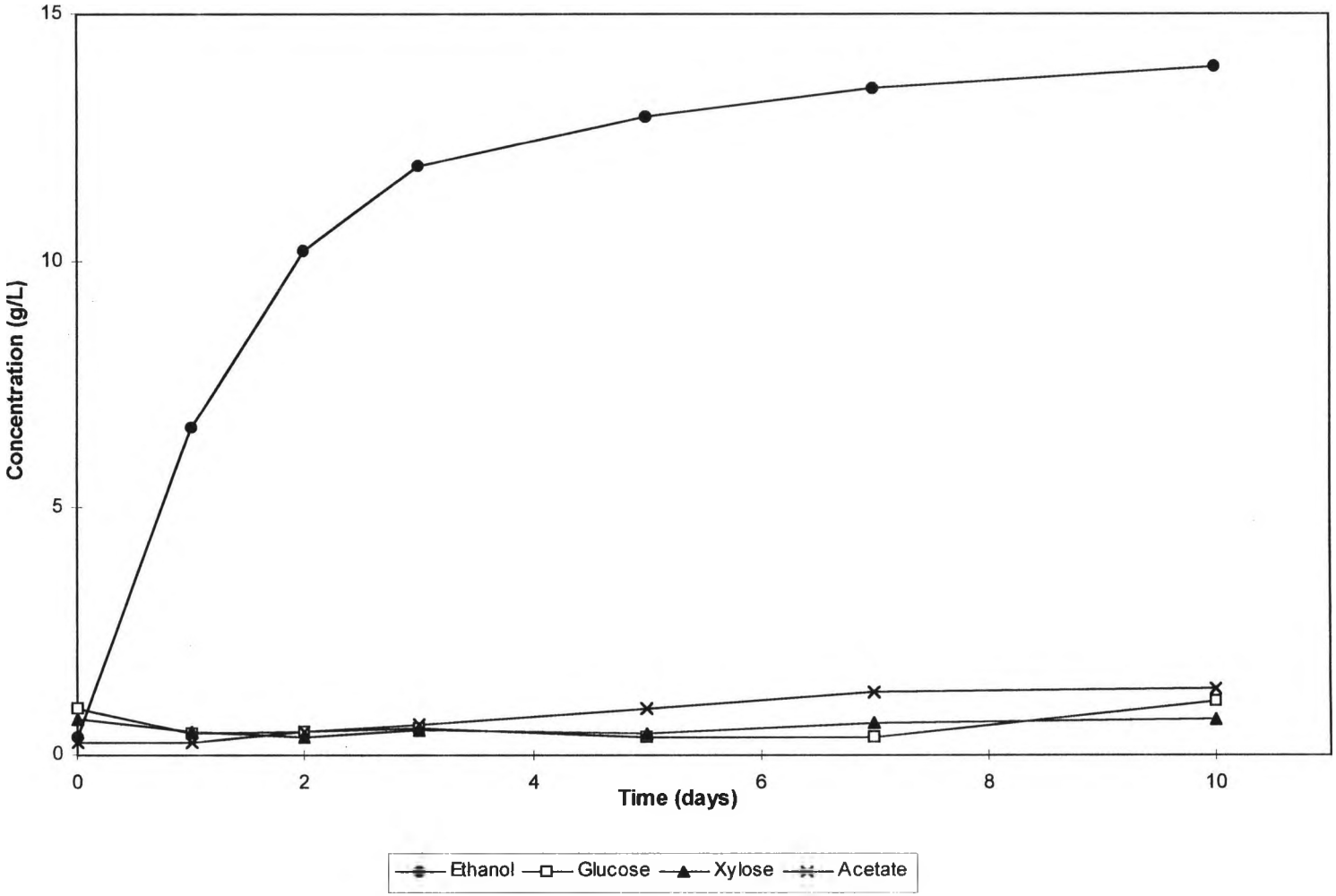



Fig. 4.22. SSCF kinetics for alpha-cellulose.

Sugar cane bagasse

The SSCF results for alkaline/peracetic pretreated sugar cane bagasse are very similar to these found for hybrid poplar. But for peracetic acid pretreated sugar cane bagasse the ethanol yields were much better than peracetic acid pretreated hybrid poplar. Inhibition effects by the pretreated bagasse is less than found by pretreated hybrid poplar, especially for pretreatment performed under higher acid concentrations (Fig. 4.23). The 6% NaOH/15% peracetic acid, 21% peracetic acid, and 60% peracetic acid pretreated bagasse samples give ethanol yields of 91.9, 91.4, and 90.7% respectively. As with hybrid poplar, 6% NaOH/15% peracetic acid pretreated bagasse has shown the best overall performance considering the satisfactory ethanol yield, very low sugar level and acetate formation during SSCF by *Z. mobilis* (Figs. 4.23 and 4.24). Fermentation kinetics for sugar cane bagasse pretreated with 21% peracetic acid are much better than observed for hybrid poplar due to negligible inhibition effects (Figs. 4.25 and 4.17). The ethanol yield is very low during the first day for 60% peracetic acid pretreated substrate but, in the second day of fermentation, the rate of ethanol formation surpass all comparative tests (Fig. 4.23). According to Fig. 4.26, high levels of glucose and xylose, 10 and 8 g/L respectively, were accumulated in the beginning of fermentation and these levels were reduced drastically to below 2 g/L during the second day fermentation. This could suggest an adaptation need for the recombinant bacteria in a highly chemically modified substrate as found in 60% peracetic acid pretreated bagasse. Raw bagasse gave a better ethanol yield than raw hybrid poplar indicating a more favorable natural enzymatic digestibility property found in most grass species (Fig 4.23 and 4.15). Fermentation kinetics for raw bagasse are shown in Fig. 4.27.

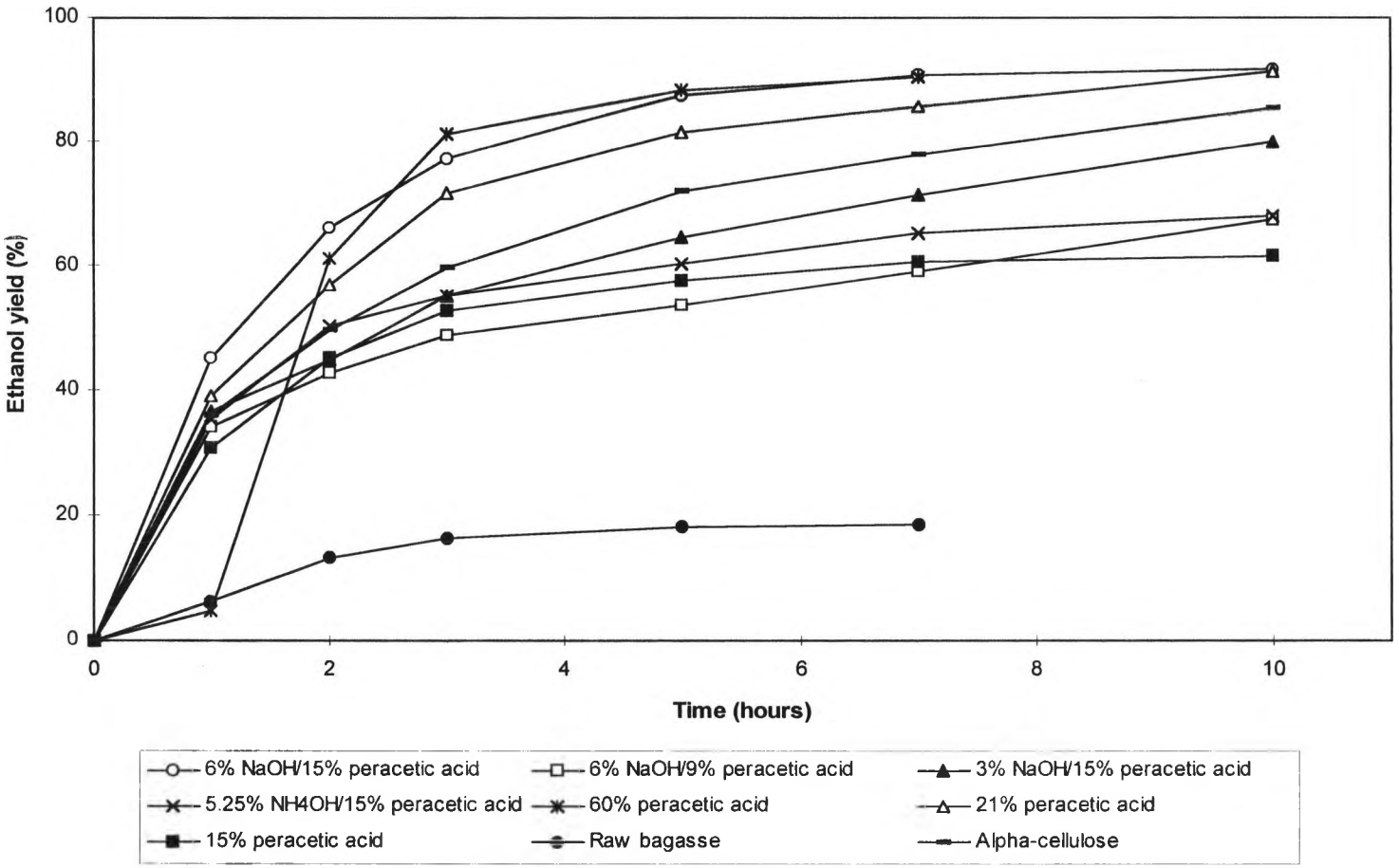



Fig. 4.23. Ethanol yield from SSCF of samples from different pretreatment conditions for sugar cane bagasse, raw bagasse and alpha-cellulose.

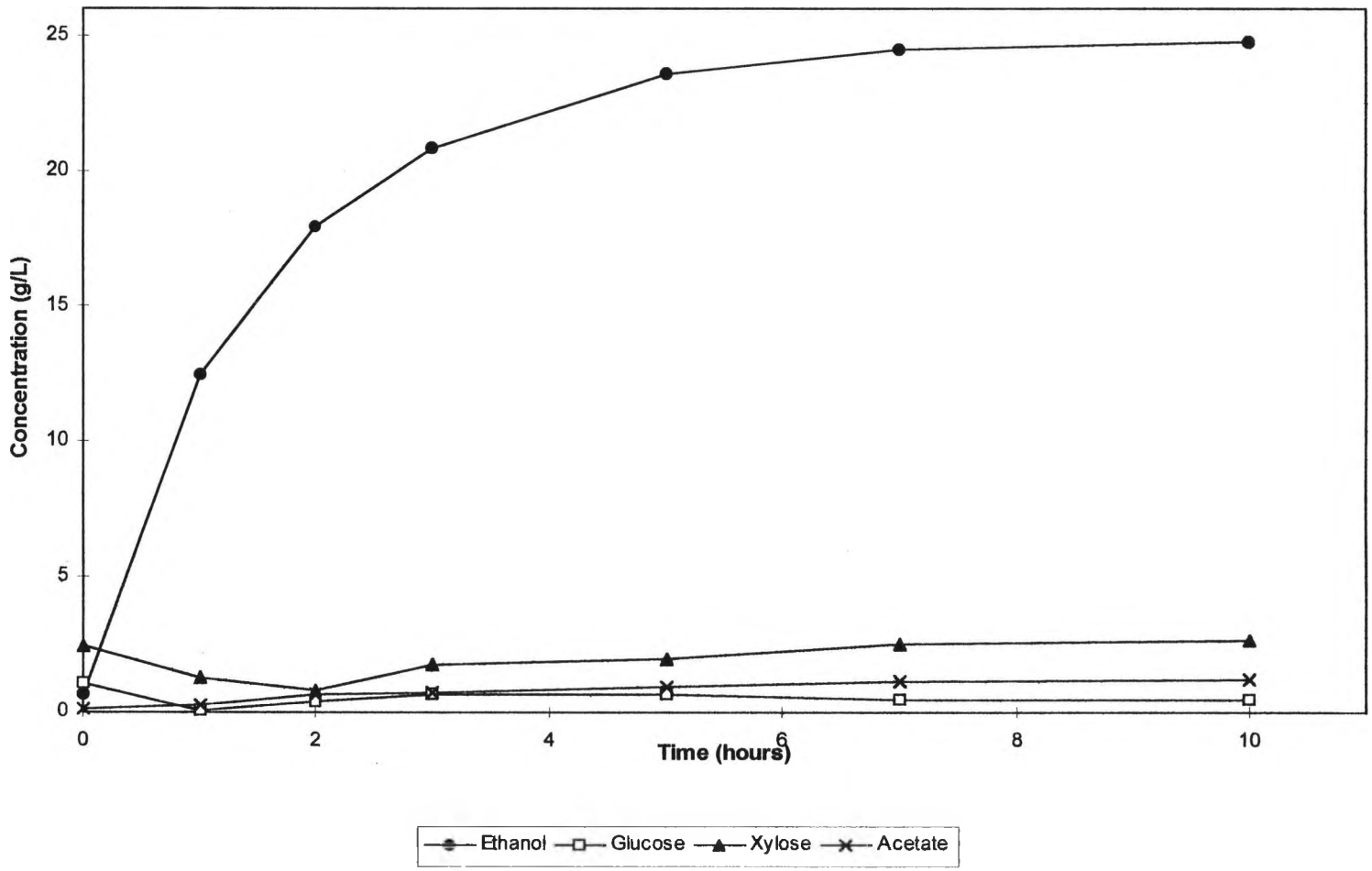



Fig. 4.24. SSCF kinetics for 6% NaOH/15% peracetic acid pretreated sugar cane bagasse.

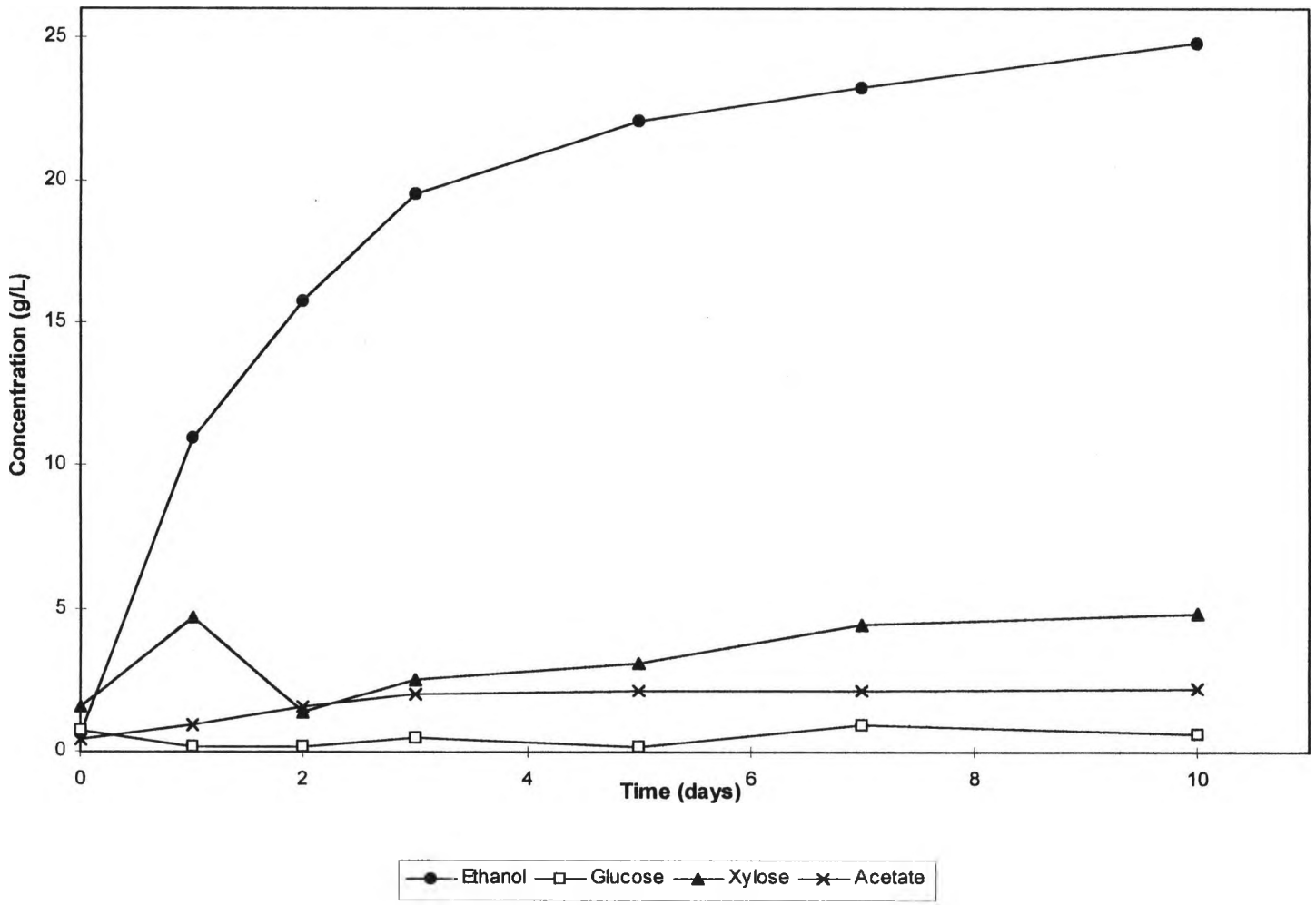



Fig. 4.25. SSCF kinetics for 21% peracetic acid pretreated sugar cane bagasse.

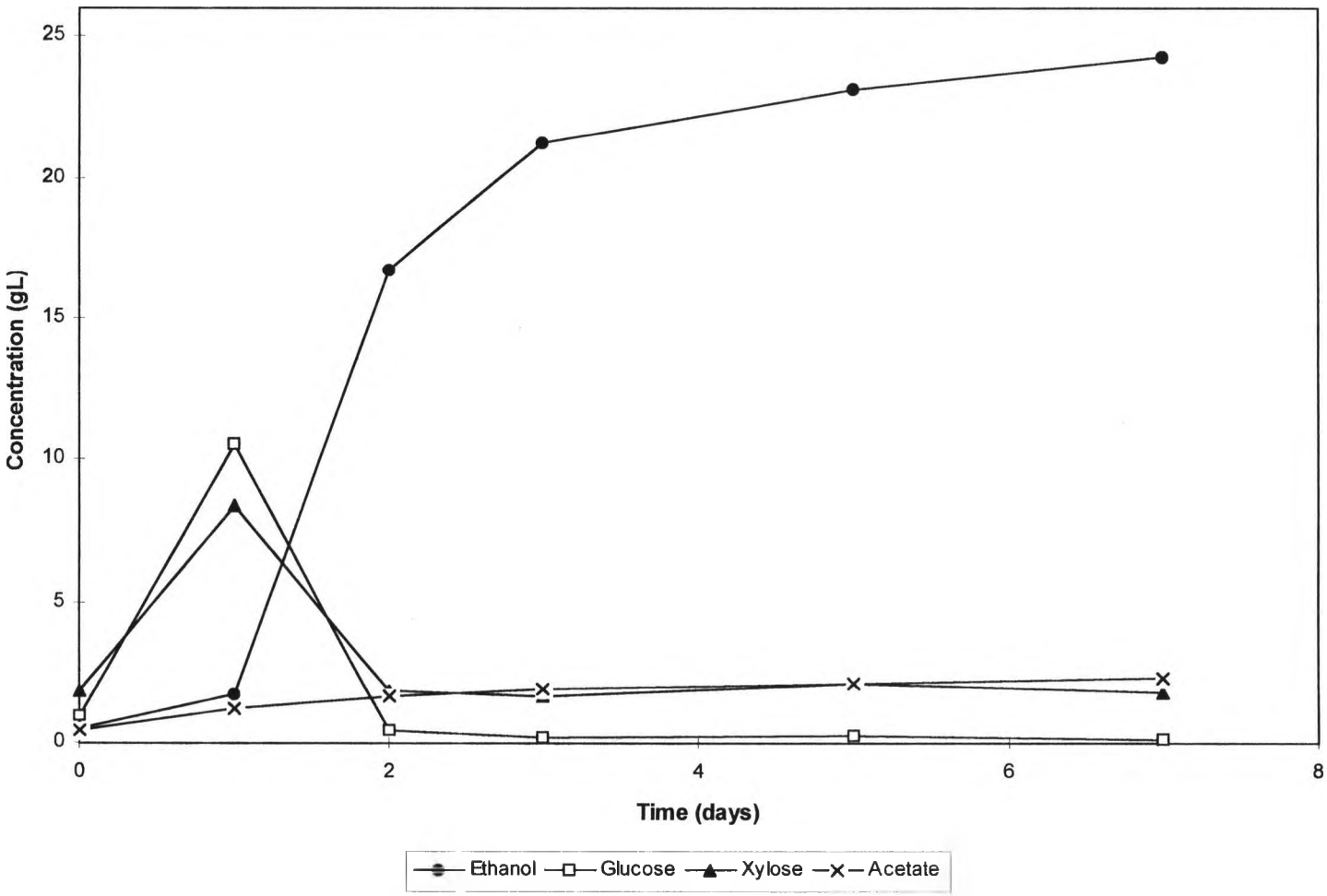



Fig. 4.26. SSCF kinetics for 60% peracetic acid pretreated sugar cane bagasse.

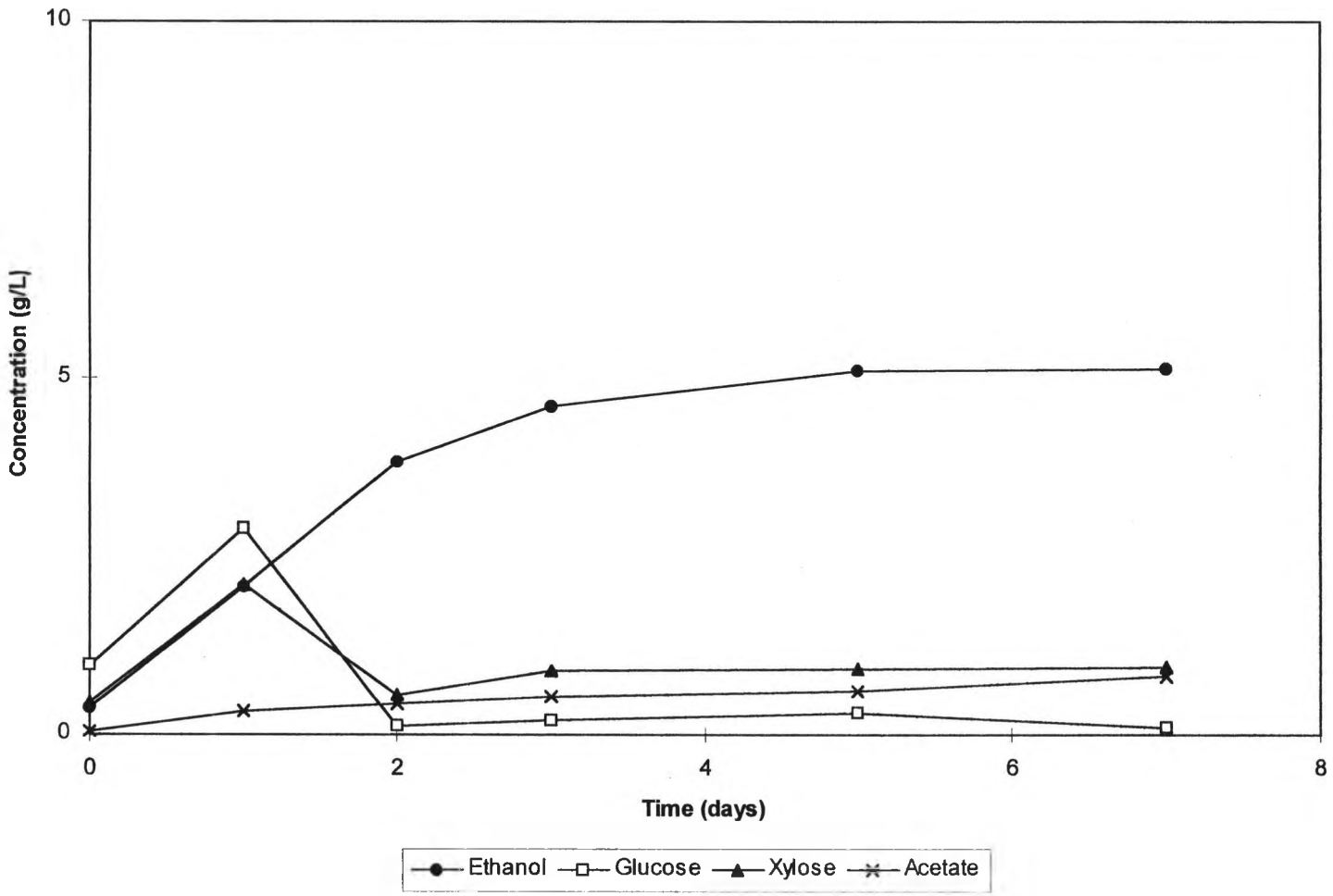



Fig. 4.27. SSCF kinetics for raw sugar cane bagasse.

# CHAPTER 5

# CONCLUSIONS AND RECOMMENDATIONS

The purpose of this research was to evaluate the effectiveness of peracetic acid as a pretreatment chemical for improving enzymatic digestibility of hybrid poplar and sugar cane bagasse. The effects of alkaline washing prior to the peracetic acid pretreatment was studied in addition to the use of the acid only. Sodium hydroxide was much more effective than ammonium hydroxide. The technical feasibility of the pretreated biomasses for ethanol fuel production was demonstrated by standard enzymatic hydrolysis and simultaneous saccharification and cofermentation (SSCF) procedures. The compiled results obtained in this research lead to the following conclusions and recommendations:

- Peracetic acid and alkaline/peracetic acid pretreatments give higher overall solid yields than conventional pretreatment methods because of the mild conditions characteristic of the process.

- Considering the high xylan content present in the peracetic acid pretreated hybrid poplar substrate, the combination of industrial enzymes SP431, a xylanase with significant cellulase activity, and Novozym 188, a β-glucosidase supplement, presented the best sugar conversion in enzymatic hydrolysis compared to other combinations. An

explanation might be the higher amount of xylanases present in this enzyme loading that facilitates the xylan degradation during hydrolysis and makes the cellulose microfibrils more accessible to cellulases. Addition of β-glucosidase avoids cellobiose accumulation and subsequent feedback inhibition.

• Peracetic acid pretreatment was effective in improving enzymatic digestibility of hybrid poplar and sugar cane bagasse. A seven-day reaction period was enough to satisfactorily treat the hybrid poplar. The minimum loading of peracetic acid for obtaining a good enzymatic hydrolysis yield in hybrid poplar and sugar cane bagasse is 21% based on oven-dry weight of biomass.

• A pre-pretreatment using a diluted (6%) sodium hydroxide solution prior to peracetic acid pretreatment resulted, in the 9 and 15% peracetic acid treated samples, in significantly better glucan and xylan conversion to simple sugars. Consequently, smaller amounts of peracetic acid are needed.

• Preliminary results indicate that reduction of acetyl groups in combination with a slight delignification are responsible for satisfactory sugar and ethanol yields. Increase in enzymatic digestibility of hybrid poplar wood and sugar cane bagasse by removal of acetyl groups from xylan has been demonstrated. Extrapolation of sugar cane bagasse data suggests that if the acetyl groups were removed totally from biomass by an efficient alkaline pre-pretreatment, a loading of 6% of peracetic acid could be enough to obtain similar results found in higher acid concentrations of 9 and 15% based on oven-dry weight of biomass.

• The technical viability of hybrid poplar and sugar cane bagasse pretreated with 6% NaOH/15% peracetic acid was verified through SSFC using a recombinant *Z. mobilis* CP4/pZB5, with an average theoretical ethanol yield of 92.3 and 91.4% respectively using 8.33 IFPU/g cellulose. The 6% NaOH/9% peracetic acid treated hybrid poplar could meet the same requirements if the enzyme loading were increased to greater than the used enzymatic loading.

• In spite of satisfactory simple sugar yields using the standard enzymatic hydrolysis tests, pretreatments using concentration of 21% or higher of peracetic acid with hybrid poplar caused growth inhibition in *Z. mobilis* during SSCF tests. Oxidized lignin by-products might be the possible cause of inhibition. Negligible inhibition has been verified for sugar cane bagasse pretreated with the same acid concentrations.

• The results presented here are preliminary and should be optimized, specially for alkaline pre-pretreatments with regard to base concentration and residence time for complete acetyl removal from the biomass. Alkaline washing after peracetic acid pretreatment helps to diminish growth inhibition in recombinant *Z. mobilis* and this post-washing procedure might be included as a part of the overall pretreatment. SSCF tests using recombinant yeast instead of bacteria should be tested with the substrates obtained here for comparison and better evaluation of pretreatment effectiveness.

## REFERENCES


Adler, E. (1977). Lignin chemistry, past, present, and future. *Wood Science and Technology*, **11**:169-218.

Albrecht, J. S. and Nicholls, G. A. (1976). Size and saccharide distribution of soluble products from stepwise-peroxyacetic acid delignification of loblolly pine. *Paperi ja Puu - Papper och Trä*, **2**:49-56.

Ando, S; Kakimoto, T.; Itoh, K.; Arai, I; Kiyoto, K.; and Hanai, S. (1988). Increased digestibility of cedar by pretreatment with peracetic acid and steam explosion. *Biotechnology and Bioengineering*, **31**:802-804.

Anonymous (1996). Kemira to build peracetic acid plant. *Chemical Week*, Jan. 3/10, p. 22.

Anonymous (1995). Kemira peracetic unit a "first". *Chemical Marketing Reporter*, Dec. 25, p. 9.

Anonymous (1991). Modern methods of analysis of wood, annual plants and lignins. Proceedings of the International Energy Agency Pre-Symposium, New Orleans, Nov. 29, 1991.

Anonymous (1965). Preparing peracetic acid from Albone hydrogen peroxide. Technical Information. E. I. DuPont de Nemours and Company. Wilmington, Delaware.

Aziz, S. and Sarkanen, K. (1989). Organosolv pulping - a review. Tappi Journal, vol. 72, no. 3, 169-175.

Bailey, A. J. (1936). Lignin in Douglas Fir - Composition of the Middle Lamella. *Industrial and Engineering Chemistry, Analytical Ed.*, **8**:52-55.

Bailey, B. K. (1996). Performance of ethanol as a transportation fuel. In, *Handbook on Bioethanol: Production and Utilization*. Wyman, C. E., Ed., Taylor & Francis Washington, DC. Pp. 1-18.

Bailey, M. J.; Biely, P.; and Poutanen, K. (1992). Interlaboratory testing of methods for assay of xylanase activity. *Journal of Biotechnology*, **23**:257-270.

Baker, A. J.; Millett, M. A.; and Satter, L.D. (1974). Wood and wood-based residues in animal feeds. In, *Cellulose Technology Research*. A. F. Turbak, Ed. ACS Symposium Series 10. Washington, D.C. Pp. 75-105.

Barbosa, M. F. S.; Beck, M. J.; Fein, J. E.; Potts, D.; and Ingram, L. O. (1992). Efficient fermentation of *Pinus sp.* Acid hydrolysates by an ethanologenic strain of *Escherichia coli. Applied and Environmental Microbiology*, **58**(4):1382-1384.

Bennani, A.; Rigal, L.; Wright, J. D.; and Grohmann, K. (1991). Refining of lignocellulose by organosolv processes. Part I: Isolation, characterization and utilization of hemicellulose extracted from Norway spruce. *Biomass and Bioenergy*, **1**(5):289-296.

Breuil, C.; Chan, M.; Gilbert, M.; and Saddler, J. N. (1992). Influence of β-glucosidase on the filter paper activity and hydrolysis of lignocellulosic substrates. *Bioresource Technology*, **39:**139-142.

Breuil, C.; Chan, M.; and Saddler, J. N. (1990). Comparison of the hydrolytic activity of commercial cellulase preparation. *Applied Microbiology and Biotecnology*, **34**:31-35.

Brink, D. L. (1994). Method of treating biomass material. U.S. Patent 5,366,558.

Brink, D. L. (1993) Method of treating biomass material. U.S. Patent 5,221,357.

Brito, L. M. R. A. (1994). Ph.D. Dissertation. Dilute phosphoric and oxalic acids as pretreatments for woody biomass prior to enzymatic hydrolysis. Colorado State University, Fort Collins, CO.

Browning, B. L. (1967). *Methods of Wood Chemistry*. Vol. II. Interscience Publishers, John Wiley & Sons, N.Y.

Burden, D. W. and Whitney, D. B. (1995). *Biotechnology: Proteins to PCR.* Birkhäuser, Boston, MA.

Carey, V. C.; Walia, S. K.; and Ingram, L. O. (1983). Expression of a lactose transposon (Tn951) in *Zymomonas mobilis. Applied Environmental Microbiology*, **46**:1163-1168.

Casey, J. P. (1980). *Pulp and Paper, Chemistry and Chemical Technology.* Wiley-Interscience Publication, John Wiley & Sons, N.Y.

Chang, M. M.; Chou,, T. Y. C.; and Tsao, G. T. (1981). Structure, pretreatment, and hydrolysis of cellulose. In, *Advances in Biochemical Engineering*, No. 20. A. Fiechter, Ed. Springer-Verlag, Berlin, Germany.

Chatters, R. M. (1963). Siliceous skeletons of wood fibers. *Forest Products Journal*, 13:368-372.

Chum, H. L.; Johnson, D. K.; Black, S.; Baker, J.; Grohmann, K.; and Sarkanen, K.V. (1988). Organosolv pretreatment for enzymatic hydrolysis of poplars: 1. Enzyme hydrolysis of cellulose residues. *Biotechnology and Bioengineering*, **31**:643-649.

Chum, H. L.; Johnson, D. K.; and Black, S. K. (1990). Organosolv pretreatment for enzymatic hydrolysis of poplars: 2. Catalyst effects and the combined severity parameters. *Industrial & Engineering Chemistry Research*, **29**:156-162.

Chum, H. L.; Douglas, L. J.; Feinberg, D. A.; and Schroeder, H. A. (1985). Evaluation of pretreatment of biomass for enzymatic hydrolysis of cellulose. Technical Report NREL/TP-231-2183 from the National Renewable Energy Laboratory Golden, CO.

Côté, W. A., Jr., ed. (1967). *Wood Ultrastructure. An Atlas of Electron Micrographs.* University of Washington Press. Seattle, WA.

Dale, B. E. (1986). Method for increasing the reactivity and digestibility of cellulose with ammonia. U.S. Patent 4,600,590.

Dale, B. E.; Henk, L. L.; and Shiang, M. (1985). Fermentation of lignocellulosic materials treated by ammonia freeze-explosion. *Development in Industrial Microbilogy*, **26**:223-233.

Dale, B. E. and Moreira, M. J. (1982). A freeze-explosion technique for increasing cellulose hydrolysis. *Biotechnology and Bioengineering Symposium*. No 12, 31-43.

Davidge, H.; Davies, A. G.; Kenyon, J.; and Mason, R. F. (1958). The oxidation of phenolic ethers with peroxyacetic acid. *Journal of the Chemical Society of London*. Part IV: 4569-4573.

Dekker, R. F. H. and Wallis, A. F. A. (1983). Enzymic saccharification of sugar cane bagasse pretreated by autrohydrolysis-steam explosion. *Biotechnology and Bioengineering*, **25**:3027-3048.

DeLong, E. A. (1981). Method of rendering lignin separable from cellulose and hemicellulose in lignocellulosic material and the product so produced. Canadian Patent 1,096,374.

Ehrman, T. (1992). Two stage sulfuric acid hydrolysis for determination of carbohydrates. Chemical Analysis & Testing Standard Procedure, No. 2. National Renewable Energy Laboratory, Golden, Colorado.

Fan, L. T.; Gharpuray, M. M.; and Lee, Y. -H. (1987). *Cellulose Hydrolysis*. Springer-Verlag, Berlin, Germany.

Fan, L. T.; Gharpuray, M. M.; and Lee, Y. -H. (1981). Evaluation of pretreatments for enzymatic conversion of agricultural residues. In, *Biotechnology Bioengineering Symposium*, No. 11. C.D. Scott, Ed. Interscience, John Wiley & Sons, N.Y. Pp. 29-45.

Farid, M. A.; Shaker, H. M.; and El-Diwany, A. I. (1983). Effect of peracetic acid, sodium hydroxide and phosphoric acid on cellulosic materials as a pretreatment for enzymatic hydrolysis. *Enzyme Microbiology and Technology*, **5**:421-424.

Farrand, J. C. and Johnson, D. (1971). Peroxyacetic acid oxidation of 4-methylphenols and their methyl ethers. *Journal of Organic Chemistry*, **36**(23):3606-3612.

Fengel, D. and Weneger, G. (1984). *Wood, Chemistry, Ultrastructure and Reactions.* Walter de Gruyter & Co., Berlin - New York.

Fengel, D. and Grosser, D. (1975). Chemische Zusammensetzung von Nadel und Laubhölzern. *Holz Roh Werkstoff*, **33**(1):32-35.

France, R. C. (1974). M.Sc. thesis. Peracetic Acid Pretreatment Alkaline Pulping. Colorado state University. Fort Collins, CO.

Freudenberg, K. and Neish, A. C. (1968). Constitution and Biosynthesis of Lignin. Springer-Verlag New York Inc.

Gharpuray, M. M.; Lee Y. H; and Fan, L.T. (1983). Structural modification of Lignocellulosics by pretreatment to enhance enzymatic hydrolysis. *Biotechnology and Bioengineering*, **25**:157-172.

Ghose, T. K. (1987). Measurement of cellulase activities. *Pure and Applied Chemistry*, **59**(2):257-268.

Glinski, A. J. and Nicholls, G. A. (1977). Peroxyacetic acid delignification of white birch and new evidence for lignin-carbohydrate bonds. *Paperi ja Puu - Papper och Trä*, **11**:745-760.

Grace, T. M.; Leopold, B; and Malcolm, E. W. (1989). Chemical reactions of wood constituents. In, *Pulp and Paper Manufacture*. Vol. 5, Alkaline Pulping. Published by The Joint Textbook Committee of the Paper Industry, TAPPI, CPPA.

Goldstein, I. S. (1983). Acid process for cellulose hydrolysis and their mechanisms. In, *Wood and Agricultural Residues: Research on Use for Feed, Fuels, and Chemicals*. E. J. Soltes, Ed. Academic Press, N.Y. Pp. 315-328.

Gould, J. M. (1987). Alkaline peroxide treatment of nonwoody lignocellulose. U.S. Patent 4,649,113.

Gould, J. M. (1985). Studies on the mechanism of alkaline peroxide delignification of agricultural residues. *Biotechnology and Bioengineering*, **27**:225-231.

Gould, J. M. (1984). Alkaline peroxide delignification of agricultural residues to enhance enzymatic saccharification. *Biotechnology and Bioengineering*, **26**:46-52.

Grohmann, K. (1993). Simultaneous saccharification and fermentation of cellulosic substrates to ethanol. In, *Bioconversion of Forest and Agricultural Plant Residues*. Saddler, J. N., Ed. Biotechnology in Agriculture Series No. 9. Wallingford, Oxon, UK. Pp. 183-209.

Grohmann, K.; Mitchell, D. J.; Himmel, M. E.; Dale, B. E; and Schroeder, H. A. (1989). The role of ester groups in resistance of plant cell wall polysaccharides to enzymatic hydrolysis. *Applied Biochemistry and Biotechnology*, **20/21**:45-61.

Grohmann, K.; Torget, R.; and Himmel, M. (1985). Optimization of dilute acid pretreatment of biomass. *Biotechnology and Bioengineering Symposium*, 15:59-80.

Heitz, M.; Capek-Menard, E.; Koeberle, P. G.; Gangne, J.; Chornet, E.; Overend; R. P.; Taylor, J. D.; and Yu, E. (1991). Fractionation of *Populus tremuloides* at the pilot plant scale: Optimization of steam preteatment conditions using the Stake II Technology. *Bioresouce Technology*, **35**:23-32.

Hinmam, N. D.; Schell, D. J.; Riley, C. J.; Bergeron, P. W.; and Walter, P. L. (1992). Preliminary estimates of the cost of ethanol production for SSF technology. *Applied Biochemistry and Biotechnology*, **34/35**:639-651.

Holztapple, M. T.; Lundeen, J. E.; Sturgis, R.; Lewis, J. E; and Dale, B. E. (1992). Pretreatment of lignocellulosic solid waste by ammonia fiber explosion (AFEX). *Applied Biochemistry and Biotechnology*, **34/35**:5-21.

Holtzapple, M. T.; Jun, J-H; Ashok, G.; Patibandla, S. L.; and Dale, B. E. (1991). The ammonia freeze explosion (AFEX) process. *Applied Biochemistry and Biotechnology,* **28/29**:59-74.

Holtzapple, M. T. and Humphrey A. E. (1984). The effects of organosolv pretreatments on the enzymatic hydrolysis of poplar. *Biotechnology and Bioengineering*, **26**:670-676.

Holtzapple, M. T. (1981). Ph.D. Dissertation. The pretreatment and enzymatic saccharification of poplar wood. University of Pennsylvania, Philadelphia, PA.

Hsu, T-A. (1996). Preteatment of biomass. In, *Handbook on Bioethanol: Production and Utilization*. Wyman, C. E., Ed., Taylor & Francis , Washington, DC. Pp. 179-212.

Immergut, E. H. (1975). Cellulose. In, *The Chemistry of Wood*. B. L. Browning, Ed., Robert E. Krieger Publishing Company, Huntington, NY, Pp.103-190.

Ingram, L. O.; Carey, V. C.; Dombek, K. M.; Holt, A. S.; Holt, W. A.; Osman, Y. A.; and Walia, S. K. (1984). Biochemical and genetic improvement of *Zymomonas mobilis. Biomass*, 6, pp. 131-143.

Janes, R. L. (1969). The chemistry of wood fibers. In, *The Pulping of Wood*. R. G. McDonald, Ed.

Jones, J. L. and Semrau, K.T. (1984). Wood hydrolysis for ethanol production - Previous experience and the economics of selected processes. *Biomass*, **5**:109-135.

Joseleau, J. P.; Comtat, J.; and Ruel, K. (1991). Chemical structure of xylans and their interaction in the plant cell walls. In, *Xylan and Xylanases*. J. Visser, M. A. Kusters-van Someren, G. Beldman, A. G. J. Voragen, Eds. Elsevier, Amsterdam, Netherlands. Pp. 1-15.

Kawamoto, H.; Chang, H.; and Jameel, H. (1994). Reaction of lignin model compounds with peracetic acid or peroxymonosulfuric acid. Proceedings of the International Symposium on Fiber Science and Technology. Oct, 26-28. Yokohama, Japan. Page 230.

Kong, F.; Engler, C. R; and Soltes, E. J. (1992). Effects of cell-wall acetate, xylan backbone, and lignin on enzymatic hydrolysis of aspen wood. *Applied Biochemistry and Biotechnology*, **34/35**:23-35.

Kong, F. (1990). M.S. Thesis. Effect of acetate and other cell wall components on enzymatic hydrolysis of aspen wood. Texas A&M University, College Station, TX.

Koran, Z.; Kokta, B. V.; Valade, J. L.; and Law, K. N. (1978). Fiber characteristics of Masonite pulp. *Pulp and Paper Canada*, **29**:T107-T113.

Kuhad, R. C.; Singh, A.; and Eriksson, K-E. (1997). Microorganisms and enzymes involved in the degradation of plant fiber cell walls. In, *Advances in Biochemical Engineering Biotechnology*. T. Scheper, Ed., Springer-Verlag, Berlin, Germany. Pp. 45-125.

Kutscha, N. P. (1968). Cell wall development in normal and compression wood of balsam fir, *Abies balsamea* (L.) Mill., Ph.D. Dissertation, State University College of Forestry at Syracuse University, Syracuse, N.Y.

Lai, Y. Z. and Sarkanen, K. V. (1971). Lignin: isolation and structural studies. In, *Lignins, Occurrence, Formation, Structure and Reactions*. Sarkanen, K. V. and Ludwig, C. H., Eds. Wiley-Interscience, N.Y. Pp. 165-240.

Lai, Y. Z. and Sarkanen, K. V. (1968). Delignification by peracetic acid. II. Comparative study on softwood and hardwood lignins. *TAPPI*, **51**(10):449-453.

Lawford, H. G. and Rousseau, J. D. (1992). The effect of lactic acid on fuel ethanol production by *Zymomonas*. *Applied Biochemistry and Biotechnology*, **34/35**:205-215.

Lee, K. J.; Skotnicki, M. L.; Tribe, D. E.; and Rogers, P. L. (1981). The effect of temperature on the kinetics of ethanol production by strains of *Zymomonas mobilis*. *Biotechnology Letters*, **3**(6):291-296.

Lima, O. G.; Araújo, J. M.; Schumacher, I. E.; and Silva, E. C. (1970). Estudos de microorganimos antagonistas presentes nas bebidas fermentadas usadas pelo povo de Recife. I. Sôbre uma variedade de *Zymomonas mobilis* (Lindner, 1928), (Kluyver e van Niel, 1936): *Zymomonas mobilis* var. *recifences* (Gonçalves de Lima, Araújo, Schumacher & Calvacanti, 1970), isolada de bebida popular denominada "caldo de cana picado." *Revista do Instituto de Antibioticos da Universidade de Recife*, **10**:3-15.

Linden, J. C. and Schroeder, H. A. (1996). Development of alternate pretreatment and biomass fractionation processes. Final Technical Progress Report. Subcontract No. XAW-4-14320-01. Contracted by the National Renewable Energy Laboratory, Golden, Colorado. Submitted by the Departments of Chemical and Bioresource Engineering, Forest Sciences, and Microbiology. Colorado State University, Fort Collins, CO.

Mackie, K. L.; Browell, H. H.; West, K. L.; and Saddler, J. N. (1985). Effect of sulphur dioxide and sulphuric acid on steam explosion of aspenwood. *Journal of Wood Chemistry and Technology*, **5**(3):405-425.

Marshall II, S. R. (1976). M.S. Thesis. Peracetic Acid Pretreatment and Alkaline Digestion of Southern Pine. Colorado State University. Fort Collins, CO.

McComb, E. A. and McCready, R. M. (1957). Determination of acetyl in pectins and in acetylated carbohydrate polymers. *Analytical Chemistry*, **29**(5):819-821.

McMillan, J. D. (1996). Hemicellulose conversion to ethanol. In, *Handbook on Bioethanol: Production and Utilization*. C. E. Wyman, Ed., Taylor & Francis, Washington, DC. Pp. 287-313.

McMillan, J. D. (1994). Pretreatment of lignocellulosic biomass. In, *Enzymatic Conversion of Biomass for Fuels Production*. M. E. Himmel, J. O. Baker, and R. P. Overend, Eds. American Chemical Society, Washington, DC. Pp. 292-324.

Meier, H. (1957). Discussion of the cell wall organization of tracheids and fibers. *Holzforschung*, **11**:41-46.

Meller, A. (1960). The chemistry of alkaline degradation of cellulose and oxidized cellulose I. *Holzforschung*, **14**:78-88.

Mitchell, D. J. (1989). M.S. Thesis. Acetyl Xylans: The Effect of Acetylation on the Enzymatic Digestion of Biomass. Colorado State University, Fort Collins, CO.

Millet, M. A.; Baker, A. J.; and Satter, L. D. (1976). Physical and chemical pretreatments for enhancing cellulose saccharification. In, *Enzymatic Conversion of Cellulosic Materials: Technology and Applications.*. Biotechnology & Bioengineering Symposium No. 6. E. L.Gaden; M. H. Mandels; E. T. Reese; and L. A. Spano, Eds. Interscience, John Wiley & Sons, N.Y., Pp. 125-153.

Millet, M. A.; Baker, A. J.; and Satter, L. D. (1975). Pretreatments to enhance chemical, enzymatic, and microbiology attack of cellulose materials. In, *Cellulose as a Chemical and Energy Resource*. Biotechnology and Bioengineering Symposium No. 5. C. R. Wilke, Ed. Interscience. John Wiley & Sons, N.Y. Pp. 193-219.

Montenecourt, B. S. (1985). *Zymomonas*, a unique genus of bacteria. In, *Biology of Industrial Microorganisms*, A. L. Demain. And N. Solomon, Eds. Benjamin-Cummings, Menlo Park, CA. Pp. 261-289.

Morrison, I. M. (1974). Structural investigations on the lignin-carbohydrate complexes of *Lolium perenne*. *Biochemical Journal*, **139**:197-204.

Myung, K. H. and Kennelly, J. J. (1992). Effect of alkaline hydrogen peroxide and peracetic acid on *in sacco* ruminal digestibility of aspen sawdust. *AJAS*, **5**(4):635-641.

Nimz, H. H.; Muladi, S; Pilz, A; Salow, H; Schöne, M.; and Schwind, H. (1992). Chlorine-free bleaching of acetosolv-pulps with ozone and peracetic acid. European Workshop on Lignocellulosic and Pulp Oxidation of Lignocellulosic Materials. Pp. 91-92.

Nimz, H. (1974). Beech lignin - proposal of a constitutional scheme. *Angewandte Chemie, International Ed.*, **13**(5):313-321.

Panshin, A. J. and Zeeuw, C. (1980). *Textbook of Wood Technology. Structure. Identification, Properties, and Uses of the Commercial Woods of the United States and Canada*. McGraw-Hill, Inc., N.Y.

Paszner, L. and Cho, H. J. (1988). High yield organosolv process for conversion of cellulosic biomass to ethanol. *Energy from Biomass and Wastes*, **12**:1297-1318.

Pettersen, R. C. (1984). The chemical composition of wood. In, *The Chemistry of Solid Wood*. R. Rowell, Ed., American Chemistry Society, Washington, D.C. Pp. 57-126.

Philipippidis, G. P. (1996). Cellulose conversion technology. In, *Handbook on Bioethanol: Production and Utilization*. C. E. Wyman, Ed., Taylor & Francis, Washington, DC. Pp. 253-285.

Philippidis, G. P.; Smith, T. K.; and Schmidt, S. L. (1993). SSF Experimental Protocols: Lignocellulosic Biomass Hydrolysis and Fermentation, No. 8. National Renewable Energy Laboratory, Golden, Colorado.

Playne, M. L. (1984). Increased digestibility of bagasse by pretreatment with alkalis and steam explosion. *Biotechnology and Bioengineering*, **26**:426-433.

Prine, G. M. and Woodard, K. R. (1993). Herbaceous energy crops in humid lower south USA. Proceedings of the First Biomass Conference of the Americas: Energy, Environmental, Agriculture, and Industry, Vol. 1, pp.278-283.

Pye, E. K. and Lora, J. H. (1991). The ALCELL process: A proven alternative to Kraft pulping. *Tappi Journal*, *74*(3):113-118.

Reichert, J. S.; McNeight, S. A.; and Rudel, H. W. (1939). Determination of hydrogen peroxide and some related oxygen compounds. *Industrial and Engineering Chemistry*, **11**(4):194-197.

Rodríguez-Vázquez, R. (1993). Ph.D. Dissertation. Chemical Pretreatment of Biomass and Comparison with Brown Rot Mechanism. Colorado State University, Fort Collins, CO.

Rogers, P.L.; Lee, K.J.; Skotnicki, M. L.; and Tribe, D. E. (1982). Ethanol production by *Zymomonas mobilis*. *Advances in Biochemical Engineering*, **23**:37-84.

Rydholm, S. A. (1965). *Pulping Process*. Interscience Publishers, NY.

Saddler, J. N.; Ramos, L. P.; and Breuil, C. (1993). Steam pretreatments of lignocellulosic residues. In, *Bioconversion of Forest and Agricultural Plant Residues*. Biotechnology in Agriculture No. 9. J. N. Saddler. Pp. 73-91.

Saddler, J. N.; Hogan, C.; Chan, M. K. -H.; and Louis-Seize, G. (1982). Ethanol fermentation of enzymatically hydrolysed pretreated wood fractions using *Trichoderma* cellulases, *Zymomonas mobilis*, and *Saccharomyces cerevisiae*. *Canadian Journal of Microbiology*, **28**:1311-1319.

Sakai, K; Kuroda, K; and Kishimoto, S. (1972). Delignification in peracetic bleaching. VI. Reaction of the beta-aryl ether type model compounds with peracetic acid. *TAPPI*, **55**:1702-1706.

Sakai, K. and Kishimoto, S. (1966). Delignification in peracetic bleaching. II. Peracetic acid oxidation of lignin. *Journal of the Japan Wood Reasearch Society*, **12** (6): 310-315.

Sarkanen, K. V. and Ludwig, C. H. (1971). *Lignins, Occurrence, Formation, Structure and Reactions*. Wiley-Interscience, N.Y.

Sarkanen, K. V. and Hergert, H. L. (1971). Lignin, classification and distribuition. In, *Lignins, Occurrence, Formation, Structure and Reactions*. K. V. Sarkanen and C. D. Ludwid, Eds. Wiley-Interscience, N.Y. Pp. 43-94.

Schöning, A. G. and Johansson, G. (1965). Absorptiometric determination of acid-soluble lignin in semichemical bisulfite pulps and in some woods and plants. *Svensk Papperstidning*, **68**(18):607-613.

Schroeder, H. A. (1994). Personal communication. Preparation and analysis of peracetic acid.

Schroeder, H. A. (1978). Wood chemistry class notes.

Schwald, W.; Breuil, C.; Brownell, H. H.; Chan, M.; and Saddler, J. N. (1989). Assessment of pretreatment conditions to obtain fast complete hydrolysis on high substrate concentrations. *Applied Biochemistry and Biotechnology*, **20/21**:29-44.

Seifert, K. (1956). Uber ein neues Verfahren zur Schnellbestimmung der Rein-Cellulose. *Das Papier* **10**:310-306.

Sjöström, E. (1993). Wood Chemistry, Fundamentals and Applications. Academic Press, Inc., N.Y.

Shafizadeh, F. (1983). Thermal conversion of cellulosic materials to fuel and chemicals. In, *Wood and Agricultural Residues. Research on Use for Feed, Fuels, and Chemicals.* J. Soltes, Ed. Academic Press, N.Y. Pp. 415-438.

Smith, D. C. C. (1955). Ester groups in lignin, *Nature*, **176**:267-268.

Soltes, J. (1989). Mechanism of plant cell wall resistance to attack by polysaccharide degrading enzymes. Final Report. Subcontract No. XK-7-07031-9, under Prime Contract No. DE-AC02-83CH10093, Conversion of Lignocellulose to Ethanol. Solar Energy Research Institute. Submitted by the Texas A&M Reseach Foundation. College Station, Texas.

Spangler, D. L. and Emert, G. H. (1985). Simultaneous saccharification / fermentation with *Zymomonas mobilis*. Biotechnology and Bioengineering, vol. 28, pp. 115-118.

Stricker, J. A.; Prine, G. M.; Woodard, K. R.; Anderson, D. L.; Shibles, D. B.; and Riddle, B. S. (1993). Production of biomass/energy crops on phosphatic clay soils in central Florida. Proceedings of the First Biomass Conference of the Americas: Energy, *Environmental, Agriculture, and Industry*, **1**:254-259.

Swings, J. and De Ley, J. (1977). The Biology of *Zymomonas. Bacteriological Reviews*, **41**:1-46.

Szmant, H. H. (1989). In Organic Building Blocks of the Chemical Industry, Wiley-Interscience, John Wiley & Sons, USA, Pp. 236-239.

Takagi, M; Abe, S.; Susuki, S.; Emert, G. H.; and Yata, N. (1977). A method for production of alcohol directly from cellulose using cellulase and yeast. Bioconversion of Cellulosic Substances into Energy Chemicals and Microbial Protein, Symposium. T. K. Ghose, Ed., ITT, New Delhi, India.

TAPPI Test Methods (1988). Fibrous Materials and Pulp Testing T1-T269; Paper and Paperboard Testing T400-7545. Vol. 1.

Taylor, J. D. (1986). Commercial update on the Stake process. In, *Biotechnology and Renewable Energy*. M. Moo-Young, S. Hasnain, and J. Lamptey, Eds. Elsevier Applied Science Publishers. Pp. 286-291.

Taylor, J. D. (1980). Continuous autohydrolysis, a key step in the economic conversion of forest and crop residues into ethanol. In, *Energy from Biomass*, Proceedings of the International Conference on Biomass. Brighton, UK, November, pp. 330-336.

Teixeira, L. C.; Linden, J. C.; and Schroeder, H. A. (1998a). Optimizing peracetic acid pretreatment conditions for improved simultaneous saccharification and cofermentation (SSCF) of woody biomass to ethanol. Biomass for Energy and Industry. 10th European Conference and Technological Exhibition. Proceedings of the International Conference. H. Kopetz, T. Weber, W. Palz, P. Chartier, G.L. Ferrero, Eds. Würzburg, Germany, pp. 134-137.

Teixeira, L. C.; Linden, J. C.; and Schroeder, H. A. (1998b). Peracetic acid as an alternative pretreatment for ethanol production from biomass. Presented at the 20th Symposium on Biotechnology for Fuels and Chemicals. Gatlinburg, Tennessee, May 3-7 (in press).

Timmel, T. E. (1967). Recent progress in the chemistry of wood hemicelluloses. *Wood Science and Technology*, **1**:45-70.

Timmel, T. E. (1965). Wood and bark polysaccharides. In, *Cellular Ultrastructure of Woody Plants*. W. A. Côté, ed. Syracuse University Press, Syracuse, NY. Pp. 127-156.

Timmel, T. E. (1964). Wood hemicellulose. *Advances in Carbohydrate Chemistry*, **19**:247-302.

Toon, S. T.; Philippidis, G. P.; Ho, N. W. Y., Chen, Z; Brainard, A.; Lumpkin, R. E.; and Riley, C. J. (1997). Enhanced cofermentation of glucose and xylose by recombinant Saccharomyces yeast strains in batch and continuous operating modes. *Applied Biochemistry and Biotechnology*, **63-65**:243-255.

Toyama, N. and Ogawa, K. (1975). Sugar production from agricultural woody wastes by saccharification with *Trichoderma viride* celullase. In, *Cellulose as a Chemical and Energy Resource*. Biotechnology and Bioengineering Symposium No. 5. C. R. Wilke ed. Published by John Wiley & Sons, New York. Pp. 225-244.

Tsao, G. T; Ladisch, M R.; Ladisch, C. M.; Hsu, T. (1981). Process for treating cellulosic materials and obtaining glucose therefrom. U.S. Patent 4,281,063.

Tsao, G. T.; Ladisch, M; Ladisch, C; Hsu, T. A.; Dale, B.; Chou, T. (1978). Fermentation substrates from cellulosic materials: Production of fermentable sugars from cellulosic materials. In, *Annual Report on Fermentation Proccess*, **2**:1-21.

Tsoumis, G. (1991). *Science and Technology of Wood. Structure, Properties, Utilization*. Van Nostrand Reinhold, N.Y.

Van Winkle, S. C. and Glasser, W. G. (1994). Chemical cellulose from steam-exploded wood by peracetic acid treatment. *Journal of Pulp and Paper Science*, **21**(2):J37-J43.

Yatagai, M. and Takashashi, T. (1980). Tropical wood extractives: Effects on durability, paint curing time and pulp sheet resin spotting. *Wood Science*, **12**(3):176-182.

Wangaard, F. F. (1992). The case for a non-fungitoxic wood preservative. International Conference on Wood Poles and Piles. Fort Collins, CO.

Wangaard, F. F. (1979). Wood: its structure and properties. *Journal of Educational Modules for Materials Science and Engineering*, **1**(3):437-534.

Waiss, A. C.; Guggolz, J.; Kohler, G. O.; Walker, H. G. (1972). Improving digestibility of straws for ruminant feed by aqueous ammonia. *Journal of Animal Science*, **35**(1):109-112.

Wayman, M. and Parekh, S. R. (1990). *Biotechnology of Biomass Converson. Fuels and Chemicals from Renewable Resources*. Prentice Hall, Englewood Cliffs, NJ.

Wilke, C. R.; Yang, R. D.; and von Stockar, U. (1976). Preliminary cost analysis for enzymatic hydrolysis of newsprint. In, *Enzymatic Conversion of Cellulosic Materials: Technology and Applications.*. Biotechnology & Bioengineering Symposium No. 6. E. L. Gaden; M. H. Mandels; E. T. Reese; and L. A. Spano, Eds. Interscience, John Wiley & Sons, N.Y. Pp. 155-175.

Wilkie, K. C. B. (1979). The hemicelluloses of grasses and cereals. *Advances in Carbohydrate Chemistry and Biochemistry*, **36**:215-264.

Wilson, S. (1994). Peroxygen technology in the chemical industry. *Chemistry & Industry*, **7**:255-258.

Wiselogel, A; Tyson, S; and Johnson, D. (1996). Biomass feedstock resources and composition. In, *Handbook on Bioethanol: Production and Utilization*. Wyman, C. E., ed., Taylor & Francis , Washington, DC. Pp. 105-118.

Wright, L. L. (1994). Production technology status of woody and herbaceous crops. *Biomass and Energy*, **6**(3):191-209.

Wu, Z. and Lee, Y. Y. (1997). Ammonia recycled percolation as a complementary pretreatment to the dilute-acid process. *Applied Biochemistry and Biotechnology*, **63-65**:21-57.

Wyman, C. E. (1996). Ethanol production from lignocellulosic biomass: overview. In, *Handbook on Bioethanol: Production and Utilization*. C. E. Wyman, Ed., Taylor & Francis , Washington, DC. Pp. 1-18.

Yoon, H. H.; Wu, Z.; and Lee, Y. Y. (1994). Ammonia-recycled percolation process for pretreatment of biomass feedstock. Sixteenth Symposium on Biotechnology for Fuels and Chemicals, Gatlinburg, TN, May 9-13.

Zang, M.; Eddy, C.; Deanda, K.; Finkelstein, M.; and Picataggio, S. (1995). Metabolic engineering of a pentose metabolism pathway in ethanogenic *Zymomonas mobilis. Science*, **267**:240-243.

Zhaoxin, L and Kumakura, M. (1995). Enzymatic hydrolysis of wheat straw irradiated by electron beam in presence of peracetic acid solution. *Isotopes Environmental Health Studies*, **31**:151-160.

# APPENDIX I

Time effect - Sugar released, partial composition and overall yield

Table 1. Glucose and xylose released after 48-hour enzymatic hydrolysis. Partial composition of peracetic acid treated hybrid poplar and overall yield of the pretreatment. Loadings or concentrations of peracetic acid were calculated on oven dry weight of wood. Pretreatments were performed at ambient temperature. Enzymatic hydrolysis tests were done at 37°C and using the enzyme combination Spenzyme/Novozym 188.

| Pretreatment conditions | Overall yield (%) | Klason lignin (%) | Soluble lignin (%) | Glucan (%) | Xylan (%) | Glucose released (g/L) after 48 hours | Xylose released (g/L) after 48 hours |
|---|---|---|---|---|---|---|---|
| 6%, 3 days | 96.4 | 23.6 | 3.4 | 41.4 | 13.7 | 1.24 | 0.41 |
| 9%, 3 days | 96.7 | 22.5 | 3.9 | 41.9 | 14.0 | 2.14 | 0.82 |
| 6%, 7 days | 97.2 | 22.0 | 3.7 | 41.5 | 13.7 | 1.20 | 0.45 |
| 9%, 7days | 94.8 | 20.5 | 4.0 | 42.0 | 14.2 | 2.50 | 1.02 |
| 6%, 14 days | 95.6 | 22.2 | 3.6 | 42.0 | 13.9 | 1.24 | 0.47 |
| 9%, 14 days | 93.7 | 19.7 | 4.1 | 43.6 | 14.7 | 2.39 | 1.01 |
| 6%, 28 days | 95.1 | 21.9 | 3.8 | 43.0 | 14.1 | 1.27 | 0.49 |
| 9%, 28 days | 93.6 | 19.6 | 4.2 | 44.3 | 14.9 | 2.40 | 1.07 |

# APPENDIX II

## Enzyme screening tests - released sugar composition and relative percentage glucan and apparent xylan conversion

Table 1. Test using SP431/Novozym 188 combination

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | nd | 0 |
| 5 | nd | 2.46 | 22.2 | 0.92 | 26.2 |
| 10 | nd | 3.44 | 31.0 | 1.50 | 42.8 |
| 24 | nd | 5.43 | 48.9 | 1.67 | 48.4 |
| 48 | nd | 6.25 | 56.5 | 2.51 | 71.8 |
| 118 | nd | 8.10 | 73.1 | 2.68 | 76.5 |

nd: not detected

Table 2. Test using SP431 only

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | 0.03 | nd | 0.1 | nd | 0 |
| 5 | 1.82 | 0.43 | 21.1 | 0.93 | 26.6 |
| 10 | 2.52 | 0.52 | 28.5 | 1.44 | 41.2 |
| 24 | 3.30 | 0.89 | 39.3 | 1.62 | 46.4 |
| 48 | 3.83 | 1.56 | 49.5 | 2.22 | 63.5 |
| 118 | 3.64 | 2.46 | 56.6 | 2.54 | 72.7 |

nd: not detected

Table 3. Test using SP431/Spezyme/Celluclast/Novozym 188 combination

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | nd | 0 |
| 5 | nd | 1.77 | 16.2 | 0.66 | 18.9 |
| 10 | nd | 3.18 | 28.7 | 1.24 | 35.3 |
| 24 | nd | 4.88 | 44.0 | 1.39 | 39.8 |
| 48 | nd | 6.09 | 54.9 | 1.65 | 47.2 |
| 118 | nd | 7.56 | 68.2 | 2.33 | 65.0 |

nd: not detected

Table 4. Test using Spezyme only

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | nd | 0 |
| 5 | 0.91 | 0.68 | 14.7 | 0.41 | 11.6 |
| 10 | 1.12 | 1.00 | 19.7 | 0.85 | 24.2 |
| 24 | 1.39 | 1.87 | 30.1 | 1.03 | 29.3 |
| 48 | 1.38 | 2.99 | 40.1 | 1.51 | 43.2 |
| 118 | 0.88 | 4.64 | 50.1 | 1.91 | 54.7 |

nd: not detected

Table 5. Test using Spezyme/Novozym 188 combination

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | nd | 0 |
| 5 | nd | 1.62 | 14.6 | 0.47 | 13.6 |
| 10 | nd | 2.69 | 24.3 | 1.02 | 28.8 |
| 24 | nd | 4.51 | 37.2 | 1.13 | 32.3 |
| 48 | nd | 5.73 | 48.2 | 1.45 | 41.5 |
| 118 | nd | 6.85 | 61.7 | 2.12 | 60.7 |

nd: not detected

Table 6. Test using Spezyme/SP431/Novozym 188 combination

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | nd | 0 |
| 5 | nd | 2.16 | 19.4 | 0.68 | 19.3 |
| 10 | nd | 3.29 | 29.6 | 1.27 | 36.3 |
| 24 | nd | 4.96 | 44.7 | 1.42 | 40.6 |
| 48 | nd | 6.23 | 56.2 | 1.77 | 50.6 |
| 118 | nd | 7.77 | 70.1 | 2.47 | 70.7 |

nd: not detected

Table 7. Test using Celluclast only

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | 0.07 | nd | 0.6 | nd | 0 |
| 5 | 0.52 | 0.63 | 10.6 | nd | 0 |
| 10 | 0.42 | 0.77 | 10.9 | 0.31 | 8.9 |
| 24 | 0.53 | 1.55 | 19.0 | 0.35 | 10.1 |
| 48 | 0.55 | 2.55 | 28.2 | 0.55 | 15.7 |
| 118 | 0.23 | 3.86 | 37.0 | 1.02 | 29.2 |

nd: not detected

Table 8. Test using Celluclast/Novozym 188 combination

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | nd | 0 |
| 5 | nd | 1.44 | 9.5 | 0.11 | 3.2 |
| 10 | nd | 1.76 | 15.8 | 0.54 | 12.2 |
| 24 | 0.15 | 2.53 | 24.6 | 0.53 | 15.5 |
| 48 | 0.27 | 3.33 | 33.5 | 0.74 | 21.1 |
| 118 | 0.33 | 4.77 | 46.2 | 1.27 | 36.3 |

nd: not detected

Table 9. Test using Celluclast/SP431/Novozym 188 combination

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | nd | 0 |
| 5 | 0.05 | 2.18 | 16.9 | 0.55 | 15.7 |
| 10 | nd | 3.22 | 25.6 | 1.12 | 32.1 |
| 24 | nd | 4.32 | 38.9 | 1.20 | 34.9 |
| 48 | nd | 5.53 | 49.8 | 1.52 | 43.5 |
| 118 | nd | 6.98 | 62.9 | 2.27 | 65.0 |

nd: not detected

# APPENDIX III

## Enzymatic hydrolysis tests - released sugar composition and relative percentage glucan and apparent xylan conversion

Table 1. Hybrid poplar treated with 0% peracetic acid (raw wood)

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | nd | 0 |
| 5 | nd | 0.23 | 2.1 | nd | 0 |
| 10 | nd | 0.29 | 2.6 | nd | 0 |
| 24 | nd | 0.44 | 4.0 | nd | 0 |
| 48 | nd | 0.54 | 4.8 | nd | 0 |
| 120 | nd | 0.76 | 6.8 | 0.23 | 6.0 |

nd: not detected

Table 2. Hybrid poplar treated with 6% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.05 | 1.3 |
| 5 | nd | 0.57 | 5.1 | 0.18 | 6.9 |
| 10 | nd | 0.77 | 6.9 | 0.24 | 9.9 |
| 24 | nd | 0.98 | 8.8 | 0.32 | 10.7 |
| 48 | nd | 1.25 | 11.3 | 0.47 | 12.3 |

nd: not detected

Table 3. Hybrid poplar treated with 9% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.06 | 1.6 |
| 5 | nd | 1.30 | 11.7 | 0.43 | 11.2 |
| 10 | nd | 1.72 | 15.5 | 0.60 | 15.6 |
| 24 | nd | 2.15 | 19.4 | 0.76 | 19.8 |
| 48 | nd | 2.50 | 22.5 | 1.02 | 26.6 |

nd: not detected

Table 4. Hybrid poplar treated with 15% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.09 | 2.3 |
| 5 | nd | 2.40 | 24.4 | 1.16 | 29.1 |
| 10 | nd | 3.70 | 33.3 | 1.29 | 32.4 |
| 24 | nd | 6.27 | 53.5 | 1.57 | 39.5 |
| 48 | nd | 7.01 | 63.3 | 1.90 | 47.8 |
| 120 | nd | 8.48 | 76.5 | 2.82 | 71.9 |

nd: not detected

Table 5. Hybrid poplar treated with 21% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.21 | 5.2 |
| 5 | nd | 4.53 | 37.7 | 2.01 | 49.5 |
| 10 | nd | 6.76 | 59.4 | 2.25 | 55.6 |
| 24 | nd | 9.26 | 83.3 | 2.86 | 70.5 |
| 48 | nd | 9.92 | 89.2 | 3.06 | 75.3 |
| 120 | nd | 10.93 | 98.3 | 3.58 | 88.2 |

nd: not detected

Table 6. Hybrid poplar treated with 30% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.35 | 9.2 |
| 5 | nd | 5.21 | 46.9 | 2.36 | 62.3 |
| 10 | nd | 8.07 | 72.7 | 2.58 | 68.2 |
| 24 | nd | 9.89 | 89.0 | 2.82 | 74.5 |
| 48 | nd | 10.12 | 91.1 | 3.00 | 79.2 |
| 120 | nd | 10.71 | 96.4 | 3.42 | 90.5 |

nd: not detected

Table 7. Hybrid poplar treated with 60% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.75 | 19.9 |
| 5 | nd | 5.30 | 47.7 | 2.53 | 66.9 |
| 10 | nd | 8.47 | 76.3 | 2.67 | 70.6 |
| 24 | nd | 10.64 | 95.8 | 2.89 | 76.4 |
| 48 | nd | 10.85 | 97.7 | 3.11 | 82.2 |
| 120 | nd | 11.38 | 102.5 | 3.47 | 91.8 |

nd: not detected

Table 8. Sugar cane bagasse treated with 0% peracetic acid (raw bagasse) 3

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.10 | 1.5 |
| 5 | nd | 1.95 | 17.6 | 0.63 | 9.5 |
| 10 | nd | 2.42 | 21.1 | 0.81 | 12.2 |
| 24 | nd | 2.68 | 24.1 | 0.98 | 14.7 |
| 48 | nd | 2.80 | 25.2 | 1.09 | 16.4 |
| 120 | nd | 2.94 | 28.8 | 1.25 | 18.8 |

nd: not detected

Table 9. Sugar cane bagasse treated with 6% peracetic acid 3

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.18 | 2.8 |
| 5 | nd | 2.52 | 22.7 | 1.07 | 16.6 |
| 10 | nd | 3.04 | 27.3 | 1.34 | 20.7 |
| 24 | nd | 3.33 | 29.9 | 1.53 | 23.7 |
| 48 | nd | 4.07 | 36.8 | 1.90 | 29.4 |
| 120 | nd | 4.35 | 39.2 | 2.18 | 33.7 |

nd: not detected

Table10. Sugar cane bagasse treated with 9% peracetic acid 3

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.19 | 2.9 |
| 5 | 0.03 | 2.86 | 26.0 | 1.32 | 20.4 |
| 10 | nd | 3.50 | 31.5 | 1.69 | 26.7 |
| 24 | nd | 4.31 | 38.8 | 2.08 | 32.1 |
| 48 | nd | 5.00 | 45.0 | 2.41 | 37.2 |
| 120 | nd | 5.50 | 49.5 | 2.86 | 43.8 |

nd: not detected

Table 11. Sugar cane bagasse treated with 15% peracetic acid 2

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.28 | 4.3 |
| 5 | 0.14 | 4.28 | 39.8 | 2.32 | 35.8 |
| 10 | nd | 5.66 | 52.0 | 2.91 | 44.9 |
| 24 | nd | 7.13 | 64.9 | 3.56 | 55.0 |
| 48 | nd | 8.29 | 74.8 | 4.06 | 62.7 |
| 120 | nd | 9.60 | 85.9 | 4.78 | 73.8 |

nd: not detected

Table 12. Sugar cane bagasse treated with 21% peracetic acid 2

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.42 | 6.9 |
| 5 | 0.36 | 5.34 | 50.6 | 3.26 | 53.2 |
| 10 | 0.31 | 7.51 | 70.4 | 3.78 | 61.7 |
| 24 | nd | 8.85 | 80.6 | 4.30 | 70.1 |
| 48 | nd | 9.67 | 87.7 | 4.64 | 75.7 |
| 120 | nd | 10.26 | 93.1 | 5.08 | 82.9 |

nd: not detected

Table 13. Sugar cane bagasse treated with 30% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.57 | 9.9 |
| 5 | 0.28 | 5.48 | 52.0 | 3.25 | 55.8 |
| 10 | 0.38 | 8.20 | 77.4 | 4.02 | 69.0 |
| 24 | 0.25 | 9.32 | 86.2 | 4.37 | 75.0 |
| 48 | 0.23 | 9.67 | 89.2 | 4.66 | 79.9 |
| 120 | nd | 10.46 | 94.2 | 4.95 | 85.0 |

nd: not detected

Table 14. Sugar cane bagasse treated with 60% peracetic acid 2

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.73 | 13.1 |
| 5 | 0.50 | 5.30 | 54.6 | 3.25 | 58.4 |
| 10 | 0.58 | 8.51 | 82.1 | 3.92 | 70.4 |
| 24 | 0.36 | 9.77 | 91.3 | 4.28 | 76.9 |
| 48 | 0.38 | 10.09 | 94.3 | 4.52 | 81.2 |
| 120 | 0.33 | 10.32 | 96.7 | 4.87 | 87.5 |

nd: not detected

Table15. Hybrid poplar treated with 6% NaOH/15% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.55 | 14.2 |
| 5 | nd | 4.80 | 43.2 | 1.62 | 41.8 |
| 10 | nd | 7.81 | 70.4 | 1.94 | 50.0 |
| 24 | nd | 8.93 | 80.4 | 2.71 | 69.8 |
| 48 | nd | 10.91 | 93.5 | 3.27 | 84.2 |
| 120 | nd | 11.12 | 100.1 | 3.81 | 98.1 |

nd: not detected

Table 16. Hybrid poplar treated with 6% NaOH/9% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.36 | 9.45 |
| 5 | nd | 3.84 | 34.5 | 1.09 | 41.8 |
| 10 | nd | 6.17 | 57.1 | 1.78 | 43.3 |
| 24 | nd | 7.74 | 69.6 | 2.55 | 66.9 |
| 48 | nd | 9.01 | 81.1 | 3.27 | 84.2 |
| 120 | nd | 10.43 | 93.9 | 3.62 | 95.0 |

nd: not detected

Table 17. Hybrid poplar treated with 3% NaOH/15% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.17 | 4.23 |
| 5 | nd | 4.13 | 37.1 | 1.15 | 28.6 |
| 10 | nd | 6.93 | 59.8 | 1.78 | 44.3 |
| 24 | nd | 8.93 | 71.4 | 2.55 | 63.6 |
| 48 | nd | 9.20 | 82.8 | 3.03 | 75.6 |
| 120 | nd | 10.38 | 94.9 | 3.72 | 92.7 |

nd: not detected

Table 18. Hybrid poplar treated with 3% NaOH/9% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.05 | 1.3 |
| 5 | nd | 2.27 | 20.4 | 0.66 | 16.8 |
| 10 | nd | 3.61 | 32.5 | 1.02 | 25.9 |
| 24 | nd | 5.58 | 50.3 | 1.56 | 35.7 |
| 48 | nd | 6.47 | 58.3 | 2.15 | 54.9 |
| 120 | nd | 7.30 | 65.7 | 2.79 | 71.1 |

nd: not detected

Table 19. Hybrid poplar treated with 14% NH4OH/15% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.17 | 4.2 |
| 5 | nd | 3.62 | 29.5 | 1.19 | 29.1 |
| 10 | nd | 5.82 | 49.3 | 1.89 | 47.5 |
| 24 | nd | 8.17 | 70.4 | 2.52 | 70.4 |
| 48 | nd | 9.33 | 80.9 | 3.13 | 76.7 |
| 120 | nd | 10.56 | 92.0 | 3.84 | 94.0 |

nd: not detected

Table 20. Hybrid poplar treated with 5.25% NH4OH/15% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.03 | 0.8 |
| 5 | nd | 3.50 | 32.0 | 0.90 | 23.1 |
| 10 | nd | 5.07 | 45.5 | 1.44 | 36.8 |
| 24 | nd | 6.84 | 61.6 | 2.47 | 63.3 |
| 48 | nd | 7.93 | 71.4 | 2.91 | 74.7 |
| 120 | nd | 9.24 | 83.1 | 3.58 | 91.8 |

nd: not detected

Table 21. Hybrid poplar treated with 5.25% NH4OH/9% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.01 | 0.3 |
| 5 | nd | 1.49 | 13.4 | 0.42 | 11.2 |
| 10 | nd | 2.23 | 20.1 | 0.66 | 17.7 |
| 24 | nd | 3.63 | 32.7 | 1.12 | 29.9 |
| 48 | nd | 4.30 | 38.7 | 1.52 | 40.5 |
| 120 | nd | 4.84 | 43.6 | 2.25 | 59.9 |

nd: not detected

Table 22. Hybrid poplar treated with 2.63% NH4OH/15% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.01 | 0.3 |
| 5 | nd | 3.61 | 31.7 | 1.00 | 25.5 |
| 10 | nd | 4.97 | 44.7 | 1.26 | 32.0 |
| 24 | nd | 6.74 | 60.6 | 2.39 | 69.0 |
| 48 | nd | 7.87 | 70.9 | 2.85 | 72.6 |
| 120 | nd | 9.10 | 82.0 | 3.56 | 90.8 |

nd: not detected

Table 23. Hybrid poplar treated with 2.63% NH4OH/9% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | nd | 0 |
| 5 | nd | 1.47 | 13.3 | 0.40 | 10.8 |
| 10 | nd | 2.16 | 19.4 | 0.65 | 17.4 |
| 24 | nd | 3.59 | 32.2 | 1.12 | 30.1 |
| 48 | nd | 4.27 | 38.4 | 1.48 | 39.7 |
| 120 | nd | 4.90 | 43.9 | 2.18 | 58.6 |

nd: not detected

Table 24. Sugar cane bagasse treated with 6% NaOH/15% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.27 | 4.5 |
| 5 | nd | 5.46 | 49.1 | 3.02 | 50.0 |
| 10 | nd | 6.89 | 62.0 | 3.56 | 59.4 |
| 24 | nd | 8.85 | 79.7 | 4.53 | 75.1 |
| 48 | nd | 9.90 | 89.1 | 5.10 | 84.5 |
| 120 | nd | 10.84 | 97.6 | 5.57 | 92.3 |

nd: not detected

Table 25. Sugar cane bagasse treated with 6% NaOH/9% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.20 | 3.3 |
| 5 | nd | 4.52 | 40.7 | 2.29 | 37.9 |
| 10 | nd | 5.49 | 49.4 | 2.82 | 46.6 |
| 24 | nd | 6.50 | 58.5 | 3.51 | 58.0 |
| 48 | nd | 7.64 | 68.8 | 3.93 | 65.0 |
| 120 | nd | 9.32 | 83.9 | 4.50 | 74.4 |

nd: not detected

Table 26. Sugar cane bagasse treated with 6% NaOH/6% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.16 | 2.64 |
| 5 | nd | 3.49 | 31.4 | 1.71 | 28.3 |
| 10 | nd | 4.28 | 38.5 | 2.19 | 36.2 |
| 24 | nd | 5.13 | 46.2 | 2.75 | 45.4 |
| 48 | nd | 5.80 | 52.2 | 2.97 | 49.1 |
| 120 | nd | 7.15 | 64.4 | 3.45 | 57.0 |

nd: not detected

Table 27. Sugar cane bagasse treated with 3% NaOH/15% peracetic acid 2

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.24 | 3.9 |
| 5 | nd | 5.14 | 46.3 | 2.82 | 46.3 |
| 10 | nd | 6.48 | 58.3 | 3.29 | 54.0 |
| 24 | nd | 8.31 | 74.8 | 4.02 | 65.9 |
| 48 | nd | 8.98 | 80.8 | 4.48 | 73.5 |
| 120 | nd | 10.29 | 92.6 | 4.81 | 78.9 |

nd: not detected

Table 28. Sugar cane bagasse treated with 3% NaOH/9% peracetic acid 2

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.17 | 2.7 |
| 5 | nd | 3.64 | 32.8 | 1.76 | 28.3 |
| 10 | nd | 4.63 | 41.7 | 2.32 | 37.2 |
| 24 | nd | 5.59 | 50.3 | 2.94 | 47.2 |
| 48 | nd | 6.34 | 57.1 | 3.27 | 52.5 |
| 120 | nd | 7.86 | 69.9 | 3.68 | 59.1 |

nd: not detected

Table 29. Sugar cane bagasse treated with 5.25% NH4OH/15% peracetic acid

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.25 | 4.0 |
| 5 | nd | 5.07 | 45.6 | 2.64 | 42.6 |
| 10 | nd | 6.39 | 57.5 | 3.29 | 53.1 |
| 24 | nd | 8.14 | 73.3 | 3.80 | 61.3 |
| 48 | nd | 8.90 | 80.1 | 4.24 | 68.4 |
| 120 | nd | 10.07 | 90.6 | 4.63 | 74.6 |

nd: not detected

Table 30. Sugar cane bagasse treated with 5.25% NaOH/9% peracetic acid 3

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.19 | 3.1 |
| 5 | nd | 3.67 | 33.0 | 1.74 | 28.0 |
| 10 | nd | 4.36 | 39.2 | 2.15 | 34.6 |
| 24 | nd | 5.33 | 48.0 | 2.76 | 44.4 |
| 48 | nd | 6.02 | 54.2 | 3.00 | 48.2 |
| 120 | nd | 7.15 | 64.4 | 3.33 | 53.5 |

nd: not detected

Table 31. Sugar cane bagasse treated with 5.25% NH4OH/6% peracetic acid 3

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.17 | 2.8 |
| 5 | nd | 3.09 | 27.8 | 1.36 | 22.0 |
| 10 | nd | 3.51 | 31.6 | 1.66 | 26.9 |
| 24 | nd | 4.26 | 38.3 | 2.17 | 35.1 |
| 48 | nd | 4.66 | 41.9 | 2.30 | 37.2 |
| 120 | nd | 5.37 | 48.3 | 2.56 | 41.4 |

nd: not detected

Table 32. Sugar cane bagasse treated with 2.63% NH4OH/15% peracetic acid 2

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.25 | 4.0 |
| 5 | nd | 5.04 | 45.4 | 2.64 | 41.9 |
| 10 | nd | 6.37 | 57.3 | 3.22 | 51.1 |
| 24 | nd | 8.10 | 72.9 | 3.73 | 59.2 |
| 48 | nd | 8.83 | 79.5 | 4.28 | 67.9 |
| 120 | nd | 9.91 | 89.2 | 4.58 | 72.7 |

nd: not detected

Table 33. Sugar cane bagasse treated with 2.63% NaOH/9% peracetic acid 3

| Time (hours) | Cellobiose (g/L) | Glucose (g/L) | Glucan conversion (%) | Xylose (g/L) | Xylan conversion (%) |
|---|---|---|---|---|---|
| 0 | nd | nd | 0 | 0.20 | 3.2 |
| 5 | nd | 3.61 | 32.5 | 1.71 | 27.3 |
| 10 | nd | 4.30 | 38.7 | 2.12 | 33.8 |
| 24 | nd | 5.30 | 47.7 | 2.70 | 43.1 |
| 48 | nd | 5.88 | 52.9 | 2.91 | 46.4 |
| 120 | nd | 7.07 | 63.6 | 3.31 | 52.8 |

nd: not detected

# APENDIX IV

SSCF kinetic tables - concentration of hydrolysed sugars and cofermented products

Table 1. 6% NaOH/15% peracetic acid hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.56 | 0.98 | 0.87 | 0.03 |
| 1 | 9.68 | 0.54 | 3.90 | 0.32 |
| 2 | 16.68 | 0.91 | 1.97 | 0.50 |
| 3 | 18.58 | 1.40 | 2.83 | 0.50 |
| 5 | 21.21 | 0.89 | 3.48 | 0.86 |
| 7 | 21.73 | 0.70 | 3.60 | 0.96 |
| 10 | 21.85 | 0.66 | 3.76 | 1.01 |

Table 2. 6% NaOH/9% peracetic acid hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.45 | 0.98 | 0.93 | 0.03 |
| 1 | 7.26 | 0.49 | 3.79 | 0.29 |
| 2 | 12.03 | 0.46 | 1.52 | 0.60 |
| 3 | 13.52 | 0.79 | 2.12 | 0.63 |
| 5 | 14.69 | 0.97 | 3.08 | 0.73 |
| 7 | 14.96 | 1.79 | 6.10 | 0.89 |
| 10 | 15.47 | 3.09 | 6.93 | 1.03 |

Table 3. 3% NaOH/15% peracetic acid hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.44 | 1.02 | 0.83 | 0.10 |
| 1 | 8.53 | 0.57 | 2.37 | 0.88 |
| 2 | 13.24 | 1.28 | 2.48 | 0.94 |
| 3 | 14.59 | 2.11 | 3.34 | 1.08 |
| 5 | 17.22 | 1.33 | 4.09 | 1.16 |
| 7 | 18.90 | 0.71 | 3.34 | 1.56 |
| 10 | 19.24 | 1.23 | 3.71 | 1.69 |

Table 4. 14% NH4OH/15% peracetic acid hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.42 | 0.95 | 0.73 | 0.15 |
| 1 | 7.37 | 0.76 | 3.21 | 1.08 |
| 2 | 10.97 | 1.43 | 2.32 | 1.09 |
| 3 | 11.88 | 1.91 | 3.21 | 1.20 |
| 5 | 13.03 | 2.58 | 4.39 | 1.31 |
| 7 | 14.83 | 1.11 | 4.18 | 1.73 |
| 10 | 15.31 | 2.09 | 5.42 | 2.00 |

Table 5. 15% peracetic acid hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.41 | 0.81 | 0.40 | 0.20 |
| 1 | 3.48 | 3.01 | 3.98 | 0.28 |
| 2 | 5.89 | 1.34 | 4.13 | 0.96 |
| 3 | 6.31 | 2.68 | 6.16 | 1.20 |
| 5 | 6.39 | 4.36 | 7.39 | 1.37 |
| 7 | 6.39 | 5.43 | 7.75 | 1.79 |
| 10 | 6.71 | 6.65 | 8.31 | 1.89 |

Table 6. 21% peracetic acid hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.39 | 0.95 | 0.72 | 0.24 |
| 1 | 5.21 | 2.25 | 5.15 | 1.35 |
| 2 | 10.61 | 1.00 | 3.29 | 1.50 |
| 3 | 11.77 | 2.31 | 4.10 | 1.77 |
| 5 | 12.02 | 5.68 | 6.49 | 1.84 |
| 7 | 11.91 | 7.21 | 5.72 | 2.39 |
| 10 | 12.04 | 8.88 | 7.09 | 2.74 |

Table 7. 60% peracetic acid hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.62 | 0.70 | 2.03 | 0.82 |
| 1 | 1.09 | 6.48 | 7.50 | 1.96 |
| 2 | 2.09 | 8.55 | 7.81 | 1.96 |
| 3 | 2.23 | 10.78 | 8.65 | 2.00 |
| 5 | 2.77 | 11.07 | 9.50 | 2.10 |
| 7 | 3.01 | 12.18 | 9.81 | 2.17 |

Table 8. Raw hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.46 | 0.61 | 0.15 | 0 |
| 1 | 0.73 | 0.14 | 0.33 | 0.20 |
| 2 | 0.82 | 0.13 | 0.25 | 0.24 |
| 3 | 0.92 | 0.17 | 0.40 | 0.28 |
| 5 | 1.30 | 0.40 | 0.48 | 0.54 |
| 7 | 1.37 | 0.35 | 0.67 | 0.56 |

Table 9. Alkaline post-washed 6% NaOH/15% peracetic acid hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.34 | 0.95 | 1.08 | 0.01 |
| 1 | 12.95 | 0.51 | 1.59 | 0.40 |
| 2 | 17.41 | 1.00 | 2.27 | 0.51 |
| 3 | 19.69 | 0.75 | 2.96 | 0.67 |
| 5 | 20.23 | 1.42 | 2.99 | 1.03 |
| 7 | 20.85 | 1.36 | 3.87 | 1.26 |
| 10 | 21.07 | 1.16 | 4.12 | 1.39 |

Table 10. Alkaline post-washed 21% peracetic acid hybrid poplar substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.40 | 0.92 | 0.64 | 0.06 |
| 1 | 10.23 | 3.01 | 3.98 | 1.33 |
| 2 | 12.76 | 1.34 | 4.13 | 1.47 |
| 3 | 14.76 | 2.48 | 3.33 | 1.73 |
| 5 | 17.50 | 1.17 | 3.40 | 1.79 |
| 7 | 19.38 | 1.70 | 3.73 | 2.50 |
| 10 | 19.17 | 1.56 | 3.53 | 2.55 |

Table 11. Alpha-cellulose substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.37 | 0.95 | 0.72 | 0.04 |
| 1 | 6.63 | 0.43 | 0.47 | 0.25 |
| 2 | 10.19 | 0.47 | 0.37 | 0.47 |
| 3 | 11.93 | 0.55 | 0.50 | 0.61 |
| 5 | 12.93 | 0.38 | 0.42 | 0.93 |
| 7 | 13.52 | 0.65 | 0.65 | 1.26 |
| 10 | 13.93 | 1.08 | 0.74 | 1.34 |

Table 12. 6% NaOH/15% peracetic acid sugar cane bagasse substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.67 | 1.09 | 2.45 | 0.12 |
| 1 | 12.48 | 0.10 | 1.32 | 0.25 |
| 2 | 17.99 | 0.41 | 0.80 | 0.65 |
| 3 | 20.87 | 0.65 | 1.80 | 0.78 |
| 5 | 23.61 | 0.66 | 1.95 | 0.98 |
| 7 | 24.49 | 0.50 | 2.50 | 1.17 |
| 10 | 24.77 | 0.45 | 2.63 | 1.21 |

Table 13. 6% NaOH/9% peracetic acid sugar cane bagasse substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.68 | 0.82 | 1.44 | 0.10 |
| 1 | 9.62 | 0.01 | 0.67 | 0.14 |
| 2 | 11.90 | 0.12 | 0.71 | 0.42 |
| 3 | 13.46 | 0.32 | 1.20 | 0.51 |
| 5 | 14.75 | 0.64 | 1.97 | 0.88 |
| 7 | 16.18 | 0.22 | 1.45 | 0.91 |
| 10 | 18.38 | 0.40 | 1.59 | 0.99 |

Table 14. 3% NaOH/15% peracetic acid sugar cane bagasse substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.63 | 1.00 | 1.94 | 0.20 |
| 1 | 10.24 | 0.02 | 1.16 | 0.32 |
| 2 | 12.44 | 0.10 | 0.94 | 0.74 |
| 3 | 15.15 | 0.36 | 1.60 | 1.03 |
| 5 | 17.68 | 0.66 | 2.82 | 1.49 |
| 7 | 19.45 | 1.07 | 3.24 | 1.65 |
| 10 | 21.67 | 0.36 | 2.84 | 1.77 |

Table 15. 5.25% NaOH/15% peracetic acid sugar cane bagasse substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.70 | 0.91 | 2.35 | 0.51 |
| 1 | 10.14 | 0.07 | 3.41 | 0.67 |
| 2 | 13.88 | 0.15 | 1.28 | 1.40 |
| 3 | 15.17 | 0.53 | 1.92 | 1.52 |
| 5 | 16.53 | 1.56 | 3.75 | 1.91 |
| 7 | 17.86 | 2.08 | 3.98 | 2.08 |
| 10 | 18.53 | 0.82 | 4.99 | 2.10 |

Table 16. 15% peracetic acid sugar cane bagasse substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.66 | 0.69 | 1.60 | 0.41 |
| 1 | 8.91 | 0.43 | 5.04 | 0.79 |
| 2 | 12.77 | 0.20 | 1.41 | 1.41 |
| 3 | 14.87 | 0.63 | 2.09 | 1.78 |
| 5 | 16.19 | 1.95 | 3.06 | 1.83 |
| 7 | 17.01 | 3.54 | 4.16 | 1.86 |
| 10 | 17.27 | 5.30 | 4.94 | 1.91 |

Table 17. 21% peracetic acid sugar cane bagasse substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.66 | 0.77 | 1.57 | 0.47 |
| 1 | 10.98 | 0.21 | 4.74 | 0.95 |
| 2 | 15.73 | 0.22 | 1.43 | 1.58 |
| 3 | 19.54 | 0.54 | 2.53 | 2.10 |
| 5 | 22.13 | 0.21 | 3.13 | 2.14 |
| 7 | 23.27 | 0.95 | 4.43 | 2.17 |
| 10 | 24.77 | 0.66 | 4.86 | 2.20 |

Table 18. 60% peracetic acid sugar cane bagasse substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.54 | 0.96 | 1.87 | 0.49 |
| 1 | 1.79 | 10.55 | 8.40 | 1.23 |
| 2 | 16.26 | 0.43 | 1.90 | 1.69 |
| 3 | 21.29 | 0.20 | 1.71 | 1.98 |
| 5 | 23.21 | 0.24 | 2.13 | 2.13 |
| 7 | 24.28 | 0.10 | 1.85 | 2.33 |

Table 19. Raw sugar cane bagasse substrate

| Time (days) | Ethanol (g/L) | Glucose (g/L) | Xylose (g/L) | Acetate (g/L) |
|---|---|---|---|---|
| 0 | 0.40 | 1.00 | 0.46 | 0.05 |
| 1 | 2.08 | 2.90 | 2.10 | 0.34 |
| 2 | 3.83 | 0.12 | 0.57 | 0.44 |
| 3 | 4.60 | 0.21 | 0.90 | 0.54 |
| 5 | 5.10 | 0.32 | 0.94 | 0.63 |
| 7 | 5.13 | 0.10 | 0.96 | 0.83 |

# APENDIX V

Additional figures of simultaneous saccharification and co-fermentation kinetics

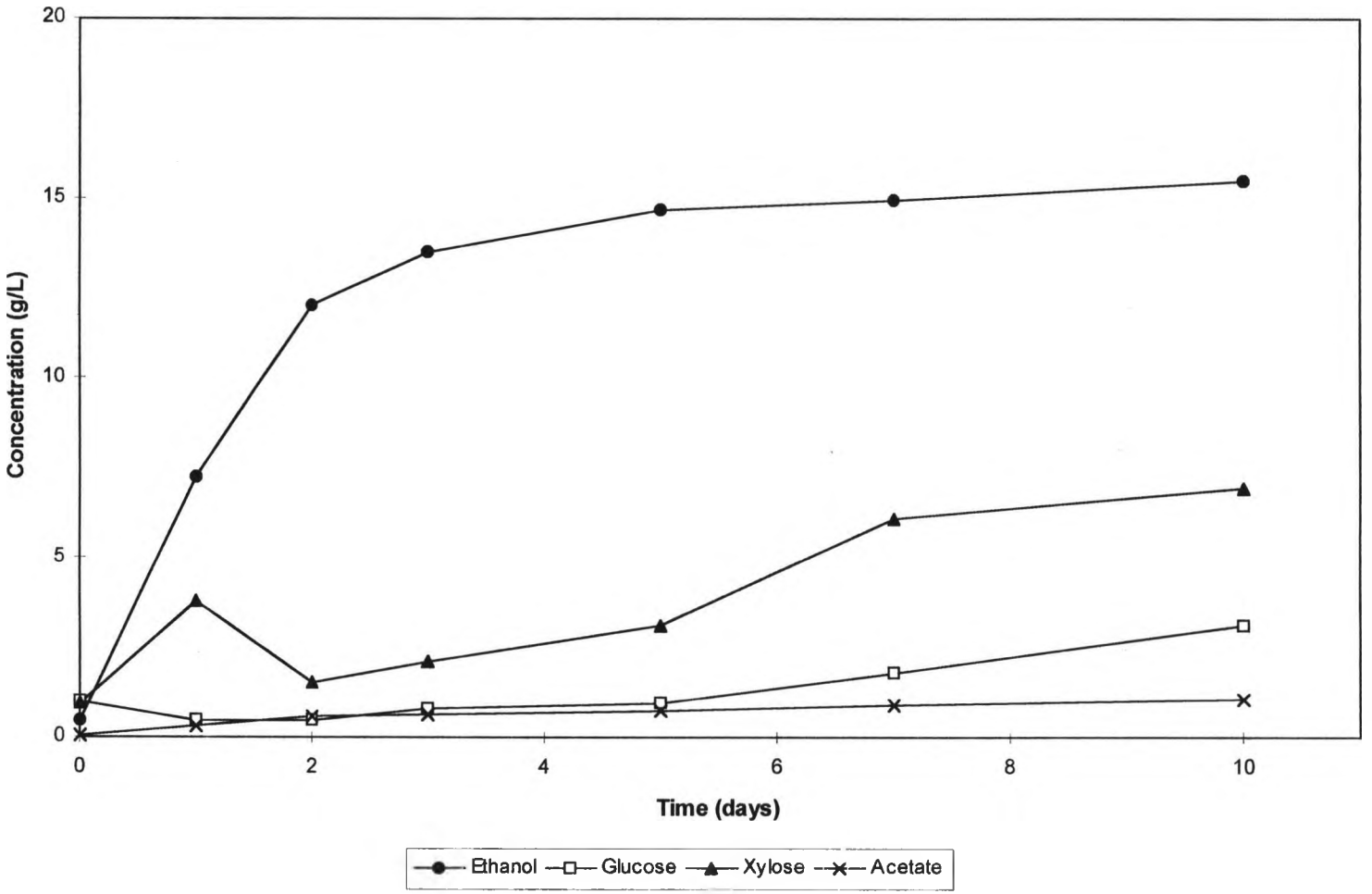



Fig. 1. SSCF kinetics for 6% NaOH/9% peracetic acid pretreated hybrid poplar.

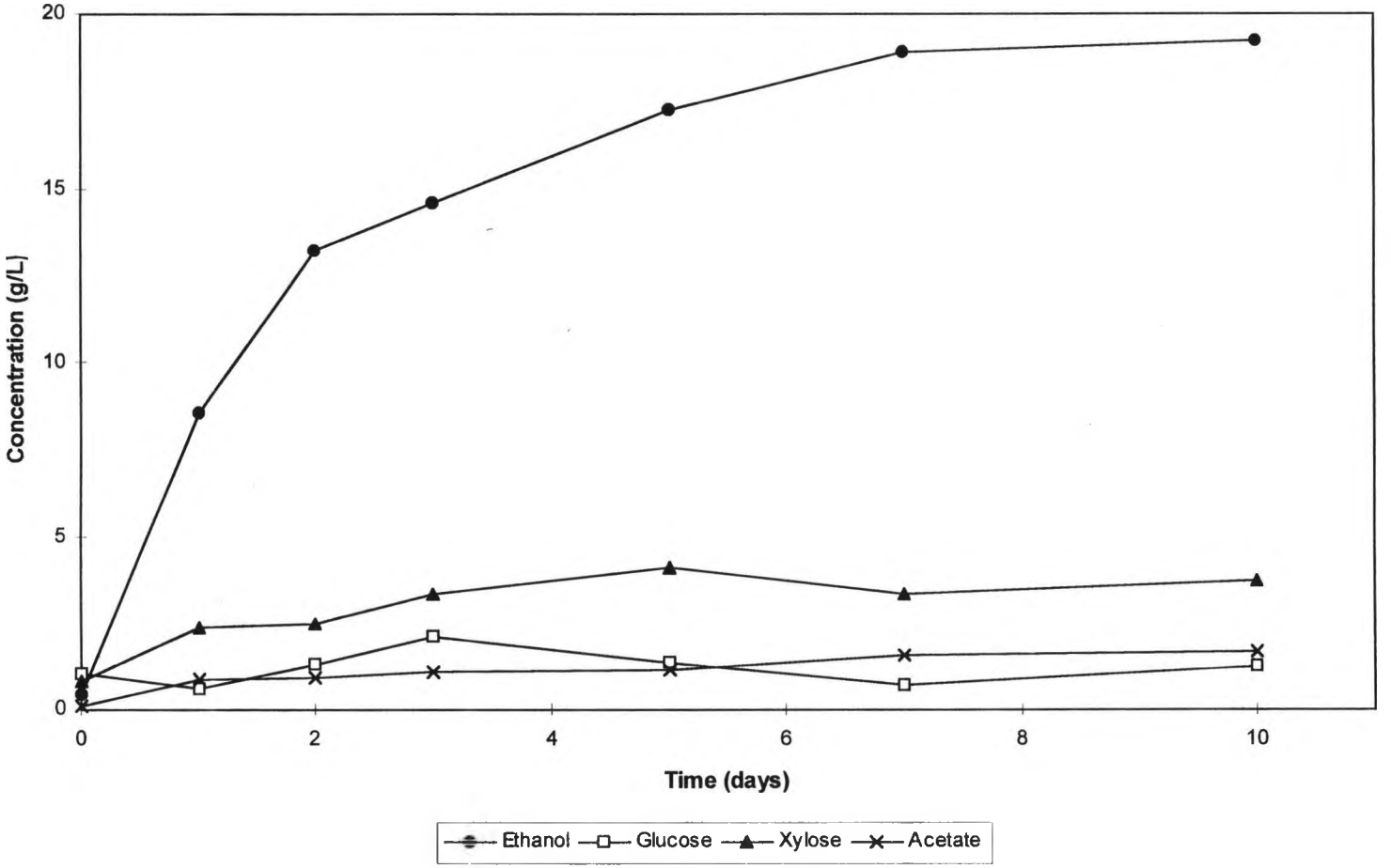



Fig. 2. SSCF kinetics for 3% NaOH/15% peracetic acid pretreated hybrid poplar.

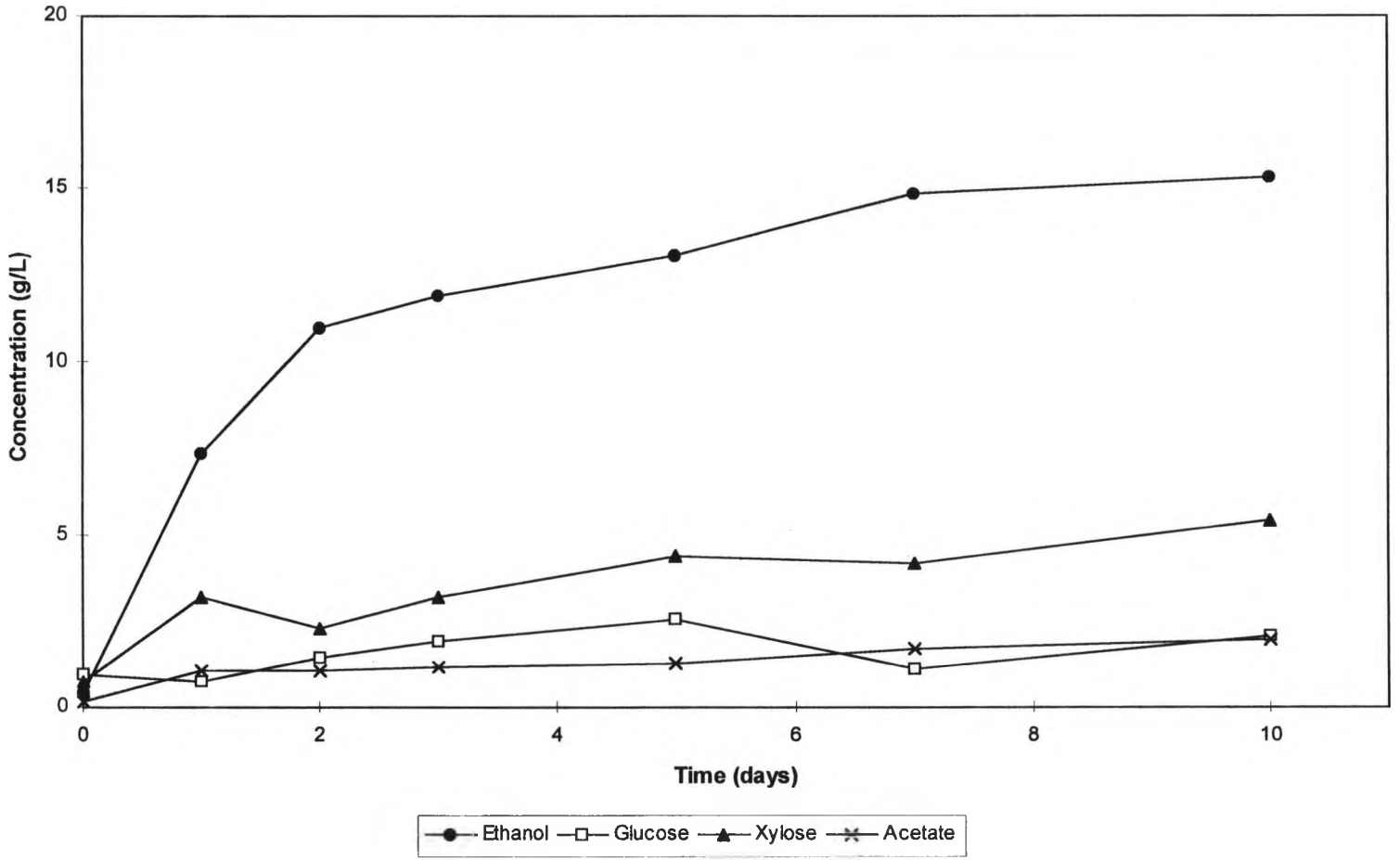



Fig. 3. SSCF kinetics for 14% NH4OH/15% peracetic acid pretreated hybrid poplar.

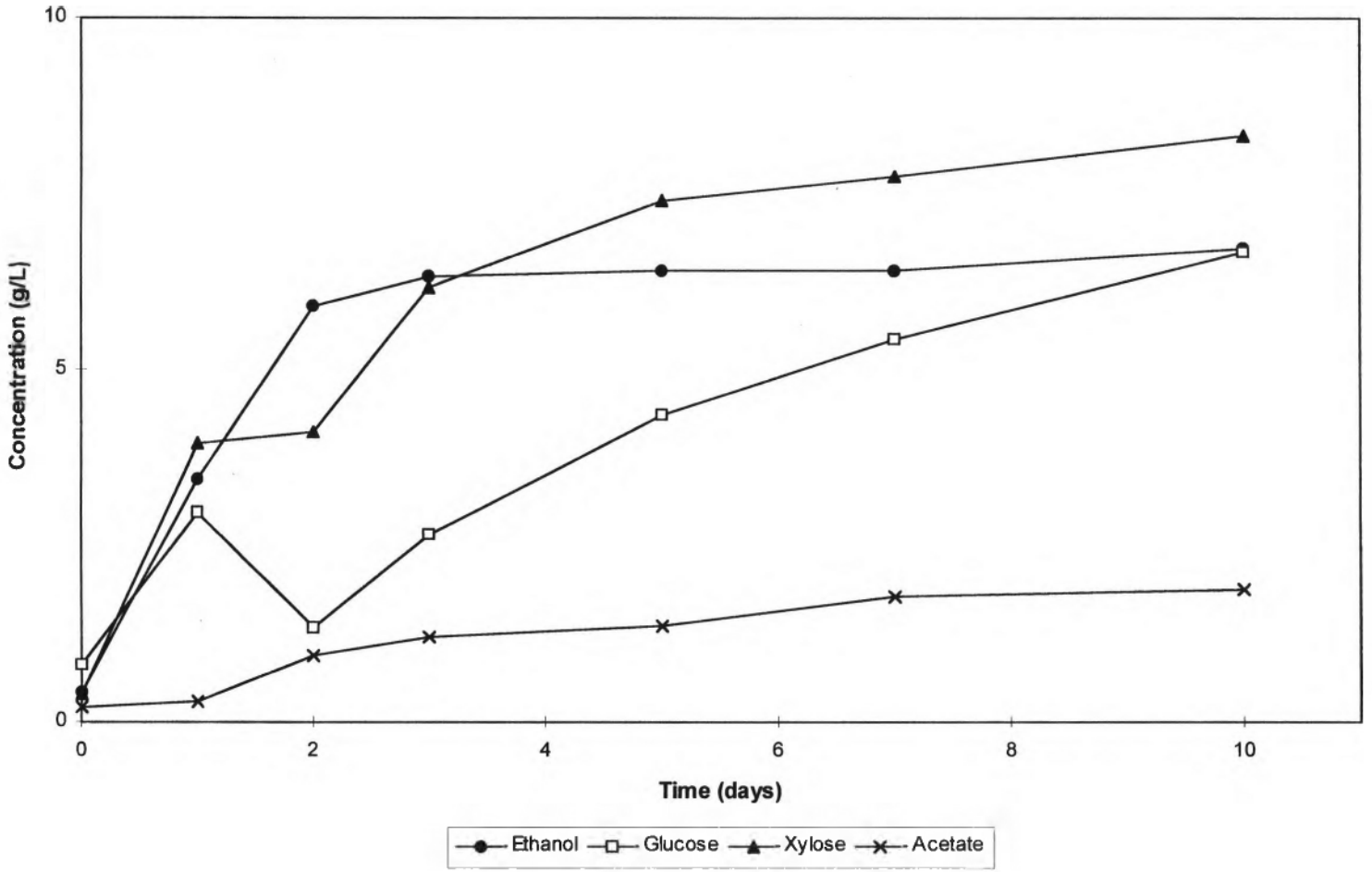



Fig. 4. SSCF kinetics for 15% peracetic acid pretreated hybrid poplar.

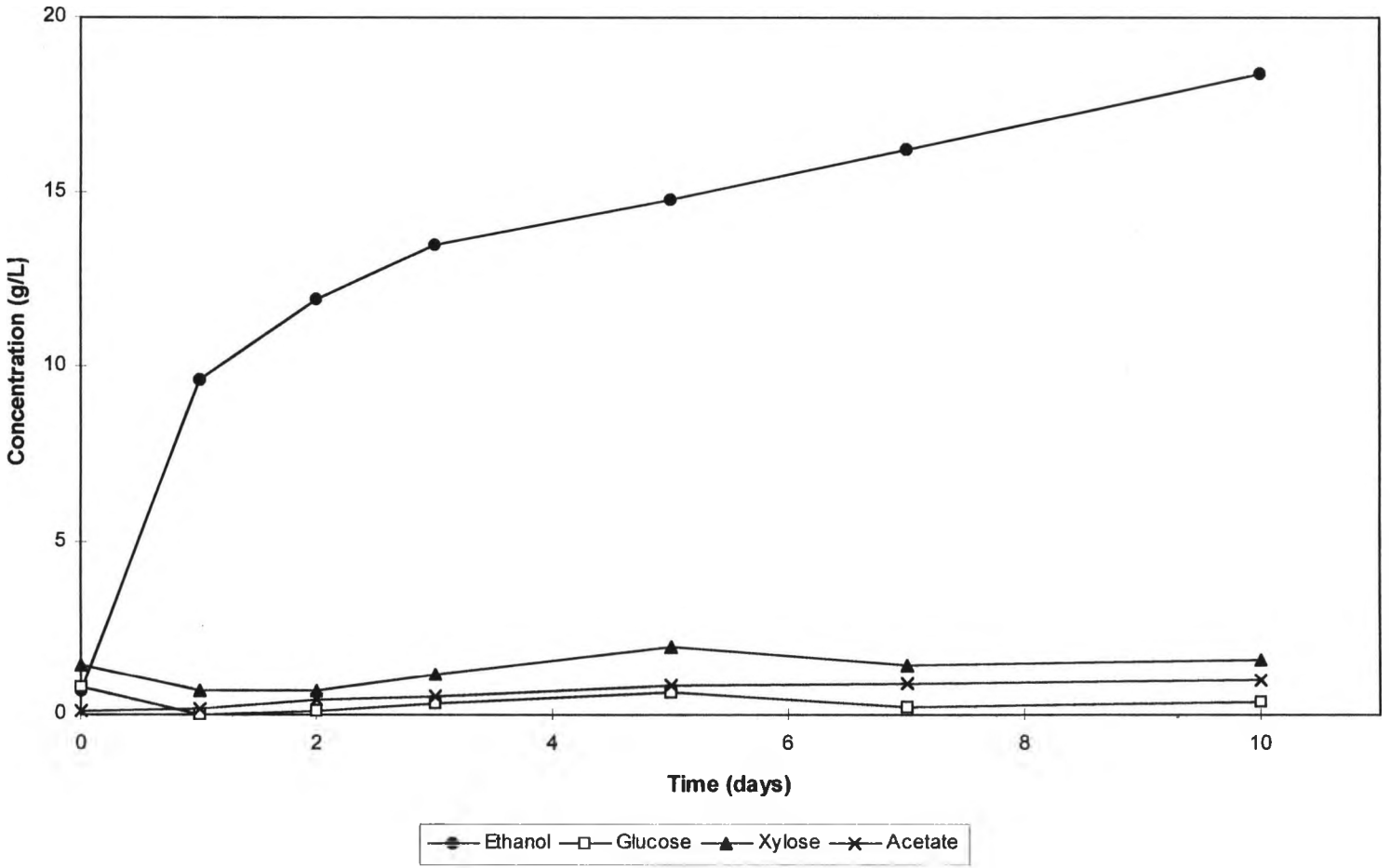



Fig. 5. SSCF kinetics for 6% NaOH/15% peracetic acid pretreated sugar cane bagasse.

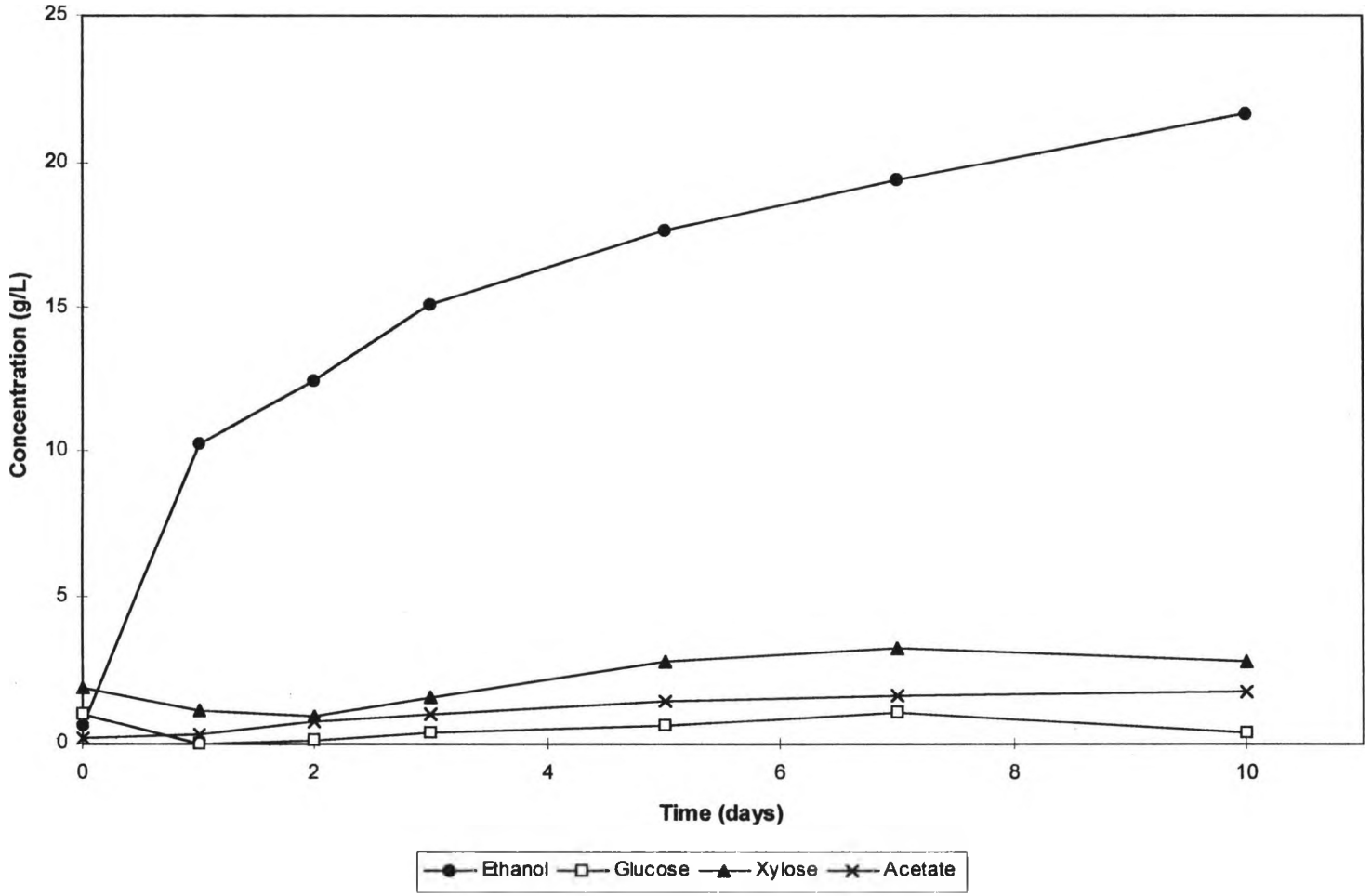



Fig. 6. SSCF kinetics for 3% NaOH/15% peracetic acid pretreated sugar cane bagasse.

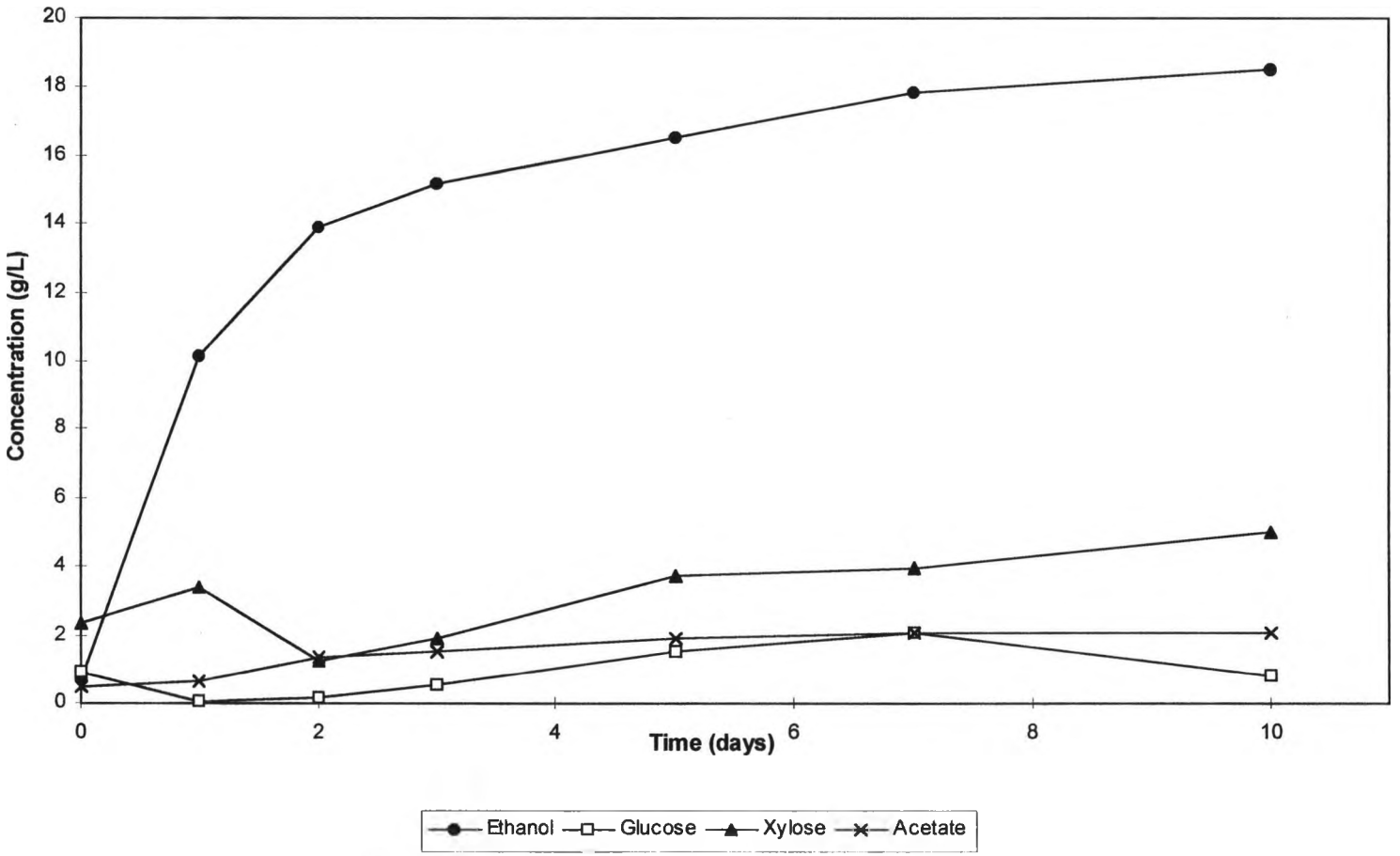



Fig. 7. SSCF kinetics for 5.25% NH4OH/15% peracetic acid pretreated sugar cane bagasse.

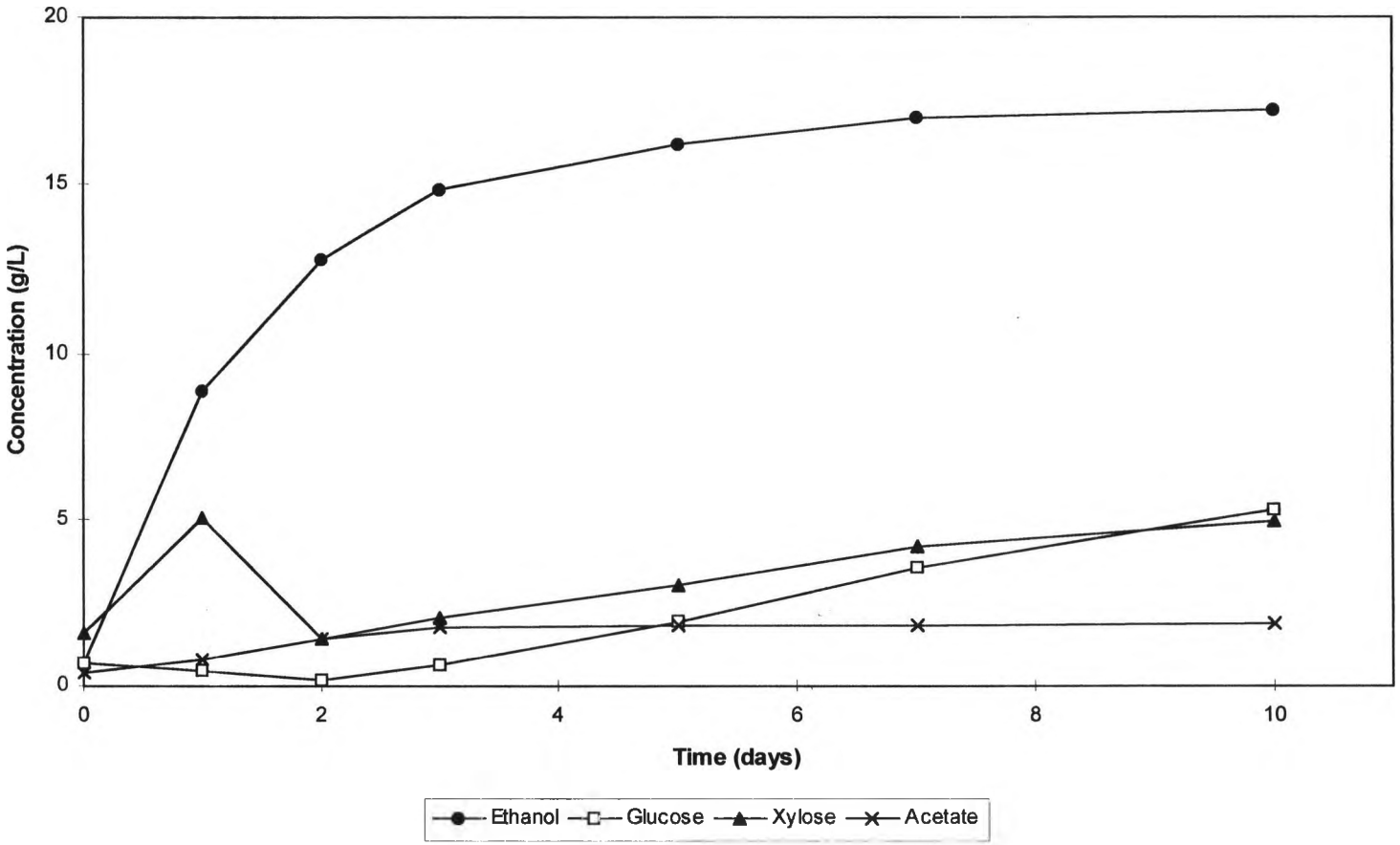



Fig. 8. SSCF kinetics for 15% peracetic acid pretreated sugar cane bagasse.

# APENDIX VI

Tables of ethanol yield from simultaneous saccharification and cofermentation tests

Table 1. 6% NaOH/15% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 22.94 | 0 | 0 |
| 1 | 22.94 | 9.12 | 39.8 |
| 2 | 22.94 | 16.12 | 70.3 |
| 3 | 22.94 | 18.02 | 78.6 |
| 5 | 22.94 | 20.65 | 90.0 |
| 7 | 22.94 | 21.17 | 92.6 |
| 10 | 22.94 | 21.29 | 92.8 |

Table 2. 6% NaOH/9% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 22.83 | 0 | 0 |
| 1 | 22.83 | 6.81 | 29.8 |
| 2 | 22.83 | 11.58 | 50.7 |
| 3 | 22.83 | 13.07 | 57.3 |
| 5 | 22.83 | 14.24 | 62.4 |
| 7 | 22.83 | 14.51 | 63.6 |
| 10 | 22.83 | 15.02 | 65.8 |

Table 3. 3% NaOH/15% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 23.13 | 0 | 0 |
| 1 | 23.13 | 8.09 | 35.0 |
| 2 | 23.13 | 12.8 | 55.3 |
| 3 | 23.13 | 14.15 | 61.2 |
| 5 | 23.13 | 16.78 | 72.6 |
| 7 | 23.13 | 18.46 | 79.8 |
| 10 | 23.13 | 19.24 | 83.2 |

Table 4. 14% NH4OH/15% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 23.25 | 0 | 0 |
| 1 | 23.13 | 6.95 | 29.9 |
| 2 | 23.13 | 10.55 | 45.4 |
| 3 | 23.13 | 11.46 | 49.3 |
| 5 | 23.13 | 12.61 | 54.2 |
| 7 | 23.13 | 14.41 | 62.0 |
| 10 | 23.13 | 14.89 | 64.1 |

Table 5. 15% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 23.08 | 0 | 0 |
| 1 | 23.08 | 3.07 | 13.3 |
| 2 | 23.08 | 5.48 | 23.7 |
| 3 | 23.08 | 5.90 | 25.6 |
| 5 | 23.08 | 5.98 | 25.9 |
| 7 | 23.08 | 5.98 | 25.9 |
| 10 | 23.08 | 6.30 | 27.3 |

Table 6. 21% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 23.21 | 0 | 0 |
| 1 | 23.21 | 4.82 | 20.8 |
| 2 | 23.21 | 10.22 | 44.1 |
| 3 | 23.21 | 11.38 | 49.1 |
| 5 | 23.21 | 11.63 | 50.1 |
| 7 | 23.21 | 11.52 | 49.6 |
| 10 | 23.21 | 11.65 | 50.2 |

Table 7. 60% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 22.78 | 0 | 0 |
| 1 | 22.78 | 0.50 | 2.2 |
| 2 | 22.78 | 1.48 | 6.5 |
| 3 | 22.78 | 1.61 | 7.1 |
| 5 | 22.78 | 2.16 | 9.5 |
| 7 | 22.78 | 2.23 | 9.8 |

Table 8. Raw hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 22.78 | 0 | 0 |
| 1 | 22.78 | 0.27 | 1.2 |
| 2 | 22.78 | 0.36 | 1.6 |
| 3 | 22.78 | 0.46 | 2.0 |
| 5 | 22.78 | 0.84 | 3.7 |
| 7 | 22.78 | 0.91 | 4.0 |

Table 9. Alkaline post-washed 6% NaOH/15% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 22.94 | 0 | 0 |
| 1 | 22.94 | 12.61 | 55.0 |
| 2 | 22.94 | 17.07 | 74.4 |
| 3 | 22.94 | 19.35 | 84.4 |
| 5 | 22.94 | 19.89 | 86.7 |
| 7 | 22.94 | 20.51 | 89.4 |
| 10 | 22.94 | 20.73 | 90.4 |

Table 10. Alkaline post-washed 21% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 23.21 | 0 | 0 |
| 1 | 23.21 | 9.63 | 41.5 |
| 2 | 23.21 | 12.36 | 53.3 |
| 3 | 23.21 | 14.36 | 61.9 |
| 5 | 23.21 | 17.10 | 73.7 |
| 7 | 23.21 | 17.98 | 77.5 |
| 10 | 23.21 | 18.77 | 80.9 |

Table 11. Alpha-cellulose substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 17.00 | 0 | 0 |
| 1 | 17.00 | 6.26 | 36.8 |
| 2 | 17.00 | 9.82 | 57.8 |
| 3 | 17.00 | 11.56 | 68.0 |
| 5 | 17.00 | 13.17 | 77.5 |
| 7 | 17.00 | 13.82 | 81.3 |
| 10 | 17.00 | 14.27 | 83.9 |

Table 12. 6% NaOH/15% peracetic acid sugar cane bagasse substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 26.22 | 0 | 0 |
| 1 | 26.22 | 11.81 | 45.0 |
| 2 | 26.22 | 17.32 | 66.1 |
| 3 | 26.22 | 20.20 | 77.0 |
| 5 | 26.22 | 22.94 | 87.5 |
| 7 | 26.22 | 23.82 | 90.8 |
| 10 | 26.22 | 24.10 | 91.9 |

Table 13. 6% NaOH/9% peracetic acid sugar cane bagasse substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 26.25 | 0 | 0 |
| 1 | 26.25 | 8.94 | 34.0 |
| 2 | 26.25 | 11.22 | 42.8 |
| 3 | 26.25 | 12.78 | 48.7 |
| 5 | 26.25 | 14.07 | 53.6 |
| 7 | 26.25 | 15.50 | 59.1 |
| 10 | 26.25 | 17.70 | 67.4 |

Table 14. 3% NaOH/15% peracetic acid sugar cane substrate substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 26.33 | 0 | 0 |
| 1 | 26.33 | 9.61 | 36.5 |
| 2 | 26.33 | 11.81 | 44.8 |
| 3 | 26.33 | 14.52 | 55.1 |
| 5 | 26.33 | 17.05 | 64.7 |
| 7 | 26.33 | 18.82 | 71.5 |
| 10 | 26.33 | 21.04 | 79.9 |

Table 15. 5.25% NH4OH/15% peracetic acid hybrid poplar substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 26.64 | 0 | 0 |
| 1 | 26.64 | 9.44 | 35.4 |
| 2 | 26.64 | 13.18 | 50.2 |
| 3 | 26.64 | 14.47 | 55.1 |
| 5 | 26.64 | 15.83 | 60.3 |
| 7 | 26.64 | 17.16 | 65.4 |
| 10 | 26.64 | 17.83 | 67.9 |

Table 16. 15% peracetic acid sugar cane bagasse substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 26.91 | 0 | 0 |
| 1 | 26.91 | 8.25 | 30.7 |
| 2 | 26.91 | 12.11 | 45.0 |
| 3 | 26.91 | 14.21 | 52.9 |
| 5 | 26.91 | 15.53 | 57.7 |
| 7 | 26.91 | 16.35 | 60.8 |
| 10 | 26.91 | 16.61 | 61.73 |

Table 17. 21% peracetic acid sugar cane bagasse substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 26.38 | 0 | 0 |
| 1 | 26.38 | 10.32 | 39.1 |
| 2 | 26.38 | 15.07 | 57.1 |
| 3 | 26.38 | 18.88 | 71.6 |
| 5 | 26.38 | 21.47 | 81.4 |
| 7 | 26.38 | 22.61 | 85.7 |
| 10 | 26.38 | 24.11 | 91.4 |

Table 18. 60% peracetic acid sugar cane bagsse substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 25.61 | 0 | 0 |
| 1 | 25.61 | 1.25 | 4.9 |
| 2 | 25.61 | 15.72 | 61.4 |
| 3 | 25.61 | 20.75 | 81.1 |
| 5 | 25.61 | 22.67 | 88.5 |
| 7 | 25.61 | 23.22 | 90.7 |

Table 19. Raw sugar cane bagasse substrate

| Time (days) | Theoretical ethanol (g/L) | Practical ethanol (g/L) | Ethanol yield (%) |
|---|---|---|---|
| 0 | 25.61 | 0 | 0 |
| 1 | 25.61 | 1.66 | 6.5 |
| 2 | 25.61 | 3.43 | 13.4 |
| 3 | 25.61 | 4.20 | 16.4 |
| 5 | 25.61 | 4.68 | 18.3 |
| 7 | 25.61 | 4.73 | 18.5 |