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Alkaline and Peracetic Acid Pretreatments of Biomass for Ethanol Production

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Abstract

Prehydrolysis with dilute acid and steam explosion constitute the most promising methods for improving enzymatic digestibility of biomass for ethanol production. Despite worldwide acceptance, these methods of pretreatment are quite expensive considering costs for the reactor, energy, and fractionation. Using peracetic acid is a lignin-oxidation pretreatment with low-energy input by which biomass can be treated in a silo-type system without need for expensive capitalization. Experimentally, ground hybrid poplar 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-dried biomass. The ratio of solution to biomass is 6:1 and a 7-d storage period at ambient temperature (20°C) has been used. As an auxiliary 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 are performed before addition of peracetic acid. The basic solutions are added to the biomass in a ratio of 14:1 solution to biomass, and mixed for 24 h at the same ambient temperature. Biomass is filtered and washed to a neutral pH before peracetic acid addition. The aforementioned procedures give high xylan content substrates as a function of the selectivity of peracetic acid for lignin oxidation and the mild conditions of the process. Consequently, xylanase/β-glucosidase combinations were more effective than cellulase preparations in hydrolyzing these materials. The pretreatment efficiency was evaluated through enzymatic hydrolysis and simultaneous saccharification and cofermentation (SSCF) tests. Peracetic acid treatment improves enzymatic digestibility of hybrid poplar and sugar cane bagasse with no need of high temperatures. Alkaline treatments are helpful in reducing peracetic acid requirements in the pretreatment.

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Index Entries: Hybrid poplar; sugar cane bagasse; peracetic acid; enzymatic hydrolysis; SSCF; *Zymomonas mobilis*; ethanol fuel.

Introduction

Biomass is basically cellulose microfibers 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. 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 has been studied for improving biomass digestibility *(1–3)*, but it has never been used at ambient temperature. 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 is not significant carbohydrate losses and furfural is not produced during the process.

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 *(4)*. The aliphatic propane side-chain is displaced to some extent during the oxidative process *(5)*. 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 (m.t.)/yr, began in 1996 in Finland *(6,7)*, using acetic acid and hydrogen peroxide raw materials supplied by the Oulu plants. In the laboratory, oxidation of acetic anhydride was used only for obtaining better peracetic acid yield, but is far too expensive for even pilot-scale evaluation of this process *(8)*. Hydrogen peroxide costs are declining and its use is environmentally safe *(8,9)*. Since acetic acid is conveniently prepared from ethanol by aerobic fermentation, an inexpensive source of acetic acid is available, especially in countries with strong ethanol production capabilities.

The feasibility of pretreating two biomass sources—hybrid poplar and sugar cane bagasse—with peracetic acid is demonstrated. Because these pretreated materials contain nearly the original hemicellulose content, combinations of commercial enzymes are investigated. The effectiveness of various pretreatment conditions is evaluated by standard enzymatic hydrolysis and simultaneous saccharification and cofermentation procedures.

Materials and Methods

Pretreatment

Hybrid poplar (*Populus deltoides* × *Populus nigra*) provided by the National Renewable Energy Laboratory (NREL); Golden, CO) and sugar cane bagasse provided by Cajun Sugar Co-op, Inc. (New Iberia, LA) were milled and screened to 20–80 mesh. The moisture content of the two air-dried biomasses were 5.6 and 7.0%, respectively. The ground biomasses were placed in plastic bags with solutions at loading of 0, 6, 9, 15, 21, 30, and 60% of peracetic acid based on oven-dried biomass weight. The solutions were added at the ratio of 6:1, liquor:wood, mixed, and stored for a 7-d period at ambient temperatures. 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 17:1 for sugar cane bagasse for 24 h at room temperature and then washed to neutral pH with distilled water using Buchner funnels. Subsequent 6, 9, and 15% of peracetic acid loadings based on oven-dried biomass were added to this material using the conditions for peracetic acid treatment previously described.

Carbohydrate Composition

Two-stage acid hydrolysis was performed for determining carbohydrate composition on peracetic acid treated samples and the raw biomass according to a standard procedure of the NREL *(10)*. Hydrolysates were analyzed using high-performance liquid chromatography (HPLC) equipped with a 300 × 7.8 mm HPX-87P Bio-Rad column coupled to a Waters Associates refractive index (RI) detector.

Enzymatic Hydrolysis

The commercial enzymes, SP431 (xylanase), Celluclast (cellulase), and Novozym 188, (β-glucosidase), used in the enzymatic hydrolysis tests were provided by Novo Nordisk Biochem North America (Franklinton, NC). The enzyme Spezyme (cellulase) was produced by Environmental Biotechnologies (Santa Rosa, CA). 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 <1 IFPU/mL for Novozym 188 using IUPAC procedure *(11)*. 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 *(12)*. 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 *(13)*.

The enzymatic hydrolysis tests according to an NREL procedure *(14)* modified without addition of yeast-peptone (YP) medium, were performed in 250-mL Erlenmeyer flasks with a biomass loading based on 1 g cellulose content to 100 mL of 0.05 *M* acetyl 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 1. Flasks were incubated at 37°C and shaken at 125 rpm. Samples for HPLC sugar analyses were collected at 0, 5, 10, 24, 48, and 120 h in a laminar flow hood (*see* Table 1).

Simultaneous Saccharification and Co-Fermentation (SSCF)

The SSCF tests were performed using 250-mL Erlenmeyer flasks with a rubber-stopper gas trap according to a modification of standard NREL procedures *(14)*. The biomass loading was based on 3 g cellulose content/100 mL of fermentation medium. Xylan content varied for each sample. Distilled water was added to each flask, the pH was adjusted to 5, and then flasks were autoclaved for 20 min. After a natural cooling, sterile distilled water was added to achieve initial weight. One mL of sterile tetracycline solution containing 1 mg of the antibiotic was added to each flask. Following addition of 10 mL of sterile 10X yeast extract medium, 10 mL of inoculum prepared as described below was added. Combinations of the enzymes Novozym 188 and SP431 (Novo Nordisk) was used at a loading of 8.33 IFPU/g of cellulose, a total of 25 IFPU for each flask. Flasks were placed in the shaker at 37°C and 150 rpm. Samples were collected at 0, 1, 2, 3, 5, 7, and 10 d using sterile technique in a laminar flow hood for later HPLC analysis. The formation of ethanol, cellobiose, glucose, xylose, and acetyl were measured by HPLC using a 300 × 7.8 mm HPX-87H Bio-Rad column.

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

Results

Peracetic acid is selective in oxidizing lignin. Only a small amount of hemicellulose is removed from both hybrid poplar and sugar cane bagasse. HPLC analysis show the peracetic pretreated samples contain most of their original xylan (Tables 2 and 3).

Table 1
Assay of Enzymatic Activities in Various Combinations
of Enzymes Used to Hydrolyze Peracetic Acid Pretreated Hybrid Poplar at the Loadings of 25 IFPU/g Cellulose

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

Table 2
Partial Composition and Yield of Pretreated Hybrid Poplar

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
6% NaOH-15% peracetic acid	50.0	17.1	15.1	3.6	77.9
6% NaOH-9% peracetic acid	46.8	15.7	12.0	3.9	84.0
3% NaOH-15% peracetic acid	47.3	16.7	17.6	3.4	84.8
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
5.25% NH_4OH-15% peracetic acid	45.7	15.7	13.1	5.0	85.4
5.25% NH_4OH-9% peracetic acid	43.9	14.5	17.1	4.8	88.9
2.63% NH_4OH-15% peracetic acid	44.9	15.5	13.9	4.9	88.2
2.63% NH_4OH-9% peracetic acid	43.6	14.3	18.7	4.4	89.8

[a]The xylan content is reported as a linear polymer not considering the side groups of 4-methyl-glucuronic acid and acetyl.

Table 3
Partial Composition and Yield of Pretreated Sugar Cane Bagasse

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
3% NaOH-15% peracetic acid	47.7	25.6	15.6	4.9	77.8
6% NaOH-9% peracetic acid	47.9	25.5	16.5	3.5	78.0
3% NaOH-9% peracetic acid	45.4	24.9	12.9	3.6	80.8
6% NaOH-6% peracetic acid	46.9	25.0	17.2	3.0	78.0
5.25% NH_4OH-15% peracetic acid	46.5	25.4	13.0	4.9	77.2
2.63% NH_4OH-15% peracetic acid	45.6	25.3	14.1	5.3	79.3
5.25% NH_4OH-9% peracetic acid	45.1	24.7	16.0	4.2	80.1
2.63% NH_4OH-9% peracetic acid	44.6	24.6	17.2	3.9	81.3
5.25% NH_4OH-6% peracetic acid	44.1	24.0	19.8	4.0	81.4

[a]The xylan content is reported as a linear polymer not considering any side groups.

Enzymatic Screening Tests

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 1 shows the behavior of 15% peracetic-treated hybrid poplar with different enzymes and their combinations. When using different cellulase combinations (e.g., Celluclast/Novozym 188 or Spezyme/Novozym 188), the results were less than satisfactory. The reason for the difference can be explained by higher xylanase activities present in the SP431/Novozym 188 combination than in the others (Table 1). These results show that the addition of Novozym 188 was a very significant improvement when compared to the use of single cellulases.

Enzymatic Hydrolysis Tests

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; the results are shown in Fig. 2. The 60% treated sample was readily converted to sugars in only 24 h. The 21% loading pretreated hybrid poplar is acceptable for attaining the requirements for SSCF *(15)* using recombinant *Z. mobilis* developed by Zhang et al. *(16)*. The data indicate 98.3% of the cellulose in the 21% loading pretreated hybrid poplar is converted to simple sugars in 120 h. The 0% treated sample, raw wood, had a very poor conversion of only 6.8% to simple sugars at the end of the hydrolysis period.

Regarding overall pretreatment yields (i.e., solid dry matter recovery), Brito obtained corresponding yields from dilute sulfuric acid prehydrolysis of hybrid poplar of less than 70% *(17)* and Schwald et al. reported 57% yields from steam-explosion pretreatment of aspen *(18)*. Losses from other dilute-acid pretreatment methods are relatively great compared to the average 85% yield obtained here (Table 2). In Table 3, the yields of pretreated bagasse are less than yields noted in Table 2, because approx 10% sucrose remains in the extracted bagasse.

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, but at 60% loading, the spectrophotometric analysis of soluble lignin at 205 nm is reduced probably because the oxidative fragmentation of the phenyl ring structures decrease the electronic resonance. The carbohydrate contents are greater at the higher loading values because of lignin solubilization. However, the relative carbohydrate losses are low

Fig. 1. *(opposite page)* Enzyme pool test for identifying the best enzyme combination using the 15% peracetic hybrid poplar acid-treated sample. An equal loading of 25.0 IFPU/g of cellulose was added to each sample. **(A)** glucan conversion as percent of total glucan. **(B)** xylan conversion as percent of apparent xylan loading (Table 2). The xylanase varied in the amounts present in the various enzyme preparations (*see* Table 1).

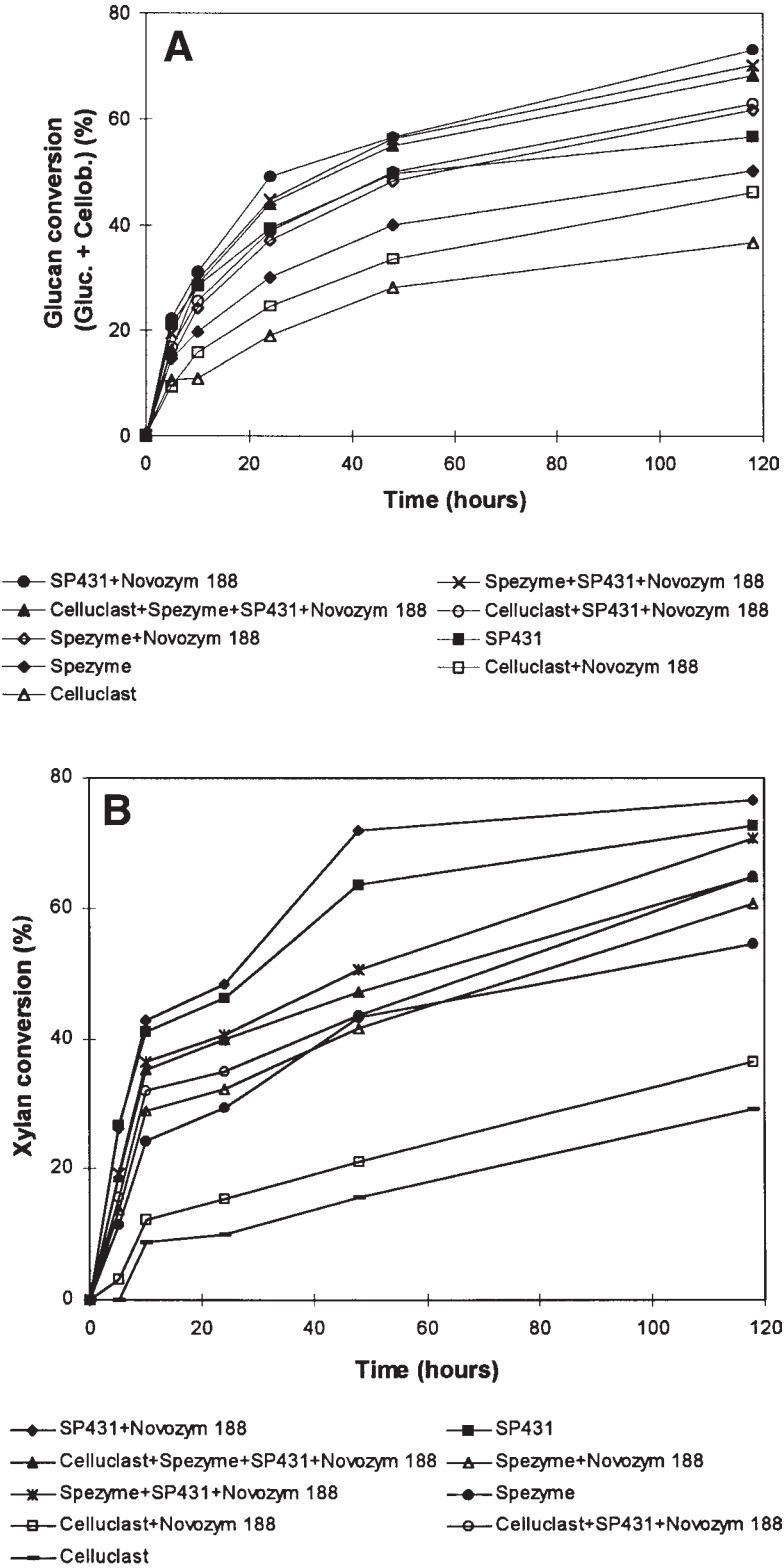
A
Glucan conversion (Gluc. + Cellob.) (%)
Time (hours)
SP431+Novozym 188
Spezyme+SP431+Novozym 188
Celluclast+Spezyme+SP431+Novozym 188
Celluclast+SP431+Novozym 188
Spezyme+Novozym 188
SP431
Spezyme
Celluclast+Novozym 188
Celluclast
B
Xylan conversion (%)
Time (hours)
SP431+Novozym 188
SP431
Celluclast+Spezyme+SP431+Novozym 188
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Fig. 1.

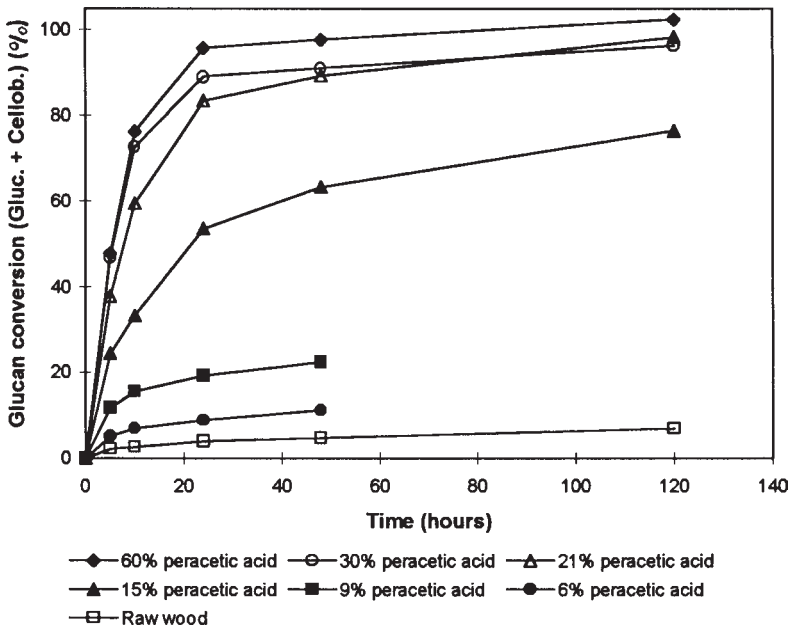

Fig. 2. Enzymatic hydrolysis of hybrid poplar treated with different loadings of peracetic acid. Six and 9% peracetic acid treated samples were hydrolyzed using the Spezyme/Novozym 188 combination.

as explained 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 2 and 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.

For sugar cane bagasse, the 21, 30, and 60% peracetic pretreated samples show the best results on biomass conversion to simple sugars (*see* Fig. 3). The 21% treated bagasse looks very acceptable for attaining the SSCF requirements. The raw bagasse had poor conversion of only 28.8% to simple sugars at the end of the hydrolysis period.

With the intent of reducing peracetic acid loading, basic pre-pretreatments were performed using 3 and 6% sodium hydroxide, and 2.63, 5.25 and 14.00% ammonium hydroxide, based on dry-wood weight of a ratio of 14:1 of solution to biomass. Only the 9 and 15% peracetic acid pretreatments were evaluated. This procedure, as shown in Figs. 4 and 5, improved the simple sugar yield when comparisons were made at 9 and 15% peracetic acid treatment without the prior use of base (Fig. 2). Respective conversions were estimated at 40% and 75% at the end of 120 hours of enzymatic hydrolysis without base pretreatments. The sodium hydroxide pre-pre-

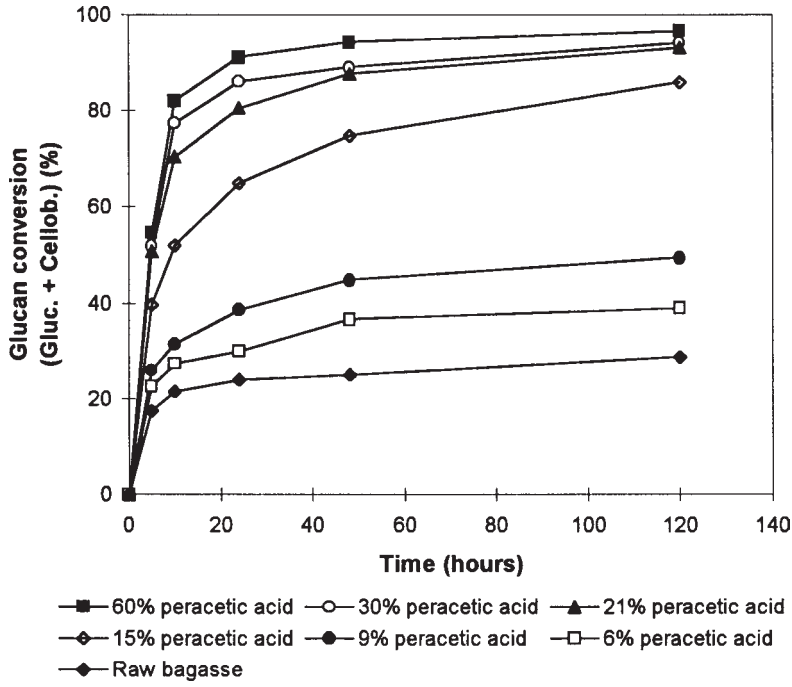

Fig. 3. Enzymatic hydrolysis of sugar cane bagasse treated with different loadings of peracetic acid. Percentages of reactants are based on oven-dry weight of biomass.

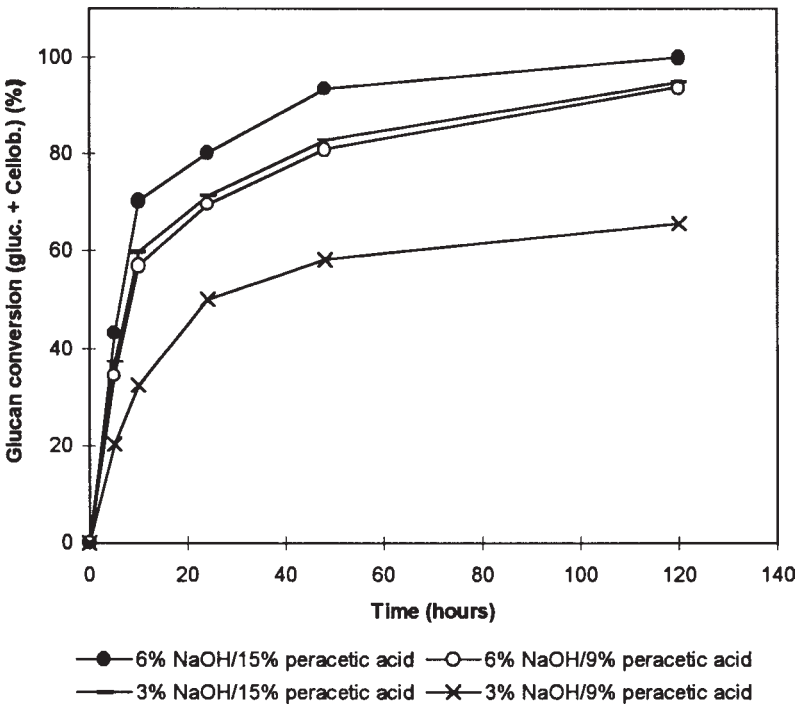

Fig. 4. Effect of sodium hydroxide pre-pretreatment followed by peracetic acid pretreatment on enzymatic hydrolysis of hybrid poplar. Percentages of reactants are based on oven-dry weight of woody biomass. The SP431/Novozym 188 combination was used.

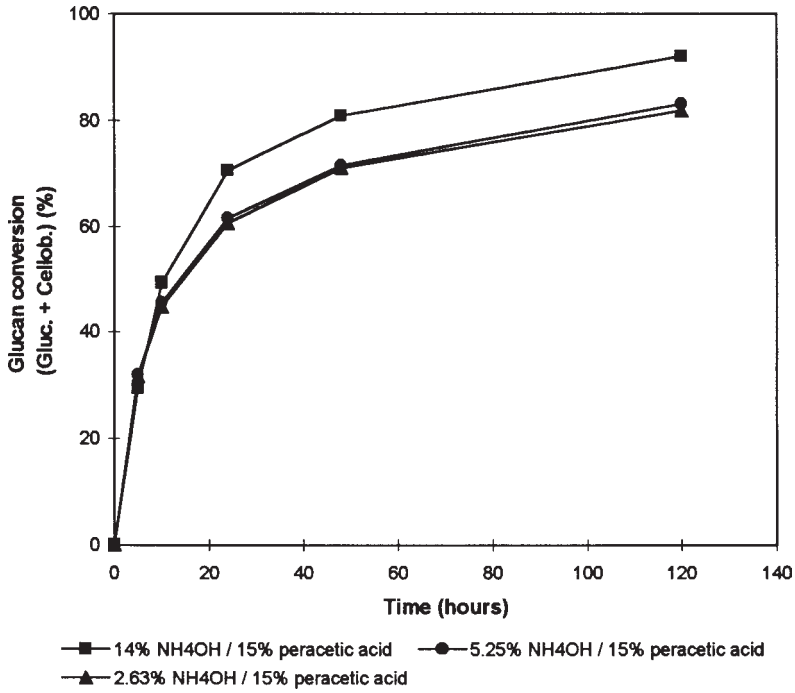

Fig. 5. Effect of ammonium hydroxide loading on hybrid poplar prior to peracetic acid pretreatment.

treated samples show satisfactory conversion in the course of 120 h. Specifically, the sample treated with 6% NaOH/9% peracetic acid (95% yield) shows better conversion than the sample treated only with 15% peracetic acid (75% yield).

According to data in Fig. 5, 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. Ammonium hydroxide, being a weak base, requires the use of that base in a higher concentration of 14% based on oven-dry weight of biomass to give a better conversion (*see* Fig. 5). Presumably, larger ammonium hydroxide concentrations would be more cost effective than 6% of sodium hydroxide, considering the possibility of recycling ammonium hydroxide.

As performed for poplar, the sugar cane bagasse was submitted to an alkaline pre-pretreatment prior to peracetic acid addition (*see* Fig. 6). Alkaline concentrations were the same used for poplar. Due to mixing difficulty, the ratio had to be increased to 17:1 of 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 h. Specifically, the sample treated with 6% NaOH/15% peracetic acid shows similar conversion when compared to the sample treated only with 21% peracetic acid.

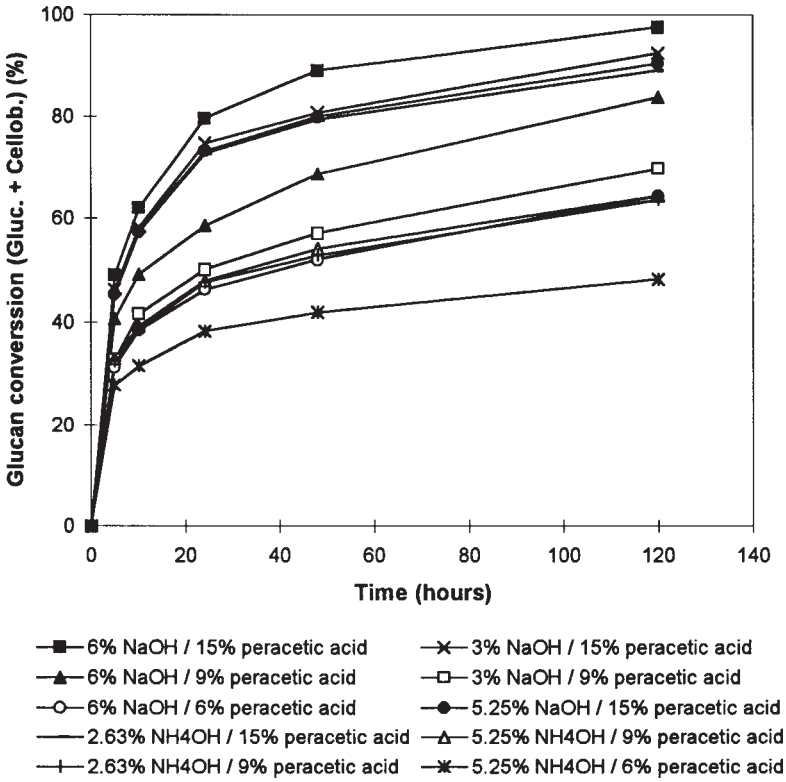

Fig. 6. Effect of sodium hydroxide and ammonium hydroxide pre-pretreatment on enzymatic hydrolysis of sugar cane bagasse followed by peracetic acid pretreatment. Percentages of reactants are based on oven-dry weight of biomass.

The survey of studies with the alkaline pre-pretreatments was not exhaustive; considerably more work is required to establish optimal conditions in combination with peracetic acid pretreatments.

SSCF

Figure 7 shows the SSCF kinetics of hybrid poplar pretreated with 6% NaOH/15% peracetic acid based on oven-dry weight of wood. The maximum ethanol yield, corresponding to 21.8 g/L or 95.0% yield, was reached in 7 d fermentation. The concentration of glucose and xylose were very low after 7 d. The concentration of xylose was higher than glucose during the process indicating that glucose is consumed before xylose by the recombinant bacteria. Because an acetyl was formed at concentrations below 1 g/L efficiency of ethanol conversion by the *Z. mobilis* fermentation with this practical substrate is quite satisfactory.

The comparison of ethanol yield among three different substrates is shown in Fig. 8. Raw hybrid poplar had very poor conversion to ethanol, α-cellulose conversion to ethanol was 75% of theoretical in 7 d and the pretreated hybrid poplar obtained an average yield of 92.3% theoretical

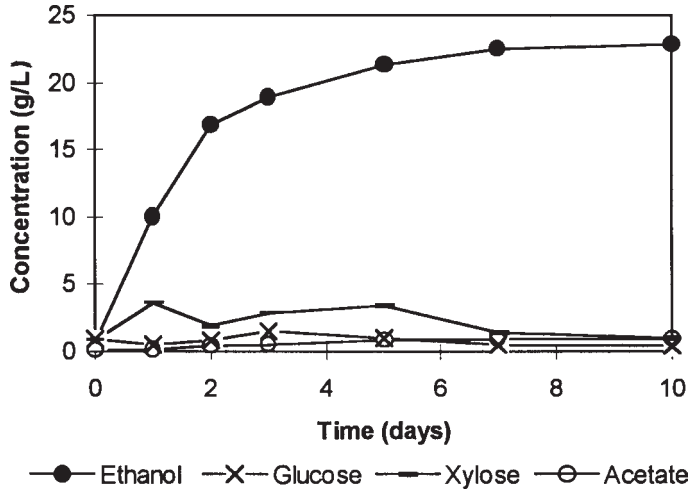

Fig. 7. Typical SSCF kinetics of hybrid poplar pretreated with 6% NaOH/15% peracetic acid based on oven-dry weight of wood.

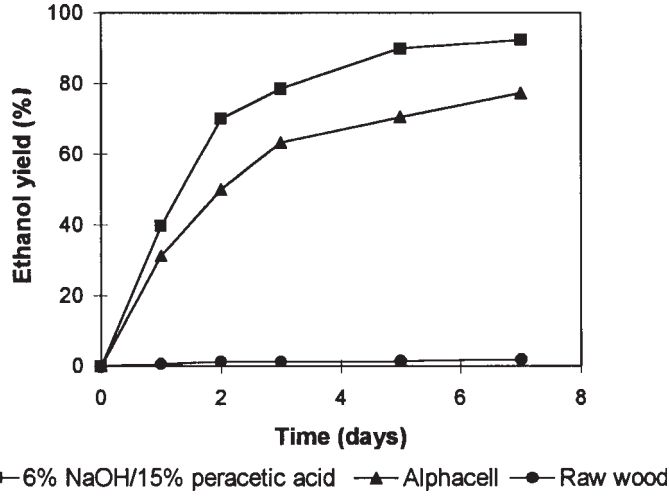

Fig. 8. Ethanol yields from SSCF of pretreated hybrid poplar, alpha-cellulose, and untreated hybrid poplar.

ethanol based on combined glucan and xylan content. High yields from the ambient temperature pretreatments are greater than those from severe high temperature pretreatments. 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 alphacell (Fig. 8), from which xylan hydrolysis products are found by HPLC (data not presented). Crystallinity might be a factor as well.

Conclusions and Discussion

Considering the high xylan content present in the peracetic acid-treated, hybrid-poplar substrate, the combination of industrial enzymes SP431 and Novozym 188 gives the best sugar conversion in enzymatic hydrolysis compared to other combinations. An explanation might be that 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 is effective in improving enzymatic digestibility of hybrid poplar and sugar cane bagasse. The loading of peracetic acid for obtaining the best enzymatic hydrolysis yield is found to be 21% based on oven-dried weight of biomass of both substrates. A pre-pretreatment using sodium-hydroxide solution prior to peracetic acid pretreatment gives, in 9 and 15% peracetic acid-treated samples, significantly better sugar production. Consequently, lower amounts of peracetic acid are needed. The 6% NaOH/15% peracetic acid pre-pretreated poplar and sugar cane bagasse samples meet SSCF requirements and the 6% NaOH/9% peracetic acid treated hybrid poplar could meet the same requirements if the enzyme loading were increased to greater than 8.33 IFPU/g cellulose.

The technical viability of hybrid poplar 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%.

Acknowledgments

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