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Simultaneous Saccharification and Cofermentation of Peracetic Acid–Pretreated Biomass

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Abstract

Previous work in our laboratories has demonstrated the effectiveness of peracetic acid for improving enzymatic digestibility of lignocellulosic materials. The use of dilute alkali solutions as a pre-pretreatment prior to peracetic acid lignin oxidation increased carbohydrate hydrolysis yields in a synergistic as opposed to additive manner. Deacetylation of xylan is easily achieved using dilute alkali solutions under mild conditions. In this article, we evaluate the effectiveness of peracetic acid combined with an alkaline pre-pretreatment through simultaneous saccharification and cofermentation (SSCF) of pretreated hybrid poplar wood and sugar cane bagasse. Respective ethanol yields of 92.8 and 91.9% of theoretical are achieved using 6% NaOH/15% peracetic acid–pretreated substrates and recombinant *Zymomonas mobilis* CP4/pZB5. Reduction of acetyl groups of the lignocellulosic materials is demonstrated following alkaline pre-pretreatments. Such processing may be helpful in reducing peracetic acid requirements. The influence of deacetylation is more significant in combined pretreatments using lower peracetic acid loadings.

Index Entries: Pretreatment; biomass; peracetic acid; *Zymomonas mobilis*; simultaneous saccharification and cofermentation; ethanol.

Introduction

The use of biomass such as lignocellulosic residues from farm, forest, and urban areas as fermentation feedstocks has been a promising research field. Hybrid poplar wood and sugar cane bagasse are often cited in the

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literature as promising sources of lignocellulosic materials. Requirements such as productivity, availability, and production costs make their use realistic. Hybrid poplar is recognized as an alternative woody crop because of its high productivity, 18–26 dry Mg/(ha · yr), in the Pacific Northwest *(1)* for large-scale production of fuels *(2)*. Sugar cane bagasse, a residue from the sugar cane industry, is available at sugar mill sites at no additional cost because harvesting, transportation, and storage costs are borne by the sugar production. In addition, Prine and Woodard *(3)* have estimated the oven-dry biomass yield for perennial tall grasses, including sugar cane, to vary from 20 to 45 Mg/(ha · yr) in the cooler subtropical to mild temperature locations to more than 60 Mg/(ha · yr) in the lower portion of the Florida peninsula. For sugar cane grown on phosphoric clay, Stricker et al. *(4)* have reported yields varying from 37 to 54 Mg/(ha · yr) in central Florida.

For 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% ethanol to gasoline increases octane number, improves engine efficiencies by excellent antiknock properties, and oxygenates gasoline *(5)*.

Biomass, by 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 largely 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, as well as decrystallization of cellulose *(6–8)*. Prehydrolysis using steam explosion and diluted acids constitute the most studied technologies. Both methods use high-temperature and high-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 *(9–13)*, but little work has been done at ambient temperature *(14,15)*. Based on a low-temperature pretreatment approach, a silo-type system designed for using peracetic acid constitutes a new alternative for pretreating biomass. Although acetic acid is released as a product of the peracetic acid reaction, 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 is quite selective toward the lignin structure. It oxidizes aromatics in lignin generating dicarboxylic acids and their lactones *(16)*. The aliphatic propane side chain is displaced, to some extent, during the oxidative pro-

cess *(17)*. Peracetic acid is produced by the reaction of hydrogen peroxide and acetic acid.

In this article, we describe additional results derived from previous publications, in which enzyme mixture optimization and relative rates of enzyme hydrolysis of variously pretreated biomass samples are given *(18,19)*. We quantify the value of deacetylation in the reduction of pretreatment chemical usage, the efficiency of enzymatic hydrolysis, and the consequent yields in simultaneous saccharification and cofermentation (SSCF). Lignocellulosic materials pretreated with peracetic acid retain most of the original xylan without significant deacetylation. The alkaline pre-pretreatments described here result in deacetylation of hemicellulose. The acetyl groups cause stereochemical impediments to enzymatic degradation as reported in the literature *(20–23)*.

Materials and Methods

The ground and 20- to 80-mesh-screened hybrid poplar wood was provided by the National Renewable Energy Laboratory (NREL), Golden, CO, and the sugar cane bagasse was provided by Cajun Sugar Co-op, New Iberia, LA. The bagasse was air-dried, milled, and screened to the same conditions as performed in hybrid poplar wood. The moisture contents were 5.6 and 7.0%, respectively. The pretreatment procedures and the analytical methods for determining the cellulose and hemicellulose content of resulting substrates are described in previous work *(19)*. Alkaline pre-pretreatments were conducted prior to application of peracetic acid using a 17:1 liquid:solid ratio. The peracetic acid was prepared in the laboratory using acetic anhydride and hydrogen peroxide. It was then mixed in stated proportions with a ratio of 6:1 liquor:biomass and stored for 7 d at ambient temperatures. The enzymatic hydrolysis tests as well as the enzyme activity assays were developed from standard procedures used at the NREL and were performed as described in previous work *(19)*. The enzyme hydrolysis test allowed determination of glucan and xylan conversion by high-performance liquid chromatography (HPLC) analysis, as given below. Triplicate analysis of holocellulose and acetyl content was done according to Browning *(24)* and McComb and McCready *(25)*, respectively.

Considerable effort was made to optimize the ratio of commercial enzymes used in the SSCF tests *(19)*. A combination of the Novo Nordisk (Franklinton, NC) enzymes Novozym 188 (which assayed at 1.0 international filter paper units [IFPU]/mL of cellulase activity and 560 IU/mL of xylanase activity and was used as 23.8% of the applied mixture) and SP431 (which assayed at 39.2 IFPU/mL of cellulase activity and 5420.0 IU/mL of xylanase activity and was used as 76.28% of the applied mixture) was used at a loading of 8.33 IFPU/g of cellulose. Enzymes were filter sterilized (0.45 μ) separately and added aseptically to the SSCF flasks. SSCFs were carried out according to a modification of standard procedures *(26)*, in that enzyme loading was reduced threefold compared to the loading used in the enzy-

matic hydrolysis tests with the intent of avoiding high accumulation of sugars in the first hours of the process. In spite of the enzyme loading being lowered threefold, each flask still had a total of 25 IFPU. The optimum temperature growth for *Zymomonas mobilis* has been demonstrated to be 30°C *(27–29)*, but 37°C, common in SSF tests, was maintained in order to avoid the consequent decrease in the enzyme activity.

Prior to SSCF, the biomass samples were exhaustively washed to remove residual acetate that was released by the alkaline pre-pretreatments. Also, the acetic acid formed from the peracetic acid reaction was removed by washing on Buchner funnels until the filtrate was pH 5.0. The amount of biomass was calculated based on 3 g of cellulose and weighed directly into a 250-mL Erlenmeyer flask to the nearest 0.01 g. Xylan content varied for each sample depending on its content in the biomass. Distilled water (approx 60 mL) was added to each flask, and the pH was adjusted to 5.0. A rubber stopper gas trap was placed in the opening of the Erlenmeyer flask. The weight of the flask and its contents was recorded. Following the addition of 10 mL of sterile 10X yeast extract medium and 10 mL of inoculum prepared as described below, sufficient sterile water was added to bring the final volume to 100 mL. Flasks were placed in a shaker at 37°C and 150 rpm (New Brunswick Scientific PsycoTherm, New Brunswick, NJ). Samples were collected at 0, 1, 2, 3, 5, 7, and 10 d and stored at –20°C for later HPLC analysis. The formation of ethanol, cellobiose, glucose, xylose, and acetate was measured by HPLC using a 300 × 7.8 mm HPX-87H Bio-Rad (Hercules, CA) organic acid column. The ethanol yields were calculated according to the following equation:

$$\text{Ethanol yield (\%)} = \frac{[\text{EtOH}]_f - [\text{EtOH}]_0}{0.51[(\text{Glucan} \times 1.111) + (\text{Xylan} \times 1.136)]} \times 100$$

in which $[EtOH]f$ = ethanol concentration at the end of cofermentation (g/L); $[EtOH]_0$ = ethanol concentration at the beginning of the cofermentation (g/L); [Glucan] = Glucan loading (g/L); and [Xylan] = Xylan loading (g/L). The practical conversion factor of 0.51, xylose to ethanol, is the same as determined for glucose according to McMillan *(30)*.

Frozen cultures of recombinant *Z. mobilis* CP4/pZB5 supplied by NREL was used for inoculum. This organism has the capability of utilizing glucose as well as xylose as fermentation substrates, which is important because peracetic acid pretreatment does not degrade hemicellulose. The inoculum preparation was performed using a medium containing 0.7% (w/v) glucose, 0.3% (w/v) xylose, 1.0% (w/v) yeast extract, 0.2% (w/v) KH_2PO_4, and 1.0 mg of tetracycline per 100 mL of medium. First, the frozen culture was transferred to a test tube containing 10 mL of sterile medium and placed in an incubator at 37°C with no shaking for 12 h. This mixture was then transferred to a 500-mL Erlenmeyer flask with 300 mL of the same medium. The inoculum was used 12–16 h after the second transfer.

Table 1
Chemical Composition of Hybrid Poplar Wood and Sugar Cane Bagasse

Component	Hybrid poplar wood (dry wt basis, %)	Sugar cane bagasse (dry wt basis, %)
Cellulose	41.7	39.6
Pentosans	18.4	29.7
Lignin		
Klason	25.0	22.2
Acid soluble	3.4	2.5
Holocellulose	70.4	74.5
Ash	0.8	4.1
Extractives	4.8	14.3

Results and Discussion

Chemical Composition of Biomass

Table 1 gives the chemical composition of the raw lignocellulosic materials hybrid poplar wood and sugar cane bagasse used in this study. The high extractive content verified in sugar cane bagasse is owing to residual sucrose left during processing of the sugar cane. Sucrose could not be specifically accounted for in the HPLC analysis; presumably sucrose was removed by washing steps prior to fermentation. Total lignin (Klason and acid soluble) and other results, excluding extractive content, are calculated based on extractive free biomass.

Pretreatment Solid Yield and Chemical Composition

Pretreatment solid yield is defined as solids remaining following pretreatment divided by original oven-dried weight. The pretreatment yields published in our previous work *(19)*, which average approx 85% for hybrid poplar and 80% for bagasse, are greater than values reported for dilute-acid and steam-explosion pretreatments. Brito *(31)* and Grohmann et al. *(7)* obtained corresponding yields from dilute sulfuric acid prehydrolysis of hybrid poplar of <70%. Schwald et al. *(32)* reported recovery yields that varied from 49.9 to 63.0%, depending on temperatures used, for steam-exploded aspen wood. Mackie et al. *(33)* presented recovery yields from 65.7 to 70.8% for the same pretreatment method and wood type. Dekker and Wallis *(34)* presented overall solid yield of approx 60% from steaming exploded sugar cane bagasse. Note that dilute-acid or steam-explosion pretreatments may solubilize hemicellulose, but this is not necessarily wasted and may be converted to ethanol after the hydrolysis liquor is detoxified and neutralized.

The relevant comparison of pretreatment types is the percentage of feedstock carbohydrate that can be made available for fermentation. According to data published in previous work *(19)*, carbohydrate content

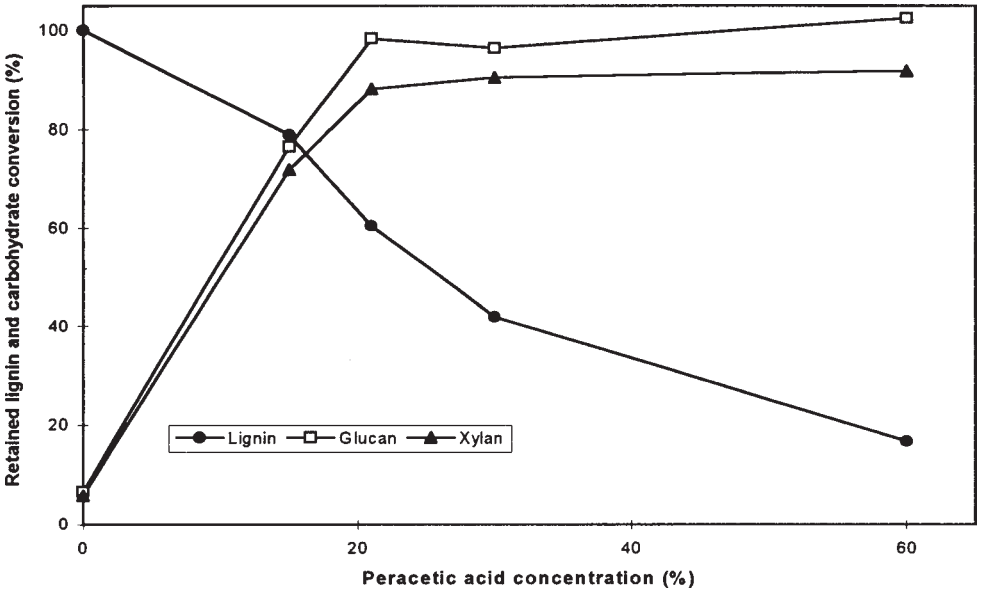

Fig. 1. Lignin retention and carbohydrate hydrolysis conversion as a function of peracetic acid concentration for hybrid poplar.

increases with the extent of delignification. Even the apparent xylan content increases with severity of the process differently from the thermal conversion processes. Klason lignin content decreases with an increase of peracetic acid concentration; soluble lignin 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 decreases the absorption at 205 nm. Figures 1 and 2 show both the glucan and xylan conversions (120 h time point; *see* ref. *19* for representative enzyme hydrolysis curves) and the extent of delignification that is also a function of peracetic acid concentration. Satisfactory sugar conversion is achieved using 20% peracetic acid loadings based on the oven-dried weight of biomass. At this condition, only 40% of lignin has been removed from pretreated hybrid poplar and 30% from pretreated bagasse. As with dilute-acid pretreatments, improved enzymatic hydrolysis of biomass that contains residual lignin occurs, because the lignin/hemicellulose matrix around the cellulose microfibril needs only to be disrupted for cellulase enzyme access to the cellulose molecules.

Synergistic Effect of Alkali Washing on Enzymatic Hydrolysis

Alkaline washing is beneficial prior to oxidative treatment by reducing requirements for peracetic acid. Tables 2 and 3 give the acetyl content in different substrates. Synergism is verified by observing the extent of enzymatic hydrolysis as a function of acetyl group removal from xylan.

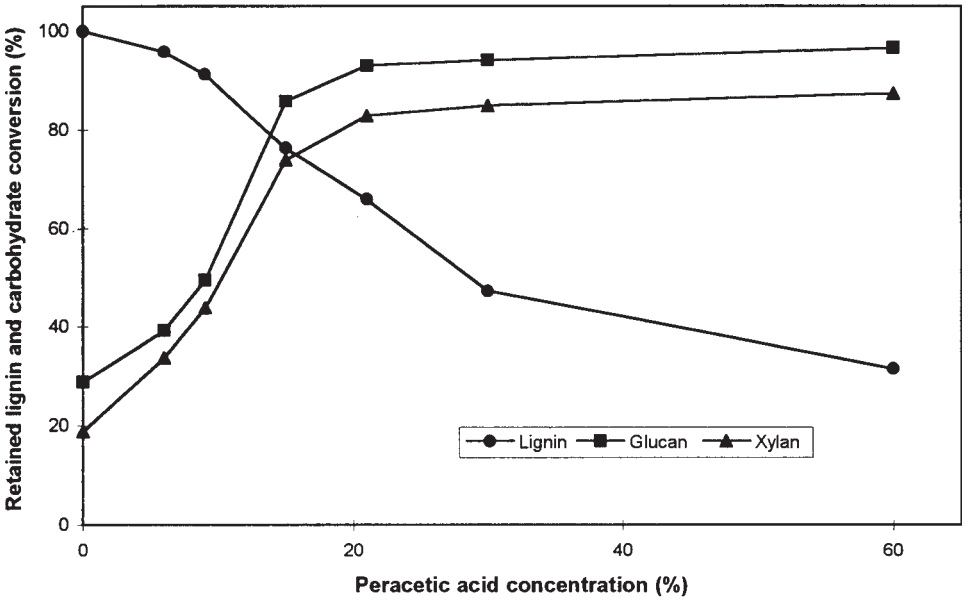

Fig. 2. Lignin retention and carbohydrate hydrolysis conversion as a function of peracetic acid concentration for sugar cane bagasse.

Table 2
Acetyl Content and Retention After Pretreatment of Hybrid Poplar[a]

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

[a]Values for the retained acetyl have been corrected to raw wood considering variations in the amount of xylan in the treated substrate.

Higher acid concentrations (15%) have less effect on enzymatic hydrolysis than lower concentrations, as verified by the slopes of the bioconversion curves (Figs. 3 and 4). The slope increases considerably, which indicates

Table 3
Acetyl Content and Retention After Pretreatment of Sugar Cane Bagasse[a]

Pretreatment condition	Acetyl 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

[a]Values for the retained acetyl have been corrected to raw biomass considering variations in the amount of xylan in the treated substrate.

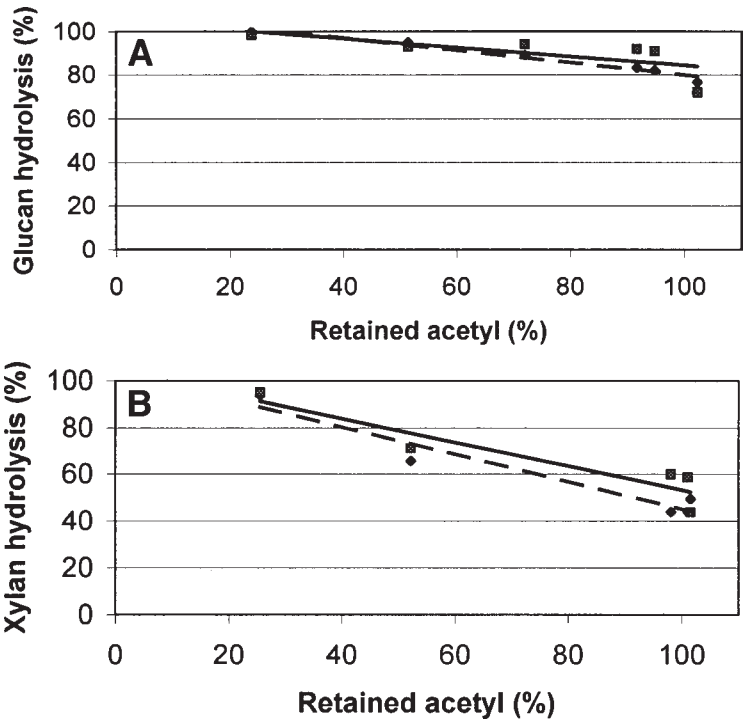

Fig. 3. Carbohydrate hydrolysis for **(A)** glucan and **(B)** xylan as a function of retained acetyl groups in pretreated hybrid poplar. ◆, 15% peracetic acid series; ■, 9% peracetic acid series.

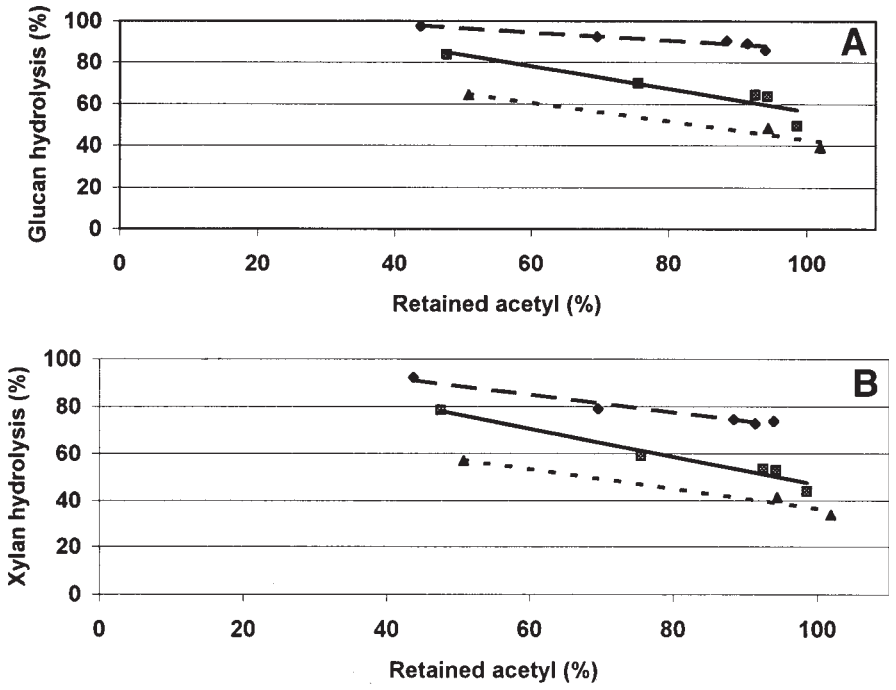

Fig. 4. Carbohydrate hydrolysis for **(A)** glucan and **(B)** xylan as a function of retained acetyl groups in pretreated sugar cane bagasse. ◆, 15% peracetic acid series; ■, 9% peracetic acid series; ▲, 6% peracetic acid series.

greater influence of the alkaline washing, when the relatively lower amounts of peracetic acid are used. Further improved sugar conversion with 6% peracetic acid would probably result if acetyl groups were further removed, e.g., by an efficient alkaline pre-pretreatment or by acetyl xylan esterase treatment prior to or simultaneous with SSCF (Fig. 4).

Simultaneous Saccharification and Cofermentation

Hybrid Poplar

Figure 5 shows the ethanol yields of hybrid poplar samples from different pretreatment conditions using the recombinant *Z. mobilis* CP4/pZB5 as described previously. The maximum average ethanol yield, corresponding to 21.3 g/L or 92.8% of theoretical yield, was reached in 10 d of fermentation in the sample pretreated in two steps with 6% NaOH/15% peracetic acid. A period of 7 d was sufficient for achieving a theoretical yield of 92.6%. This result is slightly lower than that found in the literature, 95.0% yield, for the same recombinant microorganism using a medium containing a mixture of glucose and xylose *(35)*. The slight difference in yield can be assumed to be owing 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 previously, *Z. mobilis* grows better at 30

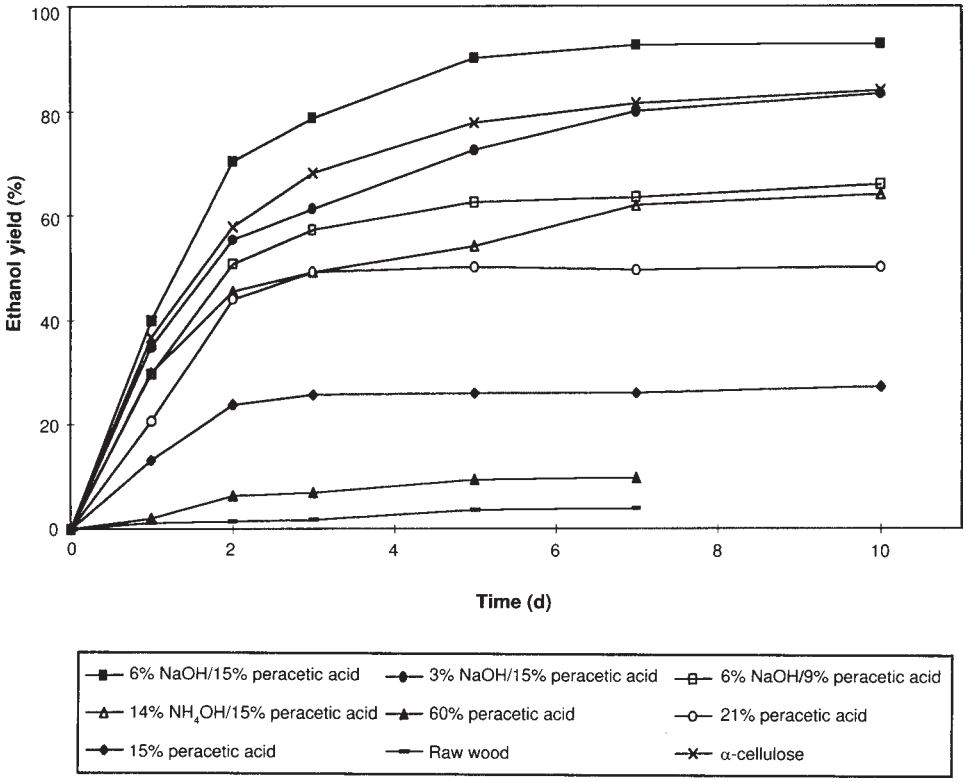

Fig. 5. Ethanol yields from SSCF from different pretreatment conditions for hybrid poplar, raw wood, and α-cellulose.

than at 37°C, the temperature used at which this species can also grow satisfactorily *(27,29,36)*. Significant decreases in both cell mass and ethanol formation have been verified for temperatures higher than 37°C in simultaneous saccharification and fermentation (SSF) tests using *Z. mobilis* ATCC 29501 *(37)*. According to Lima et al. *(38)*, the *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 (*see* Fig. 6A). The concentration of glucose and xylose were very low after 7 d, indicating a satisfactory sugar utilization during the course of the cofermentation (Fig. 6A). Saddler et al. *(36)* presented satisfactory ethanol yields of 85.4% from steam-exploded poplar with excellent glucose consumption in 6-d SSF 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 herein and a satisfactory xylose utilization (Figs. 5 and 6A) confirm the high temperature tolerance by *Z. mobilis*. Barbosa et al. *(39)* showed that yields varying from 85 to 91% from pine hemicellulose hydrolysates using a recombinant *Escherichia coli* KO11 are improved when using a commercial sugar mixture

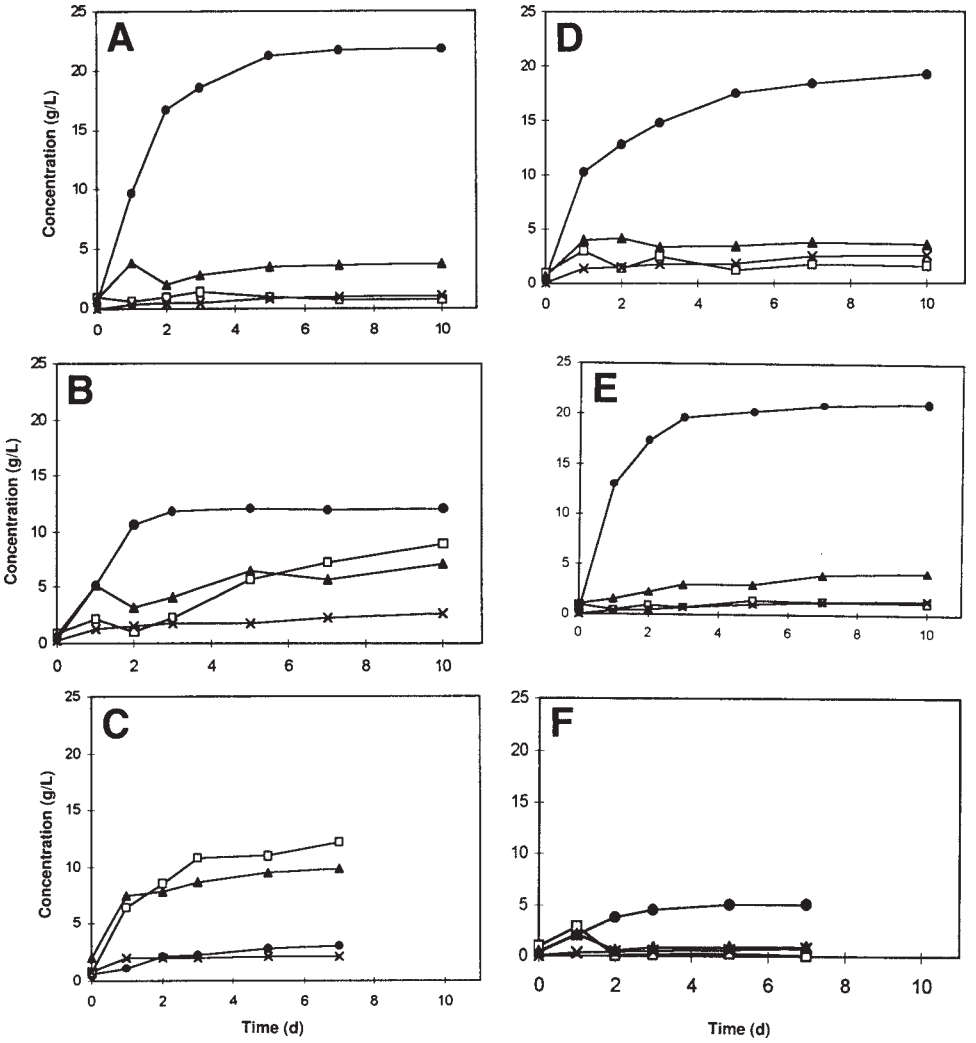

Fig. 6. SSCF kinetics for hybrid poplar wood–pretreated substrates: **(A)** 6% NaOH/15% peracetic acid; **(B)** 21% peracetic acid; **(C)** 60% peracetic acid; **(D)** 21% peracetic acid (alkaline postwashed); **(E)** 6% NaOH/15% peracetic acid (alkaline postwashed); **(F)** raw hybrid poplar. (—□—), Glucose; (—▲—), xylose; (—●—), ethanol; (—×—), acetate.

simulating hemicellulose hydrolysate. Toon et al. *(40)* 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 *(41)* reported a one-third decrease in growth yield of *Z. mobilis* ATCC 29191 when acetic acid, at a 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 to a low level of acetate during the cofermentation and conse-

quently lowered growth inhibition. Because acetate remained at concentrations below 1 g/L, the efficiency of ethanol conversion by *Z. mobilis* with this practical substrate is quite satisfactory.

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-dried wood does not improve biomass digestibility *(19)*, and, consequently, the substrate treated at that loading does not fit the SSCF tests (Fig. 5). Figure 6 presents the SSCF kinetics of variously pretreated hybrid poplar samples. When peracetic acid was used in concentrations of 21% or above, a good biomass digestibility was verified *(19)* but a satisfactory ethanol yield was not achieved (Figs. 5 and 6B). 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. 6B,C, the release of sugar was verified but unsatisfactory sugar uptake by the recombinant bacteria was 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-dried weight of wood, was performed before enzyme loading and inoculation. The biomass was mixed overnight and filtered to remove possible growth inhibitors. In two pretreatment samples, 21% peracetic acid and 6% NaOH/15% peracetic acid–pretreated hybrid poplar, better ethanol yields (80.9%) were observed for the alkaline postwashed substrate when compared with unwashed 21% peracetic acid–pretreated hybrid poplar (50.2%) (Fig. 6B,D). No improvement in the final ethanol concentration was observed for the alkaline postwashed 6% NaOH/15% peracetic acid pretreated sample. The yield decreased slightly from 92.8% of theoretical for the unwashed, to 90.4% for the alkaline postwashed sample, probably because of some carbohydrate losses during washing (Fig. 6A,E). Only a better initial ethanol formation rate and xylose uptake was noted for the alkaline postwashed 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. 5 and 6F). Based on the information presented, we speculate that additional acetyl groups are exposed and saponified by the post–peracetic acid alkaline treatment.

Ethanol yields from the ambient temperature pretreatments were greater than those from severe high-temperature pretreatments, such as used for the production of the chemical grade pulp, α-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 α-cellulose (Figs. 5 and 7F), from which xylan hydrolysis products are found by HPLC (data not presented). Crystallinity might be a factor as well. The crystallinity index (CrI) for wood pulps and chemical grade pulp varies from 60 to 70% *(42)*. Brito *(31)* reported crystallinity varying from 76 to 85% for hybrid poplar

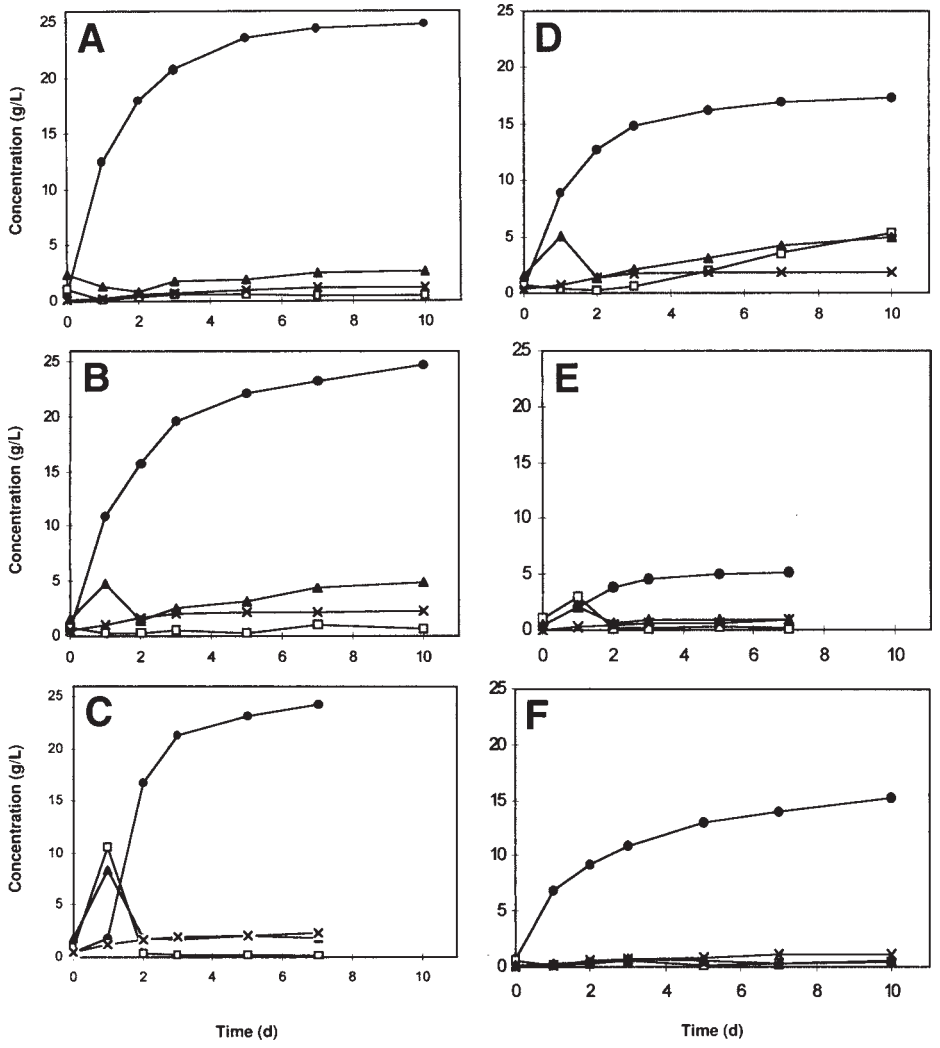

Fig. 7. SSCF kinetics for sugar cane bagasse–pretreated substrates and α-cellulose: **(A)** 6% NaOH/15% peracetic acid; **(B)** 21% peracetic acid; **(C)** 60% peracetic acid; **(D)** 15% peracetic acid; **(E)** raw bagasse; **(F)** α-cellulose. (—□—), Glucose; (—▲—), xylose; (—●—), ethanol; (—×—), acetate.

treated with phosphoric acid at high pressures and temperatures. The CrI 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 *(13)*.

Figure 7F shows fermentation kinetics for α-cellulose, a reference substrate.

Sugar Cane Bagasse

The SSCF results for alkaline/peracetic-pretreated sugar cane bagasse are quite similar to those found for hybrid poplar. However, for peracetic

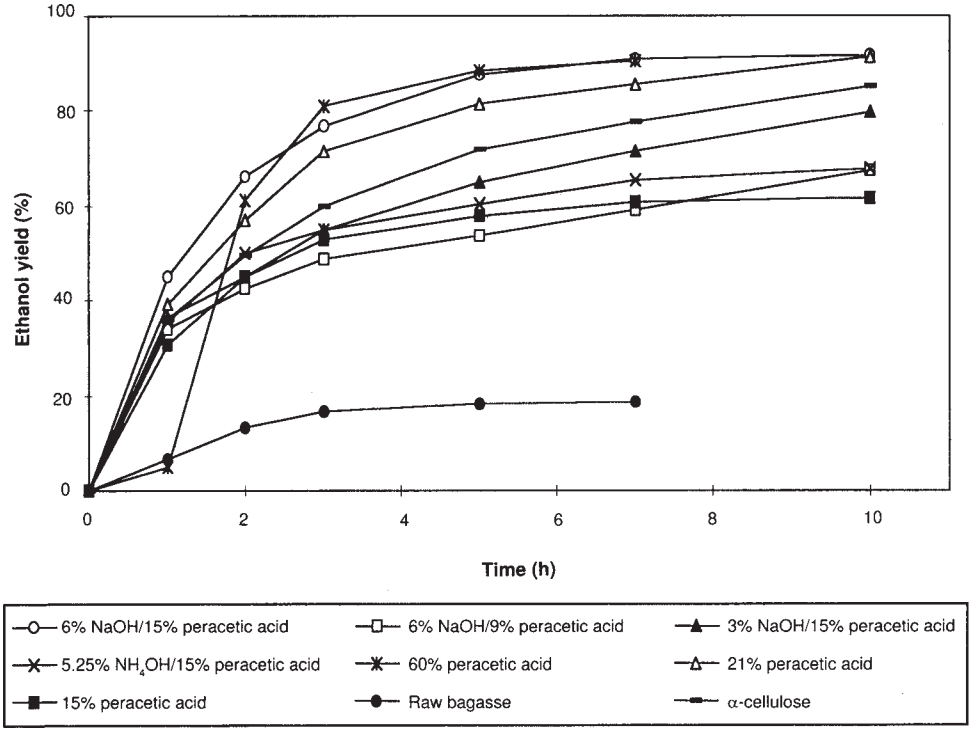

Fig. 8. Ethanol yield from SSCF from different pretreatment conditions for sugar cane bagasse, raw bagasse, and α-cellulose.

acid–pretreated sugar cane bagasse, the ethanol yields were much better than those of peracetic acid–pretreated hybrid poplar. Inhibition by the pretreated bagasse was less than that found for pretreated hybrid poplar, especially for pretreatments performed under higher acid concentrations (Fig. 8). The 6% NaOH/15% peracetic acid–, 21% peracetic acid–, and 60% peracetic acid–pretreated bagasse samples gave ethanol yields of 91.9, 91.4, and 90.7%, respectively. As with hybrid poplar, 6% NaOH/15% peracetic acid–pretreated bagasse showed the best overall performance considering the satisfactory ethanol yield during SSCF by *Z. mobilis* (Figs. 7A and 8). Fermentation kinetics for sugar cane bagasse pretreated with 21% peracetic acid were much better than observed for hybrid poplar owing to negligible inhibition effects (Fig. 7). Ethanol yield was very low during the first day for 60% peracetic acid–pretreated substrate, but, on the second day of fermentation, the rate of ethanol formation surpassed all comparative tests (Fig. 8). According to Fig. 7C, 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 of fermentation. This could suggest an adaptation was needed 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 (Figs. 6 and 7). Figure 7E presents fermentation kinetics for raw bagasse.

Conclusion

Pre-pretreatment using a diluted sodium hydroxide (about 0.5% w/w concentration) prior to peracetic acid pretreatment (9 and 15%) results in significantly more rapid glucan and xylan conversion to simple sugars than the 21, 30%, and 60% peracetic acid treatments without alkaline pre-pretreatments with similar extents of enzyme hydrolysis *(19)*. The results presented herein indicate that a reduction in acetyl groups in combination with slight delignification is responsible for satisfactory enzyme hydrolysis and SSCF ethanol yields. The technical viability of hybrid poplar wood and sugar cane bagasse pretreated with 6% NaOH/15% peracetic acid was verified through SSFC using recombinant *Z. mobilis* CP4/pZB5 and enzyme loading of 8.33 IFPU/g of cellulose, with a respective theoretical ethanol yield of 92.8 and 91.9%. Farid et al. *(10)* obtained a satisfactory and linear sugar release, almost 0.5 g of glucose/h, using a cellulase loading of 8 IFPU/g of sugar cane bagasse pretreated with peracetic acid. Complete enzymatic hydrolysis conversion of steam-pretreated aspen posttreated with hydrogen peroxide was achieved in 3 d using a loading of 10 IFPU/g of cellulose. Using a higher loading of 16 IFPU/g of cellulose, the same yield was obtained with the only difference being a time reduction, from 3 to 2 d *(43)*. Using less enzyme was also more economical based on information that production of enzymes is the most expensive step in ethanol production from lignocellulosic materials *(44)*.

In spite of satisfactory simple sugar yields using the standard enzymatic hydrolysis tests, pretreatments using a concentration of 21% or higher of peracetic acid concentrations with hybrid poplar caused growth inhibition in *Z. mobilis* during SSCF tests. Oxidized lignin by-products may possibly cause inhibition. No inhibition was observed for sugar cane bagasse pretreated with the same acid concentrations.

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