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Effect of Pretreatment on Simultaneous Saccharification and Fermentation of Hardwood into Acetone/Butanol

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ABSTRACT

The effectiveness of pretreatments on hardwood substrate was investigated in connection with its subsequent conversion by simultaneous saccharification and fermentation (SSF), using *Clostridium acetobutylicum*. The main objectives of the pretreatment were to achieve efficient separation of lignin from carbohydrates, and to obtain maximum sugar yield on enzymatic hydrolysis of pretreated wood. Two methods have given promising results: (1) supercritical CO_2—SO_2 treatment, and (2) monoethanolamine (MEA) treatment. The MEA pretreatment removed above 90% of hardwood lignin while retaining 83% of carbohydrates. With CO_2—SO_2 pretreatment, the degree of lignin separation was lower.

Under the scheme of SSF, the pretreated hardwood was converted to acetone, butanol, and ethanol (ABE) via single stage processing by cellulase enzyme system and *C. acetobutylicum* cells. The product yield in the process was such that 15 g of ABE/100 g of dry aspen wood was produced. In the overall process of SSF, the enzymatic hydrolysis was found to be the rate-limiting step. The ability of *C. acetobutylicum* to metabolize various 6-carbon and 5-carbon sugars resulted in efficient utilization of all available sugars from hardwood.

Index Entries: Pretreatment; simultaneous saccharification and fermentation; monoethanolamine; supercritical CO_2—SO_2; *C. acetobutylicum*.

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INTRODUCTION

Lignocellulosic raw material is a renewable feedstock that can be converted to fuels and chemicals. Current technology for utilization of these materials is not economically feasible. From an economic standpoint, it is important to utilize all the main components of lignocellulosics to the fullest extent. Overall bioconversion of lignocellulosics involves pretreatment, saccharification, and fermentation. Various chemical pretreatment methods have been used to increase susceptibility of cellulosics to enzymatic attack (*1–3*). On saccharification of pretreated wood by cellulase enzyme, a mixture of sugar is usually obtained. For better efficiency, it would be desirable to choose a microorganism, such as *Clostridium acetobutylicum,* that can metabolize various sugars derived from wood (*4,5*). Employing such microbe would simplify the fermentation process.

For conversion of cellulosics to chemicals, the simultaneous saccharification and fermentation (SSF) process has emerged as one of the most efficient bioconversion scheme. Takagi et al. introduced the concept of SSF that converts cellulose into chemicals in one step (*6*). SSF offers two main advantages: elimination of separate hydrolysis step and reduction in end production inhibition by sugars in saccharification. Efficiency of SSF can be greatly affected by the nature of pretreatment, since it can result in formation of toxic compounds that might be inhibitory to enzyme and microorganism.

Saddler et al. reported that unextracted steam exploded aspen contained extraneous components that were quite inhibitory for the cellulase enzyme and *K. pneumoniae* (*7*). Extraction of these inhibitory components by water resulted in separation of 75% of pentosans from cellulose rich fraction, and this pentosan stream was found to be unsuitable for further bioconversion. Mackie et al. noted that *K. pneumoniae* could not grow on water soluble fraction (containing pentosans) of SO_2-steam exploded aspen because of the presence of inhibitory substances (*8*). When organoslov pretreatment was used, delignification was accompanied by similar loss of hemicellulose (*9*).

It is well known that monoethanolamine (MEA) is an extremely efficient delignifying agent. MEA has been routinely used to estimate cellulose content of pulp (*10*). Wise et al. used pure monoethanolamine to remove 98.2% of lignin from large tooth aspen (*11*). Supercritical fluids have been used in pretreating lignocellulosics to convert them into suitable fermentation substrates. Chou employed supercritical ammonia to pretreat hardwood (*12*). Tillman and Lee used supercritical CO_2—SO_2 mixture to delignify softwood (*13*).

In seeking a pretreatment method that will remove lignin and the extraneous components while leaving all the carbohydrates (both hexosans and pentosans) intact in the substrates, we have investigated MEA pretreatment and supercritical CO_2 SO_2 pretreatment. In so doing, we have

compared these two methods for efficiency in separating carbohydrates from lignin, and converting carbohydrates into acetone and butanol under SSF scheme, using *C. acetobutylicum*.

MATERIALS AND METHODS

Microorganism and Medium

Clostridium acetobutylicum, ATCC 824, was used throughout this study. It was stored in sporulated form at 4°C on the medium containing 5%(w/v) corn mash and 0.5%(w/v) glucose. The media contained the following components in 1 L of distilled water: KH_2PO_4 0.75 g, K_2HPO_4 0.75 g, $MgSO_4 \cdot H_2O$ 0.4 g, $MnSO_4 \cdot H_2O$ 0.01 g, $FeSO_4 \cdot 7H_2O$ 0.01 g, cysteine 0.5 g, yeast extract 5 g, asparagine·H_2O 2 g, $(NH_4)_2SO_4$ 2 g.

Substrate and Enzyme

Aspen chips were obtained from the Solar Energy Research Institute, Golden, Colorado. Chips with nominal size of 0.066–0.25 in were used. Cellulase enzyme (Genencor, 85 IFPU/mL) was also provided by SERI.

Monoethanolamine(MEA) Pretreatment

Aspen chips were soaked in aqueous solution of MEA for 24 h. The concentration of MEA ranged from 5 to 50%(v/v). MEA solution was filtered, and wet chips (liquid/solid ratio of 2.5) were charged into an autoclave (Parr Instruments). Pretreatment reaction was carried out at 186°C for 3 h under 200 psig nitrogen. Chips removed from the reactor were soaked in 2% NaOH solution for 24 h to extract lignin. Several water washes were given until filtrate was colorless.

Supercritical CO_2—SO_2 Pretreatment

Aspen chips were presoaked in 2% NaOH solution for 24 h. Solution was filtered, and chips were charged to Parr autoclave. Reactor was charged with CO_2/SO_2 (with molar composition of 98%/2%) mixture. Temperature was varied from 120 to 150°C, and pressure was kept at or near 2000 psig. Reaction time was varied from 2 to 4 h. Treated chips were washed with water until filtrate became colorless.

Enzymatic Hydrolysis

Pretreated chips were added to serum bottle containing 35 mL, 0.01*M* citrate buffer (4.5 pH) to the level of 1.5 to 2%(w/v). Bottles were sealed and autoclaved at 121°C for 15 min. Cellulase (2 mL) was added, and hydrolysis was carried out in a constant temperature shaker bath (120 rpm, 50°C).

Simultaneous Saccharification and Fermentation (SSF)

Cellulosic substrate was added to serum bottle containing growth medium to the level of 5%(w/v). Bottles were sparged with nitrogen, sealed, and autoclaved at 121°C for 15 min. Medium was inoculated with *C. acetobutylicum* culture (10%v/v), and cellulase (62 IFPU/g substrate) was added. The SSF was then carried out in a constant temperature shaker bath (120 rpm, 37°C).

Analytical Methods

Sugars were analyzed by HPLC, using a column packed with Bio-Rad, Aminex Q15S resin. Fermentation products were analyzed by Gas Chromatograph (Varian Model 3700) equipped with chromosorb 101 column. Kappa number of pretreated chips was estimated by the TAPPI standard method T236 os-76. Lignin content was calculated by multiplying Kappa number by 0.15.

RESULTS AND DISCUSSION

Effect of Pretreatment on Fractionation of Hardwood

Aspen composition reported by Chum et al. was used in calculation of yields of carbohydrates and lignin (*1*). Yield of hardwood after pretreatment varied between 60 and 68%. Lignin removal varied from 50 to 91%. Table 1 summarizes the carbohydrate yields and lignin removal data for the two different pretreatment methods investigated. The effect of MEA concentration on cellulose retention was rather insignificant. There was only a slight enhancement in hemicellulose retention, with increase in MEA concentration. Most noticeable, however, was the increase in lignin removal, with increase in MEA concentration. At 50% MEA concentration, 91.2% of original lignin was removed and 83% yield of carbohydrates (81% hexosan yield, 91% pentosan yield) was achieved, confirming the high selectivity of MEA toward delignification in pretreatment process.

Lignin removal and carbohydrate yields after supercritical CO_2—SO_2 pretreatment are shown in Figs. 1 and 2, respectively. The degree of delignification increased with reaction temperature, as well as reaction time. The loss of carbohydrates (mostly hemicellulose) to wash water was significant, especially at temperatures above 130°C: The highest carbohydrate yield (83%) was obtained at 130°C, 4 h. Highest degree of lignin removal (84% of original) was achieved at 140°C and 4 h, at which point, the carbohydrate yield was 78.3%. In treatment at 130°C, 4 h, the

Table 1
Carbohydrate Yield and Lignin Removal For Pretreated Hardwood

Pretreatment	Solids Yield %[a]	Lignin Removed %[b]	Hexosan Yield %[a]	Pentosan Yield %[a]	Total Carbohydrates %[c]
MEA (%v/v)					
5	66.8	49.8	43.2	14.1	82.3
10	64.9	61.5	44.0	15.4	85.3
20	63.8	76.5	42.3	15.6	83.2
30	63.2	79.6	42.8	15.5	83.8
40	62.7	87.9	43.1	16.2	85.2
50	61.4	91.2	41.1	17.1	83.6
Supercritical SO_2–CO_2					
130°C, 4 hr	66.0	60.8	43.5	14.3	83.0
140°C, 4 hr	58.0	84.0	42.9	11.6	78.3

a based on original Aspen total dry weight.
b based on initial lignin content in Aspen.
c based on initial total carbohydrate content in Aspen.

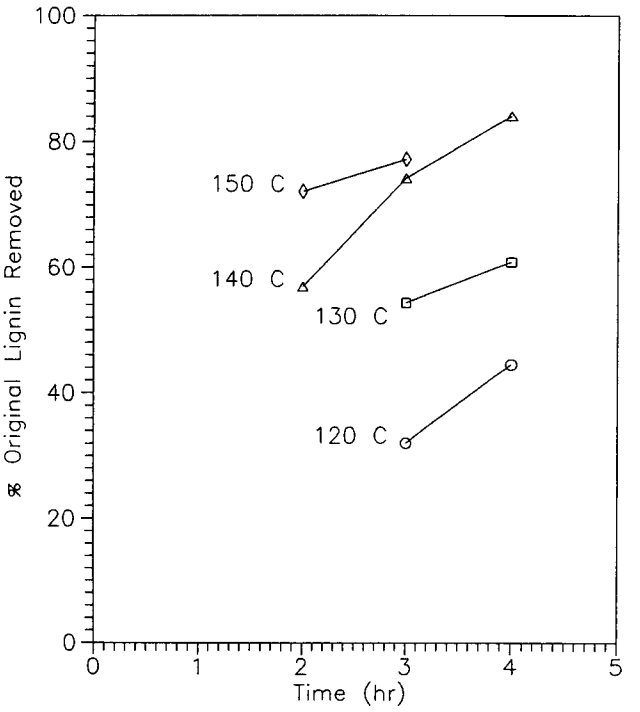

Fig. 1. Effect of supercritical CO_2—SO_2 pretreatment on lignin removal at various temperatures and reaction times.

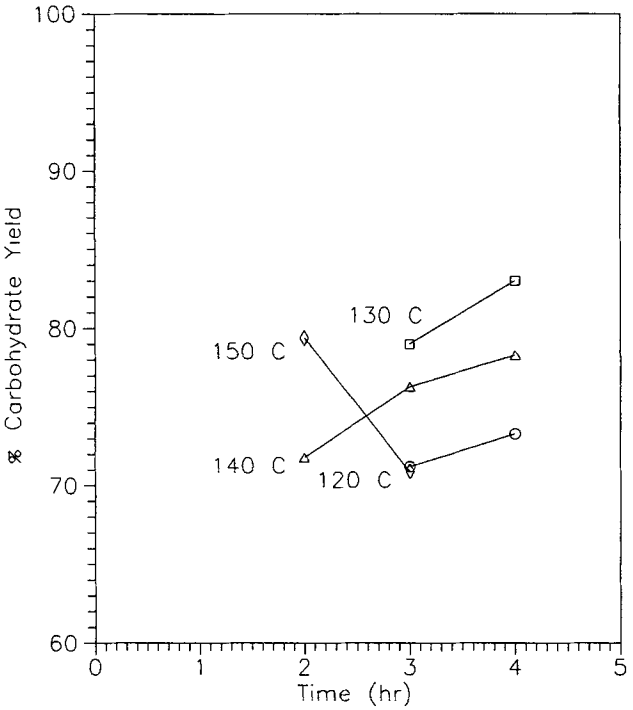

Fig. 2. Carbohydrate yields in pretreatments by supercritical CO_2—SO_2 at various temperatures and reaction times.

lignin removal was only 60.8%. These results collectively indicate that sulfur dioxide, in combination with carbon dioxide under supercritical condition, can be effectively utilized in separating lignin from carbohydrates.

In terms of the ability of fractionating hardwood components (carbohydrates and lignin), however, MEA method gave results superior to CO_2—SO_2 method. At the present time, the technology of MEA recovery is yet to be developed. Although the recovery of MEA after pretreatment is an important factor from an economic standpoint, it was beyond the scope of this investigation. If further improvement is to be made in CO_2—SO_2 method, it would be in the area of lignin removal.

Enzymatic Hydrolysis of Pretreated Aspen

Results of enzymatic hydrolysis after MEA pretreatment at various conditions are shown in Fig. 3. Glucose (as hexosan) and xylose (as pentosan) are expressed as wt% of original aspen. Initial rates of hydrolysis increased with the level MEA concentration applied during pretreatment. The terminal glucose yield was essentially the same for all levels of MEA, whereas terminal xylose yield increased slightly with MEA concentration. It seems the residual lignin in pretreated aspen affects the accessibility of the enzyme to hemicellulose. Fig. 4 shows enzymatic hydrolysis results for the supercritical CO_2—SO_2 pretreated substrates. The carbohydrate yields and lignin removal data for these experiments are listed in Table 1.

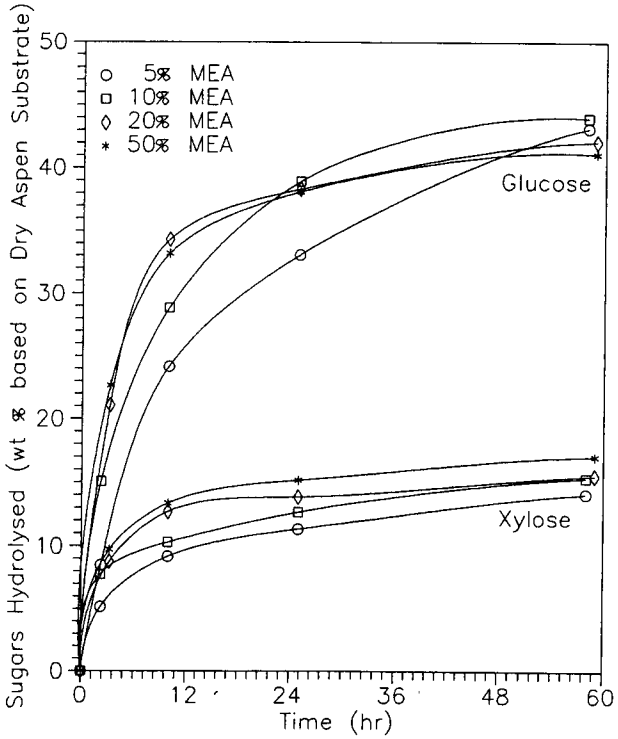

Fig. 3. Enzymatic hydrolysis of MEA pretreated aspen.

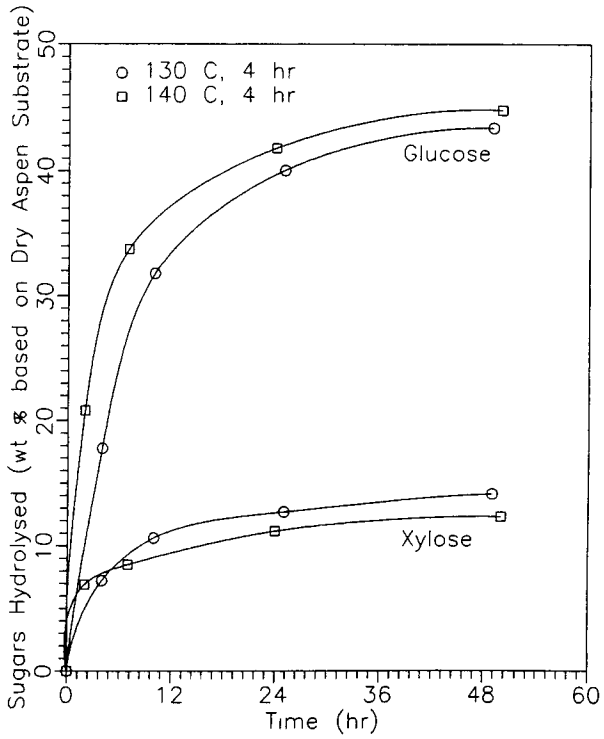

Fig. 4. Enzymatic hydrolysis of supercritical CO_2—SO_2 pretreated aspen.

Aspen treated at 140°C, 4 h showed higher initial rates than those for aspen treated at 130°C, 4 h. The final glucose yield was essentially the same for these two runs, whereas the final xylose yield was higher at 130°C than at 140°C. Obviously, the high pretreatment temperature enhances both delignification and loss of hemicellulose. Because of this, the delignification selectivity in CO_2—SO_2 pretreatment was not as high as that observed in MEA pretreatment.

SSF of Pretreated Aspen

Shown in Fig. 5 are the time course data for SSF, using MEA pretreated chips. Similar data for supercritical CO_2—SO_2 pretreatment are given in Fig. 6. Except for the initial phase in which glucose accumulation is noticeable, the SSF proceeded mostly under glucose limited condition. When sufficient amount of glucose (>15 g/L) is present, *C. acetobutylicum* does not utilize any xylose that may be present in the medium (*14*). Under glucose limited condition, however, this bacterium seems to synthesize necessary enzymes for xylose metabolism. As an evidence, it was observed in the SSF runs that both glucose and xylose were utilized simultaneously, not following a diauxic pattern. Such substrate consumption pattern is a unique feature associated with SSF scheme. It was also found that hydrolysis of glucose is the rate-limiting step in the SSF process. The fact that SSF proceeds under glucose limitation provides an additional advantage that the inhibition on cellulase enzyme is eliminated. An inherent problem also exists with SSF that the optimum temperatures of cellulase and microorganism do not usually match, which may result in reduction of enzymatic activity.

Table 2 shows the solvent production from aspen wood under various pretreatment conditions. The amount of solvent produced represents the sum of acetone, butanol, and ethanol produced. The effect of pretreatment condition was noticeable only in the initial phase of solvent production. The final solvent yields were practially the same for all runs. Amount of solvent produced varied from 22.6 to 24 g/100 g of pretreated aspen. Multiplying these values by the pulp yields of respective treatments, the amount of solvent produced were calculated to be in the range of 13.1 to 15.6 g/100 g original dry wood. The residual lignin in the pretreated substrate does not appear to inhibit the growth of the microorganism.

CONCLUSIONS

A SSF process designed to produce acetone/butanol from hardwood was investigated in relation to two different pretreatment methods. In these pretreatments, efficient fractionation of lignin from carbohydrates was achieved. Both the MEA and the supercritical CO_2—SO_2 pretreatments rendered 83% carbohydrate yield in enzymatic hydrolysis. Rates of hydrolysis increased with increase in degree of delignification. The MEA

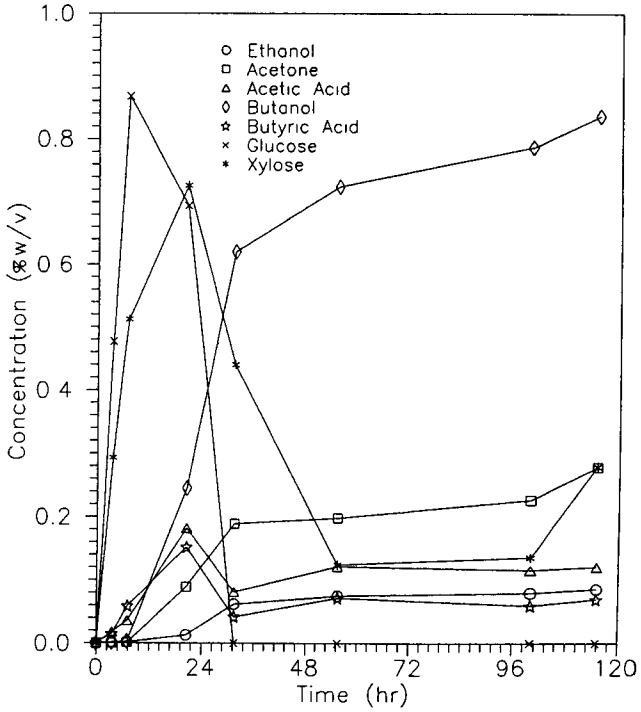

Fig. 5. Time progression of SSF, aspen wood pretreated by 50% MEA.

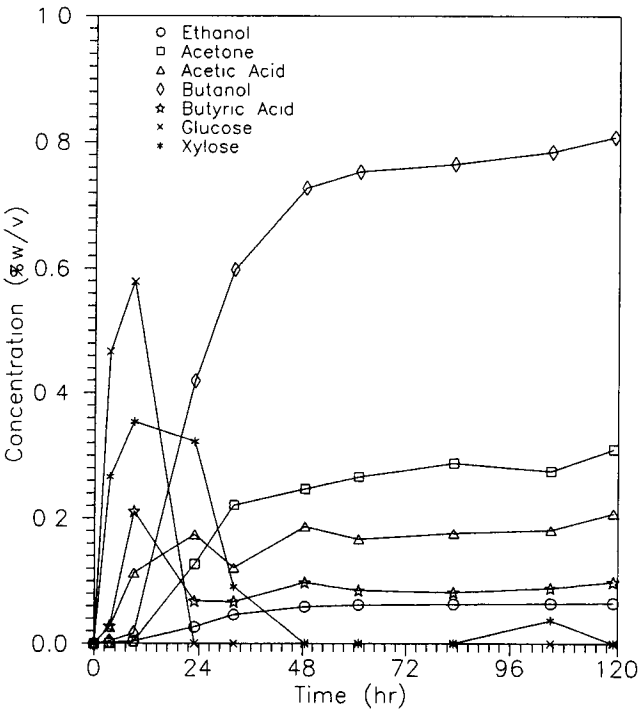

Fig. 6. Time progression of SSF, aspen wood pretreated by supercritical CO_2—SO_2 mixture at 130°C, 4 h.

Table 2
Solvent Production By *C. acetobutylicum* Grown on Pretreated Hardwood under SSF Process

Pretreatment	Solvents(acetone+butanol+ethanol)	
	g/100g pretreated aspen	g/100g dry aspen wood
MEA (%v/v)		
5	22.6	15.1
10	23.1	15.0
20	23.2	14.8
30	23.7	15.0
40	23.4	14.7
50	24.0	14.7
Supercritical SO_2–CO_2		
130°C, 4 hr	23.7	15.6
140°C, 4 hr	22.7	13.1

pretreatment facilitated high retention of carbohydrates, especially in pentosans (91%). Xylose recovery on enzymatic hydrolysis increased with increase in MEA level applied in pretreatment. The ability of *C. acetobutylicum* to utilize various sugars resulted in one step conversion of cellulosics into acetone and butanol. The pretreated substrates were converted into ABE under the scheme of SSF producing as high as 15.6 g of total solvent/100 g dry Aspen wood. Both glucose and xylose could be utilized simultaneously in the SSF mode. In the current SSF scheme, however, utilization of xylose fraction was slow, and in some cases, incomplete.

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