

Ethanol Production from Alkaline Peroxide Pretreated Enzymatically Saccharified Wheat Straw[†]

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Wheat straw used in this study contained $44.24 \pm 0.28\%$ cellulose and $25.23 \pm 0.11\%$ hemicellulose. Alkaline H_2O_2 pretreatment and enzymatic saccharification were evaluated for conversion of wheat straw cellulose and hemicellulose to fermentable sugars. The maximum yield of monomeric sugars from wheat straw (8.6%, w/v) by alkaline peroxide pretreatment (2.15% H_2O_2 , v/v; pH 11.5; 35 °C; 24 h) and enzymatic saccharification (45 °C, pH 5.0, 120 h) by three commercial enzyme preparations (cellulase, β -glucosidase, and xylanase) using 0.16 mL of each enzyme preparation per g of straw was 672 ± 4 mg/g (96.7% yield). During the pretreatment, no measurable quantities of furfural and hydroxymethyl furfural were produced. The concentration of ethanol (per L) from alkaline peroxide pretreated enzyme saccharified wheat straw (66.0 g) hydrolyzate by recombinant *Escherichia coli* strain FBR5 at pH 6.5 and 37 °C in 48 h was 18.9 ± 0.9 g with a yield of 0.46 g per g of available sugars (0.29 g/g straw). The ethanol concentration (per L) was 15.1 ± 0.1 g with a yield of 0.23 g/g of straw in the case of simultaneous saccharification and fermentation by the *E. coli* strain at pH 6.0 and 37 °C in 48 h.

Introduction

Ethanol is a renewable oxygenated fuel. In the U.S., the production of fuel ethanol from corn starch reached about 3.4 billion gallons in 2004. Developing ethanol as fuel, beyond its current role as fuel oxygenate, will require developing lignocellulosic biomass as a feedstock because of its abundance and low cost. Previously, we targeted corn fiber (obtained from corn wet-milling industries) as a model substrate for use as lignocellulosic biomass because of its high carbohydrate content (70%) containing 20% residual starch, 15% cellulose, and 35% hemicellulose, and low lignin content (<8%) (1). Corn fiber can be enzymatically saccharified to fermentable sugars with a yield of 85–100% after pretreatment with dilute acid at a moderate temperature (2). The hydrolyzates obtained from corn fiber by dilute acid pretreatment contain a mixture of sugars such as glucose, xylose, arabinose, and galactose. These sugars were successfully fermented to ethanol by mixed sugar utilizing ethanogenic recombinant bacteria such as *Escherichia coli*, *Klebsiella oxytoca*, and *Zymomonas mobilis* and yeast such as *Saccharomyces cerevisiae* (3).

In many countries, including the U.S., wheat straw is an abundant byproduct from wheat production. The average yield of wheat straw is 1.3–1.4 lb per lb of wheat grain (4). Based on the data from FAO, 627.1 million metric tons of wheat was produced in the world in 2004 (U.S. production, 58.7 million metric tons) (5). Wheat straw contains 35–45% cellulose, 20–30% hemicellulose, and 8–15% lignin and can also serve as a low-cost attractive feedstock for production of fuel alcohol. Research has been done on the separation of cellulose, hemicellulose, and lignin components from wheat straw and structural characterization of the hemicellulose fraction (6–11). Also, a few reports are available on the production of ethanol from wheat straw hydrolyzates (12–16). To our knowledge, no research paper is available on ethanol production from alkaline peroxide pretreated enzyme saccharified wheat straw hydrolyzates by the mixed sugar utilizing ethanogenic recombinant microorganisms. In the present study, conditions for obtaining a high sugar yield from wheat straw using alkaline peroxide as pretreatment option and enzymatic saccharification were examined.

The utilization of both cellulose and hemicellulosic sugars present in typical lignocellulosic biomass hydrolyzate is essential for the economical production of ethanol (17). The conventional ethanol fermenting yeast (*S. cerevisiae*) or bacterium (*Z. mobilis*) cannot ferment multiple sugar substrates to ethanol (3). *E. coli* metabolizes a wide variety of sugars. Our research unit has developed a recombinant *E. coli* (strain FBR5) that can ferment mixed multiple sugars to ethanol (18). The strain carries the plasmid pL1297, which contains the genes from *Z. mobilis* necessary for efficiently converting pyruvate into ethanol. It selectively maintains the plasmid when grown anaerobically. In the present paper, the results of alcohol production by this recombinant bacterium from alkaline peroxide pretreated and enzyme saccharified wheat straw hydrolyzates containing glucose, xylose, and arabinose are presented. Simultaneous saccharification and fermentation (SSF) of alkaline peroxide pretreated wheat straw has also been presented.

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Materials and Methods

Materials. Wheat straw was purchased from a local farmer. It was dried in a forced-air oven at 55 °C for 24 h and milled in a hammer mill to pass through a 1.27 mm screen. The milled wheat straw was stored at room temperature. Celluclast 1.5 L,

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Novozyme 188, glucose, xylose, arabinose, Tween 20, furfural, and hydroxymethyl furfural (HMF) were purchased from Sigma Chemical Co., St. Louis, MO. Viscostar 150 L was supplied by Dyadic Corp., Jupiter, FL. Aminex HPX 87P column (300 × 7.8 mm), Aminex HPX 87H column (300 × 7.8 mm), De-ashing cartridge (30 × 4.6 mm), Carbo-P micro-guard cartridge (30 × 4.6 mm), and Cation H micro-guard cartridge (30 × 4.6 mm) were purchased from Bio-Rad Laboratories, Inc., Hercules, CA. All other chemicals used were of standard analytical grades.

Alkaline Peroxide Pretreatment. Milled wheat straw was slurried in water (8.6%, w/v, unless otherwise stated) containing H₂O₂ (0–4.3%, v/v) and adjusted to pH 11.5 using NaOH and shaken in an incubator at 250 rpm at 25 or 35 °C for 3–24 h. The pretreated wheat straw was adjusted to pH 5.0 using concentrated HCl before enzymatic saccharification.

Enzyme Assays. Carboxymethyl cellulase (CMCase) and xylanase activities were assayed in a reaction mixture (0.5 mL) containing 1% (w/v) carboxymethyl cellulose and 1% (w/v) oat spelt xylan, respectively, 50 mM acetate buffer, pH 5.0, and appropriately diluted enzyme solutions. After 30 min incubation at 50 °C, the reducing sugar liberated in the reaction mixture was measured by the dinitrosalicylic acid (DNS) method (19). One unit (U) of each enzyme activity is defined as the amount of enzyme that produces 1 μmol of reducing sugar as glucose (xylose in the case of xylanase) in the reaction mixture per minute under the above specified conditions.

β-Glucosidase, β-xylosidase, and α-L-arabinofuranosidase activities were assayed in the reaction mixture (1 mL) containing 4 mM *p*-nitrophenyl β-D-glucoside, 2 mM *p*-nitrophenyl β-D-xyloside, or 1 mM *p*-nitrophenyl-α-L-arabinofuranoside, respectively, 50 mM acetate buffer, pH 5.0, and appropriately diluted enzyme solutions. After incubation at 50 °C for 30 min, the reaction was stopped by adding 1 mL of ice-cold 0.5 M Na₂CO₃, and the color that developed as a result of *p*-nitrophenol liberation was measured at 405 nm. One unit (U) of each enzyme activity is defined as the amount of enzyme that releases 1 μmol of *p*-nitrophenol per min in the reaction mixture under these assay conditions.

Enzymatic Saccharification. The enzymatic saccharification of the alkaline peroxide pretreated wheat straw was performed by shaking gently (100 rpm) at 45 °C after adjusting the pH to 5.0 with HCl and adding enzymes at each enzyme dose of 4 mL/100 g of wheat straw, unless otherwise stated for 72–120 h. Samples (1 mL) were withdrawn and kept at –20 °C before analysis.

Bacterial Strain and Preparation of Inoculum. Recombinant *E. coli* strain FBR5 was provided by Bruce S. Dien and was maintained in glycerol vials at –20 °C for use as a working stock. It was plated on Luria broth (10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl) containing 4.0 g of xylose and 20 mg of tetracycline solidified with 15 g of agar per L. Plates were incubated at 37 °C. Cells from a single well-isolated colony were inoculated into a 125 mL flask containing 100 mL of Luria broth with 20 g of xylose/L. Cultures were incubated for 24 h at 37 °C and 100 rpm and used as seed culture for fermentation experiments.

Fermentation Experiments. Batch culture experiments were carried out in pH-controlled 500 mL fleakers with a working volume of 350 mL under semianaerobic conditions essentially as described previously (20, 21). Wheat straw hydrolyzate was used as substrate. The medium was prepared by dissolving 10 g of tryptone and 5 g of yeast extract in the hydrolyzate (per L) and autoclaving at 121 °C for 15 min. A 4 M KOH solution was used for pH control. Samples were withdrawn periodically

Table 1. Commercial Enzymes Used in Alkaline Peroxide Pretreated Wheat Straw Saccharification

enzyme	activity (U/mL) ^a		
	Celluclast	Novozyme 188	Viscostar 150 L
CMCase ^b	1513	39	986
β-glucosidase	74	330	3
xylanase	905	605	32 956
β-xylosidase	15	8	68
α-L-arabino-furanosidase	8	29	58

^a At pH 5.0 and 50 °C. ^b Carboxymethyl cellulase.

to determine cell density, ethanol content, and residual sugars and stored at –20 °C prior to analysis. Base consumption and pH were also recorded. For SHF experiments, the fermentation was performed at pH 6.5, 37 °C, and 130 rpm using the liquid portion of the hydrolyzate after separating it from the solids. For SSF experiments, 2 L fermenters (Biostat B, B. Braun Biotech International, Allentown, PA) with working volumes of 1.5 L were used at pH 6.0 and 37 °C at the agitation rate of 150 rpm. The alkaline peroxide pretreated whole wheat straw hydrolyzate was added to the fermenter as substrate after adjusting the pH to 6.0 with concentrated HCl and saccharified with enzymes for 1 h before adding inoculum. Inoculum size was 5% (v/v) in both cases.

Analytical Procedures. Sugars, furfural, HMF, acetic acid, ethanol, and succinic acid were analyzed by high-pressure liquid chromatography (HPLC) (2). The separation system consisted of a solvent delivery system (P2000 pump, Spectra-Physics, San Jose, CA) equipped with an autosampler (717, Waters Chromatography Division, Millipore Corp., Milford, MA), a refractive index detector (410 differential refractometer, Waters), a dual λ absorbance detector (2487, Waters), and a computer software based integration system (Chromquest 4.0, Spectra-Physics). Two ion moderated partition chromatography columns (Aminex HPX-87P with De-ashing and Carbo-P micro-guard cartridges, Aminex HPX 87H with Cation H micro-guard cartridge) were used. The Aminex HPX-87P column was maintained at 85 °C, and the sugars were eluted with Milli-Q filtered water at a flow rate of 0.6 mL/min. The Aminex HPX-87H column was maintained at 65 °C, and the sugars, organic acids, furfural, HMF, and ethanol were eluted with 10 mM HNO₃ prepared using Milli-Q filtered water at a flow rate of 0.6 mL/min. Peaks were detected by refractive index or UV absorption (277 nm) and were identified and quantified by comparison to retention times of authentic standards (glucose, xylose, galactose, arabinose, furfural, HMF, acetic acid, succinic acid, and ethanol). Cell growth of the bacterium was monitored by measuring the optical density of the appropriately diluted culture broth at 660 nm in the case of SHF experiments.

Results and Discussion

Effect of Alkaline Peroxide Level on Pretreatment and Enzymatic Saccharification. Wheat straw used in this study contained 44.24 ± 0.28% cellulose and 25.23 ± 0.11% hemicellulose, which make up the total carbohydrate content of 69.47 ± 0.39% on an as-is basis (moisture, 8.92 ± 0.08%). The detailed composition of wheat straw has been reported in a previous paper (22). Three commercial enzyme preparations were used in this study: Celluclast (cellulase preparation), Novozyme 188 (β-glucosidase preparation), and Viscostar 150 L (xylanase preparation). The activity levels of cellulase (carboxymethyl cellulase), xylanase, β-glucosidase, β-xylosidase, and α-L-arabinofuranosidase in each of these enzyme preparations are presented in Table 1. Initially, the effects of alkaline H₂O₂ level (0–4.3%, v/v) on the pretreatment of wheat

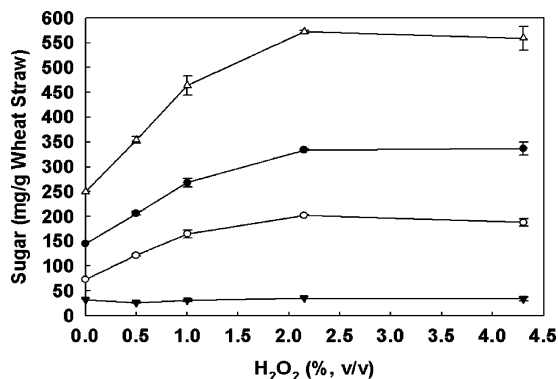


Figure 1. Effect of H₂O₂ level (0–4.3%, v/v) for the pretreatment (pH 11.5, 35 °C, 24 h) of wheat straw (8.6%, w/v) on its enzymatic saccharification (45 °C, pH 5.0, 120 h). The data presented are averages of two separate experiments. Symbols used: ●, glucose; ○, xylose; ▼, arabinose; and ▽, total sugars.

straw (8.6%, w/v) at pH 11.5 and 35 °C for 24 h were evaluated. The resultant glucose, xylose, arabinose, and total sugars yield in terms of mg per g of wheat straw after enzymatic saccharification using a cocktail of three commercial enzyme preparations (cellulase, β -glucosidase, and xylanase) at 45 °C, pH 5.0, for 120 h is shown in Figure 1. It was found that the sugar yield increased with increasing H₂O₂ up to 2.15% (v/v). There is not much difference between 2.15 and 4.3% (v/v) H₂O₂ pretreatment on each individual sugar as well as total sugar yields. Thus, it was decided to use 2.15% (v/v) H₂O₂ concentration for subsequent pretreatment studies. No galactose was detected in any of the hydrolyzates even though acid pretreatment released galactose (16 mg per g) from wheat straw (22). The detectable limit of the sugars by HPLC analysis was 25 μ g/mL. No furfural and HMF were detected in any of the peroxide pretreated wheat straw hydrolyzates. The detectable limit of both furfural and HMF by HPLC analysis was 1 μ g/mL. The enzymatic saccharification (45 °C, pH 5.0, 72 h) of pretreated wheat straw without alkaline peroxide (24 h, 35 °C, control using water instead of alkaline peroxide) using the same enzyme cocktail generated 110 \pm 7 mg of glucose, 43 \pm 4 mg of xylose, 11 \pm 1 mg of arabinose, and 4 \pm 0 mg of galactose (total sugars, 168 \pm 12 mg per g of straw).

Effect of Duration of Alkaline Peroxide Pretreatment at Two Temperatures. The effects of duration of alkaline peroxide pretreatment on the enzymatic saccharification of wheat straw at 25 and 35 °C were investigated. The results are presented in Figure 2. It is evident that the effect of pretreatment time is more pronounced at 25 °C than at 35 °C. There was an increase of formation of total sugars of 72 \pm 3 mg per g of straw by increasing the pretreatment time from 3 to 24 h at 25 °C, whereas the increase of total sugars was only 27 \pm 4 mg per g of straw within the same time period at 35 °C. However, the longer is the pretreatment time, the better is the yield of sugars by enzymatic saccharification. Galactose was not detected in any of these hydrolyzates. The reason for this is not clear.

The time course of enzymatic hydrolysis of the alkaline H₂O₂ pretreated (2.15%, v/v; pH 11.5; 35 °C, 3 h) of wheat straw is presented in Figure 3. Most of the sugars were released within 24 h. However, the sugar yield increased very slowly up to 120 h, after which there was no increase in sugar concentration. This indicates that there is a long incubation time needed for getting the maximum sugar yield under the conditions used. The addition of Tween 20 (4.3 g/L) in the reaction mixture did not increase the saccharification yield (data not shown). The effect of enzyme dose on the hydrolysis of alkaline H₂O₂ pretreated

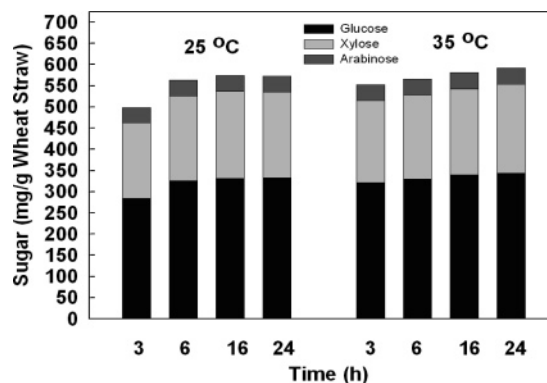


Figure 2. Effect of duration of alkaline H₂O₂ pretreatment (2.15%, v/v; pH 11.5) of wheat straw (8.6%, w/v) at two temperatures (25 and 35 °C) on its enzymatic saccharification (45 °C, pH 5.0, 120 h). The data presented are averages of two separate experiments.

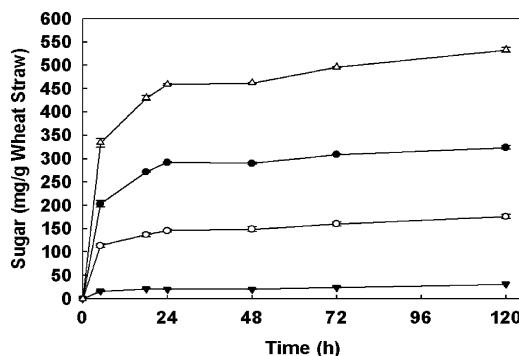


Figure 3. Time course of enzymatic hydrolysis (45 °C, pH 5.0) of alkaline H₂O₂ pretreated (pH 11.5, 35 °C, 3 h) wheat straw (8.6%, w/v) using three commercial enzyme preparations (cellulase, β -glucosidase, and xylanase). The data presented are averages of two separate experiments. Each enzyme dose is 4 mL/100 g of wheat straw. Symbols used: ●, glucose; ○, xylose; ▼, arabinose; and ▽, total sugars.

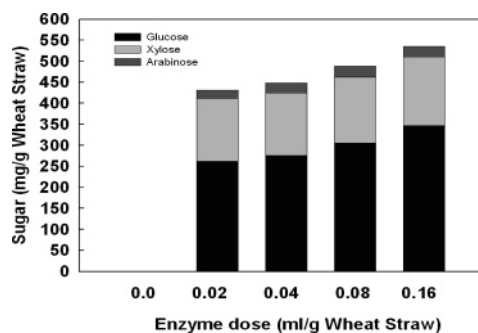


Figure 4. Effect of enzyme dose on the release of sugars from alkaline H₂O₂ pretreated (2.15%, v/v; pH 11.5; 3 h) wheat straw (8.6%, w/v). The data presented are averages of two separate experiments.

(pH 11.5, 35 °C, 3 h) wheat straw is presented in Figure 4. As expected, the sugar yield was better with higher enzyme dose, and this was especially true for cellulose hydrolysis (glucose release). We have been able to obtain a yield of total sugars of 672 \pm 4 mg per g of wheat straw pretreated with alkaline H₂O₂ (2.15%, v/v; pH 11.5; 35 °C; 24 h) after enzymatic saccharification (45 °C, pH 5.0, 120 h) using 0.16 mL of enzyme per g of straw. This is 96.7% conversion of total cellulose and hemicellulose present in the wheat straw. Also, the combination of all three enzyme preparations worked better than using one enzyme preparation or cellulase and β -glucosidase preparations (data not shown).

Attempts were made to separate the liquid from the solids after alkaline H₂O₂ pretreatment and then saccharify these two

Table 2. Ethanol Production from Wheat Straw Hydrolyzate by Recombinant *Escherichia coli* Strain FBR5 at 37 °C^a

hydrolyzate	fermentation time (h)	total sugars (g/L)	ethanol (g/L)	ethanol (g/g of sugar)	ethanol (g/g of straw)
separate hydrolysis and fermentation (SHF)	48	41.5 ± 0.9	18.9 ± 0.9	0.41	0.29
simultaneous saccharification and fermentation (SSF)	48		15.1 ± 0.1	0.33	0.23

^a The medium contained hydrolyzates from 66 g of wheat straw per L. For pretreatment, wheat straw (8.6%, w/w) was treated with 2.15% H₂O₂ (v/v) at 35 °C for 24 h. Separate enzymatic saccharification was performed using cellulase (Celluclast), β-glucosidase (Novozyme 188), and xylanase (Viscostar 150 L) at 45 °C and pH 5.0 for 120 h. Each enzyme used 4 mL/100 g of wheat straw. Fermentation experiments were performed at pH 6.5 for SHF and pH 6.0 for SSF. The data presented are averages of two separate experiments.

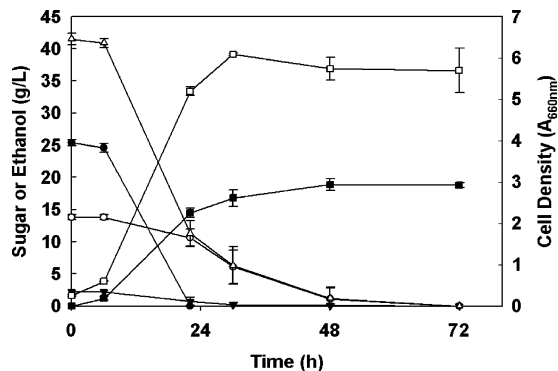


Figure 5. Time course of ethanol production by recombinant *Escherichia coli* strain FBR5 from alkaline H₂O₂ pretreated (2.15%, v/v; pH 11.5; 3 h) enzymatically saccharified (45 °C, pH 5.0, 120 h) wheat straw hydrolyzate at pH 6.5 and 37 °C. The data presented are averages of two separate experiments. Symbols used: ●, glucose; ○, xylose; ▼, arabinose; ▽, total sugars; ■, ethanol; and □, cell density.

fractions separately. The total loss of solids after the alkaline peroxide pretreatment was about 40 ± 1% (w/w). It was found that almost all of the cellulose (93%) and about 75% of the hemicellulose remained in the solid fraction after the pretreatment, and the total yield of sugars after enzymatic saccharification was not improved by performing separate hydrolysis in comparison with the whole pretreated wheat straw (data not shown).

Fermentation of Wheat Straw Hydrolyzates. The results of fermentation of alkaline peroxide pretreated and enzyme saccharified wheat straw by the recombinant *E. coli* strain FBR5 are summarized in Table 2. The bacterium produced small amounts of succinic and acetic acids as byproducts in addition to ethanol. It is clear that the separate hydrolysis and fermentation (SHF) approach worked better than the simultaneous saccharification and fermentation (SSF) method with respect to ethanol yield. This is true in the cases of both dilute acid pretreated wheat straw and rice hull (22, 23). The maximum concentration of ethanol (per L) from wheat straw (66.0 g) hydrolyzate by recombinant *E. coli* strain FBR 5 was 18.9 ± 0.9 g with a yield of 0.29 g per g of straw (0.46 g per g of available sugars in wheat straw) by SHF. For SSF, the maximum concentration of ethanol was 15.1 ± 0.1 g, which gives a yield of 0.23 g per g of straw (0.36 g per g of available sugars based on separate hydrolysis data). The time course of ethanol production by the recombinant *E. coli* strain from wheat straw hydrolyzate by SHF is shown in Figure 5. Both SHF and SSF were completed within 48 h of fermentation even though the yield of ethanol was higher in the case of SHF than SSF. The cell density (A_{660nm}) reached a maximum of 6.1 ± 0.0 in 30 h, after which it declined slowly to 5.7 ± 0.5 in 72 h in the case of SHF (Figure 5). There is little growth (A_{660nm} , 0.44 ± 0.02) of recombinant *E. coli* strain FBR 5 in the control medium where water was substituted for the hydrolyzate. No detectable metabolic product was found to be produced in the control medium by the strain.

Fang et al. demonstrated that alkaline peroxide pretreatment does not affect the structure of hemicellulose from wheat straw (11). Ahring et al. investigated the wet oxidation process as a means of solubilizing hemicellulose from wheat straw (13). The fermentation of the hemicellulose hydrolyzates was carried out using *Thermonaerobacter mathranii* strain A2M1. No significant inhibitory effect was observed. However, the solubilized hemicellulose was only partly available for fermentation by the bacterium, and both weak acid hydrolysis and enzyme treatment with Celluclast enhanced the ethanol production.

A critical problem in the fermentation of dilute acid hydrolyzates is the inability of the fermentative microorganism to withstand inhibitory compounds formed during pretreatment, and usually a detoxification step is needed to improve fermentability (24). This was also true with the fermentation of the dilute acid hydrolyzates of wheat straw (15, 22). The inhibitor problem is not pronounced in the case of alkaline peroxide pretreatment of wheat straw. Bjerre et al. reported that wet oxidation combined with base addition readily oxidizes lignin from wheat straw, facilitating the polysaccharides for enzymatic hydrolysis (25). The process water, containing dissolved hemicellulose and carboxylic acids, was found to be a direct nutrient source for fungal growth and enzyme production. Furfural and HMF were not produced during the wet oxidation treatment. Unlike corn fiber hemicellulose, which is very resistant to hydrolysis using commercial enzymes, wheat straw hemicellulose can be easily hydrolyzed enzymatically by using a single xylanase preparation (Viscostar) after alkaline peroxide pretreatment (2). Pan et al. reported that wheat straw contained 0.48% ferulic acid and 0.42% *p*-coumaric acid, and these phenolic acids had a tendency to dissolve in alkaline peroxide solution (26). This is probably the reason Viscostar 150 L performed well with respect to hemicellulose saccharification. Thygesen et al. studied the production of cellulose and hemicellulose-degrading enzymes by filamentous fungi cultivated on wet-oxidized wheat straw (27). Enzymatic hydrolysis of filter cake from wet-oxidized wheat straw for 48 h with an enzyme loading of 5 FPU/g of biomass resulted in a glucose yield of 58% (w/w) from cellulose using the enzyme produced by *Penicillium brasilianum* IBT 20888. The glucose yield from cellulose was in the range 77–79% (w/w) using higher enzyme loading (25 FPU/g of biomass).

This research demonstrates that wheat straw can be easily converted to fermentable sugars almost completely by alkaline peroxide pretreatment and enzymatic saccharification, and the generated hydrolyzate can be efficiently fermented to ethanol by using recombinant bacterium without any detoxification step generally needed for dilute acid pretreated enzymatically saccharified wheat straw hydrolyzate (22).

Acknowledgment

We thank Gregory J. Kennedy for excellent technical assistance.

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Accepted for publication December 13, 2005.

BP050310R