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Ethanol Production from Enzymatic Hydrolysates of AFEX-Treated Coastal Bermudagrass and Switchgrass

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ABSTRACT

Switchgrass and coastal bermudagrass were pretreated by ammonia fiber explosion (AFEX), and the treated materials hydrolyzed using 5 IU cellulase/g substrate. Resulting sugar solutions (2–3%, w/v) were fermented with recombinant *Klebsiella oxytoca*. Glucose was rapidly and completely fermented to ethanol, whereas xylose fermentation was slower and less complete. At higher sugar concentrations (~ 8%) glucose fermentation continued, but xylose fermentation almost ceased. Protein extraction somewhat enhanced ethanol production from coastal bermudagrass. Improved fermentation technologies and media appear necessary for practical mixed-sugar lignocellulosic hydrolyzates.

Index Entries: Ethanol; lignocellulose; cellulase enzymes; ammonia fiber explosion; *Klebsiella oxytoca*.

INTRODUCTION

Worldwide demand for energy and liquid transportation fuels continues to increase, and the demand for oxygenated fuels and octane enhancers is increasing even more rapidly (*1*). Fermentation ethanol currently supplies much of the US demand for oxy-fuels, and its use could grow substantially if production costs could be further reduced. Many millions of tons of crop residues and cellulose-containing wastes are potentially available at low cost and many more millions of tons of energy

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crops, such as switchgrass (SWG), or existing forage crops, such as coastal bermudagrass (CBG), could be provided at moderate cost if the conversion technology were more economically attractive. In particular, improved cellulose pretreatments to allow much higher yields of fermentable sugars are badly needed (*2*).

The ammonia fiber explosion (AFEX) process is a new lignocellulose pretreatment process that may offer both an effective and economically attractive means of increasing yields of fermentable sugars from lignocellulosic biomass (*3,4*). In the AFEX process, biomass is treated with liquid ammonia at moderate temperatures and high pressure for 15–30 min. The pressure is then rapidly released, literally exploding the fibrous biomass, and the ammonia evaporates and is recovered. The resulting AFEX-treated biomass is readily hydrolyzed at near theoretical yields of sugars, and the resulting sugars can be easily fermented (*5*). Preliminary economic evaluations of the AFEX process estimate its costs at about $10/dry ton of biomass treated (*6,7*). AFEX appears to be an effective and economical pretreatment. The AFEX process is currently undergoing scale-up and commercialization by RA Energy Ltd., Austin, TX.

The cost of the cellulase enzymes is widely believed to be another key hurdle for biological conversion of lignocellulosic materials to fuel ethanol (*8*). Much previous pretreatment and hydrolysis research have used enzyme loadings of 20 IU/g and higher, which is probably uneconomical for one-time enzyme use. However, a recent study shows that AFEX-treated crop residues, energy crops, and forage grasses, including SWG and CBG, can be rapidly and completely hydrolyzed at <5 IU commercial cellulase/g dry biomass (*9*).

Development of a lignocellulose-based alcohol fuels industry will also require effective use of all the biomass components. One refining approach is to coproduce valuable protein from forage crops, such as alfalfa and CBG (*10*). We recently developed conditions for AFEX pretreatment and protein extraction from CBG, which give over 80% protein extraction. The present work extends these previous studies using low enzyme level hydrolysis and subsequent ethanol fermentation of protein-extracted and unextracted CBG.

Another major challege is to produce ethanol from xylose. Nearly all ethanol fermentation studies on biomass have utilized yeasts and bacteria. Although most yeasts are unable to ferment xylose, certain yeasts, such as *S. cerevisiae*, can ferment xylulose after enzymatically converting xylose to xylulose. A few, such as *Pichia stipitis, Pachysolen tannophilus,* and *Candida shehatae*, can directly convert xylose to ethanol (*11–13*) but with less than satisfactory results to date.

A promising recent development is a genetically manipulated *K. oxytoca*. Genes encoding two essential enzymes (alcohol dehydrogenase II and pyruvate decarboxylase) in the fermentative pathway for ethanol production of *Zymomonas mobilis*, an obligately ethanologenic bacterium,

were chromosomally integrated into *K. oxytoca* (*14*). This article emphasizes sequential cellulase hydrolysis (at low enzyme levels) and ethanol fermentation by *K. oxytoca* of the sugar mixtures resulting from hydrolysis of AFEX-treated CBG and SWG.

MATERIALS AND METHODS

Biomass

Materials utilized were: (1) fertilized coastal bermudagrass, from the Texas A&M University Research Farm harvested in August 1991, 4 wk after the first cutting and (2) switchgrass (Alamo and native cultivars) from the East Texas Plant Materials Center, Nacogdoches, TX, harvested in the fall of 1992 from a 4-yr-old stand of grass. Materials were sun-dried, ground to approx 6 mm particle size, and then stored in sealed bags in a walk-in cooler at 4°C. Biomass compositions were determined by standard techniques (*15*). The CBG composition was approximately (% of dry wt): hemicellulose (35.7), cellulose (25.0), protein (10.0), lignin (6.4), ash (5.1), starch (6.4), free sugars (2.0 with 0.7 glucose), pectin (5.0), and lipid (3.0). The SWG composition (Alamo) was: cellulose (44.9), hemicellulose (31.4), protein (4.0), lignin (12.0), ash (4.6), and other (3.1).

AFEX Treatment

Previously ground and dried materials were AFEX-treated in a 4-L highly modified steel pressure vessel according to procedures described in detail previously (*3,5*). The AFEX treatment conditions selected from previous work were 2 kg ammonia:kg dry biomass, 30-min treatment time at 90°C, and 30% moisture, dry basis (*9*). Following AFEX treatment, the ammonia was allowed to evaporate overnight in a fume hood, and the treated material was then sealed in heavy plastic bags and refrigerated for later use.

Protein Extraction

Protein was extracted from AFEX-treated and untreated CBG (as a control) using a dilute (0.5 g/L) aqueous solution of calcium hydroxide at 90°C, 100 mL of solution shaken with 5 g of CBG. Protein extraction results are described elsewhere (*16*).

Hydrolysis

AFEX-treated and untreated samples were enzymatically hydrolyzed at 48°C at a solids loading of 5% (50 g biomass/L of pH 4.8 buffer solution). The enzymes used were Cytolase 300 ("cellulase") from Genencor Inc., San Francisco, CA and Novozyme 188 ("cellobiase") from Novo

Laboratories, Wilton, CT. Cellulase and cellobiase loadings were 5 IU/g dry biomass and 28.4 U/g, respectively. Hydrolysis was done in capped 500-mL fleakers agitated with a magnetic stirrer. One-millilter liquid samples were taken, placed in test tubes, capped, and placed in a boiling water bath to inactivate the enzymes. Hydrolysis and fermentation studies were carried out with the native cultivar, which gave a lower reducing sugar yield than did previous studies with the Alamo cultivar under the same AFEX treatment conditions (*9*).

Fermentation

K. oxytoca strain P_2 was provided by L. O. Ingram (University of Florida, Gainesville, FL). Cultures were maintained as frozen stocks in sterile 40% glycerol/distilled water at −20°C and on a complex medium containing either 2% glucose or 2% xylose with 40 mg/L chloramphenicol. The organism was grown at 30°C in Luria broth (*17*) consisting of tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; glucose or xylose, 20 g/L of distilled water. Luria agar was prepared by adding 25 g/L of Difco Bacto-Agar to the broth. The medium pH was adjusted to 5.5 and sterilized at 121°C for 15 min. Sugar solutions were sterilized by filtration through 0.22-μ filters. Chloramphenicol stock was prepared by dissolving 400 mg in 10 mL of 70% ethanol/distilled water (self-sterilizing). One milliliter of stock was added per liter of culture media.

Several Luria agar plates were streaked from a drop of glycerol stock to ensure good isolated colonies. The large, cream-colored, raised colonies are good ethanol producers, and were used to inoculate flasks and Petri dishes to grow the fermentation inocula. Single colonies were transferred to 50 mL of Luria broth containing 1% glucose or 1% xylose as the sole carbon source. Cultures were incubated overnight at 30°C without agitation and then mixed vigorously, and the optical density (OD) measured at 550 nm by a Beckman DU-6 spectrophotometer.

After 24 h of enzymatic hydrolysis of the substrate, the stirred 500-mL fleakers were inoculated aseptically by a (5% v/v) inoculum of *K. oxytoca*, which had an OD of 1.0 at 550 nm. The fermentation temperature and the pH were maintained at 35°C and 5.5, respectively. Fermentation media were not supplemented (*14*). Samples were taken in sterile vials at various times, placed immediately in an ice bath, and then centrifuged at 10,000*g* and 4°C for 20 min. Supernatants were stored at −20°C until required for analysis.

Trace Elements and Growth Factors

For some fermentation experiments, the effects of mineral or growth factor addition were investigated by adding: (1) 0.01% tryptone and 0.05% yeast extract from stock solutions previously sterilized at 121°C for 15 min and (2) a trace element solution consisting of 2 g citric acid/L

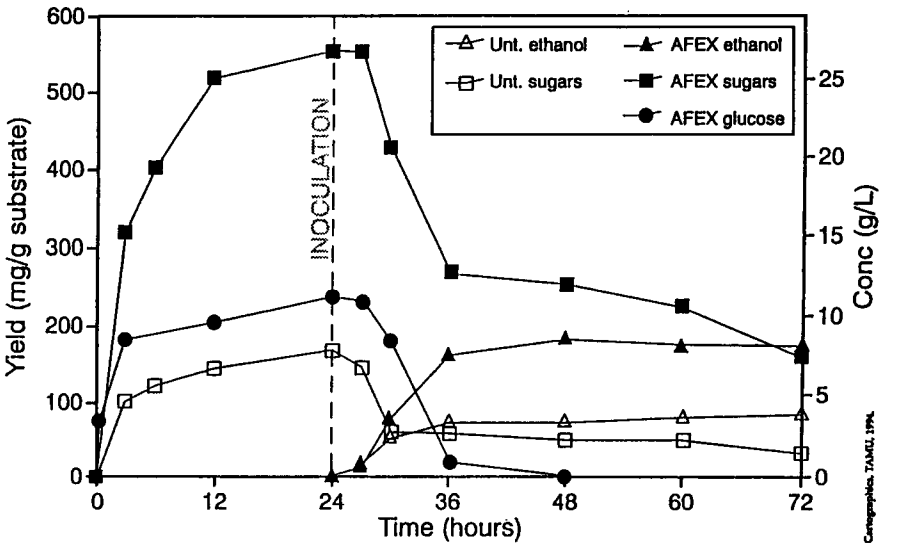

Fig. 1. Sequential saccharification and fermentation by *K. oxytoca* of AFEX-treated and untreated native SWG.

and having the following mineral concentrations $CaCl_2 \cdot 2H_2O$ (1 m*M*); $MgSO_4 \cdot 7H_2O$ (1 m*M*); $FeSO_4 \cdot 7H_2O$ (10 μ*M*); $FeCl_3 \cdot 6H_2O$ (5 m*M*); $Na_2MoO_4 \cdot 2H_2O$ (1 m*M*); $ZnSO_4 \cdot 7H_2O$ (1 m*M*); $MnSO_4 \cdot H_2O$ (2 m*M*); $CuSO_4 \cdot 5H_2O$ (1 m*M*); $CoCl_2 \cdot 6H_2O$ (1 m*M*); and $NiCl_2$ (1 m*M*). The mineral solution was sterilized by passing through a 0.22-μm membrane.

Analytical Methods

After thawing, samples were filtered through syringe filters containing a 0.22 μm nylon membrane and analyzed by DNSA (*18*) for total reducing sugars using glucose as the standard. The results are expressed as mg equivalent glucose/g of initial dry biomass. HPLC was also performed on selected samples for glucose and xylose. Samples analyzed by HPLC were eluted with deionized, degassed water through an Aminex HPX 87-P column in series with an HPX 87-C column (Bio-Rad, Richmond, CA) both heated at 85°C, and sugars and ethanol detected by a refractive index monitor (LDC Analytical, Riviera Beach, FL).

RESULTS AND DISCUSSION

Switchgrass

AFEX-treated and untreated native SWG was hydrolysed at 48°C with a low level of commercially available enzymes (5 IU/g SWG). Figure 1 shows the hydrolysis and fermentation profiles. Within 24 h, a total

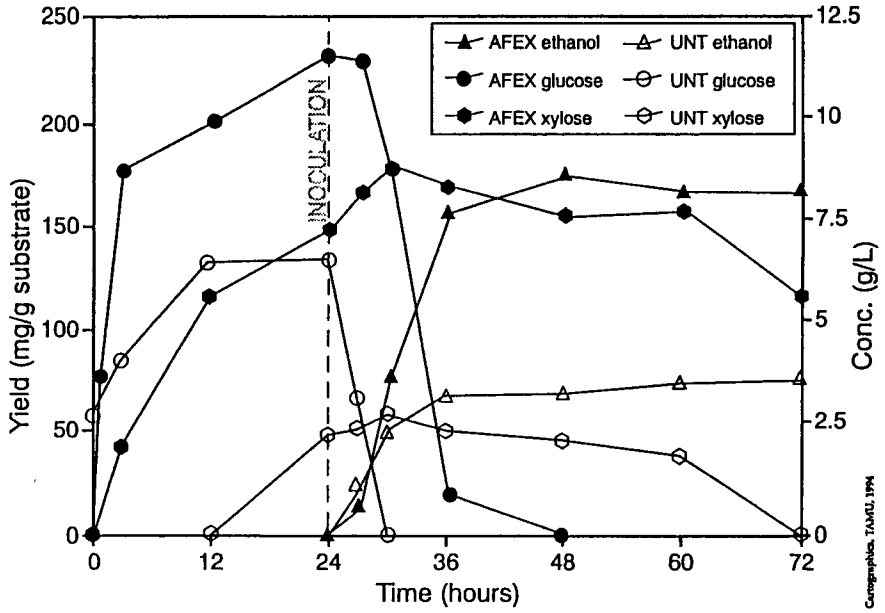

Fig. 2. Sequential saccharification and fermentation by *K. oxytoca* of AFEX-treated and untreated native SWG: glucose, xylose, and ethanol profiles.

reducing sugar yield of 546 mg/g dry substrate was obtained for AFEX-treated SWG vs 186 mg/g substrate for untreated SWG. This is lower than the 680 mg total sugars/g substrate obtained for AFEX-treated Alamo cultivar hydrolyzed at 5 IU/g (*9*). (A sample of Alamo switchgrass harvested in spring 1992 yielded over 750 mg total sugars/g substrate after AFEX; AFEX treatment conditions, like other pretreatments, should be optimized for each substrate.) The glucose yield at 24 h for AFEX-treated SWG was 231 mg/g substrate (Fig. 2). The hydrolyzate was inoculated with *K. oxytoca* (5% v/v, OD = 1) at 24 h of hydrolysis, and the fermentation conducted at pH 5.5 and 35°C. Glucose was almost completely depleted within 12 h of inoculation (Figs. 1 and 2). Comparing Figs. 1 and 2, we also see that the sum of glucose and xylose is not equal to the total sugars measured by DNSA. Other sugars (e.g., mannose, arabinose) exist in lignocellulosic biomass, and nonsugar-reducing compounds will be measured as apparent sugars by the DNSA test. This same pattern is observed when comparing Figs. 4 and 5 and Figs. 5–7.

For AFEX-treated SWG, ethanol production almost reached its maximum yield (173 mg/g substrate) within 12 h of *K. oxytoca* inoculation. Both the initial rate and final extent of hydrolysis are much lower for untreated SWG than for AFEX-treated SWG (approx 200 mg/g of glucose plus xylose in 24 h vs over 400 mg/g of glucose plus xylose in 24 h for AFEX-treated SWG). The ethanol yield, if all glucose and xylose present after 24 h hydrolysis were converted completely to ethanol, would be 209 mg ethanol/g SWG. Therefore, the experimental ethanol yield was 83% of

"theoretical." This is not a very satisfactory means of calculating ethanol yield as a percent of "theoretical" for the following reasons:

1. The hydrolysis of remaining biomass proceeds, although probably quite slowly, even after the pH is increased and the temperature dropped for the ethanol fermentation;
2. Other sugars, such as arabinose, which were not measured, can also be converted to ethanol by this organism; and
3. We did not measure the full spectrum of products of the organism.

Nonetheless, here we define "theoretical" yield as that which would be obtained if all the glucose and xylose present at 24 h of hydrolysis were converted completely to ethanol. In model systems, sugars consumed by *K. oxytoca* have been converted to ethanol at theoretical levels (*19*).

The ethanol yield for untreated SWG was 72 mg/g SWG; thus, AFEX-treated SWG gave an ethanol yield 2.4 times the ethanol yield for untreated SWG, which was lower than expected, since the total reducing sugars yield from AFEX-treated SWG was 3.3 times that from untreated SWG. A contributing factor may be the better uptake of xylose from untreated SWG vs AFEX-treated SWG (56% of the 24-h xylose content vs 40%). However, Fig. 2 shows clearly that xylose is not efficiently converted to ethanol for either treated or untreated SWG. These results may be the result of a combination of inhibition by acetic acid, ethanol, and sugar inhibition at the higher sugar concentrations plus xylose sparing caused by the fact that glucose is more than 50% of the sugar mixture (*19*). Similar results with extensively washed and extracted CBG, shown below, seem to rule out inhibition of *Klebsiella* by some product of AFEX pretreatment.

To investigate further the effects of higher sugar concentrations in real hydrolyzates on ethanol fermentation, 24-h SWG hydrolyzates were concentrated by reverse osmosis to approx 8% (total reducing sugars), and inoculated twice with a 5% inoculum (v/v, OD = 1) *K. oxytoca* (at zero time and 24 h) of fermentation. The effects of adding tryptone and minerals were also studied. Figure 3 summarizes these results. The relative merits of tryptone addition vs minerals at high sugar concentrations were not clear. However, it is clear that there was almost no xylose uptake at 8% total sugars. The fermentation with added minerals did achieve nearly theoretical ethanol yields from glucose without the confounding factor of possible continuing hydrolysis. Therefore, the addition of minerals seemed to enhance ethanol production from glucose, but did not help in increasing xylose utilization. In addition, Fig. 3 shows that at these concentrations, fermentation does not begin until 18 h after inoculation vs the almost immediate fermentation seen in Figs. 1 and 2. In fact, two inoculations were required to produce fermentation. Apparently the higher sugar concentrations (or some artifact of the concentration process) inhibit the fermentation of these sugars by this organism.

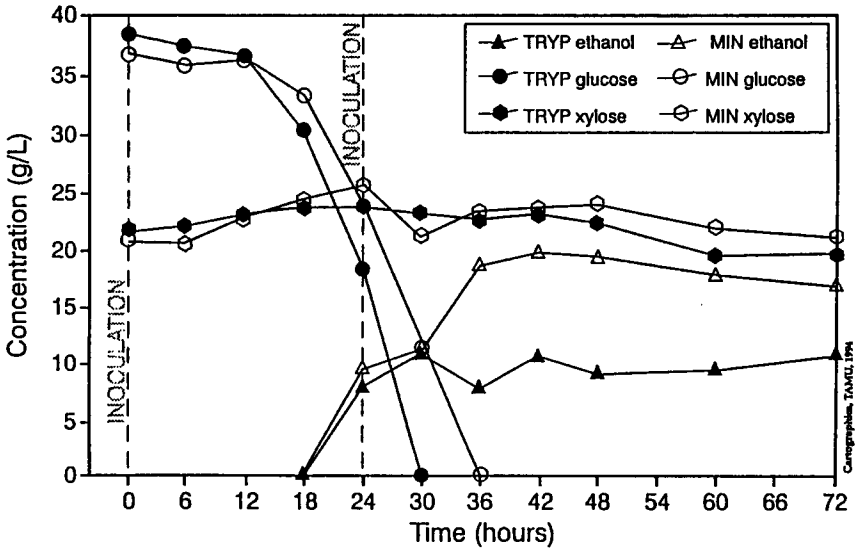

Fig. 3. Fermentation by *K. oxytoca* of concentrated (8% w/v) native SWG hydrolysates supplemented with minerals or tryptone.

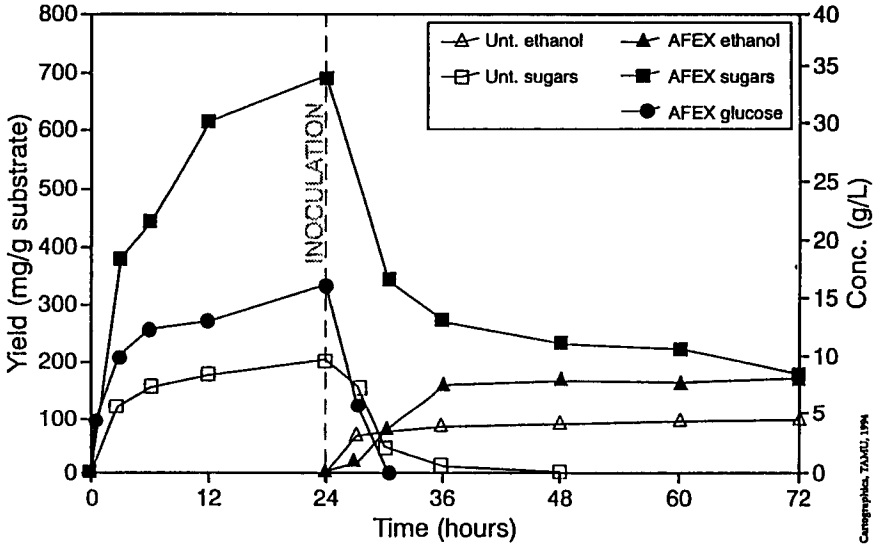

Fig. 4. Sequential saccharification and fermentation by *K. oxytoca* of unextracted, AFEX-treated and untreated CBG.

Coastal Bermudagrass

Unextracted CBG

Ethanol fermentation experiments involving AFEX-treated and untreated CBG were conducted with protein-extracted and nonextracted substrates, prepared as described above. The following discussion refers to AFEX-treated CBG unless indicated otherwise. Figure 4 shows the sequential

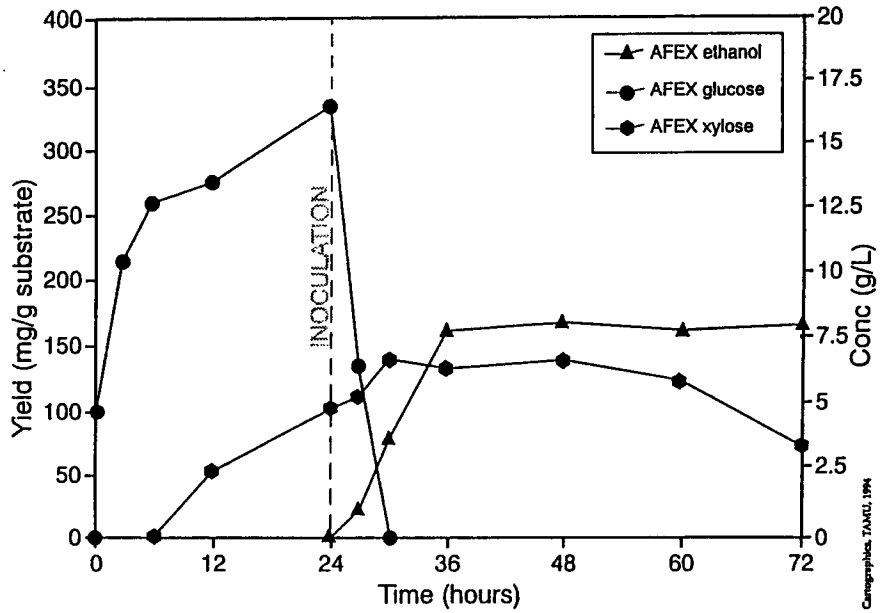

Fig. 5. Sequential saccharification and fermentation by *K. oxytoca* of unextracted, AFEX-treated and untreated CBG: glucose, xylose, and ethanol profiles.

hydrolysis and fermentation of nonextracted CBG. A total reducing sugar yield of 684 mg/g AFEX-treated CBG was obtained in 24 h, of which 331 mg/g CBG were glucose. All the glucose was consumed by *K. oxytoca* within 6 h following inoculation (Fig. 5). For AFEX-treated nonextracted CBG, about 50% of the xylose was consumed within 48 h. Xylose utilization apparently did not lead to increased ethanol production. At 24 h, approx 467 mg of glucose and xylose were potentially available for conversion to ethanol for a theoretical yield of 238 g ethanol/g AFEX-treated, nonextracted CBG. This compares with the 162 mg/g actually achieved, which is 68% of "theoretical." For untreated, nonextracted CBG, a total reducing sugar yield of 200 mg/g CBG was obtained within 24 h of hydrolysis, and all these sugars were utilized by *K. oxytoca* within 24 h of inoculation.

This ethanol yield of 162 mg/g CBG is equivalent to about 205 L ethanol/t of dry CBG. However, if all the glucose and xylose present after 24 h of hydrolysis were converted to ethanol, about 302 L/t could be produced from nonextracted, AFEX-treated CBG. For untreated, nonextracted CBG, although all the sugars were consumed, the resulting ethanol yield was only 85 mg/g CBG or a possible 108 L ethanol/t dry untreated CBG. Therefore, the ethanol yield possible from untreated, nonextracted CBG is about one-third of what is possible from nonextracted, AFEX-treated CBG.

Protein-Extracted CBG

For protein-extracted CBG (Figs. 6 and 7), the hydrolysis and fermentation profiles show similar trends. Total reducing sugars of 603 mg/g extracted CBG were produced with a glucose and xylose yield of 464 mg/g

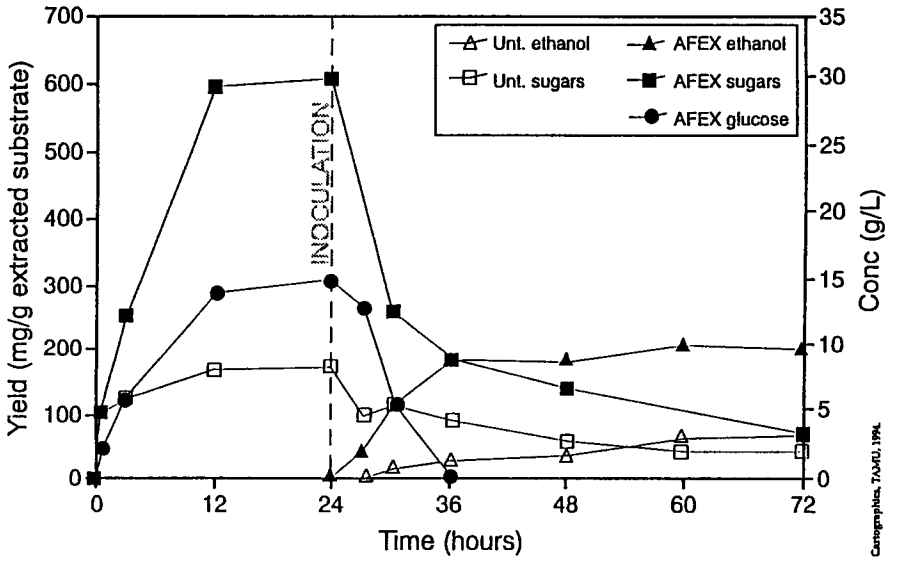

Fig. 6. Sequential saccharification and fermentation by *K. oxytoca* of extracted, AFEX-treated and untreated CBG.

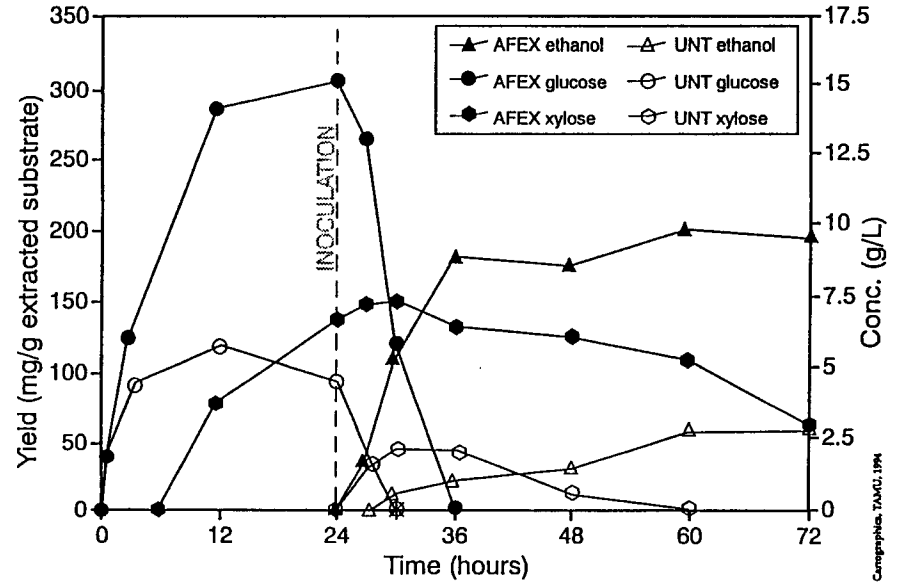

Fig. 7. Sequential saccharification and fermentation by *K. oxytoca* of extracted, AFEX-treated and untreated CBG: glucose, xylose, and ethanol profiles.

CBG. (The yield of extracted CBG is approx 0.85 g/g original CBG). Figure 7 shows that glucose was completely utilized within 12 h. Approximately 60% of the starting xylose was utilized with only a slight increase in ethanol levels. The final ethanol yield was 197 mg/g CBG (250 L/t) or 83% of the "theoretical" 300 L/t. The theoretical ethanol yield based on total glucose plus xylose was 82 mg/g CBG; hence, the yield was 67% of

Table 1
Saccharification and Fermentation Parameters
for Some Lignocellulose Hydrolysates

Parameter, mg/g subs	SWG		CBG			
			Unextracted		Extracted	
	AFEX	No AFEX	AFEX	No AFEX	AFEX	No AFEX
Ethanol	173	72	162	85	199	55
24 h DNS sugars	546	186	684	200	603	175
24 h glucose and xylose	410	182	467	n.d.	464	161
Theoretical ethanol[a]	209	93	238	n.d.	237	82
Percent of theoretical yield	83	77	68	n.d.	84	67
Percent xylose utilized (48 h)	40	100	51	n.d.	60	100

n.d. = not determined.
[a]Based on complete conversion of glucose and xylose present at 24 h.

theoretical. At low sugar concentrations, xylose is completely consumed, but theoretical ethanol yields are still not achieved. For untreated, protein-extracted CBG, Fig. 7 shows an ethanol yield of 55 mg/g CBG with glucose and xylose fully consumed.

A higher ethanol yield per gram substrate was obtained from the protein-extracted CBG than from nonextracted CBG (both AFEX-treated). Although more reducing sugars were obtained from nonextracted CBG, total glucose and xylose yields for the two cases were virtually the same, and glucose and xylose consumption were also the same. This may have been because of the extraction treatment, which improved fermentation conditions, or perhaps the slightly lower sugar concentration in the protein-extracted CBG hydrolyzate. In any event, protein extraction did not in any way inhibit ethanol fermentation and may have actually helped. Protein recovery and ethanol fermentation could consequently be integrated into a fuel and food production system. Sugar and ethanol yields for the materials studied are summarized in Table 1.

CONCLUSIONS

AFEX-treated switchgrass and coastal bermudagrass are good lignocellulosic substrates for bioconversion to ethanol at very low enzyme levels. The ethanol yields at low hydrolyzate concentrations (2–3% total

sugars) are equivalent to 200–250 L ethanol/t vs < 100 L/t from untreated materials. If all the glucose and xylose available after 24 h of hydrolysis with low enzyme levels (5 IU/gm) were consumed, 260–300 L ethanol/t might be produced. Protein extraction did not inhibit ethanol fermentation. Thus, protein recovery and ethanol fermentation could be integrated into a fuel/food/feed production system using AFEX.

In all fermentations, glucose utilization was rapid and complete, whereas xylose utilization was slow and incomplete. The maximum level of ethanol was always produced within 12 h of *K. oxytoca* inoculation. At higher hydrolyzate concentrations (about 8% total sugars), xylose uptake virtually ceased, but glucose could still be fermented to ethanol in theoretical yield, although more slowly than at lower concentrations. Xylose uptake did not necessarily mean more ethanol production. *K. oxytoca* shows great promise for fermentation of mixed biomass sugars. Our research did not optimize fermentation conditions and media, and the limitations of xylose utilization and conversion to ethanol can probably be overcome. Further work with *K. oxytoca* is needed to develop improved fermentation methods and conditions for real mixed-sugar hydrolysates, such as those available from AFEX pretreatment.

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