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Ethanol Production from AFEX-Treated Forages and Agricultural Residues

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ABSTRACT

Lignocellulosic materials derived from forages, namely timothy grass, alfalfa, reed canary grass, and agricultural residues, such as corn stalks and barley straw, were pretreated using ammonia fiber explosion (AFEX) process. The pretreated materials were directly saccharified by cellulolytic enzymes. Sixty to 80% of theoretical yield of sugars were obtained from the pretreated biomasses. Subsequent ethanolic fermentation of the hydrolysates by *Pachysolen tannophilus* ATCC 32691 resulted in 40–60% of theoretical yield after 24 h, based on the sugars present in the hydrolysates. The uptake of sugars was not complete, indicating a possible inhibitory effect on *P. tannophilus* during the fermentation of these substrates.

Index Entries: Forages; agricultural residues; AFEX; enzymatic hydrolysis; ethanolic fermentation; biofuel.

INTRODUCTION

Perennial grasses are widely available in eastern Canada. Their potential was examined with respect to the context of Quebec's transport sector *(1)*. The production of fuel ethanol from lignocellulosic materials, such as forages and agricultural residues, offers many potential economic and environmental benefits. Several methods were developed to make the production of ethanol based on lignocellulosic biomass technologically feasible, but the challenge of developing and commercializing a cost-effective process remains. One of the major problems in utilizing lignocellulosics as fermentation substrates is their resistance to hydrolysis. A wide variety

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of pretreatments have been used to reduce the particle size, lignin content *(2)*, and cellulose cristallinity to improve the surface area and the porosity of such materials, as well as their accessibility for the enzymes *(3–5)*. No single method has yet found widespread commercial application. Mechanical comminution by a combination of chipping, grinding, and milling techniques is a costly process because of its power requirements *(6)*. Various chemical treatments employing acids and bases are performed at high temperatures and pressure, and are costly. The chemicals are difficult to recover and recycle, and are often toxic or inhibitory to the subsequent fermentation step *(7)*. Steam explosion pretreatment can induce extensive hemicellulose degradation to furfural and its derivatives and lignin modification at high severity (temperature >220°C, and long reaction times) *(8–10)*. Impregnation prior to steam explosion with H_2SO_4 or SO_2 has been shown to satisfactorily enhance the selectivity of the process and favor hydrolysis over pyrolysis and degradation reactions *(8,11)*. Aqueous/steam fractionation of lignocellulosics was recently studied, and ethanol production from cellulosic fines derived from fractionated forages was evaluated and could be integrated within the "biorefinery" concept *(12)*. Novel pretreatments for biomass using ammonia were recently proposed. The ammonia recycled percolation (ARP) process *(13,14)* uses aqueous ammonia ($NH_3 \cdot H_2O$); ARP-H, a variant of the ARP process, combines the action of hydrogen peroxide to that of aqueous ammonia to modify structural features of herbaceous biomass *(15)*. Good results were obtained with ARP-H-treated corn cobs/stover mixture and switchgrass, but the efficiency of this process with other types of biomass remains to be demonstrated. The ammonia fiber explosion (AFEX) process was reported as a pretreatment method for improving the reactivity of lignocellulosics *(16)*. This technique, which combines ammoniacal hydrolysis and freezing to shatter and split plant material in relatively mild conditions, has been demonstrated to markedly improve the saccharification rates of several herbaceous crops and grasses *(17–23)*.

Within the mandate of the Canadian Green Plan Ethanol Program, the performance of the AFEX pretreatment on local biomasses was evaluated. The subsequent hydrolysis and fermentation steps of three hays (alfalfa, timothy, and reed canary grass) and two grain residues (barley straw and corn stalks) are reported here.

METHODS

Feedstock

Mature timothy grass (*Phleum pratense*, Basho cultivar), alfalfa (*Medicago sativa*, Apica cv.), and reed canary grass (*Phalaris arundinacea*, Vantage cv.) were mowed in July 1995 and stored as baled hay. Barley straw

(Hordeum vulgare) and corn stalks were baled in August and October 1995, respectively. Bales were stored at a low and stable moisture content (8% dry wt) until samples were needed for the AFEX treatment.

Enzymes

Two enzyme complexes derived from the fermentation of selected strains of *Trichoderma longibrachiatum* were purchased from Genencor International (Rochester, NY). Multifect Cellulase 300 was a soluble powder containing cellulases, β-D-glucanase, and pentosanase with mainly arabinoxylanase activity. The total declared activity was 180–190 Genencor Cellulase Units (GCU)/g of powder. Spezyme CP was a liquid solution containing cellulase activity and other combined activities (hemicellulases and pectinases), with a declared global activity of 90 GCU/mL. One GCU of Cellulase 300 was found to be equivalent to 0.8 FPU (filter paper unit).

Microorganism and Inocula

Pachysolen tannophilus ATCC 32691 was obtained from the American Type Culture Collection (Rockville, MD). Working stock cultures were grown at 30°C on agar slants containing 3g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, 20 g/L D-glucose, 20 g/L D-xylose, and 20 g/L agar. They were kept at 4°C or stored in 4% (w/v) glycerol at −80°C.

A loopful of cells from the agar slants was inoculated in 200 mL of the culture medium described previously, but without agar, autoclaved, and adjusted to pH 5.0. The 250-mL Erlenmeyer flasks were agitated at 150 rpm for 48 h at 25–27°C in a rotary incubator (Queue Orbital Shaker, Queue Systems, Parkersburg, WV). The concentration of yeasts in the culture reached 6–7 g/L. Cells were harvested by centrifugation at 16,320*g* for 10 min with an RC5C model centrifuge (Sorvall® Instruments, Dupont Canada, Mississauga, ON). The resulting pellet was resuspended in sterile dH_2O to obtain the same concentration as the final concentration of the culture broth.

AFEX Treatment

Five hundred g of previously chopped biomass (9% moisture; chopped to 4–5 mm) were humidified to 15% dry wt with dH_2O and placed in a 7.6-L stainless steel Packless autoclave (Autoclave Engineers, Erie, PA). Five hundred g of liquid ammonia (99.5% purity, obtained from Prodair, Québec) was then added. The temperature was increased to 90°C, while agitating, resulting in a pressure inside the vessel of about 3.45 MPa. After maintaining these conditions for 30 min, the pressure was suddenly released. This treatment induces structural changes within the lignocellulosic matrix and increases the specific surface area of the biomass *(16)*. The treated biomass was removed from the vessel and left overnight under a

fume hood to evaporate ammonia. A maximum of 2% w/w of ammonia used was left in the biomass. The evaporated biomass was placed in plastic bags until its hydrolysis.

Enzymatic Hydrolysis

Hydrolysis was carried out with 5 and 10% (dry basis) of nontreated and AFEX-treated material in 250-mL Erlenmeyer flasks with a working volume of 100 mL, or in 2-L Erlenmeyer flasks with a working volume of 1 L, in 0.05 *M* acetate buffer (pH 4.85). After addition of enzymes, flasks were capped and placed in a rotary incubator (Queue Systems) at 50°C and 290 rpm. The media were kept sterile with NaN_3 (0.005% w/w), except when they were followed by larger-scale fermentations. A separate flask was prepared for each sampling with the 250-mL Erlenmeyer flasks. Two-mL samples were withdrawn at specific time intervals, placed in a boiling water bath for 15 min to deactivate the enzymes, then passed through a 0.2-μm filter. A portion of 0.2 mL was used for measurement of the total reducing sugars by the DNS method *(24)* and compared to a glucose standard. Remaining filtrates were stored at −30°C for subsequent sugar analyses by gas chromatography (GC). Hydrolysis of AFEX-treated biomass was carried out by adding Multifect Cellulase 300 at 3, 5, 6.8, 10, and 15 GCU/g dry fiber (0.017, 0.028, 0.038, 0.056, and 0.083 g of Cellulase 300/g dry fiber). Concentration of Spezyme CP was varied at 0, 1, 2, and 3 μL/g dry matter (0.00, 0.09, 0.18, and 0.27 GCU/g dry fiber).

The potential maximum yield of total sugars (TS_{max}) was evaluated by strong acid hydrolysis with 24.1 *N* H_2SO_4 at 30°C for 30 min, followed by weak acid hydrolysis with 0.82 *N* H_2SO_4 at 120°C for 55 min. The sugar content was analyzed by GC, after neutralization with saturated $Ba(OH)_2$ solution and centrifugation at 12,062*g* for 10 min *(25)*. The efficiency of enzymatic hydrolysis was expressed as a percentage of saccharification, i.e., the ratio of actual sugars released (TS_t) over potential maximum yield of sugars, corrected for soluble sugars initially present (TS_i) in the nonhydrolyzed substrates. In all cases, TS_i was found negligible.

$$\text{Saccharification (\%)} = TS_t - TS_i/TS_{max} - TS_i * 100\% \quad (1)$$

When larger quantities of hydrolysates were required for subsequent fermentation, the initial suspensions of AFEX-treated biomass were not supplemented with NaN_3. The AFEX-treated biomasses were hydrolyzed at a working volume of 8–10 L in 20-L 316 stainless steel mechanically agitated bioreactors (New Brunswick Scientific, New Brunswick, NJ), placed in a water bath at 50°C with agitation at 300 rpm. The enzyme loading were 5 GCU of Cellulase 300 and 2 mL of Spezyme CP/g of dry fiber. Solid loading was fixed at 5% w/v. After 35 h of reaction, the obtained slurry was autoclaved at 100°C for 15 min to deactivate the enzymes, and

allowed to settle overnight at 4°C. The supernatant (hydrolysate) was carefully withdrawn. The remaining solid residues were freeze-dried and stored at room temperature for a subsequent evaluation in sacco and in vitro as an animal feed *(26)*.

Fermentation

Prior to the fermentation experiments, hydrolysates obtained from 20-L 316 stainless steel bioreactors were centrifuged by Alfa-Laval centrifuge (LAPX 202 Model), at 10,000 rpm, 160 mL/min, in order to remove small amounts of solid particles. The hydrolysates of AFEX-treated forages and agricultural residues contained 20–24 g/L total sugars. They were supplemented with 0.25 g/L of KCl and 0.4 g/L of H_3PO_4, then autoclaved at 120°C for 20 min. After cooling to 30°C, 2 g/L of urea and 1 mL/L of a vitamin solution (1.0 g/L thiamin HCl, 1.0 g/L calcium pantothenate, 0.6 g/L biotin, HCl 0.05 *N*) were aseptically added to the media. These media were then inoculated with a concentrated yeast solution (6–7 g/L), so that the final concentration of *P. tannophilus* in the fermentation broth was 0.5 g/L. The amounts of added nutrients were those found optimum and satisfactory for ethanol production by Beck *(27)*. The composition of the resulting medium was such that it acted as a buffer, maintaining at pH 5.0 ± 0.1 during the fermentation. Separate 150-mL Erlenmeyer flasks were prepared for each sample and filled with a working volume of 100 mL. Flasks were placed in a Model G-53 rotary shaker (New Brunswick Scientific, New Brunswick, NJ) at 75 rpm and ambient temperature (25°C). Fermentations were monitored for 2–5 d by removing 5-mL samples, which were centrifuged with an RC5C model centrifuge (Sorvall Instruments, Dupont Canada) at 16,320*g* for 10 min at 4°C. Pellets were washed with 8% w/v saline (NaCl) solution, resuspended in dH_2O, centrifuged again, and dried at 105°C until constant weight. Supernatants from the first centrifugation were analyzed for ethanol, acetaldehyde, and carbohydrates.

Analytical Methods

AFEX-treated and nontreated biomasses were analyzed for their lignin, cellulose, ash, and hemicellulose contents using methods reported elsewhere *(25)*.

Sugar analysis was performed as silylated sugars using HP 5890 GC system equipped with an FID detector, at 350°C, with He at 30 mL/min. STOX and TMSI (Pierce, Rockford, IL) were used as derivatization reagents; myo-inositol was used as internal standard. The total reducing sugars of these solutions was also determined by the DNS method of Miller *(24)*.

Ethanol and acetaldehyde were determined by GC with an FID detector, an HP 19395A headspace injector, an auto-sampler system, and an HP DB-WAX 30 m × 0.25 mm capillary column (J & W Scientific, Rancho

Cordova, CA) running at 250°C with He at 30 mL/min. Ethylformate was used as internal standard.

RESULTS

As seen in Table 1, the cellulose content of all AFEX-treated forages and agricultural residues did not change substantially from that of nontreated materials. However, the hemicelluloses lost simple sugars, or methyl and acetyl groups, during the treatment, but these could be accounted for in the water extracts. The organic degradation products from lignin could be accounted for in the ethanol–toluene extracts. The proportion of pentosans in the hemicellulose fraction of nontreated biomasses represented 62% for corn stalks, 69% for barley straw, 80% for alfalfa, 88% for timothy, and 59% for reed canary grass. The residual portion of hemicelluloses usually contains sugars such as galactose, fructose, mannose, and glucose, with glucuronic acid and its methylated derivative. Except for alfalfa, the pentosan fraction increased in the hemicelluloses of AFEX-treated biomasses (for example, to 86% for reed canary grass, and to 95% for timothy), although its proportion in the biomasses remained constant. It thus seems that the AFEX treatment mainly solubilized the sugars making up the highly branched heteropolymeric hemicelluloses. With alfalfa, the treatment conditions seem to have hydrolyzed part of the pentosans as well. The fact that the potential sugars of AFEX-treated materials were 7–11% lower than in the nontreated biomasses might reflect condensation of the soluble sugars with other molecules. The potential sugars represented more than 97.8% of the theoretical amount of glucose and xylose in the cellulose and hemicellulose fractions of all materials.

Five % (w/v) of AFEX-treated materials were subjected to hydrolysis with varying amounts of enzymes at the temperature and pH recommended by the manufacturer of enzymes. Figure 1 shows the hydrolysis profiles for AFEX-treated timothy. When the hydrolysis took place with 5 GCU Cellulase 300/g dry fiber, the addition of Spezyme CP at 2 μL/g dry fiber was found essential for an acceptable 80% of saccharification of the sugars in a reasonable reaction time of 30 h. Varying the concentration of Cellulase 300 when 2 μL of Spezyme CP was present/g of dry fiber did not improve the saccharification, except at the level of 15 GCU, at which a substantial increase in early productivity of the released sugars was found: More than 50% saccharification was obtained after only 4 h. However, this level was not judged economically viable, since it would represent a cost of $360 per metric ton of AFEX-treated biomass if the price of Cellulase 300 (commercial grade) was $6/kg. Timothy not treated by AFEX was also hydrolyzed with 5 GCU Cellulase 300 and 2 μL of Spezyme CP/g dry fiber. As in the case of all other biomasses, only 35% saccharification was obtained in about 30 h *(25)*. This proves that the AFEX treatment did provide better accessibility for the enzymes.

Table 1
Composition of Forages and Agricultural Residues Before or After AFEX-Treatment[a]

	Nontreated biomass (% dry matter)					AFEX-treated biomass (% dry matter)				
	Corn stalks	Barley straw	Alfalfa	Timothy	Reed canary grass	Corn stalks	Barley straw	Alfalfa	Timothy	Reed canary grass
α-Cellulose	35.2	32.3	27.6	34.0	27.1	33.8	32.5	28.4	34.9	28.2
Hemicelluloses	32.5	35.0	26.4	23.2	28.1	26.7	24.2	19.0	21.2	19.4
Klason lignin	8.1	6.4	6.5	11.5	15.9	5.1	3.5	5.5	8.9	11.3
Hot Water extracts	13.1	12.2	20.0	21.1	14.8	17.3	24.1	28.8	23.2	24.7
Ethanol-toluene extracts	9.0	8.4	12.6	6.2	8.1	13.2	9.9	11.4	8.0	10.6
Ash	4.8	5.9	9.0	5.9	7.9	4.6	5.6	6.6	5.4	7.0
Total	102.7	100.2	102.1	101.9	101.9	100.7	99.8	99.7	101.6	101.2
Pentosans	20.0	24.0	21.0	20.4	16.6	20.7	21.3	15.2	20.2	16.6
Potential sugars[b]	75.2	74.4	59.9	63.5	61.3	67.2	63.0	52.6	62.9	52.8

[a] Average of three samples.
[b] From acid hydrolysis.

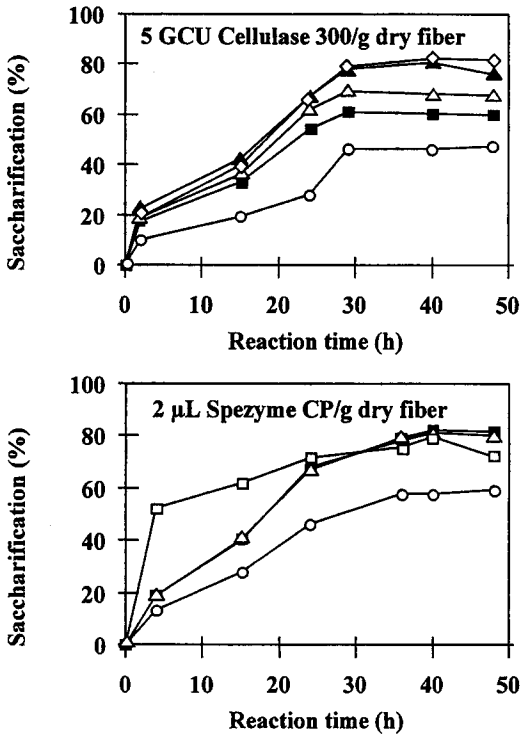

Fig. 1. Enzymatic hydrolysis of AFEX-treated timothy (5% dry basis in 250-mL Erlenmeyer flasks) with 5 GCU Cellulase 300 and Spezyme CP at: (○), 0 µL; (■), 0.5 µL; (△), 1 µL; (◇), 2 µL; (▲), 3 µL/g dry fiber; or with 2 µL Spezyme CP and Cellulase 300 at: (○), 3 GCU; (■) 5 GCU; (△), 6.8 GCU; (□), 15 GCU/g dry fiber.

Maximum saccharification of AFEX-treated alfalfa also occurred around 30 h (Fig. 2). When 2 GCU Cellulase 300 were present/g of dry fiber, saccharification reached a maximum of 65% at 2–3 µL of Spezyme CP. This was slightly superior to AFEX-treated timothy at 2 µL of Spezyme CP with 3 GCU Cellulase 300 (*see* Fig. 1). Increasing the concentration of Cellulase 300 in AFEX-treated alfalfa to 5 GCU/g dry fiber showed only a small improvement. Saccharification levels of 80% similar to those for AFEX-treated timothy were finally reached with 8 GCU Cellulase 300 and 2–3 µL of Spezyme CP.

Table 2 shows results of hydrolysis after 24 and 48 h for the other studied AFEX-treated biomasses. Hydrolysis was usually faster with 2 and 3 µL of Spezyme CP at all concentrations of Cellulase 300, but their hydrolysis profile was similar. Corn stalks seemed to be more resistant to hydrolysis, with maximum saccharification never surpassing 70%, but barley straw and reed canary grass attained more than 80%.

Simple sugars expected from the hydrolysis of AFEX-treated materials were glucose (from the cellulose portion of the fibers), and xylose, arabinose, galactose, fructose, mannose, and glucose (from the hemicellu-

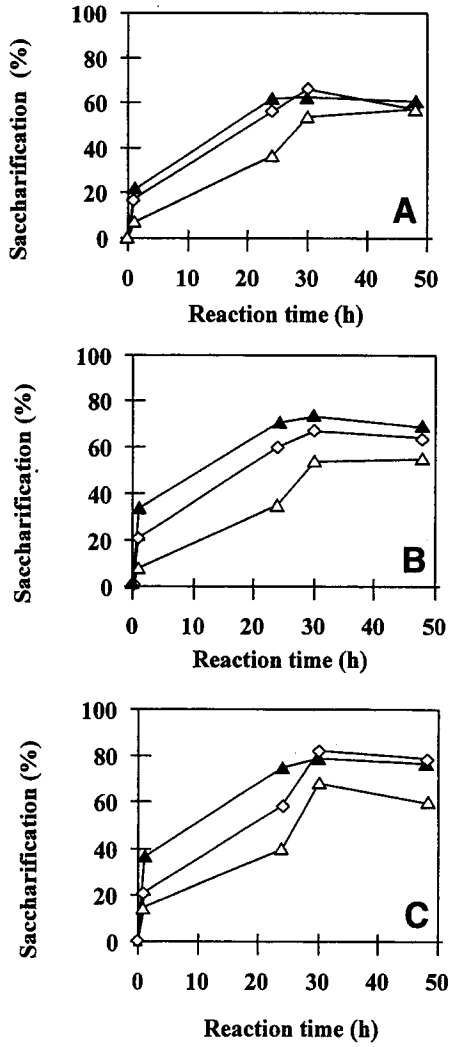

Fig. 2. Enzymatic hydrolysis of AFEX-treated alfalfa with Cellulase 300 and: (△), 1 μL; (◇), 2 μL; (▲), 3 μL of Spezyme CP/g dry fiber. **(A)** 2 GCU Cellulase 300/g dry. **(B)** 5 GCU Cellulase 300/g dry. **(C)** 8 GCU Cellulase 300/g dry.

lose portion), with a 2:1 glucose:xylose ratio. The simple sugars released during the hydrolysis of AFEX-treated timothy are shown in Fig. 3. The concentrations shown at the start of hydrolysis in Fig. 3A represent the soluble sugars. About 12 g/L of glucose were released after 40 h of hydrolysis; 4 g/L of xylose, and an almost equal quantity of arabinose and galactose, were released. Assuming that all the glucose was released from the cellulose, one can say that 65% of the cellulose and 99% of the hemicelluloses were hydrolyzed from the AFEX-treated fibers. About 1.6 g/L of the dimer cellobiose were also produced from the cellulose in the first 30 h, and contributed to 8.4% of cellulose solubilization. Then a total of 73.4%

Table 2
Enzymatic Hydrolysis of AFEX-Treated[a] Barley Straw, Corn Stalks, and Reed Canary Grass with Cellulase 300 and Spezyme CP

	Barley straw		Corn stalks		Reed canary grass	
	Saccharification (%) after:					
Enzyme loadings	24 h	48 h	24 h	48 h	24 h	48 h
2 GCU Cellulase 300:						
1 μL Spezyme CP	33.12	56.36	25.44	54.34	36.79	46.15
2 μL Spezyme CP	62.25	61.18	42.06	57.28	58.88	53.05
3 μL Spezyme CP	67.30	67.30	51.02	67.00	58.88	61.72
5 GCU Cellulase 300:						
1 μL Spezyme CP	55.83	50.48	40.15	58.95	49.06	49.37
2 μL Spezyme CP	76.40	68.07	44.62	65.72	56.81	71.69
3 μL Spezyme CP	75.71	71.74	50.31	62.46	68.01	72.69
8 GCU Cellulase 300:						
1 μL Spezyme CP	67.34	62.25	51.72	62.48	41.84	64.25
2 μL Spezyme CP	69.67	76.48	52.23	67.00	57.73	84.96
3 μL Spezyme CP	65.39	82.60	51.59	70.20	61.18	81.89

[a] 5% (dry basis) of biomass in 250-mL Erlenmeyer flasks.

of cellulose fraction were converted after 40 h. The decrease in monomer concentration after 40 h could not be blamed on the consumption by microorganisms, since NaN_3 was present in the medium. Other researchers *(28–31)* have found inhibition of exoglucanases or β-D-glucosidase by cellobiose or glucose just formed and condensation of monomers into polymeric compounds. Although the presence of lignin and solvent-extractable components, such as tannins and terpenes, could also negatively affect hydrolysis, no such inhibition was found in the aqueous hemicellulose-rich liquors derived from aqueous/steam fractionation of these forages *(12)*. Possible inhibition of hemicellulases (xylanases, β-D-xylosidase) and pectinases by xylobiose or xylose were also reported *(32)*. Figure 3B confirms the adequacy between the DNS results for total reducing sugars and the total simple sugars detected by GC.

Increasing the solids loading to 10% (w/v) during hydrolysis (Fig. 4; full symbols) from 5% (open symbols) almost reduced the saccharification by half. The studied enzyme/substrate did not seem to follow classical saturation kinetics as demonstrated by Penner and Liaw *(33)* working with microcrystalline cellulose at 1–2% w/v solids loading. At a 5% solids loading, a 25% reduction in hydrolysis occurred in the 20-L vessel, as opposed to reaction in the 250-mL flasks. This was probably caused by the presence of dead volumes in the larger vessel, resulting in less contact between the enzymes and their substrates. Using a 2-L vessel, as opposed to a 250-mL flask, had a negligible impact at the 10% solids level. This was expected,

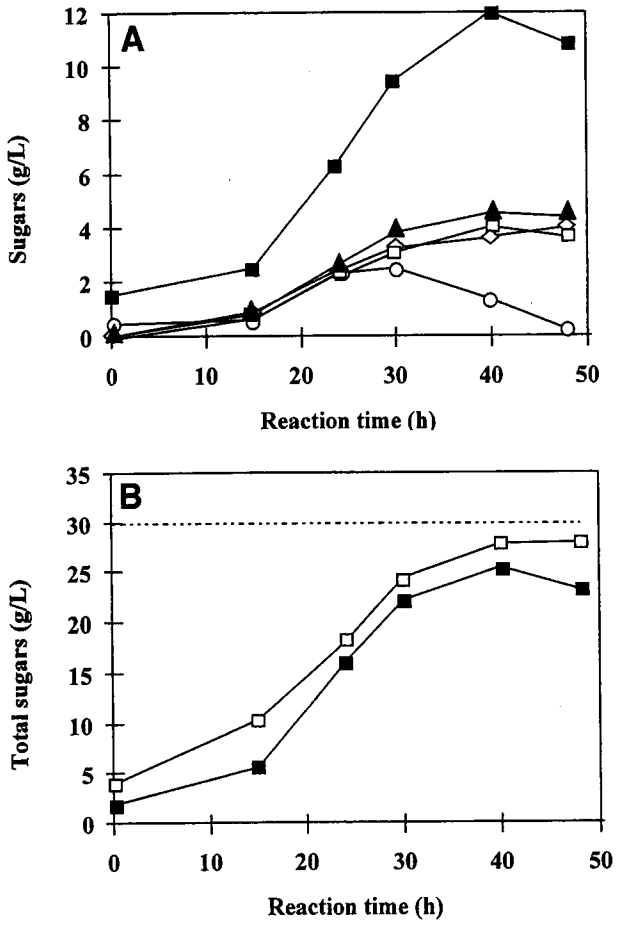

Fig. 3. Sugar profiles during hydrolysis of AFEX-treated timothy with 5 GCU of Cellulase 300 and 2 μL of Spezyme CP/g dry fiber, 5% w/v of solid loading in 250-mL flasks. Symbols: **(A)** (■), glucose; (▲), xylose; (□), arabinose; (◇), galactose; (○), cellobiose; **(B)** (□), total sugars by GC method; (■), total sugars by DNS method. Dashed line represents the potential sugars from Table 1.

since both reactors were magnetically stirred with a single bar in the bottom of the vessels.

The fermentation capabilities of *P. tannophilus* ATCC 32691 were first checked with mixtures of pure glucose and xylose (Fig. 5). When only glucose was present in the medium, the yeast converted 90% of the sugar into ethanol. Only 65% of the xylose could be converted into ethanol when xylose was the sole sugar in the medium. When xylose and glucose were mixed together, less and less ethanol could be produced out of the sugars, until a yield of 30% was obtained with 75% xylose in the mixture. This proved that catabolic repression by glucose would be present with this yeast, as investigated by Panchal et al. *(34)*. Since hydrolysates obtained from the studied AFEX-treated biomasses contained about 25 to 45% xy-

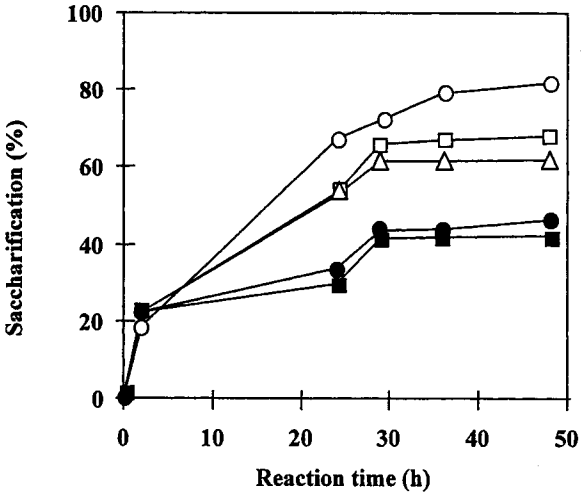

Fig. 4. Enzymatic hydrolysis of AFEX-treated timothy with 5 GCU of Cellulase 300 and 2 μL of Spezyme CP/g dry fiber. Effect of solids loading and scale of bioreactors. Symbols: (○), 5% w/v of solids in 250-mL flasks; (□), 5% w/v of solids in 2-L flasks; (△), 5% w/v of solids in 20-L bioreactors; (●), 10% w/v of solids in 250-mL flasks; (■), 10% w/v of solids in 2-L flasks.

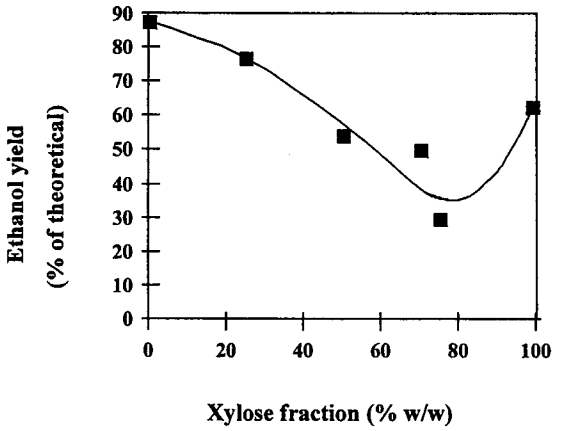

Fig. 5. Performance of *P. tannophilus* ATCC 32691 in pure glucose/xylose mixtures at 20 g/L total sugars.

lose, expected ethanol yields of 65 to 75% were deemed acceptable for this project.

Fermentation profiles of hydrolysates from AFEX-treated barley straw and corn stalks are shown in Fig. 6. The hydrolysates contained 20 g/L of total sugars. In the case of barley straw, 13 g/L sugars were converted into a little more than 6 g/L ethanol during 48 h of fermentation. There was almost no growth of the yeast *P. tannophilus*, and almost no

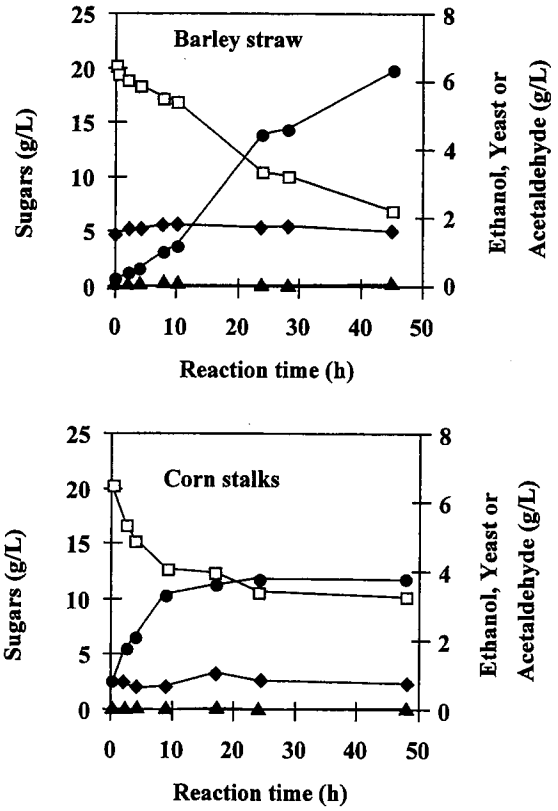

Fig. 6. Fermentation profiles of hydrolysates from AFEX-treated barley straw and corn stalks using *P. tannophilus* ATCC 32691. (□), sugars; (●), ethanol; (◆), yeast; (▲), acetaldehyde.

production of acetaldehyde, a normal byproduct formed by ethanolic yeasts. Other byproducts of ethanolic fermentation were probably produced, but were not measured. Since 1.96 g sugars are theoretically needed to produce 1 g ethanol, and 2.0 g sugars for growth of the yeast, a total of 12.2 g sugars were theoretically needed to account for the ethanol production and the growth of the yeast over that 48-h period. This represented 94% of the 13 g sugars consumed/L. The performance of *P. tannophilus* with hydrolysates from alfalfa and reed canary grass was similar to that of barley straw (results not shown). In the case of corn stalks, the consumption of sugars stopped shortly after 24 h, although half of the sugars remained with less than 3 g/L of ethanol produced. Since the conversion into ethanol represented only 65% of the theoretical yield at that time, it is possible that some inhibition occurred from the 13.2% ethanol–toluene extracts (*see* Table 1; highest value of all the AFEX-treated biomasses).

Almost 3.7 g/L ethanol were formed in a hydrolysate from timothy (Fig. 7a) in the first 24 h. This represents 26 g ethanol produced/100 g sugars consumed, or 50% theoretical yield. Some growth of the yeast simul-

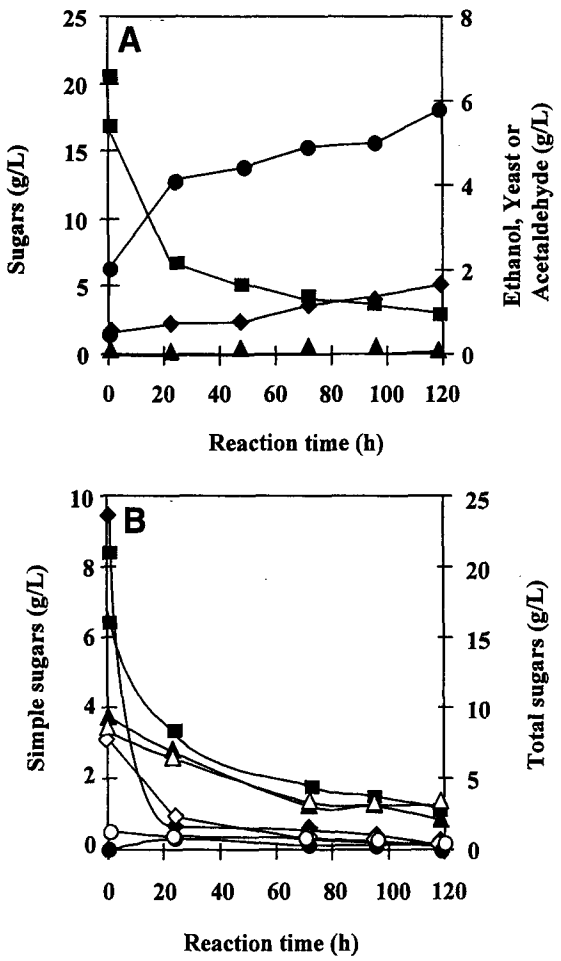

Fig. 7. Fermentation of hydrolysate from AFEX-treated timothy using *P. tannophilus* ATCC 32691. **(A)** Total sugars, ethanol, growth of yeast and acetaldehyde: (■), sugars; (●), ethanol; (◆), yeast; (▲), acetaldehyde. **(B)** Sugar profiles: (■), total sugars by GC method; (◆), glucose; (▲), xylose; (△), arabinose; (◇), galactose; (○), cellobiose; (●), xylitol.

taneously occurred (0.1 g/L). Fifty-one % of the 14.5 g/L sugars consumed accounted for the ethanol production and the growth of the yeast at that time. A small amount of acetaldehyde (no more than 0.1 g/L) was produced during the first 10 h of fermentation, but subsequently disappeared. Fermentation continued at a slower pace, until, at 120 h, almost all of the sugars had been consumed. The total amount of ethanol produced at that time was about 5.4 g/L, resulting in 53% of theoretical yield. The fate of the simple sugars during fermentation of the timothy hydrolysate of Fig. 7A is shown in Fig. 7B. Glucose was almost completely taken up in the first 24 h, and was largely responsible for the decrease in total sugars. More than one-third of the galactose was used in the same 24 h, but was

not expected to be converted into ethanol *(35,36)*. Xylose was slowly metabolized throughout fermentation, but was not converted into ethanol. In the first 24 h, 0.4 g/L xylitol was produced from xylose and represented half of the xylose decrease in the medium. Arabinose was also slowly catabolized throughout fermentation, but was not expected to be converted into ethanol *(37)*. If it were converted into arabitol, the concentration was expected to be much less than that of xylitol *(38)*, thus, not detectable. On the other hand, cellobiose was not utilized by the yeast. This behavior was similar to that of another common ethanolic yeast, *Saccharomyces cerevisiae*.

Table 3 compares the performance of *P. tannophilus* for all studied biomasses. After 24 h of fermentation, sugar uptake was the fastest in timothy hydrolysates (66% of initial sugars had been used). However, the yeast performed at less than acceptable yield (only 53% of its capacity, based on the sugars consumed). Barley straw was found to be the most performant biomass after 24 h, with almost 50% of the initial sugars converted to 4.5 g/L ethanol, and working very near full capacity (92%). Similar results were found after 48 h of fermentation. Barley straw also gave the fastest average production of ethanol: 0.19 g ethanol/(L·h) after 24 h, and 0.13 g/(L·h) after 48 h of fermentation.

DISCUSSION

Whole forages and agricultural residues were treated by AFEX, enzymatically hydrolyzed, and fermented to ethanol with a nonengineered yeast. These steps represent the goal of the Canadian Green Plan Ethanol Program of putting forward a process as environmentally friendly as possible. This placed constraints on the process: The pretreatment must not generate compounds potentially inhibitory to either the enzymes or the ethanolic yeast; the milder conditions of enzymatic hydrolysis require longer reaction contact than for acid hydrolysis, and the ethanolic yeast must be able to convert, in a single step, both hexoses and pentoses released from the enzymatic hydrolysis.

AFEX Pretreatment

All biomasses studied in this project reacted similarly when treated with liquid ammonia under pressure: The pretreatment solubilized part of the hemicellulose and partially degraded the lignin into compounds that seemed not to be detrimental to either enzymes or yeast in the conditions used. It is not known why alfalfa's hemicelluloses were more degraded than that of other biomasses. Its lignin content was initially similar to that of barley straw and was even less degraded than its barley counterpart; its hemicellulose content was similar to those of timothy and reed canary grass. The composition and structure of xylan are more complicated than those of cellulose, and can vary qualitatively and quantitatively in various grasses and cereals *(39)*. They are highly branched and substituted

Table 3
Performance of *Pachysolen tannophilus* ATCC 32691 in Hydrolysates from AFEX-Treated Forages and Agricultural Residues after 24 h and 45 h[a]

Biomass	Sugar uptake (% of initial content)	Ethanol (g/L)	Yield[b] (% theoretical)	Yield[c] (% theoretical)	Productivity (g EtOH/L.h)
Corn stalks	46.6 ***(48.9)***[d]	3.8 ***(3.7)***[d]	37 ***(37)***[d]	79 ***(75)***[d]	0.16 ***(0.08)***[d]
Barley straw	47.7 ***(65.0)***	4.5 ***(6.3)***	44 ***(62)***	92 ***(95)***	0.19 ***(0.13)***
Alfalfa	37.2 ***(54.0)***	3.8 ***(5.7)***	38 ***(56)***	100 ***(100)***	0.16 ***(0.12)***
Timothy	66.1 ***(67.9)***[d]	4.1 ***(4.4)***[d]	35 ***(37)***[d]	53 ***(55)***[d]	0.15 ***(0.08)***[d]
Reed canary grass	35.4 ***(50.4)***	3.1 ***(5.2)***	31 ***(51)***	87 ***(100)***	0.13 ***(0.11)***

[a] Values in parentheses and bold characters.
[b] Based on total initial sugars available for the fermentation.
[c] Based on consumed sugars.
[d] Value after 48 h of reaction.

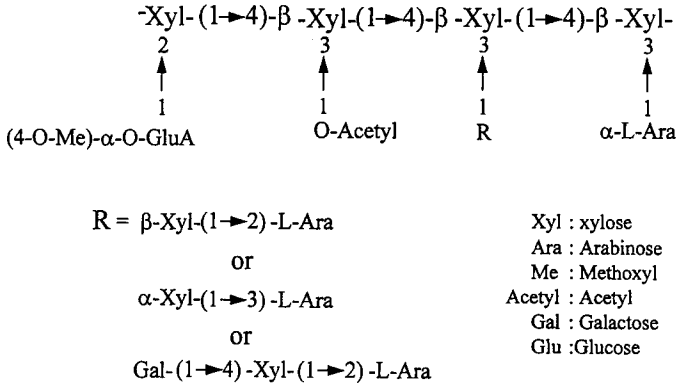

Fig. 8. Idealized structure of hemicellulose showing predominant structural features and modes of linkage.

as indicated in Fig. 8. These substitutions allow various reactions to occur in hemicelluloses.

Hemicellulases were found necessary to hydrolyze the AFEX-treated biomasses, as evidenced by the need for Spezyme CP in Fig. 1. The enzymatic activities of the enzymes used in this work was reported elsewhere *(12)*. Thus, the AFEX treatment might not have succeeded in physically separating the cellulose backbone from the hemicelluloses, or this separation occurred only at the ends of the cut fibers. Alfalfa, despite a more degraded hemicelluloses, was harder to hydrolyze than timothy or even barley straw. This seems to prove the preceeding comment.

Preliminary studies showed that the average size of the AFEX-treated biomasses' particles (in the range of 1 to 4-5 mm) had little effect on the yield of hydrolysis *(25)*. The AFEX pretreatment increased the protein degradability of the biomasses caused in part by a 1 wt% residual ammonia in them *(26)*. More than 98% of the liquid ammonia is released to the atmosphere after the AFEX treatment. It could be recompressed and recycled to make the process even more environmentally friendly. This means that the economics of recovering and reusing the 99% released after treatment should be investigated.

Hydrolysis

The AFEX pretreatment was found essential for the hydrolysis of at least 50% of the sugars in the biomasses. Alvo and Belkacemi *(40)* proved, however, that milling non-AFEX-treated biomasses down to 50 μm could yield up to 60% of saccharification. The cost of this alternative should be assessed before rejecting it. Since Cellulase 300 was found to possess a more pronounced filter paper activity than the Spezyme CP, but less β-D-xylosidase activity (result not shown), it is postulated that when Spezyme CP was added, the hemicelluloses could be opened up somewhat, giving

access to the glucanases of the Cellulase 300 enzymatic complex. However, only a little xylose was released by the process in the first 15 h of reaction, as can be seen in Fig. 3, in which its concentration was half that of glucose.

During hydrolysis of timothy, a very slight inhibition by cellobiose could be demonstrated by the slight change of slope in the concentration of glucose produced at the peak of the cellobiose release, and, possibly, by the sharp decrease in glucose prior to the complete exhaustion of the cellobiose in the hydrolysate. The similar aspect of the xylose and arabinose curves during the same period might infer a possible inhibition by xylobiose, although this compound was not assayed for in the hydrolysate. These findings are in agreement with the literature *(28,31,41,42)* although condensation of these simple sugars could have also occurred.

Late harvesting of mature forages for ethanol production could be a complementary activity for livestock farmers who usually harvest forages early because of the higher feed value of immature hay. However, the increased lignin content of mature forages makes them more resistant to hydrolysis, as shown in Table 4, in which our results are compared with that of the literature.

The solid residue after hydrolysis was evaluated by Chiquette *(26)* as a potential animal feed. *In sacco* and in vivo studies seemed to indicate improved degradability and digestibility of the AFEX-treated and partially hydrolyzed forages. The increased N-ADF content seems to prove that enzymes remained mostly with the fibers. These fibers could be recycled into the hydrolysis step to reduce the amount and cost of enzymes required. But more studies are needed to evaluate the inhibition of the enzymes during hydrolysis and their reactivation with fresh AFEX-treated forages and medium.

Fermentation

No engineered microorganisms were used in this work, despite numerous potential bacteria and yeasts in the literature *(43–47)* since the mandate included an environmentally benign aspect. *P. tannophilus* ATCC 32691 was chosen solely on the basis of its description in the catalog as being able to convert wheat straw cellulose/hemicellulose to ethanol. Many publications reported also that *P. tannophilus* was able to ferment pure xylose *(48–50)*. Its ethanol yield in enzymatic hydrolysates from AFEX-treated herbaceous crops varied from 40 to 60% of theoretical, based on total sugars. These values were similar to those obtained with many other ethanolic microorganisms capable of converting both glucose and xylose *(51,52)*. All pentose-fermenting microorganisms usually produce ethanol at a slower rate than *S. cerevisiae (53,54)*. No fermentation with *S. cerevisiae* was done, since this yeast was known not to be able to convert xylose into ethanol.

Growth of the yeast did not usually occur during the first 48 h of fermentation in the hydrolysates, whether a consumption of sugars oc-

Table 4
Relationship Between Maximum Saccharification of AFEX-Treated Lignocellulosic Substrates and Their Lignin Content Prior to Treatment

Substrate	Lignin content (% dry wt)	Maximum saccharification (% of theoretical)	Ref.
Corn stalks	8.1	67	This work
Barley straw	6.4	82	This work
Alfalfa	6.5	82	This work
Timothy	11.5	80	This work
Reed canary grass	15.9	82	This work
Bermuda costal grass	6.4	94	*(20)*
Switchgrass[a]	5.5	96	*(19)*
Switchgrass[b]	12.0	68	*(19)*
Corn fiber	7.8	>85	*(17)*
Corn fiber	1.4	100	*(19)*
Newspaper	30.0	<40	*(21)*

[a] Spring harvest.
[b] Fall harvest.

curred or not. This was a bit unexpected, since the initial inoculum concentration was only 0.5 g/L. On the other hand, reduced growth meant that more energy could be channeled into ethanol production. Hydrolysates from timothy showed growth of the yeast after the first 48 h, coupled with a slow increase in ethanol production. This showed that the AFEX treatment did not produce substantial amounts of inhibitory products. However, a scale-up of the whole process might have to include a concentration step of the hydrolysates, to decrease costs associated with the separation of dilute ethanol from the fermentation broth. The influence of the AFEX conditions on the production of certain inhibitory compounds should thus be evaluated.

Xylose was shown to be slowly consumed, with a weak production of xylitol in the early stages of fermentation. No attempts were made to measure the amount of ethanol actually produced from the conversion of xylose through the xylitol metabolic pathway.

Fermentation generated 10–15 L of stillage per L of ethanol recovered. The chemical oxygen demand value was 25,000 mg O_2/L, comparable to stillage from breweries (20,000 mg O_2/L) *(55)*.

CONCLUSIONS

Late harvesting of mature forages for ethanol production in eastern Canada could be a complementary activity for livestock farmers, who usually harvest herbaceous crops early because of the higher feed value of immature hay. However, the increased lignin content of mature forages makes them more resistant to hydrolysis.

Based on our results with barley straw (63% sugars in the AFEX-treated feedstock; 76.4% saccharification yield after 24 h of hydrolysis; 62% theoretical yield after 48 h of fermentation), an expected yield of 190 L ethanol per ton of dry biomass could be achieved with AFEX-treated and enzymatically hydrolyzed forages and agricultural residues. Between 40 and 50% of the original biomasses were hydrolyzed. The remaining portion could be used as value-added residue for animal feed.

Legislation for ethanolic microorganisms, engineered or not, is desperately needed. Without it, no recycling of the solid residue from the fermentation step would be allowed as animal feed. The nutritional value of the studied yeast, *P. tannophilus*, should be evaluated.

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