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Integrated Production of Ethanol Fuel and Protein from Coastal Bermudagrass

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ABSTRACT

The herbaceous crops that may provide fermentable carbohydrates for production of fuels and chemicals also contain 10–20% protein. Protein coproduction with biomass-derived fuels and chemicals has important advantages: (1) food and fuel production can be integrated, and (2) protein is a high-value product that may significantly improve overall process economics. We report the results of an integrated approach to producing protein and fermentable sugars from one herbaceous species, Coastal Bermudagrass (CBG). The ammonia fiber explosion (AFEX) process makes possible over 90% conversion of cellulose and hemicellulose to simple sugars (about 650 mg reducing sugars/g dry CBG) at 5 IU cellulase/g vs < 20% conversion for untreated CBG. The AFEX treatment also improves protein extraction from CBG; over 80% protein recovery is possible from AFEX-treated CBG vs about 30% recovery from untreated CBG.

Index Entries: Herbaceous energy crops; alcohol fuels; cellulase enzymes; protein recovery; Ammonia Fiber Explosion.

INTRODUCTION

Development of Integrated Process

Most biomass refining research to date has focused on individual scientific or engineering aspects of the overall processing system. Comparatively less work has been done on complete biomass refining scenarios

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and on systems analysis issues. Most biomass refining systems developed or studied to data have also emphasized wood species; complete conversion of herbaceous crops has received almost no attention. Some published studies on biomass refining of herbaceous species have noted the potential economic impact of protein recovery in such systems (*1,2*). Some of the advantages of protein recovery in a well-integrated biomass refining system are:

1. Readily available technology for protein recovery;
2. High value of protein;
3. Reduced food/fuel conflicts; and
4. Division of energy inputs between ethanol and protein products.

Protein Recovery Options

Various options exist for protein recovery from herbaceous species including:

1. Mechanical expression of a protein-rich "juice" from green biomass;
2. Separation of protein-rich leaves from stems;
3. Extraction of proteins to produce protein concentrates; and
4. Fermentation of the whole plant to produce a protein-rich fermentation residue.

Although each of these options might find use under particular circumstances, the third option is believed to be the most generally applicable because:

1. Leafy green biomass probably will not be available year round at most biomass refining facilities;
2. Leaf-stem separation is not a complete enough protein separation to achieve good economic advantages;
3. The protein bound to insoluble fermentation residues (mostly lignin) is significantly diminished in its potential feed value by association with undigestible lignin; and
4. Protein concentrates provide the most flexibility in producing foods and feeds.

Protein is a comparatively fragile component of biomass whose potential value can be significantly decreased by the various processing steps. In particular, high temperatures and extreme pH are likely to degrade protein. Therefore, many of the pretreatments used to increase the conversion of cellulose and hemicellulose to fermentable sugars may not be appropriate if protein recovery is an important process goal.

Brief Assessment of Pretreatment Options

Pretreatments to increase the conversion of cellulose to fermentable sugars have been studied for almost a century, and a complete review of this subject is outside the scope of this article. A relatively recent review of pretreatment technologies and techniques is available (*3*). A viable pretreatment must be both effective and economical. Briefly, many pretreatments are effective in facilitating cellulose conversion to fermentable sugars; however, pretreatment options are sharply constrained by the need to produce sugars at a cost of approx $0.05–0.10/pound. Many effective pretreatments fail this test; they just are not economical and have no reasonable hope of ever becoming so. In addition, many pretreatments operate under harsh conditions (e.g., steam explosion, various acid processes, and so on) that tend to degrade the sugars and biomass protein.

A new pretreatment called ammonia fiber explosion (AFEX) has recently been developed, which operates under relatively mild conditions and which appears to be cost-effective (*4*). The AFEX process consists of treating cellulosic materials (grasses, crop residues, garbage paper, and so forth) with liquid anhydrous ammonia under pressure and at moderate temperatures (ca. 30–80°C) for a few minutes and then rapidly releasing the pressure. The rapid pressure release literally blows the fiber apart, greatly increasing the surface area available for enzymatic or microbial attack (*4*). In addition, the cellulose swelling or decrystallizing effect of liquid ammonia also distends the unit cell of crystalline cellulose, opening cellulose up for hydrolysis on the molecular level (*5,6*). Over 99% of the ammonia can be readily recovered and reused.

Economic estimates of the costs of the AFEX process have been performed and appear to be <$10/ton of biomass treated (*7,8*). Essentially complete conversion of plant cellulose and hemicellulose to fermentable sugars is achieved by enzymatic hydrolysis of many AFEX-treated materials. AFEX is not yet effective on softwoods or softwood newsprint. Table 1 summarizes some of the hydrolysis results achieved thus far. Sugar solutions resulting from AFEX treatment and hydrolysis do not appear to contain fermentation inhibitors, and have been demonstrated to be fermentable to ethanol, cellulase, and single-cell protein in high yield (*6*). (Ethanol fermentation studies with glucose- and xylose-fermenting organisms are ongoing in our laboratory.) Finally, little or no decrease in the feed value of protein treated under AFEX conditions appears to occur at temperatures less than about 80°C (*9*). Therefore, the AFEX process appears to have significant potential as an effective and economical biomass pretreatment, particularly for herbaceous energy crops and other materials with low to moderate lignin contents.

The remainder of this article is devoted to exploring the potential for protein recovery from herbaceous crops with simultaneous conversion of

Table 1
Enzymatic Hydrolysis of Some AFEX-Treated and Untreated Lignocellulosics

Sample	Total sugars after hydrolysis, mg/g dry fiber		Percent of theoretical yield in 24 h
	3 Hours	24 Hours	
Alfalfa press cake-treated	342	594	96
Alfalfa press cake-untreated	—	323	65
Barley straw-treated	278	593	83
Barley straw-untreated	100	181	25
Corn stover-treated	476	679	95
Corn stover-untreated	—	201	28
Rice straw-treated	—	584	94
Rice straw-untreated	—	204	32
Wheat straw-treated	—	637	88
Wheat straw-untreated	—	150	21
Switchgrass-treated	605	802	96
Switchgrass-untreated	—	230	28

cellulose and hemicellulose to fermentable sugars. The model herbaceous species chosen is Coastal Bermudagrass previously treated using the AFEX process.

MATERIALS AND METHODS

Coastal Bermudagrass (CBG)

CBG (*Cynodon dactylon* [L] Pers.) was harvested from the Texas A&M University Research Farm in August 1991, 4 wk after the first cut. The grass was fertilized with general-purpose lawn fertilizer at a rate of about 500 kg/ha. The grass was dried in the sun to approx 15% moisture (dry basis) and ground in a Wiley mill to reduce the fiber length to about 6 mm for better handling in the AFEX pretreatment operation. A CBG compositional analysis (cellulose, hemicellulose, lignin, starch, free sugars, and ash) was performed by conventional methods (*10*), and the crude protein content (total nitrogen content × 6.25) was determined by the microKjeldahl method (*11*). The composition is (% 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).

AFEX Pretreatment

Samples of CBG (100 g) were moistened with 30 mL of distilled water and then placed in an electrically heated 4-L pressure vessel. Two parts of

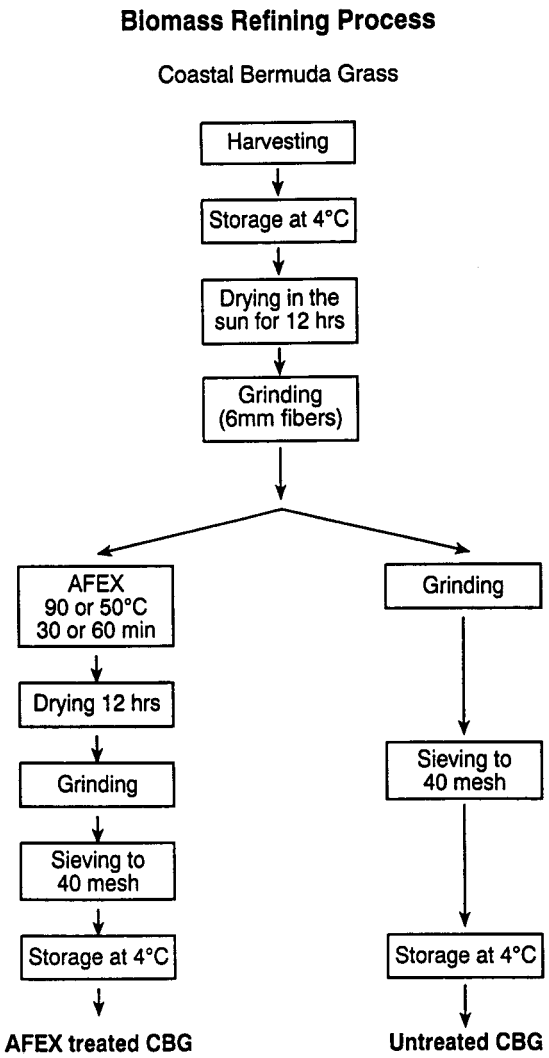

Fig. 1. Pretreatment flow sheet for Coastal Bermudagrass.

liquid anhydrous ammonia (by weight) per one part of dry grass were added. The grass was soaked with mechanical agitation for 30 min at 90°C. The high pressure was then quickly released to atmospheric pressure by opening a large ball valve connected to a 230-L blowdown tank. The ammonia vapors were absorbed into water. The grass was then removed and air-dried overnight to remove residual ammonia. About 0.6% (w/w) of ammonia remains with the fibers as measured by microKjeldahl. This ammonia is not volatile, but is soluble and is presumably the ammonium salts of plant acids. The dried AFEX-treated CBG was then ground to pass a 40-mesh sieve. The CBG pretreatment is summarized in Fig. 1.

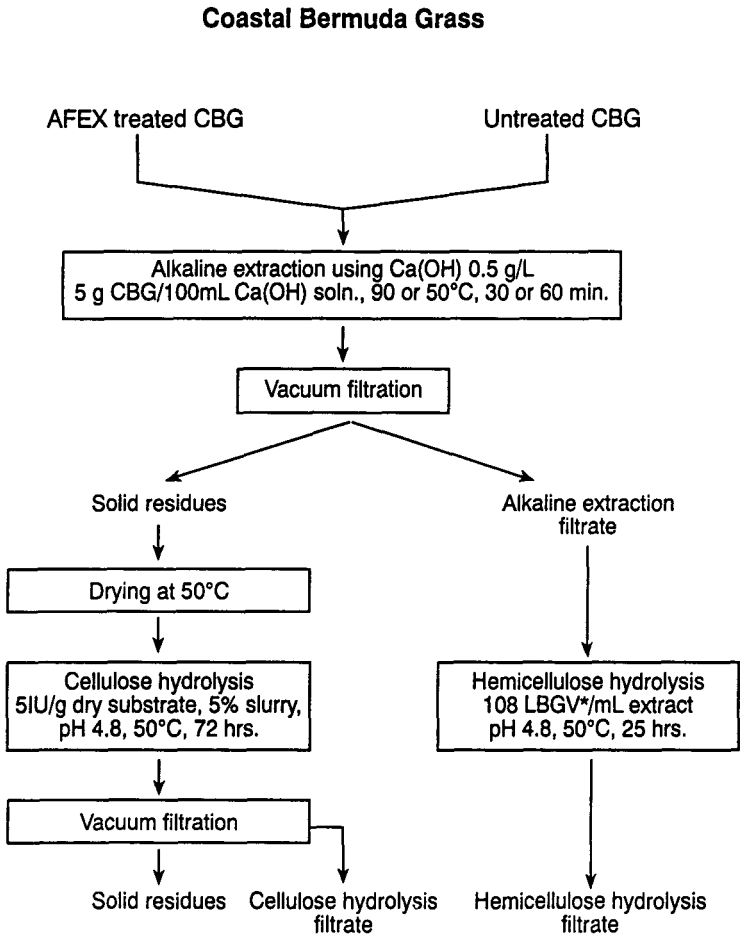

Fig. 2. Protein extraction and reducing sugar recovery process.

Protein and Reducing Sugar Recovery

The protein and reducing sugar recovery (PRSR) process is comprised of three main steps. The first step involves extraction of most of the protein by dilute alkaline solutions (*12,13*). Vacuum filtration then separates the solids from the liquid filtrate. In the second step, the solid residues are hydrolyzed enzymatically using cellulases to recover most of the reducing sugars in the CBG. Some of the carbohydrate content in the grass (mainly hemicellulose) will be solubilized by the alkaline extraction. Therefore, to increase the reducing sugar yield, the liquid fraction remaining after the alkaline extraction is hydrolyzed enzymatically using a commercial hemicellulase/cellulase mixture. The PRSR process scheme is presented in Fig. 2. An untreated portion of CBG was subjected to the PRSR process as a control to determine the effects of the AFEX treatment.

Alkaline extraction was performed with a calcium hydroxide solution, since calcium is an essential mineral nutrient. Five-grams (dry wt) samples of AFEX-treated or untreated CBG were added to 100 mL of a 0.5 g/L solution of $Ca(OH)_2$ (Sigma Co., St. Louis, MO), pH 11.5 and extracted at 90°C with shaking at 100 rpm for 1 h. After this, the solution was first vacuum-filtered using a glass-fiber filter (grade 934 AH) (Whatman, Inc., Clifton, NJ) and then passed through a 0.22-μ membrane to remove any solid particles. The liquid volume was measured and then refrigerated. The solid residues were removed from the glass-fiber filter, dried at 50°C overnight, and weighed.

The enzymatic hydrolysis of the solid residues was performed in a 5% (w/v, solid/liquid) slurry of biomass in a 0.05*M* citrate buffer, pH 4.8. Five international units of cellulase and 28.4 CBU of cellobiase/g of dry grass were used. Cellulase (Cytolase 300™, Genencor, Inc., San Francisco, CA) activity was 132 IU/g of enzyme measured with the standard filter-paper assay (*14*). Cellobiase (Novozyme 188™, Novo Laboratories, Wilton, CT) activity of 250 CBU/mL was reported by Novo Laboratories. The hydrolysis was carried out at 50°C in a 100 rpm shaking air bath for 72 h. To avoid microbial contamination, sodium azide (0.15%) was added. One-milliliter samples were taken at 0, 1, 3, 12, 24, 48, and 72 h. Samples were boiled in capped test tubes for 15 min to stop the hydrolysis and then filtered through a 0.22-μm nylon membrane.

The enzymatic hydrolysis of the liquid fraction was performed by diluting 1 part (volume) of the alkaline extract with 2 parts of 0.05*M* citrate buffer, pH 4.8 and then adding a commercial hemicellulase/cellulase mixture (Cytolase M103S™, Genencor, Inc., San Francisco, CA; 108 locust bean gum viscosity units [LBGV]/mL of alkaline extract). Cytolase M103S™ has a reported activity of 10,800 LBGV/g of enzyme. The hydrolysis was carried out at 50°C in a 100-rpm shaking air bath for 25 h. Sodium azide (0.15%) was added to avoid any contamination. One-milliliter samples were taken at 0, 1, 3, 18, and 25 h. Samples were boiled in capped test tubes for 15 min to stop the hydrolysis and then filtered through a 0.22-μm nylon membrane.

Analytical Methods

Crude protein (CP) (total nitrogen content × 6.25) in the liquid and in the solid fractions was determined with the microKjeldahl technique (*11*) using K_2SO_4/HgO as the catalyst in a 1-h digestion. Total protein in solution was determined by the Bicinchoninic Acid (BCA) assay (*15*) using as the calibration standard a major component of the grass protein: ribulose 1-5 biphosphate carboxylase (from spinach) (Sigma Co., St. Louis, MO). The reducing sugar interference in the determination of total protein concentration was corrected using 1.0 g/L glucose solution as a blank in the assay.

The amino acid composition was determined by the Pico-Tag method (*16*), which involves three main steps:

1. Hydrolysis of the protein sample with 6*N* HCl;
2. Derivatization of the amino acid with phenylisothiocyanate to produce phenylthiocarbamil amino acids; and
3. Analysis of these derivatives by reverse-phase HPLC.

This analysis was performed in the Biotechnology Support Laboratory (Department of Entomology, Texas A&M University).

Total reducing sugars were determined by the Dinitrosalycilic Acid (DNS) assay (*17*). Glucose was used as the calibration standard. Glucose levels were confirmed using a YSI glucose analyzer. Glucose and xylose were measured using HPLC. The samples were eluted with deionized water through an Aminex HPX-87P column (Bio-Rad, Richmond, CA) heated at 85°C, and detected by a refractive index monitor (LDC Analytical, Riviera Beach, FL).

EXPERIMENTAL RESULTS AND DISCUSSION

CBG Composition

The amount of protein potentially extractable from the low-quality CBG used in this study is approx 100 mg of crude protein/g of dry grass. The maximum amount of sugars that can be produced by cellulose/hemicellulose hydrolysis is 674 mg/g dry CBG. Given the reported amylase activity in the cellobiase preparation (Novozyme 188L™), hydrolysis of starch in CBG might also yield 71 mg of sugars/g dry CBG. Therefore, the total sugars potentially available from CBG, including the free sugars and starch, are 765 mg of sugars/g dry CBG. (The water added to the polysaccharide polymers cellulose, hemicellulose, and starch during hydrolysis increases the mass of the total sugar by about 10% above the mass of the polymers.)

Protein Extraction

The protein material balances for the extraction of AFEX-treated and untreated CBG are presented in Tables 2 and 3, respectively. The notation used in these tables (stream names) is shown in Fig. 3. The total nitrogen content was directly measured by the microKjeldahl method in all the inputs (I1, I2, and I3) and outputs (S1, E1, L1, L2, and L3). The protein material balance closure (ratio between the amount of nitrogen in the outputs and nitrogen in the inputs) found was 99% for the AFEX-treated CBG case and 89% for the untreated CBG case. The total nitrogen content after the AFEX pretreatment increases from 16 to 26 mg of nitrogen/g dry CBG. The amount of crude protein extracted was obtained from the CP

Table 2
Protein Material Balance for the AFEX-Treated CBG Case[a]
(Stream Descriptions Are Given in Fig. 3)

Stream	I1	I2	I3	E1	E2	L1	L2	L3	S1
Mass (g, dry wt)	5.0	—	—	5.1	3.0	—	—	—	1.3
Volume (mL)	—	60.0	140.0	—	—	70.0	45.0	210.0	—
Nitrogen content (%)	1.6	—	—	2.6	—	—	—	—	2.5
Crude protein concentration (g/L)[b]	—	2.0	1.2	—	—	5.9	3.3	2.6	—
BCA protein concentration (g/L)[c]	—	—	—	—	—	6.4	4.6	2.5	—
Crude protein yield (mg of CP/g dry CBG)	100.0	—	—	—	—	—	5.0[d]	75.0[e]	—

Total crude protein yield = 80 mg of CP/g of dry CBG (calculated with the microKjeldahl method).

Material balance closure = *N* amount in the outputs/*N* amount in the inputs = 8321.4/8365.7 = 99%.

[a]Mean values of duplicate experiments.
[b]Measured by the microKjeldahl method.
[c]Measured by the BCA protein assay.
[d,e]Subtracting the amount of CP added as cellulases or hemicellulases, respectively.

Table 3
Protein Material Balance for the Untreated CBG Case[a]
(Stream Descriptions Are Given in Fig. 3)

Stream	I1	I2	I3	E1	E2	L1	L2	L3	S1
Mass (g, dry wt)	—	—	—	5.0	3.9	—	—	—	3.2
Volume (mL)	—	78.0	154.0	—	—	77.0	78.0	231.0	—
Nitrogen content (%)	—	—	—	1.6	—	—	—	—	1.2
Crude protein concentration (g/L)[b]	—	2.0	1.2	—	—	1.6	2.2	1.4	—
BCA protein concentration (g/L)[c]	—	—	—	—	—	2.4	2.8	0.8	—
Crude protein yield (mg of CP/g dry CBG)	—	—	—	100	—	—	3.6[d]	30.3[e]	—

Total crude protein yield = 33.9 mg of CP/g of dry CBG (calculated with the microKjeldahl method).

Material balance closure = *N* amount in the outputs (g)/*N* amount in the inputs (g)= 118.7/132.4 = 89%.

[a]Mean values of duplicate experiments.
[b]Measured by the microKjeldahl method.
[c]Measured by the BCA protein assay.
[d,e]Subtracting the amount of CP added as cellulases or hemicellulases, respectively.

concentration, and the volume of the liquid fractions recovered after the alkaline extraction/hemicellulose hydrolysis and after the cellulose hydrolysis. The extraction yield was corrected by subtracting the amount of crude protein added as enzyme. The CP extraction yield obtained from AFEX-treated CBG was over twice that obtained from untreated CBG (80 vs 34 mg of CP/g dry CBG).

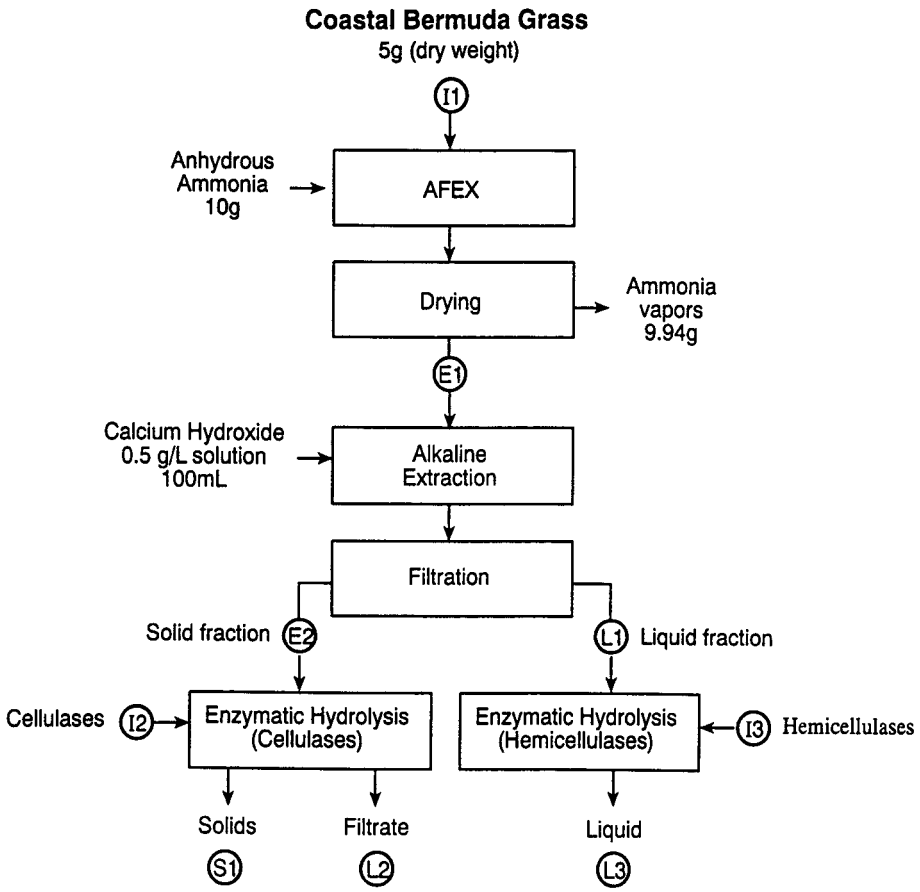

Fig. 3. Material balance framework and stream designations.

The alkaline extraction step releases most of the extractable protein from the AFEX-treated and untreated CBG. In the case of the AFEX-treated CBG, part of the nitrogen measured in the liquid sample after the alkaline extraction might be the result of the ammonia used in the pretreatment. However, the protein extraction yields seem correct for two reasons: (1) measuring total protein in solution with the BCA protein assay shows that the protein nitrogen in the samples obtained from the alkaline extraction/hemicellulose hydrolysis step is approx 90% of the total nitrogen (*see* Table 2), although reducing sugar interference was not completely avoided, and (2) assuming that all the ammonia nitrogen added was solubilized by the alkaline extraction, the corrected extraction yield of true protein (subtracting ammonia and enzyme nitrogen) would be 10–15%. This low figure seems unlikely. There is no reason to believe that AFEX-treated CBG will release less protein than untreated CBG, which has no ammonia added. Thus, we believe that the extraction yield of true protein from the AFEX-treated CBG is about 80% of the total protein potentially available, as calculated here.

The essential amino acid profile of the original protein content in untreated and AFEX-treated CBG (*see* Fig. 4a) is very similar to the previous amino acid analysis of untreated CBG (*18*). The essential amino acid composition is apparently unaffected by AFEX pretreatment. Figures 4B and 4C show the essential amino acid profile of the protein fraction recovered after alkaline extraction and cellulose hydrolysis, respectively. The amino acid profile indicates that the protein fraction recovered by alkaline extraction fulfills FAO requirements (*19*) for threonine, valine, isoleucine, leucine, and phenylalanine-tyrosine. The amino acid profile of the liquid fraction after cellulose hydrolysis satisfies FAO requirements for threonine, valine, leucine, isoleucine, and phenylalanine-tyrosine. The essential amino acid with the lowest content in the alkaline extract and in the cellulose hydrolysis filtrate is methionine. Others have also found that methionine is the limiting amino acid of protein extracts from plant leaf materials (*20,21*).

Reducing Sugar Recovery

The reducing sugar material balance for AFEX-treated and untreated CBG (based on DNSA) is presented in Tables 4 and 5. Sugar yields are expressed in mg equivalents of glucose/g of untreated CBG. The biomass conversion to reducing sugars is almost five times as much for the AFEX-treated CBG as it is for untreated CBG (94% of theoretical vs 20%). The reducing sugar yield was mostly the result of hydrolysis of the solid residues left after the alkaline extraction step. Figures 5 and 6 show the course of the enzymatic hydrolysis for the solid and liquid fractions, respectively. The glucose concentration measured by the YSI glucose analyzer and HPLC for the liquid fractions obtained after cellulose hydrolysis is about 60–65% of the total reducing sugar concentrations.

SUMMARY

High yields of both protein and sugar are possible from an integrated process involving AFEX treatment, protein extraction, and cellulose hydrolysis. This approach should be broadly applicable to herbaceous energy crops. In other "leaf protein" processes, protein recoveries are generally in the range of 50–55%. The much higher protein recoveries obtained here are believed to be the result of the intense disruptive effect of the AFEX treatment and the removal of interfering cell wall material using the cellulase enzyme complex. Very high yields of sugars and protein have been achieved at relatively low enzyme loadings and under conditions that do not seem to degrade the plant proteins. Fermentability of these sugars to ethanol and other products in high yield has already been demonstrated.

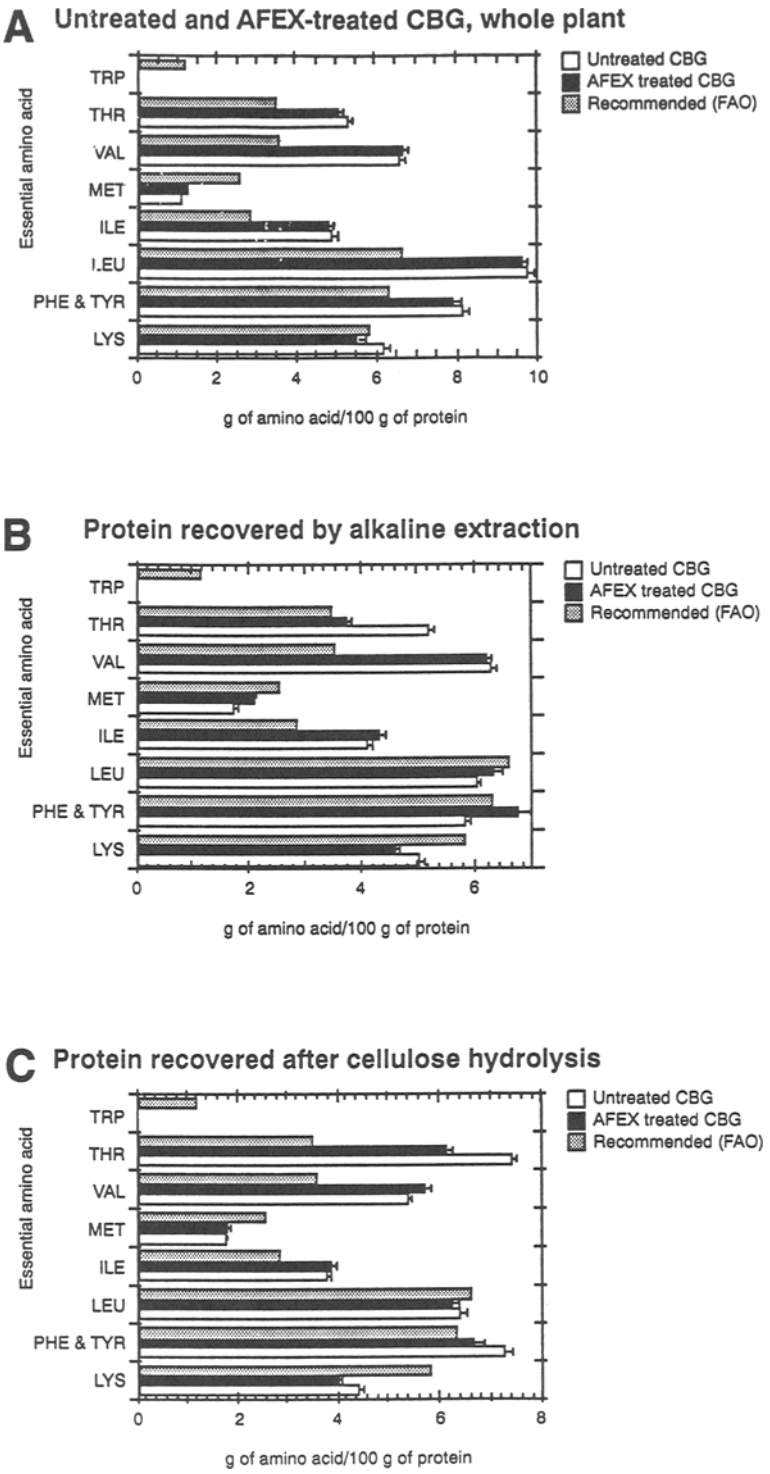

Fig. 4. Amino acid profiles of selected streams. 4A: Untreated and AFEX-treated CBG, whole plant. 4B: Protein recovered by alkaline extraction. 4C: Protein recovered after cellulose hydrolysis.

Table 4
Reducing Sugar Material Balance for the AFEX-treated CBG Case[a]
(Stream Descriptions Given in Fig. 3)

Stream	I1	I2	I3	E2	L1	L2	L3
Mass (g, dry wt)	5.0	—	—	3.0	—	—	—
Reducing sugar concentration (g/L)	—	—	—	—	0.3	32.7	4.9
Glucose concentration (g/L)	—	—	—	—	—	18.3	2.9
Reducing sugar yield (mg Eq glucose/g dry CBG)	765.0	—	—	—	—	617.2[b]	97.5[c]

Total reducing sugar yield = 714.2 mg Eq glucose/g of dry CBG (93.8% of the potential yield).
[a]Mean values of duplicate experiments.
[b,c]Subtracting the reducing sugars contained in the cellulases and hemicellulase preparation.

Table 5
Reducing Sugar Material Balance for the Untreated CBG Case[a]
(Stream Descriptions Given in Fig. 3)

Stream	I1	I2	I3	E2	L1	L2	L3
Mass (g, dry wt)	5.0	—	—	3.9	—	—	—
Reducing sugar concentration (g/L)	—	—	—	—	0.3	7.3	2.6
Glucose concentration (g/L)	—	—	—	—	—	5.9	2.5
Reducing sugar yield (mg Eq glucose/g dry CBG)	765.0	—	—	—	—	124.2[b]	30.5[c]

Total reducing sugar yield = 154.7 mg Eq glucose/g of dry CBG (20.3% of the potential yield).
[a]Mean values of duplicate experiments.
[b,c]Subtracting the reducing sugars contained in the cellulases and hemicellulase preparation.

Current fermentation studies on AFEX-derived sugars focus on xylose to ethanol. The protein extraction and fermentation process produces somewhat higher overall sugar yields under these conditions than does a simple hydrolysis after AFEX treatment. These results clearly illustrate that it is important to treat biomass refining processes as systems.

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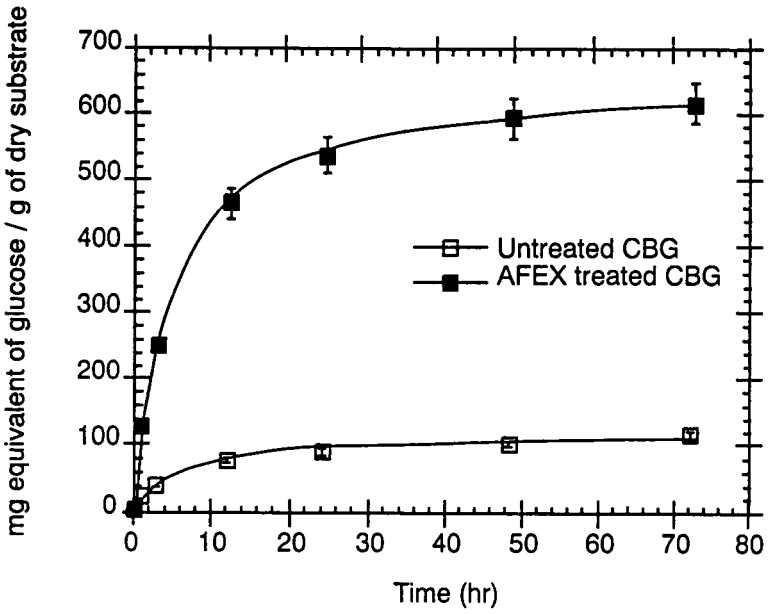

Fig. 5. Enzymatic hydrolysis of AFEX-treated and untreated CBG with protein extraction.

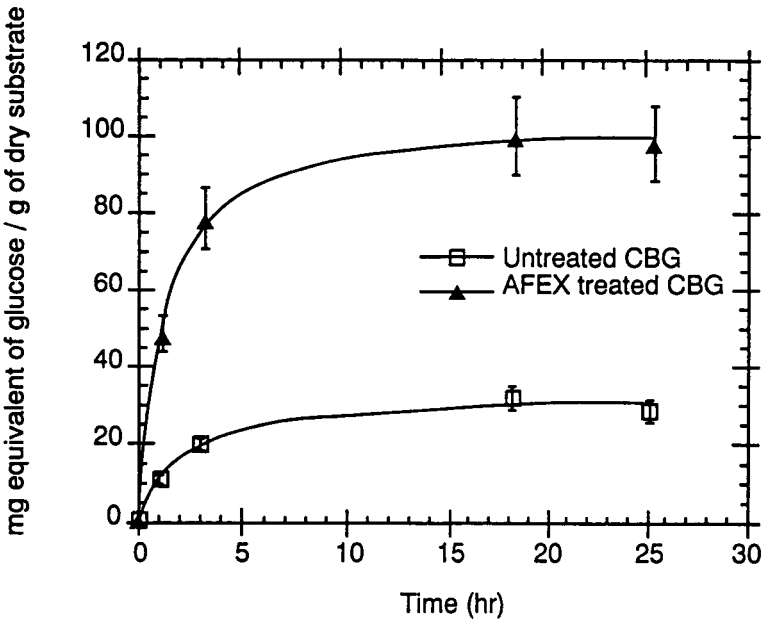

Fig. 6. Enzymatic hydrolysis of the liquid fraction recovered after alkaline extraction.

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