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Extraction of Proteins from Switchgrass Using Aqueous Ammonia within an Integrated Biorefinery

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Abstract Switchgrass (*Panicum vergatum*) is a potential feedstock for future cellulosic biorefineries. Such a feedstock may also provide protein, most likely for use as an animal feed. In this paper, we present a potential scheme for integrating fiber processing with extractions to obtain both sugar and protein products from switchgrass pretreated using Ammonia Fiber Expansion (AFEX). Solutions of 3% aqueous ammonia at pH 10.5 provided optimal extraction of proteins. Addition of the nonionic surfactant Tween-80 improved protein recovery for AFEX-treated materials. It was determined that an extraction following AFEX solubilized approximately 40% of the protein, while a subsequent hydrolysis solubilized much of the remaining protein while producing 325 g sugar per kg biomass. The remaining insoluble residue contained very little protein or ash, making it ideal for heat and power production. In contrast, an extraction following hydrolysis solubilized only 68% of the original protein in the biomass, while obtaining slightly higher sugar yields.

Keywords AFEX · Switchgrass · Extraction · Enzymatic hydrolysis · Leaf protein

Introduction

Recent concerns about the environmental, political, and economic impact of oil use have spurred renewed interest in alternative fuels for transportation. Ethanol derived from cellulosic feedstocks such as agricultural waste, wood chips, municipal waste, or forages is one particularly attractive alternative because it is domestically available, renewable, and can potentially reduce greenhouse gas emissions [1]. Although early biorefineries will likely use agricultural residue as feedstocks due to their low cost, dedicated energy crops will be necessary to reach the very high levels of ethanol production proposed in various

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studies [2]. Switchgrass (*Panicum vergatum*) is a model herbaceous energy crop and is attractive as a feed stock due to several favorable characteristics: high crop yields, low soil erosion, low water, fertilizer and pesticide requirements, ability to sequester carbon, and high genetic variability [2, 3]. Ample research has been conducted from the agricultural perspective, providing a foundation for further investigation and optimization using switchgrass for ethanol production [3].

To ferment the carbohydrates in cellulosic feedstocks into ethanol, they must first be broken down into their component sugars. However, yields from enzymatic hydrolysis are low unless the biomass first undergoes a pretreatment process. One promising method to improve the efficiency of the hydrolysis is the ammonia fiber expansion (AFEX) process. Concentrated ammonia is added to the biomass under high pressure and moderate temperatures, held for a residence time for 5 min, before rapidly releasing the pressure. This process decrystallizes the cellulose, hydrolyzes hemicellulose, removes and depolymerizes lignin, and increases the size of micropores on the cellulose surface, thereby, significantly increasing the rate of enzymatic hydrolysis [4]. Previous work has shown this process to give near theoretical yields of glucose on different types of agricultural residue [5, 6] and grasses [7, 8]. In particular, previous work has shown conversions of over 90% of the glucan and 70% of the xylan for switchgrass [9].

Although the structural carbohydrates in lignocellulosic feedstocks is the largest component in plant biomass, several other components are present as well. In an ideal biorefinery, each component would be processed into value added products [10]. In particular, proteins are a potentially valuable coproduct, which can be separated from the rest of the biomass and sold as animal feed or other value added products. Such a process could have numerous benefits, including potentially decreasing the cost of producing ethanol. Economic analyses of such integration suggest that the costs of separating out the protein residue would be vastly outweighed by the high value of the final product. Assuming high yields and selling at a price comparable to soymeal protein, Greene [2] estimates that extracting proteins from switchgrass in a mature biorefinery could reduce the selling price of ethanol from $0.52 to $0.39. Furthermore, an acre of switchgrass can produce at least as much protein as an acre of soybean, providing the opportunity to replace soy acreage with switchgrass, and thereby, increasing the total amount of biofuels able to be produced in the United States without reducing the capacity to produce animal feed [2].

There have been no reported studies of extracting proteins from switchgrass, although several other types of biomass have been considered for production of protein concentrates [10–16]. Dilute solutions of a strong alkali such as sodium hydroxide are generally used, with the pH between 8 and 12. Extractions generally range from 30 to 60 min at 10:1 or higher liquid to solid ratio. Protein yields varied considerably depending upon the types of biomass, generally resulting in high yields of protein from grains and moderate to low yields from leaf proteins. Studies with *Atriplex* leaves obtained only 41% of the total protein, while a pilot plant extracting proteins from alfalfa obtained 47% of the total protein [15, 16]. In general, it appears that simple extractions are not sufficient to obtain complete protein recovery from leafy biomass.

However, to date, very little research has been done into integrating a protein extraction process with ethanol production. De la Rosa [17] and Urribarri [18] found increases in protein yields from coastal bermuda grass and dwarf elephant grass, respectively, when undergoing ammonia pretreatment before extraction. By disrupting the lignocellulosic structure of the biomass, proteins appear to more easily diffuse out of the biomass and into solution. It may be possible to further increase yields of sugar and protein by further integration of pretreatment, extraction, and hydrolysis. Removing soluble material during

extraction may remove hydrolysis inhibitors, whereas hydrolysis of the cellulose and hemicellulose may further improve protein recovery. One particular advantage of integration is in the use of ammonia as an extraction agent. A portion of the ammonia used in the AFEX process may be diluted and used as the extraction agent before returning to the ammonia recovery system, potentially lowering overall raw material requirements. In addition, studying the process as a whole, rather than as individual operations, is critical for developing complete material balances needed to create more accurate biorefinery models.

Thus, the goal of this study was to assess the feasibility of extracting proteins from switchgrass harvested in the spring, while simultaneously producing sugars through enzymatic hydrolysis. The optimal conditions for solid/liquid extraction using aqueous ammonia were determined and compared to other solvents. Potential process flow schemes were examined with respect to their sugar and protein yields before a complete material balance of the final process was determined.

Materials and Methods

Feedstock

The feedstock used in this experiment was Alamo switchgrass obtained from Auburn University and harvested on May 22, 2005. The moisture content of the material was approximately 9%. All materials were ground to less than 2 mm before experiments.

Pretreatment

The AFEX pretreatment was performed in a 300-ml stainless steel pressure vessel. Water was mixed with the switchgrass to increase the moisture content to 80% dry weight basis. Glass spheres were added to minimize void space, thereby reducing the amount of ammonia in the gaseous state. The lid was bolted shut, and a sample cylinder loaded with 1 (±0.04) g NH_3 per g dry biomass, allowing the ammonia to be charged into the vessel. The reactor was heated using a 400-W PARR heating mantle, and allowed to stand at 100°C (± 1°C) for 5 min. The pressure was explosively released by rapidly turning the exhaust valve. The treated samples were removed and were placed in a fume hood overnight to remove residual ammonia.

Hydrolysis

The enzymatic hydrolysis procedure was based upon the LAP-009 protocol from the National Renewable Energy Laboratory [19]. Samples were hydrolyzed in Erlenmeyer flasks at 10% solid loading buffered to pH 4.8 by 1 M citrate buffer. Spezyme CP (Genencor) cellulase was loaded at 15 FPU/g glucan (31 mg protein/g glucan), and β-glucosidase (Novozyme 188) at 64 pNPGU/g glucan. All samples were incubated at 50°C with 200 rpm rotation. Sugar concentration after 168 h was determined using a Waters high performance liquid chromatograph (HPLC) system equipped with a Bio-Rad (Richmond, CA) Aminex HPX-87P carbohydrate analysis column. Degassed HPLC water with a flow rate of 0.6 ml/min was used as the mobile phase, while the temperature in the column was kept constant at 85°C.

Protein Extractions

Screening for optimal protein extraction conditions was done using a Dionex (Sunnyvale, CA) ASE 200 Accelerated Solvent Extractor. Extractions were performed at 1,500 psi, which reduces the required residence time from 30 to 3 min. Extractions were done using 11:1 (*w*/*w*) liquid/solid ratio and two separate extractions per sample. For experiments involving varying the pH, hydrochloric acid was used to reduce the pH. The pH of the solution was measured after the extraction was complete. Once the optimal extraction conditions were obtained, all further extractions were performed in flasks for 30 min with a 10:1 liquid/solid ratio, while continuously stirred. After the extraction was complete, the solids and liquids were separated using filtration. Solid cakes were washed with distilled water at approximately 10:1 liquid/solid ratio to insure that all soluble material is removed.

Due to the presence of ammonia nitrogen, both during the AFEX pretreatment and subsequent extractions, it is impossible to use standard nitrogen analysis methods (the Kjehldahl or Dumas methods) to measure total protein content. Instead, protein concentration was measured using a Pierce (Rockford, IL) bichronimic acid colorimetric assay kit using bovine serum albumin (BSA) as a standard. To reduce the effects of interfering agents such as ammonium salts, lignin components, and glucose, the proteins were first precipitated and resolubilized [20]. A 100-μl 0.15% sodium deoxycholate was added to 100 μl protein solution and was allowed to sit for 15 min. Two hundred microliters of 15% trichloroacetic acid solution was added and allowed to sit at 2°C overnight. The mixture was centrifuged at 13,000 rpm for 10 min, and the resulting pellet was washed with acetone. The pellet was resolubilized in a buffer solution containing 0.1 M Tris, 2.5 M urea, and 4% sodium dodecyl sulfate (SDS). Known concentrations of protein extracts were used to calibrate the protein recovery of this method.

Composition Analysis

The weight and moisture content of the remaining solid fraction after each processing step was measured for determining the mass balance in the system. The composition of each of these fractions was determined based upon NREL's LAP 002 protocol [19]. Ash content was determined by heating 1.5 g of biomass at 575°C for 24 h and measuring the weight loss. Water and ethanol extractives were removed using a soxhlet extraction. A portion of the extracted biomass was digested in concentrated (67%) sulfuric acid in a 10:1 liquid: solid ratio at 30°C for 1 h. The solution was diluted to 4% sulfuric acid and autoclaved at 120°C for 1 h, and then analyzed for sugar components using a Bio-Rad (Richmond, CA) Aminex HPX-87H HPLC column using sulfuric acid as the mobile phase. The acid insoluble lignin was measured as the remaining solid after hydrolysis less the ash content in the solid residue.

Results and Discussion

Composition Analysis

The composition of the switchgrass used in this study is shown in Table 1. Approximately 80% of the mass is accounted for. The remaining material is primary water soluble components, such as minor organic acids, and acid soluble lignin. The amount of protein present was lower than reported in literature for other strains of switchgrass [21]. Switchgrass grown as a biomass energy crop and harvested early in the growing season would

Table 1 Composition of Alamo (g/100 g dry matter) switchgrass used in this study.

Component	Percent value (%)
Glucan	26.4
Xylan	16.4
Arabinan	3.5
Sucrose	3.4
Protein	7.3
AI Lignin	10.8
Lipids	7.3
Ash	4.8
Total	79.9

AI Acid insoluble

likely have protein contents near 10%, and thus might be more suitable for integrated protein and sugar processing. The amount of fiber present is lower than switchgrass harvested at a later date, which seems to suggest lower sugar yields would also result from using an earlier cut. However, early cut switchgrass is less recalcitrant than that harvested in the fall (data not shown), and thus, the lower cellulose and hemicellulose content may not be a significant factor. The low amount of lignin is a promising sign, as this implies less interference with hydrolysis and fewer harmful degradation products that could inhibit sugar production or otherwise be present in the protein product. Ash content is higher than at later harvests (data not shown), as expected. It will likely be necessary to return much of this ash to the land to maintain a high quality soil.

The essential amino acid profile for switchgrass, along with literature values for corn and soy [22], is shown in Table 2. The most promising feature of switchgrass protein is the high value seen for lysine, an essential amino acid that is often the first limiting amino acid in poultry and swine diets. High values for phenylalanine and valine are also seen. Although switchgrass is somewhat deficient in leucine, arginine, and methionine, these amino acids are relatively abundant in corn. Thus, a corn-switchgrass protein diet would balance out these deficiencies, and thus might be a strong alternative to a corn–soy diet.

Extraction Optimization

Figure 1 shows the effect of the temperature of the extraction on the overall protein and mass yields. Protein yields increased significantly from 25 to 40°C, but further increases in temperature did not result in major improvements in protein yield. It is likely that most, if not all, of the proteins present in the switchgrass are in their natural state, as the harvesting and drying conditions should not have damaged them. As such, the mild temperatures should not unfold the proteins or significantly affect their solubility.

Table 2 Essential amino acid profile of Alamo switchgrass (SG) compared to literature values for soybean and corn grain [22].

Biomass	Arg	His	Ile	Leu	Lys	Met	Phe	Thr	Val
SG	2.1	1.8	3.7	5.6	7.4	0.6	9.1	4.9	6.1
Soy	7.5	2.6	4.9	7.7	6.1	1.6	5.1	4.3	5.1
Corn	2.9	1.6	4.3	16.2	1.6	2.3	5.9	3.1	4.4

Values are in g amino acid/100 g protein. Of particular note are lysine, phenylalanine, and valine, of which switchgrass is rich in, and methionine, of which switchgrass is somewhat deficient.

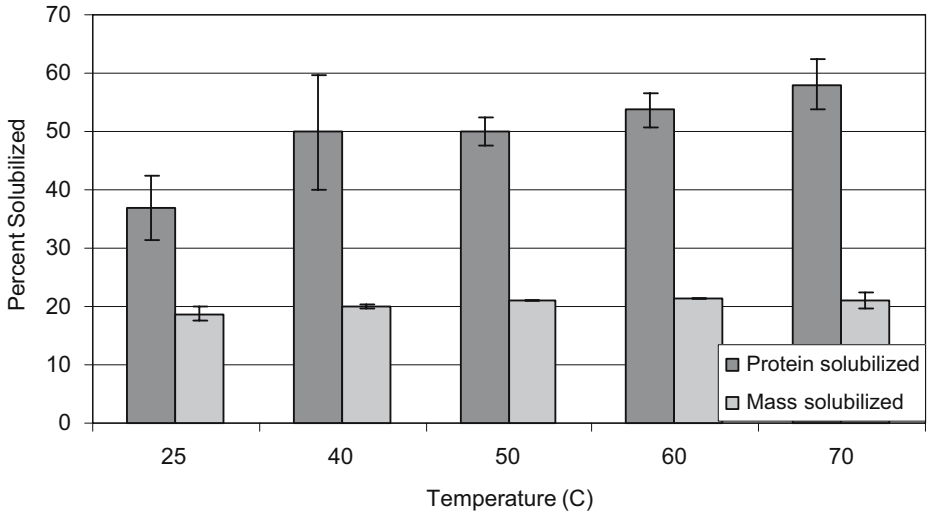

Fig. 1 Effect of extraction temperature on protein yields. All extractions were done with 3% ammonia at pH 10.5. The results are combined after two separate extractions using 11:1 liquid/solid ratio and 3 min residence time. All runs were done in duplicate, and *error bars* represent the maximum and minimum values

The effect of ammonia concentration on extraction yields is seen in Fig. 2. It is clear that some base is necessary to achieve strong yields, as the yield without the presence of ammonia was approximately 65% of those that were performed in ammonia. Protein yield remains constant from 1–3% NH4+, but then begins to drop off. This is most likely due to "salting

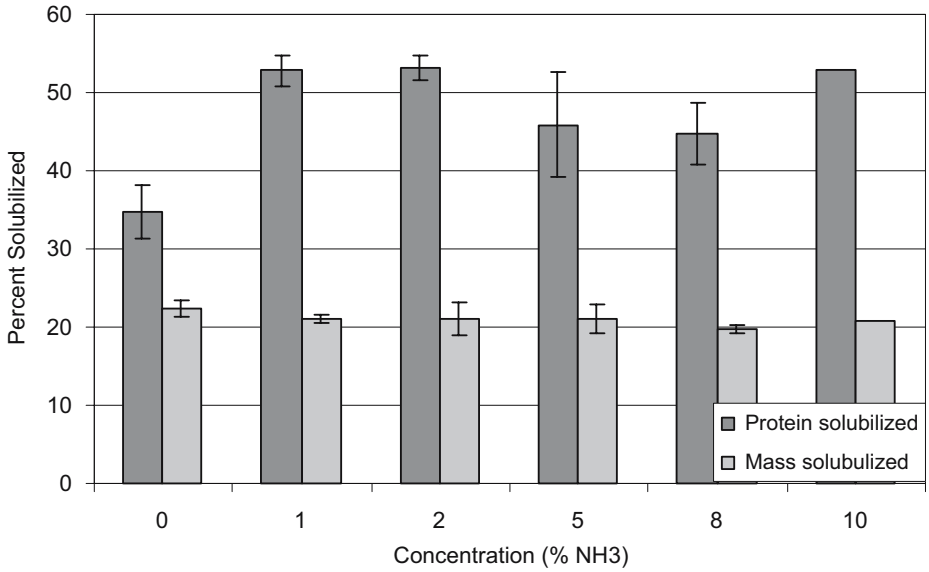

Fig. 2 Effect of ammonia concentration on protein yields. All extractions were done at 50°C and at pH 10.5 except for 0% concentration, which was performed using distilled water at 50°C. The results are combined after two separate extractions using 11:1 liquid/solid ratio and 3 min residence time. All runs were done in duplicate, and *error bars* represent the maximum and minimum values

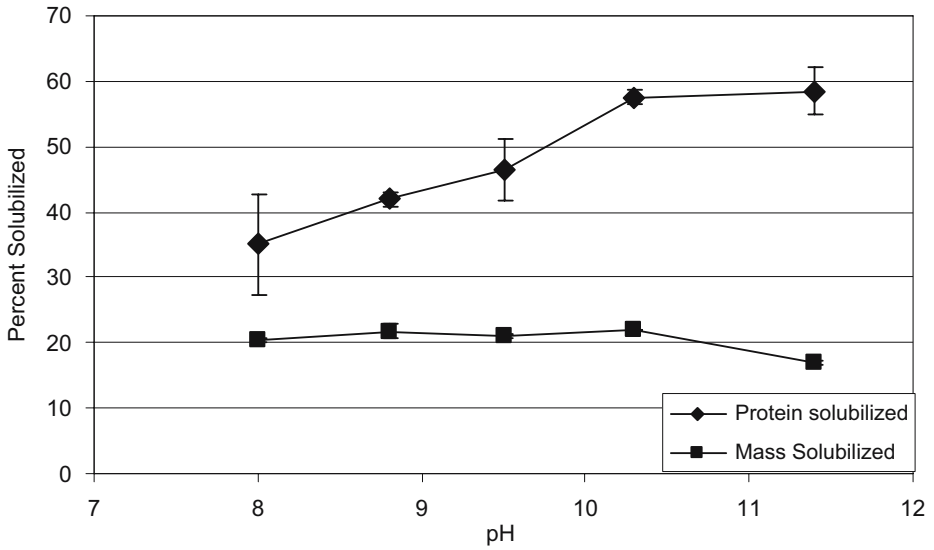

Fig. 3 Effect of extraction pH on protein yields. All extractions were done with 3% ammonia and at 25°C. The results are combined after two separate extractions using 11:1 liquid/solid ratio and 3 min residence time. All runs were done in duplicate, and *error bars* represent the maximum and minimum values

out" the protein, as the increase in salt concentration decreases the amount of water available to solubilize the protein. It is also possible that greater hydrolysis of the peptide chains is occurring at higher salt concentrations, as these smaller peptide chains would then be lost during the TCA precipitation. However, lower salt concentrations are still more desirable

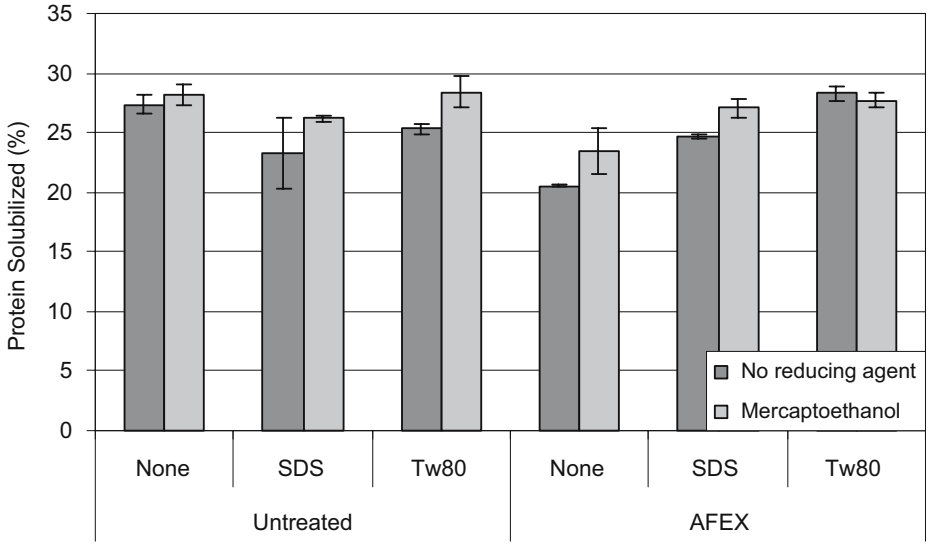

Fig. 4 Effect of reducing agents on protein yields for untreated and AFEX treated samples. All extractions were done with 3% ammonia, 25°C, and at pH 10.5. The results are combined after two separate extractions using 11:1 liquid/solid ratio and 30 min residence time. Both the ionic sodium dodecyl sulfate (SDS) and the nonionic Tween 80 (Tw80) surfactants were tested, both with and without the addition of β-mercaptoethanol. All runs were done in duplicate, and *error bars* represent the maximum and minimum values

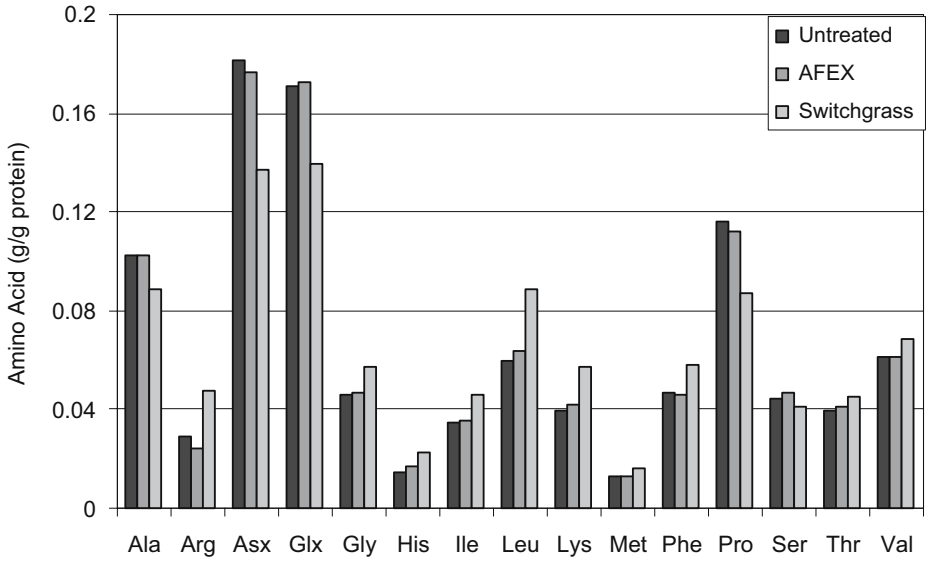

Fig. 5 Amino acid profiles for untreated protein extract, AFEX-treated protein extract, and the native switchgrass protein. Asx and Glx are a combination of aspartic acid and asparagine and glutamic acid and glutamine, respectively. Cysteine and tryptophan were not detected due to their instability during acid hydrolysis

because of both the lower cost of solvent and the relative ease of concentration of larger protein fragments compared to small peptide chains. There does not appear to be any salting in effect, likely because 1% salt solution is already a sufficient concentration to solubilize the protein. The total mass solubilized was unaffected by salt concentration, as expected.

The most significant factor in determining protein yields is the pH of the system, as seen in Fig. 3. The amount of protein extracted increased dramatically from a pH of 8 to 10.5 before leveling off. Similar trends have been seen in other types of biomass [10–16]. Most proteins have an acidic isoelectric point, the pH at which the protein will have no net charge and therefore be the least soluble in a polar medium. Thus, increasing the pH should increase protein solubility, as demonstrated here. The most alkaline solution also produced a significant drop in the total mass solubilized, a potentially useful characteristic. If there is less biomass in solution, it should be easier to purify the proteins. In addition, the biomass lost during extraction likely includes hemicellulose that could be hydrolyzed into sugars for ethanol production. Further increases in pH would require a stronger base than ammonia and might degrade the protein, and thus, were not considered.

As seen in Fig. 4, attempts were made to improve yields by the addition of the nonionic surfactant Tween 80, the ionic surfactant SDS, and β-mercaptoethanol, a reducing agent. No significant improvements can be found by the addition of either surfactant or reducing agent for the untreated switchgrass. However, adding β-mercaptoethanol and Tween 80 to AFEX treated grass did increase protein removal. This would seem to suggest that the AFEX process affects the proteins in some manner. This effect might be through the creation of sulfur–sulfur bonds, which would then be cleaved by β-mercaptoethanol, or by proteins unfolding and exposing hydrophobic sites, which can be resolubilized with surfactants. The total mass solubilized also increased with the addition of surfactants (data not shown), most likely due to interactions between the surfactants and hydrophobic portions of the biomass.

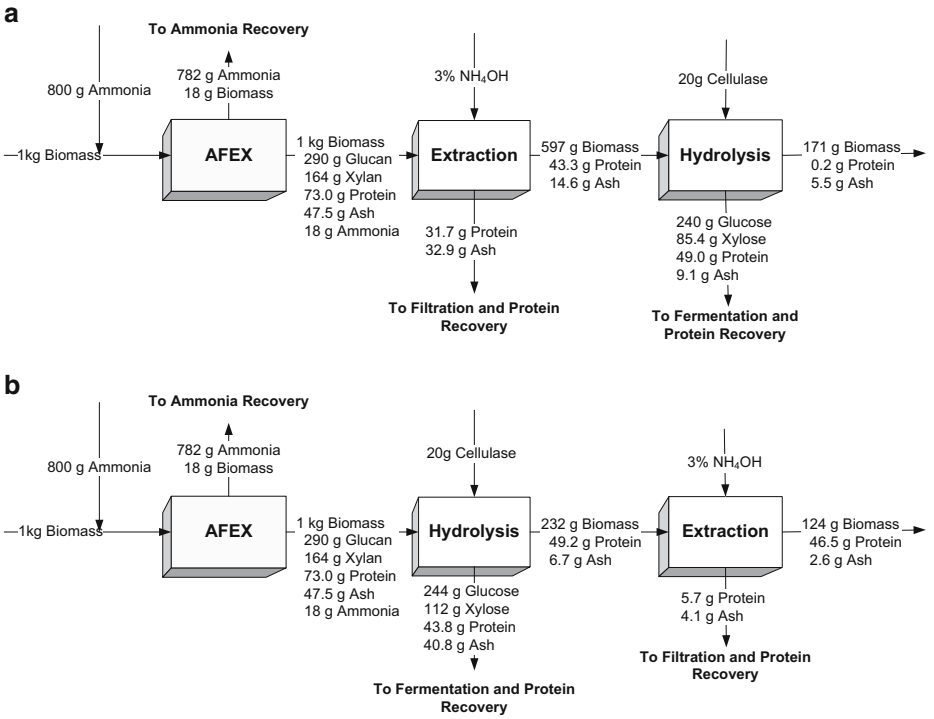

Fig. 6 **a** Process flow diagram for extraction before hydrolysis. Balances around the protein and ash content are given, as well as total mass and the amount of glucose and xylose produced. **b** Process flow diagram for extraction after hydrolysis. Balances around the protein and ash content are given, as well as total mass and the amount of glucose and xylose produced

To determine whether AFEX pretreatment affects the types of proteins recovered, the composition of the individual amino acids was determined, as seen in Fig. 5. Both the untreated and AFEX treated samples were extracted at the optimal ammonia conditions without adding surfactant or reducing agent. Although the amino acid profile for the proteins solubilized during extraction compared to the total protein from switchgrass is quite different, there is very little difference between extractions from untreated and AFEX treated grass. Although AFEX does disrupt the cellular structure of the biomass, it does not appear to release any other proteins to be available for extraction. Therefore, it appears that the structure of the plant is not a major hindrance in protein recovery, but rather, the structure of the protein itself.

Thus, optimal extraction conditions for switchgrass are approximately 3% aqueous ammonia at a pH of 10 and temperature of 40–50°C. These conditions are in line with those seen for protein extraction of other types of biomass and are the conditions used for all subsequent experiments reported here. Total protein yields are approximately 40%. However, AFEX did not appear to significantly improve yields, unlike previously reported with coastal bermuda grass [17]. It is currently unclear why this is the case.

Integration

Two potential scenarios for integrated sugar and protein recovery were studied: an extraction immediately after AFEX and an extraction immediately after hydrolysis. A third

option, extraction before AFEX, produced sugar yields far below the first two scenarios and so is not presented here. It is possible that extracting proteins and other material before AFEX changes the effects of AFEX pretreatment. AFEX produces some phenolic degradation products because of side reactions with the lignin components, and these molecules have a slight inhibitory effect on hydrolysis. It is possible that due to increasing the relative concentration of lignin in the remaining biomass, a prior extraction could produce more of these inhibitory acids. Washing the biomass after AFEX did increase the sugar yields to approximately the same level as hydrolysis without any previous extraction. However, this process was deemed to require too much water use with no clear advantage, and thus, was not studied in greater depth.

The overall mass balance for integrated sugar and protein with extraction before hydrolysis is seen in Fig. 6a. Final yields were 240 g glucose, 85.4 g xylose, and 80.7 g protein per kg dry biomass. Sugar recovery was approximately 74% of theoretical values, indicating that further improvements in sugar recovery can be made. Approximately 40% of the protein was found in the extract and 60% in the hydrolysate, demonstrating that protein must be recovered from both streams to be economical. It should be noted that the insoluble biomass was washed after hydrolysis to insure all soluble components were recovered, and thus, this may have acted as a second extraction to remove any remaining proteins bound to insoluble portions of the biomass. Total protein yield is approximately 87% of the total, taking into account both the switchgrass protein and the enzymes used in hydrolysis. However, virtually no insoluble protein remains in the biomass, thus suggesting that the remaining protein was broken down and lost at some point during the process.

Approximately 40% of the biomass is solubilized during the initial protein extraction step. It may be possible to utilize this soluble fraction of the biomass after the proteins have been removed. The protein might be concentrated and removed through ultrafiltration or heat precipitation, while the remaining solution undergoes further processing.

Most of the ash was removed from the biomass during the first extraction step. It is important to remove this ash, as the final insoluble residue would likely be burned to provide heat and power for the refinery. The ash content in switchgrass, particularly potassium, has been shown to cause problems with slagging in coal/biomass co-firing power plants. The remaining biomass contains only 3% ash, and thus, should reduce this risk in heat and power generation. It remains to be seen if the ash in the extraction step can be separated and returned to the land. The fact that most of the ash is removed during one unit operation should help keep the costs of any ash processing step low, as only one stream needs to be treated.

Approximately 17% of the biomass remains insoluble throughout this process. There is virtually no protein or ash still present in this residue, which is mostly composed of unhydrolyzed fiber and insoluble lignin. This material would likely be burned for heat and power generation in the refinery, thus, reducing natural gas or coal requirements. The lack of protein and ash would reduce the presence of NO*x* formation and slagging, respectively.

A separate balance, focusing on performing hydrolysis before extraction, is shown in Fig. 6b. Here, sugar yields were slightly higher, with a total of 356 g compared to 325 g per kg biomass using the previous approach. This is mainly due to xylan conversion, indicating that xylan oligomers were likely extracted along with protein during the initial extraction step in the previous scenario. However, although approximately 60% of the protein in the switchgrass was solubilized during hydrolysis, very little was extracted afterwards. During hydrolysis, other compounds may be produced that interfere with the colorimetric analysis, thus increasing the error involved. This mass balance, however, relies solely on the individual amino acids rather than a colorimetric response, and thus, is a more accurate representation of actual protein levels. Subsequent extractions on the final residue did not

release more than a small fraction of the residual proteins (data not shown), making it unlikely that further treatments can remove the residual protein.

The amount of insoluble material remaining is less than that of the previous scenario, indicating that less heat and power can be produced. Although less ash is present, there is still a great deal of protein remaining. Protein has lower energy content than lignin and also its combustion will generate NO*x*. Thus, due primarily to the higher protein yields, an extraction before hydrolysis is likely to be the best option despite the slightly lower sugar yields.

Conclusions

Our experimental results show that the integrated recovery of sugar and protein from early cut switchgrass appears to be a feasible approach to a cellulosic biorefinery. Ammonia has been shown to be an effective solvent for removing proteins from the biomass, thus opening up possibilities of integrating with AFEX pretreatment or providing a nitrogen source during fermentation. Integrating sugar and protein production will cause some tradeoffs, as producing maximum sugar will result in a lower protein recovery and vice versa. However, there are possibilities for overcoming these obstacles.

Further integration of these two steps is also possible. If the loss in sugar yields is due solely to oligomer loss, then using the protein extract as the hydrolysate liquid after separating the proteins would reduce these losses. This would require neutralizing the extract, but would decrease overall water use and increase sugar and therefore ethanol yields. Fortunately, as high concentrations of ammonia are not needed for the extraction as seen in Fig. 2, it is likely that the environmental and economic benefits of using the extract as the hydrolysate liquid will outweigh the cost of neutralization. In addition, the fact that there are multiple protein streams may allow further specialization. If the cellulase enzymes are still active after hydrolysis, it may be possible to concentrate and recycle them, again reducing operating costs.

It still remains to be seen if downstream processing can fully separate the proteins and sugars to produce the desired final products. This will require separating the proteins from the remaining sugar and other soluble portions of the biomass, likely through ultrafiltration. The effect of integrating extraction with hydrolysis on fermentation will also need to be determined to see if the loss of protein will adversely affect microbial growth. Steps to reduce the water use throughout the process would also improve the overall process. These steps, along with a full economic analysis, will help to determine the potential viability of producing animal feed concurrently with cellulosic products within a mature biorefinery.

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References

1. Lin, Y., & Tanaka, S. (2006). *Applied Microbiology and Biotechnology, 69*, 627–642.
2. Greene, N. (2003). Growing Energy, Natural Resources Defense Council.
3. Sanderson, M. A., Reed, R. L., McLaughlin, S. B., Wullschleger, S. D., Conger, B. V., & Parrish, D. J. (1996). *Bioresource Technology, 56*, 83–93.

4. Mosier, N., Wyman, C., Dale, B., Elander, R., Lee, Y., Holtzapple, M., et al. (2005). *Bioresource Technology, 96*, 673–686.
5. Sulbaran-de-Ferrer, B., Aristiguieta, M., Dale, B., Ferrer, A., & Ojeda-de-Rodriguez, G. (2003). *Applied Biochemistry and Biotechnology, 105–108*, 155–164.
6. Teymouri, F., Laureano-Perez, L., Alizadeh, H., & Dale, B. (2005). *Bioresource Technology, 96*, 2014–2018.
7. Holtzapple, M., Jun, J., Ashok, G., Patibandla, S., & Dale, B. (1991). *Applied Biochemistry and Biotechnology, 28–29*, 59–74.
8. Ferrer, A., Byers, F. M., Sulbarán De Ferrer, B., Dale, B. E., & Aiello, C. (2000). *Applied Biochemistry and Biotechnology, 84–86*, 163–179.
9. Alizadeh, H., Teymouri, F., Gilbert, T., & Dale, B. (2005). *Applied Biochemistry and Biotechnology, 121–124*, 1133–1141.
10. Ordonez, C., Asenjo, M., Benitez, C., & Gonzalez, J. (2001). *Bioresource Technology, 78*, 187–190.
11. Lawhon, J. (1986). US Patent #4,624,805. Nov 25, 1986.
12. El-Adaway, T., Rahma, E., El-Bedawey, A., & Gafar, A. (2001). *Food Chemistry, 74*, 455–462.
13. Park, S., & Bean, S. (2003). *Journal of Agricultural and Food Chemistry, 51*, 7050–7054.
14. Betschart, A., & Kinsella J. (1973). *Journal of Agricultural and Food Chemistry, 21*(1), 60–65.
15. Fernandez, S., Padilla, A., & Mucciarelli, S. (1999). *Plant Foods for Human Nutrition 54*, 251–259.
16. Fiorentini, R., & Galoppini, C. (1981). *Journal of Food Science 46*, 1514–1520.
17. De La Rosa, L. B., Reshamwala, S., Latimer, V., Shawky, B., Dale, B., & Stuart, E. (1994). *Applied Biochemistry and Biotechnology, 45–46*, 483–497.
18. Urribarrí, L., Ferrer, A., & Colina, A. (2005). *Applied Biochemistry and Biotechnology, 121–124*, 721–730.
19. NREL. (2004). Chemical Analysis and Testing (CAT) Standard Procedures, National Renewable Energy Laboratory.
20. Lovrien, R., & Matulis, D. (1995). *Current Protocols in Protein Science*, 3.4.1–3.4.24.
21. Madakadze, I., Stewart, K., Peterson, P., Coulman, B., & Smith, D. (1999). *Crop Science, 39*, 552–557.
22. Allan, G., Parkinson, S., Booth, M., Stone, D., Rowlamd, S., Frances, J., & Warner-Smith, R. (1999). *Aquaculture, 186*, 293–310.