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Alfalfa Fiber as a Feedstock for Ethanol and Organic Acids

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Abstract

Valuable co-products derived from fractionation of alfalfa herbage give the resulting fibrous fraction an economic advantage as a feedstock for ethanol or other organic products. Alfalfa fiber was saccharified and fermented with or without a liquid hot water (LHW) pretreatment. The LHW pretreatment hydrolyzed approximately 60% of the original fiber, yielding a high cellulose residue and a liquid extract. These yielded predominantly hexoses and pentoses, respectively, after enzymatic saccharification. Yields of ethanol and lactic acid resulting from fermentations are given.

Index Entries: Medicago sativa; pretreatment; saccharification; ethanol; lactic acid.

Introduction

The alfalfa "fiber" discussed in this paper is the result of wet fractionation of fresh alfalfa herbage into a juice fraction and a fiber fraction. Although there are a number of other uses for this fiber fraction *(1,2)*, this study deals with its conversion to ethanol and lactic acid.

Production of perennial legume crops such as alfalfa offer many advantages. These include:

1. Excellent soil and water conservation characteristics;
2. Low expenditure of nonrenewable resources for tillage and fertilizer, making production highly sustainable;
3. High per acre yields of protein and energy;

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4. Varieties adapted to a wide range of environments exist; production practices are likewise well established;
5. Use of perennial legumes in a rotation with "cash crops" such as corn and soybean can greatly reduce the need for pesticides and can actually increase yields.

With rare exceptions, however, utilization of perennial legumes, such as alfalfa, has been limited to ruminant rations by the intimate association of large quantities of fiber with the more easily digested nutrients. Two emerging technologies have improved the potential for profitably utilizing perennial legume crops for a much wider variety of products, some having high unit values. The first technology is fractionation, the separation of the herbage into two or more components. Two important conditions must be met for fractionation to be feasible and sustainable: 1. The total value of the resulting products must be greater than that of the original herbage plus the cost of processing; and 2. all fractions must have an economic value to avoid creating a waste stream. Three methods of herbage fractionation exist:

1. Wet fractionation; separation into a juice fraction and a fiber fraction;
2. Dry fractionation; separation into leaves and stems; and
3. Fractionation by passage of the whole herbage through the digestive systems of ruminant animals, leaving a high-fiber residue.

The second emerging technology is that of genetic engineering; specifically, adding genes to plants that cause them to produce economically valuable substances not normally produced. An example of this would be the production of industrially valuable enzymes in herbage *(3,4)*. There appears to be general consensus that valuable coproducts are needed to "subsidize" the ligno-cellulosic or fiber fraction for bioenergy use.

Dry fractionation of alfalfa into leaf meal and stems used as solid fuel is currently being pioneered by the Minnesota Valley Alfalfa Producers (MNVAP) and Northern States Power of Granite Falls, Minnesota. Although solid fuel yields the highest net energy and has the lowest processing cost, its use is generally limited to electric-power generation. An alternative to solid fuel is the saccharification and fermentation of the ligno-cellulosic, fiber fraction to ethanol. Although conversion of fiber to ethanol results in less total energy and is a more complicated process, its versatility and potential use as a transportation fuel make it an interesting alternative.

It has been frequently stated that the greatest single cost in producing ethanol from ligno-cellulosics is for the enzymes used to hydrolyze the fiber to fermentable sugars. It is therefore conventional wisdom that a chemical/thermal pretreatment to partially hydrolyze the fiber is necessary to improve processing economics. A number of such pretreatments have been proposed and studied. Many involve acids or bases used with elevated temperatures. Others involve softening with steam or liquid ammonia followed by an abrupt pressure drop to atmospheric. The pretreatment chosen for this study is referred to as liquid hot water (LHW) or "Aquasolv" *(5,6)*. It consists of treating the feedstock with water at 220°C,

pressurized to hold it in the liquid state, for 2 min. Advantages claimed for this process include:

1. No chemicals required that can ultimately lead to costs and waste products;
2. Almost total hydrolysis of hemicellulose; and
3. Partial hydrolysis of lignin.

Enzymes are still needed for reducing the oligomers (short chains of sugars) in the pretreatment extract to monomers (single molecules) required by the fermentation organisms. Because hydrolysis of hemicellulose leads mainly to five-carbon sugars and hydrolysis of cellulose to six-carbon sugars, the fermentation organism(s) must be capable of fermenting both types of sugars. This dual capability is not normally found but has been achieved in genetically engineered organisms.

Objectives

The primary objective of this ongoing research is to quantify the amount of ethanol and/or organic acids that can be produced from alfalfa "fiber" by enzymatic hydrolysis with or without a high-temperature pretreatment. Because of problems of inhibition described later with ethanol fermentations, and because of the impending use of lactic acid as a feedstock for biodegradable plastics, it was decided to compare the production of lactic acid from alfalfa fiber, with and without the LHW pretreatment, to that of ethanol. Such a comparison might point to possibilities for coproduction of ethanol and lactic or other organic acids to yield the highest total product value.

Methods

The alfalfa "fiber" used in this research was the result of wet fractionation in which freshly cut alfalfa herbage was macerated using a rotary impact device and was immediately dejuiced by means of a screw press. The resulting fiber contained 70–75% of the dry matter of the original herbage. It was analyzed at the U.S. Dairy Forage Research Center (Madison, WI) for neutral detergent fiber (NDF), acid detergent fiber (ADF), and detergent lignin (ADL), % nitrogen (N), and % ash by the procedures of Goering and Van Soest *(7)*. The following definitions were used: % hemicellulose = % NDF – % ADF; % cellulose = % ADF – % ADL % lignin = % ADL; % protein = % N × 6.25. Solubles were taken as the difference between the original sample weight and the sum of hemicellulose, cellulose, lignin, protein, and ash. Its average composition (*see* Fig. 1A) was 33% cellulose, 18% hemicellulose, 8% lignin, 11% protein, 9% ash, and 22% solubles (by difference). This material was air-dried and stored for future use.

The pretreatment consisted of flowing preheated water at 220°C through 30 g of sample for 2 min at the rate of approximately 370 mL/min. The water was held at sufficient pressure to insure that it remained in the

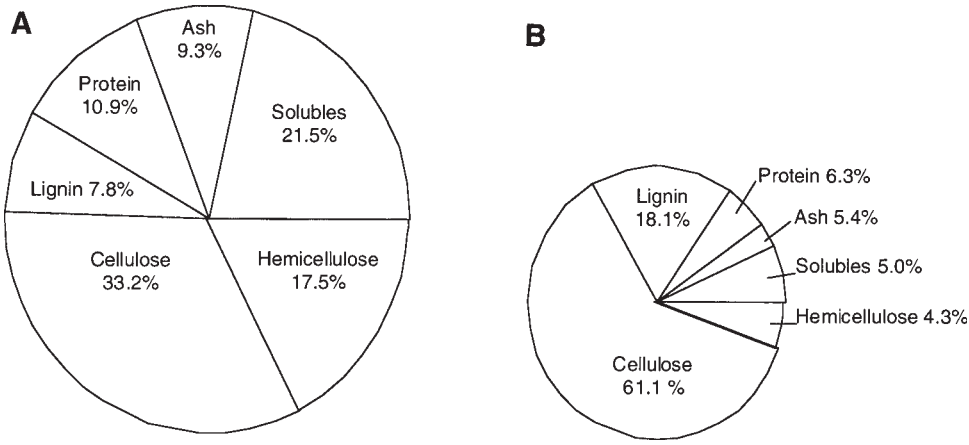

Fig. 1. **(A)** Representative composition of alfalfa "fiber" obtained by the wet fractionation process. N = 22 (circle area represents 100 g). **(B)** Representative composition of the fibrous residue resulting from the LHW treatment (3 reps of composite of 44 runs) (circle area represents 41 g, an average yield from 100 g).

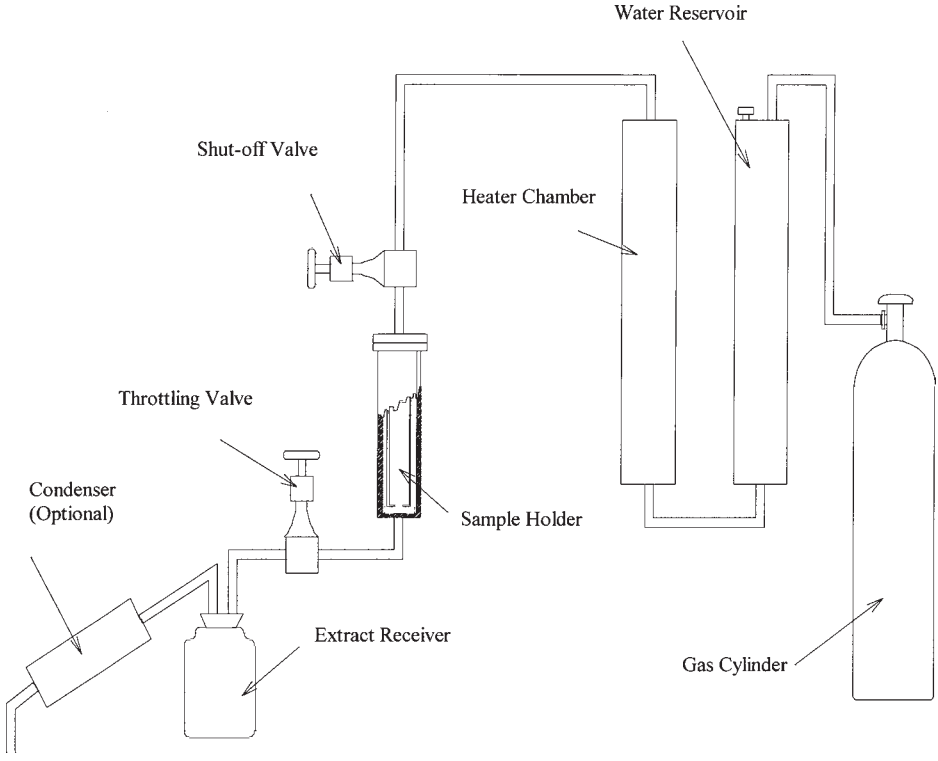

Fig. 2. Schematic of liquid hot water (LHW) treatment apparatus.

liquid phase. Figure 2 is a schematic of the apparatus. Pressure from the gas cylinder caused water to flow from the reservoir through the heater and then through the sample holder. After the sample holder, the water with

hydrolyzed dry matter was throttled through a valve to atmospheric pressure and finally flowed to the extract receiver. The pressure drop caused approximately one-third of the liquid to vaporize. After some initial treatments, a water-cooled condenser was added to allow retention of the vapor fraction for analysis. After the treatments, the fibrous residue was removed, oven-dried, and stored for further analysis or treatment. The liquid "extract" was weighed and sampled to determine dry-matter content. It was then stored under refrigeration for further use.

Pectinase (SP249) was procured from Novo (Nordisk, Franklinton, NC) and Cellulase (Multifect B) was procured from Genencore (Rochester, NY). The activity of these enzyme preparations on pectin, xylan, filter paper, carboxymethylcellulose, and microcrystalline cellulose were determined experimentally.

For enzymatic saccharification, a 40-mL reaction mixture containing 4 g of untreated alfalfa fiber (10%) in distilled water was incubated with 0.8 mL of enzyme mixture (2% v/v) containing 1:1 pectinase and cellulase at 50°C and pH 5.4, with occasional stirring. The pretreated residue (10%) was treated with cellulase alone at 2% under identical conditions.

Samples of 1.5 mL were removed periodically and centrifuged at 16000*g* for 3–4 min and clear decant samples were saved for determination of reducing sugar *(8)* and HPLC analysis. The residue left unsaccharified was dried and dry weight was determined.

Forty mL of the pretreatment extract containing 0.8 mL of enzyme mixture (2% v/v) containing 1:1 pectinase and cellulase was incubated at 50°C and pH 5.0 with occasional stirring. Samples of 1.5 mL were removed periodically and centrifuged at 16000*g* for 3–4 min and clear decant samples were saved for determination of reducing sugar *(8)* and HPLC analysis. The precipitate was dried and dry weight was determined.

The yeasts, *Candida shehatae* FPL-702 and FPL-049, employed in this work for ethanol fermentation are genetically engineered to ferment both five-carbon and six-carbon sugars. They are preserved in glycerol at –80°C. The strains were grown on YEPX agar at 32°C for 48 h containing 1.0% yeast extract, 2.0% peptone, 2.0% xylose, and 2.0% agar.

For simultaneous saccharification and fermentation (SSF), 50 mL of media containing either 5 g of untreated fiber or pretreated residue (10%) in 43 mL of water or 44 mL of pretreatment extract was placed in 125-mL Erlenmeyer flasks. The untreated fiber was mixed with 2.0 mL of enzyme mixture of cellulase and pectinase in equal quantities with the addition of 5 mL of fresh cell inoculum of *C. shehatae* (FPL 702) containing 2.5 mL of 20X yeast nitrogen base and 2.5 mL of 20 X mixture of urea and peptone and zinc (0.001% final). The cell inoculum was adjusted to concentration of 2 g/L by scooping and washing a 2-d-old culture.

Preparation of the pretreated residue and the pretreatment extract were the same as previously noted except that in the case of the former only 2.0 mL of cellulase was used and in the case of the latter only 1.0 mL of the enzyme mixture was used. The fermentation was carried out on a shaker at

100 rpm at 30°C for 5 d. Periodically 1.5 mL of samples were taken and centrifuged at 16,000*g* for 3–4 min and decant samples were saved for various analyses. The residue left unfermented was dried and dry weight was determined.

For lactic-acid fermentations, the organisms *Lactobacillus plantarum* Sp. 14431 or *L. delbrueckii* NRRL-B445 were used. They were grown on MRS agar at 37°C for 48–72 h. As in the case of ethanolic fermentations, the untreated fiber, pretreated residue, and extract were SSF using cellulases and/or pectinases along with the selected *Lactobacillus* culture. All fermentations were maintained in the range of pH 5.5–6.0 by adding $CaCO_3$. For the SSF, either the untreated fiber or the pretreated residue were added at 10% concentration to a 50 mL control volume containing 4% (v/v) of cellulase and pectinase (cellulase only for treated residue) and the *Lactobacillus* inoculum. A nutrient medium was used for fermentation of treated residue and extract, but not for untreated fiber. The process was carried out at 37–41°C on a shaker at 100 rpm for 96 h. Conditions for SSF of the extract were the same except that 44 mL (20.0 g/L solids) were added to the 50 mL control volume containing 2% v/v of the enzyme mixture.

Ethanol was estimated by gas chromatography (GC) (Hewlett Packard, Palo Alto, CA) using Poropack column with 175°C initial and final oven temperature, 275°C detector temperature, and 225°C injector temperature. The carrier gas was helium and the detector gas was hydrogen *(9)*.

The sugars were quantified by high-performance liquid chromatography (HPLC) (Hewlett Packard series 1050) with a refractive index (RI) detector using column Aminex Carbohydrate HPX 87C (300×7.8 mm) (Biorad) maintained at 85°C *(10)*. The mobile phase was degassed distilled water at a flow rate of 0.5 mL/min at a pressure of 50–55 bar.

The organic acid was determined in an HPLC *(11)* using 10N-300 column (300 × 7.8 mm) interaction chromatography with refractive index detector. The mobile phase was 0.005 *N* sulfuric acid at a flow rate of 0.4 mL/min at 65°C.

Results and Discussion

LHW Pretreatment

The division of the fiber dry matter brought about by the LHW treatment is shown in Fig. 3. Almost 60% of the dry matter was extracted, leaving ~41% of the original dry matter as fibrous residue. Only about 48% of the original dry matter was in the extract, however, leaving about 11% of the dry matter unaccounted for.

The composition of the residue resulting from the LHW treatment is shown in Fig. 1B. Because the circle areas in Fig. 1A,B are proportional to the masses of dry matter in the fiber and residue, respectively, it is possible to see approximately to what degree various components were extracted. This degree of extraction of the three major fiber constituents—cellulose,

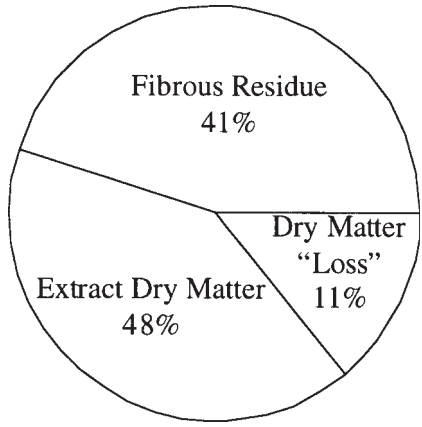

Fig. 3. LHW treatment of alfalfa fiber. Percentages of initial dry matter found in fibrous residue and extract, respectively.

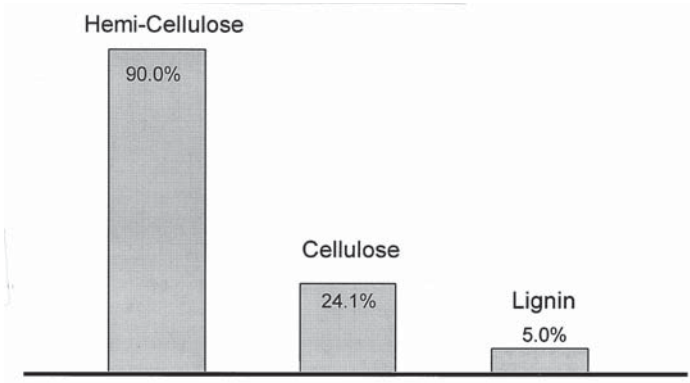

Fig. 4. LHW treatment of alfalfa fiber. Percentages of initial hemicellulose, cellulose, and lignin removed from fiber.

hemicellulose, and lignin—is shown in Fig. 4. The values given in Fig. 4 were calculated as follows:

1. It was assumed that a mass of 100 g of fiber in Fig. 1A yielded 41 g of residue in Fig. 1B.
2. To get actual component masses, the decimal fractions in Fig. 1A were multiplied by 100, whereas those in Fig. 1B were multiplied by 41.
3. The difference in mass of a component (e.g., cellulose) between Figs. 1A and 1B was divided by the original mass (Fig. 1A) and converted to a percentage.

Hemicellulose is clearly the major constituent extracted. The results differ somewhat from those reported by Van Walsum *(6)* for bagasse and aspen. They reported extraction of 98–100% of the hemicellulose, 5–7% of the cellulose, and 37–65% of the lignin. The differences may be owing to differences in the feedstock and/or slight processing parameter differences.

Table 1
Observed Production of Ethanol or Lactic Acid
from 100 U of Alfalfa Fiber With and Without LHW Pretreatment

Product	Fermentation organism	Untreated fiber 100 U	LHW Pretreated fiber 100 U before treatment	
			Residue 41 U	Extract 48 U
Ethanol	*C. shehatae* (FPL 702)	6.0 U @ 90 h SD[a] = 0.40 *n* = 3	7.3 U @ 144 h SD = 0.29 *n* = 3	2.2 U @ 66 h SD = 0.24 *n* = 3
Lactic acid	*L. delbrueckii*	35.0 U @ 48 h SD = 0.84 *n* = 3	24.3 U @ 72 h SD = 0.75 *n* = 2	21.4 U @ 48 h SD = 1.37 *n* = 2
Lactic acid	*L. plantarum*	46.5 U @ 72 h SD = 0.21 *n* = 3	23.6 U @ 96 h SD = 0.86 *n* = 2	31.2 U @ 24 h SD = 1.97 *n* = 3

[a]SD, standard deviation.

For example, it is recognized that differences in cellulose crystallinity affect hydrolysis. It is also known that lignin may be solubilized and then reprecipitated under certain conditions.

The flow of water through the apparatus of approx 370 mL/min required to maintain the target temperature diluted the extract to an average of 2.0% dry matter. This dry-matter content could be approximately doubled by adding the residue to the extract for fermentation. However, a minimum concentration of 5% ethanol is frequently considered necessary to make distillation economically feasible. This implies a sugar content of about 10% and a dry-matter content of the residue-extract mix of 20% as minima.

Ethanol

The alfalfa fiber was enzymatically saccharified and fermented with and without the LHW treatment to compare quantities of products derived by each method. Table 1 and Fig. 5 show representative quantities without pretreatment. The quantity of sugars produced was about 45% of the initial material. The rule of thumb conversion rate for sugars to ethanol is 0.5 (stoichometric = .57). This would result in the ethanol being 0.5 × 45% or 22.5% of the original fiber weight. The actual ethanol production was a disappointing 6.0% or about 27% of the expected.

In the case of fiber subjected to the LHW treatment (Table 1 and Fig. 6), the sugars resulting from enzymatic saccharification of the extract and the residue were 22.5% and 18.5% of the initial fiber, respectively. This totals to 41%, not different from the sugar yield of the fiber without pretreatment. Based on the cellulose and hemicellulose components of the residue in Fig. 1B, its sugar yield might have been expected to be about 8 U higher, ≅ 27 U. Although the exact composition of the extract is not yet known,

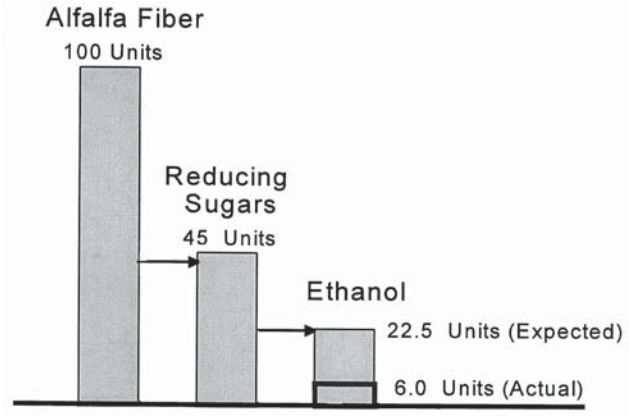

Fig. 5. Reducing sugar and ethanol yields resulting from saccharification and fermentation of 100 units of alfalfa fiber without LHW pretreatment. Fermentation organism: *C. shehatae* (FPL 702).

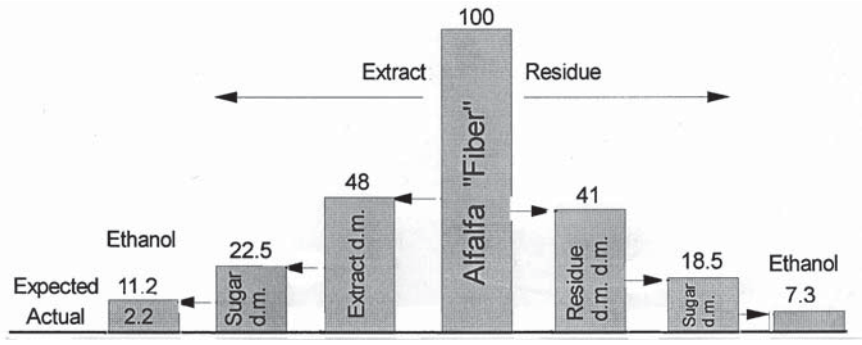

Fig. 6. Reducing sugar and ethanol yields resulting from saccharification and fermentation of 100 U of alfalfa fiber converted (left) to extract and (right) to residue by LHW pretreatment. Fermentation organism: *C. shehatae* (FPL 702).

contrary to expectations, about 40% of the extract dry matter was in the form of fine particulate or insoluble precipitate after saccharification. Based on the unavailability of this material, the sugar yield of 22.5% of the original fiber appears reasonable.

The ethanol yield of the residue-derived sugars was about 0.4, 80% of the "expected." Insignificant amounts of ethanol were produced by the fermentation of the extract-derived sugars using *C. shehatae*. Analysis of the extract for organic acids showed it to have an acetic acid level around 1%, approximately twice the level known to be inhibitory to yeasts. Acetic acid is a breakdown product of hemicellulose.

Lactic Acid

Fermentation of 100 U of the untreated fiber resulted in a maximum production of 35.0 U of lactic acid at 48h using *L. delbrueckii*, (Table 1 and

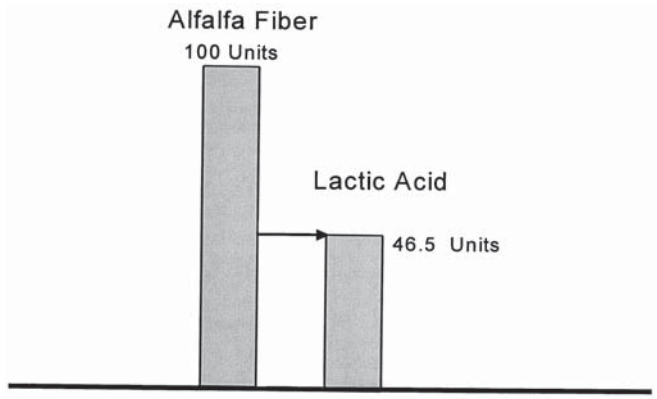

Fig. 7. Lactic acid yields resulting from saccharification and fermentation of 100 U of alfalfa fiber without LHW pretreatment. Fermentation organism: *L. delbrueckii*.

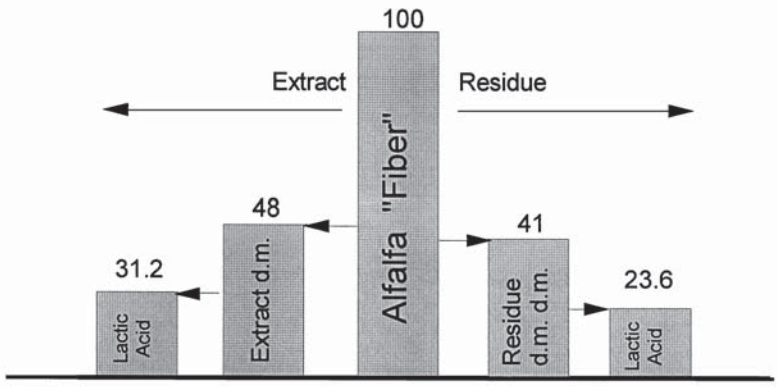

Fig. 8. Lactic acid yields resulting from saccharification and fermentation of 100 U of alfalfa fiber converted (left) to extract and (right) to residue by LHW pretreatment. Fermentation organism: *L. plantarum*.

Fig. 7) and 46.5 U at 72 h using *Lactobacillus plantarum*. When the extract and residue resulting from the LHW treatment of 100 U of fiber were individually saccharified and fermented using *Lactobacillus plantarum* the lactic acid yields were 31.2 and 23.6 U, respectively, for a total of 54.8 U. This is a difference of 8.3 U (54.8–46.5) or an increase of 18% relative to the yield from the untreated fiber (Fig. 8).

Conclusions

1. The LHW process consistently extracted about 60% of the dry matter from alfalfa fiber resulting from wet fractionation of herbage (up to 65% when .07% sulfuric acid was used).
2. The main fiber constituent extracted was hemicellulose, leaving a high-cellulose residue.

3. Commercially available enzymes were able to convert the untreated fiber and the residue and extract of the pretreated fiber to fermentable sugars, although in the case of the last two the yields were not as great as anticipated.
4. The sugars derived from the high-cellulose residue gave a reasonable ethanol yield.
5. Sugar derived from untreated fiber gave a lower ethanol yield than expected. The reason is not yet known.
6. Sugars derived from the extract were only minimally fermentable using *C. shehatae*. The reason is believed to be inhibition by acetic acid.
7. The total lactic acid produced from the extract and residue of the LHW pretreatment was 18% greater than from untreated fiber (54.8 U vs 46.5 U/100 U of feedstock). However, it is still unclear whether the cost of the LHW treatment would be justified.
8. The lactic-acid fermentations yielded significantly more product than the ethanol fermentations with *L. plantarum* giving greater yields than *L. delbrueckii*.

Acknowledgments

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