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Protein Extraction and Enzymatic Hydrolysis of Ammonia-Treated Cassava Leaves (*Manihot esculenta* Crantz)

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Abstract In the present work, cassava leaves were treated with 0.5 kg ammonia/kg dry matter at 78 °C and 30% moisture content in a 2-kg reactor. Protein extraction was carried out with a calcium hydroxide solution (pH 10) for 30 min at several temperatures (30 °C, 45 °C, 60 °C, 75 °C, and 90 °C) and solid/liquid ratios (1:10 and 1:15) in a thermostatized bath. Soluble protein content of the extracts was determined by Lowry's method. Dry substrate concentrations of 5%, 7.5%, and 10% and enzyme doses of 2 and 5 IU/g dry matter were used for the enzymatic hydrolysis in an orbital incubator at 50 °C and 100 rpm. Both cellulase and xylanase were used. Reducing sugars produced were determined with the dinitrosalicylic acid method. The highest protein extraction yield for the ammonia-treated leaves was 29.10%, which was 50% higher than with the untreated leaves (20%), and was obtained at 90 °C with a 1:10 solid/liquid ratio. The concentrate had a protein content of 36.35% and the amino acid profile was suitable for swine and poultry. The highest sugar yield was 54.72% with respect to theoretical and was obtained with 5% solids and an enzyme dose of 5 IU/g dry matter. This yield was 3.4 times higher than the yield of the untreated leaves (16.13%). These results indicate that cassava leaves have a great potential for animal feeding and ethanol production. Both protein extraction and sugar yields may be enhanced by optimizing the ammonia treatment.

Keywords Cassava leaves · Leaf protein · Sugars · Ammonia treatment

Introduction

Cassava is the world's fourth most important crop, with production estimated at 226 million tonnes in 2006, and is grown in many countries in Africa, Asia, and Latin America [1]. The

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roots are a major source of starch. Recently, cassava has been considered one of the four top crop priorities in Venezuela, which will make the quantity of cassava leaves increase. The leaves are rich in proteins (14–40%), vitamins, minerals, and carotenoids [2]; however, they are usually left in the field after cropping the roots. Generally, the roots are used as a human food, as a source of starch for the chemical industries, and as animal feed. On the other hand, cassava leaves meal has been used for single-gutted animal feeding, mainly swine, but because of the high fiber content, the percentage in the diets is very small. Attempts to industrially obtain protein concentrates from cassava leaves have failed due to low extraction yields and high content of tannins [3, 4]. In addition, the remaining fiber has a very low digestibility and poor nutritional value for ruminants.

Ammonia treatments have been successful in separating grass proteins from the fibers [5] and removing tannins [6], which increases the potential of the leaves for animal feeding, mainly swine and poultry. In addition, protein extraction leaves a solid fraction rich in cellulose which can be converted into sugars with greater yields [7] and these into ethanol by fermentation. The objective of this work was to treat cassava leaves with ammonia in order to enhance the protein extraction and the sugars yield obtained by enzymatic hydrolysis.

Material and Methods

Materials

Untreated and ammonia-treated cassava leaves (*Manihot esculenta* Crantz, Tempranita variety) were used. The raw leaves were collected at Municipio Mara, Zulia State, Venezuela, dried in a convection oven at 48 °C for 48 h, ground to a 2-mm particle size, and kept in plastic bags under refrigeration until used.

Chemical Analyses

Moisture content [8], crude protein [9], and cellulose, hemicellulose, and lignin [10] were determined in the raw and ammonia-treated materials. All the analyses were carried out in triplicate on unwashed materials both for untreated and ammonia-treated samples

Ammonia Treatment

A representative sample of 300 g of cassava leaves was processed in a 2-kg biomass pilot plant. Water (adjusted to experimental level) and liquid anhydrous ammonia were added to the sample at the selected temperature. The processing conditions were 0.5 kg ammonia/kg dry matter, 30% moisture content (w.w.b.), and 78 °C for 5 min.

Protein Extraction

A calcium hydroxide solution (pH 10) was used as the extracting agent. All the extractions were carried out in triplicate with 5 g of dry matter with constant stirring, varying solid/liquid ratio (1:10, 1:15) and temperature (30 °C, 45 °C, 60 °C, 75 °C, and 90 °C) for 30 min. Extracts were refrigerated to eliminate green proteins by precipitation [5]. After centrifugation, the white protein (true protein) present in the extracts was determined by the Lowry's modified method [11].The protein extraction yield was determined with respect to initial crude protein content.

Enzymatic Hydrolysis

A solids loading of 5%, 7.5%, and 10% *w/v* and enzyme doses (cellulase and xylanase) of 2 and 5 IU/g dry matter were used, in 500 ml Erlenmeyers containing 100 ml of 0.05 M citrate buffer (pH 4.8) placed at 50 °C for 48 h in an INNOVA 4300 incubator shaker (New Brunswick Scientific, Edison, NJ, USA) with 100 rpm [7]. Sodium azide was added for preservation (0.15%). Enzymes used were Celluclast 1.5L (Novo Nordisk, Franklinton, NC, USA), cellobiase (Novozym 188, Novo Nordisk, Franklinton, NC, USA), and Multifect XL (Genencor Internacional, Rochester, NY, USA). The enzymatic hydrolysis was applied to the ammonia-treated samples after protein extraction at optimal conditions. Sugar production was measured as reducing sugars during 48 h with the dinitrosalicylic acid method [12].

Amino Acid and Sugar Profiles

Proteins extracted from the leaves were precipitated by salting out with ammonium sulfate, dialyzed, and dried [13]. The protein concentrate was then subjected to an acid hydrolysis in a Waters Millipore Pico-Tag system [14]. The amino acid profiles were determined by high performance liquid chromatography (HPLC).

Individual sugars were measured by HPLC [7]. Standard sugars for HPLC were sucrose, glucose, xylose, arabinose, fructose, mannose, and galactose (SIGMA, MO, USA). Sugars present in the untreated sample and the treated samples (unwashed materials) before enzymatic hydrolysis were measured after a 5-h wetting period (HPLC) and subtracted from sugars measured after enzymatic hydrolysis to obtain true enzymatic sugar yields. Fiber conversion to sugars with respect to theoretical was determined based on initial cellulose and hemicellulose contents determined with the Goering and Van Soest method.

Results and Discussion

Moisture, cellulose, hemicellulose, lignin, and crude protein contents of untreated cassava leaves used in this study were 5.96%, 18.58%, 1.05%, 16.78%, and 18.60%, respectively. The crude protein content is higher than in grasses and is in the range of legumes and tubers [2]. The total neutral detergent fiber content (cellulose + hemicellulose + lignin) is 36.41%, too high for the feeding of single-gutted animals such as swine (<15%) and poultry (≤4%) [15, 16]; therefore, it is important to separate the proteins from the fiber in order to make the protein digestible. The composition of the treated leaves was similar to that of the untreated. Protein and cellulose contents remained constant and hemicellulose and lignin contents decreased about 10% (0.95% and 15.15%, respectively). In ammonia treatments applied to grasses and legumes, hemicellulose and lignin were partially solubilized, up to 50 and 30%, respectively, at optimal treatment conditions [7, 17]. In those materials, however, the lignin content was much lower (3–6%), and the treatments were more severe (greater ammonia loading, 1 kg ammonia/kg dry matter, and temperature 90 °C). In the present work, a lower ammonia loading and temperature were chosen due to the relatively high protein content of the leaves. However, although the ammonia treatment did not greatly affect the composition of the leaves, it did affect the structure since protein extraction and sugars yields increased with the treatment as described later in this section. Changes caused by alkali treatments involve breakage of ester linkages and opening of the crystalline structure of the cellulose [18].

Protein Extraction

Figure 1 shows the yields of the proteins extracted at several temperatures and solid/liquid ratios using pH 10 calcium hydroxide solution for 30 min, conditions that were previously determined according to Urribarrí et al. [5]. The yield increased as temperature increased. The solid/liquid ratio in the range studied did not affect the yield; therefore, 90 °C and a 1:10 solid/liquid ratio were chosen as optimal conditions. A higher solid/liquid ratio means lower equipment capacities and effluents, therefore, lower costs. The greatest yield for white protein extraction was 29.1%, which is 1.5-fold the extraction yield of untreated leaves, 20%. Both yield and increase in protein extraction are relatively low compared with those obtained for grasses such as dwarf elephant grass, 52.65% and 4.5-fold, respectively [5], which may be due to the higher lignin content of the cassava leaves (16.78% for cassava leaves relative to 4% for dwarf elephant grass), which suggest that treatment should be more severe, although it could damage the protein. It is important to point out that white protein content in the leaf is usually around 50% of the total protein, which indicates that the extraction of the white protein could have reached 60% of the initial content of white protein for ammonia-treated leaves. When the extracted protein was precipitated and freeze-dried, the concentrate had 35.35% protein. The amino acid profile of the protein concentrate from the ammonia-treated cassava leaves was comparable to that one of soybean meal and is shown in Table 1.

In terms of economics, 30% protein extraction looks low. However, a protein extraction yield to make specific protein extraction technologies from cassava leaves an economically attractive option has not been reported. It is important to point out that the economics of this process is based both on protein extraction and sugar yield. Certainly, the process may be optimized to increase the recovery of protein. This work is currently on its way.

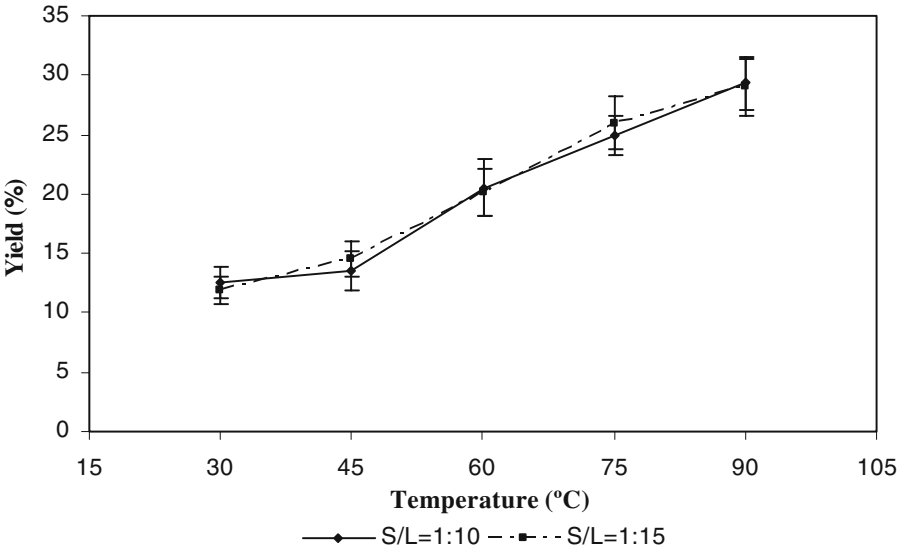

Fig. 1 Effect of temperature and solid/liquid ratio on the protein extraction yield for ammonia-treated cassava leaves at pH 10 for 30 min. *S/L* solid/liquid ratio

Table 1 Amino acids profile of the protein concentrate from ammonia-treated cassava leaves (g/16 g N).

Amino acids	Treated leaves	Poultry requirement [16]	Swine requirement [15]
Aspartic acid	14.65		
Glutamic acid	17.17		
Arginine	3.93	5.6	1.2
Lysine[a]	8.99	4.7	2.8
Methionine[a] + Cysteine	5.05	3.3	2.8
Tryptophan[a]	1.77	1	0.9
Histidine	5.62	1.4	1.1
Isoleucine[a]	9.49[b]	3.3	3.1
Leucine[a]		5.6	3.7
Phenylalanine[a]	5.48	5.6	4.4
Threonine[a]	6.19	3.1	2.8
Valine[a]	5.78	3.4	3.1

[a] Essential amino acids

[b] Isoleucine + Leucine

Enzymatic Hydrolysis

Table 2 shows the effect of solids content and enzyme dose on the sugar yield for nontreated leaves. For both enzymes doses, the highest yield was obtained for the highest solid content (10%). The yield was also higher for the greater enzyme dose as expected (>twofold). Although solid content could be increased up to 10% without negative effects on the sugar yield, the highest yield obtained (16%) was very low, as it has also been found in other nontreated materials such as grasses and legumes (20–26%) [7, 17], typical of complex lignocellulosic materials.

The ammonia-treated sample which was subjected to protein extraction at pH 10, 90 °C, and a 1:10 solid/liquid ratio for 30 min was used to investigate the optimal enzyme loading and solids content. Results are presented in Table 3.

Firstly, for an enzyme dose of 2 IU/g dry matter, the sugar yield was similar for the different solid contents. In this case, a solid content of 10% is preferred since the net sugar production will be proportional to the solid content, meaning savings in both equipment and sugar concentration.

For an enzyme dose of 5 IU/g dry matter, the highest yield was found for 5% solids, indicating that increasing the solids negatively affects sugar production and therefore sugar yield as expected for recalcitrant materials. For grasses, the highest yield has been found for

Table 2 Effect of solids content and enzyme dose on the sugar yield for nontreated leaves.

Time (h)	Yield (%)					
	2 IU/g dry matter			5 IU/g dry matter		
	5%	7.5%	10%	5%	7.5%	10%
3	0.83±0.11	0.94±0.24	0.98±0.23	1.70±0.15	2.48±0.26	1.91±0.26
6	2.12±0.21	2.35±0.16	2.55±0.18	5.12±0.17	4.52±0.16	4.32±0.23
12	3.60±0.15	4.70±0.09	3.38±0.14	11.61±0.13	7.05±0.21	8.26±0.16
24	3.97±0.28	5.50±0.14	4.73±0.20	12.81±0.22	9.39±0.19	8.72±0.24
48	5.18±0.08	6.17±0.16	8.01±0.13	13.18±0.19	16.06±0.19	16.13±0.22

Table 3 Effect of solids content and enzyme dose on the sugar yield for ammonia-treated leaves.

Time (h)	Yield (%)					
	2 IU/g dry matter			5 IU/g dry matter		
	5%	7.5%	10%	5%	7.5%	10%
3	8.17±0.15	9.61±0.72	11.31±0.46	10.80±0.00	12.88±0.56	14.15±0.29
6	12.43±0.34	15.85±0.32	16.25±1.16	17.63±1.19	20.13±0.39	18.75±0.75
12	22.78±0.46	24.96±0.49	26.36±0.57	28.12±0.55	32.95±0.00	29.11±0.70
24	34.04±0.65	32.66±0.65	39.32±0.76	43.05±1.72	44.47±1.72	45.44±0.95
48	40.65±0.85	40.64±0.38	41.15±0.39	54.72±0.53	48.85±0.95	46.67±0.91

7.5% solids (preliminary results). In several steam-exploded materials, solids concentration has been increased to 10% and high yields have been obtained [19], but this is likely due to lower amounts of polysaccharides in solution (hydrolysis of the hemicellulose during pretreatment and washing of the correspondent sugars prior to the saccharification process).

The results indicate that the sugar yield was higher for a higher enzyme dose, but the increase was lower than expected, since the enzyme dose increased 2.5 times whereas the yield increased just 1.33 times. When the solids content was very high (10%), agitation became more difficult suggesting a viscosity increase which might limit enzyme diffusion to the substrate. An increase in viscosity may occur due to an increase in water retention in ammonia-treated substrates (unpublished results) and liberation of polysaccharides (hemicelluloses) from the lignocellulosic matrix [7]. The greatest sugar yield, 54.72%, was obtained with 5% solids and 5 IU enzyme/g dry matter for 48 h.

Figure 2 shows the comparison between sugars produced in nontreated and ammonia-treated cassava leaves. The yield of reducing sugars increased from 16.13% conversion in

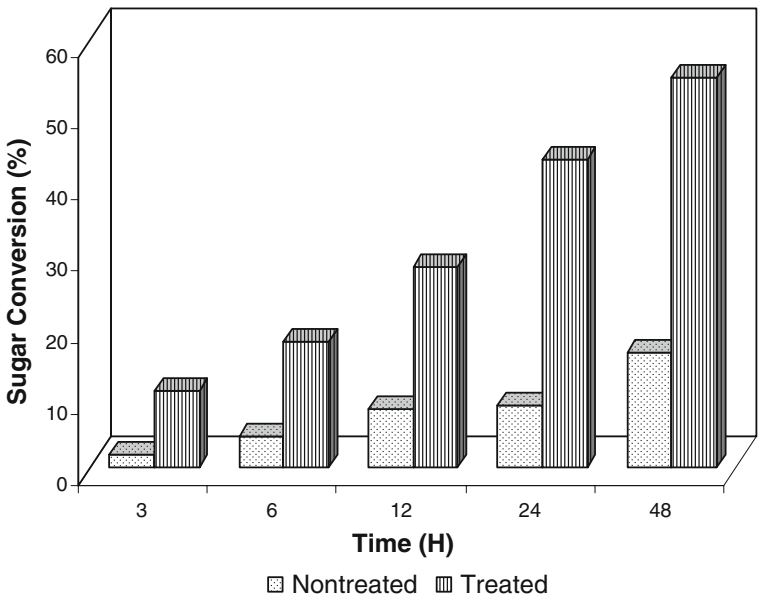

Fig. 2 Sugar yields of nontreated and ammonia-treated cassava leaves. Nontreated—10% solids and 5 IU/g enzyme dose. Treated—5% solids and 5 IU/g enzyme dose

the untreated sample (10% solids and 5 IU enzyme/g dry matter) to 54.72% conversion (5% solids and 5 IU enzyme/g dry matter) in the treated sample which corresponds to a factor of 3.4 which shows the great efficiency of the treatment even though ammonia treatment conditions were not optimized. Greater yields have been obtained in ammonia-treated grasses [7] but lignin content was much lower in the grasses and the treatment conditions were more severe. It is also possible that lignin in cassava leaves is mainly bonded by ether rather that ester linkages. Ether linkages are not broken with alkali.

The greatest yield for the treated leaves was reached at 5% solids, a lower value than for the nontreated leaves. Three facts might explain these results. First, the treatment dissolves some polysaccharides which increase viscosity; second, the treated materials have a higher water retention capacity, which also increases viscosity; and third, the treatment increases the amount of cellulose that is susceptible to enzymatic hydrolysis. In fact, there is more cellulose available in 5% treated solids than in 10% nontreated solids.

When individual sugars were determined by HPLC, only glucose, xylose, and arabinose appeared (Fig. 3). Glucose concentration increased from 28 in the untreated sample to 65 mg/g dry matter in the treated one (2.32-fold). Xylose and arabinose concentrations in the untreated sample increased from 2.1 and 1.5 mg/g dry matter, respectively, to 8.0 and 3.4 mg/g dry matter in the treated sample (3.81- and 2.27-fold, respectively).

This is the first work that shows that cassava leaves may be biorefined in order to produce a high protein concentrate and fiber with high susceptibility to enzymatic hydrolysis which may be converted into ethanol. In this way, cassava roots can still be used for human food and for native and modified starch production, whereas the leaves may be used for both animal feeding and bioenergy. As lignin remains in the process, it may be used to produce a great variety of chemicals. Although sugarcane is used in countries like Brazil for ethanol production, such approach uses land that competes with food production. The Food and Agriculture Organization of the United Nations (FAO) [20] recommends the use of the cassava roots for ethanol production. FAO [1] also calls for a significant increase in research and development on cassava to boost yields and industrial uses. On the other hand, the value of the leaves as a fertilizer is very low. Although they are generally left in

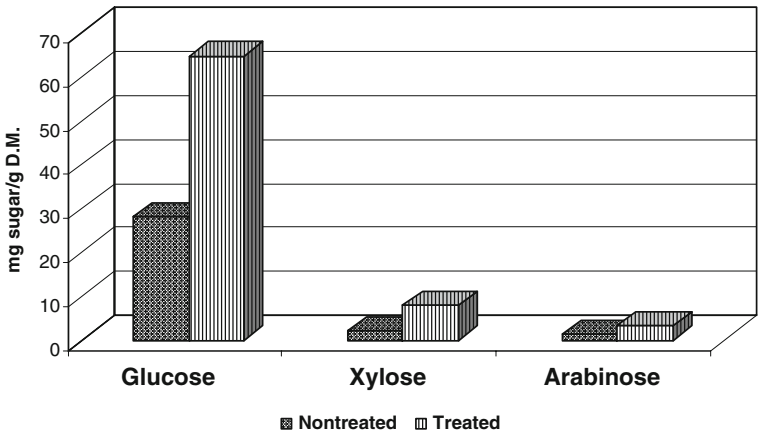

Fig. 3 Sugar profile of the hydrolysate from nontreated and ammonia-treated cassava leaves. Nontreated—10% solids and 5 IU/g enzyme dose. Treated—5% solids and 5 IU/g enzyme dose

the field, considerable amounts of fertilizers have to be added. The impact of removing cassava leaves from the field has not been addressed to the knowledge of the authors.

If the ammonia treatment conditions are optimized, the yields of protein extraction and sugar production may be further increased. Increasing the ammonia loading up to a certain level has caused increases in the sugar yield from enzymatic hydrolysis of several ammonia-treated lignocellulosic materials [7, 17], which in turn would increase ethanol production; however, there are no reports of the effect of ammonia loading on protein extraction. It is possible that protein extraction might also increase with ammonia loading, but protein quality may decrease due to degradation of amino acids by high concentrations of alkali. This remains to be investigated.

Conclusions

Chemical composition of the cassava leaves was not greatly affected by the ammonia treatment. The extraction conditions for the white proteins from cassava leaves treated with ammonia that rendered the maximum yield, 29.10%, were pH 10, 90 °C, and 1:10 solid/liquid ratio for 30 min. A suitable amino acid profile makes it a protein with great potential for the production of high quality feeds for poultry and swine. The high lignin content of the leaves, 16.78%, is likely responsible for the relatively low increase of the extraction yield compared to the untreated sample (≈50%).

The highest sugar yield was 54.72% with respect to theoretical which was 3.4 times higher than that of the untreated leaves. It could be increased with a greater ammonia loading. Sugars produced were glucose, xylose, and arabinose. The ammonia treatment was effective in increasing the extraction of white protein and sugars yield.

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