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Plant Processing by Simultaneous Lactic Acid Fermentation and Enzyme Hydrolysis

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ABSTRACT

Traditional ensiling of plant material by anaerobic lactic acid fermentation was combined with enzymatic hydrolysis (ENLAC for short) with cell wall degrading enzymes (hemicellulases, cellulases, and pectinases) to increase fiber digestibility or to increase the recovery of cell content from plants. Such findings were made using 0.015% (w/w, wet basis) Phylacell® enzyme preparation by ENLAC of corn and corn-sorghum mixtures, but not of forage grasses. Addition to alfalfa of a mixture of cell wall degrading enzymes, such as NOVO Viscozyme® together with NOVO Celluclast® each at 0.2–1.0% (w/w, wet basis), resulted in more rapid ensiling and improvement of rumen digestibility of silage by 20%. After 20 d of ensiling at 25°C when the same enzymes were added to alfalfa at the 1.0% level, protein recovery by pressing increased by 35%, β-carotene recovery by 80%, and chlorophyll/xanthophyll recovery by 30%. ENLAC with the same enzymes also increased the recovery of sclareol from muscatel sage by 400%.

Index Entries: Ensiling; enzyme hydrolysis; plant cell wall degradation; cell content recovery.

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INTRODUCTION

Plants contain valuable natural substances that are usually recovered by pressing or extraction. The recovery and yield of such substances depend on the efficient disruption of the plant cell wall. Examples are recovery of protein from alfalfa in the so-called leaf protein process, recovery of reducing sugars from sweet sorghum, and recovery of drugs and fragrances from various plants. Most of these biological substances are sensitive to harsh mechanical, chemical, or physical treatments during recovery, hence the need for a mild biological procedure.

The new biological procedure described in this article combines traditional anaerobic lactic acid fermentation, ensiling, with enzymatic hydrolysis of the plant cell wall (ENLAC for short). In ENLAC, lactic acid bacteria (LAB) produce lactic acid that partially hydrolyzes hemicellulose and pectin, and some LAB may directly degrade and utilize hemicellulose. The weakened cell wall is more accessible for enzyme attack by hemicellulases, pectinases, and cellulases in an acidic environment that favors enzyme activity and preserves valuable cell content during treatment and subsequent recovery. The ensiling conditions allow a long reaction time with very low enzyme concentrations without loss of the valuable substance to be recovered (*1–3*). ENLAC targets primarily the outer "cementing" components of the cell wall, pectin, extensin, and hemicellulose, because a partial removal of these components causes sufficient increase in cell wall permeability and weakening of cell wall structure to allow easier recovery of cell content (*4*). ENLAC is particularly suitable for plants or parts of plants with $< 10\%$ lignin content (soft stem plants, leaves, fruits, young shoots, and so on).

ENLAC has evolved from attempts to improve the ensiling process. Some newer silage additives contain either LAB inoculants, cell wall degrading enzymes, or a combination of both (*5–7*), and are used in the ensiling of a variety of crops. ENLAC has also been used to improve the recovery of protein, chlorophyll, xanthophyll, and β-carotene from alfalfa (*4*). In ENLAC, 10× less enzyme concentration was sufficient for the same degree of increase in fiber digestibility (*7*) or protein recovery (*4*) from alfalfa as by conventional 24-h enzyme treatment. In the present article, examples are presented of the successful application of ENLAC technology for ensiling and the recovery of various cell contents from plants.

MATERIALS AND METHODS

Enzyme Additives

The commercial enzymes NOVO Viscozyme® (from *Aspergillus* sp. in liquid form, containing hemicellulases, cellulases, and pectinases, 140 mg/mL total proteins, 120 FBG [Fungal β Glucanase] U/mL activity, pH optimum 3.3–5.5, temperature optimum 40–50°C) and NOVO Celluclast®

1.5 L (from *Trichoderma reesei,* in liquid form, containing mainly cellulases, 220 mg/mL total proteins, 1500 NCU [NOVO Cellulase Units]/mL, pH optimum 4.8, temperature optimum 60°C) were used in the experiments in concentrations optimized in a previous study (*4*). Phylacell® (Phylaxia Inc., Budapest, Hungary, from *Helminthosporium sativum,* containing hemicellulases, cellulases, and pectinases, in powder form, 65% total proteins, 20 CMC [carboxymethyl cellulase] U/g, pH optimum 4.9, temperature optimum 50°C) was used in large-scale ensiling studies of corn, corn-sorghum, and mixed grass forages.

ENLAC Treatment

Fourth cutting alfalfa in late vegetative growth was harvested in October 1989 in Fort Collins, Colorado and was immediately frozen. The dry matter (DM) content of the frozen alfalfa was 28.0±0.4% and contained 210 g/kg DM protein. The thawed forage was chopped to 1-in size, mixed thoroughly with silage additives, and then tightly packed into 200-mL Mason jars with tightly sealed lids, about 150 g/jar. Each treatment had three replicates; i.e., three jars were opened for assays at each designated time point. Each sample was assayed in duplicate. The control was alfalfa ensiled without additives. The zero time values represent untreated alfalfa.

Enzyme Treatment

The same chopped alfalfa as above was thoroughly mixed with enzymes, placed in a 1.0-L container and incubated in a moist chamber for 24 h with occasional stirring. Each treatment had three replicates, i.e., three samples for each time point and duplicate assays of each sample.

Cell Content Recovery

Fifty grams of treated or untreated alfalfa were mixed with 50 mL phosphate-buffered saline (PBS, pH 7.2), stored overnight in a refrigerator at 4°C, and then pressed with a French Press at 4000 psi. The pressate was used for assaying cell content recovery. For comparison, another 50 g alfalfa from the same treatment was blended with 50 mL PBS in a Waring blender for 2 min, refrigerated overnight, and then pressed at 4000 psi.

Assays

Dry matter (DM) content was determined gravimetrically after oven drying samples at 105°C. Nutrient composition in treated and untreated plant material was determined by US Standard Methods immediately after sampling (*8*). Fiber analysis was performed by the method of Van Soest and Wine (*9*). Rumen digestibility was measured *in situ* by placing 5 g of air-dried sample into a 7×12 cm nylon bag and inserting it into the rumen of a fistulated cow consuming a forage diet. The bag was incubated in the rumen for 48 h, and then the weight loss was measured (*10*).

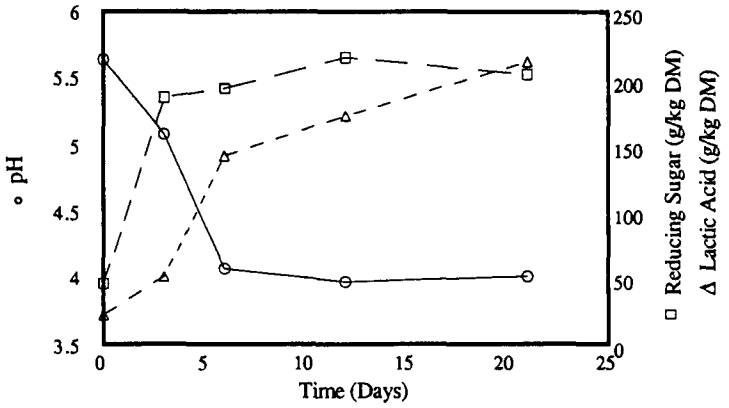

Fig. 1. Kinetics of ENLAC. Chopped alfalfa (2.5-cm size) at 28% DM content was ensiled with 1.0% (w/w, wet basis) each of Celluclast® and Viscozyme® at 25°C.

Reducing sugars, lactic acid, and pH were determined from treated or untreated alfalfa immediately after sampling. A 10-g sample was diluted with 90 mL distilled water and blended in a Waring blender for 2 min. The homogenate was filtered through Whatman paper no. 1, and the filtrate was used for assay. Reducing sugar was determined by the dinitrosalicylic acid method (*11*). Lactic acid was determined by HPLC, using a Bio-Rad Aminex HPX-87H 300×7.8 mm column with 0.008*N* H_2SO_4 as the mobile phase. The column temperature was 43°C, the pressure 1000 psi, and the flow rate 0.6 mL/min. A Waters Associates differential refractive index detector and data module were used for quantitation.

Cell content recovery was assayed from the pressates described above. Protein was determined by the Folin-Lowry procedure (*12*). Chlorophyll was determined colorimetrically (*4*). β-carotene was determined by the method of Kramer and Twigg after acetone extraction (*13*). Sclareol was determined spectrophotometrically after aqueous extraction.

RESULTS AND DISCUSSION

A comparison of the kinetics of enzyme hydrolysis of chopped alfalfa in ENLAC and enzyme treatment alone is presented in Figs. 1 and 2. In a 20-d ENLAC treatment, 210 g/kg DM reducing sugar were produced with a significant and rapid pH drop and corresponding lactic acid production (Fig. 1). In contrast, a 24-h enzyme treatment only resulted in the production of 110 g/kg DM reducing sugar with a modest drop in pH and no lactic acid production (Fig. 2). Note that the two figures are plotted on different scales.

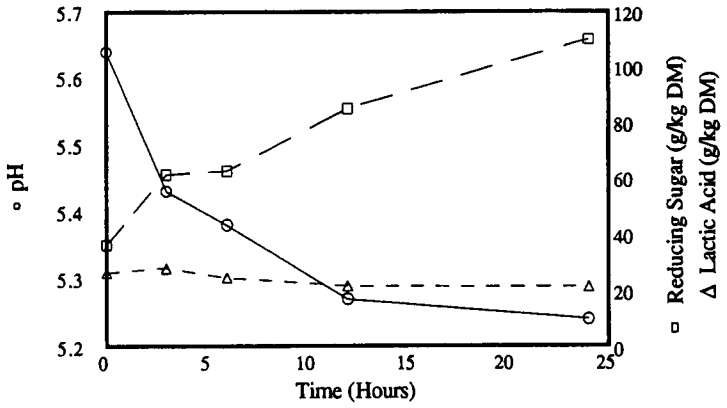

Fig. 2. Kinetics of enzyme treatment. Chopped alfalfa (2.5-cm size) at 28% DM content was treated with 1.0% (w/w, wet basis) each of Celluclast® and Viscozyme® at 25°C.

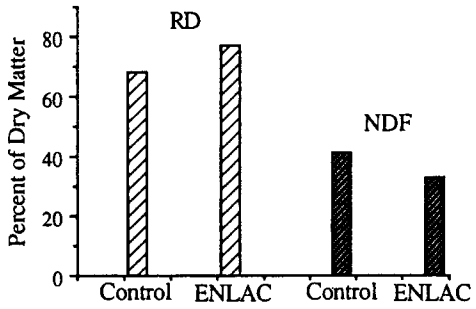

Fig. 3. Fiber digestibility of alfalfa. Chopped alfalfa (2.5-cm size) at 28% DM content was ensiled with 1.0% (w/w, wet basis) each of Celluclast® and Viscozyme® for 20 d at 25°C. RD = rumen digestibility, NDF = neutral detergent fiber.

ENLAC increased the fiber digestibility of alfalfa (Fig. 3). After 20 d of ensiling at 25°C in the presence of 1.0% (w/w, wet basis) each of Celluclast® and Viscozyme®, rumen digestibility increased from 68 to 77%, and the neutral detergent fiber (NDF) fraction decreased from 40 to 36%. The reduction of NDF and the simultaneous appearance of reducing sugars strongly suggest the partial hydrolysis of hemicellulose, the main cementing component of the cell wall. This suggestion was corroborated by the appearance of pentoses among the hydrolysis products, as reported in another study (*7*).

ENLAC was tested in large scale in Mosonmagyarovar, Hungary to improve the digestibility of corn, corn-sorghum, and grass silages. Corn and corn-surghum (70:30 mix) were ensiled in 3500-t horizontal silos for 90 d at ambient temperatures in the presence of Phylacell® enzyme and a commercial lactic acid bacteria inoculum (Medipharm Sweden, Monor

Table 1
Changes in Digestibility of Phylacell®-Treated Silages

	Digestibility[a] with Phylacel®, g/t wet wt				
	Corn silage		Corn-sorghum silage	Grass silage	
Component	34	150	150	200	4000
Organic matter	107.2	107.6	111.7	103.5	106.2
Crude protein	118.6	103.3	114.3	104.8	107.8
Crude fat	103.1	97.7	103.0	110.3	102.8
Crude fiber	122.7	114.4	123.9	102.9	106.1
N-free extract	103.4	107.2	105.7	105.5	111.7

[a]Percent change from untreated silage (100%). Phylacell® is a commercial enzyme from *Helminthosporium sativum* (Phylaxia Ltd., Hungary) applied at the concentrations of 34, 150, 200, or 4000 g/wet t silage. Silage corn, corn:sorghum 70:30 mix, or mixed forage grasses chopped to 2.5-cm size at 28% DM content were ensiled with Phylacell® and LAB inoculum at 25°C for 90 d.

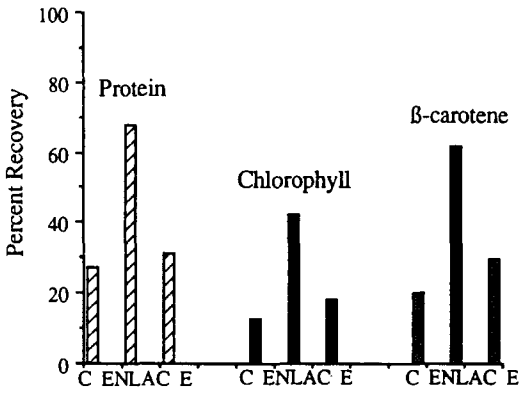

Fig. 4. Cell content recovery from alfalfa. C: Ensiled control; ENLAC: ensiled at 25°C for 14 d with 1.0% (w/w, wet basis) each of Celluclast® and Viscozyme®; E: treated with same enzymes for 24 h at 25°C to represent enzyme control. Protein content was recovered by pressing at 4000 psi.

AG, Hungary, effective total count in silage 1.0×10^5 CFU/g wet forage). First-cut mixed forage grasses were ensiled in 200-L plastic barrel silos under the same conditions as above. The results are shown in Table 1. The ENLAC treatment improved the protein and fiber digestibility of corn and corn-sorghum silages with a very modest expenditure of enzymes, 34 and 150 g/wet t silage. The ENLAC treatment did not improve the digestibility of grass silage, even at greater enzyme application rates, presumably because of the tougher cell wall structure of grasses.

ENLAC improved the recovery of protein, chlorophyll and β-carotene from alfalfa (Fig. 4). There was no change in DM content during the 20-d ensiling period (data not shown). The main benefit of ENLAC is that it allows recovery under mild conditions (low pressure, no mechanical dis-

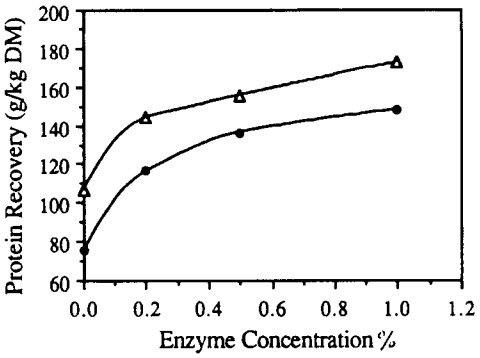

Fig. 5. Protein recovery vs enzyme concentration —△— ENLAC 14 d, 25°C; —•— enzyme treatment 24 h, 25°C to represent enzyme control. The enzyme concentration shown (w/w, wet basis) is for each the Celluclast® and the Viscozyme®.

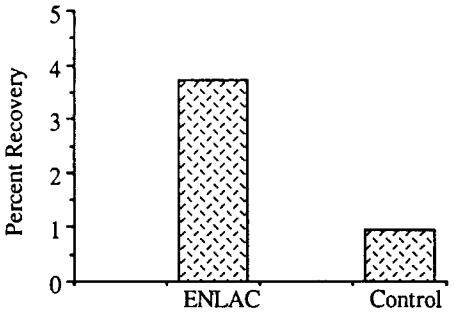

Fig. 6. Sclareol recovery from muscatel sage. Chopped sage (2.5-cm size) was ensiled at 28% DM content with 1.0% (w/w, wet basis) each of Celluclast® and Viscozyme® and Lactobacillus TUB-216 (1.0×10^6 CFU/g wet material) for 14 d at 25°C. Sclareol was recovered by aqueous extraction. Recovery is expressed as percent (w/w) of DM content.

integration of cells). The β-carotene recovery by ENLAC shown in Fig. 4 was equal to recovery after thorough blending for 10 min (mechanical disintegration) of untreated alfalfa. The amount of enzyme needed for 70% recovery of protein was about 10× less in ENLAC than in enzyme treatment alone (Fig. 5).

In another joint research project in Hungary, ENLAC was applied for the recovery of sclareol (a fragrance base) from muscatel sage (Fig. 6). ENLAC dramatically increased sclareol recovery at a low enzyme concentration (1.0% w/w, wet basis).

The examples shown above demonstrate that ENLAC is a useful plant processing technology for recovering valuable cell content or for improving the fiber digestibility of forages. The synergistic action of lactic acid fermentation and cell wall degrading enzymes and the acidic environment create favorable conditions for cell wall degradation at very low enzyme concentrations. The mechanism of cell wall degradation in ENLAC is currently being investigated in this laboratory.

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