Enhancement of Polyphenol Recovery from Rosemary (*Rosmarinus officinalis*) and Sage (*Salvia officinalis*) by Enzyme-Assisted Ensiling (ENLAC)

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The efficacy of enzyme-assisted ensiling (ENLAC) in the recovery of polyphenols from rosemary and sage was tested. Fresh rosemary and sage were chopped and ensiled in 0.5-L anaerobic jars. Treatments comprised control (no additives), 0.5% glucose and lactic acid bacteria, and 1% cellulase plus 1% hemicellulase plus pectinase. Following storage at room temperature for 45 days (experiment 1) and 26 days (experiment 2), polyphenols were extracted from the silages in ethanol either by direct blending or by cold extraction. The enzyme treatment resulted in silages with the lowest pH values, lowest fiber content, highest water-soluble sugar content, and highest polyphenol recovery; this treatment resulted in increased polyphenol recovery from rosemary and sage, by 100 and 20%, respectively. Comparison between direct blending and cold extraction revealed similar efficiency of polyphenol recovery.

Keywords: *Polyphenol recovery; rosemary; sage; ensiling*

INTRODUCTION

Recently, there has been increasing interest in the development of natural antioxidants from plants for the food industry and preventive medicine. Plant tissues are the main source of α-tocopherol, ascorbic acid, carotenoids, and phenolic compounds (Kanner et al., 1994). Flavonoids and other plant phenolics have been reported to have multiple biological effects such as antioxidant activity, anti-inflammatory action, inhibition of platelet aggregation, and antimicrobial activity [e.g., Bors and Saran (1987), Moroney et al. (1988), Pratt and Hudson (1990), and Van-Wauwe and Gossenc (1983)]. A high correlation has been found between the amounts of polyphenolics extracted from grapes, wine, and other plant material and their antioxidant activity (Kanner et al., 1994; Yi et al., 1997). Extraction of these compounds involves homogenization of the plants in the presence of an adequate solvent.

An alternative process that might improve the recovery of polyphenolics from plants is enzyme-assisted ensiling (ENLAC). Ensiling is a preservation method for moist crops. It is based on natural solid-state fermentation under anaerobic conditions, whereby lactic acid bacteria (LAB) convert water-soluble carbohydrates (WSC) into organic acids, mainly lactic acid; as a result, the pH decreases and the forage is preserved. Cell wall hydrolyzing enzymes (cellulases, hemicellulases, and pectinases) can be applied at ensiling of sugar-poor forage crops. Sugars released from the hydrolysis of the cell wall can be used by the LAB in the ensiling fermentation. In addition, the digestibility of such crops by ruminants increases as a result of cell-wall disruption [e.g., Selmer-Olsen (1994) and Weinberg et al. (1993)]. Enzyme levels commonly used in forage ensiling are 0.025−0.05% on a fresh weight basis.

The ENLAC process can also be used to improve extraction recovery of valuable substances from plants. The advantages of ENLAC in comparison with conventional methods are as follows: (1) Higher recovery yields are obtained. (2) Ensiling results in optimal pH conditions for the enzymes (4.5−5.0). (3) Preservation of the biomass by ensiling under anaerobic conditions enables long storage periods without any need for immediate processing. (4) Softening of the cell wall by ENLAC ensures mild extraction conditions.

Previous studies have proven the efficacy of ENLAC in the recovery of protein, chlorophyll, β-carotene, and xanthophyll from alfalfa and preservation of sugar in sweet sorghum (Schmidt et al., 1997; Tengerdy et al., 1992; Weinberg et al., 1990).

Rosemary and sage have been reported to have strong antioxidative activity, which is related mainly to three phenolic compounds: carnosic acid, carnosol, and rosmarinic acid. Other phenolic compounds that are potent antioxidants were also identified in rosemary and sage (Cuvelier et al., 1996). The purpose of the present study was to test the efficacy of ENLAC in the recovery of polyphenols from rosemary and sage, in comparison with direct extraction.

MATERIALS AND METHODS

Whole plant fresh rosemary (*Rosmarinus officinalis*) and sage (*Salvia officinalis*) were hand chopped to 2−3-cm pieces. After treatment application, the chopped plants were ensiled in 0.5-L anaerobic jars. Each jar was filled with ∼160 g (wet weight) of plants without a headspace. The jars were stored at room temperature (25−27 °C). There were two experiments: in the first, rosemary and sage were ensiled for 45 days, and in the second, rosemary was ensiled for 26 days.

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10.1021/jf981317+ CCC: $18.00 © 1999 American Chemical Society
Published on Web 06/26/1999

There were two jars per treatment except for the enzyme treatment in experiment 2, which comprised four jars.

At the end of the ensiling period, silage samples were homogenized immediately with a Waring blender for 1 min at room temperature in the presence of ethanol (1:9), and the concentration of polyphenols was determined according to the Folin−Ciocalteu procedure (Kanner et al., 1994). In addition, in experiment 1, a 50-g silage sample from each jar was mixed with 175 mL of ethanol and left overnight at 4 °C, after which time the ethanol was collected; the plant material was pressed out with a French press at 60 psi, and the resultant liquid was combined with the ethanol. Polyphenols in the cold extracts were determined as described above.

The following treatments were used: (1) control (no additives); (2) inoculant plus 0.5% glucose; and (3) same as treatment 2 plus 1% cellulase and 1% (hemicellulase plus pectinase) (w/wet weight). The inoculant used was Biomax Si (CHR Hansen's, Denmark), which contained 5×10^9 colony-forming units (cfu) per gram of *Lactobacillus plantarum* (manufacturer's declaration). It was applied by suspending 50 mg of the inoculant powder in 10 mL of water, spraying it over 1 kg of chopped plants, and mixing thoroughly. Thus, 2.5×10^5 cfu/g was applied.

The enzymes used were Celluclast 1.5L and Peelzyme I (Novo, Denmark, and Novo Nordisk, Switzerland, respectively). The stated activities of the enzymes were 1500 NCU (Novo cellulase units) and 26000 PG (polygalacturinase) mL^{-1}, repectively. Thus, 15 NCU and 260 PG units were added per gram of silage.

ANALYTICAL PROCEDURES

Dry matter was determined by oven-drying for 48 h at 60 °C. WSC were determined according to the phenol−sulfuric acid method, according to the procedure of Dubois et al. (1956). Lactic acid and volatile fermentation end products (ethanol and acetic, propionic, and butyric acids) were determined by gas chromatography with an FFAP (Hewlett-Packard) megabore column, over a temperature range of 45−230 °C. Cellulase activity was determined by filter paper assay, according to the method of Ghose (1987).

Fiber analysis included determination of neutral detergent fiber (NDF), acid detergent fiber (ADF), and acid detergent lignin (ADL); fiber analysis was performed according to the method of Goering and Van Soest (1970), with the Ankom filter bag system (Ankom Technology Corp., Fairport, NY).

Statistical analysis included one-way analysis of variance and Duncan's multiple-range test, performed with the GLM procedure of the Statistical Analysis System (SAS, Cary, NC).

RESULTS

The Celluclast activity determined by the filter paper assay was 147 units mL^{-1}; that is, at the 1% rate of application, 1.47 units g^{-1} fresh material was added.

Table 1 gives the chemical analyses of the fresh rosemary and sage and of their respective silages. The enzyme treatment resulted in the lowest pH values, and the highest WSC content, because of the sugar released by cell-wall hydrolysis. In the other treatments the WSCs were used in the ensiling fermentation. Ethanol was the major fermentation product, and the enzyme treatment resulted in lower ethanol contents than the control or LAB plus glucose treatments.

Table 2 gives the cell-wall content of the fresh plants and of the respective silages. The enzyme treatment resulted in significantly lower NDF and ADF contents than the other treatments. The NDF and ADF fractions represent the total cell-wall and (cell-wall minus hemicellulose) contents, respectively. The ADL fraction (lignin plus ash) was the least affected. Table 3 gives the polyphenol recoveries from the fresh plants and from the silages. Ensiling itself resulted in improved polyphenol recovery, compared with that from the fresh material (which is equivalent to the conventional extraction method) only from the rosemary in experiment 1. In this experiment, application of LAB resulted in a further improvement in polyphenol recovery, whereas the best results were obtained in silages treated with the cell wall hydrolyzing enzymes. This treatment resulted in an increase in polyphenol recovery from the rosemary by 100% in both experiments. In the second experiment with rosemary only the enzyme treatment resulted in significantly higher polyphenol recovery. With sage, both LAB plus glucose and enzyme treatments resulted in improved polyphenol recovery, but only by ~20%, as compared with the fresh material and with the control.

Table 1. Chemical Analysis of the Rosemary and Sage, Fresh Material and Silages (Grams per Kilogram of Dry Matter)[a]

treatment	pH	DM	WSC	ethanol	acetic acid	lactic acid
rosemary (expt 1)						
fresh material	6.0[a]	400	67[a]			
control	5.4[b]	392	18[b]	225	2	2
LAB + glucose	4.5[c]	395	12[b]	122	3	11
enzymes	4.2[d]	402	85[a]	87	6	19
rosemary (expt 2)						
fresh material	6.3	354	19[b]			
control	5.8	350	18[b]	18	2	8
LAB + glucose	5.3	351	14[b]	19	0	6
enzymes	5.1	376	71[a]	2	0	14
sage						
fresh material	6.1[a]	279	86[b]			
control	6.0[a]	277	28[c]	131	3	13[a]
LAB + glucose	5.3[b]	285	12[d]	115	3	17[a]
enzymes	4.5[e]	309	129[a]	55	3	6[b]

[a] DM, dry matter; WSC, water-soluble carbohydrates; LAB, lactic acid bacteria. Within a column and experiment, means followed by different letters differ significantly ($P < 0.05$).

Table 2. Cell-Wall Content of the Rosemary and Sage, Fresh Material and Silages (Grams per Kilogram of Dry Matter)

treatment	NDF	ADF	ADL
rosemary (expt 1)			
fresh material	447[a]	369[a]	194[a]
control	418[b]	337[b]	166[bc]
LAB + glucose	438[ab]	363[a]	179[ab]
enzymes	324[c]	271[c]	162[c]
rosemary (expt 2)			
fresh material	362[b]	289[a]	171[b]
control	342[c]	282[a]	185[a]
LAB + glucose	377[a]	290[a]	189[a]
enzymes	265[d]	222[b]	150[c]
sage			
fresh material	416[a]	273[a]	121[a]
control	350[b]	268[ab]	116[a]
LAB + glucose	351[b]	264[b]	98[b]
enzymes	231[c]	184[c]	94[c]

[a] DM, dry matter; LAB, lactic acid bacteria; NDF, neutral detergent fiber; ADF, acid detergent fiber; ADL, acid detergent lignin. Within a column and experiment, means followed by different letters differ significantly ($P < 0.05$).

DISCUSSION

ENLAC is a relatively simple biotechnological process that can be applied to recover valuable substances from plants for various purposes: food, feed, cosmetics, pharmaceuticals, etc. Its advantages over conventional extraction have been pointed out in the Introduction. Improved recovery of valuable substances by ENLAC from alfalfa and sorghum has been obtained in previous

Table 3. Antioxidant Recovery from Rosemary and Sage[a]

treatment	recovered polyphenols: direct blending	recovered polyphenols: cold pressing
rosemary (expt 1)		
fresh material	22.5[b]	
control	35.7[ab]	31.7[b]
LAB + glucose	43.7[ab]	34.5[ab]
enzymes	53.5[a]	56.5[a]
rosemary (expt 2)		
fresh material	25.4[b]	
control	21.8[b]	
LAB + glucose	20.1[b]	
enzymes	43.2[a]	
sage		
fresh material	64.7[b]	
control	62.3[b]	72.3
LAB + glucose	81.5[a]	73.3
enzymes	76.9[a]	80.6

[a] Results are given as polyphenol recovery (g kg^{-1} DM). DM, dry matter; FM, LAB, lactic acid bacteria. Within a column and experiment, means followed by different letters differ significantly ($P < 0.05$).

experiments (Schmidt et al., 1997; Tengerdy et al., 1992; Weinberg et al., 1990). In these experiments, application of the enzyme cocktail for 24 h without ensiling did not improve protein or chlorophyll recovery, even when lactic acid was added to decrease the pH (Weinberg et al., 1990). Therefore, such a treatment was not included in the present study. In the extraction of carotenoids from orange peels, cellulolytic and pectolytic enzymes have been applied, without ensiling, and this resulted in increased recovery (Kanner et al., 1984). It is assumed that differences in cell-wall structure between whole plants and citrus peels account for the difference in the contribution of ensiling to obtaining higher extraction yields.

In the present study the recovery of polyphenols from rosemary and sage by ENLAC was tested. These compounds were chosen as targets for the test because of their multiple biological activities, which were described in the Introduction. Ensiling of herbal plants for this purpose is a new concept, and there were no data available on the ensiling properties of rosemary and sage. Therefore, LAB plus glucose treatment was included to ensure proper ensiling fermentation. The results indicate that it is possible to ensile rosemary and sage and that there is no need for enhancement with inoculants or glucose.

Ensiling itself resulted in some cell-wall hydrolysis (Table 2); this was due to breakdown of hemicellulose under acidic conditions (Morrison, 1979). However, the best results were obtained with the enzymes. These results and also previous ones indicate that improved recovery of cell contents is associated with cell-wall hydrolysis; a cocktail of enzymes, comprising both cellulases and hemicellulases plus pectinases, is required to achieve cell-wall hydrolysis and improved recovery (Weinberg et al., 1990). Differences between experiments 1 and 2 with the rosemary could be attributed to the season of harvest (summer and winter in Israel, respectively), which affected the plant composition and, consequently, their ensiling properties.

Comparison between direct blending and cold pressing reveals similar efficiencies of polyphenol recovery by the two methods, and the results from the respective extraction methods can be viewed as replicates with regard to treatment. In the present study much higher enzyme levels than are commonly applied in the ensiling of forage crops were used, to ensure that enzyme levels were not a limiting factor in antioxidant recovery. The process should be tested with lower enzyme levels before it is scaled up for industrial use.

Recently, a process was developed by Tengerdy et al. (1997) in which in-situ-produced enzymes are used, instead of commercial enzymes. This is done by growing cellulolytic fungi (*Trichoderma*, *Gliocladium*) on straw and using the straw in silages as a source of crude enzymes, which lowers the cost of ENLAC considerably. Such a process is currently being tested in our laboratory for recovery of polyphenols from rosemary and sage.

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Received for review November 30, 1998. Revised manuscript received April 23, 1999. Accepted April 28, 1999. Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan, Israel. No. 435/98, 1998 series.

JF981317+