Fermentation of Alfalfa Silages with Ozonated Cotton Stalks Added†

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The effect on fermentation patterns and silage properties of ensiling alfalfa (A) with ozonated cotton stalks (OCS), added at 10, 20, and 30% of dry matter, was explored in laboratory silos. Silos were opened after 90 days, and the silages were analyzed. DM loss was highest for the untreated A silage (14.6%). DM loss in the A + OCS silages was 1–4%, much lower than in the wilted A silage (7.4%). Reducing sugars and fructose were the major carbohydrates fermented; pectin uronic acid (PUA) proved resistant to 90 days of fermentation. OCS is acidic (pH 2); therefore, the pH of the 10% OCS + A mixture dropped to 5.2 immediately at mixing. This allowed a fermentation to proceed, as reflected from sugar fermentation and lactic acid production (3.55%). At 20 and 30% OCS + A mixtures, pH decreased immediately to 4.47 and 4.26, respectively, so the fermentation was partly or completely inhibited. Protein, extensively degraded in the A silage, was preserved in the A + 30% OCS silage. Wilted A and A + 20% OCS silages were comparable in protecting the alfalfa forage protein.

INTRODUCTION

Green legume forage harvested at the optimal stage often cannot be ensiled directly, due to its low dry matter (DM) content. To reach the desired DM content, forage can be cut and wilted in the field. Unfortunately, wilting is weather-dependent, and therefore it is often unsuccessful. Moreover, wilting is often excessive, causing overheating in storage, which increases loss of nutrients and decreases digestibility of protein and energy (Muck, 1988). Direct ensiling of fresh legume herbage therefore is desirable and could be attained by rapidly acidifying the forage mass. This concept was the basis for a series of studies in which chemically treated lignocelluloses, characterized by a low pH but high DM and soluble-carbohydrate contents, were examined as additives for ensiling fresh alfalfa.

Adding ozonated cotton stalks (OCS) or SO_2-treated wheat straw inhibited alfalfa silage fermentation (Ben-Ghedalia and Yosef, 1989a–d) as a result of a combined action of low pH and inhibitory compounds. For instance, OCS may contain 60 g of preserving organic acids/kg, mostly formic acid (Ben-Ghedalia et al., 1982). Thus, when fresh alfalfa was mixed with OCS at a DM ratio of 60:40, the pH was immediately depressed (3.55), resulting in effective preservation of DM, protein (Ben-Ghedalia and Yosef, 1989a), and carbohydrates (Ben-Ghedalia and Yosef, 1989b). In those studies, one level of OCS, 40% of DM, was applied and the preservative effect was drastic. Whether or not lower levels of application could achieve the same effect is not known.

The objective of this study was to explore the effects of different levels of OCS application on the fermentation patterns of fresh alfalfa.

MATERIALS AND METHODS

Materials. Alfalfa (*Medicago sativa*) was harvested from a commercial field at the very beginning of flowering (<5%). Shortly after harvest, the material was transferred to the laboratory and ensiled in hermetically sealed 2-L laboratory glass silos, after being chopped into pieces 1–2 cm in length. Cotton stalks chopped to the same size and ozonated as described earlier (Ben-Ghedalia et al., 1983) served as the silage additive in this study. The batch of ozonated cotton stalks was described by Ben-Ghedalia and Yosef (1989a,b); its DM content was 50%, and its chemical composition is given in Table I.

Table I. Composition of the Ozonated Cotton Stalks (OCS) (g 100 g^{-1} DM)

constituent	OCS	constituent	OCS
neutral detergent (ND) fiber	53.4	formic acid	3.98
total N	1.10	acetic acid	1.36
ND-soluble carbohydrates	18.2	pH	1.92

Table II. Composition (g 100 g^{-1} DM) and pH of Source Materials: Alfalfa (A), Untreated or Wilted, and the Mixtures of A + Ozonated Cotton Stalks (OCS)

	treatment				
criterion	A	A + 10% OCS	A + 20% OCS	A + 30% OCS	wilted A
dry matter	24.2	24.9	26.5	28.4	44.1
neutral detergent fiber	33.6	35.1	39.8	40.5	36.2
total N	3.52	3.28	2.99	2.88	3.52
TCA-N	2.99	2.88	2.56	2.39	2.87
pH	6.18	5.20	4.55	4.17	6.25

The ensilage treatments consisted of (1) alfalfa ensiled as fresh material without any treatment or additive (A); (2) alfalfa, sun wilted to reach a DM content of 44% (wilted A); (3, 4, and 5) mixtures of fresh alfalfa plus OCS at DM ratios of 90:10 (A + 10% OCS), 80:20 (A + 20% OCS), and 70:30 (A + 30% OCS), respectively.

The above-mentioned materials and mixtures were ensiled, three replicates per treatment, for 90 days at 21–23 °C. After this period, the silos were opened to assess DM losses, recovery of soluble carbohydrates, fermentation products, and protein degradation.

Analytical Procedures. Silage samples were extracted with distilled water at 0 °C; this extract was used for pH, lactic acid (Pryce, 1969), volatile fatty acid (Ben-Ghedalia et al., 1982), and ammonia (Conway, 1957) determinations. Another portion of the silages and the corresponding source materials was freeze-dried, ground, and used for measuring the content of TCA-precipitable N, in vitro DM digestibility (Tilley and Terry, 1963), and neutral detergent fiber (NDF) (Goering and van Soest, 1970). Freeze-dried and ground (1 mm) samples of source material and silages underwent two parallel extractions: (i) to extract the fermentable sugars (total fructose and the reducing sugars), 5 g of DM was refluxed in 100 mL of 0.1 N HCl for 1 h; (ii) to extract pectin, 5 g of DM was extracted by refluxing in 100 mL of 1% ammonium oxalate solution for 1 h. Dry matter was determined by oven-drying at 105 °C. Total N and TCA-precipitated N were measured according to the Kjeldahl method. A 10% w/v

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Table III. Pectin Uronic Acid (PUA), Total Reducing Sugars, and Total Fructose Contents (g 100 g^{-1} DM) in Source Materials (Pre-ensilage) and in the Resulting Silages and Their Fermentation (%) after 90 Days of Ensilage[a]

	PUA			total reducing sugars			fructose		
treatment	pre-ensilage, g	in silage, g	fermented, %	pre-ensilage, g	in silage, g	fermented, %	pre-ensilage, g	in silage, g	fermented, %
alfalfa (A)	11.0	10.0	22.1[a]	4.07	0.78	83.6[a]	1.72	0.29	85.6[a]
A + 10% OCS	11.3	10.5	10.9[d]	4.12	1.09	74.6[a]	1.99	0.33	84.2[a]
A + 20% OCS	10.6	10.7	2.26[b]	3.99	3.88	5.96[c]	1.93	0.70	65.2[b]
A + 30% OCS	10.2	9.95	2.77[bc]	4.36	5.26	−19.4[d]	1.67	1.55	7.99[c]
wilted A	10.2	10.0	9.02[cd]	4.33	3.54	23.9[b]	2.62	1.12	60.2[b]
SEM			2.10			4.78			2.92

[a] Within column, means followed by a common superscript do not differ statistically, $P < 0.05$.

solution of trichloroacetic acid was used for the precipitation and determination of protein and related N compounds in the silages and in the source materials. Total fructose and reducing sugars were determined in the 0.1 N HCl extract according to the procedures of Boratynski (1984) and Miller (1959), respectively.

The pectin–uronic acid (PUA), representing the pectin fraction, was determined in the ammonium oxalate extract, following hydrolysis in 1 N H_2SO_4 at 100 °C for 5 h, according to the procedure of Blumenkrantz and Asboe-Hansen (1973).

Statistical Analysis. Results were statistically analyzed using a completely randomized design consisting of five treatments, with three replicates per treatment. Duncan's multiple range test was used to differentiate between means (Little and Hills, 1978).

RESULTS

The composition of source materials is shown in Table II. The alfalfa in this study was chosen to represent a leguminous, young, high-protein forage, problematic to ensile. With increasing proportions of OCS in mixtures, the content of DM and NDF was increased and that of nitrogen was decreased. Because the pH of OCS was 2 and that of alfalfa was 6.18, increasing the level of OCS in the alfalfa plus OCS mixtures resulted in immediate drops in pH down to 4.17 (A + 30% OCS).

The concentrations of water-soluble carbohydrate fractions in the source materials and in resulting silages and their fermentation percentage are presented in Table III. The reducing sugars and total fructose were the fractions that underwent large compositional changes during fermentation, in contrast to the PUA fraction which was largely recovered. In the A silos the reducing sugars and fructose were extensively degraded, but the processes were not those of a typical silage fermentation but rather those which led to the deterioration of the forage. The inclusion of 10% OCS created conditions for desirable silage fermentation. Nevertheless, in the A + 10% OCS treatment, PUA hardly changed during 90 days of fermentation; the major substrates exhausted were reducing sugars and total fructose. The A + 20% OCS treatment underwent some fermentation of total fructose but gave almost complete recovery of PUA and reducing sugars. Fermentation generally was inhibited in the A + 30% OCS silos, with minor changes in PUA and fructose and a positive balance ("negative fermentation") in reducing sugars. This issue will be addressed under Discussion. The wilted A underwent some fermentation with minor participation of PUA (9%), with some participation of the reducing sugars (24%), and massive fermentation of fructose (60%).

Table IV presents the DM losses, pH values, and fermentation end-product profiles of the silages. DM losses were highest with the A (negative control, untreated) silage (14.6%) and lowest (almost no DM losses) with the A + 30% OCS treatment. However, the 10 and 20% OCS treatments also reduced DM losses (3.5–4%) during alfalfa fermentation. They were more effective than wilting (7.5%). A + 30% OCS already at the pre-ensilage stage

Table IV. Effect of Using Ozonated Cotton Stalks (OCS) as a Silage Additive on Fermentation Characteristics of Alfalfa (A) Silages after 90 Days (g 100 g^{-1} DM)[a]

	treatment					
criterion	A	A + 10% OCS	A + 20% OCS	A + 30% OCS	wilted A	SEM
DM loss	14.6[a]	3.97[bc]	3.43[bc]	0.95[c]	7.48[b]	1.07
pH	6.06[a]	4.73[c]	4.47[d]	4.26[e]	5.38[b]	0.03
in vitro digestion	68.5[a]	71.4[b]	72.1[b]	71.2[b]	71.9[b]	0.60
lactic acid	0.83[a]	3.55[c]	1.59[b]	0.18[d]	1.58[b]	0.08
acetic acid	2.37[a]	1.65[b]	1.42[c]	1.25[c]	0.59[d]	0.07
propionic acid	0.19	0.13	0.08	0.12	0.09	0.05
butyric acid	0.39					

[a] Within row, means followed by a common superscript do not differ statistically, $P < 0.05$.

Table V. Protein Degradation after 90 Days of Fermentation of Alfalfa (A) Silages and Silages Made of Various Mixtures of A + Ozonated Cotton Stalks (OCS)[a]

	treatment					
criterion	A	A + 10% OCS	A + 20% OCS	A + 30% OCS	wilted A	SEM
TCA-precipitable N						
g 100 g^{-1} DM	0.78[a]	1.02[b]	1.20[b]	1.56[c]	1.22[b]	0.06
g 100 g^{-1} N	20.4[a]	29.4[c]	37.3[b]	52.3[d]	33.2[bc]	1.32
silage/source material	0.23[a]	0.34[c]	0.45[b]	0.66[d]	0.39[c]	0.02
NH_3 - N						
g 100 g^{-1} DM	0.53[a]	0.23[b]	0.12[c]	0.08[c]	0.15[c]	0.02
g 100 g^{-1} N	13.7[a]	6.70[b]	3.85[c]	2.79[c]	4.09[c]	0.61

[a] Within row, means followed by a common superscript do not differ statistically, $P < 0.05$.

reached a pH of 4.17 (Table II); after 90 days of preservation, its pH was 4.26. During that period, only minor changes occurred in the A + 30% OCS as reflected from the substrate fermentation balance and the low concentration (0.18%) of lactic acid in this silage. In the A silos, lactic acid content was low, pH was high (6.06), and acetic acid accumulation did not prevent butyric acid production and silage deterioration. The fermentation of reducing sugars and total fructose in A + 10% OCS (Table III) resulted in the formation of a moderate level of lactic acid (3.55%). In the A + 20% OCS silage, fermentation was partly inhibited; the level of lactic acid (1.59%) was similar to that found in the wilted A silage. The in vitro DM digestibility of the alfalfa silages was generally high.

Data on protein preservation are shown in Table V. Protein was extensively degraded in the A silage, with only 23% of the original alfalfa protein surviving the 90 days of fermentation. From this standpoint, the A + 30% OCS treatment was best, as expressed by recovery of 66% of the original TCA-precipitable N.

The A + 10% OCS treatment was inadequate to preserve

forage protein; the A + 20% OCS treatment conferred the same level of protection against protein degradation as the wilted alfalfa. Ammonia levels in silages reflected these changes mentioned above: high in A, medium in A + 10% OCS, and low in the other treatments.

DISCUSSION

The use of straw for ensiling low-DM leguminous herbage is not a new idea. Although high in DM content, straw is poor in fermentable sugars; therefore, the ensilage of alfalfa plus straw has proven unsuccessful, as shown by DM loss and digestibility measurements (Phillips and Pendlum, 1984). Singh et al. (1984) succeeded in ensiling mixtures of legume forages plus straw but only after adding molasses to the mixtures. In this context, the approach of using ozone or sulfur dioxide treated straw as a silage additive for the ensilage of legume herbage is unique from the standpoint of straw characteristics, namely, improved nutritional value, high content of DM and fermentable carbohydrates, low pH (~2), and the presence of bacteriostatic substances (Ben-Ghedalia and Yosef, 1989a–d). This package of features as a whole is quite rare among chemically treated lignocelluloses. Thus, the positive response from different levels of OCS with alfalfa, as demonstrated in the present study, was not surprising. At 10% OCS, fermentation was enhanced as reflected by exhaustion of soluble carbohydrates, production of lactic acid, and a decrease in pH (Tables III and IV). The A + 10% OCS silage was preserved with minor DM losses; however, the initial drop of pH to 5.2 at the onset of ensilage (Table II) was inadequate to prevent proteolysis (Table V). The addition of 20 and 30% OCS to alfalfa inhibited or stopped silage fermentation; the A + 30% OCS was more successful in protecting alfalfa protein.

Although the wilted A and the A + 20% OCS treatments showed similar fermentation patterns (Tables III and IV), the lower final pH and the somewhat better protein protection favor the A + 20% OCS silage. Due to the activity of plant proteases, ensiling of alfalfa can result in up to 85% of the total N being NPN (Muck, 1987). Proteases of legume-forage origin are pH-sensitive, having pH optima around 6, with activity declining linearly between pH 6 and 4 (Finley et al., 1980; McKersie, 1985).

Although at pH 4 there still was some plant protease activity, the immediate drop in pH at the onset of ensilage to pH 4.55 and 4.17 in the A + 20% OCS and A + 30% OCS treatments, respectively (Table II), probably was responsible for the protein sparing effect in these treatments. This suggestion is based on the fact that in the case of low-DM leguminous herbage most proteolysis occurs during the first day in the silo (McKersie and Buchanan-Smith, 1982; Muck, 1987). Dry matter content also is an important factor correlated with and negatively affecting plant protease activity. However, its effect is expressed within the range 50–75% DM. This could explain why the wilted A (44% DM) was inferior to the 20 and 30% OCS treatments regarding the protection of forage protein.

Pectin, as represented by PUA, is fairly resistant to silage fermentation (Ben-Ghedalia and Yosef, 1989b,d). This finding was interesting because pectin is considered the most fermentable polysaccharide in the rumen with a GIT digestibility of over 90% (Ben-Ghedalia and Miron, 1984; Ben Ghedalia et al., 1989). The recovery of more than 90% of the PUA in the A + 10% OCS and in the wilted A silages after 90 days of ensilage supports previous findings and indicates that pectin is not used by lactic acid bacteria during the ensilage of alfalfa. In the 30% OCS silage, almost no fermentation occurred. Indeed, reducing sugars increased by 19.4% after 90 days of fermentation (Table III). One possible explanation could be that organic acids present in OCS hydrolyzed labile structural monosaccharide residues from the cell walls (Table I), as suggested by Ben-Ghedalia and Yosef (1989b). We have conducted several digestibility and feeding experiments with OCS (Ben-Ghedalia et al., 1983; Solomon et al., unpublished results) with positive nutritional results and no toxic effects. Progress in ozonation technology will facilitate the application of OCS as an ensiling agent.

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