BARD

FINAL REPORT

PROJECT NO. I-1243-87

Nutritional Evaluation of Ozonated Dicot Lignocelluloses and Their Use as Silage Additives

D. Ben Ghedalia, C.R. Richardson, R.W. Tock, J. Miron

1991

3526 0

BD

Standard BARD Cover Page for Scientific Reports

Date: 15.4. 1991

BARD

P.O.BOX 6

Bet-Dagan, ISRAEL

BARD project No. IS - 1243 - 87

Title

Nutritional Evaluation of Ozonated Dicot Lignocelluloses and Their Use as Silage Additives

Investigators' Names (Principal first)	Investigators' Institutions
D. Ben-Ghedalia	ARO, Israel
C.R. Richardson	Texas Tech. U. USA
R.W. Tock	Texas Tech. U. USA
J. Miron	ARO, Israel

Project starting date: 1.1. 1989

Type of Report: Final

D. Ben-Ghedalia

Signature
Principal Investigator

Signature
Institution's Authorizing Official

630.72
BAR/ BEN ! 633.51 : 636.208.5 : 636.085.52 ;
633.31 : 574.23

TABLE OF CONTENTS

ABSTRACT

This report presents data of a two-years BARD project dedicated to: (1) Exploring ways of improving the ozonation technology aimed at the nutritional upgrading of dicotyledonous lignocelluloses; (2) studying the potential of ozonated lignocelluloses as a silage additive.

To solve the problem of ozone instability, a series of materials have been examined for their ozone stabilizing effect. The breakthrough in this area was the development of the CONOXYL-TT solution based on acetic acid and glycerol, by the US-Texas group. Decomposition of ozone in CONOXYL-TT is very slow and the cost of materials for effectively treating one metric ton of dicotyledonous lignocellulose is $2.5 in the US. By adding the additional cost components, overall treatment expenses may rise to not higher than $10/metric ton of treated lignocellulose. To the best of our knowledge this is the lowest cost for an effective treatment of dicotyledonous lignocellulose.

The potential of ozone treated cotton stalks as an additive for the ensilage of fresh alfalfa forage was studied by the Israeli group. In a series of studies with laboratory silos it was shown that ozonated cotton stalks are an excellent additive with respect to the preservation of dry matter, carbohydrates and protein of alfalfa ensiled as a fresh-cut forage. Preliminary data show high acceptability of those silages by ruminants.

INTRODUCTION

Objectives:

The objectives of this project as originally stated in the research proposal, were:

A. To improve the ozonation process.

B. To examine the potential of ozonated lignocelluloses, as a silage additive, to be used for ensiling problematic materials such as young (unwilted) alfalfa.

C. To explore the nutritional availability of silage made of unwilted young forage plus ozonated lignocellulose, in terms of energy yielding substrates and amino acids, by using GIT-cannulated ruminants.

D. To study the productive performance of ruminants fed silage made as in C and rations containing ozonated cotton by-product.

Of the above-mentioned, A & B only were approved by the BARD evaluation panel as stated in their comments:

1243

PANEL COMMENTS:

This proposal deals with azonation of dicot lignocelluloses and their nutritional evaluation.

The investigators are qualified and their facilities are adequate. However, due to the lack of an appropriate economical evaluation of the suggested azonation process, the expected benefit to agriculture is not clear.

The panel recommends to support for two (2) years a study dealing with objectives (A, B) of this proposal. This recommendation is conditional upon the participation of an expert in the field of silage microbiology.

The panel suggests consideration of other reviewers comments.

Cooperation and Research Achievements

The research proposal was jointly written while the principal investigator visited Texas. After project approval, the US and Israeli investigators met twice for the detailed planning of experiments, evaluation of results and for discussing future prospects of research and application of the ozone treatment. The major part of cooperation was planned for the 3rd year which unfortunately was not approved.

A breakthrough was achieved in the ozonation process. Ozone, which was mostly wasted during treatment according to the old method, could be stabilized by CONOXYL-TT and its oxidative potential fully applied for treating the lignocellulose. Thus, objective A has been completely achieved.

Fresh alfalfa forage can not be directly (unwilted) ensiled and is regarded as a problematic material for ensilage. There are however several advantages if the goal of direct ensilage of fresh alfalfa can be achieved. The studies on the use of ozonated cotton stalks as a silage additive had shown that by ensiling mixtures of alfalfa + OCS, energy yielding substrates and protein are well preserved and DM loss can be reduced to almost zero. Thus, Objective B has been completely achieved.

Reviewing first year results, BARD referee of the 1st annual report, strongly recommended the continuation of animal experiments (see page 4).

The results and reviewer recommendation, motivate the submission of a continual research project.

United States - Israel
Binational Agricultural Research and Development Fund
קרן דו-לאומית למחקר ולפיתוח חקלאיים של ארצות הברית וישראל
BARD - קמח

29/03/90

BEN-GHEDALIA D.
A.R.O. THE VOLCANI CENTER
INST. OF ANIMAL SCI.
POB 6
BET DAGAN 50250
ISRAEL

Dear Dr. BEN-GHEDALIA D.

Re: BARD Proposal No. IS-1243-87

Attached please find comments sent to us by a reviewer who recently evaluated the annual report for the above mentioned project.

We hope that these comments will be of interest and assistance to you.

Sincerely,

M. Shilo

Dr. Mira Shilo
Coordinator of Scientific Reports

Encls.

SUBJECT: Project - I-1243-87

I have reviewed the materials in regard to the first annual report of the above project. Clearly the investigators have made substantial progress in establishing the technology for efficient ozone treatment of high lignocellulose feedstuffs and have demonstrated the benefit of using this technology for preparing mixed silages. Plans for the next year seem quite reasonable. I look forward to seeing the nutritional quality of these ensiled products tested in animals. If this is not part of this total project, it is a strong justification for extending this work to the practical end of evaluating animal performance.

BODY

OF

REPORT

J Sci Food Agric 1989, **46**, 279–287

Ozonated Cotton Stalks as a Silage Additive: Fermentation Data on Lucerne with Particular Reference to Protein Degradation*

Daniel Ben-Ghedalia and Edith Yosef

Institute of Animal Science, Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel

(Received 2 November 1987; revised version received 29 March 1988; accepted 28 April 1988)

ABSTRACT

Fermentation patterns of lucerne silages were studied in laboratory silos. The treatments consisted of: (a) fresh (200 g kg^{-1} DM) lucerne, ensiled without any treatment (L); (b) lucerne wilted to 525 g kg^{-1} DM prior to ensilage (WL); (c) fresh lucerne + cotton stalks at a ratio of 60:40 on a dry matter (DM) basis (L+CS); and (d) fresh lucerne + ozone-treated cotton stalks at the same ratio as above (L+O$_3$). Silos were opened after 90 days and the silages analysed. The highest DM loss was found in the L silage (14·7%), whereas in the L+O$_3$ silage DM loss was practically nil. Both wilting and the addition of untreated cotton stalks proved to be effective in reducing DM losses during fermentation. The production of lactic acid and volatile organic acids in the L+O$_3$ and WL silages was lower than in the L and L+CS silages. The poorest ability to preserve forage protein was found in the L silage, in which only 28% of the protein was recovered after 90 days. The greatest ability to preserve protein was found in the L+O$_3$ silage, in which 78% of the protein was maintained. Ammonia production followed generally similar patterns. Amino acids underwent extensive degradation in the L silage. Recovery of amino acids in the WL silage was in the range 69–93%, and in the L+O$_3$ silage it was almost complete. Ozonated CS proved to be a good silage additive with respect to energy and protein preservation. Its future use in the field would allow direct ensilage of fresh leguminous material immediately after harvest, producing a high quality silage.

Key words: Lucerne silage, wilted silage, ozonated cotton stalks, additives, protein breakdown.

* Contribution from the Agricultural Research Organization, The Volcani Center, Bet Dagan 50250, Israel. No 2208-E, 1987 series.

1 INTRODUCTION

At their optimal stage of harvest, most forage crops contain about 200 g kg^{-1} dry matter (DM), whereas the minimum DM content to ensure reasonable ensilage of legumes is 400 g kg^{-1}, and 250 g kg^{-1} for grasses or cereal forages (Weissbach *et al* 1974; Bodine *et al* 1983). To reach the desired DM content, the forage is cut and wilted in the field, which means that the process is complicated since the forage cannot be ensiled directly after harvest. Unfortunately, wilting is weather dependent and presents a difficult management challenge. Moreover, a major problem involved in wilting is that of excessive wilting, which brings on the problem of excessive heating in storage. This, in turn, increases losses and decreases the digestibility of protein and energy (Thomas *et al* 1982).

Water-soluble carbohydrates (WSC) are the major substrates for anaerobic fermentation of silage. The effectiveness of the organic acids produced during fermentation in reducing the pH depends mostly on the buffering capacity (BC) of the forage at the pre-ensilage stage. Thus, the three most important factors affecting the success of the ensiling process are DM content, WSC concentration, and the BC of the forage (McDonald 1983).

From the point of view of silage making, young, high quality leguminous forage is considered to be a problematic material. For ensiling such materials, fermentation inhibitors are commonly introduced; in this class formic acid is the most widely used (Waldo *et al* 1971), due to its ability to prevent clostridial fermentation and to reduce protein and carbohydrate fermentation in the silo (McDonald 1981). However, the formic acid treated silages are still low in DM and, as found by Demarquilly and Jarrige (1970) and Wilkins *et al* (1971), DM intake is positively correlated with DM content of the silage. For this reason and others (economic and agrotechnical), the search for better additives or ensiling techniques is continuing.

The use of ozonated lignocelluloses as silage additives is suggested in this study. Ozonated lignocelluloses contain, on a DM basis, up to 35 g kg^{-1} formic acid and 25 g kg^{-1} acetic acid, and their pH is in the range 2–3 (Ben-Ghedalia *et al* 1982). The preserving power of such material containing a total of 60 g kg^{-1} of preserving acids is expected to be high. Moreover, during ozonation, cell wall (CW) structural carbohydrates are partly solubilised, so that ozonated materials are enriched with soluble carbohydrates up to a concentration of 210 g kg^{-1} (Ben-Ghedalia *et al* 1982). These substrates may serve for the initial and limited but important stage of silage fermentation in a combined, green forage plus ozonated cotton stalks (CS) system.

Finally, ozonated CS is relatively high in DM ($\sim$ 500 g kg^{-1}), so that its addition to the low-DM green forage prior to ensilage will increase silage DM content.

The objective of this research was to study the effect of ozonated CS as a silage additive on the ensilage of young, low-DM lucerne, with particular reference to protein and amino acid degradation.

2 EXPERIMENTAL

2.1 Materials

Lucerne (*Medicago sativa* L.) was harvested with hand shears from a commercial field

at the very beginning (<5%) of flowering. Shortly after harvest the material was transferred to the laboratory and ensiled in hermetically sealed 2-litre laboratory glass silos, after being chopped into pieces 1–2 cm in length. Cotton stalks chopped to the same size, either untreated or ozonated as described in a previous paper (Ben-Ghedalia and Shefet 1983), were used as an ensilage additive. The treatment consisted of:

(1) Lucerne ensiled as a fresh material without any treatment or additive (L).
(2) Lucerne, sun-wilted to reach the desired DM content of 500 g kg^{-1} (WL). Wilting was done on a very hot (38°C) day and the desired DM content was reached in less than 24 h.
(3) A mixture of lucerne + untreated CS at a DM ratio of 60:40 (L+CS).
(4) A mixture of lucerne + ozonated CS at a DM ratio of 60:40 (L+O_3).

The above mentioned materials and mixtures were ensiled, three replicates per treatment for 90 days, and kept at a temperature of 21–23°C. After this period the silos were opened to assess DM losses, fermentation products, protein degradation, and general silage characteristics.

2.2 Analytical procedures

Silage samples were extracted with distilled water at 0°C and the extract was used for pH, lactic acid (Pryce 1969), volatile fatty acid (Ben-Ghedalia and Shefet 1983) and ammonia (Conway 1947) determinations. Another portion of the silage was freeze-dried, ground, and used for measuring TCA-precipitable N, in-vitro DM digestibility (Tilley and Terry 1963), and amino acids. Ball-milled samples were hydrolysed in 6 M HCl for 22 h at 110°C under nitrogen. Samples were analysed by means of an LC-7000 Biotronic Amino Acid Analyser on a single column. 1,4-Dithiothreitol was added prior to hydrolysis to avoid methionine oxidation. An unusually high peak was obtained on the chromatogram at the identical location of phenylalanine for three of the silages. Although there was no evidence for questioning the identity of that peak, the possibility of a co-elution artifact should not be overlooked. Dry matter was determined by oven drying at 105°C and cell walls by the neutral detergent method (Goering and Van Soest 1970). Total N and TCA-precipitable N were measured by the Kjeldahl method. A 10% w/v 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.

2.3 Statistical analysis

Results were statistically analysed by using a completely randomised design consisting of four treatments and three replicates per treatment. Duncan's multiple-range test was used to differentiate between means (Little and Hills 1978).

3 RESULTS

The composition of the source materials is given in Table 1. According to the compositional data, the lucerne used in this experiment represents a young and

TABLE 1
Composition (g per 100 g DM), pH, and in-vitro dry matter digestibility (DMD) of source materials and mixtures used for ensilage

Criterion	*Lucerne (L)*	*Lucerne + cotton stalks (L+CS)*	*Lucerne + ozonated cotton stalks ($L+O_3$)*	*Wilted lucerne (WL)*
Dry matter	20·8	29·0	28·0	52·9
Cell walls	30·8	45·7	41·6	34·9
Total nitrogen	4·42	2·98	3·06	4·22
TCA-precipitable N	3·46	2·62	2·32	3·51
pH	6·35	6·19	3·55	6·40
In-vitro DMD	0·696	0·557	0·694	0·683

TABLE 2
Effect of using ozonated cotton stalks as a silage additive on fermentation characteristics of lucerne silages after 90 days

Criterion	*Treatments*				
	*L**	*L+CS**	*$L+O_3$**	*WL**	*SEM*
DM loss (%)	14·7[a]	3·11[b]	0·24[c]	5·67[b]	0·744
pH	5·60[a]	4·64[b]	3·50[c]	5·75[a]	0·044
Lactic acid[d]	4·27[a]	4·77[a]	1·55[b]	1·72[b]	0·214
Acetic acid[d]	2·48[a]	1·13[b]	0·93[b]	0·44[c]	0·100
Propionic acid[d]	0·168[a]	0·147[a]	0·113[a]	0·117[a]	0·025
Butyric acid[d]	0·160[a]	0·070[ab]	0·057[b]	0·060[ab]	0·031
Isobutyric acid[d]	0·117[a]	0·173[b]	0·153[ab]	0·263[c]	0·015
In-vitro DMD	0·661[a]	0·508[b]	0·676[c]	0·675[c]	0·021

* L = lucerne; L+CS = lucerne + cotton stalks; $L+O_3$ = lucerne + ozonated cotton stalks; WL = wilted lucerne.
[a,b,c] Means with different superscripts in the same row are significantly different, $P<0·05$.
[d] g per 100 g DM.

problematic plant material from the standpoint of ensilage. It was low in DM and CW and high in total N. By adding ozonated and untreated CS at a ratio of 40 CS: 60 lucerne (on a DM basis), the DM content of the mixtures was increased to 280–290 g kg^{-1}, but that of the total N and TCA-precipitable N was reduced, since N content in CS was 11 g kg^{-1} on a DM basis. The pH of the ozonated CS was 2·0 and its in-vitro DM digestibility 0·65; consequently, the initial pH of the $L+O_3$ mixture was 3·55 and its in-vitro DMD was the same as that of the lucerne source material (0·69).

The effects of treatments on parameters of silage fermentation are demonstrated in Table 2. DM losses are based on oven drying; therefore losses of volatiles should be borne in mind when referring to these values. The highest DM loss was found in the L silos (14·7%), whereas in the $L+O_3$ treatment DM loss was practically nil.

TABLE 3
Protein degradation after 90 days of fermentation of lucerne silage (L), wilted lucerne silage (WL), and silage consisting of lucerne+cotton stalks, untreated (L+CS) or ozonated ($L+O_3$)

Criterion	*Treatments*				
	L	*L+CS*	*L+O₃*	*WL*	*SEM*
TCA-precipitable N:					
g per 100 g DM	0·96[a]	1·32[b]	1·81[c]	2·05[d]	0·037
g per 100 g N	20·6[a]	42·7[b]	59·2[c]	46·9[d]	1·040
silage/source material	0·28[a]	0·50[b]	0·78[c]	0·58[d]	0·012
NH_3-N:					
g per 100 g DM	0·693[a]	0·263[b]	0·160[c]	0·137[c]	0·023
g per 100 g N	14·9[a]	8·53[b]	5·24[c]	3·13[d]	0·462

[a,b,c,d] Means with different superscripts in the same row are significantly different, $P<0·05$.

Both wilting and the addition of untreated CS proved to be effective in reducing DM losses during fermentation of lucerne. Ensiling the lucerne without any additive (L) resulted in a high level of organic acids, but the pH was not decreased accordingly, probably as a result of extensive protein breakdown (Table 3). The addition of untreated CS to the lucerne exerted a positive effect on fermentation, reflected in the profile of organic acids and pH, but the in-vitro DMD was significantly lower than that of the L: 0·51 vs 0·66. Fermentation in the wilted silage and in the $L+O_3$ mixture was minimal. However, whereas the reduced fermentation within the WL silage was a result of an initially high DM content, the source material of the $L+O_3$ mixture was very well preserved by the organic acids originating from the ozonated CS. Accordingly, the smell of WL silage was typical of lucerne haylage, whereas that of the $L+O_3$ silage resembled frozen herbage. The in-vitro DMD of the $L+O_3$ silage was not reduced, as compared with the original lucerne material, because of the high in-vitro DMD value (0·65) of the ozonated CS.

Data regarding the ability of the various ensilage systems to preserve the forage protein are presented in Table 3. The poorest performance in this respect was by the L silage, in which the TCA-precipitable N comprised only 20% of the total N and only 28% of that found originally in the source material. The highest protein-preserving power was found in the $L+O_3$ silage, in which 78% of the TCA-precipitable N of the source material was maintained, followed by that of the WL silage.

Data on amino acid composition of source materials and silages are shown in Table 4, and the proportion of amino acids surviving fermentation after 90 days is presented in Table 5. Amino acids underwent extensive degradation in the L silage; in some cases, for example in arginine, tyrosine and serine, 76–84% of the amino acids was degraded. The concentration of phenylalanine was increased in the L silage. Generally, the rise in phenylalanine content was characteristic for the three silages in which protein catabolism had occurred. ie L, L+CS and WL. Proline, glycine and alanine were the most stable amino acids in these treatments. Recovery

TABLE 4
Amino acid composition (g per 100 g DM) of the source materials and mixtures and the corresponding silages

Amino acid	*Treatments*							
	*L**		*L+CS**		*L+O₃**		*WL**	
	Source material	*Silage*	*Source material*	*Silage*	*Source material*	*Silage*	*Source material*	*Silage*
Threonine	1·34	0·53	0·92	0·84	0·88	0·79	1·40	1·23
Valine	1·62	1·70	1·06	1·08	0·97	0·96	1·67	1·64
Methionine	0·41	0·42	0·29	0·26	0·23	0·22	0·44	0·35
Isoleucine	1·28	1·45	0·84	0·90	0·78	0·79	0·37	0·35
Leucine	2·19	2·28	1·84	1·47	1·34	1·32	2·25	2·13
Phenylalanine	2·37	7·06	0·99	2·92	1·29	1·34	2·64	3·95
Lysine	1·60	0·56	1·07	1·08	0·95	0·85	1·69	1·29
Histidine	0·66	0·28	0·43	0·30	0·39	0·36	0·70	0·51
Arginine	1·57	0·29	1·12	0·43	0·93	0·89	1·51	1·27
Aspartic acid	2·72	0·65	2·36	2·17	1·88	1·94	3·03	2·90
Serine	1·32	0·37	0·93	0·72	0·83	0·78	1·47	1·28
Glutamic acid	2·91	1·54	2·04	1·52	1·88	1·87	2·75	2·25
Proline	1·28	1·30	0·90	0·88	0·91	0·97	1·74	2·13
Glycine	1·39	1·35	1·02	0·92	0·93	0·89	1·32	1·20
Alanine	1·62	1·89	1·15	1·21	1·01	1·00	1·58	1·53
Tyrosine	1·14	0·27	0·72	0·36	0·64	0·64	1·10	0·90

* L = lucerne; L+CS = lucerne + cotton stalks; L+O_3 = lucerne + ozonated cotton stalks; WL = wilted lucerne.

of amino acids (except phenylalanine) in the WL silage was in the range 0·69–0·93. For most of the amino acids the recovery in L+CS did not differ from that in the WL silage. Considering both the composition (Table 4) and the recovery (Table 5) of amino acids in the L+O_3 silages, forage protein seems to have been well preserved in this treatment.

4 DISCUSSION

From the nutritional standpoint, the ideal situation would be to harvest the forage at the stage of maximum net energy yield, and then to ensile it directly. However, the DM content and the general composition of leguminous forage at that stage imply that it would be difficult to ensile such a material satisfactorily without wilting or supplying an additive (Weissbach *et al* 1974). Beside the disadvantages and agrotechnical complications involved in the wilting procedure as mentioned previously, there is also the problem of loss of amino acids during wilting (Merchen and Satter 1983), and soil contamination at the harvest of the wilted material. Therefore, the use of additives is preferred in many systems. Untreated and ozonated CS have been examined as silage additives in the present study. Using untreated CS as an additive at the ratio of 40% CS + 60% lucerne resulted in a

TABLE 5
Proportion of total amino acids recovered in lucerne silages after 90 days of fermentation

Amino acid	*Treatments*				
	*L**	*L+CS**	*L+O₃**	*WL**	*SEM*
Threonine	0·340^{a}	0·887^{b}	0·913^{b}	0·830^{b}	0·027
Valine	0·893^{a}	0·987^{ab}	0·997^{b}	0·930^{ab}	0·029
Methionine	0·877^{ab}	0·850^{ab}	0·960^{b}	0·767^{a}	0·041
Isoleucine	0·967^{a}	1·040^{a}	1·023^{a}	0·933^{a}	0·034
Leucine	0·877^{a}	0·973^{a}	0·997^{a}	0·900^{a}	0·035
Phenylalanine	2·543^{a}	2·850^{a}	1·047^{b}	1·413^{c}	0·097
Lysine	0·300^{a}	1·000^{b}	0·900^{b}	0·720^{c}	0·031
Histidine	0·357^{a}	0·690^{b}	0·940^{c}	0·690^{b}	0·066
Arginine	0·157^{a}	0·403^{b}	0·973^{c}	0·797^{d}	0·036
Aspartic acid	0·277^{a}	0·893^{b}	1·040^{c}	0·903^{bc}	0·045
Serine	0·240^{a}	0·750^{b}	0·947^{c}	0·820^{b}	0·031
Glutamic acid	0·453^{a}	0·720^{b}	1·003^{c}	0·770^{b}	0·038
Proline	0·867^{a}	0·953^{a}	1·077^{ab}	1·160^{b}	0·062
Glycine	0·830^{a}	0·873^{ab}	0·970^{b}	0·857^{ab}	0·038
Alanine	0·997^{ab}	1·023^{a}	0·997^{ab}	0·913^{b}	0·032
Tyrosine	0·207^{a}	0·480^{b}	1·013^{c}	0·770^{d}	0·039

* L = lucerne; L+CS = lucerne + cotton stalks; L+O_3 = lucerne + ozonated cotton stalks; WL = wilted lucerne.
^{a,b,c,d} Means with different superscripts in the same row are significantly different, $P < 0·05$.

TABLE 6
Composition (g per 100 g OM) of untreated and ozonated cotton stalks (CS)

Criterion	*Untreated CS*	*Ozonated CS*
pH	6·72	1·92
Formic acid	—	3·98
Acetic acid	Trace	1·36
Soluble carbohydrates	9·20	21·8

Data from Ben-Ghedalia *et al* (1982).

reasonable fermentation quality but a low in-vitro DMD, since the organic matter (OM) digestibility of untreated CS is only 30% (Ben-Ghedalia and Shefet 1983). The positive effect of untreated CS as a silage additive is associated with its high content of DM and moderate content of low N cell solubles. However, the low digestibility of the L+CS silage is a serious drawback when considering CS as a silage additive.

To the best of our knowledge this is the first study reporting the use of ozonated lignocellulose as a silage additive. The rationale underlying the investigation was based on the composition and low pH of the ozonated CS, as shown in Table 6. The fermentation processes in the L+O_3 silage were very limited, as evidenced by the

low level of production of organic acids. It appears that the low-pH ozonated CS exerted a preserving effect on the lucerne and its nutrients. A forage crop containing up to 250 g kg^{-1} DM crude protein is a valuable material, and attention should be paid not only to preserving the energy but also to the amino acid component. In this regard, ozonated CS protected the forage protein well. The recovery of amino acids in the $L + O_3$ treatment after 90 days of fermentation was almost complete, and the content of TCA precipitable N was about the same as in the wilted silage. The pH values of the $L + O_3$ source material and silage were low, 3·55 and 3·50, respectively, suggesting that the ratio of ozonated CS to lucerne may be reduced to 30:70 or even 25:75.

The remarkable recoveries of phenylalanine in the L, L + CS and WL treatments deserve attention. The fact that this phenomenon is related to the treatments in which extensive protein breakdown had occurred, and not to the $L + O_3$ silage in which protein has been well preserved, implies an active synthesis of this amino acid in the silo. However, the possibility of a chromatographic co-elution artifact should not be overlooked.

Fermentation patterns of the WL silage were as expected and as reported in the literature for 50% DM lucerne silages (Bodine *et al* 1983; Kung *et al* 1984).

In summary, ozonated CS proved to be a good silage additive in the context of energy and protein preservation. Its future use in the field would allow a direct ensilage of the fresh leguminous material immediately after harvest, producing a high quality silage. In-vivo studies will be continued to evaluate comprehensively the nutritional value of silage consisting of lucerne + ozonated CS.

ACKNOWLEDGEMENTS

The authors are grateful to the members of the Metabolic Unit: A Rubinstein, R Solomon, and Y Est, for skilled technical assistance. This research was supported by BARD, the United States–Israel Binational Agricultural Research and Development Fund.

REFERENCES

Ben-Ghedalia D, Shefet G 1983 Chemical treatments for increasing the digestibility of cotton straw. 1. Effect of ozone and sodium hydroxide treatments on the digestibility of cell wall and organic matter. *J agric Sci Camb* **100** 393–400.

Ben-Ghedalia D, Shefet G, Miron J, Dror Y 1982 Effect of ozone and sodium hydroxide treatments on some chemical characteristics of cotton straw. *J Sci Food Agric* **33** 1213–1218.

Bodine A B, O'Dell G D, Moore M E, Wheat C K 1983 Effect of dry matter content and length of ensiling on quality of alfalfa silage. *J Dairy Sci* **66** 2434–2437.

Conway E J 1947 *Microdiffusion Analysis and Volumetric Error* (2nd edn). Crosby Lockwood, London.

Demarquilly C, Jarrige R 1970 The effect of method of forage conservation on digestibility and voluntary intake. *Proc 11th Int Grassld Congr* Queensland, pp 733–737.

Goering H K, Van Soest P J 1970 *Forage Fiber Analysis*. USDA Agricultural Handbook No 379. USDA, Washington, DC.

Kung L J, Grieve D B, Thomas J W, Huber J T 1984 Added ammonia or microbial inocula for fermentation and nitrogenous compounds of alfalfa ensiled at various percents of dry matter. *J Dairy Sci* **67** 299–306.

Little T M, Hills F J 1978 The Latin square design. In: *Agricultural Experimentation, Design and Analysis*. John Wiley, New York, pp 77–85.

McDonald P 1981 *The Biochemistry of Silage*. John Wiley, New York.

McDonald P 1983 The control of silage fermentation. *Res Rep Hannah Res Inst* pp 59–67.

Merchen N R, Satter L D 1983 Changes in the nitrogenous compounds and sites of digestion of alfalfa harvested at different moisture contents. *J Dairy Sci* **66** 789–801.

Pryce J D 1969 A modification of the Barker–Summerson method for the determination of lactic acid. *Analyst* **94** 1151–1152.

Thomas J W, Yu Y, Middleton T, Stallings C 1982 Estimation of protein damage. In: *Protein Requirements for Cattle*. Symp Oklahoma State University, MP-109, pp 81–98.

Tilley J M, Terry R M 1963 A two stage technique for the *in vitro* digestion of forage crops. *J Br Grassl Soc* **18** 104–111.

Waldo D R, Keys J E, Smith L W, Gordon C H 1971 Effect of formic acid on recovery, intake, digestibility and growth from unwilted silage. *J Dairy Sci* **54** 77–83.

Weissbach F, Schmidt L, Hein E 1974 Method of anticipation for the run of fermentation in silage making, based on the chemical composition of green fodder. *Proc 12th Int Grassld Congr* Moscow, pp 226–236.

Wilkins R J, Hutchinson K J, Wilson R F, Harris C E 1971 The voluntary intake of silage by sheep. 1. Interrelationships between silage composition and intake. *J agric Sci Camb* **77** 511–537.

J. Anim. Physiol. a. Anim. Nutr. 61 (1989), 105–110
Received 11. 07. 88

ISSN 0044-3565

Institute of Animal Science, A.R.O., The Volcani Center, Bet Dagan Israel

Ozonated cotton stalks as a silage additive: The participation of pectin and cell wall monosaccharides in lucerne silage fermentation

By D. Ben-Ghedalia and Edith Yosef

1 Introduction

Both the rumen and silage fermentation systems are anaerobic. However, whereas soluble carbohydrates are the major source of carbon used in silage fermentation, rumen microorganisms utilize both soluble and structural carbohydrates. Recent experiments with sheep showed that the fractional digestion rate of water-soluble sugars and pectin in the rumen is in the range of 50 to 60% h^{-1}, whereas structural polysaccharides are digested at 1 to 4% h^{-1} (Barry et al. 1984; Ulyatt et al. 1984). Thus, pectin, an α-(1–4)-poly-D-galacturonan, is rapidly degraded in the rumen, but there is little information on the fate of pectin during silage fermentation and the same is true for the cell wall monosaccharide residues. (Morrison 1979; McDonald 1981). The level of pectin in legumes may reach 15% on a DM basis, and so the content of neutral detergent-soluble carbohydrates in legumes is over 20% (Bailey 1973; Ben-Ghedalia and Miron 1984). It was shown recently that during the fermentation of young lucerne forage (20% DM), lactic acid was produced at an acceptable level (4.3% on a DM basis) although silage quality was low and fermentation losses were high (Ben-Ghedalia and Yosef 1989). Did the pectin fraction play any part in this type of fermentation? This question is intriguing, since if galacturonic acid can serve as a substrate in silage fermentation, then the introduction of a pectin-hydrolyzing agent as a preparative step prior to ensilage may open the way to the direct ensilage of unwilted legumes. However, galacturonic acid was not mentioned among the 13 monosaccharides listed by McDonald (1981) as potential substrates for lactic acid bacteria. The objective of this study was to investigate the contribution made by the uronic acid-pectin residues and cell wall monosaccharides of lucerne to the overall silage fermentation. This research is a continuation of a previous study (Ben-Ghedalia and Yosef 1988) in which we investigated the effect of using ozonated cotton stalks as a silage additive, on the fermentation of lucerne silage. Additional information on silage properties, fermentation data and DM losses can be found in that paper.

2 Experimental

2.1 Materials

Lucerne *(Medicago sativa)* was harvested with hand shears from a commercial field at the very beginning (< 5%) of flowering. Shortly after harvest the material was transferred to the laboratory and ensiled in hermetically sealed 2-liter laboratory glass silos, after being chopped into pieces 1–2 cm in length. Cotton stalks chopped to the same size, either untreated or ozonated as described in a previous paper (Ben-Ghedalia et al. 1983), were used as an ensilage additive. The composition of the untreated and the ozone-treated cotton stalks is shown in Table 1. The treatments consisted of:

Contribution from the Ag. Res. Org., The Vol. Cent. B.D., Israel. No. 2259-E, 1987 series.

1. Lucerne ensiled as a fresh material without any treatment or additive [L].
2. Lucerne, sun-wilted to reach the desired DM content of 50% [WL]. Wilting was done on a very hot (38°C) day and the desired DM content was reached in less than 24 h.
3. A mixture of lucerne + untreated CS at a DM ratio of 60/40 [L + CS].
4. A mixture of lucerne + ozonated CS at a DM ratio of 60/40 [L + O_3].

Table 1. **Composition of untreated and ozone-treated cotton stalks (g 100 g^{-1} DM)**

Constituent	Untreated cotton stalks	Ozone-treated cotton stalks
NDF	75.3	53.4
NDF-glucose polymers	33.5	29.8
NDF-non glucose polysaccharides	17.6	8.80
ND-soluble carbohydrates	8.90	18.2
Lignin (permanganate)	17.8	8.22

The above-mentioned materials and mixtures were ensiled, three replicates per treatment for 90 days, and kept at a temperature of 21–23°C. After that period the silos were opened and a large and representative portion of each silo was freeze-dried, ground, and used for the preparation of neutral detergent fibre and for assessing monosaccharide residues composition.

2.2 Analytical procedures

Neutral detergent fibres (NDF) were prepared and determined according to the method of Goering and Van Soest (1970). Monosaccharides in whole material and in NDF preparations of source materials and silages were determined in ball-milled material after hydrolysis in 12 M H_2SO_4 for 1 h at room temperature and continuing the hydrolysis in 0.5 M H_2SO_4 at 100°C for 5 h. Inositol was added as an internal standard and the released monosaccharides were determined as their alditol acetate derivatives after reduction and acetylation according to Blakeney et al. 1983. The conditions for separation by gas-liquid chromatography were those of Bacon and Gordon (1980). Uronic acids in hydrolysates were determined colorimetrically according to Blumenkrantz and Asboe-Hansen (1973).

ND-soluble monosaccharides were calculated as the difference between the monosaccharide residue content in the whole material and that of NDF.

2.3 Statistical analysis and calculations

Results were statistically analysed by using a completely randomized design consisting of four treatments and 3 replicates per treatment. Duncan's multiple-range test was used to differentiate between means (Little and Hills 1978). The percentage of monosaccharide recovery after 90 days of fermentation in the lab. silos was calculated as following:

$$\frac{\text{quantity after 90 days}}{\text{quantity at ensilage, d} = 0} \times 100.$$

3 Results

The composition of total and ND-soluble monosaccharide residues of the source materials is presented in Table 2. The lucerne contained 19.3% of neutral detergent (ND) soluble carbohydrates on a DM basis, comprising 46% of total carbohydrate. Glucose and xylose were mostly CW-bound, whereas minor sugars and uronic acids were largely (53–82%) ND-soluble. Uronic acids were the major ND-soluble monosaccharide residue in lucerne.

The addition of untreated cotton stalks to the lucerne increased the content of CW sugars and decreased the proportion of ND-soluble monosaccharide residues to 30% of the total carbohydrates. Ozonated cotton stalks changed the ratio of ND-soluble to ND-insoluble monosaccharides to 35/65. The profile of monosaccharide residues of the WL was very similar to that of the untreated lucerne. The composition of total and ND-soluble monosaccharide residues of the silages is shown in Table 3. In both the L and WL silages, ND-soluble glucose was extensively fermented and exhausted; followed by the minor sugars arabinose, mannose and galactose. The proportion of ND-soluble monosaccharide residues was reduced to 25% in the L and to 29% in the WL silages. Monosaccharide profiles of the L + O_3 treatment indicate that the fermentation in this silage was very limited. In the L + CS silage, the minor sugars-arabinose, mannose and galactose comprised the bulk of fermentable substrates, followed by ND-soluble glucose.

Table 2. **Composition (g 100 g^{-1} DM) of total (TM) and ND-soluble (SM) monosaccharide residues of source materials**

	Treatments							
	Lucerne (L)		Lucerne + cotton stalks (L + CS)		Lucerne + ozonated cotton stalks (L + O_3)		Wilted lucerne (WL)	
Monosaccharide	TM	SM	TM	SM	TM	SM	TM	SM
Glucose	21.3	6.79	24.2	3.13	23.3	3.64	20.1	6.96
Xylose	3.32	0.08	4.95	0	4.85	0.90	3.66	0.77
Arabinose	2.64	1.41	2.00	1.02	1.90	1.04	2.70	1.70
Mannose	2.18	1.41	1.67	1.00	1.54	1.03	2.33	1.72
Galactose	2.56	1.77	1.93	1.21	1.87	1.32	2.51	1.74
Uronic acids	9.56	7.83	9.11	6.67	8.78	6.73	9.87	7.98
Total	41.6	19.3	43.9	13.0	42.2	14.7	41.2	20.9

Table 3. **Composition (g 100 g^{-1} DM) of total (TM) and ND-soluble (SM) monosaccharide residues of silages after 90 days of fermentation**

	Treatments							
	Lucerne (L)		Lucerne + cotton stalks (L + CS)		Lucerne + ozonated cotton stalks (L + O_3)		Wilted lucerne (WL)	
Monosaccharide	TM	SM	TM	SM	TM	SM	TM	SM
Glucose	17.5	0.10	23.9	2.31	24.8	3.32	15.9	0
Xylose	3.60	0	6.88	0.41	5.67	1.12	3.15	0
Arabinose	1.26	0.44	1.18	0.46	1.65	0.82	1.71	0.61
Mannose	1.57	0.64	0.75	0	1.58	0.86	2.19	1.32
Galactose	1.01	0.32	1.02	0.01	1.71	1.03	1.79	0.35
Uronic acids	9.37	7.27	9.82	7.52	8.70	6.83	9.48	7.69
Total	34.3	8.77	43.6	10.7	44.1	14.0	34.2	9.97

The recovery of ND-soluble monosaccharide residues in the silages after 90 days of fermentation is shown in Table 4. The high recovery of soluble sugars in the L + O_3 implies that by mixing the low pH-ozonated cotton stalks with lucerne, the carbohydrates were preserved to a great extent. ND-soluble uronic acids, representing the pectin fraction, are

the only sugars which are hardly utilized in the fermentation process. The minor sugars-ND-soluble arabinose, mannose and galactose, were also not completely utilized in the L and WL silages.

The recovery of CW monosaccharide residues in the silages after 90 days of fermentation is shown in Table 5. It is clear that NDF-glucose and xylose residues do not participate in the fermentation process. NDF-uronic acids were apparently more extensively used than the soluble pectic acids, irrespective of treatments. NDF-arabinose in the L and L + CS silages, and NDF-galactose in the L silage, were the only CW minor sugars involved in the fermentation of lucerne silages.

Table 4. **Percent recovery of ND-soluble sugars in the silages after 90 days of fermentation**

	Treatments				
Monosaccharide	L[e]	L + CS[e]	L + O_3[e]	WL[e]	SEM
Glucose	1.25[a]	71.5[b]	94.3[c]	0.10[a]	1.09
Xylose	0.10[a]	0.10[a]	100[b]	0.10[a]	0
Arabinose	26.7[a]	43.7[b]	79.5[c]	33.9[d]	0.41
Mannose	38.7[a]	0.10[b]	84.2[c]	72.4[d]	0.49
Galactose	15.4[a]	0.80[b]	78.6[c]	19.0[d]	0.38
Uronic acids	79.2[a]	100[b]	100[b]	90.9[c]	0.59

[a, b, c, d] Means with different superscripts in the same row are statistically different, $P < 0.05$.
[e] L = lucerne; L + CS = lucerne + cotton stalks; L + O_3 = lucerne + ozonated cotton stalks; WL = wilted lucerne.

Table 5. **Percent recovery of NDF sugars in the silages after 90 days of fermentation**

	Treatments				
Monosaccharide	L[d]	L + CS[d]	L + O_3[d]	WL[d]	SEM
Glucose	101	99.9	101	100	1.05
Xylose	99.4	98.9	104	100	1.61
Arabinose	57.7[a]	71.6[b]	94.2[c]	103[c]	3.17
Mannose	99.6	100	106	103	1.09
Galactose	74.1[a]	100[b]	105[b]	96.8[b]	1.20
Uronic acids	71.2[a]	81.5[b]	82.2[b]	79.8[b]	1.63

[a, b, c] Means with different superscripts in the same row are statistically different, $P < 0.05$.
[d] L = lucerne; L + CS = lucerne + cotton stalks; L + O_3 = lucerne + ozonated cotton stalks; WL = wilted lucerne.

Discussion

Although there are a variety of pectin-rich fodders used for ensilage, including leguminous plants, orange peels and tuberous plants, information on the role of pectin in silage fermentation is very sparse indeed. Soluble glucose and fructose are known as the major substrates fermented by lactic acid bacteria during ensilage (McDonald 1981). In lucerne, pectin comprises the major part of ND-soluble monosaccharide residues, mainly as uronic acid residues (Table 2). However, the recovery of ND-soluble monosaccharides after 90 days of fermentation suggests that pectin is hardly involved in lucerne silage fermentation (Table 4). Is pectinolysis limiting the involvement of uronic acids in silage fermenta-

tion, or is this a matter of substrate-microorganisms incompatibility? This issue deserves further research; the fact that some 10% and 20% of the ND-soluble uronic acid disappeared during fermentation of the WL and L silages, respectively (Table 4), is insufficient for drawing any conclusion.

The participation of CW monosaccharide residues of perennial ryegrass in silage fermentation was studied by MORRISON (1979). Slight losses of both cellulose and hemicellulose were reported in that study as a result of microbial action. Lucerne is a leguminous plant and its CW composition and structural features are different (BEN-GHEDALIA and RUBINSTEIN 1984). Cellulose and xylans were not affected in the present study in any of the silages. However, CW arabinose and CW galactose were catabolized to some extent. MORRISON (1979) suggested that acids formed during fermentation or added for ensilage are among the factors affecting the removal or catabolism of CW components. The pH of the ozonetreated cotton stalks used as an additive in the L + O_3 treatment was 2, and consequently the pH of the source material at ensilage was 3.55 (BEN-GHEDALIA and YOSEF 1989). Nevertheless, CW arabinose and CW galactose were hardly affected in the L + O_3 silage (Table 4). It is therefore suggested that the limited catabolism of CW components in the present study was a result of microbial (enzymatic) hydrolysis.

The disappearance of 20–30% of the CW uronic acid in the silages is difficult to explain, particularly in view of the relative stability of ND-soluble uronic acid residues. Glucuronic acid is the major branching unit in hemicellulosic polysaccharides of leguminous plants (BAILEY 1973; BEN-GHEDALIA and MIRON 1984) and as such, it is the most resistant against biodegradation by rumen microbes (BEN-GHEDALIA and MIRON 1984; BEN-GHEDALIA and RUBINSTEIN 1984) and acid hydrolysis (REES 1977; SHEFET and BEN-GHEDALIA 1982). Some of our unpublished data show that neutral detergent fibre (GOERING and VAN SOEST 1970) still contains uronic acid residues which can be further removed by ammonium oxalate solution-the classical pectin solubilizer. Therefore, it is possible that the in-silo catabolism of CW uronic acid residues noted in this study is simply an artifact. Also, the possibility that ND-insoluble pectin is converted to ND-soluble pectin on ensilage should not be overlooked.

In a previous paper we showed that protein catabolism is minimal in the L + O_3 silage. The present paper shows that both the soluble and the structural carbohydrates are well preserved in a silage made of lucerne + ozonated cotton stalks.

Summary

The participation of soluble and cell wall (CW) monosaccharide residues in lucerne silage fermentation was studied in laboratory silos. The treatments consisted of: a, fresh lucerne (20% DM) ensiled without any treatment (L); b, lucerne wilted to 52.5% DM prior to ensilage (WL); c, fresh lucerne + cotton stalks at the ratio of 60/40 on a DM basis (L + CS); and d, fresh lucerne + ozone treated cotton stalks at the same ratio as above (L + O_3). Silos were opened after 90 days and the silages analysed. Uronic acid was the major neutral detergent (ND)-soluble monosaccharide residue in lucerne. However, ND-soluble glucose was the most extensively fermented sugar in both the L and WL silages, followed by arabinose, mannose and galactose. ND-soluble uronic acid was barely utilized during fermentation. Fibrilar components (glucose and xylose) did not participate in the fermentation process. The present study showed that the carbohydrates – both the soluble and the structural fractions – are well preserved in a silage made of lucerne + ozonated cotton stalks.

Zusammenfassung

Ozon-behandelte Baumwollstengel als Silagezusatz: der Einfluß von Pektin und Zellwandmonosacchariden auf die Fermentation von Luzernesilage

Die Untersuchungen wurden in Laborsilos über 90 Tage geführt, dabei wurden die folgenden Behandlungen geprüft: a. frische Luzerne (L; 20% Trockensubstanz), b. angewelkte Luzerne

(WL; 52% Trockensubstanz), c. frische Luzerne plus Baumwollstengel im Gewichtsverhältnis 60:40 auf Trockensubstanzbasis (L + CS) und d. frische Luzerne plus Ozon-behandelte Baumwollstengel (L + O_3) im Verhältnis 60:40 auf Trockensubstanzbasis. Nach der Öffnung der Silos zeigte sich, daß Uronsäure den Hauptbestandteil der ND-löslichen Monosaccharide in der Luzernesilage ausmachte. ND-lösliche Glucose war der am stärksten fermentierte Zucker in L- und WL-Silage, gefolgt von Arabinose, Mannose und Galaktose. ND-lösliche Uronsäure wurde während der Fermentation kaum genutzt. Strukturkomponenten (Glucose und Xylose) nahmen nicht am Fermentationsprozeß teil. Aus den vorliegenden Untersuchungen geht hervor, daß Kohlenhydrate sowohl in löslicher als auch strukturell gebunden, in Silagen aus Luzerne plus Ozon-behandelten Baumwollstengeln gut vor der Fermentation geschützt werden.

Acknowledgements

The authors are grateful to the staff of the Metabolic Unit, A. Rubinstein, R. Solomon and Y. Est, for skilled technical assistance. This research was supported by Bard, the United States-Israel Binational Agricultural Research and Development Fund.

References

Bacon, J. S. D.; Gordon, A. H., 1980: J. agric. Sci., Camb. *94,* 361–367.
Bailey, R. W., 1973: Structural carbohydrates. In: Butler, G. W.; Bailey, W. (Eds.). Chemistry and Biochemistry of Herbage. New York: Academic Press, pp. 157–209.
Barry, T. N.; Manley, T. R.; Duncan, S. J., 1984: J. agric. Sci., Camb. *102,* 479–486.
Ben-Ghedalia, D.; Miron, J., 1984: J. Nutr. *114,* 880–887.
Ben-Ghedalia, D.; Rubinstein, A., 1984: J. Sci. Food Agric. *35,* 1159–1164.
Ben-Ghedalia, D.; Shefet, G.; Dror, Y., 1983: J. agric. Sci., Camb. *100,* 393–400.
Ben-Ghedalia, D.; Yosef, Edith, 1989: J. Sci. Food Agric., in press.
Blakeney, A. B.; Harris, P. J.; Henry, R. J.; Stones, B. A., 1983: Carbohydr. Res. *113,* 291–299.
Blumenkrantz, N.; Asboe-Hansen, G., 1973: Anal. Biochem. *54,* 484–489.
Goering, H. K.; van Soest, P. J., 1970: Forage Fiber Analysis. USDA Agricultural Handbook, No. 379. Washington, D.C.
Little, T. M.; Hills, F. J., 1978: The Latin square design. In: Agricultural Experimentation, Design and Analysis. New York: John Wiley & Sons, pp. 77–85.
McDonald, P., 1981: The Biochemistry of Silage. New York: John Wiley & Sons.
Morrison, I. M., 1979: J. agric. Sci., Camb. *93,* 581–586.
Rees, D. A., 1977. Polysaccharide Shapes. New York: John Wiley & Sons, pp. 22–27.
Shefet, G.; Ben-Ghedalia, D., 1982: Eur. J. appl. Microbiol. Biotechnol. *15,* 47–51.
Ulyatt, M. J.; Waghorn, G. C.; John, A.; Reid, C. S. W.; Monro, J., 1984: J. agric. Sci., Camb. *102,* 645–657.

Authors' Address: Institute of Animal Science, A.R.O. The Volcani Center, Bet Dagan 50250, Israel.

A Fermentation Study on Silages Made of Various Proportions of Alfalfa Plus Ozonated Cotton Stalks

Daniel Ben-Ghedalia,* Joshua Miron, and Edith Yosef

The effect of ensiling alfalfa (A) with ozonated cotton stalks (OCS) included, on a dry matter (DM) basis, at 10, 20 and 30%, on fermentation patterns and silage properties, was explored in laboratory silos. Silos were opened after 90 days and the silages analysed. Highest DM loss was found in the untreated A silage (14.6%), whereas in the A + OCS silages DM loss was in the range of 1-4%, lower than in the A-wilted silage (7.4%). Reducing sugars and fructose were the major fermentable carbohydrates, whereas pectin uronic acid (PUA) proved resistant after 90 days of fermentation. OCS is acidic (pH 2), therefore the pH of the (10% OCS +A) mixture was dropped to 5.2 immediately at mixing, allowing a normal-type fermentation to occur during ensilage as reflected from sugars fermentation and lactic acid production (3.55%). At 20 and 30% OCS + A mixtures, pH was decreased immediately to 4.47 and 4.26, respectively and the fermentation was partly or completely inhibited. Protein was extensively degraded in the A silage and well preserved in the A+ 30% OCS silage. Wilted A and A+20% OCS silages were comparable in protecting the alfalfa forage protein.

Short title: Alfalfa + OCS Silages.

Metabolic Unit, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet-Dagan 50250, Israel.

Contribution from the ARO, The Volcani Center, Bet-Dagan Israel.

Green legume forage harvested at the optimal stage can not be ensiled directly due to it's low DM content. To reach the desired DM content the forage is cut and wilted in the field. Unfortunately, wilting is weather dependent and therefore sometimes complicated. Moreover, excessive wilting is not uncommon, causing over heating in storage, which is expressed in increased losses of nutrients and a decrease in the digestibility of protein and energy (Muck, 1988). A successful direct ensilage of fresh legume herbage is therefore desirable and could be attained by reaching a rapid acidification of the forage mass. This concept was the basis for a series of studies in which chemically-treated lignocelluloses characterized by a low pH, high DM and elevated soluble carbohydrate content were examined as additives for the ensilage of fresh alfalfa.

An inhibitory effect upon alfalfa silage fermentation was exerted by adding ozonated cotton stalks (OCS) (Ben-Ghedalia and Yosef, 1989 a,b) or by adding SO_2-treated wheat straw (Ben Ghedalia and Yosef, 1989 c,d) as a result of a combined action of low pH and inhibitory compounds. For instance, OCS may reach the content of 60g Kg^{-1} of preserving organic acids, mostly formic (Ben-Ghedalia et al. 1982). Thus by mixing fresh alfalfa with OCS at a DM ratio of 60:40 respectively, a low initial pH (3.55) was reached immediately, expressed later in an effective preservation of DM and protein (Ben-Ghedalia and Yosef, 1989a) and carbohydrates (Ben-Ghedalia and Yosef, 1989b). In those studies one level, 40% on a DM basis of OCS was applied and the preservative effect was drastic. The question is weather, lower levels of application could reach the same effect.

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 (<5%) of flowering. Shortly after being harvest the material was transferred to the laboratory and ensiled in hermetically sealed 2-liter 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 the same as described by Ben-Ghedalia and Yosef (1989a, b), and its chemical composition is given in Table 1.

The ensilage treatments consisted of:

1. Alfalfa ensiled as a fresh material without any treatment or additive (A).
2. Alfalfa, sun wilted to reach the DM content of 44% (wilted A).
3,4, and 5. Mixtures of fresh alfalfa + OCS at the following DM ratios: 90:10 (A+10% OCS); 80:20 (A+20% OCS); 70:30 (A+30% OCS).

The above-mentioned materials and mixtures were ensiled, three replicates per treatment for 90 days, and kept 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 and the extract was used for pH, lactic acid (Pryce, 1969), volatile fatty acids (Ben-Ghedalia and Shefet, 1983) and ammonia (Conway, 1957) determinations. Another portion of the silages and the corresponding source materials were freeze-dried, ground and used for measuring content of TCA-precipitable N,

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

constituent	OCS
Neutral detergent (ND) fiber	53.4
Total N	11.0
ND-soluble carbohydrates	18.2
Formic acid	3.98
Acetic acid	1.36
pH	1.92

in vitro DM digestibility (Tilley and Terry, 1963) and Neutral detergent fiber -NDF (Goering and Van-Soest, 1970). Freeze-dried and ground (1mm) samples of source material and silages underwent two parallel extractions: 1. For extracting the fermentable sugars (fructose and the reducing ones), 5g of DM were refluxed in 100 ml 0.1N HCl for 1h; 2. Pectin was extracted by refluxing 5g DM in 100 ml 1% ammonium oxalate solution for 1h. Dry matter was determined by oven drying at 105°C and total N and TCA-precipitable N were measured by the Kjeldahl method. A 10% W/V 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. Fructose and reducing sugars were determined in the 0.1N HCl extract according to the procedure of Boratynski (1987) and Miller (1959), respectively.

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

Statistical analysis. Results were statistically analysed by using a completely randomized design consisting of five treatments and 3 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 2. The alfalfa in this study was chosen to represent a leguminous, young - high protein forage, problematic for ensilage. By increasing the proportion of OCS in mixtures, the content of DM and NDF were

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

	treatments				
Criteria	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

increased and that of nitrogen was decreased. Since the pH of OCS was 2 and that of alfalfa was 6.18, increasing the level of OCS inclusion in the alfalfa + OCS mixtures, resulted in an immediate drop in pH down to 4.17 (A + 30% OCS).

The concentrations of water-soluble carbohydrate fractions in the source materials and in resulting silages, are presented in **Table 3** and their fermentation balance in **Table 4**. A general look on Table 3 shows that the reducing sugars and fructose were the fractions which 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, however 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 was effective in creating the conditions for a positive silage fermentation. Nevertheless, in A + 10% OCS treatment, PUA was hardly changed during 90 days of fermentation and the major substrates exhausted during that period were the reducing sugars and fructose. The A + 20% OCS treatment underwent some fermentation with the participation of fructose mainly, and almost the complete recovery of PUA and reducing sugars. Fermentation appears to be generally 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 in the Discussion section. The wilted A underwent some fermentation with the minor participation of PUA (9%), with some participation of the reducing sugars (24%) and massive fermentation of fructose (60%).

Table III. Contents (g 100 g^{-1} DM–) of Pectin Uronic Acid (PUA), Total Reducing Sugars and Fructose in the Source Materials (pre-ensilage), Alfalfa (A) and A + Ozonated Cotton Stalks (OCS) Mixtures, and in the Resulting Silages

	PUA		total reducing sugars		fructose	
treatments	pre-ensilage	in silage	pre-ensilage	in silage	pre-ensilage	in silage
Alfalfa (A)	11.0	10.0	4.07	0.78	1.72	0.29
A + 10% OCS	11.3	10.5	4.12	1.09	1.99	0.33
A + 20% OCS	10.6	10.7	3.99	3.88	1.93	0.70
A + 30% OCS	10.2	9.95	4.36	5.26	1.67	1.55
Wilted A	10.2	10.0	4.33	3.54	2.62	1.12

Table IV. Fermentation Balance of Pectin Uronic Acid (PUA), Total Reducing Sugars and Fructose in Untreated and Wilted Alfalfa (A) Silages and in Silages Made of Various Mixtures of A + Ozonated Cotton Stalks (OCS).

	PUA			total reducing sugars			fructose		
Treatments	pre-ensilage g	in silage g	fermented %	pre-ensilage g	in silage g	fermented %	pre-ensilage g	in silage g	fermented %
Alfalfa (A)	24.4	19.0	22.1^{a}	9.03	1.48	83.6^{a}	3.82	0.55	85.6^{a}
A + 10% OCS	26.6	23.7	10.9^{d}	9.71	2.47	74.6^{a}	4.69	0.74	84.2^{a}
A + 20% OCS	26.6	26.0	2.26^{b}	10.0	9.41	5.90^{c}	4.85	1.69	65.2^{b}
A + 30% OCS	25.3	24.6	2.77^{bc}	10.8	12.9	-19.4^{d}	4.13	3.80	7.99^{c}
Wilted A	36.6	33.3	9.02^{cd}	15.5	11.8	23.9^{b}	9.39	3.74	60.2^{b}
SEM			2.10			4.78			2.92

^{a,b,c,d} Means with different superscripts in the same column are statistically different, $P < 0.05$

Table 5 presents the DM losses, pH values and fermentation profiles of the silages. Highest DM losses were recorded with the A (negative control, untreated) silage (14.6%), and the lowest (almost no DM-losses) with the A +30% OCS treatment. However, the 10 and 20% OCS treatments were effective too in reducing DM losses (3.5-4%) during alfalfa fermentation, more effective than wilting (7.5%).
A + 30% OCS reached already at the pre-ensilage stage the pH of 4.17 (Table 2) and after 90 days of preservation pH was 4.26. During that period only minor changes had 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 the acetic acid fermentation pattern did not save the silage from butyric acid production and deterioration. The fermentation of reducing sugars and fructose in A + 10% OCS (table 4), resulted in the formation of a fair level of lactic acid (3.55%). In the A + 20% OCS silage, fermentation was partly inhibited and 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 6**. Protein was extensively degraded in the A silage, only 23% of the original alfalfa protein survived the 90 days of fermentation. From this standpoint, the A + 30% OCS treatment had the best performance, as expressed in a recovery of 66% of the original TCA-precipitable N.
The A+10% OCS treatment was inadequate in preserving the forage protein, and the A + 20% OCS treatment conferred the same level of protection against protein degradation as the wilted alfalfa.
Ammonia levels in silages were a reflection of the above-mentioned: high in A, medium in A +10% OCS and low in the other treatments.

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

	Treatments					
Criteria	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 DMD	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,b,c,d,e} Means with different superscripts in the same row are statistically different, $P<0.05$

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

	Treatments					
Criterion	A	A + 10% OCS	A + 20% OCS	A + 30% OCS	wilted A	SEM
TCA-precipitable N:						
g $100g^{-1}$ DM	0.78^{a}	1.02^{b}	1.20^{b}	1.56^{c}	1.22^{b}	0.06
g $100g^{-1}$ N	20.4^{a}	29.4^{c}	37.3^{b}	52.3^{d}	$33.2^{b,c}$	1.32
silage/source material	0.23^{a}	0.34^{c}	0.45^{b}	0.66^{d}	0.39^{c}	0.02
NH3 - N:						
g $100g^{-1}$ DM	0.53^{a}	0.23^{b}	0.12^{c}	0.08^{c}	0.15^{c}	0.02
g $100g^{-1}$ N	13.7^{a}	6.70^{b}	3.85^{c}	2.79^{c}	4.09^{c}	0.61

^{a,b,c,d} Means with different superscripts in the same row are statistically different, $P<0.05$

DISCUSSION

The use of straw for ensiling low DM leguminous herbage is not a new idea. However, straw although high in DM content is poor in fermentable sugars, therefore the ensilage of alfalfa + straw proved unsuccessful as shown by DM loss and digestibility measurements (Phillips and Pendlum, 1984). Singh et al. (1984) succeeded in ensiling mixtures of legume forages + straw, but only after adding molasses to the mixtures. In this context, the approach of using ozone or sulphur dioxide-treated straws as silage additives 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, 1989 a,b,c,d). This package of features as a whole, is quite rare among chemically treated lignocelluloses. Thus, the positive response of different levels of OCS inclusion on the ensilage of alfalfa, as demonstrated in the present study, was not surprising. At the level of 10% OCS application, natural fermentation was enhanced as reflected in soluble carbohydrates exhaustion, lactic acid production and the decrease in pH (Tables 4 and 5). The A + 10% OCS silage was good and was produced with minor DM losses, however, the initial drop of pH to 5.2 at the onset of ensilage (Table 2) was inadequate for protecting against proteolysis (Table 6). The levels of 20 and 30% OCS application inhibited or stopped silage fermentation, however 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 4 and 5), the lower final pH and the somewhat better protein protection are in favor of 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 is still some plant-proteases activity, it is suggested that 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, respectively (Table 2), was responsible for the protein sparring effect in these treatments. This suggestion is based on the fact that in the case of low DM leguminous herbage, the major part of proteolysis occurs during the first day in the silo (McKerie and Buchanan-smith, 1982; Muck, 1987). Dry matter content is also an important factor correlated with and affecting negatively plant protease activity. However, its effect is expressed within the range of 50-75% DM. this would explain why the wilted A (44% DM) was inferior to the 20 and 30% OCS treatments regarding the protection of forage protein.

It was shown previously that pectin as represented by PUA is fairly resistant to silage fermentation (Ben-Ghedalia and Yosef, 1989 b,d). This finding was interesting since pectin is considered as 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 indicate that pectin is not used by lactic acid bacteria during the ensilage of alfalfa. In the 30% OCS silage there was almost no fermentation. Nevertheless, the increase of 19.4% in reducing sugars after 90 days of fermentation (Table 4) is intriguing. One possible explanation could be the hydrolysis of labile structural monosaccharide residues from the cell walls by the

organic acids present in OCS (Table 1), as suggested by Morrison (1979).

Progress in ozonation technology based on studies at the Lubbock Technical University (R.W. Tock and C.R. Richardson, personal communication) will facilitate the application of OCS as an ensiling agent.

ACKNOWLEDGEMENT

This study was supported by the US-Israel Binational Agricultural Reseach and Development Fund (BARD), Project no. IS-1243-87.

LITERATURE CITED

Ben-Ghedalia, D.; Miron, J. The Digestion of Total and Cell Wall Monosaccharides of Alfalfa by Sheep. J. Nutr. **1984**, 114, 880-887.

Ben-Ghedalia, D.; Shefet, G.; Dror, Y. Chemical Treatments for Increasing the Digestibility of Cotton Straw. 1. Effect of Ozone and Sodium Hydroxide Treatments on Rumen Metabolism and on the Digestibility of Cell-Walls and Organic Matter. J. Agric. Sci., Camb. **1983**, 100, 393-400.

Ben-Ghedalia, D.; Shefet, G.; Miron, J.; Dror, Y. Effect of Ozone and Sodium Hydroxide Treatments on Some Chemical Characteristics of Cotton Straw. J. Sci. Food Agric. **1982**, 33, 1213-1218.

Ben-Ghedalia, D.; Yosef, E. Ozonated Cotton Stalks as a Silage Additive: Fermentation Data of Lucerne with Particular Reference to Protein Degradation. J. Sci. Food Agric. **1989**, 46, 279-287.

Ben-Ghedalia, D.; Yosef, E. Ozonated Cotton Stalks as a Silage Additive: The Participation of Pectin and Cell Wall Monosaccharides in Lucerne Silage Fermentation. J. Anim. Physiol. Anim. Nutr. **1989**, 61, 105-110.

Ben-Ghedalia, D.; Yosef, E. SO_2-Treated Straw as a Silage Additive: Fermentation Data on Lucerne with Particular Reference to Protein Degradation. Anim. Fd Sci. Technol. **1989**, 22, 247-254.

Ben-Ghedalia, D.; Yosef, E. SO_2-Treated Straw as a Silage Additive: the Participation of Soluble and Cell Wall Monosaccharide Residues in Lucerne Silage Fermentation. Anim. Fd Sci. Technol. **1989**, 22, 255-262.

Ben-Ghedalia, D.; Yosef, E.; Miron, J.; Est, Y. The Effect of Starch- and Pectin-rich Diets on Quantitative Aspects of Digestion in Sheep. Anim. Fd Sci. Technol. **1989**, 24, 289-298.

Blumenkrantz, N; Asboe-Hansen, G. New Method for Quantitative Determination of Uronic Acids. Anal. Biochem. **1973**, 54, 484-489.

Boratynski, J. Colorimetric Method for the Determination of Ketoses Using Phenol-Acetone-Boric Acid Reagent (PABR). Anal. Biochem. **1984**, 137, 528-532.

Conway, E.J. Microdiffusion Analysis and Volumetric Eror. 2nd Edit. **1957**, London, Crosby Lockwood and Sons Ltd.

Finley, J.W.; Pallavicini, C.; Kohler, G.O. Partial Isolation and Characterization of Medicago sativa Leaf Proteases. J. Sci. Food Agric. **1980**, 31, 156-

Goering, H.K.; van-Soest, P.J. Forage Fiber Analysis. USDA Agricultural Handbook, Washington DC, **1970**, No. 379, pp: 1-19.

Little, T.M.; Hills, F.J. In: Agricultural Experimentation, Design and Analysis. John Wiley, New York. **1978**, pp: 47-75.

McKersie, B.D.; Buchanan-Smith, J. Changes in the Levels of Proteolytic Enzymes in Ensiled Alfalfa Forage. Can. J. Plant Sci. **1982**, 62, 111- .

McKersie, B.D. Effect of pH on Proteolysis in Ensiled Legume Forage. Agrom. J. **1985**, 77, 81- .

Miller, G.L. Use of Dinitrosalicylic Acid Reagent for Determination of Reducing Sugars. Anal. Chem. **1959**, 31, 426-428.

Morrison, I.M. Changes in the Cell Wall Components of Laboratory Silages and the Effect of Various Additives on these Changes. J. Agric. Sci., Camb. **1979**, 93, 581-586.

Muck, R.E. Dry Matter Level Effects on Alfalfa Silage Quality. I. Nitrogen Transformations. Trans. Am. Soc. Agric. Eng. **1987**, 30, 7-12.

Muck, R.E. Factors Influencing Silage Quality and their Implications for Management. J. Dairy Sci. **1988**, 71, 2992-3002

Phillips, W.H.; Pendlum, L.C. Digestibility of Wheat and Alfalfa Silages with and without Wheat Straw. J. Anim. Sci., **1984**, 59, 476-482.

Pryce, J.D. A Modification of the Barker-Summerson Method for the Determination of Lactic Acid. Analyst, **1969**, 94, 1151-1152.

Singh, R.; Kamra, D.N.; Jakhmola, R.C. Ensiling of Leguminous Green Forages in Combination with Different Dry Roughages and Molasses. Anim. Fd Sci. Technol. **1984**, 12, 133-139.

Tilley, J.M.A.; Terry, R.A. A Two-Stage Technique for the In-vitro Digestion of Forage Crops. J. Br. Grassl. Soc., **1963**, 18, 104-111.

BARD PROPOSAL NO. IS-1243-87: NUTRITIONAL EVALUATION OF OZONATED DICOT LIGNOCELLULOSES AND THEIR USE AS SILAGE ADDITIVES

United States (Texas Tech University) Part of the Project

SUMMARY

A series of laboratory evaluations were used to refine and improve the efficiency of ozone capture for subsequent use with studies on in vitro and in vivo digestibilities. Laboratory tests were conducted to determine the effectiveness of the ozonation production and capture process. Once the overall ozonation process for treatment of biomass had been established, it was possible to demonstrate a dramatic reduction in cost. At this point, several ton quantities of the lignocellulose biomass (cotton gin trash) was produced by this chemical pretreatment for use in a lamb feeding experiment.

The improvement in the ozone based treatment process is now referred to as a Concentrated Oxygenated Liquid at Texas Tech University (CONOXYL-TT) for the predigestion of lignocellulosic materials. When CONOXYL-TT is applied to low quality roughages, it results in an increase in the bioavailability of the energy fraction of the roughage. Thus cotton gin trash (CGT), a low quality roughage, is effectively upgraded to provide an energy source for ruminants at approximately the same level as medium quality alfalfa hay. In addition, CONOXYL-TT, plus sufficient water to increase the moisture content of the CGT to sixty percent, allows this low quality lignocellulose to be

ensiled. The CONOXYL-TT treatment may also enhance the availability of the energy fraction in the high quality feed grains used in the diet. However, the use of CONOXYL for this purpose is still speculative and remains to be tested on a definitive basis.

Treatment of CGT with the CONOXYL-TT oxidizer improved ($P<.05$) in vitro organic matter digestibility by 36%, after 24 hours of incubation in buffered rumen fluid. Ensiling the CONOXYL-TT treated CGT resulted in an improvement of 45% for in vitro digestibility as compared to control CGT which had only an equivalent amount of water added. Ensiling CONOXYL-TT treated CGT resulted in 67% apparent digestibility by growing lambs when fed at a ratio of 60% ensiled CGT and 40% concentrate. Digestibility of the CONOXYL-TT treated ensiled CGT was not, however, improved by the addition to the diet of readily available sources of energy such as molasses or fat (tallow).

These data show that lignocellulosic materials can be chemically processed with CONOXYL-TT for approximately $2.50 per ton. Furthermore, the treatment process can be accomplished on site with equipment common to the feedlot industry (chemical costs only). Finally, these data indicate that the CONOXYL-TT treated CGT can be ensiled and stored without significant loss as in the gross energy content of the biomass.

INTRODUCTION

Residues of the cotton industry are typical examples of dicotlignocelluloses which are relatively high in cellulose content, and which when properly chemically predigested can serve as a major energy source for ruminants. Historically, a variety of chemical reagents and treatment methods have been tested for their potential to improve in vitro and in vivo digestibility. However, more recent chemical treatment processes have been appraised not only for their impact on digestibility, but also for additional benefits such as those derived from long term environmental effects and cost effectiveness.

Dicotlignocelluloses most abundantly available from the cotton industry in the United States are cottonseed hulls and cotton gin trash (CGT). Because of the great quantity of CGT generated from the stripping of drought tolerant varieties of cotton in Texas, readily available CGT was the lignocellulose used in these studies.

The plant components comprising cotton gin trash consists of burrs, stems and leaves, cotton lint and immature seed. The relative proportions of these components can vary considerably with the growing season and the method of harvesting. The cell walls of CGT plant components consist primarily of cellulose, hemicellulose, lignin and minerals (Ben-Ghedalia et al., 1980; Axe et al., 1982; Conner, 1985; Conner and Richardson, 1987). CGT, as well as other plant residues, change in the proportion of

total cell wall constituents as the plant matures. These morphological changes produced by aging of both the stem and leaf alter the chemical composition of the plant tissues and can result in lower digestibility when fed to ruminants (Terry and Tilley, 1964; Mowat et al., 1965).

The composition of CGT is also quite different from that of Graminae straws. This is demonstrated by the permanganete lignin (PML) content of these straws which ranges from 5 to 10%, while that for cotton gin trash is elevated to as much as 25% (Conner and Richardson, 1987). The higher degree of lignification associated with CGT is analogous to that found in woody plants. Wood fibers typically form a cohesive three-dimensional structure whose integrity is assured by large amounts of intercellular substances (Cowling and Brown, 1969). The large PML values documented for CGT, help explain the slow rate of soil decomposition for CGT and its poor degradability by ruminal microorganisms.

The similarity of CGT cell wall constituents to that of hard woods presents the opportunity to utilize oxidizing agents, similar to those used in the paper pulping industry, to improve the digestibility of CGT by degradation of the lignin fraction to expose the cellulose. A comparison between ozone and sodium hydroxide (NaOH) treated cotton straw indicated that both treatments improved organic matter digestibility and cell wall digestibility (Ben-Ghedalia et al., 1983). Conner and Richardson (1987) reported that

chemical treatments for predigesting lignocelluloses can be ranked in order of potential improvement offered, with ozone giving the greatest response. While strong oxidizers, such as ozone, offer tremendous potential to increase cellulose availability, no practical method for large-scale treatment has been developed because of the high cost, low utilization of ozone generated in the ozonation process.

Thus, the objectives of these studies were: a) to improve the ozonation process by capture and storage of the ozone as it is generated; b) to examine the potential of ozone treated biomass, as a silage additive, particularly when it is to be used in ensiling problematic materials such as cotton gin trash (CGT).

OBJECTIVE A: TO IMPROVE THE OZONATION PROCESS.

Demonstration of Storage Process For Gaseous Ozone as a Concentrated Oxygenated Liquid.

Preliminary activities were directed at technology to improve the stability of ozone (i.e. increased half life) in the presence of the gin wastes in the reactor. If this can be accomplished, then the utilization of the oxidative potential of ozone in contact with the moist biomass should be improved. Side reactions involving ozone decomposition on the walls of the reactor, self destruction, and similar unproductive mechanisms would be reduced, and direct reactions with the biomass enhanced.

The stability experiments were carried out in a batch system similar to that shown in Figure 1. In this approach, the liquid medium to be used as a carrier of the dissolved ozone to the biomass reactor was placed in a three liter (3L) plastic tank. A connection to the bottom of the tank allowed this liquid to be pumped from the container through an aspirator and back to the holding tank. The aspirator's low pressure inlet was connected to the output lead from the ozone generator. Hence, when the generator was operating, ozone and air were drawn into the circulating fluids and thoroughly mixed to enhance solution and saturation. All contact surfaces, i.e. pump, tubing, aspirator, etc., were plastic. Aliquots were then taken periodically from the 3L holding tank and checked for pH and O_3 concentration. The ozone level was determined by a standard KI procedure which

measures the presence of all oxidizing moieties. The pH was measured with a pH electrode to two decimal points. Concentration and pH values were then plotted as a function of time. With distilled water one would expect to see the pH decrease with time while the ozone level increases to a steady state saturation level.

Several different solutions were tested with this approach. Because of work at the University of Illinois with alkaline-hydrogen peroxide as a pretreatment process for roughages based on wheat straw, a solution of water and NaOH was investigated. This was done in spite of most literature references suggesting that ozone is extremely unstable in high pH, aqueous mediums. Because of synergisms reported for ozone and hydrogen peroxide, aqueous solutions of hydrogen peroxide were also tested. In addition to the hydrogen peroxide, periodic acid and glycerin were additional reagents tested in the aqueous solutions.

Discussion of Results

The experimental results of these preliminary experiments are shown on the following graphs. Both pH and the concentration for the oxidative moieties in ppm as O_3 equivalents are plotted on vertical axes with time on the horizontal axis. Care must be exercised in reading these graphs, since the scales on the axes are not consistent in range or subdivisions from graph to graph. Overall, only the solution containing glycerin was found to stabilize the ozone concentration to the point that, at ambient

temperature, the ozone concentration in the holding tank increased with time. All the other moieties enhanced the oxidative potential in the initial period, but then decayed.

Of the other liquids, distilled water with approximately 0.3% NaOH dissolved in it gave no indication of any ozone buildup over a period of three hours (Figure 2). In essence, ozone was so rapidly broken down in the high pH (pH > 12) water, that it was undetected by the KI procedure over the time span of the test. The pH also remained unchanged although it appears to increase on the graph.

The aqueous solutions of peroxide started at low pH values (pH ≈ 4) and continued to decrease, suggesting as reported in the literature, that the decomposition of ozone in water can lead to increasingly acid solutions (Figures 3 and 4). The dissolved oxidants on the other hand started at elevated concentration levels due to the peroxide's presence, but diminished in about three hours to a lower concentration and stabilized. The periodic acid, which was included as a third oxidizer source, appeared to indicate two reductions over time (Figure 5); one for peroxide and one for periodic acid, with steady-state being approached at some time after five hours.

With glycerin in solution with peroxide, the ozone (oxidizer) concentration actually increased 10% over four hours' time, but with a corresponding drop in pH from 4.1 to

2.9 (Figure 6). This graph is difficult to read since the curves cross at a common juncture.

Although these data are preliminary, they do suggest that the ozone pretreatment should take place in reactors where the pH is less than 5. Moreover, the addition of hydrogen peroxide, periodic acid, and other soluble oxidizers such as halogen oxygen complexes can significantly enhance the oxidizing potential of the reactor. The addition of these other oxidizers or stabilizers does add increased cost factors, however. On the other hand, the polyol structures such as glycerine appear to complex with ozone and shield it from premature breakdown. This too can contribute significantly to enhancement of the availability of the cellulose fraction of the raw gin wastes.

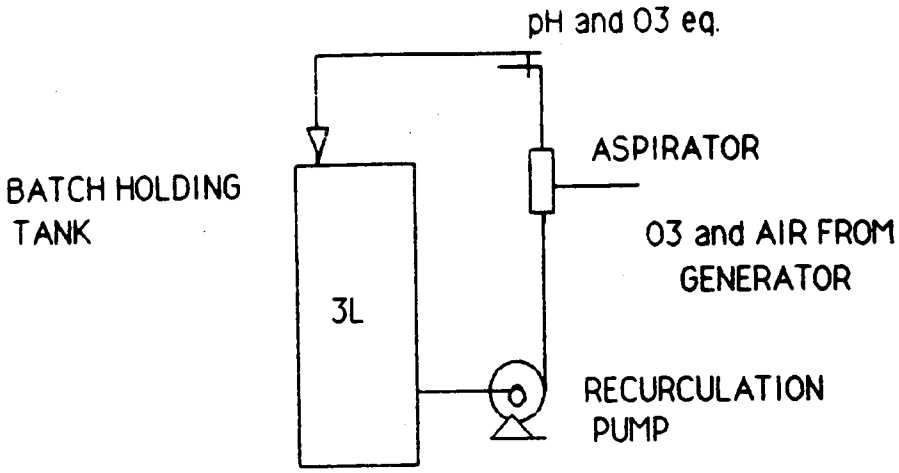


FIGURE 1; 03 Stability Test System

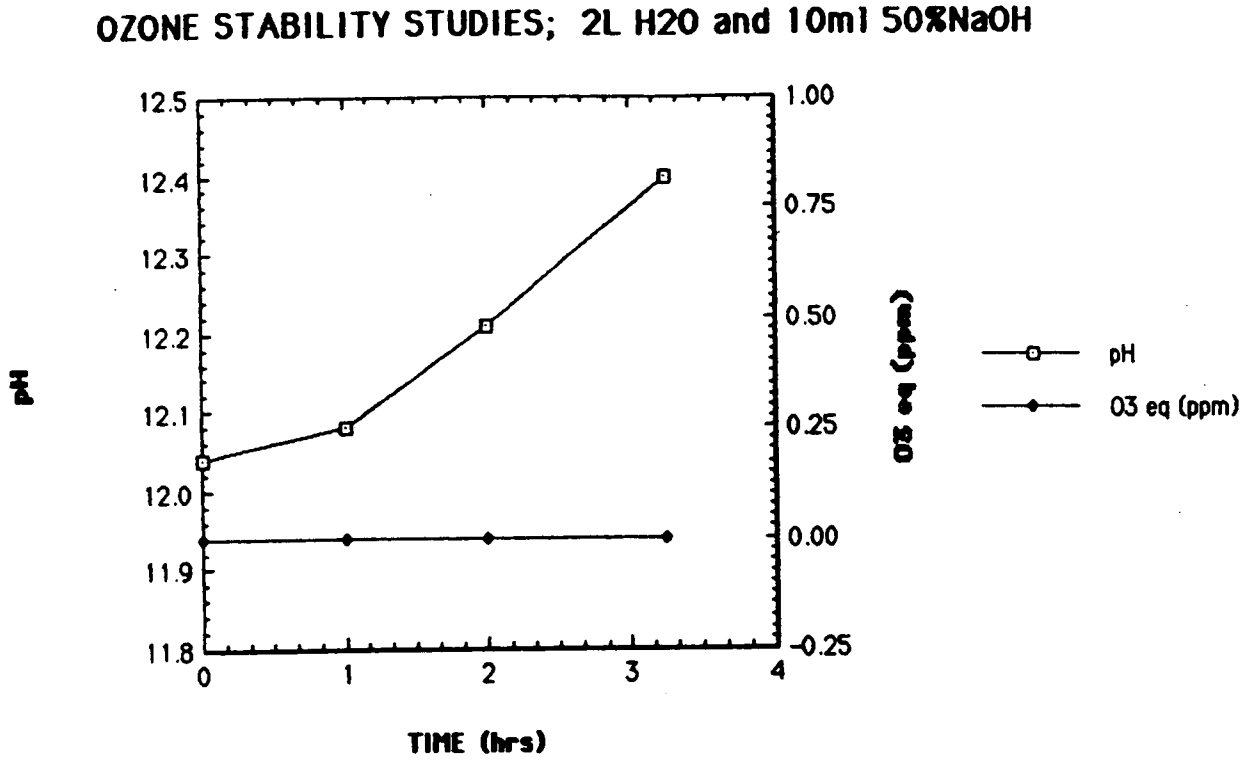

OZONE STABILITY STUDIES; 2L H20 and 10ml 50%NaOH
pH
12.5
12.4
12.3
12.2
12.1
12.0
11.9
11.8
0
1
2
3
4
TIME (hrs)
1.00
0.75
0.50
0.25
0.00
-0.25
03 eq (ppm)
pH
03 eq (ppm)

Figure 2

OZONE STABILITY STUDIES; 2L H2O and 10ml 3%H2O2

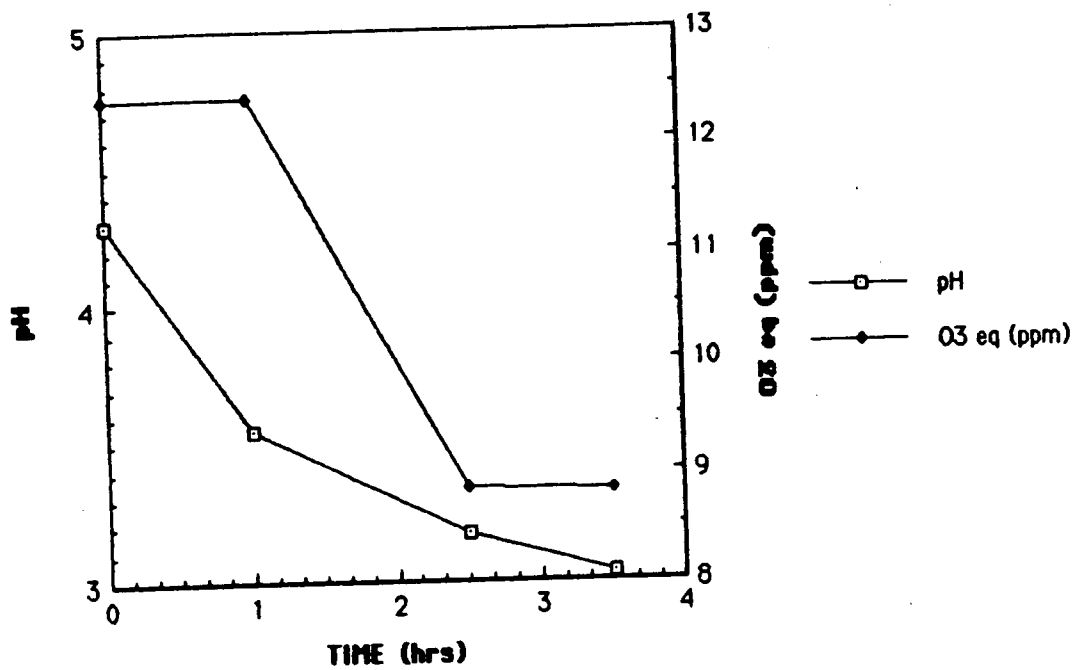


Figure 3

OZONE STABILITY STUDIES; 1L H2O and 1L 3%H2O2

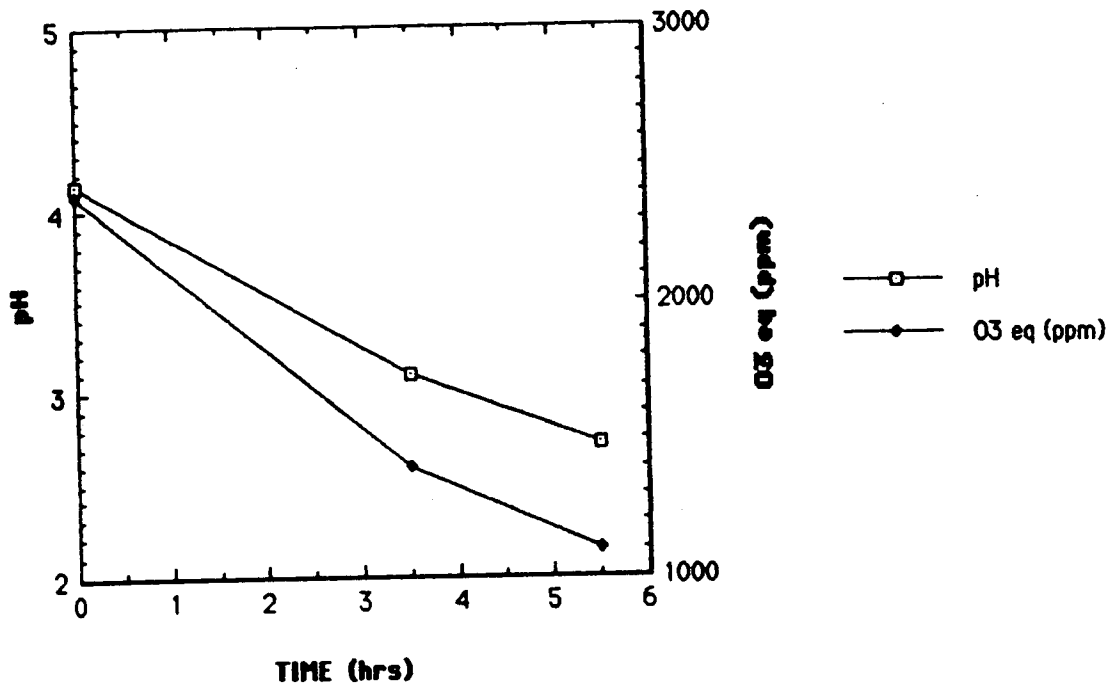


Figure 4

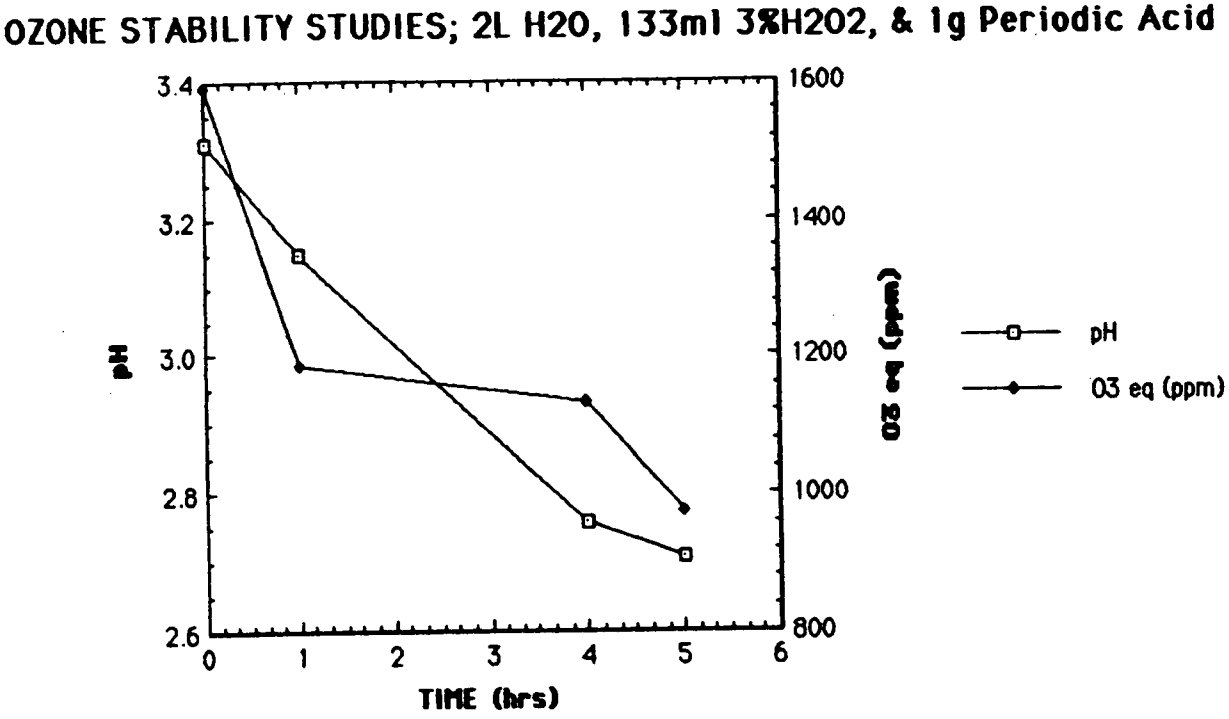

OZONE STABILITY STUDIES; 2L H20, 133ml 3%H202, & 1g Periodic Acid
3.4
3.2
3.0
2.8
2.6
pH
1600
1400
1200
1000
800
03 eq (ppm)
0
1
2
3
4
5
6
TIME (hrs)
pH
03 eq (ppm)

Figure 5

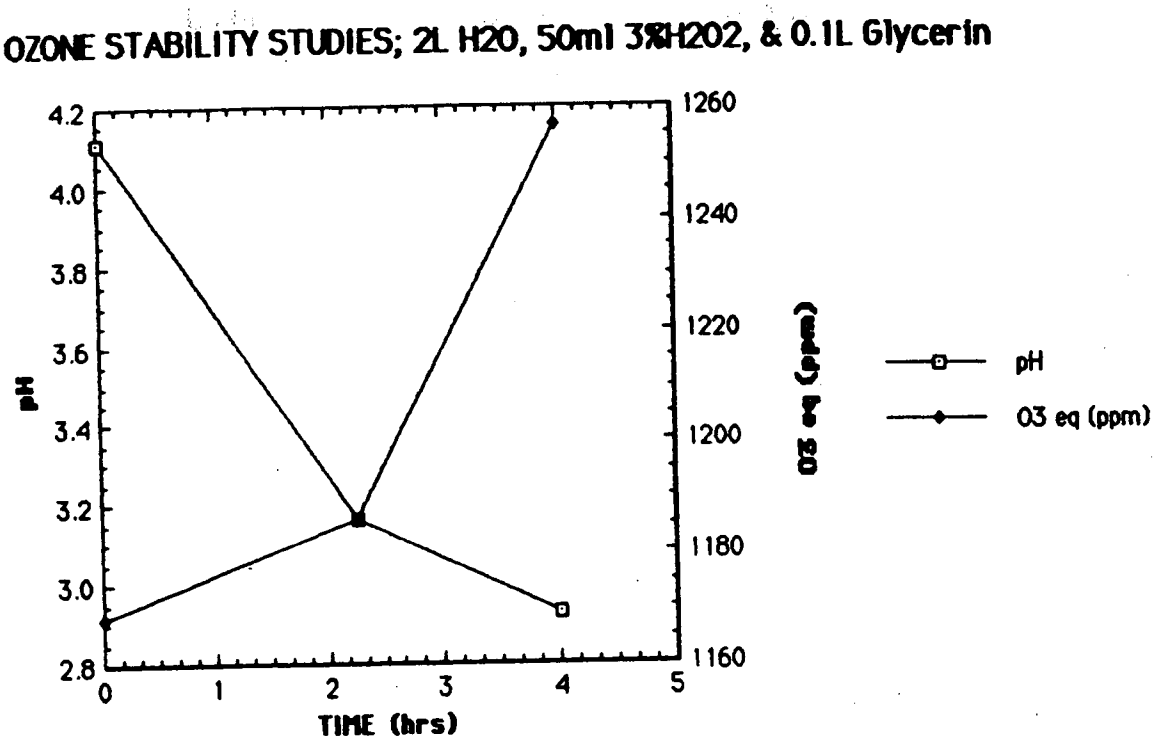
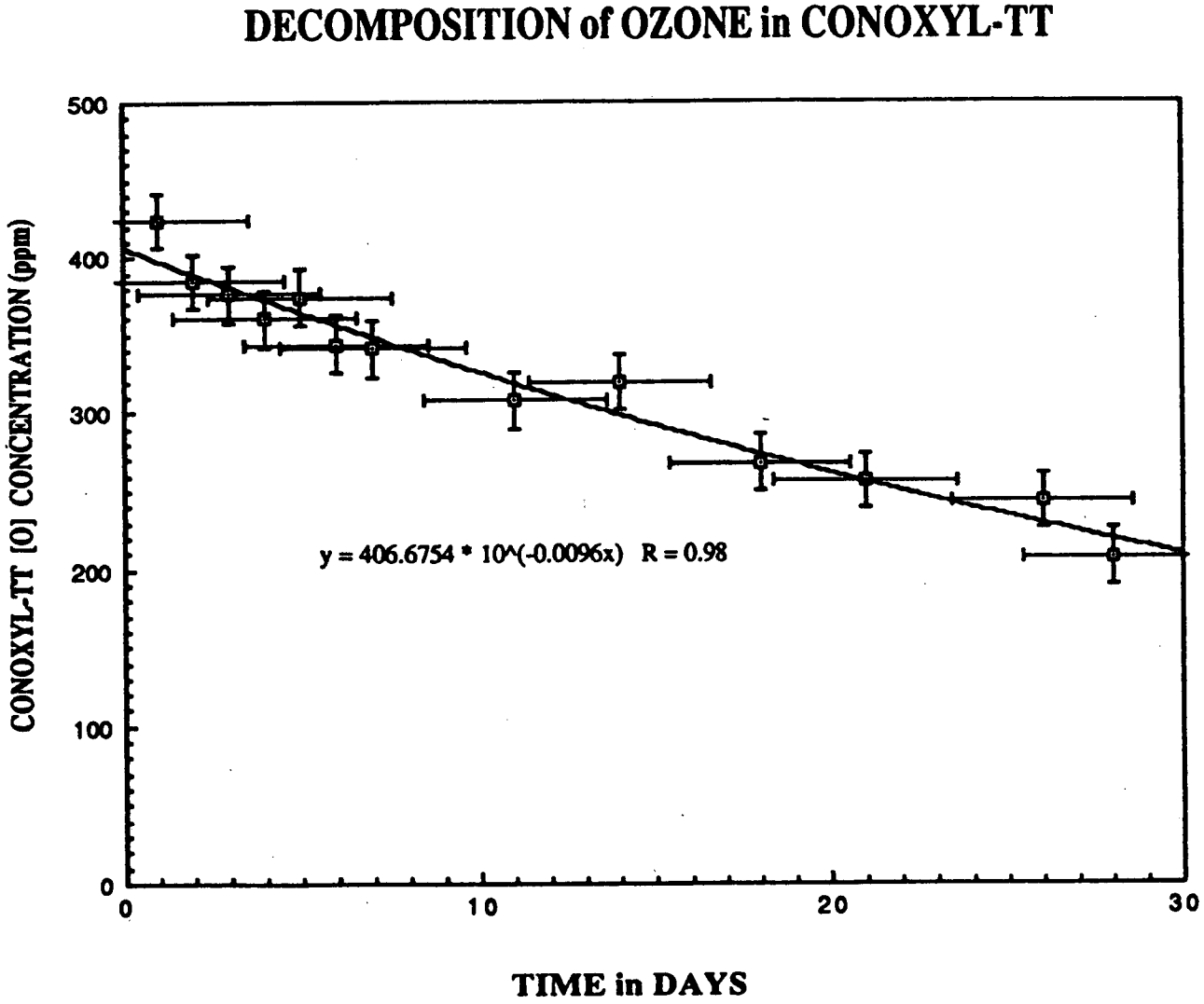

OZONE STABILITY STUDIES; 2L H2O, 50ml 3%H2O2, & 0.1L Glycerin
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
pH
1260
1240
1220
1200
1180
1160
O3 eq (ppm)
0
1
2
3
4
5
TIME (hrs)
pH
O3 eq (ppm)

Figure 6

t days	(O)ppm	ln(O)
1	424	6.05
2	385	5.95
3	376	5.93
4	360	5.89
5	374	5.92
6	344	5.84
7	341	5.83
11	308	5.73
14	320	5.70
18	268	5.59
21	256	5.55
26	244	5.50
28	208	5.34



OBJECTIVE B: TO EXAMINE THE POTENTIAL OF OZONATED LIGNOCELLULOSE, AS A SILAGE ADDITIVE, TO BE USED FOR ENSILING PROBLEMATIC MATERIALS SUCH AS COTTON GIN TRASH.

Pilot Plant Production of Concentrated Oxygenated Liquids For Treatment of Ton Quantities of Biomass.

The following procedure was followed in order to establish a standard production method for the concentrated oxidizing liquid (CONOXYL) to be used for treatment of cotton burrs prior to ensilage. The ensilage process requires high levels of moisture. This means that a ton of raw burrs with an equilibrium moisture level of 10% (200 lbs) will require an additional 2500 lbs of water to be added. Rather than add ozone-oxidizers to this total mass of water, it was decided that a concentrated, smaller volume of solution would be added at some point intermediate to the addition of the water. For example, in the prior reference to a ton of raw burrs, the 2500 lbs of make up water (300 gallons) would be added in a sequence of 1125 lbs (135 gallons); 250 lbs (30 gallons); 1125 lbs (135 gallons). The addition would be made to the burrs in a mixing truck. The first 135 gallons is to be applied with a hose over a period of 12-15 minutes and is intended to thoroughly wet and expand the lignocellulose structure of the burrs. The next 30 gallons (10% of the total water added) is the concentrated oxidizing liquid (CONOXYL). It should be applied so as to distribute the solution uniformly over the burr biomass. The intent is to open up the biomass structure and expose more of the cellulose as an energy source for the ensiling micro-organisms. This increased exposure may be created by (1) degradation of the

lignin fraction shielding the cellulose (2) swelling and rupture of both the physical and chemical bonding structure, (3) hydrolysis of the hemi-cellulose to simple sugars or (4) a combination of all these activities. The final addition of 1125 lbs (135 gallons) of water is applied like the first (135 gallon) addition over a 12-15 minute mixing span. It's purpose is to provide a high moisture environment for the ensiling activity of the micro organisms. In a more immediate sense, however, this final addition probably assists in a more uniform distribution of the COINOXYL as well as in the dispersal of reaction products caused by oxidation reactions. These reaction products are primarily carbon dioxide, and/or carboxylic acid moieties which help reduce the pH of the mixture.

CONOXYL Production

In order to produce the CONOXYL used in the experiment, a thirty-gallon plastic tank was washed and rinsed with tap water. The drum was fitted with ports to allow continuous recycling of the contents through an external loop. A small centrifugal pump was used for circulation. The external loop also contained a venturi or aspirator section and a static mixing section.

The plastic tank was filled with the following ingredients:

Basic Recipe:	28 gallons water	(234 lbm)
	700 ml glycerin	(2.0 lbm)
	470 ml glacial acetic acid	(1.0 lbm)
	4.5 liter 3% $H_2)_2$	(10.0 lbm)

The tank's ingredients were then circulated for at least 30 minutes in order to assure uniform dispersion. A mixture of ozone in air, which was generated on site, was then introduced to the

system through the low pressure connection of the aspirator or venturi. The ozone was being generated at approximately 1.1 gram per hour which amounted to less than 0.5% of the gas contacting the liquid. The contacting was done over a period of ten hours. After this period of time, the solution was taken to the TTU feedlot for treatment of the cotton burrs as described in the prior discussion.

The basis for the different ingredients in the CONOXYL solution was arrived at as a means of increasing and stabilizing the amount of ozone (O_3) dissolved in the water. Secondly, some of the ingredients assist the ozone in the degradation process when the solution is applied to the organic biomass. Hence, the presence of glycerin and the acetic acid are known to enhance ozone levels in water. They form quasi stable complexes which retard outgasing and decomposition of the dissolved O_3. The acetic acid and the hydrogen peroxide assist in the degradation of the biomass with ozone by increasing the amounts of hydroxyl and perhydroxyl radicals which form as the ozone decomposes. The ratios of ingredients used may not be optimal, but were found to be within the range of good performance based on oxidizer levels maintained over time in laboratory glassware. Additional work may well be needed to determine a better mixture. A final ingredient, periodic acid, was added to the mixture after ozonation. This addition was 30 grams, or approximately 200 ppm, to the thirty-gallon tank.

Samples of the ozonated water were taken from the thirty-gallon container after ozonation was stopped and just prior to application on the cotton burrs. A summary of the data obtained from these samples are shown in the following table.

TABLE (1) SUMMARY OF DATA GATHERED FOR THREE TREATMENTS

Liquid Sample Destriction	ph	Measured Variable [0] ppm	Mass of Cotton Gin Trash
(Quart of Concentrate)			
New Deal Research Center Water (make up water)	8.67	0.0	----
First Treatment (30 gal.)	4.42	600	1680 lbm
Second Treatment (30 gal.)	4.45	350	1260 lbm
Third Treatment (27 gal.)	5.02	478	840 lbm

TABLE (2) IN VITRO RESULTS

Treatment	[0] ppm by KI Overall Concentration	48 hr % OMD	%DM
Control	0	47.37	93
First	40	81.1	38
Second	30	80.7	41

TABLE 3. MATERIALS COSTS FOR CONOXYL-TT TREATMENT

	Ingredient	Chemical Formula	Bulk Price*
(1)	Water	H_2O	$0.10/1000 gal
(2)	Acetic acid	CH_3COOH	$0.30/lbm
(3)	Glycerin (glycerol)	$OHCH_2CHOHCH_2OH$	$0.75/lbm
(4)	Hydrogen peroxide	H_2O_2 (50% solution)	$1.00/lbm
(5)	Ozone	O_3	$2.50/lbm

*Bulk prices based on Chem Drug Rept., July 5, 1990

Based on the CONOXYL-TT formulation of 30 gallons of concentrate per ton of cotton gin trash with a background of 10% moisture. Water is added to reach the sixty percent moisture level necessary for ensilage. If ensilage is not used, only the 30 gallons of concentrate need to be added per ton of raw gin waste.

	Ingredient	CONOXYL-TT Reagents Per Ton of Cotton Gin Trash Volume or Mass Required	Unit Cost
(1)	Water	323 gallons	$0.03
(2)	Acetic acid	(470 ml) 1.0 lb	$0.30
(3)	Glycerol	(700 ml) 2.0 lb	$1.50
(4)	Hydrogen peroxide (35 solution)	(4.51) 10.0 lb	$0.30
(5)	Ozone	0.14 lb	$0.35
		Total	$2.48

In vitro and in vivo studies. A concentrated oxidizer solution (CONOXYL-TT), consisting of ozone (O_3) and other oxygen radicals (O_X), was used to predigest ground cotton gin trash. The solution was applied to the gin trash in combination with water while mixing in a auger-mixer truck. Water was then added to increase the moisture content to the 60 percent level and create a high moisture environment for the ensiling process and to assist in distribution of the CONOXYL-TT. The gin trash was treated in two batches of 1680 and 1260 pounds. For the first batch (1680 lbs), half of the water (1050 lbs) was applied first, followed by 30 gallons (250 lbs) of CONOXYL-TT at a concentration of 90 ppm. The other 1050 pounds of water was then applied. The other batch (1260 lbs) was treated in the same manner, half of the water (780 lbs), 30 gallons of CONOXYL-TT, another 780 pounds of water. However, the concentration of the CONOXYL-TT was 70 ppm for the second batch. A portion of the treated gin trash was mixed with other ration ingredients for each treatment and packed into 50-gallon drums lined with plastic. Another portion was packed into the drums without the addition of other ingredients. Drums were then sealed to create an anaerobic environment for lactic acid fermentation. The temperature of each drum was measured daily through small entry ports with a probe thermometer. Probe samples were also taken through the entry ports on days 5, 15 and 25 after sealing, for pH analysis.

The five dietary treatments are shown in table 4. All rations were isonitrogenous at 13 percent crude protein.

TABLE 4. TREATMENTS

A - Control, CONOXYL-TT treated cotton gin trash (ensiled)
B - CONOXYL-TT treated cotton gin trash with 5% added molasses (ensiled)
C - CONOXYL-TT treated cotrton gin trash with 10% added molasses (ensiled)
D - CONOXYL-TT treated cotton gin trash with 5% added fat
E - CONOXYL-TT treated cotton gin trash with 5% added fat plus 5% added molasses

TABLE 5. COMPOSITION OF DIETS

Ingredient	Silage Treatment				
	A	B	C	D	E
	%				
Gin trash silage	60	60	60	60	60
Cracked corn	24.46	20.88	15.31	14.54	8.97
Soybean meal	12.91	13.49	13.69	14.26	14.83
Molasses	0	5	10	0	5
Fat	0	0	0	5	5
Salt	.25	.25	.25	.25	.25
Trace mineral premix	.25	.25	.25	.25	.25
Vitamin premix	.20	.20	.20	.20	.20

Twenty-five crossbred wether lambs with an average weight of 75 pounds were allotted to five treatment groups by weight. Experimental units consisted of a pen of five lambs for each dietary treatment. All lambs were vaccinated against enterotoxemia and dewormed with ivermectin. The lambs were allowed to adjust to their dietary treatment for 14 days and then were fitted with fecal collection bags. A seven-day collection period followed, in which daily fecal output (grams) for each lamb was recorded. Feces for each lamb were thoroughly mixed for collection of a ten percent sample each day. After the collection period, the lambs were weighed and re-allotted to treatment groups by weight for a second adjustment and an additional collection period. Feed refusals were weighed and recorded daily for each pen.

TABLE 6. AVERAGE TEMPERATURE OF FEED IN SILOS[a]

Day	Silage (Treatment) A	B	C	D	E	SE[1]
1-5	87	89	89	86	85	1.79
6-15	84	87	86	85	86	1.14
16-20	78	80	80	77	77	1.52
21-25	72	74	75	72	73	1.30

[a]Degrees Fahrenheit

[1]Standard error of the mean

TABLE 7. AVERAGE pH OF FEED IN SILOS

	Silage (Treatment)					
Day	A	B	C	D	E	SE[1]
0[a]	6.46	6.00	5.72	5.20	5.85	.457
5	6.04	5.27	4.78	4.89	5.06	.501
15	5.52	4.45	4.41	4.48	4.52	.474
25	5.05	4.41	4.35	4.44	4.36	.297

[a]The value for day 0 was the initial pH

[1]Standard error of the mean

TABLE 8. APPARENT DIGESTIBILITY BY LAMBS FED CONOXYL-TT TREATED GIN TRASH SILAGE

Treatment	Dry Matter Intake[a]	Dry Matter Digestibility[b]
A	47.46	67.42[c]
B	48.27	57.45[d]
C	46.92	55.52[d]
D	50.14	57.65[d]
E	50.21	57.89[d]

[a]Kilograms per pen of five head, average of two replications.

[b]Percentage of dry matter.

[c,d]Means in the same column with different superscripts differ ($P<.05$). (Pooled standard error = .82) Means represent the average of two replications (pens) of five lambs each for a total of 50 animal observations.

TABLE 9. IN VITRO DIGESTIBILITY OF CONOXYL-TT TREATED COTTON GIN TRASH[a]

	48 hour OMD	96 hour OMD	SE[d]
Untreated gin trash	45.11[b]	52.94[b]	3.72
Treated gin trash	61.75[c]	64.26[c]	3.04

[a]Percentage of dry matter.

[b,c]Means in the same column with different superscripts differ ($P<.05$).

[d]Standard error of the mean. Means represent the average of twelve replications.

REFERENCES CITED

Axe, D., D. Addis, J. Clark, J. Dunbar, W. Garrett, N. Hinman and R. Zinn. 1982. Feeding value of cleaned and uncleaned gin trash. Proc. West. Sec. Am. Soc. Anim. Sci. 33:57.

Ben-Ghedalia, D., G. Shefet and Y. Dror. 1983. Chemical treatments for increasing the digestibility of cotton straw. 1. Effect of ozone and sodium hydroxide treatments on rumen metabolism and on the digestibility of cell walls and organic matter. J. Agric. Sci. 100:393.

Ben-Ghedalia, D., G. Shefet and J. Miron. 1980. Effect of ozone and ammonium hydroxide treatments on the composition and in vitro digestibility of cotton straw. J. Sci. Food Agric. 31:1337.

Conner, M. C. 1985. Utilization of chemically-treated cotton gin trash by ruminants. Ph.D. Dissertation. Texas Tech Univ., Lubbock.

Conner, M. C. and C. R. Richardson. 1987. Utilization of cotton plant residues by ruminants. J. Anim. Sci. 65:1131.

Cowling, E. B. and W. Brown. 1969. Structural features of cellulosic materials in relation to enzymatic hydrolysis. In: Robert R. Gould (Ed.). Celluloses and Their Applications. pp 152-187. Adv. in Chem. Ser. No. 95. Am. Chem. Soc., Washington, DC.

Mowat, D. N., B. R. Christie and J. E. Winch. 1965. The in vitro digestibility of plant parts of orchard grass clones with advancing stages of maturity. Can. J. Plant Sci. 45:503.

Terry, R. A. and J. M. A. Tilley. 1964. The digestibility of the leaves and stems of perennial rye grass, cocksfoot, timothy, tall fescue, lucerne, and sanfoin as measured by an in vitro procedure. J. Br. Grassl. Soc. 19:363.