In vitro stimulation of forage fiber degradation by ruminal microorganisms with *Aspergillus oryzae* fermentation extract

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Running title: Stimulation of fiber degradation with *A. oryzae*

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ABSTRACT

Aspergillus oryzae fermentation extract (Amaferm) was evaluated for its ability to influence degradation of bromegrass and switchgrass fiber fractions by mixed ruminal microorganisms in vitro. Amaferm at 0.067 mg/ml, which approximates that found in the rumen ecosystem, (0.06 mg/ml), increased the degradation of bromegrass neutral detergent fiber (NDF) by 28% after 12 h fermentations ($P < 0.01$), but had no effect at 24 or 48 h. Degradation of both the cellulose and hemicellulose fractions at 12 h were increased ($P < 0.01$). Additions of 0.08 and 8% (vol/vol) Amaferm filtrate (12.5 g/100 ml) stimulated degradation of switchgrass NDF 12 and 24%($P < 0.01$), respectively, at 12 h; while 80% filtrate decreased degradation 38%. Total anaerobes in culture tubes containing 80% filtrate were 5 times greater than controls, however, cellulolytic organisms were 3.5 times less than controls ($P < 0.05$). This suggested that the filtrate contained high concentrations of soluble substrate which did not allow the cellulolytic organisms to compete well with other populations. Remaining concentrations of esterified *p*-coumaric and ferulic acids were lower at 12 h in NDF residues from fermentations supplemented with Amaferm. Because total anaerobes were not inhibited in fermentations containing Amaferm, antibiotics are unlikely to be involved as a mode of action for increasing NDF degradation. The possibility that Amaferm contains enzymes, possibly esterases, that may play a role in stimulating the rate of fiber degradation by mixed ruminal microorganisms by removal of plant cell-wall phenolic acid esters is discussed.

INTRODUCTION

The effect of feeding microbial cultures or extracts of these cultures to ruminant animals has recently been reviewed (16). Interest in these studies originates from the concern of feeding subtherapeutic levels of antibiotics and the potential adverse affects generated. *Aspergillus oryzae* fermentation extract and *Saccharomyces cerevisiae* cultures have most commonly been fed to promote a desired response such as increased weight gain, milk production, or total tract digestibility of feed components. Enhancement of these responses have been mixed (5,8,30,31). Some studies indicate that significant improvements in the digestibility of fiber fractions can be achieved when *A. oryzae* extract is fed to ruminants (7,26). This coincides with an increase in the number of cellulolytic bacteria in the rumen (30).

Results from the effects of these supplements on in vitro studies with mixed ruminal microorganisms have also been inconclusive (16). However, several studies indicate that cellulolytic bacteria increase when in vitro samples are supplemented with *A. oryzae* extract (18,19). Newbold et al. (19) also concluded that increases in fiber digestion by animals fed *A. oryzae* extract is most likely due to stimulation of bacterial, rather than fungal or protozoal activity in the rumen. Martin and Nisbet (16) demonstrated that *A. oryzae* extract increased the growth and uptake of lactate by *Selenomonas ruminantium*. The extract appears to provide soluble factors, malate being one, that stimulate lactate utilization by *S. ruminantium*, which in turn may stabilize the rumen environment (31).

The objectives of our studies were to evaluate the effect of *A. oryzae* fermentation extract on the ability of mixed ruminal microorganisms to degrade cell wall components of bromegrass and switchgrass in vitro. Results indicate that the extract stimulates forage fiber degradation in a time-dependent manner.

MATERIALS AND METHODS

Amaferm supplement. *A. oryzae* fermentation extract (Amaferm) was supplied by BioZyme Enterprises, Inc., St. Joseph, MO. Amaferm was used in various studies as received. This was a product dried on an insoluble carrier of wheat bran and wheat middlings. It was added to in vitro digestion tubes without being heat sterilized. No degradation of forage cell walls took place in control tubes containing cell walls, Amaferm, and sterile rumen fluid (20% vol/vol) incubated for 72 h. In other studies, a filtrate of Amaferm was prepared similarly to the procedure of Nisbet and Martin (21). Amaferm, 12.5 g/100 ml McDougall's buffer (17), was mixed for 1 h on a magnetic stirrer, vacuum filtered through a Whatman no. 1 filter (Whatman Lab Sales, Hillsboro, OR), filter sterilized, and gassed with O_2 free CO_2. For unknown reasons a precipitate developed in the filtrate after 3 to 4 days, thus it was prepared fresh each time. The pH of the filtrate before gassing with CO_2 was 6.96 and after was 5.53, which may explain the precipitate. The filtrate was autoclaved (121°C, 15 min) in one experiment to determine the effect of heat on the product.

In vitro grass cell wall degradation. The effect of Amaferm on in vitro degradation by mixed ruminal organisms of bromegrass and switchgrass cell wall components (cell walls, cellulose,

hemicellulose) was determined at 0,12,24, and 48 h. These grasses were selected because they represent two major taxa of forages normally fed to ruminants and are known to differ in cell wall chemistry (15). The forages were ground to pass through a 1-mm-pore size screen prior to being used to prepare the cell walls. They were boiled for 1 h with neutral detergent, and the insoluble residues (cell walls) were extensively washed to remove detergent before drying (27).

Triplicate 0.5 g samples of each substrate at each time period were placed in 50 ml plastic culture tubes with screw caps. Each tube received 24 ml buffer (17), 6 ml of rumen fluid which was blended and strained through 2 layers of cheesecloth, and Amaferm where indicated. The rumen fluid was obtained from a steer fed 70% corn silage, 20% alfalfa hay, 9% soybean meal, trace minerals, and vitamins (13% crude protein diet). Concentrations of the branched chain acids, isobutyric and isovaleric, in culture tubes were 0.25 and 0.38 mM, respectively (9). The samples were vigorously shaken at 0800 and 1600 h daily. As each digestion period was completed 1 ml of 5% sodium azide was added to the tube to stop the fermentation. Samples were frozen and later analyzed for fiber content by the sequential detergent system (27,28). Sulfuric acid (72%) was used to solubilize cellulose and isolate crude lignin plus ash.

Total cell wall was defined as neutral detergent fiber. Hemicellulose and cellulose were calculated by weight difference as follows: neutral detergent fiber minus acid detergent fiber equaled hemicellulose, and acid detergent fiber minus acid detergent lignin equaled cellulose. Degradation was calculated

as the disappearance of the component during fermentation relative to the initial concentration. Correction was made for the addition of the components in the inoculum. Each result is the mean of three replicate tubes.

Microbiological analyses. The Hungate anaerobic culture method as described by Bryant (4) was used. Total viable and cellulolytic bacteria were determined from duplicate in vitro tubes separate from those used to analyze cell wall degradation. The medium used to determine total viable bacteria from duplicate in vitro tubes contained the following (per 100 ml): clarified ruminal fluid, 30.0 ml; glucose, cellobiose, maltose, starch, xylose, and glycerol, 0.03 g each; Trypticase (BBL Microbiology Systems, Cockeysville, MD), 0.2 g; resazurin, 0.0001 g; mineral S2, 5 ml (23); and purified agar (BBL), 1.75 g. Sodium carbonate (0.4%) and cysteine hydrochloride (0.05%) were added as sterile anaerobic solutions after the medium was autoclaved (4). Roll tubes (four replicates) were incubated at 37°C, and colonies were counted after 7 days. The composition of the cellulose agar roll tube medium was as follows (per 100 ml): clarified, preincubated ruminal fluid, 15 ml; Trypticase, 0.2 g; yeast extract, 0.05 g; mineral S2, 5 ml; cellulose (Whatman no. 1 filter paper ball milled with flint pebbles for 18 h), 0.2 g; resazurin, 0.0001 g; Na_2CO_3, 0.4 g; cysteine hydrochloride, 0.05 g; purified agar, 0.7 g. Four replicates of these tubes were incubated for 2 weeks before zones of clearing were counted.

Ferulic and *p*-coumaric acid analyses. Esterified ferulic and *p*-coumaric acid were extracted with alkali as previously published (15). The acidified sample solution was loaded on a

C_{18} solid-phase extraction column (Supelco, Inc., Bellefonte, PA), the column was washed with 2 ml of NaOH-phosphoric acid solution (pH 2.6), and the cinnamic acids were eluted with two 2.5-ml washes of 50% methanol. The eluted samples were brought to a final volume of 10 ml, filtered through a 0.2-μm filter, and stored at -20°C until analyzed. All extractions were performed in duplicate.

Identification and quantification of phenolic acids was done by LC. A Gilson gradient autoanalytical system (Gilson Medical Electronics, Inc., Middleton, WI) with a programmable dual-wavelength UV detector (Gilson Model 116) was utilized. Samples (20 μl) were injected and separated by isocratic elution through a Spherisorb-ODS, C_{18}, 5-μm column (Supelco). The solvent (97.7% water-0.3% glacial acetic acid-2% butanol) was pumped at 3 ml/min. Column temperature was maintained at 50°C. *p*-Coumaric and ferulic acids were quantified by absorbance at 320 nm.

Statistics. Fiber digestibility data were analyzed as a completely randomized design. Means were compared by the F-protected ($P < 0.05$) least significant difference method using SAS (24).

RESULTS

Initial efforts were to determine whether or not Amaferm would affect degradation of plant cell wall components when added to an in vitro system at concentrations which approximated those in the rumen. Assuming a 50 l rumen received 3 g/day, which is the manufacturer's recommended feeding level for a lactating cow, an equivalent amount in a 30 ml working volume culture tube would be 1.8 mg. This does not adjust for the 5-fold dilution of

microbial mass in our in vitro fermentations (6 ml rumen fluid in 24 ml buffer) compared to the rumen. Amaferm was added at 2 mg, (0.067 mg/ml), and at a higher concentration of 6 mg (0.2 mg/ml), per 30 ml. The results in Table 1 indicated that these concentrations increased the degradation of bromegrass NDF over the control at 12 h, but not 24 or 48 h. The increase was 28 and 33% ($P < 0.01$) for the 0.067 and 0.2 mg/ml level, respectively. Degradation of hemicellulose and cellulose fractions of NDF were increased ($P < 0.01$) by the addition of Amaferm (Fig. 1).

To verify the effect that Nisbet and Martin (21) reported, i.e., that a filtrate could be prepared from Amaferm which retained activity, we prepared a filtrate to examine its ability to increase degradation of switchgrass NDF (Fig. 2). At 12 h, with the exception of the 0.0008% level, an increasing amount of NDF was degraded with increasing levels of filtrate added up to the 8% level. At the 80% filtrate level a decrease ($P < 0.01$) in the amount of NDF degradation occurred. The 0.08 and 8% filtrate increased NDF degradation at 12 h by 12 and 24% ($P < 0.01$), respectively; while the 80% level decreased the degradation by 38%. At 24 h the levels of Amaferm filtrate had little effect with the exception of the 80% level which reduced degradation by 47%.

To determine whether or not the component in Amaferm which increased NDF degradation was heat stable, we autoclaved the filtrate and compared it to filter sterilized filtrate additions. At the two levels we examined, 8 and 80%, heat sterilized filtrate did not give a different response than the filter sterilized filtrate (Fig. 3). The 8% levels increased NDF

degradation on average 21% ($P < 0.01$), while the 80% levels decreased degradation by 64%.

Total viable and cellulolytic bacteria were enumerated (Table 2) from duplicate in vitro tubes in the previous experiment. At the 8% level of filtrate, total viable bacteria increased 3 times, 8.3×10^9 to 25.7 or 25.3×10^9 ($P < 0.05$); at the 80% level the increase was approximately 5 times ($P < 0.05$) for the filter sterilized and autoclaved filtrates (41.8×10^9). The cellulolytic bacteria increased 1.4 to 1.8 times ($P < 0.05$) at the 8% level while they decreased 3.5 times ($P < 0.05$) at the 80% level for the filter sterilized filtrate. There was little difference in the number of cellulolytic bacteria between the control and 80% level of Amaferm filtrate when the filtrate was autoclaved.

DISCUSSION

Our studies indicate that Amaferm, whether in the dry product form or as a liquid filtrate, can enhance the rate of in vitro degradation of bromegrass or switchgrass fiber fractions by mixed ruminal microorganisms (Table 1 and Fig. 2). The in vitro concentration of Amaferm used in this study, 0.067 mg/ml, approximates that which microorganisms may be exposed to in the bovine rumen. Stimulation of the rate, but not extent, of forage fiber degradation confirms the studies of Fondevila et al. (6) in which they found that Amaferm increased the rate of straw degradation in sheep but did not alter the final extent.

The data in Fig. 2 indicate that a very high concentration (relative to rumen concentration 0.067 mg/ml) of Amaferm (8%

filtrate) can be added to an in vitro digestion system without an inhibitory response being observed. Only with 80% filtrate was the NDF degradation significantly reduced (38%). Because there was no inhibitory fermentation response with 8%, we conclude that an agent such as an antibiotic, (much like feeding subtherapeutic levels of antibiotics to animals for growth efficiency) can be excluded as a factor which may explain the enhanced rate of fiber degradation by Amaferm. This is further supported by our observation of gas pressure in the in vitro tubes when opened for analyses. Those containing 80% extract had considerably higher gas pressure (greater microbial activity) than those tubes with lesser amounts of Amaferm. Our conclusion is that the 80% filtrate contained a significant amount of soluble substrate which inhibited the degradation of insoluble NDF substrate. This is supported by the data in Table 2. The total viable bacteria found in tubes supplemented with 80% filtrate increased 5 times, while the cellulolytic bacteria decreased 3.5 times. Because of the potential for significant substrate being present in the 8 and 80% filtrate levels, which would mask any trace element effect, we are unable to conclude whether or not the component which stimulates NDF degradation at the 0.067 and 0.2 mg/ml levels is heat sensitive. Newbold et al. (18) recently concluded that the mode of action of Amaferm on rumen fermentation depends on a heat-labile component. The stimulation in NDF degradation that we saw with Amaferm levels of 0.067 and 0.2 mg/ml is due to a trace component. However, the stimulation that we saw with additions of 8% Amaferm filtrate is likely due to soluble substrates being rapidly fermented by organisms including the

cellulolytic population, which increase their numbers (cellulolytic) over control samples, thus, greater amounts of NDF are degraded. Our results suggest that other studies (1,16,21) which have used Amaferm filtrates at 2 to 5% levels, may not be using physiological concentrations (0.06 mg/ml) similar to those which the microorganisms in the rumen ecosystem are exposed to; thus, such results may not be applicable to explaining what happens in the in situ environment.

Species of *Aspergillus* are known to produce a wide variety of polysaccharidase enzymes (cellulases, hemicellulases) which could influence plant cell wall degradation (22). The high fibrolytic enzyme content of *A. oryzae* is still likely the primary mode of action for the improved fiber breakdown in the rumen (20). *A. oryzae* is an aerobic organism and is not expected to multiply in the rumen (6). Whether it is viable for any period is unknown. Recently an esterase has been purified from *A. oryzae* which liberates ferulic, *p*-coumaric, and acetic acid from wheat straw (25). The phenolic acids, ferulic and *p*-coumaric, are covalently bound to cell wall polysaccharides and may act as crosslinking agents between lignin and hemicellulose (10,12). Extent of crosslinking by ferulic and *p*-coumaric acid dimers and esterification of feruloyl and *p*-coumaroyl groups to arabinoxylans have been shown to limit plant cell wall biodegradation (10,14). Ferulic acid esters of arabinoxylan are thought to limit rate of fiber degradation, but are not thought to limit extent, because of naturally occurring ruminal esterases (13). We have previously hypothesized that bacterial species capable of metabolizing phenolic monomers may act as catalysts

for forage fiber breakdown by increasing microbial access to cell wall polysaccharides (29). Similarly, high concentrations of both feruloyl and *p*-coumaroyl esterases in rumen anaerobic fungi, which can be ten-fold greater than rumen bacteria (2), may provide a unique advantage to these microorganisms for the biodegradation of phenolic containing and phenolic cross-linked arabinoxylans (3). This in turn could render the polysaccharides in cell walls more available to degradation. Once the aerobic fungi *A. oryzae* is exposed to an anaerobic environment, it may simply lyse and release esterase and potentially other enzymes which enhance at least the rate of plant cell-wall breakdown but not necessarily the extent.

With this mechanism in mind, i.e., that *A. oryzae* produces esterases which enhance cell wall degradation, we analyzed the residual concentrations of esterified *p*-coumaric and ferulic acids from bromegrass NDF fermentations with and without Amaferm supplementation (Table 1). At 12 h an increase in NDF degradation with Amaferm was observed along with a decrease in the remaining esterified *p*-coumaric and ferulic acid concentrations. At 24 and 48 h, no differences in NDF degradation with or without Amaferm were observed and little difference was observed in the remaining *p*-coumaric and ferulic acid concentrations. This suggests that esterase enzymes from Amaferm may play a role in the degradation of bromegrass fiber components. Preliminary measurement of feruloyl esterase activity (10) of Amaferm at 25° C gave an activity of 0.01 μ moles/h/mg protein. This activity should be considered as the minimal activity because it is based on the total protein

extracted from Amaferm, and it is an activity at 25°C as opposed to the temperature of the rumen. Further studies are needed to determine the survival of *A. oryzae* in the vicinity of the rumen wall where it may be able to scavenge some oxygen for short-term growth. Measurement of esterase activity from the pure culture of *A. oryzae* under anaerobic conditions is also warranted.

In summary, two important conclusions can be drawn from this study. One, *A. oryzae* fermentation extract by an as yet unknown mechanism, stimulates the rate but not extent of in vitro degradation of grass forages by mixed ruminal microorganisms. Secondly, caution should be exercised in drawing conclusions from studies which add substrate levels of fermentation extract, because the extract contains high levels of soluble components which are fermented, and thus, this is likely to mask-out the true trace element affect in the extract such as the ruminal ecosystem sees at 3 g of extract per 50 l.

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TABLE 1. Influence of Amaferm on in vitro degradation of bromegrass neutral detergent fiber (NDF) and remaining concentration of esterified *p*-coumaric (PCA) and ferulic acids (FA)

Amaferm concn (mg/ml)	Time (h)	NDF degradation (%)	Esterified monomer concn (mg/g)[a] PCA	FA
0	12	8.1[b]	3.15	2.42
0.067	12	11.3[c]	2.73	2.11
0.2	12	12.1[c]	2.57	2.09
0	24	23.0	2.75	2.09
0.067	24	22.8	2.76	2.14
0.2	24	22.9	2.72	2.13
0	48	30.7	2.86	2.04
0.067	48	30.4	2.77	2.05
0.2	48	30.3	2.61	2.00
SE		0.6		

[a]Concentration of PCA and FA in bromegrass NDF before autoclaving was 3.30 and 2.70, respectively; after autoclaving, 3.06 and 2.52, respectively. Means in these columns are the average of two analyses.

[b,c]Means in the same column with different superscripts differ ($P < 0.01$).

TABLE 2. Influence of Amaferm filtrate on total viable and cellulolytic bacteria from 12 h in vitro digestion tubes containing bromegrass NDF

Amaferm treatment and concn (%)[a]	Total bacteria (10^9/ml)	Cellulolytic bacteria (10^6/ml)
0	8.3[b]	9.7[b]
8	25.7[c]	18.0[c]
80	40.3[c]	2.8[d]
8A	25.3[c]	13.5[c]
80A	41.8[c]	8.3[b]
SE	5.3	2.5

[a] Amaferm (12.5 g/100 ml) was extracted in McDougall's buffer and filtered. Twenty four ml of this filtrate plus 6 ml rumen fluid inoculum equaled the 80% filtrate concentration (vol/vol). Amaferm treatments 8A and 80A are 8% and 80% Amaferm filtrate which were autoclaved prior to being added to in vitro digestibility tubes.

[b,c,d] Means in the same column with different superscripts differ ($P < 0.05$).

List of Figures

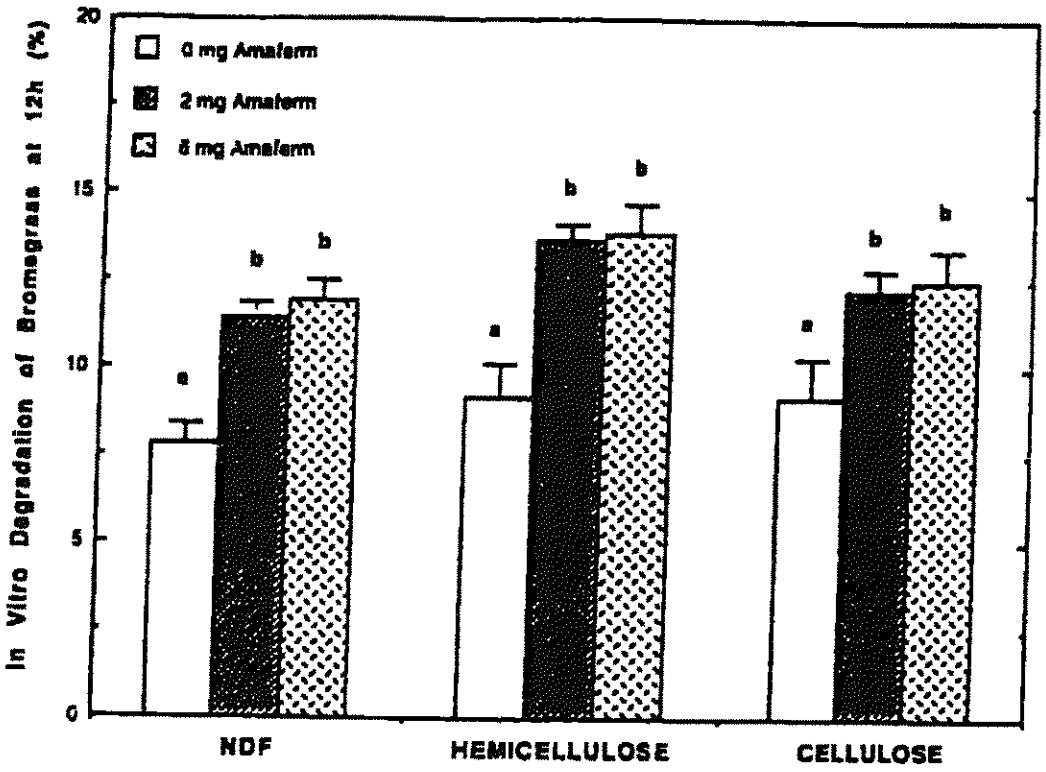
0 mg Amaferm
2 mg Amaferm
6 mg Amaferm
In Vitro Degradation of Bromegrass at 12h (%)
0
5
10
15
20
a
b
b
a
b
b
a
b
b
NDF
HEMICELLULOSE
CELLULOSE

Fig. 1

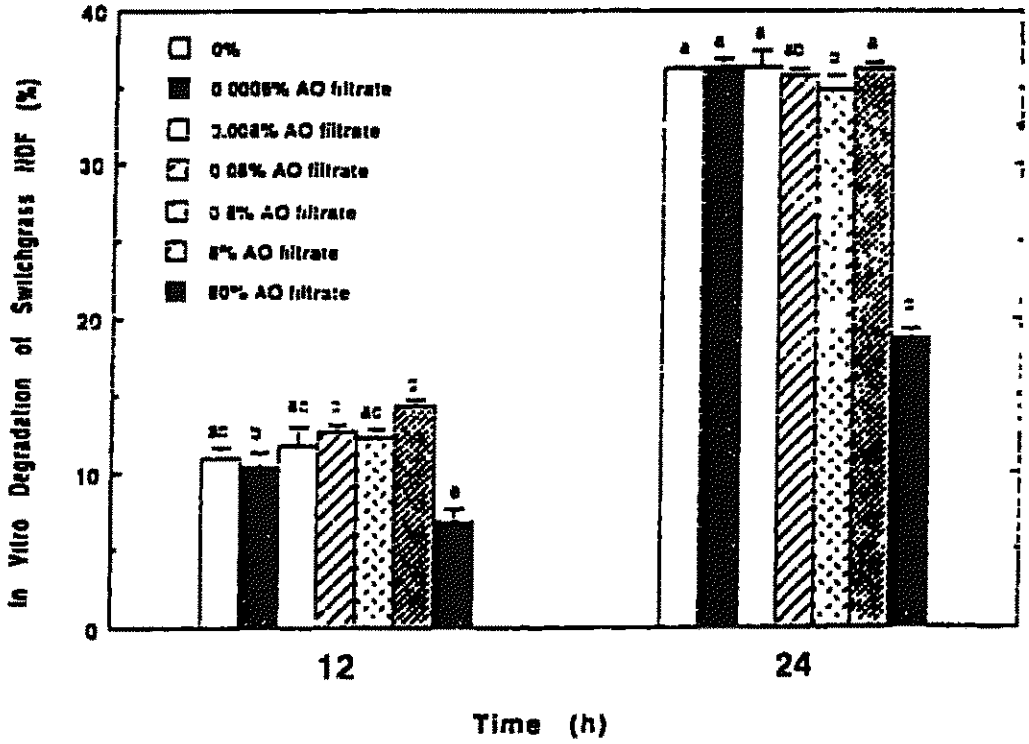
0%
0.0008% AO filtrate
0.008% AO filtrate
0.08% AO filtrate
0.8% AO filtrate
8% AO filtrate
80% AO filtrate
In Vitro Degradation of Switchgrass NDF (%)
0
10
20
30
40
12
24
Time (h)

Fig. 2

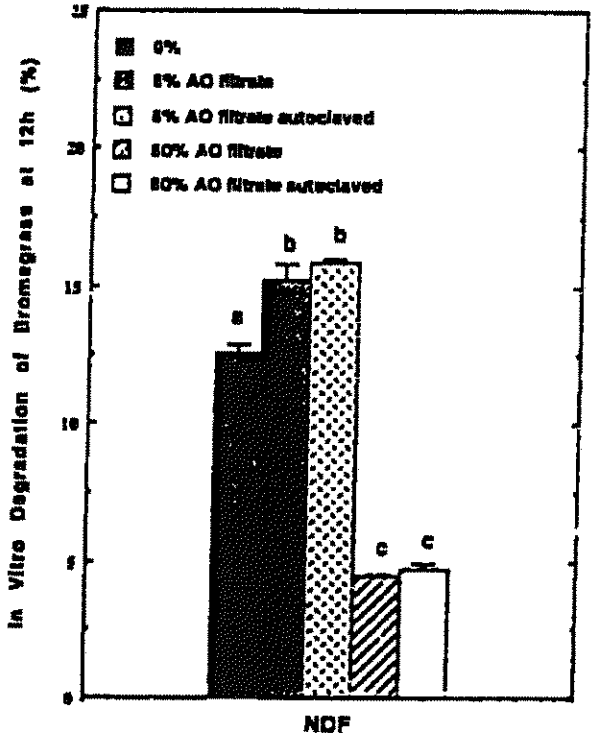
0%
5% AO filtrate
5% AO filtrate autoclaved
50% AO filtrate
50% AO filtrate autoclaved
In Vitro Degradation of Bromegrass at 12h (%)
NDF
a
b
b
c
c

Fig. 3