



## Chemical Changes during Ensilage and *In sacco* Degradation of Two Tropical Grasses: Rhodesgrass and Guineagrass Treated with Cell Wall-degrading Enzymes

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**ABSTRACT :** Effects of the cell wall-degrading enzymes derived from *Acremonium cellulolyticus* and *Trichoderma viride* on the silage fermentation and *in sacco* degradation of tropical grasses i.e. rhodesgrass (*Chloris gayana* Kunth. cv. Callide) and guineagrass (*Panicum maximum* Jacq. cv. Natsukaze) were investigated in laboratory-scale experiments. These two grasses were either treated with or without the enzymes before ensiling. Untreated rhodesgrass produced acetate fermentation silage (lactate, 13.0 g kg<sup>-1</sup> DM; acetate, 38.7 g kg<sup>-1</sup> DM) with high final pH value and NH<sub>3</sub>-N content (5.84 and 215 g kg<sup>-1</sup> DM). Addition of enzymes significantly increased (p<0.01) the lactate production (lactate, 45.6; acetate, 34.0 g kg<sup>-1</sup> DM) and decreased (p<0.01) the pH and NH<sub>3</sub>-N (4.80 and 154 g kg<sup>-1</sup> DM) in the ensiled forages when compared with the control silages. Untreated guineagrass was successfully preserved with a high lactate proportion (lactate, 45.5; acetate, 24.1 g kg<sup>-1</sup> DM), and the addition of enzymes further enhanced the desirable fermentation (lactate, 57.5 g kg<sup>-1</sup> DM; acetate, 19.4 g kg<sup>-1</sup> DM). The content of NDF was lowered (p<0.05) by enzymes in both silages, but the extent appeared greater in the enzyme-treated rhodesgrass (rhodesgrass, 48 g kg<sup>-1</sup> DM; guineagrass, 21 g kg<sup>-1</sup> DM). Changes in the kinetics of *in sacco* degradation showed that enzyme treatment increased (p<0.01) the rapidly degradable DM (rhodesgrass, 299 vs. 362 g kg<sup>-1</sup> DM; guineagrass, 324 vs. 343 g kg<sup>-1</sup> DM) but did not influence the potential degradation, lag time and degradation rate of DM and NDF in the two silages. (**Key Words :** Cell Wall-degrading Enzymes, Rhodesgrass, Guineagrass, Silage, Digestion, *In sacco*)

### INTRODUCTION

The influence of cell wall-degrading enzymes on preservation of grass and legume silages has been widely investigated. The efficacy of enzyme treatment is influenced by enzyme activity, level of application and temperature during storage (Weinberg et al., 1993; Aniwari et al., 1998; Nishino and Uchida, 1998; Jalilvand et al., 2008). Maturity and dry matter (DM) content of the crop material may also influence the effectiveness of enzyme additives (Van et al., 1989; Adogla-Bessa and Owen, 1995; Zhang et al., 1997) and the potential benefit may be increased by adding to crops with low soluble carbohydrate

content. Tropical grasses usually have a coarse, stemmy structure, with low sugar and high cell wall contents compared with temperate grasses (Catchpole and Henzel, 1971; Crowder and Chheda, 1982). Thus, when ensiling tropical grasses more air will be retained in the crop material which usually leads to unfavourable fermentation (Catchpole and Henzel, 1971).

Hydrolysis of cell wall polysaccharides by exogenous enzymes may alter the digestion of silage organic matter. Several experiments have shown that addition of enzymes to the ensiled forage could increase its digestibility as a result of enhancing cell wall digestion by microorganisms (Mchan, 1986; Tengerdy et al., 1991; Chamberlain and Robertson, 1992; Stokes, 1992; Zhang et al., 1997; Eun et al., 2007), while others demonstrated that enzyme treatment did not improve silage digestibility (Jacobs and McCallan, 1992; Weinberg et al., 1993; Sheperd et al., 1995). Effects of enzymes on the *in sacco* degradation of the silage are also variable. Several workers (Huhtanen et al., 1985; Yu Zhu et al., 1991; Sheperd et al., 1995) showed a reduction

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in degradation of NDF after treatment of enzymes, whereas others (Van Vuuren; 1989; Jacobs and Mcallan, 1991) did not find those changes due to the addition of enzymes. Nadeau et al. (1996) reported smaller effects of enzymes on digestibility of ensiled legume (lucerne) than on grass (cocksfoot) silage, whilst Yu et al. (1991) obtained the opposite results with lucerne and Italian ryegrass silage. Although a large number of studies have been carried out on the fermentation and digestion of enzyme-treated silage, little information on tropical grasses is apparently available compared with that on temperate grasses and legumes. In this study, two silages were prepared in the laboratory using rhodesgrass and guineagrass to investigate effects of cell wall-degrading enzymes on chemical composition, fermentation quality and *in sacco* degradation of the silage.

## MATERIALS AND METHODS

### Preparation of silages

Primary growth of rhodesgrass (*Chloris gayana* Kunth. cv. Callide) and guineagrass (*Panicum maximum* Jacq. cv. Natsukaze) was used in the present experiment. The herbage was both harvested at the early heading stage and wilted for about 2 h under good weather conditions. After chopping into 13 mm length with a forage cutter, the forages were ensiled in polyethylene bottles with or without cell wall-degrading enzymes. The enzymes were derived from *Acremonium cellulolyticus* and *Trichoderma viride*, and the mixture (1:2 based on avicelase activity) was added at 50 mg kg<sup>-1</sup> fresh material (FM) prior to ensiling. The activity of the enzyme mixture (at pH 4.50 and 50°C) on microcrystalline cellulose, carboxymethylcellulose and birch wood xylan was 638, 9,260 and 2,630 µmol sugar released g<sup>-1</sup> min<sup>-1</sup>, respectively (Yu et al., 1991). The amounts of forage ensiled in each bottle were 600 g and 530 g FM for rhodesgrass and guineagrass, respectively; and the tops of laboratory silos were covered with four layers of polyethylene sheet before capping. Each treatment was made in triplicate for each time and the silos were stored for 2, 5, 15 and 45 d at room temperature (20-30°C).

### Chemical analysis

Upon opening silos, the ensiled forage obtained from each bottle was thoroughly mixed and a 20 g sample was taken for the count of lactic acid bacteria using a GYP-CaCO<sub>3</sub> agar plate (Yu et al., 1991). Another 20 g sample was added to 180 ml of distilled water, and homogenised for 1 min. The subsequent filtrate was used for pH, lactic and volatile fatty acids, and NH<sub>3</sub>-N measurements (Nishino et al., 1997). For sampling the crop material, a number of subsamples were taken from the original chopped forage and then mixed well before being subdivided into small samples. Half of the divided small samples were then

discarded and the other half was again mixed and subdivided. About 100 g of representative forage was packed in a polyethylene bag and subjected to freeze-drying. The chemical composition of crop material and silage was determined on freeze-dried samples, which were ground to pass through a 1 mm screen. Kjeldahl nitrogen (N) was used to determine crude protein content. Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined according to the methods of Van Soest (1991). Water soluble carbohydrates (WSC) were determined using the anthrone method (Deriaz, 1961), and buffering capacity was measured by the titration procedure (Playne and McDonald, 1966).

### *In sacco* degradation

The 45 d freeze-dried silages were incubated *in sacco* according to the procedure described by Nocek (1988). The silages were ground to pass through a 2 mm screen, and a composite sample was made for each treatment by mixing the freeze-dried powders of triplicate jars. Three goats, each equipped with a rumen cannula, were randomly allocated for the incubation of untreated or enzyme-treated silage. The goats were offered rhodesgrass or guineagrass hay prepared from the same sward as used for silage making. The surface area and pore size of nylon bags was 110×140 mm<sup>2</sup> and 42 µm, respectively. Six bags, each containing 3 g of composite sample, were inserted in the rumen and removed sequentially after 3, 6, 12, 24, 48 and 72 h incubation. After washing the bags with a domestic washing machine until running water was clear, losses of DM and NDF were determined after drying at 60°C for 24 h.

The kinetics of *in sacco* degradation were determined according to the equation,  $p = a + b(1 - e^{-c(t-t_0)})$ , described by McDonald (1981). Non-linear regression analysis was performed to estimate the parameters, while the rapidly degradable fraction was determined as the disappearance of DM and NDF during the washing process.

### Statistical analysis

Data for silage composition are presented graphically, with SEM bars included at each treatment×time point mean (n = 3) to indicate variation. The effects of enzyme treatment on chemical composition, silage fermentation and *in sacco* degradation were subjected to one-way analysis of variance. Control means were compared with enzyme treatment means using the *t*-test of Systat (Ver 5.2) at the 0.05 probability level.

## RESULTS

Wilting increased the DM content of rhodesgrass and guineagrass from 156 to 235 g kg<sup>-1</sup> and from 221 to 284 g kg<sup>-1</sup>, respectively. The WSC content was less than 50

**Table 1.** Dry matter ( $\text{g kg}^{-1}$  fresh forage), chemical composition ( $\text{g kg}^{-1}$  DM), buffering capacity ( $\text{meq kg}^{-1}$  DM) and lactic acid bacteria count ( $\log \text{cfu g}^{-1}$  FM) of wilted rhodesgrass and guineagrass

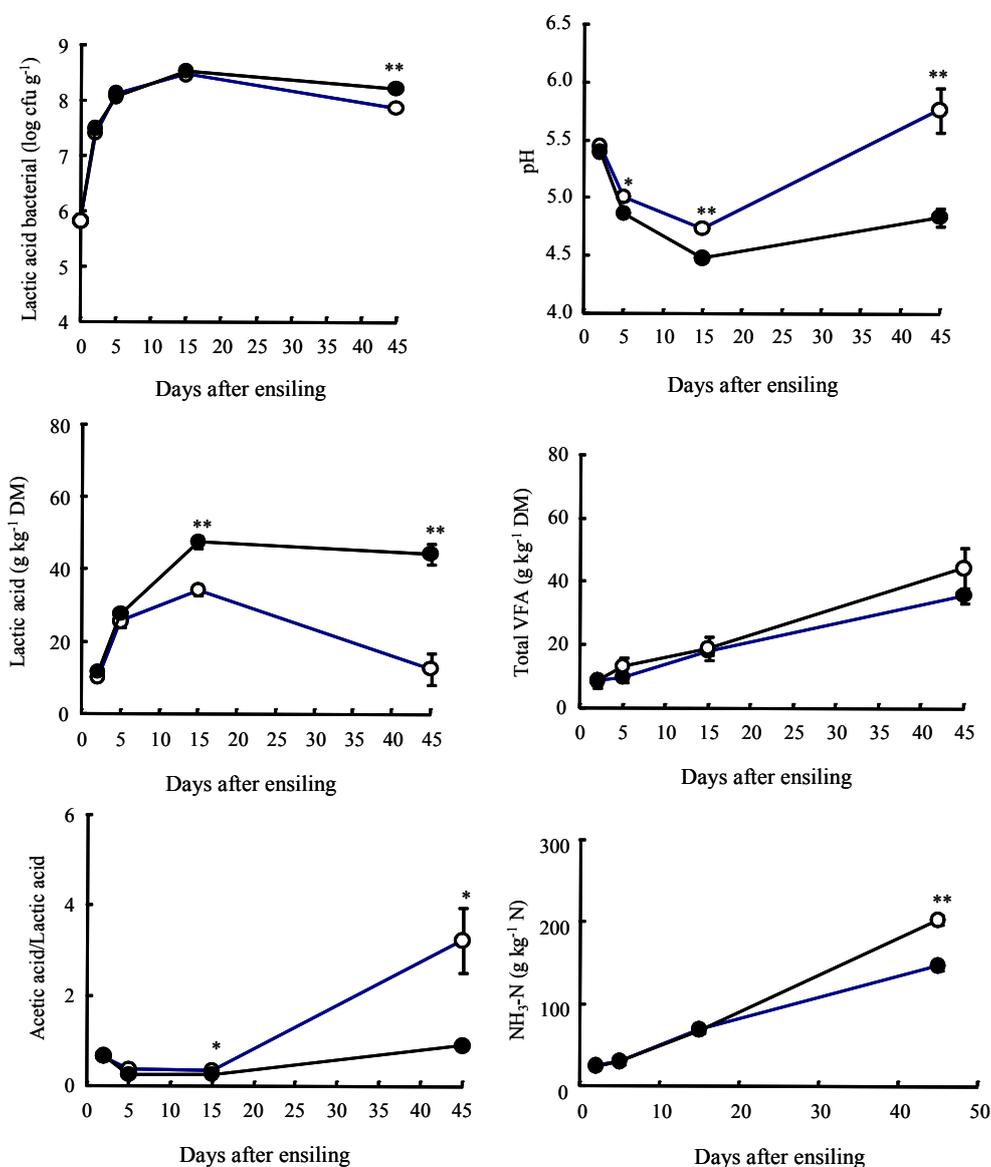
	DM	CP	NDF	ADF	ADL	WSC	BC*	LAB**
Rhodesgrass	235	161	537	314	34.0	42.0	240	5.74
Guineagrass	284	132	603	339	44.7	35.8	239	4.85

Means values from duplicate analysis. \* BC = Buffer capacity. \*\* LAB = Lactic acid bacteria.

$\text{g kg}^{-1}$  DM and the counts of lactic acid bacteria were about  $10^5 \text{ cfu g}^{-1}$  FM in both wilted forages. CP content in rhodesgrass was higher than that in guineagrass and the NDF, ADF and ADL were *vice versa* (Table 1).

The amounts of lactic acid bacteria and lactic acid increased with the ensiling time of rhodesgrass until d 15 of storage after which they decreased (Figure 1). The content

of lactic acid in the enzyme treated silage was significantly higher ( $p < 0.01$ ) than that in the control silage on d 15 ( $53.4$  vs.  $37.5 \text{ g kg}^{-1}$  DM) and d 45 ( $45.6$  vs.  $13.0 \text{ g kg}^{-1}$  DM) of ensiling. Addition of enzymes significantly decreased the pH value of the ensiled forage on d 5 ( $p < 0.05$ ), d 15 ( $p < 0.01$ ) and d 45 ( $p < 0.01$ ) when compared with the control silage. On day 45 of ensiling, a higher ratio of acetic



**Figure 1.** Changes in lactic acid bacteria count, pH value, lactic acid, total volatile fatty acid (VFA), acetate to lactate ratio and  $\text{NH}_3\text{-N}$  of rhodesgrass silage added with (●) or without (○) cell wall-degrading enzymes. Values are means and standard deviation represented by vertical bars. Asterish indicates significant difference between treatments (\*  $p < 0.05$ , \*\*  $p < 0.01$ ).

**Table 2.** Kinetics of *in sacco* degradation of rhodesgrass and guineagrass silages with or without addition of cell wall-degrading enzymes

Item	Rhodesgrass		SE	Sig.	Guineagrass		SE	Sig.
	Control	Enzyme-treated			Control	Enzyme-treated		
<b>DM</b>								
Rapidly degradable fraction (g kg <sup>-1</sup> )	299	362	1.83	**	324	343	2.30	**
Slowly degradable fraction (g kg <sup>-1</sup> )	453	378	11.1	**	412	392	8.65	NS
Potential degradation (g kg <sup>-1</sup> )	752	740	9.75	NS	736	735	8.79	NS
Rate of degradation (h <sup>-1</sup> )	0.071	0.071	0.010	NS	0.059	0.061	0.008	NS
Lag time (h)	1.99	2.85	0.31	NS	2.28	2.58	0.32	NS
<b>NDF</b>								
Rapidly degradable fraction (g kg <sup>-1</sup> )	38.0	57.3	2.97	**	49.8	60.7	3.49	NS
Slowly degradable fraction (g kg <sup>-1</sup> )	621	579	10.6	NS	601	576	13.3	NS
Potential degradation (g kg <sup>-1</sup> )	659	636	10.1	NS	651	637	11.6	NS
Rate of degradation (h <sup>-1</sup> )	0.069	0.076	0.001	NS	0.061	0.065	0.008	NS
Lag time (h)	1.96	1.75	0.19	NS	2.63	2.84	0.31	NS

SE of difference for three incubation measurements. NS = Not significant, \* p<0.05; \*\* p<0.01.

acid/lactic acid and higher NH<sub>3</sub>-N content were observed in the control silages compared to the silages treated with enzymes (p<0.05) and the NH<sub>3</sub>-N content of the control and enzyme-treated silage was 215 and 154 g kg<sup>-1</sup> of total N, respectively. Volatile fatty acid concentration increased linearly with the time of ensiling, with acetate accounting for the highest proportion in both untreated and treated silages.

Untreated guineagrass produced lactate fermentation silage (Figure 2). Although final pH (4.95) was not low, NH<sub>3</sub>-N accounted for less than 120 g kg<sup>-1</sup> N. The major volatile fatty acid was acetate in guineagrass silage, and the ratio to lactate was kept low throughout the ensiling period. Addition of enzymes significantly (p<0.01) increased the lactic acid and decreased the pH and NH<sub>3</sub>-N (p<0.01).

The NDF content was significantly (p<0.01) lowered by the added enzymes in both rhodesgrass and guineagrass silages (p<0.05) (Figures 3 and 4). ADF content was significantly (p<0.01) decreased in the ensiled rhodesgrass after enzyme treatment, but not in the guineagrass silage. The ADL content was not affected by enzymes in either of the two grass silages. A large portion of WSC rapidly disappeared during the initial ensiling period, and after 15 d of storage the content appeared unchanged in both the silages. Although differences were small, higher WSC content remained in enzyme-treated silage (p<0.05) than in the control silages irrespective of forage species.

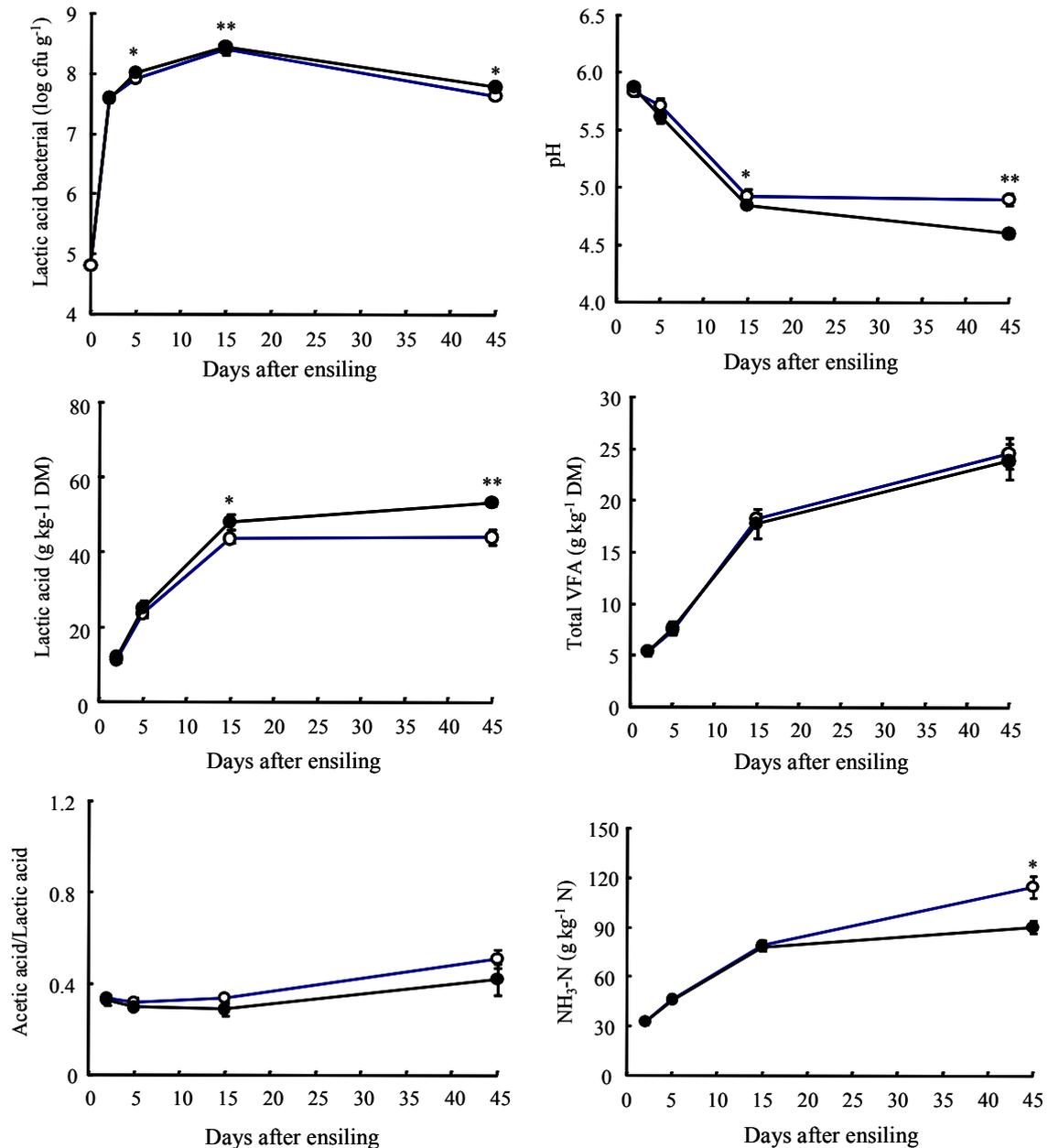
Addition of enzymes increased the rapidly degradable DM fraction of rhodesgrass silage (p<0.01), whilst it reduced the slowly degradable fraction (p<0.01). No differences were observed in potential degradable DM fraction, the rate of degradation and lag time between the enzyme-treated silages and the control silages. The rapid

degradable NDF content of the rhodesgrass silage treated with enzymes was higher than that of the control (p<0.01), but no differences were found on the remaining *in sacco* degradation parameters of NDF (p>0.05). In guineagrass silage, only the proportion of rapidly degradable fraction of DM differed between the enzyme-treated silages and the control silages and the rapidly degradable fraction of DM increased significantly (p<0.01) after enzyme treatment.

## DISCUSSION

It has been shown that ensiling tropical grasses may result in silages preserved predominantly by acetate rather than lactic acid (Catchpoole and Henzel, 1971; Uchida and Kitamura, 1987; Kim and Uchida, 1991; Ogawa, 1992). This may, in part, be due to the lack of sugar substrates, because the production of lactate can be increased by addition of readily fermentable carbohydrates (Uchida and Kitamura, 1987; Kim and Uchida, 1991). The silage made from tropical grasses is less dense and more permeable than that from temperate species (Catchpoole and Henzel, 1971), suggesting that the coarse and stemmy structure may also be responsible for acetate production.

In this study, the WSC content (<50 g kg<sup>-1</sup> DM) was probably insufficient for good fermentation in both rhodesgrass and guineagrass. An increase in acetate, coupled with a decrease in lactate, occurred in untreated rhodesgrass silage after 15 d of storage. This might be due to the action of saccharolytic and proteolytic clostridia (McDonald et al., 1991), while the production of butyric acid was low (<5 g kg<sup>-1</sup> DM) even in untreated rhodesgrass silage. Certain species of lactic acid bacteria, which could metabolize lactate to acetate under conditions of sugar



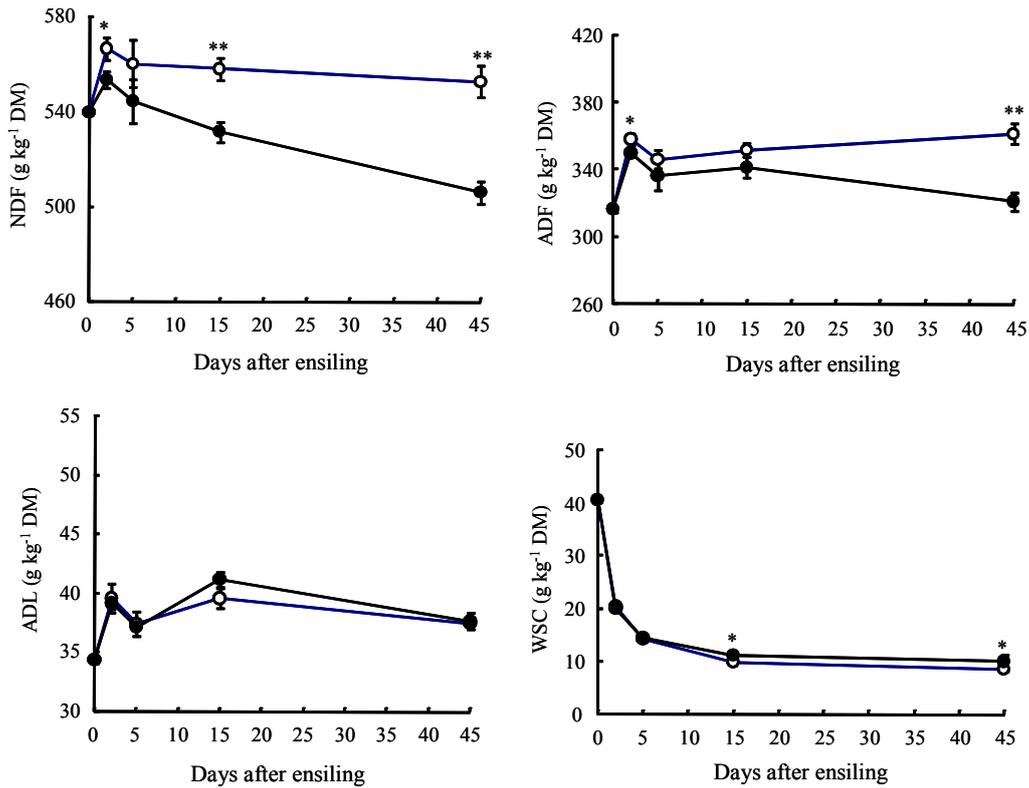
**Figure 2.** Changes in lactic acid bacteria count, pH value, lactic acid, total volatile fatty acid (VFA), acetate to lactate ratio and NH<sub>3</sub>-N of guineagrass silage added with (●) or without (○) cell wall-degrading enzymes. Values are means and standard deviation represented by vertical bars. Asterish indicates significant difference between treatments (\* p < 0.05, \*\* p < 0.01).

limitation (Lindgren et al., 1990), might also have increased in population to alter the acetate to lactate ratio in the later ensiling period. The numbers of lactic acid bacteria were relatively high in both forages at ensiling, because during wilting the population was increased from the level of 10<sup>4</sup> cfu g<sup>-1</sup> FM at harvest. The bacterial numbers were marginally higher in enzyme-treated rather than untreated silage, whilst not relating to changes in lactic acid content.

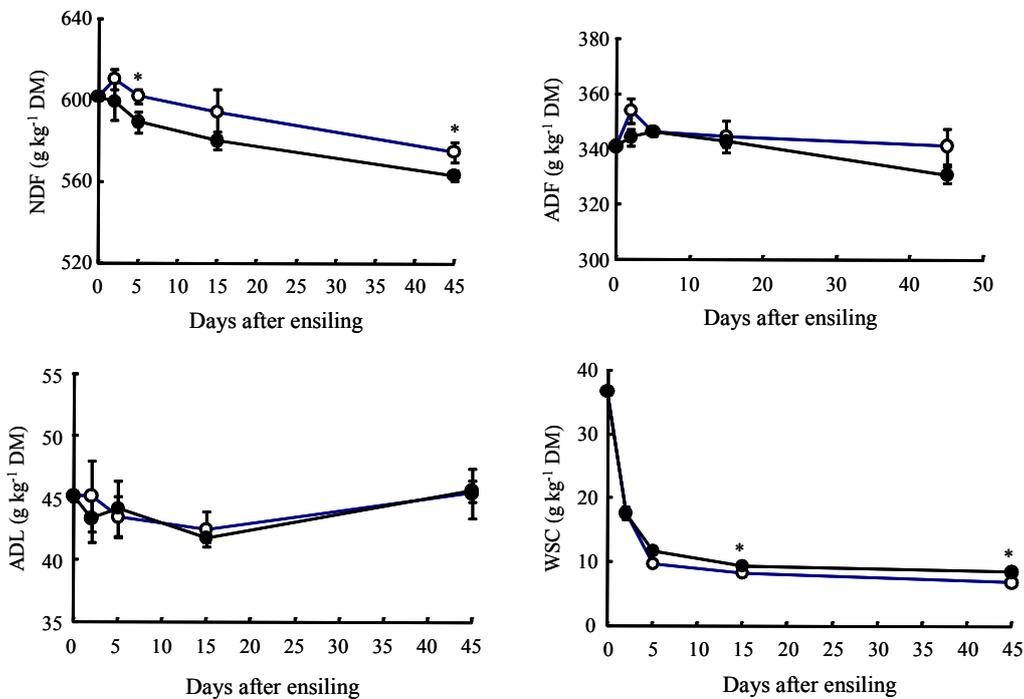
The reduction in lactate did not occur in untreated guineagrass silage, while no great differences were found in composition between the two parent crops. Ensiling at

higher DM content could lead to a sustained dominance of lactic acid in guineagrass silage. However, Yu et al. (2000) obtained lactate-rich silage from direct-cut guineagrass with low WSC content (DM, 183 g kg<sup>-1</sup>; WSC, 52.1 g kg<sup>-1</sup> DM), whereas acetate-rich silage was produced from direct-cut rhodesgrass with similar DM and WSC contents to guineagrass (DM, 188 g kg<sup>-1</sup>; WSC, 64.8 g kg<sup>-1</sup> DM).

In the present study, addition of enzymes to both of the ensiled grasses significantly decreased the pH value, NDF, ADF (not in the guineagrass) and NH<sub>3</sub>-N contents, and increased the content of lactic acid and WSC content.



**Figure 3.** Changes in neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and water soluble carbohydrates (WSC) of Rhodesgrass silage added with (●) or without (○) cell wall-degrading enzymes. Values are means and standard deviation represented by vertical bars. Asterish indicates significant difference between treatments (\* p < 0.05, \*\* p < 0.01).



**Figure 4.** Changes in neutral detergent fibre (NDF), acid detergent fibre (ADF), acid detergent lignin (ADL) and water soluble carbohydrates (WSC) of Guinea grass silage added with (●) or without (○) cell wall-degrading enzymes. Values are means and standard deviation represented by vertical bars. Asterish indicates significant difference between treatments (\* p < 0.05, \*\* p < 0.01).

Similar results were reported by Selmer et al. (1993) who investigated the effect of two commercial cellulase/hemicellulase enzymes derived from *Trichoderma reesei* on the silage fermentation of ryegrass. Jacobs and McAllan (1991) also reported that enzyme-treated silages had lower levels of cellulose, ADF and NDF than untreated and formic acid-treated silages. The extent of improved fermentation seemed to relate to the amount of cell wall hydrolysed by added enzymes. The lower response in guineagrass was probably due to more lignified cell wall composition compared with rhodesgrass. In addition, high DM content of guineagrass might have impaired the effects of enzymes, because a certain amount of water would be needed as a transport medium (Henderson and McDonal, 1977; Yu et al., 2000).

Changes in the *in sacco* degradation also appeared to depend on the extent of cell wall hydrolysis by enzymes, and the reduction in NDF resulted in the increase in rapidly degradable DM. The effects of enzymes were similar in the two silages, and mostly in agreement with those reported for Italian ryegrass silage (Yu et al., 1991). Addition of enzymes increased the rapidly degradable fraction of DM and NDF, but not the potential degradable fractions in the present study, which agrees well with the report of Moharrery et al. (2009). Previous studies also suggested that exogenous fibrolytic enzyme could increase the *in vitro* degradation of forages (Eun et al., 2007; Eun and Beauchemin, 2007; Jalilvand et al., 2008). In contrast, the amount and rate of NDF degradation were lowered when the same enzymes were added to lucerne (Yu et al., 1991; Moharrery et al., 2009), which was probably because proteolysis might have been limited to the more easily digestible components, thereby retaining the less degradable cell wall in lucerne silages. These results suggest that there are differences in the effects of enzymes on the DM digestion of grass and legume crops (Yu et al., 1991; Nadeau et al., 1996; Moharrery et al., 2009). However, differences may be marginal between temperate and tropical grasses.

## CONCLUSION

Addition of the enzymes derived from *Acremonium cellulolyticus* and *Trichoderma viride* increased the fermentation quality and WSC in the ensiled tropical grasses of rhodesgrass and guineagrass and reduced the NH<sub>3</sub>-N, NDF and ADF contents. Extent of the improved fermentation was related to the amount of cell wall hydrolysed by added enzymes. Changes in the *in sacco* degradation depend on the extent of cell wall hydrolysis by enzymes, and the reduction in NDF resulted in an increase in rapidly degradable DM. The enzyme treatment could increase the rapidly degradable DM but did not influence

the potential degradation, lag time and the degradation rate of DM and NDF in the two tropical grass silages.

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