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In vitro Effects of Exogenous Fibrolytic Enzymes on Rumen Fermentation of Wild Guinea Grass (*Panicum maximum*)

Research Article

S. Sujani[1], I.N. Pathirana[1*], R.T. Seresinhe[1] and K.B. Dassanayaka[2]

[1] Department of Animal Science, Faculty of Agriculture, University of Ruhuna, Kamburupitiya, Sri Lanka
[2] School of Land and Environment, University of Melbourne, Parkville, Victoria, Australia

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*Correspondence E-mail: thakshas@ansci.ruh.ac.lk

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ABSTRACT

This study aimed to evaluate the potential of commercial enzymes cellulase (CE), xylanase (XY) and the combination of CE + XY to improve rumen fermentation of Guinea grass ecotype 'A' (*Panicum maximum*). The experiment was carried out in a randomized complete block design. *In vitro* incubations were performed with four doses of individual enzymes and their combinations (1:1 from each enzyme) as 50, 100, 150 and 200 µL enzymes with 500 mg substrate. *In vitro* gas production (IVGP) was measured at 4 h intervals. At the end of 24 h incubation *in vitro* rumen dry matter disappearance (IVRDMD), ammonia nitrogen (NH_3-N), protozoa population and volatile fatty acid (VFA) were estimated. Supplementation with CE, XY and CE + XY significantly enhanced IVGP (control: 38.54 mL; CET1: 50.06 mL; XYT1: 54.27 mL and CET1 + XYT1: 52.77 mL) and IVRDMD (control: 46.78%; CET1: 51.21%; XYT1: 51.53% and CE1 + XY1: 52.64%). The rumen NH_3-N production was significantly increased ($P<0.05$) with XY and CE + XY (control: 100%; XYT1: 108.88%; and CET1 + XYT1: 111.6%). Though the total VFA did not exhibit any significant change, acetate production was significantly reduced by CE + XY while the same treatment enhanced the butyrate production. The alterations of acetate and propionate profiles led to the significantly decreased acetate: propionate with CE and CE + XY. Insignificant deduction of rumen protozoa population was observed with all enzyme treatments. In conclusion, the supplementation of exogenous fibrolytic enzyme could improve the rumen fermentation of Guinea grass ecotype 'A'.

KEY WORDS gas production, rumen ammonia-nitrogen, rumen dry matter disappearance, rumen protozoa, volatile fatty acid.

INTRODUCTION

One of the major constraints in tropical livestock production systems is the inferior quality of available feed resources which ultimately leads to the overall drop in animal's production efficiency. In Sri Lanka ruminants depend on year-round grazing on natural pastures especially the Guinea grass ecotype 'A' (*Panicum maximum*). It is characterized by low-digestibility and feed value due to high acid-detergent fiber and lignin content and lower crude protein level (Aganga and Tshwenyane, 2004). The inefficiency of forage utilization not only limits the available energy to the animal, but also it prompts the enteric methane production. Over the decades scientists have proposed different approaches to improve rumen digestion process in ruminants fed poor quality forages. Supplementation of ruminant feeds with exogenous fibrolytic enzymes as means of improving rumen performance has attracted a distinct attention in recent years [Positive responses in rumen fermentation have drawn with several *in vitro* (Elghandour *et al.* 2013) and *in vivo* (Khattab *et al.* 2011; Salem *et al.* 2013)] experiments. However the effects of fibrolytic enzymes vary depending on many factors such as the type of animal, dose rate, supplementation method and more specifically on

forage species. The studies conducted on Guinea grass ecotype 'A' supplemented with fibrolytic enzymes are extremely limited. Therefore this study was undertaken to disclose the potential use of exogenous fibrolytic enzymes on Guinea grass ecotype 'A' on rumen fermentation parameters under *in vitro* conditions.

MATERIALS AND METHODS

Substrate, enzymes and treatments

Guinea grass ecotype 'A' (*Panicum maximum*) was collected and used as the substrate after drying at 55 ˚C for 48 hours and grinding to pass a 1 mm screen. Proximate analysis of dry matter (238.3±3.37 g/kg), crude ash (Ash=93.53±0.35 g/kg DM), N (Kjeldahl method) were done according to the AOAC (1990). Crude protein (CP) was calculated as N × 6.25 (CP=95.9±2.1 g/kg DM). Neutral detergent fiber (NDF) was reported to be 700.03 ± 2.71 g/kg DM (Van Soest *et al*. 1991).

Two fibrolytic enzymes namely cellulase (CE; E.C. 3.2.1.4, Dyadic International, Inc., Jupiter, FL, USA) with activity of 115000 to 140000 cellulase units/g and xylanase (XY; E.C. 3.2.1.8, Dyadic International, Inc., Jupiter, FL, USA) with activity of 34000 to 41000 xylanase units/g [in a preliminary study activity of enzymes was assayed following the procedures of Wood and Bhatt (1988)] were evaluated separately and as a combination between cellulase and xylanase (1:1 w/w). Four different doses of each enzymes were evaluated [50 (CET1, XYT1 and CET1+XYT1), 100 (CET2, XYT2 and CET2+XYT2), 150 (CET3, XYT3 and CET3+XYT3) and 200 (CET4, XYT4 and CET4+XYT4) µL] along with control (C, control).

In vitro gas production technique

In vitro fermentation procedure and preparation of buffer and mineral solutions were done according to the procedures demonstrated by Menke and Steingas (1988). Samples (500 mg) of substrate were accurately weighted into glass bottles (120 mL) and supplemented with previously mentioned four doses of diluted enzyme and kept for the pre-incubation for 24 h. For the *in vitro* incubation procedure, the medium of 1 l volume was prepared with 2.5 g of tryptone (Sigma-Aldrich, Co., 3050 Spruce Street, St. Louis, MO, USA) dissolved in 500 mL distilled water, 0.125 mL of micro mineral solution, 250 mL of buffer solution and 1.25 mL of 0.1% (w/v) resazurin (Fluka AG, CH-9470 Buchs, Switzerland) solution. The medium was mixed in a container which kept in a water bath (39 ˚C) while bubbling CO_2 through the solution for 45 minutes. L-cysteine hydrochloride (0.313 g) (Sigma-Aldrich, Co., 3050 Spruce Street, St. Louis, MO, USA) and sodium sulphide (0.313 g) (Park Scientific Limited, Northampton, UK) were directly added to the medium and further bubbled with CO_2 for 15 min. At this point rumen fluid was collected from two donor heifers, maintained on natural grazing at the farm of the Faculty of Agriculture, University of Ruhuna, Kaburupitiya, Sri Lanka through an esophageal suction method. Collected rumen fluid was transferred to a pre-warmed flask and strained through four layer cheese cloth. All the laboratory handlings of rumen fluid were carried out under continuous flow of CO_2 and 39 ˚C of temperature. Prepared rumen fluid was added to the medium in a ratio of 1:4 (rumen fluid: medium) and flushing of CO_2 was continued until the solution turned to grey or clear, after which 42 mL of medium were pipetted into each incubation bottle, containing the pre-incubated substrate and the bottles were immediately crimp sealed with a rubber stopper and placed in the water bath with shaker at 39 ˚C.

The gas production was recorded at 4 h intervals for 24 h incubation period. After 24 h, bottles were removed from the shaker and placed on ice to terminate the reaction. Remaining solid portions were separately prepared to determine *in vitro* rumen dry matter disappearance (IVRDMD) while the aliquots of the filtrates were stored at 20 ˚C until analyzed for NH_3-N, protozoa [by mixing 1 mL of the filtrate with a 1 mL of 40% (w/v) formaldehyde] and VFA (by centrifuging 5 mL of aliquot at 1500×g for 10 min).

Chemical analysis

At the end of 24 h incubation, solid portions were separately analyzed to determine IVRDMD with oven dry method (55 ˚C, 48 hours). Liquid portion was analyzed for NH_3-N (Kjeltec System 1002, Tecator AB, Hoganas, Sweden) (AOAC, 1990) and protozoa were counted with Burker type hemocytometre (0.1 and 0.02 mm depth, respectively; Blau Brandw, Wertheim, Germany). Triplicate preparations of each sample were counted. Volatile fatty acid (VFA) were quantified by using high performance liquid chromatography (HPLC) (Model 5890, Series II, Avondale, PA, USA).

Experimental design and statistical analysis

Experiment was carried out in a randomized complete block design with three replicates for each treatment per run in three consecutive runs.

Gas production data were subjected to regression analysis (generalized linear Models) with SPSS (2011) to define the relationship between enzyme dose and the gas production. Data of IVRDMD, NH_3-N production, protozoan count and VFA were subjected to standard analysis of variance using the general linear model of SPSS. Significance between individual means was identified using LSD test. The significance of means were considered at ($P<0.05$). Descriptive analysis was done using microsoft excel 2010 version.

RESULTS AND DISCUSSION

In vitro gas production and rumen dry matter disappearance

According to the gas production profiles (Figure 1), IVGP was linearly inclined for the first four hours after which the rate decreased, but was in a linear manner. IVGP and IVRDMD (Table 1) were significantly higher ($P<0.05$) in all enzyme treated samples than the control.

Rumen NH_3-N production

Rumen NH_3-N synthesis was vary among enzyme treatments proclaiming the XY and the combination of CE + XY as the most potential enzymes for significant enhancements ($P<0.05$) of NH_3-N then followed by CE and Control (Table 1).

Rumen protozoa population

The results of the performed study revealed that increasing doses of added fibrolytic enzymes suppressed the rumen protozoan population with CE, XY and CE + XY even in some instances there were some increments (XYT4, CET3+XYT3), but the results seemed to be less consistent and insignificant (Table 1).

VFA profile

Total VFA and individual profiles of acetate, propionate, butyrate and acetate: propionate ratios in percentage are comprised in Table 2. Total VFA did not show any significant difference due to the enzyme supplementation. With the supplementation of CE + XY significant reduction of acetate and insignificant enhancements of propionate were observed. Significant reduction of butyrate was only gained with CE + XY. In some instances CE and CE + XY resulted in reduced acetate: propionate significantly. The butyrate production in response to CE was declined slightly and enhanced with CE + XY. Significant reduction of acetate: propionate ratio was observed with both CE and CE + XY.

In vitro gas production and rumen dry matter disappearance

Carbohydrates are fermented to volatile fatty acids, microbial cells and gases as end products. The IVGP is worthy of discussion as it helps to better quantify nutrients utilization hence can be a good indicator of digestibility, fermentability, and microbial protein production (Sommart *et al.* 2000). Referring to the Figure 1, it could be assumed that accelerated initial IVGP is due to the stimulation of initial phase degradation of substrate (Giraldo *et al.* 2008). Furthermore, it would be the most likely mode of action of enzyme and also due to the readily soluble fraction of the substrate.

Giraldo *et al.* (2008) stated that prolonged gas production trial would not make a significant effect of enzyme supplementation on fermentation parameters. This result supports the findings of previous works that most active period for a fibrolytic enzymes mixture appears up to first 12 hours of incubation (Moreno *et al.* 2007) and more extensively the action is declined drastically after 6th hour of incubation.

Based on the strong relationship between measured rumen dry matter disappearance and the gas production, regression equations have been developed and the method has been standardized (Chumpawadee *et al.* 2005). Enhanced IVRDMD should be the result of improved fiber digestibility mainly xylose (fraction of hemicellulose) and cellulose with the action of enzymes. As suggested by a previous study of Colombatto and Beauchemin (2003), enzymes could enhance IVRDMD by removing structural barriers and facilitating microbial colonization resulting increased rate of degradation, which is consistent with current research results. Through the improved IVRDMD, it can be suggested that the addition of XY enzyme promoted the release of reducing sugars, xylan with more considerably higher amounts when compared to cellulose which comparatively had low released amounts. However the lower significance associated with CE does not mean that the CE was ineffective against cellulose fraction in substrate.

Klyosov (1990) detailed the mechanical action of cellulase on cellulose with the principal process of dispersion of cellulase, which happens by adsorption of cellulases to cellulose defects. Conversing the result in present study, the significance activity of XY suggests that it has a higher resistant to degradation within rumen conditions than in CE which is less stable under rumen (Morgavi *et al.* 2000).

Rumen NH_3 –N production

Improved NH_3-N production in present study is consistent with the findings of Giraldo *et al.* (2007) and Khattab *et al.* (2011). In contrast, several *in vivo* (Beauchemin *et al.* 2003; Giraldo *et al.* 2008) and *in vitro* (Wang *et al.* 2001) researches proclaimed no effect of fibrolytic enzyme supplementation on rumen NH_3-N synthesize. As theory implies the level of ammonia in rumen liquor is an indicator of nutritional conditions. According to the existing body of knowledge, dietary protein is fermented in the rumen to simpler N compounds and re-incorporated; primarily as NH_3-N which acts as an indicator of microbial nitrogen synthesis.

As NH_3-N is the primary N source of most rumen organisms, increased NH_3-N could be resulted in improved microbial activities (Seresinhe *et al.* 2012). As several research have demonstrated high levels of rumen ammonia facilitated the mircobial activity in the rumen and accordingly protein and carbohydrate digestion will be improved.

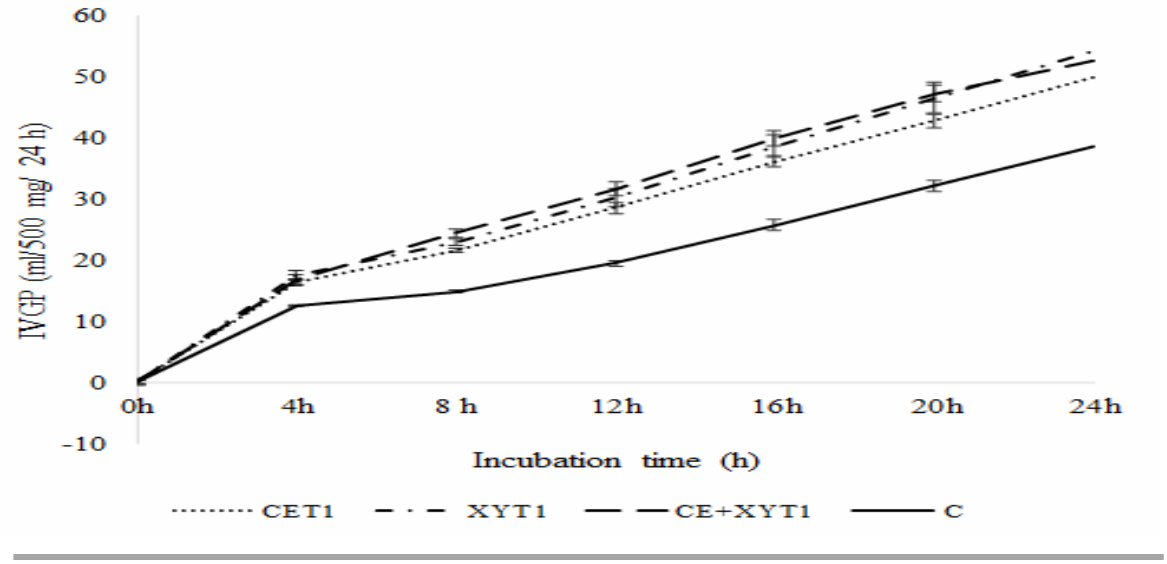

Figure 1 *In vitro* gas production profile of guinea grass (*Panicum maximum*) in response to the enzyme treatments in 24 h incubation time
CE: cellulose; XY: xylanase; CE + XY: cellulase + xylanase; C: control; CE1, XY1, CE1 + XY1: 50 µL

Table 1 Influence of cellulase (CE), xylanase (XY) and combination of cellulase and xylanase (CE+XY) enzyme supplementation on *in vitro* gas production (IVGP), rumen dry matter disappearance (IVRDMD), NH_3-N production and protozoan population after 24 h incubation

Treatment[1]	IVGP (mL/500 mg DM)[2]	IVRDMD %	NH_3-N %	Protozoa %
Control	38.54±0.92^{c}	46.78±0.43^{b}	100.00±0.00^{c}	100.00±25.8
CET1	50.06±1.11^{b}	51.21±2.00^{a}	102.14±6.35^{c}	49.84±36.00
CET2	52.11±0.87^{b}	50.29±1.45^{a}	102.72±4.52^{c}	69.96±26.00
CET3	52.11±2.51^{b}	50.85±2.34^{a}	101.84±3.22^{c}	90.09±75.00
CET4	56.39±0.14^{b}	50.35±0.27^{a}	106.78±3.24^{b}	80.18±9.00
XYT1	54.27±1.74^{b}	51.53±1.02^{a}	108.88±6.78^{b}	75.00±14.00
XYT2	58.27±1.76^{ba}	52.96±0.27^{a}	113.03±7.17^{b}	66.67±8.00
XYT3	60.28±1.99^{b}	52.13±4.82^{a}	112.46±2.58^{b}	91.67±8.00
XYT4	67.46±2.13^{a}	53.29±0.56^{a}	122.62±3.49^{a}	125.00±38.00
CET1 + XYT1	52.77±0.73^{b}	52.64±0.00^{a}	111.6±3.14^{b}	49.62±28.00
CET2 + XYT2	54.10±0.86^{b}	50.62±0.02^{a}	109.28±4.64^{b}	66.16±43.00
CET3 + XYT3	58.77±0.97^{b}	50.04±0.01^{a}	113.39±6.64^{b}	132.94±66.00
CET4 + XYT4	61.99±0.91^{b}	51.91±0.01^{a}	114.87±5.65^{b}	99.25±57.00

[1] CET1 - CET4: cellulase enzyme treatment 1 to 4; XYT1 - XYT4: xylanase enzyme treatment 1 to 4; CET1 + XYT1 - CET4 + XYT4: cellulase + xylanase treatment 1 to 4; 1, 50; 2, 100; 3, 150 and 4, 200 µL of enzyme.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Table 2 Volatile fatty acid (VFA) production of *Panicum maximum* in response to the supplementation of CE, XY and CE + XY at 24 h incubation time

Treatment[1]	Total VFA (m*M*)	Individual VFA profile (mol/100 mol)			A/P[2]
		Acetate	Propionate	Butyrate	
Control	108.30±3.30	66.56±0.51^{a}	25.03±0.43^{b}	8.23±0.51b	2.67±0.10^{b}
CET1	112.20±5.10	63.09±0.32	28.97±0.39	7.56±0.18	2.18±0.03^{b}
CET2	113.50±5.93	63.33±0.06	29.08±0.48	7.24±0.041	2.18±0.03^{b}
CET3	114.00±6.40	64.86±0.49	21.04±0.08^{c}	8.85±0.64	3.08±0.03^{a}
CET4	115.60±5.57	61.49±1.09^{b}	30.00±0.46	7.68±0.44	2.05±0.06^{b}
XYT1	114.90±3.70	66.04±1.28	23.63±1.83	10.32±0.68	2.83±0.27
XYT2	116.40±4.61	65.39±1.96	25.71±1.92	8.89±0.29	2.58±0.28
XYT3	113.00±4.53	68.01±0.33	26.24±2.47	5.75±2.56^{c}	2.64±0.26
XYT4	112.30±9.43	67.79±3.64	22.35±1.27	9.85±2.42^{b}	3.07±0.34^{a}
CET1 + XYT1	105.20±2.24	55.98±0.29^{c}	29.43±2.89	14.26±2.34^{a}	1.93±0.2^{c}
CET2 + XYT2	106.70±3.46	59.52±1.75^{c}	25.2±0.07	15.27±1.83^{a}	2.36±0.06^{b}
CET3 + XYT3	109.30±3.21	57.01±2.92^{c}	27.02±2.39	15.86±0.6^{a}	2.14±0.29^{b}
CET4 + XYT4	108.10±1.78	55.78±3.34^{c}	27.16±1.67	16.68±2.29^{a}	2.07±0.25^{b}

[1] CET1 - CET4: cellulase enzyme treatment 1 to 4; XYT1 - XYT4: xylanase enzyme treatment 1 to 4; CET1 + XYT1 - CET4 + XYT4: cellulase + xylanase treatment 1 to 4; 1, 50; 2, 100; 3, 150 and 4, 200 µL of enzyme.
[2] A/P: acetate/propionate.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).

Rumen protozoa population

Addition of fibrolytic enzymes suppressed the rumen protozoan population. It is known that protozoa represents large proportion of rumen microbial population and have an important role in rumen fermentation process by providing a considerable cellulolytic effect and pre-determining activity of bacteria. In many studies researchers found that supplementation of exogenous fibrolytic enzymes can affect both on the number of microorganisms and the composition of microbial communities in the rumen (Wang *et al*. 2001). Also Chung *et al.* (2012) reported no effect of dietary addition of enzymes on protozoan population. However, to date there are only few studies investigating the effect of the exogenous enzymes on the protozoan population and their results are uncertain.

VFA profile

Addition of CE and CE + XY resulted in enhanced propionate production and reduced acetate production which were enough to lower the acetate: propionate significantly. Generally this is an indicator of the improved nutritional value of substrate which resulted from altered growth rates of bacteria. The effects of enzyme supplementation on rumen VFA production seem to be inconsistent (Eun *at al.* 2007) in the literature, even some studies yield increased VFA with enzyme treatments Gado *et al*. (2009); Giraldo *et al.* (2007) reported an increase in the proportion of acetate in rumen fluid by fibrolytic enzymes; whereas, Yang *et al.* (1999) and Eun and Beauchemin (2007) reported no effect of fibrolytic enzymes on rumen VFA profile. Tricario and Dawson (2005) experienced that addition of xylanase and endoglucanase increased total VFA production from the fescue hay-based diet without changing the acetate to propionate ratio.

In another work, supplementation of exogenous fibrolytic enzymes to Guinea grass hay caused higher acetate and lower propionate and butyrate productions (Avellaneda *et al.* 2009). Changes in VFA proportions as a direct effect of adding exogenous fibrolytic enzymes have been reported, implying that these enzymes could affect microbial growth and / or shift the metabolic pathways by which specific microbes utilize substrates (Eun and Beauchemin, 2008).

CONCLUSION

Supplementation of exogenous fibrolytic especially the xylanase enzyme enhances rumen fermentation parameters such as *in vitro* gas production, *in vitro* rumen dry matter disappearance, rumen ammonia nitrogen production and volatile fatty acid production even though the results on rumen protozoa and volatile fatty acid seem inconsistent.

Therefore, as mirrored by results, authors would like to recommend xylanase as the enzyme with the optimum effect. However further studies, especially considering feed-specific activity of enzyme are highly recommended.

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