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Supplementation of rice straw (*Oryza sativa*) with exogenous fibrolytic enzymes improves in vitro rumen fermentation characteristics

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Abstract: A study was conducted to evaluate the use of exogenous enzymes as a potential means of improving the cell wall degradation of rice straw. The enzymes used for in vitro incubations were characterized for cellulase (CE) and xylanase (XY). Enzymes were supplemented alone (CE or XY) and as a mixture (CE + XY) in a 1:1 ratio. Five supplementation levels of enzymes including no enzyme (control) were used with 500 mg of ground (1 mm) substrate (rice straw) dry matter. Anaerobic buffer medium and strained ruminal fluid were added to the in vitro incubations in triplicate, and in vitro gas production (IVGP), in vitro rumen dry matter disappearance (IVRDMD), ruminal ammonia nitrogen (NH3-N), and volatile fatty acid (VFA) were determined. All enzyme treatments significantly increased ($P < 0.05$) IVGP and IVRDMD regardless of enzyme. The total NH_3-N in fermentation liquid significantly increased under XY and CE4 (30.65 units of cellulase), and no differences were observed for CE + XY. No significant differences were observed for molar proportions of individual VFA, acetate:propionate ratio, or nonglucogenic ratio. The results reveal that use of fibrolytic enzymes is an effective way to improve the ruminal fermentation characteristics of rice straw.

Key words: Ammonia nitrogen, cellulase, in vitro gas production, rumen dry matter disappearance, volatile fatty acid

1. Introduction

Rice is the most extensively grown cereal crop, the staple food in Sri Lanka, and an important traditional feed source for ruminants, providing a potential solution for dry season feed stress in the livestock industry (1). In Sri Lanka a large proportion of the harvested rice straw is used in the paper industry, and a small quantity of straw is used for the feeding and bedding of cattle and buffaloes; most of the straw is either ploughed in or burned directly on the field, which causes environmental pollution (2). Feeding rice straw to ruminants is constrained by several factors including low digestibility, low protein, low palatability, high crude fiber (3), and high silica content (5%–15%), which acts as an antinutrient. The poor fermentation characteristics of rice straw lead to low rates of rumen disappearance and low passage, thus reducing feed intake (4). With the emerging concerns regarding health issues stemming from animal-based food sources, interest in biological treatment methods instead of physical and chemical methods is growing. Several studies have reported that fibrolytic enzyme supplementation could increase dry matter intake, production performance, and nutrient digestibility of fibrous feeds (5–7). Despite the large number of studies conducted, most have focused on temperate feed stuffs with little attention given to tropical feed resources, especially cereal straw as from rice. For this reason, and to determine enzyme-feed specificity it is crucial to test the performance of rice straw under enzyme supplementation (5). This research was conducted to investigate the potential of fibrolytic enzyme supplementation with rice straw (*Oryza sativa*) on rumen fermentation parameters.

2. Materials and methods

2.1. Substrate preparation

The substrate (rice straw) was prepared by oven drying (55 °C, 48 h) and grinding to pass through a screen (1 mm). Organic matter and crude protein were analyzed according to the AOAC (8), and fiber fractions were determined following methods described by Van Soest et al. (9). Five hundred milligrams of substrate were used as the sample.

2.2. Enzymes and doses

The enzyme products were characterized for the activity of cellulase (CE; E.C. 3.2.1.4, Dyadic International, Inc., Jupiter, FL, USA) at 115,000–140,000 cellulase units/g and xylanase (XY; E.C. 3.2.1.8, Dyadic International, Inc., Jupiter, FL, USA) at 34,000–41,000 xylanase units/g. Supplementation levels were as follows: 0 units (control),

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7.65 units (CE1), 15.30 units (CE2), 22.95 units (CE3), 30.60 units (CE4), 2.25 units (XY1), 4.5 units (XY2), 6.75 units (XY3), and 9.0 units (XY4) and 3.825 + 1.125 units (CE1 + XY1), 7.65 + 2.25 units (CE2 + XY2), 11.475 + 3.375 units (CE3 + XY3), and 15.30 + 4.50 units (CE4 + XY4).

2.3. In vitro incubation

In vitro fermentation procedures and preparation of buffer and mineral solutions were according to Menke and Steingas (10). Samples (500 mg) of substrate were accurately weighed into glass bottles (120 mL), supplemented with the previously mentioned levels of enzymes, and preincubated for 24 h. For the in vitro incubation procedure, 1 L of medium was prepared with 2.5 g of tryptone (Sigma-Aldrich, St Louis, MO, USA) dissolved in 500 mL of distilled water, 0.125 mL of micromineral 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 kept in a water bath (39 °C) as CO_2 was bubbled through the solution for 45 min. L-cysteine hydrochloride (0.313 g) (Sigma-Aldrich) and sodium sulfide (0.313 g) (Park Scientific Limited, Northampton, UK) were added directly to the medium, and it was bubbled with CO_2 for 15 min. At this point rumen fluid was collected from two donor heifers maintained with natural grazing at the farm of the Faculty of Agriculture, University of Ruhuna, Kaburupitiya, Sri Lanka, through esophageal suction. Collected rumen fluid was transferred to a prewarmed flask and strained through four layers of cheese cloth. All laboratory handling of rumen fluid was carried out under a continuous flow of CO_2 at 39 °C. Prepared rumen fluid was added to the medium in a ratio of 1:4 (rumen fluid:medium), and CO_2 flushing was continued until the solution turned gray or clear, after which 42 mL of medium were pipetted into incubation bottles containing the preincubated substrate. The bottles were immediately crimp-sealed with a rubber stopper and placed in a water bath with a shaker at 39 °C (10).

The gas production was recorded at 4-h intervals over a 24-h incubation period. After 24 h the remaining solid portions were separately prepared to determine IVRDMD, while the aliquots of the filtrates were stored at 20 °C until analyzed for NH_3-N and VFA by centrifuging 5 mL of aliquot at 2000 × *g* for 10 min.

2.4. Experimental design and chemical and statistical analysis

The series of experiments was conducted in triplicate with a randomized complete block design (the same experiment was repeated three times and was blocked across the time period).

At 4-h intervals IVGP was measured with a calibrated glass syringe, and IVRDMD (oven dry, 55 °C, 48 h) and NH_3-N were determined with the Kjeldahl method (Kjeldahl, Distillation Unit 1002) (11). Gas chromatography (HPLC) (Model 5890, Series II, Avondale, PA, USA) was used to determine the VFA.

The nonglucogenic ratio (NGR) was estimated by using the following equation developed by Orskov et al. (11):

$$NGR = \frac{A + (2B)}{P}$$, where *A*, *P*, and *B* are moles of acetate, propionate, and butyrate, respectively.

Values are given as means ± SEM. All the parameters were subjected to standard analysis of variance (ANOVA) using the general linear model (GLM) of IBM SPSS 20 (IBM Cooperation, Somers, NY, USA). Significance between individual means was identified by the Bonferroni test. The significance of means was considered at $P < 0.05$. Descriptive analysis was done using Microsoft Excel, 2013 version.

3. Results

3.1. Proximate composition of rice straw

Substrate rice straw consisted of 88.30 ± 0.68% dry matter (DM) (fed basis), 3.90 ± 0.14% crude protein (DM basis), 70.30 ± 0.52% neutral detergent fiber (DM basis), and 40.20 ± 0.46% acid detergent fiber (DM basis).

3.2. In vitro gas production, in vitro rumen dry matter disappearance, and ammonia nitrogen

Cumulative IVGP, IVRDMD, and NH_3-N in rice straw supplemented with CE, XY, and CE + XY are shown in Table 1. All enzymatic treatments (except for CE1) enhanced the IVGP significantly ($P < 0.05$) when compared with the control, indicating active microbial fermentation. The highest IVGP was for XY supplementation followed by CE + XY and CE. The IVGP increased with increasing doses of enzymes.

IVRDMD was significantly enhanced compared to the control, whereas the highest IVDMD value was obtained with XY supplementation followed by CE + XY and CE.

NH_3-N production in rice straw with supplementation of XY was significantly enhanced, while only the highest dose of CE caused significant enhancement; CE + XY failed to exhibit any marked difference although numerical values were higher than the control value.

3.3. Volatile fatty acid

At the end of the 24-h incubation period the molar proportion of acetate, propionate, and butyrate remained unchanged in all enzyme treatments. Molar proportion of acetate was somewhat higher with CE treatment, and a slight reduction was perceived with XY and CE + XY. However, the decrease in acetate from XY and CE + XY was not enough to alter the acetate:propionate (A:P), which was similar across treatments. Very minor increases in

Table 1. In vitro cumulative gas production (IVGP), in vitro rumen dry matter disappearance (IVRDMD), and ammonia nitrogen (NH_3-N) of rice straw in response to supplementation with enzymes CE, XY, and CE + XY at different levels and 24-h incubation time.

Supplementation level	IVGP	IVRDMD%	NH_3-N (mg/L)
Control	28.21 ± 1.56^{a}	33.04 ± 0.90^{a}	438.24 ± 8.23^{a}
CE1	30.61 ± 0.47^{a}	36.20 ± 0.81^{b}	454.60 ± 1.20^{a}
CE2	37.11 ± 0.24^{b}	36.33 ± 0.75^{b}	461.20 ± 7.90^{a}
CE3	42.44 ± 2.39^{c}	36.69 ± 0.12^{b}	460.26 ± 12.53^{a}
CE4	48.16 ± 0.69^{d}	37.20 ± 0.20^{b}	482.67 ± 13.53^{b}
XY1	40.06 ± 2.19^{b}	36.31 ± 0.02^{b}	493.00 ± 27.20^{b}
XY2	46.05 ± 2.14^{c}	38.09 ± 1.04^{bc}	505.46 ± 26.14^{b}
XY3	48.77 ± 2.24^{cd}	38.82 ± 1.16^{bc}	510.00 ± 3.40^{b}
XY4	53.00 ± 2.40^{d}	39.16 ± 0.21^{c}	549.00 ± 12.77^{b}
CE1 + XY1	40.83 ± 0.53^{b}	37.58 ± 0.01^{b}	451.06 ± 30.43^{a}
CE2 + XY2	45.52 ± 2.31^{c}	37.76 ± 0.74^{bc}	443.13 ± 9.68^{a}
CE3 + XY3	48.05 ± 2.45^{cd}	38.29 ± 0.51^{c}	439.73 ± 4.08^{a}
CE4 + XY4	50.16 ± 1.09^{d}	36.89 ± 0.12^{b}	473.73 ± 4.53^{a}

Values are means of nine replicates of three runs ±SE. Means with different superscripts (a, b, c, and d) are significantly different ($P < 0.05$).
0 units (control), 7.65 units (CE1), 15.30 units (CE2), 22.95 units (CE3), 30.60 units (CE4), 2.25 units (XY1), 4.5 units (XY2), 6.75 units (XY3), and 9.0 units (XY4); 3.825 + 1.125 units (CE1 + XY1), 7.65 + 2.25 units (CE2 + XY2), 11.475 + 3.375 units (CE3 + XY3), and 15.30 + 4.50 units (CE4 + XY4).

propionate were experienced with CE and CE + XY, while XY supplementation caused an insignificant reduction compared with the control. With the effect of XY and CE + XY butyrate production was encouraged, although it was not a noticeable increment.

4. Discussion

4.1. In vitro gas production, in vitro rumen dry matter disappearance, and ammonia nitrogen

In agreement with the present results, Yang et al. (5) reported increased IVGP for rice straw supplemented with fibrolytic enzymes, while the same results were observed by Jalivaland et al. (6) when fibrolytic enzymes were added to wheat straw. In results similar to the present findings, Liu and Orskov (12) observed elevated IVGP in steam-treated rice straw supplemented with nonstarch polysaccharide enzyme. Although it is assumed that enzyme activity is suppressed after the initial phase of incubation, IVGP was higher with enzyme supplementation than in the control, which may be due to increased adsorption of enzymes to the feed during preincubation; this could increase the resistance of the enzymes to proteolysis and prolong their viability in the rumen.

Gas volume is a good parameter for predicting digestibility, VFA production, and microbial protein synthesis. As documented in previous research, IVRDMD has a high correlation with gas production (13). Enhanced IVRDMD may be due to the release of reducing sugars with direct application of enzymes to the substrate (14), partial solubility of neutral detergent fiber and acid detergent fiber (15), and better absorption of the enzyme during preincubation as it is an important prerequisite for hydrolysis (16). Forsberg et al. (16) demonstrated that release of soluble sugars would provide sufficient additional available carbohydrates to encourage rapid microbial growth, shortening the lag-time required for microbial colonization and suggesting that exogenous enzymes caused structural changes to forages that improved digestion. Yang et al. (5) and Bhasker et al. (17) confirmed these results, showing increased IVRDMD in cereal straw supplemented with cellulase enzyme and a mixture of the enzymes cellulase, hemicellulase, protease, and amylase.

Ammonia arising in the rumen is one of the end products of bacterial degradation of feedstuff protein and may be used to synthesize bacterial protein. During

Table 2. Volatile fatty acids (mol/100 mol) and stoichiometric derivations of NGR of rice straw (*Oryza sativa*) in response to supplementation with CE, XY, and CE + XY after a 24-h incubation period.

Supplementation level	A (mol/100 mol)	P (mol/100 mol)	B (mol/100 mol)	A/P (mol/100 mol)	NGR
Control	63.87 ± 2.02	24.47 ± 0.73	10.30 ± 2.48	2.66 ± 0.16	3.52 ± 0.31
CE1	65.13 ± 1.15	27.73 ± 0.96	6.82 ± 0.83	2.35 ± 0.11	2.85 ± 0.12
CE2	64.77 ± 2.03	27.67 ± 0.93	7.88 ± 1.25	2.35 ± 0.14	2.91 ± 0.09
CE3	64.98 ± 0.15	31.48 ± 4.01	6.04 ± 0.59	1.63 ± 0.70	2.61 ± 0.26
CE4	63.94 ± 2.17	26.88 ± 1.46	7.65 ± 0.32	2.40 ± 0.20	2.97 ± 0.19
XY1	62.37 ± 1.04	25.88 ± 1.81	11.74 ± 1.39	2.43 ± 0.21	3.36 ± 0.36
XY2	62.78 ± 1.67	24.51 ± 2.13	12.7 ± 1.91	2.60 ± 0.27	3.67 ± 0.45
XY3	63.79 ± 3.00	25.84 ± 3.46	10.35 ± 2.92	2.59 ± 0.47	3.47 ± 0.77
XY4	59.65 ± 3.07	22.55 ± 3.07	17.79 ± 6.14	2.69 ± 0.23	4.42 ± 1.24
CE1 + XY1	60.50 ± 0.71	27.04 ± 1.57	12.77 ± 0.64	2.25 ± 0.15	3.20 ± 0.31
CE2 + XY2	59.13 ± 1.26	28.93 ± 0.13	11.94 ± 1.13	2.04 ± 0.05	2.86 ± 0.02
CE3 + XY3	59.26 ± 0.43	26.33 ± 1.13	14.83 ± 1.59	2.25 ± 0.08	3.35 ± 0.30
CE4 + XY4	61.27 ± 0.30	24.19 ± 1.29	14.53 ± 1.80	2.54 ± 0.12	3.76 ± 0.39

Values are means of nine replicates of three runs ±SE.
A = acetate, P = propionate, B = butyrate, A/P = acetate/propionate, NGR = non-glucogenic ratio;
0 units (control), 7.65 units (CE1), 15.30 units (CE2), 22.95 units (CE3), 30.60 units (CE4), 2.25 units (XY1), 4.5 units (XY2), 6.75 units (XY3), and 9.0 units (XY4); 3.825 + 1.125 units (CE1 + XY1), 7.65 + 2.25 units (CE2 + XY2), 11.475 + 3.375 units (CE3 + XY3), and 15.30 + 4.50 units (CE4 + XY4).

ruminal digestion, microbes multiply and synthesize considerable amounts of microbial crude protein (MCP) using protein or nonprotein nitrogen (NPN) as a source of N which is provided with the diet and incorporated as NH_3-N; it is the principle N-source for ruminal microbes (18). Studies in vivo (19) and in vitro (20) found no effect of fibrolytic enzyme supplementation on ruminal NH_3-N production, while other studies showed a reduction in NH_3-N production compared to the control (21). Ammonia N concentrations required for ruminal bacteria have been estimated in several types of studies. Optimal concentrations range from 29 to 35 mg/dL (22); approximately 290 to 350 mg/L. In the present study, NH_3-N values deviate from the optimal concentration range (control: 438.24 mg/L). Girlado et al. (23) acquired 250 to 270 mg/L NH_3-N, values lower than the results in Satter and Slyter (22), with no significant differences between the control and treatments. It can be assumed that supplementation of enzymes, more specifically XY, encouraged the colonization and proliferation of the ammonia-producing bacteria.

4.2. Volatile fatty acid production

The VFA profile is inconsistent, and this is in agreement with previous studies where no major differences in VFA profile were observed (5) in cereal-straw–based diets. Although the reduction in the A:P ratio was insignificant, it generally reflects the improved nutritive value of food, thus indicating enhancement of propionate, the important precursor of glucose synthesis (24). Nonglucogenic ratio (NGR), which mirrors the utilization of VFA, is presented in Table 2. According to the literature the optimal NGR ratio for better VFA utilization is around 3.5; higher values indicate suboptimal use of VFA (25). In the current work, almost all NGR values were below 3.5, which is lower than the control (3.52) and indicates optimum utilization of VFA.

The results of the present study show that exogenous fibrolytic enzymes, more specifically xylanase, have benefits for utilization of rice straw through in vitro gas production, in vitro rumen dry matter disappearance, and ammonia nitrogen production. However, the effects on volatile fatty acid production seem indistinct, suggesting a topic for future research.

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