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Influence of cellulase or xylanase on the *in vitro* rumen gas production and fermentation of corn stover

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ABSTRACT

In vitro gas production (GP) technique was used to investigate effect of exogenous enzymes cellulase (CEL) or xylanase (XYL) at different doses on *in vitro* fermentation characteristics of corn stover. Enzymes were supplemented at 0 (control), 10, 20, 40 and 80 µg/g DM. Gas production was determined at 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 h of incubation. After 72 h, the incubation was stopped and supernatant pH was determined, and filtered to determine dry matter (DMD), neutral detergent fiber (NDFD) and acid detergent fiber (ADFD) degradabilities. Interaction effects occurred for enzyme type and dose for all measured gas parameters with exception of the lag time, DMD, organic matter degradability (OMD), NDFD, metabolizable energy (ME), short chain fatty acids (SCFA) and microbial crude protein (MCP) production. Cellulase and XYL increased GP ($P<0.05$) at different incubation hours with better results at the dose of 40 µg/g DM. The dose 80 µg XYL/g DM had the lowest GP compared to other doses. In addition, CEL and XYL decreased pH with increasing OMD, ME, SCFA and MCP production at 40 µg/g DM of corn stover. The present results suggested that the level of CEL and XYL at 40 µg/g DM have higher GP than other levels of enzymes, imply this level can be more effectively to improve rumen fermentation; however, the difference of XYL between treatments and control was less than that of CEL.

Key words: Cellulase, Corn straw, Fibrolytic enzyme, *In vitro* fermentation, Xylanase

Roughages are the main sources of feed for ruminants (Kholif *et al*. 2014). Fibrous feeds are characterized by low nutritive value. High fiber of fibrous feeds prevents the access of ruminal enzymes to the plant cell wall and reduce nutrient digestibility (Abdel-Aziz *et al*. 2015, Elghandour *et al*. 2015, Togtokhbayar*et al*. 2015). High fiber content and plant epidermal surface with a high concentration of silica prevents the access of ruminal enzymes to the plant cell wall and reduce nutrient digestibility (Khattab *et al*. 2013, Kholif *et al*. 2014). Corn stover is one of the agro-byproducts and can be used in ruminant feeding after upgrading its nutritive value (Elghandour *et al*. 2014). Hence, there is a need to develop feeding strategies that improve the nutritive value of such fibrous feeds. Using fibrolytic enzymes (e.g. cellulase, xylanase) for this purpose will prove effective.

Exogenous fibrolytic enzymes are used to improve carbohydrate and cell wall degradation of low quality feeds (Alsersy *et al*. 2015, Salem *et al*. 2015a,Valdes *et al*. 2015). The modes of actions proposed to explain the improved nutritive value of low quality feeds with high fiber contents are: enhanced attachment by rumen microorganisms (Nsereko *et al*. 2002), improving ruminal fermentation working synergetically with endogenous rumen microbial enzymes (Khattab *et al*. 2011), creation of a stable complexes between enzymes and feeds (Kung *et al*. 2000), and possible alteration in the fiber structure stimulating microbial colonization (Giraldo *et al*. 2004). The aim of the current experiment is to investigate the effect of adding increasing doses of cellulase (CEL) or xylanase (XYL) on *in vitro* fermentation kinetics and gas production (GP) of corn stover.

MATERIALS AND METHODS

Corn stover and enzyme products: Corn stover with chemical composition (g/kg DM): 959.7 organic matter (OM), 62.9 crude protein (CP), 476.7 neutral detergent fiber (NDF) and 274.4 acid detergent fiber (ADF) was used as incubation substrate. Corn stover samples were dried at 65 °C for 48 h in a forced air oven until constant weight and then ground in a Wiley mill to pass a 1 mm sieve and stored in plastic bags for subsequent determination of chemical composition and *in vitro* GP. Cellulase (CEL) and xylanase (XYL) enzymes in liquid form were tested at 0, 10, 20, 40

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and 80 µg/g DM corn stover. A stock solution of each enzyme was prepared for each treatment (10, 20, 40 and 80 mg/L), so that the intended concentration in the fermentation cultures was achieved by dispensing 1 ml of each stock solution in each serum bottle topping the sample of feed. Activities of the CEL product were 30,000 to 36,000 units of cellulase/g and 7500 to 10,000 units of ß-glucanase/g while activities of XYL were 34,000 to 41,000 units of xylanase/g, from 12,000 to 15,000 units of beta-glucanase/g and 45,000 to 55,000 units of cellulase/g. Enzyme activities were provided by manufacturer.

In vitro *incubations:* Rumen inoculum was collected before morning feeding from a Brown Swiss cow (450 kg body weight) fitted with permanent rumen cannula and fed *ad lib.* a total mixed ration made up of 1:1 concentrate and alfalfa hay, formulated to cover its nutrient requirements (NRC 2001) with a full access to fresh water.

Obtained ruminal contents were flushed with CO_2, mixed and strained through 4 layers of cheese cloth into a flask with O_2-free headspace. Corn stover (1 g) was weighed into 120 ml serum bottles with appropriate addition of enzyme doses/g DM, applied immediately before incubation with rumen buffered solution. Consequently, 10 ml of particle free ruminal fluid was added to each bottle followed by 40 ml of the buffer solution of Goering and Van Soest (1970), with no trypticase added, in a 1:4 (v/v) proportion.

Bottles (90) were used during the incubation runs (2 enzymes × 5 doses × 3 replicates × 3 runs) plus 3 bottles as blanks (i.e., buffered rumen fluid only), were incubated for 72 h. After filling all bottles with substrates and inoculum medium, bottles were immediately closed with rubber stoppers, shaken and placed in the water-bath at 39 °C. The volumes of GP were recorded at times of 2, 4, 6, 8, 10, 12, 14, 24, 36, 48 and 72 h of incubation. The GP was recorded using the pressure transducer technique of Theodorou *et al.* (1994).

Nutrient degradability: At the end of incubation after 72 h, the fermentation process was stopped by swirling the bottles in ice, and the contents of each serum bottle were filtered under vacuum through glass crucibles with a sintered filter. The obtained fermentation residues were dried at 105ºC overnight to estimate DM disappearance. Both of NDF and ADF were determined in the residues after DM determinations and DM (DMD), NDF (NDFD) and ADF (ADFD) degradability were calculated. Blanks were used to correct for substrate contamination from enzyme or ruminal fluid.

Chemical analysis: Samples of the corn stover were analyzed for DM, ash and N according to AOAC (1997). The NDF and ADF content (Van Soest *et al.* 1991) of both feed and fermentation residues were determined using an fiber analyzer unit without use of an alpha amylase but with sodium sulphite. Both NDF and ADF are expressed without residual ash.

Calculations and statistical analysis: To estimate kinetic parameters of GP, gas volumes recorded (ml/g DM) were fitted using the NLIN option of SAS (2002) according to France *et al.* (2000) model as:

$$y = b \times (1 - e^{-c(t-L)})$$

where, *y*, volume of GP at time t; *b*, asymptotic GP (ml/g DM); *c*, fractional rate of fermentation (/h); and *L* (h), discrete lag time prior to any gas is released. Metabolizable energy (ME; MJ/kg DM) and *in vitro* OM digestibility (OMD; g/kg OM) were estimated according to Menke *et al*. (1979).

Short chain fatty acid concentrations (SCFA) and microbial crude protein (MCP) productions were calculated according to Getachew *et al*. (2002).

The experimental design for the *in vitro* ruminal GP, degradability and fermentation parameters analysis was a completely random design considering enzyme type as fixed factors in the linear model within each enzyme dose using the PROC GLM option of SAS (2002). Data of each of the 3 runs within the same sample were averaged prior to statistical analysis. Mean values of each individual sample were used as the experimental unit. The statistical model was: $Y_{ijk} = \mu + Z_i + D_j + (Z \times D)_{ij} + E_{ijk}$; where $Y_{ijk,}$ every observation of the *I*th enzyme when incubated in the *j*th dose; µ, general mean; Z_i (i=1–2), enzyme effect; $D_{j,}$ enzyme dose effect (j=1–5); $(Z \times D)_{ij}$, interaction between enzyme type and enzyme dose; and E_{ijk}, experimental error. Linear and quadratic polynomial contrasts were used to examine responses to increasing addition levels of the enzymes.

RESULTS AND DISCUSSION

In vitro *gas production:* With exception of the fraction *L*, interaction effects occurred (P<0.05) for enzyme type × enzyme dose, revealing that GP is enzyme preparation and enzyme dose dependent. Enzyme dose had linear effect (P<0.05) on GP at different incubation hours with quadratic effect (P=0.041) on the fraction *c* (Table 1). Addition of CEL or XYL to corn stover increased GP (P<0.05) with greater (P<0.05) effect at the dose 40 µg/g DM. The effectiveness of enzymes depends upon substrate, enzyme specificity and enzyme dose causing variable responses with different enzyme preparations and doses (Salem *et al*. 2015b). This supports the hypothesis that a suitable enzyme dose could improve fermentation efficiency. Increased GP indicated the increased fermentable material with enzyme addition (Elghandour *et al*. 2015). Increased GP without affecting lag time could be due to release of polysaccharidase from corn stover, which provided fermentable carbohydrate to stimulate microbial growth. Another possible reason is the increased numbers of fibrolytic and nonfibrolytic bacteria in the rumen, which is very clear in the current study as the MCP production increased with enzyme addition (Nsereko *et al*. 2002). Mao *et al.* (2013) observed that addition of CEL and XYL increased numbers of total bacteria and *Fibrobacte rsuccinogens* in the incubation medium with improving *in vitro* fermentation.

The dose 80 µg XYL/g DM had the lowest GP (P<0.05) compared to other doses (Table 1); however, the higher dose

Table 1. Impact of cellulase (CEL) and xylanase (XYL) at different doses on *in vitro* gas production of corn stover at different hours of incubation

Enzyme	Dose (µg/g DM)	GP parameters			*In vitro* GP, ml/g DM at:									
		b, ml/g DM	*c*, /h	*L*, h	2 h	4 h	6 h	8 h	10 h	12 h	24 h	36 h	48 h	72 h
CEL	0	213.2	0.074	3.69a	11.3c	23.8c	42.4c	65.0c	91.5c	117.9b	175.9b	203.9b	218.0b	225.7b
	10	231.5	0.070	3.28ab	15.3b	30.4b	51.2bc	74.7bc	102.1bc	129.5ab	190.7ab	221.1ab	236.8ab	246ab
	20	227.2	0.073	3.23ab	16.0b	31.9b	53.6ab	77.8ab	104.9abc	131.6ab	191.7ab	220.3ab	234.9ab	242.8ab
	40	231.1	0.080	3.13ab	19.4a	37.9a	62.3a	88.7a	117.5a	144.9a	203.9a	230.7a	244.5a	251.5a
	80	230.7	0.074	2.61b	19.4a	37.6a	60.7ab	85.3ab	112.1ab	138.1a	196.4a	223.1ab	237.4ab	245.2ab
P value	Linear	0.067	0.939	0.055	0.001	0.001	0.003	0.005	0.013	0.022	0.030	0.029	0.030	0.034
	Quadratic	0.087	0.242	0.508	0.079	0.129	0.248	0.351	0.359	0.316	0.240	0.143	0.111	0.085
XYL	0	222.2b	0.091	3.12	17.0c	36.9b	63.4c	92.5c	121.8bc	148.3bc	202.4bc	224.7bc	235.3ab	242.4ab
	10	215.0b	0.087	2.97	19.5b	39.7b	65.2c	92.1c	118.9c	143.7c	195.7cd	219.4c	230.3b	237.0b
	20	218.8b	0.091	2.89	22.4a	45.2a	72.4b	99.9b	127.1b	152.3ab	205.0ab	227.5ab	237.6ab	243.6ab
	40	216.3b	0.096	3.15	22.6a	48.2a	77.5a	106.8a	133.9a	159.3a	211.4a	233.1a	242.8a	248.7a
	80	240.5a	0.069	2.50	14.5d	29.2c	51.4d	77.1d	104.3d	131.1d	192.8d	222.2bc	236.6ab	245.7ab
P value	Linear	0.563	0.836	0.528	<0.001	<0.001	<0.001	0.001	0.028	0.110	0.266	0.273	0.447	0.724
	Quadratic	0.294	0.071	0.910	0.707	0.125	0.060	0.024	0.010	0.006	0.001	0.007	0.031	0.063
SEM pooled		14.57	0.0020	0.235	1.60	1.05	6.72	2.17	2.60	12.92	13.47	13.61	13.82	14.06
P value														
	Enzyme	0.161	<0.001	0.089	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.001	0.021	0.364	0.632
	Dose:													
	Linear	0.258	0.852	0.153	<0.001	<0.001	<0.001	<0.001	0.001	0.005	0.013	0.013	0.017	0.031
	Quadratic	0.472	0.041	0.600	0.191	0.519	0.852	0.824	0.717	0.725	0.872	0.713	0.529	0.417
	Enzyme × Dose	0.012	<0.001	0.773	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.003	0.024	0.044	0.047

b, the asymptotic gas production; *c*, the rate of gas production; DM, dry matter; *L*, the initial delay before gas production begins. Means within in the same column with different superscripts differ significantly among treatments (P<0.05).

of CEL (i.e., 80 µg/g DM) increased GP compared to control. This result showed the difference between both enzymes, and importance to determine optimal doses of each enzyme. Increasing level of XYL (i.e., 80 µg/g DM) affected negatively on fermentation, which may be due to the fact that excessive levels of XYL prevented binding of enzymes to substrate receptors causing reduced microorganisms attachment to feeds (Treacher and Hunt 1996). Beauchemin *et al.* (2003) concluded that high levels of enzyme addition can be less effective than low levels, indicating the need to determine the optimal application rate of enzyme.

In vitro *fermentation kinetics:* Interaction effects were observed between enzyme type and enzyme dose for DMD (P=0.003), OMD (P=0.003), NDFD (P=0.031), ME (P=0.003), SCFA (P=0.003) and MCP production (P=0.003), which clearly showed the main factors affecting fermentation kinetic of corn stover with fibrolytic enzyme addition.

Addition of CEL and XYL linearly decreased (P=0.041) pH compared to control (Table 2). This may be due to greater enzymatic hydrolysis of feeds into readily fermentable substrates that depress pH when fermented. Elghandour *et al.* (2013) obtained decreased ruminal pH values when incubated 4 fibrous feeds, including corn stover, with different levels of exogenous fibrolytic enzyme. Linearly increased OMD (P=0.030), ME (P=0.030), SCFA (P=0.030) and MCP production (P=0.030) were obtained with CEL addition at 40 and 80 µg/g DM of corn stover. The dose of 40 µg XYL/g DM corn stover quadratically increased OMD (P=0.001), ME (P=0.024), SCFA production (P=0.001) and MCP production (P=0.001) compared to other doses (Table 2). Several studies showed that adding exogenous enzyme to ruminant diets increased feed digestion *in situ* (Togtokhbayar *et al*. 2015), *in vitro* (Salem *et al*. 2015b) or *in vivo* (Alsersy *et al*. 2015, Salem *et al*. 2015a, Valdes *et al*. 2015). Increased *in vitro* GP with enzyme is mainly due to increased OMD as GP is closely correlated with the amount of OM fermented (Elghandour *et al*. 2014). Increased OMD with enzyme addition may allow higher voluntary feed intake and overcome the problem of low intakes and slow digestion rates of low quality forages because long retention times of digesta in the rumen (Leng 1990). This can allow decreasing the physical rumen fill and stimulating MCP production (Oba and Allen 2000). The increase of OMD, ME and SCFA production of corn stover with the addition of CEL and XYL may be due to increased digestion and improved ruminal fermentation (Nsereko *et al*. 2002, Khattab *et al*. 2011), enhanced attachment and colonization between enzymes and the plant cell wall material (Morgavi *et al*. 2001). Our results also agree with Elghandour *et al*. (2013), who concluded that

Table 2. Impact of cellulase (CEL) and xylanase (XYL) at different doses on pH, digestion and fermentation of corn stover

Enzyme	Dose (µg/g DM)	pH	DMD, DM mg/g	OMD, DM mg/g	NDFD, DM mg/g	ADFD, mg/g DM	ME, MJ/kg DM	SCFA, mmol/g DM	MCP, mg/g DM
CEL	0	5.62a	525.0	492.4b	431.1	268.3	7.34b	3.88b	604.9b
	10	5.57b	501.8	518.9ab	431.8	271.2	7.75ab	4.21ab	632.7ab
	20	5.57b	504.5	520.5ab	435.4	270.7	7.77ab	4.23ab	634.4ab
	40	5.60ab	495.0	542.3a	432.5	270.6	8.10a	4.51a	657.3a
	80	5.56c	508.9	528.9a	438.5	266.5	7.90a	4.34a	643.3a
P value	Linear	0.041	0.160	0.030	0.574	0.539	0.030	0.030	0.030
	Quadratic	0.266	0.137	0.240	0.828	0.593	0.240	0.240	0.241
XYL	0	5.70a	548.2	539.6bc	445.1	275.8	8.06bc	4.47bc	654.5bc
	10	5.64ab	557.4	527.8cd	431.6	270.9	7.88cd	4.32cd	642.0cd
	20	5.64ab	563.4	544.2ab	440	273.7	8.13ab	4.53ab	659.3ab
	40	5.63ab	556.7	555.7a	434.1	271.9	8.31a	4.67a	671.4a
	80	5.62b	577.1	522.6d	421.6	264	7.80d	4.26d	636.6d
P value	Linear	0.022	0.140	0.266	0.330	0.616	0.269	0.267	0.269
	Quadratic	0.132	0.850	0.001	0.237	0.292	0.001	0.001	0.001
SEM pooled		0.016	15.56	16.18	14.52	12.75	1.10	0.077	16.50
P value									
	Enzyme	<0.001	0.306	0.001	0.835	0.306	0.001	0.001	0.001
	Dose:								
	Linear	0.002	0.629	0.013	0.934	0.968	0.013	0.013	0.013
	Quadratic	0.059	0.250	0.872	0.123	0.659	0.872	0.874	0.872
	Enzyme × Dose	0.455	0.003	0.003	0.031	0.459	0.003	0.003	0.003

ADFD, *in vitro* acid detergent fiber degradability; DM, dry matter; DMD, *in vitro* dry matter degradability; MCP, microbial crude protein production; ME, metabolizable energy; NDFD, *in vitro* neutral detergent fiber degradability; OMD, *in vitro* organic matter degradability; SCFA, short chain fatty acids. Means within in the same column with different superscripts differ significantly among treatments (P<0.05).

supplementation of enzymes to fibrous feeds increased *in vitro* OMD, ME and SCFA production.

It could be concluded that addition of exogenous fibrolytic enzymes cellulase or xylanase increased *in vitro* gas production and fermentation kinetics of corn stover. The optimum dose of both cellulase and xylanase is 40 µg/g DM; however, the difference of xylanase between treatments and control was less than that of cellulase.

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