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Effects of Exogenous Cellulase Source on *In Vitro* Fermentation Characteristics and Methane Production of Crop Straws and Grasses[#]

S.X. Tang[1], Y. Zou[1], M. Wang[1], A.Z.M. Salem[2,7], N.E. Odongo[3], C.S. Zhou[1], X.F. Han[1], Z.L. Tan[1,*], M. Zhang[4], Y.F. Fu[5], S.Q. Huang[6], Z.X. He[1] and J.H. Kang[1]

Institute of Subtropical Agriculture, The Chinese Academy of Sciences
Changsha P.O. Box 10, Hunan 410125, P.R. China

ABSTRACT

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In vitro fermentation experiments were conducted to investigate the effects of 3 sources of exogenous cellulase products (EC) at 4 dose rates (DR) (0, 12, 37 and 62 IU/g of DM) on degradation of forage and methane production by mixed rumen micro-organisms of goats. The maximum gas production (*Vf*) of grasses was higher ($P<0.001$) in *Neocallimastix patriciarum (*NP) group than those in *Trichoderma reesei* (TR) and *Trichoderma longibrachiatum* (TL) groups. Quadratic increases in dry matter degradation (DMD) of forage and neutral detergent fiber (NDFD) of straw were observed for all EC, with optimum DR in the low range. Supplementation of EC originated from TR and NP increased ($P<0.001$) DMD of forage compared to that from TL. Addition of EC originated from TR and NP also decreased pH value, ammonia nitrogen (NH_3-N) and methane (CH_4) production compared to that from TL. Quadratic decreases in pH value, NH_3-N and CH_4 of forage were noted for EC of TR and NP, and with optimum DR in the low range. For short chain fatty acid, the EC of NP increased total volatile fatty acid (TVFA) and acetate concentration and the ratio of acetate to propionate of forage compared with EC of TL and TR, and with optimum DR in the low to medium range. It was concluded that the source of EC differed in fiber degradation and methane emission, and with optimum DR of TR in the low range (from 12 to 37 U/g DM) in improving fiber degradation and decreasing methane emission.

Key words: Forage, Exogenous cellulase, *In vitro* fermentation, Methane production

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*Corresponding author: zltan@isa.ac.cn

[1]Key Laboratory for Agro-Ecological Processes in Subtropical Region, and Hunan Research Center of Livestock & Poultry Sciences, and South-Central Experimental Station of Animal Nutrition and Feed Science in Ministry of Agriculture, Institute of Subtropical Agriculture, The Chinese Academy of Sciences, Changsha, Hunan 410125, P.R. China; [2]Facultad de Medicina Veterinaria y Zootecnia, Universidad Autónoma del Estado de México, Estado de México, Mexico

[3]Animal Production and Health Section, Joint FAO/IAEA Division of Nuclear Techniques in Food and Agriculture, International Atomic Energy Agency, Vienna, Austria

[4]Hunan Youtell Biochemical., Ltd, Yueyang, China

[5]Wuhan Sunhy Biological Co., LTD, Wuhan, China

[6]Guangdong VTR Biological Technology Co., Ltd., Zhuhai, China; [7]Faculty of Agriculture (El-Shatby), Alexandria University, Egypt.

INTRODUCTION

Roughages, especially crop residues constitute the major ingredients of ruminants' diets in developing countries, while the complex network formed by structural carbohydrates and lignin in crop residues limits the digestibility and efficient utilization of forages by ruminants. Many attempts have been made to overcome this limitation, and the use of exogenous enzymes has been received considerable attention for many years (Tang *et al.,* 2008; Malik and Bandla, 2010; Chung *et al.,* 2012). However, the *in vitro* and *in vivo* experimental results have highly variable, the ineffectiveness (Burroughs *et al.,* 1960; Reddish and Kung, 2007) and even negative responses (Sutton *et al.,* 2003) have frequently been observed in previous studies. Meanwhile, they have suggested that the effectiveness of dietary exogenous fibrolytic enzymes supplementation depends not only on the type of ration, but also on activity of enzymes, level and mode of enzyme supplementation, and even on nature and source of enzymes. On the other hand, 15-20% of global methane (CH_4) is produced by ruminants in agriculture production systems (Ding *et al.,* 2012). Methane production from ruminants fed highly fibrous diets is higher than those from animals fed better quality forages. Recent researches have shown that supplementing fiber degrading enzymes in livestock diets may improve feed utilization by enhancing fiber degradation, reduction in methane emission per unit of animal by-products (Nsereko *et al.,* 2002; Reddish and Kung, 2007; Shojaeian and Thakur, 2007). While Chung *et al.* (2012) has reported that CH_4 emissions obviously increased when EC was added in total mixed ration at 0.5 or 1.0 mL/kg DM for dairy cows, whether it was calculated according to per kg of dry matter intake or per kg of milk production. Thus, the objective of present study was conducted to evaluate the effects of dosage and source of EC on the *in vitro* fermentation and CH_4 production from forages.

MATERIALS AND METHODS

This experiment was approved by the Animal Care Committee, Institute of Subtropical Agriculture (ISA), the Chinese Academy of Sciences, Changsha, China.

Crop straws, grasses and enzymes

Three crop straws, i.e., maize stover (Kexiangtian 1), rice straw (Xiang 125s) and wheat straw (Taishan 9818) after grain were harvested, and two grasses, i.e., Guimu 1 at late tillering and alfalfa before flowering were selected as *in vitro* fermentation substrates in this study. They were dried at 65°C for 24 h, and then ground through 1 mm sieve and stored in plastic bag for assay. Maize stover had the following chemical composition (DM basis): 5.3% CP, 63.6% NDF, and 38.6% ADF. The rice straw contained (DM basis): 6.2% CP, 63.2% NDF, and 43.4% ADF. The wheat straw was composed of (on DM basis): 8.4% CP, 68.4% NDF, and 42.6% ADF. The Guimu 1 contained (on DM basis) 8.9% CP, 61.2% NDF and 33.6% ADF, and 10.4% CP, 43.3% NDF, and 30.0% ADF were involved in alfalfa.

Exogenous cellulase (EC) were procured from *Neocallimastix patriciarum* (NP; Hunan Youtell Biochemical., Ltd. Yueyang, China), *Trichoderma Longibrachiatum* (TL; Wuhan Sunhy Biological Co., LTD, Wuhan, China) and *Trichoderma Reesei (*TR; Guangdong VTR Biological Technology Co., Ltd., Zhuhai, China), respectively. All of the EC products were powder form, and are acceptable for use in animal feeds in China.

In vitro gas production and sampling

Culture solutions, i.e., macroelement solution, buffered solution and reducing solution used for *in vitro* fermentation were prepared to form artificial salivary according to the procedures modified by Tang *et al.* (2006). The artificial salivary was anaerobic by pumping carbon dioxide for 2 h.

About 1000±20 mg sample of each straw or grass was accurately weighed into 150 mL fermentation bottles. Each sample was measured in triplicates. Exactly 0.5333 g of NP, 1.2121 g of TL and 0.8889 g of TR enzyme powder were solubilized using 5 mL of water, and 15, 46.25 and 77.5 μl of the diluted enzyme was added to the forage to achieve a DR of 12, 37 and 62 IU of concentrated enzyme product per g of forage DM. Then the samples were stored at room temperature until artificial saliva and rumen fluids were added.

Ruminal fluid was collected before morning feeding from three rumem-cannulated goats fed a corn stover based total mixed ration, and immediately transported to laboratory. Ruminal contents were strained through four layers of cheesecloth under a continuous CO_2 stream. Ten milliliters of ruminal fluids and 90 mL of artificial salivary were introduced to the bottle pre-warmed at 39°C. All bottles were connected with pressure sensors. Then, the bottles were incubated at 39°C.

The pressure in the bottles were recorded at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 36, 48 and 72 hrs during the process of *in vitro* fermentation. Undegraded residue was immediately filtered through 2 layers of nylon cloth (40-um pore size), and the gas was artificially collected with plastic syringe for CH_4 determination. The incubation solution of each sample was also sampled for ammonia nitrogen (NH_3-N) and volatile fatty acid (VFA) determination. Five milliliters of gas sample were collected and injected into the vacuum flask (Labco Exetainer, UK) for CH_4 determination.

Chemical analyses

The filtered residue was dried at 105°C for 12 h and weighed for dry matter disappearance determination. Neutral detergent fiber (NDF) content in the dried residue was determined according to the described method of Van Soest *et al.* (1991). Two milliliters of incubation solution was centrifuged at 10000×g and 4°C for 15 min, then 1.5 mL of supernatant solution was taken and 0.15 mL metaphosphoric acid was added and homogenized. The mixed solution was centrifuged at 10000×g and 4°C for 15 min

again, and the supernatant solution was used to determine VFA content with a gas chromatograph (HP5890, Agilent 5890; Agilent Technologies Co. Ltd, USA). A DB-FFAP column (30m in length with a 0.25mm i.d.) was used for the separation. The attenuation was set at a nitrogen diffluent ratio of 1:50, hydrogen flow was 30 mL/min, airflow was 365 mL/min, injector temperature was 250°C, column temperature was 150°C and detector temperature was 220°C. The N_2 was used as carrier gas at a flow rate of 0.8 mL/min. The relative response factor, representing the peak of each VFA, was calculated using the standard VFA mixture, which was chromatographed with each group of 10 samples. Total molar concentration was calculated by taking the sum of individual VFA as 100%.

For NH_3-N, 5 mL of incubation solution was centrifuged at 4000×g and 4°C for 10 min, then 2 mL of the supernatant solution was taken and mixed with 8 mL 0.2 M HCl into a tube followed by homogenization. Subsequently, 0.4 mL of the mixed solution was taken and mixed with 2 mL of sodium nitroprusside solutions (0.08 g sodium nitroprusside dissolved in 100 ml of 14% natrium salicylicum) and 2 mL of prepared solution (2 mL sodium hypochlorite solution mixed with 100 mL 0.3 M sodium hydroxide solution) into a tube followed by homogenization and equilibrated at room temperature for 10 min. The ultraviolet absorption value was recorded at 700 nm. The preparation of NH_4Cl standard solution was the same as above-mentioned procedures. The CH_4 analysis was performed by GC-flame ionization detection (FID) using gas chromatography (GC7890A, Agilent, America) equipped with a Hayesep Q packing column (2.44 M×1/8 in.×2.0 mm ID). The temperature of column and injector was respectively set at 60°C and 100°C, and held for 3 min. The N_2 was used as carrier gas at a flow rate of 21 mL/min.

Calculation and statistical analysis

During the initial stages of this work, the correlativity between the pressure in bottle and gas volume was measured at 39°C, and the regression equation was then established:

$y = (x-0.816)/0.805$ ($n=20$, $R^{2=}0.999$, $P<0.0001$),

Where, y represents gas volume (mL), x is the pressure in bottle (kPa), 0.816 and 0.805 are constant. The measured pressure was then converted to gas production (mL). *In vitro* gas production at 0, 1, 2, 3, 4, 5, 6, 8, 10, 12, 16, 24, 36, 48 and 72 hrs were fitted to Logistic-Exponential (Wang *et al.,* 2011):

$GP = Vf\ (1 - \exp\ (d - t\times k)) / (1+\exp\ (b - k\times t))$

Where, GP represents gas production at t time, Vf means the maximum gas production (mL), k represents gas production fraction (/h), b and d represent the shape of the gas production curve. The following equation: $T0.5 = In\ (\exp\ (b)+2\exp\ (d))/k$ (Wang *et al.,* 2011) was used to calculate the time ($T0.5$, h) when half of the maximum gas production reached. $FRD0 = k/1+\exp\ (b))$ (Wang *et al.,* 2013) was used to calculate the initial fractional rate of degradation (/h).

Gas production, DM and NDF disappearances were corrected by subtracting the values obtained for the blanks. Statistical analyses were performed using the MIXED procedures (SAS Institute 2001). Data were analysed separately by forage substrate as a completely randomized design. The fixed effects in the model were cellulase, dose, and the cellulase×dose interaction. Linear and quadratic effects of DR were analysed using orthogonal polynomial contrasts. Cubic effects of DR were not examined for inexplicability in biology. The IML procedure of SAS (2001) was used to correct the contrast coefficients of orthogonal polynomial. Least squares means are reported throughout the text, and significance was declared at $P<0.05$.

RESULTS

In vitro gas production parameters

For grasses, *in vitro* gas production parameters generally were not affected by EC source, except for *Vf* which increased ($P<0.001$) by 8% and 19% for grasses supplemented with NP than those supplemented with TL and TR, respectively, and the grasses supplemented with NP had the maximum *Vf* value (Table 1). Only *Vf* and *k* were influenced by the dose rate of supplemented EC. The effects of TR (linear, $P<0.001$) and NP (quadratic, $P<0.05$) inclusion on *Vf*, and TL (linear, $P=0.01$) and NP (linear, $P=0.006$) inclusion on *k* were dose-dependent. The optimum DR for NP which was considered to be the minimum dose required to elicit the greatest significant increase in gas production and gas production rate compared with the control, being at 12 IU/g of DM.

For crop straws, the EC source affected *Vf* ($P<0.001$) and *d* ($P=0.02$) values, and the lowest *Vf* was observed in TR. Relative improvements in *Vf* were 17% and 9% for TL and NP compared with TR. The effect of all EC addition on *in vitro* gas production parameters, except for *RFD0*, depended upon the DR used, as proved by significant EC×DR interactions (Table 2). The linear effects on *Vf*, *k*, *b* and *d* values to DR were found for TL, and a linear effects on *Vf*, *d* and *T0.5* values to DR were also found for NP. A linear response on *Vf*, a quadratic response on *k* and *d* values to DR were observed for TR, respectively. The optimum DR varied among EC. Low DR of NP (12 IU/g of DM) and the highest DR of TL (62 IU/g of DM) increased *Vf* by 5% and 7.0% compared with control.

DM and NDF disappearance

For grasses, the effect of each of 3 EC on DMD depended on the source of EC and the DR used, while for NDFD, EC supplementing effects were only related to DR used (Table 3). The source of EC affected ($P<0.001$) DMD, and the lowest DMD was noted in TL; NDFD in TL also decreased by margin of 7.1% and 5.5% compared with TR and NP, respectively. A quadratic response on DMD and NDFD to DR was observed for all EC except for NDFD of NP which linearly ($P=0.01$) increased NDFD of grasses. Degradation of DM and NDF of grasses were increased when EC was

supplemented. Optimum DR for improving DMD and NDFD was 12 IU/g of DM for TL, TR and NP compared with control. Improvements in DMD were 26.7, 28.1 and 24.6%, and in NDFD were 17, 24.4 and 24%, respectively.

Table 1. *In vitro* fermentation characteristics of grass supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[₸]		
		Mean	0	12	37	62		EC	DR	EC×DR
Vf, mL	TL	274[f]	281	266	272	284	8.4	P<0.001	NS	P<0.001
	TR	247[g]	281[a]	260[ab]	245[ab]	236[b]			L (P<0.001)	
	NP	295[e]	281	326	272	287			NS	
	SEM[ŧ]	5.3								
k,/h	TL	0.065	0.059[b]	0.058[ab]	0.068[a]	0.068[a]	0.0051	NS	L (P=0.01)	NS
	TR	0.062	0.059	0.050	0.067	0.068			NS	
	NP	0.069	0.059[ab]	0.061[ab]	0.064[ab]	0.082[a]			L (P=0.006)	
	SEM[ŧ]	0.00274								
b	TL	-1.78	-2.03	-2.31	-1.18	-1.86	1.333	NS	NS	NS
	TR	-1.33	-2.03	-3.73	-0.01	-0.25			NS	
	NP	-0.98	-2.03	-0.45	-3.11	0.63			NS	
	SEM[ŧ]	0.6587								
d	TL	-0.126	-0.183	-0.182	-0.087	-0.109	0.0487	NS	NS	NS
	TR	-0.106	-0.183	-0.056	-0.137	-0.126			NS	
	NP	-0.123	-0.183	0.001	-0.150	-0.221			NS	
	SEM[ŧ]	0.0191								
T0.5, h	TL	15.3	16.3	15.7	15.3	14.7	1.13	NS	NS	NS
	TR	14.9	16.3	15.3	14.6	14.8			NS	
	NP	15.5	16.3	16.8	13.5	16.1			NS	
	SEM[ŧ]	0.6485								
FRD0,/h	TL	0.036	0.029	0.034	0.036	0.037	0.0052	NS	NS	NS
	TR	0.037	0.029	0.040	0.034	0.036			NS	
	NP	0.034	0.029	0.034	0.040	0.027			NS	
	SEM[ŧ]	0.0029								

[e-g]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-b]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]*Vf*=the maximum gas production; *k*=gas production fraction; *b* and *d*=the shape of the gas production curve; *T0.5*=the time when half of the maximum gas production reached; *FRD0*=the initial fractional rate of degradation;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum (*for TL), *Trichoderma Reesei* (for TR) or *Neocallimastix patriciarum (*for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[₸]DR=dose rate; L=linear effect of DR; EC×DR=interaction between EC and DR;

[ŧ]SEM for pooled mean of EC excluding the dose rate of 0.

Table 2. *In vitro* fermentation characteristics of straws supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[₸]		
		Mean	0	12	37	62		EC	DR	EC×DR
Vf, mL	TL	275[c]	274[ab]	243[b]	290[ab]	293[a]	10.8	P<0.001	L (P=0.03)	P<0.001
	TR	235[f]	274[a]	263[ab]	216[b]	227[ab]			L (P<0.001)	
	NP	259[e]	274[ab]	289[a]	238[b]	251[ab]			L (P=0.01)	
	SEM[ŧ]	6.1								
k,/h	TL	0.059	0.062[a]	0.061[ab]	0.054[ab]	0.052[b]	0.0036	NS	L (P=0.04)	P<0.001
	TR	0.061	0.062[b]	0.043[c]	0.083[a]	0.057[b]			Q (P<0.001)	
	NP	0.061	0.062	0.057	0.061	0.065			NS	
	SEM[ŧ]	0.00225								
b	TL	0.402	0.798[a]	0.734[a]	0.383[ab]	0.091[b]	0.2509	NS	L (P=0.01)	P<0.001
	TR	0.392	0.798	-0.807	1.398	0.586			NS	
	NP	0.580	0.798	0.686	0.537	0.518			NS	
	SEM[ŧ]	0.1631								
d	TL	-0.214[e]	-0.147[a]	-0.418[b]	-0.114[a]	-0.111[a]	0.0528	P=0.02	L (P<0.001)	P<0.001
	TR	-0.358[f]	-0.147[a]	-0.153[a]	-0.674[b]	-0.248[a]			Q (P<0.001)	
	NP	-0.259[ef]	-0.147[a]	-0.142[a]	-0.242[ab]	-0.393[b]			L (P=0.01)	
	SEM[ŧ]	0.034								
T0.5, h	TL	21.4	22.0	20.6	21.8	21.7	0.73	NS	NS	P=0.01
	TR	20.5	22.0	20.2	20.3	21.2			Q (P=0.04)	
	NP	20.7	22.0[a]	23.3[a]	19.7[b]	19.2[b]			L (P<0.001)	
	SEM[ŧ]	0.4535								
FRD0,/h	TL	0.021	0.019	0.019	0.022	0.022	0.0015	NS	NS	P=0.01
	TR	0.020	0.019	0.024	0.016	0.020			NS	
	NP	0.021	0.019	0.019	0.022	0.022			NS	
	SEM[ŧ]	0.0009								

[c-f]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]*Vf*=the maximum gas production; *k*=gas production fraction; *b* and *d*=the shape of the gas production curve; *T0.5*=the time when half of the maximum gas production reached; *FRD0*=the initial fractional rate of degradation;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum (*for TL), *Trichoderma Reesei* (for TR) or *Neocallimastix patriciarum (*for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[₸]DR=dose rate; L=linear effect of DR; EC×DR=interaction between EC and DR;

[ŧ]SEM for pooled mean of EC excluding the dose rate of 0.

For crop straws, EC source only affected (P<0.001) DMD (Table 3). Difference in NDFD among 3 EC was not observed. Crop straws supplemented with EC from TR and NP increased DMD by 16.9% and 14.5% compared with those supplemented with EC from TL. All of 3 EC influence on DMD and NDFD relied on the DR used, as evidenced by significant EC×DR interaction (Table 3). A quadratic response on DMD and NDFD to DR was observed for all EC. The optimum DR in increasing DMD was 12 IU/g of DM for 3 EC, and in improving NDFD was 12 IU/g of DM for TL and NP, and 37 IU/g of DM for TR. The lowest DR of TL, TR and NP (12 IU/g of DM) increased DMD by 21.2, 19.2 and 15% compared with control. Improvements in the NDFD were 23.8% for TL, 25.7% for NP, and 31.7% for TR.

Table 3. *In vitro* disappearance of DM and NDF of forage supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[₸]		
		Mean	0	12	37	62		EC	DR	EC×DR
Grass										
DMD, %	TL	47.2[f]	35.1[c]	61.8[a]	39.3[bc]	40.6[b]	1.82	P<0.001	Q (P=0.01)	P<0.001
	TR	61.0[e]	35.1[b]	63.2[a]	60.6[a]	59.1[a]			Q (P<0.001)	
	NP	60.6[e]	35.1[b]	59.7[a]	59.1[a]	63.0[a]			Q (P<0.001)	
	SEM[t]	1.20								
NDFD, %	TL	76.8	57.6[b]	74.6[a]	76.1[a]	79.7[a]	4.18	NS	Q (P=0.03)	NS
	TR	83.9	57.6[b]	82.0[a]	87.6[a]	82.3[a]			Q (P<0.001)	
	NP	82.3	57.6[c]	81.6[ab]	75.5[b]	89.7[a]			L (P=0.01)	
	SEM[t]	2.46								
Straw										
DMD, %	TL	35.4[f]	32.5[b]	53.7[a]	26.0[c]	26.5[c]	1.87	P<0.001	Q (P=0.02)	P<0.001
	TR	52.3[e]	32.5[b]	51.7[a]	53.5[a]	51.8[a]			Q (P<0.001)	
	NP	49.9[e]	32.5[b]	47.5[a]	51.2[a]	50.9[a]			Q (P<0.001)	
	SEM[t]	1.20								
NDFD, %	TL	60.3	39.4[b]	63.2[a]	61.9[a]	55.7[a]	3.30	NS	Q (P<0.001)	P=0.02
	TR	59.4	39.4[c]	55.2[b]	65.1[a]	58.0[ab]			Q (P<0.001)	
	NP	66.2	39.4[c]	71.1[a]	58.6[b]	69.0[a]			Q (P=0.02)	
	SEM[t]	1.91								

[c-f]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]DMD=DM degradability; NDFD=NDF degradability;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum (*for TL), *Trichoderma Reesei* (for TR) or *Neocallimastix patriciarum (*for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[₸]DR=dose rate; L=linear effect of DR; EC×DR=interaction between EC and DR;

[t]SEM for pooled mean of EC excluding the dose rate of 0.

pH, NH_3-N and CH_4 production

The source of EC influenced pH value (P<0.001), concentration of NH_3-N in fermentation liquor (P=0.001) and CH_4 production (P<0.001) of grasses (Table 4). Grasses supplemented with EC of TR and NP had lower pH value, NH_3-N concentration and CH_4 production than those supplemented with EC of TL. The decreases in pH were 3.8% and 3.2%, in NH_3-N were 28% and 39%, and in CH_4 were 44% and 24% for TR and NP compared with TL. Dosage of each 3 of EC influenced pH value, NH_3-N and CH_4 production of grasses. The pH of TR and NP decreased, while that of TL increased. The optimum DR of 3 EC for decreasing pH, NH_3-N and CH_4 was 12 IU/g of DM for TL, TR and NP. Relative decreases in pH, NH_3-N and CH_4 were 6.2%, 65% and 48% for TL, 6.3%, 52% and 51% for TR, and 4.2%, 71% and 31% for NP, respectively.

Table 4. Ammonia nitrogen concentration and pH value of fermentation liquor of forage supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[₸]		
		Mean	0	12	37	62		EC	DR	EC×DR
Grass										
pH	TL	6.80[e]	6.93[a]	6.50[b]	6.94[a]	6.96[a]	0.023	P<0.001	Q (P<0.001)	P<0.001
	TR	6.54[f]	6.93[a]	6.49[b]	6.58[b]	6.55[b]			Q (P<0.001)	
	NP	6.58[f]	6.93[a]	6.64[b]	6.55[b]	6.56[b]			Q (P<0.001)	
	SEM[t]	0.013								
NH_3-N,	TL	155[e]	195[a]	69[b]	196[a]	199[a]	16.9	P=0.01	L (P=0.03)	P=0.01
mg/L	TR	111[f]	195[a]	93[b]	102[b]	138[b]			Q (P<0.001)	
	NP	95[f]	195[a]	57[c]	115[b]	114[b]			Q (P=0.01)	
	SEM[t]	10.4								
CH_4,	TL	5.20[e]	6.19[a]	3.23[b]	6.28[a]	6.08[a]	0.428	P<0.001	Q (P<0.001)	P<0.001
mmol/g DMD	TR	2.91[g]	6.19[a]	3.03[b]	2.90[b]	2.81[b]			Q (P<0.001)	
	NP	3.94[f]	6.19[a]	4.28[b]	3.83[b]	3.71[b]			Q (P=0.02)	
	SEM[t]	0.219								
Straw										
pH	TL	6.77[e]	6.87[a]	6.44[b]	6.94[a]	6.93[a]	0.019	P<0.001	Q (P<0.001)	P<0.001
	TR	6.53[g]	6.87[a]	6.48[b]	6.54[b]	6.57[b]			Q (P<0.001)	
	NP	6.60[f]	6.87[a]	6.73[b]	6.52[c]	6.54[c]			Q (P<0.001)	
	SEM[t]	0.011								
NH_3-N,	TL	143[e]	227[a]	74[c]	202[a]	153[b]	16.6	P=0.005	Q (P<0.001)	P<0.001
mg/L	TR	99[f]	227[a]	109[b]	89[b]	98[b]			Q (P<0.001)	
	NP	97[f]	227[a]	76[b]	96[b]	119[b]			Q (P<0.001)	
	SEM[t]	6.4								
CH_4,	TL	7.20[e]	7.75[a]	3.31[b]	9.15[a]	9.13[a]	0.518	P<0.001	L (P<0.001)	P<0.001
mmol/g DMD	TR	3.07[g]	7.75[a]	3.47[b]	2.79[b]	2.97[b]			Q (P<0.001)	
	NP	3.90[f]	7.75[a]	4.45[b]	3.71[b]	3.55[b]			Q (P<0.001)	
	SEM[t]	0.239								

[c-g]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]NH_3-N=ammonia nitrogen concentration; CH_4=methane production;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum* (for TL), *Trichoderma Reesei* (for TR) or *Neocallimastix patriciarum* (for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC; [¶]SEM for EC×DR;

[₸]DR=dose rate; L=linear effect of DR; EC×DR=interaction between EC and DR;

[t]SEM for pooled mean of EC excluding the dose rate of 0.

For crop straws, the differences of EC source on pH value (P<0.001), NH_3-N (P=0.005) and CH_4 (P<0.001) were observed among 3 EC (Table 4). The favorable EC in reducing pH, NH_3-N and CH4 was TR and NP which decreased pH by 3.7% and 2.6%, reduced NH_3-N by 44% and 47%, and lower CH_4 by 134% and 85% compared with TL, respectively. Dose rate response in pH, NH_3-N and CH_4 varied among EC, as evidenced by significant EC×DR interaction. Optimum DR for decreasing pH, NH_3-N and CH_4 was 12 IU/g of DM for TL, TR and NP, except for pH of NP which was 37

IU/g of DM was favorable. Relative decreases in NH_3-N and CH_4 at this DR were 67, 52 and 67% and were 57, 55 and 43%, and in pH at those DR were 6.3, 5.7 and 5.1% for TL, TR and NP compared with control, respectively.

Volatile fatty acids

For grasses, the source of EC affected volatile fatty acid concentration in fermentation liquor and the ratio of acetate to propionate, except for isobutyrate (Table 5). The grasses supplemented with EC of NP had the highest TVFA, acetate, propionate, butyrate, isobutyrate, valerate, isovalerate and the ratio of acetate to propionate. The EC of NP improved TVFA by 29 and 22%, enhanced acetate by 51 and 31%, and increased the ratio of acetate to propionate by 16% and 17% compared with TL and TR, and enhanced butyrate, isovalerate and valerate by 36, 22 and 25% compared with TR, and increased propionate by 26% compared with TL, respectively. The EC produced variable effects on short chain fatty acid across DR, indicated by the EC×DR interaction. Optimum DR for increasing TVFA and each volatile fatty acid was 37 IU/g of DM for NP compared with control, and relative improvements in these values at this DR were ranged from 20% of isobutyrate to 106% of acetate. Optimum DR for increasing acetate and propionate was 37 IU/g of DM for TR compared with control. Relative improvements in these values at this DR were 41% and 35%. The EC of TL supplementation did not increase the concentration of TVFA, each volatile fatty acid compared to control.

For crop straws, the source of EC had significant effects on TVFA ($P<0.001$), acetate ($P<0.001$), butyrate ($P<0.001$), isovalerate ($P=0.01$) and the value of A:P ($P<0.001$). The highest TVFA, acetate, butyrate and the ratio of acetate to propionate were observed in NP, and the highest isovalerate was noted in the EC of TR. The EC of NP increased ($P<0.001$) TVFA by 21% and 23%, enhanced acetate by 47% and 37% compared with TL and TR, respectively, and increased propionate by 14% compared with TL. More than 28% and 34% of butyrate, and 20% and 19% of isovalerate were produced when crop straws supplemented with the EC of TR and NP compared with those of TL. The EC produced variable effects on short chain fatty acid across DR, indicated by the EC×DR interaction. whereas the improvements in these parameters were very weak for crop straws supplemented with EC of TL and TR compared with control. The EC of NP linearly increased ($P<0.001$) TVFA, butyrate, isovalerate, valerate and the ratio of acetate to propionate, and quadratically increased acetate ($P=0.02$) and propionate ($P=0.04$) compared with control. Optimum DR for increasing the value of these parameters was 37 IU/g of DMD, except for A:P, and relative improving in the values of these parameters at this DR were ranged from 21% of valerate to 75% of acetate compared with control. The EC supplementation could not decrease the ratio of acetate to propionate.

Table 5. Volatile fatty acid concentration (mmol/l) in incubation solution of grasses supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[Ŧ]		
		Mean	0	12	37	62		EC	DR	EC×DR
TVFA	TL	18.1^{f}	17.4	18.2	16.3	19.8	1.49	P<0.001	NS	P<0.001
	TR	19.1^{f}	17.4^{ab}	19.8^{ab}	20.5^{a}	17.1^{b}			Q (P=0.02)	
	NP	23.3^{e}	17.4^{c}	18.7^{bc}	28.1^{a}	23.2^{ab}			Q (P=0.02)	
	SEM[ŧ]	0.95								
Acetate	TL	6.35^{f}	5.81	6.49	5.69	6.86	0.776	P<0.001	NS	P=0.004
	TR	7.30^{f}	5.81^{b}	7.36^{ab}	8.21^{a}	6.35^{b}			Q (P=0.003)	
	NP	9.57^{e}	5.81^{c}	7.90^{b}	11.95^{a}	8.86^{b}			Q (P=0.003)	
	SEM[ŧ]	0.497								
Propionate	TL	5.74^{f}	5.56	6.17	4.93	6.13	0.453	P=0.007	NS	P=0.01
	TR	6.66^{ef}	5.56^{b}	6.34^{b}	7.51^{a}	6.14^{b}			Q (P=0.002)	
	NP	7.22^{e}	5.56^{b}	6.48^{ab}	7.71^{a}	7.46^{a}			L (P=0.02)	
	SEM[ŧ]	0.296								
Butyrate	TL	3.46^{c}	3.38^{ab}	3.18^{b}	3.27^{b}	3.92^{a}	0.219	P<0.001	L (P=0.03)	P<0.001
	TR	2.79^{f}	3.38^{a}	3.62^{a}	2.49^{b}	2.25^{b}			L (P<0.001)	
	NP	3.80^{e}	3.38^{b}	2.45^{c}	4.92^{a}	4.02^{b}			L (P<0.001)	
	SEM[ŧ]	0.138								
Isobutyrate	TL	0.788	0.825^{ab}	0.684^{b}	0.750^{b}	0.929^{a}	0.0451	NS	Q (P=0.004)	P<0.001
	TR	0.775	0.825	0.665	0.819	0.840			NS	
	NP	0.759	0.825^{b}	0.460^{c}	0.990^{a}	0.827^{b}			L (P=0.003)	
	SEM[ŧ]	0.0273								
Isovalerate	TL	0.933^{ef}	0.975^{ab}	0.839^{b}	0.880^{b}	1.079^{a}	0.0673	P=0.02	Q (P=0.009)	P<0.001
	TR	0.803^{f}	0.975^{a}	0.976^{a}	0.680^{b}	0.754^{b}			L (P=0.002)	
	NP	0.973^{e}	0.975^{b}	0.606^{c}	1.311^{a}	1.003^{b}			L (P=0.01)	
	SEM[ŧ]	0.0409								
Valerate	TL	0.839^{ef}	0.836	0.820	0.771	0.925	0.0717	P=0.01	NS	P=0.005
	TR	0.791^{f}	0.836	0.872	0.744	0.758			NS	
	NP	0.987^{e}	0.836^{b}	0.804^{b}	1.170^{a}	0.987^{ab}			L (P=0.04)	
	SEM[ŧ]	0.0452								
A:P	TL	1.08^{f}	1.04^{ab}	1.00^{b}	1.14^{a}	1.12^{a}	0.062	P=0.003	L (P=0.03)	P<0.001
	TR	1.07^{f}	1.04	1.10	1.10	1.01			NS	
	NP	1.25^{e}	1.04^{b}	1.19^{ab}	1.43^{a}	1.14^{b}			Q (P=0.003)	
	SEM[ŧ]	0.036								

[c-f]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]TVFA=Total volatile fatty acid; A:P=ration of acetic acid to propionic acid;

[‡]TL, TR and NP were cellulose originating from *Trichoderma longibrachiatum* (for TL), *T. reesei* (for TR) or *Neocallimastix patriciarum* (for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[Ŧ]EC=exogenous cellulase; DR=dose rate; L=linear effect of DR; Q=quadratic effect of DR; NS=no significant; EC×DR=interaction between EC and DR;

[ŧ]SEM for pooled mean of EC excluding the dose rate of 0.

Table 6. Volatile fatty acid concentration (mmol/l) in incubation solution of straws supplemented with exogenous cellulase

Item[†]	Enzyme[‡]	Dose rate[§]					SEM[¶]	Significance of effect[Ŧ]		
		Mean	0	12	37	62		EC	DR	EC×DR
TVFA	TL	14.9[f]	14.7	17.8	13.3	13.8	1.01	P<0.001	NS	P<0.001
	TR	15.1[f]	14.7	16.2	14.3	14.8			NS	
	NP	18.3[e]	14.7[b]	14.3[b]	21.3[a]	19.4[a]			L (P<0.001)	
	SEM[ŧ]	0.61								
Acetate	TL	5.53[f]	4.98	6.49	4.90	5.20	0.471	P<0.001	NS	P<0.001
	TR	5.16[f]	4.98	5.66	4.88	4.95			NS	
	NP	7.60[e]	4.98[b]	5.84[b]	8.71[a]	8.25[a]			Q (P=0.02)	
	SEM[ŧ]	0.288								
Propionate	TL	5.21	4.91	5.95	4.61	5.05	0.333	NS	NS	P=0.02
	TR	4.88	4.91[ab]	5.65[a]	4.37[b]	4.62[b]			L (P=0.02)	
	NP	5.57	4.91[b]	5.25[ab]	6.17[a]	5.28[ab]			Q (P=0.04)	
	SEM[ŧ]	0.204								
Butyrate	TL	2.33[f]	2.81[a]	3.26[a]	1.91[b]	1.81[b]	0.169	P<0.001	L (P<0.001)	P<0.001
	TR	2.98[e]	2.81	3.03	2.93	2.97			NS	
	NP	3.13[e]	2.81[b]	2.02[c]	3.90[a]	3.46[ab]			L (P<0.001)	
	SEM[ŧ]	0.093								
Isobutyrate	TL	0.647	0.677	0.595	0.687	0.660	0.0337	NS	NS	P<0.001
	TR	0.649	0.677[a]	0.549[b]	0.676[a]	0.722[a]			Q (P=0.03)	
	NP	0.646	0.677[a]	0.363[b]	0.780[a]	0.795[a]			L (P<0.001)	
	SEM[ŧ]	0.0185								
Isovalerate	TL	0.645[f]	0.714[ab]	0.822[a]	0.594[bc]	0.518[c]	0.0425	P=0.01	NS	P<0.001
	TR	0.771[e]	0.714	0.730	0.772	0.810			NS	
	NP	0.766[e]	0.714[b]	0.424[c]	0.976[a]	0.897[a]			L (P<0.001)	
	SEM [6]	0.0243								
Valerate	TL	0.573	0.636	0.637	0.559	0.523	0.0362	NS	NS	P<0.001
	TR	0.642	0.636[ab]	0.562[b]	0.670[a]	0.693[a]			L (P=0.008)	
	NP	0.621	0.636[b]	0.399[c]	0.770[a]	0.694[ab]			L (P<0.001)	
	SEM[ŧ]	0.0204								
A:P	TL	1.06[f]	0.995	1.06	1.09	1.02	0.046	P<0.001	NS	P<0.001
	TR	1.05[f]	0.995[ab]	0.984[b]	1.11[a]	1.06[ab]			L (P=0.02)	
	NP	1.29[e]	0.995[b]	1.05[b]	1.38[a]	1.45[a]			L (P<0.001)	
	SEM[ŧ]	0.025								

[c-f]Means within a column for EC that do not have a common superscript differ at P<0.05;

[a-c]Means within a row for dose rates of 0 to 62 IU/g of DM that do not have a common superscript differ at P<0.05;

[†]SCFA=short chain fatty acid; A:P=ration of acetic acid to propionate;

[‡]TL, TR, and NP were cellulose originating from *Trichoderma longibrachiatum (*for TL), *T. reesei* (for TR) or *Neocallimastix patriciarum (*for NP);

[§]Dose rate as IU/g of DM forage substrate; Mean=mean for individual EC across dose rates except dose rate of 0; 0=control without added EC;

[¶]SEM for EC×DR;

[Ŧ]EC=exogenous cellulase; DR=dose rate; L=linear effect of DR; Q=quadratic effect of DR; NS=no significant; EC×DR=interaction between EC and DR;

[ŧ]SEM for pooled mean of EC excluding the dose rate of 0.

DISCUSSION

Cell wall degrading enzymes used in ruminants feedstuffs may differ in improving the degradation of forage because of the composition and the activity of enzyme. The maximum gas production of grass and crop straw differed among three EC was similar to that of Eun and Beauchemin (2007b) and Chen *et al.* (2013). Eun and Beauchemin (2007b) reported that feed enzymes originated from *Trichoderma longibrachiatum* had higher gas production than that from *Penicillum funiculosum*. In present study, crop straw or grass supplemented with EC of TL and NP had higher gas production than that of TR. Also, Chen *et al.* (2013) found that gas production at 48 h incubation time ranged from 166 to 177 mL/g for corn silage added 5 cellulase. Compose of exoglucanase, endoglucanase and xylanase, and their activity in enzyme system may be responsible for this difference. It indicated that the origination of EC should be considered when EC was used to improve *in vitro* fermentation of forage. When the gas production were fitted to the Wang *et al.* (2011) model, no obvious improvement in the maximum gas production compared with control was consistent with that of previous studies (Colombatto *et al.,* 2004; Eun and Beauchemin, 2007b; Chen *et al.,* 2013). Index of *FRD0* and *T0.5* reflect the rate of fermentation at early incubation stages of '< 12 h' and the incubation time of half of the maximum gas production reached, respectively. Supplementation of EC, although, did not increase the value of *T0.5* and *FRD0* obviously, a numerically decrease in *T0.5* and an increase in *FRD0* indicated that EC supplementation may be beneficial to improve the effect of bacteria on degradation of forage. In the other hand, *T0.5* is more than 14 h for grass and 20 h for crop straw indicated mainly degradation period of bacteria on forage is in the middle or late stages of incubation. These results also support the general agreement that enzymes increased the rate, but not the extent, of feed degradation in the rumen (Beauchemin *et al.,* 2001).

Analyzing on the data of DMD and NDFD revealed that enzyme treatments increased the degradable fraction of the forages, which is agreement with the data obtained from grass (Zhu *et al.,* 1999), alfalfa (Chen *et al.,* 1994; Eun and Beauchemin, 2007b), corn silage (Eun and Beauchemin, 2007b; Sun *et al.,* 2009; Chen *et al.,* 2013), and with our previous report (Tang *et al.,* 2008). Other studies using maize silage or corn silage have reported small increases (Chen *et al.,* 1994; Eun and Beauchemin, 2007b). Our results suggest that more macromolecular were hydrolyzed to simpler and degradable ones as enzymes were supplemented in forages. Origin of EC differed in improving DMD and NDF was also observed in previous studies (Colombatto *et al.,* 2004; Eun and Beauchemin, 2007b). Probable cause may be related to the activity of endoglucanase which hydrolyze cellulose chains at random, and exoglucanase which hydrolyze cellulose chain from the nonreducing end (Bhat and Hazlewood, 2001), in the enzyme system. Eun and Beauchemin (Eun and Beauchemin 2007b) also found endoglucanase linked to NDF degradability of alfalfa and corn silage. Significant increase in DMD or NDFD indicated catalytic affect of EC on the substrate

(Morgavi *et al.,* 2001). In any case, the outcome indicated that the fermentation efficiency of forage may be improved as enzyme used as additives in ruminant feedstuffs.

The value of pH is a main factor in reflecting internal environment of rumen. Treatments of TR and NP or EC reduced pH value of fermentation liquor to 6.49 and 6.73 compared with TL, or control suggests that TR and NP, or EC treatments could maintain more suitable condition for fermentation, and suitable for the growth of micro-organism. Final pH value of EC treatment ranged from 6.53 to 6.80, and can be considered optimal for fiber degradation in the rumen (Stewart *et al.,* 1997). TR and NP or EC treatments in forage had higher DMD than TL or control may partially due to this reason. Satter and Slyter (1974) suggested that the lowest ammonia nitrogen concentration of rumen liquor should be higher than 5 mL/dL for bacteria to get the highest growth rate. Concentration of NH_3-N in EC treatments is higher than 5 mg/dL indicated the growth rate of bacteria will not be restricted. In the other hand, ammonia nitrogen is a main source of nitrogen in the synthesis of rumen bacteria, and 18% to 100% of bacterial nitrogen is originated from ammonia nitrogen (Salter *et al.,* 1979). The treatments of EC or TR and NP had lower NH_3-N concentration than control or TL implied that forage supplemented EC or EC of TR and NP could enhance the utilization of bacteria on nitrogen.

In present study, EC or EC of TR and NP treatment significantly decreased methane production compared with control or TL. Giraldo *et al.* (2007) found that methane production was not influenced for cellulase was sprayed into the diet which was composed with forage and concentration at 70: 30. Whereas, Chung *et al.* (2012) reported that methane production, whether calculated as per kg of DM or per kg of milk, would increase when enzyme added in dairy diets at 0.5 and 1.0 mL/kg. Dong *et al.* (1999) also found methane production increased by 43% when cellulase and xylanase were added in hay. Beauchemin *et al.* (2008; 2009) reported that enzyme supplementation though absolutely increased methane production, methane production per kg of milk would decrease. Methane production decrease may be related to microflora change of methanogenium leaded by enzyme addition (Zhou *et al.,* 2011). Addition of EC leaded to methane production decrease suggested that utilization of cellulase, especially for TR and NP, in ruminant diets may be an efficient method in reducing greenhouse effect caused by CH_4 emitted from ruminant production.

Changes in TVFA and each profile of VFA corresponded to increased fiber degradation of forage. Addition of EC increased the concentration of TVFA of forage was consistent with that of previous studies (Eun and Beauchemin, 2007a; Eun and Beauchemin, 2007b; Eun and Beauchemin, 2008; Giraldo *et al.,* 2008). This indicated that the activity of bacteria in degrading fiber has been promoted by EC, especially for EC of NP. The ratio of acetate to propionate of EC treatment was not decreased, or even increased compared with control was inconsistent with that of previous studies

(Eun and Beauchemin, 2007a; 2008; Giraldo *et al.,* 2008). Eun and Beauchemin (2007b) found that the addition of single or combined enzyme numerically decreased the ratio of acetate to propionate of alfalfa. The inconsistent of enzyme addition on the changes of VFA composition may relate to the enzyme activities added, the forage substrates, diet of donor animal and donor species used. The ratio of acetate to propionate was lower for forage supplemented with EC at 37 IU/g of DM than that of higher dosage indicated that it is benefit for diets supplementing EC at lower dosage from increasing availability of glucogenic precursors to ruminants.

CONCLUSIONS

Exogenous cellulase originated from TR and NP improved *in vitro* DMD of forage, decreased pH value, NH_3-N and CH_4 production of forage compared to TL. Forage added with EC of NP had higher TVFA, acetate, propionate and the ratio of acetate to propionate compare with TL and TR. Forage supplemented with EC could improve DMD, NDFD and TVFA, and decrease pH value, NH_3-N and CH_4 production, and their optimum DR varied hardly depending upon the forage. In general, low DR of EC resulted in increase in DMD and NDFD and decrease in CH_4 and NH_3-N as that of medium and higher DR. It is recommended that the treatments of TR and NP should be further evaluated in animal feeding study.

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