Int.J.Curr.Microbiol.App.Sci (2018) *7*(10): 330-338

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 10 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.710.035

An *In vitro* Dose Optimization of Exogenous Fibrolytic Enzymes in Total Mixed Ration for Crossbred Cows

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Keywords

Exogenous fibrolytic enzymes, *in vitro* digestibility, Metabolizable energy, Microbial biomass production, Total gas production

Article Info

Accepted:
04 September 2018
Available Online:
10 October 2018

A B S T R A C T

An experiment was conducted to evaluate the levels of exogenous fibrolytic enzymes (EFE) on *in vitro* digestibility of dry matter (DM) and organic matter (OM), net gas production (NGP), metabolizable energy (ME) content, microbial biomass production (MBP) and efficiency of microbial biomass production (EMP). The total mixed ration (TMR) was prepared using sorghum hay and groundnut straw and compound concentrate mixture @ 30, 30 and 40%, respectively, to meet nutrient requirement of cow (500 kg) producing 12 kg fat corrected milk. The cellulase and xylanase was incorporated @ 0+0, 1000+2000, 1000+3000, 1000+4000, 2000+4000, 2000+6000, 2000+8000, 3000+6000, 3000+9000, 3000+12000, 4000+8000, 4000+12000, 4000+16000, 5000+10000, 5000+15000, 5000+20000, 6000+12000, 6000+18000 and 6000+24000 IU/kg TMR. The TMR with different levels of cellulase and xylanase were *in vitro* incubated to ascertain their effect on digestibility, gas production, and nutritive values. The significantly (p<0.05) higher and optimum *in vitro* digestibility of DM (66.243%) and OM (66.56%), NGP (76.48 ml/500 mg TMR) and ME (7.41 MJ/kg DM) were observed at 3000+12000 cellulase and xylanase IU/kg TMR supplementation, whereas MBP (146.73 mg/500 mg TMR) and EMP (44.02) were significantly (p<0.05) higher and optimum at C4X12 and C5X15 TMR, respectively. The MBP (145.86 mg/500 mg TMR) and EMP (43.83) also better at C3X12 TMR. The levels of cellulase 3000 + xylanase 12000 IU/kg TMR were found suitable for further *in vivo* study in crossbred cows.

Introduction

The digestion of forage in ruminants occurs through the microbial fermentation. The microbial digestion allowed ruminants to better unlock the unavailable energy in the plant cell wall components than other herbivores (Van Soest, 1994; Krause *et al.*, 2003). Thus ruminant animals are able to convert low nutritive and resistant lignocellulosic biomass to milk, meat, wool and hides (Weimer *et al.*, 2009) owing to ruminal microorganisms that synthesize and secrete β 1-4 cellulase enzyme complex. However, most forage plants are high in cell walls and low in nitrogen (N) and energy content (Romney and Gill, 2000), hence are not utilized efficiently even by ruminants. Though fibrous components in forages is importance for salivation, rumen buffering and efficient rumen fermentation (Mertens, 1997) only 10 to 35% of energy intake is utilize as net energy (Varga and Kolver, 1997). This is owing to incomplete ruminal digestion of plant cell walls (Krause *et al.*, 2003). An incorporation of exogenous fibrolytic enzymes (EFE) in feeds is one of the bio-processing tool to improve the energy availability of feeds in dairy cattle.

Positive effects of EFE include direct fibre hydrolysis, palatability improvement, changes in gut viscosity, complimentary and synergistic action with ruminal enzymes, change in the site of digestion (Beauchemin and Rode, 1996), increase in rumen bacterial colonization of the fibre substrate (Wang *et al.*, 2012), altering and thinning of fiber cell wall (van de Vyver and Cruywagen, 2013). The feeding EFE supplemented diet were reported to improve the digestibility of dry matter (DM), organic matter (OM), crude fiber, neutral detergent fiber (NDF), cellulose and hemicellulose in dairy animals (Miachieo and Thakur, 2007; Rajamma *et al.*, 2014; Morsy *et al.*, 2015). An *in vivo* study need living animals which is expensive and time consuming, where as an *in vitro* to evaluation of the effect of EFE on digestibility and nutritive value of feed is cheaper, faster and more controllable (Makkar, 2004).

It was hypothesized that EFE improve the fermentation parameters of feed. The experiment was conducted to examined the effect of supplementing various levels of EFE in TMR on *in vitro* digestibility, net gas production (NGP), metabolizable energy (ME) content, and microbial biomass production (MBP) and efficiency of microbial biomass production (EMP).

Materials and Methods

The study was conducted at Animal Nutrition Research Department, College of Veterinary Science and Animal Husbandry, Anand Agricultural University, Anand. Sorghum hay, groundnut straw and compound concentrate mixture were oven dried at 70°C and finely ground in mill using 1 mm sieve. These ingredients were mixed in ratios of 30, 30 and 40% to prepare total mixed ration (TMR). The TMR was prepared to meet nutrients requirement of dairy cow (500 kg) producing 12 kg 4% fat corrected milk (FCM) per day (NRC, 2001). The calculated nutritional values of TMR were 10.61% crude protein (CP), 6.59% digestible CP (DCP), 56.51% total digestible nutrients, and 2.05 Mcal ME/kg DM. The ingredients and TMR were analyzed for proximate constituents (AOAC, 1995), fiber fractions (Van Soest *et al.*, 1991) and calcium and phosphorus.

The cellulase and xylanase was procured from M/s Aumgene Bioscience Pvt. Ltd., Surat, Gujarat, India. The cellulase and xylanase contained 3000 and 200000 IU/g, respectively as per manufacturer's labeling. The cellulase and xylanase were incorporated at 0+0, 1000+2000, 1000+3000, 1000+4000, 2000+4000, 2000+6000, 2000+8000, 3000+6000, 3000+9000, 3000+12000, 4000+8000, 4000+12000, 4000+16000, 5000+10000, 5000+15000, 5000+20000, 6000+12000, 6000+18000 and 6000+24000 IU/kg TMR to ascertain effect on *in vitro* digestibility of DM and OM, *in vitro* NGP, ME content, MBP and EMP. The supplementation of cellulase was from 1000, 2000, 3000, 4000, 5000 and 6000 IU/kg TMR with xylanase @ 2, 3 and 4 time higher than cellulase in each TMR. The TMR with above levels of EFE was designated as C0X0, C1X2, C1X3, C1X4, C2X4, C2X6, C2X8, C3X6, C3X9, C3X12, C4X8, C4X12, C4X16, C5X10, C5X15, C5X20, C6X12, C6X18 and C6X24, respectively.

Rumen liquors were collected from three crossbred cows using stomach tube. The cows were fed individually to meet nutrients requirement (NRC, 2001) with free access to clean and wholesome water. The rumen liquor was strained through four layer muslin cloth and was termed strained rumen liquor (SRL). TMR with various levels of EFE were incubated for 48 h in quadruplet at 39±1°C for 48 h in a shaker twin water bath with 40 ml of fresh McDougall buffer and 10 ml SRL as per Menke *et al.*, (1979). After incubation, the content of each syringe was filtered through dried and pre-weighed Gooch crucible, which was again dried and weighed. Simultaneously, the blank was also run without TMR sample in quadruplet. *In vitro* NGP was taken after subtracting gas production from blank. ME (Elghandour *et al.*, 2015), MBP and EMP (Mir *et al.*, 2015) was calculated as follows:

ME (MJ/kg DM) = 2.20+0.136 Gp+0.057 CP%, (R^2=0.94)

Where, CP is crude protein% and Gp is ml of net gas production from 200 mg dry sample.

MBP= {TDOM-(2.2×net gas volume)}; TDOM = (Feed OM incubated-residue OM).

Where, TDOM is total digestible OM.

EMP= {TDOM-(2.2×net gas volume)} x 100÷TDOM

Statistical analysis of the data on DM and OM digestibility, NGP, ME, MBP and EMP were carried out using Duncan's multiple range tests (SPSS 9.00 software) as per Snedecor and Cochran, (1994).

Results and Discussion

The proximate and fiber fractions composition of ingredients and TMR utilized for study are presented in Table 1. The TMR contained 10.35% CP, 23.59% CF, 53.34% NDF and 29.92% ADF. *In vitro* DM and OM digestibility (%), NGP, ME content, MBP and EMP of TMR supplemented with various levels of EFE are presented in Table 2.

In vitro DM and OM digestibility (IVDMD and IVOMD) and net gas production (NGP)

The digestibility of DM (58.20±0.41%) and OM (58.79±0.51%) were significantly ($p<0.05$) higher at C1X2 level of EFE incorporation in TMR and also at higher levels of incorporation. However, supplementation of cellulase and xylanase (EFE) at 3000+12000 IU/kg TMR (C3X12) had improved ($p<0.05$) DM and OM digestibility (66.24±0.55% and 66.56±0.50%, respectively) than lower levels of incorporation. Further increase in level of fibrolytic enzyme did not show beneficial effect on IVDMD and IVOMD. *In vitro* net gas production followed same trend.

Optimum higher *in vitro* DM and OM digestibility was reported by Lunagariya *et al.*, (2017) on supplementation of EFE (1,4-β glucanase 800, 1(3),4-β glucanase 700 and endo 1,4-β xylanase 2700 IU/g) at 240 mg/kg TMR than lower (0, 40, 60, 80, 100, 120, 140, 160, 180, 200 and 220 mg/kg TMR) or higher (260, 280, 300, 320, 340, 360, 380 and 400 mg/kg TMR) dose rate for crossbred cows while in vitro gas production followed same trend. Rajamma *et al.*, (2015) has incorporated EFE at 2.5 g/kg DM TMR (roughage to concentrate ratio of 60:40) which resulted in higher 62.18% ($p\leq0.001$) IVDMD than control TMR (58.44%). Similarly optimum and higher IVDMD and IVOMD of TMR (having 40:40:20 ratio of concentrate, wheat straw and green oats) was reported by Miachieo and Thakur (2007) on incorporated with fibrolytic enzymes @ 1.5 g/kg than without or higher levels of EFE (3.0 g/kg DM) in TMR. Sipai *et al.,* (2013) observed higher ($p \leq 0.05$) *in vitro* gas production (96.33 ml and 96.00 ml) when dry sorghum supplemented with EFE (1:1 mixture of neutral cellulase-3000 units/g and fungal xylanase-200000 units/g) at 0.6 and 0.8%, respectively, than dry sorghum supplemented without or with lower levels (0.01, 0.1, 0.2, 0.3, and 0.5%) and higher levels (0.9% and 1%) of fibrolytic enzymes.

Similarly, significant higher *in vitro* gas production observed on EFE supplementation in roughage diet (Gemeda *et al.*, 2014), maize stover and sugarcane bagasse (Elghandour *et al.*, 2016) and sorghum straw (Elghandour *et al.*, 2013). These observations are in accordance with present study. However, when silage of corn (Faramarzi-Garmroodi *et al.*, 2013; Elghandour *et al.*, 2015), alfalfa and barley (Holtshausen *et al.,* 2011) used as substrate for sole or ingredients of total mixed ration incubation, fermentation kinetics was not improved as silage considered as predigested feed.

ME content of TMR

Average ME content of TMR was 7.28±0.02 MJ/kg DM. However, ME of TMR (6.95±0.02 MJ/kg DM) significantly improved at minimum EFE level 1000+2000 cellulase and xylanase IU/kg (C1X2) than control (C0X0).

The optimum and significantly higher ME content of TMR was 7.41±0.03 MJ/kg DM when TMR was incorporated with EFE 3000+12000 cellulase and xylanase IU/kg (C3X12). Further higher level of incorporation of EFE had shown no improvement in ME content of TMR. Lower ME content of TMR was observed than calculated ME (9.50 MJ/kg DM), however ME content of experimental TMR had shown incremental trend with increase in level of EFE incorporation.

Table.1 Composition of TMR and ingredients (% on DM basis)

Parameters	TMR	Concentrate mixture	Sorghum hay	Groundnut straw
CP	10.35±0.34	15.06	6.06	8.98
EE	3.38±0.08	6.42	1.62	1.04
CF	23.59±0.58	10.23	34.55	30.32
NFE	52.21±0.36	58.49	48.31	47.89
Total ash	10.29±0.12	9.80	9.46	11.77
Silica	2.03±0.38	1.08	3.24	2.03
Calcium	1.01±0.25	1.31	0.66	0.91
Phosphorus	0.45±0.12	0.65	0.28	0.37
NDF	53.34±1.21	26.48	72.46	69.96
ADF	29.92±0.29	11.19	48.21	36.45

TMR=total mixed ration, DM=Dry matter, CP=Crude protein, EE=Ether extract, CF=Crude fibre, NFE=Nitrogen free extract, NDF=Neutral detergent fibre, ADF=Acid detergent fibre

Table.2 Average IVDMD, IVOMD, IVNGP, ME, MBP, EMP and PF of TMR containing different levels of Exogenous fibrolytic enzymes

TMR	Particulars					
	IVDMD%	IVOMD%	IVNGP (ml/500 mg)	ME (MJ/kg DM)	MBP (mg/500 mg TMR)	EMP
C0X0	55.92^{a}±0.28	56.47^{a}±0.16	66.15^{a}±0.34	6.79 ^{a}±0.02	120.65 ^{a}±0.16	42.73 ^{a}±0.14
C1X2	58.20^{b}±0.41	58.79^{b}±0.51	68.80^{b}±0.29	6.95^{b}±0.02	125.76^{b}±2.05	42.78 ^{a}±0.34
C1X3	59.95^{c}±0.24	60.58^{c}±0.27	70.25^{c}±0.21	7.04^{c}±0.01	131.16^{c}±1.43	43.30^{abc}±0.30
C1X4	60.65^{cd}±0.26	61.30^{cd}±0.37	70.83^{cd}±0.40	7.07^{cd}±0.02	133.35^{cd}±1.11	43.51^{abc}±0.17
C2X4	61.49^{de}±0.30	62.03^{de}±0.32	71. 85de±0.53	7.13^{de}±0.03	134.52^{cd}±1.33	43.37^{abc}±0.34
C2X6	62.43^{e}±0.22	62.76^{e}±0.24	72.88^{ef}±0.18	7.19^{ef}±0.01	135.66^{d}±0.96	43.23^{abc}±0.16
C2X8	62.53 ^{e}±0.31	62.88^{e}±0.21	73.20^{ef}±0.34	7.21^{ef}±0.02	135.45^{d}±0.64	43.09^{abc}±0.16
C3X6	63.96^{f}±0.39	64.23^{f}±0.68	74.10^{fg}±0.41	7.27 ^{fg}±0.02	140.02^{e}±2.51	43.59^{bc}±0.34
C3X9	64.50 ^{f}±0.39	64.99^{f}±0.35	74.88^{g}±0.34	7.32 ^{fg} ±0.02	141.91^{ef}±0.92	43.67^{bc}±0.06
C3X12	66.24^{g}±0.55	66.56^{g}±0.50	76.48^{h}±0.45	7.41^{h}±0.03	145.86^{fg}±1.47	43.83^{bc}±0.14
C4X8	66.27^{g} ±0.18	66.60 ^{g}±0.13	76.88^{h}±0.25	7.44 ^{h}±0.02	145.06^{fg}±0.23	43.56^{bc}±0.09
C4X12	66.26^{g}±0.48	66.83 ^{g}±0.48	76.68^{h}±0.40	7.42 ^{h}±0.02	146.73^{g}±1.41	43.91^{bc}±0.11
C4X16	66.43^{g}±0.30	67.04 ^{g}±0.18	76.95^{h}±0.41	7..44 ^{h}±0.02	147.10 ^{g}±0.65	43.88^{bc}±0.21
C5X10	66.38^{g}±0.36	66.69 ^{g}±0.44	76.90^{h}±0.40	7.44 ^{h}±0.02	145.45 ^{fg}±1.50	43.62^{bc}±0.22
C5X15	66.49^{g}±0.62	67.07 ^{g}±0.53	76.80^{h}±0.69	7.43 ^{h}±0.04	147.59 ^{g}±1.03	44.02^{c}±0.11
C5X20	66.30^{g}±0.69	66.88 ^{g}±0.64	76.63^{h}±0.98	7.42 ^{h}±0.06	147.07 ^{g}±1.71	43.99^{c}±0.38
C6X12	66.37^{g}±0.24	66.89 ^{g}±0.31	76.98^{h}±0.49	7.44 ^{h}±0.03	146.30 ^{g}±1.85	43.74^{bc}±0.42
C6X18	66.49^{g}±0.33	66.84 ^{g}±0.36	76.78^{h}±0.63	7.43 ^{h}±0.04	146.50 ^{g}±0.87	43.84^{bc}±0.25
C6X24	66.47^{g}±0.37	66.89 ^{g}±0.44	77.18^{h}±0.32	7.45 ^{h}±0.02	145.79 ^{fg}±1.67	43.59^{bc}±0.25
Gen. Mean	63.86	64.33	74.27	7.28	140.10	43.54
SEM	0.38	0.37	0.38	0.02	0.95	0.06

a, b, c, d, e, f, g, h Means with different superscripts in a column for a parameter differ significantly ($p<0.05$).

IVDMD=In vitro dry matter, IVOMD=In vitro organic matter, IVNGP=In vitro net gas production, ME=metabolizable energy, MBP=Microbial biomass production, EMP=Efficiency of microbial biomass production

The ME content of TMR was linearly improved on incorporation of EFE from 40 to 240 mg/kg and shown no improvement on further incorporation of EFE from 240 to 400 mg/kg (Lunagariya *et al.*, 2017), whereas quadratic ($p<0.05$) improvement has been observed when sorghum incubated with exogenous enzymes mixture at 0, 6, 12 and 24 mg/g DM levels (Elghandour *et al.,* 2013). Similar values and non-significant ($p=0.0734$) improvement in ME content of TMR as 6.88, 6.89, 6.76 and 7.12 MJ/kg DM, respectively observed by Elghandour *et al.,* (2015) when the TMR having 50% maize silage (F) and 50% concentrate incubated without EFE, with cellulase 1 µl/g (C, 0.033 unit/g), xylanase 1 µl/g (X, 0.038 unit/g) and a mixture of cellulase and xylanase (XC, 1:1, v:v).

MBP and EMP

The MBP (mg/500 mg TMR) increased significantly at cellulose + xylanase 1000+2000 IU/kg TMR (125.76±2.05) than control (120.65±0.16). The linear improvement trend was observed with increasing level of cellulase and xylanase, but the optimum and significantly higher MBP (146.73±1.41) was achieved at 4000+12000 IU cellulase and Xylanase/kg TMR. The further higher levels of EFE were without significant improvement effect on MBP. The efficiency of microbial biomass production (EMP) was significantly higher at 3000+6000 IU cellulase and xylanase/kg TMR, however best efficiency was observed at C5X15 and C5X20 TMR supplemented with fibrolytic enzymes. The MBP values observed in present experiment are within the physiological range (100- 470 g/kg TDOM) for mixed diets (Thirumalesh and Krishnamoorthy, 2013). Lunagariya *et al.,* (2017) also reported improved trend in microbial biomass production on incorporation of EFE from 40 to 240 mg/kg TMR and further higher levels were without improvement. Elghandour *et al.,* (2015) reported non-significant ($p=0.6602$) difference in MBP when TMR (50% each of maize silage and concentrate) was incubated with cellulase 1 µl/g (C, 0.033 unit/g), xylanase 1 µl/g (X, 0.038 unit/g), a mixture of cellulase and xylanase (XC, 1:1, v:v), and control TMR, this may be due to use of pre-digested roughage (maize silage).

When TMR incorporated with cellulase and xylanase @ 3000+ 12000 IU/kg *in vitro* DM and OM digestibility and IVNGP was found to be significant and optimum (66.24±0.55%, 66.56±0.50% and 76.48±0.45 ml/500 mg, respectively) than control TMR without EFE or other levels of EFE. The optimum and higher ME (7.41±0.03 MJ/kg DM) also observed at same level of fibrolytic enzymes. The MBP (146.73±1.41 mg/500 mg TMR) and EMP (44.02±0.11) was significant higher ($p<0.05$) at incorporation cellulase and xylanase @ 4000+12000 and 5000+15000 IU/kg TMR in comparison to TMR without EFE or lower levels. Further higher levels of EFE did not shown improvement. The digestibility of DM and OM was improved positive and linearly with increment in NGT. The same correlations of gas production with digestibility of DM and OM under *in vitro* study were also reported by Blair (2011) and Mir *et al.,* (2015). The digestibility, gas production, ME, MBP and EMP was improved owing to stimulatory effect on rumen microbiota (Nsereko *et al.,* 2002), synergetic effect of EFE and rumen microbe (Morgavi *et al.,* 2000), enhance attachment of rumen microbial to feed particles (Yang *et al.,* 1999) and degrade complex fraction to simple molecules making them more available to rumen microbes by EFE (Azzaz *et al.,* 2013).

An improved ($p<0.05$) *in vitro* digestibility of DM (58.20%), OM (58.79%), and NGP (68.80 ml/500 mg TMR), ME (6.95 MJ/kg DM) and MBP (125.76 mg/500 mg TMR) was observed when cellulase and xylanase was supplemented at 1000+2000 IU/kg TMR in comparison to control whereas EMP was significantly higher at C3X6 TMR. The incorporation of cellulase and xylanase @ 3000+12000 IU/kg TMR resulted in optimum and significantly ($p<0.05$) higher *in vitro* digestibility of DM (66.24%) and OM (66.56%); NGP (76.48 ml/500mg) and ME (7.41 MJ/kg DM) compared to lower levels, whereas MBP (146.73 mg/500 mg TMR) and EMP (44.02) was optimum and significant at C4X12 and C5X15 TMR, respectively. MBP and EMP were also better at C3X12 TMR level. The digestibility, gas production and nutritive values of TMR did not show improvement with higher incorporation of cellulase and xylanase. The levels of cellulase and xylanase @ 3000+12000 IU/kg TMR were found suitable for milk production study in crossbred dairy cows.

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How to cite this article:

Lunagariya, P.M., S.V. Shah, B.R. Devalia, A.C. Patel and Pandya, P.R. 2018. An *In vitro* Dose Optimization of Exogenous Fibrolytic Enzymes in Total Mixed Ration for Crossbred Cows. *Int.J.Curr.Microbiol.App.Sci*. 7(10): 330-338.
doi: https://doi.org/10.20546/ijcmas.2018.710.035