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Effect of incorporation of exogenous fibrolytic enzymes on *in vitro* degradability of cotton straw based total mixed ration

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Abstract
The present study was carried out with the objective of to study the effect of incorporation of exogenous fibrolytic enzymes on *in vitro* degradability of cotton straw based total mixed ration. IVDMD significantly (p<0.05) increased @ 100000 & 200000 IU/kg cellulase and @ 50000 & 100000 IU/kg xylanase when supplemented alone. All levels of xylanase showed significant (p<0.05) effect on IVOMD as compared to control when supplemented alone. Cellulase also showed significant (p<0.05) effect @ 100000 & 200000 IU/kg and in combination @ 100000 IU/kg showed numerically higher IVOMD. IVTGP significantly (p<0.05) increased @ 100000 IU/kg xylanase and all combination of xylanase with 50000 IU/kg cellulase compared to control. Numerically higher gas production was observed in combination of enzymes @ 100000 IU/kg. TDOMR significantly (p<0.05) increased in combination @ 100000 and 50000 IU/kg cellulase and xylanase, respectively and also @ 200000 IU/kg of both enzymes. SCFA was significantly (p<0.05) improved @ 100000 IU/kg xylanase and combination of both enzymes @ 100000 IU/kg as well as in combination @ 100000 & 200000 IU/kg cellulase and xylanase, respectively. TVFA was numerically higher in combination @ 200000 IU/kg of both enzymes. NH_3-N was numerically higher @ 200000 IU/kg xylanase.

Keywords: Total mixed ration, fibrolytic enzymes, *In vitro*, degradability

Introduction
The livestock sector contributes 5.21% of GDP and 28.36% of the total agriculture GDP of India (Anonymous, 2020) [1]. According to the 20th livestock census, the total livestock population is 535.78 million in the country, showing an increase of 4.6 percent over the previous census. Due to the large population of livestock, a feed deficit is a major constraint to animal rearing. To increase nutrient availability, efficient crop residue utilisation has been a primary focus for farms seeking long-term profit. Roughages are lower in nutrient content but rich in fibrous portions, which are a major component of the forage dry matter. There are several methods to improve the availability of nutrients from roughages. Treating roughages or crop residues with physical processing, physico-chemical processing, adding enzymes, etc. may improve the digestibility. The fibrolytic enzymes hydrolyse the non-starch polysaccharides in the plant cell wall; they usually target the cellulose and hemicellulose (Scheller and Ulvskov, 2010) [13]. The addition of enzymes to rumen diets could increase the digestibility of fibrous feeds and thus, improve productivity and feed conversion. A synergism between exogenous enzymes and endogenous enzymes in the ruminal fluid results in increased numbers of non-fibrolytic and fibrolytic bacteria, causing increased feed digestibility and utilisation (Eun *et al.*, 2006) [6]. Keeping the above facts in view, the experiment was planned to study the effect of supplementing different combinations of exogenous fibrolytic enzymes on the cotton straw-based total mixed ration.

Materials and Methods
Preparation of TMR
Cotton straw as roughage source & commercial concentrate mixture were used as concentrate source for the experiment. Roughage and concentrate were ground to pass through a 1 mm screen using a willey mill. Total mixed rations were prepared by taking cotton straw and concentrate in the ratio of 70:30 and was used as a substrate. Different levels of exogenous fibrolytic enzymes (EFEs) in different combinations were used with 70:30 roughage

concentrate ratio. EFEs, cellulase was added at the level of 0, 50000, 100000 and 200000 IU/kg of TMR level and xylanase was added at the level of 0, 50000, 100000 and 200000 IU/Kg for each level of cellulase and all the combinations of EFEs was used for each roughage level.

Doner animals and collection of rumen liquor
Two adult Surti goats of same age and uniform conformation were selected as donor of rumen liquor for *in vitro* study. The nutrient requirements of the donor animal were met by feeding as per ICAR feeding standards (2013). Rumen liquor was collected at 2 hours post feeding with the help of stomach tube against negative pressure created by suction pump into pre-heated thermos flask. Rumen liquor was strained through four layered muslin cloth and referred as Strained Rumen Liquor (SRL).

Estimation of proximate composition and fibre fractions:
Samples of cotton straw, concentrate and prepared TMR were analysed for proximate composition as per the AOAC (2005) [2] and fibre fraction according to Goering and Van Soest (1970) [8].

Estimation of *in vitro* degradability
TMR was analysed for *in vitro* degradability as per the Tilley and Terry method (1963) [18]. About 0.5 g sample was placed in 100 ml Erlenmeyers conical flasks. Then add 40 ml CO_2 saturated phosphate-carbonate buffer and 10 ml CO_2 saturated strained rumen liquor into conical flask. Pass CO_2 gas through the content for 10 seconds to maintain an anaerobic environment. Then immediately put a stopper on the flasks. After 48 h incubation the flasks were removed from the incubator. The content of the Erlenmeyers conical flasks were carefully filtered through weighed sintered crucible (G_1). Then sintered crucibles with contents were dried into hot air oven for overnight at 100±5 °C and weighed.

Estimation of *in vitro* total gas production
TMR was analysed for *in vitro* total gas production as per the Menke and Steingass (1988) [12]. About 0.2 g of the substrate was taken in calibrated glass syringes and incubate the syringe at 39 ℃ for overnight. About 30 ml of rumen liquor buffer medium was injected to each calibrated glass syringe. The syringes were shaken gently, residual air or air bubbles were removed and then outlet was closed. Then initial level of piston was recorded and the syringes were placed in an incubator at 39 ℃ and shake periodically for 24 h. Syringes containing rumen liquor buffer solution without sample were also run as blank. The gas production was measured after 24 h of incubation.

Estimation of truly degradable organic matter in rumen (TDOMR): For the determination of TDOMR, the content of each syringe was filtered through sintered crucible (G_1). Then sintered crucibles with contents were dried into hot air oven for overnight at 100±5 °C and weighed. Then sintered crucibles were placed in one litter beaker with 100 ml of NDS and 2 ml of decahydronaphthalene. The contents were refluxed for 60 minutes at 100 °C on hot plate after boiling start. After one hours, contents were filtered through the sintered crucible (G_1) and kept in hot air oven at 100±5 °C for drying then kept in muffle furnace at 500 ºC for three hours and weighed after cooling in desiccator.

Estimation of partitioning factor (PF):

$$PF = \frac{\text{TDOMR (mg)}}{\text{Net gas production (ml)}}$$

Estimation of short chain fatty acids (SCFAs)
SCFAs (mMol/200 mg) = 0.0222* (ml gas at 24 h) – 0.00425 (Getachew *et al.*, 2002) [7]

Estimation of metabolizable energy (ME) and net energy (NE):

ME (MJ/kg DM) = 0.146 × GP + 0.007 × CP + 0.0224 × EE + 1.24

Where, GP = Net gas production (ml/200 mg DM)

Estimation of pH
pH of standard rumen liquor was determined using pen type pH meter.

Estimation of total volatile fatty acids
The TVFAs were determined by Markham's steam distillation method (Barnett and Reid, 1957) [3]. About 5 ml of SRL was treated with equal amount of saturated magnesium sulfate and filtered after 4 h. From 10 ml treated SRL 5 ml was distilled in the Markham steam distillation apparatus after adding oxalate buffer and TVFAs in the first 100ml distillate was collected in conical flask kept on ice and titrate under carbon dioxide free condition with 0.01N sodium hydroxide solution after adding 2-3 drops of phenolphthalein indicator. After titration total volatile fatty acids were calculated.

Estimation of ammonia nitrogen
Ammonia nitrogen was estimated by Conway's micro-diffusion method (Conway, 1957) [4]. 1 ml of 2 percent boric acid solution containing Tashiro's indicator was placed into the mid piece of Conway's cell. 1 ml of saturated solution of potassium carbonate was placed in outer chamber of cell. Then 1 ml of rumen liquor was placed opposite side of potassium carbonate and immediately placed glass cover plate on the Conway's cell on cover surface. The fluid of the outer chamber was mixed by rotating and then the cell was kept in the incubator at 39 ºC for 1 h. At the end of this period, the contents of inner chamber were titrated against 0.0143N sulphuric acid using micro burette.

Estimation of total nitrogen and TCA-nitrogen:
5ml of rumen liquor was precipitated with 5 ml of 20 percent trichloroacetic acid and kept overnight in refrigerated condition. Next day after centrifugation at 2000 rpm for 10 minutes, give 2-3 times wash to precipitate with distilled water to make acid free and then whole precipitate was transferred into digestion tube and proceed for digestion, distillation and titration as like CP estimation as per the AOAC (2005) [2].

Statistical analysis
The data were collected and statistically analysed by one-way analysis of variance (ANOVA) as per procedures suggested by Snedecor and Cochran (1994) [15]. Significance of mean differences were tested by Duncan's New Multiple Range Test (DNMRT) as modified by Kramer (1957) [10].

Results and Discussion

Proximate composition and fibre fractions (% DM basis) of cotton straw, concentrate and TMR is presented in Table 1. Proximate composition of cotton straw contains 91.19, 87.09, 6.00, 2.24, 41.21, 37.64 and 12.91% of DM, OM, CP, EE, CF, NFE and TA, respectively. While, fibre fractions of bajra straw contains 75.38, 55.11, 35.08, 20.27 and 14.02% of NDF, ADF, cellulose, hemicellulose and lignin, respectively. Proximate composition of concentrate contains 91.18, 90.16, 19.55, 2.65, 12.00, 55.96 and 9.84% of DM, OM, CP, EE, CF, NFE and TA, respectively. While, fibre fractions of concentrate contain 37.90, 19.75, 6.94, 18.15 and 6.25% of NDF, ADF, cellulose, hemicellulose and lignin, respectively. Proximate composition of TMR contains 91.19, 88.01, 10.78, 2.36, 37.64, 41.14 and 11.99% of DM, OM, CP, EE, CF, NFE and TA, respectively. Fibre fractions of TMR contains 64.14, 44.50, 26.63, 19.64 and 11.69% of NDF, ADF, cellulose, hemicellulose and lignin, respectively.

Table 1: Proximate composition and fibre fractions (% DMB) of feed ingredients and TMR

Proximate composition and fibre fractions	Ingredients & TMR (70:30)		
	Cotton straw	Concentrate	TMR (70:30)
DM	91.19	91.18	91.19
OM	87.09	90.16	88.01
CP	6.00	19.55	10.78
EE	2.24	2.65	2.36
CF	41.21	12.00	37.64
NFE	37.64	55.96	41.14
TA	12.91	9.84	11.99
AIA	0.91	2.80	1.47
NDF	75.38	37.90	64.14
ADF	55.11	19.75	44.50
Cellulose	35.08	6.94	26.63
Hemicellulose	20.27	18.15	19.64
Lignin	14.02	6.25	11.69
Calcium	1.79	1.68	1.76
Phosphorus	0.23	1.07	0.48

Note: AIA- acid insoluble ash, DM- dry matter, OM- organic matter, CP- crude protein, EE- ether extract, CF- crude fibre, NFE- nitrogen free extract, TA- total ash, NDF- neutral detergent fibre, ADF- acid detergent fibre.

Effect of exogenous fibrolytic enzymes on IVDMD, IVOMD and IVTGP are presented in Table 2. Statistical analysis of data revealed cellulase significantly ($p<0.05$) increased @ 200000 IU/kg (43.05±1.28%), whereas, xylanase showed significantly higher ($p<0.05$) value @ 50000 IU/kg (39.77±1.53%) and 100000 IU/kg (41.95±1.30%) compared to control when supplemented alone. In combination of both enzymes @ 50000 and 100000 IU/kg cellulase and xylanase, respectively (42.64±2.27%) showed significant effect ($p<0.05$) for xylanase compared to control and also numerically higher value among all combinations. In the present study results revealed EFEs significantly increased IVDMD. This might be occurred due to improved attachment and colonization of rumen microorganisms to the plant cell wall material. The results of the current study are consistent with those of earlier research by Lopez *et al.* (2016) [11], Sujani *et al.* (2017) [16] and Selzer *et al.* (2021) [14], stated that supplementing EFEs significantly increased IVDMD. Results revealed that cellulase showed significant effect ($p<0.05$) @ 100000 IU/kg (43.56±1.00%) and 200000 IU/kg (45.24±1.99%) and xylanase showed significant values ($p<0.05$) @ 50000 (43.23±0.97%), 100000 (44.78±0.45%) and 200000 IU/kg (41.90±0.65%) compared to control group whereas, combination of cellulase @ 50000 IU/kg and xylanase @ 100000 IU/kg (46.28±2.04%) showed higher significant value ($p<0.05$). Combination of both enzymes @ 100000 IU/kg (46.38±1.06%) showed numerically higher IVOMD. In the present study results revealed EFEs significantly increased IVOMD. This might be occurred due to improved attachment and colonization of rumen microorganisms to the plant cell wall material. The results of the current study are consistent with those of earlier research by Elghandour *et al.* (2015) [5] and Vallejo *et al.* (2016) [19], stated that supplementing EFEs significantly increased IVOMD. Results revealed that xylanase enzyme exhibited significant ($p<0.05$) @ 100000 IU/kg (21.20±0.15 ml/200 mg) compared to control when supplemented alone, whereas, combination of both enzymes @ 100000 IU/kg (24.04±2.16 ml/200 mg) showed significant effect ($p<0.05$) compared to control. All combinations of xylanase with cellulase @ 50000 IU/kg showed significant ($p<0.05$) effect on IVTGP compared to control. In the present study results revealed EFEs significantly increased IVTGP. This might be occurred due to higher digestibility of feed particles. The results of the current study are consistent with those of earlier research by Lopez *et al.* (2016) [11] and Sujani *et al.* (2016) [17], stated that supplementing EFEs significantly increased IVTGP.

Table 2: IVDMD, IVOMD and IVTGP of different treatments

Cellulase (IU/kg)	Xylanase (IU/kg)											
	IVDMD				IVOMD				IVTGP			
	0	50000	100000	200000	0	50000	100000	200000	0	50000	100000	200000
0	34.1^{Bb}	39.7^{a}	41.9^{Aa}	38.9^{ab}	36.7^{Bb}	43.2^{ABa}	44.7^{Aa}	41.9^{a}	15.1^{b}	19.4^{ab}	21.2^{a}	20.2^{ab}
50,000	31.8^{Bb}	37.0^{ab}	42.6^{Aa}	41.9^{a}	34.9^{Bb}	40.7^{ABa}	46.2^{Aa}	45.2^{a}	16.5^{b}	20.7^{a}	21.0^{a}	21.2^{a}
1,00,000	39.1^{AB}	41.1	42.3^{A}	41.6	43.5^{A}	45.1^{A}	46.3^{A}	44.7	18.1^{b}	21.2^{ab}	24.0^{a}	23.3^{ab}
2,00,000	43.0^{Aa}	36.2^{b}	35.5^{Bb}	38.2^{ab}	45.2^{Aa}	39.7^{Bb}	38.8^{Bb}	41.7^{a}	19.3	20.3	22.2	23.2^{a}

Note: Means superscripted with different superscripts within a column (A, B) and row (a, b) differ significantly from each other

IVDMD- *in vitro* dry matter degradability, IVOMD- *in vitro* organic matter degradability, IVTGP- *in vitro* total gas production

Effect of exogenous fibrolytic enzymes on TDOMR, SCFAs and ME are presented in Table 3. Results revealed that significant difference ($p<0.05$) for TDOMR was seen in the combination of both enzymes @ 100000 & 50000 IU/kg (55.03±3.46%) cellulase and xylanase, respectively compared to control. Similar result was found in the combination of both enzymes @ 200000 IU/kg (55.82±1.50%). Results revealed that xylanase showed significant effect @ 100000 IU/kg (0.46±0.01 mMol/200 mg) for SCFAs when supplemented alone compared to control. However, combinations @ 100000 & 200000 IU/kg cellulase and xylanase, respectively (0.51±0.04 mMol/200mg) and @ 100000 IU/kg cellulase and xylanase (0.52±0.04 mMol/200

mg) showed significant (p<0.05) effect. In the present study results revealed EFEs significantly increased SCFAs. This might be occurred due to higher microbial activity leads to higher digestibility of feed particles. The results of the current study are consistent with those of earlier research by Lopez *et al.* (2016) [11], stated that supplementing EFEs significantly increased SCFAs. Results revealed that xylanase showed significant effect @ 100000 IU/kg (4.46±0.02 MJ/kg DM) for ME as compared to control group when supplemented alone. However, the combination of both enzymes @ 100000 IU/kg (4.87±0.31 MJ/kg DM) and @ 100000 IU/kg cellulase & 200000 IU/kg xylanase (4.76±0.29 MJ/kg DM) showed significant (p<0.05) difference from the control group.

Table 3: TDOMR, SCFAs and ME of different treatments

Cellulase (IU/kg)	Xylanase (IU/kg)											
	TDOMR				SCFAs				ME			
	0	50000	100000	200000	0	50000	100000	200000	0	50000	100000	200000
0	43.3	44.7^{B}	46.2	45.2^{B}	0.33^{b}	0.42^{ab}	0.46^{a}	0.44^{ab}	3.5^{b}	4.2^{ab}	4.4^{a}	4.3^{ab}
50,000	45.2	48.2^{AB}	47.4	48.2^{AB}	0.36	0.45	0.46	0.46	3.7	4.3	4.4	4.4
1,00,000	48.4	55.0^{A}	52.9	53.6^{AB}	0.39^{b}	0.46^{ab}	0.52^{a}	0.51^{a}	4.0^{b}	4.4^{ab}	4.8^{a}	4.7^{a}
2,00,000	51.2	53.4^{AB}	50.2	55.8^{A}	0.42	0.44	0.48	0.51	4.1	4.3	4.6	4.7

Note: Means superscripted with different superscripts within a column (A, B) and row (a, b) differ significantly from each other

TDOMR- truly degradable organic matter in rumen, SCFAs- short chain fatty acids, ME- metabolizable energy

Effect of exogenous fibrolytic enzymes on TVFAs, NH_3-N and Total-N are presented in Table 4. Statistical analysis of data revealed no significant effect (p>0.05) was observed in terms of TVFAs, NH_3-N and Total-N as compared to control. For TVFAs numerically higher value was observed @ 100000 IU/kg cellulase (7.10±1.70 mMol/dl) and 200000 IU/kg xylanase (7.16±1.88 mMol/dl) compared to control when supplemented alone. However, the combination of both enzymes @ 200000 IU/kg (9.64±0.20 mMol/dl) showed numerically highest TVFA. For NH_3-N cellulase @ 200000 IU/kg (29.0±3.0 mg/dl) and xylanase @ 200000 IU/kg (32.0±2.0 mg/dl) showed numerically higher values. However, in combination of both enzymes @ 100000 IU/kg (30.0±4.0 mg/dl) showed numerically highest NH_3-N. For Total-N cellulase @ 200000 IU/kg (51.8±5.6 mg/dl) and xylanase @ 100000 IU/kg (46.2±7.0 mg/dl) showed numerically higher value as compared to control. However, combination of both enzymes @ 50000 IU/kg (49.0±4.2mg/dl) and @ 100000 IU/kg (49.0±7.0 mg/dl) showed numerically highest Total-N.

Table 4: TVFAs, NH_3-N and Total-N of different treatments

Cellulase (IU/kg)	Xylanase (IU/kg)											
	TVFAs				NH_3-N				Total-N			
	0	50000	100000	200000	0	50000	100000	200000	0	50000	100000	200000
0	4.36	5.58	5.98	7.16	25.0	27.0	28.0	32.0	40.6	40.6	46.2	43.4
50,000	6.04	6.24	5.92	8.80	26.0	18.0	22.0	25.0	46.2	49.0	40.6	44.8
1,00,000	7.10	7.36	6.48	6.34	24.0	26.0	30.0	28.0	43.4	43.4	49.0	43.4
2,00,000	5.84	6.04	8.70	9.64	29.0	28.0	25.0	29.0	51.8	39.2	44.8	46.2

TVFAs- total volatile fatty acids, NH_3-N- ammonia nitrogen, Total-N- total nitrogen

Effect of exogenous fibrolytic enzymes on pH, PF and TCA-N are presented in Table 5. Statistical analysis of data revealed no significant effect (p>0.05) was observed in terms of pH, PF and TCA-N as compared to control. Numerically higher value of TCA-N was observed @ 100000 IU/kg cellulase (25.20±2.80 mg/dl) and 100000 IU/kg xylanase (26.60±2.80 mg/dl) compared to control when supplemented alone. However, combination of enzymes @ 200000 IU/kg showed numerically highest value (26.60 ± 5.60 mg/dl) of TCA-N.

Table 5: pH, PF and TCA-N of different treatments

Cellulase (IU/kg)	Xylanase (IU/kg)											
	pH				PF				TCA-N			
	0	50000	100000	200000	0	50000	100000	200000	0	50000	100000	200000
0	6.8	6.9	7.0	7.0	2.87	2.30	2.17	2.26	19.6	23.8	26.6	21.7
50,000	6.9	6.9	6.9	6.8	2.74	2.33	2.26	2.29	24.5	25.2	25.9	20.3
1,00,000	6.8	6.9	6.7	6.9	2.69	2.60	2.23	2.32	25.2	25.9	23.1	25.2
2,00,000	7.0	7.0	6.9	6.9	2.65	2.65	2.25	2.40	24.5	22.4	25.9	26.6

pH- potential of hydrogen, PF- partitioning factor

Conclusions

Results of the present study indicated that supplementation of fibrolytic enzymes (Cellulase and xylanase) improve IVDMD, IVOMD, IVTGP, TDOMR, SCFAs and ME. No significant effect of exogenous fibrolytic enzymes was observed for pH, PF, TVFAs and NH_3-N. Based on overall results of the study it is inferred that the exogenous fibrolytic enzymes (cellulase and xylanase) can be supplemented @ 1,00,000 IU/Kg each in combination to improve the degradability and nutrient utilization in cotton straw based total mixed ration.

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