Effect of Diet on Enzyme Profile, Biochemical Changes and *In sacco* Degradability of Feeds in the Rumen of Buffalo

D. N. Kamra*, Sudipto Saha, Neeru Bhatt, L. C. Chaudhary and Neeta Agarwal
Microbiology Section, Centre of Advanced Studies in Animal Nutrition, Indian Veterinary Research Institute
Izatnagar-243 122, India

ABSTRACT : Four rumen fistulated Murrah buffaloes were used to study the effect of four diets differing in roughage to concentrate ratio on rumen biochemical changes, microbial enzyme profile and *in sacco* degradability of feed in a 4×4 Latin Square design. The animals were fed four diets consisting of 80:20, 70:30, 60:40 and 50:50 ratios of wheat straw and concentrate mixtures, respectively. Wheat straw and concentrate mixture were mixed with water (0.6 l/kg feed) and complete feed mixture was offered to the animals at 8:00 h and 16:00 h in two equal parts. The variation in pH of rumen liquor (difference of maximum and minimum during 0-8 h post feeding) increased with increasing level of concentrate mixture in the diet. There was no effect of diet composition on volatile fatty acids, total nitrogen and trichloro-acetic acid precipitable nitrogen in the rumen liquor, but ammonia nitrogen increased with increasing level of concentrate mixture in the ration. Major portions of all fibre degrading enzymes were present in the particulate material (PM) of the rumen contents, but protease was absent in PM fraction. The activities of micro-crystalline cellulase, acetyl esterase and protease increased with increase in the level of concentrate mixture, but the activities of other enzymes (carboxymethylcellulase, filter paper degrading activity, xylanase, β-glucosidase and β-xylosidase) were not affected. The *in sacco* degradability and effective degradability of feeds increased with increasing level of concentrate mixture in the ration. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 16, No. 3 : 374-379)***

Key Words : Rumen Enzymes, Buffalo, Enzyme Profile, ISDMD, Diet Effect, Rumen Fermentation, Roughage Effect, Cellulase, Xylanase, Acetyl Esterase

INTRODUCTION

The rumen microbial eco-system is very complex and is responsible for the bioconversion of lignocellulosic feeds into volatile fatty acids (Hungate, 1966; Hobson and Stewart, 1997) which serve as a source of energy for the ruminants. The phenotypic and genotypic diversity of the fibre degrading microbes makes it more difficult to understand the mechanism of fibre degradation in the rumen (McSweeney et al., 1999). The fibre degrading microbes (bacteria, protozoa and fungi) present in the rumen of domestic and wild ruminants excrete a large variety of different enzymes which are involved in hydrolysis of some or the other component of the fibrous portion of the roughage feeds (Agarwal et al., 1991; Borneman et al., 1991, 1992; Williams et al., 1994; Sahu and Kamra, 2002). Different strains of the same genus may have different capability to digest plant cell walls (Krause et al., 1998; Fujino et al., 1999; Lee et al., 1999; Singh et al., 2001) depending upon the individual enzyme profile of that strain. The enzyme profile of the rumen eco-system of the animals fed high roughage diets is very poorly understood as the chemical nature of feeds being offered to the animals is very complex. Thus a rate limiting step in fibre degradation *in vivo* is not well known, which impedes our ability to manipulate rumen eco-system for increasing fibre degradation. Therefore, the present experiment was conducted to study the rumen enzyme profile, biochemical changes and *in sacco* dry matter degradability of feeds on four diets differing in roughage content, so that the indicator enzymes, which are crucial for the degradability of roughage feeds, can be identified to be used in the future experiments on rumen manipulation for improving degradability of lignocellulosic feeds.

* Corresponding Author: D. N. Kamra. Tel: +91-581-442313, Fax: +91-581-447284, E-mail: kamra@ivri.up.nic.in
Received July 6, 2002; Accepted October 9, 2002

MATERIALS AND METHODS

Feeding of animals and experimental design

Four rumen fistulated Murrah buffaloes of body weight ranging from 180-242 kg, were fed four diets consisting of roughage (wheat straw) and concentrate mixture in the ratios of 80:20, 70:30, 60:40 and 50:50 respectively, in a 4×4 Latin Square design. Wheat straw and concentrate mixture, consisting of wheat bran 37, crushed maize grain 30, mustard seed cake 30, mineral mixture 2 and salt 1 parts, were mixed with water (0.6 l/kg feed) manually and offered to animals as complete feed mixtures at 8:00 h and 16:00 h in two equal parts. Each feeding period lasted 30 days, and the sampling of rumen liquor and rumen contents was done after 25 days of feeding for two consecutive days. The rumen liquor was collected at 0, 2, 4, 6 and 8 h post feeding. After recording pH the samples were pooled for the day

animal wise and used for biochemical analyses. The rumen contents sampled at 2 h post feeding were used for the estimation of various enzymes in three different fractions of rumen contents i.e. particulate material (PM), cellular (C) and extra-cellular (EC) fractions using the method described earlier (Agarwal et al., 2000). Wheat straw alone and wheat straw : concentrate mixture in the above four ratios were incubated in the rumen in nylon bags for 0, 4, 8, 12, 24, 36, 48, 72 and 96 h. Degradability of dry matter was estimated using Ørskov's model (Ørskov and McDonald, 1979).

Extraction of enzymes

The enzymes from the PM and C fractions of rumen contents were extracted as described by Hristov et al. (1999). The particulate material (5 g) was suspended in 25 ml phosphate buffer (0.1 M, pH 6.8), 5 ml of 0.4% lysozyme solution and 5 ml carbon tetrachloride were added to it. The suspension was incubated for 3 h at 40°C and the reaction was terminated by keeping it in ice followed by centrifugation at 27,000 g for 30 min at 4°C to get the clear supernatant which was used as enzyme source of particulate material (PM) of rumen contents.

For the extraction of enzymes from protozoal and bacterial fractions, both the pellets (Protozoal and Bacterial) were collectively suspended in the amount of phosphate buffer equal to the amount of extracellular fluid. To this suspension, each of 0.4% lysozyme solution and carbon tetrachloride were added at the rate of 5 ml/30 ml cell suspension and processed as described above for the particulate material. The clear supernatant thus collected was used as a source of cellular (C) enzymes.

Enzyme assay

For the estimation of carboxymethylcellulase, xylanase, and α-amylase, the reaction mixture contained : 1.0 ml phosphate buffer (0.1 M, pH 6.8), 0.5 ml carboxymethylcellulose (1%), 0.5 ml enzyme; 1.0 ml buffer, 0.5 ml xylan (0.25%), 0.5 ml enzyme; 0.5 ml buffer, 0.25 ml soluble starch (1%), 0.25 ml enzyme and incubated for 60, 15 and 30 min, respectively. The reaction was stopped by adding 3 ml of dinitrosalicylic acid reagent. For the estimation of micro-crystalline cellulase, 1% substrate (microcrystalline cellulose) suspension in the buffer was incubated at 10°C for 48 h (Tanaka et al., 1988). The 2 ml assay mixture consisted of 1.0 ml substrate suspension and 1.0 ml enzyme and incubated for 60 min with continuous gentle shaking. For filter paper degrading activity the assay mixture contained 1.0 ml buffer, 50 mg Whatman No. 1 filter paper and 1.0 ml enzyme with incubation of 60 min. The reducing sugars in all the above tests were estimated by dinitrosalicylic acid method as described by Miller (1959) taking glucose and xylose as standards. The enzyme activity was expressed as μmole of reducing sugars produced per minute under assay conditions. β-glucosidase and β-xylosidase activities were measured as described by Shewale and Sadana (1978) using p-nitrophenylglucopyranoside and p-nitrophenylxylopyranoside as substrates, respectively. The activity of acetyl esterase was estimated by the method of Huggins and Lapides (1947) with some modifications. The assay mixture contained 0.1 ml enzyme, 0.9 ml substrate (2 mM p-nitrophenyl acetate) and 1.0 ml phosphate buffer pH 6.0. After incubation of 10 min, the absorbance was recorded at 410 nm. The activities of β-glucosidase, β-xylosidase and acetyl esterase were expressed as μmole of p-nitrophenol released per minute under assay conditions. Protease activity was measured by using azocasein as substrate (Brock et al., 1982). The enzyme activity was expressed as μg protein hydrolysed per hour. For protein estimation the samples were treated with an equal amount of 20% trichloroacetic acid and the precipitate was dissolved in 1.0 M NaOH and protein was estimated as per the method of Lowry et al. (1951). Specific activities of the enzymes were expressed as units/mg protein.

Biochemical analyses

The rumen liquor samples were analysed for ammonia-N, total nitrogen and TCA-precipitable nitrogen by micro Kjeldahl method as described by AOAC (1988). Volatile fatty acids were estimated using Nucon-5765 gas chromatograph equipped with a double flame ionization detector as per method described by Cottyn and Boucque (1968) using a glass column of 4 feet length packed with chromosorb with gas flow rates for nitrogen, hydrogen and air of 30, 30 and 320 ml/min, respectively.

Statistical analysis

The experiment was conducted in a 4×4 Latin Square design and data on nutrient intake, rumen fermentation and enzyme activities were analysed by one way analysis of variance using ANOVA. The data on *in sacco* studies were analysed using the model of Ørskov and McDonald (1979). For this the Jandel Sigmastat Statistical software Version2 was used. All the statistical analyses were done as per methods described by Snedecor and Cochran (1967). Unless otherwise stated the significance was measured at $p<0.05$.

RESULTS AND DISCUSSION

The intake of dry matter by the animals of different groups did not vary much (Table 1), but crude protein and total digestible nutrients intake increased with increasing level of concentrate mixture in the ration. The results of the experiment indicated that the animals maintained a steady

Table 1. Effect of roughage:concentrate ratio on dry matter intake

	Roughage:concentrate ratio			
	80:20	70:30	60:40	50:50
Body weight (kg)	207.5 ±12.58	207.8 ±12.45	205.5 ±13.22	205.67 ±14.57
Dry matter intake				
kg/d	3.33±0.08	3.35±0.06	3.30±0.08	3.73±0.16
g/kg $W^{0.75}$	60.91±4.08	61.21±1.55	60.79±2.31	68.67±7.25
% body weight	1.63±0.14	1.62±0.07	1.62±0.08	1.88±0.23
Crude protein intake (kg/d)	0.21	0.26	0.31	0.41
Total digestible nutrient intake (kg/d)	1.53	1.64	1.71	2.05

Table 2. Effect of roughage:concentrate ratio on changes in pH of rumen liquor

Time (h)	Roughage:concentrate ratio			
	80:20	70:30	60:40	50:50
0	7.03	7.04	7.13	7.16
2	6.94	6.96	6.96	6.96
4	6.90	6.87	6.78	6.72
6	6.87	6.84	6.75	6.74
8	6.90	6.87	6.75	6.73
Variation in pH (Max-Min)	0.16	0.20	0.38	0.44

Table 3. Effect of roughage:concentrate ratio on changes in biochemical characteristics of rumen liquor

Parameters	Roughage:concentrate ratio			
	80:20	70:30	60:40	50:50
TVFA (mM/100 ml)	5.81±0.20	6.31±0.42	6.56±0.26	6.17±0.24
Molar proportion				
Acetate	74.42	73.47	73.96	71.08
Propionate	21.03	20.98	21.08	22.77
Butyrate	4.56	5.55	4.97	6.16
Total-N (mg/100 ml)	50.5±3.92	55.4±4.70	56.25±3.40	61.66±6.14
Ammonia-N (mg/100 ml)	11.71±1.41[a]	13.88±1.81[a]	17.20±3.61[ab]	24.17±4.39[b]
TCA-ppt N (mg/100 ml)	27.03±2.40	26.88±1.98	26.26±1.87	29.27±1.68

[a,b] Averages in a row with different superscripts differ significantly ($p<0.05$).

state fermentation pattern with minimal variations in the biochemical parameters with the passage of time as the animals were fed a complete diet in the form of wet mixture (wheat straw and concentrate mixed with water before offering the animals). Minimum pH was observed 6 h post feeding, but the pH never dropped to lower than 6.7 in any of the feeding schedules, which might be perhaps due to lower content of maize grain (varying between 6-15% in all the diets). Thus no adverse effect of pH on fermentation is expected as the cellulolytic microbes are inhibited if these are exposed to a pH lower than 6.3 for a considerable time (Russell and Dombrowski, 1980; Hiltner and Dehority, 1983). The drop in pH from 0 h was observed as minimum with 80:20 ratio of roughage to concentrate and the drop increased continuously with increase in the proportion of concentrate in the ration (Table 2). There was a slight increase in total volatile fatty acids production with increase in concentrate, but the differences were statistically non significant ($p>0.05$) (Table 3). The molar proportion of various volatile fatty acids was also not affected with the change in concentrate ratio in the diet. The ammonia nitrogen increased significantly ($p<0.05$) with increase in concentrate in the diet. Similarly, the levels of total nitrogen and TCA precipitable nitrogen also increased, but the differences were non-significant ($p>0.05$). The increase in nitrogen fractions might be due to increase in the nitrogen intake by the animals with increasing proportion of concentrate in the diet.

Fibre degradation in the rumen gets initiated by attachment of fibrolytic microbes with the feed particles, which is an essential pre-requisite (Akin, 1979; Morris and Cole, 1987). In an earlier experiment in our laboratory and results from the other laboratories, it has been shown that fibrolytic enzymes are maximum in the particulate fraction (partially digested fibrous feed particles in the rumen) (Williams et al., 1989; Huhtanen and Khalili, 1992; Agarwal et al., 2000; Ha et al., 2002). Similarly in the present experiment too it has been observed that major portions (>80%) of the total activity of fibrolytic enzymes were present in the particulate fraction, except protease which was absent in this fraction. This might be due to the fact that cellulose degrading bacteria are usually protease negative (Ushida et al., 1997) and majority of the feed attached bacteria are cellulose degrading bacteria. Therefore protease activity was absent in the particulate matter of the rumen content. There was no effect of diet on carboxymethyl cellulase, filter paper degrading activity, xylanase, β-glucosidase and β-xylosidase, but the total and specific activities of micro-crystalline cellulase, acetyl esterase and protease increased significantly ($p<0.05$) with increasing level of concentrate in the diet. Maximum micro-crystalline activity in the rumen contents and the PM fraction was found when the highest level of concentrate was fed, while the activities of acetyl esterase and protease were highest when the animals were fed roughage to concentrate ratio of 60:40 (Table 4). Similar results have been reported by van der Linden et al. (1984) who showed that there was an increase in the number of cellulose degrading bacteria (corresponding to increase in micro-crystalline cellulase and acetyl esterase activities in our study) with increase of corn in the diet, but the number of hemicellulose degrading bacteria was not affected (corresponding to xylanase and β-xylosidase in this experiment). On the contrary, Hristov et al. (1999) reported that there was a depression in carboxymethylcellulase and

Table 4. Effect of roughage:concentrate ratio on changes in enzyme activities of rumen liquor

Fraction	Roughage:concentrate ratio			
	80:20	70:30	60:40	50:50
		Carboxymethylcellulase		
PM*	32.19±4.08 (0.281)**	32.35±4.07 (0.300)	33.03±3.50 (0.374)	36.19±3.64 (0.430)
C	2.31±0.23 (0.179)	1.92±0.34 (0.131)	1.85±0.22 (0.101)	2.40±0.39 (0.126)
EC	0.70±0.12 (0.045)	0.51±0.06 (0.094)	0.66±0.08 (0.068)	0.78±0.18 (0.066)
Total	35.20±4.31	34.78±4.38	35.53±3.68	39.38±3.94
		Microcrystalline cellulase		
PM	11.11±1.73^{a} (0.131)	12.93±2.20^{ab} (0.144)	15.53±2.60^{ab} (0.199)	18.39±2.47^{b} (0.147)
C	1.30±0.16 (0.093)	1.47±0.27 (0.080)	1.44±0.23 (0.065)	1.53±0.48 (0.121)
EC	0.45±0.12 (0.043)	0.58±0.16 (0.042)	0.58±0.15 (0.044)	0.63±0.14 (0.064)
Total	12.86±1.90^{a}	14.98±2.50^{ab}	17.55±2.95^{ab}	20.55±2.50^{b}
		Filter paper degrading activity		
PM	7.09±1.92 (0.072)	9.08±1.18 (0.065)	9.80±1.69 (0.090)	10.05±1.28 (0.104)
C	0.53±0.14 (0.030)	0.56±0.14 (0.032)	0.82±0.13 (0.052)	0.79±0.14 (0.032)
EC	0.29±0.09 (0.016)	0.26±0.05 (0.023)	0.23±0.05 (0.021)	0.37±0.09 (0.028)
Total	7.91±1.89	9.90±1.15	10.85±1.82	11.21±1.41
		Xylanase		
PM	97.16±11.77 (1.037)	110.11±5.14 (1.045)	99.89±8.60 (1.060)	83.80±9.23 (0.811)
C	6.34±0.55 (0.344)	7.16±0.71 (0.470)	6.81±1.13 (0.477)	7.01±0.97 (0.373)
EC	3.18±0.42 (0.274)	2.98±0.31 (0.280)	3.41±0.43 (0.300)	3.66±0.59 (0.265)
Total	106.68±11.74	120.25±5.61	110.11±9.05	94.47±9.36
		Amylase		
PM	73.51±17.61 (0.621)	49.75±12.97 (0.550)	73.34±13.58 (1.011)	60.06±13.09 (0.245)
C	21.18±4.79^{b} (0.869)	10.66±1.62^{a} (0.661)	15.05±3.30^{ab} (0.632)	9.84±2.73^{a} (0.559)
EC	8.18±1.95^{ab} (0.538)	3.90±0.57^{a} (1.615)	6.49±1.61^{ab} (0.913)	12.40±4.24^{b} (0.376)
Total	102.87±16.73	64.31±11.85	94.88±15.74	82.30±15.30
		β-glucosidase		
PM	1.79±0.20 (0.025)	2.08±0.38 (0.070)	1.98±0.32 (0.025)	2.56±0.47 (0.005)
C	0.20±0.05 (0.025)	0.19±0.06 (0.011)	0.27±0.09 (0.011)	0.20±0.09 (0.012)
EC	0.29±0.13 (0.029)	0.11±0.02 (0.017)	0.13±0.02 (0.014)	0.10±0.02 (0.012)
Total	2.28±0.28	2.38±0.36	2.38±0.36	2.86±0.53
		β-xylosidase		
PM	3.36±1.00 (0.039)	2.17±0.61 (0.016)	1.52±0.14 (0.014)	1.54±0.55 (0.009)
C	0.36±0.11 (0.046)	0.23±0.09 (0.011)	0.15±0.03 (0.014)	0.15±0.02 (0.009)
EC	0.15±0.04 (0.058)	0.17±0.05 (0.023)	0.12±0.02 (0.011)	0.13±0.03 (0.015)
Total	3.87±1.15^{b}	2.57±0.54^{ab}	1.79±0.16^{a}	1.82±0.56^{ab}
		Acetyl esterase		
PM	2.11±0.39^{a} (0.014)	2.58±0.17^{ab} (0.024)	3.28±0.29^{b} (0.032)	2.59±0.13^{ab} (0.034)
C	0.24±0.03 (0.012)	0.21±0.09 (0.013)	0.26±0.02 (0.016)	0.28±0.05 (0.014)
EC	0.14±0.02 (0.019)	0.17±0.003 (0.017)	0.19±0.02 (0.030)	0.17±0.03 (0.010)
Total	2.49±0.42^{a}	2.96±0.17^{ab}	3.73±0.31^{b}	3.04±0.18^{ab}
		Protease		
PM	0	0	0	0
C	873±121 (86.62)	1,010±50 (59.70)	1,124±103 (63.38)	983±231 (47.29)
EC	454±66 (43.69)	454±57 (64.06)	565±78 (47.90)	480±135 (39.17)
Total	1,327±158^{a}	1,464±98^{ab}	1,689±175^{b}	1,463±304^{ab}

^{a,b} Averages in a row with different superscripts differ significantly ($p<0.05$).

* PM-particulate material, C-cellular fraction, EC-extra-cellular fraction.

** Values in parenthesis indicate specific activity (u/mg protein).

xylanase activities and an increase in amylase activity on a shift from forage based diet to high grain based diet. The composition of two diets in their experiment was extremely diverse containing either 83% forage or 91% grain in the two rations, while in our experiment the grain content varied only between 6-15% of the total ration in the four diets, thus there were not diverse changes in the enzyme profile and biochemical characteristics among the four groups.

The *in sacco* dry matter degradability of wheat straw was not affected with the change of diet (Figure 1a), but the *in sacco* dry matter degradability of feeds (wheat straw+concentrate mixtures) increased with increasing level of concentrate in the diet (Figure 1b). The effective degradability of concentrate+wheat straw increased significantly ($p<0.05$) with increasing level of concentrate mixture in the ration and there was no further increase when the concentrate level was increased beyond 40% in the ration (Table 5). The production of micro-crystalline cellulase and acetyl esterase increased along with an increase *in sacco* degradability and effective degradability of feed. This finding is similar to earlier reports of Silva et al. (1987) and Huhtanen and Khalili (1992) who observed a positive correlation between particle associated carboxymethylcellulase and xylanase activities with cellulose and hemicellulose degradation in the nylon bags incubated in the rumen. But in our experiment we did not find any correlation of these two enzymes with increase in degradation of feed. This might be due to the reason that the level of non fibre carbohydrates did not vary much (6-15%) in the dry matter intake of the animals on four different diets, which was too low to affect rumen fermentation and bring about drastic changes in the enzyme profile of the rumen. This was perhaps the increasing nitrogen content with every additional dose of concentrate mixture that increased the activities of some of the enzymes and degradability of feed. When the nitrogen intake reached the level, required by the animals for maintenance (at 60:40 ratio of roughage:concentrate mixture) further increase in the activities of some of these enzymes like acetyl esterase and protease was not observed.

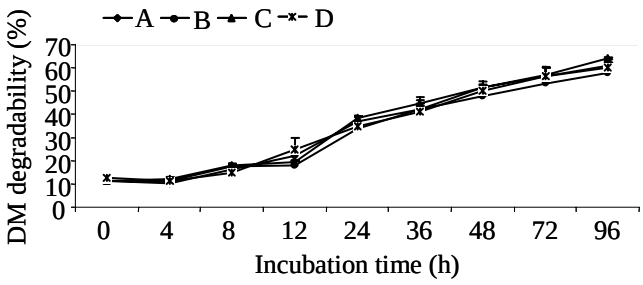

Figure 1A. *In sacco* DM degradability of wheat straw incubated in the rumen of buffaloes fed different ratios of wheat straw and concentrate mixture (A-80:20, B-70:30, C-60:40, D-50:50).

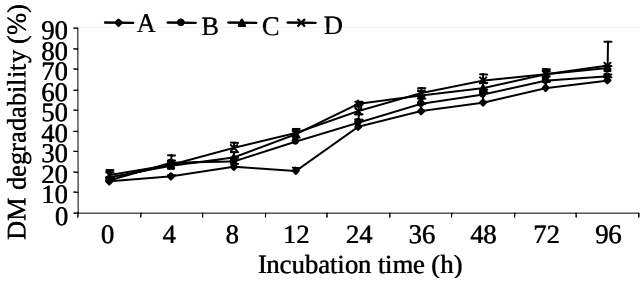

Figure 1B. *In sacco* DM degradability of complete feed incubated in the rumen of buffaloes fed on respective ratios of wheat straw and concentrate mixture (A-80:20, B-70:30, C-60:40, D-50:50).

Table 5. Effect of roughage : concentrate ratio on ISDMD and effective degradability (ED) of wheat straw and wheat: concentration ratios in rumen

	Roughage:concentrate ratio			
	80:20	70:30	60:40	50:50
Wheat straw				
a	7.21±0.91	8.72±0.92	7.54±1.01	7.92±1.27
b	60.03±2.10	57.69±4.86	61.23±3.23	61.18±0.74
c	0.026±0.003	0.024±0.003	0.026±0.003	0.023±0.003
a+b	67.24±2.75	66.41±4.66	68.77±3.99	69.09±1.88
ED (%)	27.94±1.90	26.67±0.59	26.28±0.40	26.72±2.57
Wheat straw+conc. ratios				
a	12.97±1.60	14.86±1.68	16.45±2.40	17.21±2.66
b	57.39±2.63	56.06±1.50	54.61±2.74	56.58±2.47
c	0.029±0.005	0.034±0.006	0.040±0.003	0.036±0.005
a+b	70.35±3.11	71.81±2.25	71.06±0.72	72.50±1.03
ED (%)	33.19±1.45[a]	36.93±1.81[ab]	40.49±0.47[b]	40.87±1.63[b]

[a,b] Means with different superscripts in a row differ significantly ($p<0.05$).
a-completely degradable portion, b-insoluble, but potentially degradable portion, c-rate of degradation, a+b=potential degradability, ED=effective degradability.

On the basis of the results obtained in this experiment, it can be concluded that inclusion of non fibre carbohydrates upto 15% in diet is not sufficient to bring about any significant changes in the rumen microbial ecosystem and the biochemical parameters. Thus to obtain a definite information on the indicator enzymes for fibre degradation, further experiments should be conducted by feeding diversely variable diets containing different amounts of non fibre carbohydrates in the ration.

ACKNOWLEDGEMENTS

The funds provided by National Agricultural Technology Project PSR No. 29 for this study is duly acknowledged.

REFERENCES

Agarwal, N., I. Agarwal, D. N. Kamra and L. C. Chaudhary. 2000. Diurnal variations in the activities of hydrolytic enzymes in

different fractions of rumen liquor of Murrah buffalo. J. Appl. Anim. Res. 18:73-80.

Agarwal, N., N. Kewalramani, D. N. Kamra, D. K. Agrawal and K. Nath. 1991. Hydrolytic enzymes of buffalo rumen: Comparison of cell free rumen fluid, bacterial and protozoal fractions. Buffalo J. 7:203-207.

Akin, D. E. 1979. Microscopic evaluation of fibre digestion by rumen microorganisms : A review. J. Anim. Sci. 48:701-710.

AOAC. 1988. Official Methods of Analysis, 16th edn., Association of Official Analytical Chemists, Washington, DC.

Borneman, W. S., L. G. Ljungdahl, R. D. Hartley and D. E. Akin. 1991. Isolation and characterization of p-coumaroyl esterase from the anaerobic fungus *Neocallimastix* strain MC-2. Appl. Environ. Microbiol. 57:2337-2344.

Borneman, W. S., L. G. Ljungdahl, R. D. Hartley and D. E. Akin. 1992. Purification and partial characterization of two feruloyl esterases from the anaerobic fungus *Neocallimastix* strain MC-2. Appl. Environ. Microbiol. 58:3762-3766.

Brock, F. M., C. L. Forsberg and J. G. Buchanan-Smith. 1982. Proteolytic activity of rumen microorganisms and effects of proteinase inhibitors. Appl. Environ. Microbiol. 44:561-569.

Cottyn, B. G. and C. V. Boucque. 1968. Rapid method for the gas-chromatographic determination of volatile fatty acids in rumen fluid. J. Agric. Food Chem. 16:105-107.

Fujino, Y. and K. Ushida. 1999. Plant cell wall degradation and glycanase activity of the rumen anaerobic fungus *Neocallimastix frontalis* MCH3 grown on various forages. Asian-Aus. J. Anim. Sci. 12:752-757.

Ha, J. K., S. S. Lee, M. Goto, Y.H. Moon and K. J. Cheng. 2002. Influence of tween 80 on the enzyme distribution in rumen liquor and on the growth of rumen bacteria and fungi. J. Appl. Anim. Res. 21:129-143.

Hiltner, P. and B. A. Dehority. 1983. Effect of soluble carbohydrates on digestion of cellulose by pure cultures of rumen bacteria. Appl. Environ. Microbiol. 46:642.

Hobson, P. N. and C. S. Stewart. 1997. The Rumen Microbial Ecosystem. Blackie Academic & Professional, London.

Hristov, A. N., T. A. McAllister and K. J. Cheng. 1999. Effect of diet, digesta processing, freezing and extraction procedure on some polysaccharide degrading activities of ruminal contents. Can. J. Anim. Sci. 79:73-81.

Huggins, C. and J. Lapides. 1947. Acetyl esters of p-nitrophenol as substrate for the colorimetric determination of esterase. J. Biol. Chem. 170:467-482.

Huhtanen, P. and H. Khalili. 1992. The effect of sucrose supplements on particle associated carboxymethylcellulase and xylanase activities in cattle given grass silage based diet. Brit. J. Nutr. 67:245-255.

Hungate, R. E. 1966. Rumen and its Microbes, Academic Press, New York.

Krause, D. O., R. J. Bunch, W. J. M. Smith and C. S. McSweeney. 1998. Diversity of *Ruminococcus* strains : a survey of genetic polymorphism and plant digestibility. J. Appl. Microbiol. 86: 487-495.

Lee, S. S., K. J. Shin, W. Y. Kim, J. K. Ha and I. K. Han. 1999. The rumen eco-system as a fountain source of novel enzymes. Asian-Aust. J. Anim. Sci. 12:988-1001.

Lowry, O. H., N. J. Rosenbrough, A. L. Farr and R. C. Randall. 1951. Protein measurement with the Folin-phenol reagent. J. Biol. Chem. 183:265.

McSweeney, C. S., B. P. Dalrymple, K. S. Gobius, P. M. Kennedy, D. O. Krause, R. I. Mackie and G. P. Xue. 1999. Theapplication of rumen biotechnology to improve the nutritive value of fibrous feedstuffs : pre- and post-ingestion. Livestock Prod. Sci. 59:265-283.

Miller, G. L. 1959. Modified DNS method for reducing sugars. Anal. Chem. 31:426-428

Morris, J. and O. J. Cole. 1987. Relationship between cellulolytic activity and adhesion to cellulose in *Ruminococcus albus*. J. Gen. Microbiol. 133: 53-69.

Ørskov, E. R. and I. McDonald. 1979. The estimation of protein degradability in rumen from incubation measurements weighted according to rate of passage. J. Agric. Sci. (Camb.) 92:499-503.

Russell, J. B. and D. B. Dombrowski. 1980. Effects of pH on the efficiency of growth by pure cultures of rumen bacteria in continuous culture. Appl. Environ. Microbiol. 34:604.

Sahu, N. P. and D. N. Kamra. 2002. Microbial eco-system of the gastro-intestinal tract of wild herbivorous animals. J. Appl. Anim. Res. 21:207-230.

Shewale, J. G. and J. C. Sadana. 1978. Cellulase and β-glucosidase production by a basidomycete species. Can. J. Microbiol. 24:1204-1216.

Silva, A. T., R. J. Wallace and E. R. Ørskov. 1987. Use of particle bound microbial activity to predict the rate and extent of fibre degradation in the rumen. Br. J. Nutr. 57:407-415.

Singh, B., T. K. Bhat and B. Singh. 2001. Exploiting gastro-intestinal microbes for livestock and industrial development: A review. Asian-Aust. J. Anim. Sci. 14:567-586.

Snedecor, G. W. and W. G. Cochran. 1967. In: Statistical Methods 6th ed. Oxford IBH Publishing, Calcutta.

Tanaka, M., M. Taniguchi, R. Matsuno and T. Kamikubo. 1988. Cellulases from *Eupenicillium javanicum*. Methods in Enzymology 160:251-259.

Ushida, K., H. Matsui, Y. Fujino and J. K. Ha. 1997. Role and potential of ruminal fungi in fibre digestion. A review. Asian-Aust. J. Anim. Sci. 10: 541-550.

Van der Linden, Y., N. O. van Gylswyk and H. M. Schwatz. 1984. Influence of supplementation of corn stover with corn grain on the fibrolytic bacteria in the rumen of sheep and their relation to the intake and digestion of fibre. J. Anim. Sci. 59:772-783.

Williams, A. G., S. E. Withers and C. G. Orpin. 1994. Effect of the carbohydrate growth substrate on polysaccharolytic enzyme formation by anaerobic fungi isolated from the foregut and hindgut of non ruminant herbivores and the forestomach of ruminants. Letters Appl. Microbiol. 18:147-151.

Williams, A. G., S. E. Withers and N. H. Strachan. 1989. Postprandial variations in the activity of polysaccharide degrading enzymes in microbial populations from the digesta solids and liquor fractions of rumen contents. J. Appl. Bacteriol. 66:15-26.