Effect of Cellulose Degrading Bacteria Isolated from Wild and Domestic Ruminants on *In vitro* Dry Matter Digestibility of Feed and Enzyme Production

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ABSTRACT : Cellulolytic bacterial strains have been isolated from the faeces of wild (blackbuck, *Antilope cervicapra*; nilgai, *Baselophus tragocamelus*; chinkara, *Gazella gazella*; spotted deer, *Axis axis* and hog deer, *Cervus porcinus*) and rumen liquor of domestic (sheep, *Ovis aries*) ruminants. Five best cellulose degrading bacterial isolates (*Ruminococcus* sp.) were used as microbial feed additive along with buffalo rumen liquor as inoculum to study their effect on digestibility of feed and enzyme production in *in vitro* conditions. The bacterial isolate from chinkara (CHI-2) showed the highest per cent apparent dry matter (DM) digestibility (35.40±0.60), true dry matter digestibility (40.80±0.69) and NDF (26.38±0.83) digestibility ($p<0.05$) compared to control (32.73±0.56, 36.64±0.71 and 21.16±0.89, respectively) and other isolates at 24 h of incubation with lignocellulosic feeds (wheat straw and wheat bran, 80:20). The same isolate also exhibited the highest activities of fibre degrading enzymes like carboxymethylcellulase, xylanase, β-glucosidase and acetyl esterase. The bacterial isolate from chinkara (*Gazella gazella*) appears to have a potential to be used as feed additive in the diet of ruminants for improving utilization of nutrients from lignocellulosic feeds. ***(Asian-Aust. J. Anim. Sci. 2003. Vol 17, No. 2 : 199-202)***

Key Words : Rumen Bacteria, *Ruminococcus* sp., Enzymes, Cellulose Degradation, Wild Ruminants, *In Vitro* Digestibility

INTRODUCTION

Manipulation of the rumen microbial ecosystem is one of the major thrust areas of research in animal nutrition to improve the utilization of fibrous feeds. Many researchers have reported that the digestibility of feeds in wild ruminants is higher than their domestic counterparts (Francoise Domingue et al., 1991, Latupeinissa and Dryden, 1998, Ru et al., 2002) and the information on microbiota of wild herbivores has been compiled recently in a review by Sahu and Kamra (2002). Rumen microbes of wild animals may be expected to digest fibrous feeds more efficiently as these animals have to survive in adverse conditions in the forest and have to extract energy from the poor quality feeds available to them.

Among the rumen microbes, bacteria play a major role in fermentation of feeds because of their numerical predominance and metabolic diversity (Cheng et al., 1991). They contribute a large portion (57% of total) of the cellulase enzymes present in the rumen (Agarwal et al., 1991). Selective isolation and introduction of an efficient bacterial isolate from wild ruminant into domestic ruminant may improve nutrient utilization in the later.

Therefore, present study has been conducted to evaluate the digestibility of feed and enzyme production on lignocellulosic feeds by some selected bacterial isolates from domestic and wild ruminants in *in vitro* conditions, to identify a superior bacterial strain, which can be used as a microbial feed supplement to manipulate rumen microbial ecosystem of the domestic animals.

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MATERIALS AND METHODS

Isolation of cellulolytic bacteria

Freshly voided faeces (within 2 h of defacation) were collected from wild animals like chinkara (*Gazella gazella*), nilgai (*Baselophus tragocamelus*), hog deer (*Cervus porcinus*), spotted deer (*Axis axis*), and black buck (*Antilope cervicapra*), kept under captivity at wildlife park of Indian Veterinary Research Institute, Izatnagar for the isolation of cellulolytic bacteria. Rumen liquor was collected from sheep (*Ovis aries*) from the farms of Indian Veterinary Research Institute, Izatnagar kept under stall fed conditions.

Culture medium for isolation of anaerobic cellulolytic bacteria was prepared as described by Bryant and Burkey (1953). Carboxymethylcellulose (0.5%) was used as the only energy source in the culture medium (CMC medium) for isolation of cellulose degrading bacteria. Faecal pellets were cut into two pieces with a sterile knife and the central core of the pellet was used as inoculum in the Hungate tube containing selective CMC medium and incubated for 48 h at 39°C inside the anaerobic workstation (Don Whitley, model Compact). Similarly 1ml of fresh rumen liquor (stored under oxygen free CO_2) was inoculated in CMC medium for enrichment of cellulose degrading bacteria.

After 48 h of incubation, enriched cultures were diluted up to 10^{-5} to 10^{-7} with anaerobic dilution fluid and streaked on the agar plate of selective CMC medium. Isolated colonies from the highest dilution plate were selectively picked-up and transferred into CMC broth. After 24 h of growth the culture was again streaked on the same medium and the procedure was repeated thrice to get pure cultures of bacterial isolates.

Pure cultures of the bacterial isolates were scrutinized to select only cellulolytic bacteria for further studies. Extracellular CMCase activity of the supernatant of the different cultures was tested by using Congo-red solution in the agar plate by cup diffusion method as described by Teather and Wood (1982). Those bacterial isolates were rejected which showed no zone of cellulose hydrolysis. On the basis of the morphological characteristics, biochemical tests, sugar utilization, end product formation and enzyme profile of the isolates, the five best isolates selected for this experiment have been identified as *Ruminococcus* sp. (Sahu, 2002).

***In vitro* dry matter digestibility**

Five best bacterial isolates were selected on the basis of the production of different fibrolytic enzymes like, carboxymethylcellulase, avicelase, xylanase, β-glucosidase and acetyl esterase on a medium containing lignocellulosic feeds (wheat straw and wheat bran, 80:20) (Sahu, 2002). The selected five isolates were used to study the dry matter (DM) digestibility of feed by the method described by Tilley and Terry (1963) and modified by Goering and Van Soest (1989).

One litre of Mc Dougall's buffer was heated on low flame in a 2 L capacity round bottom flask, containing the reducing agent. Oxygen free carbon dioxide was continuously bubbled through the contents of the flask. At room temperature, 37.5 ml of buffer was transferred to each flask containing 0.5 g of substrate (wheat straw and wheat bran, 80:20). Bacterial inoculum (2.5 ml culture with OD_{600} equal to 0.6) and 10 ml rumen liquor were transferred to each of the flasks under anaerobic conditions. In control 10 ml rumen liquor and 2.5 ml sterile medium were used. All the flasks were properly closed with rubber stoppers to make air tight and incubated at 24 and 48 h to study the dry matter (DM) digestibility. The residue was estimated after drying and compared with initial dry weight to calculate the DM digestibility (apparent dry matter digestibility). True dry matter digestibility (TD) and NDF (Neutral detergent fibre) digestibility were calculated (Van Soest and Robertson, 1985) as given below:

Apparent DM digestibility=(100-residue weight)/(initial weight of substrate)×100

The residue was further treated with NDF solution to determine true digestibility and NDF digestibility as given below:

True DM digestibility (TD)=(100-NDF residue)/(Initial weight of substrate)×100

NDF digestibility=(NDF at'0 h'-NDF at '24 or 48 h')/(NDF at '0 h')×100

Enzyme estimation

The contents of the above flasks were filtered through sintered glass crucible (Grade 1) and twenty ml of filtrate was collected and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was stored at -20°C and used to determine the enzyme activities like carboxymethylcellulase (CMCase) and xylanase as described earlier (Agarwal et al., 2000; Kamra et al., 2003) with a few modifications as described below. For carboxymethylcellulase and xylanase estimation, the reaction mixture contained: 1 ml phosphate buffer and 0.5 ml of either carboxy methyl cellulose (1.0% in 0.1 M phosphate buffer, pH 6.8) or xylan (0.25% in 0.1M phosphate buffer, pH 6.8) and 0.5 ml enzyme. The reaction mixture was incubated for 60 and 15 min for CMCase and xylanase respectively, at 39°C and the reducing sugars released were estimated (Miller, 1959). The activities (mIU) of enzymes were expressed as nano mole of reducing sugars produced per minute per ml of the enzyme under assay conditions.

β-glucosidase was estimated by using p-nitrophenol-β-D-glucopyranoside as substrate (Shewale and Sadana, 1978). Acetyl esterase was estimated by using p-nitrophenyl acetate as substrate (Johnson et al., 1988). The activities (mIU) of β-glucosidase and acetylesterase were expressed as nano mole of p-nitrophenol released per minute per ml of the enzyme under assay conditions.

Statistical analyses

All the data were analysed by one way ANOVA and differences between the means were compared by Duncan multiple range test as described by Snedecor and Cochran (1989).

RESULTS AND DISCUSSION

The extent of fibre degradation in the rumen depends upon the capability of individual microbes present in the microbial consortium. Inclusion of a superior fibre degrading microbe may affect positively the feed utilization. Several bacterial isolates from wild and domestic ruminants have been screened and tested for their ability to degrade cellulose in petri dish and five best strains were selected for testing their efficiency to degrade lignocellulosic feeds and fibre degrading enzyme production in *in vitro* conditions.

Table 1. Effect of addition of bacterial isolates on *in vitro* dry matter digestibility and enzyme activities at 24 h post incubation

Digestibility (%)	Control	Isolates					SEM	Sig.	P-value
		NG-3*	NG-4*	CHI-2*	BB-12*	S-14*			
Apparent DM digestibility	32.73^{ab}±0.56	30.41^{bc}±1.16	32.71^{b}±1.03	35.40^{a}±0.60	28.77^{c}±0.90	32.40^{b}±1.25	0.48	***	0.000
True DM digestibility	36.64^{b}±0.71	34.74^{b}±1.30	36.67^{b}±0.93	40.80^{a}±0.69	34.26^{b}±0.80	36.16^{b}±1.09	0.48	***	0.000
NDF digestibility	21.16^{b}±0.89	18.98^{b}±1.61	21.24^{b}±1.15	26.38^{a}±0.83	18.38^{b}±0.99	20.71^{b}±1.37	0.59	***	0.000
Enzymes (mIU/ml)									
CMCase	5.22^{bc}±0.50	6.38^{ab}±0.23	6.05^{b}±0.32	7.45^{a}±0.39	4.68^{c}±0.13	6.21^{b}±0.43	0.25	**	0.002
Xylanase	13.11^{bc}±0.50	14.37^{b}±2.18	21.16^{a}±1.15	21.16^{a}±1.31	8.07^{d}±0.66	9.34^{cd}±1.01	1.32	***	0.000
β-glucosidase	28.26^{b}±2.19	32.28^{b}±1.27	26.35^{b}±2.75	39.28^{a}±1.65	30.17^{b}±0.87	31.99^{b}±3.21	1.24	*	0.018
Acetyl esterase	50.06^{ab}±2.38	40.25^{bc}±0.36	50.43^{ab}±5.35	57.33^{a}±3.93	30.81^{c}±2.38	59.51^{a}±7.15	2.80	**	0.004

Mean values with same superscripts in a row are not significantly different ($p>0.05$).

* NG-3 and NG-4; from nilgai, CHI-2; from chinkara, BB-12: from blackbuck and S-14 from sheep.

Table 2. Effect of addition of bacterial isolates on *in vitro* dry matter digestibility and enzyme activities at 48 h post incubation

Digestibility (%)	Control	Isolates					SEM	Sig.	P-value
		NG-3*	NG-4*	CHI-2*	BB-12*	S-14*			
Apparent DM digestibility	49.17±0.82	49.51±1.97	49.03±1.43	48.91±0.83	47.73±1.03	46.61±1.71	0.53	NS	0.667
True DM digestibility	53.30±1.48	57.38±1.25	56.75±1.08	56.29±1.18	55.34±0.24	55.35±0.75	0.48	NS	0.169
NDF digestibility	41.66±1.88	46.98±1.53	46.26±1.32	45.55±1.52	44.25±0.34	44.23±0.98	0.61	NS	0.135
Enzymes (mIU/ml)									
CMCase	3.10^{bc}±0.31	2.66^{c}±0.42	3.07^{bc}±0.22	6.07^{a}±0.58	4.10^{b}±0.14	2.55^{c}±0.40	0.32	***	0.000
Xylanase	8.93±0.64	15.92±3.26	17.21±1.47	12.75±2.23	11.68±1.81	11.05±2.12	0.99	NS	0.119
β-glucosidase	58.23^{a}±2.98	21.96^{c}±1.56	20.21^{c}±3.50	39.38^{ab}±4.91	22.27^{c}±0.88	29.64^{bc}±8.04	3.58	***	0.000
Acetyl esterase	103.48^{b}±10.48	151.44^{a}±16.84	116.20^{b}±5.37	117.65^{b}±4.42	110.02^{b}±6.33	86.38^{b}±10.72	5.86	**	0.014

Mean values with same superscripts in a row are not significantly different ($p>0.05$).

* NG-3 and NG-4; from nilgai, CHI-2; from chinkara, BB-12; from blackbuck and S-14 from sheep.

Out of these best five selected strains, four were from wild ruminants (two from nilgai, one each from chinkara and blackbuck) and only one from domestic sheep. The bacterial isolate from sheep did not affect significantly any of the parameters studied in this experiment while most of the isolates from wild animals affected feed digestibility and fibrolytic enzyme production positively.

The apparent DM digestibility (AD) of the feed (a mixture of 80% wheat straw and 20% wheat bran) was affected by using different bacterial isolates as additive along with the buffalo rumen liquor as inoculum in an *in vitro* experiment. Bacterial isolate from chinkara (CHI-2) showed the highest DM digestibility followed by control and NG-4 (an isolate from nilgai). Other isolates did not exhibit any effect on AD as compared to control. The AD of BB-12 was significantly lower ($p<0.05$) than the control. The true and NDF digestibilities were significantly higher ($p<0.001$) in CHI-2 inoculated samples than the control and treatment with other bacterial isolates.

The digestive ability of the cellulolytic bacteria depends on their ability to adhere to the substrate as reported by Tarakanov and Lavlinskii (1998). They have classified 58 cellulolytic bacterial strains isolated from cow and classified them as high, medium and low activity strains, hydrolyzing the substrate in 50, 60-80 and 100 h, respectively. Thus, it appears that the isolate CHI-2 may have close substrate affinity that could induce higher AD, TD and NDF digestibilities during 24 h of incubation. But at 48 h of incubation the differences among the treatments were not significant ($p>0.05$). This might be due to accumulation of certain end products of fermentation in the reaction mixture which might have inhibited the further degradation of feed. This is also supported by a significant reduction in the activities of hydrolytic enzymes like carboxymethylcellulase and xylanase (Table 1 and 2), the key enzymes active in fibre degradation. In contrast to our results, Ha et al. (2001) reported a significant difference in rice straw degradation among different bacterial isolates at 4th and 6th day of incubation. There was no difference in digestibility ($p>0.05$) till 48 h of incubation among the bacterial isolates. This might be due to different chemical nature of the substrate used in the experiment. Forage of low nutritional value exhibited a long lag phase of cellulose digestion in *in vitro* conditions as compared to high quality forage (Baumgardt et al., 1962). In our experiment, along with wheat straw, wheat bran was also used as substrate which contains easily degradable sugars and protein which might be responsible for shortening the lag phase.

The extent of DM digestibility of different bacterial isolates was also reflected in their enzyme activities. The

carboxymethylcellulase was more in all the isolates than the control except BB-12 at 24 h of incubation. indicating that the production of CMCase was stimulated by the addition of bacterial isolates to the control reaction mixture. Higher digestibility observed in CHI-2 isolate was also accompanied with highest enzyme activities (CMCase. xylanase. β-glucosidase and acetyl esterase) at 24 h of incubation. CMCase and β-glucosidase activities were reduced at 48 h incubation, but acetyl esterase activity was increased at 48 h of incubation as compared to that at 24 h.

It appears from the results that there is a potential in the microbes of wild ruminants to be used for the manipulation of rumen fermentation in domestic animals. An isolate from chinkara showed higher DM and NDF digestibilities at 24 h of incubation with wheat straw and wheat bran (80:20) as substrate. The enzyme profile of these isolates is also better than the other isolates. Further studies are needed to find its potential as a microbial feed additive in *in vivo* experiments.

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