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Isolation, identification and characterization of rumen bacteria and estimation of their fibre degradable enzymes in yak (*Bos grunniens*)

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ABSTRACT

To utilize the plant nutrients efficiently, rumen microbes play a great role in the livestock, acting as a source of energy. Like other ruminants, the yak rumen harbours different microorganisms that are responsible for bioconversion of nutrients into a source of energy for the animals. The samples of rumen liquor were collected from the yak (*Bos grunniens*) rumen. The specific media was made to culture the rumen microbes and isolated the pure culture with serial dilution. The pure colony was identified with the help of 16S ribosomal gene sequencing. The enzyme activity of the isolates was observed by estimating the enzymes like Carboxymethyl Cellulase (CMCase) and Avicilase. The isolates were identified with the help of specific primer (*Fibrobactor succinogen*) and characterized which were closely related to *Bacteroides pyogenes* and *Ruminococcus* species. The study indicates diverse group of rumen bacterial population in yak rumen. *Fibrobactor succinogen, Ruminococcus* and *Bacteroides* species take potential roles as source of microbial enzyme for degradation of feed and fodder in yaks.

Keywords: Fibre degradable enzymes, Rumen microbes, Yak

To utilize the plant nutrients efficiently, rumen microbes have a great, important role in the livestock. Microbes survive in the rumen under different constrain which may be natural or feed associated as some of feed contains a significant amount of anti nutritional factors (Kamra *et al.* 2005). Huang *et al.* (2012) recorded a significantly different with unique characteristic of yak rumen microbiota that had less methanogenic affect that in cattle. The rumen microbes are very unique to rumen environment, identification and molecular characterization which is very essential and any constrain of contamination cannot be survived and needs their elimination. Rumen manipulation can play important roles to improve the digestibility of plant nutrients for the growth and production of livestock. In recent years many of rumen microbes were isolated, characterized by 16S ribosomal RNA gene (Das and Qin 2012). Prokaryotic diversity of the rumen of yak (*Bos grunniens*) and Jinnan cattle (*Bos taurus*) were estimated by 16S rDNA homology analysis (An *et al.* 2005). Some of microbes are identified which helps in the digestibility of rumen nutrients for improving the overall growth and production of animals (Mamen *et al.* 2010). Some of enzyme released by some rumen microbes breakdown switch grass, a renewable bio-fuel energy source (Anonymous 2011). There are some bacteria having a cellulolytic activity also posses pectinolytic, proteolytic and amylolytic activity which have important role in breaking down the complex protein into smaller peptides and amino acids involving microbes in the rumen fluids and gut fluids such as *Ruminobacter amylophilus, Selenomonas ruminantium, Succinimonas amylolytica, Streptococcus bovis, Lactobacillus* species, etc. *Prevotella ruminicola* is the proteolytic bacteria identified in the rumen fluid of cow and chicken gut fluid (Griswold and Mackie 1997). In this study the superior bacteria of rumen tried to isolate and characterized to check their potentiality to be used as feed additive.

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MATERIALS AND METHODS

The samples of rumen liquor was collected from five to six adult yaks using stomach tube from Nyukmadung farm of ICAR-NRC on Yak, Dirang situated at 9000 ft above mean sea level altitude. Isolation of bacteria was done from the collected rumen fluid periodically using standard protocol, maintaining strict anaerobic conditions.

Isolation procedure: The isolation was carried out by using the conventional techniques, of serial dilution and repeating tubing of the selectively enriched microbial cultures by using the specific media for the rumen bacteria. Strict anaerobic condition was maintained during the isolation procedure. The composition of the anaerobic growth media included:

Mineral Solution I-15 ml (KH_2PO_4 3.0 g; $(NH_4)_2SO_4$ 6.0 g; NaCl 6.0 g; $MgSO_4$ 0.6 g; $CaCl_2.2H_2O$ 0.795 g per litre) and Mineral Solution II-15 ml (K_2HPO_4 3 g/litre, 0.25 g Yeast extract, 1 g tryptone, 0.1 ml resazurine (0.1%), 0.2 ml hemin (0.05%), 0.5 g microcrystalline cellulose, 0.1 g cellobiose, 0.4 g sodium carbonate, 20 ml clear rumen fluid, 50 ml distilled water and 50 mg cysteine hydrochloride.

DNA extraction: 1.5 mL of aliquot, over night culture gram positive bacteria was centrifuged at 14,000×g overnight culture in the 1.5 ml of micro centrifuged tube and discarded the supernatant. 450 µL of sterile water was added and 50 µL EDTA to the pellet and it was vortex gently to resuspend. 50 µL of Lysozyme enzyme was added and inverted to mix and incubated at 37°C for 1.5–2 h with periodic mixing. It was centrifuged for 5 min at 14,000×g and poured off the supernatant. Gently vortexed the tubes to re-suspend the pellets in the residual fluid. Genomic lysis buffer was added (500 µL) and mixed by inverting the tube several times without vortexing. 1 µL of proteinase K solution was added for every 100 µL of Lysis buffer and incubated at 60°C for 1– 2 h (for maximum DNA recovery, it was not heated higher than 60°C). The samples were allowed to cool at room temperature. It was added 200 µL chloroform and mixed by inverting the tube several times and centrifuged for 10 min at 14,000×g. Upper clear phase was removed carefully in to a clean micro centrifuged tube. Added 50 µL of DNA stripping solution to the samples and inverted several times to mix and incubated the samples for 5–10 min at 60°C. Another, 100 µL of precipitation solution was added and mixed by inverting the tube several times. A white precipitate should be produced. If it was not produced another 50 µL aliquots of precipitation solution was added until the white precipitation was not formed. The samples were centrifuged at 14,000×g for 5 min. The supernatant was transferred to a clean tube and the genomic DNA was precipitated by adding 500 µL of Isopropanol. The tubes were inverted 10 times to precipitate the DNA. The samples were centrifuged at 14,000×g for 5 min to pellet the genomic DNA and removed the supernatant. 700 µL of 70% ethanol was added to the tube and inverted several times to the DNA pellet. It was centrifuged for minutes at 14,000×g. The tubes were decanted or pipette of the ethanol wash. The invert tube was kept on a clean absorbent surface for several minutes to allow any excess ethanol to drain away. The pellet was not dried completely because it would be difficult to rehydrate. 50 µL to 100 µL of TE buffer was added to the pellet and incubated at room temperature for at least 15 min to rehydrate. At this stage 1 µL of RNase enzyme for every 100 µL of TE buffer was added. The tube was incubated at 50–60°C for 55–60 min to speed up rehydration. The DNA was stored at 4°C and at –20°C to –80°C, for long term storage.

After extraction of DNA, the concentration was checked with the help of agarose gel electrophoresis and spectrophotometer. The polymerase chain reaction amplification of bacterial 16S rDNA was performed using the universal prokaryotic primer, (Forward-5′-AGAGTTTGATCCTCAGAACGAACGCT-3′, Reverse-5′ TACGGCTACCTTGACGACTTCACCCC-3′) for bacteria. The PCR reaction was setup using the 20 µL of total volume 1 µL of DNA template (100 ng/µl), 2 µL of 10× buffer,1.6 µL of 25 mM $MgCl_2$, 1 µL of each primer (10 µM), 0.4 µL of 10 mM dNTP mix, 0.3 µL of Taq Polymerase and 12.7 µL distilled water. The amplification was standardized for the universal primer. The amplification condition were as follows- 5 min at initial denaturation at 95°C followed by 30 cycle of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, extension at 72°C for 2 min with the last cycle followed by a 10 min extension step at 72°C. The PCR products were visualized on the agarose gel. The bands were excised and DNA was purified from the gel slices using the QUAGEN kit. The purification PCR products were send for sequencing. The sequencing was queried through the NCBI database. The isolate were again confirmed using the specific primer with the help of PCR.

Enzyme estimation

Extraction of bacterial enzymes: 5 ml of rumen culture was taken and 833 µL of Lysozyme enzyme (0.4% solution) and 833 µL of carbon tetrachloride solution. The container was placed in the shaker incubator at 39°C for 3 h and freezed the contents at –20°C for 15–20 min. It was thawed until it got liquefied. It was centrifuged at 1000 rpm for 20 min. The supernatant was collected and proceeded for immediate enzyme estimation, otherwise stored at –20°C for further use.

Enzyme estimation: To see the enzyme activity, the pure cultures were screened; the CMCase enzyme activity was determined by the amount of reducing sugar released from carboxymethyl cellulose. The mixture was made with 1.0 ml phosphate buffer (0.1 M *p*H 6.8), 0.5 ml of 1% carboxymethyl cellulose solution prepared in 0.1 M phosphate buffer (*p*H 6.8), 0.5 ml enzyme and incubated for 60 min at 39°C. The reaction was stopped and reducing sugar was determined by adding 3.0 ml of Dinitrosalicylic acid reagent (Miller *et al*. 1959). The glucose standard was made to determine the reducing sugar from carboxymethyl cellulose. The expression of glucose was determined as International Unit (IU) which is micromole of glucose released per ml per hour.

For Avicilase estimation, similar procedure was used using 1% Avicel (1 g of Avicel was suspended in 100 ml of phosphate buffer) solution and incubated at 40°C for two days for proper swelling of the substrate. The Assay mixture contained 1 ml avicel solution and 1 ml enzyme sample, with incubation time of 60 min with continuous shaking. The enzyme activity was expressed as µmol reducing sugars released per hours per ml.

RESULTS AND DISCUSSION

The isolation of rumen anaerobic bacteria was carried out using conventional techniques applying the specific media for rumen bacteria of serial dilutions with repeated tubing of the selectively enriched microbial cultures. Strict

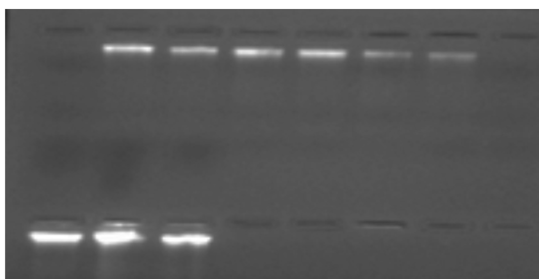

Fig. 1. Genomic DNA isolated from rumen bacterial culture from yak.

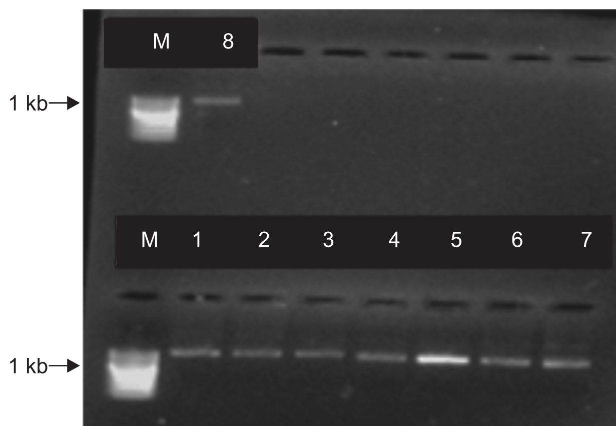

Fig. 2. Gel picture of amplification of Yak prokaryotic DNA with Primer "Upro (Universal prokaryotes)", Product size=475 bp, Marker 1000 bp plus, Name of samples: 1(Y=51), 2(Y=52), 3(Y=53), 4(Y=56), 5(Y=57), 6(Y=58), 7(Y=59), 8(Y=60).

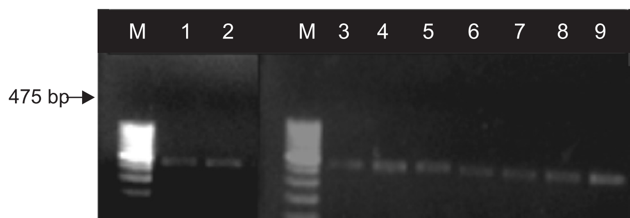

Fig. 3. Gel picture of amplification of genomic DNA by the Primer of FS (*Fibrobactor succinogen*), Product size=475 bp, Marker 100 bp plus, Name of sample: 1(Y=51), 2(Y=52), 3(Y=53), 4(Y=56), 5(Y=57), 6(Y=58), 8(Y=60).

anaerobic conditions were maintained during the isolation procedure. The rumen culture studies in Arunachali yaks showed that the yak rumen harbour some unique microbes which are essential for efficient conversion of fibrous materials in their diets. Their genomic DNA isolated from rumen liquor was observed to be homologous with *Fibrobacter succinogens.* The 16S rRNA gene diversity studies in Chinese yaks showed that microorganisms in the yak rumen were less diverse than those in cattle rumen (Dai *et al*. 2012).

Phylogenetic analysis

The isolates sequencing were compared with the available databases. The reference sequences were downloaded from the nucleotide sequences databases and used for further analysis of comparison. The obtained

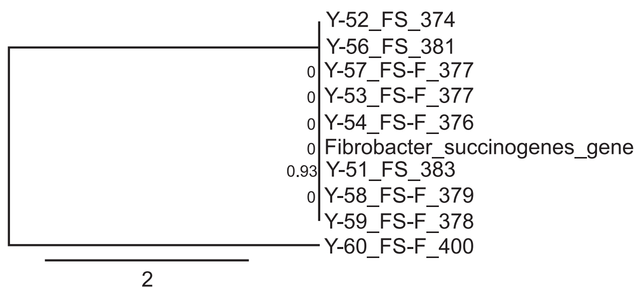

Fig. 4. Identification of isolates with help of 16s ribosomal DNA analysis.

sequences were aligned using the clustal V method of megAlign software (DNASTAR) and then phylogenetic tree was plotted.

Identification of isolate: After isolation of pure bacterial colonies, the DNA was extracted and quantified with the help of spectrophotometer. The DNA was amplified with the help of universal prokaryotic and specific primer. The required band size was obtained. The band as excised was purified and sent for sequencing. The isolates were homologous to *Fibrobacter succinogens* (Genbank Accession number dbj|AB275500.1|). Ren *et al*. (2020) recorded abundant numbers of *Ruminococcucae* and *Fibrobacter* species of bacteria in yak rumen cultures. This indicated that the microbes were of great importance for the digestion of ruminant animals which helps in growth and production of animals. *Methanobrevibacter ruminantium* was indentified in rumen fluid of cow and goat and from chicken gut and reduce carbon dioxide using hydrogen and form methane (Dighe *et al*. 2004).

Phylogenetic characterization: The phylogenetic tree represented the main groups of bacteria in the rumen of yak are *Bacteroides pyogenes* and *Ruminococcus* species. The isolates, Y-51_Upro-F_1011 (Accession No. gb|GQ898791.1|) and Y-59_Upro-F_976 (Accession No.dbj|AB510709.1|) were closely related to *Bacteroides pyogenes*. The isolate Y-56_Upro-F_956 (Accession No. gb|JX218605.1|), Y-58_Upro-F_911 (Accession No. gb|DQ256283.1|), were closely related to *Ruminococcus*. The Y-59_Upro-F_976) (Accession No. dbj|AB510709.1|-) is more closely related than Y-51_Upro-F_984 (Accession No. gb|GQ898791.1|). The isolates, Y-53_Upro-F_891 (Accession No. gb|JQ907530.1|, emb|AM884100.1|), Y-57_Upro-F_932 (Accession No. emb|HG941663.1|), Y-53_Upro-F_891

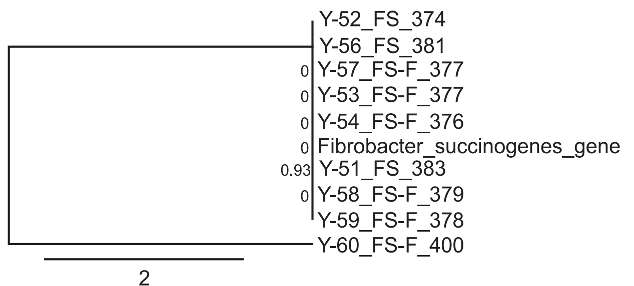

Fig. 5. Phylogenetic characterization of the isolates.

Table 1. Enzymatic analysis of bacterial isolates from yak rumen cultures

Isolates	Enzymatic activities	
	Carboxymethyl cellulase (CMCase) activity	Avicilase activity
Y-51	0.265	23.242
Y-52	0.794	12.457
Y-53	0.331	23.145
Y-54	0.265	3.457
Y-55	0.205	2.348
Y-56	0.198	6.452
Y-57	0.554	6.978
Y-58	0.661	35.483
Y-59	0.357	17.824
Y-60	0.457	25.893
Y-61	1.058	24.405

(Accession No. gb|JQ907530.1|, emb|AM884100.1|) are closely related to *Ruminococcus*. *Methanobacterium bryantii* from cow rumen was found to be more efficient methanogenic bacteria (Jarrell and Sprott 1983).

Enzyme estimation: The estimation of the enzyme activities of the isolates identified showed that most of these isolates are positive for Avicilase and Carboxymethyl cellulase activities indicating superior fibre degrading activity of these anaerobic bacteria in yak rumen.

The study indicates diverse group of rumen bacterial population in yak rumen. *Fibrobactor succinogen, Ruminococcus* and *Bacteroides* species take potential roles as source of microbial enzyme for degradation of feed and fodder in yaks.

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