

Isolation and characterization of superior rumen bacteria of cattle (*Bos taurus*) and potential application in animal feedstuff

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ABSTRACT

Rumen of cattle harbors many microorganisms responsible for bioconversion of nutrients into a source of energy for the animals. In recent years many rumen microbes have been isolated and characterized by sequence analysis of 16S ribosomal RNA gene. Some of the microbes have also been recommended as feed additives for improving the overall growth or production of animals. Rumen bacteria which have potential application in animal feed stuffs were isolated and characterized in this experiment. Isolation was carried out from the rumen of cattle (*Bos taurus*) using techniques of serial dilutions and repeated tubing of the selectively enriched microbial cultures by using the specific media for rumen bacteria. All the isolates were then screened for in vitro gas production and cellulase enzyme activity and four superior isolates were selected and characterized. There were 18.00% to 23.00% increases in gas production on addition of these isolates to the rumen fluid of cattle and there was better cellulase enzyme activity. Two isolates were identified as *Butyrivibrio fibrisolvens*, one isolate as *Streptococcus* species and one isolate as *Clostridium aminophilum*. This indicated that, these isolates are superior and may have potential to be used as microbial feed additive in ruminants if fed in higher quantity.

Keywords: Rumen; Bacteria; Feed Additive; Characterization

1. INTRODUCTION

Rumen microbes are of great importance for livestock

and they are able to utilize plant nutrients efficiently as source of energy. Microbes survive in the rumen under different constraints which may be either natural or feed associated as some of the feeds contain a significant amount of anti-nutritional factors [1]. Any contaminant which cannot survive these constraints is eliminated. Therefore, the microbes which harbor the rumen are unique and for better understanding of rumen environment, identification and molecular characterization of microbes are highly essential. Moreover, the rumen manipulation by improving the digestibility of plant nutrient is a priority area for improving the growth or production of livestock.

In recent years many rumen microbes have been isolated and characterized by sequence analysis of 16S ribosomal RNA gene. Prokaryote diversity in the rumen of yak (*Bos grunniens*) and Jinnan cattle (*Bos Taurus*) were estimated by 16S rDNA homology analysis [2]. Some of the microbes have also been recommended as feed additive for improving the overall growth or production of animals [3]. The tropical forage *Leucaena leucocephala* contains mimosine, a non-protein amino acid. When consumed by ruminants in Australia and some parts of India, DHP causes goitrogenic effects. The detoxification of the 3-hydroxy-4(1H)-pyridone (DHP) by *Synergistes jonesii*, isolated from Hawaiian cattle, is one of the examples in manipulating rumen fermentation with bacteria [4]. Similarly addition of *Megasphaera elsdenii* has also experimentally prevented acidosis in steers [5]. The enzymes produced by rumen bacteria may contribute to the breakdown of switch grass, a renewable biofuel energy source [6]. Cows in early lactation produced 5.9% more milk when enzyme mixture was added to the concentrate feed [7]. The average increase in Dry Matter Intake (DMI) was 1.0 ± 1.3 kg/day and the average increase in milk yield was 1.1 ± 1.5 kg/d. In our study some of the superior bacteria of cattle were isolated and characterized which has got potential to be used as feed additive.

2. MATERIALS AND METHODS

Isolation of bacteria was done from the rumen of cattle (*Bos Taurus*, Simmental breed) of Canada. The slaughter house of Thunder Bay (Thunder Bay Meat Processing Plant, Murille, Thunder Bay) Canada was selected for sample collection and samples were collected immediately after slaughtering of animals (5 to 6 no) in the slaughter house. About 3 to 4 samples of rumen content were collected from rumen of each animal in the slaughter house and then mixed together to form a composite sample which was brought to the laboratory in strict anaerobic conditions for further processing.

2.1. Isolation Procedure

The isolation was carried out using conventional techniques of serial dilutions and repeated tubing of the selectively enriched microbial cultures by using the specific media for rumen bacteria (**Figure 1**). Strict anaerobic conditions were maintained during the isolation procedure.

The growth media contained: 15 ml Mineral Solution I (KH_2PO_4 3.0 g; $(\text{NH}_4)_2\text{SO}_4$ 6.0 g; NaCl 6.0 g; MgSO_4 0.6 g; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 0.795 g per Litre), 15 ml Mineral Solution II (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. Fifty bacterial isolates were obtained and screened for superior isolates.

2.2. *In Vitro* Gas Production

The pure isolates were screened for *in vitro* gas production [8]. 100 ml calibrated glass syringes (haeberle Labortechnik, Germany) were used for this purpose and 24 hours gas production was recorded in triplicate for each isolate. The amount of gas produced during the incubation of isolates with rumen liquor is closely related to degradability and energetic value of isolates.

Rumen fluid was obtained from three adult Simmental breed of cattle. The rumen liquor was collected into the



Figure 1. Bacteria colonies in roll tube.

thermo flask that had been pre warmed to a temperature of 39°C. Incubation procedure was done by using special type of calibrated transparent glass syringes with fitted silicon tube. Inoculums (30 ml) containing strained rumen liquor, buffer and mineral solution under continuous flushing with CO_2 was dispensed using 50 ml plastic calibrated syringe after which 2 ml of isolates were immediately pushed by using another 10 ml syringe. The glass syringe was tapped and pushed upward by the piston in order to completely eliminate air in the inoculums. The silicon tube in the syringe was then tightened by a metal clip so as to prevent escape of gas. Incubation was carried out at $39^\circ\text{C} \pm 1^\circ\text{C}$ and the volume of gas production was measured after 24 hours of incubation. The average of the volume of gas produced from the blanks was deducted from the volume of gas produced per sample against the incubation time.

2.3. Carboxy Methyl Cellulase (CMC) Activity

The pure isolates were also screened for enzymatic activity. The CMC enzyme activity was determined by measuring the amount of reducing sugar released from carboxymethyl cellulose. The reaction mixture contained 1.0 ml phosphate buffer (0.1 M pH 6.8), 0.5 ml of 1% carboxymethyl cellulose solution prepared in 0.1 M phosphate buffer (pH 6.8), 0.5 ml enzyme and incubated for 60 minutes at 39°C. The reaction was halted and reducing sugars were determined by the addition of 3.0 ml of Dinitrosalicylic acid reagent [9]. Glucose was used as standard for determination of reducing sugars. The enzyme activity is expressed as International Unit (IU) which is micromole of glucose released per ml per hour.

2.4. Total Cellulase Activity by Filter Paper

Microplate-based filter paper assay [10] was conducted to measure total cellulase activity. Total cellulase activity is measured by the amount of glucose released from filter paper after two hrs incubation and is referred to as glucose equivalent. One FPU is defined as an average of one micromole of glucose equivalents released per min in the assay reaction.

2.5. DNA Isolation and PCR Amplification

Genomic DNA was extracted from the microbes of five isolates by using the standard kit (Nucleospin soil, manufactured by *Macherey-Nagel GmbH & Co. KG*). The DNA and its concentration were checked by agarose gel electrophoresis. Polymerase chain reaction (PCR) amplification of bacterial 16S rDNA was performed using the universal primer (forward: 5'-AGAGTTTGATCCTCAGAACGAACGCT-3' and reverse: 5'-TACGGC-

TACCTTGACGACTTCACCCC-3') of bacteria. The PCR reaction was set up in 20 µl volumes containing 1 µl template (100 ng/µl), 2 µl 10× buffer, 1.6 µl 25 mM MgCl₂, 1 µl of each primer (10 µM), 0.4 µl of 10 mM dNTP mix, 0.2 µl Taq DNA polymerase and 12.8 µl distilled water. The amplification conditions were standardized for universal primer. The amplification conditions were as follows: 3 min of initial denaturation at 95°C, followed by 30 cycles 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 product was then visualized on an agarose gel (Figures 2 and 3). The bands were excised and DNA was purified from the gel slices using the standard kit (NORGEN Biotek Corporation, ON, Canada). The purified PCR product was sent for sequencing. The sequences were queried online in the NCBI BLAST database. The isolates were again confirmed by using the specific primer of bacteria.

2.6. Phylogenetic Analysis

The sequences of the isolates were compared with those available in the database. The reference sequences were downloaded from the nucleotide sequence databases and were used in further analysis for comparison. The obtained sequences were aligned using clustal V method of megAlign software (DNASTAR) and then phylogenetic tree was plotted.

3. RESULTS AND DISCUSSION

The isolation was carried out using conventional techniques of serial dilutions and repeated tubing of the selectively enriched microbial cultures by using the specific media for rumen bacteria. Strict anaerobic conditions were maintained during the isolation procedure.

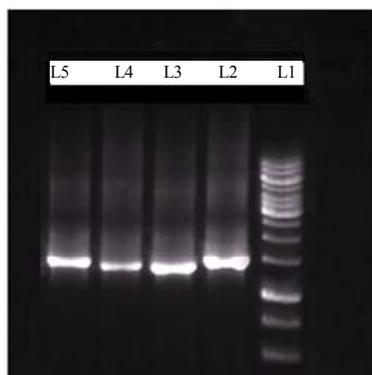


Figure 2. Agarose gel electrophoresis of PCR Products after amplification with Universal Primer (L1: 1 kb DNA Marker, L2-L5: isolates of BF12, BF14, SB13 and CA22).

3.1. In Vitro Gas Production

Four bacterial isolates *i.e.* BF12, BF14, SB13 and CA22 were observed in microscope and found to be cocci. On addition of these isolates to the rumen fluid of cattle, there was increase in gas production (Table 1). The gas production of isolates after 24 hours incubation varied from 8 to 10.5 ml/ml of isolate, highest in CA22 and lowest in BF14. It was observed that, there were 18.00% to 23.00% increases in gas production indicating release of energy on fibre-based mixed ration. In vitro gas production is the indicator of digestibility and nutritive value of feed stuffs [11,12]. Increased gas production in this experiment indicated that, these isolates may be termed as superior isolates and have potential to be used as microbial feed additives in ruminants (Table 1).

3.2. Enzymatic Analysis of Isolates

The isolates were subjected to enzymatic analysis like carboxymethyl cellulose (CMC), and total cellulose activity (filter paper activity). All the four isolates showed superior fibre degrading activity like CMC, and total cellulose activity, highest in CA22 and lowest in BF14

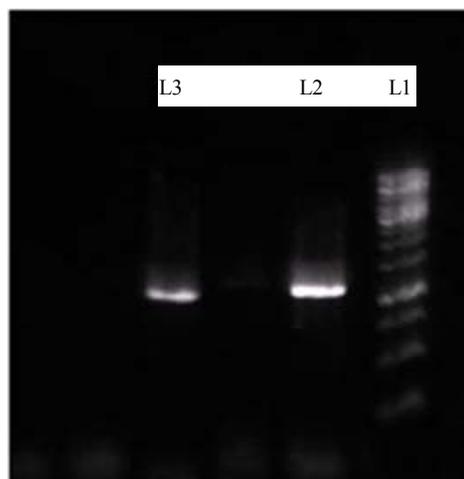


Figure 3. Agarose gel electrophoresis of PCR products amplification with specific primers of *B. fibrisolvens*. L1: 1 kb DNA Marker, L2-L3: *B. fibrisolvens* isolates (BF12, BF14).

Table 1. In vitro gas production of bacterial isolates.

Isolate no	In vitro gas production	
	Increase in gas production (ml per 1 ml isolate)	Percentage of increase in gas production (%)
BF12	10.0	22.0
BF14	8.0	18.0
SB13	9.0	20.0
CA22	10.5	23.0

(Table 2). The *in vitro* gas productions were in same order as enzymatic analysis of the isolates.

3.3. Identification of Isolates

For identification of isolates, DNA was isolated from all pure isolates and DNA was found to be sufficient for PCR reaction as quantified by spectrophotometer. The DNA was amplified by universal and specific primers of bacteria and amplification product of required band size was obtained (Figures 2 and 3). The bands were excised and purified PCR products were sent for sequencing. One isolate (BF12) was homologous to *Butyrivibrio fibrisolvens* (Genbank Accession number EU684229) where as other isolate (BF14) was homologous to *Butyrivibrio fibrisolvens* (Genbank accession number EF 427365). Isolate SB13 was homologous to *Streptococcus* species (Genbank Accession number FJ611790) where as isolate CA22 was homologous to *Clostridium aminophilum* (Genbank Accession number DQ278862). This indicated that, these isolates have potential to be used as microbial feed additive in ruminant animal for better growth and production. There is also ample scope in using the enzymes of these bacteria as microbial feed additives.

3.4. Phylogenetic Characterisation

The phylogenetic tree displayed three main groups of bacteria *i.e.* *Butyrivibrio*, *Streptococcus* and *Clostridium* under the phylum Firmicutes (Figure 4). The isolates BF12 was closely related to *Butyrivibrio fibrisolvens* isolated from sheep rumen (Accession no EU684229) and isolate no BF14 was closely related to *Butyrivibrio fibrisolvens* strain of 16S ribosomal RNA gene (Accession no EF427365). The isolate SB13 was closely related to *Streptococcus equinus* isolated from sheep rumen (Accession no AB563264) and the isolate CA22 was closely related to *Clostridium aminophilum* isolated from cow rumen (Accession no DQ278862). This shows that, three bacteria isolated in the present experiment are inhabitant of rumen.

Table 2. Enzymatic analysis of bacterial isolates.

Isolate no	Enzyme analysis	
	CMC activity	Total cellulase activity
	(MIU/ml)	(FPU/ml)
BF12	4.47	21.0
BF14	3.20	18.5
SB13	3.39	20.0
CA22	5.18	23.0

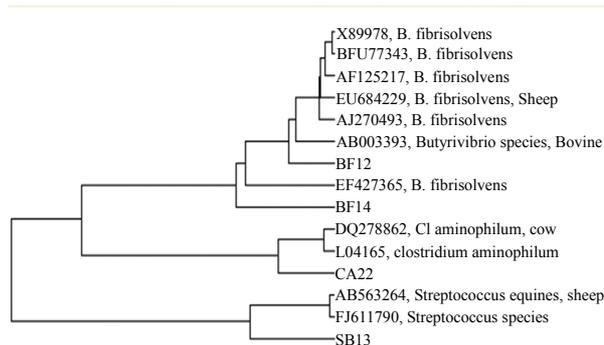


Figure 4. Phylogenetic characterization of isolates.

3.5. Potential of Rumen Bacteria as Microbial Feed Additives

Butyrivibrio species is the major cellulose and hemicellulose degrading bacteria in the rumen [13] and rapidly degrade xylan necessary for improving the overall efficiency of livestock production [14]. This may also be used as probiotics for reducing the aberrant crypt foci in the colon and rectum of mice [15]. *Butyrivibrio* species isolated in this experiment contained higher source of cellulolytic enzyme and *in vitro* gas production was increased on addition of these isolates to the rumen fluid of cattle.

Streptococcus species are the predominant cellulolytic micro-organisms that are associated with the possession of complex cellulose enzyme systems [16,17]. Some strains of streptococcus species are responsible for starch hydrolysis in the rumen [18]. Pulse dosing alone or pulse dosing with daily feeding of *Streptococcus faecium* M74 resulted in improved performance of incoming feedlot cattle [19]. *Streptococcus* species identified in this experiment are superior because of higher enzymatic activity and *in vitro* gas production. It has also been reported that, *Streptococcus equinus* are phylogenetically similar to *Streptococcus bovis* [20].

Clostridium strain IY-2 isolated from gut content of giant Panda possess cellulase activity towards untreated natural materials such as bamboo fibres, cellulose, rice straw and saw dust and comprises three major cellulases [21]. *Clostridium* isolate from swine were effective in degrading cell wall components from grasses [22]. As per the result of other worker, *Clostridium aminophilum* produces ammonia and is responsible for digestion of protein in the feed stuffs [23]. Though effectiveness depends on the strain of microbes, positive results were observed in this experiment.

4. CONCLUSION

Based on the findings of the result, it can be concluded that three species of microbes *i.e.* *Butyrivibrio fibrisol-*

vens, *Streptococcus* species and *Clostridium aminophilus* are superior and may have potential to be used as microbial feed additive as a source of microbial enzymes if fed in higher quantity. However, animal experiments are required to confirm the effectiveness of these three species of microorganism.

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