Analysis of Cellulolytic Bacterial Flora in the Rumen of Inner Mongolian Sheep

Jianguo Liu,[a,b] Zhanying Liu,[b,c,*] Yucheng Liu,[d] Min Hao,[b,c] and Xianzhi Hou [d,]

The cellulolytic bacterial flora present in the rumen of Inner Mongolian sheep are thought to have a high degree of cellulose-degrading activity because of their foraging feeding regimen. However, there are no report on the genetic and species composition of the cellulolytic bacterial flora. In this study, cellulolytic bacteria were isolated from the rumen of Inner Mongolian sheep using a combined method of transparent zone and filter paper degradation. Twenty-two strains were identified *via* morphological, physiological, and biochemical tests. Ten strains were further identified *via* DNA (G + C) mol%, together with 16S rDNA gene sequencing analysis. Four types of extracellular and total cellulase activities of representative strains were determined. The results demonstrated that the isolates included *Butyrivibrio fibrisolvens*, *Rumincoccus albus*, *R. flavefaciens*, *Fibrobacter succinogenes*, and *Clostridium polysaccharolyticum*. A big proportion of cellulolytic *Butyrivibrio fibrisolvens* was found in the rumen of Inner Mongolian sheep. This was the first study to analyze the cellulolytic bacterial flora in the rumen of foraging Inner Mongolian sheep. These results indicated that the rumen of Inner Mongolian sheep represents an attractive source for cellulolytic microorganisms and enzymes, and the research results have a certain guiding importance for the efficient degradation of cellulosic materials.

Keywords: Cellulolytic bacterial flora; Rumen; Inner Mongolian sheep

Contact information: a: Department of Environmental Science and Engineering, Inner Mongolia University of Technology, Hohehot 010051, China; b: Center for Energy Conservation and Emission Reduction in Fermentation Industry, Inner Mongolia, Hohehot 010051, China; c: School of Chemical Engineering, Inner Mongolia University of Technology, Hohehot 010051, China; d: School of Animal Science, Inner Mongolia Agricultural University, Hohhot 010018, China;
** Corresponding authors: hgxylzy2008@imut.edu.cn*

INTRODUCTION

The production of biofuels from renewable lignocellulosic biomass has received tremendous attention both in the energy industry and in academic communities worldwide based on the demand for sustainable economies and clean energy (Sanderson 2006; Lynd *et al.* 2008). Cellulosic resources, the most abundant biomass on earth, have the greatest potential to resolve the energy crisis (Sanchez and Cardona 2008; Weimer *et al.* 2015) because they can be converted to biofuels by hydrolysis and subsequent fermentation (Lane 1991; Hamelinck *et al.* 2005). Hydrolysis is a rate-limiting step because of the recalcitrance of lignocellulose. Among the various available approaches, enzymatic hydrolysis is more environmentally sound compared with acid-reliant or base-reliant hydrolytic and thermochemical processes (Hamelinck *et al.* 2005). However, the paucity of enzymes or microbes that efficiently deconstruct the plant polysaccharides represents a bottleneck for the hydrolysis of lignocellulose and therefore to the industrial-scale conversion of cellulosic biomass into biofuels (Hess *et al.* 2011). As a result, the exploration of

cellulolytic microorganisms and cellulases is important. The rumen ecosystem is the most efficient system for cellulose transformation to valuable products (Nouaille *et al.* 2009), with many rumen microbes specializing in the degradation of cellulosic plant materials (Hess *et al.* 2011). The rumen cellulolytic microorganisms, which possess very active and complex hydrolytic systems, are thus potential biocatalysts for biofuel production (Nouaille *et al.* 2009). However, most members of this complex community are not cultivable (Hess *et al.* 2011). Few studies have focused on strain isolation from the rumen due to the sensitivity to oxygen and special medium requirements (Kenters *et al.* 2011). Furthermore, molecular biology methods have demonstrated that < 1% of the known rumen microbe has been isolated (Pitta *et al.* 2014). Surveys of the 16S rDNA genes of bacteria in the rumen of various ruminants have indicated a vast diversity of bacterial genera and species that have not yet been characterized (Kenters *et al.* 2011). The development of molecular biological methods has explored the rumen microbial flora in different types of ruminants. These studies have demonstrated the structure of microbial flora to be dynamic and regulated by numerous factors, such as host, diet, physiological status, geographical location, season, and feeding regimen (Thomas *et al.* 2011; Pitta *et al.* 2014, 2016). A metagenomic study of Mehsani buffalo rumen identified a significantly higher abundance of microbiome in green roughage fed animals compared with dry roughage fed animals (Patel *et al.* 2014). Ghasemi *et al.* (2012) demonstrated that the inclusion of pistachio hulls as a replacement for alfalfa hay in the diet of sheep caused a shift in the rumen cellulolytic bacterial population (Ghasemi *et al.* 2012). There are several reports regarding the isolation of rumen bacteria from the rumen of Creole goats (Grilli *et al.* 2013), cows (Kenters *et al.* 2011; Hungate 2013), sheep (Hungate 2013), and bovines (Nyonyo *et al.* 2014), each with diverse microbial flora found in the different ruminants. The above studies suggest that the type of ruminant, diet, and geographical location affects the kind and abundance of microbial flora.

In the Inner Mongolian region, the weather is cold, dry, and the ruminant feeding regimen comprises a large number of forage species. Different ruminants in this region are thought to have microbial flora consisting of various types and proportions of cellulolytic microorganisms. Sheep is a typical ruminant of Inner Mongolia (Lim *et al.* 2015). However, to the best of the authors' knowledge, there are no reports regarding the isolation of cellulolytic bacteria in the rumen of Inner Mongolian sheep.

In this study, the authors isolated cellulolytic bacteria using a combined method and systematically identified the isolates using traditional identification and molecular biology methods. Representative strains were selected from each species, and the extracellular cellulase and total cellulase activities for four types of cellulase (filter paper enzyme, endoglucanase, exoglucanase, and β-glucosidase) were determined and compared. These findings represent the first study to analyze the cellulolytic bacterial flora in the rumen of Inner Mongolian sheep. This will be helpful to augment the pool of cellulolytic bacteria and explore new cellulolytic bacteria for biomass utilization.

EXPERIMENTAL

Materials and Methods

Medium

Medium A: The medium was prepared according to Jian Pang *et al.* (2017) and adjusted slightly. Five-hundred mL of basal medium, 165 mL of inorganic salt solution A,

165 mL of inorganic salt solution B, 169 mL of cell-free rumen fluid, and 1.0 mL of 0.1% resazurin were mixed to a total volume of 1000 mL. Basal medium was composed of 5.0 g/L $NaHCO_3$, 1.0 g/L peptone, and 1.0 g/L yeast powder. Inorganic salt solution A was composed of 3.0 g/L KH_2PO_4, 3.0 g/L $(NH4)_2SO_4$, 6.0 g/L NaCl, 0.4 g/L $CaCl_2 \cdot 2H_2O$, and 0.58 g/L $MgSO_4 \cdot 7H_2O$. Inorganic salt solution B was composed of 3.96 g/L $K_2HPO_4 \cdot 3H_2O$. Cell-free rumen fluid was prepared according to the following procedure: the rumen fluid was taken from the rumen of the Inner Mongolian sheep as the candidate and then filtered *via* a 4-layer cheese cloth. The filtrate was first centrifuged at 5,000 r/min for 15 min and then at 15000 r/min for 30 min. The supernatant was added to medium as cell-free rumen fluid.

Medium B (medium for preliminary isolation of strains): 0.05 g of sodium carboxymethyl cellulose and 0.4 g of agar were added to 10 mL of Hungate tube, and then 5 mL of medium A was added. The rest of the procedure was the same as for medium A.

Medium C (medium for secondary isolation of strains): 1 g of filter paper was put into a 20 mL serum bottle, and then 10 mL of medium A was added. Flanged butyl stoppers and aluminum crimp were used to seal up the serum bottle. All of the media were dispensed under N_2 gassing into serum bottles with aluminum crimp and flanged butyl stoppers. After the color of medium turn pale pink from red, 0.5 mL of 25 g/L cysteine hydrochloride was injected to a serum bottle. At last, serum bottles were autoclaved at 121℃ for 20 min.

Medium D (medium for cellulase enzyme activity determination of selected strains): 0.1 g cellobiose was placed in a 150 mL of serum bottle, and then 50 mL of medium A was added. The remainder of the procedure was the same as for medium A.

The initial pH value of all of the above media was about 6.8.

Strains isolation

Rumen samples were collected from six mature and healthy castrated Inner Mongolian sheep with a mean weight of 30 kg. The sheep were bought from a lab animal supplier in Hohhot (China), approved by the Inner Mongolian Agricultural University ethics committee. They were housed in individual pens and had free access to water at the animal nutrition lab in the Inner Mongolia Agricultural University in Hohhot at an average altitude of 1050 m with a middle temperature zone and continental monsoon climate (temperature from -29 °C to 31 °C). Every sheep was fitted with a permanent rumen cannula with a diet of forage to concentrate ratio of 70:30. The forage consisted of 70% mixed grass containing 11.20% corn, 6.10% wheat bran, 10.20% soybean meal, 1.25% stone powder, 0.25% $CaHPO_4$, 0.50% salt, and 0.50% compound additive. The animals were maintained on their diets for at least 1 month prior to sampling of rumen contents. After feeding at 7:00 am, the rumen contents were sampled at 9:00 am (local time). The collecting tube was 10 mm in diameter and both solid and liquid contents in the rumen were collected. Rumen samples from each sheep were transferred into a thermos, which was pre-warmed to approximately 39 °C and filled with carbon dioxide. The rumen samples from all six sheep were mixed, homogenized, serial dilution and then inoculated to medium B by Hungate roll technology for cultivation of strict anaerobes according to the method of Hungate RE (1959) [Hungate, R. E. 1969. A roll-tube method was used for cultivation of strict anaerobes. Methods Microbiol. 3B:117-132.]. Inculum was introduced to melted agar liquid by syringe injection through the rubber closure. Inculated agar tubes were rolled on ice or ice water to solidify the agar in a thin layer and are then incubated for 24 h to 48 h in an anaerobic environment (100% N_2 atmosphere in Hungate tube) at 39 °C. Serial dilutions into additional tubes can be made by syringe as desired. The colonies that

formed transparent zones on the CMC-Na agar were selected and purified to be single colonies. The isolation process was completed in an anaerobic workstation (Baker Ruskinn Bugbox). For secondary screening, each single colony was inoculated into the medium C with filter paper as the carbon source and energy source, and then incubated at 39 °C for 7 days. Strains solubilizing filter papers significantly were selected. The isolated cellulolytic strains were preserved at -80 °C with 20% glycerol as protective agent for further research.

Identification of strains

To save cost and time, the isolated strains were first identified by traditional methods (colony morphology, cell morphology, as well as physiological and biochemical characteristics), and then the selected strains were further identified according to molecular biology methods ((G + C) mol % content and 16S rDNA gene sequencing).

Cell morphologies were observed at their exponential growth phase with phase contrast microscopy (ShangHai Optical Instrument Facotry, ShangHai, China). The physiological and biochemical characteristics were determined *via* reference to Bergey's manual of systematic bacteriology (Holt *et al.* 1994). The genomic DNA was extracted using the TaKaRa bacterial DNA extraction kit (TaKaRa, Tokyo, Japan) according to the manufacturer's instructions. The (G + C) mol % of the strains was determined with an ultraviolet (UV)-1700 UV-VIS spectrophotometer (Shimadzu Corp., Kyoto, Japan). *Escherichia coli* K12 were used as the control strains. The (G + C) mol % of the test and control strains were determined using the following method: The genomic DNA was dissolved in colorimetric ware in 25 mol/L saline sodium citrate buffer (SSC) (saline sodium citrate composed of 0.15 mol/L NaCl and 0.015 mol/L sodium citrate) and then slowly heated from 25 °C (final temp) at a speed of 0.5 °C/min. The absorbance of the solution at 260 nm was continuously monitored against a blank that contained only SSC buffer. The T_m value was defined as the temperature at 50% hyperchromicity. The (G + C) mol % of the genomic DNA of the test strain was calculated using Eq. 1:

$$(G + C)\ \text{mol}\% = 51.2 + 2.08 \times (T_m\ (\text{test strain}) - T_m\ (\text{control strain})) \quad (1)$$

The 16S rDNA gene sequence identification was as follows: The 50 µL polymerase chain reaction (PCR) mixtures contained 1.0 µL of 100 ng/µL template DNA, 1.0 µL of each primer, 25.0 µL of 2 × Taq Platinum PCR MasterMix (every 0.5 mL Platinum PCR MasterMix contained 0.1 U/µL Taq Platinum polymerase, 500 µL of deoxy-ribonucleoside triphosphate (dNTP), 50 mmol/L Tris-HCl, 20 mmol/L KCl, and 4 mmol/L $MgCl_2$), and 22 µL of Milli-Q water (Millipore. MA, USA). The primers employed for the amplification were forward primer 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and reverse primer 1492R (5'-ACGGCTACCTTGTTACGACT-3') (Lane 1991). The amplification procedures were as follows: 5 min at 94 °C; 25 cycles of 30 s at 94 °C, 30 s at 55 °C to 58 °C (different temperatures were used for different strains), and 90 s at 72 °C; after the cycles, an additional 5 min at 72 °C. The PCR products were sequenced by Tiangen Biotech Co., Ltd. (Beijing, China), and the results were contrasted with the 16S rDNA gene sequences available in the Genbank from the National Center for Biotechnology Information Database. A similarity of 98% was considered as the same genera (Clarridge 2004).

Cellulase activities of selected strains

The strain was inoculated in liquid medium C with an inoculum size of 2%, and then incubated at 39 °C under anaerobic conditions for 48 h. Fermentation broth was taken,

and all samples were diluted to the same absorbance value (OD_{600}=1) to ensure the same cell concentration. Each sample was divided into two parts. One part was centrifugated at 4 °C at 6000 r/min for 15 min, and supernatant was collected for the determination of extracellular cellulase. The cells in other part were disrupted by ultrasonic waves with a power of 400 W, intermittent frequency of 0.6, and time of 25 min to obtain the total cellulase. At last, the extracellular and total cellulase activities were determined and compared. The enzymatic activities of endoglucanase, exoglucanase, glucosidase, and filter paper enzyme were determined according to the method of Ghose (1987). One unit of enzyme activity was defined as the amount of enzyme that released 1.0 μg of reducing sugar per minute. Cellulase activities of selected strains were collected from triple replicated experiments. An analysis of variance (ANOVA) for the enzyme activity was conducted using SAS software (Statistical Analysis System, SAS 9.2, Cary, NC, USA).

This study was approved by the Ethical Committee for Animal Experiments of Inner Mongolia Agricultural University (Permit Number: SYXK(Inner Mongolia)2014-0002). The animal experiment process strictly abides by the British Animals (Scientific Procedures) Act of 1986 and the European Directive 2010/63/EU.

RESULTS AND DISCUSSION

Strains Isolation

A total of 96 isolates with transparent zones were obtained from the rumen contents of Inner Mongolian sheep through plating on CMC-Na medium. Based on the calculation of the ratio of the clearance zone diameter to the colony diameter, these bacterial isolates exhibited large differences in their abilities to degrade CMC-Na, and strains with higher ratios of clearance zone diameter to colony diameter were selected for a further filter paper degradation experiment. A total of 22 isolates were obtained by observing the degree of degradation for filter paper. The degradation degree of filter paper is shown in Table 1.

Table 1. Degradation of Filter Paper

Strain	Degradation Degree of Filter Paper	Strain	Degradation Degree of Filter Paper	Strain	Degradation Degree of Filter Paper
WH-1	+++	CLQ	++	NBG	++++
WH-22	++++	VI3	+++	LBG-11	++++
WH-3	++	NBQ-1	+++	LHG	+
WG-1	+++	NDF-2	+	NDF-3	+++
WHQ	+++	LBQ-1	+	LH-1	++
LHH-1	++	LHQ-3	+	LLH	++
CCQ	+++	CBQ	+		
LYQ	+++	X6C1	+		
Note: more +'s represent more breakage					

As a result, 22 strains of cellulolytic bacteria were isolated from the rumen of Inner Mongolian sheep using the combined method of transparent zone and filter paper degradation.

Identification of Strains

The colony morphologies of the 22 strains are shown in Table 2, and the cell morphologies are shown in Table 3. The physiological and biochemical characteristics of isolates are shown in Table 4.

Table 2. Colony Morphology

Colony Parameters	WH-1, WH-22, WH-3, LHH-1, WG-1, WHQ	CCQ, LYQ	CLQ, VI3, NBQ-1, NDF-2, LBQ-1, LHQ-3, CBQ, X6C1	NBG-1, LBG-11, LHG	NDF-3, LH-1, LLH
Size(Diameter)	0.5 to 1 mm	1 to 2 mm	0.5 to 3 mm	2 to 3 mm	0.5 mm
Shape	Round	Round	Round	Round	Round
Moisture	Wet	Wet	Wet	Wet	Wet
Height	Flat	Flat	Flat	Flat	Flat
Transparency	Translucent	Translucent	Translucent	Translucent	Translucent
Color	White	Yellow	White	Milkiness	Milkiness
Edge	Regular	Irregular	Regular	Irregular	Regular
Smooth	Smooth	Smooth	Smooth	Smooth	Smooth

Table 3. Cell Morphology

Cell Parameters	WH-1, WH-22, WH-3, LHH-1, WG-1, WHQ	CCQ, LYQ,	CLQ, VI3, NBQ-1, NDF-2, LBQ-1, LHQ-3, CBQ, X6C1	NBG-1, LBG-11, LHG	NDF-3, LH-1, LLH
Size (μm)	0.4 to 0.6 × 2 to 5	0.3 to 1.5 × 0.7 to 1.8	0.3 to 1.5 × 0.7 to 1.8	0.4 to 0.8 × 0.8 to 2.0	0.6 to 1 × 3 to 6
Shape	Vibrio	Cocci	Cocci	Rod	Rod
Gram stain	Negative	Positive	Positive	Negative	Negative

Table 4(1). Physiological and Biochemical Characteristics of Isolated Strains (1)

Strain	FT	C	N	H_2S	M	Temperature Experiments					pH Experiments				
						20 °C	30 °C	37 °C	39 °C	45 °C	5.5	6.3	7.0	7.8	8.0
WH-1	Anaerobic	-	-	-	+	++	++	#	+++	+	-	-	#	-	-
WH-22	Anaerobic	-	-	-	+	++	+++	#	+++	+	+	-	#	+	-
WH-3	Anaerobic	-	-	-	+	+	++	#	+++	++	-	-	#	-	-
LHH-1	Anaerobic	-	-	-	+	++	+++	#	+++	+	-	+	#	-	-
WHQ	Anaerobic	-	-	-	+	+	++	#	+++	++	-	++	#	+	+
WG-1	Anaerobic	-	-	-	+	++	++	#	+++	+	+	++	#	+	+
NBG	Anaerobic	-	-	-	+	++	++	#	+++	++	+	+	#	+	-
LBG-11	Anaerobic	-	-	-	+	+	++	#	+++	++	-	-	#	-	-
LHG	Anaerobic	-	-	-	+	++	+++	#	+++	++	-	-	#	+	-
NDF-3	Anaerobic	-	-	-	+	++	++	+++	#	++	-	-	+	+	-
LH-1	Anaerobic	-	-	-	+	++	++	+++	#	++	+	+	#	+	-
LLH	Anaerobic	-	-	-	+	++	++	++	#	++	-	+	#	+	-
CCQ	Anaerobic	-	-	-	-	-	+++	+++	#	+	+	++	#	+	+
LYQ	Anaerobic	-	-	-	-	-	++	+++	#	+	+	++	#	+	-
CLQ	Anaerobic	-	-	-	-	-	++	++	#	-	-	+	#	+	-
VI3	Anaerobic	-	-	-	-	-	+++	+++	#	-	-	-	#	+	+

Strain	FT	C	N	H_2S	M	Temperature Experiments					pH Experiments				
						20 °C	30 °C	37 °C	39 °C	45 °C	5.5	6.3	7.0	7.8	8.0
NBQ-1	Anaerobic	–	–	–	–	++	++	+++	#	+	+	–	#	+	–
NDF-2	Anaerobic	–	–	–	–	+	++	+++	#	+	–	++	#	+	+
LBQ-1	Anaerobic	–	–	–	–	++	+++	+++	#	+++	+	++	#	+	+
LHQ-3	Anaerobic	–	–	–	–	++	++	#	#	+++	+	++	#	+	–
CBQ	Anaerobic	–	–	–	–	++	++	++	#	++	–	+	#	+	–
X6C1	Anaerobic	–	–	–	–	++	++	#	+++	++	–	++	#	+	–
Strain	FT	C	N	H_2S	M	Temperature Experiments					pH Experiments				
						20 °C	30 °C	37 °C	39 °C	45 °C	5.5	6.3	7.0	7.8	8.0
Blank		–	–	–	–	–	–	–	–	–	–	–	–	–	–

* The cell concentration decreased in an order of "#", "+++", "++", and "+"; FT: fermentation type; C: catalase; NR: nitrate reduction; H_2S: formation of H_2S; M: motility

Table 4(2). Physiological and Biochemical Characteristic of Isolated Strains (2)

Strain	G1	M1	L	A	R	S1	S2	M2	M3	F	G2	E	S3	G3	X
WH-1	+	–	–	+	–	–	+	–	–	+	–	–	–	+	+
WH-22	+	–	–	+	–	–	–	–	–	+	–	–	–	+	+
WH-3	+	–	–	+	–	–	–	–	–	+	–	+	–	+	+
LHH-1	+	–	–	+	–	–	–	–	–	+	–	+	+	+	+
WHQ-1	+	–	–	+	–	–	–	–	–	+	–	+	–	+	+
WG-1	+	–	–	+	–	–	–	–	–	+	+	+	–	+	+
NBG	+	–	+	–	–	–	+	–	–	–	–	+	–	–	–
LBG-11	+	–	+	–	–	–	–	+	–	–	–	–	–	–	–
LHG	+	–	+	+	–	–	–	–	+	–	–	–	+	–	–
NDF-3	–	–	–	+	–	–	+	+	+	–	–	+	–	–	+
LH-1	–	–	–	+	–	–	+	+	+	–	–	+	–	–	+
LLH	–	–	–	+	–	–	+	+	+	–	–	+	–	–	+
CCQ	–	–	–	–	–	–	+	–	–	–	–	+	–	–	–
LYQ	+	–	–	–	–	–	+	–	–	–	–	+	–	–	–
CLQ	+	–	–	–	–	–	–	–	–	+	–	+	–	+	+
VI3	+	–	–	–	–	–	–	–	–	+	–	–	–	–	–
NBQ-1	+	–	–	–	+	–	–	–	+	+	–	+	–	+	+
NDF-2	+	+	+	+	+	–	+	+	+	+	–	+	–	+	+
LBQ-1	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+
LHQ-3	+	+	+	+	+	+	–	+	+	+	–	+	–	+	+
CBQ	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+
X6C1	+	+	+	+	+	+	+	+	+	+	–	+	–	+	+
Blank	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Note: +: Positive; -: negative; G1: glucose; M1: mannitol; L: lactose; A: L-arabinose; R: raffinose; S1: sorbitol; S2: salicin; M2: maltose; M3: melibiose; F: fructose; G2: glycerol; E: esculin; S3: sodium lactate; G3: galactose; X: D-xylose

As shown in Tables 2 through 4, the morphological features, the physiological and biochemical characteristics indicated that approximately 22 individual strains were identified. The identification results are shown in Table 5. From the identification results,

there were six strains of *Butyrivibrio fibrisolvens*, three strains of *Fibrobacter succinogenes*, three strains of *Clostridium polysaccharolyticum*, two strains of *Ruminococcus flavefaciens*, two strains of *Ruminococcus callidu*s, and one strain of *Ruminococcus albus*, in the 22 strains of isolates. Five strains of *Ruminococcus* were only identifiable to the genus level.

Table 5. Traditional Identification Results of 22 Isolates

Genus	*Butyrivibrio*	*Ruminococcus*				*Bacteroides*	*Clostridium*
Species	*B.fibrisolvens*	*R. flavefaciens*	*R.albus*	*R. callidus*	Unknown	*F. succinogenes*	*C.Polysaccharolyticum*
Strain	WH-1, WH-2, WH-3, WG-1, WHQ, LHH-1	CCQ, LYQ	VI3	CLQ, NBQ-1	NDF-2, LBQ-1, LHQ-3, CBQ, X6C1	NBG-1, LBG-1, LHG	NDF-3, LH-1, LLH

To ensure that isolates were identified accurately, ten strains that solubilized filter paper well and represented various species were identified using molecular biology methods. The identification results of (G + C) mol% and 16S rDNA were shown in Table 6. The results showed that molecular biology-based identification was consistent with traditional methods.

Table 6. Molecular Identification of Selected Strains

Strains	Tm (°C)	(G + C) mol%	Nearest Valid Taxon	Accession Number
WH-1	87.45	40.7	*Butyrivibrio fibrisolvens*	EU106047
WH-22	87.02	39.8	*Butyrivibrio fibrisolvens*	KC438276
WG-1	86.06	37.8	*Butyrivibrio fibrisolvens*	HQ404371
WHQ	87.21	40.2	*Butyrivibrio fibrisolvens*	HQ404372
CCQ	88.65	43.2	*Ruminococcus flavefaciens*	KC438277
LYQ	87.11	40.0	*Ruminococcus flavefaciens*	KC438278
VI3	88.75	43.4	*Ruminococcus albus*	HQ404370
LBG-11	90.63	47.3	*Fibrobacter succinogenes*	KC438280
NBG	90.58	47.2	*Fibrobacter succinogenes*	KC438280
NDF-3	88.32	42.5	*Clostridium polysaccharolyticum*	HQ404373

After identification *via* traditional and molecular biology methods, there were four strains of *Butyrivibrio fibrisolvens*, two strains of *Rumincoccus flavefaciens*, two strains of *Fibrobacter succinogenes*, one strain of *Rumincoccus albus*, and one strain of *Clostridium polysaccharolyticum* in the 10 selected microbial strains.

Determination of Cellulase Activities

One strain was selected from each *R. albus*, *R. flavefaciens*, *F. succinogenes*, and *B. fibrisolvens*. The extracellular cellulase and total cellulase activities of four types of

cellulase (endoglucanase, exoglucanase, β-glucosidase, and filter paper enzyme) are shown in Table 7.

As a result, the total filter paper enzyme activity of the representative strains from *B. fibrisolvens*, *R. albus*, *R. flavefaciens*, and *F. succinogenes* were 19.48 ± 2.19 IU, 33.86 ± 3.16 IU, 32.30 ± 3.99 IU, and 47.49 ± 3.37 IU, respectively.

Table 7. Cellulase Activities for Four Strains

Strain	Filter Paperase Activity (IU)		Endoglucanase Activity (IU)		Exoglucanase Activity (IU)		β-Glucosidase Activity (IU)	
	Extracellular Cellulase Activity	Total Cellulase Activity	Extracellular Cellulase Activity	Total Cellulase Activity	Extracellular Cellulase Activity	Total Cellulase Activity	Extracellular Cellulase Activity	Total Cellulase Activity
VI3	4.37 ± 0.33^{a}	33.86 ± 3.16^{cA}	10.36 ± 1.16^{a}	66.92 ± 1.57^{c}	5.83 ± 0.52^{a}	39.76 ± 1.07^{c}	33.44 ± 1.69^{a}	204.99 ± 8.28^{c}
CCQ	4.65 ± 0.46^{a}	32.30 ± 3.99^{cA}	9.76 ± 0.58^{a}	72.52 ± 3.81^{c}	5.29 ± 0.61^{a}	37.69 ± 2.89^{c}	32.16 ± 1.87^{a}	205.46 ± 4.25^{c}
LBG-1	6.33 ± 0.22^{a}	47.49 ± 3.37^{cC}	10.32 ± 0.62^{a}	93.94 ± 5.64^{c}	6.85 ± 0.76^{a}	55.33 ± 6.21^{c}	28.32 ± 1.59^{a}	222.40 ± 11.94^{c}
WH-1	2.77 ± 0.13^{a}	19.48 ± 2.19^{cE}	4.13 ± 0.19^{a}	38.66 ± 3.78^{c}	3.29 ± 0.20^{a}	24.69 ± 2.04^{c}	17.91 ± 0.49^{a}	106.33 ± 11.74^{c}

Note: a through c: x ± s within the columns of the extracellular cellulase and total cellulase activities with lowercase superscripts are different; a through C: x ± s within the row of cellulose activity with uppercase superscripts are different; the adjacent letters are different; the interval letters are significantly different

Discussion on the Difference in Enzyme Activity

Most isolates with transparent circles on the CMC-Na plate did not effectively degrade the filter paper. The results showed that multiple bacterial isolates from the rumen of Inner Mongolian sheep degraded CMC-Na and did not degrade the filter paper because CMC-Na was degraded by endoglucanase, whereas the filter paper was degraded by the mixture or complex of endoglucanase, exoglucanase, and β-glucanase. Many carboxymethyl cellulose (CMCases) have minimal capacity to digest native insoluble cellulose, and many non-cellulolytic ruminal bacteria can hydrolyze CMC-Na even though they cannot utilize native cellulose as a substrate for growth (Avguštin *et al.* 1997; Fields *et al.* 1998). The CMCase activity was not strongly correlated with cellulose utilization (Fields *et al.* 1998) because all CMCase-positive, cellobiose-utilizing ruminal bacteria grew on β-glucan, and CMCases appear to be a mechanism for the utilization of water-soluble mixed β-glucans rather than native cellulose (Fields *et al.* 1998). The combined method of transparent zone and filter paper degradation was used in this study because the transparent zone method can save time and the filter paper degradation experiment can accurately select cellulose-solubilizing bacteria. Kong *et al.* (2012) studied the rumen of cattle fed alfalfa or triticale by fluorescence *in situ* hybridization (FISH), and the results indicated that CMC-Na-digesting bacteria contributed between 8.2% and 10.1% to the total bacterial cell numbers. In the study, some CMC-Na solubilizing bacteria were isolated from the rumen of Inner Mongolian sheep, which also indicated that rumen of Inner Mongolian sheep contained a huge pool for cellulolytic microorganisms because they,

through evolution, have a high tolerance for heterogeneous and plant polysaccharide-rich foraged materials.

The number of *B. fibrisolvens* was highest among the isolated 10 strains. Although *B. fibrisolvens* were not considered as the main cellulolytic strain in early studies (Michalet-Doreau *et al.* 2001), recent studies have verified the importance of *B. fibrisolvens* both in number and cellulolytic activity in rumen *via* molecular biology and traditional culture methods (Hess *et al.* 2011; Kenters *et al.* 2011; Nyonyo *et al.* 2013). Hess *et al.* (2011) demonstrated that the gene of *B. fibrisolvens* comprised of a substantial proportion in the metagenome of rumen and played an important role in the deconstruction of fiber *via* metagenomic analysis. In recent rumen microbiome studies (Edwards *et al*. 2017), *B. fibrisolvens* also were found as a major species. Kenters *et al.* (2011) isolated four strains of *R. albus*, six strains of *R. flavefaciens*, eleven strains of *B. fibrisolvens*, and two strains of *C. polysaccharolyticum* from the rumen of cows in New Zealand with pasture hay after 48 h of exposure to a rye-grass clover pasture. Nyonyo *et al.* (2013) isolated three strains of *Ruminococcus*, one strain of *Fibrobacter*, two strains of *Clostridium*, and twenty-one strains of *Butyrivibrio* from the rumen of Holstein cows using a novel anaerobic media. Nyonyo *et al.* (2014) isolated 129 strains from the rumen of Holstein cows, and there were twenty-two strains of *Butyrivibrio*, one strain of *Enterococcus*, five strains of *Ruminococcus*, one strain of *Clostridium* cluster IV, and three strains of *Fibrobacter*. Molecular biology has allowed more detailed and accurate analyses of rumen microbial flora.

Intra-genomic heterogeneity of 16S rDNA genes can also cause overestimation of microbiological strains (Sun *et al.* 2013). Thus, cultivation *in vitro* is still a good approach to the analysis of rumen microbial flora. As shown in Table 7, the filter paperase activity was lower than that of endoglucanase, exdoglucanase, and β-glucosidase activities in the same strain because the filter paperase activity is the sum of other three types of enzyme activities. Exoglucanase activity was close to the filter paperase enzyme activity and lower than the endoglucanase and β-glucosidase activities because exoglucanase activity was identified as the rate-limiting enzyme in cellulose degradation (Lynd *et al.* 2002). The total cellulase activity significantly increased compared with the extracellular cellulase activity ($P < 0.01$) because substantial cellulase comprises intracellular enzyme, extracellular enzyme, and enzymes that connect to cells (Weimer *et al.* 1990; Gong and Forsberg 1993). The filter paperase activity of LBG-1, VI3, CCQ, and WH-1 exhibited a decreasing trend. The filter paperase activity of LBG-1 was higher than VI3 and CCQ ($P < 0.01$), and no significant difference was identified between VI3 and CCQ ($P > 0.05$). The filter paperase activity of CCQ was higher than WH-1 ($P < 0.01$). There were different conclusions regarding the cellulase activity of *R. albus*, *R. flavefaciens*, and *F. succinogenes*. Miron (1993) reported that *F. succinogenes* S85 digested more cellulose from cellulolytic material *in vitro* than the two other predominant cellulolytic bacterial species, *R. albus* and *R. flavefaciens*. Nyonyo *et al.* (2014) isolated 129 strains from the rumen of Holstein cows. The results indicated that 51, 117, and 105 strains had filter paperase, carboxymethylcellulase, and xylanase activities, respectively, and 44 strains had both filter paperase and CMCase activities. The filter paperase activity was in an order of *R. albus*, *R. flavefaciens*, *F. succinogenes*, *Clostridium* cluster IV, and *B. fibrisolvens*. The difference in enzyme activity was caused by the difference in different strains.

CONCLUSIONS

1. Cellulolytic bacterial flora in the rumen of Inner Mongolian sheep included *B. fibrisolvens*, *R. flavefaciens*, *F. succinogenes*, *R. albus*, *R. callidus*, *C. polysaccharolyticum*. The *B. fibrisolvens* species made up the largest proportion of the microbiome.
2. The total cellulase activity of the representative strains from *B. fibrisolvens*, *R. albus*, *R. flavefaciens*, and *F. succinogenes* were high.
3. The rumen microbiome of Inner Mongolian sheep represents an important source for cellulolytic microorganisms and enzymes.

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