

# Characterization of the Rumen-Mimetic Continuous Cultivation System for Volatile Fatty Acid Production from Lignocellulosic Biomass

Hitosi Agematu<sup>1,\*</sup>, Jun Watanabe<sup>2</sup>, Takehiko Takahashi<sup>3</sup>

<sup>1</sup>Department of Applied Chemistry, Akita National College of Technology, Akita, Japan

<sup>2</sup>Department of Agribusiness, Akita Prefectural University, Ohgata, Japan

<sup>3</sup>Department of Machine Intelligence and Systems Engineering, Akita Prefectural University, Yurihonjo, Japan

## Email address:

agematu@akita-nct.ac.jp (H. Agematu)

\*Corresponding author

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**Abstract:** The rumen-mimetic continuous cultivation system (RMS) was used to produce volatile fatty acids (VFAs), such as acetic, propionic, and butyric acids, from lignocellulosic biomass. Typical 12 species of ruminal bacteria were detected in the culture broth cultivated by RMS using species-specific PCR assay. These species are known to be cellulolytic, hemicellulolytic, proteolytic, amylolytic, and lipolytic bacteria and methanogens. Therefore, RMS was characterized to be ruminal fermentation like the digestive system of roughage in the rumen of cattle. Using RMS, the ruminal bacteria were continuously cultivated for 120 days to produce VFAs. The yield of total VFAs from 20–50  $\mu$ m rice straw was 167% higher than that from 300–1000  $\mu$ m rice straw. However, the productivity was not increased by pulverization of the substrate. The yield and productivity of acetic acid from 20–50  $\mu$ m rice straw were 0.147 g/g and 34.8 mg/L/h, respectively, and those of propionic acid were 0.104 g/g and 24.6 mg/L/h, respectively. The selectivity (P/A ratio) of acetic and propionic acid production from microcrystalline cellulose at pH 6.5 and 7.0 were  $0.83 \pm 0.07$  and  $0.31 \pm 0.03$ , respectively. The ruminal bacteria could not continue to produce VFAs at pH 6.0. The yield and productivity of acetic acid from microcrystalline cellulose at pH 6.5 were 0.234 g/g and 51.7 mg/L/h, respectively, and those of propionic acid were 0.223 g/g and 49.3 mg/L/h, respectively. This study proposes that in order to increase the productivity of RMS, it is necessary to increase the ruminal bacterial biomass by recycling biomass (flocs) using a settling tank.

**Keywords:** Rumen, Ruminal Bacteria, Volatile Fatty Acid, Acetic Acid, Propionic Acid, Lignocellulosic Biomass

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## 1. Introduction

Lignocellulose is the main constituents in most plants and is comprised of cellulose (40%–60%), hemicellulose (20%–30%), and lignin (15%–30%). It is the most abundant biomass on earth. Lignocellulosic biomass is classified into four groups based on the resource: (i) municipal solid waste, (ii) agricultural residues, (iii) forest residues, and (iv) wastepaper [1]. They are inexpensive, renewable, and available everywhere. The utilization of lignocellulosic biomass has become one of the most important avenues of research in the global shift from fossil fuels to renewable resources. Lignocellulosic biomass can be converted into more than a

hundred different chemicals [2]. Among them volatile fatty acids (VFAs; mainly acetic, propionic, and butyric acids) are widely used as building block chemicals, which are employed in the manufacturing of a wide range of chemicals, pharmaceuticals, and biopolymers [1]. Therefore, biological VFAs production from lignocellulosic biomass has drawn extensive research interests to facilitate transition from an oil-based to a bio-based economy [1, 3]. However, it is economically limited by high downstream purification cost [4]. It is essentially important to produce VFAs from cheaper materials with lower purification cost. To reduce the purification cost, a fermentation broth from which VFAs can be purified at a low cost is needed. Biological VFAs

production will be addressed if the target yield, productivity, and concentration of VFAs and less by-product fermentation can be achieved in the bioprocess.

The rumen-mimetic continuous cultivation system (RMS), including an energy-saving pulverization method of the substrate, has been proposed as a bioprocess to produce VFAs from lignocellulosic biomass [5, 6]. RMS is an anaerobic continuous cultivation system operated under well-defined conditions. The anaerobic process is easy to scale-up because the process does not need sterilization and ventilation. The flow rate is controlled to maintain broth pH at 6.50 by the addition of basic artificial saliva (mineral salts solution). The digestion of substrate is conducted in a batch type fermentation process because the substrate is an insoluble solid having a specific gravity heavier than water. RMS is a mixed culture fermentation process using the rumen fluid of cattle as an inoculum. However, the microbial community structure of RMS has not yet been completely analyzed. The diversity of ruminal bacteria is known to be highly responsive to a wide spectrum of substrates [7]. The cross feeding among ruminal bacteria results in a relatively more complete conversion of lignocellulosic biomass into the final fermentation products of VFAs [8]. Therefore, if it could be confirmed that RMS recreates the same conditions as observed during the digestive process in the rumen of cattle, namely ruminal fermentation, then the high yield and productivity of VFAs and the diversity of substrate could be expected from RMS.

In this study, to analyze the microbial community structure of ruminal bacteria cultivated by RMS, the PCR assays targeting species-specific regions of 16S rDNA and genes were carried out. The effect of pulverization of substrate on the yield and productivity of VFAs was estimated. To optimize cultivation conditions, the effect of controlled pH on the selectivity of VFAs production was investigated. Furthermore, the response of ruminal bacteria to the conditions without feeding on nitrogen source was examined.

## 2. Materials and Methods

### 2.1. Sampling of Rumen Fluid

All experimental protocols were approved by the Animal Care and Use Committee of Akita Prefectural University (Ohgata, Akita, Japan). In this study, a Japanese shorthorn non-pregnant and non-lactating cow was used as the donor animal for ruminal fluid. The cow was fed 5 kg of hay (dry matter [DM] basis) and 0.65 kg of concentrate mixture (corn, buckwheat, barley, rice bran, and soybean meal; DM basis) twice daily, at 8:30 and 16:00. Approximately 1 L of rumen fluid was anaerobically collected from the cow using a stainless-steel oral catheter for cattle (Sanshin Industrial Co. Ltd., Kanagawa, Japan). The bottle was immediately filled with O<sub>2</sub>-free CO<sub>2</sub> gas and was maintained at 39°C. The rumen fluid was then transferred to the laboratory in Akita National College of Technology within 1 h.

### 2.2. Continuous Cultivation of Ruminal Bacteria Using RMS

The experimental conditions of RMS have been described in previous articles [5, 6]. Briefly, the cultivation was carried out anaerobically by intermittent ventilation with nitrogen gas at an incubation temperature of 39°C and the contents in the reactor were agitated at 30–40 rpm using impellers. The cultivation was simultaneously carried out in reactor A and B using 0.5 L of rumen fluid as a seed. The working volume of reactor A and B was 2.0 L. Twenty grams of microcrystalline cellulose (102331; Merck KGaA, Darmstadt, Germany; 20–160 µm [ $\geq 80\%$ ]) or lignocellulosic biomass powder, such as rice straw and alfalfa hay, was continuously added to the reactor to produce VFAs. These additions were carried out when the substrate was completely digested and the medium pH increased above controlled pH levels. Pulverized alfalfa hay was fed once in approximately 20 days to supplement trace nutrients for ruminal bacteria, when continuous cultivation was carried out using microcrystalline cellulose as the sole carbon source. During fermentation, the pH of the culture solution was automatically controlled so as to not fall below 6.0, 6.5, or 7.0 (representing individual reaction conditions) by addition of basic artificial saliva containing bicarbonate, phosphate, urea, and ammonium. Simultaneously, the same volume of culture suspension was withdrawn from the reactor as an effluent. To analyze the microbial community structure in the reactor, about 10 mL of the reactor contents was collected before each feeding of substrate and was preserved at -20°C. All equipment and substrates were not sterilized.

### 2.3. Pulverization of Substrates

Rice straw and alfalfa hay were pulverized using a WB-1 blender (WARING, Torrington, USA) or a vibration mill using cog-ring media, called a tandem-ring mill [9, 10], under dry conditions. The rice straw pulverized by a WB-1 blender was separated into 300–1000 µm, 100–300 µm, and less than 100 µm, based on its particle size using a sieve. The average particle diameter of the rice straw pulverized by a tandem-ring mill was 20–50 µm. The productivity (g/L/h) was expressed by product (g) per working volume (L) per digestion time (h).

### 2.4. Cultivation Without Feeding on Nitrogen Source

To investigate the effect of nitrogen source feeding on VFA production, the artificial saliva without nitrogen sources, namely urea and ammonium sulfate, was used to control the broth pH at 6.5. Twenty grams of microcrystalline cellulose was added to reactor A and B every 24 hours as a supply of carbon source, ensuring that it does not become a limiting factor for VFAs production. Twenty grams of alfalfa hay powder was added to the reactors after 6 days as a nitrogen source. The amounts of VFAs produced every 24 hours were determined.

### 2.5. High-Performance Liquid Chromatography (HPLC) Analysis

Acetic, propionic, and butyric acids in the effluent from the reactor were quantified by HPLC. The HPLC conditions and preparation of HPLC sample have been described in previous articles [5, 6].

### 2.6. Bacterial DNA Extraction

Total DNA was extracted from the rumen fluid collected from the cow and the reactor contents collected using an ISOPLANT II DNA extraction kit (Nippon Gene Co. Ltd., Tokyo, Japan) according to the manufacturer's instructions. The quantity and quality of the extracted DNA were verified by measuring the absorbance at 260 and 280 nm using a spectrophotometer, and the final concentration of DNA was adjusted to 10 ng/ $\mu$ L for DNA amplification through polymerase chain reaction (PCR).

### 2.7. 16S rDNA PCR-amplification

Seventeen species-specific PCR primer pairs, used to amplify partial 16S rDNA [11–14] and *nifH* gene [15] regions, and two genus-specific PCR primer pairs [14] were chosen (Table 1). A reaction mixture containing 20 pmol of each primer, 50 ng of template DNA, and 20  $\mu$ L of Emerald Amp PCR master mix (Takara Bio INC., Shiga, Japan) in a total volume of 40  $\mu$ L was prepared. PCR was performed by GeneAtlas 322 (ASTEC CO. LTD., Fukuoka, Japan) using the following temperature settings: an initial denaturation at 94 °C for 6 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at the temperature indicated for the primer pair (Table 1) for 30 s, and extension at 72 °C for 30 s. A final extension step at 72 °C was carried out for 10 min. The amplicons were examined by electrophoresis on 2.0% (w/v) agarose gels. Gels were stained with SYBR Green I (Takara Bio INC., Shiga, Japan) according to manufacturer's instructions and photographed with UV transillumination using a Gel Doc EZ System (Bio-Rad Laboratories, Inc., CA, USA).

### 2.8. Metabolic Flux Analysis of VFAs Production from Cellulose

The cell biomass of continuous cultivation is in a stationary phase. If the carbohydrate converted to microbial organic matter is ignored, the amounts of certain catabolic products can be stoichiometrically calculated from the molar productions of VFAs using “fermentation balance” [16]. When the productions of acetic, propionic, and butyric acids from 1 g of substrate are A, P, and B mmol, respectively, the values are calculated as follows:

Total VFAs yield (mmol/g) is  $A + P + B$ .

Glucose used (mmol/g) is  $0.5A + 0.5P + B$ .

Cellulose used (g/g) is  $(0.5A + 0.5P + B) \times 0.162$ .

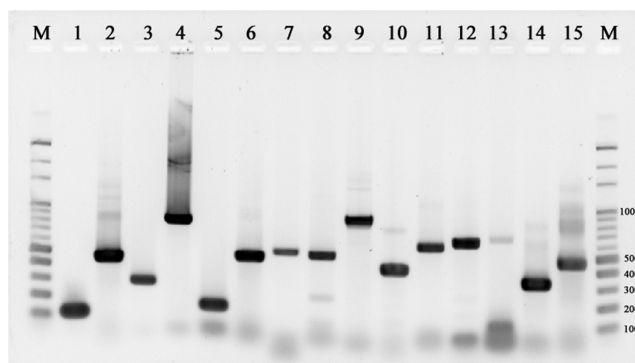
Carbon dioxide yield (mmol/g) is  $0.5A + 0.25P + 1.5B$ .

Methane yield (mmol/g) is  $0.5A - 0.25P + 0.5B$ .

NADH generated (mmol/g) is  $2A - P + 2B$ .

ATP yield (mmol/g) is  $2.5A + 2.75P + 3.5B$ .

Potential microbial yield (g/g) is  $25A + 27.5P + 35B$  (using 10 g-cell/mol-ATP).



**Figure 1.** Amplification of rumen bacterial DNA with the primer set detailed in Table 1. Lanes (size [bp]): 1, *Fibrobacter succinogenes* (120); 2, *Fibrobacter succinogenes* (446); 3, *Ruminococcus flavefaciens* (295); 4, *Ruminococcus flavefaciens* (835); 5, *Ruminococcus albus* (175); 6, *Prevotella ruminicola* (485); 7, *Prevotella bryantii* (540); 8, *Selenomonas ruminantium* (513); 9, *Streptococcus bovis* (869); 10, *Treponema bryantii* (421); 11, *Anaerobivrio lipolytica* (597); 12, *Ruminobacter amylophilus* (642); 13, *Eubacterium ruminantium* (671); 14, *Methanobrevibacter ruminantium* (336); 15, methanogen (414–438). Lane M, DNA size marker.

## 3. Results

### 3.1. Species-specific PCR Assays for Ruminal Bacteria

Ruminal bacteria were continuously cultivated for 120 days by RMS. Fourteen species-specific primer pairs recognizing 12 species of ruminal bacteria amplified the target DNA fragments from bacterial DNA extracts, while 3 primer pairs did not (Table 1). The sizes of PCR products were as expected (Figure 1). Therefore, 12 species of ruminal bacteria were detected in the culture broth. The target DNA product for *Succinivibrio dextrinosolvens* could not be detected after 12 days of cultivation. The primer pairs for *Prevotella albensis* and *Megasphaera elsdenii* did not amplify target sequences even from the total DNA extract of rumen fluid used as a seed. The other amplified target DNA products were detected until the end of cultivation.

### 3.2. Effect of Pulverization of Substrate

Twenty grams of pulverized rice straw, of varying particle size, were added to the reactor to investigate the effect of pulverization of substrate on VFAs production (Table 2). The digestion time was the time between the addition of substrate and the end of its digestion indicated by rise in pH (Figure 2). The yield of acetic acid, propionic acid, and total VFAs from 300–1000  $\mu$ m rice straw were 0.104 g/g, 0.045 g/g, and 2.46 mmol/g, respectively, while those from 20–50  $\mu$ m rice straw were 0.147 g/g, 0.104 g/g, and 4.11 mmol/g, respectively. The yield of total VFAs from 20–50  $\mu$ m rice straw was 167% higher than that from 300–1000  $\mu$ m rice straw. Therefore, the yield was increased by pulverization. The digestion time also increased due to pulverization. The productivity of acetic acid, propionic acid, and total VFAs from 300–1000  $\mu$ m rice straw

were 39.8 mg/L/h, 17.3 mg/L/h, and 0.94 mmol/L/h, mg/L/h, 24.6 mg/L/h, and 0.97 mmol/L/h, respectively. respectively, while those from 20–50  $\mu$ m rice straw were 34.8

**Table 1.** PCR primers for detection of ruminal bacteria.

Target species	Primer sequences (5'-3')	Annealing (°C)	Size (bp)	Amplification <sup>*</sup>	Reference
<i>Fibrobacter succinogenes</i>	F GTTCGGAATTACTGGGCGTAAA R CGCCTGCCCTGAACTATC	60	120	+	11
	F GGTATGGGATGAGCTTGC R GCCTGCCCTGAACTATC	60	446	+	12, 13
	F TCTGGAACGGATGGTA R CCTTTAAGACAGGAGTTTACAA	55	295	+	13
<i>Ruminococcus flavefaciens</i>	F GGACGATAATGACGGTACTT R GCAATCYGAAGTGGGACAAT	62	835	+	12, 13
	F CCCTAAAAGCAGTCTTAGTTTCG R CCTCCTTGCAGTTAGAACA	55	175	+	13
<i>Ruminococcus albus</i>	F GGTATCTTGAGTGAGTT R CTGATGGCAACTAAAGAA	53	485	+	12, 14
<i>Prevotella ruminicola</i>	F ACTGCAGCGCAACTGTCAGA R ACCTTACGGTGGCAGTGTCTC	65	540	+	12, 14
<i>Prevotella bryantii</i>	F CAGACGGCATCAGACGAGGAG R ATGCAGCACCTTCACAGGAGC	68	861	-	12
<i>Prevotella albensis</i>	F GACCGAAACTGCGATGTAGA R CGCCTCAGCGTCAGTTGTC	58	130	-	14
<i>Megasphaera elsdenii</i>	F TGCTAATACCGAATGTTG R TCCTGCACTCAAGAAAGA	57	513	+	14
<i>Selenomonas ruminantium</i>	F GACCGAAACTGCGATGTAGA R CGCCTCAGCGTCAGTTGTC	57	869	+	12, 14
<i>Streptococcus bovis</i>	F AGTCGAGCGGTAAGATTG R CAAAGCGTTTCTCTCACT	57	421	+	12, 14
<i>Treponema bryantii</i>	F TGGGTGTTAGAAATGGATTC R CTCTCCTGCACTCAAGAATT	58	597	+	12, 14
<i>Anaerovibrio lipolytica</i>	F CAACCACTCGCATTGAGA R CACTACTCATGGCAACAT	57	642	+	12, 14
<i>Ruminobacter amylophilus</i>	F TGGGTGTTAGAAATGGATTC R CTCTCCTGCACTCAAGAATT	58	854	-	12, 14
<i>Succinivibrio dextrinosolvens</i>	F GCTTCTGAAGAATCATTTGAAG R TCGTGCCCTCAGTGTCAGTGT	57	671	+	12
<i>Eubacterium ruminantium</i>	F AATATTGCAGCAGCTTACAGTGAA R TGAAAATCCTCCGCAGACC	63	336	+	15
<i>Methanobrevibacter ruminantium</i> ( <i>nifH</i> gene)	F GGTGGTGTGAGTTCACACARTAYGCWA CAGC	55	414-438	+	14
Methanogen ( <i>mcrA</i> gene)	R TTCATTGCRTAGTTWGGRTAGTT				
Genus <i>Prevotella</i>	F CACRGTAACGATGGATGCC R GGTCGGGTGTCAGACC	55	534	+	14

F, forward primer; R, reverse primer. \* this study.

**Table 2.** Production of acetic, propionic, and butyric acids from 20 g of pulverized rice straw.

Particle size ( $\mu$ m) (n <sup>a</sup> )	Digestion time (h)	Effluent (ml)	Acetic acid (mmol)	Propionic acid (mmol)	Butyric acid (mmol)	Total VFAs <sup>b</sup> (mmol)
300-1000 (3)	26.1 $\pm$ 5.3	465 $\pm$ 90	34.6 $\pm$ 7.4	12.2 $\pm$ 2.5	2.3 $\pm$ 0.4	49.1 $\pm$ 10.3
100-300 (4)	53.2 $\pm$ 6.1	579 $\pm$ 59	40.5 $\pm$ 5.2	18.5 $\pm$ 3.1	2.9 $\pm$ 0.3	61.8 $\pm$ 8.4
< 100 (3)	41.2 $\pm$ 2.0	642 $\pm$ 12	52.1 $\pm$ 3.0	15.7 $\pm$ 0.8	3.2 $\pm$ 0.1	71.0 $\pm$ 3.6
20-50 <sup>c</sup> (3)	42.3 $\pm$ 0.9	803 $\pm$ 40	49.0 $\pm$ 4.1	28.1 $\pm$ 1.3	5.0 $\pm$ 0.1	82.1 $\pm$ 5.3

Values represent means $\pm$ SE. <sup>a</sup> Numbers of separate trials; <sup>b</sup> Total VFAs is the combined amount of acetic, propionic, and butyric acids; <sup>c</sup> Average particle diameter.

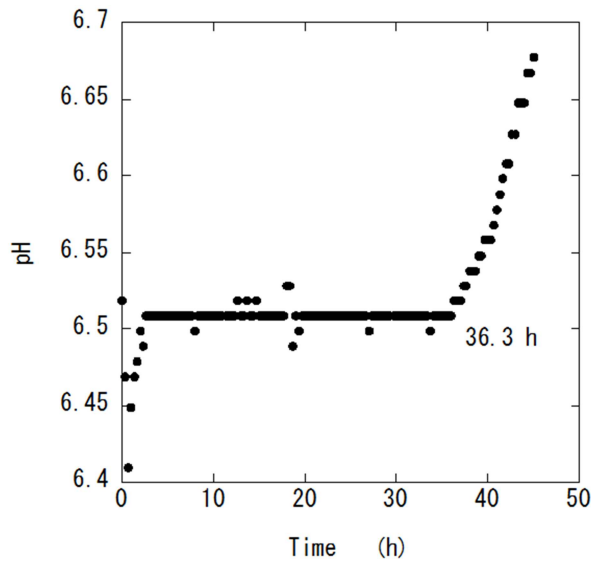
The productivity of total VFAs was not increased by pulverization.

### 3.3. Effect of Cultivation pH

Ruminal bacteria were cultivated at pH 6.0, 6.5, or 7.0 to produce VFAs from 20 grams of microcrystalline cellulose. The amounts of produced VFAs were determined (Table 3). Ruminal bacteria stably produced VFAs at pH 6.5 and 7.0. The amounts of total VFAs produced at pH 6.5 and 7.0 were

almost the same, while the selectivity (P/A ratio [mol/mol]) of acetic acid and propionic acid production at pH 6.5 and 7.0 were 0.83 $\pm$ 0.07 and 0.31 $\pm$ 0.03, respectively. The P/A ratio depended on the cultivation pH. The yield, productivity, and concentration of acetic acid at pH 6.5 were 0.234 g/g, 51.7 mg/L/h, and 3.43 g/L, respectively, while those of propionic acid at pH 6.5 were 0.223 g/g, 49.3 mg/L/h, and 3.28 g/L, respectively. The yield of methane produced theoretically at pH 6.5 and 7.0 was 1.50 and 2.48 mmol/g, respectively. It is

obvious from the metabolic flux analysis described in materials and methods section that propionic acid production and methane production are inversely related.

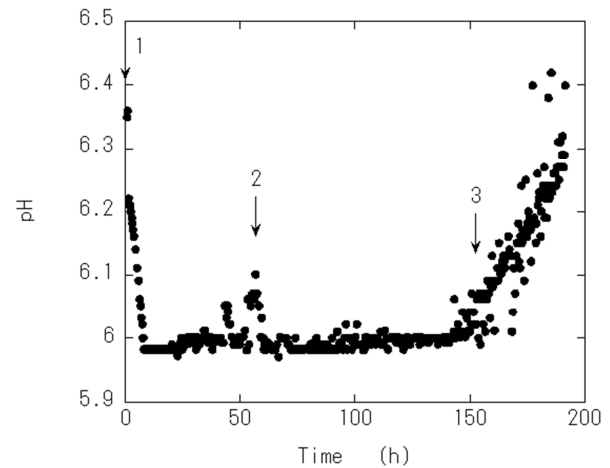


**Figure 2.** Typical pH pattern observed during the digestion of substrate. The rice straw (20 g) pulverized less than 100  $\mu\text{m}$  was added to the reactor. The pH was regulated so as not to decrease below 6.50 via the automatic addition of the artificial saliva. The pH value increased above 6.50 after 36.3 h when the digestion of rice straw ended. The digestion time of the rice straw was 36.3 h.

The concentration of total VFAs in the reactor was  $106 \pm 2.2$  mM, depending on the buffering action of artificial saliva. In the cultivation at pH 6.0, the VFAs production ceased after 150 h of the cultivation (Figure 3). Twenty grams of microcrystalline cellulose was added in the reactor (arrows 1, 2, and 3 shown in Figure 3) when the digestion of substrate ended, following by an increase in pH value above 6.0. The pH value decreased rapidly by the first (arrow 1) and the second (arrow 2) addition because of VFAs production. However, in the third (arrow 3) addition, the pH value did not decrease. This phenomenon indicated that ruminal bacteria could no longer produce VFAs.

### 3.4. Cultivation Without Feeding on Nitrogen Source

When feeding of nitrogen source was stopped, the amount of total VFAs produced in 24 h gradually decreased with cultivation time. However, VFA production continued for 6 days. When 20 grams of alfalfa hay was fed on the sixth day, the VFA production recovered sharply (Figure 4).



**Figure 3.** pH pattern observed during cultivation of ruminal bacteria at pH 6.0.

## 4. Discussion

We have previously analyzed the microbial community structure of RMS using PCR denaturing gradient gel electrophoresis (PCR-DGGE) assay, showing that the structure was relatively stable, and identified three rumen bacterial species: *Fibrobacter succinogenes*, *Prevotella ruminicola*, and *Succiniclasticum ruminis* [5]. However, the similarities of the 16S rDNA sequences of the other DGGE bands with those of known isolates were less than 90% according to BLAST searches of the GenBank database. The ruminal bacterial community appears to include a considerable proportion of unidentified bacteria [17]. Therefore, species-specific PCR assays were used to analyze the microbial community structure in this study. Furthermore, we have shown that ruminal bacteria formed flocs and biofilms on the surface of substrate to construct their community [5].

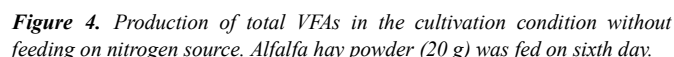
Twelve species of ruminal bacteria (1–12) detected by PCR assays are involved in the fermentation from cellulose to VFAs as shown in Figure 5. *F. succinogenes* (1), *Ruminococcus flavefaciens* (2), and *Ruminococcus albus* (3) are representative cellulolytic bacteria of the rumen [18]. All three bacteria were detected in the culture broth of the reactor. Initially, they break down cellulose into glucose. This hydrolysis is a rate-limiting step of VFAs production. They need symbiotic relationships with proteolytic bacteria because all three species are not proteolytic [8].

**Table 3.** Production of acetic, propionic, and butyric acids from 20 g of microcrystalline cellulose at pH 7.0, 6.5, or 6.0.

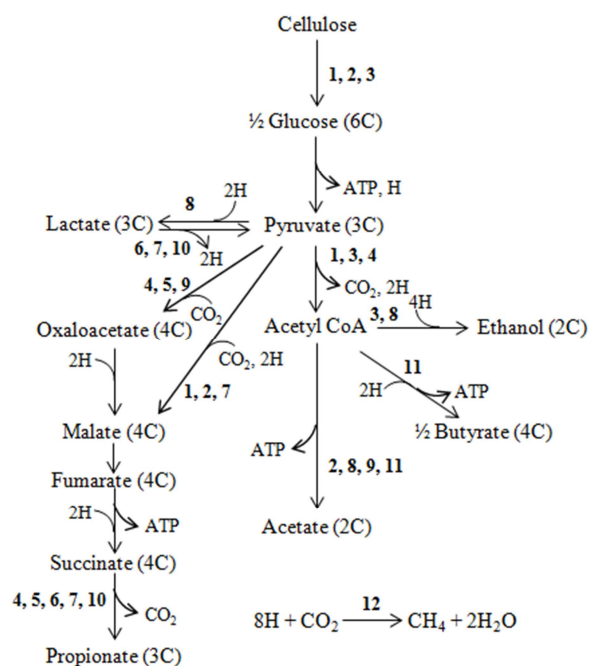
pH (n <sup>a</sup> )	Digestion time (h)	Effluent (ml)	Acetic acid (mmol)	Propionic acid (mmol)	Butyric acid (mmol)	Total VFAs <sup>b</sup> (mmol)	P/A ratio <sup>c</sup> (mol/mol)
7.0 (8)	45.1 $\pm$ 4.6	1471 $\pm$ 104	105.8 $\pm$ 8.5	33.5 $\pm$ 4.1	10.2 $\pm$ 1.0	149.5 $\pm$ 13.0	0.31 $\pm$ 0.03
6.5 (11)	48.3 $\pm$ 2.9	1371 $\pm$ 54	77.9 $\pm$ 6.0	60.3 $\pm$ 2.8	12.1 $\pm$ 1.2	150.4 $\pm$ 8.4	0.83 $\pm$ 0.07
6.0 (2)	72.7	1000	65.1	48.9	10.4	124	0.77

Values represent means $\pm$ SE. <sup>a</sup>Numbers of separate trials, <sup>b</sup>Total VFAs is the combined amount of acetic, propionic, and butyric acids.

<sup>c</sup>P/A ratio=propionic acid (mmol) / acetic acid (mmol)



most rapidly [29]. *E. ruminantium* (11) produced butyrate from two molecules of acetyl coenzyme A (acetyl-CoA) [30]. Methanogenesis is a very important metabolic activity in the rumen microbial ecosystem to eliminate reducing equivalents produced by carbohydrate-fermenting bacteria, although it is an energy and metabolic loss in VFAs production. The detection of *Methanobrevibacter ruminantium* (12) (*nifH*) and methanogens (*mcr A*) using PCR assays showed that methanogenesis was stably maintained by RMS. *M. ruminantium* is one of the most common species of methanogens isolated from the rumen [31] and grows optimally from pH 6.3 to 6.8 [32].



**Figure 5.** The roles of the detected ruminal bacteria in pyruvate metabolism and methanogenesis (adapted from [8]). 1, *F. succinogenes*; 2, *R. flavefaciens*; 3, *R. albus*; 4, *P. ruminicola*; 5, *P. bryantii*; 6, *S. ruminantium*; 7, *S. bovis*; 8, *T. bryantii*; 9, *A. lipolytica*; 10, *R. amylophilus*; 11, *E. ruminantium*; 12, *M. ruminantium*.

It is important to increase accessibility of ruminal bacteria to lignocellulosic materials and accelerate their digestion. The pulverization of lignocellulosic biomass can not only increase its accessible surface area but also decrease the crystallinity of its cellulose to digest it easily [33]. In this study, the theoretically used cellulose of microcrystalline cellulose at pH 6.5 was 0.658 g/g and the yield (g/g) was increased by pulverization of substrate (Table 2). Thus, high yield will be expected with RMS because digestible cellulose and hemicellulose contained in the substrate are completely digested by ruminal bacteria. Although the surface area of substrate was increased by pulverization, the productivity (g/L/h) of the bioprocess was not increased. The result suggested that the rate-determining factor of productivity was bacterial biomass, and not surface area of the substrate. The productivity of VFAs depends on bacterial biomass when there is enough substrate for bacteria to digest. The bacterial



biomass is almost constant in the steady state of continuous cultivation. Based on these considerations, the productivity of VFAs will be constant in most digestion time regardless of the amount of substrate. Therefore, the productivity was not increased by pulverization. To increase the productivity, it will be necessary to increase the ruminal bacterial biomass by feeding substrate containing crude protein to promote cell synthesis and/or recycling biomass (flocs) using a settling tank.

The final products of ruminal fermentation will vary according to microbiota, substrate type, substrate concentration, and fermentation conditions, particularly the pH. The variation is basically due to metabolic pathways used by different populations of ruminal bacteria to get energy (ATP) from substrate (Figure 5). In the present study, although the amount of total VFAs produced at pH 7.0 and 6.5 were almost the same, the amount of propionic acid at pH 6.5 was more than that at pH 7.0. This result indicated that the activity of ruminal bacteria that metabolized pyruvate to propionate was higher than the ones metabolizing pyruvate to acetate at pH 6.5, while the later was higher than the former at pH 7.0. The VFAs production did not continue at pH 6.0 (Figure 3). This was because cellulolytic bacteria could not grow with a low intracellular pH [34]. Therefore, the operation at pH 6.5 was chosen to produce more propionic acid from lignocellulosic biomass. The market price of propionic acid is 2.5 times higher than that of acetic acid [1]. Moreover, potential microbial yield (g/g) at pH 6.5 is theoretically more than that at pH 7.0 according to metabolic flux analysis. In another experiment using ruminal fluid collected from the same cow on another day, the P/A ratio (mol/mol) was  $1.21 \pm 0.04$  (data not shown). The result showed that the variations in ruminal fluid collected from a cow also influenced P/A ratio. Furthermore, it may be possible to increase P/A ratio by increasing the population of succinate-utilizing bacteria (Figure 5).

Most of the ruminal bacteria can use ammonia as a nitrogen source [8]. Therefore, lignocellulosic biomass not containing a nitrogen source, such as wastepaper, can be digested by ruminal bacteria via supplying ammonium. Ruminal bacteria are known to lyse even when they are growing, independent of the growth rate [35]. A nitrogen source, which is necessary for their growth, is partially provided in the form of protein by autolysis. Thus, ruminal bacteria continued to produce VFAs for 6 days without feeding of nitrogen source (Figure 4). When alfalfa hay was fed on sixth day, the VFAs production resumed to initial levels immediately. The result showed that the cell mass of ruminal bacteria scarcely decreased even if nitrogen source feeding was stopped for 6 days.

## 5. Conclusion

All ruminal bacteria needed to produce VFAs, especially acetic acid and propionic acid, from lignocellulosic biomass were stably cultivated by RMS. Therefore, RMS is ruminal fermentation effectively producing VFAs. The vast array of knowledges about ruminal fermentation in the field of animal

husbandry would help to understand the operating conditions of RMS and to develop an optimal operating strategy. The yield (g/g) was increased by pulverization of the substrate, while the productivity (g/L/h) was not. To increase the productivity, it will be necessary to increase the ruminal bacterial biomass by feeding substrate containing crude protein to promote cell synthesis and/or recycling biomass (flocs) using a settling tank. The composition of VFAs production, namely P/A ratio, depended on cultivation pH. The cultivation at pH 6.5 was suitable for propionic acid production. Because the ruminal bacteria digested most of the organic matter except for lignin to produce VFAs, carbon dioxide, and methane, the main water-soluble products in the effluent were VFAs. This is advantageous for purifying VFAs at a low cost and critical to downstream applications.

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