Characterization of highly active endoglucanases in rumen microbial community and development of a novel method for recovering rumen microorganisms with endoglucanase activity

論文内容要旨

Characterization of Highly Active Endoglucanases in Rumen Microbial Community and Development of a Novel Method for Recovering Rumen Microorganisms with Endoglucanase Activity

(ルーメン微生物群集における高活性エンドグルカナーゼの特徴解明とエンドグルカナーゼ産生微生物の新規回収方法の開発)

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CHAPTER 1. General Introduction

Ruminants are characterized by their developed forestomach that serves as one of the four stomachs of ruminants and drives microbial digestion of the ingested feed. Rumen microbial community produces various fibrolytic enzymes and efficiently convert lignocellulosic feed into volatile fatty acids (VFAs) that are major nutrient sources for the host animal. Understanding mechanisms of the polysaccharide digestion by ruminal microorganisms are essential for improving the productivity of ruminant livestock. Therefore, fibrolytic enzymes in the rumen microbial community have been investigated via genetic analysis of ruminal microorganisms (Brulc *et al.*, 2009; Dai *et al.*, 2015; Lee *et al.*, 2019) and biochemical analysis of purified fibrolytic enzyme (Deguchi *et al.*, 1991; Wood, Wilson and McCrae, 1995; Rincon *et al.*, 2001). However, the mechanisms of the polysaccharide digestion by rumen microorganisms and the highly active fibrolytic enzymes which contribute to the polysaccharide digestion are still obscure.

Lignocellulosic biomass consists primarily of the organic biopolymers (cellulose, hemicellulose, and lignin). These biopolymers bind with each other, which form a strong structure that resists disintegration and hydrolysis, thereby making lignocellulose hydrolysis a rate-limiting step in anaerobic digestion (Sawatdeenarunat *et al.*, 2015). Applications with ruminal microorganisms have demonstrated their effectiveness in degrading lignocellulosic biomass (Baba *et al.*, 2013, 2017; Zhang *et al.*, 2016). It was previously reported that pretreatment with rumen fluid enhanced methane production from paper sludge which contained high levels of lignin and ash, with the methane gas volume increasing by 3.4 times (Takizawa *et al.*, 2018). Although fibrolytic enzymes play an important role in hydrolysis of lignocellulosic biomass, the

detailed characteristics of fibrolytic enzymes during lignocellulosic biomass digestion are still obscure.

For the digestion of lignocellulosic biomass with rumen fluid, discharged rumen fluid should be preserved and transferred from slaughterhouses to the pretreatment reactor. It was previously investigated how the preservation of rumen fluid at various temperatures for 7 days affects its use in hydrolysis of waste paper, and found that the preservation of rumen fluid at 4 °C is more suitable than at either 20 and 35 °C for efficient digestion of waste paper (Takizawa *et al.*, 2019). As the next issue from the preservation of rumen fluid, a novel method to reduce the volume of rumen fluid should be developed for the efficient transportation of rumen fluid. It was hypothesized that the recovery of rumen microorganisms secreting highly active fibrolytic enzymes from rumen fluid was feasible and effective to reduce the volume of rumen fluid.

This study focused on the endoglucanase which can randomly cleave the internal beta-1,4-glycosidic bonds in amorphous regions of cellulose polymers. Here, three researches were conducted to characterize the highly active endoglucanases which contribute to the cellulose digestion in rumen microbial community and to recover the microorganisms secreting the highly active endoglucanase from rumen fluid. First, the endoglucanase activities of rumen microbial community were researched using Japanese black cattle and Holstein cattle fed various diets (Chapter 2). Secondly, it was characterized that the temporal dynamics of individual endoglucanase activity in the rumen microbial community, as well as highly active endoglucanases that contribute to cellulose digestion in the rumen microbial community (Chapter 3). Lastly, a novel method was developed to recover ruminal microorganisms producing highly active endoglucanases from rumen fluid (Chapter 4).

CHAPTER 2. Characteristics of the Endoglucanase Activity in Rumen Microbial Community

Many previous reports have suggested that the host breed and the nutritional composition of the diet, especially concentrate-to-roughage (C:R) ratio, are important factors for determining the structure of rumen microbial community (Henderson *et al.*, 2015; Li *et al.*, 2019). In this study, the endoglucanase activity was investigated by using Japanese black cattle and Holstein cow fed with various C:R ratios to characterize the highly active endoglucanase in rumen microbial community. Rumen fluid was collected from Japanese black fattening cattle, Holstein lactating cattle, Holstein dry cattle, and Japanese black breeding cattle that were fed with four C:R ratios at 82:18, 36:64, 11:89, and 0:100, respectively. The differences in VFAs, the ratio of acetic acid to propionic acid, pH value, and COD were obtained between C:R ratios. Next, the 16S rRNA gene sequencing was performed by MiSeq and revealed that the structure of rumen bacterial community and the relative abundance of *Fibrobacter* and *Succinivibrio* varied with C:R ratios (Fig. 1). Furthermore, the core genera and unclassified bacteria detected in all C:R ratios took up about 32% of all genera and about 44% of all unclassified bacteria, respectively (Fig. 2). A zymogram analysis demonstrated some differences in banding patterns of endoglucanase activity between C:R ratios (Fig. 3). Furthermore, banding patterns of endoglucanase activity at high molecular mass were different between Japanese black cattle and Holstein cattle. Meanwhile, the endoglucanases at 52 and 53 kDa in size commonly showed the strong enzyme activity. These results indicated that the endoglucanases of 52 and 53 kDa commonly contribute to the feed digestion in rumen of Japanese black cattle and Holstein cattle. It was estimated that the bacterial genera and unclassified bacteria, which were commonly

detected in cattle fed various C:R ratios, might have produced the highly active endoglucanase of 52 and 53 kDa in size in rumen microbial community. This is the first study to suggest that the common characteristics of endoglucanases among Japanese black cattle and Holstein cattle fed with various C:R ratios and their relationship with the commonly detected bacteria in rumen microbial community.

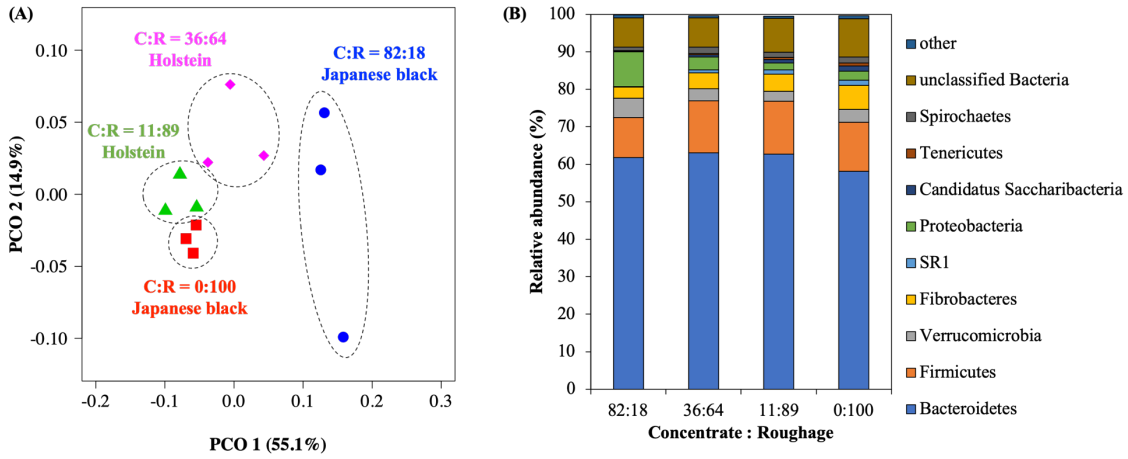

Fig. 1. Principal coordinate analysis (A) and the phylum-level analysis (B) of the rumen microbial community in Japanese black cattle and Holstein cattle fed with various concentration-to-roughage ratios. Japanese black fattening cattle, Holstein lactating cattle, Holstein dry cattle, and Japanese black breeding cattle were fed with four C:R ratios at 82:18, 36:64, 11:89, and 0:100, respectively.

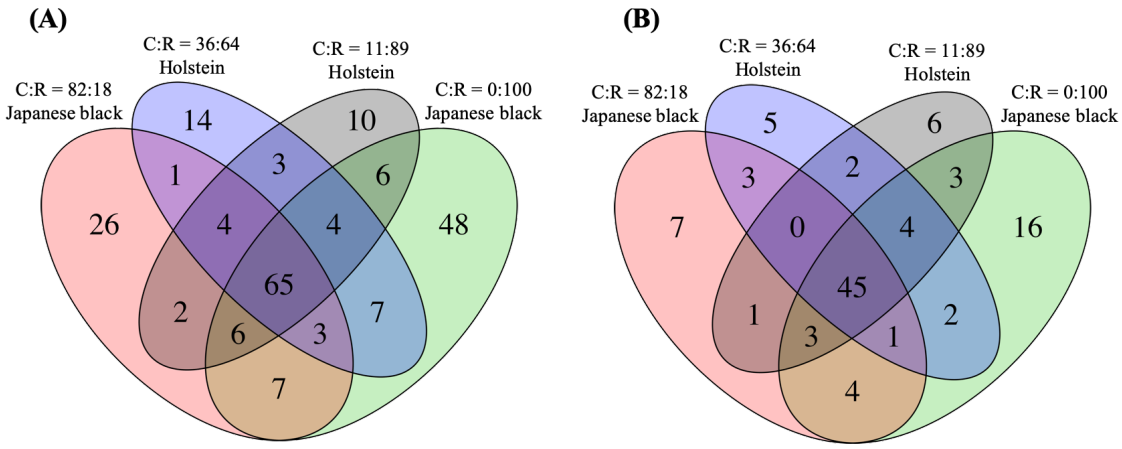

Fig. 2. Venn diagrams of the bacterial genera (A) and unclassified bacteria (B) for each concentrate-to-roughage ratio. Numbers within circles or overlapping areas indicating the number of genera in common to the corresponding concentrate-to-roughage ratios.

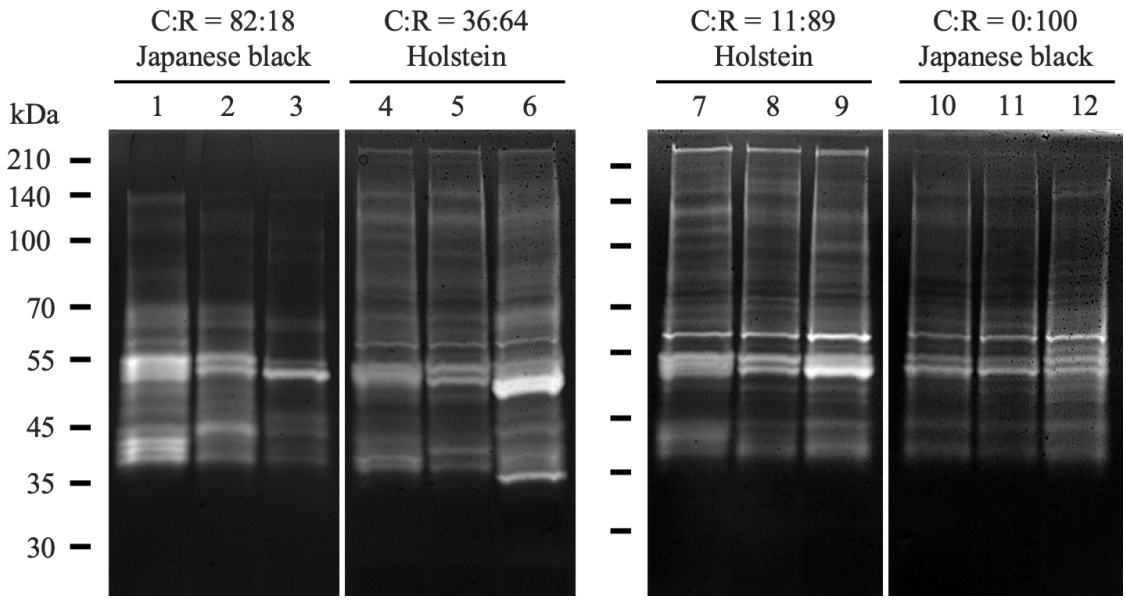

Fig. 3. Endoglucanase zymogram for rumen microbial community from cattle fed with various concentrate-to-roughage ratios.

CHAPTER 3. Temporal Dynamics of Highly Active Endoglucanases and Their Contributions to Cellulose Digestion in the Rumen Microbial Community

In chapter 2, it was demonstrated that the endoglucanases of 52 and 53 kDa in size commonly possess the high enzyme activity among cattle fed with various C:R ratios. However, many researchers have shown that the structure of rumen microbial community temporally changed during feed digestion (Piao *et al.*, 2014; Cheng *et al.*, 2017; Lee *et al.*, 2019); therefore, the highly active endoglucanases might be dynamically shifted during cellulose digestion. The aim of this study was to reveal the temporal dynamics of endoglucanase activity in rumen microbial community and the highly active endoglucanases which contribute to the cellulose digestion. Carboxymethyl cellulose was digested by the rumen microbial community. The cellulose-digestion rate increased for the first 24 h and then decreased (Fig. 4). Interestingly, the zymogram analysis showed different banding patterns corresponding to different endoglucanase activities during carboxymethyl cellulose digestion, and endoglucanases with estimated masses of 42, 50, 52, 53, and 101 kDa showed high enzyme activities in the rumen microbial community (Fig. 5). Metagenomic analysis revealed that the rumen microbial community dynamically changed for the first 24 h. Highly active endoglucanases were not identified as endoglucanases via nano-liquid chromatography/tandem mass spectrometry; however, the relative abundances of *Eudiplodinium*, unclassified Bacteroidetes, unclassified *Clostridiales* family XI, unclassified *Lachnospiraceae*, and unclassified *Sphingobacteriaceae* positively correlated with the overall endoglucanase activity (Fig. 6). These microorganisms possibly produced highly active endoglucanases. This study is the first to characterize the highly active endoglucanases in a complex rumen microbial community and their

relationships with *Eudiplodinium* and unclassified bacteria.

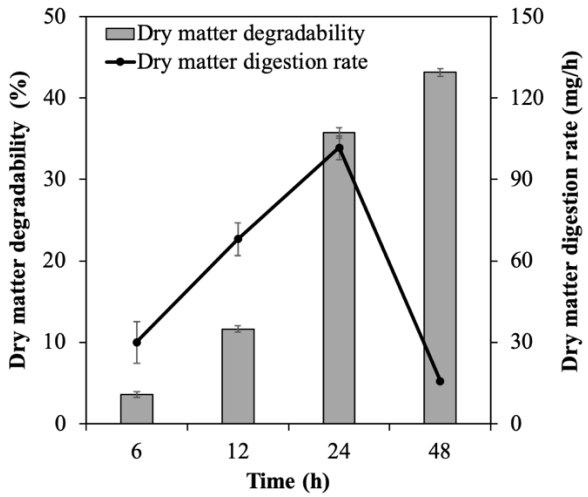

Fig. 4. Characteristics of CMC digestion in the rumen microbial community.

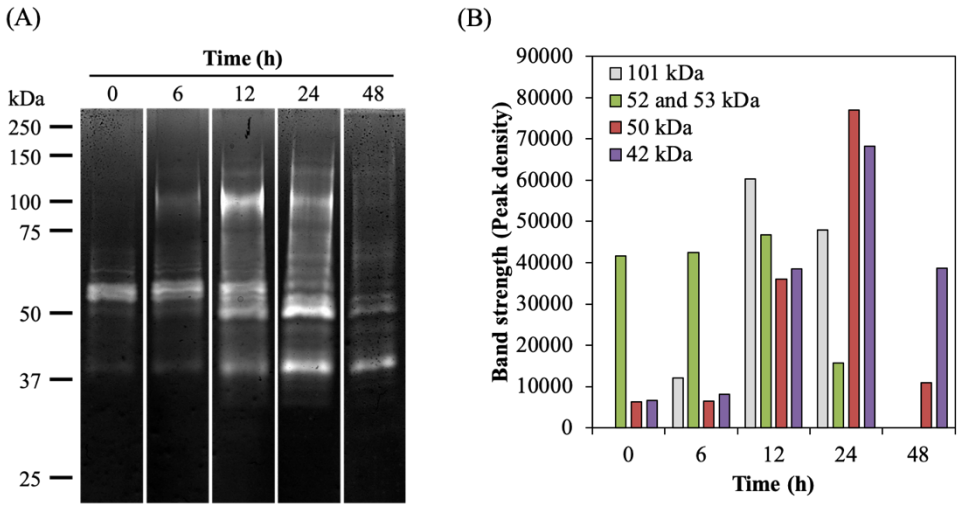

Fig. 5. Endoglucanase zymogram (A) and the peak density of highly active endoglucanases (B) during CMC digestion.

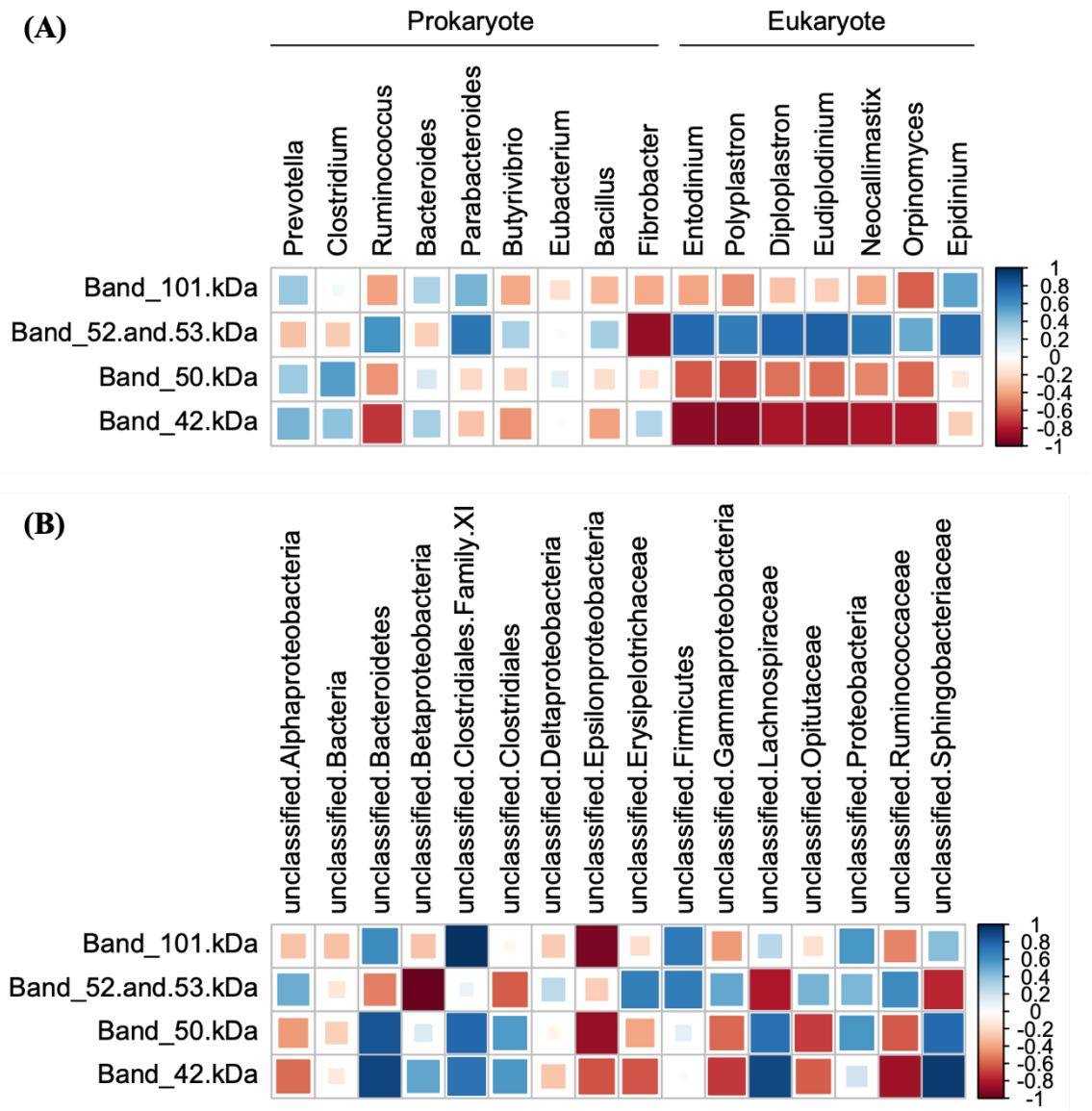

Fig. 6. Correlations of dominant cellulolytic genera (A) and unclassified microorganisms (B) with the band densities of highly active endoglucanases. The color and size of square represent each correlation coefficient. Blue shading represents a positive correlation, and red shading represents a negative correlation. A larger square represents a stronger correlation, and a smaller square represents a weaker correlation.

CHAPTER 4. Recovery of the Fibrolytic Microorganisms from Rumen Fluid by Flocculation for Simultaneous Treatment of Lignocellulosic Biomass and Volatile Fatty Acid Production

In chapter 3, it was revealed that endoglucanases at 42, 50, 52, 53, and 101 kDa possessed the highest enzyme activity in the rumen microbial community. I hypothesized that the recovery of ruminal microorganisms secreting these highly active endoglucanases effectively enables the volume reduction of rumen fluid. Toward this end, a flocculation-based method was developed with an optimized flocculant concentration required to recover ruminal microorganisms with fibrolytic activity. Rumen fluid was flocculated with poly-ferric sulfate at 0.4%, 0.7%, 1.0%, and 2.0% and with an inorganic neutral flocculant at 13.0%. Poly-ferric sulfate at 0.4%, 0.7%, and 1.0% effectively recovered ruminal microorganisms, which resulted in an 85.6%, 77.3%, and 75.6% reduction in rumen fluid volume, respectively (Fig. 7). These recovered microorganisms retained the endoglucanase activity at 52 and 53 kDa in size. In addition, recovery of ruminal microorganisms allowed for substantial reductions in the solids and organic compound concentrations of the filtrates after the flocculation. As a practical demonstration of this method, tomato leaves were treated with the flocculated rumen fluid at 37 °C for 48 h. Hydrolysis of the tomato leaves using the rumen fluid flocculated with 0.7% poly-ferric sulfate demonstrated elevated endoglucanase activity at 37, 46, 57, 61, and 66 kDa in size during treatment (Fig. 8). Furthermore, rumen fluid flocculated with 0.7% poly-ferric sulfate was preserved at 25 °C for 6 h or 24 h, which did not show the significant effects on hydrolysis of the tomato leaves. Therefore, 0.7% poly-ferric sulfate is the optimal concentration for recovering ruminal microorganisms while maintaining their fibrolytic activity. This is

the first study, indicating a novel method to efficiently recover ruminal microorganisms from huge amounts of rumen fluid. This novel method offers a practical and sustainable solution to reduce the load for wastewater treatment at slaughterhouses and in the transportation of rumen fluid.

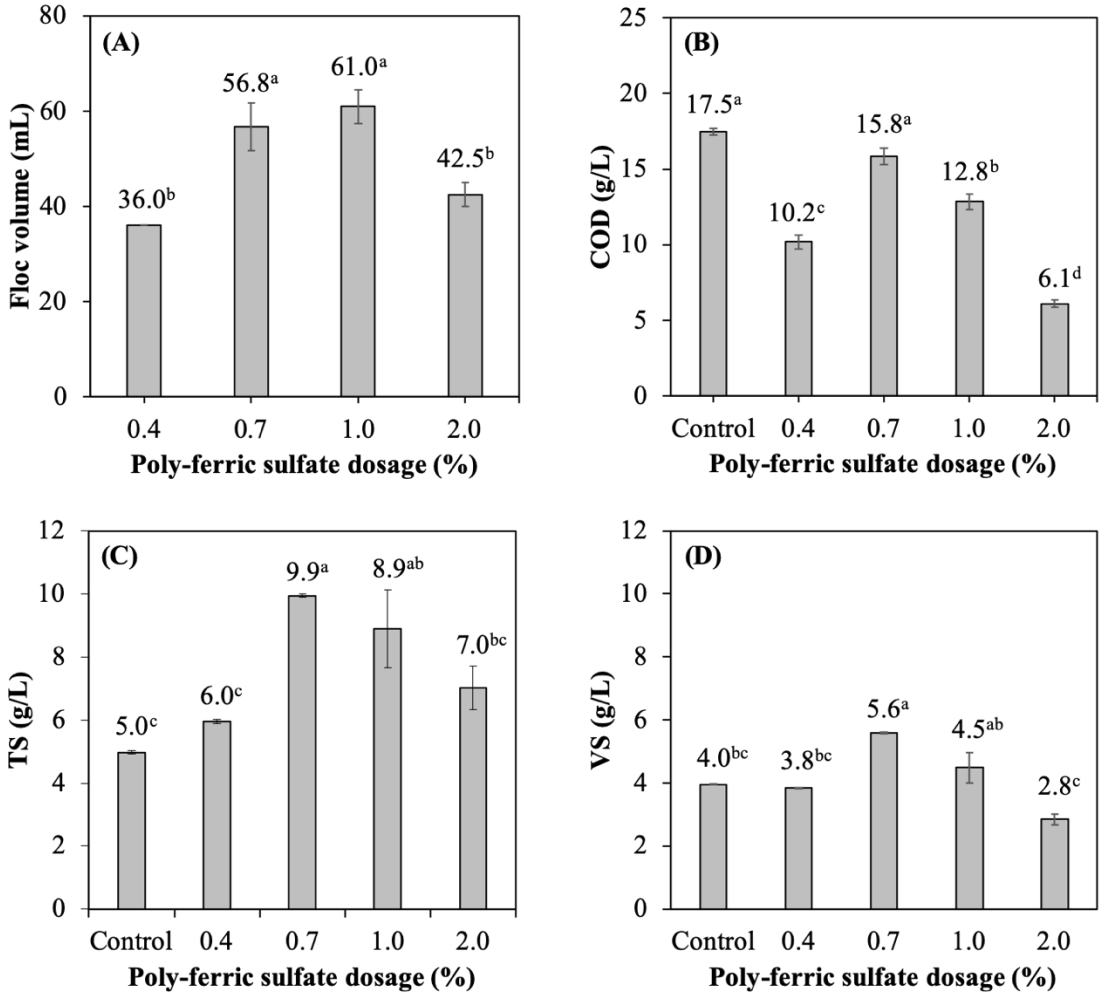

Fig. 7. Characteristics of recovered flocs. (A) Volume, (B) chemical oxygen demand, (C) total solids, and (D) volatile solids of the floc fraction. Different letters show the significant differences between poly-ferric dosages (TukeyHSD, $P < 0.05$)

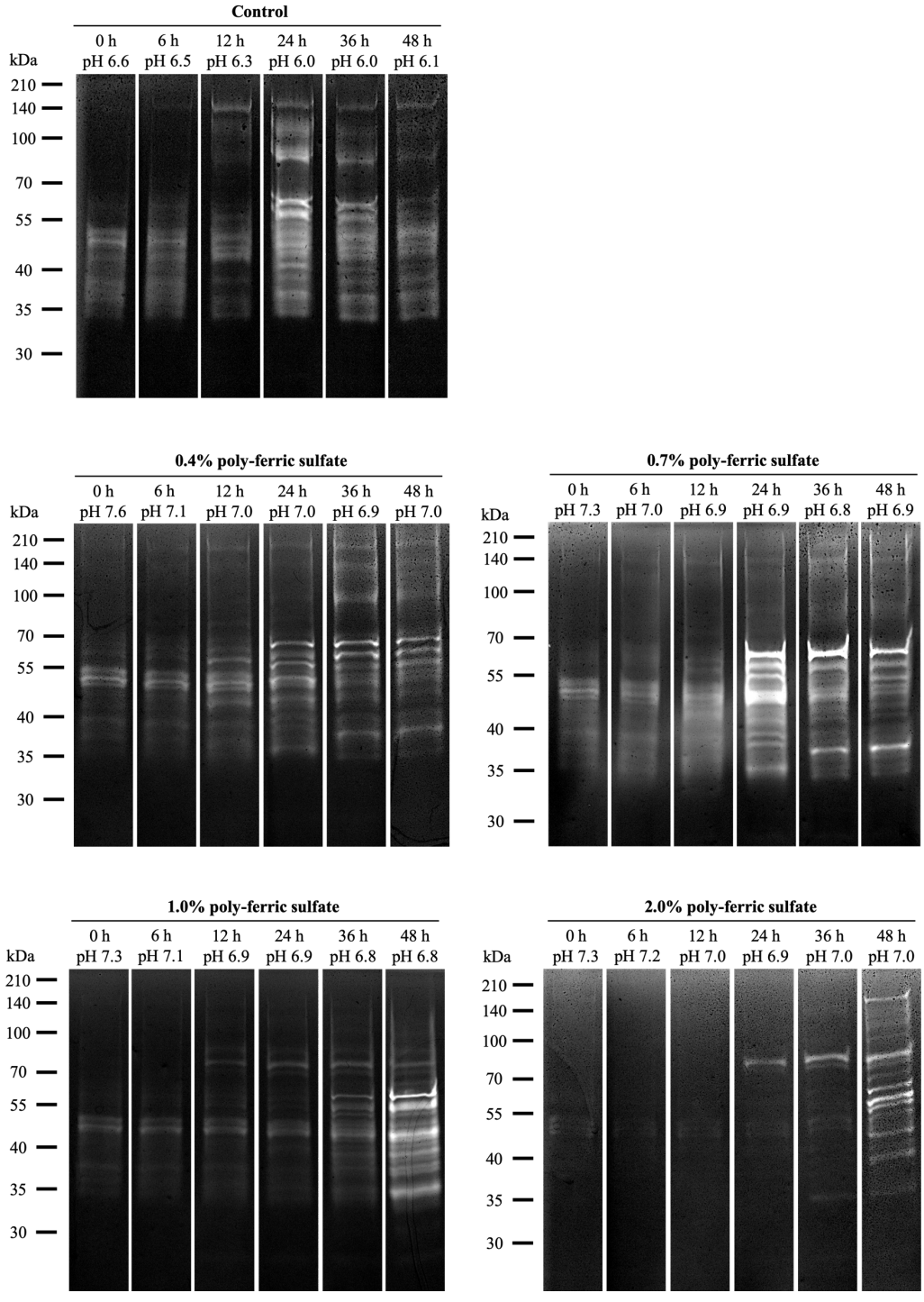

Fig. 8. Endoglucanase zymogram for the treatment of tomato leaves with flocculated rumen fluid.

CHAPTER 5. General Conclusions

Rumen microbial community produces various fibrolytic enzymes and plays an important role in lignocellulose digestion. Understanding mechanisms of the polysaccharide digestion by ruminal microorganisms are essential for improving the productivity of ruminant livestock and for enhancing the methane production from lignocellulosic biomass. For the digestion of lignocellulosic biomass with rumen fluid, the volume reduction of rumen fluid is necessary to efficiently transport huge amounts of rumen fluid from slaughterhouses to the pretreatment reactor. In this study, the highly active endoglucanases in rumen microbial community and their contribution to cellulose digestion were characterized. Furthermore, rumen microorganisms were recovered from rumen fluid while retaining their fibrolytic activity. Essential results and findings were described below;

1. The structure of rumen microbial community and the relative abundance of the core bacterial genera varied with C:R ratios. Banding patterns of endoglucanase activity were different between Japanese black cattle and Holstein fed with various C:R ratios. Meanwhile, endoglucanases of 52 and 53 kDa in size commonly possessed the high enzyme activity among Japanese black cattle and Holstein cattle fed with various C:R ratios. The commonly detected bacteria possibly produced the highly active endoglucanase of 52 and 53 kDa in size.
2. Time-course experiments showed that the dry matter-digestion rate was consistent with the endoglucanase activities. Endoglucanases at 42, 50, 52, 53, and 101 kDa possessed the highest enzyme activity in the rumen microbial community. These highly active endoglucanases positively correlated with the relative abundances of

Eudiplodinium, unclassified Bacteroidetes, unclassified *Clostridiales* family XI, unclassified *Lachnospiraceae*, and unclassified *Sphingobacteriaceae*.

3. The volume of rumen fluid was reduced by 75.6% with 0.7% poly-ferric sulfate. Rumen fluid used to treat tomato leaves for 48 h following flocculation with 0.7% poly-ferric sulfate demonstrated high levels of hydrolysis and endoglucanase activity at 37, 46, 57, 61, and 66 kDa. Treatment of tomato leaves with recovered ruminal microorganisms achieved the higher VFAs yield. Furthermore, recovered ruminal microorganisms possessed the fibrolytic activity after the preservation at 25 °C for 24 h. These results suggest that flocculation with 0.7% poly-ferric sulfate is appropriate for the effective recovery of ruminal microorganisms while maintaining fibrolytic activity.

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