Effect of Ensiling with Acremonium Cellulase, Lactic Acid Bacteria and Formic Acid on Tissue Structure of Timothy and Alfalfa

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ABSTRACT : The changes of tissue structure in timothy and alfalfa during ensiling process with silage additives; lactic acid bacteria, cellulase and formic acid, were observed with a video microscope. Stem samples were obtained from the second internode, and cut to divide into 2 pieces. One piece was for observation of ensiled material and the other was for silage. The latter piece was put into a nylon cloth bag, and ensiled with grass for 50 days in a small experimental silo. Lignification of the plant tissues was checked by acid phloroglucinol. Natural silage fermentation resulted in some degradation of less lignified parenchyma in both plant species. However, lignified sclerenchyma and vascular bundles remained intact. The cellulase enhanced the degradation of parenchyma tissue, while the formic acid suppressed the degradation. The effect of lactobacillus was small. The percentage of remained cross sectional area of stem and the loss of NDF and ADF by silage fermentation confirmed the observation. High negative correlations were obtained between the remained area and loss of fibrous components during silage fermentation in both plants, and between the loss of fibrous components and *in vitro* dry matter digestibility in timothy but not in alfalfa.
(**Key Words**: Silage, Plant Tissue, Cellulase, Lactic Acid Bacteria, Formic Acid)

INTRODUCTION

During ensiling process, complex reactions are occurred in ensiled plant material including degradation of carbohydrates and proteins in aerobic condition in an early stage and anaerobic condition thereafter. Sometimes, silage fermentation results in considerable dry matter loss (McDonald et al., 1991). It is, therefore, that some additives may be added to enhance or to control silage fermentation. There have been many studies that the effects of additives such as lactic acid bacteria, cellulase and formic acid either on degradation or on loss of nutrients during ensiling process from the chemical view point (Henderson et al., 1982, Van Vuuren et al., 1989, Ataku et al., 1993, Stokes and Chen, 1994).

There have been some histological studies on ruminal digestion of grass and straw tissues in relation to lignification (Akin and Barton, 1983, Goto et al., 1991 and Okamoto et al., 1994). However, few studies have been done on the changes of plant tissue structure during the ensiling process. In the present study, an attempt has been made to determine the effect of ensiling with additives; lactic acid bacteria, cellulase and formic acid, on the tissue structure of timothy and alfalfa with a video microscope.

MATERIALS AND METHODS

First cut timothy (*Phleum pratense*, Hokusen, heading stage) and alfalfa (*Medicago sativa*, Euver, blooming stage) were harvested from our university farm. The timothy and alfalfa were cut at about 1 cm length, and 500 g and 600 g were ensiled in a 1 liter experimental silo, respectively. For histological observation, about 2 cm length of timothy stem was cut off with a razor from the second internode from the bottom, and the corresponding part of stem was obtained from alfalfa. These stems were cut at the center and divided into two parts. One part was put into a small bag made by nylon cloth, Five bags were mixed with ensiling materials and ensiled in each silo. The other part was kept frozen until video microscope observation. Preliminary experiment indicated that freezing caused no observable change in plant structure at the magnification.

At the ensiling, additives were mixed with ensiling materials by a spray. No additives were mixed in the control silage. Lactic acid bacteria (*Lactobacillus casei*,

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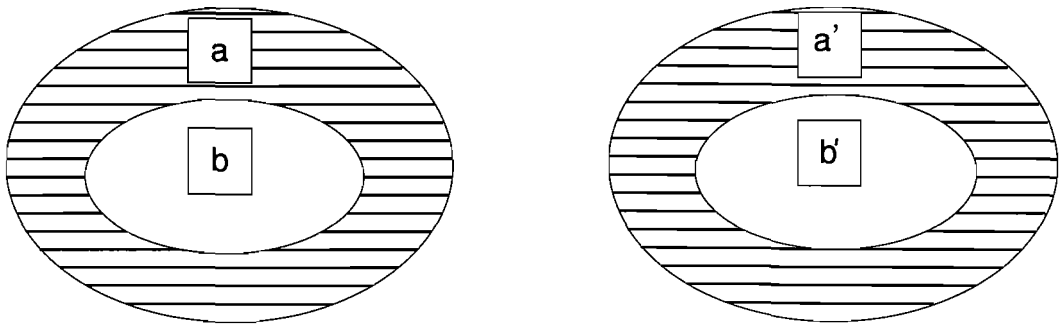
Received March 14, 1997; Accepted June 17, 1997

Snow brand seeds) was mixed at 8 ppm for LC treatment. *Acremonium* cellulase (Meiji seika) was mixed at 0.01% (AC treatment). In LC + AC treatment, both LC and AC were treated. Formic acid was mixed at 0.3% for timothy and at 0.5% for alfalfa (FA treatment). Each treatment was duplicated.

Silage were took out 50 days after ensiling for chemical analysis (WSC, NDF and ADF, Abe, 1988). Silage stem samples in nylon bag were frozen until microscope observation. Frozen stem samples were handcut in round slices having a thickness of about 0.3 mm and were adhered to slides. Histochemical reaction for lignin with acid phloroglucinol was examined with a video microscope system (Mitsubishi Kasei, Microwatcher VS-30H. Okamoto et al., 1994.). Images from the video system were recorded, and micrographs were obtained by a video printer (Sony, CVS-M3). Proportions of cross sectional area of stem sections to the area of same sections including central hollow were measured by a image analysis program (NIH Image). The percentage of the proportion for silage stem to the proportion for the corresponding ensiled stem was calculated as the remainder of the silage (figure 1). *In vitro* dry matter digestibility was determined (Abe, 1988).



Remainder (%) = (a' /a' + b')/(a/a + b) × 100

Figure 1. Calculation method of percentage of the remainder.

RESULTS

The histological changes of the cross sections of timothy stem before and after ensiling are illustrated in figure 2. Epidermis and vascular bundles of the timothy stem were lignified intensively, and some parenchyma tissues near epidermis were lignified.

After ensiling, the lignified stem tissues remained well, while inner parenchyma tissues disappeared, more or less. Most tissues remained in the FA treatment. However, most unlignified parenchyma tissues disappeared in the AC and LC + AC treatments, and vascular bundles remained as a peninsula. In the control and LC treatment, some unlignified parenchyma tissues remained.

Figure 3 illustrates the histological changes of alfalfa stem. Tissues of epidermis, sclerenchyma and vascular bundles were lignified intensively. However, degree of lignification of some pith parenchyma and cortex parenchyma tissues were less than those of other tissues.

AC and LC + AC treatments resulted in disappearance of parenchyma tissues between pith and cambium and also some cortex parenchyma. Parenchyma tissues between vascular bundles appeared to be remained. Almost no histological changes were observed in FA treatment. Control and LC treatments resulted in intermediate changes between FA and AC or LC + AC treatments.

Percentages of remainder of the silage are shown in table 1 with *in vitro* dry matter digestibility (IVDMD). The remainder values seemed to reflect above observation, and the values for AC or LC + AC treatments were tended to be less than those for the other treatments.

Chemical composition and components losses during ensiling of timothy and alfalfa are shown in table 2. FA treatment suppressed silage fermentation. More than 80% of WSC and some cell wall components were degraded in the control and LC silage. AC and LC + AC treatments resulted in more degradation in fiber components.

There were not remarkable differences in IVDMD among treatments. However, the values for AC and LC + AC treatments tended to be slightly lower than those for the other treatments.

Table 3 shows the mutual correlation among remainder percentage, components losses and IVDMD. The

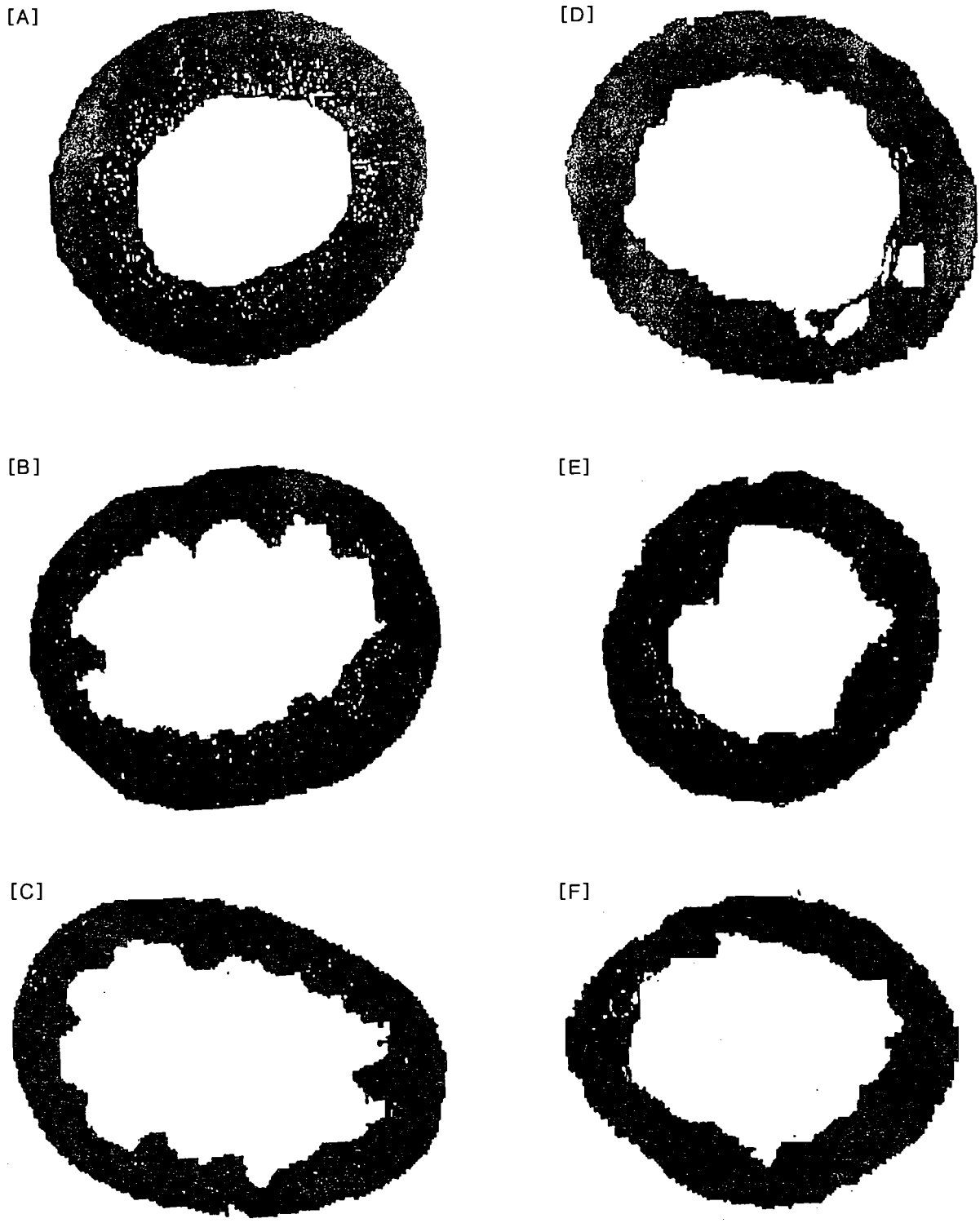


Figure 2. Plant tissues in ensiled material and silages of timothy. [A]: ensiled material, [B]: control silage, [C]: cellulase (AC) added silage, [D]: formic acid added silage, [E]: lactic acid bacteria (LC) added silage, [F]: LC and AC added silage (LC + AC).

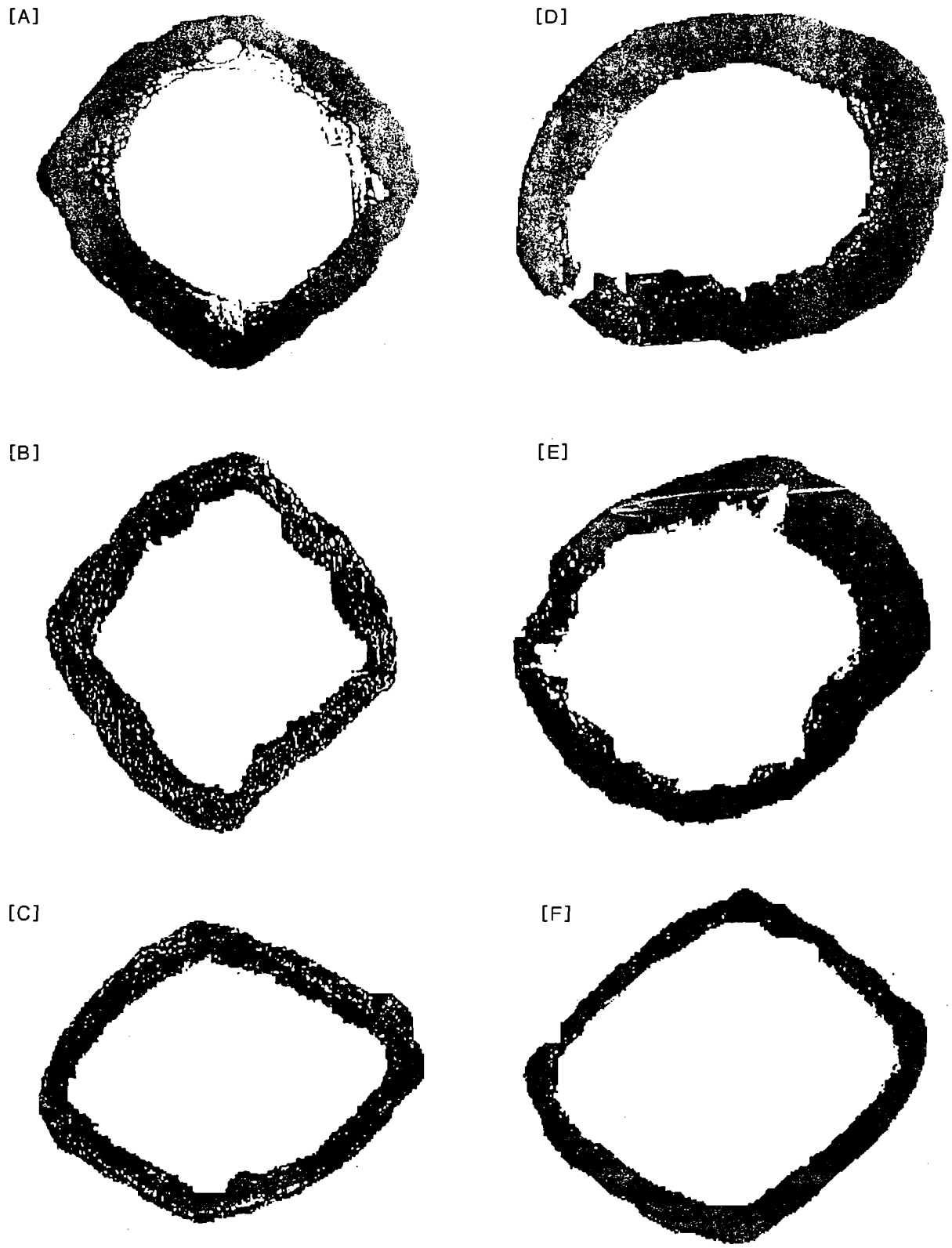


Figure 3. Plant tissues in ensiled material and silages of alfalfa. [A]: ensiled material, [B]: control silage, [C]: cellulase added silage (AC), [D]: formic acid added silage, [E]: lactic acid bacteria (LC) added silage, [F]: LC and AC added silage (LC + AC).

Table 1. Percentages of remainder and *in vitro* dry matter digestibility of the silage

		Treatments, additives					
		ensiled	control	LC	AC	LC + AC	FA
Timothy	Remainder	100.0	99.0^{ab}	96.6^{b}	71.8^{a}	73.1^{a}	86.5^{ab}
	Digestibility	77.5	74.5^{Aab}	75.4^{ABb}	74.5^{Aab}	73.4^{Aa}	77.0^{Bc}
Alfalfa	Remainder	100.0	89.5	79.0	73.7	68.6	99.6
	Digestibility	67.2	66.0	62.9	60.6	62.6	62.7

LC: lactic acid bacteria, AC: acremonium cellulase, FA: fomic acid.
A B C: $p < 0.01$, a b c d: $p < 0.05$.

Table 2. Chemical composition of ensiled materials and components losses during ensiling of timothy and alfalfa

		WSC	NDF	ADF
Timothy				
Ensiled material (% DM)		6.4	70.4	40.1
Losses(%)	control	85.2^{b}	16.6^{B}	4.5^{Aa}
	LC	84.3^{ab}	13.9^{AB}	4.7^{Aa}
	AC	80.8^{ab}	26.9^{C}	15.9^{ABb}
	LC + AC	83.0^{ab}	28.8^{C}	21.3^{Bb}
	FA	74.1^{a}	9.2^{A}	2.8^{Aa}
Alfalfa				
Ensiled material (% DM)		8.2	46.6	38.8
Losses(%)	control	83.6^{C}	5.2^{ab}	−0.7
	LC	82.4^{C}	6.5^{ab}	0.6
	AC	66.4^{B}	11.0^{ab}	4.7
	LC + AC	76.3^{BC}	14.5^{b}	8.3
	FA	14.6^{A}	−2.7^{a}	−3.7

WSC: water soluble carbohydrate
NDF: neutral detergent fiber.
ADF: acid detergent fiber. LC: lactic acid bacteria
AC: acremonium cellulase.
FA: fomic acid. A B C: $p < 0.01$, a b c d: $p < 0.05$.

remainder percentages were highly correlated with losses of NDF and ADF during ensiling in both grass species. These losses in NDF and ADF were also highly correlated with IVDMD in timothy but not in alfalfa. The remainder percentage correlated with IVDMD in timothy.

DISCUSSION

Video microscop observation for ensiling materials of both plant species showed that sclerenchyma was lignified in tensively and parenchyma was not or slightly lignified. After silage fermentation in the control and the LC treatments, parenchyma tissues decreased considerably from inner side, and the area of hollow increased. Treatments with cellulase extended the trends. However, sclerenchyma remained almost intact. The results indicated that unlignified or slightly lignified tissues were degraded more or less but intensively lignified tissues were not degraded during ensiling process. The addition of formic acid suppressed the degradation.

Staining reactions for lignin by acid phloroglucinol were too vague to detect the differences of degree of lignification by NIH image program. Therefore, it was not available that detailed analysis on the relationship

Table 3. Simple correlations between the variables of silage

		Hemicellulose	WSC	NDF	ADF	Hemicellulose	Digestibility[b)] (%)
			Losses (%)				
Timothy	Digestibility (%)	.7567**	−.5864*	−.8253**	−.7153**	−.8236**	
	remainder %[a)]	.4691*	.1934	−.6687**	−.7547**	−.473*	.5419*
Alfalfa	Digestibility (%)	.1167	.2185	−.0249	.2124	−.0828	
	remainder %[a)]	.5837*	−.4906*	−.8003**	−.7634**	−.6274*	.4138

WSC: water soluble carbohydrate, NDF: neutral detergent fiber, ADF: acid detergent fiber.
a): % remainder of material. b): *in vitro* dry matter digestibility, *$p < 0.05$, **$p < 0.01$.

between the degradation during ensiling process and the degree of lignification. However, percentage of remainder should indicate the disappearance of cross sectional area of stem during ensiling process. The percentage of remainder was decreased remarkably by the treatments with cellulase in both plant species. The results of remainder and of microscope observation indicated that cellulase enhanced the degradation of less lignified parenchyma. The addition of lactobacillus had little effect on remainder percentage in timothy but a little effect in alfalfa. The addition of formic acid resulted in higher remainder percentage, and it seemed to suppress the activities of microorganisms engaging silage fermentation.

The loss of NDF during silage fermentation was higher in timothy than in alfalfa, however, it seemed to reflect the percentage of remainder in each plant species. This point of view was supported by the high negative correlation between loss of fiber fraction and the remainder percentage. The loss of fiber fraction correlated with IVDMD in timothy but not in alfalfa. This suggested that the degraded tissues had higher digestibility than the remained tissues in timothy. However, alfalfa seemed to have more uniform digestibility between degraded and undegraded tissues.

The results in the present study indicate that silage fermentation may degrade similar part of tissues with rumen fermentation, especially when cellulase is used as a silage additives (Okamoto et al., 1994). Degradable tissues during silage fermentation are also digestible in the rumen fermentation. Cellulase may be useful to ensure silage fermentation but it also may reduce digestible fiber for the animals.

ACKNOWLEDGMENTS

This work was supported in part by a research grant from the Society for the support of Rakuno Gakuen. One of the authors, Aniwaru Aisan was supported by the Rakuno Scholarship Society and the educational board of Xinjiang Uygur of China.

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