Effects of Different Enzymes and Combined Treatments on the Microstructure of Silage Rice Straw Postprint

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Abstract

This study investigated rice straw treated with high-efficiency cellulase for silage fermentation to analyze its effects on microstructure, aiming to achieve efficient degradation of crop straw and enhance its nutritional value. The experimental design included a silage control (Group S), compound enzyme preparation treatment (Group C), pectinase + laccase treatment (Group PL), compound enzyme preparation + pectinase + laccase treatment (Group CPL), and raw material (Group M), with all groups subjected to vacuum bag silage. After 45 days of storage at room temperature, samples were collected and analyzed using laboratory detection methods, phenol-sulfuric acid method, bicinchoninic acid (BCA) method, Fourier-transform infrared spectroscopy (FTIR), X-ray diffraction analysis, and eosin-methylene blue method to determine nutrient content, fermentation quality, and microstructural changes in rice straw silage treated with different enzymes or enzyme combinations. The results demonstrated that: 1) Compared with Group S, Groups PL, C, and CPL exhibited significantly increased lactic acid (LA) content ($P<0.05$), while pH, ammonia nitrogen/total nitrogen ratio, acetic acid, and cellulose contents decreased significantly ($P<0.05$); crude protein content in Groups CPL and C decreased significantly ($P<0.05$). 2) Compared with Group S, the degree of polymerization of silage rice straw in Group PL decreased significantly ($P<0.05$) by 30.26%, intermolecular hydrogen bonding was weakened, while crystallinity and specific surface area showed no significant changes ($P>0.05$); the degree of polymerization in Group C decreased significantly ($P<0.05$) by 27.11%, intermolecular hydrogen bonding was weakened, while crystallinity and specific surface area showed no significant changes ($P>0.05$); the degree of polymerization in Group CPL decreased significantly ($P<0.05$) by 56.32%, intermolecular hydrogen bonding was weakened, with a trend toward decreased crystallinity and increased specific surface area

(P>0.05). In conclusion, Group CPL demonstrated the most effective degradation of fiber components, effectively disrupting the lignin-cellulose-hemicellulose composite structure in the cell wall, converting cellulose into utilizable sugars, enhancing the nutritional content of rice straw silage, reducing the degree of polymerization and crystallinity while increasing specific surface area, thereby improving the digestibility and utilization rate of the straw.

Full Text

Effect of Different Enzymes and Their Combinations on Microstructure of Rice Straw Silage

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Abstract

This study investigated the effects of high-efficiency cellulose-degrading enzymes on the microstructure of rice straw silage to achieve efficient degradation of crop straw and improve its nutritional value. Five treatment groups were established: silage control (S group), compound enzyme preparation (C group), pectinase + laccase (PL group), compound enzyme preparation + pectinase + laccase (CPL group), and raw material (M group). All groups were packaged in vacuum bags for ensiling. After 45 days of storage at room temperature, samples were analyzed for nutrient composition, fermentation quality, and microstructural changes using laboratory detection methods, phenol-sulfuric acid method, bicinchoninic acid (BCA) method, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction analysis, and eosin-methylene blue method.

The results showed: (1) Compared with the S group, the lactic acid (LA) content in PL, C, and CPL groups increased significantly (P<0.05), while pH, ammonia nitrogen/total nitrogen ratio, acetic acid content, and cellulose content decreased significantly (P<0.05). The crude protein (CP) content in C and CPL groups decreased significantly (P<0.05). (2) Compared with the S group, the degree of polymerization in PL group decreased significantly by 30.26% (P<0.05), with weakened intermolecular hydrogen bonding, while crystallinity and specific surface area showed no significant changes (P>0.05). The degree of polymerization in C group decreased significantly by 27.11% (P<0.05), also with weakened hydrogen bonding and no significant changes in crystallinity or specific surface area (P>0.05). The degree of polymerization in CPL group

decreased significantly by 56.32% ($P<0.05$), with weakened hydrogen bonding and a trend toward reduced crystallinity and increased specific surface area ($P>0.05$).

In conclusion, the CPL treatment showed the most effective degradation of fiber components, successfully breaking the lignin-cellulose-hemicellulose composite structure in the cell wall, converting cellulose into utilizable sugars, improving the nutritional content of rice straw silage, reducing polymerization degree and crystallinity, and increasing specific surface area, thereby enhancing the digestibility and utilization of straw.

Keywords: straw; lignocellulose; microstructure; biological treatment

Introduction

Crop straw represents an important feed resource, but its utilization as animal feed is generally low due to the complex hierarchical and aggregated structures of cellulose in the cell wall, as well as the encapsulation of cellulose by hemicellulose and lignin. Lignin binds covalently with hemicellulose, forming a natural barrier that envelops cellulose molecules and prevents digestive enzymes from accessing them, thereby limiting decomposition in the animal rumen. Numerous domestic and international scholars have conducted extensive research on the efficient utilization of straw. Currently, common processing methods to improve straw feed utilization include physical, chemical, and biological treatments. Biological treatment offers advantages such as low energy consumption, minimal pollution, and easy operation, and has attracted considerable attention for its reliance on microbial and enzymatic degradation capabilities to break down straw cell walls for use as ruminant feed.

The specific hierarchical structural characteristics of cellulose can be categorized into primary, secondary, tertiary, and quaternary structures, characterized by degree of polymerization, hydrogen bonding, crystallinity, and specific surface area, respectively. Biological treatment can alter the content of cellulose components and modify the microstructure at different hierarchical levels. The degree of cell wall disruption can reflect the effectiveness of cell wall breakdown. Therefore, identifying effective enzyme combinations to break down crop straw cell walls, alter straw physicochemical structure, and degrade cellulose, hemicellulose, and lignin into monosaccharides directly utilizable by animals will effectively improve straw feed utilization. This study used rice straw as the research material, applying different combinations of compound enzyme preparation, pectinase, and laccase for silage fermentation treatment. Laboratory detection methods, phenol-sulfuric acid method, bicinchoninic acid (BCA) method, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction analysis (XRD), and eosin-methylene blue method were employed to analyze changes in nutrient composition, fermentation quality, and microstructure of enzyme-treated rice straw silage, aiming to identify enzyme combinations capable of

breaking down the spatial structure of crop straw cell walls and provide theoretical basis for straw utilization.

1.1 Experimental Materials

Rice straw was harvested from Jilin City, Jilin Province (Daohuaxiang variety) in September 2015 after grain harvest. Compound enzyme preparation [cellulase ($\$ 10,000 U/g$) + $xylanase$(\$120,000 U/g) + β-glucanase ($\$ 40,000 U/g$)], $pectinase$($10,000 U/g$), $andlaccase$(\$10,000 U/g) in powder form were purchased from Xia Sheng Industrial Group Co., Ltd. and stored at room temperature.

1.2 Experimental Design

Five treatment groups with three replicates each were designed as shown in Table 1 . After harvest, rice straw was cut to obtain 5 cm stem segments between nodes 7-8 (600 segments total). The remaining material was chopped to 1-2 cm using a silage cutter. Compound enzyme preparation, pectinase, and laccase were dissolved in distilled water according to the combinations and dosages shown in Table 1 and sprayed evenly onto the chopped rice straw and stem segments. Moisture content was adjusted to 75%-80%. The silage control group received distilled water only without any enzymes, following the same procedure. Raw material samples were collected and stored in an ice box, then transported to the laboratory and stored at -20°C. Silage samples were stored at room temperature (25-37°C) for 45 days before opening and sampling, then immediately stored at -20°C for analysis.

Table 1 Experimental design and sample numbering

Group	Replicate	Supplemental dosage (g/kg)
Rice straw (M)	M-1, M-2, M-3	-
Silage control (S)	S-1, S-2, S-3	-
Compound enzyme preparation (C)	C-1, C-2, C-3	1.0
Pectinase + laccase (PL)	PL-1, PL-2, PL-3	0.5 + 1.0

Group	Replicate	Supplemental dosage (g/kg)
Compound enzyme preparation + pectinase + laccase (CPL)	CPL-1, CPL-2, CPL-3	1.0 + 0.5 + 1.0

2 Determination Indicators and Methods

2.1 Determination of Fermentation Quality and Nutrient Composition

Twenty grams of rice straw silage sample was mixed with 180 mL distilled water, stirred evenly, and homogenized for 1 min. The mixture was filtered through four layers of gauze and qualitative filter paper to obtain the extract. pH was measured using a pH meter (Testo 205, Germany). Ammonia nitrogen (NH_3-N) content was determined by phenol-hypochlorite colorimetry. Lactic acid (LA), acetic acid (AA), propionic acid (PA), and butyric acid (BA) contents were analyzed using a GC128 gas chromatograph with flame ionization detector (FID) (column: 2 m × 4 mm, stationary phase: Pora-pak Q, 80 mesh; column temperature: 220°C; injector and detector temperature: 260°C; N_2 flow: 65 mL/min; air flow: 550 mL/min; H_2 flow: 55 mL/min; sensitivity: 16$×10^{3}$; chart speed: 5 mm/min). Nutrient contents including dry matter (DM), cellulose (C), hemicellulose (HC), lignin (L), crude protein (CP), and total nitrogen (TN) were determined before and after treatment.

2.2 Microstructural Analysis

2.2.1 Degree of Polymerization Stem segments were ground to pass through a 200-mesh sieve. Total sugar concentration was determined by phenol-sulfuric acid method, and reducing sugar concentration was measured by BCA method. The ratio of total sugar to reducing sugar concentration was calculated as the degree of polymerization, with average degree of polymerization expressed as an integer.

2.2.2 FTIR Analysis FTIR analysis was performed using a VERTEX 70V Fourier transform infrared spectrometer (Bruker, Germany). Stem segments were ground for 1 min and passed through a 200-mesh sieve, then dried at 60°C for 12 h. One milligram of sample was mixed with 50 mg KBr, pressed at 1 MPa to form a 13 mm diameter pellet. Scanning range was 1,000-4,000 cm^{-1} with spectral resolution of 2 cm^{-1}.

2.2.3 XRD Analysis XRD analysis was conducted using a D8-Advance X-ray diffractometer (Bruker, Germany) with Cu-Kα radiation at 40 kV × 40 mA, scanning speed of 1°/min, step size of 0.04°, and 2 range of 3°-40°. Stem segments were ground for 1 min and passed through a 200-mesh sieve. Crystallinity was calculated using the formula proposed by Meyer et al.:

$$\mathrm{CrI}(\%) = \left[\frac{(I_{002} - I_{am})}{I_{002}}\right] \times 100$$

where CrI represents crystallinity, I_{002} is the maximum crystalline intensity of cellulose I at 2 = 22-23° (for cellulose II: 2 = 18-22°), and I_{am} is the minimum crystalline intensity of cellulose I at 2 = 18-19° (for cellulose II: 2 = 13-15°).

2.2.4 Specific Surface Area Specific surface area was determined by eosin-methylene blue method. Stem segments were ground to pass through a 200-mesh sieve. 0.2 g sample was suspended in 25 mL methylene blue solution and shaken at 120 r/min for 12 h at 25°C (three replicates per treatment). After standing for 15 min, the suspension was centrifuged at 10,000 r/min for 15 min. The supernatant was collected and absorbance was measured at 660 nm to determine methylene blue content. The adsorption capacity was calculated as:

$$q = \frac{(C_0 - C_t) \times V}{M}$$

where q is the methylene blue adsorption capacity (mg/g), C_0 is the initial concentration (mg/L), C_t is the concentration at time t (mg/L), V is the solution volume (L), and M is the sample mass (g).

Specific surface area was then calculated as:

$$S = q \times a$$

where S is the specific surface area (m^2/g), q is the adsorption capacity (mg/g), and a is the area covered by 1 mg methylene blue (2.45 m^2 for straw material).

2.3 Statistical Analysis

Data were initially processed using Excel 2010 and analyzed using SPSS 19.0 software. One-way ANOVA was used to test for significant differences among rice straw samples, with $P<0.05$ considered statistically significant.

3 Results

3.1 Nutrient Composition of Silage Material

As shown in Table 2 , the cellulose content of silage material was 338.84 g/kg DM, and DM content was 229.07 g/kg FM.

Table 2 Nutrient composition of silage material

Item	Content
Dry matter (g/kg FM)	229.07
Cellulose (g/kg DM)	338.84
Hemicelluloses (g/kg DM)	[value missing in original]
Lignin (g/kg DM)	[value missing in original]
Crude protein (g/kg DM)	[value missing in original]

3.2 Fermentation Quality and Nutrient Composition After Silage Treatment

As shown in Table 3 , after different silage treatments, pH decreased significantly ($P<0.05$) and lactic acid content increased significantly ($P<0.05$) compared with the S group, with the CPL group showing the highest lactic acid content. No significant differences were observed in DM content among groups ($P>0.05$). Cellulose content in PL, C, and CPL groups was significantly lower than in the S group ($P<0.05$), while lignin content showed no significant differences among groups ($P>0.05$).

Table 3 Fermentation quality and nutrient composition of rice straw after silage treatment

Item	S	PL	C	CPL	SEM	P-value
Nutrient composition						
DM (g/kg FM)	419.84	390.52	393.68	371.01	-	<0.01
C (g/kg DM)	246.19	236.81	268.91	262.33	-	<0.01
HC (g/kg DM)	[value missing]	[value missing]	[value missing]	[value missing]	-	-
L (g/kg DM)	41.01	43.67	37.56	38.53	-	<0.01
CP (g/kg DM)	[value missing]	[value missing]	[value missing]	[value missing]	-	-

Item	S	PL	C	CPL	SEM	P-value
Fermentation quality						
pH	3.73	3.65	3.64	3.66	-	<0.01
LA (g/kg DM)	34.50	37.19	36.67	52.97	-	<0.01
AA (g/kg DM)	10.34	6.18	3.71	3.18	-	<0.01
PA (g/kg DM)	1.86	[value missing]	[value missing]	[value missing]	-	<0.01
BA (g/kg DM)	0.35	0.19	[value missing]	[value missing]	-	<0.01
NH_3-N/TN	63.68	36.36	37.62	45.79	-	<0.01

3.3 Microstructural Changes

3.3.1 Degree of Polymerization As shown in Table 4 , the degree of polymerization of rice straw decreased significantly after silage treatment compared with raw material (M group) ($P<0.05$). Compared with the S group, PL, C, and CPL groups showed significant reductions in polymerization degree ($P<0.05$), with the CPL group exhibiting the lowest value.

Table 4 Degree of polymerization of rice straw

Item	M	S	PL	C	CPL	SEM	P-value
Degree of polymerization (DP)	[value missing]	[value missing]	[value missing]	[value missing]	[value missing]	-	-

3.3.2 FTIR Spectra FTIR spectra showed differences among differently treated rice straw silages (Figure 1 [Figure 1: see original paper]). Characteristic lignocellulose absorption peaks were present in raw rice straw. After silage treatment, absorption peaks at 2,921 cm^{-1} (methyl and methylene), 1,162 and 1,051 cm^{-1}, and 1,735 cm^{-1} showed significantly reduced intensity, indicating C-O-C bond cleavage and partial degradation of cellulose and hemicellulose. The benzene ring absorption peak at 1,656 cm^{-1} also weakened markedly, demonstrating that lignin underwent not only depolymerization but also partial benzene ring degradation.

Figure 1 FTIR spectra of rice straw
1. Rice straw (M); 2. Silage control (S); 3. Pectinase + laccase (PL); 4. Com-

pound enzyme preparation (C); 5. Compound enzyme preparation + pectinase + laccase (CPL). The same as below.

3.3.3 Crystallinity X-ray diffraction peak positions remained essentially unchanged after enzyme treatment, though peak intensities varied. As shown in Figure 2 [Figure 2: see original paper], crystalline diffraction peaks at 101 and 002 for PL, C, and CPL groups were significantly lower than those for S and M groups, with flatter peak shapes. All groups showed weak diffraction peak intensity at 004.

Figure 2 X-ray diffraction spectra of rice straw

As shown in Table 5 , crystallinity showed a decreasing trend after silage treatment compared with the S group, but differences were not significant (P>0.05). The CPL group exhibited the most pronounced reduction in crystallinity.

Table 5 Crystallinity of rice straw (%)

Group	CrI	SEM	P-value
[values missing]	[values missing]	-	-

3.3.4 Specific Surface Area As shown in Table 6 , no significant changes in specific surface area were observed among groups compared with the S group (P>0.05), with values ranging from 1.12 to 1.20 m^2/g. The CPL group showed the greatest increase in specific surface area.

Table 6 Specific surface area of rice straw (m^2/g)

Item	Group	SEM	P-value
Specific surface area	[values missing]	-	-

4 Discussion

4.1 Effects of Different Enzyme Treatments on Nutrient Composition and Fermentation Quality of Rice Straw Silage

Zhao et al. reported that enzyme treatment of rice straw silage did not significantly affect DM content, which is consistent with our findings, indicating that enzyme treatment largely preserves feed nutrients. This study found that cellulose content decreased to varying degrees after treatment with compound enzyme preparation, pectinase + laccase, and compound enzyme preparation + pectinase + laccase compared with the silage control group, likely because cellulose was degraded into monosaccharides or disaccharides during ensiling,

providing substrates for lactic acid bacteria fermentation. This aligns with research by Zhao et al. However, crude protein content in the compound enzyme preparation and compound enzyme preparation + pectinase + laccase groups was significantly lower than in the silage control group, which differs from results reported by Zhao et al. and Zhao et al. Tao et al. suggested that increased ammonia nitrogen content after ensiling might cause crude protein consumption, a finding similar to our results.

Numerous studies have shown that enzyme treatment of rice straw silage promotes lactic acid fermentation and improves fermentation quality. Our study found that ammonia nitrogen/total nitrogen ratio and pH decreased significantly after different enzyme treatments compared with the silage control group, indicating improved silage quality. This result is consistent with Wang' s findings. The primary purpose of adding enzymes to silage is to degrade structural carbohydrates such as cellulose and hemicellulose into soluble sugars to provide more fermentation substrates and promote lactic acid bacteria fermentation. In this study, lactic acid content increased significantly while acetic acid content decreased after enzyme treatment, indicating that homofermentative lactic acid bacteria dominated the fermentation process, producing large amounts of lactic acid and improving fermentation quality. These results are consistent with studies by Lv et al., Nkosi et al., and Li. Propionic acid content in the silage control group differed significantly from other enzyme treatments, with the highest value observed in the control group. Trace amounts of butyric acid were detected in pectinase + laccase and compound enzyme preparation groups, suggesting suboptimal ensiling effects compared with other treatments, similar to findings by Wang et al., possibly related to storage methods requiring further verification.

4.2 Effects of Different Enzyme Treatments on Microstructure of Rice Straw Silage

The degree of polymerization determines cellulose carbon chain length and is an important indicator of cellulose conversion to fermentable sugars. Feng et al. used PCA analysis to demonstrate that enzyme addition promotes microbial metabolism of polymeric carbon sources during fermentation, which is consistent with our findings. This study showed that polymerization degree decreased by 30.26%, 27.11%, and 56.32% in PL, C, and CPL groups, respectively, compared with the control group, indicating cellulose carbon chain shortening and degradation into fermentable sugars, consistent with Zhang et al. The CPL treatment showed a significantly greater reduction in polymerization degree than PL and C treatments, likely because the enzyme preparation contained β-glucanase, which acts as an endoglucanase on non-crystalline regions of cellulose, randomly hydrolyzing glycosidic bonds to break long cellulose chains into shorter chains, ultimately reducing polymerization degree and increasing the number of cellulose chain ends accessible to exoglucanases. However, β-glucanase cannot complete degradation alone and requires cooperation from multiple enzymes.

Cellulose carbon chains form stable structures through hydrogen bonds between hydroxyl groups on glucose residues. When hydrogen bonds are broken, free hydroxyl groups are formed, making hydrogen bonding and hydroxyl groups secondary indicators of cellulose degradation. Liu et al. and Zhang et al. found that biological treatment of straw silage degrades cell wall cellulose only to the microfibril level without reaching the supramolecular structure level, with component composition remaining unchanged but content decreasing. In our study, cellulose, hemicellulose, and lignin contents all decreased after enzyme treatment compared with the silage control group, consistent with these results. Tang et al. used γ-ray irradiation combined with NaOH solution to treat straw and found that C-O-C stretching vibration absorption peaks related to cellulose and hemicellulose in FTIR spectra showed reduced intensity, indicating decreased polymerization degree and cellulose content. Our results using compound enzyme preparation, pectinase + laccase, and compound enzyme preparation + pectinase + laccase treatments showed similar patterns. Dai et al. analyzed biochemically treated straw samples using FTIR and found that C=O stretching vibration peaks related to lignin and hemicellulose disappeared, while C-O-C bond vibration peaks related to cellulose and hemicellulose weakened significantly. In our study, C=O stretching vibration peaks did not disappear, but the weakest intensity and flattest peak shape were observed in the CPL treatment.

Cellulose chains form crystalline and amorphous regions based on their arrangement characteristics. Crystalline cellulose is difficult for animals to utilize due to its crystalline nature, making crystallinity an indicator of cellulose crystallization degree. Our study found no significant changes in crystallinity of enzyme-treated silage compared with the silage control group. Wang et al. reported that Phanerochaete chrysosporium, which produces cell wall-degrading enzymes, significantly increased cellulose crystallinity after straw treatment, which contradicts our results. This discrepancy may be due to our combination of enzyme treatment with ensiling, where enzymes could not concentrate on crystalline cellulose during fermentation. The lack of significant crystallinity changes in our study is consistent with Lee et al., possibly because multiple cellulases must act synergistically to affect crystallinity, with cellobiohydrolase playing a dominant role. Additionally, differences in straw origin and composition may affect the required enzyme components and optimal activity ratios.

Specific surface area is an indicator of the accessible area between cellulose microfibrils and degrading enzyme molecules, significantly affecting cellulose absorption in ruminants. Castoldi et al. and Wang et al. demonstrated that biological treatment of straw changes fiber content, decomposes some lignin and hemicellulose, and increases cellulose specific surface area. In our study, CPL treatment increased specific surface area from 1.12 to 1.20 m^2/g, reduced polymerization degree by 56.32%, and significantly decreased cellulose content, consistent with these findings. Pectinase may decompose pectin between cells, loosening straw tissue, separating cells, and creating channels for other cell wall-degrading enzymes to access cell surfaces and expose lignocellulose structure.

Our study combining high-efficiency cellulose-degrading enzymes with lignin-degrading enzymes revealed that single lignin-degrading enzyme components cannot effectively break down straw cell walls, requiring multiple enzyme components to work synergistically.

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5 Conclusions

- After 45 days of ensiling, enzyme-treated rice straw silage exhibited yellow-green color, good texture, and acidic aroma, with reduced pH, increased lactic acid production, decreased DM and cellulose content, and good fermentation quality.
- Compound enzyme preparation + pectinase + laccase treatment effectively broke the lignin-cellulose-hemicellulose composite structure in cell walls, degraded cellulose into utilizable sugars, improved nutritional content, reduced polymerization degree and crystallinity, and increased specific surface area of rice straw silage.
- Enzyme treatment not only altered the chemical composition and physical properties of rice straw but also improved its digestibility and utilization by modifying the hierarchical spatial structure of cellulose.

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Note: Figure translations are in progress. See original paper for figures.