

Effects of Microbial and Compound Enzyme Preparations on Chemical Composition and Fiber Microstructure of Silage Corn Stover (Postprint)

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Abstract

This study utilized enzyme preparations and enzyme-bacteria composite preparations for biological fermentation treatment of corn stover, analyzing their effects on fermentation quality, nutritional composition, and fiber microstructure of corn stover, aiming to explore the mechanism of improving corn stover utilization by disrupting fiber microstructure. The experiment was designed with five groups: corn stover raw material group (CS group), silage control group (CK group), compound enzyme preparation group (CPL group), compound enzyme-single live bacteria group (CPLP group), and compound enzyme-double live bacteria group (CPLB group). A vacuum packaging machine was used to evacuate air from corn stover silage bags, and after 45 days of fermentation at room temperature, the nutritional components and fermentation quality of silage corn stover in each group were analyzed. The phenol-sulfuric acid method combined with 4,4'-dicarboxylic acid-2,2'-quinoline (BCA) method, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction, and eosin-methylene blue method were employed to analyze the fiber microstructure of corn stover in each group. The results showed: 1) Compared with the CK group, the pH, ammonia nitrogen (NH₃-N/TN) ratio, and lactic acid/acetic acid ratio of the CPL group were significantly reduced ($P < 0.05$), while the lactic acid, neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose, and hemicellulose contents showed no significant difference ($P > 0.05$). The lactic acid content of the CPLB group was significantly increased ($P < 0.05$). The pH, NH₃-N/TN ratio, lactic acid/acetic acid ratio, and NDF, ADF, cellulose, and hemicellulose contents of both CPLP and CPLB groups were significantly reduced ($P < 0.05$). 2) Compared with the CK group, the tertiary structure crystallinity of the CPL group was significantly increased ($P < 0.05$), while the primary structure degree

of polymerization and quaternary structure specific surface area showed no significant difference ($P>0.05$), and the secondary structure hydrogen bonding force exhibited no obvious change. The primary structure degree of polymerization of the CPLP and CPLB groups was significantly reduced ($P<0.05$), the quaternary structure specific surface area was significantly increased ($P<0.05$), the secondary structure hydrogen bonding force was weakened, and the tertiary structure crystallinity showed no significant difference ($P>0.05$). In conclusion, compound enzyme-single live bacteria and compound enzyme-double live bacteria significantly improved the chemical composition of corn stover by reducing the primary structure degree of polymerization and the cellulose amorphous region in the tertiary structure of corn stover cellulose, weakening the intermolecular hydrogen bonding force of the secondary structure, and increasing the quaternary structure specific surface area, thereby effectively disrupting the microstructure of corn stover cell wall and improving the utilization rate of straw.

Full Text

Effects of Enzyme Preparation and Compound Enzyme-Bacterial Preparation on Chemical Composition and Fiber Microstructure of Silage Corn Stover

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Abstract: This experiment investigated the effects of enzyme preparation and enzyme-bacteria compound preparation on fermentation quality, nutritional composition, and fiber microstructure of corn stover through biological fermentation treatment, aiming to explore the mechanism for improving corn stover utilization by breaking fiber microstructure. Five experimental groups were established: corn stover raw material group (CS group), silage control group (CK group), compound enzyme preparation group (CPL group), compound enzyme-single live bacteria group (CPLP group), and compound enzyme-double live bacteria group (CPLB group). Vacuum packaging was used to remove air from corn stover silage bags. After 45 days of fermentation at room temperature, the nutritional composition and fermentation quality of silage corn stover were analyzed. The fiber microstructure was examined using phenol-sulfuric acid method combined with 4,4 -dicarboxy-2,2 -biquinoline (BCA) method, Fourier transform infrared spectroscopy (FTIR), X-ray diffraction, and eosin methylene blue method. The results showed: 1) Compared with the CK group, the CPL group exhibited significantly decreased pH, ammonia nitrogen/total nitrogen

ratio (NH -N/TN), and lactic acid/acetic acid ratio ($P < 0.05$), while lactic acid, neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose, and hemicellulose contents showed no significant differences ($P > 0.05$). The CPLB group had significantly increased lactic acid content ($P < 0.05$). The CPLP and CPLB groups showed significant reductions in pH, NH -N/TN, lactic acid/acetic acid ratio, and contents of NDF, ADF, cellulose, and hemicellulose ($P < 0.05$). 2) Compared with the CK group, the CPL group showed significantly increased crystallinity of tertiary structure ($P < 0.05$), while the degree of polymerization of primary structure and specific surface area of quaternary structure showed no significant differences ($P > 0.05$), and the hydrogen bonding force of secondary structure remained unchanged. The CPLP and CPLB groups exhibited significantly decreased degree of polymerization of primary structure ($P < 0.05$), significantly increased specific surface area of quaternary structure ($P < 0.05$), weakened hydrogen bonding force of secondary structure, and no significant difference in crystallinity of tertiary structure ($P > 0.05$). In conclusion, compound enzyme-single live bacteria and compound enzyme-double live bacteria preparations significantly improved the chemical composition of corn stover. By reducing the degree of polymerization of primary structure and the amorphous region in tertiary structure of cellulose, weakening intermolecular hydrogen bonding force of secondary structure, and increasing specific surface area of quaternary structure, these treatments effectively disrupted the microstructure of corn stover cell walls, thereby enhancing straw utilization.

Keywords: *Lactobacillus plantarum*; straw degrading enzymes; corn stover; silage; microstructure; quaternary structure

Introduction

As a major agricultural country, China is rich in crop straw resources, particularly corn stover, which serves as an important roughage source for ruminants. The straw cell wall comprises cellulose, hemicellulose, lignin, pectin, and other chemical components, with cellulose accounting for 30%-50% of the entire cell wall and serving as a direct carbon source for ruminants [1]. However, lignin, an aromatic polymer, forms a stable lignin-carbohydrate complex with hemicellulose that encapsulates cellulose and hemicellulose, preventing direct contact between rumen microorganisms and cellulose-degrading enzymes [2-3]. Effective disruption of this cell wall complex could enhance the efficacy of biological agents and improve straw utilization [2-3]. Therefore, cell wall disruption is a critical step for improving straw feed utilization, primarily aimed at breaking down or removing anti-nutritional structures, increasing accessibility for microorganisms and enzymes, and enhancing cellulose degradation rate and conversion efficiency [4].

Cellulose possesses specific primary, secondary, tertiary, and quaternary structures, characterized by degree of polymerization, hydrogen bonding force, crystallinity, and specific surface area, respectively. Changes in these parameters reflect alterations in fiber microstructure and thus indicate the extent of straw

degradation [5]. While most research has focused on improving straw nutritional quality and feed utilization, few studies have reported on the relationship between the degree of cell wall fiber structure disruption and major fiber components such as neutral detergent fiber (NDF), acid detergent fiber (ADF), cellulose, and hemicellulose during biological treatment. Analyzing fiber morphology and microstructure of straw cell walls can elucidate the molecular mechanisms underlying straw feed utilization and significantly contribute to evaluating the nutritional value of straw feed. This study used corn stover as experimental material to investigate changes in nutritional composition, fermentation quality, and fiber microstructure before and after biological fermentation, aiming to provide technical support for straw feed utilization.

1.1 Experimental Materials

Corn stover of the Jinyu 99 variety was collected from Baoding City, Hebei Province, in September 2016 after grain harvest. The collected material was dried at 65°C for 48 hours, mixed thoroughly, and ground to pass through a 40-mesh sieve for nutrient composition and microstructure analysis.

Enzyme preparations included cellulase complex [cellulase (10,000 U/g) + xylanase (120,000 U/g) + α -glucanase (40,000 U/g)], pectinase (10,000 U/g), and laccase (10,000 U/g), all purchased from Xia Sheng Industrial Group Co., Ltd. as powders stored at room temperature. Live bacterial preparations comprised *Lactobacillus plantarum* (2×10^8 CFU/g) and *Lactobacillus buchneri* (2×10^8 CFU/g), with strains preserved at the Feed Research Institute of Chinese Academy of Agricultural Sciences as freeze-dried powders stored at 4°C.

1.2 Experimental Design

Five treatment groups were established: corn stover raw material (CS), silage control (CK), cellulase complex + pectinase + laccase (CPL), cellulase complex + pectinase + laccase + *Lactobacillus plantarum* (CPLP), and cellulase complex + pectinase + laccase + *Lactobacillus plantarum* + *Lactobacillus buchneri* (CPLB).

Fresh corn stover was chopped to 1-2 cm lengths using a silage chopper. Biological agents were dissolved in distilled water according to the dosages specified in Table 1 and evenly sprayed onto the chopped material, with moisture adjusted to 65%-70%. Treated material was packed in polyethylene bags (24 cm \times 40 cm) at 1 kg per bag, vacuum-sealed (DZ-280/2SD), and stored at room temperature (25-37°C) for 45 days before sampling. The CK group received only distilled water. Additional raw corn stover samples were stored at -20°C for analysis. Each group had four replicates (three for analysis, one backup).

1.3 Analytical Methods

1.3.1 Fermentation Quality and Nutrient Composition Twenty grams of silage were homogenized with 180 mL distilled water for 1 minute and filtered through four layers of gauze. The filtrate pH was measured using a Testo 205 pH meter (Germany) [6]. Ammonia nitrogen content was determined by phenol-hypochlorite colorimetry [5]. Lactic acid, acetic acid, propionic acid, and butyric acid were analyzed by GC128 gas chromatography with flame ionization detection (FID) using a 2 m × 4 mm column packed with Pora-pak Q (80 mesh) at 120°C column temperature, 230°C detector and injector temperatures, nitrogen flow at 65 mL/min, air at 300 mL/min, hydrogen at 30 mL/min, sensitivity of 16×10^3 , and chart speed of 5 mm/min. Nutrient composition including dry matter (DM), crude protein (CP), NDF, ADF, and lignin (L) was determined by chemical analysis [7].

Cellulose (C) = ADF -acid detergent lignin (ADL)

Hemicellulose (HC) = NDF -ADF

Total nitrogen (TN) = CP/6.25

1.3.2 Microstructure Analysis Primary Structure–Degree of Polymerization: Samples were ground to 40 mesh. Total sugar content was determined by phenol-sulfuric acid method and reducing sugar content by BCA method. The degree of polymerization was calculated as the ratio of total sugar to reducing sugar [8].

Secondary Structure–FTIR Spectroscopy: Spectra were obtained using a VERTEX 70V FTIR spectrometer (Bruker, Germany) [5]. Samples (1 mg) were ground with 50 mg KBr and pressed into 13 mm pellets at 1 MPa for 30–60 seconds after drying at 65°C for 12 hours. Scanning range was 1,000–4,000 cm^{-1} with 2 cm^{-1} resolution.

Tertiary Structure–XRD Analysis: Diffraction patterns were obtained using a D8-Advance XRD instrument (Bruker, Germany) [5]. Ground samples (40 mesh) were scanned with Cu-K radiation at 40 kV × 40 mA, scanning speed of 1°/min, step size of 0.04°, and 2 θ range of 3°–40°. Crystallinity was calculated using the formula of Meyer et al. [9]:

$$CrI(\%) = \frac{I_{002} - I_{am}}{I_{002}} \times 100$$

where I_{002} is the maximum intensity of cellulose I at $2\theta = 22^\circ$ – 23° (cellulose II: 18° – 22°) and I_{am} is the minimum intensity at $2\theta = 18^\circ$ – 19° (cellulose II: 13° – 15°).

Quaternary Structure–Specific Surface Area: Determined by eosin methylene blue method [10–11]. Ground sample (0.2 g, 40 mesh) was suspended in 25 mL methylene blue solution, incubated at 25°C with shaking at 120 r/min,

centrifuged at 10,000 r/min for 15 minutes, and the supernatant absorbance measured at 660 nm. Methylene blue adsorption was calculated as:

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

where q is adsorption capacity (mg/g), C_0 and C_t are initial and final concentrations (mg/L), V is volume (L), and m is sample mass (g). Specific surface area (S) was calculated as $S = q \times a$, where $a = 2.45 \text{ m}^2/\text{mg}$ methylene blue for straw materials.

1.4 Statistical Analysis

Data were processed in Excel 2007 and analyzed by one-way ANOVA using SPSS 21.0. Significance was declared at $P < 0.05$.

Results

2.1 Nutrient Composition of Silage Corn Stover

As shown in Table 2, fermentation for 45 days significantly reduced NDF, ADF, cellulose, hemicellulose, and lignin contents in all treatment groups compared with the CS group ($P < 0.05$). The CS group had significantly higher DM, CP, NDF, ADF, cellulose, and hemicellulose contents than the CK group ($P < 0.05$). Compared with the CK group, the CPL group showed no significant differences in NDF, ADF, cellulose, hemicellulose, or lignin ($P > 0.05$), while the CPLP and CPLB groups exhibited significant reductions in NDF, ADF, cellulose, and hemicellulose ($P < 0.05$).

2.2 Fermentation Quality of Silage Corn Stover

All groups maintained pH below 4.2 after fermentation (Table 3). Compared with the CK group, the CPL group showed significantly lower NH₃-N/TN and lactic acid/acetic acid ratios ($P < 0.05$) but similar lactic acid content ($P > 0.05$). The CPLB group had significantly increased lactic acid content ($P < 0.05$). Both CPLP and CPLB groups showed significantly reduced NH₃-N/TN and lactic acid/acetic acid ratios ($P < 0.05$). No propionic or butyric acid was detected in any group.

2.3 Microstructural Changes

2.3.1 Primary Structure—Degree of Polymerization Fermentation significantly affected cellulose polymerization (Table 4). The CS group showed higher polymerization than the CK group ($P < 0.05$). Compared with the CK group, the CPL group showed no significant difference ($P > 0.05$), while CPLP and CPLB groups exhibited significant reductions of 38.92% and 35.64%, respectively ($P < 0.05$).

2.3.2 Secondary Structure—Hydrogen Bonding Force FTIR spectra are presented in Figure 1 [Figure 1: see original paper]. The CS group showed stronger -OH, -CH, and C=O absorption peaks than the CK group. After fermentation with compound enzyme, compound enzyme-single bacteria, and compound enzyme-double bacteria preparations, absorption intensities decreased at 3,386 cm^{-1} (-OH stretching), 2,909 cm^{-1} (-CH stretching), 1,098 cm^{-1} (crystalline cellulose), 1,375 cm^{-1} (-CH bending of cellulose/hemicellulose), and 1,515/1,656 cm^{-1} (lignin C=O conjugated with aromatic rings), indicating degradation of cellulose, hemicellulose, and lignin with consequent microstructural changes.

2.3.3 Tertiary Structure—Crystallinity Crystallinity increased after fermentation, following the order: CPL > CPLB > CPLP > CK > CS (Table 5). XRD patterns (Figure 2 [Figure 2: see original paper]) showed broadened diffraction peaks at crystal planes 101, 002, and 004 with enhanced intensity in CPL, CPLP, and CPLB groups, confirming crystallinity alterations.

2.3.4 Quaternary Structure—Specific Surface Area Specific surface area, a critical parameter for enzyme accessibility [10], showed no significant difference between CPL and CK groups ($P > 0.05$), while CPLP and CPLB groups exhibited significant increases ($P < 0.05$), with CPLP showing the greatest enhancement (Table 6).

Discussion

3.1 Effects of Silage Fermentation on Nutrient Composition and Microstructure

Silage effectively preserves original nutrients while softening straw [6]. In this study, CS group had 21.97%, 20.65%, 26.22%, and 22.50% higher NDF, ADF, cellulose, and hemicellulose, respectively, and 2.75% higher lignin than the CK group. Microstructurally, the CS group showed 13.18% higher polymerization, indicating tightly arranged molecular chains with more glycosidic bonds. Silage caused chain scission and reduced polymerization [12]. The broad peak at 3,386 cm^{-1} represents -OH stretching from cellulose hydrogen bonds; 2,909 cm^{-1} corresponds to cellulose -CH vibration; 1,730 cm^{-1} reflects C=O bending from hemicellulose acyl and uronic groups; while 1,515 cm^{-1} and 1,656 cm^{-1} represent lignin characteristic absorptions [13]. FTIR analysis revealed weakened -OH vibrations and reduced intensities at 2,909, 1,730, 1,515, and 1,635 cm^{-1} , consistent with nutrient composition changes. Cellulose comprises crystalline and amorphous regions—the former with tight, orderly structure resistant to hydrolysis, the latter with loose, accessible structure [14]. XRD analysis showed broadened 101 and 002 peaks and increased crystallinity from 49.50% to 50.07%, indicating preferential utilization of amorphous regions. Specific surface area increased by 2.20%, suggesting surface pore formation favorable for enzyme and microbial adsorption.

3.2 Effects of Compound Enzyme Preparation

High-quality silage is characterized by high lactic acid and low pH, NH₃-N, and butyric acid [15]. Elevated NH₃-N/TN indicates protein degradation and poor quality [11]. Wang Yurong [11] reported that cellulase complex treatment significantly reduced pH, NH₃-N, NDF, and ADF in rice straw silage. In this study, CPL treatment significantly decreased pH, lactic/acetic acid ratio, and NH₃-N/TN but did not affect lactic acid, NDF, ADF, cellulose, or hemicellulose, possibly due to material type and particle size. A lactic/acetic acid ratio >3.0 indicates homofermentative metabolism, and acetic acid inhibits fungi, improving aerobic stability [11]. Enzyme preparations specifically degrade straw structure [11,16]. Li et al. [17] demonstrated that increasing cellulase dosage progressively breaks cellulose chains and reduces polymerization. The lower polymerization in our CPL group likely resulted from endoglucanase preferentially hydrolyzing amorphous regions and randomly cleaving -1,4-glycosidic bonds [18]. FTIR revealed persistent but weakened characteristic peaks for cellulose (3,386, 2,909 cm⁻¹), hemicellulose (1,730 cm⁻¹), and lignin (1,656, 1,515 cm⁻¹), consistent with Chen [19] who reported reduced cellulose content and damaged functional groups after enzymatic treatment. Song [16] found increased porosity and substrate roughness after enzymatic hydrolysis, enhancing enzyme infiltration and adsorption. Our CPL group showed increased crystallinity and specific surface area trends, consistent with Wang et al. [20], likely because biological agents penetrated cell walls, preferentially degrading amorphous regions and dissolving lignin/hemicellulose, thereby reducing amorphous area and increasing crystallinity [19]. Zheng et al. [21] attributed increased crystallinity to orderly molecular rearrangement and secondary crystallization.

3.3 Effects of Compound Enzyme-Bacteria Preparation

Combining cellulose-degrading enzymes with lactic acid bacteria improves fermentation and preserves nutrients [22]. CPLP and CPLB treatments increased lactic acid, decreased lactic/acetic acid ratio, and significantly reduced pH and NH₃-N/TN, consistent with Wang et al. [23] and Han et al. [24]. This synergistic effect increased carbon sources for fermentation and initial lactic acid bacteria populations, enhancing silage quality. Nutrient analysis revealed significant reductions in NDF, ADF, cellulose, hemicellulose, and lignin, demonstrating that enzyme-bacteria cooperation inhibited harmful microbes and preserved nutrients, agreeing with Men [25] and Gu et al. [26]. Microstructurally, CPLP and CPLB showed significantly lower polymerization, likely due to synergistic performance enhancement. FTIR confirmed significant damage to -OH, -CH, and C=O groups, with weakened lignin aromatic skeleton vibrations at 1,515 cm⁻¹, indicating structural degradation consistent with chemical composition changes [18]. Peng [14] reported decreased crystallinity and increased surface area after fungal treatment, with hyphae penetrating crystalline regions. Our contrasting results may reflect differences in enzyme activity, dosage, or substrate lignification. The significantly increased specific surface area in CPLP and CPLB,

especially CPLP, likely resulted from glucosidic bond cleavage creating pores, consistent with Xu et al. [27] and Zeng et al. [2]. Nazarpour et al. [28] reported that fungal lignin degradation increased substrate porosity, while acetyl group removal from hemicellulose enhances internal surface area, porosity, and enzyme degradation rate [29].

Conclusion

1. Compound enzyme-live bacteria treatment of corn stover significantly reduced NDF, ADF, cellulose, and hemicellulose contents while decreasing pH, NH₃-N/TN, and lactic/acetic acid ratio, thereby improving nutritional quality.
2. Biological treatment effectively disrupted anti-nutritional structures and reduced non-crystalline regions in cellulose. Compound enzyme-live bacteria treatment significantly decreased polymerization, weakened hydrogen bonding, and increased crystallinity and specific surface area, markedly altering cellulose microstructure in straw cell walls.
3. The reduction in four fiber components correlated with decreased polymerization, weakened hydrogen bonding, and increased crystallinity and specific surface area, demonstrating that microstructural analysis can elucidate cell wall disruption at the molecular level.

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