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Effects of Different Microbial Additive Combinations on Cassava Residue Quality through Solid-State Fermentation Postprint

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Abstract

This experiment aimed to investigate the effects of combinations of *Lactobacillus buchneri* (LAB), *Aspergillus niger* (AN), *Candida tropicalis* (CT), *Bacillus subtilis* (BS), and *Lactobacillus plantarum* (LAP) on cassava residue quality and to screen for the optimal mixed microbial combination for fermentation. Cassava residue was used as the fermentation substrate, with different microbial suspensions of LAB, AN, CT, BS, and LAP combined at a 1:1 volume ratio, establishing five distinct combinations: Combination 1: LAB+AN+CT+LAP; Combination 2: LAB+AN+BS+LAP; Combination 3: LAB+CT+BS+LAP; Combination 4: AN+CT+BS+LAP; Combination 5: LAB+AN+CT+BS+LAP. Each combination was supplemented with either 1% urea or 1% urea + 0.6% brown sugar; the blank group received no additives, Control Group I received 1% urea, and Control Group II received 1% urea + 0.6% brown sugar. All groups were adjusted to approximately 65% moisture content with physiological saline and vacuum-sealed in polyethylene film bags for 10 days of fermentation. The results demonstrated: 1) Among the different microbial additive combinations for cassava residue fermentation, the five-strain combinations exhibited superior improvement in nutritional composition compared to the four-strain combinations. Specifically, Combination 5 produced the optimal fermentation results, significantly reducing the pH of fermented cassava residue ($P<0.05$) and increasing acetic acid and propionic acid contents compared to the blank group; it also yielded the lowest neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents, which were significantly lower than those of the blank and control groups ($P<0.05$); and it achieved the highest crude protein (CP) content, significantly higher than that of the blank and control groups ($P<0.05$). 2) The addition of appropriate microbial strains + urea + brown sugar during cassava residue fermentation favored propionic acid production. 3) Supplementation of the fermentation medium with urea significantly increased the CP content of

cassava residue (P<0.05). 4) The addition of urea + brown sugar during cassava residue fermentation was more effective for improving nutritional composition than urea alone. In conclusion, the combination of LAB, AN, CT, BS, and LAP with urea and brown sugar for solid-state fermentation of cassava residue can effectively improve cassava residue quality.

Full Text

Effects of Solid-State Fermentation with Different Combinations of Mixed Strains on Quality of Cassava Residue

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Abstract

This experiment investigated the effects of different microbial additive combinations—*Lactobacillus buchneri* (LAB), *Aspergillus niger* (AN), *Candida tropicalis* (CT), *Bacillus subtilis* (BS), and *Lactobacillus plantarum* (LAP)—on the quality of cassava residue, aiming to identify the optimal mixed-strain combination for fermentation. Fresh cassava residue served as the fermentation substrate. Strains were combined at a 1:1 volume ratio to create five distinct combinations: (1) LAB+AN+CT+LAP; (2) LAB+AN+BS+LAP; (3) LAB+CT+BS+LAP; (4) AN+CT+BS+LAP; and (5) LAB+AN+CT+BS+LAP. Each combination was supplemented with either 1% urea or 1% urea + 0.6% brown sugar. Two control groups were included: Control I received 1% urea only, while Control II received 1% urea + 0.6% brown sugar. A blank group received no additives. All groups were adjusted to approximately 65% moisture content with physiological saline and vacuum-sealed in polyethylene bags for 10 days of fermentation.

The results demonstrated: (1) Five-strain combinations improved cassava residue nutritional quality more effectively than four-strain combinations. Combination 5 yielded the best fermentation results, significantly reducing pH (P<0.05) while increasing acetic and propionic acid contents compared to the blank group. This combination also produced the lowest neutral detergent fiber (NDF) and acid detergent fiber (ADF) contents, which were significantly lower than those in both blank and control groups (P<0.05), and achieved the highest crude protein (CP) content, significantly exceeding all control groups (P<0.05). (2) Supplementation with mixed strains plus urea and brown sugar favored propionic acid production. (3) Urea addition significantly increased CP content in fermented cassava residue (P<0.05). (4) The combination of urea and brown sugar improved nutritional composition more effectively than urea alone. In conclusion, solid-state fermentation of cassava residue using

LAB, AN, CT, BS, and LAP combined with urea and brown sugar effectively enhances cassava residue quality.

Keywords: cassava residue; mixed microbial strains; solid-state fermentation

Introduction

Cassava residue, a byproduct of cassava starch and ethanol production, is primarily used as animal feed. Composed mainly of cellulose with minimal protein content, it serves as a common roughage. However, its high lignification degree results in poor palatability and low digestive utilization by animals. Additionally, fresh cassava residue has high moisture content, making it prone to darkening, souring, and spoilage during storage. Large quantities of cassava residue are currently discarded, representing both resource waste and environmental pollution. As cassava residue production continues to increase, its processing and utilization have become urgent issues requiring solutions.

Microbial fermentation can enhance cassava residue nutritional value by increasing crude protein content, reducing fiber content, and improving palatability while preventing mold and deterioration during storage [1-2]. Zhao et al. [3] fermented cassava residue using four microbial categories—*Aspergillus*, *Trichoderma*, *Bacillus*, and yeast—and found that mixed fermentation outperformed other combinations. Under optimal conditions (38°C, substrate-to-water ratio of 1:1.3, 4.5-day fermentation, and inoculation ratio of AN:*Trichoderma reesei*:BS:*Saccharomyces cerevisiae* = 1:1:2:1), CP content increased from 6.37% to 9.75% (dry matter basis), ether extract increased from 2.71% to 4.92%, reducing sugar reached 8.22%, and enzyme activities for filter paper cellulase, carboxymethyl cellulase, amylase, and -glucosidase reached 3.29, 4.26, 5.15, and 3.75 U/g DM, respectively. This synergistic effect likely resulted from complementary interactions among the multiple strains. Kumar and Singh [4] reported that co-culturing *A. niger* with *T. reesei* could compensate for low -glucosidase activity in *T. reesei*, thereby improving hydrolysis efficiency and substrate utilization.

Tang et al. [5] investigated *A. niger* solid-state fermentation of cassava residue and found optimal conditions of 3×10 spores/g inoculum, 4-day fermentation at 36°C, 15% rapeseed meal supplementation, and 1:1.5 substrate-to-water ratio. Under these conditions, CP increased from 8.67% to 13.48% while crude fiber decreased from 22.26% to 17.71%. Numerous studies indicate that single-strain treatment yields limited nutritional improvement and poor practical application. Mixed-strain fermentation leverages synergistic interactions among microorganisms, expanding substrate adaptability and contamination resistance while achieving superior results. However, research on strain selection and compatibility remains limited.

Aspergillus niger exhibits strong starch and cellulose degradation capacity,

producing amylase, glucoamylase, cellulase, phytase, and pectinase. *Candida tropicalis* can utilize sugars from the fermentation substrate for growth and metabolism, producing feed yeast that increases protein content. *Bacillus subtilis* secretes alkaline protease, lipase, amylase, glucoamylase, and cellulase, demonstrating excellent synergistic fermentation with *A. niger*. Additionally, *Lactobacillus buchneri* and *Lactobacillus plantarum* produce lactic acid during fermentation, reducing pH and improving palatability. However, no studies have reported on the combined treatment of cassava residue using these five specific strains.

Given that cassava residue has low nitrogen content while microbial growth requires adequate nitrogen sources, we supplemented with 1% urea as a nitrogen source. Additionally, because cassava residue has low soluble sugar concentration yet lactic acid bacteria proliferation requires certain sugar levels, we added 0.6% brown sugar to adjust soluble sugar concentration. This study evaluated different mixed-strain combinations with urea and brown sugar supplementation by measuring CP, NDF, ADF, and dry matter recovery (DMR) to identify the optimal combination for improving cassava residue quality and palatability, providing a reference for cassava residue development and utilization.

Materials and Methods

1.1 Experimental Materials Fresh cassava residue was provided by the Buffalo Breeding Farm of Guangxi Buffalo Research Institute, with nutrient composition shown in Table 1 . *Candida tropicalis*, *Aspergillus niger*, *Bacillus subtilis*, *Lactobacillus plantarum*, and *Lactobacillus buchneri* were purchased from the China Center of Industrial Culture Collection. Urea was obtained from Sichuan Meifeng Chemical Co., Ltd. (total nitrogen 46.4% on dry basis, biuret 0.9%, moisture 0.4%, particle size Φ1.18-3.35 mm 93%). Brown sugar was purchased from Guangxi State Farms Sugar Industry Group Co., Ltd., with nutritional content per 100 g: energy 1.63 MJ, carbohydrates 96.6 g, protein 0.7 g, moisture 1.9 g, and crude ash 0.8 g.

Culture media included: MRS broth for *Lactobacillus* activation and culture; MRS agar for *Lactobacillus* plate counting; malt extract broth (MEB) for *A. niger* and *C. tropicalis* activation and culture; MEB agar for fungal plate counting; glucose nutrient broth for *B. subtilis* activation and culture; and glucose nutrient agar for *B. subtilis* plate counting.

1.2.1 Strain Activation and Expansion Freeze-dried strains from ampoules were activated and inoculated at 5% into appropriate liquid media. *Candida tropicalis*, *Aspergillus niger*, and *Bacillus subtilis* were cultured at 30°C with 150 rpm shaking, while *Lactobacillus plantarum* and *Lactobacillus buchneri* were cultured anaerobically at 37°C without shaking. Inoculum concentrations were determined by plate counting.

1.2.2 Experimental Design The five strains were combined at 1:1 volume ratios to create five combinations: Combination 1 (LAB+AN+CT+LAP), Combination 2 (LAB+AN+BS+LAP), Combination 3 (LAB+CT+BS+LAP), Combination 4 (AN+CT+BS+LAP), and Combination 5 (LAB+AN+CT+BS+LAP). The experiment included a blank group (no additives), Control I (1% urea), Control II (1% urea + 0.6% brown sugar), and treatment groups.

Each treatment group consisted of 100 g dry cassava residue with 0.6% brown sugar (w/w, DM basis) to adjust soluble sugar concentration and 1% urea (w/w, DM basis) as nitrogen source. Mixed strains were added at 5% of cassava residue DM. The experimental design comprised:

- **Blank group**: Cassava residue only
- **Control I**: Cassava residue + urea
- **Control II**: Cassava residue + urea + brown sugar
- **Experimental Group I**: Cassava residue + urea + each of the five strain combinations (I1-I5)
- **Experimental Group II**: Cassava residue + urea + brown sugar + each of the five strain combinations (II1-II5)

All groups were adjusted to approximately 65% moisture with physiological saline, mixed thoroughly, vacuum-sealed in polyethylene bags, and stored at room temperature. On day 10, two bags per group were opened for sensory evaluation, pH measurement, DMR determination, and CP, NDF, and ADF analysis.

Table 2 shows the microbial populations of each inoculant, determined by pour plating.

1.3.1 Routine Analysis Dry matter, crude protein, NDF, and ADF contents were determined using conventional methods [6].

1.3.2 Sensory Evaluation Sensory evaluation followed the "Silage Quality Evaluation Standards (Trial)" issued by the Chinese Ministry of Agriculture in 1996, assessing odor, color, texture, and mold contamination. Acetic and butyric acid contents were used to evaluate fermentation grade [7].

1.3.3 pH and Quality Analysis **1.3.3.1 Sample Preparation** After opening, 35 g of each sample was placed in a 250 mL flask with 150 mL ultrapure water, extracted at 4°C for 24 h, filtered through two layers of gauze. The filtrate was used for pH and volatile fatty acid (VFA) determination. The remaining residue was dried at 65°C, ground (40-mesh), and used for routine nutrient analysis.

1.3.3.2 pH Measurement pH was measured using a HANNA HI-8424 pH meter.

1.3.3.3 VFA Determination Filtrate (0.5 mL) was mixed with 0.5 mL 8.2% metaphosphoric acid, centrifuged at 13,000 rpm for 10 min, and the supernatant was analyzed for acetic, propionic, and butyric acids using an Agilent 7890A gas chromatograph after adding crotonic acid as internal standard. Chromatographic conditions: HP-INNOWAX column (30 m × 0.25 mm × 0.25 m); temperature program: 80°C for 1 min, then 15°C/min to 170°C for 2 min; nitrogen carrier gas at 14 psi, 1.19 mL/min; hydrogen flow 40 mL/min; air flow 400 mL/min; nitrogen makeup gas 25 mL/min; injector temperature 200°C with 50:1 split ratio; FID detector at 220°C; injection volume 2 L.

1.3.3.4 DMR Calculation DMR (%) = (Mass at opening × DM% at opening) / (Initial mass × Initial DM%) × 100

1.4 Statistical Analysis Data were processed using Excel software. One-way ANOVA and Duncan' s multiple comparison tests were performed using SPSS 17.0. Significance was declared at $P < 0.05$. Results are expressed as means ± standard error.

Results

2.1 Effects of Different Microbial Additive Combinations on Fermentation pH As shown in Table 3 , all treatment groups exhibited lower pH than the blank and control groups. Group II5 showed the lowest pH, decreasing by 11.17% (P<0.05), 10.40% (P<0.05), and 10.14% (P<0.05) compared to the blank, Control I, and Control II groups, respectively. Furthermore, five-strain combinations consistently produced lower pH than their corresponding four-strain counterparts, indicating superior acidification capacity.

2.2 Effects of Different Microbial Additive Combinations on VFA Content Table 3 reveals that Group II5 achieved the highest acetic acid content, increasing by 25.28% (P<0.05), 26.96% (P<0.05), and 34.83% (P<0.05) compared to the blank, Control I, and Control II groups, respectively. Propionic acid was undetectable in the blank, Control I, Control II, and Experimental Group I, but present in all Experimental Group II treatments, with Group II5 showing the highest concentration (P>0.05). This demonstrates that combined supplementation with strains, urea, and brown sugar promotes propionic acid production. Butyric acid levels were generally higher in Experimental Group II than in other groups, while Group I5 showed the lowest butyric acid content, though differences were not significant (P>0.05).

2.3 Effects of Different Microbial Additive Combinations on Nutritional Composition Table 4 shows that all treatment groups had significantly lower DMR than the blank and control groups (P<0.05). Group II5 exhibited the lowest DMR, decreasing by 5.93% (P<0.05), 5.08% (P<0.05),

and 4.73% ($P<0.05$) compared to the blank, Control I, and Control II groups, respectively, indicating greater DM loss during fermentation. Control II had significantly lower DMR than Control I ($P<0.05$), suggesting that urea+brown sugar supplementation caused slightly greater DM loss than urea alone.

Although NDF and ADF contents did not differ significantly among treatment groups ($P>0.05$), all treatments showed significantly lower values than the blank and control groups ($P<0.05$). Group II5 achieved the lowest NDF and ADF contents, with NDF decreasing by 20.12% ($P<0.05$), 19.31% ($P<0.05$), and 18.60% ($P<0.05$), and ADF decreasing by 23.10% ($P<0.05$), 21.73% ($P<0.05$), and 22.28% ($P<0.05$) compared to the blank, Control I, and Control II groups, respectively. Five-strain combinations consistently outperformed four-strain combinations in reducing NDF and ADF. Additionally, Control II showed lower NDF than Control I, and Experimental Group II treatments generally had lower ADF and NDF than Experimental Group I, indicating that urea+brown sugar supplementation was more effective for fiber degradation than urea alone.

All treatment and control groups showed significantly higher CP content than the blank group ($P<0.05$), reaching 1.7-2.0 times the blank group value, confirming that urea supplementation significantly increases protein content. All treatment groups exceeded Control I ($P<0.05$), with Group II5 achieving the highest CP content, increasing by 102.33% ($P<0.05$), 15.74% ($P<0.05$), and 12.74% ($P<0.05$) compared to the blank, Control I, and Control II groups, respectively. Control II had significantly higher CP than Control I ($P<0.05$), demonstrating that urea+brown sugar was more effective than urea alone for protein enhancement. Five-strain combinations also outperformed four-strain combinations in increasing CP content.

Overall, urea+brown sugar supplementation improved nutritional composition more effectively than urea alone, with the five-strain combination plus urea and brown sugar producing the best results. While four-strain combinations improved nutritional quality, five-strain combinations were superior.

2.4 Evaluation of Fermentation Quality Table 5 shows that after 10 days, the blank group exhibited the poorest sensory quality with pungent alcoholic odor and brownish-yellow color, scoring 28 points. All other groups scored higher, displaying sweet-sour aroma, bright yellow color, and good structure. Treatment groups scored 49 points, higher than both control groups. Based on the evaluation criteria in Table 6 , all fermented cassava residue samples achieved Grade 1 quality.

Discussion

3.1 Effects of Different Microbial Additive Combinations on Fermentation Products pH is a critical determinant of successful feed fermentation.

Low pH inhibits harmful microorganisms, reduces protein degradation, and minimizes nutrient loss, ensuring fermentation quality [8]. High-quality fermented feed typically has pH below 4.2 [9]. In this study, all groups achieved pH < 4.2, meeting the standard for quality fermented feed. While all treatments, with or without microbial inoculation and additives, achieved acceptable pH values, supplementation with urea, brown sugar, and microbial strains further reduced pH, with five-strain combinations showing superior acidification compared to four-strain combinations.

The highest acetic and propionic acid contents in Group II5 likely resulted from robust microbial growth and enzyme secretion. Zhang et al. [10] reported that heterofermentative lactic acid bacteria such as *L. buchneri* produce not only lactic acid but also acetic acid, CO , and ethanol during fermentation. Acetic acid helps reduce pH and improves aerobic stability, which is crucial for preventing aerobic spoilage. Ma et al. [2] inoculated cassava residue with lactic acid bacteria consortium SFC-2 plus sucrose, finding increased lactic acid and reduced butyric acid, methylamine, and cyanide, thereby improving palatability and feed conversion. Our results differed, with Experimental Group II showing higher butyric acid than other groups, though levels remained low and did not compromise overall quality. These discrepancies may be attributed to different strain combinations and fermentation conditions.

3.2 Effects of Different Microbial Additive Combinations on Nutritional Composition Cassava residue is characterized by low protein and high fiber content with poor palatability. Improving its quality requires increasing protein while decreasing fiber content. Huang et al. [11] fermented cassava residue with various combinations of *A. niger*, *Saccharomyces cerevisiae*, *Aspergillus oryzae*, and probiotics (containing *B. subtilis*, *Bacillus licheniformis*, *L. plantarum*, and *Bacillus cereus*), finding that the mixed culture produced the highest CP and lowest CF and pH. Similar results were reported by Hang [12] and Okpako et al. [13].

Our findings align with these studies, demonstrating that the five-strain combination with urea and brown sugar produced the best results, with significantly higher CP and lower NDF and ADF contents. This reveals synergistic effects among *L. buchneri*, *A. niger*, *C. tropicalis*, *B. subtilis*, and *L. plantarum*. *Aspergillus niger* effectively degrades starch and cellulose, producing glucoamylase, amylase, phytase, and cellulase, while also acidifying the substrate to create favorable conditions for yeast growth [14]. *Candida tropicalis* produces feed yeast that increases CP content. *Bacillus subtilis* secretes multiple enzymes and synergizes well with *A. niger* [15]. *Lactobacillus buchneri* produces acetic acid and heterolactic fermentation products that reduce pH and improve aerobic stability. *Lactobacillus plantarum*, an anaerobic or facultative anaerobe, produces lactic acid and bacteriocins that lower pH and improve palatability while preventing spoilage. Thus, the five-strain combination not only reduces fiber content but also synthesizes microbial protein from available nutrients, thereby increasing

CP content.

Guan et al. [16] fermented cassava residue with yeast alone, mold alone, or their combination plus 7% urea, achieving 4.94-, 4.32-, and 5.72-fold increases in protein content, respectively, demonstrating that mixed cultures outperform single strains. They also noted that adding inorganic nitrogen significantly increases protein content in low-nitrogen substrates like cassava residue. Our results are consistent, showing that five-strain combinations outperformed four-strain combinations, and that all treatment and control groups had 1.7-2.0 times higher CP than the blank group.

Yang et al. [17] reported that adequate soluble sugar concentration is necessary for rapid lactic acid bacteria proliferation during feed fermentation. Our results confirm that 1% urea + 0.6% brown sugar supplementation was more effective than urea alone for improving cassava residue fermentation quality.

Group II5 showed the lowest DMR, indicating relatively high DM loss during fermentation. This aligns with Huang et al. [11] and occurs because microorganisms consume substrate carbon and nitrogen sources for growth and reproduction. More vigorous microbial activity leads to better fermentation but greater substrate consumption, consequently reducing DMR while relatively increasing CP, crude ash, and ether extract concentrations.

3.3 Evaluation of Fermentation Quality Huang et al. [18] evaluated cassava residue fermented with various combinations of brewer' s yeast, *A. niger*, and *Candida utilis* for 30 days, finding all treatments achieved superior quality in color, odor, and texture. Ma et al. [2] reported that cassava residue inoculated with SFC-2 alone scored 9 points in sensory evaluation, while appropriate sucrose supplementation improved quality scores. In our study, all groups except the blank achieved superior sensory scores, with treatment and control groups outperforming the blank group, demonstrating that microbial, urea, and brown sugar supplementation improves sensory quality.

Our sensory evaluation followed the Chinese Ministry of Agriculture' s 1996 standards, which have limitations and strong subjective components. Kaiser et al. [7] proposed an evaluation system based on acetic and butyric acid contents, excluding pH, lactic acid, and ammonia nitrogen because pH depends on forage species, chemical composition, and fermentation process. This alternative standard is valuable for studying different silage additives. Our evaluation based on acetic and butyric acid contents classified all treatments as Grade 1 quality, indicating that although butyric acid was detected in all groups, low concentrations did not compromise cassava residue quality.

Conclusions

1. Under conditions of 65% moisture, 1% urea, 0.6% brown sugar, 5% mixed-strain inoculum, and 10-day fermentation, optimal fermentation was achieved, significantly reducing pH, increasing acetic and propionic acids, decreasing NDF and ADF contents, and increasing CP content.
2. Combined supplementation with microbial strains, urea, and brown sugar promotes propionic acid production.
3. Five-strain combinations improve cassava residue nutritional quality more effectively than four-strain combinations.
4. Urea supplementation significantly increases CP content in fermented cassava residue.
5. Urea plus brown sugar supplementation is more effective than urea alone for improving cassava residue nutritional composition.

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