

Optimization of Mixed-Culture Solid-State Fermentation Conditions for Converting Baijiu Distillers' Grains into Protein Feed and Evaluation of Its Nutritional Value: Postprint

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Abstract

This experiment aimed to optimize the conditions for mixed-culture solid-state fermentation of distiller' s grains by *Geotrichum candidum*, *Aspergillus oryzae*, *Trichoderma viride*, and *Bacillus subtilis* for development as protein feed, and to evaluate its nutritional value. The four microorganisms were mixed at a ratio of 1:1:1:1 and inoculated into the culture medium at 10%. An L16(4⁵) orthogonal experimental design was adopted, with five fermentation conditions: basal substrate, urea, potassium dihydrogen phosphate, pH, and moisture content, each with four levels, resulting in 16 experimental groups. The prepared mixtures were incubated at (30±2) °C for 72 h. Range analysis of true protein and crude fiber contents before and after fermentation was performed to determine the optimal conditions, followed by comparison of changes in nutritional levels and amino acid composition of distiller' s grains before and after fermentation under the optimal conditions. The results showed: 1) The optimal fermentation conditions were: basal substrate composed of 80% distiller' s grains, 10% wheat bran, 5% corn meal, and 5% rapeseed meal; urea addition at 1.5%; potassium dihydrogen phosphate addition at 0.7%; pH 5; and moisture content at 50%, which yielded the best fermentation effect. 2) Under the optimal conditions, compared with before fermentation, the fermented distiller' s grains showed increases in true protein content by 57.85% (P<0.01); decreases in crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), and crude fat contents by 42.39%, 31.95%, 27.73%, and 21.48%, respectively (P<0.01); increases in calcium and phosphorus contents by 16.67% and 68.18%, respectively (P<0.01); and an increase in total amino acid content by 24.47%, with lysine, methionine, threonine, valine, leucine, isoleucine, and proline contents increased by 109.68%, 38.09%, 39.39%, 71.43%, 28.93%, 10.87%, and 3.70%, respectively. In conclusion, the optimal conditions for fermenting distiller' s grains with the mixed

culture of *Geotrichum candidum*, *Aspergillus oryzae*, *Trichoderma viride*, and *Bacillus subtilis* were: basal substrate composition of 80% distiller's grains, 10% wheat bran, 5% corn meal, and 5% rapeseed meal; 1.5% urea; 0.7% potassium dihydrogen phosphate; pH 5; and 50% moisture content, with the fermented product containing 24.34% true protein.

Full Text

Optimization of Mixed Bacterial Solid-State Fermentation Conditions for Distillers Grains to Produce Protein Feed and Evaluation of Nutritional Value

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Abstract

This experiment was conducted to optimize the conditions for mixed bacterial solid-state fermentation of distillers grains using *Geotrichum candidum*, *Aspergillus oryzae*, *Trichoderma viride*, and *Bacillus subtilis* to produce protein feed, and to evaluate its nutritional value. The four strains were mixed at a ratio of 1:1:1:1 and inoculated at 10% into the culture medium. An L16(5⁴) orthogonal experimental design was employed, comprising five fermentation conditions (base material, urea, potassium dihydrogen phosphate, pH, and moisture), each with four variables, resulting in 16 groups of fermentation conditions. The prepared mixtures were cultured at (30±2)°C for 72 h. Range analysis of true protein and crude fiber contents before and after fermentation was performed to determine the optimal conditions, followed by comparison of nutritional levels and amino acid composition of distillers grains between before and after fermentation under the optimal condition. The results showed: 1) The optimal fermentation condition was a base material composed of 80% distillers grains, 10% wheat bran, 5% corn flour, and 5% rapeseed meal, supplemented with 1.5% urea and 0.7% potassium dihydrogen phosphate, at pH 5 and 50% moisture. 2) Compared with unfermented distillers grains, the fermented product showed a 57.85% increase in true protein content (P<0.01), while crude fiber, acid detergent fiber, neutral detergent fiber, and ether extract contents decreased by 42.39%, 31.95%, 27.73%, and 21.48% (P<0.01), respectively. Calcium and phosphorus contents increased by 16.67% and 68.18% (P<0.01), respectively. Total amino acid content increased by 24.47%, with lysine, methionine, threonine, valine, leucine, isoleucine, and proline contents increasing by 109.68%, 38.09%, 39.39%, 71.43%, 28.93%, 10.87%, and 3.70%, respectively. In conclusion, the optimal conditions for solid-state fermentation of distillers grains using *G. candidum*, *A. oryzae*, *T. viride*, and *B. subtilis* are: base material of 80% distillers grains, 10% wheat bran, 5% corn flour, and 5% rapeseed meal, with 1.5% urea, 0.7% potassium dihydrogen phosphate, pH 5, and 50% moisture, yielding a

fermented product with 24.34% true protein content.

Keywords: distillers grains; mixed bacteria; solid-state fermentation; nutrient value; optimization

Introduction

China is a major producer of liquor, with annual distillers grains output reaching 3×10^{10} t [1]. However, the high crude fiber content and low digestibility of distillers grains, combined with extremely high moisture content that makes them prone to spoilage, pose significant challenges for their further utilization [2]. Research has shown that microorganisms can convert nutrients such as starch and sugars in distillers grains into their own metabolites while simultaneously producing enzymes like lipases, proteases, and cellulases that degrade cellulose, increase protein content, and improve overall quality. Yeast and mold combinations exhibit positive synergistic effects. Although molds such as *Aspergillus oryzae* can effectively degrade starch and cellulose into simple sugars readily utilized by yeast for synthesis of microbial protein [3], *A. oryzae* only produces exopeptidases, limiting its ability to utilize proteins in distillers grains. *Bacillus* species produce highly active endopeptidases, amylases, and lipases, and can secrete many enzymes that animals lack, including those that degrade complex carbohydrates such as pectin, glucan, and cellulose, thereby increasing amino acid content and compensating for deficiencies in other strains. *Trichoderma viride* is recognized as the best cellulase-producing organism, capable of degrading not only cellulose but also hemicellulose and pectin. Therefore, this study selected *Geotrichum candidum*, *T. viride*, *A. oryzae*, and *Bacillus subtilis* for mixed fermentation of distillers grains to optimize fermentation conditions and further enhance nutritional value.

Materials and Methods

1.1.1 Test Strains and Media Distillers grains were purchased from Ya' an Shaping Distillery with a moisture content of 63.45% and the following nutritional levels on a dry matter basis: crude protein 16.32%, true protein 15.01%, crude fiber 34.90%, acid detergent fiber 55.60%, neutral detergent fiber 65.39%, ether extract 2.58%, crude ash 12.65%, calcium 0.22%, and phosphorus 0.18%. Wheat bran, rapeseed meal, and corn flour were purchased from the experimental farm of the Institute of Animal Nutrition, Sichuan Agricultural University. *Geotrichum candidum*, *Aspergillus oryzae*, *Bacillus subtilis*, and *Trichoderma viride* were obtained from the China Industrial Microorganism Collection Center. The culture media and primary functions of the test strains are shown in Table 1 .

Table 1 Media and primary functions of test microorganisms

PDA medium preparation: 200 g peeled potatoes were chopped, added to 800

mL distilled water, and boiled on an electric stove for approximately 30 min. The mixture was filtered through 8 layers of gauze, the filtrate was retained, 20 g glucose was added, and water was added to 1,000 mL. (For solid medium, 20 g agar was added and brought to 1,000 mL.) The medium was sterilized at 121°C for 20 min.

Malt extract medium: 15 g agar and 5°Bé malt extract were brought to 1,000 mL and sterilized at 121°C for 20 min.

Beef extract starch medium: 20 g beef extract, 20 g starch, 10 g peptone, and 20 g agar were dissolved in distilled water, brought to 1,000 mL, adjusted to pH 7.2, and sterilized at 121°C for 20 min.

1.1.2 Strain Activation A sterile pipette was used to add 0.5 mL of liquid medium to the ampoule to completely dissolve the dried bacterial cells. The solution was transferred to a test tube containing 4-5 mL of liquid medium. *G. candidum* and *A. oryzae* were cultured in malt extract medium for 3-5 days, *T. viride* was cultured in PDA medium for 5-7 days, and *B. subtilis* was cultured in beef extract starch medium for 2 days. Strains were activated for 2-3 generations.

1.1.3 Microbial Seed Preparation *Trichoderma viride* was cultured on PDA slants at 28°C until mycelium covered the surface. *Aspergillus oryzae* was cultured in malt extract medium at 28°C for 5-7 days. Spores were washed with sterile water, filtered through 4 layers of lens paper, and spore suspension concentration was determined using a hemocytometer and diluted to 1×10^7 CFU/mL for later use. * *Geotrichum candidum* * was cultured in liquid medium at 28°C and 150 r/min until reaching approximately 1×10^7 CFU/mL. * *Bacillus subtilis* * was cultured in beef extract medium at 28°C and 250 r/min until reaching approximately 1×10^7 CFU/mL.

1.2.1 Experimental Design *Geotrichum candidum*, *Aspergillus oryzae*, *Trichoderma viride*, and *Bacillus subtilis* were mixed at a 1:1:1:1 ratio and inoculated at 10% into the culture medium. An L16(5^4) orthogonal experimental design was employed with five fermentation conditions: base material (A), urea (B), potassium dihydrogen phosphate (C), pH (D), and moisture (E), each with four variables, resulting in 16 groups of fermentation conditions. The L16(5^4) orthogonal experimental design scheme for fermentation conditions is shown in Table 2. The prepared mixtures were placed in a constant temperature incubator at $(30 \pm 2)^\circ\text{C}$ for 72 h, stirred every 12 h to prevent excessive internal temperature. After the experiment, a portion was immediately taken for moisture determination, and the remainder was dried for later analysis. True protein and crude fiber contents were first used as indicators to screen for optimal fermentation conditions, after which nutritional levels and amino acid contents of distillers grains were determined under the optimal conditions and compared before and after fermentation.

Table 2 L16(5^4) orthogonal testing design scheme for fermentation condition

Note: Numbers outside brackets represent coding numbers, while those inside brackets represent specific condition parameters.

1.2.2 Measurement Indicators and Methods After fermentation, samples were dried in a forced-air oven at 65°C to prepare air-dried samples. True protein content was determined by precipitating true protein with excess copper hydroxide, washing away water-soluble nitrogenous substances with water, and measuring the nitrogen content in the precipitate by the Kjeldahl method [4]. Crude fiber content was determined by acid-alkali washing. For neutral detergent fiber (NDF) and acid detergent fiber (ADF) determination, acid and neutral detergent solutions were added to the precipitate and boiled gently for 1 h, after which soluble substances were washed away with hot distilled water and the remaining material was dried at 105°C, following the method of Van Soest et al. [5]. Calcium content was determined by potassium permanganate titration, phosphorus content by molybdenum yellow spectrophotometry [6], and amino acid content by automatic amino acid analyzer (Hitachi L-8900) following the method of Zhang Liying [7].

1.3 Data Statistics and Analysis Data were initially organized using Excel 2007 and then processed and analyzed using SPSS 19.0. Experimental results were subjected to range analysis and variance analysis, with significance determined by Duncan's multiple comparison test.

Results

2.1 Range Analysis of True Protein Content in Mixed Fermentation Orthogonal Test As shown in Table 3, the effects on true protein content followed the order $B > A > E > C > D$, indicating that urea was the primary influencing factor, followed by base material, moisture, potassium dihydrogen phosphate, and pH. The optimal combination was A4B3C4D1E1. Visual analysis revealed that group 9, with the combination A4B3C2D1E2, had the highest true protein content at 23.95%, representing a 59.56% increase compared with unfermented distillers grains. This differed from the optimal combination identified by range analysis, necessitating further verification tests.

Table 3 Range analysis for true protein content in L16(5^4) orthogonal test of complex strains fermentation

Note: K1, K2, K3, and K4 represent the average values of data obtained under fermentation conditions with corresponding coding numbers; R represents the range, calculated as the difference between the maximum and minimum values of K1, K2, K3, and K4. The same applies below.

2.2 Range Analysis of Crude Fiber Content in Mixed Fermentation Orthogonal Test As shown in Table 4, the effects on crude fiber content

followed the order $A > C > B > D > E$, indicating that base material was the primary influencing factor for cellulose degradation, followed by potassium dihydrogen phosphate, urea, pH, and moisture. The optimal combination for cellulose degradation was A4B3C4D1E1, consistent with the results from true protein content screening. Visual analysis revealed that group 15 had the lowest crude fiber content at 20.23%, representing a 42.03% reduction compared with unfermented distillers grains.

Table 4 Range analysis for crude fiber content in L16(5^4) orthogonal test of complex strains fermentation

2.3 Comparison of Nutrient Levels Before and After Fermentation Under Optimal Conditions

As shown in Table 5, the true protein content of fermented distillers grains was significantly higher than that before fermentation ($P < 0.01$), increasing by 57.85% compared with the unfermented substrate. Crude fiber content was significantly lower after fermentation ($P < 0.01$), decreasing by 42.39%. Additionally, acid detergent fiber and neutral detergent fiber contents also showed significant reductions ($P < 0.01$), decreasing by 31.95% and 27.73%, respectively. Ether extract content was significantly lower after fermentation, while crude ash, calcium, and phosphorus contents were significantly higher ($P < 0.01$), increasing by 4.42%, 16.67%, and 68.18%, respectively.

Table 5 Comparison of nutrient levels before and after fermentation under optimal condition (DM basis), %

Note: Growth rate = $100 \times (\text{after fermentation} - \text{before fermentation}) / \text{before fermentation}$. The same applies below.

In the same row, values with no letter or the same letter superscripts indicate no significant difference ($P > 0.05$), different lowercase letters indicate significant difference ($P < 0.05$), and different uppercase letters indicate highly significant difference ($P < 0.01$).

2.4 Comparison of Amino Acid Composition Before and After Fermentation Under Optimal Conditions

As shown in Table 6, total amino acid content after fermentation was 16.58%, representing a 24.47% increase compared with before fermentation. Except for cysteine, which decreased by 11.43% compared with before fermentation, all other amino acid contents increased to varying degrees. Lysine showed the highest increase at 109.68%. Essential amino acids methionine, threonine, valine, leucine, isoleucine, and proline increased by 38.09%, 39.39%, 71.43%, 28.93%, 10.87%, and 3.70%, respectively. The overall increase in non-essential amino acids was slightly lower than that of essential amino acids.

Table 6 Comparison of AA composition before and after fermentation under optimal condition (DM basis)

Discussion

3.1 Optimization of Distillers Grains Fermentation Conditions Base material composition significantly affects the quality of microbial fermentation products. As a byproduct of liquor production, distillers grains have unbalanced nutrients, requiring supplementation with other raw materials. Wheat bran, corn flour, and rapeseed meal are rich in carbon sources. Appropriate addition during fermentation not only improves the nutritional quality of distillers grains but also increases substrate porosity, facilitating oxygen flow and promoting aerobic bacterial growth. Guo et al. [8] reported that adding wheat bran and corn flour significantly improved fermentation efficiency of mixed *Candida tropicalis*, *Aspergillus niger*, and *Trichoderma viride* on distillers grains. Jiao et al. [9] found that when wheat bran addition was less than 15%, crude protein content in fermentation products increased with increasing wheat bran addition. Zhang et al. [10] reported that adding 10% rapeseed meal significantly improved distillers grains fermentation. This study found that the group with 10% wheat bran, 5% corn flour, and 5% rapeseed meal showed the best fermentation results, with highest true protein content and lowest crude fiber content, significantly outperforming other groups.

Moisture is an indispensable factor for microbial growth. Microorganisms can grow on solid media when moisture content exceeds 40%. When free-flowing water is present in the medium, microbial metabolites and various enzymes can permeate and flow into the substrate, promoting microbial growth and reproduction and improving distillers grains quality. Liu et al. [11] reported that when moisture content of fermentation medium reached 50%–60%, crude protein content in the product stabilized. This study found that true protein content was highest at 50% moisture, which is also more favorable for product promotion in practical production.

Initial pH of the fermentation substrate is also an important factor affecting microbial growth. Suitable pH conditions promote microbial growth, while unsuitable conditions inhibit it. Hou et al. [12] reported that crude protein content was highest at pH 5.5 using selected strains 8505 and 8503, increasing by 23.75% compared with unfermented substrate, but decreased significantly when pH exceeded 6.0. However, Sun et al. [13] found that protein yield was highest at pH 6.31 using multi-strain solid-state mixed fermentation. Dong et al. [14] reported that the optimal pH for strain fermentation was 9.0. This study found that true protein content was highest at pH 5.0, differing from other studies, possibly due to different strains used and their varying optimal pH requirements.

Potassium serves as an activator for various important enzyme systems in organisms and maintains osmotic pressure, regulates acid-base balance, and controls water metabolism as an electrolyte. Phosphorus is an important participant in phosphorylation of biochemical reaction substrates and a component of genetic material and numerous enzymes. Therefore, this study adjusted potassium and phosphorus content by adding potassium dihydrogen phosphate. Yi et al. [15]

reported that microbial density was highest when potassium dihydrogen phosphate content was 0.6%. This study found that true protein content was highest with 0.7% potassium dihydrogen phosphate, similar to their results.

Microorganisms can utilize non-protein nitrogen and convert it into part of their metabolites; therefore, exogenous nitrogen can be added to increase true protein content in fermentation products. Li et al. [16] reported that true protein content was highest when 2% urea was added to distillers grains. Lu et al. [17] found that 3% urea was optimal for mixed fermentation with *B. subtilis* and *C. tropicalis*. However, this study found that true protein content was highest with 1.5% urea, lower than these studies, possibly due to differences in fermentation strains and raw materials. When urea content exceeded 1.5%, true protein content decreased, likely because microbial conversion of exogenous nitrogen is limited, and addition beyond the optimal amount cannot increase true protein content in fermentation products.

3.2 Effects of Optimal Fermentation Conditions on Distillers Grains Nutrient Levels

Microbial fermentation can greatly improve the nutritional value of distillers grains. Song et al. [18] used *Candida utilis*, *A. niger*, *A. oryzae*, and *B. subtilis* for combined fermentation and found that crude protein and true protein contents increased significantly while acid detergent fiber and neutral detergent fiber contents decreased. Chen [19] reported that true protein content increased by 27.44% and neutral detergent fiber decreased by 5.53% using mixed solid-state fermentation of distillers grains. Wang et al. [20] used *G. candidum* and *Candida* spp. for mixed fermentation and found that crude fiber decreased by 20.1% and ether extract decreased by 61.81%. Zhong et al. [21] used *T. viride* and *C. tropicalis* for synergistic solid-state fermentation and found increased protein content. Sun et al. [13] co-fermented distillers grains with *G. candidum*, *A. niger*, *A. oryzae*, and *C. utilis* and found increased protein content and significantly decreased crude fiber content, improving quality and nutritional value. Guo et al. [22] mixed *Lactobacillus*, *Brettanomyces*, *T. viride*, and *B. subtilis* in equal proportions and found that true protein content under optimal conditions was significantly higher than in unfermented distillers grains, with the greatest reductions in neutral detergent fiber, acid detergent fiber, and crude fiber contents.

This study found that under optimal conditions, the conventional nutrient levels of fermented distillers grains were superior to those before fermentation, with true protein content increasing by 57.85%. This increase was 269.18%, 110.82%, and 33.29% higher than the 15.67%, 27.44%, and 43.40% reported by Song et al. [18], Chen [19], and Zhong et al. [21], respectively. The crude fiber reduction was 110.89% greater than that reported by Wang et al. [20], while the ether extract reduction was 65.20% lower. The reductions in neutral detergent fiber and acid detergent fiber were 12.30% and 68.23% higher than those reported by Song et al. [18] but 22.50% and 15.56% lower than those reported by Guo et al. [22]. These differences may be due to optimal complementary effects

of metabolites among the strains selected in this study and the addition of potassium dihydrogen phosphate as an activator for various enzyme systems, resulting in greater production of proteases and cellulose-degrading enzymes. Additionally, microorganisms could utilize non-protein nitrogen from urea and rapeseed meal to convert into true protein and various small peptides, thereby increasing protein content in fermentation products. This study also found that crude ash content increased by 4.42%, calcium by 16.67%, and phosphorus by 68.18% after fermentation, possibly due to release of carbon dioxide during fermentation causing loss of organic matter and consequent relative increase in these components.

3.3 Effects of Optimal Fermentation Conditions on Distillers Grains Amino Acid Composition

Ruminant protein nutrition primarily involves degradation into amino acids for absorption and participation in metabolism. Although distillers grains are rich in amino acids, their amino acid composition is extremely unbalanced. Through microbial fermentation with sufficient carbon, nitrogen, and mineral sources, large amounts of amino acids can be synthesized. Under optimal conditions in this study, all amino acids except cysteine increased to varying degrees, particularly the limiting amino acids for ruminants—lysine, methionine, and histidine [23]—which increased by 109.68%, 38.09%, and 19.51%, respectively, compared with before fermentation. Mullins et al. [24] found that when the dietary lysine to methionine ratio was 3:1, calves showed optimal growth performance and maximum nitrogen utilization. This study found that the lysine to methionine ratio was 1.47:1.00 before fermentation and 2.24:1.00 after fermentation, with the latter being closer to the optimal ratio reported by Mullins et al. [24], indicating higher utilization value of fermented distillers grains for ruminants. Wang et al. [25] reported that the optimal lysine to threonine ratio for beef cattle diets was 1.00:0.65, similar to this study's results. Cysteine is a sulfur-containing amino acid involved in synthesis of active substances such as glutathione and taurine, playing important roles in oxidation and immunity. This study found that cysteine content decreased slightly, possibly related to its participation in physiological activities during microbial metabolism, though specific reasons require further investigation.

Comparison with FAO/WHO and egg protein ideal amino acid patterns (Table 7) revealed that the ratio of essential amino acids to total amino acids after fermentation was 0.37, close to the 0.4 proposed by FAO/WHO. The ratio of essential to non-essential amino acids was 0.59, also close to the 0.6 proposed by FAO/WHO [26]. Threonine, lysine, and isoleucine contents were 47.25%, 48.55%, and 52.25% of the FAO/WHO ideal amino acid pattern, and only 28.64%, 41.72%, and 40.98% of egg protein, considered the highest quality protein. Therefore, these three amino acids were the first, second, and third limiting amino acids in fermented distillers grains. However, phenylalanine + tyrosine content in fermented distillers grains was 104.83% of the FAO/WHO ideal amino acid pattern, superior to the FAO/WHO ideal protein, and leucine content was also close to the ideal pattern. Compared with animal nutritional

requirements, leucine and valine contents exceeded the requirements for growing layers and lean pigs (2-20 kg) [27].

Table 7 Comparison between AA composition of fermented distillers grains and ideal modes of AA

Table 8 Comparison of ideal AA balance pattern

Conclusion

1. The optimal conditions for mixed fermentation of distillers grains using *Geotrichum candidum*, *Aspergillus oryzae*, *Trichoderma viride*, and *Bacillus subtilis* are: base material composed of 80% distillers grains, 10% wheat bran, 5% corn flour, and 5% rapeseed meal, with 1.5% urea, 0.7% potassium dihydrogen phosphate, pH 5, and 50% moisture.
2. Under optimal conditions, fermented distillers grains contain 24.34% true protein, 20.06% crude fiber, and 16.58% total amino acids.

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